Supporting Information

Negating coordinative cysteine and methionine residues during metathesis of unprotected peptides

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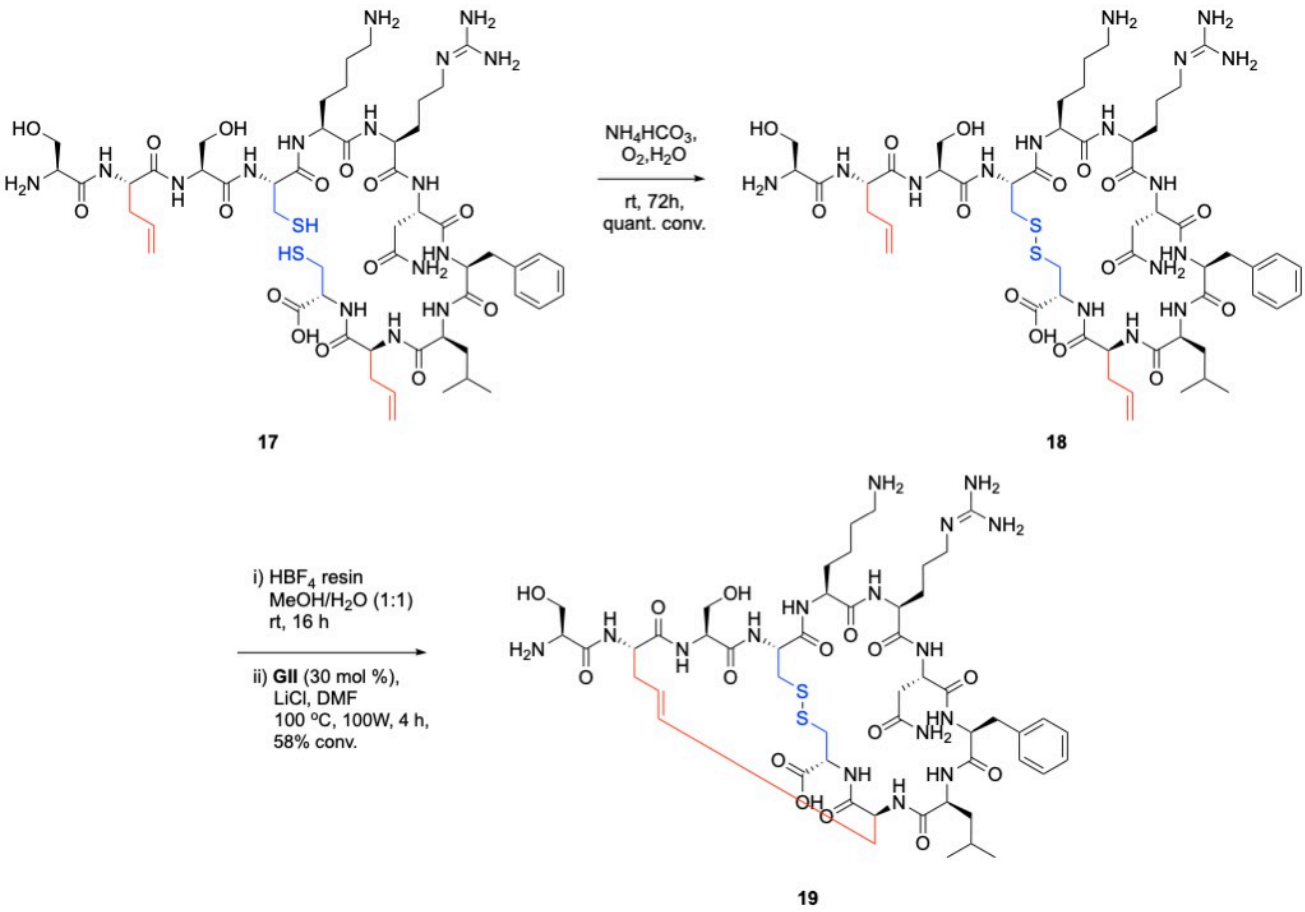
Supplementary Scheme



Scheme S1: Protecting group-free synthesis of dicarba conotoxin pc16a.

General Experimental Information

General Considerations

Manipulation of organometallic compounds was performed using standard Schlenk techniques under an atmosphere of dry nitrogen or in a nitrogen-filled drybox.

Instrumentation

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Bruker DRX400 or DRX600 spectrometers operating at 400 or 600 MHz respectively, as solutions in deuterated solvents as specified. Each resonance was assigned according to the following convention: chemical shift; multiplicity; observed coupling constants (*J* Hz); number of protons. Chemical shifts (δ), measured in parts per million (ppm), are reported relative to the residual proton peak in the solvent used as specified. Multiplicities are denoted as singlet (s), doublet (d), triplet (t), quartet (q), pentet (p), septet (sept), multiplet (m) or prefixed broad (b), or a combination where necessary.

Low resolution electrospray ionisation (ESI) mass spectra were recorded on a Micromass Platform Electrospray mass spectrometer (QMS-quadrupole mass spectrometry) as solutions in specified solvents. Spectra were recorded in positive and negative modes (ESI^+ and ESI^-) as specified.

Reverse-phase high performance liquid chromatography (RP-HPLC) was performed on Agilent 1200 series instruments. For analytical experiments, the instrument was equipped with photodiode array (PDA) detection (controlled by ChemStation software) and an automated injector (100 μL loop volume). Experiments were carried out on a Vydac C18 analytical column (4.6 mm x 250 mm, 5 μm) at a flow rate of 1.5 mL min^{-1}. For preparative runs, the instrument used multivariable wavelength (MVW) detection (controlled by ChemStation software) and an Agilent unit injector (2 mL loop volume). Experiments were carried out on a Vydac C18 preparative column (22 mm x 250 mm, 10 μm) at a flow rate of 10 mL min^{-1}. The solvent systems were buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in MeCN.

Solvents and Reagents

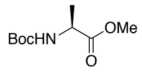
Dichloromethane (CH_2Cl_2) was supplied by Merck and distilled over CaH_2 prior to use. Acetic acid (AcOH), dichloromethane (CH_2Cl_2), dimethylformamide (DMF), ethyl acetate (EtOAc), hexane, methanol (CH_3OH) and *N*-methyl-2-pyrrolidone (NMP) were used as supplied by Merck. Human

recombinant methionine reductase sulfoxide A and B were purchased from Abcam. Trifluoroacetic acid and (1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(phenylmethylene)-(tricyclohexyl-phosphine)ruthenium (**GII**) were supplied by Sigma-Aldrich. D_2O, $(CD_3)_2SO$, C_6D_6 and $CDCl_3$ were purchased from Cambridge Isotopes Laboratory.

Experimental Section

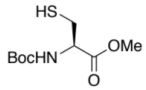
Styrene model experimental:

Methyl (*tert*-butoxycarbonyl)-L-alaninate, A



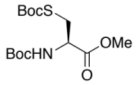
TMSCl (3.41 g, 4.00 mL, 31.4 mmol) was added to a stirred solution of MeOH (50 mL) at 0°C. After 15 mins, alanine (1.00 g, 11.2 mmol) was added, and the reaction was stirred for 16 h. The reaction mixture was concentrated under reduced pressure. The residue was taken up in CH_2Cl_2 (100 mL). Et_3N (3.41 g, 4.70 mL, 33.7 mmol) and Boc anhydride (2.45 g, 11.2 mmol) were added and the reaction was stirred at room temperature for 16 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with H_2O (2 x 100 mL) and brine (100 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica column chromatography (8 : 1 – hexanes : EtOAc) to provide **A** (2.08 g, 91%) as a colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.03 (br s, 1H), 4.33-4.30 (m, 1H), 3.74 (s, 3H), 1.44 (s, 9H), 1.38 (d, J = 7.2 Hz, 3H). All data was consistent with that previously reported.[1]

Methyl (*tert*-butoxycarbonyl)-L-cysteinate, B



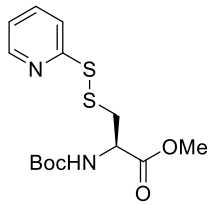
Compound **B** was prepared following a procedure developed by Boucher and coworkers.[2] The protected cystine **F** (150 mg, 320 μmol), triphenylphosphine (89.0 mg, 339 μmol) and sodium acetate (11.0 mg, 134 μmol) were suspended in a mixture of MeOH (3 mL), H_2O (1.5 mL) and glacial acetic (10 μL) and heated at reflux for 16 h. The mixture was diluted with CH_2Cl_2 (50 mL) and washed with H_2O (2 x 50 mL) and brine (50 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by silica column chromatography (6 : 1 – hexanes : EtOAc) to provide **B** (133 mg, 88%) as a colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.42-5.39 (m, 1H), 4.62-4.59 (m, 1H), 3.79 (s, 3H), 2.99-2.95 (m, 2H), 1.46 (s, 9H). All data was consistent with that previously reported.[2]

Methyl *N*,*S*-bis(*tert*-butoxycarbonyl)-L-cysteinate, C



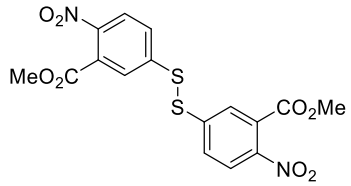
TMSCl (1.71 g, 2.00 mL, 15.7 mmol) was added to a stirred solution of MeOH (25 mL) at 0°C. After 15 mins, L-cysteine (430 mg, 3.55 mmol) was added and the reaction was stirred for 16 h. The reaction mixture was concentrated under reduced pressure and the residue was dissolved in 2:1 CH_2Cl_2/MeOH (15 mL). Et_3N (1.80 g, 2.48 mL, 17.8 mmol) and Boc anhydride (1.70 g, 7.81 mmol) were added and the reaction was stirred at room temperature for 16 h. The reaction mixture was then diluted with CH_2Cl_2 (50 mL) and washed with H_2O (2 x 50 mL) and brine (50 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by silica column chromatography (8 : 1 – hexanes : EtOAc) to provide **C** (1.06 g, 89%) as a colourless solid, mp 67.8-68.1 °C. IR (neat): 3398m, 2981m, 1751s, 1688s, 1511s, 1365m, 1210m, 1129s. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.34-5.32 (m, 1H), 4.57-4.54 (m, 1H), 3.75 (s, 3H), 3.30 (AB dd, *J* = 14.4, 4.8 Hz, 1H), 3.22 (AB dd, *J* = 14.4, 5.6 Hz, 1H), 1.49 (s, 9H), 1.44 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.1, 168.5, 155.1, 85.6, 80.1, 53.4, 52.7, 33.2, 28.4, 28.2. HRMS (ESI+, MeOH): *m/z* 336.1473 [M + H]+, $C_{14}H_{26}NO_6S$+ requires 336.1475.

Methyl *N*-(*tert*-butoxycarbonyl)-*S*-(2-thiopyridinyl)cysteinate, D



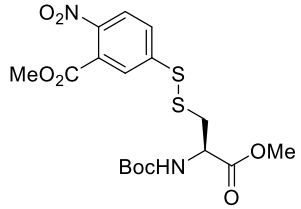
2,2'-Dipyridyldisulfide (78 mg, 356 μmol) was added to a solution of protected cysteine **B** (56 mg, 238 μmol) in MeOH (5 mL). The resulting yellow solution was stirred at room temperature for 16 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by silica column chromatography (3 : 1 – hexanes : EtOAc) to provide **D** (82 mg, quant.) as a colourless oil. IR (neat): 2976m, 1707s, 1499m, 1446m, 1417s, 1365m, 1213m, 1159s, 1114m cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.57 (d, *J* = 4.8 Hz, 1H), 7.64 (td, *J* = 7.7, 1.8 Hz, 1H), 7.53 (d, *J* = 8.1 Hz, 1H), 7.15 (dd, *J* = 7.3, 4.9 Hz, 1H), 6.60 (s, 1H), 4.58 (s, 1H), 3.73 (s, 3H), 3.42 (dd, *J* = 14.1, 5.7 Hz, 1H), 3.28 (dd, *J* = 13.9, 4.6 Hz, 1H), 1.45 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.2, 158.9, 155.6, 149.7, 137.4, 121.3, 120.9, 80.2, 53.2, 52.7, 42.4, 28.5. HRMS (ESI+, MeOH): *m/z* 345.0930 [M + H]+, $C_{14}H_{21}N_2O_4S_2$+ requires 345.0937.

Dimethyl 5,5'-disulfanediylbis(2-nitrobenzoate)



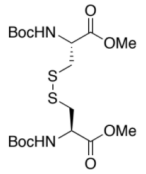
Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)) (760 mg, 1.92 mmol) was dissolved in 10% MeOH in DCM (10 mL). (Trimethylsilyl)diazomethane (2 M in hexanes) was added dropwise until the reaction mixture remained yellow. The reaction mixture was stirred at room temperature for a further 15 mins before being concentrated under reduced pressure to give the desired product (812 mg, quant.) as a yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.92 (d, *J* = 8.7 Hz, 2H), 7.75 (s, 2H), 7.68 (d, *J* = 8.7 Hz, 2H), 3.92 (s, 6H). All data was consistent with that previously reported.[3]

Methyl (R)-5-((2-((tert-butoxycarbonyl)amino)-3-methoxy-3-oxopropyl)disulfaneyl)-2-nitrobenzoate, E



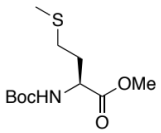
Dimethyl 5,5'-disulfanediylbis(2-nitrobenzoate) (812 mg, 1.91 mmol) was dissolved in MeOH/DCM (1 : 1, 10 mL). Neat **B** was added, and the solution stirred at room temperature for 24 hours before being concentrated under reduced pressure. The dark orange oil was purified by silica column chromatography (7 : 2 – hexanes : EtOAc) to provide **E** (689 mg, 81%) as a yellow oil. IR (neat): 3405w, 2978w, 2956w, 1737s, 1569m, 1524s, 1436m, 1340s, 1276m, 1159s, 1133m cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.96 (d, *J* = 8.5 Hz, 1H), 7.77 (s, 1H), 7.71 (d, J = 8.7 Hz, 1H), 5.32 – 5.24 (m, 1H), 4.66 – 4.54 (m, 1H), 3.95 (s, 3H), 3.76 (m, 3H), 3.22 – 3.08 (m, 2H), 1.44 (s, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.8, 165.8, 145.8, 129.1, 128.2, 127.0, 126.6, 125.3, 124.9, 80.7, 53.6, 53.0, 52.8, 41.6, 28.4. HRMS (ESI+, MeOH): *m/z* 469.0719 [M + H]+, $C_{17}H_{22}N_2O_8S_2Na$+ requires 469.0710.

Dimethyl 3,3'-disulfanediyl (2*R*,2'*R*)-bis(2-((*tert*-butoxycarbonyl)amino)-propanoate), F



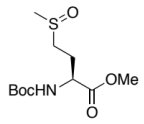
Di-Boc-L-cystine (1.00 g, 2.27 mmol) was dissolved in 10% MeOH in DCM (50 mL). (Trimethylsilyl)diazomethane (2 M in hexanes) was added dropwise until the reaction mixture remained yellow. The reaction mixture was stirred at room temperature for a further 15 mins before being concentrated under reduced pressure. The residue was purified by silica column chromatography (3 : 1 – hexanes : EtOAc) to provide **F** (872 mg, 82%) as a colourless solid, m.p. 96.5-97.6 °C, lit. m.p. 96.0-98.0 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ5.38-5.36 (m, 2H), 4.61-4.59 (m, 2H), 3.77 (s, 6H), 3.16 (d, J = 5.2 Hz, 4H), 1.45 (s, 18). All data was consistent with that previously reported.[2, 4]

Methyl (*tert*-butoxycarbonyl)-L-methioninate, G



Fmoc-Met-OH (1.00 g, 2.69 mmol) was dissolved in 10% MeOH in DCM (60 mL). (Trismethylsilyl)diazomethane (2 M in hexanes) was added dropwise until the solution remained yellow. The reaction mixture was stirred at room temperature for a further 15 mins before being concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (20 mL) and tris(2-aminoethyl)amine (3.94 g, 4.00 mL, 26.9 mmol) was added. The solution was stirred at room temperature for 1 h, after which the reaction mixture was washed with phosphate buffer (18.0 g $NaH_2PO_4.H_2O$, 6.50 g Na_2HPO_4 in 100 mL H_2O) and brine (50 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (60 mL). Et_3N (1.10 g, 1.50 mL, 10.9 mmol) and Boc anhydride (650 mg, 2.97 mmol) were added and the reaction was stirred at room temperature for 16 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with H_2O (2 x 50 mL) and brine (50 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by silica column chromatography (4 : 1 – hexanes : EtOAc) to provide **G** (554 mg, 78%) as a colourless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.10 (br s, 1H), 4.42-4.41 (m, 1H), 3.75 (s, 3H), 2.55-2.52 (m, 2H), 2.10 (s, 3H), 1.97-1.90 (m, 2H), 1.45 (s, 9H). All data was consistent with that previously reported.[5]

Methyl (2*S*)-2-((*tert*-butoxycarbonyl)amino)-4-(methylsulfinyl)butanoate, H



Compound **H** was prepared following a procedure developed by Monbaliu and coworkers.[6] A solution of $NaIO_4$ (300 mg, 1.40 mmol) in H_2O (2 mL) was added dropwise to a vigorously stirred solution of methyl (*tert*-butoxycarbonyl)-L-methioninate **G** (370 mg, 1.40 mmol) in MeOH (11 mL) at room temperature. After 12 h, the reaction mixture was filtered, and filtrate extracted with CH_2Cl_2 (2 x 50 mL). The combined extract was washed with brine (50 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by silica column chromatography (5% MeOH in EtOAc) to provide **H** (357 mg, 91%) as a colourless oil as a mixture of diasteroisomers. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.26-5.24 (m, 1H), 4.45-4.41 (m, 1H), 3.78 (s, 1.5 H), 3.77 (s, 1.5 H), 2.85-2.67 (m, 2H), 2.59 (s, 1.5 H), 2.58 (s, 1.5H), 2.40-2.32 (m, 1H), 2.15-2.04 (m, 1H), 1.45 (s, 9H). All data was consistent with that previously reported.[6]

Solid Phase Peptide Synthesis (SPPS):

Manual SPPS was performed in polypropylene Terumo syringes (5 mL) fitted with a porous (20 μm) polyethylene filter. Resin wash and filtering steps were aided by the use of a Visiprep SPE DL 24-port model vacuum manifold as supplied by Supelco. Capping reactions and cleavage mixtures were shaken on a KS125 basic KA elliptical shaker supplied by Labortechnik at 400 motions per minute.

Automated microwave-accelerated SPPS was carried out using a CEM Liberty-Discover synthesizer, involving the flow of dissolved reagents from external nitrogen pressurized bottles to a resin-containing microwave reactor vessel fitted with a porous filter. Coupling and deprotection reactions were carried out within this vessel and were aided by microwave energy. Each reagent delivery, wash, and evacuation step was carried out according to the automated protocols of the instrument (controlled by PepDriver software). In a 50 mL centrifuge tube, resin was swollen with DMF:CH_2Cl_2 (1:1; 10 mL, 1 x 60 min) and connected to the Liberty resin manifold. The Fmoc-amino acids (0.2 M in DMF), activators (0.5 M HATU in DMF), activator base (2 M DIPEA in NMP), and deprotection agent (20% v/v piperidine in DMF) were solubilized in an appropriate volume of specified solvent as calculated by the PepDriver software program. The default microwave conditions used in the synthesis of each linear peptide included: initial deprotection (36 W, 37 °C, 2 min), deprotection (45 W, 75 °C, 10 min), pre-activation (0 W, 25 °C, 2 min), and

coupling (25 W, 75 °C, 10 min), or initial deprotection (40 W, 75 °C, 0.5 min), deprotection (40 W, 75 °C, 3 min), and coupling (20 W, 75 °C, 5 min). After sequence completion, the resin-bound peptides were automatically returned to the Liberty resin manifold as a suspension in DMF:DCM (1:1) and filtered through fritted plastic syringes (5 mL) for acid-mediated cleavage.

