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

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Chapter 3

Lipophilic NOD2 ligands as immuno-stimulatory agents

3.1 Introduction

Muramyldipeptide (MDP) is the minimal structural element in the peptidoglycan (PG) of bacterial cell walls that exhibits an immunostimulatory effect.[1] However, in comparison with isolated bacterial cell wall samples the adjuvant properties of MDP are limited. For example, the immunostimulatory effect of the *Bacillus Calmette-Guerin* (BCG) cell wall is well recognized and can be partly attributed to the presence of mycolic acids, α-branched-β-hydroxy fatty acids.[2] To improve the adjuvant properties of MDP the group of Kusumoto synthesized MDP derivatives provided with various C-6-*O* fatty acid residues (*Figure 1*).[3,4,5,6] The highest adjuvant activity in the explored series of MDP derivatives were 6-*O*-mycoloyl MDP (reported as B30-MDP, **1**) and 6-*O*-stearoylated MDP derivative **2**.[7,8] Guided by these results, lipophilic esters were installed at other positions in the MDP core.[9] For instance, the MDP derivative Romurtide or MDP-Lys(L18) (**3**), equipped with a N^6-stearoyl-L-lysine on the carboxylic acid of isoglutamine shows enhanced immunostimulatory activity.[10]

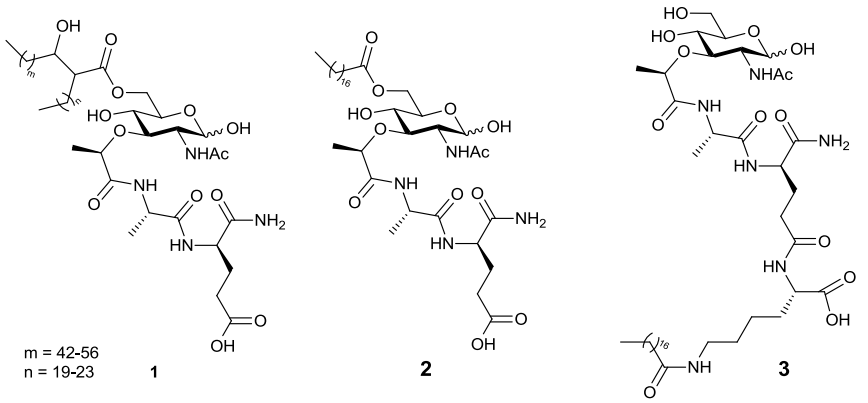


Figure 1. B30-MDP (**1**), 6-stearoyl-MDP (**2**) and romurtide (**3**).

The favorable influence of lipophilic tails on the activity of MDP derivatives was an incentive to explore whether the immunostimulatory potency of the conjugates described in Chapter 2 can be improved by the implementation of a stearoyl moiety at various positions of the MPD moiety. It was decided to prepare and evaluate MDP derivatives **4 – 6**, having a stearoyl ester at the C-6-*O*-position of glucosamine (**4**), a stearoyl amide at the anomeric spacer of glucosamine (**5**) and a N^6-stearoyl-L-lysine appendage on the isoglutamine (**6**). The most potent MDP derivative was conjugated to the antigenic peptide $DEVA_5K$ and the resulting construct was probed for antigen presentation and immunostimulatory activity.

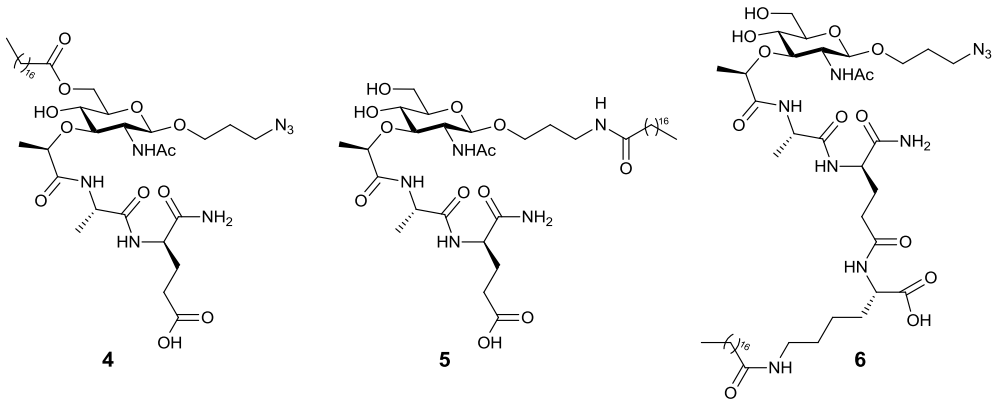


Figure 2. Target molecules **4** – **6** with lipophilic tails.

In the immunological evaluation reference compounds MDP derivative **7** and acetylated MDP derivative **8** (for synthesis see Chapter 2) were used to evaluate the potency of compounds **4** – **6**. For the evaluation of the NOD2-antigen conjugates reference conjugate **9** (for synthesis see Chapter 2), TLR2-antigen conjugate (**10**) (*Figure 3*) and antigenic peptide $DEVA_5K$ (**11**) were used.

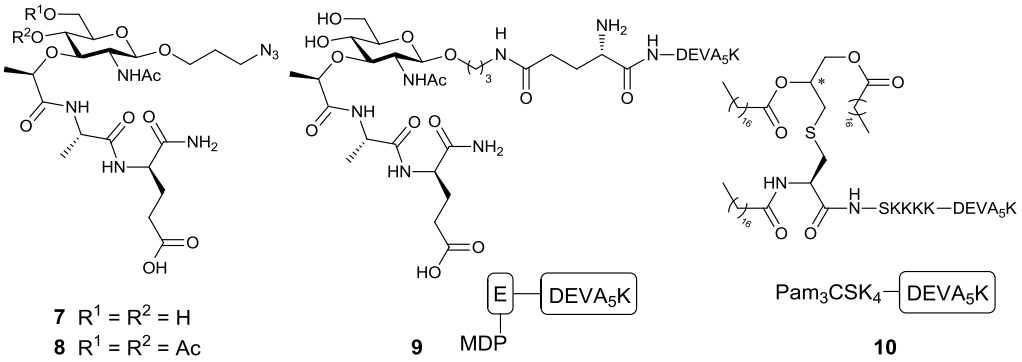
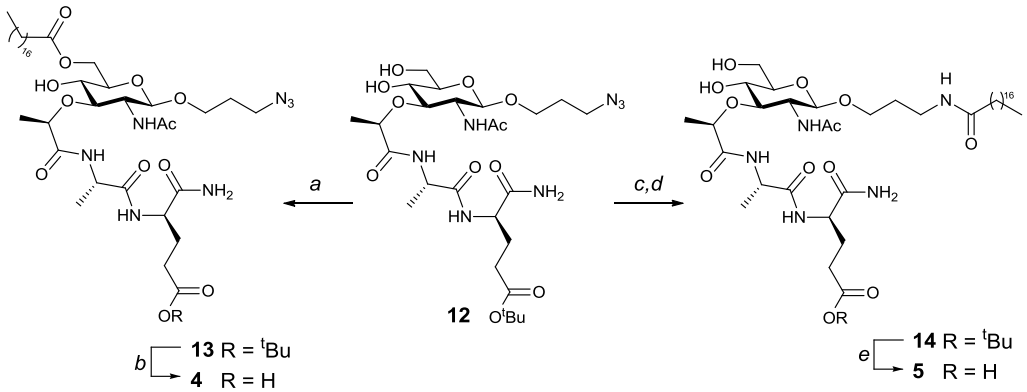


Figure 3. Reference compounds **7** – **10**.

3.2.1 Synthesis of lipophilic MDP derivatives

The syntheses of MDP derivatives **4** and **5** are depicted in *Scheme 1* and start with the common building block **12**, of which the preparation is described in Chapter 2. Regioselective acylation was achieved by reaction of compound **12** with a slight excess of stearoyl chloride in pyridine and DCM to give compound **13**. Subsequent treatment of **13** with 20% TFA in DCM gave compound **4** in a 73% yield. Only a trace amount of hydrolyzed product was observed by mass spectrometry, indicating that the electron withdrawing properties of the stearoyl ester suppressed the unwanted acid mediated hydrolysis of the azidopropanol spacer.
The synthesis of MDP derivative **5** started with a Staudinger reduction of the azide by treatment of **12** with PMe_3 in aqueous THF followed by the condensation of the obtained amine and stearic acid under influence of HATU and DiPEA. Removal of the *tert*-butyl group in compound **14** was attained by treatment with 20% TFA in DCM and subsequently precipitation with diethyl ether gave crude **5**. After re-crystallization from a mixture of chloroform, methanol and diethyl ether MDP derivative **5** was obtained in 42% yield.

Scheme 1. Synthesis of **4** and **5**.

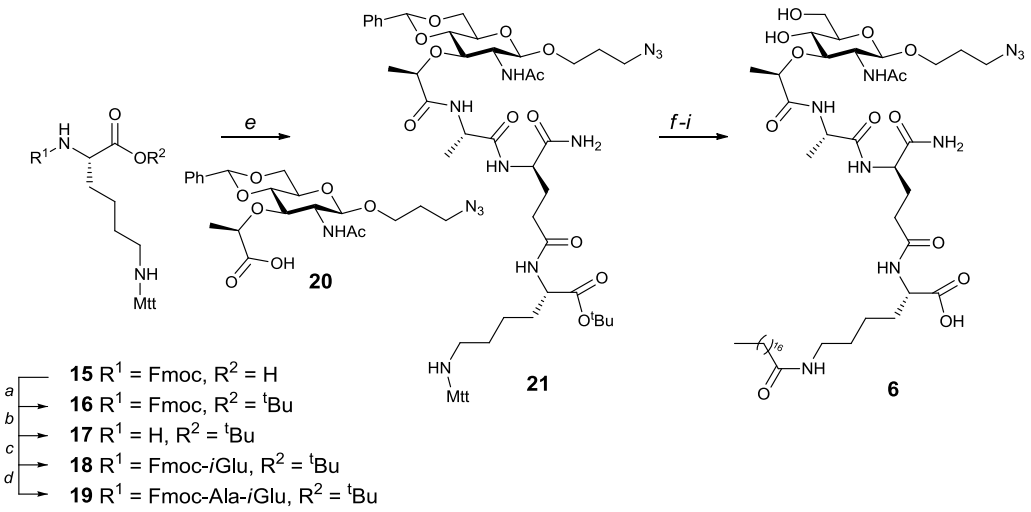


Reaction conditions: *a*) 1.1 eq stearoyl chloride, pyridine, DCM, 1 h, 63%; *b*) 20% TFA, DCM, 74%; *c*) PMe_3, THF, H_2O; *d*) HATU, DiPEA, stearicl acid, DMF, 90% over 2 steps; *e*) 10% TFA, DCM, 42%.

