SUPPORTING INFORMATION FOR:

β-Arrestin-biased agonists of the GLP-1 receptor from β-amino acid residue incorporation into GLP-1 analogues

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Materials and Methods:

Materials

Protected α-amino acids, 2-(6-chloro-1-benzotriazole-1-yl)-1,1,3,3 - tetramethylaminium hexafluorophosphate (HCTU) and 6-chloro-1-hydroxybenzotriazole (Cl-HOBt) were purchased from Chem-Impex International. Fmoc-ACPC-OH was purchased from Chem-Impex International. Fmoc-APC(Boc)-OH was synthesized as previously described.[1] ChemMatrix H-PAL amide resin was purchased from PCAS BioMatrix. All other reagents for peptide synthesis and purification were purchased from Sigma-Aldrich or Fisher Scientific.

Cell culture reagents were purchased from Gibco. Luciferase substrates (DeepBlueC and D-luciferin) were purchased from GoldBiotechnology. GLP-1(7-36)NH_2 was purchased from Anaspec. Primers were ordered from Integrated DNA Technologies.

Peptide Synthesis and Purification

Peptides were synthesized by microwave-assisted solid phase peptide synthesis[2] on ChemMatrix H-PAL amide resin based on Fmoc-protection of the main chain amino group. Coupling reactions were carried out by treating the resin with a solution of the protected amino acid, 2-(6-chloro-1-benzotriazole-1-yl)-1,1,3,3 - tetramethylaminium hexafluorophosphate (HCTU) and 0.1 M 6-chloro-1-hydroxybenzotriazole (Cl-HOBt) in the presence of *N,N*-diisopropylethylamine (DIEA) and 1-methyl-2-pyrrolidone (NMP). Each coupling was carried out using 4 equivalents of amino acid, 3.9 equivalents of HCTU and Cl-HOBt, and 8 equivalents of DIEA. Deprotection of the Fmoc group was carried out using a solution of 20% piperidine in *N,N*-dimethylformamide (DMF). Peptides were cleaved from the resin using a solution of 95% trifluoroacetic acid (TFA), 2.5% H_2O and 2.5% triisopropylsilane (TIS). Excess TFA was removed with a stream of nitrogen, and the crude peptides were precipitated by addition of cold diethyl ether.

Crude peptides were purified using HPLC. Peptide purity was assessed by analytical HPLC (BEH C18 stationary phase, 4.6 mm x 250 mm) or UPLC (BEH C18 stationary

phase, 2.1 mm x 100 mm), and peptide mass was confirmed using MALDI-TOF MS. All peptides reported here were >95% pure as determined by analytical HPLC, or >90% pure as determined by UPLC. For all HPLC and UPLC, solvent A = H_2O with 0.1% trifluroacetic acid and solvent B = acetonitrile with 0.1% trifluroacetic acid. Analytical HPLC and MALDI data for each peptide are shown in Figures S5-S19.

Molecular Biology

Human GLP-1R (SC124060) and human β-arr1 (SC303424) cDNA was purchased from Origene. Human β-arr2 N-terminally tagged with green fluorescent protein $(GFP)^2$, human GLP-1R C-terminally tagged with *Renilla* luciferase (Rluc8), and human GRK5 were kind gifts from Rasmus Jorgensen and Jakob Lerche Hansen at Novo Nordisk. Mutations (R393E, R395E) were introduced into the GFP^2-βarr2 gene using the QuikChangeXL site-directed mutagenesis kit (Strategene). A mutation in the GLP1R-Rluc8 gene (A260F) was corrected using the QuikChangeXL site-directed mutagenesis kit. A GFP^2-β-arr1 fusion protein (as reported by Jorgenson *et al*[3]) was constructed using Gibson assembly methods.[4] Briefly, the β-arr1 vector was linearized by PCR amplification, and the GFP^2 gene was cut out of the GFP^2-β-arr2 construct (by PCR amplification) with an overhang to match the linearized ends of the β-arr1 vector, to facilitate assembly of the two genes. The linearized β-arr1 and GFP^2 fragment were mixed and incubated at 50 °C for 1 hr in the presence of T5 exonuclease, *Phusion* polymerase and *Taq* ligase to facilitate a circular GFP^2-β-arr1-containing plasmid. All clones were confirmed by DNA sequencing. Primers used to facilitate mutagenesis are listed below.

GLP1R C780A:

C780A F- CTGGCCTTCTCGGTCTTATCTGAGCAATGGATC
C780A R-GATCCATTGCTCAGATAAGACCGAGAAGGCCAG

GFP2-β-Arrestin 2 R393E, R395E:

F: GTGTTTGAGGACTTTGCCGAGCTTGAGCTGAAGGGGATGAAGGA
R: TCCTTCATCCCCTTCAGCTCAAGCTCGGCAAAGTCCTCAAACAC

GFP2-β-Arrestin 1 Construction:

GFP Insert Primers (overhang in **bold**):
GFP FP 2: **ACCTCCCTGCGACCGTCGCGGACC**CCACCATGGTGAGCAAGGGCGAGGA
GFP RP 2: **CACTGGGGTCCCTTTGTCGCCCAT**GGATCCGGTACCGGAGCCCTT

βArr1 Linearization Primers:
βArr1 FP: ATGGGCGACAAAGGGACCCCAGTG
βArr1 RP: GGTCCGGGACGGTCCCAGGGAGGT

Mutagenesis Primers:
C20G F: CGACAAAGGGACCCGACTGTTCAAGAAG
C20G R: CTTCTTGAACACTCGGGTCCCTTTGTCG

Cell Culture

HEK293FT cells (Life Technologies) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 6 mM L-glutamine, 1 mM sodium

pyruvate, 0.1 mM non-essential amino acids, 1% penicillin/streptomycin and 500 μg/mL Geneticin.

HEK293 cells stably expressing the Glosensor cAMP reporter plasmid (a kind gift from Prof. Thomas Gardella) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 37 °C in 5% CO_2. Cells were grown to 70-80% confluence before transfections.

Peptide Quantification and Stock Solution Preparation

After peptides were purified and lyophilized, stock solutions were prepared, with concentration determined by UV-visible spectroscopy using the absorbance of each peptide at 280 nm. These calculations were based on $\varepsilon_{280\ nm} = 6{,}970\ M^{-1}cm^{-1}$, derived from the extinction coefficients of tryptophan ($5{,}690\ M^{-1}cm^{-1}$) and tyrosine ($1{,}280\ M^{-1}cm^{-1}$)[5] (each peptide contains one residue of each type) Once the concentration of each peptide was determined, known volumes were aliquoted and lyophilized to dry powder. These known amounts of peptide were then dissolved in DMSO to 1 mM, and the resulting stock solutions were stored at -20 °C. Commercially purchased GLP-1(7-36)NH_2 was dissolved in 0.1% aqueous AcOH to 1 mM. For cellular assays, 1 mM DMSO stocks of peptide were diluted into D-PBS buffer. When commercially purchased GLP-1(7-36)NH_2 was used to prepare assay stock solutions, DMSO was added to GLP-1(7-36)NH_2 to equal the amount of DMSO in synthetic peptide assay stock solutions, and 0.1% AcOH was added to synthetic peptides to equal the amount of 0.1% AcOH in commercial GLP-1(7-36)NH_2, such that all assay stock solutions had identical vehicles.

cAMP Production Assay Transfection Optimization

To determine the optimal amount of GLP-1R DNA in transfections, cells were plated in a 96 well plate and transfected with varying amounts (0 ng, 40 ng, 60 ng, 80 ng, 100 ng and 120 ng per well) of GLP-1R DNA, each containing a 3-fold excess of FuGeneHD. The transfections were performed as described in the main text. After 24 hours, transfection medium was removed from the plate, and replaced with DMEM supplemented with 10% FBS. After 24 more hours, the GloSensor assay was performed as described in the main text. Varying concentrations of GLP-1 were added to each transfection condition, to determine the minimum amount of GLP-1R DNA required to reach maximum luminescence in response to GLP-1 (Figure S3). Sixty ng of GLP-1R DNA per well of a 96-well plate was found to be the minimum amount of GLP-1R DNA per transfection required to achieve a wide dynamic range (the absolute luminescence maximum increased at greater amounts of GLP-1R DNA transfected, but the range of luminescence values remained similar to the range produced upon transfection of 60 ng GLP-1R DNA per well). It should be noted that at the time of this experiment, the critical importance of changing pipet tips during serial dilution of peptides had not yet been recognized, and we attribute the non-zero signal observed for GLP-1 at very low concentrations to contamination resulting from tip reuse.

