Design and synthesis of NLR and TLR based ligand-antigen conjugates
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Chapter 5

Synthesis and immunological evaluation of NOD1 ligand containing conjugates

5.1   Introduction

The family of nucleotide oligomeric domain (NOD)-like receptors consists of 22 members of which the NOD1 and NOD2 receptors are the best known. The NOD1 receptor, like the NOD2 receptor is primarily located in the cytosol of immune cells, including lymphocytes and antigen-presenting cells (APCs) and recognizes bacterial cell wall fragments. The NOD1 efficiently detects the naturally occurring muropeptide GlcNAc-MurNAc-L-Ala-D-*i*E-(2S,6R)-*meso*-DAP (GM-triDAP, **1,** figure 1)[1] and the minimal structural motive for recognition was determined to be γ-D-glutamyl-ε-meso-(2*S*,6*R*)-diaminopimelic acid[2] (D-*i*E-DAP, **2**). On the other hand, NOD1 receptors of different species recognize different structural parts of the peptidoglycan (PG). For example, the murine NOD1 receptor recognizes tracheal cytotoxin (TCT, **3**), a GlcNAc-1,6-anhydro-MurNAc-L-Ala-D-*i*E-(2S,6R)-*meso*-DAP fragment[3], whereas the human NOD1 does not.[2,4] The human NOD1 requires tripeptide L-Ala-D-*i*E-(2S,6R)-*meso*-DAP (**4**) for efficient

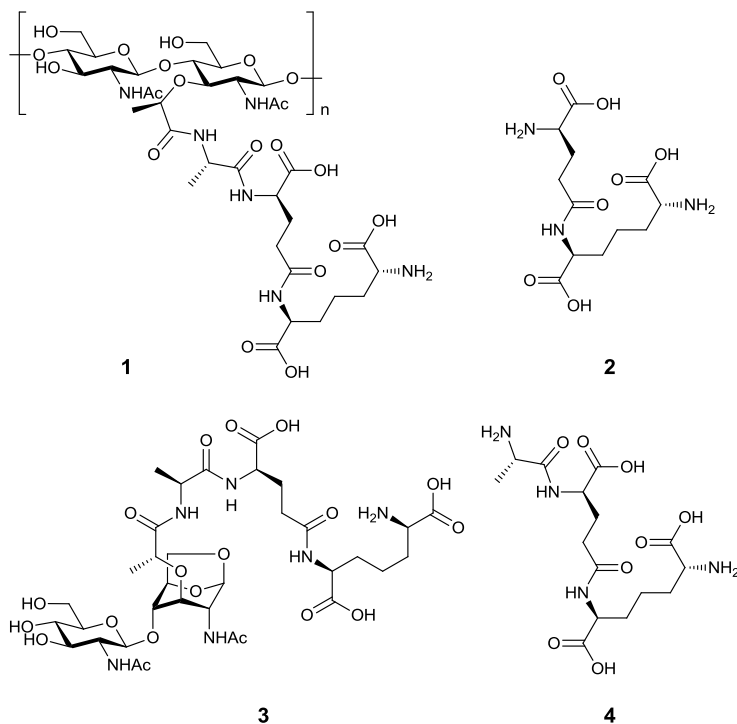recognition while the minimal peptide motive for recognition has proved to be D-*i*E-(2S,6R)-*meso*-DAP (**2**).[5]

Figure 1. GM-triDAP (**1**), minimal NOD1 agonist D-*i*E-*meso*-DAP (**2**), TCT (**3**) and L-Ala-D-*i*E-*meso*-DAP (**4**).

It was envisioned to incorporate NOD1-L D-*i*E-DAP (**2**) in similar conjugates as described for the NOD2-L muramyldipeptide (Chapter 2 – 4). In order to achieve this, mono-conjugates A and B were designed in which NOD1-L D-*i*E-DAP is connected to the either the *N*- or the *C*-terminal position of the OVA-derived peptide, respectively (*Figure 2*). The design of bis-conjugates C and D is based on the occurrence of a synergy effect when a combination of agonists of NOD1 or NOD2 and TLR2 ligand (i.e. Pam$_3$CSK$_4$) are tested simultaneously.[6,7] In bis-conjugate C both ligands are appended to the *N*-terminus of the peptide epitope whereas in conjugate D Pam$_3$CSK$_4$ is positioned at the *N*-terminus and D-*i*E-DAP at the *C*-terminus of the antigenic peptide (*Figure 2*).

A   *i*E-DAP ── epitope   B   epitope ── *i*E-DAP

C   *i*E-DAP ⟩ epitope   D   Pam₃CSK₄ ── epitope ── *i*E-DAP
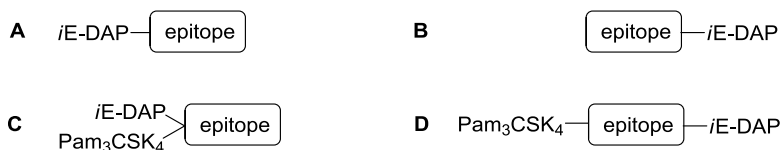   Pam₃CSK₄

Figure 2. General design of mono- and bis-conjugates.

The nature and the position of the linkage of D-*i*E-DAP to the peptide epitope were selected on the basis of reported structure activity relationship studies of derivatives of NOD1-L **2**. Boons and co-workers showed that amidation of the carboxylic acids in the isoglutamine and diaminopimelic moieties results in loss of activity.[8] With the commercially available dodecanoyl-γ-D-glutamyldiaminopimelic acid it was demonstrated that modifications of the amine function are allowed and can stimulate the NOD1 receptor at concentrations 100- to 1000-fold lower than the parent D-*i*E-(2S,6R)-*meso*-DAP.[9,10] The influence of the stereochemistry of the diaminopimelic moiety has also been investigated. Diaminopimelic acid occurs as LL-, DD-, and *meso*-DAP and individual stereoisomers have been prepared and evaluated. It was revealed that, although at very high concentrations, (2S,6R)-*meso*-DAP was most active, LL-DAP was less active whereas DD-DAP was completely inactive.[2] From a similar study with all stereoisomeric forms of D-*i*E-DAP, Inohara *et al.* concluded that all stereo-forms of D-*i*E-DAP induce a NOD1 dependent activation with D-*i*E-(2S,6R)-*meso*-DAP as the most potent isomer.[11] In addition a mixture of D-*i*E-DAP stereoisomers was compared with D-*i*E-(2S,6R)-*meso*-DAP. The mixture exhibited a potency similar to D-*i*E-(2S,6R)-*meso*-DAP, indicating that a mixture of all four stereoisomers of D-*i*E-DAP can be used as a NOD-1 ligand.[11,12] David *et al.* used racemic DAP to determine structure activity relations of the ligand for further ligation purposes.[13] They synthesized and evaluated several D-*i*E-DAP derivatives with modifications on the amine and carboxylic acid functions (*Figure 3, arrow* I – III). Their study confirmed that modifications on the glutamic acid part are restricted. The conversion of the glutamic acid α–carboxylic acid function into an amide was not tolerated (*Figure 3,* arrow II), whereas *N*-alkylation of the glutamic acid amine was accepted (*Figure 3, arrow I*). The 2,6-diaminopimelic acid part of the NOD1-L is more stringent: one amine needs to be connected with a glutamic acid moiety whereas the remaining free amine is essential for the biological activity. (*Figure 3, arrow III*).
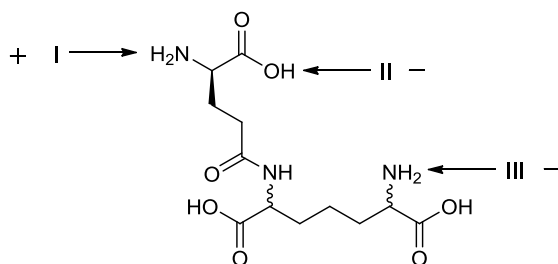
Figure 3. Positions of D-*i*E-DAP that are modified in SAR studies (+ : modifications are allowed; - : modifications are not tolerated).

