Synthetic carbohydrate ligands for immune receptors

Reintjens, N.R.M.

Synthetic carbohydrate ligands for immune receptors

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C. J. J. M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 27 februari 2020
klokke 15:00 uur

door

Niels Rafael Margaretha Reintjens

Geboren te Sittard in 1989

Promotiecommissie

Promotor	:	Prof. dr. G.A. van der Marel
Co-promotor	:	Dr. J. D. C. Codée
Overige leden	:	Prof. dr. J. Brouwer
		Prof. dr. H. S. Overkleeft
		Prof. dr. F.A. Ossendorp
		Prof. dr. A. Molinaro (University of Naples Federico II)
		Dr. M. T. C. Walvoort (University of Groningen)
		Dr. D.V. Filippov

ISBN: 978-94-028-1895-6
Cover design: Niels R. M. Reintjens & Edwin Vonk

Table of contents

Chapter 1

General introduction

Introduction

Vaccination is one of the most successful approaches for the prevention of infectious diseases as it can provide a strong and lifelong immune response against pathogens. Classical vaccines are composed of attenuated or inactivated pathogens and owe their success mainly due to humoral immune responses. Vaccines for diseases such as cancer and HIV cannot be attained by analogous procedures and their development has proven to be more challenging as cellular immune responses are required.[1] Promising strategies to generate cancer vaccines exploit cancer specific peptides, the so-called neoepitopes[2] or tumor-associated carbohydrate antigens (TACAs)[3]. Peptide (neo)epitopes can be presented to T lymphocytes (T cells) via major histocompatibility complexes class I (MHC-I) or class II (MHC-II), present on antigen presenting cells (APCs). Both MHC classes present their antigen to a T cell: MHC-I activates a cytotoxic T cell (CTL) and MHC-II engages T helper (Th) cells. CTLs can eradicate for instance (virus-) infected cells or cancerous cells, while Th cells play an important role in generating a humoral (B cell) and cellular (T cell) responses, as they secrete cytokines resulting in the

activation and proliferation of B cells and CTLs (Figure 1). To achieve an effective CTL activation and thus anti-tumor immunity, T cells require three signals for activation.[4,5] Antigen presentation by the MHC on a target cell is the first signal, followed by interaction with co-stimulatory receptors on T cells and their corresponding ligands on APCs. The secretion of cytokines, such as interleukins (ILs), is considered to be the third signal. These three signals are important as immune tolerance can occur when antigen presentation is not followed by the last two signals.[6,7] Although TACAs are uniquely or overexpressed glycans on tumor cells, the deployment of these carbohydrates for cellular immune responses requires the assistance of a Th peptide epitope as carbohydrates are poorly immunogenic and only bind to B cells. For an effective anti-tumor immune response, B cells require the help of Th cells.

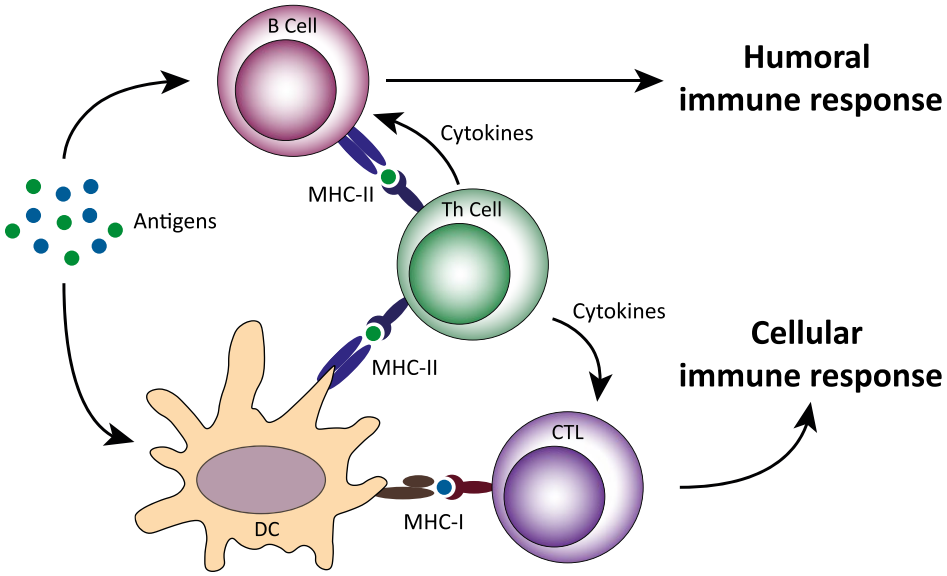


Figure 1. Schematic overview of acquiring a cellular and a humoral immune response.

Targeting antigen presenting cells

Antigen presenting cells, such as dendritic cells (DCs) play an important role in providing the previously mentioned three signals to obtain a T cell mediated immune response. To rapidly detect invading pathogens and send out “danger signals”, DCs are equipped with a row of pattern recognition receptors (PRRs).[8,9] PRRs can be divided into four main families of proteins: Toll-like receptors (TLRs), Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectins and retinoic acid-induced gene (RIG)-I-like receptors. The binding of pathogen-associated molecular patterns (PAMPS) to a PRR results in the maturation of the DC and subsequent secretion of cytokines, the

so called “danger signal”. TLRs[10,11] are transmembrane glycoproteins (Figure 2), which are either located on the outer cell membrane (TLR1, 2, 4, 5, 6, 10, 11, 12) or expressed in endosomes (TLR3, 7, 8, 9, 13). The PRRs located on the outer membrane are able to recognize bacterial and fungal components such as lipomannan, lipoteichoic acids, di- and tri-acetylated bacterial lipopeptides, lipopolysaccharides (LPS) and flagellin, whereas the ones expressed in endosomes recognize viral or microbial nucleic acids, for example ssRNA, dsRNA, and the CpG motif. Most TLRs can be found as homodimers, with the exception of a few heterodimers: TLR1/TLR2, and TLR2/TLR6. In humans, TLRs 1-10 are expressed, while in mice TLR1-9 and TLR11-13 are found. NOD-like receptors (NOD1 and NOD2)[12] are intracellular proteins that can provide an innate immune response upon detection of components of the bacterial peptidoglycan. C-type lectins[13,14] and RIG-I-like[15] receptors recognize a diverse set of carbohydrate structures and viral RNA, respectively and will not be discussed further as they are beyond the scope of this Thesis. Another group of receptors present on APCs are the Fc receptors (FcRs), which form a bridge between the humoral and the cellular immune system.[16,17] FcRs are able to recognize immune complexes (ICs), which are formed from antibodies bound to antigens, and internalize the complex via the endocytic pathway resulting in both antigen presentation and DC-maturation and thus the secretion of cytokines.

Vaccination approaches

Vaccination with solely CTL or Th epitopes is not an effective approach to induce a cellular immune response.[18] Small peptides are generally poorly immunogenic and are unable to activate the innate immune system, which may lead to tolerance.[6,7] This problem can be obviated by the application of adjuvants (Figure 2).[19,20] Two types of adjuvants[1] exist, the first of which is involved in the improvement of the delivery of the antigen to DCs, for example liposomes, virosomes, emulsions and mineral salts.[21] The most commonly used adjuvant in vaccine formulations is Alum, that is able to enhance the potency of bacterial vaccines, but lacks the ability to induce a cellular immune response.[22] The second type of adjuvants are immunostimulants, comprising PRR-ligands that can induce a danger signal, such as the production of cytokines, by binding to, for example, one of the PRRs present on APCs.

This chapter describes selected well-defined synthetic ligands that are used as immunostimulants in vaccine formulations. Next, the so-called self-adjuvanting vaccines or conjugates with a specific antigen covalently bound to one or more immunostimulants, are discussed (Figure 2).[23,24] Self-adjuvanting vaccines are promising in inducing effective anti-tumor immunity. Finally, antibody-recruiting

molecule (ARM) strategies will be discussed as another approach to attain long-lasting adaptive immunity.[25]

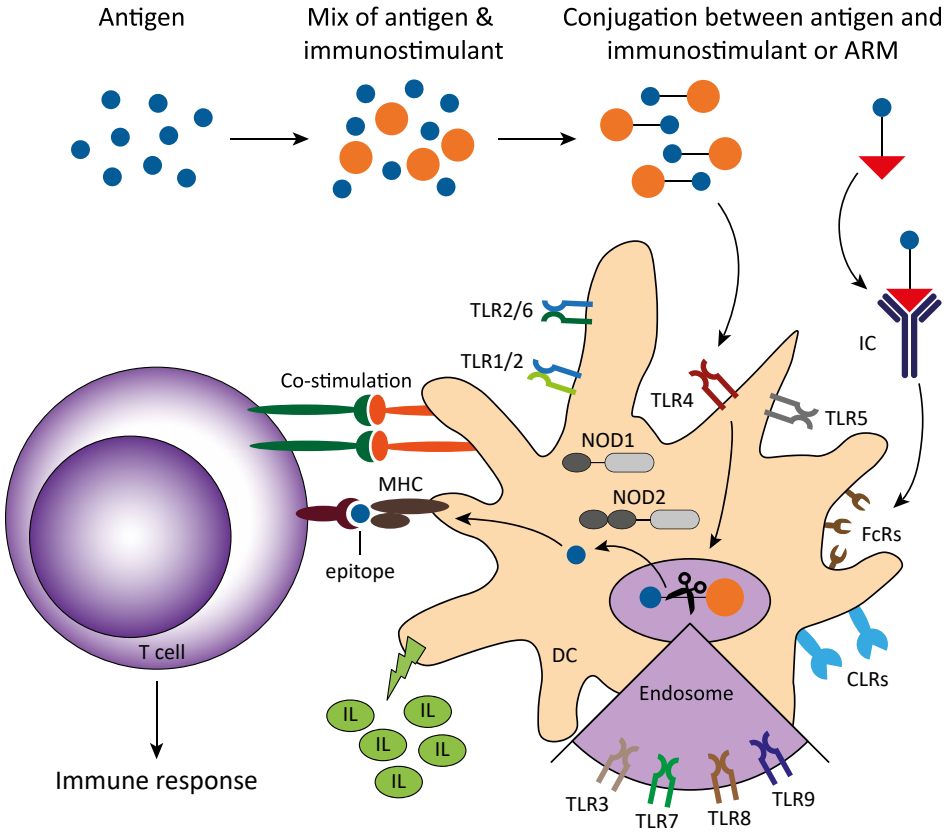


Figure 2. Schematic overview of acquiring an immune response using a conjugate by targeting DCs.

Immunostimulants

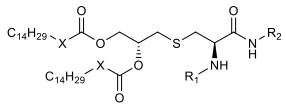
The discovery of well-defined (synthetic) PRR-ligands enables the study of structure-activity relations and the development of synthetic vaccine modalities.[11] Table 1 provides an overview of such TLR and NLR ligands that can be used as immunostimulating agents.

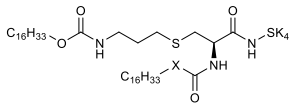
PRR	Natural ligands	Pathogen source	Synthetic ligands
TLR1/ TLR2	Triacylated lipopeptides	Bacteria	Pam_3C Pam_3CSK_4
TLR2/ TLR6	Diacylated lipopeptides	Bacteria	Pam_2CSK_4 MALP-2 FSL-1
TLR4	LPS	Gram negative bacteria	Lipid A MPL GLA AGPs
TLR7-8	ssRNA	Viruses	Imiquimod Resiquimod, 8-oxo-adenine derivatives
TLR9	CpG DNA	Bacteria, viruses	CpG ODN
NOD1	Meso-DAP	Bacteria	D-*i*E-DAP derivatives
NOD2	MDP	Bacteria	Muramyl dipeptide derivatives

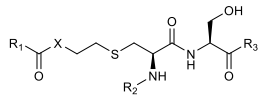
Table 1. Overview of synthetic well-defined TLR and NLR agonists.

TLR2 recognizes a wide variety of lipopeptides and lipoproteins and its specificity depends on the dimerization with either TLR1 or TLR6 as shown by the crystal structures of heterodimers TLR1/TLR2[26] and TLR2/TLR6.[27] Triacylated lipopeptides, such as Pam_3CSK_4 (**1**, Figure 3), one of the most potent TLR2 agonists to date, target TLR1/TLR2, as two lipid chains are inserted into the TLR2 pocket, while the amide-bound lipid is inserted into the TLR1 pocket. The amide-bound lipid chain also prevents the triacylated ligands from binding to TLR2/TLR6 as the hydrophobic pocket is blocked by bulky side chains. Pam_2CSK_4 (**2**) lacks the amide-bound lipid and triggers the dimerization between TLR2 and TLR6. Other diacylated lipopeptides, MALP-2 (**3**) and FSL-1 (**4**), are derived from *Mycoplasma fermentans* and *Mycoplasma salivarium* respectively and only differ in peptide composition.[28–31] The use of Pam_3CSK_4 TLR1/TLR2 agonists can improve the immune response by the production of cytokines, and its use has been shown to halt the development of cancer and can even induce tumor regression.[32] While it was shown that biological activity of Pam_3CSK_4 originates from the diasteroisomer having the RR-configuration, a diastereoisomeric mixture of Pam_3CSK_4 **1** is often employed because of synthetic ease and commercial availability. Several groups have tried to enhance the potency of TLR2 agonist **1**. A library of Pam_3CSK_4 derivatives in which the α-CH_2 of the amide lipid was replaced with an NH to form an extra hydrogen bridge with the receptor resulted in several new potent TLR2 agonists.[33] Others found that the immunological

properties strongly depend on the length of the lipid and the presence of *S-2*(*R*)-dihydroxypropyl-(*R*)-cysteine.[34–36] A chemically and metabolically more stable TLR2-ligand was made by replacing the two ester-linked palmitoyl groups with a 14-carbon chain via a carbamate linkage. The resulting ligand, SUP3 (**5**), was shown to induce a stronger antitumor response than **1**, when co-administered with different antigens.[37] One of the disadvantages of **1** is its poor solubility, and therefore Du *et al*. have generated more water soluble diacylated TLR2 agonists based on Pam_3CSK_4 introducing carbamate linkages as in **6**, which was shown to be as potent as Pam_3CSK_4.[38] The subtle change, replacing the α-CH_2 of the amide lipid in **6** for an NH (**7**), was shown to alter the binding preference of **6**/**7** from TLR1/TLR2 to TLR2/TLR6 binding. Monoacylated agonists, such as **8** and **9**, have been synthesized as well in an effort to improve the physical properties, e.g. water solubility.[39,40]







1 R_1 = $C(O)C_{15}H_{31}$, R_2 = SK_4, X = CH_2
2 R_1 = H, R_2 = SK_4, X = CH_2
3 R_1 = H, R_2 = GNNDESNISFKEK, X = CH_2
4 R_1 = H, R_2 = GDPKHPKSF, X = CH_2
5 R_1 = $C(O)C_{15}H_{31}$, R_2 = GK_3, X = NH

6 X = CH_2
7 X = NH

8 X = O, R_1 = $C_{15}H_{31}$, R_2 = H, R_3 = OMe
9 X = NH, R_1 = $OC_{16}H_{33}$, R_2 = Ac, R_3 = $NH(CH_2)_3N(CH_3)_2$

Figure 3. TLR2-ligands **1**-**9**.

TLR4 is the TLR that was first discovered in humans and it has been extensively studied. It has been shown that a complex of the lipopolysaccharide ligand and MD-2 binds to TLR4, upon which activation of the TRIF and MyD88 signaling pathways is induced. Notably, no other TLR is able to trigger both pathways.[41] TLR4 recognizes lipopolysaccharides (LPS), which are components of the Gram-negative bacteria cell membrane, and its endotoxic principle, lipid A (**10**, Figure 4), is the natural ligand for this receptor. Crystal structures have shown that six lipid chains are optimal for TLR4 activation as five are buried in the MD-2 pocket, while the remaining lipid is engaged in a hydrophobic interaction with TLR4.[42] Many groups have focused on the synthesis of lipid A agonists.[43–45] Removal of the phosphate on the anomeric position has led to the development of monophosphoryl lipid A (MPLA) derivatives, which are 1000-fold less toxic compared to lipid A and thus suitable as a vaccine adjuvant.[46,47] One of these MPLA analogues, MPL (**11**), has been used as a component of the AS04 adjuvant mixture in approved vaccines for hepatitis B and HPV.[48–50] A library of synthetic aminoalkyl glucosamine 4-phosphates (AGPs) has been developed, where the acetylated monosaccharides mimic the structure of lipid A.[51] These AGPs are not only easier to

synthesize, they also induce comparable or even enhanced immunostimulatory activities.[52] One of these AGPs, RC-529 (**12**) has been shown in human clinical trials to have an excellent safety profile. In an effort to mimic the diphosphate nature of lipid A, Lewicky *et al*. synthesized various analogues of **12** and biological evaluation of these compounds showed that **15**, which features an additional carboxylic acid moiety, has a higher potency than **13** and **14**.[53] Another structure-activity relationship study of AGPs showed that the potential of this class of agonists also relies on the length of the secondary acyl chains and the nature of the functional group on the aglycon component.[54,55] CRX-527 (**17**), wherein the L-serinyl carboxylic group mimics the anomeric phosphate of lipid A, was shown to be more potent than CRX-524 (**16**). Whereas CRX-527 (**17**) induces the production of MyD88- and TRIF-dependent cytokines, CRX-547 (**18**), containing a D-serinyl carboxylic group, was shown to be TRIF-selective.[56] TLR4 agonists, such as **19**[57] and **20**[58], with no structural similarity to lipid A also exist, and the latter has been used in vaccine modalities in combination with other TLR ligands.[59,60]

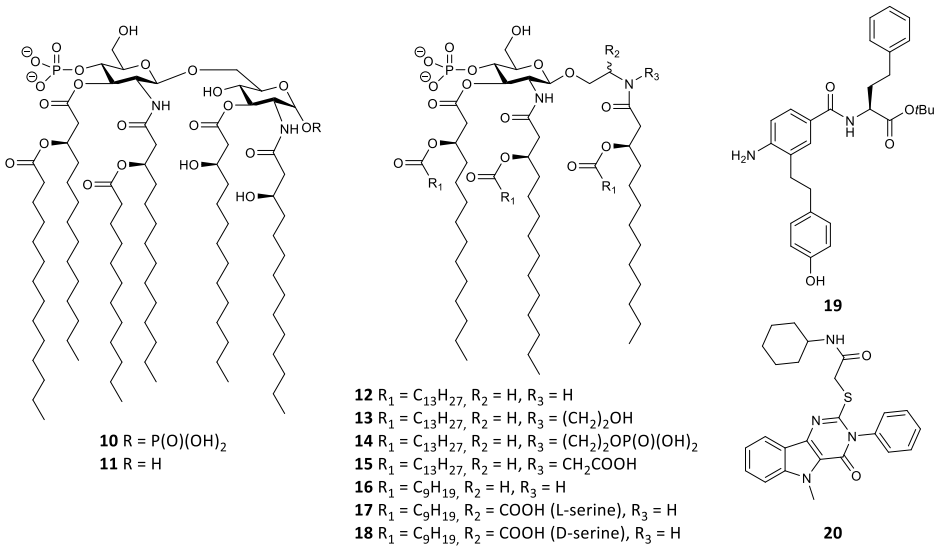
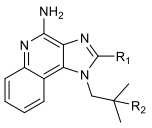


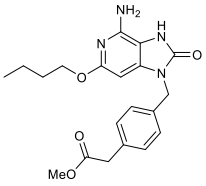
Figure 4. TLR4-ligands **10**-**20**.

TLR7 and TLR8 bind to single stranded viral RNA[61] and they have been the subject of studies to arrive at small molecule agonists (Figure 5).[62] The synthetic ligands for these TLRs are based on imidazoquinolines, for example imiquimod (**21**), resiquimod (**22**), and 8-oxo-adenine derivatives, such as **23** and **24**, and these ligands have been shown to have promising adjuvant properties.[63–65] A cream with imiquimod, suitable for external

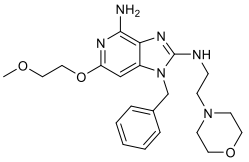
use, was shown to enhance the anti-ovalbumin antibody response in mice 100-fold compared to mice that were not given the adjuvant.[66] Bacterial DNA and synthetic oligodeoxynucleotides with an unmethylated CpG motif (CpG ODNs) are able to trigger an immune response via TLR9.[67] Vaccines containing the synthetic CpG adjuvant have been tested in preclinical studies and have shown to enhance both humoral and cellular immune responses.[68] Moreover, simultaneous administration of this adjuvant and antigens proved to be crucial to obtain significantly enhanced antibody response in a hepatitis B vaccine.[69] Phase II trials with A15 (a mixture of MPL, QS-21 and CpG 7909) have demonstrated to be a promising in the treatment of MAGE-A3 melanoma.[70,71]



21 R_1 = H, R_2 = H
22 R_1 = $CH_2O(CH_2)_2$, R_2 = OH



23

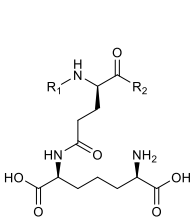


24

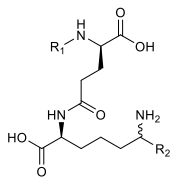
Figure 5. TLR7/8-ligands **21**-**24**.

NOD1 and NOD2, the founding members of the NOD-like receptors (NLRs) family, are able to recognize components of the bacterial peptidoglycan (PG). D-Glutamyl-meso-diaminopimelic acid, *i*E-DAP (**25**, Figure 6), has been found to be the minimal structure required for interaction with NOD1.[72] Several structure-activity relationship studies have been performed to determine what modifications on **25** are tolerated and what has to be done to increase the potency of **25**, as NOD1 ligands are generally relatively poor immune stimulatory agents. It was found that elongation with L-Ala (**26**) increased the activity of the ligand, whereas replacing glutamic acid with glutamine (**27** and **28**) decreased the NOD1 activity.[73,74] Masumoto *et al*. showed that increasing the lipophilicity of the ligand improved the immune response as the induced NOD1-dependent NF-κB activation was several 100-folds higher for **29** and **30** compared to **25**.[73] Substitution of the meso-diaminopimelic acid component of **31** with for example L-serine (**32**) was found to reduce the NOD1-agonistic activity.[75] The minimal structure that is capable of triggering NOD2 activation is muramyl dipeptide (MDP, **33**). Several groups have investigated the influence of modification at the 2-amine of MDP and a glycolylated MDP, for example **34**, was demonstrated to be more potent than **33**, containing an *N*-acetyl group.[76–78] MDP **34** has also been shown to be more efficacious in the induction of an ovalbumin specific T cell response.[76] Increasing the lipophilicity of MDP by monoacylation at the 6-*O* position with decanoic acid (**35**) or stearic acid (**36**)

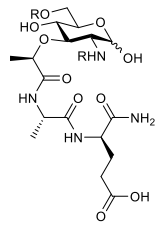
increased the activity of the ligand, while 4,6-diacetylation did not. Notably, the activity of these MDP derivatives was originally shown to originate from TLR2 and TLR4 activation rather than interaction with NOD2. Willems *et al*. have recently shown that lipophilic MDP derivatives can also act in a TLR2-independent manner.[79,80] Conjugation of MDP via its 6-*O* position with TLR2-ligand Pam_2Cys, led to dual adjuvant **37**, which enhanced the immune response compared to a mixture of the separate ligands and co-administration of **37** and a model antigen led to the induction of high antigen-specific IgA and IgG titers.[81] Mifamurtide (**38**), another conjugate between MDP and Pam_3Cys, was found to be effective against osteosarcoma and has been approved as a drug against bone cancer.[82]



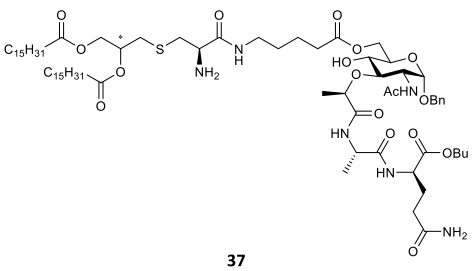
25 R_1 = H, R_2 = OH
26 R_1 = H, R_2 = OH
27 R_1 = L-Ala, R_2 = NH_2
28 R_1 = L-Ala, R_2 = NH_2



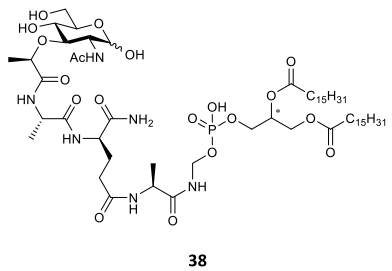
29 R_1 = $C(O)C_{14}H_{29}$, R_2 = COOH
30 R_1 = $C(O)C_{15}H_{31}$, R_2 = COOH
31 R_1 = $C(O)C_{11}H_{23}$, R_2 = COOH
32 R_1 = $C(O)C_{11}H_{23}$, R_2 = H



33 R_1 = $C(O)CH_3$, R_2 = H
34 R_1 = $C(O)CH_2OH$, R_2 = H
35 R_1 = $C(O)CH_3$, R_2 = $C(O)C_9H_{19}$
36 R_1 = $C(O)CH_3$, R_2 = $C(O)C_{17}H_{35}$



37



38

Figure 6. NOD1- and NOD2-ligands **25**-**38**.

Synergy

During an infection, a broad range of PRR-ligands are presented to the immune system which are recognized by the diverse PRRs. Immunization with different synthetic PRR-ligands can act synergistically resulting in an enhanced immunostimulatory effect and the synergistic activity of different PRR-ligands has therefore been the subject of many studies.[60,83–87] Tada *et al*. combined synthetic ligands for NOD1 and NOD2 with TLR2, TLR3, TLR4, and TLR9 ligands and found that the combination of lipid A with either MDP or FK565, an *i*E-DAP analogue, enhanced the immune response more than 1000-fold than each separate stimulant.[85] Co-stimulation with **1** (TLR2-ligand) and **31** (NOD1-

ligand) was shown to enhance the proliferation, expansion, and effector function of T cells.[88] Conjugation of two or more PRR-ligands has also been investigated, besides the previously mentioned combinations of NOD2 and TLR2 (**37** and **38**), MDP has been conjugated to lipid A analogues.[81,89,90] Synergistic effects can also become problematic and the combination of LPS and MDP has led to lethal outcomes in mice.[91]

Conjugation of antigens and immunostimulants

Our understanding of the innate and the adaptive immune system continuously grows, enabling the development of anti-cancer vaccines with enhanced immunological properties. While the administration of a mix of anti-tumor antigens and immunostimulants have led to promising results, the immunogenicity of a vaccine can even be further enhanced by the conjugation of the antigens to an immunostimulant. These "self-adjuvanting" vaccines ensure the simultaneous delivery of both components to APCs, such as DCs, thereby inducing a stronger humoral and cellular immune response.[23,92,93] As a result, the required dose can be lowered which reduces the chance of possible (toxic) side effects. The following sections of this Chapter describe a number of selected conjugates targeting APCs with the goal to either up-regulate the production of cytokines and co-stimulatory molecules or to increase the uptake of the antigens.

TLR2 based conjugates

Due to its synthetic ease and commercial availability, TLR2-ligands have been used in peptide-conjugates since 1989. Immunological evaluation of conjugate **39** (Figure 7) demonstrated for the first time the possibility of inducing an influenza virus-specific CTL response in mice.[94] This set the stage for the conjugation of other Pam_3C analogues such as Pam_3CSK_4 (**1**), which was conjugated to antigenic peptides derived from ovalbumin containing the MHC-I (SIINFEKL) and MHC-II (ISQAVHAAHAEINEAGR) epitopes in several studies.[95–97] First, Khan *et al*. synthesized conjugate **40** via solid phase peptide synthesis (SPPS) using FMOC/HCTU chemistry.[95] The conjugate induced an enhanced T cell specific response due to improved antigen presentation and DC maturation. It was also discovered that the uptake of the antigens occurred independently of TLR expression. Next, Khan *et al*. investigated the immunological behavior of the two Pam_3C diastereoisomers.[96] The IL-12 production *in vitro* was significantly higher for the *R*-conjugate **41** as compared to that of the *S*-isomer **42** indicating that the *R*-isomer induces better activation of DCs, which confirms previous studies on the chirality of Pam_3C.[98,99] However, the racemic mixture **43** was shown to be as potent as **41** in the production of IL-12 and it demonstrated an enhanced CTL

response *in vivo*. Next, the TLR2-ligand peptide conjugates **44** and **45**, in which **1** was conjugated to Th epitopes derived from ovalbumin (**44**) and the Moloney virus envelope (**45**) were studied.[97] *In vivo* experiments with **43**-**45** show enhanced CTL and Th cell responses and more efficient anti-tumor immunity when the TLR2-ligand is covalently bound to the antigen in comparison to a mixture of the ligand and the corresponding antigen. These results demonstrate the potential of PRR-ligand-peptide conjugates as both CTL and Th cell priming are necessary for cancer immunotherapy. These finding were therefore exploited in the GMP synthesis of **46** and **47**, which are used for vaccination against human papillomavirus (HPV) type 16. Herein, UPam[33], an improved Pam_3C analogue, was conjugated at the *N*-terminus of the antigenic peptide via SPPS.[100] The conjugated synthetic long peptides (SLPs) were shown to be efficiently processed by APCs and significantly enhanced the *ex vivo* stimulation of lymph node-derived T cells. The Pam_3C conjugates (**43**, **44**, **48**) were compared with the corresponding UPam analogues (**49**-**51**) to show that the latter are more potent *in vitro* and *in vivo*. In combination with photodynamic therapy tumor eradication can be induced with these conjugates.[101] Besides antigenic peptides, TLR2-ligands have also been conjugated to carbohydrate antigens, TACAs.[102] Several groups have reported the synthesis of a multicomponent vaccine, in which a TLR2-ligand is covalently bound to a B cell epitope and/or a T cell epitope.[103–108] In 2005, the group of Boons used this strategy for the development of a three component vaccine, wherein Pam_3C is conjugated to a Th epitope (YAF) and a tumor-associated Tn-antigen.[109] The latter is a B cell epitope that is overexpressed on the surface of human cancer cells. The conjugate suffered from poor solubility and conjugate **52** elicited only low titers of IgG antibodies. An improvement of this conjugate was made by replacing Pam_3C by the more potent Pam_3CSK_4 (**53**).[110] Conjugation of this ligand to a helper T cell epitope (KLFAVWKITYKDT) derived from poliovirus and a MUC1 glycopeptide B cell epitope elicited excellent high titers of IgG antibodies in mice, while its Pam_2CSK_4 analogue (**54**) gave a low immune response. The group of Kunz investigated the impact of multivalent glycopeptide antigens on the immunogenicity of a vaccine. To this end, Pam_3CSK_4 was either conjugated via SPPS or click chemistry to one, two, or four MUC1 glycopeptides containing either two Tn antigens or one STn antigen (**55**-**60**).[111] Immunological evaluation showed **60** to be a promising vaccine modality as it induced efficient killing of tumor cells.[112]

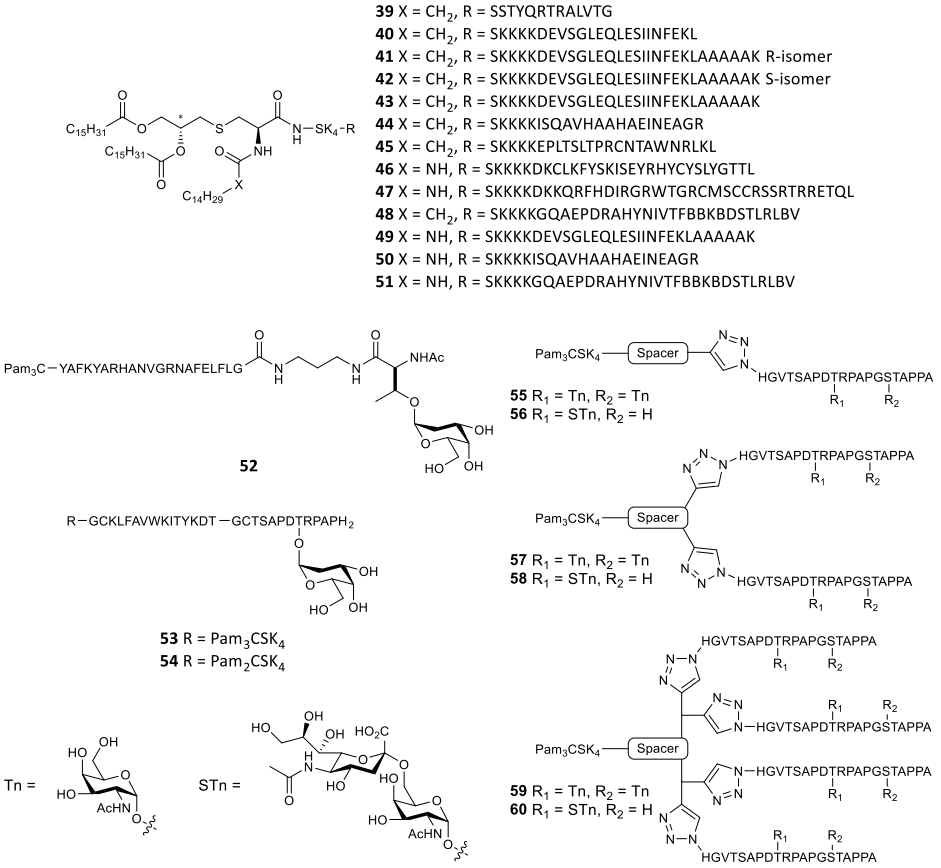


Figure 7. TLR2-conjugates **39**-**60**.

TLR4 based conjugates

Although many groups have studied the adjuvanting effect of TLR4-ligands, conjugates with a TLR4-ligand are scarce, probably because these glycolipids are not only difficult to synthesize but also the coupling to an antigen is challenging. Up to now, there is only one example of a conjugate between a protein and TLR4-ligand.[113] Schülke *et al*. coupled the detoxified TLR4-ligand, MPLA (**11**), to ovalbumin via a carbamate linkage and the resulting conjugate induced a stronger immune response compared to a mixture of unconjugated **11** and ovalbumin. Although TLR4-ligands peptide-conjugate have not been reported (See Chapter 2 of this Thesis), they have been conjugated to TACAs.[114,115] Ziaco *et al*. developed a semisynthetic strategy to obtain MPLA derivatives equipped with clickable conjugation handles (Figure 8).[116] Herein, **11** was obtained via a semisynthetic method and then subjected to a regioselective oxidation of the primary

alcohol. The carboxylic acid could then be used to install the linker. To demonstrate the potential of the clickable MPLA, they synthesized two conjugates with a Tn (**61**) and a TF antigen (**62**). Preliminary evaluation showed that the conjugates could successfully induce cytokine production. The group of Guo developed a synthetic approach to conjugate MPLA to TACAs via the anomeric position of the reducing end sugar.[117] To this end, MPLA from *Neisseria meningitidis* was elongated with a small linker and coupled to GM3 or a GM3 derivative yielding conjugate **63** and **64** respectively. Due to solubility issues, pure conjugates **63** and **64** could not be used for immunization of the mice and therefore liposomes containing the conjugates were prepared. Immunological studies showed that **64** elicited a strong immune response with the total antibody titer four times higher than that of **63**. In a follow-up study, they synthesized conjugates **65**-**68** in which MPLA is coupled via the same strategy as before to sTnNPhAc, a modified TACA.[118] All four conjugates were incorporated in liposomes and these provided a similar immune response pattern. In the series **66** was shown to be the best vaccine as it elicited the highest response and was more consistent in the production of the total amount of antibodies. Conjugate **65** was significantly less potent than **66** and **67**, demonstrating that the hydroxyl functions on the lipids play an important role in receptor binding. On the other hand, the length of the lipids and the incorporation of an additional lipid chain had a relatively small impact. Conjugation of MPLA to a Globo H antigen (**69**) provided a vaccine modality that not only induced more IgG antibodies than the corresponding KLH conjugate, it also resulted in a faster immune response.[119]

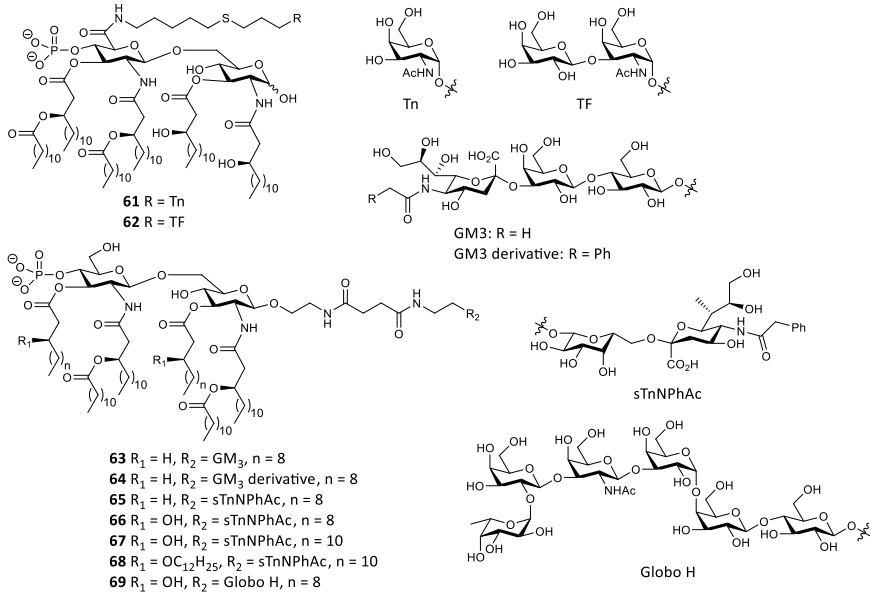
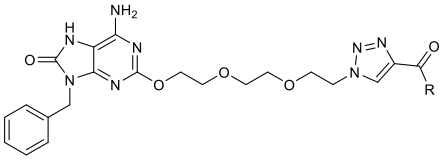


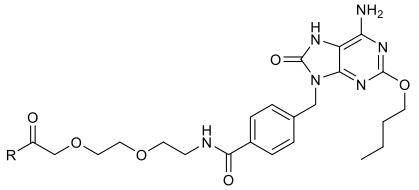
Figure 8. TLR4-conjugates **61**-**69**.

TLR7/8 based conjugates

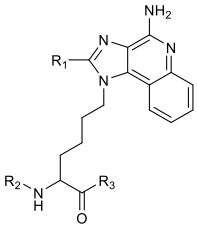
Agonists for TLR7 and TLR8 have received considerable attention in conjugation chemistry because their chemical structure presents multiple sites for functionalization. Besides their use in a mixture with a protein or conjugated to proteins[120–122], they have also been covalently linked to antigenic peptides. Weterings *et al*. combined SPPS and Cu(I) catalyzed Huisgen cycloaddition for the conjugation of 2-alkoxy-8-hydroxy adenine (TLR7-ligand) to ovalbumin-derived peptides DEVSGLEQLESIINFEKL and DEVSGLEQLESIINFEKLAAAAAK, that both contain the MHC-I epitope SIINFEKL.[123] Although improved antigen presentation was detected after stimulation of DCs with conjugates **70**-**73** (Figure 9), the conjugates lacked the ability to activate DCs as almost no IL-12 was produced. These results show how important the right conjugation site of an agonist can be, as conjugation via the benzyl moiety did result in DC maturation.[121] These findings led to the design of a TLR7-ligand, extended on the benzyl moiety, and its application in conjugates **74**-**76**, that were able to induce DC maturation.[124] T cell proliferation experiments not only showed that the conjugates perform better than a mixture of peptide and TLR7-ligand, but also that *N*-terminus conjugates (**74** and **75**) perform better than the *C*-terminus conjugate (**76**). The group of Taguchi synthesized a series of synthetic TLR7-ligand amino acids containing the imidazoquinolyl structure (**77**-**81**) of which **81** was shown to be the most potent agonist.[125] Ligand **81** was therefore selected for conjugation to either the *N*-terminus (**82**), the *C*-terminus (**83**) or both (**84**) of a peptide derived from the influenza A virus M2 protein. Immunological evaluation showed that the obtained conjugates of **81** exhibit poor adjuvanting properties.



70 R = DEVSGLEQLESIINFEKL
71 R = DEVSGLEQLESIINFEKLAAAAAK (N-terminus)
73 R = DEVSGLEQLESIINFEKLAAAAAK (C-terminus



74 R = DEVSGLEQLESIINFEKL
75 R = DEVSGLEQLESIINFEKLAAAAAK (N-terminus)
76 R = DEVSGLEQLESIINFEKLAAAAAK (C-terminus



77 R_1 = H, R_2 = H, R_3 = OH
78 R_1 = CH_3, R_2 = H, R_3 = OH
79 R_1 = C_2H_5, R_2 = H, R_3 = OH
80 R_1 = C_3H_7, R_2 = H, R_3 = OH
81 R_1 = C_4H_9, R_2 = H, R_3 = OH
82 R_1 = C_4H_9, R_2 = H, R_3 = SLP
83 R_1 = C_4H_9, R_2 = SLP, R_3 = H
84 R_1 = C_4H_9, R_2 = SLP, R_3 = SLP

SLP = SLLTEVETPIRNEWGCRCNDSSD

Figure 9. TLR7/8-conjugates **70**-**84**.

TLR9 based conjugates

Conjugation of CpG oligonucleotides (ODN) to protein antigens has been well-studied and these conjugates have been shown to enhance the immunogenicity of the antigens.[68,120,126–128] The group of Diamond synthesized a library of conjugates, combining CpG with several minimal CTL and Th epitopes.[129] These TLR9-mediated self-adjuvanting vaccines were superior in cytokine production and protection against viral infection compared to non-covalently linked mixtures of the corresponding molecules. Khan *et al*. conjugated CpG to antigenic peptides comprising the MHC-I epitope SIINFEKL and compared the resulting conjugates **85** and **86** to conjugates containing the non-stimulatory oligonucleotide GpC, **87** and **88** (Figure 10).[95] While the SIINFEKL-specific T cell response of the GpC-conjugates **87** and **88** was equal to that induced by a mixture of peptide and adjuvant, the response obtained with **85** and **86** was significantly higher, showing that the T cell response depended on the activation of DCs. A three-component vaccine containing the TLR9-ligand CpG was made by the group of Boons. Herein, CpG was conjugated to a Th epitope and a MUC1 peptide, serving as a B cell epitope. Immunization with conjugates **89** and **90** did not result in significant improvement in anticancer properties, while its Pam_3CSK_4-analogue did, which demonstrates that the choice of build-in adjuvant can be important for the efficiency of a vaccine.

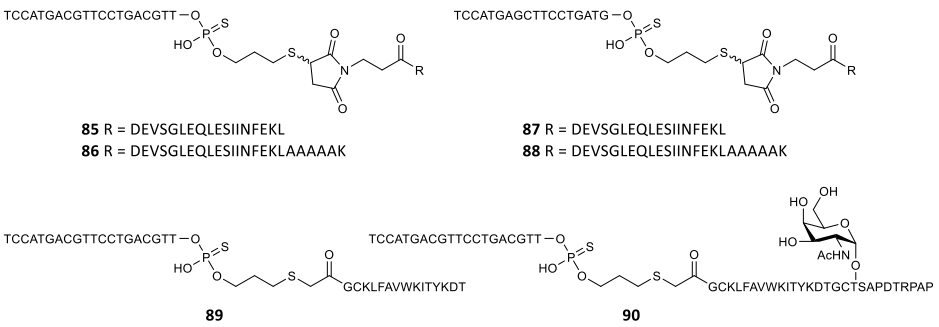


Figure 10. TLR9-conjugates **85**-**90**.

NOD2 based conjugates

In comparison to the TLR-family, NLR-ligands have been rarely used in a synthetic conjugation approach. Carelli *et al*. have coupled MDP to peptide fragments derived from the luteinizing hormone-releasing hormone (LH-RH). Besides the fact that these conjugates can potentially be used in the veterinary field for castration, they can also be exploited for the treatment of LH-RH dependent tumors.[130,131] In a three-component vaccine MDP was covalently bound to a B cell epitope derived from a growth hormone

and a T cell epitope derived from ovalbumin.[132] This conjugate was shown to produce high titers of antibody and an increased body weight for the immunized rats. Willems *et al*. reported the synthesis of a MDP building block, which is suitable for SPPS, and they generated conjugates, in which MDP was covalently connected to an ovalbumin derived model peptide containing the MHC-I epitope SIINFEKL.[133] Several conjugation sites were investigated by coupling MDP via the dipeptide to the *N*- or the *C*-terminus of the peptide giving **91** and **92** (Figure 11). Alternatively, the linker on the anomeric position of *N*-acetylglucosamine (E-MDP) was connected to the *N*- or the *C*-terminus of the same peptide giving **93** and **94**. According to the level of IL-12 production, MDP is a poor immunostimulator, while the antigen presentation induced by the conjugates was comparable to that induced by the Pam_3CSK_4 conjugate **43**. In a follow-up study[134], the synergistic acting of NOD2 and TLR2 was exploited by the assembly of bis-conjugates (**95**-**98**) containing MDP and the TLR2-ligand, Pam_3CSK_4.[85] Although all conjugates showed a strong IL-12 production, conjugate **96** proved to be the most potent in activating DCs. The latter conjugate also induced an enhanced CTL priming compared to the mono-conjugates containing either MDP or Pam_3CSK_4 indicating that these bis-conjugates could be of use for the treatment of virus infections or cancer.

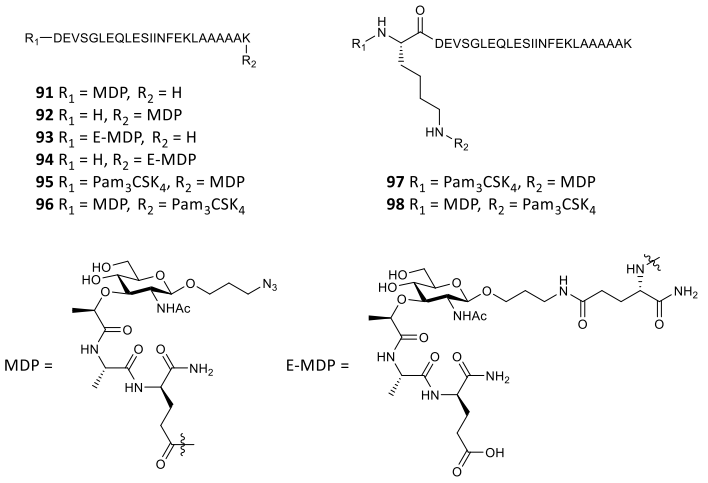


Figure 11. NOD2-conjugates **91**-**98**.

Antibody-recruiting molecules-based conjugates

Fc receptors (FcRs) on APCs are able to enhance the uptake of immune complexes, formed by antibody-recruiting molecules (ARM) bound to their designated antibody. The FcR-mediated uptake can also induce the production of cytokines resulting in enhanced processing and presentation of the antigens leading to an effective immune

response[135] and therefore ARM-conjugates have been investigated as a strategy to improve vaccines.[25] Several ARMs have been studied in vaccine formulations. Besides targeting APCs, ARM-conjugates can also be used to target tumor cells and recruit antibodies resulting in a localized cytotoxic immune response.[136–139] The group of Spiegel synthesized several bifunctional linkers (**99**-**101**, Figure 12), wherein 2,4-dinitroaniline was conjugated on one side and a target-binding molecule on the other side and could be used to either induce phagocytosis of fungi, the inactivation of HIV virus or the destruction of cancer cells.[140–142] Another ARM-strategy is based on the fact that virtually almost all people have endogenous antibodies against tetanus toxoid.[143,144] Thus, the B cell epitope of tetanus toxin, FIGITELKKLESKINKVF as part of a longer peptide, was conjugated via thiol-maleimide chemistry to SLPs containing CTL epitopes derived from either ovalbumin (**102**), cytomegalovirus (**103**) or influenza virus (**104**). The conjugates were able to induce DC and T cell activation as a result of improved antigen uptake. Anti-α-Gal antibodies represent 1-3% of all immunoglobulins and are produced by about 1% of all B cells, and these have also been explored in vaccines against HIV, lymphoma cells and influenza virus. The conjugation of α-Gal epitope (**105**) to either an HIV gp-peptide, tumor-specific antibodies or PR8 derived peptides was shown to enhance the immunogenicity of the vaccines.[145–149] One of the disadvantages of the α-Gal epitope in model vaccination studies is the need to use expensive KO mice. Chen *et al*. have demonstrated that L-rhamnose monosaccharides can be a good alternative since anti-L-rhamnose antibodies are not only one of the most abundant antibodies in humans[150,151], and wild-type mice can be used instead of KO mice.[152] Several studies exploited rhamnose-functionalized proteins[153] and liposomes[154–156] for cancer immunotherapy. Sarkar *et al*. synthesized a three-component vaccine consisting of rhamnose as ARM molecule, a Th cell epitope (YAF) and a tumor-specific antigen (Tn) using SPPS.[157] A T cell proliferation study showed that conjugate **106** was as active as conjugate **107** when using a 10-fold lower concentration, demonstrating that the addition of rhamnose-monosaccharide results in a better internalization, processing and presentation of the epitope.[157]

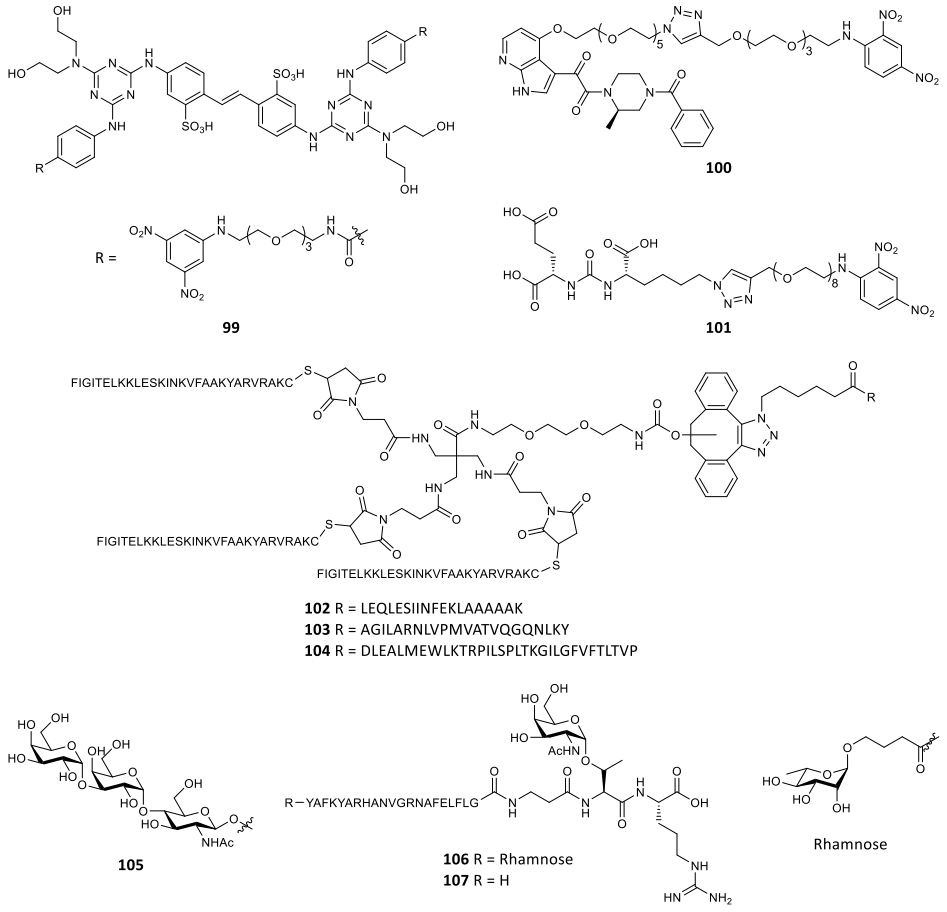


Figure 12. ARM-based conjugates **99**-**107**.

Outline of this thesis

The research described in this Thesis aims at the development of cancer vaccines by the design and synthesis of conjugates in which structurally defined adjuvants are covalently attached to antigenic peptides. **Chapter 1** has provided a concise overview of selected well-defined synthetic ligands that have been used as immunostimulants in vaccine formulations. **Chapter 2** describes the design, synthesis and immunological evaluation of four TLR4-ligands and four TLR4-ligand antigen conjugates. A new synthesis route towards (R)-3-alkyloxytetradecanoic acid is presented together with the optimized synthesis of the monophosphoryl lipid A analogue, CRX-527, in which the key step is the introduction of the lipid tails. Two different linkers between the CRX-527 ligand and an antigenic peptide are investigated and the CRX-527 ligand has been conjugated to either the *N*- or the *C*-terminus of an antigenic peptide via thiol-

maleimide-chemistry. The *in vitro* studies of the resulting ligands and "self-adjuvanting" conjugates showed that the choice of spacer type is critical to obtaining a proper immune response. The preparation of four bis-conjugates containing a NOD2-ligand and a TLR2-ligand as well as four mono-conjugates with only a NOD2-ligand is the subject of **Chapter 3**. Herein, two types of NOD2-ligands featuring either an *O*- or a *C*-MDP-moeity, with either an *N*-acetyl or an *N*-glycolyl substituent have been prepared. The *O*-MDP contains an azidopropanol spacer at the anomeric position of the glucosamine and it was covalently bound via its isoglutamic acid moiety to an antigenic peptide derived from the human papilomavirus using SPPS chemistry. The *C*-MDP derivatives were conjugated via the anomeric center of the glucosamine to the peptide using an online SPPS approach. **Chapter 4** covers the use of the mannose-6-phosphate receptor that could mediate a more efficient delivery of conjugates to the endosome to improve the immune response. To this end, two types of mannose-6-phosphonates building blocks, an *O*-analogue and a *C*-analogue, have been synthesized and conjugated to either a CTL or to a Th epitope using Cu(I) catalyzed 1,3-dipolar cycloaddition or SPPS chemistry. **Chapter 5** describes the synthesis of two *C*-rhamnose-lysine building blocks, which are suitable for SPPS chemistry. One, two, three or six *C*-rhamnose-functionalized lysines were linked at the *N*-terminus end of an antigenic peptide to investigate the multivalent effect on the binding to anti-rhamnose antibodies to obtain an improved vaccine based on the ARM-strategy. **Chapter 6** describes the synthesis of four different *C*-glycosyl functionalized lysines, the glycosidic linkage of which are stable against the acidic conditions used in SPPS. The building blocks were equipped with protecting groups that could be removed under acidic conditions, concomitantly with the cleavage of the synthesized peptides form the resin. In **Chapter 7**, the research of this Thesis is summarized and some future prospects are presented.

References

(1) Vermaelen, K. *Front. Immunol.* **2019**, *10*.
(2) Schumacher, T. N.; Schreiber, R. D. *Science.* **2015**, *348* (6230), 69–74.
(3) Heimburg-Molinaro, J.; Lum, M.; Vijay, G.; Jain, M.; Almogren, A.; Rittenhouse-Olson, K. *Vaccine* **2011**, *29* (48), 8802–8826.
(4) Kapsenberg, M. L. *Nat. Rev. Immunol.* **2003**, *3* (12), 984–993.
(5) Goral, S. *Dial. Transplant.* **2011**, *40* (1), 14–16.
(6) Schwartz, R. H. *Annu. Rev. Immunol.* **2003**, *21* (1), 305–334.
(7) Toes, R. E.; Blom, R. J.; Offringa, R.; Kast, W. M.; Melief, C. J. *J. Immunol.* **1996**, *156* (10), 3911–3918.
(8) Broz, P.; Monack, D. M. *Nat. Rev. Immunol.* **2013**, *13* (8), 551–565.
(9) Brubaker, S. W.; Bonham, K. S.; Zanoni, I.; Kagan, J. C. *Annu. Rev. Immunol.* **2015**, *33* (1), 257–290.
(10) Kawai, T.; Akira, S. *Nat. Immunol.* **2010**, *11* (5), 373–384.
(11) Hennessy, E. J.; Parker, A. E.; O'Neill, L. A. J. *Nat. Rev. Drug Discov.* **2010**, *9* (4), 293–307.
(12) Garaude, J.; Kent, A.; van Rooijen, N.; Blander, J. M. *Sci. Transl. Med.* **2012**, *4* (120), 120ra16.
(13) Osorio, F.; Reis e Sousa, C. *Immunity* **2011**, *34* (5), 651–664.

(14) van Dinther, D.; Stolk, D. A.; van de Ven, R.; van Kooyk, Y.; de Gruijl, T. D.; den Haan, J. M. M. *J. Leukoc. Biol.* **2017**, *102* (4), 1017–1034.
(15) Chan, Y. K.; Gack, M. U. *Curr. Opin. Virol.* **2015**, *12*, 7–14.
(16) Takai, T. *Nat. Rev. Immunol.* **2002**, *2* (8), 580–592.
(17) Baker, K.; Rath, T.; Pyzik, M.; Blumberg, R. S. *Front. Immunol.* **2014**, *5*.
(18) Zwaveling, S.; Mota, S. C. F.; Nouta, J.; Johnson, M.; Lipford, G. B.; Offringa, R.; van der Burg, S. H.; Melief, C. J. M. *J. Immunol.* **2002**, *169* (1), 350–358.
(19) Skwarczynski, M.; Toth, I. *Chem. Sci.* **2016**, *7* (2), 842–854.
(20) Reed, S. G.; Orr, M. T.; Fox, C. B. *Nat. Med.* **2013**, *19* (12), 1597–1608.
(21) Temizoz, B.; Kuroda, E.; Ishii, K. J. *Int. Immunol.* **2016**, *28* (7), 329–338.
(22) Kool, M.; Fierens, K.; Lambrecht, B. N. *J. Med. Microbiol.* **2012**, *61* (Pt 7), 927–934.
(23) Zom, G. G. P.; Khan, S.; Filippov, D. V.; Ossendorp, F. In *Advances in Immunology*; **2012**; pp 177–201.
(24) Liu, H.; Irvine, D. J. *Bioconjug. Chem.* **2015**, *26* (5), 791–801.
(25) McEnaney, P. J.; Parker, C. G.; Zhang, A. X.; Spiegel, D. A. *ACS Chem. Biol.* **2012**, *7* (7), 1139–1151.
(26) Jin, M. S.; Kim, S. E.; Heo, J. Y.; Lee, M. E.; Kim, H. M.; Paik, S.-G.; Lee, H.; Lee, J.-O. *Cell* **2007**, *130* (6), 1071–1082.
(27) Kang, J. Y.; Nan, X.; Jin, M. S.; Youn, S.-J.; Ryu, Y. H.; Mah, S.; Han, S. H.; Lee, H.; Paik, S.-G.; Lee, J.-O. *Immunity* **2009**, *31* (6), 873–884.
(28) Okusawa, T.; Fujita, M.; Nakamura, J. -i.; Into, T.; Yasuda, M.; Yoshimura, A.; Hara, Y.; Hasebe, A.; Golenbock, D. T.; Morita, M.; *et al*. *Infect. Immun.* **2004**, *72* (3), 1657–1665.
(29) Cataldi, A.; Yevsa, T.; Vilte, D. A.; Schulze, K.; Castro-Parodi, M.; Larzábal, M.; Ibarra, C.; Mercado, E. C.; Guzmán, C. A. *Vaccine* **2008**, *26* (44), 5662–5667.
(30) Takeuchi, O.; Kaufmann, A.; Grote, K.; Kawai, T.; Hoshino, K.; Morr, M.; Muhlradt, P. F.; Akira, S. *J. Immunol.* **2000**, *164* (2), 554–557.
(31) Mühlradt, P. F.; Kiess, M.; Meyer, H.; Süssmuth, R.; Jung, G. *Infect. Immun.* **1998**, *66* (10), 4804–4810.
(32) Zhang, Y.; Luo, F.; Cai, Y.; Liu, N.; Wang, L.; Xu, D.; Chu, Y. *J. Immunol.* **2011**, *186* (4), 1963–1969.
(33) Willems, M. M. J. H. P.; Zom, G. G.; Khan, S.; Meeuwenoord, N.; Melief, C. J. M.; van der Stelt, M.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; *et al*. *J. Med. Chem.* **2014**, *57* (15), 6873–6878.
(34) Müller, S. D. .; Müller, M. R.; Huber, M.; Esche, U. v. .; Kirschning, C. J.; Wagner, H.; Bessler, W. G.; Mittenbühler, K. *Int. Immunopharmacol.* **2004**, *4* (10–11), 1287–1300.
(35) Buwitt-Beckmann, U.; Heine, H.; Wiesmuller, K.-H.; Jung, G.; Brock, R.; Ulmer, A. J. *FEBS J.* **2005**, *272* (24), 6354–6364.
(36) Spohn, R.; Buwitt-Beckmann, U.; Brock, R.; Jung, G.; Ulmer, A. J.; Wiesmüller, K.-H. *Vaccine* **2004**, *22* (19), 2494–2499.
(37) Guo, X.; Wu, N.; Shang, Y.; Liu, X.; Wu, T.; Zhou, Y.; Liu, X.; Huang, J.; Liao, X.; Wu, L. *Front. Immunol.* **2017**, *8*.
(38) Du, X.; Qian, J.; Wang, Y.; Zhang, M.; Chu, Y.; Li, Y. *Bioorg. Med. Chem.* **2019**.
(39) Agnihotri, G.; Crall, B. M.; Lewis, T. C.; Day, T. P.; Balakrishna, R.; Warshakoon, H. J.; Malladi, S. S.; David, S. A. *J. Med. Chem.* **2011**, *54* (23), 8148–8160.
(40) Salunke, D. B.; Connelly, S. W.; Shukla, N. M.; Hermanson, A. R.; Fox, L. M.; David, S. A. *J. Med. Chem.* **2013**, *56* (14), 5885–5900.
(41) Shimazu, R.; Akashi, S.; Ogata, H.; Nagai, Y.; Fukudome, K.; Miyake, K.; Kimoto, M. *J. Exp. Med.* **1999**, *189* (11), 1777–1782.
(42) Park, B. S.; Lee, J.-O. *Exp. Mol. Med.* **2013**, *45* (12), e66–e66.
(43) Molinaro, A.; Holst, O.; Di Lorenzo, F.; Callaghan, M.; Nurisso, A.; D'Errico, G.; Zamyatina, A.; Peri, F.; Berisio, R.; Jerala, R.; *et al*. *Chem. - A Eur. J.* **2015**, *21* (2), 500–519.
(44) Zamyatina, A. *Beilstein J. Org. Chem.* **2018**, *14*, 25–53.
(45) Gao, J.; Guo, Z. *Med. Res. Rev.* **2018**, *38* (2), 556–601.
(46) Qureshi, N.; Takayama, K.; Ribi, E. *J. Biol. Chem.* **1982**, *257* (19), 11808–11815.
(47) Behzad, H.; Huckriede, A. L. W.; Haynes, L.; Gentleman, B.; Coyle, K.; Wilschut, J. C.; Kollmann, T. R.; Reed, S. G.; McElhaney, J. E. *J. Infect. Dis.* **2012**, *205* (3), 466–473.
(48) Beran, J. *Expert Opin. Biol. Ther.* **2008**, *8* (2), 235–247.
(49) Schwarz, T. F.; Spaczynski, M.; Schneider, A.; Wysocki, J.; Galaj, A.; Schulze, K.; Poncelet, S. M.;

Catteau, G.; Thomas, F.; Descamps, D. *Hum. Vaccin.* **2011**, *7* (9), 958–965.
(50) Garçon, N.; Di Pasquale, A. *Hum. Vaccin. Immunother.* **2017**, *13* (1), 19–33.
(51) Johnson, D. A.; Gregory Sowell, C.; Johnson, C. L.; Livesay, M. T.; Keegan, D. S.; Rhodes, M. J.; Terry Ulrich, J.; Ward, J. R.; Cantrell, J. L.; Brookshire, V. G. *Bioorg. Med. Chem. Lett.* **1999**, *9* (15), 2273–2278.
(52) Persing, D. H.; Coler, R. N.; Lacy, M. J.; Johnson, D. A.; Baldridge, J. R.; Hershberg, R. M.; Reed, S. G. *Trends Microbiol.* **2002**, *10* (10 Suppl), S32-7.
(53) Lewicky, J. D.; Ulanova, M.; Jiang, Z.-H. *Bioorg. Med. Chem.* **2013**, *21* (8), 2199–2209.
(54) Stöver, A. G.; Da Silva Correia, J.; Evans, J. T.; Cluff, C. W.; Elliott, M. W.; Jeffery, E. W.; Johnson, D. A.; Lacy, M. J.; Baldridge, J. R.; Probst, P.; *et al*. *J. Biol. Chem.* **2004**, *279* (6), 4440–4449.
(55) Cluff, C. W.; Baldridge, J. R.; Stover, A. G.; Evans, J. T.; Johnson, D. A.; Lacy, M. J.; Clawson, V. G.; Yorgensen, V. M.; Johnson, C. L.; Livesay, M. T.; *et al*. *Infect. Immun.* **2005**, *73* (5), 3044–3052.
(56) Bowen, W. S.; Minns, L. A.; Johnson, D. A.; Mitchell, T. C.; Hutton, M. M.; Evans, J. T. *Sci. Signal.* **2012**, *5* (211), ra13.
(57) Wang, Y.; Su, L.; Morin, M. D.; Jones, B. T.; Whitby, L. R.; Surakattula, M. M. R. P.; Huang, H.; Shi, H.; Choi, J. H.; Wang, K.; *et al*. *Proc. Natl. Acad. Sci.* **2016**, *113* (7), E884–E893.
(58) Chan, M.; Hayashi, T.; Mathewson, R. D.; Nour, A.; Hayashi, Y.; Yao, S.; Tawatao, R. I.; Crain, B.; Tsigelny, I. F.; Kouznetsova, V. L.; *et al*. *J. Med. Chem.* **2013**, *56* (11), 4206–4223.
(59) Goff, P. H.; Hayashi, T.; Martínez-Gil, L.; Corr, M.; Crain, B.; Yao, S.; Cottam, H. B.; Chan, M.; Ramos, I.; Eggink, D.; *et al*. *J. Virol.* **2015**, *89* (6), 3221–3235.
(60) Tom, J. K.; Dotsey, E. Y.; Wong, H. Y.; Stutts, L.; Moore, T.; Davies, D. H.; Felgner, P. L.; Esser-Kahn, A. P. *ACS Cent. Sci.* **2015**, *1* (8), 439–448.
(61) Heil, F. *Science.* **2004**, *303* (5663), 1526–1529.
(62) Czarniecki, M. *J. Med. Chem.* **2008**, *51* (21), 6621–6626.
(63) Kurimoto, A.; Hashimoto, K.; Nakamura, T.; Norimura, K.; Ogita, H.; Takaku, H.; Bonnert, R.; McInally, T.; Wada, H.; Isobe, Y. *J. Med. Chem.* **2010**, *53* (7), 2964–2972.
(64) Jin, G.; Wu, C. C. N.; Tawatao, R. I.; Chan, M.; Carson, D. A.; Cottam, H. B. *Bioorg. Med. Chem. Lett.* **2006**, *16* (17), 4559–4563.
(65) Weterings, J. J.; Khan, S.; van der Heden van Noort, G. J.; Melief, C. J. M.; Overkleeft, H. S.; van der Burg, S. H.; Ossendorp, F.; van der Marel, G. A.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2009**, *19* (8), 2249–2251.
(66) Johnston, D.; Bystryn, J.-C. *Vaccine* **2006**, *24* (11), 1958–1965.
(67) Krieg, A. M. *Annu. Rev. Immunol.* **2002**, *20* (1), 709–760.
(68) Shirota, H.; Klinman, D. M. *Expert Rev. Vaccines* **2014**, *13* (2), 299–312.
(69) Mutwiri, G. K.; Nichani, A. K.; Babiuk, S.; Babiuk, L. A. *J. Control. Release* **2004**, *97* (1), 1–17.
(70) McQuade, J. L.; Homsi, J.; Torres-Cabala, C. A.; Bassett, R.; Popuri, R. M.; James, M. L.; Vence, L. M.; Hwu, W.-J. *BMC Cancer* **2018**, *18* (1), 1274.
(71) Kruit, W. H. J.; Suciu, S.; Dreno, B.; Mortier, L.; Robert, C.; Chiarion-Sileni, V.; Maio, M.; Testori, A.; Dorval, T.; Grob, J.-J.; *et al*. *J. Clin. Oncol.* **2013**, *31* (19), 2413–2420.
(72) Chamaillard, M.; Hashimoto, M.; Horie, Y.; Masumoto, J.; Qiu, S.; Saab, L.; Ogura, Y.; Kawasaki, A.; Fukase, K.; Kusumoto, S.; *et al*. *Nat. Immunol.* **2003**, *4* (7), 702–707.
(73) Masumoto, J.; Yang, K.; Varambally, S.; Hasegawa, M.; Tomlins, S. A.; Qiu, S.; Fujimoto, Y.; Kawasaki, A.; Foster, S. J.; Horie, Y.; *et al*. *J. Exp. Med.* **2006**, *203* (1), 203–213.
(74) Wolfert, M. A.; Roychowdhury, A.; Boons, G.-J. *Infect. Immun.* **2007**, *75* (2), 706–713.
(75) Agnihotri, G.; Ukani, R.; Malladi, S. S.; Warshakoon, H. J.; Balakrishna, R.; Wang, X.; David, S. A. *J. Med. Chem.* **2011**, *54* (5), 1490–1510.
(76) Coulombe, F.; Divangahi, M.; Veyrier, F.; de Léséleuc, L.; Gleason, J. L.; Yang, Y.; Kelliher, M. A.; Pandey, A. K.; Sassetti, C. M.; Reed, M. B.; *et al*. *J. Exp. Med.* **2009**, *206* (8), 1709–1716.
(77) Chen, K.-T.; Huang, D.-Y.; Chiu, C.-H.; Lin, W.-W.; Liang, P.-H.; Cheng, W.-C. *Chem. - A Eur. J.* **2015**, *21* (34), 11984–11988.
(78) Melnyk, J. E.; Mohanan, V.; Schaefer, A. K.; Hou, C.-W.; Grimes, C. L. *J. Am. Chem. Soc.* **2015**, *137* (22), 6987–6990.
(79) Uehori, J.; Fukase, K.; Akazawa, T.; Uematsu, S.; Akira, S.; Funami, K.; Shingai, M.; Matsumoto, M.; Azuma, I.; Toyoshima, K.; *et al*. *J. Immunol.* **2005**, *174* (11), 7096–7103.
(80) Willems, M. M. J. H. P.; Zom, G. G.; Meeuwenoord, N.; Khan, S.; Ossendorp, F.; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V.; Codée, J. D. C. *ChemMedChem* **2016**, *11* (2), 190–198.

(81) Pavot, V.; Rochereau, N.; Rességuier, J.; Gutjahr, A.; Genin, C.; Tiraby, G.; Perouzel, E.; Lioux, T.; Vernejoul, F.; Verrier, B.; *et al*. *J. Immunol.* **2014**, *193* (12), 5781–5785.
(82) Nardin, A.; Lefebvre, M.; Labroquere, K.; Faure, O.; Abastado, J. *Curr. Cancer Drug Targets* **2006**, *6* (2), 123–133.
(83) Kawai, T.; Akira, S. *Immunity* **2011**, *34* (5), 637–650.
(84) Mutwiri, G.; Gerdts, V.; van Drunen Littel-van den Hurk, S.; Auray, G.; Eng, N.; Garlapati, S.; Babiuk, L. A.; Potter, A. *Expert Rev. Vaccines* **2011**, *10* (1), 95–107.
(85) Tada, H.; Aiba, S.; Shibata, K.-I.; Ohteki, T.; Takada, H. *Infect. Immun.* **2005**, *73* (12), 7967–7976.
(86) Uehara, A.; Yang, S.; Fujimoto, Y.; Fukase, K.; Kusumoto, S.; Shibata, K.; Sugawara, S.; Takada, H. *Cell. Microbiol.* **2004**, *7* (1), 53–61.
(87) Tom, J. K.; Albin, T. J.; Manna, S.; Moser, B. A.; Steinhardt, R. C.; Esser-Kahn, A. P. *Trends Biotechnol.* **2019**, *37* (4), 373–388.
(88) Mercier, B. C.; Ventre, E.; Fogeron, M.-L.; Debaud, A.-L.; Tomkowiak, M.; Marvel, J.; Bonnefoy, N. *PLoS One* **2012**, *7* (7), e42170.
(89) Satoru, I.; Yoshio Kumazawa; Kazutoshi Sai; Chiaki Nishimura; Mitsunobu Nakatsuka; J. Yuzuru, H.; Akihiro Yamamoto; Makoto Kiso; Akira Hasegawa. *Int. J. Immunopharmacol.* **1988**, *10* (4), 339–346.
(90) Ogawa, Y.; Kitagawa, M.; Fujishima, Y.; Kiso, M.; Hasegawa, A.; Ishida, H.; Azuma, I. *Agric. Biol. Chem.* **1989**, *53* (4), 1025–1036.
(91) Takada, H.; Galanos, C. *Infect. Immun.* **1987**, *55* (2), 409–413.
(92) Ignacio, B. J.; Albin, T. J.; Esser-Kahn, A. P.; Verdoes, M. *Bioconjug. Chem.* **2018**, *29* (3), 587–603.
(93) Xu, Z.; Moyle, P. M. *Bioconjug. Chem.* **2018**, *29* (3), 572–586.
(94) Deres, K.; Schild, H.; Wiesmüller, K.-H.; Jung, G.; Rammensee, H.-G. *Nature* **1989**, *342* (6249), 561–564.
(95) Khan, S.; Bijker, M. S.; Weterings, J. J.; Tanke, H. J.; Adema, G. J.; van Hall, T.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; van der Marel, G. A.; *et al*. *J. Biol. Chem.* **2007**, *282* (29), 21145–21159.
(96) Khan, S.; Weterings, J. J.; Britten, C. M.; de Jong, A. R.; Graafland, D.; Melief, C. J. M.; van der Burg, S. H.; van der Marel, G.; Overkleeft, H. S.; Filippov, D. V.; *et al*. *Mol. Immunol.* **2009**, *46* (6), 1084–1091.
(97) Zom, G. G.; Khan, S.; Britten, C. M.; Sommandas, V.; Camps, M. G. M.; Loof, N. M.; Budden, C. F.; Meeuwenoord, N. J.; Filippov, D. V; van der Marel, G. A.; *et al*. *Cancer Immunol. Res.* **2014**, *2* (8), 756–764.
(98) Makimura, Y.; Asai, Y.; Taiji, Y.; Sugiyama, A.; Tamai, R.; Ogawa, T. *Clin. Exp. Immunol.* **2006**, *146* (1), 159–168.
(99) Asai, Y.; Makimura, Y.; Ogawa, T. *J. Med. Microbiol.* **2007**, *56* (4), 459–465.
(100) Zom, G. G.; Welters, M. J. P.; Loof, N. M.; Goedemans, R.; Lougheed, S.; Valentijn, R. R. P. M.; Zandvliet, M. L.; Meeuwenoord, N. J.; Melief, C. J. M.; de Gruijl, T. D.; *et al*. *Oncotarget* **2016**, *7* (41).
(101) Zom, G. G.; Willems, M. M. J. H. P.; Khan, S.; van der Sluis, T. C.; Kleinovink, J. W.; Camps, M. G. M.; van der Marel, G. A.; Filippov, D. V.; Melief, C. J. M.; Ossendorp, F. *J. Immunother. Cancer* **2018**, *6* (1), 146.
(102) McDonald, D. M.; Byrne, S. N.; Payne, R. J. *Front. Chem.* **2015**, *3*.
(103) Kaiser, A.; Gaidzik, N.; Becker, T.; Menge, C.; Groh, K.; Cai, H.; Li, Y.-M.; Gerlitzki, B.; Schmitt, E.; Kunz, H. *Angew. Chemie Int. Ed.* **2010**, *49* (21), 3688–3692.
(104) Wilkinson, B. L.; Malins, L. R.; Chun, C. K. Y.; Payne, R. J. *Chem. Commun.* **2010**, *46* (34), 6249.
(105) Thompson, P.; Lakshminarayanan, V.; Supekar, N. T.; Bradley, J. M.; Cohen, P. A.; Wolfert, M. A.; Gendler, S. J.; Boons, G.-J. *Chem. Commun.* **2015**, *51* (50), 10214–10217.
(106) Martínez-Sáez, N.; Supekar, N. T.; Wolfert, M. A.; Bermejo, I. A.; Hurtado-Guerrero, R.; Asensio, J. L.; Jiménez-Barbero, J.; Busto, J. H.; Avenoza, A.; Boons, G.-J.; *et al*. *Chem. Sci.* **2016**, *7* (3), 2294–2301.
(107) Abdel-Aal, A.-B. M.; Lakshminarayanan, V.; Thompson, P.; Supekar, N.; Bradley, J. M.; Wolfert, M. A.; Cohen, P. A.; Gendler, S. J.; Boons, G.-J. *ChemBioChem* **2014**, *15* (10), 1508–1513.
(108) Shi, L.; Cai, H.; Huang, Z.-H.; Sun, Z.-Y.; Chen, Y.-X.; Zhao, Y.-F.; Kunz, H.; Li, Y.-M. *ChemBioChem* **2016**, *17* (15), 1412–1415.
(109) Buskas, T.; Ingale, S.; Boons, G.-J. *Angew. Chemie Int. Ed.* **2005**, *44* (37), 5985–5988.
(110) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G.-J. *Nat. Chem. Biol.* **2007**, *3* (10), 663–667.
(111) Cai, H.; Huang, Z.; Shi, L.; Zhao, Y.; Kunz, H.; Li, Y. *Chem. – A Eur. J.* **2011**, *17* (23), 6396–6406.

(112) Cai, H.; Sun, Z.-Y.; Chen, M.-S.; Zhao, Y.-F.; Kunz, H.; Li, Y.-M. *Angew. Chemie Int. Ed.* **2014**, *53* (6), 1699–1703.
(113) Schülke, S.; Vogel, L.; Junker, A.-C.; Hanschmann, K.-M.; Flaczyk, A.; Vieths, S.; Scheurer, S. *J. Immunol. Res.* **2016**, *2016*, 1–8.
(114) Lewicky, J. D.; Ulanova, M.; Jiang, Z.-H. *ChemistrySelect* **2016**, *1* (5), 906–910.
(115) Li, Q.; Guo, Z. *Molecules* **2018**, *23* (7), 1583.
(116) Ziaco, M.; Górska, S.; Traboni, S.; Razim, A.; Casillo, A.; Iadonisi, A.; Gamian, A.; Corsaro, M. M.; Bedini, E. *J. Med. Chem.* **2017**, *60* (23), 9757–9768.
(117) Wang, Q.; Zhou, Z.; Tang, S.; Guo, Z. *ACS Chem. Biol.* **2012**, *7* (1), 235–240.
(118) Zhou, Z.; Mondal, M.; Liao, G.; Guo, Z. *Org. Biomol. Chem.* **2014**, *12* (20), 3238–3245.
(119) Zhou, Z.; Liao, G.; Mandal, S. S.; Suryawanshi, S.; Guo, Z. *Chem. Sci.* **2015**, *6* (12), 7112–7121.
(120) Wille-Reece, U.; Wu, C. -y.; Flynn, B. J.; Kedl, R. M.; Seder, R. A. *J. Immunol.* **2005**, *174* (12), 7676–7683.
(121) Gao, D.; Liu, Y.; Diao, Y.; Gao, N.; Wang, Z.; Jiang, W.; Jin, G. *ACS Med. Chem. Lett.* **2015**, *6* (3), 249–253.
(122) Wu, C. C. N.; Hayashi, T.; Takabayashi, K.; Sabet, M.; Smee, D. F.; Guiney, D. D.; Cottam, H. B.; Carson, D. A. *Proc. Natl. Acad. Sci.* **2007**, *104* (10), 3990–3995.
(123) Weterings, J. J.; Khan, S.; van der Heden, G. J.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; van der Burg, S. H.; Ossendorp, F.; van der Marel, G. A.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2006**, *16* (12), 3258–3261.
(124) Gential, G. P. P.; Hogervorst, T. P.; Tondini, E.; van de Graaff, M. J.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2019**.
(125) Fujita, Y.; Hirai, K.; Nishida, K.; Taguchi, H. *Amino Acids* **2016**, *48* (5), 1319–1329.
(126) Cho, H. J.; Takabayashi, K.; Cheng, P.-M.; Nguyen, M.-D.; Corr, M.; Tuck, S.; Raz, E. *Nat. Biotechnol.* **2000**, *18* (5), 509–514.
(127) Kramer, K.; Young, S. L.; Walker, G. F. *ACS Omega* **2017**, *2* (1), 227–235.
(128) Heit, A.; Schmitz, F.; O'Keeffe, M.; Staib, C.; Busch, D. H.; Wagner, H.; Huster, K. M. *J. Immunol.* **2005**, *174* (7), 4373–4380.
(129) Daftarian, P.; Sharan, R.; Haq, W.; Ali, S.; Longmate, J.; Termini, J.; Diamond, D. J. *Vaccine* **2005**, *23* (26), 3453–3468.
(130) Carelli, C.; Audibert, F.; Gaillard, J.; Chedid, L. *Proc. Natl. Acad. Sci.* **1982**, *79* (17), 5392–5395.
(131) Carelli, C.; Ralamboranto, L.; Audibert, F.; Gaillard, J.; Briquelet, N.; Dray, F.; Fafeur, V.; Haour, F.; Chedid, L. *Int. J. Immunopharmacol.* **1985**, *7* (2), 215–224.
(132) Carelli, C.; Guillon, C.; Gobert, M. G. *Biomed. Pharmacother.* **2001**, *55* (7), 404–412.
(133) Willems, M. M. J. H. P.; Zom, G. G.; Meeuwenoord, N.; Ossendorp, F. A.; Overkleeft, H. S.; van der Marel, G. A.; Codée, J. D. C.; Filippov, D. V. *Beilstein J. Org. Chem.* **2014**, *10*, 1445–1453.
(134) Zom, G. G.; Willems, M. M. J. H. P.; Meeuwenoord, N. J.; Reintjens, N. R. M.; Tondini, E.; Khan, S.; Overkleeft, H. S.; van der Marel, G. A.; Codee, J. D. C.; Ossendorp, F.; *et al*. *Bioconjug. Chem.* **2019**, *30* (4), 1150–1161.
(135) Yada, A.; Ebihara, S.; Matsumura, K.; Endo, S.; Maeda, T.; Nakamura, A.; Akiyama, K.; Aiba, S.; Takai, T. *Cell. Immunol.* **2003**, *225* (1), 21–32.
(136) Cioca, D. P.; Deak, E.; Cioca, F.; Paunescu, V. *J. Immunother.* **1997**, *29* (1), 41–52.
(137) Dhodapkar, K. M.; Krasovsky, J.; Williamson, B.; Dhodapkar, M. V. *J. Exp. Med.* **2002**, *195* (1), 125–133.
(138) Sheridan, R. T. C.; Hudon, J.; Hank, J. A.; Sondel, P. M.; Kiessling, L. L. *ChemBioChem* **2014**, *15* (10), 1393–1398.
(139) Jakobsche, C. E.; Parker, C. G.; Tao, R. N.; Kolesnikova, M. D.; Douglass, E. F.; Spiegel, D. A. *ACS Chem. Biol.* **2013**, *8* (11), 2404–2411.
(140) Chirkin, E.; Muthusamy, V.; Mann, P.; Roemer, T.; Nantermet, P. G.; Spiegel, D. A. *Angew. Chemie Int. Ed.* **2017**, *56* (42), 13036–13040.
(141) Parker, C. G.; Domaoal, R. A.; Anderson, K. S.; Spiegel, D. A. *J. Am. Chem. Soc.* **2009**, *131* (45), 16392–16394.
(142) Murelli, R. P.; Zhang, A. X.; Michel, J.; Jorgensen, W. L.; Spiegel, D. A. *J. Am. Chem. Soc.* **2009**, *131* (47), 17090–17092.
(143) Mangsbo, S. M.; Fletcher, E. A. K.; van Maren, W. W. C.; Redeker, A.; Cordfunke, R. A.; Dillmann, I.; Dinkelaar, J.; Ouchaou, K.; Codee, J. D. C.; van der Marel, G. A.; *et al*. *Mol. Immunol.* **2018**, *93*, 115–

124.
(144) Fletcher, E. A. K.; van Maren, W.; Cordfunke, R.; Dinkelaar, J.; Codee, J. D. C.; van der Marel, G.; Melief, C. J. M.; Ossendorp, F.; Drijfhout, J. W.; Mangsbo, S. M. *J. Immunol.* **2018**, *201* (1), 87–97.
(145) Naicker, K. P.; Li, H.; Heredia, A.; Song, H.; Wang, L.-X. *Org. Biomol. Chem.* **2004**, *2* (5), 660–664.
(146) Perdomo, M. F.; Levi, M.; Sallberg, M.; Vahlne, A. *Proc. Natl. Acad. Sci.* **2008**, *105* (34), 12515–12520.
(147) Abdel-Motal, U. M.; Wang, S.; Awad, A.; Lu, S.; Wigglesworth, K.; Galili, U. *Vaccine* **2010**, *28* (7), 1758–1765.
(148) Sianturi, J.; Manabe, Y.; Li, H.-S.; Chiu, L.-T.; Chang, T.-C.; Tokunaga, K.; Kabayama, K.; Tanemura, M.; Takamatsu, S.; Miyoshi, E.; *et al*. *Angew. Chemie Int. Ed.* **2019**, *58* (14), 4526–4530.
(149) Abdel-Motal, U. M.; Guay, H. M.; Wigglesworth, K.; Welsh, R. M.; Galili, U. *J. Virol.* **2007**, *81* (17), 9131–9141.
(150) Huflejt, M. E.; Vuskovic, M.; Vasiliu, D.; Xu, H.; Obukhova, P.; Shilova, N.; Tuzikov, A.; Galanina, O.; Arun, B.; Lu, K.; *et al*. *Mol. Immunol.* **2009**, *46* (15), 3037–3049.
(151) Oyelaran, O.; McShane, L. M.; Dodd, L.; Gildersleeve, J. C. *J. Proteome Res.* **2009**, *8* (9), 4301–4310.
(152) Chen, W.; Gu, L.; Zhang, W.; Motari, E.; Cai, L.; Styslinger, T. J.; Wang, P. G. *ACS Chem. Biol.* **2011**, *6* (2), 185–191.
(153) Zhang, H.; Wang, B.; Ma, Z.; Wei, M.; Liu, J.; Li, D.; Zhang, H.; Wang, P. G.; Chen, M. *Bioconjug. Chem.* **2016**, *27* (4), 1112–1118.
(154) Sarkar, S.; Salyer, A. C. D.; Wall, K. A.; Sucheck, S. J. *Bioconjug. Chem.* **2013**, *24* (3), 363–375.
(155) Karmakar, P.; Lee, K.; Sarkar, S.; Wall, K. A.; Sucheck, S. J. *Bioconjug. Chem.* **2016**, *27* (1), 110–120.
(156) Hossain, M. K.; Vartak, A.; Karmakar, P.; Sucheck, S. J.; Wall, K. A. *ACS Chem. Biol.* **2018**, *13* (8), 2130–2142.
(157) Sarkar, S.; Lombardo, S. A.; Herner, D. N.; Talan, R. S.; Wall, K. A.; Sucheck, S. J. *J. Am. Chem. Soc.* **2010**, *132* (48), 17236–17246.

Chapter 2

*Conjugation ready monophosphoryl lipid A-analogues for self-adjuvanting cancer peptide vaccines**

Introduction

Immunotherapy is a powerful, emerging strategy that makes use of the body's own immune system to combat malignant cancer cells. One approach is to train the immune system to recognize and destroy cancer cells through vaccination, by targeting cancer specific epitopes, such as neoantigens[1] or tumor-associated carbohydrate antigens (TACAs)[2]. To optimally direct an immune reaction against these antigens, adjuvants can be used to activate antigen presenting cells, such as dendritic cells (DCs) and macrophages, to enhance and polarize the T cell response. DCs express pattern recognition receptors (PRRs)[3], through which they recognize invading pathogens and initiate an immune response.[4] Ligands for these PRRs can be used as adjuvants and many well-defined molecular PRR-ligands have been explored for their application in cancer vaccine modalities and various classes of PRR-receptors have been targeted, including the Toll-like receptors (TLRs)[5], C-type lectins[6], and Nucleotide binding Oligomerization Domain-like receptors[7,8]. To further improve vaccine activity, the antigen and adjuvants have been combined in covalent constructs, delivering "self-

*The data presented in this Chapter were gathered in collaboration with Elena Tondini, Nico J. Meeuwenoord, Fabrizio Chiodo, Elko Peterse, Ferry A. Ossendorp, Herman S. Overkleeft, Dmitri V. Filippov, Gijsbert A. van der Marel and Jeroen D. C. Codée.

adjuvanting" vaccines.[9,10] Several TLR agonists[9,11,12] have been conjugated to antigenic peptides, including ligands for TLR2[13–15], TLR7[16,17] and TLR9[18,19], yielding vaccine modalities with improved activity with respect to their non-conjugated counterparts. TLR4-ligands have so far not been explored in peptide-conjugate vaccine modalities. TLR4 can recognize lipopolysaccharides (LPS) and in particular lipid A (Figure 1), which can be found on the cell surface of Gram-negative bacteria.[20] Lipid A can form a complex with MD-2[21], which then binds to TLR4, resulting in the activation of the TRIF and the MyD88 signaling pathways, which induces the release of cytokines and chemokines.[22] Due to its high toxicity, lipid A cannot be used *in vivo*, but removal of the anomeric phosphate provides monophosphoryl lipid A (MPLA, Figure 1), which is significantly less toxic while maintaining the immunostimulatory activity.[23,24] MPLA has therefore been used as an adjuvant in various vaccines, and its use has been approved for human use.[25,26] It is part of the AS04 adjuvant mixture (in which it is combined with aluminum hydroxide or phosphate) in commercially available Human Papillomavirus and Hepatitis B vaccines.[27] The group of Guo, has introduced MPLA for use in several covalent glycoconjugate vaccines, in which MPLA was conjugated to a TACA or a synthetic bacterial glycan.[28–32] These latter conjugates were able to elicit a robust IgG antibody response, critical for effective anti-bacterial vaccination.[32]

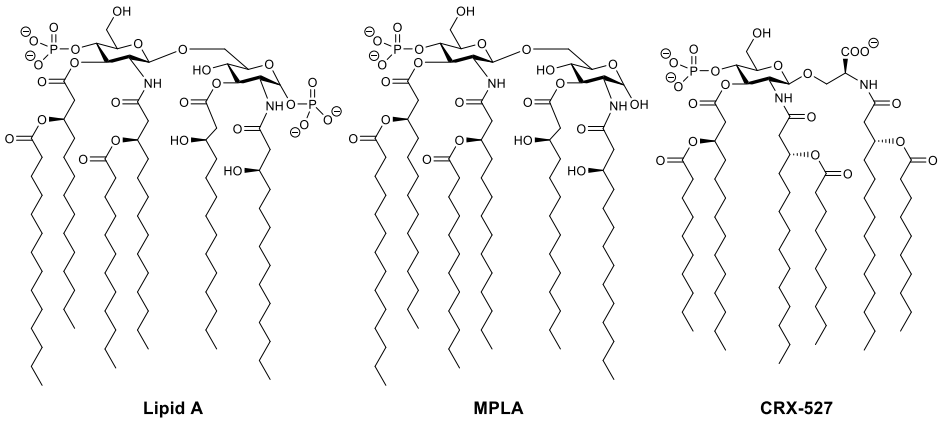


Figure 1. Structures of Lipid A and MPLA of *Salmonella enterica* serotype minnesota Re 595, and CRX-527.

One of the challenges in the generation of MPLA conjugates is to obtain sufficient quantities of the vaccine due to their complex structure and physical properties.[33–35] Based on the structure of lipid A, a new class of potent monosaccharide adjuvants has been developed, the aminoalkyl glucosamine 4-phosphates (AGPs), in which the reducing end glucosamine of lipid A is replaced for an acylated serine residue.[36] These AGPs are easier to synthesize in comparison to other MPLA derivatives, while

maintaining their well-defined and powerful immunostimulatory properties.[37–40] AGPs have been shown to be efficacious adjuvants and to be clinically safe, resulting in its use in a Hepatitis B vaccine.[41] CRX-527 (Figure 1) has been established as one of the most potent AGPs.[37]

This chapter describes the design, synthesis and immunological evaluation of TLR4-ligands **1**-**4** and the novel TLR4-ligand peptide conjugates **5**-**8** (Figure 2). A spacer equipped CRX-527 was used as the TLR4-ligand, which was conjugated with an ovalbumin derived peptide, $DEVA_5K$, comprising the MHC-I epitope SIINFEKL embedded in a longer peptide motif, serving as a model antigen in the peptide conjugates **5**-**8**. The $DEVA_5K$-peptide was equiped with a thiol functionality either at the *N*- or the *C*-terminal end and the TLR4-ligand was provided with a maleimide functionality to allow their union through thiol-maleimide chemistry. The required maleimide was installed via a linker at the *C*-6 position of the glucosamine residue in CRX-527, as this is the same position to which bacterial *O*-antigens are attached to LPS in the bacterial cell wall.[36] Previous work on anti-bacterial MPLA conjugate vaccines has shown that the adjuvant can be modified at this position without compromising adjuvant activity.[32] Two type of linkers at the *C*-6 position of CRX-527 were evaluated: an hydrophobic alkyl linker (A) and a hydrophilic triethylene glycol (TEG) linker (B). These linkers were connected to the 6-OH of CRX-527 through an ester bond,[32] or *via* a more stable amide bond to an hereto installed 6-NH_2 functionality.

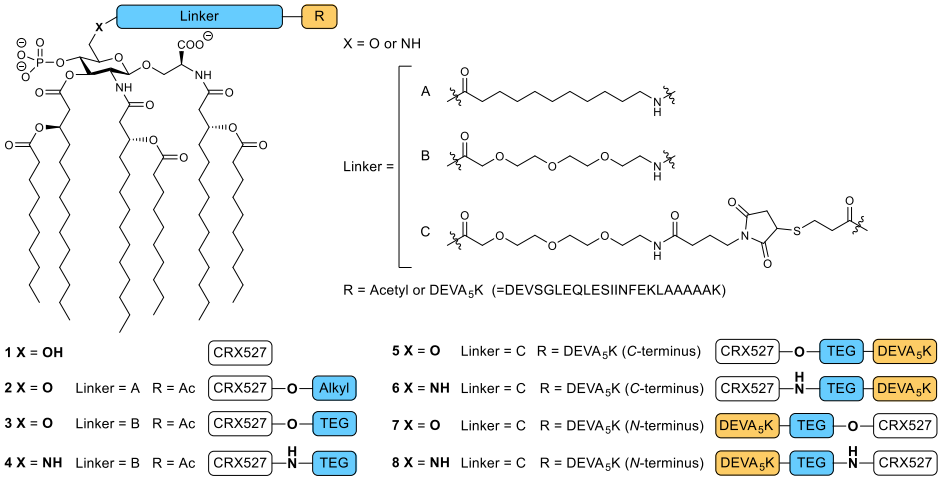
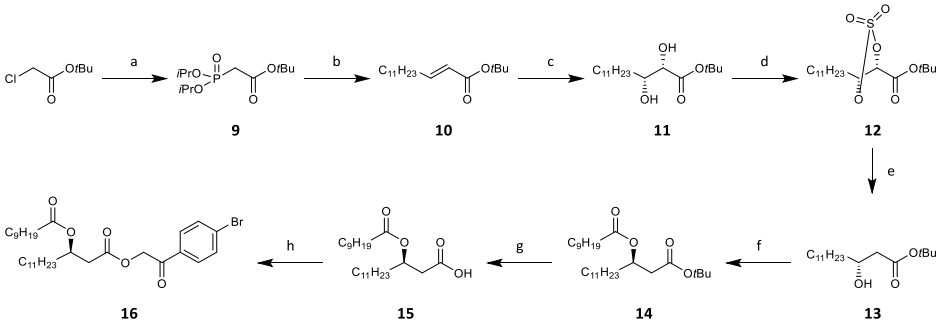


Figure 2. Structures of TLR4-ligands **1**-**4** and TLR4-ligand conjugates **5**-**8**.

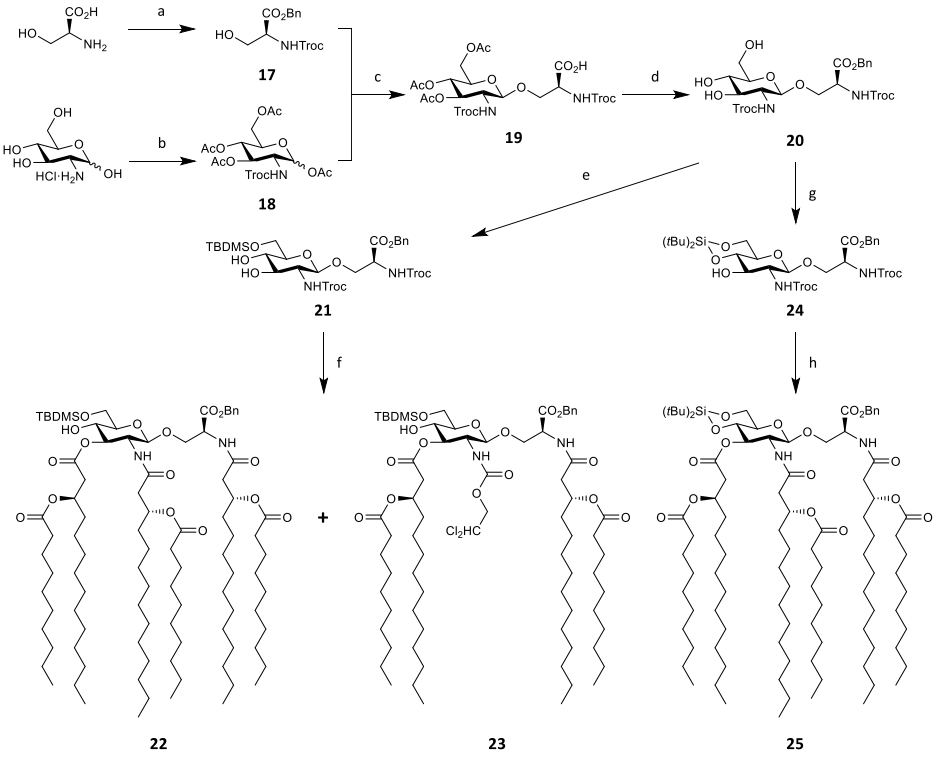
Results and Discussion

For the synthesis of ligands **1**-**4**, first the route towards (R)-3-alkyloxytetradecanoic acid **15** was optimized, to allow for a large scale synthesis (Scheme 1). Previous routes turned out to give lower yields, partly due to the formation of side products and the associated difficult separations.[42,43] The synthesis starts with the conversion of *tert*-butyl 2-chloroacetate into Horner-Wadsworth-Emmons (HWE) reagent **9**, which was obtained by vacuum distillation. Next, the HWE reaction of **9** with dodecanal led to the predominant formation of E-alkene **10** in 96% (64 mmol scale).[44] Further scaling-up of this reaction (560 mmol) led to a drop in yield (78%) due to the difficult separation of the two isomeric alkenes. Sharpless asymmetric dihydroxylation of ester **10** with OsO_4 in the presence of $(DHQD)_2PHAL$ gave diol **11** in 98% (the ee was determined at a later stage of the synthesis). Diol **11** was treated with thionyl chloride and pyridine, followed by oxidation of the intermediate cyclic sulfite with $NaIO_4$ and ruthenium trichloride to give cyclic sulfate **12**.[45–47] Regioselective nucleophilic opening of the cyclic sulfate with sodium borohydride and acidic hydrolysis of the obtained sulfate ester afforded alcohol **13**.[45] Crucial to the removal of the sulfate ester is the use of exactly two equivalents of H_2SO_4, since the use of a larger excess leads to hydrolysis of the *tert*-butyl ester. Acetylation of the hydroxyl group in **13** with decanoyl chloride, pyridine and a catalytic amount of DMAP gave **14** and subsequent TFA mediated removal of the *tert*-butyl group gave fatty acid **15** in 64% yield over 9 steps. Conversion of acid **15** into *p*-bromophenacyl ester **16** was performed to determine the *ee*, which turned out to be 98.6%.



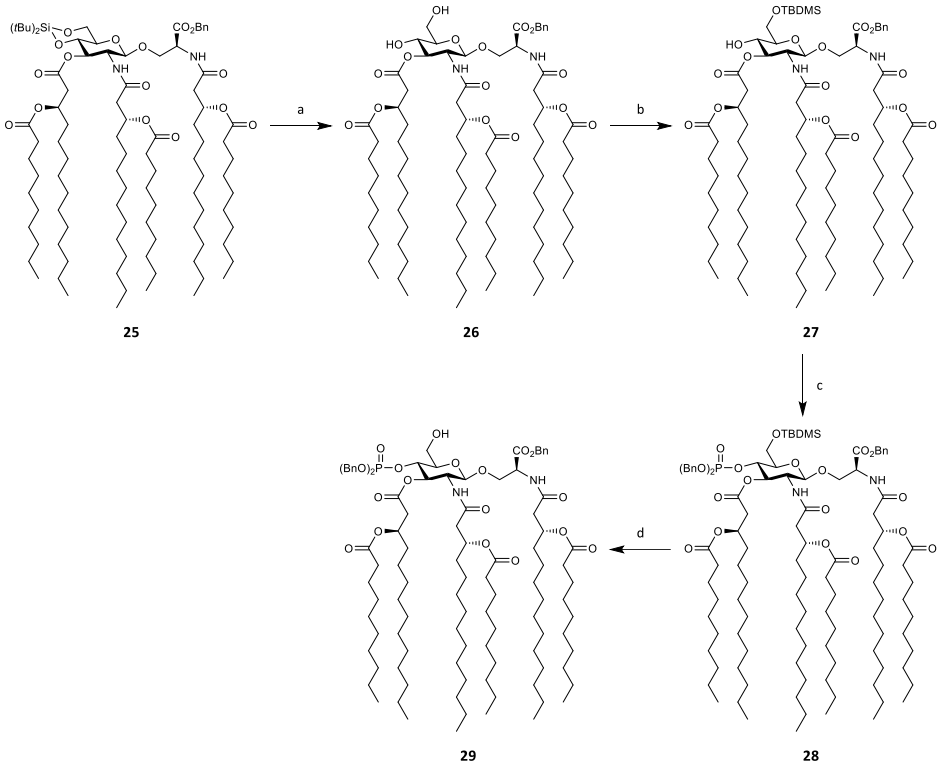
Scheme 1. Synthesis of chiral fatty acid **15**. *Reagents and conditions*: a) $P(OiPr)_3$, 130°C, quant.; b) dodecanal, *n*-BuLi, THF, 96%; c) $K_3Fe(CN)_6$, K_2CO_3, $[(DHQD)_2PHAL]$, OsO_4, methanesulfonamide, H_2O/*t*-BuOH, 98%; d) *i*. $SOCl_2$, pyridine, EtOAc, 0°C; *ii*. $RuCl_3$, $NaIO_4$, CCl_4/MeCN/H_2O, 95% over two steps; e) *i*. $NaBH_4$, DMF, 0°C; *ii*. H_2SO_4 (2 eq.), H_2O (2 eq.), THF, 0°C, quant. over two steps; f) decanoyl chloride, DMAP, pyridine, 0°C, 89%; g) TFA, DCM, 92%; h) 2,4'-dibromoacetophenone, Et_3N, EtOAc, 48%.

The synthesis of the required glucosaminyl serine synthon is depicted in Scheme 2.[40] First, Troc-protected serine **17** was generated by masking the amino group of benzyl L-serine with a 2,2,2-trichloroethoxycarbonyl group.[48] Next, glucosamine **18** was assembled by treatment of glucosamine with Troc-chloride and subsequent acetylation of the hydroxyl groups. Coupling of **17** and **18** under the influence of boron trifluoride etherate proceeded in a completely β-selective manner to give a mixture of the desired product and unreacted donor **18**. The mixture could be separated after hydrogenolysis of the benzyl ester giving acid **19** in 63%, on 170 mmol scale. Next all acetyls were removed with ammonia in methanol, before the benzyl ester was re-installed using phase transfer conditions to yield triol **20**. Regioselective introduction of the *tert*-butyl dimethyl silyl (TBDMS) group at the 6-OH gave compound **21**[39,40] in 81%. In previous AGP syntheses TBDMS-protected glucosamine **21** has been successfully used for the regioselective introduction of the fatty acid at the 3-OH and after Troc removal on both liberated amines.[39,40] In line with these studies, the 3-OH in **21** was acetylated and the Troc protecting groups were removed using Zn/AcOH. However, the last step was accompanied by partial reduction of the Troc groups to give 1,2-dichloroethoxycarbonyl groups. This led to the formation of several side products, such as **23**, during the acylation step and it proved to be impossible to remove these side products at any stage of the synthesis. The possibility that these side products can function as TLR4 antagonists, because of the lower number of fatty acid chains, necessitated the exploration of a different route. Therefore, building block **24**, bearing a 4,6-*O*-silylidene group, was synthesized from **20**. The removal of the Troc protecting groups of **24**, was followed by *N*,*N*,*O*-triacetylation with **15** in the presence of EDC·MeI and catalytic DMAP (0.03 eq.). Using column chromatography it was possible to separate the desired compound from the side products, derived from partial reduction of the Troc-groups, and **25** was obtained in 62% over two steps (3.0 mmol scale). Scale-up of the procedure (9.5 mmol) led to a slightly diminished yield of 57%. Of note, it was observed that increasing of the amount of DMAP (>0.1 eq.) in the acylation step, led to partial β-elimination of the fatty acids.



Scheme 2. Synthesis of building block **25**. *Reagents and conditions*: a) *i*. *p*-toluenesulfonic acid, CCl_4/benzyl alcohol, 100°C; *ii*. succinimidyl-2,2,2-trichloroethyl carbonate, Et_3N, DCM, 40% over two steps; b) *i*. 2,2,2-trichloroethoxycarbonyl chloride, $NaHCO_3$, H_2O; *ii*. Ac_2O, pyridine, 66% over two steps; c) *i*. $BF_3{\cdot}OEt_2$, DCM, 0°C to rt; *ii*. H_2, Pd/C, THF, 63% over two steps; d) *i*. NH_4OH, MeOH; *ii*. BnBr, TBAB, DCM/$NaHCO_3$ (aq. sat.), 79% over two steps; e) TBDMSCl, pyridine, 81%; f) *i*. **15**, EDC·MeI, DMAP, DCM, 84%; *ii*. Zn dust, AcOH; *iii*.) **15**, EDC·MeI, DCM; g) $(t\text{Bu})_2Si(OTf)_2$, DMF, -40°C, 94%; h) *i*. Zn dust, AcOH; *ii*.) **15**, EDC·MeI, DMAP, DCM, 62% over two steps.