To obtain the third MDP derivative **6**, fully protected tripeptide **19** was synthesized starting from Fmoc-lysine **15** (*Scheme 2*). Fmoc-Lys(Mtt)-OH **15** was converted into *tert*-butyl ester **16** in quantitative yield. The Fmoc group of **16** was selectively removed with DBU in the presence of octanethiol to give amine **17** in 50% yield. The condensation of **17** with N-Fmoc-D-isoglutamine under influence of HATU and DiPEA gave dipeptide **18** in 77% yield. In a one pot procedure **18** was deprotected and condensed with Fmoc-alanine resulting in

fully protected tripeptide **19** in 70% yield after flash column chromatography. In a similar one pot procedure was peptide **19** coupled with MurNAc (**20**, for synthesis see Chapter 2) to give fully protected **21**. The 4-methyltrityl (Mtt) group at the lysine side chain of **21** was removed with 3% TFA in DCM. This acid treatment was accompanied by partial benzylidene cleavage. Condensation of stearic acid with the free amine of the lysine residue under influence of HATU and DiPEA was followed by treatment with a solution of 20% TFA and 2.5% TIS in dry DCM to remove the remaining benzylidene and *tert*-butyl ester. Target compound **6** was obtained in low yield after RP-HPLC/Ms purification, which yield can be explained by acid mediated hydrolysis of the anomeric spacer. Nonetheless, a sufficient amount of compound **6** was obtained (2.9 mg) and its synthesis was not further optimized.

Scheme 2. Synthesis of **6**.



Reaction conditions: *a*) Boc_2O, cat DMAP, ^tBuOH, THF, quant; *b*) cat. DBU, octanethiol, DCM, 50%; *c*) HATU, DiPEA, Fmoc-*i*-D-Gln-OH, DCM, 77%; *d*) DBU, HOBt, Fmoc-Ala-OH, HATU, DiPEA, DCM, 70%; e) DBU, HOBt, MurNAc, HATU, DiPEA, DMF, 60%; *f*) 3% TFA, 2% TIS; *g*) stearic acid, HATU, DiPEA, DMF; *h*) 20% TFA, 2.5% TIS, DCM; *i*) RP-HPLC/Ms, 5% over 4 steps.

3.2.2 Immunological evaluation of lipophilic MDP derivatives

Examples of MDP derivatives equipped with lipophilic tails that show improved immunostimulatory activity in comparison with non-lipophilic MDP analogues have been reported.[10-21] To examine the potency of the here presented lipophilic NOD2-L, compounds **4** – **6** were submitted to the same immunological assays as described in Chapter 2. The NOD2 immunostimulatory potency of the conjugates was assessed in a NOD2 transfected human embryonic kidney (HEK) cell line (293HEK) and the immunostimulatory activity of the conjugates was evaluated on the basis of their ability to induce maturation of dendritic cells (DCs). In addition to the IL-12p40 production assay (as described in Chapter 2) the upregulation of cell surface markers (CD40, CD86) was studied by staining with fluorescently labeled antibodies as another measure of DC maturation.
Uehori *et al*. showed that in human DCs lipophilic MDP derivatives induce DC maturation, potentially *via* TLR2 and TLR4.[12] The group of Boons reported that PG-fragments can be recognized by TLR2.[17] To investigate the possibility that the constructs **4** – **6** are recognized by TLR2, the TLR2 immunostimulatory potency of these constructs was probed as well. To determine whether TLR2 is involved in sensing of the constructs, both a wild type (WT) and a TLR2 knockout (TLR2-KO) bone marrow derived cell (BMDC) line are used in a IL-12p40 assay. There is no functional TLR2 in the TLR2-KO cell line and the difference in IL-12 production between the WT and TLR2-KO cell lines reflects the involvement of TLR2 in the recognition of the lipophilic MDP-derivatives being studied.
The data from NOD2 immunostimulatory assay with NOD2 transfected HEK cells (*Figure 4A*) show that stearoyl containing MDP derivatives **4**, **5** and **6** exhibit an increased activity in comparison to less lipophilic reference compounds **7** and **8**. The activity of **4**, **5**, and **6** was also assessed at lower concentrations revealing compound **4** as the most potent agonist (*Figure 4B*). These results are in agreement with the reported literature in which the same trend was observed for Romurtide (**3**)[21-23] and B30-MDP (**1**).[3,8,24-26] The increased lipophilicity may be related to an improved uptake of the ligand, which results in a better availability for ligating to the cytosolic NOD2 receptor.

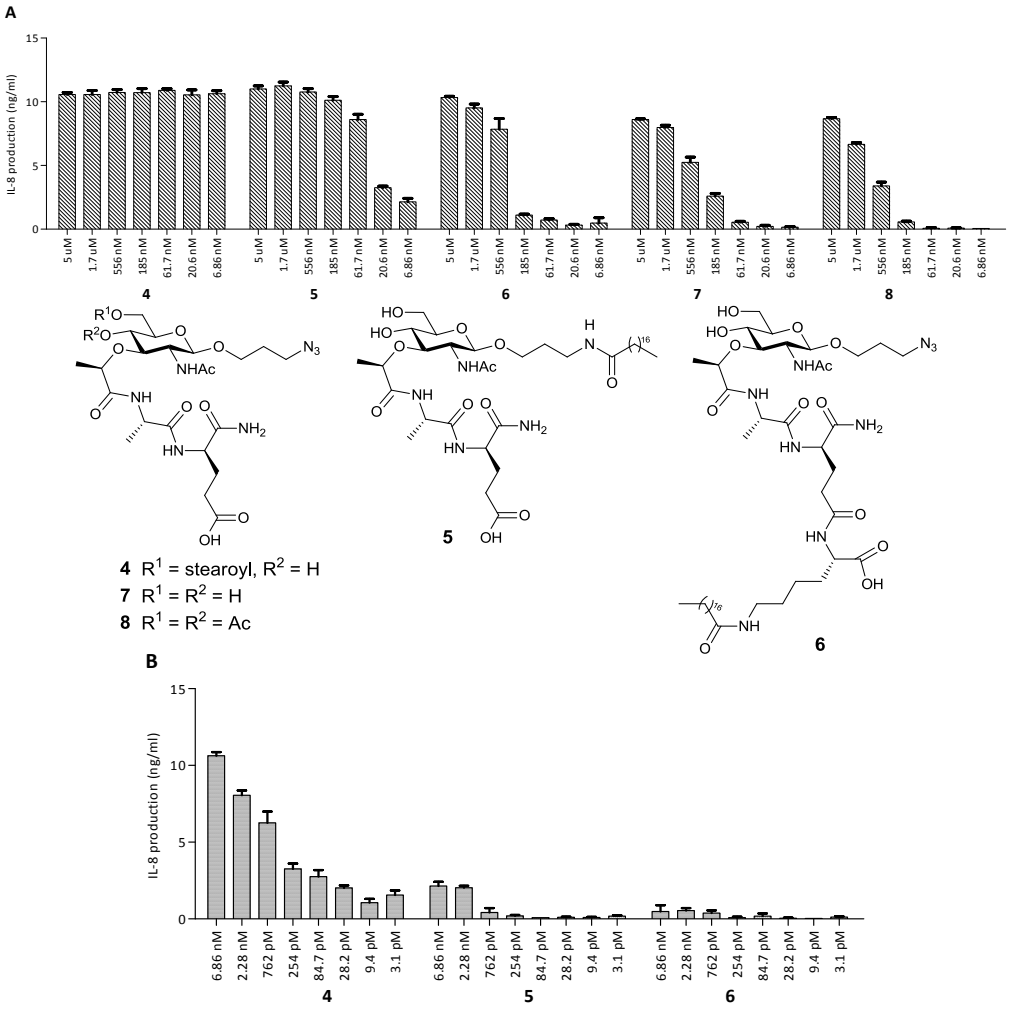


Figure 4. Potency of NOD2 ligands **4 – 8** at 5 µM – 6 nM (A) and 6 nM – 3 pM (B) to stimulate NOD2 transfected HEK cells.

Next, the influence of the lipophilic MDP derivatives on the maturation of DCs was investigated in a similar way as described in Chapter 2. Thus, the lipophilic MDP derivatives **4** – **6** were compared with **7** and **8** in an IL 12 production assay (*Figure 5*). The results show the same trend as observed in the NOD2 HEK assay: the stearoyl containing MDP derivatives **4**, **5** and **6** are more potent than their non-lipophilic derivatives **7** and **8**, and is in agreement with the results reported previously for different lipophilic MDP derivatives.[12]

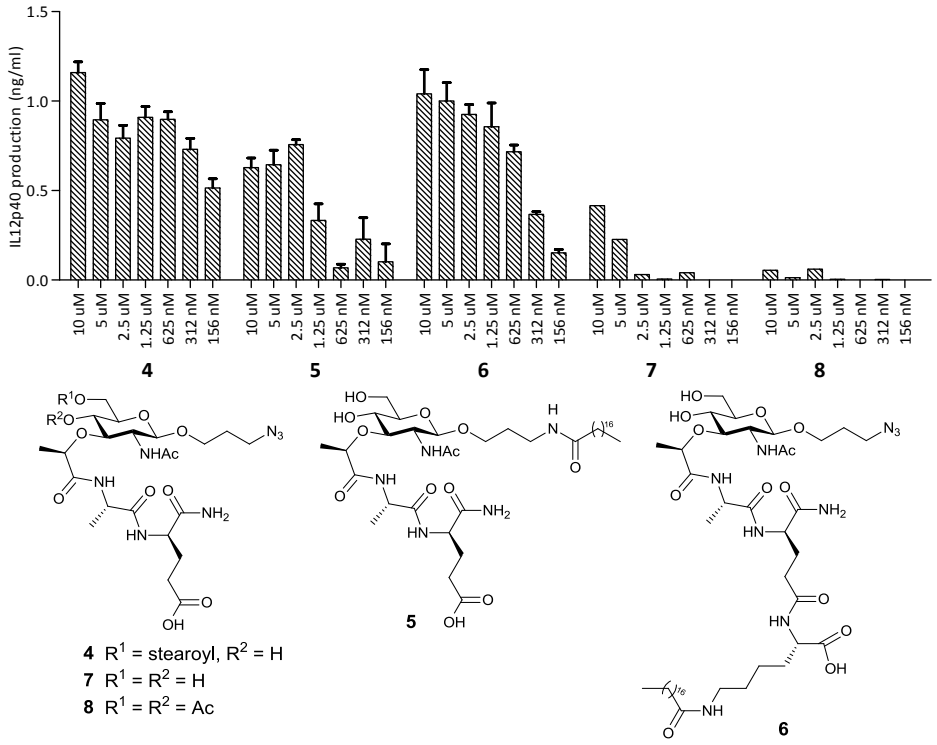


Figure 5. DC maturation potency of NOD2 ligand derivatives **4 – 8**.

