Self adjuvanting immunopeptides : design and synthesis

Gential, G.P.P.

Chapter 4: Synthesis of TLR-7 peptide conjugates

Toll-like receptors (TLRs) are part of the mammalian innate immune system that forms the first line of defence against pathogens by recognising pathogen associated molecular patterns (PAMPs).[1] Upon recognition of a specific PAMP by the corresponding TLR, a signal transduction pathway is started that activates the immune system. Ten different human TLRs can be discerned that are situated either at the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or in intracellular (TLR3, TLR7, TLR8 and TLR9) compartments.[2] Each TLR recognizes PAMPs of certain structural identity and intracellular TLRs bind to nucleic acids of various origin. TLR8 binds to viral and bacterial RNA.[3, 4] TLR9 recognizes bacterial and viral single stranded DNA.[5] Single-stranded RNA from viruses is the ligand of TLR7.[6] As modulators of the immune system TLR ligands are important drug targets and much research has been directed to the development of small-molecule TLR agonists.[7] Imiquimod is the first example of a TLR7 agonist[8] that is used for topical treatment of a variety of skin diseases.[9, 10] In the last decade structure and activity studies of small molecules have resulted in specific and non-specific agonists for TLR7 and TLR8. Well known examples are represented by imidazoquinolines (e.g. imiquimod) and 8-hydroxyadenine derivatives.[11, 12] These agonists, either as such or conjugated to other molecular entities have been explored as adjuvants in the framework of the development of new vaccines and immunotherapies.[11, 13, 14] For example, to improve the potency and/or to prevent toxic side effects TLR7 ligands are conjugated to macromolecules such as phospholipids, polysaccharides and peptides.[15, 16] One approach toward the development of fully synthetic vaccine modalities is directed to the design, synthesis and evaluation of conjugates, comprising a structurally defined TLR agonist, covalently connected to an oligopeptide epitope. Several of these conjugates exhibit improved immunological properties in comparison with a mixture of the composing components.[17-19] Such conjugates in which a TLR7 ligand is covalently connected to an antigenic peptide have been prepared as well. The group of Filippov synthesized derivatives (**1b-d**, Figure 1) of the TLR7 ligand 9-benzyl-8-hydroxy-2-alkoxy-adenine (**1a**), suitable for conjugation.[20] The azide was used to covalently attach the ligand to the well-known CTL (cytotoxic T lymphocyte) epitope (SIINFEKL) by copper(I) catalysed cycloaddition to give conjugates such as **2**. Although these conjugates give rise to enhanced antigen presentation in vitro, they unexpectedly lack the ability to induce maturation of dendritic cells (DC). The latter action is crucial for the induction of T-cell mediated immunity. The position in the TLR7 agonist molecule to which the peptide epitope is attached proved to be of prime importance for the immunological activity of the resulting conjugate. It was reported that attachment of the 8-hydroxyadenine based TLR7 agonist via its 9-benzyl moiety to an oligopeptide did mediate DC activation.[15]

This chapter describes the synthesis of conjugates comparable to the ones described previously by others for different antigens. In the conjugates described here (Figure 1, **3 - 5**) the TLR7 ligand 9-benzyl-8-hydroxy-2-butoxy-adenine is covalently connected with its 9-benzyl moiety to the CTL (SIINFEKL) epitope. A derivative of 9-benzyl-8-hydroxy-2-butoxy-adenine having a carboxyl function at the para position of the benzyl moiety was used for the covalent attachment of the TLR7 ligand to the N-terminus of OVA-derived peptide DEVSGLEQLESIINFEKL to give conjugate **3**. The same TLR7 ligand was installed at both the N-terminus and at the side chain of the C-terminal lysine residue to give conjugates **4** and **5**, respectively. The A_5K extension on the C terminus of the peptide is introduced to allow a comparison of the immunological activity of the conjugates having the TLR7 agonist installed at either the N- terminus or close to the C-terminus of the epitope containing peptide. It is not excluded that a TLR ligand at the C-terminus of DEVSGLEQLESIINFEKL will preclude the antigen presentation. An ethylene glycol spacer, exhibiting minimal steric hindrance is selected for its favourable influence on the solubility, as was shown in former studies (see Chapter 3).

