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Synthetic peptides as tools in chemical immunology
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6

Synthesis of peptides containing a combination of free and 2-*trans*-cyclooctene carbamate protected lysine residues

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Introduction

Dissociative bio-orthogonal reactions are a quickly developing field of chemical biology.[1] In these reactions, a protected bioactive molecule – where the protecting group blocks the biological function of the molecule in some manner – are removed using a bio-compatible chemical reaction. This restores the molecule's biological activity, giving chemical control over the activity of a molecule inside a living system. The very first example of such a reaction was

shown in 2006, when Streu and Meggers showed intracellular cleavage of an Alloc protecting group using a ruthenium complex.[2] Since then, a large amount similar, bio-compatible bond cleavage reactions have been developed, both using transition metal catalysis and organic reactions.[3] Using these reactions, spatial and/or temporal control over biological functions, such as receptor-ligand interactions[4] or cytotoxic activity[5,6] has been achieved.

Among the currently known dissociative bio-orthogonal reactions, the "click-to-release"-reaction, first demonstrated by Robillard,[6] has become one of the main reactions of choice. The "click" part of the name is the inverse electron demand Diels-Alder (IEDDA) reaction between a 2-*trans*-cyclooctene (2-TCO) (Figure 1, I) and a 1,2,4,5-tetrazine. In this reaction an unstable dihydropyridazine intermediate (II) is formed, which can adopt a different tautomeric form (III). This tautomer can eliminate the leaving group on the allylic position of the TCO, "releasing" a molecule (IV), and forming a pyridazine (V) as a byproduct. Different functional groups have been successfully uncaged in this manner, among them amines,[6–9] carboxylic acids[10] and alcohols.[11,12]

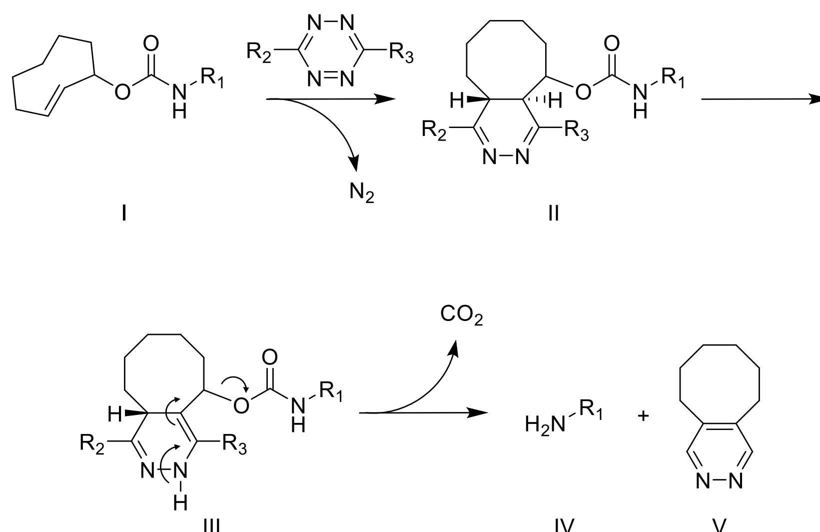


Figure 1. Mechanism of the click-to-release reaction between an amine protected with an allylic TCO-carbamate and a 1,2,4,5-tetrazine. In this reaction, the TCO (I) reacts with a tetrazine to form the click-product (II) with gaseous nitrogen as the byproduct. This intermediate tautomerizes to a different dihydropyridazine (III) which eliminates the allylic carbamate. After CO_2 release, the deprotected amine (IV) is formed, as well as a byproduct (V).

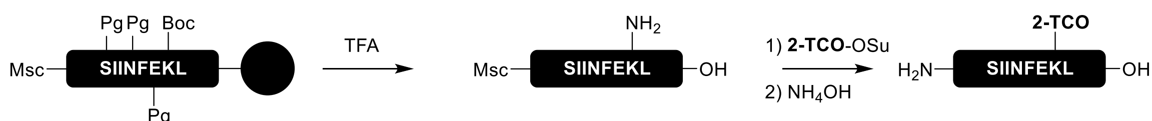
The reaction has properties that make it favorable for use as a biocompatible deprotection reaction. Firstly, the uncaging reaction has fast kinetics.[13] Secondly, the tetrazine reagents have low toxicity and good bioavailability.[5,14] As a result, the system has been successfully used for the *in vivo* deprotection of a kinase enzyme,[14] for the dissociation of antibody-drug conjugates[15], and is even pursued in a Phase I clinical trial for localized drug activation, using a TCO caged form of the anticancer drug doxorubicin.[16]

Allylic trans-cyclooctenes have also been used for the protection of peptides. For example, to shield a T-cell epitope from recognition by its cognate T-cell clone. Addition of tetrazine (*in*

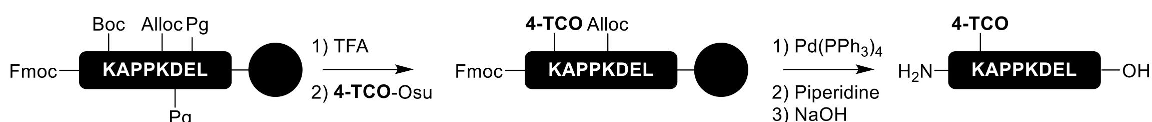
vitro or *in vivo*) allowed for the controlled activation of the T-cell in space and time.[17] Further expansion of this approach to more complicated peptides has been hindered by the extreme acid-lability of the trans-cyclooctene moiety.[18,19] As any concentration of trifluoroacetic acid (TFA; commonly employed as a deprotection reagent in the solid support synthesis of peptides) led to the partial deprotection and/or isomerization to the unreactive *cis*-isomer,[4,19] on-resin modification of lysine residues during Fmoc-SPPS (solid phase peptide synthesis) with TCO was not viable. Therefore, the only known approaches were post-synthetic modification of lysine sidechains with TCO reagents. Using either a compatible protection group to temporarily block the N-terminus, like the Msc-group used by van de Gracht *et al.*[17] (Figure 2A) or a permanent modification of the N-terminus, for instance acetylation,[20] peptides bearing a single lysine function could be modified. However, none of these strategies provided the necessary selectivity to modify peptides containing multiple lysine functions. The lack of a good protecting group strategy has limited the effective use of TCO-modified peptides to those containing only a single lysine residue.

