# Supplemental Methods and Characterization for:

## A Potent α/β-Peptide Analogue of GLP-1 with Prolonged Action In Vivo

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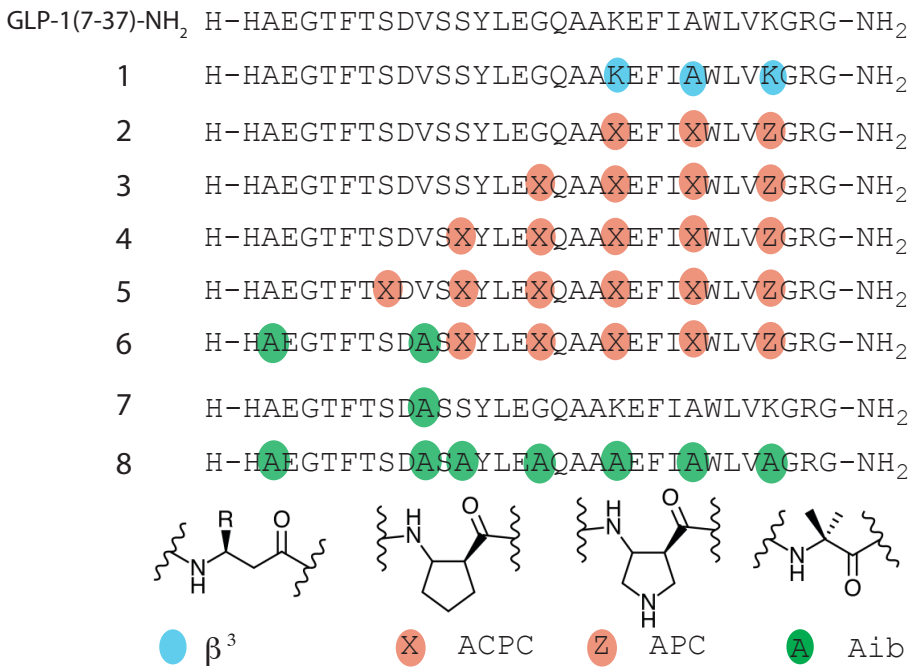
## Peptide Synthesis and Purification

Peptides were synthesized on NovaPEG rink amide resin (NovaBiochem) using previously reported microwave-assisted solid-phase conditions, based on Fmoc protection of main chain amino groups.[1] Peptides were synthesized on a scale of 25 μmoles. Briefly, 4 equivalents (100 μmoles) of protected amino acids were activated with 100 μmoles of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and of N-hydroxybenzotriazole (HOBt) in the presence of 200 μmoles N,N-diisopropylethylamine (DIEA). The growing peptide chain was deprotected using 2 mL of 20% piperdine in DMF. Cyclic β-amino acids were prepared using previously published methods.[2,3] Protected $\beta^3$-homoamino acids were purchased from PepTech.

After synthesis, the peptides were cleaved from the resin and side chains were deprotected using 2 mL trifluoroacetic acid (TFA), 50 uL water, and 50 uL triisopropylsilane for three hours. The TFA solution was dripped into cold ether to precipitate the deprotected peptide. Peptides were purified on a prep-C18 column using Reverse Phase-HPLC. Purity was assessed by RP-HPLC (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile, C18 analytical column (4.6 X 250 mm), flow rate 1 mL/min, gradient 10-60% B solvent over 50 minutes). Masses were measured by MALDI-TOF-MS.

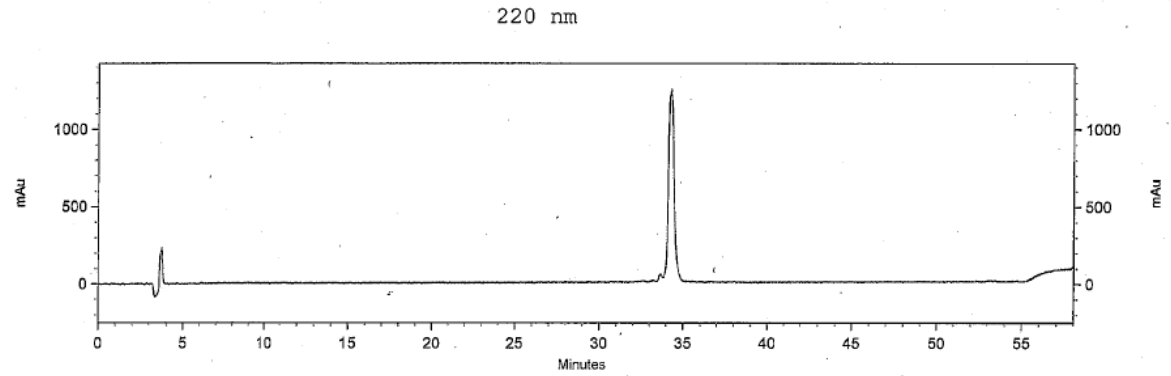
**Supplementary Figure 1**: Peptide key for supplementary information.