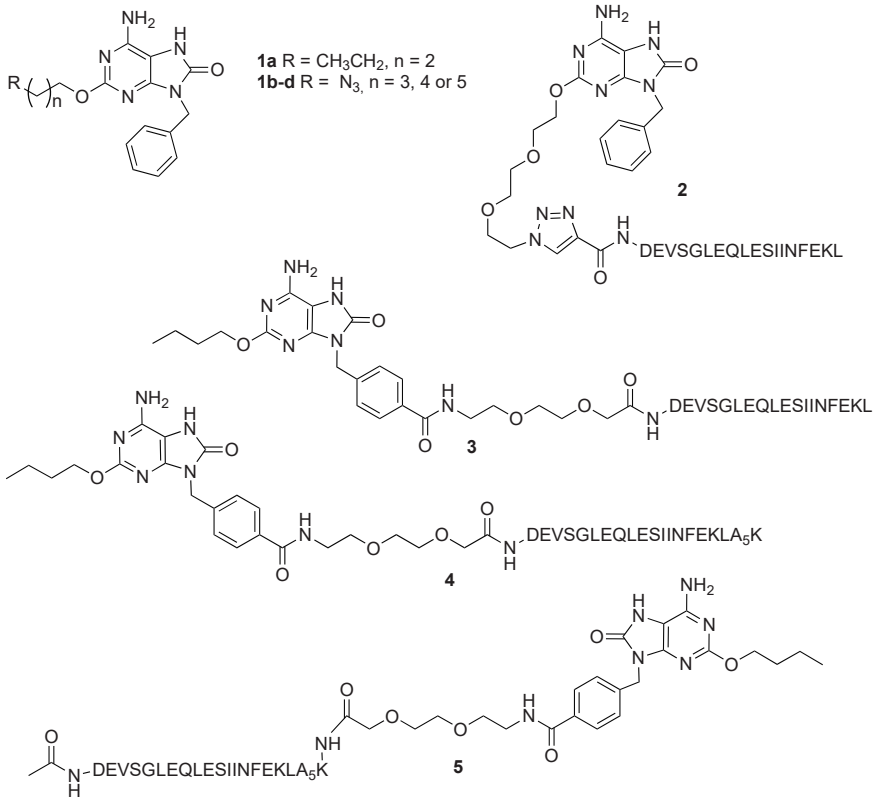
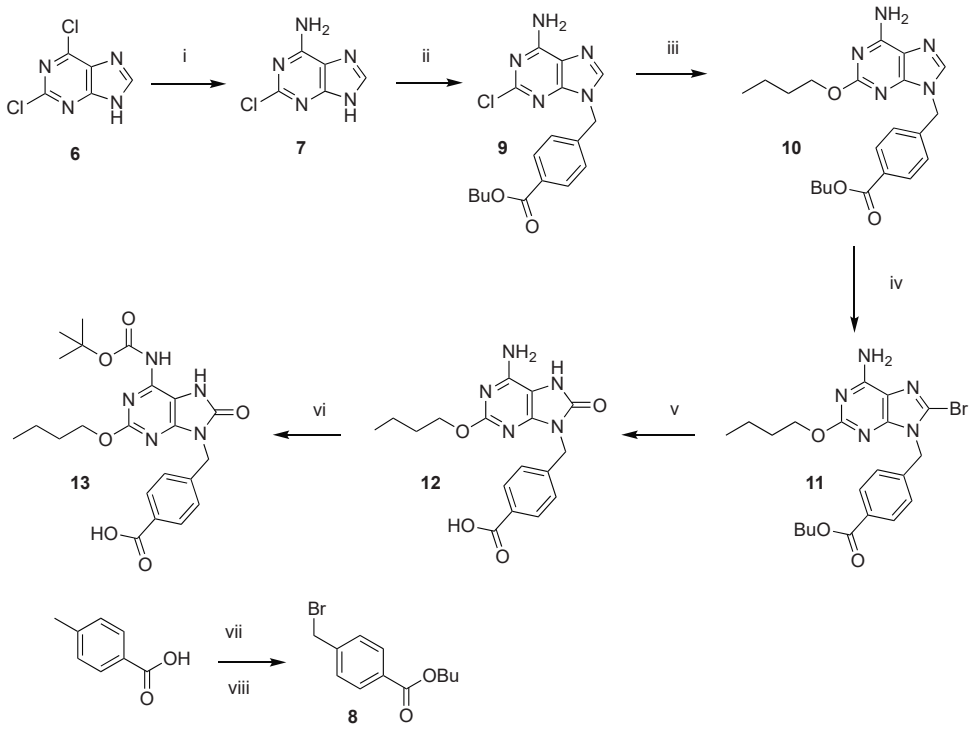


Figure 1. TLR7 ligand (**1**) and its conjugate (**2**) synthesized previously by Weterings *et al.* and the target TLR7 conjugates (**3**, **4** and **5**) described n this Chapter.

