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Synthesis and evaluation of peptide and nucleic acid based Toll-like receptor ligands

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Chapter 3

7-Hydro-8-oxo-adenine peptide conjugates via Huisgen Cycloaddition¹

Introduction

The ability of Toll-like receptor 7 (TLR7) to bind small drug-like compounds such as imidazoquinolines,² guanine derivatives³ and 7-hydro-8-oxo-adenines⁴ is described in **Chapter 2**. It has previously been discovered that covalent attachment of Toll-like receptor ligands (TLR-L) to antigenic proteins enables the development of model vaccines with enhanced immunogenicity in comparison to a mixture of the two components.^{5,6} The general idea behind such conjugates is that optimal immune response against a specific MHC class I peptide epitope can be achieved when both the epitope and the TLR ligand are brought to the same dendritic cell population simultaneously. Ideally, both cytokine production and antigen presentation are upregulated and the measurements of these parameters provide a guideline on the possibility of the peptide-TLR-L constructs as leads for vaccine development. Specifically, lipopeptides⁷ (peptides containing a palmitoylated cysteine residue) and nucleopeptides⁸ (hybrids of peptides and CpG phosphorothioate DNA) have been reported as structurally defined constructs designed for TLR-2 and TLR-9 recognition, respectively. With these constructs, both cytokine production and antigen presentation were elevated in comparison with their separate (non-covalently linked) counterparts. Whether TLR-L-antigenic peptide conjugates aimed at TLR7 and based on imidazoquinoline-derived TLR-7

ligands are feasible constructs for vaccine development is subject of the work described in this chapter.

