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# Lipophilic Muramyl Dipeptide–Antigen Conjugates as Immunostimulating Agents

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Muramyl dipeptide (MDP) is the smallest peptidoglycan fragment capable of triggering the innate immune system through interaction with the intracellular NOD2 receptor. To develop synthetic vaccine modalities composed of an antigenic entity (typically a small peptide) and a molecular adjuvant with well-defined activity, we previously assembled covalent MDP–antigen conjugates. Although these were found to be capable of stimulating the NOD2 receptor and were processed by dendritic cells (DCs) leading to effective antigen presentation, DC maturation—required for an apt immune response—could not be achieved with these conjugates. To improve the efficacy of these vaccine modalities, we equipped the MDP moiety with lipophilic tails, well-known modifications to enhance the immune-stimulatory activity of MDPs. Herein we report the design and synthesis of a lipophilic MDP–antigen conjugate and show that it is a promising vaccine modality capable of stimulating the NOD2 receptor, maturing DCs, and delivering antigen cargo into the MHC-I cross-presentation pathway.

The development of agonists and antagonists to stimulate or block specific pathogen recognition receptors (PRRs) of the innate immune system is an important approach to modulate the mammalian immune system.<sup>[1]</sup> In the form of either stand-alone entities or as part of larger (synthetic) constructs, PRR agonists can be used as molecular adjuvants to trigger a well-defined innate immune response. A variety of different PRRs have been discovered over the years, including the families of Toll-like receptors (TLRs), RIG-like receptors (RLRs), C-type lectin receptors (CLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs), each of which recognize specific pathogen-associated molecular patterns (PAMPs). Many of these PAMPs are components of the bacterial cell wall, such as

lipopolysaccharides, lipoteichoic acids, lipoproteins, and peptidoglycan fragments. Although the exact mode of action of lipoteichoic acids is still under debate, it is well established that lipopolysaccharide exerts its activity through the binding of its core disaccharide (Lipid A) to TLR4. Similarly, lipoproteins and lipopeptides are known to stimulate TLR2, and the synthetic immunostimulatory agent *S*-(2,3-bisphosphatidyl)-*N*-palmitoylcysteine (Pam<sub>3</sub>Cys) is one of the most well-used triggering agents of the innate immune system.<sup>[2]</sup> Besides its use as an additive to various vaccine formulations, Pam<sub>3</sub>Cys has also found numerous applications as a covalently linked adjuvant. In particular, it has attracted considerable attention in the development of synthetic anticancer vaccines.<sup>[3]</sup> We previously showed that covalent attachment of Pam<sub>3</sub>Cys to a synthetic peptide antigen (both to ovalbumin as a model and relevant melanoma- and lymphoma-specific peptide sequences) can lead to enhanced antigen uptake, stimulation of dendritic cells (DCs), and increased antigen presentation by these cells.<sup>[4]</sup> In the same vein, we recently explored the use of muramyl dipeptide (MDP) in synthetic covalent molecular adjuvant–antigen conjugates to stimulate the NOD2 receptor.<sup>[5]</sup> MDP (**1**, Figure 1) is composed of *N*-acetylmuramic acid with an *L*-alanine-*D*-isoglutamine dipeptide attached to the muramic acid at the lactic acid moiety. It is the smallest peptidoglycan fragment recognized by the cytosolic NOD2 receptor and can serve as an innate immune system potentiator, although the molecular details behind the recognition of MDP by NOD2 are currently unclear.<sup>[6]</sup> Unfortunately, covalently linking MDP (either through the anomeric center of the muramic acid or the *D*-isoglutamine  $\gamma$ -carboxylate group) to a peptide antigen did not lead to a potent self-adjuvanting vaccine modality. Although we were able to show that the conjugates were taken up and properly processed by DCs leading to presentation of the incorporated MHC-I epitope, the constructs did not activate DCs.<sup>[5]</sup>

To improve the adjuvant properties of MDP, various derivatives have been generated and evaluated. These studies have revealed lipophilic MDP derivatives as potent immunostimulatory agents.<sup>[7]</sup> Initial work in this area was reported by Kusumoto and co-workers, who disclosed that the incorporation of a fatty acid at the C6 hydroxy group, as in 6-*O*-stearoyl-MDP **2** (Figure 1), leads to enhanced activity.<sup>[8]</sup> Over the years various potent MDPs have been developed, including the commercially available MDP derivatives romurtide (**3**), with an *N*<sup>6</sup>-stearoyl-*L*-lysine residue attached to the *D*-isoglutamine  $\gamma$ -carboxylate, and murabutide (**4**), featuring a butyl ester functionality.

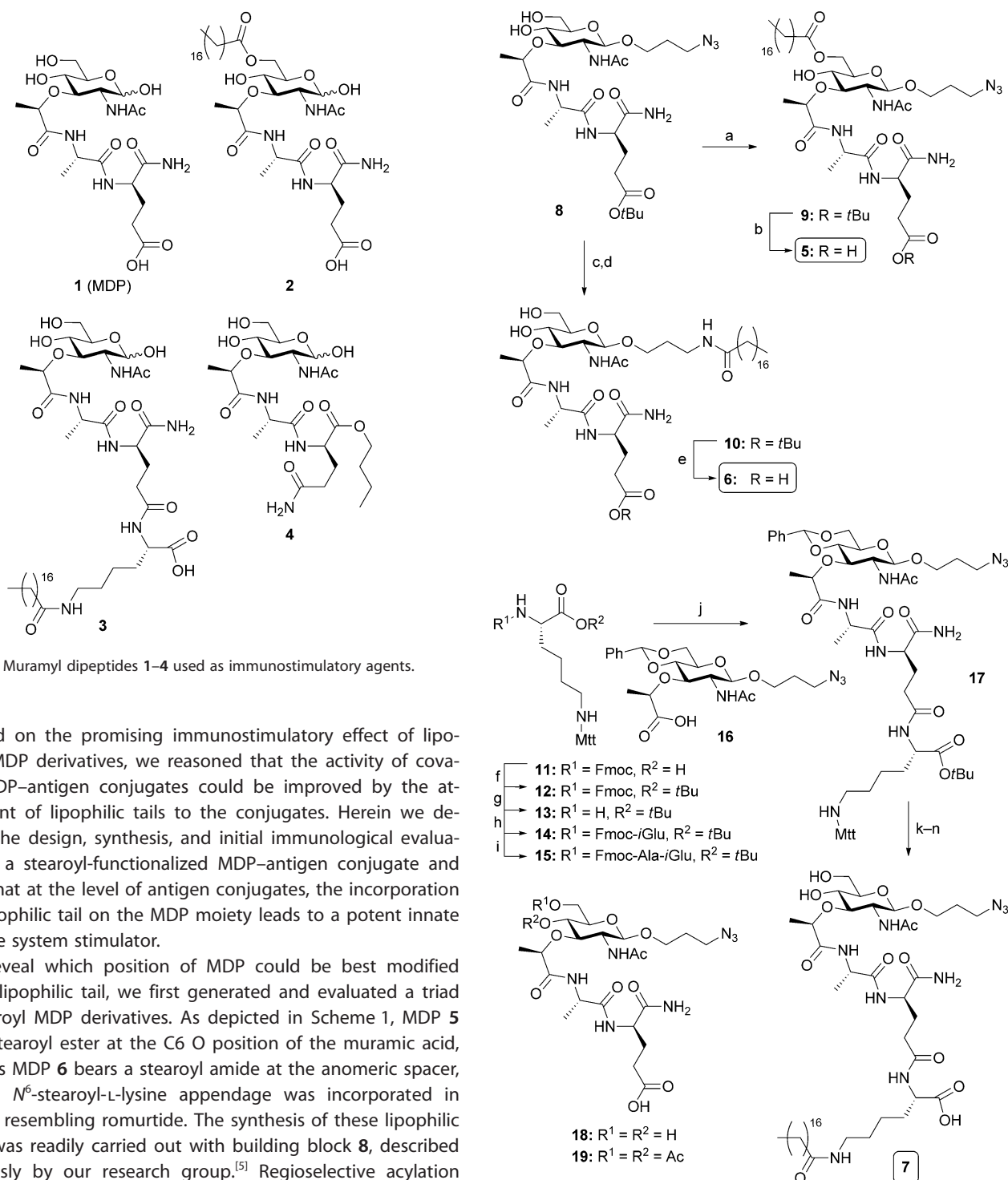
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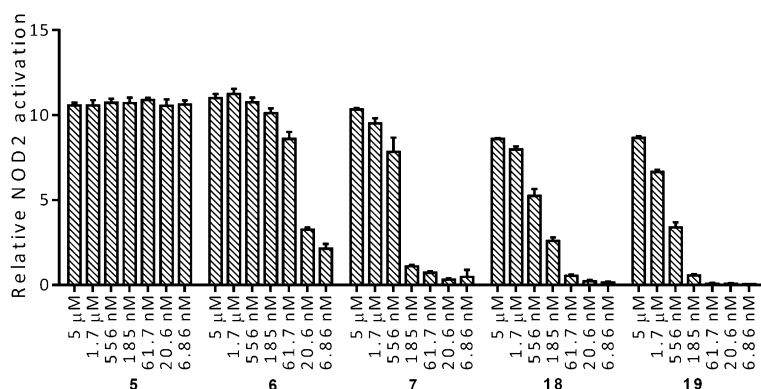


