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A Template-Induced *Incipient* Collagen-Like Triple Helical Structure

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Supplementary Material

Synthesis

Stepwise peptide synthesis methods in solution were used to prepare the template derivative KTA-(Gly-OH)$_3$ and the tripeptide building block Boc-Gly-Pro-Hyp(OBz)-OH. Active ester (p-nitrophenyl ester) method and coupling reagents (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) and N-hydroxybenzotriazole (HOBt)) were used for the peptide couplings in solution. The polypeptide chains (Gly-Pro-Hyp)$_n$ were assembled on a 4-methylbenzhydrylamine (MBHA) resin by segment condensation method using diisopropylcarbodiimide (DIC) and HOBt as the coupling reagents and dichloromethane (DCM) as the solvent.

A direct method was developed to couple the templates and the peptide chains on resin. The peptide chains were assembled on a resin (MBHA). Before the removal of the peptides from the resin, the template derivatives were coupled to the N-termini of these peptide chains using DIC and HOBt as the coupling reagents and 25% DMF in DCM as the solvents. In order to obtain a high yield of the desired three-chain product and minimize side products, such as the one- and two-chain compounds, the template derivative was used as the limiting reagent. The reactions were monitored by the Kaiser test and normally they were allowed to proceed three days. The HF cleavage method was used to remove the

products from the resin. Reverse phase HPLC was utilized to separate the template-assembled compounds and determine their purity. Mass spectroscopy and NMR were used to verify the structures. The yields were calculated based on the template used.

KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$. Boc-Gly-Pro-Hyp(OBz)-MBHA (0.3 mmol based on resin substitution level) was prepared by solid phase segment condensation method (the segment was the tripeptide Boc-Gly-Pro-Hyp(OBz)-OH). The Boc group was removed using a solution of 30% TFA in DCM (20 mL) and anisol (1.0 mL) was added as the scavenger. The resin was washed with DCM (2 x 10 mL), MeOH (2 x 10 mL), 10% TEA in DCM (2 x 10 mL) and DCM (2 x 10 mL) to give Gly-Pro-Hyp(OBz)-MBHA. KTA-(Gly-OH)$_3$ (35 mg, 0.08 mmol) and HOBt (50 mg) were added to the vessel and 25% DMF was added to help dissolve the HOBt. Then, 1.0 M DIC in DCM (4 mL, 0.4 mmol) was added. The Kaiser test showed the absence of free amines after 3 d. The resin was washed by DCM and MeOH several times and dried in vacuum overnight. The HF cleavage methods were carried out to remove the peptide from the resin, and the resulting mixture of resin and product was washed with a hexane and Et_2O mixture. The product was separated from the resin by extracting with H_2O. The solution was then lyophilized. The crude material was purified by preparatory RP-HPLC to give the final product as a white powder (55 mg, 56% yield based on the template used). The analytical HPLC profile showed a single homogeneous peak with RT = 13.2 min (no TFA, 7-50% B in 30 min). ESI-MS (M+H) calcd for $C_{54}H_{81}N_{15}O_{18}$ 1229, obsd 1229. ^{1}H-NMR (500 MHz, D_2O, 27 °C, using H_2O at 5°C to obtain NH signals, DQF-COSY) δ 8.84 (m, 3H, Gly-NH), 8.57 (m, 3H, spacer Gly-NH), 7.89 (m, 3H, terminal NH_2), 7.33 (m, 3H, terminal NH_2), 4.61 (m, 3H, Hyp-γ), 4.49 (t, 3H, Hyp-α), 4.06 (q, 6H, Gly-α), 3.86-3.70 (m, 12H, spacer Gly-a and Hyp-δ), 3.67-3.54 (m, 6H, Pro-δ), 2.65 (d, 3H, methylene-equatorial), 2.37-2.26 (m, 6H, Pro-β and Hyp-β), 2.10-2.00 (m, 9H, Pro-γ and Hyp-β), 1.97-1.90 (m, 3H, Pro-β), 1.33 (d, 3H, methylene-axial), 1.26 (s, 9H, methyl).

