Supporting Information File 1
defor

Design, automated synthesis and immunological evaluation of NOD2-ligand–antigen conjugates

Marian M. J. H. P. Willems¹, Gijs G. Zom², Nico Meeuwenoord¹, Ferry A. Ossendorp², Herman S. Overkleeft¹, Gijsbert A. van der Marel¹, Jeroen D. C. Codée*¹ and Dmitri V. Filippov*¹

Address: ¹Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands and ²Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre, P. O. Box 9600, 2300 RC Leiden, The Netherlands

Email: Jeroen D. C. Codée - jcodee@chem.leidenuniv.nl; Dmitri V. Filippov - filippov@chem.leidenuniv.nl

*Corresponding author

Full experimental details and characterization of all new compounds

Experimental section
All reagents and solvents used in the solid phase peptide synthesis were purchased from Bachem and Biosolve and used as received. Palmitoyl-Cys((RS)-2,3-di(palmityloxy)propyl)-OH was purchased from Bachem, Fmoc-amino acids from
Novabiochem and HATU from Tebu Bio. Tentagel based resins were ordered from Rapp Polymere. Light petroleum ether with a boiling range of 40–60 °C was used. All other solvents used under anhydrous conditions were stored over 4 Å molecular sieves except for methanol, which was stored over 3 Å molecular sieves. Solvents, used for work–up and silica gel column chromatography were of technical grade and distilled before use. All other solvents were used without further purification. Reactions were monitored by TLC–analysis or LC/MS analysis. LC/MS was conducted on a JASCO system using an Alltima C_{18} analytical column (4.6 × 50 mm, 5 μm particle size, flow 1.0 mL/min), Alltima CN analytical column (4.6 × 50 mm, 3 μm particle size, flow 1.0 mL/min) or a Alltima C_{4} analytical column (4.6 × 50 mm, 5 μm particle size, flow 1.0 mL/min). Absorbance was measured at 214 and 256 nm. Solvent system: A: 100% water, B: 100% MeCN, C: 1% aq. TFA. Gradients of MeCN in 10% C were applied over 15 minutes unless stated otherwise. Purifications were conducted on the Gilson GX-281 preparative RP-HPLC system, supplied with a semipreparative Alltima CN column (10 × 250 mm, 5 μm particle size, flow 5.0 mL/min.) or semi preparative Alltima C_{4} column (10 × 250, 5 μm particle size, flow 5.0 mL/min.). Solvent system: A: 0.1% aq. TFA and B: MeCN. Gradients of 10–90% MeCN were applied over 3 CV over 15 min unless stated otherwise. The UV absorption was measured at 214 and 256 nm. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in H_{2}O/MeCN; 50/50: v/v and 0.1% formic acid) on a mass spectrometer Thermo Finnigan LTQ Orbitrap equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 523 K) with resolution R = 60000 at m/z 400 (mass range m/z = 150–2000 ) and dioctylphthalate (m/z = 391.28428) as lock mass. Optical rotations were measured on a Propol automatic polarimeter (sodium D–line, λ = 589 nm). Specific rotations [α]_{D} are given in degree per centimeter and the concentration c is given in mg/mL in the specific solvent. Maturation and B3Z presentation results were analyzed with Graphpad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA. IR spectra were recorded on a Perkin Elmer Paragon 1000 FTIR spectrometer.

3-Azidopropyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (7)

Oxazoline 6 (4.4 g, 10 mmol) and azidopropanol (4.6 g, 46 mmol) were dissolved in freshly distilled DCM (75 mL, 0.13 M) and stirred over molecular sieves under argon
atmosphere at rt for 40 minutes. TMSOTf (1.7 mL, 9.4 mmol) was added portion-wise over 5 days. To monitor the progress of the reaction a sample of the crude reaction mixture was concentrated and taken up in CDCl₃ to analyse the sample by NMR spectroscopy. Upon completion, the reaction was quenched with TEA, filtered over Celite® and purified by flash chromatography (1:1 → 7:3 EtOAc:PE) to yield compound 7 as a white amorphous solid (3.72 g, 8.63 mmol, 83%). Rₛ = 0.4 (9 : 1 EtOAc : MeOH); [α]₀D = -0.05 (c = 1, DCM); ¹H NMR (400 MHz, CDCl₃) δ 5.37 (t, J = 9.9 Hz, 1H, H-3), 5.06 (t, J = 9.6 Hz, 1H, H-4), 4.82 (d, J = 8.3 Hz, 1H, CH, H-1), 4.30 (dd, J = 12.2, 5.0 Hz, 1H, CH₂, H-6), 4.21 – 4.07 (m, 1H, CH₂, H-6), 4.01 – 3.88 (m, 2H, CH₂, C₃H₆N₃), 3.70 – 3.65 (m, 1H, CH, H-5), 3.40 (t, J = 6.4 Hz, 2H, CH₂, C₃H₆N₃), 2.13 – 1.95 (m, 12H, CH₃, Ac, CH₃, NAc), 1.95 – 1.83 (m, J = 6.4 Hz, 2H, CH₂, C₃H₆N₃); ¹³C NMR (100 MHz, CDCl₃) δ 170.4 (C=O), 170.3 (C=O), 170.1 (C=O), 169.0 (C=O), 100.4 (CH, C-1), 72.1 (CH, C-3), 71.1 (CH, C-5), 68.6 (CH, C-4), 65.9 (CH₂, C₃H₆N₃), 61.9 (CH₂, C-6), 53.9 (CH, C-2), 47.6 (CH₂, C₃H₆N₃), 28.5 (CH₂, C₃H₆N₃), 20.7 (CH₃, NAc), 20.3 (CH₃, Ac), 20.2 (CH₃, Ac), 20.2 (CH₃, Ac); IR (cm⁻¹): 3275, 2098, 1745, 1639, 1224; HRMS [M+H⁺] Calcd. for C₁₇H₂₆N₄O₉ 431.17705, found 431.17725.

3-Azidopropyl 2-acetamido-2-deoxy-β-D-glucopyranoside (8)

Compound 7 (31.6 g, 73.4 mmol) was dissolved in MeOH (750 mL, 0.1 M) and NaOMe (0.71 g, 13 mmol) was added. The resulting solution was stirred at rt for 20 h. The reaction mixture was quenched with Amberlite® H⁺ resin. Filtration and concentration in vacuo yielded compound 10 as a white amorphous solid (22 g, 73 mmol). Rₛ = 0.1 (9 : 1 EtOAc : MeOH); [α]₀D = -0.07 (c = 1, MeOH);

¹H NMR (400 MHz, MeOD) δ 4.35 (d, J = 8.4 Hz, 1H, CH, H-1), 3.94 – 3.81 (m, 2H, CH₂ H-6, CH₂, C₃H₆N₃), 3.63 – 3.47 (m, 3H, CH, H-3, CH₂ H-6, CH₂, C₃H₆N₃), 3.40-3.18 (m, 5H, CH, H-4, CH, H-5, CH, H-2, CH₂, C₃H₆N₃) 1.95 (s, 3H, CH₃, NAc), 1.78 – 1.75 (m, 2H, CH₂, C₃H₆N₃); ¹³C NMR (100 MHz, MeOD) δ 173.7 (C=O), 102.8 (CH, C-1), 77.9 (CH, C-5), 76.0 (CH, C-3), 72.1 (CH, C-4), 67.1 (CH₂O, C₃H₆N₃), 62.8 (CH₂, C-6), 57.3 (CH, C-2), 49.0 (CH₂, C₃H₆N₃), 30.1 (CH₂, C₃H₆N₃), 23.0 (CH₃, NAc); IR (cm⁻¹): 3255, 2092, 1651, 1552; HRMS Calcd. for [C₁₁H₂₀N₄O₆+ H⁺] 305,14556, found 305,14575.
3-Azidopropyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-D-glucopyranoside (9)