**Scheme 1.** Reagents and conditions: a) stearyl chloride (1.1 equiv), pyridine, CH<sub>2</sub>Cl<sub>2</sub>, RT, 1 h, 63%; b) TFA (20%), CH<sub>2</sub>Cl<sub>2</sub>, RT, 4 h, 74%; c) PMe<sub>3</sub>, THF, H<sub>2</sub>O, RT, 4 h; d) HATU, DiPEA, stearic acid, DMF, RT, 18 h, 90% (two steps); e) TFA (10%), CH<sub>2</sub>Cl<sub>2</sub>, RT, 5 h, 42%; f) Boc<sub>2</sub>O, DMAP (cat.), *t*BuOH, THF, RT, 18 h, quant.; g) DBU (cat.), octanethiol, CH<sub>2</sub>Cl<sub>2</sub>, RT, 3 h, 50%; h) HATU, DiPEA, Fmoc-D-iGln-OH, CH<sub>2</sub>Cl<sub>2</sub>, RT, 18 h, 77%; i) DBU, HOBt, Fmoc-Ala-OH, HATU, DiPEA, CH<sub>2</sub>Cl<sub>2</sub>, RT, 18 h, 70%; j) DBU, HOBt, MurNAc, HATU, DiPEA, DMF, RT, 18 h, 60%; k) TFA (3%), TIS (2%), RT, 1.5 h; l) stearic acid, HATU, DiPEA, DMF, RT, 18 h; m) TFA (20%), TIS (2.5%), CH<sub>2</sub>Cl<sub>2</sub>, RT, 3 h; n) RP-HPLC/MM, 5% (four steps).

quent trituration of the mixture with diethyl ether to give crude **6**.

After crystallization from a mixture of chloroform, methanol, and diethyl ether, MDP **6** was obtained in 42% yield. To obtain the third MDP derivative **7**, fully protected tripeptide **17** was synthesized by starting from Fmoc-lysine **11**. Fmoc-Lys(Mtt)-OH **11** was converted into *tert*-butyl ester **12** in quantitative yield, after which the Fmoc group was selectively removed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in the presence of octanethiol to give amine **13**. The condensation of **13** with *N*-Fmoc-D-isoglutamine under influence of HATU and DiPEA gave dipeptide **14** in 77% yield. In a one-pot procedure **14** was deprotected and condensed with Fmoc-L-alanine, resulting in fully protected tripeptide **15** in 70% yield after flash column chromatography. In a similar one-pot procedure peptide **15** was coupled with muramic acid **16**<sup>[5]</sup> to give fully protected MDP derivative **17**. The 4-methyltrityl (Mtt) group at the lysine side chain of **17** was removed with 3% TFA in dichloromethane. This acid treatment was accompanied by partial benzylidene cleavage. The crude mixture was treated with stearic acid, HATU, and DiPEA to allow acylation of the lysine amine residue. Subsequent treatment of the product with a solution of 20% TFA and 2.5% triisopropylsilane (TIS) in dry dichloromethane to remove the remaining benzylidene and *tert*-butyl ester gave target compound **6** in low yield after RP-HPLC purification. Notably, the overall yields of MDP derivatives **6** and **7** are influenced by hydrolysis of the anomeric functionality of the MDP moiety during the acidic reaction steps.<sup>[5]</sup> Placement of an electron-withdrawing acyl group at the C6 hydroxy group (as in **5**) of the MDP moiety protects the anomeric acetal from degradation.

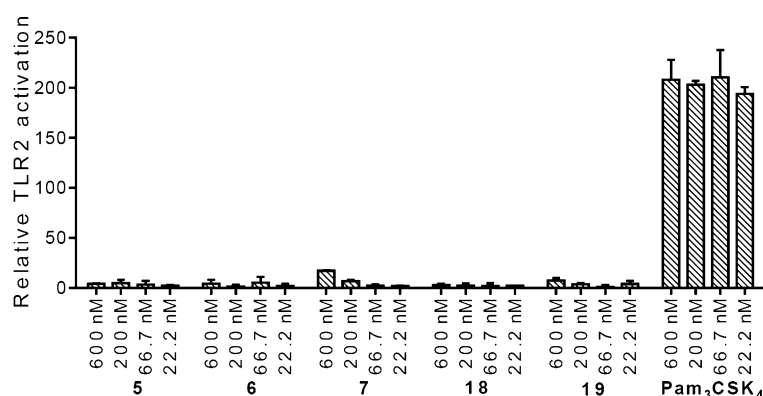
The immunostimulatory activity of the new lipophilic MDP derivatives **5**, **6**, and **7** together with the relevant reference compounds **18** and **19** (Scheme 1) was next determined. The NOD2 immunostimulatory potency of the MDP derivatives was assessed in a NOD2-transfected human embryonic kidney (HEK) cell line (HEK293). As shown in



**Figure 2.** NOD2-stimulatory activity of MDP derivatives **5**, **6**, **7**, **18** and **19**. Activation is depicted as the fold increase in IL-8 production over medium control. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in two additional experiments.

Figure 2, the lipophilic MDP derivatives **5** and **6** exhibit higher activity than the reference compounds without the stearyl group, in line with previous studies on romurtide (**3**) and other lipophilic MDP derivatives.<sup>[7–10]</sup> Lipophilic MDP derivative **7** proved to be less active than **5** and **6**. C6-O-stearyl MDP **5** appeared to be the most potent of the three lipophilic compounds. The increased activity of **5** and **6** may be related to improved uptake of the ligand, which results in greater availability of the ligand for the NOD2 receptor. None of the MDP derivatives induced activation of non-transfected control HEK293 cells (Supporting Information Figure S1).

Previously, TLR2 was indicated to play a role in the immunostimulatory activity of monoacyl MDP derivatives;<sup>[11]</sup> therefore, we evaluated **5–7** and **18** alongside Pam<sub>3</sub>Cys (a TLR2-dependent agonist) on TLR2-transfected HEK cells. From Figure 3 it is clear that the lipophilic MDP derivatives are unable to

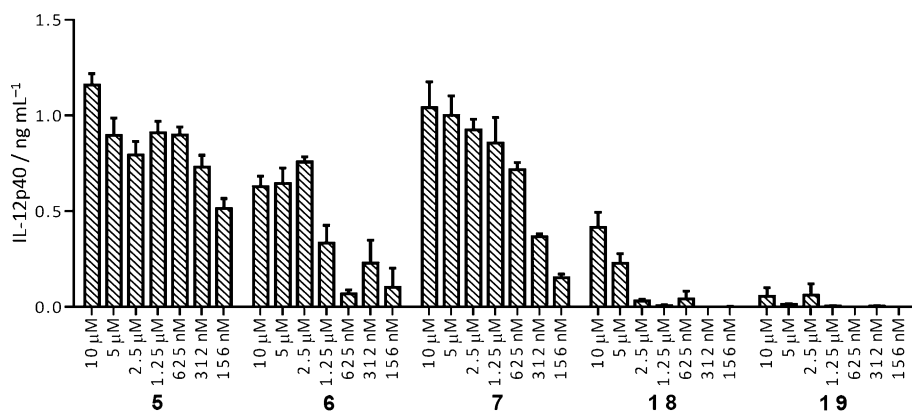


**Figure 3.** TLR2-stimulatory activity of MDP derivatives **5**, **6**, **7**, **18** and **19**. Activation is depicted as the fold increase in IL-8 production over medium control. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in two additional experiments.

stimulate the TLR2 HEK cells in the production of pro-inflammatory cytokines, in contrast to Pam<sub>3</sub>Cys and TNF $\alpha$ . Together, the assays indicate that the lipophilic MDP derivatives can act as TLR2-independent immunostimulatory agents.

Next, the immunostimulatory activity of the lipophilic MDP derivatives on murine DCs from C57BL/6 mice was investigated using an IL-12 production assay (Figure 4). The results show the same trend as observed in the NOD2 HEK assay: the stearyl-containing MDP derivatives **5** and **6** are more potent than their non-lipophilic counterparts **18** and **19**. Lipophilic **7** also outperformed control compounds **18** and **19** in this assay. Again, MDP **5** appeared to be the most potent of the three lipophilic MDPs. The DC maturation potency of MDP derivatives **5–7** was corroborated by the ability of these to up-regulate the cell-surface markers CD40 and CD86 (Supporting Information Figure S2).

Overall, the immunological assays show that lipophilic MDP derivatives **5**, **6**, and **7** are more potent than the parent MDPs **18** and **19**. No involvement of



**Figure 4.** DC activation potency of MDP derivatives **5**, **6**, **7**, **18** and **19**. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in three additional experiments.

TLR2 could be detected for the compound. Lipophilic MDP derivative **5** shows the highest immunostimulatory activity of the series, and therefore we continued with the incorporation of this ligand into an MDP–antigen conjugate.

