Design and synthesis of NLR and TLR based ligand-antigen conjugates

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Chapter 2

Synthesis and biological evaluation of NOD2-Ligand-Antigen conjugates

2.1 Introduction

In recent years the study of pattern recognition receptors (PRRs) and associated ligands has evolved tremendously. In the 1970s only the peptidoglycan (PG) in Freund's adjuvant was recognized to have adjuvant properties. The discovery of Toll-like receptors (TLRs)[1] in the late 1990s had a major impact on the field of immunology. This is reflected in the exploration of conjugates consisting of a PRR-Ligand (PRR-L) covalently bound to antigenic proteins and oligopeptides in the development of new (semi)-synthetic vaccine modalities.[2,3] For instance, the group of Boons investigated a three-component conjugate containing a tumor-associated glycopeptide and a T cell epitope (YAFKYARHANVGRNAFELFL) covalently bound to a TLR2 ligand (TLR2-L).[4] Furthermore they reported a vaccine candidate consisting of a TLR2-L, a helper T-cell epitope and a MUC-1 glycopeptide B-cell epitope.[5] The latter construct elicit high IgG antibody responses, which have the ability to recognize cancer cells. Kahn *et al.* reported on the design, synthesis and immunological evaluation of constructs of TLR

ligands covalently linked to a long peptide harboring a major histocompatibility complex (MHC) class I specific epitope. The structurally defined TLR2-L Pam_3CSK_4[6], TLR7-L 7-hydroxy-8-oxo-adenine[7] and TLR9-L CpG DNA were covalently bound to a model antigen, an ovalbumin derived peptide comprising the MHC I epitope SIINFEKL, embedded in a longer peptide motif (DEVSGLEQLESIINFEKLAAAAAK, $DEVA_5K$).[6,8] It was revealed that conjugates, in which the TLR-L Pam_3CSK_4 or CpG DNA were incorporated, showed an increased uptake of conjugated peptide, intact DC maturation potential and enhanced antigen presentation in comparison with the mixture of the single peptide and ligands.[7,9]

In the late 1990s the cytosolic NOD (Nucleotide-binding Oligomerization Domain-containing protein) receptors[10] NOD1[11] and NOD2[12] were discovered to be intracellular PRRs.[13] NOD2 recognizes specific parts of PG (*Figure 1*), found in the bacterial cell wall. PG consists of a polysaccharide chain of β(1-4) linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) of which the lactic acid is connected to a peptide. The minimal structural element of PG required for activation of the intracellular protein NOD2 is *N*-acetylmuramyl-L-alanine-D-isoglutamine (MDP, **1**).[14-17]

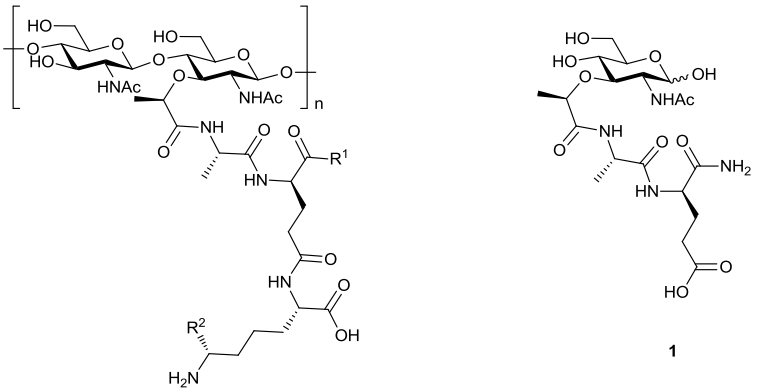


Figure 1. PG of Gram-positive or Gram-negative bacteria and muramyldipeptide (MDP) **1**.

Based on the effectiveness of the above mentioned TLR2-L and TLR9-L conjugates, it was hypothesized that constructs consisting of a NOD2 ligand (NOD2-L) covalently bound to the ovalbumin derived model peptide ($DEVA_5K$) could lead to similar enhanced immunological activity. Important issues in the design of such conjugates are the selection of a suitable MDP derivative and the position of the covalent linkage between this NOD2-L and the peptide epitope.

Little is known about conjugates of MDP and antigenic peptides and the only reports date from the early 1980s concerning the synthesis and evaluation of mixtures of conjugates.[18-20] Besides, the nature of the interaction between MDP and NOD2 receptor at the molecular level has not been established yet. The following linkages between MDP and the antigenic peptide are considered (*Figure* 2). In the antigenic peptide, the *N*-terminus (the left side, *Figure 2A*) and *C*-terminus (the right side, *Figure 2B*) are obvious choices. In MDP the anomeric centre of the sugar (*Figure2 C,* I) and the carboxylic acid function of isoglutamine (*Figure 2C,* II) are viable conjugation sites.

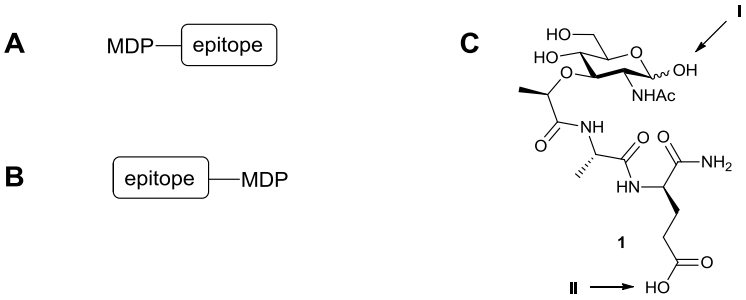


Figure 2. General design of NOD2-ligand-antigen conjugates; **A)** *N*-terminal conjugated NOD2-L-antigen conjugate, ***B)*** *C*-terminal conjugated NOD2-L-antigen conjugate; **C)** Conjugation positions of MDP (**1**) at the anomeric position (I) of MurNAc or acid residue of the isoglutamine (II).

It is known that the size and orientation of the aglycon installed at the anomeric centre of MDP influences the immunological activity (*Figure 2C,* I).[21, 18] On the basis of these data an 3-azidopropanol spacer was selected as conjugation handle with minimal steric bulk. This anomeric 3-azidopropanol handle on the MurNAc moiety needs to have a beta configuration since this anomer is reported to be higher in activity than the corresponding alpha anomer.[21]
The second possible location to conjugate MDP to the antigenic peptide is the carboxylic acid function of the isoglutamine residue (*Figure 2C,* II). Since Li *et al.* reported that condensation of this acid with an unnatural amine does not affect the immunological properties of MDP.[21] They have described a conjugate of the anticancer drug Paclitaxel with MDP, which not only shows antitumor activity but also immunostimulatory effects.[22] Also, Murabutide, a glutamine-*n*-butyl ester derivative of MDP and a commercially available immunomodulator supports the notion that modifications on the isoglutamine are allowed.[23]

Thus, NOD2-L antigen conjugates **2** – **5** were selected as target molecules (*Figure 3*). In conjugate **2** the carboxylic acid function of the isoglutamine of the NOD2-L is linked to the *N*-terminal amine of the antigenic peptide. In conjugate **3** the same acid function of the NOD2-L connects to the *C*-terminal lysine of the

antigenic peptide. The 3-azidopropanol spacers at the anomeric centre of MurNAc in conjugate **2** and **3** remain unmodified. In conjugates **4** and **5** on the other hand the 3-azidopropanol is functionalized with glutamic acid allowing conjugation to the antigenic peptide at the *N*-terminus for conjugate **4** and at the *C*-terminus for conjugate **5**.

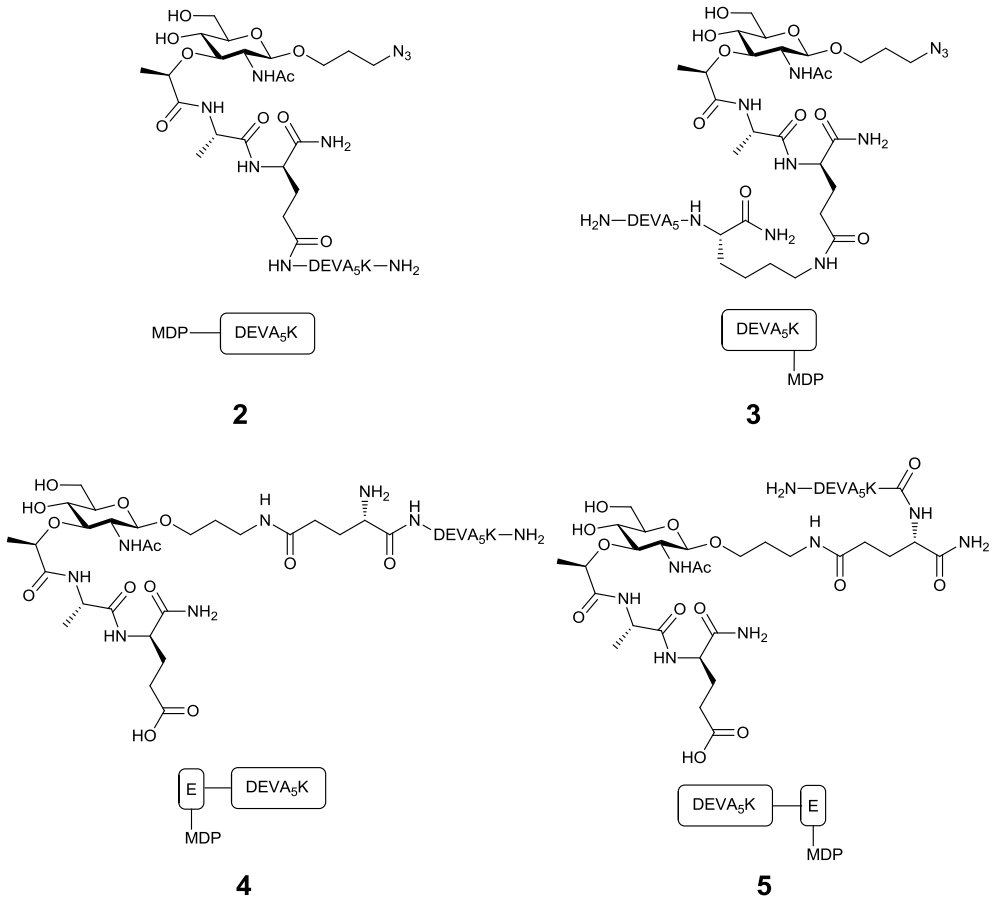


Figure 3. NOD2-L-antigen conjugates **2 – 5**, $DEVA_5K$ = DEVSGLEQLESIINFEKLAAAAAK.

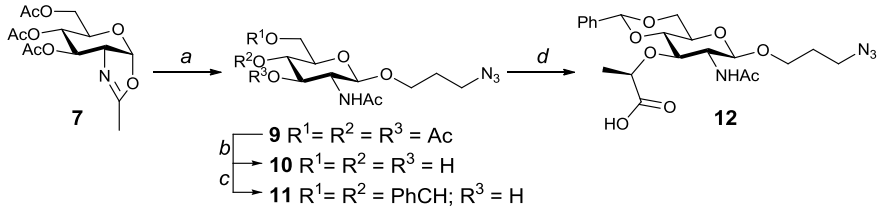
The next sections describe the synthesis and biological evaluation of NOD2-L antigen conjugates **2 – 5** together with the non-conjugated MDP azidopropanol ligand **25** as a relevant reference compound.

2.2 Synthesis of NOD2-L-antigen conjugates

The NOD2-L-antigen conjugates **2** – **5** were prepared using an automated solid phase peptide synthesis (SPPS) protocol. In all these syntheses commercially available Tentagel S RAM resin and amino acids were applied. Building block **12**, required for the assembly of conjugates **2** and **3** was accessed by solution phase synthesis (*Scheme 1*). The same holds for the synthesis of advanced building block **21** that is needed for conjugates **4** and **5** (*Scheme 3*).

The MurNAc **12** is equipped with a 3-azidopropanol spacer at the anomeric position (*Scheme 1*). This spacer was affixed using an oxazoline mediated glycosylation procedure.[24-26] Oxazoline **7**, prepared from fully acetylated glucosamine was purified with Et_3N neutralized silica and subsequently treated with 3-azido-propanol (**8**) and TMSOTf to give **9** in good yield. Deacetylation of compound **9** and subsequent installation of the benzylidene protective group gave alcohol **11**. Condensation of **11** with (S)-2-chloro-propanoic acid in the presence of sodium hydride resulted in the formation of protected MurNAc **12** in 93% yield.[26,27,28]

Scheme 1. Synthesis of building block **12**.

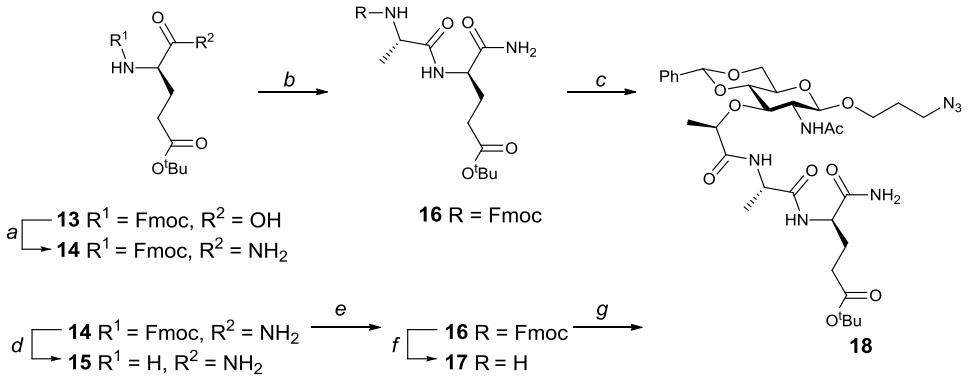


Reaction conditions: *a*) TMSOTf, DCM, 3-azidopropanol (**8**), 83%; *b*) cat. NaOMe, MeOH, quant.; *c*) CSA, $PhCH(OMe)_2$, MeCN, DMF, 86%; *d*) 3-chloropropionic acid, NaH, 1,4-dioxane, 93%.

Compound **18**, an intermediate en route to building block **21** and reference compound **25** was prepared from building block **12** and dipeptide **16** (*Scheme 2*). Reaction of Fmoc protected *tert*-butyl glutamic acid **14** with di-*tert*-butyl dicarbonate and ammonolysis of the intermediate anhydride resulted in **14**.[29] In a one-pot procedure compound **14** was deprotected with DBU and after quenching the reaction mixture with HOBt, the free amine of **14** was condensed with Fmoc-protected alanine to give compound **16** in 82% yield. The same procedure was used to condense MurNAc **12** with dipeptide **16**, using the more reactive coupling reagent HATU. Despite the low solubility of MurNAc **12** and dipeptide **16**, building block **18** was obtained in 70% yield.

In an alternative procedure (*Reaction conditions d – g, Scheme 2*) the Fmoc group in **14** was removed by DBU in the presence of ethanethiol as a scavenger to give amine **15** in 98% isolated yield.[30] Compound **15** was then condensed with Fmoc-protected alanine to give compound **16** in 68% yield. Subsequently the Fmoc deprotection step in the presences of octanethiol was repeated resulting in amine **17** in 62% isolated yield. Although the following coupling with MurNAc **12** proceeded smoothly, this procedure was no improvement in comparison with the one-pot procedure. The isolation and purification process of key intermediate **18** was substantially facilitated by the finding that the compound coulf be efficiently precipitated from a solvent mixture of MeOH, DCM and diethyl ether.