Note on cAMP assays: in all experiments comparing GLP-1 and α/β-peptide **4**, the maximum response for **4** was lower than that for GLP-1. However, the degree to which the maximum response for **4** was diminished relative to GLP-1 varied between experiments. Experiments in which the reduction in maximum response for **4** was

most dramatic (e.g., 40% of GLP-1's maximum response, versus 80% of GLP-1's maximum response) tended to have particularly high signal, suggesting that the activity of this peptide may depend on receptor expression levels, which vary in these experiments due to the need for transient transfection before each experiment. We are currently investigating the underlying reason for this inconsistency in maximum response. None of the other peptides discussed in this paper displayed variation in maximum response.

Bioluminescence Resonance Energy Transfer Transfection Optimization

To determine the optimal ratio of GLP1R-RLuc8 DNA to GFP^2-β-arr1 DNA or GFP^2-β-arr2(R393E, R395E) DNA, cells were plated in 6 well plates and transfected with varying amounts (0 ng, 670 ng, 1340 ng, 2010 ng, 2680 ng and 3350 ng) of GFP^2-β-arr1 DNA or GFP^2-β-arr2(R393E, R395E) DNA, while GLP1R-Rluc8 DNA and GRK5 DNA were kept constant at 100 ng each. Twenty-four hours after transfection, each well (containing different ratios of GLP1R-Rluc8 DNA to GFP^2-β-arr1 DNA or GFP^2-β-arr2(R393E, R395E) DNA) was plated into two columns of a 96-well plate. After 24 hours, the $BRET^2$ signal was measured for each condition in the presence and absence of GLP-1 (1 μM) (experimental details of BRET assay are described in the main text). For the β-arr1 BRET assay, the optimal transfection ratio was found to be 20:1:1 GFP^2-βarr1:GLP1R-Rluc8:GRK5 DNA, and for the β-arr2 BRET assay, the optimal transfection ratio was found to be 30:1:1 GFP^2-β-arr2(R393E, R395E:GLP1R-Rluc8:GRK5 DNA. BRET data for transfection ratio optimization experiments are shown in Figure S4A-B.

Supplementary Figures

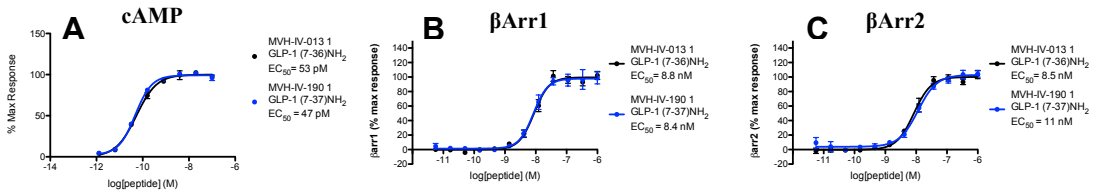


Figure S1. GLP-1R activation by GLP-1(7-36)NH_2 and GLP-1(7-37)NH_2. Data points are from one experiment with duplicate measurements. Concentration-response curves for peptide-induced activation of GLP-1R in (A) $G\alpha_s$ activation, as measured by the luciferase-based GloSensor cAMP reporter assay. (B) β-arrestin-1 recruitment, as measured using a β-arrestin-1 BRET assay. (C) β-arrestin-2 recruitment, as measured using a β-arrestin-2 BRET assay. The β-arrestin-2 plasmid contains (R393E, R395E) mutations, which prevent receptor internalization. Curves were generated by fitting the data to a sigmoidal dose-response curve with a variable slope in Prism 5.

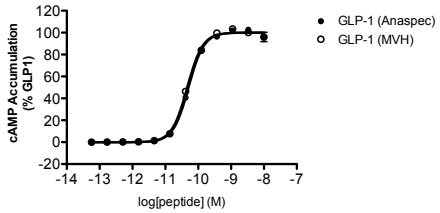


Figure S2. Comparison of purchased (Anaspec) GLP-1(7-36)NH_2 and GLP-1(7-36)NH_2 prepared in our laboratory. Data points are from one experiment with duplicate measurements. Concentration-response curves for peptide-induced activation of GLP-1R in $G\alpha_s$ activation, as measured by the luciferase-based GloSensor cAMP reporter assay. Curves were generated by fitting the data to a sigmoidal dose-response curve with a variable slope in Prism 5.

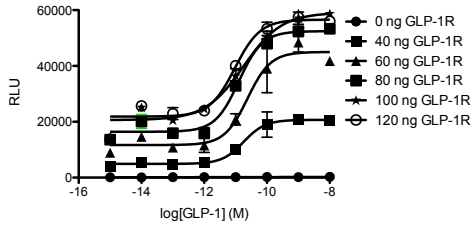


Figure S3. Transfection ratio optimization for GloSensor cAMP assays. Cells transfected with different amounts of GLP-1R DNA were treated with either GLP-1(7-36)NH_2 or vehicle, and the luminescence signal was measured to determine at which amount of GLP-1R DNA the maximum luminescence in response to GLP-1 plateaued. Curves were generated by fitting the data to a sigmoidal dose-response curve with a variable slope in Prism 5.

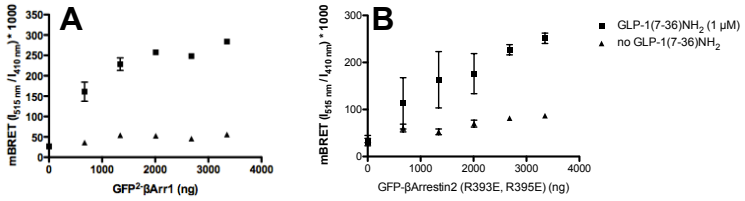


Figure S4. Transfection ratio optimization for BRET assays for (A) GFP^2-β-arrestin-1 and (B) GFP^2-β-arrestin-2 (R393E, R395E). Cells transfected with different ratios of fluorescent acceptor DNA and luminescent donor DNA were treated with either GLP-1(7-36)NH_2 or vehicle, and the BRET signal was measured to determine which ratio of acceptor to donor produced a maximal signal and the least fluctuation between replicates.

Figure S5. UPLC trace and MALDI-TOF spectrum for GLP-1(7-36)NH_2

UPLC: Gradient = 10 – 60 % solvent B over 5 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3297.7, observed monoisotopic $[M+H]^+$ = 3297.9

