Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Bicycle Synthesis Through Peptide Macrocyclization Using Aziridine Aldehydes Followed by Late Stage Disulfide Bond Installation

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Supporting Information

Contents

Section 1 – General Information

1.1. Reagents

2-chlorotrityl chloride resin, coupling reagents, and all Fmoc-protected amino acids were purchased from AAPPTec Inc. (Louisville, KY, U.S.A.). 2,2,2-trifluoroethanol was purchased from Oakwood Products (West Columbia, SC, U.S.A.). All other commercial reagents and solvents were purchased from commercial sources and used as received, unless otherwise noted.

1.2. Chromatography

Flash column chromatography was carried out using Silicycle Inc. (Quebec City, QC, Canada) 230-400 mesh silica gel, unless otherwise noted. Thin-layer chromatography (TLC) was performed on Macherey Nagel pre-coated glass backed TLC plates (SIL G/UV254, 0.25 mm) and visualized using a UV lamp (254 nm) or potassium permanganate. All reactions involving chemical transformations on peptides were analyzed using analytical high-performance liquid chromatography-mass spectrometry (HPLC/MS) with an Agilent Technologies (Mississauga, ON, Canada) 1200 Series liquid chromatography system equipped with G1312A Binary Pump, G1329A Thermostatted Autosampler, G1316A Column Thermostat, G1314D Variable Wavelength Detector (UV detection at 214 nm), and G6130B Single Quadrupole LC/MS. Analytical HPLC/MS separations were performed with an Agilent Poroshell 120 EC-C18 2.7 µm, 4.6 x 50 mm column, and a linear gradient of 5% - 95% acetonitrile (with 0.1% formic acid) in water (with 0.1% formic acid) over eight minutes. Purification of final peptide products were performed with semi-preparative HPLC/MS using an Agilent 1260 Infinity Series liquid chromatography system equipped with G1311A Quaternary Pump, G1310A Isocratic Pump, two G1364C Automatic Fraction Collectors, G2258A Dual Loop Autosampler PS, G1315C Diode Array Detector (UV detection at 214 nm), and G6130B Single Quadrupole LC/MS. Semi-preparative HPLC/MS separations were performed with a Phenomenex® (Torrance, CA, U.S.A.) Jupiter 4µ Proteo 90A 4 µm, 10.0 x 250 mm column, and a linear gradient of 30 – 70% acetonitrile (with 0.1% formic acid) in water (with 0.1% formic acid) over 25 minutes.

1.3. Nuclear Magnetic Resonance Spectroscopy

Proton (^{1}H NMR), carbon (^{13}C NMR), gCOSY, zTOCSY, gc2HSQCse, and gcHMBC spectra were recorded on an Agilent DD2 500 MHz spectrometer with an Agilent HC 5-mm XSens cryogenically-cooled probe. 1D ^{1}H NMR spectra were acquired over a 8012.8 Hz spectral window with 65k points and 16 transients. 1D ^{13}C NMR spectra were acquired over a 30487.8 Hz spectral window with 131k points and 2000 transients. 2D ^{1}H/^{1}H gCOSY and ^{1}H/^{1}H zTOCSY spectra were acquired with 256 increments, a 1.0 s d1 delay, and one transient. 2D ^{1}H/^{13}C gc2HSQCse spectra were acquired with 256 increments, a 1.0 s d1 delay, and eight transients. For all 2D experiments, the F2 spectral window and number of points were automatically minimized by the acquisition software. All pulse sequences used were provided by Agilent. All NMR spectra were Fourier transformed, manually phase corrected, and auto baseline corrected using a Whittaker Smoother method in MestReNova v. 8.0.0 (Santiago de Compostela, Spain). ^{1}H NMR spectra chemical shifts (δ) are reported in parts per million (ppm) referenced to residual protonated solvent peak (MeOD, δ = 3.31). Spectral data is reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, ddt = doublet of doublet of triplets, dtd= doublet of triplet of doublets, m = multiplet, br = broad), coupling constant (*J*) in Hertz (Hz), and integration. ^{13}C NMR spectra chemical shifts (δ) are reported in parts per million (ppm) were referenced to carbon resonances in the NMR solvent (MeOD, δ = 49.00).

1.4. High-Resolution Mass Spectrometry

High resolution mass spectra were obtained on an ABI/Sciex™ (Framingham, MA, U.S.A.) Qstar mass spectrometer with ESI source, MS/MS and accurate mass capabilities.

Section 2 – General Procedures

2.1. Solid-Phase Peptide Synthesis

All linear peptides were prepared on 2-chlorotrityl chloride resin (theoretical loading of 0.84 mmol g^{-1}) using *N*-Fmoc-amino acids. The 2-chlorotrityl chloride resin was loaded with the C-terminal *N*-Fmoc-amino acid according to a literature procedure[1] that was modified to perform double coupling of the C-terminal amino acid. All other *N*-Fmoc-amino acids and 6-chloro-3-((dimethylamino)(dimethyliminio)methyl)-1H-benzo[d][1,2,3]triazol-3-ium-1-olate hexafluorophosphate(V) (HCTU) were prepared as 0.5 M solutions in *N*-methyl-2-pyrrolidone (NMP) with addition of dropwise *N*,*N*-dimethylformamide (DMF) to solubilize, if necessary. *N*,*N*-diisopropylethylamine (DIPEA) was prepared as a 2.0 M solution in NMP. Piperidine was prepared as a 20% solution in NMP. Solid-phase peptide synthesis was performed using an AAPPtec Apex 396 automated multiple peptide synthesizer equipped with a 40 vessel classic reactor assembly containing approximately 0.15 mmol of loaded resin per vessel. Coupling steps were performed as single couplings with sequential addition of 4 eq. of *N*-Fmoc-amino acid, 10 eq. of DIPEA, and 4 eq. of HCTU as solutions in NMP, followed by shaking at 475 rpm for 1h at room temperature. Fmoc deprotection steps were performed by addition of 40 eq. of 20% piperidine in NMP, followed by shaking at 475 rpm for 5 min, repeated twice, with 30 seconds of wait time in between each the three shaking periods. Coupling steps were followed by wash steps using DMF, repeated once. Fmoc deprotection steps were followed by wash steps using DMF, repeated once, followed by NMP, repeated once. A final wash step was performed at the end of the synthesis using methanol (MeOH), repeated once. To explore on-resin disulfide formation, the final Fmoc deprotection step was omitted from the synthesis sequence, and the resin was obtained directly from the reaction vessels. For all other experiments, the peptides were cleaved from resin by addition of 25% 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) in dichloromethane (DCM) then shaken at 475 rpm for 1 hour, repeated once, followed by a rinse with 25% HFIP in DCM without

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[1] Barlos, K.; et al., *Tetrahedron Lett.*, **1989**, *30*, 3943-3946.

shaking. The cleavage mixtures were concentrated using a Genevac EZ-2 Plus centrifugal evaporator. The peptides were then triturated once with *tert*-butyl methyl ether (TBME), then twice with diethyl ether (Et_2O). The residual solvent was removed with N_2 stream and the peptides were stored overnight under reduced pressure in a dessicator containing anhydrous calcium sulfate and 3% cobalt(II) chloride (Drierite™) and subsequently at -20 °C.

2.2. Synthesis of Aziridine-Containing Peptide Macrocycles

(2R,4R,5S)-2-((S)-aziridin-2-yl)-3-oxa-1-azabicyclo[3.1.0]hexan-4-ol ((S)-aziridine-2-carboxaldehyde dimer) was prepared according to a literature procedure.[2] Additionally, aziridine-2-carboxaldehyde dimer was purified by silica gel flash column chromatography with a short silica gel pad using ethyl acetate (EtOAc):acetonitrile (MeCN):MeOH:water (28:2:1:1). Fractions containing the desired compound were combined and concentrated under reduced pressure to yield a white solid. Side-chain protected linear peptide (1 eq.) and (S)-aziridine-2-carboxaldehyde dimer (0.51 eq.) were weighed in a 20 mL glass vial equipped with stir bar. 2,2,2-trifluoroethanol (TFE, 0.1 M) was added, and the resultant solution/suspension stirred vigorously. *tert*-Butyl isocyanide (1.01 eq.) was added, and the reaction was stirred at room temperature for 3 – 5 hours until complete consumption of linear peptide as demonstrated by LC-MS analysis. The solvent was removed under reduced pressure using a Genevac EZ-2 Plus centrifugal evaporator and triturated once with TBME. The aziridine-containing peptide macrocycles were used in the next step without further purification.