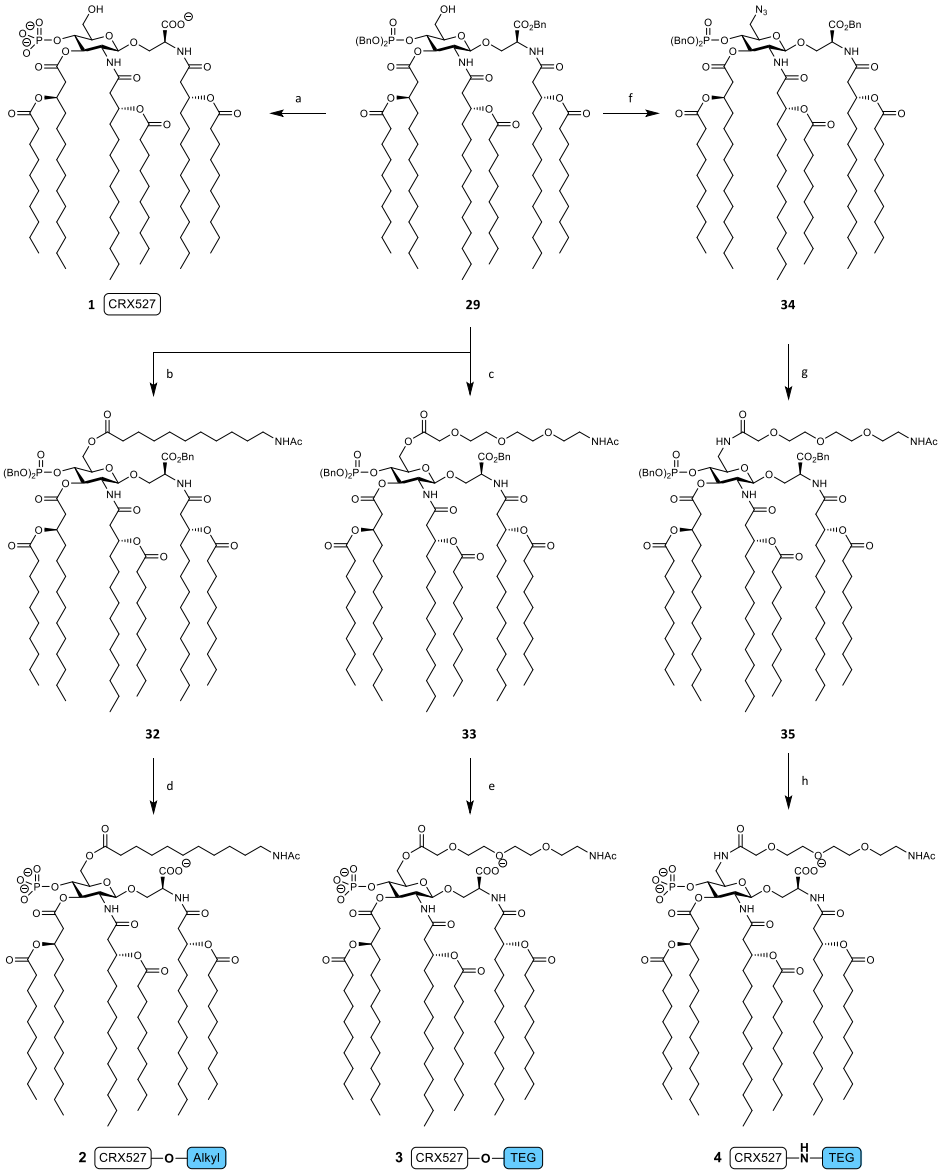
In the final stage of the synthesis of key building block **29** (Scheme 3), the silylidene was removed using $HF{\cdot}Et_3N$ to give diol **26**, of which the primary alcohol was protected with a TBDMS group. The dibenzyl phosphotriester at the *C*-4 of **27** was installed using dibenzyl di-*iso*-propylphosphoramidite and tetrazole, followed by oxidation of the intermediate phosphite triester with 3-chloroperbenzoic acid yielding **28**. Final desilylation with TFA gave key building block **29** in 13% yield over 14 steps from commercially available D-glucosamine.



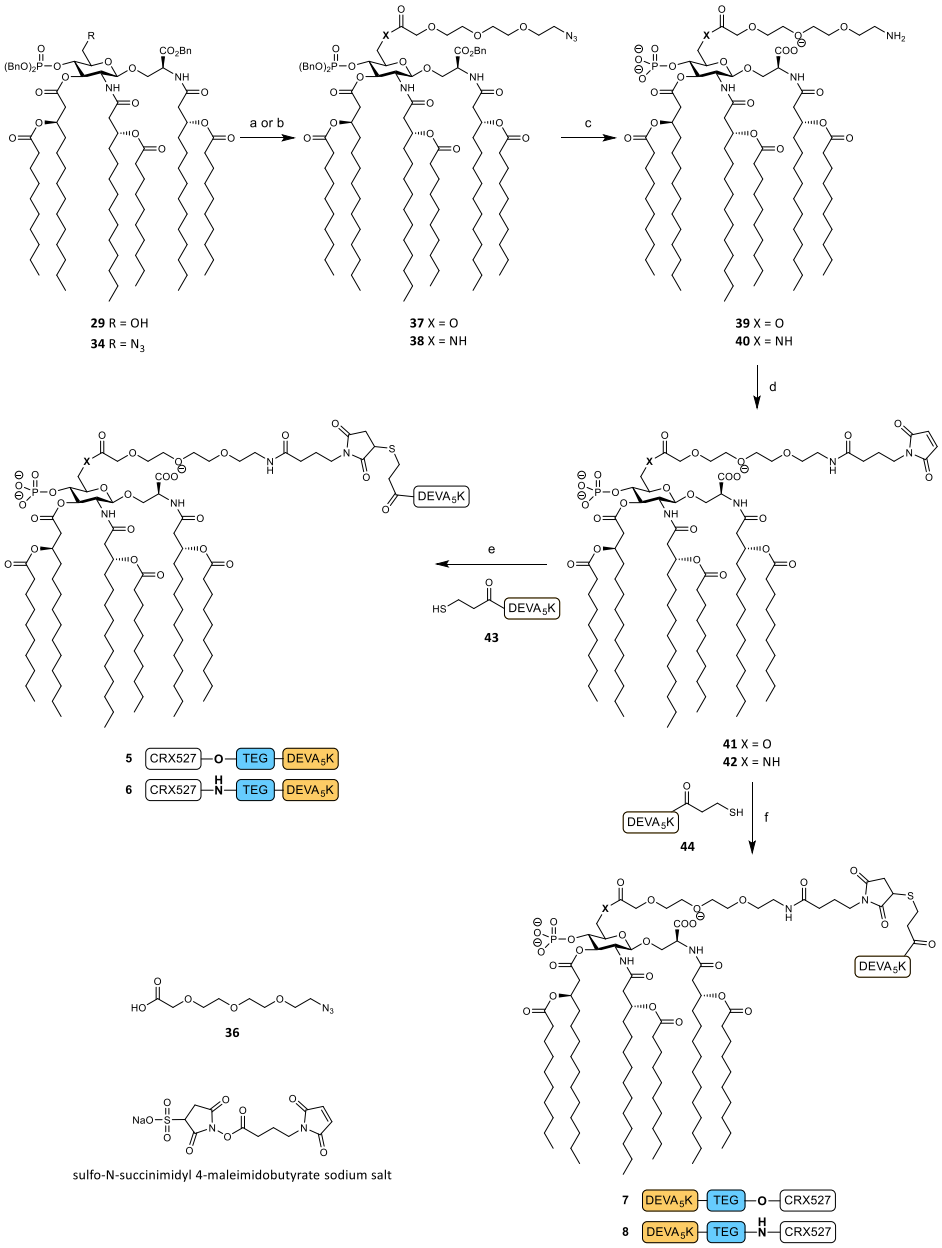
Scheme 3. Synthesis of key building block **29**. *Reagents and conditions*: a) HF·Et_3N. THF, 0°C, 92%; b) TBDMSCl, pyridine, 88%; c) *i*. dibenzyl *N*,*N*-diisopropylphosphoramidite, tetrazole, DCM, 0°, 1h; *ii*. 3-chloroperbenzoic acid, quant. over two steps; d) TFA, DCM, 84%.

With the common starting compound **29** available in sufficient amounts, attention was directed to the assembly of ligands **1**-**4,** having either an alkyl linker or a triethylene glycol (TEG) linker (Scheme 4). These ligands are needed as reference compounds in the immunological evaluation to determine the potential influence of the linker at the *C*-6 position of CRX-527. Debenzylation of **29** using Pd/C gave the original CRX-527 (**1**). Elongation of **29** with the *N*-acetylated linkers **30** and **31**, under influence of EDC·MeI and DMAP furnished **32** and **33**, in 88% and 74% yield, respectively. After hydrogenation of **32** and **33** ligands **2** and **3** were obtained. Contrary to Guo and coworkers, who noticed the 6-*O* ester bond is unstable, no hydrolysis of esters **2** and **3** is observed.[32] Ligand **4** was obtained by conversion of the primary alcohol of **29** into azide **34** using Mitsunobu conditions, reduction of the azide with Zn/NH_4Cl and condensation of the amine with linker **31** to give **35** in 40% yield over two steps. A final hydrogenation step yielded ligand **4** in 61%.

At this stage, the synthesis of the TLR4-ligand peptide conjugates **5**-**8** was undertaken (Scheme 5). Based on preliminary immunological evaluation of the ligands **1**-**4** (*vide infra*) the TEG linker was used for the assembly of the peptide antigen conjugates. The ester linker in **37** was introduced by EDC mediated condensation of **29** and linker **36** to deliver **37** in 80% yield. The amide analogue was synthesized by reduction of the azide in CRX-527 derivative **34** and condensation with linker **36** to give **38** in 56% over two steps. The maleimide functionalized TLR4-ligands **41** and **42** were acquired by hydrogenation of compounds **37** and **38** and treatment of the thus obtained **39** and **40** with sulfo-*N*-succinimidyl 4-maleimidobutyrate sodium salt. The $DEVA_5K$, antigenic peptides having a thiol function at the *N*-terminus (**43**) and at the *C*-terminus (**44**) were synthesized to allow the conjugation of a TLR4-ligand at either side of the peptide. Using a semi-automated solid phase peptide chemistry protocol, the peptides were assembled on a Tentagel S Ram resin. The thiol function in peptide **43** was introduced by a HCTU mediated condensation of the immobilized oligopeptide with 3-(tritylthio)propionic acid. This procedure could also be applied to peptide **44** by the incorporation of MMT protected lysine at the start of the sequence and bocylation of the *N*-terminal amine at the end of the solid phase peptide synthesis. Thiol functionalized peptides **43** or **44** were purified to homogeneity by RP-HPLC. The thiol-maleimide coupling was performed by dissolving **43** or **44** in DMF/H_2O followed by the addition of a solution of maleimide **41** or **42** in $CHCl_3$. After shaking for two days, LC-MS analysis confirmed full conversion of the maleimide. Initially RP-HPLC purification of the crude conjugates was performed, but ester conjugates **5** and **7** turned out to be unstable and hydrolysis of the *C*-6-ester took place during lyophilization of the purified conjugates, likely as the result of the presence of minimal amounts of acid or base. Therefore, a new purification method without any base or acid was used. Herein, the crude mixture was added to a prepacked C18 column and eluted with several solvent systems (see experimental section for details). This resulted in pure *N*-terminus conjugates **5** and **6**, and *C*-terminus conjugates **7** and **8**, of which integrity and purity were shown by analysis with LC-MS and MALD-TOF MS (Figure 3).



Scheme 4. Synthesis of TLR4-ligands **1**-**4**. *Reagents and conditions*: a) H_2, Pd/C, THF, 89%; b) **30**, EDC·MeI, DMAP, DCE, 88%; c) **31**, EDC·MeI, DMAP, DCE, 74%; d) H_2, Pd/C, THF, 56%; e) H_2, Pd/C, THF, 66%; f) PPh_3, DEAD, DPPA, THF, 67%; g) *i*. Zn, NH_4Cl, DCM/MeOH/H_2O; *ii*. **31**, EDC·MeI, DMAP, DCE, 40% over two steps; h) H_2, Pd/C, THF, 61%.



Scheme 5. Synthesis of TLR4-ligand peptide conjugates **5**-**8**. *Reagents and conditions*: a) **36**, EDC·MeI, DMAP, DCE, **37**: 80%; b) *i*. Zn, NH_4Cl, DCM/MeOH/H_2O; *ii*. **36**, EDC·MeI, DMAP, DCE, **38**: 56% over two steps; c) H_2, Pd/C, THF (**39**: 77%, **40**: 83%); d) sulfo-*N*-succinimidyl 4-maleimidobutyrate sodium salt, Et_3N, DCM or DCE (**41**: 84%, **42**: 81%); e) **43**, DMF/$CHCl_3$/H_2O, 48h (**5**: 52%, **6**: 54%); f) **44**, DMF/$CHCl_3$/H_2O, 48h (**7**: 57%, **8**: 42%).

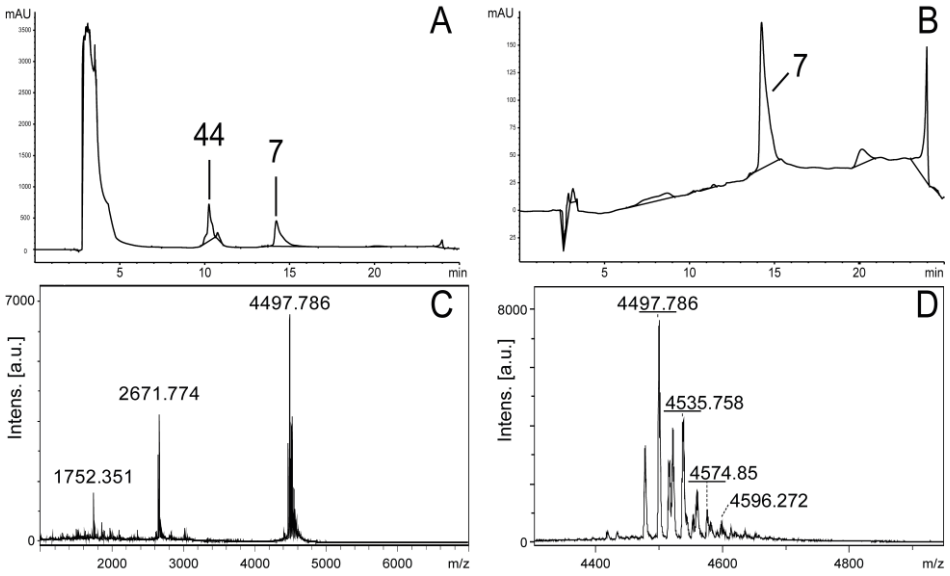


Figure 3. LC-MS traces of crude *C*-terminus conjugate **7** (A) and after purification (B) and the MALDI analysis of **7** (C and D).

Biological evaluation

Immunological evaluation of TLR4-ligands **1**-**4** and conjugates **5**-**8** was performed by assessing their ability to induce maturation of dendritic cells and antigen presentation by DCs *in vitro*.[49] For this purpose, peptide $DEVA_5K$, **45**, TLR2-ligand Pam_3CSK_4, **46**,[50] and TLR2-ligand conjugate **47** were used as relevant reference compounds (Figure 4B).[51] The outline of the experiments is depicted in Figure 4A. Binding of the conjugate to the TLR4/MD-2 complex triggers the production of inflammatory cytokines, such as interleukin-12 (IL-12) and maturation of the DCs. After uptake, the conjugates have to be processed to enable the presentation of the peptide antigen by MHC-I molecules. Binding to the T cell receptor and simultaneous activation by the excreted proinflammatory cytokines then results in the maturation $CD8^+$ T cells.[52] To probe activation of the DCs by the TLR4-ligands and conjugates, DCs were stimulated for 24h with the compounds and the amount of IL-12p40 was measured. First, the activating capacity of the CRX-527 ligand (**1**) and its close analogues **2**-**4**, was tested. As can be seen in Figure 4C the negative controls, DMSO and peptide **45**, show no activity. Stimulation of the DCs with ligands **1**, **3** and **4** gave higher IL-12 secretion than commercially available MPLA or the TLR2-ligand **46**, while ligand **2** proved incapable of DC-activation. Possibly, the hydrophobic linker leads to different binding to the MD-2/TLR4 pocket, preventing activation of the required signaling cascades. The DC-activating capacity of compounds **1**, **3** and **4** shows that functionalization at the *C*-6

position with a hydrophilic linker does not inhibit binding of the ligand to the receptor. Next, the conjugates **5**-**8** were evaluated for DC activation as shown in Figure 4D. The activity of the ester-linked conjugates, **5** and **7** is similar to the activity of the ligands **1** and **3**, while for the amide linked conjugates a slight decrease in activity was observed upon conjugation to the peptide. No difference in activity was observed between the *N*- or the *C*-terminal $DEVA_5K$ conjugates. Finally, the conjugates **5**-**8** and a mixture of peptide **45** and CRX-527 ligand **1** were evaluated for antigen presentation using a SIINFEKL-specific T cell hybridoma assay. Figure 4E shows that exposure of DCs to the ester conjugates **5** and **7**, leads to a relatively low level of antigen presentation, similar to the level induced by the stand-alone antigen and the mixture of the antigen and CRX-527 **1**. Notably, incubation with the amide conjugates **6** and **8** resulted in higher antigen presentation. It is possible that hydrolysis takes place for the ester conjugates **5** and **7** prior to uptake in the DCs, leading to diminished uptake of the peptide part of the conjugate, leading to a lower antigen presentation. The *in vitro* experiments show that the amide conjugates, **6** and **8**, are processed more efficiently than the ester conjugates, **5** and **7**, and a mix of **1** and **45**. The conjugates are not as potent however, as the TLR2-ligand conjugate **47** in terms of antigen presentation *in vitro*.

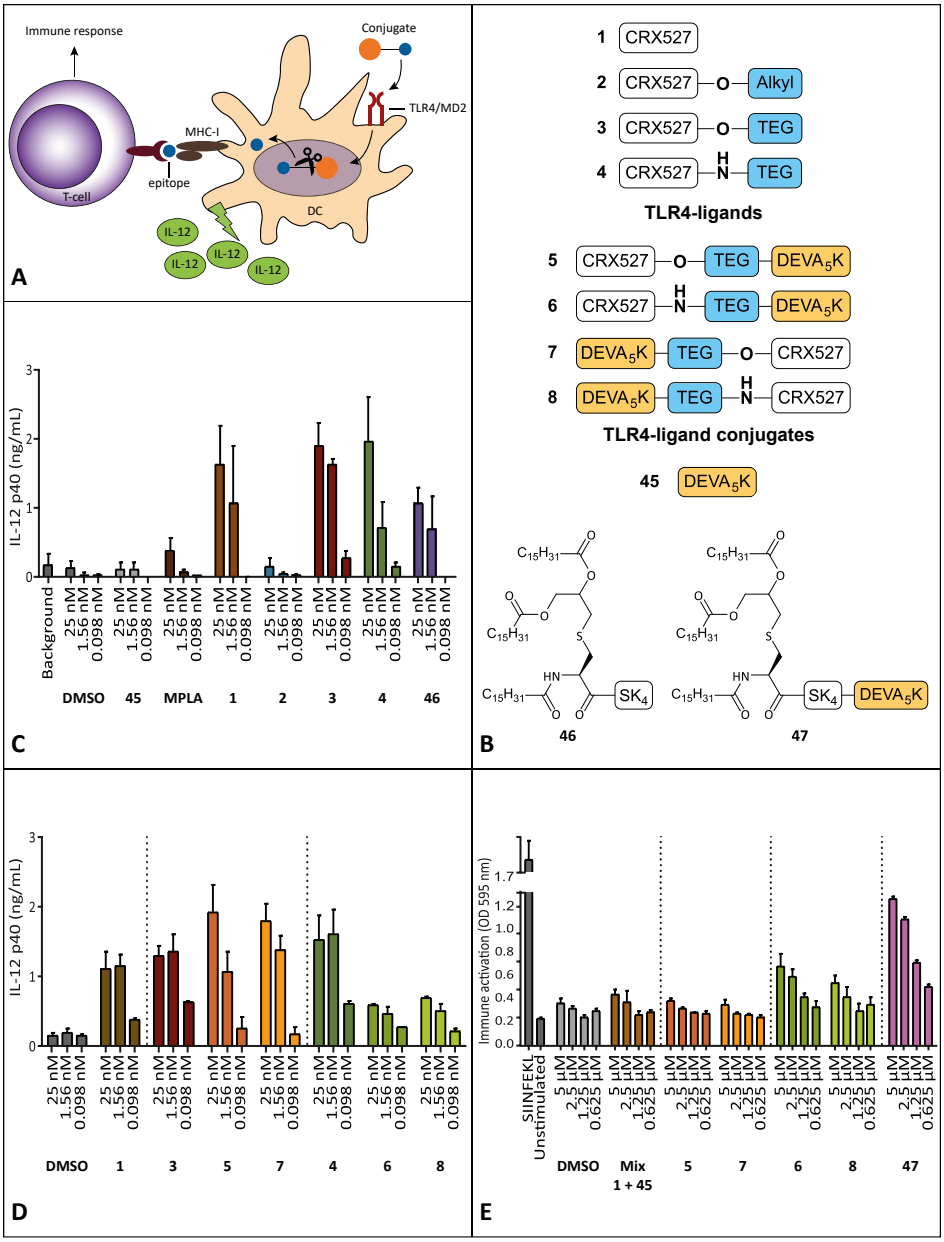


Figure 4. A) Schematic overview of stimulation of a DC with a conjugate leading to an immune response; B) Overview of the TLR4-ligands, TLR4-ligand peptide conjugates and reference compounds used in the *in vitro* experiments; C) DC activation of ligands **1**-**4** and **46**; D) DC activation of conjugates **5**-**8** and ligands **1**, **3** and **4**; E) Antigen presentation of conjugates **5**-**8**, mix of ligand **1** and peptide **45**.[49]

Conclusion

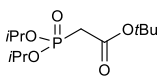
This chapter describes the first synthesis of four TLR4-ligand peptide-conjugates. In these model vaccine constructs, CRX-527, a potent MPLA analogue, was covalently linked to the *N*- or the *C*-terminal end of the $DEVA_5K$ peptide, harboring the MHC-I epitope SIINFEKL, through two different linking moieties to provide the "self adjuvanting" conjugates **5**-**8**. In order to acquire these conjugates, an efficient synthetic route was developed to generate multi-gram amounts of (R)-3-alkyloxytetradecanoic acid **15**. These chiral lipids were used in combination with a silylidene protected glucosaminyl serine building block to provide *N*,*N*,*O*-triacetylated CRX-527 derivative **29**. Different linker systems and connection modes were probed to conjugate the peptide antigen and TLR4-ligands. The conjugates with an ester bond at the *C*-6 position of CRX-527 (**5** and **7**) turned out to be relatively labile, prohibiting HPLC purification. A manual reversed phase chromatography purification protocol allowed for the purification of the conjugates delivering the pure conjugates. Biological evaluation of the ligands showed that the use of a hydrophobic linker led to an inactive ligand, while the presence of a hydrophilic linker at the *C*-6 position did not adversely affect the activity and led to the induction of IL-12 production. Stimulation of DCs with ester conjugates, **5** and **7**, resulted in higher IL-12 production than activation of the cells by the amide conjugates **6** and **8**. In contrast, conjugates **6** and **8** showed to give better antigen presentation *in vitro*. No significant difference was found between the *N*-terminus and *C*-terminus conjugates. The results presented in this Chapter show that TLR4-ligand-antigen conjugates are promising self-adjuvanting vaccine modalities and warrant the evaluation of their activity in *in vivo* experiments.

Experimental

All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AV-300 (300/75 MHz), AV-400 (400/100/162 MHz) spectrometer, a Bruker AV-500 Ultrashield (500/126/202 MHz) spectrometer, a Bruker AV-600 (600/151 MHz) or a Bruker AV-850 (850/214 MHz) and all individual signals were assigned using 2D-NMR

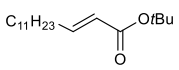
spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column or a Vydac 219TP 5 µm Diphenyl, 150 x 4.6 mm column with a flow of 1, 0.8 or 0.7 ml/min. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. Peptides, TLR2-ligand and conjugate were purified with a Gilson GX-281 preparative HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column or a Vydac 219TP 5 µm Diphenyl, 250 x 10 mm column. Peptide fragments were synthesized with automated solid phase peptide synthesis on an Applied Biosystems 433A Peptide Synthesizer. Optical rotations were measured on an Anton Paar Modular Circular Polarimeter MCP 100/150. High resolution mass spectra were recorded on a Synapt G2-Si or a Q Exactive HF Orbitrap equipped with an electron spray ion source positive mode. Mass analysis of the TLR4-ligands and TLR4-ligand conjugates was performed on an Ultraflextreme MALDI-TOF or a 15T MALDI-FT-ICR MS system. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR. Unprotected lipid A derivatives were dissolved in a mixture of $CDCl_3$/MeOD 5/1 v/v for NMR analysis. DC activation and B3Z assay results were analysed with GraphPad Prism version 7.00 for Windows, GraphPad Software. FA = fatty acid.

tert-Butyl 2-(diisopropoxyphosphoryl)acetate (9)



A mixture of *tert*-butyl chloroacetate (0.12 L, 0.81 mol, 1.0 eq.) and triisopropyl phosphite (0.22 L, 0.90 mol, 1.1 eq.) was heated to 150°C for 3 hours, after which it was cooled down to room temperature. After purification by vacuum distillation (14 mbar, 95 °C) compound **9** was obtained in quantitative yield (245 g) as a transparent oil, which was used without further purification. $[\alpha]_D^{20}$ -1.0° (*c* = 1.3, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 4.57 – 4.43 (m, 2H, 2x CH *i*Pr), 2.60 (d, 2H, *J* = 21.5 Hz, CH_2), 1.23 (s, 9H, 3x CH_3 *t*Bu), 1.11 (dd, 12H, *J* = 6.3, 2.8 Hz, 4x CH_3 *i*Pr); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 164.6, 164.6 (C=O), 81.3 (C_q *t*Bu), 70.8, 70.7 (CH *i*Pr), 37.0, 35.7 (CH_2), 27.6 (CH_3 *t*Bu), 23.7, 23.7, 23.5, 23.5 (CH_3 *i*Pr); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ 18.85; FT-IR (neat, cm^{-1}): 2980, 2935, 1728, 1457, 1387, 1369, 1287, 1258, 1173, 1142, 1104, 985, 904, 889, 823, 755, 701, 617, 507; HRMS: $[M+Na]^+$ calcd. for $C_{12}H_{25}O_5PNa$: 303.1332, found 303.1337.

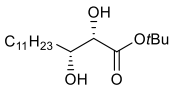
tert-Butyl (_E_)-2-tetradecanoate (10)



Compound **9** (18.1 g, 64.4 mmol, 1.3 eq.) was co-evaporated with toluene (2x), dissolved in THF (0.16 L) under an argon atmosphere and *n*-BuLi (1.6 M in hexane, 40.0 mL, 64.0 mmol 1.3 eq.) was added under an argon flow. After 1 hour, a solution of dodecanal (12.0 mL, 49.8 mmol, 1 eq.) in THF (40 mL) was added and the mixture was stirred at room temperature overnight. The obtained yellow reaction mixture was quenched by addition of H_2O and extracted with Et_2O (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (0→3% Et_2O in pentane) yielded the title compound (13.5 g, 47.8 mmol, 96%). R_f: 0.71 (19.5/0.5

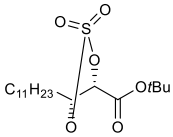
pentane/Et_2O); $[\alpha]_D^{25}$ -0.96° (*c* = 1.2, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 6.84 (dt, 1H, *J* = 15.6, 6.9 Hz, *H*C=CH), 5.71 (dt, 1H, *J* = 15.6, 1.6 Hz, HC=C*H*), 2.18 – 2.09 (m, 2H, CH_2), 1.46 (s, 9H, *t*Bu) , 1.44 – 1.37 (m, 2H, CH_2), 1.29 – 1.20 (m, 16H, 8x CH_2), 0.86 (d, 3H, *J* = 8.0 Hz, CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 166.3 (C=O), 148.3, 123.0 (C=C), 80.0 (C_q *t*Bu), 32.2, 32.0, 30.4, 29.8, 29.7, 29.7, 29.5, 29.5, 29.3 (CH_2), 28.3 (CH_3 *t*Bu), 28.2, 22.8 (CH_2), 14.2 (CH_3); FT-IR (neat, cm^{-1}): 2926, 2855, 2361, 1717, 1654, 1458, 1392, 1367, 1289, 1256, 1154, 1127, 979, 854; HRMS: $[M+H]^+$ calcd. for $C_{18}H_{36}O_2$: 283.26316, found 283.26289.

tert-Butyl (2*S*, 3*R*)-2,3-dihydroxytetradecanoate (11)



To a mixture of *t*BuOH/H_2O (1/1 v/v, 0.18 L) were the following chemicals subsequently added: $K_3[Fe(CN)_6]$ (35.5 g, 106 mmol, 3.0 eq.), K_2CO_3 (14.6 g, 106 mmol, 3.0 eq.), $[(DHQD)_2PHAL]$ (0.29 g, 0.35 mmol, 0.01 eq.), aq. OsO_4 (0.14 M, 1.6 mL, 0.22 mmol, 0.006 eq.), and methanesulfonamide (3.41 g, 35.1 mmol, 1.0 eq.). The reaction mixture was cooled to 0°C and thoroughly stirred for 25 minutes, followed by addition of a solution of compound **10** (9.97 g, 35.2 mmol, 1 eq.) in DCM (8.0 mL). The reaction mixture was stirred at 5°C overnight, after which TLC analysis showed complete conversion of the starting material and it was quenched by the addition of sodium thiosulfate pentahydrate (53.3 g, 215 mmol, 7.0 eq.). After 30 minutes vigorously stirring, the suspension was diluted with H_2O and extracted with EtOAc (3x). The combined organic layers were washed with 2 M KOH (2x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (5→10% EtOAc in pentane) afforded the title compound (10.1 g, 31.9 mmol, 91%). R_f: 0.54 (1/1 pentane/EtOAc); $[\alpha]_D^{25}$ +4.4° (*c* = 0.85, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 3.96 (d, 1H, *J* = 2.3 Hz, CH), 3.82 (td, 1H, *J* = 6.8, 2.1 Hz, CH), 1.63 – 1.54 (m, 2H, CH_2), 1.53 – 1.43 (m, 11H, CH_2, 3x CH_3 *t*Bu), 1.37 – 1.18 (m, 16H, 8x CH_2), 0.91 – 0.83 (m, 3H, CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.1 (C=O), 83.3 (C_q *t*Bu), 73.3 (CH), 72.9 (CH), 34.1, 32.1, 29.8, 29.8, 29.7 29.7, 29.5 (CH_2), 28.2 (CH_3 *t*Bu), 25.9, 22.8 (CH_2), 14.3 (CH_3); FT-IR (neat, cm^{-1}): 3457, 2924, 2854, 1732, 1459, 1369, 1256, 1162, 1135, 849; HRMS: $[M+Na]^+$ calcd. for $C_{18}H_{36}O_4Na$: 339.2506, found 339.2511.

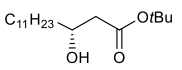
tert-Butyl (4*S*, 5*R*)-5-undecyl-1,3,2-dioxathiolane-4-carboxylate-2,2-dioxide (12)



Diol **11** (118 g, 373 mmol, 1 eq.) was dissolved in EtOAc (0.92 mL) and cooled to 0°C. Pyridine (75 mL, 0.93 mol, 2.5 eq.) was added, followed by slow addition of thionyl chloride (30 mL, 0.41 mol, 1.1 eq.) resulting in a white suspension. After 45 minutes, TLC analysis showed complete conversion of the starting material and the reaction was quenched by the addition of H_2O. The organic layer was washed with H_2O (2x) and the combined water layers were extracted with EtOAc (2x). The combined organic layers were dried over Na_2SO_4, filtered, concentrated *in vacuo* and co-evaporated with toluene (2x). The obtained cyclic sulfite was used without further purification by dissolving in a mixture of CCl_4/CH_3CN (1/1 v/v, 1.3 L). The solution was cooled to 0°C, followed by the addition of $RuCl_3 \cdot H_2O$ (7.74 g, 37.3 mmol, 0.1 eq.), $NaIO_4$ (202 g, 935 mmol, 2.5 eq.) and H_2O (1.3 L) subsequently. The black suspension was

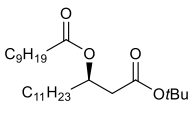
allowed to warm-up to room temperature and after stirring for 1.5 hours, the dark brown mixture was filtered twice over celite and a Whatmann-filter. The residu was washed with DCM and the combined filtrates were diluted with H_2O and brine. The aqueous layers were extracted with DCM (3x) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (2→40% Et_2O in pentane) gave the title compound (127 g, 336 mmol, 90%). R_f: 0.48 (9/1 pentane/Et_2O); $[\alpha]_D^{25}$ +35.3° (*c* = 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 4.92 – 4.85 (m, 1H, CH), 4.74 (d, 1H, *J* = 7.4 Hz, CH), 2.03 – 1.87 (m, 2H, CH_2), 1.59 – 1.40 (m, 12H, CH_2, CH_3 *t*Bu), 1.40 – 1.18 (m, 16H, 8x CH_2), 0.87 (t, 3H, *J* = 6.8 Hz, CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 163.9 (C=O), 85.5 (C_q *t*Bu), 84.4 (CH), 80.3 (CH), 33.2, 32.0, 29.7, 29.5, 29.4, 29.3, 29.0 (CH_2), 28.0 (CH_3 *t*Bu), 24.9, 22.8 (CH_2), 14.2 (CH_3); FT-IR (neat, cm^{-1}): 2925, 2855, 1764, 1737, 1459, 1396, 1372, 1257, 1210, 1154, 1047, 951, 904, 835, 724, 650, 530; HRMS: $[M+Na]^+$ calcd. for $C_{18}H_{34}O_6SNa$: 401.1968, found 401.1974.

tert-Butyl (*R*)-3-hydroxytetradecanoate (13)



A solution of cyclic sulfate **12** (127 g, 336 mmol, 1.0 eq.) in DMF (0.84 L) was cooled to 0°C, followed by the addition of $NaBH_4$ (14.9 g, 394 mmol, 1.17 eq.). The reaction mixture was allowed to warm-up to room temperature and after 1.5 hours the reaction was quenched with acetone, concentrated *in vacuo* and co-evaporated with toluene. The resulting sulfate was dissolved THF (0.84 mL) and cooled to 0°C. H_2O (12 mL, 0.67 mol, 2.0 eq.) and concentrated H_2SO_4 (36 mL, 0.67 mol, 2.0 eq.) were added to the solution. After the reaction mixture was vigorously stirred for 2 hours, the reaction was neutralized by the addition of Et_3N and sat. aq. $NaHCO_3$. The reaction mixture was further diluted with brine and extracted with Et_2O (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (2→10% EtOAc in pentane) yielded compound **13** (101 g, 335 mmol, Quant.). R_f: 0.26 (9/1 pentane/Et_2O); $[\alpha]_D^{25}$ -14.7° (*c* = 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 3.98 – 3.88 (m, 1H, CH), 3.07 (s, 1H, OH), 2.46 – 2.24 (m, 2H, 2x CH_2), 1.56 – 1.34 (m, 13H, 2x CH_2, 3x CH_3 *t*Bu), 1.34 – 1.15 (m, 16H, 8x CH_2), 0.86 (t, 3H, *J* = 6.8 Hz, CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 172.8 (C=O), 81.3 (C_q *t*Bu), 68.2 (CH), 42.4, 36.6, 32.0, 29.8, 29.7, 29.5 (CH_2), 28.2 (CH_3 *t*Bu), 25.6, 22.8 (CH_2), 14.3 (CH_3); FT-IR (neat, cm^{-1}): 3455, 2924, 2854, 1730, 1458, 1393, 1368, 1256, 1153, 954, 844; HRMS: $[M+Na]^+$ calcd. for $C_{18}H_{36}O_3Na$: 323.2557, found 323.2561.

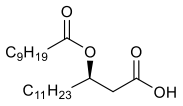
tert-Butyl (*R*)-3-(decanoyloxy)tetradecanoate (14)



A solution of compound **13** (10.2 g, 34.1 mmol, 1.0 eq.) in pyridine (85 mL) was cooled to 0°C under an argon atmosphere. Decanoyl chloride (10.8 mL, 51.0 mmol, 1.5 eq.) and DMAP (0.42 g, 3.4 mmol, 0.1 eq.) were added and after 45 minutes the resulting yellow suspension was allowed warm-up to room temperature. After 30 minutes, TLC analysis showed complete conversion of the starting material and the mixture was concentrated *in vacuo*. After purification by column chromatography (0→5% Et_2O in pentane), the title compound (14.5 g, 31.9 mmol, 94%) was obtained as a transparent oil. R_f: 0.78 (9/1

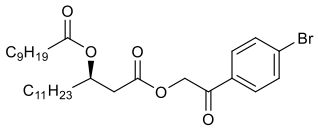
pentane/Et_2O); $[\alpha]_D^{25}$ +1.4° (*c* = 0.91, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.23 – 5.14 (m, 1H, CH), 2.53 – 2.34 (m, 2H, CH_2), 2.24 (t, 2H, *J* = 7.5 Hz, CH_2), 1.65 – 1.48 (m, 4H, 2x CH_2), 1.41 (s, 9H, 3x CH_3 *t*Bu), 1.35 – 1.14 (m, 30H, 15x CH_2), 0.85 (t, 6H, *J* = 6.8 Hz, 2x CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.2 (C=O), 169.8 (C=O), 80.8 (C_q *t*Bu), 70.6 (CH), 40.7, 34.6, 34.1, 32.0, 32.0, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.3 (CH_2), 28.1 (CH_3 *t*Bu), 25.2, 25.1, 22.8 (CH_2), 14.2 (CH_3); FT-IR (neat, cm^{-1}): 2925, 2855, 1738, 1466, 1368, 1153; HRMS: $[M+Na]^+$ calcd. for $C_{28}H_{54}O_4Na$: 477.3914, found 477.3924.

(*R*)-3-(decanoyloxy)tetradecanoic acid (15)



Compound **14** (117.7 g, 258.9 mmol, 1 eq.) was dissolved in DCM (0.43 L) and cooled to 0°C. TFA (0.12 L, 1.57 mol, 6.0 eq.) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was subsequently concentrated *in vacuo* and co-evaporated several times with toluene. Purification by column chromatography (0→10% MeOH in DCM) gave acid **15** (96.5 g, 242 mmol, 93%). R_f: 0.63 (9/1 DCM/MeOH); $[\alpha]_D^{20}$ +4.0° (*c* = 2.0, DCM); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.20 (p, 1H, *J* = 7.0, 6.4 Hz, CH), 2.67 – 2.51 (m, 2H, CH_2), 2.27 (t, 2H, *J* = 7.5 Hz, CH_2), 1.70 – 1.51 (m, 4H, 2x CH_2), 1.39 – 1.16 (m, 30H, 15x CH_2), 0.87 (t, 6H, *J* = 6.8 Hz, 2x (CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 176.9 (C=O), 173.4 (C=O), 70.1 (CH), 39.0, 34.6, 34.1, 32.0, 32.0, 29.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.2, 25.2, 25.1, 22.8, 22.8 (CH_2), 14.2 (CH_3); FT-IR (neat, cm^{-1}): 2923, 2854, 1740, 1714, 1466, 1378, 1163, 1109, 722; HRMS: $[M+Na]^+$ calcd. for $C_{24}H_{46}O_4Na$: 421.3388, found 421.3289.

2-(4-Bromophenyl)-2-oxoethyl-(*R*)-3-(decanoyloxy)tetradecanoate (16)



Acid **15** (0.29 g, 0.98 mmol, 1 eq.) was dissolved in EtOAc (5.0 mL), followed by the addition of 2,4'-dibromoacetophenone (0.31 g, 1.1 mmol, 1.1 eq.) and Et_3N (0.15 mL, 1.1 mmol, 1.1 eq.). The reaction mixture was stirred overnight, after which it was diluted with H_2O and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (5→30% EtOAc in pentane) afforded the title compound (0.28 g, 0.47 mmol, 48%, ee = 98.55%). R_f: 0.17 (9/1 pentane/Et_2O); $[\alpha]_D^{25}$ -1.4° (*c* = 0.28, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.73 (d, 2H, *J* = 8.5 Hz, Ar), 7.59 (d, 2H, *J* = 8.5 Hz, Ar), 5.32 – 5.22 (m, 3H, CH_2, CH), 2.77 – 2.64 (m, 2H, CH_2), 2.28 (t, 2H, *J* = 7.5 Hz, CH_2), 1.69 – 1.53 (m, 4H, 2x CH_2), 1.37 – 1.13 (m, 30H, 15x CH_2), 0.90 – 0.79 (m, 6H, 2x CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 190.9, 173.3, 169.9 (C=O), 132.9 (C_q Ar), 132.2, 129.3 (Ar), 129.2 (C_q Ar), 70.1 (CH), 66.0, 38.9, 34.5, 34.1, 32.0, 31.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.4, 29.4, 29.4, 29.4, 29.2, 25.2, 25.0, 22.7 (CH_2), 14.2 (CH_3); FT-IR (neat, cm^{-1}): 2925, 2854, 1739, 1708, 1588, 1164, 1072, 972; HRMS: $[M+Na]^+$ calcd. for $C_{32}H_{51}O_5BrNa$: 617.2812, found 617.2824.

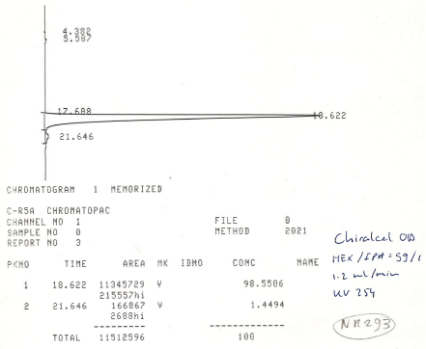
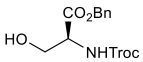


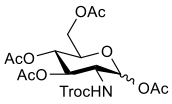
Figure 5. Analysis of *ee* of compound **16**.

Benzyl *N*-trichloroethoxycarbonyl-L-serinate (17)



L-Serine (49.6 g, 472 mmol, 1.0 eq.) was dissolved in a mixture of CCl_4/benzyl alcohol (1/1 v/v, 0.46 L). *p*-Toluenesulfonic acid (96.6 g, 508 mmol, 1.1 eq.) was added and the white suspension was heated to 100°C using a Dean-Stark apparatus. After stirring overnight, a clear solution was obtained, which was cooled down to room temperature before concentrating *in vacuo*. The residue was dissolved in DCM and washed with sat. aq. $NaHCO_3$ (3x). The organic layer was extracted with 1 M HCl (3x) and the combined aqueous layers were concentrated *in vacuo*. Co-evaporation with toluene yielded the intermediate as a white solid (46.6 g, 201 mmol), which was dissolved in DCM (1.0 L) under an argon atmosphere. Succinimidyl-2,2,2-trichloroethyl carbonate[48] (61.5 g, 212 mmol, 1.05 eq.) was added to the reaction mixture, followed by the addition of Et_3N (42 mL, 0.30 mol, 1.5 eq.) under a flow of argon. After 1 hour, TLC analysis showed complete conversion of the starting material and the reaction mixture was washed with 1 M HCl (1x) and H_2O (1x). The aqueous layers were extracted with DCM (1x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→100% EtOAc in pentane) yielded the title compound (69.4 g, 187 mmol, 40% over two steps). HRMS: $[M+H]^+$ calcd. for $C_{13}H_{15}O_5NCl_3$: 370.00103, found 370.00105. Analytic data were in agreement with reported data.[48]

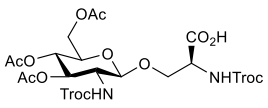
Acetyl 3,4,6-tri-*O*-acetyl-2-*N*-trichloroethoxycarbonyl-α/β-D-glucopyranoside (18)



$NaHCO_3$ (144 g, 1.65 mol, 3.0 eq.) and 2,2,2-trichloroethoxycarbonyl chloride (93 mL, 0.68 mol, 1.2 eq.) were added to a solution of D-glucosamine·HCl (0.12 kg, 0.55 mol, 1.0 eq.) in H_2O (1.1 L). The reaction was stirred vigorously at room temperature overnight, after which the resulting white suspension was filtered and the residue was washed with cold H_2O. The white solid was co-evaporated with toluene (3x) before dissolving in pyridine (0.60 L). The reaction mixture was cooled to 0°C and Ac_2O (0.30 L, 3.2 mol, 5.8 eq.) was added. The reaction mixture was allowed to warm-up to room temperature and stirred overnight. The reaction mixture was cooled to 0°C, quenched by the addition of H_2O and subsequently diluted with EtOAc. The organic layer was washed several times with 1 M HCl, dried over $MgSO_4$, filtered and concentrated *in vacuo*. TLC analysis showed no

full conversion, therefore the oil was dissolved in pyridine (0.60 L) and cooled to 0°C. Ac_2O (0.45 L, 4.8 mol, 8.7 eq.) was added and after 30 minutes the mixture was allowed to warm-up to room temperature. After 2.5 hours TLC analysis showed full conversion. The reaction was quenched by the addition of MeOH and concentrated *in vacuo*. Co-evaporation with toluene (3x) gave compound **18** (189 g, 362 mmol, 66%), which was used without further purification. R_f: 0.20 (7/3 pentane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 6.16 (d, 1H, *J* = 3.8 Hz, H-1), 5.43 (d, 1H, *J* = 9.5 Hz, NH), 5.27 – 5.17 (m, 1H, H-3), 5.13 (t, 1H, *J* = 9.9 Hz, H-4), 4.76 (d, 1H, *J* = 12.1 Hz, C*H*H Troc), 4.56 (d, 1H, *J* = 12.1 Hz, C*H*H Troc), 4.23 – 4.11 (m, 2H, H-2, C*H*H-6), 4.02 – 3.94 (m, 2H, H-5, C*H*H-6), 2.13 (s, 3H, CH_3 Ac), 2.02 (s, 3H, CH_3 Ac), 1.98 – 1.96 (m, 6H, 2x CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 171.2, 170.7, 169.2, 168.7 (C=O Ac), 154.1 (C=O Troc), 95.3 (C_q Troc), 90.4 (C-1), 74.6 (CH_2 Troc), 70.3 (C-3), 69.6 (C-5), 67.6 (C-4), 61.5 (CH_2-6), 53.1 (C-2), 20.9, 20.7, 20.6, 20.5 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3329, 2958, 2258, 2126, 1742, 1536, 1432, 1368, 1212, 1172, 1141, 1123, 1095, 1080, 1031, 1012, 952, 910, 820, 728, 681, 648, 599, 568, 526, 475; HRMS: $[M+Na]^+$ calcd. for $C_{17}H_{22}Cl_3NO_{11}Na$: 544.0151, found 544.0159.

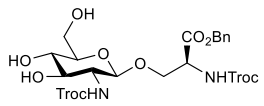
_N_-trichloroethoxycarbonyl-_O_-[3,4,6-tri-_O_-acetyl-2-_N_-trichloroethoxycarbonyl-β-D-glucopyranosyl]-L-serine (19)



Compounds **18** (88.9 g, 170 mol, 1.0 eq.) and **17** (69.3 g, 187 mmol, 1.1 eq.) were co-evaporated with toluene (2x) under an argon atmosphere and dissolved in DCM (0.28 L). The mixture was cooled to 0°C, followed by the slow addition of $BF_3{\cdot}OEt_2$ (42 mL, 0.34 mol, 2.0 eq.). The mixture was allowed to warm-up to room temperature and stirred for an additional 48 hours. The mixture was quenched with Et_3N and washed with sat. aq. $NaHCO_3$ (1x). The aqueous layer was extracted with DCM (3x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→100% EtOAc in pentane) gave an oil (117 g), which was a mixture of unreacted donor **18** and benzyl *N*-trichloroethoxycarbonyl-*O*-[3,4,6-tri-*O*-acetyl-2-*N*-trichloroethoxycarbonyl-β-D-glucopyranosyl]-L-serinate. After co-evaporating with toluene (3x) under an argon atmosphere, the oil was dissolved in THF (1.2 L), followed by the addition of Pd/C (10%, 11.7 g). The black suspension was purged with argon for 15 minutes, followed by purging with $H_{2(g)}$ and after 15 minutes a $H_{2(g)}$-filled balloon was applied. After stirring at room temperature overnight, the mixture was filtered over a Whatmann-filter and concentrated *in vacuo*. Purification by column chromatography (10→100% acetone in pentane) yielded the title compound (79.7 g, 107 mmol, 63% yield over two steps) and unreacted donor **18** (18.5 g, 35.4 mmol). R_f: 0.13 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +11.3° (*c* = 0.23, $CHCl_3$); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 5.31 – 5.21 (m, 1H, H-3), 4.98 (t, 1H, *J* = 9.7 Hz, H-4), 4.86 (d, 1H, *J* = 5.1 Hz, C*H*H Troc), 4.83 – 4.74 (m, 2H, 2x C*H*H Troc), 4.74 – 4.68 (m, 2H, H-1, C*H*H Troc), 4.41 (d, 1H, *J* = 5.0 Hz, CH serine), 4.31 (dd, 1H, *J* = 12.4, 4.6 Hz, C*H*H-6), 4.25 – 4.07 (m, 2H, C*H*H-6, C*H*H serine), 3.95 (dd, 1H, *J* = 10.5, 3.9 Hz, C*H*H serine), 3.85 – 3.73 (m, 1H, H-5), 3.66 – 3.52 (m, 1H, H-2), 2.07 (s, 3H, CH_3 Ac), 2.00 (s, 3H, CH_3 Ac), 1.97 (s, 3H, CH_3 Ac); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 172.5, 172.4, 171.7 (C=O Ac), 171.3 (C=O serine), 156.7, 156.5 (C=O Troc), 101.9 (C-

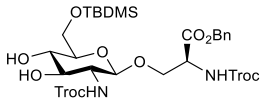
1), 97.1 (C_q Troc), 75.7, 75.4 (CH_2 Troc), 73.7 (C-3), 73.0 (C-5), 70.2 (C-4), 70.1 (CH_2 serine), 63.1 (CH_2-6), 57.1 (C-2), 55.7 (CH serine), 20.6 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3340, 2958, 1744, 1532, 1369, 1232, 1170, 1102, 1048, 819, 769, 734, 569; HRMS: $[M+Na]^+$ calcd. for $C_{21}H_{26}Cl_6N_2O_{14}Na$: 762.9407, found 762.9416.

Benzyl *N*-trichloroethoxycarbonyl-*O*-[2-*N*-trichloroethoxycarbonyl-β-D-glucopyranosyl]-L-serinate (20)



Compound **19** (79.6 g, 107 mmol, 1.0 eq.) was dissolved in MeOH (1.1 L) and NH_4OH (13.4 M, 73.5 mL, 985 mmol, 9.2 eq.) was added. After two days stirring at room temperature, TLC analysis showed complete conversion of the starting material. The reaction mixture was concentrated *in vacuo* and co-evaporated with toluene. The obtained oil was dissolved in a DCM/sat. aq. $NaHCO_3$ mixture (1/1 v/v, 2.6 L), after which tetrabutylammonium bromide (34.9 g, 108 mmol, 1.0 eq.) and benzyl bromide (64 mL, 0.54 mol, 5.0 eq.) were added. The reaction mixture was stirred overnight. The layers were separated and the aqueous layer was extracted with $CHCl_3$ (2x) and DCM (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→100% EtOAc in pentane, then 20% MeOH in EtOAc) afforded compound **20** (44.2 g, 65.5 mmol, 79% yield over two steps). R_f: 0.49 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -15.2° (*c* = 0.48, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.41 – 7.28 (m, 5H, Ar), 5.25 – 5.12 (m, 2H, CH_2 Bn), 4.85 (d, 1H, *J* = 12.2 Hz, C*H*H Troc), 4.79 – 4.69 (m, 3H, C*H*H Troc, CH_2 Troc), 4.49 (t, 1H, *J* = 4.4 Hz, CH serine), 4.45 (d, 1H, *J* = 8.2 Hz, H-1), 4.24 (dd, 1H, *J* = 10.2, 5.2 Hz, C*H*H serine), 3.93 – 3.83 (m, 2H, C*H*H serine, C*H*H-6), 3.67 (dd, 1H, *J* = 11.8, 5.5 Hz, C*H*H-6), 3.45 (dd, 1H, *J* = 10.2, 8.2 Hz, H-3), 3.41 – 3.33 (m, 1H, H-2), 3.31 – 3.21 (m, 2H, H-4, H-5); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 171.2 (C=O serine), 157.1, 156.5 (C=O Troc), 137.0 (C_q Ar), 129.6, 129.3, 129.1 (Ar), 102.8 (C-1), 96.8 (C_q Troc), 78.0 (C-5), 75.6 (CH_2 Troc), 75.5 (C-3), 72.0 (C-4), 69.6 (CH_2 serine), 68.2 (CH_2 Bn), 62.7 (CH_2-6), 58.9 (C-2), 56.2 (CH serine); FT-IR (neat, cm^{-1}): 3423, 2955, 2487, 1729, 1431, 1332, 1293, 1173, 1060, 820, 731, 569; HRMS: $[M+Na]^+$ calcd. for $C_{22}H_{26}Cl_6N_2O_{11}Na$: 726.9560, found 726.9576.

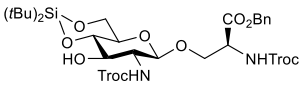
Benzyl *N*-trichloroethoxycarbonyl-*O*-[6-*O*-*tert*-butyldimethylsilyl-2-*N*-trichloroethoxycarbonyl-β-D-glucopyranosyl]-L-serinate (21)



Compound **20** (44.2 g, 62.5 mmol, 1.0 eq.) was dissolved in pyridine (0.30 L) and *tert*-butyldimethylsilyl chloride (14.6 g, 96.9 mmol, 1.5 eq.) was added. After 3 hours, TLC analysis showed complete conversion of the starting material and the reaction mixture was diluted with EtOAc. The organic layer was washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Coevaporation with toluene (2x) and purification by column chromatography (20→100% EtOAc in pentane, then 20% MeOH in EtOAc) yielded the title compound (42.3 g, 51.4 mmol, 82%) as a white foam. R_f: 0.72 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -18.2° (*c* = 0.71, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.41 – 7.30 (m, 5H, Ar), 6.15 (d, 1H, *J* = 7.8 Hz, NH serine), 5.63 (d, 1H, *J* = 7.0 Hz, NH GlcN), 5.20 (s, 2H, CH_2 Bn), 4.83

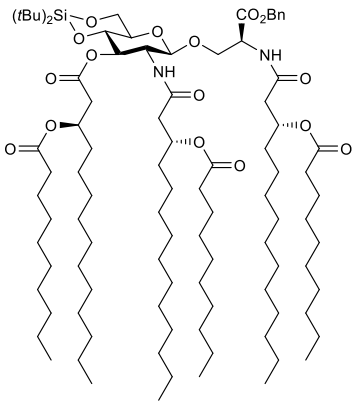
– 4.68 (m, 4H, 2x CH_2 Troc), 4.59 – 4.50 (m, 2H, H-1, CH serine), 4.24 (dd, 1H, *J* = 10.5, 4.2 Hz, C*H*H serine), 3.92 – 3.84 (m, 2H, C*H*H-6, C*H*H serine), 3.81 (dd, 1H, *J* = 10.5, 5.7 Hz, C*H*H-6), 3.75 – 3.59 (m, 3H, H-3, 2x OH), 3.59 – 3.49 (m, 1H, H-4), 3.37 – 3.25 (m, 2H, H-2, H-5), 0.89 (s, 9H, 3x CH_3 TBDMS), 0.12 – 0.05 (m, 6H, 2x CH_3 TBDMS); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 169.4 (C=O serine), 155.2, 154.7 (C=O Troc), 135.2 (C_q Ar), 128.7, 128.6, 128.3 (Ar), 100.6 (C-1), 95.5, 95.4 (C_q Troc), 74.9, 74.8 (CH_2 Troc), 74.3 (C-3), 74.3 (C-5), 73.6 (C-4), 68.7 (CH_2 serine), 67.8 (CH_2 CO_2Bn), 64.5 (CH_2-6), 57.6 (C-2), 54.5 (CH serine), 25.9, 18.3, -5.3 (CH_3 TBDMS); FT-IR (neat, cm^{-1}): 3341, 2954, 2930, 2857, 1733, 1531, 1462, 1389, 1253, 1203, 1165, 1062, 950, 836, 778, 733, 698, 569; HRMS: $[M+Na]^+$ calcd. for $C_{28}H_{40}Cl_6N_2O_{11}SiNa$: 841.0425, found 841.0437.

Benzyl *N*-trichloroethoxycarbonyl-*O*-[4,6-*O*-di-*tert*-butylsilylidene-2-*N*-trichloroethoxycarbonyl-β-D-glucopyranosyl]-L-serinate (24)



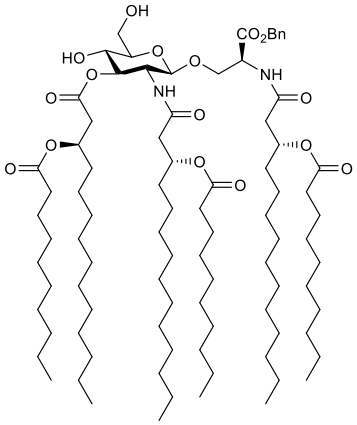
A solution of compound **20** (2.01 g, 2.84 mmol, 1.0 eq.) in DMF (14 mL) was cooled to -40°C. Di-*tert*-butylsilanediyl-bistriflate (0.92 mL, 3.1 mmol, 1.1 eq.) was added drop-wise. After one hour, the reaction was allowed to warm-up to room temperature and stirred overnight. The reaction mixture was quenched by the addition of pyridine (1.6 mL, 19.9 mmol, 7.0 eq.). The mixture was diluted with Et_2O and the organic layer was washed with H_2O (1x) and sat. aq. $NaHCO_3$ (3x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (2→3% acetone in DCM), the title compound (2.07 g, 2.44 mmol, 86%) was obtained as a white foam. R_f: 0.60 (1/1 pentane/Et_2O); $[\alpha]_D^{25}$ -24.0° (*c* = 0.86, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.38 – 7.28 (m, 5H, Ar), 6.30 (s, 1H, NH serine), 5.80 (d, 1H, *J* = 7.7 Hz, NH GlcN), 5.23 – 5.12 (m, 2H, CH_2 Bn), 4.81 – 4.65 (m, 5H, H-1, 2x CH_2 Troc), 4.53 (dt, 1H, *J* = 7.8, 3.4 Hz, CH serine), 4.25 (dd, 1H, *J* = 10.3, 3.3 Hz, C*H*H serine), 4.14 (dd, 1H, *J* = 10.1, 5.0 Hz, C*H*H-6), 3.89 – 3.80 (m, 2H, C*H*H-6, C*H*H serine), 3.80 – 3.72 (m, 1H, H-3), 3.67 (t, 1H, *J* = 8.9 Hz, H-4), 3.42 – 3.34 (m, 1H, H-5), 3.34 – 3.25 (m, 1H, H-2), 3.22 (br, 1H, OH), 1.04 (s, 9H, 3x CH_3 *t*Bu), 0.97 (s, 9H, 3x CH_3 *t*Bu); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 169.3 (C=O serine), 154.6, 154.5 (C=O Troc), 135.1 (C_q Ar), 128.6, 128.5, 128.2 (Ar), 100.8 (C-1), 95.5, 95.4 (C_q Troc), 77.4 (C-4), 74.6 (CH_2 Troc), 73.5 (C-3), 70.3 (C-5), 68.9 (CH_2 serine), 67.6 (CH_2 Bn), 66.0 (CH_2-6), 57.4 (H-2), 54.5 (CH serine), 27.4, 27.0 (CH_3 *t*Bu), 22.6, 19.9 (C_q *t*Bu); FT-IR (neat, cm^{-1}): 3340, 2935, 2886, 2860, 1730, 1523, 1473, 1387, 1365, 1336, 1243, 1201, 1160, 1076, 1009, 943, 909, 826, 765, 730, 697, 653, 618, 569, 476; HRMS: $[M+Na]^+$ calcd. for $C_{30}H_{42}Cl_6N_2O_{11}SiNa$: 867.0581, found 867.0599.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4,6-*O*-di-*tert*-butylsilylidene-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (25)



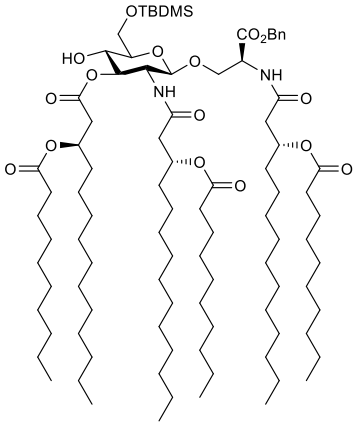
To a solution of compound **24 (**2.55 g, 3.00 mmol, 1.0 eq.) in THF (30 mL) was added activated zinc (4.0 g, 61 mmol, 20 eq.) and AcOH (0.69 mL, 12 mmol, 4.0 eq.) under an argon atmosphere. The suspension was stirred for 25 minutes and the mixture was subsequently sonicated for 5 min. The mixture was stirred again for 25 min, followed by sonicating for 5 minutes. TLC and LC-MS analysis showed complete conversion of the starting material. The suspension was filtered over a Whatmann filter and the residue was washed with DCM and EtOAc. The combined filtrates were concentrated *in vacuo*, co-evaporated with toluene (3x) and the obtained solid was dissolved in EtOAc. The solution was subsequently washed with 0.1 M HCl (1x), sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. A mixture of the obtained yellow oil and acid **15** (5.39 g, 13.5 mmol, 4.5 eq.) was co-evaporated with toluene (1x) and dissolved in DCM (30 mL) under an argon atmosphere. EDC·MeI (4.01 g, 13.5 mmol, 4.5 eq.) and DMAP (11 mg, 90 µmol, 0.03 eq.) were added and the reaction mixture was stirred 4 hours, after which the mixture was concentrated *in vacuo*. Several purifications by column chromatography (2→20% EtOAc in DCM + 0.1% Et_3N and 0→10% acetone in DCM + 0.1% Et_3N) gave compound **25** (3.07 g, 1.87 mmol, 62% over two steps) as a white foam. R_f: 0.58 (95/5 DCM/acetone); $[\alpha]_D^{25}$ -15.4° (*c* = 0.50, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 7.37 – 7.27 (m, 5H, Ar), 7.01 (d, 1H, *J* = 7.8 Hz, NH serine), 6.27 (d, 1H, *J* = 8.3 Hz, NH GlcN), 5.22 – 5.05 (m, 5H, 3x CH FA, CH_2 Bn), 5.06 – 4.98 (m, 1H, H-3), 4.72 – 4.66 (m, 2H, H-1, CH serine), 4.21 (dd, 1H, *J* = 10.7, 3.0 Hz, C*H*H serine), 4.14 (dd, 1H, *J* = 10.2, 5.0 Hz, C*H*H serine), 3.88 – 3.77 (m, 3H, H-4, C*H*H-6, C*H*H serine), 3.73 – 3.65 (m, 1H, H-2), 3.44 – 3.37 (m, 1H, H-5), 2.67 – 2.20 (m, 12H, 6x CH_2 FA), 1.71 – 1.50 (m, 12H, 6x CH_2 FA), 1.40 – 1.17 (m, 90H, 45x CH_2 FA), 1.02 (s, 9H, 3x CH_3 *t*Bu), 0.94 (s, 9H, 3x CH_3 *t*Bu), 0.87 (t, 18H, *J* = 6.7 Hz, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 173.9, 173.8, 173.3, 170.6, 170.3, 170.2 (C=O FA), 169.5 (C=O serine), 135.5 (C_q Ar), 128.6, 128.4, 128.1 (Ar), 101.6 (C-1), 75.1 (C-4), 74.4 (C-3), 71.5, 71.5 (CH FA), 70.8 (C-5), 70.1 (CH FA), 68.8 (CH_2 serine), 67.3 (CH_2 Bn), 66.3 (CH_2-6), 54.7 (C-2), 52.8 (CH serine), 42.2, 41.3, 39.2, 34.7, 34.6, 34.6, 34.5, 34.0, 32.1, 32.0, 32.0, 29.9, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.3 (CH_2 FA), 27.5, 27.0 (CH_3 *t*Bu), 25.5, 25.4, 25.2, 25.2, 25.1, 22.8 (CH_2 FA), 22.7, 20.0 (C_q *t*Bu), 14.2 (CH_3 FA); FT-IR (neat, cm^{-1}): 3285, 3068, 2956, 2923, 2854, 1734, 1652, 1540, 1450, 1466, 1378, 1364, 1246, 1173, 1075, 1030, 1011, 837, 827, 769, 723, 696, 652, 581, 463; HRMS: $[M+H]^+$ calcd. for $C_{96}H_{173}N_2O_{16}Si$: 1638,2549, found 1638.2493.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (26)



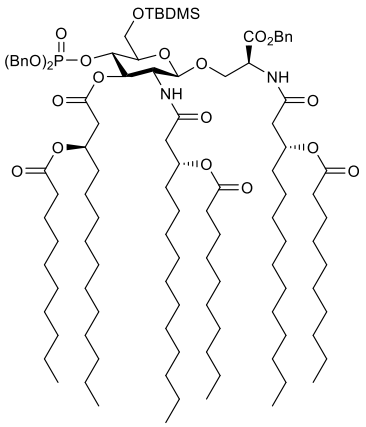
Compound **25** (1.92 g, 1.17 mmol, 1.0 eq.) was dissolved in THF (12 mL) under an argon atmosphere and cooled to 0°C. HF·Et_3N (0.58 mL, 3.6 mmol, 3.0 eq.) was added and the reaction mixture was stirred for 1.5 h, after which TLC analysis showed complete conversion of the starting material. The reaction was quenched with sat. aq. $NaHCO_3$, diluted with EtOAc and washed with brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (0→4% MeOH in DCM) yielded the title compound (1.61 g, 1.08 mmol, 92%). R_f: 0.64 (8/2 DCM/acetone); $[\alpha]_D^{25}$ -11.0° (*c* = 0.71, $CHCl_3$/MeOH 1/1); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.41 – 7.24 (m, 5H, Ar), 7.04 (d, 1H, *J* = 7.8 Hz, NH serine), 6.38 (d, 1H, *J* = 8.5 Hz, NH GlcN), 5.23 – 5.03 (m, 5H, 3x CH FA, CH_2 Bn), 4.96 (t, 1H, *J* = 8.0 Hz, H-3), 4.74 – 4.64 (m, 1H, CH serine), 4.57 (d, 1H, *J* = 8.2 Hz, H-1), 4.24 – 4.12 (m, 1H, C*H*H serine), 3.93 – 3.80 (m, 2H, C*H*H serine, C*H*H-6), 3.80 – 3.67 (m, 2H, H-2, C*H*H-6), 3.67 – 3.52 (m, 2H, H-4, OH), 3.39 – 3.26 (m, 1H, H-5), 2.76 (br, 1H, OH), 2.67 – 2.35 (m, 6H, 3x CH_2 FA), 2.35 – 2.18 (m, 6H, 3x CH_2 FA), 1.72 – 1.46 (m, 12H, 6x CH_2 FA), 1.46 – 0.95 (m, 90H, 45x CH_2 FA), 0.86 (t, 18H, *J* = 6.7 Hz, 6x CH_3 FA).^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.6, 174.0, 173.8, 171.6, 170.4, 170.2 (C=O FA), 169.8 (C=O serine), 135.4 (C_q Ar), 128.6, 128.4, 128.1 (Ar), 101.3 (C-1), 76.0 (C-3), 75.8 (C-5), 71.5, 71.3, 71.2 (CH FA), 69.3 (C-4), 68.7 (CH_2 serine), 67.3 (CH_2 Bn), 62.2 (CH_2-6), 54.0 (C-2), 52.9 (CH serine), 42.0, 41.3, 40.3, 34.8, 34.7, 34.6, 34.5, 34.5, 32.0, 32.0, 32.0, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 29.3, 29.2, 25.4, 25.4, 25.3, 25.1, 25.1, 25.1, 22.8 (CH_2 FA), 14.2 (CH_3 FA); FT-IR (neat, cm^{-1}): 3509, 3285, 3092, 2956, 2922, 2853, 1733, 1708, 1647, 1553, 1499, 1467, 1419, 1378, 1307, 1250, 1176, 1130, 1102, 1046, 1003, 964, 906, 722, 696, 673, 597, 510, 478; HRMS: $[M+H]^+$ calcd. for $C_{88}H_{157}N_2O_{16}$: 1498.15276, found 1498.15332.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[6-*O*-*tert*-butyldimethylsilyl-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (27)



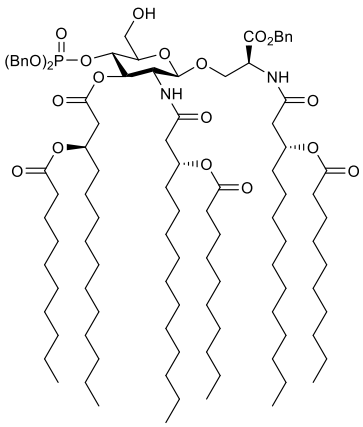
TBDMSCl (290 mg, 1.92 mmol, 1.5 eq.) was added to a solution of compound **26** (1.81 g, 1.21 mmol, 1.0 eq) in pyridine (8.0 mL). After stirring at room temperature for 3 hours, TLC analysis showed complete conversion of the starting material. The reaction mixture was diluted with EtOAc, washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (2x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (5→20% EtOAc in toluene) afforded the title compound (1.71 g, 1.06 mmol, 88%). R_f: 0.43 (6/3 toluene/EtOAc); $[\alpha]_D^{25}$ -10.0° (*c* = 0.47, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.34 – 7.23 (m, 5H, Ar), 7.09 (d, 1H, *J* = 7.7 Hz, NH serine), 6.40 (d, 1H, *J* = 8.3 Hz, NH GlcN), 5.19 – 5.04 (m, 5H, 3x CH FA, CH_2 Bn), 5.01 – 4.92 (m, 1H, H-3), 4.73 – 4.64 (m, 1H, CH serine), 4.58 (d, 1H, *J* = 8.0 Hz, H-1), 4.19 (dd, 1H, *J* = 10.8, 3.1 Hz, C*H*H serine), 3.90 – 3.75 (m, 3H, C*H*H serine, CH_2-6), 3.75 – 3.65 (m, 1H, H-2), 3.65 – 3.56 (m, 1H, H-4), 3.53 (d, 1H, *J* = 2.6 Hz, OH), 3.37 – 3.27 (m, 1H, H-5), 2.69 – 2.18 (m, 12H, 6x CH_2 FA), 1.73 – 1.45 (m, 12H, 6x CH_2 FA), 1.45 – 1.02 (m, 90H, 45x CH_2 FA), 0.93 – 0.74 (m, 27H, 6x CH_3 FA, 3x CH_3 TBDMS), 0.03 (s, 6H, 2x CH_3 TBDMS); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.0, 173.7, 173.7, 171.2, 170.3, 170.2 (C=O FA), 169.5 (C=O serine), 135.5 (C_q Ar), 128.5, 128.2, 127.9 (Ar), 101.2 (C-1), 75.7 (C-3), 74.8 (C-5), 71.5, 71.3, 70.8 (CH FA), 70.6 (C-4), 68.4 (CH_2 serine), 67.1 (CH_2 Bn), 63.8 (CH_2-6), 54.0 (C-2), 52.8 (CH serine), 41.9, 41.2, 39.9, 34.6, 34.5, 34.5, 31.9, 31.9, 31.9, 29.8, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.2, 29.2 (CH_2 FA), 25.8 (CH_3 TBDMS), 25.4, 25.2, 25.0, 25.0, 22.7, 18.2 (CH_2 FA)), 14.1 (CH_3 FA), -5.4, -5.5 (CH_3 TBDMS); FT-IR (neat, cm^{-1}): 3284, 3094, 2955, 2920, 2852, 1729, 1645, 1538, 1498, 1467, 1419, 1378, 1322, 1250, 1211, 1179, 1139, 1067, 1006, 965, 909, 837, 816, 778, 749, 722, 695, 668, 560, 555, 498, 480, 463; HRMS: $[M+H]^{2+}$ calcd. for $C_{94}H_{171}N_2O_{16}Si$: 806.11962, found 806.34730.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-bis(benzyloxy)phosphoryl-6-*O*-*tert*-butyldimethylsilyl-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (28)



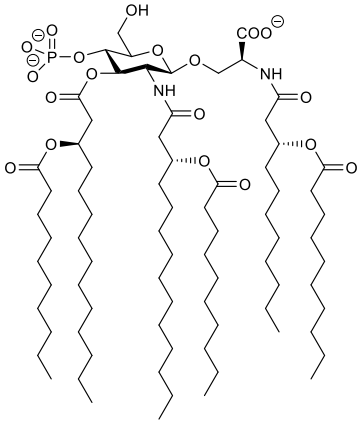
Compound **27** (1.71 g, 1.06 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry DCM (18 mL). Dibenzyl diisopropylaminephosphoramidite (0.70 mL, 1.9 mmol, 1.8 eq.) and tetrazole (186 mg, 2.65 mmol, 2.5 eq.) were added. After stirring for 35 minutes, the reaction mixture was cooled to 0°C, followed by the addition of m-CPBA (0.74 g, 3.0 mmol, 2.8 eq.). After 40 minutes, TLC analysis showed complete conversion into the phosphate. The reaction was diluted with aq. sat. $NaHCO_3$ and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (0→20% EtOAc in toluene) and several size exclusions (DCM/MeOH: 1/1) gave compound **28** in quantitative yield (2.00 g). R_f: 0.77 (2/1 toluene/EtOAc); $[\alpha]_D^{25}$ +3.6° (*c* = 1.1, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.39 – 7.23 (m, 15H, Ar), 7.13 (d, 1H, *J* = 8.0 Hz, NH serine), 6.41 (d, 1H, *J* = 7.4 Hz, NH GlcN), 5.32 (dd, 1H, *J* = 10.5, 9.0 Hz, H-3), 5.22 – 5.11 (m, 5H, 3x CH FA, CH_2 CO_2Bn), 4.97 (d, 3H, *J* = 7.8 Hz, H-1, 2x C*H*H dibenzyl phosphate), 4.91 (d, 2H, *J* = 7.7 Hz, 2x C*H*H dibenzyl phosphate), 4.77 – 4.70 (m, 1H, CH serine), 4.37 (q, 1H, *J* = 9.0 Hz, H-4), 4.26 (dd, 1H, *J* = 11.2, 3.2 Hz, C*H*H serine), 3.94 – 3.85 (m, 1H, C*H*H-6), 3.81 (dd, 1H, *J* = 11.2, 2.6 Hz, C*H*H serine), 3.72 (dd, 1H, *J* = 11.9, 5.1 Hz, C*H*H-6), 3.51 – 3.36 (m, 2H, H-2, H-5), 2.68 (dd, 1H, *J* = 14.8, 6.1 Hz, C*H*H FA), 2.59 – 2.46 (m, 2H, 2x C*H*H FA), 2.46 – 2.18 (m, 9H, 1x C*H*H FA, 4x CH_2 FA), 1.74 – 1.43 (m, 12H, 6x CH_2 FA), 1.43 – 1.14 (m, 90H, 45x CH_2 FA), 0.95 – 0.77 (m, 27H, 6x CH_3 FA, 3x CH_3 TBDMS), 0.03 – -0.06 (m, 6H, 2x CH_3 TBDMS); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.9, 173.7, 173.7, 171.0, 170.3, 170.3 (C=O FA), 169.6 (C=O), 135.6 (C_q Ar), 128.7, 128.7, 128.7, 128.6, 128.4, 128.1, 128.1, 128.0 (Ar), 100.4 (C-1), 75.4, 75.4 (C-5), 74.0, 73.9 (C-4), 73.1, 73.0 (C-3), 71.4, 71.1, 70.3 (CH FA), 69.7, 69.6, 69.6, 69.5 (CH_2 dibenzyl phosphate), 69.0 (CH_2 serine), 67.3 (CH_2 CO_2Bn), 62.0 (CH_2-6), 55.9 (C-2), 52.9 (CH serine), 41.7, 41.2, 39.8, 34.8, 34.7, 34.6, 34.6, 32.1, 32.0, 29.9, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4 (CH_2 FA), 25.9 (CH_3 TBDMS), 25.5, 25.4, 25.3, 25.2, 25.2, 25.1, 22.8, 18.4 (CH_2 FA), 14.2 (CH_3 FA), -5.0, -5.2 (CH_3 TBDMS); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -1.64; FT-IR (neat, cm^{-1}): 3306, 2924, 2854, 1735, 1660, 1541, 1499, 1465, 1379, 1251, 1164, 1108, 1013, 903, 837, 779, 733, 696, 600, 502; HRMS: $[M+H]^+$ calcd. for $C_{108}H_{184}N_2O_{19}PSi$: 1872.29947, found 1872.30474.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-bis(benzyloxy)phosphoryl-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (29)



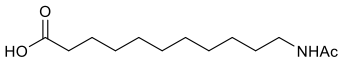
TFA (0.81 mL, 11 mmol, 10 eq.) was added to a solution of phosphate **28** (2.00 g, 1.06 mmol, 1.0 eq.) in DCM (21 mL) at 0°C. After 20 minutes, the resulting yellow solution was allowed to warm-up to room temperature and stirred for an additional 3 hours. TLC analysis showed complete conversion and the reaction was quenched with aq. sat. $NaHCO_3$ at 0°C. The reaction mixture was further diluted with H_2O and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (10→50% EtOAc in toluene), compound **29** (1.57 g, 0.893 mmol, 84%) was obtained as a white foam. R_f: 0.70 (1/1 pentane/EtOAc); $[\alpha]_D^{25}$ +2.2° (*c* = 0.33, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.38 – 7.24 (m, 15H, Ar), 7.19 – 7.08 (m, 1H, NH serine), 6.57 (d, 1H, *J* = 7.7 Hz, NH GlcN), 5.36 (t, 1H, *J* = 9.7 Hz, H-3), 5.23 – 5.07 (m, 5H, 3x CH FA, CH_2 CO_2Bn), 5.05 – 4.88 (m, 5H, H-1, 2x CH_2 dibenzyl phosphate), 4.76 – 4.67 (m, 1H, CH serine), 4.44 (q, 1H, *J* = 9.3 Hz, H-4), 4.28 – 4.18 (m, 1H, C*H*H serine), 3.93 – 3.84 (m, 1H, C*H*H serine), 3.82 – 3.71 (m, 3H, CH_2-6, OH), 3.60 – 3.51 (m, 1H, H-2), 3.37 (d, 1H, *J* = 9.7 Hz, H-5), 2.69 (dd, 1H, *J* = 14.7, 6.1 Hz, C*H*H FA), 2.54 (dd, 1H, *J* = 14.7, 5.8 Hz, C*H*H FA), 2.43 – 2.23 (m, 8H, 4x CH_2 FA), 2.20 (t, 2H, *J* = 7.5 Hz, CH_2 FA), 1.74 – 1.48 (m, 12H, 6x CH_2 FA), 1.48 – 1.06 (m, 90H, 45x CH_2 FA), 0.94 – 0.80 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.5, 173.4, 173.1, 170.6, 170.1, 169.7 (C=O FA), 169.4 (C=O serine), 135.3, 135.2, 135.2, 135.1, 135.1 (C_q Bn), 128.8, 128.7, 128.6, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.8, 127.8 (Ar), 100.4 (C-1), 74.7, 74.7 (C-5), 73.1, 73.0 (C-4), 72.3, 72.2 (C-3), 71.2, 70.8 (CH FA), 69.9, 69.9 (CH_2 dibenzyl phosphate), 69.8 (CH FA), 68.7 (CH_2 serine), 67.0 (CH_2 CO_2Bn), 60.3 (CH_2-6), 55.1 (C-2), 52.8 (CH serine), 41.4, 41.0, 39.0, 34.4, 34.3, 34.2, 31.8, 31.8, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 25.2, 25.2, 25.0, 24.9, 24.9, 22.6, 22.6 (CH_2 FA), 14.0 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -0.05; FT-IR (neat, cm^{-1}): 3317, 3066, 2956, 2923, 2853, 1733, 1654, 1640, 1541, 1499, 1466, 1456, 1379, 1238, 1166, 1128, 1106, 1080, 1034, 1016, 914, 736, 696, 602, 531, 498; HRMS: $[M+H]^+$ calcd. for $C_{102}H_{170}N_2O_{19}P$: 1758,2130, found 1758.2065.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (1)



After co-evaporating with toluene (3x) under an argon atmosphere, compound **29** (21.7 mg, 12.3 µmol, 1.0 eq.) was dissolved in THF (1.0 mL), followed by the addition of Pd/C (10%, 21 mg). A $H_{2(g)}$-filled balloon was applied on the obtained black suspension. After stirring at room temperature for 3 hours, the mixture was filtered over a Whatmann filter. The filter was washed with DCM, followed by the addition of Et_3N (3.4 µL, 24 µmol, 2.0 eq.). After mixing for 5 minutes, the clear solution was concentrated *in vacuo* and purified by size exclusion (DCM/MeOH: 1/1). Lyophilization gave compound **1** (18.2 mg, 12.2 µmol, 99%) as a white solid. ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC) δ 5.20 – 5.15 (m, 1H, CH FA), 5.14 – 5.07 (m, 3H, H-3, 2x CH FA), 4.54 – 4.49 (m, 2H, H-1, CH serine), 4.19 – 4.08 (m, 2H, H-4, C*H*H serine), 3.88 (d, *J* = 13.1 Hz, 1H, C*H*H-6), 3.84 – 3.78 (m, 1H, C*H*H serine), 3.72 – 3.66 (m, 2H, H-2, C*H*H-6), 3.28 (d, *J* = 9.8 Hz, 1H, H-5), 2.63 – 2.45 (m, 4H, 2x CH_2 FA), 2.40 (dd, *J* = 14.7, 7.3 Hz, 1H, C*H*H FA), 2.29 (dd, *J* = 14.7, 5.7 Hz, 1H, C*H*H FA), 2.28 – 2.20 (m, 6H, 3x CH_2 FA), 1.60 – 1.47 (m, 12H, 6x CH_2 FA), 1.30 – 1.15 (m, 90H, 45x CH_2 FA), 0.85 – 0.81 (m, 18H, 6x CH_3 FA); ^{13}C NMR ($CDCl_3$, 214 MHz, HSQC) δ 173.8, 173.8, 173.7, 170.8, 170.6, 170.5 (C=O), 100.7 (C-1), 75.3 (C-5), 73.5 (C-3), 71.1, 70.8 (CH FA), 70.3 (C-4), 70.1 (CH FA), 69.2 (CH serine), 59.9 (CH_2-6), 54.1 (C-2), 52.6 (CH serine), 41.0, 40.5, 38.8, 34.4, 34.4, 34.2, 34.1, 34.0, 31.8, 31.8, 31.8, 31.8, 29.6, 29.6, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.4, 29.3, 29.3, 29.3, 29.3, 29.2, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 25.2, 25.1, 25.1, 24.9, 24.9, 22.6, 22.5, 22.5 (CH_2 FA), 13.9 (CH_3 FA); ^{31}P NMR ($CDCl_3$, 202 MHz, HMBC) δ 2.40; HRMS: $[M+H]^+$ calcd. for $C_{81}H_{152}N_2O_{19}P$: 1488.0721, found 1488.0725.

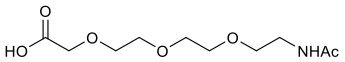
11-Acetamidoundecanoic acid (30)



11-Bromo undecanoic acid (2.87 g, 10.8 mmol, 1.0 eq.) was dissolved in aq. ammonium hydroxide (30%, 80 mL). After stirring overnight, the mixture was concentrated *in vacuo*. Co-evaporation with toluene afforded the amine as a white solid (3.16 g, quant.). The amine (0.61 g, 3.0 mmol, 1.0 eq.) was dissolved in pyridine (4.3 mL) and cooled to 0°C. Ac_2O (0.85 mL, 9.0 mmol, 3.0 eq.) and DMAP (cat.) were added and the solution was allowed to warm-up to room temperature and stirred for 1 hour. The reaction mixture was quenched with H_2O, concentrated *in vacuo* and co-evaporated with toluene (4x). The residue was dissolved in DCM and washed with 1 M HCl. The aqueous layer was extracted with DCM (3x) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Crystallization in DCM/pentane yielded the title compound (0.41 g, 1.7 mmol, 56%) as a white solid. R_f: 0.43 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -1.9° (*c* = 0.47, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC):

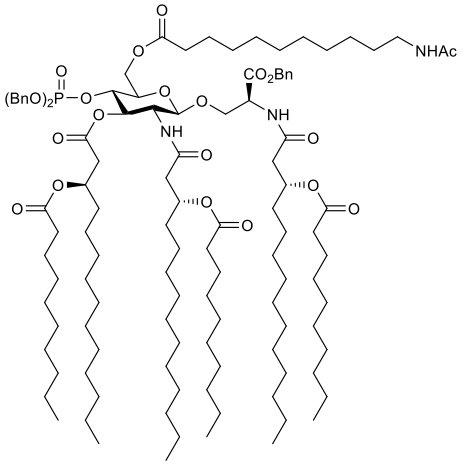
δ 5.65 (s, 1H, NH), 3.23 (q, 2H, *J* = 6.9 Hz, C*H*$_2$NHAc), 2.34 (t, 2H, *J* = 7.5 Hz, CH_2), 1.99 (s, 3H, CH_3 Ac), 1.68 – 1.58 (m, 2H, CH_2), 1.53 – 1.44 (m, 2H, CH_2), 1.35 – 1.23 (m, 12H, 6x CH_2); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 178.5 (C=O), 170.6 (C=O Ac), 39.9 (*C*H_2NHAc, 34.1, 29.6, 29.4, 29.3, 29.2, 29.2, 29.0, 26.9, 24.8 (CH_2), 23.4 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3289, 3086, 2916, 2850, 1695, 1641, 1543, 1470, 1434, 1372, 1298, 1245, 1217, 1192, 927, 721, 610; HRMS: $[M+Na]^+$ calcd. for $C_{13}H_{25}NO_3Na$: 266.1727, found 266.1731.

2-Oxo-6,9,12-trioxa-3-azatetradecan-14-oic acid (31)



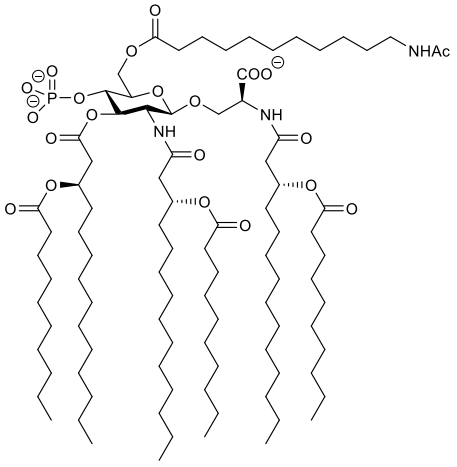
To a solution of *tert*-butyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (0.58 g, 2.0 mmol, 1.0 eq.) in THF (15 mL) was added PPh_3 (0.68 g, 2.6 mmol, 1.3 eq.). After stirring for 24 hours, H_2O (0.1 mL, 5.2 mmol, 2.6 eq.) were added and the mixture was continued to stir for two more days. The reaction mixture was diluted with H_2O, washed with toluene (2x) and the aqueous layer was concentrated *in vacuo*. The obtained amine was dissolved in pyridine (3.0 mL), cooled to 0°C, followed by the addition of Ac_2O (1.3 mL, 14 mmol, 7.0 eq.) and DMAP (27 mg, 0.22 mmol, 0.11 eq.). The reaction mixture was allowed to warm-up to room temperature and stirred for 1 hour, after which the reaction was quenched with H_2O at 0°C and concentrated *in vacuo*. This gave acetyl (0.54 g, 1.77 mmol, 89% over two steps) as an oil. The intermediate (0.22 g, 0.70 mmol, 1.0 eq.) was dissolved in DCM (3.5 mL) and cooled to 0°C. TFA (1.1 mL, 14 mmol, 20 eq.) was added and after 30 minutes the mixture was allowed to warm-up to room temperature. R_f: 0.46 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -1.7° (*c* = 0.81, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 10.98 (s, 1H, OH), 6.80 (s, 1H, NH), 4.09 (s, 2H, CH_2), 3.68 (dd, 2H, *J* = 5.6, 3.1 Hz, CH_2), 3.66 – 3.51 (m, 6H, 3x CH_2), 3.49 (t, 2H, *J* = 5.1 Hz, CH_2), 3.36 (q, 2H, *J* = 5.2 Hz, C*H*$_2$NHAc), 1.95 (s, 3H, CH_3); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 172.3, 171.7 (C=O), 70.9, 70.4, 70.3, 69.9, 69.6, 68.5 (CH_2), 39.4 (*C*H_2NHAc), 22.7 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3330, 2874, 1731, 1620, 1552, 1433, 1375, 1351, 1293, 1221, 1099, 939, 882, 723, 678, 560, 543; HRMS: $[M+Na]^+$ calcd. for $C_{10}H_{19}NO_6Na$: 272.1105, found 272.1112.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-[bis(benzyloxy)phosphoryl]-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-6-*O*-(11-acetamidoundecanoyl)-β-D-glucopyranosyl]-L-serinate (32)



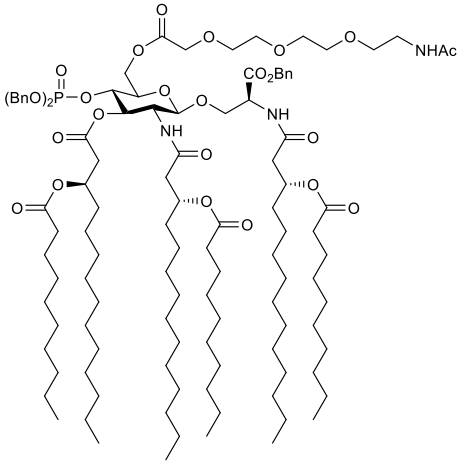
Compound **29** (57.6 mg, 32.8 µmol, 1.0 eq) and acid **30** (20.8 mg, 85.5 µmol, 2.6 eq.) were co-evaporated twice with toluene under an argon atmosphere before being dissolved in dry DCE (0.5 ml). The solution was cooled to 0°C, followed by the addition of EDC·MeI (20.8 mg, 68.6 µmol, 2.1 eq.) and DMAP (5.3 mg, 43 µmol, 1.3 eq.). The obtained yellow suspension was allowed to warm-up to room temperature and was stirred overnight. The white suspension was diluted with aq. sat. $NaHCO_3$ and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→60% EtOAc in pentane) yielded the title compound (57.4 mg, 28.9 µmol, 88%). R_f: 0.34 (1/1 pentane/EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 7.37 – 7.21 (m, 15H, Ar), 7.10 (d, 1H, *J* = 7.9 Hz, NH serine), 6.44 (d, 1H, *J* = 7.1 Hz, NH GlcN), 5.67 (br, 1H, NHAc), 5.33 (d, 1H, *J* = 9.4 Hz, H-3), 5.22 – 5.10 (m, 5H, 3x CH FA, CH_2 CO_2Bn), 5.00 – 4.93 (m, 3H, H-1, 2x C*H*H dibenzyl phosphate), 4.89 (d, 2H, *J* = 7.7 Hz, 2x C*H*H dibenzyl phosphate), 4.72 (d, 1H, *J* = 7.9 Hz, CH serine), 4.43 – 4.32 (m, 2H, H-4, C*H*H-6), 4.29 – 4.23 (m, 1H, C*H*H serine), 4.11 (dd, 1H, *J* = 12.3, 5.1 Hz, C*H*H-6), 3.82 (d, 1H, *J* = 9.5 Hz, C*H*H serine), 3.57 (dd, 1H, *J* = 8.9, 4.1 Hz, H-5), 3.46 – 3.37 (m, 1H, H-2), 3.25 – 3.17 (m, 2H, CH_2NHAc), 2.67 (dd, 1H, *J* = 14.8, 6.1 Hz, C*H*H FA), 2.57 – 2.46 (m, 2H, 2x C*H*H FA), 2.41 – 2.18 (m, 11H, CH_2 linker, 4x CH_2 FA, C*H*H FA), 1.96 (s, 3H, CH_3 Ac), 1.71 – 1.41 (m, 16H, 2x CH_2 linker, 6x CH_2 FA), 1.39 – 1.11 (m, 102H, 6x CH_2 linker, 45x CH_2 FA), 0.92 – 0.81 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 173.8, 173.7, 173.7, 173.5, 171.1, 170.4, 170.2, 170.1 (C=O FA, linker, Ac), 169.5 (C=O serine), 135.5, 135.5, 135.5, 135.5, 135.5 (C_q Ar), 128.8, 128.7, 128.7, 128.6, 128.5, 128.2, 128.1, 128.1 (Ar), 100.5 (C-1), 74.0, 73.9 (C-4), 72.6, 72.6 (C-5), 72.5 (C-3), 71.4, 71.0, 70.4 (CH FA), 69.8, 69.8 (CH_2 dibenzyl phosphate), 69.2 (CH_2 serine), 67.3 (CH_2 CO_2Bn), 62.2 (CH_2-6), 55.8 (C-2), 52.8 (CH serine), 41.7, 41.2 (CH_2 FA), 39.8 (*C*H_2NHAc), 34.7, 34.6, 34.6 (CH_2 FA), 34.0 (CH_2 linker), 32.0, 32.0 (CH_2 FA), 29.9 (CH_2 linker), 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.7, 29.6, 29.5, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.4, 29.3, 29.3, 29.1 (CH_2 FA), 27.0 (CH_2 linker), 25.5, 25.4, 25.2, 25.2, 25.1 (CH_2 FA), 24.8 (CH_2 linker), 23.5 (CH_3 Ac), 22.8, 22.8 (CH_2 FA), 14.2 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ -1.32; FT-IR (neat, cm^{-1}): 3313, 3066, 2955, 2921, 2852, 1731, 1649, 1541, 1499, 1466, 1456, 1378, 1274, 1212, 1169, 1110, 1013, 906, 796, 732, 696, 603, 527, 501, 460; HRMS: $[M+H]^{2+}$ calcd. for $C_{115}H_{193}N_3O_{21}P$: 992.19657, found 992.19715.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-6-_O_-(11-acetamidoundecanoyl)-β-D-glucopyranosyl]-L-serine (2)



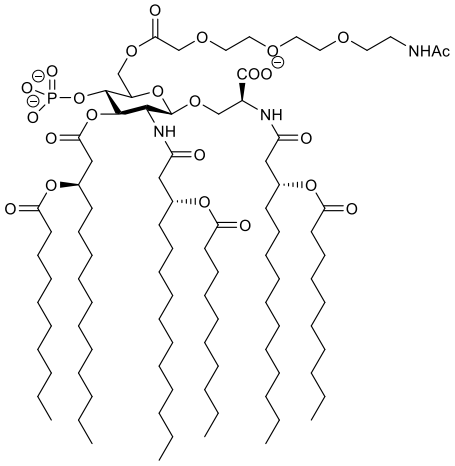
Compound **32** (26.7 mg, 13.5 µmol, 1 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in THF (1.0 mL). Pd/C (10%, 20.8 mg) was added and the reaction mixture was stirred for 2.5 hours at room temperature under a blanket of $H_{2(g)}$. The black suspension was filtered over a Whatmann filter and the filter was washed with $CHCl_3$. Et_3N (4.0 µL, 28.6 µmol, 2.1 eq.) was added to the combined filtrates, mixed for 5 minutes and the solution was concentrated *in vacuo*. After purification by size exclusion (DCM/MeOH: 1/1) and lyophilization, compound **2** (12.0 mg, 7.00 µmol, 52%) was obtained as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz, HH-COSY, HSQC): δ 5.21 – 5.15 (m, 2H, CH FA), 5.15 – 5.06 (m, 2H, H-3, CH FA), 4.60 (d, 1H, *J* = 7.0 Hz, H-1), 4.57 – 4.53 (m, 1H, CH serine), 4.49 (d, 1H, *J* = 11.0 Hz, C*H*H-6), 4.22 – 4.07 (m, 3H, H-4, C*H*H-6, C*H*H serine), 3.71 – 3.62 (m, 3H, H-2, H-5, C*H*H serine), 3.11 (t, 2H, *J* = 7.2 Hz, CH_2NHAc), 2.62 – 2.54 (m, 3H, CH_2 FA, C*H*H FA), 2.50 (dd, 1H, *J* = 14.6, 5.8 Hz, C*H*H FA), 2.40 (dd, 1H, *J* = 14.6, 7.3 Hz, C*H*H FA), 2.34 – 2.21 (m, 9H, C*H*H FA, 3x CH_2 FA, CH_2 linker), 1.90 (s, 3H, CH_3 Ac), 1.62 – 1.48 (m, 14H, 6x CH_2 FA, CH_2 linker), 1.46 – 1.41 (m, 2H, CH_2 linker), 1.33 – 1.15 (m, 102H, 45x CH_2 FA, 6x CH_2 linker), 0.83 (t, 18H, *J* = 6.9 Hz, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 151 MHz, HSQC): δ 174.5, 174.2, 174.0, 171.7, 171.0, 170.9, 170.7 (C=O), 100.1 (C-1), 73.5 (C-3), 73.3, 73.3 (C-5), 71.8, 71.7 (C-4), 71.3, 71.2, 70.5 (CH FA), 68.7 (CH_2 serine), 63.9 (CH_2-6), 54.3 (C-2), 52.6 (CH serine), 41.2, 40.7 (CH_2 FA), 39.8 (*C*H_2NHAc), 39.3, 34.7, 34.6, 34.6, 34.5, 34.5, 34.4, 34.2, 32.1, 32.1, 32.1, 32.0, 29.9, 29.9, 29.9, 29.9, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.3, 29.2, 27.0, 25.5, 25.4, 25.4, 25.2, 25.2, 25.0, 22.8, 22.8 (CH_2 FA, CH_2 linker), 22.6 (CH_3 Ac), 14.2 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 0.59; HRMS: $[M+H]^+$ calcd. for $C_{94}H_{175}N_3O_{21}P$: 1713.2450, found 1713.2458.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-[bis(benzyloxy)phosphoryl]-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-6-*O*-(13-acetamido-3-oxo-2,5,8,11-tetraoxatridecyl)-β-D-glucopyranosyl]-L-serinate (33)



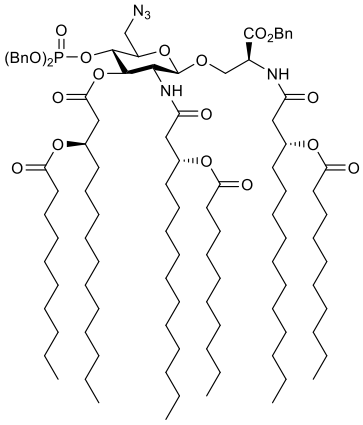
Compound **29** (49.6 mg, 28.2 µmol, 1.0 eq) and acid **31** (22.5 mg, 90.3 µmol, 3.2 eq.) were co-evaporated twice with toluene under an argon atmosphere before being dissolved in dry DCE (0.43 ml). The solution was cooled to 0°C, followed by the addition of EDC·MeI (17.7 mg, 59.6 µmol, 2.1 eq.) and DMAP (2.2 mg, 18 µmol, 0.6 eq.). The obtained yellow suspension was allowed to warm-up to room temperature and was stirred overnight. The resulting white suspension was diluted DCM (0.6 mL) and stirred for an additional 4 hours. The reaction mixture was subsequently diluted with aq. sat. $NaHCO_3$ and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→30% acetone in DCM) yielded the title compound (41.3 mg, 20.8 µmol, 74%). R_f: 0.65 (6/4 DCM/acetone); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.40 – 7.21 (m, 15H, Ar), 7.10 (d, 1H, *J* = 7.9 Hz, NH serine), 6.46 (d, 1H, *J* = 7.4 Hz, NH GlcN), 6.30 (br, 1H, NHAc), 5.33 (dd, 1H, *J* = 10.3, 9.0 Hz, H-3), 5.22 – 5.09 (m, 5H, 3x CH FA, CH_2 CO_2 Bn), 4.99 (d, 2H, *J* = 8.1 Hz), H-1, 4.95 (d, 3H, *J* = 8.6 Hz, 2x C*H*H dibenzyl phosphate), 4.88 (d, 2H, *J* = 7.8 Hz, 2x C*H*H dibenzyl phosphate), 4.76 – 4.68 (m, 1H, CH serine), 4.44 – 4.31 (m, 2H, H-4, C*H*H-6), 4.29 – 4.18 (m, 2H, C*H*H-6, C*H*H serine), 4.12 (s, 2H, CH_2 linker), 3.83 (dd, 1H, *J* = 11.3, 2.6 Hz, C*H*H serine), 3.70 – 3.57 (m, 9H, H-5, 4x CH_2 linker), 3.53 (t, 2H, *J* = 5.0 Hz, CH_2 linker), 3.47 – 3.37 (m, 3H, H-2, CH_2NHAc), 2.68 (dd, 1H, *J* = 14.9, 5.9 Hz, C*H*H FA), 2.57 – 2.42 (m, 2H, 2x C*H*H FA), 2.40 – 2.18 (m, 9H, C*H*H FA, 4x CH_2 FA), 1.96 (s, 3H, CH_3 Ac), 1.71 – 1.50 (m, 12H, 6x CH_2 FA), 1.50 – 1.15 (m, 90H, 45x CH_2 FA), 0.90 – 0.83 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.9, 173.9, 173.7, 171.1, 170.4, 170.2, 170.0 (C=O FA, linker, Ac), 169.5 (C=O serine), 135.5, 135.5, 135.4, 135.4, 135.4 (C_q Ar), 128.9, 128.8, 128.8, 128.7, 128.7, 128.5, 128.2, 128.2 (Ar), 100.6 (C-1), 73.7, 73.7 (C-4), 72.4, 72.4, 72.3 (C-3, C-5), 71.5, 71.1 (CH FA), 70.9, 70.6, 70.6 (CH_2 linker), 70.3 (CH FA), 70.3, 69.9 (CH_2 linker), 69.9, 69.9, 69.8, 69.8 (CH_2 dibenzyl phosphate), 69.2 (CH_2 serine), 68.4 (CH_2 linker), 67.4 (CH_2 CO_2Bn), 62.5 (CH_2-6), 55.7 (C-2), 52.9 (CH serine), 41.7, 41.3, 39.7 (CH_2 FA), 39.4 (*C*H_2NHAc), 34.8, 34.8, 34.7, 34.6, 34.6, 32.1, 32.0, 29.9, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 25.5, 25.4, 25.3, 25.2, 25.1, 23.3, 22.8, 22.8 (CH_2 FA), 14.2 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -1.15; FT-IR (neat, cm^{-1}): 3301, 2923, 2853, 1734, 1654, 1541, 1457, 1378, 1265, 1152, 1111, 1012, 905, 736, 697, 601, 501; HRMS: $[M+H]^+$ calcd. for $C_{112}H_{181}N_3O_{24}P$: 1989.32367, found 1989.32201.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-6-_O_-(13-acetamido-3-oxo-2,5,8,11-tetraoxatridecyl)-β-D-glucopyranosyl]-L-serine (3)



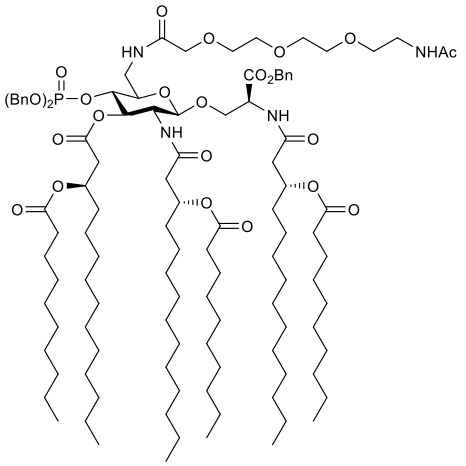
Compound **31** (19.7 mg, 9.90 µmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in THF (1.0 mL). Pd/C (10%, 19.8 mg) was added and the reaction mixture was stirred for 3 hours at room temperature under a blanket of $H_{2(g)}$. The black suspension was filtered over a Whatmann filter. The filter was washed with $CHCl_3$ and Et_3N (3.0 µL, 22 µmol, 2.2 eq.) was added to the combined filtrates. The solution was mixed for 5 minutes and concentrated *in vacuo*. After purification by size exclusion (DCM/MeOH: 1/1) and lyophilization, compound **3** (6.7 mg, 3.9 µmol, 39%) was obtained as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz, HH-COSY, HSQC): δ 5.22 – 5.15 (m, 2H, 2x CH FA), 5.15 – 5.04 (m, 2H, H-3, CH FA), 4.60 – 4.47 (m, 3H, H-1, CH serine, C*H*H-6), 4.33 – 4.12 (m, 5H, H-4, C*H*H-6, C*H*H serine, CH_2 linker), 3.78 – 3.71 (m, 3H, H-2, C*H*H serine, C*H*H linker), 3.69 – 3.53 (m, 10H, H-5, 4x CH_2 linker, C*H*H linker), 3.44 –3.32 (m, 2H, C*H*$_2$NHAc), 2.68 – 2.46 (m, 4H, 2x CH_2 FA), 2.41 (dd, 1H, *J* = 14.5, 7.2 Hz, C*H*H FA), 2.30 (dd, 1H, *J* = 14.5, 5.7 Hz, C*H*H FA), 2.28 – 2.19 (m, 6H, 3x CH_2 FA), 1.95 (s, 3H, CH_3 Ac), 1.63 – 1.46 (m, 12H, 6x CH_2 FA), 1.33 – 1.15 (m, 90H, 45x CH_2), 0.83 (t, 18H, *J* = 7.0 Hz, 6x CH_3); ^{13}C-APT NMR ($CDCl_3$, 151 MHz, HSQC): δ 174.1, 174.0, 173.8, 172.6, 171.2, 170.8, 170.7 (C=O), 100.6 (C-1), 73.9 (C-3), 72.7, 72.7 (C-5), 71.3 (CH FA), 71.1 (C-4, CH FA), 70.4 (CH_2 linker), 70.3 (CH FA), 70.1, 69.9 (CH_2 linker), 69.4 (CH_2 serine, CH_2 linker), 68.5 (CH_2 linker), 62.5 (CH_2-6), 53.8 (C-2), 52.4 (CH serine), 41.4, 40.7, 39.3 (CH_2 FA), 39.1 (*C*H_2NHAc), 34.7, 34.6, 34.6, 34.5, 34.5, 34.3, 32.1, 32.1, 32.0, 32.0, 29.9, 29.9, 29.9, 29.9, 29.8, 29.8, 29.8, 29.8, 29.7, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.3, 25.5, 25.4, 25.3, 25.2, 25.2, 25.2, 22.8, 22.8 (CH_2 FA), 22.6 (CH_3 Ac), 14.1 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 1.52; HRMS: $[M+H]^+$ calcd. for $C_{91}H_{169}N_3O_{24}P$: 1719.18282, found 1719.18284.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[6-azide-4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (34)



After co-evaporating with toluene (2x), compound **29** (82 mg, 47 µmol, 1.0 eq.) was dissolved in THF under an argon atmosphere. PPh_3 (48 mg, 0.18 mmol, 3.9 eq.) was added and the reaction mixture was cooled to -20 °C. DEAD (15 µL, 96 µmol, 2.0 eq.) and DPPA (20.5 µL, 96 µmol, 2.0 eq.) were added subsequently and the stirring was continued for 1 hour, followed by the addition of DEAD (15 µL, 96 µmol, 2.0 eq.) and DPPA (20.5 µL, 96 µmol, 2.0 eq.). After stirring for 1 hour at -20°C, the reaction mixture was slowly warmed-up to room temperature overnight. The mixture was concentrated *in vacuo*. Purification by column chromatography (10→30% EtOAc in pentane) afforded the title compound (56 mg, 31 µmol, 66%). R_f: 0.52 (7/3 pentane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.38 – 7.24 (m, 15H, Ar), 7.02 (d, 1H, *J* = 8.1 Hz, NH serine), 6.44 (d, 1H, *J* = 7.4 Hz, NH GlcN), 5.34 (dd, 1H, *J* = 10.5, 8.9 Hz, H-3), 5.21 – 5.12 (m, 5H, 3x CH FA, CH_2 CO_2Bn), 5.03 (d, 1H, *J* = 8.2 Hz, H-1), 5.00 – 4.94 (m, 2H, 2x C*H*H Bn), 4.91 (dd, 2H, *J* = 7.9, 3.2 Hz, 2x C*H*H Bn), 4.78 – 4.70 (m, 1H, CH serine), 4.32 – 4.21 (m, 2H, H-4, C*H*H serine), 3.85 (dd, 1H, *J* = 11.2, 2.8 Hz, C*H*H serine), 3.55 – 3.48 (m, 1H, H-5), 3.46 – 3.37 (m, 2H, H-2, C*H*H-6), 3.28 (dd, 1H, *J* = 13.4, 6.2 Hz, C*H*H-6), 2.67 (dd, 1H, *J* = 14.9, 6.2 Hz, C*H*H FA), 2.55 – 2.44 (m, 2H, 2x C*H*H FA), 2.41 – 2.19 (m, 9H, C*H*H FA, 4x CH_2 FA), 1.71 – 1.43 (m, 12H, 6x CH_2 FA), 1.37 – 1.14 (m, 90H, 45x CH_2 FA), 0.93 – 0.81 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.8, 173.7, 171.1, 170.3, 170.0 (C=O FA), 169.5 (C=O serine), 135.5, 135.5, 135.4 (C_q Bn), 128.9, 128.8, 128.8, 128.7, 128.6, 128.4, 128.3, 128.3, 128.2 (Ar), 100.3 (C-1), 74.5, 74.5 (C-5), 74.0, 73.9 (C-4), 72.3, 72.3 (C-3), 71.4, 71.0, 70.3 (CH FA), 70.0, 70.0, 69.9, 69.9 (CH_2 dibenzyl phosphate), 69.2 (CH_2 serine), 67.3 (CH_2 CO_2Bn), 55.8 (C-2), 52.8 (CH serine), 50.7 (CH_2N_3), 41.6, 41.2, 39.8, 34.7, 34.6, 34.6, 34.6, 32.0, 32.0, 29.9, 29.8, 29.8, 29.8, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 25.5, 25.4, 25.3, 25.2, 25.2, 25.1, 22.8, 22.8 (CH_2 FA), 14.2 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -1.03; FT-IR (neat, cm^{-1}): 3276, 2992, 2853, 2361, 2102, 1733, 1654, 1543, 1498, 1457, 1274, 1248, 1171, 1108, 1010, 904, 734, 696, 601, 506; HRMS: $[M+H]^+$ calcd. for $C_{102}H_{169}N_5O_{18}P$: 1783.22059, found 1783.22059.