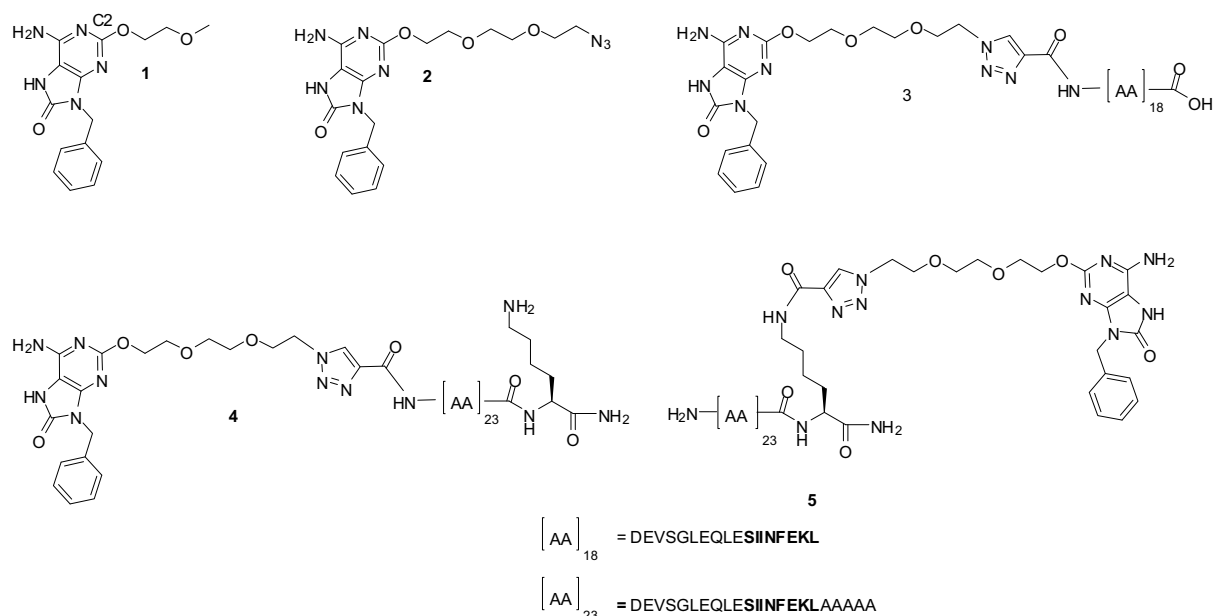


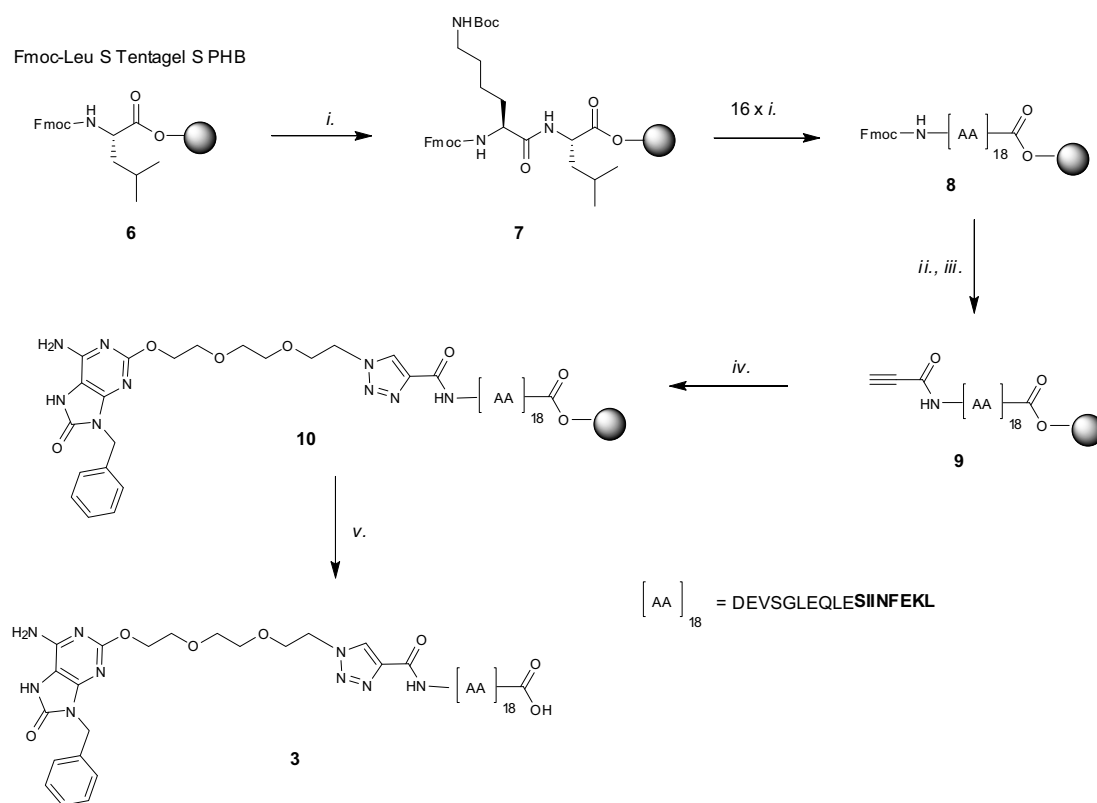
Figure 3.1: 2-alkoxy-7-hydro-8-oxo-adenine derivatives and peptide conjugates thereof.

The target peptide-TLR7-L constructs are depicted in **Figure 3.1** and are based on azide-containing 7-hydro-8-oxo-adenine derivative **2**. In the previous chapter it was revealed that compound **2**, a closely related compound in comparison with the literature TLR7 agonist **1**^{4,9} (with the only difference in the nature of the alkoxy substituents at C2), is a moderately potent TLR7 agonist. The azide in **2** allows conjugation to acetylene-modified peptides in a copper (I)-catalyzed Huisgen [3+2] cycloaddition reaction.¹⁰⁻¹³ This chemoselective transformation does not necessitate any protective group manipulation on the exocyclic amino group of oxo-adenine **2** and enables a straightforward synthetic entry to the target peptide derivatives **3**, **4**, and **5** (**Figure 3.1**) with the TLR7-L attached to either the C- or the N-terminus of the peptide. As model peptide an ovalbumin-derived peptide containing the major histocompatibility complex class I (MHC I) epitope SIINFEKL was selected.

The primary structure of the selected model peptides reflects earlier findings¹⁴ indicating that for optimal antigen presentation the CTL-epitope (SIINFEKL in this study) is best embedded in a longer peptide motif. This allows for the specific targeting of the epitope to professional antigen presenting cells (APCs) and (after proteolytic processing) exposure on MHC class I molecules via the cross-presentation pathway. An important feature in the processing of polypeptides towards MHC class I epitopes is the involvement of the proteasome, which is

responsible for the generation of the final C-terminal residue (here L in SIINFEEKL). A peptide epitope targeted for presentation in the MHC class I route should not interfere with this process. In practice this entails that either the conjugate terminates in the correct C-terminal residue or has a C-terminal peptide extension motif. Conjugate **3** meets the first criterion (it features the SIINFEEKL motif at the C-terminus) thereby allowing the conjugation of the adenine moiety to the N-terminus. Compounds **4** and **5** containing the penta-alanine elongated peptide, suitable for proteasomal processing, are modified with the adenine moiety **2** at the N- and C-terminus respectively. The assembly of conjugates **3**, **4** and **5** (Figure 3.1) based on Huisgen [3+2] cycloaddition requires the availability of an azide-containing TLR7 agonist. Chronologically, compound **2** was the first of the azide series described in Chapter 2 to be finished and tested positively in a TLR7-specific cytokine production assay. Therefore, the conjugates **3**, **4** and **5** are based on this azide.

Results and Discussion



Scheme 3.1

Reagents and Conditions: *i*) A) 20% piperidine in NMP, B) Fmoc-AA-OH, HCTU, DiPEA in NMP; C) Ac₂O/DiPEA in NMP; *ii*) 20 % piperidine in NMP; *iii*) propiolic acid, HCTU, DiPEA in NMP; *iv*) CuI, **2**, DiPEA in NMP; *v*) TFA/TIS/H₂O (95/2.5/2.5), RP-HPLC.