2.3. Aziridine Ring-Opening of Aziridine-Containing Peptide Macrocycles with Sodium Azide

The aziridine-containing peptide macrocycle was dissolved in a mixture of MeCN:DMF (1:1, 0.04 M) followed by the addition of sodium azide (10 eq.). The reactions were then heated to 65 °C with stirring for 4 hours. The solvent was partially removed under reduced pressure using a Genevac EZ-2 Plus centrifugal evaporator. The remaining mixture in DMF was diluted with ten volumes of water, and

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[2] Hili, R. and Yudin, A. K. *J. Am. Chem. Soc.* **2006**, *128*, 14772-14773.

extracted five times with EtOAc. The organic extracts were combined, dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The azide ring-opened macrocycles were used in the next step without further purification.

2.4. Solution-Phase Oxidative Deprotection of *S*-Protected Peptide Macrocycles

The azide ring-opened peptide macrocycle was dissolved in TFA (0.002 M), followed by addition of anisole (2 eq.) and thallium(III) trifluoroacetate ($Tl(TFA)_3$, 1.5 eq.). The reactions were stirred at room temperature for 4 hours. Next, the solvent was removed under reduced pressure using a Genevac EZ-2 Plus centrifugal evaporator, and the peptide macrocycles were triturated once with TBME. The crude material was then loaded onto celite and subjected to silica gel chromatography using a CombiFlash® Rf system equipped with a RediSep Rf Gold® Normal-Phase Silica 4 g column and a gradient of 0 – 50% MeOH in EtOAc as eluent. Following concentration under reduced pressure, the roughly purified product was then subjected to semi-preparative HPLC/MS purification as described in section 1.2. The fractions containing the desired mass as determined by ESI-MS were combined and lyophilized overnight.

Section 3 – Disulfide Formation before Head-to-Tail Peptide Macrocyclization

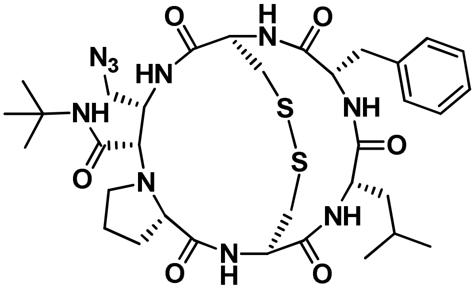
Procedure for the On-Resin Oxidative Deprotection of Fmoc-PCLFC Using Iodine

2-chlorotrityl resin-bound Fmoc-PC(Trt)LFC(Trt) or Fmoc-PC(Acm)LFC(Acm) was swelled in DCM:MeOH:H_2O (15:6:1, at 18 mL/g) for 60 minutes. 8 eq. of I_2 in DCM and 8 eq. of DIPEA were then added, and left to stand for 45 minutes. The reaction mixture was then drained, and the resin rinsed three times with DMF, twice with THF:AcOH:DIPEA (7:2:1), twice with DMF, and twice with DCM. A small portion of the resin was isolated from which the peptide was cleaved using 1:3 HFIP:DCM and analyzed by LC-MS.

Procedure for the On-Resin Oxidative Deprotection of Fmoc-PCLFC Using Thallium Trifluoroacetate

$Tl(TFA)_3$ (1.5 eq.) was dissolved in DMF (0.06 M), added to 2-chlorotrityl resin-bound Fmoc-PC(Trt)LFC(Trt) or Fmoc-PC(Acm)LFC(Acm), and shaken for two hours at room temperature. The resin was then rinsed three times with DMF, three times with DCM, and three times with MeOH. A small portion of the resin was isolated from which the peptide was cleaved using 1:3 HFIP:DCM and analyzed by LC-MS. The disulfide derived from Fmoc-PC(Acm)LFC(Acm) showed cleanest conversion into the disulfide. Thus, the remainder of the oxidized Fmoc-PCLFC resin was treated with 20% piperidine in DMF (10 mL/g resin) for 30 minutes, then rinsed three times with DMF, three times with DCM, and three times with MeOH. The disulfide-containing peptide was then cleaved from resin by treatment with 1:3 HFIP:DCM (10 mL/g resin) for 1h and concentrated using a Genevac EZ-2 Plus centrifugal evaporator. The peptide was then triturated once with TBME. The residual solvent was removed with N_2 stream and the peptide was stored overnight under reduced pressure in a dessicator containing anhydrous calcium sulfate and 3% cobalt(II) chloride (Drierite$^{\text{TM}}$) and subsequently at -20 °C.

Section 4 – Head-to-Tail Peptide Macrocyclization before Disulfide Formation



(3R,6S,9S,12R,15S,16S,20aS)-15-(azidomethyl)-9-benzyl-N-(tert-butyl)-6-isobutyl-1,4,7,10,13-pentaoxoicosahydro-3,12-(methanodithiomethano)pyrrolo[1,2-a][1,4,7,10,13,16]hexaazacyclooctadecine-16-carboxamide (1)

PC(Acm)LFC(Acm) linear peptide was prepared as described section 2.1. PC(Acm)LFC(Acm) (0.6 mmol, 1 eq.) was subjected to macrocyclization with (S)-aziridine-2-carboxaldehyde dimer (0.45 mmol, 0.75 eq.) and *tert*-butyl isocyanide (1.2 mmol, 2 eq.) in TFE (6 mL, 0.1 M) as described in Section 2.2 to yield 500 mg of crude material in 97% yield. Crude aziridine-containing macrocyclic PC(Acm)LFC(Acm) (0.58 mmol, 1 eq.) was subjected to aziridine ring-opening with sodium azide (5.8 mmol, 10 eq.) in 1:1 MeCN:DMF (7.25 mL each, 0.04 M) as described in section 2.3 to yield 185 mg of crude material in 35% yield. Finally, azide aziridine ring-opened PC(Acm)LFC(Acm) macrocycle (0.2 mmol, 1 eq.) was subjected to oxidative *S*-deprotection using Tl(TFA)$_3$ (0.4 mmol, 2 eq.) in a 20:1 TFA:anisole solution (100 mL, 0.002 M) as described in Section 2.4 to afford 12 mg of a **1** as a white powder in 8% yield after column chromatography and semi-preparative HPLC/MS purification.

^{1}H NMR (500 MHz, Methanol-d_4)

δ 7.30 – 7.18 (m, 5H), 4.22 (ddd, *J* = 7.0, 5.4, 4.0 Hz, 1H), 3.95 (t, *J* = 7.5 Hz, 1H), 3.75 (dd, *J* = 15.2, 5.3 Hz, 1H), 3.62 – 3.54 (m, 2H), 3.51 – 3.32 (m, 4H), 3.27 – 3.13 (m, 5H), 2.17 – 2.06 (m, 1H), 1.95 (m, 1H), 1.85 (m, 1H), 1.68 (m, 1H), 1.36 (s, 12H), 0.87 (d, *J* = 6.1 Hz, 1H), 0.81 (d, *J* = 6.2 Hz, 1H).

