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Design of azidoproline containing gluten peptides to suppress CD4+ T-cell responses associated with Celiac disease

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Abstract—Celiac disease is an intestinal disease caused by intolerance for gluten, a common protein in food. A life-long gluten-free diet is the only available treatment. As it is well established that the interaction between proline-rich gluten derived peptides and the human HLA-DQ2 molecules induces immune responses that lead to disease development, we have now designed a series of gluten peptides in which proline residues were replaced by azidoprolines. These peptides were found to bind to HLA-DQ2 with an affinity similar to that of the natural gluten peptide. Moreover, some of these peptides were found to be non-immunogenic and block gluten induced immune responses. These can thus serve as lead compounds for the development of HLA-DQ2 blocker peptides.

1. Introduction

Celiac disease (CD) is the most prevalent food-related disease in the Western World. CD is caused by a misdirected immune response towards gluten, storage proteins present in food sources such as wheat, barley and rye.[1] CD patients, estimated to encompass one percent of the total Western population, may suffer from a variety of symptoms, ranging from mild (chronic diarrhoea) to serious (growth retardation in children, neurological disorders). Fortunately, CD is not a life-threatening disease. Upon diagnosis, prescription of a gluten-free diet is usually sufficient to reverse the disease. Indeed, a life-long gluten-free diet is the only therapy for CD patients currently available, but living by the regime of a life-long gluten-free diet is burdensome as wheat and related cereals are very commonly used in the food industry. Moreover, the presence of gluten in food is not always obvious. Alternative therapies are therefore of interest, for instance drugs that suppress immune response elicited by gluten peptides, enabling CD patients to temporarily ingest gluten-containing foods.

The physiological processes that are at the basis of CD have been subject to intensive studies in recent years. Although we do not yet have a complete picture of all factors that contribute to CD, the outcome of these studies allows the design of strategies that may lead to new therapies. Briefly, the current view on how CD evolves is as follows. The relatively high occurrence of glutamine (up to 35% of the amino acid residues) and proline (up to 15%) in gluten proteins makes them relatively resistant to proteolytic activities.[2,3] As a consequence, ingestion of gluten-rich food leads to a relative abundance of gluten oligopeptides in the gut and gastrointestinal tract. Humans that are susceptible to CD have specific HLA alleles, either HLA-DQ2 or HLA-DQ8,[4,5] that weakly bind some gluten peptides. HLA-molecules play an essential role in immunity against pathogens. Their function is to bind peptides derived from proteins of (intracellular) pathogens and to display these peptides on the cell surface of infected cells in order to alert the immune system to the presence of such pathogens. Upon specific recognition of these HLA–peptide complexes by white blood cells an immune response is triggered to eliminate both the pathogen and infected cells. While the immune system should only respond to complexes

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between HLA-molecules and peptides derived from pathogens, occasionally it responds erroneously which can have grave consequences. Autoimmune diseases like type I diabetes and rheumatoid arthritis are most likely caused by immune responses towards HLA-molecules that have bound a tissue specific peptide, resulting in tissue specific damage. In the case of Celiac disease it is well established that an immune response to HLA-molecules that have bound harmless food derived peptides plays a critical role in disease development. The HLA–gluten peptide complexes are recognized by specific $CD4^+$ T-cells, leading to an undesired immune response. As a consequence, tissue damage of the small intestine occurs, which results in the release of intracellular tissue transglutaminase. As its physiological function, tissue transglutaminase catalyses the formation of isopeptide linkages between glutamine residues and lysine residues, thereby creating a covalent network of proteins that helps in repair of tissue damage. Confronted with a high concentration of glutamine-rich gluten peptides, tissue transglutaminase will take on specific glutamine residues and transform these into the corresponding glutamates.[6,7] As HLA-DQ2 and -DQ8 are known to preferentially bind peptides that harbour negatively charged amino acids, some of these glutamate-containing peptides bind to HLA-DQ2 or HLA-DQ8 with a much-enhanced affinity compared to their glutamine counterparts,[8–10] with an amplified immune response as a result.[6,7,11–16] From this sequence of events several potential therapeutic strategies appear feasible. The accelerated processing of gluten proteins can be achieved by the administration of proteases tailored to act on gluten proteins in the acidic environment of the gut.[3] Small molecule tissue transglutaminase inhibitors may partially suppress formation of toxic gluten epitopes and thereby prevent immune response directed against gluten peptides. Complete silencing of the undesired immune response may be effected by the design of compounds that have high affinity for CD-related HLA-DQ molecules and thereby prohibit T-cell recognition of the formed complexes. In our research after potential CD therapeutics we opted for the HLA-DQ blocker approach. We here report our first results in the design of gluten-based peptides that have good affinity for HLA-DQ2 and that, when bound, defy recognition by gluten-specific $CD4^+$ T-cell clones isolated from CD patients. Our strategy can be summarized as follows. In the first step we selected a gluten peptide that binds to HLA-DQ2 with high affinity. We then established which residues are essential for HLA-DQ2 recognition (the anchor residues) and which residues are accessible for chemical modification. These were subsequently substituted for the corresponding azide-modified amino acids that are then available for modification with a variety of functionalities that may help in prohibiting T-cell recognition, for instance by the introduction of steric bulk. The resulting peptides were then assessed on their ability to (a) bind to HLA-DQ2 and (b) block T-cell recognition. The most promising constructs were finally assessed on their down-modulating ability of gluten peptide elicited immune response in a competition experiment that includes a gluten peptide with high HLA-DQ2 affinity.