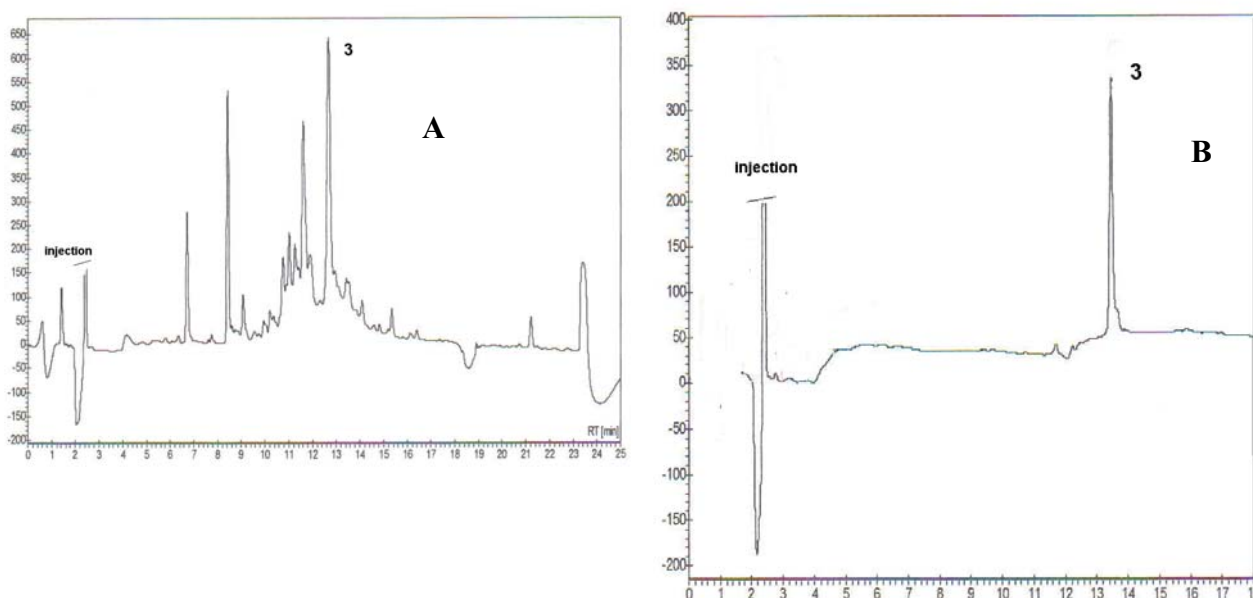
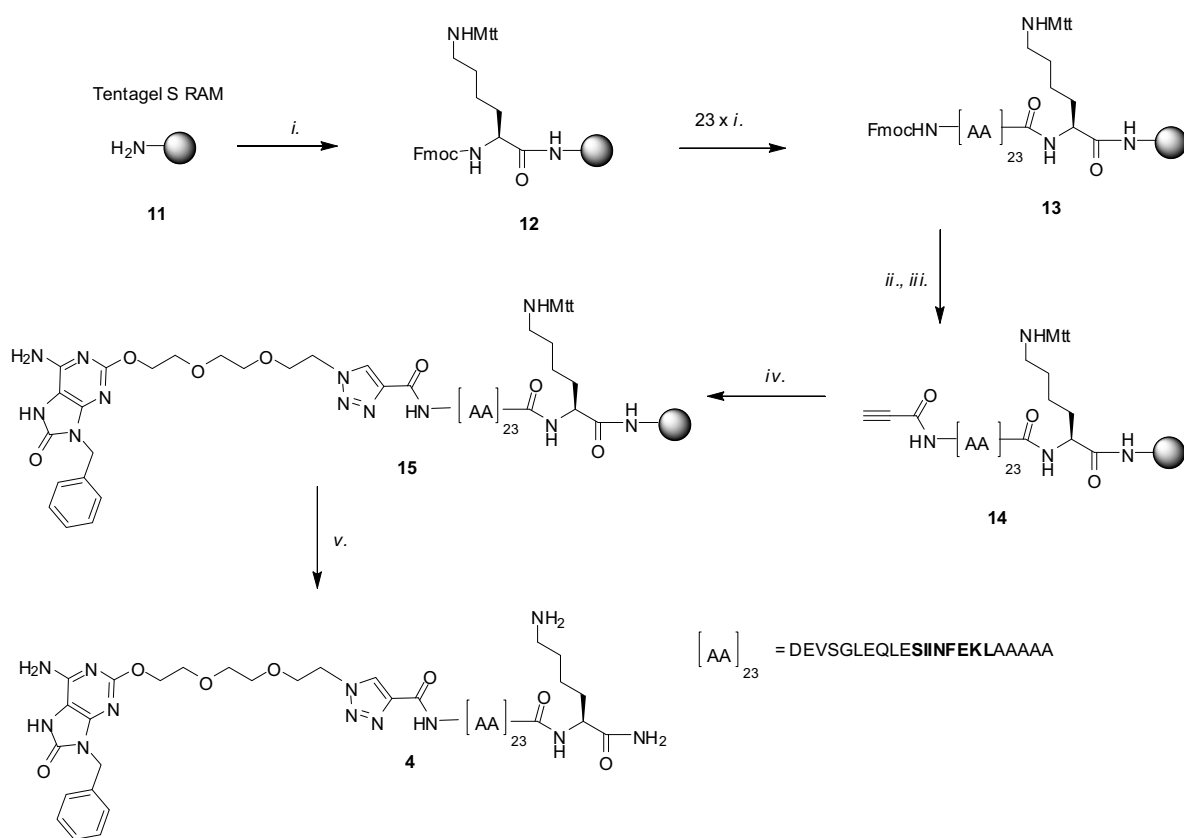


Figure 3.2 A) RP HPLC trace (UV 214 nm) of crude oxo-adenyl peptide **3** after TFA/TIS/H₂O mediated cleavage/deprotection and precipitation from diethyl ether. B) RP HPLC trace (UV 214 nm) of purified **3**.

