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-\textit{i}E-(2S,6R)-meso-DAP (GM-triDAP, 1, figure 1)\textsuperscript{1} and the minimal structural motive for recognition was determined to be \textit{γ}-D-glutamyl-\textit{ε}-meso-(2S,6R)-diaminopimelic acid\textsuperscript{2} (D-\textit{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-\textit{i}E-(2S,6R)-\textit{meso}-DAP fragment\textsuperscript{3}, whereas the human NOD1 does not.\textsuperscript{2,4} The human NOD1 requires tripeptide L-Ala-D-\textit{i}E-(2S,6R)-\textit{meso}-DAP (4) for efficient
recognition while the minimal peptide motive for recognition has proved to be D-iE-(2S,6R)-meso-DAP (2).\(^5\)

![Chemical structures](image)

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

It was envisioned to incorporate NOD1-L D-iE-DAP (2) in similar conjugates as described for the NOD2-L muramyl-dipeptide (Chapter 2 – 4). In order to achieve this, mono-conjugates A and B were designed in which NOD1-L D-iE-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.\(^5,7\) 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-iE-DAP at the C-terminus of the antigenic peptide (Figure 2).
The nature and the position of the linkage of D-iE-DAP to the peptide epitope were selected on the basis of reported structure activity relationship studies of derivatives of NOD1-L. Boons and co-workers showed that amidation of the carboxylic acids in the isoglutamine and diaminopimelic moieties results in loss of activity. 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-iE-(2S,6R)-meso-DAP. 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. From a similar study with all stereoisomeric forms of D-iE-DAP, Inohara et al. concluded that all stereo-forms of D-iE-DAP induce a NOD1 dependent activation with D-iE-(2S,6R)-meso-DAP as the most potent isomer. In addition a mixture of D-iE-DAP stereoisomers was compared with D-iE-(2S,6R)-meso-DAP. The mixture exhibited a potency similar to D-iE-(2S,6R)-meso-DAP, indicating that a mixture of all four stereoisomers of D-iE-DAP can be used as a NOD-1 ligand. David et al. used racemic DAP to determine structure activity relations of the ligand for further ligation purposes. They synthesized and evaluated several D-iE-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 2.** General design of mono- and bis-conjugates.
Figure 3. Positions of D-iE-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-iE-DAP ligand. In all conjugates the D-iE-DAP moiety is linked to the OVA-derived peptide DEVA₅K via an additional glutamic acid thereby leaving the essential carboxylic acid of the glutamic acid in D-iE-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 Pam₃CSK₄-DEVA₅K (8) and the DEVA₅K 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-iE-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.\(^\text{14,15}\) 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.

Reaction conditions: a) tBuAc, HClO₄, 32%; b) Boc-ON, DCM, 54%, 13) 22%, 11) 11%; c) Fmoc-D-Glu(OH)-O₄Bu, 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% H₂O. 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 Ac₂O and DiPEA. Removal of the Fmoc-protective group was followed by elongation of the thus obtained resin with the sequence DEVA₅K 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 Pam₃CSK₄ 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% H₂O; 
d) RP-HPLC; 
e) Fmoc SPPS cycle for SK₄; 
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 DEVA₃K; 
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).
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-iE-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-iE-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-d-iE-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 Pam3Cys-SK4 in the constructs.
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.
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Figure 8. Antigen presentation of conjugates 2 – 5 and reference compound 9.

