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

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Chapter 4

Synthesis and immunological evaluation of NOD2/TLR2-ligand-antigen bis-conjugates

4.1 Introduction

Pattern recognition receptors (PRRs) of the innate immune system, such as TLRs and NLRs recognize specific pathogen-associated molecular patterns (PAMPs). For a number of PRRs the exact structure of the corresponding PAMP has been established. One of the most studied PRR ligands is the synthetic triacylated lipoprotein Pam_3CSK_4, an agonist of TLR2. In Chapter 2 and 3 the development of spacer containing MDP derivatives, as potential agonists of the NOD2, a prominent member of the NLR family is described. It was also investigated whether conjugates of these NOD2 ligands and the antigenic OVA-derived peptide $DEVA_5K$ exhibit an improved immunological profile as was discovered with similar conjugates of Pam_3CSK_4.[1] It turned out that a mono-conjugate in which a lipophilic MDP derivative is incorporated retained activity as NOD2 receptor ligand, was able to induce DC maturation and showed the ability to sustain antigen presentation by DCs.It is likely that for the optimal activation of the immune system activation more than one PRR of the innate immune system

needs to be triggered. However, not much is known on the joint effect of PRRs and this is particular true for the molecular details of the collaboration of PRRs.[2] It has been reported that TLR2 and NOD2 agonists can act synergistically.[3-8] It is also known that TLR2 recognizes whole peptidoglycan (PG)[9] whereas PG-fragments are recognized by NOD2. Separate activation of TLR2 and NOD2 leads to the production of the same cytokines. This suggests that their signaling can be linked and that their respective extra- and intra-cellular responses can be coordinated.[10]
Based on these findings and the results obtained with NOD2-L-antigen and TLR2-L-antigen mono-conjugates (see Chapter 2 and 3) a new class of conjugates was envisioned. These bis-conjugates comprise an antigenic peptide covalently bound to both a TLR2-L and a NOD2-L. Pam_3CSK_4 was selected as TLR2-L and MDP as NOD2 ligand. For the reason that the attachment position of the ligands to the antigenic peptide may affect the immunological activity three types of bis-conjugates are considered (*Figure 1,* A – C). In conjugate A Pam_3CSK_4 is connected to the *N*-terminus and MDP to *C*-terminus of the antigenic peptide. Conversely, in conjugate B, the MDP is positioned on the *N*-terminus and the Pam_3CSK_4 on the *C*-terminus. Conjugate C has both TLR2 and NOD2 ligands attached to the *N*-terminus of the peptide.

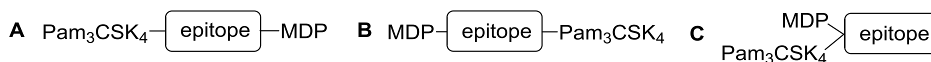


Figure 1. General design bis-conjugates A – C.

In this Chapter the synthesis and biological evaluation of conjugates **1** – **6** are described (*Figure 2*). In type A conjugate **1**, the $DEVA_5K$ peptide is elongated at the *N*-terminus with the TLR2-L, Pam_3CSK_4 while the side chain of the *C*-terminal lysine is connected with the isoglutamine of spacer containing MDP. Conversely, in conjugate **2** of type B the isoglutamine of MDP is attached to the *N*-terminus of the peptide epitope and Pam_3CSK_4 is bound to the side chain of the *C*-terminal lysine of the antigenic peptide. The type C conjugates **3** and **4** contain $DEVA_5K$ that is provided with an additional lysine at the *N*-terminus to allow conjugation of both ligands *via* either the side chain or α–amino function of this lysine. For conjugate **3** the α–amino function is elongated with the TLR2-L while the NOD2-L is connected to the side chain of lysine. In comparison with conjugate **3**, the linkages of the ligands with the epitope in conjugate **4** are reversed. In conjugates **5** and **6** the MDP derivative is connected *via* its anomeric spacer with the peptide epitope through the use of a ‘click’ reaction. For type C conjugate **5**, the use of a strained cyclooctyne at the anomeric spacer of MDP the Cu-free click reaction as conjugation method. Type B conjugate **6** is special as the MDP

moiety is provided with a lipophilic stearoyl at the 6-OH. The lipophilic MDP is conjugated to the *N*-terminus of the peptide via a standard click reaction while the TLR2-L is bound to the *C*-terminal lysine.

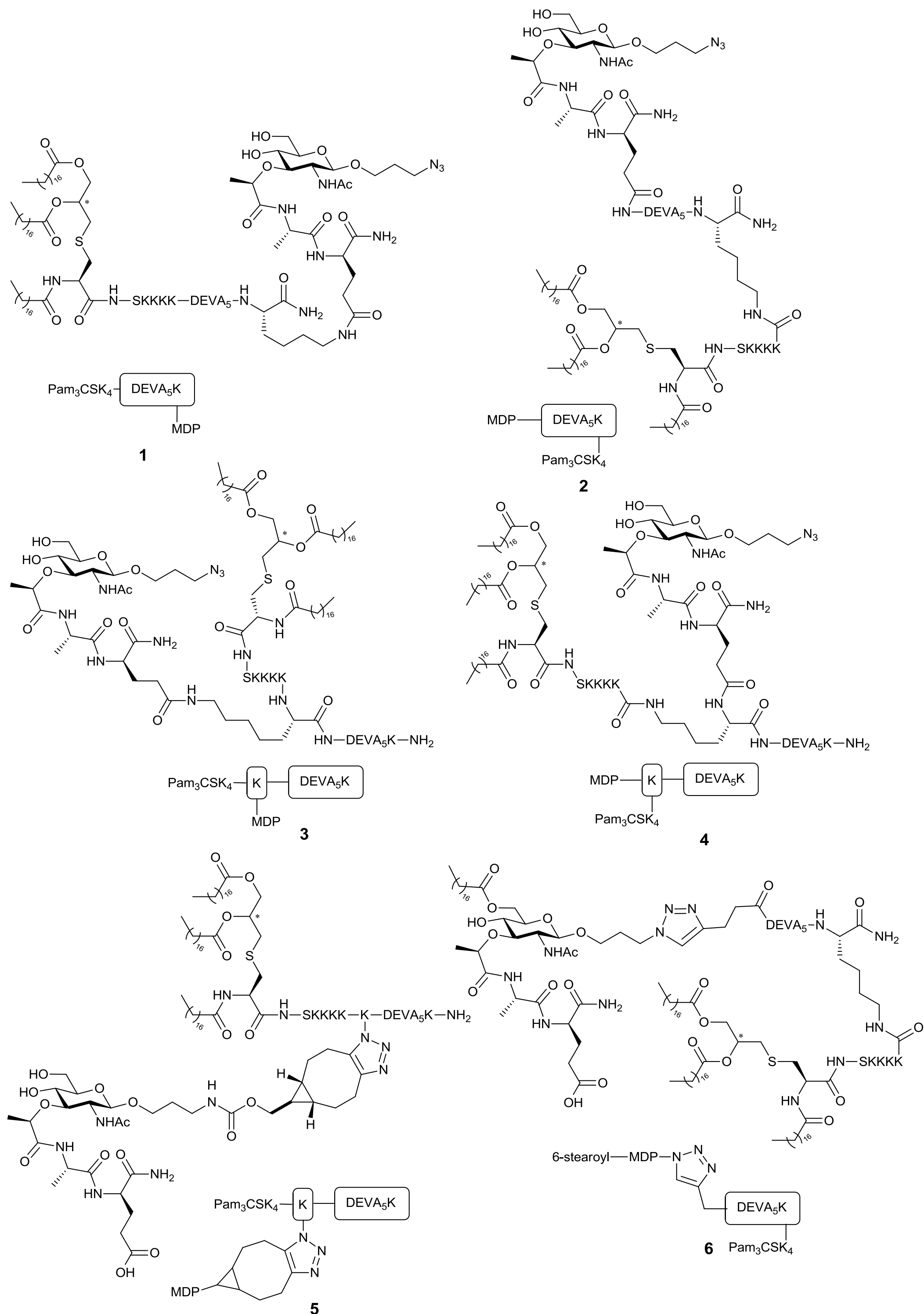


Figure 2. Target molecules **1 – 4**.

For the biological evaluation of bis-conjugates **1** – **6**, peptide epitope **21**, TLR2-L conjugates **22** and **23**, lipophilic NOD2-L conjugate **25** and MDP derivatives **24** and **26** were used as reference compounds (*Figure 3*).

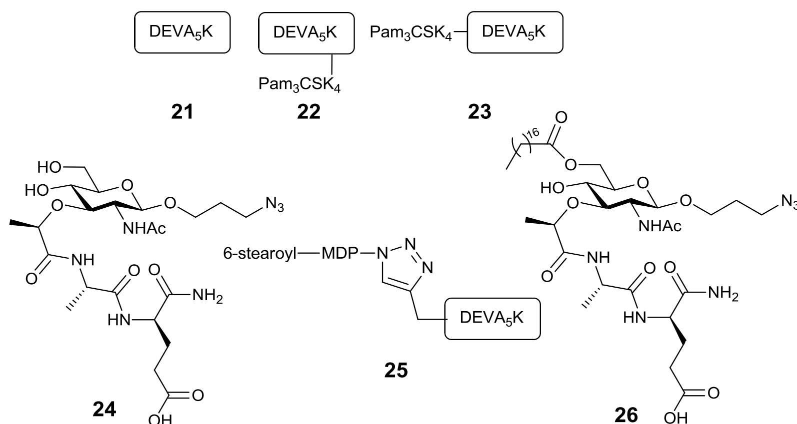


Figure 3. Reference compounds **21** – **26**.