TFA Cleavage Procedure

A small aliquot of resin-bound peptide (approximately 3 mg) was suspended in cleavage solution (1 mL; 95:2.5:2.5; TFA:TIPS:water) and shaken gently for 2 h. The mixture was filtered through a fritted syringe and the beads rinsed with TFA (1 × 0.2 mL). The filtrate was concentrated under a constant stream of air, and the resultant oil was induced to precipitate in ice-cold Et_2O (1 mL). Cleaved peptides were collected by centrifugation (3 × 5 min) and dried for analysis by analytical RP-HPLC and mass spectrometry. For full-scale resin cleavages, 10 mL of cleavage solution was used, and after 4 h, the resin was rinsed with TFA (3 × 1 mL). The filtrate was concentrated under a constant stream of air, and the resultant oil was induced to precipitate in ice-cold Et_2O (30−35 mL). Collection by centrifugation was carried out over 5 × 6 min spin times. Cleaved peptides were collected by centrifugation at a speed of 6000 rpm on a Hermle Z200A centrifuge supplied by Medos, or at a speed of 6000 rpm on a TMC-1 mini centrifuge supplied by Thermoline.

Counter Ion Exchange of Peptides:

Counter ion exchange was performed according to a modified procedure outlined by Alcalde and Dinarès.[7] In a sintered funnel, wet strongly basic ion exchange Amberlite IRA-400(OH) (5 g) was washed with H_2O (50 mL) and H_2O:MeOH (1:1) (50 mL). A solution of 1% acid in 1:1 H_2O:MeOH was slowly passed through the resin until the eluent had the same pH value as the original selected acid solution. The resin was then transferred to a round-bottom flask and stirred in the 1% aqueous solution (50 mL) for a further 4 h at room temperature. The resin was filtered and washed generously with both H_2O:MeOH (1:1) (200 mL) and H_2O (500 mL) and allowed to air dry. In a separate round-bottom flask, the ion exchange resin was added to a stirred solution of peptide substrate in H_2O:MeOH (1:1) (5-10 mL) at rt. After 16 h, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The efficiency of the ion exchange was monitored by ^{19}F NMR spectroscopy.[7]

Microwave accelerated solution phase RCM:

RCM was carried out on a CEM Discover system fitted with the Benchmate option. Reactions were performed in 10 mL high pressure glass microwave vessels fitted with self-sealing Teflon septa as a

pressure relief device. A microwave reactor vessel was loaded with substrate, deoxygenated solvent, deoxygenated chaotropic salt solution, and **GII** in an inert (nitrogen) environment. The system was sealed, and the reaction mixture irradiated with microwave energy while being stirred at a specified temperature for the specified period of time. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure and then taken up in MeOH (0.1 mL) and precipitated with Et_2O (1 mL). Peptides were collected by centrifugation (1 × 5 min) and analysed by RP-HPLC and mass spectrometry.

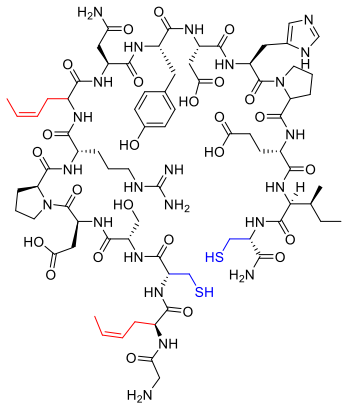
General Procedure for solution phase RCM:

A Schlenk vessel was loaded with substrate, deoxygenated solvent, deoxygenated chaotropic salt solution, and **GII** in an inert (nitrogen) environment. The reaction mixture was stirred at a specified temperature for the specified period of time under a positive flow of nitrogen. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure, taken up in a small amount of MeOH and precipitated with Et_2O. Peptides were collected by centrifugation (1 × 5 min) and analysed by RP-HPLC and mass spectrometry.

Experimental methods and product characterization:

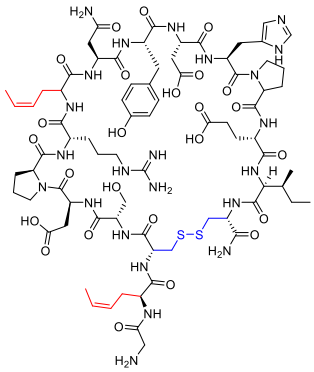
(*S*,*Z*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)hex-4-enoic acid was prepared according to Robinson *et al*.[8] and incorporated into peptide sequences using the SPPS method described below.

[2,8]-Z-Crt-[3,16]-Cys Conotoxin Vc1.1, 9



Synthesis of the linear sequence **9** was performed according to the microwave accelerated SPPS procedure described previously using Fmoc-Rink amide resin (300 µmol). Following synthesis, the peptide underwent acid-mediated cleavage to provide a crude off-white solid (625 mg). Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 609.8 $[M+ 3H]^{3+}$, $(C_{77}H_{118}N_{23}O_{25}S_2)^{3+}$ theoretical 609.6; 914.2 $[M + 2H]^{2+}$, $(C_{77}H_{117}N_{23}O_{25}S_2)^{2+}$ theoretical 913.9. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 18.2 min.

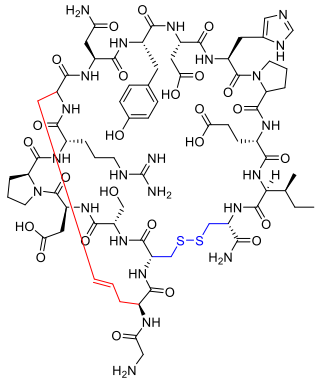
[2,8]-Z-Crt-[3,16]-cystino Conotoxin Vc1.1, 10



The crude linear peptide **9** (300 µmol) in H_2O (10 mL) was added to a stirred solution of 0.12 M NH_4HCO_3 (814 mL) at r.t. under a constant stream of air. After 16 h the reaction mixture was acidified to pH 2 with glacial AcOH and then purified by RP-HPLC (Agilent Vydac C18 preparative column, 15-50% buffer B over 40 min, t_R = 15.2 min). Selected fractions were combined and lyophilised to give the target peptide **10** as a colourless solid (38.9 mg). Mass spectrum (ESI+,

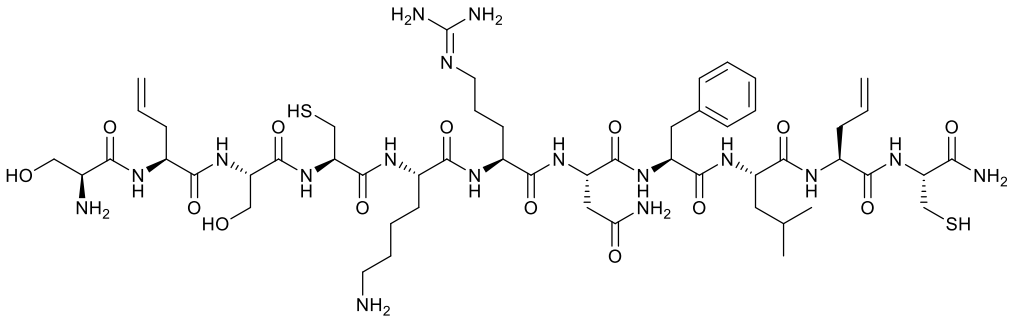
MeCN:H_2O:HCOOH): *m/z* 609.1 $[M+ 3H]^{3+}$, $(C_{77}H_{116}N_{23}O_{25}S_2)^{3+}$ theoretical 608.9; 913.0 $[M + 2H]^{2+}$, $(C_{77}H_{115}N_{23}O_{25}S_2)^{2+}$ theoretical 912.9. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 17.8 min. ^{1}H-NMR (600 MHz, D_2O): 7.28 (s, 1H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.85 (d, *J* = 8.4 Hz, 2H), 5.69-5.63 (m, 2H), 5.38-5.28 (m, 2H), 4.61-4.55 (m, 2H), 4.48-4.38 (m, 6H), 4.32-4.24 (m, 4H), 3.92-3.81 (m, 5H), 3.68-3.59 (m, 2H), 3.29-3.16 (m, 5H), 3.11-2.95 (m, 5H), 2.85-2.82 (m, 1H), 2.78-2.70 (m, 4H), 2.58-2.45 (m, 6H), 2.34-2.27 (m, 2H), 2.16-2.10 (m, 1H), 2.04-1.74 (m, 12H), 1.68-1.62 (m, 2H), 1.59-1.49 (m, 8H), 1.29 (t, *J* = 7.2 Hz, 1H), 1.24-1.18 (m, 1H), 0.93 (d, *J* = 6.6 Hz, 3H), 0.87-0.82 (m, 6H), 28 protons not observed.

[2,8]-Dicarba-[3,16]-cystino Conotoxin Vc1.1, 11



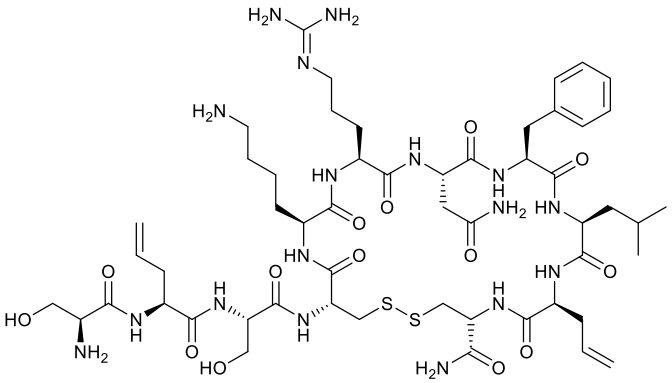
Peptide **10** was subjected to HBF_4 ion exchange conditions followed by the solution phase RCM procedure described previously under the following conditions: peptide **10.**HBF_4 (6.4 mg, 3.3 µmol), DMF (0.5 mL), 0.4 M LiCl in DMF (0.05 mL), GII (0.80 mg, 0.94 µmol), 100 °C, 4 h. RP-HPLC and mass spectral analysis of the peptide supported formation of the required unsaturated carbocycle **11** in quantitative conversion. The crude product was purified by RP-HPLC (Agilent Vydac C18 preparative column, 10-30% buffer B over 30 min, t_R = 16.2 min). Selected fractions were combined and lyophilised to give the target peptide **11** as a colourless solid (1.5 mg, 24%, 6:4 cis and trans). Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 590.6 $[M+ 3H]^{3+}$, $(C_{73}H_{108}N_{23}O_{25}S_2)^{3+}$ theoretical 590.3; 885.2 $[M + 2H]^{2+}$, $(C_{73}H_{107}N_{23}O_{25}S_2)^{2+}$ theoretical 884.9. RP-HPLC (Agilent Vydac C18 analytical column, 10-20% neutral buffer B over 30 min): t_R = 15.0 and 17.5 min. All data was consistent with the literature.[9]

[2,10]-Agl-[4,11]-Cys Conotoxin pc16a, 17



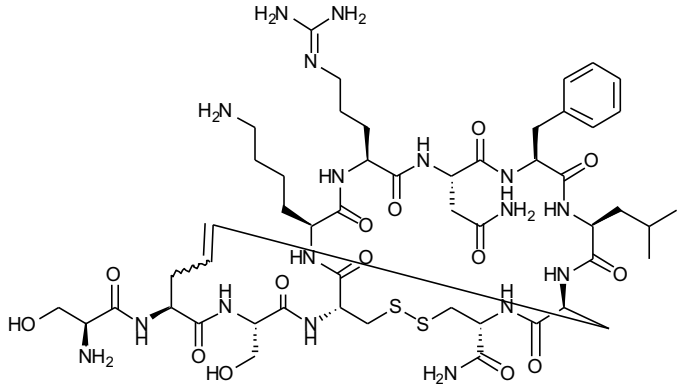
Synthesis of the linear sequence **17** was performed according to the microwave accelerated SPPS procedure described previously using Fmoc-Rink amide resin (0.2 mmol). Following synthesis, the peptide underwent acid-mediated cleavage to provide a crude off-white solid (299 mg). Mass spectrum (ESI+, MeCN:H_2O): *m/z* 417.8 $[M+ 3H]^{3+}$, $(C_{53}H_{90}N_{17}O_{14}S_2)^{3+}$ theoretical 417.5, *m/z* 626.0 $[M+ 2H]^{2+}$, $(C_{53}H_{89}N_{17}O_{14}S_2)^{2+}$ theoretical 625.8. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 18.6 min.

[2,10]-Agl-[4,11]-cystino Conotoxin pc16a, 18



The crude linear peptide **17** (150 mg) in H_2O (5 mL) was added to a stirred solution of 0.1 M NH_4HCO_3 (500 mL) at r.t. under a constant stream of air. After 72 h the reaction mixture was acidified to pH 3 with glacial AcOH and then purified by RP-HPLC (Agilent Vydac C18 preparative column, 10-50% buffer B over 40 min, t_R = 16.0 min). Selected fractions were combined and lyophilised to give the target peptide **18** as a colourless solid (27 mg). Mass spectrum (ESI+, MeCN:H_2O): *m/z* 625.0 $[M+ 2H]^{2+}$, $(C_{53}H_{87}N_{17}O_{14}S_2)^{2+}$ theoretical 624.8. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 20.1 min

[2,10]-Dicarba-[4,11]-cystino Conotoxin pc16a, 19



Peptide **18** was subjected to HBF_4 ion exchange conditions followed by the microwave accelerated solution phase RCM procedure described previously under the following conditions: peptide **18.**HBF_4 (2.5 mg, 2.0 μmol), DMF (1.1 mL), 0.4 M LiCl in DMF (0.15 mL), GII (0.51 mg, 0.60 μmol), 100 W, 100 °C, 4 h. RP-HPLC and mass spectral analysis of the peptide supported formation of the required unsaturated carbocycle **19** in 58% conversion as a 1:1 mixture of *E*- and *Z*-isomers.

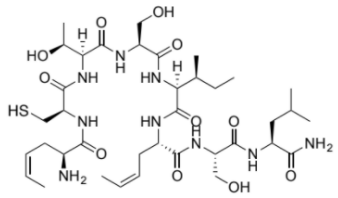
19(I): RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 19.3 min. Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 611.0 $[M + 2H]^{2+}$, $(C_{51}H_{81}N_{17}O_{14}S_2)^{2+}$ theoretical 610.8. ^{1}H-NMR (600 MHz, D_2O): δ 7.41 (t, *J* = 7.4 Hz, 2H), 7.35 (d, *J* = 7.3 Hz, 1H), 7.33 (d, *J* = 7.9 Hz, 2H), 5.58 (dt, *J* = 14.8, 7.2 Hz, 1H), 5.45 (dt, *J* = 15.2, 7.6 Hz, 1H), 5.16 (d, *J* = 15.8 Hz, 1H), 4.68 – 4.62 (m, 1H), 4.62 – 4.56 (m, 1H), 4.28 (dd, *J* = 9.5, 5.7 Hz, 1H), 4.22 (d, *J* = 10.6 Hz, 1H), 4.11 (t, *J* = 7.6 Hz, 1H), 4.04 (dd, *J* = 10.2, 6.1 Hz, 1H), 3.91 (dd, *J* = 10.6, 6.0 Hz, 1H), 3.77 (t, *J* = 10.2 Hz, 1H), 3.34 – 3.13 (m, 8H), 3.02 – 2.93 (m, 5H), 2.80 (dd, *J* = 15.7, 5.4 Hz, 1H), 2.64 (t, *J* = 9.9 Hz, 1H), 2.60 – 2.52 (m, 1H), 2.52 – 2.44 (m, 1H), 2.35 (q, *J* = 10.9 Hz, 1H), 1.74 (q, *J* = 8.1 Hz, 2H), 1.71 – 1.63 (m, 2H), 1.54 – 1.37 (m, 3H), 1.32 (d, *J* = 13.6 Hz, 2H), 0.92 (d, *J* = 5.8 Hz, 1H), 0.89 (d, *J* = 6.6 Hz, 3H), 0.82 (d, *J* = 6.5 Hz, 3H), 30 protons not observed.

19(II): RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 21.0 min. Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 610.9 $[M + 2H]^{2+}$, $(C_{51}H_{81}N_{17}O_{14}S_2)^{2+}$ theoretical 610.8. ^{1}H-NMR (600 MHz, D_2O): δ 7.45 – 7.29 (m, 5H), 5.66 (s, 1H), 5.54 (s, 1H), 4.44 (dd, *J* = 28.8, 17.0 Hz, 2H), 4.22 – 4.11 (m, 2H), 4.08 (q, *J* = 7.1 Hz, 1H), 4.03 – 3.96 (m, 1H), 3.85 (d, *J* = 46.4 Hz, 5H), 3.37 (dd, *J* = 14.8, 10.8 Hz, 2H), 3.30 – 3.20 (m, 2H), 3.15 (ddd, *J* = 32.6, 14.2, 7.0 Hz, 2H), 3.04 – 2.68 (m, 7H), 2.32 (s, 1H), 2.16 – 1.54 (m, 4H), 1.47 (t, *J* = 12.0 Hz, 3H), 1.39 – 1.21 (m, 2H), 0.90 (q, *J* = 7.2 Hz, 6H), 0.82 (q, *J* = 6.7 Hz, 3H), 30 protons not observed.

[1,6]-Dicarba-[2]-Ala Human Insulin A chain (6-13), 2

Prepared according to the method of Robinson *et al*..[10]

[1,6]-Z-Crt-[2]-Cys Human Insulin A Chain (6-13), 3

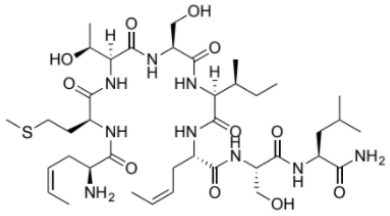


Synthesis of the linear sequence **3** was performed according to the microwave accelerated SPPS procedure described previously using Fmoc-Rink amide resin (100 μmol). Following synthesis, the peptide underwent acid-mediated cleavage to provide an off-white solid. The crude peptide was purified by RP-HPLC (Agilent Vydac C18 preparative column, 10-40% buffer B over 45 min, t_R = 24.1 min). Selected fractions were combined and lyophilised to give the target peptide **3** as a colourless solid (14.8 mg). Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 844.4 [M + H]+, ($C_{37}H_{66}N_9O_{11}S$) theoretical 844.5. RP-HPLC (Agilent Vydac C18 analytical column, 20-50% buffer B over 30 min): t_R = 7.0 min. ^{1}H-NMR (600 MHz, $(CD_3)_2SO$): 8.09 (d, *J* = 7.8 Hz, 1H), 7.99 (d, *J* = 7.8 Hz, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 7.89-7.83 (m, 3H), 7.23 (s, 2H), 7.08 (s, 2H), 5.63-5.58 (m, 1H), 5.50-5.45 (m, 2H), 5.38-5.30 (m, 2H), 5.12-4.98 (m, 3H),4.64-4.62 (m, 1H), 4.43-4.40 (m, 2H), 4.33-4.28 (m, 4H), 4.22-4.18 (m, 3H), 3.65-3.57 (m, 2H), 2.42-2.39 (m, 1H), 2.30-2.26 (m, 1H), 1.78-1.73 (m, 2H), 1.66-1.55 (m, 6H), 1.52-1.46 (m, 3H), 1.43-1.39 (m, 2H), 1.10-1.07 (m, 1H), 1.05 (d, *J* = 6.6 Hz, 4H), 0.88 (d, *J* = 6.6 Hz, 6H), 0.84-0.80 (m, 9H).