^{13}C NMR (126 MHz, Methanol-d_4)

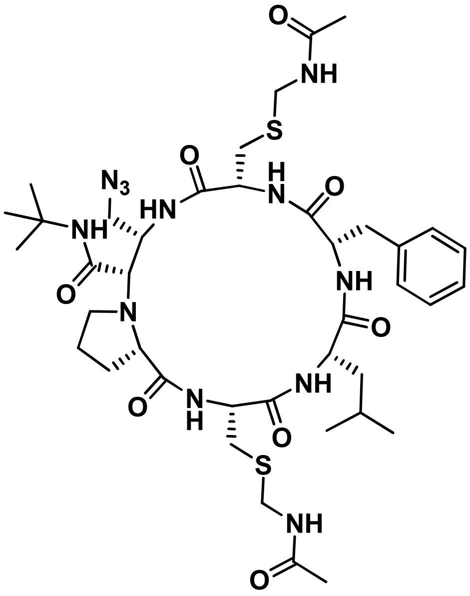
δ 176.9, 174.4, 174.0, 172.2, 172.1, 171.9, 130.4, 129.5, 127.9, 115.8, 66.6, 66.3, 56.8, 54.3, 52.7, 52.6, 52.2, 51.6, 49.5, 49.3, 49.1, 49.0, 40.9, 40.4, 31.0, 28.8, 28.7, 25.7, 25.4, 22.9, 22.5.

HRMS (ESI)

Calculated for $C_{38}H_{56}N_{12}O_8S_2$ ($M+H^+$):	759.3429
Found:	759.3423

Retention Time (Analytical HPLC/MS)

6.99 min (5% MeCN isocratic from 0.00 – 1.00 min, 5% – 95% MeCN linear gradient from 1.00 – 9.00 min, 95% MeCN isocratic from 9.00 – 10.00 min)



N,N'-(((((3R,6S,9S,12R,15S,16S,20aS)-15-(azidomethyl)-9-benzyl-16-(tert-butylcarbamoyl)-6-isobutyl-1,4,7,10,13-pentaoxoicosahydropyrrolo[1,2-a][1,4,7,10,13,16]hexaazacyclooctadecine-3,12-diyl)bis(methylene))bis(sulfanediyl))bis(methylene))diacetamide (PC(Acm)LFC(Acm)-N_3)

PC(Acm)LFC(Acm) linear peptide was prepared as described section 2.1. PC(Acm)LFC(Acm) (0.6 mmol, 1 eq.) was subjected to macrocyclization with (S)-aziridine-2-carboxaldehyde dimer (0.45 mmol, 0.75 eq.) and *tert*-butyl isocyanide (1.2 mmol, 2 eq.) in TFE (6 mL, 0.1 M) as described in Section 2.2 to yield 500 mg of crude material in 97% yield. Crude aziridine-containing macrocyclic PC(Acm)LFC(Acm) (0.58 mmol, 1 eq.) was subjected to aziridine ring-opening with sodium azide (5.8 mmol, 10 eq.) in 1:1 MeCN:DMF (7.25 mL each, 0.04 M) as described in section 2.3 and subsequently purified by semi-preparative HPLC-MS to yield 16.35 mg of PC(Acm)LFC(Acm)-N_3 as a yellow solid in 3% overall yield over two steps.

^{1}H NMR (500 MHz, Methanol-d_4)

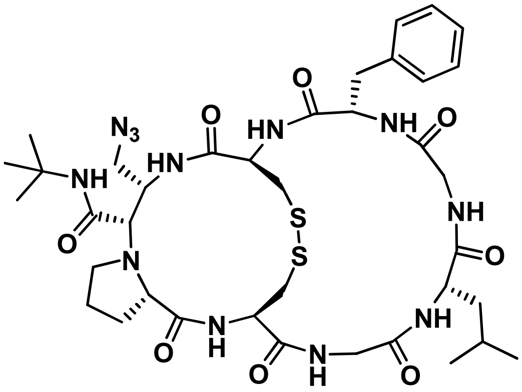
δ 7.39 – 7.20 (m, 5H), 6.84 (s, 1H), 4.76 (dd, *J* = 11.9, 3.8 Hz, 1H), 4.69 (dd, *J* = 8.9, 4.5 Hz, 1H), 4.60 (d, *J* = 13.9 Hz, 1H), 4.53 (dd, *J* = 7.3, 3.9 Hz, 1H), 4.34 (ddd, *J* = 11.0, 5.7, 2.9 Hz, 1H), 4.30 (dt, *J* = 9.4, 6.2 Hz, 1H), 4.26 (s, 2H), 4.19 (d, *J* = 5.3 Hz, 1H), 4.17 (d, *J* = 2.6 Hz, 1H), 3.71 – 3.63 (m, 2H), 3.38 – 3.32 (m, 2H), 3.27 – 3.12 (m, 3H), 3.10 – 2.94 (m, 3H), 2.89 (ddd, *J* = 13.9, 3.9, 1.1 Hz, 1H), 2.21 – 2.09 (m, 1H), 1.99 (s, 3H), 1.97 (s, 3H), 1.91 – 1.78 (m, 4H), 1.74 (ddd, *J* = 13.9, 12.0, 3.7 Hz, 1H), 1.63 – 1.50 (m, 1H), 1.33 (s, 9H), 1.05 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (126 MHz, Methanol-d_4)

δ 175.6, 175.5, 172.1, 171.8, 171.3, 171.1, 170.2, 170.2, 169.2, 136.2, 128.8, 128.3, 126.8, 66.0, 61.2, 58.6, 56.2, 52.9, 51.9, 51.6, 50.4, 48.8, 48.7, 45.9, 43.0, 40.8, 40.8, 35.7, 31.9, 31.4, 29.6, 27.2, 25.0, 23.2, 22.7, 21.4, 21.3, 19.8.

Retention Time (Analytical HPLC/MS)

6.13 min (5% MeCN isocratic from 0.00 – 1.00 min, 5% – 95% MeCN linear gradient from 1.00 – 9.00 min, 95% MeCN isocratic from 9.00 – 10.00 min)



(3R,9S,15S,18R,21S,22S,26aS)-21-(azidomethyl)-15-benzyl-N-(tert-butyl)-9-isobutyl-1,4,7,10,13,16,19-heptaoxohexacosahydro-3,18-(methanodithiomethano)pyrrolo[1,2-a][1,4,7,10,13,16,19,22]octaazacyclotetracosine-22-carboxamide (2)

PC(Acm)GLGFC(Acm) linear peptide was prepared as described section 2.1. PC(Acm)GLGFC(Acm) (0.85 mmol, 1 eq.) was subjected to macrocyclization with (S)-aziridine-2-carboxaldehyde dimer (0.43 mmol, 0.51 eq.) and *tert*-butyl isocyanide (0.86 mmol, 1.01 eq.) in TFE (8.5 mL, 0.1 M) as described in Section 2.2. Crude aziridine-containing macrocyclic PC(Acm)GLGFC(Acm) was subjected to aziridine ring-opening with sodium azide (8.5 mmol, 10 eq.) in 1:1 MeCN:DMF (10.6 mL each, 0.04 M) as described in section 2.3 to yield 304.4 mg of crude material in 35% yield over two steps. Finally, azide aziridine ring-opened PC(Acm)GLGFC(Acm) macrocycle (0.30 mmol, 1 eq.) was subjected to oxidative *S*-deprotection using anisole (0.60 mmol, 2 eq.) and Tl(TFA)$_3$ (0.45 mmol, 1.5 eq.) in TFA (120 mL, 0.002 M) as described in Section 2.4 to afford 6.8 mg of a **2** as a white powder in 3% yield after column chromatography and semi-preparative HPLC/MS purification.