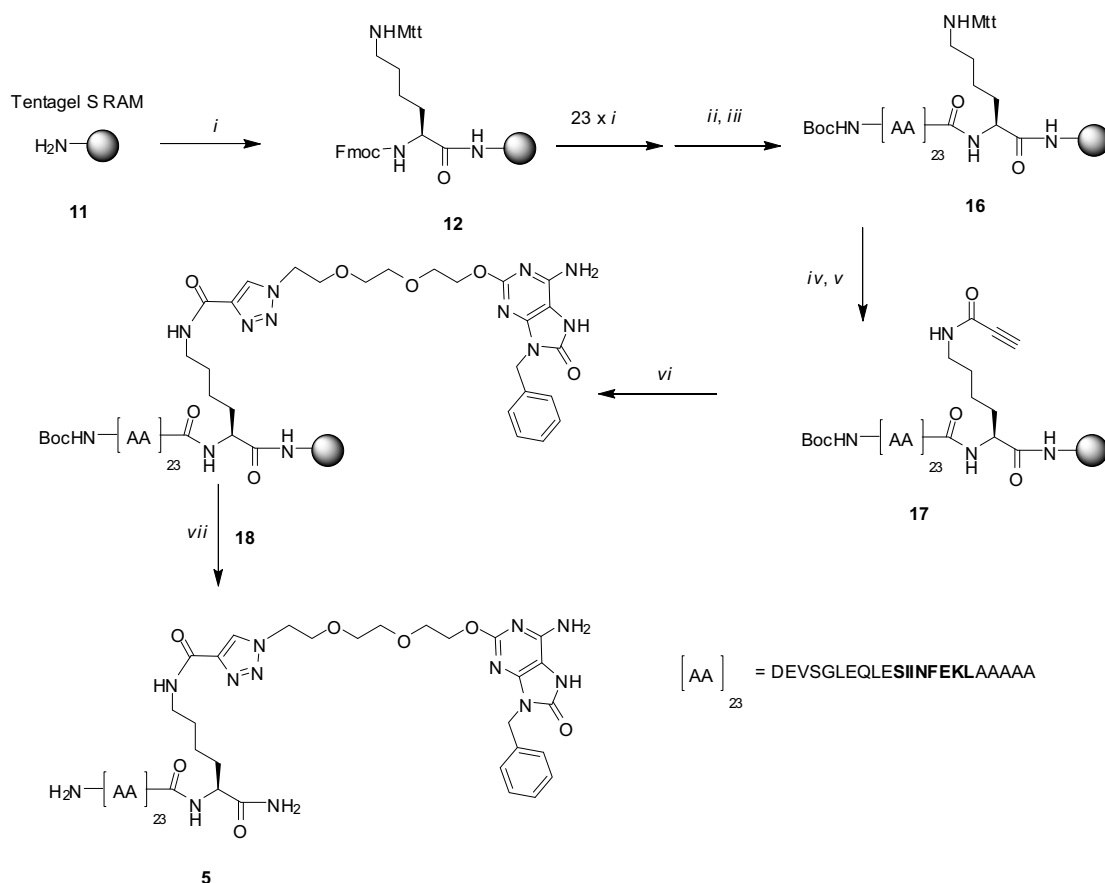
The synthesis of **3** (**Scheme 3.1**) starts with the stepwise assembly of the peptide chain on the commercially available TFA sensitive Tentagel S PHB resin preloaded with Fmoc-Leu-OH (**6**). Repetition of the coupling cycle, entailing cleavage of the N^α-Fmoc-groups with piperidine and introduction of the amino acids, as well as the electron-deficient propiolic acid residue, using HCTU assisted¹⁵ activation led to immobilized **9**. Subsequent attachment of the oxo-adenine moiety was executed by treatment (48 h.) of resin **9** suspended in NMP with alkyl azide functionalized building block **2** in the presence of CuI and DIPEA. Target compound **3** was released from the solid support and simultaneously deprotected by treatment¹⁶ with TFA and isolated by diethyl ether precipitation. Crude oxo-adenylated peptide **3** was analyzed by LC-MS using a C₁₈-column. No trace of the propiolyl peptide was detected, suggesting that the cycloaddition reaction went to completion (**Figure 3.2**). Purification using a semi-preparative RP HPLC column gave homogeneous **3** in 4.3% overall yield.



Scheme 3.2

Reagents and Conditions: *i*) A) 20% piperidine in NMP, B) Fmoc-AA-OH, HCTU, DiPEA in NMP; C) Ac_2O /DiPEA in NMP; *ii*) 20 % piperidine, NMP; *iii*) propionic acid, HCTU, DiPEA in NMP; *iv*) compound **2**, CuI, DiPEA in NMP; *v*) TFA/TIS/ H_2O (95/2.5/2.5) RP-HPLC.

