Towards peptide based therapeutics-applications in celiac disease and infectious diseases

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Chapter 4

Modification of a High Affinity HLA-DQ2 Binding Peptide

Manuscript in preparation:
Kapoerchan, V. V.; Overhand, M.; Van der Marel, G. A.; Koning, F.; Overkleeft, H. S. 'The development of highly efficient HLA-DQ2 blockers by the modification of high affinity HLA-DQ2 binding peptides'.

Introduction

Celiac disease (CD) is the most prevalent food-related disease in the Western world. CD patients may suffer from chronic diarrhea and neurological disorders and in children, growth retardation is often observed.[1] These symptoms are caused by a misdirected immune response towards dietary gluten. The only therapy available today is adhering to a gluten-free diet, but this diet regime is burdensome and has a large impact on the quality of life.[2] Furthermore, the presence of gluten in food is not always obvious and so-called 'gluten-free' products sometimes still contain traces of gluten.[3] Therefore, alternative therapies to prevent the immune response elicited by gluten are of interest. One of the key elements in the pathogenesis of CD is the HLA-DQ2 protein, a MHC class II molecule that binds to certain peptides produced after proteolytic cleavage of the gluten proteins. This peptide/HLA-DQ2 complex is then recognized by gluten-specific $CD4^+$ T cells, leading to an immune response. A possible strategy to prevent this process is blocking of the HLA-DQ2 molecule with a peptide that has a higher binding affinity for HLA-DQ2 than the gluten peptides, but when in complex is not recognized by the $CD4^+$ T cells.

In Chapter 2, it was shown that a peptide derived from a known antigenic gluten peptide could be modified with a large side chain and that this peptide was not

recognized by $CD4^+$ T cells when in complex with HLA-DQ2. However, this peptide does not represent an optimal blocker. Because of the comparable binding affinity of the synthetic peptide with the native α-9, it is not able to completely prevent the binding of gluten peptides to HLA-DQ2, which means that the gluten-directed immune response cannot be inhibited completely. Therefore, the same strategy as described in Chapter 2 should be applied to a peptide with a higher binding affinity for HLA-DQ2, which could lead to more efficient blockers for this protein. In this Chapter, three high affinity peptide sequences are modified *via* the incorporation of azide containing amino acids as described in Chapter 2. The binding affinity of these peptides is determined.

Design and synthesis of target peptides and building blocks

In Chapter 3, peptide sequence **4** (Table 1) was identified with a much higher affinity (~60 times) for HLA-DQ2 as compared to the natural α-9 peptide. Two other peptide sequences found, **2** and **3**, also displayed higher affinity for HLA-DQ2.

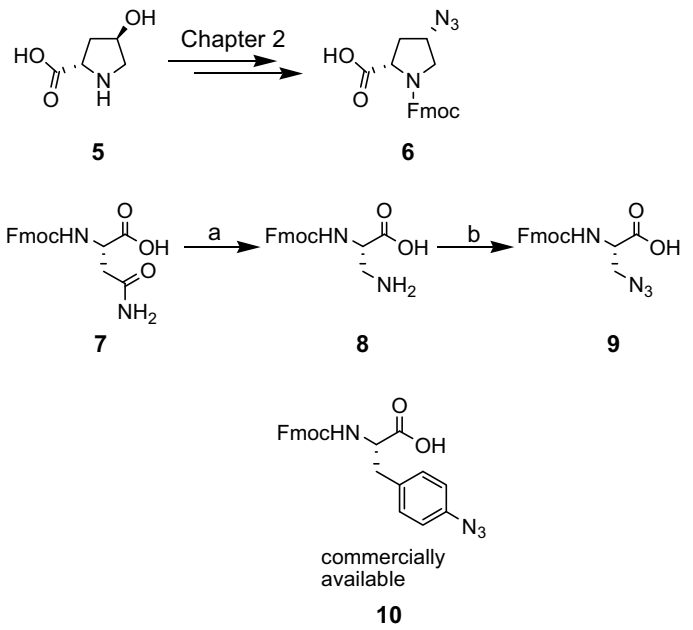
Table 1 The final high affinity peptides **2**-**4** identified compared to the gluten sequence **1** used in Chapter 2. Residues to be modified are indicated in bold.