2. Results

2.1. Design of target compounds

We selected the gluten peptide QLQ**PFPQPELPY**PQ (**1**) as our starting point. The epitope (embedded in bold) in this peptide is a high affinity HLA-DQ2 binder.[17] Further, a high-resolution crystal structure of a complex of HLA-DQ2 and a related, truncated peptide (QLQPFPQPELPY) has recently become available.[18] From this structure it is apparent that the side-chain functionalities of the proline residues at positions P3 and P5 of the epitope are not involved in HLA-DQ2 binding and therefore amenable to substitution with either (4*S*)-4-azido-L-proline (**2**) or (4*R*)-4-azido-L-proline (**3**, Fig. 1), followed by functionalization.

Both the Fmoc-protected azidoprolines, which can be used in Fmoc-based SPPS, are readily accessible from commercially available (4*R*)-4-hydroxy-L-proline.[19–21]

2.2. Protection of the N-terminus of peptides and synthesis of target compounds

As our first research objective we set out to study the influence of a variety of caps that block the N-terminus in derivatives of **1** on HLA-DQ2 binding. It has long been established that the Fmoc-based solid phase synthesis of oligopeptides having a glutamine residue at its N-terminus is hampered by the formation of the corresponding N-terminal pyroglutamate during acidic cleavage from the resin (**4** and **5**, Scheme 1) and subsequent HPLC purification under standard (acidic) conditions.[22]

In order to circumvent this potential complication, we synthesized, by means of standard Fmoc-based solid phase peptide synthesis, N-terminal modified gluten peptides **6**–**10**. We found that these peptides bind to HLA-DQ2 with about equal affinity as gluten epitope α-9, a truncated version of gluten peptide **1** (Table 1).

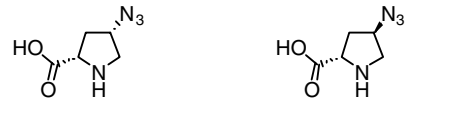
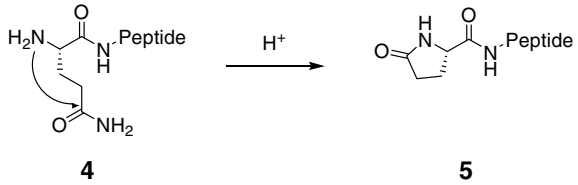
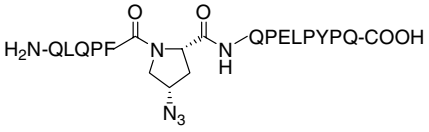
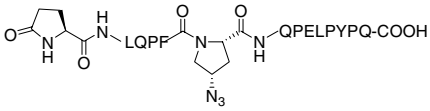
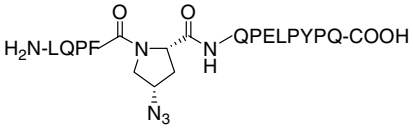
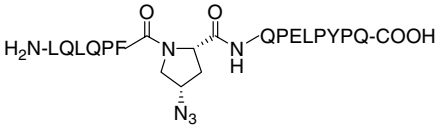
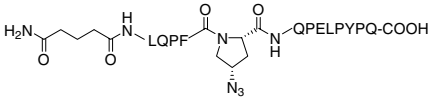
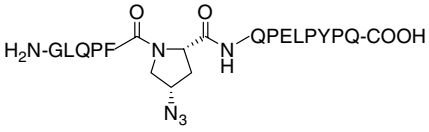


Figure 1. Azidoprolines used for modification of peptides.



Scheme 1. Pyroglutamate formation from glutamine under acidic conditions.

Table 1. HLA-DQ2 binding affinity of modified gluten peptides with modified N-termini

	Structure	IC_{50} (μm)
6		10.7
7		5.9
8		6.9
9		8.6
10		7.2
11		9.4
α-9	QLQPFPQPELPY	17.2

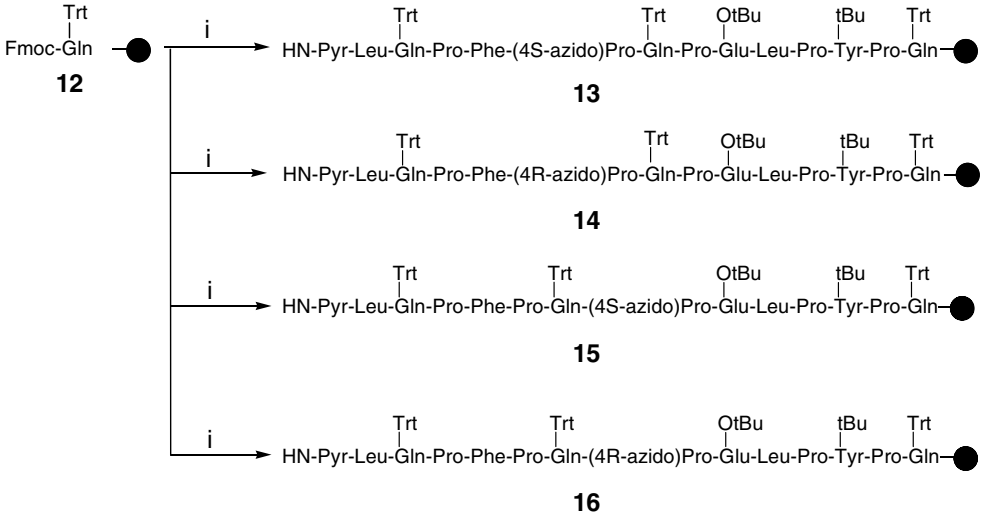
Based on these results and for practical purposes we chose to introduce a pyroglutamate residue as the replacement of the N-terminal glutamine residue in our ensuing studies.

Introduction of the diastereoisomeric set of Nα-F moc protected azidoprolines **2** and **3** (synthesized according to literature procedures)[19–21] at either p3 or p5 by means of Fmoc-based SPPS starting from loaded Wang resin **12** led to immobilized and fully protected peptides **13**–**16** (Scheme 2).