4.2 Synthesis of NOD2-L-TLR-L-antigen bis-conjugates

The syntheses of conjugates **1** – **6** were largely carried out with an automated peptide synthesizer, using Fmoc chemistry, the condensing agent HCTU and Tentagel-S-Ram as solid support. Commercially available protected amino acids and palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH were used. The syntheses of bis-conjugates **1** – **4** are depicted in *Scheme 1* and require the availability of MurNAc derivative **9**, the preparation of which is described in Chapter 2.

The syntheses of bis-conjugates **1** and **2** started with SPPS to resin bound peptide **7**, having a *C*-terminal lysine equipped with the orthogonal and acid labile methyltrityl (Mtt) protective group allowing *C*-terminal extensions at a later stage of the synthesis. To obtain conjugate **1**, TLR2-L Pam_3CSK_4 was attached to the *N*-terminus by elongation of peptide **7** with the SKKKK motive using a standard SPPS cycle, followed by a manual coupling with palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH under influence of PyBOP and DiPEA resulting in immobilized peptide **8**.

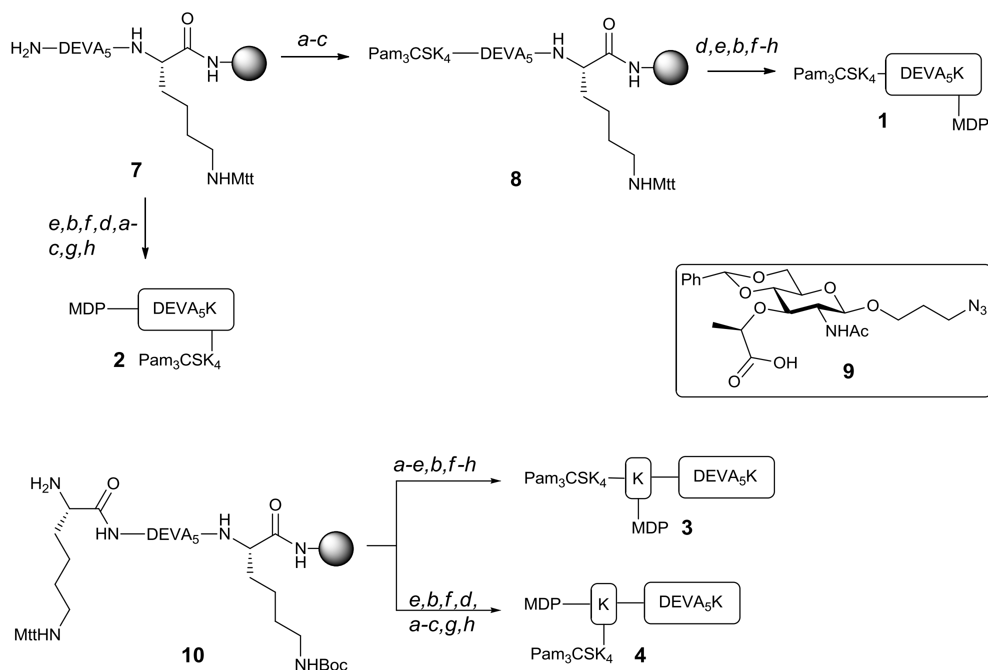
Subsequently, immobilized peptide **8** was treated with a solution of 3% TFA in DCM to selectively remove the Mtt protective group at the side chain of the *C*-terminal lysine. To install the NOD2-L at this position, the free amine was consecutively elongated with Fmoc-isoglutamine, Fmoc-Ala-OH and MurNAc derivative **9**. The coupling of **9** was executed twice using HATU instead of HCTU as condensing agent. Conjugate **1** was simultaneously deprotected and cleaved from the resin with a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 60 minutes. The conjugate was precipitated with diethyl ether and purified by RP-HPLC yielding 2.5 mg conjugate **1** in 2% overall yield.

To synthesize bis-conjugate **2**, the NOD2-L was appended at the *N*-terminus of immobilized **7** by the above-described elongation with Fmoc-isoglutamine, Fmoc-Ala-OH and MurNAc **9**. Next the Mtt protective group at the side chain of the *C*-terminal lysine was selectively removed and the released amino group was extended with Pam_3CSK_4 by the same sequence of reactions as described for conjugate **1**. The thus obtained immobilized **2** was cleaved from the solid support, deprotected and purified using the conditions as described for conjugate **1** to give 0.94 mg conjugate **2** in 1% overall yield.

The routes of synthesis to conjugates **3** and **4** have the immobilized peptide **10,** prepared by standard Fmoc SPPS, in common. The side chain of the *N*-terminal lysine in **10** is protected with the orthogonal Mtt protective group, permitting the successive addition of the TLR2 and NOD2 ligands. The sequence of reactions to install both the TLR2-L and NOD2-L in **3** and **4** was adopted from the synthesis of conjugate **1**. To obtain **3**, Pam_3CSK_4 was added to the free α amine of **10**, followed by selective removal of the Mtt and appendage of NOD2-L at the side chain of lysine. To obtain conjugate **4**, NOD2-L was added to the free α amine of **10**, followed by selective removal of the Mtt and attachment of Pam_3CSK_4 at the side chain of lysine. Acid treatment of the immobilized and protected precursors of **3** and **4** led, after precipitation and purification, to target conjugates **3** and **4**, both in 1% overall yield.

The relatively low overall yields obtained for the syntheses of conjugates **1** – **4** can be explained by the concurrent hydrolysis of the azidopropyl group at the anomeric center of glucosamine of the NOD2-L. Apart from this loss, the hydrolyzed side product hampered the final purification because of a small difference in retention time between the target conjugates **1** – **4** and their corresponding side products.

Scheme 1. Synthesis of conjugates **1** – **4**.

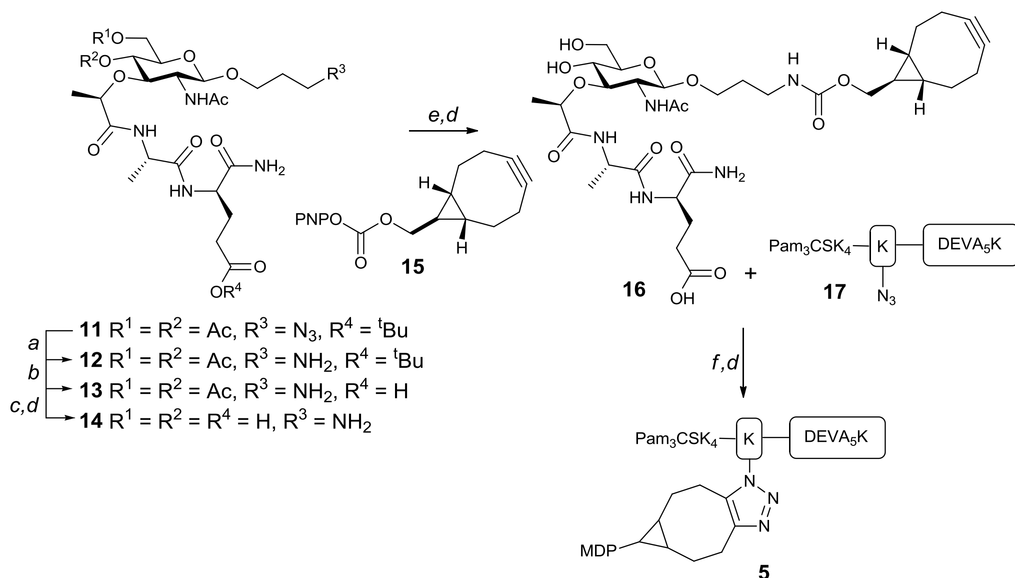


Reaction conditions: *a*) Fmoc SPPS cycle for SK_4; *b*) 20% piperidine, NMP; *c*) palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, PyBOP, DiPEA; *d*) 3% TFA, DCM; *e*) Fmoc SPPS cycle Fmoc-Ala-iGlu(NH_2)-OH; *f*) **9**, HATU, DiPEA, NMP; *g*) 95% TFA, 2.5% TIS, 2.5% H_2O; *h*) RP-HPLC, yield conjugates: **1**) 2.5 mg, 2%, **2**) 0.94 mg, 1%, **3**) 1.2 mg, 1%, **4**) 1.5 mg, 1%.

For the assembly of conjugate **5** the Cu-free click reaction was applied as conjugation method to circumvent the hydrolysis of the anomeric linked MDP-moiety (*Scheme 2*).[11,12] This reaction was executed after the SPPS procedure by reaction of purified TLR2-L-antigen conjugate **17** with purified NOD2-L **16**, equipped with a strained cyclooctyne. In this manner it is circumvented that the acid sensitive NOD2-L is subjected to the acidic conditions required for deprotection and cleavage at the end of the SPPS procedure. The synthesis of **16** started with MDP derivative **11**, the preparation of which is described in Chapter 2. Staudinger reduction of the azide in **11** to give amine **12** was followed by removal of the *tert*-butyl ester by treatment of **12** with 20% TFA in DCM to give compound **13** in 90% yield. Finally the acetyl groups in **13** were cleaved by treatment with a solution of ammonia in methanol (4M) for 18 hours. Purification by RP-HPLC furnished unprotected MDP **14** in 41%. Next the strained cyclooctyne moiety, described by van Delft *et al.*,[12] was installed with the aid of active ester **15**, prepared from the corresponding free alcohol and an excess of 4-nitrophenyl chloroformate, to give key building block **16**. To this end ester **15**

was added portions wise to **14**, using pyridine as base to give after RP-HPLC purification alkyne functionalized MDP **16** in 22%. The SPPS of conjugate **17** started with the elongation of peptide **7** with Fmoc–Lys(N_3)–OH[13] to introduce the azide needed for the click reaction. The subsequent attachment of Pam_3CSK_4 was performed as described for the conjugates described above. TLR2-L conjugate **17** was obtained in 3% yield after RP-HPLC purification. The Cu-free click reaction between **16** and **17** was executed in MeCN : ^{t}BuOH : H_2O (1 : 1 : 1), a solvent mixture chosen to dissolve **17**. Lipopeptide **17** was added to a solution of MDP derivative **16** (0.07 M and 1.75 equivalents). The reaction was monitored by mass spectrometric analysis and after 24 hours another 1.75 equivalents of **16** were added to drive the reaction to completion. After an additional 24 h the reaction was diluted and subsequently purified by RP-HPLC resulting in compound **5** in 10% yield.