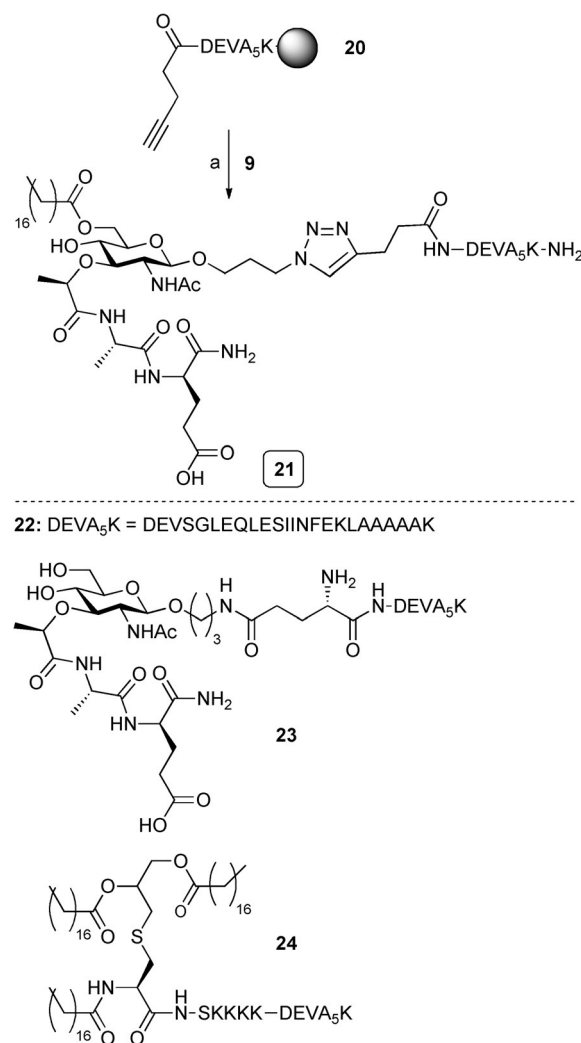
The design of conjugate **21**, in which the potent lipophilic MDP derivative **5** is connected to the antigenic DEVA<sub>5</sub>K peptide (an ovalbumin-derived antigenic peptide harboring the MHC-I epitope SIINFEKL), is based on our earlier findings that covalent attachment of a peptide epitope to the anomeric center of the sugar moiety in an MDP derivative does not interfere with its biological activity. The presence of the azide function in **9**, the protected precursor building block of **5**, allows the application of a copper-mediated ‘click’ reaction for conjugation to the antigenic peptide, functionalized with an alkyne reactive group.

To facilitate the removal of the copper salts required for the click reaction, we decided to perform the reaction on resin. The required immobilized peptide **20** was synthesized by functionalization of immobilized DEVA<sub>5</sub>K peptide with 4-pentynoic acid (Scheme 2). The key click reaction was executed by dissolving MDP building block **9** in *N,N*-dimethylformamide (DMF) followed by the addition of aqueous stock solutions of copper(II) sulfate (100 mM) and sodium ascorbate (200 mM) and addition to the resin, followed by heating at 40 °C. The progress of the click reaction was monitored by the cleavage and deprotection of aliquots of resin that were analyzed by LC–MS. The reaction required six days at 40 °C to reach completion. Finally, the immobilized conjugate was deprotected and cleaved from the resin using a mixture of 95% TFA, 2.5% TIS, and 2.5% H<sub>2</sub>O. The lipophilic MDP–antigen conjugate **21** was obtained by precipitation with diethyl ether and subsequent purification by RP–HPLC. The conjugate was obtained in 30% yield, which represents a major improvement over the yields we obtained for MDP–antigen conjugates lacking the C6 ester, because in these cases the acidic cleavage/deprotection conditions caused significant hydrolysis at the anomeric center of the MDP moiety.

The immunostimulatory activity of lipophilic MDP–antigen conjugate **21** was evaluated using the same assays as described in Figures 2–4. Thus, the NOD2-stimulatory activity of

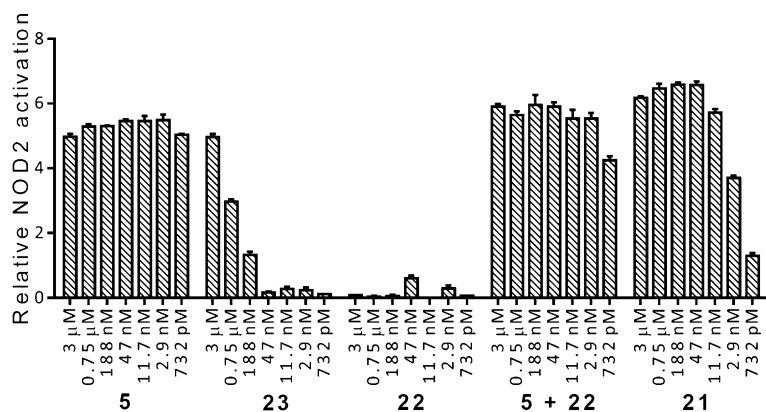
the conjugate was tested in NOD2-transfected HEK293 cells, and DC activation was evaluated by determining the level of IL-12 production upon stimulation of a murine DC cell line. In these assays we used the non-conjugated lipophilic MDP **5** and the peptide antigen **22** as control compounds. We also included the non-lipophilic MDP–antigenic peptide conjugate **23** we previously studied and Pam<sub>3</sub>Cys–antigen conjugate **24**, as reference compounds. Finally, the level of antigen presenta-

tion was assessed by exposing DCs to **21** in a SIINFEKL-specific T-cell hybridoma assay. The results of the NOD2 stimulation and DC activation assays are depicted in Figures 5 and 6, re-



**Scheme 2.** Reagents and conditions: a) 1. CuSO<sub>4</sub> (10%), sodium ascorbate, DMF, 60 °C, 6 days; 2. TFA (95%), TIS (2.5%), H<sub>2</sub>O (2.5%), RT, 1 h; 3. RP–HPLC 30%.





**Figure 5.** NOD2-stimulatory activity of the stearyl-MDP antigen conjugate. Activation is depicted as the fold increase in IL-8 production over medium control. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in two additional experiments.

spectively. Conjugates **21** and MDP **5** show similar levels of activity in the stimulation of NOD2-HEK293 cells, indicating that covalent attachment of the antigenic peptide to the MDP does not adversely affect the interaction with the NOD2 receptor. The stearyl tail on the MDP ligand has a beneficial effect on

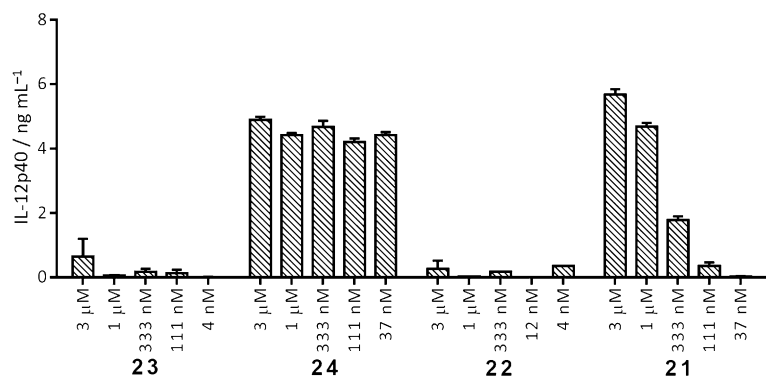
the activity of the conjugate as judged from the higher activity of **21** with respect to its non-lipophilic counterpart **23**. The activity of conjugate **21** is of similar magnitude as a mixture of MDP **5** and the antigenic peptide DEVA<sub>5</sub>K **22**.

The results of the DC stimulation assay, depicted in Figure 6, reveal that the lipophilic MDP-antigen conjugate **21**, in contrast to its inactive non-lipophilic counterpart **23**, is indeed capable of inducing the activation of DCs as judged from the amount of IL-12 production. With respect to Pam<sub>3</sub>Cys-DEVA<sub>5</sub>K conjugate **24**, the stearyl-MDP-DEVA<sub>5</sub>K conjugate shows somewhat diminished activity.

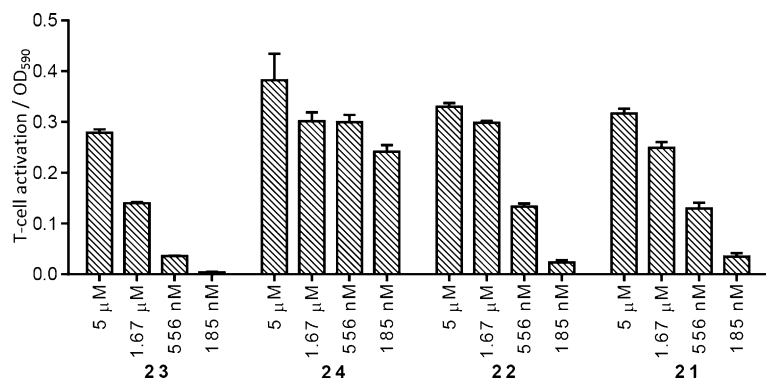
Finally, conjugate **21** was tested for its ability to induce MHC class I-mediated antigen presentation of the ovalbumin-derived SIINFEKL epitope by DCs. Figure 7 shows that the peptide of conjugate **21** is presented at a level similar to that of reference compounds **22–24**. Also in this assay the TLR2-based conjugate **24** is somewhat more active than conjugate **21**.