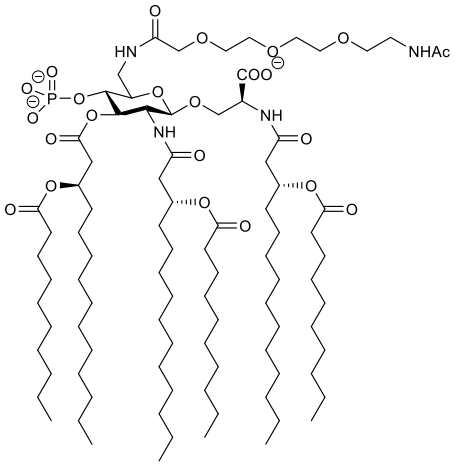
Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-[bis(benzyloxy)phosphoryl]-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-6-*N*-(13-acetamido-3-oxo-5,8,11-trioxa-2-azatridecyl)-β-D-glucopyranosyl]-L-serinate (35)



Compound **34** (23.6 mg, 13.2 µmol, 1.0 eq.) was dissolved in a mixture of DCM/MeOH/H_2O (1,1,0.1 v/v/v, 1.2 mL). Activated zinc powder (9.1 mg, 0.15 mmol, 11.6 eq.) and NH_4Cl (7.9 mg, 0.15 mmol, 11.2 eq.) were added and the reaction mixture was stirred for 6 hours. The reaction mixture was subsequently diluted with DCM and washed with aq. sat. $NaHCO_3$ (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained amine (13.2 µmol, 1.0 eq.) and acid **31** (10.6 mg, 42.5 µmol, 3.2 eq.) were co-evaporated with toluene (2x) under an argon atmosphere before being dissolved in dry DCE (0.4 ml). The solution was cooled to 0°C, followed by the addition of EDC·MeI (8.5 mg, 29 µmol, 2.2 eq.) and DMAP (0.7 mg, 6 µmol, 0.4 eq.). The resulting yellow suspension was allowed to warm-up to room temperature and was stirred overnight. The obtained white suspension was diluted with aq. sat. $NaHCO_3$ and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→40% acetone in DCM) and size exclusion (DCM/MeOH: 1/1) afforded the title compound (10.6 mg, 5.33 µmol, 40% over two steps). R_f: 0.44 (6/4 DCM/acetone); ^{1}H NMR ($CDCl_3$, 600 MHz, HH-COSY, HSQC): δ 7.41 – 7.37 (m, 1H, NH-6), 7.37 – 7.23 (m, 16H, NH serine, Ar), 6.60 (t, 1H, *J* = 4.9 Hz, NHAc), 6.41 (d, 1H, *J* = 7.8 Hz, NH GlcN), 5.26 – 5.09 (m, 6H, H-3, 3x CH-FA, CH_2 CO_2Bn), 5.02 (d, 2H, *J* = 8.1 Hz, 2x C*H*H dibenzyl phosphate), 4.98 – 4.89 (m, 2H, 2x C*H*H dibenzyl phosphate), 4.82 (d, 1H, *J* = 8.2 Hz, H-1), 4.77 – 4.72 (m, 1H, CH serine), 4.26 – 4.13 (m, 2H, H-4, C*H*H serine), 4.07 – 3.97 (m, 3H, C*H*H-6, CH_2 linker), 3.85 (dd, 1H, *J* = 11.2, 3.1 Hz, C*H*H serine), 3.69 – 3.47 (m, 11H, H-2, 5x CH_2 linker), 3.42 – 3.35 (m, 3H, H-5, CH_2NHAc), 3.16 – 3.10 (m, 1H, C*H*H-6), 2.68 (dd, 1H, *J* = 14.8, 6.1 Hz, C*H*H FA), 2.56 (dd, 1H, *J* = 14.8, 6.1 Hz, C*H*H FA), 2.40 – 2.19 (m, 10H, 5x CH_2 FA), 1.94 (s, 3H, Ac), 1.79 – 1.49 (m, 12H, 6x CH_2 FA), 1.48 – 1.10 (m, 90H, 45x CH_2 FA), 0.90 – 0.85 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 151 MHz, HSQC): δ 173.9, 173.8, 173.7, 170.9, 170.6, 170.5, 170.5, 170.2 (C=O FA, linker), 169.7 (C=O serine), 135.6, 135.5, 35.5, 135.5, 135.5 (C_q Ar), 128.9, 128.8, 128.8, 128.8, 128.7, 128.6, 128.4, 128.3, 128.2 (Ar), 100.6 (C-1), 75.1, 75.1 (C-4), 73.2, 73.2 (C-5), 72.4, 72.4 (C-3), 71.5, 71.2 (CH FA), 70.9, 70.8, 70.6, 70.4 (CH_2 linker), 70.3 (CH FA), 70.2 (CH_2 linker), 70.0, 70.0, 70.0 (CH_2 dibenzyl phosphate), 69.1 (CH_2 serine), 67.4 (CH_2 CO_2Bn), 55.4 (C-2), 52.9 (CH serine), 41.8, 41.1, 39.6, 39.4 (CH_2 FA), 39.3 (CH_2-6), 34.8, 34.8, 34.6, 34.6, 32.1, 32.0, 32.0, 29.9, 29.9, 29.8, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 25.5, 25.4, 25.3, 25.2, 25.2, 25.2 (CH_2 FA), 23.2 (CH_3 Ac), 22.8, 22.8 (CH_2 FA), 14.3 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -0.81; FT-IR (neat, cm^{-1}): 2924, 2854,

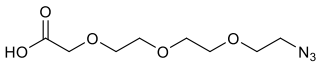
2016, 1734, 1663, 1547, 1466, 1163, 1016, 743, 698, 604, 595; HRMS: $[M+H]^{2+}$ calcd. for $C_{112}H_{188}N_4O_{23}P$: 994.1699, found 994.1779.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-6-_N_-(13-acetamido-3-oxo-5,8,11-trioxa-2-azatridecyl)-β-D-glucopyranosyl]-L-serine (4)



Compound **35** (10.1 mg, 5.08 µmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in THF (1.0 mL). Pd/C (10%, 21 mg) was added and the reaction mixture was stirred for 3.5 hours at room temperature under a blanket of $H_{2(g)}$. The black suspension was filtered over a Whatmann filter and the filter was washed with $CHCl_3$. Et_3N (1.5 µL, 10.8 µmol, 2.1 eq.) was added to the combined filtrates. The solution was mixed for 5 minutes and concentrated *in vacuo*. After purification by size exclusion (DCM/MeOH: 1/1) and lyophilization, the title compound (5.8 mg, 3.4 µmol, 67%) was obtained as a white solid; ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC) δ 5.11 – 5.06 (m, 1H, CH FA), 5.07 – 5.03 (m, 1H, CH FA), 5.03 – 4.97 (m, 1H, CH FA), 4.97 – 4.93 (m, 1H, H-3), 4.45 (s, 1H, CH serine), 4.39 (d, *J* = 7.6 Hz, 1H, H-1), 4.02 (d, *J* = 9.9 Hz, 1H, C*H*H serine), 4.00 – 3.87 (m, 3H, H-4, CH_2 linker), 3.84 – 3.78 (m, 1H, C*H*H-6), 3.69 (d, *J* = 8.3 Hz, 1H, C*H*H serine), 3.64 – 3.47 (m, 9H, H-2, 4x CH_2 linker), 3.45 – 3.39 (m, 2H, CH_2 linker), 3.36 (s, 1H, H-5), 3.27 – 3.24 (m, 2H, CH_2NHAc), 3.23 – 3.20 (m, 1H, C*H*H-6), 2.54 – 2.49 (m, 1H, C*H*H FA), 2.49 – 2.42 (m, 2H, 2x C*H*H FA), 2.42 – 2.38 (m, 1H, C*H*H FA), 2.29 (dd, *J* = 14.6, 7.2 Hz, 1H, C*H*H FA), 2.20 (dd, *J* = 14.6, 5.6 Hz, 1H, C*H*H FA), 2.18 – 2.09 (m, 6H, 3x CH_2 FA), 1.84 (s, 3H, CH_3 Ac), 1.51 – 1.37 (m, 12H, 6x CH_2 FA), 1.21 – 1.06 (m, 90H, 45x CH_2 FA), 0.74 (t, *J* = 7.2 Hz, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 214 MHz, HSQC): δ 173.7, 173.7, 170.9, 170.7 (C=O), 100.6 (C-1), 73.2, 73.2 (C-3/4/5), 71.1, 71.0 (CH FA), 70.9, 70.9 (CH FA), 70.6 (CH_2 linker), 70.1, 70.1 (CH FA), 69.9, 69.9, 69.8, 69.7 (CH_2 linker), 69.1 (CH_2 serine), 53.8 (C-2), 52.6 (CH serine), 41.1, 40.4 (CH_2 FA), 39.0 (*C*H_2NHAc), 39.0 (CH_2-6), 34.4, 34.4, 34.3, 34.2, 34.1, 31.8, 31.8, 29.6, 29.6, 29.6, 29.5, 29.4, 29.4, 29.4, 29.3, 29.2, 29.2, 29.1, 29.1, 25.2, 25.1, 25.1, 24.9, 22.5 (CH_2 FA), 13.9 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 1.19; HRMS: $[M+H]^+$ calcd. for $C_{91}H_{170}N_4O_{23}P$: 1718.19880, found 1718.19982.

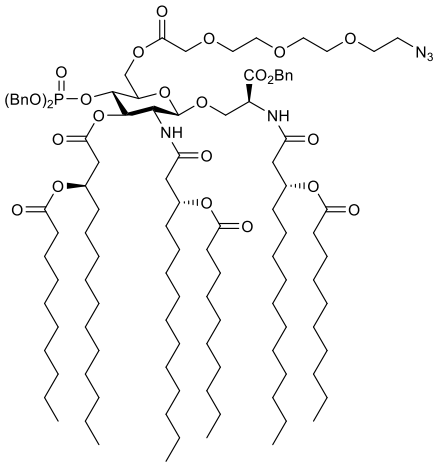
2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)acetic acid (36)



A mixture of 2-(2-(2-chloroethoxy)ethoxy)ethan-1-ol (4.39 g, 26.1 mmol, 1.0 eq.) and sodium azide (3.39 g, 52.1, 2.0 eq.) in DMF (35 mL) was heated to 90°C and stirred overnight. The suspension was cooled down to room temperature and concentrated *in vacuo*. The residue was dissolved in H_2O and extracted with EtOAc (3x).

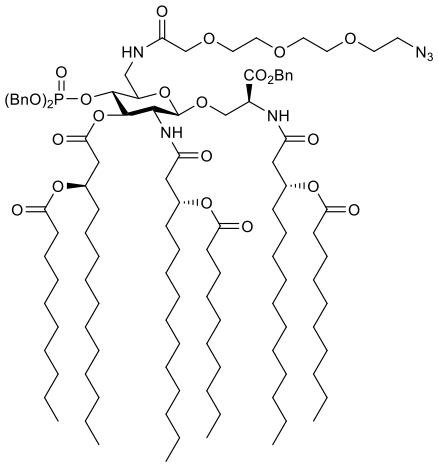
The combined organic layers were washed with brine (1x), dried over $MgSO_4$, filtered and concentred *in vacuo*, which yielded the azide in quantitative yield. After co-evaporation with toluene (2x) under an argon atmosphere, the oil was dissolved in THF (0.13 L) and cooled to 0°C. NaH (2.1 g, 52 mmol, 2.0 eq.) was added under an argon flow. The reaction mixture was stirred for 30 minutes, followed by the addition of *tert*-butyl bromoacetate (9.6 mL, 66 mmol, 2.5 eq.). After the reaction mixture was stirred at room temperature overnight, the reaction was quenched with MeOH and concentrated *in vacuo*. The residue was dissolved in DCM, filtered over celite and concentrated *in vacuo*. Purification by column chromatography (20→50% EtOAc in pentane) yielded methyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (3.90 g, 15.8 mmol, 61% over two steps). ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 4.07 (s, 2H, CH_2), 3.68 – 3.53 (m, 13H, 5x CH_2, CH_3), 3.29 (t, 2H, *J* = 4.8 Hz, CH_2N_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.7 (C=O), 70.7, 70.5, 70.5, 69.9, 68.4 (CH_2), 51.6 (CH_3), 50.5 (CH_2N_3). Methyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (3.90 g, 15.8 mmol, 1.0 eq.) was dissolved in a mixture of THF/MeOH/H_2O (7/2/1 v/v/v, 50 mL). LiOH (0.97 g, 41 mmol, 2.6 eq.) was added and the suspension was heated to 50°C for 2 hours. The reaction mixture was cooled to room temperature, acidified with 1 M HCl to pH = 2/3, diluted with H_2O and extracted several times with EtOAc and DCM. The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Co-evaporation with toluene (1x) and purification by column chromatography (50→100% EtOAc in pentane, then 20% MeOH in EtOAc) yielded the title compound (3.90 g, 15.8 mmol, 61%). R_f: 0.72 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -0.29° (*c* = 1.7, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz, HH-COSY, HSQC): δ 10.67 (br, 1H, OH), 4.15 (s, 2H, CH_2), 3.77 – 3.57 (m, 10H, 5x CH_2), 3.40 – 3.31 (m, 2H, CH_2N_3); ^{13}C-APT NMR ($CDCl_3$, 75 MHz, HSQC): δ 174.0 (C=O), 71.2, 70.6, 70.6, 70.4, 70.4, 70.0, 68.5 (CH_2), 50.6 (CH_2N_3); FT-IR (neat, cm^{-1}): 2873, 2102, 1744, 1286, 1120, 935, 855; HRMS: $[M+Na]^+$ calcd. for $C_8H_{15}N_3O_5Na$: 256,09039, found 256.09016.

Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[6-*O*-(11-azido-3,6,9-trioxaundecanoyl)-4-*O*-bis(benzyloxy)phosphoryl-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serinate (37)



A solution of alcohol **29** (0.57 g, 0.32 mmol, 1.0 eq.) and acid **36** (0.19 g, 0.81 mmol, 2.5 eq.) was cooled to 0 °C under an argon atmosphere. EDC·MeI (0.20 g, 0.67 mmol, 2.0 eq.) and DMAP (2.0 mg, 16 µmol, 0.05 eq.) were added. After stirring for 15 minutes, the reaction mixture was allowed to warm-up to room temperature and stirring continued for 3 hours. Silica was added and the suspension was concentrated *in vacuo*. Purification by column chromatography (20→50% EtOAc in Toluene + 0.1% Et_3N) gave the title compound (0.51 g, 0.26 mmol, 80%). R_f: 0.68 (1/1 pentane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.36 – 7.17 (m, 15H, Ar), 7.09 (d, 1H, *J* = 7.9 Hz, NH serine), 6.45 (d, 1H, *J* = 7.4 Hz, NH GlcN), 5.36 – 5.27 (m, 1H, H-3), 5.20 – 5.06 (m, 5H, 3x CH FA, CH_2 CO_2Bn), 4.97 (d, 1H, *J* = 8.2 Hz, H-1), 4.92 (d, 2H, *J* = 8.7 Hz, 2x C*H*H dibenzyl phosphate), 4.85 (d, 2H, *J* = 7.8 Hz, 2x C*H*H dibenzyl phosphate), 4.73 – 4.66 (m, 1H, CH serine), 4.41 – 4.29 (m, 2H, H-4, C*H*H-6), 4.26 – 4.16 (m, 2H, C*H*H-6, C*H*H serine), 4.09 (s, 2H, CH_2 linker), 3.85 – 3.78 (m, 1H, C*H*H serine), 3.68 – 3.54 (m, 13H, H-5, 5x CH_2 linker), 3.44 – 3.36 (m, 1H, H-2), 3.33 – 3.28 (m, 2H, CH_2N_3), 2.66 (dd, 1H, *J* = 14.9, 6.0 Hz, C*H*H FA), 2.55 – 2.42 (m, 2H, 2x C*H*H FA), 2.38 – 2.17 (m, 9H, CHH FA, 4x CH_2 FA), 1.69 – 1.39 (m, 12H, 6x CH_2 FA), 1.37 – 1.10 (m, 90H, 45x CH_2 FA), 0.90 – 0.78 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.7, 173.6, 173.4, 170.9, 170.2, 170.0, 169.8 (C=O FA, C=O linker), 169.3 (C=O serine), 135.4, 135.3, 135.3, 135.2, 135.2 (C_q Ar), 128.6, 128.6, 128.5, 128.4, 128.3, 128.0, 128.0 (Ar), 100.4 (C-1), 73.5, 73.5 (C-4), 72.2, 72.2, 72.1 (C-3, C-5), 71.3, 70.8 (CH FA), 70.7, 70.5 (CH_2 linker), 70.1 (CH FA), 69.9 (CH_2 linker), 69.7, 69.6, 69.6 (CH_2 dibenzyl phosphate), 69.0 (CH_2 serine), 68.2 (CH_2 linker), 67.1 (CH_2 CO_2Bn), 62.2 (CH_2-6), 55.5 (C-2), 52.7 (CH serine), 50.5 (CH_2N_3), 41.5, 41.1, 39.6, 34.5, 34.4, 34.4, 31.9, 31.8, 29.7, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 29.2, 29.1, 25.3, 25.2, 25.0, 25.0, 24.9, 22.6, 22.6 (CH_2 Fa), 14.0 (CH_3 Fa); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ -1.34; FT-IR (neat, cm^{-1}): 3312, 2923, 2854, 2103, 1734, 1658, 1538, 1457, 1378, 1273, 1154, 1114, 1014, 736, 697, 601, 498; HRMS: $[M+H]^+$ calcd. for $C_{110}H_{183}N_5O_{23}P$: 1973.3036, found 1973.30465.

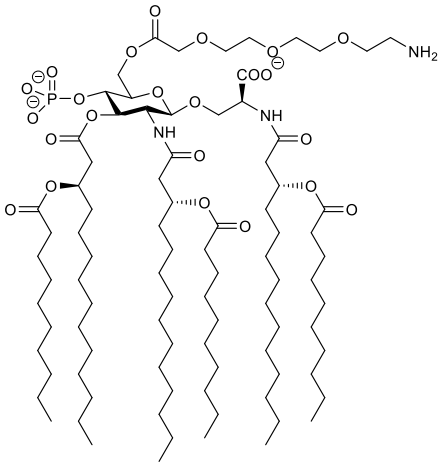
Benzyl *N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-*O*-[4-*O*-[bis(benzyloxy)phosphoryl]-2-*N*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-3-*O*-[(*R*)-3-(decanoyloxy)tetradecanoyl]-6-*N*-(13-acetamido-3-oxo-5,8,11-trioxa-2-azatridecyl)-β-D-glucopyranosyl]-L-serinate (38)



Azide **34** (76 mg, 43 µmol, 1.0 eq.) was dissolved in a mixture of DCM/MeOH/H_2O (1/1/0.1 v/v/v, 3.8 mL). Freshly prepared activated zinc powder (30 mg, 0.47 mmol, 11 eq.) and NH_4Cl (23.1 mg, 0.43 mmol, 11 eq.) were added and the suspension was stirred vigorously for 5.5 hours. The reaction mixture was subsequently diluted with DCM and washed with sat. aq. $NaHCO_3$ (1x). The aqueous layer was extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The obtained amine and acid **36** (27 mg, 0.12 mmol, 2.8 eq.) were dissolved in DCE (1.3 mL) under an argon atmosphere and cooled to 0°C. EDC·MeI (26 mg, 87 µmol, 2.0 eq.) and DMAP (2.8 mg, 23 µmol, 0.53 eq.) were added and the reaction mixture was allowed to warm-up to room temperature overnight. The white suspension was diluted with DCM and washed with sat. aq. $NaHCO_3$ (1x). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Purification by column chromatography (30→70% EtOAc in pentane + 0.1% Et_3N) and size exclusion (DCM/MeOH: 1/1) afforded compound **38** (48 mg, 24 µmol, 56% over two steps). R_f: 0.63 (9/1 DCM/aceton). ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC): δ 7.39 – 7.36 (m, 1H, NH-6), 7.36 – 7.26 (m, 16H, NH serine, Ar), 6.36 (d, 1H, *J* = 7.8 Hz, NH GlcN), 5.23 – 5.19 (m, 2H, H-3, C*H*H CO_2Bn), 5.19 – 5.16 (m, 1H, CH FA), 5.15 – 5.10 (m, 3H, 2x CH FA, C*H*H CO_2Bn), 5.02 (d, 2H, *J* = 8.1 Hz, 2x C*H*H dibenzyl phosphate), 4.96 (dd, 1H, *J* = 11.6, 8.4 Hz, C*H*H dibenzyl phosphate), 4.91 (dd, 1H, *J* = 11.6, 7.2 Hz, C*H*H dibenzyl phosphate), 4.81 (d, 1H, *J* = 8.2 Hz, H-1), 4.76 – 4.73 (m, 1H, CH serine), 4.23 (dd, 1H, *J* = 11.4, 2.9 Hz, C*H*H serine), 4.16 (q, 1H, *J* = 9.2 Hz, H-4), 4.01 (d, 1H, *J* = 15.6 Hz, C*H*H linker), 3.99 – 3.96 (m, 1H, C*H*H-6), 3.95 (d, 1H, *J* = 15.5 Hz, C*H*H linker), 3.86 (dd, 1H, *J* = 11.3, 3.3 Hz, C*H*H serine), 3.68 – 3.57 (m, 10H, 5x CH_2 linker), 3.52 – 3.47 (m, 1H, H-2), 3.33 – 3.30 (m, 3H, H-5, CH_2N_3), 3.12 – 3.08 (m, 1H, C*H*H-6), 2.68 (dd, 1H, *J* = 14.8, 6.3 Hz, C*H*H FA), 2.57 (dd, 1H, *J* = 14.7, 6.1 Hz, C*H*H FA), 2.38 – 2.25 (m, 8H, 4x CH_2 FA), 2.21 (t, 2H, *J* = 7.6 Hz, CH_2 FA), 1.68 –1.37 (m, 12H, 6x CH_2 FA), 1.37 – 1.14 (m, 90H, 45x CH_2 FA), 0.90 – 0.83 (m, 18H, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 214 MHz, HSQC): δ 173.9, 173.8, 173.6, 170.8, 170.5, 170.3, 170.1 (C=O FA, linker), 169.7 (C=O serine), 135.6, 135.5, 135.5, 135.5, 135.5 (C_q Ar), 128.8, 128.8, 128.7, 128.7, 128.7, 128.6, 128.4, 128.3, 128.2 (Ar), 100.5 (C-1), 74.9, 74.9 (C-4), 73.2, 73.2 (C-5), 72.3, 72.3 (C-3), 71.5, 71.1 (CH FA), 71.1, 70.7, 70.7, 70.5 (CH_2 linker), 70.2 (CH FA), 70.1 (CH_2 linker), 70.0, 69.9 (CH_2 dibenzyl phosphate), 69.2 (CH_2 serine), 67.3 (CH_2 CO_2Bn), 55.4 (C-2), 52.9 (CH serine), 50.7 (CH_2N_3), 41.8, 41.0, 39.5 (CH_2 FA), 39.1 (CH_2-6), 34.7, 34.7, 34.6, 34.6, 34.6, 32.1, 32.0, 32.0, 29.9, 29.8, 29.8, 29.8, 29.8, 29.8, 29.8, 29.7, 29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 25.5, 25.4, 25.3, 25.2, 25.2, 25.1, 22.8, 22.8

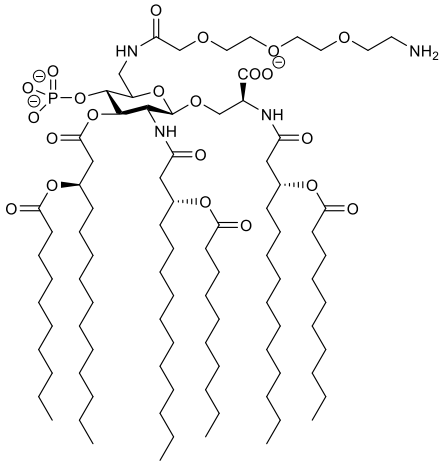
(CH_2 FA), 14.3, 14.2, 14.2 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ -0.78; FT-IR (neat, cm^{-1}): 3303, 2924, 2854, 2101, 1735, 1664, 1536, 1457, 1272, 1163, 1112, 1014, 735, 697; $[M+H]^+$ calcd. for $C_{110}H_{184}N_6O_{22}P$: 1972.31958, found 1972.31915.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[6-_O_-(11-amino-3,6,9-trioxaundecanoyl)-4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (39)



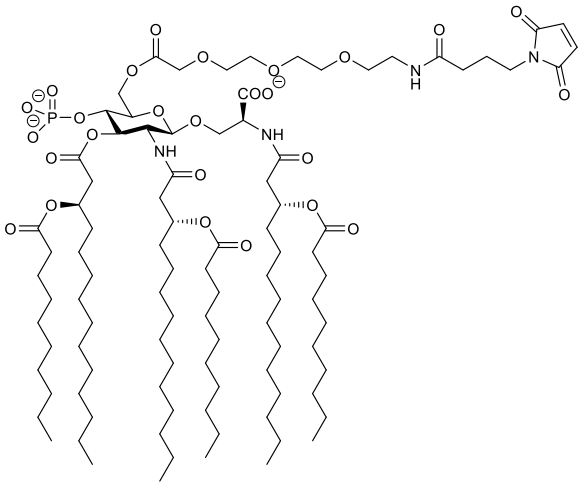
After co-evaporating with toluene (3x) under an argon atmosphere, compound **37** (0.51 g, 0.26 mmol, 1.0 eq.) was dissolved in dry THF (2.6 mL), followed by the addition of Pd/C (10%, 53 mg). A $H_{2(g)}$-filled balloon was applied on the obtained black suspension. After stirring at room temperature overnight, the black suspension was filtered over washed silica. The silica was washed $CHCl_3$, followed by the addition of Et_3N (0.14 mL, 1.0 mmol, 4 eq.). After mixing for 10 minutes, the clear solution was concentrated *in vacuo*. Lyophilization gave compound **39** (0.34 g, 0.20 mmol, 77%) as a white solid. ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC): δ 5.08 – 5.02 (m, 3H, 3x CH FA), 4.99 (t, 1H, *J* = 9.4 Hz, H-3), 4.53 (d, 1H, *J* = 9.6 Hz, C*H*H-6), 4.45 (d, 1H, *J* = 7.8 Hz, H-1), 4.33 (s, 1H, CH serine), 4.22 – 4.14 (m, 2H, C*H*H-6, C*H*H serine), 4.11 (q, 1H, *J* = 9.5 Hz, H-4), 4.07 – 3.92 (m, 3H, CHH serine, CH_2 linker), 3.74 – 3.69 (m, 1H, C*H*H linker), 3.68 – 3.48 (m, 11H, H-2, H-5, C*H*H linker, 4x CH_2 linker), 2.97 (s, 2H, CH_2NH_2), 2.56 (dd, 1H, *J* = 16.4, 6.7 Hz, C*H*H FA), 2.48 – 2.41 (m, 2H, 2x C*H*H FA), 2.38 – 2.30 (m, 2H, 2x C*H*H FA), 2.20 (dd, 1H, *J* = 14.7, 5.6 Hz, C*H*H FA), 2.18 – 2.11 (m, 6H, 3x CH_2 FA), 1.53 – 1.37 (m, 12H, 6x CH_2 FA), 1.25 – 1.03 (m, 90H, 45x CH_2 FA), 0.74 (t, 18H, *J* = 7.2 Hz, 6x CH_2 FA); ^{13}C-APT NMR ($CDCl_3$, 214 MHz, HSQC): δ 173.7, 173.6, 173.5, 170.9, 170.5, 170.1 (C=O), 100.3 (C-1), 73.8 (C-3), 72.9 (C-5), 71.1 (CH FA), 70.8, 70.8 (C-4), 70.8 (CH FA), 70.5, 70.2, 70.1, 70.1 (CH_2 linker), 70.1 (CH FA), 69.8, 69.8, 69.4 (CH_2 linker), 68.0 (CH_2 serine), 66.9 (CH_2 linker), 62.4 (CH_2-6), 53.7 (C-2), 53.1 (CH serine), 40.9, 40.8 (CH_2 FA), 38.9 (CH_2NH_2), 38.9, 34.4, 34.3, 34.2, 34.1, 34.0, 31.8, 31.8, 31.8, 31.8, 29.6, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 25.2, 25.2, 25.1, 25.0, 24.9, 22.5, 22.5 (CH_2 FA), 13.9 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 0.40; MALDI-FT-ICR MS (m/z): $[M+H]^+$ calcd. for $C_{89}H_{167}N_3O_{23}P$: 1677.1723, found 1677.1561.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[6-_N_-(11-amino-3,6,9-trioxaundecanoyl)-4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (40)



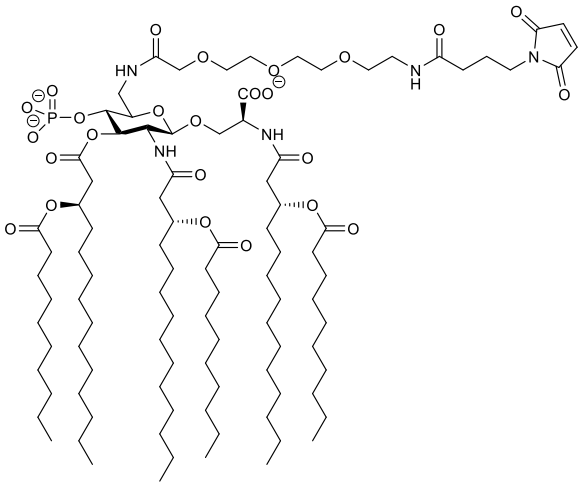
Compound **38** (21.4 mg, 10.8 µmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in THF (1.0 mL). Pd/C (10%, 21.1 mg) was added and the reaction mixture was stirred for 5.5 hours at room temperature under a blanket of $H_{2(g)}$. The black suspension was filtered over a Whatmann filter. The filter was washed with $CHCl_3$ and Et_3N (3.0 µL, 22 µmol, 2.0 eq.) was added to the combined filtrates. The solution was mixed for 5 minutes and concentrated *in vacuo*. Purification by size exclusion (DCM/MeOH: 1/1) afforded compound **40** (15.1 mg, 9.0 µmol, 83%). ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC): δ 5.17 – 5.07 (m, 2H, 2x CH FA), 5.07 – 4.96 (m, 2H, H-3, CH FA), 4.54 (s, 1H, CH serine), 4.27 (d, 1H, *J* = 7.8 Hz, H-1), 4.10 – 3.98 (m, 2H, C*H*H serine, C*H*H linker) 3.98 – 3.91 (m, 2H, H-4, C*H*H linker), 3.83 (d, 1H, *J* = 15.4 Hz, C*H*H-6), 3.77 (dd, 1H, *J* = 11.8, 4.2 Hz, C*H*H serine), 3.74 – 3.69 (m, 1H, H-2), 3.69 – 3.51 (m, 10H, 5x CH_2 linker), 3.45 – 3.40 (m, 1H, H-5), 3.09 – 3.03 (m, 1H, C*H*H-6), 3.02 – 2.99 (m, 2H, C*H*$_2$NH$_2$), 2.60 – 2.49 (m, 4H, 2x CH_2 FA), 2.34 (dd, 1H, *J* = 14.4, 7.1 Hz, C*H*H FA), 2.22 – 2.10 (m, 7H, C*H*H FA, 3x CH_2 FA), 1.51 – 1.38 (m, 12H, 6x CH_2 FA), 1.21 – 1.05 (m, 90H, 45x CH_2 FA), 0.74 (t, 18H, *J* = 7.2 Hz, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 214 MHz, HSQC): δ 174.0, 173.7, 173.3, 170.9 (C=O), 100.6 (C-1), 73.8 (C-3), 72.9 (C-5), 71.7 (C-4), 71.1 (CH FA), 70.9, 70.8 (CH_2 linker), 70.7 (CH FA), 69.8 (CH_2 serine), 69.7 (CH FA), 69.6, 69.5, 69.1 (CH_2 linker), 66.1, 56.4, 53.1 (C-2), 52.3 (CH serine), 46.1, 46.1, 41.1, 40.0 (CH_2 FA), 39.1 (*C*H$_2$NH$_2$), 38.9 (CH_2-6), 34.4, 34.4, 34.3, 34.2, 34.0, 31.8, 31.8, 31.8, 31.8, 31.8, 29.7, 29.7, 29.6, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.3, 29.3, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 29.0, 25.3, 25.2, 25.1, 25.1, 25.0, 25.0, 24.9, 22.6 (CH_2 FA), 13.9 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 162 MHz, HMBC): δ 1.91; MALDI-FT-ICR MS (m/z): $[M+H]^+$ calcd. for $C_{89}H_{168}N_4O_{22}P$: 1676.1882, found 1676.1736.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[6-_O_-(11-_N_-(4-maleimidobutanoyl)-11-amino-3,6,9-trioxa-undecanoyl)-4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (41)



Amine **39** (32.9 mg, 19.6 µmol, 1.0 eq.) was dissolved in DCM (1.6 mL), followed by the addition of sulfo-*N*-succinimidyl 4-maleimidobutyrate sodium salt (9.1 mg, 23.8 µmol, 1.2 eq.) and Et_3N (16.4 µL, 0.12 mmol, 6.0 eq.). After stirring for 4 hours, DCM (0.8 mL) was added to the obtained white suspension and the mixture was stirred overnight. The reaction mixture was diluted with brine and extracted with DCM (3x). The combined organic layers were concentrated *in vacuo*. Purification by size exclusion (DCM/MeOH: 1/1) afforded compound **41** (30.3 mg, 16.4 µmol, 84%). ^{1}H NMR ($CDCl_3$, 600 MHz, HH-COSY, HSQC): δ 6.64 (s, 2H, *H*C=C*H*), 5.11 – 5.01 (m, 3H, CH FA), 4.98 (t, 1H, *J* = 9.0 Hz, H-3), 4.63 – 4.54 (m, 1H, C*H*H-6), 4.45 – 4.32 (m, 2H, H-1, C*H*H-6), 4.28 (s, 1H, CH serine), 4.20 – 3.91 (m, 5H, H-4, CH_2 serine, CH_2 linker), 3.71 – 3.33 (m, 14H, H-2, H-5, 6x CH_2 linker), 3.24 – 3.17 (m, 2H, CH_2 linker), 2.56 (dd, 1H, *J* = 16.1, 7.0 Hz, C*H*H FA), 2.51 – 2.38 (m, 2H, 2x C*H*H FA), 2.38 – 2.28 (m, 2H, 2x C*H*H FA), 2.21 (dd, 1H, *J* = 14.6, 5.3 Hz, C*H*H FA), 2.17 – 2.11 (m, 6H, 3x CH_2 FA), 2.08 (t, 2H, *J* = 7.1 Hz, CH_2 linker), 1.82 – 1.72 (m, 2H, CH_2 linker), 1.51 – 1.37 (m, 12H, 6x CH_2 FA), 1.26 – 1.02 (m, 90H, 45x CH_2 FA), 0.73 (t, 18H, *J* = 7.0 Hz, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 151 MHz, HSQC): δ 173.8, 173.7, 173.6, 171.3, 171.1, 171.0, 170.9, 170.2 (C=O), 134.2 (C=C), 100.7 (C-1), 73.5 (C-3), 72.9 (C-5), 71.0 (CH FA), 70.9 (C-4), 70.8 (CH FA), 70.6, 70.3, 70.3, 70.2 (CH_2 linker), 70.1 (CH FA), 69.9, 69.7, 69.5, 69.5, 69.1, 69.0, 68.3 (CH_2 linker), 68.0 (CH_2 serine), 62.7 (CH_2-6) 53.6 (C-2), 53.3 (CH serine), 41.0, 40.7, 39.1, 39.0, 39.0, 38.7, 37.1, 36.9, 34.4, 34.3, 34.2, 34.2, 34.1, 32.6, 31.8, 31.8, 31.8, 31.8, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.3, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 25.2, 25.2, 25.1, 25.0, 24.9, 24.2, 22.6, 22.5 (CH_2 FA, CH_2 linker), 13.9 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 0.82; HRMS: $[M+H]^+$ calcd. for $C_{97}H_{174}N_4O_{26}P$: 1842.21484, found 1842.21478.

_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-_O_-[6-_N_-(11-_N_-(4-maleimidobutanoyl)-11-amino-3,6,9-trioxa-undecanoyl)-4-_O_-phosphoryl-2-_N_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-3-_O_-[(_R_)-3-(decanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-L-serine (42)



A solution of amine **40** in DCE (22.4 mM, 375 µL, 1.0 eq.) to an eppendorf tube containing sulfo-*N*-succinimidyl 4-maleimidobutyrate sodium salt (4.1 mg, 10.7 µmol, 1.2 eq.) and Et_3N (7.0 µL, 50 µmol, 6.0 eq.). After mixing overnight at 850 rpm, the mixture was diluted with brine and extracted with DCM (3x). The combined organic layers were concentrated *in vacuo*. Purification by size exclusion (DCM/MeOH: 1/1) afforded the title compound (12.5 mg, 6.8 µmol, 81%). ^{1}H NMR ($CDCl_3$, 850 MHz, HH-COSY, HSQC): δ 6.64 (s, 2H, *H*C=C*H*), 5.13 – 5.00 (m, 3H, 3x CH FA), 4.97 – 4.89 (m, 1H, H-3), 4.35 (d, 1H, *J* = 7.7 Hz, H-1), 4.23 (s, 1H, CH serine), 3.99 – 3.88 (m, 3H, H-4, C*H*H serine, CH_2 linker), 3.76 – 3.69 (m, 1H, C*H*H-6), 3.69 – 3.61 (m, 2H, H-2, C*H*H serine), 3.61 – 3.48 (m, 7H, 3x CH_2 linker, C*H*H linker), 3.48 – 3.39 (m, 4H, 2x CH_2 linker), 3.39 – 3.33 (m, 2H, H-5, C*H*H linker), 3.26 – 3.19 (m, 1H, C*H*H-6), 2.54 (dd, 1H, *J* = 16.2, 7.2 Hz, C*H*H FA), 2.49 – 2.39 (m, 2H, 2x C*H*H FA), 2.39 – 2.34 (m, 1H, C*H*H FA), 2.30 (dd, 1H, *J* = 14.6, 7.4 Hz, C*H*H FA), 2.20 (dd, 1H, *J* = 14.6, 5.4 Hz, C*H*H FA), 2.18 – 2.10 (m, 6H, 3x CH_2 FA), 2.08 (t, 1H, *J* = 7.2 Hz, CH_2 linker), 1.78 – 1.73 (m, 2H, CH_2 linker), 1.51 – 1.37 (m, 12H, 6x CH_2 FA), 1.26 – 1.05 (m, 90H, 45x CH_2 FA), 0.73 (t, 18H, *J* = 7.2 Hz, 6x CH_3 FA); ^{13}C-APT NMR ($CDCl_3$, 214 MHz, HSQC): δ 173.9, 173.9, 173.8, 173.6, 171.2, 171.1, 170.9, 170.4 (C=O), 134.2 (C=C), 100.9 (C-1), 73.9 (C-5), 73.5 (C-3), 72.0 (C-4), 71.0, 70.8 (CH FA), 70.3 (CH_2 linker), 70.1 (CH FA), 70.0, 69.5, 69.3, 69.3 (CH_2 serine, CH_2 linker), 53.6 (C-2, CH serine), 41.0, 40.7, 39.0, 39.0 (CH_2 FA), 38.7 (CH_2-6), 34.4, 34.3, 34.2, 32.7, 31.8, 31.8, 31.8, 31.8, 30.7, 29.6, 29.6, 29.6, 29.6, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.3, 29.3, 29.3, 29.2, 29.2, 29.2, 29.1, 29.1, 29.1, 25.2, 25.2, 25.1, 25.0, 25.0, 24.9, 24.3, 22.6, 22.5 (CH_2 FA, CH_2 linker), 13.9 (CH_3 FA); ^{31}P-APT NMR ($CDCl_3$, 202 MHz, HMBC): δ 1.24; HRMS: $[M+H]^+$ calcd. for $C_{97}H_{175}N_5O_{25}P$: 1841.23083, found 1841.23006.

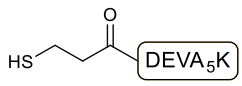
Automated solid phase synthesis general experimental information

The automated solid-phase peptide synthesis was performed on a 250 µmol scale on a Protein Technologies Tribute-UV IR Peptide Synthesizer applying Fmoc based protocol starting from Tentagel S RAM resin (loading 0.22 mmol/g). The synthesis was continued with Fmoc-amino acids specific for each peptide. The consecutive steps performed in each cycle for HCTU chemistry on 250 µmol scale: 1) Deprotection of the Fmoc-group with 20% piperidine in DMF for 10 min; 2) DMF wash; 3) Coupling of the appropriate

amino acid using a four-fold excess. Generally, the Fmoc amino acid (1.0 mmol) was dissolved in 0.2 M HCTU in DMF (5 mL), the resulting solution was transferred to the reaction vessel followed by 0.5 mL of 0.5 M DIPEA in DMF to initiate the coupling. The reaction vessel was then shaken for 30 min at 50°C; 4) DMF wash; 5) capping with 10% Ac_2O in 0.1 M DIPEA in DMF; 6) DMF wash; 7) DCM wash. Aliquots of resin of the obtained sequences were checked on an analytical Agilent Technologies 1260 Infinity system with a Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column or a Vydac 219TP 5 µm Diphenyl, 150 x 4.6 mm column with a 1 ml/min flow. The Fmoc amino acids applied in the synthesis were: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(MMT)-OH, Fmoc-Phe-OH, Fmoc-Ser(O*t*Bu)-OH Fmoc-Val-OH.

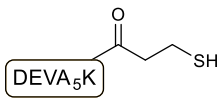
General procedure for cleavage from the resin, deprotection and purification
30 µmol resin was washed with DMF, DCM and dried after the last synthesis step followed by a treatment for 180 minutes with 0.6 mL cleavage cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The suspension was filtered, the resin was washed with 0.6 mL of the cleavage cocktail, and the combined TFA solutions were added dropwise to cold Et_2O and stored at -20°C overnight. The obtained suspension of the product in Et_2O was centrifuged, Et_2O was removed and the precipitant was dissolved in $CH_3CN/H_2O/t$BuOH (1/1/1 v/v/v) or DMSO/$CH_3CN/H_2O/t$BuOH (3/1/1/1 v/v/v/v). Purification was performed on a Gilson GX-281 preparative RP-HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column or a Vydac 219TP 5 µm Diphenyl, 250 x 10 mm column.

3-Mercaptoproponamide-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (43)



Tentagel S Ram resin loaded with H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT) on 70 µmol scale was washed with DMF (5x), followed by the addition of a solution of 3-(tritylthio)propionic acid (52 mg, 150 µmol, 2.1 eq.) and HCTU (58 mg, 140 µmol, 2.0 eq.) in DMF (1.4 mL) and DIPEA (49 µL, 280 µmol, 4.0 eq.). The reaction vessel was shaken overnight at 850 rpm, after which it was washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O/1,2-ethanedithiol (94/2.5/2.5/1 v/v/v/v, 2.8 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **43** (14 mg, 5.3 µmol, 8%) was obtained as a white solid. LC-MS: Rt = 5.15 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1317.7 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{115}H_{191}N_{29}O_{39}S$: 1317.17819, found 1317.17784.

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-(N_ε-(3-mercaptoproponamide))-Lys-NH_2 (44)



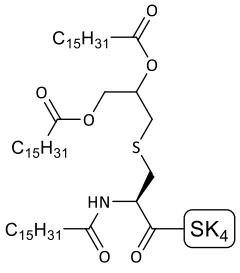
Tentagel S Ram resin loaded with H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT) on 50 µmol scale was washed with DMF (5x), treated with a mixture of Boc_2O (0.11 g, 0.50 mmol, 10 eq.) in 0.1 M DIPEA in DMF (0.5 mL) for one hour, and washed with DMF (3x) and DCM (3x). The peptide was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 15 mL) over 5 minutes. The resin was washed subsequently with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL), DCM (3x) and DMF (3x). A solution of 3-(tritylthio)propionic acid (49 mg, 140 µmol, 2.0 eq.) and HCTU (58 mg, 140 µmol, 2.0 eq.) in DMF (1.4 mL) and DIPEA (49 µL, 280 µmol, 4.0 eq.) were added. The reaction vessel was shaken overnight at 850 rpm, after which it was washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O/1,2-ethanedithiol (95/2/2/1 v/v/v/v, 2.8 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **44** (7.9 mg, 3.0 µmol, 6%) was obtained as a white solid. LC-MS: Rt = 5.31 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1317.2 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{115}H_{191}N_{29}O_{39}S$: 1317.17819, found 1317.17916.

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (45)

DEVA$_5$K

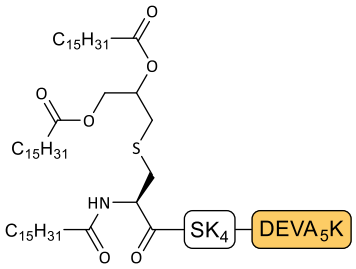
Tentagel S Ram resin loaded with H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT) on 30 µmol scale was washed with DCM (5x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2/2/1 v/v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **45** (12 mg, 4.7 µmol, 16%) was obtained as a white solid. LC-MS: Rt = 4.82 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1273.7 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{112}H_{187}N_{29}O_{38}$: 1273.17904, found 1273.17779.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-NH_2 (46)



Reference compound was synthesized according to literature.[53] LC-MS: Rt = 13.02 min (Diphenyl Vydac, 10 - 90% CH_3CN, 21 min); ESI-MS: m/z 1509.1 $[M+H]^+$; HRMS: $[M+H]^+$ calcd. for $C_{81}H_{158}N_{11}O_{12}S$: 1509.18067, found 1509.18052.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (47)

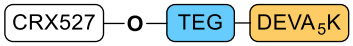


Tentagel S Ram resin loaded with H-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT) on 50 µmol scale was washed with DMF (5x), followed by the addition of a solution of 3-(palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl) (91 mg, 100 µmol, 2.0 eq.) and HCTU (41 mg, 100 µmol, 2.0 eq.) in DMF/DCM (1/1 v/v, 1.0 mL) and DIPEA (35 µL, 200 µmol, 4.0 eq.). The reaction vessel was shaken overnight at 850 rpm, after which it was washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v/v) (2.0 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **47** (9.7 mg, 2.4 µmol, 5%) was obtained as a white solid. LC-MS: Rt = 12.31 min (Diphenyl Vydac, 10 - 90% CH_3CN, 21 min); ESI-MS: m/z 1346.7 $[M+H]^{3+}$; HRMS: $[M+H]^{3+}$ calcd. for $C_{193}H_{342}N_{39}O_{50}S$: 1346.17073, found 1346.17063.

General purification method for CRX-527-*O*-conjugates.
A C18 column was washed subsequently with CH_3CN, MeOH, DCM/MeOH (1/1 v/v), MeOH, CH_3CN, CH_3CN/H_2O, H_2O. The reaction mixture was added on the column and the Eppendorf was rinsed with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.50 mL), which was also added on the C18 column. The column was subsequently flushed with 6 mL of the follow solvent systems: H_2O, CH_3CN/H_2O (1/1 v/v), CH_3CN, DMSO, CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v) and collected in Eppendorfs containing 1.0 mL of each solvent system. The column was then flush with MeOH (6.0 mL), followed by DCM/MeOH (1/1 v/v, 6.0 mL), which were collected in separate flasks, concentrated *in vacuo* at 35°C and lyophilized by dissolving in CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v), yielding the conjugate as a white solid.

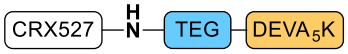
General purification method for CRX-527-*NH*-conjugates.
A C18 column was washed subsequently with CH_3CN, MeOH, DCM/MeOH (1/1 v/v), MeOH, CH_3CN + 0.1% TFA, CH_3CN/H_2O (1/1 v/v) + 0.1% TFA, H_2O + 0.1% TFA. The reaction mixture was added on the column and the Eppendorf was rinsed with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.50 mL), which was also added on the C18 column. The column was subsequently flushed with 6 mL of the follow solvent systems: H_2O + 0.1% TFA, CH_3CN/H_2O (1/1 v/v) + 0.1% TFA, CH_3CN + 0.1% TFA, DMSO, CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v) and collected in Eppendorfs containing 1.0 mL of each solvent system. The column was then flush with MeOH (6.0 mL), followed by DCM/MeOH (1/1 v/v, 6.0 mL), which were collected in separate flasks, concentrated *in vacuo* at 35°C and lyophilized by dissolving in CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v), yielding the conjugate as a white solid.

***N-Terminus 6-O*-DEVA$_5$K conjugate (5)**



Thiol-peptide **43** (1.4 mg, 0.53 µmol, 1.5 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/1 v/v, 114 µl) in an Eppendorf tube. A solution of compound **41** (5.0 mM, 114 µL, 0.36 µmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 48 hours. LC-MS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.80 mL) and sonicated for 5 minutes. After purification using a C18 column, LC-MS analysis showed that the DCM/MeOH (1/1 v/v) flush contained pure conjugate. After lyophilization, conjugate **5** (0.86 mg, 0.19 µmol, 52%) was obtained as a white solid. LC-MS: Rt = 14.27 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.8 mL/min); ESI-MS: m/z 1500.2 $[M+Na]^{3+}$; MALDI-TOF MS (m/z): $[M+2Na]^+$ calcd. for $C_{212}H_{362}N_{33}O_{65}SPNa_2$: 4522.406, found 4531.383.

***N*-Terminus 6-*NH*-DEVA$_5$K conjugate (6)**

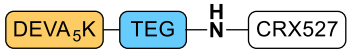


Thiol-peptide **43** (3.65 mg, 1.39 µmol, 1.5 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/0.5 v/v, 277 µl) in an Eppendorf tube. A solution of compound **42** (5.0 mM, 182 µL, 0.91 µmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 48 hours. LC-MS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.59 mL) and sonicated for 5 minutes. After purification using a C18 column, LC-MS analysis showed that the MeOH and DCM/MeOH (1/1 v/v) flush contained pure conjugate. After lyophilization, conjugate **6** (2.19 mg, 0.49 µmol, 54%) was obtained as a white solid. LC-MS: Rt = 16.85 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.7 mL/min); ESI-MS: m/z 1499.9 $[M+Na]^{3+}$; MALDI-TOF MS (m/z): $[M+2Na]^+$ calcd. for $C_{212}H_{363}N_{34}O_{64}SPNa_2$: 4521.422, found 4530.340.

***C*-Terminus 6-*O*-DEVA$_5$K conjugate (7)**

Thiol-peptide **44** (2.1 mg, 0.80 µmol, 1.5 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/0.5 v/v, 161 µl) in an Eppendorf tube. A solution of compound **41** (5.0 mM, 107 µL, 0.53 µmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 48 hours. LC-MS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.80 mL) and sonicated for 5 minutes. After purification using a C18 column, LC-MS analysis showed that the MeOH and DCM/MeOH (1/1 v/v) flush contained pure conjugate. After lyophilization, conjugate **7** (1.37 mg, 0.30 µmol 57%) was obtained as a white solid. LC-MS: Rt = 14.24 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.8 mL/min); ESI-MS: m/z 1508.6 $[M+2Na]^{3+}$; MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{212}H_{362}N_{33}O_{65}SPNa$: 4499.417, found 4497.786.

_C_-Terminus 6-_NH_-DEVA$_5$K conjugate (8)



Thiol-peptide **44** (2.0 mg, 0.76 µmol, 1.5 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/0.5 v/v, 152 µl) in an Eppendorf tube. A solution of compound **42** (5.0 mM, 101 µL, 505 nmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 48 hours. LC-MS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.80 mL) and sonicated for 5 minutes. After purification using a C18 column, LC-MS analysis showed that the DCM/MeOH (1/1 v/v) flush contained pure conjugate. After lyophilization, conjugate **8** (0.94 mg, 0.21 µmol, 42%) was obtained as a white solid. LC-MS: Rt = 16.90 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.7 mL/min); ESI-MS: m/z 1507.4 $[M+2Na]^{3+}$; MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{212}H_{363}N_{34}O_{64}SPNa$: 4498.433, found 4508.766.

Cell culture

The D1 cell line is a growth factor-dependent immature spleen-derived DC cell line from C57BL/6 (H-2^b) mice. D1 cells were cultured as described elsewhere.[54] B3Z is a $CD8^+$ T cell hybridoma specific for the H-2Kb CTL-epitope SIINFEKL of ovalbumin and was cultured in IMDM medium (Lonza, Basel, Switzerland) supplemented with 8% FCS (Greiner, Kremsmünster, Austria), penicillin and streptomycin, glutamine (Gibco, Carlsbad, CA, USA), β-mercaptoethanol (Merck, Kenilworth, NJ USA), and hygromycin B (AG Scientific Inc, San Diego, CA, USA) to maintain expression of the beta-galactosidase reporter gene.

In vitro DC maturation assay

50.000 D1 cell were seeded in 96-well round bottom plates (Corning, Amsterdam, The Netherlands) in 100 µl of R1 supplemented IMDM medium and 100 µl of 2 times concentrated test compounds were added. After 24 hours of incubation at 37°C, supernatant was taken from the wells for ELISA analysis (BioLegend, San Diego, USA) to measure the amount of produced IL-12p40.

In vitro antigen presentation assay

50.000 D1 cells were seeded in 96-well flat bottom plates and pulsed for 2 hours with the indicated test compounds. After 2 hours, cells were washed once with fresh medium and 50.000 B3Z were added per well and incubated with the pulsed D1 cells overnight. The following day, TCR activation triggered by recognition of the SIINFEKL epitope was detected by measurement of absorbance at 595 nm upon color conversion of chlorophenol red-β-D-galactopyranoside (Calbiochem®, Merck, Bullington MA, USA).

Footnotes and References

(1) Schumacher, T. N.; Schreiber, R. D. *Science.* **2015**, *348* (6230), 69–74.
(2) Heimburg-Molinaro, J.; Lum, M.; Vijay, G.; Jain, M.; Almogren, A.; Rittenhouse-Olson, K. *Vaccine* **2011**, *29* (48), 8802–8826.
(3) Brubaker, S. W.; Bonham, K. S.; Zanoni, I.; Kagan, J. C. *Annu. Rev. Immunol.* **2015**, *33* (1), 257–290.
(4) Iwasaki, A.; Medzhitov, R. *Nat. Immunol.* **2004**, *5* (10), 987–995.

(5) Kawai, T.; Akira, S. *Nat. Immunol.* **2010**, *11* (5), 373–384.
(6) van Dinther, D.; Stolk, D. A.; van de Ven, R.; van Kooyk, Y.; de Gruijl, T. D.; den Haan, J. M. M. *J. Leukoc. Biol.* **2017**, *102* (4), 1017–1034.
(7) Garaude, J.; Kent, A.; van Rooijen, N.; Blander, J. M. *Sci. Transl. Med.* **2012**, *4* (120), 120ra16.
(8) Zom, G. G.; Willems, M. M. J. H. P.; Meeuwenoord, N. J.; Reintjens, N. R. M.; Tondini, E.; Khan, S.; Overkleeft, H. S.; van der Marel, G. A.; Codee, J. D. C.; Ossendorp, F.; *et al*. *Bioconjug. Chem.* **2019**, *30* (4), 1150–1161.
(9) Zom, G. G. P.; Khan, S.; Filippov, D. V.; Ossendorp, F. In *Advances in Immunology*; 2012; pp 177–201.
(10) Liu, H.; Irvine, D. J. *Bioconjug. Chem.* **2015**, *26* (5), 791–801.
(11) Moyle, P. M.; Toth, I. *ChemMedChem* **2013**, *8* (3), 360–376.
(12) Ignacio, B. J.; Albin, T. J.; Esser-Kahn, A. P.; Verdoes, M. *Bioconjug. Chem.* **2018**, *29* (3), 587–603.
(13) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G.-J. *Nat. Chem. Biol.* **2007**, *3* (10), 663–667.
(14) Khan, S.; Weterings, J. J.; Britten, C. M.; de Jong, A. R.; Graafland, D.; Melief, C. J. M.; van der Burg, S. H.; van der Marel, G.; Overkleeft, H. S.; Filippov, D. V.; *et al*. *Mol. Immunol.* **2009**, *46* (6), 1084–1091.
(15) Abdel-Aal, A.-B. M.; Zaman, M.; Fujita, Y.; Batzloff, M. R.; Good, M. F.; Toth, I. *J. Med. Chem.* **2010**, *53* (22), 8041–8046.
(16) Weterings, J. J.; Khan, S.; van der Heden, G. J.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; van der Burg, S. H.; Ossendorp, F.; van der Marel, G. A.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2006**, *16* (12), 3258–3261.
(17) Fujita, Y.; Hirai, K.; Nishida, K.; Taguchi, H. *Amino Acids* **2016**, *48* (5), 1319–1329.
(18) Kramer, K.; Young, S. L.; Walker, G. F. *ACS Omega* **2017**, *2* (1), 227–235.
(19) Daftarian, P.; Sharan, R.; Haq, W.; Ali, S.; Longmate, J.; Termini, J.; Diamond, D. J. *Vaccine* **2005**, *23* (26), 3453–3468.
(20) Molinaro, A.; Holst, O.; Di Lorenzo, F.; Callaghan, M.; Nurisso, A.; D'Errico, G.; Zamyatina, A.; Peri, F.; Berisio, R.; Jerala, R.; *et al*. *Chem. - A Eur. J.* **2015**, *21* (2), 500–519.
(21) Shimazu, R.; Akashi, S.; Ogata, H.; Nagai, Y.; Fukudome, K.; Miyake, K.; Kimoto, M. *J. Exp. Med.* **1999**, *189* (11), 1777–1782.
(22) Dobrovolskaia, M. A.; Vogel, S. N. *Microbes Infect.* **2002**, *4* (9), 903–914.
(23) Qureshi, N.; Takayama, K.; Ribi, E. *J. Biol. Chem.* **1982**, *257* (19), 11808–11815.
(24) Evans, J. T.; Cluff, C. W.; Johnson, D. A.; Lacy, M. J.; Persing, D. H.; Baldridge, J. R. *Expert Rev. Vaccines* **2003**, *2* (2), 219–229.
(25) Alving, C. R.; Peachman, K. K.; Rao, M.; Reed, S. G. *Curr. Opin. Immunol.* **2012**, *24* (3), 310–315.
(26) Casella, C. R.; Mitchell, T. C. *Cell. Mol. Life Sci.* **2008**, *65* (20), 3231–3240.
(27) Garçon, N.; Di Pasquale, A. *Hum. Vaccin. Immunother.* **2017**, *13* (1), 19–33.
(28) Wang, Q.; Zhou, Z.; Tang, S.; Guo, Z. *ACS Chem. Biol.* **2012**, *7* (1), 235–240.
(29) Zhou, Z.; Mondal, M.; Liao, G.; Guo, Z. *Org. Biomol. Chem.* **2014**, *12* (20), 3238–3245.
(30) Zhou, Z.; Liao, G.; Mandal, S. S.; Suryawanshi, S.; Guo, Z. *Chem. Sci.* **2015**, *6* (12), 7112–7121.
(31) Liao, G.; Zhou, Z.; Suryawanshi, S.; Mondal, M. A.; Guo, Z. *ACS Cent. Sci.* **2016**, *2* (4), 210–218.
(32) Wang, L.; Feng, S.; Wang, S.; Li, H.; Guo, Z.; Gu, G. *J. Org. Chem.* **2017**, *82* (23), 12085–12096.
(33) Zamyatina, A. *Beilstein J. Org. Chem.* **2018**, *14*, 25–53.
(34) Fujimoto, Y.; Shimoyama, A.; Saeki, A.; Kitayama, N.; Kasamatsu, C.; Tsutsui, H.; Fukase, K. *Mol. Biosyst.* **2013**, *9* (5), 987.
(35) Li, Q.; Guo, Z. *Molecules* **2018**, *23* (7), 1583.
(36) Johnson, D. A.; Gregory Sowell, C.; Johnson, C. L.; Livesay, M. T.; Keegan, D. S.; Rhodes, M. J.; Terry Ulrich, J.; Ward, J. R.; Cantrell, J. L.; Brookshire, V. G. *Bioorg. Med. Chem. Lett.* **1999**, *9* (15), 2273–2278.
(37) Stöver, A. G.; Da Silva Correia, J.; Evans, J. T.; Cluff, C. W.; Elliott, M. W.; Jeffery, E. W.; Johnson, D. A.; Lacy, M. J.; Baldridge, J. R.; Probst, P.; *et al. J. Biol. Chem.* **2004**, *279* (6), 4440–4449.
(38) Cluff, C. W.; Baldridge, J. R.; Stover, A. G.; Evans, J. T.; Johnson, D. A.; Lacy, M. J.; Clawson, V. G.; Yorgensen, V. M.; Johnson, C. L.; Livesay, M. T.; *et al*. *Infect. Immun.* **2005**, *73* (5), 3044–3052.
(39) Bazin, H. G.; Bess, L. S.; Livesay, M. T.; Ryter, K. T.; Johnson, C. L.; Arnold, J. S.; Johnson, D. A. *Tetrahedron Lett.* **2006**, *47* (13), 2087–2092.
(40) Bazin, H. G.; Murray, T. J.; Bowen, W. S.; Mozaffarian, A.; Fling, S. P.; Bess, L. S.; Livesay, M. T.;

Arnold, J. S.; Johnson, C. L.; Ryter, K. T.; *et al*. *Bioorg. Med. Chem. Lett.* **2008**, *18* (20), 5350–5354.
(41) Dupont, J.; Altclas, J.; Lepetic, A.; Lombardo, M.; Vázquez, V.; Salgueira, C.; Seigelchifer, M.; Arndtz, N.; Antunez, E.; von Eschen, K.; *et al*. *Vaccine* **2006**, *24* (49–50), 7167–7174.
(42) Oikawa, M.; Kusumoto, S. *Tetrahedron: Asymmetry* **1995**, *6* (4), 961–966.
(43) de Jong, N. R. Leiden University, **2013**.
(44) The use of a wittig reagent, lead to the formation of more Z-alkene, which was difficult to seperate from the desired E-alkene using column chromatography.
(45) Gao, Y.; Sharpless, K. B. *J. Am. Chem. Soc.* **1988**, *110* (22), 7538–7539.
(46) Kim, S.; Lee, S.; Lee, T.; Ko, H.; Kim, D. *J. Org. Chem.* **2006**, *71* (22), 8661–8664.
(47) Oldendorf, J.; Haufe, G. *European J. Org. Chem.* **2006**, *2006* (19), 4463–4472.
(48) Paquet, A. *Can. J. Chem.* **1982**, *60* (8), 976–980.
(49) Tondini, E. Leiden University Medical Centre, unpublished Thesis.
(50) Weterings, J. J. Leiden University, **2008**.
(51) Khan, S.; Bijker, M. S.; Weterings, J. J.; Tanke, H. J.; Adema, G. J.; van Hall, T.; Drijfhout, J. W.; Melief, C. J. M.; Overkleeft, H. S.; van der Marel, G. A.; *et al*. *J. Biol. Chem.* **2007**, *282* (29), 21145–21159.
(52) Trinchieri, G. *Nat. Rev. Immunol.* **2003**, *3* (2), 133–146.
(53) Geert-Jan Boons, Margaretha Wolfert, Sandra J. Gendler, Vani Lakshminarayanan, P. A. C. 20150299290, **2015**.
(54) Winzler, C.; Rovere, P.; Zimmermann, V. S.; Davoust, J.; Rescigno, M.; Citterio, S.; Ricciardi-Castagnoli, P. *Adv. Exp. Med. Biol.* **1997**, *417*, 59–64.

Chapter 3

*Synthesis of O- and C-muramyl dipeptide–antigen conjugates**

Introduction

Currently, much effort is directed to improve and develop therapeutic cancer vaccines.[1] Cancer specific epitopes, such as neoantigens[2] or tumor-associated carbohydrate antigens[3] are not actively targeted to and taken up by antigen presenting cells or elicit poor immunological responses. Therefore these are aided by adjuvants to enhance the immune response. Among the first was Freund's adjuvant: a water-in-oil emulsion of heat-killed mycobacteria resulting in a mixture of bacterial components, which turned out too toxic for human use. So far, only alum salts, oil-in-water emulsions, virosomes and a mixture of alum and monophosphoryl lipid A (AS04) have been licensed for human use.[4] Although alum has proven its ability to enhance the potency of bacterial vaccines (requiring a humoral response), it cannot be used in cancer vaccines as it is unable to induce a cell-mediated immune response.[5] One of the strategies to enhance the immunogenicity of cancer vaccines is the employment of conjugates in which the antigen is covalently bound to an adjuvant.[6–8] In the search for suitable adjuvants, pathogen-associated molecular patterns have been extensively explored, as they bind

*The data presented in this Chapter were gathered in collaboration with Tony S. Koemans, Nick Zilverschoon, Nico J. Meeuwenoord, Stefan van der Vorm, Herman S. Overkleeft, Dmitri V. Filippov, Gijsbert A. van der Marel and Jeroen D. C. Codée.

to pattern recognition receptors (PRRs), for example Toll-like receptors[9,10], which play an important role in activating our immune system. The Nucleotide binding Oligomerization Domain (NOD)-like receptors represent an intracellular PRR family recognizing specific parts of the bacterial cell wall peptidoglycan (PG).[11] Freund's adjuvant lends its adjuvant activity from many components of the PG of the cell wall of bacteria present in the mixture.[12] The PG polymer consists of repeating disaccharide units of β-(1,4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid, where the muramic acid is elongated with a peptide (Figure 1). NOD-1 is able to recognize and bind to D-glutamyl-meso-diaminopimelic acid (*i*E-DAP) and muramyl dipeptide (MDP) is the minimal structure of a NOD-2 ligand (Figure 1). MDP generally contains an *N*-acetyl group at the *C*-2 of the muramic acid residue (MDP(Ac)), but the PG of mycobacteria and actinobacteria contains MDP bearing a *N*-glycolyl moiety, MDP(Gly).

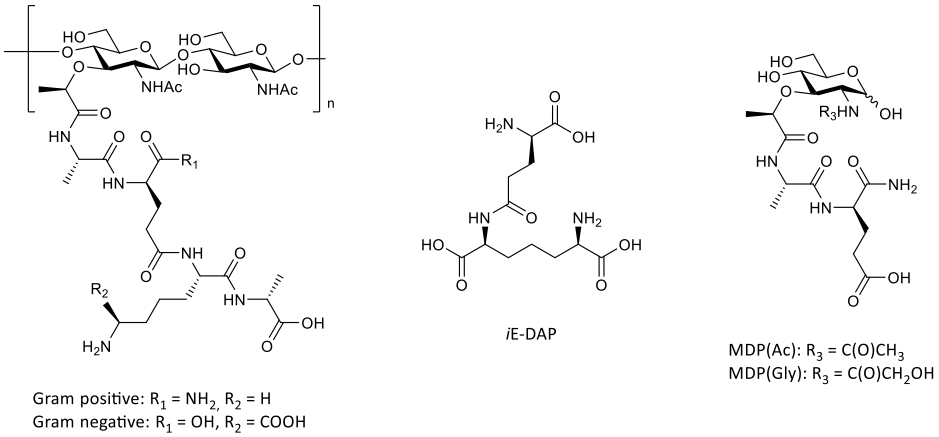


Figure 1. PG structures of Gram-positive or Gram-negative bacteria, NOD-1 ligand *i*E-DAP and NOD-2 ligands MDP(Ac) and MDP(Gly).

Willems *et al*. synthesized a set of conjugates, wherein the NOD-2 ligand, MDP(Ac), was covalently linked to an ovalbumin-derived peptide, harboring the MHC-I epitope SIINFEKL.[13] Immunological evaluation of these conjugates indicated that the conjugates were internalized and processed, but they were unable to effectively induce maturation of dendritic cells (DCs). Incubation with a combination of PRR ligands can act synergistically[14–16] to produce an enhanced immune response and synergy between NOD-2 and TLR2 ligands have been reported by several groups.[17–19] Therefore, several bis-conjugates containing both an MDP(Ac) and a TLR2-ligand (Pam_3CSK_4) were synthesized.[20] These bis-conjugates improved the maturation of DCs leading to the proper activation of antigen-specific T cells.

In this Chapter, a set of MDP-human papillomavirus (HPV) conjugates is explored. HPV16 is one of the two types of HPV that are responsible for cervical cancer and the HPV16-derived peptide, GQAEPDRAHYNIVTFBBKBDSTLRLBV, contains both a MHC-I and a MHC-II epitope. To prevent disulfide formation, the cysteine residues in this sequence are replace for (S)-2-aminobutiric acid residues (B). Besides the MDP(Ac) ligand, the MDP(Gly) is also used for conjugation as it has been shown to be more potent than MDP(Ac).[12,21,22] In the first part of this Chapter, the work of Zom *et al*.[20] is extended by conjugation of MDP(Ac) and MDP(Gly) to HPV16 via the carboxylic acid function of the D-isoglutamine of the MDP moiety using solid phase peptide synthesis (SPPS). Bis-conjugates carrying the TLR2-ligand, Pam_3CSK_4, in addition to the NOD2-ligand and the peptide antigen, have shown that good immunostimulatory properties are obtained using this conjugation site.[20] This lead to the design of the first generation mono- and bis-conjugates **1**-**4**. Herein, a MDP building block **9a** or **9b** with a 3-azidopropanol linker at the anomeric position (*O*-MDP) was coupled to the peptide at the *N*-terminus and Pam_3CSK_4 via the *C*-terminal lysine (Figure 2). The anomeric 3-azidopropanol can be used for conjugation of MDP to additional peptides, fluorophores and other moieties at a later stage.

Previous work has shown that the glycosidic linkage of the *O*-MDP in the previously described peptide conjugates is relatively labile and that hydrolysis of this linkage can take place during acidic cleavage of the conjugates from the solid phase resin.[13] The second part of this Chapter therefore describes the synthesis of a *C*-glycoside analogue of MDP, *C*-MDP, of which the anomeric linkage is stable against the acidic conditions used in SPPS as the exocyclic oxygen is replace with a CH_2. Two lysine building blocks provided with a *C*-MDP were designed for application in SPPS, thereby facilitating the incorporation of MDP into peptides. One of the opportunities of these building blocks is the conjugation of MDP via the anomeric position as this was previously shown to be an ideal conjugation site.[13,22] This resulted in the design of the second generation mono- and bis-conjugates **5**-**8** depicted in Figure 2. Both the *O*-MDP building blocks (**9a** and **9b**) and the *C*-MDP building blocks (**10a** and **10b**) are protected with acid-labile benzylidene, *p*-methoxybenzyl and *tert*-butyl groups to ensure the simultaneous deprotection and cleavage of the conjugate from the resin in the final stage of the SPPS.

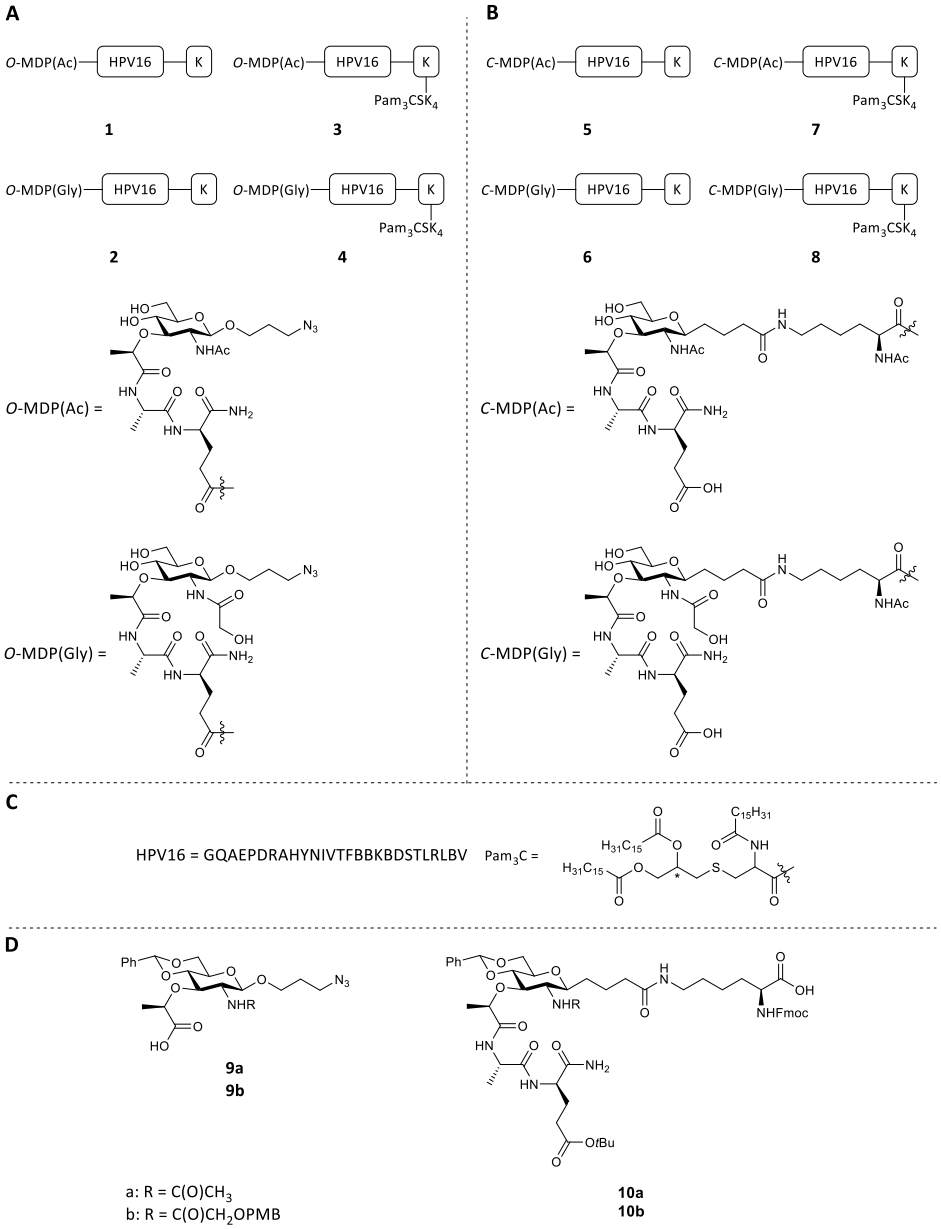
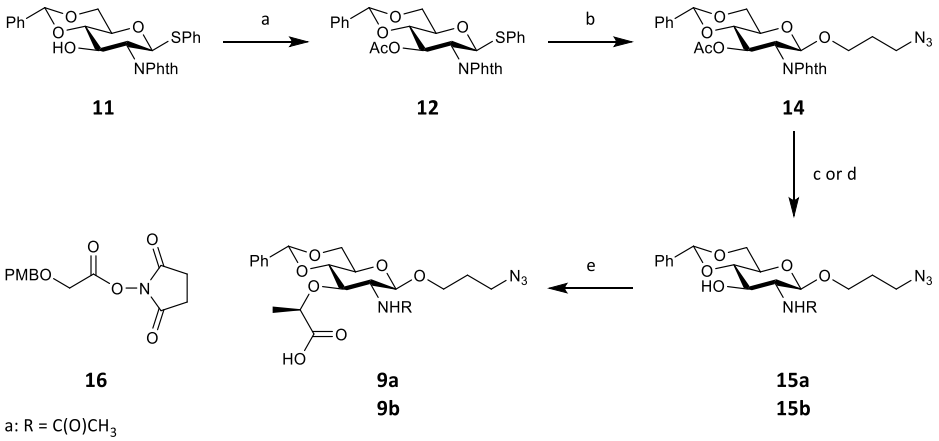


Figure 2. Structures of A) the 1st generation *O*-MDP conjugates **1**-**4**; B) the 2nd generation *C*-MDP-conjugates **5**-**8**; C) HPV16 and Pam_3C structure; D) the *O*-MDP building blocks, **9a** and **9b**, and *C*-MDP building blocks, **10a** and **10b**.

Results and Discussion

1st generation: *O*-MDP conjugates

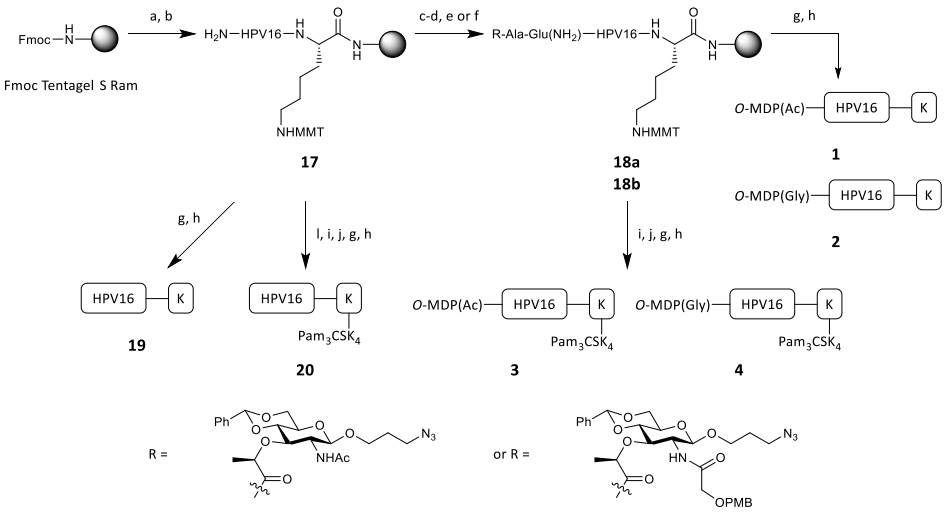
An optimized synthesis route[13] towards *O*-MDP building blocks **9a** and **9b** is shown in Scheme 1, wherein a phthaloyl protected amine was used as a participating protecting group and as a precursor for the *N*-acetyl and *N*-glycolyl functionalities at a later stage of the synthesis. Synthesis of the building blocks starts with the acetylation of **11**[23], followed by NIS/TMSOTf-mediated glycosylation of donor **12** with 3-azidopropanol **13**. Due to the neighboring group participation of the bulky *N*-phthaloyl group, only formation of β-product **14** was observed during the glycosylation. Treatment of **14** with ethylene diamine (50 eq.) at 90°C removed the *N*-phthaloyl and the acetyl groups. The obtained amine could then be selectively acetylated with $NaHCO_3$ and Ac_2O to give compound **15a** in 81% yield over two steps. For the selective glycolylation, the activated ester **16** was used in combination with Et_3N to deliver **15b** in 78% over two steps. Alkylation of **15a** and **15b** with (S)-(-)-2-chloropropionic acid with sodium hydride gave crystalline SPPS building blocks **9a** and **9b**.



Scheme 1. Synthesis of buildingblocks **9a** and **9b**. *Reagents and conditions*: a) Ac_2O, pyridine, DMAP, DCM, quant.; b) 3-azidopropanol (**13**), NIS, TMSOTf, DCM, 86%; c) *i*. ethylene diamine, EtOH, 90°C; *ii*. Ac_2O, $NaHCO_3$, THF/H_2O, **15a**: 81% over two steps; d) *i*. ethylene diamine, EtOH, 90°C; *ii*. *N*-succinimidyl-(*p*-methoxybenzyloxy)acetate (**16**), Et_3N, DCM, **15b**: 78% over two steps; e) (S)-(-)-2-chloropropionic acid, NaH, DMF, **9a**: 83%, **9b**: 86%.

With *O*-MDP(Ac) building block **9a** and *O*-MDP(Gly) building block **9b** in hand, the synthesis of the mono- and bis-conjugates **1**-**4** was undertaken (Scheme 2), which was carried out with an automated peptide synthesizer. Immobilized peptide **17** was

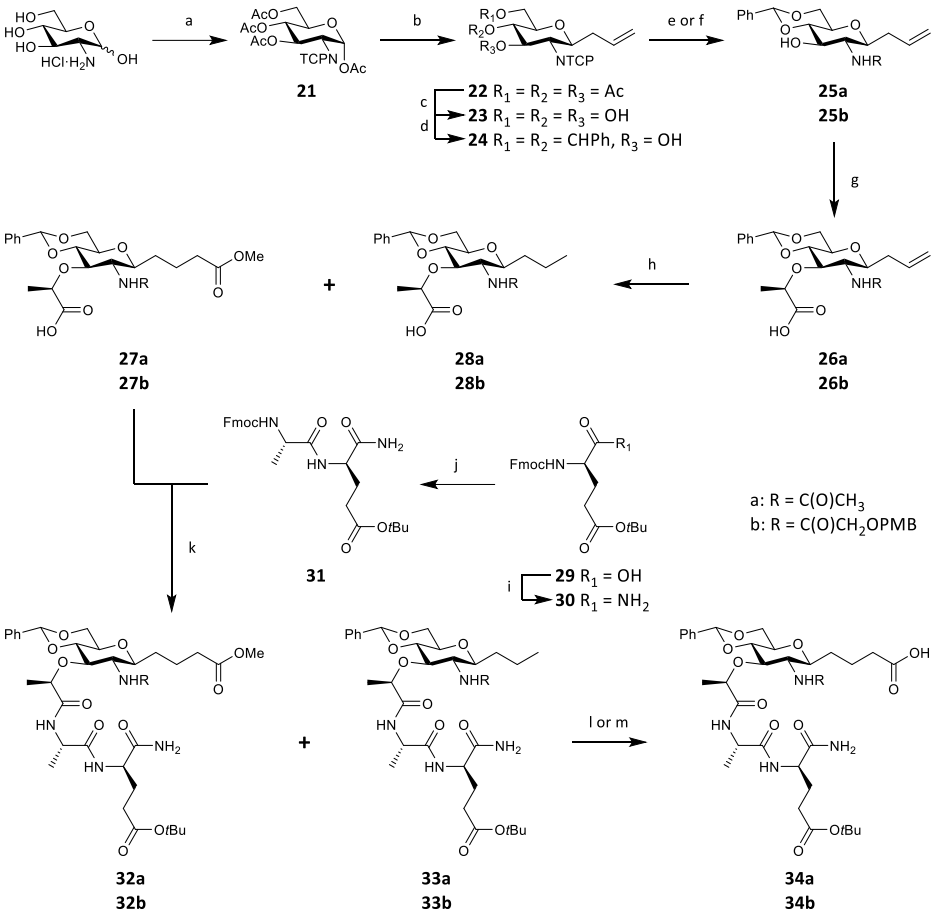
prepared with standard SPPS HCTU/Fmoc chemistry on a Tentagel S Ram solid support. Peptide **17** was elongated with Fmoc-Ala-OH, Fmoc-Glu(NH_2)-OH and **9a** or **9b**. The obtained peptides **18a** and **18b** were cleaved from the resin by treatment with a cocktail of TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 60 minutes. Longer reaction times lead to substantial hydrolysis of MDP-azidopropyl spacer, a side reaction previously also observed by Willems *et al*.[13] The mono-conjugates were precipitated with Et_2O and purified by RP-HPLC yielding 5.4 mg **1** and 14.7 mg **2** in 3% and 8% respectively. To obtain bis-conjugates **3** and **4**, the MMT protecting group at the *C*-terminal lysine of immobilized peptides **18a** and **18b** was selectively removed with a cocktail of TFA/TIS/DCM (2/2/96 v/v/v). The obtained amino groups were extended with SK_4 using the automated peptide synthesizer, followed by manual coupling with palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH overnight. The peptides were then cleaved from the solid support and purification by RP-HPLC lead to **3** (5.2 mg, 1% yield) and **4** (3.3 mg, 1% yield).[24] Treatment of immobilized peptide **17** with a cocktail of TFA/TIS/H_2O (95/2.5/2.5 v/v/v) gave reference peptide **19** (9.4 mg, 10%). Besides, capping of immobilized peptide **17** with an acetyl, followed by MMT removal and elongation with Pam_3CSK_4 gave reference TLR2L-conjugate **20** (5.5 mg, 2%).



Scheme 2. Solid phase peptide synthesis of *O*-MDP mono- and bis-conjugates **1**-**4** and reference compound **19**. *Reagents and conditions*: a) 20% piperidine, DMF; b) Fmoc SPPS cycle for GQAEPDRAHYNIVTFBBKBDSTLRLBVK; c) Fmoc-*i*-D-Glu(NH_2)-OH, HCTU, DIPEA, DMF; d) Fmoc-L-Ala-OH, HCTU, DIPEA, DMF; e) **9**, HCTU, DIPEA, DMF; f) **9b**, HCTU, DIPEA, DMF; g) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 1h; i) RP-HPLC; i) TFA/TIS/DCM (2/2/96 v/v/v); j) Fmoc SPPS cycle for SK_4; k) palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, HCTU, DIPEA, DMF/DCM; Yield conjugates: **1**) 5.4 mg, 3%; **2**) 14.7 mg, 8%; **3**) 5.2 mg, 1%; **4**) 3.3 mg, 1%; **19**) 9.4 mg, 10%; **20**) 5.5 mg, 2%.

2nd generation: *C*-MDP conjugates

Synthesis of the 2nd generation MDP-conjugates **5**-**8**, required the SPPS building blocks **10a** and **10b**. Their synthesis starts with the installation of a TCP protecting group on commercially available glucosamine, followed by acetylation giving donor **21** (Scheme 3). Fuchss *et al*. reported a synthesis of **22** in which they first transformed acetyl donor **21** into the corresponding α-fluoride, which was then used to stereoselectively install the *C*-allyl group.[25] To shorten the synthesis of **22**, donor **21** was used directly for the *C*-glycosylation. Sonication of **21** with allyltrimethylsilane (5.0 eq.), and $BF_3{\cdot}OEt_2$ (5.0 eq.) and TMSOTf (1.0 eq.), generating the strong Lewis acid $BF_2OTf{\cdot}OEt_2$ *in situ*,[26] delivered the *C*-glycoside **22** in 58% yield on 40 mmol scale. Deacetylation with *in situ* generated HCl (0.8 eq.) gave triol **23** in 94%. The use of more equivalents of HCl, or the use of sodium methoxide resulted in lower yields as ring opening of the TCP protecting group was observed. Subsequent installation of the benzylidene protecting group gave alcohol **24** in 87%. Removal of the TCP protecting group with ethylene diamine, followed by selective acetylation or glycolylation gave **25a** and **25b** in 83% and 98% respectively. Alkylation of **25a** and **25b** with (S)-(-)-2-chloropropionic acid provided the acids **26a** and **26b**. The next step entailed cross metathesis with methyl acrylate and subsequent reduction of the double bond to obtain **27a** and **27b**. Initial metatheses in DCM or DCE proceeded very sluggishly due to the poor solubility of the starting materials. Switching to THF as reaction solvent and the addition of CuI with heating to 60°C increased the conversion as indicated by NMR analysis. Voigtritter *et al*. have shown that the addition of CuI increases the reaction rate by stabilization of the catalyst by the iodine ion and simultaneous scavenging of the phosphine ligand.[27] However, even under these forcing conditions the cross-metatheses did not go to full completion, and the starting alkenes and α,β-unsaturated ester products could not be separated with column chromatography. Reduction of the double bonds in the metatheses product mixture was carried out with $NaBH_4$ and ruthenium trichloride[28] to give compound **27a**, contaminated with reduced starting material **28a**. Also reduction of the corresponding glycolyl derivative gave a mixture of target **27b** and side-product **28b**. Because purification was impossible, both mixtures (**27a/ 28a** and **27b/ 28b**) were condensed with dipeptide **31**, to generate the protected *C*-MDP building blocks **32a** and **32b**. The required dipeptide **31** was obtained by treatment of Fmoc protected *tert*-butyl glutamic acid **29** with di-*tert*-butyl dicarbonate, followed by NH_4HCO_3 mediated conversion of the intermediate anhydride to give amide **30** in 96% yield over two steps. Removal of the Fmoc-group in amino acid **30** with DBU, quenching with HOBt and coupling of the resulting free amine with Fmoc protected alanine afforded dipeptide **31** in 73% yield after crystallization.

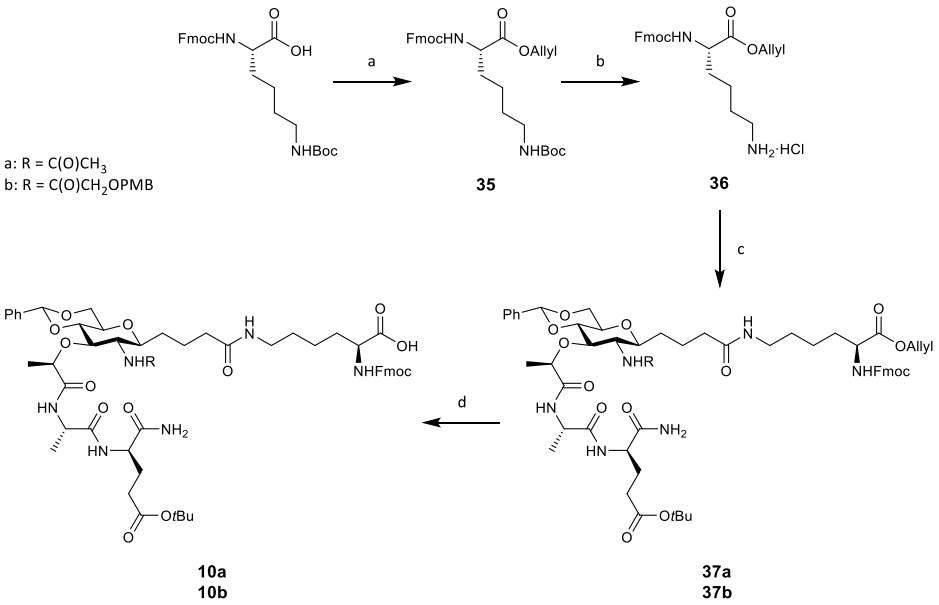


Scheme 3. Synthesis of compounds **34a** and **34b**. *Reagents and conditions*: a) *i*. tetrachlorophthalic anhydride, NaOMe, MeOH, 50°C; *ii*. Ac_2O, pyridine, 51% over two steps; b) allyltrimethylsilane, $BF_3{\cdot}OEt_2$, TMSOTf, MeCN, 58%; c) AcCl, MeOH, 94%; d) benzaldehyde dimethylacetal, *p*-toluenesulfonic acid, DMF/MeCN, 60°C, 87%; e) *i*. ethylene diamine, EtOH, 90°C; *ii*. Ac_2O, $NaHCO_3$, THF/H_2O, **25a**: 83% over two steps; f) *i*. ethylene diamine, EtOH, 90°C; *ii*. *N*-succinimidyl-(*p*-methoxybenzyloxy)acetate (**16**), Et_3N, DCM, **25b**: 98% over two steps; g) (S)-(-)-2-chloropropionic acid, NaH, DMF, **26a**: 95%, **26b**: 91%; h) *i*. methyl acrylate, CuI, Grubbs 2nd gen. catalyst, THF, 40°C; *ii*. $NaBH_4$, $RuCl_3$, MeOH, THF, 40°C, **27a**: 64% over two steps, **27b:** 69% over two steps; i) Boc_2O, NH_4HCO_3, pyridine, dioxane, 96%; j) *i*. DBU, DCM; *ii*. HOBt, Fmoc-L-Ala-OH, EDC·HCl, DIPEA, DCM, 73%; k) *i*. DBU, DMF; *ii*. HOBt, **27a** or **27b**, HCTU, DIPEA, **32a**: quant. over two steps, **32b**: 89% over two steps; l) LiOH, H_2O_2, MeOH, room temperature, 5 h, **34a**: 73%; m) LiOH, H_2O_2, THF/H_2O, 0°C, 8 h, **34b**: 92%.

The same one-pot procedure was used for the coupling of dipeptide **31** to the acids **27a/28a** and **27b/28b** resulting in **32a** and **32b,** still inseparable from the corresponding side products **33a** and **33b**, respectively.[29] To obtain acids **34a** and **34b**, the methyl esters in **32a** and **32b** were carefully hydrolysed to prevent hydrolysis of the *tert*-butyl ester. To this end **32a** was treated with a mixture of LiOH and H_2O_2 in MeOH, yielding

34a in 73%. Because these conditions did not completely convert **32b** into the corresponding acid, the hydrolysis was performed in a THF/H_2O mixture at 0°C, resulting in **34b** in 92%. At this stage compounds **34a/b** were separated from the propyl side products **33a/b**.

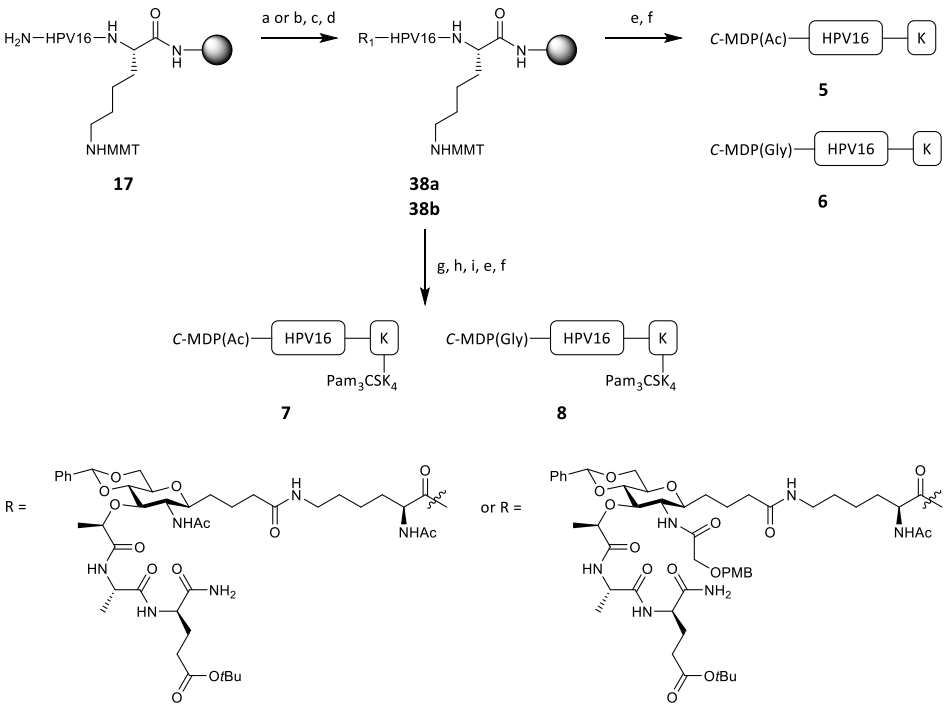
Next, both free acids **34a** and **34b** were condensed with protected lysine **36**, generated from Fmoc-Lys(Boc)-OH by an allylation-debocylation sequence, using HCTU and DIPEA, to give **37a** and **37b** in 85% and 76% yield, respectively (Scheme 4). Deprotection of the allyl ester in **37a** and **37b** was performed using $Pd(PPh_3)_4$ as catalyst and $PhSiH_3$ as scavenger providing the final SPPS building blocks **10a** and **10b** in 81% and 93%.



Scheme 4. Synthesis of SPPS building blocks **10a** and **10b**. *Reagents and conditions*: a) Ag_2CO_3, AllylBr, DMF, quant.; b) 4 M HCl in dioxane, 97%; c) **34a** or **34b**, HCTU, DIPEA, DMF, **37a**: 85%, **37b**: 76%); d) $Pd(PPh_3)_4$, $PhSiH_3$, DMF, **10**a: 81%, **10b**: 93%.

At this stage the solid phase synthesis of mono- and bis-conjugates **5**-**8** was undertaken (Scheme 5). To this end, the immobilized peptide **17** (Scheme 2) was elongated with **10a** or **10b** and the resulting peptides were cleaved from the resin by treatment with a cocktail of TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 3 hours. Precipitation of the peptides from Et_2O, followed by RP-HPLC purification gave target conjugates **5** (7.2 mg, 6% yield) and **6** (9.2 mg, 6% yield). Bis-conjugates **7** and **8** were synthesized by deprotection of the MMT group of the *C*-terminal lysine in peptides **38a** and **38b**, followed by elongation

with SK_4 using the automated synthesizer. After manual coupling with palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH overnight, the peptides were cleaved from the solid support, after which purification by RP-HPLC led to **7** (2.3 mg, 0.6% yield) and **8** (1.4 mg, 0.4% yield) respectively.



Scheme 5. Solid phase peptide synthesis of *C*-MDP mono- and bis-conjugates **5**-**8**. *Reagents and conditions*: a) **10a**, HCTU, DIPEA, DMF; b) **10b**, HCTU, DIPEA, DMF; c) 20% piperidine, DMF, d) Ac_2O, DIPEA, DMF; e) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 3h; f) RP-HPLC; g) TFA/TIS/DCM (2/2/96 v/v/v); h) Fmoc SPPS cycle for SK_4; i) palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, HCTU, DIPEA, DMF/DCM. Yield conjugates: **5**) 7.2 mg, 6%; **6**) 9.2 mg, 6%; **7**) 2.3 mg, 0.6%; **8**) 1.4 mg, 0.4%.

Conclusion

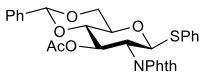
The synthesis of *O*-MDP and *C*-MDP building blocks, carrying either an *N*-acetyl or an *N*-glycolyl group and their incorporation in novel HPV16-conjugates is described. A crucial step in the synthesis of the *C*-MDP building blocks entailed the Grubbs cross metathesis, to functionalize the anomeric *C*-allyl moiety. Due to the poor solubility of the compounds this turned out to be a challenging transformation, giving rise to side products that could only be separated from the target compounds at a late stage of the synthesis.

The applicability of the novel *C*-MDP building blocks has been demonstrated in the assembly of four peptide-antigen conjugates. The acid stability of the *C*-MDP enables conjugation via the anomeric position of the MDP building block and its use in online solid phase syntheses of MDP functionalized oligopeptides. The ease of incorporation of the building block will allow the future generation of conjugates carrying multiple MDP moieties. As the building block can be incorporated in the peptide sequences through standard automated SPPS, all other types of conjugation chemistry remain available for the attachment of additional PRR-ligands, targeting entities and or fluorophores. The immunological properties of the prepared conjugates are presently under investigation.

Experimental

All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H and ^{13}C spectra were recorded on a Bruker AV-400 (400/100 MHz) spectrometer or a Bruker AV-500 Ultrashield (500/126 MHz) spectrometer and all individual signals were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column or a Vydac 219TP 5 µm Diphenyl, 150 x 4.6 mm. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. Peptides, TLR2-ligand and conjugate were purified with a Gilson GX-281 preparative HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column, a Vydac 219TP 5 µm Diphenyl, 250 x 10 mm column or a Cosmosil 5C4-MS 250 x 10 mm column. Peptide fragments were synthesized with automated solid phase peptide synthesis on an Applied Biosystems 433A Peptide Synthesizer. Optical rotations were measured on an Anton Paar Modular Circular Polarimeter MCP 100/150. High resolution mass spectra were recorded on a Synapt G2-Si or a Q Exactive HF Orbitrap equipped with an electron spray ion source positive mode. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR.

Phenyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (12)

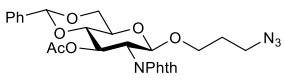


To a solution of phenyl 3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside[23] (46 g, 93 mmol, 1.0 eq.) in DCM (0.37 L) was added DMAP (2.4 g, 19 mmol, 0.2 eq.), pyridine (23 mL, 0.29 mol, 3.0 eq.) and Ac_2O (13 mL, 0.14 mol, 1.5 eq.). The reaction was stirred for 5.5 hours, after which TLC analysis showed full conversion of the starting material. The mixture was diluted with EtOAc and subsequently washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Crystallization in pentane/Et_2O gave compound **12** in quantitative yield (49 g) as white crystals. R_f: 0.70 (1/1 pentane/EtOAc); $[\alpha]_D^{20}$ +29.5° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 7.90 – 7.86 (m, 2H, Ar), 7.78 – 7.73 (m, 2H, Ar), 7.47 – 7.43 (m, 2H, Ar), 7.41 – 7.37 (m, 2H, Ar), 7.37 – 7.34 (m, 3H, Ar), 7.30 – 7.25 (m, 3H, Ar), 5.90 (t, 1H, *J* = 9.5, 9.0, 0.9 Hz, H-3), 5.83 (d, 1H, *J* = 10.6, 0.9 Hz, H-1), 5.54 (s, 1H, CH benzylidene), 4.46 – 4.41 (m, 1H, C*H*H-6), 4.39 – 4.33 (m, 1H, H-2), 3.87 – 3.73 (m, 3H, H-4, H-5, C*H*H-6), 1.88 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 170.3, 168.0, 167.4 (C=O), 137.0 (C_q Ar), 134.6, 134.4, 133.2 (Ar), 131.8, 131.3 (C_q Ar), 131.3, 129.3, 129.2, 128.5, 128.4, 126.4, 123.9, 123.8 (Ar), 101.8 (CH benzylidene), 84.0 (C-1), 79.2 (C-4), 70.7, 70.7 (C-3, C-5), 68.7 (CH_2-6), 54.4 (C-2), 20.7 (CH_3 Ac); FT-IR (neat, cm^{-1}): 2877, 1776, 1742, 1716, 1584, 1479, 1440, 1382, 1294, 1220, 1094, 1033, 1013, 995, 965, 917, 893, 872, 827, 794, 749, 720, 699, 659, 643, 610,530, 477; HRMS: $[M+Na]^+$ calcd. for $C_{29}H_{25}NO_7SNa$: 554.1249, found 554.1251.

3-Azidopropanol (13)

NaN_3 (40 g, 0.60 mol, 2.0 eq.) was added to a solution of 3-bromopropanol (28 mL, 0.30 mol, 1.0 eq.) in DMF (0.50 L) under argon atmosphere. The reaction mixture was heated to 70°C. After stirring for 72 hours, the reaction was cooled to 0°C and diluted with H_2O. The mixture was extracted with Et_2O (4x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→50% Et_2O in pentane) yielded the title compound (19 g, 0.19 mol, 64%) as a transparent liquid. R_f: 0.69 (pentane/EtOAc: 3/7); $[\alpha]_D^{20}$ -0.5° (*c* = 1.0, DCM).^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 3.91 (s, 1H, OH), 3.46 (t, 2H, *J* = 6.3 Hz, C*H*$_2$OH), 3.20 (t, 2H, *J* = 6.8 Hz, CH_2, CH_2N_3), 1.68 – 1.50 (m, 2H, CH_2); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 58.6 (CH_2, CH_2OH), 47.7 (CH_2N_3), 30.9 (CH_2); FT-IR (neat, cm^{-1}): 3349, 2946, 2880, 2092, 1456, 1344, 1260, 1049, 967, 902, 639, 557, 513.

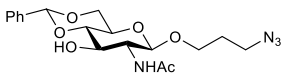
3-Azidopropyl-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (14)



A mixture of compound **12** (20.7 g, 38.4 mmol, 1.0 eq.) and alcohol **13** (5.4 mL, 59 mmol, 1.5 eq.) was co-evaporated with toluene (3x) under argon atmosphere. The mixture was dissolved in dry DCM (0.40 L), followed by the addition of 3 Å flame dried molecular sieves and NIS (10.8 g, 49.1 mmol, 1.2 eq.). After 1 hour, TMSOTf (0.70 mL, 3.9 mmol, 0.10 eq.) was added and the reaction was continued to stir for 2.5 hours, after which TLC analysis showed full conversion of the starting material. The reaction was cooled to

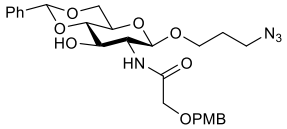
0°C, quenched with sat. aq. $NaHCO_3$, diluted with EtOAc and washed with sat. aq. $NaHCO_3$ (2x) and sat. aq. Na_2SO_4 (2x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→100% DCM in pentane, followed by 0→2% EtOAc in DCM) gave the title compound **14** (13.8 g, 26.4 mmol, 69%) as a white solid. R_f: 0.50 (2/98 EtOAc/DCM); $[\alpha]_D^{20}$ -9.8° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.93 – 7.79 (m, 2H, Ar), 7.78 – 7.67 (m, 2H, Ar), 7.49 – 7.42 (m, 2H, Ar), 7.40 – 7.30 (m, 3H, Ar), 5.90 (dd, 1H, *J* = 10.4, 8.8 Hz, H-3), 5.54 (s, 1H, CH benzylidene), 5.45 (d, 1H, *J* = 8.4 Hz, H-1), 4.41 (dd, 1H, *J* = 10.3, 4.3 Hz, C*H*H-6), 4.30 (dd, 1H, *J* = 10.4, 8.4 Hz, H-2), 3.94 – 3.69 (m, 4H, H-4, H-5, C*H*H-6, C*H*H $C_3H_6N_3$), 3.59 – 3.48 (m, 1H, C*H*H $C_3H_6N_3$), 3.24 – 3.07 (m, 2H, CH_2, $C_3H_6N_3$), 1.88 (s, 3H, CH_3 Ac), 1.81 – 1.58 (m, 2H, CH_2, $C_3H_6N_3$); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.1 (C=O), 136.9 (C_q Ar), 129.1, 128.2, 126.2, 123.6 (Ar), 101.6 (CH benzylidene), 98.7 (C-1), 79.2 (C-4), 69.7 (C-3), 68.6 (CH_2-6), 66.6 (CH_2 $C_3H_6N_3$), 66.2 (C-5), 55.3 (C-2), 47.8, 28.8 (CH_2 $C_3H_6N_3$), 20.5 (CH_3 Ac); FT-IR (neat, cm^{-1}): 2883, 2098, 1776, 1742, 1716, 1469, 1386, 1225, 1104, 1084, 1033, 999, 970, 872, 764, 722, 700, 665, 530; HRMS: $[M+Na]^+$ calcd. for $C_{26}H_{26}N_4O_8Na$: 545.1648, found 545.1646.

3-Azidopropyl-2-*N*-acetyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (15a)



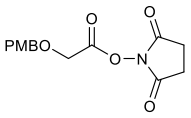
Compound **33** (2.6 g, 5.0 mmol, 1.0 eq.) was suspended in EtOH (50 mL). Ethylene diamine (17 mL, 0.25 mol, 50 eq.) was added and the reaction was heated to 90°C for 100 minutes, after which the mixture was the concentrated *in vacuo*. The residue was purified by column chromatography (1→10% MeOH in DCM). The obtained free amine was co-evaporated with dioxane (2x) and dissolved in a mixture of H_2O/THF (1/1 v/v, 40 mL). The mixture was cooled to 0°C, followed by the addition of Ac_2O (2.4 mL, 25 mmol, 5.0 eq.) and $NaHCO_3$ (4.2 g, 50 mmol, 10 eq.). The suspension was further diluted with THF (4.0 mL) and after stirring at room temperature for 72 hours, TLC analysis showed full conversion of the intermediate. The reaction mixture was diluted with EtOAc and washed with H_2O (1x), 1 M HCl (1x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude was purified by crystallization from DCM/MeOH/pentane, yielding compound **15a** (1.6 g, 4.1 mmol, 81%) as a white solid. R_f: 0.68 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ -74.0° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.52 – 7.46 (m, 2H, Ar), 7.37 – 7.31 (m, 3H, Ar), 5.60 (s, 1H, CH benzylidene), 4.53 – 4.49 (m, 1H, H-1), 4.29 (dd, 1H, *J* = 10.3, 4.9 Hz, C*H*H-6), 3.95 – 3.87 (m, 1H, C*H*H $C_3H_6N_3$), 3.85 – 3.73 (m, 3H, C*H*H-6, H-5, H-2), 3.62 – 3.49 (m, 2H, H-3, C*H*H $C_3H_6N_3$), 3.47 – 3.36 (m, 3H, H-4, CH_2 $C_3H_6N_3$), 1.99 (s, 3H, CH_3 Ac), 1.85 – 1.75 (m, 2H, CH_2-$C_3H_6N_3$); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 173.7 (C=O), 139.1 (C_q Ar), 129.9, 129.0, 127.5 (Ar), 103.4 (C-1), 102.9 (CH benzylidene), 82.9 (C-4), 72.5 (C-3), 69.7 (CH_2-6), 67.7 (CH_2 $C_3H_6N_3$), 67.4 (C-5), 58.0 (C-2), 49.1, 30.1 (CH_2 $C_3H_6N_3$), 23.0 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3266, 2871, 2103, 1659, 1627, 1555, 1034, 756, 698, 473; HRMS: $[M+Na]^+$ calcd. for $C_{18}H_{24}N_4O_6Na$: 415.1588, found 415.15873.