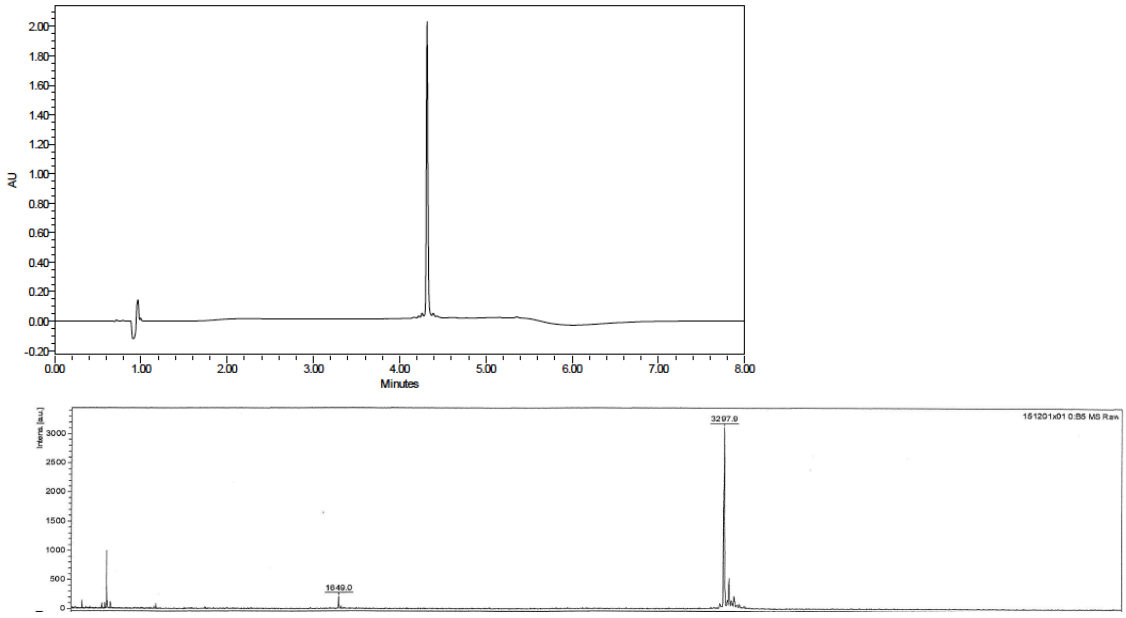
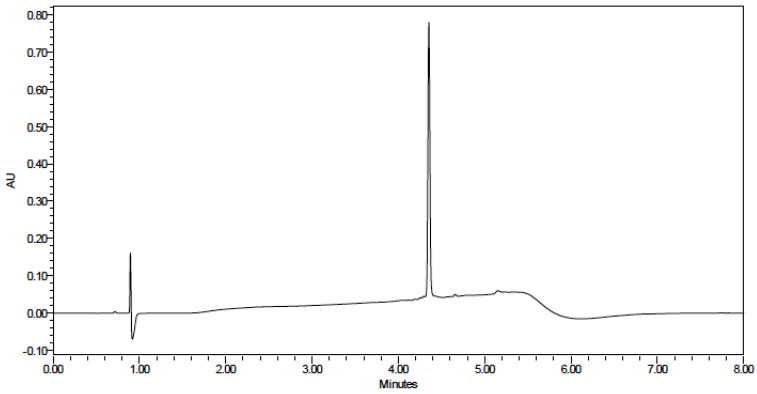


Figure S6. UPLC trace and MALDI-TOF spectrum for GLP-1(7-37)NH_2

UPLC: Gradient = 10 – 60 % solvent B over 5 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3353.7, observed monoisotopic $[M+H]^+$ = 3353.4



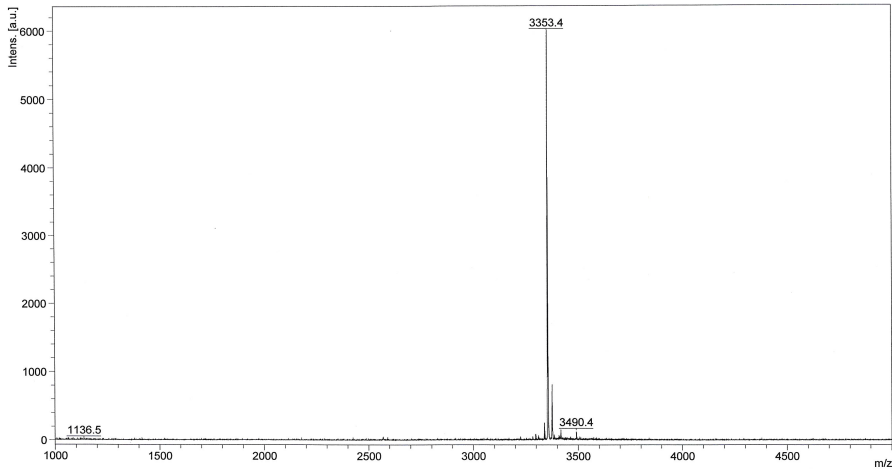


Figure S7. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **1**.

HPLC: Gradient = 10 – 90 % solvent B over 80 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3359.6, observed monoisotopic $[M+H]^+$ = 3359.8

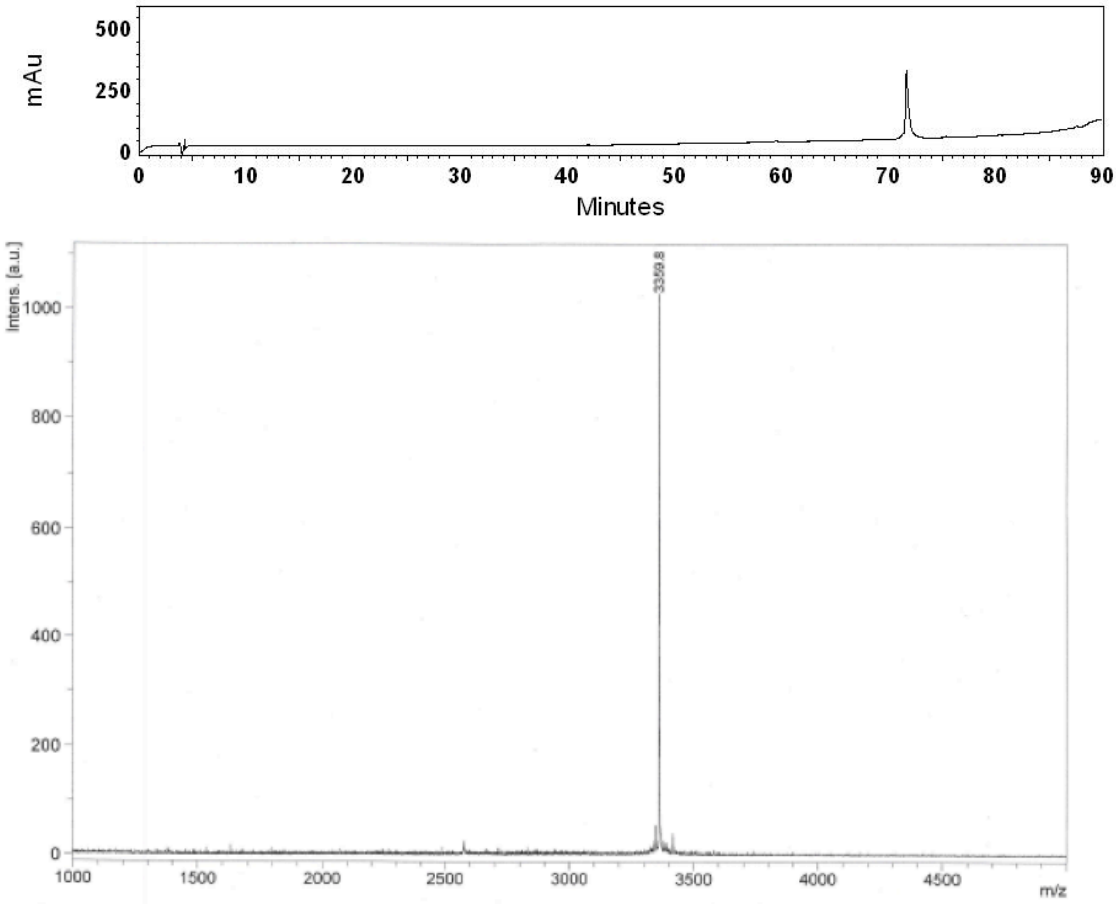
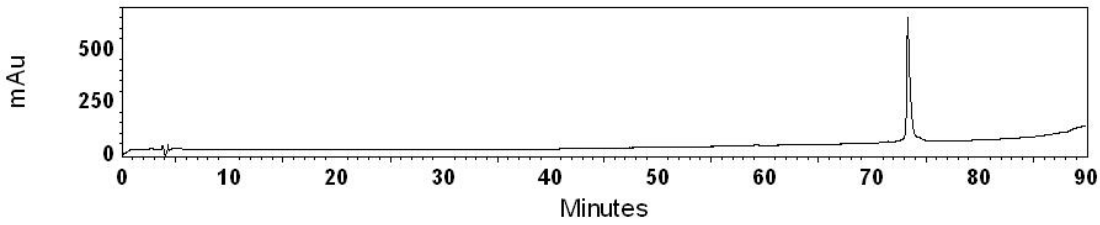


Figure S8. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **2**.

