Self adjuvanting immunopeptides : design and synthesis

Gential, G.P.P.

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Chapter 1: Introduction

The mammalian immune system consists of two interdependent parts, namely the innate and adaptive immune system.[1-3] Adaptive immune responses can be divided in humoral (antibody) and cytotoxic (cellular) responses. B cells, T cells, and dendritic cells (DCs) are involved in generating these immune responses that ultimately can lead to the ability of the host to identify and memorize specific pathogens. Whilst cytotoxic T cells (CTLs) are key players in cellular responses and B cells mediate humoral responses each of these responses require T-helper (Th) cells. Th cells release cytokines, soluble proteins that can induce activation and proliferation of CTLs as well as B cell antibody class switching. Two major subtypes of T-helper cells are Th1 cells and Th2 cells. The cells of Th1-type produce the cytokine interferon-gamma and are involved in combatting intracellular pathogens. Th2-cells produce interleukin-4, -5, and -13 and help combatting extracellular pathogens. Antigen presenting cells (APCs) such as DCs and macrophages present peptides derived from pathogens within the cell on major histocompatibility complex class I (MHC class I) molecules.[4, 5] Recognition of peptides, derived from viral proteins and that are presented by MHC class I molecules, by CTLs initiates a cellular cytotoxic response which can eradicate for instance virus-infected cells. Peptides derived from extracellular pathogens are presented by APCs on major histocompatibility complex class II (MHC class II) molecules. Upon recognition by T-helper cells an activation process is initiated through which these specific T-helpers respond to B cells that have taken up the same antigen and therefore display the same MHC II-peptide complex. This interaction result in differentiation of B cells into plasma cells that secrete antigen-specific antibodies which can neutralize for instance bacterial pathogens.[6]

The above brief impression of immune responses indicates the importance of peptide dependent recognition processes for controlling extracellular and intracellular infection. However, single pathogen-derived peptides epitopes are by themselves not effective in inducing an immune response.[7, 8] Peptides are poorly immunogenic because peptides do not function as danger signals that activate the innate immune system, which is required for inducing adequate adaptive immune responses. Induction of an effective adaptive immune response therefore requires signals of the innate immune system to activate antigen presenting cells which can strongly

stimulate peptide-specific T cells. In modern vaccination technologies combinations of defined molecules stimulating both the innate and adaptive responses are used.[9, 10]

The innate immune system forms the first line of defence against pathogenic invaders by recognising pathogen associated molecular patterns (PAMPs) or microbe-associated-molecular-pattern (MAMPs) with the aid of pathogen recognizing receptors (PRRs). Toll-like receptors (TLRs) and NOD-like receptors (NLRs), RIG-like receptors (RLRs) and C-type lectins are part of the innate immune system.

Toll-like receptors (TLRs)[11] are a family of membrane bound glycoproteins that have been studied most among the PRRs.[12] Upon recognition of a specific PAMP by the corresponding TLR, a signal transduction pathway is started that activates specialized cells of both the innate and adaptive immune system, leading eventually to eradication of the pathogen. Ten different human TLRs can be discerned that are expressed in different ratios by immune and epithelial cells, while mice has 12 receptors (TLR1-9+TLR11-TLR13). To detect pathogens that are present in the extracellular environment as well as the internalized ones, both human and murine TLRs are situated at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in the various intracellular (TLR3, TLR7, TLR8 and TLR9) compartments (Figure 1). Each TLR recognizes PAMPs with certain structural identity.[13] TLR2 along with TLR1 or TLR6 binds a wide variety of microbial membrane components such as peptidoglycans. Bacterial lipopolysaccharide is the ligand for TLR4 while TLR5 recognizes bacterial flagellin. TLRs inside the cell are specialized in recognizing nucleic acids of different origin. TLR3 recognizes viral double-stranded RNA and also small self-RNAs derived from damaged cells. TLR8 binds to viral and bacterial single-stranded RNA. TLR9 recognizes bacterial and viral single stranded unmethylated CpG-DNA. Single-stranded RNA from viruses is the ligand of TLR7. The finding, that TLR agonists are capable of linking the innate and adaptive immune system, make the study toward TLRs and the corresponding ligands important for the development of prophylactic and therapeutic vaccines. This has stimulated structure and activity studies of TLR ligands, leading to several well-defined small molecular modulators (mostly agonists but also antagonists). In particular, structurally defined ligands for TLR2, TLR4, TLR7 and TLR9 have been developed.[14, 15]

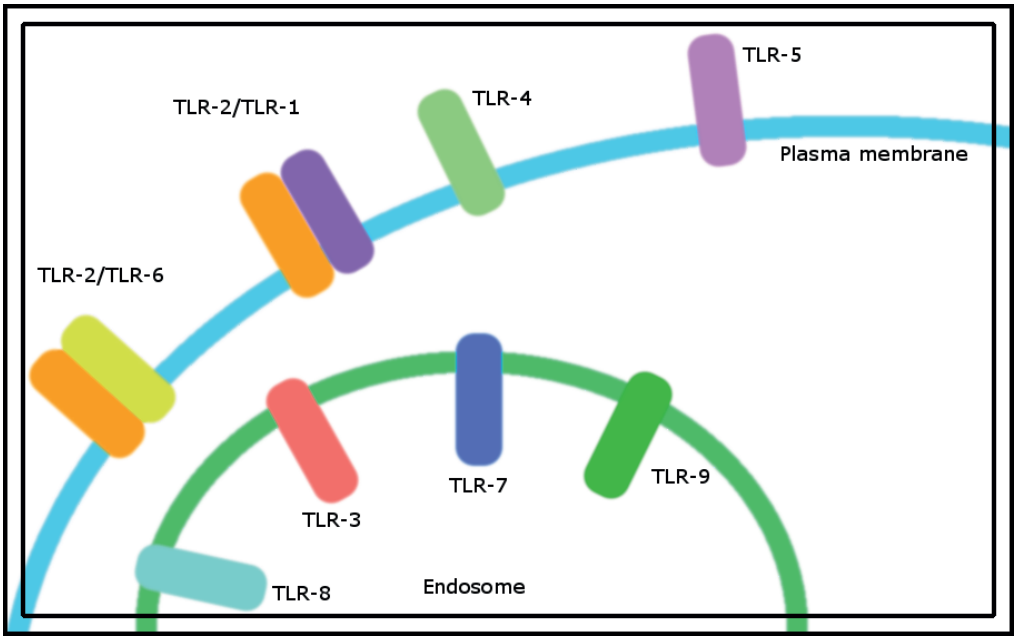

Figure 1. Schematic view of the location of the various TLRs

Lipopeptides and lipoteichoic acids originating from gram positive bacteria are the naturally occurring agonists for TLR2. Heterodimerization of TLR2 with TLR1 or TLR6 is a prerequisite for recognition of bacterial lipoproteins or lipopeptides. Structure-activity studies have revealed that synthetic Pam_3CSK_4, (Figure 2) the structure of which is based on triacylated lipopeptide derived from *Escherichia coli* membrane protein[16,17], targets specifically heterodimeric TLR1/TLR2.[18] Pam_2CSSNA (Figure 2) and macrophage-activating lipopeptide (MALP-2) are examples of synthetic accessible agonists for the TLR2/TLR6 combination.[19] Further studies have led to, amongst others, water soluble and structurally less complex TLR2 agonists.[20-22] Double-stranded viral RNA, the natural ligand for TLR3, can be replaced by polyinosinic–polycytidylic acid (poly I:C).[23] Homodimerisation of TLR3 occurs and it appears that an (I:C) oligomer of at least 100 base pairs is needed for a sufficient immune response. Also, double-stranded RNA mimics are explored as adjuvants.[23] Lipopolysaccharides (LPS) originating from Gram-negative bacteria are the naturally occurring agonists of TLR4.[24] Lipid A is an important part of LPS and a lot of structure and activity studies have resulted in synthetic compounds which can serve as either antagonist or agonist for TLR4.[25] Mono-phosphoryl lipid A (MPLA), a lipid A derivative from Salmonella enterica that has stimulatory properties but lacks endotoxicity and pyrogenicity, is approved as a human vaccine adjuvant.[26] While the natural agonists for TLR7 is single-stranded RNA from viruses[27], several structural defined small molecules have been discovered that can function as ligands for TLR7/8, such as dimidazoquinolines and adenine derivatives (Figure 2).[15, 28-30] TLR9 is the only receptor that recognizes synthetic ssDNA fragments. Specific oligodeoxynucleotides with CpG motifs and a nuclease-resistant phosphorothioate backbone function as agonist of TLR9 (Figure 2).[31, 32] Up to now TLR10 is the only receptor without a known ligand or signalling function.[33] TLR agonists are used in the development of new immune therapeutics[14, 34, 35] while TLR antagonists are explored for the treatment of autoimmune diseases such as rheumatoid arthritis.[36]

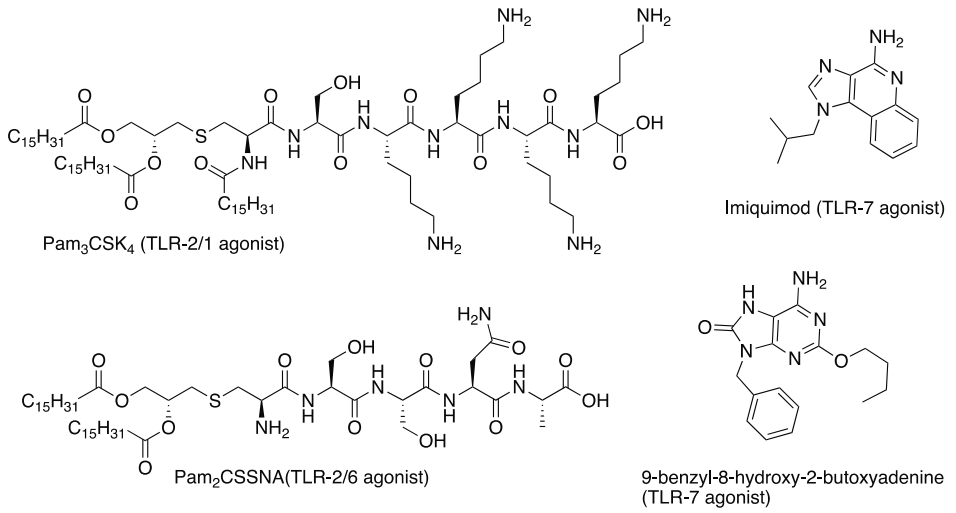

Figure 2. Examples of TLR-2/1, TLR2/6, TLR7 and TLR9 agonists

Self adjuvanting TLR peptide conjugates

With the objective to develop new classes of vaccines with more precise characteristics and new applications, considerable research is devoted to the use of agonistic ligands of PRRs and in particular TLRs.[37] For instance, individual TLR ligands have been investigated as adjuvants with improved properties.[15] TLR agonists have also been used in the quest toward fully synthetic vaccines.[38, 39] The role of oligopeptides in recognition processes inherent to immune responses makes peptide epitopes essential components of these types of vaccines.[9] The inability of oligopeptides to induce sufficient immune responses requires the presence of either an adjuvant or a suitable TLR agonist.[9] Both antigenic proteins and epitopes embedded in synthetic long peptides (SLP) in combination with specific TLR agonists have been evaluated for their immunological properties.[40, 41] In particular ligands of TLR1/2[35, 42], TLR2/6[43], TLR3[44], TLR4[45-47], TLR7[48] and TLR9[49, 50] were evaluated. In the course of these studies it was discovered that conjugates in which a peptide epitope is covalently attached to a specific TLR agonist proved to be more potent than just a mixture of the same TLR agonist and the epitope.[8, 31, 35] Several examples of these potential vaccines, termed "self adjuvanting peptide conjugates" have been reported and this chapter presents a selected number of examples of peptide conjugates that target different TLRs.

TLR-2 targeting peptide conjugate

Already in 1989, the group of Rammensee reported the synthesis and immunological evaluation of a conjugate (**1**, Figure 3) consisting of Pam_3CSS and the peptide epitope TYQRTRALVTG, derived

from the nucleoprotein of influenza virus.[51] Conjugate **1** was assembled with the aid of an automated SPPS procedure, using Fmoc-chemistry. This group of Rammensee showed for the first time that priming of virus-specific cytotoxic T cells, which is an important event in the immune response against viral infections, can be induced *in vivo* with conjugate **1**. With the objective to attain TLR-2 ligands with improved properties several groups designed and synthesized analogues of Pam_3C.[16, 52-54] Evaluation of their immunological properties resulted in Pam_3CSK_4 as a potent TLR-2 agonist with increased solubility by virtue of the hydrophilic lysine residues.[54] Khan *et al.* prepared conjugates (e.g. **2**, Figure 3) composed of $CD8^+$ cytotoxic T-lymphocyte SIINFEKL epitope (a model MHC I epitope derived from ovalbumin and often used in immunology studies in mice or murine-derived tissue) covalently linked to the ligand Pam_3CSK_4.[31] Immunological evaluation showed that this conjugate was able to induce DC maturation to the same amount as the single Pam_3CSK_4 ligand. Importantly, in comparison with a mixture of the free ligand and the peptide epitope, conjugate **2** showed not only enhanced MHC class I antigen presentation but also enhanced antigen uptake resulting in a robust and systemic response of specific T-cells. Interestingly, the enhanced uptake was found to be independent of the expression of cell-surface TLR2.[31] These studies were expanded with the synthesis and evaluation of three different conjugates containing the ovalbumin derived CTL epitope DEVSGLEQLESIINFEKLAAAAAK, the ovalbumin derived Th epitope ISQAVHAAHAEINEAGR and the Moloney virus envelope derived Th epitope.[35] The outcome of the *in vivo* studies shows that the conjugates of type **2** have superior capacity to prime both CTL ($CD8^+$) and T-helper ($CD4^+$) cells in mice as compared to a mixture of the corresponding free epitope and the free Pam_3CSK_4 ligand. In addition, vaccination with these conjugates leads to efficient induction of antitumor immunity in mice challenged with aggressive transplantable melanoma or lymphoma.[35] The same group investigated the influence of the chiral centre in the glycerol moiety of the Pam_3CSK_4 ligand on the immunological properties of conjugates of type **2**.[55] Although both the R- and S-stereoisomers were internalized into cells to similar extent in a clathrin- and caveolin-dependent manner the R–stereoisomer was not only superior in facilitating activation and maturation of dendritic cells but also in induction of specific CTLs ($CD8^+$ T-cells).[55] All these conjugates were accessible via an automated on-line solid phase peptide synthesis (SPPS) approach using Fmoc-chemistry.

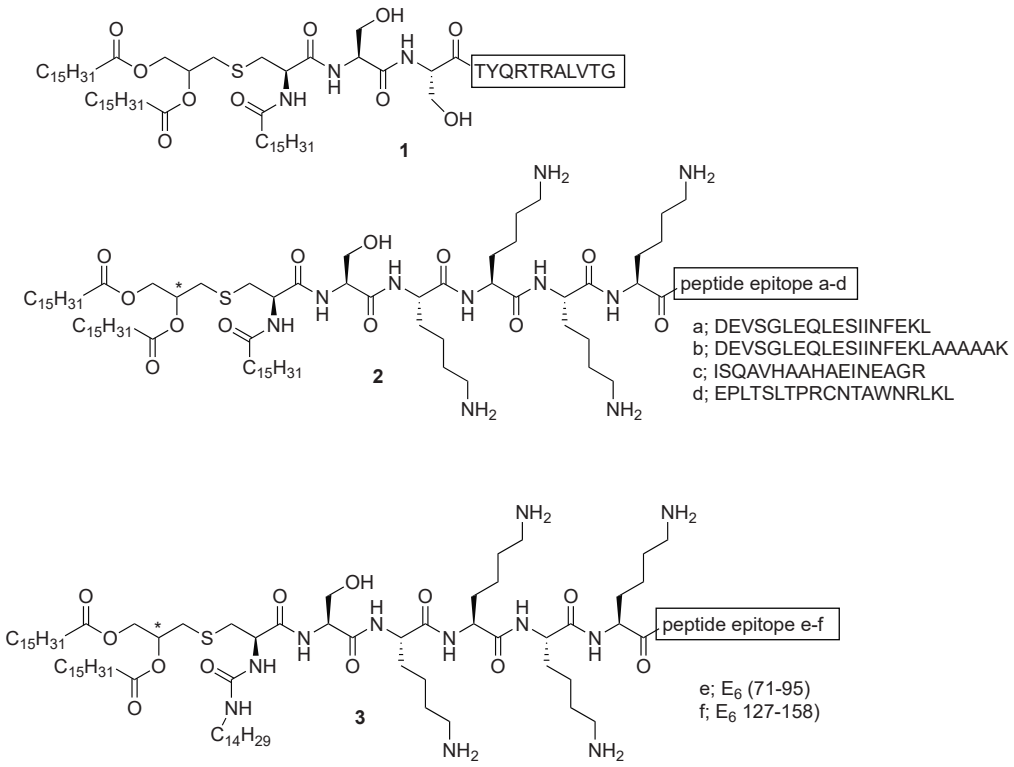

Figure 3. Examples of conjugates comprising a TLR-2/1 ligand and a synthetic long peptide epitope. Synthetic Pam_3Cys-lipopetides are mixtures of epimers at the glycerol residue (indicated by asterisk).

Guided by an X-ray structure of the TLR1/TLR2 dimer co-crystallized with the Pam_3C -ligand, Willems *et al.* designed a new and improved Pam_3CSK_4 ligand termed UPam, in which the cysteine amide bond was replaced by an urea linkage.[42] With the aid of an automated SPPS and using Fmoc-chemistry the new TLR2 ligand was incorporated into a conjugate, containing human papillomavirus type 16 (HPV16)-encoded synthetic long peptide epitopes to give conjugates **3** (Figure 3).[56] It was shown that these conjugates can activate both circulating and lymph node derived tumor specific T-cells.[56]

While gram-negative bacterial lipoproteins are provided with three fatty acid residues, gram-positive bacterial lipoproteins contain two fatty acid chains.[57] It was established that Pam_2Cys functions as a TLR-2/6 ligand.[58] Jackson *et al.* have prepared and evaluated a number of fully synthetic conjugates, composed of a helper (Th) T cell epitope, a target epitope and *S*-[2,3-bis(palmitoyloxy)propyl]cysteine as (Pam_2Cys) ligand (**4** in Figure 4).[59] In conjugates of type **4** two different Th peptide sequences were combined with sequences of various MHC-class I restricted target epitopes, such as the TYQRTRALV sequence derived from influenza virus and the SIINFEKL model epitope. In conjugates of type **4**, the Th epitope is situated at the N-terminal end and the target epitope is positioned at the C-terminal end.[59] In the first stage of the on-line SPPS toward conjugates **4** immobilized peptide **6** is assembled having the epitopes separated by a single lysine (K) residue, of which the amino group in the side chain was protected with the orthogonal Mtt group. The TLR ligand was next installed by selective removal of the mild acid labile Mtt group in immobilized peptide **6**. To improve the immunogenicity of the conjugate the released amino function in the lysine side chain was first elongated with two serine residues and subsequently with Pam_2Cys.[59] Removal of the protecting groups and cleavage of the conjugate from the solid

support gave conjugates of type **4**. Immunological evaluation indicate that these conjugates were able to induce both humoral and cellular immunity, thereby potentially provide protection against viral or bacterial infection.[59]

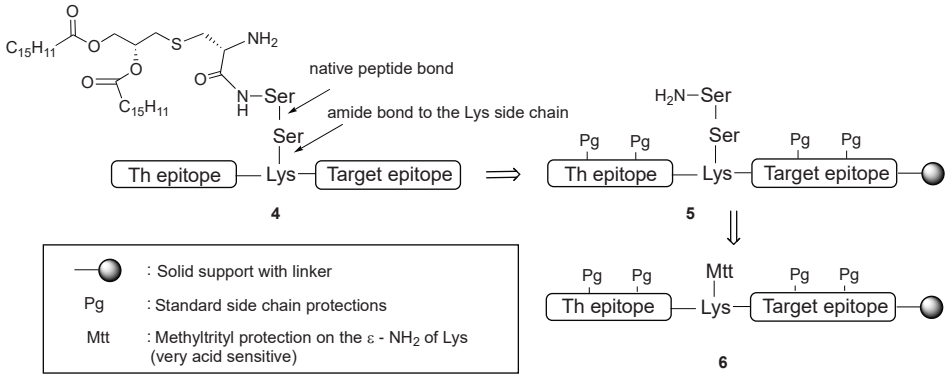

Figure 4. Retro synthesis and the generic structure of branched lipopeptide conjugates that contain TLR-2/TLR6 ligand, as developed by Jackson *et al.*[59]

Although the synthetic method fulfilled well for several conjugates, the overall yield and quality of the final conjugate was inadequate for conjugates in which the peptide epitope probably could adopt a specific tertiary/quarternary structure. The construction of a new class of conjugates, composed of a Th epitope, a CTL epitope and Pam_2Cys, was investigated by a modular approach that is terminated by a block coupling.[60] As branched conjugates showed more favorable immunological properties than their linear counterparts, the Pam_2Cys ligand was appended to the N terminal end of the Th epitope to give a lipopeptide that was coupled to a separately prepared target epitope.[60] Three different reactions for the final block coupling were explored (Figure 5). The participating reactive functional groups were installed at the N-terminal end of both the target epitope and the lipopeptide composed of the Th epitope and the Pam_2Cys. An oxime linkage was introduced by the reaction of the aldehyde in target epitope **7** with hydroxyl amine of lipopeptide **8** to give conjugate **9** (Figure 5). In the second conjugation strategy the bromo acetyl at the N-terminus of target epitope **10** reacts with the terminal cysteine in lipopeptide **11** to furnish conjugate **12** with a thioether linkage.[60]

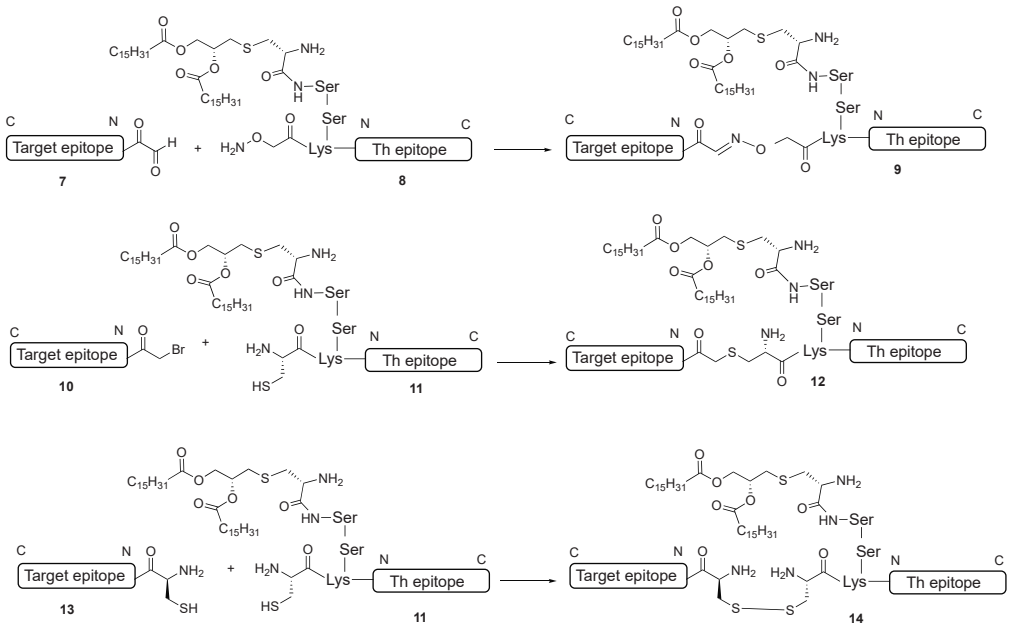

Figure 5. Three coupling strategies towards branched TLR2/TLR6-ligand peptide conjugates

Lipopeptide **11** was also used in the third strategy, in which a disulfide linkage was introduced by a reaction with the terminal cysteine in **13** to provide conjugate **14**. All three reactions proceeded successfully to provide the final lipidated peptide in sufficient quality while stepwise solid phase synthesis as previously described failed. It appears that alkylation of the bromoacetylated peptide with cysteine, leading to conjugates of type **12** is the most efficient out of the three strategies. Although, the non-natural thioether bond formed between the target epitope and the rest of the construct tends to decrease the processability of the conjugate by the proteasome, all constructs could induce significant immune response.[60]

Prior to the above described modular approach, the oxime ligation approach was also used in the synthesis of self adjuvanting immunopeptides **15** by Rose *et al.* as depicted in Figure 6. An important aspect of these conjugates is the presence of several copies of the peptide antigen on a multifunctional core.[61] Conjugates with multivalent epitopes often showed an increase in immunogenicity. The multiple antigen peptide system **16** that uses an oligomeric branching lysine was selected as a core. Construct **16** was prepared by SPPS using SASRIN resin and Fmoc-chemistry. After six coupling cycles the TLR2/1 ligand, Pam_3Cys-OH, could be condensed to the side chain of the terminal lysine after selective cleavage of the orthogonal Dde group with hydrazine.[61]

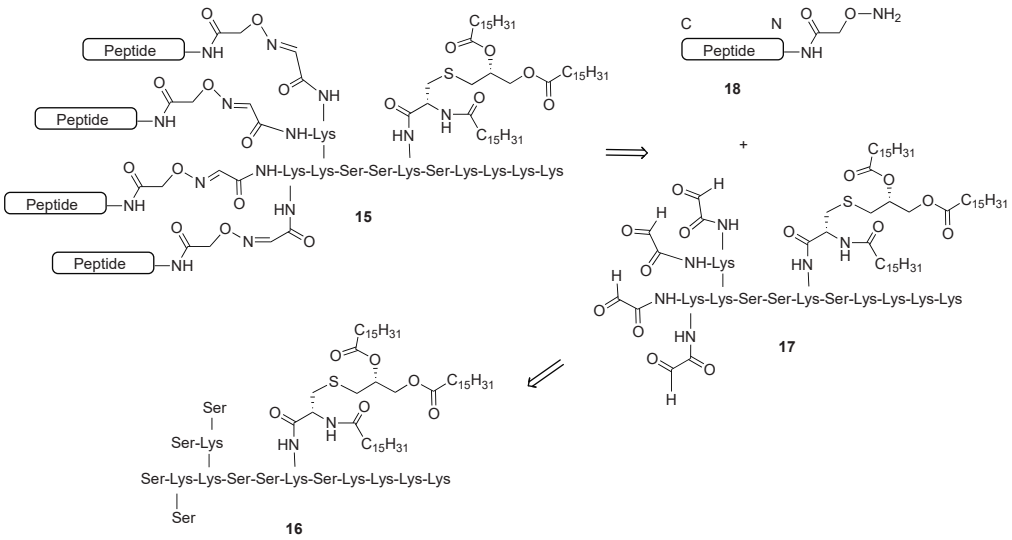

Figure 6. TLR2/TLR1 ligand peptide conjugate bearing multiple peptides

Next, the synthesis was continued by elongation with two serine residues and one lysine residue. The lysine at the N terminus was fully deprotected and the released alpha amine and the epsilon amine were simultaneously condensed with two protected lysines. Subsequent deprotection of both amines in the lysines allowed the coupling of four serine residues. Finally, removal of the protecting groups, cleavage from the solid support and purification furnished core **16**. The aldehyde functions were produced by reaction of the 1,2-amino alcohols in the N-terminal serine residues with sodium periodate to give construct **17**. Peptide **18** was separately assembled by standard SPPS, in which the final coupling entails the introduction of the hydroxyl amine moiety by reaction with Boc-aminooxyacetyl N-hydroxysuccinimide ester. Zeng *et al.* completed the synthesis by condensation of aminooxyacetyl peptide **18** and template **17** provided with four aldehydes to give immunopeptides **15**.[61]

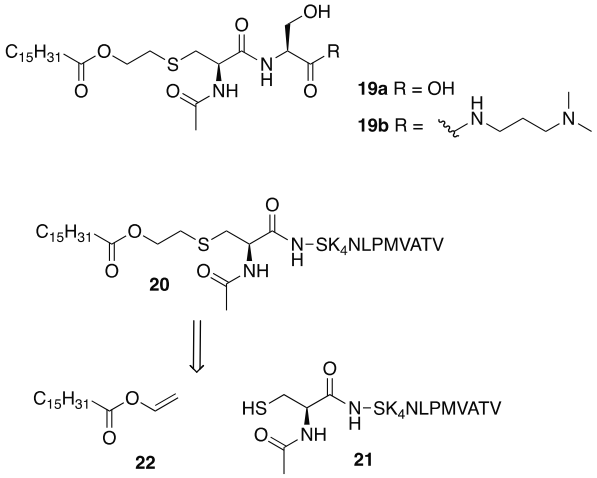

Figure 7. Simplified monoacyl lipopeptide **19** and retro synthesis of the incorporation of this ligand in antigenic peptide conjugate **20**.

The group of David explored structure-activity relationships of several immunostimulatory TLR agonists, including TLR2 ligands.[20-22] These studies led, among other findings, to the interesting discovery of monoacyl lipopeptide **19a**, a simplified TLR2 ligand which unexpectingly showed exclusive human TLR2 agonistic activity (Figure 7). With the objective to increase the water solubility of this ligand compound **19b** was found as a stable, water soluble, highly potent, human specific TLR agonist. Brimble *et al.* applied ligand **19a** in the construction of conjugate **20**, via an innovative synthetic approach.[62] Most of the reported preparations to these type of molecules use a convergent synthesis, in which a specific building block was pre-synthesized and then coupled to an amino acid or an oligopeptide. The group of Brimble developed a thiol-ene coupling procedure which does not require any separately prepared building block.[62] The thioylated peptide **21** and vinyl palmitate **22** were irradiated with UV light in presence of 2,2-dimethoxy-2-phenylacetophenone (DMPA) as photo-initiator, leading to over 90% conversion. This new self adjuvanting peptide conjugate **20** prove to be remarkably potent, but its exact target, either TLR1/2 or TLR2/6 heterodimer, and also the reason for the specificity of **20** for human TLR2 remain unclear.[22]

TLR-7 targeting peptide conjugate

Ligands of the TLR 7 and/or TLR-8 receptor are intensively investigated and several small molecule agonists[15] have been discovered and immunologically evaluated in a mixture with a protein or conjugated to a proteins[63], antibodies[64], lipids[65] or other entities.[66] Also a few conjugates in which a TLR7 ligand is covalently connected to an antigenic peptide are also reported.[67] Fujita *et al.* reported the synthesis of partially protected 6-(4-amino-2-butyl-imidazoquinolyl)-norleucine **23**, the structure of which was based on the TLR7/8 ligand imidazoquinoline (Figure 8).[68] This modified amino acid could be applied in SPPS, using Fmoc chemistry and Rink-amide PEG MBHA resin. This led to the assembly of peptide conjugates **24a** and **24b**, in which the TLR7/8 ligand was attached to the N- and C-terminal end of the peptide M2e antigen of influenza A virus. The produced conjugates led to a poorly antigenic peptide with self-adjuvanting properties.[68]

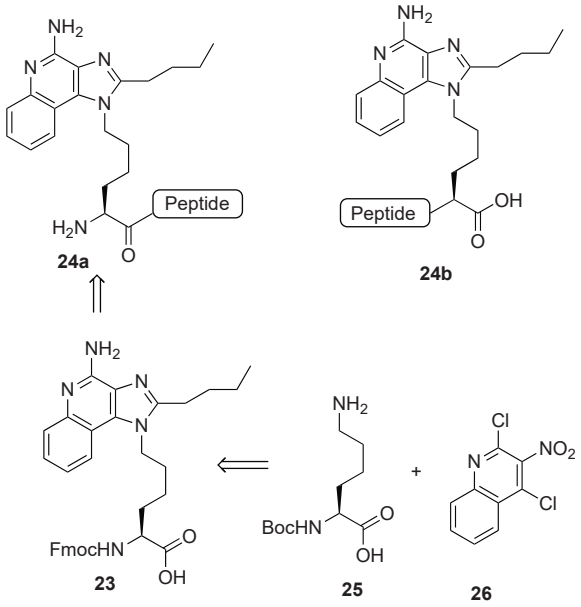

Figure 8. Retro synthesis of TLR-7 agonist peptide conjugate

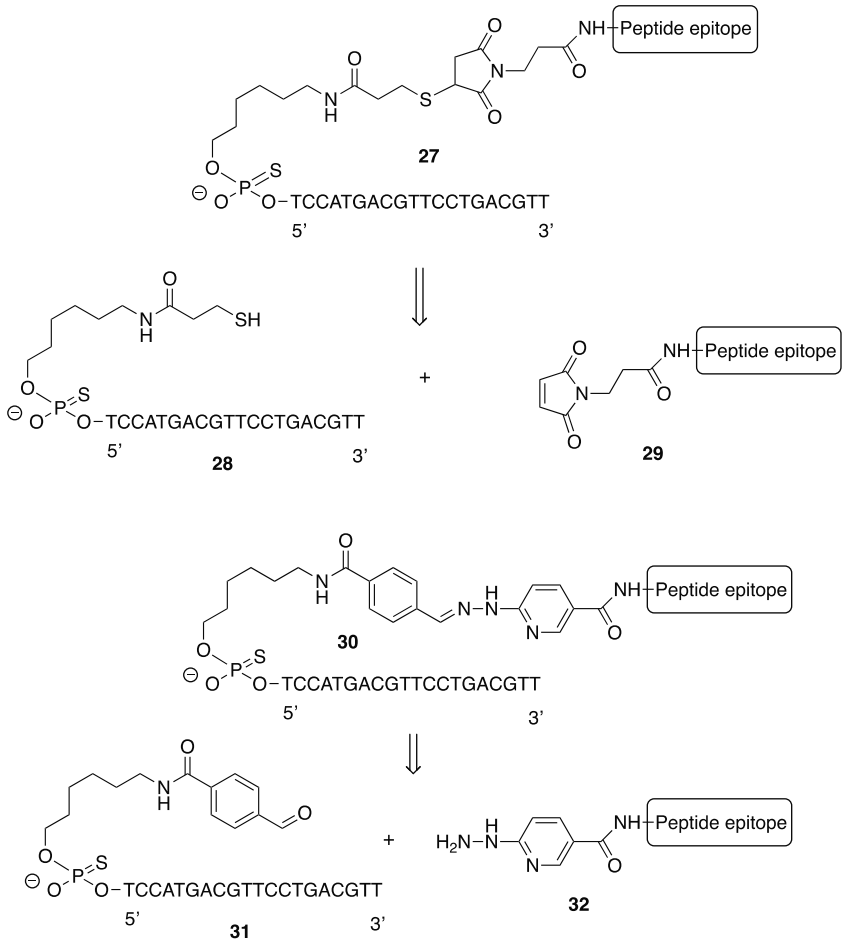

Figure 9. Synthesis approaches toTLR9 peptide conjugates

TLR-9 targeting peptide conjugate

CpG, an oligodeoxynucleotide fragment of specific sequence and length, is an agonist for TLR-9[31,69,70] In order to obtain a TLR9 peptide conjugate several convergent synthesis approaches are explored in which the CpG oligonucleotide with a reactive group at the 5'-end is coupled in solution with a selected peptide epitope provided at the N- or C-terminal end with a corresponding reactive group.[50] Both the functionalized CpG fragment and the functionalized peptide are prepared via a standard solid phase procedures and purified before conjugation. Diamond *et al.* successfully assembled self adjuvanting immuno peptides (**29**) using a peptide epitope bearing a maleimide moiety on the N terminus (**27**) and a CpG oligonucleotide, having a thiol function at the 5'-end (**28** Figure 9).[50] This stategy was applied using various relevant peptide epitopes in order to synthesize a library of TLR-9 mediated self adjuvanting vaccine candidates. In another strategy a hydrazine reactive group in the peptide (**32**) was combined with an aldehyde at the 5'-end of the CpG oligonucleotide (**31**) to give a conjugate with a hydrazone

linkage (**30**, Figure 9) The obtained conjugate proved to be more potent than just a mixture of the CpG and the immunogenic peptide.[50]

Conclusion

TLRs are very attractive drug targets that are intensively investigated not only for the development of new adjuvants for improved vaccines but also in the search for new classes of vaccines, such as cancer vaccines. In this respect multiple studies have been directed to design and optimize specific small molecule agonists for these PRRs. Besides, antagonists of TLRs may be applied for the treatment of autoimmune diseases. Furthermore structurally defined TLR ligands are explored in the search for fully synthetic vaccines. The first steps to the development of such vaccines are the here described conjugates comprising TLR ligand(s) and peptide epitope(s). From a synthesis point of view multiple challenges remain such as to overcome the low solubility of these conjugates and the development of improved functionalization methods (post synthetic labelling, introduction of multiple orthogonal handles).

Outline of this thesis

Chapter 2 describes a post-synthetic methodology to introduce a fluorescent label in highly lipophilic, Pam_3Cys based conjugates, consisting of the TLR-2 ligand covalently connected to an immunogenic peptide. The fluorescent labels were appended to the peptide part of the conjugate with the aid of a strain promoted [3+2] azide-alkyne cycloaddition. The prepared fluorescent lipopeptides triggered DCs maturation in TLR-2-dependent way. Furthermore, the conjugates labelled with label Cy-5 could be successfully used in confocal microscopy studiesand were taken up by dendritic cells in a TLR-independent manner. In **Chapter 3** a synthesis is discussed of a structurally simple human specific TLR-2 ligand with diminished lipophilicity, as compared to Pam_3Cys. Conjugation of such moiety to peptide is studied and optimized to produce human specific analogues of the conjugates described in **Chapter 2** with higher solubility and an equal propensity to activate TLR-2. The synthesis of a newly designed TLR-7 agonist is demonstrated in **Chapter 4** as well as the synthesis of a selection of self-adjuvanting immunogenic peptides that contain a model MHC-I epitope (SIINFEKL). Such constructs are designed in a way similar to that described in **Chapter 2** and **Chapter 3**. A biocompatible methodology to reduce an azide in a side chains of peptides is described in **Chapter 5** with a particular focus on side reaction occurring during the reduction. A selection of phosphines is evaluated for their capacity to reduce the azide functionality in a peptide context and under biocompatible aqueous conditions. The pH dependency of the product ratio has been investigated as well. **Chapter 6** describes the development of a convergent synthesis of the naturally occurring conjugate between the 5'-terminal fragment of genomic RNA from Coxsackie virus and the full-length viral genome-linked protein (VPg). Towards this end, a novel solid-phase methodology has been developed, which is based on the 5'-O-levulinyl ester as the temporal protection in the synthesis of the target RNA-oligonucleotide attached to a pentapeptide fragment from the VPg.

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Chapter 2: Synthesis and evaluation of fluorescent Pam_3Cys peptide conjugates

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Introduction

Conjugated cancer vaccines have attracted much attention as a promising lead for innovative therapeutic interventions[1–5]. A particular flavour of conjugated vaccines, that has been extensively investigated through the years, comprises a structurally defined construct of a Toll-like receptor agonist covalently attached to a synthetic peptide, that contains a T-cell epitope, either model or tumor associated[6]. It has been discovered that a conjugate of this kind show improved T-cell priming and tumor protection when compared to a mixture of the individual antigenic peptide and Toll-like receptor agonist[7,8]. The usefulness of such synthetic peptide based conjugates in tumor vaccination has been demonstrated as well. A commonly used agonist in these studies is a lipopeptide known as Pam_3CysSK_4 that binds to TLR2/TLR1[9–11]. This compound has been derived from the N-terminus of a bacterial lipoprotein of, among others, *E.coli*[12]. Notably, Pam_3CysSK_4 when applied as a component of a vaccine candidate either covalently attached to a longer peptide sequence or simply admixed with a peptide, is often present as a mixture of R- and S-epimers at the glycerol moiety, while it is known that the R-epimer is the biologically active one[13].

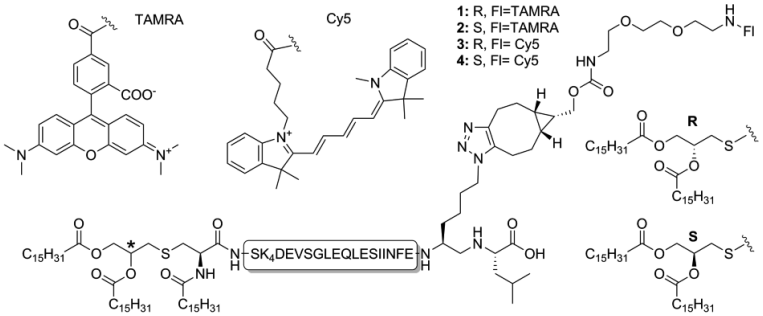
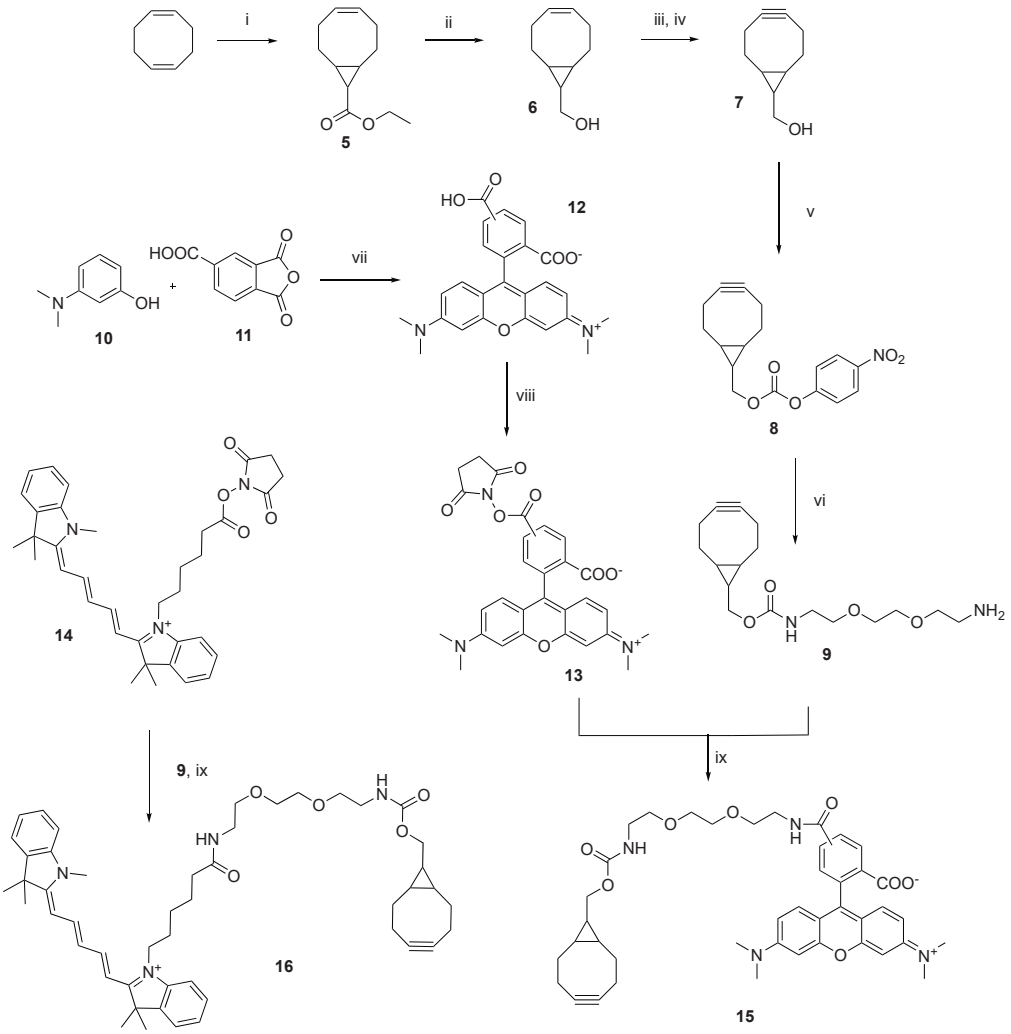


Figure 1. Target labelled Pam_3Cys-lipopeptides



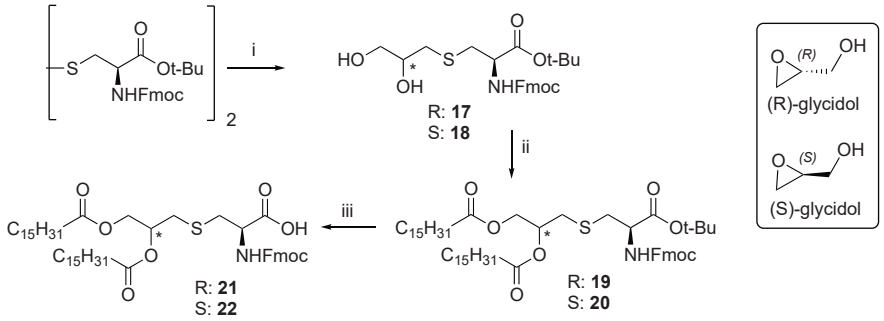
Scheme 1. Synthesis of the reactive dyes **15** and **16** functionalized with a strained alkyne. Reagents and conditions: i) $N_2CH_2C(O)OEt$, $Cu(C_5H_7O_2)_2$, EtOAc,78%, ii) $LiAlH_4$, THF/Et_2O, 91%, iii) Br_2, DCM, iv) KOtBu, THF, 35%, v) p-$NO_2PhOC(O)Cl$, DCM, 59%, vi) 1,8-diamino-3,6-dioxaoctane, NEt_3, DMF, 76%, vii) Cat. H_2SO_4, AcOH, reflux, 30% viii) SuOH, DIC, DMF, ix) D*i*PEA, DMF.

With the aid of non-labelled Pam_3CysSK_4 conjugates it has been shown that R-epimer of Pam_3Cys is indeed the one responsible for dendritic cell (DC) maturation and the S-epimer is inactive while the cellular uptake remained unaffected by the chirality of the glycerol moiety of the Pam_3Cys residue, as judged by the level of the antigen presentation by DC's[13]. In this chapter, it is shown that fluorescently labelled and chirally pure Pam_3Cys-lipopeptides represent useful tools in the studies of antigen processing because these constructs allow a visual evaluation of the antigen uptake irrespective of the DC-maturation status. Towards this end conjugates **1**-**4** (Figure 1) with the fluorescent label covalently attached to the modified side chain of a lysine residue in the commonly used model MHC-I epitope (SIINFEKL) have been synthesized. This design of the

labelled construct proved to be successful in studies preceding this one and that involved the monitoring of the intracellular trafficking of Pam_3Cys-lipopeptides as mixtures of epimers at C-2 of the glycerol moiety[7]. To be able to vary the type of fluorophore more readily a convergent approach based on copper free click chemistry[14–16] has been chosen in the present work. The DC-maturation capacity of the constructs has been evaluated and the uptake of these was studied using confocal microscopy.

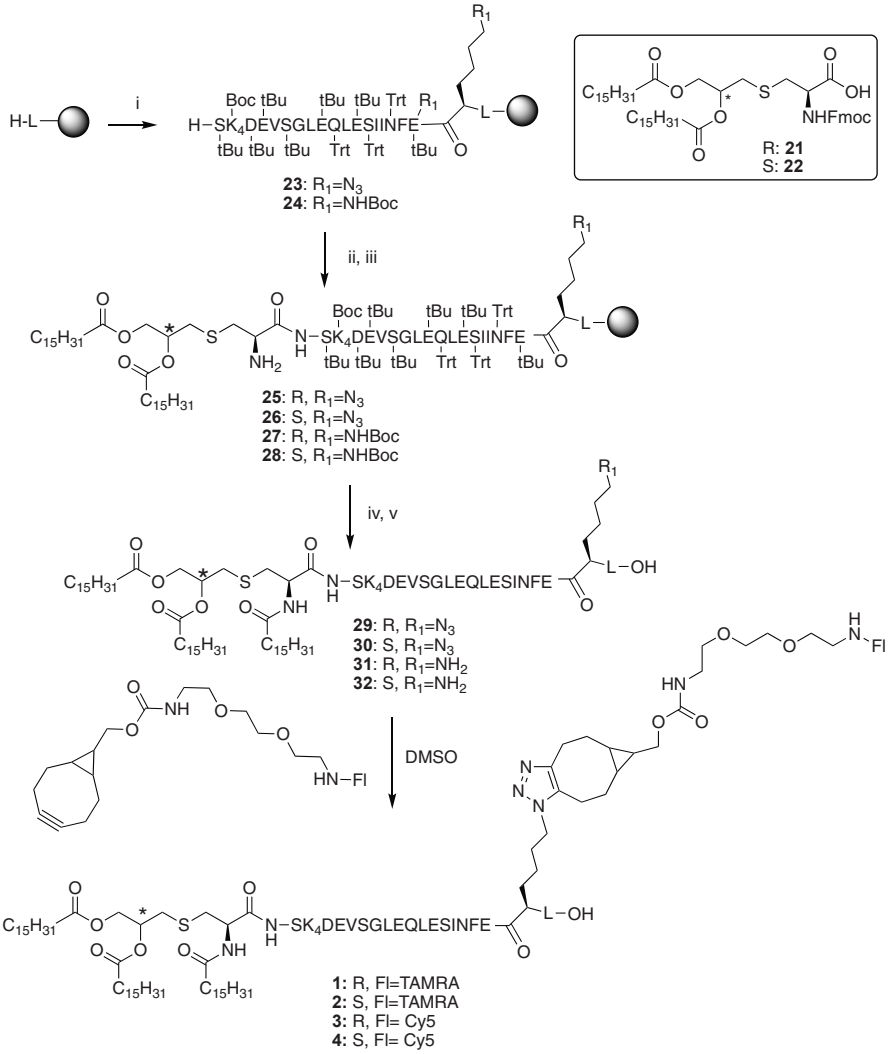
Results and discussion

The key step of the convergent synthesis of conjugates **1** – **4** in which the fluorescent labels are appended to the peptide with the aid of strain promoted [3+2] azide alkyne cycloaddition (Scheme 3) required the availability of azide containing lipopeptides (**29**, **30**) and dyes functionalized with a strained alkyne (**15**, **16**, scheme 1). The lipopeptides **29** and **30** were accessible via standard Fmoc-based solid phase synthesis using chirally pure Fmoc-Pam_2Cys-OH building blocks prepared as described in Scheme 2. The click-reaction prevents the use of a copper catalyst and requires the availability of the bifunctional (1R,8S)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (BCN) linker **9** to which fluorescent labels of choice can be attached via amide bond formation. The synthesis of BCN linker **9** (Scheme 1) is based on the coupling of known BCN 4-nitrophenyl carbonate (**8**) with 1,8-diamino-3,6-dioxaoctane. The reported procedure for the synthesis of BCN alcohol (**6**)[17] commences with cyclopropanation of 1,5-cyclooctadiene through a rhodium tetraacetate mediated Simmons-Smith type reaction to provide exo-**5** (28%) and endo-**5** isomers (58%). Although this step has been reported to be efficient, the cheaper copper acetoacetonate was evaluated as catalyst, in order to facilitate the scaling up of the synthesis. By using ethyl acetate instead of DCM to bring the reaction to higher temperature endo-**5** and exo-**5** could be obtained in 18% and in 58% yield, respectively. A notable difference with the rhodium catalyzed reaction is the appearance of exo-**5** as a major isomer (lowest running spot on TLC). The rest of the synthesis was performed without any major changes with respect to the literature procedure except that the exo-isomer was used to proceed with the synthesis. Ester **5** was reduced using $LiAlH_4$ in a mixture of Et_2O and THF to give BCN alcohol **6**. Subsequent bromination of the double bond in **6** using Br_2 followed by double elimination of bromide from the crude dibromide intermediate generated alkyne **7** in 35% yield. Treatment of **7** with p-nitrophenylchloroformate, followed by addition of 1,8-diamino-3,6-dioxaoctane to the resulting carbonate gave target bifunctional BCN linker **9** in 11% overall yield based on ethyl diazo acetate.