After that, the DC maturation potency of MDP derivatives **4 – 6** was assessed on the ability of these to up-regulate the cell surface receptors CD40 and CD86 in cultured DCs (D1 line). The efficiency of compounds **4 – 6** was again tested against reference compounds **7** and **8** (*Figure 6*). The lipophilic MDP derivatives **4**, **5** and **6** showed higher upregulation of the cell surface markers than the non-lipophilic compounds **7** and **8**. These results are again in line with the reported data.[12] It is evident from *Figure 6* that the position of the lipophilic chain at the MDP core structure influences the activity because compounds **4** and **6** are more potent than **5**. In contrast to the results from IL-12 production in the TLR2-KO assay (*Figure 7*) compounds **4** and **6** were found to be more potent than TLR2-L Pam_3CSK_4 in upregulation of cell surface markers on DCs.

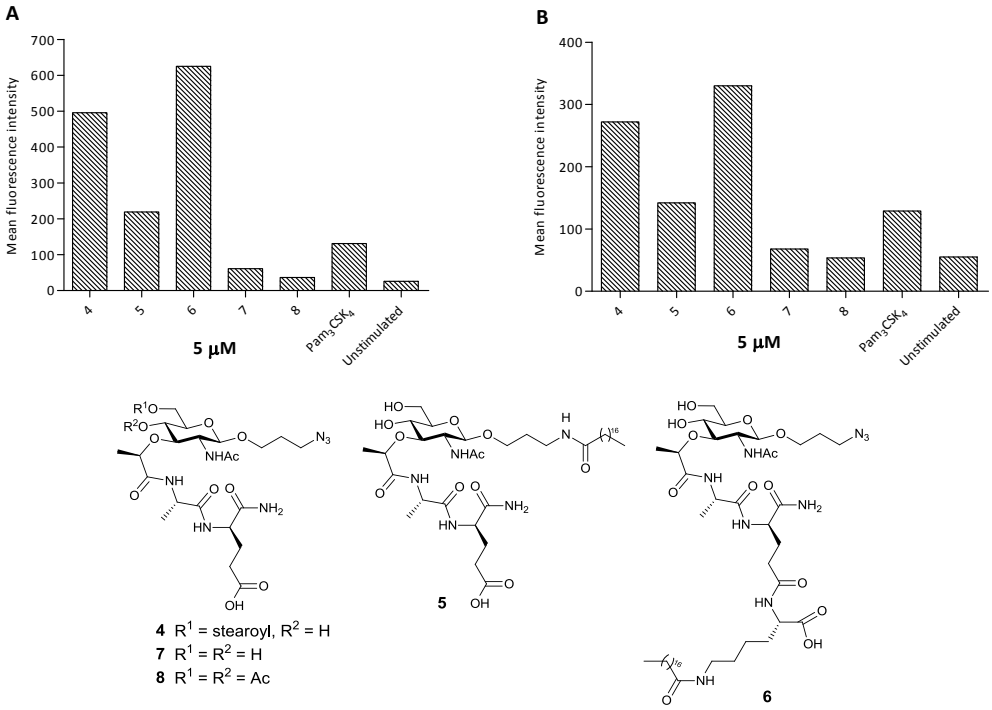


Figure 6. CD40 (A) and CD86 (B) upregulation by MDP derivatives **4** – **6**.

The TLR2 immunostimulatory potency of MDP derivatives **4** – **6** was tested by comparing the activity of the constructs in the WT BMDCs, with those derived from TLR2-KO mice (*Figure 7*). MDP derivative **7** and TLR2-L Pam_3CSK_4 were used as reference compounds and the TLR9-L CpG was used as a control compound.[27] In the WT BMDC (white bars, *Figure 7*) compound **7** shows cytokine production close to background levels as was also observed for D1 DCs. Compounds **4** – **6** show a significant amount of IL-12 production, although the NOD2 ligands are less active than TLR2-L Pam_3CSK_4. In the TLR2-KO cell line (black bars, *Figure 7*) all tested compounds were inactive suggesting a TLR2 immunostimulatory activity of the lipophilic MDP derivatives as previously indicated.[12]

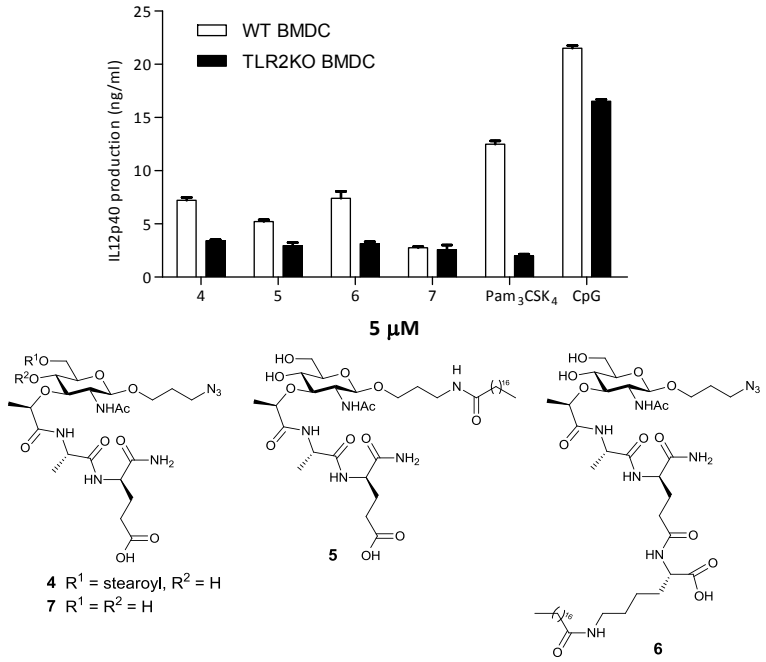


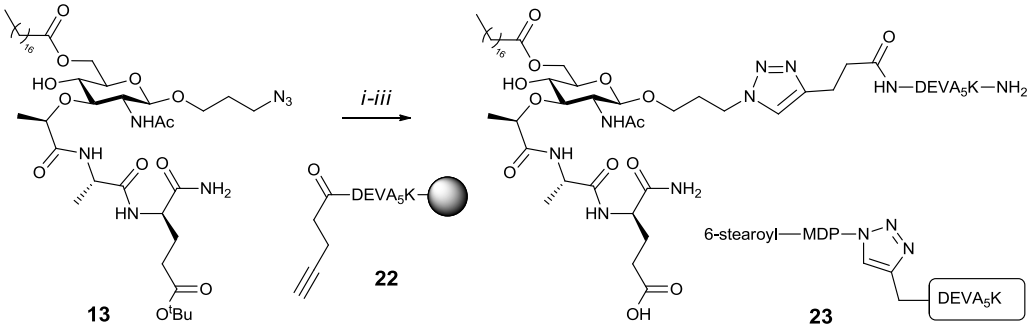
Figure 7. IL-12 inducing potency of NOD2 ligands **4** – **7** in wild-type and TLR2 KO BMDC.

Overall, the immunological assays show that lipophilic MDP derivatives **4**, **5** and **6** are more potent than the parent MDP derivatives described in Chapter 2. The data presented here imply an involvement of TLR2 in the recognition of lipophilic MDP derivatives since TLR2-KO cells proved to be irresponsive to the stimulation with **4** – **6**. However, as indicated by the upregulation of CD40 and CD86 in BMDCs, it is not excluded that an additive signaling pathway involving NOD2 is also operational. It can be concluded from the data presented above that lipophilic MDP derivative **4** shows the highest immunostimulatory activity of the series and therefore this particular ligand was selected to be incorporated into a NOD2-L-antigen conjugate.

3.3.1 Synthesis of NOD2-L-conjugate with antigenic peptide

Most potent lipophilic MDP derivative **4** was chosen to be incorporated into an MDP-antigen conjugate. In Chapter 2 it was shown that covalent attachment of a peptide epitope to the spacer at the anomeric center of the sugar moiety in a MDP derivative does not interfere with its biological activity. The presence of the azide function in **13**, the protected precursor of **4**, allows the application a copper mediated 'click' reaction for conjugation with the antigenic peptide. To facilitate the removal of the copper salts the click reaction was performed on resin. The required immobilized peptide **22** was synthesized by functionalization of immobilized $DEVA_5K$ peptide with 4-pentynoic acid (*Scheme 3*). The key 'click' reaction was executed as follows: compound **13** was dissolved in DMF and the resin **22** was added, followed by the addition of aqueous stock solutions of $CuSO_4$ (100 mM) and sodium ascorbate (200 mM) and the resulting suspension was heated to 60°C. The progress of the 'click' reaction was monitored by the cleavage and deprotection of aliquots of resin that were analyzed by mass spectrometry and it took 5 days at 60°C to reach completion. Finally, immobilized conjugate **23** was deprotected and cleaved from the resin with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O and the crude peptide was precipitated with diethyl ether. The lipophilic NOD2-L-antigen **23** was obtained in 30% yield after purification by RP-HPLC.

Scheme 3. Synthesis of conjugate **23**.



***Reaction conditions**: i*) 10% $CuSO_4$, sodium ascorbate, DMF, 60 °C, 5 days; *ii*) 95% TFA, 2.5% TIS, 2.5% H_2O; *iii*) RP-HPLC, 30%.