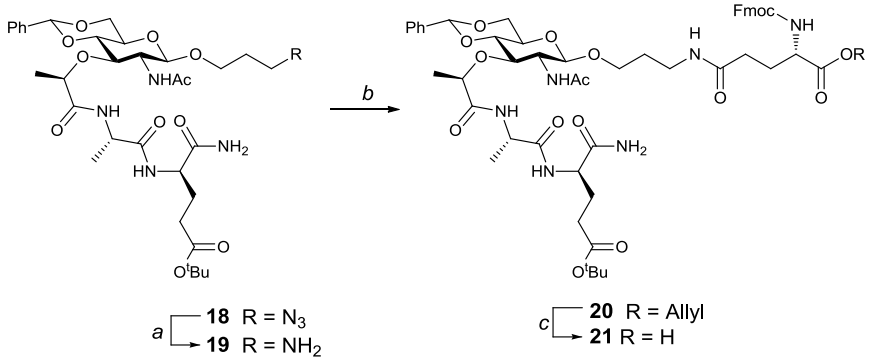
Scheme 2. Synthesis of building block **18**.



Reaction conditions: *a*) Boc_2O, NH_4HCO_3, pyridine, 1,4-dioxane, >99%; *b*) 1) DBU, HOBt, DCM, 2) Fmoc-L-Ala-OH, EDC, DiPEA, DCM, 82%; *c*) 1) **16**, DBU, HOBt, DCM, 2) **12**, HATU, DiPEA, DCM, 70%; *d*) cat. DBU, EtSH, THF, 98%; *e*) Fmoc-L-Ala-OH, EDC, HOBt, DiPEA, DCM, 68%; *f*) cat. DBU, octanethiol, THF, 62%; *g*) **12**, HATU, DiPEA, DMF, 80%.

The synthesis of building block **21** started with the reduction of the azide in compound **18** with PMe_3, to afford compound **19** in 80% yield (*Scheme 3*).[31] Subsequently the condensation of **19** and Fmoc protected glutamic acid allyl ester under influence of HATU and DiPEA gave the orthogonally protected compound **20** in 57%. To make **20** suitable for coupling, the allyl protective group was removed with Bu_3SnH and $Pd(PPh_3)_4$ under acidic conditions yielding compound **21** in 72%.[32]

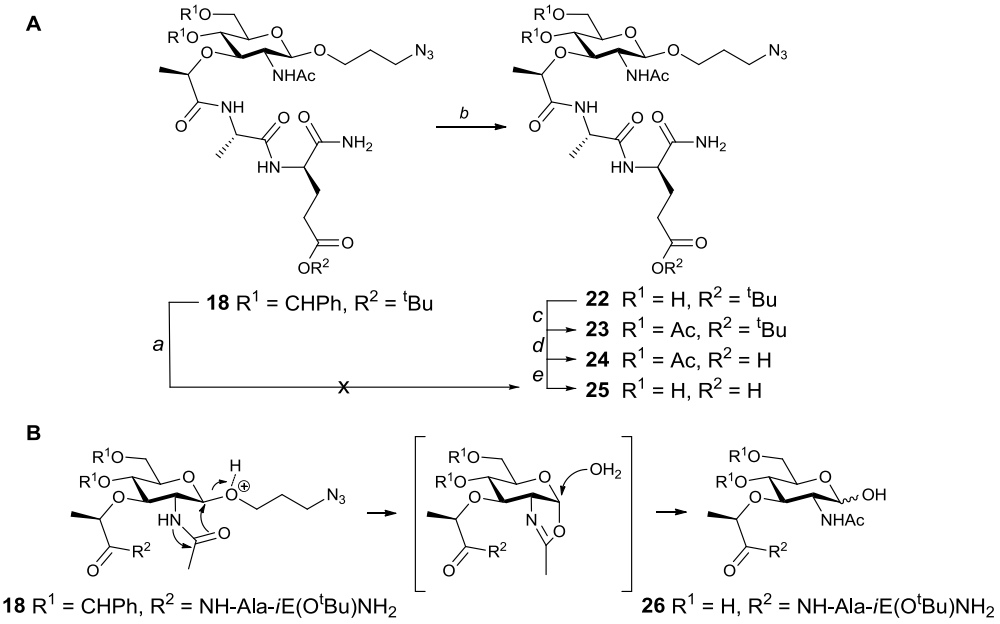
Scheme 3. Synthesis of **21**.



Reaction conditions: *a*) Me_3P (solution in THF), DMF, THF, 80%; *b*) Fmoc-Glu-(OH)-OAllyl, HATU, DiPEA, DMF, 57%; *c* $Pd(PPh_3)_4$, Bu_3SnH, AcOH, DMF, 72%.

For the immunological evaluation of conjugates **2** – **5** relevant reference compounds are needed. The presence of the 3-azidopropanol spacer in the conjugates **2** and **3** and the lack thereof in natural MDP (**1**) makes compound **25** a suitable reference compound. In principle acidic removal of the Boc and benzylidene protecting groups in **18** could lead to reference **25** (*Scheme 4A*). However, treatment of **18** with a solution of 20% TFA in DCM was accompanied by hydrolysis of the glycosidic linkage. The acid mediated removal of the 3-azidopropanol spacer can be explained by the intermediate formation of an oxazoline (*Scheme 4B*). To suppress acid-mediated hydrolysis of key intermediate **18** the attention was directed to a stepwise deprotection procedure in which the more acid labile benzylidene group was first replaced by electron withdrawing acetyl groups. This protective group manipulation increases the acid stability of the glycosidic linkage and permits the removal of the Boc group using more stringent conditions. Thus, treatment of **18** with a solution of 60% aqueous acetic acid in the presence of two equivalents of neopentyl glycol at 60^{o}C and careful monitoring of the reaction progress gave compound **22** in 88% yield. Acetylation of **22** quantitatively gave compound **23**. Protection of the glycosidic linkage by the installment of electron withdrawing groups proved to be successful, and the treatment of **23** with 20% TFA in DCM resulted in compound **24** in 82% yield. During the reaction only a minimal amount of hydrolysis was observed. The synthesis was followed by the treatment of **24** with ammonia in MeOH and purification using HW40 gel filtration resulted in reference compound **25** in 87% yield.

Scheme 4. Synthesis of reference compound **25**.

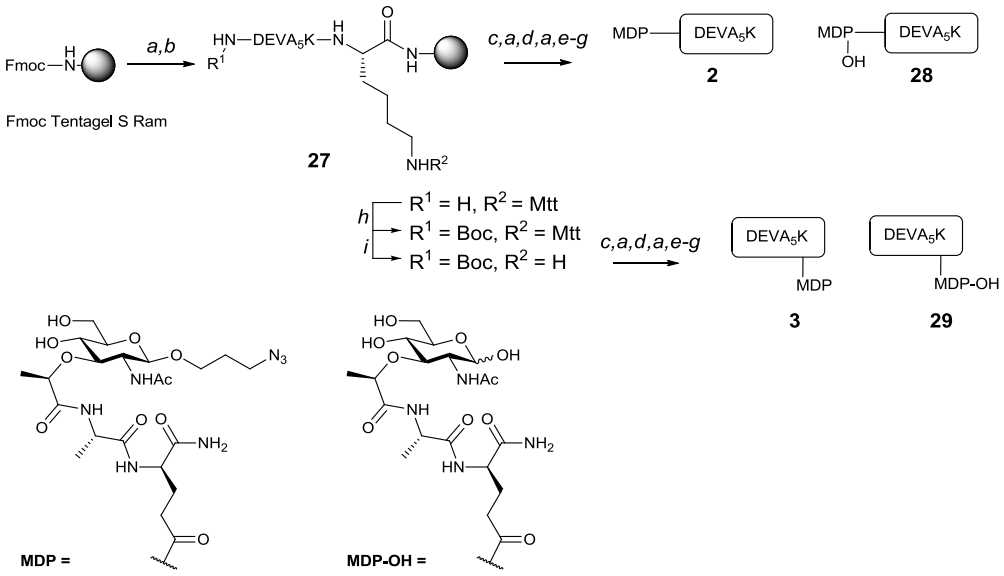


Reaction conditions: **A** *a*) 20% TFA, DCM; *b*) 60% AcOH, H_2O, neopentylglycol, 88%; *c*) Ac_2O, pyridine, quant; *d*) 20% TFA, DCM, 82%; *e*) NH_4, MeOH, HW40 gel filtration, 87%; **B** Mechanism acid hydrolysis of C-1 hydroxyl of **18**.

With MurNAc building block **12** in hand, the solid phase peptide syntheses of the NOD2-L-antigen conjugates **2** and **3** were undertaken (*Scheme 5*). Commercially available Fmoc protected amino acids, equipped with standard acid labile protective groups were used. The side chain of the *C*-terminal lysine of the antigenic peptide was protected with the methyl trityl (Mtt) protective group, allowing the modification of both *N*- or *C*-terminal end at the final stage of the synthesis. In a standard elongation cycle using HCTU as a coupling reagent, acetic anhydride as capping reagent and piperidine to remove the Fmoc-group immobilized peptide **27** was assembled with the aid of fully automated peptide synthesis. To obtain conjugate **2** with the NOD2-L on the *N*-terminus of the peptide, peptide **27** was consecutively elongated with Fmoc-isoglutamine, Fmoc-alanine and MurNAc **12**. The coupling of MurNAc **12** involved a double coupling protocol using HATU instead of HCTU as condensing agent. The thus obtained fully protected and immobilized precursor was treated with a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 104 minutes to give, after precipitation in Et_2O, conjugate **2** and the hydrolyzed conjugate **28** in a 1 : 1 ratio. Purification by RP-HPLC resulted in the isolation of 1.3 mg pure conjugate **2** (2% yield).

In the next target conjugate **3**, MDP occupies the *C*-terminal position of the antigenic peptide. To obtain **3**, the *N*-terminus of immobilized peptide **27** was protected with the Boc-group by treatment with 1 M Boc_2O in NMP and two equivalents of DiPEA for 1 hour (*Scheme 5*). Next, the resin was treated with a solution of 3 % TFA in DCM to selectively remove the Mtt protective group from the side chain of the *C*-terminal lysine. The resulting free amine was consecutively elongated with Fmoc-isoglutamine, Fmoc-alanine and MurNAc **12** as described for conjugate **2**. Subsequently, the resin was subjected to the cleavage cocktail (95% TFA, 2.5% TIS and 2.5% H_2O) for 104 minutes to give conjugate **3** and the hydrolyzed conjugate **29** after precipitation in a 1 : 1 ratio. Purification by RP-HPLC gave 1.2 mg pure conjugate **3** (2% yield).

Scheme 5. SPPS of conjugates **2** and **3**.



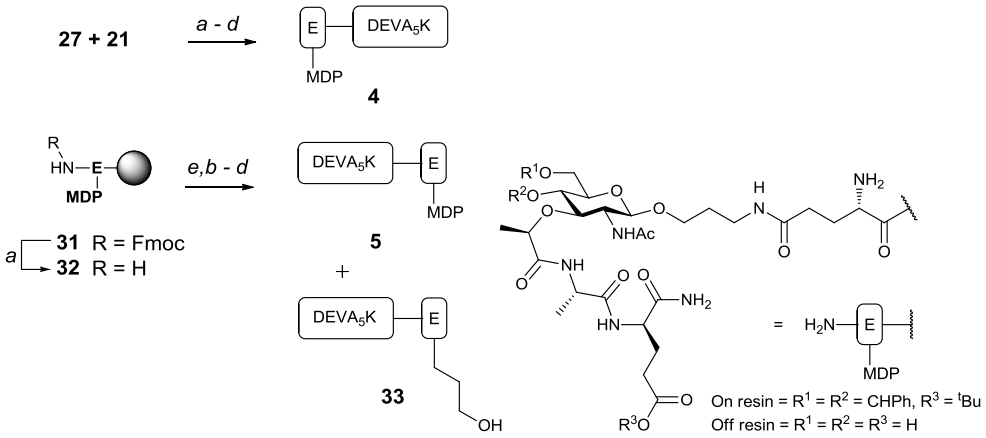
Reaction conditions: a) 20% piperdine, NMP; *b*) Fmoc SPPS $DEVA_5K$; *c*) Fmoc-*i*-D-Gln-OH, HCTU, DiPEA, NMP; *d*) Fmoc-L-Ala-OH, HCTU, DiPEA, NMP; *e*) **12**, HATU, DiPEA, NMP; *f*) 95% TFA, 2.5% H_2O, 2.5% TIS; *g*) RP-HPLC; *h*) Boc_2O, NMP, DiPEA; *i*) 1% TFA, DCM. Yield conjugates: **2**) 1.3 mg, 2%; **3**) 1.2 mg, 2%; **28**) 1.5 mg, 2%; **29**) 1.0 mg, 2%.

The conjugates **4** and **5** were obtained by the application of advanced building block **21** (*Scheme 6*). En route to **4**, peptide **27** was elongated with **21** in a coupling cycle using HATU as a coupling reagent. The solid phase synthesis was concluded with the removal of the Fmoc protective group resulting in the partially protected immobilized precursor of **4**. To reduce the acid hydrolysis of conjugates **4** during the removal of the protecting groups and cleavage from the solid support, the treatment time with the standard cleavage cocktail was decreased to 60 minutes. The conjugate was precipitated by the addition of Et_2O

and cooling at -20°C. Purification by RP-HPLC gave pure conjugate **4** in 1.8 mg in 2% yield.

To synthesize the *C*-terminal functionalized conjugate **5**, the Tentagel S RAM resin first was condensed with building block **21**. It turned out to be difficult to couple **21** to the resin. Changing the solvent mixture to 20% DMSO in NMP led to **31** with a moderate coupling efficiency of 56% as judged from the Fmoc-cleavage test with an aliquot of resin. The Fmoc protective group of **31** was removed and the resin was elongated by automated SPPS with the $DEVA_5K$ motive resulting in the immobilized conjugate **5**. The same deprotection and cleavage conditions as described for **4** were used to isolate conjugate **5**. Purification by RP-HPLC gave pure conjugate **5** in 4.8 mg and 6% overall yield. The treatment of the resin with the cleavage cocktail by a shorter reaction time reduced the hydrolysis of the conjugates. In the synthesis of conjugate **5** the isolation of hydrolyzed compound **33** yielded 2.8 mg resulting in an improved 3 : 2 ratio.

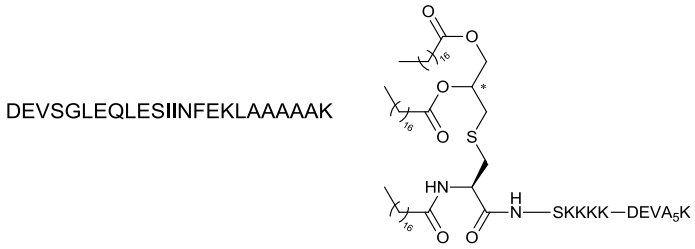
***Scheme* 6**. SPPS of conjugates **4** and **5**.



Reaction conditions: a) HATU, DiPEA, NMP; *b*) 20% piperdine, NMP; *c*) 95% TFA, 2.5% H_2O, 2.5% TIS; *d*) RP-HPLC; *e*) Fmoc SPPS $DEVA_5K$. Yield conjugates: **4**) 1.8 mg, 2%; **5**) 4.8 mg, 6%; **33**) 2.8 mg, 4%.