Peptide				*p1*	*p2*	*p3*	*p4*	*p5*	*p6*	*p7*	*p8*	*p9*		
1	Pyr[a]	L	Q	P	F	**P**	Q	P	E	L	P	Y	P	Q
2	A	D	A	F	V	**A**	E	Y	E	P	V	L	A	A
3	A	D	A	F	R	**A**	E	Y	E	E	W	L	A	A
4	A	D	A	Y	D	**Y**	E	S	E	E	L	F	A	A

[a] Pyr = L-Pyroglutamic acid

With the three new sequences in hand, modifications were planned as follows. As described in Chapter 2, the amino acid occupying the p3 position can be replaced with its corresponding azide containing analog. Then, the azide functionality was modified by means of a Cu(I) catalyzed Huisgen cycloaddition between an azide and a functionalized alkyne, yielding a 1,4-disubstituted triazole.[4] Thus, peptide **1** was modified with Fmoc-(*4S*)-4-azido-L-proline **6** (Scheme 1). Fmoc-azido-L-alanine (**9**) and Fmoc-4-azido-L-phenylalanine (**10**) would replace the Ala residue in **2** and **3** and the Tyr residue in **4**, respectively. Azidoproline **6** was synthesized from (*4R*)-4-hydroxy-L-proline **5** as described in Chapter 2 and Fmoc-azido-L-alanine **9** was prepared by performing a diazotransfer on Fmoc-Dpr-OH (**8**), which in turn was prepared via a Hofmann rearrangement of Fmoc-L-asparagine **7** (Scheme 1).[5] Fmoc-4-azido-L-phenylalanine **10** is commercially available.

Scheme 1 Azido-amino acids used as replacement for natural amino acids.

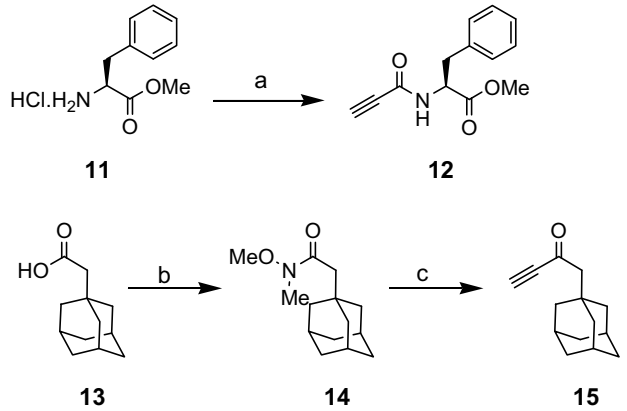


5 **6** **7** **8** **9** **10**

Reagents and conditions: a) bis(trifluoroacetoxy)iodobenzene, pyridine, MeCN/DMF/H_2O (1:2:1), rt, 70h, 67%. b) Imidazole-1-sulfonyl azide.HCl, Et_3N, $CuSO_4$, MeOH, rt, 3 h, 95%.

As the 'click' reaction gives the best results using electron-poor alkynes,[6] ynones **12** and **15** (Scheme 2) were chosen for modifying the azide functionality.

Scheme 2 Synthesis of ynones used for the 'click' reaction.



11 **12** **13** **14** **15**

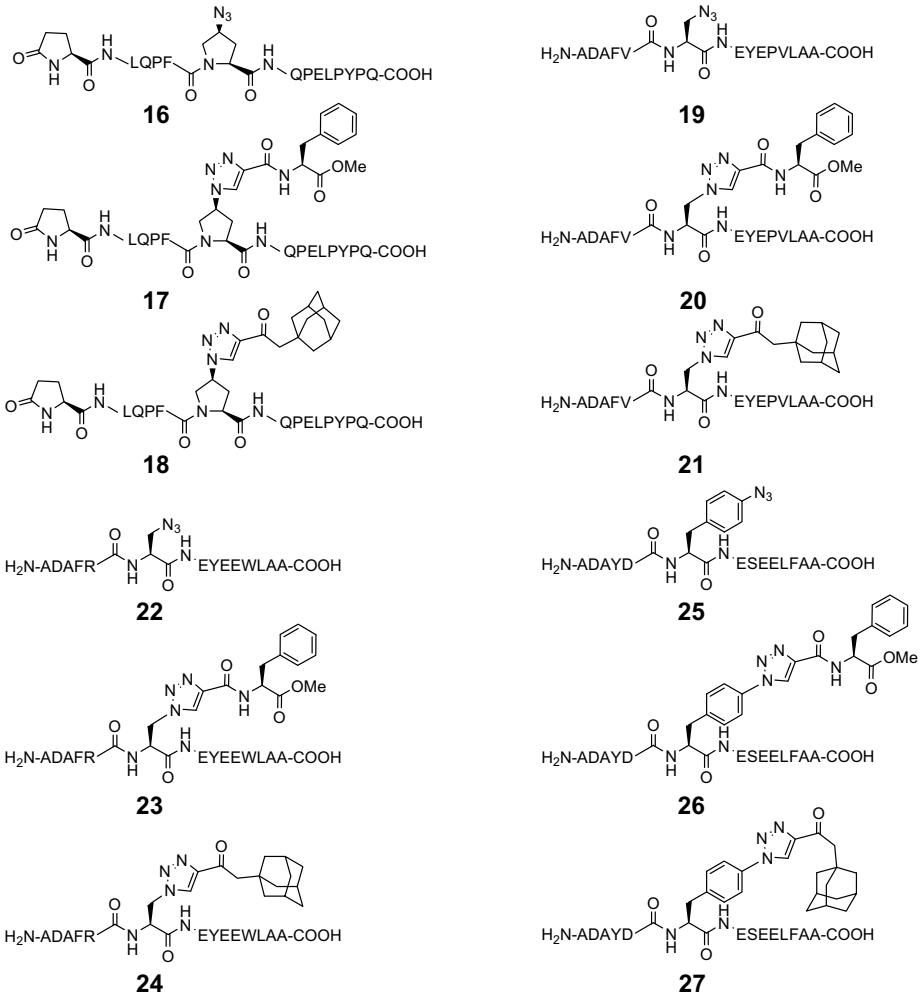
Reagents and conditions: a) Propiolic acid, Et_3N, DIC, DCM, rt, 3 h, 45%. b) NH(Me)OMe.HCl, EDC, NMM, DCM, -15 ºC to rt, 20 h, 90%. c) HC≡CMgBr, THF, 0 ºC to rt, 24 h, 78%.