Scheme 2. Synthesis of enantiopure Pam_2Cys building block. Reagents and conditions: i) 1) Zn, H_2SO_4, HCl, MeOH, 2) (R) : (R)-Glycidol, RT, 85%; (S) : (S)-Glycidol, RT, 82%, ii) Palmitic acid, DIC, DMAP, DCM, 89-91%, iii) TFA, RT, 91-96%.

With the availability of BCN linker **9** the fluorescent labels TAMRA and Cy5 can be connected to the amine in bifunctional linker **9**. In order to allow optimization of the click reaction sufficient quantities of the relatively stable TAMRA dye should be available. Hence, using a slightly modified procedure from the literature[18] TAMRA was prepared and coupled to BCN linker **9** on mmol scale (Scheme 1). Sulfuric acid mediated condensation of dimethylaminophenol **10** with trimellitic anhydride **11** in acetic acid instead of butyric acid proceeded smoothly to give **12** as a mixture of regioisomers. Crude **12** was precipitated from diethyl ether and the obtained partially purified compound was converted into hydroxysuccinimide ester **13.** Subsequently BCN linker (**9**) was added to the reaction mixture to give fluorescent reagent **15.** After HPLC-purification TAMRA reagent **15** could be obtained as a single isomer in high purity in a low overall yield. The corresponding Cy5 reagent **16** was prepared according to the same procedure using the commercially available hydroxysuccinimide ester of Cy5 **14** and the crude product was immediately used in the ensuing cycloaddition.



Scheme 3. Synthesis of labelled labelled Pam_3Cys-lipopeptides. Reagents and conditions: i) SPPS Fmoc automated synthesis, ii) **21** or **22**, HCTU, D*i*PEA, NMP, iii) 20% piperidine, NMP, iv) PamCl, Pyridine/DCM, v) 95% TFA, 2.5% TIS, 2.5% H_2O. ON = overnight

Synthesis of chirally pure Pam_2Cys building blocks **21**(R) and **22**(S), as shown in Scheme 2, is essentially as reported previously[19]. The disulfide bridge was reduced using activated zinc powder and subsequently enantiopure glycidol (R or S) was added in a one-pot procedure yielding corresponding diols **17** and **18**. Esterification with palmitic acid using carbodiimide as condensating agent was followed by deprotection of the *tert*-butyl ester with neat TFA to give the building blocks **21**(R) and **22**(S) in 57% and 59% overall yield respectively.
Having all building blocks in hand the R- and S-Pam_3CysSK_4 peptide conjugates **29-30** were assembled by standard solid-phase peptide synthesis SPPS using Fmoc-chemistry (Scheme 3). Commercially available suitably protected amino acids were applied while Fmoc-azidonorleucine was prepared based on a published procedure[20]. Automated SPPS was performed until the azide containing peptide **23** was reached. The optically pure R- and S-Pam_3CysSK_4 moieties were appended manually to immobilized peptide fragment **23** using modified cysteine building blocks **21**(R) and **22**(S), respectively and HCTU as a coupling agent. The known[13] lipopeptides **31**-**32** were prepared alongside to be used as controls. This manual coupling saved building blocks as only 1.2 eq **21** and **22** in an overnight reaction instead of the standard 5 eq for 1h could be used. Ensuing, Fmoc deprotection with piperidine was followed by coupling with 10 eq of palmitoyl chloride. Finally, TFA mediated removal of the side chain protecting groups and concomitant cleavage from resin yielded the lipopeptides **29-30**. It is important to note that lipopeptides **29-30** are poorly soluble in both aqueous and organic solvents and pure DMSO is needed for further processing. The use of DMSO brings along precautions as the oxidative power of DMSO together with traces of acid or water may induce oxidation of the thioethers in **29** and **30**. After purification by HPLC the azide containing Pam_3CysSK_4 peptide conjugates **29**(R) and **30**(S) were labelled with TAMRA and Cy-5. The azide containing conjugate (**29-30**) was dissolved in dry DMSO and TAMRA reagent (**15**) was added in 1:1 ratio. After overnight stirring at room temperature, LCMS analysis showed complete conversion of the starting peptides and the untreated reaction mixture was immediately used for purification by preparative RP HPLC, yielding the labelled lipopeptides **1-2**. Introduction of the Cy5-fluorophore with crude reagent **16** using the same procedure, as described for the TAMRA dye (**15**), gave after HPLC purification the labelled lipopeptides **3-4**.

Biological evaluation

Immunological evaluation of labelled conjugates **1-4** started with assessing murine DC-maturation upon exposure to the conjugates as well as relevant reference compounds. DCs were stimulated for 48h with either the R-Pam_3Cys or the S-Pam_3Cys and DC maturation was measured by IL-12 production (Figure 2). Cells treated with R-Pam_3Cys containing lipopeptide (**31**) produced significantly higher amounts of IL-12 compared to the S-Pam_3Cys based counterpart (**32**). Similar results were found with the compounds labelled with Cy5 or TAMRA, showing intact immunogenicity of the fluorophore-labelled conjugates **1** and **3**.

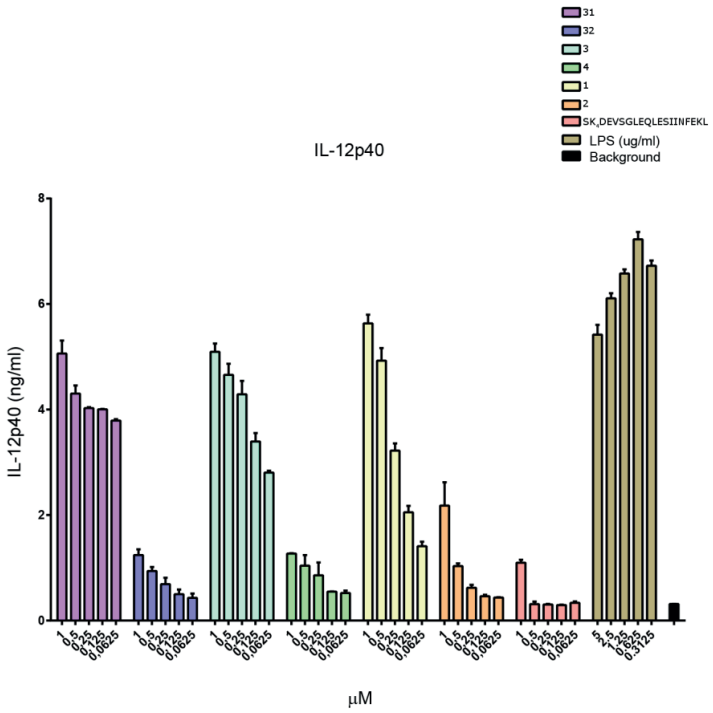


Figure 2. Activation of dendritic cells. DCs were stimulated with titrated amounts of either R-Pam$_3$Cys, S-Pam$_3$Cys (in labelled (**1**-**4**) or non-labelled (**31**-**32**) form; μM), LPS (positive control; μg/ml) or peptide (negative control) for 48h. Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA. One representative from three independent experiments is shown.

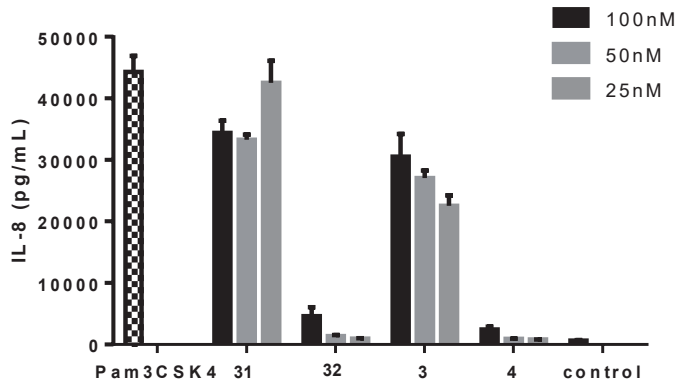


Figure 3. Ability of immunogenic lipopeptides in triggering human IL-8 production via TLR-2. (a) HEK TLR-2 cells were incubated with compounds **31**, **32**, **3** and **4** (100-25nM) or 100ng/mL Pam$_3$CysSK$_4$ for 24 h. Error bars represent SD.

To corroborate the TLR-2 dependent activation of DC's by the fluorescent conjugates the compounds were next assessed using HEK-cells transfected with TLR2. The level of IL-8 produced in the assay reflects the capacity of the conjugates to activate the receptor. The results (Figure 3) show the ability of compounds **31** and **3** to trigger human TLR-2. Compound **31** showed a similar behaviour to the natural TLR-2 ligand Pam_3CysSK_4 while compound **3** showed a lower ability in triggering TLR-2 especially at lower concentration (25nM). Compounds **32** and **4** showed no ability in triggering human TLR-2. To control the receptor specificity of immunogenic lipopeptides for TLR-2, HEK cells expressing TLR-4 were stimulated with compounds **31**, **32**, **3** and **4** (Figure 4). None of the compounds were able to trigger human TLR-4 showing not only the high specificity of the immunogenic lipopeptides for TLR-2 but also the absence of any inadvertent LPS contamination in the samples of the TLR-2 activating conjugates of this study (**3**, **4**, **31**, **32**).

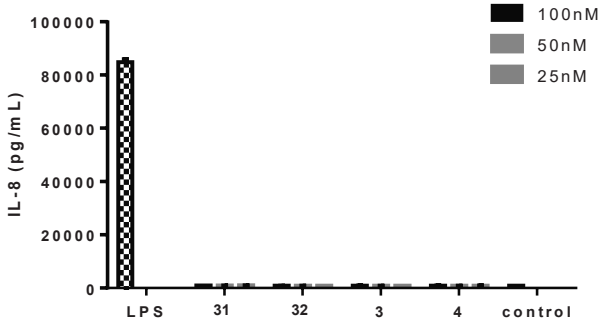


Figure 4. Pam-conjugates do not activate TLR-4. HEK TLR-4 cells were incubated with compounds **31**, **32**, **3** and **4** (100-25nM) or 10 ng/mL LPS for 24 h. Untreated cells were used as control. Supernatants were subsequently analyzed for IL-8 production by ELISA. The graphs are representative of two different independent experiments performed in duplicate.

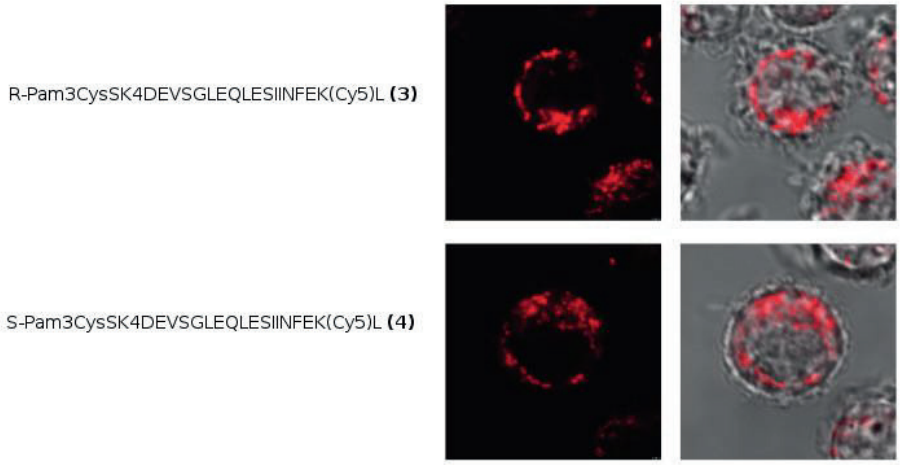


Figure 5. Uptake of Pam-conjugates by dendritic cells. DCs were incubated for 15 min with compounds 3 or 4 (1μM). The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy with Leica system settings as described.[20] The images are representative for multiple cells in at least 3 experiments.

The uptake of **3** and the **4** was measured with confocal microscopy. After 15 min, both compounds were efficiently internalized by murine DCs (shown in red and overlay with DC) and accumulated in hot spots surrounding the nucleus (Figure 5). Similar as have already been reported, no differences in localization or uptake intensity were observed[7,13].

Conclusion

Summarizing, using strain-promoted [3+2]cycloaddition a small set of fluorescent Pam_3Cys-based lipopeptides (**1-4**) has been successfully synthesized and compared to known immunogenic compounds (LPS, **31**, **32**). The R- and S-epimer of Pam_3Cys in the prepared fluorescent lipopeptides triggered DCs maturation in TLR-2-dependent manner and at approximately the same level as their unlabelled analogues. However, the poor aqueous solubility of the conjugates containing TAMRA (**1** and **2**) precluded the use of those for microscopy studies. This indicates that attaining sufficient solubility remains a major challenge in the synthesis of Pam_3Cys-based constructs labelled with fluorophores. Nevertheless, conjugate **3** (R-epimer) and conjugate **4** (S-epimer), both labelled with Cy-5, could be successfully used for confocal microscopy and were taken up by dendritic cells to the same extent. This result corroborates previous findings that suggested a TLR-independent uptake of the peptides conjugated to a TLR-ligand.

Experimental

General methods: All reactions were carried out in oven-dried (110 °C) glassware. Solvents were removed under reduced pressure using standard rotary evaporator. "Dry solvents" were dried over activated 4A° molecular sieves for at least 15 hours before use. All other chemicals were used as received. Thin layer chromatography analysis was performed on pre-coated silica gel 60 plates (Merck) and irradiated with UV light (λ=254 nm), sprayed with a staining solution of $KMnO_4$ (5 g), K_2CO_3 (25 g) in distilled water (1 L) followed by heating at around 150 °C. TLC-MS analyses were executed on a CAMAG TLC interface

connected to an Agilent 6130 Quadrupole or API 165 mass spectrometer. One- and two-dimensional ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz and 100 MHz for respectively ^{1}H and ^{13}C nuclei) instrument, with chemical shift (δ) in ppm relative to tetramethylsilane (TMS: ^{1}H, δ: 0 ppm). Spectra were recorded at room temperature. Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ: 589 nm). Infrared spectra were recorded on a Shimadzu FTIR-8300 and absorbance bands are reported in cm^{-1}. LC-MS measurements were done on an API 3000 Alltech 3300 with a Grace Vydac 214TP 4,6 mm x 50 mm C4 column and preparative high pressure liquid chromatography was conducted on a Gilson GX281 with an automatic fraction collector and Grace Vydac 214TP 10 mm x 250 mm C4 column or Gemini 5u C18 110A 250x10.0 mm. Buffer A: 0.1% TFA in MilliQ water, Buffer B: ACN. Solid phase peptide synthesis (SPPS) was carried out with an ABI 433A peptide synthesizer.

IL-12p40 ELISA

D1 dendritic cells (immature splenic DCs line derived from B6(H-2^{b}) mice were plated in a 96-wells plate and incubated with the compounds for 48h as indicated in the figure legends[21]. Supernatants were collected and tested with ELISA for IL-12p40 using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12p40 mAb (clone C15.6, Biolegend). Detection Ab: biotinylated rat anti-mouse IL-12p40 mAb (clone C17.8, Biolegend). Streptavidin-Poly-HRP (Sanquin) and 3,3',5,5' Tetramethylbenzidine (Sigma-Aldrich) were used as enzyme and substrate, respectively.

Confocal microscopy

D1 DCs were incubated with 1 μM **3** or **4** for 15min at 37 ˚C and washed with culture medium. The cells were plated out into glass-bottom Petri dishes (MatTek) and imaged using the Leica SP5-STED with a 63x objective lens. Differential interference contrast (DIC) was used to image cell contrast. Images were acquired in 10x magnification and processed with Leica LAS AF Lite software.

Activity assay on transfected TLR-2/4 HEK cells assay

Human TLR-expressing HEK cells were cultured in DMEM medium enriched with Penicillin/Streptomycin/Glutamine and 1% FCS. HEK TLR-2 and HEK TLR-4 cells were cultured in the presence of G418 (Geneticin, 0.5 mg/mL). Suspensions of 100 μL cells (1.10^{6} cells/mL) were stimulated for 24h with compounds **31**, **32**, **3** and **4** or appropriate control TLR ligands Pam_3CysSK_4 (100 ng/mL) for TLR-2, LPS, 10 ng/mL for TLR-4. Supernatants were subsequently analyzed for IL-8 production by ELISA.

(1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl 3,6,9-trioxa-12-azadodecylcarbamate (9)

To a solution of 1,5-cyclooctadiene (100 mL, 0.816 mol) and $Cu(C_5H_7O_2)_2$ (525 mg, 2 mmol) in EtOAc (50 mL) was added dropwise in 3 h a solution of ethyl diazoacetate (10.5 mL, 100 mmol) in EtOAc (50 mL). This solution was stirred overnightunder reflux. EtOAc was evaporated and the excess of cyclooctadiene was removed by filtration over a glass filter filled with silica and elution with EtOAc:heptane, 1:200. The filtrate was concentrated in vacuo and the residue was purified by column chromatography on silica gel to afford ***endo*-5** (3.5 g, 18 %) and ***exo*-5** (10.1 g, 58%) and mixed isomers (1.6g, 8.1%) as colorless oils. The rest of the synthesis was performed starting with ***exo*-5** as published.[17]

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 5.53 (m,1H), 3.90-3.88 (d, 2H), 3.55-3.44 (m, 8H), 3.30 (m, 2H), 2.33-2.05 (m, 8H), 1.28 (m, 2H), 0.67-0.58 (m, 3H)

^{13}C-NMR (100 MHz): ($CDCl_3$) δ: 156.88, 98.75, 73.00, 70.23, 70.14, 70.12, 68.93, 41.49, 40.72, 33.26, 23.73, 22.79, 21.36

HRMS: $[M+H]^{+}$: 325.21218 found: 325.21157

5-carboxy-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (12)

Dimethyl aminophenol (6.9 g, 50 mmol) and trimellitic anhydride (4.8 g, 25 mmol) were dissolved in AcOH (400 ml). After adding a catalytic amount of conc. H_2SO_4 (0.5 mL) the mixture was refluxed overnight. Reaction mixture was concentrated to a small volume and diethyl ether (200 mL) was added. Filtration of the precipitate yielded 2.7 g (30 %) of a mixture containing desired of regio-isomers **12**.

LCMS: RT (C_{18} column, 10%B-90%B, 13min grad): 5.28 min, 5.46 min

$[M+H]^{+}$:431.7

((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3',6'-bis(dimethylamino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxamido)ethoxy)ethoxy)ethyl)carbamate (15)

Regioisomeric mixture of TAMRA **12** (0.1 mmol, 0.043 g) was suspended in DMF(1 mL). N-hydroxy succinimide (0.1 mmol, 0.011 g) and DIC (0.1 mmol, 0.015 mL) was added and the reaction was stirred overnight at room temperature. Mixture was flushed over silica filter a concentrated. Regioisomeric mixture of TAMRA-OSu (0.056 mmol, 0.03 g) was dissolved in DMF (0.5 mL). D*i*PEA (0.056 mmol, 0.01 mL) and **9** (0.05 mmol, 0.0138 g) were added and the mixture was stirred overnight at room temperature. The crude product was directly purified by HPLC (C_{18} column, 20%-55%B, 30 min grad) yielding pure **15**.

LCMS: RT: (C_{18} column, 10%B-90%B, 13min grad): 5.28 min
$[M+H]^+$: 737.4

1-(1-((1*R*,8*S*,9*r*)-bicyclo[6.1.0]non-4-yn-9-yl)-3,14-dioxo-2,7,10-trioxa-4,13-diazanonadecan-19-yl)-3,3-dimethyl-2-((1*E*,3*E*)-5-((*E*)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium (16)
Cy5-OSu (1.6 µmol, 1 mg) was dissolved in DMF (1.5 mL). D*i*PEA (as 0.1M solution in DMF, 1.6 µmol, 16 µL) and **9** (0.015 mmol, 0.0041 g) were added and the mixture was stirred overnight at room temperature. The remaining of Cy5-OSu was quenched using 1,8-diamino-3,6-dioxaoctane (0.015 µmol, 2 µL) for 2h. The crude product was directly used without further purification.
LCMS: RT: (C_{18} column, 10%B-90%B, 13 min grad): 4.10 min
$[M+H]^+$: 789.6

N-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2R)-propyl]-(R)-cysteine tert-butyl ester (17)
$(Fmoc\text{-}Cys\text{-}OtBu)_2$ (1.64 mmol, 1.31 g) was dissolved in DCM (12.9 mL). Zinc dust (11.37 mmol, 0.74 g) and H_2SO_4/HCl/MeOH (5.5 mL, 1/7/100) were added and the reaction mixture was stirred at RT. After 15 min, (R)-Glycidol (16.56 mmol, 1.11 mL) was added, the resulting mixture was stirred for 5 h at 40°C. The reaction mixture was filtered and then concentrated under vacuum until half of the volume. The crude was diluted (EtOAc) and washed (10% aq. $KHSO_4$). The aqueous layer was back extracted with EtOAc three times. The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Silica gel column chromatography (50-80 % EtOAc in PE) yielded compound **17**(2.01 mmol, 0.9544 g, 61.3%).
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.76 (d, *J* = 7.5 Hz, 2H),7.69 – 7.58 (m, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 6.20 (d, *J* = 8.1 Hz, 1H), 4.61 – 4.48 (m, 1H), 4.40 (d, *J* = 7.3 Hz, 2H), 4.24 (t, *J* = 7.2 Hz, 2H), 3.90 – 3.78 (m, 1H), 3.70 - 3.57 (m, 2H), 3.01 (qd, *J* = 14.0, 5.8 Hz, 2H), 2.83 – 2.56 (m, 2H), 1.50 (s, 9H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:170.00, 156.24, 143.83, 141.28, 127.79, 127.15, 125.20, 120.05, 83.04, 71.18, 67.25, 65.24, 54.62, 47.07, 36.29, 35.44, 28.02
IR:3360, 1732.08, 1699.29, 1527.62, 1220.94, 758.02
α_D: -1.6°

N-Fluorenylmethoxycarbonyl-S-[2,3-dihydroxy-(2S)-propyl]-(R)-cysteine tert-butyl ester (18)
$(Fmoc\text{-}Cys\text{-}OtBu)_2$ (1 mmol, 0.797 g) was dissolved in DCM (7.9 mL). Zinc dust (6.92 mmol, 0.45 g) and a solution of H_2SO_4/HCl/MeOH (1/7/100) (3.37 mL) was added and the reaction mixture was stirred at RT. After 15 min (S)-glycidol (10.1 mmol, 0.75 g, 0.7 mL) was added to the flask and the mixture was stirred for 5 h under reflux (40 °C). The reaction mixture was then filtered, concentrated and diluted with EtOAc. The solution was washed (10% aq. $KHSO_4$) and the aqueous layer was back extracted 3 times (EtOAc). The organic layer was then dried ($MgSO_4$), filtered and concentrated. The crude was purified by column chromatography (50-80 % EtOAc/PE), compound **18** (1 mmol, 0.478 g, 50 %) was obtained.
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.78 (d, *J* = 7.4 Hz, 2H), 7.64 (d, J = 7.4 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.33 (t, *J* = 7.3 Hz, 2H), 6.02 (d, *J* = 8.1 Hz, 1H), 4.56 – 4.47 (m, 1H), 4.41 (d, *J* = 7.3 Hz, 2H), 4.25 (t, *J* = 7.2 Hz, 2H), 3.84 (m, 1H), 3.70 – 3.58 (m, 2H), 3.01 (qd, *J* = 14.0, 5.8 Hz, 2H), 2.84 – 2.57 (m, 2H), 1.51 (s, 9H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:169.8, 156.2, 143.8, 141.3, 127.8, 127.1, 125.2, 120.0, 83.1, 70.8, 67.3, 65.1, 54.6, 47.1, 36.4, 35.7, 28.0
IR:3350.35, 2933.73, 1697.36, 1149.57, 758.02.
α_D: +3°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine tert-Butyl ester (19)

Compound **17** (11.5 mmol, 5.44 g) was dissolved in dry DCM (150 mL) under argon condition. Consecutively palmitic acid (36.8 mmol, 9.42 g), DIC (44.85 mmol, 5.65 g, 7 mL) and DMAP (4.6 mmol, 0.56 g) were added. The reaction mixture was stirred over the weekend at RT. Glacial acetic acid (86.25 mmol, 5.2 g, 5 mL) was added and the solution was stirred for 15 min, then filtered and concentrated. Crystallization in DCM/MeOH (1/19) yielded compound **19** (8.9 mmol, 8.46 g, 77 %).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.80 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H,), 7.43 (t, *J* = 7.4 Hz, 2H), 7.35 (t, *J* = 7.4 Hz, 2H), 5.74 (d, *J* = 7.6 Hz, 1H), 5.19 (dd, *J* = 6.4, 3.7 Hz, 1H), 4.54 (dt, *J* = 7.7, 5.1 Hz, 1H), 4.46 – 4.34 (m, 2H), 4.27 (t, *J* = 7.2 Hz, 2H), 4.19 (dd, *J* = 12.0, 6.0 Hz, 1H), 3.08 (qd, *J*= 13.7, 5.0 Hz, 2H), 2.80 (d, *J* = 6.4 Hz, 2H), 2.33 (q, *J* = 7.3 Hz, 4H), 1.70 – 1.55 (m, 4H), 1.52 (s, 9H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:173.4,173.1,169.5, 155.7, 143.8, 141.3, 127.7, 127.1, 125.2, 120.0, 83.0, 70.2, 67.2, 63.5, 54.3, 47.1, 35.4, 34.3, 33.3, 32.0,29.7, 29.7, 28.0, 24.9, 22.7, 14.2

IR:3356.14, 2916.37, 2848.86, 1732.08, 1699.29, 1527.62, 1157.29, 736.81

α_D: -1.2°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2S)-propyl]-(R)-cysteine tert-Butyl ester (20)

18 (1 mmol, 0.478 g) was dissolved in dry THF (15 mL). Palmitic acid (4 mmol, 1.024 g), DIC (4.5mmol, 0.567 g ,0.7 mL) and DMAP (0.40 mmol, 0.05 g) were added. The mixture was stirred at RT for 5h. Then glacial acetic acid was added (8 mmol, 0.48 g, 0.5 mL) and the reaction mixture was stirred for 15 min at RT. The reaction mixture was then filtered and concentrated. Purification of the crude by crystallization (DCM/MeOH (1/19)) yielded **20** (1 mmol, 1.236 g, quant).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H,), 7.43 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.75 (d, *J* = 7.6 Hz, 1H), 5.19 (dd, *J* = 6.4, 3.7 Hz, 1H,), 4.56 (dt, *J* = 7.7, 5.1 Hz, 1H), 4.39 (m, 2H), 4.27 (t, *J* = 7.2 Hz, 2H), 4.18 (dd, *J* = 12.0, 6.0 Hz, 1H), 3.08 (qd, *J* = 13.7, 5.0 Hz, 2H), 2.80 (d, *J* = 6.4 Hz, 2H), 2.31 (q, *J* = 7.3 Hz, 4H), 1.65 – 1.55 (m, 4H,), 1.53 (s, 9H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:173.4, 173.0, 169.5, 155.7, 143.8, 141.3, 127.7, 127.1, 125.2, 120.0, 83.0, 70.3, 67.3, 63.4, 54.3, 47.1, 35.4, 34.3, 33.2, 32.0, 29.7, 29.7, 28.0 24.9, 22.7, 14.2

IR:3398, 2916.37, 2848.86,1737.86, 1701.22, 1531.48,1155.36, 736.81

α_D: -2.6°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2R)-propyl]-(R)-cysteine (21)

TFA (130.7 mmol, 14.9 g, 9.1 mL) was added to compound **7** (1.2 mmol, 1.1 g) and the solution was stirred for 1h at RT. Co-evaporation of TFA with toluene (3 x 10 mL) resulted in a crude compound. Purification of the crude by column chromatography (15%-20% EtOAc in PE/ 1% AcOH) yielded the final building block **21**, (R)-Pam_2CysOH (1.03 mmol, 0.9195 g, 86 %).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.88 (d, *J* = 7.9 Hz, 1H), 5.21 (dd, *J* = 6.3, 3.7 Hz, 1H,), 4.70 (q, *J* = 6.1 Hz, 1H), 4.48 – 4.34 (m, 2H), 4.27 (t, *J* = 7.1 Hz, 2H), 4.19 (dd, *J* = 11.9, 6.1 Hz, 1H) 3.15 (ddd, *J* = 46.3, 14.0, 5.5 Hz, 2H), 2.81 (d, *J* = 6.4 Hz, 2H), 2.33 (q, *J* = 7.7 Hz, 4H), 1.62 (d, *J* = 7.1 Hz, 4H), 1.29 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:174.3, 173.6, 173.5, 156.0, 143.7, 141.3, 127.8, 127.1, 125.2, 120.0, 70.3, 67.5, 63.6, 53.7, 47.1, 34.1, 33.0, 32.0, 29.8, 29.7, 24.9, 22.7, 14.2.

IR: 2916.37, 2848.86, 1730.15, 1521.84, 1168.86, 763.81

α_D: +8.2°

N-Fluorenylmethoxycarbonyl-S-[2,3-bis(palmitoyloxy)-(2S)-propyl]-(R)-cysteine (22)

TFA (1146.68 mmol, 130.72 g, 85.6 mL) was added to compound **20** (10.52 mmol, 10 g) under stirring. After 1 h the reaction mixture was co-evaporated with toluene (3x 90 mL). Purification was done via column chromatography (10% EtOAc/pentane, glacial AcOH 1%) and yielded compound **22** (S)-Pam_2CysOH (8.38 mmol, 7.49 g, 80%).

^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 7.79 (d, *J* = 7.5 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 2H), 7.43 (t, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 5.84 (d, *J* = 7.9 Hz, 1H), 5.21 (dd, *J* = 6.3, 3.7 Hz, 1H,), 4.71 (q, *J* = 6.1 Hz, 1H), 4.46 – 4.35 (m, 2H), 4.27 (t, *J* = 7.1 Hz, 2H), 4.17 (dd, *J* = 11.9, 6.1 Hz, 1H), 3.16 (ddd, *J* = 46.3, 14.0, 5.5 Hz, 2H), 2.88 – 2.72 (m, 2H), 2.33 (q, *J* = 7.7 Hz, 4H), 1.62 (d, *J* = 7.1 Hz, 4H), 1.28 (s, 48H), 0.91 (t, *J* = 6.7 Hz, 6H).

^{13}C-NMR (100 MHz): ($CDCl_3$) δ:174.4, 173.7, 173.4, 155.9, 143.7, 141.3, 127.8, 127.1, 125.2, 120.0, 70.3, 67.5, 63.6, 53.5, 47.1, 34.1, 32.9, 32.0, 29.8, 29.7, 24.9, 22.7, 14.2
IR:2916.37, 2848.86, 1701.22, 1521.84, 1151.50, 736.81
α_D: +10.6°

Imidazole-1-sulfonyl azide hydrochloride[22]
Sulfuryl chloride (16.1 mL, 200 mol) was added drop-wise to an ice-cooled suspension of NaN_3 (13.0 g, 200 mmol) in MeCN (200 mL) and the mixture stirred overnight at room temperature. Imidazole (25.9 g, 380 mmol) was added portion-wise to the ice-cooled mixture and the resulting slurry stirred for 3 h at room temperature. The mixture was diluted with EtOAc (400 mL), washed with H_2O (2 × 400 mL) then saturated aqueous $NaHCO_3$ (2 × 400 mL), dried over $MgSO_4$ and filtered. A solution of HCl in EtOH (obtained by the drop-wise addition of AcCl (21.3 mL, 300 mmol) to ice-cooled dry ethanol (75 mL)] was added drop-wise to the filtrate with stirring, the mixture chilled in an ice-bath, filtered and the filter cake washed with EtOAc (3 × 100 mL) to give Imidazole-1-sulfonyl azide.HCl as colorless needles (24.9 g, 59 %).

Fmoc-azidonorleucine
Fmoc-lysine.HCl (19.7 mmol, 7.98 g) was dissolved in 8:2 MeOH:H_2O (150 mL). $CuSO_4$ (0.5 mmol, 80 mg), $NaHCO_3$ (90 mmol, 7.56 g) and HCl.imidazole-1-sulfonyl azide (24 mmol, 5.0 g) was added. After overnight stirring, 3M HCl (aq) (30 mL) was added to acidify the solution to pH=2. The methanol was evaporated *in vacuo* and the mixture was diluted with EtOAc and consequently washed with 10% $KHSO_4$ (aq) (3x) and brine (1x). The solution was dried ($MgSO_4$), filtrated and concentrated. The crude was adsorbed on Celite and purified by silica gel column chromatography (From 9:1 Pentane :EtOAc to 5:5 Pentane :EtOAc, Δ=5%). Fmoc-azidonorleucine (14.8 mmol, 5.84 g) was obtained with an 75% yield.
HRMS: $[M+H]^+$: 395.17120 (calculated 395.17138)
^{1}H-NMR (400 MHz): ($CDCl_3$) δ: 9.38 (s, 1H), 7.74 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 5.8 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (m, 2H), 5.47 (d, *J* = 33.8 Hz, 1H), 4.51 (m, 1H), 4.39 (m, 2H), 4.20 (t, *J* = 6.8 Hz, 1H), 3.22 (m, 2H), 1.99 – 1.82 (m, 0.5H), 1.80 – 1.65 (m, 0.5H), 1.58 (m, 2H), 1.52 – 1.38 (m, 2H).
^{13}C-NMR (100 MHz): ($CDCl_3$) δ:176.38, 156.31, 143.82, 141.35, 127.83, 127.15, 125.12,120.09, 67.19, 53.62, 51.09, 47.14, 31.81,28.38, 22.51
IR: 2949, 2093,1694

R- Pam$_3$CysSK$_4$DEVSGLEQLESIINFEK(N$_3$)L (29)
The SK_4DEVSGLEQLESIINFEK(N_3)L (**23**) peptide was prepared by applying Fmoc-protocol starting from Tentagel S RAM resin (loading 0.23 mmol/g) on a ABI-433 peptide synthesizer. To a mixture of resin bound peptide (0.05 mmol,0.228 g) in NMP/DCM (1:1) was added (R,R)-Fmoc-Pam$_2$Cys-OH (**21**) (0.125 mmol, 0.111 g), PyBOP (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The D*i*PEA was added in two times, first an amount of 125 µL, after 10 min another amount of 125 µL. The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed three times with DCM and the Fmoc was cleaved with 20% piperidine/DMF (3 times, 5 min each). The free amine was palmitoylated by adding palmitic chloride (0.3 mmol, 0.137 g, 151 µL) in pyridine/DCM (1:1), the mixture was stirred for 2.5 h. The solution was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Purification of the conjugate was done by adding the crude to cold diethyl ether/n-pentane (1:1) (14 mL) and centrifugation (4000 rpm, 5min) was performed. The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure lipopeptide fractions were collected and concentrated by freeze-drying. This yielded conjugate **29** in 12% yield.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+3H]^{3+}$: 755.09364 (calculated :755.09397)

S-Pam$_3$CysSK$_4$DEVSGLEQLESIINFEK(N$_3$)L (30)
Compound **30** was synthesized (11% yield) using method described above using (S,R)-Fmoc-Pam$_2$Cys-OH **22** instead.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.3min
HRMS: $[M+2H]^{2+}$: 1791.60431 (calculated :1791.60281)

R- Pam_3CysSK_4DEVSGLEQLESIINFEKL (31)
Compound **31**(15% yield) was synthesized using method described above for **29** using Fmoc-Lys(Boc)-OH instead of Fmoc-Nle(N_3)-OH.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+2H]^{2+}$: 1778.61622 (calculated :1778.60756)

S-Pam_3CysSK_4DEVSGLEQLESIINFEKL (32)
Compound **32** (13% yield) was synthesized using method described above for **30** using Fmoc-Lys(Boc)-OH instead of Fmoc-Nle(N_3)-OH.
LC:RT: (C_4 column, 50%B-90%B, 13min grad): 8.4min
HRMS: $[M+2H]^{2+}$: 1778.61283 (calculated :1778.60756)
R-Pam_3CysSK_4DEVSGLEQLESIINFEK(TAMRA)L (1)
Compound **29** (0.22 µmol, 0.8 mg) was dissolved in dry DMSO (100 µL). TAMRA-BCN **15** (0.3 µmol, 0.22 mg) in dry DMSO (100 µL) was added to the solution. The reaction mixture was stirred at RT for 1 week. After HPLC purification, **1** (0.1 µmol, 0.43 mg) was obtained in 45% yield.
LCMS: RT: (C_4 column, 50%B->90%B, 13min grad): 7.9min
$[M+3H]^{3+}$: 1441.0
S- Pam_3CysSK_4DEVSGLEQLESIINFEK(TAMRA)L (2)
Compound **2** was synthesized (37% yield) using method described above starting with **30** instead of **29**.
LCMS: RT: (C_4 column, 50%B->90%B, 13min grad): 7.9min
$[M+3H]^{3+}$: 1441.3
R- Pam_3CysSK_4DEVSGLEQLESIINFEK(Cy5)L (3)
Compound **3** was synthesized as **1** using method described above using Cy5-BCN **16** instead of TAMRA-BCN **15** (52% yield).
LCMS:RT: (C_4 column, 50%B->90%B, 13min grad): 8.3min
$[M+3H]^{3+}$: 1458.0
S- Pam_3CysSK_4DEVSGLEQLESIINFEK(Cy5)L (4)
Compound **4** was synthesized using method described above starting with **30** instead of **29** (48% yield).
LCMS:RT: (C_4 column, 50%B->90%B, 13min grad): 8.3min
$[M+3H]^{3+}$: 1457.4

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Chapter 3: Design, synthesis and immunological evaluation of simplified self-adjuvanting TLR-2 stimulating peptides

This chapter is a part of a patent application: "Adjuvant compounds", NL2018803, filing date 27-04-**2017**)

Introduction

The human immune system consists of two interdependent parts, namely the adaptive and the innate system. As part of the innate immune system dendritic cells (DCs) express pattern recognition receptors (PRRs) that can bind non-self molecular structures, termed pathogen associated molecular patterns (PAMPs). Upon binding of a PAMP to the corresponding PRR, signal transduction pathways are initiated that ultimately lead to adaptive immune responses. The best known PRRs are the Toll-like receptors and in humans ten distinct TLRs have been identified.[1] Lipopeptides derived from the outer membrane of *Escherichia coli* are well known agonists for the TLR-2.[2-5] Currently, the synthetic Pam_3CysSK_4 lipopeptide, one of the most potent agonist for TLR-2, is applied in the development of new vaccines. For instance, conjugates of immunogenic peptides with Pam_3CysSK_4 resulted in highly potent self-adjuvanting vaccines conjugates.[6-9] However, the widening of the applicability of this class conjugates is hindered by solubility issues. Conjugates of Pam_3Cys ligand and antigenic peptides exhibit regularly unwanted aggregation or insolubility in mixtures of DMSO and/or aqueous buffers, by which the immunological evaluation can be hampered. This is certainly true when these conjugates are combined with labels or other PRR ligands (see Chapter 2). Although several studies towards the optimization of TLR2 ligands have been reported a break-through in terms of solubility came from the group of David[10-12], who developed mono-and bis-palmitoylated cysteines derivatives as potent TLR-2 ligands (**1** and **2**, respectively, Figure 1). Remarkable is the finding that their optimal ligand **1** is only active on human TLR-2 and is completely inactive in a murine model. As part of a new peptide lipidation method the group of Brimble[13] prepared and evaluated a conjugate of TLR-2 ligand **1** and peptide epitope NLVPMVATV derived from the cytomegalovirus. Based on their results, and the efforts of the group of Filippov on the development of new TLR2 ligands and the corresponding conjugates with antigenic peptides[14] five new TLR-2 ligands (**3**, **4**, **5**, **6** and **7**), two new conjugates (**8** and **9**) and conjugates labeled with the fluorophore Cy5 (**10** and **11**) were designed, prepared and evaluated (Figure 1).

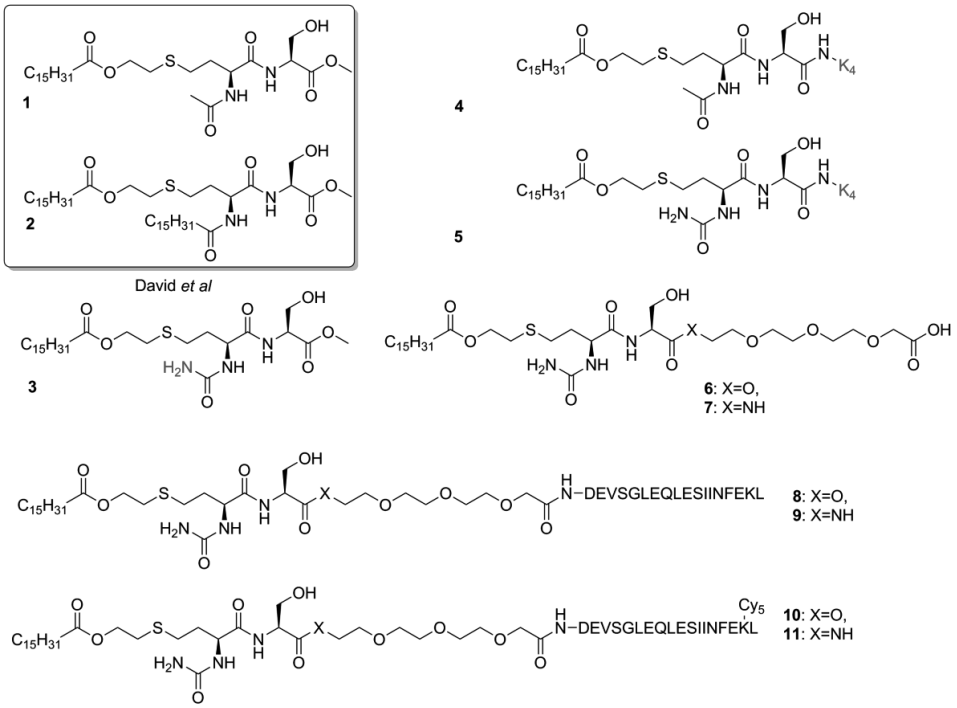
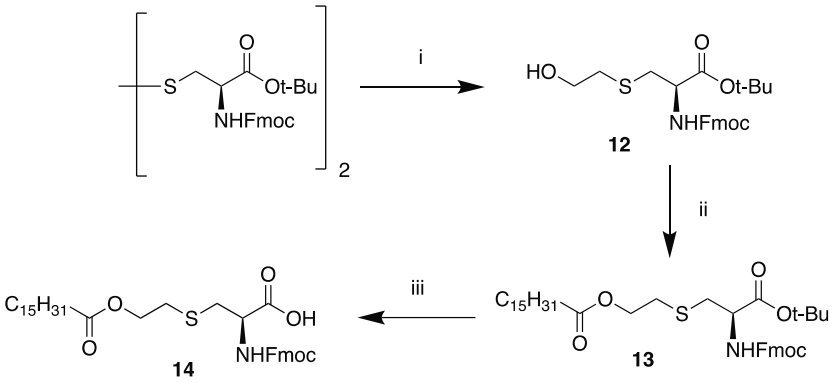


Figure 1. Target self-adjuvanting simplified TLR-2 lipopeptide.

The choice of the urea moiety in ligand **3** is based on the favorable influence of this functionality in the recently developed TLR-2 ligand, termed UPam[14]. In ligands **4** and **5** a tetra-lysine (K_4) segment, a frequently used solubility handle in the field of TLR-2 ligands, was appended. A triethylene glycol (TEG) segment with the same numbers of atoms as tetra-lysine was included as another solubility handle in the ligands **6** and 7[15]. Guided by the outcome of immunological evaluation, the latter ligands were coupled via an ester or amide linkage to MHC 1 epitope DEVSGLEQLESIINFEKL to give conjugates **8** and **9**, respectively. In addition, the corresponding labeled conjugates **10** and **11** were prepared. In this chapter, the synthesis of the prepared ligands and conjugates as well as their preliminary immunological evaluation are described.

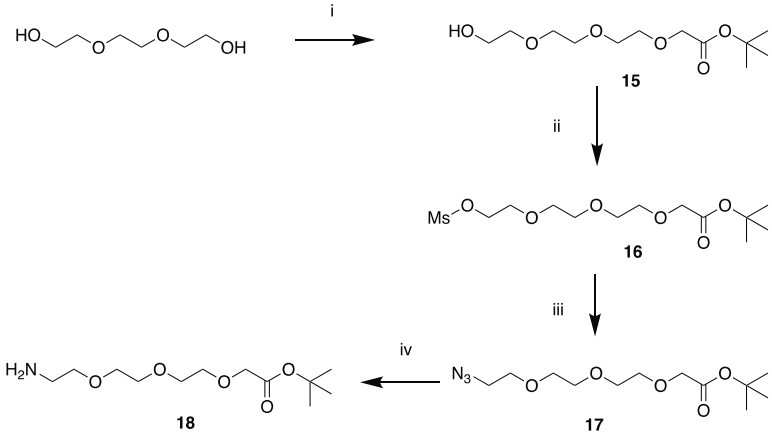
Results and discussion

To arrive at the projected ligands and conjugates (Figure 1), attention was first directed to the synthesis of cysteine derivative **14** (Scheme 1) and TEG spacers **15** and **18** (Scheme 2). Toward cysteine derivative **14** a published procedure was slightly adapted.[16]



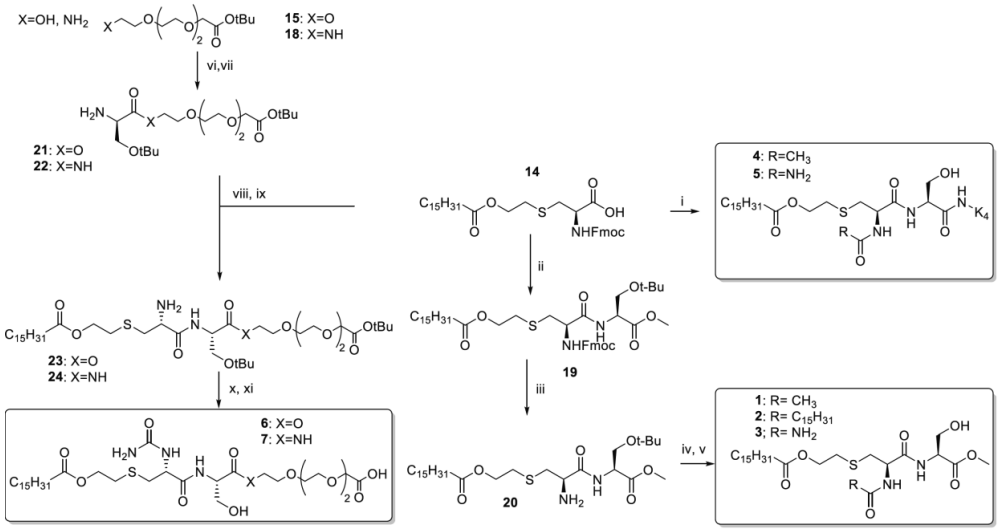
Scheme 1. Synthesis of simplified TLR-2 ligand building block. i) 1) Zn, H_2SO_4, HCl, MeOH 2) Oxirane, RT, 50% ii) Palmitic acid, DIC, DMAP, DCM, 88% iii) TFA, RT, 90%.

In a one pot-procedure commercially available Fmoc-Cysteine-tBu was first reduced using activated zinc in an acidic environment and upon completion of the reduction oxirane was added at 0°C to give alcohol **12** in 50% yield (Scheme 1). Subsequent esterification of **12** with palmitic acid to **13** using diisopropyl carbodiimide (DIC) and DMAP proceeded smoothly. Finally, tBu ester in **13** was cleaved with neat TFA to give target cysteine derivative **14** in 40% overall yield.