A synthetic approach, whereby (longer) peptides can be synthesized that contain both a 2-TCO-protected lysine, as well as unreacted lysines, is therefore an unmet chemical need. Particularly, as this would allow the extension of the above T-cell activation study, to also study the processing of long peptide antigens by antigen presenting cells, as was previously done using a Staudinger-reduction approach.[21] La-Venia *et al.*[19] (Figure 2B) have developed an approach, where short peptides containing multiple lysines carrying a non-allylic TCO group (4-TCO), were synthesized. This method was based on an acid-mediated global deprotection of the synthesized peptide on resin, followed by introduction of the 4-TCO onto the lysine sidechain and NaOH mediated release of the peptide from resin. Additional lysine residues could be protected using either the Alloc group or as a trifluoroacetate amide, which remain stable during acidic deprotection but could be removed orthogonally to the 4-TCO later using either $Pd(PPh_3)_4$ with morpholine (for Alloc) or NaOH (for the trifluoroacetamide). The palladium treatment would likely result in the removal of an allylic TCO, rendering the approach less promising for use with 2-TCO protecting groups. The use of trifluoroacetamide protection could be a promising approach for the synthesis of 2-TCO protected peptides. Since it is known 2-TCO groups are stable to nucleophilic base,[17] deprotection of amines, as well as cleavage of the resin linker, with NaOH should be possible. However, it is known that nucleophilic base mediated cleavage from SPPS resin is often problematic on longer peptides or peptides containing a sterically hindered C-terminal amino acid.[22,23] This method has not been tested on longer peptides and is incompatible with peptides carrying groups labile to nucleophilic base. Therefore, an alternative strategy that could utilize the regular, highly efficient acidic resin cleavage used in standard Fmoc-SPPS would greatly benefit the development of more complex *trans*-cyclooctene protected peptides.

A) van de Gracht *et al.* (2018):



B) La-Venia *et al.* (2021):



C) This chapter:

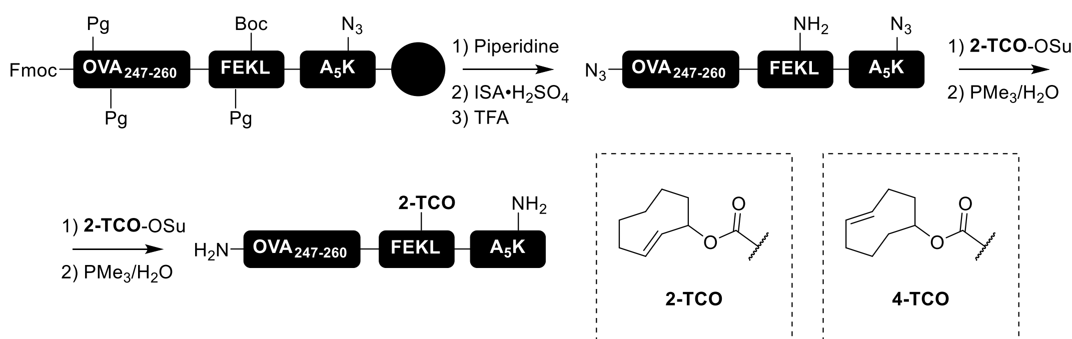


Figure 2. Overview of published methods to synthesize TCO functionalized peptides. A) Hybrid synthesis using the base-labile Msc group as temporary N-terminal protecting group[17] b) Full on-resin method published by Vrabel to introduce 4-TCO groups onto peptides.[19] C) Novel hybrid approach described in this chapter. Here, azide groups are used to temporary mask amine functions.

It was here postulated that an azide protection strategy could be used (Figure 2C). Lysine residues not intended for 2-TCO protection could be introduced as the commercially available azidolysine building block. For the N-terminus, multiple on-resin diazotransfer reactions have been reported, using either triflic azide[24] or the more easily handled imidazole-1-sulfonyl azide (ISA).[25,26] This second reagent was first described by Goddar-Borger and Stick as the HCl salt,[27] however the later reported bisulfate salt[28] is preferred since the hydrochloride salt has been reported to decompose to HN_3 over time. Hansen and co-workers have reported a protocol for on-resin diazotransfer where ISA·H_2SO_4 is used in DMF with DiPEA as base.[25] Alternatively, the α-azido forms of some amino acids are also commercially available. The azide is stable during basic Fmoc-deprotection and acidic resin cleavage,[29] and can be reduced to the corresponding amine using a mild phosphine reduction, often called the Staudinger reduction,[30] a transformation successfully used before on synthetic peptides.[31,32]

In this chapter, a novel approach is described that allows the facile incorporation of a 2-TCO (from this point on simply referred to as TCO) protected lysine in a peptide containing multiple lysine residues. This is achieved by masking all amines as azides during SPPS, except the lysine to which the TCO carbamate is introduced, which is Boc protected as h during normal SPPS

(Figure 2C). Introduction of the TCO happens after full peptide assembly and cleavage from the resin, completely avoiding acid when the TCO group is present, while still utilizing the standard, optimized, Fmoc-SPPS protocols. Here, it is shown that TCO is compatible with this azide reduction, enabling facile synthesis of peptides containing a combination of TCO-protected and free lysine residues.

Results and Discussion

To validate the utility of azide as a protective group of amines in peptides during the synthesis of TCO protected peptides, a model peptide was designed that contained both an azidolysine, free lysine and azido-N-terminus (**3**, Figure 3). The peptide was synthesized using manual SPPS on Tentagel S RAM resin. For the introduction of the precious azidolysine building block on the resin two equivalents (in relation to the manufacturer specified resin loading), instead of the typical five, were utilized. The rest of the peptide was synthesized using standard Fmoc-SPPS conditions. After synthesis of the full resin bound hexapeptide **1**, the N-terminal amine was masked as an azide using the protocol of Hansen *et al.*[25], using ISA·H_2SO_4 in DMF with DiPEA as base. This method was preferred over aqueous diazotransfer reactions,[26,33] since the solubility of fully protected peptides in aqueous mixtures was expected to be poor.