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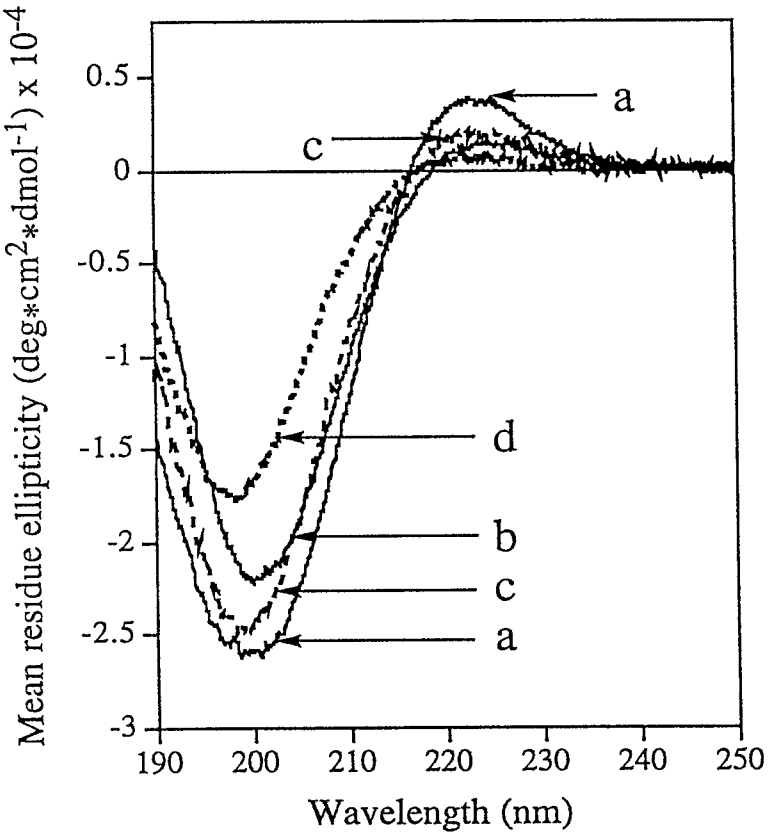
KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH$_2$]$_3$. Boc-(Gly-Pro-Hyp(OBz))$_3$-MBHA (0.25 mmol based on resin substitution level) was prepared by the solid phase segment condensation method. The Boc group was removed using a solution of 30% TFA in DCM (15 mL) and anisol (1.0 mL) was added as the scavenger. The resin was washed with DCM (2 x 10 mL), MeOH (2 x 10 mL), 10% TEA in DCM (2 x 10 mL) and DCM (2 x 10 mL) to give (Gly-Pro-Hyp(OBz))$_3$-MBHA. KTA-(Gly-OH)$_3$ (35 mg, 0.08 mmol) and HOBt (50 mg) were added to the vessel and 25% DMF in DCM (15 mL) was used as the solvent Then, 1.0 M DIC in DCM (4 mL, 0.4 mmol) was added. The Kaiser test showed the absence of free amines after 3 d. The resin was washed by DCM and MeOH several times and dried in vacuum overnight. The HF cleavage methods were carried out to remove the peptide from the resin, and the resulting mixture of resin and product was washed with a hexane and Et_2O mixture. The product was separated from the resin by extracting with H_2O. The solution was lyophilized (130 mg). This crude material was purified by preparatory RP-HPLC to give the final product as a white powder (78 mg, 35% yield based on the template used). The analytical HPLC profile showed a single homogeneous peak with RT = 15.7 min (no TFA, 8-50% B in 25 min). ESI-MS (M+H) calcd for $C_{126}H_{183}N_{33}O_{42}$ 2833, obsd 2832.