5.4 Conclusion

This Chapter describes the syntheses of D-/E-DAP derivatives 6 and 7 as well as mono-conjugates 2 and 3 and bis-conjugates 4 and 5, in which D-/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 L- DD- and meso-isomers. To assign the experimental data, the major rotamer is described and the following nomenclature for the DAP residue was used:

\[
\begin{align*}
\text{NH} & \quad \beta \\
\alpha & \quad \gamma \\
\beta' & \quad \alpha'
\end{align*}
\]

Di-tert-butyl 2,6-Amino-heptanedioate (11)

2,6-diamino pimelic acid (1.9 g, 10 mmol) was suspended in 1BuAc (50 mL, 0.2 M) and cooled to 0 °C. HClO₄ (5.7 mL, 60 mmol, 72% solution in H₂O) was added dropwise over 1 h. The resulting mixture was stirred for 48 h at ambient temperature. The clear solution was diluted with 10 % K₂CO₃ (aq) solution (5 mL) and brought to pH 9 with solid K₂CO₃ followed by an extraction with DCM. The combined organic layers were dried (MgSO₄) and concentrated in vacuo to yield the title compound (0.98 g, 32%). Rf = 0.77 (1 : 1 1BuOH : MeCN : H₂O); ¹H NMR (400 MHz, CDCl₃) δ 3.24 (t, 2H, J= 5.2 Hz, α + α’ CH, DAP), 1.65 – 1.61 (m, 4H, β + β’ CH₃H₂, DAP), 1.56 – 1.50 (m, 2H, γ CH₂, DAP), 1.44 (s, 18 H, CH₃, 1Bu); ¹³C NMR (100 Hz) : δ 175.1 (C=O), 80.6 (C₆ tBu), 54.7 (α CH), 54.6 (α’ CH), 34.6 (β CH₂), 27.9 (CH₃ 1Bu), 21.6 (γ CH₂); IR (cm⁻¹): 2978, 1705, 1500, 1392, 1149; LC/Ms: Rt = 3.07 min (C₁₈ Altitma, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C₁₅H₃₀N₂O₄ + H]⁺ 303.22783, found 303.22781.

Di-tert-butyl 2-Amino-6-((tertbutyloxy carbonyl)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 °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 (CHCl₃ : 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 CHCl₃ in 54% yield. Rf = 0.42 (9 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, CDCl₃) 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’ CH₃H₂, DAP, β + β’ CH₃H₂, DAP), 1.49 – 1.42 (m, 20 H, CH₃, 1Bu, γ CH₂, DAP), 1.44 (s, 9H, CH₃, 1Bu); ¹³C NMR (101 MHz, CDCl₃) δ 175.1 (C=O), 171.9 (C=O), 155.4 (C=O), 81.7 (C₆), 81.0 (C₆), 79.5 (C₆), 54.8 (α CH, DAP), 53.8 (α’ CH, DAP), 34.5 (β CH₂, DAP), 32.7 (β’ CH₂, DAP), 32.6 (β’ CH₂, DAP), 28.3 (CH₃, 1Bu), 28.03 (CH₃, 1Bu), 27.99 (CH₃, 1Bu), 21.4 (γ CH₂, DAP), 21.3 (γ CH₂, DAP); IR (cm⁻¹): 2978, 1724, 1367, 1149; LC/Ms: Rt = 6.69 min (C₁₈ Altitma, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C₂₀H₃₈N₂O₆ + H]⁺ 403.28026, found 403.27938.

Di-tert-butyl 2,6-di-((tertbutyloxy carbonyl)amino)heptanedioate (13)

Title compound was isolated as side product of previous described reaction. Flash column chromatography with pure CHCl₃ resulted 0.369 g (1.3 mmol, 22 mol %). Rf = 0.88 (9 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, CDCl₃) δ 5.09 (s, 1H, NH), 4.21 – 4.06 (m, 2H, α + α’ CH, DAP), 1.86 – 1.71 (m, 2H, β + β’ CH₃H₂, DAP), 1.70 – 1.55 (m, 2H, β + β’ CH₃H₂, DAP), 1.45 (m, 40H, 120
CH₃, tert-Bu, γ CH₂, DAP). ¹³C NMR (101 MHz, CDCl₃) δ 171.7 (C=O), 155.4 (C=O), 81.7 (C₆), 79.5 (C₆), 53.6 (α + α' CH, DAP), 32.4 (β + β' CH₂, DAP), 32.3 (β + β' CH₂, DAP), 28.2 (CH₃, tert-Bu), 27.9 (CH₃, tert-Bu), 21.0 (γ CH₂, DAP), 20.7 (γ CH₂, DAP); IR (cm⁻¹): 2978, 1708, 1499, 1365, 1149; LC/MS: Rt = 11.01 min (C₁₈ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C₁₃H₄₆N₂O₈ + H]⁺ 503.3269, found 503.3265.