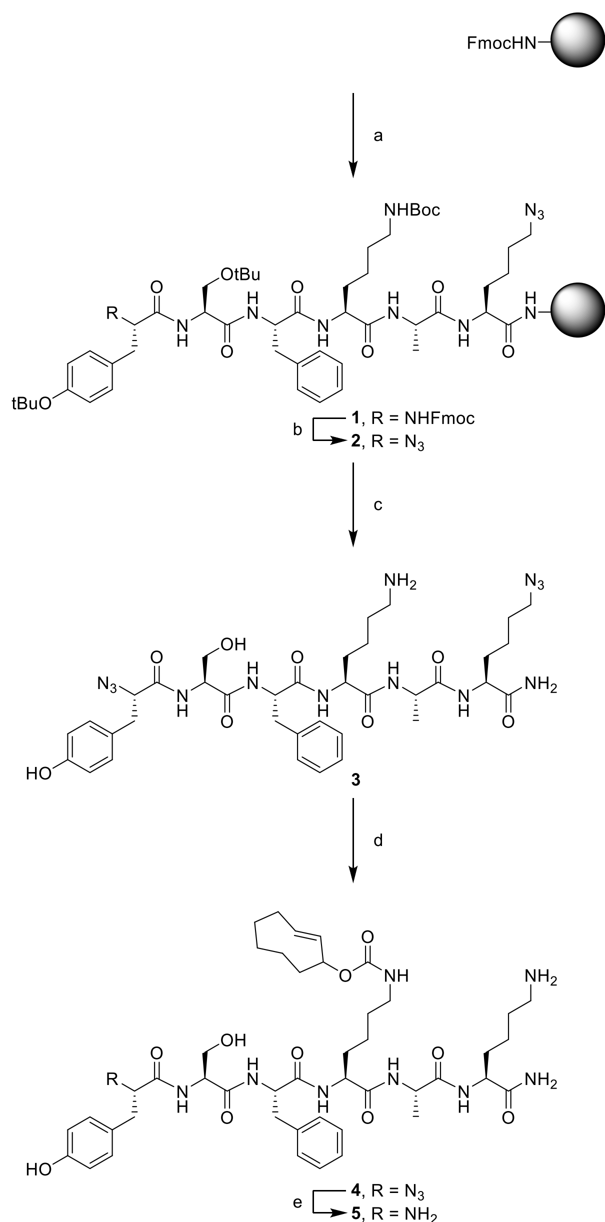


Figure 3. Synthesis of example TCO caged peptide **5**. Reagents and conditions: a) Fmoc-SPPS b) i) 20 % v/v piperidine, DMF ii) ISA·H_2SO_4, DiPEA c) TFA, TIS, H_2O d) TCO-OSu, DiPEA, DMF e) i) PMe_3, H_2O, DMF ii) RP-HPLC, 2.1%

This diazotransfer reaction with 3 equivalents of ISA·H_2SO_4 and 5 equivalents of DiPEA, gave full conversion to resin-bound peptide **2**, as determined by LC-MS analysis of the crude peptide after acidic cleavage of a small amount of resin. After full resin liberation and global deprotection with a standard TFA cocktail (95:2.5:2.5, TFA/TIS/H_2O) the crude peptides **3** was precipitated from cold diethyl ether and residual TFA removed by co-evaporation with toluene. To introduce the 2-TCO carbamate, a small excess of TCO-OSu together with 5 equivalents of DiPEA were dissolved in DMF and added to the crude peptide. After two hours, LC-MS analysis indicated complete conversion to the TCO protected peptide **4**. The final step was the reduction of the azides. For this, a solution of PMe_3 in THF was added, to form the iminophosphorane intermediate, followed by water, to hydrolyze this intermediate to the

corresponding amine. After one hour, full consumption of peptide **4** was confirmed by LC-MS and the crude peptide was precipitated. RP-HPLC was carried out, using acetic acid instead of TFA as the pH modulator in the aqueous buffer, and peptide **5** was obtained in 2.1% yield.

The integrity of the TCO cage was validated by deprotection of a small aliquot of the purified peptide with 3,6-dimethyl-1,2,4,5-tetrazine followed by LC-MS analysis, which confirmed complete removal of the TCO-group, indicating that none had isomerized to the non-reactive *cis*-cyclooctene by-product.

With the successful application of both the diazotransfer reaction and Staudinger reduction established, the method was next used to synthesize an immuno-relevant peptide. The model antigen $OVA_{247-264}A_5K$, a peptide derived from chicken ovalbumin, C-terminally extended with the amino acid sequence AAAAAK, is used to study the antigen processing and presentation of MHC class I restricted antigens.[34,35] This peptide contains the MHC class I restricted epitope $OVA_{257-264}$ (SIINFEKL),[36] an often used model antigen to study T-cell activation with murine immune cells. Previously, antigen processing studies have been carried out by masking the lysine in this epitope with TCO. The second lysine on the C-terminus of the $OVA_{247-264}A_5K$ peptide precluded a similar approach to study processing of the extended peptide. Using the azide based protecting group strategy enables the synthesis of this complex, TCO protected peptide.

The synthesis was again initiated with the coupling of Fmoc-Lys(N_3)-OH onto Tentagel S RAM resin using two equivalents of amino acid and HCTU as the coupling agent. The rest of the peptide was assembled using an automated peptide synthesizer to yield resin bound peptide **6** (Figure 4). After Fmoc removal, the resin was treated again with ISA·H_2SO_4 and DiPEA in DMF. After a TFA mediated test cleavage, the main peak in the chromatogram could be assigned to peptide **8**, confirming that formation of the N-terminal azide had proceeded to near completion. Since some peptide with an unreacted N-terminal amine was still present, the resin-bound peptide was treated with a solution of Ac_2O and DiPEA in DMF to acetylate any remaining free amines.

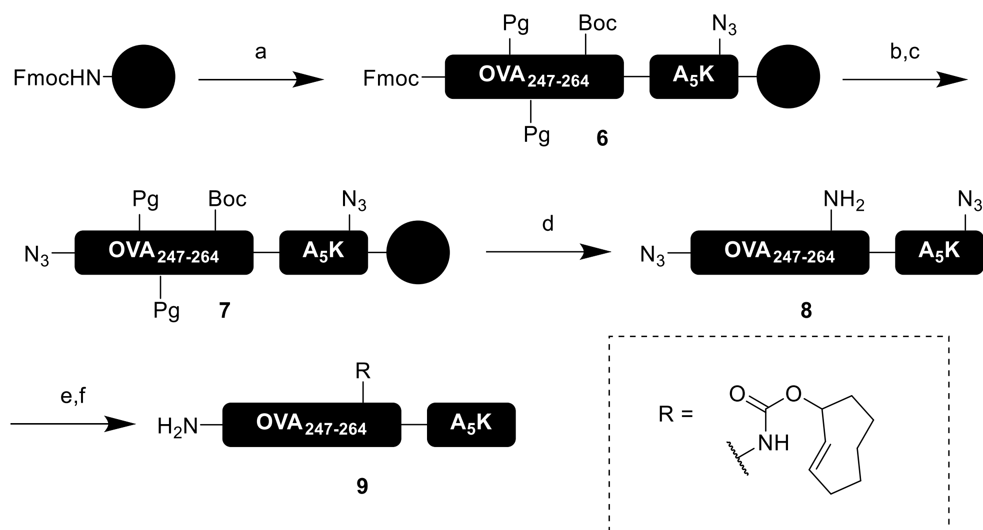


Figure 4. Synthesis $OVA_{247\text{-}264}A_5K$, bearing an allylic TCO on Lys_{263} (**11**). Reagents and conditions: a) Fmoc-SPPS b) 20% piperidine (v/v), DMF c) $ISA{\cdot}H_2SO_4$, DiPEA, DMF d) TFA, TIS, H_2O e) TCO-OSu, DiPEA, DMF f) i) PMe_3, H_2O, DMF ii) RP-HPLC, 5.4%