Scheme 2. Synthesis of TEG spacer. i) t-Butyl bromoacetate, NaH, TBAI, THF, RT, 40% ii) MsCl, TEA, DCM, RT, qt. iii) NaN_3, DMF, 60°C, 96% iv) 1) PPh_3, THF, RT 2) H_2O, RT, 45%

The preparation of the TEG spacer **18**, as illustrated in Scheme 2, started with a Williamson alkylation of triethylene glycol, with t-butyl bromoacetate in the presence of a catalytic amount of TBAI to yield the alcohol TEG spacer **15**. The alkylation was accompanied by the formation of substantial amounts of bis-substituted product. The synthesis of the amino TEG spacer **18** began with mesylation of **15**, followed by substitution with sodium azide to give compound **17**. Staudinger reduction of the azide in **17** led to isolation of the amine TEG spacer **18** in a moderate yield.



Scheme 3. Synthesis of TLR-2 ligands. i) Fmoc SPPS; TFA/TIS/H_2O (95/2.5/2.5) ii) H-Ser(tBu)-OMe.HCl, DIC, HOBt, Et_3N, DCM, 88% iii) 2% DBU, 2% Piperidine, DMF, RT, 88% iv) R= $C_{15}H_{31}$: Acetic anhydride, TEA, DCM, RT, 94%; R= $C_{15}H_{31}$: Palmitoyl chloride, pyridine, RT, qt.; R = NH_2: TMS-CN, i-PrOH, DCM, RT, qt. v) TFA, RT, 62%-94% vi) X = OH: Fmoc-Ser(tBu)-OH, DIC, DMAP, DCM, RT, qt.; X =NH_2: Fmoc-Ser(tBu)-OH, DIC, HOBt, DCM, RT, 47% vii) 2% DBU, 2% piperidine, DMF, RT, **21**:76%, **22** :qt. viii) DIC, HOBt, DCM, RT, X = O: 71%, X = NH: qt. ix) DBU, octanethiol, DCM, RT, **23**: 90%, **24** :88% x) TMS-CN, *i*-PrOH, DCM, RT, X = O: 90%, X = NH: 82% Xi) TFA/TIS/H_2O (95/2.5/2.5) RT, **6**: 80%, **7**:71%.

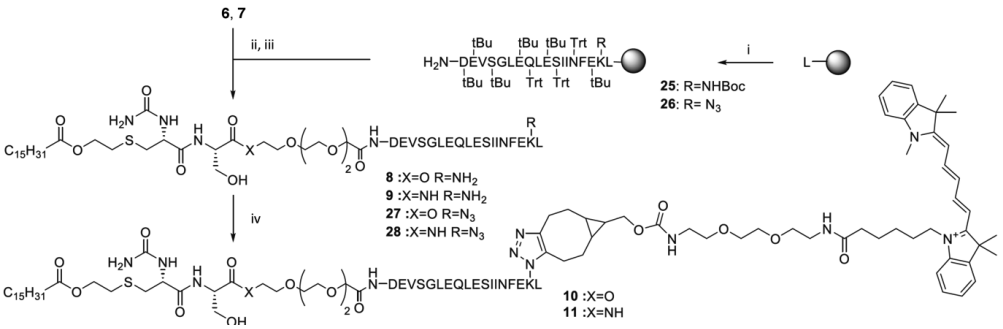
The reference ligands of the group of David (**1** and **2**) and the urea modified version **3** were prepared starting from cysteine derivative **14** (Scheme 3). In a standard procedure **14** was coupled with NH_2-Ser(tBu)-OMe using DIC and HOBt as coupling agents to give **19**. Ensuing Fmoc cleavage with a mixture of piperidine and DBU in dry DMF to prevent cleavage of the methyl ester gave common precursor **20**. Acylation of amine **20** with acetic anhydride or palmitoyl chloride, and subsequent cleavage of the tBu ether with neat TFA provided compounds **1** and **2**, respectively. En route to modified version **3** the urea moiety was installed by treatment of **20** with TMS isocyanate in the presence of isopropanol. Although the role of isopropanol is unclear, it appears to be mandatory for the success of this reaction. Similarly, to the synthesis of **1** and **2**, tBu ester was removed using neat TFA to afford ligand **3**.

Cysteine derivative **14** was also used in the solid phase peptide synthesis (SPPS) to tetra-lysine containing ligands **4** and **5**. With the aid of an automated peptide synthesizer and standard Fmoc chemistry immobilized SK_4 was prepared using Tentagel S RAM resin, Fmoc-Lys(Boc) and Fmoc-Ser(Ot Bu)-OH. Subsequent manual condensation of building block **14** with immobilized SK_4 under influence of PyBop was followed by Fmoc removal to give the immobilized peptide having a free amine. Acetylation of this amine with acetyl chloride and TFA/TIS mediated protecting group removal and cleavage from the solid support led the isolation of ligand **4**. Alternatively, the same immobilized peptide with a free amine was treated with TMS isocyanate as described for the conversion of **20** into **3** and subsequent TFA/TIS treatment gave after purification ligand **5**.

The ligands **6** and **7** were prepared in solution phase. First TEG spacers **15** and **18** were elongated with a serine moiety by a DIC mediated condensation with Fmoc-Ser(tBu)-OH, followed by Fmoc cleavage to afford **21** and **22**. Next cysteine derivative **14** was coupled to the free amine in **21** and **22** by the same sequence of events except that octanethiol was used as a scavenger during Fmoc

cleavage. Installation of the urea moiety at the newly obtained amine was performed as described above and acidic cleavage of the tBu groups yielded ligands **6** and **7**.

Having ligands **6** and **7** in hand the SPPS assembly of conjugates **8-9** and **27-28** was undertaken (Scheme 4). Immobilized peptides **25** and **26**, having a Boc protected lysine or an azidonorleucine incorporated were prepared with standard SPPS. Ligands **6** and **7** were appended manually by pre-activation with HCTU. The progress of the reaction was monitored with the aid of the Kaiser test. Upon completion of the synthesis, removal of the protecting groups and cleavage from the resin with a TFA cocktail led after HPLC purification to the isolation of both the conjugates **8-9** and the conjugates **27-28**, having an azido group. Conjugates **27** and **28** were labelled with Cy5-BCN in DMSO as described Chapter 2.



Scheme 4. Synthesis of labelled and unlabeled TLR2-ligand conjugates. i) Fmoc SPPS ii) HCTU, DIPEA, NMP iii) TFA/TIS/H_2O (95/2.5/2.5) iv) Cy5-BCN, DMSO.

Preliminary immunological evaluation was performed using two different cell types, TLR-2 transfected HEK cells and isolated human DC. Figure 2 shows that the ligands, provided with a TEG spacer (**6** and **7**) show enhanced capacity to activate both TLR2 HEK cells (Figure 2A) and human monocyte-derived dendritic cells (Figure 2B). Their TLR2-mediated IL production (IL-8 and IL-12 respectively) was improved compared to the original ligand of the group of David (compound **1**) and also to a chirally pure version of the classical Pam_3CSK_4 (Pam-R) ligand. The TEG spacer outperforms the usual K4 solubility handle as incorporated in ligand **5**. On the basis of these data it was decided to prepare and evaluate conjugates **8** and **9**, in which ligands **6** and **7** are covalently connected to the N-terminus of MHC 1 epitope DEVSGLEQLESIINFEKL as well as the corresponding Cy_5 labeled conjugates **10** and **11**. As shown in Figure 3A and 3B, using both HEK cells and human DC the IL production (IL-8 and IL-12 respectively) of the unlabeled conjugates remain largely intact while a decrease is seen with the labelled conjugates **10** and **11**. The IL production of the positive controls (Pam-R ligand and LPS) have the same order of magnitude.

Next conjugates **8** and **9** were evaluated for antigen presentation and conjugates **10** and **11** for uptake in dendritic cells. Antigen presentation was determined using SIINFEKL-specific T cells which are activated by antigen presentation by dendritic cells. Murine dendritic cells were first incubated with the conjugates **8** and **9** and subsequently with specific T cells. Figure 4 shows moderate antigen presentation by the DC. This could be expected as maturation of the murine DC does not occur with these human-specific TLR2 ligand. For the same reason the mouse bio-active Pam-R conjugate shows a much stronger antigen presentation in this assay. The uptake by

dendritic cells was analyzed using the Cy5 fluorophore-labeled conjugate compound **10**. Figure 4 shows efficient engulfment of the compounds in both human (Figure 5A) and murine dendritic cells (Figure 5B) in similar endo-lysosomal compartments as shown in earlier studies with Pam_3CSK_4-peptide conjugates[14]. This efficient uptake in mouse dendritic cells is in accordance with the previous findings of Khan *et al.* that uptake of Pam-based lipopeptides by DC is independent of TLR2 triggering.[17]

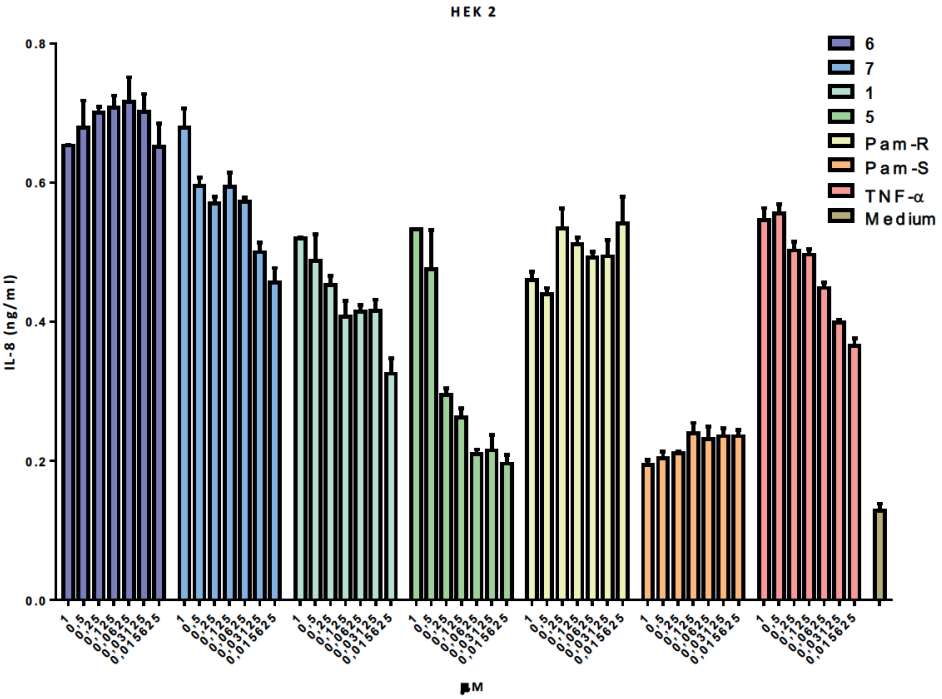


Figure 2A. Ability of the lipophilic ligands in triggering human IL-8 production via TLR-2. (a) HEK TLR-2 cells were incubated with titrated amounts of compounds **1**, **5**, **6**, **7**; R-Pam_3Cys, TNF-alpha (positive controls), S-Pam_3Cys (negative control) for 24 h. Error bars represent SD.

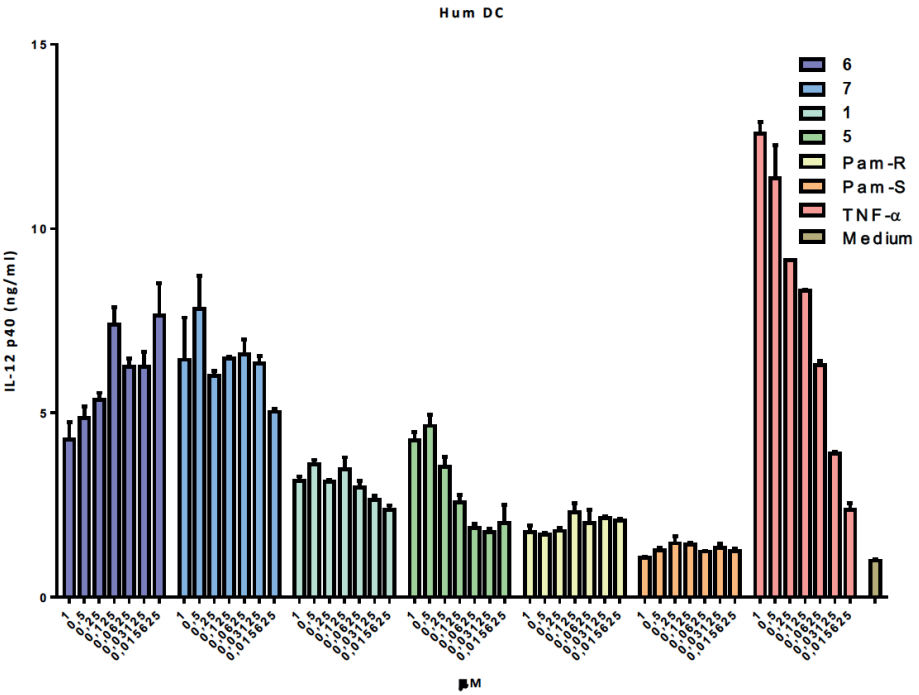


Figure 2B. Activation of human dendritic cells. DCs were stimulated with titrated amounts of compounds **1**, **5**, **6**, **7**; R-Pam$_3$Cys, TNF-alpha (positive controls), S-Pam$_3$Cys (negative control) for 48h. Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA. One representative from three independent experiments is shown.

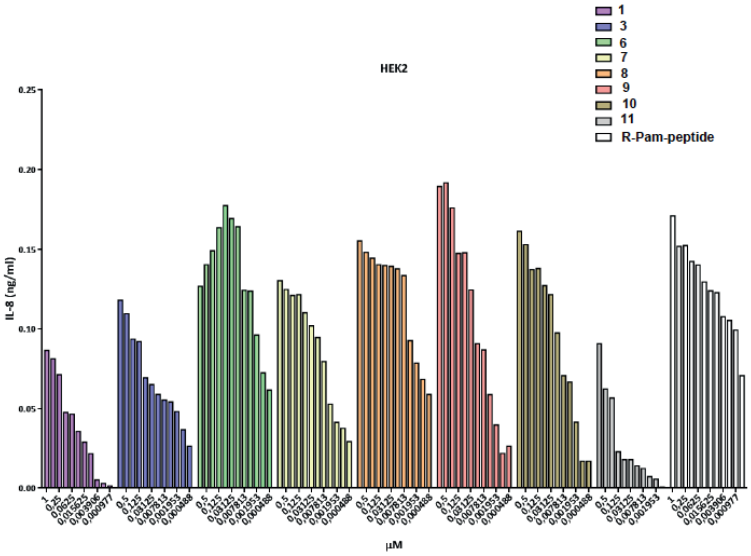


Figure 3A. Ability of the lipophilic ligands and lipopeptides in triggering human IL-8 production via TLR-2. (a) HEK TLR-2 cells were incubated with titrated amounts of compounds **1**, **3**, **6** – **11** or R-Pam3Cys, (positive control) for 24 h.

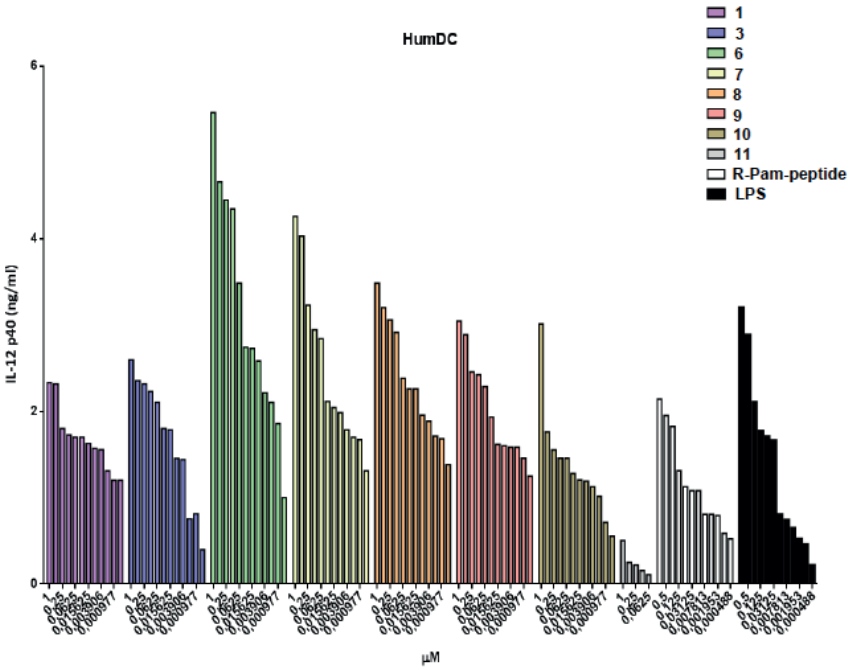


Figure 3B. Activation of human dendritic cells. DCs were stimulated with titrated amounts of compounds **1**, **3**, **6** – **11**; R-Pam$_3$Cys-lipopeptide, LPS (positive controls). Supernatants were harvested and analyzed for IL-12 cytokine secretion by ELISA as in Figure 2B.

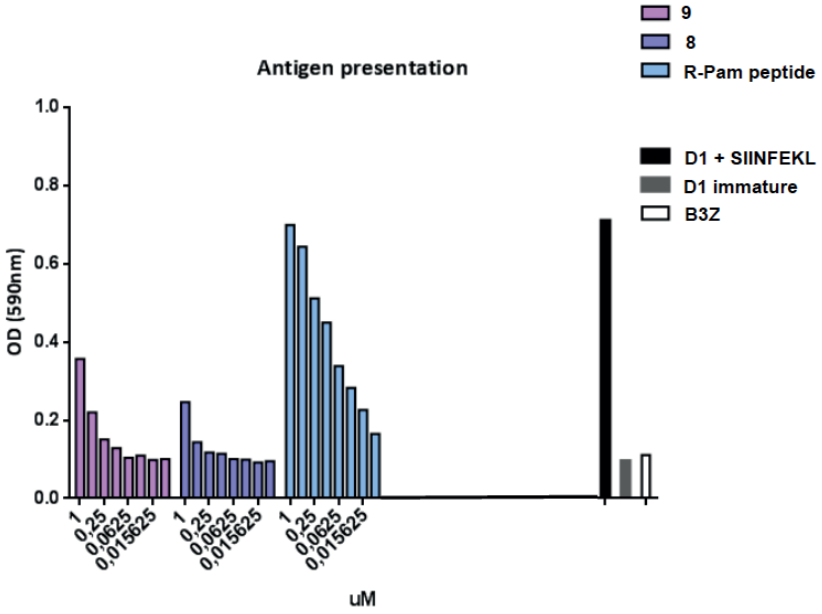


Figure 4. Antigen presentation of SIINFEKL MHC-I epitope by Dendritic cells loaded with lipopeptides **8** and **9.**

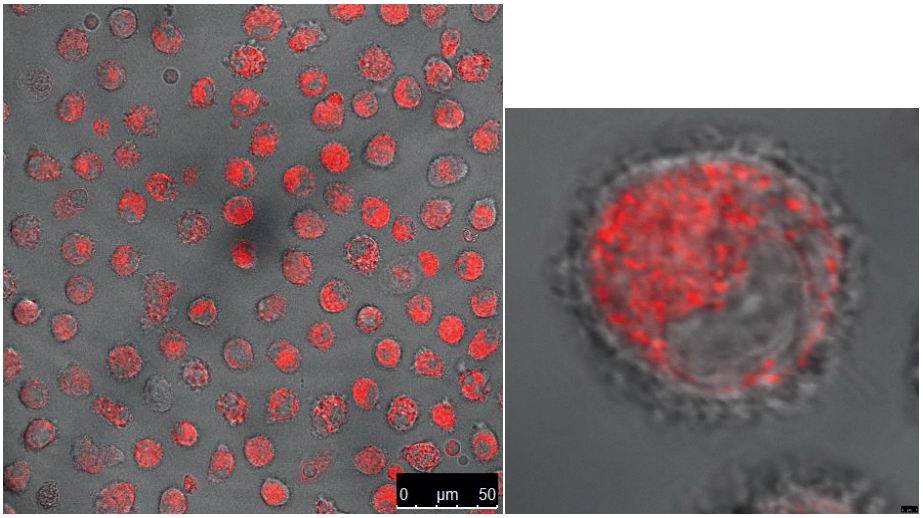


Figure 5A. Uptake of conjugate **10** by human moDC. The cells were incubated for 15 min with compound **10** (1µM). The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy. The images are representative for multiple cells in at least 3 experiments.

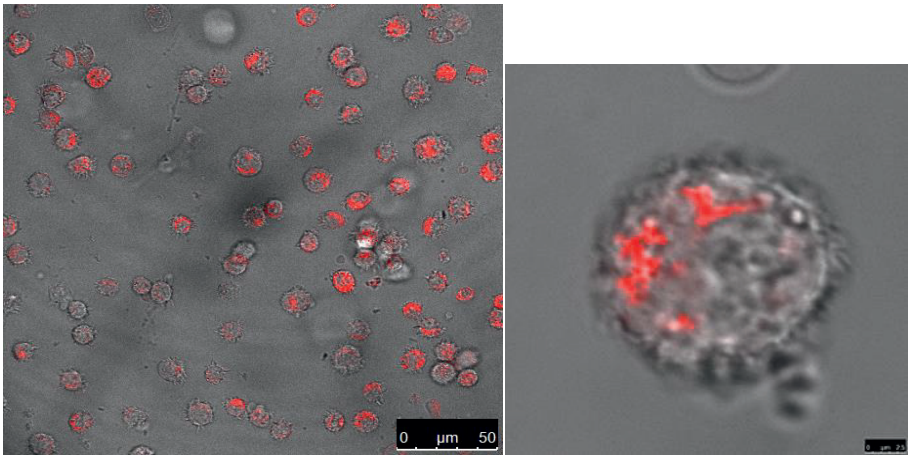


Figure 5B. Uptake of conjugate **10** by mouse D1-cells. The cells were incubated for 15 min with compound **10** (1µM). The uptake and localization of the compounds were analyzed with confocal laser scanning microscopy. The images are representative for multiple cells in at least 3 experiments.

Conclusion

This chapter describes the synthesis of five new TLR-2 ligands (**3**, **4**, **5**, **6** and **7**), two new conjugates (**8** and **9**) and the corresponding conjugates labeled with the fluorophore Cy5 (**10** and **11**). Preliminary immunological evaluation of ligands **5**, **6** and **7** shows that the presence of 4 lysines (K_4) as a solubility handle next to the serine in TLR-2 ligand **5** does not contribute favorably to the IL production. It can be hypothesized that steric hindrance contributes to this loss of activity. Ligands **6** and **7** which are provided with a less sterically demanding triethylene glycol spacer retain the water solubility and are much more potent than their lysine alternative (**5**). Attachment

of Ligands **6** and **7** to the N terminus of the DEVSGLEQLESIINFEKL model epitope furnished conjugates **8** and **9.** The agonistic activity of **8** and **9** remains mainly intact with conjugate **8** being slightly more active than amide analogue **9**. Internalization of the labeled conjugate **10** could be properly visualized on both human monocyte derived DC and mouse dendritic cells. These results are an incentive to synthesize and evaluate conjugates similar to **8** provided with a human specific epitope to allow the assessment of antigen presentation by DCs

Experimental

All solvents used under anhydrous conditions were stored over 4Å molecular sieves, except for methanol, which was stored over 3Å molecular sieves. Solvents used for workup and column chromatography were of technical grade from Sigma Aldrich and used directly. Unless stated otherwise, solvents were removed by rotary evaporation under reduced pressure at 40°C. Reactions were monitored by TLC-analysis using Merck 25 DC plastikfolien 60 F254 with detection by spraying with 1% $KMnO_4$, 10% Na_2CO_3 (aq) (unless stated otherwise) followed by charring at approx. 150°C. Column chromatography was performed on Fluka silicagel (0.04 – 0.063 mm). Analytical LC/MS was conducted on a JASCO system using an Alltima C_{18} analytical column (5µ particle size, flow: 1.0 ml/min), on which the absorbance was measured at 214 and 254 nm. Solvent system for LC/MS: A: 100% water, B: 100% acetonitrile, C: 1% TFA. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 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 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.2842) as a "lock mass". The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H and ^{13}C NMR spectra were recorded with a Brüker AV 400 (400/100 MHz) and all individual signal were assigned using 2D-NMR spectroscopy. Chemical shifts are given in ppm (δ) relative to TMS (0 ppm) and coupling constants are given in Hz. Optical rotations were measured in $CHCl_3$. IR spectra were recorded on a Shimadzu FTIR-8300 and are reported in cm^{-1}. Specific rotation was measured in chloroform at 10 mg/mL concentration.

N-Fluorenylmethoxycarbonyl-S-[2 hydroxy ethyl]-(R)-cysteine tert-Butyl ester (12) To a solution of protected cysteinedisulfide (1.04 mmol, 835 mg) in THF (10 mL) was added zinc powder (7 mmol, 455 mg, <10 µm) and a 100:7:1 solution of MeOH:37% HCl:98% H_2SO_4 (5 mL). The mixture was stirred for 15 min at RT. Oxirane (10 mmol, 0.51 mL) was added at 0°C and the mixture was stirred overnight at RT. TLC analysis (2:8 EA:pentane, R_f=0,7) showed complete conversion and the reaction mixture was filtrated and concentrated *in vacuo*. The crude was dissolved in EtOAc and washed with 10% $KHSO_4$ (aq). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. Purification by silica gel column chromatography (4:6 EA:pentane, R_f=0,4) yielded compound **12** (1.07 mmol, 477 mg) in a 50% yield.
IR(cm^{-1}): 2917,2850, 1742, 1660, 1463.
HRMS $[M+H]^+$: 444.18392 (calculated), 444.18436 (measured).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.3 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 5.90 (d, *J* = 7.9 Hz, 1H), 4.55 – 4.45 (m, 1H), 4.39 (m, 2H), 4.22 (t, *J* = 7.0 Hz, 1H), 3.77 – 3.66 (m, 2H), 3.06 – 2.87 (m, 2H), 2.80 – 2.65 (m, 2H), 1.47 (s, 9H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 169.75, 156.00, 143.85, 141.31, 127.75, 127.11, 125.15, 120., 83.04, 67.15, 60.90, 54.62, 47.14, 36.31, 35.04, 28.02,
N-Fluorenylmethoxycarbonyl-S-[2 palmitoyloxy ethyl]-(R)-cysteine tert-Butyl ester (13)
Compound **8** (2.23 mmol, 987 mg) was dissolved in dry DCM (30 mL). Palmitic acid (6.69 mmol, 1.72 g), DIC (8.92 mmol, 1.41 mL) and DMAP (1.1 mmol, 0.14 g) were added. The mixture was stirred overnight at RT under argon atmosphere. TLC analysis (4:6 EA:pentane, R_f=0.4) showed complete conversion. Glacial acetic acid (1 mL) was added and the mixture was stirred for 15 min at RT. The reaction mixture was filtrated and concentrated *in vacuo*. The crude was dissolved in PE and filtrated. The filtrate was purified

by silica gel column chromatography (5:95 EA:pentane, R_f=0.15) and compound **9** (1.97 mmol, 1.34 g) was obtained with a 88% yield.

$[\alpha]_D$: +2°

IR(cm^{-1})= 3333, 2916, 2850, 1733, 1699, 1532.

HRMS $[M+H]^+$: 682.41354 (measured), 682.41359 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.61 (d, *J* = 7.3 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 5.73 (d, *J* = 7.6 Hz, 1H), 4.52 (dt, *J* = 7.5, 4.8 Hz, 1H), 4.46 – 4.28 (m, 2H), 4.22 (m, 3H), 3.04 (m, 2H), 2.77 (t, *J* = 6.6 Hz, 2H), 2.32 – 2.20 (m, 2H), 1.58 (d, *J* = 6.8 Hz, 2H), 1.45 (d, *J* = 26.7 Hz, 9H), 1.21 (m, 24H), 0.94 – 0.78 (t, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.61, 169.61, 155.75, 143.83, 141.33, 127.76, 127., 125.17, 120.02, 82.99, 67.18, 63.13, 54.38, 47.16, 34.92, 34.20, 31.99, 31.43, 29.76, 29.73, 29.68, 29.54, 29.44, 29.34, 29.20, 28.03, 24.94, 22.77, 14.22

N-Fluorenylmethoxycarbonyl-S-[2 palmitoyloxy ethyl]-(R)-cysteine (14)

Compound **9** (0.47 mmol, 0.32 g) was dissolved in neat TFA (5 mL) and stirred for 1 hour at RT. TLC analysis (10% EtOAc in PE, R_f= 0.3) showed complete conversion. The reaction mixture was concentrated and co-evaporated with toluene *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (15:85 EA:pentane + 1% acetic acid, R_f=0.15). Compound **10** (0.42 mmol, 0.26 g) was obtained with a 90% yield.

$[\alpha]_D$: +12.4°

IR(cm^{-1})= 3317, 2500-3200, 2916,2848, 1732, 1691, 1537

HRMS $[M+H]^+$: 626.36067 (measured), 626.35099 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H, COOH), 7.75 (d, *J* = 7.5 Hz, 2H, C-H Fmoc), 7.60 (d, *J* = 6.2 Hz, 2H C-H Fmoc), 7.39 (t, *J* = 7.4 Hz, 2H C-H Fmoc), 7.30 (t, *J* = 7.4 Hz, 2H C-H Fmoc), 5.79 (d, *J* = 7.8 Hz, 1H, N_{25}), 4.66 (m, 1H, C_2), 4.50 – 4.33 (m, 2H, C_{23}), 4.31 – 4.08 (m, 3H, C_{5+24}), 3.09 (m, 2H, C_3), 2.77 (t, *J* = 6.4 Hz, 2H, C_4), 2.28 (t, *J* = 7.6 Hz, 2H, C_7), 1.57 (m, 2H, C_8), 1.24 (m, 24H, $C_{9\text{-}20}$), 0.88 (t, *J* = 6.8 Hz, 3H, C_{21}).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.61 ($C_{1,6}$), 174.11 ($C_{1,6}$), 156.11 (C_{22}), 143.72 (C_q Fmoc), 141.40 (C_q Fmoc), 127.87 (C-H Fmoc), 127.20 (C-H Fmoc), 125.21 (C-H Fmoc), 120.12 (C-H Fmoc), 67.51 (C_{23}), 63.23 (C_5), 53.73 (C_2), 47.17 (C_{24}), 34.48 (C_3), 34.29 (C_7), 32.05, 31.35 (C_4), 29.83 ($C_{9\text{-}19}$), 29.79 ($C_{9\text{-}19}$), 29.75 ($C_{9\text{-}19}$), 29.61 ($C_{9\text{-}19}$), 29.49 ($C_{9\text{-}19}$), 29.40 ($C_{9\text{-}19}$), 29.26 ($C_{9\text{-}19}$), 24.99 (C_8), 22.82 (C_{20}), 14.27 (C_{21}).

$HO\text{-}(CH_2CH_2O)_3CH_2C(O)OtBu$ (15)

Triethyleneglycol (40 mmol, 5.3 mL) was dissolved in dry THF (200 mL) under argon atmoshphere. Sodium hydride (0.84 g, 21 mmol) was added at 0°C. Tetrabutylammonium iodide (2.0 mmol, 0.37 g) was added. tert-Butyl bromoacetate (20 mmol, 3.0 mL) was added and the reaction was stirred overnight at RT under argon atmosphere. The reaction mixture was filtrated and the THF was evaporated. The crude was adsorbed on Celite and purified by silica gel column chromatography (8:2 EA:pentane). Compound **15** (7.7 mmol, 2.0 g) was obtained with a 40% yield.

IR(cm^{-1})=2873(C-H, stretch), 1742(C=O, stretch).

HRMS $[M+H]^+$: 265.16298 (measured), 265.16456 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.02 (s, 2H), 3.78 – 3.66 (m, 10H), 3.61 (dd, *J* = 5.3, 3.8 Hz, 2H), 1.48 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.67, 81.72(C_{10}), 72.66, 70.62, 70.60, 70.49, 70.23, 68.97, 61.65, 28.10.

$MsO\text{-}(CH_2CH_2O)_3CH_2C(O)OtBu$ (16)

Compound **15** (4.73 mmol, 1.25 g) was dissolved in DCM (50 mL). TEA (9.46 mmol, 1.30 mL) was added and the mixture was cooled to 0°C. MsCl (5.31 mmol, 0.41 mL) was slowly added. The mixture was heated to RT and stirred for 3 hours. TLC analysis (EA) indicated complete conversion. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$ (aq) (3x), 10% $NaHCO_3$ (aq) (3x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was purified by silica gel column chromatography (5:5 EA:pentane → 8:2 EA:pentane, Δ=10%). Compound **16** (4.25 mmol, 1.45 g) was obtained with a 90% yield.

IR(cm^{-1})=2872(C-H, stretch), 1742(C=O, stretch).

HRMS $[M+H]^+$: 343.14186 (measured), 343.14211 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.01 (s, 2H), 3.80 – 3.76 (m, 2H), 3.73 – 3.65 (m, 8H), 3.09 (s, 3H), 1.48 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.58, 81.61, 70.68, 70.60, 70.53, 70.52, 69.37, 69.01, 68.96, 37.70, 28.11.

N_3-$(CH_2CH_2O)_3CH_2C(O)OtBu$ (17)

Compound **16** (2.96 mmol, 1.01 g) was dissolved in DMF (30 mL). Sodium azide (9 mmol, 585 mg) was added and the mixture was heated to 60°C. After 4 hours of stirring TLC analysis (8:2 EA:pentane) showed complete conversion. The mixture was diluted with EA and washed with 5% $NaHCO_3$ (aq) (4x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was purified by silica gel column chromatography (4:6 EA:pentane → 8:2 EA:pentane, Δ=10%). Compound **17** (2.65 mmol, 766 mg) was obtained with a 90% yield.

IR(cm^{-1})=2869(C-H, stretch), 2099(N=N=N, stretch), 1745(C=O, stretch).

HRMS $[M+Na]^+$: 312.15314 (measured), 312.15299 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.03 (s, 2H), 3.76 – 3.65 (m, 10H), 3.44 – 3.35 (m, 2H), 1.48 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.56, 81.42, 70.61, 70.58, 70.56, 70.54, 69.94, 68.92, 50.58, 28.01 .

NH_2-$(CH_2CH_2O)_3CH_2C(O)OtBu$ (18)

Compound **17** (2.22 mmol, 642 mg) was dissolved in dry THF (25 mL) under argon atmosphere. Triphenylphosphine (2.7 mmol, 707 mg) was added and the mixture was stirred for 20 hours. TLC analysis (6:4 pentane:EA) indicated complete conversion. Water was added until white crystals were formed in the solution. The mixture was diluted with DCM and washed with 10% $NaHCO_3$ (aq) (3x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was absorbed on Celite and purified by silica gel column chromatography (1% MeOH in DCM + 1% TEA → 10% MeOH in DCM + 1 % TEA, Δ=2.5%). Compound **18** (1 mmol, 264 mg) was obtained with a 45% yield.

IR(cm^{-1})= 3400(1° N-H, stretch), 2874(C-H, stretch), 1742(C=O, stretch).

HRMS $[M+H]^+$: 264.18073 (measured), 264.18055 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (t, *J* = 5.5 Hz, 1H), 4.04 (s, 2H), 3.71 (m, 4H), 3.63 (s, 4H), 3.55 (t, *J* = 5.2 Hz, 2H), 3.37 (m, 2H), 1.48 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.79, 81.84, 70.80, 70.76, 70.51, 70.48, 70.17,69.06,40.19, 28.22

FmocNH-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe (19)

Compound **14** (1.27 mmol, 794 mg) was dissolved in dry DMF (10 mL). H-Ser(OtBu)-OMe.HCl (1.52 mmol, 321 mg) was added. A solution of TEA (1.91 mmol, 0.26 mL) in DMF (10 mL) was slowly added to the reaction mixture. HOBt (1.91 mmol, 258 mg) and DIC (1.9 mmol, 0.30 mL) were added and the mixture was stirred at RT for 2 hours under argon atmosphere. TLC analysis (3:7 EA:pentane, R_f=0.2, ninhydrine) showed complete conversion. The crude was dissolved in DCM and washed with H_2O. The solution was dried ($MgSO_4$), filtrated, concentrated and co-evaporated with toluene *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (2:8 EA:pentane, R_f=0.15). **19** (1.12 mmol, 873 mg) was obtained with a 88% yield.

$[\alpha]_D$: +8°

IR(cm^{-1})= 3298, 2918,2848, 1734, 1660, 1531

HRMS $[M+H]^+$: 783.46038 (measured), 783.46126 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.29 (t, *J* = 7.4 Hz, 3H), 5.99 (d, *J* = 7.1 Hz, 1H), 4.69 (dd, *J* = 5.1, 3.1 Hz, 1H), 4.54 – 4.30 (m, 3H), 4.23 (m, 3H), 3.82 (dd, *J* = 9.1, 2.7 Hz, 1H), 3.72 (s, 3H), 3.57 (dd, *J* = 9.1, 3.0 Hz, 1H), 2.98 (d, *J* = 3.9 Hz), 2.84 (s, 2H), 2.29 (t, *J* = 7.6 Hz, 2H), 1.59 (dd, *J* = 14.0, 7.0 Hz, 2H), 1.26 (m, 24H), 1.12 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.66,170.43, 170.14, 155.91, 143.76, 141.26, 127.72, 127.07, 125.15, 119.97, 73.56, 67.27, 62.92, 61.62, 54.14, 53.21, 52.43, 47.08, 34.91, 34.16, 31.93, 30.99, 29.70, 29.66, 29.62,29.48,29.37, 29.29, 29.15, 27.26, 24.88, 22.70, 14.15.

NH_2-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe (20)

19 (0.50 mmol, 0.39 g) was dissolved in dry DMF (10 mL). A solution of 2% piperidine and 2%DBU in DMF (10 mL) was slowly added. The mixture was stirred for 15 min at RT) under argon atmosphere. TLC analysis (95:5 DCM:MeOH, R_f=0.8, ninhydrine) showed complete conversion. The mixture was taken up in EA and washed with 10% $KHSO_4$(aq)(2x) and H_2O(2x). The solution was dried ($MgSO_4$), filtrated, concentrated and co-evaporated with toluene *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column

chromatography (99% DCM, 1% MeOH + 0.1% TEA, R_f=0.1). Compound **20** (0.44 mmol, 0.25 g) was obtained with an 88% yield.
$[\alpha]_D$: -49.2°
IR(cm^{-1})= 3178,3082, 2917, 2851, 1744, 1677, 1660, 1464
HRMS $[M+H]^+$: 561.39046 (measured), 561.39318 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (d, *J* = 8.5 Hz, 1H), 4.67 (dt, *J* = 8.6, 3.2 Hz, 1H), 4.22 (t, *J* = 6.7 Hz, 2H), 3.83 (dd, *J* = 9.1, 3.2 Hz, 1H), 3.75 (s, 3H), 3.56 (m, 2H), 3.09 (dd, *J* = 13.6, 3.8 Hz, 1H), 2.83 – 2.74 (m, 3H), 2.32 (t, *J* = 7.6 Hz, 2H), 1.62 (m, 2H), 1.27 (s, 24H), 1.15 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.68,173.26, 170.92, 73.47, 63.12, 62.06, 54.19, 52.76, 52.41, 37.93, 34.25, 32.00, 30.68, 29.76, 29.73, 29.68, 29.54, 29.43, 29.35, 29.21, 27.39, 24.98, 22.77, 14.20

AcNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(tBu)-OMe
Compound **20** (0.17 mmol, 95 mg) was dissolved in dry DCM (2 mL). A solution of TEA (0.25 mmol, 36 µL) in dry DCM (1 mL) was slowly added. Acetic anhydride (0.34 mmol, 32 µL) was added and the mixture was stirred overnight at RTunder argon atmosphere. TLC analysis (93:7 DCM:MeOH, R_f=0,3, ninhydrine) showed complete conversion. The mixture was taken up in DCM and washed with a saturated solution of NH_4Cl (aq). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was dissolved in DCM and purified by silica gel column chromatography (99:1 DCM:MeOH, R_f=0.15, ninhydrine). **AcNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(tBu)-OMe** (0.16 mmol, 96 mg) was obtained with a 94% yield.
$[\alpha]_D$: +17.8°
IR(cm^{-1})= 3285, 2916, 2849, 1737, 1660
HRMS $[M+H]^+$: 603.40167 (measured), 603.40375 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (d, *J* = 8.2 Hz, 1H), 6.77 (d, *J* = 7.4 Hz, 1H), 4.72 – 4.60 (m, 2H), 4.26 (t, *J* = 6.6 Hz, 2H), 3.83 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.75 (s, 3H), 3.57 (dd, *J* = 9.1, 3.3 Hz, 1H), 3.03 – 2.80 (m, 4H), 2.32 (t, *J* = 7.6 Hz, 2H), 2.04 (s, 3H), 1.61 (m, 2H), 1.35 – 1.20 (m, 24H), 1.14 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.71, 170.44, 170.34, 170.02, 73.57, 62.83, 61.54, 53.24, 52.46, 52.44, 34.59, 34.22, 31.95, 30.97, 29.72, 29.68, 29.64, 29.50, 29.39, 29.31, 29.18, 27.29, 24.91, 23.11, 22.72, 14.16 .
$C_{15}H_{31}$C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(tBu)-OMe
Compound **20** (0.17 mmol, 95 mg) was dissolved in dry pyridine (2 mL). Palmitoyl chloride (0.21 mmol, 63 µL) was added and the mixture was stirred at RT under argon atmosphere for 45 min. TLC analysis (93:7 DCM:MeOH, R_f=0.3, ninhydrine) showed complete conversion. The reaction mixture was taken up in DCM and washed with 10% $KHSO_4$ (aq). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was dissolved in DCM and purified by silica gel column chromatography (99.5:0.5 DCM:MeOH → 99:1 DCM:MeOH, R_f=0.1). **$C_{15}H_{31}$C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(tBu)-OMe** (0.17 mmol, 0.13 g) was obtained with a quantitative yield.
$[\alpha]_D$: +10.6°
IR(cm^{-1})= 3283, 2915, 2848, 1737, 1639
HRMS $[M+H]^+$: 799.62276 (measured), 799.62285 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, *J* = 8.2 Hz, 1H), 6.74 (d, *J* = 7.4 Hz, 1H), 4.71 – 4.62 (m, 2H), 4.26 (t, *J* = 6.6 Hz, 2H), 3.83 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.74 (s, 3H), 3.57 (dd, *J* = 9.1, 3.3 Hz, 1H), 3.00 – 2.82 (m, 4H), 2.32 (m, 2H), 2.28 – 2.20 (m, 2H), 1.69 – 1.56 (m, 4H), 1.37 – 1.20 (m, 48H), 1.14 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 6H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.64, 173.20, 170.51, 170.34, 73.54, 62.84, 61.47, 53.23, 52.40, 52.22, 36.46, 34.57, 34.17, 33.97, 31.91, 30.90, 29.69,29.65, 29.63, 29.49, 29.47, 29.35, 29.28, 29.26, 29.15, 27.23, 25.57, 24.87, 22.68, 14.10
NH_2C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(tBu)-OMe
Compound **20** (0.39 mmol, 0.22 g) was dissolved in dry DCM (10 mL). Isopropanol (8.0 mmol, 0.61 mL) was added. 85% (Trimethylsilyl)isocyanate (4.0 mmol, 0.65 mL) was added and the mixture was stirred overnight at RT under argon atmosphere. TLC analysis (1:1 EA:pentane + 1% TEA) indicated completion of

reaction. Celite was added to the reaction mixture and the DCM was evaporated *in vacuo*. The adsorbed crude was purified by silica gel column chromatography (1:1 EA:pentane +1% TEA → 95:5 EA:MeOH + 1% TEA). **$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe** (0.40 mmol, 0.24 g) was obtained with a quantitative yield.

$[\alpha]_D$: +10.3°

IR(cm^{-1})=2916, 2849, 1733, 1645,

HRMS $[M+H]^+$: 604.39816 (measured), 604.39900 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, *J* = 8.1 Hz, 1H), 6.46 (d, *J* = 7.9 Hz, 1H), 5.15 (m, 1H), 4.69 – 4.54 (m, 2H), 4.31 – 4.18 (m, 2H), 3.80 (dd, *J* = 9.1, 3.3 Hz, 1H), 3.74 (s, 3H), 3.58 (dd, *J* = 9.2, 3.5 Hz, 1H,), 2.94 (m, 2H), 2.83 (m, 2H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.60 (m, 2H), 1.35 – 1.20 (m, 24H), 1.14 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.99,171.66, 170.62, 158.57, 73.70, 63.01, 61.68, 53.44, 53.33, 52.51, 35.35, 34.29, 32.00, 31.13, 29.77, 29.74, 29.57, 29.44, 29.38, 29.25, 27.34, 24.97, 22.76, 14.20.

AcNH-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-OMe (1)

AcNH-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe (0.24 mmol, 145 mg) was dissolved in TFA (5 mL) and was stirred at RT. After 30 min the mixture was dropped in Et_2O and precipitated overnight at -20° C. Compound **1** (0.15 mmol, 82 mg) was obtained with a 62% yield.

$[\alpha]_D$: +7.0°

IR(cm^{-1})= 3281, 2914, 2849, 1737, 1630.

HRMS $[M+H]^+$: 547,33966 (measured), 547.34115 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 7.9 Hz, 1H), 6.91 (d, *J* = 7.5 Hz, 1H), 4.71 (dd, *J* = 13.7, 6.8 Hz, 1H), 4.67 – 4.61 (m, 1H), 4.33 – 4.16 (m, 2H), 3.94 (ddd, *J* = 25.2, 11.6, 3.5 Hz, 2H), 3.78 (s, 3H), 2.96 (qd, *J* = 13.9, 6.5 Hz, 2H), 2.81 (t, *J* = 6.7 Hz, 2H), 2.32 (t, *J* = 7.6 Hz, 2H), 2.05 (s, 3H), 1.68 – 1.54 (m, 2H), 1.36 – 1.19 (m, 24H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.17, 171.31, 170.71, 170.64, 62.88, 62.61, 55.12, 52.85, 34.56, 34.34, 32.04, 31.14, 29.81, 29.78, 29.74, 29.61, 29.48, 29.40, 29.27, 25.00, 23.10, 22.81, 14.24

$C_{15}H_{31}$C(O)NH-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-OMe (2)

$C_{15}H_{31}$C(O)NH-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe (0.31 mmol, 245 mg) was dissolved in TFA (5 mL) and was stirred at RT. After 30 min the mixture was dropped in Et_2O and precipitated over weekend at -20°C. Compound **2** (0.29 mmol, 217 mg) was obtained with a 94% yield.

$[\alpha]_D$: +2.9°

IR(cm^{-1})= 3313, 2910, 2848, 1739, 1639

HRMS $[M+H]^+$: 743.56003 (measured), 743.56025 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ7.61 (d, *J* = 7.7 Hz, 1H), 6.87 (d, *J* = 7.3 Hz, 1H), 4.77 – 4.58 (m, 2H), 4.36 – 4.15 (m, 2H), 3.96 (m, 2H), 3.80 (d, *J* = 10.2 Hz, 3H), 3.05 – 2.89 (m, 2H), 2.82 (t, *J* = 6.6 Hz, 2H), 2.29 (m, 4H), 1.61 (m, 4H), 1.37 – 1.16 (m, 48H), 0.88 (t, *J* = 6.6 Hz, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.95, 174.34, 170.83, 170.50, 62.88, 62.61, 55.14, 52.91, 52.77, 36.47, 34.43, 34.36, 32.05, 31.15, 29.83, 29.79, 29.63, 29.49, 29.42, 29.36, 29.28, 25.75, 25.00, 22.82, 14.24 .

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-OMe (3)

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(tBu)-OMe (0.37 mmol, 225 mg) was dissolved in TFA (5 mL) and was stirred at RT. After 30 min the mixture was co-evaporated with toluene *in vacuo*. After silica gel column chromatography purification (1% MeOH in EA → 4% MeOH in EA) compound **3** (0.33 mmol, 180 mg) was obtained with a 89% yield.

$[\alpha]_D$: +3.3°

IR(cm^{-1})= 3286, 2916, 2849, 1742, 1639

HRMS $[M+H]^+$: 548.33519 (measured), 548.33640 (calculated).

^{1}H NMR (400 MHz, DMSO) δ 8.37 (d, *J* = 7.8 Hz, 1H), 6.25 (d, *J* = 8.7 Hz, 1H), 5.68 (s, 2H), 5.08 (t, *J* = 5.6 Hz,

1H), 4.49 – 4.39 (m, 1H), 4.36 (m, 1H), 4.21 – 4.06 (m, 2H), 3.71 (m, 1H), 3.67 – 3.56 (m, 4H,), 2.83 (dd, *J* = 13.7, 5.2 Hz, 1H), 2.75 (m, 2H), 2.65 (dd, *J* = 13.7, 7.7 Hz, 1H), 2.28 (t, *J* = 7.4 Hz, 2H), 1.59 – 1.45 (m, 2H), 1.33 – 1.15 (m, 24H), 0.85 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ 172.76, 171.34, 170.74, 157.95, 62.88, 61.17, 54.61, 52.25, 51.84, 34.89, 33.40, 31.29, 29.99, 29.04, 28.88, 28.70, 28.44, 24.42, 22.09, 13.96

AcNH-Cys(EtOC(O)$C_{15}H_{31}$)-SerLysLysLysLys-C(O)NH_2 (4)

The SK_4 peptide was prepared by applying Fmoc based protocol starting from Tentagel S RAM resin (4.35 g; loading 0.23 mmol/g) on a AB peptide synthesizer. The amino acids used were Fmoc-Lys(Boc) and Fmoc-Ser(Ot Bu)-OH. To a mixture of resin bound peptide (SK_4) (0.05 mmol; 0.228 g) in NMP/DCM (1:1) was added **14**(0.125 mmol; 0.78 g), PyBop (0.175 mmol; 0.091 g) and DiPEA 1M (0.25 mmol; 0.032 g; 250 µL). The DiPEA was added in two times, first an amount of 125 µL, after 10 min another amount of 125 µL. The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed three times with DCM and the Fmoc was cleaved with 20% piperidine/DMF (3 times 5 min). The now free amine acetylated by adding acetyl chloride (0.5 mmol; 0.039 g; 35 µL) in pyridine/DCM (1:1), the mixture was stirred for 2.5 h. The solution was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TiS/H2O (95/2.5/2.5) (2h). Purification of the conjugate was done by adding the crude to cold diethylether/n-pentane (1:1) (14 mL) and centrifugation (4000 rpm, 5min) was performed. The precipitate was dissolved in magic (MeCN/t BuOH/H2O) (1:3:1). This solution was then subjected to semi-prep HPLC, pure lipopeptide fractions were collected and concentrated by freeze-drying. This yielded conjugate **4**(5.6 µmol; 5.8 mg; 7.7%). LCMS: 10-90% C18, Rt = 6.4 min.