In summary, the synthesis and immunological evaluation of three lipophilic MDP derivatives (**5**, **6**, and **7**) were described, and the functionalized muramyl dipeptides were evaluated as

a starting point for the development of covalent MDP-antigen conjugates. The most potent of the three, MDP **5**, featuring a C6 *O*-stearyl ester and an anomeric azidopropyl handle, was conjugated using 'click' chemistry to the antigenic peptide DEVA<sub>5</sub>K to obtain a MDP-antigen conjugate **21**. Immunological evaluation of this conjugate showed the desired improvement in in vitro immunological potency relative to non-lipophilic MDP-antigen constructs described previously.<sup>[5]</sup> It appears that innate immune activation occurs through stimulation of the NOD2 receptor. On the basis of these favorable properties, conjugate **21** is a suitable candidate for follow-up research in human DCs and in vivo assays. It is also an excellent starting point to investigate conjugates that encompass multiple PRR ligands, capable of simultaneously triggering various types of receptors of the innate immune system.<sup>[12]</sup>



**Figure 6.** DC activation by the stearyl-MDP antigen conjugate. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in three additional experiments.



**Figure 7.** Antigen presentation by DC of the stearyl-MDP antigen conjugate. T-cell activation is depicted as OD values at  $\lambda$  590 nm. Error bars represent standard error of the mean of triplicates. Highly similar results were obtained in two additional experiments.

## Experimental Section

**3-Azidopropyl-2-*N*-acetamide-3-*O*-(*R*)-1-carboxyethyl-L-alanylacetamide-5-*O*-*tert*-butoxy-D-isoglutaminyl)-2-deoxy-6-*O*-stearyl- $\beta$ -D-glucopyranoside (**9**):** Compound **8** (0.21 g, 0.33 mmol) was dissolved in warm pyridine (1 mL) and diluted with CH<sub>2</sub>Cl<sub>2</sub> (2.3 mL, 0.05 M). A stock solution of stearic acid chloride in CH<sub>2</sub>Cl<sub>2</sub> (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<sub>3</sub>/MeOH 9:0 to 9:1) resulted in compound **9** as a white solid (91 mg, 0.10 mmol, 63%). *R*<sub>f</sub> = 0.6 (9:1 CHCl<sub>3</sub>/MeOH); [ $\alpha$ ]<sub>D</sub><sup>20</sup> = −6.5 (*c* = 0.34, 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (400 MHz, MeOD):  $\delta$  = 4.41–4.33 (m, 3H, CH, H-1, CH,  $\alpha$ -D-Gln, CH<sub>2</sub>, H-6), 4.31–4.20 (m, 2H, CH<sub>2</sub>, H-6,

CH, lactic acid), 4.23–4.16 (m, 1 H, CH, Ala), 3.93–3.85 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.83–3.74 (m, 1 H, CH, H-2), 3.63–3.57 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.53–3.41 (m, 3 H, C), 3.40–3.33 (m, 2 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.40–2.31 (m, 4 H, CH<sub>2</sub>, γ-D-iGln, CH<sub>2</sub>, stearyl), 2.28–2.15 (m, 1 H, CH, β-D-iGln), 1.94 (s, 3 H, CH<sub>3</sub>, NAc), 1.92–1.76 (m, 3 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH, β-D-iGln), 1.70–1.63 (m, 2 H, CH<sub>2</sub>, stearyl), 1.46 (s, 9 H, tBu), 1.42 (d, *J* = 6.1 Hz, 3 H, CH<sub>3</sub>, lactic acid), 1.37 (d, *J* = 6.7 Hz, 3 H, CH<sub>3</sub>, Ala), 1.35–1.21 (m, 28 H, CH<sub>2</sub>, stearyl), 0.89 ppm (t, *J* = 6.8 Hz, 3 H, CH<sub>3</sub>, stearyl); <sup>13</sup>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, C1), 81.4 (CH, C4), 80.7 (C<sub>q</sub>, tBu), 76.7 (CH, C3), 73.3 (CH, lactic acid), 69.2 (CH, C5), 65.6 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 63.1 (CH<sub>2</sub>, C6), 54.8 (CH, C2), 51.9 (CH, α-D-iGln), 49.0 (CH, Ala), 48.0 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 33.7 (CH<sub>2</sub>, stearyl), 31.5 (CH<sub>2</sub>, γ-D-iGln), 31.3 (CH<sub>2</sub>, stearyl), 29.2 (CH<sub>2</sub>, stearyl), 28.9 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 28.9 (CH<sub>2</sub>, stearyl), 28.7 (CH<sub>2</sub>, stearyl), 28.6 (CH<sub>2</sub>, stearyl), 27.4 (CH<sub>3</sub>, tBu), 26.5 (CH<sub>2</sub>, β-D-iGln), 24.5 (CH<sub>2</sub>, stearyl), 22.2 (CH<sub>2</sub>, stearyl), 22.2 (CH<sub>3</sub>, NAc), 18.1 (CH<sub>3</sub>, lactic acid), 16.5 (CH<sub>3</sub>, Ala), 13.5 ppm (CH<sub>3</sub>, stearyl); IR: ν̄ = 3282, 2916, 2850, 2098, 1635 cm<sup>-1</sup>; LC-MS: t<sub>R</sub> = 7.58 min (Alltima C<sub>4</sub>, 10–90% MeCN, 15 min run); HRMS calcd for [C<sub>44</sub>H<sub>79</sub>N<sub>7</sub>O<sub>12</sub> + H]<sup>+</sup> 898.58595, found: 898.58689.

**3-Azidopropyl-2-N-acetamide-3-O-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl)-2-deoxy-6-O-stearyl-β-D-glucopyranoside (5):** Compound **9** (31 mg, 35 μmol) was treated with a mixture of 20% TFA in CH<sub>2</sub>Cl<sub>2</sub> (0.35 mL, 0.1 M) and stirred for 4 h at room temperature. The solution was concentrated in vacuo, and the compound was purified by flash column chromatography (8:2 CHCl<sub>3</sub>/MeOH + 1% AcOH) to yield **5** (21 mg, 25 μmol, 74%). *R*<sub>f</sub> = 0.2 (9:1 CHCl<sub>3</sub>/MeOH); [α]<sub>D</sub><sup>20</sup> = −4.0 (*c* = 0.2, 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (600 MHz, MeOD): δ = 4.37 (d, *J* = 8.6 Hz, 1 H, CH, H-1), 4.26–4.21 (m, 1 H, CH, α-D-iGln), 4.19–4.16 (m, 1 H, CH, lactic acid), 3.92–3.88 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.80–3.76 (m, 1 H, CH, H-2), 3.62–3.53 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.50–3.41 (m, 3 H, CH, H-3, CH, H-4, CH, H-5), 3.19–3.15 (m, 2 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.45–2.37 (m, 2 H, CH<sub>2</sub>, stearyl), 2.34 (t, *J* = 7.7 Hz, 2 H, CH<sub>2</sub>, γ-D-iGln), 2.25–2.17 (m, 1 H, CH<sub>2</sub>, β-D-iGln), 1.93 (s, 3 H, CH<sub>3</sub>, NAc), 1.88–1.75 (m, 3 H, CH<sub>2</sub>, β-D-iGln, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 1.72–1.61 (m, 2 H, CH<sub>2</sub>, stearyl), 1.40 (d, *J* = 7.1 Hz, 3 H, CH<sub>3</sub>, lactic acid), 1.35 (d, *J* = 6.7 Hz, 3 H, CH<sub>3</sub>, Ala), 1.20–1.15 (m, 28 H, CH<sub>2</sub>, stearyl), 0.86 ppm (t, *J* = 7.0 Hz, 3 H, CH<sub>3</sub>, stearyl); <sup>13</sup>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, C1), 81.4 (CH, C4), 73.3 (CH, lactic acid), 69.2 (CH, C5), 65.7 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 63.1 (CH<sub>2</sub>, C6), 54.7 (CH, C2), 52.1 (CH, α-D-iGln), 49.0 (CH, Ala), 46.1 (CH<sub>2</sub>, stearyl), 33.7 (CH<sub>2</sub>, stearyl), 31.5 (CH<sub>2</sub>, stearyl), 29.9 (CH<sub>2</sub>, γ-D-iGln), 29.2 (CH<sub>2</sub>, stearyl), 29.0 (CH<sub>2</sub>, stearyl), 28.9 (CH<sub>2</sub>, stearyl), 28.8 (CH<sub>2</sub>, stearyl), 28.7 (CH<sub>2</sub>, stearyl), 28.6 (CH<sub>2</sub>, stearyl), 26.3 (CH<sub>2</sub>, β-D-iGln), 24.5 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 22.2 (CH<sub>2</sub>, stearyl), 22.2 (CH<sub>2</sub>, stearyl), 22.2 (CH<sub>3</sub>, NAc), 18.1 (CH<sub>3</sub>, lactic acid), 16.4 (CH<sub>3</sub>, Ala), 13.4 ppm (CH<sub>3</sub>, stearyl); IR: ν̄ = 3278, 2916, 2850, 2098, 1643 cm<sup>-1</sup>; LC-MS: t<sub>R</sub> = 4.22 min (Alltima C<sub>4</sub> Vidac, 10–90% MeCN, 15 min run); HRMS calcd for [C<sub>40</sub>H<sub>71</sub>N<sub>7</sub>O<sub>12</sub> + H]<sup>+</sup> 842.52335, found: 842.52397.