Scheme 2. Synthesis of conjugate **5**.

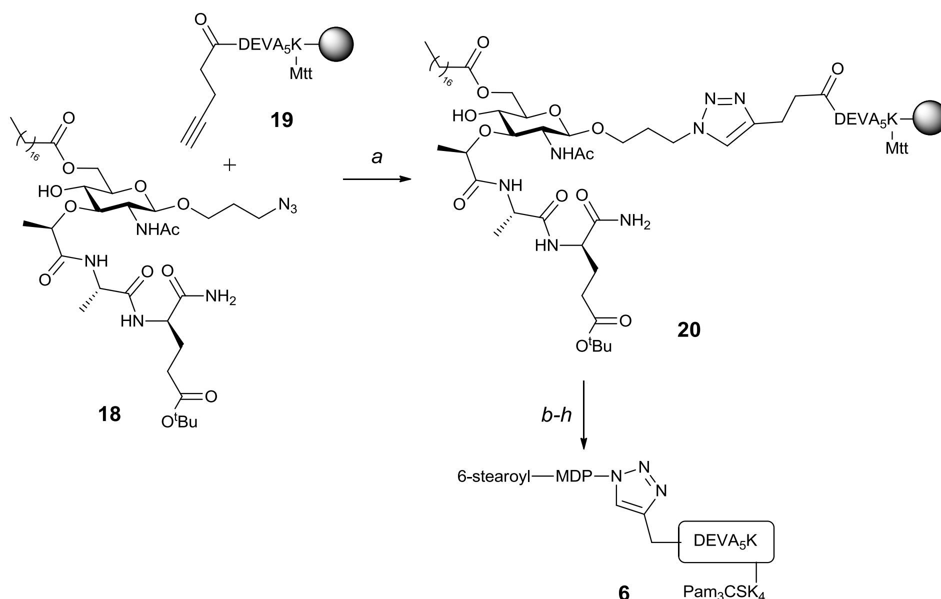


***Reaction conditions**: a*) Me_3P, DMF/THF, quant.; *b*) 20% TFA, DCM, 90%; *c*) ammonia in MeOH; *d*) RP-HPLC, **14**) 41%, **16**) 22%; **5**) 0.22 mg, 10%; *e*) **15**, DMF, pyridine; *f*) MeCN, H_2O, ^{t}BuOH, 2d.

Chapter 3 describes that mono-conjugates comprising the $DEVA_5K$ epitope and a lipophilic NOD2-L exhibit an improved immunological activity with respect to non-lipophilic counterparts. This finding was an incentive for the design and synthesis of bis-conjugate **6**, having a stearoyl moiety at the C-6-OH of the NOD2-L (*Scheme 3*). The copper mediated click reaction was selected to link lipophilic MDP derivative **18** (the synthesis of which is described in Chapter 3) and alkyne functionalized immobilized peptide **19**, prepared by condensation of

immobilized **7** with pentynoic acid. Guided by the assumption that the stearoyl group in the lipophilic MDP derivative **18** would suppress unwanted glycosidic bond hydrolysis and that palmitoylated cysteine in the TLR2-L would not be beneficial for the copper mediated click reaction, the following route of synthesis to conjugate **6** was executed: Azide **18** was reacted with alkyne resin **19** in the presence of copper sulfate and sodium ascorbate for 5 days at 50°C (monitored by LC-MS) to give immobilized **20**. The free 4-*O*-hydroxyl in the glucosamine moiety of **20** requires protection to prevent side reactions during the installation of Pam_3CSK_4. The standard capping step with Ac_2O and DiPEA is prohibited by the presence of the stearoyl at the 6-OH and protection with a Boc-group was selected. Therefore resin **20** was treated with a solution of Boc_2O (1 M) and DiPEA (0.03 M) in NMP for one hour. Next, the Mtt group at the *C*-terminus was selectively removed followed by the addition of Pam_3CSK_4 as described for the other conjugates. Target conjugate **6** was obtained in 1% yield after the regular deprotection and purification procedure, as described above for conjugates **1** and **2**.

Scheme 3. Synthesis of conjugate **6**.



Reaction conditions: *a*) 10% $CuSO_4$, 1 eq sodium ascorbate, DMF, 50 °C, 5d, rt; *b*) 1M Boc_2O, 2eq DiPEA, NMP 1h; *c*) 3% TFA, DCM; *d*) SPPS SK_4 with Boc_2O/DiPEA capping; *e*) 20% piperidine, NMP; *f*) palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, PyBOP, DiPEA, NMP; *g*) 95% TFA, 2.5% TIS, 2.5% H_2O; *h*) RP-HLPC, 1.3 mg, 1%.

4.3 Immunological evaluation of NOD2/TLR-L-antigen bis-conjugates

The immunostimulatory properties of bis-conjugates **1** – **6** were determined with the assays described in Chapters 2 and 3, namely, NOD2 activity in transfected HEK cells, maturating capacity in DCs, TLR2 specificity in wild-type (WT) and TLR2 knockout (KO) DCs and antigen presentation. The NOD2 specificity of the conjugates is probed in a stable HEK293 cell line transfected with NOD2 by measuring the interleukin IL-8 production. The maturating capacity of the conjugates is assessed using murine D1 DCs by quantifying IL-12p40 production. To determine whether TLR2 is involved in sensing of the constructs both a WT and a TLR2-KO bone marrow derived dendritic cell (BMDC) line are used, again measuring the induced IL-12p40 production. The levels of antigen presentation by DCs exposed to the conjugates is assessing using a $CD8^+$ T cell hybridoma assay equipped with a β-galactosidase construct to measure the levels of antigen presentation colorimetrically.

The bis-conjugates **1** – **6** were administered to NOD2 transfected HEK cells to test the potency of the conjugates to activate the NOD2 receptor. NOD2 ligand **24** and TNFα were used as a positive control (*Figure 3 A and B*) and NOD2 mono-conjugates (known to be inactive, Chapter 2) were used as a negative control (data not shown). The conjugates **1** – **6** were measured in two separate assays depicted in *Figure 3A* and *3B*. As shown in *Figure 3A* NOD2/TLR2-conjugates **1** – **4** expressed certain level of NOD2 immunostimulatory activity. Conjugate **2** and **4** proved to be most active whereas compounds **1** and **3** were roughly as active as **24**. These results contrast those reported in Chapter 2 in which the MDP mono-conjugates were inactive as NOD2 agonists. The differences in activity observed between the Pam_3Cys containing MDP-peptide conjugates and conjugates containing only MDP indicate that a better uptake of the former. The presence of the lipophilic TLR2 ligand may be responsible for the increase in NOD2 immunostimulatory capacity of the bis-conjugates. These encouraging results did not translate, unfortunately, to bis-conjugate **6** containing a lipophilic derivative of MDP in addition to Pam_3Cys. As shown in *Figure 3B*, compound **6** is a poorer NOD2 agonist than the parent conjugate **25** containing 6-stearyl-MDP only. Such loss of activity may be a result of insufficient solubility of the construct **6** or other unknown effects. It can be inferred from a comparison of the NOD2 stimulating activity of **6** that the conjugation method does not improve the potency of the conjugate. The activity of bis-conjugate **5** is comparable to the activity of **24**.