NH_2C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-SerLysLysLysLys-C(O)NH_2 (5)

To synthesize **5**, the coupling of building block **14** to the pentapeptide SK_4 was done under the same conditions described by the coupling procedure for compound **4**. After the coupling, DCM wash and the cleavage of the Fmoc, the free amine was treated with TMS-isocyanate (0.5 mmol; 0.058 gr; 68 µL) and isopropanol (1 mmol; 0.06 g; 76 µL) in DCM. Further procedure and purification was done according to the same method explained for compound **4**. This provided compound **5** (2.9 µmol; 3.0 mg; 4%). LCMS: 10-90% C18, Rt = 6.3 min.

FmocNH-Ser(OtBu)-O($CH_2CH_2O)_3CH_2$C(O)OtBu

Compound **15** (2.8 mmol, 1.6 g) was dissolved in dry DCM (30 mL). Fmoc-Ser(OtBu)-OH (3.38 mmol, 1.29 g), DIC (3.36 mmol, 0.52 mL) and DMAP (0.3 mmol, 38 mg) were added and the mixture was stirred for 4 hours at RT under argon atmosphere. TLC-MS indicated complete conversion. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$(aq)(3x), 10% $NaHCO_3$(aq)(3x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was dissolved in THF and the urea byproduct was removed by crystallization at -20°C. The crude was further purified by silica gel column chromatography (8:2 pentane:EA→ 7:3 pentane:EA, R_f=0.3). Product (1.74 g, 2.77 mmol) was obtained with a quantitative yield.

$[\alpha]_D$: +6.33°

IR(cm^{-1})=2974, 2873, 1738, 1722, 1717

HRMS [M+H]$^+$: 630.32709 (measured), 630.32727 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.63 (t, *J* = 7.1 Hz, 2H), 7.40 (t, 2H), 7.31 (t, 2H), 5.75 (d, *J* = 8.9 Hz, 1H), 4.56 – 4.49 (m, 1H), 4.48 – 4.07 (m, 5H), 4.01 (s, 2H), 3.86 (dd, *J* = 9.0, 2.8 Hz, 1H), 3.76 – 3.54 (m, 11H), 1.47 (s, 9H), 1.16 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.62, 169.64, 156.11, 143.99, 143.81, 141.27, 127.70, 127.08,125.21, 125.17, 119.97, 81.55, 73.47, 70.69, 70.62, 70.57, 69.00, 68.98, 67.16, 64.54, 62.11, 54.67, 47.14, 28.12, 27.35

NH_2-Ser(OtBu)-O($CH_2CH_2O)_3CH_2$C(O)OtBu (21)

FmocNH-Ser(OtBu)-O($CH_2CH_2O)_3CH_2$C(O)OtBu (1.629 g, 2.59 mmol) was dissolved in dry DMF (10 mL). A mixture of 2:2 piperidine and DBU (v:v) in dry DMF (30 mL) was added. The mixture was stirred for 15 minutes at RT under argon atmosphere. TLC (6:4 pentane:EA) indicated complete conversion. The reaction mixture was diluted with EA and washed with 0.25% $KHSO_4$(aq) (3x), 10% $NaHCO_3$(aq) (3x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was adsorbed

on Celite and purified with silica gel column chromatography (DCM →95:5 DCM:MeOH, Δ= 0.5%). Compound **21** (1.97 mmol, 802 mg) was obtained with a 76% yield.
$[\alpha]_D$: -8.95°
IR(cm^{-1})= 2974,2872, 1742
HRMS $[M+H]^+$: 408.25702 (measured), 408.25919 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 4.29 (m, 2H), 4.02 (s, 2H), 3.76 – 3.51 (m, 13H), 1.48 (s, 9H), 1.09 (s, 9H,).
^{13}C NMR (101 MHz, $CDCl_3$) δ 174.22, 169.74, 81.72, 73.23, 70.81, 70.72, 70.65, 70.33, 69.12, 69.09, 64.12, 63.83, 55.26, 28.22, 27.57

FmocNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-O($CH_2CH_2O)_3CH_2C$(O)OtBu
Compound **21** (0.70 mmol, 0.29 g) was dissolved in dry DMF (5 mL). Compound **14** (0.50 mmol, 0.31 g), HOBt (0.70 mmol, 94 mg) and DIC (0.70 mmol, 0.11 mL) was added. The mixture was stirred overnight at RT under argon atmosphere. TLC-MS indicated complete conversion. The mixture was diluted with EA and washed with 10% $NaHCO_3$(aq) (2x), 10% $KHSO_4$(aq) (2x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was dissolved in THF and the urea byproduct was removed by crystallization at -20°C. The crude was adsorbed on Celite and purified by silica gel column chromatography (8:2 pentane:EA→ 5:5 pentane:EA, Δ=10%). Product (0.35 mmol, 0.36 g) was obtained with a 71% yield.
$[\alpha]_D$: +4.83°
IR(cm^{-1})= 2923, 2852, 1733, 1739
HRMS $[M+H]^+$: 1015.59320 (measured), 1015.59234 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.30 (m, 3H), 5.88 (d, *J* = 6.5 Hz, 1H), 4.70 (dt, *J* = 8.2, 3.0 Hz, 1H), 4.48 – 4.34 (m, 3H), 4.32 – 4.17 (m, 5H), 4.01 (s, 2H), 3.86 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.75 – 3.65 (m, 10H), 3.59 (dd, *J* = 9.1, 3.1 Hz, 1H), 2.98 (s, 2H), 2.86 (s, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.59 (m, 2H), 1.47 (s, 9H), 1.27 (d, *J* = 22.4 Hz, 24H), 1.13 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.79, 170.01, 169.75, 143.85, 141.41, 127.85, 127.21, 125.27, 125.22, 81.71, 73.69, 70.83, 70.73, 70.69, 70.66, 69.13, 69.06, 67.38, 64.70, 63.02, 61.72, 54.25, 53.37, 47.23, 35.06, 34.31, 32.05, 31.13, 29.82, 29.78, 29.75, 29.60, 29.48, 29.42, 29.29, 28.24, 27.44, 25.01, 22.82, 14.25 .

NH_2-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-O($CH_2CH_2O)_3CH_2C$(O)OtBu (23)
FmocNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-O($CH_2CH_2O)_3CH_2C$(O)OtBu (0.29 mmol, 0.29 g) was dissolved in dry DCM (5 mL). Octanethiol (1.5 mmol, 0.25 mL), then DBU (0.03 mmol, 5 µL) was added and the mixture was stirred overnight at RT. TLC (8:2 EA:pentane) indicated complete conversion. Celite was added to the reaction mixture and the DCM was evaporated *in vacuo*. The adsorbed crude was purified by silica gel column chromatography (EA + 1% TEA → 9:1 EA:MeOH + 1% TEA). Compound **23**(0.26 mmol, 203 mg) was obtained with a 90% yield.
$[\alpha]_D$: -41.42°
IR(cm^{-1})=2918, 2850, 1742, 1677, 1662.
HRMS $[M+H]^+$: 793.52330 (measured), 793.52426 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, *J* = 8.6 Hz, 1H), 4.69 (dt, *J* = 8.6, 3.2 Hz, 1H), 4.30 (t, 2H), 4.22 (t, *J* = 6.7 Hz, 2H), 4.02 (d, *J* = 2.5 Hz, 2H), 3.85 (dd, *J* = 9.0, 3.1 Hz, 1H), 3.75 – 3.66 (m, 10H), 3.63 – 3.52 (m, 2H), 3.09 (dd, *J* = 13.6, 3.8 Hz, 1H), 2.82 – 2.70 (m, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.67 – 1.56 (m, 2H), 1.48 (s, 9H), 1.36 – 1.21 (m, 23H), 1.13 (d, *J* = 9.3 Hz, 9H), 0.88 (t, *J* = 6.8 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.61, 173.24, 170.35, 169.65, 81.55, 73.37, 72.58, 70.74, 70.66, 70.62, 70.35, 69.03, 64.51, 63.06, 62.02, 54.16, 52.72, 37.88, 34.21, 31.95, 30.63, 29.72, 29.68, 29.64, 29.50, 29.39, 29.31, 29.17, 28.14, 27.38, 24.94, 22.72, 14.17

NH_2C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-O($CH_2CH_2O)_3CH_2C$(O)OtBu
Compound **23** (0.23 mmol, 0.18 g) was dissolved in dry DCM (20 mL). Isopropanol (4.6 mmol, 0.35 mL) was added. 85% (Trimethylsilyl)isocyanate (2.3 mmol, 0.37 mL) was added and the mixture was stirred

overnight at RT under argon atmosphere. TLC (EA, R_f=0.5, ninhydrine) indicated complete conversion. The mixture was diluted with DCM and washed with 10% $NaHCO_3$ (aq) (2x), 10% $KHSO_4$ (aq) (2x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (8:2 EA:pentane + 1% TEA). Product (0.21 mmol, 0.18 g) was obtained with a 90% yield.

$[\alpha]_D$: +3°

IR(cm^{-1})=2917, 2849, 1739, 1733, 1641.

HRMS $[M+H]^+$: 836.52912 (measured), 836.53007 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ7.41 (d, *J* = 8.2 Hz, 1H), 6.15 (d, *J* = 7.6 Hz, 1H), 4.98 (s, 2H), 4.64 (dt, *J* = 8.1, 3.3 Hz, 1H), 4.51 (dd, *J* = 13.5, 6.4 Hz, 1H), 4.37 – 4.19 (m, 4H), 4.05 – 4.00 (m, 2H), 3.84 (dd, *J* = 9.1, 3.3 Hz, 1H), 3.75 – 3.62 (m, 10H), 3.59 (dd, *J* = 9.1, 3.4 Hz, 1H), 3.08 – 2.89 (m, 2H), 2.87 – 2.81 (m, 2H), 2.32 (t, 2H), 1.65 – 1.56 (m, 3H), 1.47 (s, 9H), 1.35 – 1.21 (m, 24H), 1.15 (s, 9H), 0.88 (t, *J* = 6.9 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.03, 171.33, 170.11, 158.33, 73.66, 70.83, 70.68, 70.65, 70.60, 69.06,64.71, 63.04, 61.71, 53.62, 53.45, 35.11, 34.35, 32.04, 31.14, 29.82, 29.78, 29.61, 29.48, 29.42, 29.29, 28.24, 27.45, 25.02, 22.81, 14.25

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-$O(CH_2CH_2O)_3CH_2C(O)OH$ (6)

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OtBu)-$O(CH_2CH_2O)_3CH_2C(O)OtBu$ (50 µmol, 42 mg) was dissolved in dry DCM (2 mL). TFA (2 mL) was added and the mixture was stirred for 1 hour at RT under argon atmosphere. LC-MS indicated complete conversion. The reaction mixture was dropped in a tube with Et_2O (9 mL) and left at -20°C overnight. The tube was centrifuged and the precipitate was collected. Compound **6** (39 µmol, 28 mg) was obtained with an 80% yield.

$[\alpha]_D$: -6.3°

IR(cm^{-1})= 3291, 2916(C-H, stretch), 2849, 1739, 1641.

HRMS $[M+H]^+$: 724.40325 (measured), 724.40487 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (s, 1H), 6.53 (s, 1H), 4.67 (s, 2H), 4.43 (s, 1H), 4.37 – 4.07 (m, 5H,), 3.95 (m, 2H), 3.69 (d, *J* = 26.1 Hz, 10H), 2.97 (s, 2H), 2.79 (s, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.59 (m, 2H,), 1.25 (m, 24H), 0.88 (t, *J* = 6.7 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 174.07, 70.49, 68.98, 63.17, 34.34, 32.06, 31.04, 29.85, 29.80, 29.66, 29.50, 29.33, 25.04, 22.83, 14.27.

FmocNH-Ser(OtBu)-$NH(CH_2CH_2O)_3CH_2C(O)OtBu$

Compound **18** (0.79 mmol, 0.17 g) was dissolved in dry DCM (30 mL). Fmoc-Ser(OtBu)-OH (1 mmol, 383 mg), DIC (1 mmol, 0.16 mL) and HOBt (1 mmol, 134 mg) were added and the mixture was stirred for 4 hours at RT under argon atmosphere. TLC-MS indicated complete conversion. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$(aq)(3x), 10% $NaHCO_3$(aq)(3x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (8:2 pentane:EA→ 5:5 pentane:EA, Δ=10%). Product (0.37 mmol, 235 mg) was obtained with a 47% yield. R_f= 0.5 at 8:2 EA:pentane.

$[\alpha]_D$: +15.8°

IR(cm^{-1})=2974, 2868, 1723, 1717, 1668.

HRMS $[M+H]^+$: 629.34200 (measured), 629.34326 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.4 Hz, 2H), 7.61 (d, *J* = 6.8 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.3 Hz, 2H), 7.09 (s, 1H), 5.85 (s, 1H), 4.39 (d, *J* = 4.6 Hz, 2H), 4.23 (t, *J* = 6.9 Hz, 2H), 4.00 (s, 2H), 3.84 – 3.73 (m, 1H), 3.73 – 3.53 (m, 10H), 3.53 – 3.44 (m, 2H), 3.40 (t, *J* = 8.1 Hz, 1H), 1.46 (s, 9H,), 1.20 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.38, 141.40, 127.83, 127.19, 125.26, 120.10, 70.77, 70.66, 70.62, 70.43,69.93, 69.08, 62.07, 54.68, 47.28, 39.54, 28.23, 27.53

NH_2-Ser(OtBu)-$NH(CH_2CH_2O)_3CH_2C(O)OtBu$

FmocNH-Ser(OtBu)-$NH(CH_2CH_2O)_3CH_2C(O)OtBu$ (0.16 mmol, 0.10 g) was dissolved in dry DCM (5 mL). Octanethiol (0,80 mmol. 0,14 mL) was added. DBU (0,02 mmol, 3 µL) was added and the mixture was stirred at RT overnight under argon atmosphere. TLC analysis (8:2 EA:pentane) indicated completion of reaction. The DCM was evaporated *in vacuo* and the mixture was diluted with EA. The crude was purified by silica gel column chromatography (EA + 1% TEA → 9:1 EA:MeOH + 1% TEA). Compound **22** (0.16 mmol, 65 mg) was obtained with a quantitative yield.

$[\alpha]_D$: -9.1°
IR(cm^{-1})=2973, 2930, 2872, 1746, 1661.
HRMS $[M+H]^+$: 407.27390 (measured), 407.27518 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (s, 1H), 4.02 (s, 2H), 3.74 – 3.54 (m, 11H), 3.47 (m, 4H), 1.48 (s, 9H), 1.19 (s, 9H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.25,169.68, 81.69, 73.40, 70.70, 70.59, 70.54, 70.29, 69.96, 69.01, 64.07, 55.52, 38.91, 28.14, 27.55.

FmocNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-NH($CH_2CH_2O)_3CH_2C$(O)OtBu
Compound **22** (0.28 mmol, 0.11 g) was dissolved in dry DCM (10 mL). Compound **14** (0,25 mmol, 156 mg), HOBt (0.30 mmol, 40 mg) and DIC (0.30 mmol, 50 µL) were added and the mixture was stirred overnight at RT under argon atmosphere. TLC analysis (EA) indicated completion of reaction. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$ (aq) (3x), 10% $NaHCO_3$ (aq) (3x) and brine (1x). The solution was dried (Na_2SO_4), filtrated and evaporated *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (8:2 pentane:EA→EA). product (0.25 mmol, 0.25 g) was obtained with a quantitative yield. R_f= 0.6 EA.
$[\alpha]_D$: +7.3°
IR(cm^{-1})=3300, 2920, 2820, 1736, 1733, 1641
HRMS $[M+H]^+$: 1014.60823 (measured), 1014.60832 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.43 – 7.26 (m, 5H), 7.12 (t, *J* = 5.2 Hz, 1H), 5.96 (d, *J* = 7.0 Hz, 1H), 4.51 – 4.31 (m, 4H), 4.30 – 4.18 (m, 3H), 4.01 (s, 2H), 3.81 (dd, *J* = 8.5, 3.3 Hz, 1H), 3.73 – 3.54 (m, 10H), 3.50 – 3.42 (m, 2H), 3.42 – 3.35 (m, 1H), 3.06 – 2.90 (m, 2H), 2.82 (s, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.60 (m, 2H), 1.47 (s, 9H), 1.21-1.35 (m, 24H), 1.17 (s, 9H), 0.88 (t, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.60, 171.07, 169.95, 169.69, 143.72, 143.69, 141.26, 127.72, 127.07, 125.12, 119.97, 81.55, 73.97, 70.65, 70.53, 70.49, 70.27, 69.69, 68.94, 67.26, 62.83, 61.31, 54.44, 53.45, 47.08, 39.42, 34.68, 34.13, 31.91, 31.00, 29.67, 29.64, 29.60, 29.46, 29.34 , 29.28, 29.14, 28.08, 27.36, 24.86, 22.67, 14.18 .

NH_2-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-NH($CH_2CH_2O)_3CH_2C$(O)OtBu (24)
FmocNH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-NH($CH_2CH_2O)_3CH_2C$(O)OtBu (0.22 mmol, 0.23 g) was dissolved in dry DCM (1 mL) . Octanethiol (1.1 mmol, 0.19 mL) was added. DBU (22 µmol, 3.3 µL) was added and the mixture was stirred for 3 hours at RT under argon atmosphere. TLC analysis (EA) indicated completion of reaction. Celite was added and the DCM was evaporated *in vacuo*. The crude was purified by silica gel column chromatography (1:1 EA:pentane→ 9:1 EA:MeOH +1% TEA). Compound **24** (0.20 mmol, 154 mg) was obtained with an 88% yield.
$[\alpha]_D$: -1.1°
IR(cm^{-1})=2923, 2852, 1734, 1647.
HRMS $[M+H]^+$: 792.53907 (measured), 792.54024 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, *J* = 7.3 Hz, 1H), 7.09 (t, *J* = 5.3 Hz, 1H), 4.42 (td, *J* = 7.3, 4.2 Hz, 1H), 4.22 (t, *J* = 6.8 Hz, 2H) 4.02 (s, 2H), 3.76 (dd, *J* = 8.7, 4.2 Hz, 1H), 3.67-3.56 (dd, *J* = 23.1, 3.7 Hz, 11H), 3.48 (t, *J* = 4.8 Hz, 2H), 3.39 (t, *J* = 8.1 Hz, 1H), 3.06 (dd, *J* = 13.5, 4.0 Hz, 1H), 2.77 (dt, *J* = 10.3, 4.1 Hz, 3H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.60 (m, *J* = 14.4, 7.3 Hz, 3H), 1.48 (s, 9H), 1.26 (s, 24H), 1.20 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H)
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.56, 170.18, 169.61, 81.60, 73.88, 70.66, 70.53, 70.29, 69.83, 68.95, 62.98,61.57, 54.30, 52.93, 39.36, 37.86, 31.91, 30.64, 29.68, 29.64, 29.60, 29.46, 29.35, 29.27, 29.13, 28.11, 27.40, 24.89, 22.68, 14.13.

NH_2C(O)NH-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OtBu)-NH($CH_2CH_2O)_3CH_2C$(O)OtBu
Compound **24** (0.18 mmol, 142 mg) was dissolved in dry DCM (10 mL). Isopropanol (3.5 mmol, 0.27 mL) was added. 85% (Trimethylsilyl)isocyanate (1.8 mmol, 0.28 mL) was added and the mixture was stirred overnight at RT under argon atmosphere. Celite was added and the DCM was evaporated *in vacuo*. The adsorbed crude was purified by silica gel column chromatography (8:2 EA:pentane + 1% TEA → 95:5 EA:MeOH + 1% TEA). Product (0.14 mmol, 0.12 g) was obtained with a 82% yield. R_f=0.5 EA.

$[\alpha]_D$: +10
IR(cm^{-1})=2917, 2849, 1733, 1634
HRMS $[M+H]^+$: 835.54504 (measured), 835.54606 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (t, *J* = 6.1 Hz, 2H), 6.35 (d, *J* = 6.6 Hz, 1H), 5.54 (s, 2H), 4.51 (m, 2H), 4.22 (t, *J* = 6.7 Hz, 2H), 4.02 (s, 2H), 3.82 (dd, *J* = 8.9, 3.7 Hz, 1H), 3.73 – 3.55 (m, 11H), 3.45 (dd, *J* = 8.9, 5.7 Hz, 1H), 3.36 (dt, *J* = 12.7, 3.7 Hz, 1H), 2.95 (m, 2H), 2.79 (t, *J* = 6.7 Hz, 2H), 2.31 (m, *J* = 7.6 Hz, 2H), 1.65 – 1.55 (m, 2H), 1.48 (s, 9H), 1.35 – 1.21 (m, 24H), 1.16 (s, 9H), 0.88 (t, *J* = 6.9 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.80, 171.34, 170.34, 169.86, 159.15, 82.09, 73.62, 70.69, 70.67, 70.37, 70.28, 69.89, 69.00, 62.91, 61.62, 54.07, 53.52, 39.62, 34.63, 34.21, 31.95, 30.85, 29.73, 29.70, 29.53, 29.39, 29.34, 29.21, 28.16, 27.43, 24.93, 22.72, 14.17

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-$NH(CH_2CH_2O)_3CH_2C(O)OH$ (7)

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OtBu)-$NH(CH_2CH_2O)_3CH_2C(O)OtBu$ (0.14 mmol, 0.11 g) was dissolved in dry DCM (2mL). TFA (2 mL) was added and the mixture was stirred for 1 hour. The TFA was evaporated *in vacuo*. The crude was dissolved in DCM and dropped in a tube with Et_2O (35 mL). Compound **7** (0.10 mmol, 74 mg) was obtained with a 71% yield.
$[\alpha]_D$: -2°
IR(cm^{-1})=3282, 2917, 2849, 1729, 1638
HRMS $[M+H]^+$: 723.42011 (measured), 723.42086 (calculated).
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, *J* = 5.7 Hz, 1H), 7.89 (s, 1H), 6.66 (s, 1H), 4.58 (s, 2H), 4.21 (m, 4H), 3.86 (d, *J* = 29.0 Hz, 2H), 3.65 (dd, *J* = 38.0, 20.2 Hz, 10H), 3.48 (m, 2H), 2.93 (s, 2H), 2.79 (t, *J* = 6.2 Hz, 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.58 (m, 2H), 1.23 (m, 24H), 0.88 (t, *J* = 6.7 Hz, 3H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 173.99, 173.77, 172.33, 170.67, 160.19, 70.68, 70.45, 70.14, 69.61, 68.44, 63.02, 62.57, 55.65, 53.85, 39.66, 34.70, 34.28, 32.04, 30.83, 29.82, 29.78, 29.65, 29.48, 29.32, 25.00, 22.80, 14.24 .

NH_2-DEVSGLEQLESIINFEKL-resin bound (25)

Preloaded leucine resin (0.05 mmol) was subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building block (NovaBiochem, 0.25 mmol, 5eq), HCTU as an activating agent, and Fmoc cleavage as the final step.

NH_2-DEVSGLEQLESIINFEK(^{N3})L-resin bound (26)

Peptide-resin **26** was synthesized using the same procedure than **25** using Fmoc-azidonorleucine instead of Fmoc-Lys(Boc)-OH for the first coupling.

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-$O(CH_2CH_2O)_3CH_2C(O)NH$-DEVSGLEQLESIINFEKL-OH (8)

Resin **25** (12 µmol) was put in a syringe and suspended in NMP until resin appeared sufficiently swollen. **6**(12 µmol, 10 mg) was preactivated with HCTU (12 µmol, 5 mg) to form a 0,2M solution, which was added to **25**. A solution of 1M DIPEA in NMP (12 µL) was added and the syringe was shaken for 15 min, after which 1M DIPEA in NMP (12 µL) was added again. The syringe was shaken overnight, when a Kaiser test indicated completion of reaction. The resin was washed with DCM (3x) after which a solution of TFA/TIS/H_2O (95/2.5/2.5) was added. The syringe was shaken for 75 min and the solution was dropped in Et_2O. After overnight precipitation and HPLC purification compound **8**(0.6 µmol, 1.7 mg) was obtained.
HRMS [(M+2H)/2]: 1385.23537 (measured),1385.22598(calculated)

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-$NH(CH_2CH_2O)_3CH_2C(O)NH$-DEVSGLEQLESIINFEKL-OH (9)

The procedure used to synthesize **8** was also used to obtain **9**. **7**(48 µmol, 35 mg) was used instead of **6**, thus **9** (1.5 µmol, 4.3 mg) was obtained.
HRMS [(M+2H)/2]:1384,74236 (measured). 1384,73397(calculated).

$NH_2C(O)NH$-Cys($EtOC(O)C_{15}H_{31}$)-Ser(OH)-$O(CH_2CH_2O)_3CH_2C(O)NH$-DEVSGLEQLESIINFEK(^{N3})L-OH (27)

26 bound (20 µmol) was put in a syringe and suspended in NMP until resin appeared sufficiently swollen. **6** (20 µmol, 14 mg) was preactivated with HCTU (20 µmol, 8.3 mg) to form a 0.2M solution, which was added to **26**. A solution of 1M DIPEA in NMP (20 µL) was added and the syringe was shaken for 15 min, after which 1M DIPEA in NMP (20 µL) was added again. The syringe was shaken overnight, when a Kaiser test indicated completion of reaction. The resin was washed with DCM (3x) after which a solution of

TFA/TIS/H_2O (95/2.5/2.5) was added. The syringe was shaken for 75 min and the solution was dropped in Et_2O. After overnight precipitation and HPLC purification compound **27** (1.4 µmol, 4.0 mg) was obtained.

$NH_2C(O)NH$-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OH)-NH($CH_2CH_2O)_3CH_2C(O)$NH-DEVSGLEQLESIINFEK(^{N3})L-OH (28)

The procedure used to synthesize **27** was also used to obtain **28**. **7**(20 µmol, 14 mg) was used instead of **6**, thus **28** (0.75 µmol, 2.09 mg) was obtained.

$NH_2C(O)NH$-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OH)-O($CH_2CH_2O)_3CH_2C(O)$NH-DEVSGLEQLESIINFEK(^{Cy5})L-OH (10)

Compound **27** (0.3 µmol, 0.8 mg) was dissolved in dry DMSO (100 µL). Cy5-BCN (1.08 µmol, 0.9 mg) in dry DMSO (100 µL) was added to the solution. The reaction mixture was stirred at RT for 1 week. After HPLC purification, **10** (30 nmol, 1 mg) was obtained.

HRMS [(M+1H)/2]: 1792.97317 (measured),1792.96897 (calculated).

$NH_2C(O)NH$-Cys(EtOC(O)$C_{15}H_{31}$)-Ser(OH)-NH($CH_2CH_2O)_3CH_2C(O)$NH-DEVSGLEQLESIINFEK(^{Cy5})L-OH (11)

Compound **28** (0.3 µmol, 0.8 mg) was dissolved in dry DMSO (100 µL). Cy5-BCN (0.36 µmol, 0.3 mg) in dry DMSO (100 µL) was added to the solution. The reaction mixture was stirred at RT for 1 week. After HPLC purification, **11** (0.1 µmol, 3.5 mg) was obtained.

HRMS [(M+1H)/2]: 1791.47912(measured),1791.471375 (calculated).

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Chapter 4: Synthesis of TLR-7 peptide conjugates

Toll-like receptors (TLRs) are part of the mammalian innate immune system that forms the first line of defence against pathogens by recognising pathogen associated molecular patterns (PAMPs).[1] Upon recognition of a specific PAMP by the corresponding TLR, a signal transduction pathway is started that activates the immune system. Ten different human TLRs can be discerned that are situated either at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in intracellular (TLR3, TLR7, TLR8 and TLR9) compartments.[2] Each TLR recognizes PAMPs of certain structural identity and intracellular TLRs bind to nucleic acids of various origin. TLR8 binds to viral and bacterial RNA.[3, 4] TLR9 recognizes bacterial and viral single stranded DNA.[5] Single-stranded RNA from viruses is the ligand of TLR7.[6] As modulators of the immune system TLR ligands are important drug targets and much research has been directed to the development of small-molecule TLR agonists.[7] Imiquimod is the first example of a TLR7 agonist[8] that is used for topical treatment of a variety of skin diseases.[9, 10] In the last decade structure and activity studies of small molecules have resulted in specific and non-specific agonists for TLR7 and TLR8. Well known examples are represented by imidazoquinolines (e.g. imiquimod) and 8-hydroxyadenine derivatives.[11, 12] These agonists, either as such or conjugated to other molecular entities have been explored as adjuvants in the framework of the development of new vaccines and immunotherapies.[11, 13, 14] For example, to improve the potency and/or to prevent toxic side effects TLR7 ligands are conjugated to macromolecules such as phospholipids, polysaccharides and peptides.[15, 16] One approach toward the development of fully synthetic vaccine modalities is directed to the design, synthesis and evaluation of conjugates, comprising a structurally defined TLR agonist, covalently connected to an oligopeptide epitope. Several of these conjugates exhibit improved immunological properties in comparison with a mixture of the composing components.[17-19] Such conjugates in which a TLR7 ligand is covalently connected to an antigenic peptide have been prepared as well. The group of Filippov synthesized derivatives (**1b-d**, Figure 1) of the TLR7 ligand 9-benzyl-8-hydroxy-2-alkoxy-adenine (**1a**), suitable for conjugation.[20] The azide was used to covalently attach the ligand to the well-known CTL (cytotoxic T lymphocyte) epitope (SIINFEKL) by copper(I) catalysed cycloaddition to give conjugates such as **2**. Although these conjugates give rise to enhanced antigen presentation in vitro, they unexpectedly lack the ability to induce maturation of dendritic cells (DC). The latter action is crucial for the induction of T-cell mediated immunity. The position in the TLR7 agonist molecule to which the peptide epitope is attached proved to be of prime importance for the immunological activity of the resulting conjugate. It was reported that attachment of the 8-hydroxyadenine based TLR7 agonist via its 9-benzyl moiety to an oligopeptide did mediate DC activation.[15]

This chapter describes the synthesis of conjugates comparable to the ones described previously by others for different antigens. In the conjugates described here (Figure 1, **3 - 5**) the TLR7 ligand 9-benzyl-8-hydroxy-2-butoxy-adenine is covalently connected with its 9-benzyl moiety to the CTL (SIINFEKL) epitope. A derivative of 9-benzyl-8-hydroxy-2-butoxy-adenine having a carboxyl function at the para position of the benzyl moiety was used for the covalent attachment of the TLR7 ligand to the N-terminus of OVA-derived peptide DEVSGLEQLESIINFEKL to give conjugate **3**. The same TLR7 ligand was installed at both the N-terminus and at the side chain of the C-terminal lysine residue to give conjugates **4** and **5**, respectively. The A_5K extension on the C terminus of the peptide is introduced to allow a comparison of the immunological activity of the conjugates having the TLR7 agonist installed at either the N- terminus or close to the C-terminus of the epitope containing peptide. It is not excluded that a TLR ligand at the C-terminus of DEVSGLEQLESIINFEKL will preclude the antigen presentation. An ethylene glycol spacer, exhibiting minimal steric hindrance is selected for its favourable influence on the solubility, as was shown in former studies (see Chapter 3).

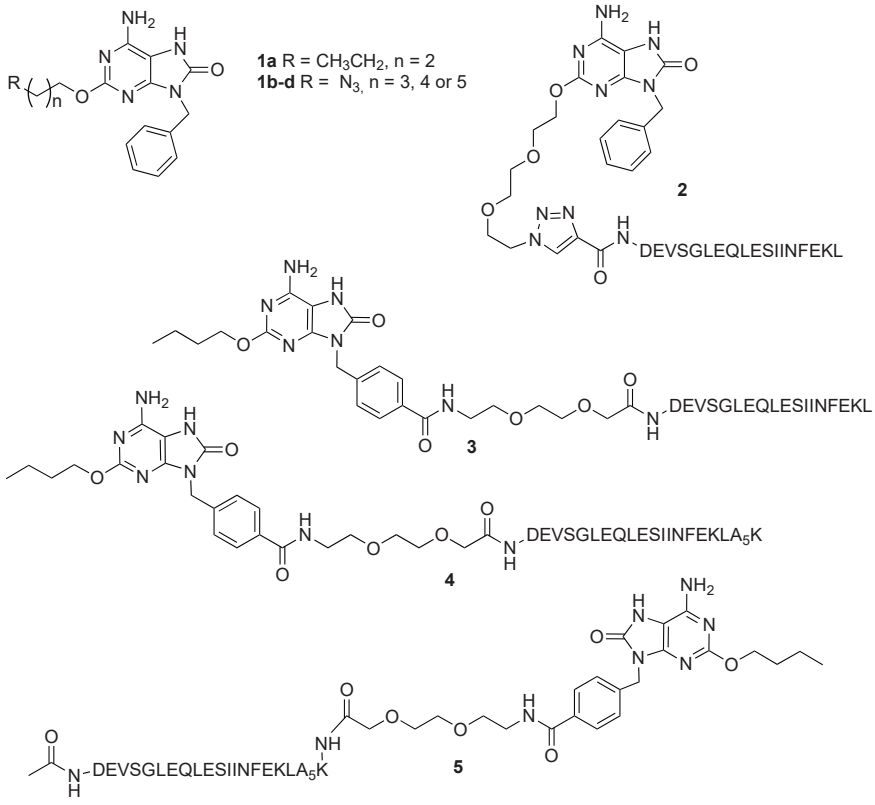
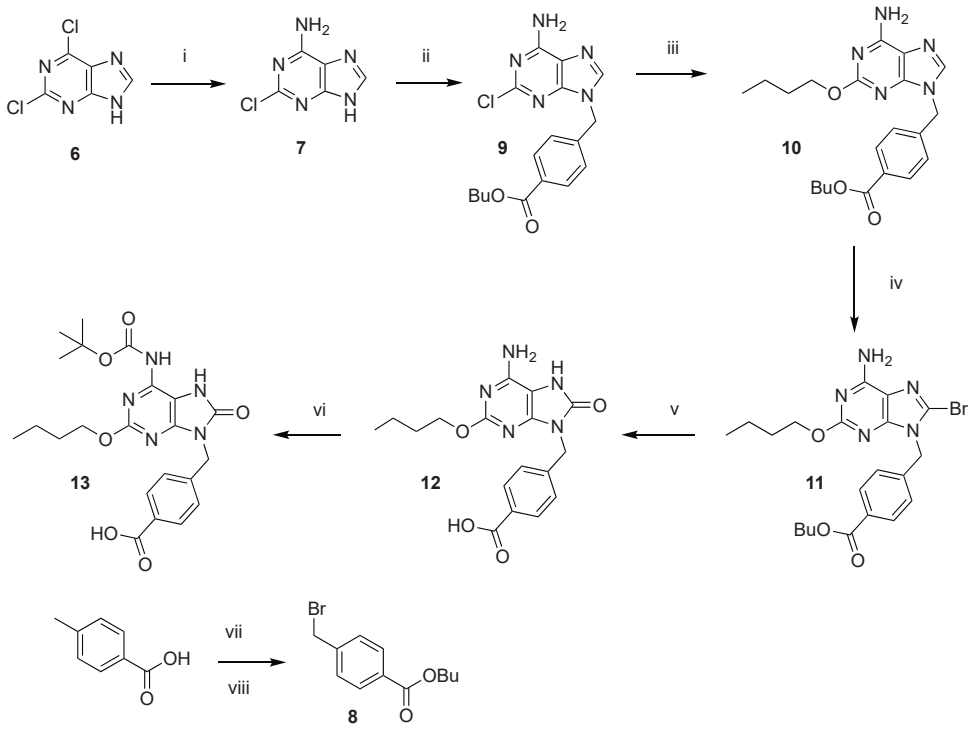


Figure 1. TLR7 ligand (**1**) and its conjugate (**2**) synthesized previously by Weterings *et al.* and the target TLR7 conjugates (**3**, **4** and **5**) described n this Chapter.

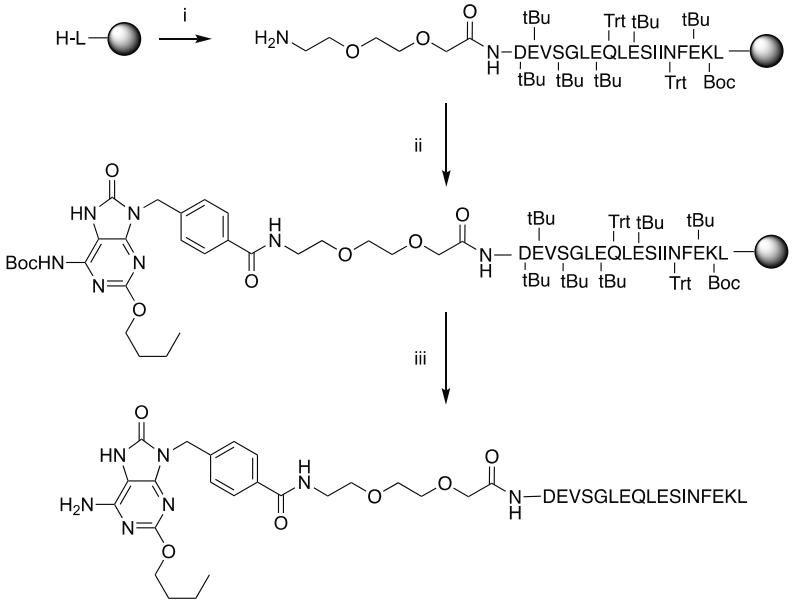
Result and discussion

The assembly of the projected conjugates (**3-5**) was performed with the aid of an automated synthesizer using a solid phase peptide synthesis (SPPS) protocol. To achieve this, the synthesis of partially protected and functionalized TLR7 ligand **13** was undertaken by adaptation of published procedures (Scheme 1).[11, 20-22] In the first step of the synthetic route to **13** commercially available 2,6-dichloropurine **6** was treated with ammonia/methanol to give in a regioselective manner 2-chloroadenine **7**. Next selective benzylation of the N9 position in 2-chloroadenine **7** was explored. The required alkylating reagent **8** was prepared by acid mediated condensation of p-toluic acid and n-butanol, followed by radical bromination of the obtained ester, using N-bromosuccinimide and AIBN. It was established that benzylation of 2-chloroadenine **7** with bromide **8** using 1 M TBAF in THF outperformed other applied bases. For instance, the selectivity and yield dropped when K_2CO_3 was used as a base. It of interest that the quality of the TBAF/THF solution, probably its water content, can influence the yield of benzylation of the N9 position of the adenine derivative. The following nucleophilic aromatic substitution of 2-chloroadenine derivative **9** with sodium n-butoxide at 120^{0}C was accompanied by partial hydrolysis of the butyl ester.



Scheme 1. Synthesis of compound **13**. *Reagent and conditions:* i) Sat. NH_3 in MeOH, 100°C. 88% ii) **8**, 1M TBAF in THF, RT, 92% iii) 1) NaH, n-BuOH, 120°C 2) H_2SO_4, 80 C°, 62% iv) Br_2, DCM, RT, 76% v) 1) NaOMe, MeOH, 65°C 2) 37% HCl, RT 3) 1M KOH, H_2O, RT, 53% vi) BocON, TEA, H_2O/Dioxane 1:1, RT 10%.vii) H_2SO_4, n-BuOH, 120°C, 94% viii) NBS, AIBN, CCl_4, 80°C, 73%

The butyl ester was recovered by acidifying the crude reaction mixture with H_2SO_4 and heating it at 80°C for 4 hours to give adenine derivative **10**. Despite the fact that the conversion of **9** into **10** was executed several times, a yield higher than 65% could not be obtained. Electrophilic aromatic substitution of **10**, using elementary bromine led to **11**. Subsequent direct hydrolysis of bromine at the C8 position in **11** with NaOH was unsuccessful. Fortunately, treatment of **11** with sodium methanolate, followed by acid mediated hydrolysis of the produced methyl ether and, finally, saponification of the ester gave adenine derivative **12.** Compound **12** was insoluble in most solvents and was isolated by precipitation in an acidic environment followed by collection of the sticky precipitate by centrifugation, washing the precipitate with dilute HCl (aq) and drying. The impracticability of **12** urged the protection of the exocyclic amine function in **12** with the Boc-group. Using BocON as a protecting reagent the installation of the protecting group proceeded sluggishly, requiring an excess of reagents and long reaction times. After five days of stirring at room temperature with a ten-fold excess of reagents, LC-MS analysis indicated ~50% conversion and after purification **13** was obtained in only 10% yield. The moderate nucleophilicity of aromatic amines and the low solubility of both starting compound **12** and BocON may be an explanation for this disappointing result. Although the reduced nucleophilicity of the exocyclic amine in **12** should also reduce or even prevent side reactions during the use of **12** in SPPS, the low solubility of precursor **12** in organic solvents effectively precluded the application of this building block in peptide synthesis. Therefore, despite the low yield at the protection stage, building block **13** was used in the forthcoming SPPS.



Scheme 2. Synthesis of conjugate **3**. *Reagents and conditions:* i) standard SPPS synthesis ii) **13**, HCTU, Dipea, NMP iii) TFA/H_2O/TIS (95/2.5/2.5)

SPPS of conjugate **3** was performed using Tentagel® S PHB resin, Fmoc chemistry, HCTU as condensing agent and commercially available protected amino acid building blocks (Scheme 2). After completion of the DEVSGLEQLESIINFEKL sequence, the Fmoc group at the N terminus of the immobilized oligopeptide was removed and Fmoc-PEG-COOH linker was introduced and

deprotected using the same conditions as use for the Fmoc amino acids. In the final step Boc-protected ligand **13** was the coupled to the released amine using HCTU as a coupling agent. Finally, removal of all protecting groups and cleavage from the resin was achieved with a TFA cocktail to give after HPLC purification target conjugate **3** in a yield of 10%. Conjugate **4** (see figure 1) was prepared and purified using the same procedure as described for conjugate **3** but starting with with Tentagel® S RAM amide resin, to which Fmoc-Lys(MMT)-OH was coupled as the first amino acid, and was isolated in 12%. The synthesis of conjugate **5** was started from Fmoc-Lys(MMT)-Tentagel® S RAM using the same standard Fmoc peptide synthesis protocol as for **3** and **4**. After completion of the DEVSGLEQLESIINFEKLA_5K sequence, the N-terminal amine was acetylated and the MMT side chain protective group in the C-terminal lysine was cleaved. Next, the introduction of Fmoc-PEG-COOH linker and the removal of the Fmoc group was followed by the coupling of Boc-protected ligand **13**. Removal of all protecting groups and cleavage from the resin, as described above, gave after purification conjugate **5** in 10% yield.

Conclusion

This chapter describes the functionalisation of the TLR 7 ligand 9-benzyl-8-hydroxy-2-butoxy-adenine with a carboxyl function at the para position of the benzyl moiety. This functionalized ligand (**12**) was insoluble in various solvents and to make it suitable for SPPS the Boc protected derivative (**13**) was prepared. Although the synthesis of building block **13** has been successfully achieved, optimization is needed as the installation of the Boc group was low yielding. Application of building block **13** in the on line SPPS resulted in the isolation of the conjugates **3**, **4** and **5**. Biological evaluation of these conjugates is in progress. Further achievement would be to label these conjugates using method described in chapter two to allow confocal microscopy upon DC stimulation with such constructs.

Experimental section

General procedures

All reactions were carried out at room temperature and under nitrogen atmosphere unless stated otherwise. All solvents were dried and stored using activated 4Å or 3Å molecular sieves. TLC monitoring was performed using Merck aluminum sheets (60 F_{254}). Compounds where visualized by UV detection if applicable at 254 nm and by spraying with 20% H_2SO_4 in EtOH, or $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at 150°C. Flash column chromatography was performed using Fluka silica gel (0.04 – 0.063 mm). LC-MS analysis was performed on a 50 x 4.60 mm Gemini C18/3 µm column (detection at 214 and 254 nm), coupled to an ESI mass spectrometer with a solvent system of B over A where A is H_2O and B is MeCN and 0.1% TFA with a linear gradient as specified. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 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 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.2843) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H and Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard or the residual peak of the deuterated solvent. Coupling constants are given in Hz and peak assignment was done using COSY and HSQC spectra. FT-IR spectra were recorded on a Shimadzu IRAffinity-1 FT-IR spectrometer.

Butyl 4-methylbenzoate

p-Toluic acid (390 mmol, 53.03 g) was dissolved in dry n-BuOH (500 mL). H_2SO_4 (35 mmol, 1.9 mL) was added. The mixture was refluxed at 110°C for 5 hours. TLC analysis (2:8 EA:Pnt) showed complete conversion of the reaction. n-BuOH was evaporated *in vacuo* and the reaction mixture was diluted with DCM and consequently washed with 10% $NaHCO_3$ (aq). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. Excess n-BuOH was removed by co-evaporation with toluene. butyl 4-methylbenzoate (366 mmol, 70.4 g) was obtained with a 94% yield.

IR (cm^{-1}): 2957 (C-H alkane, stretch), 1713 (C=O, stretch).

HRMS $[M+H]^+$: 193.12231 (calculated),

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 8.2 Hz, 2H), 7.22 (t, *J* = 14.0 Hz, 2H), 4.29 (t, *J* = 6.6 Hz, 2H), 2.37 (s, 3H), 1.73 (dt, *J* = 14.5, 6.7 Hz, 2H), 1.52 – 1.40 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.67, 143.33, 129.53, 128.99, 127.78, 64.58, 30.81, 21.56, 19.29, 13.75.

Butyl 4-(bromomethyl)benzoate (**8**)

Butyl 4-methylbenzoate (366 mmol, 70.4 g) was dissolved in CCl_4 (350 mL) under argon atmosphere. NBS (402 mmol, 71.6 g), AIBN (30 mmol, 4.92 g) was added. The reaction mixture was slowly heated to 90°C and refluxed for 4 hours. TLC analysis (5:95 Et_2O:Pnt) indicated complete conversion. The solvent was evaporated *in vacuo*. The mixture was diluted with EA and washed with H_2O. The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (0.25% Et_2O in Pnt → 1% Et_2O in Pnt, Δ=0.25%). Compound **8** (266 mmol, 71.8 g) was obtained with a 73% yield.

IR (cm^{-1}): 3197 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1713 (C=O, stretch).

HRMS $[M+H]^+$: 271.11816 (measured), 271.03282 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.3 Hz, 2H), 7.42 (d, *J* = 8.2 Hz, 2H), 4.46 (s, 2H,), 4.31 (m, 2H), 1.81 – 1.66 (m, 2H), 1.53 – 1.36 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 165.92, 142.52, 130.39, 129.97, 128.98, 64.90, 32.29, 30.75, 19.28, 13.79.

2-chloro-9H-purin-6-amine (**7**)

2,6-Dichloropurine (153.6 mmol, 29.04 g) was dissolved in 28% NH_3 in MeOH (150 mL) in an autoclave. The autoclave was sealed and heated to 100°C and stirred for 20 hours. The reaction mixture was filtrated and the residue was washed with MeOH (3x). Compound **7** (130.5 mmol, 22.12 g) was obtained with a 85% yield.

IR (cm^{-1}): 3273 (N-H, stretch), 3106, 2959, 1678, 1611.

HRMS $[M+H]^+$: 170.02369 (measured), 170.02280 (calculated).

^{1}H NMR (400 MHz, DMSO) δ 12.04 (s, 1H), 7.68 (s, 1H), 7.19 (s, 2H).

Butyl 4-((6-amino-2-chloro-9H-purin-9-yl)methyl)benzoate (**9**)

Compound **7** (4.50 mmol, 0.765 g) was dissolved in 1M TBAF in THF under argon atmosphere. Compound **8** (9.03 mmol, 2.45 g) was added and the reaction mixture was stirred overnight. TLC-MS indicated complete conversion of the reaction. The reaction mixture was concentrated *in vacuo* and consequently diluted with EA. The solution was washed with 10% $NaHCO_3$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (1% MeOH in DCM → 5% MeOH in DCM, Δ=1%). Compound **9** (4.15 mmol, 1.492 g) was obtained with a 92% yield.

IR (cm^{-1}): 3297 (N-H, stretch), 3122 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1736 (C=O, stretch), 1597 (C-C ring, stretch).

HRMS $[M+H]^+$: 360.12143 (measured), 360.12218 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.3 Hz, 2H), 7.73 (s, 1H), 7.33 (d, *J* = 8.3 Hz, 2H), 6.20 (s, 2H), 5.40 (s, 2H), 4.32 (t, *J* = 6.6 Hz, 2H), 1.74 (m, 2H), 1.53 – 1.40 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 156.31, 140.63, 139.92, 130.51, 127.81, 65.22, 47.09, 30.84, 19.38, 13.88.

Butyl 4-((6-amino-2-butoxy-9H-purin-9-yl)methyl)benzoate (**10**)

Compound **9** (16.8 mmol, 6.03 g) was suspended in n-BuOH (170 mL). NaH (168 mmol, 6.75 g) was slowly added at 0°C. The mixture was heated to 120°C and refluxed overnight. TLC analysis (5% MeOH in DCM) indicated complete disappearance of the starting material. The mixture was cooled to 0°C and H_2SO_4 (185 mmol, 9.8 mL) was slowly added. The mixture was heated to 90°C and stirred for 3 hours. TLC analysis

indicated complete conversion. The solvent was evaporated *in vacuo*. The mixture was diluted with EA and washed with 10% $NaHCO_3$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (1% MeOH in DCM). Compound **10** (10.5 mmol, 4.18 g) was obtained with a 62% yield.