Compound 8 (2.7 g, 8.6 mmol) was co-evaporated with DMF and dissolved in dry MeCN/DMF (3:1, 90 mL, 0.1 M). Benzaldehyde dimethyl acetal (1.9 mL, 13 mmol) and CSA (0.40 g, 1.7 mmol) were added and the resulting solution was stirred at rt for 18 h. The reaction was quenched with TEA, concentrated in vacuo and crystallized (DMC, MeOH, PE). Compound 9 was obtained as a white solid (2.90 g, 7.39 mmol, 86%); Rf = 0.6 (1 : 9 MeOH : DCM); [α]D = -0.27 (c = 1, 1 : 1 DCM : MeOH); 1H NMR (400 MHz, DMSO-D6) 7.86 (d, J = 8.8 Hz, NH), 7.49 – 7.35 (m, 5H, CH, Ar), 5.60 (s, 1H, CH, benzylidene acetal), 5.33 (d, J = 4.9 Hz, 1H, NH), 4.46 (d, J = 8.4 Hz, 1H, H-1), 4.20 (dd, J = 10.1, 4.8 Hz, 1H, H-6), 3.81 – 3.68 (m, 2H, CH2, C3H6N3), 3.66 – 3.28 (m, 6H, CH, H-3, CH, H-4, CH, H-5, CH2, C3H6N3), 1.83 (s, 3H, CH3, NAc), 1.77 – 1.67 (m, 2H, CH2, C3H6N3); 13C NMR (100 MHz, DMSO-D6) δ 169.4 (C=O), 137.8 (Cq, CHPh), 128.1 (CH, CHPh), 126.5 (CHPh), 101.7 (CH, CHPh), 100.8 (CH, C-1), 81.3 (CH, C-3), 70.5 (CH, C-5), 67.9 (CH2, C-6), 66.0 (CH, C-4), 65.7 (CH2, C3H6N3), 56.2 (CH, C-2), 47.5 (CH2, C3H6N3), 28.6 (CH2, C3H6N3), 23.1 (CH3, NAc); IR (cm⁻¹): 3275, 2870, 2100, 1624, 1552; HRMS Calcd. for [C18H24N4O6 + H]+ 393.1768 found 393.17673.

3-Azidopropyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-((R)-1-carboxyethyl)-β-D-glucopyranoside (10)

Compound 9 (0.8 g, 2.0 mmol) was suspended in 1,4-dioxane (30 mL, 0.07 M) and dissolved upon heating to 95 °C. NaH (0.32 g, 60% in oil, 8.0 mmol) was added and the resulting solution was stirred at 95 °C for 1 h. The solution was cooled to 65 °C and a stock solution of (S)-2-chloropropanoic acid (0.26 mL, 3.0 mmol, in 5 mL 1,4-dioxane) was added. The solution was stirred at 65 °C for 1.5 h. The reaction was quenched with MeOH and the pH was adjusted to pH ~ 3 with 1 M HCl. The product was extracted with DCM, dried (MgSO4) and concentrated in vacuo. Crystallization (CHCl3/EtOAc/PE) yielded compound 10 as an off-white solid (0.64 g, 1.4 mmol, 69%). Rf = 0.43 (1 : 9 MeOH : EtOAc); [α]D = -0.21 (c = 1, MeOH); 1H NMR (400 MHz, 1 : 1 MeOD : CDCl3) δ 7.51 – 7.45 (m, 2H, CH, Ar), 7.41 – 7.45 (m, 3H, CH, Ar), 5.59 (s, 1H, CH, benzylidene acetal), 4.57 (d, J = 8.4 Hz, 1H, CH, H-1), 4.45 (dd, J = 7.2 Hz, 14.0 Hz, 1H, CH, lactic acid), 4.34 (dd, J = 4.8 Hz, 10.4 Hz, 1H, CH2, H-6), 3.97 -3.91 (m, 1H, CH2, C3H6N3), 3.86 – 3.78 (m, 2H, H3, H6), 3.72 – 3.68 (m,
2H, CH, H2, H4), 3.61 – 3.56 (m, 1H, CH₂, C₃H₆N₃), 3.48 – 3.41 (m, 1H, CH, H-5), 3.40 (t, J = 6.4 Hz, 2H, CH₂, C₃H₆N₃), 2.03 (s, 3H, CH₃, NAc), 1.89 – 1.78 (m, 2H, CH₂, C₃H₆N₃), 1.40 (d, J = 6.8 Hz, 3H, CH₃, lactic acid); ¹³C NMR (100 MHz) δ 175.8 (C=O), 172.4 (C=O), 136.9 (C₉, Ar), 128.8 (CH, Ar), 128.0 (CH, Ar), 125.6 (CH, Ar), 102.0 (CH, benzylidene acetal), 101.0 (CH, C-1), 82.1 (CH, C-4), 77.1 (CH, C-3), 75.4 (CH, Lactic acid), 68.4 (CH₂, C-6), 66.2 (CH₂, C₃H₆N₃), 65.9 (CH, C-5), 55.4 (CH, C-2), 47.7 (CH₂, C₃H₆N₃), 28.7 (CH₂, C₃H₆N₃), 22.6 (CH₃ NAc), 18.5 (CH₃, lactic acid); IR (cm⁻¹): 3275, 2098, 1709, 1656, 1556; HRMS Calcd. for [C₂₁H₂₈N₄O₈]+ H⁺ 465.19774, found 465.19799.