The protected peptide **7** was then cleaved from resin and deprotected (with the exception of the azides) and precipitated from cold diethyl ether, producing crude **8**. Residual TFA was removed by toluene co-evaporation. Then, a mixture of TCO-OSu and 20 equivalents of DiPEA in DMF were added to the peptide, in order to introduce the TCO onto the free lysine residue. After 2 hours, LC-MS analysis indicated the formation of the TCO conjugated intermediate. Finally, the azides were reduced using PMe_3 and water. After one hour, LC-MS analysis indicated complete reduction of both azides. Crude peptide **9** was recovered from solution by ether precipitation and the desired TCO protected peptide was isolated using RP-HPLC in 5.4% yield.

In a similar manner, a TCO protected peptide was also made from the C-terminally non-extended peptide $OVA_{247\text{-}264}$ (Figure 5). The critical lysine within the $OVA_{257\text{-}264}$ peptide, Lys_{263}, was again caged with an allylic TCO carbamate. This peptide was synthesized on Tentagel S Ac resin, to produce the C-terminal carboxylic acid needed for epitope binding to the MHC complex.[37] Standard Fmoc-SPPS produced resin-bound peptide **10**. Initial attempts to introduce the N-terminal azide via the described diazotransfer reaction with $ISA{\cdot}H_2SO_4$ in DMF yielded poor conversion. An alternative approach was taken, where a solution of $ISA{\cdot}H_2SO_4$ in water, using Na_2CO_3 as base, was used. This resulted in complete conversion of the N-terminal amine into an azide. TCO conjugation and Staudinger reduction were carried out as before, resulting in the isolation of peptide **12** in a rather poor 1.4% yield.

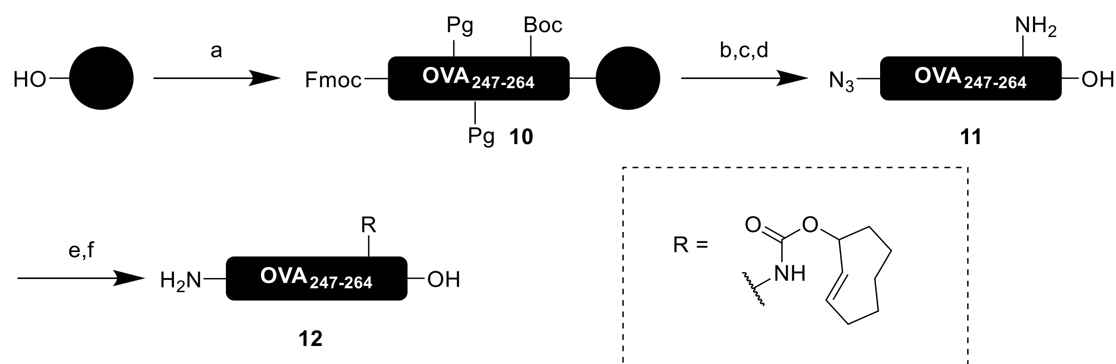


Figure 5. Synthesis of TCO protected $OVA_{247-264}$, peptide **14**. Reagents and conditions: a) Fmoc-SPPS b) 20% (v/v) piperidine, DMF c) ISA·H_2SO_4, DiPEA, DMF d) TFA, TIS, H_2O e) TCO-OSu, DiPEA, DMF f) i) PMe_3, H_2O, DMF ii) RP-HPLC, 1.4%.

The TCO-caged $OVA_{247-264}A_5K$ peptide **9** was tested in a T-cell activation assay. In this assay, the dendritic cell-line D1 is preincubated with varying concentration of either peptide **9** or the non-protected control. After two hours, the caged peptides were uncaged by the addition of 10 µM tetrazine **13**[38] (Figure 6A) for one hour. Then, the D1 cells were co-cultured with B3Z T-cell hybridoma cells.[39] In these cells, the expression of the enzyme LacZ is under the control of the nuclear factor of activated T cells (NF-AT) promotor, meaning LacZ production is induced when the cells become activated by T-cell receptor (TCR) stimulation. LacZ mediated hydrolysis of the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) was then used as a reporter of T-cell activation, by measuring absorption at 570 nm.[40] The response to 100 nM $OVA_{247-264}$ was used as a positive control (Figure 6B).

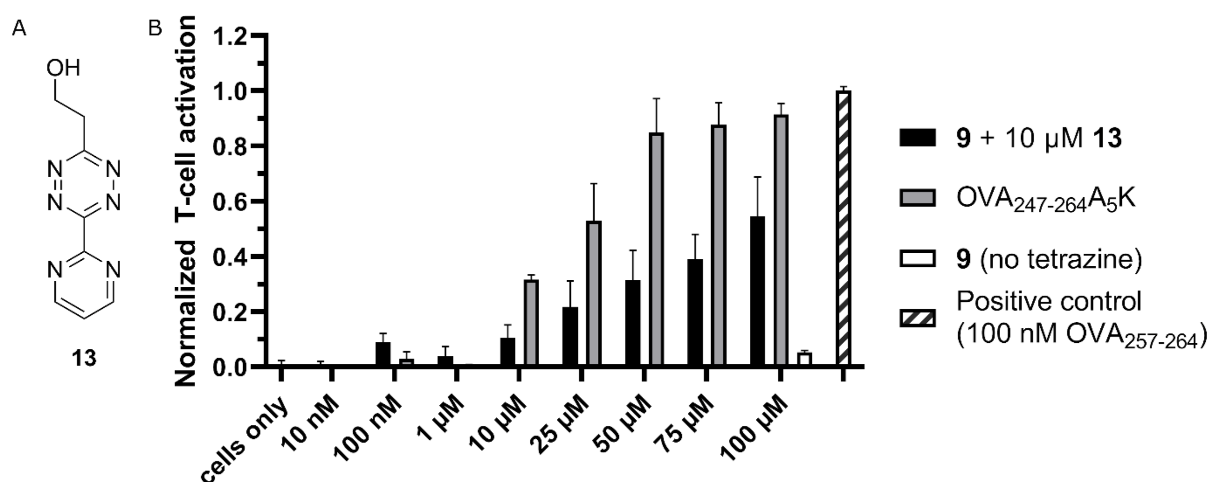


Figure 6. A) Structure of tetrazine **13**, used in the on-cell uncaging experiment. B) Graph of the normalized T-cell response measured for either peptide **9** (with or without uncaging with tetrazine **13**) or the non-caged control $OVA_{247-264}A_5K$. T-cell assays were carried out by N.A.M. Ligthart (Leiden University).