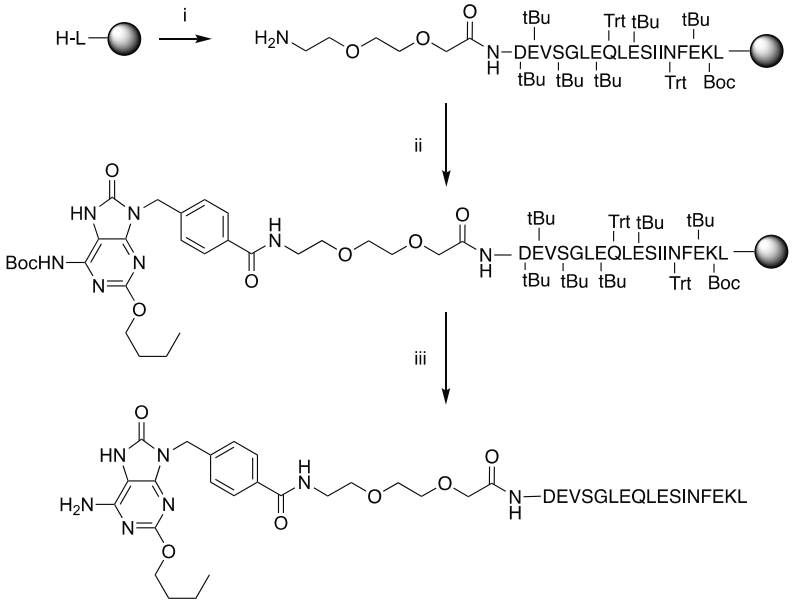
Result and discussion

The assembly of the projected conjugates (**3-5**) was performed with the aid of an automated synthesizer using a solid phase peptide synthesis (SPPS) protocol. To achieve this, the synthesis of partially protected and functionalized TLR7 ligand **13** was undertaken by adaptation of published procedures (Scheme 1).[11, 20-22] In the first step of the synthetic route to **13** commercially available 2,6-dichloropurine **6** was treated with ammonia/methanol to give in a regioselective manner 2-chloroadenine **7**. Next selective benzylation of the N9 position in 2-chloroadenine **7** was explored. The required alkylating reagent **8** was prepared by acid mediated condensation of p-toluic acid and n-butanol, followed by radical bromination of the obtained ester, using N-bromosuccinimide and AIBN. It was established that benzylation of 2-chloroadenine **7** with bromide **8** using 1 M TBAF in THF outperformed other applied bases. For instance, the selectivity and yield dropped when K_2CO_3 was used as a base. It of interest that the quality of the TBAF/THF solution, probably its water content, can influence the yield of benzylation of the N9 position of the adenine derivative. The following nucleophilic aromatic substitution of 2-chloroadenine derivative **9** with sodium n-butoxide at 120^{0}C was accompanied by partial hydrolysis of the butyl ester.



Scheme 1. Synthesis of compound **13**. *Reagent and conditions:* i) Sat. NH_3 in MeOH, 100°C. 88% ii) **8**, 1M TBAF in THF, RT, 92% iii) 1) NaH, n-BuOH, 120°C 2) H_2SO_4, 80 C°, 62% iv) Br_2, DCM, RT, 76% v) 1) NaOMe, MeOH, 65°C 2) 37% HCl, RT 3) 1M KOH, H_2O, RT, 53% vi) BocON, TEA, H_2O/Dioxane 1:1, RT 10%.vii) H_2SO_4, n-BuOH, 120°C, 94% viii) NBS, AIBN, CCl_4, 80°C, 73%

The butyl ester was recovered by acidifying the crude reaction mixture with H_2SO_4 and heating it at 80°C for 4 hours to give adenine derivative **10**. Despite the fact that the conversion of **9** into **10** was executed several times, a yield higher than 65% could not be obtained. Electrophilic aromatic substitution of **10**, using elementary bromine led to **11**. Subsequent direct hydrolysis of bromine at the C8 position in **11** with NaOH was unsuccessful. Fortunately, treatment of **11** with sodium methanolate, followed by acid mediated hydrolysis of the produced methyl ether and, finally, saponification of the ester gave adenine derivative **12.** Compound **12** was insoluble in most solvents and was isolated by precipitation in an acidic environment followed by collection of the sticky precipitate by centrifugation, washing the precipitate with dilute HCl (aq) and drying. The impracticability of **12** urged the protection of the exocyclic amine function in **12** with the Boc-group. Using BocON as a protecting reagent the installation of the protecting group proceeded sluggishly, requiring an excess of reagents and long reaction times. After five days of stirring at room temperature with a ten-fold excess of reagents, LC-MS analysis indicated ~50% conversion and after purification **13** was obtained in only 10% yield. The moderate nucleophilicity of aromatic amines and the low solubility of both starting compound **12** and BocON may be an explanation for this disappointing result. Although the reduced nucleophilicity of the exocyclic amine in **12** should also reduce or even prevent side reactions during the use of **12** in SPPS, the low solubility of precursor **12** in organic solvents effectively precluded the application of this building block in peptide synthesis. Therefore, despite the low yield at the protection stage, building block **13** was used in the forthcoming SPPS.