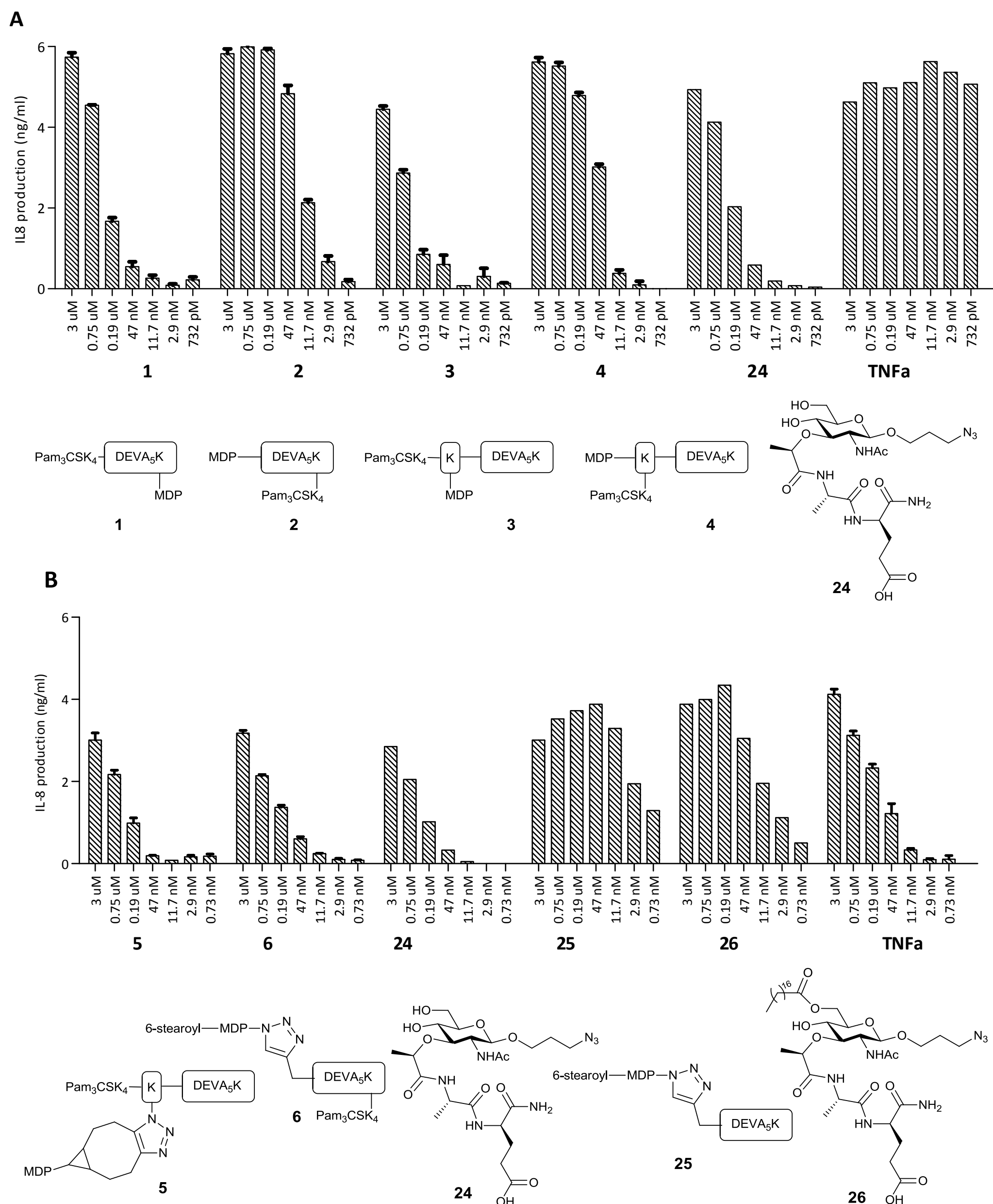


Figure 3. NOD2 immunostimulatory potency of conjugates (transfected HEK-cells) A) **1** – **4**; B) **5** and **6** and reference compounds **24** – **26**.

Next, bis-conjugates **1** – **6** were tested in a D1 DC maturation assay using the IL-12p40 production as the read out (*Figure 4 A – C*). As evident from *Figure 4 A, B* and *C* conjugates **1** – **5** gave rise to cytokine production similar to TLR2-L-conjugates **22** and **23** and only subtle differences in maturation capacity were observed between different conjugates. Therefore no additional effect of the NOD2 ligand was apparent. Notably, conjugate **6** containing lipophilic 6-stearoyl MDP proved to be significantly less active than **22** and **23**, showing approximately 15 times lower induction of IL-12 at 12 nM (*Figure 4C*). Conjugates **3** and **4** are of equal potency and both are more active than conjugate **1**. As is apparent from *Figure 4A* and *4C* that the best results were obtained when MDP is attached at the *N*-terminus of the peptide and Pam_3CSK_4 is connected to the side chain of Lys of the peptide (**2** and **3**). It can be also surmised from the comparison of **5** and **3** presented in *Figure 4B* that the bulky linker in conjugate **5**, which resulted from the addition of azide to cycloalkyne in the 'click'-conjugation step, does not have a negative effect on the potency of the construct. Compounds **3** and **5** proved to be equally active.

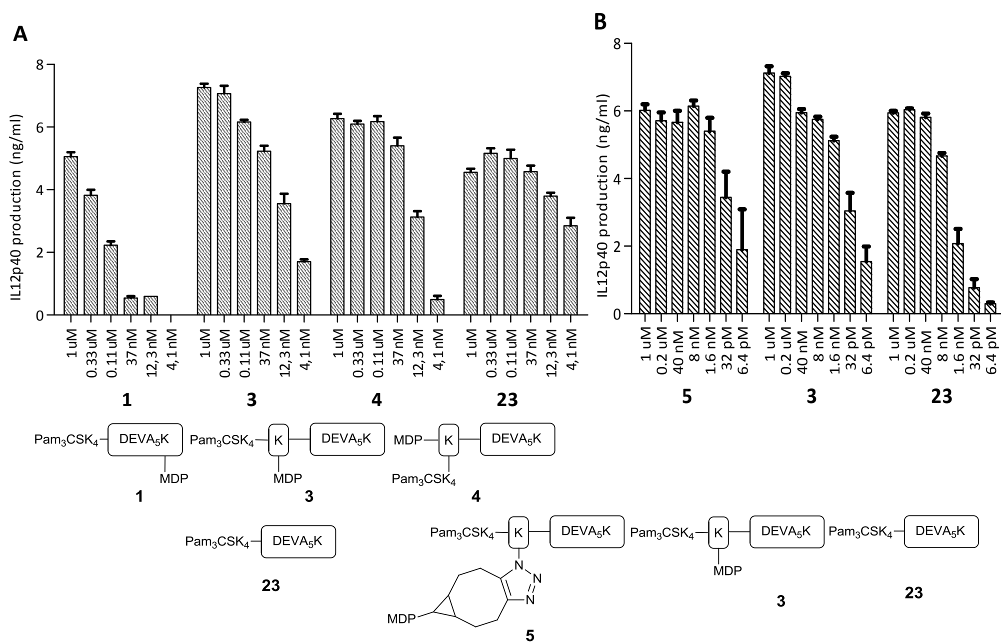
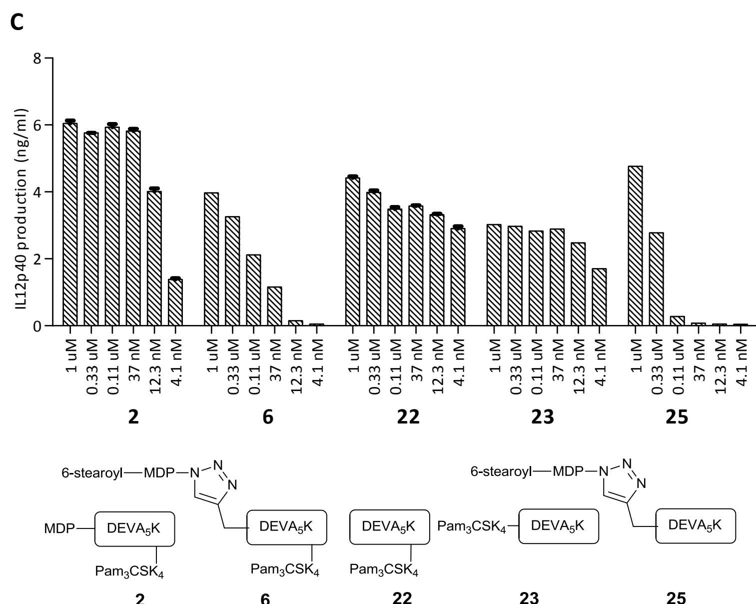


Figure 4. DC maturation of A) conjugates **1**, **3**, **4** B) conjugates **3** and **5** and corresponding references.



Continue Figure 4. DC maturation of C) conjugates **6** and **3** and corresponding references.

In an attempt to detect an effect of the MDP-moiety on the immunostimulatory capacity of the bis-conjugates, constructs **1** – **6** to activate DCs, activation of wild type and TLR2 KO (*Figure 5 A and B*) was tested. Stimulatory activity found in the TLR2-KO cells can be attributed to stimulation of NOD2. The conjugates **22** – **26** and Pam_3CSK_4 and CpG[14] were used as reference compounds. The amount of produced IL-12 was determined at two different concentrations of conjugates. *Figure 5A* depicts the activity of the compounds at 0.3 uM and *Figure 5B* at 0.75 µM. It can be concluded that all Pam_3Cys based conjugates (**1** - **3**, **5**, **6**, **22**, **23**) show maturating activity in the WT BMDC (white bars) at both concentrations, although to a different extent. In turn, the constructs containing only a NOD2 ligand (**24**, **25**, **26**) proved to be inactive. The lack of activity in the TLR2-KO BMDC (black bars) for any of the tested compounds demonstrates that the immunostimulatory effect of the bis-conjugates is indeed predominantly TLR2 dependent. It is noteworthy that at 0.75 µM bis-conjugates **2**, **3**, **5** and **6** showed higher maturating capacity than the constructs containing TRL-2 ligand alone (**22** and **23**). The latter finding points to a possible synergistic effect between NOD2 and TLR2 receptor ligands.