In the absence of tetrazine, very low levels of T-cell activation were observed when cells were treated with 100 µM of peptide **9**. When tetrazine **13** was added, T-cell activation following a clear dose-response relation was found, with the highest concentration of 100 µM showing nearly 60% of the level of T-cell activation of non-caged $OVA_{247-264}A_5K$.

Over the last two decades, it has become apparent that the direct conjugation of an innate immune activating molecule to a peptide vaccine can greatly enhance its immunogenicity.[41] To this end, Toll-like receptor (TLR) ligands are often conjugated to synthetic peptides.[42,43] This results in activation and maturation of dendritic cells and improved cross-presentation of the T-cell epitope.[34]

Studying the processing and presentation of these peptide conjugate vaccines is crucial to their further development. The development of model constructs that give chemical control over the antigen presentation kinetics would be very useful. Here, as a proof of concept, such a peptide was synthesized, based on the $OVA_{247-264}A_5K$ model antigen. This antigenic peptide can be conjugated to the hTLR2 ligand mini-uPam to enhance its immunogenicity. This ligand is a derivative of the TLR2 ligand Pam_3SK_4, a small lipopeptide, optimized for lower lipophilicity.[44] Since this mini-uPam moiety contains multiple ester functionalities, the synthetic strategies previously reported based on NaOH labile protecting groups, were not feasible for the synthesis of such a conjugate. The Staudinger-reduction based approach shown here, however, has no such limitations, enabling the synthesis of **18**.

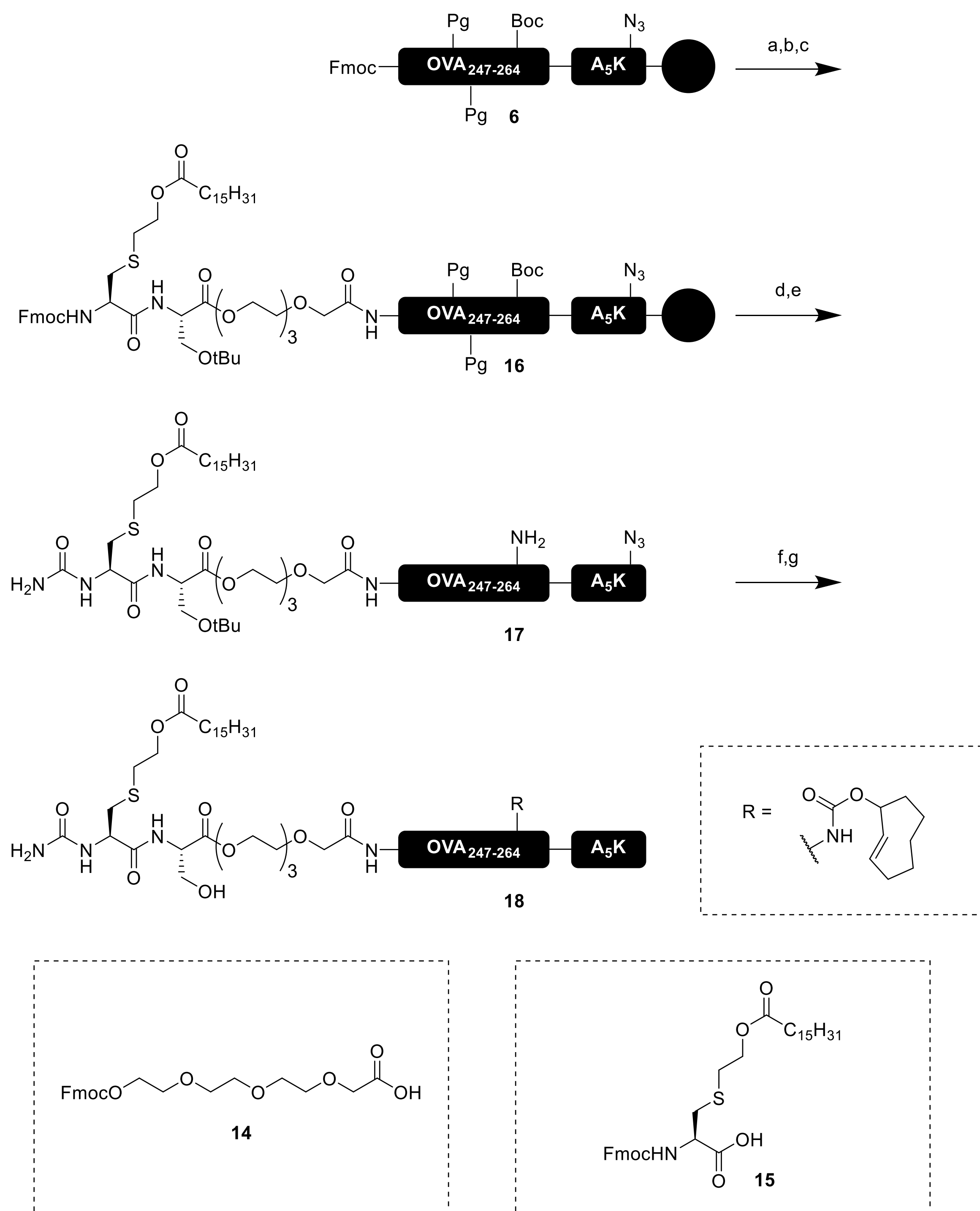


Figure 7. Synthesis of TCO-protected model conjugate vaccine **19**. Reagents and conditions: a) i) 20% (v/v) piperidine, DMF ii) **15**, HCTU, DiPEA, DMF b) i) 20% (v/v) piperidine, DMF ii) Fmoc-Ser(tBu)-OH, DIC, DMAP, DMF c) i) 20% (v/v) piperidine, DMF ii) **16**, HCTU, DiPEA, DMF d) 20% (v/v) piperidine, DMF ii) TMS-NCO, iPrOH, DCM e) TFA, TIS, H_2O f) TCO-Osu, DiPEA, DMF g) i) PMe_3, H_2O, DMF ii) RP-HPLC, 7.9%