2.3 Immunological evaluation of NOD2-L-antigen conjugates

First, the NOD2 immunostimulatory activity of the conjugates **2** – **5** was assessed in an assay, using a stable NOD2 transfected human embryonic kidney (HEK) cell line (293HEK), in which the level of interleukin 8 (IL-8) production reflects the NOD2 activity of the compound. For this purpose, peptide $DEVA_5K$ (**34**)[6] and TLR2-L-antigen conjugate (**35**)[6] are used as relevant reference compounds (*Figure 4*).



34 **35**

Figure 4. Reference compounds: peptide **34** and TLR2-L-conjugate **35**.

The immunostimulatory potential of the conjugates was evaluated on the basis of their ability to induce maturation of dendritic cells (DCs) and their ability to support antigen presentation upon uptake by DCs. To monitor the ability of the conjugates to activate DCs, the level of IL-12p40 production upon stimulation of DCs with the conjugates is measured. Interleukin 12 (IL-12) secretion by DCs is a good indication of the immunostimulatory potential of a compound, since this cytokine is important for the development of T-cell based immune responses. DCs are important antigen presenting cells, as they can efficiently take up exogenous antigens and present it not only in MHC class II, but also in MHC class I, a process known as cross-presentation. In this way, DCs are able to present exogenously derived peptides to both CD4+ T cells and CD8+ T cells. The MHC class I molecule K^b on DCs derived from C57BL/6 mice is able to present the SIINFEKL epitope ($OVA_{257-264}$), which is embedded in the long OVA peptide. Antigen presentation can be measured colorimetrically in a SIINFEKL-specific T-cell hybridoma assay.

The NOD2 activating capacity of the conjugates **2** – **5** was tested using the NOD2 HEK cell line in which MDP derivative **25**, peptide **34** and TLR2-L conjugate **35** were included (*Figure 4*). MDP derivative **25**, having the anomeric azidopropyl spacer was used as a positive control; peptide **34** and TLR2-L-antigen conjugate **35** were used as negative controls as can be seen in *Figure 5*. MDP derivative **25**

showed a substantial amount of IL-8 production confirming that the β-azidopropanol modification on the anomeric position is accepted.[33,34] Of conjugates **2** – **5** the conjugates **2** and **3** showed activity close to background levels. In contrast conjugates **4** and **5** induced IL-8 production at a level comparable to MDP derivative **25**. The difference in the activity of conjugates **2** and **3** compared to **4** and **5** indicates that the attachment point of the NOD2-L to the antigenic peptide is important. Conjugation at the C-1 hydroxyl of MDP as in conjugates **4** and **5** is more favorable than conjugation to the side chain of isoglutamic acid (**2**, **3**). The negative controls, peptide **34** and conjugate **35**, show no activity indicating that the activities of **4** and **5** are a consequence of the presence of the NOD2-L in these conjugates. Notably, the location of the ligand on either the *C*- or *N*-terminus of the peptide (**4** vs. **5**) does not seem to affect NOD2 stimulating activity, although *N*-terminal conjugation appears more active than *C*-terminal conjugated MDP.

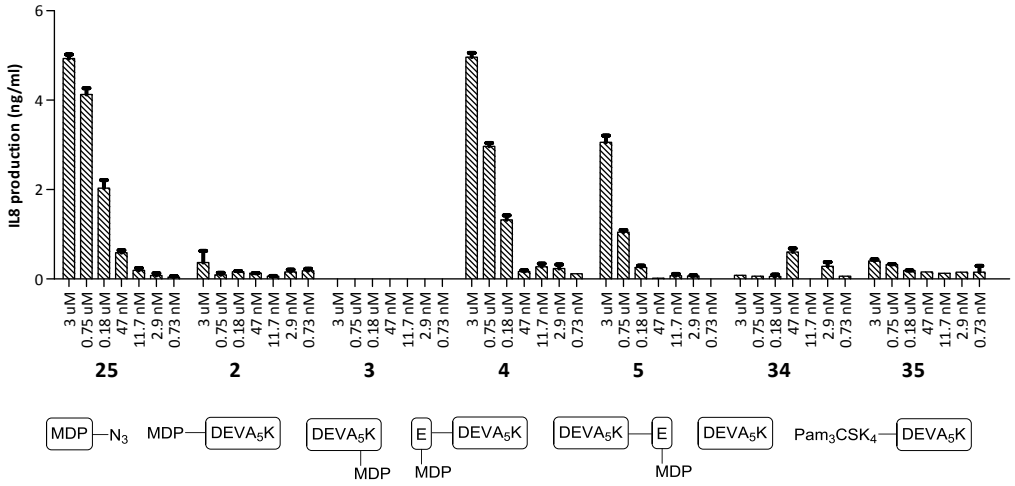


Figure 5. Potency of the NOD2-L antigen conjugates **2** – **5** in NOD2 transfected HEK cells.

The ability of the conjugates **2** – **5** to induce the maturation of DCs was evaluated by interleukin 12 (IL-12p40) production by the cultured murine dendritic cell line D1.[35] The DCs were stimulated with conjugates **2** – **5**, peptide reference **34** and TLR2-L-antigen conjugate **35**. The latter is known to induce DC maturation and thus serves as a positive control. In *Figure 6* it is shown that conjugates **2** – **5** produce amounts of IL-12 that are close to background level. In agreement with the results of the NOD2 transfected HEK cells conjugates **4** and especially **5** are more active than **2** and **3** indicating that the ligation method of MDP affects the NOD activity of the constructs. Notably, reference TLR2-L-conjugate **35** is much more potent than NOD2 conjugates **4** and **5**. A possible

explanation for the lack of strong maturating activity of the conjugates **4** and **5** is that these conjugates are not effectively internalized and therefore only a very low concentration reaches the cytosol resulting in a poor stimulation of the NOD2 receptor as was also suggested by Lee and co-workers.[36]

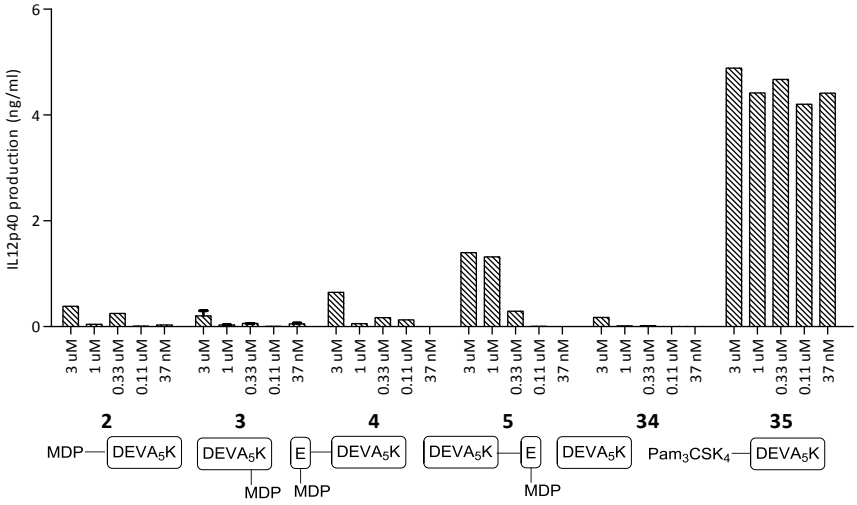


Figure 6. DC activation of the conjugates **2** – **5** and reference compounds **34** and **35**.

Finally, the influence of the NOD2-L modification on the MHC I-mediated presentation of the antigenic peptide was investigated in an assay using a SIINFEKL-specific T-cell hybridoma assay. The DCs were treated with constructs **2** – **5** and reference compounds **34** and **35** (*Figure 7*). All constructs gave rise to antigen presentation which indicates that the presentation is not affected by the condensation of an MDP to ether C- or N-terminus of the antigenic peptide. Known conjugate **35** containing TLR-2 ligand Pam_3CSK_4 showed, as was expected, an increased level of antigen presentation in comparison to peptide **34**.[37]

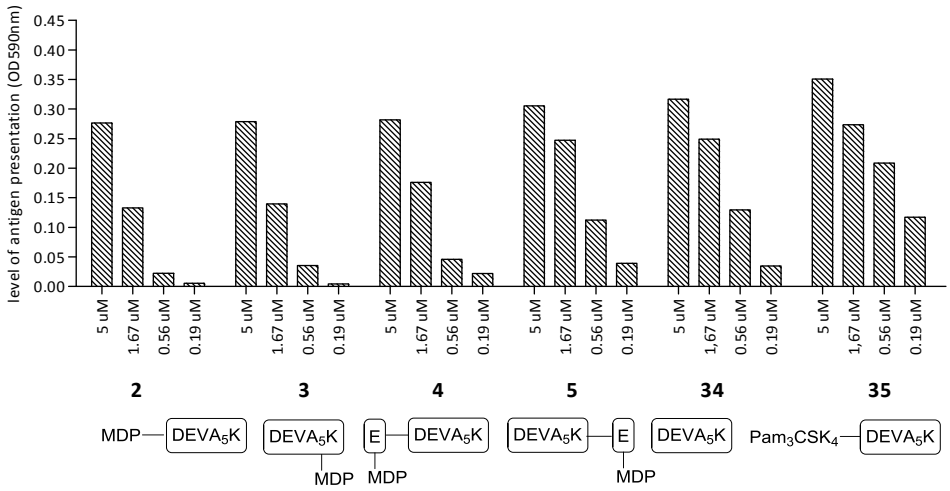


Figure 7. Antigen presentation of the conjugates **2 – 5** and reference compounds **34** and **35**.

Collectively, the data obtained from the assays described in this Chapter indicate that the NOD2-L based conjugates **2** - **5** are poor maturating agents for dendritic cells while antigen presentation from the peptide part of the constructs is retained. Since the NOD-2 activity of constructs **4** and **5** is intact as determined by the assay using the NOD-2 transfected cell line, the overall failure of the described construct to maturate DCs is probably not caused by the lack of receptor binding. The NOD2 receptor is located in the cytosol and the mechanism how putative NOD2 ligand are internalized, processed and presented to the receptor is not fully understood yet.[36] There are indications that PG fragments are internalized *via* endocytosis and subsequently processed and transported to cytosol. Therefore we assume that the poor activity of the NOD-2-L conjugates is due to inability of these to reach the cytosol of the DC. Despite the moderate immuno stimulatory potential of MDP based conjugates in DC based assays (usually high µM ranges, see also Chapter 3) these compounds are known to be useful as synthetic adjuvants.[18,19,38] The antigen presentation assay indicates that the NOD2 modified peptides are processed correctly and presented by the MHC class I molecule.

2.4 Conclusion

This Chapter describes the synthesis of conjugates **2** – **5** that comprise a *C*- or *N*-terminal ligation of the NOD2-L MDP to an antigenic peptide *via* either the isoglutamine function or the C-1 hydroxyl of MDP and evaluation of the immunostimulatory potential of these. During the synthesis the glycosidic bond of NOD2-L turned out to be sensitive to the acidic deprotection conditions used, resulting in partial hydrolysis of the aglycon. Nevertheless, pure MDP derivatives could be isolated. Further the hydrolysis could be suppressed by the use of temporary electron withdrawing acyl groups on the 4,6-hydroxyls of the glucosamine moiety.

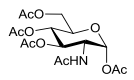
The immunostimulatory potential of the prepared conjugates **2** – **5** proved to be poor in the *in vitro* assays described here. In contrast conjugation of the NOD2 ligands to the antigenic peptide did not hamper the antigen presentation, although the conjugates show a marginal DC maturation potential. It is not excluded, however, that an assay in a different type of system, for example in *in vivo* or using human DCs could have a different outcome since it is known that there are subtle differences in NOD sensitivity between human or murine DCs known. In addition, improved NOD2-ligands based on lipophilic MDP derivatives can possibly be used because such compounds can display a more favorable uptake profile than the relatively polar MDP-conjugates (**2** – **5**) described here. Design, synthesis and immunological evaluation of such constructs are described in Chapter 3.

2.5 Experimental section

All reagents and solvents used in the solid phase peptide synthesis were purchased from Bachem and Biosolve and used as received. Palmitoyl-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-OH was purchased from Bachem, Fmoc-amino acids from Novabiochem and HATU from Tebu Bio. Tentagel based resins were ordered from Rapp Polymere. Light petroleum ether with a boiling range of 40–60 ºC was used. All other solvents used under anhydrous conditions were stored over 4Å molecular sieves except for methanol, which was stored over 3Å molecular sieves. Solvents used for work–up and silica gel column chromatography were of technical grade and distilled before use. All other solvents were used without further purification. Reactions were monitored by TLC–analysis or LC/MS analysis. LC/MS was conducted on a JASCO system using an Alltima C_{18} analytical column (4.6 x 50 mm, 5 µm particle size, flow 1.0 mL/min.), Alltima CN analytical column (4.6 x 50 mm, 3 µm particle size, flow 1.0 mL/min.) or a Alltima C_4 analytical column (4.6 x 50 mm, 5 µm particle size, flow 1.0 mL/min.). Absorbance was measured at 214 and 256 nm. Solvent system: A: 100% water, B: 100% MeCN, C: 1% aq. TFA. Gradients of MeCN in 10% C were applied over 15 minutes unless stated otherwise. Purifications were conducted on the Gilson GX-281 preparative RP-HPLC system, supplied with a semi preparative Alltima CN column (10 x 250 mm, 5 µm particle size, flow 5.0 mL/min.) or semi preparative Alltima C_4 column (10 x 250, 5 µm particle size, flow 5.0 mL/min.). Solvent system: A: 0.1 % aq. TFA and B: MeCN. Gradients of 10 – 90% MeCN were

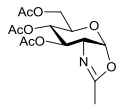
applied over 3 CV over 15 min. unless stated otherwise. The UV absorption was measured at 214 and 256 nm. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in H_2O/MeCN; 50/50: v/v and 0.1% formic acid) on a mass spectrometer Thermo Finnigan LTQ Orbitrap equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 523 K) with resolution R = 60000 at m/z 400 (mass range m/z = 150–2000) and dioctylphthalate (m/z = 391.28428) as lock mass. Optical rotations were measured on a Propol automatic polarimeter (sodium D–line, λ = 589 nm). Specific rotations $[\alpha]^D$ are given in degree per centimeter and the concentration c is given in mg/ml in the specific solvent. Maturation and B_3Z presentation results were analyzed with Graphpad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA. IR spectra were recorded on a Perkin Elmer Paragon 1000 FT–IR Spectrometer.

Acetyl-3,4,6-tri-*O*-acetyl-2-*N*-acetamide-2-deoxy-D-glucopyranoside (6)



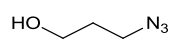
D-Glucosamine (0.22 kg, 1.0 mol) was co-evaporated with pyridine and dissolved in pyridine (650 mL, 1.5 M). Acetic anhydride (0.6 L, 6.0 mol) was added portion wise to the cooled solution (0 °C). The resulting solution was stirred at 4°C for 5 days. The reaction was quenched by adding ice water. The product was extracted with EtOAc (3 x 100 mL) and the organic layer was washed with 2M aq. HCl (4 x 400 mL), sat. aq. $NaHCO_3$ (150 mL), brine (200 mL), dried with $NaSO_4$ and concentrated *in vacuo*. Crystallization (EtOAc : PE) resulted in compound **6** as a white solid (0.31 kg, 0.80 mol, 80%). R_f = 0.4 (1 : 4 PE : EtOAc); $[\alpha]^D$ = 0.46 (c = 1, DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.17 (d, *J* = 3.6 Hz, 1H, H-1), 5.66 (d, 1H, *J* = 8.8 Hz, NHAc), 5.64 - 5.19 (m, 2H, H-4, H-3), 4.49 (td, 1H, *J* = 3.2 Hz, 8.8 Hz, H-2), 4.25 (dd, *J* = 4.0 Hz, 10.4 Hz, 2H, H-6), 4.00 – 3.96 (m, 1H, H-5), 2.20 (s, 3H, CH_3, Ac), 2.09 (s, 3H, CH_3, Ac), 2.06 (s, 3H, CH_3, Ac), 2.05 (s, 3H, CH_3, Ac), 1.94 (s, 3H, CH_3, NAc); ^{13}C NMR (100 Hz, $CDCl_3$) δ 171.6 (C=O), 170.6 (C=O), 169.9 (C=O), 169.0 (C=O), 168.6 (C=O), 90.6 (CH, C-1), 70.6 (CH, C-3), 69.7 (CH, C-5), 67.6 (CH, C-4), 61.5 (CH_2, C-6), 51.0 (CH, C-2), 23.0 (CH_3, NAc), 20.9 (CH_3, Ac), 20.6 (CH_3, Ac), 20.6 (CH_3, Ac), 20.5 (CH_3, Ac).