**Stearyl-(3-amidopropyl)-2-N-acetamide-2-deoxy-3-O-((R)-1-carboxyethyl-L-alanylacetamide-5-O-tert-butoxy-D-isoglutaminyl)-β-D-glucopyranoside (10):** To a stirred solution of compound **8** (0.27 g, 0.43 mmol) in THF (4 mL) was added H<sub>2</sub>O (0.4 mL) and PMe<sub>3</sub> (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<sub>3</sub> to 9:1 CHCl<sub>3</sub>/MeOH) and size-exclusion chromatography (LH-20, 1:1 CH<sub>2</sub>Cl<sub>2</sub>/MeOH) resulting in

compound **10** as a white solid (0.21 g, 0.24 mmol, 90%). *R*<sub>f</sub> = 0.5 (9:1 CHCl<sub>3</sub>/MeOH); [α]<sub>D</sub><sup>20</sup> = −4.4 (*c* = 0.5, 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (400 MHz, MeOD): δ = 4.42–4.32 (m, 2 H, H-1, CH, α-D-iGln), 4.21–4.11 (m, 2 H, CH, lactic acid, CH, Ala), 3.94–3.85 (m, 2 H, CH, H-6, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.85–3.69 (m, 2 H, CH, H-2, CH, H-6), 3.44 (m, 4 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH, H-3, CH, H-4), 3.32–3.30 (m, 1 H, CH, H-5), 3.21–2.99 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.34 (t, *J* = 7.5 Hz, 2 H, CH<sub>2</sub>, γ-D-iGln), 2.25–2.14 (m, 3 H, CH<sub>2</sub>, stearyl, CH β-D-iGln), 1.96 (s, 3 H, NAc), 1.94–1.83 (m, 1 H, CH, β-D-iGln), 1.80–1.65 (m, 2 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 1.64–1.56 (m, 2 H, CH<sub>2</sub>, stearyl), 1.50–1.40 (m, 12 H, CH<sub>3</sub>, tBu, CH<sub>3</sub>, lactic acid), 1.38 (d, *J* = 6.6 Hz, 3 H, CH<sub>3</sub>, Ala), 1.36–1.18 (m, 28 H, CH<sub>2</sub>, stearyl), 0.89 ppm (t, *J* = 6.5 Hz, 3 H, CH<sub>3</sub>, stearyl); <sup>13</sup>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, C1), 81.6 (CH, C3), 80.4 (C<sub>q</sub>, tBu), 76.5 (CH, lactic acid), 75.6 (CH, C5), 69.0 (CH, C4), 66.5 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 61.0 (CH<sub>2</sub>, C6), 54.5 (CH, C2), 51.72 (CH, α-D-iGln), 35.7 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 35.7 (CH<sub>2</sub>, stearyl), 31.3 (CH<sub>2</sub>, stearyl), 31.1 (CH<sub>2</sub>, γ-D-iGln), 29.0 (CH<sub>2</sub>, stearyl), 28.9 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 28.9 (CH<sub>3</sub>, tBu), 28.8 (CH<sub>2</sub>, β-D-iGln), 28.7 (CH<sub>2</sub>, stearyl), 26.3 (CH<sub>2</sub>, stearyl), 25.4 (CH<sub>3</sub>, NAc), 18.0 (CH<sub>3</sub>, lactic acid), 16.3 (CH<sub>3</sub>, Ala), 13.2 ppm (CH<sub>3</sub>, stearyl); IR: ν̄ = 2386, 2920, 2850, 1635, 1066 cm<sup>-1</sup>; LC-MS: t<sub>R</sub> = 6.70 min (CN Alltima, 10–90% MeCN, 15 min run); HRMS calcd for [C<sub>44</sub>H<sub>81</sub>N<sub>5</sub>O<sub>12</sub> + H]<sup>+</sup> 872.59545, found: 872.59691.

**Stearyl-(3-amidopropyl)-2-N-acetamide-2-deoxy-3-O-((R)-1-carboxyethyl-L-alanylacetamide-D-isoglutaminyl)-β-D-glucopyranoside (6):** Compound **10** (58 mg, 67 μmol) was dissolved in 10% TFA in CH<sub>2</sub>Cl<sub>2</sub> (4 mL, 0.02 M) and stirred for 5 h. The crude compound was precipitated out of solution (Et<sub>2</sub>O) and purified by flash column chromatography (CHCl<sub>3</sub>/MeOH 9:0 to 8:2 with 2% AcOH). The title compound **6** was obtained as a white solid (42 mg, 0.052 mmol, 42%). *R*<sub>f</sub> = 0.3 (9:1 CHCl<sub>3</sub>/MeOH + 1% AcOH); [α]<sub>D</sub><sup>20</sup> = −8.9 (*c* = 0.27, 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ = 4.37 (d, *J* = 8.4 Hz, 1 H, H-1), 4.30–4.50 (under H<sub>2</sub>O peek, 1 H, CH, lactic acid, CH, α-D-iGln), 4.25–4.21 (m, 1 H, CH Ala), 3.90–3.86 (m, 4 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH<sub>2</sub>, H-6, CH, H-2), 3.52–3.48 (m, 3 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH, H-3, CH, H-4), 3.35–3.32 (m, 2 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH, H-5), 3.20–3.16 (m, 1 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.40–2.35 (m, 2 H, CH<sub>2</sub>, γ-D-iGln), 2.23–2.16 (m, 3 H, CH<sub>2</sub>, stearyl, CH<sub>2</sub>, β-D-iGln), 1.98–1.86 (m, 7 H, CH<sub>3</sub>, NAc, CH<sub>2</sub>, stearyl, CH<sub>2</sub>, β-D-iGln), 1.73–1.83 (m, 2 H, CH<sub>2</sub>, stearyl), 1.65–1.61 (t, *J* = 7.2 Hz, 2 H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 1.46 (d, *J* = 7.2 Hz, 3 H, CH<sub>3</sub>, lactic acid), 1.43 (d, *J* = 7.2 Hz, 3 H, CH<sub>3</sub>, Ala), 1.31–1.24 (m, 18 H, CH<sub>2</sub>, stearyl), 0.89 ppm (t, *J* = 7.2 Hz, 3 H, CH<sub>3</sub>, stearyl); <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>): 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, C1), 81.8 (CH, C3), 76.5 (CH, Ala), 75.6 (CH, C5), 70.5 (CH, C4), 66.3 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 60.7 (CH<sub>2</sub>, C6), 52.4 (CH, α-D-iGln), 48.7 (CH, lactic acid), 47.1 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 35.5 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 35.4 (CH<sub>2</sub>, stearyl), 32.4 (CH<sub>2</sub>, γ-D-iGln), 31.2 (CH<sub>2</sub>, stearyl), 28.9 (CH<sub>2</sub>, stearyl), 28.9 (CH<sub>2</sub>, stearyl), 28.6 (CH<sub>2</sub>, stearyl), 28.6 (CH<sub>2</sub>, stearyl), 27.2 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 26.9 (CH<sub>2</sub>, β-D-iGln), 25.3 (CH<sub>2</sub>, stearyl), 21.9 (CH<sub>2</sub>, stearyl), 21.7 (CH<sub>3</sub>, NAc), 17.8 (CH<sub>3</sub>, lactic acid), 16.1 (CH<sub>3</sub>, Ala), 12.91 ppm (CH<sub>3</sub>, stearyl); IR: ν̄ = 3275, 2916, 2850, 1635, 1543 cm<sup>-1</sup>; LC-MS: t<sub>R</sub> = 6.173 min (Alltima CN, 10–90% MeCN, 15 min run); HRMS calcd for [C<sub>40</sub>H<sub>73</sub>N<sub>5</sub>O<sub>12</sub> + H]<sup>+</sup> 816.53285, found: 816.53265.