With these data in mind the mono- and bis-conjugates **2 – 5** were designed (*Figure 4*). A commercially available mixture of 2,6-diaminopimelic acids comprising LL-, DD- and *meso*-isomers was used as starting compound to obtain the D-*i*E-DAP ligand. In all conjugates the D-*i*E-DAP moiety is linked to the OVA-derived peptide DEVA$_5$K *via* an additional glutamic acid thereby leaving the essential carboxylic acid of the glutamic acid in D-*i*E-DAP intact. All conjugates (**2 – 5**) were evaluated in immunological assays, using compound **6** and **7** as references (*Figure 4*). Non-lipophilic **6** and lipophilic **7** ligands contain the minimal NOD-1 binding motive, which is also incorporated in the conjugates **2 – 5**. Known TLR2-L-antigen conjugate Pam$_3$CSK$_4$-DEVA$_5$K (**8**) and the DEVA$_5$K peptide (**9**) were used as additional reference compounds.
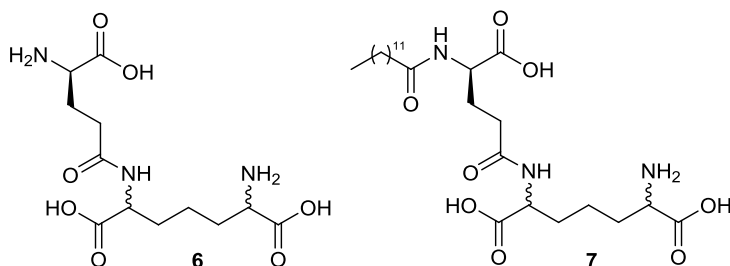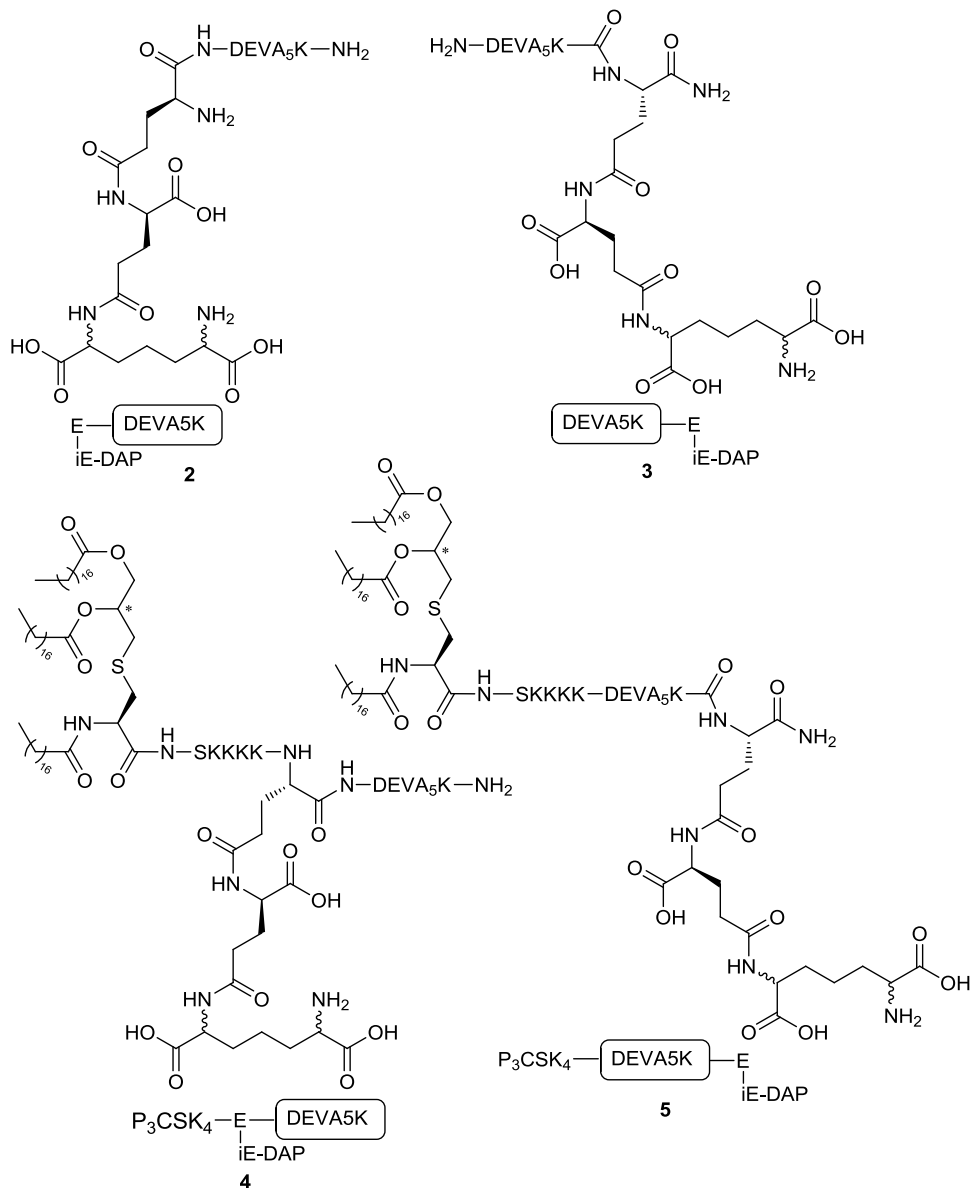

**Figure 4.** Target conjugates **2 – 5** and reference compounds **6** and **7**..

Continue Figure 4. Target conjugates **2 – 5** and reference compounds **6** and **7**..

5.2   Synthesis of NOD1-L and NOD1-L-antigen conjugates

Conjugates **2 – 5** were constructed *via* a solid phase peptide synthesis protocol, using commercially available Fmoc protected amino acids and Tentagel resin together with D-*i*E-DAP derivative **17**, which was synthesized in solution. The synthesis of building block **17** was started with a commercially available stereoisomeric mixture of 2,6-diaminopimelic acids (**10**, *Scheme 1*). In a Taschner reaction **10** was converted in doubly *tert*-butyl-protected **11** in a moderate yield.[14,15] Subsequently, one of the free amines was masked with a Boc-protective group using 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON). Compound **12** was isolated in 54% yield after flash column chromatography alongside 21% of the starting material and 22% of the bis-bocylated **13**. The remaining free amine of **12** was condensed with Fmoc and *tert*-butyl protected D-glutamic acid in the presence of BOP and DiPEA resulting in compound **14** in good yield. Compound **14** was treated with DBU in the presence of ethanethiol to remove the Fmoc-protective group to give compound **15** in moderate yield. Then the free amine was condensed with Fmoc-protected glutamine bearing the acid labile 2-phenylisopropyl (Pp) ester resulting in compound **16**. The Pp-ester could be selectively removed in the presence of the *tert*-butyl esters and Boc groups by treatment of **16** with a solution of 4% TFA in DCM for 15 minutes to give after purification by flash column chromatography target building block **17** in 72% yield.

Scheme 1. Synthesis of SPPS building block **17**.
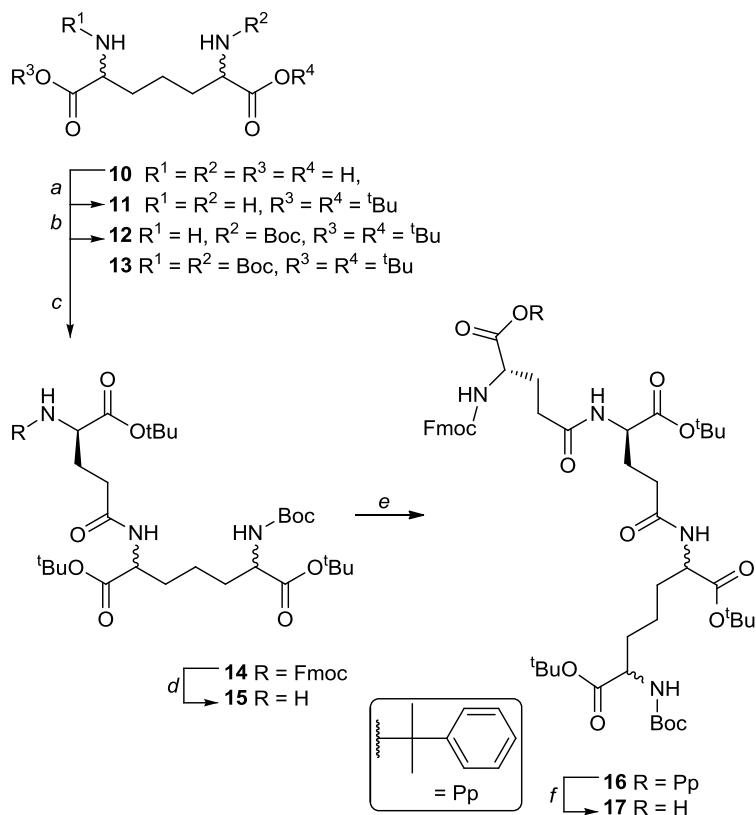
Reaction conditions: *a*) ^{t}BuAc, HClO$_4$, 32%; *b*) Boc-ON, DCM, **12**) 54%, **13**) 22%, **11**) 11%; *c*) Fmoc-D-Glu(OH)-O^{t}Bu, BOP, DMF, DiPEA, 1h, rt, 80%; *d*) DBU, EtSH, DCM, 66%; *e*) Fmoc-Glu (OH)-OPp, HATU, DiPEA, DMF, 68%; *f*) 4% TFA, DCM, 72%.