[1,6]-Dicarba-[2]-Cys Human Insulin A chain (6-13), 5

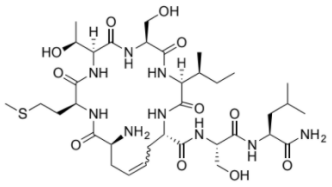
Linear peptide **3** was subjected to HBF_4 ion exchange conditions followed by the solution phase RCM procedure described previously under the following conditions: linear peptide **3.**HBF_4 (15.5 mg), DMF (1 mL), 0.4 M LiCl in DMF (0.1 mL), GII (4.1 mg), 100 °C, 4 h. RP-HPLC and mass spectral analysis of the crude peptide showed only starting peptide **3**.

[1,6]-Z-Crt-[2]-Met Human Insulin A Chain (6-13), 4



Synthesis of the linear sequence **4** was performed according to the microwave accelerated SPPS procedure described previously using Fmoc-Rink amide resin (300 μmol). Following synthesis, the peptide underwent acid-mediated cleavage to provide an off-white solid. The crude peptide was purified by RP-HPLC (Agilent Vydac C18 preparative column, 15-45% buffer B over 40 min, t_R = 21.2 min). Selected fractions were combined and lyophilised to give the target peptide **4** as a colourless solid (17.2 mg). Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 872.5 [M + H]+, ($C_{39}H_{70}N_9O_{11}S$) theoretical 872.5; 894.5 $[M + Na]^+$, ($C_{39}H_{69}N_9O_{11}SNa$) theoretical 894.5. RP-HPLC (Agilent Vydac C18 analytical column, 15-50% buffer B over 35 min): t_R = 13.1 min. ^{1}H-NMR (600 MHz, $(CD_3)_2SO$): 8.62 (d, *J* = 8.4 Hz, 1H), 8.10 (br s, 2H), 7.99-7.97 (m, 1H), 7.94-7.91 (m, 1H), 7.88-7.85 (m, 2H), 7.23 (br s, 1H), 7.08 (s, 1H), 5.63-5.58 (m, 1H), 5.50-5.46 (m, 1H), 5.36-5.29 (m, 2H), 5.12-5.11 (m, 1H), 4.99 (d, *J* = 4.8 Hz, 2H), 4.58-4.54 (m, 1H), 4.42-4.39 (m, 1H). 4.33-4.28 (m, 3H), 4.21-4.18 (m, 3H), 4.01-3.97 (m, 1H), 3.86 (br s, 1H), 3.65-3.55 (m, 4H), 3.52-3.48 (m, 2H), 2.31-2.26 (m, 1H), 1.99-1.94 (m, 1H), 1.81-1.73 (m, 2H), 1.63-1.38 (m, 12H), 1.04 (d, *J* = 6.6 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 6H), 0.83-0.79 (m, 12H).

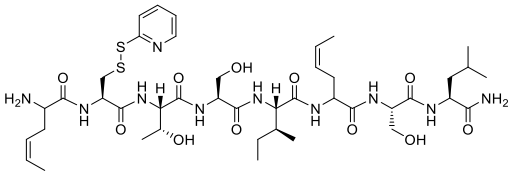
[1,6]-Dicarba-[2]-Met Human Insulin A Chain (6-13), 6



Linear peptide **4** was subjected to HBF_4 ion exchange conditions followed by the solution phase RCM procedure described previously under the following conditions: linear peptide **4.**HBF_4 (9.2 mg, 9.3 μmol), DMF (1 mL), 0.4 M LiCl in DMF (0.1 mL), GII (2.4 mg, 2.8 μmol), 100 °C, 4 h. RP-HPLC and mass spectral analysis of the peptide supported formation of the required unsaturated carbocycle **6** in 40% conversion as a mixture of *E*- and *Z*-isomers. RP-HPLC (Agilent Vydac C18 analytical column, 15-50% buffer B over 35 min): t_R = 7.9 and 8.3 min. Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 816.5 [M + H]+, ($C_{35}H_{61}N_9O_{11}S$) theoretical 816.4. ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ 8.85 (br s, 1H), 8.49 (br s, 1H), 8.22 (br s, 2H), 8.09 (br s, 1H), 7.77 (d, *J* = 8.0 Hz,

1H), 7.73 – 7.55 (m, 2H), 7.40 – 7.34 (m, 1H), 7.20 (d, *J* = 7.7 Hz, 1H), 7.12 (s, 1H), 7.07 (s, 1H), 5.66 – 5.55 (m, 2H), 5.52 – 5.46 (m, 1H), 4.52 – 4.42 (m, 2H), 4.38 (br s, 1H), 4.26 – 4.18 (m, 1H), 4.16 – 4.05 (m, 3H), 4.04 – 3.94 (m, 2H), 3.91 – 3.83 (m, 1H), 3.84 – 3.52 (m, 3H), 2.72 – 2.64 (m, 1H), 2.39 – 2.29 (m, 1H), 2.16 – 2.08 (m, 1H), 1.88 – 1.71 (m, 2H), 1.66 – 1.57 (m, 1H), 1.50 (t, *J* = 7.3 Hz, 2H), 1.42 – 1.33 (m, 2H), 1.10 (d, *J* = 6.2 Hz, 4H), 0.87 (d, *J* = 6.6 Hz, 4H), 0.84 – 0.76 (m, 12H).

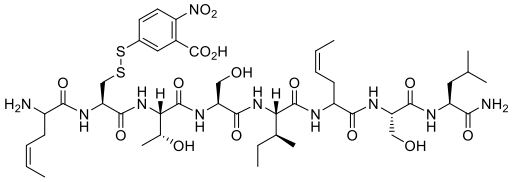
[1,6]-Z-Crt-[2]-CysS(SPy) Human Insulin A Chain (6-13), 7



Crude linear peptide **3** (8.4 mg) was suspended in Millipore water (6.4 mL), MeCN (2 mL), buffer A (1 mL) and MeOH (2 mL). A solution of 2,2'-dipyridyldisulfide (0.84 mL, 4 mM in MeOH) was added and the reaction mixture was stirred at r.t. for 3 h then lyophilised. The resulting solid was washed with ice cold Et_2O (15 mL) and peptides were collected by centrifugation (3 × 10 min). The crude peptide was purified by RP-HPLC (Agilent Vydac C18 preparative column, 15-50% buffer B over 35 min, t_R = 15.9 min). Selected fractions were combined and lyophilised to give the target peptide **7** as a colourless solid. Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 953.6 [M + H]+, ($C_{42}H_{69}N_{10}O_{11}S_2$) theoretical 953.2.

Linear peptide **7** was subjected to HBF_4 ion exchange conditions followed by the optimized solution phase RCM procedure. The target metathesis product **8** could not be identified post-metathesis and only the starting peptide **7** was recovered.

Synthesis and attempted RCM of [1,6]-Z-Crt-[2]-CysS(SNB) Human Insulin A Chain (6-13), 16

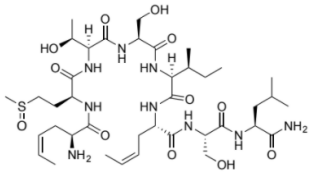


Crude linear peptide **3** (19.7 mg) was suspended in MeCN (1 mL), buffer A (19 mL) and MeOH (2 mL). A solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (3.5 mL, 4mM in MeOH) was added and the reaction mixture was stirred at r.t. for 3 h then lyophilised. The crude peptide was purified by

RP-HPLC (Agilent Vydac C18 preparative column, 15-60% buffer B over 90 min, t_R = 33.0 min). Selected fractions were combined and lyophilised to give the target peptide as a colourless solid **16**. RP-HPLC (Agilent Vydac C18 analytical column, 15-50% buffer B over 35 min, t_R = 18.3 min). Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 1041.5 [M + H]+, ($C_{44}H_{69}N_{10}O_{15}S_2$) theoretical 1041.4, *m/z* 1063.4 [M + Na]+, ($C_{44}H_{68}N_{10}O_{15}S_2Na$) theoretical 1063.4.

Linear peptide **16** (75% purity) was subjected to HBF_4 ion exchange conditions followed by the optimized solution phase RCM procedure. The target metathesis product could not be identified in the resultant complex reaction mixture.

[1,6]-Z-Crt-[2]-Met(O) Human Insulin A Chain (6-13), 14

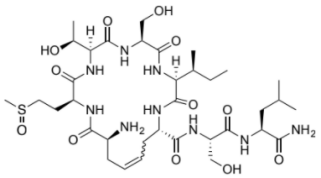


Synthesis of the linear sequence **14** was performed according to the microwave accelerated SPPS procedure described previously using Fmoc-Rink amide resin (300 μmol) and Fmoc-L-methionine-D,L-sulfoxide. Following synthesis, the peptide underwent acid-mediated cleavage to provide an off-white solid. The crude peptide was purified by RP-HPLC (Agilent Vydac C18 preparative column, 15-50% buffer B over 40 min, t_R = 15.2 min). Selected fractions were combined and lyophilised to give the target peptide **14** as a colourless solid (13.9 mg). Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 888.8 [M + H]+, ($C_{39}H_{70}N_9O_{12}S$) theoretical 888.5. RP-HPLC (Agilent Vydac C18 analytical column, 15-50% buffer B over 35 min): t_R = 10.4 min. ^{1}H-NMR (600MHz, $(CD_3)_2SO$): δ 8.59-8.49 (m, 1H), 8.14-8.10 (m, 1H), 7.97-7.96 (m, 1H), 7.91 (d, *J* = 8.4 Hz, 1H), 7.89-7.84 (m, 3H), 7.22 (s, 1H), 7.07 (s, 1H), 5.60-5.54 (m, 1H), 5.49-5.44 (m, 1H), 5.37-5.27 (m, 3H), 5.10 (t. *J* = 5.4 Hz, 1H), 5.00-4.99 (m, 1H), 4.98-4.96 (m, 1H), 4.62-4.60 (m, 1H), 4.42-4.39 (m, 1H), 4.33-4.27 (m, 3H), 4.21-4.17 (m, 2H), 4.01-3.98 (m, 1H), 3.64-3.54 (m, 3H), 3.51-3.47 (m, 1H), 2.83-2.75 (m, 2H), 2.71-2.61 (m, 1H), 2.49-2.42 (m, 2H), 2.36-2.22 (m, 1H), 2.09-1.99 (m, 2H), 1.95-1.87 (m, 2H), 1.76-1.72 (m, 2H), 1.62-1.59 (m, 1H), 1.57-1.52 (m, 6H), 1.51-1.45 (m, 3H), 1.42-1.38 (m, 2H), 1.10-1.07 (m, 1H), 1.04 (d, *J* = 6.0 Hz, 3H), 0.87 (d, *J* = 6.0 Hz, 3H), 0.83-0.77 (m, 9H).

Oxidation of [1,6]-Z-Crt-[2]-Met Human Insulin A Chain (6-13) 4 to [1,6]-Z-Crt-[2]-Met(O) Human Insulin A Chain (6-13) 14

Oxidation of the linear methionine-containing insulin fragment **4** was carried out according to a modified procedure outlined by Lecommandoux and coworkers.[11] Insulin fragment **4** was dissolved in 30% H_2O_2 and 1% AcOH in water (1.5 mL) and stirred at 0 °C for 15 mins. The reaction was quenched with a few drops of 1 M sodium thiosulfate. A small aliquot was taken, and RP-HPLC and mass spectral analysis of the peptide supported formation of the required peptide **14** with quantitative conversion. Mass spectrum (ESI+, MeCN, H_2O, HCOOH): *m/z* 888.8 [M + H]+, ($C_{39}H_{70}N_9O_{12}S$) theoretical 888.5. RP-HPLC (Agilent Vydac C18 analytical column, 15-50% buffer B over 35 min): t_R = 10.3 min.

[1,6]-Dicarba-[2]-Met(O) Human Insulin A Chain (6-13), 15



Linear peptide **14** was subjected to HBF_4 ion exchange conditions followed by the solution phase RCM procedure described previously under the following conditions: linear peptide **14.**HBF_4 (10.9 mg, 10.9 μmol), DMF (1 mL), 0.4 M LiCl in DMF (0.1 mL), GII (2.8 mg, 3.3 μmol), 100 °C, 4 h. RP-HPLC and mass spectral analysis of the peptide supported formation of the required unsaturated carbocycle **15** in 80% conversion (isomers inseparable). Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 416.7 $[M + 2H]^{2+}$, ($C_{35}H_{64}N_9O_{12}S$) theoretical 416.7; 832.4 [M + H]+, ($C_{35}H_{62}N_9O_{12}S$) theoretical 832.4. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 35 min): t_R = 14.8 min and 15.0 min. ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ 8.97 – 8.92 (m, 1H), 8.51 (s, 1H), 8.25 (br s, 2H), 8.11 (br s, 1H), 7.77 (d, *J* = 8.1 Hz, 1H), 7.70 – 7.57 (m, 2H), 7.38 (t, *J* = 9.4 Hz, 0H), 7.20 (d, *J* = 7.8 Hz, 1H), 7.12 (s, 1H), 7.07 (s, 1H), 5.71 – 5.52 (m, 1H), 5.51 – 5.46 (m, 1H), 4.53 – 4.40 (m, 2H), 4.40 – 4.33 (m, 1H), 4.25 – 4.18 (m, 1H), 4.17 – 3.93 (m, 4H), 3.90 – 3.84 (m, 1H), 3.82 – 3.61 (m, 1H), 2.86 – 2.79 (m, 1H), 2.76 – 2.63 (m, 1H), 2.39 – 2.31 (m, 1H), 2.27 – 2.16 (m, 1H), 2.00 – 1.87 (m, 1H), 1.84 – 1.74 (m, 1H), 1.67 – 1.56 (m, 1H), 1.50 (t, *J* = 7.3 Hz, 2H), 1.41 – 1.32 (m, 1H), 1.11 (d, *J* = 6.2 Hz, 4H), 0.87 (d, *J* = 6.5 Hz, 4H), 0.83 – 0.77 (m, 12H).

Reduction of [1,6]-Dicarba-[2]-Met(O) Human Insulin A Chain (6-13) 15 to [1,6]-Dicarba-[2]-Met Human Insulin A Chain 6

Method 1

Enzyme catalyzed reduction of methionine sulfoxide **15** to methionine fragment **6** was carried out according to a modified procedure by Deming and coworkers.[12] Insulin fragment **15** was diluted in Millipore water (1.2 mL) containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 30 mM KCl, 20 mM DTT, and 2.5 μg of each of methionine sulfoxide reductase A and methionine sulfoxide reductase B. A control sample was also prepared as above but without the addition of the enzymes. The reactions were placed in a 37 °C water bath for 16 h. A small aliquot was then removed and RP-HPLC and mass spectral analysis of the reaction mixture supported formation of the reduced methionine peptide **6** with 67% conversion. Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 816.5 [M + H]+, ($C_{35}H_{61}N_9O_{11}S$) theoretical 816.4. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 17.9 and 18.4 min. Addition of further enzyme, reagents, adjustment of reaction pH, and/or longer reaction time did not lead to further reaction conversion.

Method 2

Chemical reduction of methionine sulfoxide **15** to methionine fragment **6** was carried out according to a modified procedure by Beck and Jung.[13] Insulin fragment **15** was dissolved in TFA (3 mL) in a 15 mL falcon tube. Ethane-1,2-dithiol (45 μL) and bromotrimethylsilane (45 μL) were added to the solution and the reaction stirred at room temperature for 15 minutes. The reaction mixture was then concentrated under flow of N_2. The peptide material was precipitated *via* the addition of ice cold Et_2O (10 mL) then collected by centrifugation (5 min at 6000 rpm). RP-HPLC and mass spectral analysis of the crude product supported formation of the reduced methionine peptide **6** with quantitative conversion. Mass spectrum (ESI+, MeCN:H_2O:HCOOH): *m/z* 816.3 [M + H]+, ($C_{35}H_{61}N_9O_{11}S$) theoretical 816.4. RP-HPLC (Agilent Vydac C18 analytical column, 5-35% buffer B over 30 min): t_R = 17.9 and 18.4 min.

Theoretical methodology

All studied compounds were optimized with ωB97XD[14]/aug-cc-pVDZ and a conductor-like polarisable continuum model (CPCM)[15] with water as solvent. All optimisations were performed with Gaussian 16.[16]

Table S1. Geometric parameters and molecular orbital energies of lone pairs on sulfur atoms (HOMO and HOMO-1).

Compound	R(S-S), Å	∠(C_1-S-S)[a]	∠(C_2-S-S)	∠(C_1-S-S-C_2)	HOMO, eV	HOMO-1, eV
B	…	96.5[b]	…	…	-8.9	…
C	…	100.0[c]	…	…	-9.3	…
D	2.07	105.5	103.6	87.9	-8.5	-9.1
E	2.08	103.6	102.6	87.6	-9.0	-9.3
F	2.08	104.0	103.2	84.0	-8.8	-9.0

[a] In case of two substituents – aliphatic and aryl, C_1 is aliphatic and C_2 is aryl; [b] C_1-S-H; [b] C_1-S-C($OOCH_3$)

Figure S1. Gaussian 16 archive entries for optimized compounds **B**, **C**, **D**, **E** and **F** together with their ball-and-stick structures (atoms labeled) and HOMO images.