Scheme 2. Synthesis of conjugate **3**. *Reagents and conditions:* i) standard SPPS synthesis ii) **13**, HCTU, Dipea, NMP iii) TFA/H_2O/TIS (95/2.5/2.5)

SPPS of conjugate **3** was performed using Tentagel® S PHB resin, Fmoc chemistry, HCTU as condensing agent and commercially available protected amino acid building blocks (Scheme 2). After completion of the DEVSGLEQLESIINFEKL sequence, the Fmoc group at the N terminus of the immobilized oligopeptide was removed and Fmoc-PEG-COOH linker was introduced and

deprotected using the same conditions as use for the Fmoc amino acids. In the final step Boc-protected ligand **13** was the coupled to the released amine using HCTU as a coupling agent. Finally, removal of all protecting groups and cleavage from the resin was achieved with a TFA cocktail to give after HPLC purification target conjugate **3** in a yield of 10%. Conjugate **4** (see figure 1) was prepared and purified using the same procedure as described for conjugate **3** but starting with with Tentagel® S RAM amide resin, to which Fmoc-Lys(MMT)-OH was coupled as the first amino acid, and was isolated in 12%. The synthesis of conjugate **5** was started from Fmoc-Lys(MMT)-Tentagel® S RAM using the same standard Fmoc peptide synthesis protocol as for **3** and **4**. After completion of the DEVSGLEQLESIINFEKLA_5K sequence, the N-terminal amine was acetylated and the MMT side chain protective group in the C-terminal lysine was cleaved. Next, the introduction of Fmoc-PEG-COOH linker and the removal of the Fmoc group was followed by the coupling of Boc-protected ligand **13**. Removal of all protecting groups and cleavage from the resin, as described above, gave after purification conjugate **5** in 10% yield.

Conclusion

This chapter describes the functionalisation of the TLR 7 ligand 9-benzyl-8-hydroxy-2-butoxy-adenine with a carboxyl function at the para position of the benzyl moiety. This functionalized ligand (**12**) was insoluble in various solvents and to make it suitable for SPPS the Boc protected derivative (**13**) was prepared. Although the synthesis of building block **13** has been successfully achieved, optimization is needed as the installation of the Boc group was low yielding. Application of building block **13** in the on line SPPS resulted in the isolation of the conjugates **3**, **4** and **5**. Biological evaluation of these conjugates is in progress. Further achievement would be to label these conjugates using method described in chapter two to allow confocal microscopy upon DC stimulation with such constructs.

Experimental section

General procedures

All reactions were carried out at room temperature and under nitrogen atmosphere unless stated otherwise. All solvents were dried and stored using activated 4Å or 3Å molecular sieves. TLC monitoring was performed using Merck aluminum sheets (60 F_{254}). Compounds where visualized by UV detection if applicable at 254 nm and by spraying with 20% H_2SO_4 in EtOH, or $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at 150°C. Flash column chromatography was performed using Fluka silica gel (0.04 – 0.063 mm). LC-MS analysis was performed on a 50 x 4.60 mm Gemini C18/3 µm column (detection at 214 and 254 nm), coupled to an ESI mass spectrometer with a solvent system of B over A where A is H_2O and B is MeCN and 0.1% TFA with a linear gradient as specified. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 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 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.2843) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H and Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard or the residual peak of the deuterated solvent. Coupling constants are given in Hz and peak assignment was done using COSY and HSQC spectra. FT-IR spectra were recorded on a Shimadzu IRAffinity-1 FT-IR spectrometer.

Butyl 4-methylbenzoate

p-Toluic acid (390 mmol, 53.03 g) was dissolved in dry n-BuOH (500 mL). H_2SO_4 (35 mmol, 1.9 mL) was added. The mixture was refluxed at 110°C for 5 hours. TLC analysis (2:8 EA:Pnt) showed complete conversion of the reaction. n-BuOH was evaporated *in vacuo* and the reaction mixture was diluted with DCM and consequently washed with 10% $NaHCO_3$ (aq). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. Excess n-BuOH was removed by co-evaporation with toluene. butyl 4-methylbenzoate (366 mmol, 70.4 g) was obtained with a 94% yield.

IR (cm^{-1}): 2957 (C-H alkane, stretch), 1713 (C=O, stretch).

HRMS $[M+H]^+$: 193.12231 (calculated),

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, *J* = 8.2 Hz, 2H), 7.22 (t, *J* = 14.0 Hz, 2H), 4.29 (t, *J* = 6.6 Hz, 2H), 2.37 (s, 3H), 1.73 (dt, *J* = 14.5, 6.7 Hz, 2H), 1.52 – 1.40 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.67, 143.33, 129.53, 128.99, 127.78, 64.58, 30.81, 21.56, 19.29, 13.75.