Both mono-conjugate **2** and bis-conjugate **4** were synthesized starting from immobilized peptide **18**, obtained by Fmoc solid phase peptide synthesis (*Scheme 2*). Compound **18** was elongated with building block **17** under the influence of HATU as a coupling reagent to give the fully protected immobilized precursor of target conjugate **2**. Subsequent removal of the protective groups and cleavage from the solid support was achieved by sequentially treating the resin with piperidine and 95% TFA, 2.5% TIS and 2.5% H_2O. The crude product was precipitated with diethyl ether and purified by RP-HPLC resulting in mono-conjugate **2** in 4% overall yield. To obtain bis-conjugate **4** with both ligands at the *N*-terminus of the peptide epitope, immobilized **18** was consecutively elongated with building block **17**, four times with Fmoc-Lys-OH, followed by Fmoc-Ser-OH and Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH. Removal of the protecting groups, cleavage from the solid support and purification was accomplished by the same sequence of events as described for **2**, to give bis-conjugate **4** in 4% overall yield. The synthesis of mono-conjugate **3** and bis-conjugate **5**, both provided with the NOD1-L on the *C*-terminus, started with the coupling of advanced building block **17** on Tentagel resin using HATU as condensation agent. Before continuation of the synthesis the efficiency of this condensation reaction was determined with the aid of a Fmoc loading-test. A 76% coupling efficiency was considered as sufficient and the remaining free amines were capped by treatment with Ac_2O and DiPEA. Removal of the Fmoc-protective group was followed by elongation of the thus obtained resin with the sequence $DEVA_5K$ in nine coupling cycles using the corresponding Fmoc protected amino acids to give the fully protected and immobilized precursor of conjugate **3**. This resin was also used to obtain bis-conjugate **5** and sequential elongation with the amino acids required for the installation of TLR2-L Pam_3CSK_4 resulted in the fully protected and immobilized precursor of conjugate **5**. Both resins were subjected to the same deprotection, cleavage and purification procedure as described above, to give mono-conjugate **3** and bis-conjugate **5** in respectively 7% and 2% overall yield.

Scheme 2. Synthesis of NOD1-L **2** and **3** conjugates and NOD1-L-TLR2-L conjugates **4** and **5**.
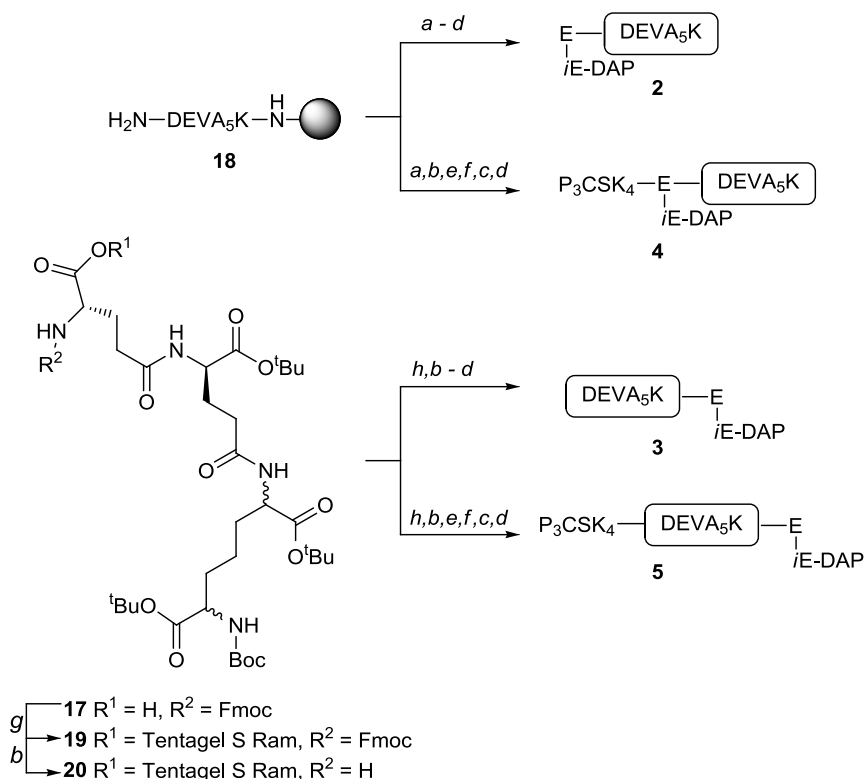
Reaction conditions: *a*) **17**, HATU, DiPEA, NMP; *b*) 20% piperidine, NMP; *c*) 95% TFA, 2.5% TIS, 2.5% H$_2$O; *d*) RP-HPLC; *e*) Fmoc SPPS cycle for SK$_4$; *f*) Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, PyBOP, DiPEA; *g*) Tentagel S Ram, HATU, DiPEA, NMP, 76% loading; *h*) Fmoc SPPS cycle for DEVA$_5$K; Yield conjugates: **2**) 4%; **3**) 7%; **4**) 4%; **5**) 2%.

Both reference ligands **6** and **7** were synthesized in solution, using **15** as starting compound (*Scheme 3*). Treatment of **15** with a solution of 20% TFA in DCM removed the *tert*-butyl and Boc-protective groups and purification by HW40 gel filtration gave compound **6** in 32 % yield. Ligand **7** was prepared in 44% yield by the following sequence of events: Condensation of **15** with lauric acid in the presence of HATU and DiPEA, purification of the obtained intermediate by gel filtration (1 : 1 DCM : MeOH), subsequently removal of all protective groups with a solution of 20% TFA in DCM and finally purification by gel filtration (HW40, 0.15 M ammonium acetate, 20% MeCN).

Scheme 3. Synthesis of NOD1-L references **6** and **7**.
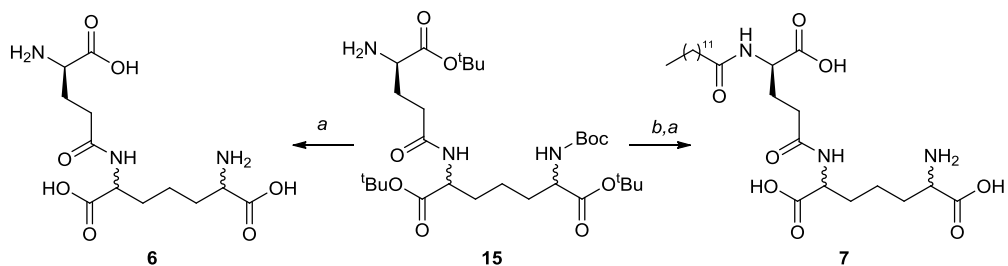
Reaction conditions: *a*) 20% TFA, DCM, **6**) 32%; **7**) 44%; *b*) Lauric acid, HATU, DIPEA, DMF 51%.

5.3   Immunological evaluation of the NOD1-L conjugates 2 – 5

The immunological activity of NOD1-L and NOD1-L/TLR2-L conjugates **2 – 5** was determined with the aid of the same assays as described in Chapter 2 – 4. First, the NOD1 immunostimulatory activity of the constructs was evaluated in NOD1 transfected HEK293 cells. Next, DC maturation was assessed in cultured murine D1 DCs using cytokine IL-12p40 production as the read-out signal. In addition, antigen presentation was measured in a T-cell hybridoma assay.

The NOD1 immunostimulatory capacity of conjugates **2 – 5**, together with references **6** and **7** was tested in a NOD1 HEK assay (*Figure 6*). TNFα was used as a positive control and known TLR2-L-antigen conjugate **8** as a negative control. As evident from *Figure 6* both compound **6** and **7** are active as determined by specific induction of IL-8, **7** being approximately 20 times more active at 0.7 nM. These results are in line with the findings of Girardin *et al.* who described D-*i*E-*meso*-DAP (**6**) to be a weak NOD1 agonist in transfected HEK-cells and they attributed this to an ineffective cytosolic uptake of this compound.[5] Both mono-conjugates **2** and **3** and bis-conjugates **4** and **5**, however, exhibited a very low cytokine production, indicating that covalent attachment of the DEVA$_5$K antigen is detrimental for the binding capacity of D-*i*E-DAP to NOD1.
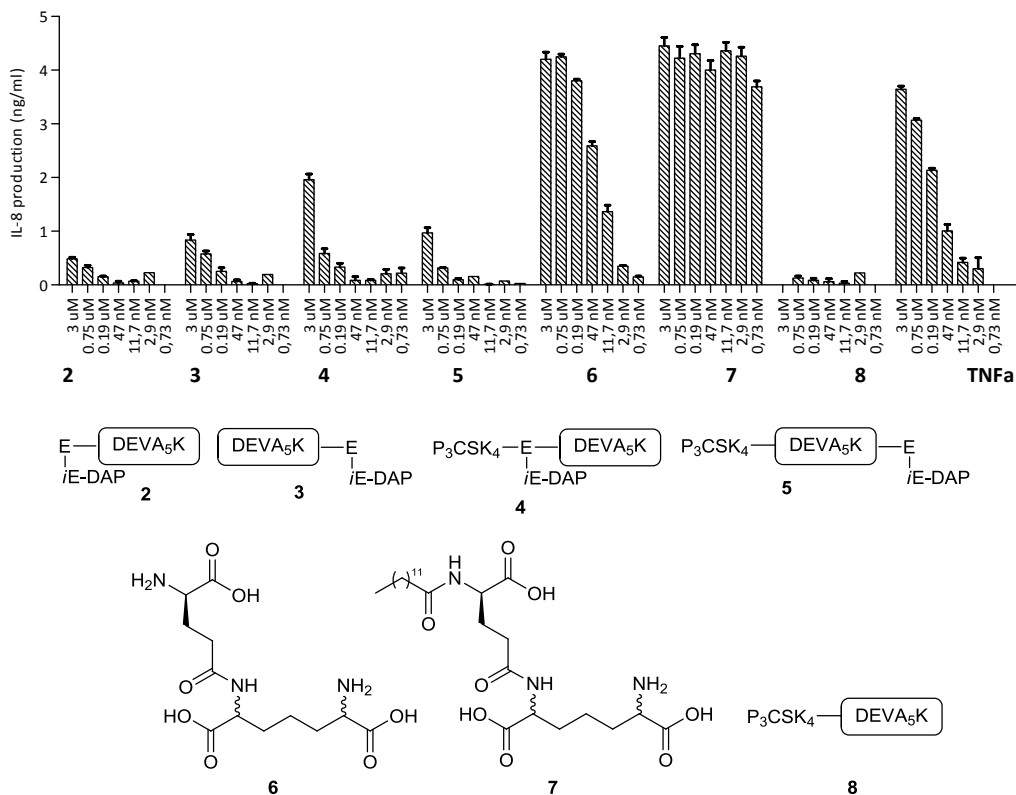
Figure 6. Potency of conjugates **2 – 5** and NOD1 ligands **6**, **7** and reference compound **8** in NOD1 transfected HEK cells.