Phenylalanine-derived ynone **12** is described in Chapter 2, adamantane-derived ynone **15** was chosen because of the bulkiness of the adamantane and because of the absence of amide bonds as compared to **12**. This would reduce any proteolytic breakdown of the side chain *in vivo*. Ynone **12** was synthesized by coupling propiolic acid to the HCl salt of L-phenylalanine methyl ester **11** (see Chapter 2) and ynone **15** was synthesized by a Grignard addition on Weinreb amide **14**, which could be prepared from adamantaneacetic acid **13** (Scheme 2).

Synthesis of the peptide series

With all building blocks in hand, the series of peptides was synthesized following the same procedure as described in Chapter 2. The series thus generated is depicted in Figure 1.

Figure 1 Series of p3-modified peptides.



Binding affinity of the modified peptides for HLA-DQ2

First, the binding affinity of peptides **16**-**27** was evaluated. For comparison, the unmodified peptides **2**-**4** and α-9 were taken as reference. The results are presented in Tables 2 and 3. For the analogs of the α-9 derived peptide **1** (Table 2) it was previously observed that attachment of a side chain did not have much effect on the binding properties (see Chapter 2) and this is also observed for peptides **16**-**18**. It was expected that the same would be true for the modified superbinder peptides, as a non-anchor amino acid was modified and therefore the side chain would not interact with the HLA-DQ2 binding pocket. Looking at the results in Table 3, it becomes clear that although the 'superbinders' display decreased binding affinity upon modification, as compared to the unmodified sequences, the modified peptides still bind to the HLA-DQ2 protein with good affinity. The modified peptide with the highest affinity is peptide **26**, containing a phenylalanine side chain on an azido-phenylalanine residue.

Table 2 The affinity of modified peptides **16**-**18** and α-9 for HLA-DQ2. The peptides were tested with biotin-EPRAP as indicator peptide.

Peptide	*IC_{50} (μM)*
α-9	19.3
16	18.1
17	12.7
18	17.8

Table 3 The binding affinity for HLA-DQ2 of superbinder peptides **2**-**4** and **19**-**27**. Each peptide was tested in at least three independent experiments, the averaged result of all experiments is shown. The peptides were tested against biotin-ADWFRAEYEEVI as indicator. The modified peptide with the highest binding affinity is indicated in italic.

Peptide	*IC_{50} (μM)*	*Peptide*	*IC_{50} (μM)*	*Peptide*	*IC_{50} (μM)*
2	0.7	**3**	17.9	**4**	1.2
19	4.3	**22**	17.7	**25**	4.6
20	3.1	**23**	8.7	**26**	*2.1*
21	16.2	**24**	12.0	**27**	13.4

Another finding is that in all cases the adamantane-modified peptides have a lower affinity towards HLA-DQ2 than their phenylalanine counterparts (compare, for instance **17** with **18**, or **23** with **24**).

The α-9 and derived peptides **16**-**18** were tested against a different indicator peptide than the superbinder peptides **2**-**4** and **19**-**27** (EPRAP and ADWFRAEYEEVI,

respectively), as described in Chapter 3. The use of different indicators has as a consequence that the binding affinities of the α-9 derived peptides cannot be compared directly with the superbinder peptides. However, as it is estimated that the 'superbinders' have at least a 60-fold higher binding affinity than α-9 it can be concluded that the modified 'superbinders' **19**-**27** are still better HLA-DQ2 binders than the α-9 derived peptides **16**-**18**, although compounds **19**-**27** displayed slightly decreased affinity compared to the unmodified peptides.