Butyl 4-(bromomethyl)benzoate (**8**)

Butyl 4-methylbenzoate (366 mmol, 70.4 g) was dissolved in CCl_4 (350 mL) under argon atmosphere. NBS (402 mmol, 71.6 g), AIBN (30 mmol, 4.92 g) was added. The reaction mixture was slowly heated to 90°C and refluxed for 4 hours. TLC analysis (5:95 Et_2O:Pnt) indicated complete conversion. The solvent was evaporated *in vacuo*. The mixture was diluted with EA and washed with H_2O. The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (0.25% Et_2O in Pnt → 1% Et_2O in Pnt, Δ=0.25%). Compound **8** (266 mmol, 71.8 g) was obtained with a 73% yield.

IR (cm^{-1}): 3197 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1713 (C=O, stretch).

HRMS $[M+H]^+$: 271.11816 (measured), 271.03282 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.3 Hz, 2H), 7.42 (d, *J* = 8.2 Hz, 2H), 4.46 (s, 2H,), 4.31 (m, 2H), 1.81 – 1.66 (m, 2H), 1.53 – 1.36 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 165.92, 142.52, 130.39, 129.97, 128.98, 64.90, 32.29, 30.75, 19.28, 13.79.

2-chloro-9H-purin-6-amine (**7**)

2,6-Dichloropurine (153.6 mmol, 29.04 g) was dissolved in 28% NH_3 in MeOH (150 mL) in an autoclave. The autoclave was sealed and heated to 100°C and stirred for 20 hours. The reaction mixture was filtrated and the residue was washed with MeOH (3x). Compound **7** (130.5 mmol, 22.12 g) was obtained with a 85% yield.

IR (cm^{-1}): 3273 (N-H, stretch), 3106, 2959, 1678, 1611.

HRMS $[M+H]^+$: 170.02369 (measured), 170.02280 (calculated).

^{1}H NMR (400 MHz, DMSO) δ 12.04 (s, 1H), 7.68 (s, 1H), 7.19 (s, 2H).

Butyl 4-((6-amino-2-chloro-9H-purin-9-yl)methyl)benzoate (**9**)

Compound **7** (4.50 mmol, 0.765 g) was dissolved in 1M TBAF in THF under argon atmosphere. Compound **8** (9.03 mmol, 2.45 g) was added and the reaction mixture was stirred overnight. TLC-MS indicated complete conversion of the reaction. The reaction mixture was concentrated *in vacuo* and consequently diluted with EA. The solution was washed with 10% $NaHCO_3$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was adsorbed on Celite and purified by silica gel column chromatography (1% MeOH in DCM → 5% MeOH in DCM, Δ=1%). Compound **9** (4.15 mmol, 1.492 g) was obtained with a 92% yield.

IR (cm^{-1}): 3297 (N-H, stretch), 3122 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1736 (C=O, stretch), 1597 (C-C ring, stretch).

HRMS $[M+H]^+$: 360.12143 (measured), 360.12218 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 8.3 Hz, 2H), 7.73 (s, 1H), 7.33 (d, *J* = 8.3 Hz, 2H), 6.20 (s, 2H), 5.40 (s, 2H), 4.32 (t, *J* = 6.6 Hz, 2H), 1.74 (m, 2H), 1.53 – 1.40 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 156.31, 140.63, 139.92, 130.51, 127.81, 65.22, 47.09, 30.84, 19.38, 13.88.

Butyl 4-((6-amino-2-butoxy-9H-purin-9-yl)methyl)benzoate (**10**)

Compound **9** (16.8 mmol, 6.03 g) was suspended in n-BuOH (170 mL). NaH (168 mmol, 6.75 g) was slowly added at 0°C. The mixture was heated to 120°C and refluxed overnight. TLC analysis (5% MeOH in DCM) indicated complete disappearance of the starting material. The mixture was cooled to 0°C and H_2SO_4 (185 mmol, 9.8 mL) was slowly added. The mixture was heated to 90°C and stirred for 3 hours. TLC analysis

indicated complete conversion. The solvent was evaporated *in vacuo*. The mixture was diluted with EA and washed with 10% $NaHCO_3$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (1% MeOH in DCM). Compound **10** (10.5 mmol, 4.18 g) was obtained with a 62% yield.

IR (cm^{-1}): 3282 (N-H, stretch), 3110 (C-H aromatic, stretch), 2955 (C-H alkane, stretch), 1736 (C=O, stretch), 1597 (C-C ring, stretch).

HRMS $[M+H]^+$: 398.21702 (measured), 398.21867 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.1 Hz, 2H), 7.58 (s, 1H), 7.32 (d, 2H), 5.33 (s, 2H), 4.31 (t, *J* = 6.6 Hz, 4H), 1.84 – 1.66 (m, 4H), 1.47 (m, 4H), 0.96 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.17, 162.48, 156.50, 140.79, 130.56, 130.26, 127.73, 67.24, 65.09, 46.69 , 31.15, 30.80, 19.35, 13.96, 13.83.