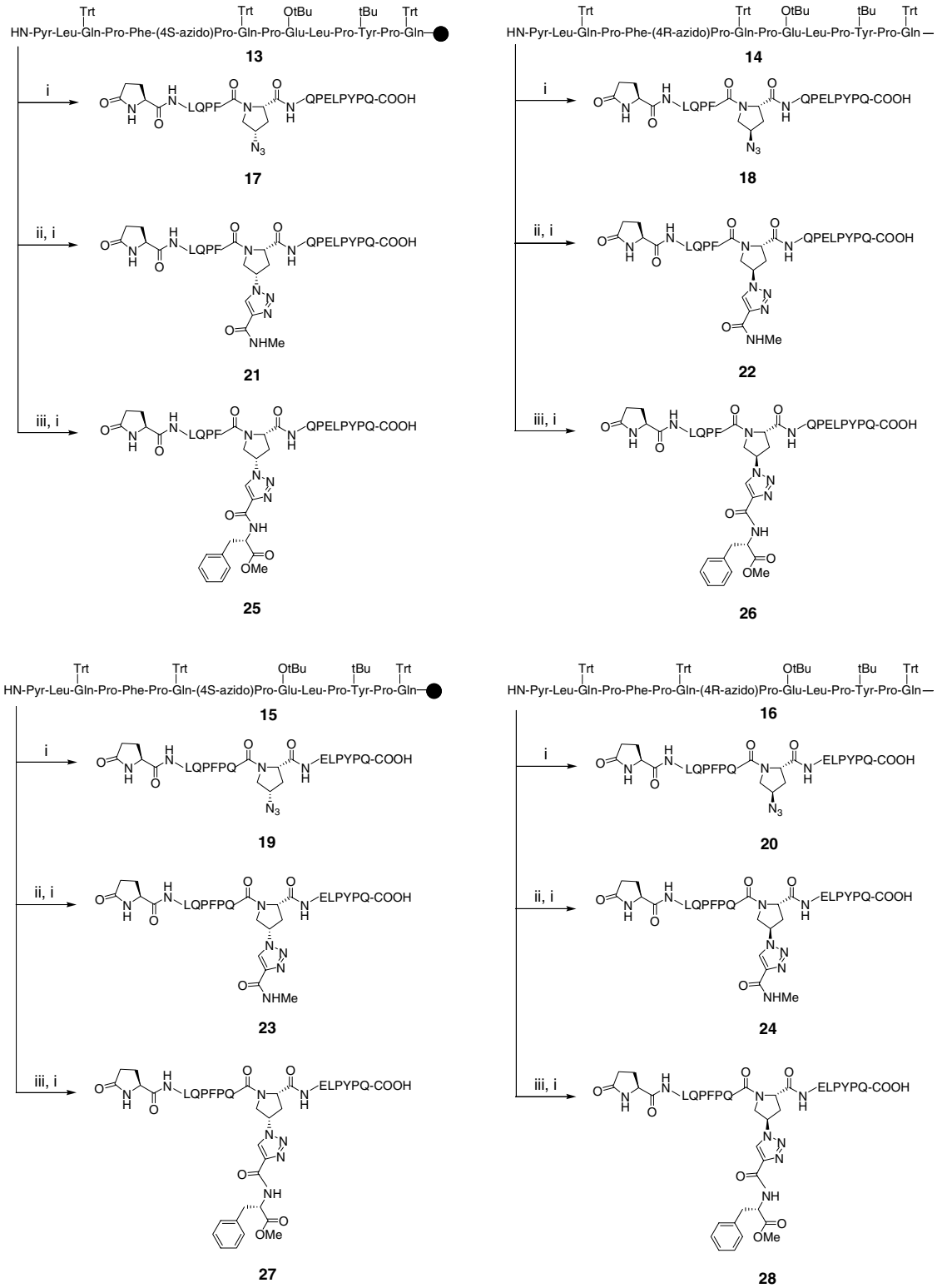
The immobilized peptides were divided into three equal portions. One portion of each immobilized compound was cleaved from the resin to give, after HPLC purification, azide-modified gluten epitopes **17**–**20**. The second portion of each was functionalized with *N*-methylacrylamide in an on-resin Cu(I)-catalysed Huisgens [2+3]-cycloaddition (the most famous of the 'click' reactions) to give modified gluten epitopes **21**–**24**. The third portion of each was modified using the same reaction, but with *N*-acryl-L-phenylalanine methyl ester as the acetylene component, yielding modified gluten epitopes **25**–**28** (Scheme 3). The whole sequence of SPPS/on-resin cycloaddition/cleavage/HPLC purification proceeded uneventfully, giving the homogeneous target compounds in yields ranging from 11% to 48%.

2.3. Assessment of HLA-DQ2 binding affinity and T-cell stimulation capability of the synthesized peptides

The affinity of the modified gluten epitopes **17**–**28** to bind to HLA-DQ2 was assessed in a competition assay. To this end, 96-well plates coated with HLA-DQ specific monoclonal antibodies were treated with lysate from EBV-transformed B-cells expressing HLA-DQ2. After removal of the unbound proteins the immobilized HLA-DQ2 molecules were exposed to one of the modified gluten epitopes **17**–**28** at different concentrations, together with a biotinylated peptide (biotin-EEPRAPWIEQEGPEYWDQE) as the reporter peptide. The amount of reporter peptide bound is detected by subsequent treatment with europium–streptavidin, followed by fluorescence readout. In this assay, enhanced binding of a modified peptide is reflected by reduced amounts of reporter peptide, with less fluorescence as a result.



Scheme 2. Synthesis of immobilized peptides **13**–**16** with one proline residue replaced by an azidoproline residue. Reagents and conditions: (i) Repeating cycle of (a) Fmoc-deprotection (20% piperidine in NMP), 15 min; (b) coupling of amino acids: 5 equiv Fmoc-aa-OH, 5 equiv HCTU, 10 equiv DIPEA, 2 h; (c) capping: 0.5 M acetic anhydride, 0.125 M DIPEA in NMP, 5 min.