3-Azidopropyl-4,6-*O*-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-β-D-glucopyranoside (15b)



Compound **14** (4.2 g, 8.1 mmol, 1.0 eq.) was suspended in EtOH (80 mL). Ethylene diamine (27 mL, 0.40 mol, 50 eq.) was added and the reaction was heated to 90°C for 2 hours, after which the mixture was concentrated *in vacuo*. Purification by column chromatography (1→10% MeOH in DCM) yielded the desired free amine, which was co-evaporated with dioxane (2x) under argon atmosphere and dissolved in DCM (40 mL). Compound **16** (3.28 g, 11.2 mmol, 1.4 eq.) and Et_3N (1.7 mL, 12 mmol, 1.5 eq.) were added. After stirring overnight, the reaction was washed with sat. aq. $NaHCO_3$ (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude was purified by crystallization from DCM/pentane, yielding the title compound **15b** (3.3 g, 6.3 mmol, 78%) as a white solid. R_f: 0.80 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ -38.0° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 500 MHz, HH-COSY, HSQC): δ 7.52 – 7.47 (m, 2H, Ar), 7.37 – 7.30 (m, 5H, Ar), 6.95 – 6.90 (m, 2H, Ar), 5.60 (s, 1H, CH benzylidene), 4.63 – 4.60 (m, 1H, H-1), 4.58 – 4.52 (m, 2H, CH_2 glycol), 4.29 (dd, 1H, *J* = 10.3, 5.0 Hz, C*H*H-6), 4.00 – 3.91 (m, 2H, CH_2 PMB), 3.91 – 3.84 (m, 3H, H-2, H-3, C*H*H $C_3H_6N_3$), 3.84 – 3.77 (m, 4H, C*H*H-6, CH_3 PMB), 3.61 – 3.51 (m, 2H, H-4, C*H*H $C_3H_6N_3$), 3.49 – 3.42 (m, 1H, H-5), 3.39 – 3.32 (m, 2H, CH_2, $C_3H_6N_3$), 1.82 – 1.75 (m, 2H, CH_2, $C_3H_6N_3$); ^{13}C-APT NMR (MeOD, 126 MHz, HSQC): δ 173.1 (C=O), 161.2, 139.1 (C_q Ar), 131.0, 130.5, 129.9, 129.0, 127.5, 114.9 (Ar), 103.1 (C-1), 102.9 (CH benzylidene), 83.0 (C-4), 74.0 (CH_2 glycol), 72.2 (C-3), 69.8 (CH_2 PMB), 69.7 (CH_2-6), 67.6 (C-5), 67.5 (CH_2 $C_3H_6N_3$), 57.6 (C-2), 55.7 (CH_3 PMB), 49.1, 30.1 (CH_2 $C_3H_6N_3$); FT-IR (neat, cm^{-1}): 3676, 2972, 2097, 1660, 1514, 1454, 1381, 1250, 1175, 1089, 1033, 754, 700; HRMS: $[M+Na]^+$ calcd. for $C_{26}H_{32}N_4O_8Na$: 551.2112, found 551.21124.

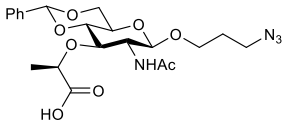
***N*-succinimidyl-(*p*-methoxybenzyloxy)acetate (16)**



Methyl glycolate (5.0 g, 55 mmol, 1.0 eq.) was dissolved in DMF (0.50 L) and cooled to 0°C after which sodium hydride (60% dispersion in mineral oil, 3.3 g, 83 mmol, 1.5 eq.) was added. After 20 minutes, *p*-methoxybenzyl chloride (11 mL, 83 mmol, 1.5 eq.) was added and the reaction mixture was allowed to warm-up to room temperature overnight. The reaction was cooled to 0°C, quenched with MeOH/H_2O and diluted with Et_2O. The obtained mixture was washed with H_2O (3x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was dissolved in a mixture of ethanol/H_2O (7/1 v/v, 160 mL), followed by the addition of $LiOH{\cdot}H_2O$ (5.8 g. 0.14 mol, 2.5 eq.) at 0°C. After stirring overnight, the solution was diluted with H_2O. The mixture was acidified with 1 M HCl to pH = 5 and extracted with DCM (2x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (2→10% methanol in DCM) afforded (*p*-Methoxybenzyloxy) acetic acid (6.4 g, 32 mmol, 59%) as a yellow oil. Rf: 0.4 (1/9 MeOH/DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.29 (d, 2H, Ar), 6.90 (d, 2H, Ar), 4.58 (s, 2H, CH_2 Glycolyl), 4.11 (s, 2H, CH_2 PMB), 3.81 (s, 3H, CH_3 PMB); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 175.5 (C=O), 159.7 (C_q Ar), 130.6, 129.8 (Ar), 128.7 (C_q Ar), 114.1 (Ar), 73.2 (CH_2 Glycolyl), 66.3 (CH_2 PMB), 55.4 (CH_3 PMB); FT-IR (neat, cm-1): 2937, 2838, 1726,

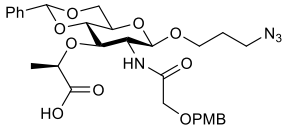
1611, 1586, 1513, 1464, 1441, 1301, 924, 817, 759, 735, 669, 637, 580, 518; HRMS: $[M+Na]^+$ calcd. for $C_{10}H_{12}O_4Na$: 219.0634, found 219.0632. (*p*-Methoxybenzyloxy) acetic acid (4.7 g, 24 mmol, 1.0 eq.) was dissolved in MeCN (0.24 L), followed by the addition of DCC (3.7 mL, 24 mmol, 1.0 eq.) and *N*-hydroxylsuccinimide (4.1 g, 36 mmol, 1.5 eq.). After 16 hours, TLC analysis showed full conversion of the starting material and the reaction mixture was filtered over celite and concentrated *in vacuo*. Purification by column chromatography (20→50% EtOAc in pentane) gave the title compound (5.90 g, 20.1 mmol, 85%) as a white solid. R_f: 0.28 (3/2 pentane/EtOAc); $[\alpha]_D^{20}$ +5.8° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.30 (d, 2H, Ar), 6.90 (d, 2H, Ar), 4.61 (s, 2H, 2H, CH_2 Glycolyl), 4.40 (s, 2H, CH_2 PMB), 3.81 (s, 3H, CH_3 PMB), 2.85 (s, 4H, CH2 succinimide); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 168.9, 166.1 (C=O), 159.8, 130.8 (C_q Ar), 130.1, 130.0, 128.4, 114.1, 113.9 (Ar), 73.3 (CH_2 Glycolyl), 64.5 (CH_2 PMB), 55.4 (CH_3 PMB), 25.7 (CH_2 Succinimide) FT-IR (neat, cm^{-1}): 2939, 1706, 1612, 1586, 1514, 1465, 1429, 1303, 1247, 1213, 1176, 1109, 1031, 818, 761, 715, 656, 579, 521; HRMS: $[M+Na]^+$ calcd. for $C_{14}H_{15}NO_6Na$: 316.0797, found 316.0802.

3-Azidopropyl-2-*N*-acetyl-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside (9a)



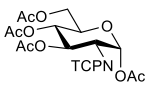
Compound **15a** (1.5 g, 3.8 mmol, 1.0 eq.) was co-evaporated with dioxane (3x) under argon atmosphere and dissolved in DMF (19 mL). The mixture was cooled to 0°C and sodium hydride (60% dispersion in mineral oil, 0.75 g, 19 mmol, 5.0 eq.) was added. After stirring for 1 hour, (S)-(-)-2-chloropropionic acid (0.71 mL, 8.3 mmol, 2.2 eq.) was slowly added. After 2 hours, sodium hydride (60% dispersion in mineral oil, 0.76 g, 19 mmol, 5.0 eq.) was added and the mixture was allowed to warm-up to room temperature overnight, after which TLC analysis showed full conversion of the starting material. The reaction mixture was cooled to 0°C, slowly quenched with H_2O, acidified with 1 M HCl to pH = 4 and extracted with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by crystallization in DCM/MeOH/pentane, gave compound **9a** (1.47 g, 3.16 mmol, 83%) as white crystals. R_f: 0.57 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ -46.5° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.49 – 7.44 (m, 2H, Ar), 7.40 – 7.34 (m, 3H, Ar), 5.63 (s, 1H, CH benzylidene), 4.56 (d, 1H, *J* = 7.7 Hz, H-1), 4.39 (q, 1H, *J* = 6.9 Hz, CH lactic acid), 4.29 (dd, 1H, *J* = 10.3, 5.0 Hz, C*H*H-6), 3.94 – 3.86 (m, 1H, C*H*H $C_3H_6N_3$), 3.85 – 3.70 (m, 3H, H-2, H-3, C*H*H-6), 3.70 – 3.64 (m, 1H, H-4), 3.61 – 3.54 (m, 1H, C*H*H $C_3H_6N_3$), 3.50 – 3.41 (m, 1H, H-5), 3.38 (t, 2H, *J* = 6.6 Hz, $C_3H_6N_3$), 1.99 (s, 3H, CH_3 Ac), 1.86 – 1.73 (m, 2H, CH_2, $C_3H_6N_3$), 1.33 (d, 3H, *J* = 6.9 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.7, 173.9 (C=O), 139.1 (C_q Ar), 130.0, 129.2, 127.2 (Ar), 103.4 (C-1), 102.5 (CH_2 benzylidene), 83.6 (C-4), 79.6 (C-3), 77.0 (CH lactic acid), 69.6 (CH_2-6), 67.5 (CH_2 $C_3H_6N_3$), 67.3 (C-5), 56.6 (C-2), 49.1, 30.1 (CH_2 $C_3H_6N_3$), 23.2 (CH_3 Ac), 19.4 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 3269, 2876, 2104, 1712, 1657, 1562, 1452, 1374, 1308, 1177, 1120, 1095, 1013, 966, 748, 695; HRMS: $[M+Na]^+$ calcd. for $C_{21}H_{19}N_4O_8Na$: 465.1980, found 465.19795; LC-MS: Rt = 6.36 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

3-Azidopropyl-4,6-*O*-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside (9b)



Compound **15b** (2.6 g, 5.0 mmol, 1.0 eq.) was co-evaporated with dioxane (3x) under argon atmosphere and dissolved in DMF (20 mL). The mixture was cooled to 0°C and sodium hydride (60% dispersion in mineral oil, 1.0 g, 25 mmol, 5.0 eq.) was added. After stirring for 1 hour, (S)-(-)-2-chloropropionic acid (0.94 mL, 11 mmol, 2.2 eq.) was slowly added. After 1 hour, sodium hydride (60% dispersion in mineral oil, 1.0 g, 25 mmol, 5.0 eq.) was added and the mixture was allowed to warm-up to room temperature overnight, after which TLC analysis showed full conversion of the starting material. The reaction mixture was cooled to 0°C, slowly quenched with H_2O, acidified with 1 M HCl to pH = 4 and extracted with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by crystallization in DCM/MeOH/Pentane afforded compound **9b** (2.6 g, 4.3 mmol, 86%) as white crystals. R_f: 0.57 (1/9 DCM/MeOH); $[\alpha]_D^{20}$ -34.5° (*c* = 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.48 – 7.42 (m, 2H, Ar), 7.41 – 7.35 (m, 3H, Ar), 7.29 – 7.24 (m, 3H, Ar), 7.06 (d, 1H, *J* = 7.9 Hzn NH), 6.89 (d, 2H, Ar), 5.55 (s, 1H, CH benzylidene), 4.83 (d, 1H, *J* = 8.3 Hz, H-1), 4.58 – 4.47 (m, 2H, CH_2 Glycol), 4.47 – 4.38 (m, 1H CH lactic acid), 4.35 (dd, 1H, *J* = 10.5, 5.0 Hz, C*H*H-6), 4.16 – 3.87 (m, 4H, H-3 CH_2 PMB, C*H*H $C_3H_6N_3$), 3.83 – 3.74 (m, 4H, C*H*H-6, CH_3 PMB), 3.68 – 3.53 (m, 3H, H-2, H-4, C*H*H $C_3H_6N_3$), 3.53 – 3.43 (m, 1H, H-5), 3.34 (t, 2H, *J* = 6.6 Hz, $C_3H_6N_3$), 1.91 – 1.74 (m, 2H, CH_2, $C_3H_6N_3$), 1.42 (d, 3H, *J* = 7.0 Hz, CH_3 lactic acid); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.6, 171.8 (C=O), 159.8, 137.1 (C_q Ar), 130.0, 129.3 (Ar), 129.0 (C_q Ar), 128.5, 126.0, 120.3, 114.2 (Ar), 101.5 (CH_2 benzylidene), 100.8 (C-1), 82.4 (C-4), 78.1 (C-3), 76.2 (CH lactic acid), 73.4 (CH_2 glycol), 69.3 (CH_2 PMB), 68.8 (CH_2-6), 66.7 (CH_2 $C_3H_6N_3$), 66.0 (C-5), 56.6 (C-2), 56.6 (CH_3 PMB), 48.1, 29.1 (CH_2 $C_3H_6N_3$), 19.1, (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 2973, 2099, 1659, 1514, 1454, 1381, 1250, 1177, 1091, 1033, 751, 699; HRMS: $[M+Na]^+$ calcd. for $C_{29}H_{37}N_4O_{10}Na$: 601.2504, found 601.25021; LC-MS: Rt = 7.78 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

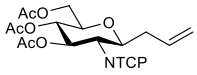
1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-α-D-glucopyranoside (21)



Glucosamine hydrochloride (21.6 g, 100 mmol, 1.0 eq.) was added to a solution of sodium methoxide (1.0 M in MeOH, 0.10 L, 1.0 eq.) at room temperature and the obtained solution was stirred for 10 minutes, followed by the addition of tetrachlorophthalic anhydride (14.3 g, 50.0 mmol, 0.5 eq.). After 20 minutes, additional tetrachlorophthalic anhydride (14.3 g, 50.0 mmol, 0.5 eq.) and Et_3N (10 mL, 0.10 mol, 1.0 eq.) were added and the reaction was stirred at 50°C for 20 minutes. The mixture was concentrated *in vacuo*. The residue was dissolved in pyridine (98 mL), followed by slow addition of Ac_2O (0.15 L, 1.6 mol, 16.0 eq.). The resulting mixture was stirred for 16 hours at room temperature, after which it was poured into ice water (0.15 L) and extracted with DCM (3x). The combined organic layers were subsequently washed with a 1 M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered, concentrated *in vacuo* and co-evaporated with toluene (1x). Recrystallization in MeOH yielded the title compound (31.4 g, 51.0 mmol, 51%) as a white solid. R_f: 0.6 (3/2 pentane/EtOAc); $[\alpha]_D^{20}$ = +96.6° (*c*

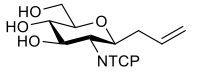
= 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 6.48 (dd, 1H, *J* = 11.5, 9.1 Hz, H-3), 6.24 (d, 1H, *J* = 3.4 Hz, H-1), 5.15 (t, 1H, *J* = 10.1, 9.0 Hz, H-4), 4.70 (dd, 1H, *J* = 11.5, 3.4 Hz, H-2), 4.38 – 4.27 (m, 2H, H-5, C*H*H-6), 4.13 (dd, 1H, *J* = 12.2, 1.8 Hz, C*H*H-6), 2.11 (s, 3H, CH_3 Ac), 2.08 (s, 3H, CH_3 Ac), 2.05 (s, 3H, CH_3 Ac), 1.90 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.8, 169.9, 169.8, 169.6 (C=O), 140.9 (C_q Ar), 130.3, 126.8 (C-Cl), 90.6 (C-1), 70.4 (C-5), 69.3 (C-4), 67.0 (C-3), 61.5 (CH_2-6), 53.5 (C-2), 21.1, 20.9, 20.8, 20.8 (CH_3 Ac); FT-IR (neat, cm^{-1}): 2965, 1750, 1731, 1385, 1370, 1219, 1154, 1081, 1040, 1015, 922, 794, 752, 740, 603, 540, 485; HRMS: $[M+Na]^+$ calcd. for $C_{22}H_{19}Cl_4NO_{11}Na$ 635.9610, found 635.9617.

3-*C*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranosyl)-1-propene (22)



Compound **21** (24.6 g, 40.0 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in acetonitrile (0.24 L) and cooled to 0°C. Allyltrimethylsilane (32 mL, 0.20 mol, 5.0 eq.) was added, followed by slow addition of TMSOTf (7.2 mL, 40 mmol, 1.0 eq.) and $BF_3{\cdot}OEt_2$ (25 mL, 0.20 mol, 5.0 eq.). The yellow suspension was sonicated for 90 minutes and stirred for an additional hour at room temperature. The resulting brown solution was cooled to 0°C and quenched with Et_3N to pH = 7. The reaction was diluted with EtOAc, washed with sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo.* Purification column chromatography (10→50% Et_2O in pentane) yielded the title compound (13.9 g, 23.2 mmol, 58%) as a white foam. R_f: 0.5 (1/1 pentane/Et_2O); $[\alpha]_D^{20}$ = +74.4° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.78 – 5.65 (m, 2H, H-3, CH_2-C*H*=CH_2), 5.12 (t, 1H, *J* = 10.2 Hz, H-4), 5.00 – 4.87 (m, 2H, CH_2-CH=C*H*$_2$), 4.47 – 4.34 (m, 1H, H-1), 4.27 (dd, 1H, *J* = 12.3, 4.9 Hz, C*H*H-6), 4.21 (t, 1H, *J* = 10.2 Hz, H-2), 4.10 (dd, 1H, *J* = 12.3, 2.3 Hz, C*H*H-6), 3.79 – 3.73 (m, 1H, H-5), 2.26 (t, 2H, *J* = 6.8 Hz, C*H*$_2$-CH=CH_2), 2.09 (s, 3H, CH_3 Ac), 2.01 (s, 3H, CH_3 Ac), 1.86 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.9, 170.8, 169.6, 163.5, 162.8 (C=O), 140.9, 140.6 (C_q Ar), 132.5 (CH_2-*C*H=CH_2), 130.2, 130.0, 127.1, 126.8 (C-Cl), 118.1 (CH_2-CH=*C*H$_2$), 75.8 (C-5), 74.0 (C-1), 71.9 (C-3), 69.0 (C-4), 62.4 (CH_2-6), 55.3 (C-2), 36.8 (*C*H$_2$-CH=CH_2), 20.9, 20.7, 20.6 (CH_3 Ac); FT-IR (neat, cm^{-1}): 2957, 1782 1746, 1724, 1384, 1370, 1352, 1226, 1150, 1047, 908, 791, 753, 740, 603; HRMS: $[M+H]^+$ calcd. for $C_{23}H_{22}Cl_4NO_9$ 596.0043, found 596.0045.

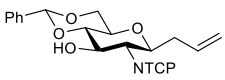
3-*C*-(2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranosyl)-1-propene (23)



Acetyl chloride (1.6 mL, 23 mmol, 0.8 eq.) was added to a solution of compound **22** (17.1 g, 28.7 mmol, 1.0 eq.) in a mixture of DCM/MeOH (1:4 v/v, 0.29 L) at 0°C. After stirring for 1 hour, reaction mixture was allowed to warm-up to room temperature and stirred for 72 hours. The mixture was diluted with toluene (30 mL) and concentrated *in vacuo*. The residue was co-evaporated with toluene (2x) and purified by column chromatography (1→10% MeOH in DCM) to obtain the title compound (12.7 g, 26.9 mmol, 94%) as a white solid. R_f: 0.5 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = +34.7° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.76 – 5.63 (m, 1H, CH_2-C*H*=CH_2), 4.88 (t, 2H, CH_2-CH=C*H*$_2$),

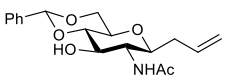
4.24 (t, 1H, *J* = 10.5, 8.8 Hz, H-3), 4.21 – 4.13 (m, 1H, H-1), 3.91 (t, 1H, *J* = 10.3, 10.3 Hz, H-2), 3.86 – 3.77 (m, 2H, C*H*$_2$-6), 3.56 (t, 1H, *J* = 9.2, 9.2 Hz, H-4), 3.52 – 3.43 (m, 3H, OH), 3.40 (dt, 1H, *J* = 9.6, 3.2, 3.2 Hz, H-5), 2.27 – 2.10 (m, 2H, C*H*$_2$-CH=CH$_2$); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 163.9, 163.8 (C=O), 140.4, 140.4 (C_q Ar), 133.4 (CH$_2$-*C*H=CH$_2$), 130.1, 129.7, 127.3, 127.3 (C-Cl), 117.5 (CH$_2$-CH=*C*H$_2$), 79.2 (C-5), 74.2 (C-1), 71.8 (C-3), 71.4 (C-4), 62.0 (CH$_2$-6), 57.5 (C-2), 37.2 (*C*H$_2$-CH=CH$_2$); FT-IR (neat, cm^{-1}): 3378, 2929, 1779, 1718, 1387, 1370, 1351, 1299, 1202, 1142, 1085, 1000, 919, 791, 753, 740, 643; HRMS: [M+Na]$^+$ calcd. for $C_{17}H_{15}Cl_4NO_6Na$ 491.9551, found 491.9551.

3-*C*-(4,6-di-*O*-benzylidene-2-deoxy-2-tetrachlorophthalimido-β-D-glucopyranosyl)-1-propene (24)



Compound **23** (10.6 g, 22.5 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in a mixture of DMF/acetonitrile (9:1 v/v, 113 mL). Benzylaldehyde dimethyl acetal (6.9 mL, 45 mmol, 2.0 eq.) and *p*-toluenesulfonic acid (0.43 g, 2.3 mmol, 0.1 eq.) were added and the mixture was heated to 60°C. After stirring overnight, the mixture was cooled to 0°C and quenched with Et_3N to pH = 7. The solution was diluted with EtOAc and the organic layer was washed with H_2O (3x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→40% Et_2O in pentane) gave compound **24** (11.0 g, 19.7 mmol, 87%) as a white solid. R_f: 0.8 (1/1 pentane/Et_2O); $[\alpha]_D^{20}$ = +17.5° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.39 – 7.27 (m, 5H, Ar), 5.78 – 5.64 (m, 1H, CH$_2$-C*H*=CH$_2$), 5.49 (s, 1H, CH benzylidene), 4.94 (t, 2H, *J* = 9.4 Hz, CH$_2$-CH=C*H*$_2$), 4.60 (t, 1H, *J* = 10.2, 9.0 Hz, H-3), 4.33 (dd, 1H, *J* = 10.2, 4.7 Hz, C*H*H-6), 4.30 – 4.22 (m, 1H, H-1), 4.05 (t, 1H, *J* = 10.2 Hz, H-2), 3.70 (t, 1H, *J* = 10.1 Hz, C*H*H-6), 3.64 – 3.55 (m, 1H, H-5), 3.48 (t, 1H, *J* = 9.1 Hz, H-4), 3.19 (s, 1H, OH), 2.30 – 2.17 (m, 2H, C*H*$_2$-CH=CH$_2$); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 163.7, 163.2 (C=O), 140.4, 140.3 (C-Cl), 136.9 (C_q Ar), 133.0 (CH$_2$-*C*H=CH$_2$), 130.1, 129.8 (C-Cl), 129.3, 128.3 (Ar), 127.1, 127.1 (C-Cl), 126.0 (Ar), 117.7 (CH$_2$-CH=*C*H$_2$), 101.7 (CH benzylidene), 82.5 (C-4), 75.0 (C-1), 70.1 (C-5), 68.8 (CH$_2$-6), 68.6 (C-3), 57.1 (C-2), 37.0 (*C*H$_2$-CH=CH$_2$); FT-IR (neat, cm^{-1}): 3485, 2864, 1779, 1720, 1371, 1351, 1300, 1203, 1124, 1096, 988, 918, 790, 753, 740, 699, 643; HRMS: [M+H]$^+$ calcd. for $C_{24}H_{20}Cl_4NO_6$ 558.0039, found 558.0047.

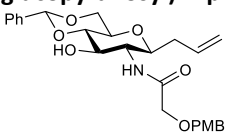
3-*C*-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-β-D-glucopyranosyl)-1-propene (25a)



To a solution of compound **24** (3.8 g, 6.8 mmol, 1.0 eq.) in EtOH (70 mL) was added ethylenediamine (23 mL, 0.34 mol, 50 eq.) and the reaction was heated to 90°C. After 16 hours, the reaction mixture was diluted with toluene and concentrated *in vacuo*. The residue was co-evaporated with toluene (3x) and imbedded on silica gel. Purification by column chromatography (2→5% MeOH in DCM) gave 3-*C*-(4,6-di-*O*-benzylidene-2-deoxy-2-amine-β-D-glucopyranosyl)-1-propene (1.92 g, 6.59 mmol) as a yellow solid. R_f: 0.42 (1/9 MeOH/DCM). The obtained amine (1.92 g, 6.59 mmol, 1.0 eq.) was dissolved in a mixture of THF/H_2O (1/1 v/v, 50 mL). Sodium bicarbonate (5.6 g, 66 mmol, 10 eq.) and Ac_2O (3.1 mL, 33 mmol, 5.0 eq.) were added. The mixture was stirred at room temperature for 4 days, after which the reaction mixture was diluted with EtOAc. The

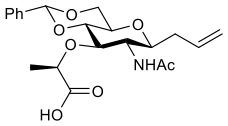
obtained suspension was filtered and the obtained pure title compound was collected as a white solid. The filtrate was washed with sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The remaining crude product was crystallized using DCM/MeOH/pentane giving compound **25a**. The remaining residue was imbedded on silica and purified by column chromatography (2→6% MeOH in DCM). The combined title compound (1.87 g, 5.63 mmol, 83% over two steps) was collected as a white solid. R_f: 0.5 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -38.5° (*c* = 0.3, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.53 – 7.46 (m, 2H, Ar), 7.38 – 7.30 (m, 3H, Ar), 5.93 – 5.80 (m, 1H, CH_2-C*H*=CH_2), 5.59 (s, 1H, CH benzylidene), 5.03 (t, 2H, *J* = 17.5, 8.7 Hz, CH_2-CH=C*H*$_2$), 4.25 (dd, 1H, *J* = 10.3, 5.0 Hz, C*H*H-6), 3.75 (q, 2H, *J* = 11.5, 10.0 Hz, H-2, C*H*H-6), 3.66 (t, 1H, *J* = 9.7, 8.9 Hz, H-3), 3.50 (t, 1H, *J* = 9.1 Hz, H-4), 3.47 – 3.34 (m, 2H, H-1, H-5), 2.41 – 2.29 (m, 1H, C*H*H-CH=CH_2), 2.26 – 2.13 (m, 1H, C*H*H-CH=CH_2), 1.99 (s, 3H, C*H*$_3$ Ac); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 173.7 (C=O), 139.2 (C_q Ar), 135.7 (CH_2-C*H*=CH_2), 129.9, 129.0, 127.5 (Ar), 117.2 (CH_2-CH=*C*H_2), 102.9 (CH benzylidene), 83.2 (C-4), 80.4 (C-1), 73.9 (C-3), 71.8 (C-5), 69.8 (CH_2-6), 57.2 (C-2), 37.7 (*C*H_2-CH=CH_2), 22.9 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3380, 2361, 1630, 1377, 1125, 1033, 999, 698; HRMS: $[M+H]^+$ calcd. for $C_{18}H_{24}NO_5$ 334.1655, found 334.1654.

3-*C*-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-β-D-glucopyranosyl)-1-propene (25b)



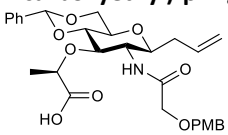
A mixture of 3-*C*-(4,6-di-*O*-benzylidene-2-deoxy-2-amine-β-D-glucopyranosyl)-1-propene (see synthesis of **25a**) (6.13 g, 21.0 mmol, 1.0 eq.), compound **16** (7.13 g, 24.3 mmol, 1.2 eq.) and Et_3N (4.2 mL, 32 mmol, 1.5 eq.) in DCM (0.10 L) stirred for 16 hours under an argon atmosphere. The reaction was washed with sat. aq. $NaHCO_3$ (1x) and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (40→100% EtOAc in pentane) yielded compound **25b** (9.66 g, 20.6 mmol, 98%) as a white solid. R_f: 0.4 (3/7 pentane/EtOAc); $[\alpha]_D^{20}$ = -43.3° (*c* = 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.52 – 7.46 (m, 2H, Ar), 7.40 – 7.31 (m, 3H, Ar), 7.28 – 7.22 (m, 2H, Ar), 6.93 – 6.87 (m, 2H, Ar), 6.53 (d, 1H, *J* = 8.9 Hz, NH), 5.89 – 5.76 (m, 1H, CH_2-C*H*=CH_2), 5.53 (s, 1H, CH benzylidene), 5.11 – 5.00 (m, 2H, CH_2-CH=C*H*$_2$), 4.50 (q, 2H, *J* = 11.1, 3.6 Hz, CH_2 glycolyl), 4.31 (dd, 1H, *J* = 10.4, 5.0 Hz, C*H*H-6), 3.99 (q, 2H, *J* = 15.3, 14.2, 4.0 Hz, CH_2 PMB), 3.90 – 3.74 (m, 5H, H-2, H-4, CH_3 PMB), 3.70 (t, 1H, *J* = 10.3 Hz, C*H*H-6), 3.54 – 3.44 (m, 2H, H-1, H-3), 3.44 – 3.35 (m, 1H, H-5), 2.42 – 2.32 (m, 1H, C*H*H_2-CH=CH_2), 2.32 – 2.15 (m, 1H, C*H*H-CH=CH_2); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.9 (C=O), 159.9, 137.3 (C_q Ar), 133.9 (CH_2-*C*H=CH_2), 129.9, 129.3 (Ar), 128.8 (C_q Ar), 128.4, 126.5 (Ar), 117.6 (CH_2-CH=*C*H_2), 114.2 (Ar), 101.9 (CH benzylidene), 82.0 (C-4), 78.6 (C-1), 73.5 (CH_2 glycolyl), 73.4 (C-3), 70.3 (C-5), 69.2 (CH_2 PMB), 68.9 (CH_2-6), 55.4 (CH_3 PMB), 55.4 (C-2), 36.5 (*C*H_2-CH=CH_2); FT-IR (neat, cm^{-1}): 3386, 2862, 2360, 1666, 1612, 1514, 1454, 1250, 1097, 1033, 822, 763, 700; HRMS: $[M+H]^+$ calcd. for $C_{26}H_{32}NO_7$ 470.2180, found 470.2177.

3-*C*-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranosyl)-1-propene (26a)



Compound **25a** (2.76 g, 8.28 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere and dissolved in DMF (41 mL). The solution was cooled to 0°C and NaH (60% dispersion in mineral oil, 1.66 g, 42 mmol, 5.1 eq.) was added. The mixture was stirred at 0°C for 30 minutes before dropwise addition of (S)-(-)-2-chloropropionic acid (1.6 mL, 18.7 mmol, 2.3 eq.). After stirring for an additional 30 minutes at 0°C, NaH (60% dispersion in mineral oil, 1.66 g, 42 mmol, 5.1 eq.) was added and the mixture was allowed to slowly warm-up to room temperature overnight. The reaction mixture was diluted with DCM, cooled to 0°C and quenched with H_2O. The suspension was acidified with 1 M HCl to pH = 1 and extracted with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Precipitation from DCM with pentane gave the title compound (3.20 g, 7.89 mmol, 95%) as a white solid. R_f: 0.3 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -56.6° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.43 (m, 2H, Ar), 7.40 – 7.31 (m, 3H, Ar), 5.92 – 5.80 (m, 1H, CH_2-C*H*=CH_2), 5.62 (s, 1H, CH benzylidene), 5.02 (t, 2H, *J* = 17.4, 9.3 Hz, CH_2-CH=C*H*$_2$), 4.40 (q, 1H, *J* = 6.9 Hz, CH lactic acid), 4.25 (dd, 1H, *J* = 10.4, 5.0 Hz, C*H*H-6), 3.79 – 3.71 (m, 2H, H-2, H-3), 3.70 – 3.60 (m, 2H, H-4, C*H*H-6), 3.49 – 3.35 (m, 2H, H-1, H-5), 2.40 – 2.31 (m, 1H, CH*H*-CH=CH_2), 2.24 – 2.14 (m, 1H, CH*H*-CH=CH_2), 1.99 (s, 3H, CH_3 Ac), 1.33 (d, 3H, *J* = 6.9 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.9 (C=O lactic acid), 174.1 (C=O Ac), 139.2 (C_q Ar), 135.7 (CH_2-*C*H=CH_2), 129.9 , 129.2, 127.2 (Ar), 117.3 (CH_2-CH=*C*H$_2$), 102.5 (CH benzylidene), 84.1 (C-4), 81.1 (C-3), 80.6 (C-1), 76.9 (CH lactic acid), 71.5 (C-5), 69.7 (CH_2-6), 55.8 (C-2), 37.7 (*C*H$_2$-CH=CH_2), 23.1 (CH_3 Ac), 19.4 (CH_3 PMB); FT-IR (neat, cm^{-1}): 2871, 1654, 1552, 1103, 1033, 1011, 696; HRMS: $[M+H]^+$ calcd. for $C_{21}H_{28}NO_7$ 406.1861, found 406.1872.

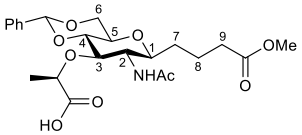
3-*C*-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranosyl)-1-propene (26b)



Compound **25b** (9.48 g, 20.2 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere and dissolved in DMF (0.10 L). The solution was cooled to 0°C, NaH (60% dispersion in mineral oil, 4.04 g, 0.10 mol, 5.0 eq.) was added and the mixture was stirred at 0°C for 30 minutes. (S)-(-)-2-chloropropionic acid (3.8 mL, 44.4 mmol, 2.2 eq.) was added dropwise and stirring was continued for 30 minutes at 0°C. After addition of NaH (60% dispersion in mineral oil, 4.04 g, 0.10 mol, 5.0 eq.), the mixture was stirred for another 15 minutes at 0°C before being allowed to warm-up to room temperature. After stirring for 16 hours, TLC analysis showed full conversion of the starting material, the reaction mixture was diluted with DCM, cooled to 0°C and quenched with H_2O. The suspension was acidified with 1 M HCl to pH = 1 and extracted with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Precipitation from DCM with pentane gave the title compound as a white solid (10.0 g, 18.5 mmol, 91%). R_f: 0.6 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -33.0° (*c* = 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.48 – 7.41 (m, 2H, Ar), 7.40 – 7.32 (m, 3H, Ar), 7.25 (d, 2H, *J* = 8.5 Hz, Ar), 6.98 (d, 1H, *J* = 8.0 Hz, NH), 6.88 (d, 2H, *J*

= 8.6 Hz, Ar), 5.87 – 5.74 (m, 1H, CH_2-C*H*=CH_2), 5.54 (s, 1H, CH benzylidene), 5.09 – 5.01 (m, 2H, CH_2-CH=C*H*$_2$), 4.51 (q, 2H, *J* = 11.2, 9.3, 3.8 Hz, CH_2 glycolyl), 4.45 (q, 1H, *J* = 6.8, 4.6 Hz, CH lactic acid), 4.31 (dd, 1H, *J* = 10.5, 5.0 Hz, C*H*H-6), 4.00 (s, 2H, CH_2 PMB), 3.84 – 3.74 (m, 5H, H-2, H-3, CH_3 PMB), 3.71 (t, 1H, *J* = 10.3 Hz, C*H*H-6), 3.64 – 3.54 (m, 2H, H-1, H-4), 3.44 – 3.36 (m, 1H, H-5), 2.40 – 2.31 (m, 1H, C*H*H-CH=CH_2), 2.28 – 2.18 (m, 1H, C*H*H-CH=CH_2), 1.41 (d, 3H, *J* = 6.9 Hz, CH_3 lactic acid); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 175.9 (C=O lactic acid), 171.6 (C=O glycolyl), 159.8, 137.2 (C_q Ar), 133.9 (CH_2-*C*H=CH_2), 129.9, 129.2 (Ar), 128.9 (C_q Ar), 128.5, 126.0 (Ar), 117.6 (CH_2-CH=*C*H_2), 114.2 (Ar), 101.3 (CH benzylidene), 82.8 (C-4), 79.7 (C-3), 78.8 (C-1), 75.7 (CH lactic acid), 73.4 (CH_2 glycolyl), 70.3 (C-5), 69.2 (CH_2 PMB), 68.9 (CH_2-6), 55.4 (CH_3 PMB), 54.6 (C-2), 36.6 (*C*H_2-CH=CH_2), 19.1 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 2938, 1514, 1250, 1105, 1055, 1033, 1011; HRMS: $[M+H]^+$ calcd. for $C_{29}H_{36}NO_9$ 542.2385, found 542.2386.

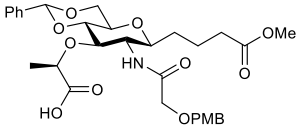
Methyl 4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranosyl)-butanoate (27a)



Compound **26a** (2.35 g, 5.79 mmol, 1.0 eq.) was co-evaporated with dioxane (2x) and THF (1x) under an argon atmosphere before being dissolved in THF (58 mL). Methyl acrylate (1.5 mL, 16.2 mmol, 2.8 eq.) and copper iodide (0.17 g, 0.87 mmol, 0.15 eq.) were added, followed by the addition of Grubbs 2nd generation catalyst (0.51 g, 0.58 mmol, 0.1 eq.). After shielding the flask from light with aluminium foil, the reaction was heated to 40°C for 48 h. The reaction mixture was concentrated *in vacuo* and co-evaporated with toluene (3x) under an argon atmosphere and dissolved in THF (23 mL). The solution was purged with argon for 5 minutes. Ruthenium trichloride (0.26 g, 1.16 mmol, 0.2 eq.) and $NaBH_4$ (0.70 g, 18.5 mmol, 3.3 eq.) were added and an empty balloon was connected to the reaction. The mixture was cooled to 0°C before dropwise addition of MeOH (6.7 mL). The reaction was stirred at 40°C for 3 hours. After completion of the reaction determined by LC-MS, the reaction was cooled to 0°C, quenched with H_2O and diluted with DCM. The mixture was acidified with 1 M HCl to pH = 1, and the aqueous layer was extracted with DCM (3x). The combined organic layers were washed with brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (2→10% MeOH in DCM + 0.1% AcOH) and recrystallization (DCM/pentane) to obtain a mixture of compound **27a** (1.73 g, 3.71 mmol, 64%) and **28a** (0.50 g, 1.24 mmol). Analysis given for title compound only. R_f: 0.3 (1/9 MeOH/DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.43 (m, 2H, Ar), 7.40 – 7.31 (m, 3H, Ar), 5.61 (s, 1H, CH benzylidene), 4.40 (q, 1H, *J* = 6.9 Hz, CH lactic acid), 4.25 (dd, 1H, *J* = 10.3, 5.0 Hz, C*H*H-6), 3.77 – 3.68 (m, 2H, H-2, C*H*H-6), 3.68 – 3.60 (m, 5H, H-3, H-4, OCH_3), 3.43 – 3.36 (m, 2H, H-1, H-2), 2.35 – 2.28 (m, 2H, CH_2-9), 1.99 (s, 3H, CH_3 Ac), 1.88 – 1.76 (m, 1H, C*H*H-8), 1.69 – 1.56 (m, 2H, C*H*H-7, C*H*H-8), 1.49 – 1.37 (m, 1H, C*H*H-7), 1.33 (d, 3H, *J* = 6.8 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 177.0, 175.7, 174.1 (C=O), 139.2 (C_q Ar), 129.9, 129.1, 127.2 (Ar), 102.5 (CH benzylidene), 84.0 (C-4), 81.0 (C-3), 80.7 (C-1), 77.0 (CH lactic acid), 71.6 (C-5), 69.8 (CH_2-6), 56.0 (C-2), 52.0 (OCH_3), 34.5 (C-9), 32.5 (C-7), 23.1 (CH_3 Ac), 22.1 (C-8), 19.5 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 2950, 1737, 1651, 1552, 1372, 1103, 1055, 1033,

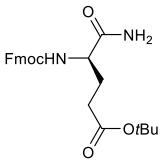
1012, 697; HRMS: $[M+H]^+$ calcd. for $C_{23}H_{32}NO_9$ 466.2072, found 466.2076; LC-MS: Rt = 5.81 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

Methyl 4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranosyl)-butanoate (27b)



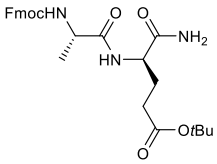
After co-evaporation with toluene (2x) and THF (1x) under an argon atmosphere, compound **26b** (0.27 g, 0.50 mmol, 1.0 eq.) was dissolved in THF (5.0 mL). Methyl acrylate (0.21 mL, 1.4 mmol, 2.8 eq.) and copper iodide (15 mg, 0.08 mmol, 0.15 eq.) were added, followed by the addition of Grubbs 2nd generation catalyst (43 mg, 0.05 mmol, 0.1 eq.). The flask was shielded from light with aluminium foil, heated to 40°C and stirred overnight. The reaction mixture was concentrated *in vacuo* and co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in THF (1.9 mL) and the solution was purged with argon for 5 minutes. Ruthenium trichloride (33 mg, 0.16 mmol, 0.3 eq.) and $NaBH_4$ (61 mg, 1.6 mmol, 3.2 eq.) were added. An empty balloon was put on the reaction flask. After cooling to 0°C, MeOH (0.58 mL) was slowly added and the reaction was stirred at 40°C. After 3 hours, LC-MS analysis showed full conversion of the starting material. The reaction was quenched with H_2O and diluted with DCM. The mixture was acidified with 1 M HCl to pH = 1. The aqueous layer was extracted with DCM (3x). The combined organic layers were washed with brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (2→10% MeOH in DCM + 0.1% AcOH) gave a mixture of compound **27b** (0.21 g, 0.35 mmol, 69%) and compound **28b** (0.04 g, 0.08 mmol). Analysis given for title compound only. R_f: 0.5 (1/9 MeOH/DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.48 – 7.41 (m, 2H, Ar), 7.38 – 7.29 (m, 5H, Ar), 6.93 – 6.87 (m, 2H, Ar), 5.60 (s, 1H, CH benzylidene), 4.54 (q, 2H, *J* = 14.2, 12.0, 11.5 Hz, CH_2 glycolyl), 4.33 (q, 1H, *J* = 6.8 Hz, CH lactic acid), 4.24 (dd, 1H, *J* = 10.3, 4.9 Hz, C*H*H-6), 3.97 (q, 2H, *J* = 15.2, 14.9, 6.2 Hz, CH_2 PMB), 3.89 – 3.70 (m, 6H, H-2, H-3, H-6, CH_3 PMB), 3.67 (t, 1H, *J* = 8.9 Hz, H-4), 3.62 (s, 3H, OCH_3), 3.52 – 3.44 (m, 1H, H-1), 3.44 – 3.36 (m, 1H, H-5), 2.30 (t, 2H, *J* = 7.2 Hz, CH_2-9), 1.87 – 1.74 (m, 1H, C*H*H-8), 1.68 – 1.51 (m, 2H, C*H*H-7, C*H*H-8), 1.51 – 1.37 (m, 1H, C*H*H-7), 1.33 (d, 3H, *J* = 6.9 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 175.7 (C=O), 161.1, 139.2 (C_q Ar), 130.9 (Ar), 130.7 (C_q Ar), 129.9, 129.1, 127.2, 114.8 (Ar), 102.5 (CH benzylidene), 83.6 (C-4), 80.6 (C-3), 80.4 (C-1), 74.1 (CH_2 glycolyl), 71.8 (C-5), 69.8 (CH_2-6), 69.8 (CH_2 PMB), 55.9 (C-2), 55.7 (CH_3 PMB), 52.0 (OCH_3), 34.5 (C-9), 32.5 (C-7), 22.0 (C-8), 19.7 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 3676, 2988, 2901, 2361, 2342, 1735, 1654, 1514, 1455, 1394, 1250, 1175, 1077, 752, 699, 668; HRMS: $[M+H]^+$ calcd. for $C_{31}H_{40}NO_{11}$ 602.2596, found 602.2606; LC-MS: Rt = 7.55 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

Fmoc-*i*-D-Gln(O*t*Bu)-NH_2 (30)



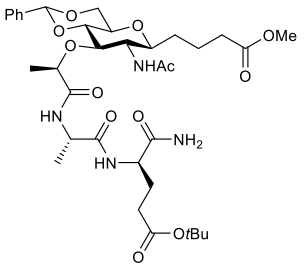
Fmoc-D-Glu(O*t*Bu)-OH (8.5 g, 20 mmol, 1.0 eq.) was dissolved in dioxane (0.20 L) followed by the addition of ammonium bicarbonate (7.2 g, 90 mmol, 4.5 eq.), di-*tert*-butyl dicarbonate (5.9 g, 27 mmol, 1.35 eq.) and pyridine (2.5 mL, 31 mmol, 1.55 eq.). After stirring at room temperature for 16 hours, the mixture was cooled to 0°C and quenched with H_2O. The aqueous layer was extracted with EtOAc (3x). The combined organic layers were washed with H_2O (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Recrystallization in MeOH gave compound **30** (8.6 g, 19 mmol, 96%) as a white solid. R_f: 0.3 (3/7 pentane/EtOAc); ^{1}H NMR (DMSO, 400 MHz, HH-COSY, HSQC): δ 7.89 (d, 2H, *J* = 7.8, 0.9 Hz, Ar), 7.73 (dd, 2H, *J* = 7.4, 4.9 Hz, Ar), 7.42 (t, 3H, *J* = 7.5, 1.2 Hz, Ar, NH), 7.32 (t, 3H, Ar, N*H*H), 6.14 (s, 1H, NH*H*), 4.35 – 4.13 (m, 3H, CH Fmoc, CH_2 Fmoc), 4.00 – 3.86 (m, 1H, CH *i*-D-Gln), 2.22 (t, 2H, *J* = 7.9 Hz, CH_2 γ–*i*-D-Gln), 1.98 – 1.81 (m, 1H, C*H*H β-*i*-D-Gln), 1.81 – 1.62 (m, 1H, C*H*H β-*i*-D-Gln), 1.39 (s, 9H, 3x CH_3 *t*Bu), 1.36 (s, 4H); ^{13}C-APT NMR (DMSO, 101 MHz, HSQC): δ 173.4, 171.7, 156.0 (C=O), 143.9, 143.8, 140.7 (C_q Ar), 127.7, 127.1, 125.4, 120.2 (Ar), 79.7 (C_q *t*Bu), 65.6 (CH_2 Fmoc), 53.7 (CH *i*-D-Gln), 46.7 (CH Fmoc), 31.5 (CH_2 γ–*i*-D-Gln), 27.8 (CH_3 *t*Bu), 27.3 (CH_2 β-*i*-D-Gln); FT-IR (neat, cm^{-1}): 2988, 2361, 2342, 1684, 1394, 1250, 1066, 668; HRMS: $[M+H]^+$ calcd. for $C_{24}H_{29}N_2O_5$ 425.2071, found 425.2068.

Fmoc-L-Ala-*i*-D-Gln(O*t*Bu)-NH_2 (31)



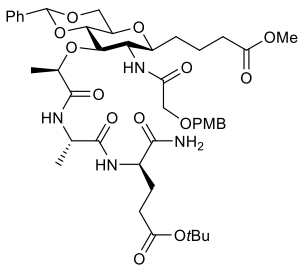
Compound **30** (8.12 g, 19.1 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere and dissolved in DCM (0.19 L). DBU (2.9 mL, 19.1 mmol, 1.0 eq.) was added and the mixture was stirred for 20 minutes. To quench the reaction, HOBt (12.9 g, 84.2 mmol, 4.4 eq.) was added and stirred for 20 minutes. Fmoc-L-Ala-OH (7.12 g, 23.0 mmol, 1.2 eq.), EDC·HCl (4.44 g, 23.0 mmol, 1.2 eq.) and DIPEA (19.3 mL, 111 mmol, 5.8 eq.) were added and stirring was continued for 16 hours. 1 M HCl was added and the resulting suspension was filtered. The filtrate was extracted with DCM (3x). The combined organic layers were washed with sat. aq. $NaHCO_3$ (3x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Recrystallization (EtOAc/pentane) gave the title compound (6.93 g, 14.0 mmol, 73%) as a white solid. R_f: 0.2 (3/7 pentane/EtOAc); ^{1}H NMR (DMSO, 400 MHz, HH-COSY, HSQC): δ 8.09 – 8.03 (m, 1H, NH), 7.88 (d, 2H, *J* = 7.5 Hz, Ar), 7.84 (dt, 1H, *J* = 7.6, 1.0 Hz, NH), 7.72 (t, 2H, *J* = 6.6 Hz, Ar), 7.62 (d, 1H, *J* = 7.1 Hz, NH), 7.41 (t, 2H, *J* = 7.4, 1.2 Hz, Ar), 7.37 – 7.29 (m, 2H, Ar), 7.27 (s, 1H, N*H*H), 7.14 (s, 1H, N*H*H), 4.33 – 4.10 (m, 3H, CH *i*-D-Gln, CH Fmoc, CH_2 Fmoc), 4.06 (p, 1H, *J* = 7.2 Hz, CH L-Ala), 2.23 – 2.11 (m, 2H, CH_2 γ–*i*-D-Gln), 2.05 – 1.87 (m, 1H, C*H*H β-*i*-D-Gln), 1.77 – 1.63 (m, 1H, C*H*H β-*i*-D-Gln), 1.36 (d, 9H, *J* = 10.7 Hz, 4x CH_3 *t*Bu), 1.22 (d, 3H, *J* = 7.0 Hz, CH_3 L-Ala); ^{13}C-APT NMR (DMSO, 101 MHz, HSQC): δ 173.1, 172.6, 171.7, 171.6, 155.9 (C=O), 143.9, 143.8, 142.6, 140.8, 139.5, 137.5 (C_q Ar), 129.0, 127.7, 127.3, 127.1, 125.3, 125.3, 121.4, 120.1, 120.1 (Ar), 79.7 (C_q *t*Bu), 65.8 (CH_2 Fmoc), 51.4 (CH *i*-D-Gln), 50.3 (CH L-Ala), 46.6 (CH Fmoc), 31.2 (CH_2 γ–*i*-D-Gln), 27.7 (CH_3 *t*Bu), 27.2 (CH_2 β-*i*-D-Gln), 18.0 (CH_3 L-Ala); FT-IR (neat, cm^{-1}): 3286, 2975, 1726, 1692, 1675, 1644, 1539, 1448, 1367, 1329, 1259, 1153, 1121, 1085, 1045, 981, 850, 756, 737, 621, 590, 550; HRMS: $[M+H]^+$ calcd. for $C_{27}H_{34}N_3O_6$ 496.2442, found 496.2443.

Methyl 4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-butanoate (32a)



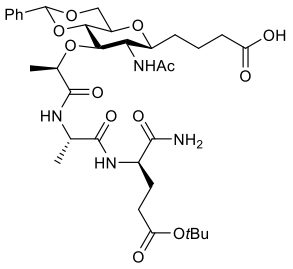
To a solution of compound **31** (3.72 g, 7.50 mmol, 1.5 eq.) in DMF (67 mL) was added DBU (1.2 mL, 8.0 mmol, 1.6 eq.) and the solution was stirred at room temperature for 1 hour. The reaction was quenched by addition HOBt (2.7 g, 17.6 mmol, 3.4 eq.) and the mixture was stirred for 20 minutes. A mixture of compound **27a** (1.79 g, 3.85 mmol, 0.75 eq.) and compound **28a** (0.52 g, 1.28 mmol, 0.25 eq.) was added, followed by the addition of HCTU (2.48 g, 6.0 mmol, 1.2 eq.) and DIPEA (3.5 mL, 20 mmol, 3.9 eq.). The reaction mixture was stirred for overnight after which TLC analysis showed full conversion of the starting material. The reaction mixture was diluted with DCM and washed with brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was embedded on QuadraSil® aminopropyl and purification by column chromatography (2→10% MeOH in DCM) gave a mixture of compound **32a** and compound **33a** in quantitative yield (3.83 g) as a white solid. R_f: 0.4 (1/9 MeOH/DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.51 – 7.44 (m, 2H, Ar), 7.39 – 7.31 (m, 3H, Ar), 5.63 (s, 1H, CH benzylidene), 4.35 (dd, 1H, *J* = 9.6, 4.6 Hz, CH *i*-D-Gln), 4.31 – 4.21 (m, 2H, C*H*H-6, CH L-Ala), 4.17 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.87 (t, 1H, *J* = 9.6 Hz, H-2), 3.76 (t, 1H, *J* = 10.2 Hz, C*H*H-6), 3.71 – 3.58 (m, 5H, H-3, H-4, OCH_3), 3.47 – 3.34 (m, 2H, H-1, H-5), 2.39 – 2.23 (m, 4H, CH_2-9, CH_2 γ-*i*-D-Gln), 2.23 – 2.10 (m, 1H, C*H*H β-*i*-D-Gln), 1.96 (s, 3H, CH_3 Ac), 1.91 – 1.76 (m, 2H, C*H*H-8, C*H*H β-*i*-D-Gln), 1.70 – 1.57 (m, 2H, C*H*H-8, C*H*H-7), 1.57 – 1.41 (m, 10H, C*H*H-7, 3x CH_3 *t*Bu), 1.41 – 1.35 (m, 3H, CH_3 L-Ala), 1.35 – 1.28 (m, 3H, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 175.7, 175.0, 173.7 (C=O), 139.1 (C_q Ar), 129.9, 129.1 , 127.3 (Ar), 102.6 (CH benzylidene), 82.8 (C-4), 82.0 (C-3), 81.8 (C_q *t*Bu), 80.6 (C-1), 79.0 (CH lactic acid), 71.8 (C-5), 69.7 (CH_2-6), 56.1 (C-2), 53.5 (OCH_3), 52.0 (CH *i*-D-Gln), 50.7 (CH L-Ala), 34.5 (C-9), 32.6 (C-7), 32.2 (CH_2 γ-*i*-D-Gln), 28.3 (CH_3 *t*Bu), 28.3 (CH_2 β-*i*-D-Gln), 23.2 (CH_3 Ac), 22.0 (C-8), 19.7 (CH_3 lactic acid), 17.9 (CH_3 L-Ala); FT-IR (neat, cm^{-1}): 3280, 1731, 1643, 1544, 1369, 1155; HRMS: $[M+H]^+$ calcd. for $C_{35}H_{53}N_4O_{12}$ 721.3655, found 721.3664. *Data given for title compound only.

Methyl 4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-butanoate (32b)



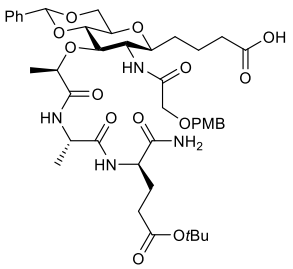
Compound **31** (3.73 g, 7.52 mmol, 1.5 eq.) was dissolved in DMF (67 mL). DBU (1.2 mL, 8.0 mmol, 1.6 eq.) was added and the reaction was stirred at room temperature for 1 hour. After quenching with HOBt (0.18 g, 1.35 mmol, 3.5 eq.), the suspension was stirred for 20 minutes. A mixture of compound **27b** (2.43 g, 4.04 mmol, 0.80 eq.) and compound **28b** (0.58 g, 1.0 mmol, 0.20 eq.) was added, followed by the addition of HCTU (2.5 g, 6.0 mmol, 1.2 eq.) and DIPEA (3.5 mL, 20 mmol, 4.0 eq.). The reaction mixture was stirred overnight. Upon completion of the reaction determined by TLC analysis, the reaction mixture was diluted with DCM and washed with brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was embedded on QuadraSil® aminopropyl and purification by column chromatography (2→6% MeOH in DCM) gave a mixture of compound **32b** (3.08 g, 3.60 mmol, 89%) and compound **33b** (0.67 g, 0.84 mmol). R_f: 0.6 (1/9 MeOH/DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.43 (m, 2H, Ar), 7.39 – 7.27 (m, 5H, Ar), 6.95 – 6.89 (m, 2H, Ar), 5.60 (s, 1H, CH benzylidene), 4.53 (q, 2H, *J* = 11.9, 11.4, 3.9 Hz, CH_2 glycolyl), 4.34 (dd, 1H, *J* = 9.5, 4.7 Hz, CH *i*-D-Gln), 4.30 – 4.20 (m, 2H, C*H*H-6, CH L-Ala), 4.16 (q, 1H, *J* = 6.8 Hz, CH lactic acid), 4.02 – 3.91 (m, 3H, H-2, CH_2 PMB), 3.82 – 3.69 (m, 5H, H-3, C*H*H-6, CH_3 PMB), 3.68 – 3.58 (m, 4H, H-4, OCH_3), 3.54 – 3.46 (m, 1H, H-1), 3.47 – 3.37 (m, 1H, H-5), 2.35 – 2.25 (m, 4H, CH_2-9, CH_2 γ-*i*-D-Gln), 2.21 – 2.11 (m, 1H, C*H*H β-*i*-D-Gln), 1.89 – 1.75 (m, 2H, C*H*H-8, C*H*H β-*i*-D-Gln), 1.70 – 1.49 (m, 2H, C*H*H-7, C*H*H-8), 1.49 – 1.37 (m, 10H, C*H*H-7, 3x CH_3 *t*Bu), 1.37 – 1.26 (m, 6H, CH_3 lactic acid, CH_3 L-Ala); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.1, 175.6, 175.5, 174.9, 173.7, 173.2 (C=O), 161.1, 139.1 (C_q Ar), 130.9 (Ar), 130.4 (C_q Ar), 129.9, 129.1, 127.2, 114.9 (Ar), 102.5 (CH benzylidene), 82.6 (C-4), 81,8 (C_q *t*Bu), 81.5 (C-3), 80.0 (C-1), 79.0 (CH lactic acid), 74.0 (CH_2 glycolyl), 71.7 (C-5), 69.7 (CH_2 PMB), 69.7 (CH_2-6), 55.8 (C-2), 55.7 (CH_3 PMB), 53.4 (CH *i*-D-Gln), 52.0 (OCH_3), 50.6 (CH L-Ala), 34.4 (CH_2 γ-*i*-D-Gln), 32.7 (C-9), 32.2 (C-7), 28.3 (CH_3 *t*Bu), 28.2 (CH_2 β-*i*-D-Gln), 21.9 (C-8), 19.8 (CH_3 lactic acid), 18.0 (CH_3 L-Ala); FT-IR (neat, cm^{-1}): 2360, 1665, 1515, 1250, 1103, 1038; HRMS: $[M+H]^+$ calcd. for $C_{43}H_{61}N_4O_{14}$ 857.4179, found 857.4201. *Data given for title compound only.

4-*C*-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-butanoic acid (34a)



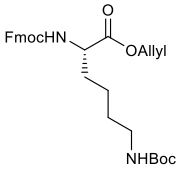
The previously obtained mixture of compound **32a** (0.37 g, 0.51 mmol, 0.72 eq.) and compound **33a** (0.13 g, 0.20 mmol, 0.28 eq) was dissolved in MeOH (23 mL). LiOH (91 mg, 2.2 mmol, 3.0 eq.) and a 35% H_2O_2 in H_2O solution (0.69 mL, 7.9 mmol, 11 eq.) were added. After 8 hours of stirring, the reaction mixture was acidified with acetic acid to pH = 1. Toluene (30 mL) was added and the solution was concentrated *in vacuo*. Recrystallization (MeOH/DCM/Et_2O) gave the title compound (0.26 g, 0.37 mmol, 73%) as a white solid. R_f: 0.6 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -21.2° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.51 – 7.44 (m, 2H, Ar), 7.39 – 7.30 (m, 3H, Ar), 5.62 (s, 1H, CH benzylidene), 4.34 (dd, 1H, J = 9.7, 4.5 Hz, CH *i*-D-Gln), 4.31 – 4.21 (m, 2H, CHH-6, CH L-Ala), 4.15 (q, 1H, J = 6.6 Hz, CH lactic acid), 3.85 (t, 1H, J = 9.6 Hz, H-2), 3.75 (t, 1H, J = 10.2 Hz, C*H*H-6), 3.68 – 3.57 (m, 2H, H-3, H-4), 3.46 – 3.36 (m, 2H, H-1, H-5), 2.36 – 2.23 (m, 2H, CH_2 γ-*i*-D-Gln), 2.23 – 2.10 (m, 3H, CH_2-9, C*H*H β-*i*-D-Gln), 1.96 (s, 3H, CH_3 Ac), 1.88 – 1.75 (m, 2H, C*H*H-8, C*H*H β-*i*-D-Gln), 1.69 – 1.56 (m, 2H, C*H*H-8, C*H*H-7), 1.44 (s, 9H, 3x CH_3 *t*Bu), 1.42 – 1.36 (m, 4H, CH_3 L-Ala, C*H*H-7), 1.32 (d, 3H, J = 6.7 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 175.7, 175.1, 173.7, 173.7 (C=O), 139.2 (C_q Ar), 129.9, 129.1, 127.3 (Ar), 102.6 (CH benzylidene), 82.8 (C-4), 82.3 (C-3), 81.8 (C_q *t*Bu), 80.5 (C-5), 79.1 (CH lactic acid), 71.8 (C-1), 69.8 (CH_2-6), 56.5 (C-2), 53.5 (CH *i*-D-Gln), 50.8 (CH L-Ala), 38.7 (C-9), 32.9 (C-7), 32.7 (CH_2 γ-i-D-Gln), 28.3 (CH_3 *t*Bu), 28.2 (CH_2 β-i-D-Gln), 23.7 (C-8), 23.3 (CH_3 Ac), 19.7 (CH_3 lactic acid), 17.9 (CH_3 L-Ala); FT-IR (neat, cm^{-1}): 3274, 2360, 1643, 1562, 1423, 1369, 1153, 1105, 1038, 1028, 694; HRMS: $[M+H]^+$ calcd. for $C_{34}H_{51}N_4O_{12}$ 707.3498, found 707.3515; LC-MS: Rt = 5.25 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

4-*C*-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-butanoic acid (34b)



LiOH (0.57 g, 13.6 mmol) and a 35% H_2O_2 in H_2O solution (4.35 mL, 50.6 mmol) were dissolved in H_2O (25.7 mL). A previously obtained mixture of compound **32b** (0.44 g, 0.51 mmol, 0.86 eq.) and compound **33b** (66 mg, 83 µmol, 0.14 eq) was dissolved in THF (5.1 mL) and cooled to 0°C, followed by the addition of prepared $LiOH/H_2O_2$ solution (3.4 mL). The reaction was stirred at 0°C for 11 hours and subsequently quenched with AcOH to pH = 1. The mixture was diluted with toluene, concentrated *in vacuo* and recrystallization (MeOH/DCM/Et_2O) gave acid **34b** (0.40 g, 0.47 mmol, 92%) as a white solid. R_f: 0.6 (1/9 MeOH/DCM). $[\alpha]_D^{20}$ = -15.5° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.43 (m, 2H, Ar), 7.39 – 7.28 (m, 5H, Ar), 6.95 – 6.88 (m, 2H, Ar), 5.61 (s, 1H, CH benzylidene), 4.53 (s, 2H, CH_2 glycolyl), 4.33 (dd, 1H, *J* = 9.5, 4.6 Hz, CH *i*-D-Gln), 4.30 – 4.19 (m, 2H, C*H*H-6, CH L-Ala), 4.16 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.98 (t, 1H, *J* = 9.9 Hz, H-2), 3.93 (s, 2H, CH_2 PMB), 3.82 – 3.71 (m, 5H, H-3, CH*H*-6, CH_3 PMB), 3.64 (t, 1H, *J* = 9.2 Hz, H-4), 3.55 – 3.47 (m, 1H, H-1), 3.47 – 3.38 (m, 1H, H-5), 2.33 – 2.23 (m, 4H, CH_2-9, CH_2 γ-*i*-D-Gln), 2.22 – 2.10 (m, 1H, C*H*H β-*i*-D-Gln), 1.89 – 1.75 (m, 2H, C*H*H-8, C*H*H β-*i*-D-Gln), 1.70 – 1.54 (m, 2H, C*H*H-8, C*H*H-7), 1.51 – 1.37 (m, 10H, C*H*H-7, 3x CH_3 *t*Bu), 1.33 (dd, 6H, *J* = 7.0, 1.3 Hz, CH_3 lactic acid, CH_3 L-Ala); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 177.2, 176.2, 175.5, 174.9, 173.7, 173.2 (C=O), 161.1, 139.1 (C_q Ar), 130.9 (Ar), 130.4 (C_q Ar), 129.9, 129.1, 127.2, 114.9 (Ar), 102.6 (CH benzylidene), 82.6 (C-4), 81.8 (C_q *t*Bu), 81.6 (C-3), 80.1 (C-1), 79.0 (CH lactic acid), 74.0 (CH_2 glycolyl), 71.7 (C-5), 69.8, 69.7 (CH_2-6, CH_2 PMB), 55.9 (C-2), 55.7 (CH_3 PMB), 53.5 (CH *i*-D-Gln), 50.6 (CH L-Ala), 34.6 (CH_2 γ-*i*-D-Gln), 32.7 (C-9), 32.3 (C-7), 28.3 (CH_3 *t*Bu), 28.2 (CH_2 β-*i*-D-Gln), 22.0 (C-8), 19.8 (CH_3 lactic acid), 18.0 (CH_3 L-Ala); FT-IR (neat, cm^{-1}): 3319, 2974, 2360, 2342, 1663, 1515, 1454, 1394, 1250, 1154, 1076, 668; HRMS: $[M+H]^+$ calcd. for $C_{42}H_{59}N_4O_{14}$ 843.4023, found 843.4047; LC-MS: Rt = 6.59 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

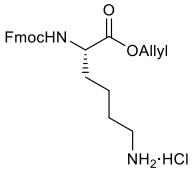
Fmoc-L-Lys(Boc)-OAllyl (35)



A solution of Fmoc-Lys(Boc)-OH (4.70 g, 10.0 mmol, 1.0 eq.) in DMF (40 mL) under an argon atmosphere was cooled to 0°C. Silver carbonate (3.59 g, 13.0 mmol, 1.3 eq.) was added and the mixture was stirred for 15 minutes at 0°C, followed by the addition of allyl bromide (3.98 mL, 46.0 mmol, 4.6 eq.). The reaction mixture was allowed to warm-up to room temperature and stirred for 2.5 hours, before TLC analysis showed that the reaction was complete. The suspension was filtered, diluted with Et_2O, washed with a 10 wt% $KHSO_4$ solution (2x) and H_2O (2x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→60% EtOAc in pentane) gave the title compound in quantitative yield (5.52 g) as a yellow solid. R_f: 0.7 (3/2 pentane/EtOAc); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.80 (d, 2H, *J* = 7.6, 0.9 Hz, Ar), 7.67 (t, 2H, *J* = 6.4,

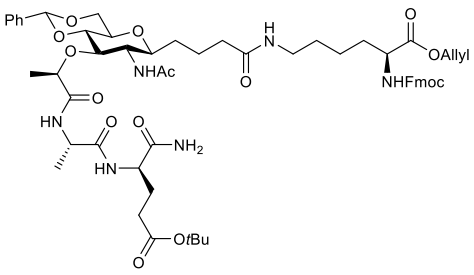
0.9 Hz, Ar), 7.39 (t, 2H, *J* = 7.5 Hz, Ar), 7.31 (t, 2H, *J* = 7.5, 1.2 Hz, Ar), 6.00 – 5.86 (m, 1H, CH_2-C*H*=CH_2), 5.32 (dd, 1H, *J* = 17.2, 1.6 Hz, CH_2-CH=C*H*H), 5.21 (dd, 1H, *J* = 10.5, 1.3 Hz, CH_2-CH=C*H*H), 4.62 (dd, 2H, *J* = 5.6, 1.4 Hz, C*H*$_2$-CH=CH_2), 4.41 – 4.29 (m, 2H, CH_2 Fmoc), 4.25 – 4.13 (m, 2H, CH Fmoc, CH L-Lys), 3.03 (t, 2H, *J* = 7.1 Hz, CH_2 ε-Lys), 1.89 – 1.78 (m, 1H, C*H*H β-Lys), 1.75 – 1.63 (m, 1H, C*H*H β-Lys), 1.56 – 1.33 (m, 13H, CH_2 γ-Lys, CH_2 δ-Lys, 3x CH_3 *t*Bu); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 173.8, 158.7 (C=O), 145.3, 145.1, 142.6 (C_q Ar), 133.4 (CH_2-*C*H=CH_2), 128.8, 128.2, 128.2, 126.3, 126.2, 120.9 (Ar), 118.6 (CH_2-CH=*C*H_2), 79.9 (C_q *t*Bu) 68.0 (CH_2 Fmoc), 66.7 (*C*H_2-CH=CH_2), 55.5 (CH L-Lys), 48.4 (CH Fmoc), 41.0 (CH_2 ε-Lys), 32.2 (CH_2 β-Lys), 30.5 (CH_2 δ-Lys), 28.8 (CH_3 *t*Bu), 24.2 (CH_2 γ-Lys); FT-IR (neat, cm^{-1}): 3341, 2937, 1710, 1522, 1451, 1366, 1250, 1173, 760, 741; HRMS: $[M+Na]^+$ calcd. for $C_{29}H_{36}N_2O_6Na$ 531.2471, found 531.2475.

Fmoc-L-Lys-OAllyl (36)



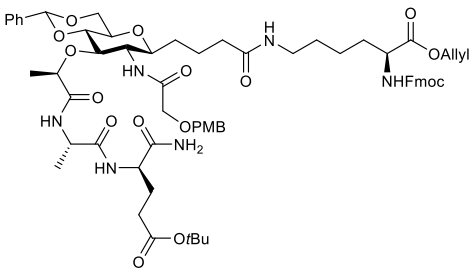
Compound **35** (5.01 g, 10 mmol, 1.0 eq.) was cooled to 0°C. 4 M HCl in dioxane (25 mL, 10 eq.) was added and the reaction was stirred at 0°C. After complete solvation of the starting material, the ice bath was removed and the clear solution was stirred for 1 hour at room temperature. The mixture was diluted with toluene (5 mL) and concentrated *in vacuo*. Co-evaporation with toluene (3x) and purification by column chromatography (4→16% MeOH in DCM) gave the title compound (3.95 g, 9.70 mmol, 97%) as a white solid. R_f: 0.2 (1/9 MeOH/DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.79 (d, 2H, *J* = 7.6, 1.0 Hz, Ar), 7.66 (t, 2H, *J* = 15.4, 7.8 Hz, Ar), 7.39 (t, 2H, Ar), 7.31 (t, 2H, *J* = 14.9 Hz, Ar), 6.00 – 5.85 (m, 1H, CH_2-C*H*=CH_2), 5.32 (dq, 1H, *J* = 17.2, 1.6 Hz, CH_2-CH=C*H*H), 5.21 (dq, 1H, *J* = 10.5, 1.4 Hz, CH_2-CH=C*H*H), 4.62 (dt, 2H, *J* = 5.6, 1.5 Hz, C*H*$_2$-CH=CH_2), 4.45 – 4.29 (m, 2H, CH_2 Fmoc), 4.25 – 4.16 (m, 2H, CH Fmoc, CH L-Lys), 2.97 – 2.84 (m, 2H, CH_2 ε-Lys), 1.94 – 1.79 (m, 1H, C*H*H β-Lys), 1.79 – 1.55 (m, 3H, C*H*H β-Lys, CH_2 δ-Lys), 1.55 – 1.25 (m, 2H, CH_2 γ-Lys); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 173.5, 158.7 (C=O), 145.3, 145.1, 142.6 (C_q Ar), 133.3 (CH_2-*C*H=CH_2), 128.8, 128.2, 128.1, 126.2, 126.2, 120.9 (Ar), 118.7 (CH_2-CH=*C*H_2), 67.9 (CH_2 Fmoc), 66.8 (*C*H_2-CH=CH_2), 55.2 (CH L-Lys), 48.4 (CH Fmoc), 40.5 (CH_2 ε-Lys), 31.9 (CH_2 β-Lys), 28.0 (CH_2 δ-Lys), 23.9 (CH_2 γ-Lys); FT-IR (neat, cm^{-1}): 2944, 1716, 1648, 1609, 1520, 1478, 1450, 1412, 1331, 1248, 1195, 1170, 1121, 1047, 987, 936, 782, 760, 738, 621, 541; HRMS: $[M+H]^+$ calcd. for $C_{24}H_{29}N_2O_4$ 409.2122, found 409.2129.

N_α-Fmoc-N_ε-[butan-4-*C*-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-allyl ester (37a)



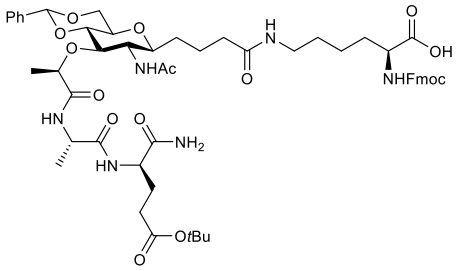
Compound **34a** (0.21 g, 0.30 mmol, 1.0 eq.) and compound **36** (0.18 g, 0.45 mmol, 1.5 eq.) were co-evaporated with toluene (3x) under an argon atmosphere and dissolved in DMF (12 mL). HCTU (0.15 g, 0.36 mmol, 1.2 eq.) and DIPEA (78 µL, 0.45 mmol, 3.0 eq.) were added and the mixture was stirred for 3 hours. The reaction was diluted with Et_2O and the precipitate was collected by filtration. Recrystallization (MeOH/DCM/Et_2O) gave the title compound (0.28 g, 0.26 mmol, 87%) as a white solid. R_f: 0.4 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -18.6° (*c* = 1.0, DCM/MeOH: 1/1); ^{1}H NMR (MeOD/CD_2Cl_2 1/1, 400 MHz, HH-COSY, HSQC): δ 7.78 (d, 2H, *J* = 7.5 Hz, Ar), 7.65 (dd, 2H, *J* = 7.6, 4.1 Hz, Ar), 7.48 – 7.43 (m, 2H, Ar), 7.40 (t, 2H, *J* = 7.4 Hz, Ar), 7.37 – 7.28 (m, 5H, Ar), 5.98 – 5.85 (m, 1H, CH_2-C*H*=CH_2), 5.55 (s, 1H, CH benzylidene), 5.32 (d, 1H, *J* = 17.1 Hz, CH_2-CH=C*H*H), 5.22 (d, 1H, *J* = 10.5 Hz, CH_2-CH=C*H*H), 4.62 (d, 2H, *J* = 5.4 Hz, C*H*$_2$-CH=CH_2), 4.45 – 4.30 (m, 3H, CH *i*-D-Gln, CH_2 Fmoc), 4.30 – 4.16 (m, 4H, C*H*H-6, CH L-Lys, CH Fmoc, CH L-Ala), 4.13 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.90 – 3.79 (m, 1H, H-2), 3.68 (t, 1H, *J* = 10.3 Hz, C*H*H-6), 3.63 – 3.54 (m, 2H, H-3, H-4), 3.43 – 3.33 (m, 2H, H-1, H-5), 3.15 (t, 2H, *J* = 7.0 Hz, CH_2 ε-L-Lys), 2.30 (t, 2H, *J* = 7.6 Hz, CH_2 γ-*i*-D-Gln), 2.21 – 2.10 (m, 3H, CH_2-9, C*H*H β-*i*-D-Gln), 1.93 (s, 3H, CH_3 Ac), 1.90 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, C*H*H β-L-Lys, C*H*H-8), 1.75 – 1.46 (m, 6H, C*H*H β-L-Lys, C*H*H-8, CH_2-7, CH_2 γ-L-Lys), 1.42 (s, 9H, 3x CH_3 *t*Bu), 1.37 (d, 5H, *J* = 7.0 Hz, CH_2 δ-L-Lys, CH_3 L-Ala), 1.32 (d, 3H, *J* = 6.8 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD/CD_2Cl_2 1/1, 101 MHz, HSQC): δ 175.4, 175.2, 175.0, 174.1, 173.4, 173.3, 173.0 (C=O), 157.8, 144.6, 142.0, 138.2 (C_q Ar), 132.6 (CH_2-*C*H=CH_2), 129.6, 128.8, 128.4, 127.8, 127.7, 126.7, 125.8, 125.8, 120.6 (Ar), 118.7 (CH_2-CH=*C*H_2), 102.0 (CH benzylidene), 82.2 (C-4), 81.6 (C_q *t*Bu), 81.4 (C-3), 80.1 (C-1), 78.5 (CH lactic acid), 71.1 (C-5), 69.4 (CH_2-6), 67.6 (CH_2 Fmoc), 66.5 (*C*H_2-CH=CH_2), 55.6 (C-2), 54.9 (CH L-Lys), 52.9 (CH *i*-D-Gln), 50.1 (CH L-Ala), 47.9 (CH Fmoc), 39.6 (CH_2 ε-L-Lys), 36.7 (C-9), 32.3 (CH_2 γ-*i*-D-Gln), 31.9 (C-7), 29.5 (CH_2 γ-L-Lys), 28.2 (CH_3 *t*Bu), 27.7 (CH_2 β-*i*-D-Gln), 23.7 (CH_2 δ-L-Lys), 23.2 (CH_3 Ac), 22.6 (C-8), 19.5 (CH_3 L-Ala), 17.6 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 3281, 1728, 1642, 1541, 1451, 1369, 1275, 1153, 1105, 1028, 741, 696; HRMS: $[M+H]^+$ calcd. for $C_{58}H_{77}N_6O_{15}$ 1097.5442, found 1097.5452; LC-MS: Rt = 7.30 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

N_α-Fmoc-N_ε-[butan-4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-allyl ester (37b)



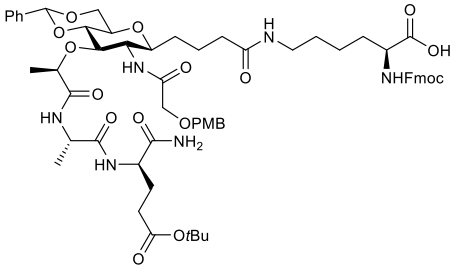
Compound **34b** (1.19 g, 1.41 mmol, 1.0 eq.) and compound **36** (0.87 g, 2.12 mmol, 1.5 eq.) were co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in DMF (14 mL), followed by the addition of HCTU (0.71 g, 1.70 mmol, 1.2 eq.) and DIPEA (0.37 mL, 2.12 mmol, 3.0 eq.). The mixture was stirred 3 hours and subsequently diluted with Et_2O to precipitate the product. The precipitate was filtered and purification by recrystallization (MeOH/DCM/Et_2O) and column chromatography (5→15% MeOH in DCM) gave the title compound (1.32 g, 1.07 mmol, 76%) as a white solid. R_f: 0.6 (1/9 MeOH/DCM); $[\alpha]_D^{20}$ = -13.6° (*c* = 1.0, DCM/MeOH: 1/1); ^{1}H NMR (MeOD/CD_2Cl_2 1/1, 400 MHz, HH-COSY, HSQC): δ 7.77 (d, 2H, *J* = 6.9 Hz, Ar), 7.65 (dd, 2H, *J* = 7.6, 4.4 Hz, Ar), 7.48 – 7.24 (m, 11H, Ar), 6.90 (d, 2H, *J* = 9.0 Hz, Ar), 5.97 – 5.84 (m, 1H, CH_2-C*H*=CH_2), 5.55 (s, 1H, CH benzylidene), 5.35 – 5.26 (m, 1H, CH_2-CH=C*H*H), 5.26 – 5.18 (m, 1H, CH_2-CH=C*H*H), 4.49 (d, 2H, *J* = 2.8 Hz, CH_2 glycolyl), 4.45 – 4.14 (m, 7H, C*H*H-6, CH *i*-D-Gln, CH L-Lys, CH Fmoc, CH_2 Fmoc, CH L-Ala), 4.11 (q, 1H, *J* = 6.6 Hz, CH lactic acid), 3.99 – 3.87 (m, 3H, H-2, CH_2 PMB), 3.78 (s, 3H, CH_3 PMB), 3.70 (q, 2H, *J* = 10.0, 9.4 Hz, H-3, H-4), 3.58 (t, 1H, *J* = 9.2 Hz, C*H*H-6), 3.50 – 3.35 (m, 2H, H-1, H-5), 3.18 – 3.09 (m, 2H, CH_2 ε-L-Lys), 2.29 (t, 2H, *J* = 8.0 Hz, CH_2 γ-*i*-D-Gln), 2.20 – 2.08 (m, 3H, CH_2-9, C*H*H β-*i*-D-Gln), 1.90 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, C*H*H β-L-Lys, C*H*H-8), 1.75 – 1.45 (m, 5H, C*H*H β-L-Lys, C*H*H-8, C*H*H-7, CH_2 γ-L-Lys), 1.45 – 1.35 (m, 12H, C*H*H-7, CH_2 δ-L-Lys, 3x CH_3 *t*Bu), 1.32 (t, 6H, *J* = 7.2 Hz, CH_3 lactic acid, CH_3 L-Ala); ^{13}C-APT NMR (MeOD/CD_2Cl_2 1/1, 126 MHz, HSQC): δ 175.4, 175.1, 174.9, 174.8, 174.1, 174.0, 173.5, 173.4, 172.3 (C=O), 160.5, 157.9, 144.8, 144.7, 142.1, 138.3 (C_q Ar), 132.7 (CH_2-*C*H=CH_2), 130.5 (Ar), 129.9 (C_q Ar), 129.6, 128.8, 128.4, 127.8, 127.8, 126.8, 125.9, 125.8, 120.6 (Ar), 118.7 (CH_2-CH=*C*H_2), 114.6 (Ar), 102.1 (CH benzylidene), 82.1 (C-4), 81.6 (C_q *t*Bu), 81.1 (C-3), 79.6 (C-1), 78.5 (CH L-Ala), 73.8 (CH_2 glycolyl), 71.2 (C-5), 69.6 (CH_2 PMB), 69.4 (CH_2-6), 67.6 (CH_2 Fmoc), 66.5 (*C*H_2-CH=CH_2), 55.7 (CH_3 PMB), 55.5 (C-2), 54.9 (CH L-Lys), 53.0 (CH *i*-D-Gln), 50.1 (CH lactic acid), 48.0 (CH Fmoc), 39.6 (CH_2 ε-L-Lys), 36.7 (C-9), 32.4 (CH_2 γ-*i*-D-Gln), 32.0 (CH_2 β-L-Lys), 32.0 (CH_2 γ-L-Lys), 29.5 (CH_2 δ-L-Lys), 28.2 (CH_3 *t*Bu), 27.8 (CH_2 β-*i*-D-Gln), 23.7 (C-7), 22.6 (C-8), 19.6 (CH_3 L-Ala), 17.7 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 3315, 2937, 1729, 1691, 1644, 1537, 1451, 1368, 1253, 1105, 1129, 845, 741, 696; HRMS: $[M+H]^+$ calcd. for $C_{66}H_{85}N_6O_{17}$ 1233.5966, found 1233.5964; LC-MS: Rt = 8.80 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

N_α-Fmoc-N_ε-[butan-4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine (10a)



Compound **37a** (0.54 g, 0.49 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in DMF (20 mL) and cooled to 0°C. Phenylsilane (0.12 mL, 0.98 mmol, 2.0 eq.) and $Pd(PPh_3)_4$ (59 mg, 0.05 mmol, 0.1 eq.) were added and the reaction was stirred at 0°C for 30 minutes. Upon completion of the reaction determined by LC-MS, Et_2O was added to precipitate the crude product. After filtration, the precipitate was purified by recrystallization (MeOH/DCM/Et_2O) to yield the title compound (0.42 g, 0.40 mmol, 82%) as a pale yellow solid. $[\alpha]_D^{20}$ = -10.2° (*c* = 1.0, DCM/MeOH: 1/1); ^{1}H NMR (MeOD/CD_2Cl_2 1/1, 500 MHz, HH-COSY, HSQC): δ 7.78 (d, 2H, *J* = 7.6 Hz, Ar), 7.65 (t, 2H, *J* = 7.0 Hz, Ar), 7.48 – 7.42 (m, 2H, Ar), 7.42 – 7.36 (m, 2H, Ar), 7.36 – 7.27 (m, 5H, Ar), 5.55 (s, 1H, CH benzylidene), 4.42 – 4.30 (m, 3H, CH L-Lys, CH *i*-D-Gln, CH Fmoc), 4.28 – 4.15 (m, 4H, C*H*H-6, CH_2 Fmoc, CH L-Ala), 4.13 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.88 – 3.80 (m, 1H, H-2), 3.69 (t, 1H, *J* = 10.3 Hz, C*H*H-6), 3.62 – 3.55 (m, 2H, H-3, H-4), 3.37 (q, 2H, *J* = 11.2, 9.2 Hz, H-1, H-5), 3.16 (t, 2H, *J* = 6.9 Hz, CH_2 ε-L-Lys), 2.33 – 2.27 (m, 2H, CH_2 γ-*i*-D-Gln), 2.20 – 2.11 (m, 3H, C*H*H β-*i*-D-Gln, CH_2-9), 1.94 (s, 3H, CH_3 Ac), 1.90 – 1.76 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 β-L-Lys), 1.76 – 1.46 (m, 6H, CH_2 γ-L-Lys, CH_2 δ-L-Lys, CH_2-8), 1.41 (s, 11H, CH_2-7, 3x CH_3 *t*Bu), 1.37 (d, 3H, *J* = 7.1 Hz, CH_3 L-Ala), 1.32 (d, 3H, *J* = 6.7 Hz, CH_3 lactic acid); ^{13}C-APT NMR (MeOD/CD_2Cl_2 1/1, 126 MHz, HSQC): δ 175.4, 175.2, 175.0, 174.1, 173.4, 173.0 (C=O), 144.8, 144.7, 142.0, 138.3 (C_q Ar), 129.6, 128.8, 128.4, 127.8, 126.7, 125.8, 120.6 (Ar), 102.1 (CH benzylidene), 82.2 (C-4), 81.6 (C_q *t*Bu), 81.5 (C-3), 80.0 (C-1), 78.5 (CH lactic acid), 71.2 (C-5), 69.4 (CH_2-6), 67.5 (CH_2 Fmoc), 55.7 (C-2), 54.9 (CH L-Lys), 53.0 (CH *i*-D-Gln), 50.2 (CH L-Ala), 47.9 (CH Fmoc), 39.7 (CH_2 ε-L-Lys), 36.7 (C-9), 32.4 (CH_2 γ-*i*-D-Gln), 31.9 (C-7), 29.5 (CH_2 γ-L-Lys), 28.2 (CH_3 *t*Bu), 27.8 (CH_2 β-*i*-D-Gln), 23.6 (CH_2 δ-L-Lys), 23.2 (CH_3 Ac), 22.6 (C-8), 19.5 (CH_3 L-Ala), 17.6 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 3300, 2934, 1656, 1537, 1451, 1369, 1252, 1154, 1104, 1029, 742, 698; HRMS: $[M+H]^+$ calcd. for $C_{55}H_{73}N_6O_{15}$ 1057.5129, found 1057.5153; LC-MS: Rt = 7.37 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

N_α-Fmoc-N_ε-[butan-4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine (10b)



Compound **37b** (0.75 g, 0.60 mmol, 1.0 eq.) was co-evaporated with toluene (3x) under argon atmosphere. The residue was dissolved in DMF (12 mL) and cooled to 0°C. Phenylsilane (0.15 mL, 1.20 mmol, 2.0 eq.) and $Pd(PPh_3)_4$ (69.0 mg, 0.06 mmol, 0.1 eq.) were added and the reaction was stirred at 0°C. After 30 minutes, TLC showed full conversion of the starting material. Et_2O was added to precipitate the crude product and after filtration, the precipitate was purified by recrystallization (MeOH/DCM/Et_2O) to give the title compound (0.62 g, 0.52 mmol, 87%) as a pale yellow solid. R_f: 0.2 (1/9 MeOH/DCM); $[\alpha]_D^{25}$ = -18.7° (*c* = 0.30, DCM/MeOH: 1/1); ^{1}H NMR (MeOD/CD_2Cl_2 1/1, 500 MHz, HH-COSY, HSQC): δ 7.77 (d, 2H, *J* = 7.6 Hz, Ar), 7.65 (t, 2H, *J* = 7.0 Hz, Ar), 7.50 – 7.42 (m, 3H, Ar), 7.42 – 7.36 (m, 3H, Ar), 7.36 – 7.30 (m, 3H, Ar), 7.30 – 7.25 (m, 3H, Ar), 6.90 (d, 2H, *J* = 8.6 Hz, Ar), 5.54 (s, 1H, CH benzylidene), 4.49 (q, 2H, *J* = 12.0, 11.6, 3.3, 2.8 Hz, CH_2 glycolyl), 4.42 – 4.29 (m, 3H, CH_2 Fmoc, CH *i*-D-Gln), 4.29 – 4.15 (m, 4H, C*H*H -6, CH Fmoc, CH L-Lys, CH L-Ala), 4.11 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.98 – 3.86 (m, 3H, H-2, CH_2 PMB), 3.77 (s, 3H, CH_3 PMB), 3.69 (q, 2H, *J* = 10.9, 10.3 Hz, H-3, C*H*H -6), 3.58 (t, 1H, *J* = 9.2 Hz, H-4), 3.45 (t, 1H, *J* = 9.2 Hz, H-1), 3.42 – 3.36 (m, 1H, H-5), 3.19 – 3.10 (m, 2H, CH_2 ε-L-Lys), 2.33 – 2.23 (m, 2H, CH_2 γ-*i*-D-Gln), 2.18 – 2.09 (m, 3H, C*H*H β-*i*-D-Gln, CH_2-9), 1.89 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, CH_2-8), 1.74 – 1.45 (m, 5H, CH_2 β-L-Lys, C*H*H γ-L-Lys, CH_2 δ-L-Lys), 1.41 (s, 12H, CH_2-7, C*H*H γ-L-Lys, 3x CH_3 *t*Bu), 1.32 (dd, 6H, *J* = 9.7, 6.9 Hz, CH_3 lactic acid, CH_3 L-Ala); ^{13}C-APT NMR (MeOD/CD_2Cl_2 1/1, 126 MHz, HSQC): δ 175.4, 175.1, 174.9, 173.4, 172.3 (C=O), 160.5, 144.7, 142.0, 138.3 (C_q Ar), 130.5 (Ar), 129.8 (C_q Ar), 129.6, 128.8, 128.4, 127.8, 126.7, 125.8, 120.6, 114.6 (Ar), 102.1 (CH benzylidene), 82.0 (C-4), 81.6 (C_q *t*Bu), 81.1 (C-3), 79.6 (C-1), 78.5 (CH lactic acid), 73.8 (CH_2 glycol), 71.1 (C-5), 69.5 (CH_2 PMB), 69.4 (CH_2-6), 67.5 (CH_2 Fmoc), 55.7 (CH_3 PMB), 55.4 (C-2), 53.1 (CH L-Lys), 53.0 (CH *i*-D-Gln), 50.2 (CH L-Ala), 47.9 (CH Fmoc), 39.7 (CH_2 ε-L-Lys), 36.7 (C-9), 33.2 (CH_2 γ-*i*-D-Gln), 32.4 (CH_2 β-L-Lys), 32.0 (CH_2 γ-L-Lys), 29.5 (CH_2 δ-L-Lys), 28.2 (CH_3 *t*Bu), 27.7 (CH_2 β-*i*-D-Gln), 23.6 (C-7), 22.5 (C-8), 19.5 (CH_3 L-Ala), 17.7 (CH_3 lactic acid); FT-IR (neat, cm^{-1}): 33141, 2934, 1658, 1514, 1451, 1368, 1249, 1153, 1102, 1029, 847, 760, 742, 699, 621; HRMS: $[M+H]^+$ calcd. for $C_{63}H_{81}N_6O_{17}$ 1193.5653, found 1193.5674; LC-MS: Rt = 8.03 min (Gemini C_{18}, 10 - 90% MeCN, 12.5 min run).

Automated solid phase synthesis general experimental information

The automated solid-phase peptide synthesis was performed on a 250 µmol scale on a Protein Technologies Tribute-UV IR Peptide Synthesizer applying Fmoc based protocol starting from Tentagel S Ram resin (loading 0.22 mmol/g). The synthesis was continued with Fmoc-amino acids specific for each peptide. The consecutive steps performed in each cycle for HCTU chemistry on 250 µmol scale: 1) Deprotection of the Fmoc-group with 20% piperidine in DMF for 10 min; 2) DMF wash; 3) Coupling of the appropriate amino acid using a four-fold excess. Generally, the Fmoc amino acid (1.0 mmol) was dissolved in 0.2 M HCTU in DMF (5 mL), the resulting solution was transferred to the reaction vessel followed by 0.5 mL of 0.5 M DIPEA in DMF to initiate the coupling. The reaction vessel was then shaken for 30 min at 50°C; 4) DMF wash; 5) capping with 10% Ac_2O in 0.1 M DIPEA in DMF; 6) DMF wash; 7) DCM wash. Aliquots of resin of the obtained sequences were checked on an analytical Agilent Technologies 1260 Infinity system with a Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column or a Vydac 219TP 5 µm Diphenyl, 150 x 4.6 mm column with a 1 ml/min flow. The Fmoc amino acids applied in the synthesis were: Fmoc-Abu-OH, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(MMT)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(O*t*Bu)-OH Fmoc-Thr(O*t*Bu)-OH, Fmoc-Tyr(O*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Val-Thr(psiMe,Mepro)-OH and Fmoc-Asp(O*t*Bu)-Ser(psiMe,Mepro)-OH.

General procedure for cleavage from the resin, deprotection and purification

30 µmol resin was washed with DMF, DCM and dried after the last synthesis step followed by a treatment for 180 minutes with 0.6 mL cleavage cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The suspension was filtered, the resin was washed with 0.6 mL of the cleavage cocktail, and the combined TFA solutions were added dropwise to cold Et_2O and stored at -20°C overnight. The obtained suspension of the product in Et_2O was centrifuged, Et_2O was removed and the precipitant was dissolved in CH_3CN/H_2O/*t*BuOH (1/1/1 v/v/v) or DMSO/CH_3CN/H_2O/*t*BuOH (3/1/1/1 v/v/v/v). Purification was performed on a Gilson GX-281 preparative RP-HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column or a Vydac 219TP 5 µm Diphenyl, 250 x 10 mm column.

3-Azidopropyl-MDP(Ac)-Ala-*i*-D-Gln-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_2 (1)

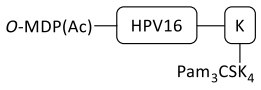
Tentagel S Ram resin loaded with Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(Boc)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 50 µmol scale was elongated with Fmoc-*i*-D-Gln-OH (74 mg, 0.20 mmol, 4.0 eq), Fmoc-L-Ala-OH (63 mg, 0.20 mmol, 4.0 eq) and compound **9a** (70 mg, 0.15 mmol, 3.0 eq.) with standard HCTU/Fmoc cycle. The resin was washed with DCM and treated with the standard cleavage cocktail for 60 minutes. The suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **1** (5.4 mg, 1.5 µmol, 3%) was obtained as a white solid. LC-MS: Rt = 5.03 min (C18 Gemini, 10 - 90% MeCN, 15 min

run); ESI-MS: m/z 1829.0 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{160}H_{264}N_{48}O_{50}$: 914.48922, found 914.48996.

3-Azidopropyl-MDP(Gly)-Ala-*i*-D-Gln-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_2 (2)

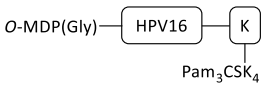
Tentagel S Ram resin loaded with Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(Boc)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 50 µmol scale was elongated with Fmoc-*i*-D-Gln-OH (74 mg, 0.20 mmol, 4.0 eq), Fmoc-L-Ala-OH (63 mg, 0.20 mmol, 4.0 eq) and compound **9b** (91 mg, 0.20 mmol, 4.0 eq.) with standard HCTU/Fmoc cycle. The resin was washed with DCM and treated with the standard cleavage cocktail for 60 minutes. The suspension was filtered and the product waconjugate **2** (14.7 mg, 3.8 µmol, 8%) was obtained as a white solid. LC-MS: Rt = 7.33 min (C18 Gemini, 10 - 50% MeCN, 15 min run); ESI-MS: m/z 1836.8 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{160}H_{264}N_{48}O_{51}$: 918.48795, found 918.48729.

3-Azidopropyl-MDP(Ac)-Ala-*i*-D-Gln-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (3)



50 µmol of crude [3-Azidopropyl-2-*N*-acetyl-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside]-Ala-*i*-D-Gln-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT)-Tentagel S Ram (see synthesis of compound **1**) was washed with DCM (3x) and treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 15 mL) over 5 minutes. The resin was subsequently washed with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL) and DCM (5x). The peptide was elongated on 25 µmol scale with Ser(*t*Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc cycle on the peptide synthesizer concluding with a final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was washed with DMF (5x) and treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (46 mg, 50 µmol, 2.0 eq.) in the presence of HCTU (21 mg, 50 µmol, 2.0 eq.) and DIPEA (18 µL, 0.10 mmol, 4.0 eq.) in DMF/DCM (1/1 v/v, 0.5 mL) overnight. The resin was washed with DMF (3x), DCM (3x) and treated with the standard cleavage cocktail for 60 minutes. The suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **3** (5.2 mg, 1.0 µmol, 1%) was obtained as a white solid. LC-MS: Rt = 12.50 min (Vydac 219TP 5 µm Diphenyl, 10 - 90% MeCN, 21 min run); ESI-MS: m/z 1716.9 $[M+H]^{2+}$; HRMS: $[M+H]^{6+}$ calcd. for $C_{241}H_{420}N_{58}O_{62}S$: 858.51972, found 858.51999.

3-Azidopropyl-MDP(Gly)-Ala-*i*-D-Gln-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (4)



50 µmol of crude [3-Azidopropyl-4,6-*O*-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside]-Ala-*i*-D-Gln-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT)-Tentagel S Ram (see synthesis of compound **2**) was washed with DCM (3x) and treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 15 mL) over 5 minutes. The resin was subsequently washed with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL) and DCM (5x). The peptide was elongated with Ser(*t*Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc cycle on the peptide synthesizer concluding with a final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was washed with DMF (5x) and treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (91 mg, 0.10 mmol, 2.0 eq.) in the presence of HCTU (41 mg, 0.10 mmol, 2.0 eq.) and DIPEA (35 µL, 0.20 mmol, 4.0 eq.) in DMF/DCM (1/1 v/v, 1.0 mL) overnight. The resin was washed with DMF (3x), DCM (3x) and treated with the standard cleavage cocktail for 60 minutes. The suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **4** (3.3 mg, 0.64 µmol, 1%) was obtained as a white solid. LC-MS: Rt = 12.04 min (Vydac 219TP 5 µm Diphenyl, 10 - 90% MeCN, 21 min run); ESI-MS: m/z 1722.2 $[M+H]^{2+}$; HRMS: $[M+H]^{6+}$ calcd. for $C_{241}H_{420}N_{58}O_{63}S$: 861.18553, found 861.18634.

***N*$_\alpha$-Acetyl-*N*$_\varepsilon$-[butan-4-(2-deoxy-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_2 (5)**

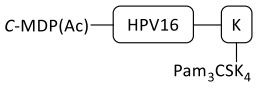
Tentagel S Ram resin loaded with H-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 70 µmol scale was washed with DMF (5x), followed by the addition of a solution of compound **10a** (148 mg, 140 µmol, 2.0 eq.) and HCTU (58 mg, 140 µmol, 2.0 eq.) in a mixture of DMF/DMSO (1.4 mL/0.4 mL) and DIPEA (49 µL, 280 µmol, 4.0 eq.). The reaction vessel was shaken overnight, after which it was washed with DMF (5x), treated with 20% piperidine in DMF (2x 1.4 mL, 10 min), washed with DMF (5x), treated with a mixture of 10% Ac_2O in 0.1 M DIPEA in DMF (2x 1.4 mL, 20 min) and washed with DMF (3x) and DCM (3x). The resin dried on air and split in two portions of which 30 µmol of the crude was washed with DCM (3x). After treatment with a standard cleavage cocktail (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the standard cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **5** (7.2 mg, 1.9 µmol, 6%) was obtained as a white

solid. LC-MS: Rt = 7.38 min (C18 Gemini, 10 - 50% MeCN, 15 min run); ESI-MS: m/z 1907.2 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{169}H_{279}N_{47}O_{53}$: 953.76399, found 953.76373.

N_α-Acetyl-N_ε-[butan-4-(2-deoxy-2-*N*-(2-hydroxyacetamide)-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_2 (6)

Tentagel S Ram resin loaded with H-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 40 µmol scale was washed with DMF (5x), followed by the addition of a solution of compound **10b** (95 mg, 80 µmol, 2.0 eq.) and HCTU (34 mg, 82 µmol, 2.0 eq.) in DMF (0.8 mL) and DIPEA (28 µL, 160 µmol, 4.0 eq.). The reaction vessel was shaken overnight, after which it was washed with DMF (5x), treated with 20% piperidine in DMF (2x 0.8 mL, 10 min), washed with DMF (5x), treated with a mixture of 10% Ac_2O in 0.1 M DIPEA in DMF (2x 0.8 mL, 20 min) and washed with DMF (3x) and DCM (3x). After treatment with a standard cleavage cocktail (1.6 mL) for 3 hours the suspension was filtered and the residue was washed with the standard cleavage cocktail (1.6 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **6** (9.2 mg, 2.4 µmol, 6.1%) was obtained as a white solid. LC-MS: Rt = 7.89 min (C18 Gemini, 10 - 50% MeCN, 15 min run); ESI-MS: m/z 1914.9 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{169}H_{279}N_{47}O_{54}$: 957.76271, found 957.76246.

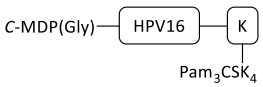
N_α-Acetyl-N_ε-[butan-4-(2-deoxy-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (7)



70 µmol of crude N_α-Ac-N_ε-[butan-4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT)-Tentagel S Ram (see synthesis of compound **5**) was washed with DCM (3x) and treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 20 mL) over 10 minutes. The resin was washed with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL) and DCM (5x). The peptide was elongated with Ser(*t*Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc chemistry on the peptide synthesizer concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3 x 3 min). The resin was treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (128 mg, 0.14 mmol, 2.0 eq.) in the presence of HCTU (59 mg, 0.14 mmol, 2.0 eq.) and DIPEA (49 µL, 0.28 mmol, 4.0 eq.) in DMF/DCM (1/1 v/v, 1.4 mL) overnight. After treatment with a standard cleavage cocktail (2.8 mL) for 3 hours the suspension

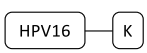
was filtered and the residue was washed with the standard cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **7** (2.3 mg, 433 nmol, 0.6%) was obtained as a white solid. LC-MS: Rt = 12.31 min (Vydac 219TP 5 µm Diphenyl, 10 - 90% MeCN, 21 min run); ESI-MS: m/z 1769.4 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{250}H_{433}N_{57}O_{65}S$: 1326.55070, found 1326.55125.

N_α-Acetyl-N_ε-[butan-4-(2-deoxy-2-*N*-(2-hydroxyacetamide)-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (8)



70 µmol of crude N_α-Ac-N_ε-[butan-4-(4,6-*O*-di-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranosyl)-amide]-L-lysine-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT)-Tentagel S Ram (see synthesis of compound **6**) was washed with DMF (3x) and DCM (3x). The resin was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 30 mL) over 10 minutes. The resin was washed with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL) and DCM (5x). The peptide was elongated with Ser(*t*Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc chemistry on the peptide synthesizer concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3 x 3 min). The resin was treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (128 mg, 0.14 mmol, 2.0 eq.) in the presence of HCTU (59 mg, 0.14 mmol, 2.0 eq.) and DIPEA (49 µL, 0.28 mmol, 4.0 eq.) in DMF/DCM (1/1 v/v, 1.4 mL) overnight. After treatment with a standard cleavage cocktail (2.8 mL) for 3 hours the suspension was filtered and the residue was washed with the standard cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **8** (1.4 mg, 263 nmol, 0.4%) was obtained as a white solid. LC-MS: Rt = 12.21 min (Diphenyl Vydac, 10 - 90% CH_3CN, 21 min); ESI-MS: m/z 1774.7 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{250}H_{433}N_{57}O_{66}S$: 1330.54942, found 1330.54563.

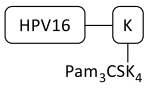
Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_2 (19)



Tentagel S Ram resin loaded with H-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 30 µmol scale was washed with DCM (5x). After treatment with a standard cleavage cocktail (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the standard cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and

lyophilisation, conjugate **19** (9.4 mg, 3.0 µmol, 10%) was obtained as a white solid. LC-MS: Rt = 4.85 min (C18 Gemini, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 1549.8 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{138}H_{229}N_{41}O_{40}$ 775.17809, found 775.17790.

Acetyl-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (20)



Tentagel S Ram resin loaded with H-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 50 µmol scale was washed with DMF (5x), treated with a mixture of 10% Ac_2O in 0.1 M DIPEA in DMF (2x 1.0 mL, 20 min) and washed with DMF (3x) and DCM (3x). The resin was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 20 mL) over 10 minutes. The resin was washed with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL) and DCM (5x). The peptide was elongated with Ser(*t*Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc chemistry on the peptide synthesizer concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3 x 3 min). The resin was treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (92 mg, 0.10 mmol, 2.0 eq.) in the presence of HCTU (42 mg, 0.10 mmol, 2.0 eq.) and DIPEA (35 µL, 0.20 mmol, 4.0 eq.) in DMF/DCM (1/1 v/v, 1.0 mL) overnight. After treatment with a standard cleavage cocktail (2.0 mL) for 3 hours the suspension was filtered and the residue was washed with the standard cleavage cocktail (2.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **20** (5.5 mg, 1.2 µmol, 2%) was obtained as a white solid. LC-MS: Rt = 12.17 min (Diphenyl Vydac, 10 - 90% CH_3CN, 21 min); ESI-MS: m/z 1545.1 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{221}H_{385}N_{51}O_{53}S$ 1158.46744, found 1158.46863.

Footnotes and References

(1) Vermaelen, K. *Front. Immunol.* **2019**, *10*.
(2) Schumacher, T. N.; Schreiber, R. D. *Science.* **2015**, *348* (6230), 69–74.
(3) Heimburg-Molinaro, J.; Lum, M.; Vijay, G.; Jain, M.; Almogren, A.; Rittenhouse-Olson, K. *Vaccine* **2011**, *29* (48), 8802–8826.
(4) Xu, Z.; Moyle, P. M. *Bioconjug. Chem.* **2018**, *29* (3), 572–586.
(5) Kool, M.; Fierens, K.; Lambrecht, B. N. *J. Med. Microbiol.* **2012**, *61* (Pt 7), 927–934.
(6) Liu, H.; Irvine, D. J. *Bioconjug. Chem.* **2015**, *26* (5), 791–801.
(7) Zom, G. G. P.; Khan, S.; Filippov, D. V.; Ossendorp, F. In *Advances in Immunology*; **2012**; pp 177–201.
(8) Ingale, S.; Wolfert, M. A.; Gaekwad, J.; Buskas, T.; Boons, G.-J. *Nat. Chem. Biol.* **2007**, *3* (10), 663–667.
(9) Kawai, T.; Akira, S. *Nat. Immunol.* **2010**, *11* (5), 373–384.
(10) Ignacio, B. J.; Albin, T. J.; Esser-Kahn, A. P.; Verdoes, M. *Bioconjug. Chem.* **2018**, *29* (3), 587–603.
(11) Philpott, D. J.; Sorbara, M. T.; Robertson, S. J.; Croitoru, K.; Girardin, S. E. *Nat. Rev. Immunol.* **2014**, *14* (1), 9–23.
(12) Behr, M. A.; Divangahi, M. *Curr. Opin. Microbiol.* **2015**, *23*, 126–132.
(13) Willems, M. M. J. H. P.; Zom, G. G.; Meeuwenoord, N.; Ossendorp, F. A.; Overkleeft, H. S.; van der Marel, G. A.; Codée, J. D. C.; Filippov, D. V. *Beilstein J. Org. Chem.* **2014**, *10*, 1445–1453.