^{1}H NMR (500 MHz, Methanol-d4)

δ 7.31 – 7.15 (m, 5H), 4.73 (dd, *J* = 12.2, 4.9 Hz, 1H), 4.60 (dd, *J* = 11.2, 3.7 Hz, 1H), 4.57 (dd, 1H), 4.37 (dd, *J* = 12.0, 4.2 Hz, 1H), 4.28 (td, *J* = 7.0, 3.8 Hz, 1H), 4.10 (d, *J* = 16.9 Hz, 1H), 3.80 (d, *J* = 15.0 Hz, 1H), 3.68 (d, *J* = 10.0 Hz, 2H), 3.66 (d, *J* = 3.2 Hz, 1H), 3.65 – 3.61 (m, 1H), 3.58 (dd, *J* = 12.1, 4.9 Hz, 1H), 3.46 – 3.32 (m, 6H), 3.05 (dd, *J* = 15.1, 4.2 Hz, 1H), 2.93 (td, *J* = 9.7, 5.6 Hz, 1H), 2.85 (dd, *J* = 14.4, 11.1 Hz, 1H), 2.18 (tdd, *J* = 12.4, 9.9, 6.8 Hz, 1H), 1.96 – 1.89 (m, 1H), 1.88 – 1.80 (m, 1H), 1.72 – 1.61 (m, 1H), 1.61 – 1.53 (m, 2H), 1.49 (q, *J* = 8.4, 7.5 Hz, 1H), 1.40 (s, 9H), 0.93 (dd, *J* = 6.1, 4.3 Hz, 6H).

^{13}C NMR (126 MHz, Methanol-d4)

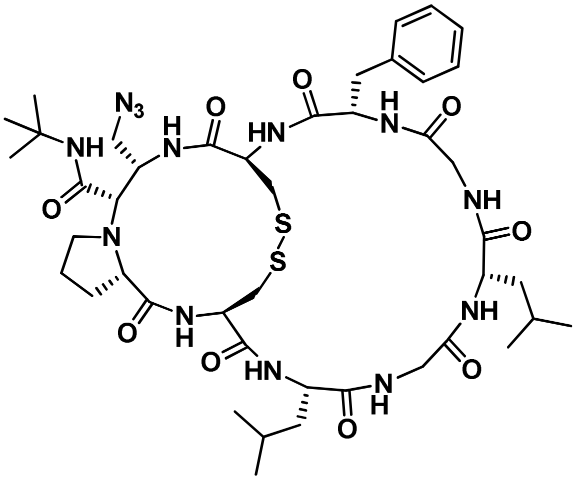
δ 179.1, 174.4, 173.8, 172.9, 172.1, 171.9, 171.1, 170.9, 139.0, 130.0, 129.5, 127.7, 71.5, 68.2, 60.5, 56.2, 54.4, 53.5, 53.1, 52.9, 52.6, 44.9, 44.5, 44.0, 39.2, 37.3, 34.3, 32.3, 28.9, 28.8, 26.1, 25.6, 23.3, 23.0.

HRMS (ESI)

Calculated for $C_{38}H_{56}N_{12}O_8S_2$ (M+H$^+$):	873.3858
Found:	873.3831

Retention Time (Analytical HPLC/MS)

7.22 min (5% MeCN isocratic from 0.00 – 1.00 min, 5% – 95% MeCN linear gradient from 1.00 – 9.00 min, 95% MeCN isocratic from 9.00 – 10.00 min)



(3R,6S,12S,18S,21R,24S,25S,29aS)-24-(azidomethyl)-18-benzyl-N-(tert-butyl)-6,12-diisobutyl-1,4,7,10,13,16,19,22-octaoxooctacosahydro-1H-3,21-(methanodithiomethano)pyrrolo[1,2-a][1,4,7,10,13,16,19,22,25]nonaazacycloheptacosine-25-carboxamide (3)

PC(Acm)LGLGFC(Acm) linear peptide was prepared as described section 2.1. PC(Acm)LGLGFC(Acm) (0.42 mmol, 1 eq.) was subjected to macrocyclization with (S)-aziridine-2-carboxaldehyde dimer (0.21 mmol, 0.51 eq.) and *tert*-butyl isocyanide (0.42 mmol, 1.01 eq.) in TFE (4.2 mL, 0.1 M) as described in Section 2.2. Crude aziridine-containing macrocyclic PC(Acm)LGLGFC(Acm) was subjected to aziridine ring-opening with sodium azide (4.2 mmol, 10 eq.) in 1:1 MeCN:DMF (5.2 mL each, 0.04 M) as described in section 2.3 to yield 176.4 mg of crude material in 38% yield over two steps. Finally, azide aziridine ring-opened PC(Acm)LGLGFC(Acm) macrocycle (0.16 mmol, 1 eq.) was subjected to oxidative *S*-deprotection using anisole (0.32 mmol, 2 eq.) and Tl(TFA)$_3$ (0.24 mmol, 1.5 eq.) in TFA (62 mL, 0.002 M) as described in Section 2.4 to afford 9.7 mg of a **3** as a white powder in 6% yield after column chromatography and semi-preparative HPLC/MS purification.

^{1}H NMR (500 MHz, Methanol-d4)

δ 7.33 – 7.11 (m, 5H), 4.71 (t, *J* = 7.5 Hz, 1H), 4.66 (dd, *J* = 10.0, 4.9 Hz, 1H), 4.41 (td, *J* = 7.2, 4.4 Hz, 1H), 4.27 (dd, *J* = 9.5, 5.2 Hz, 1H), 4.20 (s, 2H), 4.14 (d, *J* = 17.2 Hz, 1H), 3.88 – 3.81 (m, 1H), 3.74 – 3.70 (m, 1H), 3.67 (dd, *J* = 3.9, 1.5 Hz, 1H), 3.65 – 3.60 (m, 1H), 3.57 (dd, *J* = 16.7, 1.6 Hz, 1H), 3.53 – 3.32 (m, 6H), 3.28 – 3.24 (m, 2H), 3.17 (dd, *J* = 13.9, 10.3 Hz, 1H), 3.01 (q, *J* = 7.8, 7.1 Hz, 1H), 2.23 – 2.10 (m, 1H), 1.95 (dt, *J* = 12.2, 4.4 Hz, 1H), 1.82 (ddd, *J* = 13.4, 6.8, 3.8 Hz, 2H), 1.80 – 1.60 (m, 5H), 1.59 – 1.50 (m, 1H), 1.40 – 1.36 (m, 9H), 0.97 – 0.90 (m, 12H).

^{13}C NMR (126 MHz, Methanol-d4)

δ 177.4, 174.7, 172.9, 171.6, 171.1, 171.0, 170.7, 170.4, 169.7, 137.9, 128.8, 128.1, 126.2, 68.8, 66.3, 55.8, 54.3, 53.2, 53.0, 52.6, 52.4, 51.9, 51.4, 51.3, 43.3, 42.3, 39.5, 38.5, 34.7, 30.6, 27.5, 24.7, 24.6, 24.5, 22.3, 21.9, 20.7, 20.5.

HRMS (ESI)

Calculated for $C_{38}H_{56}N_{12}O_8S_2$ (M+H$^+$):	986.4699
Found:	986.4685

Retention Time (Analytical HPLC/MS)

7.39 min (5% MeCN isocratic from 0.00 – 1.00 min, 5% – 95% MeCN linear gradient from 1.00 – 9.00 min, 95% MeCN isocratic from 9.00 – 10.00 min)

Section 5 – Assay Yield Determination

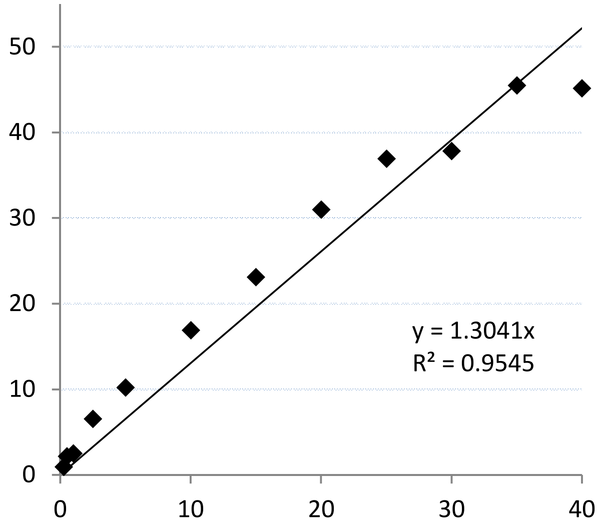
5.1 Calibration Curve for Macrocyclization and Aziridine Ring-Opening of PC(Acm)LFC(Acm)