Butyl 4-((6-amino-8-bromo-2-butoxy-9H-purin-9-yl)methyl)benzoate (**11**)

Compound **10** (1.21 mmol, 480 mg) was dissolved in DCM. Bromine (23.4 mmol, 1.21 mL) was slowly added at 0°C. The reaction mixture was heated to RT and stirred for 70 minutes. TLC analysis (3% MeOH in DCM) indicated complete conversion of the reaction. The reaction was quenched with a saturated solution of $Na_2S_2O_3$ (aq) at 0°C. The mixture was diluted with DCM and washed with H_2O (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (1% MeOH in DCM). Compound **11** (0.92 mmol, 437 mg) was obtained with a 76% yield.

IR (cm^{-1}): 3320 (N-H, stretch), 3196 (C-H aromatic, stretch), 2957 (C-H alkane, stretch), 1722 (C=O, stretch), 1652 (N-H, bend), 1589 (C-C ring, stretch).

HRMS $[M+H]^+$: 478.12507 (measured), 478.12713 (calculated).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.3 Hz, 2H), 7.38 (d, *J* = 8.3 Hz, 2H), 6.60 (s, 2H), 5.34 (s, 2H), 4.31 (m, 4H), 1.81 – 1.67 (m, 4H), 1.54 – 1.39 (m, 4H), 0.96 (m, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 166.07, 162.24, 155.57, 152.75, 140.09, 130.31, 129.99, 127.69, 123.98, 115.93, 67.16, 64.93, 46.86, 31.01, 30.68, 19.24, 19.22, 13.87, 13.73.

4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid (**12**)

Compound **11** (3.8 mmol, 1.8 g) was dissolved in 5M NaOMe in MeOH (10 mL) under argon atmosphere. The mixture was refluxed for 4 hours at 65°C, after which LC-MS indicated complete conversion of the reaction. The solvent was evaporated *in vacuo* and 37% HCl (aq) (20 mL) was slowly added at 0°C. LC-MS indicated complete disappearance of starting material after overnight stirring at RT. The solvent was evaporated *in vacuo* and 1M KOH (aq) (50mL) was added. After overnight stirring, LC-MS indicated complete conversion to the product. The solution was acidified with a 1M HCl until a white crash was formed. The solution was centrifuged and the residu was washed with 1M HCl (3x) and consequently dried *in vacuo*. Compound **12** (2.0 mmol, 787 mg) was obtained with a 53% yield.

IR (cm^{-1}): 3418 (N-H, stretch), 3168 (O-H, stretch), 1689 (C=O acid, stretch).

HRMS $[M+H]^+$: 358.15009 (measured), 358.15098 (calculated).

^{1}H NMR (400 MHz, DMSO) δ 10.96 (s, 1H), 7.90 (t, *J* = 8.7 Hz, 2H), 7.38 (t, *J* = 9.6 Hz, 2H), 4.95 (s, 2H), 4.20 (t, *J* = 6.6 Hz, 2H), 1.61 (dt, *J* = 14.5, 6.7 Hz, 2H), 1.40 – 1.27 (m, 2H), 0.87 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (100 MHz, DMSO) δ 167.45, 152.23, 149.08, 142.16, 130.36, 130.04, 127.95, 98.67, 67.44, 42.78, 30.77, 19.06, 14.11.

4-((2-butoxy-6-((tert-butoxycarbonyl)amino)-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid (**13**)

Compound **12** (1.5 mmol, 530 mg)was dissolved in 1:1 H_2O:Dioxane (10 mL). TEA (4.5 mmol, 0.63 mL) and BocON (20 mmol, 4.9 g) were added to the mixture. The reaction mixture was stirred for 4 days at RT. The reaction mixture was diluted with DCM and washed with 10% $KHSO_4$ (aq) (3x). The solution was dried ($MgSO_4$), filtrated and concentrated *in vacuo*. The crude was purified by silica gel column chromatography (5% MeOH in DCM). Compound **13** (0.15 mmol, 68 mg) was obtained with a 10% yield.

IR (cm^{-1}): 3431(N-H, stretch), 3163 (C-H aromatic, stretch), 2958 (C-H alkane, stretch), 1753 (C=O, stretch), 1720 (C=O, stretch), 1637 (C=O, stretch).

^{1}H NMR (400 MHz, DMSO) δ 7.90 (d, *J* = 8.3 Hz, 2H), 7.41 (d, *J* = 8.3 Hz, 2H), 7.06 (s, 2H), 4.93 (s, 2H), 4.16 (t, *J* = 6.6 Hz, 2H), 1.66 – 1.57 (m, 2H), 1.54 (s, 9H), 1.41 – 1.28 (m, 2H), 0.88 (dd, *J* = 9.1, 5.7 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ 167.06, 161.19, 150.55, 150.18, 149.62, 149.15, 141.16, 130.03, 129.62, 127.58, 96.42, 85.19, 66.25, 42.74, 30.52, 27.65, 18.72, 13.72.