Circular Dichroism Measurements

Circular dichroism (CD) measurements were carried out on a Cary 61 spectropolarimeter which was modified by replacing the original Pockel cell with a 50 KHz photoelastic modulator (Hinds International FS-5/PEM-80). The original Cary linear polarizer was replaced with a MgF2 linear polarizer supplied by AVIV, Inc. A EGG Princeton Applied Research model 128A lock in amplifier is used to integrate the phase detected output of the original end-on PMT and preamp. System automation, multiple scan signal averaging, and base line subtraction were accomplished with an AT286 PC interfaced directly to both the Cary 61 and the 128A amplifier. The system software and custom hardware interfaces

were designed by Allen MicroComputer Services Inc. and the UC San Diego Department of Chemistry & Biochemistry Computer Facility.

CD spectra were recorded in a 0.5 mm cell by averaging 10 scans from 190-300 nm with a scan rate of 1.0 nm per second. The samples were stored in a refrigerator (4 °C) 3 d for equilibration of triple helix formation before CD experiments and another 2 h at the specified temperature before acquiring data.



Suppl.-Figure 1

Suppl.-Figure 1 shows the CD spectra of **KTAg-3,3** in H_2O (0.2 mg/mL) at 20 °C (a) and 60 °C (b), **Ac-3** in H_2O (0.2 mg/mL) at 20 °C (c), and **KTAg-1,3** in H_2O (0.2 mg/mL) at 20 °C (d). All these CD spectra are similar in spectral positions and shapes and they resemble those of collagen. However, a significant difference can be found in the

relative intensities of the positive peak over negative peak. We denote this relative intensity parameter as **Rpn**. The **Rpn** values for **KTAg-3,3**, **KTAg-1,3** and **Ac-3** in H_2O (0.2 mg/mL) at 20 °C are 0.14, 0.05 and 0.08 respectively. Based on the published CD spectra, the **Rpn** values for collagen and $(Gly\text{-}Pro\text{-}Hyp)_{10}$ in H_2O are both estimated to be 0.13. Therefore, the CD spectrum of **KTAg-3,3** in H_2O at 20 °C is consistent with a triple helical conformation. At 60 °C which is above the melting temperature of **KTAg-3,3** in H_2O according to Figure 3, the CD spectrum of **KTAg-3,3** (b) (**Rpn** = 0.06) is very similar to those of **Ac-3** and **KTAg-1,3** in H_2O at 20 °C (0.2 mg/mL). These results indicate that the triple helical conformation of **KTAg-3,3** has been denatured at 60 °C to polyproline-II-like structures.

Optical Rotation Measurements

Optical rotations were measured with a Perkin-Elmer 241 Polarimeter equipped with a Model 900 isotemp refrigerator circulator (Fisher Scientific). Data were collected at 365 nm (Hg). All samples were measured at a concentration of 0.2 mg/mL. The solutions were stored in a refrigerator (4 °C) 3 d for the equilibration of triple helix formation. Before recording an optical rotation, the sample was equilibrated 1 h at the initial temperature. At each subsequent temperature point, the sample was allowed to equilibrate until the optical rotations were time-independent.

NMR Spectroscopy

The compounds synthesized as described were dissolved in H_2O and kept at 5 °C at least 24 h prior to any experiment, in order to allow for a proper equilibration of the sample. The NMR samples were prepared in H_2O/D_2O (9:1) and in D_2O with a peptide concentration in the range 1.43-4 mg/mL. The pH is in the range 3.3-3.5 (direct pH meter reading without correction for isotope effects).