**Fmoc-d-isoGln(Ot-Bu)-NH₂ (12)**

To a stirred solution of Fmoc-d-Glu(Ot-Bu)-OH (0.9 g, 2.0 mmol) in 1,4-dioxane (20 mL, 0.1M) was added NH₄HCO₃ (0.71 g, 9.0 mmol), Boc₂O (0.58 g, 2.7 mmol) and pyridine (0.25 mL, 3.1 mmol). After 24 h the solution was diluted with EtOAc/H₂O and washed with water. The organic layer was dried (NaSO₄) and concentrated in vacuo. Crystallization (MeOH) yielded compound 12 (0.62 g, 1.5 mmol, 73%). Rf = 0.5 (7 : 3 EtOAc : PE); [α]₁D = -0.6 (c = 1, CHCl₃); ¹H NMR (400 MHz, DMSO-D₆) δ 7.89 (d, J = 7.5 Hz, 2H, CH, Fmoc), 7.75 – 7.72 (m, 2H, CH, Fmoc), 7.43 – 7.28 (m, 4H, CH, Fmoc), 7.43 – 7.28 (m, 4H, CH, Fmoc), 7.06 (s, 1H, NH₂), 4.34 – 4.17 (m, 3H, CH, Fmoc, CH₂, Fmoc), 3.97 – 3.94 (m, 1H, CH, α i-d-Gln), 2.22 (t, J = 7.8 Hz, 2H, CH₂, γ i-d-Gln), 1.94 – 1.84 (m, 1H, CH, β i-d-Gln), 1.78 – 1.67 (m, J = 13.7 Hz, CH, β i-d-Gln), 1.39 (s, 9H, CH₃, ¹Bu); ¹³C NMR (100 MHz, DMSO-D₆) δ 173.4 (C=O), 171.7 (C=O), 156.0 (C=O), 143.8 (C₉, Fmoc), 140.7 (C, Fmoc), 127.7 (CH, Fmoc), 127.1 (CH, Fmoc), 125.4 (CH, Fmoc), 120.1 (CH, Fmoc), 79.7 (C₉, ¹Bu), 65.64 (CH₂, Fmoc), 53.7 (CH, α i-d-Gln), 46.7 (CH, Fmoc), 31.5 (CH₂, γ i-d-Gln), 27.8 (CH₃, ¹Bu), 27.3 (CH₂, β i-d-Gln); IR (cm⁻¹): 33387, 3329, 1720, 1689, 1532; LC/MS: Rt = 9.24 min (C₁₈ Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₂₁H₂₈N₂O₅ + H⁺] 425.20710, found 425.20706.

**Fmoc-L-Ala-d-isoGln(Ot-Bu)-NH₂ (13)**

To a stirred solution of compound 12 (2.0 g, 4.8 mmol) in DCM (40 mL, 0.73 M) was added DBU (0.71 mL, 4.8 mmol). After 20 min HOBt (2.9 g, 21 mmol) was added. Subsequently Fmoc-Ala-OH (1.8 g, 5.7 mmol), EDC (1.1 g, 5.7 mmol) and DiPEA
(4.7 mL, 28 mmol) were added. The resulting solution was stirred for 18 h, washed with 1 M HCl (3 x 5 mL), sat. NaHCO₃ (3 x 5 mL), brine (3 x 5 mL), dried (NaSO₄) and concentrated in vacuo. Precipitation from EtOAc/PE resulted in compound 16 as a white solid (1.93 g, 3.89 mmol, 82%). Rf = 0.8 (8 : 2 EtOAc : PE); [α]D = 0.8 (c = 1, CHCl₃); 1H NMR (400 MHz, DMSO-D₆) δ 8.04 (d, J = 8.2 Hz, 1H, NH), 7.90 (d, J = 7.5 Hz, 2H, CH, Fmoc), 7.73 (t, J = 6.6 Hz, 2H, CH, Fmoc), 7.61 (d, J = 7.0 Hz, 1H, NH), 7.47 – 7.32 (m, 4H, CH, Fmoc), 7.14 (s, 1H, NH₂), 4.34 – 4.12 (m, 4H, CH, α i-D-Gln, CH₂, Fmoc, CH, Fmoc), 4.08 – 4.04 (m, 1H, CH, Fmoc), 2.18 (t, J = 7.8 Hz, 2H, γ CH₂, i-D-Gln), 1.99 – 1.94 (m, J = 14.4, 7.7 Hz, CH₂, β i-D-Gln), 1.72 – 1.69 (m, 1H CH₂, β i-D-Gln), 1.36 (s, 9H, CH₃, tBu), 1.26 – 1.14 (m, 3H, CH₃, Ala); 13C NMR (100 MHz, DMSO-D₆) δ 173.1 (C=O), 172.6 (C=O), 171.6 (C=O), 155.9 (C=O), 143.9 (C=O), 140.7 (C=O), 127.7 (CH, Fmoc), 127.1 (CH, Fmoc), 125.3 (CH, Fmoc), 120.1 (CH, Fmoc), 79.7 (C₃H₆N₃O₆), 65.7 (CH₂, Fmoc), 51.4 (CH, α i-D-Gln), 50.3 (CH, α Ala), 46.6 (CH, Fmoc), 31.2 (CH₂, γ i-D-Gln), 27.7 (CH₃, tBu), 27.2 (CH₂, β i-D-Gln), 18.0 (CH₃, Ala); IR (cm⁻¹): 3286, 1641, 1541, 1257; LC/MS: Rt = 6.09 min (C₁₈ Alltima, 0 – 50% MeCN, 15 min run); HRMS Calcd. for [C₂₇H₃₃N₃O₆ + H]^+ 496.24421, found 496.24396.

Muramyl dipeptide derivative 14

To a stirred solution of compound 13 (50 mg, 0.1 mmol) in DMF (2 mL, 0.08 M) was added DBU (14 µL, 90 µmol). After 10 min HOBT (56 mg, 0.42 mmol) was added. A mixture of compound 10 (35 mg, 77 µmol) in DMF (0.5 mL, 0.15 M), HATU (29 mg, 7.7 µmol) and DiPEA (80 µL, 0.46 mmol) was added to the solution. The resulting solution was stirred for 18 h at rt. The solution was diluted 10 times (DCM), washed with 1 M HCl (3 x 5 mL), sat. NaHCO₃ (3 x 5 mL), brine (3 x 5 mL), dried (NaSO₄) and concentrated. The crude compound was purified by flash chromatography (98:2 → 9:1 DCM : MeOH) to obtain title compound 14 as an amorphous solid (50 mg, 70 µmol, 70%). Rf = 0.6 (9 : 1 CHCl₃ : MeOH); [α]D = -11.4 (c = 0.44, 1 : 1 MeOH : CHCl₃); 1H NMR (400 MHz, MeOD) δ 7.54 (d, J = 4.9 Hz, 1H, NHAc), 7.47 (m, 2H, CH, Ar), 7.42 – 7.31 (m, 3H, CH, Ar), 5.58 (s, 1H, CH, benzylidene acetal), 4.59 (d, J = 7.8 Hz, 1H, CH, H-1), 4.41 – 4.31 (m, 2H, CH, α i-D-Gln, CH₂, H-6), 4.31 – 4.21 (m, 1H, CH, α Ala), 4.14 (q, J = 6.7 Hz, 1H, CH, lactic acid), 3.99 – 3.89 (m, 1H, CH₂, C₃H₆N₃), 3.89 – 3.74 (m, 3H, CH, H-2, CH, H-3, CH₂, C₃H₆N₃), 3.71 – 3.55 (m, 2H, CH, H-4, CH₂, H-6), 3.52 – 3.42 (m, 1H, CH, H-5), 3.39 (t, J = 6.6 Hz, 2H, CH₂,
C₃H₆N₃), 2.35 – 2.31 (m, 2H, CH₂, γ i-D-Gln), 2.25 – 2.11 (m, 1H, CH₂, β i-D-Gln), 1.99 (s, 3H, CH₃, NAc), 1.95 – 1.75 (m, 3H, CH₂, C₃H₆N₃, CH₂, β i-D-Gln), 1.44 (s, 9H, CH₃, ¹Bu), 1.40 (d, J = 7.1 Hz, 3H, CH₃, lactic acid), 1.36 (d, J = 6.7 Hz, 3H, CH₃, Ala); ¹³C NMR (100 MHz, MeOD) δ 175.0 (C=O), 174.8 (C=O), 173.7 (C=O), 173.2 (C=O), 172.7 (C=O), 137.6 (C₆H₅, Ar), 129.5 (CH, Ar), 128.7 (CH, Ar), 126.4 (CH, Ar), 102.1 (CH, benzylidene acetal), 101.9 (CH, C-1), 81.6 (C₆H₅, ¹Bu), 81.5 (CH, C-4), 79.7 (CH, C-3), 78.3 (CH, lactic acid), 69.0 (CH₂, C₃H₆N₃), 66.8 (CH₂, C₃H₆N₃), 66.5 (CH, C-5), 56.3 (CH, C-2), 52.7 (CH, α i-D-Gln), 49.9 (CH, α Ala), 48.4 (CH₂, C₃H₆N₃), 32.2 (CH₂, γ i-D-Gln), 29.4 (CH₂, C₃H₆N₃), 28.2 (CH₃, ¹Bu), 27.5 (CH₂, β i-D-Gln), 23.2 (CH₃, NAc), 19.6 (CH₃, lactic acid), 17.7 (CH₃, Ala); IR (cm⁻¹): 3286, 2094, 1647, 1535; LC/MS: Rt = 7.62 min (C₁₈ Altimax), 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₃3H₄9N₇O₁₁ + H⁺] 720.35628, found 720.35639.