The ability of the NOD1-L and NOD1/TLR2-L conjugates **2 – 5** to induce DC maturation was determined by measuring the interleukin-12 (IL-12p40) production in D1 DCs. The DCs were stimulated with conjugates **2 – 5**, using free ligands **6** and **7**, TLR2-L conjugate **8** and epitope **9** as references (*Figure 7*). Only the IL-12p40 production at 1 µM is shown because the same trend was observed for all concentrations. As evident from the maturation capacity of **2**, **3**, **6** and **7**, the D-*i*E-DAP moiety is a poor DC maturation inducer. The level of cytokine production effected by bis-conjugates **4** and **5** is comparable to that induced by TLR2-L conjugate **8** and can be attributed to the presence of TLR2 ligand Pam$_3$Cys-SK$_4$ in the constructs.
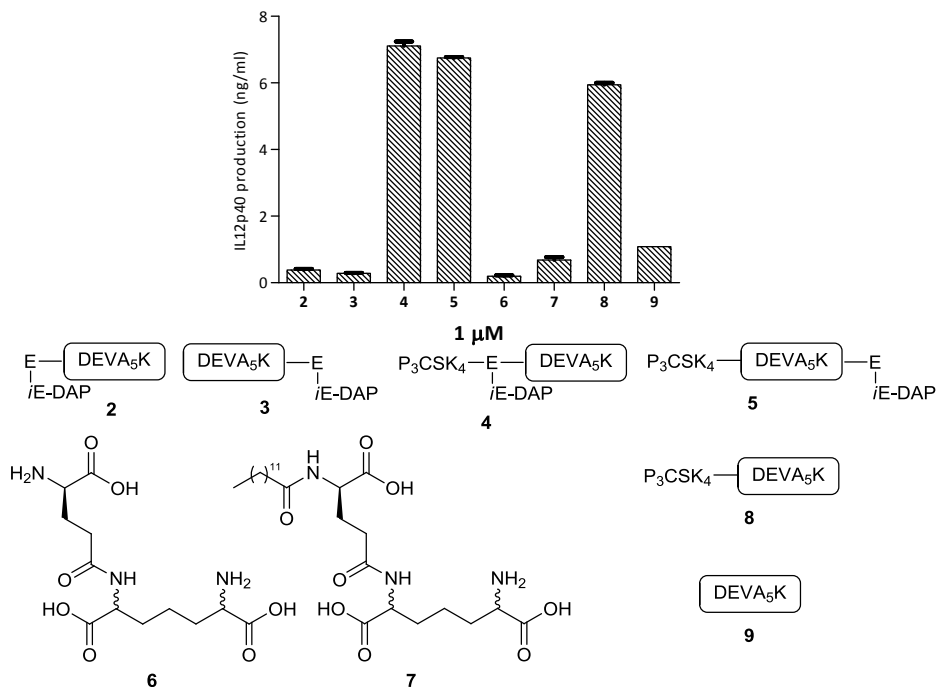
Figure 7. DC maturation at 1 µM of conjugates **2 – 5** and reference compound **6 – 9**.

Finally, the MHC I mediated presentation of the OVA-derived SIINFEKL epitope was studied using the NOD1 and NOD1/TLR2 constructs **2 – 5**. The level of antigen presentation was measured together with reference compound **9** (*Figure 8*). All constructs give rise to antigen presentation, indicating that the NOD1 modification does not hamper the presentation of the epitope by MHC class I molecules, though *N*-terminal conjugation is favorable to *C*-terminal conjugation as antigen presentation of the latter constructs is significantly lower in both cases.

Figure 8. Antigen presentation of conjugates **2 – 5** and reference compound **9**.
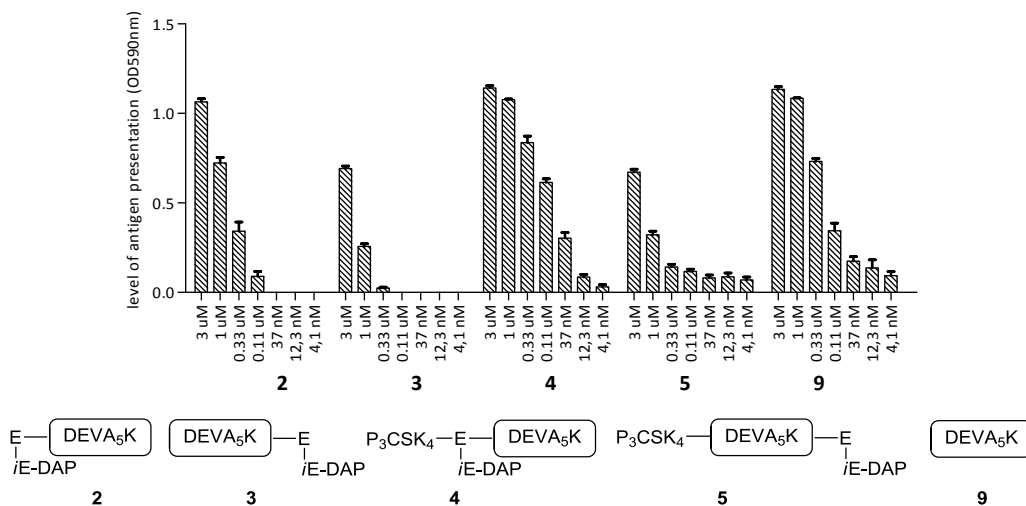
5.4 Conclusion

This Chapter describes the syntheses of D-*i*E-DAP derivatives **6** and **7** as well as mono-conjugates **2** and **3** and bis-conjugates **4** and **5**, in which D-*i*E-DAP is incorporated as a mixture of all four stereoisomers at the chiral centers of DAP. Compound **6** and **7** proved to be active as NOD1 ligands but were unable to mature dendritic cells. Conjugates **2** and **3** (in agreement with the results obtained on free ligand **6**) showed no DC maturation activity, whereas NOD1/TLR2 bis-conjugates **4** and **5** were found to cause maturation in D1 DCs. The DC maturation of **4** and **5** can be ascribed to a TLR2-specific activity. Conjugates **2 – 5** were able to sustain antigen presentation though recognition by the NOD1 receptor was lost.

5.5   Experimental section

The procedure for the automated solid phase peptide synthesis was used as described in **Chapter 2**. 2,6-diaminopimelic acid was purchased from Sigma Aldrich and Fmoc-Glu(OH)OPp from Bachem. As reported the synthesis of the NOD1-L was based on 2,6-diaminopimelic acid that consists of a stereomeric mixture of LL- DD- and *meso*-isomers. To assign the experimental data, the major rotamer is described and the following nomenclature for the DAP residue was used:
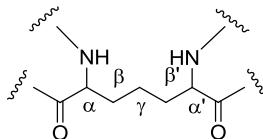

### Di-*tert*-butyl 2,6-Amino-heptanedioate (11)
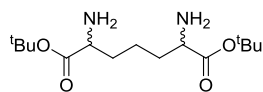
2,6-diamino pimelic acid (1.9 g, 10 mmol) was suspended in ^{t}BuAc (50 mL, 0.2 M) and cooled to 0 ^{o}C. $HClO_4$ (5.7 mL, 60 mmol, 72% solution in H_2O) was added dropwise over 1 h. The resulting mixture was stirred for 48 h at ambient temperature. The clear solution was diluted with 10 % K_2CO_3 (aq) solution (5 mL) and brought to pH 9 with solid K_2CO_3 followed by an extraction with DCM. The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo* to yield the title compound (0.98 g, 32%). R_f = 0.77 (1 : 1 : 1 ^{t}BuOH : MeCN : H_2O); ^{1}H NMR (400 MHz, CDCl$_3$) : δ 3.24 (t, 2H, J= 5.2 Hz, α + α' CH, DAP), 1-65 – 1.61 (m, 4H, β + β' CH$_A$H$_B$, DAP), 1.56 – 1.50 (m, 2H, γ CH$_2$, DAP), 1.44 (s, 18 H, CH$_3$, ^{t}Bu); ^{13}C NMR (100 Hz) : δ 175.1 (C=O), 80.6 (C$_q$ tBu), 54.7 (α CH), 54.6 (α' CH), 34.6 (β CH$_2$), 34.6 (β' CH$_2$), 27.9 (CH$_3$ ^{t}Bu), 21.6 (γ CH$_2$); IR (cm^{-1}): 2978, 1705, 1500, 1392, 1149; LC/Ms: Rt = 3.07 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for $[C_{15}H_{30}N_2O_4 + H]^+$ 303.22783, found 303.22781.