(14) Fritz, J. H.; Girardin, S. E.; Fitting, C.; Werts, C.; Mengin-Lecreulx, D.; Caroff, M.; Cavaillon, J.-M.; Philpott, D. J.; Adib-Conquy, M. *Eur. J. Immunol.* **2005**, *35* (8), 2459–2470.
(15) Mutwiri, G.; Gerdts, V.; van Drunen Littel-van den Hurk, S.; Auray, G.; Eng, N.; Garlapati, S.; Babiuk, L. A.; Potter, A. *Expert Rev. Vaccines* **2011**, *10* (1), 95–107.
(16) Schwarz, H.; Posselt, G.; Wurm, P.; Ulbing, M.; Duschl, A.; Horejs-Hoeck, J. *Immunobiology* **2013**, *218* (4), 533–542.
(17) Pavot, V.; Rochereau, N.; Rességuier, J.; Gutjahr, A.; Genin, C.; Tiraby, G.; Perouzel, E.; Lioux, T.; Vernejoul, F.; Verrier, B.; *et al*. *J. Immunol.* **2014**, *193* (12), 5781–5785.
(18) Natsuka, M.; Uehara, A.; Shuhua Yang; Echigo, S.; Takada, H. *Innate Immun.* **2008**, *14* (5), 298–308.
(19) Tada, H.; Aiba, S.; Shibata, K.-I.; Ohteki, T.; Takada, H. *Infect. Immun.* **2005**, *73* (12), 7967–7976.
(20) Zom, G. G.; Willems, M. M. J. H. P.; Meeuwenoord, N. J.; Reintjens, N. R. M.; Tondini, E.; Khan, S.; Overkleeft, H. S.; van der Marel, G. A.; Codee, J. D. C.; Ossendorp, F.; *et al*. *Bioconjug. Chem.* **2019**, *30* (4), 1150–1161.
(21) Coulombe, F.; Divangahi, M.; Veyrier, F.; de Léséleuc, L.; Gleason, J. L.; Yang, Y.; Kelliher, M. A.; Pandey, A. K.; Sassetti, C. M.; Reed, M. B.; *et al*. *J. Exp. Med.* **2009**, *206* (8), 1709–1716.
(22) Chen, K.-T.; Huang, D.-Y.; Chiu, C.-H.; Lin, W.-W.; Liang, P.-H.; Cheng, W.-C. *Chem. - A Eur. J.* **2015**, *21* (34), 11984–11988.
(23) de la Fuente, J. M.; Penadés, S. *Synthesis of Le X-Neoglycoconjugate to Study Carbohydrate-Carbohydrate Associations and Its Intramolecular Interaction*; **2002**; Vol. 13.
(24) Anomeric hydrolysis was observed for the conjugates 2-4 using LC-MS analysis, however it was not possible to determine the amount of hydrolyzed product as it was inseparable from the desired conjugate.
(25) Fuchss, T.; Schmidt, R. R. *Synthesis (Stuttg).* **1998**, *1998* (05), 753–758.
(26) Myers, E. L.; Butts, C. P.; Aggarwal, V. K. *Chem. Commun.* **2006**, No. 42, 4434–4436.
(27) Voigtritter, K.; Ghorai, S.; Lipshutz, B. H. *J. Org. Chem.* **2011**, *76* (11), 4697–4702.
(28) Sharma, P. K.; Kumar, S.; Kumar, P.; Nielsen, P. *Tetrahedron Lett.* **2007**, *48* (49), 8704–8708.
(29) At this stage, the remaining ruthenium impurities were removed by embedding the compound on QuadraSil® aminopropyl, followed by column chromatography.

Chapter 4

*Synthesis of multivalent MPR ligand–antigen conjugates**

Introduction

Carbohydrates play an important role in many biological processes, such as cell-cell communication, pathogen recognition and protein folding. Mannose-6-phosphate (M6P), a D-mannopyranose bearing a phosphate group at *C*-6 position, serves as a signaling moiety on the termini of glycan branches mounted on newly synthesized proteins in the trans-Golgi network (TGN) and it is essential for the transportation of these enzymes to the late endosomes and lysosomes. The mannose-6-phosphate receptor (MPR), a P-type lectin, plays an important role in this transportation through binding to the M6P.[1,2] There are two members of this lectin family: the cation-independent mannose-6-phosphate receptor (CI-MPR) and the cation-dependent mannose-6-phosphate receptor (CD-MPR). The first one is a ~300 kDa transmembrane glycoprotein that consists of 15 extracellular repeating domains (Figure 1A), has a low affinity for M6P and is able to bind to both M6P and other ligands, such as insulin growth factor II (IGFII).[3–5] On the other hand, the CI-MPR has high affinity for ligands containing multiple M6Ps due to the two M6P binding domains (3 and 9), which can

*The data presented in this Chapter were gathered in collaboration with Christopher Vis, Toroa McGlinn, Nico J. Meeuwenoord, Tim P. Hogervorst, Herman S. Overkleeft, Dmitri V. Filippov, Gijsbert A. van der Marel and Jeroen D. C. Codée.

simultaneously bind two M6Ps. Binding to the two M6P domains can also lead to dimerization between two receptors. The group of Berkowitz studied the ligand-receptor interactions with mono- and bivalent probes and two dimeric models for multivalent binding of the receptor were suggested: the "ladder" and the "hook" model (Figure 1A and 1B, respectively).[6,7] The CD-MPR is a ~46 kDa transmembrane protein that consists of only one M6P binding site. It does not bind to IGFII and can be found as a dimer (Figure 1C). A small fraction of MPRs can be found on the cell surface, where only CI-MPR binds and internalizes M6P-bound enzymes (Figure 1D).[8] The optimal binding of the CD-MPR and domain 9 of the CI-MPR is reported to be at a pH of 6.0-6.5 and 6.4-6.5, while domain 3 of the CI-MPR binds at a higher pH of 6.9-7.0. These observations explain why only the CI-MPR binds M6P-bound enzymes at the cell surface (pH = ~7.4).[3,9,10]

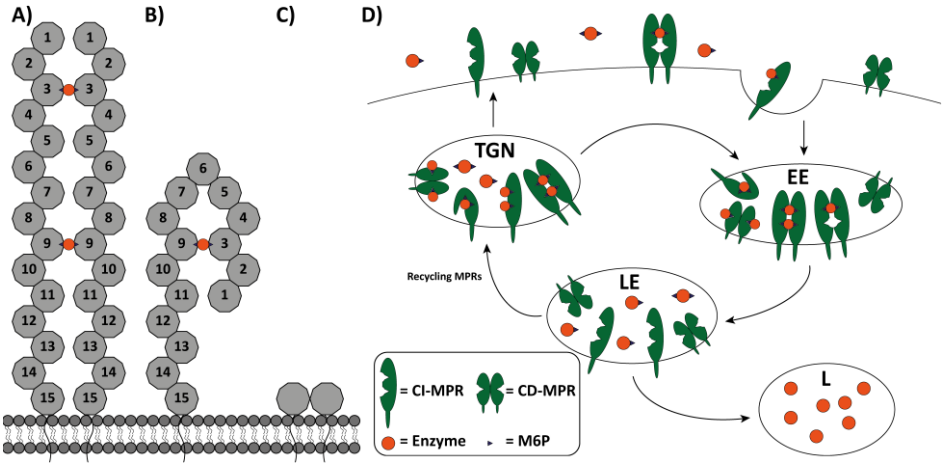


Figure 1. Proposed binding models and schematic representations of: A) Bivalent M6P ligand bound to two monomers of CI-MPR; B) Bivalent M6P ligand bound to the same receptor; C) CD-MPR; D) MPR-pathway: lysosomal enzymes in the TGN are transported to the early endosome. From there, the enzymes are transported to the late endosome, where the enzyme is dissociated from the MPR. The enzymes are packaged into the lysosome and the MPR is recycled back to the TGN, from where a small portion is moved to the cell surface. LE = late endosome; EE = early endosome, L = lysosome.

The MPR can be used as an efficient tool for targeted delivery to the endosomes as the conjugation of M6P analogues to acid α-glucosidase improved the treatment of the lysosomal myopathy Pompe disease[11]. The MPR has also been exploited in the delivery of doxorubicin via mannose-6-phosphate-modified human serum albumin as carrier or *N*-hexanoyl-D-*erythro*-sphingosine with M6P-functionalized liposomes.[12,13] Hogendoorn *et al.* have reported the synthesis of a M6P cluster attached to a covalent cathepsin inhibitor and a fluorescent BODIPY dye, which was used to label proteases

along the endocytic pathway.[14] It was shown that the MPR can also be exploited as an effective pathway for immunogenicity.[15] This led to the idea that conjugate vaccines in which a M6P moiety is covalently bound to an epitope will have improved uptake ensuring a more efficient delivery to the endosomes. One of the potential drawbacks of the use of M6P is dephosphorylation by endogenous phosphatases. To prevent this, several M6P analogues have previously been evaluated, such as a malonyl ether and a malonate or a *C*-phosphonate ester.[6,16] The *C*-phosphonate proved to be a stable and effective replacement for the phosphate monoester.

This chapter describes the incorporation of a cluster of mannose-6-phosphonates (M6Po) in two types of peptide antigen-conjugates (**1**-**8**, Figure 2), wherein two different M6Po's (**9** and **10,** respectively) are conjugated to either the *N*- or the *C*-terminus of a synthetic long peptide (SLP). In the first type of conjugates (**1**-**4**), $DEVA_5K$ was used as SLP. This model epitope contains the MHC-I antigen SIINFEKL, incorporated in a longer peptide motif DEVSGLEQLESIINFEKLAAAAAK. The α-propargyl mannose-6-phosphonate (*O*-M6Po) building block (**9**) was developed since an α-configured linker is tolerated by the MPR.[7,14] The alkyne function in building block **9** allows the copper mediated 1,3-dipolar cycloaddition to azide-functions in the SLP.[17] As it was shown that bi- or multivalent M6P-ligands are more effective in binding the CI-MPR than ligands with only one M6P, six M6Po's were incorporated in each conjugate.[3,18,19] The objective to enhance the immune response after transferring the conjugate to the endosomes by the MPR, led to the design of bis-conjugates (**2** and **4**) in which a Toll-like receptor ligand 7 (TLR7L) is added at either the *N*- or the *C*-terminus of the SLP. In the second type of conjugates (**5**-**8**) the ovalbumin derived (HAAHA) peptide ISQAVHAAHAEINEAGRK, which contains an MHC-II epitope, was used as SLP. *C*-M6Po building block **10** was developed as a building block that can be used in the online solid phase peptide synthesis (SPPS) of conjugates **5**-**8**. This building block is a *C*-analogue of M6Po, in which the anomeric oxygen is replaced with a CH_2, preventing hydrolysis by the acidic conditions used in SPPS. An additional advantage of the SPPS compatible building blocks is the possibility to prepare conjugates of peptides that are not suitable for copper mediated 1,3-dipolar cycloaddition. For example, the HAAHA peptide, used for the second type of conjugates, contains two histidines, which can coordinate to copper and thereby inhibit the reduction of Cu(II) to Cu(I).[20] The SPPS building block **10** is equipped with acid labile protecting groups, which will be removed at the end of the SPPS concomitantly with the other acid labile peptide protecting groups and release of the peptide from the resin.

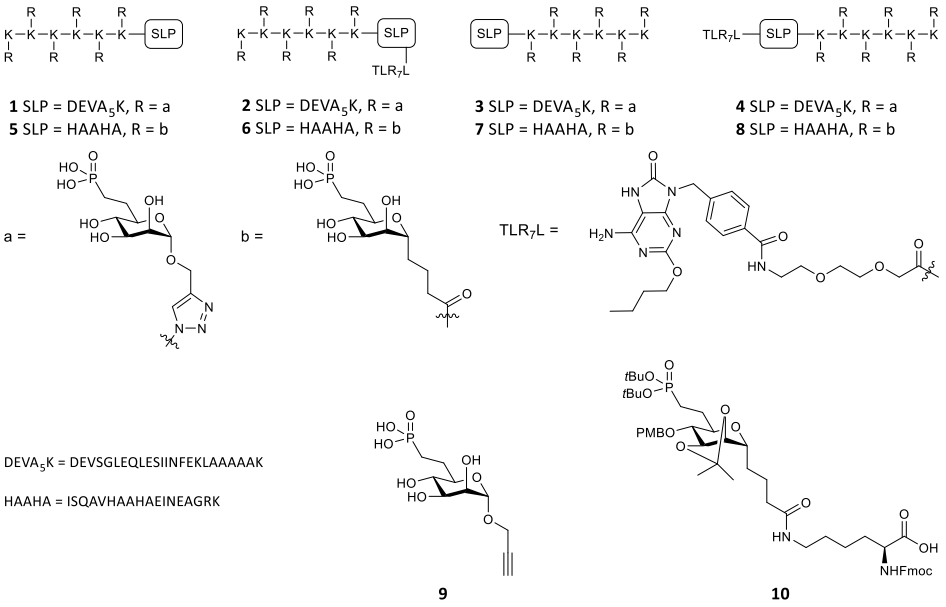


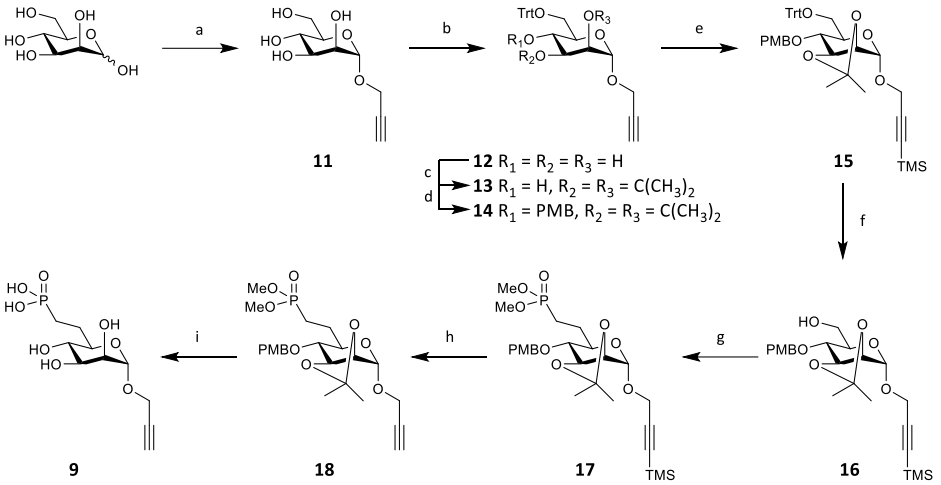
Figure 2. Structures of the 1st type *O*-M6Po conjugates **1**-**4**, the 2nd type *C*-M6Po conjugates **5**-**8** and building blocks **9** and **10**.

Results and Discussion

***O*-M6Po-SIINFEKL conjugates**

The first type of *O*-M6Po conjugates comprises the six-fold addition of α-propargyl mannose-6-phosphonate (*O*-M6Po) building block (**9**) to azide containing peptides. Synthesis of the required building block **9** started with the acetylation of D-mannose, followed by $BF_3{\cdot}OEt_2$ catalyzed glycosylation of propargyl alcohol and deacetylation to give crystalline compound **11** (Scheme 1). Subsequently, the following protective group manipulations were performed: tritylation of the primary 6-OH in **11**, isopropylidation of **12**, and *p*-methoxybenzylation of **13** to give fully protected mannose **14**. The presence of the isopropylidene group proved to be an essential conformational lock, preventing intramolecular cyclization during the installation of the phosphonate.[21–23] Prior to the installation of the phosphonate, the terminal alkyne in **14** was protected with a TMS group using TMSCl and *n*-BuLi at -78°C. Removal of the trityl in thus obtained **15** with a catalytic amount of *p*-toluenesulfonic acid in DCM/MeOH was accompanied by partial removal of the isopropylidene ketal. Reinstallation of the isopropylidene and subsequent deprotection of the mixed acetal formed on the primary alcohol gave **16** in 98% over three steps. Alcohol **16** was converted into a triflate using Tf_2O and pyridine at -40°C. A fast work-up was necessary due to the instability of the formed triflate.[24]

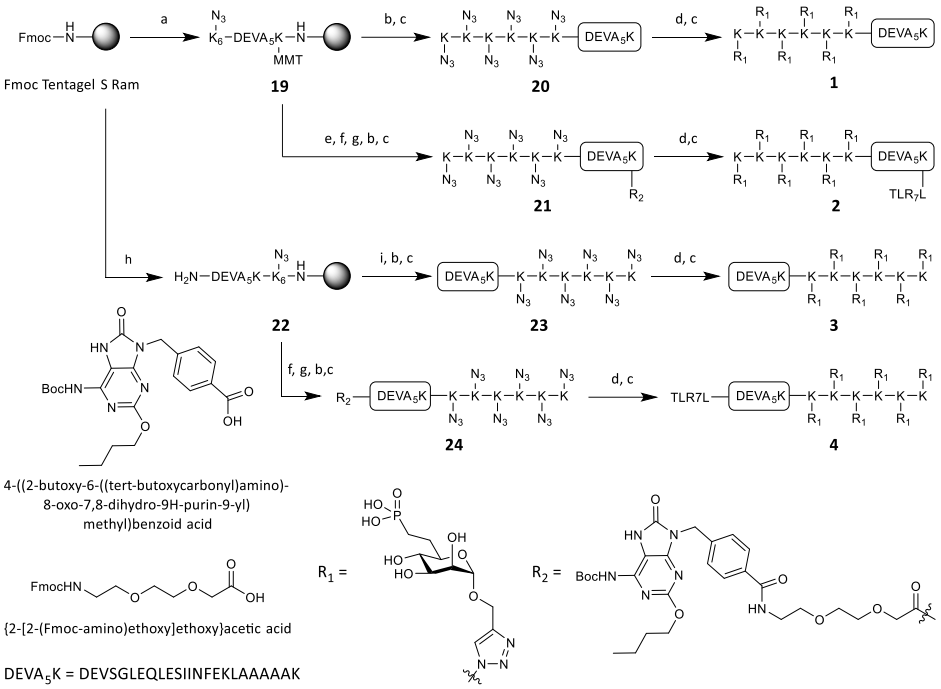
The obtained crude triflate was added to a solution of deprotonated dimethyl methylphosphonate in THF at -70°C, giving compound **17** in 72% over two steps. Removal of the TMS protecting group gave **18**, which was transformed into key building block **9** by a two-step deprotection sequence. In the first step, the phosphonate was deprotected using TMSBr. Next, the *p*-methoxybenzyl and isopropylidene were removed by treatment of the intermediate with AcOH/H_2O at 90°C. Compound **9** was obtained in 27% over 15 steps starting from D-mannose.



Scheme 1. Synthesis of alkyne building block **9**. *Reagents and conditions*: a) *i*. Ac_2O, pyridine; *ii*. propargyl alcohol, $BF_3{\cdot}OEt_2$, 50°C; *iii*. NaOMe, MeOH, 70% over three steps; b) TrtCl, Et_3N, DMF, 60°C, 83%; c) *p*-toluenesulfonic acid, 2,2-dimethoxypropane, 87%; d) *p*-methoxybenzyl chloride, NaH, DMF, 95%; e) TMSCl, *n*-BuLi, THF, -78°C, 97%; f) *i*. *p*-toluenesulfonic acid, DCM/MeOH; *ii*. *p*-toluenesulfonic acid, 2,2-dimethoxypropane; *iii*. 1 M HCl, EtOAc, 0°C, 98% over three steps: g) *i*. Tf_2O, pyridine, DCM, -40°C; *ii*. *n*-BuLi, dimethyl methylphosphonate, THF, -70°C to -50°C, 72% over two steps; h) TBAF, THF, quant.; i) *i*. TMSBr, pyridine, MeCN; *ii*. AcOH/H_2O, 90°C, 81% over two steps.

Next the assembly of the (*O*-M6Po)$_6$-SIINFEKL conjugates was undertaken. Immobilized peptides **19** and **22** were prepared through standard SPPS HCTU/Fmoc chemistry using Tentagel S Ram as solid support (Scheme 2). TFA/TIS/H_2O (95/2.5/2.5 v/v/v) treatment removed all protecting groups and cleaved the peptides from the resin to give peptides **20** and **23** in 1% and 6%, respectively, after purification. Alternatively, the MMT protecting group at the *C*-terminal lysine of **19** was selectively deprotected with a cocktail of TFA/TIS/DCM (2/2/96 v/v/v) and the released amine was subsequently coupled with the spacer {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid and the Boc-protected TLR7-ligand building block.[25] After deprotection, release from the resin and RP-HPLC purification peptides **21** and **24** were both obtained in 2% yield. Coupling of *O*-

M6Po building block **9** to peptides **20**, **21**, **23** and **24** was performed with a cocktail containing $CuSO_4$, sodium ascorbate and tris(benzyltriazolylmethyl)amine in DMSO/H_2O.[26] The addition of a 20 mM Tris/150 mM NaCl buffer was essential to get conversion of the starting peptides. After RP-HPLC, conjugates **1**-**4** were obtained in 5% (0.3 mg), 18% (0.9 mg), 18% (1.0 mg) and 31% (3.3 mg) respectively.

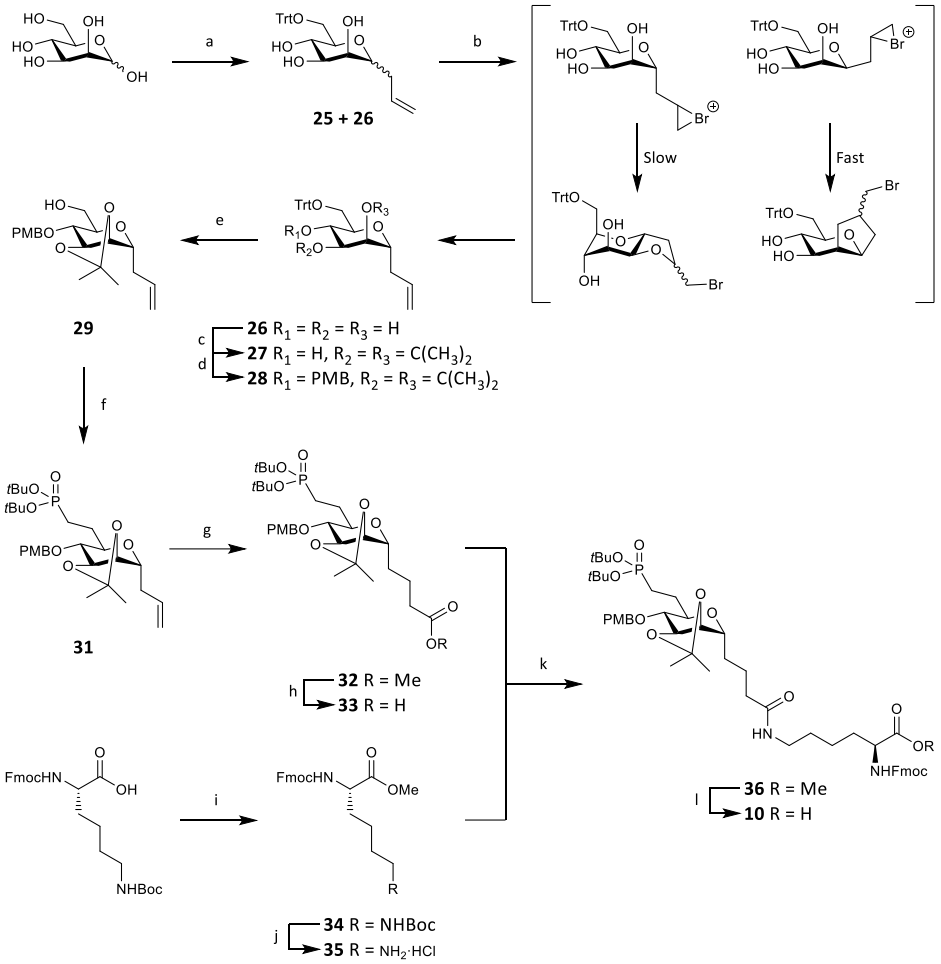


Scheme 2. Solid phase peptide synthesis of *O*-M6Po conjugates **1**-**4**. *Reagents and conditions*: a) *i*. 20% piperidine, DMF; *ii*. Fmoc SPPS cycle for K(N_3)-K(N_3)-K(N_3)-K(N_3)-K(N_3)-K(N_3)-DEVSGLEQLESIINFEKLAAAAAK; *iii*. 20% piperidine, DMF; *iv*. Ac_2O, DIPEA, DMF; b) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 3h; c) RP-HPLC; d) **9**, 20 mM Tris/150 mM NaCl buffer, $CuSO_4$/NaAsc/TBTA, H_2O/DMSO; e) TFA/TIS/DCM (2/2/96 v/v/v); f) *i*. {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid, HCTU, DIPEA, DMF; *ii*. 20% piperidine, DMF; g) 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid, HCTU, DIPEA, DMF; h) *i*. 20% piperidine, DMF; *ii*. Fmoc SPPS cycle for DEVSGLEQLESIINFEKLAAAAAK-K(N_3)-K(N_3)-K(N_3)-K(N_3)-K(N_3)-K(N_3); *iii*. 20% piperidine, DMF; i) Ac_2O, DIPEA, DMF; Yield peptides and conjugates: **20**) 4.6 mg, 1%; **21**) 4.0 mg, 2%; **23**) 17.4 mg, 6%; **24**) 8.2 mg, 2%; **1**) 0.3 mg, 5%; **2**) 1.0 mg, 18%; **3**) 0.9 mg, 18%; **4**) 3.3 mg, 31%.

***C*-M6Po-HAAHA conjugates**

The second type of *C*-M6Po conjugates was generated by an online SPPS synthesis using *C*-M6Po building block **10**, which was assembled as depicted in Scheme 3. The first step in the synthesis of **10** comprised the *C*-allylation at the anomeric position of mannose. Girard *et al*. achieved a stereoselective allylation using a Sakurai-type reaction on per-

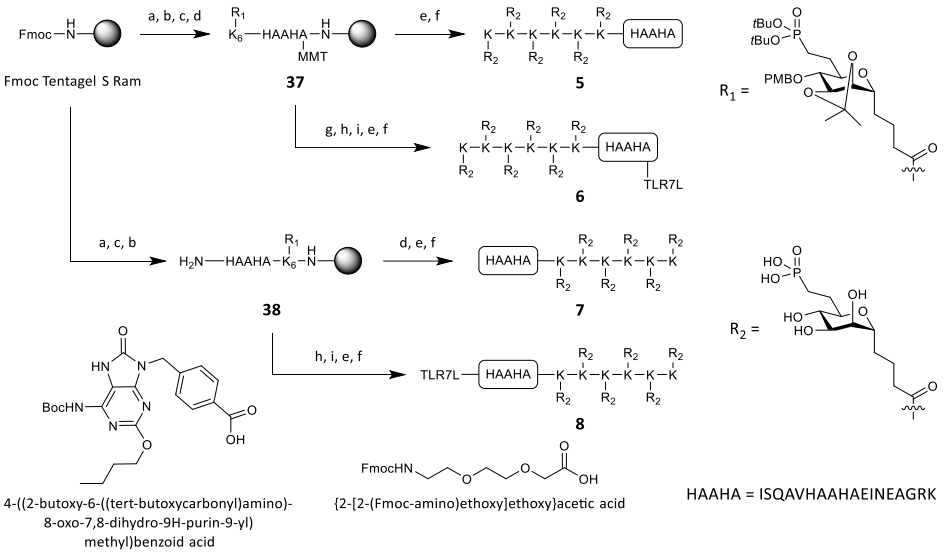
benzylated methyl α-D-mannopyranoside.[27] However, debenzylation and purification turned out to be problematic when this was performed on large scale. Therefore an alternative procedure was followed in which D-mannose was acetylated and treated with a mixture of allyltrimethylsilane, $BF_3{\cdot}OEt_2$, and TMSOTf in MeCN to give the desired allyl mannoside as a 4.2/1 α/β mixture. Known methods[28,29] to separate the α/β mixture of *C*-allyl mannose could not be reproduced on large scale, and therefore the following procedure was developed. The primary alcohol in the crude α/β-*C*-allyl mannose was protected with a trityl, to produce a mixture of **25** and **26** (55% yield over 4 steps). Next, the two anomers were treated with *N*-bromosuccinimide in THF. The formed bromonium ion allows a fast, intramolecular cyclization of the β-mannose while the α-mannose cyclizes slowly due to the unfavorable ring flip, necessary for nucleophilic attack of the 2-*O* at the bromonium ion. After column purification pure α-*C*-allyl mannose **26** was recovered in 91%. Protection of the *C*-2-OH and *C*-3-OH with an isopropylidene ketal was followed by installation of a *p*-methoxybenzyl at the *C*-4-OH to give compound **28**. Removal of the trityl group was performed with the same conditions used in the synthesis of **16**, giving **29** in 72% over three steps. Conversion of **29** to the primary triflate, followed by nucleophilic attack of the anion of di-*tert*-butyl methylphosphonate **30**[30] gave phosphonate **31** in 72% over two steps on 3 mmol scale. The yield dropped to 49% when increasing the scale to 30 mmol due to the instability of the primary triflate. Cross metathesis with methyl acrylate, followed by the reduction of the double bond with $NaBH_4$ and ruthenium trichloride gave compound **32**.[31,32] Hydrolysis of the obtained methyl ester using LiOH gave acid intermediate **33**. Although carboxylic acid **33** is suitable for SPPS the spacer length was adjusted to that of the first type of conjugates. For this purpose, Fmoc-L-Lys-OMe was condensed with **33** under the influence of HCTU and DIPEA to give **34** in 86%. Hydrolysis of **34** with LiOH at 0°C, left the Fmoc group unaffected and gave SPPS building block **10** in 80%.



Scheme 3. Synthesis of building block compound **10**. *Reagents and conditions*: a) *i*. Ac_2O, pyridine, *ii*. allyltrimethylsilane, $BF_3{\cdot}OEt_2$, TMSOTf, MeCN; *iii*. NaOMe, MeOH; *iv*. TrtCl, Et_3N, DMF, 60°C, 55% over four steps; b) *N*-bromosuccinimide , THF, 3 h, 91%; c) *p*-toluenesulfonic acid, 2,2-dimethoxypropane, 93%; d) *p*-methoxybenzyl chloride, NaH, DMF, 97%; e) *i*. *p*-toluenesulfonic acid, DCM/MeOH; *ii*. *p*-toluenesulfonic acid, 2,2-dimethoxypropane; *iii*. 1 M HCl, EtOAc, 0°C, 75% over three steps; f) *i*. Tf_2O, pyridine, DCM, -40°C; *ii*. *n*-BuLi, di-*tert*-butyl methylphosphonate (**30**), THF, -70°C to -50°C, 72% over two steps; g) *i*. methyl acrylate, CuI, Grubbs 2nd gen. catalyst, DCE, 60°C; *ii*. $NaBH_4$, $RuCl_3$, MeOH, DCE, 45°C, 72% over two steps; h) LiOH, THF/H_2O, quant; i) MeI, K_2CO_3, DMF, 93%; j) 4 M HCl in dioxane, dioxane, 98%; k) HCTU, DIPEA, DMF, 86%; l) LiOH, THF/H_2O, 0°C, 80%.

With the desired building block **10** in hand, conjugates **5**-**8** were prepared using semi-automated SPPS (Scheme 4). Tentagel S Ram resin was elongated with ISQAVHAAHAEINEAGRK using automated SPPS, wherein the lysine(MMT) at the *C*-terminus will be used for elongation at a later stage of the synthesis. Six subsequent

manual coupling and Fmoc removal cycles with building block **10** gave immobilized peptide **37**. Peptide **38**, bearing the *C*-M6P cluster at the *C*-terminal end was generated by assembling the hexa-*C*-M6P peptide through manual couplings of building block **10**, followed by automated SPPS to assemble the rest of the peptide. Immobilized and protected peptides **37** and **38** were deprotected and simultaneously cleaved from the resin with the TFA/TIS cocktail to furnish conjugates **5** and **7** after purification by RP-HPLC in 10% and 8% yield respectively, showing the apt behavior of **10** in SPPS. To obtain conjugate **6**, bearing the TLR7-ligand, the MMT-group in **37** was selectively removed with a cocktail of AcOH/TFE/DCM (1/2/7 v/v/v). The obtained free amine was elongated with the spacer {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid and TLR7-ligand 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid to give, after removal of all the protecting groups, cleavage from the resin and finally RP-HPLC purification bis-conjugate **6** in 2% yield. The same sequence of events was applied to the *N*-terminal amine in immobilized peptide **38** to afford bis-conjugate **8** in 8% yield.



Scheme 4. Solid phase peptide synthesis of *C*-M6Po conjugates **5**-**8**. *Reagents and conditions*: a) 20% piperidine, DMF; b) Fmoc SPPS cycle for ISQAVHAAHAEINEAGRK; c) *i*. **10**, HCTU, DIPEA, DMF; *ii*. 20% piperidine, DMF; *iii*. repeat of *i*. and *ii*.; d) Ac_2O, DIPEA, DMF; e) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 3h; f) RP-HPLC; g) AcOH/TFE/DCM (1/2/7 v/v/v); h) *i*. {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid, HCTU, DIPEA, DMF; *ii*. 20% piperidine, DMF; i) 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid, HCTU, DIPEA, DMF; Yield conjugates: **5**) 13.3 mg, 10%; **6**) 11.0 mg, 8%; **7**) 3.1 mg, 2%; **8**) 17.0 mg, 11%.

Conclusion

This chapter describes the synthesis of two type of peptide conjugates (**1**-**4** and **5**-**8**, respectively) having a hexavalent M6Po cluster incorporated at either the *N*- or the *C*-terminus. These ligands for the mannose-6-phosphate receptors were designed and synthesized to improve the uptake of these conjugates resulting in an enhanced immunogenicity. To prevent dephosphorylation by endogenous phosphatases, *C*-phosphonates are applied in the conjugates. The assembly of the first type uses copper mediated 1,3-dipolar cycloaddition (click chemistry) to append six *O*-M6Po residues to the separately prepared peptides in one event. For this purpose *O*-M6Po building block **9** was developed. The second type comprises an online synthesis of the conjugates requiring a mannose building block with an anomeric center, resisting the acid conditions of SPPS. A new method was found to synthesize an α-*C*-allyl-mannoside that was further elaborated to building block **10**. The designed *C*-M6P building block **10** proved to be well suited for SPPS allowing for the streamlined assembly of the conjugates. To further improve the immune response bis-conjugates were assembled provided with a TLR7-ligand. Presently, the immunological evaluation is ongoing.

Experimental

All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker AV-400 (400/100/162 MHz) spectrometer or a Bruker AV-500 Ultrashield (500/126/202 MHz) spectrometer and all individual signals were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. Peptides and conjugates were purified with a Gilson GX-281 preparative HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column. Peptide fragments were synthesized with automated solid phase peptide synthesis on an Applied Biosystems 433A Peptide

Synthesizer. Optical rotations were measured on an Anton Paar Modular Circular Polarimeter MCP 100/150. High resolution mass spectra were recorded on a Synapt G2-Si or a Q Exactive HF Orbitrap equipped with an electron spray ion source positive mode. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR.

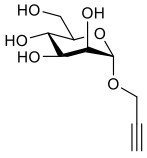
Automated solid phase synthesis general experimental information

The automated solid-phase peptide synthesis was performed on a 250 µmol scale on a Protein Technologies Tribute-UV IR Peptide Synthesizer applying Fmoc based protocol starting from Tentagel S RAM resin (loading 0.22 mmol/g). The synthesis was continued with Fmoc-amino acids specific for each peptide. The consecutive steps performed in each cycle for HCTU chemistry on 250 µmol scale: 1) Deprotection of the Fmoc-group with 20% piperidine in DMF for 10 min; 2) DMF wash; 3) Coupling of the appropriate amino acid using a four-fold excess. Generally, the Fmoc amino acid (1.0 mmol) was dissolved in 0.2 M HCTU in DMF (5 mL), the resulting solution was transferred to the reaction vessel followed by 0.5 mL of 0.5 M DIPEA in DMF to initiate the coupling. The reaction vessel was then shaken for 30 min at 50°C; 4) DMF wash; 5) capping with 10% Ac_2O in 0.1 M DIPEA in DMF; 6) DMF wash; 7) DCM wash. Aliquots of resin of the obtained sequences were checked on an analytical Agilent Technologies 1260 Infinity system with a Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column. The Fmoc amino acids applied in the synthesis were: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(N_3)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(MMT)-OH, Fmoc-Phe-OH, Fmoc-Ser(O*t*Bu)-OH Fmoc-Val-OH.

General procedure for cleavage from the resin, deprotection and purification

30 µmol resin was washed with DMF, DCM and dried after the last synthesis step followed by a treatment for 180 minutes with 0.6 mL cleavage cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The suspension was filtered, the resin was washed with 0.6 mL of the cleavage cocktail, and the combined TFA solutions were added dropwise to cold Et_2O and stored at -20°C overnight. The obtained suspension of the product in Et_2O was centrifuged, Et_2O was removed and the precipitant was dissolved in $CH_3CN/H_2O/$*t*BuOH (1/1/1 v/v/v) or DMSO/$CH_3CN/H_2O/$*t*BuOH (3/1/1/1 v/v/v/v). Purification was performed on a Gilson GX-281 preparative RP-HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column.

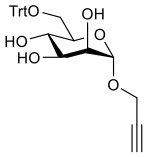
Propargyl α-D-mannopyranoside (11)



To a solution of D-mannose (28.1 g, 156 mmol, 1.0 eq.) in pyridine (0.22 L) was added acetic anhydride (0.10 L, 1.1 mol, 6.8 eq.) and DMAP (0.37 g, 3.0 mmol, 0.02 eq.) at 0°C. After 30 minutes the solution was allowed to warm-up to room temperature and stirred for an additional 5.5 hours. The reaction mixture was cooled to 0°C and quenched by slowly adding MeOH. The mixture was diluted with EtOAc, washed with 1 M HCl (3x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Co-evaporation with toluene (2x) under an argon atmosphere yielded acetyl 2,3,4,6-tetra-*O*-acetyl-α/β-D-mannoside as an oil, which was dissolved in DCM (0.45 L). Propargyl alcohol (14.0 mL, 240 mmol, 1.5

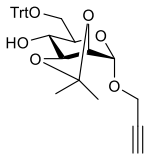
eq.) and $BF_3{\cdot}OEt_2$ (30.0 mL, 239 mmol, 1.5 eq.) were added and the solution was stirred overnight at 50°C. TLC analysis showed complete conversion and the reaction mixture was cooled to 0°C, followed by quenching with Et_3N to pH 8. The dark solution was washed with sat. aq. $NaHCO_3$ (1x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by flash column chromatography (50→100% Et_2O in pentane) yielded propargyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (55.3 g, 143 mmol), which was dissolved in MeOH (0.30 L). Sodium methoxide (5.4 M in MeOH, 11 mL, 60 mmol, 0.40 eq.) was added and the solution was stirred for 30 minutes, after which it was acidified by the addition of amberlite H^+ resin. The mixture was filtered and concentrated *in vacuo*. Crystallization by EtOH/pentane afforded the title compound (22.9 g, 105 mmol, 67% over three steps) as a white solid. R_f: 0.39 (1/4 MeOH/DCM); $[\alpha]_D^{25}$ +145° (*c* = 0.53, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 4.96 (d, 1H, *J* = 1.6 Hz, H-1), 4.27 (d, 2H, *J* = 2.4 Hz, CH_2 propargyl), 3.84 (dd, 1H, *J* = 11.8, 2.3 Hz, C*H*H-6), 3.80 (dd, 1H, *J* = 3.2, 1.7 Hz, H-2), 3.74 – 3.65 (m, 2H, H-3, C*H*H-6), 3.62 (t, 1H, *J* = 9.4 Hz, H-4), 3.51 (ddd, 1H, *J* = 8.7, 5.8, 2.2 Hz, H-5), 2.86 (t, 1H, *J* = 2.4 Hz, CH propargyl); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 99.7 (C-1), 80.0 (C_q propargyl), 76.0 (CH propargyl), 75.0 (C-5), 72.4 (C-3), 72.0 (C-2), 68.4 (C-4), 62.8 (C-6), 54.8 (CH_2 propargyl); FT-IR (neat, cm^{-1}): 3370, 2931, 2584, 1982, 1639, 1365, 1263, 1132, 1058, 1007, 965, 912, 880, 812, 685, 515; HRMS: $[M+Na]^+$ calcd. for $C_9H_{14}O_6Na$: 241.0688, found 241.0684.

Propargyl 6-*O*-trityl-α-D-mannopyranoside (12)



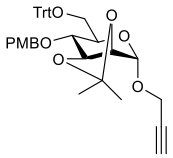
Trityl chloride (57.2 g, 205 mmol, 1.5 eq.) and Et_3N (46 mL, 0.33 mol, 2.5 eq.) were added to a solution of compound **11** (29.0 g, 133 mmol, 1.0 eq.) in DMF (0.44 L). The mixture was heated to 60°C for 4 hours, followed by addition of trityl chloride (38.1 g, 137 mmol, 1.0 eq.) and Et_3N (28 mL, 0.20 mol, 1.5 eq.). After stirring for one hour, TLC analysis showed complete conversion of the starting material and the reaction mixture was cooled to room temperature. The mixture was diluted with EtOAc, washed with H_2O (3x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (30→100% EtOAc in pentane), the title compound (50.4 g, 109 mmol, 82%) was obtained as a white foam. R_f: 0.65 (1/4 pentane/EtOAc); $[\alpha]_D^{25}$ +45.3° (*c* = 0.91, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.53 – 7.45 (m, 6H, Ar), 7.38 – 7.30 (m, 6H, Ar), 7.30 – 7.22 (m, 3H, Ar), 4.96 (d, 1H, *J* = 1.2 Hz, H-1), 4.44 – 4.30 (m, 2H, CH_2 propargyl), 3.81 – 3.75 (m, 1H, H-2), 3.67 (ddd, 1H, *J* = 9.1, 7.1, 1.7 Hz, H-5), 3.55 (ddd, 1H, *J* = 9.5, 6.3, 3.5 Hz, H-3), 3.52 – 3.42 (m, 1H, H-4), 3.36 – 3.28 (m, 2H, C*H*H-6, OH), 3.26 (d, 1H, *J* = 6.4 Hz, OH), 3.18 (dd, 1H, *J* = 9.9, 7.0 Hz, C*H*H-6), 3.11 (d, 1H, *J* = 5.0 Hz, OH), 2.78 (t, 1H, *J* = 2.4 Hz, CH propargyl); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 145.2 (C_q Trt), 129.6, 128.8, 128.0 (Ar), 99.1 (C-1), 87.2 (C_q Trt), 80.0 (C_q propargyl), 76.1 (CH propargyl), 73.3 (C-5), 72.5 (C-3), 71.2 (C-2), 68.6 (C-4), 64.7 (CH_2-6), 54.4 (CH_2 propargyl); FT-IR (neat, cm^{-1}): 3412, 3290, 3059, 3033, 2928, 2119, 1597, 1490, 1449, 1377, 1320, 1221, 1184, 1134, 1074, 1049, 1005, 986, 900, 844, 810, 765, 748, 702, 650, 633, 582, 531; HRMS: $[M+Na]^+$ calcd. for $C_{28}H_{28}O_6Na$: 483.1784, found 483.1780.

Propargyl 2,3-di-*O*-isopropylidene-6-*O*-trityl-α-D-mannopyranoside (13)



To a solution of compound **12** (50.4 g, 109 mmol, 1.0 eq.) in 2,2-dimethoxypropane (0.55 L) was added *p*-toluenesulfonic acid (3.22 g, 16.9 mmol, 0.15 eq.) at 0°C. After stirring for 1.5 hours, TLC analysis showed complete conversion of the starting material. The mixture was quenched by the addition of Et_3N (8 mL), diluted with brine and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→50% Et_2O in pentane) and crystallization in DCM/pentane yielded compound **13** (47.7 g, 95.4 mmol, 87%) as a white solid. R_f: 0.20 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ +23.0° (*c* = 0.67, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.52 – 7.45 (m, 6H, Ar), 7.38 – 7.30 (m, 6H, Ar), 7.30 – 7.24 (m, 3H, Ar), 5.21 (s, 1H, H-1), 4.50 – 4.35 (m, 2H, CH_2 propargyl), 4.14 (d, 1H, *J* = 5.6 Hz, H-2), 3.98 – 3.92 (m, 1H, H-3), 3.69 – 3.61 (m, 1H, H-5), 3.49 – 3.42 (m, 1H, H-4), 3.34 (dd, 1H, *J* = 10.1, 1.7 Hz, C*H*H-6), 3.24 – 3.14 (m, 2H, C*H*H-6, OH), 2.79 (t, 1H, *J* = 2.4 Hz, CH propargyl), 1.45 (s, 3H, CH_3 isopropylidene), 1.32 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 145.1 (C_q Trt), 129.5, 128.8, 128.1 (Ar), 110.0 (C_q isopropylidene), 96.2 (C-1), 87.3 (C_q Trt), 79.7 (C_q propargyl), 79.6 (C-3), 76.4 (C-2, CH propargyl), 70.9 (C-5), 69.9 (C-4), 64.3 (C-6), 54.5 (CH_2 propargyl), 28.2, 26.5 (CH_3 isopropylidene); FT-IR (neat, cm^{-1}): 3279, 2935, 1490, 1448, 1374, 1225, 1168, 1136, 1103, 1075, 1047, 1029, 992, 918, 898, 851, 822, 786, 767, 743, 705, 696, 650, 634, 583, 543, 532, 471; HRMS: $[M+Na]^+$ calcd. for $C_{31}H_{32}O_6Na$: 523.2097, found 523.2095.

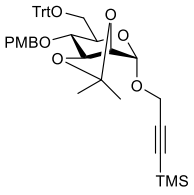
Propargyl 2,3-di-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-mannopyranoside (14)



After co-evaporation with toluene (1x) under an argon atmosphere, compound **13** (47.7 g, 95.4 mmol, 1.0 eq.) was dissolved in DMF (0.48 L) and cooled to 0°C. Sodium hydride (60% dispersion in mineral oil, 4.59 g, 115 mmol, 1.2 eq.) and *p*-methoxybenzyl chloride (15.6 mL, 115 mmol, 1.2 eq.) were added to the mixture. After 3 hours stirring at 0°C, the suspension was allowed to warm-up to room temperature and stirred for an additional 2 hours. The reaction mixture was quenched by the addition of MeOH at 0°C, diluted with H_2O and extracted with EtOAc (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (5→40% Et_2O in pentane) and crystallization in DCM/pentane the title compound (56.4 g, 90.9 mmol, 95%) was obtained as a white solid. R_f: 0.50 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ +29.8° (*c* = 0.94, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.49 – 7.43 (m, 6H, Ar), 7.36 – 7.25 (m, 9H, Ar), 6.94 – 6.89 (m, 2H, Ar), 6.79 – 6.74 (m, 2H, Ar), 5.24 (s, 1H, H-1), 4.59 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.47 – 4.34 (m, 2H, CH_2 propargyl), 4.29 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.21 – 4.15 (m, 2H, H-2, H-3), 3.76 (s, 3H, CH_3 PMB), 3.69 (ddd, 1H, *J* = 10.2, 6.2, 1.6 Hz, H-5), 3.46 (dd, 1H, *J* = 10.3, 6.4 Hz, H-4), 3.38 (dd, 1H, *J* = 10.1, 1.7 Hz, C*H*H-6), 3.09 (dd, 1H, *J* = 10.1, 6.3 Hz, C*H*H-6), 2.78 (t, 1H, *J* = 2.4 Hz, CH propargyl), 1.49 (s, 3H, CH_3 isopropylidene), 1.35 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 160.1 (C_q PMB), 145.0 (C_q Trt), 131.2 (C_q PMB), 130.5, 129.6, 128.8, 128.1, 114.4 (Ar), 110.1 (C_q isopropylidene), 96.2 (C-1), 87.2 (C_q Trt), 79.7 (C_q propargyl), 79.5 (C-3), 76.4 (C-2), 76.4

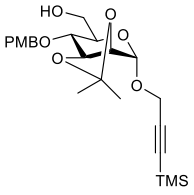
(CH propargyl), 76.3 (C-4), 73.1 (CH_2 PMB), 69.7 (C-5), 64.1 (CH_2-6), 55.8 (CH_3 PMB), 54.6 (CH_2 propargyl), 28.2, 26.5 (CH_3 isopropylidene); FT-IR (neat, cm^{-1}): 3286, 1612, 1586, 1513, 1490, 1449, 1372, 1302, 1245, 1220, 1171, 1145, 1076, 1059, 1029, 998, 915, 899, 863, 821, 777, 765, 737, 699, 644, 632, 587, 550, 518, 468; HRMS: $[M+Na]^+$ calcd. for $C_{39}H_{40}O_7Na$: 643.2672, found 643.2677.

Trimethylsilylpropargyl 2,3-di-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-mannopyranoside (15)



Compound **14** (38.1 g, 61.3 mmol, 1.0 eq.) was co-evaporated twice with toluene under an argon atmosphere and dissolved in THF (0.61 L). The solution was cooled to -78°C, followed by the addition of *n*-butyllithium (1.6 M in hexane, 46 mL, 74 mmol, 1.2 eq.). After 15 minutes, TMSCl (12 mL, 95 mmol, 1.5 eq.) was added dropwise to the pink mixture. The resulting yellow mixture was allowed to warm-up to -50°C over two hours and the reaction was quenched by the addition of sat. aq. NH_4Cl. The mixture was diluted with EtOAc and washed with sat. aq. NH_4Cl (1x) and sat. aq. $NaHCO_3$ (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (2→15% Et_2O in pentane) gave compound **15** in quantitative yield (44.1 g). R_f: 0.38 (9/1 pentane/Et_2O); $[\alpha]_D^{25}$ +33.8° (*c* = 0.45, $CHCl_3$); ^1H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.49 – 7.43 (m, 6H, Ar), 7.36 – 7.24 (m, 9H, Ar), 6.94 – 6.89 (m, 2H, Ar), 6.79 – 6.73 (m, 2H, Ar), 5.24 (s, 1H, H-1), 4.59 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.47 – 4.34 (m, 2H, CH_2 propargyl), 4.30 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.21 – 4.15 (m, 2H, H-2, H-3), 3.75 (s, 3H, CH_3 PMB), 3.71 – 3.65 (m, 1H, H-5), 3.48 (dd, 1H, *J* = 10.4, 6.2 Hz, H-4), 3.39 (dd, 1H, *J* = 10.0, 1.7 Hz, C*H*H-6), 3.10 (dd, 1H, *J* = 10.0, 6.2 Hz, C*H*H-6), 1.50 (s, 3H, CH_3 isopropylidene), 1.35 (s, 3H, CH_3 isopropylidene), 0.15 (s, 9H, 3x CH_3 TMS); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 160.1 (C_q PMB), 145.0 (C_q Trt), 131.2 (C_q PMB), 130.4, 129.6, 128.8, 128.1, 114.4 (Ar), 110.1 (C_q isopropylidene), 101.7 (C≡C), 96.3 (C-1), 92.4 (C≡C), 87.3 (C_q Trt), 79.5 (C-3), 76.5 (C-2), 76.3 (C-4), 73.1 (CH_2 PMB), 69.7 (C-5), 64.1 (CH_2-6), 55.8 (CH_3 PMB), 55.3 (CH_2 propargyl), 28.3, 26.6 (CH_3 isopropylidene), -0.1 (CH_3 TMS); FT-IR (neat, cm^{-1}): 3059, 3033, 2988, 2934, 2179, 1613, 1587, 1514, 1491, 1449, 1381, 1372, 1302, 1248, 1221, 1171, 1146, 1082, 1031, 999, 966, 946, 899, 846, 763, 747, 708, 633, 588, 551, 522, 475; HRMS: $[M+Na]^+$ calcd. for $C_{42}H_{48}O_7SiNa$: 715.3067, found 715.3068.

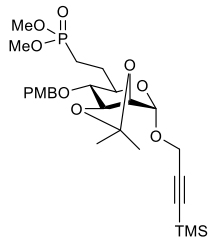
Trimethylsilylpropargyl 2,3-di-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-α-D-mannopyranoside (16)



A mixture of compound **15** (42.8 g, 61.3 mmol, 1.0 eq.), *p*-toluenesulfonic acid (4.76 g, 24.5 mmol, 0.4 eq.) in DCM/MeOH (2/1 v/v, 0.42 L) was stirred at room temperature for 1.5 hours, after which TLC analysis showed complete conversion of the starting material. The reaction mixture was quenched by the addition of sat. aq. $NaHCO_3$ (0.50 L) and extracted with EtOAc (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained residue was dissolved in a mixture of 2,2-dimethoxypropane/DCM (4/1, 0.35 L). *p*-Toluenesulfonic acid (1.17 g, 6.02 mmol, 0.1 eq.) was added and the mixture was

stirred at room temperature for 25 minutes. The reaction mixture was quenched with sat. aq. $NaHCO_3$ (0.50 L) and extracted with EtOAc (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained intermediates were dissolved in EtOAc (0.28 L) and cooled to 0°C. 1 M HCl (30 mL) was added and the mixture was allowed to warm-up to room temperature. After stirring for 1 hour, TLC analysis showed complete conversion and the reaction was quenched with sat. aq. $NaHCO_3$ (0.50 L) at 0°C, followed by extraction with EtOAc (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (20→60% Et_2O in pentane) yielded the title compound (26.0 g, 57.8 mmol, 94%) as an oil. R_f: 0.47 (4/1 pentane/EtOAc); $[\alpha]_D^{25}$ +79.6° (*c* = 0.57, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.31 – 7.23 (m, 2H, Ar), 6.90 – 6.81 (m, 2H, Ar), 5.23 (s, 1H, H-1), 4.82 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.56 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.36 – 4.29 (m, 1H, H-3), 4.23 (d, 2H, *J* = 2.3 Hz, CH_2 propargyl), 4.18 (d, 1H, *J* = 6.2 Hz, H-2), 3.86 – 3.81 (m, 1H, C*H*H-6), 3.80 (s, 3H, CH_3 PMB), 3.76 – 3.68 (m, 1H, C*H*H-6), 3.64 – 3.57 (m, 1H, H-5), 3.56 – 3.49 (m, 1H, H-4), 1.94 (br, 1H, OH), 1.52 (s, 3H, CH_3 isopropylidene), 1.38 (s, 3H, CH_3 isopropylidene), 0.17 (s, 9H, 3x CH_3 TMS); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 159.4, 130.3 (C_q PMB), 129.8, 113.9 (Ar), 109.6 (C_q isopropylidene), 100.1 (C≡C), 95.7 (C-1), 92.3 (C≡C), 78.8 (C-3), 75.8 (C-2), 75.7 (C-4), 72.7 (CH_2 PMB), 69.0 (C-5), 62.6 (CH_2-6), 55.4 (CH_3 PMB), 55.1 (CH_2 propargyl), 28.1, 26.5 (CH_3 isopropylidene), -0.1 (CH_3 TMS); FT-IR (neat, cm^{-1}): 3493, 2936, 2178, 1613, 1587, 1514, 1457, 1372, 1302, 1246, 1220, 1171, 1142, 1075, 1033, 994, 965, 948, 914, 842, 788, 760, 737, 701, 650, 637, 580, 515; HRMS: $[M+Na]^+$ calcd. for $C_{23}H_{34}O_7SiNa$: 473.1971, found 473.1968.

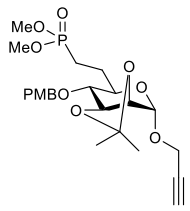
Trimethylsilylpropargyl 6-deoxy-2,3-di-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-6-dimethoxyphosphonomethyl-α-D-mannopyranoside (17)



Alcohol **16** (9.05 g, 20.1 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry DCM (0.10 L). The solution was cooled to -40°C, followed by the addition of pyridine (4.0 mL, 50.0 mmol, 2.5 eq.) and trifluoromethanesulfonic anhydride (5.1 mL, 30.3 mmol, 1.5 eq.). After stirring for 80 minutes at -40°C, the mixture was diluted with cold DCM and washed with cold sat. aq. $CuSO_4$ (1x), cold sat. aq. $NaHCO_3$ (1x) and cold brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* at 30°C. The obtained triflate was co-evaporated with toluene (2x) under an argon atmosphere and used without further purification. Dimethyl methylphosphonate (10 g) was co-evaporated with toluene (2x) under an argon atmosphere. 3.0 equivalents of dimethyl methylphosphonate (7.65 g, 60.8 mmol) were dissolved in dry THF (40 mL) and cooled to -70°C, followed by the addition of *n*-butyllithium (1.6 M in hexane, 38 mL, 60.8 mmol, 3.0 eq.). After 1.5 hours, a solution of the obtained triflate in dry THF (2x 17 mL) was added via a canula over 30 minutes. The reaction mixture was allowed to warm-up to -50°C over 2 hours. The reaction was subsequently quenched by the addition of a solution of AcOH in THF (60 mL, 2 M, 6.0 eq.) and diluted with EtOAc. The organic layer was washed with sat. aq. $NaHCO_3$ (1x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by

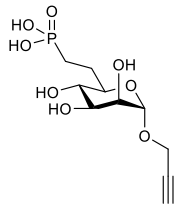
column chromatography (5→50% acetone in DCM + 0.1% Et_3N) afforded the title compound (7.31 g, 13.1 mmol, 65%) as an oil. R_f: 0.55 (4/1 DCM/acetone); $[\alpha]_D^{25}$ +70.6° (*c* = 0.49, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.04 (d, 2H, *J* = 8.6 Hz, Ar), 6.63 (d, 2H, *J* = 8.6 Hz, Ar), 4.94 (s, 1H, H-1), 4.59 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.31 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.03 (t, 1H, *J* = 6.3 Hz, H-3), 3.98 (d, 2H, *J* = 10.6 Hz, CH_2 propargyl), 3.95 – 3.90 (m, 1H, H-2), 3.54 (s, 3H, CH_3 PMB), 3.50 (s, 3H, OCH_3), 3.47 (s, 3H, OCH_3), 3.34 – 3.24 (m, 1H, H-5), 3.00 (dd, 1H, *J* = 9.9, 7.0 Hz, H-4), 1.97 – 1.83 (m, 1H, C*H*H-6), 1.73 – 1.58 (m, 1H, C*H*H-7), 1.58 – 1.38 (m, 2H, C*H*H-6, C*H*H-7), 1.28 (s, 3H, CH_3 isopropylidene), 1.14 (s, 3H, CH_3 isopropylidene), -0.04 (s, 9H, 3x CH_3 TMS); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 158.8, 129.7 (C_q Ar), 129.2, 113.2 (Ar), 108.7 (C_q isopropylidene), 99.8 (C≡C), 94.8 (C-1), 91.4 (C≡C), 78.1 (C-3), 77.6 (C-4), 75.2 (C-2), 71.7 (CH_2 PMB), 67.6, 67.4 (C-5), 54.6 (CH_3 PMB), 54.3 (CH_2 propargyl), 51.8, 51.7, 51.7, 51.7 (OCH_3), 27.6, 25.8 (CH_3 isopropylidene), 24.0 (CH_2-6), 20.5, 19.1 (CH_2-7), -0.7 (CH_3 TMS); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 35.0; FT-IR (neat, cm^{-1}): 2176, 1612, 1586, 1515, 1458, 1372, 1302, 1245, 1220, 1170, 1140, 1062, 1029, 916, 842, 808, 760, 736, 701, 636, 584, 523, 486; HRMS: $[M+Na]^+$ calcd. for $C_{26}H_{41}O_9PSiNa$: 579.2155, found 579.2158.

Propargyl 6-deoxy-2,3-di-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-6-di-methoxyphosphonomethyl-α-D-mannopyranoside (18)



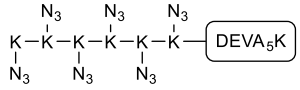
TBAF (1 M in THF, 24.5 mL, 2.0 eq.) was added to a solution of compound **17** (6.86 g, 12.1 mmol, 1.0 eq.) in THF (60 mL) at 0°C. After stirring for 15 minutes, TLC analysis showed complete conversion of the starting material. The mixture was diluted with EtOAc and washed with sat. aq. $NaHCO_3$ (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (5→15% acetone in DCM + 0.1% Et_3N) gave compound **18** in quantitative yield (5.94 g). R_f: 0.44 (4/1 DCM/acetone); $[\alpha]_D^{25}$ +67.9° (*c* = 1.3, $CHCl_3$); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.25 (m, 2H, Ar), 6.84 (d, 2H, *J* = 8.0 Hz, Ar), 5.16 (s, 1H, H-1), 4.79 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.50 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.27 – 4.21 (m, 1H, H-3), 4.18 (dd, 2H, *J* = 8.1, 2.4 Hz, CH_2 propargyl), 4.12 (d, 1H, *J* = 5.6 Hz, H-2), 3.77 (s, 3H, CH_3 PMB), 3.71 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.54 – 3.46 (m, 1H, H-5), 3.21 (dd, 1H, *J* = 9.8, 7.0 Hz, H-4), 2.44 (t, 1H, *J* = 2.4 Hz, CH propargyl), 2.17 – 2.05 (m, 1H, C*H*H-6), 1.97 – 1.79 (m, 1H, C*H*H-7), 1.79 – 1.60 (m, 2H, C*H*H-6, C*H*H-7), 1.48 (s, 3H, CH_3 isopropylidene), 1.34 (s, 3H, CH_3 isopropylidene); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.3, 130.2 (C_q Ar), 129.8, 113.8 (Ar), 109.4 (C_q isopropylidene), 95.4 (C-1), 78.6 (C-3), 78.5 (C_q propargyl), 78.4 (C-4), 75.7 (C-2), 75.3 (CH propargyl), 72.5 (CH_2 PMB), 68.3, 68.1 (C-5), 55.3 (CH_3 PMB), 54.2 (CH_2 propargyl), 52.4, 52.4 (OCH_3), 28.1, 26.3 (CH_3 isopropylidene), 24.6 (CH_2-6), 21.1, 19.7 (CH_2-7); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 35.2; FT-IR (neat, cm^{-1}): 3280, 2936, 1612, 1514, 1458, 1373, 1302, 1244, 1221, 1171, 1140, 1064, 1031, 916, 853, 810, 637, 591, 521; HRMS: $[M+Na]^+$ calcd. for $C_{23}H_{33}O_9PNa$: 507.1760, found 507.1760.

Propargyl 6-deoxy-6-phosphonomethy*l*-*α*-*D*-mannopyranoside (9)



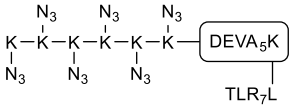
Compound **18** (1.0 g, 2.1 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry MeCN (25 mL). Pyridine (1.8 mL, 22 mmol, 11 eq.) was added and the solution was cooled to 0°C. TMSBr (5.6 mL, 42 mmol, 20 eq.) was added and a glass stopper was put on the flask. After stirring at 0°C for 2 hours, the mixture was quenched with pyridine and diluted with H_2O. The obtained mixture was concentrated *in vacuo* and co-evaporated with dioxane (2x). The residue was dissolved in EtOAc and washed with brine (2x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained intermediate (R_f = 0.14 (MeOH/DCM: 2/3 v/v) was dissolved in a mixture of AcOH/Milli-Q H_2O (1/1, v/v, 25 mL) and heated to 90°C for 8 hours, after which it was cooled to room temperature. The reaction mixture was diluted with Milli-Q H_2O and concentrated *in vacuo*. After co-evaporation with Milli-Q H_2O (5x), the residue was dissolved in Milli-Q H_2O, washed with DCM (4x) and concentrated *in vacuo*. Lyophilization yielded the title compound (0.50 g, 1.7 mmol, 81%) as a white solid. R_f: 0.40 (3/2 DCM/MeOH); ^{1}H NMR (D_2O, 500 MHz, HH-COSY, HSQC): δ 4.97 (d, 1H, *J* = 1.7 Hz, H-1), 4.36 – 4.23 (m, 2H, CH_2 propargyl), 3.92 (dd, 1H, *J* = 3.4, 1.8 Hz, H-2), 3.73 (dd, 1H, *J* = 9.3, 3.5 Hz, H-3), 3.60 – 3.47 (m, 2H, H-4, H-5), 2.90 (t, 1H, *J* = 2.4 Hz, CH propargyl), 2.13 – 2.00 (m, 1H, C*H*H-6), 1.90 – 1.75 (m, 1H, C*H*H-7), 1.75 – 1.52 (m, 2H, C*H*H-6, C*H*H-7); ^{13}C-APT NMR (D_2O, 126 MHz, HSQC): δ 98.7 (C-1), 78.9 (C_q propargyl), 76.3 (CH propargyl), 73.0, 72.9 (C-5), 70.4 (C-3), 70.0 (C-4), 70.0 (C-2), 54.6 (CH_2 propargyl), 25.2 (CH_2-6), 24.4, 23.3 (CH_2-7); ^{31}P-APT NMR (D_2O, 162 MHz, HMBC): δ 26.1; HRMS: $[M+Na]^+$ calcd. for $C_{10}H_{17}O_8PNa$: 319.0559, found 319.0566.

Ac-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (20)



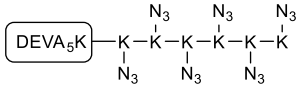
100 µmol of crude H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was elongated with Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was treated with a mixture of Ac_2O/DMF/DIPEA (2x 2.0 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (4.0 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (4.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **20** (4.6 mg, 1.3 µmol, 1%) was obtained as a white solid. LC-MS: Rt = 6.91 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1757.0 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{150}H_{249}N_{53}O_{45}$: 1756.44071, found 1756.44051.

Ac-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-NH_2 (21)



50 µmol of crude H-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was elongated with Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was treated with a mixture of Ac_2O/DMF/DIPEA (2x 1.0 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 15 mL) over 5 minutes. The resin was washed subsequently with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL), DCM (3x) and DMF (3x). A solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (41 mg, 106 µmol, 2.1 eq.) and HCTU (42 mg, 101 µmol, 2.0 eq.) in DMF (1.0 mL) and DIPEA (35 µL, 201 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (1.0 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (47 mg, 102 µmol, 2.0 eq.) and HCTU (42 mg, 100 µmol, 2.0 eq.) and DIPEA (35 µL, 200 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (2.0 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **21** (4.0 mg, 1.1 µmol, 2%) was obtained as a white solid. LC-MS: Rt = 7.13 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1999.8 $[M+H]^{2+}$; HRMS: $[M+H]^{3+}$ calcd. for $C_{173}H_{278}N_{59}O_{51}$: 1332.69857, found 1332.69879.

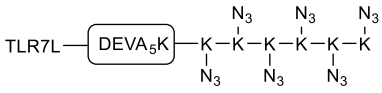
Ac-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-NH_2 (23)



100 µmol of crude H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was elongated with Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was treated with a mixture of Ac_2O/DMF/DIPEA (2x 2.0 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (4.0 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (4.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **23** (17.4 mg, 5.5 µmol, 6%) was obtained as a white solid. LC-MS: Rt = 7.11 min (C18 Gemini, 10 - 90% MeCN, 11

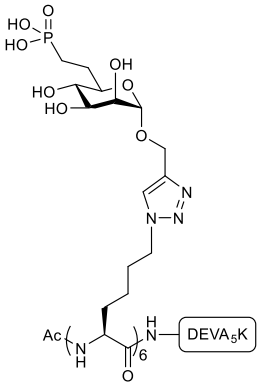
min run); ESI-MS: m/z 1757.4 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{150}H_{249}N_{53}O_{45}$: 1756.44071, found 1756.44041.

(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-NH_2 (24)



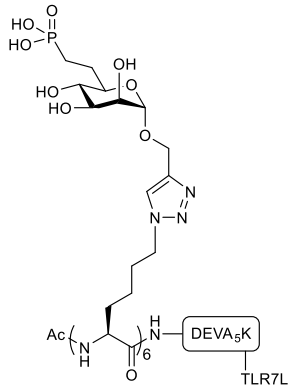
50 µmol of crude H-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was elongated with Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). The resin was washed with DMF (5x), followed by the addition of a solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (41 mg, 106 µmol, 2.1 eq.) and HCTU (42 mg, 101 µmol, 2.0 eq.) in DMF (1.0 mL) and DIPEA (35 µL, 201 µmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (1.0 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (47 mg, 102 µmol, 2.0 eq.) and HCTU (42 mg, 100 µmol, 2.0 eq.) and DIPEA (35 µL, 200 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (2.0 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.0 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **24** (8.2 mg, 2.1 µmol, 2%) was obtained as a white solid. LC-MS: Rt = 7.11 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1978.7 $[M+H]^{2+}$; HRMS: $[M+H]^{3+}$ calcd. for $C_{171}H_{276}N_{59}O_{50}$: 1318.69505, found 1318.69517.

Ac-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (1)



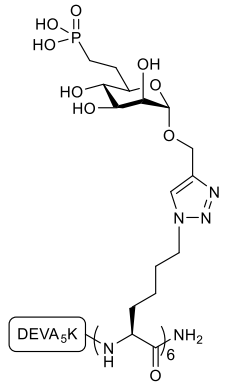
Azide **20** (4.24 mg, 1.21 µmol, 1.0 eq.) was dissolved in DMSO (641 µL), followed by the addition of a solution of compound **9** in DMSO (91.1 mM, 120 µL, 10.9 µmol, 9.0 eq.). 20 mM Tris/150 mM NaCl buffer (787 µL) and click mix (24 µL, 26 mg/mL $CuSO_4$ in H_2O, 120 mg/mL NaAsc in H_2O, 52 mg/mL TBTA in DMSO) were added. The reaction vessel was shaken for 3 hours, after which LC-MS analysis showed complete conversion. The reaction was quenched by the addition of EDTA (6.6 µL, 0.5 M in H_2O) and mixed for 15 minutes. After purification by RP-HPLC and lyophilisation, compound **1** (0.3 mg, 64 nmol, 5%) was obtained as a white solid. LC-MS: Rt = 4.56 min (C18 Gemini, 10 - 90% MeCN, 11 min run); HRMS: $[M+H]^{3+}$ calcd. for $C_{210}H_{352}N_{53}O_{93}P_6$: 1763.42844, found 1763.42972.

Ac-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-NH_2 (2)



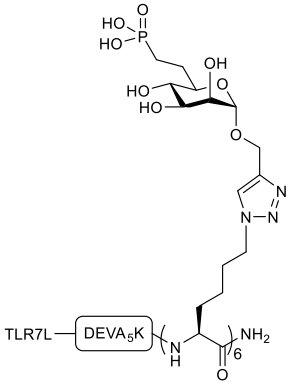
Azide **21** (3.7 mg, 0.93 µmol, 1.0 eq.) was dissolved in DMSO (491 µL), followed by the addition of a solution of compound **9** in DMSO (91.1 mM, 92 µL, 8.33 µmol, 9.0 eq.). 20 mM Tris/150 mM NaCl buffer (604 µL) and click mix (18 µL, 26 mg/mL $CuSO_4$ in H_2O, 120 mg/mL NaAsc in H_2O, 52 mg/mL TBTA in DMSO) were added. The reaction vessel was shaken for 3 hours, after which LC-MS analysis showed complete conversion. The reaction was quenched by the addition of EDTA (5.0 µL, 0.5 M in H_2O) and mixed for 15 minutes. After purification by RP-HPLC and lyophilisation, compound **2** (1.0 mg, 165 nmol, 18%) was obtained as a white solid. LC-MS: Rt = 4.89 min (C18 Gemini, 10 - 90% MeCN, 11 min run); HRMS: $[M+H]^{3+}$ calcd. for $C_{233}H_{380}N_{59}O_{99}P_6$: 1924.83078, found 1924.83260.

Ac-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-NH_2 (3)



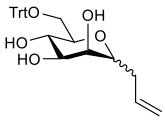
Azide **23** (3.2 mg, 0.92 µmol, 1.0 eq.) was dissolved in DMSO (480 µL), followed by the addition of a solution of compound **9** in DMSO (91.1 mM, 100 µL, 9.11 µmol, 9.0 eq.). 20 mM Tris/150 mM NaCl buffer (600 µL) and click mix (18 µL, 26 mg/mL $CuSO_4$ in H_2O, 120 mg/mL NaAsc in H_2O, 52 mg/mL TBTA in DMSO) were added. The reaction vessel was shaken for 2.5 hours, after which LC-MS analysis showed complete conversion. The reaction was quenched by the addition of EDTA (10 µL, 0.5 M in H_2O) and mixed for 15 minutes. After purification by RP-HPLC and lyophilisation, compound **3** (0.9 mg, 0.17 µmol, 18%) was obtained as a white solid. LC-MS: Rt = 4.45 min (C18 Gemini, 10 - 90% MeCN, 11 min run); HRMS: $[M+H]^{3+}$ calcd. for $C_{210}H_{352}N_{53}O_{93}P_6$: 1763.42844, found 1763.43069.

(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-Lys(M6Po)-NH_2 (4)



Azide **24** (7.4 mg, 1.86 µmol, 1.0 eq.) was dissolved in DMSO (989 µL), followed by the addition of a solution of compound **9** in DMSO (91.1 mM, 184 µL, 16.8 µmol, 9.0 eq.). 20 mM Tris/150 mM NaCl buffer (1.21 mL) and click mix (36 µL, 26 mg/mL $CuSO_4$ in H_2O, 120 mg/mL NaAsc in H_2O, 52 mg/mL TBTA in DMSO) were added. The reaction vessel was shaken for 3 hours, after which LC-MS analysis showed complete conversion. The reaction was quenched by the addition of EDTA (10 µL, 0.5 M in H_2O) and mixed for 15 minutes. After purification by RP-HPLC and lyophilisation, compound **4** (3.3 mg, 0.58 µmol, 31%) was obtained as a white solid. LC-MS: Rt = 4.71 min (C18 Gemini, 10 - 90% MeCN, 11 min run); HRMS: $[M+H]^{4+}$ calcd. for $C_{231}H_{379}N_{59}O_{98}P_6$: 1433.37226, found 1433.37178.

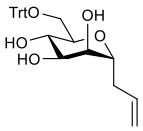
3-(6-*O*-trityl-α/β-D-mannopyranosyl)-1-propene (25 + 26)



D-Mannose (52.3 g, 302 mmol, 1.0 eq.) was dissolved in pyridine (0.43 L) and the reaction mixture was cooled to 0°C. Acetic anhydride (0.20 L, 2.1 mol, 7.0 eq.) and DMAP (3.69 g, 30.2 mmol, 0.1 eq.) were added. After stirring for 25 minutes, the solution was allowed to warm-up to room temperature and stirring was continued overnight. The mixture was subsequently cooled to 0°C and quenched with MeOH. The solution was diluted with EtOAc and washed with 1 M HCl (5x). The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was co-evaporated with toluene (2x), which gave acetyl 2,3,4,6-tetra-*O*-acetyl-α/β-D-mannopyranoside as a clear oil which solidified on bench in quantitative yield (124 g). The intermediate was co-evaporated with toluene (2x) and dissolved in MeCN (1.20 L) under an argon atmosphere. After cooling the mixture to 0°C, allyltrimethylsilane (95 mL, 0.62 mol, 2.0 eq.), $BF_3{\cdot}OEt_2$ (0.19 L, 1.5 mol, 4.9 eq.) and TMSOTf (11 mL, 62 mmol, 0.2 eq.) were added, respectively. After stirring for 30 minutes, the reaction mixture was allowed to warm-up to room temperature and stirring continued for 3 days. The reaction mixture was cooled to 0°C, diluted with EtOAc and quenched with Et_3N to pH 8. The organic layer was washed with sat. aq. $NaHCO_3$ (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→60% Et_2O in pentane) gave a mixture (86.3 g) of 3-(α/β-D-mannopyranosyl)-1-propene and unreacted acetyl 2,3,4,6-tetra-*O*-acetyl-α/β-D-mannopyranoside. After dissolving the mixture in MeOH (0.60 L), sodium methoxide (5.4 M in MeOH, 22 mL, 0.12 mol, 0.4 eq.) was added and the solution was stirred for 1.5 hours. TLC analysis showed complete conversion into a lower running spot (R_f = 0.19 (MeOH/DCM: 1/9 v/v) and the reaction was quenched using amberlite H^+ resin to pH 2-3. The reaction mixture was filtered and concentrated *in vacuo*, which gave a mixture of the fully deacetylated intermediates (47.2 g, max. 231 mmol) as an oil. After co-

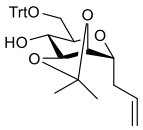
evaporating with dioxane (1x) under an argon atmosphere, the residue was dissolved in DMF (0.77 L). Trityl chloride (100 g, 348 mmol, 1.5 eq.) and Et_3N (80 mL, 0.57 mol, 2.5 eq.) were added and the suspension was heated to 60°C. After stirring for 2.5 h, TLC analysis showed complete conversion of the starting material. The reaction mixture was cooled to room temperature, diluted with H_2O and extracted with EtOAc (2x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. After purification by column chromatography (20→40% EtOAc in pentane) compounds **25** and **26** (74.5 g, 167 mmol, 55% over 4 steps) were obtained as a foam with an α/β ratio of 4.2/1. R_f: 0.46 (1/4 pentane/EtOAc); See compound **26** for analysis.

3-(6-*O*-trityl-α-D-mannopyranosyl)-1-propene (26)



A solution of compound **25** and **26** (31.4 g, 70.3 mmol, 1.0 eq., α/β: 4.2/1) and *N*-bromosuccinimide (6.3, 35 mmol, 0.5 eq.) in THF (0.70 L) was stirred for 2 h, after which LC-MS analysis showed complete conversion of the β-mannose. The mixture was quenched by the addition of sat. aq. $Na_2S_2O_3$ (0.50 L). After stirring for an additional 10 minutes, the mixture was further diluted with sat. aq. $Na_2S_2O_3$ (0.25 L) and extracted with DCM (1x). The organic layer was washed with sat. aq. $NaHCO_3$ (1x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→60% acetone in DCM + 0.1% Et_3N) yielded the title compound (23.1 g, 51.7 mmol, 91%) as a white foam. R_f: 0.42 (7/3 DCM/acetone); $[\alpha]_D^{25}$ -18.2° (*c* = 0.72, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.54 – 7.44 (m, 6H, Ar), 7.36 – 7.29 (m, 6H, Ar), 7.29 – 7.23 (m, 3H, Ar), 6.04 – 5.90 (m, 1H, CH_2-C*H*=CH_2), 5.26 – 5.11 (m, 2H, CH_2-CH=C*H*$_2$), 3.88 (ddd, 1H, *J* = 9.5, 5.4, 2.5 Hz, H-1), 3.71 – 3.64 (m, 2H, H-2, H-5), 3.60 (ddd, 1H, *J* = 9.3, 6.0, 3.5 Hz, H-4), 3.45 – 3.37 (m, 1H, H-3), 3.25 – 3.12 (m, 3H, CH_2-6, OH), 3.05 (t, 2H, *J* = 4.6 Hz, 2x OH), 2.60 – 2.51 (m, 1H, C*H*H-CH=CH_2), 2.35 – 2.27 (m, 1H, C*H*H-CH=CH_2); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 145.3 (C_q Trt), 136.3 (CH_2-*C*H=CH_2), 129.6, 128.8, 128.0 (Ar), 117.3 (CH_2-CH=*C*H$_2$), 87.2 (C_q Trt), 77.4 (C-1), 74.4 (C-5), 72.5 (C-4), 71.6 (C-2), 69.7 (C-3), 65.1 (CH_2-6), 34.4 (*C*H$_2$-CH=CH_2); FT-IR (neat, cm^{-1}): 3402, 3060, 2928, 1708, 1643, 1597, 1490, 1449, 1221, 1073, 1033, 989, 901, 827, 765, 748, 701, 633, 529; LC-MS: Rt = 7.15 min (Gemini C_{18}, 10 – 90% MeCN, 11 min run); HRMS: $[M+Na]^+$ calcd. for $C_{28}H_{30}O_5Na$: 469.1991, found 496.1991.

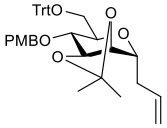
3-(2,3-*O*-isopropylidene-6-*O*-trityl-α-D-mannopyranosyl)-1-propene (27)



Compound **26** (43.9 g, 98.3 mmol, 1.0 eq.) was dissolved in 2,2-dimethoxypropane (0.50 L) and cooled to 0°C. *p*-Toluenesulfonic acid (2.88 g, 15.1 mmol, 0.15 eq.) was added and the reaction mixture was stirred for 10 minutes, after which TLC analysis showed complete conversion of the starting material. The reaction was quenched by the addition of Et_3N (7 mL), diluted with DCM and washed with a mixture of sat. aq. $NAHCO_3$/brine (1/1, v/v, 1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→50% Et_2O in pentane + 0.1% Et_3N) gave compound **27** (44.6 g, 89.1 mmol, 91%) as a clear oil. R_f: 0.24 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ -15.7° (*c* = 0.19, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.44 (m, 6H, Ar), 7.36 – 7.30 (m, 6H, Ar), 7.29 – 7.23 (m, 3H, Ar), 6.02 – 5.91 (m, 1H, CH_2-C*H*=CH_2),

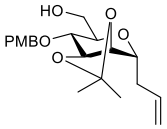
5.22 – 5.07 (m, 2H, CH_2-CH=CH_2), 4.07 – 3.98 (m, 2H, H-2, H-3), 3.92 – 3.86 (m, 1H, H-1), 3.73 – 3.66 (m, 1H, H-4), 3.59 – 3.53 (m, 1H, H-5), 3.25 – 3.20 (m, 2H, C*H*H-6, OH), 3.16 (dd, 1H, *J* = 10.0, 5.7 Hz, C*H*H-6), 2.40 (t, 2H, *J* = 6.9 Hz, CH_2-CH=CH_2), 1.39 (s, 3H, CH_3 isopropylidene), 1.30 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 145.2 (C_q Trt), 135.9 (CH_2-*C*H=CH_2), 129.6, 128.8, 128.1 (Ar), 117.5 (CH_2-CH=*C*H_2), 109.8 (C_q isopropylidene), 87.2 (C_q Trt), 79.3 (C-3), 77.1 (C-2), 74.4 (C-5), 73.6 (C-1), 69.6 (C-4), 64.5 (CH_2-6), 37.7 (*C*H_2-CH=CH_2), 28.1, 25.9 (CH_3 isopropylidene); FT-IR (neat, cm^{-1}): 2936, 1612, 1514, 1458, 1373, 1302, 1244, 1221, 1171, 1140, 1064, 1031, 916, 853, 810, 637, 591, 521; HRMS: $[M+Na]^+$ calcd. for $C_{31}H_{34}O_5Na$: 509.2304, found 509.2305.

3-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-mannopyranosyl)-1-propene (28)



After co-evaporating with toluene (2x) under an argon atmosphere, compound **27** (49.9 g, 102.5 mmol, 1.0 eq.) was dissolved in DMF (0.50 L) and cooled to 0°C. Sodium hydride (60% dispersion in mineral oil, 4.95 g, 123 mmol, 1.2 eq.) and *p*-methoxybenzyl chloride (17.0 mL, 125 mmol, 1.2 eq.) were added and the suspension was allowed to warm-up up to room temperature after 20 minutes. After stirring at room temperature for an additional hour, TLC analysis showed complete conversion of the starting material. The mixture was quenched by the addition of MeOH at 0°C, diluted with Et_2O and washed with H_2O (2x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (5→20% Et_2O in pentane + 0.1% Et_3N) yielded the title compound (60.3 g, 99.4 mmol, 97%) as a clear oil. R_f: 0.63 (pentane/Et_2O); $[\alpha]_D^{25}$ +12.7° (*c* = 0.67, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.49 – 7.44 (m, 6H, Ar), 7.35 – 7.24 (m, 9H, Ar), 6.96 – 6.92 (m, 2H, Ar), 6.80 – 6.75 (m, 2H, Ar), 6.06 – 5.93 (m, 1H, CH_2-C*H*=CH_2), 5.24 – 5.10 (m, 2H, CH_2-CH=CH_2), 4.62 (d, 1H, *J* = 11.0 Hz, C*H*H PMB), 4.31 – 4.21 (m, 2H, H-3, C*H*H PMB), 4.08 (dd, 1H, *J* = 6.4, 5.4 Hz, H-2), 3.99 – 3.92 (m, 1H, H-1), 3.75 (s, 3H, CH_3 PMB), 3.70 – 3.60 (m, 2H, H-4, H-5), 3.31 (dd, 1H, *J* = 9.9, 2.1 Hz, C*H*H-6), 3.08 (dd, 1H, *J* = 9.8, 5.0 Hz, C*H*H-6), 2.42 (t, 2H, *J* = 6.9 Hz, CH_2-CH=CH_2), 1.46 (s, 3H, CH_3 isopropylidene), 1.34 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 160.1 (C_q PMB), 145.1 (C_q Trt), 135.9 (CH_2-*C*H=CH_2), 131.3 (C_q PMB), 130.5, 129.6, 128.8, 128.1 (Ar), 117.6 (CH_2-CH=*C*H_2), 114.4 (Ar), 109.9 (C_q isopropylidene), 87.2 (C_q Trt), 79.2 (C-3), 77.2 (C-2), 76.5 (C-4), 73.7 (C-1), 73.2 (CH_2 PMB), 73.0 (C-5), 64.4 (CH_2-6), 55.8 (CH_3 PMB), 37.4 (*C*H_2-CH=CH_2), 28.1, 26.0 (CH_3 isopropylidene); FT-IR (neat, cm^{-1}): 2987, 2934, 1613, 1514, 1491, 1449, 1381, 1302, 1247, 1212, 1172, 1069, 1034, 1002, 915, 868, 822, 765, 747, 704, 633, 518; HRMS: $[M+Na]^+$ calcd. for $C_{39}H_{42}O_6Na$: 629.2879, found 629.2881.

3-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-α-D-mannopyranosyl)-1-propene (29)



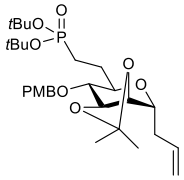
A solution of compound **28** (60.3 g, 99.4 mmol, 1.0 eq.) and *p*-toluenesulfonic acid (7.70 g, 39.7 mmol, 0.4 eq.) in DCM/MeOH (2/1 v/v, 0.66 L) was stirred for one hour, after which TLC analysis showed complete conversion of the starting material. The reaction mixture was quenched by the addition of sat. aq. $NaHCO_3$ (0.50 L) and extracted with EtOAc (3x).

The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained residue was dissolved in a mixture of 2,2-dimethoxypropane/DCM (4/1, 0.50 L). *p*-Toluenesulfonic acid (1.93 g, 9.94 mmol, 0.1 eq.) was added and the mixture was stirred for 5 minutes, after which it was quenched with sat. aq. $NaHCO_3$ (0.50 L) and extracted with EtOAc (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The obtained intermediates were dissolved in EtOAc (0.45 L) and cooled to 0°C. 1 M HCl (45 mL) was added and after 30 minutes the mixture was allowed to warm-up to room temperature. After stirring for an additional 30 minutes, TLC analysis showed complete conversion and the reaction was quenched with sat. aq. $NaHCO_3$ (0.50 L) at 0°C. The organic layer was separated and the aqueous layer was extracted with EtOAc (1x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (20→70% Et_2O in pentane) the title compound (35.2 g, 96.6 mmol, 97%) was obtained as a clear oil. R_f: 0.53 (2/3 pentane/Et_2O); $[\alpha]_D^{25}$ +29.8° (*c* = 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.30 – 7.24 (m, 2H, Ar), 6.90 – 6.84 (m, 2H, Ar), 5.88 – 5.76 (m, 1H, CH_2-C*H*=CH_2), 5.17 – 5.09 (m, 2H, CH_2-CH=C*H*$_2$), 4.81 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.55 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.30 (t, 1H, *J* = 6.7 Hz, H-3), 4.07 (dd, 1H, *J* = 6.4, 4.5 Hz, H-2), 3.96 – 3.90 (m, 1H, H-1), 3.80 (s, 3H, CH_3 PMB), 3.73 (dd, 1H, *J* = 11.5, 3.0 Hz, C*H*H-6), 3.67 – 3.54 (m, 2H, H-4, C*H*H-6), 3.53 – 3.45 (m, 1H, H-5), 2.43 – 2.31 (m, 2H, C*H*$_2$-CH=CH_2), 1.97 (br, 1H, OH), 1.52 (s, 3H, CH_3 isopropylidene), 1.38 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 159.5 (C_q PMB), 133.9 (CH_2-*C*H=CH_2), 130.2 (C_q PMB), 129.9 (Ar), 118.0 (CH_2-CH=*C*H$_2$), 113.9 (Ar), 109.4 (C_q isopropylidene), 78.7 (C-3), 76.3 (C-2), 75.9 (C-4), 72.7 (C-1), 72.7 (CH_2 PMB), 71.9 (C-5), 62.9 (CH_2-6), 55.4 (CH_3 PMB), 36.4 (*C*H$_2$-CH=CH_2), 27.9, 25.9 (CH_3 isopropylidene); FT-IR (neat, cm^{-1}): 3480, 2985, 2935, 1642, 1612, 1587, 1514, 1457, 1381, 1302, 1245, 1218, 1168, 1139, 1062, 1034, 992, 918, 863, 821, 638, 582, 515; HRMS: $[M+Na]^+$ calcd. for $C_{20}H_{28}O_6Na$: 387.1784, found 387.1786.

Di-*tert*-butyl methylphosphonate (30)

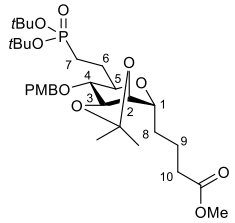
To a solution of di-*tert*-butyl phosphite (26 g, 0.13 mol, 1.0 eq.) in THF (0.25 L) was added slowly a solution of *n*-butyllithium (1.6 M in hexane, 99 ml, 0.16 mol, 1.2 eq.) at -78°C under an argon atmosphere. After stirring for 1 h, a solution of iodomethane in THF (2 M, 85 ml, 0.17 mol, 1.3 eq.) was added and the reaction was allowed to warm up to room temperature overnight. Concentration *in vacuo* and purification by column chromatography (0→50% EtOAc in pentane) yielded the title compound as a slightly yellow liquid (21.7 g, 104 mmol, 80%). ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 1.50 (s, 18H, 6x CH_3 *t*Bu), 1.42 (d, 3H, *J* = 17.4 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 101 MHz, HSQC): δ 81.4, 81.3 (C_q *t*Bu), 30.4 (CH_3 *t*Bu), 17.1, 15.7 (CH_3); ^{31}P NMR (CDCl3, 162 MHz): δ 21.9; FT-IR (neat, cm^{-1}): 2980, 1370, 1310, 1257, 1173, 1040, 983, 771; HRMS: $[M+Na]^+$ calcd. for $C_9H_{21}O_3PNa$: 231.11205, found 231.11206.

3-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-1-propene (31)



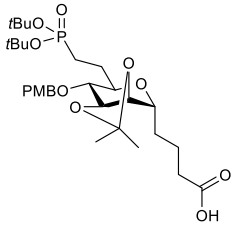
Alcohol **29** (1.11 g, 3.04 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry DCM (15 mL). The solution was cooled to -40°C, followed by the addition of pyridine (0.6 mL, 7.4 mmol, 2.4 eq.) and trifluoromethanesulfonic anhydride (0.8 mL, 4.8 mmol, 1.6 eq.). After stirring for one hour at -40°C, TLC analysis showed complete conversion of the starting material. The reaction mixture was diluted with cold DCM and washed with cold sat. aq. $CuSO_4$ (1x), cold sat. aq. $NaHCO_3$ (1x) and cold brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* at 30°C. The obtained triflate was co-evaporated with toluene (2x) under an argon atmosphere and used without further purification. Di-*tert*-butyl methylphosphonate **30** (4 g) was co-evaporated with toluene (2x) under an argon atmosphere. Di-*tert*-butyl methylphosphonate **30** (1.85 g, 8.88 mmol, 2.9 eq.) was dissolved in dry THF (6.0 mL) and cooled to -78°C, followed by the addition of *n*-butyllithium (1.6 M in hexane, 5.7 mL, 9.1 mmol, 3.0 eq.). After two hours, a solution of the crude triflate in dry THF (2x 2.5 mL) was added via a canula over 10 minutes. The reaction mixture was allowed to warm-up to -50°C over 2.5 hours. The reaction was subsequently quenched by the addition of a solution of AcOH in THF (36 1 M, 14 mL) and diluted with EtOAc. The organic layer was washed with sat. aq. NaHCO (1x), dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (5→25% acetone in DCM + 0.1% Et_3N) and size exclusions (DCM/MeOH: 1/1) gave the title compound (1.22 g, 2.20 mmol, 72%). R_f: 0.52 (9/1 DCM/acetone); $[\alpha]_D^{25}$ +0.87° (*c* = 0.11, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.27 (d, 2H, *J* = 4.2 Hz, Ar), 6.88 – 6.82 (m, 2H, Ar), 5.88 – 5.73 (m, 1H, CH_2-C*H*=CH_2), 5.14 – 5.02 (m, 2H, CH_2-CH=C*H*$_2$), 4.80 (d, 1H, *J* = 11.0 Hz, C*H*H PMB), 4.55 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.23 (t, 1H, *J* = 6.4 Hz, H-3), 4.03 (dd, 1H, *J* = 6.2, 4.9 Hz, H-2), 3.85 – 3.76 (m, 4H, H-1, CH_3 PMB), 3.45 – 3.31 (m, 2H, H-4, H-5), 2.43 – 2.24 (m, 2H, C*H*$_2$-CH=CH_2), 2.07 – 1.79 (m, 2H, CH_2-6/7), 1.76 – 1.54 (m, 2H, CH_2-6/7), 1.47 (s, 21H, CH_3 isopropylidene, 6x CH_3 *t*Bu), 1.37 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 159.4 (C_q PMB), 134.2 (CH_2-*C*H=CH_2), 130.3 (C_q PMB), 129.9 (Ar), 117.7 (CH_2-CH=*C*H$_2$), 113.9 (Ar), 109.3 (C_q isopropylidene), 81.4, 81.3 (C_q *t*Bu), 79.1 (C-4), 78.5 (C-3), 76.4 (C-2), 72.7 (CH_2 PMB), 72.3, 72.2 (C-5), 71.9 (C-1), 55.4 (CH_3 PMB), 36.7 (*C*H$_2$-CH=CH_2), 30.6, 30.5 (CH_3 *t*Bu), 28.0 (CH_3 isopropylidene), 27.4, 26.1, 26.1 (CH_2-6/7), 26.0 (CH_3 isopropylidene), 25.9 (CH_2-6/7); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 24.1; FT-IR (neat, cm^{-1}): 2979, 2934, 1643, 1613, 1586, 1514, 1458, 1393, 1369, 1302, 1244, 1219, 1171, 1077, 1036, 972, 916, 867, 822, 737, 696, 639, 519, 486; HRMS: $[M+H]^+$ calcd. for $C_{29}H_{48}O_8P$: 555.30813, found 555.30800.

Methyl 4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-butanoate (32)



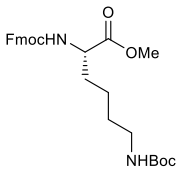
Compound **31** (8.26 g, 14.9 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere, before being dissolved in dry DCE (0.15 L). Methyl acrylate (3.8 mL, 42 mmol, 2.8 eq.), CuI (0.43 g, 2.3 mmol, 0.15 eq.) and Grubbs 2nd generation catalyst (0.76 g, 0.89 mmol, 0.06 eq.) were added and the flask was covered in aluminum foil. The suspension was heated to 60°C and stirred for 48 hours, after which it was concentrated *in vacuo* and co-evaporated with toluene (5x) under an argon atmosphere. The residue was dissolved in dry DCE (75 mL) and cooled to 0°C. Two empty balloons were placed on the flask, followed by the addition of ruthenium trichloride (0.59 g, 2.8 mmol, 0.19 eq.) and $NaBH_4$ (1.80 g, 47.6 mmol, 3.2 eq.). Methanol (12.0 mL, 0.3 mol, 20 eq.) was carefully added to the suspension over 30 minutes, after which the mixture was allowed to warm-up up to room temperature over 15 minutes. The mixture was subsequently heated to 45°C for 5.5 hours. The reaction mixture was cooled to room temperature, diluted with brine and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→30% acetone in pentane + 0.1% Et_3N) afforded compound **32** (6.59 g, 10.7 mmol, 72% over two steps). R_f: 0.73 (7/3 DCM/acetone); $[\alpha]_D^{25}$ +15.5° (*c* = 0.53, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.23 (d, 2H, *J* = 8.7 Hz, Ar), 6.83 – 6.77 (m, 2H, Ar), 4.75 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.50 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.17 (t, 1H, *J* = 6.4 Hz, H-3), 3.93 (dd, 1H, *J* = 6.2, 4.9 Hz, H-2), 3.73 (s, 3H, CH_3 PMB), 3.71 – 3.65 (m, 1H, H-1), 3.60 (s, 3H, OCH_3), 3.36 – 3.27 (m, 2H, H-4, H-5), 2.32 – 2.23 (m, 2H, CH_2-10), 2.02 – 1.89 (m, 1H, C*H*H-6/7/8/9), 1.89 – 1.69 (m, 2H, CH_2-6/7/8/9), 1.69 – 1.49 (m, 5H, CH_2-6/7/8/9), 1.47 (s, 3H, CH_3 isopropylidene), 1.45 – 1.40 (m, 18H, 6x CH_3 *t*Bu), 1.31 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.6 (C=O), 159.2, 130.2 (C_q PMB), 129.7, 113.7 (Ar), 109.1 (C_q isopropylidene), 81.4, 81.3, 81.3, 81.3 (C_q *t*Bu), 78.8 (C-4), 78.3 (C-3), 76.9 (C-2), 72.5 (CH_2 PMB), 71.9, 71.8 (C-5), 71.7 (C-1), 55.2 (CH_3 PMB), 51.5 (OCH_3), 33.5 (CH_2-10), 31.2 (CH_2-9), 30.4, 30.4, 30.3, 30.3 (CH_3 *t*Bu), 27.8 (CH_3 isopropylidene), 27.1, 26.0, 26.0 (CH_2-6 or CH_2-7), 25.8 (CH_3 isopropylidene), 25.6 (CH_2-6 or CH_2-7), 21.0 (CH_2-8); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 24.5; FT-IR (neat, cm^{-1}): 2979, 2935, 1737, 1612, 1514, 1457, 1393, 1369, 1302, 1244, 1219, 1170, 1082, 1037, 1006, 974, 918, 866, 823, 791, 739, 695, 520, 487; HRMS: $[M+Na]^+$ calcd. for $C_{31}H_{51}O_{10}PNa$: 637.3118, found 637.3124.

4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-butanoic acid (33)



Methyl ester **32** (6.59 g, 10.7 mmol, 1.0 eq.) was dissolved in in a mixture of THF/H_2O (4/1, v/v, 0.10 L). LiOH (1.35 g, 32 mmol, 3.0 eq.) was added and the mixture was stirred for 7 hours, after which TLC analysis showed complete conversion of the starting material. The reaction mixture was cooled to 0°C, acidified with 3 M HCl to pH = 4-5 and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The title compound was obtained in quantitative yield (6.65 g) and used without further purification. R_f: 0.28 (7/3 DCM/acetone); $[\alpha]_D^{25}$ +20.0° (*c* = 0.18, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.27 (d, 2H, J = 8.7 Hz, Ar), 6.85 (d, 2H, J = 8.6 Hz, Ar), 4.80 (d, 1H, J = 11.1 Hz, C*H*H PMB), 4.54 (d, 1H, J = 11.1 Hz, C*H*H PMB), 4.22 (t, 1H, J = 6.6 Hz, H-3), 3.97 (t, 1H, J = 6.1 Hz, H-2), 3.79 (s, 3H, CH_3 PMB), 3.73 – 3.65 (m, 1H, H-1), 3.43 – 3.34 (m, 2H, H-4, H-5), 2.35 (t, 2H, J = 7.2 Hz, CH_2-10), 2.03 – 1.57 (m, 8H, CH_2-6/7/8/9), 1.56 – 1.42 (m, 21H, CH_3 isopropylidene, 6x CH_3 *t*Bu), 1.36 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 176.1 (C=O), 159.4, 130.4 (C_q PMB), 129.9, 113.9 (Ar), 109.4 (C_q isopropylidene), 82.4, 82.3, 82.3, 82.2 (C_q *t*Bu), 79.1 (C-4), 78.7 (C-3), 77.3 (C-2), 72.6 (CH_2 PMB), 72.4, 72.2 (C-5), 71.7 (C-1), 55.4 (CH_3 PMB), 34.1 (CH_2-10), 31.9 (CH_2-8), 30.5, 30.5, 30.5, 30.5 (CH_3 *t*Bu), 27.9 (CH_3 isopropylidene), 27.0, 26.1, 26.1 (CH_2-6/7), 25.7 (CH_3 isopropylidene), 25.5 (CH_2-6/7), 21.2 (CH_2-9); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 24.8; FT-IR (neat, cm^{-1}): 2980, 2935, 1724, 1613, 1586, 1514, 1458, 1394, 1370, 1302, 1245, 1217, 1158, 1081, 1037, 980, 918, 867, 822, 793, 735, 701, 661, 519, 486; HRMS: $[M+Na]^+$ calcd. for $C_{30}H_{49}O_{10}PNa$: 623.2961, found 623.2971.

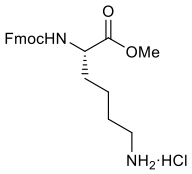
Fmoc-L-Lys(Boc)-OMe (34)



K_2CO_3 (16.8 g, 0.122 mol, 3.0 eq.) was added to a solution of Fmoc-L-Lys(Boc)-OH (18.8 g, 40 mmol, 1.0 eq.) in DMF (0.20 L). The mixture was cooled to 0°C and MeI (7.5 mL, 0.12 mol, 3.0 eq.) was slowly added. The reaction mixture was allowed to warm-up to room temperature and stirred for 2 hours. The reaction mixture was quenched with H_2O and the obtained solution was extracted with Et_2O (5x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→100% Et_2O in pentane) gave the title compound (17.9 g, 37.1 mmol, 93%). R_f: 0.60 (2/8 pentane/Et_2O); $[\alpha]_D^{20}$ -6.0° (*c* = 1.0, DCM); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.80 (d, 2H, *J* = 7.5 Hz, Ar), 7.71 – 7.64 (m, 2H, Ar), 7.42 – 7.36 (m, 2H, Ar), 7.35 – 7.27 (m, 2H, Ar), 4.41 – 4.31 (m, 2H, CH_2 Fmoc), 4.22 (t, 1H, *J* = 7.0 Hz, CH Fmoc), 4.15 (dd, 1H, *J* = 9.3, 4.8 Hz, CH-L-Lys), 3.71 (s, 3H, OCH_3), 3.03 (t, 2H, *J* = 6.8 Hz, CH_2 ε-L-Lys), 1.87 – 1.76 (m, 1H, *CH*H β-L-Lys), 1.73 – 1.62 (m, 1H, *CH*H β-L-Lys), 1.51 – 1.35 (m, 13H, CH_2 γ-L-Lys, CH_2 δ-L-Lys, 3x CH_3 Boc); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 174.7, 158.7 (C=O), 145.3, 145.2, 142.6 (C_q Ar), 128.8, 128.2, 126.2, 120.9 (Ar), 67.9 (CH_2 Fmoc), 55.4 (CH-Lys), 52.7 (OCH_3), 48.4 (CH_2 Fmoc, 41.0 (CH_2 ε-L-Lys), 32.2 (CH_2 β-L-Lys) , 30.5 (CH_2 γ-L-Lys), 28.8 (CH_3 Boc), 24.2 (CH_2 δ-L-Lys); FT-IR (neat, cm^{-1}): 2973, 1687, 1421, 1365,

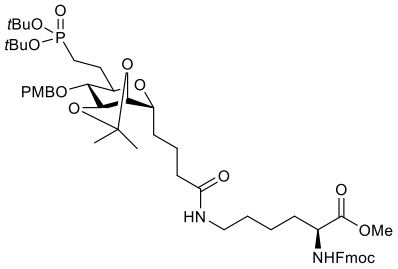
1167, 1046, 759, 739; HRMS: $[M+H]^+$ calcd. for $C_{27}H_{35}N_2O_6$: 483.24896, found 483.24895.

Fmoc-L-Lys-OMe·HCl (35)



Compound **34** (17.8 g, 37 mmol, 1.0 eq.) was suspended in dioxane (10 mL) and cooled to 0°C, followed by the addition of 4 M HCl in dioxane (90 mL). The reaction mixture was stirred for 3.5 hours and the mixture was concentrated *in vacuo*. Crystallization with dioxane/EtOAc/pentane gave the title compound (15.2 g, 36.3 mmol, 98%) as a white solid. R_f: 0.14 (9/1 DCM/MeOH); $[\alpha]_D^{20}$ +3.8° (*c* = 2.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.79 (d, 2H, *J* = 7.5 Hz, Ar), 7.71 – 7.62 (m, 2H, Ar), 7.43 – 7.35 (m, 2H, Ar), 7.35 – 7.27 (m, 2H, Ar), 4.40 (dd, 1H, *J* = 10.6, 6.9 Hz, *CH*H Fmoc), 4.33 (dd, 1H, *J* = 10.5, 6.9 Hz, *CH*H Fmoc), 4.23 – 4.15 (m, 2H, CH Fmoc, CH-L-Lys), 3.71 (s, 3H, OCH_3), 2.95 – 2.87 (m, 2H, CH_2 ε-L-Lys), 1.92 – 1.81 (m, 1H, *CH*H β-L-Lys), 1.77 – 1.61 (m, 3H, *CH*H β-Lys, CH_2 γ-L-Lys), 1.52 – 1.39 (m, 2H, CH_2 δ-L-Lys).^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 174.3, 158.7 (C=O), 145.3, 145.1, 142.6 (C_q Ar), 128.8, 128.2, 128.1, 126.2, 126.2, 120.9 (Ar), 67.9 (CH_2 Fmoc), 55.1 (CH-Lys), 52.8 (OCH_3 Lysine), 48.4 (CH_2 Fmoc), 40.5 (CH_2 ε-Lys), 32.0 (CH_2 β-Lys), 28.0 (CH_2 γ-Lys), 23.8 (CH_2 δ-Lys); FT-IR (neat, cm^{-1}): 3302, 2862, 1725, 1689, 1582, 1544, 1478, 1466, 1447, 1396, 1355, 1306, 1289, 1274, 1239, 1209, 1171, 1149, 1135, 1109, 1083, 1047, 1023, 1007, 959, 928, 894, 785, 757, 739, 657, 620, 594, 533, 499, 462; HRMS: $[M+H]^+$ calcd. for $C_{22}H_{27}N_2O_4$: 383.19653, found 383.19633.

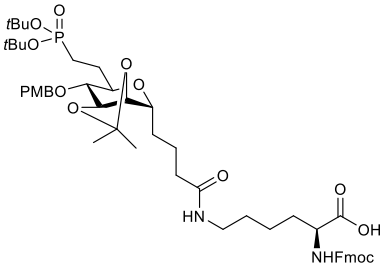
***N*$_\alpha$-Fmoc-*N*$_\varepsilon$-[butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-amide]-L-lysine-methyl ester (36)**



Compound **33** (1.78 g, 2.97 mmol, 1.0 eq.) and lysine **35** (1.39 g, 3.32 mmol, 1.12 eq.) were dissolved in DMF (15 mL). HCTU (1.47 g, 3.55 mmol, 1.2 eq.) and DIPEA (1.6 mL, 9.2 mmol, 3.0 eq.) were added and the solution was stirred for 2 hours. The reaction mixture was diluted with EtOAc and was washed with 1 M HCl (1x), sat. aq. $NaHCO_3$ (1x), brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→100% acetone in DCM) gave compound **36** (2.25 g, 2.33 mmol, 78%) as an oil. R_f: 0.31 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +14.7 (*c* = 0.44, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz, HH-COSY, HSQC): δ 7.76 (d, 2H, *J* = 7.5 Hz, Ar), 7.61 (t, 2H, *J* = 8.1 Hz, Ar), 7.40 (t, 2H, *J* = 7.4 Hz, Ar), 7.31 (t, 2H, *J* = 7.5 Hz, Ar), 7.29 – 7.23 (m, 2H, Ar), 6.85 (d, 2H, *J* = 8.5 Hz, Ar), 6.68 (br, 1H, NH), 5.60 (d, 1H, *J* = 8.1 Hz, N*H*Fmoc), 4.78 (d, 1H, *J* = 11.2 Hz, C*H*H PMB), 4.52 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.45 – 4.38 (m, 1H, C*H*H Fmoc), 4.38 – 4.30 (m, 2H, CH L-Lys, C*H*H Fmoc), 4.26 – 4.18 (m, 2H, H-3, CH Fmoc), 3.95 (t, 1H, *J* = 6.2 Hz, H-2), 3.79 (s, 3H, CH_3 PMB), 3.74 (s, 3H, OCH_3), 3.63 – 3.55 (m, 1H, H-1), 3.42 – 3.32 (m, 2H, H-4, H-5), 3.32 – 3.20 (m, 1H, C*H*H ε-L-Lys), 3.20 – 3.12 (m, 1H, C*H*H ε-L-Lys), 2.25 – 2.13 (m, 2H, CH_2-10), 1.99 – 1.52 (m, 10H, 5x CH_2-6/7/8/9, β/γ/δ-L-Lys), 1.51 – 1.43 (m, 23H, 1x CH_2-6/7/8/9, β/γ/δ-L-Lys,

CH_3 isopropylidene, 6x CH_3 *t*Bu), 1.40 – 1.32 (m, 5H, 1x CH_2-6/7/8/9, β/γ/δ-L-Lys, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 126 MHz, HSQC): δ 173.1 (C=O), 159.4 (C_q PMB), 156.2 (C=O), 144.1, 143.9, 141.4 (C_q Fmoc), 130.4 (C_q PMB), 129.9, 127.8, 127.2, 125.3, 125.2, 120.1, 113.9 (Ar), 109.4 (C_q isopropylidene), 81.8, 81.8, 81.7, 81.6 (C_q *t*Bu), 79.1 (C-4), 78.6 (C-3), 78.6 (C-2), 72.8, 72.6 (C-5), 72.6 (CH_2 PMB), 71.6 (C-1), 67.1 (CH_2 Fmoc), 55.4 (CH_3 PMB), 53.9 (CH L-Lys), 52.5 (OCH_3), 47.3 (CH Fmoc), 39.0 (CH_2 ε-L-Lys), 36.2 (CH_2-10), 32.1 (CH_2 β-L-Lys), 31.9 (CH_2 δ-L-Lys), 30.6, 30.5, 30.5 (CH_3 *t*Bu), 29.2, 27.8 (CH_3 isopropylidene), 27.2, 26.2, 26.2, 26.0 (CH_2-6/7), 25.6 (CH_3 isopropylidene), 22.6 (CH_2 γ-L-Lys), 22.2 (CH_2-9); ^{31}P-APT NMR ($CDCl_3$, 162 MHz): δ 24.3; FT-IR (neat, cm^{-1}): 3281, 2980, 2935, 1721, 1650, 1613, 1514, 1451, 1370, 1246, 1172, 1082, 1037, 981, 916, 867, 823, 760, 732, 646, 621, 538; HRMS: $[M+Na]^+$ calcd. for $C_{52}H_{73}N_2O_{13}PNa$: 987.4748, found 987.4761.