All NMR experiments were carried out on an AMX-500 Bruker spectrometer. The 1D spectra were collected using 16K points, a spectral width of 5507 or 7508 Hz, 80 scans and a relaxation delay of 1.3 s. The two dimensional homonuclear experiments phase-sensitive double quantum filtered correlation spectroscopy (DQF-COSY), nuclear Overhauser enhancement spectroscopy (NOESY), total correlation spectroscopy (TOCSY) and rotating frame nuclear Overhauser spectroscopy (ROESY) were run using the time proportional phase incrementation method (TPPI). NOESY spectra were obtained at mixing times of 150 and 300 ms. ROESY experiments were carried out with a mixing time of 200 ms and a spin locking field of 2.5 KHz. The 2D spectra were recorded with 256-400 t_1 increments and 2K-4K data points for the t_2 dimension with a spectral width of 5507 Hz. All the NMR experiments were processed using the program FELIX. An exponential multiplication function was used in the processing of one dimensional spectra. The baseline was corrected with the ABL flatten algorithm and the cubic spline algorithm. The integrals were normalized using the peaks in the spectral region from 1.5 ppm to 3 ppm which contains a known number of protons from the Pro $C_\beta H$, the Pro $C_\gamma H$ and the Hyp $C_\beta H$. In the case of the KTA analogs this spectral region includes also the equatorial methylene protons of the KTA ring. Two dimensional spectra were enhanced using a phase-shifted sine-bell window function and zero-filled resulting in final matrices of 2K x 2K or 4K x 2K data points.

The spin systems of the compounds studied were identified using DQF-COSY and TOCSY spectra. Glycine residues could be recognized by their typical connectivity pattern with a pair of nondegenerate $C_\alpha H$ resonances and the amide proton resonance which clearly distinguish them from Pro and Hyp. The connectivity of Pro and Hyp are similar, but Hyp can be readily distinguished from Pro on the basis of its $C_\gamma H$ resonance which is shifted significantly downfield by the OH group. [27] The sequential connectivities between Gly and Pro, Pro and Hyp (and also Hyp and Gly in the **KTAg-3,3** and **Ac-3**) of all set of resonances are consistent with the ROESY and NOESY spectra. See also ref. 17.

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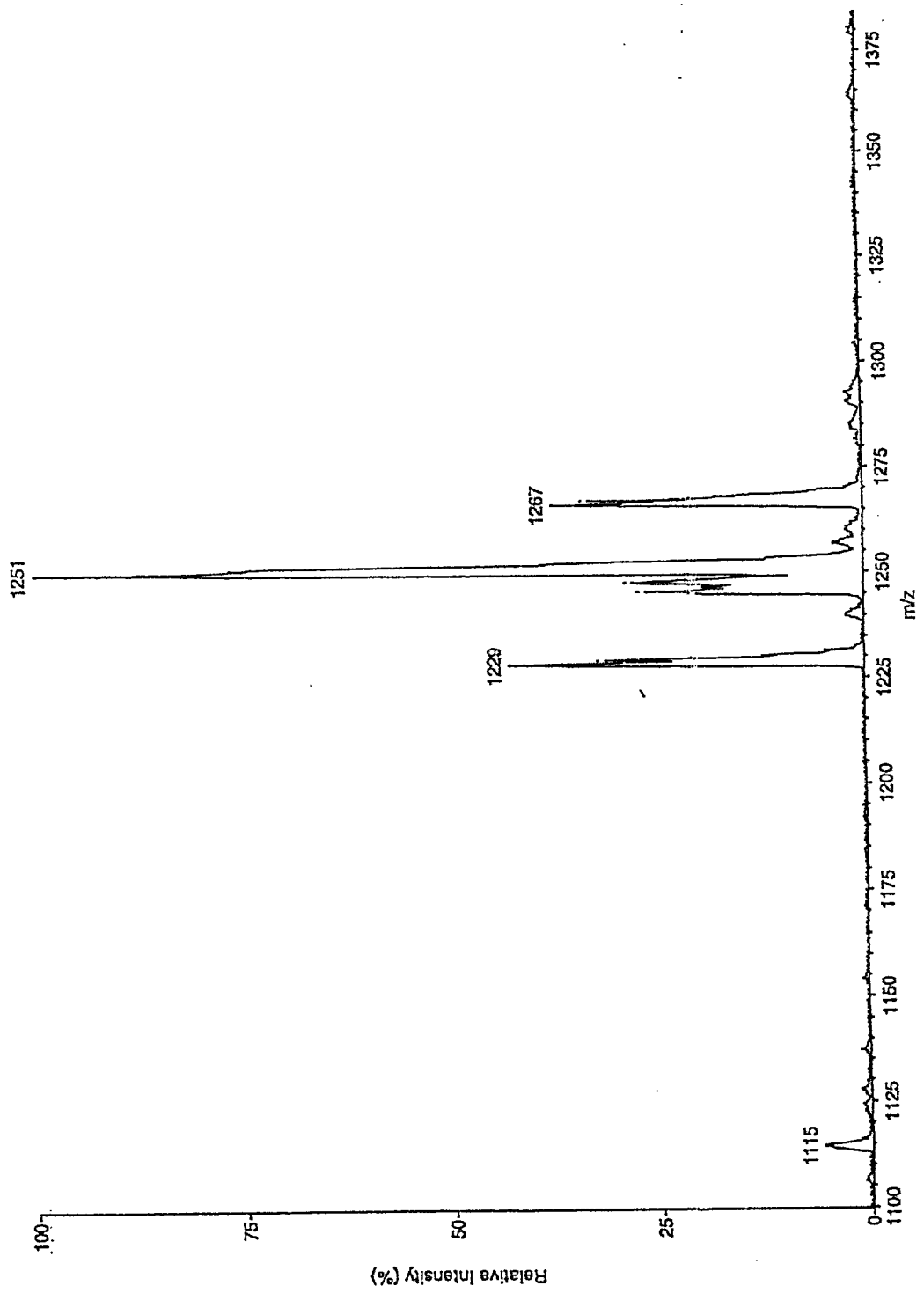