3.3.2 Immunological evaluation of NOD2-L-antigen conjugate

To test the immunostimulatory activity of lipophilic NOD2-L-antigen conjugate **23** assays similar to those described in section **2.2.2** were used. Thus, NOD2 immunostimulatory activity of the conjugate was tested in transfected HEK cells, DC maturation was evaluated by determining the levels of IL12 upon stimulation of a D1 cell line with conjugate **23** and, finally, the level of antigen presentation by DCs exposed to **23** was evaluated in a T-cell hybridoma assay (*cf*. Chapter 2).
In the first place, conjugate **23** was tested on the NOD2 transfected HEK cell line to check its potency as NOD2 ligand (*Figure 8*). Peptide $DEVA_5K$ **11** was used as a negative control and non-lipophilic NOD2-L-antigen conjugate **9** (see Chapter 2) and stearoyl NOD2-L **4** were used as reference compounds. Conjugate **23** and NOD2-L **4** show similar levels of activity in the activation of the NOD2 HEK cells. The lipophilic tail on the MDP ligand also has a positive effect on the activity of the MDP moiety when incorporated in a peptide conjugate as judged from the higher activity of **23** with respect to **9**. It was found that the activity of conjugate **23** is of similar magnitude as a mixture of ligand **4** and the antigenic peptide $DEVA_5K$ (**11**).

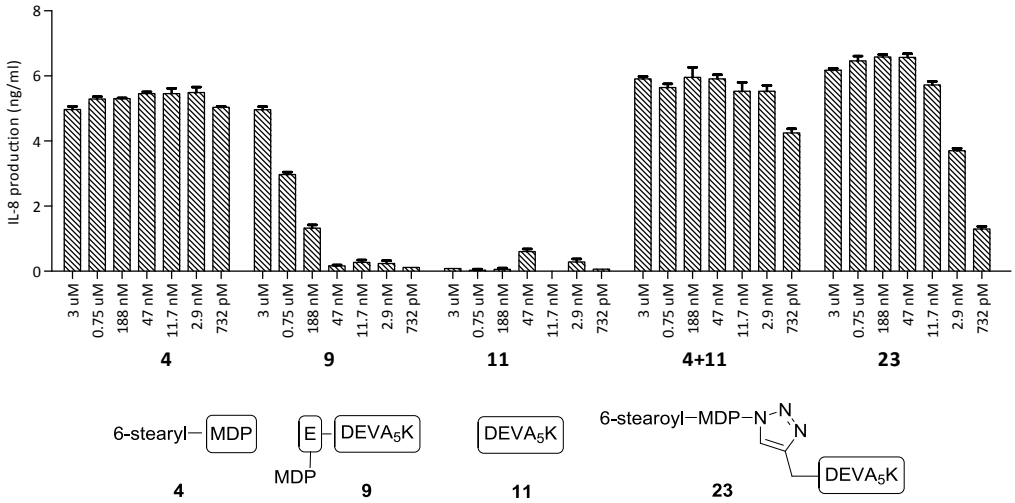


Figure 8. The NOD2 potency of conjugate **23** compared to ligand **4** and compounds **9** and **11**.

Secondly, conjugate **23** was submitted to a DC maturation assay in which the conjugate was compared with non-lipophilic conjugate **9** and known TLR2-L conjugate **10** (*Figure 9*). It was found that lipophilic NOD2-antigen conjugate **23** induces DCs to produce more cytokine IL-12p40 than non-lipophilic conjugate **9**. TLR2 based conjugate **10** is more active than conjugate **23**, as determined by the level of IL-12 produced upon the stimulation of DCs with these two compounds.

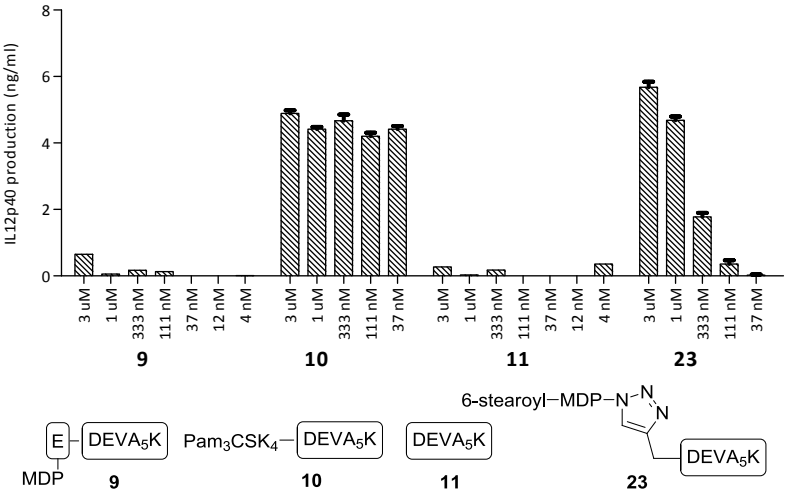


Figure 9. DC maturation of NOD2-L conjugates **23** and **9** and reference compounds **10** and **11**.

Finally, conjugate **23** was tested for its ability to induce the MHC class I-mediated antigen presentation of the OVA-derived SIINFEKL-epitope by DCs. *Figure 10* shows that the peptide of conjugate **23** is presented at a level comparable to reference compounds **9 – 11**. Also in this assay the TLR2 based conjugate **10** is more active than conjugate **23**.

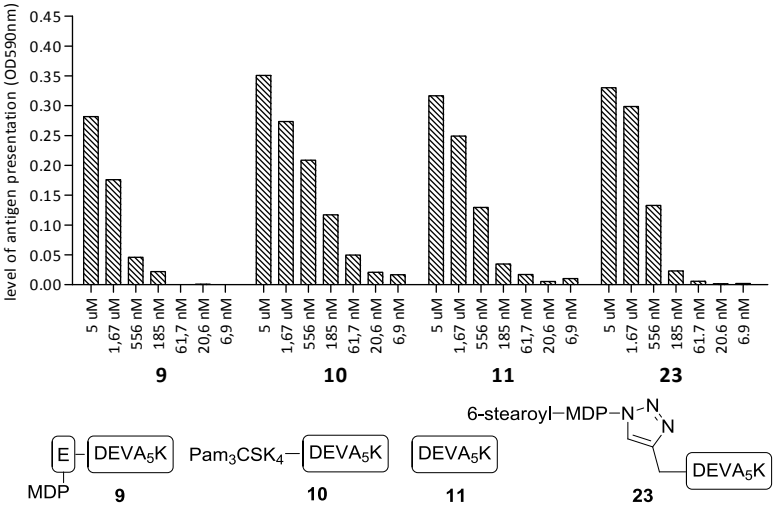


Figure 10. Antigen presentation of NOD2 ligand conjugates and reference compounds.

Overall, conjugate **23** retained activity as NOD2 receptor ligand and is capable of DC-maturation. In the *in vitro* antigen presentation assay the peptide epitope in **23** showed the ability to be presented by DCs.

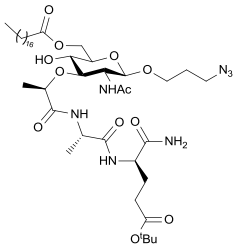
3.4 Conclusion

The synthesis and immunological evaluation of the lipophilic MDP derivatives **4**, **5** and **6** are described. Compound **4** turned out to be the most potent NOD2-L of the series of lipophilic MDP derivatives. To exploit the potency of the ligand, MDP derivative **4** was conjugated *via* 'click' chemistry to the antigenic peptide $DEVA_5K$ to obtain a novel NOD2-L-antigen conjugate (**23**). Immunological evaluation of conjugate **23** showed the desired improvement in *in vitro* immunological potency as compared to non-lipophilic constructs described in Chapter 2. At present it is not possible to deduce which PRR is responsible for the activity of the compound and the results presented here indicate that both the NOD2 as well as TLR2 receptors can be involved. On the basis of these favorable properties conjugate **23** represent an interesting candidate for the follow-up research in human DCs and *in vivo* assays.

3.5 Experimental section

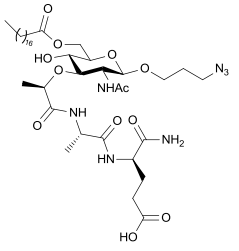
All reagents and solvents used in the solid phase peptide synthesis were purchased from Bachem and Biosolve and used as received. The analyses were performed as described in the Experimental section of Chapter 2.

3-Azidopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-2-deoxy-6-*O*-stearoyl-β-D-glucopyranoside (13)



Compound **12** (0.21 g, 0.33 mmol) was dissolved in warm pyridine (1 mL) and diluted with DCM (2.3 mL, 0.05 M). A stock solution of stearic acid chloride in DCM (0.5 mL, 0.35 M) was added. The resulting mixture was stirred for 3 h at rt, quenched with MeOH and concentrated *in vacuo*. Purification by flash column chromatography ($CHCl_3$: MeOH 9 : 0 → 9 : 1) resulted in compound **13** as a white solid (91 mg, 0.10 mmol, 63%). R_f = 0.6 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = -6.5 (c = 0.34, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, MeOD) δ 4.41 – 4.33 (m, 3H, CH, H-1, CH, α *i*-D-Gln, CH_2, H-6), 4.31 – 4.20 (m, 2H, CH_2, H-6, CH, lactic acid), 4.23 – 4.16 (m, 1H, CH, Ala), 3.93 – 3.85 (m, 1H, CH_2, $C_3H_6N_3$), 3.83 – 3.74 (m, 1H, CH, H-2), 3.63 - 3.57 (m, 1H, CH_2, $C_3H_6N_3$), 3.53 – 3.41 (m, 3H, C), 3.40 – 3.33 (m, 2H, CH_2, $C_3H_6N_3$), 2.40 – 2.31 (m, 4H, CH_2, γ *i*-D-Gln, CH_2, stearoyl), 2.28 – 2.15 (m, 1H, CH, β *i*-D-Gln), 1.94 (s, 3H, CH_3, NAc), 1.92 – 1.76 (m, 3H, CH_2, $C_3H_6N_3$, CH, β *i*-D-Gln), 1.70 – 1.63 (m, 2H, CH_2, stearoyl), 1.46 (s, 9H, ^{t}Bu), 1.42 (d, *J* = 6.1 Hz, 3H, CH_3, lactic acid), 1.37 (d, *J* = 6.7 Hz, 3H, CH_3, Ala), 1.35 – 1.21 (m, 28H, CH_2, stearoyl), 0.89 (t, *J* = 6.8 Hz, 3H, CH_3, stearoyl); ^{13}C NMR (101 MHz, MeOD) δ 174.0 (C=O), 174.2 (C=O), 174.1 (C=O), 173.3 (C=O), 172.4 (C=O), 171.7 (C=O), 100.7 (CH, C-1), 81.4 (CH, C-4), 80.7 (C_q, ^{t}Bu), 76.7 (CH, C-3), 73.3 (CH, lactic acid), 69.2 (CH, C-5), 65.6 (CH_2, $C_3H_6N_3$), 63.1 (CH_2, C-6), 54.8 (CH, C-2), 51.9 (CH, α *i*-D-Gln), 49.0 (CH, Ala), 48.0 (CH_2, $C_3H_6N_3$), 33.7 (CH_2, stearoyl), 31.5 (CH_2, γ *i*-D-Gln), 31.3 (CH_2, stearoyl), 29.2 (CH_2, stearoyl), 28.9 (CH_2, $C_3H_6N_3$), 28.9 (CH_2, stearoyl), 28.7 (CH_2, stearoyl), 28.6 (CH_2, stearoyl), 27.4 (CH_3, ^{t}Bu), 26.5 (CH_2, β *i*-D-Gln), 24.5 (CH_2, stearoyl), 22.2 (CH_2, stearoyl), 22.2 (CH_3, NAc), 18.1 (CH_3, lactic acid), 16.5 (CH_3, Ala), 13.5 (CH_3, stearoyl); IR (cm^{-1}): 3282, 2916, 2850, 2098, 1635; LC/MS: Rt = 7.58 min (Alltima C_4, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{44}H_{79}N_7O_{12} + H]^+$ 898.58595 found 898.58689.