Di-tert-butyl-2-(4-H-fluoren-9-yl)methoxy)carbonylamino-5-tert-butoxyo-α-isoglutamyl)-6-((tert-butoxycarbonylamino)heptanedioate (H-o-ιe-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-o-Glu-O'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 : 1), washed with 1M HCl (3 x 5 mL), sat. aqueous NaHCO₃ (3 x 5 mL), Brine (3 x 5 mL), dried (MgSO₄) and concentrated in vacuo. The crude mixture was purified by flash column chromatography (PE -> 7 : 3 PE : EtOAc) to result in the title compound (1.36 g, 1.68 mmol) in 90% yield. R₁ = 0.76 (7 : 3 EtOAc : PE); ¹H 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, CH₂, Fmoc), 4.42 - 4.31 (m, 2H, α CH, DAP, CH₂, Fmoc), 4.22 (t, J = 6.9 Hz, 1H, CH, Fmoc), 4.18 - 4.05 (m, 1H, CH, α i-o-Glu), 3.98 (q, J = 4.9, 4.0 Hz, 1H, α CH, DAP), 2.45 - 2.24 (m, 2H, CH₂, γ i-o-Glu), 2.24 - 2.10 (m, 1H, CH₂, β i-o-Glu), 1.99 - 1.87 (m, 1H, CH₂, β i-o-Glu), 1.87 - 1.72 (m, 2H, β + β' CH₂, DAP), 1.72 - 1.56 (m, 2H, β + β' CH₃, DAP), 1.53 - 1.38 (m, 40H, CH₃, tert-Bu, γ CH₂, DAP); ¹³C NMR (101 MHz, CDCl₃) δ 171.7 (C=O), 155.4 (C=O), 81.7 (C₆), 158.6 (C₆), 158.0 (C₆), 145.3 (C₆), 145.1 (C₆), 142.5 (C₆), 128.8 (CH, Fmoc), 128.1 (CH, Fmoc), 126.3 (CH, Fmoc), 126.2 (CH, Fmoc), 120.9 (CH, Fmoc), 82.8 (C₆, tert-Bu), 82.7 (C₆), 82.5 (C₆, tert-Bu), 80.4 (C₆, tert-Bu), 68.0 (CH, α i-o-Glu), 55.6 (α CH, DAP), 54.4 (α' CH, DAP), 48.6 (CH₂, Fmoc), 48.3 (CH₂, γ i-o-Glu), 33.1 (β CH₂, DAP), 32.2 (β' CH₂, DAP), 28.8 (CH₃, tert-Bu), 28.6 (CH₂, β i-o-Glu), 28.3 (CH₂, tert-Bu), 23.2 (γ CH₂, DAP); IR (cm⁻¹): 2978, 1712, 1508, 1367, 1350, 1217, 1149; LC/MS: Rt = 12.04 min (C₁₈ Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for [C₁₃H₄₆N₂O₁₁ + H]⁺ 810.45354, found 810.45447.