ESI-MS of KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$

Observed $(M+H)^+ = 1229$, $(M+Na)^+ = 1251$, $(M+K)^+ = 1267$

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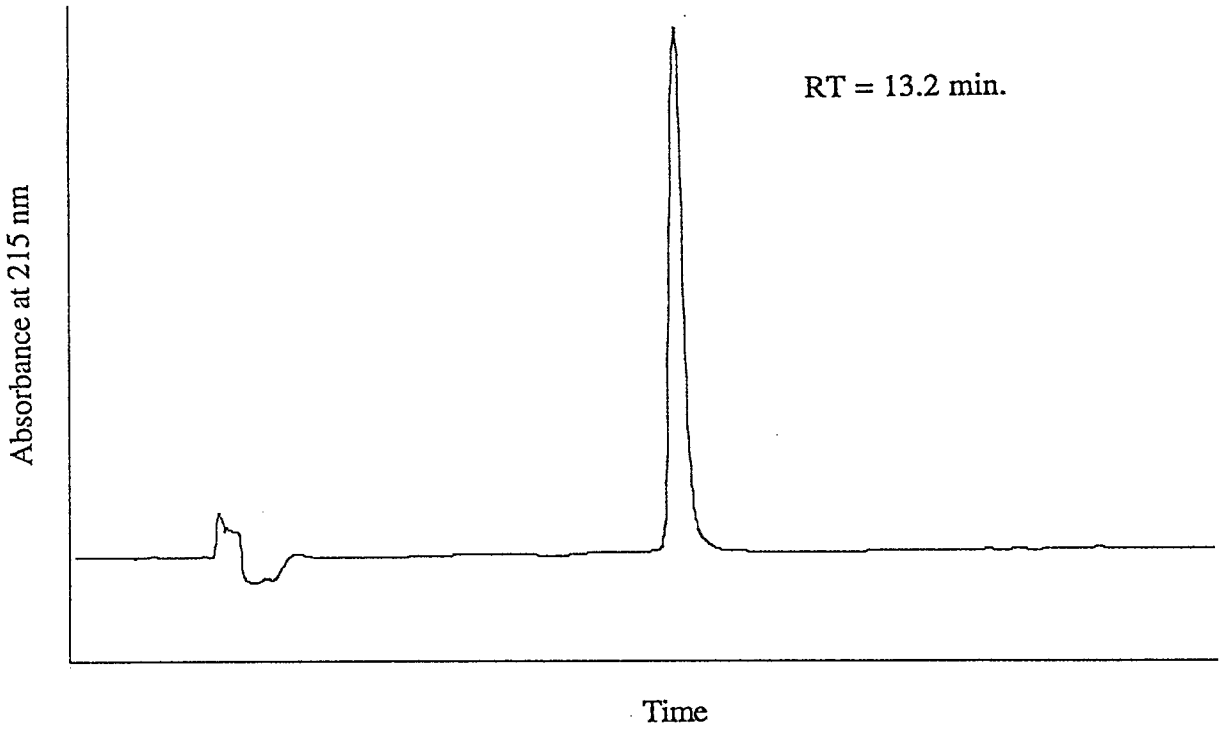