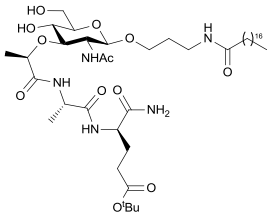
3-Azidopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-6-*O*-stearoyl-β-D-glucopyranoside (4)



Compound **13** (31 mg, 35 µmol) was treated with a mixture of 20% TFA in DCM (0.35 mL, 0.1 M) and stirred for 4 h at ambient temperature. The solution was concentrated *in vacuo* and the compound was purified by flash column chromatography (8 : 2 $CHCl_3$: MeOH + 1% AcOH) yielding **4** (21 mg, 25 µmol, 74%). R_f = 0.2 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = -4.0 (c = 0.2, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (600 MHz, MeOD) δ 4.37 (d, *J* = 8.6 Hz, 1H, CH, H-1), 4.26 – 4.21 (m, 1H, CH, α *i*-D-Gln), 4.19 – 4.16 (m, 1H, CH, lactic acid), 3.92 – 3.88 (m, 1H, CH_2, $C_3H_6N_3$), 3.80 – 3.76 (m, 1H, CH, H-2), 3.62 – 3.53 (m, 1H, CH_2, $C_3H_6N_3$), 3.50 – 3.41 (m, 3H, CH, H-3, CH, H-4, CH, H-5), 3.19 – 3.15 (m, 2H, CH_2, $C_3H_6N_3$), 2.45 – 2.37 (m, 2H, CH_2, stearoyl), 2.34 (t, *J* = 7.7 Hz, 2H, CH_2, γ *i*-D-Gln), 2.25 – 2.17 (m, 1H, CH_2, β *i*-D-Gln), 1.93 (s, 3H, CH_3, NAc), 1.88 – 1.75 (m, 3H, CH_2, β *i*-D-Gln, CH_2, $C_3H_6N_3$), 1.72 – 1.61 (m, 2H, CH_2, stearoyl), 1.40 (d, *J* = 7.1 Hz, 3H, CH_3, lactic acid), 1.35 (d, *J* = 6.7 Hz, 3H, CH_3, Ala), 1.20 -1.35 (m, 28H, CH_2, stearoyl), 0.86 (t, *J* = 7.0 Hz, 3H, CH_3, stearoyl); ^{13}C NMR (151 MHz, MeOD) δ 175.1 (C=O), 174.2 (C=O), 174.2 (C=O), 174.1 (C=O), 173.3 (C=O), 171.7 (C=O), 100.7 (CH, C-1), 81.4 (CH, C-4), 73.3 (CH, lactic acid), 69.2 (CH, C-5), 65.7 (CH_2, $C_3H_6N_3$), 63.1 (CH_2, C-6), 54.7 (CH, C-2), 52.1 (CH, α *i*-D-Gln), 49.0 (CH, Ala), 46.1 (CH_2, stearoyl),

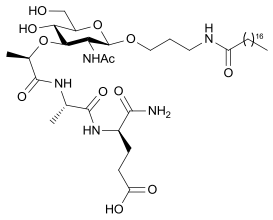
33.7 (CH_2, stearoyl), 31.5 (CH_2, stearoyl), 29.9 (CH_2, γ *i*-D-Gln), 29.2 (CH_2, stearoyl), 29.0 (CH_2, stearoyl), 28.9 (CH_2, stearoyl), 28.8 (CH_2, stearoyl), 28.7 (CH_2, stearoyl), 28.6 (CH_2, stearoyl), 26.3 (CH_2, β *i*-D-Gln), 24.5 (CH_2, $C_3H_6N_3$), 22.2 (CH_2, stearoyl), 22.2 (CH_2, stearoyl), 22.2 (CH_3, NAc), 18.1 (CH_3, lactic acid), 16.4 (CH_3, Ala), 13.4 (CH_3, stearoyl); IR (cm^{-1}): 3278, 2916, 2850, 2098, 1643; LC/MS: Rt = 4.22 min (Alltima C_4 Vidac, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{40}H_{71}N_7O_{12}+H]^+$ 842.52335 found 842.52397.

Stearoyl-(3-amidopropyl)-2-*N*-acetamide-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (14)



To a stirred solution of compound **12** (0.27 g, 0.43 mmol) in THF (4 mL) was added H_2O (0.4 mL) and PMe_3 (0.52 mL, 1.0 M in toluene). After stirring for 4 h the mixture was concentrated and dissolved in DMF (4.0 mL). To the mixture was added HATU (0.20 g, 0.52 mmol), DiPEA (0.22 mL, 1.3 mmol) and stearic acid (0.13 g, 0.52 mmol). The mixture was stirred for 18 h. The solution was concentrated *in vacuo* and purified by flash column chromatography ($CHCl_3$ → 9 : 1 $CHCl_3$: MeOH) and size exclusion chromatography (LH-20, 1 : 1 DCM : MeOH) resulting in the title compound **14** as a white solid (0.21 g, 0.24 mmol, 90%). R_f = 0.5 (9 : 1 $CHCl_3$: MeOH); $[\alpha]^D$ = -4.4 (c = 0.5, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, MeOD) δ 4.42 – 4.32 (m, 2H, H-1, CH, α *i*-D-Gln), 4.21- 4.11 (m, 2H, CH, lactic acid, CH, Ala), 3.94 – 3.85 (m, 2H, CH, H-6, CH_2, $C_3H_6N_3$), 3.85 – 3.69 (m, 2H, CH, H-2, CH, H-6), 3.44 (m, 4H, CH_2, $C_3H_6N_3$, CH, H-3, CH, H-4), 3.32 – 3.30 (m, 1H, CH, H-5), 3.21 – 2.99 (m, 1H, CH_2, $C_3H_6N_3$), 2.34 (t, *J* = 7.5 Hz, 2H, CH_2, γ *i*-D-Gln), 2.25 – 2.14 (m, 3H, CH_2, stearoyl, CH β *i*-D-Gln), 1.96 (s, 3H, NAc), 1.94 – 1.83 (m, 1H, CH, β *i*-D-Gln), 1.80- 1.65 (m, 2H, CH_2, $C_3H_6N_3$), 1.64 – 1.56 (m, 2H, CH_2, stearoyl), 1.50 – 1.40 (m, 12H, CH_3, ^{t}Bu, CH_3, lactic acid), 1.38 (d, *J* = 6.6 Hz, 3H, CH_3, Ala), 1.36 – 1.18 (m, 28H, CH_2, stearoyl), 0.89 (t, *J* = 6.5 Hz, 3H, CH_3, stearoyl); ^{13}C NMR (101 MHz, MeOD) δ 174.5 (C=O), 174.1 (C=O), 174.1 (C=O), 173.1 (C=O), 172.1 (C=O), 171.7 (C=O), 100.73 (CH, C-1), 81.6 (CH, C-3), 80.4 (C_q, ^{t}Bu), 76.5 (CH, lactic acid), 75.6 (CH, C-5), 69.0 (CH, C-4), 66.5 (CH_2, $C_3H_6N_3$), 61.0 (CH_2, C-6), 54.5 (CH, C-2), 51.72(CH, α *i*-D-Gln), 35.7 (CH_2, $C_3H_6N_3$), 35.7 (CH_2, stearoyl), 31.3 (CH_2, stearoyl), 31.1 (CH_2, γ *i*-D-Gln), 29.0 (CH_2, stearoyl), 28.9 (CH_2, $C_3H_6N_3$), 28.9 (CH_3, ^{t}Bu), 28.8 (CH_2, β *i*-D-Gln), 28.7 (CH_2, stearoyl), 26.3 (CH_2, stearoyl), 25.4 (CH_3, NAc), 18.0 (CH_3, lactic acid), 16.3 (CH_3, Ala), 13.2 (CH_3, stearoyl); IR (cm^{-1}): 2386, 2920, 2850, 1635, 1066; LC/MS: Rt = 6.70 min (CN Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{44}H_{81}N_5O_{12}+H]^+$ 872.59545, found 872.59691.