Muramyl dipeptide derivative 15

Compound 14 (2.7 g, 3.8 mmol) was co-evaporated with DMF and dissolved in DMF (25 mL), diluted with THF (12 mL, 0.1 M) and the reaction mixture was stirred for 3 h with PME₃ (7.5 mL, 1M in THF). The solution was concentrated in vacuo yielding the free amine (2.1 g, 3.0 mmol, 80%). Rᵣ = 0.2 (8 : 2 CHCl₃ : MeOH +2% AcOH); [α]₀ = -19 (c = 0.19, 1 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, DMSO-d₆) δ 8.31 (s, 1H, NH), 8.12 (d, J = 8.2 Hz, 1H, NH), 8.02 (d, J = 9.1 Hz, 1H, NH), 7.94 (s, 1H, NH₂), 7.73 (t, J = 3.7 Hz, 1H, NH), 7.49 – 7.30 (m, 5H, CH, Ar), 7.11 (s, 1H, NH₂), 5.75 (s, 1H, CH, benzylidene acetal), 4.47 (d, J = 8.3 Hz, 1H, CH, H-1), 4.27 – 4.19 (m, 2H, CH, α i-D-Gln, CH₂, H-6), 4.18 – 4.04 (m, 2H, CH, lactic acid, CH, Ala), 3.82 – 3.72 (m, 3H, CH, H-2, CH₂, H-6, CH₂, C₃H₆N₃), 3.69 – 3.38 (m, 4H, CH, H-3, CH, H-4, CH, H-5, CH₂, C₃H₆N₃), 2.63 (t, J = 6.9 Hz, 2H, CH₂, C₃H₆N₃), 2.17 (t, J = 7.9 Hz, 2H, CH₂, γ i-D-Gln), 1.99 – 1.87 (m, 1H, CH₂, β i-D-Gln), 1.82 (s, 3H, NAc), 1.74 – 1.55 (m, 1H, CH₂, β i-D-Gln), 1.36 (s, 9H, CH₃, ¹Bu), 1.26 – 1.18 (m, 6H, CH₃, lactic acid, CH₃, Ala); ¹³C NMR (100 MHz, DMSO-d₆) δ 171.86 (C=O), 171.24 (C=O), 171.86 (C=O), 171.67 (C=O), 137.63 (C₆H₅, Ar), 128.73 (CH, Ar), 128.21 (CH, Ar), 127.90 (CH, Ar), 125.92 (CH, Ar), 101.54 (CH, C-1), 100.20 (CH, benzylidene acetal), 80.37 (CH, C-3), 79.77 (C₆H₅, ¹Bu), 79.09 (CH, C-4), 77.38 (CH, lactic acid), 67.87 (CH₂, C-6), 66.91 (CH₂, C₃H₆N₃), 65.69 (CH, C-5), 54.73 (CH, C-2), 51.54 (CH, α i-D-Gln), 48.35 (CH, Ala), 37.84 (CH₂, C₃H₆N₃), 31.29 (CH₂, C₃H₆N₃), 31.07 (CH₂, β i-D-Gln), 27.78 (CH₃, ¹Bu), 27.22 (CH₂, γ i-D-Gln), 23.09 (CH₃, NAc), 19.08
(CH₃, lactic acid), 18.27 (CH₃, Ala); IR (cm⁻¹): 3267, 1724, 1639, 1539, 1369; LC/MS: Rt = 5.66 min (Alltima C₁₈, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₃₃H₅₁N₅O₁₁ + H]⁺ 694.36578, found 694.36615.

To the primary amine (2.1 g, 2.9 mmol), dissolved in DMF (39 mL, 0.07 M), were added Fmoc-Glu-OAllyl (1.3 g, 3.2 mmol), HCTU (1.3 g, 3.2 mmol) and DiPEA (1.4 mL, 8.7 mmol). The solution was stirred for 18 h and concentrated in vacuo. Precipitation from CHCl₃ /MeOH/Et₂O yielded the MDP derivative 15 (1.8 g, 1.7 mmol, 57%). Rf = 0.8 (8 : 2 CHCl₃ : MeOH); [α]D = -23 (c = 0.28, 1 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, DMSO-D₆) δ 8.09 (d, J = 8.3 Hz, 1H, NH), 7.95 – 7.75 (m, 5H, CH, Ar, NH), 7.71 (d, J = 7.4 Hz, 2H, CH, Ar), 7.48 – 7.26 (m, 2H, CH, Fmoc), 7.09 (s, 1H, NH₂, i-d-Gln) 5.96 – 5.80 (m, 1H, CH, allyl), 5.35 – 5.16 (m, 2H, CH₂, allyl), 4.45 (d, J = 8.3 Hz, 1H, CH, H-1), 4.36 – 4.18 (m, 5H, CH₂, H-6, CH, lactic acid, CH₂, Fmoc, CH₂, Fmoc), 4.18 – 4.09 (m, 1H, CH, α Glu), 4.10 – 4.00 (m, 2H, C₃H₆N₃, CH₂, β i-D-Glu, CH₂, γ Glu), 3.81 – 3.71 (m, 9H, CH₃, tBu), 3.69 – 3.57 (m, 2H, CH, H-3, CH, H-4), 3.49 – 3.28 (under H₂O signal, CH₂, C₃H₆N₃, CH, H-5), 3.19 – 2.94 (m, 2H, CH₂, C₃H₆N₃), 2.24 – 2.10 (m, 4H, CH₂, γ i-D-Glu, CH₂, γ Glu), 2.05 – 1.86 (m, 2H, CH₂, β i-D-Glu, CH₂, β Glu), 1.91 – 1.73 (m, 4H, CH₃, NAc, CH₂, β i-D-Gln), 1.73 – 1.63 (m, 1H, CH₂, β Glu), 1.58 (t, J = 6.4 Hz, 2H, CH₂, C₃H₆N₃), 1.36 (s, 9H, CH₃, tBu), 1.24 – 1.19 (m, J = 16.7, 6.8 Hz, 6H, CH₃, lactic acid, CH₃, Ala); ¹³C NMR (100 MHz, DMSO-D₆) δ 173.1 (C=O), 172.1 (C=O), 171.9 (C=O), 171.8 (C=O), 171.6 (C=O), 169.9 (C=O), 156.2 (C=O), 143.8 (Cq), 140.8 (Cq), 137.6 (Cq), 132.4 (CH, allyl), 128.8 (CH, Ar), 128.2 (CH, Ar), 127.7 (CH, Ar), 127.2 (CH, Ar), 125.9 (CH, Ar), 125.3 (CH, Ar), 120.2 (CH, Ar), 117.8 (CH₂, allyl), 101.6 (CH, C-1), 100.2 (CH, benzylidene acetal), 80.4 (CH, C-3), 79.7 (Cq, tBu), 79.1 (CH, C-4), 77.4 (CH, lactic acid), 67.8 (CH₂, H-6), 66.9 (CH₂, C₃H₆N₃), 65.8 (CH₂, Fmoc), 65.7 (CH, C-5), 64.9 (CH₂, allyl), 54.7 (CH, C-2), 53.6 (CH, α i-D-Gln), 51.5 (CH, α Glu), 48.7 (CH, Fmoc), 46.7 (CH, Ala), 35.6 (CH₂, C₃H₆N₃), 31.6 (CH₂, γ i-D-Gln), 31.3 (CH₂, γ Glu), 29.3 (CH₂, C₃H₆N₃), 27.8 (CH₃, tBu), 27.2 (CH₂, β, Glu), 26.7 (CH₂, β i-D-Gln), 23.1 (CH₃, NAc), 19.1 (CH₃, lactic acid), 18.2 (CH₃, Ala); IR (cm⁻¹): 3278, 1728, 1639, 1539, 1369; LC/MS: Rt = 9.20 min (Alltima C₁₈, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₅₆H₇₂N₆O₁₆ + H]⁺ 1085.50776, found 1085.50731.
Muramyl dipeptide derivative 16