3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-1-*O*,2-*N*-methylidene-α-D-glucopyranoside (7)



Compound **6** (5.0 g, 13 mmol) was co-evaporated with dichloroethane and dissolved in dry dichloroethane (64 mL, 0.5 M) under argon atmosphere. TMSOTf was added portion wise (2.6 mL, 14 mmol) and the resulting solution was stirred at 65°C for 20 hours. A sample of the crude reaction mixture was concentrated dissolved in $CDCl_3$ to follow the completion of the reaction by NMR. After completion, the mixture was quenched with TEA, concentrated, and purified by flash chromatography (1 : 2 Toluene : EtOAc, 0.5% TEA). Compound **7** was obtained as a brown oil (4.40 g, 10.4 mmol, 81%). R_f = 0.3 (1 : 2 toluene : EtOAc, 0.5% TEA); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.97 (d, *J* = 7.6 Hz, 1H, H-1) 5.26 (t, *J* = 2.4 Hz, 1H, H-3), 4.94 (d, *J* = 9.2 Hz, 1H, H-4), 4.17 (d, *J* = 4.8 Hz, 2H, H-6), 4.14 (d, *J* = 6.0 Hz, 1H, H-2), 3.62 – 3.58 (m, 1H, H-5), 2.11 (s, 3H, CH_3, NAc), 2.09 (s, 3H, CH_3, Ac), 2.08 (s, 3H, CH_3, Ac), 2.06 (s, 3H, CH_3, Ac); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3 (C=O), 169.3 (C=O), 169.0 (C=O), 166.4 (C=N), 99.2 (CH, C-1), 70.2 (CH, C-3), 68.2 (CH, C-4), 67.7 (CH, C-5), 64.8 (CH, C-2), 63.1 (CH_2, C-6), 20.7 (CH_3, Ac), 20.6 (CH_3, Ac), 20.5 (CH_3, Ac), 13.7 (CH_3, NAc); HRMS Calcd. for $[C_{29}H_{45}N_7O_{13} + H]^+$ 700.31481, found 700.31490.

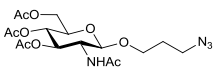
3-Azidopropanol (8)



3-bromo-1-azidopropanol (4.2 mL, 46 mmol) was added to a stirred solution of NaN_3 (12 g, 0.19 mol) in H_2O (20 mL) and acetone (95 mL) (2 : 8, 0.4 M). After stirring at rt for 7 days, the solution was diluted with H_2O and extracted with DCM (4 x 100 mL). The organic layer was dried ($NaSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography yielded compound **8** as a yellow oil (4.39 g, 43.5 mmol, 94%). R_f = 0.3 (8 : 2 PE : EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) : δ 3.69 (t, *J* = 6.0 Hz, 2H, CH_2, HOCH_2), 3.42 (t, *J*

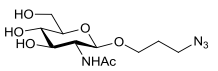
= 6.8 Hz, 2H, CH_2, CH_2N_3), 3.27 (s, 1H, OH), 1.82 (m, 2H, CH_2, $CH_2CH_2CH_2$); ^{13}C NMR (100 MHz) : δ 59.2 (CH_2, $HOCH_2$), 48.0 (CH_2, CH_2N_3), 31.2 (CH_2, $CH_2CH_2CH_2$).

3-Azidopropyl-2-*N*-acetamide-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (9)



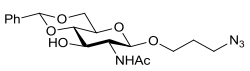
Compound **7** (4.4 g, 10 mmol) and compound **8** (4.6 g, 46 mmol) were dissolved in freshly distilled DCM (75 mL, 0.13 M) with molecular sieves under argon atmosphere. The resulting solution was stirred at rt for 40 minutes. TMSOTf (1.7 mL, 9.4 mmol) was added portion wise over 5 days to drive the reaction to completion. A sample of the crude reaction mixture was concentrated an dissolved in $CDCl_3$ to assure the completion of the reaction by NMR. The reaction was quenched with TEA, filtered over Celite ® and purified by flash chromatography (1 : 1 → 7 : 3 EtOAc : PE). Concentration yielded compound **9** as a white solid (3.72 g, 8.63 mmol, 83%). R_f = 0.4 (9 : 1 EtOAc : MeOH); $[\alpha]^D$ = -0.05 (c = 1, DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (t, *J* = 9.9 Hz, 1H, H-3), 5.06 (t, *J* = 9.6 Hz, 1H, H-4), 4.82 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.30 (dd, *J* = 12.2, 5.0 Hz, 1H, CH_2, H-6), 4.21 – 4.07 (m, 1H, CH_2, H-6), 4.01 – 3.88 (m, 2H, CH_2, $C_3H_6N_3$, CH, H-2), 3.87 – 3.83 (m, 1H, CH_2, $C_3H_6N_3$), 3.70 – 3.65 (m, , 1H, CH, H-5), 3.40 (t, *J* = 6.6 Hz, 2H, CH_2, $C_3H_6N_3$), 2.13 – 1.95 (m, 12H, CH_3, Ac, CH_3, NAc), 1.93 – 1.83 (m, *J* = 6.4 Hz, 2H, CH_2, $C_3H_6N_3$);^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4 (C=O), 170.3 (C=O), 170.1 (C=O), 169.0 (C=O), 100.4 (CH, C-1), 72.1 (CH, C-3), 71.1 (CH, C-5), 68.6 (CH, C-4), 65.9 (CH_2, $C_3H_6N_3$), 61.9 (CH_2, C-6), 53.9 (CH, C-2), 47.6 (CH_2, $C_3H_6N_3$), 28.5 (CH_2, $C_3H_6N_3$), 20.7 (CH_3, NAc), 20.3 (CH_3, Ac), 20.2 (CH_3, Ac), 20.2 (CH_3, Ac); IR (cm^{-1}) 3275, 2098, 1745, 1639, 1224; HRMS $[M+H^+]$ Calcd. for $C_{17}H_{26}N_4O_9$ 431.17705, found 431.17725.

3-Azidopropyl-2-*N*-acetamide-2-deoxy-β-D-glucopyranoside (10)



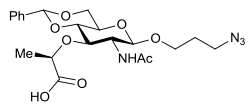
Compound **9** (31.6 g, 73.4 mmol) was dissolved in MeOH (750 mL, 0.1 M) and NaOMe (0.71 g, 13 mmol) was added. The resulting solution was stirred at rt for 20 h. The reaction mixture was quenched with Amberlite® H^+ resin. Filtration and concentration *in vacuo* yielded compound **10** as a white solid (22 g, 73 mmol, quant). R_f = 0.1 (9 : 1 EtOAc : MeOH); $[\alpha]^D$ = -0.07 (c = 1, MeOH);
^{1}H NMR (400 MHz, MeOD) δ 4.35 (d, *J* = 8.4 Hz, 1H, CH, H-1), 3.94 – 3.81 (m, 3H, CH_2 H-6, CH_2, $C_3H_6N_3$), 3.68 – 3.47 (m, 4H, CH, H-3, CH, H-4, CH, H-5, CH_2, $C_3H_6N_3$), 1.95 (s, 3H, CH_3, NAc), 1.78 – 1.75 (m, 2H, CH_2, $C_3H_6N_3$); ^{13}C NMR (100 MHz, MeOD) δ 173.7 (C=O), 102.8 (CH, C-1), 77.9 (CH, C-5), 76.0 (CH, C-3), 72.1 (CH, C-4), 67.1 (CH_2O, $C_3H_6N_3$), 62.8 (CH_2, C-6), 57.3 (CH, C-2), 49.0 (CH_2, CH_2N_3), 30.1 (CH_2, $C_3H_6N_3$), 23.0 (CH_3, NAc); IR (cm^{-1}): 3255, 2092, 1651, 1552; HRMS Calcd. for $[C_{11}H_{20}N_4O_6 + H]^+$ 305,14556, found 305,14575.

3-Azidopropyl-2-*N*-acetamide-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (11)



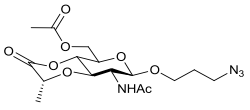
Compound **10** (2.7 g, 8.6 mmol) was co-evaporated in DMF and dissolved in dry MeCN : DMF (3 : 1, 90 mL, 0.1 M). Benzaldehyde dimethyl acetal (1.9 mL, 13 mmol) and CSA (0.40 g, 1.7 mmol) were added and the resulting solution was stirred at rt for 18 h. The reaction was quenched with TEA, concentrated *in vacuo* and re-crystallized (DCM, MeOH, PE). Compound **11** was obtained as a white solid (2.90 g, 7.39 mmol, 86%); R_f = 0.6 (1 : 9 MeOH : DCM); $[\alpha]^D$ = -0.27 (c = 1, 1 : 1 DCM : MeOH); ^{1}H NMR (400 MHz, DMSO-D_6) 7.49 – 7.35 (m, 5H, CH, Ar), 5.60 (s, 1H, CH, benzylidene acetal), 5.33 (d, *J* = 4.9 Hz, 1H, NH), 4.46 (d, *J* = 8.4 Hz, 1H, H-1), 4.20 (dd, *J* = 10.1, 4.8 Hz, 1H, H-6), 3.81 – 3.68 (m, 2H, CH_2, H-6, CH_2, $C_3H_6N_3$), 3.66 – 3.28 (m, 6H, CH, H-3, CH, H-4, CH, H-5, CH_2, $C_3H_6N_3$), 1.83 (s, 3H, CH_3, NAc), 1.77 – 1.67 (m, 2H, CH_2, $C_3H_6N_3$); ^{13}C NMR (100 MHz, DMSO-D_6) δ 169.4 (C=O), 137.8 (C_q, CHPh), 128.1 (CH, CHPh), 126. 5 (CHPh), 101.7 (CH, CHPh), 100.8 (CH, C-1), 81.3 (CH, C-3), 70.5 (CH, C-5), 67.9 (CH_2, C-6), 66.0 (CH, C-4), 65.7 (CH_2, $C_3H_6N_3$), 56.2 (CH, C-2), 47.5 (CH_2, $C_3H_6N_3$), 28.6 (CH_2, $C_3H_6N_3$), 23.1 (CH_3, NAc); IR (cm^{-1}): 3275, 2870, 2100, 1624, 1552; HRMS Calcd. for $[C_{18}H_{24}N_4O_6 + H]^+$ 393.17686 found 393.17673.

3-Azidopropyl-2-*N*-acetamide-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl)-β-D-glucopyranoside (12)



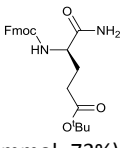
Compound **11** (0.8 g, 2.0 mmol) was suspended in 1,4-dioxane (30 mL, 0.07 M) and dissolved under heating to 95°C. NaH (0.32 g, 60% in oil, 8.0 mmol) was added and the resulting solution was stirred at 95°C for 1 h. The solution was cooled to 65°C and a stock solution of (S)-2-chloro-propanoic acid (0.26 mL, 3.0 mmol, 5 mL 1,4-dioxane) was added. The solution was stirred at 65°C for 1.5 h. The reaction was quenched with MeOH and pH was adjusted to pH ~ 3 with 1M HCl. The product was extracted with DCM, dried ($MgSO_4$) and concentrated *in vacuo*. Crystallization ($CHCl_3$: EtOAc : PE) yielded compound **12** as an off-white solid (0.64 g, 1.4 mmol, 69%). R_f = 0.43 (1 : 9 MeOH : EtOAc); $[\alpha]^D$ = -0.21 (c = 1, MeOH); ^{1}H NMR (400 MHz, 1 : 1 MeOD : $CDCl_3$) δ 7.51 – 7.45 (m, 2H, CH, Ar), 7.41 – 7.45 (m, 3H, CH, Ar), 5.59 (s, 1H, CH, benzylidene acetal), 4.57 (d, *J* = 8.4 Hz, 1H, CH, H-1), 4.45 (dd, *J* = 7.2 Hz, 14.0 Hz, 1H, CH, lactic acid), 4.34 (dd, *J* = 4.8 Hz, 10.4 Hz, 1H, CH_2, H-6), 3.97 -3.91 (m, 1H, CH_2, $C_3H_6N_3$), 3.86 – 3.78 (m, 2H, H3, H6), 3.72 – 3.68 (m, H2, H4), 3.61 – 3.56 (m, 1H, CH_2, $C_3H_6N_3$), 3.48 – 3.41 (m, 1H, CH, H-5), 3.40 (t, *J* = 6.4 Hz, 2H, CH_2, $C_3H_6N_3$), 2.03 (s, 3H, CH_3, NAc), 1.89 – 1.78 (m, 2H, CH_2, $C_3H_6N_3$), 1.40 (d, *J* = 6.8 Hz, 3H, CH_3, lactic acid); ^{13}C NMR (100 MHz) δ 175.8 (C=O), 172.4 (C=O), 136.9 (C_q, Ar), 128.8 (CH, Ar), 128.0 (CH, Ar), 125.6 (CH, Ar), 102.0 (CH, benzylidene acetal), 101.0 (CH, C-1), 82.1 (CH, C-4), 77.1 (CH, C-3), 75.4 (CH, Lactic acid), 68.4 (CH_2, C-6), 66.2 (CH_2, $C_3H_6N_3$), 65.9 (CH, C-5), 55.4 (CH, C-2), 47.7 (CH_2, $C_3H_6N_3$), 28.7 (CH_2, $C_3H_6N_3$), 22.6 (CH_3 NAc), 18.5 (CH_3, lactic acid); IR (cm^{-1}): 3275, 2098, 1709, 1656, 1556; HRMS Calcd. for $[C_{21}H_{28}N_4O_8 + H]^+$ 465.19774, found 465.19799.