Conjugates **4** and **5** were prepared in a related fashion as follows. Copper(I) catalyzed cycloaddition of azide **2** to alkyne functionalized peptide-resin **14**, obtained via Fmoc-based SPPS starting with immobilized Fmoc-Lys(Mtt) Tentagel S PHB **12** (Scheme 3.2), gave after TFA treatment and purification conjugate **4** in an overall yield of 11.3%.



Scheme 3.3

Reagents and Conditions: *i*) A) 20% piperidine in NMP, B) Fmoc-AA-OH, HCTU, DiPEA in NMP; C) Ac₂O/DiPEA in NMP; *ii*) 20 % piperidine, NMP; *iii*) Boc₂O, DiPEA in NMP; *iv*) 1% TFA/DCM; *v*) propiolic acid, HCTU, DiPEA in NMP; *vi*) compound **2**, CuI, DiPEA in NMP; *vii*) TFA/TIS/H₂O, RP-HPLC.

Alternatively (**Scheme 3.3**), Fmoc-Lys(Mtt) resin **12** was converted into immobilized **17** by the following procedure: HCTU/DiPEA assisted Fmoc-SPPS, Boc₂O/DiPEA treatment to block the *N*-terminus, 1% TFA/DCM¹⁷ treatment to selectively cleave the Mtt group from the side-chain of the C-terminal lysine residue and finally HCTU/DiPEA mediated introduction of the propiolic acid residue. Subsequent CuI catalyzed Huisgen cycloaddition followed by TFA treatment and RP HPLC purification furnished **5** in 2.9% overall yield. The purity of the conjugates **4** and **5** was ascertained by LCMS and the relevant traces are depicted in **Figure 3.3**.

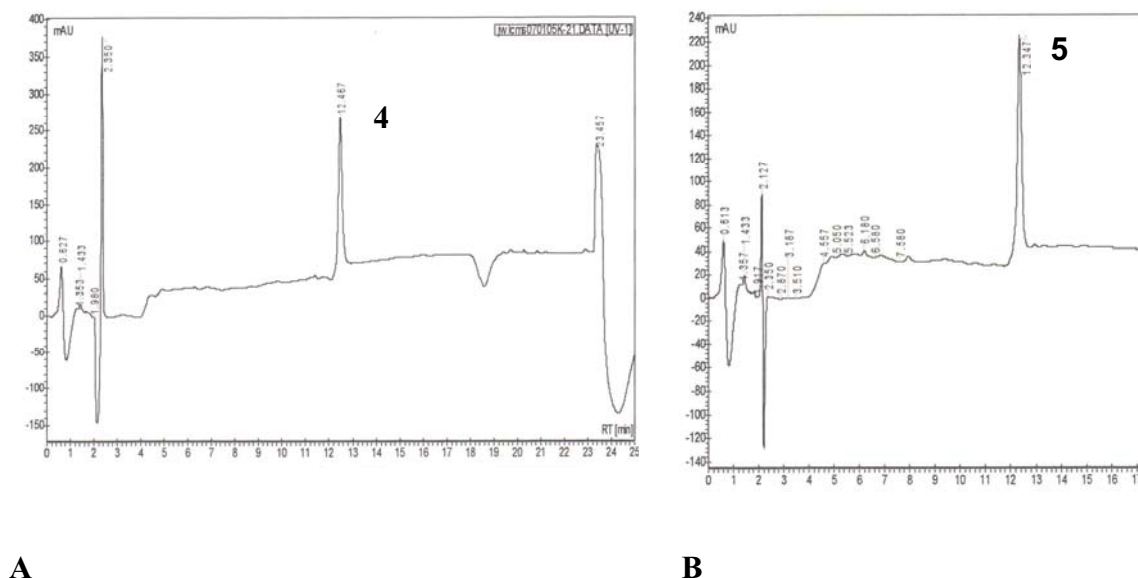


Figure 3.3: RP HPLC trace (UV 214 nm) of purified **4** (A) and **5** (B)

The ability of the pure synthetic 7-hydro-8-oxo-adenine derivative **2** and the modified peptides **3**, **4** and **5** to induce production of the T helper cell (Th1)-activating cytokine, interleukin-12 (IL-12p40) was investigated. The known⁹ derivative **1** and the established TLR7 ligand² R848 (resiquimod) were included in the assays as reference compounds. Bone marrow derived dendritic cells were incubated with the indicated substances, and IL-12p40 production was measured after either 24hrs or 48hrs in the culture supernatant. As evident from **Figure 3.4** both **1** and **2** efficiently induce IL12p40 production. Conjugation of the peptides to the TLR7 ligand (compounds **3**, **4** and **5**) led to complete abolishment of the IL12p40 production.