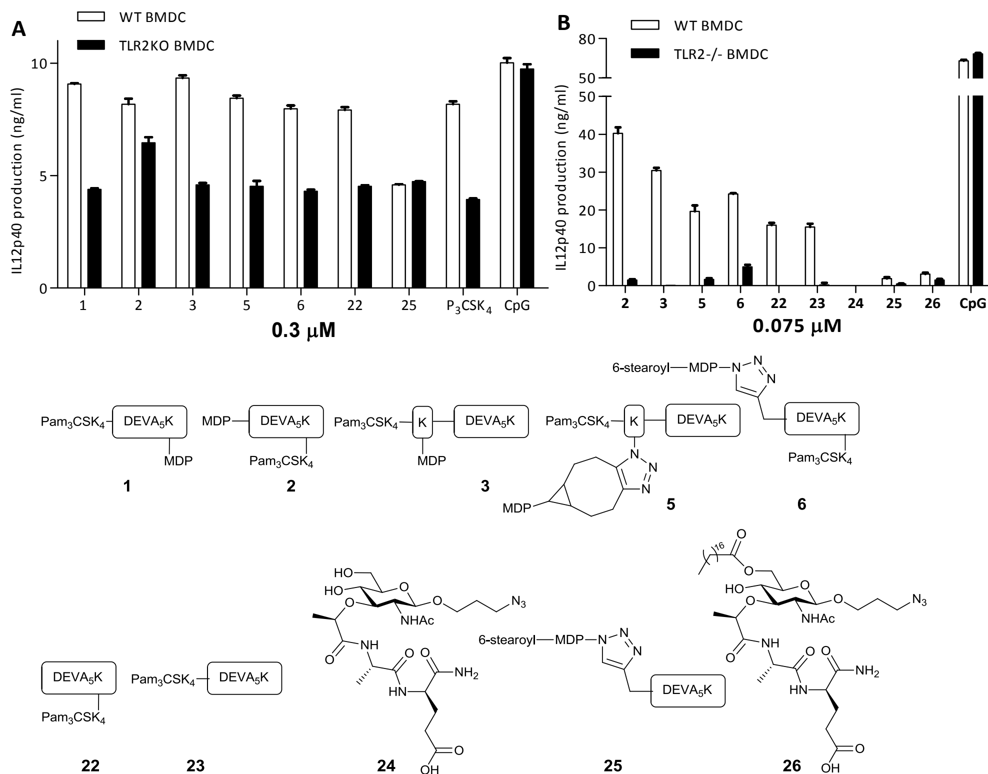


Figure 5. IL-12p40 production by TLR2 knockout BMDC and WT BMDC of A) conjugates **1**, **3** – **6** at 0.3 µM**;** B) conjugates **2**, **3**, **5**, **6** at 0.075 µM and corresponding reference compounds **22** – **26** and Pam_3CSK_4.

Finally, bis-conjugates **1** – **6** were tested with respect to their ability to sustain MHC class I antigen presentation in DCs (*Figure 6 A and B*). Peptide **21** was used as a positive control and conjugates **22**, **23** and **25** were used as relevant references. All tested conjugates showed detectable antigen presentation at the concentrations of 0.2 µM and higher, except for **1**, which showed virtually no antigen presentation below 0.56 µM. None of the tested compounds outperformed the reference conjugates **22** and **23**, which contain only a TLR2 ligand, although conjugates **2** and **6** came close to the presentation level of the references (60 to 75%, as compared to **22**, at 37 nM). Interestingly, in terms of antigen presentation no significant difference is observed between conjugate **5** and **3** (*cf*. *Figure 6B*), this indicates that the presence of cycloalkyl moiety in **5** is of no consequence for the biological activity of the construct. It can be concluded that compound **2**, which contains MDP at the *N*-terminus of the peptide and Pam_3CysSK_4 at the side chain of the *C*-terminal lysine, is the best bis-conjugate in this assay.

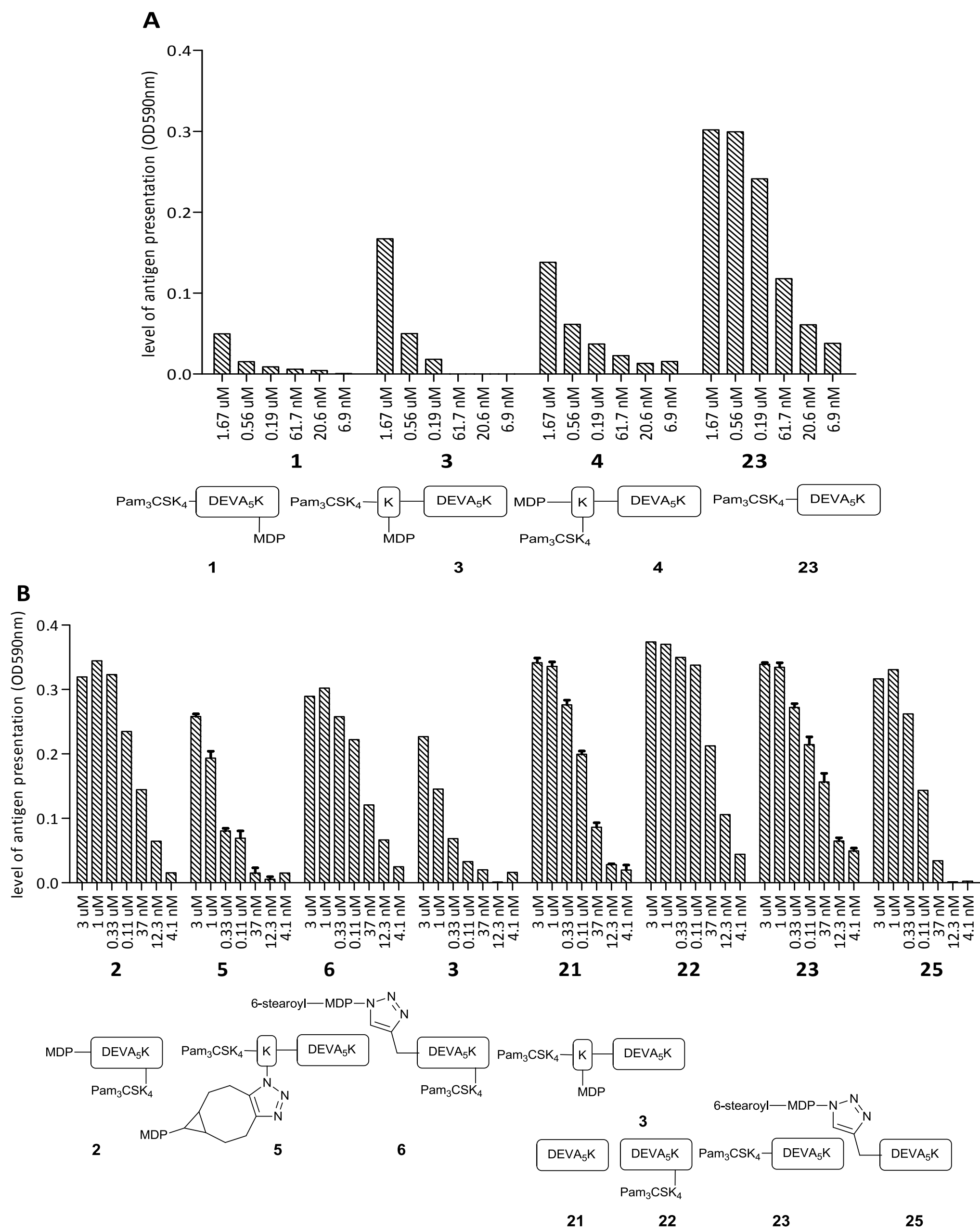


Figure 6. Antigen presentation of A) conjugates **1**, **3**, **4**; B) conjugates **2**, **3**, **5** and **6** and corresponding reference compounds. **21** – **23** and **25**. .

Collectively the presented biological data indicate that all synthesized bis-conjugates **1** – **6** are recognized by the NOD2 receptor and are able to facilitate MHC class I antigen presentation *in vitro*. Furthermore, the conjugates were found to mature dendritic cells already at nanomolar concentrations. There is an indication that a positive or additive effect between the TLR2-L and NOD2-L with respect to the maturation is observed on the primary DC line (BMDC).

4.4 Conclusion

This Chapter described the synthesis and immunological evaluation of conjugates **1** – **6** containing both a NOD2-L and a TLR2-L. The hoped-for synergistic effect of the two PRR ligands attached to the same antigenic peptide was not clearly shown with respect to DC-maturation potency and antigen presentation ability of the constructs. The immunostimulatory capacity of the studied conjugates seems mostly TLR2 driven, notwithstanding the observation of somewhat higher DC-maturation capacity of the bis-conjugates in comparison to the TLR-L-conjugates in the BMDC assay. Overall, compound **2** seems to be the best conjugate in terms of the immunostimulatory potency and antigen presentation. This conjugate therefore is the most promising construct to be studied in the future.

4.5 Experimental section

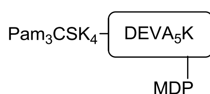
All reagents and solvents used in the solid phase peptide synthesis were purchased from Bachem and Biosolve and used as received. The analyses were performed as described in the experimental of Chapter 2.

General procedure for automated solid phase synthesis
For the detailed description about the solid phase synthesis see the experimental of Chapter 2.

General procedure coupling Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH
Swollen 25 µmol resin loaded with peptide as mentioned was treated with a stock solution of palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (0.18 M)and PyBOP (0.22 M) in DCM : NMP (2 : 1, 1 mL). The resulting mixture was treated with 25 µmol DiPEA at t = 0 and t = 15 min. The resulting suspension was reacted by shaking for 18 h followed by NMP and DCM wash.

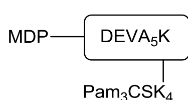
General procedure for cleavage from the resin, deprotection and purification
For the cleavage and deprotection of the conjugates described in this chapter a reaction time of 60 min instead of 104 min was used. To purify the conjugates, all the precipitates were dissolved in a 1 : 1: 1 mixture of MeCN, ^{t}BuOH and H_2O and sonificated for 15 minutes. The mixtures were centrifuged and filtered before injection on the RP-HPLC. A detailed description about the cleavage from the resin, deprotection and purification by RP-HPLC is reported in the experimental of Chapter 2.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(*i*-D-Glu-Ala-MurNAc)-NH$_2$ (1)



25 µmol of crude H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(O^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt)-Tentagel-S-Ram was elongated with Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard HCTU/Fmoc chemistry concluding in final Fmoc removal with a solution of 20% piperidine in NMP (4 x 3 min). The resin was treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH in the presence of PyBOP and DiPEA in NMP : DCM (1 mL) for 18 h. The resin was washed (NMP, DCM) and treated with a solution of 3% TFA in DCM. The resin was elongated with standard Fmoc/HCTU chemistry for Fmoc-*i*-D-Gln(OH)-NH$_2$, Fmoc-Ala-OH. The synthesis was completed with a double coupling of a two-fold excess of compound **9** pre-activated with HATU and DiPEA. To conclude, the resin was treated with a standard cleavage cocktail for 60 minutes and precipitated with Et_2O. After purification by RP-HPLC title compound **1** was obtained in 2.52 mg (0.55 µmol, 2.2%); LC/MS: Rt = 6.99 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4595.75 $[M+H]^+$; HRMS Calcd. for $[C_{215}H_{374}N_{46}O_{60}S_1 + H]^{4+}$ 1149.69245, found 1149.69466.