HPLC: Gradient = 10 – 90 % solvent B over 80 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3413.7, observed monoisotopic $[M+H]^+$ = 3414.1



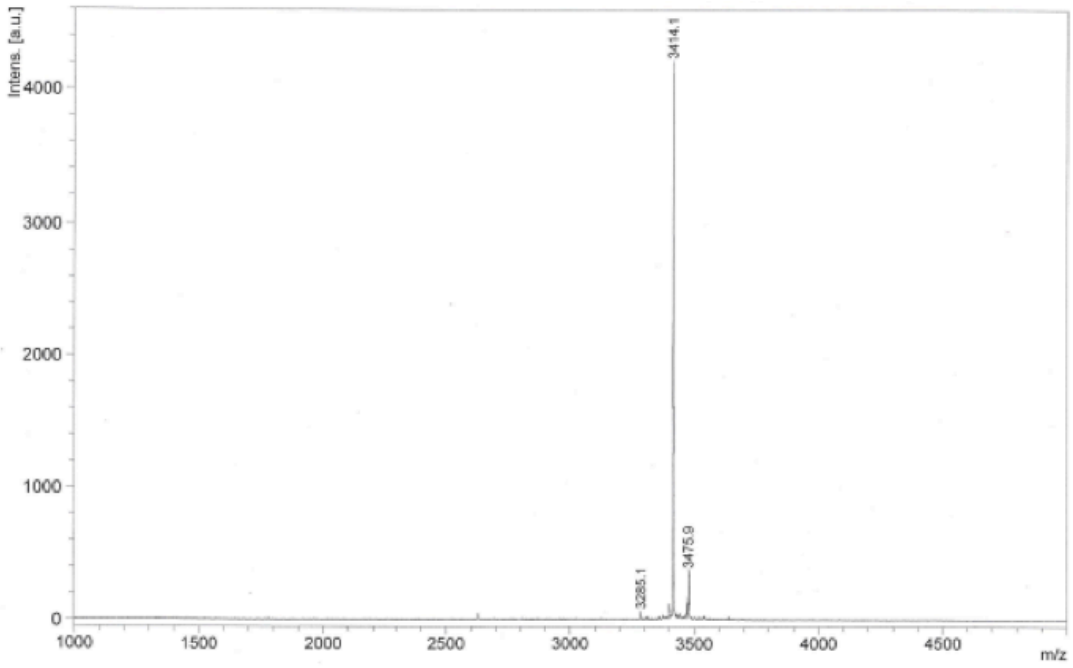


Figure S9. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **3**.

HPLC: Gradient = 10 – 90 % solvent B over 80 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3437.7, observed monoisotopic $[M+H]^+$ =3437.7

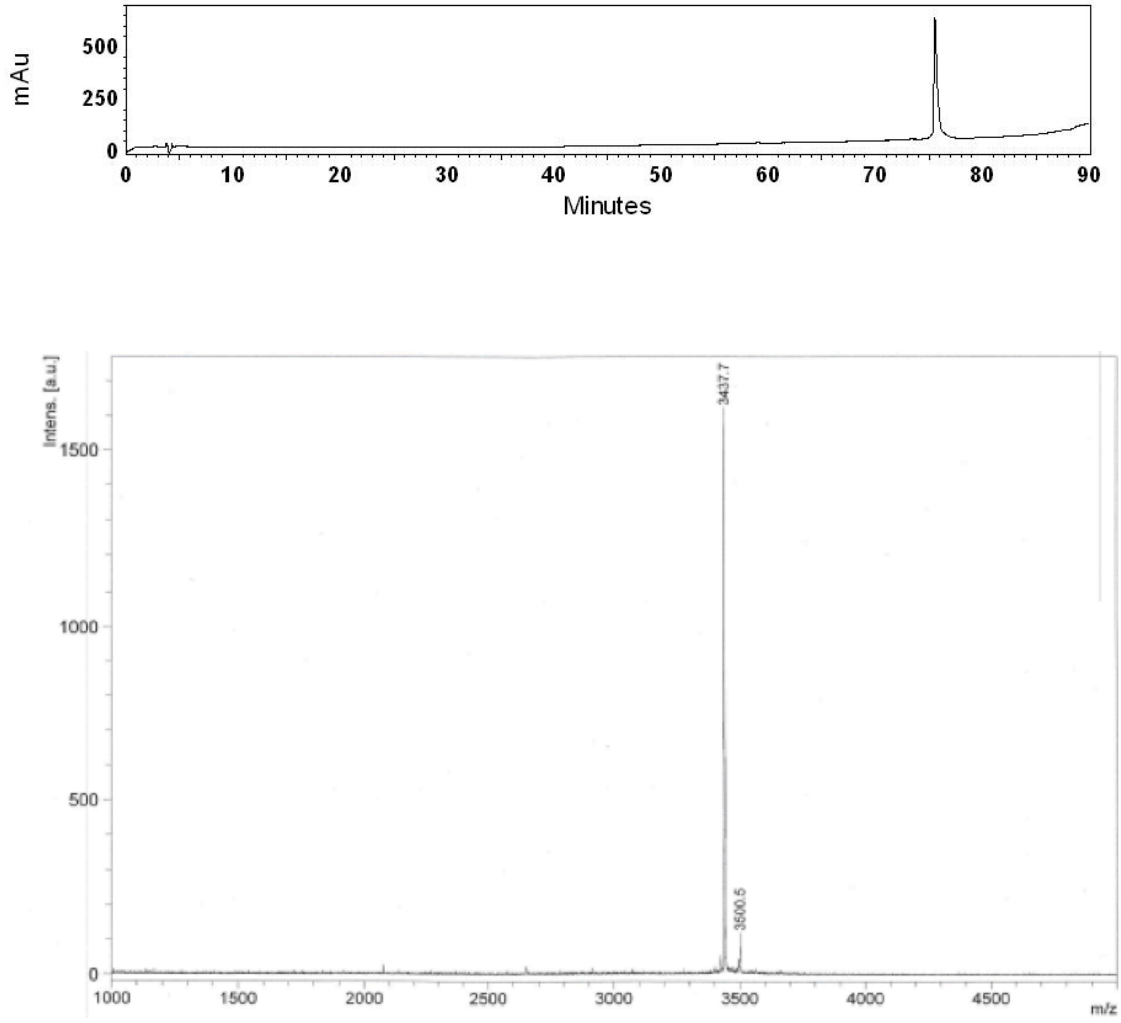
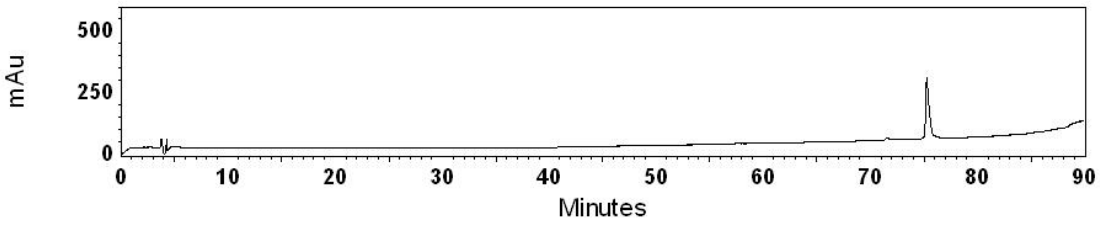


Figure S10. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **4**.