Conjugate 3: Tentagel® S PHB Resins preloaded with Fmoc-Leu or with Fmoc-Lys-(Boc) was subjected to solid phase Fmoc-peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **3** in 10% yield. LC-MS (10%-90% gradient); Rt = 5.63; ESI-MS: m/z 1274.3 (exact mass), 1274.7 (most abundant); calculated for $[C_{114}H_{175}N_{27}O_{39} + 2H]^{2+}$ 1274.13 (exact mass), 1274.64 (most abundant).

Conjugate 4: Tentagel® S RAM resin was coupled to Fmoc-Lys-(MMT)-OH and subjected to solid phase Fmoc-peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **4** in 12% yield. LC-MS (10%-90% gradient); Rt = 5.41; ESI-MS: m/z 1515.9 (most abundant); calculated for $[C_{135}H_{213}N_{35}O_{44} + 2H]^{2+}$ 1515.28 (exact mass), 1515.78 (most abundant).

Conjugate 5: Tentagel® S RAM resin was coupled to Fmoc-Lys-(MMT)-OH and subjected to solid phase Fmoc peptide synthesis using standard Fmoc protected amino acid building block (Nova Biochem, 0.25 mmol, 5 eq), HCTU as an activating agent, and Fmoc cleavage as the final step. Terminal amine was capped using standard capping conditions (5% Ac_2O in NMP). MMT side chain protective group was cleaved from the resin-bound peptide using 5% TFA in DCM (three times, 5 min). Fmoc-PEG-COOH was introduced and deprotected using the same condition as standard Fmoc amino acid. To a mixture of resin bound peptide (0.05 mmol,0.228 g) in NMP/DCM (1:1) was added compound **13** (0.125 mmol, 0.111 g), HCTU (0.175 mmol, 0.091 g) and D*i*PEA 1M (0.25 mmol, 0.032 g, 250 µL). The reaction mixture was then stirred overnight on the orbital shaker at rt. The resin was washed with DCM three times and the resulting conjugate was cleaved off the resin by adding TFA/TIS/H_2O (95/2.5/2.5) (2h). Precipitation of the conjugate was done by adding the TFA solution to cold diethyl ether/n-pentane (1:1) (14 mL) and subsequent centrifugation (4000 rpm, 5 min). The precipitate was dissolved in dry DMSO. This solution was then subjected to semi-preparative HPLC, pure peptide conjugate fractions were collected and concentrated by freeze-drying. This yielded conjugate **5** in 10% yield. LC-MS (10%-90% gradient); Rt = 5.59; ESI-MS: m/z 1536.8 (most abundant); calculated for $[C_{137}H_{215}N_{35}O_{45} + 2H]^{2+}$ 1536.29 (exact mass), 1536.79 (most abundant).

References

[1] Kawai, T., and Akira, S. (2011) Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity, *Immunity 34*, 637-650.

[2] Jimenez-Dalmaroni, M. J., Gerswhin, M. E., and Adamopoulos, I. E. (2016) The critical role of toll-like receptors - From microbial recognition to autoimmunity: A comprehensive review, *Autoimmun. Rev. 15*, 1-8.

[3] Jung, S., von Thulen, T., Laukemper, V., Pigisch, S., Hangel, D., Wagner, H., Kaufmann, A., and Bauer, S. (2015) A Single Naturally Occurring 2 '-O-Methylation Converts a TLR7-and TLR8-Activating RNA into a TLR8-Specific Ligand, *Plos One 10*.

[4] Leslie, A. J., Pichulik, T., Khatamzas, E., Mayer, A., McMichael, A., and Simmons, A. (2011) Early Phosphorylation Events Induced in Dendritic Cells by the HIV Derived TLR8 Ligand ssRNA40, *AIDS Res. Hum. Retroviruses 27*, A65-A66.

[5] Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995) Cpg Motifs in Bacterial-DNA Trigger Direct B-Cell Activation, *Nature 374*, 546-549.

[6] Gantier, M. P., Tong, S., Behlke, M. A., Xu, D., Phipps, S., Foster, P. S., and Williams, B. R. G. (2008) TLR7 is involved in sequence-specific sensing of single-stranded RNAs in human macrophages, *J. Immunol. 180*, 2117-2124.

[7] Czarniecki, M. (2008) Small Molecule Modulators of Toll-like Receptors, *J. Med. Chem. 51*, 6621-6626.

[8] Gibson, S. J., Lindh, J. M., Riter, T. R., Gleason, R. M., Rogers, L. M., Fuller, A. E., Oesterich, J. L., Gorden, K. B., Qiu, X. H., McKane, S. W., Noelle, R. J., Miller, R. L., Kedl, R. M., Fitzgerald-Bocarsly, P., Tomai, M. A., and Vasilakos, J. P. (2002) Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod, *Cell. Immunol. 218*, 74-86.