Di-*tert*-butyl 2-Amino-6-((tertbutoxycarbonyl)amino)heptanedioate (12)
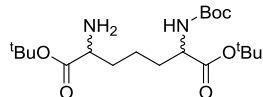
Compound **11** (1.87 g, 6.17 mmol) was dissolved in dry DCM (60 mL, 0.1 M) and cooled down to 0 ^{o}C. The resulting solution (pH 9) was stirred with Boc-ON (1.52 g, 6.17 mmol) for 2 h. The mixture was stirred for another 1.5 h at ambient temperature. When the formation of **13** was observed the reaction mixture was reduced in volume and purified by flash chromatography (CHCl$_3$: MeOH). Title compound (1.34 g, 3.32 mmol) was separated from starting material (21 mol %) and compound **13** (22 mol%) with 1 % MeOH in CHCl$_3$ in 54% yield. R_f = 0.42 (9 : 1 CHCl$_3$: MeOH); ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.08 (d, *J* = 7.7 Hz, 1H, NH), 4.21 – 4.07 (m, 1H, α' CH), 3.30 (dd, *J* = 11.3, 5.2 Hz, 1H, α CH), 1.80 – 1.50 (m, 4H , β + β' CH$_A$H$_B$, DAP, β + β' CH$_A$H$_B$, DAP), 1.49 – 1.42 (m, 20 H, CH$_3$, ^{t}Bu, γ CH$_2$, DAP), 1.44 (s, 9H, CH$_3$, ^{t}Bu); ^{13}C NMR (101 MHz, CDCl$_3$) δ 175.1 (C=O), 171.9 (C=O), 155.4 (C=O), 81.7 (C$_q$), 81.0 (C$_q$), 79.5 (C$_q$), 54.8 (α CH, DAP), 53.8 (α' CH, DAP), 34.5 (β CH$_2$, DAP), 32.7 (β' CH$_2$, DAP), 32.6 (β' CH$_2$, DAP), 28.3 (CH$_3$, ^{t}Bu), 28.03 (CH$_3$, ^{t}Bu), 27.99 (CH$_3$, ^{t}Bu), 21.4 (γ CH$_2$, DAP), 21.3 (γ CH$_2$, DAP); IR (cm^{-1}): 2978, 1724, 1367, 1149; LC/Ms: Rt = 6.69 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for $[C_{20}H_{38}N_2O_6 + H]^+$ 403.28026, found 403.27938.

Di-*tert*-butyl 2,6-di-((tertbutoxycarbonyl)amino)heptanedioate (13)
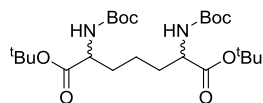
Title compound was isolated as side product of previous described reaction. Flash column chromatography with pure CHCl$_3$ resulted 0.369 g (1.3 mmol, 22 mol %). R_f = 0.88 (9 : 1 CHCl$_3$: MeOH); ^{1}H NMR (400 MHz, CDCl$_3$) δ 5.09 (s, 1H, NH), 4.21 – 4.06 (m, 2H, α + α' CH, DAP), 1.86 – 1.71 (m, 2H, β + β' CH$_A$H$_B$, DAP), 1.70 – 1.55 (m, 2H, β + β' CH$_A$H$_B$, DAP), 1.45 (m, 40H,

CH$_3$, ^{t}Bu, γ CH$_2$, DAP). ^{13}C NMR (101 MHz, CDCl$_3$) δ 171.7 (C=O), 155.4 (C=O), 81.7 (C$_q$), 79.5 (C$_q$), 53.6 (α + α' CH, DAP), 32.4 (β + β' CH$_2$, DAP), 32.3 (β + β' CH$_2$, DAP), 28.2 (CH$_3$, ^{t}Bu), 27.9 (CH$_3$, ^{t}Bu), 21.0 (γ CH$_2$, DAP), 20.7 (γ CH$_2$, DAP); IR (cm^{-1}): 2978, 1708, 1499, 1365, 1149; LC/Ms: Rt = 11.01 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{25}$H$_{46}$N$_2$O$_8$ + H]$^+$ 503.33269, found 503.33265.

Di-tertbutyl-2-(4-9H-fluoren-9-yl)methoxy)carbonyl)amino-5-tert-butyloxy-D-isoglutamyl)-6-((tert-butoxycarbonyl)amino)heptanedioate (H-D-*i*E-DAP) (14)
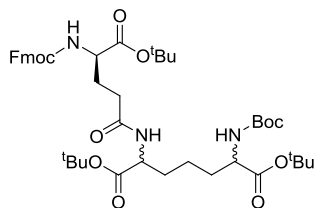
To a solution of compound **12** (1.3 mg, 3.23 mmol) dissolved in dry DMF (25 mL, 0.12 M) was added Fmoc-D-Glu-O^{t}Bu (1.65 g, 3.88 mmol), BOP (1.72 g, 3.88 mmol) and DiPEA (1.6 mL, 9.7 mmol). The resulting mixture was stirred for 1 h (TLC, 7 : 3 PE : EtOAc). The mixture was diluted with EtOAc (1 : 10), washed with 1M HCl (3 x 5 mL), sat. aqueous NaHCO$_3$ (3 x 5 mL), Brine (3 x 5 mL), dried (MgSO$_4$) and concentrated *in vacuo*. The crude mixture was purified by flash column chromatography (PE -> 7 : 3 PE : EtOAc -> 1 : 1 PE : EtOAc) to result in the title compound (1.36 g, 1.68 mmol) in 90% yield. R$_f$ = 0.76 (7 : 3 EtOAc : PE); ^{1}H NMR (400 MHz, MeOD) δ 8.16 (m, 1H, NH), 7.80 (d, *J* = 7.5 Hz, 2H, CH, Fmoc), 7.69 (t, *J* = 7.0 Hz, 2H, CH, Fmoc), 7.45 (d, *J* = 8.2 Hz, 1H, NH), 7.39 (t, *J* = 7.4 Hz, 2H, CH, Fmoc), 7.32 (t, *J* = 7.4 Hz, 2H, CH, Fmoc), 6.80 (d, *J* = 7.5 Hz, 1H, NH), 4.45 − 4.36 (m, 1H, CH$_2$, Fmoc), 4.42 − 4.31 (m, 2H, α' CH, DAP, CH$_2$, Fmoc), 4.22 (t, *J* = 6.9 Hz, 1H, CH, Fmoc), 4.18 − 4.05 (m, 1H, CH, α *i*-D-Glu), 3.98 (q, *J* = 4.9, 4.0 Hz, 1H, α CH, DAP), 2.45 − 2.24 (m, 2H, CH$_2$, γ *i*-D-Glu), 2.24 − 2.10 (m, 1H, CH$_2$, β *i*-D-Glu), 1.99 − 1.87 (m, 1H, CH$_2$, β *i*-D-Glu), 1.87 − 1.72 (m, 2H, β + β' CH$_A$H$_B$, DAP), 1.72 − 1.56 (m, 2H, β + β' CH$_A$H$_B$, DAP), 1.53 − 1.38 (m, 40H, CH$_3$, ^{t}Bu, γ CH$_2$, DAP); ^{13}C NMR (101 MHz, MeOD) δ 174.8 (C=O), 173.6 (C=O), 172.8 (C=O), 158.6 (C=O), 158.0 (C=O), 145.3 (C$_q$), 145.1 (C$_q$), 142.5 (CH$_2$, Fmoc), 128.8 (CH, Fmoc), 128.1 (CH, Fmoc), 126.3 (CH, Fmoc), 126.2 (CH, Fmoc), 120.9 (CH, Fmoc), 82.8 (C$_q$, ^{t}Bu), 82.7 (C$_q$, ^{t}Bu), 82.5 (C$_q$, ^{t}Bu), 80.4 (C$_q$, ^{t}Bu), 68.0 (CH, α *i*-D-Glu), 55.6 (α CH, DAP), 54.4 (α' CH, DAP), 48.6 (CH$_2$, Fmoc), 48.3 (CH$_2$, γ *i*-D-Glu) , 33.1 (β CH$_2$, DAP), 32.2 (β' CH$_2$, DAP), 28.8 (CH$_3$, ^{t}Bu), 28.6 (CH$_2$, β *i*-D-Glu), 28.3 (CH$_3$, ^{t}Bu), 23.2 (γ CH$_2$, DAP); IR (cm^{-1}): 2978, 1712, 1508, 1367, 1350, 1217, 1149; LC/Ms: Rt = 12.04 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{44}$H$_{63}$N$_3$O$_{11}$ + H]$^+$ 810.45354, found 810.45447.