3-Azidopropyl-2-*N*-acetamide-6-*O*-acetyl-2-deoxy-3,4-(1-metyl-2-carbolactone)-β-D-glucopyranoside (36)[20]



Compound **12** (50 mg, 0.11 mmol) was stirred in DCM (1.9 mL, 6.0 mM) with TFA (0.1 mL, 5%) for 3 h at ambient temperatures. The reaction mixture was quenched with TEA to pH ~ 10, concentrated *in vacuo* and co-evaporated with toluene. The crude reaction mixture was dissolved in a one to one mixture of DCM and pyridine (2 mL, 6.0 mM). Ac_2O (0.1 mL) was added and the resulting solution was stirred for 16 h. The solution was diluted with DCM, washed with sat. $CuSO_4$, H_2O and brine, dried ($NaSO_4$) and concentrated. Purification by flash column chromatography (9 : 1 EtOAc : MeOH) yielded compound **36** (40 mg, 0.1 mmol, 93%). R_f = 0.5 (9 : 1 EtOAc : MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.86 (d, *J* = 7.6 Hz, 1H, NH), 4.97 (d, *J* = 8.2 Hz, 1H, CH, H-1), 4.69 (q, *J* = 7.0 Hz, 1H, CH, lactone), 4.48 (dd, *J* = 12.3, 2.1 Hz, 1H, CH_2, H-6), 4.40 – 4.31 (m, 1H, CH, H-3), 4.31 – 4.19 (m, 2H, CH, H-4, CH_2, H-6), 3.97 – 3.92 (m, 1H, CH_2, $C_3H_6N_3$), 3.78 – 3.74 (m, 1H, CH, H-5), 3.65 – 3.59 (m, 1H, CH_2, $C_3H_6N_3$), 3.44 – 3.32 (m, 3H, CH_2, $C_3H_6N_3$, CH, C-2), 2.11 (s, 3H, CH_3, Ac), 2.03 (s, 3H, CH_3, NAc), 1.96 – 1.76 (m, 2H, CH_2, $C_3H_6N_3$), 1.51 (d, *J* = 7.0 Hz, 3H, CH_3, Me); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.8 (C=O), 170.5 (C=O), 169.2 (C=O), 99.9 (CH, C-1), 75.7 (CH, C-4), 71.3 (CH, α H lactone), 70.9 (CH, C-5), 69.4 (CH, C-3), 66.7 (CH_2, $C_3H_6N_3$), 61.5 (CH_2, C-6), 55.6 (CH, C-2), 48.0 (CH_2, $C_3H_6N_3$), 28.9 (CH_2, $C_3H_6N_3$), 23.5 (CH_3, NAc), 20.8 (CH_3, Ac), 17.8 (CH_3, Me); LC/MS: Rt = 5.41 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{16}H_{24}N_4O_8 + H]^+$ 401.15877, found 401.16653.

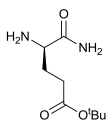
Fmoc-*i*-D-Gln(O^{t}Bu)-NH$_2$ (14)



To a stirred solution of Fmoc-D-Glu(O^{t}Bu)-OH (0.9 g, 2.0 mmol) in 1,4-dioxane (20 mL, 0.1M) was added NH_4HCO_3 (0.71 g, 9.0 mmol), Boc_2O (0.58 g, 2.7 mmol) and pyridine (0.25 mL, 3.1 mmol). After 24 h the solution was diluted with EtOAc : H_2O and washed with water. The organic layer was dried ($NaSO_4$) and concentrated *in vacuo*. Crystallization (MeOH) yielded compound **14** as white crystals (0.62 g, 1.5 mmol, 73%). R_f = 0.5 (7 : 3 EtOAc : PE); $[\alpha]^D$ = -0,6 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-D_6) δ

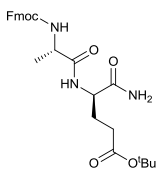
7.89 (d, *J* = 7.5 Hz, 2H, CH, Fmoc), 7.75 – 7.72 (m, 2H, CH, Fmoc), 7.43 – 7.28 (m, 4H, CH, Fmoc), 7.06 (s, 1H, NH_2), 4.34 – 4.17 (m, 3H, CH, Fmoc, CH_2, Fmoc), 3.97 – 3.94 (m, 1H, CH, α *i*-D-Gln), 2.22 (t, *J* = 7.8 Hz, 2H, CH_2, γ *i*-D-Gln), 1.94 – 1.84 (m, 1H, CH, β *i*-D-Gln), 1.78 – 1.67 (m, *J* = 13.7, CH, β *i*-D-Gln), 1.39 (s, 9H, CH_3, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-D_6) δ 173.4 (C=O), 171.7 (C=O), 156.0 (C=O), 143.8 (C_q, Fmoc), 140.7 (C, Fmoc), 127.7 (CH, Fmoc), 127.1 (CH, Fmoc), 125.4 (CH, Fmoc), 120.1 (CH, Fmoc), 79.7 (C_q, ^{t}Bu), 65.64 (CH_2, Fmoc), 53.7 (CH, α *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); IR (cm^{-1}): 33387, 3329, 1720, 1689, 1532; LC/MS: Rt = 9.24 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{24}H_{28}N_2O_5 + H]^+$ 425.20710, found 425.20706.

NH_2-*i*-D-Gln(O^{t}Bu)-NH_2 (15)



To compound **14** (15 g, 35 mmol) dissolved in THF (0.4 L, 0.1 M) was added EtSH (25 mL, 350 mmol) and a catalytic amount of DBU (0.16 mL, 1.1 mmol). The resulting solution was concentrated *in vacuo* after 3 h. Purification by flash chromatography resulted in compound **15** (4.4 g, 22 mmol, 62%). R_f = 0.05 (8 : 2 EtOAc : PE); $[\alpha]^D$ = -3.6 (c = 1, MeOH); ^{1}H NMR (400 MHz, DMSO-D_6) δ 7.29 (s, 1H, NH_2), 6.94 (s, 1H, NH_2), 3.06 (dd, *J* = 7.9, 5.2 Hz, 1H, CH, α *i*-D-Gln), 2.26 – 2.20 (m, 2H, CH_2, γ *i*-D-Gln), 1.81 – 1.70 (m, 1H, CH_2, β *i*-D-Gln), 1.61 – 1.47 (m, 1H, CH_2, β *i*-D-Gln), 1.39 (s, 9H, CH_3, ^{t}Bu); ^{13}C NMR (100 MHz, DMSO-D_6) δ 177.4 (C=O), 174.5 (C=O), 65.0 (C_q), 55.6 (CH, α *i*-D-Gln), 29.4 (CH_2, γ *i*-D-Gln), 27.8 (CH_3, ^{t}Bu), 25.4 (CH_2, β *i*-D-Gln); IR (cm^{-1}): 3402, 1716, 1670, 1369; LC/MS: Rt = 5.17 min (C_{18} Alltima, 0 – 50% MeCN, 15 min run); HRMS Calcd. for $[C_9H_{18}N_2O_3 + H]^+$ 203.13902, found 203.13896.

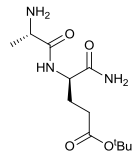
Fmoc-L-Ala-*i*-D-Gln(O^{t}Bu)-NH_2 (16)



Method A: To a stirred solution of compound **14** (2.0 g, 4.8 mmol) in DCM (40 mL, 0.73 M) was added DBU (0.71 mL, 4.8 mmol). After 20 min HOBt (2.9 g, 21 mmol) was added. Subsequently added were Fmoc-Ala-OH (1.8 g, 5.7 mmol), EDC (1.1 g, 5.7 mmol) and DiPEA (4.7 mL, 28 mmol). The resulting solution was stirred for 18 h, washed with 1 M HCl (3 x 5 mL), sat. $NaHCO_3$ (3 x 5 mL), brine (3 x 5 mL), dried ($NaSO_4$) and concentrated *in vacuo*. Re-crystallization (EtOAc : PE) resulted in compound **16** as a white solid (1.93 g, 3.89 mmol, 82%).

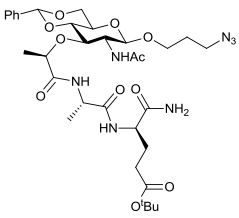
Method B: To a stirred solution of Fmoc-Ala-OH (12 g, 38 mmol), EDC (13 g, 70 mmol), HOBt (12 g, 88 mmol) and DiPEA (23 mL, 0.14 M) in DCM (0.4 mL, 0.1 M) was added compound **15** (7.2 g, 35 mmol). The resulting solution was stirred for 18 h. The solution was washed with HCl (1M), dried ($MgSO_4$) and concentrated *in vacuo*. Crystallization (EtOAc : PE) yielded compound **16** as a white solid (13 g, 26 mmol, 68%). R_f = 0.8 (8 : 2 EtOAc : PE); $[\alpha]^D$ = 0.8 (c = 1, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-D_6) δ 8.04 (d, *J* = 8.2 Hz, 1H, NH), 7.90 (d, *J* = 7.5 Hz, 2H, CH, Fmoc), 7.73 (t, *J* = 6.6 Hz, 2H, CH, Fmoc), 7.61 (d, *J* = 7.0 Hz, 1H, NH), 7.47 – 7.32 (m, 4H, CH, Fmoc), 7.26 (s, 1H, NH_2), 7.14 (s, 1H, NH_2), 4.34 – 4.12 (m, 4H, CH, α *i*-D-Gln, CH_2, Fmoc, CH, Fmoc), 4.08 – 4.04 (m, *J* = 16.4, 14.2, 7.1 Hz, 1H, α CH Ala), 2.18 (t, *J* = 7.8 Hz, 2H, γ CH_2, *i*-D-Gln), 1.99 – 1.94 (m, *J* = 14.4, 7.7 Hz, CH_2, β *i*-D-Gln), 1.72 – 1.69 (m, 1H CH_2, β *i*-D-Gln), 1.36 (s, 9H, CH_3, ^{t}Bu), 1.26 – 1.14 (m, 3H, CH_3, Ala); ^{13}C NMR (100 MHz, DMSO-D_6) δ 173.1 (C=O), 172.6 (C=O), 171.6 (C=O), 155.9 (C=O), 143.9 (C_q, Fmoc), 140.7 (C_q, Fmoc), 127.7 (CH, Fmoc), 127.1 (CH, Fmoc), 125.3 (CH, Fmoc), 120.1 (CH, Fmoc), 79.7 (C_q, ^{t}Bu), 65.7 (CH_2, Fmoc), 51.4 (CH, α *i*-D-Gln), 50.3 (CH, α 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, Ala); IR (cm^{-1}): 3286, 1641, 1541, 1257; LC/MS: Rt = 6.09 min (C_{18} Alltima, 0 – 50% MeCN, 15 min run); HRMS Calcd. for $[C_{27}H_{33}N_3O_6 + H]^+$ 496.24421, found 496.24396.

NH_2-L-Ala-*i*-D-Gln(O^tBu)-NH_2 (17)



To a stirred solution of compound **16** (10 g, 20 mmol) and octanethiol (30 mL, 0.17 mol) in DCM (0.2 L, 0.1 M) was added a catalytic amount of DBU. The solution was concentrated after 18 h and purified by flash chromatography (PE : EtOAc → EtOAc : MeOH) yielding compound **17** (3.6 g, 13 mmol, 62%); R_f: 0.1 (8 : 2 EtOAc : PE); $[\alpha]^D$ = 4.0 (c = 0.45, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (s, 2H, NH_2), 7.51 (s, 1H, NH), 6.78 (s, 2H, NH_2), 4.36 – 4.34 (m, 1H, CH, α *i*-D-Gln), 3.71 – 3.66 (m, 1H, CH, Ala), 2.35 3.32 (m, 2H, CH_2, γ *i*-D-Gln), 2.15 – 2.07 (m, 1H, CH_2, β *i*-D-Gln), 1.97 – 1.89 (m, 1H, CH_2, β *i*-D-Gln), 1.43 (s, 9H, CH_3, ^{t}Bu), 1.35 (d, *J* = 6.8 Hz, 3H, CH_3, Ala); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.1 (C=O), 174.7 (C=O), 172.4 (C=O), 80.8 (C_q, ^{t}Bu), 52.5 (CH, Ala), 49.7 (CH, α *i*-D-Gln), 31.5 (CH_2, γ *i*-D-Gln), 27.8 (CH_3 ^{t}Bu), 27.0 (CH_2, β *i*-D-Gln), 20.2 (CH_3, Ala); IR (cm^{-1}): 1728, 1666, 1543, 1257; LC/MS: Rt = 4.32 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{12}H_{23}N_3O_4 + H]^+$ 274.17613, found 274.17615.

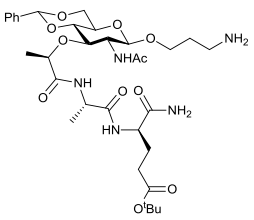
3-Azidopropyl-2-*N*-acetamide-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (18)



Method A: To a stirred solution of compound **16** (50 mg, 0.1 mmol) in DMF (2 mL, 0.08 M) was added DBU (14 µL, 90 µmol). After 10 min HOBt (56 mg, 0.42 mmol) was added. A mixture of compound **12** (35 mg, 77 µmol) in DMF (0.5 mL, 0.15 M), HATU (29 mg, 7.7 µmol) and DiPEA (80 µL, 0.46 mmol) was added to the solution. The resulting solution was stirred for 18 h at rt. The solution was diluted 10 times (DCM), washed with 1M HCl (3 x 5 mL), sat. $NaHCO_3$ (3 x 5 mL), brine (3 x 5 mL), dried ($NaSO_4$) and concentrated. The crude compound was purified by flash chromatography (98:2 → 9:1 DCM : MeOH) to obtain title compound **18** as a white solid (50 mg, 70 µmol, 70%).

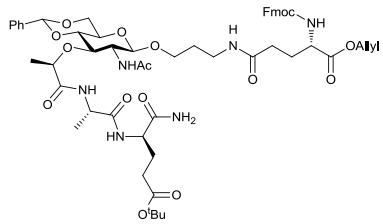
Method B: In DMF (180 mL, 0.1 M) was dissolved compound **17** (5.0 g, 18 mmol), compound **12** (7.0 g, 15 mmol), HATU (8.9 g, 23 mmol) and DiPEA (9.3 mL, 56 mmol). The resulting solution was stirred for 18 h and concentrated *in vacuo*. The crude compound was purified by re-crystallization (DCM : PE) to result compound **18** (10.7 g, 14.6 mmol, 80%). R_f = 0.6 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = -11.4 (c = 0.44, 1 : 1 MeOH : $CHCl_3$); ^{1}H NMR (400 MHz, MeOD) δ 7.54 (d, *J* = 4.9 Hz, 1H, NHAc), 7.47 (m, 2H, CH, Ar), 7.42 – 7.31 (m, 3H, CH, Ar), 5.58 (s, 1H, CH, benzylidene acetal), 4.59 (d, *J* = 7.8 Hz, 1H, CH, H-1), 4.41 – 4.31 (m, 2H, CH, α *i*-D-Gln, CH_2, H-6), 4.31 – 4.21 (m, 1H, CH, α Ala), 4.14 (q, *J* = 6.7 Hz, 1H, CH, lactic acid), 3.99 – 3.89 (m, 1H, CH_2, $C_3H_6N_3$), 3.89 – 3.74 (m, 3H, CH, H-2, CH, H-3, CH_2, $C_3H_6N_3$), 3.71 – 3.55 (m, 2H, CH, H-4, CH_2, H-6), 3.52 – 3.42 (m, 1H, CH, H-5), 3.39 (t, *J* = 6.6 Hz, 2H, CH_2, $C_3H_6N_3$), 2.35 – 2.31 (m, 2H, CH_2, γ *i*-D-Gln), 2.25 – 2.11 (m, 1H, CH_2, β *i*-D-Gln), 1.99 (s, 3H, CH_3, NAc), 1.95 – 1.75 (m, 3H, CH_2, $C_3H_6N_3$, CH_2, β *i*-D-Gln), 1.44 (s, 9H, CH_3, ^{t}Bu), 1.40 (d, *J* = 7.1 Hz, 3H, CH_3, lactic acid), 1.36 (d, *J* = 6.7 Hz, 3H, CH_3, Ala); ^{13}C NMR (100 MHz, MeOD) δ 175.0 (C=O), 174.8 (C=O), 173.7 (C=O), 173.2 (C=O), 172.7 (C=O), 137.6 (C_q, Ar), 129.5 (CH, Ar), 128.7 (CH, Ar), 126.4 (CH, Ar), 102.1 (CH, benzylidene acetal), 101.9 (CH, C-1), 81.6 (C_q, ^{t}Bu), 81.5 (CH, C-4), 79.7 (CH, C-3), 78.3 (CH, lactic acid), 69.0 (CH_2, C-6), 66.8 (CH_2, $C_3H_6N_3$), 66.5 (CH, C-5), 56.3 (CH, C-2), 52.7 (CH, α *i*-D-Gln), 49.9 (CH, α Ala), 48.4 (CH_2, $C_3H_6N_3$), 32.2 (CH_2, γ *i*-D-Gln), 29.4 (CH_2, $C_3H_6N_3$), 28.2 (CH_3, ^{t}Bu), 27.5 (CH_2, β *i*-D-Gln), 23.2 (CH_3, NAc), 19.6 (CH_3, lactic acid), 17.7 (CH_3, Ala); IR (cm^{-1}): 3286, 2094, 1647, 1535; LC/MS: Rt = 7.62 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{33}H_{49}N_7O_{11} + H]^+$ 720.35628, found 720.35639.