HPLC: Gradient = 10 – 90 % solvent B over 80 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3439.8, observed monoisotopic $[M+H]^+$ = 3438.4



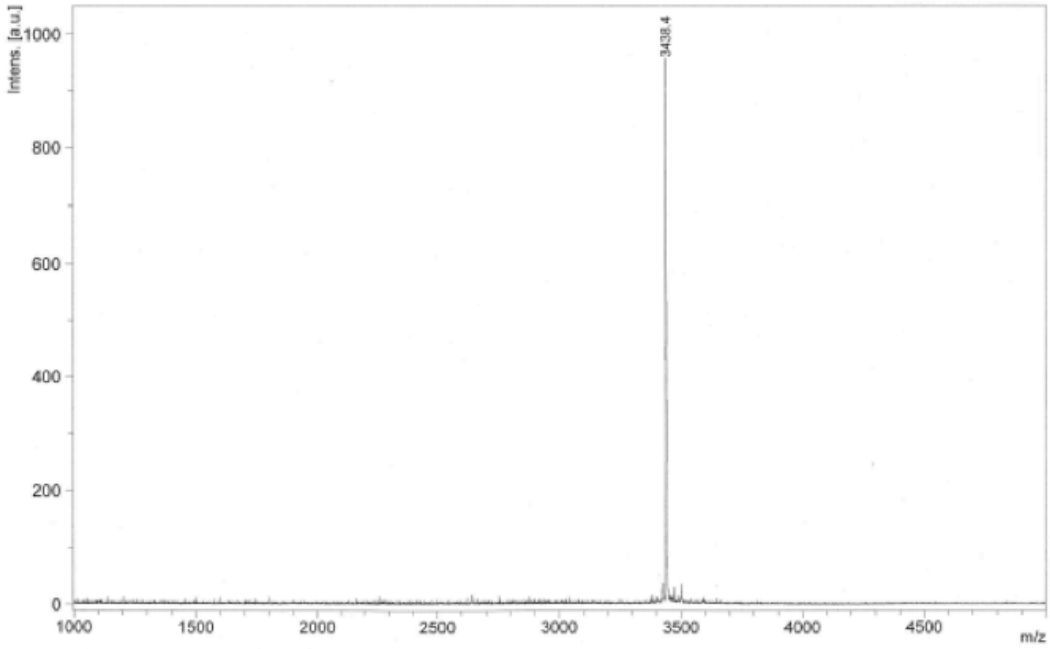


Figure S11 . Analytical HPLC trace and MALDI-TOF spectrum for α-peptide **5**.

HPLC: Gradient = 40 – 90 % solvent B over 50 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3339.7, observed monoisotopic [M+H]+ = 3339.4

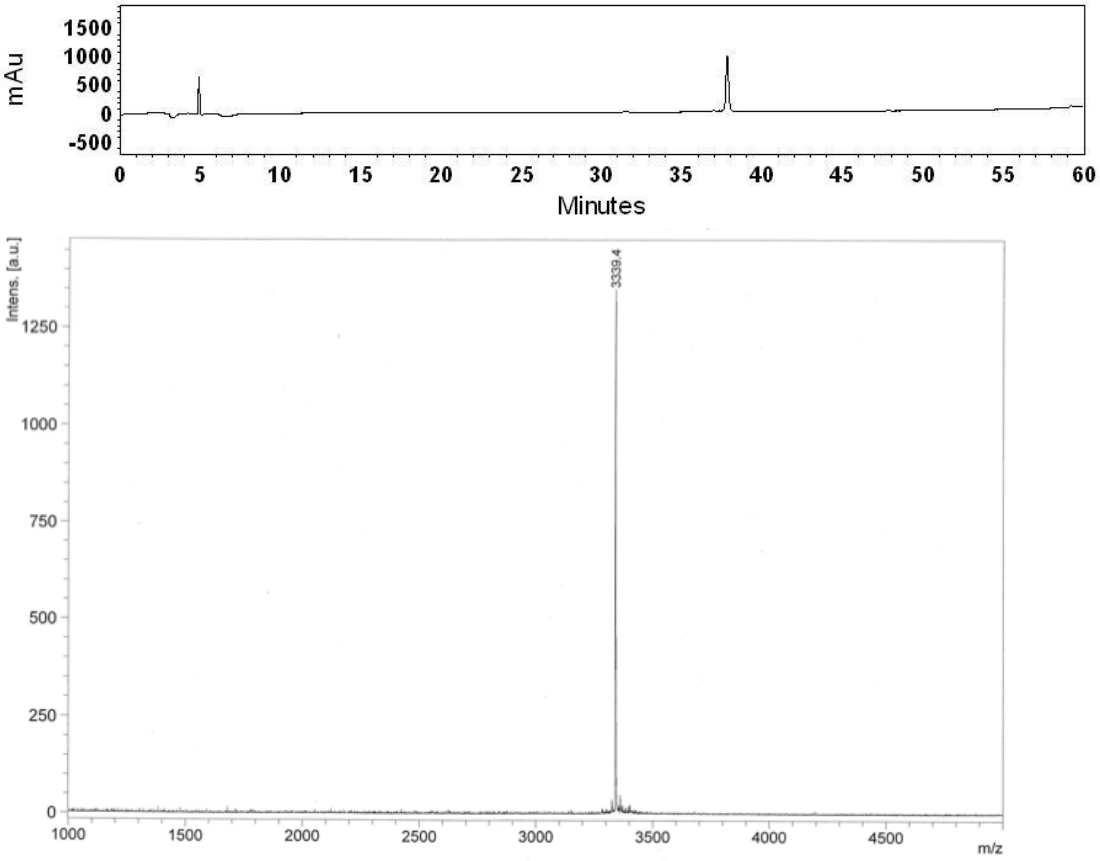


Figure S12. Analytical HPLC trace and MALDI-TOF spectrum for α-peptide **6**.

HPLC: Gradient = 40 – 90 % solvent B over 50 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3354.8, observed monoisotopic $[M+H]^+$ = 3354.0

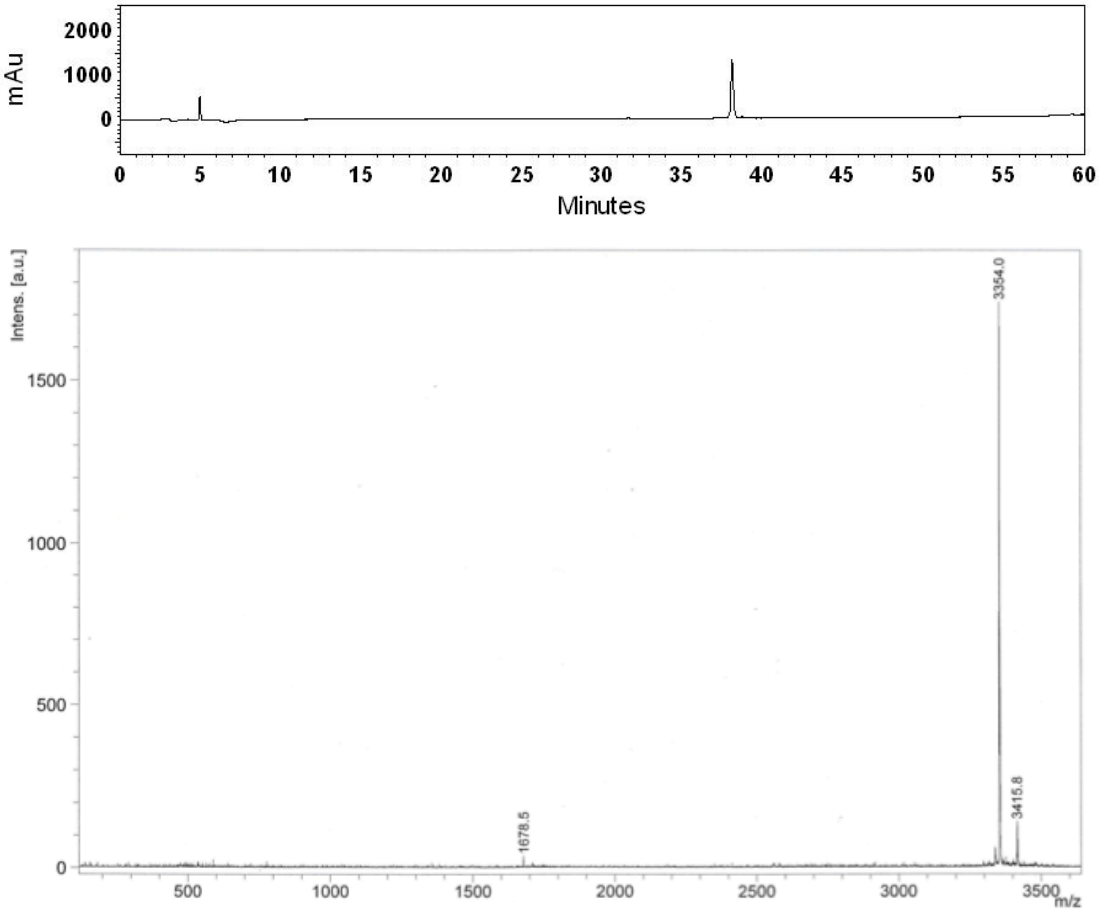
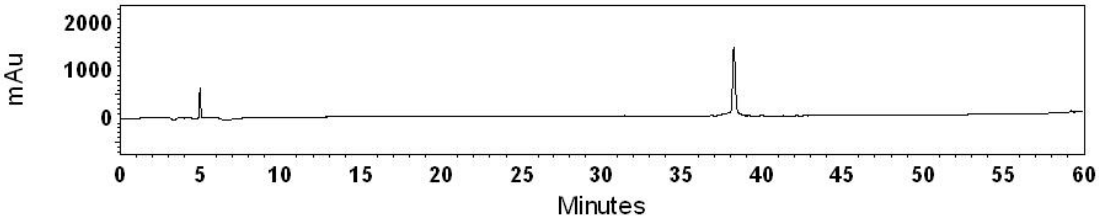


Figure S13. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **7**.