Di-tertbutyl-2-(4-Amino-5-tert-butyloxy-D-isoglutamyl)-6-((tert-butoxycarbonyl)amino)heptanedioate (15)
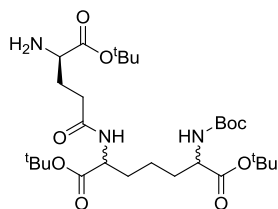
Compound **14** (1.8 g, 2.2 mmol) dissolved in DCM (22 mL, 0.1 M) was reacted with EtSH (0.63 mL, 8.8 mmol) and DBU (0.26 mL, 1.7 mmol) for 2.5 h. The solution was concentrated *in vacuo* and purified by flash column chromatography (PE -> 1 : 1 PE : EtOAc -> EtOAc -> 8 : 2 EtOAc : MeOH). The title compound was obtained in 94% (1.22 g, 2.1 mmol). R$_f$ = 0.3 (9 : 1 CHCl$_3$: MeOH); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.08 − 6.98 (m, 1H, NH), 5.26 (t, *J* = 9.6 Hz, 1H, NH), 4.46 − 4.37 (m, 1H, α CH, DAP), 4.22 − 4.08 (m, 1H, CH, α *i*-D-Glu), 4.02 − 3.95 (m, 2H, NH$_2$), 3.60 − 3.49 (m, 1H, α' CH, DAP), 2.51 − 2.26 (m, 2H, CH$_2$, γ *i*-D-Glu), 2.22 − 2.11 (m, 1H, CH$_2$, β *i*-D-Glu), 1.93 − 1.72 (m, 3H, CH$_2$, β *i*-D-Glu, β + β' CH$_A$H$_B$, DAP), 1.72 − 1.56 (m, 2H, β + β' CH$_A$H$_B$, DAP), 1.56 − 1.33 (m, 38H, CH$_3$, ^{t}Bu, γ CH$_2$, DAP); ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.6 (C=O), 172.2 (C=O), 172.2 (C=O), 172.1 (C=O), 171.8 (C=O), 171.7 (C=O), 171.6 (C=O), 171.4 (C=O), 171.0 (C=O), 155.4 (C=O), 155.4 (C=O), 82.1 (C$_q$, ^{t}Bu), 81.8 (C$_q$, ^{t}Bu), 81.7 (C$_q$, ^{t}Bu), 81.6 (C$_q$, ^{t}Bu), 79.4 (C$_q$, ^{t}Bu), 1 53.8 (α CH, DAP), 53.6 (α' CH, DAP), 53.6 (CH, α *i*-D-Glu), 52.4 (α'CH, DAP), 32.3 (CH$_2$, γ *i*-D-Glu), 32.2 (β CH$_2$, DAP), 31.7 (β' CH$_2$, DAP), 29.3 (CH$_2$, β *i*-D-Glu), 28.2 (CH$_3$, ^{t}Bu), 27.8 (CH$_3$, ^{t}Bu), 21.1 (γ CH$_2$, DAP); IR (cm^{-1}): 2978, 1716, 1367, 1248, 1149; LC/Ms: Rt = 7.03 min

(C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{29}$H$_{53}$N$_3$O$_9$ + H]$^+$ 588.38546, found 588.385.

Di-tertbutyl-2-((4-(9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-dimethylphenyloxy-isoglutamyl)-4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-tert-butyloxyl-D-isoglutamyl)-6-((tert-butoxycarbonyl)amino)heptanedioate (16)
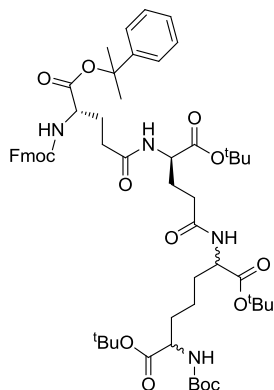
Compound **15** (0.23 g, 0.40 mmol) was co-evaporated with DMF. Fmoc-Glu(OH)OPp (0.39 g, 0.80 mmol) was dissolved in DMF (3.5 mL, 0.12 M) and pre-activated with HATU (0.32 g, 0.79 mmol) and DiPEA (0.03 mL, 2.0 mmol). The solution was added to compound **15** and stirred for 18 h at ambient temperatures. The reaction mixture was concentrated *in vacuo* and purified over gel filtration (LH20, 1 : 1, DCM : MeOH). The title compound was obtained in 90% (0.44 g, 0.36 mmol). R$_f$ = 0.5 (9 : 1 CHCl$_3$: MeOH); ^{1}H NMR (400 MHz, MeOD) δ 7.77 (d, *J* = 7.5 Hz, 2H, CH, Ar), 7.70 – 7.65 (m, 2H, CH, Ar), 7.44 – 7.34 (m, 4H, CH, Ar), 7.33 – 7.25 (m, 4H, CH, Ar), 7.24 – 7.19 (m, 1H, CH, Ar), 4.50 – 4.42 (m, 2H, CH$_2$, Fmoc), 4.37 – 4.16 (m, 4H, CH, Fmoc, α' CH, DAP, CH, α *i-L*-Glu, CH, α *i-D*-Glu, CH), 3.99 (s, 1H, α CH, DAP), 2.46 – 2.28 (m, 4H, CH$_2$, γ *i-L*-Glu, CH$_2$, γ *i-D*-Glu), 2.26 – 2.20 (m, 1H, CH$_2$, γ *i-D*-Glu), 2.19 – 2.11 (m, 2H, CH$_2$, β *i-L*-Glu, CH$_2$, β *i-D*-Glu), 2.05 – 1.87 (m, 2H, CH$_2$, β *i-L*-Glu, CH$_2$, β *i-D*-Glu), 1.85 – 1.65 (m, 8H, CH$_3$, OPp, β + β' C<u>H</u>$_A$H$_B$, DAP), 1.70 – 1.55 (m, 2H, β + β' CH$_A$<u>H</u>$_B$, DAP), 1.54 – 1.37 (m, 38H, CH$_3$, ^{t}Bu, γ CH$_2$, DAP); ^{13}C NMR (101 MHz, MeOD) δ 172.8 (C=O), 172.7 (C=O), 172.6 (C=O), 172.6 (C=O), 172.5 (C=O), 171.6 (C=O), 171.5 (C=O), 170.8 (C=O), 170.4 (C=O), 170.4 (C=O), 170.2 (C=O), 156.5 (C$_q$), 155.8 (C$_q$), 144.5 (C$_q$), 143.2 (C$_q$), 143.0 (C$_q$), 140.5 (C$_q$), 127.4 (CH, Ar), 126.8 (CH, Ar), 126.3 (CH, Ar), 126.2 (CH, Ar), 124.3 (CH, Ar), 124.3 (CH, Ar), 123.4 (CH, Ar), 119.0 (CH, Ar), 82.4 (C$_q$, ^{t}Bu), 81.2 (C$_q$, ^{t}Bu), 81.0 (C$_q$, ^{t}Bu), 80.9 (C$_q$, ^{t}Bu), 78.7 (C$_q$, ^{t}Bu), 66.2 (CH$_2$, Fmoc), 53.7 (α CH, DAP), 53.5 (CH, α *i-D*-Glu), 52.3 (CH, α *i-L*-Glu), 52.2 (α' CH, DAP), 46.4 (CH, Fmoc), 31.3 (CH$_2$), 31.2 (CH$_2$), 31.1 (CH$_2$), 30.6 (CH$_2$), 30.5 (CH$_2$), 30.3 (CH$_2$), 27.3 (CH$_3$), 27.2 (CH$_3$), 27.0 (CH$_3$), 26.7 (CH$_3$), 26.6 (CH$_3$), 21.1 (γ CH$_2$, DAP); IR (cm^{-1}): 3225, 2931, 1730, 1365, 1219, 1153; LC/Ms: Rt = 5.747 min (C$_{18}$ Alltima, 70 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{58}$H$_{80}$N$_4$O$_{14}$ +Na]$^+$ 1079.55632, found 1079.55665.