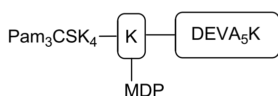
MurNAc-Ala-*i*-D-Gln-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH$_2$ (2)



25 µmol of crude H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt)-Tentagel-S-Ram was elongated with standard Fmoc/HCTU chemistry for Fmoc *i*-D-Gln(OH)-NH$_2$, Fmoc-Ala-OH. The synthesis was completed with a double coupling using a two-fold excess of compound **9** (pre-activated with HATU and DiPEA). The resin was treated with a cleavage cocktail of 3% TFA in DCM and was elongated with Fmoc-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc). The resin was treated

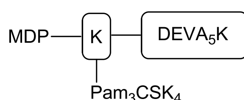
with a solution of 20% piperidine in NMP (4 x 3 min). The synthesis was proceeded by treating the resin with a solution of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH in NMP : DCM (1 : 1) in the presence of PyBOP and DiPEA for 18 h. The resin was washed (NMP, DCM) and treated with the previously described peptide cleavage and deprotection conditions for 60 minutes. Purification of the precipitated peptide by RP-HPLC resulted compound **2** (0.94 mg, 0.20 µmol, 0.8%); LC/MS: Rt = 7.06 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4595.75 $[M+H]^+$; HRMS Calcd. for $[C_{215}H_{374}N_{46}O_{60}S_1 + H]^{3+}$ 1532.58750, found 1532.58780.

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



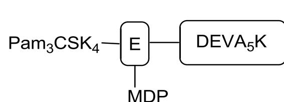
25 µmol of crude H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc)-Tentagel-S-Ram was elongated with Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Mtt)-OH according to described HCTU/Fmoc chemistry followed by a coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH in the presence of PyBOP and DiPEA. The resin was treated with a cleavage cocktail of 3% TFA in DCM and was elongated with standard Fmoc/HCTU chemistry for Fmoc *i*-D-Gln(OH)-NH_2 and Fmoc-Ala-OH. The synthesis was completed with a double coupling using a two-fold excess of compound **9** pre-activated with HATU and DiPEA. The resin was treated with the previously described cleavage and deprotection method for 60 min. Purification by RP-HPLC resulted in compound **3**. (1.2 mg, 0.25 µmol, 1.1%); LC/MS: Rt = 6.27 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4723.84 $[M+H]^+$; HRMS Calcd. for $[c_{221}H_{386}N_{48}O_{61}S_1 + H]^{3+}$ 1575.28582, found 1575.28635.

MurNAc-Ala-*i*-D-Gln-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4)



25 µmol of crude N-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc)-Tentagel-S-Ram was elongated with standard Fmoc/HCTU chemistry for Fmoc-Lys(Mtt)-OH, Fmoc-*i*-D-Gln(OH)-NH_2 and Fmoc-Ala-OH. The resin was elongated with a double coupling using a two-fold excess of compound **9** pre-activated with HATU and DiPEA. The resin was treated with a cleavage cocktail of 3% TFA in DCM and the resin was elongated with Fmoc-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc). The resin was treated with a solution of 20% piperidine in NMP. The synthesis was continued by treating the resin with a coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH in the presence of PyBOP and DiPEA. After the wash steps (NMP, DCM) the resin was treated with the previously described cleavage and deprotection cocktail for 60 min. Purification by RP-HPLC resulted compound **4**. (1.5 mg, 0.28 µmol, 1.1%); LC/MS: Rt = 6.55 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4723.84 $[M+H]^+$; HRMS Calcd. for $[c_{221}H_{386}N_{48}O_{61}S_1 + H]^{3+}$ 1575.62004, found 1575.62140.

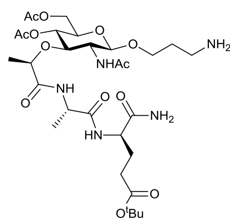
Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Glu(MurNAc-Ala-i-d-Gln)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (5)



Fifty µMol H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(^{t}Bu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) was treated with a mixture of compound **16** (88 mg, 85 µmol), HATU (33 mg, 85 µmol) and DiPEA (40 µL, 0.25 mmol) in NMP (0.5 mL) for 18 h. The resin was washed (NMP, DCM) and treated with a solution of 20% piperidine in NMP. The resin was washed (NMP, DCM, Et_2O) and 25 µmol resin was

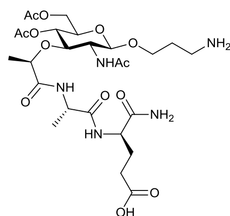
elongated with standard Fmoc/HCTU chemistry for Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc). The resin was washed (NMP, DCM) and treated with a solution of 20% piperidine in NMP (4 x 3 min). After the final Fmoc deprotection the sequence was elongated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH following standard procedures. Treatment of the resin with previously described cleavage and deprotection conditions for 60 min and precipitation method resulted in a precipitate of the crude conjugate. The crude conjugate (46 mg) was dissolved in AcOH (600 µL) and diluted with pyridine (900 µL). The mixture was reacted for 1 h with hydrazine hydrate (36 µL, 0.5 M). The sample was diluted 1 : 2 (reaction mixture : (^{t}BuOH : H_2O : MeCN)) and purified without further workup treatment. Purification by RP-HPLC resulted in compound **5**. (3.1 mg, 0.66 µmol, 3%); LC/MS: Rt = 6.44 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4698.80 $[M+H]^+$; HRMS Calcd. for $[C_{220}H_{383}N_{45}O_{63}S_1 + H]^{3+}$ 1566.93852, found 1566.93869.

3-Aminopropyl-2-*N*-acetamide-4,6-di-*O*-acetyl-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (12)



Compound **11** (1.1 g, 1.5 mmol) was co-evaporated with DMF (3x) and dissolved in 1.5 : 1 mixture of DMF : THF (16 mL, 0.1 M). The solution was treated for 3h with PMe_3 (3 mL, 1 M solution in THF) and concentrated in vacuo. Title compound was obtained in quantitative yield with traces of PMe_3O. ^{1}H NMR (400 MHz, MeOD) δ 4.97 (t, *J* = 9.5 Hz, 1H, CH, H-4), 4.50 (d, *J* = 8.4 Hz, 1H, CH, H-1), 4.44 – 4.31 (m, 1H, CH, α *i*-D-Gln), 4.26 (dd, *J* = 12.2, 4.6 Hz, 1H, CH_2, H-6), 4.20 – 4.16 (m, 1H, CH, Ala), 4.13 – 4.05 (m, 2H, CH_2, H-6, CH, lactic acid), 3.95 – 3.86 (m, 2H, CH_2, $C_3H_6NH_2$, CH, H-2), 3.75 (t, *J* = 9.2 Hz, 1H, CH, H-3), 3.71 – 3.64 (m, 1H, CH, H-5), 3.61 – 3.58 (m, 1H, CH_2, $C_3H_6NH_2$), 2.81 – 2.78 (m, 2H, CH_2, $C_3H_6NH_2$), 2.38 – 2.30 (m, 2H, CH_2, γ *i*-D-Gln), 2.27 – 2.16 (m, 1H, CH_2, β *i*-D-Gln), 2.12 (s, 3H, CH_3, Ac), 2.10 (s, 3H, CH_3, Ac), 2.02 – 1.84 (m, 4H, CH_3, NAc, CH_2, β *i*-D-Gln), 1.82 – 1.71 (m, 2H, CH_2, $C_3H_6NH_2$), 1.50 – 1.39 (m, 12H, CH_3, ^{t}Bu, CH_3, lactic acid), 1.29 (d, *J* = 6.6 Hz, 3H, CH_3, Ala); ^{13}C NMR (101 MHz, MeOD) δ 174.2 (C=O), 173.3 (C=O), 172.8 (C=O), 172.2 (C=O), 171.7 (C=O), 170.7 (C=O), 169.7 (C=O), 100.3 (CH, C-1), 80.5 (C_q, ^{t}Bu), 79.1 (CH, C-3), 77.5 (CH, lactic acid), 71.1 (CH, C-5), 69.2 (CH, C-4), 67.2 (CH_2, $C_3H_6NH_2$), 61.9 (CH_2, C-6), 55.2 (CH, C-2), 51.7 (CH, α *i*-D-Gln), 49.1 (CH, Ala), 38.0 (CH_2, $C_3H_6NH_2$), 31.2 (CH_2, γ *i*-D-Gln), 30.5 (CH_2, $C_3H_6NH_2$), 27.3 (C_q, ^{t}Bu), 26.3 (CH_2, β *i*-D-Gln), 22.0 (CH_3, NAc), 20.1 (CH_3, Ac), 19.9 (CH_3, Ac), 18.2 (CH_3, lactic acid), 16.3 (CH_3, Ala); IR (cm^{-1}): 3400, 1732, 1653, 1539; LC/MS: Rt = 4.75 min (Alltima C_{18}, 10 –90% MeCN, 15 min run); HRMS Calcd. for $[C_{30}H_{51}N_5O_{13} + H]^+$ 690.35561, found 690.35571.