Scheme 3. Generation of modified gluten peptides **17**–**28** with functionalized prolines. Reagents and conditions: (i) TFA/H2O/TIS, 95:2.5:2.5 (v/v/v), 2 h; (ii) 4 equiv *N*-methylacrylamide, 4 equiv CuI, 20% DIPEA/NMP (v/v), 48 h; (iii) 4 equiv *N*-acryl-L-phenylalanine methyl ester, 4 equiv CuI, 20% DIPEA/NMP (v/v), 48 h.

The binding affinity of compounds **17**–**28** in general appeared to be in the same range as the parent peptide epitope, but there are some differences in potency (Table 2). For instance, whereas peptide **25** displays the highest affinity, the diastereomer **26** is the weakest binder of this series. This might result from a 'steric clash' of the large side chain of the peptide with the surroundings of the HLA-DQ2 binding pocket. Another interesting finding is that the compounds modified with *N*-methylacrylamide appear to be worse HLA-DQ2 binders than both

Table 2. Binding affinities for HLA-DQ2 of modified gluten peptides **17**–**28**

Peptide	IC_{50} (μM)
17	3.2
18	5.5
19	2.4
20	2.3
21	4.6
22	5.8
23	9.0
24	5.8
25	***1.3***
26	***29.2***
27	2.3
28	3.2
α-9	2.5

Each peptide was tested in at least three independent experiments. The averaged results of all experiments are shown. The peptides with the highest and lowest binding affinity, are indicated by italicized bold numbers.

the azide precursor and the phenylalanine-modified analogue (compare for instance the binding affinities of **19**, **23** and **27**). Additionally, those peptides derived from p3-*cis*-azidoproline show higher binding affinities for HLA-DQ2 than their *trans*-azidoproline counterparts (compare for instance **17** and **18**).[23]

All modified peptides were subsequently assessed for their T-cell stimulating activity. For this purpose, HLA-DQ2 expressing cells were mixed with T-cell clones specific for the α-9-gluten peptide (α-9 = QLQPFPQPELPY), in the presence of 33 μM modified peptide or 33 μM α-9-gluten peptide, respectively. After incubation, ^{3}H-labelled thymidine was added to the mixture. As this radioactive DNA base is incorporated in dividing cells, the radioactivity measured directly indicates the amount of T-cells which have proliferated upon stimulation with modified peptides.

The experiment was carried out with T-cell clones from several human donors that are all CD patients. As can be seen in Figure 2, each donor displays a different stimulation pattern. This is not unexpected, as it is known that CD patients can respond to a wide variety of gluten epitopes and not every patient responds equally to the same set of epitopes. The position of the modifications, either on p3 or p5 of the epitope, does not make much difference in T-cell stimulating activity (only one of the four T-cell clones displays better inhibition by p3- than p5 substituted peptides). In general, however, the peptides resulting from *cis*-azidoproline incorporation show less T-cell stimulating activity than their *trans*-azidoproline counterparts (compare, for instance, **17** and **18**, or **21** and **22**). Also, peptides containing large side chains display the least T-cell stimulating activity.

Overall, peptide **25** turns out to be the best lead for further studies as this peptide combines a high affinity for HLA-DQ2 with almost no T-cell stimulating activity.

2.4. Assessment of HLA-DQ2 blocking capability of a modified gluten peptide

Although the abovementioned results look promising, nothing can be said yet about the ability of these peptides to act as a blocker for HLA-DQ2. The T-cell proliferation assays were performed in the absence of natural gluten peptides, but a 'blocker' should be able

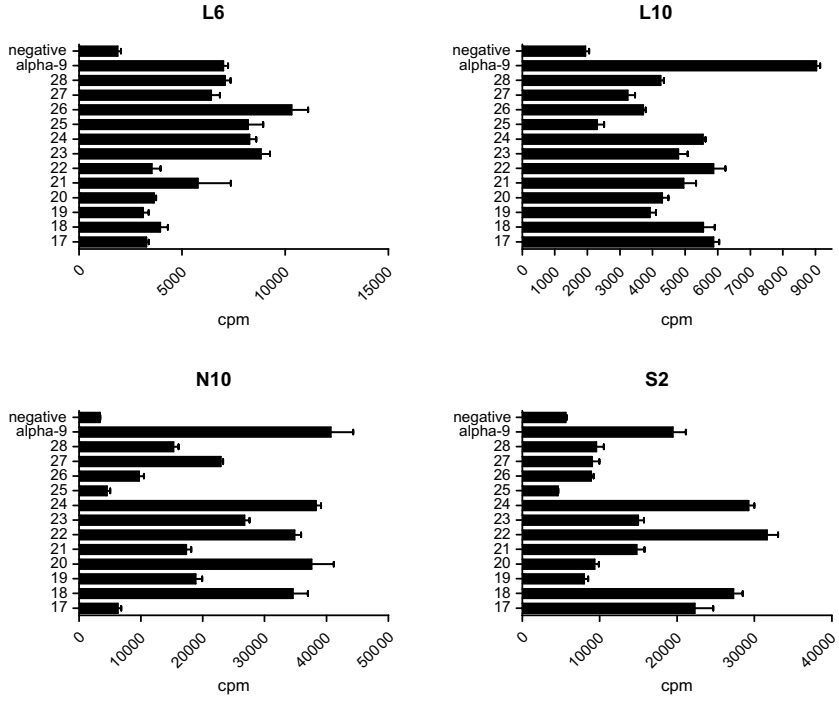


Figure 2. T-cell stimulatory activity of modified gluten peptides **17**–**28**. T-cells were stimulated with 33 μM of the respective peptide. The data shown are from one representative experiment that was repeated three times.