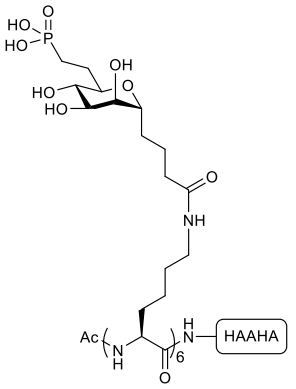
N_α-Fmoc-N_ε-[butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-amide]-L-lysine (10)



Compound **36** (2.20 g, 2.28 mmol, 1.0 eq.) was dissolved in THF (23 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 15 mL, 4.5 mmol, 2.0 eq.) was added and the mixture was stirred vigorously for 40 minutes, after which the mixture was acidified by the addition of 1 M HCl to pH = 3-4 and diluted with brine. The mixture was extracted with EtOAc (2x) and the combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (2→10% MeOH in DCM + 0.1% AcOH), the title compound (1.68 g, 1.77 mmol, 78%) was obtained as a white foam. R_f: 0.36 (9/1 DCM/MeOH + 0.1% AcOH); $[\alpha]_D^{25}$ +31.9° (*c* = 0.32, DCM); ^{1}H NMR (MeOD, 500 MHz, HH-COSY, HSQC): δ 7.78 (d, 2H, *J* = 7.6 Hz, Ar), 7.67 (t, 2H, *J* = 8.4 Hz, Ar), 7.38 (td, 2H, *J* = 7.4, 1.1 Hz, Ar), 7.30 (td, 2H, *J* = 7.5, 1.2 Hz, Ar), 7.28 – 7.23 (m, 2H, Ar), 6.89 – 6.84 (m, 2H, Ar), 4.75 (d, 1H, *J* = 11.3 Hz, CH_2 PMB), 4.53 (d, 1H, *J* = 11.3 Hz, CH_2 PMB), 4.35 (dd, 2H, *J* = 7.0, 2.4 Hz, CH_2 Fmoc), 4.23 – 4.18 (m, 2H, H-3, CH Fmoc), 4.13 (dd, 1H, *J* = 9.2, 4.6 Hz, CH L-Lys), 4.00 – 3.96 (m, 1H, H-2), 3.77 (s, 3H, CH_3 PMB), 3.71 – 3.65 (m, 1H, H-1), 3.40 – 3.33 (m, 2H, H-4, H-5), 3.19 – 3.13 (m, 2H, CH_2 ε-L-Lys), 2.22 – 2.15 (m, 2H, CH_2-10), 1.99 – 1.38 (m, 35H, 7x CH_2-6/7/8/9, β/γ/δ-L-Lys, CH_3 isopropylidene, 6x CH_3 *t*Bu), 1.33 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR (MeOD, 126 MHz, HSQC): δ 175.6, 160.8 (C=O), 158.6 (C_q PMB), 145.4, 145.2, 142.6 (C_q Fmoc), 131.6 (C_q PMB), 130.9, 128.8, 128.2, 128.2, 126.3, 126.2, 121.0, 114.7 (Ar), 110.3 (C_q isopropylidene), 83.5, 83.5, 83.5, 83.5 (C_q *t*Bu), 79.7 (C-4), 79.7 (C-3), 78.2 (C-2), 73.2 (CH_2 PMB), 73.2 (C-1), 72.8, 72.7 (C-5), 67.9 (CH_2 Fmoc), 55.7 (CH_3 PMB), 55.5 (CH Fmoc), 48.4 (CH L-Lys), 40.1 (ε-L-Lys), 36.6 (CH_2-10), 32.5 (CH_2 β-L-Lys), 32.3 (CH_2 δ-L-Lys), 30.8, 30.8, 30.7, 30.7, 30.0 (CH_3 *t*Bu), 28.2 (CH_3 isopropylidene), 27.6, 27.1, 27.1, 26.4 (CH_2-6/7), 25.9 (CH_3 isopropylidene), 24.3 (CH_2 γ-L-Lys), 23.2 (CH_2-9); ^{31}P-APT NMR (MeOD, 202 MHz, HMBC): δ 25.7; FT-IR (neat, cm^{-1}): 3301, 2980, 2935, 1716, 1650, 1613, 1514, 1451, 1394, 1370, 1246, 1161, 1081, 1038, 985, 912, 866, 823, 760, 730, 647, 621, 540;

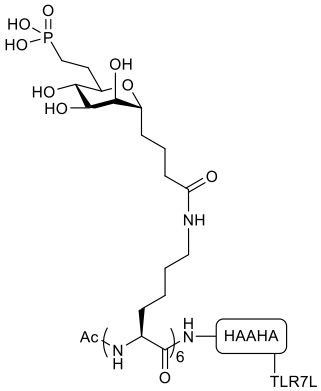
LC-MS: Rt = 8.72 min (C18 Gemini, 10 - 50% MeCN, 11 min run); HRMS: $[M+Na]^+$ calcd. for $C_{51}H_{71}N_2O_{13}PNa$: 973.4591, found 973.4603.

Ac-[Lys(butan-4-(6-deoxy-*6*-phosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-NH$_2$ (5)



100 µmol of crude H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-Tentagel S Ram was washed with DMF (5x) and elongated by the addition of a solution of acid **10** (0.19 g, 0.20 mmol, 2.0 eq.) and HCTU (84 mg, 0.20 µmol, 2.0 eq.) in DMF (2.0 mL) and DIPEA (70 µL, 0.40 mmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (2.0 mL, 2x 20 min) and washed with DMF (5x). This was repeated another 5 times, after which the resin was treated with a mixture of Ac_2O/DMF/DIPEA (2x 2.0 mL, 20 min), and washed with DMF (3x) and DCM (3x). 30 µmol of crude Ac-[Lys(butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-*α*-D-mannopyranosyl)-amide)]$_6$-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-Tentagel S Ram was taken and the peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **5** (13.3 mg, 2.9 µmol, 10%) was obtained as a white solid. LC-MS: Rt = 6.19 min (C18 Gemini, 0 - 50% MeCN, 11 min run); ESI-MS: m/z 1525.6 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{184}H_{325}N_{41}O_{80}P_6$: 1143.77568, found 1143.77633.

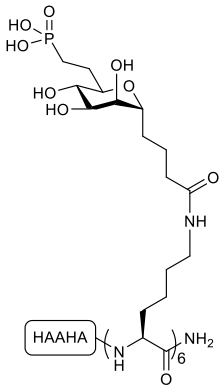
Ac-[Lys(butan-4-(6-deoxy-*6*-phosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-NH$_2$ (6)



40 µmol of crude Ac-[Lys(butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-Tentagel S Ram was treated with a continuous flow of a mixture of AcOH/TFE/DCM (1/2/7 v/v/v, 30 mL) over 15 minutes. The resin was washed subsequently with DCM (5x), AcOH/TFE/DCM (1/2/7 v/v/v, 4 mL), DCM (5x), 1 M DIPEA in NMP (2x 3 mL), DCM (3x) and DMF (3x). A solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (34 mg, 88 µmol, 2.2

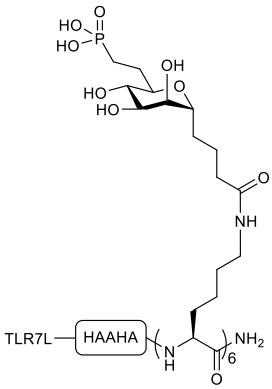
eq.) and HCTU (34 mg, 82 µmol, 2.1 eq.) in DMF (0.8 mL) and DIPEA (28 µL, 0.16 mmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.8 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (38 mg, 83 µmol, 2.1 eq.) and HCTU (34 mg, 82 µmol, 2.1 eq.) and DIPEA (28 µL, 0.16 mmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (3x), DCM (3x) and the peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.6 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.6 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **6** (3.1 mg, 0.61 µmol, 2%) was obtained as a white solid. LC-MS: Rt = 5.73 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 1686.9 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{207}H_{353}N_{47}O_{86}P_6$: 1264.82744, found 1264.82866.

Ac-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-Lys(butan-4-(6-deoxy-*6*-phosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-NH_2 (7)



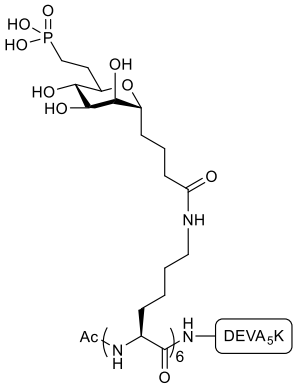
100 µmol of H-Tentagel S Ram was washed with DMF (5x) and elongated by the addition of a solution of acid **10** (0.19 g, 0.20 mmol, 2.0 eq.) and HCTU (84 mg, 0.20 mmol, 2.0 eq.) in DMF (2.0 mL) and DIPEA (70 µL, 0.40 mmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (2.0 mL, 2x 20 min) and washed with DMF (5x). This was repeated another 5 times, after which the resin was elongated using the synthesizer with Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in DMF (3x 3 min). 30 µmol of crude H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-[Lys(butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-*α*-D-mannopyranosyl)-amide)]$_6$-Tentagel S Ram was treated with a mixture of Ac_2O/DMF/DIPEA (2x 1.2 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **7** (11.0 mg, 2.4 µmol, 8%) was obtained as a white solid. LC-MS: Rt = 6.00 min (C18 Gemini, 0 - 50% MeCN, 11 min run); ESI-MS: m/z 1525.6 $[M+H]^{3+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{184}H_{325}N_{41}O_{80}P_6$: 1143.77568, found 1143.77518.

(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-[Lys(butan-4-(6-deoxy-*6*-phosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-NH$_2$ (8)



30 µmol of crude H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-[Lys(butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Tentagel S Ram was treated with a solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (27 mg, 70 µmol, 2.3 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) in DMF (0.6 mL) and DIPEA (21 µL, 0.12 mmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.6 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (29 mg, 63 µmol, 2.1 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) and DIPEA (21 µL, 0.12 mmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (3x), DCM (3x) and the peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **8** (17.0 mg, 3.4 µmol, 11%) was obtained as a white solid. LC-MS: Rt = 5.91 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 1673.1 $[M+H]^{2+}$; HRMS: $[M+H]^{4+}$ calcd. for $C_{205}H_{351}N_{47}O_{85}P_6$: 1254.32479, found 1254.32528.

Ac-[Lys(butan-4-(6-deoxy-*6*-phosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH$_2$ (43)



100 µmol of crude H-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT) was washed with DMF (5x) and elongated by the addition of a solution of acid **10** (0.19 g, 0.20 mmol, 2.0 eq.) and HCTU (84 mg, 0.20 µmol, 2.0 eq.) in DMF (2.0 mL) and DIPEA (70 µL, 0.40 mmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (2.0 mL, 2x 20 min) and washed with DMF (5x). This was repeated another 5 times. 40 µmol of crude H-[Lys(butan-4-(6-deoxy-2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-di-*tert*-butoxyphosphonomethyl-α-D-mannopyranosyl)-amide)]$_6$-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram

was treated with a mixture of Ac_2O/DMF/DIPEA (2x 0.8 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.6 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.6 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **43** (8.2 mg, 1.6 µmol, 4%) was obtained as a white solid. LC-MS: Rt = 4.74 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1740.1 $[M+H]^{2+}$; HRMS: $[M+H]^{3+}$ calcd. for $C_{216}H_{376}N_{41}O_{93}P_6$: 1739.47874, found 1739.47817.

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (39)

DEVA$_5$K

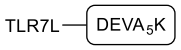
See compound **45**, chapter 2. LC-MS: Rt = 4.88 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1273.7 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{112}H_{187}N_{29}O_{38}$: 1273.17904, found 1273.17779.

Ac-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-NH_2 (40)

DEVA$_5$K
|
TLR7L

30 µmol of crude H-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was was treated with a mixture of Ac_2O/DMF/DIPEA (2x 0.6 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 11 mL) over 15 minutes. The resin was washed subsequently with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL), DCM (3x) and DMF (3x). A solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (24 mg, 62 µmol, 2.1 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) in DMF (0.6 mL) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.6 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (28 mg, 61 µmol, 2.0 eq.) and HCTU (26 mg, 62 µmol, 2.1 eq.) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **40** (9.4 mg, 3.1 µmol, 10%) was obtained as a white solid. LC-MS: Rt = 5.41 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1536.9 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{137}H_{217}N_{35}O_{45}$: 1536.28784, found 1536.28769.

(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-NH_2 (41)

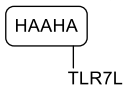


30 µmol of crude H-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Lys(N_3)-Asp(O*t*Bu)-Glu(O*t*Bu)-Val-Ser(*t*Bu)-Gly-Leu-Glu(O*t*Bu)-Gln(Trt)-Leu-Glu(O*t*Bu)-Ser(*t*Bu)-Ile-Ile-Asn(Trt)-Phe-Glu(O*t*Bu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(MMT)-Tentagel S Ram was washed with DMF (5x), followed by the addition of a solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (25 mg, 65 µmol, 2.2 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) in DMF (0.6 mL) and DIPEA (21 µL, 121 µmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.6 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (28 mg, 61 µmol, 2.0 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **41** (4.1 mg, 1.4 µmol, 5%) was obtained as a white solid. LC-MS: Rt = 5.34 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1516.0 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{135}H_{215}N_{35}O_{44}$: 1515.78424, found 1515.78242.

Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-NH_2 (42)

Tentagel S Ram resin loaded with H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-NH_2 on 30 µmol scale was washed with DCM (5x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2/2/1 v/v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **42** (16.8 mg, 8.8 µmol, 30%) was obtained as a white solid. LC-MS: Rt = 3.70 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 1901.0 $[M+H]^{+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{80}H_{135}N_{29}O_{25}$: 951.00865, found 951.00848.

Ac-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-NH_2 (43)



30 µmol of crude H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-Tentagel S Ram was was treated with a mixture of Ac_2O/DMF/DIPEA (2x 0.6 mL, 20 min), and washed with DMF (3x) and DCM (3x). The peptide was treated with a continuous flow of a mixture of TFA/TIS/DCM (96/2/2 v/v/v, 11 mL) over 15 minutes. The resin was washed subsequently with DCM (5x), TFA/TIS/DCM (96/2/2 v/v/v, 2 mL), DCM (5x), 1 M DIPEA in NMP (2 mL), DCM (3x) and DMF (3x). A solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (24 mg, 62 µmol,

2.1 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) in DMF (0.6 mL) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.6 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (28 mg, 61 µmol, 2.0 eq.) and HCTU (26 mg, 62 µmol, 2.1 eq.) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **43** (7.6 mg, 3.1 µmol, 10%) was obtained as a white solid. LC-MS: Rt = 6.47 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 1214.3 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{105}H_{165}N_{35}O_{32}$: 1214.11745, found 1214.11682.

(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)-N-(2-(2-(2-amino-2-oxoethoxy)ethoxy)ethyl)benzamide)-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-NH_2 (44)

TLR7L—(HAAHA)

30 µmol of crude H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-Tentagel S Ram was washed with DMF (5x), followed by the addition of a solution of {2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (24 mg, 62 µmol, 2.1 eq.) and HCTU (25 mg, 60 µmol, 2.0 eq.) in DMF (0.6 mL) and DIPEA (21 µL, 121 µmol, 4.0 eq.). The suspension was shaken overnight. The resin was washed with DMF (5x), treated with 20% piperidine in DMF (0.6 mL, 2x 20 min) and washed with DMF (5x). A solution of 4-((2-butoxy-6-((*tert*-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoid acid (28 mg, 61 µmol, 2.0 eq.) and HCTU (26 mg, 60 µmol, 2.0 eq.) and DIPEA (21 µL, 121 µmol, 4.0 eq.) were added and the suspension was shaken overnight. The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (1.2 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (1.2 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **44** (10.3 mg, 4.3 µmol, 14%) was obtained as a white solid. LC-MS: Rt = 6.17 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 1193.7 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{103}H_{163}N_{65}O_{31}$: 1193.11216, found 1193.11176.

Footnotes and References

(1) Dahms, N. *Biochim. Biophys. Acta - Gen. Subj.* **2002**, *1572* (2–3), 317–340.
(2) Ghosh, P.; Dahms, N. M.; Kornfeld, S. *Nat. Rev. Mol. Cell Biol.* **2003**, *4* (3), 202–213.
(3) Tong, P. Y.; Gregory, W.; Kornfeld, S. *J. Biol. Chem.* **1989**, *264* (14), 7962–7969.
(4) Kang, J. X.; Bell, J.; Beard, R. L.; Chandraratna, R. A. *Cell Growth Differ.* **1999**, *10* (8), 591–600.
(5) Godár, S.; Hořejší, V.; Weidle, U. H.; Binder, B. R.; Hansmann, C.; Stockinger, H. *Eur. J. Immunol.* **1999**, *29* (3), 1004–1013.
(6) Berkowitz, D. B.; Maiti, G.; Charette, B. D.; Dreis, C. D.; MacDonald, R. G. *Org. Lett.* **2004**, *6* (26), 4921–4924.
(7) Fei, X.; Connelly, C. M.; MacDonald, R. G.; Berkowitz, D. B. *Bioorg. Med. Chem. Lett.* **2008**, *18* (10), 3085–3089.

(8) Prydz, K.; Brändli, A. W.; Bomsel, M.; Simons, K. *J. Biol. Chem.* **1990**, *265* (21), 12629–12635.
(9) Marron-Terada, P. G.; Hancock, M. K.; Haskins, D. J.; Dahms, N. M. *Biochemistry* **2000**, *39* (9), 2243–2253.
(10) Watanabe, H.; Grubb, J. H.; Sly, W. S. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (20), 8036–8040.
(11) El Cheikh, K.; Basile, I.; Da Silva, A.; Bernon, C.; Cérutti, P.; Salgues, F.; Perez, M.; Maynadier, M.; Gary-Bobo, M.; Caillaud, C.; *et al*. *Angew. Chemie Int. Ed.* **2016**, *55* (47), 14774–14777.
(12) Greupink, R.; Bakker, H. I.; Bouma, W.; Reker-Smit, C.; Meijer, D. K. F.; Beljaars, L.; Poelstra, K. **2006**.
(13) Minnelli, C.; Cianfruglia, L.; Laudadio, E.; Galeazzi, R.; Pisani, M.; Crucianelli, E.; Bizzaro, D.; Armeni, T.; Mobbili, G. *J. Drug Target.* **2018**, *26* (3), 242–251.
(14) Hoogendoorn, S.; van Puijvelde, G. H. M.; Kuiper, J.; van der Marel, G. A.; Overkleeft, H. S. *Angew. Chemie Int. Ed.* **2014**, *53* (41), 10975–10978.
(15) Parra-López, C. A.; Lindner, R.; Vidavsky, I.; Gross, M.; Unanue, E. R. *J. Immunol.* **1997**, *158* (6), 2670–2679.
(16) Barragan-Montero, V.; Awwad, A.; Combemale, S.; de Santa Barbara, P.; Jover, B.; Molès, J.-P.; Montero, J.-L. *ChemMedChem* **2011**, *6* (10), 1771–1774.
(17) This conjugation strategy was chosen, since the synthesis of a phosphonate in the presence of an azide was unsuccessful.
(18) Distler, J. J.; Guo, J. F.; Jourdian, G. W.; Srivastava, O. P.; Hindsgaul, O. *J. Biol. Chem.* **1991**, *266* (32), 21687–21692.
(19) York, S. J.; Arneson, L. S.; Gregory, W. T.; Dahms, N. M.; Kornfeld, S. *J. Biol. Chem.* **1999**, *274* (2), 1164–1171.
(20) Zhou, J.; Xu, K.; Zhou, P.; Zheng, O.; Lin, Z.; Guo, L.; Qiu, B.; Chen, G. *Biosens. Bioelectron.* **2014**, *51*, 386–390.
(21) Mikkelsen, L. M.; Krintel, S. L.; Jiménez-Barbero, J.; Skrydstrup, T. *J. Org. Chem.* **2002**, *67* (18), 6297–6308.
(22) Liu, L.; Wang, C.-Q.; Liu, D.; He, W.-G.; Xu, J.-Y.; Lin, A.-J.; Yao, H.-Q.; Tanabe, G.; Muraoka, O.; Xie, W.-J.; *et al*. *Org. Lett.* **2014**, *16* (19), 5004–5007.
(23) Unpublished results.
(24) Decomposition was observed when concentrating in vacuo at 40°C instead of 30°C.
(25) Gential, G. P. P.; Hogervorst, T. P.; Tondini, E.; van de Graaff, M. J.; Overkleeft, H. S.; Codée, J. D. C.; van der Marel, G. A.; Ossendorp, F.; Filippov, D. V. *Bioorg. Med. Chem. Lett.* **2019**.
(26) Liu, Q.; Kistemaker, H. A. V.; Bhogaraju, S.; Dikic, I.; Overkleeft, H. S.; van der Marel, G. A.; Ovaa, H.; van der Heden van Noort, G. J.; Filippov, D. V. *Angew. Chemie Int. Ed.* **2018**, *57* (6), 1659–1662.
(27) Girard, C.; Miramon, M.-L.; de Solminihac, T.; Herscovici, J. *Carbohydr. Res.* **2002**, *337* (19), 1769–1774.
(28) Nicolaou, K. C.; Hwang, C. K.; Duggan, M. E. *J. Am. Chem. Soc.* **1989**, *111* (17), 6682–6690.
(29) Kramer, J. R.; Deming, T. J. *J. Am. Chem. Soc.* **2010**, *132* (42), 15068–15071.
(30) It was found to be important to co-evaporate the phosphonate reagents with toluene before treatment with *n*-BuLi.
(31) Sharma, P. K.; Kumar, S.; Kumar, P.; Nielsen, P. *Tetrahedron Lett.* **2007**, *48* (49), 8704–8708.
(32) Voigtritter, K.; Ghorai, S.; Lipshutz, B. H. *J. Org. Chem.* **2011**, *76* (11), 4697–4702.

Chapter 5

*Synthesis of C-rhamnoside–antigen conjugates to recruit anti-rhamnose antibodies for vaccine delivery**

Introduction

Tumor-associated carbohydrate antigens or cancer neo-epitopes are used in cancer vaccination strategies to trigger T helper cell responses and induce cytotoxic effector T cells. Vaccination with short peptides, that can be presented by major histocompatibility complex (MHC) mole

cules, can lead to immunological tolerance instead of immunity.[1,2] Therefore, longer peptide sequences that cannot bind directly to MHC and require intracellular processing by antigen presenting cells are generally used in peptide vaccine constructs.[3] Several methods have been developed to enhance the immunogenicity of synthetic long peptide (SLPs). To activate the immune system and up-regulate the production of inflammatory cytokines, the antigens are administered with an adjuvant[4–6], for example Toll-like receptor ligands. This can be achieved either by mixing the antigen and adjuvant or by generating (covalent) antigen-adjuvant conjugates. Enhanced immune activity can also be achieved by stimulating antigen uptake by antigen presenting cells, such as dendritic cells (DCs) by targeting uptake receptors on DCs for example by using

*The data presented in this Chapter were gathered in collaboration with Nick Zilverschoon, Robert A. Cordfunke, Jan Wouter Drijfhout, Herman S. Overkleeft, Dmitri V. Filippov, Gijsbert A. van der Marel and Jeroen D. C. Codée.

C-type lectin ligands.[7] The uptake of antigens can also be improved using antibody-recruiting molecules (ARM). This strategy is depicted in Figure 1 and builds on the formation of an immune complex of the antigen conjugate with pre-existing circulating antibodies, instead of binding directly to a DC surface receptor. The formed complex can then bind to Fcγ receptors[8] on the DCs leading to enhanced uptake. After internalization, the antigens are processed and the epitope is presented to T cells resulting in T cell mediated immune response.

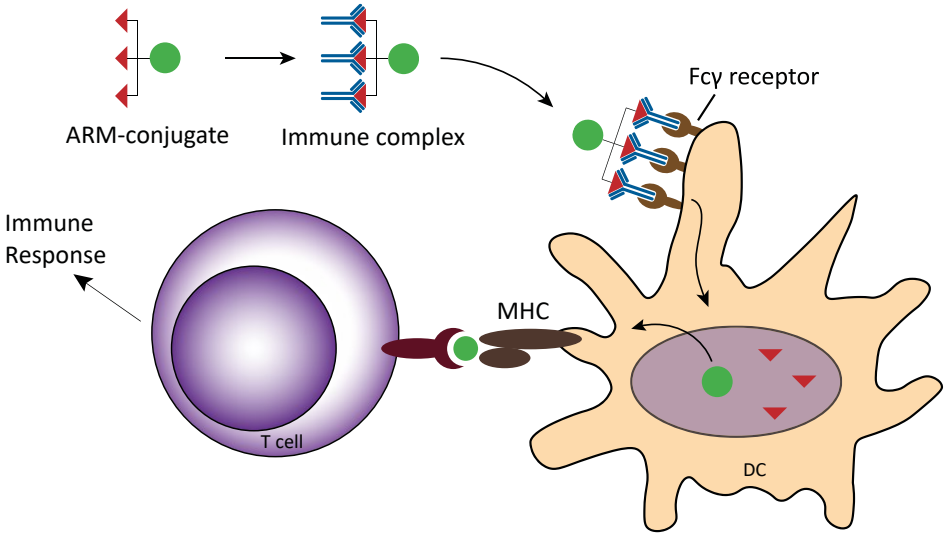


Figure 1. Mechanism of action of ARM-conjugates.

The α-Gal epitope (Figure 2) plays a crucial role in organ xenotransplantation, as it represents a highly immunogenic trisaccharide structure, against which most individuals have a naturally acquired high antibody titer. Therefore it has been explored in model ARM-conjugate systems for vaccination against for example the influenza virus and the HIV gp120 protein.[9–11] This epitope was even used to make cancer cells susceptible to lysis.[12] Other ARM-based strategies use 2,4-dinitroaniline analogues[13–15] (Figure 2) or tetanus toxoid epitopes[16,17] to enhance the immunogenicity of vaccines. Screening of human serum against broad carbohydrate antigen microarrays has shown that anti-L-rhamnose antibodies are amongst the most abundant circulating antibodies in human blood.[18,19] Several studies have exploited this abundance and used rhamnose-functionalized peptides[20], proteins[21] and liposomes[22–24] to be used in cancer immunotherapy. These studies have demonstrated that the xenoantigen L-rhamnose is an excellent alternative to the α-Gal epitope in model vaccination studies, especially as wild-type mice can be used instead of KO mice.[25]

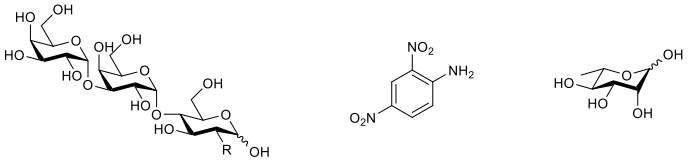


Figure 2. Structure of ARM-molecules, α-Gal-epitope, 2,4-dinitroaninine and L-rhamnose.

This Chapter describes the design and synthesis of conjugates **1**-**7** consisting of rhamnose and an ovalbumin derived peptide LEQLESIINFEKLAAAAAK, harboring the MHC-I epitope SIINFEKL to be used as model antigen (Figure 3). Functionalization of the peptide with one, two, three or six rhamnose monosaccharides will allow one to investigate the effect of multivalent binding to anti-rhamnose antibodies and the effect thereof on the immunogenicity of the vaccine. To generate these constructs two lysine building blocks **8** and **9**, equipped with an L-rhamnose-*C*-glycoside are designed to allow for application in an online solid phase peptide synthesis (SPPS) protocol. The *C*-rhamnosidic linkage in the building blocks is stable against the acidic conditions used in SPPS, while the *p*-methoxybenzyl protecting groups were chosen for their acid lability to obtain a fully deprotected conjugate after cleaving the peptides from the resin after SPPS. The building blocks **8** and **9** only differ in the length of the spacer bridging the *C*-rhamnoside and the lysine moiety.

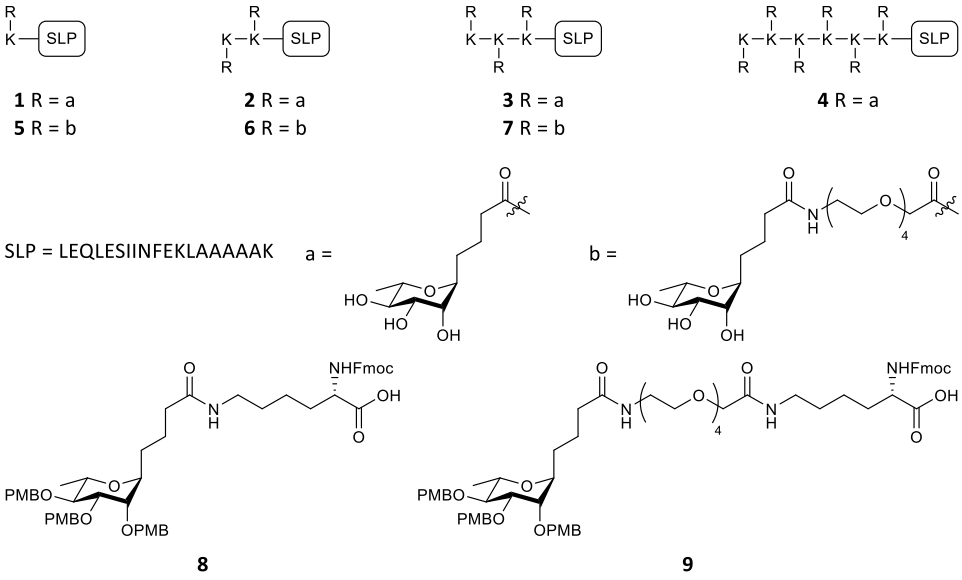


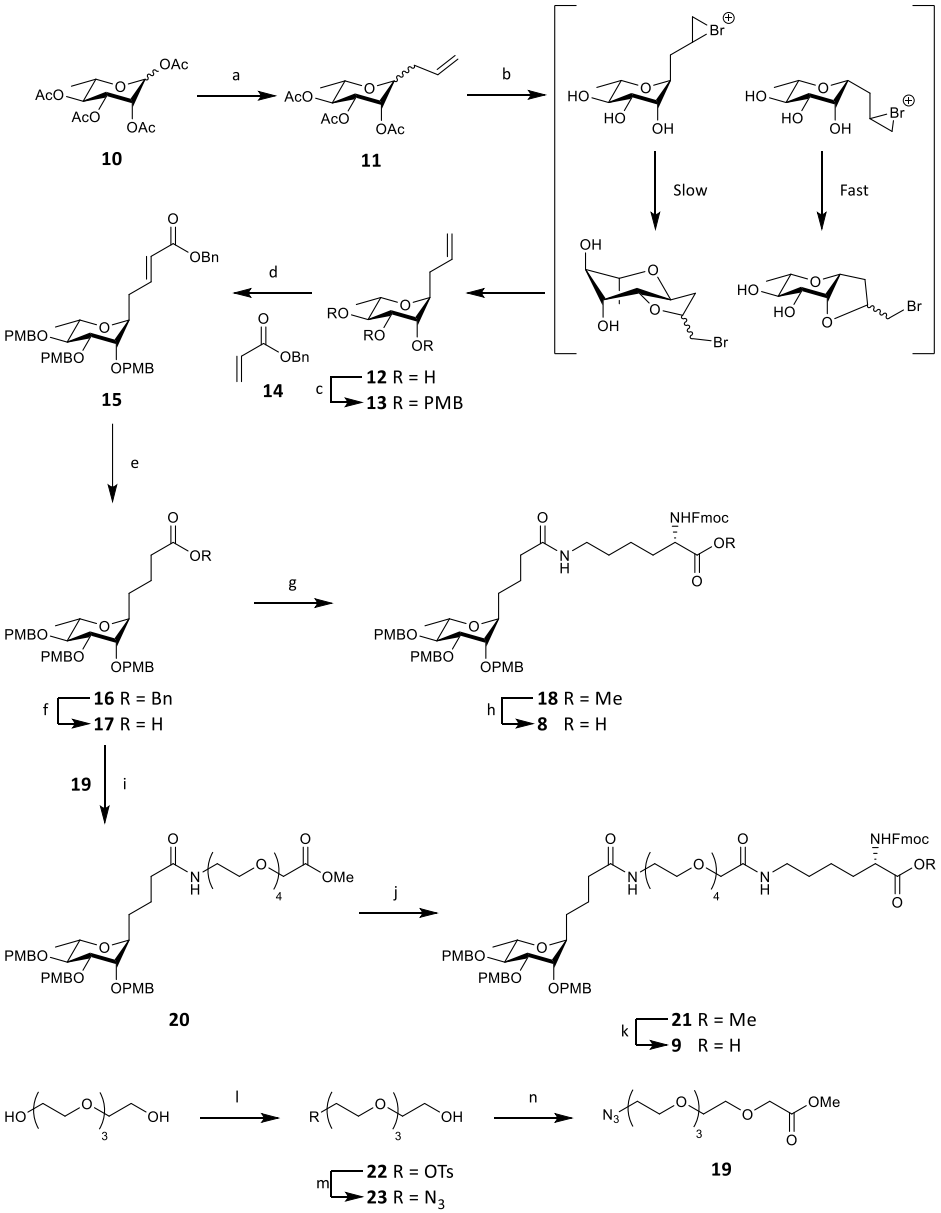
Figure 3. Structures of *C*-rhamnose conjugates **1-7** and SPPS building blocks **8** and **9**.

Results & Discussion

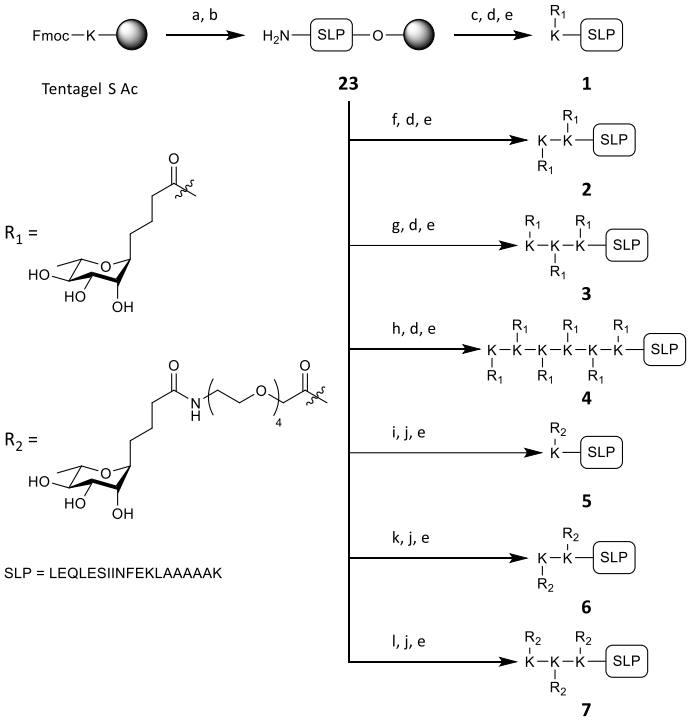
Synthesis of SPPS building blocks **8** and **9** started with the preparation of *C*-rhamnose **12** (Scheme 1). Treatment of acetylated rhamnose **10** with allyltrimethylsilane and *in situ* generated $BF_2OTf{\cdot}OEt_2$ as Lewis acid[26] afforded the desired allyl rhamnoside **11** as an inseparable 5/1 α/β-mixture. Therefore compound **11** was deacetylated using sodium methoxide and an intramolecular cyclization was induced by the addition of *N*-bromosuccinimide. A nucleophilic attack of the *C*-2-OH on the formed bromonium ion of the β-rhamnose occurred fast, while the α-rhamnose reacted slowly due to energetically unfavorable $^1C_4$ conformation and the formation of the *trans*-fused 5,6-bicyclic ring system, which is necessary for α-cyclization as shown in Scheme 1. The cyclized product and the unreacted α-rhamnose could be readily separated via column chromatography giving pure α-compound **12** in 86% over three steps. Close monitoring of the reaction progress was required as too short reaction times led to incomplete conversion of the β-rhamnose while longer reaction times decreased the yield due to cyclization of the α-rhamnose. Next, triol **12** was alkylated with *p*-methoxybenzyl chloride in the presence of sodium hydride to provide the fully protected *C*-rhamnoside **13**. Installation of the required acid functionality was achieved by a cross-metathesis with benzyl acrylate **14**, which was followed by reduction of the obtained alkene (**15**) with $NaBH_4$ and ruthenium trichloride and saponification of the so formed benzyl ester **16**.[27] To acquire an orthogonal protected SPPS building block, acid **17** and Fmoc-L-lysine-OMe[28] were coupled under the influence of HCTU and DIPEA. Subsequent careful hydrolysis of the methyl ester **18** with LiOH at 0°C gave Fmoc-protected *C*-rhamnose-functionalized lysine building block **8**. As the multivalent binding of the anti-rhamnose antibodies may be dependent on the length of the spacers between the *C*-rhamnosides, the lysine building block **9** was prepared. Spacer **19** was synthesized by successively subjecting tetraethylene glycol to mono-tosylation, azide substitution and alkylation with methyl bromoacetate. After reduction of the azide in **19** by hydrogenation, the produced amine was directly condensed with acid **17** to give rhamnoside **20** in 82%. Scale-up (5 mmol) of this coupling decreased the yield dramatically to 11% due to the formation of side-products and the difficult separation via column chromatography. After saponification of methyl ester **20**, the formed acid was coupled with Fmoc-L-lysine-OMe to give **21** and the methyl ester was carefully hydrolyzed with LiOH yielding SPPS rhamnose-functionalized lysine building block **9**.

With rhamnose-functionalized lysine building blocks **8** and **9** in hand, the SPPS of conjugates **1**-**7** was undertaken (Scheme 2). For the automated synthesis of LEQLESIINFEKLAAAAAK, Tentagel S Ac resin was used. The obtained immobilized

peptide **23** was elongated at the *N*-terminus with one, two, three or six rhamnose-functionalized lysines to obtain conjugates **1**-**4**. The coupling was performed with three equivalents of **8** in the presence of PyBOP as condensing agent and NMM as base at room temperature for two hours. After RP-HPLC purification conjugates **1**-**4** were obtained in respectively 19% (4.5 mg), 16% (4.5 mg), 10% (3.2 mg) and 6% (2.6 mg) yield. The high yields show that **8** is an excellent SPPS building block and the compatibility of the designed protecting group strategy with the SPPS approach. Unfortunately, elongation of peptide **23** with **9** proved to be more difficult and the PyBOP mediated couplings did not go to completion, not even by increasing the reaction time and temperature. Replacing PyBOP with the more reactive reagent HATU and performing the reaction at 39°C overnight did provide full conversion of the starting peptide. Coupling of **9** once, twice or thrice to immobilized peptide **23** afforded conjugates **5**-**7** in respectively 15% (2.0 mg), 2% (0.9 mg) and 4% (0.4 mg). The low yields of **6** and **7** can be caused by reaction of HATU with the *N*-terminal amine, preventing further elongation of the immobilized peptide. This side reaction was confirmed with MALDI analysis.



Scheme 1. Synthesis of SPPS building blocks **8** and **9**. *Reagents and conditions*: a) allyltrimethylsilane, $BF_3{\cdot}OEt_2$, TMSOTf, MeCN, 86%; b) *i*. NaOMe, MeOH; *ii*. *N*-bromosuccinimide, THF, 3 h, then $Na_2S_2O_3$, 79% over two steps; c) *p*-methoxybenzyl chloride, NaH, TBAI, DMF, 80%; d) benzyl acrylate (**14**), Grubbs 2nd gen. catalyst, DCM, 50°C, 92%; e) $NaBH_4$, ruthenium trichloride, MeOH, DCE, 40°C, 93%; f) LiOH, THF/MeOH/H_2O, 40°C, 96%; g) Fmoc-L-Lys-OMe, HCTU, DIPEA, DMF, 80%; h) LiOH, THF/H_2O, 0°C, 71%; i) *i*. **19**, Pd/C, H_2, THF; *ii*. HCTU, DIPEA, DMF, 82% over two steps; j) *i*. LiOH, THF/H_2O; *ii*. Fmoc-L-Lys-OMe, HCTU, DIPEA, DMF, 89% over two steps; k) LiOH, THF/H_2O, 0°C, 66%; l) TsCl, Et_3N, DCM, 93%; m) NaN_3, DMF, 90°C, 96%; n) methyl bromoacetate, NaH, THF, 83%.



Scheme 2. Synthesis of conjugates **1**-**7**. *Reagents and conditions*: a) Fmoc SPPS cycle for LEQLESIINFEKLAAAAA; b) 20% piperidine, NMP; (c) *i*. **8**, PyBOP, NMM, NMP; *ii*. 20% piperidine, NMP; d) TFA/TIS/H_2O (93/2/5 v/v/v); e) RP-HPLC; f) repeat conditions c two times; g) repeat conditions c three times; h) repeat conditions c six times; i) *i*. **9**, HATU, NMM, NMP; *ii*. 20% piperidine, NMP; j) TFA/H_2O (95/5 v/v/v); k) repeat conditions i two times; l) repeat conditions i three times. Yield conjugates: **1**) 4.5 mg, 19%; **2**) 4.5 mg, 16%; **3**) 3.2 mg, 10%; **4**) 2.6 mg, 6%; **5**) 2.0 mg, 15%; **6**) 0.9 mg, 2%; **7**) 0.4 mg, 4%.

Conclusion

This Chapter describes the synthesis of seven novel rhamnose-peptide conjugates using an SPPS approach in which the rhamnosides were incorporated by an online assembly process. In these rhamnose constructs, designed as model vaccines, one, two, three or six *C*-rhamnose-functionalized lysines were linked to the *N*-terminus end of an antigenic peptide containing the MHC-I epitope, SIINFEKL. To enable the online SPPS two building blocks **8** and **9**, differing in spacer length were prepared using α-*C*-rhamnose intermediate for which an efficient synthesis has been developed. While conjugates **1**-**4** could be obtained in high yield using building block **8**, the condensation reactions using **9** proceeded less efficiently. By the use of the condensing agent HATU together with relatively long reaction times and an increased reaction temperature conjugates **5**-**7** were obtained, albeit in relatively low yields. The immunological evaluation of the conjugates is ongoing.

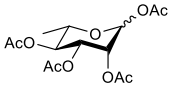
Experimental

All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H and ^{13}C spectra were recorded on a Bruker AV-400 (400/100 MHz) spectrometer and all individual signals were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. High resolution mass spectra were recorded on a Synapt G2-Si or a Q Exactive HF Orbitrap equipped with an electron spray ion source positive mode. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR.

Automated solid phase synthesis general experimental information

The synthesis of LEQLESIINFEKLAAAAAK was performed as has been described before by Hiemstra *et al.*[29] In short, the peptides were synthesized using solid-phase peptide synthesis on a Tentagel S Ac resin (Rapp, Tübingen) using a Syro II peptide synthesizer (MultiSyntech, Witten, Germany). Normal couplings (1.5 h - 2 h) were performed using Fmoc amino acids carrying acid labile side chain protection groups (were required). Activation of Fmoc amino acids was performed with PyBOP and NMM unless stated otherwise. Fmoc deprotection was performed with 20% piperidine in NMP. Washings were performed with NMP. Cleavage from the resin and side chain deprotection was performed with TFA/TIS/H_2O (93/2/5 v.v.v) unless stated otherwise. Purification was performed with RP-HPLC (C18). Analysis of the purified peptide was performed with UPLC-MS (Acquity, Waters) and showed the expected molecular masses. Building bock

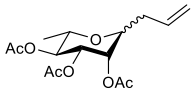
Acetyl 2,3,4-tri-*O*-acetyl-α/β-L-rhamnopyranoside (10)



A solution of L-rhamnose monohydrate (8.3 g, 51 mmol, 1.0 eq.) in pyridine (70 mL) was cooled to 0°C, followed by the addition of Ac_2O (31 mL, 0.35 mol, 6.9 eq.). The reaction was allowed to warm-up to room temperature overnight, after which it was quenched with methanol at 0°C and diluted with EtOAc. The organic layer was washed with 1 M HCl (3x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Co-evaporation with toluene gave the title compound (16 g, 48 mmol, 94%, α/β ratio: 8/1) as a transparent sticky oil. R_f: 0.40 (7/3 pentane/EtOAc); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.99 (d, 1H, *J* = 1.9 Hz,

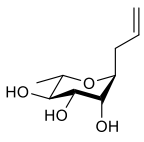
H-1), 5.28 (dd, 1H, *J* = 10.1, 3.5 Hz, H-3), 5.24 – 5.21 (m, 1H, H-4), 5.10 (t, 1H, *J* = 9.9 Hz, H-2), 3.96 – 3.87 (m, 1H, H-5), 2.14 (m, 6H, *J* = 4.6 Hz, 2x CH_3 Ac), 2.04 (s, 3H, CH_3 Ac), 1.98 (s, 3H, CH_3 Ac), 1.21 (d, 3H, *J* = 6.2 Hz, CH_3-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.2, 169.9, 169.9, 168.5 (C=O), 90.7 (C-1), 70.5 (C-2), 68.8 (C-3), 68.8 (C-5), 68.7 (C-4), 21.0, 20.9, 20.9, 20.8 (CH_3 Ac), 17.5 (CH_3-6); FT-IR (neat, cm^{-1}): 2987, 1743, 1433, 1369, 1209, 1181, 1147, 1086, 1052, 1025, 969, 947, 909, 888, 840, 783, 736, 698, 601, 563, 533, 511, 499, 480; $[M+Na]^+$ calcd. for $C_{14}H_{20}O_9Na$: 355.1005, found 355.1010. *NMR analysis only given for the α-anomer.

3-(2,3,4-tri-*O*-acetyl-α/β-L-rhamnosyl)-1-propene (11)



After co-evaporating with toluene (4x), compound **10** (44.7 g, 134 mmol, 1.0 eq.) was dissolved in dry MeCN (0.25 L) under an argon atmosphere, followed by the addition of allyltrimethylsilane (44 mL, 0.28 mol, 2.0 eq.). After cooling the mixture to 0°C, $BF_3{\cdot}OEt_2$ (35 mL, 0.28 mmol, 2.0 eq.) and TMSOTf (2.3 mL, 13 mmol, 0.10 eq.) were added and the reaction was allowed to warm-up to room temperature overnight. Upon completion determined by TLC analysis, the reaction was cooled to 0°C and slowly quenched with Et_3N. The mixture was diluted with sat. aq. $NaHCO_3$ and extracted with EtOAc (2x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10%→16% EtOAc in pentane) gave compound **11** (36.2 g, 115 mmol, 86%, α/β ratio: 5/1) as a sticky yellow oil. R_f: 0.40 (7/2 pentane/EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.75 – 5.58 (m, 1H, CH_2-C*H*=CH_2), 5.13 – 5.07 (m, 2H, H-2, H-3), 5.06 – 5.00 (m, 1H, CH_2-CH=C*H*H), 4.97 – 4.85 (m, 2H, H-4, CH_2-CH=C*H*H), 3.88 – 3.81 (m, 1H, H-1), 3.70 – 3.61 (m, 1H, H-5), 2.48 – 2.38 (m, 1H, C*H*H-CH=CH_2), 2.35 – 2.23 (m, 1H, C*H*H-CH=CH_2), 2.01 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.90 (s, 3H, Ac), 1.12 (d, 3H, *J* = 6.3 Hz, CH_3-6).^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.5, 170.3, 170.0 (C=O), 133.0 (CH_2-*C*H=CH_2), 118.3 (CH_2-CH=*C*H_2), 74.5 (C-1), 71.6 (C-4), 70.5 (C-3), 69.2 (C-2), 68.3 (C-5), 33.8 (*C*H_2-CH=CH_2), 21.1, 21.0, 20.8 (CH_3 Ac), 17.7 (CH_3-6); FT-IR (neat, cm^{-1}): 2983, 2361, 1745, 1371, 1222, 1051, 668; HRMS: $[M+Na]^+$ calcd. for $C_{15}H_{22}O_7Na$: 337.1263, found 337.1264. *Only data given for the α-anomer.

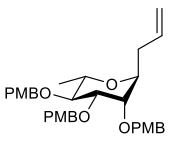
3-(α-L-rhamnosyl)-1-propene (12)



Compound **11** (36.3 g, 115 mmol, 1.0 eq., α/β ratio: 5/1) was co-evaporated with toluene (3x) under argon atmosphere and dissolved in MeOH (0.58 L). Sodium methoxide (5.4 M in MeOH, 2.2 mL, 12 mmol, 0.1 eq.) was added and the solution was stirred for two hours, after which TLC analysis showed complete conversion of the starting material. The reaction mixture was acidified by the addition of amberlite H^+ resin, filtered and concentrated *in vacuo*. The obtained residue was co-evaporated with toluene (1x) under argon atmosphere and dissolved in THF (1.2 L). *N*-bromosuccinimide (10 g, 55 mmol, 0.48 eq.) was added and the reaction was allowed to stir for 3 hours, after which the reaction was quenched with an aqueous solution of $Na_2S_2O_3$ (4.4 M, 40 mL). The mixture was further diluted with toluene and concentrated *in vacuo*. The crude product was imbedded on silica and purified by column chromatography (2→8% MeOH in DCM) yielded compound **12** (14.2 g, 75.4 mmol, 79%) as a white solid. R_f: 0.24 (9/1

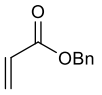
DCM/MeOH); $[\alpha]_D^{20}$ +18.0° (*c* = 1.0, MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 5.89 – 5.75 (m, 1H, CH_2-C*H*=CH_2), 5.17 – 5.03 (m, 2H, CH_2-CH=C*H*$_2$), 3.91 – 3.82 (m, 1H, H-1), 3.81 – 3.75 (m, 1H, H-2), 3.65 (dd, 1H, *J* = 8.9, 3.4 Hz, H-3), 3.54 – 3.45 (m, 1H, H-5), 3.40 (t, 1H, *J* = 8.9 Hz, H-4), 2.54 – 2.42 (m, 1H, C*H*H-CH=CH_2), 2.38 – 2.26 (m, 1H, C*H*H-CH=CH_2), 1.24 (d, 3H, *J* = 6.1 Hz, CH_3-6); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 135.9 (CH_2-*C*H=CH_2), 117.4 (CH_2-CH=*C*H$_2$), 78.5 (C-1), 74.3 (C-4), 72.4 (C-3), 72.3 (C-2), 71.0 (C-5), 34.7 (*C*H$_2$-CH=CH_2), 18.3 (CH_3-6); FT-IR (neat, cm^{-1}): 3371, 2977, 2934, 2361, 1644, 1418, 1253, 1140, 1057, 981, 916, 825, 779, 668, 550; HRMS: $[M+Na]^+$ calcd. for $C_9H_{16}O_4Na$: 211.0946, found 211.0944.

3-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-1-propene (13)



Compound **12** (1.92 g, 10.2 mmol, 1.0 eq.) was co-evaporated with toluene (1x) under argon atmosphere and dissolved in DMF (0.10 L). Sodium hydride (60% dispersion in mineral oil, 1.47 g, 36.5 mmol, 3.6 eq.) was added at 0°C. After 20 minutes, *p*-methoxybenzyl chloride (5.0 mL, 37 mmol, 3.6 eq.) and TBAI (0.38 g, 1.0 mmol, 0.1 eq.) were added. The reaction was allowed to warm-up to room temperature. After 6 hours, another portion of sodium hydride (60% dispersion in mineral oil, 0.40 g, 10 mmol, 1.0 eq.) was added and the reaction was allowed to stir overnight. The reaction mixture was quenched with MeOH at 0°C, diluted with H_2O and extracted with DCM. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→20% Et_2O in pentane) gave compound **13** (4.4 g, 8.0 mmol, 79%) as a white solid. R_f: 0.84 (8/2 pentane/EtOAc); $[\alpha]_D^{20}$ +20.0° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.32 (d, 2H, Ar), 7.27 (d, 4H, Ar), 6.93 – 6.84 (m, 6H, Ar), 5.79 – 5.66 (m, 1H, CH_2-C*H*=CH_2), 5.08 – 4.98 (m, 2H, CH_2-CH=C*H*$_2$), 4.78 (d, 1H, *J* = 10.8 Hz, C*H*H PMB), 4.65 – 4.49 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.05 – 3.97 (m, 1H, H-1), 3.79 (s, 9H, 3x CH_3 PMB), 3.73 (dd, 1H, *J* = 7.9, 3.1 Hz, H-3), 3.70 – 3.65 (m, 1H, H-4), 3.62 (t, 1H, *J* = 3.3 Hz, H-5), 3.60 – 3.54 (m, 1H, H-2), 2.42 – 2.32 (m, 1H, C*H*H-CH=CH_2), 2.30 – 2.20 (m, 1H, C*H*H-CH=CH_2), 1.34 (d, 3H, *J* = 6.3 Hz, H-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 159.1 (C_q Ar), 134.2 (CH_2-*C*H=CH_2), 130.5, 130.3, 130.2 (C_q Ar), 129.5, 129.5, 129.3, 128.3 (Ar), 116.9 (CH_2-CH=*C*H$_2$), 113.6, 113.6, 113.5 (Ar), 79.5 (C-2), 77.3 (C-3), 74.5 (C-5), 74.0 (CH_2 PMB), 72.7 (C-1), 71.4, 71.1 (CH_2 PMB), 69.5 (C-4), 64.4 (CH_2 PMB), 55.0 (CH_3 PMB), 34.2 (*C*H$_2$-CH=CH_2), 17.9 (C-6); FT-IR (neat, cm^{-1}): 2934, 2836, 2360, 1641, 1612, 1586, 1512, 1464, 1421, 1358, 1302, 1245, 1173, 1079, 1033, 917, 820, 783, 755, 710, 668, 637, 587, 517; HRMS: $[M+Na]^+$ calcd. For $C_{33}H_{40}O_7Na$: 571.2672, found 571.2670.

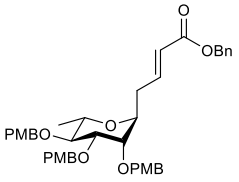
Benzyl acrylate (14)



Acrylic acid (19 mL, 0.28 mol, 1.0 eq.) was dissolved in DMF (0.56 L), followed by the addition of benzyl bromide (37 mL, 0.30 mol, 1.1 eq.) and K_2CO_3 (78 g, 0.56 mol, 2.0 eq.). The suspension was heated to 45°C overnight. The mixture was cooled to room temperature, diluted with brine and extracted with EtOAc (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (0→10% Et_2O in pentane) afforded the title compound (32 g, 0.20 mol, 71%) as a transparent oil. R_f: 0.88 (9/1 pentane/EtOAc);

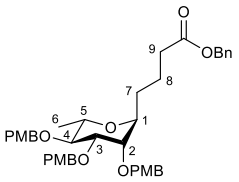
$[\alpha]_D^{20}$ +1.3° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.43 – 7.31 (m, 5H, Ar), 6.47 (dd, 1H, *J* = 17.3, 1.5 Hz, C*H*=CH), 6.23 – 6.13 (m, 1H, CH_2=C*H*), 5.86 (dd, 1H, *J* = 10.4, 1.4 Hz, C*H*=CH), 5.22 (s, 2H, CH_2 Bn); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 166.1 (C=O), 135.9 (C_q Ar), 131.2 (*C*H_2=CH), 128.7, 128.4, 128.4, 128.3 (CH_2=*C*H , Ar) 66.4 (CH_2 Bn); FT-IR (neat, cm^{-1}): 3035, 1724, 1635, 1498, 1456, 1407, 1372, 1296, 1269, 1176, 1049, 984, 809, 751, 698.

Benzyl but-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-cis/trans-2-enoate (15)



Compound **13** (25.1 g, 45.8 mmol, 1.0 eq.) and benzyl acrylate **14** (19.3 mL, 128 mmol, 2.8 eq.) were co-evaporated with toluene (1x) under argon atmosphere. The mixture was dissolved in DCM (0.23 L) and the flask was shielded from light with aluminum foil. Grubbs 2nd gen. catalyst (0.78 g, 0.92 mmol, 0.02 eq.) was added and the reaction was continued to reflux overnight at 50°C. Upon completion determined by TLC analysis, the reaction mixture was filtered over Celite® and concentrated *in vacuo*. Purification by column chromatography (10→25% EtOAc in pentane) gave compound 1**5** (28.9 g, 42.2 mmol, 92%) as a white solid. R_f: 0.22 (7/3 pentane/EtOAc); $[\alpha]_D^{20}$ -12.4° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.45 – 7.31 (m, 5H, Ar), 7.31 – 7.17 (m, 7H, CH_2-C*H*=CH, Ar), 7.00 – 6.82 (m, 6H, Ar), 5.87 (d, 1H, CH_2-CH=C*H*), 5.20 (s, 2H, CH_2 Bn), 4.68 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.61 – 4.47 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.11 – 4.01 (m, 1H, H-1), 3.81 (s, 9H, 3x CH_3 PMB), 3.77 – 3.68 (m, 2H, H-3, H-5), 3.58 – 3.49 (m, 2H, H-2, H-4), 2.53 – 2.38 (m, 2H C*H*$_2$-CH=CH), 1.34 (d, 3H, *J* = 6.5 Hz, CH_3-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 166.0 (C=O), 159.3 (C_q Ar), 145.6 (CH_2-*C*H=CH), 136.1, 130.5, 130.2, 130.0 (C_q Ar), 129.8, 129.6, 129.5, 128.6, 128.2 (Ar), 123.0 (CH_2-CH=*C*H), 113.8 (Ar), 78.8 (C-2), 76.1 (C-3), 75.0 (C-4), 73.5, 71.7, 71.2 (CH_2 PMB), 70.9 (C-5), 70.3 (C-1), 66.1 (CH_2 Bn), 55.2 (CH_3 PMB), 33.3 (*C*H_2-CH=CH), 17.7 (CH_3-6); FT-IR (neat, cm^{-1}): 2934, 2836, 1717, 1655, 1612, 1586, 1512, 1456, 1376, 1302, 1247, 1211, 1172, 1111, 1080, 1033, 820, 753, 698, 589, 518; HRMS: $[M+Na]^+$ calcd. for $C_{41}H_{46}O_9Na$: 705.3040, found 705.3052.

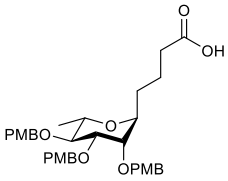
Benzyl 4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-butanoate (16)



Compound **15** (15.4 g, 22.6 mmol, 1.0 eq.) was co-evaporated with toluene (1x) under argon atmosphere and dissolved in DCE (90 mL). Ruthenium trichloride (0.89 g, 4.2 mmol, 0.19 eq.) was added and the argon balloon was replaced with an empty balloon. The reaction was cooled to 0°C, $NaBH_4$ (2.7 g, 72.3 mmol, 3.2eq.) was added after which MeOH (9.15 mL) was carefully added. The mixture was heated to 40°C for 4.5 hours, subsequently quenched with MeOH at 0°C. The reaction mixture was diluted with DCM and washed with brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (1→4% acetone in DCM) yielded the title compound (13.3 g, 22.6 mmol, 86%) as a transparent sticky oil. R_f: 0.22 (7/3 pentane/EtOAc); $[\alpha]_D^{20}$ +24.0° (*c* = 2.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.39 – 7.18 (m, 11H, Ar), 6.92 – 6.80 (m, 6H, Ar), 5.11 (s, 2H, CH_2 Bn), 4.74 (d,

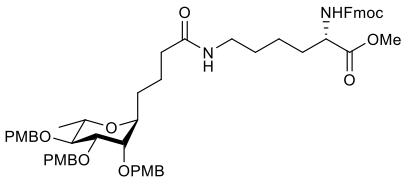
1H, *J* = 10.7 Hz, C*H*H PMB), 4.63 – 4.47 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 3.93 – 3.84 (m, 1H, H-1), 3.80 (s, 9H, 3x CH_3 PMB), 3.65 (dd, 1H, *J* = 7.8, 3.1 Hz, H-3), 3.60 – 3.47 (m, 3H, H-2, H-4, H-5), 2.34 (t, 2H, *J* = 7.1 Hz CH_2-9), 1.81 – 1.66 (m, 1H, C*H*H-8), 1.66 – 1.53 (m, 2H, C*H*H-8, C*H*H-7), 1.42 – 1.33 (m, 1H, C*H*H-7), 1.30 (d, 3H, CH_3-6, *J* = 6.1 Hz); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.3 (C=O), 159.3, 159.3, 136.1, 130.5 (C_q Ar), 129.7, 129.5, 128.7, 128.3, 128.3, 113.9, 113.8, 113.8 (Ar), 79.9 (C-2), 78.2 (C-3), 75.6 (C-4), 74.3 (CH_2 PMB), 73.2 (C-1), 71.8, 71.4 (CH_2 PMB), 69.5 (C-5), 66.3 (CH_2 Bn), 55.4 (CH_3 PMB), 33.9 (CH_2-9), 28.7 (CH_2-7), 21.5 (CH_2-8), 18.2 (CH_3-6); FT-IR (neat, cm^{-1}): 2934, 2836, 1733, 1611, 1586, 1512, 1456, 1421, 1567, 1301, 1245, 1172, 1109, 1079, 1032, 820, 752, 699, 637, 581, 516; HRMS: $[M+Na]^+$ calcd. for $C_{41}H_{48}O_9Na$: 707.3196, found 707.3216.

3-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-butanoic acid (17)



Compound **16** (22.7 g, 33.2 mmol, 1.0 eq.) was dissolved in a mixture of THF/MeOH/H_2O (7/2/1 v/v/v, 0.11 L). The reaction was cooled to 0°C and $LiOH{\cdot}H_2O$ (3.48 g, 83 mmol, 2.5 eq.) was added. The reaction was heated to 40°C for 4 hours, after which TLC analysis showed full conversion of the starting material. The reaction mixture was acidified with 1 M HCl to pH = 4-5 and extracted with DCM (2x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (1→20% acetone in DCM + 0.1% AcOH) addorded the title compound (18.3 g, 31 mmol, 96%) as a sticky yellow oil. R_f: 0.32 (4/1 DCM/acetone); $[\alpha]_D^{20}$ +37.0° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.30 – 7.18 (m, 6H, Ar), 6.90 – 6.81 (m, 6H, Ar), 4.72 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.58 – 4.48 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 3.92 – 3.85 (m, 1H, H-1), 3.80 (s, 9H, 3x CH_3 PMB), 3.67 (dd, 1H, *J* = 7.7, 3.1 Hz, H-3), 3.63 – 3.56 (m, 1H, H-5), 3.54 – 3.48 (m, 2H, H-2, H-4), 2.32 (t, 2H, *J* = 7.1 Hz, CH_2-9), 1.77 – 1.63 (m, 1H, C*H*H-8), 1.63 – 1.51 (m, 2H, C*H*H-8, C*H*H-7), 1.47 – 1.36 (m, 1H, C*H*H-7), 1.30 (d, 3H, *J* = 6.3 Hz, CH_3-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 178.9 (C=O), 159.3, 130.7, 130.6, 130.4 (C_q PMB), 129.8, 129.7, 129.5, 113.9, 113.9 (Ar), 79.7 (C-2), 77.9 (C-3), 75.6 (C-4), 74.2 (CH_2 PMB), 73.0 (C-1), 71.8, 71.4 (CH_2 PMB), 69.6 (C-5), 55.4 (CH_3 PMB), 33.5 (CH_2-9), 28.7 (CH_2-7), 21.2 (CH_2-8), 18.1 (CH_3-6); FT-IR (neat, cm^{-1}): 2934, 2836, 1721, 1707, 1611, 1586, 1512, 1463, 1359, 1302, 1245, 1173, 1108, 1077, 1032, 819, 756, 710, 637, 584, 516; HRMS: $[M+Na]^+$ calcd. for $C_{34}H_{42}O_9Na$: 617.2727, found 617.2736.

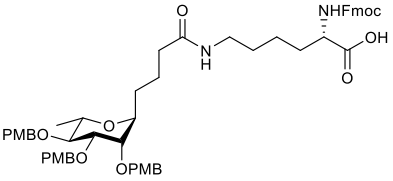
N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-L-lysine-methyl ester (18)



Compound **17** (3.0 g, 5.0 mmol, 1.0 eq.) and compound Fmoc-L-Lys-OMe·HCl (2.4 g, 6.3 mmol, 1.3 eq.) were co-evaporated with toluene (2x) under argon atmosphere. The mixture was dissolved in DMF (25 mL). HCTU (2.49 g, 6.0 mmol, 1.2 eq.) and DIPEA (2.6 mL, 15 mmol, 3.0 eq.) were subsequently added at 0°C. The reaction was allowed to warm-up to room temperature and stirred for 5 hours. The reaction mixture was quenched

with H_2O and diluted with EtOAc. The organic layer was subsequently washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→70% acetone in DCM) gave compound **18** (3.8 g, 4.0 mmol, 80%) as a white solid. R_f: 0.69 (4/1 DCM/acetone); $[\alpha]_D^{20}$ -33.0° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.78 – 7.72 (m, 2H, Ar), 7.64 – 7.57 (m, 2H, Ar), 7.43 – 7.36 (m, 2H, Ar), 7.34 – 7.18 (m, 9H, Ar, Ar), 6.89 – 6.81 (m, 6H, Ar), 5.63 (t, 1H, *J* = 5.8 Hz, NH), 5.53 (d, 1H, *J* = 8.3 Hz, N*H*Fmoc), 4.72 (d, 1H, *J* = 10.8 Hz, C*H*H PMB), 4.60 – 4.45 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.45 – 4.31 (m, 3H, 3H, CH_2 Fmoc, CH L-Lys), 4.22 (t, 1H, *J* = 7.1 Hz, CH Fmoc), 3.94 – 3.84 (m, 1H, H-1), 3.79 (s, 9H, 3x CH_3 PMB), 3.74 (s, 3H, OCH_3), 3.66 (dd, 1H, *J* = 7.7, 3.1 Hz, H-3), 3.63 – 3.55 (m, 1H, H-5), 3.54 – 3.49 (m, 2H, H-2, H-4), 3.21 (q, 2H, *J* = 6.7 Hz, CH_2 ε-L-Lys), 2.10 (t, 2H, *J* = 7.0 Hz, CH_2-9), 1.92 – 1.78 (m, 1H, C*H*H-8), 1.76 – 1.33 (m, 9H, C*H*H-8, CH_2-7, 3x CH_2 β/γ/δ-L-Lys), 1.30 (d, 3H, *J* = 6.2 Hz, CH_3-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.0, 172.8 (C=O), 159.3, 159.2, 156.1, 143.9, 143.8, 141.3, 130.7, 130.5, 130.5 (C_q Ar), 129.6, 129.4, 127.8, 127.1, 125.1, 120.0, 113.8, 113.7 (Ar), 79.7 (C-2), 77.9 (C-3), 75.6 (C-4), 74.1 (CH_2 PMB), 73.1 (C-1), 71.7, 71.3 (CH_2 PMB), 69.5 (C-5), 67.0 (CH_2 Fmoc), 55.3 (CH_3 PMB), 53.7 (CH L-Lys), 52.5 (OCH_3), 47.2 (CH Fmoc), 39.0 (CH_2 ε-L-Lys), 36.1 (CH_2-9), 32.1 (CH_2-7), 29.1, 28.7, 22.5, 22.1 (CH_2-8, CH_2 β/γ/δ-L-Lys), 17.9 (CH_3-6); FT-IR (neat, cm^{-1}): 3331, 2935, 1752, 1650, 1612, 1513, 1451, 1302, 1248, 1174, 1081, 1034, 847, 760, 742, 563; HRMS: $[M+Na]^+$ calcd. for $C_{56}H_{66}N_2O_{12}Na$: 981.4513, found 981.4545; LC-MS: Rt = 6.35 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

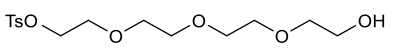
N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-L-lysine (8)



Compound **18** (2.8 g, 2.9 mmol, 1.0 eq.) was dissolved in THF (40 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 19 mL, 5.7 mmol, 2.0 eq.) was slowly added. After 40 minutes, the solution was diluted with EtOAc and acidified with 1 M HCl. The aqueous layer was extracted with EtOAc (2x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (15→25% acetone in DCM + 0.1% AcOH) afforded the title compound (1.94 g, 2.06 mmol, 71%) as a white solid. R_f: 0.26 (4/1 DCM/acetone + 0.1% AcOH); $[\alpha]_D^{20}$ +39.0° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.76 (d, 2H, *J* = 7.6 Hz, Ar), 7.62 (t, 2H, *J* = 7.3 Hz, Ar), 7.43 – 7.36 (m, 2H, Ar), 7.35 – 7.18 (m, 9H, Ar, Ar), 6.91 – 6.82 (m, 6H, Ar), 5.89 (t, 1H, *J* = 5.8 Hz, NH), 5.78 (d, 1H, *J* = 7.9 Hz, N*H*Fmoc), 4.71 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.61 – 4.46 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.44 – 4.34 (m, 3H, CH_2 Fmoc, CH L-Lys), 4.22 (t, 1H, *J* = 7.0 Hz CH Fmoc), 3.96 – 3.88 (m, 1H, H-1), 3.81 (s, 9H, 3x CH_3 PMB), 3.73 – 3.62 (m, 2H, H-3, H-5), 3.59 – 3.50 (m, 2H, H-2, H-4), 3.31 – 3.17 (m, 2H, CH_2 ε-L-Lys), 2.16 (t, 2H, CH_2-9), 1.95 – 1.84 (m, 1H, C*H*H-8), 1.83 – 1.75 (m, 1H, C*H*H-8), 1.74 – 1.64 (m, 1H, C*H*H-7), 1.64 – 1.35 (m, 7H, C*H*H-7, 3x CH_2 β/γ/δ-L-Lys), 1.32 (d, 3H, *J* = 6.4 Hz, CH_3-6); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.2, 173.7 (C=O), 159.4, 159.3, 156.2, 144.0, 143.9, 141.4, 130.5, 130.4, 129.8 (C_q Ar), 129.6, 127.8, 127.2, 125.3, 120.1,

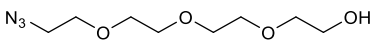
113.9 (Ar), 79.3 (C-2), 77.3 (C-3), 75.6 (C-4), 74.0 (CH_2 PMB), 72.7 (C-1), 71.8, 71.5 (CH_2 PMB), 70.0 (C-5), 67.1 (CH_2 Fmoc), 55.4 (CH_3 PMB), 53.6 (CH L-Lys), 47.2 (CH Fmoc), 39.1 (CH_2 ε-L-Lys), 36.1 (CH_2-9), 31.8 (CH_2-7), 29.0, 28.7, 22.3, 22.0 (CH_2-8, CH_2 β/γ/δ-L-Lys), 17.9 (CH_3-6); FT-IR (neat, cm^{-1}): 2930, 1719, 1612, 1512, 1451, 1302, 1247, 1174, 1080, 1034, 821, 741; HRMS: $[M+Na]^+$ calcd. for $C_{55}H_{64}N_2O_{12}Na$: 967.4357, found 967.4385; LC-MS: Rt = 9.38 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (22)



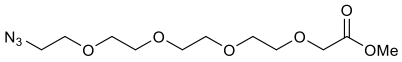
Triethylamine (10 mL, 74 mmol, 1.5 eq.) and *p*-toluenesulfonyl chloride (9.5 g, 50 mmol, 1.0 eq.) were added to a solution of tetraethyleneglycol (86 mL, 0.50 mol, 10 eq.) in DCM (62 mL) under an argon atmosphere. After stirring overnight, the reaction mixture was washed with H_2O (1x) and the aqueous layer was extracted with DCM (1x). The combined organic layers were washed three times with an aqueous solution of citric acid (0.28 M, 0.28 L). After concentration of the organic layer *in vacuo*, the title compound (16 g, 46 mmol, 93%) was obtained as a yellow oil. R_f: 0.76 (1/9 pentane/EtOAc); $[\alpha]_D^{20}$ +19.0° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.61 (d, 2H, *J* = 7.8 Hz, Ar), 7.19 (d, 2H, *J* = 7.9 Hz, Ar), 3.99 (t, 2H, *J* = 4.7 Hz, CH_2), 3.54 – 3.48 (m, 4H, 2x CH_2), 3.45 (s, 4H, 2x CH_2), 3.43 – 3.35 (m, 6H, 3x CH_2), 3.16 (s, 1H, OH), 2.27 (s, 3H, CH_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 144.5, 132.4 (C_q Ar), 129.5, 127.5 (Ar), 72.1, 70.1, 69.9, 69.8, 69.0, 68.1, 61.1 (CH_2), 21.2 (CH_3); FT-IR (neat, cm^{-1}): 2876, 1598, 1453, 1354, 1189, 1176, 1096, 1009, 922, 817, 776, 664, 555; HRMS: $[M+Na]^+$ calcd. for $C_{15}H_{23}N_3O_6SNa$: 373.1308, found 373.1132.

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (23)



NaN_3 (6.0 g, 92 mmol, 2.0 eq.) was added to a solution of compound **22** (16 g, 46 mmol, 1.0 eq.) in DMF (62 mL) and the obtained suspension was heated to 90°C overnight. The reaction mixture was diluted with H_2O and extracted with DCM (2x) and EtOAc (2x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo,* which gave compound **23** (10 g, 44 mmol, 96%) as a yellow oil. R_f: 0.22 (1/9 pentane/EtOAc); $[\alpha]_D^{20}$ +7.5° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 3.59 – 3.48 (m, 12H, 6x CH_2), 3.44 (dd, 2H, *J* = 5.4, 3.9 Hz, C*H*$_2$OH), 3.24 (t, 2H, CH_2N_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 72.3 (CH_2OH), 70.3, 70.2, 70.2, 69.9, 69.7, 61.2 (CH_2), 50.3 (CH_2N_3); FT-IR (neat, cm^{-1}): 2988, 1748, 1434, 1371, 1217, 1182, 1149, 1055, 1027, 973, 889, 601, 563, 501; HRMS: $[M+Na]^+$ calcd. for $C_8H_{17}N_3O_4Na$: 242.1117, found 242.1118.

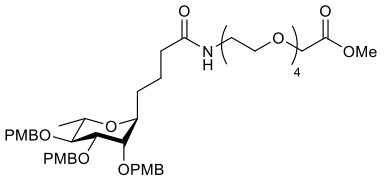
Methyl 14-azido-3,6,9,12-tetraoxatetradecanoate (19)



Compound **23** (2.2 g, 9.7 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under argon atmosphere and dissolved in THF (50 mL). The solution was cooled to 0°C and sodium hydride (60% dispersion in mineral oil, 0.78 g, 19 mmol, 2.0 eq.) was added. After stirring for 15 minutes, methyl bromoacetate (2.4 mL, 25 mmol, 2.6 eq.) was added and the mixture was allowed to warm-up to room temperature overnight. The reaction

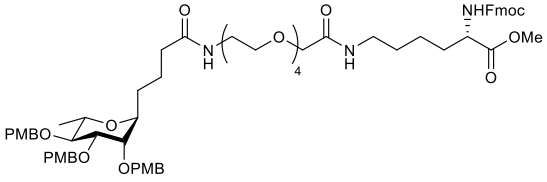
mixture was quenched with MeOH at 0°C and concentrated *in vacuo*. Purification by column chromatography (20→80% EtOAc in pentane) yielded compound **19** (2.3 g, 8.0 mmol, 83%) as a yellow oil. R_f: 0.51 (1/9 pentane/EtOAc); $[\alpha]_D^{20}$ +14.0° (*c* = 1.0, DCM); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 4.17 (s, 2H, CH_2), 3.77 – 3.71 (m, 5H, CH_2, OCH_3), 3.71 – 3.63 (m, 12H, 6x CH_2), 3.38 (t, 2H, *J* = 5.6, 4.5 Hz, CH_2N_3); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 171.0 (C=O), 71.0, 70.8, 70.8, 70.7, 70.2, 68.8 (CH_2), 51.9 (CH_3), 50.8 (CH_2N_3); FT-IR (neat, cm^{-1}): 2870, 2103, 1755, 1439, 1349, 1285, 1211, 1121, 942, 853, 706, 558; HRMS: $[M+Na]^+$ calcd. For $C_{11}H_{21}N_3O_6Na$: 314.1328, found 314.1331.

Methyl 3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate (20)



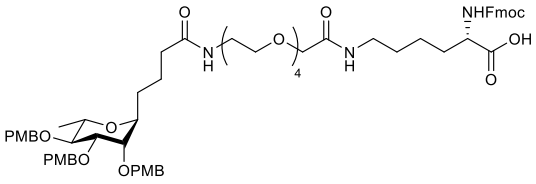
Compound **19** (0.18 g, 0.67 mmol, 3.0 eq.) was co-evaporated with toluene (3x) under argon atmosphere and dissolved in dry THF (6.5 mL). Pd/C (10%, 18 mg) was added and a $H_{2(g)}$-filled balloon replaced the argon balloon. The reaction was allowed to stir for 5 hours. The mixture was filtered over a Whatmann-filter and concentrated *in vacuo*. The obtained amine and was co-evaporated with toluene (2x) under argon atmosphere. Compound **17** (0.14 g, 0.22 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under argon atmosphere and dissolved in DMF (1.0 mL), followed by the addition of HCTU (0.11 g, 0.27 mmol, 1.2 eq.). After 15 minutes a solution of the obtained amine in DMF (0.20 mL) and DIPEA (0.11 mL, 0.69 mmol, 3.0 eq.) were added and the reaction mixture was stirred for 75 minutes. The reaction was quenched with 1 M HCl at 0°C and diluted with EtOAc. The organic layer was subsequently washed with 1 M HCl (1x) and brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (10→40% acetone in DCM) yielded the title compound (0.15 g, 0.18 mmol, 82% over two steps) as a sticky oil. R_f: 0.25 (4/1 DCM/acetone); $[\alpha]_D^{20}$ +44.3° (*c* = 2.0, DCM); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.31 – 7.25 (m, 4H, Ar), 7.25 – 7.19 (m, 2H, Ar), 6.92 – 6.84 (m, 6H, Ar), 6.53 (t, 1H, NH), 4.66 (d, 1H, *J* = 10.6 Hz, C*H*H PMB), 4.55 – 4.45 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.09 (s, 2H, CH_2 spacer), 3.89 – 3.82 (m, 1H, H-1), 3.77 (s, 9H, 3x CH_3 PMB), 3.70 (dd, 1H, *J* = 8.0, 3.2 Hz, H-3), 3.67 (s, 3H, OCH_3), 3.63 – 3.59 (m, 3H, H-4, CH_2 spacer), 3.58 – 3.55 (m, 2H, CH_2 spacer), 3.55 – 3.50 (m, 9H, H-5, 4x CH_2 spacer), 3.45 (t, 2H, *J* = 5.6 Hz, CH_2 spacer), 3.37 (t, 1H, *J* = 7.9 Hz, H-2), 3.29 (q, 2H, *J* = 5.6 Hz, CH_2 spacer), 2.12 (t, 2H, CH_2-9), 1.69 – 1.55 (m, 2H, C*H*H-8, C*H*H-7), 1.55 – 1.44 (m, 1H, C*H*H-8), 1.44 – 1.33 (m, 1H, C*H*H-7), 1.23 – 1.16 (m, 3H, CH_3-6); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 173.6, 171.8 (C=O), 160.2, 132.1, 131.9, 131.9 (C_q Ar), 130.6, 130.5, 130.5, 114.6, 114.6, 114.5 (Ar), 80.7 (C-2), 79.2 (C-3), 77.3 (C-4), 74.6 (CH_2 PMB), 74.1 (C-1), 72.0, 71.9 (CH_2 PMB), 71.4, 71.1, 71.0, 70.9, 70.4 (CH_2 spacer), 69.9 (C-5), 68.9 (CH_2 spacer), 55.9 (CH_3 PMB), 52.2 (OCH_3), 39.8 (CH_2 spacer), 36.2 (CH_2-9), 29.3 (CH_2-7), 22.9 (CH_2-8), 18.7 (CH_3-6); FT-IR (neat, cm^{-1}): 3331, 2872, 1754, 1716, 1648, 1607, 1585, 1512, 1460, 1351, 1301, 1250, 1170, 1101, 1031, 827, 770, 698, 582; HRMS: $[M+Na]^+$ calcd. for $C_{45}H_{63}O_{14}Na$: 864,4146, found 864.4169; LC-MS: Rt = 4.14 min (Gemini C_{18}, 50-90% MeCN, 11 min run).

N_α-Fmoc-N_ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate]-L-lysine-methyl ester (21)



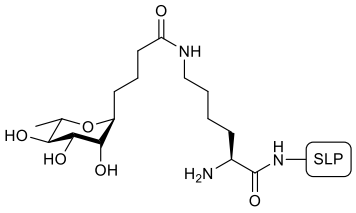
Compound **20** (0.48 g, 0.57 mmol, 1.0 eq.) was dissolved in a mixture of THF/H_2O (4/1 v/v, 5.7 mL) mixture and cooled to 0°C. LiOH·H_2O (73 mg, 1.7 mmol, 3.0 eq.) was added and the reaction was allowed to warm-up to room temperature and stirred for 40 minutes. The reaction mixture was diluted with EtOAc, acidified with 1 M HCl and subsequently washed with brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The obtained intermediate was co-evaporated with toluene (1x) under argon atmosphere and dissolved in DMF (2.9 mL). Fmoc-L-Lys-OMe·HCl (0.29 g, 0.69 mmol, 1.2eq.), HCTU (0.28 g, 0.68 mmol, 1.2eq.) and DIPEA (0.30 mL, 1.7 mmol, 3.0 eq.) were subsequently added. After 2 hours, the reaction mixture was diluted with EtOAc and quenched with 1 M HCl. The organic layer was washed with 1 M HCl (1x), sat. aq. $NaHCO_3$ (1x) and brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20→70% acetone in DCM) gave compound **21** (0.61 g, 0.51 mmol, 89%) as a sticky oil: R_f: 0.40 (8/1 DCM/acetone); $[\alpha]_D^{20}$ +24.3° (*c* = 2.0, DCM); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.85 – 7.78 (m, 2H, Ar), 7.71 – 7.63 (m, 2H, Ar), 7.45 – 7.37 (m, 2H, Ar), 7.36 – 7.29 (m, 2H, Ar), 7.29 – 7.23 (m, 4H, Ar), 7.23 – 7.18 (m, 2H, Ar), 7.16 (d, 1H, *J* = 7.8 Hz, NH), 6.91 – 6.82 (m, 6H, Ar), 6.54 (t, 1H, *J* = 5.8 Hz, NH), 6.27 (d, 1H, *J* = 7.9 Hz, N*H*Fmoc), 4.65 (d, 1H, *J* = 10.7 Hz, C*H*H PMB), 4.54 – 4.43 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.36 – 4.30 (m, 2H, CH_2 Fmoc), 4.24 – 4.18 (m, 1H, CH Fmoc), 4.15 – 4.06 (m, 1H, CH L-Lys), 3.90 – 3.81 (m, 3H, H-1, CH_2 spacer), 3.80 – 3.72 (m, 9H, 3x CH_3 PMB), 3.72 – 3.67 (m, 1H, H-3), 3.65 (s, 3H, OCH_3), 3.60 (t, 1H, *J* = 3.3 Hz, H-4), 3.59 – 3.47 (m, 13H, H-5, 6x CH_2 spacer), 3.46 (d, 2H, *J* = 5.2 Hz, CH_2 spacer), 3.37 (t, 1H, *J* = 7.9 Hz, H-2), 3.31 – 3.25 (m, 2H, CH_2 spacer), 3.24 – 3.12 (m, 2H, CH_2 ε-L-Lys), 2.15 – 2.10 (m, 2H, CH_2-9), 1.81 – 1.71 (m, 1H, C*H*H-8), 1.69 – 1.28 (m, 9H, C*H*H-8, CH_2-7, CH_2 β/γ δ-L-Lys), 1.19 (d, 3H, *J* = 3.1 Hz, CH_3-6); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 173.9, 173.5, 170.7 (C=O), 160.1, 157.1, 145.0, 142.1, 132.0, 131.8 (C_q Ar), 130.4, 128.6, 128.0, 126.1, 120.9, 114.5 (Ar), 80.6 (C-2), 79.2 (C-3), 77.3 (C-4), 74.5 (CH_2 PMB), 74.0 (C-1), 72.0, 71.8, 71.6, 71.1 (CH_2 spacer), 71.0, 70.9, 70.8 (CH_2 PMB), 70.7 (CH_2 spacer), 70.3 (CH_2 Fmoc), 69.8 (C-5), 67.1 (CH_2 Fmoc), 55.8 (CH_3 PMB), 55.0 (CH L-Lys), 52.7 (OCH_3), 47.9 (CH Fmoc), 39.7 (CH_2 spacer), 38.7, (CH_2 ε-L-Lys), 36.2 (CH_2-9), 31.6 (CH_2-7), 29.8, 29.7, 29.2, 23.5 (CH_2 β/γ/δ-L-Lys), 22.9 (CH_2-8), 18.6 (CH_3-6); FT-IR (neat, cm^{-1}): 3333, 2934, 1719, 1662, 1611, 1514, 1451, 1249, 1173, 1107, 1034, 822, 742; HRMS: $[M+Na]^+$ calcd. for $C_{66}H_{85}N_3O_{17}Na$: 1214.5777, found 1214.5812; LC-MS: Rt = 9.23 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

N_α-Fmoc-N_ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate]-L-lysine (9)



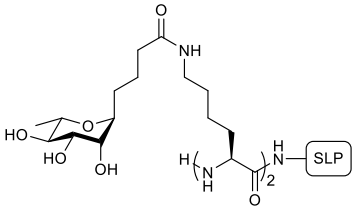
Compound **21** (0.34 g, 0.28 mmol, 1.0 eq.) was dissolved in THF (4.0 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 1.9 mL, 0.57 mmol, 2.0 eq.) was slowly added. After 45 minutes, the reaction mixture was diluted with EtOAc and acidified 1 M HCl to pH = 4/5. The organic layer was washed with brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (2→20% methanol in DCM) gave compound **9** (0.19 g, 0.16 mmol, 66%) as a sticky oil. R_f: 0.77 (4/1 DCM/MeOH); $[\alpha]_D^{20}$ +30.3° (*c* = 2.0, DCM); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.80 (d, 2H, *J* = 7.5 Hz, Ar), 7.71 – 7.59 (m, 2H, Ar), 7.46 – 7.11 (m, 10H, Ar,), 6.92 – 6.78 (m, 6H, Ar), 6.66 (s, 1H, NH), 6.19 (d, 1H, *J* = 7.7 Hz, N*H*Fmoc), 4.64 (d, 1H, *J* = 10.7 Hz, C*H*H PMB), 4.53 – 4.40 (m, 5H, 2x CH_2 PMB, C*H*H PMB), 4.31 (d, 2H, *J* = 7.0 Hz, CH_2 Fmoc), 4.20 (t, 1H, *J* = 7.1 Hz, CH Fmoc), 4.14 – 4.04 (m, 1H, CH L-Lys), 3.85 (s, 3H, H-1, CH_2 spacer), 3.80 – 3.72 (m, 9H, 3x CH_3 PMB), 3.70 – 3.66 (m, 1H, H-3), 3.60 (t, 1H, *J* = 3.2 Hz, H-5), 3.55 – 3.48 (m, 10H, 5x CH_2 spacer), 3.43 (t, 2H, *J* = 5.6 Hz, CH_2 spacer), 3.39 – 3.32 (m, 2H, H-2, H-4), 3.31 – 3.24 (m, 2H, CH_2 spacer), 3.22 – 3.13 (m, 2H, CH_2 ε-L-Lys), 2.16 – 2.07 (m, 2H, CH_2-9), 1.85 – 1.73 (m, 1H, C*H*H-8), 1.73 – 1.30 (m, 9H, C*H*H-8, CH_2-7, CH_2 β/γ/δ-L-Lys), 1.18 (d, 3H, *J* = 6.3 Hz, CH_3-6); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 159.9, 141.8, 131.7, 131.6, 131.5 (C_q Ar), 130.3, 130.2, 129.6, 128.9, 128.4, 127.8, 125.9, 120.7, 114.3, 114.2 (Ar), 80.4 (C-2), 78.9 (C-3), 77.0 (C-4), 74.2 (CH_2 PMB), 73.8 (C-1), 71.7, 71.6, 71.3 (CH_2 spacer), 70.8, 70.7 (CH_2 PMB), 70.5, 70.4, 70.1 (CH_2 spacer), 69.6 (C-5), 66.9 (CH_2 Fmoc), 55.6 (CH_3 PMB), 54.0 (CH L-lys), 47.7 (CH Fmoc), 39.5 CH_2 spacer, 38.6 (CH_2 ε-L-Lys), 36.0 (CH_2-9), 31.5 (CH_2-7), 29.5, 28.9, 23.2 (CH_2 β/γ/δ-L-Lys), 22.7 (CH_2-8), 18.3 (CH_3-6); FT-IR (neat, cm^{-1}): 3321, 2932, 1715, 1657, 1611, 1585, 1512, 1451, 1301, 1246, 1173, 1081, 1036, 821, 760, 733, 701, 662, 621, 583, 543, 515; HRMS: $[M+Na]^+$ calcd. for $C_{65}H_{83}N_3O_{17}Na$: 1200,5620, found 1200.5673; LC-MS: Rt = 8.79 min (Gemini C_{18}, 10-90% MeCN, 12.5 min run).

Lys(N_ε-[butan-4-(α-L-rhamnosyl)-amide])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (1)



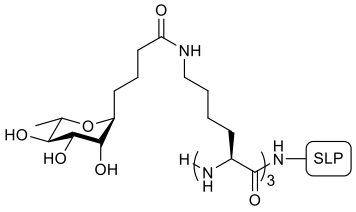
Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 10 µmol scale was elongated with **8** (3.0 eq., two hours coupling time). After cleaving from the resin, the peptide was purified by RP-HPLC. After lyophilisation, conjugate **1** (4.5 mg, 1.9 µmol, 19%) was obtained as a white solid. UPLC-MS: Rt = 3.56 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{109}H_{184}N_{25}O_{35}Na$: 2403.3, found 2403.6; HRMS: $[M+H]^{3+}$ calcd. for $C_{109}H_{186}O_{35}N_{25}$: 801.78422, found 801.78483.

Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (2)



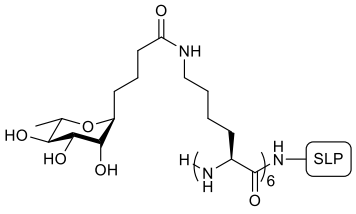
Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 10 µmol scale was elongated two times with **8** (3.0 eq., two hours coupling time). After cleaving from the resin, the peptide was purified by RP-HPLC. After lyophilisation, conjugate **2** (4.5 mg, 1.6 µmol, 16%) was obtained as a white solid. UPLC-MS: Rt = 3.49 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{125}H_{222}N_{27}O_{41}Na$: 2747.5, found 2749.5; HRMS: $[M+H]^{3+}$ calcd. for $C_{125}H_{214}O_{41}N_{27}$: 916.51580, found 916.51645.

Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (3)



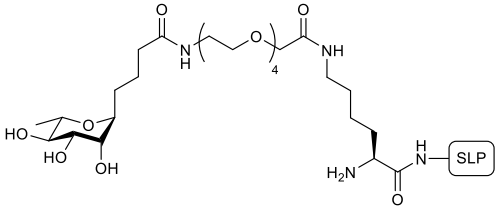
Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 10 µmol scale was elongated three times with **8** (3.0 eq., two hours coupling time). After cleaving from the resin, the peptide was purified by RP-HPLC. After lyophilisation, conjugate **3** (3.2 mg, 1.0 µmol, 10%) was obtained as a white solid. UPLC-MS: Rt = 3.47 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{141}H_{240}N_{29}O_{47}Na$: 3091.7, found 3095.5; HRMS: $[M+H]^{3+}$ calcd. for $C_{141}H_{242}O_{47}N_{29}$: 1031.24738, found 1031.25004.

Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Lys(*N*ε-[butan-4-(α-L-rhamnosyl)-amide])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (4)



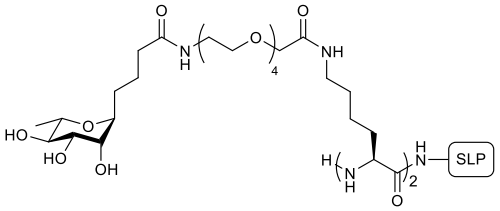
Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 10 µmol scale was elongated six times with **8** (3.0 eq., two hours coupling time). After cleaving from the resin, the peptide was purified by RP-HPLC. After lyophilisation, conjugate **4** (2.6 mg, 0.63 µmol, 6%) was obtained as a white solid. UPLC-MS: Rt = 3.34 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{189}H_{324}N_{35}O_{65}Na$: 4124.9, found 4128.7; HRMS: $[M+H]^{3+}$ calcd. for $C_{189}H_{326}O_{65}N_{35}$: 1375.44212, found 1375.44806.

Lys(*N*ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (5)



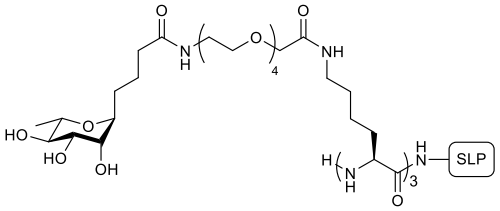
Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 5 µmol scale was elongated with **9** (3.0 eq.). Compound **9** (50 µL, 0.3 M in NMP) was preactivated by the addition of a solution of HATU (45µL, 0.67 M in NMP) and NMM (7 µL). The mixture was added to the resin and heated overnight to 39°C. MALDI analysis showed complete conversion of the starting peptide. Fmoc was cleaved using 3x 20% piperidine in NMP (400 µL) for 2, 5 and 10 min at RT. The resin was washed with NMP and DCM. The peptide was cleaved from the resin using TFA/H_2O (19/1 v/v, 1.0 mL, 3 h) and the peptide was precipitated in pentane/Et_2O (1/1 v/v, 12 mL). The precipitate was purified by RP-HPLC. After lyophilisation, conjugate **5** (2.0 mg, 0.76 µmol, 15%) was obtained as a white solid. UPLC-MS: Rt = 3.55 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{119}H_{203}N_{26}O_{40}Na$: 2636.5, found 2638.5; HRMS: $[M+H]^{3+}$ calcd. for $C_{119}H_{205}O_{40}N_{26}$: 879.49300, found 879.49355.

Lys(*N*ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Lys(*N*ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (6)



Two times elongation with **9** was performed using the conditions described for compound **5**. The synthesis was performed three times on 5 µmol scale with Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK. The combined precipitation was purified over RP-HPLC. After lyophilisation, conjugate **6** (0.9 mg, 0.28 µmol, 2%) was obtained as a white solid. UPLC-MS: Rt = 3.49 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{145}H_{251}N_{29}O_{51}Na$: 3214.8, found 3215.3; HRMS: $[M+H]^{3+}$ calcd. for $C_{145}H_{252}O_{51}N_{29}$: 1071.93335, found 1071.93421.

Lys(N_ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Lys(N_ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Lys(N_ε-[3,6,9,12-tetraoxatetra-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-α-L-rhamnosyl)-amide]-decanoate])-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-OH (7)



Tentagel S Ac resin loaded with LEQLESIINFEKLAAAAAK on 2.5 µmol scale was elongated three times with **9** (3.0 eq.). Compound **9** (25 µL, 0.6 M in NMP) was preactivated by the addition of a solution of HATU (22.5 µL, 0.67 M in NMP) and NMM (1.8 µL). The mixture was added to the resin and heated overnight to 39°C. MALDI analysis showed still starting peptide and the mixture was heated for an additional 4 hours at 43°C. Fmoc was cleaved using 3x 20% piperidine in NMP (300 µL) for 2, 5 and 10 min at RT. For the second coupling, compound **9** (25 µL, 0.6 M in NMP) was preactivated by the addition of a solution of HATU (22.5 µL, 0.67 M in NMP) and NMM (1.8 µL). The mixture was added to the resin and heated overnight to 39°C. Fmoc was cleaved using 3x 20% piperidine in NMP (300 µL) for 2, 5 and 10 min at RT. For the third coupling, compound **9** (27 µL, 0.6 M in NMP) was preactivated by the addition of a solution of HATU (20 µL, 0.67 M in NMP) and NMM (1.8 µL). The mixture was added to the resin and heated overnight to 39°C. Fmoc was cleaved using 3x 20% piperidine in NMP (300 µL) for 2, 5 and 10 min at RT. The resin was washed with NMP, DCM and Et_2O. The peptide was cleaved from the resin using TFA/H_2O (19/1 v/v, 0.5 mL, 4 h) and the peptide was precipitated in pentane/Et_2O (1/1 v/v, 10 mL). The precipitation was purified over RP-HPLC. After lyophilisation, conjugate **7** (0.4 mg, 0.11 µmol, 4%) was obtained as a white solid. UPLC-MS: Rt = 3.47 min (ACQUITY UPLC BEH C18, 5 - 100% MeCN, 10 min run); MALDI-TOF MS (m/z): $[M+Na]^+$ calcd. for $C_{171}H_{297}N_{32}O_{62}Na$: 3791.1, found 3793.7; HRMS: $[M+H]^{3+}$ calcd. for $C_{171}H_{299}O_{62}N_{32}$: 1264.37370, found 1264.37536.

Footnotes and References

(1) Toes, R. E.; Blom, R. J.; Offringa, R.; Kast, W. M.; Melief, C. J. *J. Immunol.* **1996**, *156* (10), 3911–3918.
(2) Bijker, M. S.; van den Eeden, S. J. F.; Franken, K. L.; Melief, C. J. M.; van der Burg, S. H.; Offringa, R. *Eur. J. Immunol.* **2008**, *38* (4), 1033–1042.
(3) Zwaveling, S.; Mota, S. C. F.; Nouta, J.; Johnson, M.; Lipford, G. B.; Offringa, R.; van der Burg, S. H.; Melief, C. J. M. *J. Immunol.* **2002**, *169* (1), 350–358.
(4) Vermaelen, K. *Front. Immunol.* **2019**, *10*.
(5) Temizoz, B.; Kuroda, E.; Ishii, K. J. *Int. Immunol.* **2016**, *28* (7), 329–338.
(6) Ignacio, B. J.; Albin, T. J.; Esser-Kahn, A. P.; Verdoes, M. *Bioconjug. Chem.* **2018**, *29* (3), 587–603.
(7) van Dinther, D.; Stolk, D. A.; van de Ven, R.; van Kooyk, Y.; de Gruijl, T. D.; den Haan, J. M. M. *J. Leukoc. Biol.* **2017**, *102* (4), 1017–1034.
(8) Takai, T. *Nat. Rev. Immunol.* **2002**, *2* (8), 580–592.
(9) Abdel-Motal, U.; Wang, S.; Lu, S.; Wigglesworth, K.; Galili, U. *J. Virol.* **2006**, *80* (14), 6943–6951.
(10) Abdel-Motal, U. M.; Guay, H. M.; Wigglesworth, K.; Welsh, R. M.; Galili, U. *J. Virol.* **2007**, *81* (17), 9131–9141.
(11) Perdomo, M. F.; Levi, M.; Sallberg, M.; Vahlne, A. *Proc. Natl. Acad. Sci.* **2008**, *105* (34), 12515–12520.
(12) Carlson, C. B.; Mowery, P.; Owen, R. M.; Dykhuizen, E. C.; Kiessling, L. L. *ACS Chem. Biol.* **2007**, *2* (2),

119–127.
(13) McEnaney, P. J.; Parker, C. G.; Zhang, A. X.; Spiegel, D. A. *ACS Chem. Biol.* **2012**, *7* (7), 1139–1151.
(14) Jakobsche, C. E.; Parker, C. G.; Tao, R. N.; Kolesnikova, M. D.; Douglass, E. F.; Spiegel, D. A. *ACS Chem. Biol.* **2013**, *8* (11), 2404–2411.
(15) Parker, C. G.; Domaoal, R. A.; Anderson, K. S.; Spiegel, D. A. *J. Am. Chem. Soc.* **2009**, *131* (45), 16392–16394.
(16) Fletcher, E. A. K.; van Maren, W.; Cordfunke, R.; Dinkelaar, J.; Codee, J. D. C.; van der Marel, G.; Melief, C. J. M.; Ossendorp, F.; Drijfhout, J. W.; Mangsbo, S. M. *J. Immunol.* **2018**, *201* (1), 87–97.
(17) Mangsbo, S. M.; Fletcher, E. A. K.; van Maren, W. W. C.; Redeker, A.; Cordfunke, R. A.; Dillmann, I.; Dinkelaar, J.; Ouchaou, K.; Codee, J. D. C.; van der Marel, G. A.; *et al*. *Mol. Immunol.* **2018**, *93*, 115–124.
(18) Huflejt, M. E.; Vuskovic, M.; Vasiliu, D.; Xu, H.; Obukhova, P.; Shilova, N.; Tuzikov, A.; Galanina, O.; Arun, B.; Lu, K.; *et al*. *Mol. Immunol.* **2009**, *46* (15), 3037–3049.
(19) Oyelaran, O.; McShane, L. M.; Dodd, L.; Gildersleeve, J. C. *J. Proteome Res.* **2009**, *8* (9), 4301–4310.
(20) Sarkar, S.; Lombardo, S. A.; Herner, D. N.; Talan, R. S.; Wall, K. A.; Sucheck, S. J. *J. Am. Chem. Soc.* **2010**, *132* (48), 17236–17246.
(21) Zhang, H.; Wang, B.; Ma, Z.; Wei, M.; Liu, J.; Li, D.; Zhang, H.; Wang, P. G.; Chen, M. *Bioconjug. Chem.* **2016**, *27* (4), 1112–1118.
(22) Sarkar, S.; Salyer, A. C. D.; Wall, K. A.; Sucheck, S. J. *Bioconjug. Chem.* **2013**, *24* (3), 363–375.
(23) Li, X.; Rao, X.; Cai, L.; Liu, X.; Wang, H.; Wu, W.; Zhu, C.; Chen, M.; Wang, P. G.; Yi, W. *ACS Chem. Biol.* **2016**, *11* (5), 1205–1209.
(24) Hossain, M. K.; Vartak, A.; Karmakar, P.; Sucheck, S. J.; Wall, K. A. *ACS Chem. Biol.* **2018**, *13* (8), 2130–2142.
(25) Chen, W.; Gu, L.; Zhang, W.; Motari, E.; Cai, L.; Styslinger, T. J.; Wang, P. G. *ACS Chem. Biol.* **2011**, *6* (2), 185–191.
(26) Myers, E. L.; Butts, C. P.; Aggarwal, V. K. *Chem. Commun.* **2006**, No. 42, 4434–4436.
(27) Sharma, P. K.; Kumar, S.; Kumar, P.; Nielsen, P. *Tetrahedron Lett.* **2007**, *48* (49), 8704–8708.
(28) See compound **35** of Chapter 4.
(29) Hiemstra, H. S.; Duinkerken, G.; Benckhuijsen, W. E.; Amons, R.; de Vries, R. R. P.; Roep, B. O.; Drijfhout, J. W. *Proc. Natl. Acad. Sci.* **1997**, *94* (19), 10313.

Chapter 6

*Synthesis of C-glycosyl amino acid building blocks suitable for solid phase peptide synthesis**

Introduction

Carbohydrates are involved in various inter- and intracellular recognition events and can be recognized by lectins leading to a row of biological processes. Lectins can function as pattern recognition receptors playing a role in innate immunity by promoting the secretion of cytokines and in adaptive immunity by contributing to endocytosis.[1,2] Examples of these receptors are the C-type lectins DC-SIGN and the mannose receptor, which are both present on dendritic cells. Since the binding interactions between lectins and their carbohydrate binding partners are often relatively weak, strong interactions therefore depend on multivalent binding. A lot of research has been devoted to the design, synthesis and evaluation of multivalent carbohydrate structures such as polymers, glycoconjugates and dendrimers to effect strong lectin binding for example to develop new therapeutics and more efficient vaccine therapies.[2] For instance, mannosylated polymers and peptides have been used as therapeutics against HIV, SARS and influenza virus.[3,4] These multivalent conjugates can not only be tailored to effectively mimic complex glycan structures[5–7], also their

*The data presented in this Chapter were gathered in collaboration with Nico J. Meeuwenoord, Herman S. Overkleeft, Gijsbert A. van der Marel and Jeroen D. C. Codée.

physical properties can be changed and tuned.[4,8,9] In this respect, the development of an automated solid phase assembly approach to deliver a coherent row of multivalent glycoconjugates will be beneficial. Ponader *et al.* developed a solid phase method to obtain homo- and hetero-multivalent glycooligomers using alkyne-functionalized building block, that were functionalized by a Cu-catalyzed cycloaddition with mannose, galactose or glucose synthons equipped with an azide.[10] However, *O*-glycosides[11] can be degraded enzymatically and are generally not stable enough to withstand the acidic conditions used in solid phase peptide synthesis. Therefore several groups have worked on the synthesis of *C*-glycosides[12,13] and their incorporation into a *C*-glycosyl amino acid building blocks[14–17] allowing an online solid phase peptide synthesis (SPPS) of glycopeptides. This chapter expands the library of *C*-glycoside functionalized lysine building blocks described in Chapters 3, 4 and 5 of this Thesis (Figure 1A) with α-mannose **1**, β-*N*-acetylglucosamine **2**, β- and α-galactose **3** and **4** functionalized lysine synthons (Figure 1B). These building blocks are suitable for Fmoc SPPS chemistry, and can be used for the synthesis of homo- and heteromultivalent glycomimetics. Comparable with the *C*-glycosides described in earlier Chapters, the route of synthesis to building blocks **1**-**4** comprised the key reactions shown in Figure 1C: introduction of the anomeric *C*-allyl group, cross metathesis to install the carboxylic acid and condensation with a suitably protected lysine. The monosaccharides are protected with acid-labile trityl, *p*-methoxybenzyl, isopropylidene, and benzylidene groups to allow a one-step protocol in the final stage of the SPPS that simultaneously removes all protecting groups and releases the glycopeptides or glycoclusters from the resin.