[9] Gaitanis, G., Mitsou, G., Tsiouri, G., Alexis, I., and Bassukas, I. D. (2010) Cryosurgery During Imiquimod Cream Treatment ("Immunocryosurgery") for Bowen's Disease of the Skin: A Case Series, *Acta Derm-Venereol 90*, 533-534.

[10] Mirer, E., El Sayed, F., Ammoury, A., Lamant, L., Messer, L., and Bazex, J. (2006) Treatment of mammary and extramammary Paget's skin disease with topical imiquimod, *J Dermatol Treat 17*, 167-171.

[11] Kurimoto, A., Hashimoto, K., Nakamura, T., Norimura, K., Ogita, H., Takaku, H., Bonnert, R., McInally, T., Wada, H., and Isobe, Y. (2010) Synthesis and Biological Evaluation of 8-Oxoadenine Derivatives as Toll-like Receptor 7 Agonists Introducing the Antedrug Concept, *J. Med. Chem. 53*, 2964-2972.

[12] Jin, G. Y., Wu, C. C. N., Tawatao, R. I., Chan, M., Carson, D. A., and Cottam, H. B. (2006) Synthesis and immunostimulatory activity of 8-substituted amino 9-benzyladenines as potent Toll-like receptor 7 agonists, *Bioorg. Med. Chem. Lett. 16*, 4559-4563.

[13] Moyle, P. M., and Toth, I. (2013) Modern Subunit Vaccines: Development, Components, and Research Opportunities, *Chemmedchem 8*, 360-376.

[14] Wille-Reece, U., Wu, C. Y., Flynn, B. J., Kedl, R. M., and Seder, R. A. (2005) Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8(+) T cell responses, *J. Immunol. 174*, 7676-7683.

[15] Wang, X. D., Gao, N. N., Diao, Y. W., Liu, Y., Gao, D., Li, W., Wan, Y. Y., Zhong, J. J., and Jin, G. Y. (2015) Conjugation of toll-like receptor-7 agonist to gastric cancer antigen MG7-Ag exerts antitumor effects, *World J Gastroentero 21*, 8052-8060.

[16] Chan, M., Hayashi, T., Kuy, C. S., Gray, C. S., Wu, C. C. N., Corr, M., Wrasidlo, W., Cottam, H. B., and Carson, D. A. (2009) Synthesis and Immunological Characterization of Toll-Like Receptor 7 Agonistic Conjugates, *Bioconjugate Chem. 20*, 1194-1200.

[17] Zom, G. G. P., Khan, S., Filippov, D. V., and Ossendorp, F. (2012) TLR Ligand-Peptide Conjugate Vaccines: Toward Clinical Application, In *Advances in Immunology , Vol 114: Synthetic Vaccines*, pp 177-201.

[18] Khan, S., Bijker, M. S., Weterings, J. J., Tanke, H. J., Adema, G. J., van Hall, T., Drijfhout, J. W., Melief, C. J. M., Overkleeft, H. S., van der Marel, G. A., Filippov, D. V., van der Burg, S. H., and Ossendorp, F. (2007) Distinct uptake mechanisms but similar intracellular processing of two different Toll-like receptor ligand-peptide conjugates in dendritic cells, *J. Biol. Chem. 282*, 21145-21159.

[19] Daftarian, P., Sharan, R., Haq, W., Ali, S., Longmate, J., Termini, J., and Diamond, D. J. (2005) Novel conjugates of epitope fusion peptides with CpG-ODN display enhanced immunogenicity and HIV recognition, *Vaccine 23*, 3453-3468.

[20] Weterings, J. J., Khan, S., van der Heden, G. J., Drijfhout, J. W., Melief, C. J. M., Overkleeft, H. S., van der Burg, O. H., Ossendorp, F., van der Marel, G. A., and Filippov, D. V. (2006) Synthesis of 2-alkoxy-8-hydroxyadenylpeptides: Towards synthetic epitope-based vaccines, *Bioorg. Med. Chem. Lett. 16*, 3258-3261.

[21] Akinbobuyi, B., Byrd, M. R., Chang, C. A., Nguyen, M., Seifert, Z. J., Flamar, A. L., Zurawski, G., Upchurch, K. C., Oh, S., Dempsey, S. H., Enke, T. J., Le, J., Winstead, H. J., Boquin, J. R., and Kane, R. R. (2015) Facile syntheses of functionalized toll-like receptor 7 agonists, *Tetrahedron Lett. 56*, 458-460.

[22] Weterings, J. J., Khan, S., van der Heden van Noort, G. J., Melief, C. J. M., Overkleeft, H. S., van der Burg, S. H., Ossendorp, F., van der Marel, G. A., and Filippov, D. V. (2009) 2-Azidoalkoxy-7-hydro-8-oxoadenine derivatives as TLR7 agonists inducing dendritic cell maturation, *Bioorg. Med. Chem. Lett. 19*, 2249-2251.