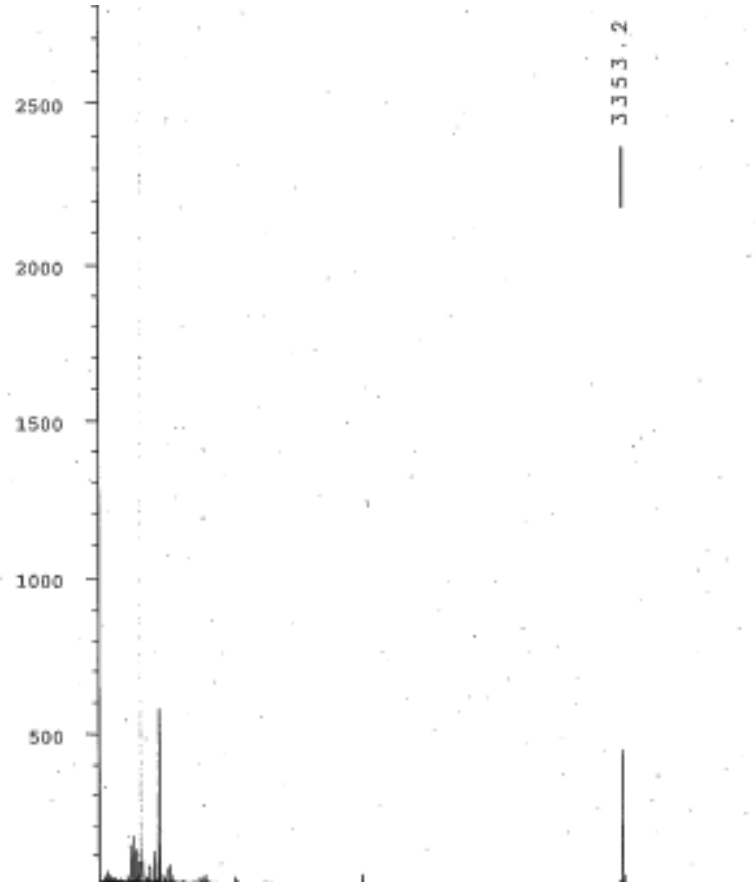


**Supplementary Table 1**: MALDI-TOF-MS data; expected and observed values (monoisotopic $[M+H]^+$).

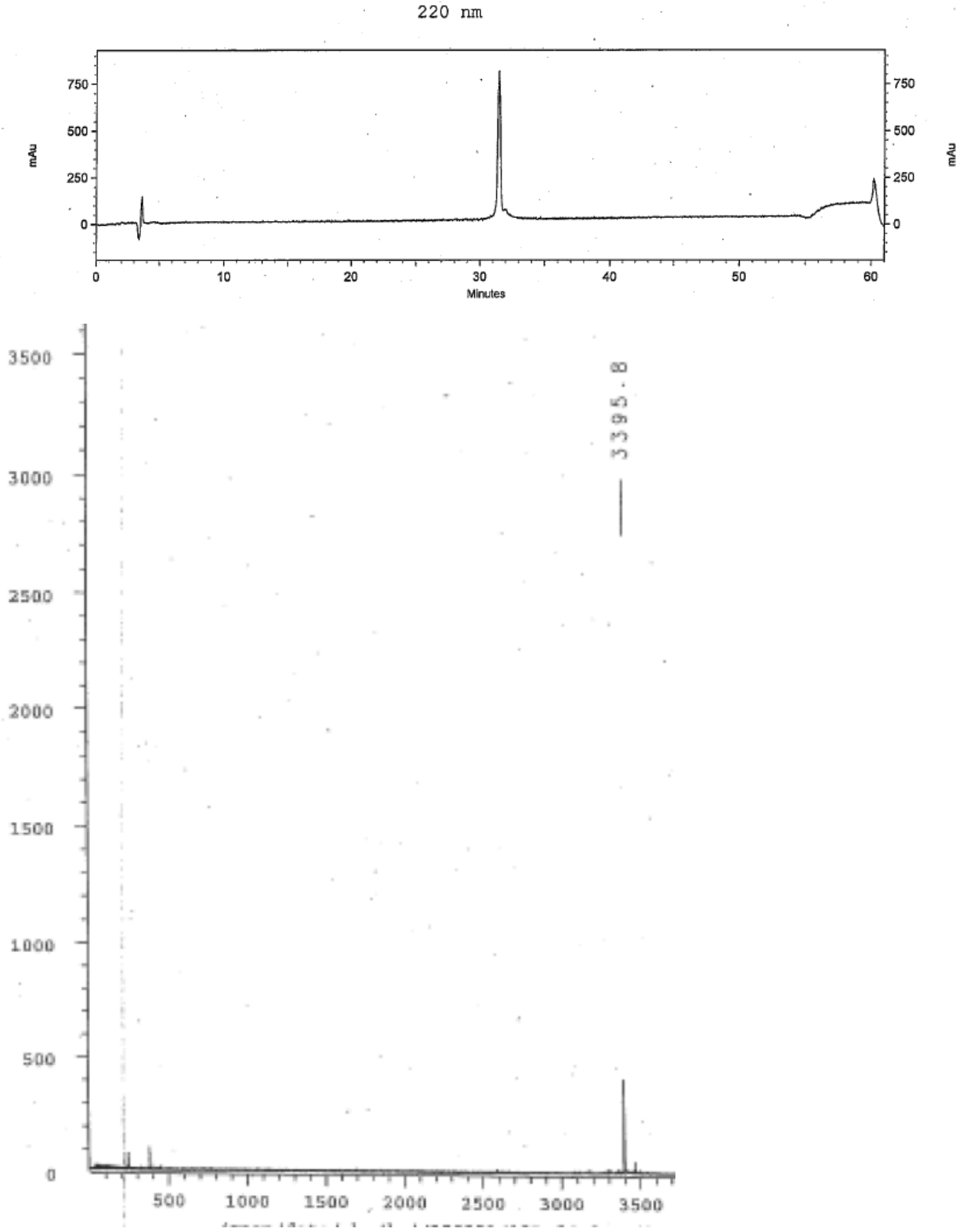
| | Expected Mass (M+H) (m/z) | Observed Mass (m/z) |
|---|---|---|
| GLP-1(7-37)-$NH_2$ | 3353.7 | 3353.2 |
| 1 | 3395.7 | 3395.8 |
| 2 | 3360.6 | 3361.8 |
| 3 | 3414.7 | 3414.7 |
| 4 | 3438.7 | 3439.1 |
| 5 | 3462.8 | 3463.3 |
| 6 | 3438.7 | 3438.3 |
| 7 | 3339.6 | 3341.0 |
| 8 | 3307.6 | 3307.9 |