Di-tert-butyl-2-(4-Amino-5-tert-butoxyo-α-isoglutamyl)-6-((tert-butoxycarbonylamino)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). R₂ = 0.3 (9 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, CDCl₃) 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-o-Glu), 4.02 - 3.95 (m, 2H, NH₂), 3.60 - 3.49 (m, 1H, α' CH, DAP), 2.51 - 2.26 (m, 2H, CH₂, γ i-o-Glu), 2.22 - 2.11 (m, 1H, CH₃, β i-o-Glu), 1.93 - 1.72 (m, 3H, CH₃, β i-o-Glu), 1.72 - 1.56 (m, 2H, β + β' CH₃, DAP), 1.56 - 1.33 (m, 38H, CH₃, tert-Bu, γ CH₂, DAP); ¹³C NMR (101 MHz, CDCl₃) 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 (C₆, tert-Bu), 81.8 (C₆, tert-Bu), 81.7 (C₆, tert-Bu), 81.6 (C₆, tert-Bu), 79.4 (C₆), 1.538 (α CH, DAP), 53.6 (α' CH, DAP), 53.6 (α' CH, DAP), 52.4 (α' CH, DAP), 32.3 (CH₂, γ i-o-Glu), 32.2 (β CH₂, DAP), 31.7 (β' CH₂, DAP), 29.3 (CH₂, β i-o-Glu), 28.2 (CH₃, tert-Bu), 27.8 (CH₃, tert-Bu), 21.1 (γ CH₂, DAP); IR (cm⁻¹): 2978, 1716, 1367, 1248, 1149; LC/MS: Rt = 7.03 min
Di-tertbutyl-2-((4-(9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-dimethylphenoxo-isoglutamyl-4-(((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-tert-butylxoxy-o-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). Rf = 0.5 (9 : 1 CHCl3 : MeOH); 1H 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, CH2, Fmoc), 4.37 – 4.16 (m, 4H, CH, Fmoc, α’ CH, DAP, CH, α i-L-Glu, CH, α i-o-Glu, CH), 3.99 (s, 1H, CH, DAP), 2.46 – 2.28 (m, 4H, CH2, γ i-L-Glu, CH2, γ i-o-Glu), 2.26 – 2.20 (m, 1H, CH2, γ i-o-Glu), 2.19 – 2.11 (m, 2H, CH2, β i-L-Glu, CH2, β i-o-Glu), 2.05 – 1.87 (m, 2H, CH2, β i-L-Glu, CH2, β i-o-Glu), 1.85 – 1.65 (m, 8H, CH3; OPp, β +’ CH3HB, DAP), 1.70 – 1.55 (m, 2H, β +’ CH3HB, DAP), 1.54 – 1.37 (m, 38H, CH3; 13C 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), 171.5 (C=O), 170.8 (C=O), 170.4 (C=O), 170.4 (C=O), 170.2 (C=O), 156.5 (Cg), 155.8 (Cg), 144.5 (Cg), 143.2 (Cg), 143.0 (Cg), 140.5 (Cg), 127.4 (CH, Ar), 126.8 (CH, Ar), 126.3 (CH, Ar), 126.2 (CH, Ar), 124.3 (CH, Ar), 123.4 (CH, Ar), 119.0 (CH, Ar), 82.4 (Cq1, Bu), 81.2 (Cq2, Bu), 81.0 (Cq3, Bu), 80.9 (Cq4, Bu), 78.7 (Cq5, Bu), 66.2 (CH2, Fmoc), 53.7 (α CH, DAP), 53.5 (CH, α i-o-Glu), 52.3 (CH, α i-L-Glu), 52.2 (α’ CH, DAP), 46.4 (CH, Fmoc), 31.3 (CH2), 31.2 (CH2), 31.1 (CH3), 30.6 (CH3), 30.5 (CH3), 30.3 (CH3), 27.3 (CH3), 27.2 (CH3), 27.0 (CH3), 26.7 (CH3), 26.6 (CH3), 21.1 (y CH2, DAP); IR (cm−1): 3225, 2931, 1730, 1365, 1219, 1153; LC/MS: Rt = 5.747 min (C18 Alltima, 70 – 90% MeCN, 15 min run); HRMS Calcd. for [C58H80N204 +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-butylxoxy-o-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). Rf = 0.6 (8 : 2 CHCl3 : MeOH); 1H NMR (400 MHz, CDCl3) 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, CH2, 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, CH2, α, i-o-Glu), 4.14 – 4.08 (m, 1H, α’ CH, DAP), 2.50 – 2.12 (m, 8H, CH2, γ i-L-Glu, CH2, γ i-o-Glu, CH2, β i-L-Glu, CH2, β i-o-Glu), 2.00 – 1.95 (m, 1H, CH2, γ i-o-Glu), 1.83 – 1.53 (m, 4H, β +’ CH2, DAP), 1.53 – 1.35 (m, 38H, CH3, 13C NMR (101 MHz, CDCl3) δ 173.8 (C=O), 172.9 (C=O), 172.4 (C=O), 171.4 (C=O), 170.8 (C=O), 156.1 (Cq), 155.5 (Cq), 143.7 (Cq), 143.6 (Cq), 141.0 (Cq), 127.5 (CH, Ar), 126.9 (CH, Ar),
125.0 (CH, Ar), 119.7 (CH, Ar), 82.2 (C₂), 82.0 (C₂), 81.7 (C₂), 79.5 (C₂), 66.9 (CH₂, Fmoc), 53.6 (CH), 53.1 (CH), 52.6 (CH), 52.4 (CH), 46.9 (CH, Fmoc), 32.0 (CH₂) 31.9, (CH₂) 31.7 (CH₂), 31.5 (CH₂), 29.4 (CH₂), 29.1 (CH₂), 28.1 (CH₂), 27.8 (CH₃), 21.0 (ν CH₃, DAP); IR (cm⁻¹): 2978, 1712, 1519, 1219, 1149; LC/Ms: Rt = 6.87 min (C₁₈ Alltima, 50 - 90% MeCN, 15 min run); HRMS Calcd. for [C₄⁹H₇₀N₄O₁₄ + H]⁺ 939.49707.