Compound **B**:

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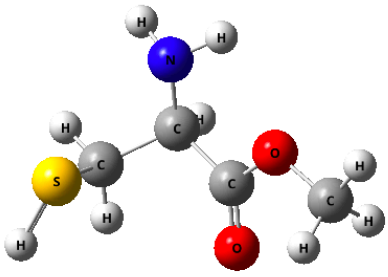

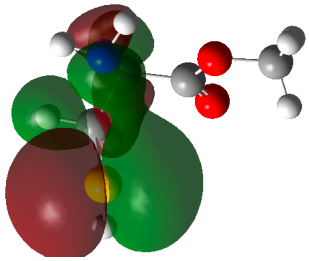

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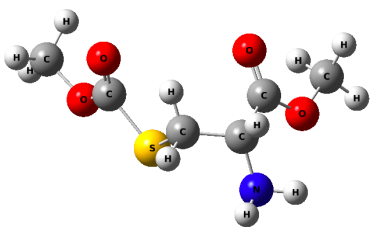

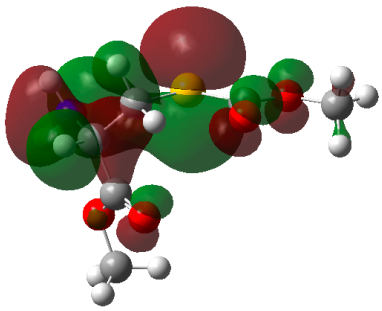

Compound **D**:


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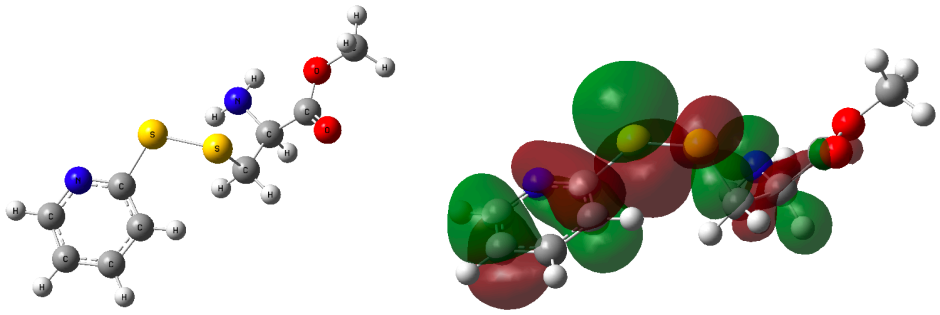

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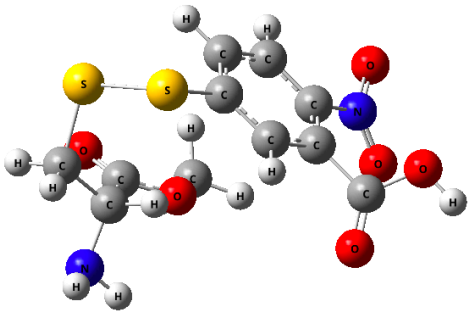

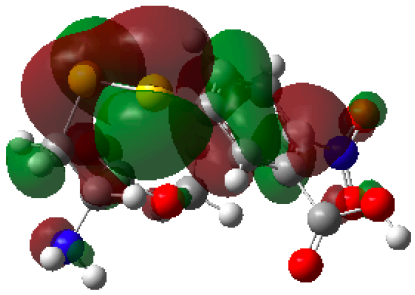

Compound **F**:


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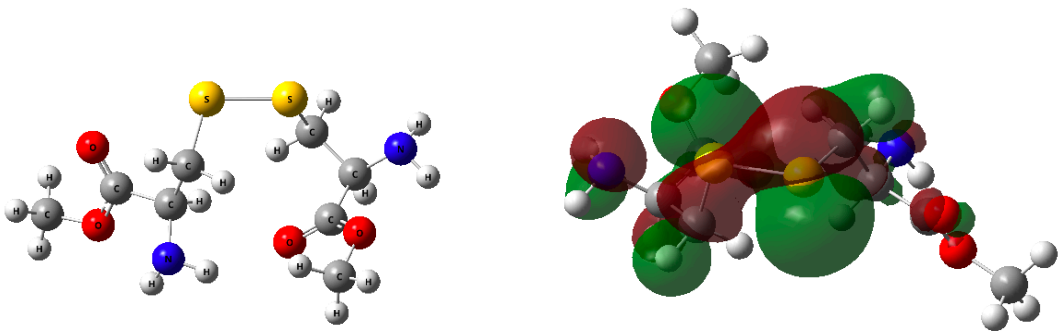

References:

1. O. Krenk, J. Kratochvíl, M. Šoulák, V. Buckta, J. Kuneš, L. Nováková, M. Ghavre and M. Pour, *Eur. J. Org. Chem.,* 2015, 5414.
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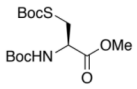
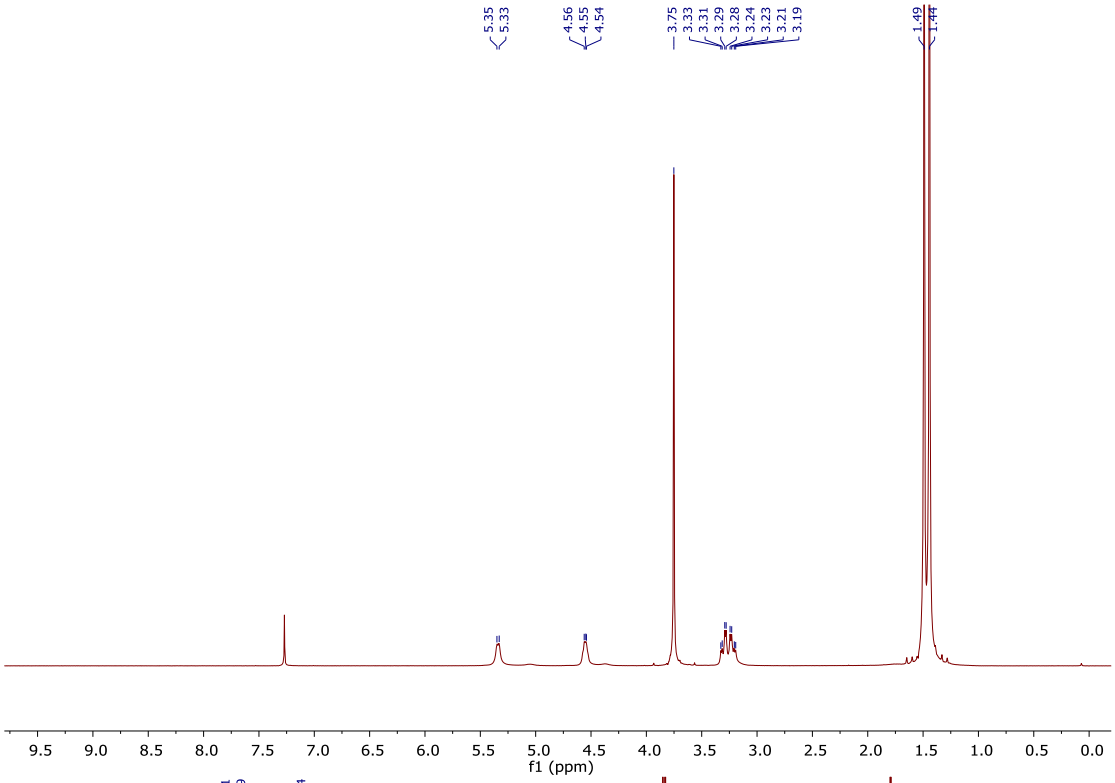
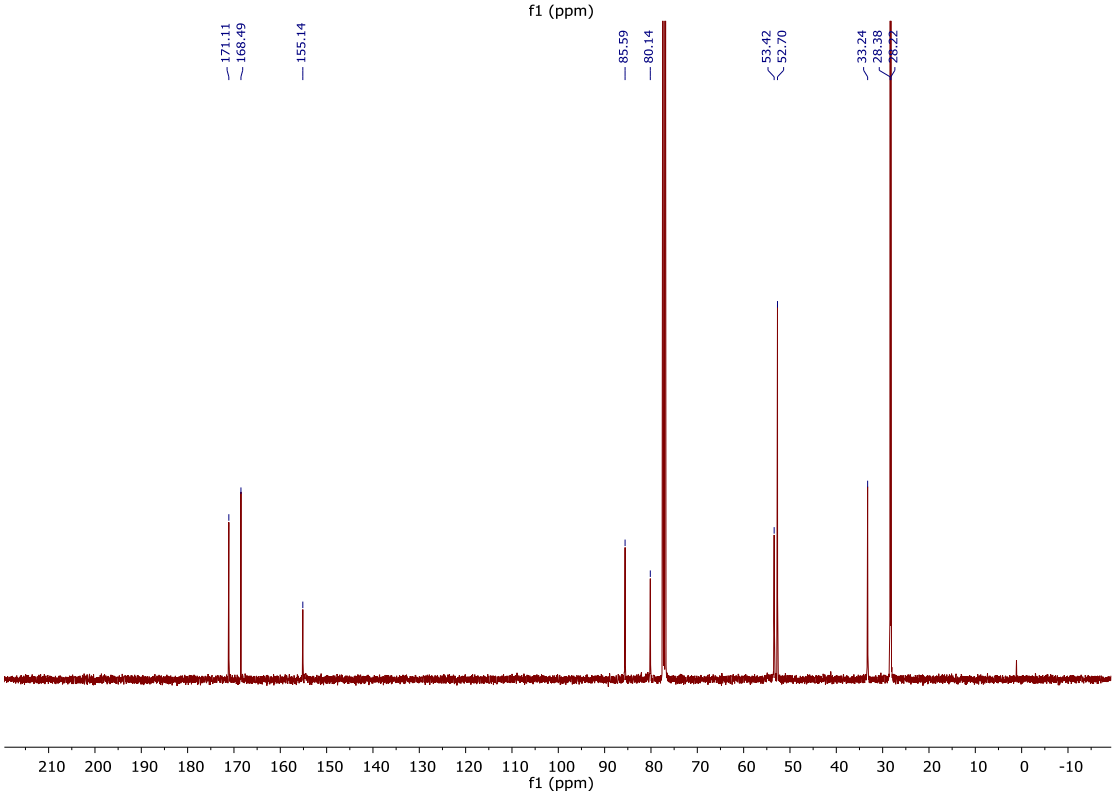
Supporting Spectra

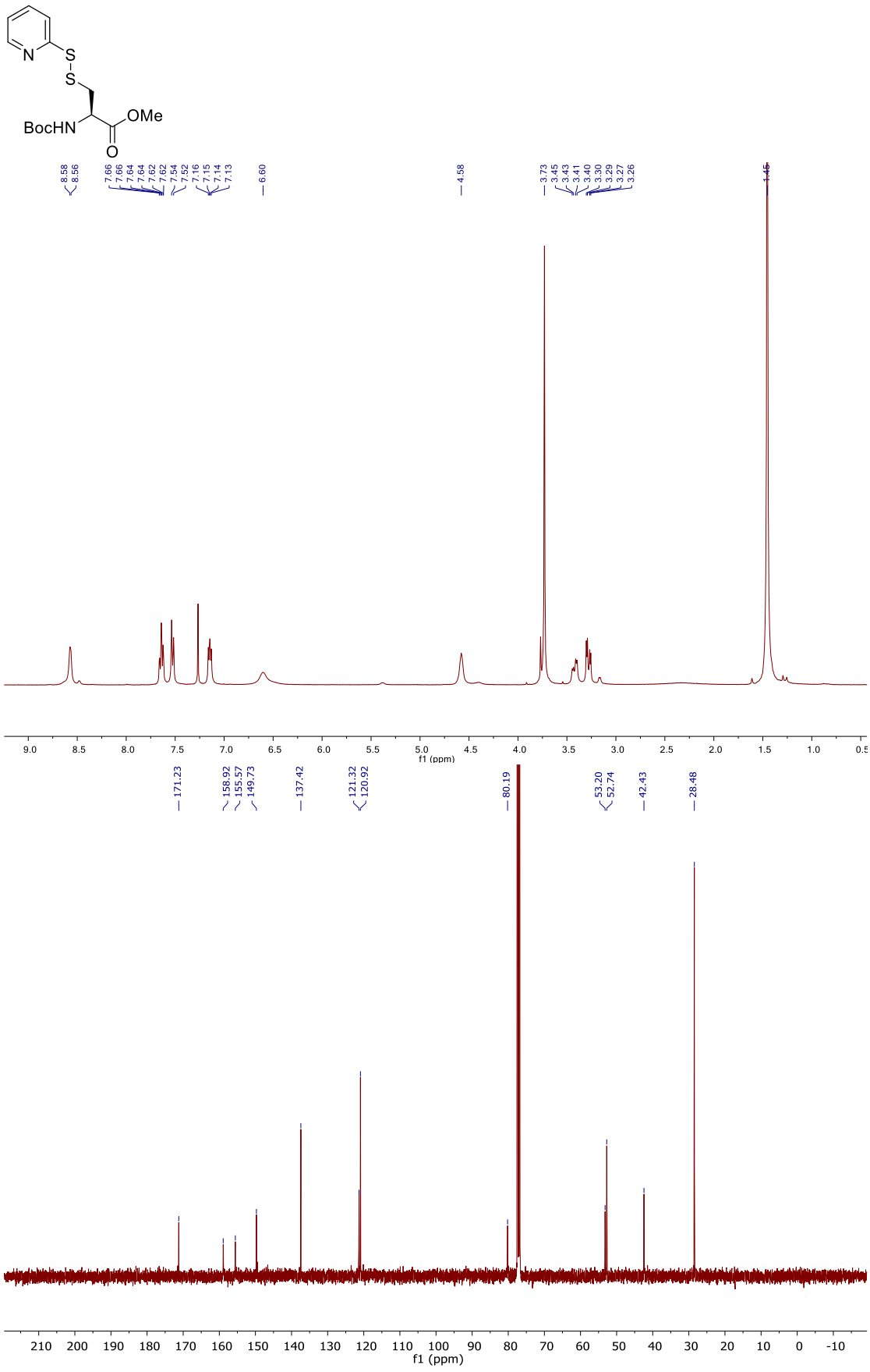
Methyl *N*,*S*-bis(*tert*-butoxycarbonyl)-L-cysteinate, C



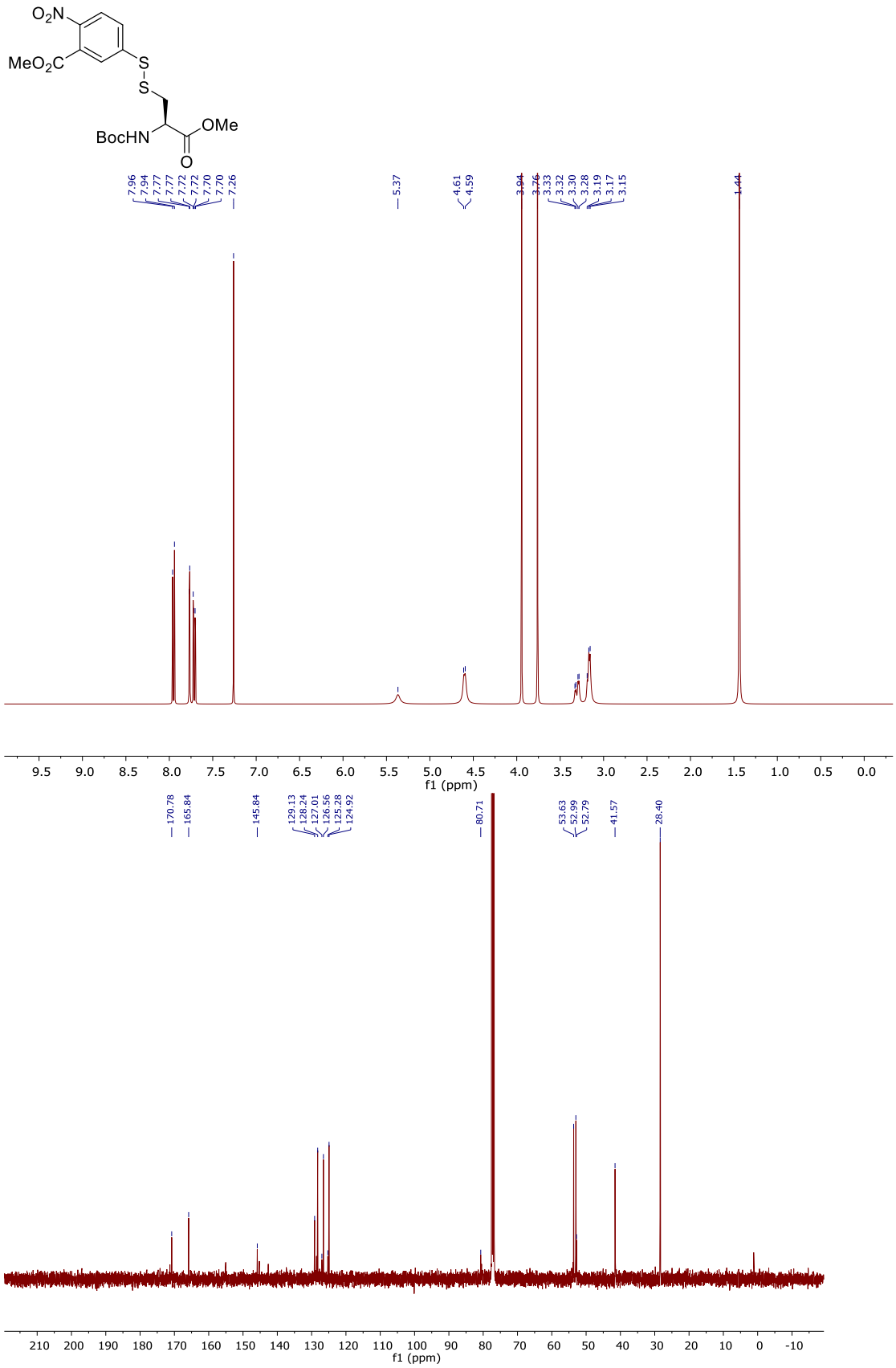




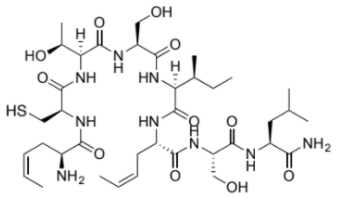
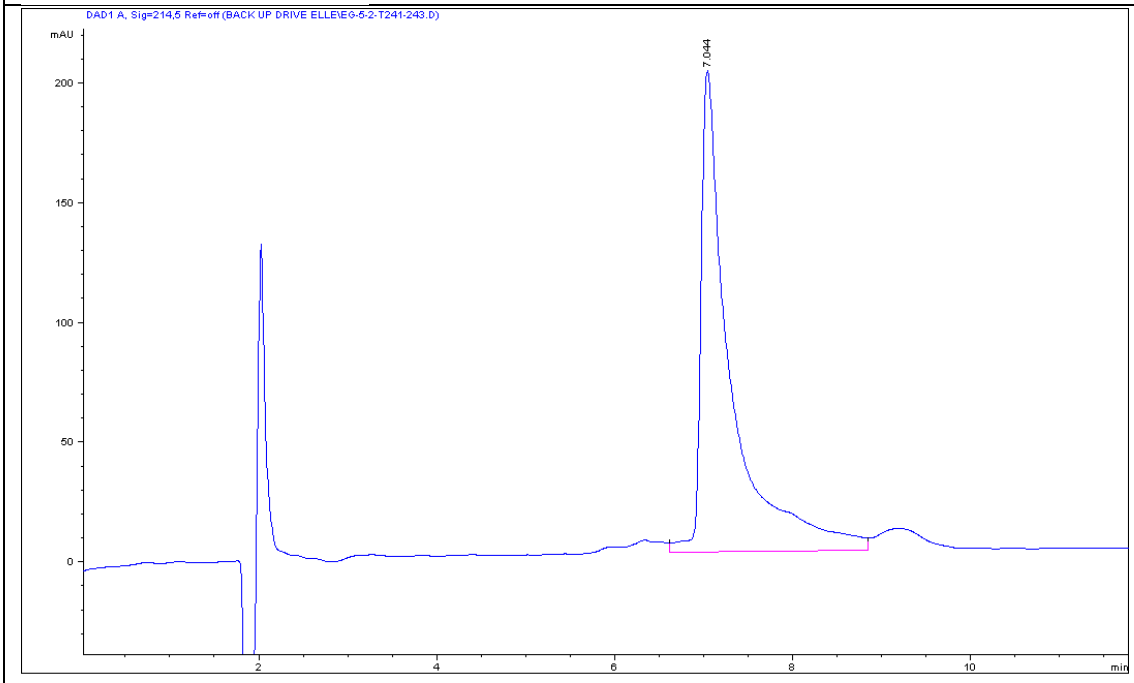
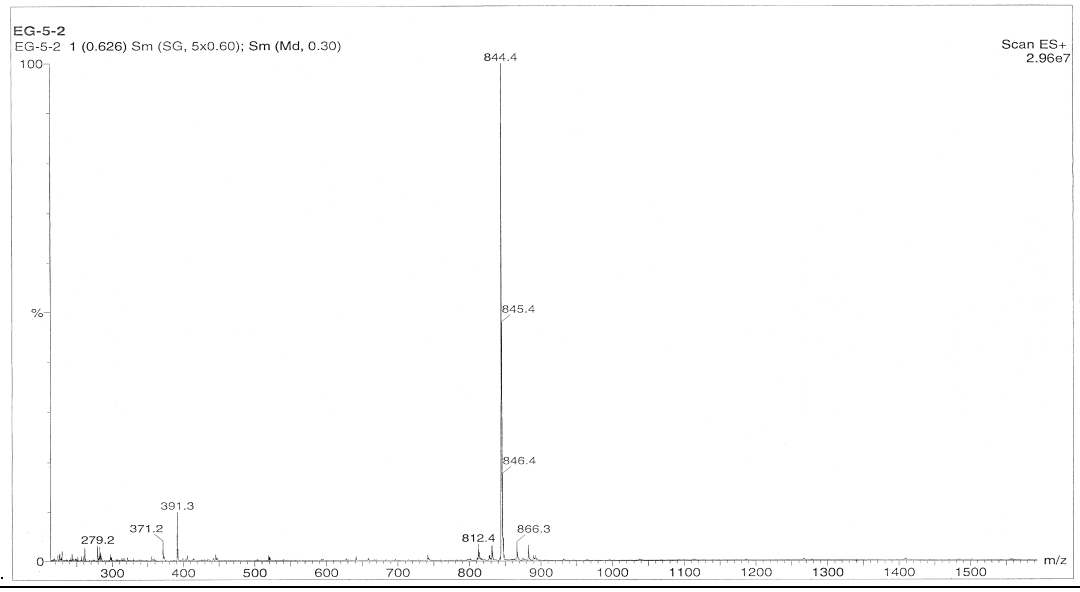
Methyl *N*-(*tert*-butoxycarbonyl)-*S*-(2-thiopyridinyl)cysteinate, D