Linear PC(Acm)LFC(Acm) was subjected to macrocyclization and aziridine ring-opening with sodium azide as described in Section 2. The azide-functionalized compound (PC(Acm)LFC(Acm)-N_3) was purified by semi-preparative HPLC-MS to obtain PC(Acm)LFC(Acm)-N_3 which was then characterized by NMR spectroscopy. A stock solution of PC(Acm)LFC(Acm)-N_3 (16.35 mg) was prepared as a 0.1 M solution in HFIP (181.0 μL). A 0.01 M peptide solution in HFIP was prepared by diluting 10 μL of the 0.1 M peptide solution with 90 μL of HFIP. Caffeine (19.42 mg) was prepared as a 0.1 M solution in HFIP (1 mL). Various dilutions were then prepared to a final volume of 20 μL in microcentrifuge tubes with 10 μL of 0.1 M caffeine, with peptide stock solution and HFIP volumes according to the table below.

Final [PC(Acm)LFC(Acm)-N_3] (mM)	[Stock PC(Acm)LFC(Acm)-N_3] (M)	Volume of Stock PC(Acm)LFC(Acm)-N_3 (μL)	Volume of HFIP (μL)
40	0.1	8	2
35		7	3
30		6	4
25		5	5
20		4	6
15		3	7
10		2	8
5		1	9
2.5		0.5	9.5
1	0.01	2	8
0.5		1	9
0.25		0.5	9.5

HPLC-MS samples were prepared by combining 5 μL of each caffeine/peptide dilution with 495 μL of 1:1 MeCN:H_2O. Each sample was then analyzed by analytical HPLC-MS (Agilent Poroshell 120 EC-C18 2.7 μm, 4.6 x 50 mm column; 30% isocratic MeCN in water from 0.00 – 0.30 min, 30 – 95% MeCN in water from 0.30 – 4.00 min, 30% isocratic MeCN in water from 4.00 – 5.00 min), with selected ion monitoring for the masses corresponding to PC(Acm)LFC(Acm)-N_3 peptide (m/z + 1 = 903.4) and caffeine (m/z + 1 = 195.1). For each sample, the mass peaks corresponding to the desired peptide/caffeine masses were integrated in ChemStation. Next, the peptide mass area was divided by the caffeine mass area to obtain the P/C ratio, which was plotted in a scatterplot on the y-axis with the final [PC(Acm)LFC(Acm)-N_3] on the x-axis. A least squares line was fitted to the data with the x,y-intercept set to (0, 0) to obtain a line with the equation y = 1.3041x (Eq. 1) and an R^2 of 0.9545.

Final [PCLFC-N_3] (mM)	**PCLFC-N_3 Area**	**Caffeine Area**	**P/C Ratio**
40	68296800	1512640	45.15073
35	67800700	1490620	45.4849
30	59827100	1581710	37.82432
25	63204700	1713100	36.89493
20	55407200	1788080	30.98698
15	45574600	1975460	23.07037
10	36159900	2143350	16.87074
5	19332700	1898360	10.1839
2.5	12314700	1889410	6.517749
1	4821140	1932260	2.495078
0.5	3440420	1602370	2.147082
0.25	2159950	2385670	0.905385



5.2 Assay Yield for PCLFC Macrocyclization and Aziridine Ring-Opening

Linear PC(Acm)LFC(Acm) was purified by semi-preparative HPLC-MS to obtain 75.7 mg of linear peptide, which was then subjected to macrocyclization followed by aziridine ring-opening in a telescopic synthesis as described in Section 2. When the reaction was complete (as determined by analytical LC-MS), 5 µL of the reaction was combined with 495 µL of 0.5 mM caffeine in 1:1 MeCN:H_2O and analyzed by analytical HPLC-MS using the same conditions described in section 5.1. The area under the PC(Acm)LFC(Acm)-N_3 peak was 31813770 while the area under the caffeine peak was 662204, giving a P/C ratio of 48.04. Substitution into Eq. 1 gives an x-value of 36.8 mM, which corresponds to an assay yield of 92.1% (assuming a theoretical 100% yield of 40 mM).

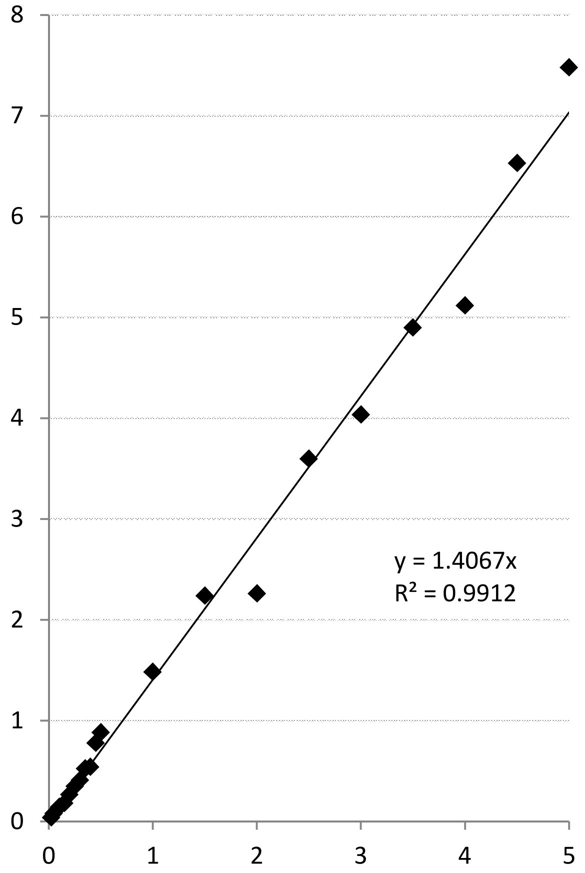
5.3 Calibration Curve for Deprotective Oxidation of PC(Acm)LFC(Acm)-N_3

A stock solution of **1** (PCLFC-SS, 8.46 mg) was prepared as a 0.1 M solution in HFIP (111.5 µL). A 0.01 M peptide solution in HFIP was prepared by diluting 10 µL of the 0.1 M peptide solution with 90 µL of HFIP. A 0.001 M peptide solution in HFIP was prepared by diluting 10 µL of the 0.01 M peptide solution with 90 µL of HFIP. Caffeine (8.46 mg) was prepared as a 0.1 M solution in HFIP (1 mL). Various dilutions were then prepared to a final volume of 20 µL in microcentrifuge tubes with 10 µL of 0.1 M caffeine, with peptide stock solution and HFIP volumes according to the table below.

Final [PCLFC-SS] (mM)	[Stock PCLFC-SS] (M)	Volume of Stock PCLFC-SS (µL)	Volume of HFIP (µL)
5.0	0.1	1	9
4.5	0.01	9	1
4.0		8	2
3.5		7	3
3.0		6	4
2.5		5	5
2.0		4	6
1.5		3	7
1.0		2	8
0.5		1	9
0.45	0.001	9	1
0.40		8	2
0.35		7	3
0.30		6	4
0.25		5	5
0.20		4	6
0.15		3	7
0.10		2	8
0.05		1	9
0.025		0.5	9.5

HPLC-MS samples were prepared, analyzed, and processed in Section 5.1, except for using selected ion monitoring for PCLFC-SS (m/z + 1 = 759.3) and caffeine (m/z + 1 = 195.1). A least squares line was fitted to the data with the x,y-intercept set to (0, 0) to obtain a line with the equation $y = 1.4067x$ (Eq. 2) and an R^2 of 0.9912.