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

E → [DEVA₃K]

50 µmol S RAM Tentagel (0.25 mmol/g) loaded with H-Asp(O'Bu)-Glu(O'Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O'Bu)-Gln(Trt)-Leu-Glu(O'Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-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) in NMP (2 mL, 0.1 M) for 18 h, drained and washed (DCM, NMP). The resin was treated with a solution of Ac₂O (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 Et₂O) 25 µmol of the resin was treated with a mixture of 95% TFA, 2.5% TIS and 2.5% H₂O for 104 min. The resin was filtrated and the peptide was precipitated with Et₂O. The resulting crude product was washed with Et₂O and purified by RP-HPLC resulting in the title compound (3.24 mg, 1.1 µmol, 4.3%); LC/Ms: Rt = 6.36 min (C₁₈ Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 2976.52 [M+H⁺]; HRMS Calcd. for [C₁₂₀H₂₁₁N₃₃O₅₉ + H]²⁺ 1488.76549, found 1488.76564.


E → [DEVA₃K]

25 µmol S RAM Tentagel (0.25 mmol/g) loaded with NH₂-Glu(EDAP)-Asp(O'Bu)-Glu(O'Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O'Bu)-Gln(Trt)-Leu-Glu(O'Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-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 continued 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 Et₂O the resin was treated with a mixture of 95% TFA, 2.5% TIS and 2.5% H₂O for 104 min. The resin was filtrated and the peptide was precipitated with Et₂O. The resulting crude product was washed with Et₂O and purified by RP-HPLC resulting in the title compound (4.22 mg, 0.94 µmol, 3.8%); LC/Ms: Rt = 6.36 min (C₁₄ Alltima, 50 - 90% MeCN, 15 min run); ESI-MS: m/z 4468.67 [M+H⁺]; HRMS Calcd. for [C₂₁₀H₃₆₅N₄₂O₃₅S + H]⁺ 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-Lys-Glu(o-/Glu-DAP)-NH₂ (3)**

E → [DEVA₃K]

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 M) 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 Ac₂O (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'Bu)-Glu(O'Bu)-Val-Ser(tBu)-Gly-Leu-Glu(O'Bu)-Gln(Trt)-Leu-Glu(O'Bu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-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...
The reaction mixture was washed with Et₂O and purified by RP-HPLC resulting in the title compound (5.44 mg, 1.8 µmol, 7.3%); LC/MS: Rt = 5.52 min (C₄ Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 4468.67 [M+H]⁺; HRMS Calcd. for [C₂₁₀H₃₆₅N₄₃O₅₉S + H]⁺ 1490.22932, found 1490.23265.