**Fmoc-Lys(Mtt)-OtBu (12):** To Fmoc-Lys(Mtt)-OH (**11**) (1.0 g, 1.6 mmol), dissolved in a mixture of tBuOH and THF (20 mL, 1:1, 0.1 M), was added Boc<sub>2</sub>O (0.45 mL, 2.1 mmol) and an catalytic amount of DMAP. After 18 h the solution was concentrated in vacuo to obtain the title compound **12** (1.0 g, 1.6 mmol) in quantitative yield. *R*<sub>f</sub> = 0.9 (8:2 EtOAc/PE + 0.5% TEA); [α]<sub>D</sub><sup>20</sup> = 4.2 (*c* = 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, [D<sub>6</sub>]DMSO): δ = 7.89 (d, *J* = 7.5 Hz, 2 H, CH, Ar), 7.72 (d, *J* = 7.4 Hz, 2 H, CH, Ar), 7.65 (d, *J* = 7.8 Hz, 2 H, 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<sub>2</sub>, Fmoc), 4.22 (t,  $J=7.0$  Hz, 1H, CH,  $\alpha$ -Lys), 3.95–3.83 (m, 1H, CH, Fmoc), 2.31 (s, 3H, CH<sub>3</sub>, Me), 1.97 (s, 1H, NH), 1.66–1.41 (m, 4H, CH<sub>2</sub>, Lys), 1.43–1.32 ppm (m, 11H, CH<sub>3</sub>, tBu, CH<sub>2</sub>, Lys); <sup>13</sup>C NMR (101 MHz, [D<sub>6</sub>]DMSO):  $\delta=172.1$  (C=O), 156.5 (C=O), 146.9 (C<sub>q</sub>, Ar), 144.3 (C<sub>q</sub>, Ar), 144.2 (C<sub>q</sub>, Ar), 143.7 (C<sub>q</sub>, Ar), 141.2 (C<sub>q</sub>, Ar), 135.4 (C<sub>q</sub>, 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<sub>q</sub>, tBu), 70.6 (CH<sub>2</sub>, Fmoc), 66.0, 54.8 (CH,  $\alpha$ -Lys), 47.1 (CH, Fmoc), 43.6 (CH<sub>2</sub>, Lys), 31.3 (CH<sub>2</sub>, Lys), 29.9 (CH<sub>2</sub>, Lys), 28.1 (CH<sub>3</sub>, tBu), 23.8 (CH<sub>2</sub>, Lys), 21.0 ppm (CH<sub>3</sub>, Me); IR:  $\tilde{\nu}=3333, 2974, 1600, 1450$  cm<sup>-1</sup>; LC-MS:  $t_R=9.45$  min (Alltima C<sub>18</sub>, 10–90 MeCN); HRMS calcd for [C<sub>46</sub>H<sub>83</sub>N<sub>9</sub>O<sub>13</sub>+H]<sup>2+</sup> 341.18798, found: 341.18405.

**NH<sub>2</sub>-Lys(Mtt)-OtBu (13):** Compound **12** (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 to 20% MeOH in EtOAc, neutralized with 2% TEA) to yield compound **13** (0.4 g, 0.8 mmol, 50%).  $R_f=0.1$  (8:2 EtOAc/PE, 1% TEA);  $[\alpha]_D^{20}=1.8$  ( $c=1.0$ , CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, MeOD):  $\delta=7.45$  (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<sub>2</sub>), 3.29 (t,  $J=6.3$  Hz, 1H, CH,  $\alpha$ -Lys), 2.28 (s, 3H, CH<sub>3</sub>, Me), 2.15 (t,  $J=6.7$  Hz, 2H, CH<sub>2</sub>, Lys), 1.72–1.58 (m, 2H, CH<sub>2</sub>, Lys), 1.58–1.49 (m, 4H, CH<sub>2</sub>, Lys), 1.45 (s, 9H, CH<sub>3</sub>, tBu), 1.43–1.34 ppm (m, 2H, CH<sub>2</sub>, Lys); <sup>13</sup>C NMR (101 MHz, MeOD):  $\delta=174.1$  (C=O), 145.7 (C<sub>q</sub>, Ar), 142.5 (C<sub>q</sub>, Ar), 134.9 (C<sub>q</sub>, Ar), 127.9 (CH, Ar), 127.7 (CH, Ar), 126.9 (CH, Ar), 125.4 (CH, Ar), 80.8 (C<sub>q</sub>, tBu), 69.9 (C<sub>q</sub>, Me), 53.6 (CH,  $\alpha$ -Lys), 42.7 (CH<sub>2</sub>, Lys), 33.9 (CH<sub>2</sub>, Lys), 29.7 (CH<sub>2</sub>, Lys), 26.9 (CH<sub>3</sub>, tBu), 22.5 (CH<sub>2</sub>, Lys), 19.8 ppm (CH<sub>3</sub>, Me); IR:  $\tilde{\nu}=3255, 3055, 2924, 1728, 1654, 1597$  cm<sup>-1</sup>; LC-MS:  $t_R=6.36$  min (Alltima C<sub>18</sub>, 10–90 MeCN); HRMS calcd for [C<sub>30</sub>H<sub>38</sub>N<sub>2</sub>O<sub>2</sub>+H]<sup>+</sup> 459.30061, found: 459.30052.

**Fmoc-D-Gln(Lys(Mtt)-OtBu)-NH<sub>2</sub> (14):** Compound **13** (0.4, 0.8 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (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-D-iGln-OH (0.33 g, 0.89 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (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 to 8:2 EtOAc/PE, neutralized with 2% TEA) to yield compound **14** (0.5 g, 0.6 mmol, 77%).  $R_f=0.5$  (8:2 EtOAc/PE, 1% TEA);  $[\alpha]_D^{20}=-6$  ( $c=1.0$ , 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta=7.94$  (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<sub>2</sub>, Fmoc, CH,  $\alpha$ -D-iGln), 4.22–4.14 (m, 1H, CH,  $\alpha$ -Lys), 2.37–2.21 (m, 5H, CH<sub>3</sub>, Me Mtt, CH<sub>2</sub>,  $\gamma$ -D-iGln), 2.18–2.04 (m, 3H, CH<sub>2</sub>,  $\gamma$ -Lys, CH<sub>2</sub>,  $\beta$ -D-iGln), 1.98–1.83 (m, 2H, CH<sub>2</sub>,  $\beta$ -D-iGln), 1.80–1.62 (m, 1H, CH<sub>2</sub>,  $\beta$ -Lys), 1.61–1.53 (m, 1H, CH<sub>2</sub>,  $\beta$ -Lys), 1.55–1.30 ppm (m, 13H, CH<sub>3</sub>, tBu, CH<sub>2</sub>,  $\delta$ -Lys, CH<sub>2</sub>,  $\epsilon$ -Lys); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>):  $\delta=174.5$  (C=O), 173.1 (C=O), 171.8 (C=O), 156.6 (C=O), 145.9 (C<sub>q</sub>), 143.5 (C<sub>q</sub>), 143.3 (C<sub>q</sub>), 142.8 (C<sub>q</sub>), 140.9 (C<sub>q</sub>), 135.3 (C<sub>q</sub>), 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<sub>q</sub>, Mtt), 77.3 (C<sub>q</sub>, tBu), 66.6 (CH<sub>2</sub>, Fmoc), 53.4 (CH,  $\alpha$ -D-iGln), 53.0 (CH,  $\alpha$ -Lys), 46.8 (CH, Fmoc), 42.9 (CH<sub>2</sub>,  $\gamma$ -Lys), 31.9 (CH<sub>2</sub>,  $\gamma$ -D-iGln), 31.5 (CH<sub>2</sub>,  $\beta$ -Lys), 30.0 (CH<sub>2</sub>,  $\epsilon$ -Lys), 29.2 (CH<sub>2</sub>,  $\beta$ -D-iGln), 27.5 (CH<sub>3</sub>, tBu), 23.0 (CH<sub>2</sub>,  $\delta$ -Lys), 20.4 ppm (CH<sub>3</sub>, Me Mtt); IR:  $\tilde{\nu}=3302, 2981, 1646, 1523, 1388$  cm<sup>-1</sup>; LC-MS:  $t_R=8.53$  min (Alltima C<sub>18</sub>, 10–90 MeCN); HRMS calcd for [C<sub>50</sub>H<sub>56</sub>N<sub>4</sub>O<sub>6</sub>+H]<sup>+</sup> 809.42726, found: 809.42802.

**Fmoc-Ala-D-Gln(Lys(Mtt)-OtBu)-NH<sub>2</sub> (15):** Compound **14** (0.5 g, 0.6 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (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. Then 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 CH<sub>2</sub>Cl<sub>2</sub> (3 mL, 0.2 M) was added. The resulting solution was stirred for 18 h and decreased in volume (to ~1.5 mL). Purification by flash column chromatography (1:1 PE/EtOAc to 5% MeOH in EtOAc, 2% TEA) gave the title compound **15** (0.38 g, 0.43 mmol, 70%).  $R_f=0.1$  (8:2 EtOAc/PE, 1% TEA);  $[\alpha]_D^{20}=-4.0$  ( $c=0.1$ , 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta=7.73$  (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<sub>2</sub>, Fmoc, CH, Fmoc), 4.21–4.01 (m, 3H, CH,  $\alpha$ -D-iGln, CH,  $\alpha$ -Lys, CH, Ala), 2.31–2.26 (m, 5H, CH<sub>3</sub>, Mtt, CH<sub>2</sub>,  $\gamma$ -D-iGln), 2.24–2.12 (m, 2H, CH<sub>2</sub>,  $\beta$ -D-iGln, CH<sub>2</sub>,  $\gamma$ -Lys), 2.06–1.95 (m, 1H, CH<sub>2</sub>,  $\beta$ -D-iGln), 1.81–1.68 (m, 1H, CH<sub>2</sub>,  $\beta$ -Lys), 1.65–1.58 (m, 1H, CH<sub>2</sub>,  $\beta$ -Lys), 1.55–1.40 (m, 11H, CH<sub>3</sub>, tBu, CH<sub>2</sub>,  $\epsilon$ -Lys), 1.37–1.25 (m, 2H, CH<sub>2</sub>,  $\delta$ -Lys), 1.25 ppm (d,  $J=7.2$  Hz, 3H, CH<sub>3</sub>, Ala); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>):  $\delta=174.2$  (C=O), 173.7 (C=O), 173.1 (C=O), 171.9 (C=O), 156.3 (C=O), 145.8 (C<sub>q</sub>), 143.3 (C<sub>q</sub>), 142.6 (C<sub>q</sub>), 140.8 (C<sub>q</sub>), 135.2 (C<sub>q</sub>), 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<sub>q</sub>, Mtt), 70.1 (C<sub>q</sub>, tBu), 66.5 (CH<sub>2</sub>, Fmoc), 52.8 (CH, Ala), 51.9 (CH,  $\alpha$ -Lys), 50.5 (CH,  $\alpha$ -D-iGln), 46.6 (CH, Fmoc), 42.8 (CH<sub>2</sub>,  $\gamma$ -Lys), 31.6 (CH<sub>2</sub>,  $\gamma$ -D-iGln), 31.2 (CH<sub>2</sub>,  $\beta$ -Lys), 29.7 (CH<sub>2</sub>,  $\epsilon$ -Lys), 27.9 (CH<sub>2</sub>,  $\beta$ -D-iGln), 27.3 (CH<sub>2</sub>,  $\delta$ -Lys), 22.9 (CH<sub>3</sub>, tBu), 20.2 (CH<sub>3</sub>, Mtt), 13.4 ppm (CH<sub>3</sub>, Ala); IR:  $\tilde{\nu}=3425, 3062, 1647, 1504$  cm<sup>-1</sup>; LC-MS:  $t_R=8.57$  min (Alltima C<sub>18</sub>, 10–90 MeCN); HRMS calcd for [C<sub>53</sub>H<sub>61</sub>N<sub>5</sub>O<sub>7</sub>+H]<sup>+</sup> 880.46438, found: 880.46576.