RP-HPLC Profile of KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$

7-50% B in 25 minutes. Retention time RT = 13.2 minute
Vydac column, C-18, 25 x 0.46 cm, 1.0 ml/min.
Solvent A: H_2O; Solvent B: CH_3CN

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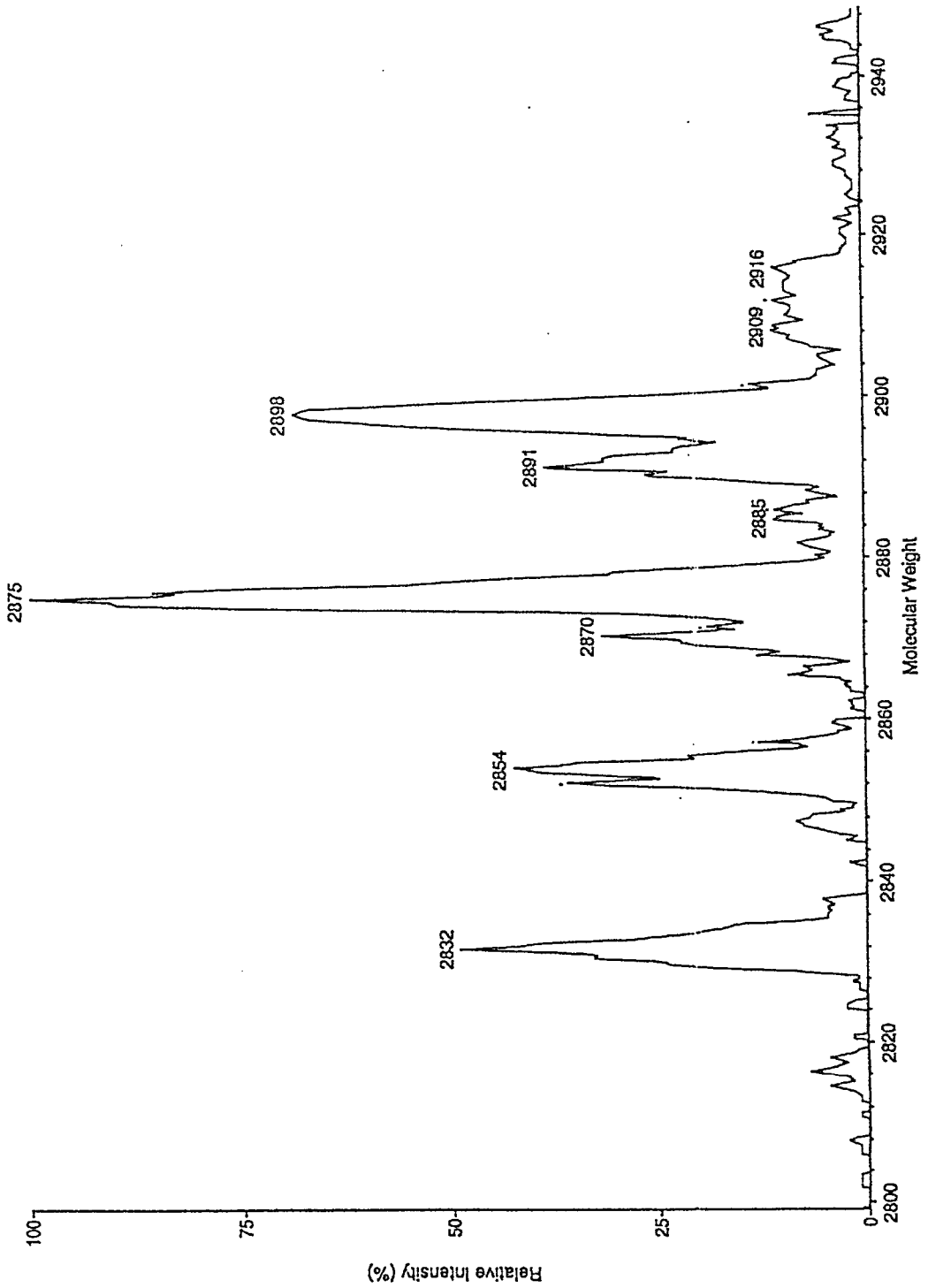
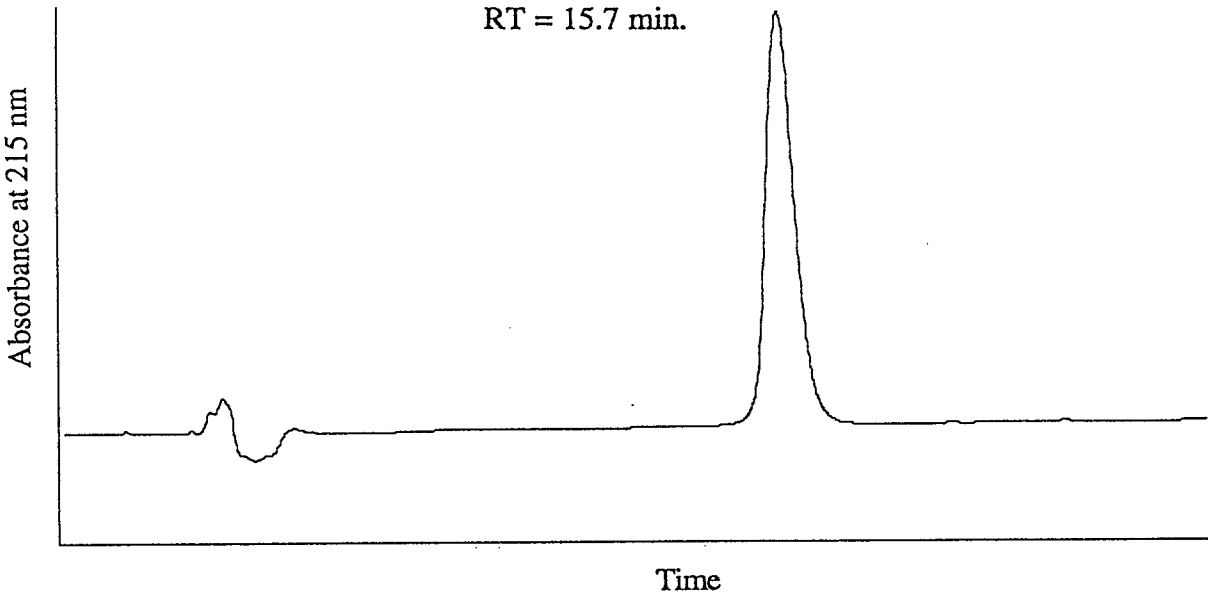


ESI-MS of KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH$_2$]$_3$

Observed $(M+H)^+ = 2832$, $(M+Na)^+ = 2854$, $(M+K-H)^+ = 2870$, $(M+2Na-2H)^+ = 2875$, $(M+3Na-3H)^+ = 2898$

J-5157-10



RP-HPLC Profile of KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH_2]$_3$

8-50% B in 25 minutes. Retention time RT = 15.7 minute
Vydac column, C-18, 25 x 0.46 cm, 1.0 ml/min.
Solvent A: H_2O; Solvent B: CH_3CN