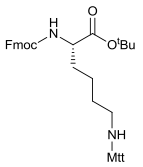
Stearoyl-(3-amidopropyl)-2-*N*-acetamide-2-deoxy-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-β-D-glucopyranoside (5)



Compound **14** (58 mg, 67 µmol) was dissolved in 10% TFA in DCM (4 mL, 0.02 M) and stirred for 5h. The crude compound was precipitated out of solution (Et_2O) and purified by flash column chromatography ($CHCl_3$: MeOH 9:0 → 8:2 with 2% AcOH). The title compound **5** was obtained as a white solid (42 mg, 0.052 mmol, 42%). R_f = 0.3 (9 : 1 $CHCl_3$: MeOH + 1% AcOH) ; $[\alpha]^D$ = -8.9 (c = 0.27, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.37 (d, *J* = 8.4 Hz, 1H, H-1), 4.30 – 4.50 (under H_2O peek, 1H, CH, lactic acid, CH, α *i*-D-Gln), 4.25 – 4.21 (m, 1H, CH Ala), 3.90-3.86 (m, 4H, CH_2, $C_3H_6N_3$, CH_2, H-6, CH, H-2), 3.52-3.48 (m, 3H, CH_2, $C_3H_6N_3$, CH, H-3, CH, H-4), 3.35-3.32 (m, 2H, CH_2, $C_3H_6N_3$, CH, H-5), 3.20 – 3.16 (m, 1H, CH_2, $C_3H_6N_3$), 2.40 – 2.35 (m, 2H, CH_2, γ *i*-D-Gln), 2.23 – 2.16 (m, 3H, CH_2, stearoyl, CH_2, β *i*-D-Gln), 1,98-1.86 (m, 7H, CH_3, NAc, CH_2, stearoyl, CH_2, β *i*-D-Gln), 1.73 – 1.83 (m, 2H, CH_2, stearoyl), 1.65 – 1.61 (t, *J* = 7.2 Hz, 2H, CH_2, $C_3H_6N_3$), 1.46 (d, *J* = 7.2, Hz, 3H, CH_3, lactic acid), 1.43 (d, *J* = 7.2 Hz, 3H, CH_3, Ala), 1.31-1.24 (m, 18H, CH_2, stearoyl), 0.89 (t, *J* = 7.2 Hz, 3H, CH_3, stearoyl); ^{13}C NMR (151 MHz,

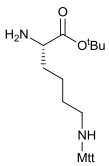
$CDCl_3$) δ 175.0 (C=O), 174.5 (C=O), 174.0 (C=O), 173.2 (C=O), 173.2 (C=O), 171.9 (C=O), 100.6 (CH, C-1), 81.8 (CH, C-3), 76.5 (CH, Ala), 75.6 (CH, C-5), 70.5 (CH, C-4), 66.3 (CH_2, $C_3H_6N_3$), 60.7 (CH_2, C-6), 52.4 (CH, α *i*-D-Gln), 48.7 (CH, lactic acid), 47.1 (CH_2, $C_3H_6N_3$), 35.5 (CH_2, $C_3H_6N_3$), 35.4 (CH_2, stearoyl), 32.4 (CH_2, γ *i*-D-Gln), 31.2 (CH_2, stearoyl), 28.9 (CH_2, stearoyl), 28.9 (CH_2, stearoyl), 28.6 (CH_2, stearoyl), 28.6 (CH_2, stearoyl), 27.2 (CH_2, $C_3H_6N_3$), 26.9 (CH_2, β *i*-D-Gln), 25.3 (CH_2, stearoyl), 21.9 (CH_2, stearoyl), 21.7 (CH_3, NAc), 17.8 (CH_3, lactic acid), 16.1 (CH_3, Ala), 12.91 (CH_3, stearoyl); IR (cm^{-1}): 3275, 2916, 2850, 1635, 1543; LC/MS: Rt = 6.173 min (Alltima CN, 10 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{40}H_{73}N_5O_{12} + H]^+$ 816.53285, found 816.53265.

Fmoc-Lys(Mtt)-O^{t}Bu (16)



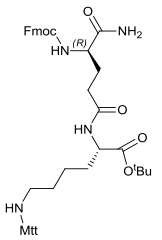
To Fmoc-Lys(mtt)-OH (**15**) (1.0 g, 1.6 mmol), dissolved in a mixture of ^{t}BuOH and THF (20 mL, 1 : 1, 0.1 M) was added Boc_2O (0.45 mL, 2.1 mmol) and an a catalytic amount of DMAP. After 18 h the solution was concentrated *in vacuo* to obtain the title compound **16** (1.0 g, 1.6 mmol) in quantitative yield. R_f = 0.9 (8 : 2 EtOAc : PE + 0.5% TEA); $[\alpha]^D$ = 4.2 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, DMSO-D_6) δ 7.89 (d, *J* = 7.5 Hz, 2H, CH, Ar), 7.72 (d, *J* = 7.4 Hz, 2H, CH, Ar), 7.65 (d, *J* = 7.8 Hz, 2H, CH, Ar), 7.47 – 7.37 (m, 10H, CH, Ar), 7.34 – 7.22 (m, 6H, CH, Ar), 7.18 – 7.05 (m, 2H, CH, Ar), 7.08 (d, *J* = 8.1 Hz, 2H, CH, Ar), 4.35 – 4.26 (m, 2H, CH_2, Fmoc), 4.22 (t, *J* = 7.0 Hz, 1H, CH, α Lys), 3.95 – 3.83 (m, 1H, CH, Fmoc), 2.31 (s, 3H, CH_3, Me), 1.97 (s, 1H, NH), 1.66 – 1.41 (m, 4H, CH_2, Lys), 1.43 – 1.32 (m, 11H, CH_3, ^{t}Bu, CH_2, Lys); ^{13}C NMR (101 MHz, DMSO-D_6) δ 172.1 (C=O), 156.5 (C=O), 146.9 (C_q, Ar), 144.3 (C_q, Ar), 144.2 (C_q, Ar), 143.7 (C_q, Ar), 141.2 (C_q, Ar), 135.4 (C_q, Ar), 129.4 (CH, Ar), 128.8 (CH, Ar), 128.7 (CH, Ar), 128.1 (CH, Ar), 128.0 (CH, Ar), 127.8 (CH, Ar), 127.5 (CH, Ar), 126.4 (CH, Ar), 125.8 (CH, Ar), 125.7 (CH, Ar), 121.8 (CH, Ar), 120.6 (CH, Ar), 80.8 (C_q, ^{t}Bu), 70.6 (CH_2, Fmoc), 66.0, 54.8 (CH, α Lys), 47.1 (CH, Fmoc), 43.6 (CH_2, Lys), 31.3 (CH_2, Lys), 29.9 (CH_2, Lys), 28.1 (CH_3, ^{t}Bu), 23.8 (CH_2, Lys), 21.0 (CH_3, Me); IR (cm^{-1}): 3333, 2974, 1600, 1450; LC/MS: Rt = 9.45 min (Alltima C_{18}, 10 – 90 MeCN); HRMS Calcd. for $[C_{46}H_{83}N_9O_{13} + H]^{2+}$ 341.18798, found 341.18405.

NH_2-Lys(Mtt)-O^{t}Bu (17)



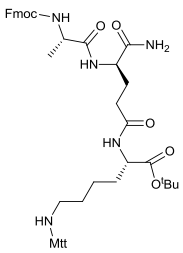
Compound **16** (1.0 g, 1.6 mmol) was dissolved in THF (16 mL) and treated with a catalytic amount of DBU and octanethiol (2.7 mL, 16 mmol) for 3 h. After concentration *in vacuo,* purification by flash column chromatography (1 : 1 PE : EtOAc → 20% MeOH in EtOAc, neutralized with 2% TEA) yielding compound **17** (0.4 g, 0.8 mmol, 50%). R_f = 0.1 (8 : 2 EtOAc : PE, 1% TEA); $[\alpha]^D$ = 1.8 (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, MeOD) δ 7.45 (d, *J* = 8.0 Hz, 4H, CH, Ar), 7.33 (d, *J* = 8.2 Hz, 2H, CH, Ar), 7.23 (t, *J* = 7.6 Hz, 4H, CH, Ar), 7.18 – 7.11 (m, 2H, CH, Ar), 7.06 (d, *J* = 8.1 Hz, 2H, CH, Ar), 4.44 – 4.62 (m, 2H, NH_2), 3.29 (t, *J* = 6.3 Hz, 1H, CH, α Lys), 2.28 (s, 3H, CH_3, Me), 2.15 (t, *J* = 6.7 Hz, 2H, CH_2, Lys), 1.72 – 1.58 (m, 2H, CH_2, Lys), 1.58 – 1.49 (m, 4H, CH_2, Lys), 1.45 (s, 9H, CH_3, ^{t}Bu), 1.43 – 1.34 (m, 2H, CH_2, Lys). ^{13}C NMR (101 MHz, MeOD) δ 174.1 (C=O), 145.7 (C_q, Ar), 142.5 (C_q, Ar), 134.9 (C_q, Ar), 127.9 (CH, Ar), 127.7 (CH, Ar), 126.9 (CH, Ar), 125.4 (CH, Ar), 80.8 (C_q, ^{t}Bu), 69.9 (C_q, Me), 53.6 (CH, α Lys) 42.7 (CH_2, Lys), 33.9 (CH_2, Lys), 29.7 (CH_2, Lys), 26.9 (CH_3, ^{t}Bu), 22.5 (CH_2, Lys), 19.8 (CH_3, Me);); IR (cm^{-1}): 3255, 3055, 2924, 1728, 1654, 1597; LC/MS: Rt = 6.36 min (Alltima C_{18}, 10 – 90 MeCN); HRMS Calcd. for $[C_{30}H_{38}N_2O_2 + H]^+$ 459.30061, found 459.30052.