Final [PCLFC-SS] (mM)	PCLFC-SS Area	Caffeine Area	P/C Ratio
5	21813600	2915830	7.481095
4.5	17705500	2710810	6.531443
4	15507600	3029590	5.118712
3.5	12984900	2649720	4.90048
3	12337900	3057440	4.035369
2.5	11193900	3110960	3.598214
2	8986180	3973460	2.26155
1.5	7091430	3165960	2.239899
1	4763020	3210150	1.483738
0.5	2848910	3220130	0.884719
0.45	2335880	2999190	0.778837
0.4	1615990	2990150	0.540438
0.35	1420780	2714180	0.523466
0.3	1414400	3435000	0.411761
0.25	1085580	3089060	0.351427
0.2	938636	3521320	0.266558
0.15	669547	3669040	0.182486
0.1	422615	2909020	0.145277
0.05	231580	2902720	0.07978
0.025	132595	3268090	0.040573



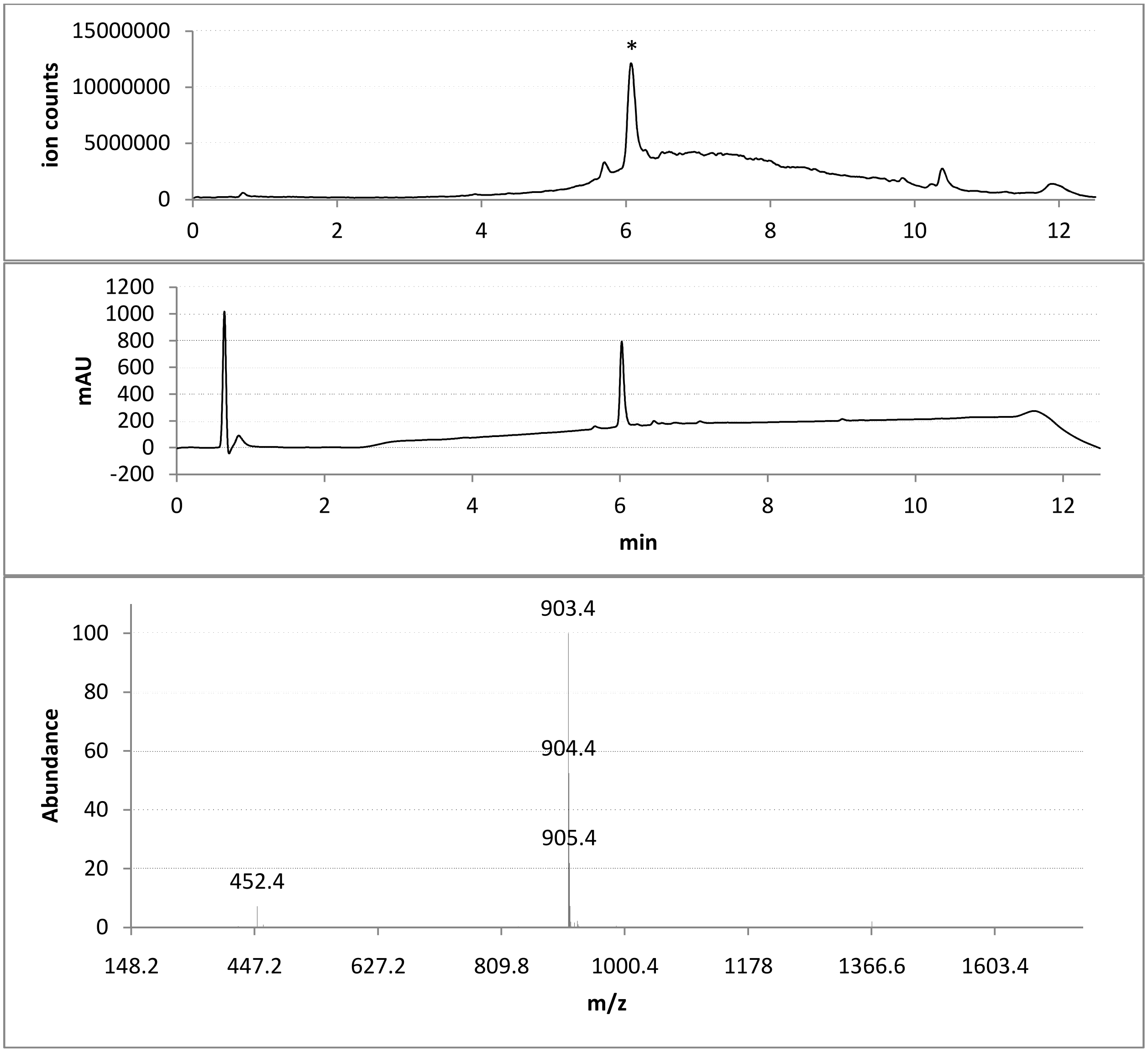
5.4 Assay Yield for Oxidative Acm-Deprotection of PC(Acm)LFC(Acm)-N_3

PC(Acm)LFC(Acm)-N_3 was purified by semi-preparative HPLC-MS to obtain 15.59 mg of peptide, which was then subjected to oxidative Acm-deprotection as described in Section 2. When the reaction was complete (as determined by analytical LC-MS), the solvent (TFA) was removed by centrifugal evaporation. Residual TFA was removed via azeotrope with approximately 10 mL chloroform, repeated five times in total. The residue was dissolved in 10 mL of 1:1 MeCN:H_2O, frozen, and lyophilized overnight. This process was repeated one additional time. The solid was then dissolved in 1:1 MeCN:H_2O using the same volume as the TFA used for oxidative deprotection. 5 µL of the solution was combined with 495 µL of 0.5 mM caffeine in 1:1 MeCN:H_2O and analyzed by analytical HPLC-MS using the same conditions described in section 5.1. The area under the PCLFC-SS peak was 3077710 while the area under the caffeine peak was 1368190, giving a P/C ratio of 2.25. Substitution into Eq. 2 gives an x-value of 1.6 mM, which corresponds to an assay yield of 80.0% (assuming a theoretical 100% yield of 2 mM).