3-Aminopropyl-2-*N*-acetamide-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (19)



Compound **18** (2.7 g, 3.8 mmol) was co-evaporated with DMF and dissolved in DMF (25 mL), diluted with THF (12 mL, 0.1 M) and the reaction mixture was stirred for 3 h with PMe_3 (7.5 mL, 1M in THF). The solution was concentrated *in vacuo* yielding compound **19** (2.1 g, 3.0 mmol, 80%). R_f = 0.2 (8 : 2 $CHCl_3$: MeOH +2% AcOH); $[\alpha]^D$ = -19 (c = 0.19, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, DMSO-D_6) δ 8.31 (s, 1H, NH), 8.12 (d, *J* = 8.2 Hz, 1H, NH), 8.02 (d, *J* = 9.1 Hz, 1H, NH), 7.94 (s, 1H, NH_2), 7.73 (t, *J* = 3.7 Hz, 1H, NH), 7.49 – 7.30 (m, 5H, CH, Ar), 7.11 (s, 1H, NH_2), 5.75 (s, 1H, CH, benzylidene acetal), 4.47 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.27 – 4.19 (m, 2H, CH, α *i*-D-Gln, CH_2, H-6), 4.18 – 4.04 (m, 2H, CH, lactic acid, CH, Ala), 3.82 – 3.72 (m, 3H, CH, H-2, CH_2, H-6, CH_2, $C_3H_6N_3$), 3.69 – 3.38 (m, 4H, CH, H-3, CH, H-4, CH, H-5, CH_2, $C_3H_6N_3$), 2.63 (t, *J* = 6.9 Hz, 2H, CH_2, $C_3H_6N_3$), 2.17 (t, *J* = 7.9 Hz, 2H, CH_2, γ *i*-D-Gln), 1.99 – 1.87 (m, 1H, CH_2, β *i*-D-Gln), 1.82 (s, 3H, NAc), 1.74 – 1.55 (m, 1H, CH_2, β *i*-D-Gln), 1.36 (s, 9H, CH_3, ^{t}Bu), 1.26 – 1.18 (m, 6H, CH_3, lactic acid, CH_3, Ala); ^{13}C NMR (100 MHz, DMSO-D_6) δ 173.17 (C=O), 172.14 (C=O), 171.86 (C=O), 171.67 (C=O), 137.63 (C_q, Ar), 128.73 (CH, Ar), 128.21 (CH, Ar), 127.90 (CH, Ar), 125.92 (CH, Ar), 101.54 (CH, C-1), 100.20 (CH, benzylidene acetal), 80.37 (CH, C-3), 79.77 (C_q, ^{t}Bu), 79.09 (CH, C-4), 77.38 (CH, lactic acid), 67.87 (CH_2, C-6), 66.91 (CH_2, $C_3H_6N_3$), 65.69 (CH, C-5), 54.73 (CH, C-2), 51.54 (CH, α *i*-D-Gln), 48.35 (CH, Ala), 37.84 (CH_2, $C_3H_6N_3$), 31.29 (CH_2, $C_3H_6N_3$), 31.07 (CH_2, β *i*-D-Gln), 27.78 (CH_3, ^{t}Bu), 27.22 (CH_2, γ *i*-D-Gln), 23.09 (CH_3, NAc), 19.08 (CH_3, lactic acid), 18.27 (CH_3, Ala); IR (cm^{-1}): 3267, 1724, 1639, 1539, 1369; LC/MS: Rt = 5.66 min (Alltima C_{18}, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{33}H_{51}N_5O_{11} + H]^+$ 694.36578, found 694.36615.

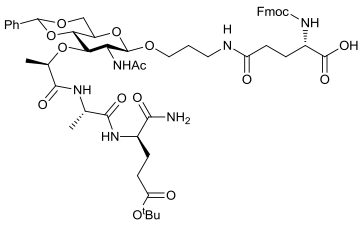
***N*-(9-fluorenylmethoxycarbonyl)-L-glutaminyl-((3-Aminopropyl)-2-*N*-acetamide-4,6-*O*-benzylidene-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside) allyl ester (20)**



To compound **19** (2.1 g, 2.9 mmol) dissolved in DMF (39 mL, 0.07 M) were added Fmoc-Glu-OAllyl (1.3 g, 3.2 mmol), HCTU (1.3 g, 3.2 mmol) and DiPEA (1.4 mL, 8.7 mmol). The solution was stirred for 18 h and concentrated *in vacuo*. Crystallization and re-crystalisation ($CHCl_3$: MeOH : Et_2O) yielded the compound **20** (1.8 g, 1.7 mmol, 57%). R_f = 0.8 (8 : 2 $CHCl_3$: MeOH); $[\alpha]^D$ = -23 (c=0.28, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, DMSO-D_6) δ 8.09 (d, *J* = 8.3 Hz, 1H, NH), 7.95 – 7.75 (m, 5H, CH, Ar, NH), 7.71 (d, *J* = 7.4 Hz, 2H, CH, Ar), 7.48 – 7.26 (m, 2H, CH, Fmoc), 7.09 (s, 1H, NH_2, *i*-D-Gln) 5.96 – 5.80 (m, 1H, CH, allyl), 5.68 (s, 1H, CH, benzylidene acetal), 5.35 – 5.16 (m, 2H, CH_2, allyl), 4.57 (d, *J* = 5.2 Hz, 2H, CH_2 allyl), 4.45 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.36 – 4.18 (m, 5H, CH_2, H-6, CH, lactic acid, CH_2, Fmoc, CH, Fmoc), 4.18 – 4.09 (m, 1H, CH, α Glu), 4.10 – 4.00 (m, 2H, CH, Ala, CH, α *i*-D-Gln), 3.81 – 3.71 (m, *J* = 18.4, 9.0 Hz, 3H, CH_2, $C_3H_6N_3$, CH_2, H-6, CH, H-2), 3.69 – 3.57 (m, 2H, CH, H-3, CH, H-4), 3.49 – 3.28 (under H_2O signal, CH_2, $C_3H_6N_3$, CH, H-5), 3.19 – 2.94 (m, 2H, CH_2, $C_3H_6N_3$), 2.24 – 2.10 (m, 4H, CH_2, γ *i*-D-Glu, CH_2, γ Glu), 2.05 – 1.86 (m, 2H, CH_2, β *i*-D-Glu, CH_2, β Glu), 1.91 – 1.73 (m, 4H, CH_3, NAc, CH_2, β *i*-D-Gln), 1.73 – 1.63 (m, 1H, CH_2, β Glu), 1.58 (t, *J* = 6.4 Hz, 2H, CH_2, $C_3H_6N_3$), 1.36 (s, 9H, CH_3, ^{t}Bu), 1.24 – 1.19 (m, *J* = 16.7, 6.8 Hz, 6H, CH_3, lactic acid, CH_3, Ala); ^{13}C NMR (100 MHz, DMSO-D_6) δ 173.1 (C=O), 172.1 (C=O), 171.9 (C=O), 171.8 (C=O), 171.6 (C=O), 171.1 (C=O), 169.9 (C=O), 156.2 (C=O), 143.8 (C_q), 140.8 (C_q), 137.6 (C_q), 132.4 (CH, allyl), 128.8 (CH, Ar), 128.2 (CH, Ar), 127.7 (CH, Ar), 127.2 (CH, Ar), 125.9 (CH, Ar), 125.3 (CH, Ar), 120.2 (CH, Ar), 117.8 (CH_2, allyl), 101.6 (CH, C-1), 100.2 (CH, benzylidene acetal), 80.4 (CH, C-3), 79.7 (C_q, ^{t}Bu), 79.1 (CH, C-4), 77.4 (CH, lactic acid), 67.8 (CH_2, H-6), 66.9 (CH_2, $C_3H_6N_3$), 65.8 (CH_2, Fmoc), 65.7 (CH, C-5), 64.9 (CH_2, allyl), 54.7 (CH, C-2), 53.6 (CH, α *i*-D-Gln), 51.5 (CH, α Glu), 48.7 (CH, Fmoc), 46.7 (CH, Ala), 35.6 (CH_2, $C_3H_6N_3$), 31.6 (CH_2,

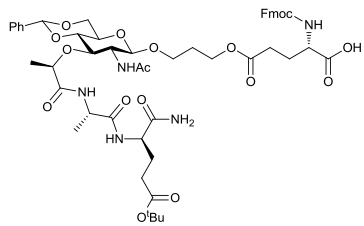
γ *i*-D-Gln), 31.3 (CH_2, γ Glu), 29.3 (CH_2, $C_3H_6N_3$), 27.8 (CH_3, ^tBu), 27.2 (CH_2, β, Glu), 26.7 (CH_2, β *i*-D-Gln), 23.1 (CH_3, NAc), 19.1 (CH_3, lactic acid), 18.2 (CH_3, Ala); IR (cm^{-1}): 3278, 1728, 1639, 1539, 1369; LC/MS: Rt = 9.20 min (Alltima C_{18}, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{56}H_{72}N_6O_{16} + H]^+$ 1085.50776, found 1085.50731.

_N_-(9-fluorenylmethoxycarbonyl)-L-glutaminyl-((3-Aminopropyl)-2-_N_-acetamide-4,6-_O_-benzylidene-2-deoxy-3-_O_-((R)-1-carboxyethyl-L-alanyl-acetamide-5-_O_-_tert_-butoxy-D-isoglutaminyl)-β-D-glucopyranoside) (21)



To compound **20** (0.2 g, 0.2 mmol) dissolved in DMF (4 mL, 0.05 M) was added AcOH (50 µL, 0.85 mmol), Bu_3SnH (0.1 mL, 0.4 mmol) and $Pd(PPH_3)_4$ (8 mg, 7 µmol). The resulting solution was stirred for 1.5 h. The compound **21** was precipitated by adding Et_2O. Re-crystalisation ($CHCl_3$: MeOH : Et_2O) yielded compound **21** (0.14 g, 0.13 mmol, 72%). R_f = 0.3 (8 : 2 $CHCl_3$: MeOH); $[\alpha]^D$ = -20.0 (c = 0.75, $CHCl_3$: MeOH); ^1H NMR (600 MHz, DMSO-D_6) δ 8.07 (d, *J* = 8.2 Hz, 1H, NH), 7.95 – 7.85 (m, 3H, CH, CHPh, NH), 7.78 (s, 1H, NH), 7.72 (d, *J* = 7.3 Hz, 2H, NH), 7.66 – 7.58 (m, 3H, CH, Ar), 7.58 – 7.52 (m, 1H, NH), 7.46 – 7.24 (m, 14H, CH, Ar), 7.07 (s, 1H, NH), 5.68 (s, 1H, CH, benzylidene acetal), 4.46 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.32 – 4.17 (m, 5H, CH_2, H-6, CH_2, $C_3H_6N_3$, CH_2, Fmoc, CH, Ala, CH, α Glu), 4.15 – 4.11 (m, 1H, CH, α *i*-D-Gln), 4.06 (q, *J* = 6.5 Hz, 1H, CH, lactic acid), 3.94 – 3.92 (m, 1H, CH, Fmoc), 3.82 – 3.69 (m, 3H, CH, H-2, CH_2, H-6, CH_2, $C_3H_6N_3$), 3.66 – 3.60 (m, 2H, CH, H-3, CH, H-4), 3.55 – 3.33 (m, 2H, CH, H-5, CH_2, $C_3H_6N_3$), 3.10 – 2.99 (m, 2H, CH_2, $C_3H_6N_3$), 2.21 – 2.15 (m, 4H, CH_2, γ *i*-D-Gln, CH_2, γ Glu), 2.03 – 1.86 (m, 2H, CH_2 β *i*-D-Gln, CH_2 β Glu), 1.92 – 1.72 (m, 4H, CH_3, NAc, CH_2, β Glu), 1.68 – 1.57 (m, 1H, CH_2, β *i*-D-Gln), 1.56 – 1.51 (m, 2H, CH_2 $C_3H_6N_3$), 1.36 (s, 9H, CH_3, ^tBu), 1.24 (d, *J* = 6.9 Hz, 3H, CH_3, lactic acid), 1.20 (d, *J* = 6.6 Hz, 3H, CH_3, Ala); ^{13}C NMR (151 MHz, DMSO-D_6) δ 173.1 (C=O), 172.1 (C=O), 171.8 (C=O), 171.6 (C=O), 171.4 (C=O), 169.9 (C=O), 156.1 (C=O), 143.8 (C_q, Fmoc), 140.7 (C_q, Fmoc), 137.6 (C_q, benzylidene acetal), 132.1 (CH, Ar), 131.5 (CH, Ar), 131.5 (CH, Ar), 128.8 (CH, Ar), 128.8 (CH, Ar), 128.8 (CH, Ar), 128.1 (CH, Ar), 127.7 (CH, Ar), 127.1 (CH, Ar), 125.9 (CH, Ar), 125.3 (CH, Ar), 120.1 (CH, Ar), 101.5 (CH, C-1), 100.1 (CH, benzylidene acetal), 80.3 (CH, C-4), 79.7 (C_q, ^tBu), 79.1 (CH, C-3), 77.3 (CH, lactic acid), 67.8 (CH_2, C-6), 66.9 (CH_2, $C_3H_6N_3$), 65.7 (CH_2, Fmoc), 65.6 (CH, C-5), 54.7 (CH, C-2), 53.6 (CH, Fmoc), 51.5 (CH, α *i*-D-Gln), 48.3 (CH, Ala), 46.7 (CH, α Glu), 35.5 (CH_2, $C_3H_6N_3$), 31.8 (CH_2, γ Glu), 31.2 (CH_2, γ *i*-D-Gln), 29.3 (CH_2, $C_3H_6N_3$), 27.7 (CH_3 ^tBu), 27.2 (CH_2, β Glu, CH_2, β *i*-D-Gln), 23.0 (CH_3, NAc), 19.0 (CH_3, lactic acid), 18.2 (CH_3, Ala); IR (cm^{-1}): 3282, 1720, 1639, 1539, 1369; LC/MS: Rt = 8.85 min (Alltima C_{18}, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{53}H_{68}N_6O_{16} + H]^+$ 1045.47646, found 1045.47762.