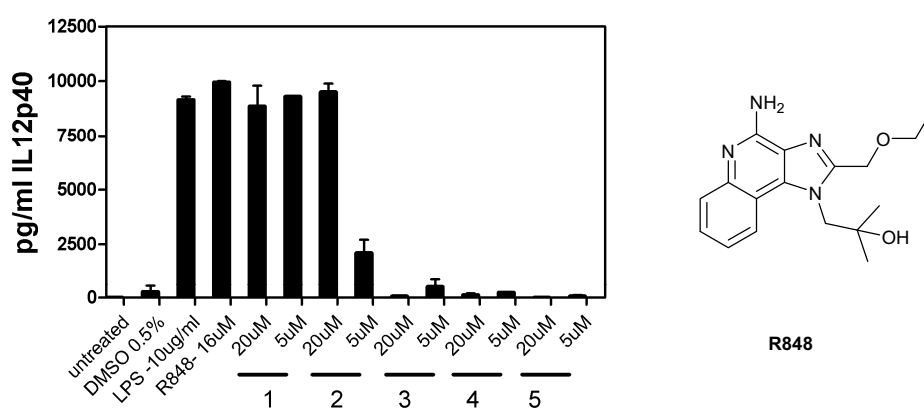


Figure 3.4 A) Potency of 7-hydro-8-oxo-adenine derivatives in dendritic cells, activation determined by the level of induced IL12p40 after 24 hrs. B) Resiquimod-R848

Next, the effect of the oxo-adenine modification on MHC I-mediated presentation of the SIINFEKL epitope was investigated. To this end, DCs were treated *in vitro* with either TLR7-L-conjugate **3** or the free parent peptide in a mixture with compound **2** and the level of antigen presentation was determined by a T-cell hybridoma assay. The results are depicted in **Figure 3.5**. A pronounced enhancement in antigen presentation was observed with the conjugate peptide compared to the mixture. Exactly the same effect was observed when conjugates **4** and **5** were compared to the parent peptide mixed with **2**.

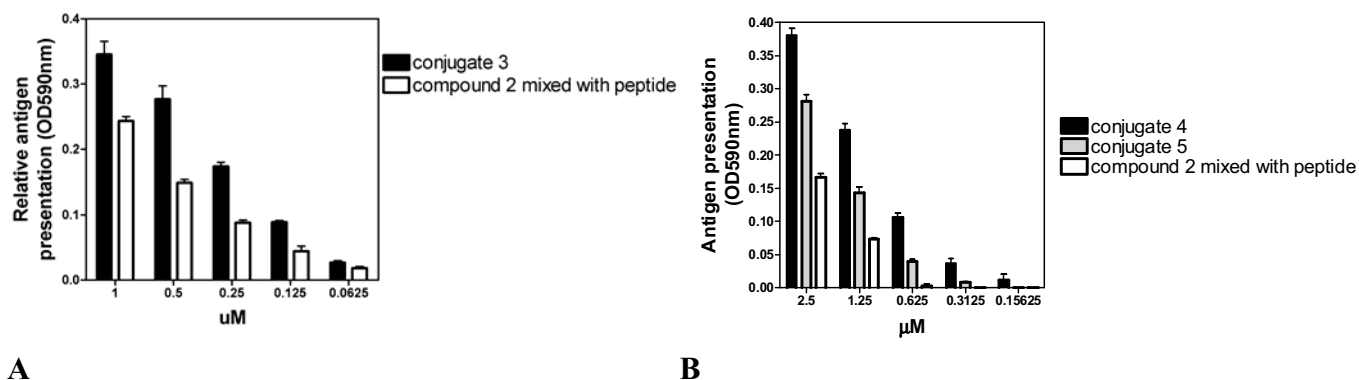


Figure 3.5 A) Comparing MHC class I antigen presentation of conjugated and non-conjugated peptide **3**. B) Comparing MHC class I antigen presentation of conjugated and non-conjugated peptides **4** and **5**.

In summary, an efficient solid-phase synthesis of three different 2-alkoxy-7-hydro-8-oxo-adenyl peptide conjugates (**3**, **4** and **5**) is presented. In comparison with a mixture of their individual components, these conjugates give rise to enhanced antigen presentation *in vitro*, but lack the ability to induce DC activation. As the *in vitro* antigen presentation assay is independent of the maturation status of the antigen-presenting cells, the enhancement of the antigen presentation of the conjugates may be explained by the improved targeting of the conjugate to the DCs compared to the free peptide. The lack of DC activation by the conjugates is likely due to either the poor binding of the conjugated ligand to TLR7 as a result of steric hindrance by the peptide moiety, the impaired intracellular trafficking of the conjugated ligand compared to free **2**, or both. According to this line of reasoning the stimulatory capacity of the conjugates can be restored by the introduction of a cleavable linker between the TLR-ligand and the peptide moiety allowing release of the ligand after internalization. The internalization itself can be improved by inclusion of functionalities known to enhance endosomal uptake.