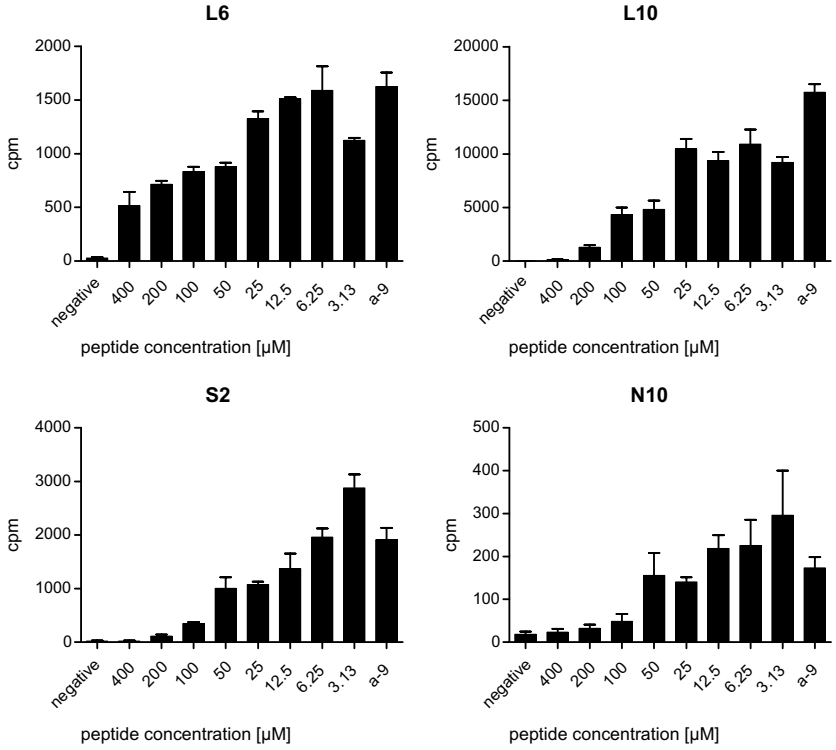


Figure 3. Blocking of gluten peptide binding by modified gluten peptide **25**. T-cells were stimulated with 4 μM α-9 peptide, which represents a suboptimal concentration. The data shown are from one representative experiment that was repeated three times.

to compete with those same gluten peptides in such a way that no gluten peptides can bind to HLA-DQ2. Therefore, T-cell proliferation experiments were performed, using lead peptide **25** to block T-cell responses in the presence of natural gluten peptide α-9 in a suboptimal concentration of 4 μM to allow blocking effects to be seen. Due to the low concentration of α-9 the cpm values for most of the clones are much lower than in the previous experiments. As can be seen in Figure 3, peptide **25** is indeed able to block T-cell proliferation in the presence of α-9, although still at fairly high concentrations (200–400 μM). This can be explained by the fact that peptide **25** shows an affinity for HLA-DQ2 in the same micromolar range as the α-9 peptide.

3. Discussion and conclusion

In summary, we have demonstrated that blocking of HLA-DQ2-specific T-cell proliferation has potential as a therapeutic strategy to treat CD patients. A similar strategy has been reported by Sollid and coworkers.[24] Gluten peptides can be modified at specific positions without impairing their affinity for HLA-DQ2. Depending on the nature of the modification HLA-DQ2-specific T-cell proliferation can be prohibited both in the absence and in the presence of native gluten peptides. Clearly, the ability of our best compound to compete with the unmodified peptide is not nearly good enough to proceed with our current constructs in a drug development programme. Most likely, the binding affinity of an effective T-cell blocker for HLA-DQ2 should be around 30-fold better. We, and others,[24,25] are currently searching for such peptides. Once these are identified we will adapt our strategy of introducing azide functionalities at predetermined sites, followed by [2+3]-cycloaddition, with the ultimate goal to arrive at modified peptides that can compete with native gluten peptides for HLA-DQ2 and, once bound, prohibit recognition by HLA-DQ2-specific T-cells.

4. Experimental

4.1. General

Reagents and solvents were used as provided, unless stated otherwise. Peptides were synthesized on solid support (Wang resin, NovaBiochem, 100–200 Mesh, theoretical loading 1.1 mmol g^{-1}) on a CS 336 system 3 peptide synthesizer using Fmoc-based peptide synthesis methods and commercially available Fmoc amino acids.

LC/MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 and 254 nm) coupled to a Perkin Elmer Sciex API 165 mass instrument with a custom-made Electronspray Interface (ESI). An analytical Gemini C_{18} column (Phenomenex, 50×4.60 mm, 3 μm) was used in combination with buffers A: H2O, B: MeCN and C: 1.0% aq TFA.

For RP-HPLC purification of the peptides, a Gilson GX-281 automated HPLC system (Gilson), supplied with a semi-preparative Gemini C_{18} column (Phenomenex, 150×21.2 mm, 5 μm) or a BioCAD 'Vision'

automated HPLC system (PerSeptiveBiosystems, Inc.) supplied with a semi-preparative Gemini C_{18} column (Phenomenex, 150 × 21.2 mm, 5 μm), were used. The applied buffers were A: H2O, B: MeCN and C: 0.1 M ammonium acetate or 1.0% aq TFA.

HRMS was measured on a Finnigan LTQ Orbitrap system.

Peptides **6**–**11** and **17**–**28** adopt multiple conformers in solution as observed in their ^{13}C and ^{1}H NMR spectra (Bruker DMX-600, 9:1 H_2O/D_2O). This complicated the accurate assignment of the NMR data and in view of this the NMR data are not included in the manuscript.

4.2. Peptide synthesis

4.2.1. First residue attachment (12). Wang resin (1.2 g) was swollen in dichloromethane. After removal of the solvent, a solution of Fmoc-Gln(Trt)-OH (3.66 g, 6 mmol) and *N*,*N*′-dimethylaminopyridine (DMAP, 73.3 mg, 0.6 mmol) in 30 mL of 1:1 (v/v) *N*-methyl-2-pyrrolidinone (NMP)/DCM was added to the resin. *N*,*N*′-diisopropylcarbodiimide (DIC, 0.93 mL, 6 mmol) was added and the mixture shaken for 16 h.

