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Synthesis of 2-alkoxy-8-hydroxyadenylpeptides: Towards synthetic epitope-based vaccines

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Abstract—The preparation of three different 2-alkoxy-8-hydroxyadenylpeptide conjugates has been accomplished by solid-phase synthesis combined with 'on-resin' Cu(I) catalyzed Huisgen cycloaddition. The immunogenicity of the compounds has been evaluated in IL-12 production and antigen presentation assays.

Toll-like receptors (TLRs) are key targets in the development of well-defined, potent and selective vaccines.[1] TLRs recognize specific molecules associated with common pathogens, such as bacteria and viruses, and elicit an inflammatory response.[2] A range of TLRs (TLR1 through TLR11) have been discovered, however the exact molecular identity of only a limited number of their ligands has been established.[3] The most notable structurally defined ligands are lipopeptides having a terminal *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2R)-propyl]cysteine (Pam_3Cys-OH) moiety, a ligand of TLR2[4], and oligodeoxynucleotides containing the CpG motif, a ligand of TLR9.[5] Interestingly, TLR7, that is expressed intracellularly in the endosomes, can bind small drug-like compounds such as imidazoquinolines[6] and guanine derivatives.[7]

It has been previously 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.[8,9] Up to now, however, only lipopeptides[10a] (peptides containing a palmitoylated cysteine residue) and nucleopeptides[10b] (hybrids of peptides and CpG phosphorothioate DNA) have been reported as structurally defined constructs designed for TLR-2 and TLR-9 recognition, respectively.

In this paper, we present the synthesis and biological evaluation of hydroxyadenylpeptide hybrid structures **3**, **4** and **5** (Fig. 1) containing the well-known major histocompatibility complex (MHC) class I epitope from ovalbumin (SIINFEKL, Figure 1, sequence in bold).

The potent interferon-alpha inducer 2-alkoxy-8-hydroxy adenine derivative **1** (Fig. 1)[11,12] is thought to act as a TLR7 ligand (TLR7-L). Structure–activity studies[11] justify alteration of the 2-substituent in **1** such as in alkylazide derivative **2** which is suitable for chemoselective attachment to alkyne functionalized peptides through copper catalyzed Huisgen cycloaddition.[13,14] This chemoselective transformation does not necessitate any protective group manipulation on the exocyclic amino group of hydroxyadenine and enables a straightforward synthetic entry to the target peptide derivatives **3**, **4** and **5** (Fig. 1) with the TLR7-L attached to either the C- or the N-terminus of the peptide.

The primary structure of the selected model peptides reflects earlier findings[15] indicating that for an optimal

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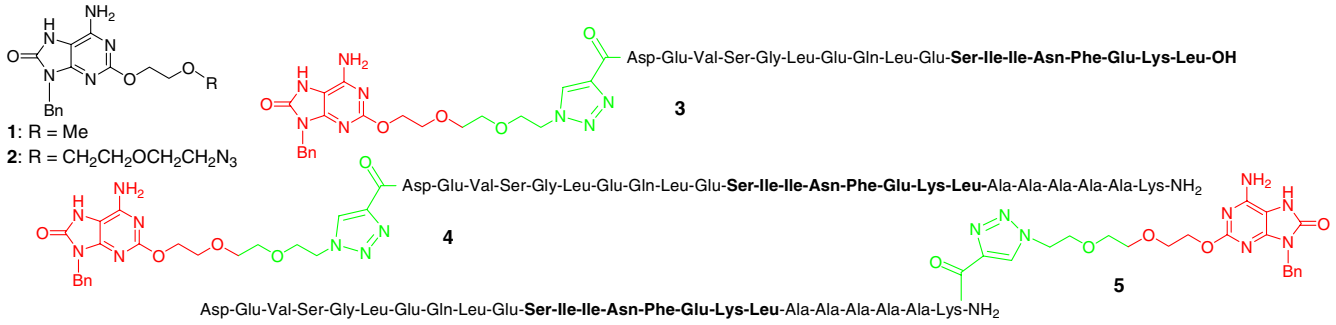
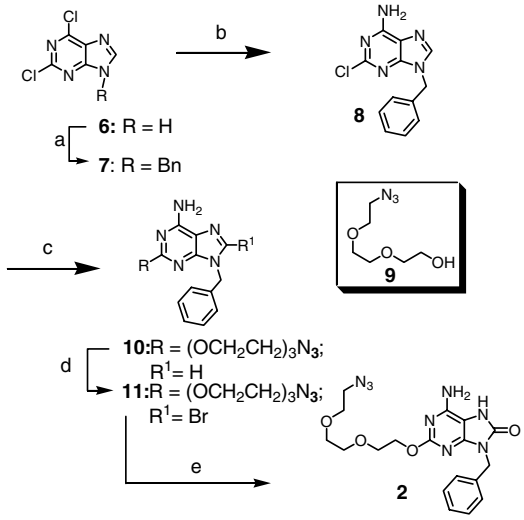


Figure 1. 2-Alkoxy-8-hydroxyadenine derivatives and peptide conjugates thereof.

immune response a CTL (cytotoxic T lymphocyte)-epitope (SIINFEKL in this study) has to be embedded in a longer peptide motif. This results in predominant presentation of the epitope (after proteolytic processing) on professional antigen-presenting cells (such as dendritic cells) and allows for exposure to the immune system in the context of MHC class I molecules. 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 C-terminus of the epitope (here L in SIINFEKL). 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 SIINFEKL motif at the C-terminus) thereby allowing the introduction 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 at the N- and C-termini, respectively.