Di-tertbutyl 2-((4-(9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-hydroxy-isoglutamyl)-4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-tert-butyloxyl-D-isoglutamyl)-6-((tert-butoxycarbonyl)amino)heptanedioate (17)
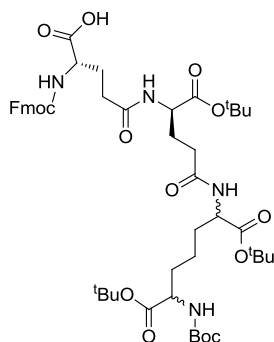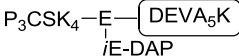

Compound **16** (0.75 g, 0.71 mmol) was treated for 15 min with a solution of 4% TFA in DCM (18 mL, 0.04 M). The reaction mixture was concentrated *in vacuo* and purified by flash column chromatography (1 : 1 PE : EtOAc -> 1 : 2 PE : EtOAc). The title compound was obtained in 72% (0.48 g, 0.51 mmol). R$_f$ = 0.6 (8 : 2 CHCl$_3$: MeOH); ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.38 (s, 1H, OH), 7.73 (d, *J* = 7.5 Hz, 2H, CH, Fmoc), 7.61 (t, *J* = 6.7 Hz, 2H, CH, Fmoc), 7.36 (t, *J* = 7.4 Hz, 2H, CH, Fmoc), 7.32 – 7.24 (m, 2H, CH, Fmoc), 7.23 – 7.13 (m, 1H, NH), 6.97 – 6.86 (m, 1H, NH), 6.21 (t, *J* = 6.3 Hz, 1H, NH), 5.36 – 5.20 (m, 1H, NH), 4.44 – 4.38 (m, 4H, CH, Fmoc, CH$_2$, Fmoc, CH, α CH, DAP), 4.36 (d, *J* = 7.1 Hz, 1H, CH, α, *i-L*-Glu), 4.20 (t, *J* = 7.1 Hz, 1H, CH$_2$, α, *i-D*-Glu), 4.14 – 4.08 (m, 1H, α' CH, DAP), 2.50 – 2.12 (m, 8H, CH$_2$, γ *i-L*-Glu, CH$_2$, γ *i-D*-Glu, CH$_2$, β *i-L*-Glu, CH$_2$, β *i-D*-Glu), 2.00 – 1.95 (m, 1H, CH$_2$, γ *i-D*-Glu), 1.83 – 1.53 (m, 4H, β + β' CH$_2$, DAP), 1.53 – 1.35 (m, 38H, CH$_3$, ^{t}Bu, γ CH$_2$, DAP); ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.8 (C=O), 172.9 (C=O), 172.4 (C=O), 171.4 (C=O), 170.8 (C=O), 156.1 (C$_q$), 155.5 (C$_q$), 143.7 (C$_q$), 143.6 (C$_q$), 141.0 (C$_q$), 127.5 (CH, Ar), 126.9 (CH, Ar),

125.0 (CH, Ar), 119.7 (CH, Ar), 82.2 (C_q), 82.0 (C_q), 81.7 (C_q), 79.5 (C_q), 66.9 (CH_2, Fmoc), 53.6 (CH), 53.1 (CH), 52.6 (CH), 52.4 (CH), 46.9 (CH, Fmoc), 32.0 (CH_2) 31.9, (CH_2) 31.7 (CH_2), 31.5 (CH_2), 29.4 (CH_2), 29.1 (CH_2), 28.1 (CH_3), 27.8 (CH_3), 21.0 (γ CH_2, DAP); IR (cm^{-1}): 2978, 1712, 1519, 1219, 1149; LC/Ms: Rt = 6.87 min (C_{18} Alltima, 50 - 90% MeCN, 15 min run); HRMS Calcd. for $[C_{49}H_{70}N_4O_{14} + H]^+$ 939.49613, found 939.49707.

H-Glu(D-*i*Glu-DAP)-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH₂ (2)

E—| DEVA₅K |
|
*i*E-DAP

50 µmol S RAM Tentagel (0.25 mmol/g) loaded with H-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) was treated with compound **17** (0.19 g, 0.2 mmol), HATU (74 mg, 0.18 mmol) and DiPEA (0.15 mL, 0.6 mmol) dissolved in NMP (2 mL, 0.1 M). The resulting mixture was reacted for 18 h, drained and washed (DCM, NMP). Subsequently, the resin was treated with a solution of Ac_2O (0.5 M) in presence of DiPEA (0.5 mmol) in NMP (3 x 15 min), washed with NMP and DCM. The resin was treated with a solution of 20% piperidine in NMP (5 x 3 min). After a thorough wash (NMP, DCM and Et_2O) 25 µmol of the resin was treated with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O for 104 min. The resin was filtrated and the peptide was precipitated with Et_2O. The resulting crude product was washed with Et_2O and purified by RP-HPLC resulting in the title compound (3.24 mg, 1.1 µmol, 4.3%); LC/Ms: Rt = 5.61 min (C_{18}, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 2976.52 $[M+H]^+$; HRMS Calcd. for $[C_{129}H_{211}N_{33}O_{47} + H]^{2+}$ 1488.76549, found 1488.76564.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Glu(D-*i*Glu-DAP)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH₂ (4)

P₃CSK₄—E—| DEVA₅K |
|
*i*E-DAP

25 µmol S RAM Tentagel (0.25 mmol/g) loaded with NH_2-Glu(*i*EDAP)-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) was elongated with Ser(^tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) by Fmoc : HCTU automated synthesis concluded with the removal of the final Fmoc. The synthesis was proceeded with the coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)OH (50 mg, 50 µmol), with PyBOP (26 mg, 50 µmol) and DiPEA (2 x 8.5 µL, 2 x 25 µmol) in NMP : DCM (1 : 1, 0.5 mL, 0.1 M) for 18 h. After a thorough wash with NMP, DCM and Et_2O was the resin treated with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O for 104 min. The resin was filtrated and the peptide was precipitated with Et_2O. The resulting crude product was washed with Et_2O and purified by RP-HPLC resulting in the title compound (4.22 mg, 0.94 µmol, 3.8%); LC/Ms: Rt = 6.36 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 4468.67 $[M+H]^+$; HRMS Calcd. for $[C_{210}H_{365}N_{43}O_{59}S + H]^{3+}$ 1490.22932, found 1490.23100.

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Glu(D-*i*Glu-DAP)-NH₂ (3)

| DEVA₅K |—E
|
*i*E-DAP

50 µmol S RAM Tentagel (0.25 mmol/g) was treated with a solution of 20% piperidine in NMP (5 x 3 min). The deprotected resin was reacted with compound **17** (0.19 g, 0.2 mmol), HATU (74 mg, 0.18 mmol) and DiPEA (0.15 mL, 0.6 mmol) in NMP (2 mL, 0.1 M) for 18 h. A small aliquot of resin (5 mg scale) was treated with a 20% piperidine in NMP solution (1 Ml) and diluted with EtOH (25 mL). A fluorimetric analysis of the solution resulted in a calculated loading of 76%. Subsequently, the resin treated with a solution Ac_2O (0.5 M) in the presence of DiPEA (0.03 M) in NMP (3 x 15 min), then a NMP and DCM wash step, followed by the treatment of a solution of 20% piperidine in NMP (5 x 3 min). The resin was elongated with Asp(O^tBu)-Glu(O^tBu)-Val-Ser(tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc) by Fmoc and HCTU automated synthesis and concluded with the removal of the final Fmoc. After a thorough washing

step (NMP, DCM and Et_2O) was 25 μmol of the resin treated with a mixture of 5% TFA, 2.5% TIS and 2.5% H_2O for 104 min. The resin was filtrated and the crude product was precipitated with Et_2O. The resulting crude product was washed with Et_2O and purified by RP-HPLC resulting in the title compound (5.44 mg, 1.8 μmol, 7.3%); LC/Ms: Rt = 5.52 min (C_4 Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 2976.52 [M+H]$^+$; HRMS Calcd. for $[C_{129}H_{211}N_{33}O_{47} + H]^{2+}$ 1488.76549, found 1488.76577.

Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-Glu(D-*i*Glu-DAP)-NH₂ (5)

P₃CSK₄ — [DEVA₅K] — E
                              |
                          *i*E-DAP

25 μmol S RAM Tentagel (0.25 mmol/g) loaded with H-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O^{t}Bu)-Gln(Trt)-Leu-Glu(O^{t}Bu)-Ser(^{t}Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Boc)Glu(*i*-D-E-DAP)-NH₂ was elongated with Ser(tBu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc) by Fmoc/HCTU automated synthesis and concluded with removal of the final Fmoc. The synthesis was proceeded with the coupling of Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)OH (50 mg, 50 μmol), with PyBOP (26 mg, 50 μmol) and DiPEA (2 x 8.5 μL, 2 x 25 μmol) in NMP : DCM (1 : 1, 0.5 mL, 0.1 M) for 18 h. After thoroughly washed with NMP, DCM and Et_2O the resin was treated with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O for 104 min. The resin was filtrated and the peptide was precipitated with Et_2O. The resulting crude product was washed with Et_2O and purified by RP-HPLC resulting in the title compound was obtained in 2.07 mg (1.8 %, 0.46 μmol); LC/Ms: Rt = 6.76 min (C_4 Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 4468.67 [M+H]$^+$; HRMS Calcd. for $[C_{210}H_{365}N_{43}O_{59}S + H]^{3+}$ 1490.22932, found 1490.23265.