Section 6 – Analytical HPLC/MS Traces

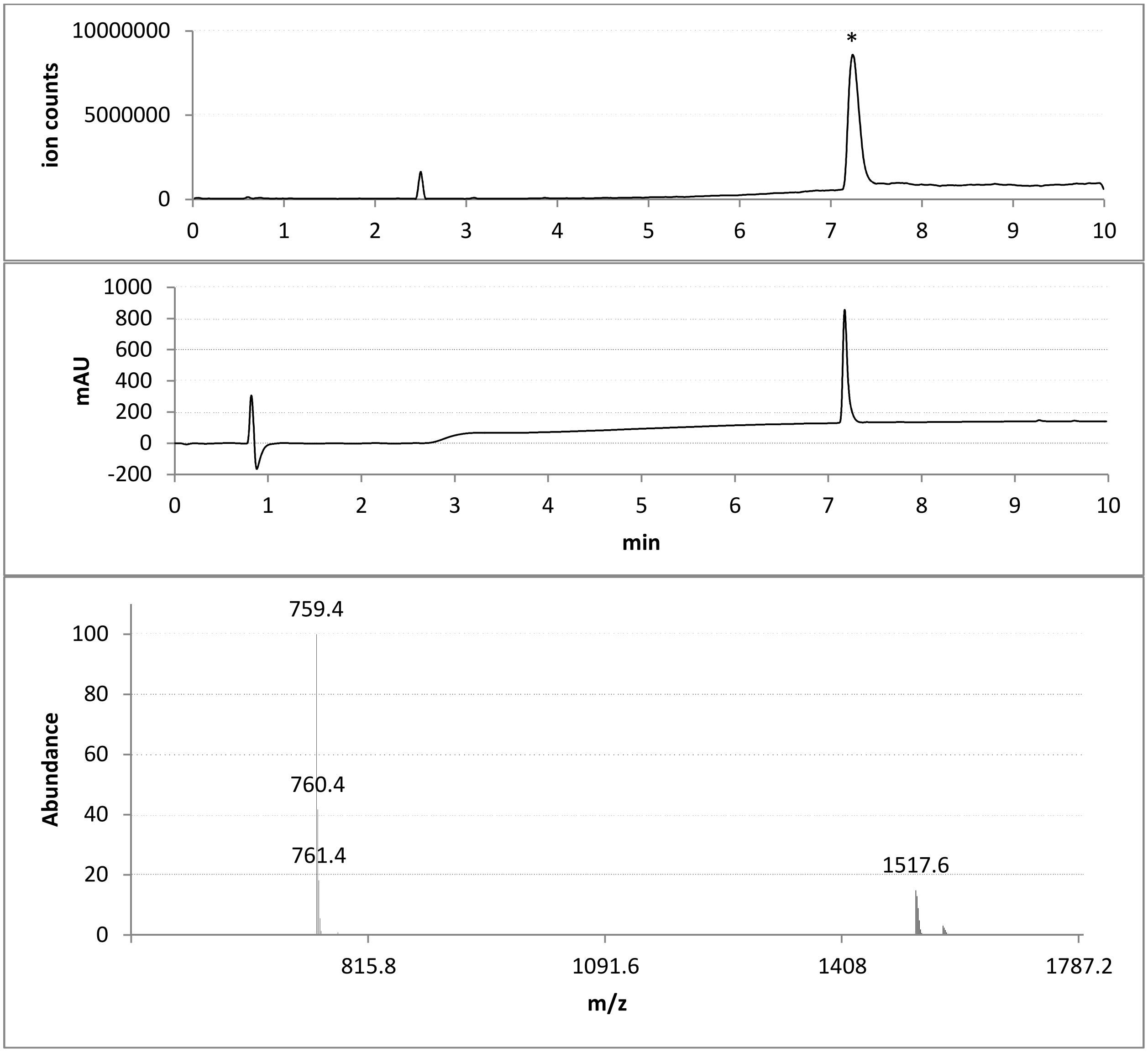
Desired compounds represented in LC peaks designated with an asterisk, followed by the UV trace and MS spectrum of the corresponding LC peak.

LC/MS, UV, and MS Traces of Crude Compound 1 Intermediate after Aziridine Ring-Opening with Sodium Azide



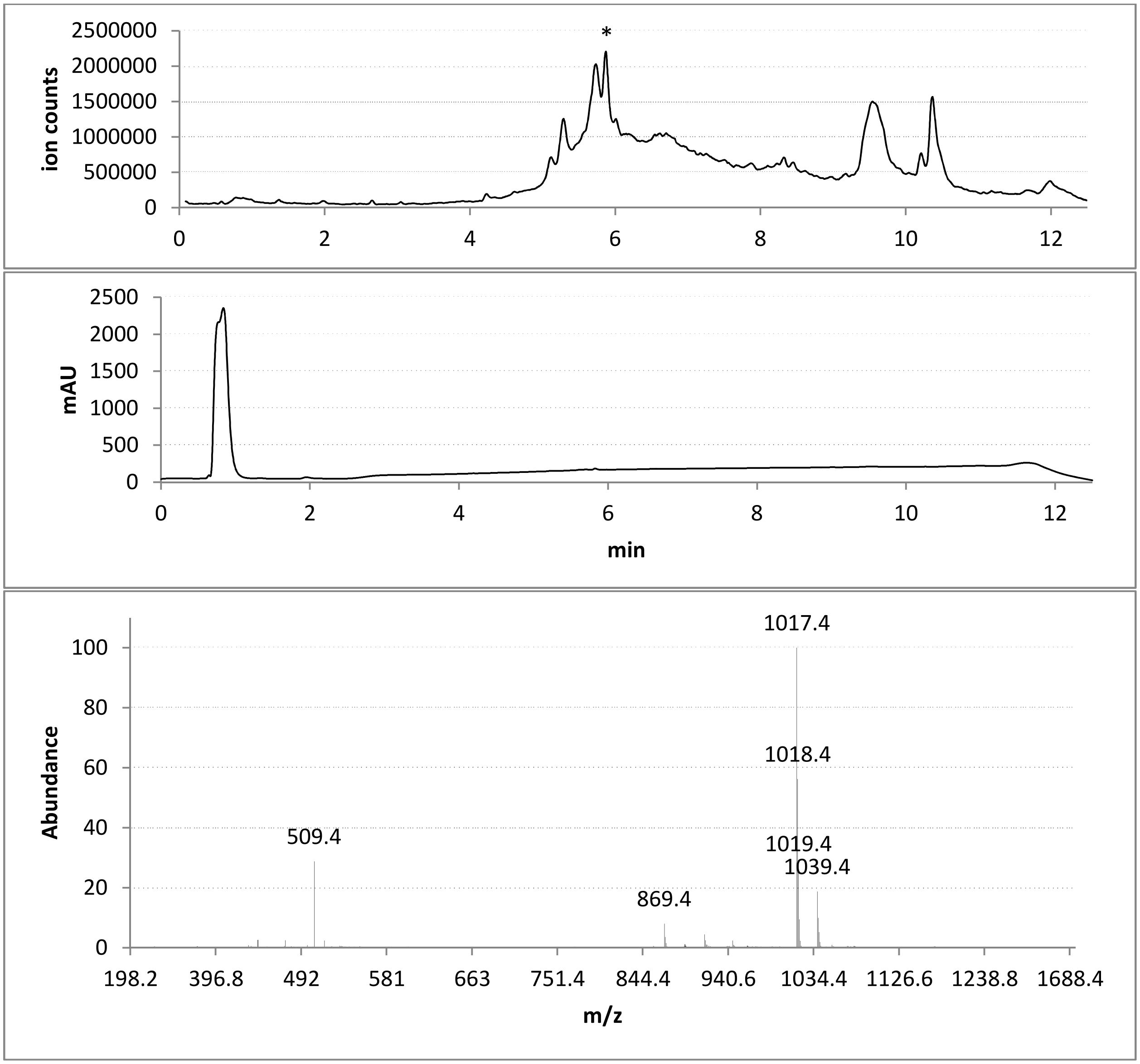
$m/z + H^{+} = 903.4$, $\frac{m/z\ +2H^{+}}{2} = 452.4$ observed

LC/MS, UV, and MS Traces of Compound 1 after Oxidative Deprotection with Thallium Trifluoroacetate