Solvents were removed, the resin was washed (NMP) and dried (DCM). The loading of **12** was determined to be 0.68 mmol g^{-1}.

4.2.2. Elongation of peptides. Repeated coupling cycles consisting of (a) Fmoc-deprotection (20% piperidine in NMP, 15 min). (b) amino acid coupling (five equivalents of Fmoc-protected amino acid, five equivalents of HCTU as coupling reagent, 10 equivalents of diisopropylethylamine (DIPEA) as base, 2 h coupling time, single couplings) and (c) capping (0.5 M acetic anhydride, 0.125 M DIPEA in NMP, 5 min) were used for elongation of the peptide chain.

The azidoproline building blocks were manually incorporated (four equivalents of Fmoc-(4*R*)-4-hydroxy-L-proline or Fmoc-(4*S*)-4-hydroxy-L-proline, four equivalents of PyBOP, eight equivalents of DIPEA, 16 h coupling time), after which the remaining amino acids were attached in an automated fashion.

N-terminal pyroglutamate residues were manually attached using four equivalents of L-pyroglutamic acid ((5*S*)-2-pyrrolidone-5-carboxylic acid), four equivalents of PyBOP, four equivalents of *N*-hydroxybenzotriazole, eight equivalents of DIPEA and double coupling, 3 h per coupling.

The following commercially available amino acid building blocks were used: Fmoc-Pro-OH, Fmoc-Tyr (^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Phe-OH, and Fmoc-Gly-OH.

Manual incorporation of standard amino acids was performed similar to attachment of Fmoc-azidoprolines, using a shorter coupling time of 3 h.

4.2.3. Attachment of side chains via 'click' chemistry, general procedure. Dry resin **13** (load 0.26 mmol g^{-1}, 77.5 mg, 20.2 μmol peptide) was washed three times with NMP. A suspension of *N*-methylacrylamide (6.7 mg, 81 μmol), copper iodide (15.4 mg, 81 μmol) in 640 μL of 20% DIPEA in NMP was added to the resin and the mixture shaken for 48 h. Solvents were removed and the resin washed with NMP.

4.2.4. Cleavage of peptides from resin and purification. If necessary, the Fmoc-protecting group was removed prior to cleavage by shaking the resin in 20% piperidine in NMP (v/v) for 15 min, followed by washing four times with NMP. Peptides were cleaved from the resin using 95:2.5:2.5 (v/v/v) TFA/water/tri-isopropylsilane (shaking for 2 h) and precipitated in a cold 1:1 (v/v) pentane/diethyl ether solution. After centrifugation, the supernatant was removed, the peptide pellet was resuspended in fresh diethyl ether and spinned down again. This was repeated two times to remove final traces of TFA. The final pellet was air-dried, analysed by LC/MS and purified by RP-HPLC. The fractions containing the pure product were concentrated and freeze-dried to yield the pure peptides.

4.3. Peptide binding assay

A binding assay was performed essentially as described previously applying minor modifications.[17] Ninety-six well FluoroNunc™ plates were coated with the HLA-DQ-specific monoclonal antibody SPV-L3, 1 μg/well in 100 μL of carbonate buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6) for 2 h at 37 °C, subsequently blocked for 2 h at 37 °C with 1% BSA in PBS.

HLA-DR3/DQ2 positive EBV-transformed B-cells were lysed in 50 mM Tris–HCL, 150 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, 0.5% NP-40 and protease inhibitor mix (Complete™, Roche) at 4 °C, at a concentration of 4×10^6 cells per 1 mL lysis buffer. Subsequently, nuclei and cell debris was removed by centrifugation (4 °C, 2000*g*, 15 min). Such prepared lysates were mixed with an equal volume of ice-cold 1% solution of BSA in PBS and 100 μL aliquots were pipetted into the SPV-L3 coated wells. After overnight incubation at 4 °C the plates were washed and 50 μL binding buffer (0.1% NP-40, 0.1% Tween, 33.6 mM citric acid, 72 mM Na_2HPO_4, pH 5.5, and Complete™ protease inhibitor mix) was added to each well. A titration range of peptides to be tested (concentration range 600–0.04 μM) were prepared in 10% DMSO containing a fixed amount of the biotin-labelled indicator peptide EEPRAPWIEQEGPEYWDQE (EPRAP), if not otherwise mentioned) at a concentration of 600 nM. Subsequently, 50 μL of the samples was applied to the SPV-L3/HLA-DQ2 coated plates. Following a 48-h incubation at 37 °C each well was washed extensively. Subsequently, 100 μL of 100 nM streptavidin–europium in assay buffer (both Wallac) was added and incubated for 45 min at RT. After extensive washing, 150 μL/well of enhancement solution (Wallac) was applied and the plates were read in a time resolved fluorimeter (1234, Wallac) 15–30 min thereafter. IC_{50} values were calculated

based on the observed competition between the test peptides and biotin-labelled indicator peptides and indicate the concentration of the tested peptide required for half maximal inhibition of the binding of the indicator peptide. Experiments were carried out in triplicate.

4.4. T-cell proliferation assays

Proliferation assays were performed in triplicate in 150 μL RPMI supplemented with 10% human serum in 96-well, flat-bottom plates using 10,000 α-9-specific T-cells stimulated with 100,000 irradiated HLA-DQ2-matched allogenic PBMCs, in the presence of 33 μM modified peptide or 33 μM α-9-gluten peptide (α-9 = QLQPFPQPELPY), respectively.

After 48 h at 37 °C, cultures were pulsed with 0.5 μCi of 3H-thymidine and harvested 18 h later.[11]

Competition assays were performed as above, but using increasing concentrations of lead peptide **25** in the presence of natural gluten peptide α-9 in a suboptimal concentration of 4 μM.