The synthesis of the TLR-conjugate peptide (Figure 7) was started from resin-bound peptide **6**. This peptide was manually elongated with PEG spacer **14**, followed by liberation of the Fmoc-protected alcohol. This alcohol was condensed with Fmoc-Ser(tBu)-OH, mediated by DIC and catalytic DMAP. Next, the peptide was further elongated using cysteine building block **15**,

producing resin-bound peptide **16**. This was followed, after Fmoc removal, by formation of an N-terminal urea using a mixture of TMS-isocyanate and isopropanol in DCM, as reported by van de Ende *et al.*[44] This conversion proceeded sluggishly, with a single treatment not yielding full conversion of the amine. A second, longer treatment of the resin with the same TMS-isocyanate solution did fully convert the N-terminal amine to the urea. This peptide was then released from resin using the standard TFA cocktail (95:2.5:2.5, TFA/TIS/H_2O) and precipitated from cold diethyl ether, yielding crude peptide **17**. Again, residual TFA was removed by toluene co-evaporation, and DiPEA and TCO-OSu in DMF were added to the crude peptide. The reaction followed by LC-MS analysis. After complete consumption of **17** was observed, Staudinger reduction was initiated, producing crude **18**. RP-HPLC yielded the desired TCO protected conjugate peptide in 7.9% yield. The biological evaluation of this construct and their associated controls was not performed due to time restrictions.

Conclusion

The synthesis of peptides containing a 2-TCO protected lysine residue together with additional lysine residues is complicated. This is primarily due to the high acid sensitivity of the *trans*-cyclooctene group. Here, a synthetic method for such peptides was developed. By using azide groups as amine protecting groups, the lysine for TCO-modification could be selectively deprotected and modified. Subsequent Staudinger reduction allowed the unmasking of the azide protected amine groups.

This was exemplified with the synthesis of the model antigen peptide $OVA_{247-264}A_5K$, bearing a TCO group on the Lys_{263} residue, while leaving the C-terminal lysine unprotected. The compatibility of the approach with other functionalities was further exemplified by the synthesis of a TCO-caged mini-uPam-$OVA_{247-264}A_5K$ conjugate peptide. The base-labile ester groups in this peptide would prevent the synthesis using existing methods.

An initial T-cell activation assay using the TCO-caged $OVA_{247-264}A_5K$ peptide (**9**) showed very low levels of background activation, indicating the TCO cage is capable of blocking T-cell activation. Treatment with tetrazine **13** resulted in T-cell activation, indicating both successful deprotection of the epitope and that these protected peptides are processed and presented by immune cells. These results will enable the further study of the antigen processing and presentation of longer peptides.

Experimental

General procedure for automated SPPS

Peptides were synthesized using automated Fmoc-SPPS on a Liberty Blue^{tm} automated microwave peptide synthesizer (CEM corporation). Synthesis was performed 100 µmol scale on Tentagel S RAM resin (loading 0.20-0.25 mmol/g, Rapp Polymere GmbH, Germany). Resin was first swollen for 5 minutes in DMF prior to amino acid coupling. Activation was achieved using DIC/Oxyma coupling as is recommended by the manufacturer. The following amino acids were used: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu), Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(N_3)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH. All amino acids were obtained from Novabiochem except Fmoc-Lys(N_3)-OH which was synthesized in house.[45] Standard coupling was achieved using 5 equivalents amino acid as a 0.2 M amino acid/DMF solution, 5 equivalents DIC as a 0.5 M of DIC/DMF solution and 5 equivalents Oxyma as a 1 M Oxyma/DMF solution (also containing 0.2 M DiPEA), at 90°C for 2 minutes. Standard Fmoc deprotection was achieved by 20% (v/v) piperidine in DMF at 90°C for 90 seconds, repeated once. To analyze the quality of the peptide, a small amount of resin (~1mg) was treated with 200 µL of a TFA cocktail (95:2.5:2.5, TFA/H_2O/TIS) for 2 hours, after which the TFA was filtered into 800 µL of ice cold Et_2O. After five minutes the formed precipitate was collected by centrifugation and the supernatant discarded. The pellet was dissolved in 200 µL 1:1:1 H_2O/MeCN/tBuOH and subjected to LC-MS analysis. Peptides were characterized using electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector on an analytical C18 column (Phenomenex, 3 µm, 110 Å, 50 mm × 4.6 mm) in combination with buffers A (H_2O), B (MeCN), and C (1% aq TFA). Quality of crude was evaluated with a linear gradient of 10-90% B with a constant 10% C over 9 minutes, unless stated otherwise.

General procedures for manual SPPS

Manual elongation of peptides was carried out in a fritted syringe at either 100 or 20 µmol scale. Fmoc deprotection was achieved using 20 % (v/v) piperidine in DMF in two steps, reacting 3 and 7 minutes respectively. All amino acids were coupled using 5 equivalents of amino acid together with 5 equivalents of HCTU (as a 0.5 M solution) and 10 equivalents DiPEA for 45 minutes, except for Fmoc-Lys(N_3)-OH, which was coupled using 2 equivalents together with 2 equivalents HCTU (as a 0.2 M in DMF solution) and 4 equivalents of DiPEA for 90 minutes. Analysis of the quality of the resin-bound peptide was carried out as above.

General procedure for peptide purification

Crude peptides were dissolved in a mixture of DMSO:H_2O:MeCN:tBuOH (typically 3:1:1:1) and subjected to RP-HPLC purification on a Agilent 1260 Infinity II semiprep system. This machine was equipped with a Nucleodur C18 gravity column (5 µm, 250 x 10.0 mm) using a flow of 5 mL/min and buffers A = 1% AcOH in H_2O and B = MeCN. Fraction collection was triggered by detection of the target peptide mass on a coupled mass detector (Agilent InfinityLab LC/MSD XT). Quality of purified peptides was determined using an electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector on an analytical C18 column (Phenomenex, 3 µm, 110 Å, 50 mm × 4.6 mm) in combination with buffers A (H_2O), B (MeCN),

and C (1% aq TFA). Quality of the peptides was evaluated with a linear gradient of 10-90% B with a constant 10% C over 9 minutes.