Fmoc-D-Gln(Lys(Mtt)-O^{t}Bu)-NH$_2$ (18)



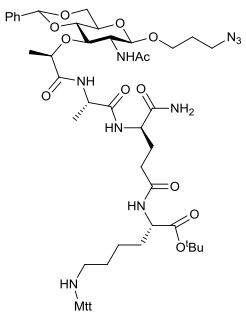
Compound **17** (0.4, 0.8 mmol) was dissolved in DCM (4 mL, 0.2 M) and added was a solution of HATU (0.46 g, 1.2 mmol), DiPEA (0.53 mL, 3.2 mmol) and Fmoc-*i*-*D*-Gln-OH (0.33 g, 0.89 mmol) in DCM (4 mL, 0.2 M). The mixture was stirred for 18 h. The solution was concentrated *in vacuo* and purified by flash column chromatography (1 : 1 → 8 : 2 EtOAc : PE, neutralized with 2% TEA) yielding compound **18** (0.5 g, 0.6 mmol, 77%). R_f = 0.5 (8 : 2 EtOAc : PE, 1% TEA); $[\alpha]^D$ = -6 (c = 1.0, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H), 7.74 (d, *J* = 7.4 Hz, 2H, CH, Ar), 7.58 (d, *J* = 4.1 Hz, 3H, CH, Ar), 7.43 (d, *J* = 7.7 Hz, 4H, CH, Ar), 7.41 – 7.19 (m, 14H, CH, Ar), 7.19 – 7.10 (m, 2H, CH, Ar), 7.05 (d, *J* = 7.9 Hz, 2H, CH, Ar), 4.47 – 4.25 (m, 4H, CH, Fmoc, CH_2, Fmoc, CH, α *i*-D-Gln,), 4.22 -4.14 (m, 1H, CH, α Lys), 2.37 – 2.21 (m, 5H, CH_3, Me Mtt, CH_2, γ *i*-D-Gln), 2.18 – 2.04 (m, 3H, CH_2, γ Lys, CH_2, β *i*-D-Gln), 1.98 – 1.83 (m, 2H, CH_2, β *i*-D-Gln), 1.80 – 1.62 (m, 1H, CH_2, β Lys), 1.61 – 1.53 (m, 1H, CH_2, β Lys), 1.55 – 1.30 (m, 13H, CH_3 ^{t}Bu, CH_2, δ Lys, CH_2, ε Lys); ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.5 (C=O), 173.1 (C=O), 171.8 (C=O), 156.6 (C=O) 145.9 (C_q), 143.5 (C_q), 143.3 (C_q), 142.8 (C_q), 140.9 (C_q), 135.3 (C_q), 128.2 (CH, Ar), 128.1 (CH, Ar), 127.3 (CH, Ar), 126.7 (CH, Ar), 125.8 (CH, Ar), 124.7 (CH, Ar), 119.6 (CH, Ar), 81.7 (C_q, Mtt), 77.3 (C_q, ^{t}Bu), 66.6 (CH_2, Fmoc), 53.4 (CH, α *i*-D-Gln), 53.0 (CH, α Lys), 46.8 (CH, Fmoc), 42.9 (CH_2, γ Lys), 31.9 (CH_2, γ *i*-D-Gln), 31.5 (CH_2, β Lys), 30.0 (CH_2, ε Lys), 29.2 (CH_2, β *i*-D-Gln), 27.5 (CH_3, ^{t}Bu), 23.0 (CH_2, δ Lys), 20.4 (CH_3, Me Mtt); IR (cm^{-1}): 3302, 2981, 1646, 1523, 1388; LC/ MS: Rt = 8.53 min (Alltima C_{18}, 10 – 90 MeCN); HRMS Calcd. for $[C_{50}H_{56}N_4O_6 + H]^+$ 809.42726, found 809.42802.

Fmoc-L-Ala-D-Gln(Lys(Mtt)-O^{t}Bu)-NH$_2$ (19)



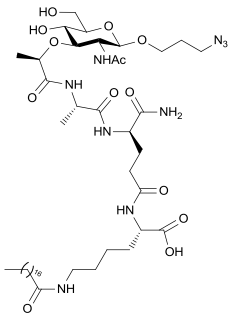
Compound **18** (0.5 g, 0.6 mmol) was dissolved in DCM (3 mL, 0.2 M) and treated with DBU (0.09 mL, 0.62 mmol). After 20 min HOBt (0.33 g, 0.62 mmol) was added. Added was a solution of HATU (0.26 g, 0.68 mmol), Dipea (0.61 mL, 3,72 mmol) and Fmoc-Ala-OH (0.21 g, 0.68 mmol) in DCM (3 mL, 0.2 M). The resulting solution was stirred for 18 h and reduced in volume. Purification by flash column chromatography (1 : 1 PE : EtOAc → 5% MeOH in EtOAc, 2% TEA) gave the title compound (0.38 g, 0.43 mmol, 70%). R_f = 0.1 (8 : 2 EtOAc : PE, 1% TEA); $[\alpha]^D$ = -4.0 (c = 0.1, 1 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 7.4 Hz, 1H, CH, Ar), 7.61 (d, *J* = 6.4 Hz, 1H, CH, Ar), 7.51 – 7.20 (m, 5H, CH, Ar), 7.20 – 7.10 (m, 1H, CH, Ar), 7.06 (d, *J* = 7.7 Hz, 1H, CH, Ar), 4.52 – 4.25 (m, 3H, CH_2, Fmoc, CH, Fmoc), 4.21 – 4.01 (m, 3H, CH, α *i*-D-Gln, CH, α Lys, CH, Ala), 2.31 – 2.26 (m, 5H, CH_3, Mtt, CH_2, γ *i*-D-Gln), 2.24 – 2.12 (m, 2H, CH_2, β *i*-D-Gln, CH_2, γ Lys), 2.06 – 1.95 (m, 1H, CH_2, β *i*-D-Gln), 1.81 – 1.68 (m, 1H, CH_2, β Lys), 1.65 – 1.58 (m, 1H, CH_2, β Lys), 1.55 – 1.40 (m, 11H, CH_3, ^{t}Bu, CH_2, ε Lys), 1.37 – 1.25 (m, 2H, CH_2, δ Lys), 1.25 (d, *J* = 7.2 Hz, 3H, CH_3, Ala); ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.2 (C=O), 173.7 (C=O), 173.1 (C=O), 171.9 (C=O), 156.3 (C=O), 145.8 (C_q), 143.3 (C_q), 142.6 (C_q), 140.8 (C_q), 135.2 (C_q), 128.1 (CH, Ar), 127.9 (CH, Ar), 127.2 (CH, Ar), 126.6 (CH, Ar), 125.7 (CH, Ar), 124.5 (CH, Ar), 119.4 (CH, Ar), 81.5 (C_q, Mtt), 70.1 (C_q, ^{t}Bu), 66.5 (CH_2, Fmoc), 52.8 (CH, Ala), 51.9 (CH, α Lys), 50.5 (CH, α *i*-D-Gln), 46.6 (CH, Fmoc), 42.8 (CH_2, γ Lys), 31.6 (CH_2, γ *i*-D-Gln), 31.2 (CH_2, β Lys), 29.7 (CH_2, ε Lys), 27.9 (CH_2, β *i*-D-Gln), 27.3 (CH_2, δ Lys), 22.9 (CH_3 ^{t}Bu), 20.2 (CH_3, Mtt), 13.4 (CH_3, Ala); IR (cm^{-1}): 3425, 3062, 1647, 1504; LC/MS: Rt = 8.57 min (Alltima C_{18}, 10 – 90 MeCN); HRMS Calcd. for $[C_{53}H_{61}N_5O_7 + H]^+$ 880.46438, found 880.46576.

3-Azidopropyl-2-*N*-acetamide-4,6-*O*-Aridene-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl-1-*O*-*tert*-butoxy-6-*N*-monomethoxytrityl-L-lysinyl)-2-deoxy-β-D-glucopyranoside (21)



Compound **19** (0.38 g, 0.43 mmol) dissolved in DMF (2 mL, 0.2 M) and was treated with DBU (0.06 mL, 0.43 mmol). After 20 min HOBt (0.23 g, 1.7 mmol) was added. Added a solution of HATU (0.16 g, 0.43 mmol), DiPEA (0.20 mL, 1.3 mmol) and compound **20** (0.22 g, 0.47 mmol) in (2 mL, 0.2 M). The resulting mixture was stirred for 18 h. The title compound was obtained by precipitation out of solution with Et_2O and recrystalization (DCM : MeOH : PE) (0.29 g, 0.26 mmol, 60%). R_f = 0.3 (8 : 2 $CHCl_3$: MeOH +2% AcOH); $[\alpha]^D$ = -10 (c = 0.5, 1: 1 $CHCl_3$: MeOH); ^1H NMR (400 MHz, DMSO-D_6) δ 8.41 (d, *J* = 4.2, Hz, 1H, NH), 8.31 (s, 1H, NH), 8.22 (dd, *J* = 8.4, 1.3 Hz, 1H, NH), 8.10 (d, *J* = 8.1 Hz, 2H, NH_2), 8.06 (d, *J* = 7.4 Hz, 2H, NH_2), 7.98 (d, *J* = 9.1 Hz, 2H, NH_2), 7.79 (d, *J* = 7.8 Hz, 2H, CH, Ar), 7.52 – 7.30 (m, 2H, CH, Ar), 7.30 – 7.19 (m, 11H, CH, Ar), 7.19 – 7.13 (m, 2H, CH, Ar), 7.07 (d, *J* = 7.9 Hz, 2H, CH, Ar), 5.69 (s, 1H, CH, benzylidine acetal), 4.48 (d, *J* = 8.2 Hz, 1H, CH, H-1), 4.31 – 4.18 (m, 2H, CH_2, lactic acid, CH_2, Ala), 4.07 – 3.11(under H_2O peek, 6H, CH, α *i*-D-Gln, CH, α Lys, CH_2, $C_3H_6N_3$, CH_2, H-6), 2.69 – 2.64 (m, 2H, CH_2, $C_3H_6N_3$), 2.24 (s, 3H, CH_3, Mtt), 2.18 – 2.08 (m 2H, CH_2, δ Lys), 1.91 – 1.85(m, 4H, CH_2, γ *i*-D-Gln, CH_2, β Lys), 1.81 (s, 3H, CH_3, NAc), 1.77 – 1.45 (m, 10H, CH_2, γ Lys, CH_2, ε Lys, CH_2, β *i*-D-Gln, CH_2, $C_3H_6N_3$), 1.35 (s, 9H, CH_3, ^{t}Bu), 1.26 – 1.15 (m, 6H, CH_3, lactic acid, CH_3, Ala); ^{13}C NMR (101 MHz, DMSO-D_6) δ 173.2 (C=O), 171.9 (C=O), 171.5 (C=O), 169.7 (C=O), 165.4 (C=O), 146.4 (C_q), 143.3 (C_q), 137.6 (C_q), 135.0 (C_q), 128.8 (CH, Ar), 128.3 (CH, Ar), 128.2 (CH, Ar), 128.2 (CH, Ar), 127.6 (CH, Ar), 127.4 (CH, Ar), 125.94 (CH, Ar), 125.8 (CH, Ar), 124.7 (CH, Ar), 123.3 (CH, Ar), 119.1 (CH, Ar), 118.5 (CH, Ar), 110.5 (CH, Ar), 101.5 (CH, H-1), 100.1 (CH, benzylidine acetal), 80.3 (CH, C-3), 78.9 (CH, α lactic acid), 77.3 (CH, C-5), 70.1 (C_q, Mtt), 65.7 (C_q, ^{t}Bu), 65.6 (CH, C-4), 54.7 (CH, C-2), 53.4 (CH_2, $C_3H_6N_3$), 52.6 (CH, α *i*-D-Gln), 52.2 (CH, α Lys), 48.1 (CH, Ala), 47.9 (CH_2, $C_3H_6N_3$), 47.5 (CH_2, C-6), 37.7 (CH_2, γ Lys), 31.7 (CH_2, $C_3H_6N_3$), 28.3 (CH_2, γ *i*-D-Gln), 27.7 (CH_2, β Lys), 25.9 (CH_2, ε Lys), 23.4 (CH_2, β *i*-D-Gln), 23.0 (CH_3, NAc), 20.5 (CH_3, Mtt), 19.0 (CH_3, lactic acid), 18.9 (CH_2, δ Lys), 18.3 (CH_3, Ala); IR (cm^{-1}): 3101, 2098, 1647, 1527, 1384; LC/MS: Rt = 7.42 min (Alltima C_{18}, 10 – 90 MeCN); HRMS Calcd. for $[C_{59}H77N_9O_{12} + H]^+$ 1104.57645, found 1104.57742.