HPLC: Gradient = 40 – 90 % solvent B over 50 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3376.7, observed monoisotopic $[M+H]^+$ = 3377.0



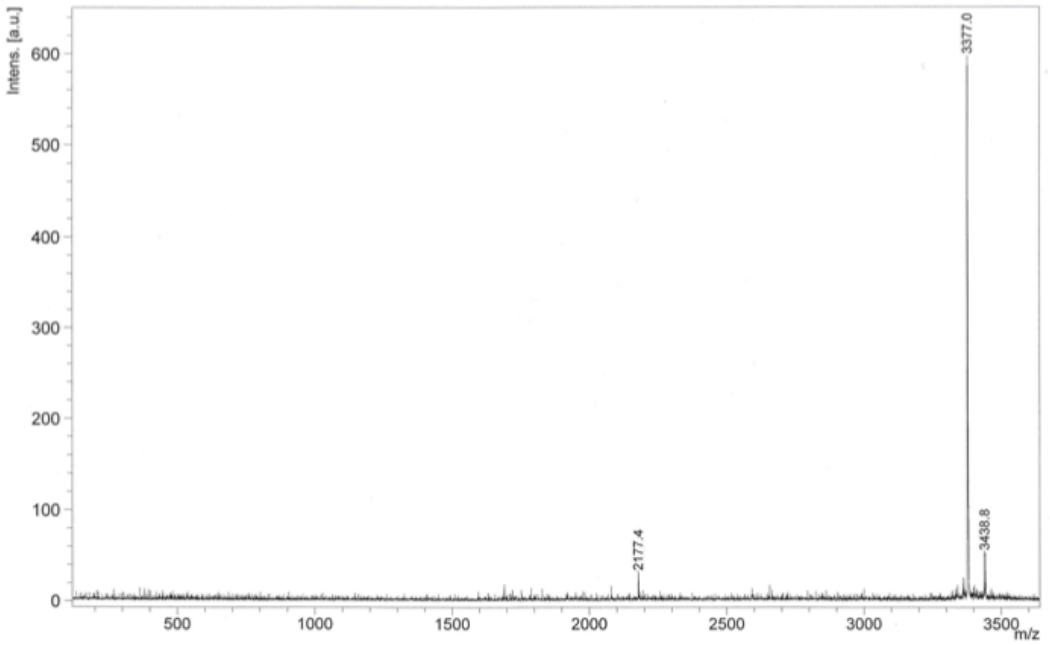


Figure S14. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **8**.

HPLC: Gradient = 40 – 90 % solvent B over 50 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3413.7, observed monoisotopic $[M+H]^+$ = 3414.0

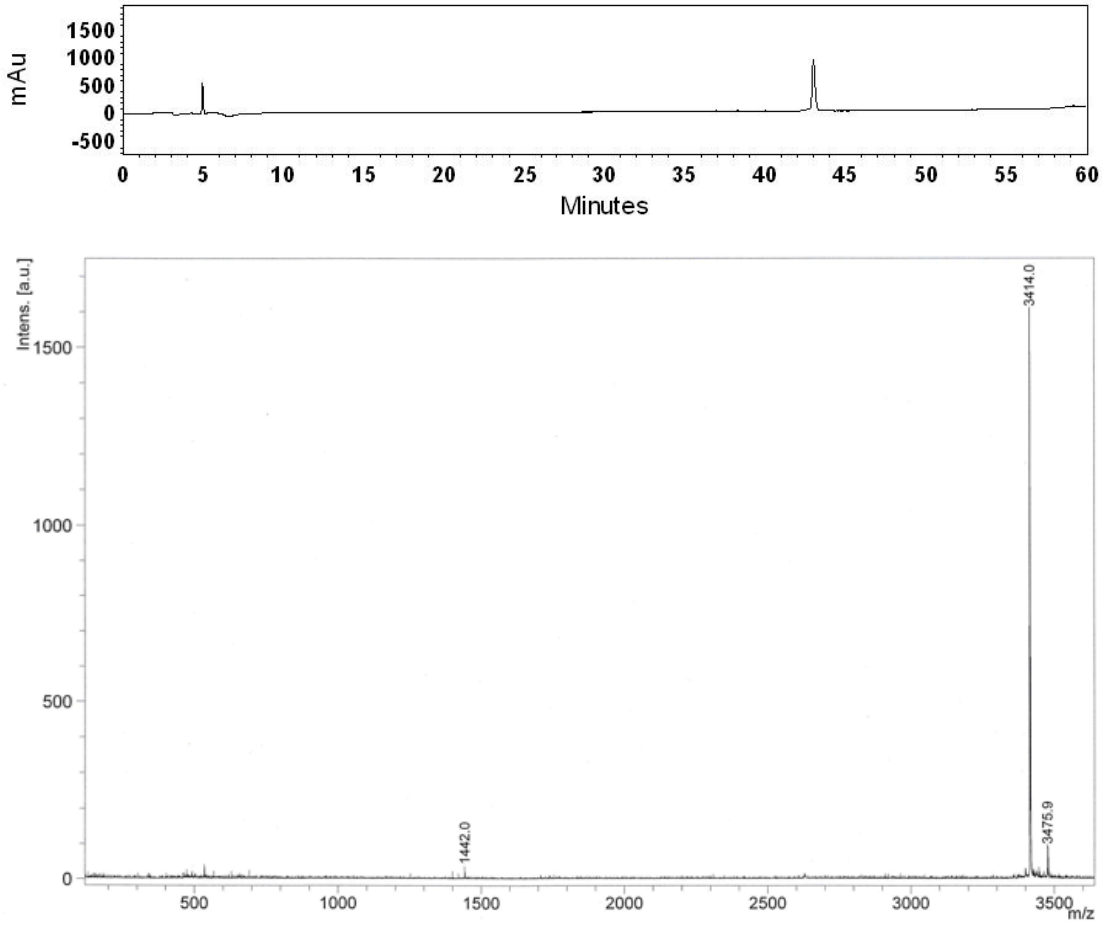
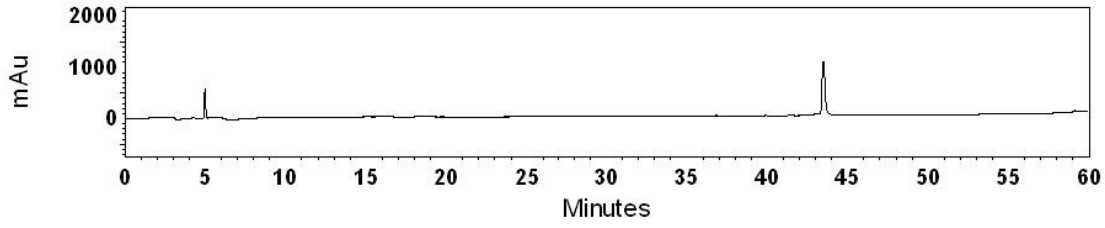


Figure S15. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **9**.