Fmoc-Tyr(tBu)-Ser(tBu)-Phe-Lys(Boc)-Ala-Lys(N_3)-RAM-Tentagel S (1)

The peptide was synthesized on a 50 µmol scale using the general manual SPPS procedures. **LC-MS** RT = 6.1 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+H]^+$ = 990.48 observed M/z = 990.47

N_3-Tyr-Ser-Phe-Lys-Ala-Lys(N_3)-NH_2 (3)

25 µmol of resin bound peptide **1** was swelled in DMF for 30 minutes followed by Fmoc removal according to the general procedure. ISA·H_2SO_4 (3 eq, 21 mg, 75 µmol) and DiPEA (5 eq, 21.8 µL, 125 µmol) were dissolved in anhydrous DMF (1 mL) and added to the drained resin. The resin was reacted under gentle agitation for 1 hour, after which the resin was drained and thoroughly washed with DMF (3 x 5 mL) and DCM (3 x 5 mL). Next, a TFA cocktail was added (1 mL, 95:2.5:2.5 TFA/TIS/H_2O) to the drained resin and this suspension was incubated for 1 hour, before draining the TFA into a centrifuge tube containing ice cold Et_2O (10 mL). The obtained suspension was kept on ice for 15 minutes followed by centrifugation. The supernatant was discarded and the pellet washed with Et_2O (2 mL). The crude peptide was transferred to a roundbottom flask with MeOH and concentrated *in vacuo*. This yielded 18.1 mg (22.8 µmol) of crude peptide **3**. **LC-MS** RT = 5.0 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+H]^+$ = 794.40 observed M/z = 794.27

Tyr-Ser-Phe-Lys(TCO)-Ala-Lys-NH_2 (5)

The crude peptide **3** (22.8 µmol) was co-evaporated with toluene (3 x 1 mL) and put under N_2. TCO-OSu (1.1 eq, 6.7 mg, 25 µmol) and DiPEA (5 eq, 20 µL, 115 µmol) were dissolved in anhydrous DMF (2.3 mL) and this solution was added to the flask containing the crude peptide. After 2 hours, LC-MS analysis indicates full conversion to the TCO protected peptide. **LC-MS** RT = 6.3 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+H]^+$ = 946.48 observed M/z = 946.17 This intermediate was then treated with a 1 M solution of PMe_3 in THF (10 eq, 230 µL, 0.23 mmol) followed by the addition of H_2O (100 eq, 41 µL, 2.3 mmol). After 1 hour LC-MS analysis indicated full consumption of the intermediate. RP-HPLC followed by lyophilization yielded peptide **5** in 2.1 % yield (0.42 mg, 0.47 µmol). **LC-MS** RT = 4.9 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+H]^+$ = 894.50, $[M+2H]^{2+}$ = 447.75 observed M/z = 894.40, 447.60 **HRMS** (ESI) m/z: $[M + 2H^+]$ calcd $C_{45}H_{67}N_9O_{10}$ 894.5084, found 894.5086

Fmoc-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(N_3)-RAM-Tentagel S (6)

Synthesis was carried out on a 100 µmol scale. The initial Fmoc-Lys(N_3)-OH was coupled using manual SPPS procedures. The peptide was then elongated using automatic SPPS. **LC-MS** RT = 6.7 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1397.70 observed M/z = 1397.53

N_3-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(N_3)-NH_2 (8)

25 µmol of resin bound peptide **6** was swelled in DMF for 30 minutes followed by Fmoc removal according to the general procedure. ISA·H_2SO_4 (3 eq, 21 mg, 75 µmol) and DiPEA (5 eq, 21.8 µL, 125 µmol) were dissolved in anhydrous DMF (1 mL) and added to the drained resin. The resin was reacted under gentle agitation for 1 hour, after which the resin was drained and thoroughly washed with DMF

(5 x 5 mL). The remaining free N-terminal amines were acetylated using a solution of 10% (v/v) Ac_2O and 5% (v/v) DiPEA in DMF (1 mL total) for 15 minutes, followed by thorough washing with DMF (3 x 5 mL) and DCM (3 x 5 mL). Next, a TFA cocktail was added (1 mL, 95:2.5:2.5 TFA/TIS/H_2O) to the drained resin and this suspension was incubated for 1 hour, before draining the TFA into a centrifuge tube containing ice cold Et_2O (10 mL). The obtained suspension was kept on ice for 15 minutes followed by centrifugation. The supernatant was discarded and the pellet washed with Et_2O (2 mL). The crude peptide was transferred to a roundbottom flask with MeOH and concentrated *in vacuo*. This yielded 41.7 mg (15.2 µmol) of crude peptide **8**. **LC-MS** RT = 6.1 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1299.65 observed M/z = 1299.47

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys(TCO)-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (9)

The crude peptide **8** (15.2 µmol) was co-evaporated with toluene (3 x 1 mL) and put under N_2. TCO-OSu (1.1 eq, 4.5 mg, 16.7 µmol) and DiPEA (20 eq, 53 µL, 300 µmol) were dissolved in anhydrous DMF (1.5 mL) and this solution was added to the flask containing the crude peptide. After 2 hours, LC-MS analysis indicates full conversion to the TCO protected peptide. **LC-MS** RT = 6.8 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1375.70 observed M/z = 1375.67. This intermediate was then treated with a 1 M solution of PMe_3 in THF (10 eq, 160 µL, 0.16 mmol) followed by the addition of H_2O (100 eq, 29 µL, 1.6 mmol). After 1 hour LC-MS analysis indicated full conversion to the desired peptide. RP-HPLC followed by lyophilization yielded peptide **9** in 5.4 % yield (2.20 mg, 0.82 µmol). **LC-MS** RT = 5.9 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1349.71 observed M/z = 1349.53 **HRMS** (ESI) m/z: $[M + 2H^+]$ calcd $C_{121}H_{197}N_{29}O_{40}$ 1349.2209, found 1349.2212

Fmoc-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-AC-Tentagel S (10)

Tentagel S AC resin (100 µmol, 400 mg, 0.25 mmol/g) was swelled in DMF for 30 minutes. Fmoc-Leu-OH (4 eq, 141 mg, 0.4 mmol) was preactivated with DIC (6 eq, 92 µL, 0.6 mmol) in a 10 mM solution of DMAP in DMF for 1 minute. This solution was added to the drained resin and reacted overnight. The resin was washed with DMF (5 x 10 mL) and capped by treatment of a solution of 10% (v/v) Ac_2O and 5% (v/v) DiPEA in DMF (1 mL) containing 10 mM DMAP for 15 minutes. The resin was again washed (5 x 10 mL) and elongated using the general automated Fmoc-SPPS methods. **LC-MS** RT = 6.4 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1143.56 observed M/z = 1143.40.