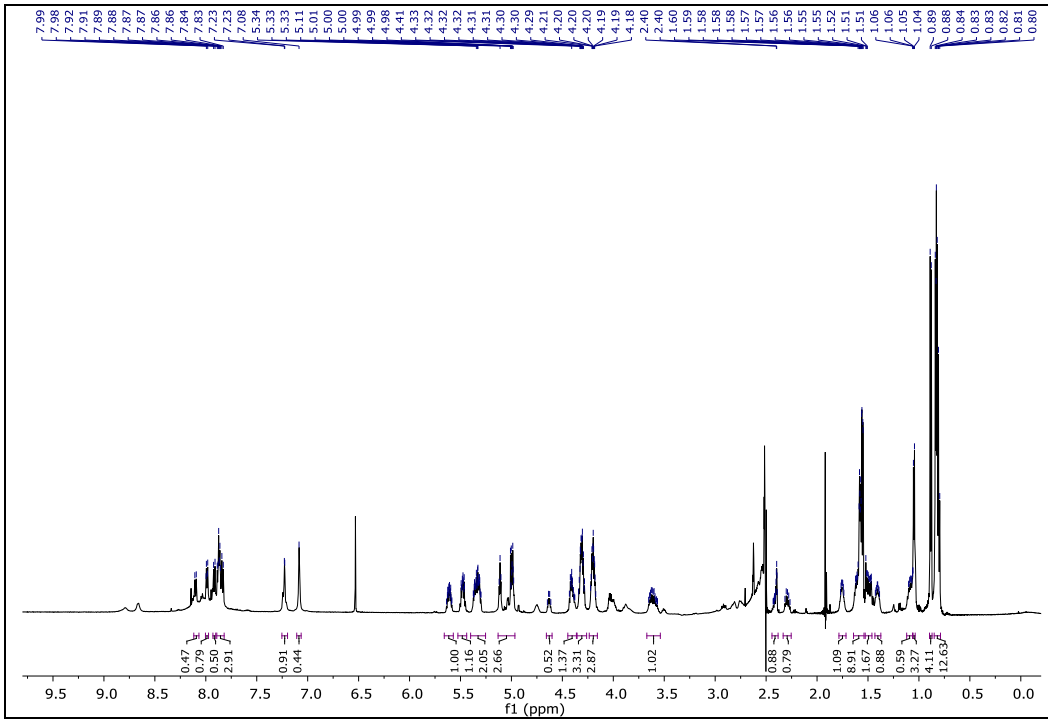


Methyl (*R*)-5-((2-((*tert*-butoxycarbonyl)amino)-3-methoxy-3-oxopropyl)disulfaneyl)-2-nitrobenzoate, E



[1,6]-Z-Crt-[2]-Cys Human Insulin A Chain (6-13), 3

	**3**
	Purified peptide
	



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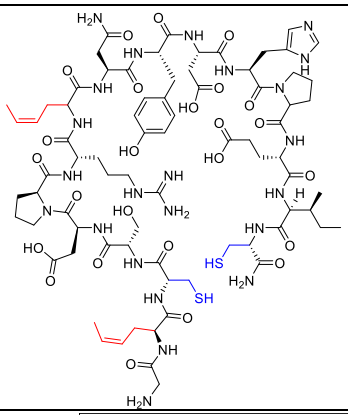
[2,8]-Z-Crt-[3,16]-Cys Conotoxin Vc1.1, 9



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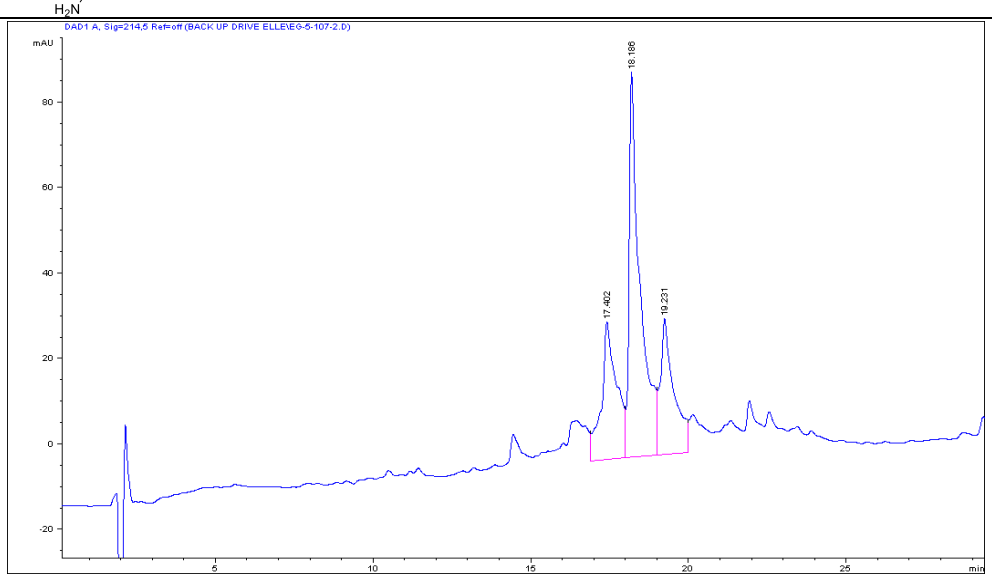
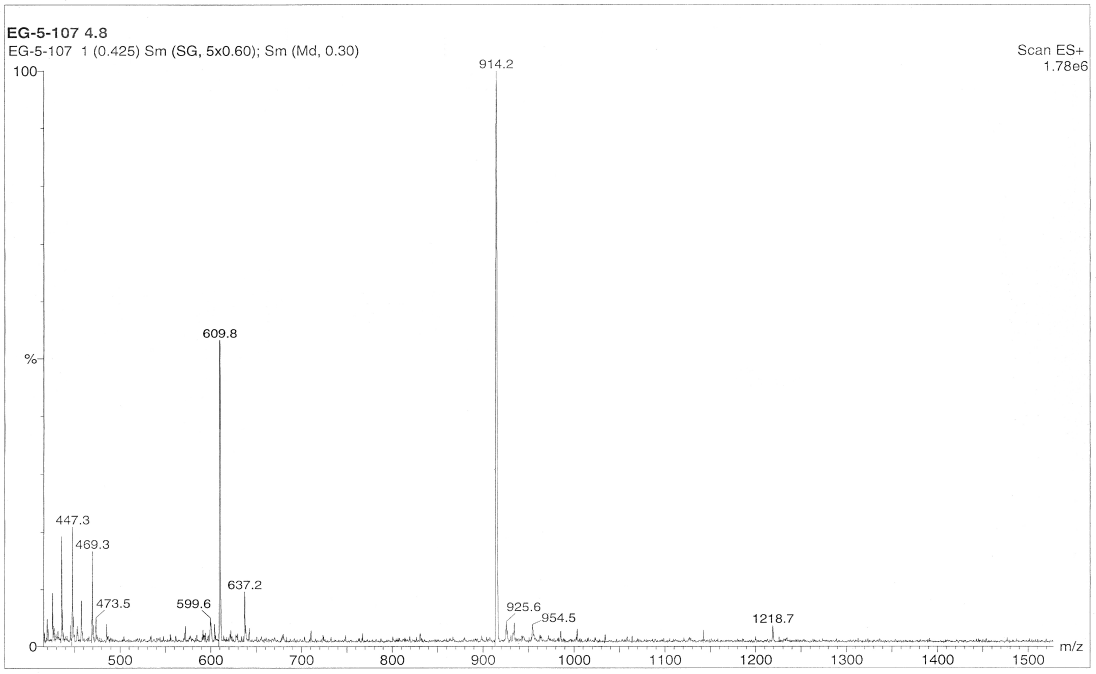
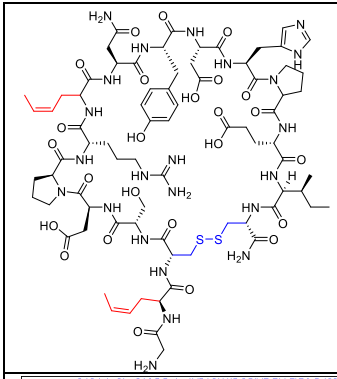


Crude peptide



9

[2,8]-Z-Crt-[3,16]-cystino Conotoxin Vc1.1, 10



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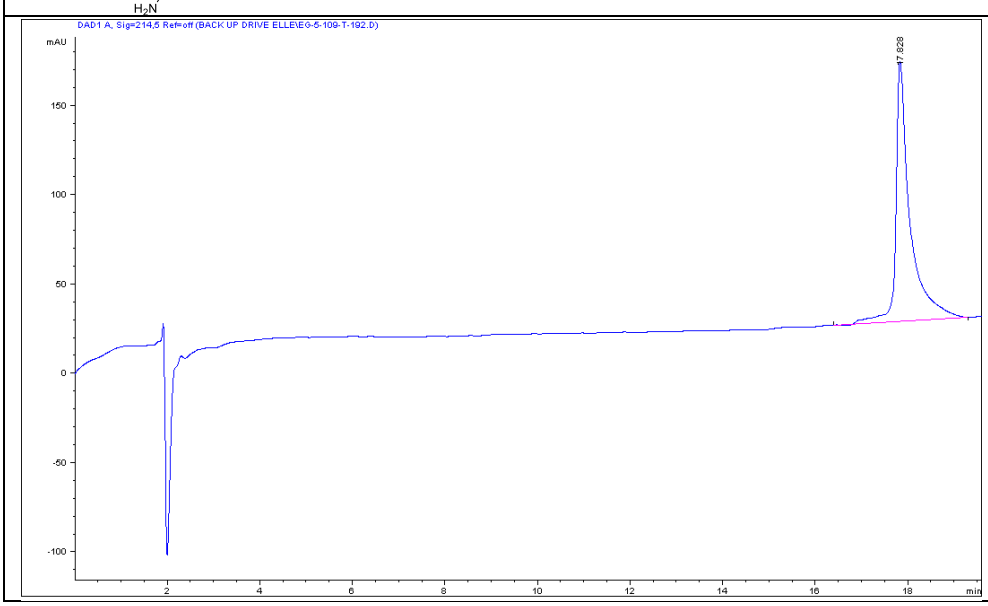
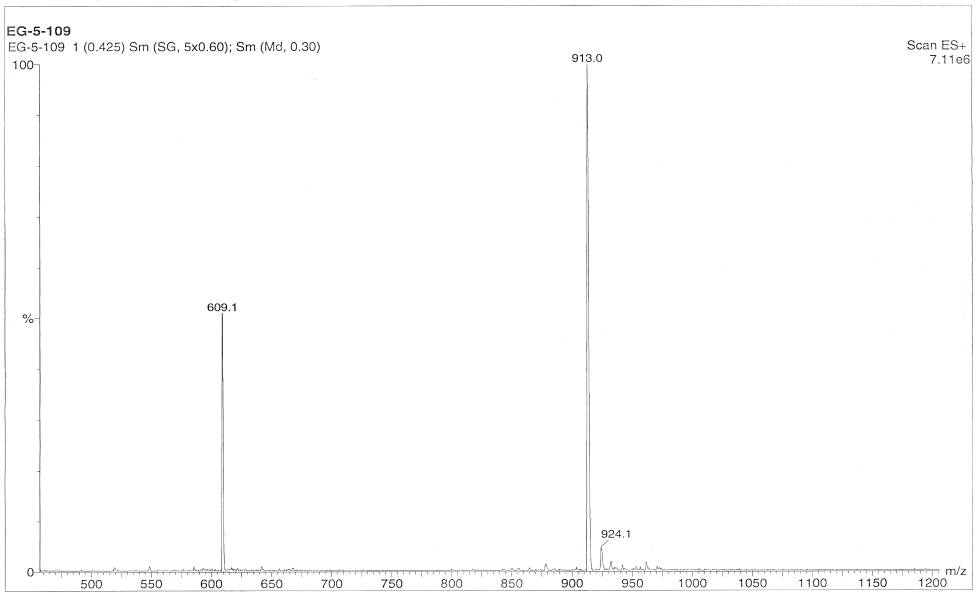
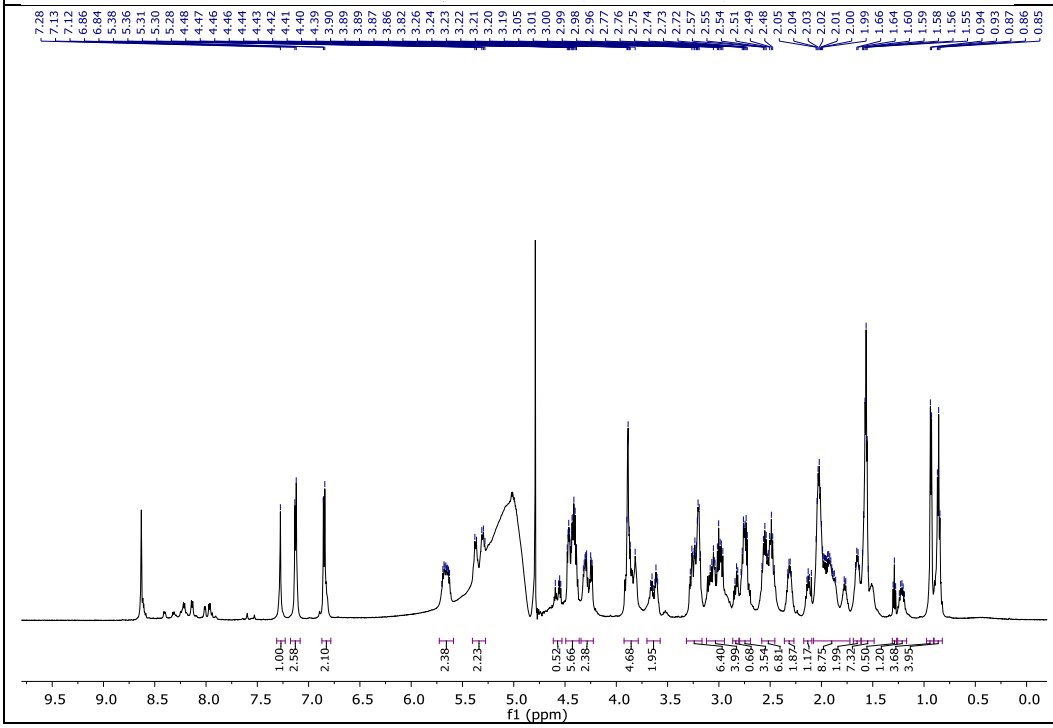


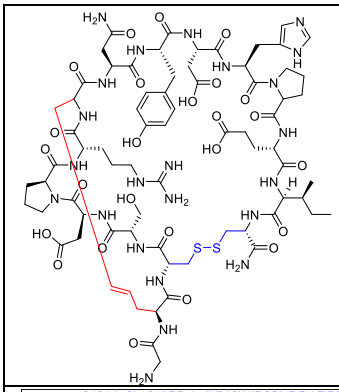
Purified Peptide, post S-S oxidation

10


EG-5-109
EG-5-109 1 (0.425) Sm (SG, 5x0.60); Sm (Md, 0.30)
Scan ES+
7.11e6
100
%
0
913.0
609.1
924.1
m/z
500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200


7.28 7.13 7.12 6.86 6.84 5.38 5.36 5.31 5.30 5.28 4.48 4.47 4.46 4.46 4.44 4.43 4.42 4.41 4.40 4.39 3.90 3.89 3.89 3.87 3.86 3.82 3.26 3.24 3.23 3.22 3.21 3.20 3.19 3.05 3.01 3.00 2.99 2.98 2.96 2.77 2.76 2.75 2.74 2.73 2.72 2.57 2.55 2.54 2.51 2.49 2.48 2.05 2.04 2.03 2.02 2.01 2.00 1.99 1.66 1.64 1.60 1.59 1.58 1.56 1.55 0.94 0.93 0.87 0.86 0.85
1.00 2.58 2.10 2.38 2.23 0.52 5.66 2.38 4.68 1.95 6.40 3.99 0.68 3.54 6.81 1.87 1.17 8.75 1.99 7.32 0.50 1.20 3.68 3.95
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