4.5. Analytical data for synthesized peptides

4.5.1. Peptide 6. Following the general procedure for peptide synthesis and cleavage, **6** was obtained from **12** as a white solid (3 mg, 1.7 μmol, 18%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 5.93 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 862.4 $[M+2H]^{2+}$; 1723.2 $[M+H]^{+}$. Exact mass: calculated for $[C_{80}H_{116}N_{21}O_{22}Na]^{2+}$: 872.9245. Found: 872.9211 $[M+H+Na]^{2+}$.

4.5.2. Peptide 7. Following the general procedure for peptide synthesis and cleavage, **7** was obtained from **12** as a white solid (3.4 mg, 2.0 μmol, 19%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.54 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 853.4 $[M+2H]^{2+}$; 1706.2 $[M+H]^{+}$. Exact mass: calculated for $[C_{80}H_{113}N_{20}O_{22}Na]^{2+}$: 864.4113; $[C_{80}H_{112}N_{20}O_{22}Na_2]^{2+}$: 875.4022. Found: 864.4171 $[M+H+Na]^{2+}$; 875.4071 $[M+2Na]^{2+}$.

4.5.3. Peptide 8. Following the general procedure for peptide synthesis and cleavage, **8** was obtained from **12** as a white solid (4.46 mg, 2.8 μmol, 38%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/1% aq TFA for purification.

LC/MS retention time: 5.89 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 798.0 $[M+2H]^{2+}$; 1595.4 $[M+H]^{+}$. Exact mass: calculated for $[C_{75}H_{108}N_{19}O_{20}Na]^{2+}$: 808.8952; $[C_{75}H_{107}N_{19}O_{20}Na_2]^{2+}$: 819.8862. Found: 808.8947 $[M+H+Na]^{2+}$; 819.8863 $[M+2Na]^{2+}$.

4.5.4. Peptide 9. Following the general procedure for peptide synthesis and cleavage, **9** was obtained from **12** as a white solid (3.82 mg, 2.1 μmol, 17%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/1% aq TFA for purification.

LC/MS retention time: 6.25 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 918.8 $[M+2H]^{+}$; 1836.6 $[M+H]^{+}$. Exact mass: calculated for $[C_{86}H_{127}N_{22}O_{23}Na]^{2+}$: 929.4666. Found: 929.4656 $[M+H+Na]^{2+}$.

4.5.5. Peptide 10. Following the general procedure for peptide synthesis and cleavage, **10** was obtained from **12** as a white solid (2.88 mg, 1.69 μmol, 17%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/1% aq TFA for purification.

LC/MS retention time: 6.48 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 854.8 $[M+2H]^{2+}$; 1708.2 $[M+H]^{+}$. Exact mass: calculated for $[C_{80}H_{115}N_{20}O_{22}Na]^{2+}$: 865.4191; $[C_{80}H_{114}N_{20}O_{22}Na_2]^{2+}$: 876.4101. Found: 865.4168 $[M+H+Na]^{2+}$; 876.4049 $[M+2Na]^{2+}$.

4.5.6. Peptide 11. Following the general procedure for peptide synthesis and cleavage, **11** was obtained from **12** as a white solid (4.83 mg, 2.9 μmol, 29%), using an HPLC gradient of 70:20:10 → 40:50:10 H_2O/MeCN/1% aq TFA for purification.

LC/MS retention time: 5.93 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 826.8 $[M+2H]^{2+}$; 1652.4 $[M+H]^{+}$. Exact mass: calculated for $[C_{77}H_{111}N_{20}O_{21}Na]^{2+}$: 837.4060. Found: 837.4083 $[M+H+Na]^{2+}$.

4.5.7. Peptide 17. Following the general procedure for peptide synthesis and cleavage, **17** was obtained from **13** (load 0.26 mmol g^{-1}, 76.9 mg, 20 μmol) as a white solid (16.3 mg, 9.6 μmol, 48%), using an HPLC gradient of 68:22:10 → 60:30:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.49 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 853.6 $[M+2H]^{2+}$; 1706.4 $[M+H]^{+}$. Exact mass: calculated for $[C_{80}H_{114}N_{20}O_{22}]^{2+}$: 853.4203. Found: 853.4193 $[M+2H]^{2+}$.

4.5.8. Peptide 18. Following the general procedure for peptide synthesis and cleavage, **18** was obtained from **14** (load 0.25 mmol g^{-1}, 75.3 mg, 18.8 μmol) as a white solid (11.1 mg, 6.5 μmol, 34%), using an HPLC gradient of 70:20:10 → 60:30:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.43 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 853.8 $[M+2H]^{2+}$; 1706.2 $[M+H]^{+}$. Exact mass: calculated for $[C_{80}H_{114}N_{20}O_{22}]^{2+}$: 853.4203. Found: 853.4191 $[M+2H]^{2+}$.

4.5.9. Peptide 19. Following the general procedure for peptide synthesis and cleavage, **19** was obtained from **15** (load 0.27 mmol g^{-1}, 74.5 mg, 20.1 μmol) as a white solid (11.81 mg, 6.9 μmol, 34%), using an HPLC gradi-

ent of 68:22:10 → 59:31:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.58 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 853.2 $[M+2H]^{2+}$; 1706.4 $[M+H]^+$. Exact mass: calculated for $[C_{80}H_{114}N_{20}O_{22}]^{2+}$: 853.4203. Found: 853.4190 $[M+2H]^{2+}$.