**Supplementary Figure 2**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of GLP-1(7-37)-$NH_2$.

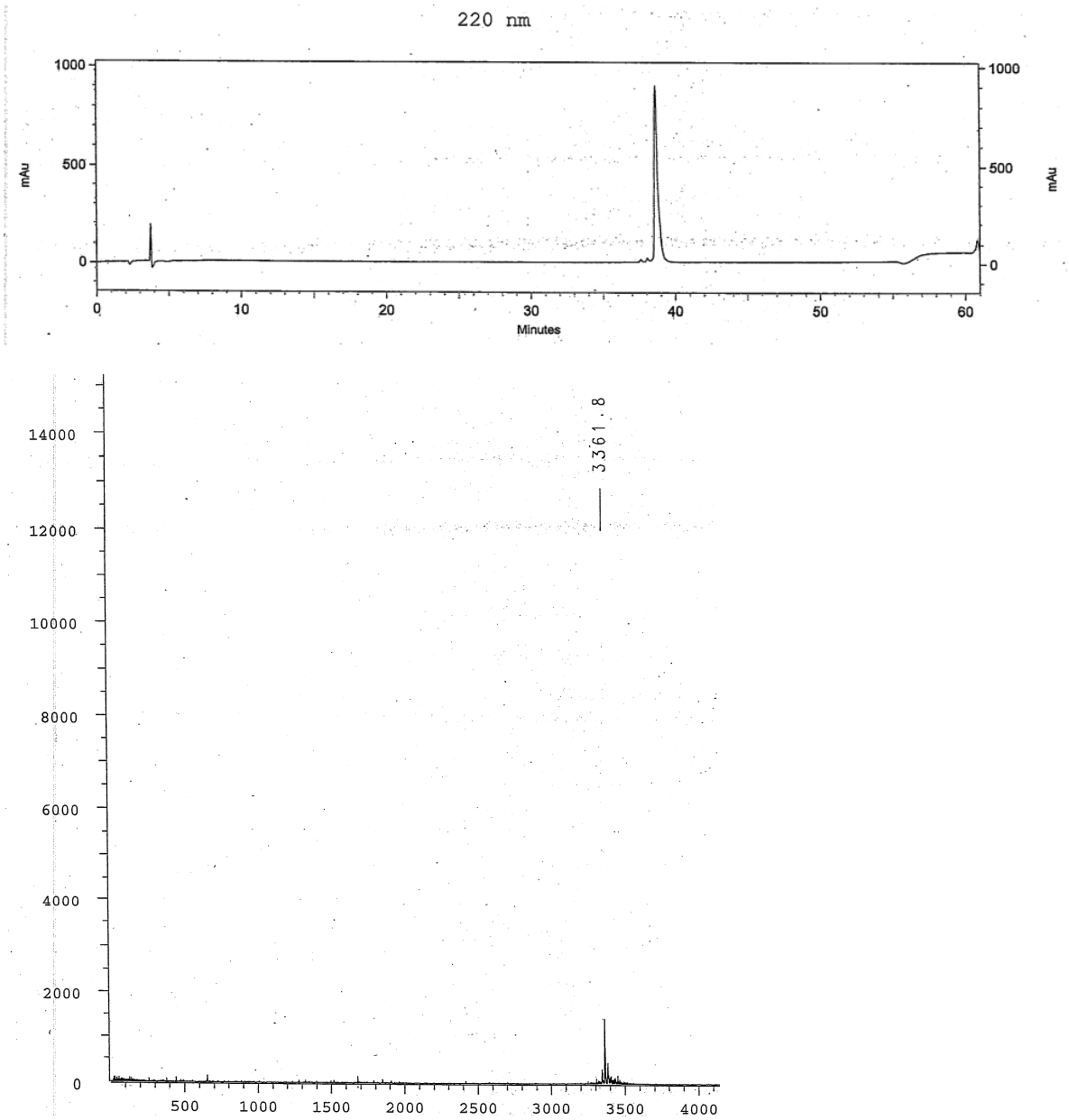


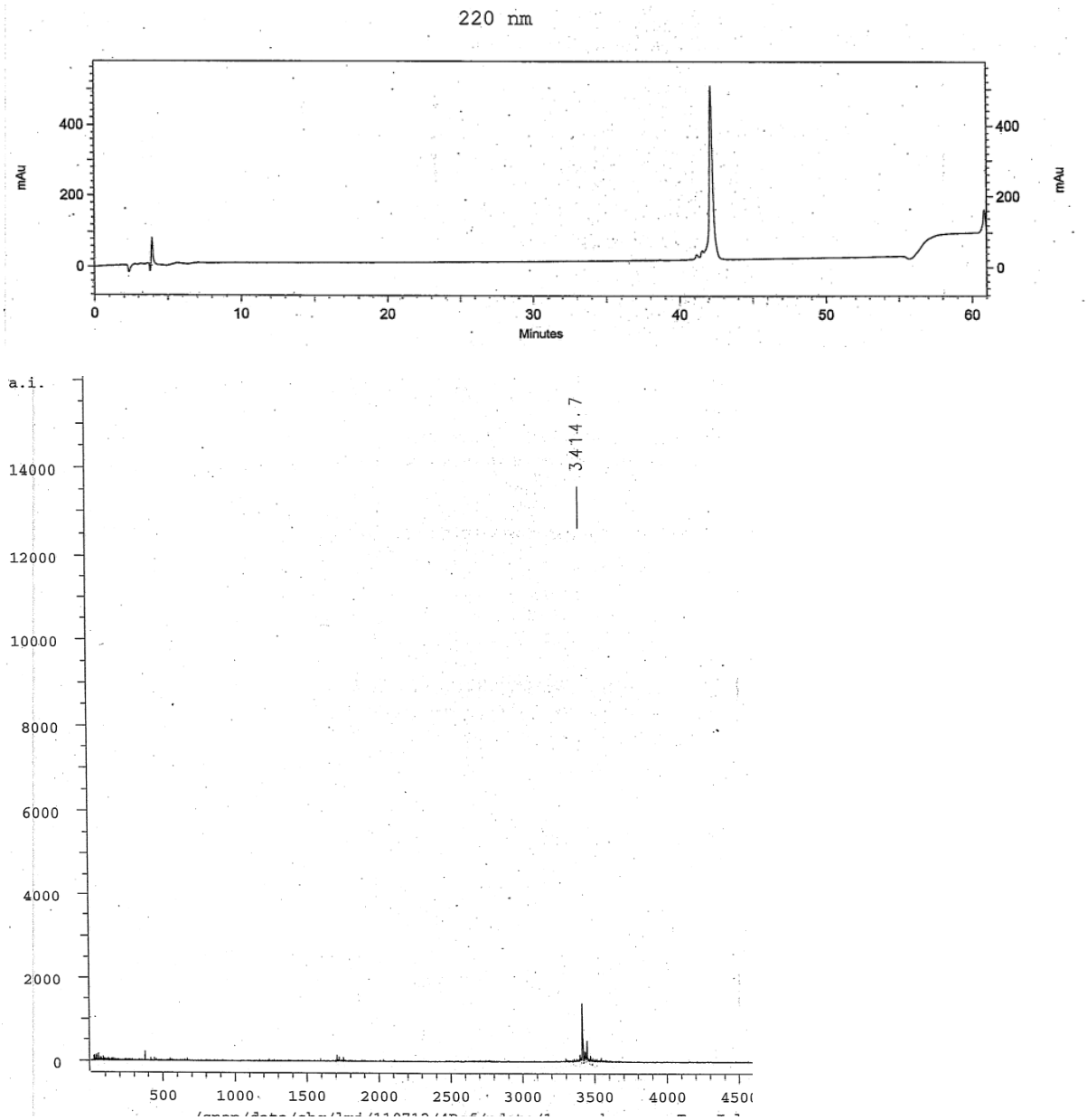
**Supplementary Figure 3**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **1**.



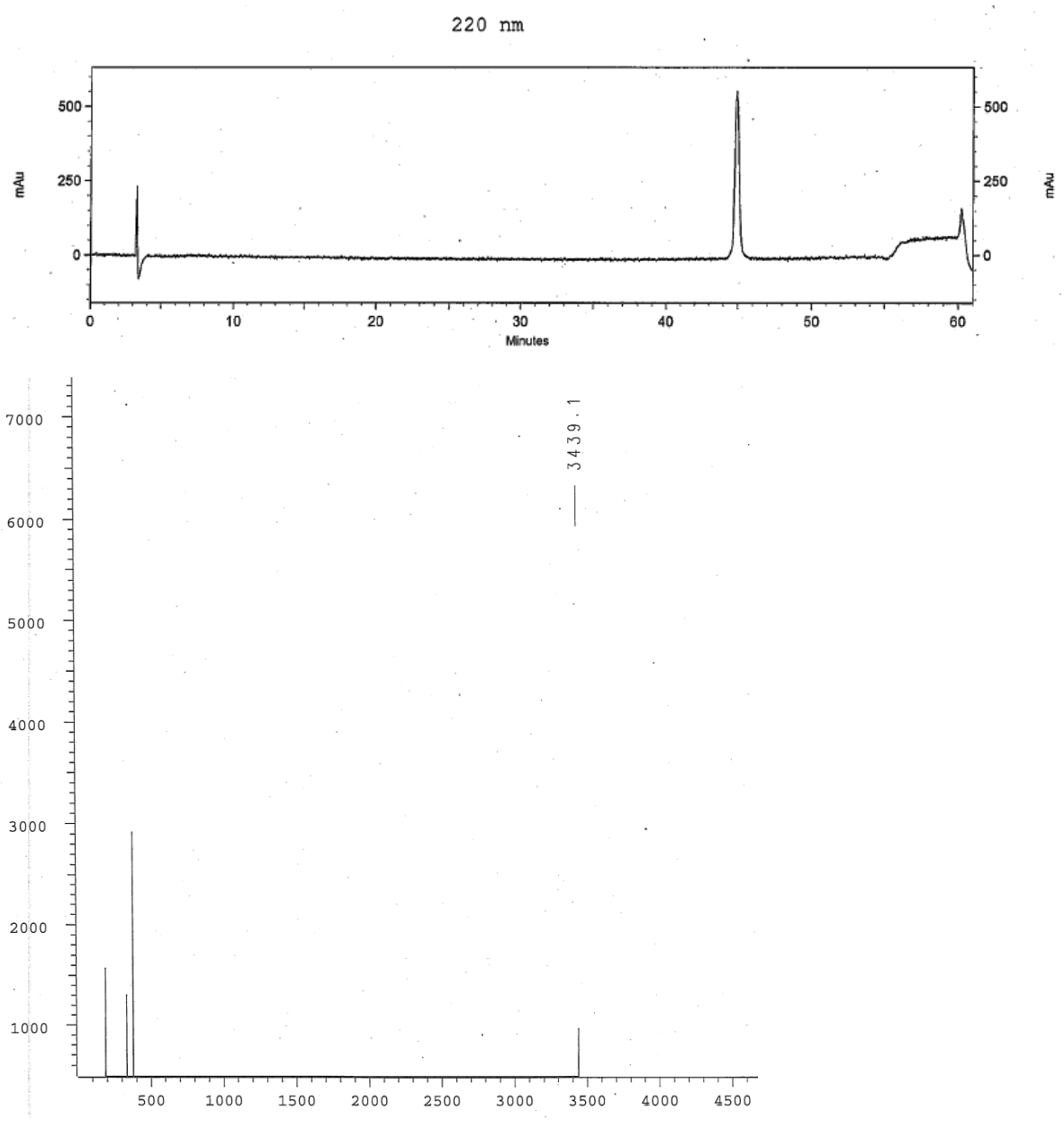
**Supplementary Figure 4**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **2**.