2-o-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 Et₂O. Purification by gel filtration (HW40, 0.15 M ammonium acetate) and lyophilization resulted in compound 6 (25.5 mg, 0.08 mmol, 32%). Rᵣ = 0.1 (8 : 2 CHCl₃ : MeOH); ¹H NMR (600 MHz, D₂O) δ 4.08 – 3.96 (m, 1H, αDCH, 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, CH₂, γ i-D-Glu), 2.12 – 1.95 (m, 2H, CH₂, β i-D-Glu), 1.85 – 1.66 (m, 3H, β ’CH₂H₃, DAP, β CH₂H₃, DAP), 1.66 – 1.54 (m, 1H, β ’CH₂H₃, DAP), 1.43 – 1.22 (m, 2H, γ CH₂, DAP), 13C NMR (151 MHz, D₂O) 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), 175.1 (C=O), 56.1 (CH), 56.0 (CH), 56.0 (CH), 55.6 (CH), 55.3 (CH), 55.1 (CH), 32.7 (CH₂), 32.3 (CH₂), 32.1 (CH₂), 32.0 (CH₂), 31.1 (CH₂), 31.1 (CH₂), 27.4 (CH₂), 27.3 (CH₂), 22.2 (CH₂), 22.0 (CH₂); IR (cm⁻¹): 2930, 1558, 1394; LC/MS: Rt = 0.81 min (C₁₈ Alltima, 0 - 20% MeCN, 15 min run); HRMS Calcd. for [C₁₂H₂₁N₃O₃S + H]⁺ 320.14523, found 320.14531.

2-(4-Lauroyl-5-tertbutyloxy-o-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 (DMF : MeOH) yielded compound 21 (85 mg, 0.11 mmol, 51%). Rᵣ = 0.9 (9 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, CDCl₃) 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, CH₂, γ i-D-Glu, CH₂, β i-D-Glu, CH₂, lauroyl), 1.99 – 1.56 (m, 8H, CH₂, lauroyl, β +

Chapter 5
Synthesis and biological evaluation of NOD1 ligand containing conjugates

\[ \beta' \text{CH}_4\text{H}_8, \text{DAP, CH}_2, \beta \text{-i-Glu}, 1.54 - 1.36 \text{ (m, 38H, CH}_3, ^1\text{Bu, } \beta + \beta' \text{CH}_4\text{H}_8, \text{DAP), 1.31 - 1.20 \text{ (m, 16H, CH}_2, \text{lauroyl, } \gamma \text{CH}_2, \text{DAP), 0.88 (t, J = 6.8 Hz, 3H, CH}_3, \text{lauroyl); } ^{13}\text{C NMR (101 MHz, CDCl}_3) \delta 173.5 \text{ (C=O), 172.1 (C=O), 171.9 (C=O), 171.8 (C=O), 171.4 (C=O), 171.3 (C=O), 171.2 (C=O), 155.4 (C=O), 82.2 (C}_o, ^1\text{Bu), 82.0 (C}_o, ^1\text{Bu), 81.7 (C}_o, ^1\text{Bu), 79. (C}_o, ^1\text{Bu), 53.6 (CH, DAP), 52.5 (CH, DAP), 52.1 (CH, DAP), 52.0 (CH, } \alpha \text{-i-Glu), 36.5 (CH}_2, \gamma \text{-i-Glu), 32.4 (CH}_2, \text{DAP), 32.3 (CH}_2, \text{DAP), 31.8 (CH}_2, \text{lauroyl), 29.5 (CH}_2, \text{lauroyl), 29.3 (CH}_2, \text{lauroyl), 29.2 (CH}_2, \text{lauroyl), 28.4 (CH}_2 \beta \text{-i-Glu), 28.2 (CH}_3, ^1\text{Bu), 27.9 (CH}_3, ^1\text{Bu), 25.5 (CH}_2, \text{lauroyl), 22.5 (CH}_2, \text{lauroyl), 21.2 (CH}_2, \text{DAP), 21.0 (CH}_2, \text{DAP), 14.0 (CH}_3, \text{lauroyl); IR (cm}^{-1}) : 2926, 1728, 1653, 1531, 1367, 1153; \text{LC/Ms: } \text{Rt} = 9.16 \text{ min (C}_{18} \text{Alltima, 70 - 90% MeCN, 15 min run); HRMS Calcd. for } [\text{C}_{41}\text{H}_{75}\text{N}_3\text{O}_{10} + \text{Na}]^+ 792.53447, \text{ found 792.53463.}
\]