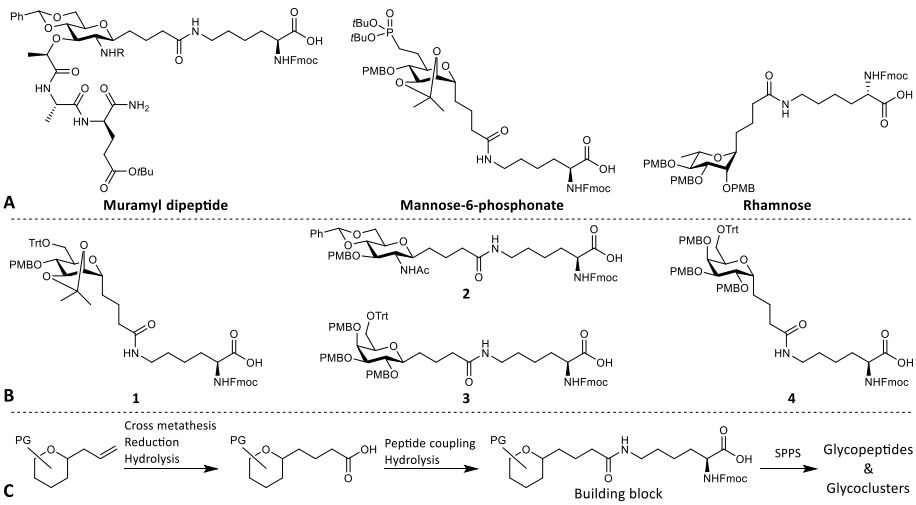


Figure 1. A) Structures of the *C*-glycoside SPPS building blocks described in previous Chapters of this Thesis; B) The SPPS building blocks **1**-**4** described in this Chapter; C) Key steps in the synthesis of the *C*-glycosidic SPPS building blocks; PG: protecting group.

Results & Discussion

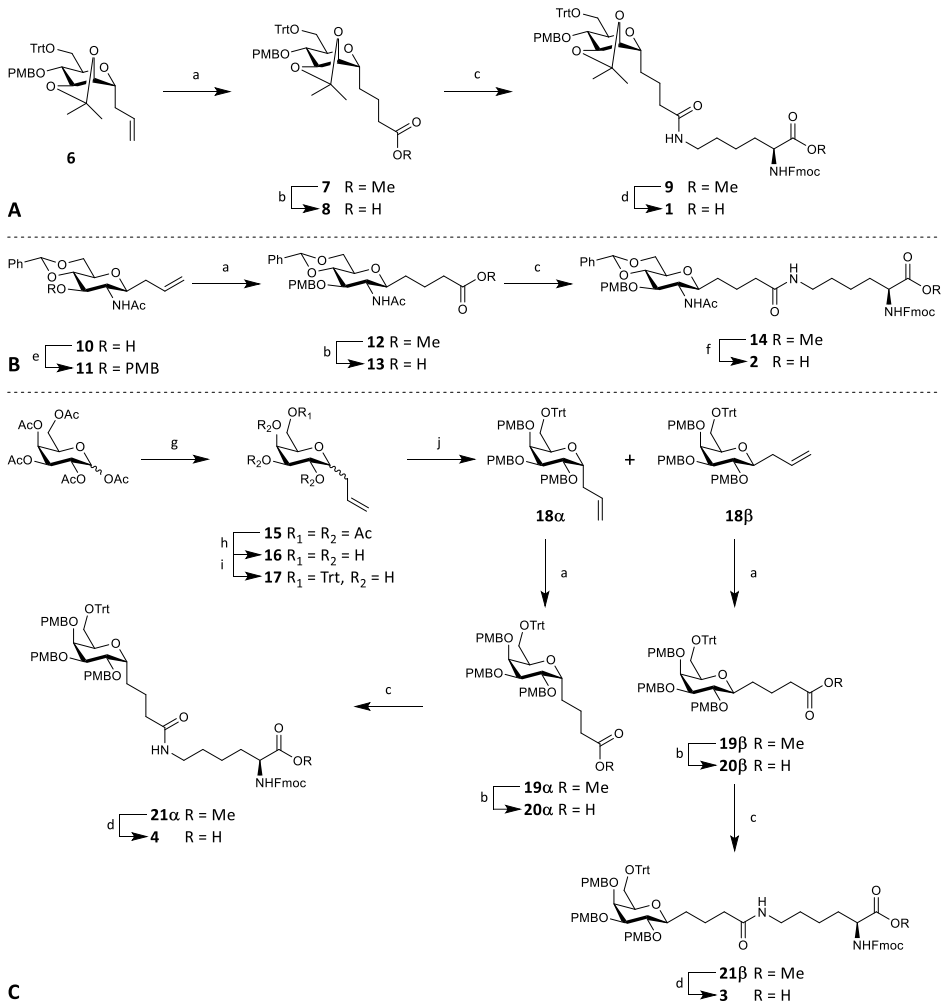
Mannose SPPS building block **1** was synthesized from fully protected allyl-*C*-mannose **6**[18], obtained as described in Chapter 4. Cross metathesis of **6** with methyl acrylate under influence of Grubbs 2nd generation catalyst was followed by reduction of the obtained α,β-unsaturated ester with $NaBH_4$ and ruthenium trichloride to give compound **7** in 73% yield over two steps (Scheme 1A).[19,20] Saponification of the methyl ester using LiOH yielded acid **8**, which was condensed with Fmoc-L-lysine-OMe[21] under the influence of HCTU and DIPEA to give **9** in 95% yield. Compound **9** was treated with LiOH at 0°C to obtain to SPPS building block **1**.

En route to GlcNAc SPPS building block **2**, the *C*-3-OH of *N*-acetyl *C*-allyl glucosamine **10**[22] (obtained as described in Chapter 3) was alkylated by treatment with *p*-methoxybenzyl-2,2,2-trichloroacetimidate and a catalytic amount of TfOH (Scheme 1B). The installation of the PMB-protecting group using sodium hydride and the alkyl chloride was low yielding, because of the presence of the *N*-acetyl function. Subsequently, a cross metathesis with methyl acrylate, reduction of the resulting double bond and hydrolysis of the obtained methyl ester led to acid **13** in 68% yield over three steps. Also, the coupling with Fmoc-L-lysine-OMe, as performed for the synthesis of **1**, went well and compound **14** was isolated in 78% yield after crystallization. However, selective hydrolysis of the methyl ester proved challenging due to the poor solubility of **14**. To solve this problem, the reaction was performed at room temperature, while closely monitoring the conversion with LC-MS. As partial Fmoc cleavage could not be prevented, the mixture was quenched with 1 M HCl and then treated with $NaHCO_3$, Fmoc *N*-hydroxysyccinimide ester to reinstall the Fmoc-protecting group. Subsequent precipitation with Et_2O and recrystallization from MeOH/DCM/Et_2O gave SPPS building block **2** in 91% yield.

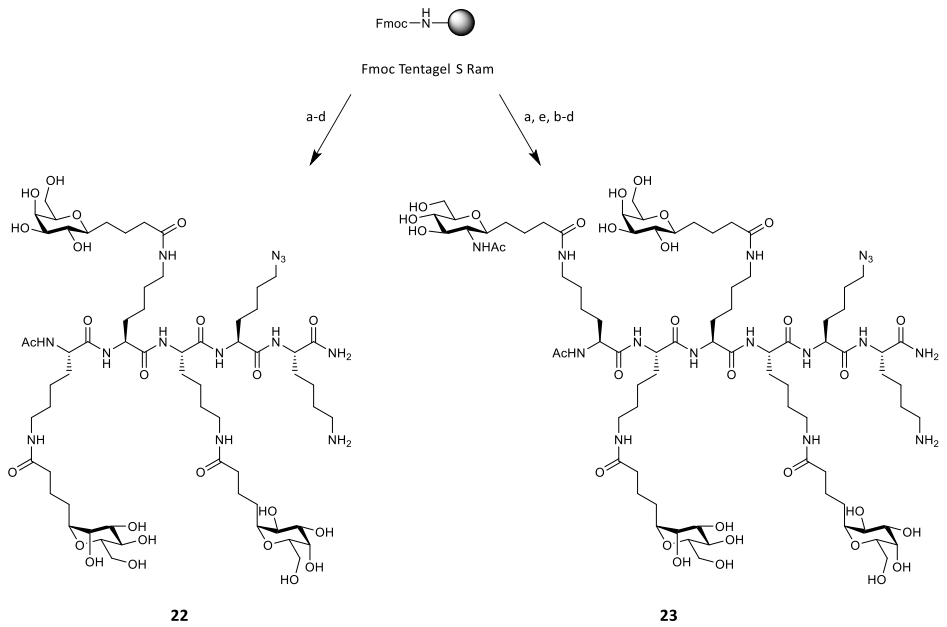
The synthesis of galactose SPPS building blocks **3** and **4** starts with the *C*-glycosylation of acetyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose with allyltrimethylsilane (Scheme 1C). Performing this reaction in MeCN gave **15** as an 6/1 α/β mixture (98% yield), while in nitromethane an 2/1 α/β mixture of (89% yield) was obtained. These reactions show, in line with literature precedent[23,24] that neighbouring group participation of the *C*-2-*O*-acetate is not a decisive factor in determining the stereochemical outcome of these reactions. The preference for the formation of the α-product, can be accounted for by the reactivity of the galactopyranosyl oxocarbenium ion.[25] Deacetylation of **15** with sodium methoxide and subsequent tritylation of the primary alcohol with TrtCl and Et_3N, produced compound **17** as an inseparable α/β mixture.[26] Alkylation of **17** with *p*-

methoxybenzyl chloride and separation of the anomers by chromatography yielded the individual α-anomer **18α** and β-anomer **18β**. Both anomers were subjected to the previously described cross metathesis, reduction of the double bond and saponification of the methyl ester to furnish acids **20α** and **20β**. The acids were condensed with Fmoc-L-lysine-OMe in the presence of HCTU and DIPEA to give **21α** and **21β**, which were carefully hydrolyzed with LiOH at 0°C to prevent Fmoc cleavage, providing galactose SPPS building blocks **3** and **4**.[27]

With the four *C*-glycoside SPPS building blocks (α-Man **1**, β-GlcNAc **2**, β-Gal **3**, α-Gal **4**) in hand the SPPS of glycopeptides **22** and **23**, which also feature an 6-azido lysine and a lysine to introduce further functionalities in the constructs, was undertaken (Scheme 2). Initial experiments showed that the condensations of building block **2** did not proceed well due to solubility issues. Therefore, pentamer **22** was synthesized first using the automated solid phase peptide synthesizer on Tentagel S Ram resin. The obtained immobilized peptide was then cleaved from the resin by treatment with a cocktail of TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 3 hours. Precipitation of the peptide from Et_2O, followed by RP-HPLC purification gave **22** (3.4 mg, 5% yield). For hexamer **23**, the immobilized peptide was treated with a mixture of **2**, HCTU and DIPEA in DMSO for two hours at 50°C. After a test cleavage, LC-MS showed that some unreacted pentamer was still present and therefore the resin was treated one more time with the previously mentioned mixture and shaken overnight. After cleaving from the resin and HPLC purification, **23** was obtained in 2% yield.



Scheme 1. Synthesis of mannose SPPS building blocks **1**-**4**. *Reagents and conditions*: a) *i*. methyl acrylate, CuI, Grubbs 2nd gen. catalyst, DCE, 50°C; *ii*. $NaBH_4$, $RuCl_3$, MeOH, DCE, 45°C, **7**: 73%, **12** 68%, **19α** 73%, **19β** 68% over two steps; b) LiOH, THF/H_2O/MeOH or THF/H_2O, 40°C, **8**: 96%, **13**: 100%, **20α**: 92%, **20β**: 96%; c) Fmoc-L-Lys-OMe, HCTU, DIPEA, DMF, **9**: 95%, **14**: 78%, **21α**: 90%, **21β**: 93%; d) LiOH, THF/H_2O, 0°C, **1**: 57%, **3**: 48%, **4**: 46%; e) *p*-methoxybenzyl-2,2,2-trichloroacetimidate, TfOH, THF, 78%; f) *i*. LiOH, THF/H_2O; *ii*. 1 M HCl; *iii*. $NaHCO_3$, Fmoc *N*-hydroxysyccinimide ester, 91%; g) allyltrimethylsilane, $BF_3{\cdot}OEt_2$, CH_3NO_2, 89%; h) NaOMe, MeOH, 91%; i) TrtCl, Et_3N, DMF, 60°C, 79%; j) *p*-methoxybenzyl chloride, NaH, DMF, **18α**: 52%, **18β**: 28%.



Scheme 2. SPPS synthesis of glycopeptides **22** and **23**. *Reagents and conditions*: a) *i*. 20% piperidine, DMF; *ii*. Fmoc SPPS cycle for Lys(α-*C*-Man)-Lys(β-*C*-Gal)-Lys(α-*C*-Gal)-Lys(N_3)-Lys(Boc); *iii*. 20% piperidine, DMF; b) Ac_2O, DIPEA, DMF; c) TFA/TIS/H_2O (95/2.5/2.5 v/v/v), 3h; d) RP-HPLC; e) *i*. **2**, HCTU, DIPEA, DMSO, 50°C, 2h; *ii*. **2**, HCTU, DIPEA, DMSO, overnight; **22**) 3.4 mg, 5%; **23**) 1.8 mg, 2%.

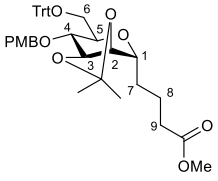
Conclusion

The synthesis of four *C*-glycosyl lysine building blocks and their application in SPPS is described. Key steps in the synthesis of the glycosyl amino acid are the installation of a *C*-allyl functionality on the carbohydrate and the ensuing Grubbs cross metathesis with methyl acrylate and subsequent reduction of the double bond. The building blocks were equipped with solely acid labile protecting groups to allow their concomitantly with release of the peptide from the resin, increasing assembly efficiency. Synthesis of a model penta- and hexapeptide showed that these building blocks are well suited to be used in an online solid phase peptide synthesis protocol. Despite its poor solubility, the GlcNAc building block could be coupled using DMSO as a solvent and coupling reactions at elevated temperature to provide the desired hexamer. The developed protecting group strategy allows one to combine the building blocks with many other functionalities in the target peptides, such as azide and alkyne click handles. The building blocks described in this Chapter will enable the rapid assembly of libraries of well-defined homo- and heteromultivalent glycopeptides and glycoclusters.

Experimental

All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H and ^{13}C spectra were recorded on a Bruker AV-400 (400/100 MHz) or a Bruker AV-500 Ultrashield (500/126 MHz) spectrometer and all individual signals were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. Automated solid phase peptide synthesis was performed on an Applied Biosystems 433A Peptide Synthesizer. The glycopeptides were purified with a Gilson GX-281 preparative HPLC with a Gemini-NX 5u, C18, 110 Å, 250 x 10.0 mm column with NH_4OAc. Optical rotations were measured on an Anton Paar Modular Circular Polarimeter MCP 100/150. High resolution mass spectra were recorded on a Q Exactive HF Orbitrap equipped with an electron spray ion source positive mode. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR.

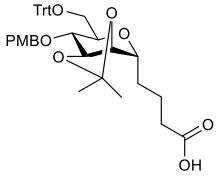
Methyl 4-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-*O*-trityl-*α*-D-mannopyranosyl)-butanoate (7)



Compound **6**[18] (5.7 g, 9.4 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere, before being dissolved in dry DCE (0.10 L). Methyl acrylate (2.4 mL, 26 mmol, 2.8 eq.), CuI (0.28 g, 1.5 mmol, 0.16 eq.) and Grubbs 2nd generation catalyst (0.32 g, 0.38 mmol, 0.04 eq.) were added and the flask was covered in aluminum foil. The suspension was heated to 50°C and stirred for 48 hours, after which it was concentrated *in vacuo* and co-evaporated with toluene (3x). Purification by column chromatography (10→70% Et_2O in pentane) afforded the intermediate (4.9 g, 7.4 mmol, 1.0 eq.), which was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry DCE (37 mL). Two empty balloons were placed on the flask, followed by the addition of ruthenium trichloride (0.29 g, 1.4 mmol, 0.19 eq.) and $NaBH_4$ (0.89 g, 24 mmol, 3.2 eq.) at 0°C. Methanol (6.0 mL, 0.15 mol, 20 eq.) was carefully added to the suspension over 20 minutes, after which the mixture was allowed to warm-up up to room temperature over 20 minutes. The mixture was subsequently heated to 45°C for 4 hours. The reaction mixture was cooled to room temperature, diluted with brine, filtered over

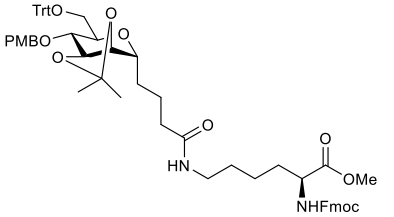
celite and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (20→60% Et_2O in pentane) gave compound **7** (4.6 g, 6.9 mmol, 73% over two steps). R_f: 0.22 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ +16.1° (*c* = 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.56 – 7.50 (m, 6H, Ar), 7.37 – 7.31 (m, 6H, Ar), 7.31 – 7.27 (m, 3H, Ar), 7.03 – 6.98 (m, 2H, Ar), 6.81 – 6.76 (m, 2H, Ar), 4.74 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.38 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.31 (t, 1H, *J* = 6.9 Hz, H-3), 4.08 (t, 1H, *J* = 6.4 Hz, H-2), 3.92 (q, 1H, *J* = 6.3 Hz, H-1), 3.84 – 3.69 (m, 5H, H-4, H-5, CH_3 PMB), 3.64 (s, 3H, OCH_3), 3.43 (dd, 1H, *J* = 9.9, 2.1 Hz, C*H*H-6), 3.23 (dd, 1H, *J* = 9.9, 5.0 Hz, C*H*H-6), 2.44 (t, 2H, *J* = 7.3 Hz, CH_2-9), 2.11 – 1.99 (m, 1H, C*H*H-8), 1.91 – 1.79 (m, 1H, C*H*H-8), 1.79 – 1.68 (m, 2H, CH_2-7), 1.55 (s, 3H, CH_3 isopropylidene), 1.42 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.8 (C=O), 159.1, 144.0, 130.1 (C_q Ar), 129.7, 128.8, 127.8, 126.9, 113.6 (Ar), 109.2 (C_q isopropylidene), 86.4 (C_q Trt), 78.8 (C-3), 77.1 (C-2), 75.5 (C-4), 72.7 (C-5), 72.7 (CH_2 PMB), 72.5 (C-1), 63.4 (CH_2-6), 55.2 (CH_3 PMB), 51.5 (OCH_3), 33.8 (CH_2-9), 32.0 (CH_2-7), 27.7, 25.6 (CH_3 isopropylidene), 21.0 (CH_2-8); FT-IR (neat, cm^{-1}): 2987, 2934, 1736, 1612, 1514, 1491, 1449, 1380, 1302, 1246, 1217, 1162, 1071, 1034, 1002, 900 867, 822, 766, 748, 705, 633; HRMS: $[M+Na]^+$ calcd. for $C_{41}H_{46}O_8Na$: 689.30849, found 689.30821.

4-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-*O*-trityl-*α*-D-mannopyranosyl)-butanoic acid (8)



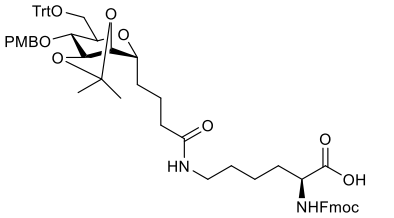
Methyl ester **7** (4.6 g, 6.9 mmol, 1.0 eq.) was dissolved in in a mixture of THF/H_2O/MeOH (7/1/2, v/v/v, 35 mL). LiOH (0.87 g, 21 mmol, 3.0 eq.) was added and the mixture was heated to 40°C for 8 hours, after which TLC analysis showed complete conversion of the starting material. The reaction mixture was cooled to 0°C, acidified with 1 M HCl to pH = 6, diluted with H_2O and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The title compound was obtained (4.3 g, 6.6 mmol, 96%) and used without further purification. R_f: 0.85 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +16.0° (*c* = 0.43, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.51 – 7.43 (m, 6H, Ar), 7.34 – 7.20 (m, 9H), 7.01 – 6.92 (m, 2H, Ar), 6.79 – 6.71 (m, 2H, Ar), 4.69 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.34 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.26 (t, 1H, *J* = 6.9 Hz, H-3), 4.03 (t, 1H, *J* = 6.5 Hz, H-2), 3.91 – 3.81 (m, 1H, H-1), 3.79 (s, 3H, CH_3 PMB), 3.78 – 3.65 (m, 2H, H-4, H-5), 3.38 (dd, 1H, *J* = 9.9, 2.1 Hz, C*H*H-6), 3.18 (dd, 1H, *J* = 9.9, 4.9 Hz, C*H*H-6), 2.43 (t, 2H, *J* = 7.2 Hz, CH_2-9), 2.00 (m, 1H, C*H*H-8), 1.85 – 1.63 (m, 3H, C*H*H-8, CH_2-7), 1.50 (s, 3H, CH_3 isopropylidene), 1.38 (s, 3H, CH_3 isopropylidene); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 179.2 (C=O), 159.2, 144.1, 130.2 (C_q Ar), 129.8, 128.9, 127.9, 127.0, 113.7 (Ar), 109.4 (C_q isopropylidene), 86.5 (C_q Trt), 78.8 (C-3), 77.1 (C-2), 75.6 (C-4), 72.9 (C-5), 72.8 (CH_2 PMB), 72.6 (C-1), 63.4 (CH_2-6), 55.4 (CH_3 PMB), 33.8 (CH_2-9), 32.0 (CH_2-7), 27.8, 25.7 (CH_3 isopropylidene), 20.8 (CH_2-8); FT-IR (neat, cm^{-1}): 3058, 2987, 2934, 1707, 1612, 1586, 1513, 1490, 1449, 1381, 1302, 1245, 1216, 1160, 1068, 1034, 1002, 900, 866, 822, 777, 765, 737, 703, 644, 632; HRMS: $[M+Na]^+$ calcd. for $C_{40}H_{44}O_8Na$: 675.29284, found 675.29260.

N_α-Fmoc-N_ε-[butan-4-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-*O*-trityl-*α*-D-mannopyranosyl)-amide]-L-lysine-methyl ester (9)



Compound **8** (3.8 g, 5.8 mmol, 1.0 eq.) and Fmoc-L-lysine-OMe[21] (2.9 g, 7.0 mmol, 1.2 eq.) were dissolved in DMF (30 mL). HCTU (2.9 g, 7.0 mmol, 1.2 eq.) and DIPEA (3.0 mL, 17 mmol, 3.0 eq.) were added and the solution was stirred for 4 hours. The reaction mixture was diluted with EtOAc and was washed with 1 M HCl (1x), sat. aq. $NaHCO_3$ (1x), brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (2→30% acetone in DCM) yielded the title compound (5.6 g, 5.5 mmol, 95%). R_f: 0.38 (9/1 DCM/acetone); $[\alpha]_D^{25}$ +11.5° (*c* = 0.33, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.78 – 7.74 (m, 2H, Ar), 7.63 – 7.58 (m, 2H, Ar), 7.48 – 7.42 (m, 6H, Ar), 7.42 – 7.37 (m, 2H, Ar), 7.34 – 7.21 (m, 11H, Ar), 6.96 – 6.91 (m, 2H, Ar), 6.75 – 6.70 (m, 2H, Ar), 5.43 (d, 1H, *J* = 8.2 Hz, NH), 5.31 (t, 1H, *J* = 5.8 Hz, N*H*Fmoc), 4.68 (d, 1H, *J* = 10.9 Hz, C*H*H PMB), 4.47 – 4.35 (m, 2H, CH_2 Fmoc), 4.34 – 4.19 (m, 4H, H-3, CH L-Lys, C*H*H PMB, CH Fmoc), 3.99 (t, 1H, *J* = 6.7 Hz, H-2), 3.83 – 3.76 (m, 4H, H-1, CH_3 PMB), 3.74 (s, 3H, CH_3 PMB), 3.71 – 3.66 (m, 2H, H-4, H-5), 3.37 (dd, 1H, *J* = 9.9, 1.4 Hz, C*H*H-6), 3.18 – 3.11 (m, 1H, C*H*H-6), 3.07 – 2.93 (m, 2H, CH_2 ε-L-Lys), 2.22 – 2.14 (m, 2H, CH_2-9), 1.92 – 1.81 (m, 2H, CH_2-8), 1.79 – 1.53 (m, 4H, CH_2-7, 1x CH_2 β/γ/δ-L-Lys), 1.49 (s, 3H, CH_3 isopropylidene), 1.36 (s, 3H, CH_3 isopropylidene), 1.25 – 1.19 (m, 4H, 2x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 172.8, 159.2 (C=O), 156.1, 144.0, 143.9, 141.4, 130.1 (C_q Ar), 129.8, 128.9, 127.9, 127.8, 127.2, 125.2, 125.2, 120.1, 113.7 (Ar), 109.4 (C_q isopropylidene), 86.6 (C_q Trt), 78.9 (C-3), 77.2 (C-2), 75.5 (C-4), 73.1 (C-5), 72.7 (CH_2 PMB), 72.7 (C-1), 67.0 (CH_2 Fmoc), 63.4 (CH_2-6), 55.3 (CH_3 PMB), 53.8 (CH Fmoc), 52.5 (OCH_3), 47.3 (CH L-Lys), 38.9 (CH_2 ε-L-Lys), 36.1 (CH_2-9), 32.1 (CH_2-7), 31.9, 29.0 (CH_2 β/γ/δ-L-Lys), 27.7, 25.5 (CH_3 isopropylidene), 22.5 (CH_2 β/γ/δ-L-Lys), 21.9 (CH_2-8); FT-IR (neat, cm^{-1}): 3315, 2935, 1722, 1654, 1612, 1514, 1449, 1380, 1247, 1213, 1175, 1077, 1033, 849, 761, 740, 704, 633, 563; HRMS: $[M+Na]^+$ calcd. for $C_{62}H_{68}O_{11}N_2Na$: 1039.47153, found 1039.47134.

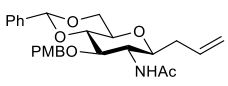
N_α-Fmoc-N_ε-[butan-4-(2,3-*O*-isopropylidene-4-*O*-*p*-methoxybenzyl-*6*-*O*-trityl-*α*-D-mannopyranosyl)-amide]-L-lysine (1)



Compound **9** (3.05 g, 3.00 mmol, 1.0 eq.) was dissolved in THF (30 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 20 mL, 6.0 mmol, 2.0 eq.) was added and the mixture was stirred vigorously for 30 minutes, after which the mixture was diluted with EtOAc and acidified by the addition of 1 M HCl to pH = 3-4. The mixture was washed with brine (1x) and the aqueous layer was extracted with EtOAc (1x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (1→8% MeOH in DCM) the title compound (1.73 g, 1.72 mmol, 57%) was obtained as a white foam. R_f: 0.61 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +20.8° (*c* = 0.62, $CHCl_3$); ^{1}H NMR (MeOD, 400 MHz, HH-COSY,

HSQC): δ 7.77 (d, 2H, *J* = 7.5 Hz, Ar), 7.69 – 7.63 (m, 2H, Ar), 7.45 – 7.34 (m, 8H, Ar), 7.33 – 7.18 (m, 11H, Ar), 6.95 – 6.88 (m, 2H, Ar), 6.75 – 6.68 (m, 2H, Ar), 4.61 (d, 1H, *J* = 11.0 Hz, C*H*H PMB), 4.34 (d, 2H, *J* = 7.0 Hz, CH_2 Fmoc), 4.29 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.25 – 4.16 (m, 2H, H-3, CH Fmoc), 4.09 (dd, 1H, *J* = 9.4, 4.6 Hz, CH L-Lys), 4.01 (t, 1H, *J* = 6.4 Hz, H-2), 3.81 (dd, 1H, *J* = 7.7, 5.3 Hz, H-1), 3.75 (s, 3H, CH_3 PMB), 3.69 (dd, 1H, *J* = 9.5, 7.3 Hz, H-4), 3.62 – 3.55 (m, 1H, H-5), 3.35 – 3.30 (m, 1H, C*H*H-6), 3.11 (t, 2H, *J* = 6.7 Hz, CH_2 ε-L-Lys), 3.05 (dd, 1H, *J* = 9.9, 5.2 Hz, C*H*H-6), 2.26 – 2.16 (m, 2H, CH_2-9), 1.98 – 1.86 (m, 1H, C*H*H-8), 1.86 – 1.68 (m, 2H, C*H*H-8, C*H*H-7), 1.68 – 1.56 (m, 3H, C*H*H-7, 1x CH_2 β/γ/δ-L-Lys), 1.50 – 1.30 (m, 10H, 2x CH_2 β/γ/δ-L-Lys, 2x CH_3 isopropylidene); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 175.8, 160.7 (C=O), 145.3, 142.6, 131.3 (C_q Ar), 130.8, 130.0, 128.8, 128.2, 128.1, 126.3, 120.9, 114.5 (C_q Ar), 110.5 (C_q isopropylidene), 87.8 (C_q Trt), 80.0 (C-3), 78.4 (C-2), 76.6 (C-4), 74.0 (C-5), 73.8 (C-1), 73.6 (CH_2 PMB), 67.8 (CH_2 Fmoc), 64.6 (CH_2-6), 55.7 (CH_3 PMB), 55.4 (CH L-Lys) 48.4 (CH Fmoc), 40.1 (CH_2 ε-L-Lys), 36.8 (CH_2-9), 32.9 (CH_2-7), 30.3, 29.9 (CH_2 β/γ/δ-L-Lys), 28.0, 25.7 (CH_3 isopropylidene), 24.3 (CH_2 β/γ/δ-L-Lys), 23.3 (CH_2-8); FT-IR (neat, cm^{-1}): 3330, 2934, 1716, 1612, 1513, 1449, 1381, 1302, 1246, 1213, 1179, 1160, 1067, 1033, 1002, 900, 865, 822, 760, 735, 701, 646, 632, 621, 516; LC-MS: Rt = 13.48 min (Vydac 219TP 5 µm Diphenyl, 10 - 90% MeCN, 21 min run); ESI-MS: m/z 1025.4 $[M+Na]^+$; HRMS: $[M+H]^+$ calcd. for $C_{61}H_{67}O_2N_{11}$: 1003.47394, found 1003.47380.

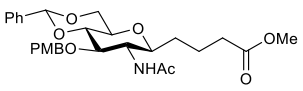
3-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-glucopyranosyl)-1-propene (11)



Alcohol **10**[28] (1.6 g, 4.9 mmol, 1.0 eq.) was co-evaporated with toluene (1x) under an argon atmosphere, followed by the addition of dry THF (0.12 L) and *p*-methoxybenzyl-2,2,2-trichloroacetimidate (3.1 mL, 15 mmol, 3.0 eq.). The suspension was cooled to 0°C and a solution of TfOH in THF (0.01 M, 50 mL, 0.5 mmol, 0.10 eq.) was added. After 15 minutes, the mixture was allowed to warm up to room temperature and stirred for 3 hours. The obtained solution was neutralized with Et_3N and diluted with EtOAc and washed with sat. aq. $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was embedded on silica and purified by column chromatography (2→20% acetone in DCM). The title compound was obtained (1.7 g, 3.8 mmol, 78%) as a white solid. R_f: 0.45 (9/1 DCM/acetone); $[\alpha]_D^{25}$ -58.4° (*c* = 0.32, $CHCl_3$/MeOH 1/1); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.53 – 7.48 (m, 2H, Ar), 7.42 – 7.35 (m, 3H, Ar), 7.25 – 7.21 (m, 2H, Ar), 6.89 – 6.84 (m, 2H, Ar), 5.88 – 5.75 (m, 1H, CH_2-C*H*=CH_2), 5.58 (s, 1H, CH benzylidene), 5.15 – 5.00 (m, 3H, NH, CH_2-CH=C*H*$_2$), 4.82 (d, 1H, *J* = 11.8 Hz, C*H*H PMB), 4.61 (d, 1H, *J* = 11.7 Hz, C*H*H PMB), 4.32 (dd, 1H, *J* = 10.4, 5.0 Hz, C*H*H-6), 3.80 (s, 3H, CH_3 PMB), 3.77 – 3.64 (m, 4H, H-2, H-3, H-4, C*H*H-6), 3.51 – 3.44 (m, 1H, H-1), 3.44 – 3.37 (m, 1H, H-5), 2.39 – 2.31 (m, 1H, C*H*H-CH=CH_2), 2.28 – 2.18 (m, 1H, C*H*H-CH=CH_2), 1.90 (s, 3H, CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.3 (C=O), 159.5, 137.6 (C_q Ar), 134.4 (CH_2-*C*H=CH_2), 130.6 (C_q Ar), 130.2, 129.1, 128.4, 126.1 (Ar), 117.2 (CH_2-CH=*C*H_2), 113.9 (Ar), 101.2 (CH benzylidene), 83.0 (C-4), 79.2 (C-1), 77.8 (C-3), 73.5 (CH_2 PMB), 70.5 (C-5), 69.0 (CH_2-6), 55.4 (CH_3 PMB), 55.1 (C-2), 36.6 (*C*H_2-CH=CH_2), 23.7 (CH_3 Ac); FT-IR (neat, cm^{-1}): 3277, 2871, 1651, 1615, 1555, 1514, 1454, 1371, 1302, 1249, 1172, 1132, 1102, 1034, 1015,

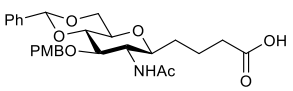
962, 919, 821, 749, 697, 592, 516; HRMS: $[M+H]^+$ calcd. for $C_{26}H_{32}O_6N$: 454.22241, found 454.22225.

Methyl 4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-glucopyranosyl)-butanoate (12)



Compound **11** (1.4 g, 3.1 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in dry DCE (31 mL). Methyl acrylate (0.78 mL, 8.6 mmol, 2.8 eq.), CuI (90 mg, 0.47 mmol, 0.15 eq.) and Grubbs 2nd generation catalyst (0.26 g, 0.31 mmol, 0.10 eq.) were added and the flask was covered in aluminum foil. The suspension was heated to 50°C overnight, after which it was concentrated *in vacuo* and co-evaporated with toluene (3x) under an argon atmosphere. The residue was dissolved in dry DCE (31 mL) and cooled to 0°C. Ruthenium trichloride (0.12 g, 0.58 mmol, 0.19 eq.) and $NaBH_4$ (0.37 g, 9.8 mmol, 3.2 eq.) were added, and an empty balloon was placed on the flask. Methanol (2.5 mL, 61 mmol, 20 eq.) was carefully added to the suspension over 20 minutes, after which the mixture was allowed to warm-up up to room temperature over 25 minutes. The mixture was subsequently heated to 45°C for 7 hours. The reaction mixture was cooled to room temperature, diluted with brine and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (8→15% acetone in DCM) gave compound **12** (1.1 g, 2.1 mmol, 68% over two steps). R_f: 0.41 (8/2 DCM/acetone); $[\alpha]_D^{25}$ -56.5° (*c* = 0.72, $CHCl_3$/MeOH 1/1); ^1H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.51 (dd, 2H, *J* = 7.7, 1.7 Hz, Ar), 7.42 – 7.35 (m, 3H, Ar), 7.23 (d, 2H, *J* = 8.6 Hz, Ar), 6.88 – 6.83 (m, 2H, Ar), 5.57 (s, 1H, CH benzylidene), 5.15 (d, 1H, *J* = 9.0 Hz, NH), 4.81 (d, 1H, *J* = 11.8 Hz, C*H*H PMB), 4.60 (d, 1H, *J* = 11.8 Hz, C*H*H PMB), 4.30 (dd, 1H, *J* = 10.5, 5.0 Hz, C*H*H-6), 3.79 (s, 3H, CH_3 PMB), 3.77 – 3.55 (m, 7H, H-2, H-3, H-4, C*H*H-6, OCH_3), 3.40 – 3.30 (m, 2H, H-1, H-5), 2.31 – 2.25 (m, 2H, CH_2-9), 1.89 (s, 3H, CH_3 Ac), 1.87 – 1.76 (m, 1H, C*H*H-8), 1.66 – 1.54 (m, 2H, C*H*H-7, C*H*H-8), 1.50 – 1.38 (m, 1H, C*H*H-7); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.1 (C=O), 170.3, 159.4, 137.6, 130.6 (C_q Ar), 130.1, 129.0, 128.4, 126.1, 113.9 (Ar), 101.2 (CH benzylidene), 83.0 (C-4), 79.6 (C-1), 77.9 (C-3), 73.4 (CH_2 PMB), 70.4 (C-5), 69.0 (CH_2-6), 55.4 (CH_3 PMB), 55.0 (C-2), 51.6 (OCH_3), 33.8 (CH_2-9), 31.4 (CH_2-7), 23.7 (CH_3 Ac), 21.1 (CH_2-8); FT-IR (neat, cm^{-1}): 3269, 2877, 1741, 1648, 1558, 1514, 1452, 1367, 1324, 1247, 1200, 1171, 1132, 1092, 1032, 957, 858, 822, 749, 695, 618; HRMS: $[M+H]^+$ calcd. for $C_{28}H_{36}O_8N_1$: 514.24354, found 514.24353.

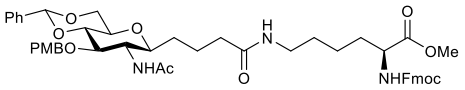
4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-glucopyranosyl)-butanoic acid (13)



To a white suspension of methyl ester **12** (1.0 g, 2.0 mmol, 1.0 eq.) in THF/H_2O/MeOH (7/1/2, v/v/v, 20 mL) was added LiOH (0.26 g, 6.2 mmol, 3.1 eq.). The mixture was heated to 40°C for 3 hours, after which TLC analysis showed complete conversion of the starting material. The reaction was diluted with EtOAc and acidified with 1 M HCl to pH = 6-7, followed by the extraction of the aqueous layer with EtOAc (1x) and DCM (1x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in*

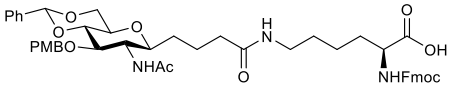
vacuo. The title compound was obtained (1.0 g, 2.0 mmol, 100%) and used without further purification. R_f: 0.45 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ -54.3° (*c* = 0.28, $CHCl_3$/MeOH 1/1); ^{1}H NMR (DMSO, 400 MHz, HH-COSY, HSQC): δ 11.99 (s, 1H, OH), 7.89 (d, 1H, *J* = 9.0 Hz, NH), 7.47 – 7.34 (m, 5H, Ar), 7.18 (d, 2H, *J* = 8.7 Hz, Ar), 6.85 (d, 2H, *J* = 8.7 Hz, Ar), 5.68 (s, 1H, CH benzylidene), 4.64 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.51 (d, 1H, *J* = 11.4 Hz, C*H*H PMB), 4.21 (dd, 1H, *J* = 10.1, 4.9 Hz, C*H*H-6), 3.77 – 3.53 (m, 7H, H-2, H-3, H-4, C*H*H-6, CH_3 PMB), 3.40 – 3.26 (m, 2H, H-1, H-5), 2.18 (t, 2H, *J* = 7.2 Hz, CH_2-9), 1.83 (s, 3H, CH_3 Ac), 1.73 – 1.62 (m, 1H, C*H*H-8), 1.58 – 1.41 (m, 2H, C*H*H-7, C*H*H-8), 1.34 – 1.22 (m, 1H, C*H*H-7); ^{13}C-APT NMR (DMSO, 101 MHz, HSQC): δ 174.4 (C=O), 169.2, 158.6, 137.8, 130.9 (C_q Ar), 129.0, 128.7, 128.1, 126.0, 113.4 (Ar), 100.0 (CH benzylidene), 81.4 (C-4), 79.7 (C-1), 79.0 (C-3), 72.9 (CH_2 PMB), 70.0 (C-5), 68.0 (CH_2-6), 55.0 (CH_3 PMB), 53.9 (C-2), 33.5 (CH_2-9), 30.8 (CH_2-7), 22.9 (CH_3 Ac), 20.8 (CH_2-8); FT-IR (neat, cm^{-1}): 2870, 2428, 1725, 1597, 1516, 1489, 1366, 1302, 1274, 1251, 1223, 1172, 1136, 1109, 1088, 1041, 1020, 966, 923, 822, 747, 694, 606, 539; HRMS: $[M+H]^+$ calcd. for $C_{27}H_{34}O_8N_1$: 500.22789, found 500.22784.

N_α-Fmoc-N_ε-[butan-4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-glucopyranosyl)-amide]-L-lysine-methyl ester (14)



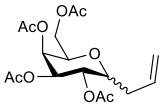
Acid **13** (0.97 g, 1.9 mmol, 1.0 eq.) and Fmoc-L-lysine-OMe[21] (0.98 g, 2.3 mmol, 1.2 eq.) were dissolved in DMF (20 mL), followed by the addition of HCTU (0.96 g, 2.3 mmol, 1.2 eq.) and DIPEA (1.0 mL, 5.7 mmol, 3.0 eq.). The reaction was stirred at room temperature for 4 hours, after which Et_2O was added. The precipitation was filtered and recrystallized with MeOH/DCM/Et_2O to obtain compound **14** (1.3 g, 1.5 mmol, 78%). R_f: 0.59 (9/1 DCM/MeOH). $[\alpha]_D^{25}$ -35.0° (*c* = 0.34, $CHCl_3$/MeOH 1/1); ^{1}H NMR (DMSO, 400 MHz, HH-COSY, HSQC): δ 7.92 – 7.86 (m, 3H, Ar, N*H*Ac), 7.79 (d, 1H, *J* = 7.8 Hz, N*H*Fmoc), 7.77 – 7.69 (m, 3H, Ar, NH), 7.45 – 7.36 (m, 7H, Ar), 7.33 (t, 2H, *J* = 7.4 Hz, Ar), 7.18 (d, 2H, *J* = 8.6 Hz, Ar), 6.84 (d, 2H, *J* = 8.6 Hz, Ar), 5.68 (s, 1H, CH benzylidene), 4.65 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.52 (d, 1H, *J* = 11.4 Hz, C*H*H PMB), 4.35 – 4.26 (m, 2H, CH_2 Fmoc), 4.26 – 4.16 (m, 2H, CH Fmoc, C*H*H-6), 4.04 – 3.96 (m, 1H, CH L-Lys), 3.75 – 3.69 (m, 4H, C*H*H-6, CH_3 PMB), 3.69 – 3.55 (m, 6H, H-2, H-3, H-4, OCH_3), 3.38 – 3.26 (m, 2H, H-1, H-5), 3.06 – 2.94 (m, 2H, CH_2 ε-L-Lys), 2.00 (t, 2H, *J* = 7.2 Hz, CH_2-9), 1.83 (s, 3H, CH_3 Ac), 1.74 – 1.18 (m, 10H, CH_2-7, CH_2-8, 3x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR (DMSO, 101 MHz, HSQC): δ 173.0, 171.8 (C=O), 169.2, 158.6, 156.1, 143.8, 143.8, 140.8, 137.8, 130.9 (C_q Ar), 129.0, 128.7, 128.1, 127.7, 127.1, 126.0, 125.3, 120.1, 113.4 (Ar), 100.0 (CH benzylidene), 81.5 (C-4), 79.7 (C-1), 79.1 (C-3), 72.9 (CH_2 PMB), 70.0 (C-5), 68.0 (CH_2-6), 65.6 (CH_2 Fmoc), 55.0 (CH_3 PMB), 54.0 (C-2), 53.8 (CH L-Lys), 51.9(OCH_3), 46.7 (CH Fmoc), 38.1 (CH_2 ε-L-Lys), 35.4 (CH_2-9), 31.1, 30.3, 28.7, 23.0 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys), 22.9 (CH_3 Ac), 21.8 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); FT-IR (neat, cm^{-1}): 3298, 2867, 1687, 1636, 1547, 1514, 1452, 1370, 1250, 1177, 1133, 1088, 1031, 819, 735, 695, 621, 539; HRMS: $[M+H]^+$ calcd. for $C_{49}H_{58}O_{11}N_3$: 864.40659, found 864.40649.

N_α-Fmoc-N_ε-[butan-4-(2-deoxy-2-*N*-acetyl-4,6-*O*-di-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-glucopyranosyl)-amide]-L-lysine (2)



To a suspension of compound **14** (0.92 g, 1.1 mmol, 1.0 eq.) in THF (21 mL) was added a solution of LiOH (0.30 M, 7.1 mL, 2.1 mmol, 2.0 eq.) at room temperature. After stirring for 50 minutes, the obtained clear solution was neutralized with 1 M HCl (2.2 mL, 2.2 mmol, 2.0 eq.). $NaHCO_3$ (0.36 g, 4.3 mmol, 4.0 eq.) and Fmoc *N*-hydroxysyccinimide ester (0.72 g, 2.1 mmol, 2.0 eq.) were added and the mixture was stirred vigorously for 2 hours. Upon completion of the reaction determined by LC-MS, Et_2O was added at 0°C to precipitate the crude product. After filtration, the precipitate was purified by recrystallization (MeOH/DCM/Et_2O) to yield the title compound (0.88 g, 1.0 mmol, 91 %) as a white solid. R_f: 0.40 (85/15 DCM/MeOH); $[\alpha]_D^{25}$ +15.5° (*c* = 1.0, $CHCl_3$/MeOH 1/1); ^{1}H NMR (MeOD/$CDCl_2$: 1/1 v/v, 400 MHz, HH-COSY, HSQC): δ 7.80 – 7.74 (m, 2H, Ar), 7.68 – 7.60 (m, 2H, Ar), 7.51 – 7.44 (m, 2H, Ar), 7.43 – 7.32 (m, 5H, Ar), 7.35 – 7.26 (m, 2H, Ar), 7.23 – 7.15 (m, 2H, Ar), 6.86 – 6.78 (m, 2H, Ar), 5.58 (s, 1H, CH benzylidene), 4.75 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.59 – 4.54 (m, 1H, C*H*H PMB), 4.37 – 4.28 (m, 2H, CH_2 Fmoc), 4.31 – 4.18 (m, 2H, C*H*H-6, CH Fmoc), 4.02 (dd, 1H, *J* = 7.0, 5.0 Hz, CH L-Lys), 3.81 – 3.72 (m, 4H, H-2, CH_3 PMB), 3.74 – 3.59 (m, 3H, H-3, H-4, C*H*H-6), 3.44 – 3.32 (m, 2H, H-1, H-5), 3.20 – 3.08 (m, 2H, CH_2 ε-L-Lys), 2.13 (t, 2H, *J* = 7.6 Hz, CH_2-9), 1.91 (s, 3H, CH_3 Ac), 1.86 – 1.29 (m, 10H, CH_2-7, CH_2-8, 3x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 178.6, 175.2, 172.6 (C=O), 160.0, 157.3, 144.9, 144.7, 142.0, 138.5, 131.5 (C_q Ar), 130.2, 129.5, 128.8, 128.3, 127.7, 126.8, 125.8, 120.5, 114.2 (Ar), 101.9 (CH benzylidene), 83.3 (C-4), 80.4 (C-3), 79.8 (C-1), 74.6 (CH_2 PMB), 71.1 (C-5), 69.5 (CH_2-6), 67.3 (CH_2 Fmoc), 57.0 (CH L-Lys), 55.6 (CH_3 PMB), 55.4 (C-2), 48.0 (CH Fmoc), 39.8 (CH_2 ε-L-Lys), 36.6 (CH_2-9), 33.4, 32.0, 29.5, 25.7, 23.4 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys), 23.0 (CH_3 Ac), 22.6 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); FT-IR (neat, cm^{-1}): 3286, 1638, 1547, 1513, 1451, 1370, 1249, 1176, 1135, 1088, 1030, 820, 737, 696, 621, 541; LC-MS: Rt = 7.75 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 872.4 $[M+Na]^+$; HRMS: $[M+H]^+$ calcd. for $C_{48}H_{56}O_{11}N_3$: 850.39094, found 850.39103.

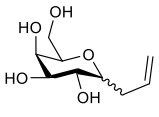
3-(2,3,4,6-tetra-*O*-acetyl-α/β-D-galactopyranosyl)-1-propene (15)



Acetyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose (23.7 g, 60.8 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in CH_3NO_2 (0.24 L). Allyltrimethylsilane (20 mL, 0.13 mol, 2.1 eq.) was added, followed by the addition of $BF_3{\cdot}OEt_2$ (23 mL, 0.18 mol, 3.0 eq.) at 0°C. The yellow solution was allowed to stir at room temperature for 3 days. The reaction was quenched by the addition of sat. aq. $NaHCO_3$ at 0°C, diluted with EtOAc and washed with brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→50% Et_2O in pentane) afforded the title compound (20.1 g, 54.0 mmol, 89%) as a yellow oil with an α/β ratio of 2/1. R_f: 0.41 (1/1 pentane/Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 5.80 – 5.69 (m, 1H, CH_2-C*H*=CH_2), 5.43 – 5.38 (m, 1H, H-4), 5.26 (dd, 1H, *J* = 9.3, 5.0 Hz, H-2), 5.20 (dd, 1H, *J* = 9.4, 3.2 Hz, H-3), 5.12 – 5.06 (m, 2H, CH_2-CH=C*H*$_2$), 4.33 – 4.25 (m, 1H, H-1), 4.24 – 4.14 (m, 1H, C*H*H-6), 4.14 – 4.01 (m, 2H,

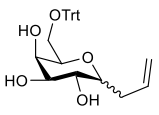
H-5, C*H*H-6), 2.54 – 2.38 (m, 1H, C*H*H-CH=CH_2), 2.35 – 2.21 (m, 1H, C*H*H-CH=CH_2), 2.11 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 2.05 – 2.00 (m, 6H, 2x CH_3 Ac); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 170.7, 170.2, 170.1, 170.0 (C=O), 133.4 (CH_2-*C*H=CH_2), 117.8 (CH_2-CH=*C*H_2), 71.6 (C-1), 68.4 (C-5), 68.0 (C-2), 67.8 (C-3), 67.7 (C-4), 61.6 (CH_2-6), 31.0 (*C*H_2-CH=CH_2), 20.9, 20.9, 20.8, 20.8, 20.8 (CH_3 Ac); FT-IR (neat, cm^{-1}): 2978, 1740, 1644, 1434, 1369, 1212, 1044, 909, 601; HRMS: $[M+Na]^+$ calcd for $C_{17}H_{24}O_9Na$: 395.1318, found 395.1316. *NMR analysis only given for the α-anomer.

3-(α/β-D-galactopyranosyl)-1-propene (16)



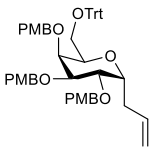
Compound **15** (20.0 g, 53.8 mmol, 1.0 eq.) was dissolved in MeOH (0.11 L). Sodium methoxide (5.4 M in MeOH, 4.0 mL, 22 mmol, 0.40 eq.) was added and the solution was stirred for 3 hours, after which it was acidified by the addition of amberlite H^+ resin. The mixture was filtered and concentrated *in vacuo*. The title compound (10.0 g, 49.2 mmol, 91%) was obtained as a yellow foam and used without further purification. R_f: 0.13 (9/1 DCM/MeOH); ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 5.93 – 5.82 (m, 1H, CH_2-C*H*=CH_2), 5.17 – 5.06 (m, 2H, CH_2-CH=C*H*$_2$), 4.03 – 3.93 (m, 2H, H-1, H-2), 3.93 – 3.85 (m, 1H, H-3), 3.80 – 3.62 (m, 4H, H-4, H-5, CH_2-6), 2.52 – 2.32 (m, 2H, C*H*$_2$-CH=CH_2); ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 136.7 (CH_2-*C*H=CH_2), 116.9 (CH_2-CH=C*H*$_2$), 75.6 (C-1), 74.0, 71.9 (C-4, C-5), 70.1 (C-3), 70.0 (C-2), 61.9 (CH_2-6), 31.0 (*C*H_2-CH=CH_2); FT-IR (neat, cm^{-1}): 3352, 2919, 1642, 1416, 1073, 914, 515; HRMS: $[M+Na]^+$ calcd for $C_9H_{16}O_5Na$: 227.0895, found 227.0894. *NMR analysis only given for the α-anomer.

3-(6-*O*-trityl-α/β-D-galactopyranosyl)-1-propene (17)



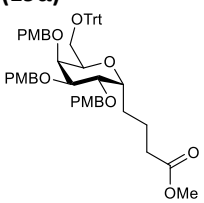
Trityl chloride (21 g, 75 mmol, 1.3 eq.) and Et_3N (17 mL, 0.12 mol, 2.5 eq.) were added to a solution of compound **16** (10.0 g, 48.9 mmol, 1.0 eq.) in DMF (0.16 L). The mixture was heated to 60°C overnight. The mixture was diluted with EtOAc and washed with brine (3x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (30→100% EtOAc in pentane) gave the title compound (17.3 g, 38.7 mmol, 79%). R_f: 0.36 (3/7 pentane/EtOAc); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.50 – 7.44 (m, 6H, Trt), 7.36 – 7.29 (m, 6H, Trt), 7.29 – 7.23 (m, 3H, Trt), 6.04 – 5.87 (m, 1H, CH_2-C*H*=CH_2), 5.24 – 5.03 (m, 2H, CH_2-CH=C*H*$_2$), 3.96 – 3.89 (m, 1H, H-1), 3.89 – 3.83 (m, 1H, H-5), 3.81 – 3.72 (m, 2H, H-2, H-4), 3.64 – 3.53 (m, 1H, H-3), 3.33 – 3.12 (m, 3H, C*H*H-6, 2x OH), 3.11 – 3.04 (m, 1H, C*H*H-6), 2.91 – 2.82 (m, 1H, OH), 2.52 – 2.42 (m, 1H, C*H*H-CH=CH_2), 2.39 – 2.29 (m, 1H, C*H*H-CH=CH_2); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 145.3 (C_q Trt), 137.0 (CH_2-*C*H=CH_2), 129.6, 128.8, 128.0 (Ar), 116.9 (CH_2-CH=*C*H_2), 87.3 (C_q Trt), 75.4 (C-1), 71.4 (C-5), 70.8 (C-3), 70.2, 69.7 (C-2, C-4), 64.3 (CH_2-6),30.2 (*C*H_2-CH=CH_2); FT-IR (neat, cm^{-1}): 3391, 3059, 2929, 1642, 1597, 1490, 1448, 1265, 1222, 1153, 1069, 988, 901, 823, 762, 737, 704, 650, 632, 580, 536; HRMS: $[M+Na]^+$ calcd for $C_{28}H_{30}O_5Na$: 469.1991, found 469.1988. *NMR analysis only given for the α-anomer.

3-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-galactopyranosyl)-1-propene (18α)



Triol **17** (16.4 g, 36.8 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere and dissolved in DMF (0.37 L). Sodium hydride (60% dispersion in mineral oil, 5.3 g, 0.13 mol, 3.5 eq.) was added at 0°C over 30 minutes. After 1 hour, *p*-methoxybenzyl chloride (18 mL, 0.13 mol, 3.5 eq.) and tetrabutylammonium iodide (1.4 g, 3.8 mmol, 0.10 eq.) were added. Another portion of sodium hydride (60% dispersion in mineral oil, 2.3 g, 58 mmol, 1.6 eq.) was added after 1 hour and the miture was stirred at room temperature overnight. The reaction mixture was quenched with MeOH at 0°C, diluted with Et_2O and washed H_2O (3x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (10→30% Et_2O in pentane) gave compound **18α** (15.5 g, 19.2 mmol, 52%) and compound **18β** (8.23 g, 10.3 mmol, 28%). Analysis α-compound: R_f: 0.26 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ +35.9° (*c* = 0.44, $CHCl_3$); ^{1}H NMR (CD_3CN, 500 MHz, HH-COSY, HSQC): δ 7.47 – 7.41 (m, 6H, Ar), 7.34 – 7.21 (m, 11H, Ar), 7.09 (dd, 4H, *J* = 19.9, 8.5 Hz, Ar), 6.90 (d, 2H, *J* = 8.6 Hz, Ar), 6.86 – 6.81 (m, 4H, Ar), 5.87 – 5.76 (m, 1H, CH_2-C*H*=CH_2), 5.17 – 5.03 (m, 2H, CH_2-CH=C*H*$_2$), 4.52 – 4.42 (m, 5H, 2x CH_2 PMB, 1x C*H*H PMB), 4.34 (d, 1H, *J* = 11.2 Hz, C*H*H PMB), 4.09 – 4.03 (m, 1H, H-5), 3.91 (dd, 1H, *J* = 4.3, 3.0 Hz, H-4), 3.82 – 3.74 (m, 11H, H-1, H-3, 3x CH_3 PMB), 3.62 – 3.55 (m, 2H, H-2, C*H*H-6), 3.17 (dd, 1H, *J* = 10.6, 3.2 Hz, C*H*H-6), 2.45 – 2.36 (m, 1H, C*H*H-CH=CH_2), 2.36 – 2.27 (m, 1H, C*H*H-CH=CH_2); ^{13}C-APT NMR (CD_3CN, 126 MHz, HSQC): δ 160.7, 160.6, 160.5, 145.7 (C_q Ar), 136.8 (CH_2-*C*H=CH_2), 132.3, 132.1, 130.8, 130.5, 130.2, 129.9, 128.9, 128.1 (Ar), 117.2 (CH_2-CH=*C*H_2), 115.0, 114.9 (Ar), 87.6 (C_q Trt), 77.2 (C-2), 76.8 (C-3), 75.3 (C-4), 74.4 (C-5), 73.4, 73.0 (CH_2 PMB), 71.2 (C-1), 62.5 (CH_2-6), 56.2 (CH_3 PMB), 33.8 (*C*H_2-CH=CH_2); FT-IR (neat, cm^{-1}): 2934, 2906, 2836, 1612, 1586, 1513, 1491, 1464, 1449, 1355, 1302, 1247, 1173, 1091, 1034, 995, 916, 821, 765, 748, 707, 649, 633, 568, 516; HRMS: $[M+Na]^+$ calcd for $C_{52}H_{54}O_8Na$: 829.3716, found 829.3735.

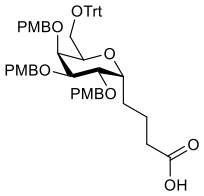
Methyl 4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-galactopyranosyl)-butanoate (19α)



Compound **18α** (15.2 g, 18.8 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere before being dissolved in dry DCE (0.19 L). Methyl acrylate (4.8 mL, 53 mmol, 2.8 eq.), CuI (0.54 g, 2.8 mmol, 0.15 eq.) and Grubbs 2nd generation catalyst (0.63 g, 0.74 mmol, 0.04 eq.) were added and the flask was covered in aluminum foil. The suspension was heated to 50°C for 48 hours, after which it was concentrated *in vacuo* and co-evaporated with toluene (2x) under an argon atmosphere. The obtained residue was dissolved in dry DCE (0.10 L) and cooled to 0°C. Two empty balloons were placed on the flask, followed by the addition of ruthenium trichloride (0.74 g, 3.6 mmol, 0.19 eq.) and $NaBH_4$ (2.3 g, 61 mmol, 3.2 eq.). Methanol (15 mL, 0.37 mol, 20 eq.) was carefully added to the suspension over 20 minutes, after which the mixture was allowed to warm-up up to room temperature over 30 minutes. The mixture was subsequently heated to 45°C for 6 hours. The reaction mixture was cooled to room temperature, diluted with brine and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and

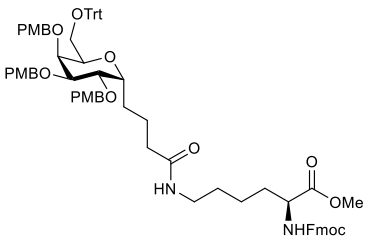
concentrated *in vacuo*. Purification by column chromatography (20→70% Et_2O in pentane) gave compound **19α** (11.8 g, 13.6 mmol, 73% over two steps). R_f: 0.43 (1/1 pentane/ Et_2O); $[\alpha]_D^{25}$ +23.0° (*c* = 0.43, $CHCl_3$); ^1H NMR (CD_3CN, 500 MHz, HH-COSY, HSQC): δ 7.44 (d, 6H, *J* = 8.1 Hz, Ar), 7.30 (t, 6H, *J* = 7.4 Hz, Ar), 7.28 – 7.20 (m, 5H, Ar), 7.13 (d, 2H, *J* = 8.4 Hz, Ar), 7.06 (d, 2H, *J* = 8.4 Hz, Ar), 6.89 (d, 2H, *J* = 8.6 Hz, Ar), 6.84 (dd, 4H, *J* = 8.6, 3.2 Hz, Ar), 4.54 – 4.41 (m, 5H, 2x CH_2 PMB, 1x C*H*H PMB), 4.34 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 3.97 – 3.92 (m, 1H, H-5), 3.86 (t, 1H, *J* = 3.4 Hz, H-4), 3.81 – 3.76 (m, 9H, 3x CH_3 PMB), 3.73 – 3.68 (m, 2H, H-1, H-3), 3.61 (s, 3H, OCH_3), 3.60 – 3.56 (m, 1H, H-2), 3.55 – 3.50 (m, 1H, C*H*H-6), 3.17 (dd, 1H, *J* = 10.5, 3.3 Hz, C*H*H-6), 2.33 (t, 2H, *J* = 7.2 Hz, CH_2-9), 1.76 – 1.58 (m, 2H, C*H*H-7, C*H*H-8), 1.58 – 1.45 (m, 2H, C*H*H-7, C*H*H-8); ^{13}C-APT NMR (CD_3CN, 126 MHz, HSQC): δ 174.8 (C=O), 160.7, 145.7 (C_q Ar), 132.4, 132.1, 130.8, 130.5, 130.3, 129.8, 128.9, 128.1, 115.0, 114.9 (Ar), 87.7 (C_q Trt), 77.5 (C-2), 77.4 (C-3), 75.6 (C-4), 73.9 (C-5), 73.4, 73.4, 73.3 (CH_2 PMB), 71.6 (C-1), 63.0 (CH_2-6), 56.2 (CH_3 PMB), 52.0 (OCH_3), 34.7 (CH_2-9), 28.0, 22.5 (CH_2-7, CH_2-8); FT-IR (neat, cm^{-1}): 2949, 1736, 1612, 1513, 1449, 1302, 1248, 1173, 1090, 1034, 821, 748, 707, 633; HRMS: $[M+Na]^+$ calcd. for $C_{54}H_{58}O_{10}Na$: 889.39222, found 889.39203.

4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-galactopyranosyl)-butanoic acid (20α)



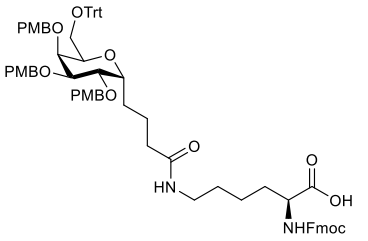
19α (11.7 g, 13.5 mmol, 1.0 eq.) was dissolved in in a mixture of THF/H_2O (4/1, v/v, 0.14 L), followed by the addition of LiOH (1.7 g, 41 mmol, 3.0 eq.). The mixture was heated to 40°C for 30 hours. The reaction mixture was cooled to 0°C, acidified with 3 M HCl to pH = 4/5, diluted with H_2O and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The title compound was obtained (10.5 g, 12.4 mmol, 92%) and used without further purification. R_f: 0.84 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +25.6° (*c* = 0.90, $CHCl_3$); ^1H NMR (CD_3CN, 500 MHz, HH-COSY, HSQC): δ 7.51 – 7.47 (m, 6H, Ar), 7.33 (t, 6H, *J* = 7.4 Hz, Ar), 7.30 – 7.24 (m, 5H, Ar), 7.18 (d, 2H, *J* = 8.5 Hz, Ar), 7.10 (d, 2H, *J* = 8.6 Hz, Ar), 6.92 (d, 2H, *J* = 8.6 Hz, Ar), 6.90 – 6.84 (m, 4H, Ar), 4.59 – 4.46 (m, 5H, 2x CH_2 PMB, 1x C*H*H PMB), 4.39 (d, 1H, *J* = 11.1 Hz, C*H*H PMB), 4.04 – 3.97 (m, 1H, H-5), 3.94 – 3.90 (m, 1H, H-4), 3.84 – 3.77 (m, 10H, H-1, 3x CH_3 PMB), 3.76 (dd, 1H, *J* = 6.9, 2.8 Hz, H-3), 3.69 – 3.64 (m, 1H, H-2), 3.64 – 3.58 (m, 1H, C*H*H-6), 3.24 (dd, 1H, *J* = 10.5, 3.4 Hz, C*H*H-6), 2.38 (t, 2H, *J* = 7.0 Hz, CH_2-9), 1.83 – 1.68 (m, 2H, C*H*H-7, C*H*H-8), 1.64 – 1.55 (m, 2H, C*H*H-7, C*H*H-8); ^{13}C-APT NMR (CD_3CN, 126 MHz, HSQC): δ 175.7 (C=O), 160.5, 160.4, 160.3, 145.5 (C_q Ar), 132.2, 132.0, 132.0, 130.6, 130.4, 130.1, 129.7, 128.8, 128.0, 114.9, 114.8, 114.8 (Ar), 87.6 (C_q Trt), 77.4 (C-2, C-3), 75.6 (C-4), 73.7 (C-5), 73.3 (CH_2 PMB), 71.7 (C-1), 63.0 (CH_2-6), 56.1, 56.1 (CH_3 PMB), 34.4 (CH_2-9), 27.8, 22.4 (CH_2-7, CH_2-8); FT-IR (neat, cm^{-1}): 2937, 1707, 1612, 1513, 1449, 1302, 1248, 1173, 1087, 1034, 821, 707, 633; HRMS: $[M+Na]^+$ calcd. for $C_{53}H_{56}O_{10}Na$: 875.37657, found 875.37656.

N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-galactopyranosyl)-amide]-L-lysine-methyl ester (21α)



Compound **01α** (4.3 g, 5.0 mmol, 1.0 eq.) and Fmoc-L-lysine-OMe[21] (2.5 g, 6.0 mmol, 1.2 eq.) were dissolved in DMF (25 mL). HCTU (2.5 g, 6.0 mmol, 1.2 eq.) and DIPEA (2.6 mL, 15 mmol, 3.0 eq.) were added and the solution was stirred for 2 hours. The reaction mixture was diluted with EtOAc and was washed with 1 M HCl (1x), sat. aq. $NaHCO_3$ (1x), brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (40→80% EtOAc in pentane) gave compound **21α** (5.5 g, 4.5 mmol, 90%) as an oil. R_f: 0.63 (3/7 pentane/EtOAc); $[\alpha]_D^{25}$ +23.3° (*c* = 0.46, $CHCl_3$); ^{1}H NMR (CD_3CN, 500 MHz, HH-COSY, HSQC): δ 7.82 (d, 2H, *J* = 7.5 Hz, Ar), 7.65 (d, 2H, *J* = 7.1 Hz, Ar), 7.46 – 7.38 (m, 8H, Ar), 7.36 – 7.17 (m, 13H, Ar), 7.11 (d, 2H, *J* = 8.4 Hz, Ar), 7.05 (d, 2H, *J* = 8.5 Hz, Ar), 6.87 (d, 2H, *J* = 8.6 Hz, Ar), 6.82 (d, 4H, *J* = 8.5 Hz, Ar), 6.15 (br, 1H, NH), 5.91 (br, 1H, N*H*Fmoc), 4.51 – 4.39 (m, 5H, 2x CH_2 PMB, 1x C*H*H PMB), 4.39 – 4.29 (m, 3H, C*H*H PMB, CH_2 Fmoc), 4.23 (t, 1H, *J* = 6.8 Hz, CH Fmoc), 4.15 – (m, 1H, CH L-Lys), 3.96 – 3.90 (m, 1H, H-5), 3.86 – 3.82 (m, 1H, H-4), 3.81 – 3.75 (m, 9H, 3x CH_3 PMB), 3.72 – 3.63 (m, 5H, H-1, H-3, OCH_3), 3.59 – 3.48 (m, 2H, H-2, C*H*H-6), 3.17 – 3.08 (m, 3H, C*H*H-6, CH_2 ε-L-Lys), 2.13 (t, 2H, *J* = 6.9 Hz, CH_2-9), 1.82 – 1.27 (m, 10H, 2x CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR (CD_3CN, 126 MHz, HSQC): δ 145.7, 142.5 (C_q Ar), 130.8, 130.5, 130.3, 129.9, 129.0, 128.9, 128.3, 128.2, 126.4, 121.2, 118.2, 115.0, 114.9 (Ar), 87.7 (C_q Trt), 77.5 (C-2, C-3), 75.6 (C-4), 74.0 (C-5), 73.4, 73.2 (CH_2 PMB), 71.7 (C-1), 67.6 (CH_2 Fmoc), 63.0 (CH_2-6), 56.2 (CH_3 PMB), 55.4 (CH L-Lys), 52.8 (OCH_3), 48.5 (CH Fmoc), 39.6 (CH_2 ε-L-Lys), 37.1 (CH_2-9), 32.3, 30.2, 28.3, 23.8, 23.4 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); FT-IR (neat, cm^{-1}): 2950, 1723, 1653, 1612, 1514, 1450, 1248, 1174, 1088, 1034, 823, 743, 707; HRMS: $[M+H]^+$ calcd. for $C_{75}H_{81}O_{13}N_2$: 1217.57332, found 1217.57311.

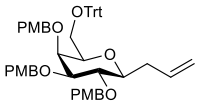
N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-α-D-galactopyranosyl)-amide]-L-lysine (4)



Compound **21α** (4.8 g, 4.0 mmol, 1.0 eq.) was dissolved in THF (55 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 27 mL, 8.1 mmol, 2.0 eq.) was added and the suspension was stirred vigorously for 1 hour, after which the obtained solution was acidified by the addition of 1 M HCl to pH = 5-6 and diluted with brine. The mixture was extracted with EtOAc (1x) and the organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (2→6% MeOH in DCM), the title compound (2.2 g, 1.8 mmol, 46%) was obtained as a white foam. R_f: 0.70 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +15.8° (*c* = 1.1, $CHCl_3$); ^{1}H NMR (CD_3CN, 500 MHz, HH-COSY, HSQC): δ 7.80 (d, 2H, *J* = 7.4 Hz, Ar), 7.64 (d, 2H, *J* = 7.1 Hz, Ar), 7.46 – 7.36 (m, 8H, Ar), 7.34 – 7.17 (m, 13H, Ar), 7.10 (d, 2H, *J* = 8.3 Hz, Ar), 7.04 (d, 2H, *J* = 8.4 Hz, Ar), 6.84 (dd, 6H, *J* = 23.1, 8.0 Hz, Ar), 6.23 (br, 1H, NH), 5.92

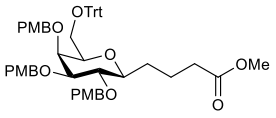
(br, 1H, N*H*Fmoc), 4.51 – 4.38 (m, 5H, 2x CH_2 PMB, 1x C*H*H PMB), 4.38 – 4.29 (m, 3H, C*H*H PMB, CH_2 Fmoc), 4.21 (t, 1H, *J* = 6.7 Hz, CH Fmoc), 4.11 (br, 1H, CH L-Lys), 3.97 – 3.90 (m, 1H, H-5), 3.88 – 3.82 (m, 1H, H-4), 3.82 – 3.73 (m, 9H, 3x CH_3 PMB), 3.73 – 3.65 (m, 2H, H-1, H-3), 3.60 – 3.48 (m, 2H, H-2, C*H*H-6), 3.23 – 3.07 (m, 3H, C*H*H-6, CH_2 ε-L-Lys), 2.17 – 2.10 (m, 2H, CH_2-9), 1.84 – 1.27 (m, 10H, 2x CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR (CD_3CN, 126 MHz, HSQC): δ 174.1, 160.7 (C=O), 160.5, 160.5, 145.6, 145.4, 145.4, 142.4, 132.4, 132.1 (C_q Ar), 130.8, 130.5, 130.3, 129.9, 129.0, 128.9, 128.3, 128.2, 126.4, 121.1, 115.0, 114.9 (Ar), 87.7 (C_q Trt), 77.5, 77.3 (C-2, C-3), 75.6 (C-4), 74.0 (C-5), 73.4, 73.3, 73.2 (CH_2 PMB), 71.7 (C-1), 67.6 (CH_2 Fmoc), 63.0 (CH_2-6), 56.2 (CH_3 PMB), 55.2 (CH L-Lys), 48.4 (CH Fmoc), 39.7 (CH_2 ε-L-Lys), 37.1 (CH_2-9), 32.3, 30.1, 28.2, 23.8, 23.4 (CH_2-7/8, 3x CH_2 β/γ/δ-L-Lys); FT-IR (neat, cm^{-1}): 2935, 1720, 1612, 1513, 1449, 1302, 1248, 1174, 1088, 1034, 822, 761, 743, 707, 633; LC-MS: Rt = 7.68 min (Vydac 219TP 5 µm Diphenyl, 50 - 90% MeCN, 21 min run); ESI-MS: m/z 1225.5 $[M+Na]^+$; HRMS: $[M+H]^+$ calcd. for $C_{74}H_{79}O_{13}N_2$: 1203.55767, found 1203.55754.

3-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-β-D-galactopyranosyl)-1-propene (18β)



See experimental of compound **18α**. Purification gave compound **18β** (8.23 g, 10.3 mmol, 28%). Analysis β-compound: R_f: 0.33 (7/3 pentane/Et_2O); $[\alpha]_D^{25}$ +9.3° (*c* = 0.52, $CHCl_3$); ^{1}H NMR (CD_3CN, 400 MHz, HH-COSY, HSQC): δ 7.47 – 7.41 (m, 6H, Ar), 7.38 – 7.20 (m, 13H, Ar), 6.99 – 6.95 (m, 2H, Ar), 6.92 – 6.85 (m, 4H, Ar), 6.79 – 6.74 (m, 2H, Ar), 5.94 – 5.82 (m, 1H, CH_2-C*H*=CH_2), 5.13 – 4.97 (m, 2H, CH_2-CH=C*H*$_2$), 4.78 (d, 1H, *J* = 10.4 Hz, C*H*H PMB), 4.72 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.67 (d, 1H, *J* = 10.7 Hz, C*H*H PMB), 4.60 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.51 (d, 1H, *J* = 10.5 Hz, C*H*H PMB), 4.27 (d, 1H, *J* = 10.7 Hz, C*H*H PMB), 3.93 (dd, 1H, *J* = 2.9, 1.1 Hz, H-4), 3.82 – 3.71 (m, 9H, 3x CH_3 PMB), 3.63 – 3.54 (m, 2H, H-3, H-5), 3.43 (t, 1H, *J* = 9.3 Hz, H-2), 3.34 – 3.19 (m, 2H, H-1, C*H*H-6), 2.89 (dd, 1H, *J* = 9.3, 5.6 Hz, C*H*H-6), 2.60 – 2.49 (m, 1H, C*H*H-CH=CH_2), 2.23 – 2.11 (m, 2H, C*H*H-CH=CH_2); ^{13}C-APT NMR (CD_3CN, 101 MHz, HSQC): δ 160.3, 160.2, 160.1, 145.1 (C_q Ar), 136.5 (CH_2-*C*H=CH_2), 132.0, 131.9, 131.9 (C_q Ar), 130.7, 130.6, 130.5, 129.6, 128.8, 128.1 (Ar), 116.9 (CH_2-CH=*C*H$_2$), 114.7, 114.5, 114.4 (Ar), 87.5 (C_q Trt), 85.2 (C-3), 79.7 (C-1), 78.9 (C-2), 78.0 (C-5), 75.4 (C-4), 75.3, 74.9, 72.3 (CH_2 PMB), 64.7 (CH_2-6), 55.9, 55.9 (CH_3 PMB), 37.1 (*C*H_2-CH=CH_2); FT-IR (neat, cm^{-1}): 2907, 1613, 1583, 1513, 1491, 1449, 1362, 1302, 1248, 1173, 1076, 1034, 915, 821, 747, 707, 633, 518; HRMS: $[M+Na]^+$ calcd for $C_{52}H_{54}O_8Na$: 829.3716, found 829.3740.

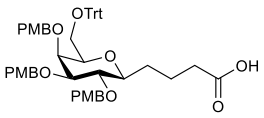
Methyl 4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-β-D-galactopyranosyl)-butanoate (19β)



Allyl **18β** (8.0 g, 9.9 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under an argon atmosphere before being dissolved in dry DCE (0.10 L). Methyl acrylate (2.6 mL, 29 mmol, 2.9 eq.), CuI (0.29 g, 1.5 mmol, 0.15 eq.) and Grubbs 2nd generation catalyst (0.34 g, 0.40 mmol, 0.04 eq.) were added and the flask was covered in aluminum foil. The suspension was heated to 50°C for 48 hours, after which it was concentrated *in vacuo* and co-evaporated with toluene (2x) under an argon atmosphere. The obtained residue was dissolved in dry DCE (50 mL) and cooled to 0°C.

Two empty balloons were placed on the flask, followed by the addition of ruthenium trichloride (0.39 g, 1.9 mmol, 0.19 eq.) and $NaBH_4$ (1.2 g, 32 mmol, 3.2 eq.). Methanol (8.0 mL, 0.18 mol, 20 eq.) was carefully added to the suspension over 30 minutes, after which the mixture was allowed to warm-up up to room temperature over 15 minutes. The mixture was subsequently heated to 45°C for 5 hours. The reaction mixture was cooled to room temperature, diluted with brine and extracted with DCM (3x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. NMR analysis showed still 20% alkene present, therefore the 2nd step was repeated using the same reaction conditions and heated for 7 hours. Purification by column chromatography (20→70% Et_2O in pentane) afforded the title compound (5.8 g, 6.7 mmol, 68% over two steps); R_f: 0.44 (1/1 pentane/ Et_2O); $[\alpha]_D^{25}$ +2.9° (*c* = 0.84, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.43 – 7.39 (m, 6H, Ar), 7.32 – 7.21 (m, 13H, Ar), 7.08 – 7.03 (m, 2H, Ar), 6.91 – 6.84 (m, 4H, Ar), 6.76 – 6.71 (m, 2H, Ar), 4.84 (d, 1H, *J* = 10.4 Hz, C*H*H PMB), 4.72 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.67 – 4.59 (m, 2H, CH_2 PMB), 4.54 (d, 1H, *J* = 10.4 Hz, C*H*H PMB), 4.45 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 3.88 (dd, 1H, *J* = 2.7, 1.0 Hz, H-4), 3.81 (d, 6H, *J* = 3.7 Hz, 2x CH_3 PMB), 3.78 (s, 3H, CH_3 PMB), 3.61 (s, 3H, OCH_3), 3.58 – 3.49 (m, 2H, H-2, H-3), 3.49 – 3.42 (m, 1H, C*H*H-6), 3.32 (t, 1H, *J* = 6.2 Hz, H-5), 3.16 – 3.04 (m, 2H, H-1, C*H*H-6), 2.30 (t, 2H, *J* = 7.3 Hz, CH_2-9), 1.92 – 1.79 (m, 2H, C*H*H-7, C*H*H-8), 1.75 – 1.63 (m, 1H, C*H*H-8), 1.52 – 1.40 (m, 1H, C*H*H-7); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.1 (C=O), 159.3, 159.2, 159.0 (C_q Ar), 144.0, 130.9, 130.7, 130.7, 129.9, 129.7, 129.2, 128.7, 127.9, 127.0, 113.8, 113.5 (Ar), 86.8 (C_q Trt), 84.7 (C-3), 79.3 (C-1), 78.8 (C-2), 77.5 (C-5), 75.1 (CH_2 PMB), 73.8 (C-4), 73.8, 72.0 (CH_2 PMB), 63.3 (CH_2-6), 55.3, 55.3, 55.3 (CH_3 PMB), 51.4 (OCH_3), 34.0 (CH_2-9), 31.1 (CH_2-7), 21.3 (CH_2-8); FT-IR (neat, cm^{-1}): 2949, 1736, 1612, 1586, 1513, 1491, 1449, 1362, 1302, 1248, 1173, 1076, 1033, 822, 748, 707, 633; HRMS: $[M+Na]^+$ calcd. for $C_{54}H_{58}O_{10}Na$: 889.39222, found 889.39207.

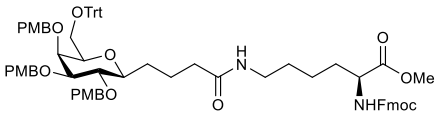
4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-β-D-galactopyranosyl)-butanoic acid (20β)



Compound **19β** (5.8 g, 6.7 mmol, 1.0 eq.) was dissolved in in a mixture of THF/H_2O (4/1, v/v, 65 mL), followed by the addition of LiOH (0.85 g, 20 mmol, 3.0 eq.). The mixture was heated to 40°C for 30 hours. The reaction mixture was cooled to 0°C, acidified with 3 M HCl to pH = 4/5, diluted with H_2O and extracted with DCM (2x). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The title compound was obtained (5.5 g, 6.4 mmol, 96%) and used without further purification. R_f: 0.89 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +3.6° (*c* = 0.53, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.44 – 7.39 (m, 6H, Ar), 7.34 – 7.20 (m, 13H, Ar), 7.09 – 7.04 (m, 2H, Ar), 6.93 – 6.82 (m, 4H, Ar), 6.77 – 6.71 (m, 2H, Ar), 4.84 (d, 1H, *J* = 10.4 Hz, C*H*H PMB), 4.73 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.68 – 4.60 (m, 2H, CH_2 PMB), 4.54 (d, 1H, *J* = 10.5 Hz, C*H*H PMB), 4.45 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 3.89 (dd, 1H, *J* = 2.7, 1.0 Hz, H-4), 3.82 (s, 3H, CH_3 PMB), 3.80 – 3.76 (m, 6H, 2x CH_3 PMB), 3.60 – 3.41 (m, 3H, H-2, H-2, H-3, C*H*H-6), 3.31 (t, 1H, *J* = 6.2 Hz, H-5), 3.17 – 3.06 (m, 2H, H-1, C*H*H-6), 2.34 (t, 2H, *J* = 7.2 Hz, CH_2-9), 1.93 – 1.81 (m, 2H, C*H*H-8, C*H*H-7), 1.74 – 1.62 (m, 1H, C*H*H-8), 1.52 – 1.42 (m, 1H, C*H*H-7); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 179.2 (C=O), 159.4, 159.3, 159.1, 144.1, 131.0, 130.8, 130.7 (C_q Ar), 130.0, 129.8, 129.3, 128.8,

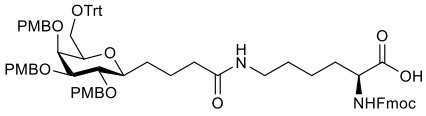
128.0, 127.1, 113.9, 113.6 (Ar), 86.9 (C_q Trt), 84.7 (C-3), 79.4 (C-1), 78.7 (C-2), 77.6 (C-5), 75.2, 73.9 (CH_2 PMB), 73.9 (C-4), 72.1 (CH_2 PMB), 63.3 (CH_2-6), 55.4, 55.4, 55.4 (CH_3 PMB), 33.9 (CH_2-9), 31.0 (CH_2-7), 21.1 (CH_2-8); FT-IR (neat, cm^{-1}): 2935, 1707, 1612, 1586, 1513, 1449, 1302, 1247, 1173, 1075, 1033, 821, 748, 706, 633; HRMS: $[M+Na]^+$ calcd. for $C_{53}H_{56}O_{10}Na$: 875.37657, found 875.37650.

N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-β-D-galactopyranosyl)-amide]-L-lysine-methyl ester (21β)



Compound **20β** (3.4 g, 4.0 mmol, 1.0 eq.) and Fmoc-L-lysine-OMe[21] (2.0 g, 4.8 mmol, 1.2 eq.) were dissolved in DMF (20 mL). HCTU (2.0 g, 4.8 mmol, 1.2 eq.) and DIPEA (2.1 mL, 12 mmol, 3.0 eq.) were added and the solution was stirred for 2 hours. The reaction mixture was diluted with EtOAc and was washed with 1 M HCl (1x), sat. aq. $NaHCO_3$ (1x), brine (1x). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification by column chromatography (30→80% EtOAc in pentane) gave compound **21β** (4.5 g, 3.7 mmol, 93%) as an oil. R_f: 0.53 (4/6 pentane/EtOAc); $[\alpha]_D^{25}$ +8.7° (*c* = 0.38, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.77 – 7.72 (m, 2H, Ar), 7.61 (dd, 2H, *J* = 7.6, 3.1 Hz, Ar), 7.42 – 7.36 (m, 8H, Ar), 7.34 – 7.17 (m, 15H, Ar), 7.05 – 7.00 (m, 2H, Ar), 6.90 – 6.81 (m, 4H, Ar), 6.74 – 6.69 (m, 2H, Ar), 5.49 (t, 1H, *J* = 5.8 Hz, NH), 5.40 (d, 1H, *J* = 8.2 Hz, N*H*Fmoc), 4.82 (d, 1H, *J* = 10.4 Hz, C*H*H PMB), 4.72 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.67 – 4.58 (m, 2H, CH_2 PMB), 4.52 (d, 1H, *J* = 10.5 Hz, C*H*H PMB), 4.45 – 4.33 (m, 3H, C*H*H PMB, CH_2 Fmoc), 4.32 – 4.25 (m, 1H, CH L-Lys), 4.22 (t, 1H, *J* = 7.0 Hz, CH Fmoc), 3.86 – 3.82 (m, 1H, H-4), 3.82 – 3.75 (m, 9H, 3x CH_3 PMB), 3.73 (s, 3H, OCH_3), 3.59 – 3.40 (m, 3H, H-2, H-3, C*H*H-6), 3.35 (t, 1H, *J* = 6.0 Hz, H-5), 3.19 – 2.93 (m, 4H, H-1, C*H*H-6, CH_2 ε-L-Lys), 2.26 – 2.08 (m, 2H, CH_2-9), 1.91 – 1.69 (m, 4H, CH_2-7, CH_2-8), 1.61 – 1.40 (m, 2H, 1x CH_2 β/γ/δ-L-Lys), 1.30 – 1.15 (m, 4H, 2x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 173.3, 159.4 (C=O), 159.1, 156.2, 144.0, 143.9, 141.4, 130.9, 130.7, 130.7 (C_q Ar), 129.9, 129.8, 129.3, 128.8, 128.0, 127.8, 127.2, 127.2, 125.2, 120.1, 113.9, 113.9, 113.6 (Ar), 86.9 (C_q Trt), 84.7 (C-3), 79.7 (C-1), 78.8 (C-2), 77.7 (C-5), 75.2 (CH_2 PMB), 73.9 (C-4), 73.9, 72.1 (CH_2 PMB), 67.1 (CH_2 Fmoc), 63.7 (CH_2-6), 55.4, 55.4 , 55.4 (CH_3 PMB), 53.8 (CH L-Lys), 52.5 (OCH_3), 47.3 (CH Fmoc), 38.9 (CH_2 ε-L-Lys), 36.6 (CH_2-9), 32.1 (CH_2 β/γ/δ-L-Lys), 30.8 (CH_2-7), 29.1, 22.6 (CH_2 β/γ/δ-L-Lys), 22.5 (CH_2-8); FT-IR (neat, cm^{-1}): 3330, 2936, 1722, 1652, 1612, 1586, 1513, 1449, 1302, 1247, 1174, 1076, 1033, 900, 822, 761, 740, 706, 651, 633, 558; HRMS: $[M+H]^+$ calcd. for $C_{75}H_{81}O_{13}N_2$: 1217.57332, found 1217.57314.

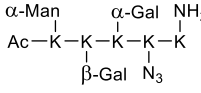
N_α-Fmoc-N_ε-[butan-4-(2,3,4-tri-*O*-*p*-methoxybenzyl-6-*O*-trityl-β-D-galactopyranosyl)-amide]-L-lysine (3)



Compound **21β** (4.0 g, 3.3 mmol, 1.0 eq.) was dissolved in THF (37 mL) and cooled to 0°C. A solution of LiOH in H_2O (0.30 M, 22 mL, 6.6 mmol, 2.0 eq.) was added and the suspension was stirred vigorously for 75 minutes, after which the obtained solution was acidified by the addition of 1 M HCl to pH = 5-6 and diluted with brine. The mixture was extracted

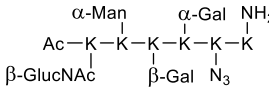
with EtOAc (2x) and the organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. After purification by column chromatography (2→8% MeOH in DCM), the title compound (1.9 g, 1.58 mmol, 48%) was obtained as a white foam. R_f: 0.64 (9/1 DCM/MeOH); $[\alpha]_D^{25}$ +35.6° (*c* = 0.41, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz, HH-COSY, HSQC): δ 7.74 (d, 2H, *J* = 7.6 Hz, Ar), 7.60 (t, 2H, *J* = 7.2 Hz, Ar), 7.44 – 7.33 (m, 8H, Ar), 7.33 – 7.18 (m, 15H, Ar), 7.05 – 6.99 (m, 2H, Ar), 6.93 – 6.87 (m, 2H, Ar), 6.87 – 6.81 (m, 2H, Ar), 6.76 – 6.69 (m, 2H, Ar), 5.78 – 5.67 (m, 2H, NH, N*H*Fmoc), 4.84 (d, 1H, *J* = 10.5 Hz, C*H*H PMB), 4.74 (d, 1H, *J* = 11.2 Hz, C*H*H PMB), 4.65 (s, 2H, CH_2 PMB), 4.55 (d, 1H, *J* = 10.5 Hz, C*H*H PMB), 4.44 (d, 1H, *J* = 11.3 Hz, C*H*H PMB), 4.36 (d, 2H, *J* = 7.2 Hz, CH_2 Fmoc), 4.33 – 4.25 (m, 1H, CH L-Lys), 4.20 (t, 1H, *J* = 7.1 Hz, CH Fmoc), 3.84 (d, 1H, *J* = 2.8 Hz, H-4), 3.83 – 3.73 (m, 9H, 3x CH_3 PMB), 3.63 (t, 1H, *J* = 9.3 Hz, H-2), 3.50 (dd, 1H, *J* = 9.3, 2.8 Hz, H-3), 3.43 (dd, 1H, *J* = 9.6, 6.4 Hz, C*H*H-6), 3.32 (t, 1H, *J* = 6.2 Hz, H-5), 3.19 – 3.10 (m, 2H, H-1, C*H*H ε-L-Lys), 3.10 – 2.95 (m, 2H, C*H*H-6, C*H*H ε-L-Lys), 2.30 – 2.11 (m, 2H, CH_2-9), 1.92 – 1.37 (m, 6H, CH_2-7, CH_2-8, 1x CH_2 β/γ/δ-L-Lys), 1.36 – 1.09 (m, 4H, 2x CH_2 β/γ/δ-L-Lys); ^{13}C-APT NMR ($CDCl_3$, 101 MHz, HSQC): δ 174.0, 159.3 (C=O), 159.3, 159.2, 156.1, 144.1, 143.9, 141.4, 130.6, 130.4 (C_q Ar), 130.0, 129.3, 128.7, 128.0, 127.8, 127.2, 127.2, 125.3, 120.0, 113.9, 113.9, 113.6 (Ar), 87.0 (C_q Trt), 84.5 (C-3), 80.2 (C-1), 78.5 (C-2), 77.7 (C-5), 75.2 (CH_2 PMB), 74.0 (C-4), 73.9, 72.2 (CH_2 PMB), 67.0 (CH_2 Fmoc), 63.6 (CH_2-6), 55.4, 55.3 (CH_3 PMB), 53.6 (CH L-Lys), 47.2 (CH Fmoc), 38.9 (CH_2 ε-L-Lys), 36.4 (CH_2-9), 31.7 (CH_2 β/γ/δ-L-Lys), 30.3 (CH_2-7), 28.8, 22.9 (CH_2 β/γ/δ-L-Lys), 21.8 (CH_2-8); FT-IR (neat, cm^{-1}): 2935, 1717, 1612, 1586, 1512, 1449, 1302, 1246, 1173, 1153, 1074, 1032, 900, 821, 760, 735, 704, 651, 633, 621, 541, 516; LC-MS: Rt = 7.96 min (Vydac 219TP 5 µm Diphenyl, 50 - 90% MeCN, 21 min run); ESI-MS: m/z 1225.6 $[M+Na]^+$; HRMS: $[M+H]^+$ calcd. for $C_{74}H_{79}O_{13}N_2$: 1203.55767, found 1203.55765.

Acetyl-Lys(*N*ε-[butan-4-(α-D-mannosyl)-amide])-Lys(*N*ε-[butan-4-(β-D-galactosyl)-amide])-Lys(*N*ε-[butan-4-(α-D-galactosyl)-amide])-Lys(N_3)-Lys-NH_2 (22)



Tentagel S Ram resin on 100 µmol scale was treated with 20% piperidine in DMF for 10 min and subsequently elongated with Fmoc-Lys(Boc)-OH, Fmoc-Lys(N_3)-OH, **4**, **3** and **1** using 2.0 equivalents of each amino acid and two hours coupling time at 50°C. After deprotection of the Fmoc, 50 µmol of the resin was capped by treatment with a mixture of 20% Ac_2O in 0.1 M DIPEA in DMF. The resin was washed with DCM and treated with the standard cleavage cocktail (TFA/TIS/H_2O, 95/2.5/2.5 v/v/v, 2.0 mL) for three hours. The suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **22** (3.4 mg, 2.4 µmol, 5%) was obtained as a white solid. LC-MS: Rt = 6.88 min (C18 Gemini, 5 - 20% MeCN, 15 min run); ESI-MS: m/z 1422.8 $[M+H]^+$; HRMS: $[M+H]^{2+}$ calcd. for $C_{62}H_{113}N_{13}O_{24}$: 711.90052, found 711.89978.

Acetyl-Lys(*N*ε-[butan-4-(2-deoxy-2-*N*-acetyl-β-D-glucosyl)-amide])-Lys(*N*ε-[butan-4-(α-D-mannosyl)-amide])-Lys(*N*ε-[butan-4-(β-D-galactosyl)-amide])-Lys(*N*ε-[butan-4-(α-D-galactosyl)-amide])-Lys(N_3)-Lys-NH_2 (23)



The previously obtained 50 µmol pentamer on resin (see **22**) was treated with a mixture of **2** (2.0 eq.), HCTU (2.0 eq.), DIPEA (4.0 eq.) in DMSO (2.0 mL) at 50 °C for two hours. LC-

MS analysis showed the presence of pentamer. Therefore the resin was treated with the same mixture at room temperature overnight. After deprotection of the Fmoc, the resin was capped by treatment with a mixture of 20% Ac_2O in 0.1 M DIPEA in DMF. The resin was washed with DCM and treated with the standard cleavage cocktail (TFA/TIS/H_2O, 95/2.5/2.5 v/v/v, 2.0 mL) for three hours. The suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, conjugate **23** (1.8 mg, 1.0 µmol, 2%) was obtained as a white solid. LC-MS: Rt = 7.33 min (C18 Gemini, 5 - 20% MeCN, 15 min run); ESI-MS: m/z 1824.0 $[M+H]^+$; HRMS: $[M+H]^{2+}$ calcd. for $C_{80}H_{144}N_{16}O_{31}$: 912.50862, found 912.50813.

Footnotes and References

(1) Osorio, F.; Reis e Sousa, C. *Immunity* **2011**, *34* (5), 651–664.
(2) van Dinther, D.; Stolk, D. A.; van de Ven, R.; van Kooyk, Y.; de Gruijl, T. D.; den Haan, J. M. M. *J. Leukoc. Biol.* **2017**, *102* (4), 1017–1034.
(3) Becer, C. R.; Gibson, M. I.; Geng, J.; Ilyas, R.; Wallis, R.; Mitchell, D. A.; Haddleton, D. M. *J. Am. Chem. Soc.* **2010**, *132* (43), 15130–15132.
(4) Kantchev, E. A. B.; Chang, C.-C.; Chang, D.-K. *Biopolymers* **2006**, *84* (2), 232–240.
(5) Russo, L.; Cipolla, L. *Chem. - A Eur. J.* **2016**, *22* (38), 13380–13388.
(6) Russo, L.; Gautieri, A.; Raspanti, M.; Taraballi, F.; Nicotra, F.; Vesentini, S.; Cipolla, L. *Carbohydr. Res.* **2014**, *389*, 12–17.
(7) Grant, D. A. W.; Kaderbhai, N. *Biochem. J.* **1986**, *234* (1), 131–137.
(8) Piccirillo, G.; Pepe, A.; Bedini, E.; Bochicchio, B. *Chem. - A Eur. J.* **2017**, *23* (11), 2648–2659.
(9) Jones, M. W.; Otten, L.; Richards, S.-J.; Lowery, R.; Phillips, D. J.; Haddleton, D. M.; Gibson, M. I. *Chem. Sci.* **2014**, *5* (4), 1611–1616.
(10) Ponader, D.; Maffre, P.; Aretz, J.; Pussak, D.; Ninnemann, N. M.; Schmidt, S.; Seeberger, P. H.; Rademacher, C.; Nienhaus, G. U.; Hartmann, L. *J. Am. Chem. Soc.* **2014**, *136* (5), 2008–2016.
(11) Asahina, Y.; Kawakami, T.; Hojo, H. *European J. Org. Chem.* **2019**, *2019* (9), 1915–1920.
(12) Koester, D.; Holkenbrink, A.; Werz, D. *Synthesis (Stuttg).* **2010**, *2010* (19), 3217–3242.
(13) Yang, Y.; Yu, B. *Chem. Rev.* **2017**, *117* (19), 12281–12356.
(14) Dondoni, A.; Marra, A. *Chem. Rev.* **2000**, *100* (12), 4395–4422.
(15) Ji, P.; Zhang, Y.; Wei, Y.; Huang, H.; Hu, W.; Mariano, P. A.; Wang, W. *Org. Lett.* **2019**, *21* (9), 3086–3092.
(16) Palomo, C.; Oiarbide, M.; Landa, A.; González-Rego, M. C.; García, J. M.; González, A.; Odriozola, J. M.; Martín-Pastor, M.; Linden, A. *J. Am. Chem. Soc.* **2002**, *124* (29), 8637–8643.
(17) Gustafsson, T.; Hedenström, M.; Kihlberg, J. *J. Org. Chem.* **2006**, *71* (5), 1911–1919.
(18) See compound **28** of Chapter 4.
(19) Sharma, P. K.; Kumar, S.; Kumar, P.; Nielsen, P. *Tetrahedron Lett.* **2007**, *48* (49), 8704–8708.
(20) Voigtritter, K.; Ghorai, S.; Lipshutz, B. H. *J. Org. Chem.* **2011**, *76* (11), 4697–4702.
(21) See compound **35** of Chapter 4.
(22) See compound **22a** of Chapter 3.
(23) Zeng, Y.; Ning, J.; Kong, F. *Carbohydr. Res.* **2003**, *338* (4), 307–311.
(24) Saada, M.-C.; Ombouma, J.; Montero, J.-L.; Supuran, C. T.; Winum, J.-Y. *Chem. Commun.* **2013**, *49* (50), 5699.
(25) Hansen, T.; Lebedel, L.; Remmerswaal, W. A.; van der Vorm, S.; Wander, D. P. A.; Somers, M.; Overkleeft, H. S.; Filippov, D. V.; Désiré, J.; Mingot, A.; *et al*. *ACS Cent. Sci.* **2019**, *5* (5), 781-788.
(26) Treatment of this mixture with *N*-bromosuccinimide in THF did not result in selective cyclization as was shown in Chapter 4 and 5 for respectively mannose and rhamnose.
(27) The low yields (48% and 46%) can be explained by partly removal of the Fmoc during the reaction and the trityl during the work-up.
(28) See compound **9a** of Chapter 3.

Chapter 7

Summary and future prospects

Summary

One of the main challenges in the development of an effective anti-cancer vaccine is the generation of an adequate and directed cellular immune response. Therefore, much attention has been directed to the construction of vaccine modalities that target dendritic cells (DCs), as these interact with both B cells and T cells, mediating humoral and cellular immune responses. DCs express pathogen recognition receptors (PRRs), that recognize pathogen associated molecular patterns, and Fc receptors that can bind antibodies complexed to antigens on the pathogens. These two types of receptors have been exploited in the development of novel vaccine modalities.[1,2] Ligands for PRRs, such as Toll-like receptors (TLRs) and Nucleotide binding oligomerization domain (NOD)-like receptors, can be used as vaccine adjuvants as they can induce maturation of DCs, stimulate the production of co-stimulatory molecules, and upregulate antigen presentation via MHC molecules. Combinations of specific antigens and selected PRR-ligands are widely investigated in vaccine modalities to generate a directed and improved immune response. Special examples are represented by the development of

synthetic peptide conjugate vaccines, in which a peptide antigen is covalently connected to one or more structurally defined PRR ligands. In the second strategy, Fc receptors are exploited as they bind to an immune complex, which is formed by binding of an antibody to an antibody-recruiting molecule (ARM). Antigens equipped with ARMs can thus be taken up more efficiently by DCs leading to enhanced antigen presentation and a more adequate immune response. The conjugation of a PRR-ligand or an ARM to an antigen is thus a much explored strategy to enhance the immunogenicity of vaccines and selected examples of immunostimulants and vaccine conjugates have been described in **Chapter 1**.

Chapter 2 describes the design, synthesis and immunological evaluation of four TLR4-ligand peptide-conjugates. In these conjugates CRX-527, a monophosphoryl lipid A analogue, was used as the built-in adjuvant, as it represents a powerful TLR4 stimulating agent, of which the mechanism of action is well described. In the generation of the conjugates several synthetic challenges had to be overcome. First, the route of synthesis towards CRX-527 was optimized and a route was developed that allowed the multi-gram scale synthesis of the required (R)-3-alkyloxytetradecanoic acids. The introduction of the chiral fatty acid on the glucosaminyl serine building block is a key step, and a silylidene protected glucosamine building block is introduced in Chapter 2 that allows for the successful incorporation of multiple fatty acids in the target structure and enables an effective purification of the generated lipid carrying carbohydrate. To investigate the influence of the linker on the immunological properties of CRX-527, three different ligands were generated, equipped with a hydrophobic or a hydrophilic linker, connected to the *C*-6 position of the glucosamine core via an ester or amide bond. The ligand equipped with a hydrophobic linker was incapable of inducing the production of the pro-inflammatory interleukin-12 (IL-12), likely because the addition of the extra fatty tail to the structure prevents proper binding to the MD2-TLR4 complex. Therefore, the ligand equipped with a hydrophilic spacer was used to generate conjugates with the ovalbumin derived $DEVA_5K$ peptide, containing the MHC-I epitope SIINFEKL. Using thiol/maleimide chemistry, four conjugates were assembled in which the ligand was either connected to the *N*- or the *C*-terminus of the peptide. A manual reversed phase chromatography protocol had to be developed, as the ester bonds at the *C*-6 position of CRX-527 turned out to be acid and base labile, prohibiting HPLC purification. Stimulation of DCs with the four new conjugates resulted in a higher IL-12 production for the ester conjugates, while the amide conjugates showed enhanced antigen presentation *in vitro*. The four new conjugates prove to be promising

self-adjuvanting vaccine modalities and further *in vivo* evaluation of the conjugates is currently ongoing.

Chapter 3 describes the exploitation of muramyl dipeptide (MDP), a NOD2 ligand, in four MDP-HPV-conjugates and four MDP/TLR2-ligand bisconjugates. With the aid of solid phase peptide synthesis (SPPS), a suitably protected *O*-MDP building block, containing an *N*-acetyl or an *N*-glycolyl group, was connected via its isoglutamic acid moiety to an HPV-16 derived peptide, containing both an MHC-I and an MHC-II epitope. An orthogonal protected lysine at the *C*-terminus of the immobilized peptide was used to introduce the TLR2-ligand, Pam_3CSK_4, leading to the projected bis-conjugates. In the second part of this Chapter, two *C*-glycoside MDP analogues bearing either an *N*-acetyl or *N*-glycolyl moiety, were synthesized. Key steps in the synthesis of these two *C*-MDP building blocks are the installation of the double bond on the glucosamine core, a Grubbs cross metathesis to install the carboxylic acid conjugation handle, and the subsequent reduction of the obtained alkene. The acid stability of the *C*-MDP analogues allowed their coupling to the immobilized peptide, via a spacer at the anomeric position, using a SPPS approach, leading to new NOD2-ligand HPV-conjugates and bisconjugates.

The mannose-6-phosphate receptor (MPR) is able to recognize and bind mannose-6-phosphate present on newly synthesized proteins and to deliver these to the endosomes. It also shuttles to the cell surface where it can take up mannose-6-phosphate carrying cargo. It was speculated that this pathway could be exploited to enhance the uptake of mannose-6-phosphate bearing peptide antigens, thereby leading to a stronger immune response. **Chapter 4** describes the synthesis of two mannose-6-phosphonate building blocks, an *O*-analogue (*O*-M6Po) equipped with an anomeric alkyne spacer and a *C*-mannosyl analogue (*C*-M6Po) equipped with an anomeric lysine spacer. In these building blocks the natural phosphate at the 6-position of the mannose moiety has been replaced with a *C*-phosphonate to prevent dephosphorylation by phosphatases. The installation of the phosphonate turned out to be critically depended on the protecting groups present on the mannosyl synthon, and it was revealed that an 2,3-*O*-isopropylidene group was required to prevent an intramolecular cyclization side reaction. Six *O*-M6Po and *C*-M6Po building blocks were incorporated at the *N*-terminus or *C*-terminus of the antigenic peptides, containing either a CTL epitope or a Th epitope, resulting in four multivalent M6Po-conjugates. The six *O*-M6Po residues were appended in one event to the separately prepared peptides by a copper mediated 1,3-dipolar cycloaddition reaction. The *C*-M6P building block

proved to be well suited for SPPS allowing an online SPPS of the projected conjugates. With the objective to further enhance the immunogenicity, four bisconjugates were designed and synthesized, in which not only the M6Po-ligands were incorporated but also a TLR7-ligand to ensure the activation of endosomal expressed TLR7.

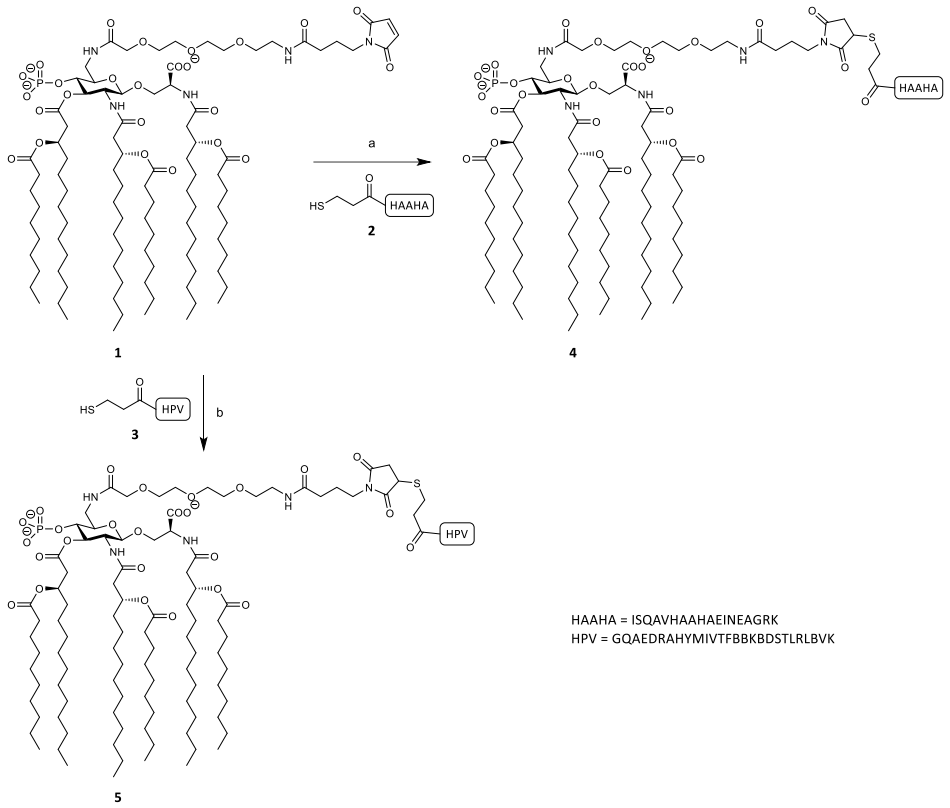
Chapter 5 describes the synthesis of seven novel rhamnose-peptide conjugates via an online SPPS. The design of these multivalent conjugates is based on rhamnose as an antibody recruiting molecule to generate antibody complexes that can be taken up by DCs through binding to Fc receptors. The generated conjugates carried one, two, three or six rhamnose moieties connected to an ovalbumin derived peptide LEQLESIINFEKLAAAAAK, harboring the MHC-I epitope SIINFEKL. An acid stabile *C*-analogue of rhamnose was used in the synthesis of *C*-rhamnose building blocks in which the rhamnose monosaccharide was connected to a lysine residue through a *C*-butanoic acid or an extended PEG-spacer, leading to two new building blocks suitable for SPPS chemistry.