2-D-isoglutamyl-6-amino-heptanedioate (6)
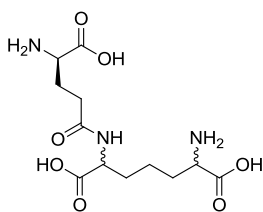
Compound **15** (0.12 g, 0.25 mmol) was stirred for 5 h in a 20% solution of TFA in DCM (10 mL, 0.02 m). The reaction mixture was concentrated *in vacuo* and co-evaporated with Et_2O. Purification by gel filtration (HW40, 0.15 M ammonium acetate) and lyophilization resulted in compound **6** (25.5 mg, 0.08 mmol, 32%). R_f = 0.1 (8 : 2 $CHCl_2$: MeOH); ^{1}H NMR (600 MHz, D_2O) δ 4.08 – 3.96 (m, 1H, α CH, DAP), 3.66 (q, *J* = 6.5 Hz, 1H, CH, α *i*-D-Glu), 3.63 – 3.61 (m, 1H, α' CH, DAP), 2.45 – 2.28 (m, 2H, CH_2, γ *i*-D-Glu), 2.12 – 1.95 (m, 2H, CH_2, β *i*-D-Glu), 1.85 – 1.66 (m, 3H, β + β' C<u>H</u>A<u>H</u>B, DAP, β CHA<u>H</u>B, DAP), 1.66 – 1.54 (m, 1H, β' CHA<u>H</u>B, DAP), 1.43 – 1.22 (m, 2H, γ CH_2, DAP); ^{13}C NMR (151 MHz, D_2O) δ 180.1 (C=O), 180.0 (C=O), 175.7 (C=O), 175.2 (C=O), 175.2 (C=O), 175.2 (C=O), 175.1 (C=O), 175.1 (C=O), 56.1 (CH), 56.0 (CH), 57.0 (CH), 56.0 (CH), 55.6 (CH), 55.3 (CH), 55.1 (CH), 32.7 (CH_2), 32.3 (CH_2), 32.1 (CH_2), 32.0 (CH_2), 31.1 (CH_2), 31.1 (CH_2), 27.4 (CH_2), 27.3 (CH_2), 22.2 (CH_2), 22.2 (CH_2), 22.0 (CH_2); IR (cm^{-1}): 2930, 1558, 1394; LC/Ms: Rt = 0.81 min (C_{18} Alltima, 0 - 20% MeCN, 15 min run); HRMS Calcd. for $[C_{12}H_{21}N_3O_7 + H]^+$ 320.14523, found 320.14531.

2-(4-Lauroyl-5-tertbutyloxy-D-isoglutamyl)-6-((tert-butoxycarbonyl)amino)heptanedioate (21)
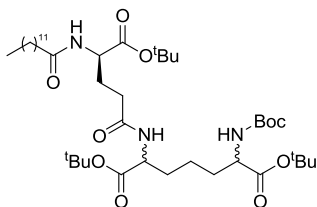
To a mixture of compound **15** (0.1 g, 0.2 mmol) co-evaporated and dissolved in DMF (1.6 mL, 0.13 M) was added Lauric acid (0.05 g, 0.3 mmol), HATU (0.1 g, 0.2 mmol) and DiPEA (0.11 mL, 0.65 mmol). The reaction mixture was stirred for 1 h and concentrated *in vacuo*. Purification by gel filtration (DCM : MeOH) yielded compound **21** (85 mg, 0.11 mmol, 51%). R_f = 0.9 (9 : 1 $CHCl_3$: MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 – 7.01 (m, 1H, NH), 6.71 – 6.47 (m, 1H, NH), 5.31 – 5.02 (m, 1H, NH), 4.64 – 4.54 (m, 1H, α CH, DAP), 4.48 – 4.36 (m, 1H, CH, α *i*-D-Glu), 4.20 – 4.03 (m, 1H, α' CH, DAP), 2.39 – 2.11 (m, 6H, CH_2, γ *i*-D-Glu, CH_2, β *i*-D-Glu, CH_2, lauroyl), 1.99 – 1.56 (m, 8H, CH_2, lauroyl, β +

β' C$\underline{H}_A$H$_B$, DAP, CH$_2$, β i-D-Glu), 1.54 – 1.36 (m, 38H, CH$_3$, ^{t}Bu, β + β' CH$_A\underline{H}_B$, DAP), 1.31 – 1.20 (m, 16H, CH$_2$, lauroyl, γ CH$_2$, DAP), 0.88 (t, J = 6.8 Hz, 3H, CH$_3$, lauroyl); ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.5 (C=O), 172.1 (C=O), 171.9 (C=O), 171.8 (C=O), 171.4 (C=O), 171.3 (C=O), 171.2 (C=O), 155.4 (C=O), 82.2 (C$_q$, ^{t}Bu), 82.0 (C$_q$, ^{t}Bu), 81.7 (C$_q$, ^{t}Bu), 79. (C$_q$, ^{t}Bu), 53.6 (CH, DAP), 52.5 (CH, DAP), 52.1 (CH, DAP), 52.0 (CH, α i-D-Glu), 36.5 (CH$_2$, γ i-D-Glu), 32.4 (CH$_2$, DAP), 32.3 (CH$_2$, DAP), 31.8 (CH$_2$, lauroyl), 29.5 (CH$_2$, lauroyl), 29.3 (CH$_2$, lauroyl), 29.2 (CH$_2$, lauroyl), 28.4 (CH$_2$ β i-D-Glu), 28.2 (CH$_3$, ^{t}Bu), 27.9 (CH$_3$, ^{t}Bu), 25.5 (CH$_2$, lauroyl), 22.5 (CH$_2$, lauroyl), 21.2 (CH$_2$, DAP), 21.0 (CH$_2$, DAP), 14.0 (CH$_3$, lauroyl; IR (cm^{-1}): 2926, 1728, 1653, 1531, 1367, 1153; LC/Ms: Rt = 9.16 min (C$_{18}$ Alltima, 70 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{41}$H$_{75}$N$_3$O$_{10}$ + Na]$^+$ 792.53447, found 792.53463.

2-Lauroyl- D-isoglutamyl-6-amino-heptanedioate (7)
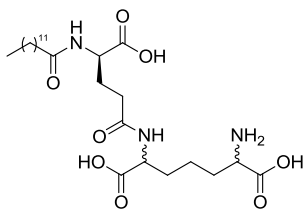
Compound **21** (85 mg, 0.11 mmol) was stirred for 5 h in a 20% solution of TFA in DCM (1.2 mL, 0.05 m). The reaction mixture was concentrated *in vacuo* and purified by gel filtration (HW40, 0.15 M ammonium acetate, 20% MeCN). Title compound was obtained in 44% (24 mg, 0.05 mmol). R$_f$ = 0.1 (9 : 1 CHCl$_3$: MeOH); ^{1}H NMR (600 MHz, D$_2$O) δ 4.21 – 4.09 (m, 2H, α CH, DAP, CH, α i-D-Glu), 3.68 – 3.62 (m, 1H, α' CH, DAP), 2.31- 2.25 (m, 2H, CH$_2$, γ i-D-Glu), 2.24 – 2.16 (m, 2H, CH$_2$, lauroyl), 2.12 – 2.01 (m, 1H, CH$_2$, β i-D-Glu), 1.98 – 1.81 (m, 2H, CH$_2$, β i-D-Glu, β + β' C$\underline{H}_A$H$_B$, DAP), 1.79 – 1.70 (m, 1H, β CH$_A\underline{H}_B$, DAP), 1.67 – 1.57 (m, 1H, β' CH$_A\underline{H}_B$, DAP), 1.54 – 1.40 (m, 2H, CH$_2$, lauroyl), 1.39 – 1.31 (m, 2H, γ CH$_2$, DAP), 1.29 – 1.12 (m, 18H, CH$_2$, lauroyl), 0.84 – 0.72 (t, J = 6.8 Hz, 3H, CH$_3$, lauroyl); ^{13}C NMR (151 MHz, D$_2$O) δ 178.9 (C=O), 177.3 (C=O), 177.3 (C=O), 175.8 (C=O), 175.6 (C=O), 55.5 (CH), 55.3 (CH), 54.9 (CH), 36.8 (CH$_2$), 33.2 (CH$_2$), 32.6 (CH$_2$), 32.1 (CH$_2$), 31.9 (CH$_2$), 31.1 (CH$_2$), 30.6 (CH$_2$), 30.2 (CH$_2$), 30.1 (CH$_2$), 29.9 (CH$_2$), 29.8 (CH$_2$), 28.7 (CH$_2$), 26.5 (CH$_2$), 23.3 (CH$_2$), 22.1 (CH$_2$), 14.6 (CH$_3$, lauroyl); IR (cm^{-1}): 2924, 1573, 1404, 1342; LC/Ms: Rt = 6.83 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C$_{24}$H$_{43}$N$_3$O$_8$ + H]$^+$ 501.31299, found 501.31193.

Immunological assays

The assays were used in a method similar to the assays described in Chapters 2 – 4.

NOD1-HEK293 activation

The NOD1 HEK cells were used in a method similar to NOD2 HEK cells. For detailed information see the experimental section of Chapter 2.

5.6   References and notes

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