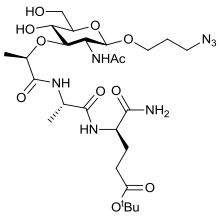
_N_-(9-fluorenylmethoxycarbonyl)-L-glutaminyl-((3-hydroxypropyl)-2-_N_-acetamide-4,6-_O_-benzylidene-2-deoxy-3-_O_-((R)-1-carboxyethyl-L-alanyl-acetamide-5-_O_-_tert_-butoxy-D-isoglutaminyl)-β-D-glucopyranoside) (37)



Compound **37** was isolated as side product in the reaction performed in MeOH : $CHCl_3$: THF (1 : 0.2 : 1). Purification by flash column chromatography yielded title compound (8 mg, 20% of reaction). R_f = 0.15 (8 : 2 $CHCl_3$: MeOH); ^1H NMR (400 MHz, DMSO-D_6) δ 8.09 (d, *J* = 8.3 Hz, 1H, NAc), 7.89 (d, *J* = 7.5 Hz, 2H, Fmoc), 7.71 (d, *J* = 6.3 Hz, 2H, Fmoc), 7.48 – 7.26 (m, 16H, Fmoc, CH, Ar), 7.07 (s, 1H, NH, amide *i*-D-Gln), 5.68 (s, 1H, CH, benzylidene acetal), 4.51 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.30 – 4.22 (m, 4H, CH_2, C-6, CH_2, $C_3H_6N_3$, CH, α *i*-D-Gln, CH, α Glu), 4.21 – 4.12 (m, 1H, CH, α lactic acid), 4.07 – 4.00 (m, 3H, CH, Ala, CH_2, $C_3H_6N_3$), 3.82 – 3.70 (m, 3H, CH, H-2, CH_2, H-6, CH_2, $C_3H_6N_3$), 3.66 – 3.52 (m, 2H, CH, H-3, CH, H-4), 3.60 – 3.21 (under H_2O signal, CH, H-5), 2.34

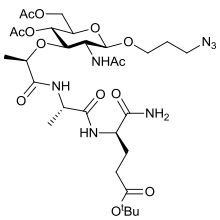
(t, *J* = 7.0 Hz, 2H, CH_2, Glu), 2.17 (t, *J* = 7.9 Hz, 2H, CH_2, *i*-D-Glu), 2.06 – 1.86 (m, 2H, CH_2, β *i*-D-Gln), 1.79 (s, 3H, CH_3, NAc), 1.73 – 1.60 (m, 3H, CH_2, β *i*-D-Gln, CH_2, β Glu), 1.36 (s, 9H, CH_3, ^tBu), 1.25 – 1.15 (m, 6H, CH_3, lactic acid, CH_3, Ala); ^{13}C NMR (100 MHz, DMSO-D_6) δ 173.5 (C=O), 172.9 (C=O), 172.5 (C=O), 172.2 (C=O), 172.0 (C=O), 170.2 (C=O), 156.3 (C=O), 144.4 (C_q, Ar), 144.3 (C_q, Ar), 141.0 (C_q, Ar), 138.0 (C_q, Ar), 129.2 (CH, Ar), 128.6 (CH, Ar, Fmoc), 128.1 (CH, Ar), 127.5 (CH, Ar), 126.3 (CH, Fmoc), 125.7 (CH, CH, Fmoc), 120.6 (CH, CH, Fmoc), 101.9 (CH, C-1), 100.6 (CH, benzylidene acetal), 80.7 (CH, C-3), 80.1 (C_q, ^tBu), 79.5 (CH, C-4), 77.8 (CH, α lactic acid), 68.2 (CH_2, C-6), 66.1 (CH, C-5), 65.97 (CH_2, $C_3H_6N_3$), 61.1 (CH_2, $C_3H_6N_3$), 55.1 (CH, C-2), 51.9 (CH, Ala), 48.7 (CH, α Glu, 47.1 (CH, α *i*-D-Gln), 31.7 (CH_2, γ *i*-D-Gln), 30.6 (CH_2, γ Glu), 28.8 (CH_2, β Glu), 28.2 (CH_3, ^tBu), 27.6 (CH_2, β *i*-D-Gln), 23.3 (CH_3, NAc), 19.5 (CH_3, lactic acid), 18.6 (CH_3, Ala); LC/MS: Rt = 8.85 min (Alltima C_{18}, 10 – 90% MeCN, 15 min run).

3-Azidopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (22)



Compound **18** (0.21 g, 0.29 mmol) was suspended in 60% AcOH in H_2O (3.0 mL, 0.1 M) and stirred with neopentylglycol (60 mg, 0.58 mmol) at 65 ^0C for 3 h. The solution was diluted with H_2O, concentrated *in vacuo* and co-evaporated (toluene). Purification by flash chromatography (9 : 1 DCM : MeOH) resulted in the title compound **22** as a white solid (0.17 g, 0.26 mmol, 88%). R_f = 0.2 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = 3.0 (c = 0.2, 1: 1 $CHCl_3$: MeOH);
^1H NMR (600 MHz, DMSO-D_6) δ 4.31 – 4.20 (m, 2H, CH, H-1, CH, lactic acid), 4.18 – 4.08 (m, 2H, CH, Ala, CH, α *i*-D-Gln), 3.78 – 3.75 (m, 1H, CH_2, $C_3H_6N_3$), 3.72 – 3.66 (m, 1H, CH_2, H-6), 3.60 (q, *J* = 9.2 Hz, 1H, CH, H-2), 3.54 – 3.43 (m, 2H, CH_2, H-6, CH_2. $C_3H_6N_3$), 3.41 – 3.26 (m, 3H, CH, H-3, CH_2, $C_3H_6N_3$), 3.26 – 3.21 (m, 1H, CH, H-4), 3.16 -1.13 (m, 1H, CH, H-5), 2.19 (t, 2H, *J* =5.2 Hz, CH_2, γ *i*-D-Gln), 1.97 – 1.91 (m, 1H, CH_2, β *i*-D-Gln), 1.78 (s, 3H, HNAc), 1.74 – 1.67 (m, 3H, CH_2, β *i*-D-Gln, CH_2, $C_3H_6N_3$), 1.39 (s, 9H, CH_3, ^tBu), 1.26 – 1.24 (m, 6H, CH_3, lactic acid, CH_3, Ala); ^{13}C NMR (100 MHz, DMSO-D_6) δ = 173.1 (C=O), 172.3 (C=O), 172.1 (C=O), 171.6 (C=O), 169.3 (C=O), 100.8 (CH, C-1), 82.2 (CH, C-3), 76.8 (CH, C-5), 76.7 (CH_2, C-6), 69.3 (CH, C-4), 65.2 (CH_2, $C_3H_6N_3$), 60.8 (CH_2, C-6), 51.5 (CH, Ala), 48.2 (CH, α *i*-D-Gln), 47.5 (CH_2, $C_3H_6N_3$), 31.2 (CH_2, γ *i*-D-Gln), 28.5 (CH_2, $C_3H_6N_3$), 27.7 (CH_3, ^tBu), 27.1 (CH_2, β *i*-D-Gln), 23.0 (CH_3, HNAc), 19.0 (CH_3, lactic acid), 18.1 (CH_3, Ala); IR (cm^{-1}): 3278, 2098, 1643, 1539, 1369; LC/MS: Rt = 4.52 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{26}H_{45}N_7O_{11} + H]^+$ 632,32498, found 632,32516.

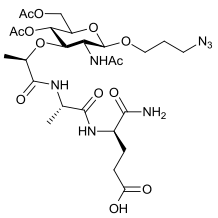
3-Azidopropyl-2-*N*-acetamide-4,6-di-*O*-acetyl-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (23)



Compound **22** (0.21 g, 0.33 mmol) was co-evaporated with pyridine, dissolved in warm pyridine (0.92 mL, 11 mmol) and diluted with 1,4-dioxane (3 mL, 0.1 M). Ac_2O (0.27 mL, 2.9 mmol) was added and the solution was stirred for 48 h. The reaction mixture was quenched with MeOH and concentrated *in vacuo* yielding compound **23** in quantitative yield. R_f = 0.7 (9 : 1 $CHCl_3$: MeOH + 1% AcOH); $[\alpha]^D$ = 1.5 (c = 0.65, 1 : 1 $CHCl_3$: MeOH); ^1H NMR (400 MHz, MeOD) δ 4.92 (t, *J* = 9.4 Hz, 1H, CH, H-4), 4.45 (d, *J* = 8.1 Hz, 1H, CH, H-1), 4.30 (dd, *J* = 9.3, 4.6 Hz, 1H, CH, α *i*-D-Gln), 4.20 (dd, *J* = 12.2, 4.8 Hz, 1H, CH_2, H-6), 4.16 – 4.04 (m, 2H, CH_2, H-6, CH, lactic acid), 3.98 (q, *J* = 6.7 Hz, 1H, CH, Ala), 3.92 – 3.83 (m, 1H, CH_2, $C_3H_6N_3$), 3.78 -3.71 (m, 2H, CH, H-2, CH, H-3), 3.63 - 3.59 (m, 1H, CH, H-5), 3.56 – 3.52 (m, 1H, CH_2, $C_3H_6N_3$), 3.33 (t, *J* = 6.6 Hz, 2H, CH_2, $C_3H_6N_3$), 2.30 – 2.25 (m, 2H, CH_2, γ *i*-D-Gln), 2.22 – 2.09 (m, 1H, CH_2, β *i*-D-Gln), 2.07 (s, 3H,CH_3, Ac), 2.05 (s, 3H, CH_3, Ac), 1.88 (s, 3H, CH_3, NAc), 1.85-1.75 (m, 3H, CH_2, $C_3H_6N_3$, CH_2, β *i*-D-Gln), 1.50 – 1.537 (m, *J* = 6.2 Hz, 12H, CH_3, ^tBu, CH_3, lactic acid), 1.23 (d, *J* = 6.8 Hz, 3H, CH_3, Ala);
^{13}C NMR (100 MHz, MeOD) δ 174.2 (C=O), 173.5 (C=O), 172.8 (C=O), 172.3 (C=O), 171.6 (C=O),

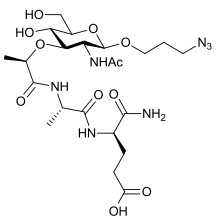
170.9 (C=O), 169.8 (C=O), 100.5 (CH, C-1), 80.6 (C_q, ^{t}Bu), 79.3 (CH, C-3), 77.7 (CH, lactic acid), 71.2 (CH, C-5), 69.2 (CH, C-4), 65.9 (CH_2, $C_3H_6N_3$), 62.0 (CH_2, C-6), 55.5 (CH, C-2), 51.8 (CH, α *i*-D-Gln), 49.3 (CH, Ala), 47.5 (CH_2, $C_3H_6N_3$), 31.3 (CH_2, γ *i*-D-Gln), 28.5 (CH_2, $C_3H_6N_3$), 27.3 (CH_3, ^{t}Bu), 26.4 (CH_2, β *i*-D-Gln), 22.9 (CH_3, NAc), 20.9 (CH_3, Ac), 20.8 (CH_3, Ac), 19.1 (CH_3, lactic acid), 17.3 (CH_3, Ala); IR (cm^{-1}): 3275, 2098, 1639, 1091, 694; LC/MS: Rt = 6.42 min (Alltima C_{18}, 10 –90% MeCN, 15 min run); HRMS Calcd. for $[C_{30}H_{49}N_7O_{13} + H]^+$ 716.34611 found 716.34653.

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



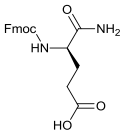
Compound **23** (73 mg, 0.11 mmol) was dissolved in a mixture of 20% TFA in DCM (1 mL, 0.1 M) and stirred for 2.5 h at ambient temperature. The compound was precipitated using Et_2O. The resulting solid was purified by flash column chromatography (9 : 1 $CHCl_3$: MeOH + 1% AcOH) yielding compound **24** (54 mg, 80 µmol, 82%). R_f = 0.2 (9 : 1 $CHCl_3$: MeOH + 1% AcOH); $[\alpha]^D$ = -5.2 (c = 0.27, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, MeOD) δ 4.96 (t, *J* = 9.6 Hz, 1H, CH, H-4), 4.46 (d, *J* = 8.4 Hz, 1H, CH, H-1), 4.37 – 4.35 (m, 1H, CH, α *i*-D-Gln), 4.26 (dd, *J* = 12.3, 4.6 Hz, 1H, CH_2, H-6), 4.19 (q, *J* = 7.1 Hz, 1H, CH, lactic acid), 4.11 (dd, *J* = 12.3, 2.1 Hz, 1H, CH_2, H-6), 4.08 – 4.03 (m, 1H, CH, Ala), 3.96 – 3.86 (m, 2H, CH_2, $C_3H_3N_3$, CH, H-2), 3.77 – 3.67 (m, 2H, CH, H-3, CH, H-5), 3.70 – 3.59 (m, 1H, CH_2, $C_3H_3N_3$), 3.41 – 3.33 (m, 2H, CH_2, $C_3H_3N_3$), 2.38 (t, *J* = 7.6 Hz, 2H, CH_2, γ *i*-D-Gln), 2.30 – 2.19 (m 1H, CH_2, β *i*-D-Gln), 2.12 (s, 3H, Ac), 2.07 (s, 3H, CH_3, Ac), 1.97 – 1.88 (m, 4H, CH_3, NAc, CH_2, β *i*-D-Gln), 1.88 – 1.72 (m, 2H, CH_2, $C_3H_3N_3$), 1.41 (d, *J* = 7.1 Hz, 3H, CH_3, lactic acid), 1.27 (d, *J* = 6.7 Hz, 3H, CH_3, Ala); ^{13}C NMR (100 MHz, MeOD) δ 176.4 (C=O), 175.9 (C=O), 175.0 (C=O), 174.7 (C=O), 173.4 (C=O), 172.6 (C=O), 171.7 (C=O), 102.0 (CH, C-1), 80.8 (CH, C-3), 79.1 (CH, lactic acid), 72.4 (CH, C-5), 70.5 (CH, C-4), 67.3 (CH_2, $C_3H_3N_3$), 63.2 (CH_2, C-6), 56.5 (CH, C-2), 53.3 (CH, α *i*-D-Gln), 50.6 (CH, Ala), 48.4 (CH_2, $C_3H_3N_3$), 31.0 (CH_2, γ *i*-D-Gln), 29.7 (CH_2, $C_3H_3N_3$), 27.6 (CH_2, β *i*-D-Gln), 23.1 (CH_3, NAc), 21.1 (CH_3, Ac), 20.9 (CH_3, Ac), 19.4 (CH_3, lactic acid), 17.5 (CH_3, Ala); IR (cm^{-1}): 3294, 2098, 1654, 1535; LC/MS: Rt = 4.85 min (Alltima C_{18}, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{26}H_{41}N_7O_{13} + H]^+$, 660.28351 found 660.28379.