Conclusion

In this Chapter, peptides **2**-**4**, with a high affinity for HLA-DQ2, were modified using azido-amino acids and 'click' chemistry. The modified peptides displayed similar binding affinity for HLA-DQ2 as compared to their unmodified counterparts. It was also found that a phenylalanine side chain is more beneficial to binding affinity than an adamantane side chain. This might be due to the fact that the phenylalanine side chains are able to form hydrogen bonds as compared to the adamantane side chains. It is encouraging that the attachment of large side chains to peptides with a high binding affinity for HLA-DQ2 does not have any significant effect on their binding affinity, however, it remains to be seen whether these peptides are recognized by T cells. Incorporation of unnatural amino acids at the p1 position of peptide **26** as described in Chapter 3 may improve the binding affinity for HLA-DQ2. If this modified peptide is not recognized by gluten-specific T cells, such a peptide represents a promising lead towards highly efficient HLA-DQ2 blockers.

Experimental section

General

Reagents and solvents were used as provided, unless stated otherwise. THF was distilled over $LiAlH_4$ prior to use. Reactions were carried out under inert conditions and ambient temperature, unless stated otherwise.

Prior to performing a reaction, traces of water were removed from the starting materials by repeated coevaporation with anhydrous 1,4-dioxane or anhydrous toluene. These solvents were stored over 4 Å molsieves. Reactions were monitored by thin layer chromatography on aluminum coated silica sheets (Merck, silica 60 F254), using visualization by spraying with a solution of 25 g $(NH4)_2MoO_4$, 10 g $(NH_4)_4Ce(SO_4)_4$ in 100 ml H_2SO_4 and 900 ml H_2O, or a solution of 20% H_2SO_4 in ethanol, followed by charring at ~150 °C. Column chromatography was carried out with silica gel (Screening Devices bv, 40-63 μm particle size, 60 Å), using technical grade solvents. NMR spectra were recorded at 298K on a Bruker AV400 using deuterated solvents. All carbon spectra are proton-decoupled. CD_3OD was used as provided, to $CDCl_3$ tetramethylsilane was added as an internal standard. Chemical shifts (δ) are given in ppm, in ^{13}C spectra relative to the solvent peaks of $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm), in ^1H spectra either relative to tetramethylsilane (0 ppm) or relative to the solvent peak of CD_3OD (3.30 ppm). Coupling constants are given in Hz. IR spectra were recorded on a Perkin Elmer Paragon 1000 FT-IR Spectrometer. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in H_2O/MeCN; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylpthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations were measured on a Propol automatic polarimeter (sodium D-line, λ = 589 nm). Specific rotations (α_D) are given in degree per centimeter and the concentration c is given in grams per 100 ml in the specified solvent. Peptides were synthesized on solid support (Wang resin, NovaBiochem, 100-200 Mesh, or pre-loaded Fmoc-Ala-Wang resin (NovaBiochem, 100-200 Mesh) and amino acids were coupled manually using Fmoc based peptide synthesis methods and commercially available Fmoc amino acids. Fmoc-4-azido-phenylalanine was purchased from Bachem. Peptides α-9 and 2-4 were kindly provided by Jan Wouter Drijfhout of the IHB group, Leiden University Medical Centre, the Netherlands. 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 Electrospray Interface (ESI). An analytical Gemini C_{18} column (Phenomenex, 50 x 4.60 mm, 3 micron) was used in combination with buffers A: H_2O, B: MeCN and C: 1.0% aq. TFA. Alternatively, a Finnigan Surveyor HPLC system with a Gemini C_{18} 50 × 4.6 mm column (3 micron, Phenomenex, Torrance, CA, USA) (detection at 200-600 nm), coupled to a Thermo Finnigan LCQ Advantage Max mass spectrometer (Breda, The Netherlands) with electrospray ionization (ESI; system 1) was used. For RP-HPLC purification of the peptides, a Gilson GX-281 automated HPLC system (Gilson), supplied with a preparative Gemini C_{18} column (Phenomenex, 150 x 21.2 mm, 5 micron) was used. The applied buffers were A: 0.1% aq. TFA or 10 mM aq. NH_4OAc and B: MeCN.

Peptide binding assay

A binding assay was performed essentially as described previously applying minor modifications.[7] 96-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 and 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 x 10^6 cells per 1 ml lysis buffer. Subsequently, nuclei and cell debris

were removed by centrifugation (4°C, 2000G, 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) was prepared in 10% DMSO containing a fixed amount of the biotin-labeled indicator peptide (EEPRAPWIEQEGPEYWDQE (EPRAP) or ADWFRAEYEEVI) at a concentration of 1.2 µM. 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 minutes 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 minutes thereafter.
IC_{50} values were calculated based on the observed competition between the test peptides and biotin-labeled 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 triplicates.