**3-Azidopropyl-2-N-acetamide-4,6-O-aridene-3-O-((R)-1-carboxy-ethylalanylacetamide-D-isoglutaminyl-1-O-tert-butoxy-6-N-monomethoxytritylsyl)-2-deoxy- $\beta$ -D-glucopyranoside (17):** Compound **15** (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. Then a solution of HATU (0.16 g, 0.43 mmol), DiPEA (0.20 mL, 1.3 mmol), and compound **16** (0.22 g, 0.47 mmol) in DMF (2 mL, 0.2 M) was added. The resulting mixture was stirred for 18 h. The title compound **17** was obtained by precipitation out of solution with Et<sub>2</sub>O and recrystallization (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/PE) (0.29 g, 0.26 mmol, 60%).  $R_f=0.3$  (8:2 CHCl<sub>3</sub>/MeOH + 2% AcOH);  $[\alpha]_D^{20}=-10$  ( $c=0.5$ , 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (400 MHz, [D<sub>6</sub>]DMSO):  $\delta=8.41$  (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<sub>2</sub>), 8.06 (d,  $J=7.4$  Hz, 2H, NH<sub>2</sub>), 7.98 (d,  $J=9.1$  Hz, 2H, NH<sub>2</sub>), 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, benzylidene acetal), 4.48 (d,  $J=8.2$  Hz, 1H, CH, H-1), 4.31–4.18 (m, 2H, CH<sub>2</sub>, lactic acid, CH<sub>2</sub>, Ala), 4.07–3.11 (under H<sub>2</sub>O peek, 6H, CH,  $\alpha$ -D-iGln, CH,  $\alpha$ -Lys, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>, CH<sub>2</sub>, H-6), 2.69–2.64 (m, 2H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.24 (s, 3H, CH<sub>3</sub>, Mtt), 2.18–2.08 (m, 2H, CH<sub>2</sub>,  $\delta$ -Lys), 1.91–1.85 (m, 4H, CH<sub>2</sub>,  $\gamma$ -D-iGln, CH<sub>2</sub>,  $\beta$ -Lys), 1.81 (s, 3H, CH<sub>3</sub>, NAc), 1.77–1.45 (m, 10H, CH<sub>2</sub>,  $\gamma$ -Lys, CH<sub>2</sub>,  $\epsilon$ -Lys, CH<sub>2</sub>,  $\beta$ -D-iGln, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 1.35 (s, 9H, CH<sub>3</sub>, tBu), 1.26–1.15 ppm (m, 6H, CH<sub>3</sub>, lactic acid, CH<sub>3</sub>, Ala); <sup>13</sup>C NMR (101 MHz, [D<sub>6</sub>]DMSO):  $\delta=173.2$  (C=O), 171.9 (C=O), 171.5 (C=O), 169.7 (C=O), 165.4 (C=O), 146.4 (C<sub>q</sub>), 143.3 (C<sub>q</sub>), 137.6 (C<sub>q</sub>), 135.0 (C<sub>q</sub>), 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, benzylidene acetal), 80.3 (CH, C3), 78.9 (CH,  $\alpha$ -lactic acid), 77.3 (CH, C5), 70.1 (C<sub>q</sub>, Mtt), 65.7 (C<sub>q</sub>, tBu), 65.6 (CH, C4), 54.7 (CH, C2), 53.4 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 52.6 (CH,  $\alpha$ -D-iGln), 52.2 (CH,  $\alpha$ -Lys), 48.1 (CH, Ala), 47.9 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 47.5 (CH<sub>2</sub>, C6), 37.7 (CH<sub>2</sub>,  $\gamma$ -Lys), 31.7 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>),



28.3 (CH<sub>2</sub>, γ-D-iGln), 27.7 (CH<sub>2</sub>, β Lys), 25.9 (CH<sub>2</sub>, ε Lys), 23.4 (CH<sub>2</sub>, β-D-iGln), 23.0 (CH<sub>3</sub>, NAc), 20.5 (CH<sub>3</sub>, Mtt), 19.0 (CH<sub>3</sub>, lactic acid), 18.9 (CH<sub>2</sub>, δ Lys), 18.3 ppm (CH<sub>3</sub>, Ala); IR:  $\tilde{\nu}$  = 3101, 2098, 1647, 1527, 1384 cm<sup>-1</sup>; LC-MS:  $t_R$  = 7.42 min (Alltima C<sub>18</sub>, 10–90 MeCN); HRMS calcd for [C<sub>59</sub>H<sub>77</sub>N<sub>9</sub>O<sub>12</sub> + H]<sup>+</sup> 1104.57645, found: 1104.57742.

**3-Azidopropyl-2-N-acetamide-3-O-((R)-1-carboxyethyl-L-alanyl-acetamide-D-isoglutaminyl-6-N-stearoyl-L-lysiny)-2-deoxy-β-D-glucopyranoside (7):** Compound **17** (71 mg, 0.06 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (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 by precipitation by the addition of Et<sub>2</sub>O. 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 was precipitated from the solution by the addition of Et<sub>2</sub>O and re-crystallized (CH<sub>2</sub>Cl<sub>2</sub>/MeOH/Et<sub>2</sub>O). Subsequently the crude compound (22 mg, 0.21 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (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 the addition of Et<sub>2</sub>O (2 mL). Purification by RP-HPLC-MS (Vidac C<sub>4</sub>) gave compound **7** (2.9 mg, 3.0 μmol, 5% over four steps).  $R_f$  = 0.2 (8:2 CHCl<sub>3</sub>/MeOH + 2% AcOH); [ $\alpha$ ]<sub>D</sub><sup>20</sup> = -10.0 (c = 0.04, 1:1 CHCl<sub>3</sub>/MeOH); <sup>1</sup>H NMR (600 MHz, [D<sub>6</sub>]DMSO):  $\delta$  = 8.20 (d,  $J$  = 7.6 Hz, 1H, NH, D-iGln), 7.79 (d,  $J$  = 9.0 Hz, 1H, NHAc), 7.72 (d,  $J$  = 7.6 Hz, 1H, NH, Ala), 7.59 (s, 1H, NH<sub>2</sub>, amide D-iGln), 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-D-iGln), 3.85 (d,  $J$  = 5.6 Hz, 1H, CH, α Lys), 3.76–3.74 (m, 1H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.69–3.65 (m, 1H, CH<sub>2</sub>, H-6), 3.61–3.51 (m, 2H, CH, H-2, CH<sub>2</sub>, H-6), 3.51–3.41 (m, 2H, CH, H-3, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 3.37–3.22 (m, 3H, CH<sub>2</sub>, δ Lys, CH, H-4), 3.16–3.13 (m, 1H, CH, H-5), 3.00–2.95 (m, 2H, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 2.21–2.15 (m, 2H, CH<sub>2</sub>, γ Lys), 2.02 (t,  $J$  = 7.5 Hz, 2H, CH<sub>2</sub>, γ-D-iGln), 1.99–1.91 (m, 1H, CH<sub>2</sub>, β-D-iGln), 1.88–1.79 (m, 1H, CH<sub>2</sub>, β-D-iGln), 1.77 (s, 3H, CH<sub>3</sub>, NAc), 1.76–1.72 (m,  $J$  = 13.0, 6.6 Hz, 2H, CH<sub>2</sub>, ε Lys), 1.69–1.61 (m, 1H, CH<sub>2</sub>, β Lys), 1.48 (m, 3H, CH<sub>2</sub>, β Lys, CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 1.39–1.31 (m, 2H, CH<sub>2</sub>, stearoyl), 1.31–1.17 (m, 36H, CH<sub>3</sub>, Ala, CH<sub>3</sub>, lactic acid, CH<sub>2</sub>, stearoyl), 0.86 ppm (t,  $J$  = 6.9 Hz, 3H, CH<sub>3</sub>, stearoyl); <sup>13</sup>C NMR (151 MHz, [D<sub>6</sub>]DMSO):  $\delta$  = 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, C1), 81.61 (CH, C3), 76.86 (CH, C5), 76.41 (CH, lactic acid), 69.54 (CH, C4), 64.97 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 60.8 (CH<sub>2</sub>, C6), 54.3 (CH, C2), 54.2 (CH, α Lys), 52.5 (CH, α-D-iGln), 48.2 (CH, Ala), 47.5 (CH<sub>2</sub>, δ Lys), 38.4 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 35.3 (CH<sub>2</sub>, γ-D-iGln), 32.0 (CH<sub>2</sub>, β Lys, CH<sub>2</sub>, γ Lys), 31.1 (CH<sub>2</sub>, stearoyl), 29.0 (CH<sub>2</sub>, ε Lys), 28.8 (CH<sub>2</sub>, stearoyl), 28.7 (CH<sub>2</sub>, stearoyl), 28.7 (CH<sub>2</sub>, stearoyl), 28.6 (CH<sub>2</sub>, stearoyl), 28.5 (CH<sub>2</sub>, stearoyl), 28.4 (CH<sub>2</sub>, stearoyl), 28.3 (CH<sub>2</sub>, stearoyl), 27.0 (CH<sub>2</sub>, β-D-iGln), 25.1 (CH<sub>2</sub>, C<sub>3</sub>H<sub>6</sub>N<sub>3</sub>), 22.8 (CH<sub>3</sub>, NAc), 22.7 (CH<sub>2</sub>, stearoyl), 21.8 (CH<sub>2</sub>, stearoyl), 18.7 (CH<sub>3</sub>, lactic acid), 17.7 (CH<sub>3</sub>, Ala), 13.7 ppm (CH<sub>3</sub>, stearoyl); IR:  $\tilde{\nu}$  = 3280, 2850, 1635, 1543 cm<sup>-1</sup>; LC-MS:  $t_R$  = 2.20 min (Alltima C<sub>18</sub>, 70–90% MeCN, 15 min run); HRMS calcd for [C<sub>46</sub>H<sub>83</sub>N<sub>9</sub>O<sub>13</sub> + H]<sup>+</sup> 970.61831, found: 970.61952.