N_3-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-OH (11)

Resin bound peptide **10** (25 µmol) was swelled in DMF, followed by removal of the Fmoc using standard conditions. The resin was thoroughly washed with water (3 x 5 mL). A solution containing ISA·H_2SO_4 (3 eq, 21 mg, 75 µmol) and Na_2CO_3 (4.5 eq, 12 mg, 113 µmol) in H_2O (1 mL) was added and the diazotranfer was allowed to proceed under gentle agitation for 16 hours. The resin was washed with H_2O (3 x 5 mL), MeOH (3 x 5 mL) and DCM (3 x 5 mL) before a TFA cocktail (1 mL, 95:2.5:2.5 TFA/TIS/H_2O) was added to the drained resin. This suspension was incubated for 1 hour, before draining the TFA into a centrifuge tube containing ice cold Et_2O (10 mL). The obtained suspension was kept on ice for 15 minutes followed by centrifugation. The supernatant was discarded and the pellet washed with Et_2O (2 mL). The crude peptide was transferred to a roundbottom flask with MeOH and

concentrated *in vacuo*. This yielded 47.6 mg (22.8 µmol) of the crude peptide **11**. **LC-MS** RT = 5.8 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1045.02 observed M/z = 1045.33.

Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys(TCO)-Leu-OH (12)

The crude peptide **11** (22.8 µmol) was co-evaporated with toluene (3 x 1 mL) and put under N_2. TCO-OSu (1.1 eq, 5.5 mg, 20.7 µmol) and DiPEA (10 eq, 33 µL, 190 µmol) were dissolved in anhydrous DMF (1.9 mL) and this solution was added to the flask containing the crude peptide. After 2 hours, LC-MS analysis indicates full conversion to the TCO protected peptide. **LC-MS** RT = 6.8 min (C18, 50-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1121.06 observed M/z = 1120.90. This intermediate was then treated with a 1 M solution of PMe_3 in THF (10 eq, 190 µL, 0.19 mmol) followed by the addition of H_2O (100 eq, 34 µL, 1.9 mmol). After 2 hours LC-MS analysis indicated full conversion to the desired peptide. RP-HPLC followed by lyophilization yielded peptide **12** in 1.4 % yield (0.70 mg, 0.32 µmol). **LC-MS** RT = 6.3 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1108.07 observed M/z = 1108.27 **HRMS** (ESI) m/z: $[M + 2H^+]$ calcd $C_{100}H_{159}N_{21}O_{35}$ 1108.0726, found

Fmoc-Cys(C_2H_4OPam)-Ser(tBu)-O-TEG-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Gly-Leu-Glu(OtBu)-Gln(Trt)-Leu-Glu(OtBu)-Ser(tBu)-Ile-Ile-Asn(Trt)-Phe-Glu(OtBu)-Lys(Boc)-Leu-Ala-Ala-Ala-Ala-Ala-Lys(N_3)-RAM-Tentagel S (16)

The building blocks **14** and **15** were synthesized as described in van de Ende et al.[44] and provided by M.M.E. Isendoorn (Leiden university). Resin-bound peptide **6** (25 µmol) was swelled in DMF and the Fmoc group was removed using the general method. The peptide was manually elongated with compound **14** (4 eq, 43 mg, 100 µmol) mediated by HCTU (4 eq, 200 µL of a 0.5 M solution in DMF) and DiPEA (8 eq, 35 µL, 200 µmol), reacted for 45 min. This was followed by Fmoc removal and the obtained resin-bound ester was condensed with Fmoc-Ser(tBu)-OH (4 eq, 38 mg, 100 µmol) mediated by DIC (6 eq, 23 µL, 150 µmol) in DMF (200 µL) containing 0.1 eq DMAP (12.5 µM), reacted for 1.5 h. The Fmoc was again removed and building block **15** (2 eq, 31 mg, 50 µmol) was coupled, mediated by HCTU (2 eq, 200 µL 0.25 M) and DiPEA (4 eq, 17.4 µL, 100 µmol) for 1.5 hour. The resin was washed with DMF (3 x 5 mL) and DCM (3 x 5 mL), drained, and stored at -20°C until further use. **LC-MS** RT = 9.8 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1728.89 observed M/z = 1729.20.

NH_2CO-Cys(C_2H_4OPam)-Ser-O-TEG-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(N_3)-NH_2 (17)

The resin bound peptide **16** was swelled in DMF for 30 minutes followed by Fmoc removal using the standard method. The N-terminal urea was introduced by treating the resin with a mixture of TMS-NCO (6 eq, 20 µL, 150 µmol) and isopropanol (12 eq, 22.8 µL, 300 µmol) in DCM (2.5 mL) for 2.5 hour. This step was repeated once to ensure full conversion. Next, a TFA cocktail was added (1 mL, 95:2.5:2.5 TFA/TIS/H_2O) to the drained resin and this suspension was incubated for 1 hour, before draining the TFA into a centrifuge tube containing ice cold Et_2O (10 mL). The obtained suspension was kept on ice for 15 minutes followed by centrifugation. The supernatant was discarded and the pellet washed with Et_2O (2 mL). The crude peptide was transferred to a roundbottom flask with MeOH and concentrated *in vacuo*. This yielded 33.6 mg (10.3 µmol) of crude peptide **17**. **LC-MS** RT = 8.4 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1639.36 observed M/z = 1639.60.

NH_2CO-Cys(C_2H_4OPam)-Ser-O-TEG-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys(TCO)-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (18)

The crude peptide **17** (10.3 µmol) was co-evaporated with toluene (3 x 1 mL) and put under N_2. TCO-OSu (1.2 eq, 3.2 mg, 12.4 µmol) and DiPEA (20 eq, 36 µL, 200 µmol) were dissolved in anhydrous DMF (1.0 mL) and this solution was added to the flask containing the crude peptide. After 2 hours, LC-MS analysis indicates full conversion to the TCO protected peptide. **LC-MS** RT = 7.8 min (C18, 50-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1715.41 observed M/z = 1715.67. This intermediate was then treated with a 1 M solution of PMe_3 in THF (10 eq, 100 µL, 0.10 mmol) followed by the addition of H_2O (100 eq, 18 µL, 1.0 mmol). After 1 hour LC-MS analysis indicated full conversion to the desired peptide. RP-HPLC followed by lyophilization yielded peptide **18** in 7.9 % yield (2.75 mg, 0.81 µmol). **LC-MS** RT = 8.6 min (C18, 10-90% B over 9 minutes) **LRMS** calcd $[M+2H]^{2+}$ = 1702.41 observed M/z = 1702.27 **HRMS** (ESI) m/z: $[M + 2H^+]$ calcd $C_{154}H_{256}N_{32}O_{51}S$ 1701.9144, found 1701.9153

T-cell activation assay

This assay was carried out as described in van de Gracht et al.[17]

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