Experimental Section

All reagents and solvents used in the solid phase peptide synthesis, with the exception of HCTU, were from Biosolve (The Netherlands) and used as received. HCTU was purchased from IRIS Biotech GmbH (Germany). Fmoc-amino acids were from SENN Chemicals or from MultiSynTech GmbH (Germany). Tentagel based resins were bought at Rapp Polymere GmbH (Germany). Analytical LC/MS was conducted on a JASCO system using an Alltima C₁₈ analytical column (5µm particle size, flow: 1.0 mL/min). Absorbance was measured at 214 and 254 nm. Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA/H₂O. Gradients of B in 10% C were applied over 26 minutes unless stated otherwise. Purifications were conducted on a BioCAD “Vision” automated HPLC system (PerSeptive Biosystems, inc.), supplied with a semipreparative Alltima C₁₈ column (5µm particle size, running at 4mL/min). Solvent system: A: 100% water, B: 100% acetonitrile, C: 1% TFA/H₂O. Gradients of B in 10% C were applied over 3 CV unless stated otherwise. The UV absorption was measured with a Varian DMS 200 UV visible spectrophotometer. MALDI-TOF spectra were recorded on a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems, inc.).

The solid-phase peptide synthesis was performed on a CS Bio 336 automated instrument (CS Bio, California, USA) applying Fmoc based protocol starting from either preloaded Fmoc-Leucine-PHB-Tentagel resin or from Tentagel–RAM resin. The synthesis was performed on a 50 µmol scale according to established methods.¹⁹ The consecutive steps performed in each cycle were:

- 1) Deprotection of the Fmoc-group with 20% piperidine in NMP for 15 min; 2) DMF wash;
- 3) Coupling of the appropriate amino acid applying 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 M DIPEA in NMP to initiate the coupling. The reaction vessel was then shaken for 45 min; 4) DMF wash; 5) Capping with 0.5 M NMP solution of acetic anhydride in the presence of 0.5 mmol DIPEA;
- 6) DMF wash.

The Fmoc amino acids applied in the syntheses were: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Phe-OH, Fmoc-Ser-OH and Fmoc-Val-OH.

Propiolyl-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(O^tBu)-Lys(Boc)-Leu-Tentagel-S-PHB (9),

Propiolyl-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(O^tBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt)-Tentagel-S-Rink Amide (14)

Peptide synthesis was carried out on a 50 μ mol scale using a CS Bio 336 automated instrument applying Fmoc based protocol starting from either preloaded Fmoc-Leu-PHB-Tentagel resin (**6**, loading 0.25 mmol/g) or from Tentagel S RAM resin **11** (loading 0.25 mmol/g). The resin, after final Fmoc deprotection, was washed with DMF and suspended in DMF (3 mL). Propiolic acid (20 eq.) and HCTU (20 eq.) were transferred to the suspension after which DiPEA (40 eq.) was added and the mixture was shaken for 2 hours. A Kaiser test²⁰ on a small aliquot of resin **9** or **14** revealed complete coupling. To control the quality of the prepared compounds **9** and **14** small aliquot of the resin was transferred to a glass tube and treated for 2 hours with a cleavage cocktail TFA/TIS/H₂O (95/2.5/2.5). The solution was filtered into cold diethylether and the resin was washed with neat TFA. The diethylether was centrifuged, removed and the precipitate was washed with diethylether. After centrifugation, the ether was removed and the precipitate was air dried and dissolved in ^tBuOH/ACN/H₂O, 3/1/1. (cleaved peptide **9**) LCMS: 10-70% B, Rt = 12.0 min; ESI-MS: [M+H]⁺: 2116.4 (calculated. 2116.1), [M+H]²⁺: 1058.5 (calculated 1058.5). (cleaved peptide **14**) LCMS: 10-90% B, Rt = 4.89 min; ESI-MS: [M+H]⁺: 2598.2 (calculated 2598.3), [M+H]²⁺: 1300.2 (calculated 1299.7).

Boc-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn-(Trt)-Phe-Glu(O^tBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Propiolyl)-Tentagel-S-Rink amide (17)

200 mg of H-Asp(O^tBu)-Glu(O^tBu)-Val-Ser(^tBu)-Gly-Leu-Glu(O^tBu)-Gln(Trt)-Leu-Glu(O^tBu)-Ser(^tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(O^tBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Mtt)-Tentagel-S-RAM (theoretical loading 0.136 mmol/g) was suspended in 2 mL of a 1M Boc₂O solution in NMP and stirred for 1 h., 18.4 eq. of DiPEA was added (82 μ L, 0.5 mmol) and the mixture was shaken for another hour. The solution was removed by filtration and the resin was washed with NMP, DCM and dried. TNBS test showed full Boc protection. The 4-methyltrityl (Mtt) was removed by treatment of the resin with 1% TFA/DCM (2 mL, 2 min., 12 times) after which the resin was washed with DCM and DiPEA/NMP (1:9). A TNBS test²¹ revealed free amines. The resin was suspended in 2 mL NMP and HCTU (20 eq., 414 mg, 1 mmol), Propiolic acid (20 eq., 1 mmol, 62 μ L) and DiPEA (21 eq., 1.05 mmol, 85 μ L) were added. After 1 h. the resin was filtered and washed with NMP and DCM, next the propiolic acid coupling was repeated again for 1 h. The resin was washed with NMP and DCM and dried. A small aliquot of the resin was subjected to a Kaiser test which revealed complete coupling giving **17**. To control the quality of **17** a small aliquot of the resin was transferred to a glass tube and treated for 2 hours with a cleavage cocktail TFA/TIS/H₂O (95/2.5/2.5). The solution was filtered into

cold diethylether and the resin was washed with neat TFA. The diethylether was centrifuged, removed and the precipitate was washed with diethylether. After centrifugation, the ether was removed and the precipitate was air dried and dissolved in ⁴BuOH/ACN/H₂O, 3/1/1. (cleaved peptide **17**) LCMS: 10-90% B, Rt = 6.35 min; ESI-MS: [M+H]²⁺: 1299.6 (calculated. 1299.7).