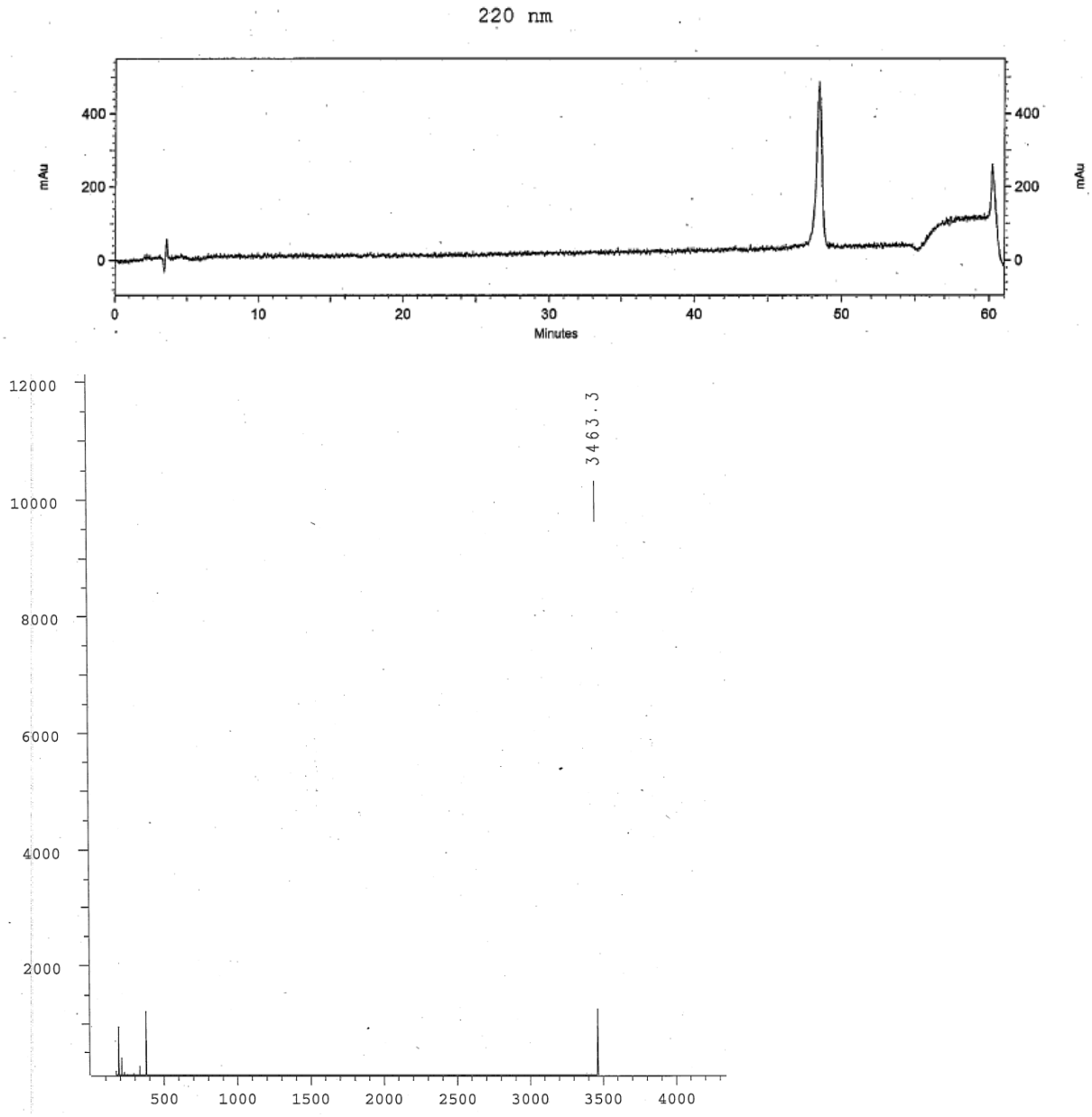
**Supplementary Figure 5**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **3**.



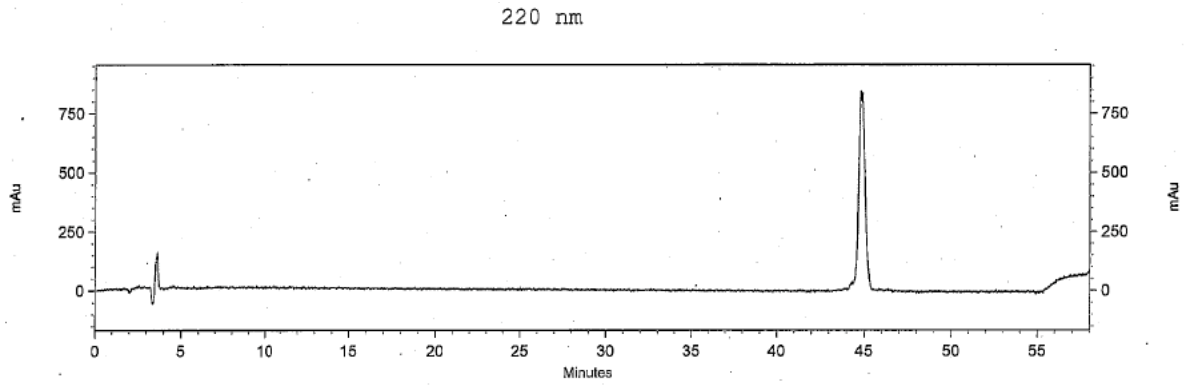
**Supplementary Figure 6**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **4**.