[2,8]-Dicarba-[3,16]-cystino Conotoxin Vc1.1, 11



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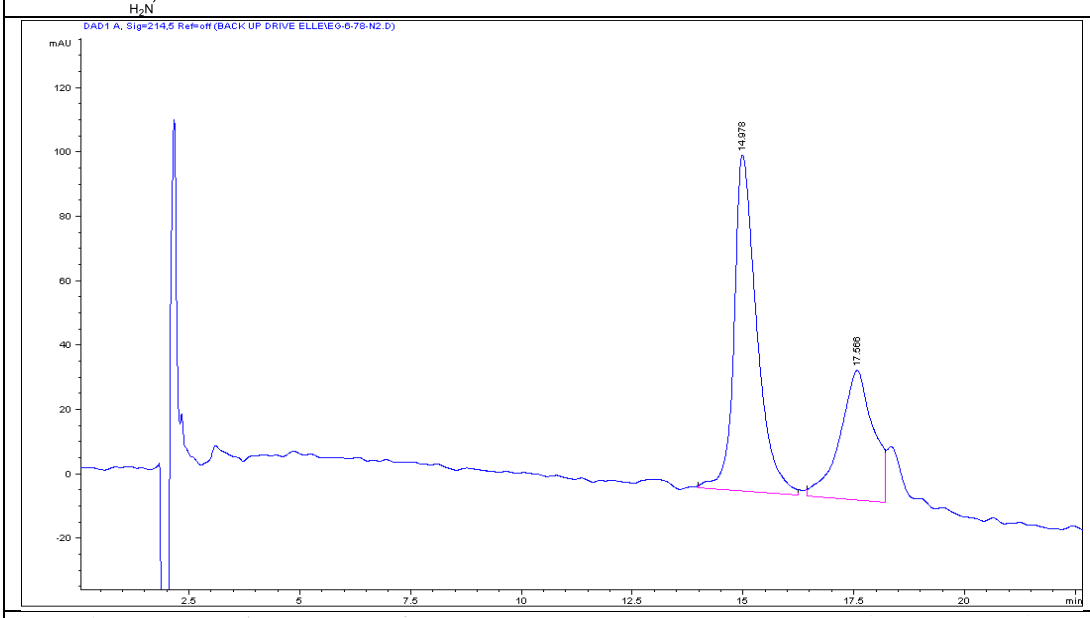
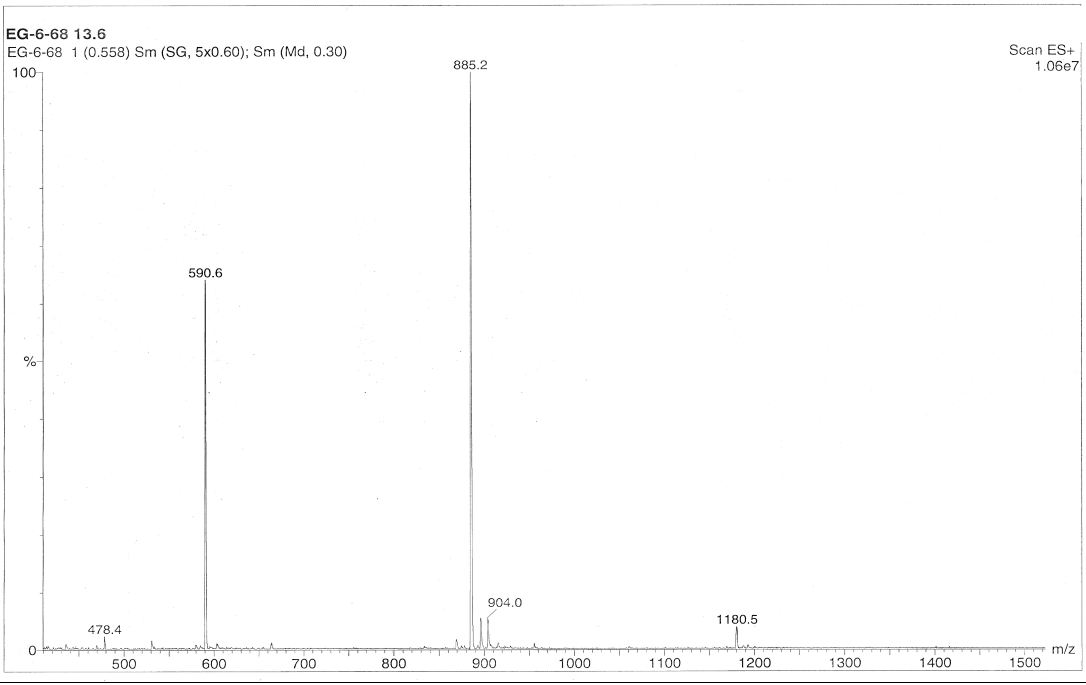
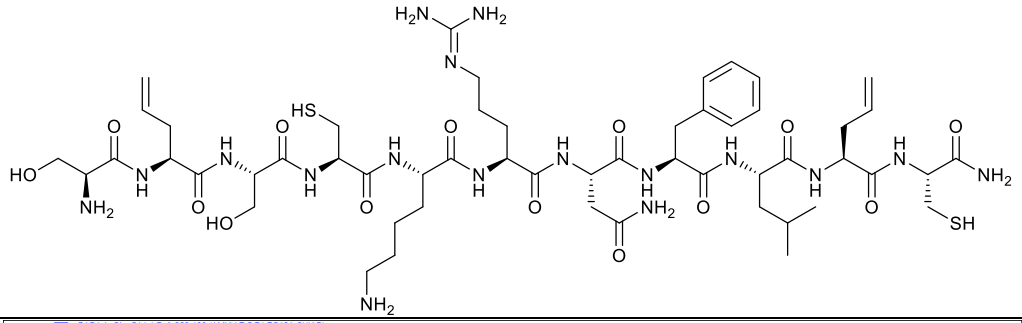
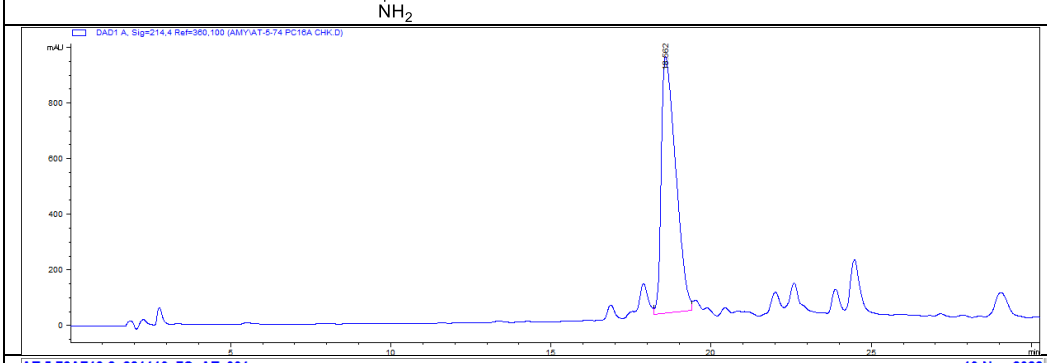
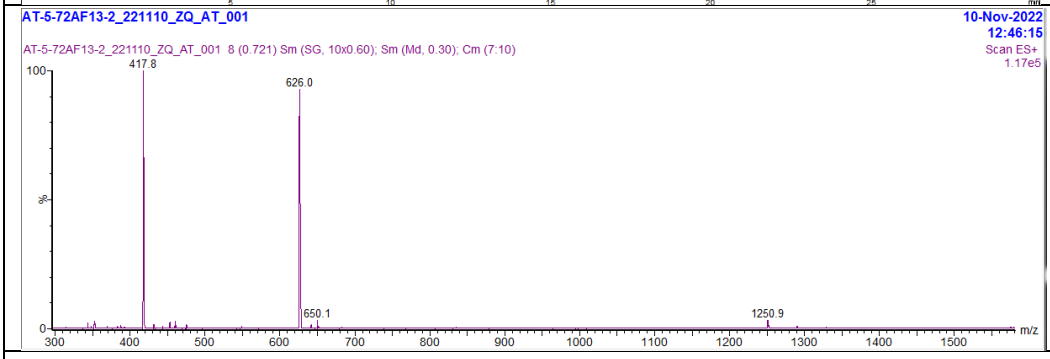


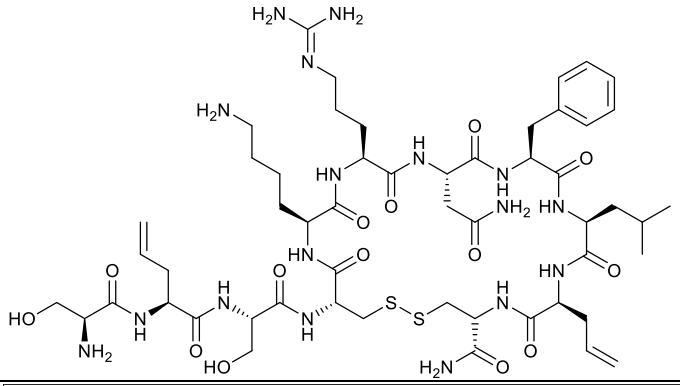
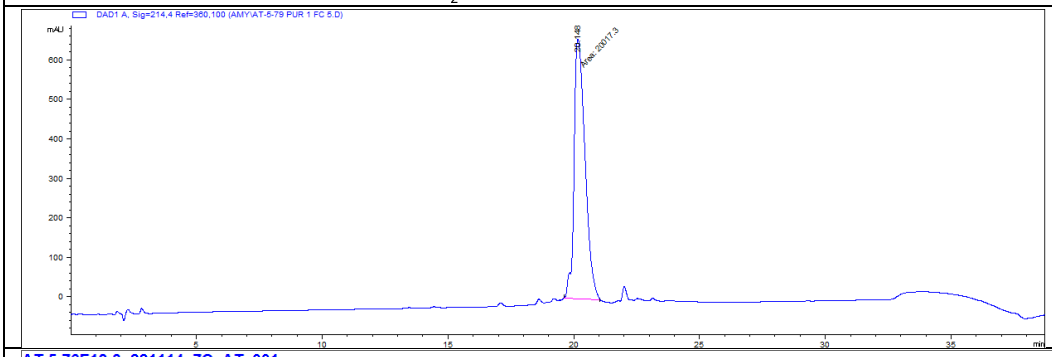
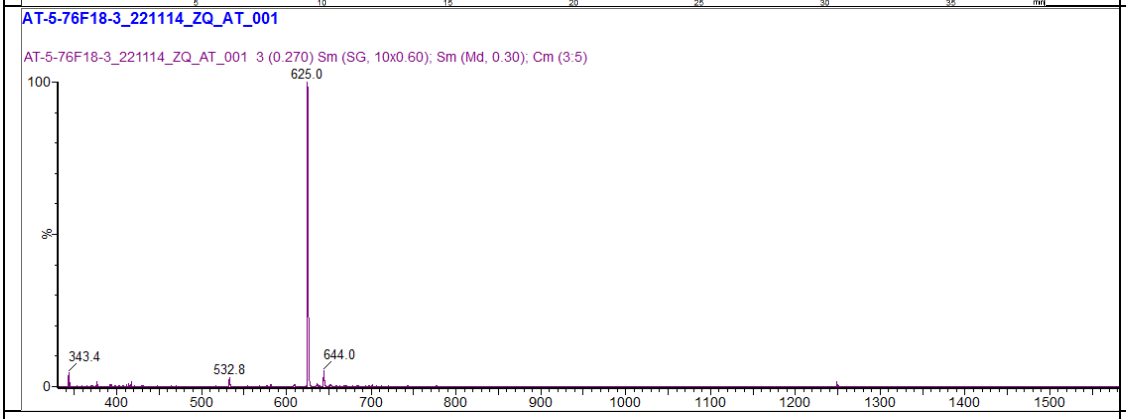
Crude reaction mixture: *E*/*Z*- product isomers



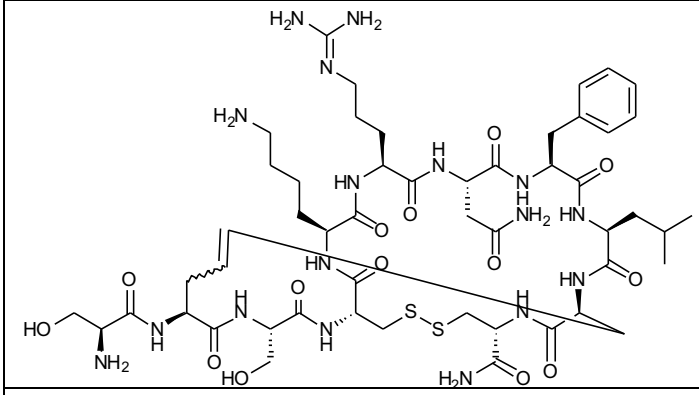
[2,10]-Agl-[4,11]-Cys Conotoxin pc16a

	17
	Crude Peptide
	

[2,10]-Agl-[4,11]-cystino Conotoxin pc16a

	**18**
	Purified peptide post SS oxidation
	

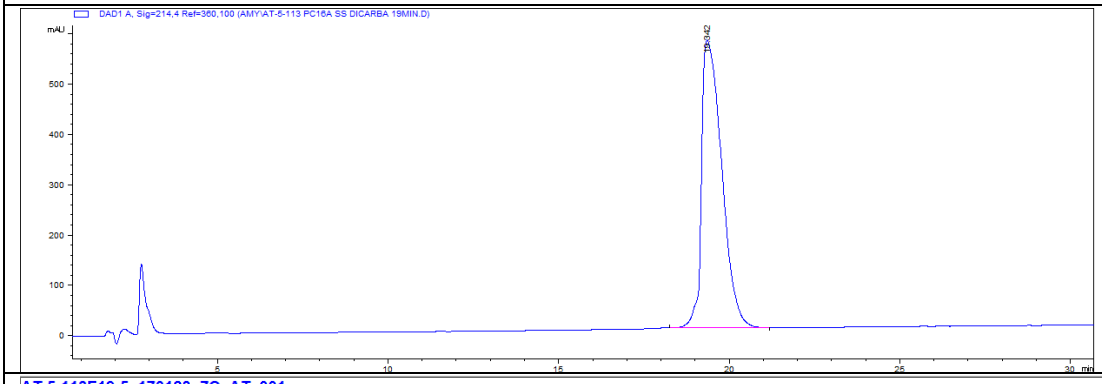
[2,10]-Dicarba-[4,11]-cystino Conotoxin pc16a

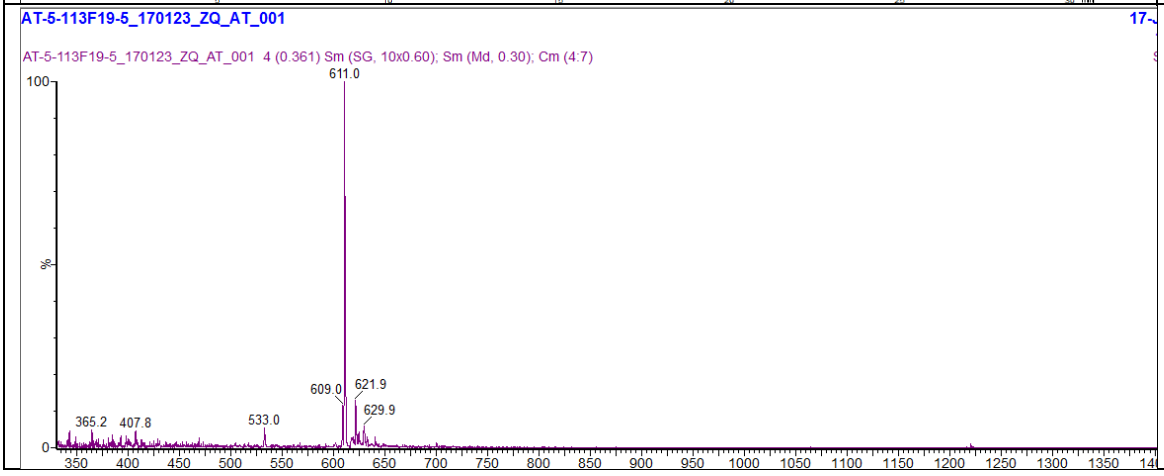


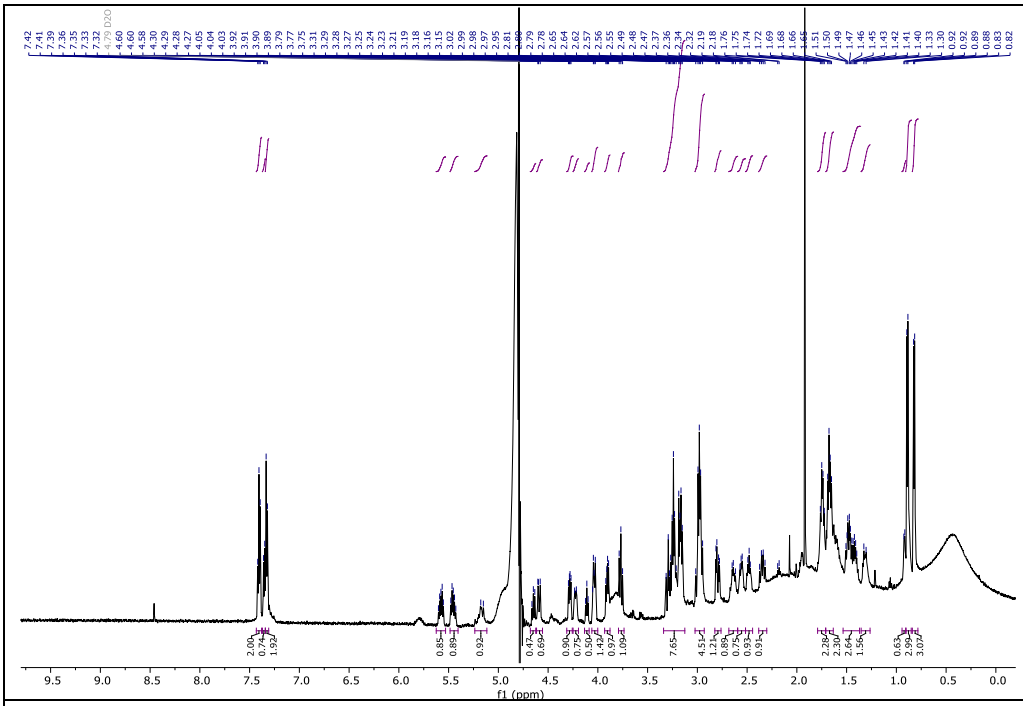
19

Isomer(I)

Purified Peptide



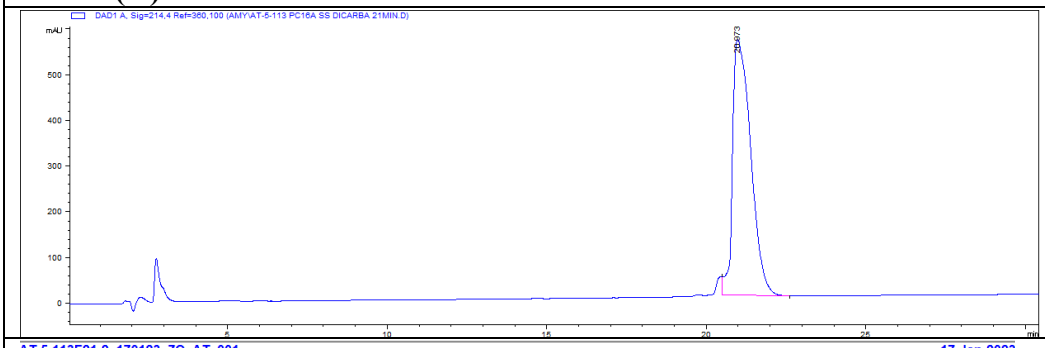




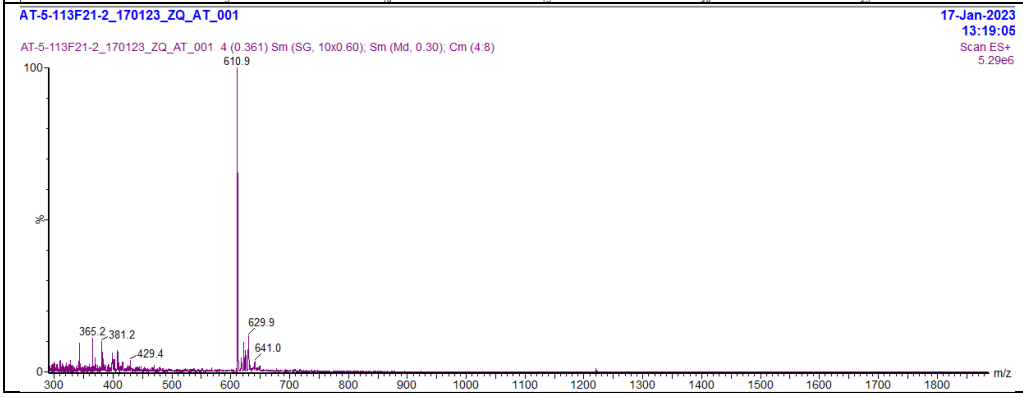
19

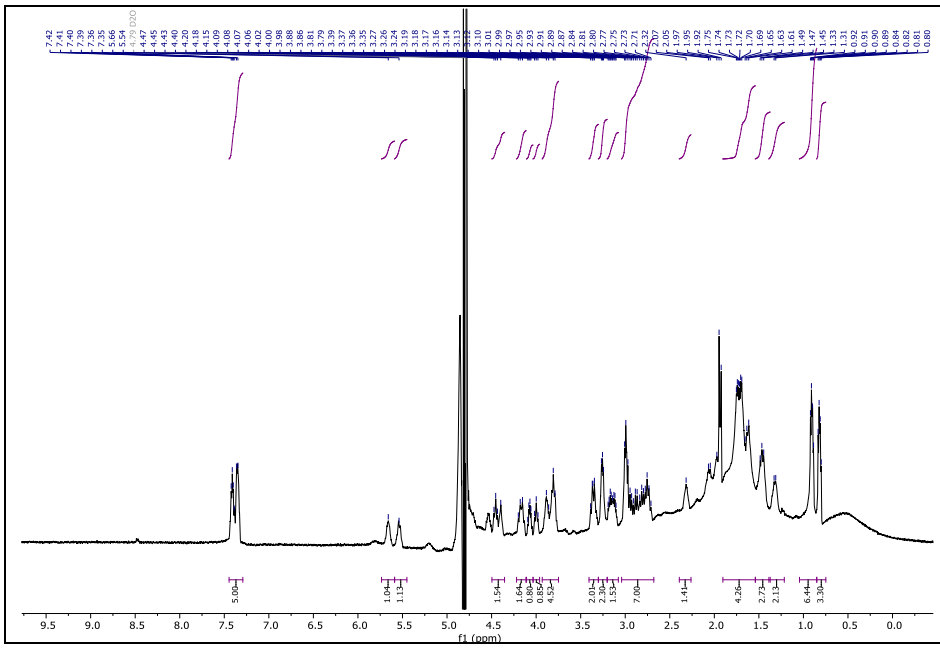
Isomer(II)