Peptide Synthesis

a) First residue attachment for peptides 16-19, general procedure
Wang resin (1.2 g, 0.6 mmol) was swollen in DCM. After removal of the solvent, a solution of Fmoc-Gln(Trt)-OH (3.66 g, 6 mmol) and DMAP (73.3 mg, 0.6 mmol) in 30 ml of 1:1 (v/v) NMP/DCM was added to the resin. DIC (0.93 ml, 6 mmol) was added and the mixture shaken for 16 h. Solvents were removed, the resin was washed (NMP and DCM) and air-dried. The loading was determined by Fmoc-test.

b) Elongation of peptides
Peptides were elongated using standard Fmoc SPPS methods using repeating cycles of i) Fmoc deprotection (20% piperidine/NMP, 15 min). ii) Peptide coupling (4 eq Fmoc-AA-OH, 4 eq HCTU, 4.8 eq DIPEA, NMP, 2h). Azido-amino acids were attached using 2 equivalents of azido-amino acid, 2 equivalents of HCTU and 2.4 equivalents of DIPEA, and 18 h coupling. N-terminal pyroglutamic acid residues were attached using 4 equivalents of L-pyroglutamic acid, 4 equivalents of HCTU, 4.8 equivalents of DIPEA, NMP, and double coupling, 2 h per coupling.

c) Functionalization of side chains via 'click' chemistry, general procedure
The dry resin was washed three times with NMP. A suspension of the appropriate ynone (4 eq), copper(I) iodide (4 eq) in 3.2 ml of 20% DIPEA in NMP was added to the resin and the mixture shaken for 48 hours. Solvents were removed and the resin washed with NMP.

d) Cleavage of peptides from resin and purification
Peptides were cleaved from the resin by shaking for 2 h in 95:2.5:2.5 (v/v/v) TFA/H_2O/TIS and precipitated in a cold 1:1 (v/v) pentane/diethyl ether mixture. 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, analyzed by LC-MS and purified by RP-HPLC. The fractions containing the pure product were pooled, concentrated and freeze-dried to yield the pure peptides.

Synthesis and analytical data of building blocks and peptides

N_α-(9-fluorenylmethoxycarbonyl)-L-diaminopropionic acid (8)
To a solution of PIFA (24.5 g, 57 mmol) in MeCN (60 ml) was added Fmoc-Asn-OH (18.3 g, 51.8 mmol) and DMF (120 ml) and the mixture was stirred until all solids were dissolved. Subsequently,

H_2O (60 ml) was added and after stirring for another 15 min pyridine (4.6 ml, 57 mmol) was added dropwise. The reaction was stirred for 70 h, during which slow CO_2 evolution was observed. Solvents were evaporated and the resulting yellow oil was crystallized from H_2O to yield pure **8** as white crystals (11.3 g, 34.8 mmol, 67%).
^{1}H NMR (CD_3OD, 400 MHz): δ 7.75 (d, *J* = 7.5 Hz, 2H, CH benzene ring); 7.63 (d, *J* = 7.2 Hz, 2H, CH benzene ring); 7.35 (t, *J* = 7.4 Hz, 2H, CH benzene ring); 7.26 (t, *J* = 7.4 Hz, 2H, benzene ring); 4.40-4.31 (m, 3H, H_α, CH_2 Fmoc); 4.19 (t, *J* = 6.87 Hz, 1H, CH Fmoc); 3.36 (dd, *J* = 12.9 Hz, *J* = 6.4 Hz, 1H, $H_{\beta A}$); 3.16 (dd, *J* = 12.3 Hz, *J* = 7.6 Hz, 1H, $H_{\beta B}$). ^{13}C NMR (CD_3OD, 100 MHz): δ 145.2 (C_q benzene ring); 142.6 (C_q Fmoc); 128.8 (CH benzene ring); 128.2 (CH benzene ring); 126.2 (CH benzene ring); 120.9 (CH benzene ring); 68.4 (CH_2 Fmoc); 53.4 (C_α); 48.3 (CH Fmoc); 41.9 (C_β). LC-MS retention time: 5.58 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 327.00 $[M + H]^+$.