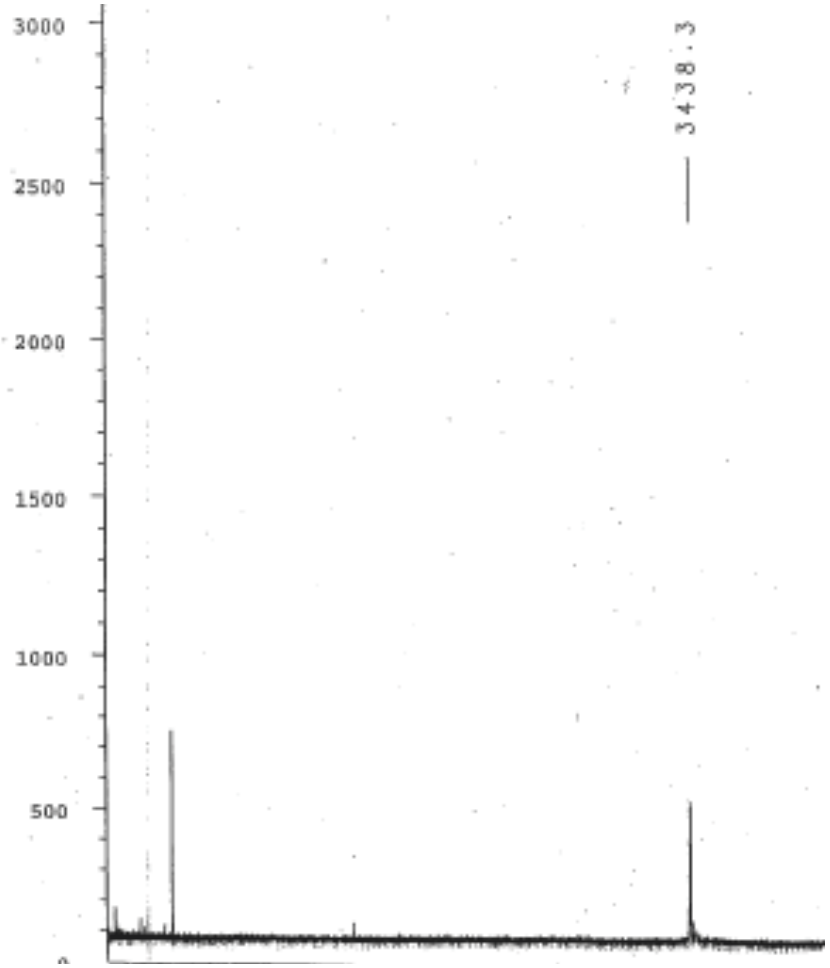


**Supplementary Figure 7**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **5**.

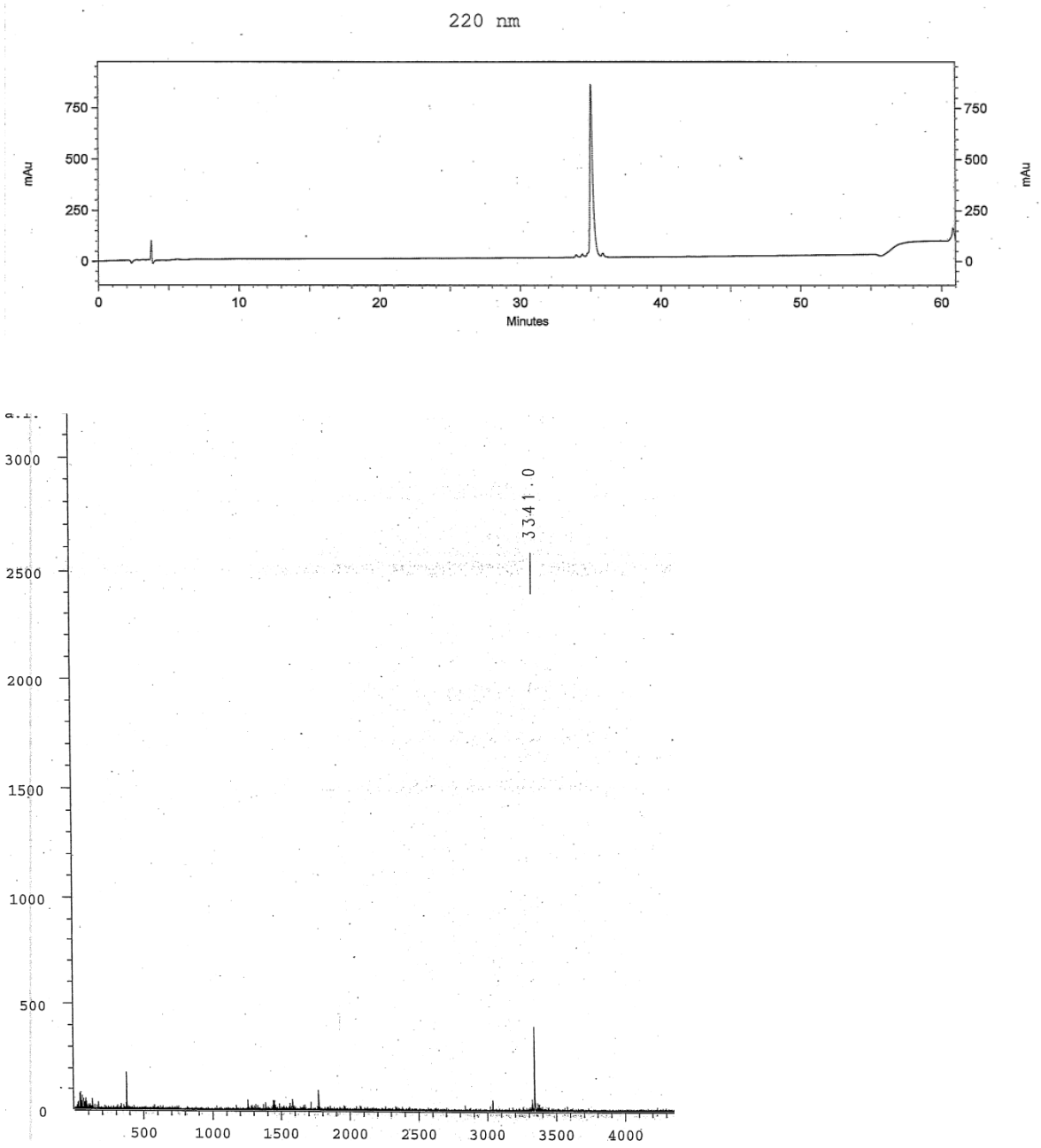


**Supplementary Figure 8**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of α/β-peptide **6**.

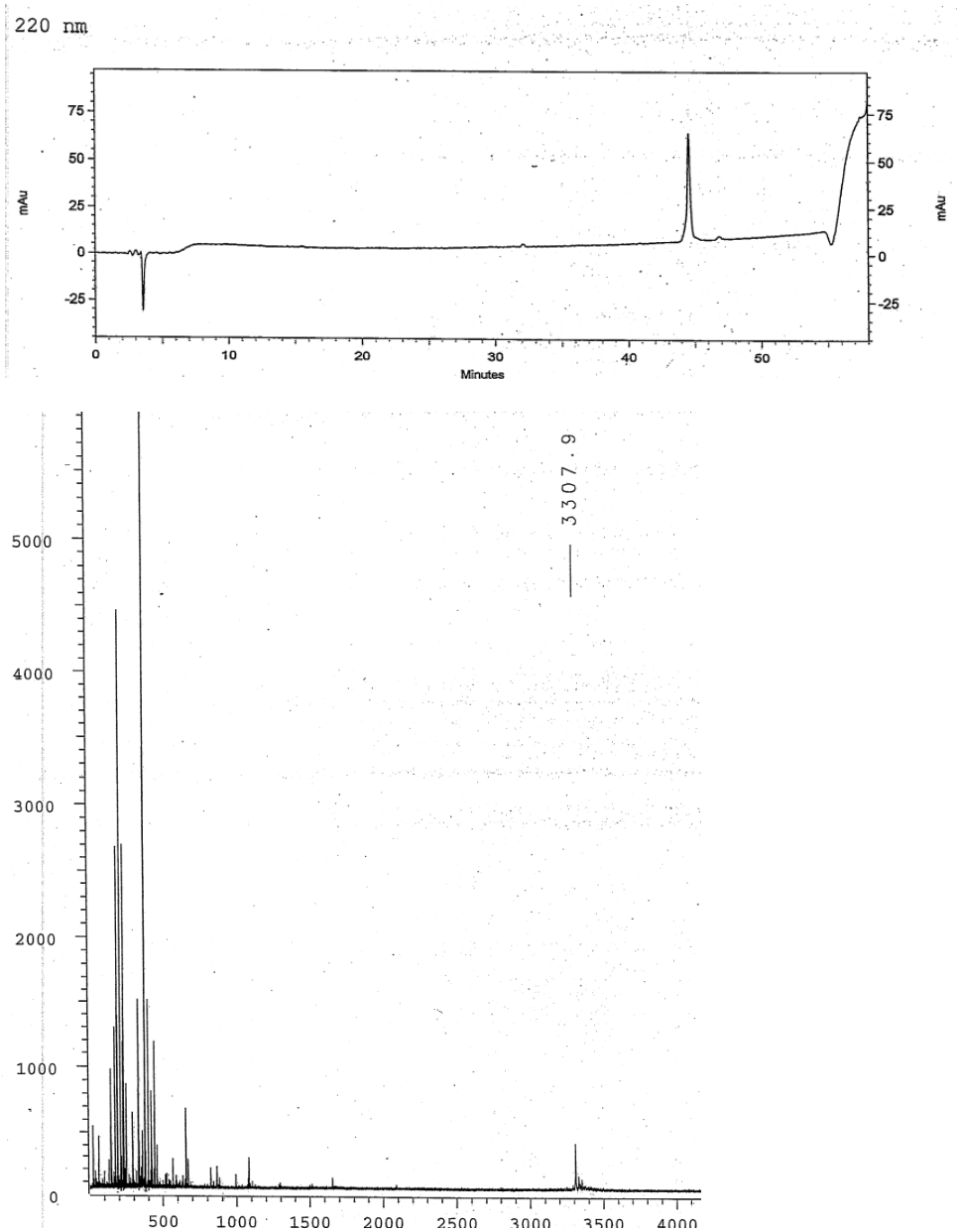


**Supplementary Figure 9**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of peptide **7**.



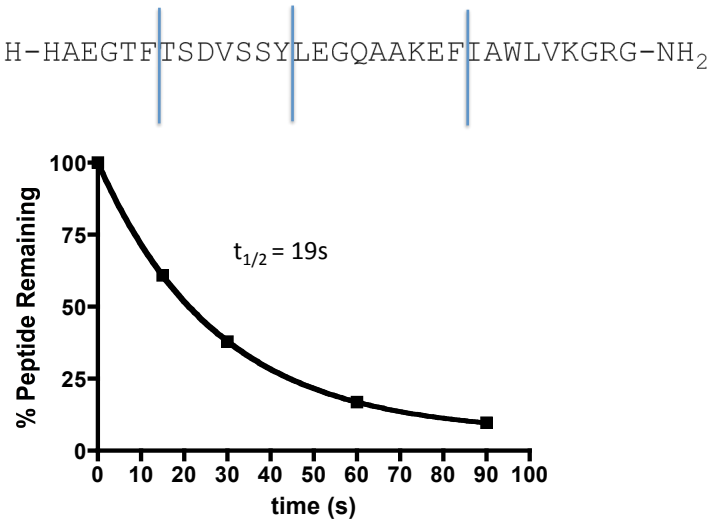
**Supplementary Figure 10**: Analytical HPLC trace (top) and MALDI-TOF spectrum (bottom) of peptide **8**.