19



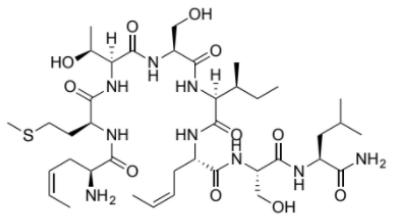
Purified Peptide



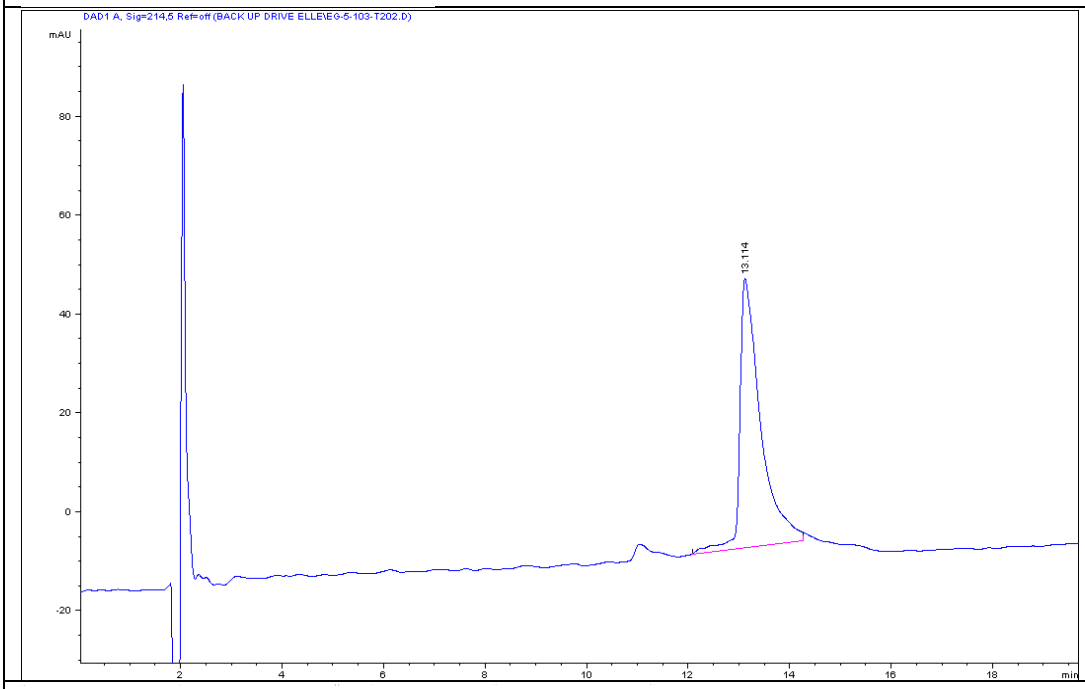


19

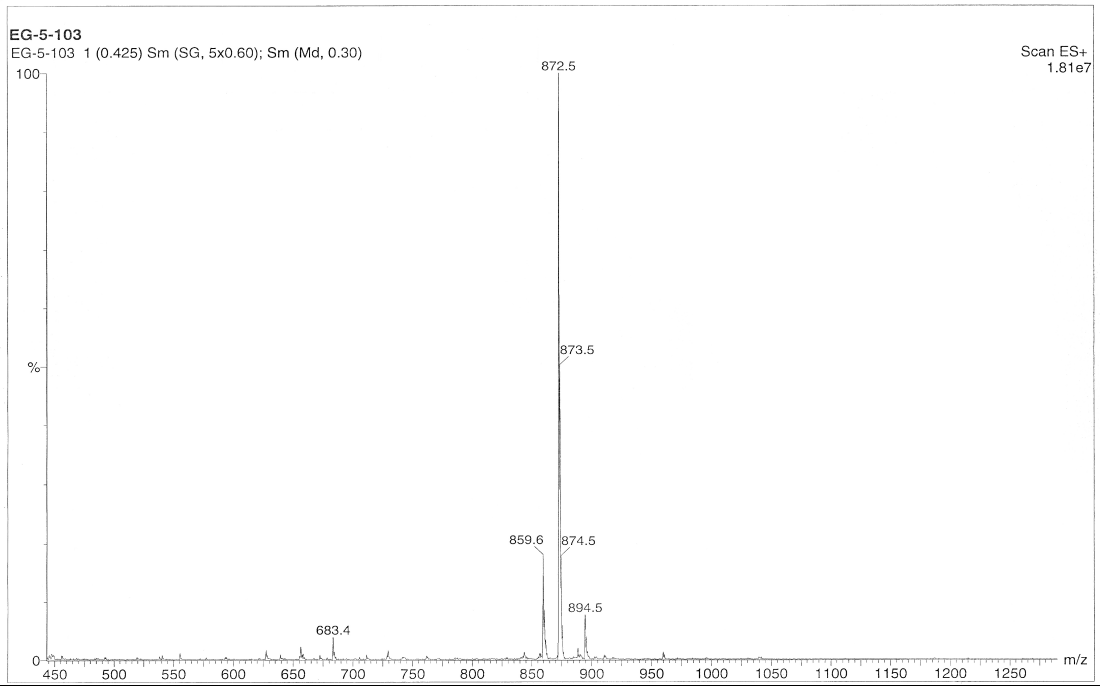
[1,6]-Z-Crt-[2]-Met Human Insulin A Chain (6-13), 4

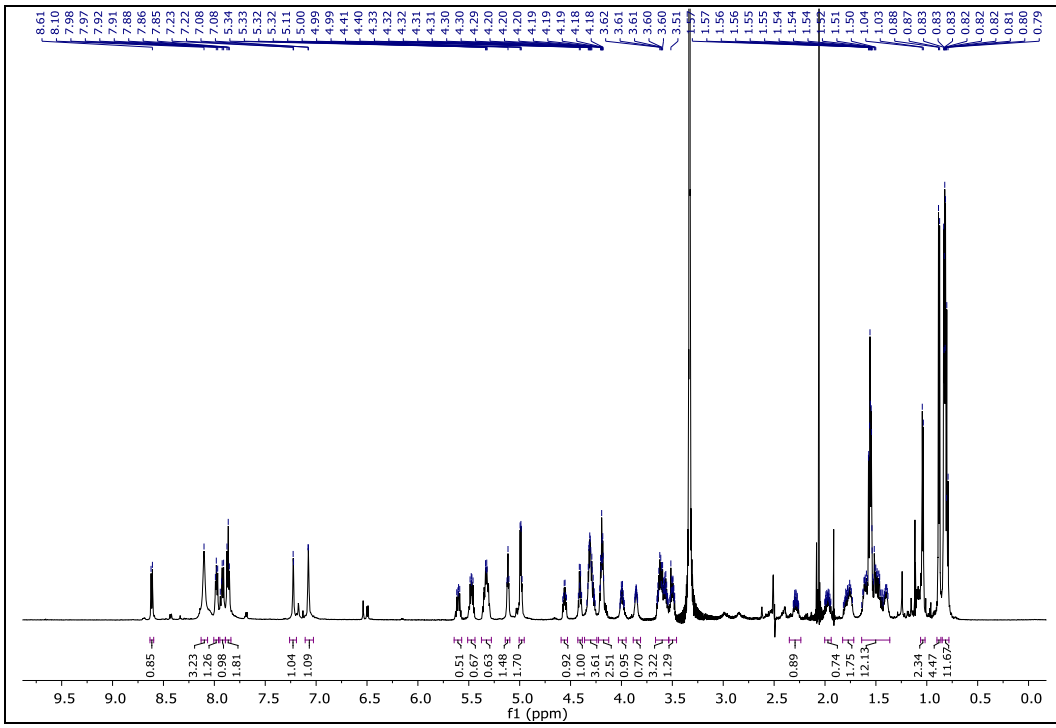


4



Purified peptide




8.61
8.10
7.98
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7.86
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7.23
7.22
7.08
7.08
5.34
5.33
5.32
5.32
5.11
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4.18
4.18
3.62
3.61
3.61
3.60
3.60
3.51
1.57
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1.54
1.52
1.51
1.50
1.04
1.03
0.88
0.87
0.83
0.83
0.83
0.82
0.82
0.82
0.81
0.80
0.79
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3.23
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12.13
2.34
4.47
11.67
9.5
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8.5
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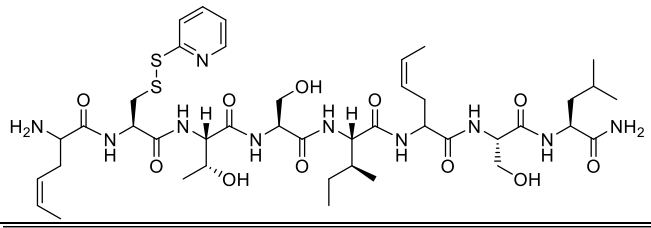
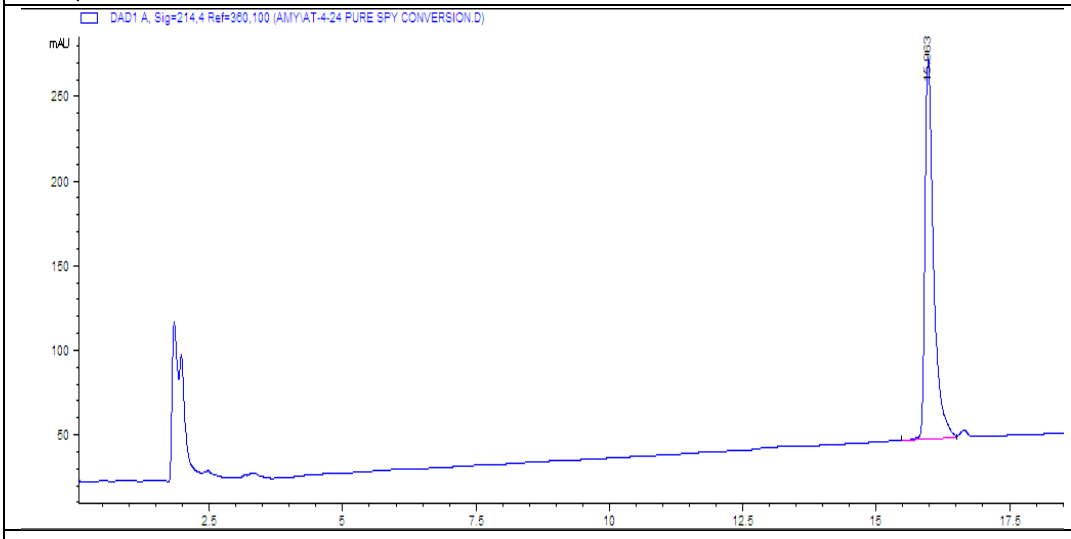
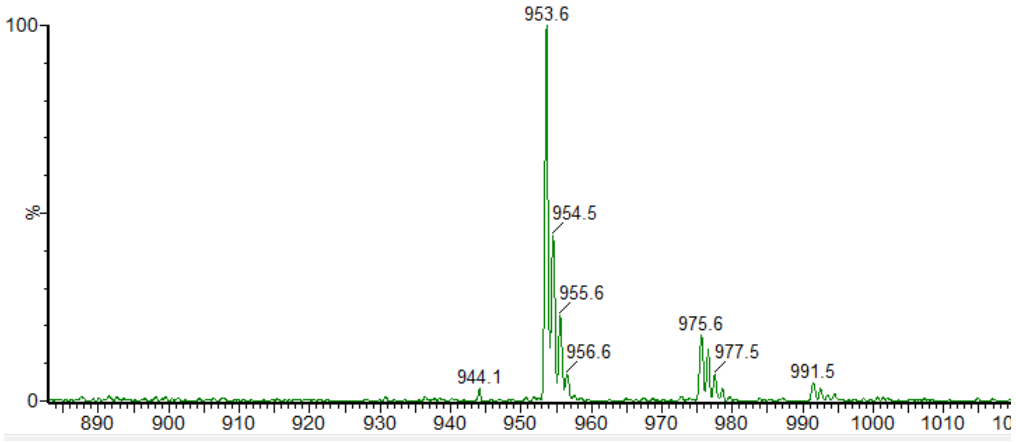
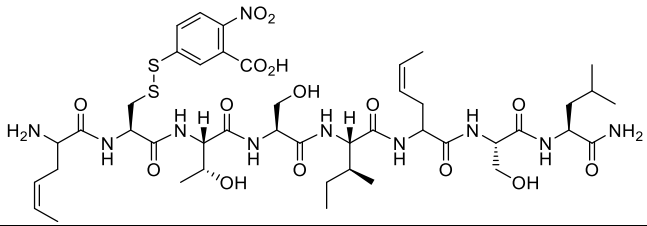
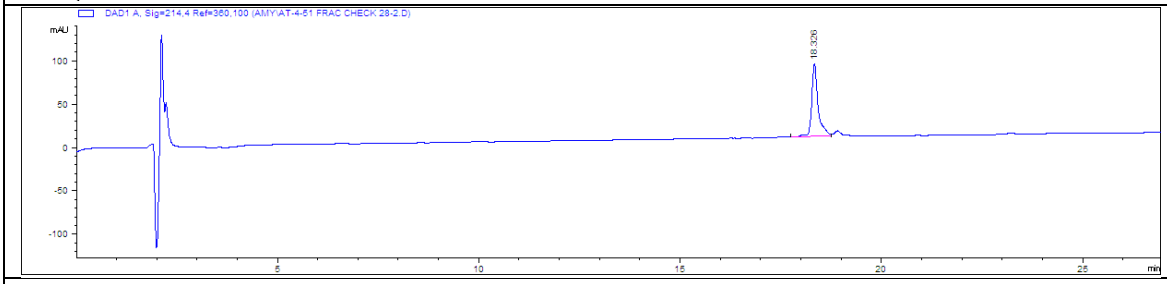
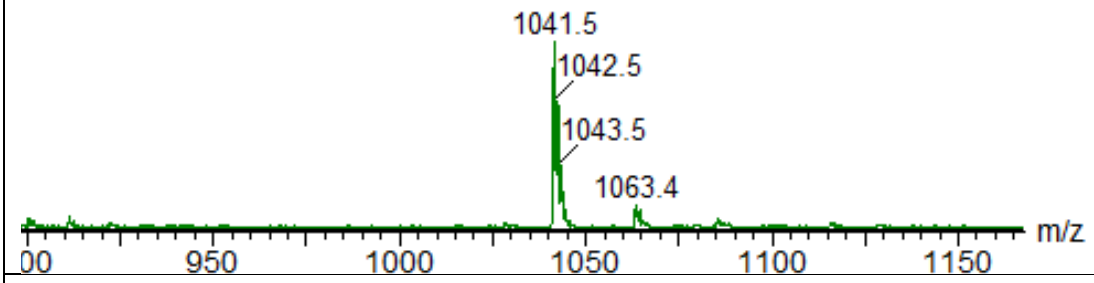
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

4

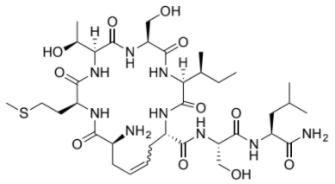
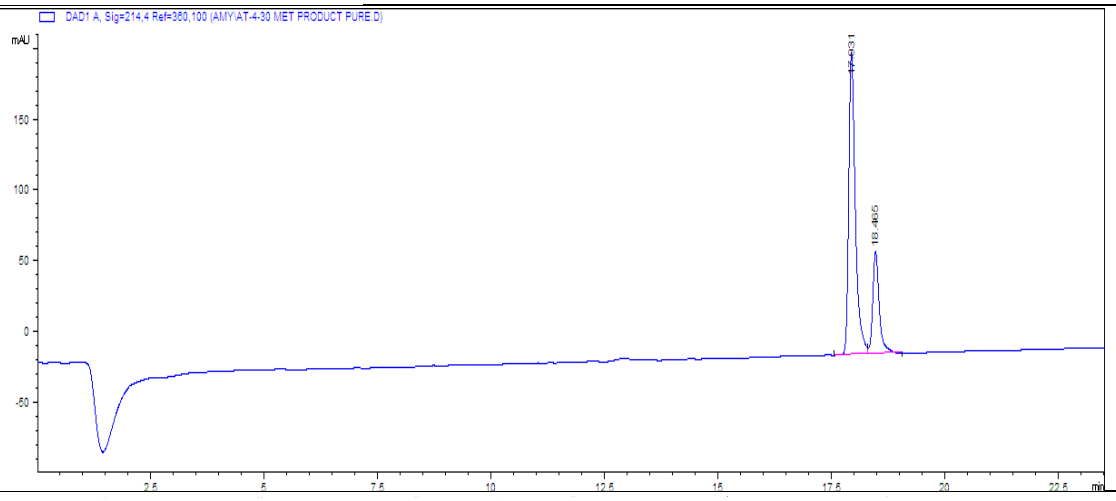
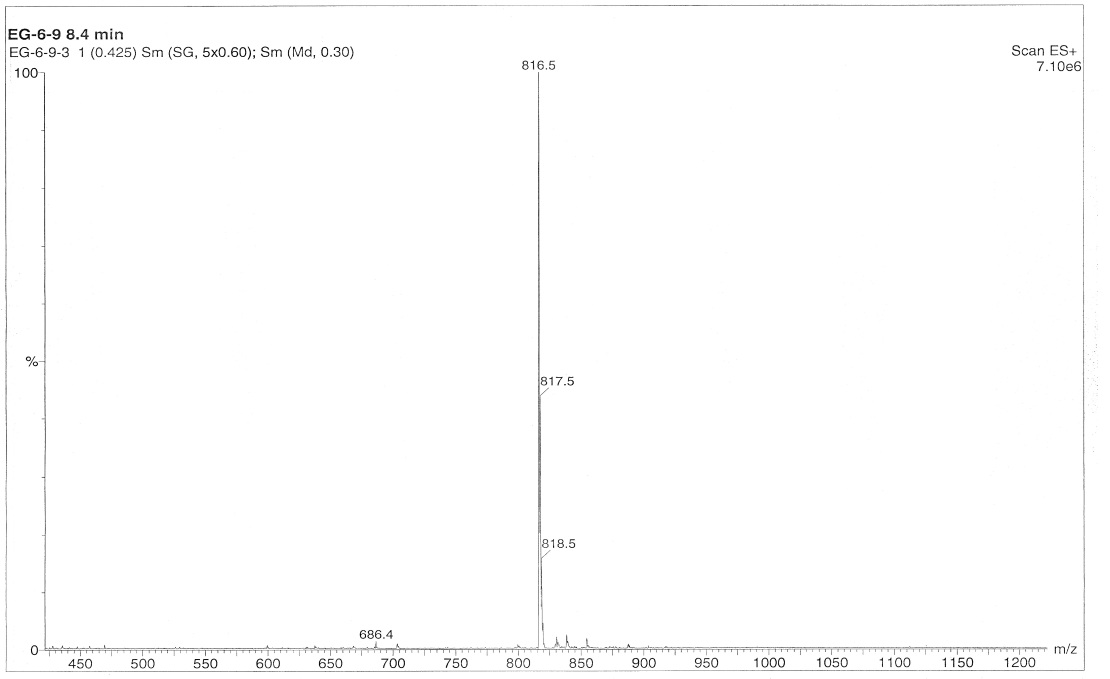
[1,6]-Z-Crt-[2]-CysS(SPy) Human Insulin A Chain (6-13), 7