HPLC: Gradient = 40 – 90 % solvent B over 50 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3453.7, observed monoisotopic [M+H]+ = 3453.8



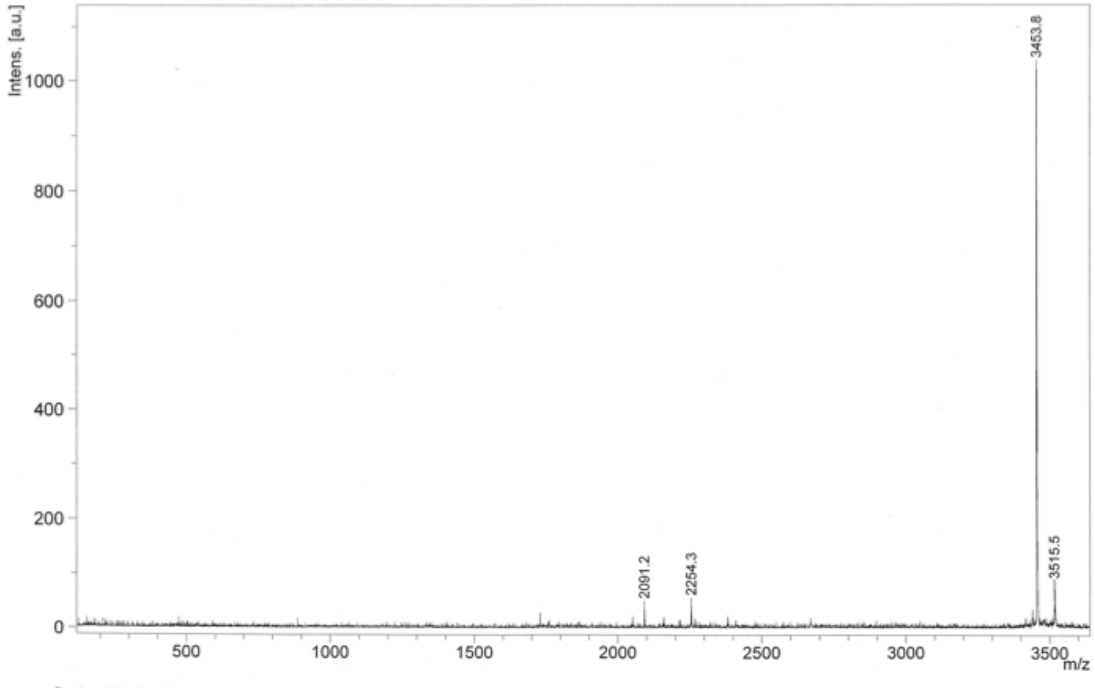
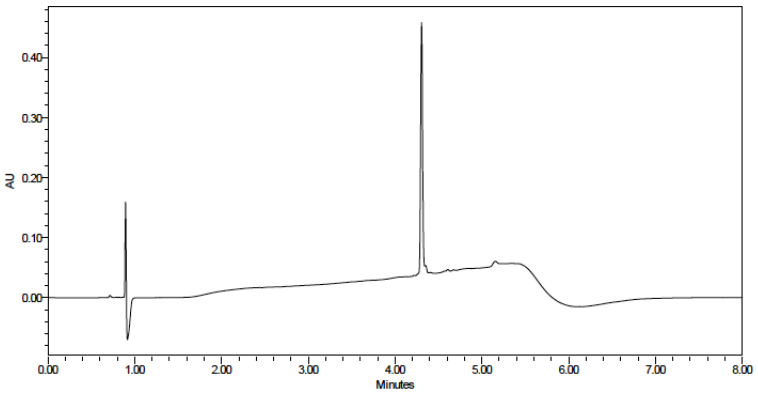


Figure S16. Analytical HPLC trace and MALDI-TOF spectrum for α/β-peptide **10**.

UPLC: Gradient = 10-95 % solvent B over 6 minutes
MALDI-TOF: Calculated monoisotopic $[M+H]^+$ = 3364.7, observed monoisotopic [M+H]+ = 3363.9



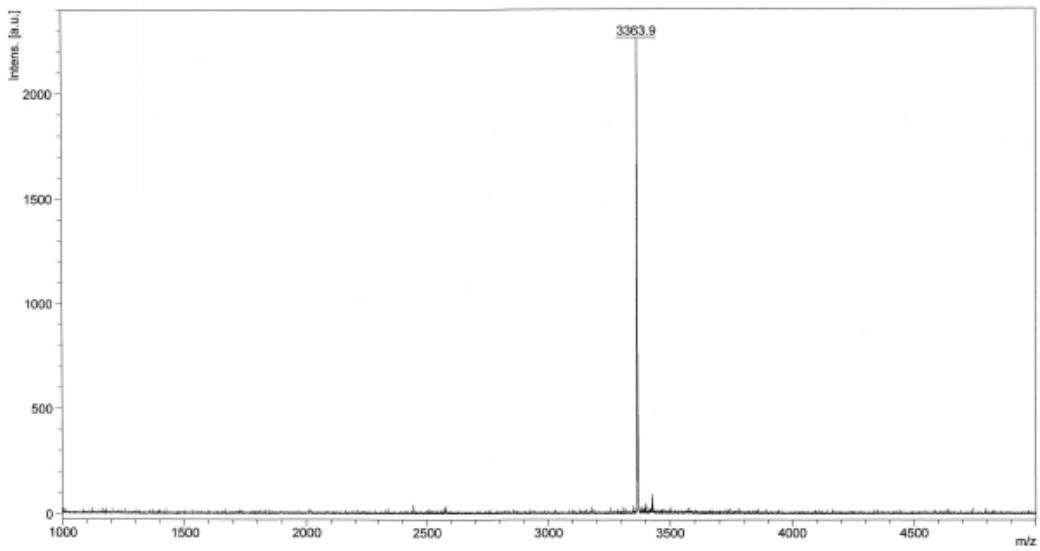
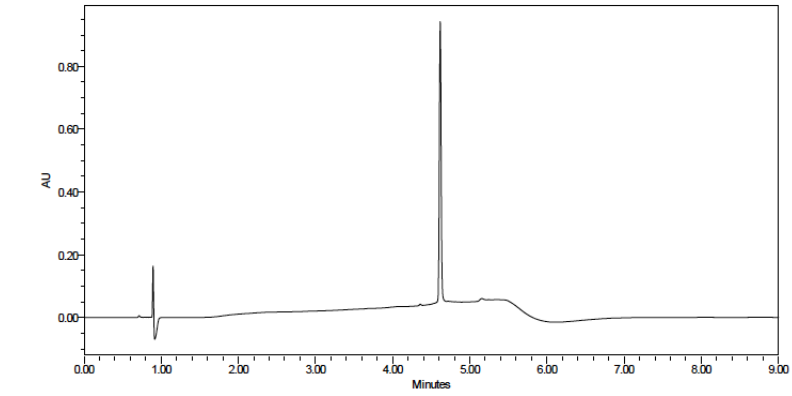


Figure S17. UPLC trace and MALDI-TOF spectrum for α/β-peptide **11**.

UPLC: Gradient = 10-95% solvent B over 6 minutes
MALDI-TOF: Calculated monoisopotic $[M+H]^+$ = 3431.7, observed monoisotopic [M+H]+ = 3431.5