To compound 15 (0.2 g, 0.2 mmol) dissolved in DMF (4 mL, 0.05 M) was added AcOH (50 µL, 0.85 mmol), Bu₃SnH (0.1 mL, 0.4 mmol) and Pd(PPh₃)₄ (8 mg, 7 µmol). The resulting solution was stirred for 1.5 h. Crude compound 15 was precipitated by adding Et₂O. A second precipitation (CHCl₃/MeOH/Et₂O) yielded compound 21 (0.14 g, 0.13 mmol, 72%). Rₛ = 0.3 (8 : 2 CHCl₃ : MeOH); [α]D = -20.0 (c = 0.75, CHCl₃ : MeOH); ¹H NMR (600 MHz, DMSO-D₆) δ 8.07 (d, J = 8.2 Hz, 1H, NH), 7.95 – 7.85 (m, 3H, CH, CHPh, NH), 7.78 (s, 1H, NH), 7.72 (d, J = 7.3 Hz, 2H, NH), 7.66 – 7.58 (m, 3H, CH, Ar), 7.58 – 7.52 (m, 1H, NH), 7.46 – 7.24 (m, 14H, CH, Ar), 7.07 (s, 1H, NH), 5.68 (s, 1H, benzylidene acetal), 4.46 (d, J = 8.3 Hz, 1H, CH, H-1), 4.32 – 4.17 (m, 5H, CH₂, H-6, CH₂, C₃H₆N₃, CH₂, Fmoc, CH, Ala, CH, α Glu), 4.15 – 4.11 (m, 1H, CH, α i-d-Gln), 4.06 (q, J = 6.5 Hz, 1H, CH, lactic acid), 3.94 – 3.92 (m, 1H, CH, Fmoc), 3.82 – 3.69 (m, 3H, CH₂, C₃H₆N₃), 3.66 – 3.60 (m, 2H, CH₂, Ar-3, CH₂, Ar-4), 3.55 – 3.33 (m, 2H, CH₂, C₃H₆N₃), 3.10 – 2.99 (m, 2H, CH₂, C₃H₆N₃), 2.21 – 2.15 (m, 4H, CH₂, CH₂, CH₂, C₃H₆N₃), 1.92 – 1.72 (m, 4H, CH₂, C₃H₆N₃), 1.68 – 1.57 (m, 1H, CH₂, β i-d-Gln), 1.56 – 1.51 (m, 2H, CH₂ C₃H₆N₃), 1.36 (s, 9H, CH₃, tBu), 1.20 (d, J = 6.9 Hz, 3H, CH₃, lactic acid), 1.20 (d, J = 6.6 Hz, 3H, CH₃, Ala); ¹³C NMR (151 MHz, DMSO-D₆) δ 173.1 (C=O), 172.1 (C=O), 171.8 (C=O), 171.6 (C=O), 171.4 (C=O), 169.9 (C=O), 156.1 (C=O), 143.8 (C₄, Fmoc), 140.7 (C₄, Fmoc), 137.6 (C₄, benzylidene acetal), 132.1 (CH, Ar), 131.5 (CH, Ar), 131.5 (CH, Ar), 128.8 (CH, Ar), 128.8 (CH, Ar), 128.8 (CH, Ar), 128.1 (CH, Ar), 127.7 (CH, Ar), 127.1 (CH, Ar), 125.9 (CH, Ar), 125.3 (CH, Ar), 120.1 (CH, Ar), 101.5 (CH, C=1), 100.1 (CH, benzylidene acetal), 80.3 (CH, C=4), 79.7 (C₄, tBu), 79.1 (CH, C=3), 77.3 (CH, lactic acid), 67.8 (CH₂, C=6), 66.9 (CH₂, C₃H₆N₃), 65.7 (CH₂, Fmoc), 65.6 (CH, C=5), 54.7 (CH, C=2), 53.6 (CH, Fmoc), 51.5 (CH, α i-d-Gln), 48.3 (CH, Ala), 46.7 (CH, α Glu), 35.5 (CH₂, C₃H₆N₃), 31.8 (CH₂, γ Glu), 31.2 (CH₂, γ i-d-Gln), 29.3 (CH₂, C₃H₆N₃), 27.7 (CH₃ tBu), 27.2 (CH₂, β Glu, CH₂, β i-d-Gln), 23.0 (CH₃, NAc), 19.0 (CH₃, lactic acid), 18.2 (CH₃, Ala); IR (cm⁻¹): 3282, 1720, 1639, 1539, 1369; LC/MS: Rt = 8.85 min (Alltima C₁₈, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₅₅H₆₈N₆O₁₆ + H]⁺ 1045.47646, found 1045.47762.
Muramyl dipeptide derivative 17

Compound 14 (0.21 g, 0.29 mmol) was suspended in 60% AcOH in H₂O (3.0 mL, 0.1 M) and stirred with neopentylglycol (60 mg, 0.58 mmol) at 65 °C for 3 h. The solution was diluted with H₂O, concentrated in vacuo and co-evaporated (toluene). Purification by flash chromatography (9:1 DCM/MeOH) resulted in compound 17 as a white solid (0.17 g, 0.26 mmol, 88%). Rₜ = 0.2 (9 : 1 CHCl₃ : MeOH); [α]D = 3.0 (c = 0.2, 1 : 1 CHCl₃ : MeOH); 