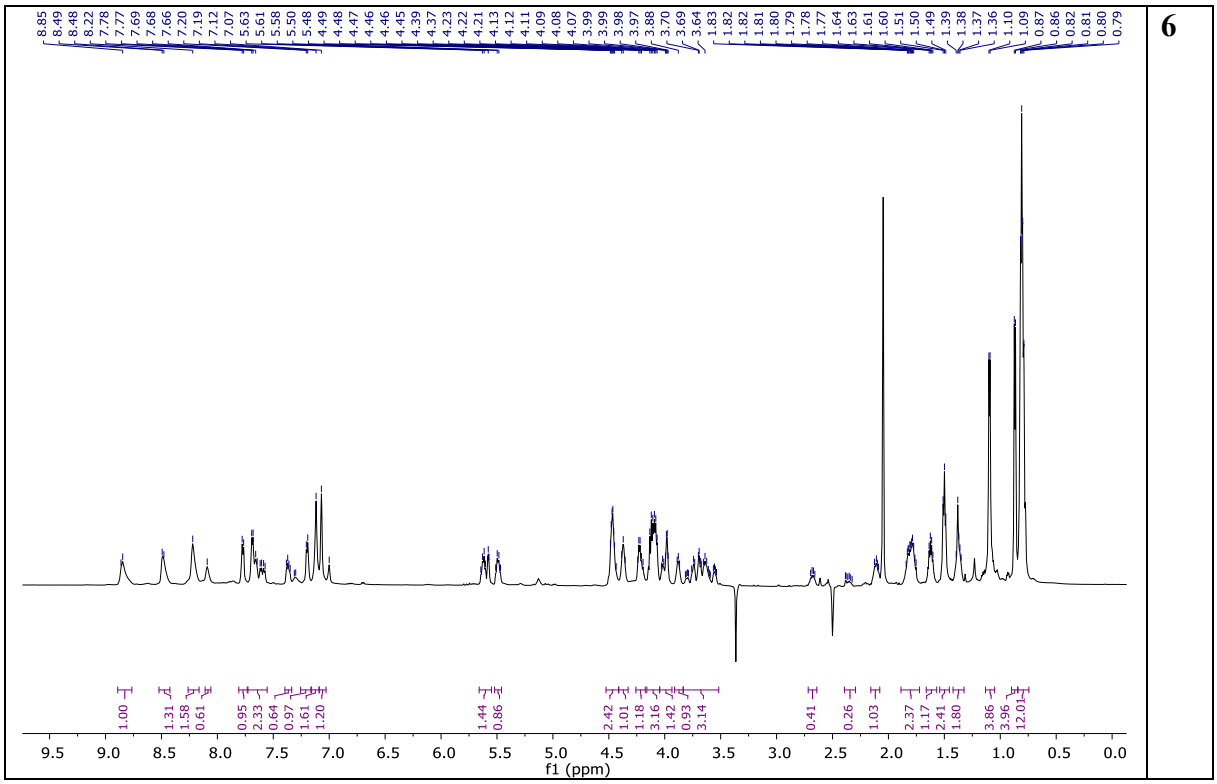
	7
	Purified peptide
	

[1,6]-Z-Crt-[2]-CysS(SNB) Human Insulin A Chain (6-13) 16

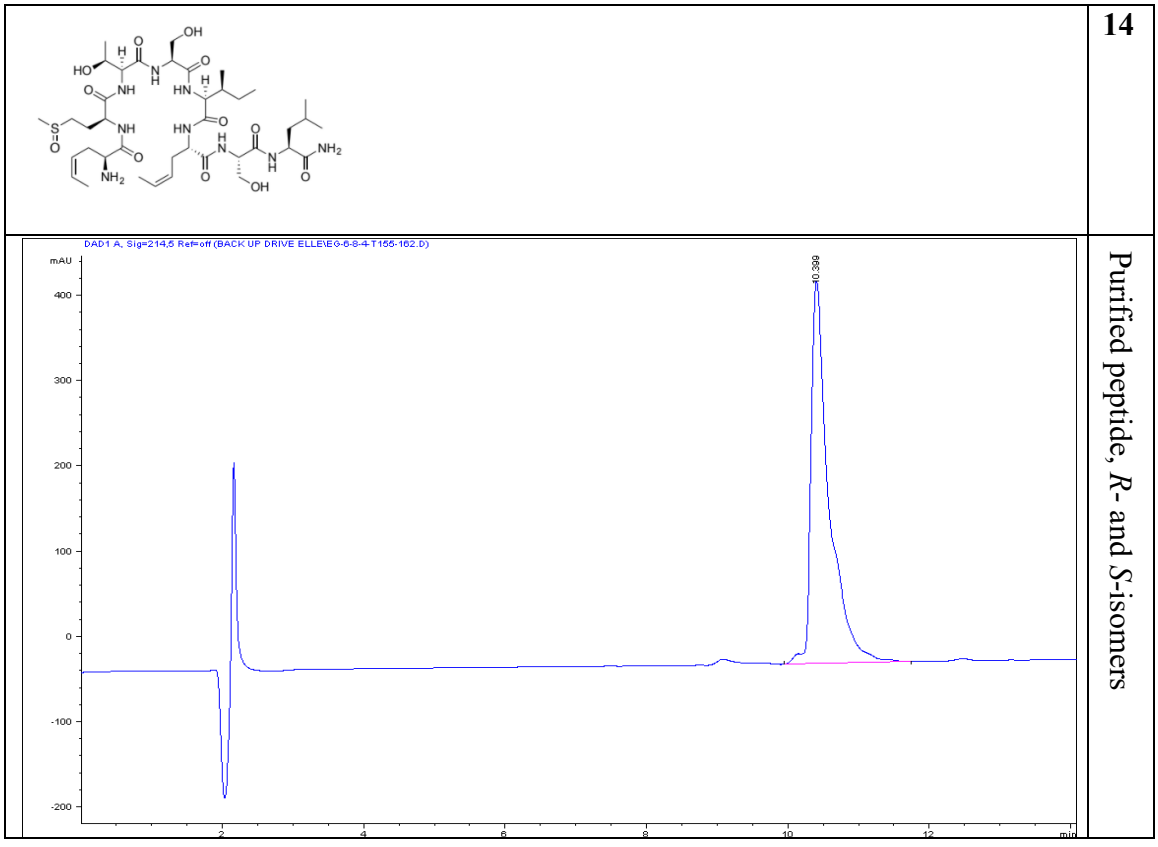
	**16**
	Purified peptide
	

[1,6]-Dicarba-[2]-Met Human Insulin A Chain (6-13), 6

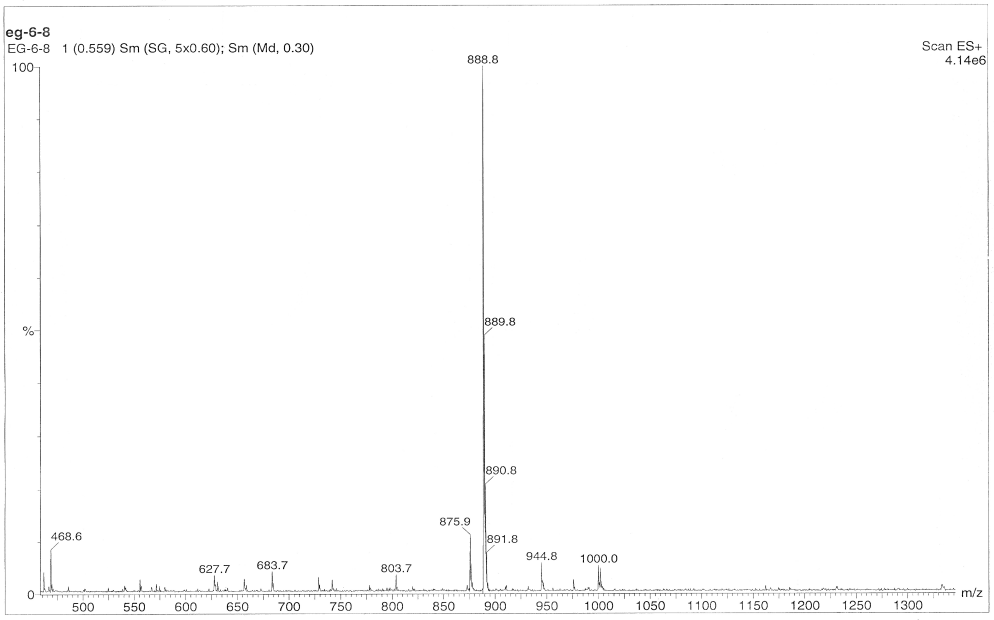
	6
	Purified peptide, *E*- and Z-isomers
	

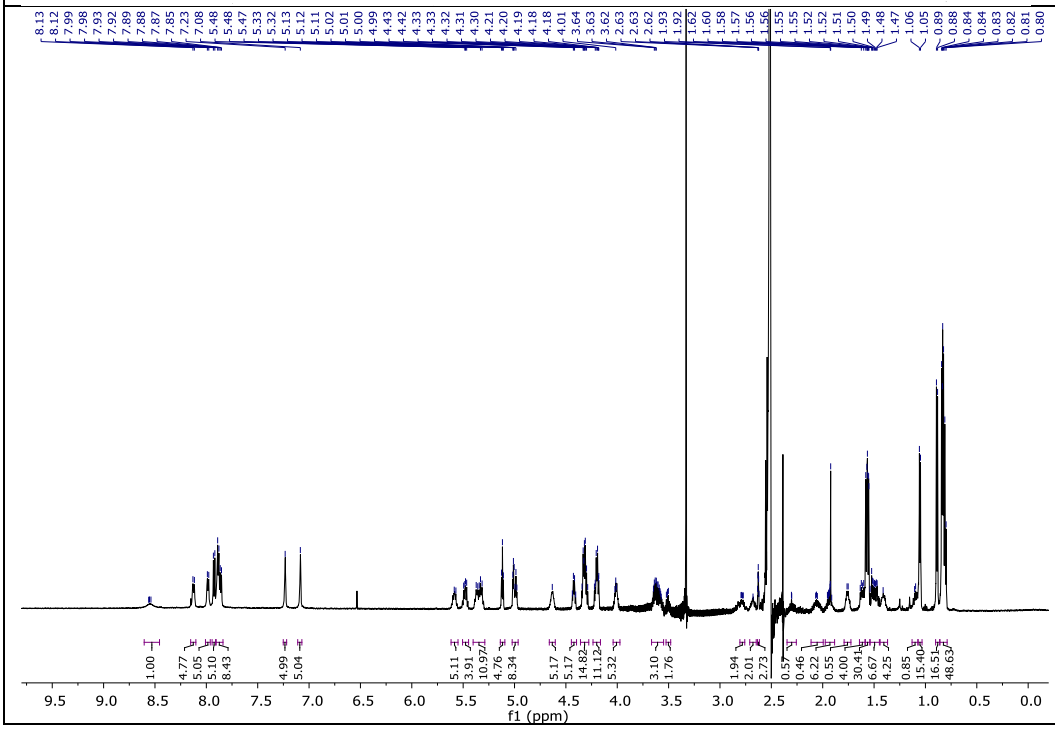


[1,6]-Z-Crt-[2]-Met(O) Human Insulin A Chain (6-13), 14

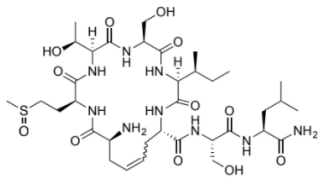
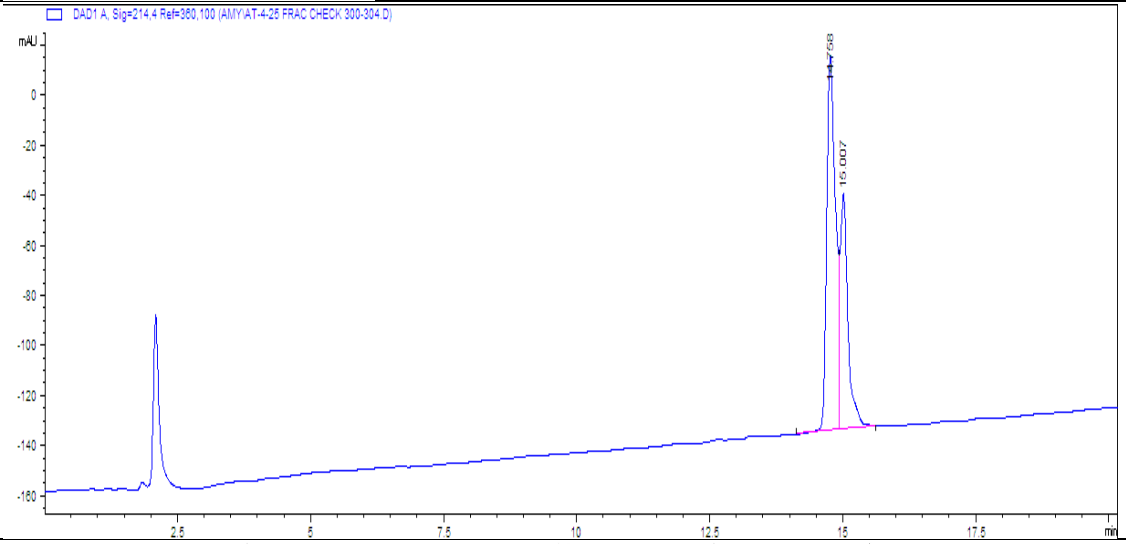
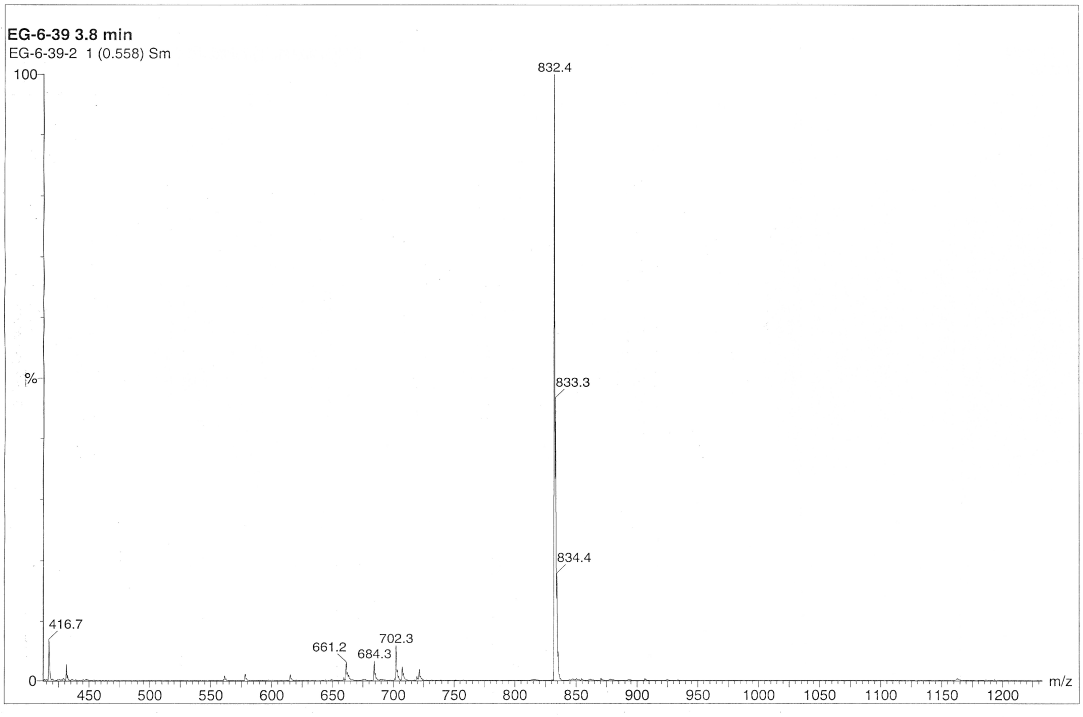


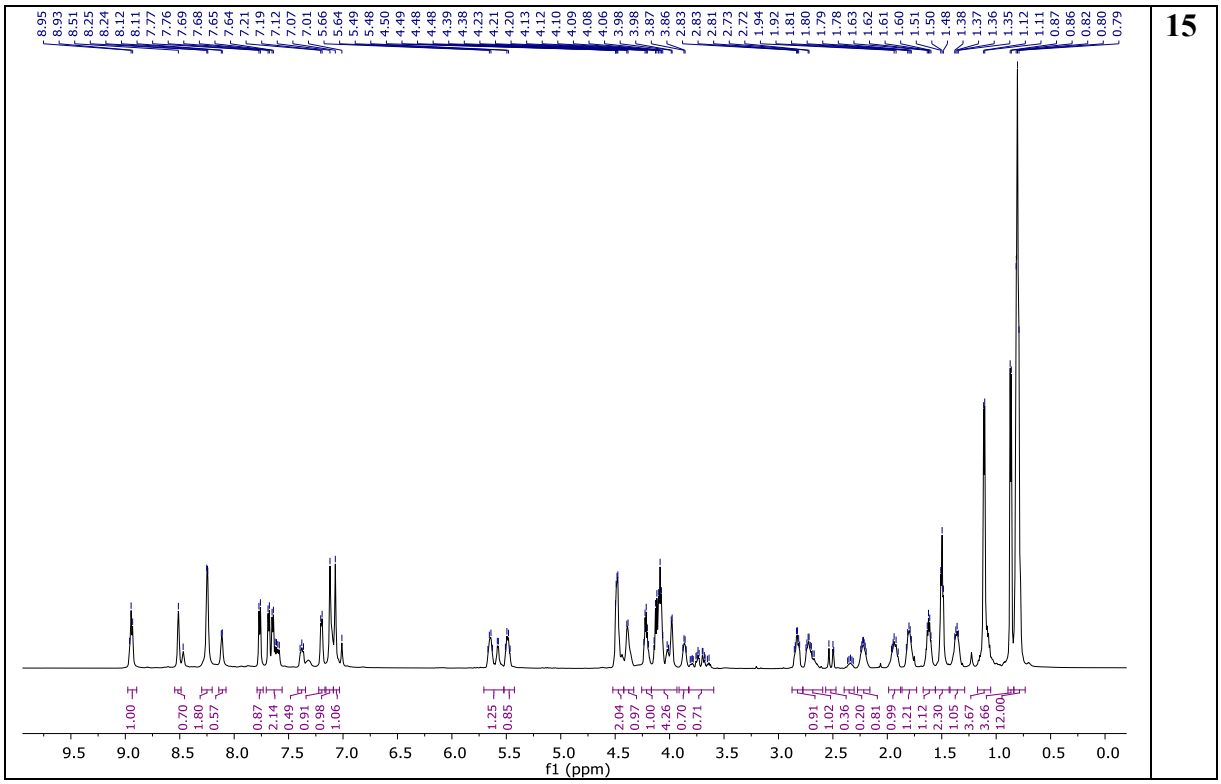
14


eg-6-8
EG-6-8 1 (0.559) Sm (SG, 5x0.60); Sm (Md, 0.30)
Scan ES+
4.14e6
100
%
0
888.8
889.8
890.8
875.9
891.8
468.6
627.7
683.7
803.7
944.8
1000.0
m/z
500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250 1300


f1 (ppm)
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

[1,6]-Dicarba-[2]-Met(O) Human Insulin A Chain (6-13), 15

	**15**
	Purified peptide, *R*- and *S*-isomers
	


15
f1 (ppm)