3-Azidopropyl-2-*N*-acetamide-3-*O*-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl-6-*N*-stearoyl-L-lysinyl)-2-deoxy-β-D-glucopyranoside (6)



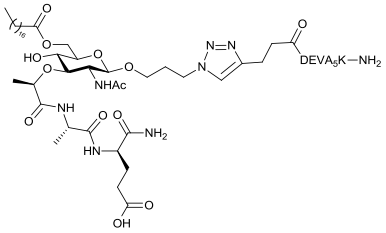
Compound **21** (71 mg, 0.06 mmol) was dissolved in DCM (3 mL, 0.02 M) with 3% TFA (0.06 mL) and TIS (0.06 mL, 2%). The mixture was stirred for 1.5 h. The crude compound was obtained to preciptate out of solution with Et_2O. To the crude mixture (83 mg, 0.07 mmol) dissolved in DMF (7 mL, 0.01 M) was added HATU (0.03 mg, 0.07 mmol), DiPEA (40 µL, 0.23 mmol) and stearic acid (19 mg, 77 µmol). The mixture was stirred for 18 h. The crude compound precipitated out of solution with Et_2O and re-crystalized (DCM : MeOH : Et_2O). Subsequently the crude compound (22 mg, 0.21 mmol) was dissolved in DCM (1.6 mL) with 20% TFA (0.4 mL) and 2.5% TIS (0.05 mL). The resulting mixture was stirred for 3 h. The compound was precipitated from the mixture by addition of Et_2O (2 mL). Purification by RP-HPLC/MS (Vidac C_4) gave compound **6** (2.9 mg, 3.0 µmol, 5% over 4 seps). R_f = 0.2 (8 : 2 $CHCl_3$: MeOH + 2% AcOH); $[\alpha]^D$ = -10.0 (c = 0.04, 1 : 1 $CHCl_3$: MeOH); ^1H NMR (600 MHz, DMSO-D_6) δ 8.20 (d, *J* = 7.6 Hz, 1H, NH, *i*-D-Gln), 7.79 (d, *J* = 9.0 Hz, 1H, NHAc), 7.72 (d, *J* = 7.6 Hz, 1H, NH, Ala), 7.59 (s, 1H, NH_2, amide *i*-D-Gln), 7.34 (s, 1H, OH), 7.26 (d, *J* = 7.0 Hz, 1H, NH, Lys), 6.84 (s, 1H, OH), 4.29 (d, *J* = 8.3 Hz, 1H, CH, H-1), 4.28 – 4.17 (m, 2H, CH, Ala, CH, lactic acid), 4.16 – 4.09 (m, 1H, CH, α *i*-D-Gln), 3.85 (d, *J* = 5.6 Hz, 1H, CH, α Lys), 3.76 – 3.74 (m,

1H, CH_2, $C_3H_6N_3$), 3.69 – 3.65 (m, 1H, CH_2, H-6), 3.61 – 3.51 (m, 2H, CH, H-2, CH_2, H-6), 3.51 – 3.41 (m, 2H, CH, H-3, CH_2, $C_3H_6N_3$), 3.37 – 3.22 (m, 3H, CH_2, δ Lys, CH, H-4), 3.16 – 3.13 (m, 1H, CH, H-5), 3.00 – 2.95 (m, 2H, CH_2, $C_3H_6N_3$), 2.21 – 2.15 (m, 2H, CH_2, γ Lys), 2.02 (t, *J* = 7.5 Hz, 2H, CH_2, γ *i*-D-Gln), 1.99 – 1.91 (m, 1H, CH_2, β *i*-D-Gln), 1.88 – 1.79 (m, 1H, CH_2, β *i*-D-Gln), 1.77 (s, 3H, CH_3, NAc), 1.76 – 1.72 (m, *J* = 13.0, 6.6 Hz, 2H, CH_2, ε Lys), 1.69 – 1.61 (m, 1H, CH_2, β Lys), 1.48 (m, 3H, CH_2, β Lys, CH_2, $C_3H_6N_3$), 1.39 – 1.31 (m, 2H, CH_2, stearoyl), 1.31 – 1.17 (m, 36H, CH_3, Ala, CH_3, lactic acid, CH_2, stearoyl), 0.86 (t, *J* = 6.9 Hz, 3H, CH_3, stearoyl); ^{13}C NMR (151 MHz, DMSO-D_6) δ 174.0 (C=O), 173.1 (C=O), 172.4 (C=O), 171.9 (C=O), 171.7 (C=O), 170.6 (C=O), 169.1 (C=O), 100.8 (CH, C-1), 81.61 (CH, C-3), 76.86 (CH, C-5), 76.41 (CH, lactic acid), 69.54 (CH, C-4), 64.97 (CH_2, $C_3H_6N_3$), 60.8 (CH_2, C-6), 54.3 (CH, C-2), 54.2 (CH, α Lys), 52.5 (CH, α *i*-D-Gln), 48.2 (CH, Ala), 47.5 (CH_2, δ Lys), 38.4 (CH_2, $C_3H_6N_3$), 35.3 (CH_2, γ *i*-D-Gln), 32.0 (CH_2, β Lys, CH_2, γ Lys), 31.1 (CH_2, stearoyl), 29.0 (CH_2, ε Lys), 28.8 (CH_2, stearoyl), 28.7 (CH_2, stearoyl), 28.7 (CH_2, stearoyl), 28.6 (CH_2, stearoyl), 28.5 (CH_2, stearoyl), 28.4 (CH_2, stearoyl), 28.3 (CH_2, stearoyl), 27.0 (CH_2, β *i*-D-Gln), 25.1 (CH_2, $C_3H_6N_3$), 22.8 (CH_3, NAc), 22.7 (CH_2, stearoyl), 21.8 (CH_2, stearoyl), 18.7 (CH_3, lactic acid), 17.7 (CH_3, Ala), 13.7 (CH_3, stearoyl); IR (cm^{-1}): 3280, 2850, 1635, 1543; LC/MS: Rt = 2.20 min (Alltima C_{18}, 70 – 90% MeCN, 15 min run); HRMS Calcd. for $[C_{46}H_{83}N_9O_{13} + H]^+$ 970.61831, found 970.61952.

Pentynoyl-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(Boc)-tentagel resin (22)

50 µmol resin loaded with NH_2-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(O^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-lys(Boc) was swollen in NMP. The resin was reacted with 4-pentynoic acid (24 mg, 0.25 mmol), HCTU (0.10 g, 0.25 mmol) and DiPEA (0.1 mL, 0.5 mmol) dissolved in NMP (0.5 mL, 0.1 M) for 16h. The resin was treated with Boc_2O (3 mL, 1 M in NMP) and DiPEA (0.2 µL, 0.1 mmol) for 2 h. A small aliquot of resin was cleaved from the resin with standard cleavage conditions. Compound **27** was formed on resin as confirmed by mass spectrometry. LC/MS : Rt = 6.39 min (C_{18} Alltima, 10 – 90% MeCN, 15 min run); ESI-MS: m/z 2626.38 $[M+H]^+$.

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-NH_2)-3-*O*-((R)-1-carboxyethyl-L-Ala-D-Gln(OH)-NH_2)-2-*N*-acetyl-6-O-stearoyl-D-glucopyranoside (23)



12.5 µmol resin loaded with pentynoyl-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(Boc) **22** was swollen in DMF. A stock solution of compound **13** (22.4 mg, 25 µmol), $CuSO_4$ (3.75 µmol, 37.5 µL, 100 mM) and sodium ascorbate (25 µmol, 125 µL, 200 mM) in DMF (0.5 mL, 0.03 M) was added to the resin and stirred for 6 days at 40^{o}C. Treating the resin with standard cleavage conditions for 60 min. and purification resulted title compound (14 mg, 4.0 µmol, 30%); LC/MS: Rt = 9.47 min (C_4 Vidac, 10 - 60% MeCN, 15 min run); ESI-MS: m/z 3467.89 $[M+H]^+$; HRMS Calcd. for $[C_{157}H_{260}N_{36}O_{51} + H]^{2+}$ 1734.45167, found 1734.45227.

Biological assays

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

Cell culture
For detailed information see the experimental section of Chapter 2.[28,29,30]

In vitro DC maturation assay
Test compounds were titrated in a 96-wells plate (Corning, Amsterdam, The Netherlands) in complete IMDM medium. Next, D1 cells, wild-type BMDC or TLR2 knockout BMDC from C57BL/6 mice were harvested and counted, and subsequently transferred to the 96-wells plates containing the test compound titrations, using approximately 40.000 cells per well. After 24 hours of incubation at 37°C, supernatant was taken from the wells for ELISA analysis (BioLegend, San Diego, USA) in which the amount of produced IL-12p40 was measured. After 48 hours of incubation at 37°C, the cells were stained for CD40 and CD86 (eBioscience, Vienna, Austria) for FACS analysis.

In vitro antigen presentation assay
For detailed information see the experimental section of Chapter 2.[30]

Flow cytometry analysis
Cells used for cell surface staining were washed with PBS-EDTA 5 mM and stained with monoclonal antibodies (eBioscience, Vienna, Austria) directed against CD40 and CD86 for 30 minutes. After washing away the antibodies, flow cytometry analyses were performed on a FACS Calibur cytometer (BD Biosciences, Breda, The Netherlands).

3.6 References and notes

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