Chapter 6 describes the design and synthesis of four *C*-glycosyl lysine building blocks, which can be used in standard SPPS. These building blocks are functionalized with either an α-mannose, β-*N*-acetyl-glucosamine, β-galactose or α-galactose residue and the synthons were protected with acid-labile protecting groups. The protective group pattern allows the concomitant removal of all acid labile (glyco)peptide protecting groups as well as the release of the peptide from the resin, at the final stage of the SPPS. Key steps in the synthesis of these building blocks are the Grubbs cross-metathesis, reduction of the double bond and subsequent hydrolysis of the methyl ester to allow the connection at the side chain of lysine.

Future prospects

The TLR4-ligands described in **Chapter 2** can be further exploited for incorporation in new conjugates with other epitopes. The first endeavors that have been undertaken include conjugation to the ovalbumin derived (HAAHA) peptide, which contains an MHC-II epitope, and the HPV-16 derived peptide, harboring both MHC-I and MHC-II epitopes (Scheme 1). The latter can be used as a vaccine against the human papillomavirus (HPV), which is responsible for cervical cancer. Both conjugates were synthesized via thiol-maleimide coupling of ligand **1** to peptides **2** and **3** (Scheme 1). The purification method developed in Chapter 2, only proved effective for the purification of conjugate **4** and therefore conjugate **5** was purified by RP-HPLC.

Conjugates **4** and **5** were obtained in respectively 40% (1.4 mg) and 54% (2.2 mg). At present, the immunological evaluation of these two conjugates is ongoing.



Scheme 1. Synthesis of TLR4-ligand peptide conjugates **4** and **5**. *Reagents and conditions*: a) **2**, DMF/$CHCl_3$/H_2O, 72h, 40%; b) **3**, DMF/$CHCl_3$/H_2O, 72h, 54%.

Tada *et al*. have studied the synergy between different PRR ligands and they discovered that a mixture of TLR4-ligands and NOD-ligands can enhance the immune response with over a 1000-fold compared to stimulation with the separate ligands.[3] Therefore, it would be interesting to synthesize bisconjugate **6** (Figure 1) in which the TLR4-ligand (**Chapter 2**), the NOD2 ligand MDP (**Chapter 3**) and an antigenic peptide are covalently connected. To further enhance the immunogenicity a third PRR-ligand may be incorporated, by coupling the TLR2-ligand Pam_3CSK_4 after deprotection of an MMT-protected lysine at the *C*-terminus, resulting in triple-conjugate **7**. This conjugate contains all the main components of the bacterial cell wall that are recognized by PRRs: TLR4, TLR2 and NOD2. The orthogonally protected *C*-MDP building blocks, described in **Chapter 3** can also be used to introduce multiple MDPs in an antigenic peptide, as in

conjugates **8** and **9** (Figure 1). The multivalent MDP-conjugates could lead to better binding to NOD2, thereby enhancing the potency of the conjugate. In order to bind to the NOD2 receptor, the MDP has to enter the cytosol and to improve this transport a cell-penetrating peptide (CPP), such as GRKKRRQRRRPSQ, could be incorporated in the conjugates as shown in **10** and **11**.[4,5]

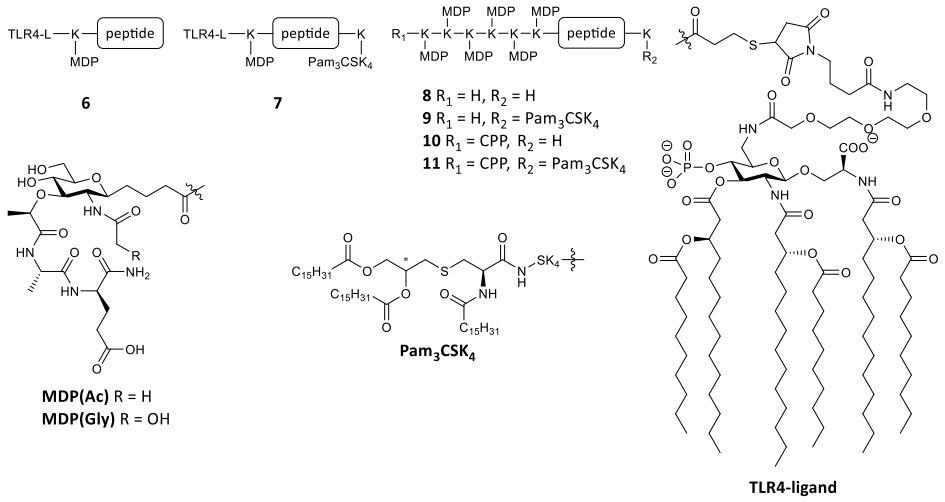


Figure 1. Proposed conjugates **6**-**11**.

Gold nanoparticles (AuNPs) have been shown to be effective carriers to improve vaccine efficacy.[6,7] AuNPs offer the possibility to combine various components needed to develop a vaccine in a controlled way and thereby deliver all immunological signals in a single well-defined system.[8] The repetitive surface organisation of AuNP facilitates phagocytosis, resulting in better processing of the antigens and subsequent activation of the immune system.[9,10] The potential of AuNPs as candidates for vaccine carriers is exemplified by their use in vaccine-modalities against *Streptococcus pneumonia*, HIV or cancer.[10–13] Therefore, it is interesting to use the NOD-2 ligands, described in **Chapter 3**, for the development of MDP-coated AuNPs. The multivalency of AuNPs[14] leads to clusters of several MDP molecules on the surface that may resemble MDP "clusters" of the bacterial cell wall. To address the influence of particle size[15] and ligand density on the immunoactivity of MDP-coated AuNPs, four 5 nm particles (**12**-**15**, Figure 1) were prepared with either a low or a high concentration of MDP(Ac) and MDP(Gly), and for the generation of 2 nm particles (**16**-**19**, Figure 2), two different ratios (1/1 and 1/9) between MDP and glucose, as an inert "inner component", were used.

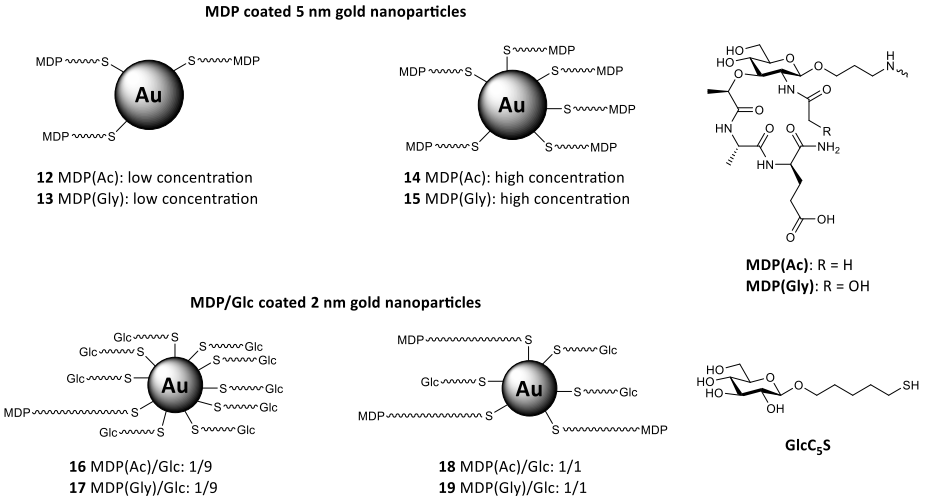
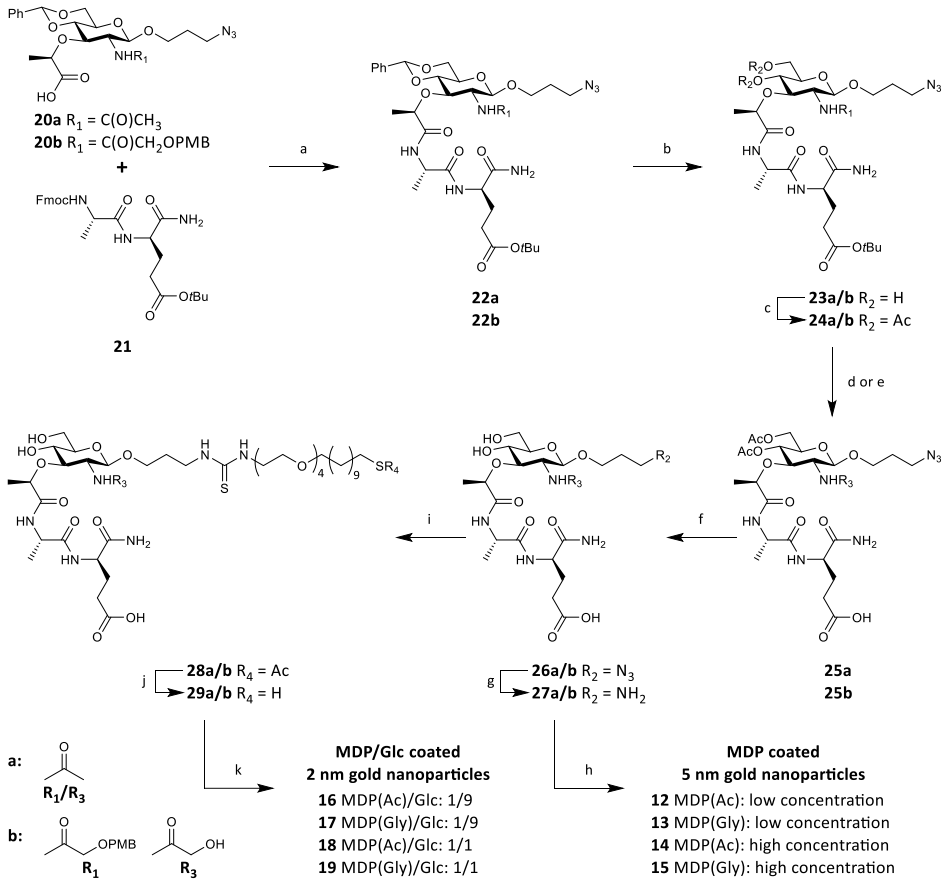


Figure 2. Gold nanoparticles **6**-**13** functionalized with MDP(Ac) and MDP(Gly).

The synthesis of the MDP coated AuNPs required the availability of amine functionalized MDP derivatives **27a** and **27b** (Scheme 2). Removal of Fmoc-group in dipeptide **21**[16] with DBU and quenching with HOBt, was followed by an HCTU and DIPEA mediated condensation of the resulting free amine with acids **20a** and **20b**[17] to give **22a** and **22b**. As an one-pot removal of the benzylidene and *tert*-butyl groups with TFA can lead to hydrolysis at the anomeric position, the following protective group manipulation was undertaken.[18] First, the benzylidene in **22a** and **22b** was selectively removed in AcOH/H_2O using neopentyl glycol as scavenger yielding **23a** and **23b** in respectively 78% and 85% yield. Acetylation of diol **23a** and **23b** stabilizes the anomeric position allowing the removal of the *tert*-butyl and *p*-methoxybenzyl groups with TFA to give **25a** and **25b**. For the synthesis of **25b**, triethylsilane was used to scavenge the released *p*-methoxybenzyl cations. Deacetylation with ammonia in methanol and purification by HW-40 gel filtration afforded azides **26a** and **26b** in 74% and 65% yield. Hydrogenation of these azides with Pd/C gave amines **27a** and **27b**, which were used for the coating of the 5 nm AuNPs. Commercially available 5 nm AuNPs functionalized with PEG-*N*-hydroxysuccinimide linkers were treated with amines **27a** and **27b** at two different concentrations (15 mM and 30 mM) to obtain AuNPs coated with different amounts of MDP(Ac) and MDP(Gly). The mixture was shaken for 2.5 hours and the remaining OSu esters were quenched with ethanolamine. The AuNPs were filtered over 30 KDa cut-off centrifugal filters against water giving AuNPs **12**-**15**. For the preparation of the 2 nm AuNPs, MDP derivatives **29a** and **29b** with a thiol functionality are required.



Scheme 2. Synthesis of MDP coated AuNPs **12**-**19**. *Reagents and conditions*: a) *i*. DBU, HOBt; *ii*. **20a** or **20b**, HCTU, DIPEA, DMF, **22a**: 85%, **22b**: 89%; b) neopentyl glycol, H_2O, AcOH, 65°C, **23a**: 78%, **23b**: 85%; c) Ac_2O, pyridine, dioxane, **24a**: 90%, **24b**: 84%; d) TFA, DCM, **25a**: 77%; e) TFA, triethylsilane, DCM, **25b**: 94%; f) NH_4OH, MeOH, **26a**: 74%, **26b**: 65%; g) Pd/C, H_2O, **27a**: 53%, **27b**: 87%; h) 5 nm-cytodiagnostics NHS-activated gold nanoparticles, H_2O, then ethanolamine; i) 1-isothiocyanate-3,6,9,12-tetraoxa-23-thioacetyltricosane, H_2O/*t*BuOH/CH_3CN, **28a**: 75%, **28b**: 74%; j) sodium methoxide, MeOH, **29a**: 89%, **29b**: 90%; k) $GlcC_5S$, $HAuCl_4$, $NaBH_4$, MeOH/H_2O.

Therefore, amines **27a** and **27b** were treated with 1-isothiocyanate-3,6,9,12-tetraoxa-23-thioacetyltricosane to give **28a** and **28b** 75% and 74% yield. The introduced linker was selected to ensure flexibility, favoring the presentation of MDP on the AuNPs as previously reported.[14] The free thiol was acquired after deacetylation in the presence of sodium methoxide yielding **29a** and **29b**. The 2 nm AuNPs were prepared using a reported protocol[19], in which an inner glucose-derivative was used to modulate the ligand-density on the particles as this has been shown to increase the water-solubility of the particles and the glucose functionalized particles show no cytotoxicity in different

cell lines.[20] AuNPs **16**-**19** were obtained by adding a methanol solution of **29a** or **29b** and the glucose derivative $GlcC_5S$ (Figure 2) to an aqueous solution of tetrachloroauric acid ($HAuCl_4$) followed by treatment with sodium borohydride. These particles are suitable for studying the multivalent effect of MDP in the induction of an immune response and are a set-up for AuNPs loaded with MDP and other immunostimulants, such as TLR2 and TLR4 ligands, and T cell epitopes to be used as cancer vaccines.

The mannose-6-phosphate receptor (MPR) can be used as an efficient tool to deliver targets to endosomes. The M6Po building blocks, described in **Chapter 4**, could be used to improve existing therapies for the treatment of lysosomal storage diseases, for example Fabry and Pompe disease. Upon implementation via click chemistry on azide functionalized enzymes[21,22], the M6Po moieties can act as a homing device in the replacement enzyme therapy.[23] TLR7 and TLR9 are expressed in the endosomes, indicating that conjugates consisting of M6Po together with a TLR7-ligand (**30**) or a TLR9-ligand (**31**) (Figure 3), could facilitate the uptake of the conjugates and thereby improve the potency of the ligands as immunostimulants. The *C*-glycosyl lysine building blocks, described in **Chapter 5** and **Chapter 6** are meant for the synthesis of homo- and heteromultivalent glycopeptides or glycoclusters via a SPPS approach.[24] Their ability to mimic glycans can be used to induce binding of vaccines to lectins leading to the enhancement of the immune response (**32**) or as carrier for drug delivery (**33**).[25–27] The incorporation of a glycosylated lysine in a glycopeptide such as **34** can be a starting point for an enzymatic transglycosylation procedure to glycans, for example $Man_9GlcNAc_2$-glycopeptide **35**. Moreover, glycosylated peptides can be used as mimics of HIV-1 envelope proteins such as gp41 and gp120, thereby interfering with the binding and entry of the virus, thus resulting in a possible treatment of HIV.[28,29]

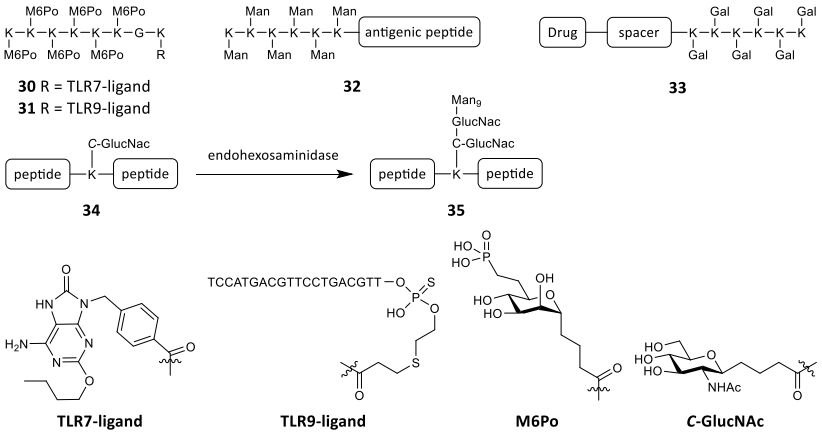
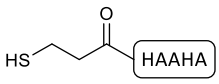


Figure 3. Proposed conjugates **30**-**35**.

Experimental

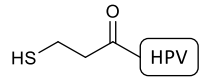
All reagents were of commercial grade and used as received unless stated otherwise. Reaction solvents were of analytical grade and when used under anhydrous conditions stored over flame-dried 3Å molecular sieves. All moisture and oxygen sensitive reactions were performed under an argon atmosphere. Column chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). For TLC analysis, pre-coated silica gel aluminum sheets (Merck, silica gel 60, F254) were used with detection by UV-absorption (254/366 nm) where applicable. Compounds were visualized on TLC by UV absorption (245 nm), or by staining with one of the following TLC stain solutions: $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L), $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) and 10% H_2SO_4 in H_2O; bromocresol (0.4 g/L) in EtOH; $KMnO_4$ (7.5 g/L), K_2CO_3 (50 g/L) in H_2O. Staining was followed by charring at ~150°C. ^1H and ^{13}C spectra were recorded on a Bruker AV-400 (400/100 MHz) spectrometer, a Bruker AV-500 Ultrashield (500/126 MHz) spectrometer, a Bruker AV-600 (600/151 MHz) or a Bruker AV-850 (850/214 MHz) and all individual signals were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) in $CDCl_3$ or via the solvent residual peak. Coupling constants (*J*) are given in Hz. LC-MS analysis were done on an Agilent Technologies 1260 Infinity system with a C18 Gemini 3 µm, C18, 110 Å, 50 x 4.6 mm column or a Vydac 219TP 5 µm Diphenyl, 150 x 4.6 mm column with a flow of 0.5 ml/min. Absorbance was measured at 214 nm and 256 nm and an Agilent Technologies 6120 Quadrupole mass spectrometer was used as detector. HPLC purification was performed with a Gilson GX-281 with a Gemini 5u, C6-Phenyl, 110 Å, 250 x 10.0 mm column. High resolution mass spectra were recorded on a Synapt G2-Si equipped with an electron spray ion source positive mode. Infrared spectra were recorded on a Perkin Elmer Spectrum 2 FT-IR. Mass analysis of the TLR4-ligand conjugates was performed on a 15T MALDI-FT-ICR MS system.

3-Mercaptoproponamide-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys-NH_2 (2)



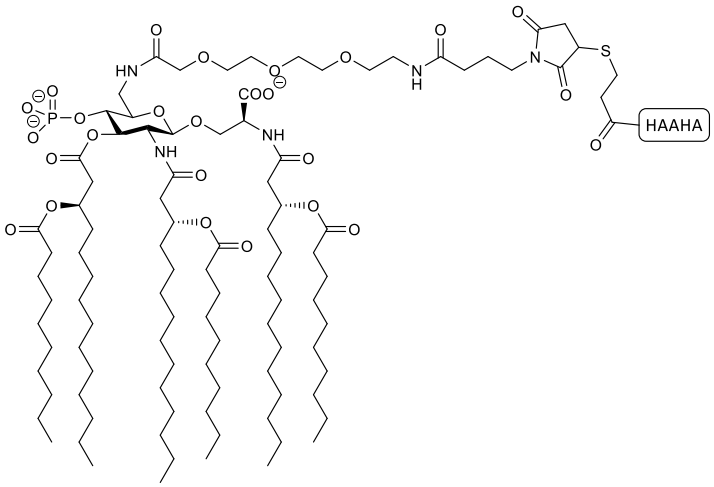
Tentagel S Ram resin loaded with H-Ile-Ser(O*t*Bu)-Gln(Trt)-Ala-Val-His(Trt)-Ala-Ala-His(Trt)-Ala-Glu(O*t*Bu)-Ile-Asn(Trt)-Glu(O*t*Bu)-Ala-Gly-Arg(Pbf)-Lys(MMT)-NH_2 on 70 µmol scale was washed with DMF (5x), followed by the addition of a solution of 3-(tritylthio)propionic acid (51 mg, 150 µmol, 2.1 eq.) and HCTU (58 mg, 140 µmol, 2.0 eq.) in DMF (1.4 mL) and DIPEA (49 µL, 280 µmol, 4.0 eq.). The reaction vessel was shaken overnight at 850 rpm, after which it was washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (2.8 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **2** (22 mg, 11 µmol, 16%) was obtained as a white solid. LC-MS: Rt = 5.34 min (C18 Gemini, 10 - 50% MeCN, 11 min run); ESI-MS: m/z 995.2 $[M+H]^{2+}$; HRMS: $[M+H]^{2+}$ calcd. for $C_{83}H_{139}N_{29}O_{26}S$: 995.00779, found 995.00816.

3-Mercaptoproponamide-Gly-Gln-Ala-Glu-Pro-Asp-Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe-Abu-Abu-Lys-Abu-Asp-Ser-Thr-Leu-Arg-Leu-Abu-Val-Lys-NH_{22} (3)



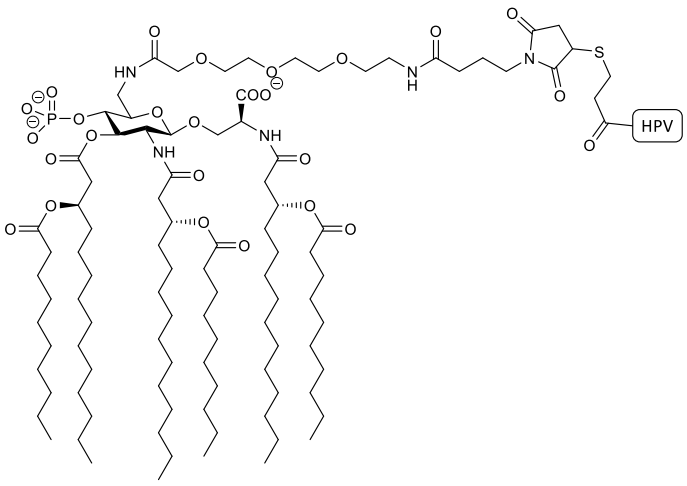
Tentagel S Ram resin loaded with H-Gly-Gln(Trt)-Ala-Glu(O*t*Bu)-Pro-Asp(O*t*Bu)-Arg(Pbf)-Ala-His(Trt)-Tyr(O*t*Bu)-Asn(Trt)-Ile-Val-Thr(psiMe,Mepro)-Phe-Abu-Abu-Lys(O*t*Bu)-Abu-Asp(O*t*Bu)-Ser(psiMe,Mepro)-Thr(O*t*Bu)-Leu-Arg(Pbf)-Leu-Abu-Val-Lys(MMT) on 70 µmol scale was washed with DMF (5x), followed by the addition of a solution of 3-(tritylthio)propionic acid (50 mg, 150 µmol, 2.1 eq.) and HCTU (58 mg, 140 µmol, 2.0 eq.) in DMF (1.4 mL) and DIPEA (49 µL, 280 µmol, 4.0 eq.). The reaction vessel was shaken overnight at 850 rpm, after which it was washed with DMF (3x) and DCM (3x). The peptide was cleaved from the resin after treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) (2.8 mL) for 3 hours the suspension was filtered and the residue was washed with the cleavage cocktail (2.8 mL). The product was precipitated with Et_2O. After purification by RP-HPLC and lyophilisation, compound **2** (7.5 mg, 2.4 µmol, 3%) was obtained as a white solid. LC-MS: Rt = 4.97 min (C18 Gemini, 10 - 90% MeCN, 11 min run); ESI-MS: m/z 1593.9 $[M+H]^{2+}$; HRMS: $[M+H]^{5+}$ calcd. for $C_{141}H_{234}N_{41}O_{41}S$: 637.94358, found 637.94327.

***N*-Terminus 6-*NH*-HAAHA conjugate (4)**



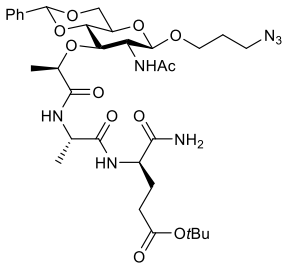
Thiol-peptide **2** (2.71 mg, 1.36 µmol, 1.5 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/0.5 v/v, 272 µl) in an Eppendorf tube. A solution of compound **1** (5.0 mM, 182 µL, 0.91 µmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 48 hours. LCMS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of CH_3CN/*t*BuOH/MilliQ H_2O (1/1/1 v/v/v, 0.55 mL) and sonicated for 5 minutes. After purification using a C18 column[30], LC-MS analysis showed that the MeOH and DCM/MeOH (1/1 v/v) flush contained pure conjugate. After lyophilization, conjugate **4** (1.4 mg, 0.36 µmol, 40%) was obtained as a white solid. LC-MS: Rt = 15.95 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.5 mL/min). ESI-MS: m/z 1916.4 $[M+H]^{2+}$. MALDI-FT-ICR MS (m/z): $[M+H]^{+}$ calcd. for $C_{238}H_{404}N_{46}O_{66}SP$: 5025,9124, found 5025.7939.

_N_-Terminus 6-_NH_-HPV16 conjugate (5)



Thiol-peptide **3** (5.79 mg, 1.82 µmol, 2.0 eq.) was dissolved in a mixture of DMF/MilliQ H_2O (4/0.5 v/v, 273 µl) in an Eppendorf tube. A solution of compound **1** (5.0 mM, 180 µL, 0.91 µmol, 1.0 eq.) was added and the mixture was shaken at 850 rpm for 3 days. LC-MS analysis showed complete conversion of the starting material. The reaction mixture was diluted with a mixture of DMSO (0.55 mL) and sonicated for 5 minutes. After purification by RP-HPLC (Gemini, C6-phenyl) and lyophilization, conjugate **5** (2.2 mg, 0.49 µmol, 54%) was obtained as a white solid. LC-MS: Rt = 15.09 min (Diphenyl Vydac, 10 - 90% IPA, 25 min run, 0.5 mL/min). ESI-MS: m/z 1676.9 $[M+H]^{3+}$. MALDI-FT-ICR MS (m/z): $[M+H]^+$ calcd. for $C_{108}H_{312}N_{34}O_{51}SP$: 3829.2319, found 3829.1598.

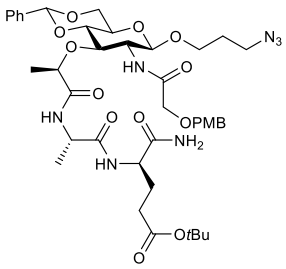
3-Azidopropyl-2-_N_-acetyl-4,6-_O_-benzylidene-2-deoxy-_O_-((R)-1-carboxyethyl-L-alanyl-acetamide-5-_O_-_tert_-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (22a)



Fmoc-L-Ala-_i_-D-Gln(O_t_Bu)-NH_2[16] (0.15 g, 0.30 mmol, 1.5 eq.) was dissolved in DMF (2.0 mL) and DBU (45 µL, 0.30 mmol, 1.5 eq.) was added. After 1 hour, HOBt (0.11 g, 0.74 mmol, 3.5 eq.) was added and the reaction continued to stir for 1 hour. 3-Azidopropyl-2-_N_-acetyl-4,6-_O_-benzylidene-2-deoxy-3-_O_-((R)-1-carboxyethyl)-β-D-glucopyranoside[31] (92 mg, 0.20 mmol, 1.0 eq.), HCTU (98 mg, 0.24 mmol, 1.2 eq.) and DIPEA (0.14 mL, 0.80 mmol, 4.0 eq.) were added and the reaction was stirred overnight. The reaction mixture was diluted with DCM, quenched with H_2O and washed with brine (1x). The aqueous layer was extracted with DCM (2x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated _in vacuo_. Purification by column chromatography (2→20% methanol in DCM) yielded the title compound (0.22 g, 0.17 mmol, 85%) as a white solid. R_f: 0.34 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 500 MHz, HH-COSY, HSQC): δ 7.51 – 7.45 (m, 2H, Ar), 7.39 – 7.32 (m, 3H, Ar), 5.64 (s, 1H, CH benzylidene), 4.53 (d, 1H, _J_ = 8.4 Hz, H-1), 4.38 – 4.25 (m, 3H, C_H_-6, CH L-Ala, CH _i_-D-Gln), 4.17 (q, 1H, _J_ = 6.7 Hz, CH lactic acid), 3.95 – 3.88 (m, 2H, H-2, C_H_H $C_3H_6N_3$), 3.82 (t, 1H, _J_ = 10.2 Hz, C_H_H-6), 3.76 – 3.66 (m, 2H, H-3, H-5), 3.64 – 3.56 (m, 1H, C_H_H $C_3H_6N_3$), 3.51 – 3.44 (m, 1H, H-4), 3.41 – 3.36 (m, 2H, CH_2 $C_3H_6N_3$), 2.34 – 2.27 (m, 2H, CH_2 γ-_i_-D-Gln), 2.23 – 2.11 (m, 1H, C_H_H β-_i_-D-Gln), 1.98 (s, 3H, CH_3 Ac), 1.88 – 1.76 (m,

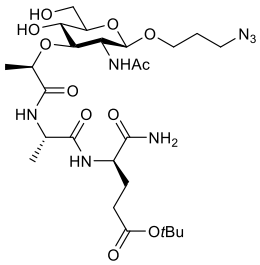
3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.44 (s, 9H, 3x CH_3 *t*Bu), 1.39 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.32 (d, 3H, *J* = 6.7 Hz, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 126 MHz, HSQC): δ 176.2 (C=O), 175.6 (C=O), 174.9 (C=O), 173.7 (C=O), 139.0 (C_q Ar), 130.0, 129.2, 127.3 (Ar), 103.3 (C-1), 102.7 (CH_2 benzylidene), 82.5 (C_q *t*Bu), 81.9 (C-3), 80.7 (C-5), 79.0 (CH lactic acid), 69.6 (CH_2-6), 67.5 (CH_2 $C_3H_6N_3$), 67.5 (C-4), 56.8 (C-2), 53.5 (CH *i*-D-Gln), 50.7 (CH L-Ala), 49.2 (CH_2 $C_3H_6N_3$), 32.7 (CH_2 γ-*i*-D-Gln), 30.1 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.3 (CH_2 β-*i*-D-Gln), 23.4 (CH_3 Ac), 19.8 (CH_3 lactic acid), 18.0 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 2388, 2105, 1730, 1635, 1474, 1428, 1368, 1154, 1101, 1016, 751, 698. $[M+Na]^+$ calcd. for $C_{33}H_{49}N_7O_{11}Na$: 742.3382, found 742.33818.

3-Azidopropyl-4,6-*O*-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (22b)



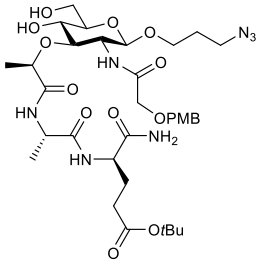
Fmoc-L-Ala-*i*-D-Gln(O*t*Bu)-NH_2[16] (1.2 g, 2.4 mmol, 1.5 eq.) was dissolved in DMF (21 mL) and DBU (0.36 mL, 2.4 mmol, 1.5 eq.) was added. After 80 minutes, the reaction was quenched with HOBt (0.86 g, 5.6 mmol, 3.5 eq.) and stirred for 1 hour. 3-Azidopropyl-4,6-*O*-benzylidene-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside[32] (0.96 g, 1.6 mmol, 1.0 eq.), HCTU (0.80 g, 1.9 mmol, 1.2 eq.) and DIPEA (1.1 mL, 6.3 mmol, 4.0 eq.) were subsequently added and the mixture was stirred overnight. After diluting with DCM, the organic layer was washed with H_2O (1x), brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (2→10% methanol in DCM) gave compound **22b** (1.6 g, 1.4 mmol, 89%) as a white solid. R_f: 0.34 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.51 – 7.43 (m, 2H, Ar), 7.39 – 7.28 (m, 5H, Ar), 6.98 – 6.89 (m, 2H, Ar), 5.64 (s, 1H, CH benzylidene), 4.65 – 4.59 (m, 1H, H-1), 4.54 (s, 2H, CH_2 glycol), 4.36 – 4.28 (m, 2H, C*H*H-6, CH *i*-D-Gln), 4.27 – 4.20 (m, 1H, CH L-Ala), 4.20 – 4.13 (m, 1H, CH lactic acid), 4.07 – 3.99 (m, 1H, H-2), 3.94 (d, 2H, *J* = 7.7 Hz, CH_2 PMB), 3.91 – 3.82 (m, 3H, H-4, C*H*H-6, C*H*H $C_3H_6N_3$), 3.80 (s, 3H, CH_3 PMB), 3.70 (t, 1H, *J* = 9.2 Hz, H-3), 3.62 – 3.54 (m, 1H, H-5), 3.52 – 3.44 (m, 1H, C*H*H $C_3H_6N_3$), 3.38 – 3.27 (m, 2H, CH_2 $C_3H_6N_3$), 2.37 – 2.31 (m, 2H, CH_2 γ-*i*-D-Gln), 2.24 – 2.14 (m, 1H, C*H*H β-*i*-D-Gln) 1.89 – 1.73 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.43 (s, 9H, 3x CH_3 *t*Bu), 1.33 (d, 6H, *J* = 7.0 Hz, CH_3 lactic acid, CH_3 L-Ala).^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.2, 175.5, 174.9, 173.8, 173.4 (C=O), 139.0 (C_q Ar), 131.0 (Ar), 130.4 (C_q Ar), 130.0, 129.2, 127.3, 114.9 (Ar), 103.0 (CH benzylidene), 102.7 (C-1), 82.4 (C-3), 81.8 (C_q *t*Bu), 80.4 (C-5), 79.1 (CH lactic acid), 73.9 (CH_2 glycol), 69.9 (CH_2 PMB), 69.6 (CH_2 $C_3H_6N_3$), 67.5 (CH_2-6), 67.4 (C-4), 56.4 (C-2), 55.7 (CH_3 PMB), 53.5 (CH *i*-D-Gln), 50.6 (CH L-Ala), 49.1 (CH_2 $C_3H_6N_3$), 32.7 (CH_2 γ-*i*-D-Gln), 30.1 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.2 (CH_2 β-*i*-D-Gln), 19.8 (CH_3 lactic acid), 18.0 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3283, 2978, 2100, 1663, 1514, 1455, 1370, 1259, 1156, 1093, 1033, 749, 696. $[M+Na]^+$ calcd. for $C_{41}H_{57}N_7O_{13}Na$: 878.9328, found 878.3937.

3-Azidopropyl-2-*N*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (23a)



Compound **22a** (75 mg, 0.11 mmol, 1.0 eq.) was dissolved in a 60% AcOH/H_2O (1.2 mL) solution. Neopentylglycol (25 mg, 0.24 mmol, 2.2 eq.) was added and the suspension was heated to 65°C for 5 hours, after which TLC analysis showed full conversion of the starting material. The reaction mixture was diluted with H_2O and co-evaporated with toluene (3x). Purification by column chromatography (2→20% methanol in DCM) afforded compound **23a** (56 mg, 88 µmol, 78%) as a white solid. R_f: 0.10 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 4.39 – 4.31 (m, 2H, H-1, CH *i*-D-Gln), 4.30 – 4.17 (m, 2H, CH lactic acid, CH L-Ala), 3.99 – 3.91 (m, 1H, C*H*H $C_3H_6N_3$), 3.89 (dd, 1H, *J* = 11.9, 2.3 Hz, C*H*H-6), 3.84 – 3.77 (m, 1H, H-2), 3.70 (dd, 1H, *J* = 12.0, 5.7 Hz, C*H*H-6), 3.61 – 3.51 (m, 1H, C*H*H $C_3H_6N_3$), 3.48 – 3.41 (m, 2H, H-3, H-4), 3.35 (s, 2H, CH_2 $C_3H_6N_3$), 3.33 – 3.26 (m, 1H, H-5), 2.36 – 2.29 (m, 2H, CH_2 γ-*i*-D-Gln), 2.25 – 2.14 (m, 1H, C*H*H β-*i*-D-Gln), 1.94 (s, 3H, CH_3 Ac), 1.90 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.45 (s, 9H, 3x CH_3 *t*Bu), 1.41 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.37 (d, 3H, *J* = 6.7 Hz, CH_3 L-Ala).^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.2, 176.0, 175.2, 173.7, 173.5 (C=O), 102.7 (C-1), 84.0 (C-3), 81.8 (C_q *t*Bu), 78.8 (CH lactic acid), 77.8 (C-5), 70.8 (C-4), 67.1 (CH_2 $C_3H_6N_3$), 62.7 (CH_2-6), 56.4 (C-2), 53.5 (CH *i*-D-Gln), 50.7 (CH L-Ala), 49.2 (CH_2 $C_3H_6N_3$), 32.6 (CH_2 γ-*i*-D-Gln), 30.0 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.2 (CH_2 β-*i*-D-Gln), 23.3 (CH_3 Ac), 19.6 (CH_3 lactic acid), 17.8 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3299, 2980, 2935, 2097, 1651, 1535, 1451, 1369, 1311, 1256, 1155, 1109, 1076, 948, 845, 628. $[M+Na]^+$ calcd. for $C_{26}H_{45}N_7O_{11}Na$: 654.3069, found 654.30600.

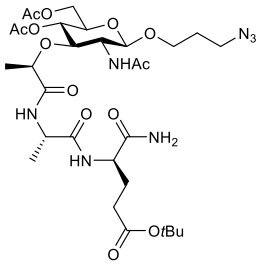
3-Azidopropyl-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (23b)



Compound **22b** (0.85 g, 1.0 mmol, 1.0 eq.) was dissolved in a 60% AcOH/H_2O (10 mL) solution. Neopentylglycol (0.21 g, 2.0 mmol, 2.0 eq.) was added and the suspension was heated to 65°C for 4 hours. The reaction mixture was diluted with H_2O and co-evaporated with toluene (3x). Purification by column chromatography (2→20% methanol in DCM) yielded compound **23b** (0.65 g, 0.85 mmol, 85%) as a white solid. R_f: 0.19 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.33 – 7.27 (m, 2H, Ar), 6.94 – 6.89 (m, 2H, Ar), 4.52 (d, 2H, *J* = 1.4 Hz, CH_2 glycol), 4.47 (d, 1H, *J* = 8.4 Hz, H-1), 4.36 – 4.31 (m, 1H, CH *i*-D-Gln), 4.28 – 4.18 (m, 2H, CH lactic acid, CH L-Ala), 3.97 – 3.87 (m, 5H, H-2, H-3, C*H*H-6, C*H*H $C_3H_6N_3$, CH_2 PMB), 3.79 (s, 3H, CH_3 PMB), 3.71 (dd, 1H, *J* = 12.0, 5.7 Hz, *CH*H-6), 3.63 – 3.51 (m, 2H, H-3, C*H*H $C_3H_6N_3$), 3.49 – 3.42 (m, 1H, H-4), 3.36 – 3.29 (m, 3H, H-5, CH_2 $C_3H_6N_3$), 2.35 – 2.27 (m, 2H, CH_2 γ-*i*-D-Gln), 2.24 – 2.13 (m, 1H, C*H*H β-*i*-D-Gln), 1.91 – 1.80 (m, 1H C*H*H β-*i*-D-Gln), 1.80 – 1.72 (m, 2H, CH_2 $C_3H_6N_3$), 1.43 (s, 9H, 3x CH_3 *t*Bu), 1.41 – 1.32 (m, 6H, CH_3 lactic acid, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 101 MHz, HSQC):

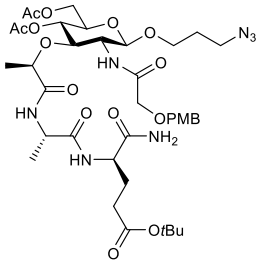
176.2, 175.7, 175.0, 173.7, 173.0 (C=O), 161.0 (C_q Ar), 130.9 (Ar), 130.4 (C_q Ar), 114.9 (Ar), 102.2 (C-1), 83.4 (C-3), 81.8 (C_q *t*Bu), 78.7 (CH lactic acid), 77.7 (C-5), 73.9 (CH_2 glycol), 70.8 (C-4), 69.8 (CH_2 PMB), 67.1 (CH_2 $C_3H_6N_3$), 62.7 (CH_2-6), 56.1 (C-2), 55.7 (CH_3 PMB), 53.5 (CH *i*-D-Gln), 50.6 (CH L-Ala), 49.8 (CH_2 $C_3H_6N_3$), 32.6 (CH_2 γ-*i*-D-Gln), 30.0 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.1 (CH_2 β-*i*-D-Gln), 19.5 (CH_3 lactic acid), 17.8(CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3316, 2932, 2098, 1662, 1515, 1454, 1369, 1250, 1156, 1077, 637. $[M+Na]^+$ calcd. for $C_{34}H_{53}N7O_{13}Na$: 790.8238, found 790.3620.

3-Azidopropyl-2-*N*-acetyl-4,6-di-*O*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (24a)



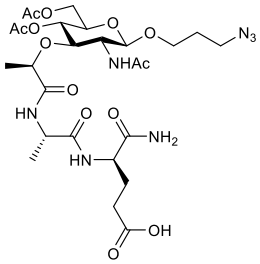
Diol **23a** (0.54 g, 0.84 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under argon atmosphere and dissolved in dioxane (8.5 mL). Pyridine (2.3 mL, 28 mmol, 33 eq.) and Ac_2O (0.7 mL, 7.4 mmol, 8.8 eq.) were added and the mixture was stirred overnight. The reaction was quenched with methanol at 0°C, diluted with toluene and concentrated *in vacuo*. Purification by column chromatography (2→20% methanol in DCM) afforded the title compound (0.55 g, 0.76 mmol, 90%) as a white solid. R_f: 0.50 (1/9 MeOH/DCM). H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 4.97 (t, 1H, *J* = 10.0, 9.1 Hz, H-4), 4.44 (d, 1H, *J* = 8.4 Hz, H-1), 4.33 (dd, 1H, *J* = 9.7, 4.5 Hz, CH *i*-D-Gln), 4.26 (dd, 1H, *J* = 12.3, 4.7 Hz, C*H*H-6), 4.20 (q, 1H, *J* = 7.1 Hz, CH L-Ala), 4.11 (dd, 1H, *J* = 12.3, 2.4 Hz, C*H*H-6), 4.05 (q, 1H, *J* = 6.7 Hz, CH lactic acid), 3.94 – 3.86 (m, 2H, H-2, C*H*H $C_3H_6N_3$), 3.76 – 3.68 (m, 2H, H-3, H-5), 3.62 – 3.54 (m, 1H, C*H*H $C_3H_6N_3$), 3.38 (t, 2H, *J* = 6.6, 1.0 Hz, CH_2 $C_3H_6N_3$), 2.35 – 2.27 (m, 2H, CH_2 γ-*i*-D-Gln), 2.25 – 2.14 (m, 1H, C*H*H β-*i*-D-Gln), 2.11 (s, 3H, CH_3 Ac), 2.06 (s, 3H, CH_3 Ac), 1.93 (s, 3H, CH_3 Ac), 1.89 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.45 (s, 9H, 3x CH_3 *t*Bu), 1.42 (d, 3H, *J* = 7.2 Hz, CH_3 lactic acid), 1.29 (d, 3H, *J* = 6.8 Hz, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.3, 175.2, 175.2, 173.7, 173.5, 172.4, 171.6 (C=O), 102.5 (C-1), 81.8 (C_q *t*Bu), 81.3 (C-3), 79.5 (CH lactic acid), 73.0 (C-5), 71.0 (C-4), 67.4 (CH_2 $C_3H_6N_3$), 63.5(CH_2-6), 56.9 (C-2), 53.5 (CH *i*-D-Gln), 51.0 (CH L-Ala), 49.1 (CH_2 $C_3H_6N_3$), 32.6 (CH_2 γ-*i*-D-Gln), 30.1 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.1 (CH_2 β-*i*-D-Gln), 23.2 (CH_3 Ac), 21.0 (CH_3 Ac), 20.7 (CH_3 Ac), 19.5 (CH_3 lactic acid), 17.6 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3299, 2980, 2099, 1746, 1661, 1536, 1371, 1242, 1156, 1116, 1045, 602. $[M+Na]^+$ calcd. for $C_{30}H_{50}N_7O_{13}Na$: 716.3461, found 716.3491.

3-Azidopropyl-4,6-di-*O*-acetyl-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (24b)



Diol **23b** (0.65 g, 0.85 mmol, 1.0 eq.) was co-evaporated with toluene (2x) under argon atmosphere and dissolved in dioxane (8.5 mL), followed by the addition of pyridine (2.3 mL, 28 mmol, 33 eq.) and Ac_2O (0.7 mL, 7.4 mmol, 8.8 eq.). After stirring for 24 hours, the reaction was quenched with methanol at 0°C, diluted with toluene and concentrated *in vacuo*. Purification by column chromatography (2→20% methanol in DCM) yielded the title compound (0.61 g, 0.72 mmol, 84%) as a white solid. R_f: 0.5 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 7.33 – 7.27 (m, 2H Ar), 6.96 – 6.89 (m, 2H, Ar), 4.97 (t, 1H, *J* = 10.0, 9.1 Hz, H-4), 4.56 – 4.50 (m, 3H, H-1, CH_2 glycol), 4.33 (dd, 1H, *J* = 9.7, 4.6 Hz, CH *i*-D-Gln), 4.26 (dd, 1H, *J* = 12.2, 4.8 Hz, C*H*H-6), 4.17 (q, 1H, *J* = 7.1 Hz, CH L-Ala), 4.14 – 3.99 (m, 3H, C*H*H-6, H-2, CH lactic acid), 3.94 – 3.83 (m, 4H, H-3, CH_2 PMB, C*H*H $C_3H_6N_3$), 3.79 (s, 3H, CH_3 PMB) 3.74 – 3.68 (m, 1H, H-5), 3.61 – 3.53 (m, 1H, C*H*H $C_3H_6N_3$), 3.38 – 3.31 (m, 2H, CH_2 $C_3H_6N_3$), 2.34 – 2.27 (m, 2H, CH_2 γ-*i*-D-Gln), 2.24 – 2.14 (m, 1H, C*H*H β-*i*-D-Gln), 2.10 (s, 3H, CH_3 Ac), 2.05 (s, 3H CH_3 Ac), 1.89 – 1.75 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.44 (s, 9H, 3x CH_3 *t*Bu), 1.37 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.30 (d, 3H, *J* = 6.7 Hz, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.2, 175.0, 174.9, 173.7, 173.0, 172.4, 171.5 (C=O), 161.1 (C_q Ar), 130.9 (Ar), 130.4 (C_q Ar), 114.9 (Ar), 102.2 (C-1), 81.8 (C_q *t*Bu), 80.7 (C-3), 79.4 (CH lactic acid), 73.9 (CH_2 glycol), 73.0 (C-5), 71.0 (C-4), 69.8 (CH_2 PMB), 67.5 (CH_2 $C_3H_6N_3$), 63.5 (CH_2-6), 56.5 (C-2), 55.7 (CH_3 PMB), 53.4 (CH *i*-D-Gln), 50.9 (CH L-Ala), 49.1 (CH_2 $C_3H_6N_3$), 32.6 (CH_2 γ-*i*-D-Gln), 30.0 (CH_2 $C_3H_6N_3$), 28.3 (CH_3 *t*Bu), 28.1 (CH_2 β-*i*-D-Gln), 21.0 (CH_3 Ac), 20.7 (CH_3 Ac), 19.3 (CH_3 lactic acid), 17.6 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3316, 2979, 2098, 1741, 1664, 1515, 1453, 1369, 1245, 1155, 1113, 1040, 848, 602. $[M+Na]^+$ calcd. for $C_{38}H_{57}N_7O_{15}Na$: 874,8978, found 874.3834

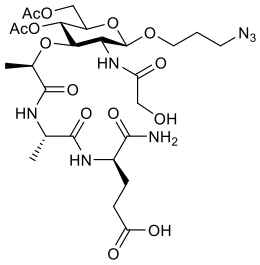
3-Azidopropyl-2-*N*-acetyl-4,6-di-*O*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (25a)



Compound **24a** (0.49 g, 0.75 mmol, 1.0 eq.) was co-evaporated with toluene (1x) under argon atmosphere and dissolved in DCM (5.0 mL). TFA (1.2 mL, 15 mmol, 20 eq.) was added and the reaction was allowed to stir for 4.5 hours, after which TLC analysis showed full conversion of the starting material. The reaction was cooled to 0°C, followed by the addition of Et_2O. The resulting white precipitation was filtered, washed with Et_2O and purified by column chromatography (2→20% methanol in DCM), which gave compound **25a** (0.38 g, 0.58 mmol, 77%) as a white solid. R_f: 0.38 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 4.97 (dd, 1H, *J* = 10.1, 9.1 Hz, H-4), 4.44 (d, 1H, *J* = 8.4 Hz, H-1), 4.36 (dd, 1H, *J* = 9.6, 4.5 Hz, CH *i*-D-Gln), 4.29 – 4.18 (m, 2H, C*H*H-6, CH lactic acid), 4.11 (dd, 1H, *J* = 12.2, 2.4 Hz, C*H*H-6), 4.05 (q, 1H, *J* = 6.7 Hz, CH L-Ala),

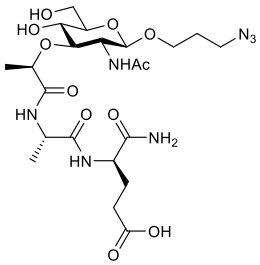
3.93 – 3.86 (m, 2H, C*H*H $C_3H_6N_3$, H-2), 3.75 – 3.68 (m, 2H, H-3, H-5), 3.62 – 3.54 (m, 1H, C*H*H $C_3H_6N_3$), 3.41 – 3.35 (m, 2H, CH_2 $C_3H_6N_3$), 2.38 (t, 2H, *J* = 7.5 Hz, CH_2 γ-*i*-D-Gln), 2.27 – 2.16 (m, 1H, C*H*H β-*i*-D-Gln), 2.11 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.91 – 1.74 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 $C_3H_6N_3$), 1.42 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.29 (d, 3H, *J* = 6.8 Hz, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 175.2, 175.2, 173.5, 172.4, 171.6 (C=O), 102.5 (C-1), 81.3 (C-3), 79.5 (CH lactic acid), 73.0 (C-5), 71.0 (C-4), 67.4 (CH_2 $C_3H_6N_3$), 63.5 (CH_2-6), 56.9 (C-2), 53.7 (CH *i*-D-Gln), 51.0 (CH L-Ala), 49.1 (CH_2 $C_3H_6N_3$), 30.1 (CH_2 γ-*i*-D-Gln), 28.2 (CH_2 $C_3H_6N_3$), 26.8 (CH_2 β-*i*-D-Gln) 23.2, 20.9, 20.7 (Ac), 19.4 (CH_3 lactic acid), 17.5 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 2988, 2407, 2100, 1743, 1645, 1435, 1374, 1242, 1066, 467. $[M+Na]^+$ calcd. for $C_{26}H_{41}N_7O_{13}Na$: 682.6398, found 682.2667.

3-Azidopropyl-4,6-di-*O*-acetyl-2-deoxy-2-*N*-((*p*-methoxybenzyl)oxy)acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (25b)



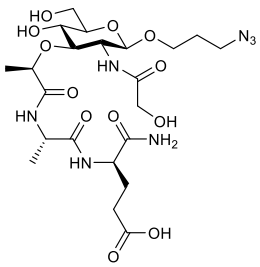
Compound **24b** (0.30 g, 0.36 mmol, 1.0 eq.) was co-evaporated with toluene (1x) under argon atmosphere and dissolved in DCM (2.4 mL). TFA (0.55 mL, 7.2 mmol, 20 eq.) and triethylsilane (0.17 mL, 1.1 mmol, 3.0 eq.) were subsequently added and the reaction was allowed to stir for 6 hours, after which TLC analysis showed full conversion of the starting material. The reaction was cooled to 0°C, followed by the addition of Et_2O. The resulting white precipitation was filtered, washed with Et_2O and purified by column chromatography (5→20% methanol in DCM + 0.1% AcOH), which gave compound **25b** (0.26 g, 0.34 mmol, 94%) as a white solid. R_f: 0.33 (1/9 MeOH/DCM). ^{1}H NMR (MeOD, 400 MHz, HH-COSY, HSQC): δ 5.05 – 4.95 (m, 1H, H-4), 4.57 (d, 1H, *J* = 8.3 Hz, H-1), 4.36 (dd, 1H, *J* = 9.7, 4.5 Hz, CH *i*-D-Gln), 4.30 – 4.19 (m, 2H, C*H*H-6, CH L-Ala), 4.16 – 4.07 (m, 2H, C*H*H-6, CH lactic), 4.06 – 3.95 (m, 3H, CH_2 glycol, H-2), 3.93 – 3.83 (m, 2H, H-3, C*H*H $C_3H_6N_3$), 3.78 – 3.70 (m, 1H, H-5), 3.63 – 3.55 (m, 1H, C*H*H $C_3H_6N_3$), 3.42 – 3.33 (m, 2H, CH_2 $C_3H_6N_3$), 2.39 (t, 2H, *J* = 7.5 Hz, CH_2 γ-*i*-D-Gln), 2.29 – 2.16 (m, 1H, C*H*H β-*i*-D-Gln), 2.11 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.97 – 1.85 (m, 1H, C*H*H β-*i*-D-Gln), 1.84 – 1.76 (m, 2H, CH_2 $C_3H_6N_3$), 1.40 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.28 (d, 3H, *J* = 6.7 Hz, CH_3 L-Ala). ^{13}C-APT NMR (MeOD, 101 MHz, HSQC): δ 176.3, 175.6, 175.1, 175.0, 172.4, 171.7 (C=O), 102.2 (C-1), 80.7 (C-3), 79.2 (CH lactic acid), 72.9 (C-5), 71.1 (C-4), 67.5 (CH_2 $C_3H_6N_3$), 63.5 (CH_2-6), 62.8 (CH_2 glycol), 56.4 (C-2), 53.7 (CH *i*-D-Gln), 50.8 (CH L-Ala), 49.1 (CH_2 $C_3H_6N_3$), 31.3 (CH_2 γ-*i*-D-Gln), 30.0 (CH_2 $C_3H_6N_3$), 28.0 (CH_2 β-*i*-D-Gln), 21.0 (CH_3 Ac), 20.7 (CH_3 Ac), 19.3 (CH_3 lactic acid), 17.6 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3331, 2939, 2412, 2100, 1728, 1651, 1434, 1373, 1241, 1114, 1044, 800, 721, 603. $[M+Na]^+$ calcd. for $C_{26}H_{41}N_7O_{14}Na$: 698.6388, found 698.2614.

3-Azidopropyl-2-*N*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-l-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (26a)



Compound **25a** (0.15 g, 0.23 mmol, 1.0 eq.) was dissolved in a solution of ammonia in MeOH (0.7 M, 7.6 mL). After 2 days the reaction was diluted with water and co-evaporated with water (3x). The residue was purified by HW-40 gel filtration. Co-evaporation with water (5x) and lyophilization yielded the title compound (0.10 g, 0.17 mmol, 74%) as a white solid. R_f: 0.25 (4/6 MeOH/DCM). ^{1}H NMR (D_2O, 400 MHz, HH-COSY, HSQC): δ 4.43 (d, 1H, *J* = 8.5 Hz, H-1), 4.28 (dd, 1H, *J* = 9.7, 4.7 Hz, CH *i*-D-Gln), 4.26 – 4.20 (m, 1H, CH lactic acid), 4.20 – 4.14 (m, 1H, CH L-Ala), 3.98 – 3.87 (m, 2H, C*H*H-6, C*H*H $C_3H_6N_3$), 3.81 – 3.69 (m, 2H, H-2, C*H*H-6), 3.65 – 3.58 (m, 1H, C*H*H $C_3H_6N_3$), 3.54 – 3.40 (m, 3H, H-3, H-4, H-5), 3.37 – 3.29 (m, 2H, CH_2 $C_3H_6N_3$), 2.31 (t, 2H, CH_2 γ-*i*-D-Gln), 2.18 – 2.06 (m, 1H, C*H*H β-*i*-D-Gln), 1.94 (s, 3H, Ac), 1.92 – 1.85 (m, 1H, C*H*H β-*i*-D-Gln), 1.84 – 1.75 (m, 2H, CH_2 $C_3H_6N_3$), 1.40 (d, 3H, *J* = 7.2 Hz, CH_3 lactic acid), 1.34 (d, 3H, *J* = 6.8 Hz, CH_3 L-Ala). ^{13}C-APT NMR (D_2O, 101 MHz, HSQC): δ 179.7, 176.3, 175.7, 175.2, 174.0 (C=O), 101.2 (C-1), 82.7 (C-3), 78.3 (CH lactic acid), 75.6 (C-5), 68.8 (C-4), 67.1 (CH_2 $C_3H_6N_3$), 60.7 (CH_2-6), 55.0 (C-2), 53.1 (CH *i*-D-Gln), 49.8 (CH L-Ala), 47.8 (CH_2 $C_3H_6N_3$), 32.3 (CH_2 γ-*i*-D-Gln), 28.1 (CH_2 $C_3H_6N_3$), 27.1 (CH_2 β-*i*-D-Gln), 22.2 (Ac), 18.7 (CH_3 lactic acid), 16.5 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3267, 3074, 2936, 2883, 2099, 1646, 1548, 1448, 1398, 1375, 1309, 1258, 1161, 1108, 1074, 1056, 947, 899, 869, 673, 622, 576. $[M+Na]^+$ calcd. for $C_{22}H_{37}N_7O_{11}Na$: 598.2449, found 598.2449.

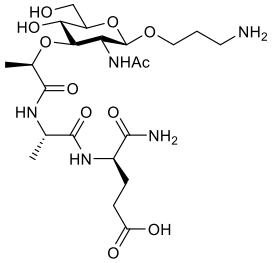
3-Azidopropyl-2-*N*-(2-hydroxyacetamide)-2-deoxy-3-*O*-((R)-1-carboxyethyl-l-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (26b)



Compound **25b** (125 mg, 0.19 mmol, 1.0 eq.) was dissolved in a solution of ammonia in MeOH (0.7 M, 6.2 mL). After 2 days the reaction was diluted with water and co-evaporated with water (3x). The residue was purified by HW-40 gel filtration and co-evaporated with water (5x). Lyophilization yielded compound **26b** (82 mg, 0.12 mmol, 65%) as a white solid. R_f: 0.24 (4/6 MeOH/DCM). ^{1}H NMR (D_2O, 500 MHz, HH-COSY, HSQC): δ 4.55 (d, 1H, *J* = 8.5 Hz, H-1), 4.31 (dd, 1H, *J* = 9.7, 4.7 Hz, CH *i*-D-Gln), 4.27 – 4.18 (m, 2H, CH lactic acid, CH L-Ala), 4.10 – 3.99 (m, 2H, CH_2 Glycol), 3.98 – 3.91 (m, 2H, C*H*H-6, C*H*H $C_3H_6N_3$), 3.88 (dd, 1H, *J* = 10.1, 8.5 Hz, H-2), 3.76 (dd, 1H, *J* = 12.4, 5.6 Hz, C*H*H-6), 3.68 – 3.61 (m, 2H, H-3, C*H*H $C_3H_6N_3$), 3.54 (t, 1H, *J* = 10.0, 8.6 Hz, H-4), 3.50 – 3.44 (m, 1H, H-5), 3.38 – 3.29 (m, 2H, CH_2 $C_3H_6N_3$), 2.40 – 2.34 (m, 2H, CH_2 γ-*i*-D-Gln), 2.19 – 2.11 (m, 1H, C*H*H β-*i*-D-Gln), 1.98 – 1.89 (m, 1H, C*H*H β-*i*-D-Gln), 1.86 – 1.78 (m, 2H, CH_2 $C_3H_6N_3$), 1.41 (d, 3H, *J* = 7.2 Hz, CH_3 lactic acid), 1.35 (d, 3H, *J* = 6.8 Hz, CH_3 L-Ala). ^{13}C-APT NMR (D_2O, 126 MHz, HSQC): δ 179.0, 176.2, 175.7, 175.2 (C=O), 101.0 (C-1), 82.2 (C-3), 77.9 (CH lactic acid), 75.7 (C-5), 68.9 (C-4), 67.3 (CH_2 $C_3H_6N_3$), 61.1 (CH_2 Glycol), 60.8 (CH_2-6), 54.9 (C-2), 53.1 (CH *i*-D-Gln), 49.8 (CH L-Ala), 47.9 (CH_2 $C_3H_6N_3$), 31.7 (CH_2 γ-*i*-D-Gln), 28.1 (CH_2 $C_3H_6N_3$), 26.8 1 (CH_2 β-*i*-D-Gln), 18.7 (CH_3 lactic acid), 16.6 (CH_3 L-Ala). FT-IR (neat), cm^{-1}: 3279,

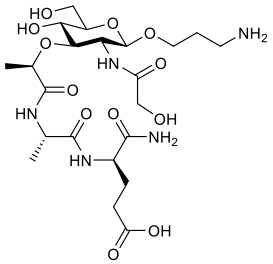
2987, 2102, 1652, 1544, 1406, 1255, 1078, 674, 626. $[M+Na]^+$ calcd. for $C_{22}H_{37}N_7O_{12}Na$: 614.2398, found 614.2398.

3-Aminopropyl-2-*N*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (27a)



Azide **26a** (17 mg, 30 µmol, 1.0 eq.) was dissolved in milli-Q H_2O (1.0 mL), followed by the addition of Pd/C (10%, 22 mg). The black suspension was purged with argon and after 15 minutes a $H_{2(g)}$-filled balloon was applied. The mixture was stirred for 3 hours, filtered over a Whatmann-filter and concentrated *in vacuo*. Purification by HW-40 gel filtration and lyophilization yielded the title compound (8.7 mg, 16 µmol, 53%) as a white solid. ^{1}H NMR (D_2O, 400 MHz, HH-COSY, HSQC): δ 4.43 (d, 1H, *J* = 8.5 Hz, H-1), 4.29 – 4.17 (m, 3H, CH *i*-D-Gln, CH L-Ala, CH lactic), 4.03 – 3.95 (m, 1H, C*H*H C_3H_8N), 3.91 (dd, 1H, *J* = 12.2, 2.0 Hz, C*H*H-6), 3.82 – 3.70 (m, 2H, H-2, C*H*H-6), 3.70 – 3.61 (m, 1H, C*H*H C_3H_8N), 3.56 – 3.41 (m, 3H, H-3, H-4, H-5), 3.02 (t, 2H, *J* = 7.1 Hz, CH_2NH_2), 2.24 (t, 2H, *J* = 7.3 Hz, CH_2 γ-*i*-D-Gln), 2.15 – 2.02 (m, 1H, C*H*H β-*i*-D-Gln), 1.98 – 1.83 (m, 6H, C*H*H β-*i*-D-Gln, CH_2 C_3H_8N, CH_3 Ac), 1.40 (d, 3H, *J* = 7.1 Hz, CH_3 lactic acid), 1.34 (d, 3H, *J* = 6.7 Hz, CH_3 L-Ala). ^{13}C-APT NMR (D_2O, 101 MHz, HSQC): δ 181.4, 176.6, 175.7, 175.1, 174.3 (C=O), 101.3 (C-1), 82.4 (C-3), 78.2 (CH lactic acid), 75.6 (C-5), 69.0 (C-4), 68.0 (CH_2 C_3H_8N), 60.7 (CH_2-6), 55.0 (C-2), 53.5 (CH *i*-D-Gln), 49.8 (CH L-Ala), 37.6 (CH_2NH_2), 33.7 (CH_2 γ-*i*-D-Gln), 27.8 (CH_2 C_3H_8N), 26.9 (CH_2 β-*i*-D-Gln), 22.2 (CH_3 Ac), 18.8 (CH_3 lactic acid), 16.7 (CH_3 L-Ala). $[M+H]^+$ calcd. for $C_{22}H_{44}N_5O_{11}$: 550.2724, found 550.2720.

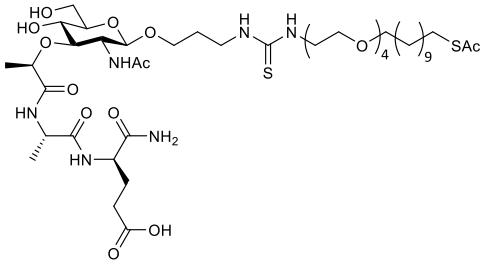
3-Aminopropyl-2-*N*-(2-hydroxyacetamide-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (27b)



Azide **26b** (23 mg, 39 µmol, 1.0 eq.) was dissolved in milli-Q H_2O (1.0 mL), followed by the addition of Pd/C (10%, 20 mg). The black suspension was purged with argon and after 15 minutes a $H_{2(g)}$-filled balloon was applied. The mixture was stirred for 3 hours, filtered over a Whatmann-filter and concentrated *in vacuo*. Purification by HW-40 gel filtration and lyophilization afforded compound **27b** (19 mg, 34 µmol, 87%) as a white solid. ^{1}H NMR (D_2O, 400 MHz, HH-COSY, HSQC): δ 4.51 (d, 1H, *J* = 8.5 Hz, H-1), 4.27 – 4.19 (m, 3H, CH *i*-D-Gln, CH L-Ala, CH lactic), 4.03 (d, 2H, *J* = 1.7 Hz, CH_2 glycol), 4.01 – 3.94 (m, 1H, C*H*H C_3H_8N), 3.94 – 3.83 (m, 2H, H-2, C*H*H-6), 3.74 (dd, 1H, *J* = 12.3, 5.6 Hz, C*H*H-6), 3.70 – 3.59 (m, 2H, H-3, C*H*H C_3H_8N), 3.56 – 3.49 (m, 1H, H-4), 3.49 – 3.43 (m, 1H, H-5), 3.03 (t, 2H, *J* = 7.1 Hz, CH_2NH_2), 2.27 – 2.21 (m, 2H, CH_2 γ-*i*-D-Gln), 2.13 – 2.03 (m, 1H, C*H*H β-*i*-D-Gln), 1.96 – 1.85 (m, 3H, C*H*H β-*i*-D-Gln, CH_2 C_3H_8N), 1.38 (d, 3H, *J* = 7.3 Hz, CH_3 lactic acid), 1.33 (d, 4H, *J* = 6.7 Hz, CH_3 L-Ala). ^{13}C-APT NMR (D_2O, 101 MHz, HSQC): δ 181.3, 176.5, 175.6, 175.4, 175.1 (C=O), 101.0 (C-1), 81.9 (C-3), 77.8 (CH lactic acid), 75.6 (C-5), 68.9 (C-4), 67.8 (CH_2 C_3H_8N), 61.0 (CH_2 glycol), 60.6 (CH_2-6), 54.7 (C-2), 53.5 (CH *i*-D-Gln), 49.7 (CH L-Ala), 37.5 (CH_2NH_2), 33.5 (CH_2 γ-*i*-D-Gln), 27.6 (CH_2 C_3H_8N), 26.6 (CH_2 β-

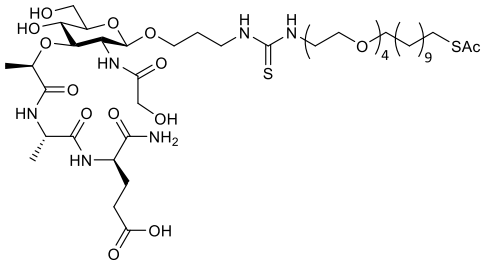
i-D-Gln), 18.7 (CH_3 lactic acid), 16.6 (CH_3 L-Ala). $[M+H]^+$ calcd. for $C_{22}H_{40}N_5O_{12}$: 566.2673, found 566.2676.

S-(1-(2-*N*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside)-5-thioxo-9,12,15,18-tetraoxa-4,6-diazanonacosan-29-yl) ethanethioate (28a)



A solution of 1-isothiocyanate-3,6,9,12-tetraoxa-23-thioacetyltricosane (2.2 mg, 4.7 µmol, 2.0 eq.) in a mixture of H_2O/*t*BuOH/CH_3CN (1/1/1 v/v/v, 0.20 mL) was added to amine **27a** (1.3 mg, 2.4 µmol, 1.0 eq.) in an Eppendorf tube. Et_3N (1.1 µL, 7.9 µmol, 3.3 eq.) was added and the mixture was shaken overnight. The reaction mixture was concentrated and the crude was washed with Et_2O (5x). After lyophilization, the title compound (1.9 mg, 1.8 µmol, 75%) was obtained as a white solid. ^{1}H NMR (D_2O, 500 MHz): δ 4.47 (d, 1H, *J* = 8.7 Hz), 4.33 (dd, 1H, *J* = 9.7, 4.8 Hz), 4.30 – 4.18 (m, 2H), 3.95 – 3.87 (m, 2H), 3.86 – 3.73 (m, 3H), 3.71 – 3.58 (m, 16H), 3.56 – 3.50 (m, 3H), 3.50 – 3.42 (m, 4H), 2.85 (t, 2H, *J* = 7.2 Hz), 2.41 (t, 2H, *J* = 7.9 Hz), 2.35 – 2.31 (m, 3H), 2.22 – 2.11 (m, 1H), 1.99 – 1.90 (m, 4H), 1.85 – 1.78 (m, 2H), 1.60 – 1.52 (m, 4H), 1.43 (d, 3H, *J* = 7.1 Hz), 1.40 – 1.25 (m, 17H). $[M+H]^+$ calcd. for $C_{44}H_{81}N_6O_{16}$: 1013.5150, found 1013.5158.

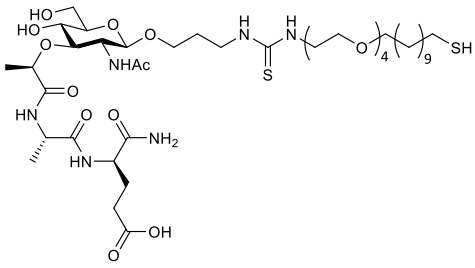
S-(1-(2-*N*-(2-hydroxyacetamide)-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside)-5-thioxo-9,12,15,18-tetraoxa-4,6-diazanonacosan-29-yl) ethanethioate (28b)



A solution of amine **27b** (4.4 mg, 7.8 µmol, 1.0 eq.) in a mixture of H_2O/*t*BuOH/CH_3CN (1/1/1 v/v/v, 0.39 mL) was added to 1-isothiocyanate-3,6,9,12-tetraoxa-23-thioacetyltricosane (7.2 mg, 15.6 µmol, 2.0 eq.) in an Eppendorf tube. Et_3N (4.4 µL, 31 µmol, 4.0 eq.) was added and the mixture was shaken overnight. The reaction mixture was concentrated and the crude was washed with Et_2O (5x). After lyophilization, compound **28b** (5.9 mg, 5.8 µmol, 74%) was obtained as a white solid. ^{1}H NMR (D_2O, 600 MHz, HH-COSY, HSQC): δ 4.59 (d, 1H, *J* = 8.5 Hz, H-1), 4.37 – 4.20 (m, 3H, CH, CH *i*-D-Gln, CH L-Ala, CH lactic), 4.15 – 4.00 (m, 2H, CH_2 glycol), 3.98 – 3.86 (m, 4H, H-2, C*H*H-6, 2x C*H*H linker), 3.84 – 3.77 (m, 1H, C*H*H-6), 3.74 – 3.57 (m, 20H, H-3, H-4, 2x C*H*H linker, 8x CH_2 linker), 3.53 – 3.46 (m, 3H, H-5, CH_2 linker), 2.88 (t, 2H, *J* = 7.2 Hz, C*H*$_2$SAc), 2.41 – 2.36 (m, 2H, CH_2 γ-*i*-D-Gln), 2.36 – 2.33 (m, 3H, CH_3 Ac), 2.22 – 2.11 (m, 1H, C*H*H β-*i*-D-Gln), 2.01 – 1.92 (m, 1H, C*H*H β-*i*-D-Gln), 1.89 – 1.81 (m, 2H, CH_2 linker), 1.62 – 1.54 (m, 4H, 2x CH_2 linker), 1.44 (d, 3H, *J* = 7.2 Hz, CH_3 lactic acid), 1.42 – 1.28 (m, 17H, CH_3 L-Ala, 7x CH_2 linker). ^{13}C-APT NMR (D_2O, 151 MHz, HSQC): δ 177.1,

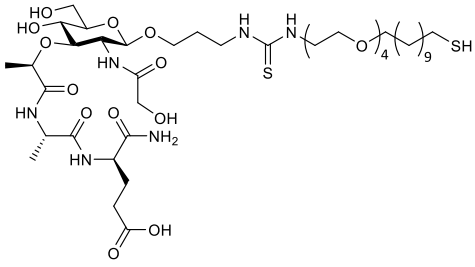
176.5, 176.0, 176.0 (C=O), 101.9 (C-1), 83.0 (C-3), 78.7 (CH lactic acid), 76.7 (C-5), 72.1, 71.0, 70.9, 70.9, 70.7, 70.2 (CH_2 linker), 69.8 (C-4), 62.2 (CH_2 glycol), 61.8 (CH_2-6), 55.8 (C-2), 54.1 (CH *i*-D-Gln), 50.7 (CH L-Ala), 32.9 (CH_2 γ-*i*-D-Gln), 31.3 (CH_3 Ac), 30.7, 30.6, 30.5, 30.5, 30.4, 30.2, 30.0, 29.8, 27.9 (CH_2 linker), 27.0 (CH_2 β-*i*-D-Gln), 19.7 (CH_3 lactic acid), 17.7 (CH_3 L-Ala). $[M+Na]^+$ calcd. for $C_{44}H_{80}N_6O_{17}Na$: 1051.4919, found 1051.4924.

1-(3-(2-*N*-acetyl-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside) propyl)-3-(23-mercapto-3,6,9,12-tetraoxatricosyl)thiourea (29a)



Sodium methoxide (5.4 M in MeOH, 0.2 µL, 1.1 µmol, 0.6 eq.) was added to a solution of compound **28a** (1.9 mg, 1.8 µmol, 1.0 eq.) in MeOH (0.18 mL). After stirring for 1.5 hours, the reaction mixture was quenched using amberlite H^+ resin to pH = 5, filtered and concentrated *in vacuo*. Lyophilization yielded compound **29a** (1.6 mg, 1.6 µmol, 89%) as a white solid. ^{1}H NMR (D_2O, 600 MHz): δ 4.49 (d, 1H, *J* = 8.3 Hz), 4.41 – 4.32 (m, 1H), 4.32 – 4.20 (m, 2H), 3.98 – 3.89 (m, 2H), 3.87 – 3.75 (m, 3H), 3.75 – 3.59 (m, 16H), 3.59 – 3.43 (m, 7H), 2.57 – 2.36 (m, 5H), 2.24 – 2.15 (m, 1H), 2.03 – 1.92 (m, 4H), 1.90 – 1.79 (m, 2H), 1.66 – 1.55 (m, 4H), 1.48 – 1.25 (m, 20H). $[M+H]^+$ calcd. for $C_{42}H_{79}N_6O_{15}$: 971.5045, found 971.5045.

1-(3-(2-*N*-(2-hydroxyacetamide)-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside) propyl)-3-(23-mercapto-3,6,9,12-tetraoxatricosyl)thiourea (29b)



Sodium methoxide (5.4 M in MeOH, 0.4 µL, 2.2 µmol, 0.4 eq.) was added to a solution of compound **29b** (5.9 mg, 5.8 µmol) in MeOH (0.58 mL). After stirring for 1.5 hours, the reaction mixture was quenched using amberlite H^+ resin to pH = 5, filtered and concentrated *in vacuo*. Lyophilization yielded the title compound (5.2 mg, 5.2 µmol, 90%) as a white solid. ^{1}H NMR (D_2O, 850 MHz, HH-COSY, HSQC): δ 4.59 (d, 1H, *J* = 8.3 Hz, H-1), 4.38 (dd, 1H, *J* = 9.7, 4.8 Hz, CH *i*-D-Gln), 4.27 (dd, 2H, *J* = 27.2, 6.9 Hz, , CH L-Ala, CH lactic), 4.08 (dd, 2H, *J* = 41.2, 17.2 Hz, CH_2 glycol), 3.94 (d, 3H, *J* = 8.4 Hz, H-2, C*H*H-6, C*H*H linker), 3.84 – 3.75 (m, 2H, C*H*H-6, C*H*H linker), 3.75 – 3.54 (m, 20H, H-3, H-4, 2x C*H*H linker, 8x CH_2 linker), 3.54 – 3.44 (m, 3H, H-5, CH_2 linker), 2.53 (t, 2H, *J* = 7.1 Hz, CH_2SH), 2.51 – 2.44 (m, 2H, CH_2 γ-*i*-D-Gln), 2.27 – 2.18 (m, 1H, C*H*H β-*i*-D-Gln), 2.01 – 1.94 (m, 1H, C*H*H β-*i*-D-Gln), 1.89 – 1.80 (m, 2H, CH_2 linker), 1.75 – 1.67 (m, 1H, C*H*H linker), 1.66 – 1.57 (m, 3H, C*H*H linker, CH_2 linker), 1.47 – 1.41 (m, 4H, 2x CH_2 linker), 1.41 – 1.26 (m, 20H, CH_3 lactic acid, CH_3 L-Ala, 7x CH_2 linker). ^{13}C-APT NMR (D_2O, 214

MHz, HSQC): δ 177.8, 176.7, 176.5, 175.9 (C=O), 101.8 (C-1), 83.0 (C-3), 78.6 (CH lactic acid), 76.6 (C-5), 72.1, 71.0, 70.9, 70.7 (CH_2 linker), 69.8 (C-4), 62.1 (CH_2 glycol), 61.7 (CH_2-6), 55.7 (C-2), 53.5 (CH *i*-D-Gln), 50.6 (CH L-Ala), 35.0 (CH_2 linker), 31.2 (CH_2 γ-*i*-D-Gln), 31.1, 30.7, 30.7, 30.7, 30.6, 30.4, 30.2, 29.4, 27.1 (CH_2 linker), 27.0 (CH_2 β-*i*-D-Gln), 25.3 (CH_2SH), 19.6 (CH_3 lactic acid), 17.6 (CH_3 L-Ala). $[M+Na]^+$ calcd. for $C_{42}H_{78}N_6O_{16}Na$: 1009.4813, found 1009.4811.

General 5 nm AuNP procedure

NHS-functionalized 5 nm gold nanoparticles (0.5 mg) were dissolved in 100 µL of reaction buffer (pH=8) and split equally over two Eppendorf tube. The amine (0.5 M solution in resuspension buffer (pH=8)) was added to an Eppendorf and the reaction was mixed for 2.5 h at room temperature, before quenching the reaction with ethanolamine (5 µL, 50 mM). After 20 minutes, the mixture was diluted with H_2O (400 µL) and filtered over a 30 KDa cut-off centrifugal filters (6x 400 µL). The AuNPs were lyophilized and stored at 4°C. Before performing cellular/biological experiments the 5 nm-particles were dissolved in H_2O and diluted to reach an OD of 0.4 reading the plasmon UV resonance at 520 nm.

5 nm AuNP containing 15 mM MDP(Ac) (12)

The title compound was prepared by the general procedure for 5 nm AuNP. Amine **27a** (1.5 µL from a 0.5 M solution in resuspension buffer (pH=8)) was used for the synthesis.

5 nm AuNP containing 15 mM MDP(Gly) (13)

The title compound was prepared by the general procedure for 5 nm AuNP. Amine **27b** (1.5 µL from a 0.5 M solution in resuspension buffer (pH=8)) was used for the synthesis.

5 nm AuNP containing 30 mM MDP(Ac) (14)

The title compound was prepared by the general procedure for 5 nm AuNP. Amine **27a** (3.0 µL from a 0.5 M solution in resuspension buffer (pH=8)) was used for the synthesis.

5 nm AuNP containing 30 mM MDP(Gly) (15)

The title compound was prepared by the general procedure for 5 nm AuNP. Amine **27b** (3.0 µL from a 0.5 M solution in resuspension buffer (pH=8)) was used for the synthesis.

2 nm AuNP: 1/9 MDP(Ac)/GlcC$_5$S (16)

A solution of compound **29a** (138 µL from a 7.5 mM solution in MeOD) and $GlcC_5S$ (127 µL from a 72.6 mM solution in MeOD) was diluted with MeOD (336 µL). ^1H NMR analysis was performed to confirm the 1/9 MDP(Ac)/$GlcC_5S$ ratio. The thiols (10.3 µmol) were diluted to a concentration of 12 µM in MeOH. $HAuCl_4$ (137 µL from a 25 mM solution in H_2O) was added, followed by the addition of freshly prepared solution of $NaBH_4$ (3x 24 µL from a 1.0 M solution in H_2O). The obtained dark suspension was shaken vigorously for 2 hours at room temperature, after which the shaking was stopped to have a precipitate at the bottom of the Eppendorf tube. EtOH (200 µL) was added, followed by centrifugation to wash the nanoparticles from the unreacted thiols and salts. The solvent was removed and the black solids were washed with MeOH (5x 1.0 mL). The

supernatant was dissolved in H_2O (100 µL) and filtered over a 3 KDa cut-off centrifugal filters (5x 400 µL). After lyophilization, the particles (0.13 mg) were analyzed by NMR in D_2O with an internal standard (0.05 wt. % TSP) showing 0.15 µmol MDP (the integrals of H-1, CH *i*-D-Gln, CH L-Ala, CH lactic were used for this measurement). The unreacted thiols from the MeOH/EtOH washes were analyzed by NMR to confirm the ligand ratio after the nanoparticles formation.

2 nm AuNP: 1/9 MDP(Gly)/GlcC_5S (17)

A solution of compound **29b** (135 µL from a 18.8 mM solution in MeOD) and GlcC_5S (312 µL from a 72.6 mM solution in MeOD) was diluted with MeOD. 1H NMR analysis was performed to confirm the 1/9 MDP(Gly)/GlcC_5S ratio. The thiols (25.3 µmol) were diluted to a concentration of 12 µM in MeOH. $HAuCl_4$ (337 µL from a 25 mM solution in H_2O) was added, followed by the addition of freshly prepared solution of $NaBH_4$ (3x 60 µL from a 1.0 M solution in H_2O). The obtained dark suspension was shaken vigorously for 2 hours at room temperature, after which the shaking was stopped to have a precipitate at the bottom. EtOH (400 µL) was added, followed by centrifugation to wash the nanoparticles from the unreacted thiols and salts. The solvent was removed and the black solid was washed with MeOH (5x 1.0 mL). The solid was then dissolved in H_2O (100 µL) and filtered over a 3 KDa cut-off centrifugal filters (5x 400 µL). After lyophilization, the particles (0.95 mg) were analysed by NMR in D_2O with an internal standard (0.05 wt. % TSP) showing 0.40 µmol MDP (the integrals of H-1, CH *i*-D-Gln, CH L-Ala, CH lactic were used for this measurement). The unreacted thiols from the MeOH/EtOH washes were analyzed by NMR to confirm the ligand ratio after the nanoparticles formation.

2 nm AuNP: 1/1 MDP(Ac)/GlcC_5S (18)

A solution of compound **29a** (300 µL from a 2.1 mM solution in MeOD) and GlcC_5S (31.1 µL from a 19.6 mM solution in MeOD) was diluted with MeOD (269 µL). ^{1}H NMR analysis was performed to confirm the 1/1 MDP(Ac)/GlcC_5S ratio. The thiols (1.22 µmol) were diluted to a concentration of 12 µM in MeOH. $HAuCl_4$ (16.3 µL from a 25 mM solution in H_2O) was added, followed by the addition of freshly prepared solution of $NaBH_4$ (2x 4.3 µL from a 1.0 M solution in H_2O). The obtained dark suspension was shaken vigorously for 2 hours at room temperature, after which the shaking was stopped, the mixture was diluted with EtOH to a total volume of 2.0 mL and stored at 0°. After centrifugation, the solvent was removed and the black solids were washed to remove the unreacted thiols and salts subsequently with cold EtOH and a mixture of cold EtOH/MeOH (1/1 v/v, 1.0 mL). The supernatant was dissolved in H_2O (200 µL) and filtered over a 3 KDa cut-off centrifugal filters (5x 400 µL). After lyophilization, the particles* were analyzed by NMR in D_2O with an internal standard (0.05 wt. % TSP) showing 0.11 µmol MDP (the integrals of H-1, CH *i*-D-Gln, CH L-Ala, CH lactic were used for this measurement). The unreacted thiols from the MeOH/EtOH washes were analyzed by NMR to confirm the ligand ratio after the nanoparticles formation. *The amount was too low for accurate measurement on the balance.

2 nm AuNP: 1/1 MDP(Gly)/Glc (19)

A solution of compound **29b** (400 µL from a 6.2 mM solution in MeOD) and $GlcC_5S$ (126.5 µL from a 19.6 mM solution in MeOD) was diluted with MeOD (75 µL). ^1H NMR analysis was performed to confirm the 1/1 MDP(Gly)/$GlcC_5S$ ratio. The thiols (4.98 µmol) were diluted to a concentration of 12 µM in MeOH. $HAuCl_4$ (66.4 µL from a 25 mM solution in H_2O) was added, followed by the addition of freshly prepared solution of $NaBH_4$ (4x 8.7 µL from a 1.0 M solution in H_2O). The obtained dark suspension was shaken vigorously for 2 hours at room temperature, after which the shaking was stopped, the mixture was diluted with EtOH to a total volume of 2.0 mL and stored at 0°. After centrifugation, the solvent was removed and the black solids were washed to remove the unreacted thiols and salts subsequently with cold EtOH (1.0 mL) and a mixture of cold EtOH/MeOH (1/1 v/v, 1.0 mL). The supernatant was dissolved in H_2O (200 µL) and filtered over a 3 KDa cut-off centrifugal filters (5x 400 µL). After lyophilization, the particles (0.34 mg) were analyzed by NMR in D_2O with an internal standard (0.05 wt. % TSP) showing 0.35 µmol MDP (the integrals of H-1, CH *i*-D-Gln, CH L-Ala, CH lactic were used for this measurement). The unreacted thiols from the MeOH/EtOH washes were analyzed by NMR to confirm the ligand ratio after the nanoparticles formation.

Footnotes and References

(1) Brubaker, S. W.; Bonham, K. S.; Zanoni, I.; Kagan, J. C. *Annu. Rev. Immunol.* **2015**, *33* (1), 257–290.
(2) McEnaney, P. J.; Parker, C. G.; Zhang, A. X.; Spiegel, D. A. *ACS Chem. Biol.* **2012**, *7* (7), 1139–1151.
(3) Tada, H.; Aiba, S.; Shibata, K.-I.; Ohteki, T.; Takada, H. *Infect. Immun.* **2005**, *73* (12), 7967–7976.
(4) Yang, N. J.; Hinner, M. J. 2015; pp 29–53.
(5) Kersemans, V.; Cornelissen, B. *Pharmaceuticals* **2010**, *3* (3), 600–620.
(6) Pati, R.; Shevtsov, M.; Sonawane, A. *Front. Immunol.* **2018**, *9*.
(7) Köping-Höggård, M.; Sánchez, A.; Alonso, M. J. *Expert Rev. Vaccines* **2005**, *4* (2), 185–196.
(8) You, C.-C.; Chompoosor, A.; Rotello, V. M. *Nano Today* **2007**, *2* (3), 34–43.
(9) Tao, W.; Ziemer, K. S.; Gill, H. S. *Nanomedicine* **2014**, *9* (2), 237–251.
(10) Safari, D.; Marradi, M.; Chiodo, F.; Th Dekker, H. A.; Shan, Y.; Adamo, R.; Oscarson, S.; Rijkers, G. T.; Lahmann, M.; Kamerling, J. P.; *et al*. *Nanomedicine* **2012**, *7* (5), 651–662.
(11) Chiodo, F.; Marradi, M.; Calvo, J.; Yuste, E.; Penadés, S. *Beilstein J. Org. Chem.* **2014**, *10*, 1339–1346.
(12) Ojeda, R.; de Paz, J. L.; Barrientos, A. G.; Martín-Lomas, M.; Penadés, S. *Carbohydr. Res.* **2007**, *342* (3–4), 448–459.
(13) Brinãs, R. P.; Sundgren, A.; Sahoo, P.; Morey, S.; Rittenhouse-Olson, K.; Wilding, G. E.; Deng, W.; Barchi, J. J. *Bioconjug. Chem.* **2012**, *23* (8), 1513–1523.
(14) Marradi, M.; Chiodo, F.; García, I.; Penadés, S. *Chem. Soc. Rev.* **2013**, *42* (11), 4728.
(15) Fallarini, S.; Paoletti, T.; Battaglini, C. O.; Ronchi, P.; Lay, L.; Bonomi, R.; Jha, S.; Mancin, F.; Scrimin, P.; Lombardi, G. *Nanoscale* **2013**, *5* (1), 390–400.
(16) See compound **31** of Chapter 3.
(17) See compounds **9a** and **9b** of Chapter 3.
(18) Willems, M. M. J. H. P.; Zom, G. G.; Meeuwenoord, N.; Ossendorp, F. A.; Overkleeft, H. S.; van der Marel, G. A.; Codée, J. D. C.; Filippov, D. V. *Beilstein J. Org. Chem.* **2014**, *10*, 1445–1453.
(19) Chiodo, F.; Marradi, M. 2015; pp 159–171.
(20) Arnáiz, B.; Martínez-Ávila, O.; Falcon-Perez, J. M.; Penadés, S. *Bioconjug. Chem.* **2012**, *23* (4), 814–825.
(21) van Kasteren, S. I.; Kramer, H. B.; Jensen, H. H.; Campbell, S. J.; Kirkpatrick, J.; Oldham, N. J.; Anthony, D. C.; Davis, B. G. *Nature* **2007**, *446* (7139), 1105–1109.
(22) Kang, J.-Y.; Shin, K. K.; Kim, H. H.; Min, J.-K.; Ji, E. S.; Kim, J. Y.; Kwon, O.; Oh, D.-B. *Sci. Rep.* **2018**, *8* (1), 8730.

(23) El Cheikh, K.; Basile, I.; Da Silva, A.; Bernon, C.; Cérutti, P.; Salgues, F.; Perez, M.; Maynadier, M.; Gary-Bobo, M.; Caillaud, C.; *et al*. *Angew. Chemie Int. Ed.* **2016**, *55* (47), 14774–14777.
(24) Ponader, D.; Maffre, P.; Aretz, J.; Pussak, D.; Ninnemann, N. M.; Schmidt, S.; Seeberger, P. H.; Rademacher, C.; Nienhaus, G. U.; Hartmann, L. *J. Am. Chem. Soc.* **2014**, *136* (5), 2008–2016.
(25) Rauen, J.; Kreer, C.; Paillard, A.; van Duikeren, S.; Benckhuijsen, W. E.; Camps, M. G.; Valentijn, A. R. P. M.; Ossendorp, F.; Drijfhout, J. W.; Arens, R.; *et al*. *PLoS One* **2014**, *9* (8), e103755.
(26) Bundle, D.; Paszkiewicz, E.; Elsaidi, H.; Mandal, S.; Sarkar, S. *Molecules* **2018**, *23* (8), 1961.
(27) Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N. K. *Biomaterials* **2012**, *33* (16), 4166–4186.
(28) Chen, R.; Tolbert, T. J. *J. Am. Chem. Soc.* **2010**, *132* (9), 3211–3216.
(29) Aussedat, B.; Vohra, Y.; Park, P. K.; Fernández-Tejada, A.; Alam, S. M.; Dennison, S. M.; Jaeger, F. H.; Anasti, K.; Stewart, S.; Blinn, J. H.; *et al*. *J. Am. Chem. Soc.* **2013**, *135* (35), 13113–13120.
(30) See Chapter 2 for the described purification conditions.
(31) See compound **9a** of Chapter 3.
(32) See compound **9b** of Chapter 3.

Nederlandse samenvatting

Synthetische koolhydraat liganden voor immuunreceptoren

Het onderzoek, dat in dit proefschrift wordt beschreven, richt zich op de synthese van specifieke koolhydraten en conjugaten daarvan, die gebruikt kunnen worden voor de ontwikkeling van nieuwe vaccins tegen kanker. Bij deze veelbelovende immunotherapie wordt het immuunsysteem zodanig geactiveerd dat tumorcellen worden aangevallen en gedood. Noodzakelijk is dat het immuunsysteem niet alleen wordt getraind om de tumor te herkennen maar dat er tevens een cellulaire immuunrespons opgewekt wordt, waarbij cytotoxische T-cellen en helper T-cellen worden geactiveerd om de kankercellen te doden. Het antigeen is de component in een vaccin, dat zorgt voor de specificiteit terwijl een adjuvant zorg draagt, dat het immuunsysteem geactiveerd wordt en er een cellulaire immuunrespons wordt opgewekt. Centraal hierbij staan dendritische cellen, die ziekteverwekkers zoals bacteriën en virussen kunnen herkennen, en het immuunsysteem kunnen activeren om deze pathogenen te bestrijden. Dendritische cellen bezitten een aantal receptoren, zoals Toll-like receptoren (TLRs) en NOD-like receptoren (NLRs), die elk specifieke moleculaire structuren, die behoren bij ziekteverwekkers, kunnen herkennen. Zodra zo'n moleculaire structuur, ook wel ligand genoemd, aan een van de receptoren van de dendritische cellen bindt, wordt een alarmsignaal afgegeven wat een proces op gang brengt dat de twee typen T-cellen kan activeren en daarmee de cellulaire immuunrespons op gang brengt. Deze moleculaire structuren werken dus om de vaccins te verbeteren en worden ook wel adjuvans (van het Latijnse *adjuvare*, dat helpen betekent) genoemd. Door het ontwerpen en synthetiseren van conjugaten, waarin een peptide antigeen covalent is gebonden met één of meerdere liganden voor receptoren op dendritische cellen, kunnen nieuwe vaccins worden verkregen.

Een andere manier om dendritische cellen te activeren, is het gebruik van antilichaam-recruterende moleculen. Deze moleculen vormen met al aanwezige antilichamen een immuuncomplex dat vervolgens kan binden aan Fc-receptoren op dendritische cellen. Door het ontwerpen en synthetiseren van conjugaten waarin een peptide antigeen covalent gebonden is aan antilichaam-recruterende moleculen kan een vaccin worden verkregen dat de gewenste immuunrepons opwekt. **Hoofdstuk 1** behandelt verschillende voorbeelden van adjuvanten en immunologisch actieve conjugaten als mogelijke vaccins.

Hoofdstuk 2 beschrijft de synthese en de immunologische evaluatie van vier TLR4-ligand peptide-conjugaten. Het TLR4-ligand CRX-527 is een potent lipide A analogon (een molecuul dat sterk lijkt op een stukje van de bacteriële celwand van een ziekteverwekker), dat geschikt kan worden gemaakt voor conjugatie met een antigeen peptide. Hiervoor werd niet alleen de syntheseroute naar CRX-527 geoptimaliseerd, maar ook een efficiënte multi-gram synthese van de bijbehorende vetstaart ontwikkeld. Het bleek dat er gebruik gemaakt moest worden van een speciale silylidene beschermgroep om de vetstaarten goed te kunnen introduceren in het molecuul. Om te kunnen conjugeren werd CRX-527 uitgerust met een hydrofobe of een hydrofiele linker, die door middel van een ester of een amide binding op de *C*-6 positie van glucosamine werd geïnstalleerd. Bij de immunologische evaluatie van de losse CRX-527 liganden werd aangetoond dat de linker een belangrijk effect kan hebben. Zo zorgde de hydrofobe linker ervoor dat het er geen pro-inflammatoire cytokines werden geproduceerd, wat er op duidt dat het gemaakte CRX-527 ligand niet goed bindt aan de TLR4 receptor. Op grond hiervan werd de hydrofiele linker gekozen om CRX-527 covalent te binden met het $DEVA_5K$ modelpeptide, dat een MHC-I epitoop bevat. Het ligand werd door middel van thiol/maleimide chemie geconjugeerd aan de *N*-terminus of de *C*-terminus van dit antigene peptide. Uit de immunologische evaluatie bleek dat de ester conjugaten beter zijn in het activeren van de dendritische cellen dan de amide conjugaten, maar dat laatstgenoemden daarentegen voor een betere antigeen presentatie zorgen.

Hoofdstuk 3 beschrijft de synthese van vier conjugaten, waarbij het NOD2-ligand, muramyl dipeptide (MDP, een molecuul dat veel voorkomt in de bacteriële celwand) covalent is gebonden aan de *N*-terminus van een HPV-16 peptide epitoop, afkomstig van het human papilloma virus (HPV), dat baarmoederhalskanker veroorzaakt. Er zijn vier bisconjugaten gesynthetiseerd, waarbij naast MDP aan de *N*-terminus het TLR2-ligand, Pam_3CSK_4, aan de *C*-terminus van het HPV-16 peptide is geconjugeerd. Voor de eerste generatie (bis)conjugaten werd een *O*-MDP bouwsteen met een *N*-acetyl of *N*-glycolyl groep gekoppeld via een isoglutaminezuur aan het antigene peptide met behulp van vaste drager chemie. Voor de synthese van de tweede generatie (bis)conjugaten werden twee bouwstenen, bestaande uit een MDP analogon met een *C*-glycosidische binding en voorzien van een *N*-acetyl of *N*-glycolyl groep, bereid. Belangrijke reacties in de synthese van deze *C*-MDP bouwstenen zijn: 1. Het introduceren van een anomere allyl; 2. een alkeenmetathese en 3. het reduceren van de verkregen alkeen. Het MDP *C*-analogon is stabiel onder de zure condities die behoren bij de geautomatiseerde vaste drager peptide chemie, waardoor de MDP

bouwstenen konden worden gekoppeld aan het vaste drager gebonden peptide en er geen extra conjugatie stappen nodig zijn na het assembleren van het peptide.

De mannose-6-fosfaat receptoren (MPR) zijn P-type lectines, die mannose-6-fosfaat glycanen binden, die aanwezig zijn op eiwitten in het Golgicomplex, waarna deze eiwitten naar de endosomen worden getransporteerd. Deze lectines komen ook voor op de buitenkant van cellen en kunnen dus gebruikt worden om een lading, voorzien van een mannose-6-fosfaat, de cel in te brengen. **Hoofdstuk 4** beschrijft de synthese van twee mannose-6-fosfonaat (M6Po) bouwstenen, die beide in plaats van een fosfaat monoester een gestabiliseerde *C*-fosfonaat functie hebben, waardoor defosforylering door fosfatase enzymen wordt verhinderd. Bij de introductie van de *C*-fosfonaat functie bleek het gebruik van de isopropylidene beschermgroep in het mannose derivaat essentieel om ongewenste intramoleculaire cyclisatie te voorkomen. De *O*-M6Po bouwsteen is een *O*-mannoside met een anomere *O*-propargyl groep, die gebruikt wordt voor koper-gekatalyseerde azide–alkyn cycloadditie (een "klik-reactie"). De *C*-M6Po bouwsteen is een *C*-mannoside, die via een *C*-glycosidische band is uitgerust met een lysine spacer en daardoor gebruikt kan worden in een geautomatiseerd vaste drager peptide synthese protocol. Omdat lectines meestal meerdere bindingsdomeinen voor koolhydraat structuren bezitten zijn de liganden in een multivalente manier ingebouwd in de doelmoleculen. Zowel de *O*-M6Po als de *C*-M6Po bouwsteen is zes keer gekoppeld op de *N*- of de *C*-terminus van een antigeen peptide met behulp van respectievelijk "klik" chemie en vaste drager chemie. De aldus verkregen conjugaten kunnen door de MPR gebonden en opgenomen worden en zo een verbeterde immuunreactie opwekken. Daarnaast zijn er ook vier bisconjugaten met een TLR7-ligand gemaakt wat de immuunrepons verder kan verbeteren.

Hoofdstuk 5 beschrijft de synthese van twee *C*-rhamnose gefunctionaliseerde lysine bouwstenen, die met behulp van vaste drager chemie eenmaal, tweemaal, driemaal, of zesmaal covalent gebonden zijn aan het antigene peptide LEQLESIINFEKLAAAAAK, dat een MHC-I klasse epitoop bevat. De multivalente presentatie van rhamnose in deze conjugaten kan de binding met anti-rhamnose antilichamen bewerkstelligen waardoor een immuuncomplex wordt gevormd dat vervolgens aan de Fc-receptor op dendritische cellen kan binden met een verhoogde immuunreactie tot gevolg. De gebruikte bouwstenen verschillen in de linker maar hebben een zuur-stabiele *C*-glycosidische binding en zijn beschermd met de zuur-labiele *p*-methoxybenzyl groepen waardoor ze uitermate geschikt zijn voor vaste drager peptide chemie.

Hoofdstuk 6 beschrijft de synthese van vier *C*-glycosides: α-mannose, β-*N*-acetyl-glucosamine, β-galactose en α-galactose, die beschermd zijn met zuur-labiele beschermgroepen en gekoppeld zijn aan lysine. Deze bouwstenen kunnen worden gebruikt om glycopeptiden te synthetiseren met behulp van geautomatiseerde peptide synthese op een vaste drager, want de *C*-glycosidische bindingen zijn zuurstabiel terwijl de verwijdering van de beschermgroepen samen met de afsplitsing van het glycopeptide van de vaste drager kan plaatsvinden. Tijdens de synthese van deze bouwstenen zijn de volgende reacties belangrijk: de alkeenmetathese met behulp van een Grubbs katalysator, de reductie van het gevormde alkeen en de verzeping van de methyl ester om koppeling met de zijketen van de lysine te kunnen bewerkstelligen.

List of publications

Conjugation ready monophosphoryl lipid A-analogues for self-adjuvanting cancer peptide vaccines
Niels R.M. Reintjens, Elena Tondini, Na-Rae de Jong, Nico J. Meeuwenoord, Fabrizio Chiodo, Elko Peterse, Herman S. Overkleeft, Dmitri V. Filippov, Gijsbert A. van der Marel, Ferry A. Ossendorp and Jeroen D. C. Codée.

Manuscript in preparation

Dual Synthetic Peptide Conjugate Vaccine Simultaneously Triggers TLR2 and NOD2 and Activates Human Dendritic Cells
Gijs G. Zom, Marian M. J. H. P. Willems, Nico J. Meeuwenoord, Niels R. M. Reintjens, Elena Tondini, Selina Khan, Herman S. Overkleeft, Gijsbert A. van der Marel, Jeroen D. C. Codee, Ferry Ossendorp and Dmitri V. Filippov.

Bioconjugate Chemistry, 2019, 30, 1150−1161

The Cyanopivaloyl Ester: A Protecting Group in the Assembly of Oligorhamnans
Volbeda A.G, Reintjens N.R.M., Overkleeft H.S., van der Marel G.A. and Codée J.D.C.

European journal of Organic Chemistry, 2016, 31, 5282-5293

Stabilization of the Low-Spin State in a Mononuclear Iron(II) Complex and High-Temperature Cooperative Spin Crossover Mediated by Hydrogen Bonding
Zheng S., Reintjens N.R.M., Siegler M.A., Roubeau O., Bouwman E., Rudavskyi A., Havenith R.W.A. and Bonnet S

Chemistry - A European Journal 2016, 22, 331–339

Automated solid-phase synthesis of hyaluronan oligosaccharides
Walvoort, M.T.C., Volbeda, G.A., Reintjens, N.R.M., van den Elst, H., Plante, O.J., Overkleeft, H.S., van der Marel, G.A. and Codée, J.D.C.

Organic Letters 2012, 14, 14: 3776-3779

Curriculum vitae – Nederlands

Niels R.M. Reintjens werd op 28 november 1989 geboren te Sittard. Na het behalen van het gymnasium diploma (profiel Natuur&Techniek) aan het Graaf Huyn College in 2008, werd begonnen aan de bacheloropleiding Molecular Science & Technology aan de Universiteit Leiden en de Technische Universiteit Delft, gevolgd door de masteropleiding Chemistry (Ontwerp & Synthese). Ter afronding van de bachelor werd een onderzoeksstage uitgevoerd bij de vakgroep Bio-organische Synthese. Dit project getiteld "Development of glucosamine derivatives for the automated synthesis of hyaluronan" werd begeleid door dr. M.T.C. Walvoort, prof. dr. G.A. van der Marel en dr. J.D.C. Codée. Bij dezelfde vakgroep werd als onderdeel van de masterstudie onder begeleiding van dr. A.G. Volbeda een nieuwe participerende pivaloyl groep ontwikkeld en gebruikt voor de synthese van repeterende rhamnose tri- en tetrasacchariden. Een tweede masterstage werd bij de vakgroep Metalen in katalyse, biomimetica en anorganische materialen uitgevoerd onder begeleiding van dr. S. Zeng, dr. S. Bonnet en prof. dr. E. Bouwman, waarbij onderzoek werd verricht aan mononucleaire Fe(II) spin crossover complexen.

In december 2013 werd het onderzoek beschreven in dit proefschrift verricht in de vakgroep Bio-organische Synthese onder supervisie van prof. dr. G.A. van der Marel en dr. J.D.C. Codée. Gedeelten van dit onderzoek werden gepresenteerd middels posterpresentaties op het 19^{e} European Carbohydrate Symposium 2017 te Barcelona, de NWO-CHAINS conferentie 2017 te Veldhoven en het 29^{e} Internationale Carbohydrate Symposium 2018 te Lissabon. Op de NWO-CHAINS conferentie werd de poster bekroond met de prijs voor beste poster. Mondelinge presentaties werden gegeven op de NWO-CHAINS conferentie 2018 en het 20^{e} European Carbohydrate Symposium 2019 in Leiden. Als onderdeel van het traject "Doctors voor de klas" werd in de periode 2015-2017 met succes de educatieve master behaald, waarvoor in diezelfde periode een twee jaar durende part-time stage is gevolgd op het Rijnlands Lyceum te Oegstgeest onder leiding van drs. I.L.M. de Herder, M.Ed. C.D. Ellison en drs. E.T. Stoutjesdijk. Samen met G.J.M. Groenewold MSc, heeft hij de small private online course "Moderne chemie is Overal" ontwikkeld.

Vanaf 1 juni 2019 is de auteur van dit proefschrift als post-doctoraal onderzoeker werkzaam in de vakgroep van prof. dr. A.J. Minnaard aan de universiteit van Groningen.

Curriculum vitae – English

Niels R.M. Reintjens was born in Sittard on November 28th 1989. In 2008, he graduated from the high school Graaf Huyn College (VWO) and subsequently started the bachelor study Molecular Science & Technology at Leiden University and Technological University Delft, followed by the master study Chemistry (major Design & synthesis). A bachelor research internship was performed in the group Bio-organic Synthesis group concerning the development of glucosamine derivatives for the automated synthesis of hyaluronan under the supervision of dr. M.T.C. Walvoort, prof. dr. G.A. van der Marel and dr. J.D.C. Codée. As part of his master program, two research internships were performed. In the Bio-organic Synthesis group under the supervision of dr. A.G. Volbeda, prof. dr. G.A. van der Marel and dr. J.D.C. Codée, a new participating pivaloyl protecting group was developed and used in the synthesis of repeating tri- and tetrarhamnosides. In his second internship in the group Metals in Catalysis, Biomimetics & Inorganic Materials bapphen-based mononuclear Fe(II) spin crossover complexes were investigated under the supervision of dr. S. Zeng, dr. S. Bonnet and prof. dr. E. Bouwman.

In December 2013, the research described in this Thesis was started under the supervision of prof. dr. G.A. van der Marel and dr. J.D.C. Codée in the Bio-organic Synthesis group of Leiden University. Parts of the research described herein, were presented by poster presentations at the 19th European Carbohydrate Symposium 2017 in Barcelona, the NWO-CHAINS conference 2017 in Veldhoven and the 29th International Carbohydrate Symposium 2018 in Lisbon. At the NWO-CHAINS conference, the poster was awarded the prize for best poster. Oral presentations were given at the NWO-CHAINS conference 2018 and the 20th European Carbohydrate Symposium 2019 in Leiden. As part of the “Doctors in the classroom” trajectory, a second master degree in education was obtained in 2017 after a two-year part-time internship at the Rijnlands Lyceum in Oegstgeest under the supervision of drs. I.L.M. de Herder, M.Ed. C.D. Ellison and drs. E.T. Stoutjesdijk. Together with drs. G.J.M. Groenewold, he developed a small private online course “Moderne chemie is Overal”.

As of June 2019, the author of this Thesis is employed as a postdoctoral fellow in the Chemical Biology group of prof. dr. A.J. Minnaard at the University of Groningen.