Synthesis of conjugates **3** and **4**

A 1.5 mL stock solution (0.15 mmol of crude **2** in NMP (20% DiPEA) was added to 100 mg of resin (**9**, theoretical loading 0.136 mmol/g or resin **14**, theoretical loading 0.15 mmol/g). CuI (0.05 mmol, 9.8 mg) was added and the mixture was shaken for 48 h. The mixture was filtered and the remaining resin was washed with 0.5% Et₂NCSSNa/0.5% DiPEA in DMF, DMF, DCM and dried and transferred to an ice cooled glass tube. The conjugate was cleaved off the resin using TFA/TIS/H₂O (95/2.5/2.5) for 2h at room temperature. The suspension was filtered into cold diethyl ether and the resin was washed with neat TFA. The resulting suspension was centrifuged and the diethyl ether layer was decanted. The precipitate was dried by airflow and dissolved in 30% hexafluoroisopropanol/water.

(**3**) Purification by RP-HPLC (gradient: in 0.5 CV from 0-30% B, followed by 30-40%B in 3CV) yielded 1.65 mg (0.65 μmol, 4.3% based on theoretical loading of resin: 0.15 mmol/g) of white solid. LCMS: 10-70% B, Rt = 13.44 min; ESI-MS: [M+H]⁺: 2530.6 (calculated. 2530.5), [M+H]²⁺: 1265.6 (calculated. 1265.2). MALDI-TOF [M+H]⁺: 2533.69 (calculated. 2530.5).

(**4**) Purification by RP-HPLC (gradient: 0.25 CV 0% B followed 22-50% B in 4 CV) yields 4.61 mg (1.53 μmol, 11.3% based on theoretical loading of resin: 0.136 mmol/g) of white solid. LCMS: 10-70% B, Rt = 12.47 min; ESI-MS: [M+H]²⁺: 1506.8 (calculated. 1506.5), [M+H]³⁺: 1005.0 (calculated 1005.0).

Synthesis of conjugate **5**

Resin **17** (200 mg, theoretical loading: 0.136 mmol/g) was suspended in 20% DiPEA/NMP (3 mL) and crude **2** 141 mg (0.34 mmol) was added. Copper(I)iodide was added (0.1 mmol, 19.6 mg) and the mixture was shaken for 48 hours. The mixture was filtered and the remaining resin was washed with 0.5% Et₂NCSSNa/0.5% DiPEA in DMF, DMF, DCM, dried and transferred to an ice cooled glass tube. The conjugate was cleaved from the resin using TFA/TIS/H₂O (95/2.5/2.5) for 2 h. at room temperature. The suspension was filtered into cold diethyl ether and the resin was washed with neat TFA. The resulting suspension was centrifuged and the diethyl ether layer was decanted. The precipitate was dried by air flow and dissolved in 30% hexafluoroisopropanol/water.

(**5**) Purification by RP-HPLC (gradient: 0.25 CV 0% B followed 22-50% B in 4 CV) yields 2.38 mg (0.79 μmol, 2.9% based on theoretical loading of resin: 0.136 mmol/g) of white solid. LCMS: 10-70% B, Rt = 12.35 min; ESI-MS: [M+H]²⁺: 1506.8 (calculated 1506.5), [M+H]³⁺: 1005.2 (calculated 1005.0)

Dendritic cells and IL12 ELISA

Freshly isolated DC were cultured from bone marrow cells of C57BL/6 mice as described.²² BMDC (4×10^4) were plated into a 96-well round bottom plate, and incubated for 24 h. or 48 h. with the indicated compounds. Supernatants were harvested and tested for IL-12 p40/p70 content using a standard sandwich ELISA. Coating Ab: rat anti-mouse IL-12 p40/p70 mAb (clone C15.6; BD PharMingen). Detection Ab: biotinylated rat anti-mouse IL-12 p40/p70 mAb (clone C17.8; BD PharMingen). Streptavidin-HRP and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively.

MHC-class I-restricted antigen presentation assay

Dendritic cells were incubated for 2 h with either the peptide-conjugate **3**, **4**, **5** or the mixture of the parent peptide (DEVSGLEQLESIINFEKL) and the TLR 7 ligand **2**, at the indicated concentrations. Cells were then washed 5x with medium before the T-cell hybridoma B3Z cells were added and incubated for 16 h. Antigen presentation of the ovalbumin cytotoxic T-cell epitope, SIINFEKL in H-2K^b was detected by activation of B3Z cells measured by a colorimetric assay using chlorophenol red- β -D-galactopyranoside as substrate to detect *lacZ* activity in B3Z lysates as described.²³

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