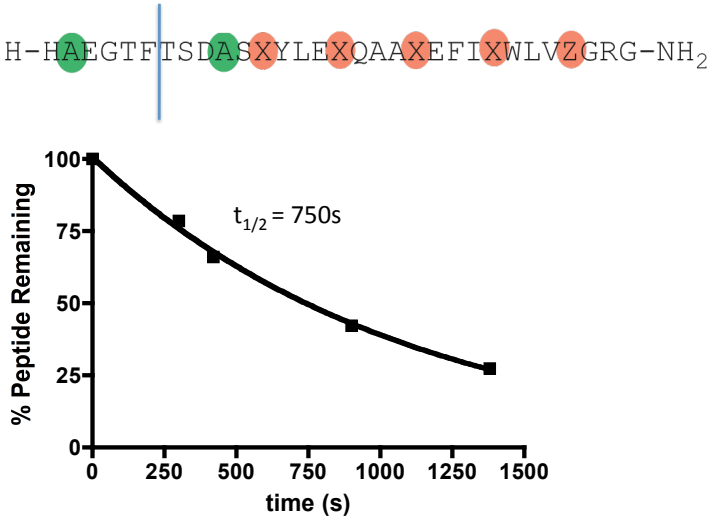
**Methods for Protease Assay**

We used an HPLC assay to assess protease stability.[4] Peptide solutions were prepared in water, and their concentration was determined by UV-vis. Two nmoles of peptide was distributed into 1.7 mL eppendorf tubes and lyophilized (6 eppendorf tubes were prepared for each peptide, 3 replicates per protease). Peptides were reconstituted in 40 μL of TBS pH 8.0 (resulting concentration of peptide 40 μM) before protease was added. Chymotrypsin was purchased from Promega (catalog # V1062) and Neprilysin was purchased from Reprokine (catalog # RKP08473). Both came in pre-weighed vials (25 μg of chymotrypsin and 20 μg of NEP), and 100 μL of water was added to form the stock solution of the respective protease. For the chymotrypsin test, 40 μL of TBS buffer was added to 2 nmoles of lyophilized peptide, and 10 μL of 250 μg/mL chymotrypsin was added (final conc 50 μg/mL). For the NEP test, 40 μL of TBS buffer was added to 2 nmoles of lyophilized peptide, and 10 μL of 200 μg/mL of NEP was added (final concentration 40 μg/mL). Both reactions were at room temperature. The reaction was timed and quenched by removing 10 μL of solution and pipetting it into 100 μL of 1%TFA solution (5 data points could be taken per sample). A portion (100 μL) of the quenched reaction solution was injected into an HPLC (see peptide synthesis, analytical conditions), and peaks were analyzed. The time course of peptide degradation was experimentally determined by integrating the area of each peak in a series of HPLC traces. The final 10 μL of the quenched reaction solution was used to acquire MALDI-TOF and MS-MS data for identification of each peptide fragment. Each protease test was repeated a second time to ensure the half-life obtained was reproducible with the given conditions. Shown below are time course data for peptide degradation (exponential decay curves and half lives were calculated using GraphPad Prism) as well as the protease cleavage sites in each substrate (solid lines in peptide key; based on MS data).

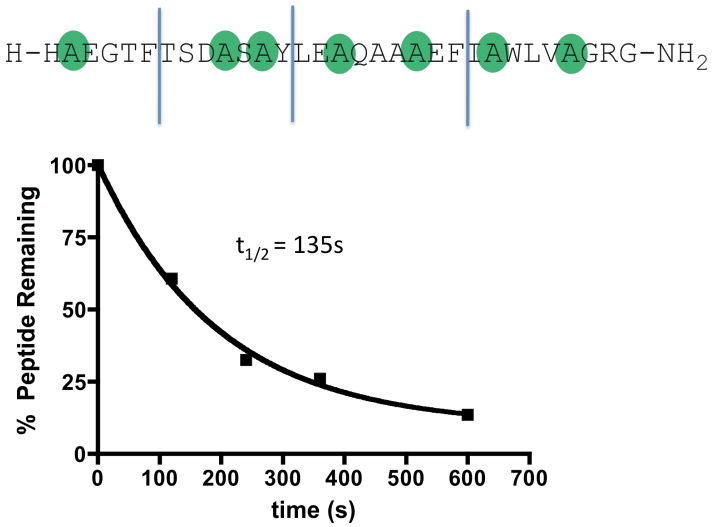
**Supplementary Figure 11**: Protease assay of 40 μM GLP-1(7-37)$NH_2$ with 50 μg/mL chymotrypsin in TBS pH 8.0 at room temperature. Major backbone cut sites, as determined by MALDI-TOF-MS, are indicated with vertical blue lines.



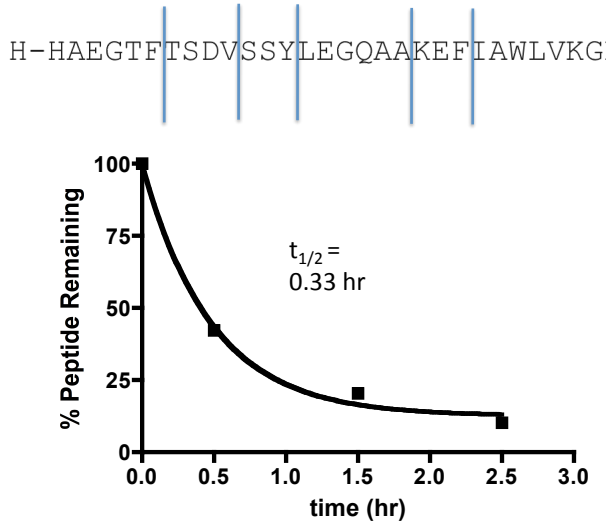

**Supplementary Figure 12**: Protease assay of 40 μM α/β-peptide **6** with 50 μg/mL chymotrypsin in TBS pH 8.0 at room temperature. Major backbone cut site, as determined by MALDI-TOF-MS, is indicated with a vertical blue line.



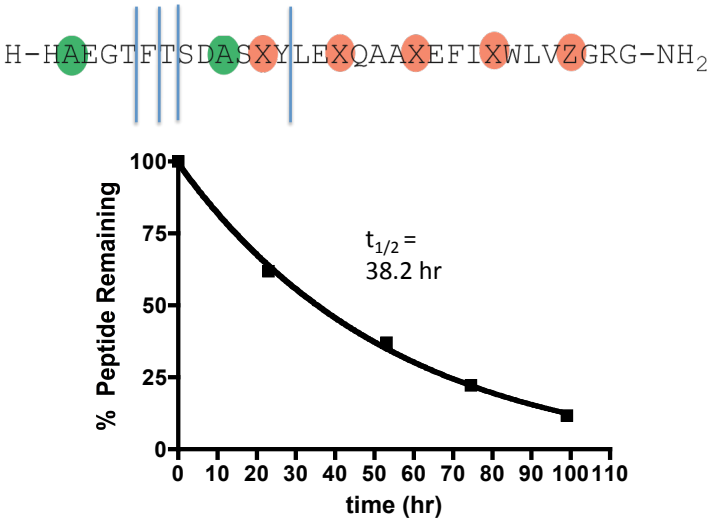

**Supplementary Figure 13**: Protease assay of 40 μM peptide **8** with 50 μg/mL chymotrypsin in TBS pH 8.0 at room temperature. Major backbone cut sites, as determined by MALDI-TOF-MS, are indicated with vertical blue lines.



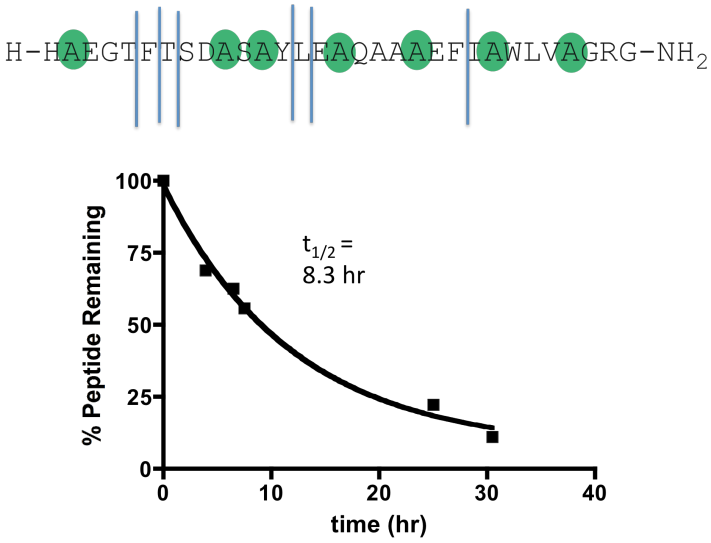

**Supplementary Figure 14**: Protease assay of 40 μM GLP-1(7-37)$NH_2$ with 40 μg/mL neprilysin in TBS pH 8.0 at room temperature. Major backbone cut sites, as determined by MALDI-TOF-MS, are indicated with vertical blue lines.