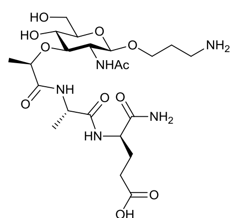
3-Aminopropyl-2-*N*-acetamide-4,6-di-*O*-acetyl-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (13)



Compound **12** (0.35 g, 0.5 mmol) was dissolved in DCM (3 mL, 0.1 M) and treated with 20% TFA (0.7 mL) for 3 h. The title compound was obtained by precipitation using Et_2O and re-crystallized ($CHCl_3$: MeOH : Et_2O) in 90% (0.3 g, 0.45 mmol). R_f = 0.15 (8 : 2 $CHCl_3$: MeOH); $[\alpha]^D$= -5.19 (c = 0.27, DCM : MeOH); ^{1}H NMR (400 MHz, MeOD) δ 4.97 (t, *J* = 9.6 Hz, 1H, CH, H-4), 4.41 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.38 – 4.34 (m, 1H, CH, α *i*-D-Gln), 4.32 – 4.18 (m, 2H, CH_2, H-6, CH, lactic acid), 4.16 – 4.05 (m, 2H, CH_2, H-6, CH, Ala), 4.01 – 3.84 (m, 2H, CH_2, $C_3H_6NH_2$, CH, H-2), 3.77 – 3.60 (m, 4H, CH, H-3, CH, H-5, CH_2, $C_3H_6NH_2$), 3.03 (t, *J* = 6.3 Hz, 2H, CH_2, $C_3H_6NH_2$), 2.37 (t, *J* = 7.5 Hz, 2H, CH_2, γ *i*-D-Gln), 2.26 – 2.14 (m, 1H, CH_2, β *i*-D-Gln), 2.11 (s, 3H, CH_3, Ac), 2.05 (s, 3H, CH_3, Ac), 1.98 – 1.82 (m, 4H, CH_3, NAc, CH_2 β *i*-D-Gln), 1.40 (d, *J* = 7.1 Hz, 3H, CH_3, lactic acid), 1.28 (d, *J* = 6.7 Hz, 3H, CH_3, Ala); ^{13}C NMR (101 MHz, MeOD) δ 175.8 (C=O), 175.7 (C=O), 174.8 (C=O), 174.6 (C=O), 173.7 (C=O), 172.0 (C=O), 171.1 (C=O), 102.3 (CH, C-1), 80.2 (CH, C-3), 78.8 (CH, lactic acid), 72.6 (CH, C-5), 70.8 (CH, C-4), 68.3 (CH_2, $C_3H_6NH_2$), 62.8 (CH_2, C-6), 56.2 (CH, C-2), 53.2 (CH_2, α *i*-D-Gln), 50.4 (CH, Ala), 39.0 (CH_2, $C_3H_6NH_2$), 30.7 (CH_2, γ *i*-D-Gln), 27.8 (CH_2, β *i*-D-Gln), 22.8 (CH_3, NAc), 20.4

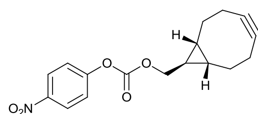
(CH_3, Ac), 20.2 (CH_3, Ac), 19.1 (CH_3, lactic acid), 17.1 (CH_3, Ala); IR (cm^{-1}): 3585, 1732, 1643, 1534, 1234; LC/MS: Rt = 0.98 min (Alltima C_{18}, 10 –90% MeCN, 15 min run); HRMS Calcd. for $[C_{26}H_{43}N_5O_{13} + H]^+$ 634.29301, found .634.29307

3-Aminopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (14)



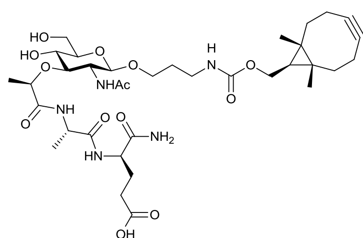
Compound **13** (0.18 g, 0.28 mmol) was stirred for 18 h in NH_3 in MeOH (28 mL, 4 M). The reaction mixture was concentrated in vacuo and purified by RP-HPLC (^tBuOH : H_2O : MeOH, C_{18} column) yielding title compound (0.06 g, 0.12 mmol, 41%). ^1H NMR (400 MHz, D_2O) δ 4.46 (d, *J* = 8.5 Hz, 1H, CH, H-1), 4.40 – 4.34 (m, 1H, CH, α *i*-D-Gln), 4.26 – 4.21 (m, 2H, CH, lactic acid, CH, Ala), 4.05 – 3.96 (m, 1H, CH_2, $C_3H_6NH_2$), 3.97 – 3.91 (m, 1H, CH_2, H-6), 3.85 – 3.77 (m, 2H, CH, H-2, CH_2, H-6), 3.69 (t, *J* = 7.6 Hz, 1H, CH_2, $C_3H_6NH_2$), 3.61 – 3.53 (m, 2H, CH, H-3, CH, H-4), 3.51 – 3.43 (m, 1H, CH, H-5), 3.07 (t, *J* = 7.0 Hz, 2H, CH_2, $C_3H_6NH_2$), 2.49 (t, *J* = 7.3 Hz, 2H, CH_2, γ *i*-D-Gln), 2.28 – 2.19 (m, 1H, CH_3, β *i*-D-Gln), 2.03 – 1.88 (m, 4H, CH_3, NAc, CH_2, β *i*-D-Gln), 1.43 (d, *J* = 7.2 Hz, 3H, CH_3, lactic acid), 1.37 (d, *J* = 6.8 Hz, 3H, CH_3, Ala); ^{13}C NMR (101 MHz, D_2O) δ 176.8 (C=O), 175.9 (C=O), 175.7 (C=O), 175.1 (C=O), 174.2 (C=O), 101.2 (CH, C-1), 82.5 (CH, C-3), 78.2 (CH, lactic acid), 75.5 (CH, C-5), 68.8 (CH, C-4), 67.3 (CH_2, $C_3H_6NH_2$), 60.5 (CH_2, C-6), 55.0 (CH, C-2), 52.5 (CH, α *i*-D-Gln), 49.7 (CH, Ala), 37.5 (CH_2, $C_3H_6NH_2$), 30.0 (CH_2, γ *i*-D-Gln), 26.6 (CH_2, β *i*-D-Gln), 22.1 (CH_3, NAc), 18.7 (CH_3, lactic acid), 16.5 (CH_3, Ala); IR (cm^{-1}); LC/MS: Rt = 3.16 min (Alltima C_{18}, 0 –20% MeCN, 15 min run); HRMS Calcd. for $[C_{22}H_{39}N_5O_{11} + H]^+$ 550.27188, found 550.27162.

(1α,8α,9β)-bicyclo[6.1.0]non-4-yn-9-ylmethyl 4-nitrophenyl ester (15)



Compound **15** was prepared according to literature.[12] ^1H NMR (400 MHz, $CDCl_3$) δ 8.31 – 8.24 (m, 2H, CH, Ar), 7.44 – 7.35 (m, 2H, CH, Ar), 2.45 (dd, *J* = 13.3, 2.4 Hz, 2H, CH_2), 2.38 – 2.25 (m, 2H, CH_2), 2.22 – 2.10 (m, 2H, CH_2), 1.51 – 1.33 (m, 2H, CH_2), 0.94 – 0.78 (m, 1H, CH); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.8 (C=O), 155.4 (C_q), 152.3 (C_q), 145.1 (C_q), 125.2 (CH, Ar, 125.0 (CH, Ar), 121.6 (Ar) 121.5 (CH, Ar), 98.4 (CH_2), 73.7 (CH_2), 60.1 (CH_2), 32.9 (CH_2), 23.0 (CH), 22.7 (CH), 21.0 (CH_2).

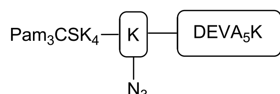
(1α,8α,9α)-bicyclo[6.1.0]non-4-yne-9-methyl-3-carbamate-3-propyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside (16)



Compound **14** (36 mg, 66 µmol) was stirred in DMF (3 mL, 0.03 M) with DiPEA (0.02 mL, 98 µmol). Dropwise was added insitu prepared *p*-methoxy phenyl carbamate **15** (82 mg, 131 µmol, 1 mL DMF) and stirred for 18 h. The mixture was concentrated in vacuo and purified by RP-HPLC/Ms (C_{18} column, 23-32 % MeCN) under basic conditions (Ammonium acetate, 0.15 mM), and freeze-dried twice. Title compound was obtained in 22% (10.45 mg, 0.014 mmol). ^1H NMR (600 MHz, D_2O) δ 4.54 (d, *J* = 8.5 Hz, 1H, CH, H-1), 4.40 – 4.34 (m, 1H, CH, α *i*-D-Gln), 4.35 (m, 1H, CH, lactic acid), 4.32 – 4.26 (m, 1H, CH, Ala), 4.1 – 4.05 (m 2H, CH_2, cyclooctyne), 4.04 – 3.96 (m, 2H, CH_2, $C_3H_6NH_2$, CH_2, H-6), 3.92 – 3.87 (m, 1H, CH, H-2), 3.84 (dd, *J* = 12.4, 5.7 Hz, 1H, CH_2, H-6), 3.72 – 3.66 (m, Hz, 1H, CH_2, C_3H_6NH), 3.64 – 3.57 (m, 2H, CH, H-3, CH, H-4), 3.57 – 3.50 (m, 1H, CH, H-5), 3.27 – 3.19 (m, 2H, CH_2, C_3H_6NH), 2.50 – 2.45 (m, 2H, CH_2, cyclooctyne), 2.43 – 2.39 (m, 2H, CH_2, γ *i*-D-Gln), 2.39 – 2.32 (m, 2H,CH_2, cyclooctyne), 2.27 – 2.18 (m, 3H, CH_2, β *i*-D-Gln, CH_2, cylooctyne), 2.08 – 1.98 (m, 4H, CH_3, NAc, CH_2, β *i*-D-Gln), 1.86 – 1.79 (m, 2H, CH_2, C_3H_6NH), 1.52 (d, *J* = 7.2 Hz, 3H, CH_3, lactic acid), 1.50 – 1.42 (m,