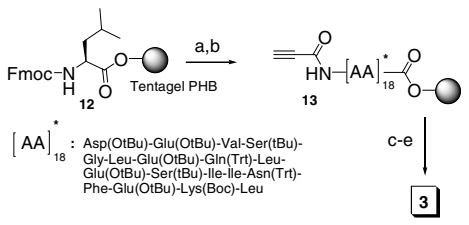
The assembly of conjugates **3**, **4** and **5** (Fig. 1) requires the availability of azide derivative **2** (Scheme 1). The synthesis of **2** is based on procedures published for the preparation of related 9-alkyl-2-alkoxyadenines[11,12] and commences with N-alkylation of 2,6-dichloropurine (**6**) with benzyl bromide under the agency of TBAF[16] to give 9-benzyl-2,6-dichloropurine **7** together with the 7-benzylated isomer. Chromatographic separation led to the isolation of both isomers in 61% (**7**) and 36% yield, respectively (Scheme 1).[17] Regioselective ammonolysis by heating of **7** with methanolic ammonia in a sealed tube under microwave irradiation for 15 h at 120 °C yielded 6-amino-2-chloropurine **8** in 97% yield. Treatment of **8** with 2-[2-(2-azidoethoxy)ethoxy]ethanol[18] (**9**) and NaH afforded the corresponding 2-alkoxyadenine **10**. Selective bromination at the C(8)-position was carried out with bromine to provide **11** in 78% yield.

Treatment of **11** with 3 N HCl at reflux gave 9-benzyl-8-hydroxy-2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy) adenine **2**. Compound **2** was found to be of satisfactory purity (62%, RP-HPLC) and contained as detectable side products only compounds lacking the azidotriethyleneglycol tail, and we therefore opted to use the mixture in the solid-phase Huisgen reaction without purification.



Scheme 1. Synthesis of the key building block **2**. Reagents and conditions: (a) BnBr, TBAF, 2 h, 61%; (b) NH_3/MeOH, 120 °C, 15 h, 97%; (c) **9**, DME, reflux, NaH, 69%; (d) Br_2/DCM, 2 h, 78%; (e) 3 N HCl, reflux, 3 days.

The synthesis of **3** (Scheme 2) started with a stepwise assembly of the peptide chain on the commercially available TFA sensitive Tentagel S PHB resin preloaded with Fmoc-Leu-OH (**12**). 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 (2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophospate) assisted[19] activation led to immobilized **13**. Subsequent attachment of the hydroxyadenine moiety was executed by treatment

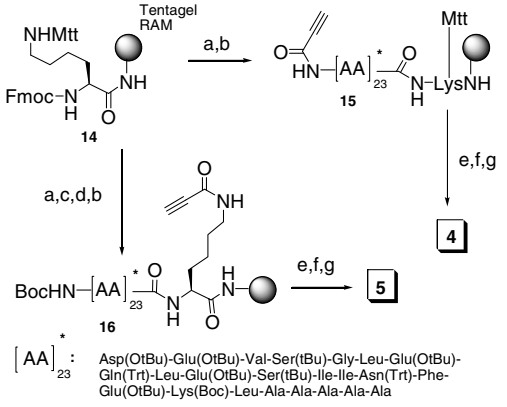


Scheme 2. Solid-phase synthesis of compound **3**. Reagents and conditions: (a) SPPS; (b) propiolic acid, HCTU, DiPEA; (c) **2**, CuI, DiPEA, NMP; (d) TFA/TIS(triisopropylsilane)/H_2O (95:2.5:2.5); (e) RP-HPLC.