4.5.10. Peptide 20. Following the general procedure for peptide synthesis and cleavage, **20** was obtained from **16** (load 0.27 mmol g^{-1}, 74.6 mg, 20.1 μmol) as a white solid (10.48 mg, 6.1 μmol, 31%), using an HPLC gradient of 68:22:10 → 60:30:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.80 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 853.8 $[M+2H]^{2+}$; 1706.4 $[M+H]^+$. Exact mass: calculated for $[C_{80}H_{114}N_{20}O_{22}]^{2+}$: 853.4203; $[C_{80}H_{113}N_{20}O_{22}Na]^{2+}$: 864.4113. Found: 853.4193 $[M+2H]^{2+}$; 864.4098 $[M+H+Na]^{2+}$.

4.5.11. Peptide 21. Following the general procedure for peptide synthesis and cleavage, **21** was obtained from **13** (load 0.26 mmol g^{-1}, 77.5 mg, 20.2 μmol) as a white solid (10.9 mg, 6.1 μmol, 30%), using an HPLC gradient of 71:19:10 → 60:30:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.13 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 895.4 $[M+2H]^{2+}$; 1789.4 $[M+H]^+$. Exact mass: calculated for $[C_{84}H_{119}N_{21}O_{23}]^{2+}$: 894.9388; $[C_{84}H_{118}N_{21}O_{23}Na]^{2+}$: 905.9298. Found: 894.9376 $[M+2H]^{2+}$; 905.9285 $[M+H+Na]^{2+}$.

4.5.12. Peptide 22. Following the general procedure for peptide synthesis and cleavage, **22** was obtained from **14** (load 0.25 mmol g^{-1}, 75.1 mg, 18.8 μmol) as a white solid (13.89 mg, 7.8 μmol, 41%), using an HPLC gradient of 71:19:10 → 61:29:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 5.98 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 895.4 $[M+2H]^{2+}$; 1789.2 $[M+H]^+$. Exact mass: calculated for $[C_{84}H_{119}N_{21}O_{23}]^{2+}$: 894.9388. Found: 894.9373 $[M+2H]^{2+}$.

4.5.13. Peptide 23. Following the general procedure for peptide synthesis and cleavage, **23** was obtained from **15** (load 0.27 mmol g^{-1}, 75 mg, 20.2 μmol) as a white solid (8.73 mg, 4.9 μmol, 24%), an HPLC gradient of 71:19:10 → 62:28:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 5.99 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 895.2 $[M+2H]^{2+}$; 1789.2 $[M+H]^+$. Exact mass: calculated for $[C_{84}H_{119}N_{21}O_{23}]^{2+}$: 894.9388. Found: 894.9366 $[M+2H]^{2+}$.

4.5.14. Peptide 24. Following the general procedure for peptide synthesis and cleavage, **24** was obtained from **16** (load 0.27 mmol g^{-1}, 74 mg, 20 μmol) as a white solid (6.57 mg, 3.7 μmol, 18%), an HPLC gradient of 71:19:10 → 63:27:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 6.0 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 895.4 $[M+2H]^{2+}$; 1789.2 $[M+H]^+$. Exact mass: calculated for $[C_{84}H_{119}N_{21}O_{23}]^{2+}$: 894.9388; $[C_{84}H_{118}N_{21}O_{23}Na]^{2+}$: 905.9298. Found: 894.9374 $[M+2H]^{2+}$; 905.9287 $[M+H+Na]^{2+}$.

4.5.15. Peptide 25. Following the general procedure for peptide synthesis and cleavage, **25** was obtained from **13** (load 0.26 mmol g^{-1}, 77.4 mg, 20.1 μmol) as a white solid (4.59 mg, 2.4 μmol, 12%), using an HPLC gradient of 63:27:10 → 51:39:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 8.24 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 969.4 $[M+2H]^{2+}$; 1937.2 $[M+H]^+$. Exact mass: calculated for $[C_{93}H_{127}N_{21}O_{25}]^{2+}$: 968.9651. Found: 968.9637 $[M+2H]^{2+}$.

4.5.16. Peptide 26. Following the general procedure for peptide synthesis and cleavage, **26** was obtained from **14** (load 0.25 mmol g^{-1}, 63.2 mg, 15.8 μmol) as a white solid (3.53 mg, 1.8 μmol, 12%), using an HPLC gradient of 63:27:10 → 52:38:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 7.95 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 969.8 $[M+2H]^{2+}$; 1937.2 $[M+H]^+$. Exact mass: calculated for $[C_{93}H_{127}N_{21}O_{25}]^{2+}$: 968.9651; $[C_{93}H_{126}N_{21}O_{25}Na]^{2+}$: 979.9560. Found: 968.9637 $[M+2H]^{2+}$; 979.9532 $[M+H+Na]^{2+}$.

4.5.17. Peptide 27. Following the general procedure for peptide synthesis and cleavage, **27** was obtained from **15** (load 0.27 mmol g^{-1}, 74.6 mg, 20.1 μmol) as a white solid (4.14 mg, 2.1 μmol, 11%), using an HPLC gradient of 63:27:10 → 51:39:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 8.03 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 969.6 $[M+2H]^{2+}$; 1937.2 $[M+H]^+$. Exact mass: calculated for $[C_{93}H_{127}N_{21}O_{25}]^{2+}$: 968.9651. Found: 968.9634 $[M+2H]^{2+}$.

4.5.18. Peptide 28. Following the general procedure for peptide synthesis and cleavage, **28** was obtained from **16** (load 0.27 mmol g^{-1}, 74.5 mg, 20.1 μmol) as a white solid (4.53 mg, 2.3 μmol, 12%), using an HPLC gradient of 63:27:10 → 51:39:10 H_2O/MeCN/0.1 M NH_4OAc for purification.

LC/MS retention time: 8.08 min (10–50% MeCN, 13.5 min run). Mass (ESI): *m/z* 969.4 $[M+2H]^{2+}$; 1937.2 $[M+H]^+$. Exact mass: calculated for $[C_{93}H_{127}N_{21}O_{25}]^{2+}$: 968.9651. Found: 968.9638 $[M+2H]^{2+}$.

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