IR (cm^{-1}): 3282 (N-H, stretch), 3110 (C-H aromatic, stretch), 2955 (C-H alkane, stretch), 1736 (C=O, stretch), 1597 (C-C ring, stretch).

HRMS $[M+H]^+$: 398.21702 (measured), 398.21867 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.1 Hz, 2H), 7.58 (s, 1H), 7.32 (d, 2H), 5.33 (s, 2H), 4.31 (t, *J* = 6.6 Hz, 4H), 1.84 – 1.66 (m, 4H), 1.47 (m, 4H), 0.96 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.17, 162.48, 156.50, 140.79, 130.56, 130.26, 127.73, 67.24, 65.09, 46.69 , 31.15, 30.80, 19.35, 13.96, 13.83.

Butyl 4-((6-amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl)benzoate (**11**)

Compound **10** (1.21 mmol, 480 mg) was dissolved in DCM. Bromine (23.4 mmol, 1.21 mL) was slowly added at 0°C. The reaction mixture was heated to RT and stirred for 70 minutes. TLC analysis (3% MeOH in DCM) indicated complete conversion of the reaction. The reaction was quenched with a saturated solution of $Na_2S_2O_3$ (aq) at 0°C. The mixture was diluted with DCM and washed with H_2O (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (1% MeOH in DCM). Compound **11** (0.92 mmol, 437 mg) was obtained with a 76% yield.

IR (cm^{-1}): 3320 (N-H, stretch), 3196 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1722 (C=O, stretch), 1652 (N-H, bend), 1589 (C-C ring, stretch).

HRMS $[M+H]^+$: 478.12507 (measured), 478.12713 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.3 Hz, 2H), 7.38 (d, *J* = 8.3 Hz, 2H), 6.60 (s, 2H), 5.34 (s, 2H), 4.31 (m, 4H), 1.81 – 1.67 (m, 4H), 1.54 – 1.39 (m, 4H), 0.96 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.07, 162.24, 155.57, 152.75, 140.09, 130.31, 129.99, 127.69, 123.98, 115.93, 67.16, 64.93, 46.86, 31.01, 30.68, 19.24, 19.22, 13.87, 13.73.

4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid (**12**)

Compound **11** (3.8 mmol, 1.8 g) was dissolved in 5M NaOMe in MeOH (10 mL) under argon atmosphere. The mixture was refluxed for 4 hours at 65°C, after which LC-MS indicated complete conversion of the reaction. The solvent was evaporated *in vacuo* and 37% HCl (aq) (20 mL) was slowly added at 0°C. LC-MS indicated complete disappearance of starting material after overnight stirring at RT. The solvent was evaporated *in vacuo* and 1M KOH (aq) (50mL) was added. After overnight stirring, LC-MS indicated complete conversion to the product. The solution was acidified with a 1M HCl until a white crash was formed. The solution was centrifuged and the residu was washed with 1M HCl (3x) and consequently dried *in vacuo*. Compound **12** (2.0 mmol, 787 mg) was obtained with a 53% yield.

IR (cm^{-1}): 3418 (N-H, stretch), 3168 (O-H, stretch), 1689 (C=O acid, stretch).

HRMS $[M+H]^+$: 358.15009 (measured), 358.15098 (calculated).

^{1}H NMR (400 MHz, DMSO) δ 10.96 (s, 1H), 7.90 (t, *J* = 8.7 Hz, 2H), 7.38 (t, *J* = 9.6 Hz, 2H), 4.95 (s, 2H), 4.20 (t, *J* = 6.6 Hz, 2H), 1.61 (dt, *J* = 14.5, 6.7 Hz, 2H), 1.40 – 1.27 (m, 2H), 0.87 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, DMSO) δ 167.45, 152.23, 149.08, 142.16, 130.36, 130.04, 127.95, 98.67, 67.44, 42.78, 30.77, 19.06, 14.11.

4-((2-butoxy-6-((tert-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid (**13**)

Compound **12** (1.5 mmol, 530 mg)was dissolved in 1:1 H_2O:Dioxane (10 mL). TEA (4.5 mmol, 0.63 mL) and BocON (20 mmol, 4.9 g) were added to the mixture. The reaction mixture was stirred for 4 days at RT. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (5% MeOH in DCM). Compound **13** (0.15 mmol, 68 mg) was obtained with a 10% yield.

IR (cm^{-1}): 3431(N-H, stretch), 3163 (C-H aromatic, stretch), 2958 (C-H alkane, stretch), 1753 (C=O, stretch), 1720 (C=O, stretch), 1637 (C=O, stretch).

^{1}H NMR (400 MHz, DMSO) δ 7.90 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 8.3 Hz, 2H), 7.06 (s, 2H), 4.93 (s, 2H), 4.16 (t, *J* = 6.6 Hz, 2H), 1.66 – 1.57 (m, 2H), 1.54 (s, 9H), 1.41 – 1.28 (m, 2H), 0.88 (dd, *J* = 9.1, 5.7 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ 167.06, 161.19, 150.55, 150.18, 149.62, 149.15, 141.16, 130.03, 129.62, 127.58, 96.42, 85.19, 66.25, 42.74, 30.52, 27.65, 18.72, 13.72.

Conjugate 3: Tentagel® S PHB Resins preloaded with Fmoc-Leu or with Fmoc-Lys-(Boc) was subjected to solid phase Fmoc-peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **3** in 10% yield. LC-MS (10%-90% gradient); Rt = 5.63; ESI-MS: m/z 1274.3 (exact mass), 1274.7 (most abundant); calculated for $[C_{114}H_{175}N_{27}O_{39} + 2H]^{2+}$ 1274.13 (exact mass), 1274.64 (most abundant).

Conjugate 4: Tentagel® S RAM resin was coupled to Fmoc-Lys-(MMT)-OH and subjected to solid phase Fmoc-peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **4** in 12% yield. LC-MS (10%-90% gradient); Rt = 5.41; ESI-MS: m/z 1515.9 (most abundant); calculated for $[C_{135}H_{213}N_{35}O_{44} + 2H]^{2+}$ 1515.28 (exact mass), 1515.78 (most abundant).

Conjugate 5: Tentagel® S RAM resin was coupled to Fmoc-Lys-(MMT)-OH and subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Terminal amine was capped using standard capping conditions (5% Ac_2O in NMP). MMT side chain protective group was cleaved from the resin-bound peptide using 5% TFA in DCM (three times, 5 min). Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol,0.228 g) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **5** in 10% yield. LC-MS (10%-90% gradient); Rt = 5.59; ESI-MS: m/z 1536.8 (most abundant); calculated for $[C_{137}H_{215}N_{35}O_{45} + 2H]^{2+}$ 1536.29 (exact mass), 1536.79 (most abundant).

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Chapter 5: Phosphine reactivity towards azides in water: Reduction versus hydrolysis

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Introduction

In the field of bioorganic synthesis, azides are commonly used as masked functionalities to protect amines. Azides are easily introduced by substitution reactions or can be obtained from amines by diazo transfer. Azides can withstand a variety of reaction conditions and are easily converted into amines by several types of reduction, including the Staudinger reaction[1,2]. Reaction of an azide with a trialkyl- or triarylphosphine proceeds via an iminophosphorane that upon hydrolysis leads to the corresponding amine (Figure 1). The combination of azide and Staudinger reaction was given a reappraisal by the emergence of bioorthogonal chemistry[3]. This field of research aims to the selective detection of a specific biomolecule in living or biological systems[4]. To attain this goal the biomolecule of interest should be provided with a reactive group that is inert in biological systems but selectively reacts with a reporter group under physiological conditions[5,6]. The azide function is small, relatively stable, abiotic and essentially non-cytotoxic while incorporation of an azide into the biomolecule of interest allows a number of selective reactions with probes such as reporter molecules. There are three bioorthogonal reactions of which the copper-catalyzed [3+2] Huisgen cycloaddition[7] and strain-promoted [3+2] cycloaddition[8] of azides with alkynes lead to triazoles. In contrast, the Staudinger–Bertozzi ligation, the first bioorthogonal reaction, connects the biomolecule and the reporter group via an amide linkage. These favorable properties make azides the most widely used reactive group in the field of *in vivo* bioorthogonal chemistry. On the other hand the conversion of an azide into an amine in biological systems or the use of an azide as a bioorthogonal protecting group has not been studied yet.

With the ultimate goal to develop a latent epitope that is not recognized by its cognate T-cell, an azide as an amino protecting group is incorporated in a CD8+ T-cell MHC class I peptide epitope. Upon presentation of this undetectable epitope by antigen presenting cells, Staudinger reduction of the azide in the epitope by a trialky- or triaryl phosphorus reagent would liberate the amine and produces the native MHC-I/peptide epitope that can activate the T-cell. This chapter describes a study towards the aqueous Staudinger reduction of an azide to an amine that is installed in an oligopeptide. The well-studied epitope **5** (SIINFEKL) from chicken egg white ovalbumine, was chosen as a model substrate to study the efficiency of the reduction. This model epitope has been successfully used in the field of cancer vaccines[9,10] and is relatively easy to synthesize on large scale. Moreover, the lysine in the SIINFEKL sequence is not involved in the formation of MHC complex[11,12], and can be used to replace the native amine by an azide to give target oligopeptide SIINFEK*L (K* = azido-norleucine) **4** as a model substrate to explore the Staudinger reduction[13]. For the Staudinger reaction three phosphines were selected on the basis of their solubility in water and their compatibility with the cell media. One of the most used water soluble phosphines is tris(2-carboxyethyl)phosphine (TCEP, **1**). In addition, sulfonylated triphenylphosphine ($PPh_3(SO_3Na)_3$, **2**) and sodium hypophosphite ($NaPO_2H_2$, **3**) were selected (Figure 2).

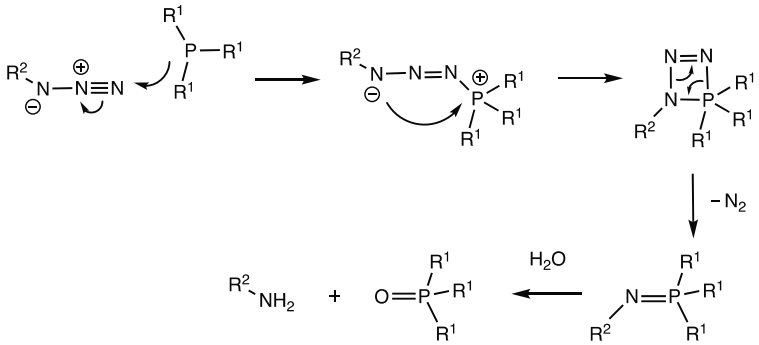


Figure 1. Staudinger reduction mechanism

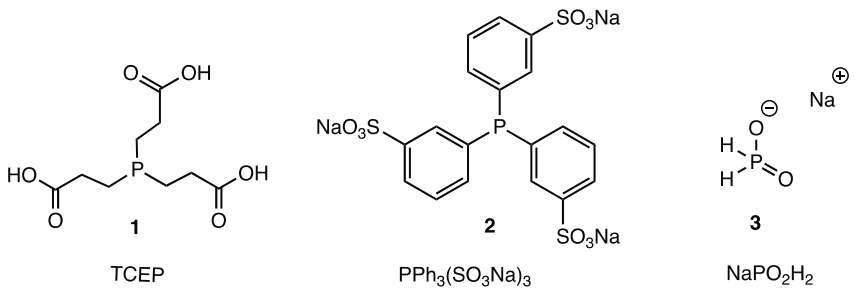


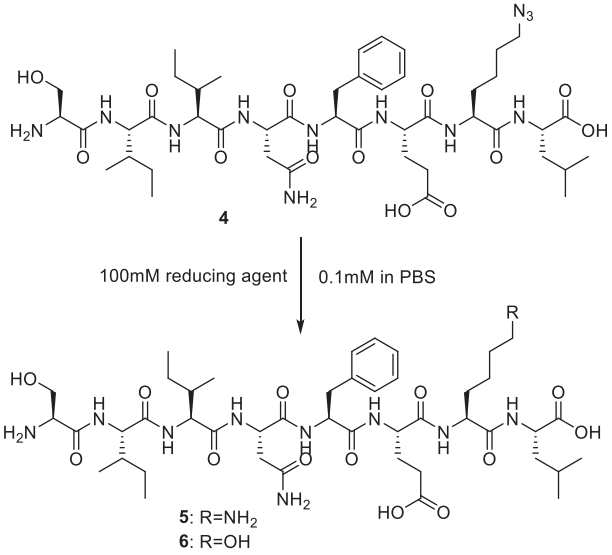
Figure 2. Reducing agents selected for this study

Results and discussion

The first step in the construction of target oligopeptide SIINFEK*L **4** involves the synthesis of Fmoc protected azido lysine (K* or 6-azido-norleucine) as described in Chapter 2. Having all protected amino acids available, azido containing peptide **4** and reference (SIINFEKL) peptide **5** were assembled by a standard solid phase peptide synthesis protocol using Fmoc chemistry, Tentagel®

S PHB Resin preloaded with leucine and HCTU as condensating agent. After cleavage from the solid support and purification by RP HPLC oligopeptide **4** and **5** were obtained in 21% and 19 % yield, respectively.

To explore the Staudinger reduction event of the azide in **4** into amine in **5**, conditions required for on-cell-surface reduction were mimicked by selecting a ratio of 1:1000 for peptide **4** and phosphine reagent (Scheme 1). Although the concentration of the reductive agent looks extreme, it was shown that cells can tolerated it with an acceptable level of survivability[14].



Scheme 1. Azide reduction in PBS is accompanied by hydrolysis

In practice this means that the use of 0.1 µM azido-containing peptide **(4)** and 100mM of the respective phosphine (**1**, **2** or **3**, Figure 2) allowed an easy monitoring of the progress of reaction by standard LC-MS analysis. In the first experiment peptide (**4**) was treated with TCEP (**1**) in PBS buffer (pH=7.4-7.6). Starting peptide (**4**, blue) disappeared within 95 min as depicted in Figure 3. Surprisingly, apart from native peptide (**5**, green) another major (**6**, red) product and a minor product (**7**, yelllow) were formed. Repetition of this experiment on large scale to allow HPLC purification led to the isolation of homogenous **5** and **6**.

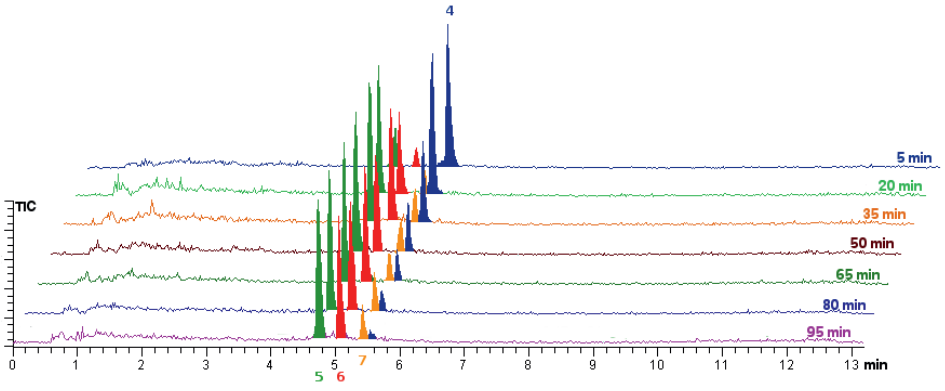


Figure 3. Reduction of peptide **4** by TCEP in PBS (1:1000). Peak intensity corresponds to the amount of compound present in the reaction mixture. While starting material (**4,** blue) disappears as the reaction proceeds, several new species: **5** (green), **6** (red) and **7** (yellow) are formed.

Mass spectroscopic analysis showed that, the mass of **6** (red) is one dalton higher than native peptide **5** (green) suggesting that product **6** is provided with 6-hydroxy-norleucine instead of lysine. To confirm this assumption NMR studies were performed. Comparison of HSQC-specta of **4**, **5** and **6** showed the following features (Figure 4). The blue cross-peak in the spectrum of SIINFEK*L **4** corresponds to the ε-hydrogen/carbon coupling in the 6-azido-norleucine. In the similar spectra of both reference and isolated SIINFEKL peptide (**5**), this signal is lacking and shifted to the new cross-peak (green), which corresponds to ε-hydrogen of lysine. Also in the NMR spectra of **6** the ε-hydrogen/carbon (red) is shifted to the range of 60-65 ppm in carbon NMR confirming that **6** is provided with a 6-hydroxyl-norleucine instead of lysine. The mass spectrometry and NMR spectroscopic data point to the fact that TCEP (**1**) not only reduces the azide into an amine but also hydrolyses the azide to an alcohol under the conditions applied.

Although the isolated amount of minor impurity **7** was not enough for NMR analysis, it is hypothesized (on the basis of LC MS data) that the hydrolysis is accompanied by elimination to give **7**, having a double bond between the δ- and ε-carbon.

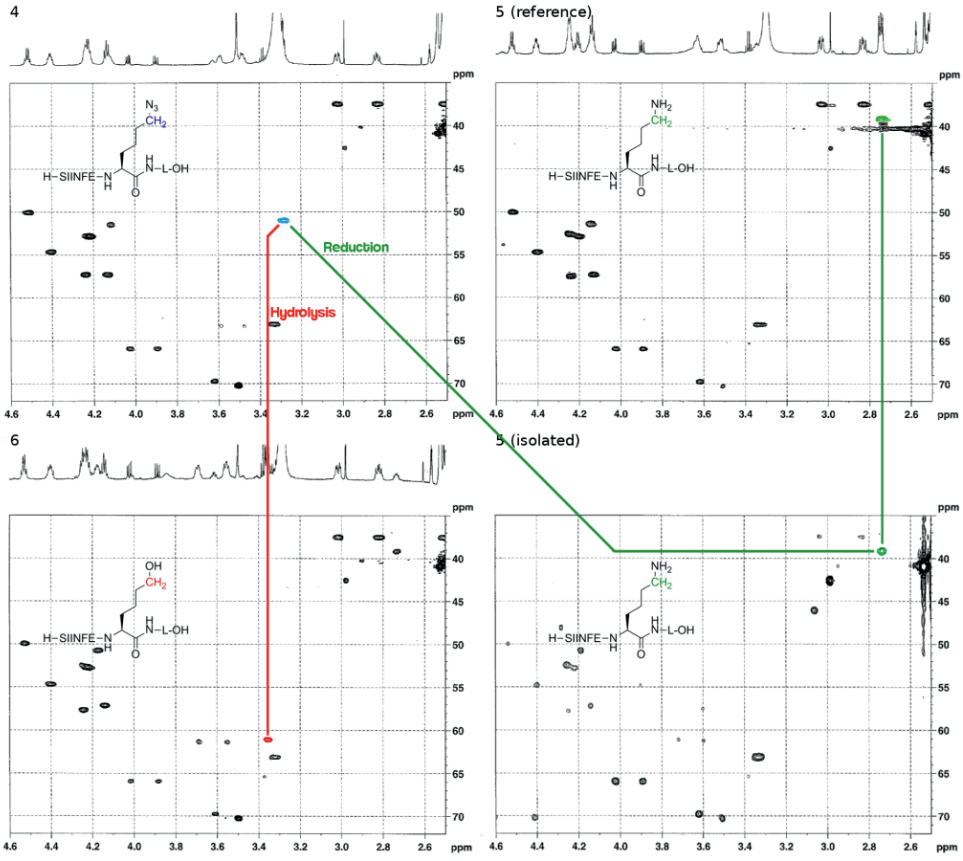


Figure 4. HSQC NMR in *d*-DMSO of isolated peptides.

Next the reduction of the azide in **4** was studied with sulfonylated triphenylphosphine ($PPh_3(SO_3Na)_3$ **2** and sodium hypophosphite ($NaPO_2H_2$) **3**. Interestingly, $PPh_3(SO_3Na)_3$ **2** in PBS buffer (pH 7.2-7.4) did not reduce the azide to an amine and yielded predominantly hydrolysis product **6** and putative elimination product **7** (Figure 5). In addition, the reaction rate has also significantly decreased and more impurities were found (peaks with higher retention time, Figure 5).

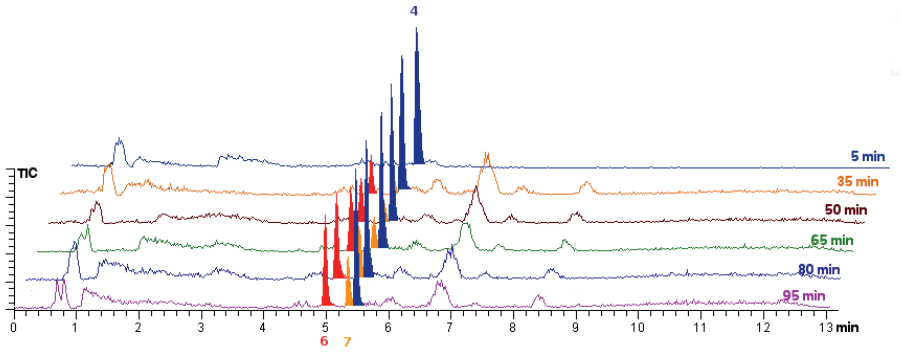


Figure 5. Reduction of azidated petide **4** using $PPh_3(SO_3Na)_3$ in PBS

No conversion was observed upon treatment of peptide **4** with $NaPO_2H_2$ (**3**) in PBS buffer for 1.5 h. With these results in hand the influence of the pH on reactions with both TCEP **1** and sulfonylated triphenylphosphine **2** was studied, using the procedure described above and different buffers (from pH 2 to pH 12).

The rate of the reaction of TCEP **1** with peptide **4** proved to be pH dependent without affecting product ratios. Contrary, the pH dramatically influenced the product distribution in the reaction of $PPh_3(SO_3Na)_3$ **2** with azidated peptide **4**. As depicted in Figure 6, at pH 12, only reduction of azide **4** into amine **5** was observed while at pH 7.4 and below, mainly hydrolysis occurred to give **6** along with the formation of minor amounts of elimination product **7**.

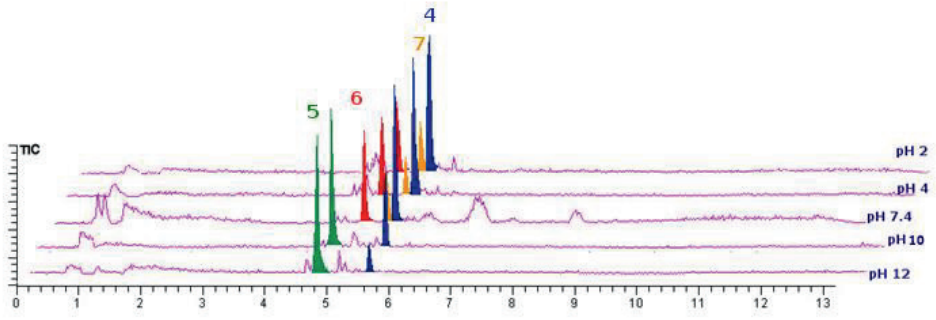


Figure 6. Reduction of **4** using $PPh_3(SO_3Na)_3$ at different pH in phosphate buffer.

As not only the pH but also the type of buffer may influence the Staudinger reaction of azidated peptide **4** with $PPh_3(SO_3Na)_3$, **2** similar experiments were executed using TRIS/HCl based buffer and a pH-range from 8.9 to 7.4. As depicted in figure 7, at pH 7.4 and pH 8, hydrolysis product **6** is still the major product, along with minor amount of **7**. At pH 8.5, amine **5** has become the major product, while minor amounts of **6** and **7** can still be observed. Increasing the pH to 8.9 shows only formation of amine **5** without any detectable amount of ether the hydrolysis product **6** or alkene **7**.

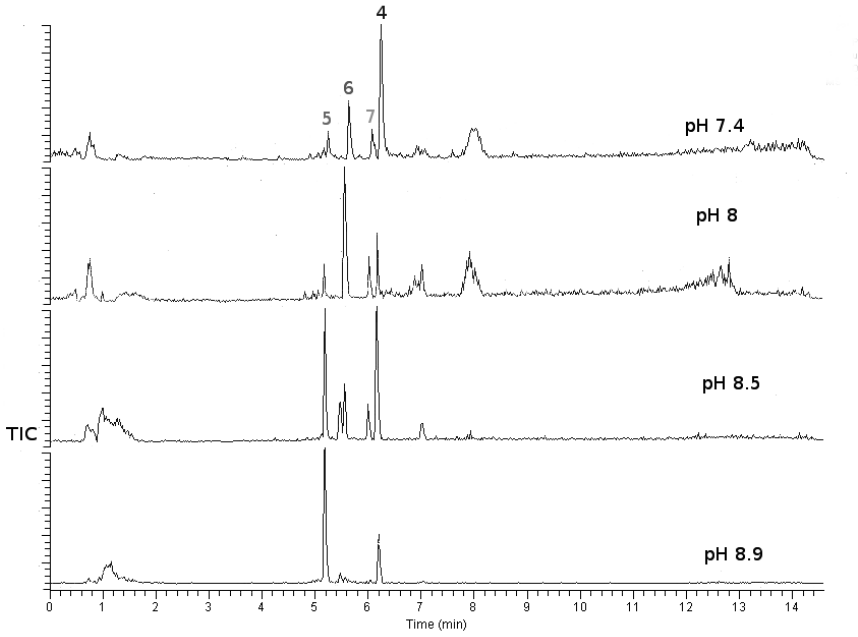


Figure 7. pH dependency using **2** as reductive agent for TRIS-buffer.

Concurrent hydrolysis during the Staudinger reduction of azides to amines with trialkyl- or triarylphosphines has not been reported. An indication for a possible mechanism is given by the group of Raines[15]. Guided by the versatility of diazo compounds they develop a procedure to prepare diazo compounds from azides and specific phosphines. Adopting their proposed mechanism, the putative formation of diazo compounds under acidic conditions, subsequent hydrolysis and elimination is a reasonable assumption and explains the formation of peptides **6** and **7** (Figure 8). The absence of **6** at high pH points to the same mechanism by which the standard Staudinger reduction of an azide to an amine is explained. Under basic conditions iminophosphorane is formed from the initial phosphazene intermediate via nitrogen elimination and subsequent hydrolysis produces an amine (Figure 8).

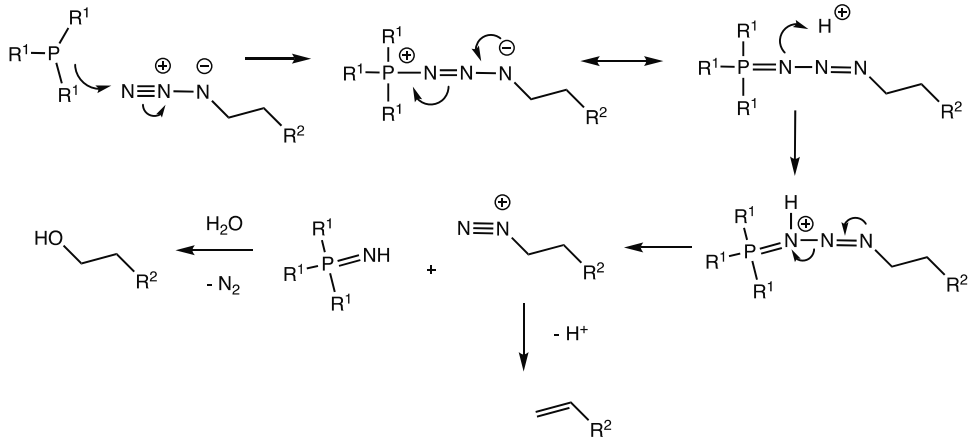


Figure 8. Proposed mechanism for the hydrolysis/elimination

Conclusion

As part of a study to explore azides as bioorthogonal protecting groups the azidated peptide epitope **4** was prepared and subjected to a reaction with three different phosphines in aqueous media. Tris(2-carboxyethyl)phosphine **1** and sulfonylated triphenylphosphine **2** transformed the azide in **4** into the expected amine **5**, but surprisingly also into several side products (**6** and **7**). Using spectroscopic analysis the major side product was identified as hydroxyl compound **6**. By exploring the reaction at different pH it was shown that using phosphine **1**, the product ratio could not be influenced. Contrary to this, phosphine **2** at either acidic or neutral pH favored hydrolysis /elimination (**6** / **7**). Under basic conditions the reduction of the azide into the amine prevailed. A mechanism is proposed explaining the formation of side products **6** and **7**.

Experimental

General: Chemicals were purchased from sigma Aldrich and Acros Organics; HPLC solvent were purchased from Biosolve. Endotoxin-free PBS was Gibco-brand purchased from Life Technologies. Peptide synthesis was performed on Applied Biosystem 433A Peptide synthesizer. LC-MS analysis was performed on a JASCO HPLC-system (detecting simultaneously at 214 and 254 nm) equipped with an analytical Gemini C_{18} column (4.6 mmD × 50 mmL, 3 µ particle size) in combination with buffers A: H_2O, B: MeCN and C: 0.5% aq. TFA. A PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer) was used as the mass detector. Alternatively, a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to surveyor HPLC system (Thermo Finnegan) was used. An analytical Gemini C_{18}. HPLC purification were performed on a Gilson GX-281 HPLC system. A preparative Gemini C_{18} (Phenomex, 150 x 21.2 mm, 5 micron) was used in combination with eluents A: 0.1M aq TFA and B: ACN as a solvent system. ^{1}H and ^{13}C were recorded on Bruker Ascend 850. Chemical shift (δ) of ^{1}H and ^{13}C spectra are relative to tetramethylsilane.

General procedure for reduction assay:

In 700 µL of PBS was added 100 µL of 1mM SIINFEK*L solution in PBS. Then 200 µL of 0.5 M solution of either desired phosphine or sodium hypophosphite was added and the reaction was performed at room temperature without stirring. Monitoring the reaction was done using LCMS every 15 min.

General procedure pH dependency assay:

In 700 µL of desired buffer was added 100 µL of 1mM SIINFEK*L solution in milliQ water. Then 200 µL of 0.5 M solution of of either desired phosphine or sodium hypophosphite was added and the reaction was performed at room temperature without stirring. Monitoring the reaction was done using LCMS after 3hours.

Peptide Synthesis:

Tentagel® S PHB Resins Preloaded with leucine was subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building block (NovaBiochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. The resin was washed with DCM, and shaken in a solution of TFA/H_2O/TIS (95%/2.5%/2.5%). The solution was separated from the resin by filtration and transferred into cold Et_2O followed by centrifugation (4400 rpm, 5 min). Decantation of the supernatant afforded the crude peptide which was purified by RP HPLC (10% → 50% ACN in 0.1% aq TFA) yielding **4** and **5**.

SIINFEK*L **4**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 6.1 min

[M+H]$^+$: 989.4 ; 21% yield

HRMS:[M+1]$^+$: 989.54146 found: 989.53982

SIINFEKL **5**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 5.2 min

[M+H]$^+$: 963.6 Yield 19% yield

HRMS:[M+1]$^+$: 963.55096 found: 963.54944

SIINFEOL **6**

LCMS:RT: (C_{18}column, 10%B-90%B, 13min grad): 5.5 min

[M+H]$^+$: 964.3

Buffers:

Table 1 Compositions of phosphate buffers for different pH

pH	Solution A	Solution B
2	0.2M KCl (0.5mL)	0.2M HCl (0.13mL)
6	0.1M KH_2PO_4 (10mL)	0.1M HCl (0.02mL)
10	0.05M $NaHCO_3$ (1mL)	0.1M NaOH (0.214mL)
12	0.2M KCl (0.5mL)	0.2M NaOH (0.12mL)

Table 2 Compositions of TRIS buffer for different pH

pH	0.1M Tris	0.1M HCl	MilliQ water
7.4	0.5mL	0.42mL	0.08mL
8	0.5mL	0.292mL	0.208mL
8.5	0.5mL	0.147mL	0.253mL
8.9	0.5mL	0.007mL	0.497mL

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Chapter 6: Towards convergent synthesis of viral VPg proteins linked to RNA

Presented at Europic2018, 3-7 June 2018, Egmond aan Zee, The Netherlands: Gential, G. P. P. *et al.* General methodology for the chemical synthesis of polynucleotidylated picornaviral genome-linked proteins, *Poster C06*.

Introduction

Nucleoproteins are naturally occurring polymers in which an hydroxy amino acid in a protein or peptide is covalently linked via a phosphodiester bond to the terminal hydroxyl of DNA or RNA[1]. Representatives of this class of hybrid biopolymers are widely found among different families of viruses, such as picornaviruses [2,3]. Members of the family of picornaviruses, such as polio- and coxsackievirus, have vertebrates as host and are associated with a number of diseases. Picornaviruses are RNA viruses the initiation of translation of which proceeds via a unique mechanism. Nucleoproteins of picornaviruses termed VPg (viral protein genome-linked) act as primer of RNA synthesis and a lot of research has been devoted to elucidate this complex mechanism at a molecular level[4–9]. In line with these investigations and to obtain useful molecular tools[10] attention was directed to the development of synthetic procedures to fragments of nucleoproteins. The main issue toward the assembly of fragments of nucleoproteins is to make compatible the chemistry of oligo(deoxy)nucleotides and oligopeptides. The automated synthesis of both oligonucleotides and oligopeptides are at a high level and within certain limits of size and composition all oligopeptides and oligonucleotides can be prepared. However, both the basic conditions inherent to the oligonucleotide synthesis and the acidic conditions belonging to oligopeptide synthesis can cause side reactions, which are detrimental for the nucleopeptide. Several synthetic approaches to nucleopeptides, including solution and solid phase approaches have been reported. Obviously, a solid phase procedure is most convenient to acquire nucleopeptides in which both the peptide and nucleotide part have a length larger than two amino acids and two nucleotides. The synthesis of DNA nucleopeptides is extensively investigated and several procedures have been published. For instance, the group of Grandas reported a solid phase synthesis of DNA nucleopeptides with both an extended peptide and oligodeoxynucleotide

part[11–13]. Nucleopeptides, varying in length and composition, in which the oligopeptide was covalently linked via a phosphodiester bond to the terminal 3' of the oligodeoxynucleotide, were prepared via an on-line solid phase approach. In contrast, the synthesis of RNA nucleopeptides is less explored. In one approach nucleotide amino acid building blocks were applied toward the solid phase synthesis of RNA nucleopeptides in which the oligopeptide is provided with a mono- or dinucleotide[14–18]. Recently van der Heden van Noort *et al* reported an automated sequential solid phase approach towards viral RNA-nucleopeptides[19]. In viral RNA nucleopeptides a hydroxy amino acid in the peptide is covalently linked via a phosphodiester bond to the terminal 5'-hydroxyl of RNA. As shown in Figure 1, to attain an on-line solid phase synthesis of a viral RNA nucleopeptide, first the oligopeptide was assembled on a HMBA resin followed by extension of the immobilized peptide with the RNA fragment. While common Fmoc protected amino acid building blocks were used for the peptide synthesis unconventional RNA building blocks were applied for the ensuing RNA synthesis. In these building blocks, the DMTr group, as a temporary protective group, is positioned at the 3' hydroxyl while the 2-cyanoethyl phosphoramidite function is installed at the 5' hydroxyl. A virus derived pentapeptide bearing a 9-mer oligonucleotide on the tyrosine was prepared [20].

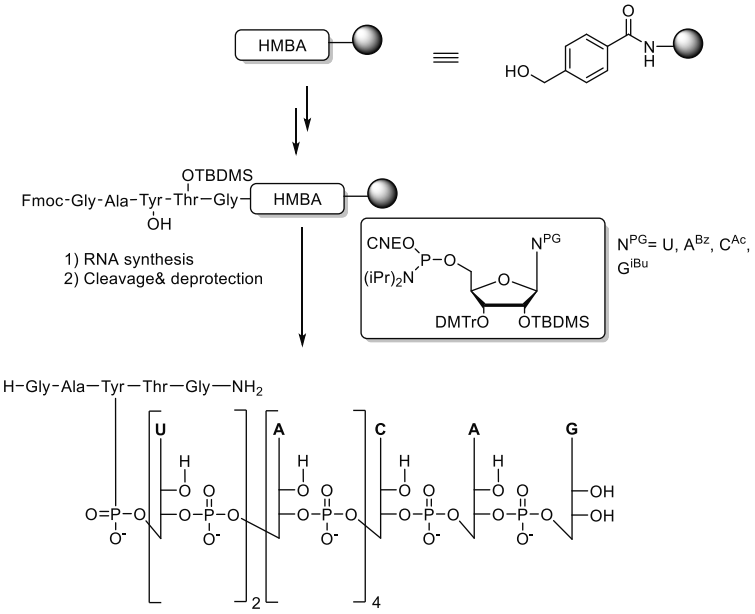


Figure 1. Oligonucleopeptide synthesis on peptide side chain using DMTr as a temporary protective group by van der Heden van Noort[20].

Although this method proved to be very powerful, the repetitive acid mediated cleavage of the temporary DMTr protective group forbid the use of an acid cleavable linker on the resin and therefor only nucleopeptides having carboxamide on the C-terminus are accessible. Nucleopeptides with a C-terminal carboxylic acid are more favorable and allow a solution phase block condensation with a separate oligopeptide to construct ultimately the complete native VPg nucleopeptide of picornaviruses. Bearing this goal in mind it was decided to adjust the method of van der Heden van Noort *et al.*[20] and coxsackievirus VPg **1** was chosen as potential target compound (Figure 2). Retrosynthetic analysis shows that complete VPg of this virus can be obtained by solution phase block condensation of peptide **3** and nucleopeptide **2**, both of which can be assembled by automated solid phase synthesis. Suitable Fmoc amino acid building blocks

for the solid phase synthesis of nucleopeptide **3** should be minimal protected to avoid acidic deprotection conditions incompatible with RNA. Consequently, lysine side chains are protected as TFA amides, which can be cleaved by ammonia treatment, a commonly used deprotection reagent for oligonucleotides. The oligonucleotide in nucleopeptide **2** was appended using nucleotide building blocks (**31-34**) in which the orthogonal acid cleavable DMT ether was replaced by the hydrazine cleavable levulinic ester (Lev-group). This modification of protective group strategy permits the application of HMBP resin that is provided with a mild acid cleavable linker, leading upon deprotection at the end of the synthesis to a native carboxylic acid on C terminus of the (nucleo)peptide.

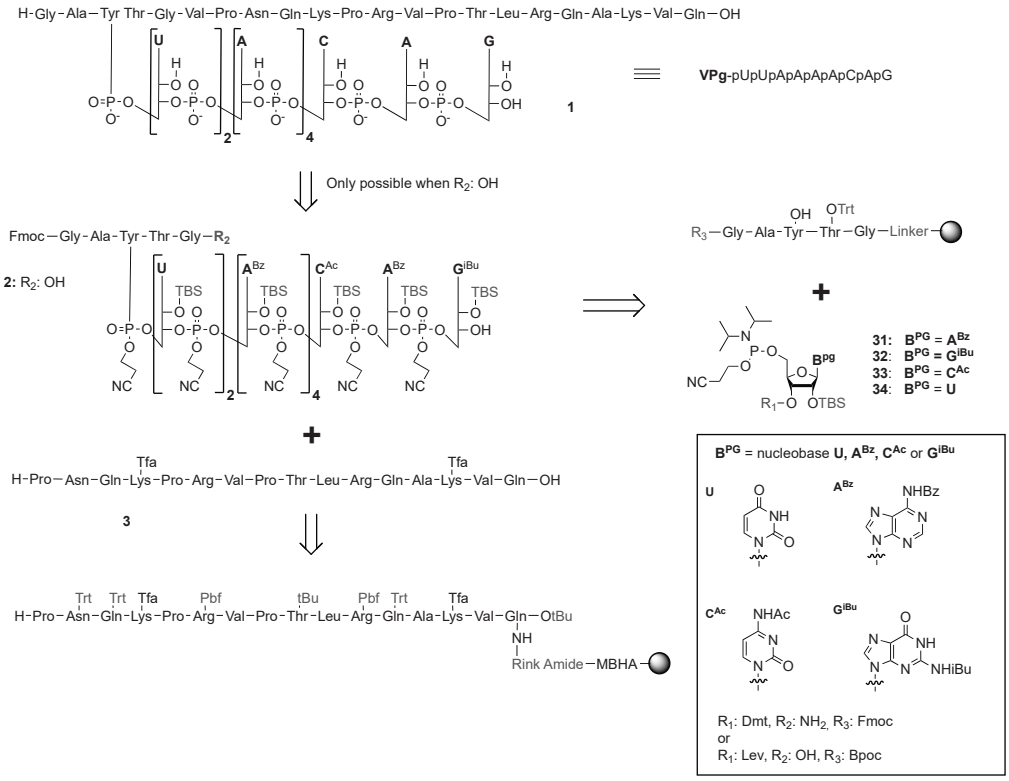


Figure 2. Retrosynthesis of the coxackie VPg using Lev as a temporary protective group.

This chapter describes the synthesis of nucleotide building blocks (**31-34**, Figure 2) and the application of these building blocks in the synthesis of partially protected nucleopeptides (**4-6**, Figure 3)**.** Two of the three obtained nucleopeptides were used in a block coupling with peptide **3** to give nucleopeptides **4** and **5** with extended peptide part.

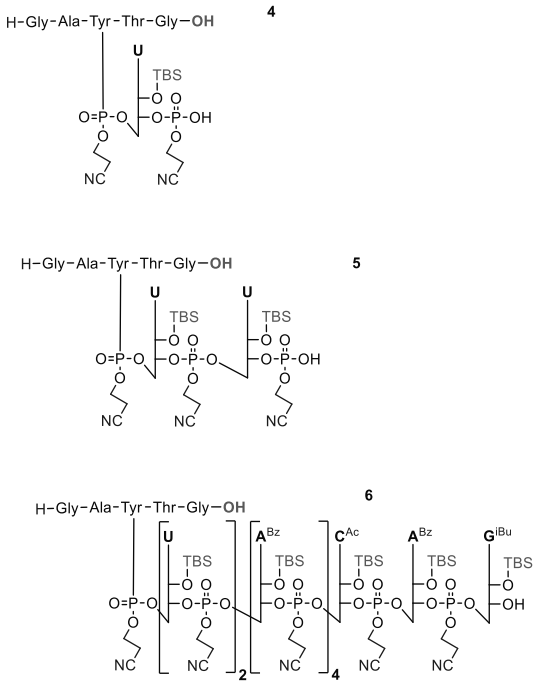
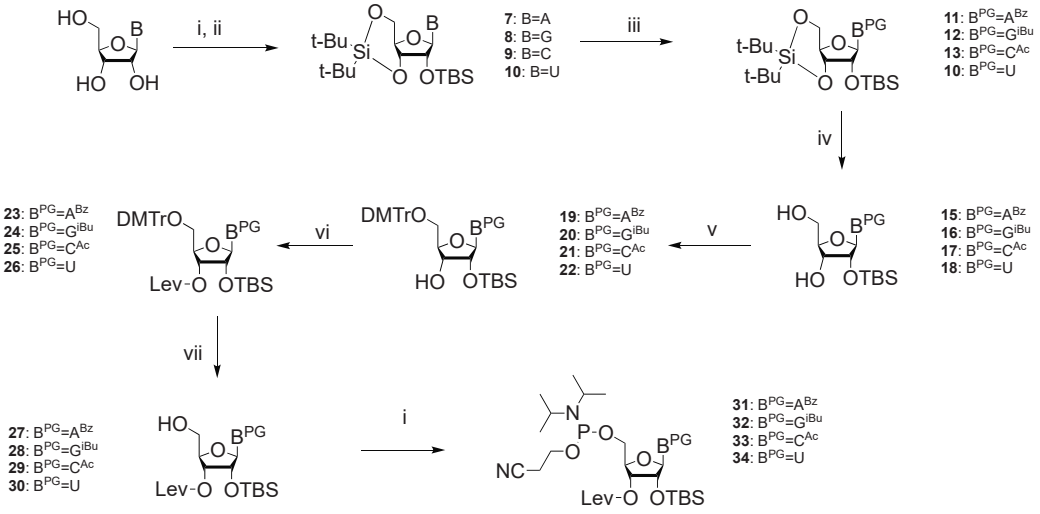
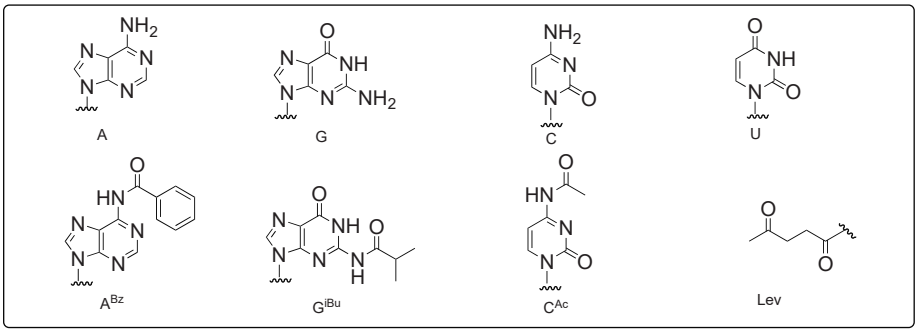


Figure 3. Target compounds. Protected nucleopeptide intermediate towards coxackie virus VPg.

Result and discussion

The syntheses of the 5'-phosphoramidite nucleoside building blocks, having the lev group as temporary protection (**31-34**) are depicted in Scheme 1. Starting from the commercially available standard ribonucleosides adenosine, guanosine, cytosine and uridine (A,G, C, U) two different silyl groups were introduced in a one-pot procedure to protect all the hydroxyl groups in the ribose moiety. The 5'- and 3'- hydroxyl functions were silylated by a reaction of the ribonucleoside with di-*tert*-butyl silyl dichloride under influence of silver nitrate. Upon completion of this reaction additional amounts of silver nitrate, pyridine and tert-butyldimethylsilyl chloride were added to install the tert-butyldimethylsilyl (TBS) ether at the 2'- hydroxyl function. In an alternative one-pot procedure to introduce the same protection group pattern di-tert-butylsilyl bis(trifluoromethanesulfonate) was applied to protect the 5'- and 3'- hydroxyl functions, while the combination tert-butyldimethylsilyl chloride and imidazole was used to introduce the TBS at the 2'-position. The exocyclic amino functions of adenosine, guanosine and cytosine were protected as amides. Benzoylation of adenosine **7** with benzoyl chloride and pyridine led to N,N'-dibenzoyl adenosine and subsequent ammonia treatment gave the required fully protected adenosine derivative **11**. N2-Isobutyryl-guanosine derivative **12** was obtained by reaction of the exocyclic amine in **8** with isobutyryl chloride (iBuCl) in pyridine. The exocyclic amine function in cytosine derivative **9** was acetylated with acetic anhydride in pyridine. The fully protected ribonucleosides derivatives (**10-13**) were further processed by the following sequence of reactions. The 5' and 3' hydroxyl group in **10-13** were deprotected using HF-Pyridine at 0°C. Depending on the nucleobase this reaction took 1 to 2h and in all cases no unwanted TBDMS cleavage was observed. Selective introduction of the DMTr group at the 5' hydroxyl was followed by carbodiimide mediated esterification of 3' hydroxyl with levulinic acid. The introduction of the Lev group proceeded more efficient with DIC than with EDC.HCl and more importantly migration of TBDMS was not observed.



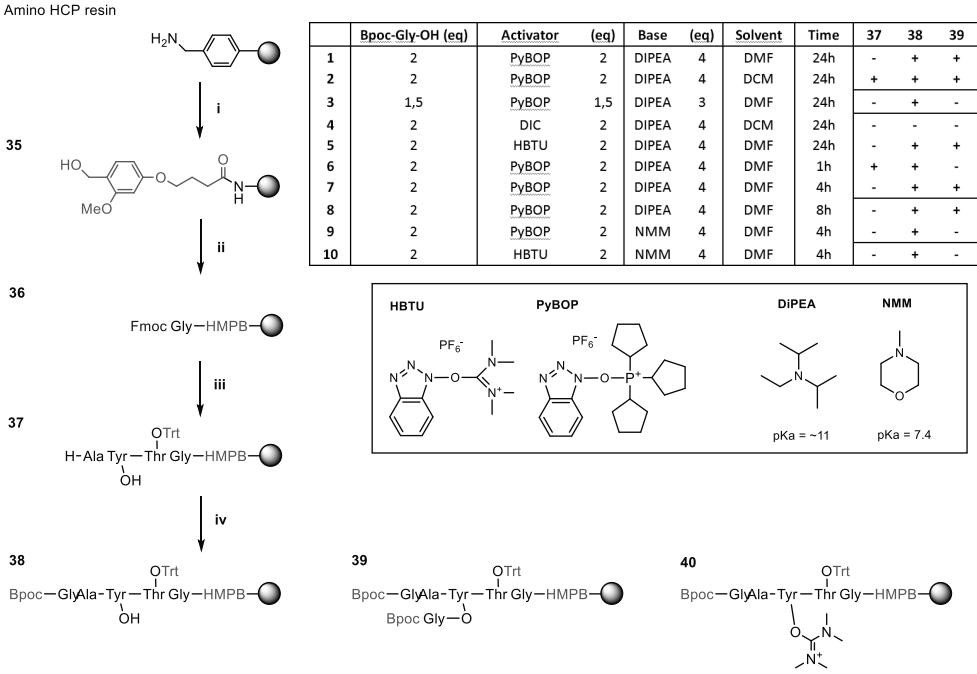


Scheme 1. (i) **7,8,9**: $(t\text{-Bu})_2Si(OTf)_2$, DMF, 0 °C, 30 min; **10**: $(t\text{-Bu})_2SiCl_2$, $AgNO_3$, DMF, 0 °C, 30 min (ii) **7,8,9**: TBS-Cl, Imidazole, 0 °C to rt, o.n. ; **10**: TBS-Cl, Pyridine, $AgNO_3$, 0 °C to rt, 2 hrs, (iii) **11**: Bz-Cl, Pyr/DCM, rt, 3 h, then conc. NH_4OH, -10 °C to rt, o.n. **12**: iBu-Cl, Pyr, -20 °C, 2 h **13**: Ac_2O, Pyr, 0 °C to rt, 2 h (iv) HF-Pyr, DCM, 0 °C, **15**, **18**: 1h, **16**, **17**: 2h; (v) DMTr-Cl, Pyr, 0 °C, o.n. (vi) Lev-OH, DIC, DMAP cat., DCM, rt, o.n. (vii) TsOH, DCM/MeOH, 0 °C, 10 min, (viii) PAM-Cl, TEA, DCM, rt, 15 min

To prevent TBDMS migration a catalytic amount of DMAP as a nucleophilic catalyst and DCM as solvent were used instead of DMF and bases such as triethylamine. The final two steps to the four nucleotide building blocks (**31-34**) comprise removal of the DMT group with diluted solution of *p*TsA in DCM/MeOH and reaction of the free 5' hydroxyl with 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite in the presence of N,N-diisopropylethylamine. Summarizing all 5'-phosphoramidite nucleoside building blocks, having the lev group as temporary protection (**31-34**) were prepared in an efficient manner and on sufficient scale to execute the automated solid phase synthesis.