**Pentynoyl-Asp(OrBu)-Glu(OrBu)-Val-Ser(OrBu)-Gly-Leu-Glu(OrBu)-Gln(Trt)-Leu-Glu(OrBu)-Ser(Trt)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-(Ala)<sub>5</sub>-Lys(Boc)-tentagel resin (20):** 50 μmol resin loaded with NH<sub>2</sub>-Asp(OrBu)-Glu(OrBu)-Val-Ser(OrBu)-Gly-Leu-Glu(OrBu)-Gln(Trt)-Leu-Glu(OrBu)-Ser(OrBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-(Ala)<sub>5</sub>-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 16 h. Capping was performed by treating the resin with Boc<sub>2</sub>O (3 mL, 1 M in NMP) and DiPEA (0.2 μL, 0.1 mmol) for 2 h. A small aliquot of resin

was cleaved under standard cleavage conditions confirming the formation of the pentynoylated peptide.

**1-β-(3-Azidopropyltriazole-ethyl-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-(Ala)<sub>5</sub>-Lys-NH<sub>2</sub>)-3-O-((R)-1-carboxyethyl-L-Ala-D-Gln(OH)-NH<sub>2</sub>)-2-N-acetyl-6-O-stearoyl-D-glucopyranoside (21):** 12.5 μmol resin loaded with pentynoyl-Asp(OrBu)-Glu(OrBu)-Val-Ser(OrBu)-Gly-Leu-Glu(OrBu)-Gln(Trt)-Leu-Glu(OrBu)-Ser(Trt)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-(Ala)<sub>5</sub>-Lys(Boc) 20 was swollen in DMF. A stock solution of compound **9** (22.4 mg, 25 μmol), CuSO<sub>4</sub> (3.75 μmol, 37.5 μL, 100 mM in H<sub>2</sub>O) and sodium ascorbate (25 μmol, 125 μL, 200 mM in H<sub>2</sub>O) in DMF (0.5 mL, 0.03 M) was added to the resin and stirred for six days at 40 °C. Treating the resin with standard cleavage conditions for 60 min and purification resulted in title compound **21** (14 mg, 4.0 μmol, 30%); LC-MS:  $t_R$  = 9.47 min (C<sub>4</sub> Vidac, 10–60% MeCN, 15 min run); ESI-MS:  $m/z$  3467.89 [M + H]<sup>+</sup>; HRMS calcd for [C<sub>157</sub>H<sub>260</sub>N<sub>36</sub>O<sub>51</sub> + H]<sup>2+</sup> 1734.45167, found: 1734.45227.

**Cell culture:** The D1 cell line is a growth-factor-dependent immature spleen-derived DC cell line from C57BL/6 (H-2b) mice. D1 cells were cultured as described.<sup>[13]</sup> The B3Z hybridoma is cultured in complete IMDM supplemented with 500 μg mL<sup>-1</sup> hygromycin.<sup>[14]</sup> HEK293 cells stably transfected with NOD2 or TLR2 (Invivogen, Toulouse, France) were cultured in complete IMDM supplemented with 10 μg mL<sup>-1</sup> blasticidin (NOD2) or 500 μg mL<sup>-1</sup> geneticin (TLR2).

**NOD2-HEK293 activation:** Test compounds were titrated in a 96-well plate, and ~50 000 NOD2-HEK293 cells were subsequently added per well. After 24 h of incubation at 37 °C, the supernatant was taken from all wells. The amount of IL-8 produced by the NOD2-HEK293 cells is a measure of NOD2-mediated activation. The concentration of IL-8 in the supernatant was determined using an IL-8 ELISA kit (Sanquin, Amsterdam, The Netherlands).

**In vitro DC stimulation assay:** Test compounds were titrated in a 96-well plate (Corning, Amsterdam, The Netherlands) in complete IMDM. Next, D1 cells from C57BL/6 mice were harvested and counted, and subsequently transferred to the 96-well plates containing the test compound titrations, using ~40 000 cells per well. After 24 h of incubation at 37 °C, the supernatant was taken from the wells for ELISA analysis (BioLegend, San Diego, CA, USA) in which the amount of IL-12p40 produced was measured. After 48 h of stimulation, the cells were stained with fluorescently labeled antibodies (eBioscience, Vienna, Austria) directed against co-stimulatory markers CD86 and CD40 and analyzed by flow cytometry.

**Cytokine ELISA:** To determine the concentrations of murine and human cytokines in culture supernatants, we made use of an enzyme-linked immunosorbent assay (ELISA). In short, NUNC Maxi-Sorp plates were coated overnight at 4 °C with a purified antibody specific for either human IL-8 (3.5 μg mL<sup>-1</sup>; clone BH0814, BioLegend) or murine IL-12p40 (1 μg mL<sup>-1</sup>; clone C15.6, BioLegend). The next day plates were washed with PBS with 0.05% Tween 20, and subsequently blocked for 1 h at 37 °C using PBS containing 1% BSA and 0.05% Tween 20. The plates were washed, and 50 μL supernatant or recombinant protein standard was added to each well. After incubation for 1.5 h at 37 °C, the plates were washed again, and 50 μL of biotinylated antibody (2 μg mL<sup>-1</sup>) specific for either human IL-8 (clone BH0840, BioLegend) or murine IL-12p40 (clone C17.8, BioLegend) was added to all wells. The plates were incubated for 1 h at RT and subsequently washed. Next, 50 μL of diluted streptavidin-HRP (BioLegend) was added according to the manufacturer's instructions. After 30 min incubation at RT, the plates were washed, and TMB substrate (Sigma-Aldrich) was added to all wells. The blue colorization process was stopped by

the addition of H<sub>2</sub>SO<sub>4</sub>. The colorization was measured spectrophotometrically at  $\lambda$  450 nm.

**In vitro antigen presentation assay:** B3Z is a CD8<sup>+</sup> T-cell hybridoma specific for the H-2K<sup>b</sup> CTL epitope SIINFEKL of ovalbumin. B3Z expresses the *lacZ* reporter gene of *Escherichia coli*, which is under the regulation of the NFAT element from the IL-2 promoter. Therefore, TCR triggering of this T cell leads to transcription of the *lacZ* reporter gene, the product of which is able to convert the chromogenic substrate CPRG (chlorophenol red- $\beta$ -D-galactopyranoside). This conversion was measured by absorbance spectrophotometry at  $\lambda$  590 nm.<sup>[14]</sup> Experimentally, 50 000 DCs per well were loaded overnight with the indicated compounds in titrating doses. The following day, the compounds were washed from the DC using complete culture medium. The B3Z hybridoma cells were added to all wells at 50 000 cells per well. After overnight incubation at 37 °C, the plate was centrifuged and the supernatant was aspirated. A buffer containing the aforementioned substrate CPRG (final concentration: 100  $\mu$ g mL<sup>-1</sup>) was added to all wells and incubated at 37 °C for several hours. Colorization of the supernatant was measured spectrophotometrically at  $\lambda$  590 nm.

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**Keywords:** conjugates • innate immunity • muramyl dipeptides • NOD2 receptor • vaccines

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