**Supplementary Figure 15**: Protease assay of 40 μM α/β-peptide **6** with 40 μg/mL neprilysin in TBS pH 8.0 at room temperature. Major backbone cut sites, as determined by MALDI-TOF-MS, are indicated with vertical blue lines.



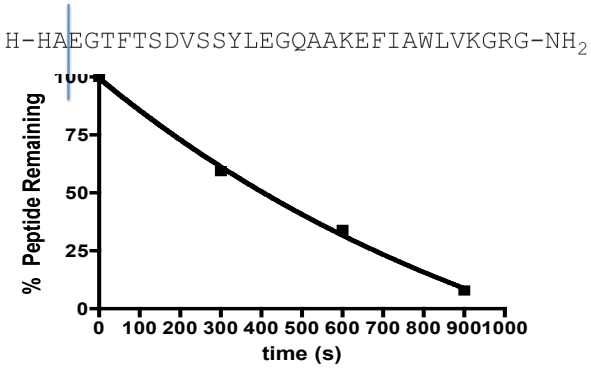

**Supplementary Figure 16**: Protease assay of 40 μM peptide **8** with 40 μg/mL neprilysin in TBS pH 8.0 at room temperature. Major backbone cut sites, as determined by MALDI-TOF-MS, are indicated with vertical blue lines.




Analogues of GLP-1(7-37)-$NH_2$ that contain Aib at position 8 are reported not to be substrates for DPP-4;[4] therefore, we expected that α/β-peptide **6** would not be a DPP-4 substrate.

To test this hypothesis, we purchased DPP-4 from EMD Biosciences (catalogue # 317640) as a stock solution in 20 mM Tris-HCl, 5 mM CaCl , 1 $\mu$M ZnCl , 0.05% $NaN_3$ pH 8.0. A 10 $\mu$L aliquot of this stock solution was added to 2 nmoles of GLP-1(7-37)-$NH_2$ or α/β-peptide **6** dissolved in 40 $\mu$L of TBS buffer, pH 8.0. The DPP-4 reaction was allowed to proceed at 37ºC (higher than for the other proteases because of the low DPP-4 concentration). The half-life of GLP-1(7-37)-$NH_2$ was 13.5 minutes under these conditions (data shown below). The solution containing α/β-peptide **6** and DPP-4 was monitored for 7 days; no cleavage was detected during this time.

**Supplementary Figure 17**: Protease assay of 40 μM GLP-1(7-37)-$NH_2$ with DPP-4(EMD Biosciences, catalogue # 317640) in TBS pH 8.0 at 37ºC. Major backbone cut site, as determined by MALDI-TOF-MS, is indicated with the vertical blue line.

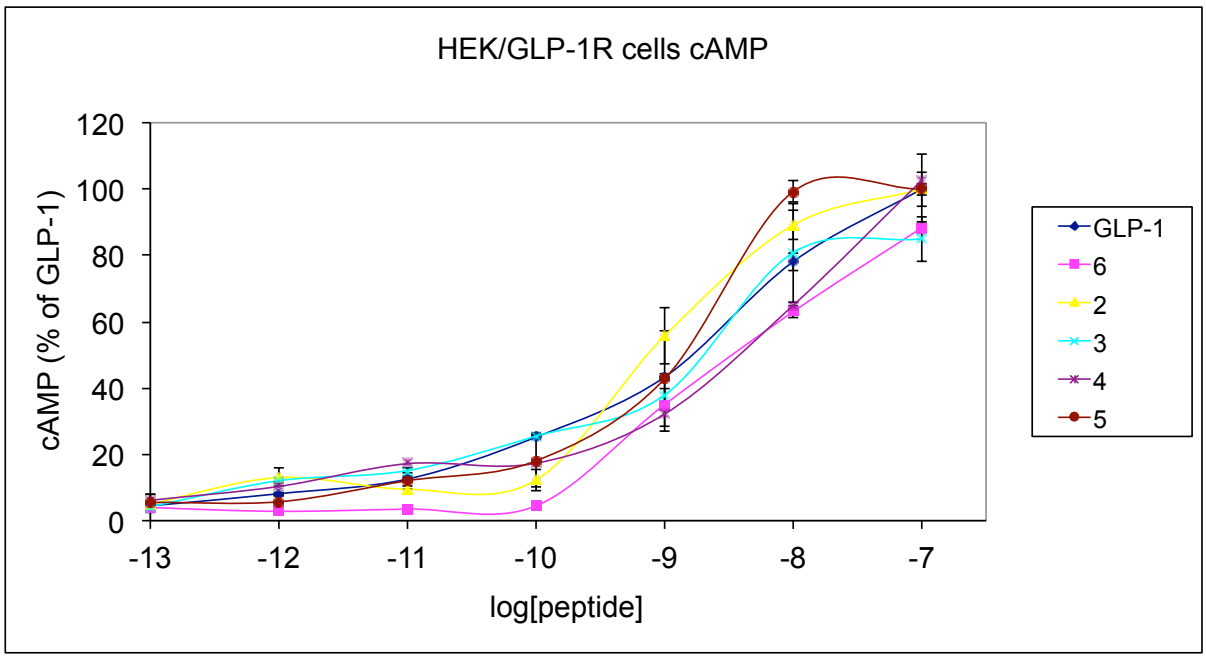



**Method for Adenylyl Cyclase Assay**

HEK293 cells stably expressing the GLP-1R[5] were seeded in 24-well plates in DMEM medium containing 10% FBS. Cells were incubated with 0.5 pCi of [$^3$H]adenine in fresh medium at 37 ºC for 2 h. Cells were then treated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) in fresh medium for 15 min and incubated in the presence of the indicated concentrations of GLP-1 analogs for 20 min at 37 ºC. The reaction was terminated by the addition of 1.2 M trichloroacetic acid (TCA), followed by neutralization with 4 N KOH. Cyclic AMP was isolated by the two-column chromatographic method.[6] Radioactivity was counted in a scintillation counter. $EC_{50}$ values were determined by sigmoidal curve fitting using GraphPad Prism version 5.0.

**Supplementary Figure 18:** GLP-1R activation, as measured by cAMP production, for GLP-1(7-36)-$NH_2$ and α/β-peptides **2-6**.

| | AVERAGES at M Concentrations | | | | | | | $EC_{50}$ (nM) |
|---|---|---|---|---|---|---|---|---|
| | -13 | -12 | -11 | -10 | -9 | -8 | -7 | |
| **GLP-1** | 4.463112 | 8.121376 | 12.55169 | 25.1881 | 43.30864 | 78.0983 | 100 | **1.6±0.2** |
| **6** | 3.831377 | 2.812731 | 3.503578 | 4.469353 | 35 | 63.0221 | 88.32258 | **3.2±0.3** |
| **2** | 5.122 | 12.98423 | 9.462563 | 12.26458 | 55.7495 | 89.12877 | 99.98469 | **0.7±0.1** |
| **3** | 4.23 | 12.05 | 15.12 | 25.49186 | 37.89101 | 80.8351 | 84.96815 | **2.0±0.1** |
| **4** | 6.14 | 10.32 | 17.1878 | 17.32045 | 32.14895 | 64.92325 | 102.6341 | **4.0±0.5** |
| **5** | 5.687831 | 5.687831 | 12.05 | 18.04762 | 42.7672 | 99.2328 | 100 | **1.6±0.2** |




**Supplementary Figure 19:** GLP-1R activation, as measured by cAMP production, of additional α- and α/β-peptides analogues of GLP-1(7-37)-$NH_2$. The $EC_{50}$ value for the positive control, GLP-1(7-36)-$NH_2$, is shown for comparison.

| | | $EC_{50}$ (nM) |
|---|---|---|
| GLP-1 | H-HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-$NH_2$ | 1.6 |
| | H-HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-$NH_2$ | >100 |
| | H-HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-$NH_2$ | >100 |
| | H-HAEGTFTSDASSYLEGQAAKEFIAWLVKGRG-$NH_2$ | >100 |
| | H-HAEGTFTSDASSYLEGQAAKEFIAWLVKGRG-$NH_2$ | 1.6 |