¹H NMR (600 MHz, DMSO-D₆) δ 4.31 – 4.20 (m, 2H, CH, H-1, CH, lactic acid), 4.18 – 4.08 (m, 2H, CH, Ala, CH, α i-D-Gln), 3.78 – 3.75 (m, 1H, CH₂, C₃H₆N₃), 3.72 – 3.66 (m, 1H, CH₂, H-6), 3.60 (q, J = 9.2 Hz, 1H, CH, H-2), 3.54 – 3.43 (m, 2H, CH₂, H-6, CH₂, C₃H₆N₃), 3.41 – 3.26 (m, 3H, CH, H-3, CH₂, C₃H₆N₃), 3.26 – 3.21 (m, 1H, CH, H-4), 3.16 – 1.13 (m, 1H, CH, H-5), 2.19 (t, 2H, J =5.2 Hz, CH₂, γ i-D-Gln), 1.97 – 1.91 (m, 1H, CH₂, β i-D-Gln), 1.78 (s, 3H, HNAc), 1.74 – 1.67 (m, 3H, CH₂, β i-D-Gln, CH₂, C₃H₆N₃), 1.39 (s, 9H, CH₃, tBu), 1.26 – 1.24 (m, 6H, CH₃, lactic acid, CH₃, Ala); 

¹³C NMR (100 MHz, DMSO-D₆) δ = 173.1 (C=O), 172.3 (C=O), 172.1 (C=O), 171.6 (C=O), 169.3 (C=O), 100.8 (CH, C-1), 82.2 (CH, C-3), 76.8 (CH, C-5), 76.7 (CH₂, C-6), 69.3 (CH, C-4), 65.2 (CH₂, C₃H₆N₃), 60.8 (CH₂, C-6), 51.5 (CH, Ala), 48.2 (CH, α i-D-Gln), 47.5 (CH₂, C₃H₆N₃), 31.2 (CH₂, γ i-D-Gln), 28.5 (CH₂, C₃H₆N₃), 27.7 (CH₃, tBu), 27.1 (CH₂, β i-D-Gln), 23.0 (CH₃, HNAc), 19.0 (CH₃, lactic acid), 18.1 (CH₃, Ala); IR (cm⁻¹): 3278, 2098, 1643, 1539, 1369; LC/MS: Rt = 4.52 min (C₁₈ Alltima, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₂₆H₄₅N₇O₁₁ + H]⁺ 632,32498, found 632,32516.

Muramyl dipeptide derivative 18

Compound 18 (0.21 g, 0.33 mmol) was co-evaporated with pyridine, dissolved in warm pyridine (0.92 mL, 11 mmol) and diluted with 1,4-dioxane (3 mL, 0.1 M). Ac₂O (0.27 mL, 2.9 mmol) was added and the solution was stirred for 48 h. The reaction mixture was quenched with MeOH and concentrated in vacuo and co-evaporated with toluene to yield crude compound 18. Rₜ = 0.7 (9 : 1 CHCl₃ : MeOH + 1% AcOH); [α]D = 1.5 (c = 0.65, 1 : 1 CHCl₃ : MeOH); 

¹H NMR (400 MHz, MeOD) δ 4.92 (t, J = 9.4 Hz, 1H, CH, H-4), 4.45 (d, J = 8.1 Hz, 1H, CH, H-1), 4.30 (dd, J = 9.3, 4.6 Hz, 1H, CH, α i-D-Gln), 4.20 (dd, J = 12.2, 4.8 Hz, 1H, CH₂, H-6), 4.16 – 4.04 (m, 2H, CH₂, H-6, CH, lactic acid), 3.98 (q, J = 6.7 Hz, 1H, CH, Ala), 3.92 – 3.83 (m, 1H, CH₂, C₃H₆N₃), 3.78 -3.71 (m, 2H, CH, H-2, CH, H-3), 3.63 -3.59 (m,
Muramyl dipeptide derivative 19

Compound 18 (73 mg, 0.11 mmol) was dissolved in a mixture of 20% TFA in DCM (1 mL, 0.1 M) and stirred for 2.5 h at ambient temperature. The compound was precipitated using Et2O. The resulting solid was purified by flash column chromatography (9:1 CHCl3/MeOH + 1% AcOH) yielding compound 19 (54 mg, 80 µmol, 82%). Rf = 0.2 (9 : 1 CHCl₃ : MeOH + 1% AcOH); [α]D = -5.2 (c = 0.27, 1 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, MeOD) δ 4.96 (t, J = 9.6 Hz, 1H, CH, H-4), 4.46 (d, J = 8.4 Hz, 1H, CH, H-1), 4.37 – 4.35 (m, 1H, CH, α i-d-Gln), 4.26 (dd, J = 12.3, 4.6 Hz, 1H, CH₂, H-6), 4.19 (q, J = 7.1 Hz, 1H, CH, lactic acid), 4.11 (dd, J = 12.3, 2.1 Hz, 1H, CH₂, H-6), 4.08 – 4.03 (m, 1H, CH, Ala), 3.96 – 3.86 (m, 2H, CH₂, C₃H₅N₃, CH, H-2), 3.77 – 3.67 (m, 2H, CH, H-3, CH, H-5), 3.70 – 3.59 (m, 1H, CH₂, C₃H₅N₃), 3.41 – 3.33 (m, 2H, CH₂, C₃H₅N₃), 2.38 (t, J = 7.6 Hz, 2H, CH₂, γ i-d-Gln), 2.30 – 2.19 (m 1H, CH₂, β i-d-Gln), 2.12 (s, 3H, Ac), 2.07 (s, 3H, CH₃, Ac), 1.97 – 1.88 (m, 4H, CH₃, NAc, CH₂, β i-d-Gln), 1.88 – 1.72 (m, 2H, CH₂, C₃H₅N₃), 1.41 (d, J = 7.1 Hz, 3H, CH₃, lactic acid), 1.27 (d, J = 6.7 Hz, 3H, CH₃, Ala); ¹³C NMR (100 MHz, MeOD) δ 176.4 (C=O), 175.9 (C=O), 175.0 (C=O), 174.7 (C=O), 173.4 (C=O), 172.6 (C=O), 171.7 (C=O), 102.0 (CH, C-1), 80.8 (CH, C-3), 79.1 (CH, lactic acid), 72.4 (CH, C-5), 70.5 (CH, C-4), 67.3 (CH₂, C₃H₅N₃), 63.2 (CH₂, C-6), 56.5 (CH, C-2), 53.3 (CH, α i-d-Gln), 50.6 (CH, Ala), 48.4 (CH₂, C₃H₅N₃), 31.0 (CH₂, γ i-d-Gln).
29.7 (CH₂, C₃H₃N₃), 27.6 (CH₂, β i-D-Gln), 23.1 (CH₃, NAc), 21.1 (CH₃, Ac), 20.9 (CH₃, Ac), 19.4 (CH₃, lactic acid), 17.5 (CH₃, Ala); IR (cm⁻¹): 3294, 2098, 1654, 1535; LC/MS: Rt = 4.85 min (Alltima C₁₈, 10 – 90% MeCN, 15 min run); HRMS Calcd. for [C₂₆H₄₁N₇O₁₃ + H]⁺, 660.28351 found 660.28379.