Optimisation of the first nucleotide synthesis cycle.

Having the respective protected amino acid and nucleotide building blocks available, attention was directed to assessment of the appropriateness of these building blocks for an efficient automated solid phase synthesis of nucleopeptides.

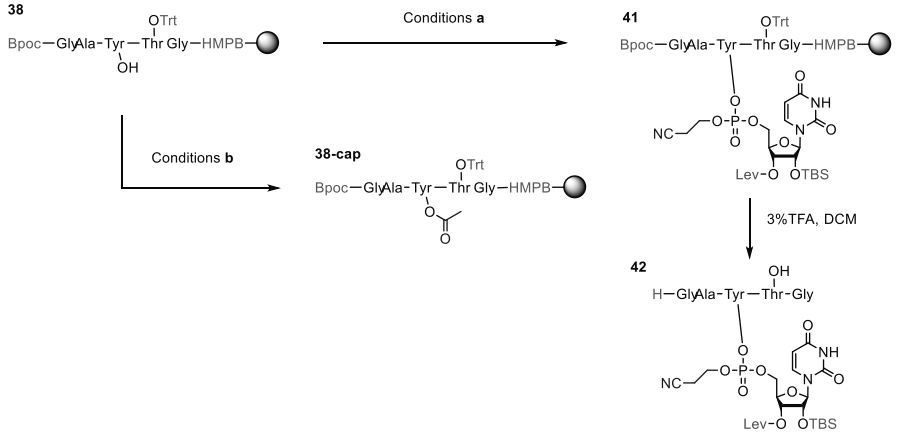


	Bpoc-Gly-OH (eq)	Activator	(eq)	Base	(eq)	Solvent	Time	37	38	39	40
1	2	PyBOP	2	DIPEA	4	DMF	24h	-	+	+	-
2	2	PyBOP	2	DIPEA	4	DCM	24h	+	+	+	-
3	1,5	PyBOP	1,5	DIPEA	3	DMF	24h	-	+	-	-
4	2	DIC	2	DIPEA	4	DCM	24h	-	-	-	-
5	2	HBTU	2	DIPEA	4	DMF	24h	-	+	+	+
6	2	PyBOP	2	DIPEA	4	DMF	1h	+	+	-	-
7	2	PyBOP	2	DIPEA	4	DMF	4h	-	+	+	-
8	2	PyBOP	2	DIPEA	4	DMF	8h	-	+	+	-
9	2	PyBOP	2	NMM	4	DMF	4h	-	+	-	-
10	2	HBTU	2	NMM	4	DMF	4h	-	+	-	-

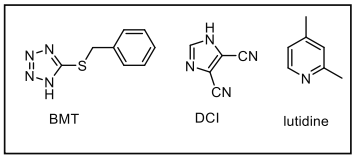
Scheme 2. Synthesis (i) HMPB-linker, PyBOP, HOBt, DiPEA, DMF, rt, 5 hrs, then (ii) Fmoc-Gly-OH, DIC, DMAP, DCM, rt, o.n.; (iii) SPPS: (a) piperidine/NMP (1:4, v:v), rt, 5 min; (b) Fmoc–AA–OH, HCTU, DiPEA, rt, 1 hr; (c) Ac_2O, DiPEA, NMP, rt, 1 min; (iv) table.

In the first instance the viability of the peptide synthesis was evaluated as the applied Fmoc amino acid building blocks were minimally protected not only to avoid harsh acidic deprotection conditions but also to provide an unprotected tyrosine hydroxyl group in the immobilized oligopeptide for oligonucleotide extension (Scheme 2). Moreover, to allow the projected block coupling of the nucleopeptide and peptide, protective group manipulations are required. Immobilized tetrapeptide Ala-Tyr-Thr-Gly (**37**) was prepared with the aid of commercially available Fmoc amino acids using HMPB resin and standard peptide chemistry (from **35** to **37**, Scheme 2). Although the tyrosine with an unprotected phenolic hydroxyl function was incorporated, the immobilized tetrapeptide **37** was synthesized without noticeable difficulties. A necessity to allow extension of the immobilized nucleopeptide with an oligoribonucleotide moiety comprises the replacement of the Fmoc group by the Bpoc group because the Fmoc will not survive the repeated cleavage of the Lev group at the 3' position during the RNA synthesis. However, the coupling of **37** with Bpoc-glycine to give **38** went problematic and gave **39** as the result of a double incorporation of Bpoc-glycine together with uronium side product **40** as identified after cleavage from the solid support (Scheme 2).

A number of different coupling condition were tested and monitored by HPLC after analytical cleavage. As depicted in the table of Scheme 2, using only 1.5 eq of both protected glycine and coupling agents (conditions 3) prevented any side reaction to happen. Using the less basic NMM instead of the common D*i*PEA also improved the rate of the reaction and the quality of the target peptide.



a	Nucleotide	(eq)	Activator	(eq)	Time	38	41
1	34	6	BMT	18	20min	+	++
2	34	6	BMT	18	60min	+	++
3	34	3	BMT	9	60min	++	++
4	34	6	DCI	18	20min	+	++
5	34	7	BMT	24	20min	-	+++
- = not observed, + = minor, ++ = major, +++= single product							

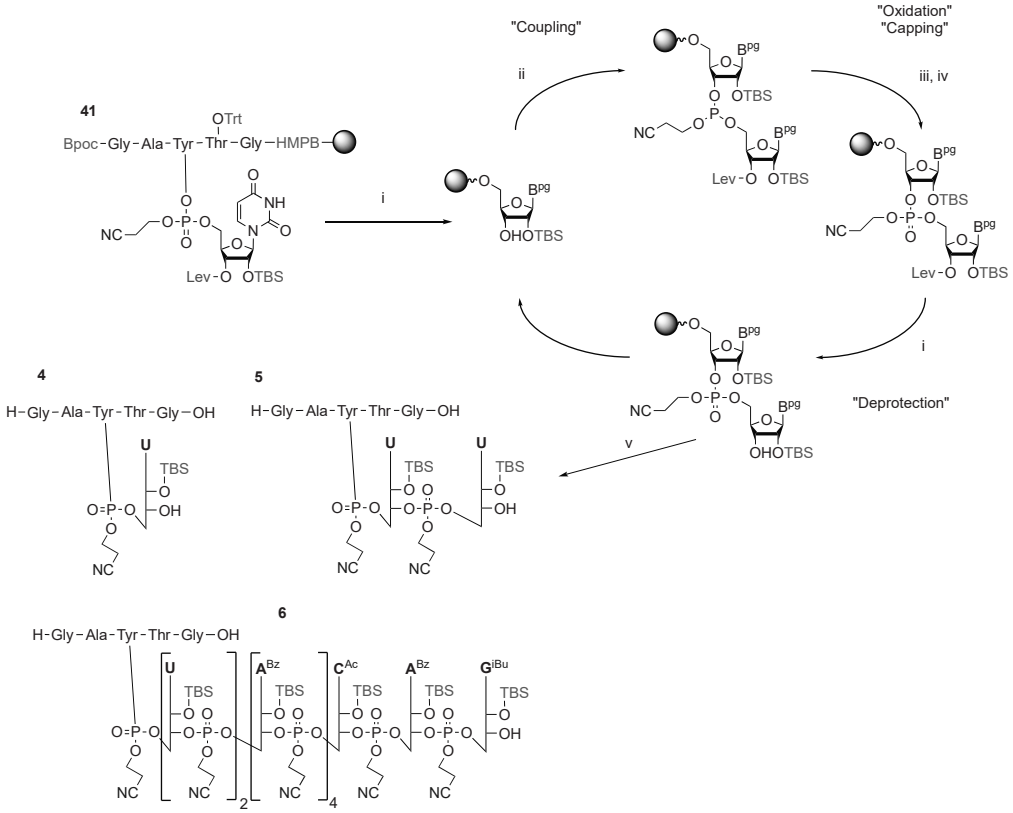


b	reagent	(eq)	Activator	(eq)	Base	(eq)	Solvent	Time	38	38-cap
1	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	20min	++	-
2	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	60min	++	-
3	Acetic anhydride	318	Me-Im	376	Lutidine	388	THF	120min	++	-
4	Acetic anhydride	80			DIPEA	24	DMF	10min	+	+
5	Acetic anhydride	80			DIPEA	24	DMF	30min	+	+
6	Acetic anhydride	80			DIPEA	24	DMF	60min	+	+
7	Acetic anhydride	80	DMAP	.cat	DIPEA	24	DMF	30min	-	++
8	Acetyl Chloride	20			DIPEA	48	DMF	30min	-	++
- = not observed, + = observed, ++ = single product										

Scheme 3. Coupling of the first nucleoside on the peptide side chain

After the quality of immobilized peptide **38** was established the extension of the free tyrosine hydroxyl group with nucleotides was investigated using phosphoramidite building blocks **31-34**. The number of equivalents of building block **31-34**, the nature and the number of equivalents of the activator (BMT or CM) and the reaction time were varied (Scheme 3) The quality of the products was established by HPLC analysis after cleavage from the solid support. It turned out that 7 eq of nucleotide amidite were necessary using BMT as activator for complete conversion toward nucleopeptide **41** (table a, condition 5). In line with these results it appeared that the capping step did not proceed without difficulties. As illustrated in the table b, various conditions were assessed of which condition 7 using Ac_2O in the presence of DMAP and DiPEA or condition 8 using AcCl with DiPEA proved to be the most effective. At the end of the synthesis nucleopeptide **41** was released from the solid support and the Bpoc and Trt groups were removed by treatment with 3% TFA in DCM to give partially protected nucleopeptide **42** as analyzed by HPLC-MS.

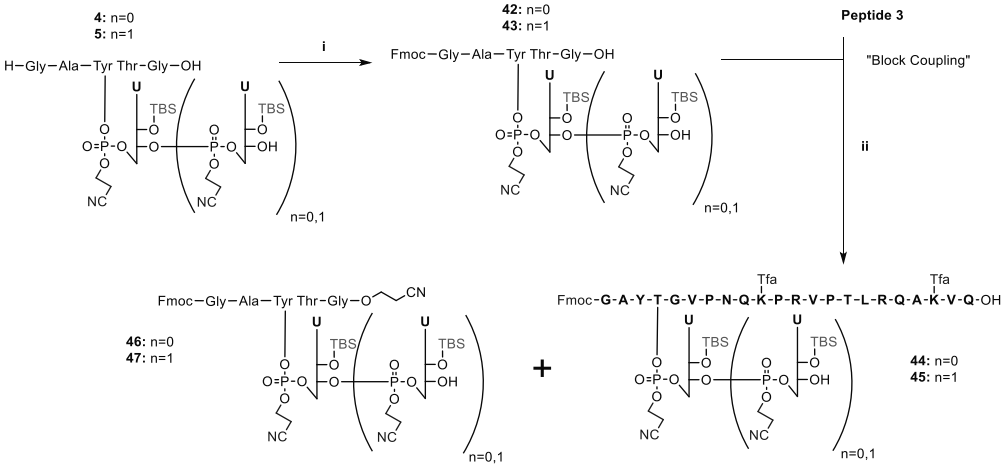
Oligonucleopeptide synthesis or target compound 4, 5 and 6.



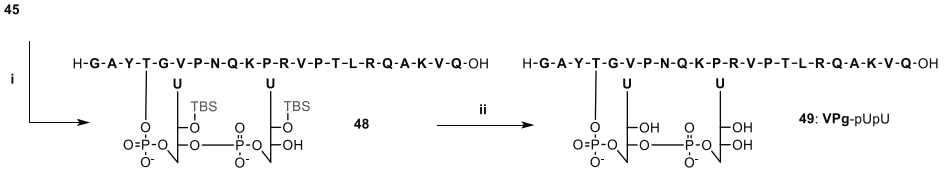
Scheme 4. (i) Hydrazine, THF/Pyr/AcOH, rt, 20 min; (ii) **31, 32, 33, 34**, BMT, dioxane/ACN, rt, 20/30/30/20 min; (iii) I_2, THF/Pyr/H2O, rt, 1.5 min; (iv) Ac_2O, MeIm, 2,6-lutidine, NMP, rt, 30 sec (v) 3% TFA/DCM, rt, 5 min.

In order to prepare partialy protected nucleopeptide **4** (Scheme 4), provided with one uridine moiety, the lev group at the 3' position in immobilized **41** should be removed without harming the intrigity of the TBS at the 2' position. After extensive optimization the lev group at the 3' position was removed using hydrazine monohydrate in a THF/pyridine/acetic acid solvent mixture for 20 min. It was established that under these conditions migration of TBDMS group from 2' to 3' hydroxyl did not take place. Subsequent mild acidic treatment with 3%TFA in DCM cleaved the product from the solid support and removed the Trt and Bpoc groups to provide the partially deprotected nucleopeptide **4**. Further extension of the RNA chain was investigated and the synthesis of partially protected nucleopeptides **5** and **6** was undertaken. Removal of the lev group from immobilized **41** was followed by the elongation of the oligonucleotide, comprising amidite coupling, oxidation of the intermediate phosphite and capping of the remaining hydroxyl groups. Depending on the nature of the nucleobase the coupling time was adjusted, being 20 min for the uridine and adenosine building blocks (**31** and **34**) and 30 min for the cytosine and guanosine building blocks (**32** and **33**). Partially protected target nucleopeptide **5** and **6** were obtained after treatment with 3% TFA in DCM analyzed by LCMS.

Preliminary studies on further block coupling



Scheme 5. (i) Fmoc-OSu. DiPEA, DMF, rt, o.n.; (ii) (a) PyBOP, HOBt, DiPEA, DMF, rt, 25 min; then (b) peptide **3**, see Figure 2, DMF, rt, o.n.



Scheme 6. (i) NH_4OH aq./dioxane (1:1, v:v), rt, 3 days; (ii) (a) TEA/TEA*3HF/DMF (2:3:4, v:v:v), rt, o.n; then (b) NH_4HCO_3 aq.

Finally, the viability of the projected block coupling was investigated by the condensation of nucleopeptide **5** (slightly contaminated with **4**) with 17 mer oligopeptide **3** (Scheme 5). This oligopeptide (see Figure 2), in which the side chain amino groups of the lysine moieties are protected with trifluoroacetyl groups, was obtained by standard solid phase peptide synthesis, using Tentagel S RAM**,** followed by purification with HPLC. The free N terminus of nucleopeptides **5** was reprotected with the Fmoc group using FmocOSu and DiPEA to give **43** that was used without further purification. Block coupling was achieved by preactivation of the glycine moiety in the nucleopeptides **43** using PyBOP for 25 min, followed by the addition of oligopeptide **3**. The reaction was monitored by LCMS and after overnight **45** was isolated, along with the cyanoethanol ester of the starting product (i.e. **47**). Complete deprotection was achieved using aqueous ammonia followed by TEA*HF and NH_4HCO_3 treatement. HPLC purification yielded compound **49** (**VPgpUpU**) as determined by mass-spectroscopic and chromatographic methods.

Conclusion

An efficient synthesis of 5' phosphoramidite ribonucleotide (A, C, G and U) building blocks having an orthogonal levunilic ester protective group at the 3'-position is described. Application of mimimally protected amino acid building blocks together with the newly prepared 5'-phosphoramidite ribonucleotide building blocks in an optimized automated solid support

synthesis makes available partially propected nucleopeptides comprising up to a pentapeptide and nonanucleotide. The viability of the partially protected nucleopeptides to participate in a block coupling with a partially protected peptide was ascertained by block condensation to give VPgpUpU (**49**).

Experimental

5',3'-Si(tBu)$_2$-2'-TBDMS-Uridine (10)

0.995 mmol (0.243g) of uridine was, after co-evaporation with 1,4-dioxane, dissolved in 5 mL of dry DMF, 2.44 mmol (0.414g) of $AgNO_3$ and 1.4 mmol (0.30 mL, 0.30g) of $(t\text{-}Bu)_2SiCl_2$ were added respectively and the mixture was stirred for 30 minutes at 0°C. Reaction completion was checked by TLC (Rf around 0.3 in 5% MeOH/DCM). Then 2.94 mmol (0.500g) of $AgNO_3$ and 5.0 mmol (0.40 mL, 0.39g) of Pyridine were added and the mixture was stirred for 15 minutes at 0 °C before adding 1.50 mmol (0.226g) of TBDMS-Cl and stirring for another 2 hours. Reaction completion was checked by TLC (Rf around 0.75 at 25% EtOAc/DCM, 0.85 at 50% EtOAc/DCM). 30 mL of EtOAc was added to the mixture and filtrated through celite (20mL of EtOAc was added during this process). The organic layer was extracted two times using sat. $KHSO_4$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0:100 to 20:80 EtOAc/DCM eluent, resulting in 0.444g (0.890mmol, 89.0%) of Compound **10** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.746 (s, 1H, NH), 7.260 ($CHCl_3$), 7.257 (d, *J* = 8.0 Hz, 1H, H^6), 5.751 (dd, *J* = 8.1, 1.3 Hz, 1H, H^5), 5.653 (s, 1H, $H^{1'}$), 4.493 (dd, *J* = 9.2, 5.1 Hz, 1H, $H^{5'a}$), 4.288 (d, *J* = 4.6 Hz, 1H, $H^{2'}$), 4.158 (td, *J* = 10.2, 5.1 Hz, 1H, $H^{4'}$), 3.968 (t, *J* = 9.4 Hz, 1H, $H^{5'b}$), 3.863 (dd, *J* = 9.6, 4.7 Hz, 1H, $H^{3'}$), 1.040, 1.012 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.922 (s, 9H, 2'-O-Si-tBu), 0.177, 0.132 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 163.70 (CO, C^2), 149.91 (CO, C^4), 139.55 (C^6), 102.47 (C^5), 94.07 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.14 ($C^{3'}$), 75.43 ($C^{2'}$), 74.63 ($C^{4'}$), 67.68 ($C^{5'}$), 27.58, 27.07 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.95 (CH_3, 2'-O-Si-tBu), 22.89, 20.45, 18.36 (3x C_q, Si-tBu), -4.19, -4.92 (2x CH_3, 2'-O-Si-Me_2).

IR: 2932, 2886, 2859, 1690, 1454, 1261, 1165, 1055, 1001, 826, 777, 750, 650.

HRMS: $[C_{23}H_{42}N_2O_6Si_2+H]^+$: found 499.2650, calculated 499.2654.

5'-OH-3'-OH-2'-TBDMS-Uridine (18)

0.523 mmol (0.261g) of Compound **10** was dissolved in 2.5 mL of DCM, 2.8 mmol (0.50mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.45 in 75% EtOAc/DCM, 0.60 in 100% EtOAc). The mixture was washed two times with H_2O, sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50% to 100% EtOAc/DCM eluent resulting in 0.172g (0.480 mmol, 91.8%) of Compound **18** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 8.988 (s, 1H, NH), 7.594 (d, *J* = 8.1 Hz, 1H, H^6), 7.260 ($CHCl_3$), 5.758 (dd, *J* = 8.1, 1.9 Hz, 1H, H^5), 5.586 (d, *J* = 5.2 Hz, 1H, $H^{1'}$), 4.596 (t, *J* = 5.2 Hz, 1H, $H^{2'}$), 4.206 (dd, *J* = 8.4, 4.0 Hz, 1H, $H^{3'}$), 4.145 (dd, *J* = 5.2, 2.2 Hz, 1H, $H^{4'}$), 3.956 (d, *J* = 12.1 Hz, 1H, $H^{5'a}$), 3.813 (dd, *J* = 11.9, 4.6 Hz, 1H, $H^{5'b}$), 2.986 (d, *J* = 3.7 Hz, 1H, $OH^{5'}$), 2.710 (d, *J* = 4.1 Hz, 1H, $OH^{3'}$), 0.898 (s, 9H, 2'-O-Si-tBu), 0.097, 0.084 (2x s, 6H, 2'-O-SiMe_2). **^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ**: 163.14 (CO, C^2), 150.40 (CO, C^4), 142.49 (C^6), 102.81 (C^5), 93.07 ($C^{1'}$), 85.52 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.24 ($C^{2'}$), 70.96 ($C^{3'}$), 62.30 ($C^{5'}$), 25.77 (CH_3, 2'-O-Si-tBu), 18.10 (C_q, 2'-O-Si-tBu), -4.71, -5.00 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2930, 1694, 1462, 1379, 1258, 1157, 1061, 837, 783, 758.

HRMS: $[C_{15}H_{26}N_2O_6Si+H]^+$: found 359.1634, calculated 359.1633.

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Uridine (22)

0.480 mmol (0.172g) of Compound **18** was dissolved in 2 mL of dry pyridine, 0.576 mmol (0.195g) of 4,4'-Dimethoxytritylchloride was added at -10 °C and the mixture was stirred for 5 nights. Reaction completion was checked by neutralized TLC (Rf around 0.25 in 25% EtOAc/PE). The mixture was concentrated using rotary evaporation before being redissolved in EtOAc. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column

chromatography using an 0% to 50% EtOAc/PE eluent resulting in 0.250g (0.378 mmol, 78.9%) of Compound **22** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.779 (s, 1H, NH), 7.961 (d, *J* = 8.2 Hz, 1H, H^6),), 7.384 (d, *J* = 7.2 Hz, 2H, $H^{arom, ortho}$, DMTr), 7.34-7.20 (m, 9H, H^{arom}, H^{ortho}, H^{meta}, H^{para}, DMTr), 7.260 ($CHCl_3$), 6.849 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.966 (d, *J* = 2.9 Hz, 1H, $H^{1'}$), 5.314 (d, *J* = 8.1 Hz, 1H, H^5), 4.36 (m, 2H, $H^{2'}$, $H^{3'}$), 4.12 (m, 1H, $H^{4'}$), 3.793 (s, 6H, OMe, DMTr), 3.57-3.45 (m, 2H, $H^{5'a}$, $H^{5'b}$), 2.644 (s, 1H, $OH^{3'}$), 0.913 (s, 9H, 2'-O-Si-tBu), 0.198, 0.169 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 163.72 (CO, C^2), 158.80, 158.72 (2x C_q, DMTr), 150.50 (CO, C^4), 144.38 (C_q, DMTr), 140.33 (C^6), 135.24, 135.04 (2x C_q, DMTr), 130.24, 130.14 (2x CH^{arom}, DMTr), 128.16 (CH^{arom}, DMTr), 128.11 (CH^{arom}, DMTr), 127.28 (CH^{arom}, DMTr), 113.39 (2x CH^{arom}, DMTr), 102.37 (C^5), 88.79 ($C^{1'}$), 87.25 (C_q, DMTr), 83.58 ($C^{4'}$), 77.48, 77.16, 76.85 ($CHCl_3$), 76.41 ($C^{2'}$), 70.48 ($C^{3'}$), 62.36 ($C^{5'}$), 55.31 (OMe, DMTr), 25.75 (CH_3, 2'-O-Si-tBu), 18.09 (C_q, 2'-O-Si-tBu), -4.55, -5.13 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2928, 1684, 1506, 1456, 1250, 1175, 1115, 1034, 827, 779, 756, 700.

HRMS: $[C_{36}H_{44}N_2O_8Si+Na]^+$: found 683.2757, calculated 683.2759.

5'-DMTr-3'-Lev-2'-TBDMS-Uridine (26)

0.378 mmol (0.250g) of Compound **22** was dissolved in 2mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 0.5 mmol (0.05mL, 0.06g) of levulinic acid and 0.64 mmol (0.10mL, 0.081g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by normal TLC (Rf around 0.65 in 50% EtOAc/PE). The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 25% to 100% EtOAc/PE eluent resulting in 0.249g (0.328 mmol, 86.8%) of Compound **26** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.941 (br, 1H, NH), 7.905 (d, *J* = 8.2 Hz, 1H, H^6), 7.40-7.20 (m, 9H, H^{arom}, H^{ortho}, H^{meta}, H^{para}, DMTr), 7.260 ($CHCl_3$), 6.854 (d, *J* = 8.7 Hz, 4H, H^{arom}, DMTr), 6.002 (d, *J* = 4.8 Hz, 1H, $H^{1'}$), 5.349 (t, *J* = 4.6 Hz, 1H, H3'), 5.311 (d, *J* = 8.1 Hz, 1H, H^5), 4.521 (t, *J* = 4.8 Hz, 1H, $H^{2'}$), 4.26 (m, 1H, $H^{4'}$), 3.787 (s, 6H, OMe, DMTr), 3.539 (d, *J* = 11.2, 2.0 Hz, 1H, $H^{5'a}$), 3.448 (dd, *J* = 10.8, 1.2 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-C$\underline{H}_2$C$\underline{H}_2$-R^2, Lev), 2.194 (s, 3H, CH_3, Lev), 0.870 (s, 9H, 2'-O-Si-tBu), 0.106, 0.088 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.22 (CO, Lev), 171.82 (CO, Lev), 163.63 (CO, C^2), 158.76, 158.72 (2x C_q, DMTr), 150.66 (CO, C^4), 144.19 (C_q, DMTr), 140.07 (C^6), 134.95, 134.83 (2x C_q, DMTr), 130.21, 130.09 (2x CH^{arom}, DMTr), 128.08 (CH^{arom}, DMTr), 127.26 (CH^{arom}, DMTr), 113.36 (2x CH^{arom}, DMTr), 102.50 (C^5), 88.43 ($C^{1'}$), 87.42 (C_q, DMTr), 81.16 ($C^{4'}$), 77.48, 77.16, 76.85 ($CHCl_3$), 74.45 ($C^{2'}$), 72.22 ($C^{3'}$), 62.28 ($C^{5'}$), 55.31 (OMe, DMTr), 37.65 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.86 (CH_3, Lev), 27.80 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.50 (CH_3, 2'-O-Si-tBu), 17.88 (C_q, 2'-O-Si-tBu), -5.00, -5.24 (2x CH_3, 2'-O-Si-Me_2).

IR: 2953, 2928, 2855, 1684, 1506, 1456, 1250, 1175, 1153, 1032, 829, 778, 756, 702.

HRMS: $[C_{41}H_{50}N_2O_{10}Si+Na]^+$: found 781.3128, calculated 781.3127.

5'-OH-3'-Lev-2'-TBDMS-Uridine (30)

8.554 mmol (6.492g) of Compound **26** was dissolved in 50mL DCM/MeOH (7:3 v:v), 85.54 mmol (16.27g, monohydrate) of diluted *p*-toluenesulfonic acid (8.14 wt% in DCM/MeOH(7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.45 in 100% EtOAc). Finally, the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 20% to 100% EtOAc/PE eluent resulting in 3.678g (8.056 mmol, 94.2%) of Compound **30** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.517 (s, 1H, NH), 7.808 (d, *J* = 8.1 Hz, 1H, H^6), 7.260 ($CHCl_3$), 5.740 (d, *J* = 8.0 Hz, 1H, H^5), 5.662 (d, *J* = 4.8 Hz, 1H, $H^{1'}$), 5.172 (t, *J* = 4.6 Hz, 1H, H3'), 4.562 (t, *J* = 4.8 Hz, 1H, $H^{2'}$), 4.210 (m, 1H, $H^{4'}$), 3.929 (d, *J* = 12.1 Hz, 1H, $H^{5'a}$), 3.764 (d, *J* = 12.2 Hz, 1H, $H^{5'b}$), 2.5-2.9 (m, 4H, R^1-C$\underline{H}_2$C$\underline{H}_2$-R^2, Lev), 2.191 (s, 3H, CH_3, Lev), 0.840 (s, 9H, 2'-O-Si-tBu), 0.039, 0.028 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.81 (CO, Lev) 172.44 (CO, Lev), 163.67 (CO, C^2), 150.50 (CO, C^4), 141.71 (C^6), 102.51 (C^5), 91.75 ($C^{1'}$), 82.93 ($C^{4'}$), 77.47, 77.16, 76.83 ($CHCl_3$), 73.50 ($C^{2'}$), 72.04 ($C^{3'}$), 61.49

($C^{5'}$), 37.84 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.93 (CH_3, Lev), 27.89 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.59 (CH_3, 2'-O-Si-tBu), 17.98 (C_q, 2'-O-Si-tBu), -5.05, -5.12 (2x CH_3, 2'-O-Si-Me_2).

IR: 3055, 2953, 2928, 2884, 2857, 1684, 1462, 1387, 1258, 1155, 1109, 1088, 835, 779, 760.

HRMS: $[C_{20}H_{32}N_2O_8Si+H]^+$: found 457.1998, calculated 457.2001.

**The solution was prepared using 85.54 mmol (16.27g) p-toluenesulfonic acid monohydrate in 200mL DCM/MeOH (7:3 v:v), resulting in a 8.14 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Uridine (34)

1.80 mmol (0.821g) of Compound **30** was dissolved in 20mL of dry DCM, 2.9 mmol (0.29g, 0.40mL) of Triethylamine and 2.2 mmol (0.53g, 0.50mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.85 in 100% EtOAc). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:20:79 to 1:50:49 TEA:EtOAc:Hex eluent resulting in 1.162g (1.769 mmol, 98.2%) of Compound **34** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.34 (br, 1H, NH), 7.84, 7.78 (d+d, 1H, *J* = 8.2 Hz, H^6) 7.260 ($CHCl_3$), 5.99, 5.98 (d+d, 1H, *J* = 6.1 + 5.7 Hz, $H^{1'}$), 5.73, 5.71 (d+d, 1H, *J* = 3.6 + 3.7 Hz, H^5), 5.21, 5.12 (t+dd, 1H, *J* = 4.4 + 4.9, 3.4 Hz, $H^{3'}$), 4.34-4.23 (m, 2H, $H^{2'}$ + $H^{4'}$), 4.00-3.70 (m, 4H, $H^{5'}$, NC-$C\underline{H_2}$-CH_2-OR), 3.65-3.50 (m, 2H, CH, $\underline{iPr_2}$NR), 2.9-2.5 (m, 6H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev, NC-$CH_2$-$C\underline{H_2}$-OR), 2.18, 2.17 (s+s, 3H, CH_3, Lev) , 1.22-1.15 (m, 12H, CH_3, $\underline{iPr_2}$NR) 0.82, 0.81 (s+s, 9H, 2'-O-Si-tBu), 0.01--0.02 (m, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.39, 206.33 (CO, Lev) 171.94, 171.89 (CO, Lev), 163.39, 163.35 (C^2), 150,60, 150.58 (C^4), 140.04, 139.93 (C^6), 117.49, 117.41 (CN), 102.76, 102.66 (C^5), 88.25, 88.05 ($C^{1'}$), 81.79, 81.72, 81.70, 81.62 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.32, 74.18 ($C^{2'}$), 72.50, 72.06 ($C^{3'}$), 63.08, 62.92, 62.83, 62.67 ($C^{5'}$), 58.70, 58.60, 58.48, 58.38 (NC-$\underline{C}H_2$-CH_2-OR), 43.36, 43.26, 43.23, 43.14 (2x CH, $\underline{iPr_2}$NR), 37.79, 37.74 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.90, 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.50 (CH_3, 2'-O-Si-tBu), 24.92, 24.88, 24.85, 24.81, 24.77, 24.70 (4x CH_3, $\underline{iPr_2}$NR), 20.57, 20.49, 20.45, 20.38 (NC-CH_2-$\underline{C}H_2$-OR), 17.91, 17.90 (C_q, Si-tBu), -5.08, -5.11, -5.20, -5.25 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.62, 148.60.

IR: 2965, 2930, 2859, 1744, 1717, 1684, 1456, 1379, 1364, 1253, 1200, 1180, 1155, 1125, 1103, 1076, 1045, 978, 858, 837, 808, 779, 729, 677, 640.

HRMS: $[C_{37}H_{54}N_7O_8PSi+H]^+$: found 657.3077, calculated 657.3079.

5',3'-Si(tBu)$_2$-2'-TBDMS-Adenosine (7)

10 mmol (2.672g) of Adenosine was dissolved in 20 mL of dry DMF, 12.3 mmol (4.0 mL, 5.40g) of (t-Bu)$_2$Si(OTf)$_2$ was added at 0 °C and the mixture was stirred for 30 minutes. Reaction completion was checked by TLC (Rf around 0.3 at 6% MeOH/DCM). Then 50.7 mmol (3.45g) of Imidazole and 12.3 mmol (1.85g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.3 at 75% EtOAc/PE). 120 mL of H_2O was added to the mixture and extracted three times using Et_2O. The organic layer was dried using $MgSO_4$. Purification was performed with silica column chromatography using a 30:80 to 100:0 EtOAc/PE eluent, resulting in 3.77g (7.22mmol, 72.2%) of Compound **7** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 8.278 (s, 1H, H^2), 7.824 (s, 1H, H^8), 7.260 ($CHCl_3$), 6.780 (s, 2H, NH_2), 5.901 (s, 1H, $H^{1'}$), 4.600 (d, *J* = 4.7Hz, 1H, $H^{2'}$), 4.511 (dd, *J* = 9.5, 4.7 Hz, 1H, $H^{3'}$), 4.460 (dd, *J* = 9.1, 5.1 Hz, 1H, $H^{5'a}$), 4.189 (td, *J* = 10.1, 5.1 Hz, 1H, $H^{4'}$), 4.008 (dd, *J* = 10.3, 9.3 Hz, 1H, $H^{5'b}$), 1.043, 1.011 (2x s, 18H, 5'-3'-O-Si-tBu$_2$), 0.896 (s, 9H, 2'-O-Si-tBu), 0.132, 0.116 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 156.06 (C^6), 153.16 (C^2), 149.21 (C^4), 138.60 (C^8), 120.23 (C^5), 92.42 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.87 ($C^{3'}$), 75.50 ($C^{2'}$), 74.70 ($C^{4'}$), 67.86 ($C^{5'}$), 27.54, 27.08 (2x CH_3, 5'-3'-O-Si-tBu$_2$), 25.96 (CH_3, 2'-O-Si-tBu), 22.79, 20.39, 18.36 (3x C_q, Si-tBu), -4.24, -4.91 (2x CH_3, 2'-O-Si-Me_2).

IR: 3312, 3157, 2932, 2887, 2859, 1672, 1601, 1128, 1063, 1003, 827, 754, 651.

HRMS: $[C_{24}H_{43}N_5O_4Si_2+H]^+$: found 522.2922, calculated 522.2926.

5',3'-Si(tBu)$_2$-2'-TBDMS-Adenosine(Bz) (11)

7.22 mmol (3.59g) of Compound **7** was dissolved in 50mL of dry pyridine/DCM (1:4 v:v), 30.2 mmol (3.5mL, 4.24g) of Benzoylchloride was added and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.9 in 50% EtOAc/PE). Then 20 mL of conc. NH_4OH was added at -10 °C and the

mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.6 in 50% EtOAc/PE). Finally, the mixture pH was lowered to 5 using 18mL of concentrated HCl. 60 mL of H_2O and 20 mL of DCM were added before partitioning layers. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 1:5 to 2:5 EtOAc/Hex eluent resulting in 3.404g (5.44mmol, 75.3%) of Compound **11** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.807 (br, 1H, NH), 8.689 (s, 1H, H^2), 8.019 (s, 1H, H^8), 8.010 (d, *J* = 8.7 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.535 (t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.445 (t, *J* = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.969 (s, 1H, $H^{1'}$), 4.593 (d, *J* = 4.6 Hz, 1H, $H^{2'}$), 4.474 (dd, *J* = 9.2, 5.1 Hz, 1H, $H^{3'}$), 4.429 (dd, *J* = 9.6, 4.6 Hz, 1H, $H^{5'a}$), 4.218 (td, *J* = 10.1, 5.1 Hz, 1H, $H^{4'}$), 4.007 (dd, *J* = 10.0, 9.2 Hz, 1H, $H^{5'b}$), 1.048, 1.018 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.913 (s, 9H, 2'-O-Si-tBu), 0.153, 0.132 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 165,27 (CO, Bz), 152.64 (C^2), 151.12 (C^6), 149.92 (C^4), 141.05 (C^8), 133.63 (C_q^{ipso}, Bz), 132.72, 128.71, 128.11 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.66 (C^5), 92.47 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.89 ($C^{3'}$), 75.51 ($C^{2'}$), 74.76 ($C^{4'}$), 67.78 ($C^{5'}$), 27.49, 27.03 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.91 (CH_3, 2'-O-Si-tBu), 22.77, 20.36, 18.32 (3x C_q, 3x Si-tBu), -4.24, -4.96 (2x CH_3, 2'-O-SiMe_2).

IR: 2932, 2886, 2859, 1697, 1609, 1582, 1454, 1250, 1138, 1057, 826, 752, 652.

HRMS: $[C_{31}H_{47}N_5O_5Si_2+H]^+$: found 626.3190, calculated 626.3188.

5'-OH-3'-OH-2'-TBDMS-Adenosine(Bz) (15)

5.232 mmol (3.275g) of compound **11** was dissolved in 25 mL of DCM, 22 mmol (4.0mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 60 minutes. Reaction completion was checked by TLC (Rf around 0.3 in 100% EtOAc). The mixture was washed with sat. $NaHCO_3$ followed by brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/EtOAc eluent resulting in 2.499g (5.146 mmol, 98.4%) of Compound **15** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.568 (br, 1H, NH), 8.706 (s, 1H, H^2), 8.029 (s, 1H, H^8), 7.969 (d, *J* = 4.7 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.521(t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz), 7.428 (t, *J* = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.904 (d, *J* = 10.9 Hz, 1H, $OH^{5'}$) 5.793 (d, *J* = 7.1 Hz, 1H, $H^{1'}$), 5.029 (dd, *J* = 7.1, 4.8 Hz, 1H, $H^{2'}$), 4.307 (d, *J* = 4.8 Hz, 1H, $H^{3'}$), 4.261 (s, 1H, $H^{4'}$), 3.891 (d, *J* = 12.7 Hz, 1H, $H^{5'a}$), 3.695 (t, *J* = 11.7 Hz, 1H, $H^{5'b}$), 3085 (s, 1H, $OH^{3'}$), 0.719 (s, 9H, 2'-O-Si-tBu), -0.242, -0.437 (2x s, 6H, 2'-O-SiMe_2). **^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ**: 164.83 (CO, Bz)), 152.29 (C^2), 150.51 (C^6), 150.47 (C^4), 142.95 (C^8), 133.46 (C_q^{ipso}, Bz), 132.86, 128.76, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.19 (C^5), 91.01 ($C^{1'}$), 87.42 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.46 ($C^{2'}$), 72.54 ($C^{3'}$), 63.03 ($C^{5'}$), 25.45 (CH_3, 2'-O-Si-tBu), 17.75 (C_q, Si-tBu), -5.34, -5.44 (2x CH_3, 2'-O-Si-Me_2).

IR: 3289, 2949, 2928, 2886, 1699, 1609, 1582, 1456, 1249, 1088, 835, 779, 706, 644 **HRMS:** $[C_{23}H_{31}N_5O_5Si+H]^+$: found 486.2167, calculated 486.2167

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Adenosine(Bz) (19)

2.158 mmol (1.048g) of compound **15** was dissolved in 5 mL of dry pyridine, 2.68 mmol (0.907g) of dimethoxytritylchloride was added at -10 °C and the mixture was stirred overnight. Reaction completion was checked by neutralized TLC (Rf around 0.75 in 100% EtOAc). Finally, the reaction was quenched using 0.5mL of MeOH. The mixture was concentrated using rotary evaporation before being extracted in DCM/H_2O. The organic layer was washed with sat. $NaHCO_3$ followed by brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 30% to 100% EtOAc/PE eluent resulting in 1.613g (2.046 mmol, 94.9%) of compound **19** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.053 (s, 1H, NH), 8.735 (s, 1H, H^2), 8.236 (s, 1H, H^8), 8.028 (d, *J* = 7.3 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.607(t, *J* = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.523 (t, *J* = 7.5 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.450 (d, *J* = 7.2 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.339 (d, *J* = 8.5 Hz, 4H, H^{arom}, DMTr), 7.276 (t, *J* = 7.0 Hz, 2H, H^{arom}, DMTr^{meta}), 7.260 ($CHCl_3$), 7.216 (t, *J* = 7.1 Hz, 1H, $H^{arom,\ para}$, DMTr), 6.816 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 6.111 (d, *J* = 5.3 Hz, 1H, $H^{1'}$), 5.028 (t, *J* = 5.1 Hz, 1H, $H^{2'}$), 4.369 (dd, *J* = 8.0, 4.0 Hz, 1H, $H^{3'}$), 4.290 (q, *J* = 3.4 Hz, 1H, $H^{4'}$), 3.780 (s, 6H, OMe, DMTr), 3.550 (dd, *J* = 10.7, 3.1 Hz, 1H, $H^{5'a}$), 3.399 (dd, *J* = 10.7, 3.4 Hz, 1H, $H^{5'b}$), 2.735 (d, *J* = 4.1 Hz, 1H, $OH^{3'}$), 0.843 (s, 9H, 2'-O-Si-tBu), -0.003, -0.142 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 164.62 (CO, Bz), 158.72 (C_q, DMTr), 152.98 (C^2), 151.79 (C^6), 149.69 (C^6), 144.63 (C_q, DMTr), 141.83 (C^2), 135.71 (C_q, DMTr), 133.84 (C_q^{ipso}, Bz), 132.92 (CH^{arom}, Bz), 130.20 (CH^{arom}, DMTr), 129.03 (CH^{arom}, Bz), 128.26, 128.06, 127.94 (3x CH^{arom}, 2x DMTr, 1x Bz), 127.21 (CH^{arom},

DMTr), 123.33 (C^5), 113.36 (CH^{arom}, DMTr), 88.56 ($C^{1'}$), 86.84 (C_q, DMTr), 84.42 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.83 ($C^{2'}$), 71.68 ($C^{3'}$), 63.43 ($C^{5'}$), 55.37 (OMe, DMTr), 25.70 (CH_3, 2'-O-Si-tBu), 18.03 (C_q, Si-tBu), -4.80, -5.02 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2905, 2857, 2835, 1699, 1607, 1580, 1506, 1456, 1246, 1175, 1029, 883, 781, 752, 702, 644

HRMS: $[C_{44}H_{49}N_5O_7Si+H]^+$: found 788.3480, calculated 788.3474.

5'-DMTr-3'-Lev-2'-TBDMS-Adenosine(Bz) (23)

1.548 mmol (1.220g) of compound **19** was dissolved in 8 mL of dry DCM, 0.17 mmol (0.021g) of 4-dimethylaminopyridine, 2.0 mmol (0.20mL, 0.23g) of levulinic acid and 1.9 mmol (0.30mL, 0.24g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by neutralized TLC (Rf around 0.75 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 30% to 45% EtOAc/PE eluent resulting in 1.165g (1.315 mmol, 84.9%) of Compound **23** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.241 (s, 1H, NH), 8.737 (s, 1H, H^2), 8.235 (s, 1H, H^8), 8.038 (d, J = 7.4 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.594 (t, J = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.510 (t, J = 7.5 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.442 (d, J = 7.3 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.331 (dd, J = 8.8, 1.5 Hz, 4H, H^{arom}, DMTr),7.282 (t, J = 7.0 Hz, 2H, $H^{arom,\ meta}$, DMTr), 7.260 ($CHCl_3$), 7.220 (t, J = 7.2 Hz, 1H, $H^{arom,\ para}$, DMTr),6.821 (d, J = 8.5 Hz, 4H, H^{arom}, DMTr), 6.126 (d, J = 6.4 Hz, 1H, $H^{1'}$), 5.476 (dd, J = 5.0 Hz, 1H, $H^{3'}$), 5.115 (dd, J = 6.2, 5.3 Hz, 1H, $H^{2'}$), 4.335 (q, J= 2.9 Hz, 1H, $H^{4'}$), 3.777 (s, 6H, OMe, DMTr),3.560 (dd, J = 10.7, 3.1 Hz, 1H, $H^{5'a}$), 3.418 (dd, J = 10.7, 3.3 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev), 2.201 (s, 3H, CH_3, Lev) , 0.726 (s, 9H, 2'-O-Si-tBu), -0.007 (TMS), -0.024, -0.265 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.34 (CO, Lev) 171.80 (CO, Lev), 164.74 (CO, Bz), 158.72 (C_q, DMTr), 152.95 (C^2), 151.93 (C^6), 149.73 (C^4), 144.48 (C_q, DMTr), 141.55 (C^8), 135.50 (C_q, DMTr), 133.82 (C_q^{ipso}, Bz), 132.87 (CH^{arom}, Bz), 130.20 (CH^{arom}, DMTr), 128.95 (CH^{arom}, Bz), 128.23, 128.10, 127.99 (3x CH^{arom}, 2x DMTr, 1x Bz), 127.17 (CH^{arom}, DMTr), 123.06 (C^5), 113.39 (CH^{arom}, DMTr), 88.08 ($C^{1'}$), 87.06 (C_q, DMTr), 82.43 ($C^{3'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.49 ($C^{2'}$), 73.26 ($C^{4'}$), 63.23 ($C^{5'}$), 55.34 (OMe, DMTr), 37.81 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.99 (CH_3, Lev), 27.91 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.46 (CH_3, 2'-O-Si-tBu), 17.83 (C_q, Si-tBu), -5.11, -5.29 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2899, 2856, 2837, 1744, 1715, 1607, 1580, 1506, 1456, 1248, 1175, 1153, 1030, 835, 779, 704 **HRMS:** $[C_{49}H_{55}N_5O_9Si+H]^+$: found 886.3849, calculated 886.3842.

5'-OH-3'-Lev-2'-TBDMS-Adenosine(Bz) (27)

1.315 mmol (1.003g) of Compound **23** was dissolved in 10mL DCM/MeOH (7:3 v:v), 13.19 mmol (2.509g, monohydrate) of diluted *p*-toluenesulfonic acid (6.27 wt% in DCM/MeOH(7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.4 in 100% EtOAc). Finally, the mixture was quenched using sat. $NaHCO_3$. The organic layer was washed with sat. $NaHCO_3$ followed by Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 80% to 100% EtOAc/PE eluent resulting in 0.663g (1.138 mmol, 86.6%) of Compound **27** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.284 (s, 1H, NH), 8.760 (s, 1H, H^2), 8.043 (s, 1H, H^8), 8.002 (d, J = 7.5 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.577 (t, J = 7.4 Hz, 1H, $H^{arom,\ para}$, Bz) 7.485 (t, J = 7.6 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 5.994 (d, J = 10.1 Hz, 1H, $OH^{5'}$), 5.815 (d, J = 7.7 Hz, 1H, $H^{1'}$), 5.476 (d, J = 5.2 Hz, 1H, $H^{3'}$), 5.114 (dd, J = 7.6, 5.2 Hz, 1H, $H^{2'}$), 4.303 (s, 1H, $H^{4'}$), 3.939 (d, J = 12.9 Hz, 1H, $H^{5'a}$), 3.779 (dd, J = 11.5, 9.8 Hz, 1H, $H^{5'b}$), 2.9-2.5 (m, 4H, R^1-$C\underline{H_2}C\underline{H_2}$-$R^2$, Lev), 2.184 (s, 3H, CH_3, Lev), 0.661 (s, 9H, 2'-O-Si-tBu), 0.041 (TMS), -0.153, -0.479 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.36 (CO, Lev) 171.79 (CO, Lev), 164.59 (CO, Bz), 152.43 (C^2), 150.57 (C^6), 150.48 (C^4), 143.03 (C^8), 133.52 (C_q^{ipso}, Bz), 133.02, 128.95, 127.97 (C^{ortho}, C^{meta}, C^{para}, Bz), 124.37 (C^5), 91.26 ($C^{1'}$), 86.00 ($C^{3'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.28 ($C^{2'}$), 73.08 ($C^{4'}$), 62.89 ($C^{5'}$), 37.85 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.87 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.39 (CH_3, 2'-O-Si-tBu), 17.76 (C_q, Si-tBu), -5.28, -5.84 (2x CH_3, 2'-O-Si-Me_2).