N_α-(9-fluorenylmethoxycarbonyl)-azido-L-alanine (9)
Free amine **8** (1.35 g, 4.13 mmol) was dissolved in MeOH (20 ml) and to this solution a few drops of a sat. aq. $CuSO_4$ solution was added. Imidazole-1-sulfonyl azide.HCl[8] was added and this mixture was stirred for 3 h. The reaction mixture was concentrated, the residue taken up in H_2O, and acidified to pH = 1. The aqueous layer was extracted three times with EtOAc, the combined organic layers dried ($MgSO_4$), filtered and concentrated. The resulting yellow oil was purified by column chromatography (0 → 40% EtOAc/light petroleum) to yield pure **9** as off-white solids (1.37 g, 3.9 mmol, 95%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.02 (br s, 1H, COOH); 7.75 (d, *J* = 7.5 Hz, 2H, CH benzene ring); 7.59 (d, *J* = 7.2 Hz, 2H, CH benzene ring); 7.39 (t, *J* = 7.4 Hz, 2H, CH benzene ring); 7.30 (t, *J* = 7.4 Hz, 2H, CH benzene ring); 4.61-4.54 (m, 1H, H_α); 4.47-4.36 (m, 2H, CH_2 Fmoc); 4.22 (t, *J* = 6.6 Hz, 1H, CH Fmoc); 3.79 (ddd, *J* = 16.1 Hz, *J* = 13.Hz, *J* = 4.2 Hz, 2H, H_β). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.1 (C=O carboxylic acid); 156.0 (C=O Fmoc); 143.5 (C_q benzene ring); 141.3 (C_q benzene ring); 127.8 (CH benzene ring); 127.1 (CH benzene ring); 125.0 (CH benzene ring); 120.0 (CH benzene ring); 67.5 (CH_2 Fmoc); 53.6 (C_α); 52.2 (C_β); 47.0 (CH Fmoc). IR (neat): 3311.7; 2926.1; 2109.7; 1717.9; 1689.1; 1537.8; 1534.0; 1464.7; 1450.4; 1418.4; 1335.9; 1255.0; 1180.4; 1105.6; 1091.5; 1047.9; 995.7; 924.7; 774.0; 757.4; 735.9; 644.3; 620.5; 542.1. $[\alpha]_D^{20}$: +18.0 (c = 0.5, $CHCl_3$). LC-MS retention time: 8.29 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 326.93 $[M - N_2 + H]^+$.

N-methoxy-N-methyl-1-adamantylacetamide (14)
Adamantaneacetic acid **13** (5.8 g, 30 mmol) was dissolved in DCM (120 ml) and cooled to -15 ºC. To this chilled solution N,O-dimethylhydroxylamine.HCl (4.4 g, 45 mmol), NMM (5.0 ml, 45 mmol) and EDC (8.6 g, 45 mmol) were added and the resulting mixture stirred for 20 h at room temperature. After addition of 18 ml cold 1 M aq. HCl the layers were separated and the aqueous layer extracted with DCM. The combined organic layers were washed with sat. aq. $NaHCO_3$, dried ($MgSO_4$), filtered and concentrated. The crude product was subjected to column chromatography (0 → 30% EtOAc/light petroleum) and the pure product was obtained as a white solid (6.4 g, 27 mmol, 90%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.66 (s, 3H, OCH_3); 3.17 (s, 3H, CH_3); 2.19 (s, 2H, CH_2-adamantane); 1.95 (s, 3H, CH adamantane); 1.67 (d, *J* = 2.6 Hz, 10H, CH_2 adamantane). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 60.3 (OCH_3); 58.3 (CH_2-adamantane); 42.2 (CH_2 adamantane); 36.3 (CH_2 adamantane); 33.0 (C_q adamantane); 31.1 (CH_3); 28.3 (CH adamantane). IR (neat): 2899.4; 2847.0; 1655.9; 1449.6; 1408.3; 1380.0; 1360.0; 1315.4; 1181.1; 1096.1; 1009.3; 985.2; 978.4; 945.5; 821.5; 772.0; 723.2; 601.6; 535.2. LC-MS retention time: 8.87 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 238.20 $[M + H]^+$; 475.00 $[2M + H]^+$. Exact mass: calculated for $[C_{14}H_{24}NO_2]^+$: 238.18016. Found: 238.18017 $[M + H]^+$.

4-Adamantyl-but-1-yn-3-one (15)
Weinreb amide **15** (1.0 g, 4.4 mmol) was dissolved in freshly distilled THF (45 ml) and the solution was cooled to 0 ºC. Ethynyl magnesium bromide (as 0.5 M solution in THF, 13.1 ml, 6.6 mmol) was added and the mixture stirred at room temperature for 24 h. The reaction mixture was poured into sat. aq. NH_4Cl and the aqueous layer extracted thrice with diethyl ether. The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The crude yellow-orange oil was purified by column chromatography (0% → 5% EtOAc/light petroleum) and pure **15** was obtained as a white solid (0.69 g, 3.4 mmol, 78%).
^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.26 (s, 1H, HC≡C); 2.38 (s, 2H, CH_2-adamantane); 1.98 (br s, 3H, CH adamantane); 1.72-1.63 (m, 12H, CH_2 adamantane). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 186.8 (C=O); 83.4 (**C**≡CH); 78.1 (C≡**C**H); 58.8 (CH_2-adamantane); 42.4 (CH_2 adamantane); 36.6 (CH_2 adamantane); 34.3 (C_q adamantane); 28.5 (CH_2 adamantane).