**Azidopropyl muramyl dipeptide 20**

Compound 19 (15 mg, 23 µmol) was dissolved in a solution of 7 M ammonia in MeOH (1.5 mL). The solution was stirred for 5 h at ambient temperature. The reaction mixture was concentrated and purified over HW40 gel filtration chromatography (0.15 M, ammonium acetate). After lyophilization compound 25 was obtained as a white solid (12 mg, 20 µmol, 87%).

Rf = 0.2 (8 : 2 CHCl₃ : MeOH + 2% AcOH); [α]D = -12.5 (c = 0.02, 1 : 1 CHCl₃ : MeOH); ¹H NMR (400 MHz, D₂O) δ 4.56 (d, J = 8.5 Hz, 1H, CH, H-1), 4.47 – 4.43 (m, 1H, CH, α i-D-Gln), 4.38 – 4.26 (m, 2H, CH, lactic acid, CH, Ala), 4.09 – 4.03 (m, 1H, CH₂, C₃H₆N₃), 4.01 (d, J = 1.9 Hz, 1H, CH₂, H-6), 3.93 – 3.87 (m, 1H, CH, H-2), 3.84 (d, J = 5.5 Hz, 1H, CH₂, H-6), 3.82 – 3.72 (m, 1H, CH₂, C₃H₆N₃), 3.65 – 3.52 (m, 3H, H-3, CH, H-4, CH, H-5), 3.31 – 3.27 (m, 2H, CH₂, C₃H₆N₃), 2.50 (t, J = 7.3 Hz, 2H, CH₂, γ i-D-Gln), 2.33 – 2.20 (m, 2H, CH₂, β i-D-Gln), 2.11 – 2.01 (m, 4H, CH₂, β i-D-Gln, 3H, CH₃, NAc), 1.81 -1.72 (m, 2H, CH₂, C₃H₆N₃), 1.53 (d, J = 7.2 Hz, 3H, CH₃, lactic acid), 1.46 (d, J = 6.8 Hz, 3H, CH₃, Ala). ¹³C NMR (151 MHz, D₂O) δ 179.5 (C=O), 177.1 (C=O), 176.7 (C=O), 176.1 (C=O), 175.0 (C=O), 102.2 (CH, C-1), 83.7 (CH, C-3), 79.3 (CH, lactic acid), 76.5 (CH, C-5), 69.8 (CH, C-4), 68.1 (CH₂, C₃H₆N₃), 61.6 (CH₂, C-6), 56.0 (CH, C-2), 53.9 (CH, α i-D-Gln), 50.8 (CH, Ala), 49.0 (CH₂, C₃H₆N₃), 32.4 (CH₂, γ i-D-Gln), 29.1 (CH₂, β i-D-Gln), 27.6 (CH₂, C₃H₆N₃), 23.2 (CH₃, NAc), 19.7 (CH₃ lactic acid), 17.5 (CH₃, Ala); IR (cm⁻¹): 3310, 2101, 1645, 1464; LC/MS: Rt = 8.07 min (Alltima C₁₈, 0 – 20% MeCN, 15 min run); HRMS Calcd. for [C₂₂H₃₇N₇O₁₁ + H]⁺, 576.26238 found 576.26243.

**Automated synthesis of MDP-peptide conjugates**

**General procedure for automated solid-phase synthesis**

The solid-phase peptide synthesis was performed on 50 µmol or 25 µmol scale according to established methods [1] on an ABI 433A (Applied Biosystems) automated instrument applying an Fmoc based protocol starting from Tentagel-S-
RAM resin (loading 0.23 mmol/g). The consecutive steps performed in each cycle for HCTU chemistry on 50 µmol scale:

1. Deprotection of the Fmoc-group with 20% piperidine in NMP for 15 min; 2) NMP wash; 3) Coupling of the appropriate amino acid using a five-fold excess. Generally, the Fmoc amino acid (0.25 mmol) was dissolved in 0.25 M HCTU in NMP (1 mL), the resulting solution was transferred to the reaction vessel followed by 0.5 mL of 1.0 M DiPEA in NMP to initiate the coupling. The reaction vessel was shaken for 30 min; 4) NMP wash; 5) capping with 0.5 M acetic anhydride in NMP in presence of 0.5 mmol DiPEA; 6) NMP wash; 7) DCM wash.

The consecutive steps performed in each cycle for HATU chemistry:

1. Deprotection of the Fmoc-group with 20% piperidine in NMP for 15 min; 2) NMP wash; 3) Coupling of the appropriate amino acid using a two-fold or five-fold excess. Generally, the Fmoc amino acid (0.1 mmol or 0.25 mmol) and HATU (0.15 mmol or 0.2 mmol) was dissolved in 1.0 M DiPEA in NMP (0.25 mL or 0.5 mL). The resulting solution was pre-activated for 1 min and transferred to the reaction vessel to initiate the coupling. The reaction vessel was shaken for 60 min; 4) NMP wash; 5) capping with 0.5 M acetic anhydride in NMP in presence of 0.5 mmol DiPEA; 6) NMP wash; 7) DCM wash.

Aliquots of resin of the obtained sequences were checked by HPLC using an analytical Alltima C_{18} column (4.6 × 50 mm, 5 µm particle size, flow 1.0 mL/min.). The Fmoc amino acids applied in the synthesis were: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(Ot-Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(Or-Bu)-OH, Fmoc-Gly-OH, Fmoc-Glu(Ot-Bu)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(MMt)-OH, Fmoc-Phe-OH, Fmoc-Ser(t-Bu)-OH and Fmoc-Val-OH.

General procedure for cleavage from the resin, deprotection and purification

The resin was washed with NMP, DCM and dried after the last synthesis step. Next it was treated with 5 mL cleavage cocktail (95% TFA, 2.5% TIS and 2.5% H_{2}O) for 104 min. The suspension was filtered, the resin was washed with neat TFA and the product was precipitated with Et_{2}O out of the TFA solution. The suspension of the
product in Et$_2$O was centrifuged, Et$_2$O removed and the precipitate was washed with Et$_2$O (3×). The final precipitate was air dried and dissolved in AcOH/H$_2$O (1:1) or MeCN/H$_2$O/t-BuOH (1:1:1) followed by RP-HPLC purification.