IR: 3254, 3167, 3123, 3065, 2953, 2928, 2859, 1695, 1609, 1580, 1454, 1152, 1093, 1080, 860, 837, 775, 727, 700, 650, 635

HRMS: $[C_{28}H_{37}N_5O_7Si+H]^+$: found 584.2535, calculated 584.2535

**The solution was prepared using 13.19 mmol (2.509g) p-toluenesulfonic acid monohydrate in 40mL DCM/MeOH (7:3 v:v), resulting in a 6.27 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Adenosine(Bz) (31)

1.138 mmol (0.663g) of Compound **27** was dissolved in 10mL of dry DCM, 2.5 mmol (0.25g, 0.35mL) of Triethylamine and 1.8 mmol (0.42g, 0.40mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by neutralized TLC (Rf around 0.80 in 100% EtOAc, 0.30 in 50% EtOAc/PE, 0.75 in DCM). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:99 TEA:DCM eluent resulting in 0.727g (0.927 mmol, 81.5%) of Compound **31** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)δ: 9.222 (br, 1H, NH), 8.780 (s, 1H, H^2), 8.466, 8.415 (s+s, 1H, H^8), 8.010 (d, *J* = 7.4 Hz, 2H, $H^{arom,\ ortho}$, Bz), 7.569 (t, *J* = 7.8 Hz, 1H, $H^{arom,\ para}$, Bz) 7.488 (t, *J* = 6.7 Hz, 2H, $H^{arom,\ meta}$, Bz), 7.260 ($CHCl_3$), 6.159, 6.135 (d+d, *J* = 6.3 + 5.7 Hz, 1H, $H^{1'}$), 5.400, 5.342 (dd+dd, *J* = 4.9, 3.4 + 5.0, 2.6 Hz, 1H, $H^{3'}$), 4.888, 4.814 (t+t, *J* = 5.6 + 5.4 Hz, 1H, $H^{2'}$), 4.40-4.35 (m, 1H, $H^{4'}$), 4.0-3.7 (m, 4H, $H^{5'}$, NC-$\underline{CH_2}$-CH_2-OR), 3.7-3.5 (m, 2H, CH, $\underline{iPr_2}$NR), 2.9-2.5 (m, 6H, R^1-$\underline{CH_2}\underline{CH_2}$-$R^2$, Lev, NC-$CH_2$-$\underline{CH_2}$-OR), 2.182 (s, 3H, CH_3, Lev) , 1.22-1.16 (m, 12H, CH_3, $\underline{iPr_2}$NR) 0.727, 0.707 (s+s, 9H, 2'-O-Si-tBu), -0.032 (TMS), -0.064, -0.091, -0.267, -0.282 (2x s+s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)δ: 206.21, 206.18 (CO, Lev) 171.84 (CO, Lev), 164.71 (CO, Bz), 152.91 (C^2), 151.98, 151.83 (C^6), 149.65 (C^4), 141.71, 141.44 (C^8), 133.85 (C_q^{ipso}, Bz), 132.77, 128.87, 127.92 (C^{ortho}, C^{meta}, C^{para}, Bz), 123.08, 123.02 (C^5), 117.57 (CN), 88.34, 87.97 ($C^{1'}$), 82.56, 82.47, 82.39 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.01, 74.94 ($C^{2'}$), 73.28, 72.70 ($C^{3'}$), 63.08, 62.90, 62.72, 62.57 ($C^{5'}$), 58.78, 58.57 (NC-$\underline{C}H_2$-CH_2-OR), 43.36, 43.24, 43.12 (2x CH, $\underline{iPr_2}$NR), 37.80, 37.77 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 29.90 (CH_3, Lev), 27.89, 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.42 (CH_3, 2'-O-Si-tBu), 24.90, 24.84, 24.79, 24.72 (4x CH_3, $\underline{iPr_2}$NR), 20.48, 20.42, 20.41, 20.35 (NC-CH_2-$\underline{C}H_2$-OR), 17.80 (C_q, Si-tBu), -5.19, -5.28, -5.35, -5.40 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.20, 149.05.

IR: 2965, 2930, 2886, 2858, 1744, 1716, 1609, 1582, 1454, 1250, 1155, 1028, 837, 779, 708, 679

HRMS: $[C_{37}H_{54}N_7O_4PSi+H]^+$: found 784.3615 , calculated 784.3619.

5',3'-Si(tBu)$_2$-2'-TBDMS-Cytidine (9)

1.99 mmol (0.485g) of cytidine was, after two co-evaporations in 1,4-dioxane, dissolved in 10 mL of dry DMF, 2.30 mmol (0.75 mL, 1.01g) of $(t\text{-}Bu)_2Si(OTf)_2$ was added at 0 °C and the mixture was stirred for 100 minutes. Reaction completion was checked by TLC (Rf around 0.40 at 10% MeOH/DCM). Then 10.1 mmol (0.691g) of Imidazole and 3.09 mmol (0.465g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.50 at 10% MeOH/DCM, 0.30 at 100% EtOAc, 0.20 at 80% EtOAc/DCM). Reaction was not fully completed, so 1.1 mmol (0.076g) of Imodazole and 0.989 mmol (0.149g) of TBDMS-Cl were added and the mixture was stirred for another 4 hours with no result. The mixture was quenched with 1 mL MeOH, and concentrated, before being redissolved in 30 mL DCM and washed with 20 mL H_2O, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using using an 80:20 to 100:0 EtOAc:DCM eluent resulting in 0.895g (1.80 mmol, 89.9%) of compound **9** as a white solid.

^{1}H NMR (399 MHz, $CDCl_3$, 330K) δ: 8.67 (br, 1H, NH_2), 7.34 (s, 1H, H^6), 7.26 ($CHCl_3$), 6.50 (br, 1H, H^5), 5.65 (s, 1H, $H^{1'}$), 4.47 (dd, *J* = 8.1, 4.7 Hz, 1H, $H^{5'a}$), 4.24 - 4.12 (m, 2H, $H^{2'}$, $H^{4'}$), 3.95 (t, *J* = 9.5 Hz, 1H, $H^{5'b}$), 3.77 (d, *J* = 6.0 Hz, 1H, $H^{3'}$), 1.01, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.90 (s, 9H, 2'-O-Si-tBu), 0.13, 0.10 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (100 MHz, $CDCl_3$, 330K) δ: 163.86 (C^2), 153.64 (C^4), 140.16 (C^6), 96.64 (C^5), 93.98 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.09 ($C^{3'}$), 75.69 ($C^{2'}$), 74.79 ($C^{4'}$), 67.74 ($C^{5'}$), 27.60, 27.08 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.97 (CH_3, 2'-O-Si-tBu), 22.81, 20.39, 18.30 (3x C_q, Si-tBu), -4.19, -4.84 (2x CH_3, 2'-O-Si-Me_2).

IR: 2932, 2895, 2884, 2859, 17.22, 1645, 1472, 1055, 826, 779, 752, 650.

HRMS: $[C_{23}H_{43}N_3O_5Si_2+H]^+$: found 498.2810, calculated 498.2814

5',3'-Si(tBu)$_2$-2'-TBDMS-Cytidine(Ac) (13)

1.159 mmol (0.570g) of Compound **9** was, after one co-evaporation with pyridine, dissolved in 5mL of dry pyridine, 2.9 mmol (0.27 mL, 0.29g) of acetic anhydride was added at 0 °Cand the mixture was stirred for

2 hours whiel warming to room temperature. Reaction completion was checked by TLC (Rf around 0.70 at 10% MeOH/DCM). The mixture was concentrated, co-evaporated with toluene and washed withsat. NH_4Cl, sat. $NaHCO_3$, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using using an 0% to 2% MeOH/DCM eluent resulting in 0.614g (1.137 mmol, 98.1%) of Compound **13** as a white solid.

^{1}H NMR (300 MHz, $CDCl_3$, 293.7K) δ: 10.46 (s, 1H, NH), 7.71 (d, *J* = 7.6 Hz, 1H, H^6), 7.44 (d, *J* = 7.6 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.71 (s, 1H, $H^{1'}$), 4.55 (dd, *J* = 9.2, 5.2 Hz, 1H, $H^{5'a}$), 4.34 – 4.21 (m, 2H, $H^{2'}$, $H^{4'}$), 4.00 (t, *J* = 10.3 Hz, 1H, $H^{5'b}$), 3.79 (dd, *J* = 9.7, 4.3 Hz, 1H, $H^{3'}$), 2.32 (s, 3H, CH_3, Ac), 1.02, 1.02 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.94 (s, 9H, 2'-O-Si-tBu), 0.22, 0.15 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (75 MHz, $CDCl_3$, 293.7K) δ: 171.68 (CO, Ac), 163.39 (C^2), 154.71 (C^4), 143.30 (C^6), 97.01 (C^5), 94.38 ($C^{1'}$), 77.59, 77.16, 76.74 ($CHCl_3$), 75.82 ($C^{3'}$), 75.46, 74.87 ($C^{2'}$, $C^{4'}$), 67.88 ($C^{5'}$), 27.61, 27.08 (2x CH_3, 5'-3'-O-Si-tBu_2), 26.01 (CH_3, 2'-O-Si-tBu), 25.11 (CH_3, Ac), 22.92, 20.47, 18.34 (3x C_q, Si-tBu), -4.16, -4.80 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2934, 2895, 2859, 1659, 1493, 1248, 1165, 1053, 997, 827, 779, 650.

HRMS: $[C_{25}H_{45}N_3O_6Si_2+H]^+$: found 540.2916, calculated 540.2920

5'-OH-3'-OH-2'-TBDMS-Cytidine(Ac) (17)

0.936 mmol (0.505g) of Compound **13** was dissolved in 5.0 mL of DCM, 3.85 mmol (0.70mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.45 in 10% MeOH/DCM). The mixture was washed withsat. NH_4Cl, sat. $NaHCO_3$, Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/DCM eluent resulting in 0.307g (0.768 mmol, 82.1%) of Compound **17** as a clear solid.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.27 (s, 1H, NH), 8.35 (d, *J* = 7.5 Hz, 1H, H^6), 7.39 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.67 (d, *J* = 2.3 Hz, 1H, $H^{1'}$), 4.57 (s, 1H, $OH^{5'}$), 4.45 (m, 1H, $H^{2'}$), 4.24 (dd, *J* = 11.2, 5.9 Hz, 1H, $H^{4'}$), 4.08 (d, *J* = 6.0 Hz, 1H, $H^{3'}$), 3.99 (d, *J* = 11.7 Hz, 1H, $H^{5'a}$), 3.84 (d, *J* = 8.5 Hz, 1H, $H^{5'b}$), 3.04 (d, *J* = 6.7 Hz, 1H, $OH^{3'}$), 2.21 (s, 3H, CH_3, Ac), 0.87 (s, 9H, 2'-O-Si-tBu), 0.12, 0.07 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 171.40 (CO, Ac), 163.14 (C^2), 155.58 (C^4), 146.67 (C^6), 97.07 (C^5), 93.29 ($C^{1'}$), 85.10 ($C^{2'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 75.34 ($C^{4'}$), 69.19 ($C^{3'}$), 60.54 ($C^{5'}$), 25.77 (CH_3, 2'-O-Si-tBu), 24.84 (CH_3, Ac), 18.04 (C_q, Si-tBu), -4.58, -5.20 (2x CH_3, 2'-O-Si-Me_2).

IR: 2951, 2930, 2889, 2857, 1647, 1491, 1248, 1113, 1059, 827, 779.

HRMS: $[C_{17}H_{29}N_3O_6Si+H]^+$: found 400.1889, calculated 400.1898

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS-Cytidine(Ac) (21)

7.544 mmol (3.015g) of Compound **17** was dissolved in 40 mL of dry Pyridine, 9.238 mmol (3.130g) of 4,4'-Dimethoxytritylchloride was added at 0 °C and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.80 in 10% MeOH/DCM, 0.60 in 100% EtOAc). Finally the reaction was quenched by adding 1 mL MeOH. The mixture was concentrated using rotary evaporation. Purification was performed with neutralized silica column chromatography using an 2:20:78 to 2:98:0 TEA:EtOAc:PE eluent resulting in 4.802g (6.842 mmol, 90.7%) of Compound **21** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ:10.47 (s, 1H, NH), 8.47 (d, *J* = 7.5 Hz, 1H, H^6), 7.44 (d, *J* = 7.3 Hz, 2H, H^{ortho}, DMTr), 7.40 – 7.20 (m, 7H, H^{arom}, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 7.15 (d, *J* = 7.5 Hz, 1H, H^5), 6.88 (d, *J* = 8.8 Hz, 4H, H^{arom}, DMTr), 5.91 (d, *J* = 0.9 Hz, 1H, $H^{1'}$), 4.38 (s, 1H, $H^{3'}$), 4.29 (dd, *J* = 4.6, 0.7 Hz, 1H, $H^{2'}$), 4.11 (d, *J* = 7.9 Hz, 1H, $H^{4'}$), 3.82 (2x s, *J* = 2.0 Hz, 6H, OMe, DMTr), 3.60 (dd, *J* = 11.1, 1.7 Hz, 1H, $H^{5'a}$), 3.54 (dd, *J* = 11.2, 2.7 Hz, 1H, $H^{5'b}$), 2.45 (d, *J* = 8.8 Hz, 1H, $OH^{3'}$), 2.29 (s, 3H, CH_3, Ac), 0.94 (s, 9H, 2'-O-Si-tBu), 0.30, 0.19 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 171.13 (CO, Ac), 163.33 (C^2), 158.77 (C_q, DMTr), 155.07 (C^4), 144.90 (C^6), 144.36, 135.55, 135.29 (3x C_q, DMTr), 130.19, 128.24, 128.13, 127.25, 113.41 (5x CH^{arom}, DMTr), 96.98 (C^5), 90.76 ($C^{1'}$), 87.20 (C_q, DMTr), 83.16 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.76 ($C^{2'}$), 69.14 ($C^{3'}$), 61.60 ($C^{5'}$), 55.32 (CH_3, OMe, DMTr), 25.91 (CH_3, 2'-O-Si-tBu), 24.93 (CH_3, Ac), 18.15 (C_q, Si-tBu), -4.27, -5.35 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2949, 2928, 2897, 2855, 2839, 1667, 1609, 1489, 1115, 814, 787.

HRMS: $[C_{38}H_{47}N_3O_8Si+H]^+$: found 702.3210, calculated 702.3205

5'-DMTr-3'-Lev-2'-TBDMS-Cytidine(Ac) (25)

5.733 mmol (4.038g) of Compound **21** was dissolved in 25mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 7.7 mmol (0.78mL, 0.89g) of Levulinic acid and 7.34 mmol (1.15mL, 0.927g) of Diisopropylcarbodiimide were added respectively and the mixture was stirred for 5 hours. Reaction completion was checked by TLC (Rf around 0.80 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ dried using $MgSO_4$ and concentrated using rotary evaporation. The mixture was redissolved in THF, centrifuged and the solution was collected. Purification was performed with neutralized silica column chromatography using an 1:30:79 to 1:99:0 TEA:EtOAc:PE eluent resulting in 4.398g (5.497 mmol, 95.6%) of Compound **25** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.31 (s, 1H, NH), 8.44 (d, *J* = 7.5 Hz, 1H, H^6), 7.40 (d, *J* = 8.7 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.40 – 7.20 (m, 7H, H^{arom}, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 7.13 (d, *J* = 7.5 Hz, 1H, H^5), 6.87 (d, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.92 (d, *J* = 2.1 Hz, 1H, $H^{1'}$), 5.18 (dd, *J* = 7.7, 4.3 Hz, 1H, $H^{3'}$), 4.50 (dd, *J* = 4.2, 2.1 Hz, 1H, $H^{2'}$), 4.37 (d, *J* = 7.7 Hz, 1H, $H^{4'}$), 3.81 (2x s, 6H, OMe, DMTr), 3.63 (dd, *J* = 11.3, 2.0 Hz, 1H, $H^{5'a}$), 3.41 (dd, *J* = 11.4, 2.2 Hz, 1H, $H^{5'b}$), 2.85 – 2.45 (m, 4H, R^1-C<u>H$_2$C</u><u>H$_2$</u>-R^2, Lev), 2.29 (s, 3H, CH_3, Ac), 2.19 (s, 3H, CH_3, Lev), 0.87 (s, 9H, 2'-O-Si-tBu), 0.18, 0.05 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 206.05 (CO, Lev), 171.90 (CO, Lev), 171.09 (CO, Ac), 163.24 (C^2), 158.80 (C_q, DMTr), 155.09 (C^4), 144.71 (C^6), 144.20, 135.28, 135.16 (3x C_q, DMTr), 130.23, 128.22, 128.18, 127.29, 113.45 (5x CH^{arom}, DMTr), 97.00 (C^5), 91.08 ($C^{1'}$), 87.40 (C_q, DMTr), 80.36 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.79 ($C^{2'}$), 70.89 ($C^{3'}$), 61.10 ($C^{5'}$), 55.36 (CH_3, OMe, DMTr), 37.69 (R^1-<u>C</u>H$_2$CH$_2$-R^2, Lev), 29.95 (CH_3, Lev), 27.80 (R^1-CH$_2$<u>C</u>H$_2$-R^2, Lev), 25.70 (CH_3, 2'-O-Si-tBu), 25.02 (CH_3, Ac), 18.03 (C_q, Si-tBu), -4.61, -5.43 (2x CH_3, 2'-O-Si-Me_2). **IR:** 2953, 2928, 2855, 1717, 1667, 1489, 1248, 1175, 1155, 1117, 1031, 1005, 829, 779.

HRMS: $[C_{43}H_{53}N_3O_{10}Si+H]^+$: found 800.3578, calculated 800.3573

5'-OH-3'-Lev-2'-TBDMS-Cytidine(Ac) (29)

5.363 mmol (4.290g) of Compound **25** was dissolved in 50mL DCM/MeOH (7:3 v:v), 58.88 mmol (11.20g, monohydrate) of diluted *p*-toluenesulfonic acid (7.80 wt% in DCM/MeOH (7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.45 in 100% EtOAc). Finally the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50:0 to 100:0 EtOAc:DCM eluent resulting in 2.450g (4.920 mmol, 91.8%) of Compound **29** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.07 (s, 1H, NH), 8.22 (d, *J* = 7.5 Hz, 1H, H^6), 7.44 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.62 (d, *J* = 3.8 Hz, 1H, $H^{1'}$), 5.18 (t, *J* = 5.1 Hz, 1H, $H^{3'}$), 4.72 (t, *J* = 4.3 Hz, 1H, $H^{2'}$), 4.27 (d, *J* = 5.4 Hz, 1H, $H^{4'}$), 3.97 (d, *J* = 12.9 Hz, 1H, $H^{5'a}$), 3.75 (d, *J* = 12.7 Hz, 1H, $H^{5'b}$), 2.90 – 2.50 (m, 4H, R^1-C<u>H$_2$C</u><u>H$_2$</u>-R^2, Lev), 2.27 (s, 3H, CH_3, Ac), 2.18 (s, 3H, CH_3, Lev), 0.85 (s, 9H, 2'-O-Si-tBu), 0.05, 0.03 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) δ: 206.54 (CO, Lev), 172.57 (CO, Lev), 171.26 (CO, Ac), 163.24 (C^2), 155.32 (C^4), 146.73 (C^6), 97.10 (C^5), 94.22 ($C^{1'}$), 83.17 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 73.34 ($C^{2'}$), 71.70 ($C^{3'}$), 61.20 ($C^{5'}$), 37.90 (R^1-<u>C</u>H$_2$CH$_2$-R^2, Lev), 29.92 (CH_3, Lev), 27.93 (R^1-CH$_2$<u>C</u>H$_2$-R^2, Lev), 25.69 (CH_3, 2'-O-Si-tBu), 25.03 (CH_3, Ac), 18.03 (C_q, Si-tBu), -4.85, -5.16 (2x CH_3, 2'-O-Si-Me_2).

IR: 3291, 2951, 2928, 2857, 1717, 1486, 1231, 1155, 1111, 837, 779.

HRMS: $[C_{22}H_{35}N_3O_8Si+H]^+$: found 498.2261, calculated 498.2266

**The solution was prepared using 58.88 mmol (11.20g) p-toluenesulfonic acid monohydrate in 120mL DCM/MeOH (7:3 v:v), resulting in a 9.33 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS-Cytidine(Ac) (33)

1.00 mmol (0.500g) of Compound 2**9** was dissolved in 10mL of dry DCM, 1.4 mmol (0.15g, 0.20mL) of Triethylamine and 1.1 mmol (0.26g, 0.25mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.60 in 100% EtOAc). Finally, the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:25:74 to 1:79:20 TEA:EtOAc:Hex eluent resulting in 0.576g (0.825 mmol, 82.5%) of Compound **33** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 293.7K) δ: 10.14, 10.09 (s+s, 1H, NH), 8.43, 8.36 (d+d, *J* = 7.5 + 7.6 Hz, 1H, H^6), 7.39 (d, *J* = 7.5 Hz, 1H, H^5), 7.26 ($CHCl_3$), 5.94, 5.91 (d+d, *J* = 2.9, 2.4 Hz, 1H, $H^{1'}$), 5.07, 4.99 (dd+dd, *J* = 7.2,

4.5 + 6.6, 4.6 Hz, 1H, $H^{3'}$), 4.46 – 4.34 (m, 2H, $H^{2'}$, $H^{4'}$), 4.14 – 3.72 (m, 4H, $H^{5'}$, NC-CH_2-CH_2-OR), 3.68 - 3.53 (m, 2H, CH, iPr_2NR), 2.85 – 2.48 (m, 6H, R^1-CH_2CH_2-R^2, Lev, NC-CH_2-CH_2-OR), 2.28 (s, 3H, CH_3, Ac), 2.18, 2.17 (s+s, 3H, CH_3, Lev), 1.27 – 1.12 (m, 12H, CH_3, iPr_2NR), 0.87, 0.86 (s+s, 9H, 2'-O-Si-tBu), 0.13, 0.12, 0.02, 0.01 (2x s+s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 293.7K) *δ*: 206.25, 206.13 (CO, Lev), 171.94, 171.87 (CO, Lev), 171.35 (CO, Ac), 163.21, 163.13 (C^2), 155.19, 155.14 (C^4), 144.95, 144.84 (C^6), 117.65, 115.59 (CN), 96.71, 96.62 (C^5), 91.04, 90.88 ($C^{1'}$), 80.95, 80.87 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.85, 74.78 ($C^{2'}$), 71.22, 70.53 ($C^{3'}$), 61.86, 61.69, 61.31, 61.14 ($C^{5'}$), 58.97, 58.76, 58.72, 58.51 (NC-CH_2-CH_2-OR), 43.43, 43.33, 43.31, 43.21 (2x CH, iPr_2NR), 37.82, 37.73 (R^1-CH_2CH_2-R^2, Lev), 29.96 (CH_3, Lev), 27.87, 27.82 (R^1-CH_2CH_2-R^2, Lev), 25.70 (CH_3, 2'-O-Si-tBu), 25.09, 25.07 (CH_3, Ac), 24.91, 24.89, 24.84, 24.82, 24.77, 24.74 (4x CH_3, iPr_2NR), 20.54, 20.46, 20.42, 20.34 (NC-CH_2-CH_2-OR), 18.05 (C_q, Si-tBu), -4.69, -5.36, -5.43 (2x CH_3, 2'-O-Si-Me_2).

^{31}P NMR (162 MHz, $CDCl_3$, 293.7K) *δ*: 149.83, 148.69.

IR: 2965, 2930, 2886, 2857, 1719, 1667, 1492, 1364, 1231, 1155, 1117, 1043, 812, 779, 731.

HRMS: $[C_{31}H_{52}N_5O_9PSi+H]^+$: found 698.3343, calculated 698.3345

5',3'-Si(tBu)$_2$-2'-TBDMS- Guanosine (8)

10.1 mmol (3.04g) of guanosine hydrate was, after three co-evaporations in 1,4-dioxane, dissolved in 50 mL of dry DMF, 11.0 mmol (3.60 mL, 4.86g) of (t-Bu)$_2$Si(OTf)$_2$ was added at 0 °C and the mixture was stirred for 40 minutes. Reaction completion was checked by TLC (Rf around 0.45 at 10% MeOH/DCM). Then 50.1 mmol (3.41g) of Imidazole and 15.1 mmol (2.28g) of TBDMS-Cl were added and the mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf around 0.55 at 10% MeOH/DCM). The mixture was quenched with 1mL MeOH and cooled to 0 °C. Purification was performed by filtering the suspension and washing the product residue with cold MeOH, resulting in 4.570g (8.498mmol, 84.2%) of Compound **8** as a white solid.

^{1}H NMR (400 MHz, DMSO, 297.3K)*δ*: 10.67 (s, 1H, NH), 7.91 (s, 1H, H^8), 6.36 (s, 2H, NH_2), 5.72 (s, 1H, $H^{1'}$), 4.56 (s, 1H, $H^{2'}$), 4.34 (dd, *J* = 7.6, 3.5 Hz, 1H, $H^{5'a}$), 4.28 (dd, *J* = 8.7, 5.2 Hz, 1H, $H^{3'}$), 4.02 – 3.88 (m, 2H, $H^{5'b}$, $H^{4'}$), 3.35 (s, HOD, H_2O), 2.50 (s, DMSO), 1.06, 1.00 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.86 (s, 9H, 2'-O-Si-tBu), 0.09, 0.07 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, DMSO, 297.3K)*δ*:156.68 (CO, C^6), 153.77 (C^2), 150.77 (C^4), 135.61 (C^8), 116.56 (C^5), 90.08 ($C^{1'}$), 75.68 ($C^{3'}$), 74.73 ($C^{2'}$), 73.91 ($C^{4'}$), 66.97 ($C^{5'}$), 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89 (DMSO), 27.33, 26.86 (2x CH_3, 5'-3'-O-Si-tBu_2), 25.72 (CH_3, 2'-O-Si-tBu), 22.25, 20.00, 18.05 (3x C_q, Si-tBu), -4.57, -5.11 (2x CH_3, 2'-O-Si-Me_2).

IR: 3474, 3292, 3159, 3140, 3088, 3019, 2936, 2884, 2856, 2816, 2778, 2714, 1688, 1167, 1049, 899, 833, 777. **HRMS:** $[C_{24}H_{43}N_5O_5Si_2+H]^+$: found 538.2873, calculated 538.2876

5',3'-Si(tBu)$_2$-2'-TBDMS- Guanosine(iBu) (12)

8.498 mmol (4.570g) of compound **8** was, after one co-evaporation with pyridine, dissolved in 80mL of dry Pyridine, 21.5 mmol (2.25 mL, 2.29g) of isobutyrylchloride as added at -20 °Cand the mixture was stirred for 1 hours. Reaction completion was checked by TLC (Rf around 0.80 at 10% MeOH/DCM). The mixture was quenched with 10 mL of MeOH and left to warm to room temperature for 1 hour. The mixture was concentrated before being redissolved in MeOH and cooled to 0°C. Purification was performed by filtering the suspension and washing the product residue with cold MeOH, resulting in 5.060g (8.324 mmol, 98.0%) of Compound **12** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K)*δ*: 11.97 (s, 1H, NH), 7.96 (s, 1H, NH), 7.73 (s, 1H, H^8), 7.26 ($CHCl_3$), 5.79 (s, 1H, $H^{1'}$), 4.50 (dd, *J* = 9.2, 4.8 Hz, 1H, $H^{5'a}$), 4.42 (s, 1H, $H^{2'}$), 4.30-4.15 (m, 2H, $H^{3'}$, $H^{4'}$), 4.00 (t, *J* = 9.6 Hz, 1H, $H^{5'b}$), 2.62 (dt, *J* = 13.6, 6.8 Hz, 1H, CH, iBu), 1.30, 1.28 (2x d, *J* = 3.3 + 3.2 Hz, 6H, CH_3, iBu), 1.07, 1.04 (2x s, 18H, 5'-3'-O-Si-tBu_2), 0.94 (s, 9H, 2'-O-Si-tBu), 0.16, 0.15 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K)*δ*: 177.88 (CO, iBu), 155.20 (CO, C^6), 147.25, 147.25 (C^4, C^2), 136.71 (C^8), 121.89 (C^5), 91.99 ($C^{1'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 76.19 ($C^{3'}$), 76.00 ($C^{2'}$), 74.78 ($C^{4'}$), 67.96 ($C^{5'}$), 36.84 (CH, iBu), 27.62, 27.13 (2x CH_3, 5'-3'-O-Si-tBu_2), 26.06 (CH_3, 2'-O-Si-tBu), 22.97, 20.49 (2x C_q, Si-tBu), 19.16, 19.00 (2x CH_3, iBu), 18.54 (C_q, Si-tBu), -4.03, -4.81 (2x CH_3, 2'-O-Si-Me_2).

IR: 3472, 3291, 3154, 3140, 3088, 3024, 2934, 2886, 2859, 2818, 2778, 2714, 1688, 1674, 1597, 1471, 1142, 1053, 829, 779, 673.

HRMS: $[C_{28}H_{49}N_5O_6Si_2+H]^+$: found 608.3293, calculated 608.3294

5'-OH-3'-OH-2'-TBDMS- Guanosine(iBu) (16)

0.181 mmol (0.110g) of Compound **12** was dissolved in 5.0 mL of DCM, 0.83 mmol (0.15mL) of diluted HF-Pyridine* was added dropwise at 0 °C and the mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf around 0.40 in 5% MeOH/DCM). The mixture was washed withsat. $KHSO_4$ and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 0% to 10% MeOH/DCM eluent resulting in 0.078g (0.167 mmol, 92.2%) of Compound **16** as a white solid.

^{1}H NMR (400 MHz, DMSO, 297.3K) *δ:* 12.08 (s, 1H, NH), 11.69 (s, 1H, NH), 8.28 (s, 1H, H^8), 5.86 (d, *J* = 6.8 Hz, 1H, $H^{1'}$), 5.11 (t, *J* = 5.4 Hz, 1H, $OH^{5'}$), 5.06 (d, *J* = 4.6 Hz, 1H, $OH^{3'}$), 4.54 (dd, *J* = 6.7, 4.9 Hz, 1H, $H^{2'}$), 4.09 (td, *J* = 4.7, 2.2 Hz, 1H, $H^{4'}$), 3.98 – 3.94 (m, 1H, $H^{4'}$), 3.70 – 3.50 (m, 2H, $H^{5'}$), 3.36 (s, HOD, H_2O), 2.76 (hept, *J* = 6.8 Hz, 1H, CH, iBu), 2.50 (DMSO), 1.12, 1.10 (2x s, 6H, CH_3, iBu), 0.71 (s, 9H, 2'-O-Si-tBu), -0.08, -0.20 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, DMSO, 297.3K) *δ:* 180.16 (CO, iBu), 154.82 (CO, C^6), 149.04 (C^2), 148.22 (C^4), 137.48 (C^8), 119.95 (C^5), 86.32 ($C^{4'}$), 85.90 ($C^{1'}$), 76.43 ($C^{2'}$), 70.82 ($C^{3'}$), 61.42 ($C^{5'}$), 40.14, 39.94, 39.73, 39.52, 39.31, 39.10, 38.90 (DMSO), 34.75 (CH, iBu), 25.50 (CH_3, 2'-O-Si-tBu), 18.91, 18.80 (2x CH_3, iBu), 17.77 (C_q, Si-tBu), -4.96, -5.45 (2x CH_3, 2'-O-Si-Me_2).

IR: 3474, 3291, 3154, 3142, 2930, 2882, 2859, 2779, 2714, 1682, 1601, 1252, 1142, 1090, 1049, 835, 781, 673. **HRMS:** $[C_{20}H_{33}N_5O_6Si+H]^+$: found 468.2267, calculated 468.2273

**70% HF-Pyridine contains 1mol HF per 28.57g at d=1.1g/mL, or 38.5M. 6:1 dilution in pyridine is 5.5M, or 0.1818mL/mmol.*

5'-DMTr-3'-OH-2'-TBDMS- Guanosine(iBu) (20)

1.43 mmol (0.680g) of Compound **16** was dissolved in 10 mL of dry Pyridine, 1.68 mmol (0.596g) of 4,4'-Dimethoxytritylchloride was added at 0 °C and the mixture was stirred overnight. Reaction completion was checked by TLC (Rf around 0.85 in 100% EtOAc). Finally the reaction was quenched by adding 0.5 mL MeOH. The (yellow) mixture was concentrated using rotary evaporation and co-evaporated with toluene (solution started to turn orange) before being redissolved in DCM (adding slight amount of sat. $NaHCO_3$ made it turn yellow again). The organic layer was washed with sat. $NaHCO_3$ and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 2:0:98 to 2:98:0 TEA:EtOAc:PE eluent resulting in 0.726g (0.943 mmol, 66.1%) of Compound **20** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) *δ:* 12.04 (s, 1H, NH), 8.04 (s, 1H, NH), 7.86 (s, 1H, H^8), 7.56 (d, *J* = 7.0 Hz, 1H, H^{ortho}, DMTr), 7.41 (dd, *J* = 8.8, 7.5 Hz, 4H, H^{arom}, DMTr), 7.30 – 7.10 (m, 3H, H^{meta}, H^{para}, DMTr), 7.26 ($CHCl_3$), 6.79 (t, *J* = 8.9 Hz, 4H, H^{arom}, DMTr), 5.76 (d, *J* = 7.3 Hz, 1H, $H^{1'}$), 5.21 (dd, *J* = 7.3, 5.3 Hz, 1H, $H^{2'}$), 4.34 (dd, *J* = 5.2, 1.2 Hz, 1H, $H^{3'}$), 4.23 (s, 1H, $H^{4'}$), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.56 (dd, *J* = 10.7, 1.6 Hz, 1H, $H^{5'a}$), 3.07 (dd, *J* = 10.8, 2.7 Hz, 1H, $H^{5'b}$), 2.91 (s, 1H, $OH^{3'}$), 1.53 (hept, *J* = 6.7 Hz, 1H, CH, iBu), 0.83 (d, *J* = 7.0 Hz, 3H, CH_3, iBu), 0.81 (s, 9H, 2'-O-Si-tBu), 0.60 (d, *J* = 6.9 Hz, 3H, CH_3, iBu), 0.01, -0.19 (2x s, 6H, 2'-O-$SiMe_2$).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) *δ:* 178.75 (CO, iBu), 158.83 (C_q, DMTr), 155.57 (C^6), 148.40 (C^2), 147.33 (C^4), 145.22 (C_q, DMTr), 139.21 (C^8), 136.19, 135.65 (2x C_q, DMTr), 130.06, 128.17, 128.02, 127.26 (4x CH^{arom}, DMTr), 122.52 (C^5), 113.38 (CH^{arom}, DMTr), 88.23 ($C^{1'}$), 86.26 (C_q, DMTr), 84.59 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.47 ($C^{2'}$), 71.28 ($C^{3'}$), 63.78 ($C^{5'}$), 55.33 (CH_3, OMe, DMTr), 35.93 (CH, iBu), 25.59 (CH_3, 2'-O-Si-tBu), 18.52, 18.48 (2x CH_3, iBu), 17.93 (C_q, Si-tBu), -5.00, -5.04 (2x CH_3, 2'-O-Si-Me_2).

IR: 3368, 3157, 3057, 3036, 2949, 2928, 2855, 2835, 1674, 1605, 1508, 1248, 1175, 1142, 1096, 1034, 781, 831, 702.

HRMS: $[C_{41}H_{51}N_5O_8Si+H]^+$: found 770.3583, calculated 770.358

5'-DMTr-3'-Lev-2'-TBDMS- Guanosine(iBu) (24)

0.858 mmol (0.661g) of Compound **20** was dissolved in 5mL of dry DCM, a catalytic amount of 4-dimethylaminopyridine, 1.5 mmol (0.15mL, 0.17g) of Levulinic acid and 1.1 mmol (0.18mL, 0.15g) of

Diisopropylcarbodiimide were added respectively and the mixture was stirred overnight. Reaction completion was checked by normal TLC (Rf around 0.80 in 100% EtOAc). The organic layer was washed with sat. $NaHCO_3$ dried using $MgSO_4$ and concentrated using rotary evaporation. The mixture was redissolved in THF, centrifuged and the solution was collected. Purification was performed with neutralized silica column chromatography using an 1:30:79 to 1:99:0 TEA:EtOAc:PE eluent resulting in 0.586g (0.675 mmol, 78.6%) of Compound **24** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 11.94 (s, 1H, NH), 7.79 (s, 1H, H^8), 7.58 (d, *J* = 7.0 Hz, 2H, $H^{arom,\ ortho}$, DMTr), 7.42 (t, *J* = 8.6 Hz, 4H, H^{ortho}, DMTr), 7.30 – 7.10 (m, 3H, H^{meta}, H^{para}, DMTr), 6.78 (dd, *J* = 11.8, 8.9 Hz, 4H, H^{arom}, DMTr), 5.69 (d, *J* = 7.7 Hz, 1H, $H^{1'}$), 5.50 (dd, *J* = 5.4, 1.5 Hz, 1H, $H^{3'}$), 5.33 (dd, *J* = 7.6, 5.5 Hz, 1H, $H^{2'}$), 4.19 (d, *J* = 1.3 Hz, 1H, $H^{4'}$), 3.76, 3.74 (2x s, 6H, OMe, DMTr), 3.57 (dd, *J* = 10.7, 1.5 Hz, 1H, $H^{5'a}$), 3.06 (dd, *J* = 10.8, 2.4 Hz, 1H, $H^{5'b}$), 2.9 – 2.50 (m, 4H, R^1-C$\underline{H_2}$C$\underline{H_2}$-R^2, Lev), 2.17 (s, 3H, CH_3, Lev), 1.31 (m, 1H, CH, iBu), 0.80 (d, *J* = 6.8 Hz, 3H, CH_3, iBu), 0.74 (s, 9H, 2'-O-Si-tBu), 0.55 (d, *J* = 6.9 Hz, 3H, CH_3, iBu), 0.02, -0.23 (2x s, 6H, 2'-O-SiMe_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.40 (CO, Lev), 178.40 (CO, iBu), 171.72 (CO, Lev), 158.86 (C_q, DMTr), 155.53 (C^6), 148.25 (C^2), 147.08 (C^4), 145.30 (C_q, DMTr), 139.37 (C^8), 136.19, 135.54 (2x C_q, DMTr), 130.10, 128.20, 128.00, 127.30 (4x CH^{arom}, DMTr), 122.79 (C^5), 113.41 (CH^{arom}, DMTr), 88.45 ($C^{1'}$), 86.38 (C_q, DMTr), 82.51 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 72.76 ($C^{2'}$), 72.68 ($C^{3'}$), 63.41 ($C^{5'}$), 55.33 (CH_3, OMe, DMTr), 37.76 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 35.99 (CH, iBu), 29.94 (CH_3, Lev), 27.86 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.46 (CH_3, 2'-O-Si-tBu), 18.44, 18.43 (2x CH_3, iBu), 17.82 (C_q, Si-tBu), -5.00, -5.29 (2x CH_3, 2'-O-Si-Me_2

IR: 3150, 3059, 3036, 2949, 2928, 2887, 2857, 1674, 1605, 1508, 1250, 1175, 1152, 1090, 1032, 833, 781, 702. **HRMS:** $[C_{46}H_{57}N_5O_{10}Si+H]^+$: found 868.3953, calculated 868.3947

5'-OH-3'-Lev-2'-TBDMS- Guanosine(iBu) (28)

0.635 mmol (0.551g) of Compound **24** was dissolved in 5mL DCM/MeOH (7:3 v:v), 6.15 mmol (1.17g, monohydrate) of diluted *p*-toluenesulfonic acid (7.80 wt% in DCM/MeOH (7:3 v:v))* was added at 0 °C and the mixture was stirred for 10 minutes. Reaction completion was checked by TLC (Rf around 0.30 in 100% EtOAc). Finally the mixture was quenched using sat. $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with silica column chromatography using an 50:50:0 (via 100:0:0) to 95:0:5 EtOAc:DCM:MeOH eluent resulting in 0.342g (0.604 mmol, 95.0%) of compound **28** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 12.26 (s, 1H, NH), 9.22 (s, 1H, NH), 7.78 (s, 1H, H^8), 7.26 ($CHCl_3$), 5.66 (d, *J* = 7.3 Hz, 1H, $H^{1'}$), 5.42 (dd, *J* = 5.4, 0.8 Hz, 1H, $H^{3'}$), 5.21 (d, *J* = 8.2 Hz, 1H, $OH^{5'}$), 4.89 (dd, *J* = 7.1, 5.5 Hz, 1H, $H^{2'}$), 4.24 (s, 1H, $H^{4'}$), 3.93 (dd, *J* = 12.3, 1.8 Hz, 1H, $H^{5'a}$), 3.82 – 3.71 (m, 1H, $H^{5'b}$), 2.90 – 2.50 (m, 6H, R^1-C$\underline{H_2}$C$\underline{H_2}$-R^2, Lev, CH, iBu), 2.19 (s, 3H, CH_3, Lev), 1.24, 1.23 (2x d, *J* = 3.0, 2.8 Hz, 6H, CH_3, iBu), 0.69 (s, 9H, 2'-O-Si-tBu), -0.14, -0.36 (2x s, 6H, 2'-O-Si-Me_2).

^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.52 (CO, Lev), 179.27 (CO, iBu), 171.94 (CO, Lev), 155.33 (C^6), 147.98 (C^2), 147.32 (C^4), 139.26 (C^8), 122.70 (C^5), 90.35 ($C^{1'}$), 84.69 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 73.84 ($C^{3'}$), 73.30 ($C^{2'}$), 62.54 ($C^{5'}$), 37.86 (R^1-$\underline{C}H_2CH_2$-R^2, Lev), 36.36 (CH, iBu), 29.94 (CH_3, Lev), 27.89 (R^1-$CH_2\underline{C}H_2$-R^2, Lev), 25.43 (CH_3, 2'-O-Si-tBu), 19.16, 18.91 (2x CH_3, iBu), 17.81 (C_q, Si-tBu), -5.25, -5.60 (2x CH_3, 2'-O-Si-Me_2).

IR: 3159, 2930, 2886, 2857, 1674, 1603, 1557, 1402, 1252, 1153, 1096, 837, 779.

HRMS: $[C_{25}H_{39}N_5O_8Si+H]^+$: found 566.2637, calculated 566.2641

**The solution was prepared using 6.15 mmol (1.17g) p-toluenesulfonic acid monohydrate in 15mL DCM/MeOH (7:3 v:v), resulting in a 7.80 wt% solution.*

5'-PAM(CNE)-3'-Lev-2'-TBDMS- Guanosine(iBu) (32)

0.604 mmol (0.342g) of compound **28** was dissolved in 6mL of dry DCM, 1.1 mmol (0.11g, 0.15mL) of triethylamine and 0.72 mmol (0.17g, 0.16mL) of 2-Cyanoethyl-N,N-diisopropylchlorophosphoramidite were added respectively and the mixture was stirred for 10 minutes. Reaction completion was checked by normal TLC (Rf around 0.45 in 100% EtOAc). Finally the mixture was quenched using aqueous 5wt% $NaHCO_3$. The mixture was partitioned and the organic layer was washed with Brine and dried using $MgSO_4$. Purification was performed with neutralized silica column chromatography using an 1:20:79 to 1:99:0 TEA:EtOAc:Hex eluent resulting in 0.360g (0.469 mmol, 77.7%) of Compound **32** as a white foam.

^{1}H NMR (400 MHz, $CDCl_3$, 297.3K) δ: 12.06 (br, 1H, NH), 8.90 (br, 1H, NH), 8.05, 8.00 (s+s, 1H, H^8), 7.26 ($CHCl_3$), 5.77 (d, *J* = 6.6 Hz, 1H), 5.44 – 5.38 (m, 1H, $H^{3'}$), 4.89, 4.69 (dd+t, *J* = 6.8, 5.3 + 5.8 Hz, 1H, $H^{2'}$), 4.32 – 4.25 (m, 1H, $H^{4'}$), 4.00 – 3.75 (m, 4H, $H^{5'}$, NC-CH_2-CH_2-OR), 3.67 – 3.54 (m, 2H, CH, iPr_2NR), 2.90 – 2.50 (m, 7H, R^1-CH_2CH_2-R^2, Lev, NC-CH_2-CH_2-OR, CH, iBu), 2.21 (s, 3H, CH_3, Lev), 1.29 – 1.09 (m, 18H, CH_3, iPr_2NR, CH_3, iBu), 0.73, 0.71 (s+s, 9H, CH_3, 2'-O-Si-tBu), -0.07, -0.08 (s+s, 3H, 2'-O-Si-Me), -0.28 (s, 3H, 2'-O-Si-Me).
^{13}C NMR (101 MHz, $CDCl_3$, 297.3K) δ: 206.65, 206.54 (CO, Lev), 178.77, 178.72 (CO, iBu), 171.97, 171.90 (CO, Lev), 155.72, 155.67 (C^6), 148.61, 148.48 (C^2), 147.72, 147.69 (C^4), 138.00 (C^8), 121.72, 121.66 (C^5), 117.85, 117.77 (CN), 88.01, 87.85 ($C^{1'}$), 82.57, 82.52, 82.48, 82.43 ($C^{4'}$), 77.48, 77.16, 76.84 ($CHCl_3$), 74.97, 74.46 ($C^{2'}$), 73.95, 73.12 ($C^{3'}$), 63.64, 63.47, 63.29, 63.14 ($C^{5'}$), 58.56, 58.44, 58.35, 58.22 (NC-CH_2-CH_2-OR), 43.48, 43.35, 43.30, 43.17 (2x CH, iPr_2NR), 37.89 (R^1-C$H_2$$CH_2$-$R^2$, Lev), 36.44, 36.41 (CH, iBu), 30.00 (CH_3, Lev), 27.86 (R^1-CH_2CH_2-R^2, Lev), 25.44 (CH_3, 2'-O-Si-tBu), 24.96, 24.89, 24.86, 24.84, 24.77 (4x CH_3, iPr_2NR), 20.67, 20.59, 20.57, 20.50 (NC-CH_2-CH_2-OR), 19.19, 19.17, 18.98, 18.95 (2x CH_3, iBu), 17.87, 17.86 (C_q, Si-tBu), -5.11, -5.21, -5.40, -5.42 (2x CH_3, 2'-O-Si-Me_2).
^{31}P NMR (162 MHz, $CDCl_3$) δ: 149.06, 148.98.
IR: 3078, 2965, 2930, 2886, 2857, 1719, 1667, 1624, 1557, 1493, 1364, 1310, 1231, 1180, 1155, 1117, 1043, 999, 978, 829, 779, 731.
HRMS: $[C_{34}H_{56}N_7O_9PSi+H]^+$: found 766.3719, calculated 766.3719

Compound 35: **HO-HMPB-HCP**

210-263 µmol (1.051 g) of Amino HCP resin (200-250 µmol/g) was put in a 20mL filter-syringe and swelled in 4 mL of dry DMF. Then a mixture of 649 µmol (156mg) of HMPB-linker, 659 µmol (343 mg) of PyBOP and 799 µmol (108mg) of HOBt dissolved in 6 mL of dry DMF was added followed by 1.44 mmol (186mg, 0.250mL) of DIPEA and the mixture was shaken for 5 hours at room temperature. The solution was drained from the resin and the resin was washed two times with DMF and three times with DCM.

Compound 36: **Fmoc-Gly-HMPB-HCP**

210-263 µmol of HO-HMPB-HCP resin **35** was put in a 10mL filter-syringe and swelled in 10 mL of dry DCM. Then 1.05 mmol (313mg) of Fmoc-Gly-OH, 1.29 mmol (163mg, 0.200mL) DIC and a catalytic amount (4 flakes) of DMAP were added and the mixture was shaken for one night at room temperature. The solution was drained from the resin and the resin was washed two times with DCM, three times with DMF and three times with DCM yielding 1.228g (95.6% via mass analysis) of Fmoc-Gly-HMPB-HCP resin **36**, with an estimated load (via mass analysis) of 239 µmol/g. **Analysis**:***Mass-based***: The dry resin appeared to have a mass of 1.228 g, which is an increase of 177 mg. The increase of resin should ideally result in a load of 294 µmol on 1.228 g, which is a load of 239 µmol/g. ***Fmoc-based UV/Vis***: 2.26 mg of Fmoc-Gly-HMPB-HCP resin **36** was taken and dissolved in 1mL of Piperidine/DMF (1:4, v:v). The mixture was left for 30 minutes at room temperature before adding 9 mL of MeOH. The resulting solution was filtrated to remove resin particles possibly interfering with the measurement. Three measurements were performed at 300nm;1,2: Sample vs Solution Sample(piperidine/DMF/MeOH (1:4:45)) resulting in A values of 0.548 and 0.552;3: Sample vs Sample (in reference cuvette) resulting in an A value of -0.005. The correct A value should be approximately (0.548 + 0.552) / 2 – (-0.005) = 0.555. To obtain the load from this A value we used the formula:

$$\mathbf{L}_{(mmol/g)} = (\mathbf{A} * \mathbf{Volume}_{(mL)}) / (\mathbf{C}_{(fmoc\ =\ 7800)} * \mathbf{Mass}_{(g)})$$

Using this formula we obtained a load of $\mathbf{L}_{(mmol/g)}$ = (0.555 * 10 mL) / (7.8 * 2.26 mg) = 0.315 mmol/g.

Compound 37: **H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP**

98.9 µmol (414 mg) of Fmoc-Gly-HMPB-HCP resin **36** (239 µmol/g) elongated using Solid Phase Peptide Synthesis (SPPS) yielding 97.9 µmol (464 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** (211 µmol/g). **Analysis**: **H-Ala-Tyr-Thr-Gly-OH** 2.5 µmol (10 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitatewas observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:**

R_t4.94 min **MS:**$[C_{18}H_{26}N_4O_7+H]^+$: found 411.07, calculated 411.19. $[2C_{18}H_{26}N_4O_7+H]^+$: found 821.13, calculated 821.37. $[3C_{18}H_{26}N_4O_7+H]^+$: found 1230.93, calculated 1231.55.

SPPS Prodcedure: Solid Phase Peptide Synthesis was performed using Fmoc-based strategy, with each cycle containing an *Fmoc deprotection*, *amino acid coupling* and *capping* step. Amino acids used: Fmoc-Thr(OTrt)-OH, Fmoc-Tyr-OH and Fmoc-Ala-OH. After each step the resin washed multiple times with NMP. An extra Fmoc deprotection cycle was introduced at the end to remove the final Fmoc protective group. As a final step the resin was washed multiple times with DCM and dried in open air. **Fmoc deprotection**: Four consecutive times 8mL of a solution of 20% Piperidine in NMP per gram resin (≈200 µmol) wes added to the resin and shaken for 4 minutes each. **Amino acid coupling**: 5 equivalents of AA were preactivated using 5 equivalents of HCTU (0.25M in NMP), 10 equivalents of DiPEA (1.0M in NMP) and NMP (4 mL / gram resin (≈200 µmol)) before being added to the resin and shaken for 1 hour. **Capping**: 20 equivalents of Ac_2O (0.5M in NMP) and 10 equivalents of DiPEA (1.0M in NMP) were added to the resin and shaken for 3 minutes.