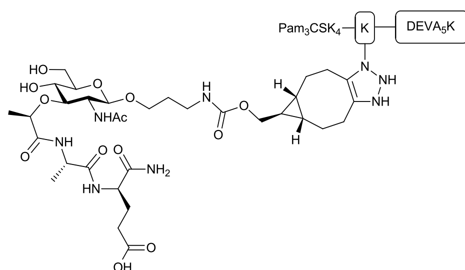
5H, 3H, CH_3, Ala, CH_2, cyclooctyne), 0.91 – 0.79 (m, CH, cyclooctyne); ^{13}C NMR (151 MHz, D2O) δ 181.0 (C=O), 177.3 (C=O), 176.7 (C=O), 176.1 (C=O), 175.0 (C=O), 159.8 (C=O), 102.2 (C-1), 101.3 (C_q), 83.6 (C-3), 79.2 (CH, lactic acid), 76.6 (C-5), 70.7 (C-4), 69.9 (CH_2, cyclooctyne), 68.7 (CH_2, C_3H_6NH), 61.7 (CH_2, C-6), 56.0 (C-2), 54.2 (CH, α *i*-D-Gln), 50.7 (CH, Ala), 38.2 (CH_2, C_3H_6NH), 33.7 (CH_2, cyclooctyne), 33.5 (CH_2, γ *i*-D-Gln), 29.8 (CH_2, C_3H_6NH), 28.2 (CH_2, β *i*-D-Gln), 24.3 (CH, cyclooctyne), 23.5 (CH, cyclooctyne), 23.2 (CH_3, NAc), 21.7 (CH_2, cyclooctyne), 20.0 (CH_3, lactic acid), 17.6 (CH_3, Ala); LC/MS: Rt = 8.64 min (Alltima C_{18}, 0 – 50% MeCN, 15 min run); HRMS Calcd. for $[C_{33}H_{51}N_5O_{13} + H]^+$ 726.35561, found 726.35591.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Lys(N_3)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (17)



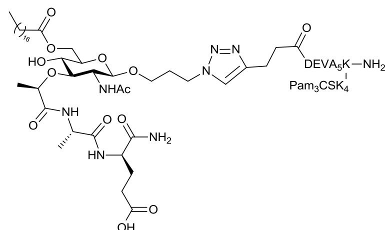
On 50 µmol H-Ser(O^tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(N_3)-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc)-NH was synthesized on S Ram Tentagel (0.25 mmol/g) using standard Fmoc chemistry incorporating an azido-lysine. After the final Fmoc deprotection the sequence was elongated with PyBOP coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH following standard procedures. Treating the resin with standard cleavage conditions and precipitation (105 min., 2.5% TIS : 2.5% H_2O : 95% TFA, Et_2O) yielded a precipitate. Purification by RP-HPLC resulted in compound **24** (11.4 mg, 1.44 µmol, 3%); LC/MS: Rt = 7.09 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4192.59 $[M+H]^+$; HRMS Calcd. for $[c_{199}H_{349}N_{43}O_{51}S_1 + H]^{2+}$ 1398.20113, found 1398.20212.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Lys(triazole-(1α,8α,9α)-bicyclo[6.1.0]non-4-ene-9-methyl-3-carbamate-3-propyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-β-D-glucopyranoside)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (5)



Compound **16** (2.48 mg, 3.34 µmol) was dissolved in 50 µL $^tBuOH : H_2O$: MeOH (0.07 M). Compound **17** (2.32 mg, 0.48 µmol) was dissolved in 25 µL stock solution of compound **16**. The resulting mixture was shaken for 24 h at ambient temperature. To complete the reaction another 25 µL stock solution of compound **16** was added. The mixture was stirred for another 24 h. Purification by RP-HPLC resulted compound **5**. (0.22 mg, 45 nmol, 10%); LC/MS: Rt = 6.28 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4917.94 $[M+H]^+$; HRMS Calcd. for $[C_{232}H_{400}N_{48}O_{64}S + H]^{3+}$ 1640.31817, found 1640.31827.

1-β-(3-Azidopropyl-triazole-ethyl-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-lys-(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Lys)-NH_2)-3-*O*-((R)-1-carboxyethyl-L-Ala-D-Gln(OH)-NH_2)-2-*N*-acetyl-6-O-stearoyl-D-glucopyranoside (6)

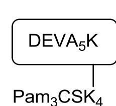


25 µmol Tentagel S Ram resin loaded with pentynoyl-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt) (**19**) was swollen in DMF. A stock solution of compound **18** (45 mg, 25 µmol), $CuSO_4$ (3.75 µmol, 75 µL, 100 mM) and sodium ascorbate (25 µmol, 250 µL, 200 mM) in DMF (1.0 mL) was added to the resin and stirred for 5 days at 50°C. The resin was thoroughly washed with DMF (3x 10 min), DMF : H_2O (1 : 1, 3 x 10 min), H_2O (2 x 10 min), pyridine : H_2O (2 : 8, 3 x 10 min) and DMF (3 x 10 min). The resin was treated with 1M Boc_2O in NMP for 15 min, then added 2 eq. of DiPEA and reacted for another hour. The resin was treated with a cleavage cocktail of 3% TFA in DCM, washed (NMP, DCM) and elongated with Ser(^tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with Fmoc/HCTU chemistry. Between the coupling steps the resin was treated with a solution of Boc_2O (1M), Ac_2O (0.05 M) and DiPEA (0.03 M) in NMP (2 mL) for 1 h. After the final Fmoc deprotection the sequence was elongated with a double coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH following standard procedures. The resin was treated with standard cleavage conditions for 70 min. Purification by RP-HPLC resulted in compound **6**. (1.3 mg, 0.23 µmol, 1%); LC/MS: Rt = 8.38 min (C_4 Vidac, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4961.05 $[M+H]^+$; HRMS Calcd. for $[C_{238}H_{414}N_{46}O_{63}S_1 + H]^{3+}$ 1654.02040, found 1654.02013.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (21)

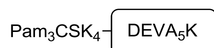
The synthesis of compound **21** on 25 µmol scale (Tentagel S Ram, 0.23 mmol/g) resulted after RP-HPLC purification in 8.59 mg (3.1 µmol, 12%); LC/MS: Rt = 8.87 min (C_{18} Alltima, 10 - 50% MeCN, 15 min run); ESI-MS: *m/z* 2546.35 $[M+H]^+$; HRMS Calcd. for $[C_{112}H_{185}N_{29}O_{38} + H]^{2+}$ 1273.68050, found 1273.68089.

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (22)



Tentagel S Ram resin loaded with H-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) on 25 µmol was treated 1M Boc_2O in NMP for 15 min followed by addition of 2 eq. of DiPEA and reacted for another hour. The resin was washed with NMP and DCM. The resin was treated with a cleavage cocktail of 3% TFA in DCM followed by a coupling sequence to elongate the resin with Ser(^tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc). The resin was treated with a solution of 20% piperidine in NMP (4 x 3 min). The resin was treated with Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH in the presence of PyBOP and DiPEA in a NMP : DCM mixture (1 : 1) for 18 h. The resin was washed (NMP, DCM) and treated with standard cleavage and deprotection conditions. Purification by RP-HPLC yielded compound **22**. (0.63 mg, 0.16 µmol, 0.62%); LC/MS: Rt = 6.93 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4038.50 $[M+H]^+$; HRMS Calcd. for $[C_{193}H_{339}N_{39}O_{50}S + H]^{2+}$ 1346.83932, found 1346.84366.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (23)



On 25 µmol scale Fmoc based solid phase synthesis was applied resulting H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt)-Tentagel-S-Ram. The resin was treated 1M BOC_2O in NMP for 15 min followed by addition of 2 eq. of DiPEA and reacted for another hour. The resin was washed with NMP and DCM. The resin was treated with a cleavage cocktail of 3% TFA in DCM (3 x 3 min). The resin was elongated with Fmoc-Ser(tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) with standard SPPS coupling cycles, concluding with the removal of the *N*-terminal Fmoc. The resin was elongated with Palmitoyl-Cys((RS)-2,3-di(palimioyloxy)-propyl)-OH (46 mg, 50 µmol) in DCM : NMP (1 mL) in the presence of PyBOP (26 mg, 50 µmol) and DiPEA (2 x 8.5 µL, 2 x 25 µmol). Treatment of the resin with the cleavage cocktail and purification by RP-HPLC resulted in compound **23**. (8.2 mg, 2.0 µmol, 8%); LC/MS: Rt = 7.09 min (C_4 Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 4038.50 $[M+H]^+$; HRMS Calcd. for $[C_{193}H_{339}N_{39}O_{50}S + H]^{3+}$ 1346.83932, found 1346.79996.

Immunological assays

Cell culture
For detailed information see the Experimental section of Chapter 2.

In vitro DC maturation assay
For detailed information see the experimental section of Chapter 3.

NOD2-HEK293 activation
For detailed information see the experimental section of Chapter 2.

In vitro antigen presentation assay
For detailed information see the experimental section of Chapter 2.

4.6 References and notes

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(14) CpG is used as an control on the livability of the DCs.