Tentagel S Ram resin loaded with H-Asp($t$-Bu)-Glu($t$-Bu)-Val-Ser($t$-Bu)-Gly-Leu-Glu($t$-Bu)-Gln(Trt)-Leu-Glu($t$-Bu)-Ser($t$-Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Lys(Boc) (25 µmol) was elongated with Fmoc-δ-isoGln-OH and Fmoc-Ala-OH using a standard HCTU/Fmoc cycle. The synthesis was completed with a double coupling using two-fold excess of compound 10, pre-activated with HATU and DiPEA. After treatment with the standard cleavage cocktail for 60 min. the suspension was filtered and the product was precipitated with Et$_2$O. After purification by RP-HPLC, compound 2 and hydrolyzed compound 22 were isolated. Compound 2 was obtained in 1.3 mg (0.41 µmol, 2%); LC/MS: Rt = 6.22 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 3103.60 [M+H]$^+$; HRMS Calcd. for [C$_{134}$H$_{220}$N$_{36}$O$_{48}$ + H]$^{2+}$ 1552.30276, found 1552.30469; Compound 22 was isolated in 1.5 mg (0.38 µmol, 2%); LC/MS: Rt = 6.05 min (C$_{18}$ Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 3020.55 [M+H]$^+$; HRMS Calcd. for [C$_{131}$H$_{215}$N$_{34}$O$_{48}$ + H]$^{2+}$ 1510.77860, found 1510.78070

H-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Lys-(δ-isoGln-Ala-3-azidopropyl-MurNAc)NH$_2$ (3)

Tentagel S Ram resin loaded with H-Asp($t$-Bu)-Glu($t$-Bu)-Val-Ser($t$-Bu)-Gly-Leu-Glu($t$-Bu)-Gln(Trt)-Leu-Glu($t$-Bu)-Ser($t$-Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Lys(Boc) (25 µmol) was treated 1 M Boc$_3$O in NMP for 15 min followed by addition of 2 equiv of DiPEA. After one hour the resin was washed with NMP and DCM. The resin was treated with a cleavage cocktail of 3% TFA in DCM followed by a coupling sequence of Fmoc-δ-isoGln-OH and Fmoc-Ala-OH with a standard HCTU/Fmoc cycle. The synthesis was completed with a double coupling of a two-fold excess of compound 10 pre-activated with HATU and DiPEA. After treatment with the standard cleavage cocktail for 60 min. the suspension was filtered and the product was precipitated using Et$_2$O. After HPLC purification, both compound 3 and hydrolyzed compound 25 were isolated.
Compound 3 was obtained in 1.2 mg (0.34 µmol, 2%); LC/MS: Rt = 6.16 min (C<sub>18</sub> Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 3103.60 [M+H]<sup>+</sup>; HRMS Calcd. for [C<sub>134</sub>H<sub>220</sub>N<sub>36</sub>O<sub>4</sub> + H]<sup>2+</sup> 1552.30276, found 1552.30434. Compound 25 was obtained in 1.0 mg (0.39 µmol, 2%); LC/MS: Rt = 6.02 min (C<sub>18</sub> Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 3020.55 [M+H]<sup>+</sup>; HRMS Calcd. for [C<sub>131</sub>H<sub>215</sub>N<sub>33</sub>O<sub>48</sub> + H]<sup>2+</sup> 1510.77860, found 1510.78070.


Resin 21 was treated with a mixture of compound 16 (53 mg, 50 µmol), HCTU (21 mg, 50 µmol) and DiPEA (18 µL, 0.1 mmol) in NMP (0.5 mL) for 18 h. The resin was washed and treated with a solution of 20% piperidine in NMP followed by a wash step (NMP, DCM, Et<sub>2</sub>O). Treatment with the cleavage cocktail for 60 min and purification resulted in compound 4 (1.8 mg, 0.53 µmol, 2%), LC/MS: Rt = 8.78 min (C<sub>18</sub> Alltima, 10 - 50% MeCN, 15 min run); ESI-MS: m/z 3206.65 [M+H]<sup>+</sup>; HRMS Calcd. for [C<sub>139</sub>H<sub>229</sub>N<sub>35</sub>O<sub>51</sub> + H]<sup>2+</sup> 1603.82882, found 1603.82944.


40 µmol Sieber Amide resin (0.2 mmol/g) was treated with 20% piperidine in NMP (3 × 3 min), washed with NMP and treated with a mixture of compound 16 (107 mg, 102 µmol), DiPEA (67 µL, 406 µmol) and HCTU (76 mg, 184 µmol) in 4:1 NMP/DMSO. The resulting suspension was shaken for 18 h, washed with NMP, DCM and Et<sub>2</sub>O. An aliquot of resin (5 mg) was treated with 20% piperidine (1 mL) for 20 min. The solution was diluted with EtOH (25 mL) and absorbance at 300 nm was measured. The Fmoc-test revealed a 56% loading. The resin was treated with a capping solution (0.5 M Ac<sub>2</sub>O, 0.05 M DiPEA, NMP, 3 × 15 min) and elongated with the standard Fmoc based SPPS protocol to H-Asp(Or-Bu)-Glu(Or-Bu)-Val-Ser(t-Bu)-Gly-Leu-Glu(Or-Bu)-Gln(Trt)-Leu-Glu(Or-Bu)-Ser(t-Bu)-Ile-Ile-Asn(Trt)-Phe-Glu-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Lys(Boc) concluding with a final Fmoc deprotection. 20 µmol resin was treated with standard cleavage conditions. After
purification compound 5 and hydrolyzed analogue 28 were isolated. Compound 5 was obtained in 4.8 mg (1.4 µmol, 6%). LC/MS: Rt = 5.70 min (C\textsubscript{18} Altima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 3206.65 [M+H]\textsuperscript{+}; HRMS Calcd. for [C\textsubscript{139}H\textsubscript{229}N\textsubscript{35}O\textsubscript{51} + H]\textsuperscript{2+} 1603.8282, found 1603.82943. Compound 28 was isolated in 2.8 mg (0.94 µmol, 4%); LC/MS: Rt = 5.82 min (C\textsubscript{18} Altima, 10 - 90% MeCN, 15 min run); ESI-MS: m/z 2731.45 [M+H]\textsuperscript{+}; HRMS Calcd. for [C\textsubscript{120}H\textsubscript{198}N\textsubscript{31}O\textsubscript{41} + H]\textsuperscript{3+} 911.15363, found 911.15694.

**Immunological assays**

**Cell culture**
The D1 cell line is a growth factor-dependent immature spleen-derived DC cell line from C57BL/6 (H-2\textsubscript{b}) mice. D1 cells were cultured as described [2]. The B3Z hybridoma is cultured in complete IMDM medium supplemented with 500 µg/mL hygromycin [3]. NOD2-HEK293 cells were cultured in complete IMDM medium, supplemented with 10 µg/ml blasticidin.

**NOD2-HEK293 activation**
The human NOD2-receptor expressing the HEK293 cell-line was obtained from Invivogen (Toulouse, France). Test compounds were titrated in a 96-wells plate and approximately 50,000 NOD2-HEK293 cells were subsequently added per well. After 24 hours 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 for 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 maturation assay**
Test compounds were titrated in a 96-wells plate (Corning, Amsterdam, The Netherlands) in complete IMDM medium. Next, D1 cells from C57BL/6 mice were harvested and counted, and subsequently transferred to the 96-wells plates containing the test compound titrations, using approximately 40,000 cells per well. After 24 hours of incubation at 37 °C, supernatant was taken from the wells for ELISA analysis (BioLegend, San Diego, USA) in which the amount of produced IL-12p40 was measured.
In vitro antigen presentation assay

B3Z is a CD8+ T-cell hybridoma specific for the H-2Kb 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 gene product of which is able to convert the chromogenic substrate CPRG (Chlorophenolred-β-D-galactopyranoside). This conversion is measured by absorbance spectrophotometry at a wavelength of 590 nm [3].

References