2-Lauroyl-i-isoglutamyl-6-amino-heptanedioate (7)

\[ \text{Compound } 21 \text{ (85 mg, 0.11 mmol) was stirred for 5 h in a 20\% solution of TFA in DCM (1.2 mL, 0.05 m). The reaction mixture was concentrated in vacuo and purified by gel filtration (HW40, 0.15 M ammonium acetate, 20\% MeCN). Title compound was obtained in 44\% (24 mg, 0.05 mmol). R}_f = 0.1 (9 : 1 \text{CHCl}_3 : \text{MeOH); } ^1\text{H NMR (600 MHz, D}_2\text{O) } \delta 4.21 - 4.09 \text{ (m, 2H, } \alpha \text{CH, DAP, CH, } \alpha \text{-i-D-Glu), 3.68 - 3.62 (m, 1H, } \alpha' \text{CH, DAP), 2.31 - 2.25 (m, 2H, CH}_2, \gamma \text{-i-D-Glu), 2.24 - 2.16 (m, 2H, CH}_2, \text{lauroyl), 2.12 - 2.01 (m, 1H, CH}_2, \beta \text{i-o-Glu), 1.98 - 1.81 (m, 2H, CH}_2, \beta \text{i-o-Glu, } \beta + \beta' \text{CH}_4\text{H}_8, \text{DAP), 1.79 - 1.70 (m, 1H, } \beta \text{CH}_4\text{H}_8, \text{DAP), 1.67 - 1.57 (m, 1H, } \beta' \text{CH}_4\text{H}_8, \text{DAP), 1.54 - 1.40 (m, 2H, CH}_2, \text{lauroyl), 1.39 - 1.31 (m, 2H, } \gamma \text{CH}_2, \text{DAP), 1.29 - 1.12 (m, 18H, CH}_3, \text{lauroyl), 0.84 - 0.72 (t, J = 6.8 Hz, 3H, CH}_3, \text{lauroyl); } ^{13}\text{C NMR (151 MHz, D}_2\text{O) } \delta 178.9 (C=O), 177.3 (C=O), 177.3 (C=O), 175.8 (C=O), 175.6 (C=O), 55.5 (CH), 55.3 (CH), 54.9 (CH), 36.8 (CH}_2, 33.2 (CH}_2, 32.6 (CH}_2, 32.1 (CH}_2, 31.9 (CH}_2, 31.1 (CH}_2, 30.6 (CH}_2, 30.2 (CH}_2, 30.1 (CH}_2, 29.9 (CH}_2, 29.8 (CH}_2, 28.7 (CH}_2, 26.5 (CH}_2, 23.3 (CH}_2, 22.1 (CH}_2, 14.6 (CH}_3, \text{lauroyl); IR (cm}^{-1}) : 2924, 1573, 1404, 1342; \text{LC/Ms: } \text{Rt} = 6.83 \text{ min (C}_{18} \text{Alltima, 10 - 90% MeCN, 15 min run); HRMS Calcd. for } [\text{C}_{24}\text{H}_{43}\text{N}_3\text{O}_8 + \text{H}]^+ 501.31299, \text{ found 501.31193.}
\]

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