Peptide 16
Following the general procedure for cleavage, **16** was obtained from Pyr-LQPF-**6**-QPELPYPQ-Wang resin (loading 0.28 mmol g^{-1}, 357 mg, 100 μmol) as a white powder (11.4 mg, 6.7 μmol, 7%) using an HPLC gradient of 80:20 → 65:35 of buffers A: 10 mM aq. NH_4OAc and B: MeCN for purification.
LC-MS retention time: 5.58 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 853.53 $[M + 2H]^{2+}$; 1705.67 $[M + H]^+$. Exact mass: calculated for $[C_{80}H_{114}N_{20}O_{22}]^{2+}$: 853.42028; $[C_{80}H_{113}N_{20}O_{22}]^+$: 1705.83328. Found: 853.42085 $[M + 2H]^{2+}$; 1705.83414 $[M + H]^+$.

Peptide 17
Following the general procedures for *on resin* 'click' chemistry and cleavage, **17** was obtained from Pyr-LQPF-**6**-QPELPYPQ-Wang resin (loading 0.28 mmol g^{-1}, 357 mg, 100 μmol) as a white powder (35.7 mg, 18.4 μmol, 18%) using an HPLC gradient of 80:20 → 60:40 of buffers A: 10 mM aq. NH_4OAc and B: MeCN for purification.
LC-MS retention time: 6.51 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 969.33 $[M + 2H]^{2+}$; 1937.40 $[M + H]^+$. Exact mass: calculated for $[C_{93}H_{127}N_{21}O_{25}]^{2+}$: 968.96505; $[C_{93}H_{126}N_{21}O_{25}]^+$: 1936.92282. Found: 968.96598 $[M + 2H]^{2+}$; 1936.92254 $[M + H]^+$.

Peptide 18
Following the general procedures for *on resin* 'click' chemistry and cleavage, **18** was obtained from Pyr-LQPF-**6**-QPELPYPQ-Wang resin (loading 0.28 mmol g^{-1}, 357 mg, 100 μmol) as a white powder (30.7 mg, 16.1 μmol, 16%) using an HPLC gradient of 80:20 → 40:60 of buffers A: 10 mM aq. NH_4OAc and B: MeCN for purification.
LC-MS retention time: 7.30 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 955.00 $[M + 2H]^{2+}$; 1908.67 $[M + H]^+$. Exact mass: calculated for $[C_{94}H_{132}N_{20}O_{23}]^{2+}$: 954.48816; $[C_{94}H_{131}N_{20}O_{23}]^+$: 1907.96905. Found: 954.48924 $[M + 2H]^{2+}$; 1907.96889 $[M + H]^+$.

Peptide 19
Following the general procedure for cleavage, **19** was obtained from Boc-ADAFV-**9**-EYEPVLAA-Wang resin (loading 0.22 mmol g^{-1}, 228 mg, 50 μmol) as a white powder (5 mg, 3.3 μmol, 7%) using an HPLC gradient of 75:25 → 60:40 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 5.45 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 753.80 $[M + 2H]^{2+}$; 1506.73 $[M + H]^+$. Exact mass: calculated for $[C_{68}H_{101}N_{17}O_{22}]^{2+}$: 753.86481; $[C_{68}H_{100}N_{17}O_{22}]^+$: 1506.72233. Found: 753.86512 $[M + 2H]^{2+}$; 1506.72234 $[M + H]^+$.

Peptide 20
Following the general procedures for *on resin* 'click' chemistry and cleavage, **20** was obtained from Boc-ADAFV-**9**-EYEPVLAA-Wang resin (loading 0.22 mmol g^{-1}, 228 mg, 50 μmol) as a white powder (4.5 mg, 2.6 μmol, 5%) using an HPLC gradient of 75:25 → 50:50 of buffers A: 0.1% aq. TFA and B: MeCN for purification.

LC-MS retention time: 6.22 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 869.67 [M + 2H]$^{2+}$; 1738.73 [M + H]$^{+}$. Exact mass: calculated for [$C_{81}H_{114}N_{18}O_{25}$]$^{2+}$: 869.40958; [$C_{81}H_{113}N_{18}O_{25}$]$^{+}$: 1737.81188. Found: 869.41014 [M + 2H]$^{2+}$; 1737.81283 [M + H]$^{+}$.

Peptide 21
Following the general procedures for *on resin* 'click' chemistry and cleavage, **21** was obtained from Boc-ADAFV-**9**-EYEPVLAA-Wang resin (loading 0.22 mmol g^{-1}, 228 mg, 50 μmol) as a white powder (2.5 mg, 1.5 μmol, 3%) using an HPLC gradient of 65:35 → 50:50 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 6.87 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 855.27 [M + 2H]$^{2+}$; 1708.73 [M + H]$^{+}$. Exact mass: calculated for [$C_{82}H_{116}N_{17}O_{23}$]$^{2+}$: 854.93269; [$C_{82}H_{115}N_{17}O_{23}$]$^{+}$: 1708.85810. Found: 854.93331 [M + 2H]$^{2+}$; 1708.85971 [M + H]$^{+}$.