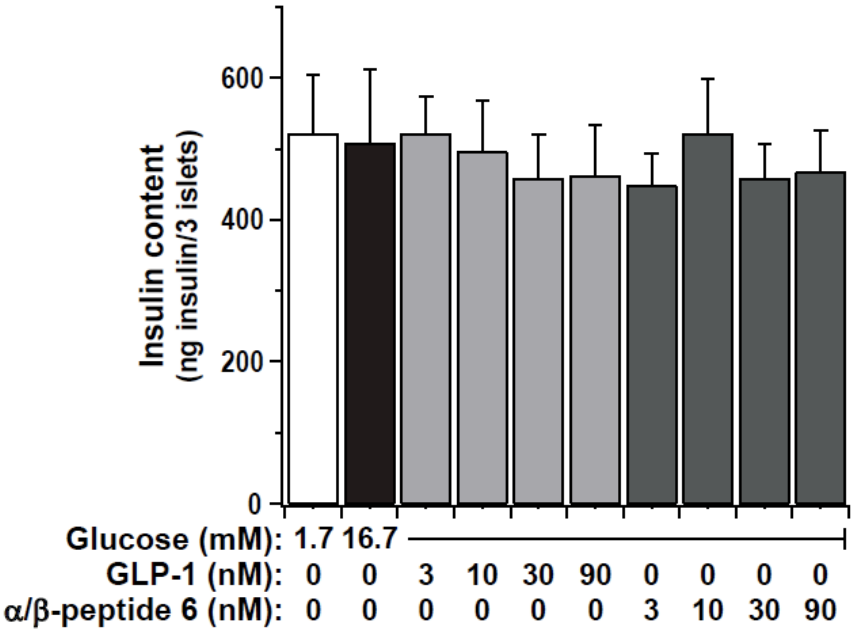

**Method for detecting Insulin Secretion of Isolated Islet Cells from Mice**

Ex vivo insulin secretion studies were performed as previously described.[7,8] Briefly, intact pancreatic islets were isolated from ~10 week-old male B6 mice using a collagenase digestion procedure.[9] Islets are individually hand-picked using a stereomicroscope with trans-illumination to ensure that healthy, clean and acinar-free islets were used for study. To monitor glucose-stimulated insulin secretion (GSIS), 3 individual islets were placed into mesh baskets made from 12 X 75 mm borosilicate tubes. Each tube had a ~5 mm hole cut in the bottom that was covered with a 62 $\mu$m mesh (Tetko). Islet selection was conducted at room temperature in Krebs Ringer

Bicarbonate Buffer (KRB) containing 16.7 mM glucose, and 0.2% RIA-grade BSA (Sigma). The mesh-bottom tube containing 3 islets was transferred to a 16X100 mm tube containing 1.0 mL KRB containing 1.7 mM glucose, and 0.5% RIA-grade BSA, and pre-incubated at 37°C for 45 mins. Following the pre-incubation period, the mesh tubes were transferred to a fresh 16 X 100 mm tube containing 1.0 mL KRB containing glucose, GLP-1 or the α/β-peptide 6 at the indicated concentrations, and incubated for an additional 45 mins to allow for insulin secretion. At the end of the 45 min incubation period, the islets and media were collected from each tube and used for insulin measurements by ELISA. Insulin secretion is expressed as the fraction of total insulin present for each sample ($insulin_{medium}/insulin_{total}$).

**Supplementary Figure 20:** (Upper) Insulin secretion from mouse pancreatic islets stimulated with external GLP-1(7-36)-$NH_2$ or α/β-peptide **6**; insulin was measured by ELISA. (Lower) Insulin content of islets after treatment with glucose and GLP-1(7-36)-$NH_2$ or α/β-peptide **6**. The data show that insulin content in all cases is indistinguishable from insulin content of islets that have not been exposed to a GLP-1R agonist.

| | | Insulin Content | |
|---|---|---|---|
| condition | nM concentration of peptide | average (ng/3 islets) | sem |
| Low glucose (LG) | 0 | 522.7 | 81.0 |
| High glucose (HG) | 0 | 508.9 | 103.5 |
| HG + 3nM GLP-1 | 3 | 522.1 | 51.8 |
| HG + 10nM GLP-1 | 10 | 496.4 | 70.9 |
| HG + 30nM GLP-1 | 30 | 458.6 | 61.0 |
| HG + 90nM GLP-1 | 90 | 463.1 | 70.3 |
| HG + 3nM α/β–peptide 6 | 3 | 448.3 | 44.6 |
| HG + 10nM α/β–peptide 6 | 10 | 521.4 | 76.4 |
| HG + 30nM α/β–peptide 6 | 30 | 459.3 | 46.8 |
| HG + 90nM α/β–peptide 6 | 90 | 467.5 | 58.5 |




**Method for Glucose Tolerance Test**

GLP-1(7-37)-$NH_2$, exendin-4 or α/β-peptide **6** was administered to mice by interperitoneal (i.p.) injection at a 1 mg/kg dose, or lower for α/β-peptide **6**, using an injection volume of 10 mL/kg body mass. Each peptide was first dissolved in prefiltered DMSO at 10 mg/mL concentration, then diluted >20-fold with TBS buffer, pH 7.4 (final DMSO conc. = <5%). Glucose was administered by i.p. injection with a sterile-filtered 30% D-glucose-saline solution at a 1.5 g/kg dose using a 5 mL/kg injection volume.

Thirteen-week-old male C57BL/6J mice (n = 4) were fasted overnight on wood chip bedding for 15 hours prior to the experiment. Blood glucose levels were monitored from a tail tip bleed using an ACCU-CHEK Aviva blood glucose meter. Fasting glucose levels were measured at 75 minutes prior to the glucose injection (t = -75 min), and the compound injection was performed 60 minutes prior to the glucose injection (t = -60 min). Glucose levels were measured immediately prior to the glucose injection (t = 0 min) to assess any changes in the baseline glucose caused by peptide administration. Blood glucose levels were monitored at 30, 60, 90, and 120 minutes after injection of glucose.

Five hours after peptide injection, the mice received a second injection of 1.5 g/kg glucose. Blood glucose was monitored at 30 min after this second glucose injection. Mice were sacrificed by $CO_2$ inhalation at the conclusion of the GTT.

**Supplementary Figure 21:** Blood glucose levels in mice measured during i.p. glucose tolerance test.

| | Average Blood Glucose Level (mg/dL) | | | | | |
|---|---|---|---|---|---|---|
| **Time (min)** | -60.0 | 0.0 | 30.0 | 60.0 | 90.0 | 120.0 |
| Vehicle | 122.8 | 126.0 | 342.5 | 210.3 | 163.5 | 139.3 |
| GLP-1(7-37)-NH2 | 108.0 | 81.8 | 132.8 | 99.3 | 74.8 | 86.0 |
| Exendin-4 | 118.0 | 89.5 | 123.5 | 87.0 | 67.5 | 70.0 |
| α/β-Peptide 6 (1mg/kg) | 107.8 | 101.3 | 139.8 | 103.0 | 86.3 | 87.5 |
| α/β-Peptide 6 (0.1mg/kg) | 123.5 | 103.5 | 147.8 | 110.0 | 95.5 | 92.3 |
| α/β-Peptide 6 (0.01mg/kg) | 102.0 | 108.8 | 297.3 | 191.0 | 150.3 | 127.0 |
| α/β-Peptide 6 (0.001mg/kg) | 117.5 | 123.5 | 291.3 | 214.0 | 158.8 | 139.3 |

Average Blood Glucose Level at 30min with Standard Deviation of the Mean (SDOM)

| | AUC | SDOM |
|---|---|---|
| Vehicle | 331.3 | 22.3 |
| GLP-1(7-37)-NH2 | 250.5 | 41.6 |
| Exendin-4 | 118.8 | 8.8 |
| α/β-Peptide 6 (1mg/kg) | 144.3 | 20.7 |
| α/β-Peptide 6 (0.1mg/kg) | 202.5 | 11.8 |
| α/β-Peptide 6 (0.01mg/kg) | 306.0 | 21.5 |
| α/β-Peptide 6 (0.001mg/kg) | 323.3 | 16.1 |

**References**

(1) Horne, W. S.; Price, J. L.; Gellman, S. H.: Interplay among side chain sequence, backbone composition, and residue rigidification in polypeptide folding and assembly. *Proc Natl Acad Sci USA* **2008**, *105*, 17205-17205.

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