Compound 38: Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP

180 µmol (853 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCPresin **37** (211 µmol/g) was taken and put in a 20mL filter syringe and 374 µmol (185 mg, DHCA salt) Bpoc-Gly-OH was added as a dry powder. 375 µmol (195 mg) of PyBOP and 737 µmol (74.5 mg, 81.0 µl) of NMM as a solution in 10 ml dry DMF were added and the mixture was shaken for 6 hours at room temperature. The solution was drained from the resin and the resin was washed three times with DMF and three times with DCM yielding 166 µmol (833 mg of Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** (199 µmol/g).

Analysis:**H-Gly-Ala-Tyr-Thr-Gly-OH** 2.0 µmol (10 mg) of Bpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%)**LC:**R_t4.41 min **MS:**$[C_{20}H_{29}N_5O_8+H]^+$: found 468.13, calculated 468.21. $[2C_{20}H_{29}N_5O_8+H]^+$: found 935.13, calculated 935.41. $[3C_{20}H_{29}N_5O_8+H]^+$: found 1402.07, calculated 1402.61. $[4C_{20}H_{29}N_5O_8+H]^+$: found 1870.13, calculated 1869.81.

Testing procedure: Reagents and conditions 5.3 µmol (25 mg) of H-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **37** was taken and put in a 2 mL filter syringe and **a** equivalents of Bpoc-Gly-OH were added as a dry powder. Then **b** equivalents of **B** and **c** equivalents of **C** as a 1 mL solution in **d** were added and the mixture was shaken for **e** hours at room temperature. The solution was drained from the resin and the resin was washed three times with DMF and three times with DCM.

	a (eq)	B	b(eq)	C	c(eq)	d	e	37	38	39	40
1	2	PyBOP	2	DIPEA	4	DMF	24h	-	+	+	-
2	2	PyBOP	2	DIPEA	4	DCM	24h	+	+	+	-
3	1,5	PyBOP	1,5	DIPEA	3	DMF	24h	-	+	-	-
4	2	DIC	2	DIPEA	4	DCM	24h	-	-	-	-
5	2	HBTU	2	DIPEA	4	DMF	24h	-	+	+	+
6	2	PyBOP	2	DIPEA	4	DMF	1h	+	+	-	-
7	2	PyBOP	2	DIPEA	4	DMF	4h	-	+	+	-
8	2	PyBOP	2	DIPEA	4	DMF	8h	-	+	+	-
9	2	PyBOP	2	NMM	4	DMF	4h	-	+	-	-
10	2	HBTU	2	NMM	4	DMF	4h	-	+	-	-

Compound 41: Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(Otrt)-Gly-HMPB-HCP

49.8 µmol (250 mg) ofBpoc-Gly-Ala-Tyr-Thr(OTrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 10 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10

minutes. The solvent was drained and the resin was washed three times with ACN. Then 346 µmol (227 mg) of uridine **34** (4.0 mL of a 0.087 M solution in ACN/Dioxane (1:1, v:v)) and 1.2 mmol (0.23 g) of BMT (4.0 mL of a 0.30M solution in ACN) were added and the mixture was shaken for 30 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then two consecutive times 375 µmol of I_2 (7.5 mL of a 0.05M solution in THF/Pyr/H_2O (7:2:1, v:v:v)) was added and the mixture was shaken for 60 seconds at room temperature before draining the solution from the resin. The resin was washed three times with ACN and three times with DCM yielding 49.8 µmol of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41**.

Analysis: **H-Gly-Ala-Tyr(OpUlev)-Thr-Gly-OH** 10 mg (≈2.0 µmol) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:** R_t8.62 min **MS:** $[C_{43}H_{63}N_8O_{18}PSi+H]^+$: found 1039.40, calculated 1039.38.

Testing procedure: Reagents and conditions 5.0 µmol (25 mg) of Bpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then **a** equivalents of **A** and **b** equivalents of **B** were added as stock solutions of 0.3M and 0.1M respectively and the mixture was shaken for **c** minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then two consecutive times **d** equivalents of **D** were added as stock solution of 0.05M and the mixture was shaken for **e** seconds at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM.

	A	a(eq)	B	b(eq)	c	38	41
1	34	6	BMT	18	20min	+	++
2	34	6	BMT	18	60min	+	++
3	34	3	BMT	9	60min	++	++
4	34	6	DCI	18	20min	+	++
5	34	7	BMT	24	20min	-	+++
- = not observed, + = minor, ++ = major, +++= single product							

Compound 38-cap: Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP

2.0 µmol (10 mg) ofBpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **38** (199 µmol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then 190 µmol (19.4mg, 18 µl) of Ac_2O and 48 µmol (6.2 mg, 8.4 µl) of DiPEA (0.5 mL of a freshly made DMF/Ac_2O/DiPEA (500:19:9, v:v:v) solution) were added and the mixture was shaken for 60 minutes at room temperature.The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM yielding 2.0 µmol of Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP resin **38-cap**.

Analysis:**H-Gly-Ala-Tyr(OAc)-Thr-Gly-OH**

2.0 µmol of Bpoc-Gly-Ala-Tyr(OAc)-Thr(Otrt)-Gly-HMPB-HCP resin **38-cap** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90 → 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) **LC:** R_t6.03 min

MS:$[C_{22}H_{31}N_5O_9+H]^+$: found 510.20, calculated 510.22. $[2C_{22}H_{31}N_5O_9+H]^+$: found 1019.20, calculated 1019.43. $[3C_{22}H_{31}N_5O_9+H]^+$: found 1529.07, calculated 1528.64.

Testing procedure: Reagents and conditions 2.0 µmol (10 mg) of Bpoc-Gly-Ala-Tyr(OH)-Thr(Otrt)-Gly-HMPB-HCP resin **35** (199 umol/g) was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 10 minutes. The solvent was drained and the resin was washed three times with ACN. Then **a** equivalents of **A**, **b** equivalents of **B** and **c** equivalents of **C** were added as stock solutions in a volume of **d** in **e** and the mixture was shaken for **f** minutes at room temperature.The solution was drained from the resin and the resin was washed three times with ACN and three times wih DCM.

	B	b(eq)	C	c(eq)	A	a(eq)	d (mL)	e	f	38	38-cap
1	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	20min	++	-
2	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	60min	++	-
3	Acetic anhydride	318	Me-Im	376	Lutidine	388	0.6	THF	120min	++	-
4	Acetic anhydride	80			DIPEA	24	0.5	DMF	10min	+	+
5	Acetic anhydride	80			DIPEA	24	0.5	DMF	30min	+	+
6	Acetic anhydride	80			DIPEA	24	0.5	DMF	60min	+	+
7	Acetic anhydride	80	DMAP	.cat	DIPEA	24	0.5	DMF	30min	-	++
8	Acetyl Chloride	20			DIPEA	48	0.5	DMF	30min	-	++
	- = not observed, + = observed, ++ = single product										

Compound 4: H-Gly-Ala-Tyr(OpU)-Thr-Gly-OH

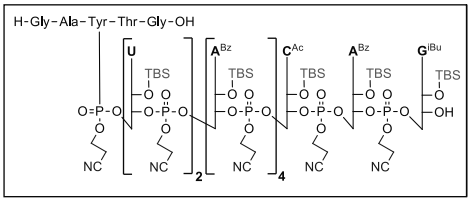
25 mg (≈ 5.0 µmol)ofBpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 20 minutes. The solvent was drained and the resin was washed three times with ACN. Then 375 µmol of hydrazine (1.5 mL of a 0.25 M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times with DCM. **Analysis**: Around 5.0 µmol Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3% TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The 800 µL of product containing solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight resulting in 1.18 mg (1.25 µmol, 25%) of Compound **4** as a white fluffy solid. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:**R_t5.36 min **MS:**$[C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 941.33, calculated 941.35. $[2C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 1882.20, calculated 1881.69.

Compound 5: H-Gly-Ala-Tyr(OpUpU)-Thr-Gly-OH

25 mg (≈ 5.0 µmol)ofBpoc-Gly-Ala-Tyr(OpUlev)-Thr(OTrt)-Gly-HMPB-HCP resin **41** was taken and put in a 2 mL filter syringe. ACN was added and the resin was allowed to swell while being shaken for 20 minutes. The solvent was drained and the resin was washed three times with ACN. Then 375 µmol of hydrazine (1.5

mL of a 0.25M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN. Then 79 µmol (52 mg) of uridine **34** (0.60 mL of a 0.13 M solution in ACN/Dioxane (1:1, v:v)) and 90 µmol (15 mg) of BMT (0.30 mL of a 0.30M solution in ACN) were added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed four times with ACN. Then two consecutive times 75 µmol of I_2 (1.5 mL of a 0.05 M solution in THF/Pyr/H_2O (7:2:1, v:v:v)) was added and the mixture was shaken for 60 seconds at room temperature before draining the solution from the resin. The resin was washed three times with ACN and three times with DCM.Then 375 µmol of hydrazine (1.5 mL of a 0.25 M solution in THF/Pyr/AcOH (5:3:2, v:v:v)) was added and the mixture was shaken for 20 minutes at room temperature. The solution was drained from the resin and the resin was washed three times with ACN and three times with DCM yielding a mixture of Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin and Bpoc-Gly-Ala-Tyr(OpUpU)-Thr(OTrt)-Gly-HMPB-HCP resin.

Analysis: The mixture of Bpoc-Gly-Ala-Tyr(OpU)-Thr(OTrt)-Gly-HMPB-HCP resin and Bpoc-Gly-Ala-Tyr(OpUpU)-Thr(OTrt)-Gly-HMPB-HCP resin was taken and put in a 2mL filter syringe. 300 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 5 mL of cold Et_2O, followed by 300 µL 3%TFA/DCM and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The 800 uL product containing solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding 3.48 mg (estimated 2.0 - 2.5 µmol, 40% - 50%) of a mixture of Compound **4** and Compound **5** as a white fluffy solid.**LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 90→ 40% ; ACN: 0 → 50% ; aq. 0.5% TFA: 10%) Compound **4**: **LC:**R_t8.12 min **MS:** $[C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 941.27, calculated 941.35. $[2C_{38}H_{57}N_8O_{16}PSi+H]^+$: found 1882.07, calculated 1881.69.Compound **5**: **LC:**R_t9.74 min **MS:**$[C_{56}H_{85}N_{11}O_{24}P_2Si_2+H]^+$: found 1414.40, calculated 1414.49.



Compound 6: H-Gly-Ala-Tyr(OpUpUpApApApApCpApG)-Thr-Gly-OH

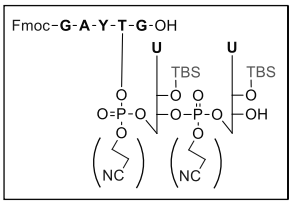
50 mg (≈10 µmol) of Bpoc-Gly-Ala-Tyr(OpUlev)-Thr(Otrt)-Gly-HMPB-HCPresin **41** was loaded in the DNA/RNA-synthesizer and elongated in 8 cycles to a 5'-pUpUpApApApApCpApG-3' sequence, with each cycle containing a *Levulinyl deprotection step*, *nucleotide coupling step, oxidation step*, and *capping step*. Nucleotides used: uridine **34** ,adenosine **31**, cytidine **33** and guanosine**32**. After each step the resin washed multiple times with ACN. An extra levulinyl deprotection cycle was introduced at the end to remove the final Levulinyl protective group. As a final step the resin was washed multiple times with DCM and dried in open air.

Sequence no.	**a** (times)	**b**	**B** (solvent)
1	2	PAM-U	ACN/ Dioxane (1:1, v:v)
2, 3, 4, 5, 7	2	PAM-A	Dioxane
6	3	PAM-C	ACN/ Dioxane (1:1, v:v)
8	3	PAM-G	ACN/ Dioxane (1:1, v:v)

Nucleotide coupling: **a** consecutive times the resin was brought in contact for 10 minutes with 30 µmol of nucleotide **b** (0.3 mL of a 0.1M solution in **B**)and 90 µmol of BMT activator (0.3 mL of a 0.3M solution in ACN). **Oxidation:**Three consecutive times the resin was brought in contact for 30 seconds with 50 µmol of I_2 (1.0 mL of a 0.05 M solution in THF/Pyr/H_2O (7:2:1, v:v:v)). **Capping**: Two consecutive times the resin was brought in contact for 30 seconds with 1.2 mmol N-Methyl-Imidazole (0.5 mL of a 20% solution in THF) and 1 mmol *tert*-butylphenoxyacetic anhydride (or alternatively Ac_2O) + 1.2 mmol lutidine (0.5mL of a 20% + 30% solution in THF).

Analysis: 20 mg (≈4.0µmol) resin was taken and put in a 2mL filter syringe. 600 µL 3%TFA/DCM was added and left for 5 minutes. The solvent was dropped into at tube containing 10mL of cold Et_2O/pentane (1:1, v:v), followed by 600 µL 3%TFA/DCM and 1 mL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 5 minutes at -20 °C and centrifuged for 5 minutes.

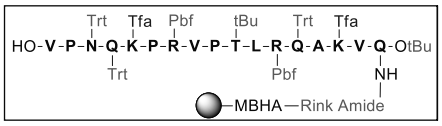
The supernatant was removed and the residue was dissolved in 2 mL of 0.1 M NH_4OAc/ACN (1:1, v:v) (noted the residue is not soluble in either H_2O or ACN, only a mixture of both). 100 µL was taken and diluted with 1mL of H_2O for LC-MS analysis.The 1.9 mL solution and remaining LC-MS solution were combined and transfered to an eppendorf. The eppendorf was cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding 7.5 mg (estimated 1.4µmol, 35%) of a mixture of fragment intermediates and Compound **6** as a white fluffy solid. **LC-MS analysis:** (Gemini 3u C_{18} 110A analytical column, linear gradient in 12.5minutes; H_2O: 40 → 0% ; ACN: 50 → 90% ; aq. 0.1M NH_4OAc: 10%) **LC:** R_t8.4 – 10.6 min. **MS:** $[C_{229}H_{316}N_{51}O_{75}P_9Si_9+H]^{3+}$: m/z found 1839.3, calculated 1838.6



Compound 43: Fmoc-Gly-Ala-Tyr(OpUpU)-Thr-Gly-OH An eppendorf containing 2.8µmol (3.2 mg) of Compound **5*** was taken and 5.5 µmol (1.9 mg) of Fmoc-OSu (250 µL of a freshly made 22 mM solution in DMF) followed by 11.0 µmol (1.42 mg, 1.82 µl) of DiPEA (250 µL of a 44 mM solution in DMF) and the mixture was left to react overnight at room temperature. 5 µL was taken and added to50 µL of H_2O/ACN/tBuOH (1:1:1, v:v:v) for LC-MS analysis. The LC-MS showed succesfull protection of the N-terminus amine, resulting in full conversion of Compound **5** into Compound **43****.**LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **44'**: **LC:** R_t6.83 min **MS:** $[C_{50}H_{64}N_7O_{18}PSi+H]^+$: found 1110.33, calculated 1110.39. Compound **44**: **LC:** R_t7.21 min **MS:**$[C_{53}H_{67}N_8O_{18}PSi+H]^+$: found 1163.33, calculated 1163.42. Compound **45'**: **LC:**R_t7.76 min **MS:** $[C_{56}H_{85}N_{11}O_{26}P_2Si_2+H]^+$: found 1583.27, calculated 1583.53.Compound **45**: **LC:** R_t7.88 min **MS:** $[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^+$: found 1636.40, calculated 1636.55.

As a mixture of Compound **4 and **5** consisting primarily of **5**. The amount of µmol was determined by mass as if it was solely Compound **5***

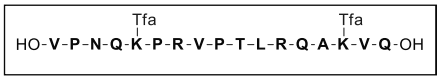
As a mixture of Compound **42'** (= **42** – 1 cyanoethyl), **42**, Compound **43'** (= **43** – 1 cyanoethyl) and Compound **43



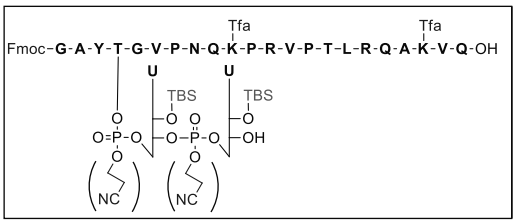
H-Val-Pro-Asn(Trt)-Gln(Trt)-Lys(Tfa)-Pro-Arg(Pbf)-Val-Pro-Thr(tBu)-Leu-Arg(Pbf)-Gln(Trt)-Ala-Lys(Tfa)-Gln(NH-Rink Amide-MBHA-PS)-OtBu 99.8 µmol (128 mg) of H_2N-Rink Amide-MBHA-PS (780 µmol/g) elongated using Solid Phase Peptide Synthesis (SPPS) yielding 89.3 µmol (425 mg, 89.5%) of H-Val-Pro-Asn(Trt)-Gln(Trt)-Lys(Tfa)-Pro-Arg(Pbf)-Val-Pro-Thr(tBu)-Leu-Arg(Pbf)-Gln(Trt)-Ala-Lys(Tfa)-Gln(NH-Rink Amide-MBHA-PS)-OtBu (210 µmol/g).

SPPS procedure: Solid Phase Peptide Synthesis was performed using Fmoc based strategy, with each cycle containing an *Fmoc deprotection*, *amino acid coupling* and *capping* step. Amino acids used: Fmoc-Glu-OtBu, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Thr(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH and Fmoc-Lys(Tfa)-OH. After each step the resin washed multiple times with NMP. An extra Fmoc deprotection cycle was introduced at the end to remove the final Fmoc protective group. As a final step the resin was washed multiple times with DCM and dried in open air. **Fmoc deprotection**: Four consecutive times 3.2mL of a solution of 20% Piperidine in NMP per100 mg resin (78 µmol) was added to the resin and shaken for 4 minutes each. **Amino acid coupling**: 5 equivalents of AA were preactivated using 5 equivalents of HCTU (0.25M in NMP), 10 equivalents of DiPEA (1.0M in NMP) and NMP (1.56 mL / 100 mg resin (78 µmol)) before being added to the resin and shaken for 1 hour. **Capping**: 20 equivalents of Ac_2O (0.5M in NMP) and 10 equivalents of DiPEA (1.0M in NMP) were added to the resin and shaken for 3 minutes.

Analysis: 2.1 µmol (10 mg) of peptide resin was taken and put in a 2 mL filter syringe. 500 µL TFA/TIS/H_2O (95/2.5/2.5, v:v:v) was added and left for 30 minutes. The solvent was dropped into at tube containing 10 mL of cold Et_2O, followed by 500 µL TFA/TIS/H_2O (95:2.5:2.5, v:v:v) and 500 µL of Et_2O which were also collected in the same tube. A white solid precipitate was observed. The tube was cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residue was dissolved in 1 mL of H_2O. 200 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** R_t4.48 min **MS:** $[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.60, calculated 1076.57.



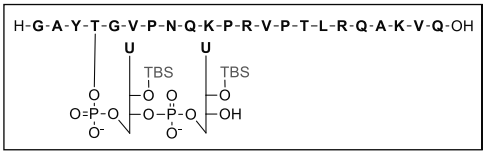
H-Val-Pro-Asn-Gln-Lys(Tfa)-Pro-Arg-Val-Pro-Thr-Leu-Arg-Gln-Ala-Lys(Tfa)-Gln-OH (3) 87.2 µmol (415 mg) of resin bound Compound **5** was taken and put in a 20mL filter syringe. 5.0 mL TFA:TIS:H_2O (95:2.5:2.5, v:v:v) was added and left for 30 minutes. The solvent was dropped into at tube containing 40 mL of cold Et_2O, followed by 5 mL TFA:TIS:H_2O (95:2.5:2.5, v:v:v) which was dropped into a second tube containing 40 mL of cold Et_2O. Crashing out of compound was observed in both tubes, though as expected much more in the first tube. The tubes were cooled for 30 minutes at -20 °C and centrifuged. The supernatant was removed and the residues were partially dissolved in 5 mL of H_2O, resulting in a slurry. 100 µL was taken and diluted with 1mL of H_2O for LC-MS analysis. The remaining slurry was stored at -20 °C. **Purification:**HPLC purification was performed by diluting the slurry 1:1 with DMSO allowing the slurry to form a properly dissolved clear solution. The solution was then purified by HPLC and the obtained fractions transferered to 20 mL tubes, cooled using liquid nitrogen for 2 minutes and freezedried overnight yielding Compound **3** as a white fluffy solid.**HPLC**: (linear gradient in15 minutes;H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC**: R_t 11 min. **Pre-HPLC LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%)**LC:**R_t4.48 min **MS:**$[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.60, calculated 1076.57. **Post-HPLC LC-MS analysis:**(Gemini 3u C_{18} 110A analytical column, linear gradient in 12.5minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.1M NH_4OAc: 10%) **LC:**R_t4.3 min. **MS**:$[C_{90}H_{149}F_6N_{29}O_{25}+H]^{+}$: found 2151.5, calculated 2152.13. $[C_{90}H_{149}F_6N_{29}O_{25}+2H]^{2+}$: found 1076.9, calculated 1076.57.



Compound 45: An eppendorf containing Compound **43*** was taken and 5.69 µmol (2.96 mg) PyBOP, 5.6 µmol (0.76 mg) HOBt (100 µl of a freshly made solution of 57 mM PyBOP and 56 mM HOBtin DMF) and 5.5 µmol (0.76 mg, 0.91 µl) of DiPEA (125 µL of a 44 mM solution in DMF) were added and the mixture was allowed to preactivate for 25 minutes before adding 5.6 µmol (12 mg) of peptide **3** predissolved in 200 µL DMF. The final mixture was left to react overnight at room temperature, but showed no notable difference after 2 hours. 5 µL was taken and added to 50 µL of H_2O/ACN/tBuOH (1:1:1, v:v:v) for LC-MS analysis. The LC-MS analysis showed moderate conversion of Compound **43** into Compound **45****. **Purification:** The resulting mixture was diluted with a mixture of H_2O/ACN/tBuOH (1:1:1, v:v:v) before being injected in the **HPLC.** Fractions containing Compound **45'** and **45** were collected and put together. Finally the product containing solution was concentrated using rotary evaporation. **Pre-HPLC LC-MS analysis:**(Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%). Compound **46'**: **LC:** R_t7.12 min **MS:** $[C_{53}H_{67}N_8O_{18}PSi+H]^{+}$: found 1163.40, calculated 1163.42. Compound **46**: **LC:** R_t 7.45 min **MS:** $[C_{56}H_{70}N_9O_{18}PSi+H]^{+}$: found 1216.47, calculated 1216.44. Compound **47'**: **LC:** R_t8.04 min **MS:**$[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^{+}$: found 1636.40, calculated 1636.55.Compound **47**: **LC:** R_t8.04 min **MS:**$[C_{71}H_{95}N_{11}O_{26}P_2Si_2+H]^{+}$: found 1689.33, calculated 1689.58.Compound **44'**: **LC:** R_t6.09 min **MS:**$[C_{140}H_{211}F_6N_{36}O_{42}PSi+2H]^{2+}$: found 1622.73, calculated 1622.75. Compound **44**: **LC:** R_t6.26 min **MS:**$[C_{143}H_{243}F_6N_{37}O_{42}PSi+2H]^{2+}$: found 1649.27, calculated 1648.76. Compound **45'**: **LC:** R_t6.64 min **MS:**$[C_{158}H_{239}F_6N_{39}O_{50}P_2Si_2+2H]^{2+}$: found 1859.07, calculated 1859.32.Compound **45**: **LC:** R_t6.70 min **MS:**$[C_{161}H_{242}F_6N_{40}O_{50}P_2Si_2+2H]^{2+}$: found 1885.80, calculated 1885.33.

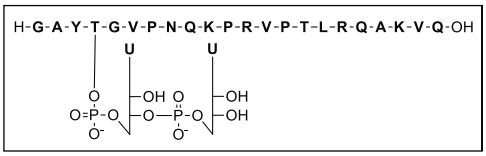
As a mixture of Compound **42, Compound **43'** (= **45** – 1 cyanoethyl) and Compound **43**.*

***As a mixture of Compound **44'** (= **44** – 1 cyanoethyl), **44**, Compound **45'** (= **45** – 1 cyanoethyl) and Compound **45**.*



Compound 48: VPg-pUpU Compound **45***, was dissolved in 0.5 mL of of 30-33% sat. NH_4OH and left for 4 days at room temperature. Finally the mixture concentrated, redissolved in aq. 0.1M NH_4OAc and concentrated again. Finally it was redissolved 1 mL of H_2O and 50 uL was taken for LC-MS analysis showing

full conversion of Compound **45** and **45'** into Compound **48**. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **48**: **LC:** R_t5.09 min **MS:** $[C_{136}H_{228}N_{38}O_{46}P_2Si_2+2H]^{2+}$: found 1625.73, calculated 1625.29. $[C_{136}H_{228}N_{38}O_{46}P_2Si_2+H+F_3CCOOH]^{2+}$: found 1681.73, calculated 1682.29.
As a mixture of compound **45' (= **47** – 1 cyanoethyl) and compound **45**.*



Compound 49: Previously obtained Compound **48** was treated with 3.1 mmol of HF (0.5 mL of TEA/TEA*3HF/DMF (2:3:4, v:v:v)) and the mixture was left overnight at room temperature. Then 2.9 mmol of $NH_4^+HCO_3^-$ (1.5 mL of a 15 wt% solution) was added dropwise with increasing speed. **Purification:** The solution was desalted using ion exchange column chromatography using an aq. NH_4HCO_3 eluent. After LC-MS analysis the obtained fractions were concentrated, redissolved in 1 mL of H_2O/ACN/tBuOH (1:1:1, v:v:v) and freezedried to remove NH_4HCO_3 salt. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80→ 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) Compound **49**: **LC:** R_t5.10 min **MS:** $[C_{124}H_{200}N_{38}O_{46}P_2+2H]^{2+}$: found 1511.53, calculated 1511.20 $[C_{124}H_{200}N_{38}O_{46}P_2+H+F_3CCOOH]^{2+}$: found 1566.87, calculated 1568.20 $[C_{124}H_{200}N_{38}O_{46}P_2+3H]^{3+}$: found 1007.67, calculated 1007.81.

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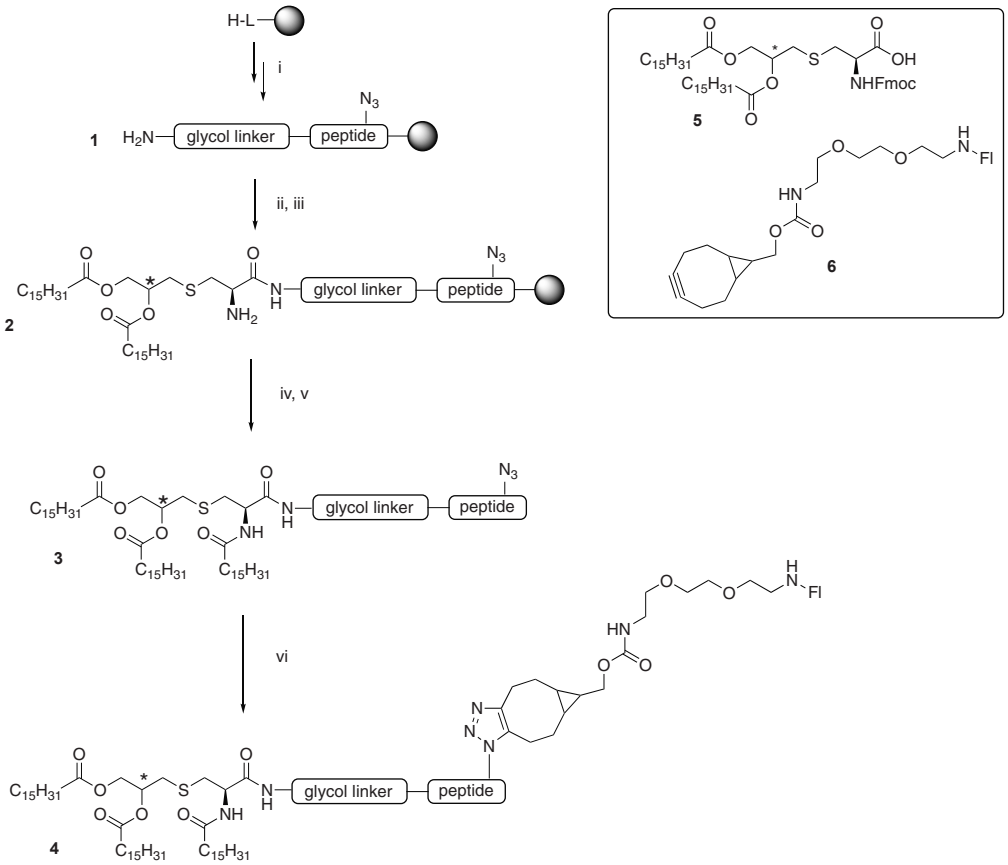
Chapter 7: Summary and future prospects

This thesis describes the design and synthesis of various bio-conjugates of a broad biological significance. All compounds share a peptide chain modified with different biomolecular entities such as nucleic acids, lipids or a small heterocyclic moiety. In the synthetic approaches described in Chapters 3 and 4, the modification could be introduced via a suitable building block using a peptide coupling on solid-phase. Alternatively, more sophisticated methods such as copper-free click chemistry (Chapter 2) could be deployed when necessary. A combined approach that involved both step-wise solid phase synthesis and block-coupling in solution was used for the synthesis of fluorescently labelled immunogenic lipopeptides (Chapter 2) and native oligoribonucleotide-peptide conjugates (Chapter 6). The well-defined conjugates described in this thesis could be applied as model compounds, references or molecular probes for the investigation of intracellular processes. For instance, TLR self-adjuvanting immunogenic peptides described in chapters 2, 3 and 4 might serve for the elucidation of antigen routing and processing within dendritic cells, while azide modified peptides (Chapter 5) could enhance the understanding of antigen presentation on cells surface.

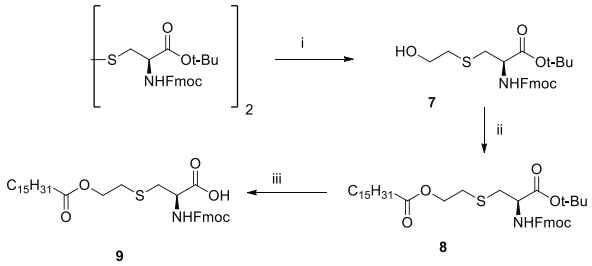
Chapter 1 represents an overview of the synthesis of selected examples of synthetic immunogenic TLR-L-peptide conjugates that were designed as vaccine candidates.

Chapter 2 describes the synthesis of fluorescently labelled conjugates of TLR2 agonist Pam_3Cys and antigenic peptides. Using Fmoc-azidonorleucine in place of Fmoc-Lysine(Boc) in SPPS, unlabelled precursor-conjugates could be isolated. Copper-free click chemistry was then applied to attain a number of fluorescent conjugates. Although the constructs performed as expected during biological assays, they suffer from very poor solubility. The water solubility of such constructs could be improved in the future through the incorporation of an ethylene glycol moiety (PEG-linker) in the peptide sequence to enhance water solubility of the overall construct (Scheme 1).



Scheme 1. Convergent synthesis of labelled Pam_3Cys-lipopeptides. Reagents and conditions: i) SPPS Fmoc automated synthesis, ii) **5**, HCTU, D*i*PEA, NMP, iii) 20% piperidine, NMP, iv) PamCl, Pyridine/DCM, v) 95% TFA, 2.5% TIS, 2.5% H_2O, vi) **6**, DMSO

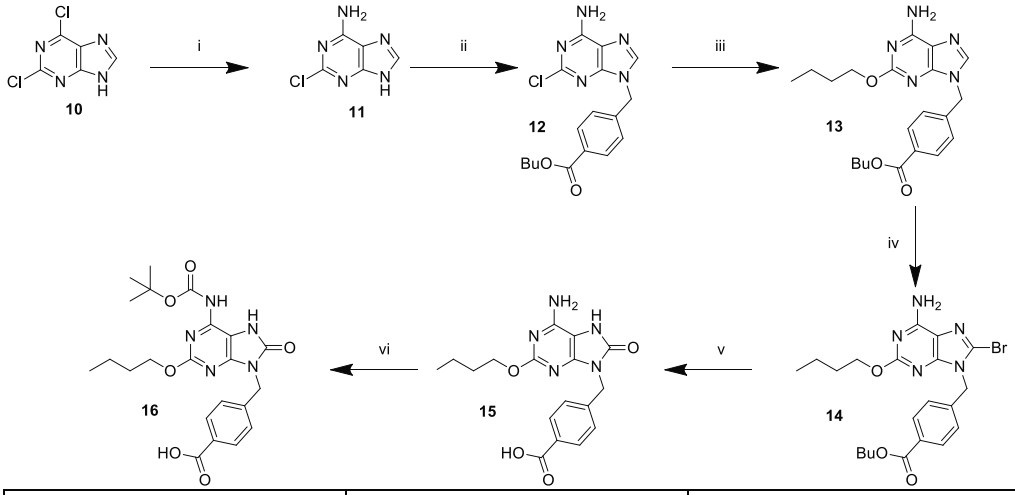
It is known that an ethylene glycol moiety ("glycol linker" Scheme 1) can be introduced between Pam_3Cys-Ser and the peptide sequence via standard peptide coupling conditions and using a commercially available ethylene glycol Fmoc-amino acid.[1] Using different R-groups as labels, a wide variety of labelled immunogenic peptide may be obtainable via this synthesis route. **Chapter 3** describes the design and synthesis of a peptide conjugate containing a simplified TLR-2 agonist ("mini-Pam"). Compared to the original ligand Pam_3Cys, two palmitic chains are removed, while the immunogenic activity of the agonist is retained. (see **9**, Scheme 2). Fluorescently labelled conjugates based on the "mini-Pam" connected to the peptide via a short PEG-linker could be obtained without constrains with regard to solubility in aqueous media. If this agonist is further adopted as a drug candidate, a transfer from lab-scale synthesis to industrial process would be necessary.



	Current	Potential substitutes
Step ii, Coupling agent/base	DIC/DMAP(cat)	T3P, EDCI, CDI / DiPEA, TEA, NMO
Step ii, Solvent	DCM	THF, MeTHF, EtOAc, iPrOAc
Step iii, Acid	TFA	pTSA
Step iii, Solvent	None	Toluene

Scheme 2. Synthesis of simplified TLR-2 ligand building block, Reagents and conditions: i) 1) Zn, H_2SO_4, HCl, MeOH 2) Oxirane, 50% ii) Palmitic acid, DIC, DMAP, DCM, 88% iii) TFA, 90%

Despite a very straightforward synthesis of compound **9**, each intermediate is purified via column chromatography, and evaporated to dryness. For an industrial process a crystallization procedure would need to be developed for each intermediate. When not achievable, using the crude as a stock solution in a suitable solvent for the next step might be a back-up solution. Because DMAP is toxic and DCM is a class 3 solvent, alternative conditions should be investigated. Alternative for DCM could be THF, Me-THF or EtOAc, depending on solubility, and condensing agent DIC catalysed with DMAP may be substituted with propylphosphonic anhydride (T3P) and NMO. The Fmoc stability under those conditions is yet to be assessed. Although, very efficient at small scale, neat TFA is not preferred at large scale. Different organic acids such as p-TSA could be employed. Chapter **4** describes the synthesis of a derivative of a known TLR-7 agonist, 2-alkoxy-9-benzyl-8-oxoadenine, that would be suitable for the conjugation to immunogenic peptides. The original structure of the pharmacofore was not alerted and the anchor site known not to disrupt the biological activity of the agonist was chosen. As depicted in scheme **3** compound **16** can be synthesized from compound **10** in 6 steps. Due to the overall poor solubility of this construct, substituting column chromatography for crystallizations appears a feasible option in an industrial process. Step i condition are readily scalable, yet, lower temperature of reaction would allow standard reactor to be used rather than an autoclave. In step ii, although TBAF is not preferred on scale, it appears difficult to substitute it for a different base without putting at risk the regioselectivity of the alkylation. In step iii, due to safety concern, it would be preferable to use commercially available sodium n-butoxide in butanol rather generating it *in-situ* by the use of sodium hydride. Step iv, consisting of a bromination in DCM, would benefit from a different source of bromine. However, alernatives for these conditions are difficult to find. Despite a long and tedious work up in step v, compound **15** should be easily isolable by crystallization due to its very poor solubility. Other options using Boc_2O rather than BocON could be explored in step vi. Homogenous conditions in THF/Water, or Schotten-Baumann conditions could perform as an alternative for the Boc protection.

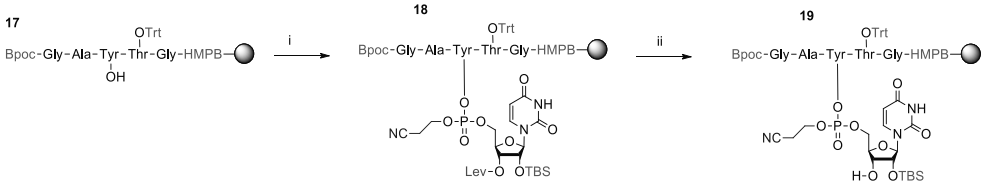


	Current	Potential substitutes
Step i, temperature	120°C	60-120°C
Step iii, reagent	NaH, n-BuOH	n-BuONa in butanol
Step vi, Reagent	BocO**N**	Boc_2O
Step vi, solvents	H_2O/Dioxane	H_2O/THF, H_2O/MeTHF, H_2O/EtOAc

Scheme 3. Synthesis of compound **16**. *Reagent and conditions:* i) Sat. NH_3 in MeOH, 100°C. 88% ii), 1M TBAF in THF, Bromo methyl, butyl benzoate, RT, 92% iii) 1) NaH, n-BuOH, 120°C 2) H_2SO_4, 80 C°, 62% iv) Br_2, DCM, RT, 76% v) 1) NaOMe, MeOH, 65°C 2) 37% HCl, RT 3) 1M KOH, H_2O, RT, 53% vi) BocON, TEA, H_2O/Dioxane 1:1, RT 10%.

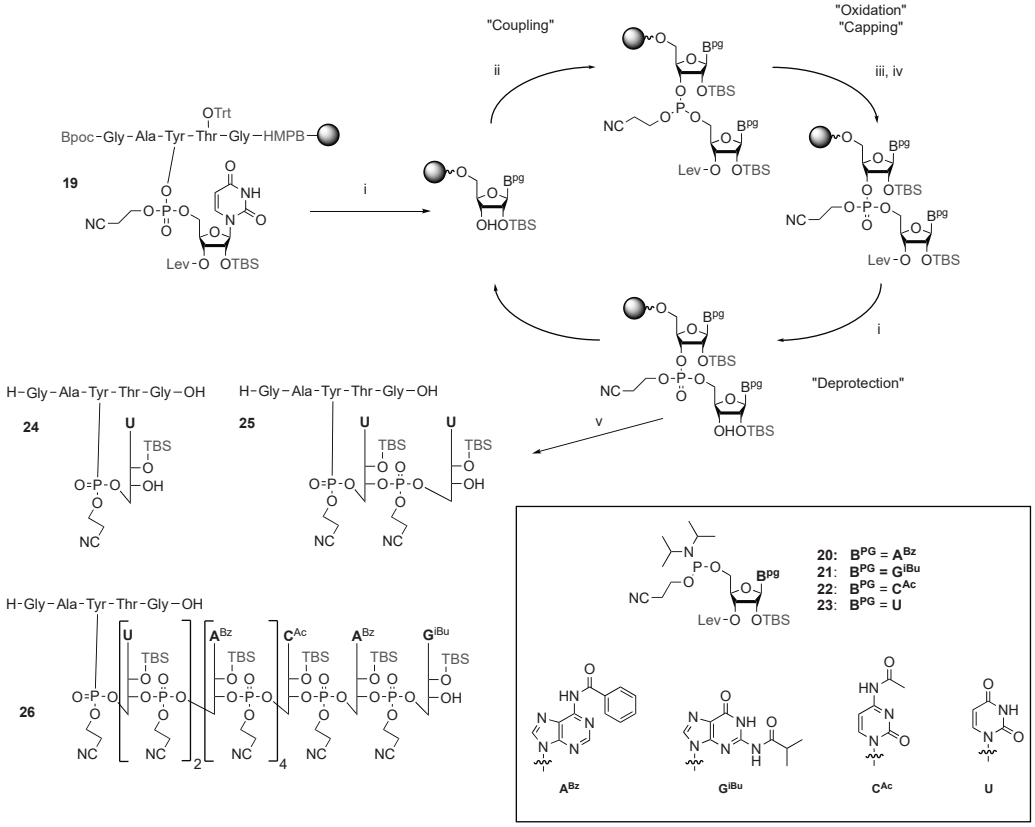
Chapter 5 describes the synthesis of an azidated peptide and the influence of the type of phosphine and the pH on the outcome of the Staudinger reduction in aqueous conditions. With a careful choice of the phosphine and the pH, it appears possible to control the obtained product. High pH resulted in reduction of the azide to the corresponding amine, while low pH resulted in hydrolysis to a product bearing an alcohol function. The structures of both products could be determined by 2D HSQC NMR. A negligible amount of the postulated eliminated product could be detected as well. As shown by Pawlak *et al.*[2], Staudinger reduction could be used to deprotect an epitope on cell surface, making it available for recognition by other immune cells. In order to expand this technology to other amino acids, azidoalanine could be used as a protected serine. Although, a larger amount of the eliminated product is to be expected, it would broaden the range of epitopes that can be masked by such process. **Chapter 6** describes the automated synthesis of a natural nucleopeptide, occurring in the genome of Coxsackie virus. The four necessary building blocks were made in a similar fashion, using a 7-step synthesis. Despite being non-optimized, the proposed synthesis gave access to sufficient material to initiate automated solid support synthesis of the targeted oligonucleopeptide. Although the first phosphoramidite coupling on the side chain of the immobilized peptide **17** proved to be troublesome, the use of a large excess of the nucleoside phosphoramidite in combination of BMT as a base yielded after oxidation the pentapeptide **18** as depicted in scheme **4**. Cleavage of the Lev group without migration of the TBDMS group could be achieved after extended screening of conditions. Although, performed in near neutral buffered condition, TBDMS migration has been observed in many occasions in such cis diol systems. Complete deprotection on the 3'

hydroxyl could be achieved using 75 equivalent excess of hydrazine in AcOH, pyridine and THF for 20 min to give nucleopeptide **19**.



Scheme 4. Synthesis of partially protected nucleopeptide **19** *Reagent and conditions:* i) 1) BMT, 5'-PAM-3'-Lev-2'-TBDMS uridine, ACN/Dioxane, 2) I_2, THF/Pyr/H2O, rt, 1.5 min ii) (TEA/TEA*3HF/DMF (2:3:4, v:v:v), rt, o.n

By repetitive cycles, as depicted in Scheme **5**, nucleopeptides of various sizes, **24-26,** could be synthesized and isolated. Subsequently, **24** and **25** were successfully coupled to the C-terminal peptide of the native VPg fragment to generate the rudimentary native nucleopeptides VPgpU and VPg-pUpU. The future coupling of nucleopeptide **26** to VPg-derived sequence will provide a synthetic Picornavirus nucleopeptide of unprecedented complexity.



Scheme 5. Synthesis of compounds **24** -**26**. *Reagents and conditions:* (i) Hydrazine, THF/Pyr/AcOH, rt, 20 min; (ii) **20**, **21**, **22**, **23,** BMT, dioxane/ACN, rt, 20/30/30/20 min; (iii) I_2, THF/Pyr/H2O, rt, 1.5 min; (iv) Ac_2O, MeIm, 2,6-lutidine, NMP, rt, 30 sec (v) 3% TFA/DCM, rt, 5 min.

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Résumé

Dans cette thèse est décrit le design et la synthèse de plusieurs bio-conjugués aux origines et applications diverses. Tous les composés ont en commun une chaine peptidique modifiée au moyen d'acides nucléiques, lipides ou autre fragment hétérocycliques. Décrites dans les chapitres 3 et 4, les modifications ont pu être induites par le choix des building block utilisé lors de la synthèse sur support solide. L'utilisation de méthodes plus sophistiquées telle que la chimie « click » sans catalyse au cuivre purent aussi être mises à profit comme illustré dans le chapitre 2. Une approche combinant synthèse sur support solide et block coupling permit la synthèse et l'isolation de lipopeptides fluorescent (chapitre 2) et de conjugués ARN-peptides impliqués dans la réplication de certains virus (chapitre 6). Les conjugués décrits dans cette thèse pourraient être utilisés comme modèles, références ou outils dans l'études de mécanismes intra cellulaire. En effet, les conjugués immunogéniques présentés dans les chapitres 2, 3 et 4 pourraient contribuer à l'élucidation des mécanismes liés au métabolisme des antigènes à l'intérieur des cellules dendritique ; les peptides modifiés par un azoture (chapitre 5), la compréhension du mécanisme de présentation sur la surface cellulaire.

Le **chapitre 1** recense et présente la synthèse de quelques exemples de conjugués immunogéniques ciblant les récepteurs Toll-like, reconnu comme de potentiels futur vaccins.

Le **chapitre 2** décrit la synthèse de conjugués fluorescent utilisant l'agoniste au TLR-2 Pam3Cys et un fragment antigénique. Substituant la Fmoc-Lysine(Boc) par de la Fmoc-Azidonorleucine durant la synthèse sur support solide, des précurseurs non fluorescents ont pu être isolés. Tirant profit de la méthode de couplage « Click » non catalysé au cuivre, ceux-ci purent être marqués avec plusieurs composés fluorescents. Bien que remplissant leur fonction lors des test biologiques, ces conjugués possèdent une faible solubilité en milieu aqueux. Cette propriété pourrait être altérée par l'introduction d'un fragment d'éthylène glycol dans la chaine peptidique afin d'augmenter la solubilité du conjugué.

Le **chapitre 3** décrit le design et la synthèse d'une version simplifiée des conjugués décrits dans le **chapitre 2**. Comparativement, deux chaines palmitiques purent être enlevé, tout en gardant les propriétés agonistiques du Pam3Cys. Des versions fluorescentes puent être isolées usant de méthodes similaires a celles décrites dans le **chapitre 2**. Cette simplification eut pour effet d'augmenter drastiquement la solubilité en milieu aqueux. Cependant, ces conjugués simplifiés ne possèdent plus d'activités sur les TLR-2 autre qu'humain, et de fait, peuvent compliquer les tests biologiques.

Le **chapitre 4** décrit la synthèse d'un agoniste des TLR-7. Afin de préserver l'activité, la structure n'a pas été modifiée mais un nouveau point d'ancrage a été introduit hors de la partie purine du composé. Une synthèse en 6 étapes est nécessaire à l'obtention d'un fragment utilisable lors de synthèse sur support solide. Néanmoins, du fait d'une solubilité médiocre dans la plupart des solvants, il apparait nécessaire d'effectuer un travail d'optimisation de la synthèse afin d'avoir accès a de plus larges quantités de cet agoniste.

Le **chapitre 5** décrit la synthèse d'un peptide portant un azoture et l'influence du choix de phosphine et de pH lors de la réduction. Grace a un choix précis des conditions de réaction, il apparait possible de contrôler le ratio des produits obtenu. Lorsque effectuée en milieu basique, l'amine résultant de la réduction de Staudinger sera majoritairement obtenu. Cependant, si effectué en milieu acide, l'hydrolyse de l'intermédiaire sera majoritaire et donnera lieu a un alcool en place de l'amine. Ces conclusions purent être confirmée par 2D HSQC RMN.

Le **Chapitre 6** décrit la synthèse automatique d'un nucleopeptide naturel présent dans le génome des virus Coxsackie. Les building block nécessaire ont put être synthétisé via une synthèse similaire, en 7 étapes. Bien que non optimisée, une quantité suffisante de chaque building block put être préparée afin de poursuivre le développement. Bien que le premier couplage du premier building block sur le fragment peptidique nécessite des conditions particulières, plusieurs conjugués purent être synthétisés. Afin d'éviter une migration du groupe TBDMS durant la déprotection du groupement Lev, plusieurs conditions furent testées et il apparait qu'un large excès d'hydrazine, ainsi qu'un court temps de réaction permet la déprotection complète sans qu'aucune migration ne soit observée. Deux nucleopeptides portant un ou deux building block purent être conjugués à un peptide afin de générer un fragment du VPg.

Curriculum vitae

Geoffroy Gential was born october 3rd 1988 at St priest en Jarez in France. He completed CPGE PCSI/PC course (Classe preparatoire au concours des grandes ecoles, Physique Chimie, Sciences industrielles) in 2006, and ENSCCF (École nationale supérieure de chimie de Clermont-Ferrand) in 2008. He did a first 3 month internship in 2011 at Leiden university on the synthesis of adenosine building block, then a second internship in 2011 on solution phase synthesis of oligonucleotide fragments and the design and synthesis of orthogonal adamantane based protective group. He graduated as fine and industrial organic chemistry engineer in 2011 (master degree). He pursued his studies at Leiden university as PhD student under supervision of Dmitri Filippov and Prof. Gijs van der Marel. He presented a poster about his research at symposium “design and synthesis” (October 2012 and November 2013, Lunteren) and CHAINS 2014 (2014, Veldhoven). He participated at HRSMC summer school “New vistas for organic synthesis” (June 2013, Maastricht). In June 2016, he joined Alcami in Weert as Chemist I in process development. Since December 2017, he works as Senior Scientist at Ardena (previously Chemconnection) in Oss.