3-Azidopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (25)



Compound **24** (15 mg, 23 µmol) was dissolved in a solution of 7M ammonia in MeOH (1.5 mL). The solution was stirred for 5 h at ambient temperature. The reaction mixture was concentrated and purified over HW40 gel filtration chromatography (0.15 M, ammonium acetate). After lyophilization compound **25** was obtained as a white solid (12 mg, 20 µmol, 87%). R_f = 0.2 (8 : 2 $CHCl_3$: MeOH + 2% AcOH); $[\alpha]^D$ = -12.5 (c = 0.02, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, D_2O) δ 4.56 (d, *J* = 8.5 Hz, 1H, CH, H-1), 4.47 – 4.43 (m, 1H, CH, α *i*-D-Gln), 4.38 – 4.26 (m, 2H, CH, lactic acid, CH, Ala), 4.09 – 4.03 (m, 1H, CH_2, $C_3H_6N_3$), 4.01 (d, *J* = 1.9 Hz, 1H, CH_2, H-6), 3.93 – 3.87 (m, 1H, CH, H-2), 3.84 (d, *J* = 5.5 Hz, 1H, CH_2, H-6), 3.82 – 3.72 (m, 1H, CH_2, $C_3H_6N_3$), 3.65 – 3.52 (m, 3H, H-3, CH, H-4, CH, H-5), 3.31 – 3.27 (m, 2H, CH_2, $C_3H_6N_3$), 2.50 (t, *J* = 7.3 Hz, 2H, CH_2, γ *i*-D-Gln), 2.33 – 2.20 (m, 2H, CH_2, β *i*-D-Gln), 2.11 – 2.01 (m, 4H, CH_2, β *i*-D-Gln, 3H, CH_3, NAc), 1.81 -1.72 (m, 2H, CH_2, $C_3H_6N_3$), 1.53 (d, *J* = 7.2 Hz, 3H, CH_3, lactic acid), 1.46 (d, *J* = 6.8 Hz, 3H, CH_3, Ala). ^{13}C NMR (151 MHz, D_2O) δ 179.5 (C=O), 177.1 (C=O), 176.7 (C=O), 176.1 (C=O), 175.0 (C=O), 102.2 (CH, C-1), 83.7 (CH, C-3), 79.3 (CH, lactic acid), 76.5 (CH, C-5), 69.8 (CH, C-4), 68.1 (CH_2, $C_3H_6N_3$), 61.6 (CH_2, C-6), 56.0 (CH, C-2), 53.9 (CH, α *i*-D-Gln), 50.8 (CH, Ala), 49.0 (CH_2, $C_3H_6N_3$), 32.4 (CH_2, γ *i*-D-Gln), 29.1 (CH_2, β *i*-D-Gln), 27.6 (CH_2, $C_3H_6N_3$), 23.2 (CH_3, NAc), 19.7 (CH_3 lactic acid), 17.5 (CH_3, Ala); IR (cm^{-1}): 3310, 2101, 1645, 1464; LC/MS: Rt = 8.07 min (Alltima C_{18}, 0 – 20% MeCN, 15 min run); HRMS Calcd. for $[C_{22}H_{37}N_7O_{11} + H]^+$, 576.26238 found 576.26243.

Fmoc-*i*-D-Gln-(OH) (30)



To a stirred solution of compound **14** (2.0 g, 4.7 mmol) in DCM (40 mL, 0.12 M) was added TFA (10 mL, 25 v/v%). After 4 h the solution was concentrated in *vacuo*. Co-evaporation with toluene results compound **30** as a white solid (1.4 g, 3.8 mmol, 80%). R_f = 0.2 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = 0.8 (c = 0.25, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (DMSO-D_6, 400 MHz): δ = 7.89 – 7.30 (m, 8H, CH, Fmoc), 7.2 (s, 1H, NH), 4.31 – 4.21 (m, 3H, CH_2, Fmoc, CH, α *i*-D-Gln), 4.0 - 3.91 (m, 1H, CH, Fmoc), 2.25 (t, *J* = 7.6 Hz, 2H, CH_2, γ *i*-D-Gln), 1.95 – 1.87 (m, 1H, CH_2, β *i*-D-Gln), 1.80 – 1.72 (m, 1H, CH_2, β *i*-D-Gln); ^{13}C NMR (100 MHz): δ = 174 (C=O), 173.6 (C=O), 156.0 (C=O), 143.9 (CH, Fmoc), 143.8 (CH, Fmoc), 140.8 (C_q, Fmoc), 127.7 (CH, Fmoc), 127.2 (CH, Fmoc), 125.4 (CH, Fmoc) 120.2 (CH, Fmoc), 65.7 (CH_2, Fmoc), 53.9 (CH, α *i*-D-Gln), 46.7 (CH, Fmoc), 30.4 (CH_2, γ *i*-D-Gln), 27.3 (CH_2, β *i*-D-Gln); IR (cm^{-1}): 3300, 1707, 1685, 1647, 1257; LC/MS: Rt = 12.5 min (C_{18} Alltima, 0 - 20% MeCN, 15 min run); HRMS Calcd. for $[C_{20}H_{20}N_2O_5 + H]^+$ 369.14450, found 369.14458.

Synthesis of MDP-peptide conjugates

General procedure for automated solid phase synthesis

The solid-phase peptide synthesis was performed on a 50 µmol or 25 µmol scale according to established methods[39] on an ABI 433A (Applied Biosystems) automated instrument applying Fmoc based protocol starting from Tentagel-S-RAM resin (loading 0.23 mmol/g) or loaded with described peptide. The synthesis was continued with Fmoc-amino acids specific for each peptide. The consecutive steps performed in each cycle for HCTU chemistry on 50 µmol scale:

1. Deprotection of the Fmoc-group with 20% piperidine in NMP for 15 min; 2) NMP wash; 3) Coupling of the appropriate amino acid using a five-fold excess. Generally, the Fmoc amino acid (0.25 mmol) was dissolved in 0.25 M HCTU in NMP (1 mL), the resulting solution was transferred to the reaction vessel followed by 0.5 mL of 1.0 M DiPEA in NMP to initiate the coupling. The reaction vessel was than shaken for 30 min; 4) NMP wash; 5) capping with 0.5 M acetic anhydride in NMP in presence of 0.5 mmol DiPEA; 6) NMP wash; 7) DCM wash.

The consecutive steps performed in each cycle for HATU chemistry:

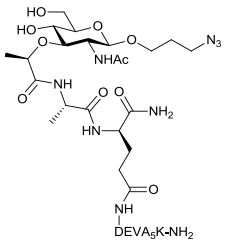
1. Deprotection of the Fmoc-group with 20% piperidine in NMP for 15 min; 2) NMP wash; 3) Coupling of the appropriate amino acid using a two-fold or five-fold excess. Generally, the Fmoc amino acid (0.1 mmol or 0.25 mmol) and HATU (0.15 mmol or 0.2 mmol) was dissolved in 1.0 M DiPEA in NMP (0.25 mL or 0.5 mL). The resulting solution was pre-activated for 1 min and transferred to the reaction vessel to initiate the coupling. The reaction vessel was than shaken for 60 min; 4) NMP wash; 5) capping with 0.5 M acetic anhydride in NMP in presence of 0.5 mmol DiPEA; 6) NMP wash; 7) DCM wash.

Aliquots of resin of the obtained sequences were checked on an analytical Alltima C_{18} column (4.6 x 50 mm, 5 µm particle size, flow 1.0 mL/min.). The Fmoc amino acids applied in the synthesis were: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(MMt)-OH, Fmoc-Phe-OH, Fmoc-Ser(^{t}Bu)-OH and Fmoc-Val-OH.

General procedure for cleavage from the resin, deprotection and purification

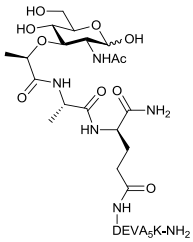
Fifty µmol resin was washed with NMP, DCM and dried after the last synthesis step followed by a treatment for 104 min. with 5 mL cleavage cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The suspension was filtered, the resin was washed with neat TFA and the product was precipitated with Et_2O out of the TFA solution. The suspension of the product in Et_2O was centrifuged, Et_2O removed and the precipitated was washed in Et_2O again. The washing was repeated (3x). The final precipitate was air dried and dissolved in AcOH : H_2O (1 : 1) or MeCN : H_2O : ^{t}BuOH (1 : 1 : 1) followed by purification on the Gilson preparative RP-HPLC system .

3-Azidopropyl-MurNAc-Ala-*i*-D-Gln-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (2)



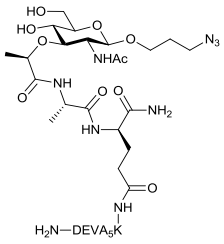
Tentagel S Ram resin loaded with H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) on 25 µmol scale was elongated with Fmoc-*i*-D-Gln-OH (**30**) and Fmoc-Ala-OH with standard HCTU/Fmoc cycle. The synthesis was completed with a double coupling of a two-fold excess of compound **12** pre-activated with HATU and DiPEA. After treatment with a standard cleavage cocktail for 60 min. the suspension was filtered and the product was precipitated with Et_2O. After purification by RP-HPLC, both compound **2** and hydrolyzed compound **28** were isolated. Compound **2** was obtained in 1.3 mg (0.41 µmol, 2%); LC/MS: Rt = 6.22 min (C_{18} Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 3103.60 $[M+H]^+$; HRMS Calcd. for $[C_{134}H_{220}N_{36}O_{48} + H]^{2+}$ 1552.30276, found 1552.30469;

MurNAc-Ala-*i*-D-Gln-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 (28)



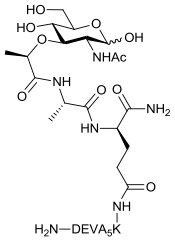
Title compound **28** was isolated in 1.5 mg (0.38 µmol, 2%); LC/MS: Rt = 6.05 min (C_{18} Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 3020.55 $[M+H]^+$; HRMS Calcd. for $[C_{131}H_{215}N_{33}O_{48} + H]^{2+}$ 1510.77860, found 1510.78070.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-(*i*-D-Gln-Ala-3-azidopropyl-MurNAc)NH_2 (3)



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-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) on 25 µmol scale was treated 1M BOC_2O in NMP for 15 min followed by addition of 2 eq. of DiPEA and reacted for another hour. The resin was washed with NMP and DCM. The resin was treated with a cleavage cocktail of 3% TFA in DCM followed by a coupling sequence of compound **30** and Fmoc-Ala-OH with a standard HCTU/Fmoc cycle. The synthesis was completed with a double coupling of a two-fold excess of compound **12** pre-activated with HATU and DiPEA. After treatment with a standard cleavage cocktail for 60 min., the suspension was filtered and the product was precipitated with Et_2O. After purification, both compound **3** and hydrolyzed compound **29** were isolated. Compound **3** was obtained in 1.2 mg (0.34 µmol, 2%); LC/MS: Rt = 6.16 min (C_{18} Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 3103.60 $[M+H]^+$; HRMS Calcd. for $[C_{134}H_{220}N_{36}O_4 + H]^{2+}$ 1552.30276, found 1552.30434.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-(*i*-D-Gln-Ala-MurNAc)NH_2 (29)



Compound **29** was obtained in 1.0 mg (0.39 µmol, 2%); LC/MS: Rt = 6.02 min (C_{18} Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 3020.55 $[M+H]^+$; HRMS Calcd. for $[C_{131}H_{215}N_{33}O_{48} + H]^{2+}$ 1510.77860, found 1510.78070.

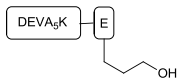
H-Glu(3-azidopropyl-MurNAc-Ala-*i*-D-Gln)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4)

25 umol H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) was treated with a mixture of compound **21** (53 mg, 50 µmol), HCTU (21 mg, 50 µmol) and DiPEA (18 µL, 0.1 mmol) in NMP (0.5 mL) for 18 h. The resin was washed and treated with a solution of 20% piperidine in NMP followed by a wash step (NMP, DCM, Et_2O). Treatment with cleavage cocktail for 60 min and purification resulted in compound **4**. The hydrolyzed compound was not isolated. (1.8 mg, 0.53 µmol, 2%), LC/MS: Rt = 8.78 min (C_{18} Alltima, 10 - 50% MeCN, 15 min run); ESI-MS: *m/z* 3206.65 $[M+H]^+$; HRMS Calcd. for $[C_{139}H_{229}N_{35}O_{51} + H]^{2+}$ 1603.82882, found 1603.82944.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-*i*-D-Gln(3-azidopropyl-MurNAc-Ala-D-iGln)NH_2 (5)

40 umol Sieber Amide resin (0.2 mmol/g) was treated with 20% piperidine in NMP (3 x 3 min), washed with NMP and reacted with a mixture of compound **21** (107 mg, 102 µmol), DiPEA (67 µL, 406 µmol) and HCTU (76 mg, 184 µmol) in 4 : 1 NMP : DMSO. The resulting solution was reacted for 18 h, washed with NMP, DCM and Et_2O. An aliquot of resin (5mg) was treated with 20% piperidine (1 mL) for 20 min. The solution was diluted with EtOH (25 mL) and absorbance at 300 nm was measured. The Fmoc-test revealed a 56% loading. The resin was treated with a capping solution (0.5 M Ac_2O, 0.05 M DiPEA, NMP, 3 x 15 min) and elongated with the standard Fmoc based SPPS protocol to H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) concluding with a final Fmoc deprotection. 20 umol Resin was treated with standard cleavage conditions. After purification compound **5** and hydrolyzed analogue **30** were isolated. Compound **5** was obtained in 4.8 mg (1.4 µmol, 6%). LC/MS: Rt = 5.70 min (C_{18} Alltima, 10 - 90% MECN, 15 min run); ESI-MS: *m/z* 3206.65 $[M+H]^+$; HRMS Calcd. for $[C_{139}H_{229}N_{35}O_{51} + H]^{2+}$ 1603.82882, found 1603.82943.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Glu(3-propanol)NH_2 (31)



The hydrolyzed analogue of **3** was isolated as a side product after the acid treatment of the resin.in 2.8 mg (0.94 µmol, 4%); LC/MS: Rt = 5.82 min (C_{18} Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 2731.45 $[M+H]^+$; HRMS Calcd. for $[C_{120}H_{198}N_{31}O_{41} + H]^{3+}$ 911.15363, found 911.15694.

Immunological assays

NOD2-HEK293 activation

The human NOD2-receptor expressing HEK293 cell-line was obtained from Invivogen (Toulouse, France). Test compounds were titrated in a 96-wells plate as described above. Subsequently, approximately 50.000 NOD2-HEK293 cells were added per well. After 24 hours of incubation at 37°C, the supernatant was taken from all wells. The amount of IL-8 produced by the NOD2-HEK293 cells is a measure for activation. Therefore, IL-8 production was determined using an IL-8 ELISA-kit (Sanquin, Amsterdam, The Netherlands).

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.[35]

The B3Z hybridoma is cultured in complete IMDM medium supplemented with 500 µg/ml hygromycin.[40]

In vitro DC maturation assay

Test compounds were titrated in a 96-wells plate (Corning, Amsterdam, The Netherlands) in complete IMDM medium. Next, D1 cells from C57BL/6 mice were harvested and counted, and subsequently transferred to the 96-wells plates containing the test compound titrations, using approximately 40.000 cells per well. After 24 hours of incubation at 37°C, supernatant was taken from the wells for ELISA analysis (BioLegend, San Diego, USA) in which the amount of produced IL-12p40 was measured.

In vitro antigen presentation assay

B3Z is a $CD8^+$ T-cell hybridoma specific for the H-$2K^b$ CTL-epitope SIINFEKL of ovalbumin. B3Z expresses the lacZ reporter gene of *Escherichia coli* which is under the regulation of the NFAT element from the IL-2 promoter. Therefore, TCR triggering of this T-cell leads to transcription of the lacZ reporter gene, the gene product of which is able to convert the chromogenic substrate CPRG (Chlorophenolred-β-D-galactopyranoside). This conversion is measured by absorbance spectrophotometry at a wavelength of 590 nm.[40]

2.6 References and notes

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(28) Care should be taken with the presence of a free C-4 hydroxyl in MurNAc derivatives because basic conditions can lead to lactonisation as is illustrated by the isolation of compound **36**, after acid treatment and subsequent acetylation of **12**. See for the synthesis compound **36** in the experimental section.

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