(48 h) of resin **13** suspended in NMP with alkylazide functionalized building block **2** in the presence of CuI and DiPEA. Target compound **3** was released from the solid support and simultaneously deprotected by treatment[20] with TFA and isolated by ether precipitation. Crude hydroxyadenylated peptide **3** was analyzed by LC–MS using a C_{18}-column. No trace of the propiolyl peptide was detected, suggesting that the cycloaddition reaction went to completion. Purification using a semi-preparative RP-HPLC column gave homogeneous **3** in 4.3% overall yield.[21]

After the successful completion of the synthesis of conjugate **3**, very similar methodology was adopted for the preparation of the structurally different conjugates **4** and **5** (Fig. 1). Copper(I) catalyzed cycloaddition of azide **2** to alkyne functionalized peptide resin **15** (Scheme 3), obtained via SPPS initiated on immobilized Fmoc-Lys(Mtt) residue (**14**, Scheme 3), gave after TFA treatment and purification conjugate **4** in an overall yield of 11.3%.[21] Alternatively, Fmoc-Lys(Mtt) resin **14** was converted into immobilized **16** by the following procedure: HCTU/DiPEA assisted Fmoc-SPPS, Boc_2O/DiPEA treatment to block the N-terminus, 1% TFA/DCM[22] 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.[21]

We next investigated the ability of the pure synthetic 8-hydroxyadenine 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). The known[11] derivative **1** and the established TLR7 ligand[6] R848 were included in the assays as reference compounds. Bone-marrow derived dendritic cells (DCs) were incubated with the indicated substances and IL-12p40 production in the culture supernatant was measured after either 24 or 48 h. As evident from Figure 2 both **1** and **2** efficiently induce IL-12p40



Scheme 3. Solid-phase synthesis of compounds **4** and **5**. Reagents and conditions: (a) SPPS; (b) propiolic acid, HCTU, DiPEA; (c) Boc_2O, DiPEA; (d) 1% TFA/DCM; (e) **2**, CuI, DiPEA, NMP; (f) TFA/TIS/H_2O (95:2.5:2.5); (g) RP-HPLC.

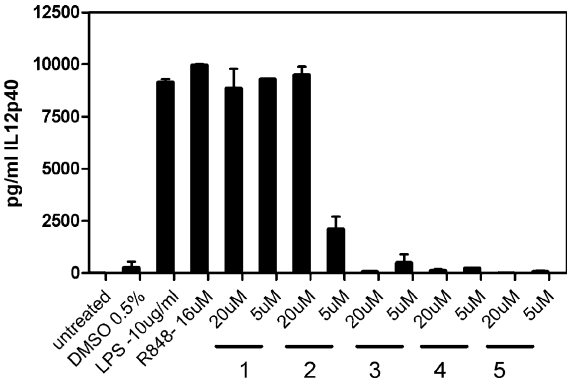


Figure 2. Potency of 8-hydroxyadenine derivatives in dendritic cells, activation determined by the level of induced IL-12p40.

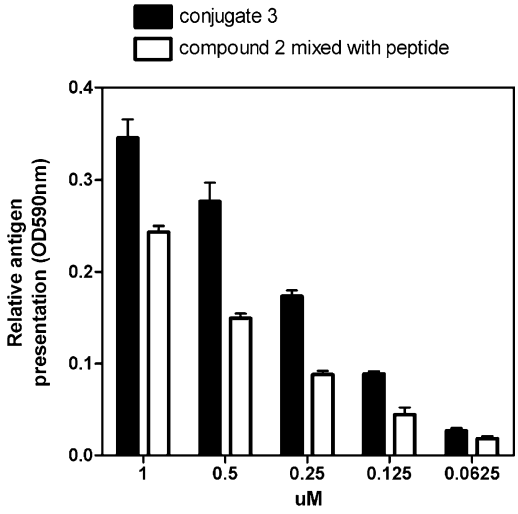


Figure 3. Comparing MHC class I antigen presentation of conjugated and non-conjugated peptides. Dendritic cells were incubated for 2 h in the presence of either conjugate **3** or a mixture of compound **2** with 18 meric parent peptide containing the cytotoxic T-cell epitope SIINFEKL. Subsequently cells were washed and incubated with B3Z T-cell hybridoma for 24 h before quantification of the T-cell response.

production. Conjugation of the peptides to the TLR7 ligand (compounds **3**, **4** and **5**) led to complete abolishment of the IL-12p40 production. Next, we investigated the effect of the hydroxyadenine modification on the antigen presentation. As evident from Figure 3, specific T cell response against the SIINFEKL epitope can be elicited when DCs are treated with either TLR7-L-conjugate **3** or the free parent peptide in a mixture with compound **2**. 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**.[23]

In summary, an efficient solid-phase synthesis of three different 2-alkoxy-8-hydroxy 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 of 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 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.

The design, synthesis and evaluation of such next-generation conjugates are now in progress and will be reported in due course.

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Supplementary data

Detailed experimental procedures and characterization for all new compounds. This material is available free of charge via the Internet at http://www.elsevier.com. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.034.

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Compound **3**: LC/MS 10–70% B, t_R = 13.44 min. ESI-MS: $[M+H]^+$: 2530.6 (calcd 2530.5).
Compound **4**: LC/MS 10–70% B, t_R = 12.47 min; ESI-MS: $[M+H]^{2+}$: 1506.8 (calcd 1506.5).
Compound **5**: LC/MS: 10–70% B, t_R = 12.35 min; ESI-MS: $[M+H]^{2+}$: 1506.8 (calcd 1506.5).
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