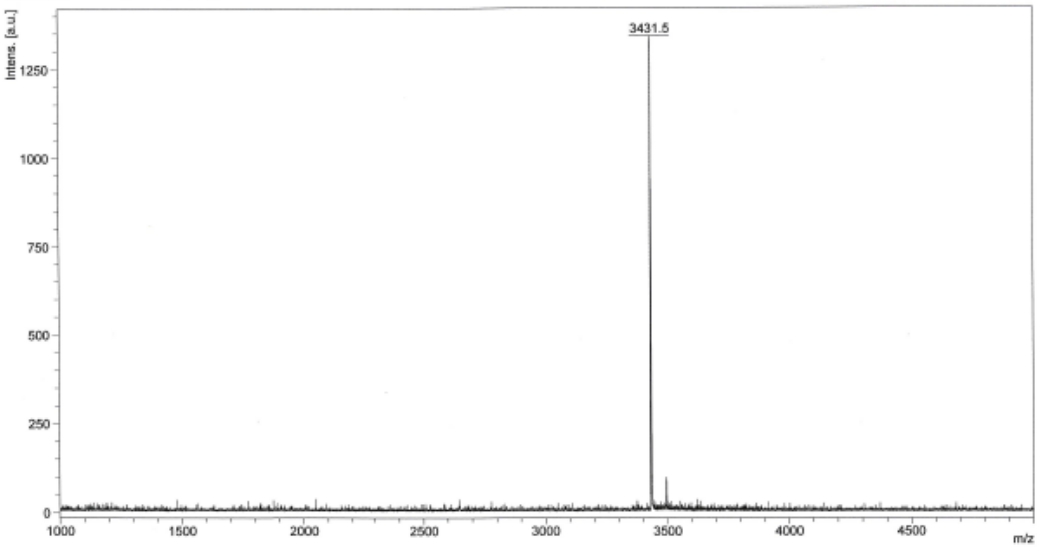
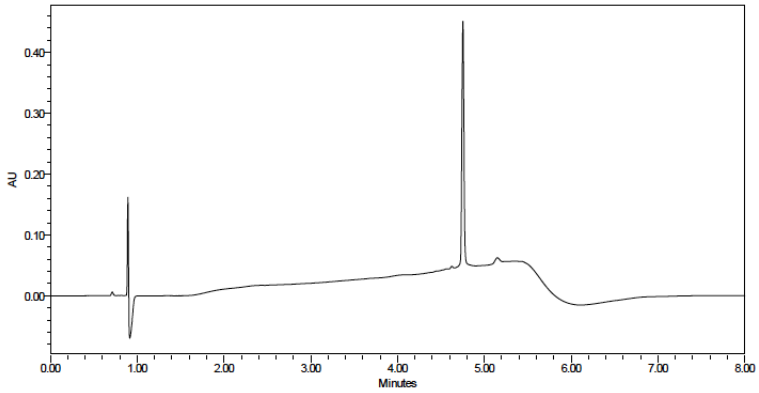


Figure S18. UPLC trace and MALDI-TOF spectrum for α/β-peptide **12**.

UPLC: Gradient = 10-95% solvent B over 6 minutes
MALDI-TOF: Calculated monoisopotic $[M+H]^+$ = 3390.7, observed monoisotopic [M+H]+ = 3390.6



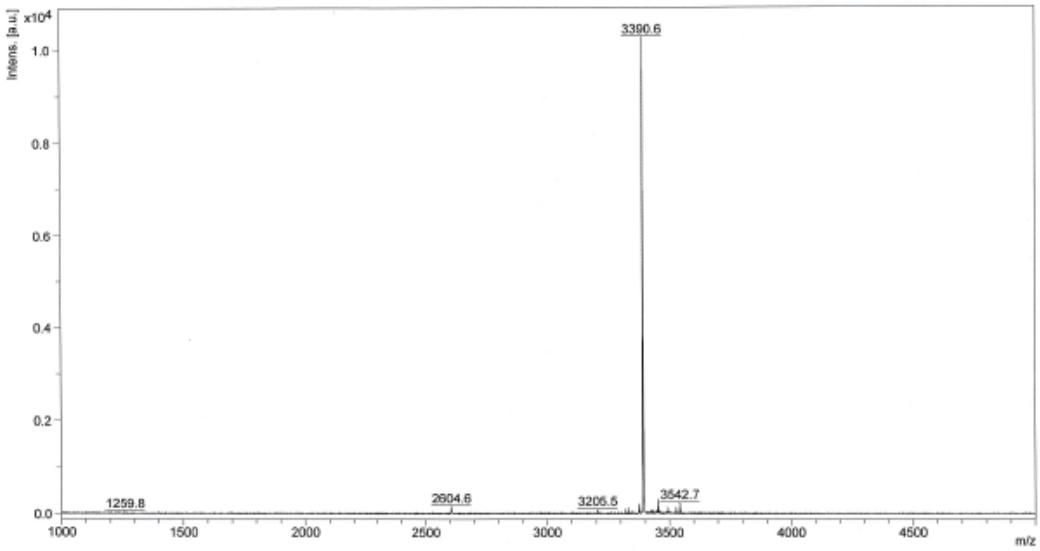
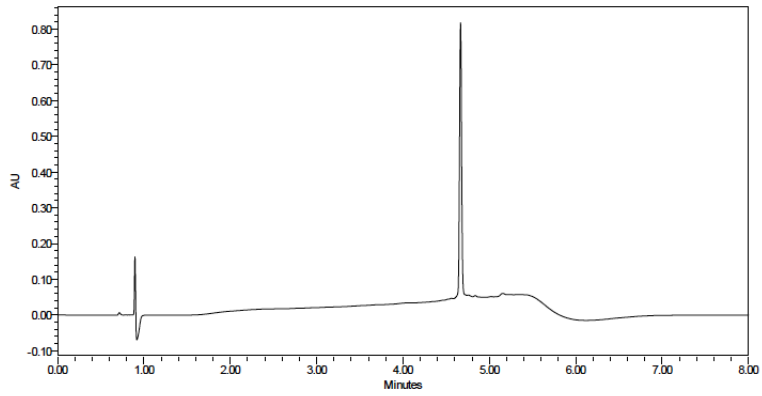
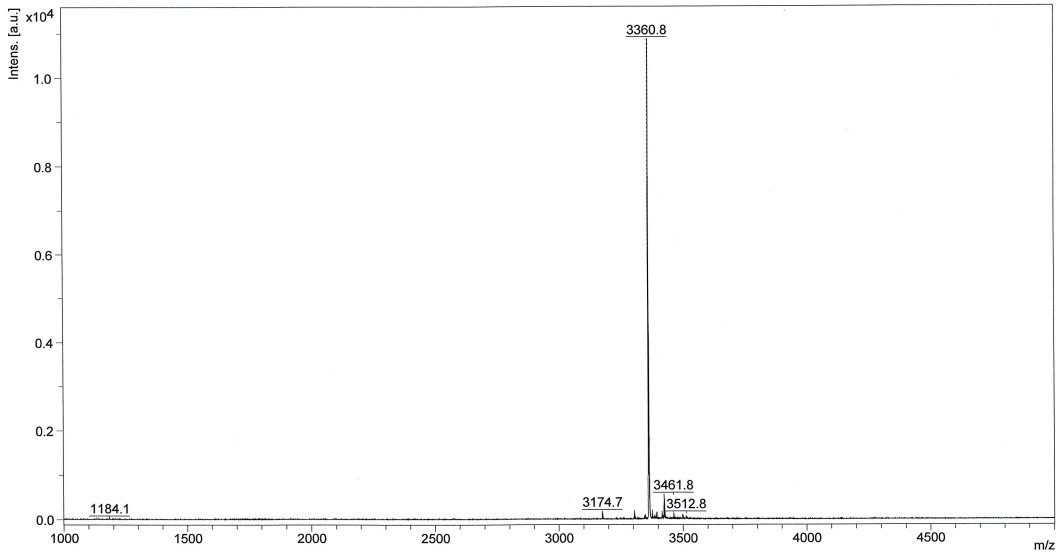


Figure S19. UPLC trace and MALDI-TOF spectrum for α/β-peptide **13**.

UPLC: Gradient = 10-95% solvent B over 6 minutes
MALDI-TOF: Calculated monoisopotic $[M+H]^+$ = 3360.7, observed monoisotopic [M+H]+ = 3360.8





References

1. Lee, H.S.; LePlae, P.R.; Porter, E.P.; Gellman, S.H. *J. Org. Chem.* **2001***, 66*, 3597 - 3599.

2. Horne, W.S.; Price J.L.; Gellman, S.H. *Proc Natl Acad Sci USA* **2008,** *105*, 9151–9156.

3. Jorgenson, R.; Kubale, V.; Vrecl, M.; Schwartz, T.W.; Elling, C.E. *J. Pharmacol. Exp. Ther.* **2007**, *342*, 148-154.

4. Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A., 3rd; Smith, H. O. *Nat. Meth.* **2009***, 6*, 343-345.

5. Gill S.C.; von Hippel, P.H. *Anal. Biochem.* **1989** *182*, 319 - 326.