Peptide 22
Following the general procedure for cleavage, **22** was obtained from Boc-ADAFR-**9**-EYEEWLAA-Wang resin (loading 0.064 mmol g^{-1}, 250 mg, 16 μmol) as a white powder (2.3 mg, 1.4 μmol, 9%) using an HPLC gradient of 80:20 → 60:40 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 5.46 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 842.00 [M + 2H]$^{2+}$; 1682.73 [M + H]$^{+}$. Exact mass: calculated for [$C_{75}H_{105}N_{21}O_{24}$]$^{2+}$: 841.88152; [$C_{75}H_{104}N_{21}O_{24}$]$^{+}$: 1682.75576. Found: 841.88206 [M + 2H]$^{2+}$; 1682.75797 [M + H]$^{+}$.

Peptide 23
Following the general procedures for *on resin* 'click' chemistry and cleavage, **23** was obtained from Boc-ADAFR-**9**-EYEEWLAA-Wang resin (loading 0.064 mmol g^{-1}, 250 mg, 16 μmol) as a white powder (4.9 mg, 2.6 μmol, 16%) using an HPLC gradient of 70:30 → 55:45 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 6.05 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 957.47 [M + 2H]$^{2+}$; 1914.93 [M + H]$^{+}$. Exact mass: calculated for [$C_{88}H_{118}N_{22}O_{27}$]$^{2+}$: 957.42629; [$C_{88}H_{117}N_{22}O_{27}$]$^{+}$: 1913.84530. Found: 957.42706 [M + 2H]$^{2+}$; 1913.84673 [M + H]$^{+}$.

Peptide 24
Following the general procedures for *on resin* 'click' chemistry and cleavage, **24** was obtained from Boc-ADAFR-**9**-EYEEWLAA-Wang resin (loading 0.064 mmol g^{-1}, 250 mg, 16 μmol) as a white powder (3.1 mg, 1.7 μmol, 10%) using an HPLC gradient of 70:30 → 50:50 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 6.49 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 1885.93 [M + H]$^{+}$. Exact mass: calculated for [$C_{89}H_{123}N_{21}O_{25}$]$^{2+}$: 942.94940; [$C_{89}H_{122}N_{21}O_{25}$]$^{+}$: 1884.89152. Found: 942.95001 [M + 2H]$^{2+}$; 1884.89162 [M + H]$^{+}$.

Peptide 25
Following the general procedure for cleavage, **25** was obtained from Boc-ADAYD-**10**-ESEELFAA-Wang resin (loading 0.32 mmol g^{-1}, 156 mg, 50 μmol) as a white powder (3.1 mg, 1.9 μmol, 4%) using an HPLC gradient of 80:20 → 55:45 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 5.57 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 1619.53 [M + H]$^{+}$. Exact mass: calculated for [$C_{71}H_{97}N_{17}O_{27}$]$^{2+}$: 809.83644; [$C_{71}H_{96}N_{17}O_{27}$]$^{+}$: 1618.66561. Found: 809.83696 [M + 2H]$^{2+}$; 1618.66690 [M + H]$^{+}$.

Peptide 26
Following the general procedures for *on resin* 'click' chemistry and cleavage, **26** was obtained from Boc-ADAYD-**10**-ESEELFAA-Wang resin (loading 0.32 mmol g^{-1}, 156 mg, 50 μmol) as a white

powder (2.3 mg, 1.3 μmol, 3%) using an HPLC gradient of 75:25 → 50:50 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 6.22 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 1849.80 $[M + H]^+$. Exact mass: calculated for $[C_{84}H_{110}N_{18}O_{30}]^{2+}$: 925.38121; $[C_{84}H_{109}N_{18}O_{30}]^+$: 1849.75517. Found: 925.38211 $[M + 2H]^{2+}$; 1849.75657 $[M + H]^+$.

Peptide 27
Following the general procedures for *on resin* 'click' chemistry and cleavage, **27** was obtained from Boc-ADAYD-**10**-ESEELFAA-Wang resin (loading 0.32 mmol g^{-1}, 187 mg, 60 μmol) as a white powder (2.0 mg, 1.1 μmol, 2%) using an HPLC gradient of 65:35 → 50:50 of buffers A: 0.1% aq. TFA and B: MeCN for purification.
LC-MS retention time: 6.52 min (10 → 90% MeCN, 15 min run). Mass (ESI): m/z 912.07 $[M + 2H]^{2+}$; 1820.80 $[M + H]^+$. Exact mass: calculated for $[C_{85}H_{115}N_{17}O_{28}]^{2+}$: 910.90432; $[C_{85}H_{114}N_{17}O_{28}]^+$: 1820.80137. Found: 910.90514 $[M + 2H]^{2+}$; 1820.80231 $[M + H]^+$.

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