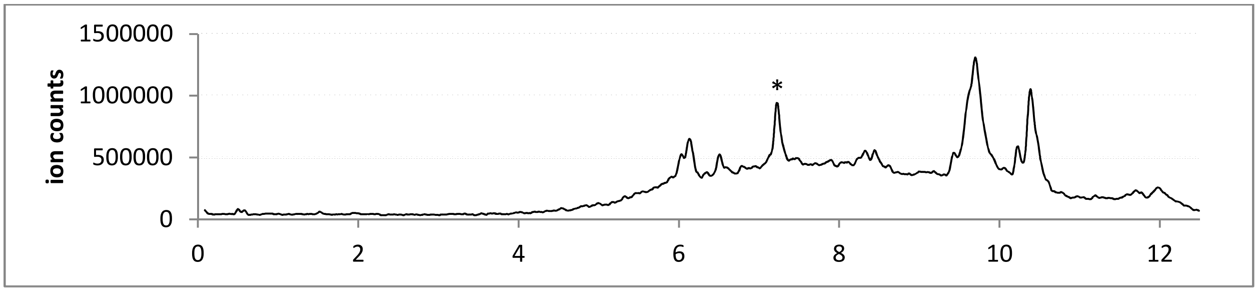
m/z + H^+ = 759.4 observed

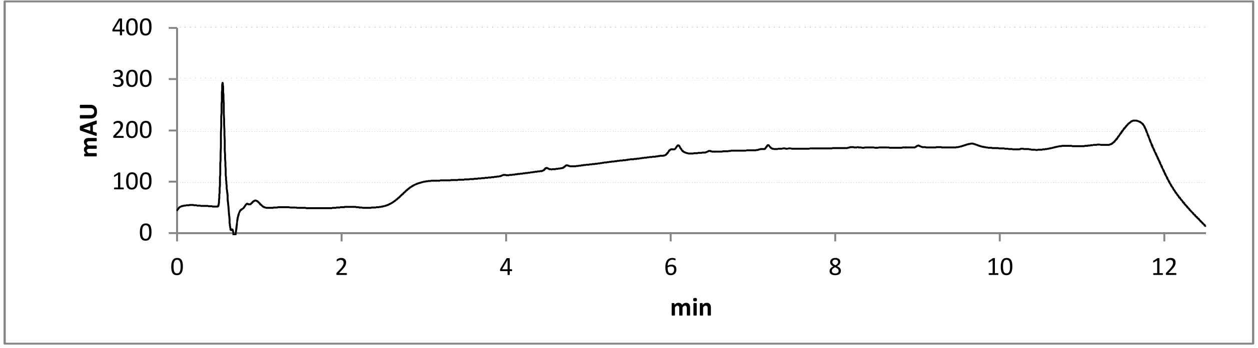
LC/MS, UV, and MS Traces of Crude Compound 2 Intermediate after Aziridine Ring-Opening with Sodium Azide

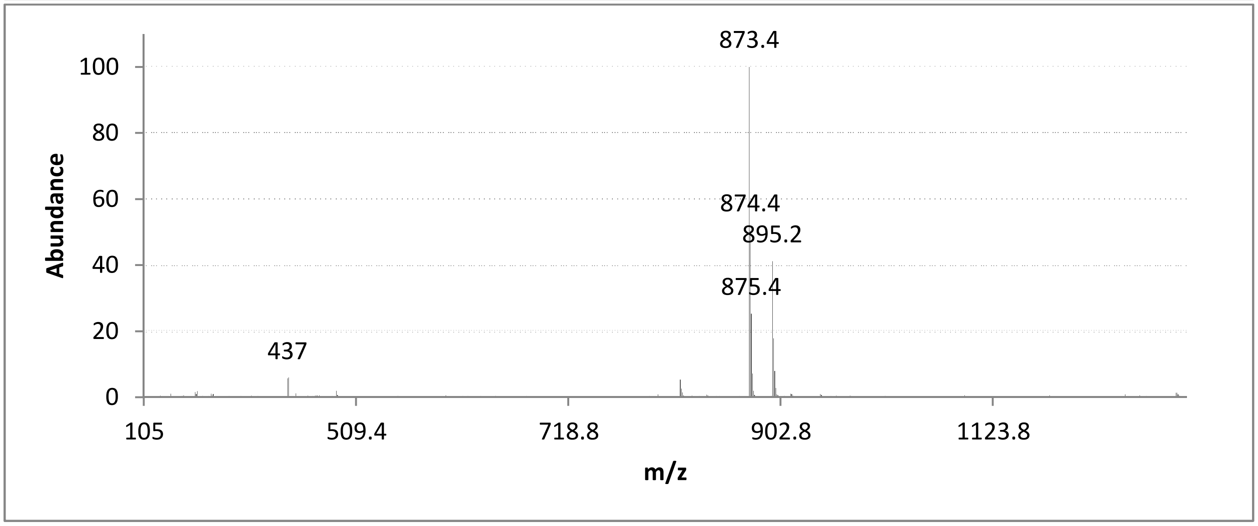


$\mathrm{m/z} + \mathrm{H}^+ = 1017.4$, $\mathrm{m/z} + \mathrm{Na}^+ = 1039.4$, $\frac{\mathrm{m/z}\ + 2\mathrm{H}^+}{2} = 509.4$ observed

LC/MS, UV, and MS Traces of Crude Compound 2 after Oxidative Deprotection with Thallium Trifluoroacetate

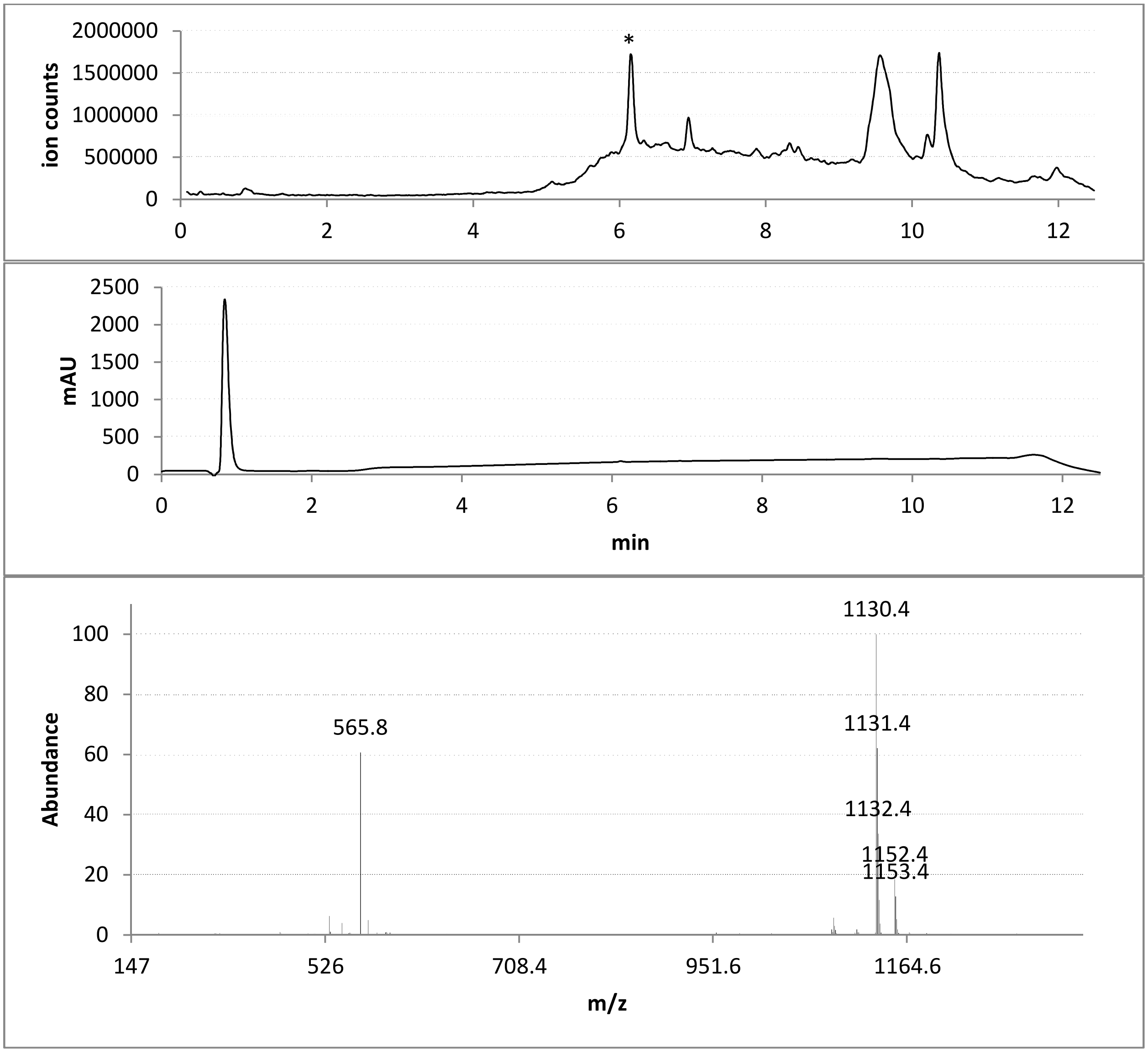






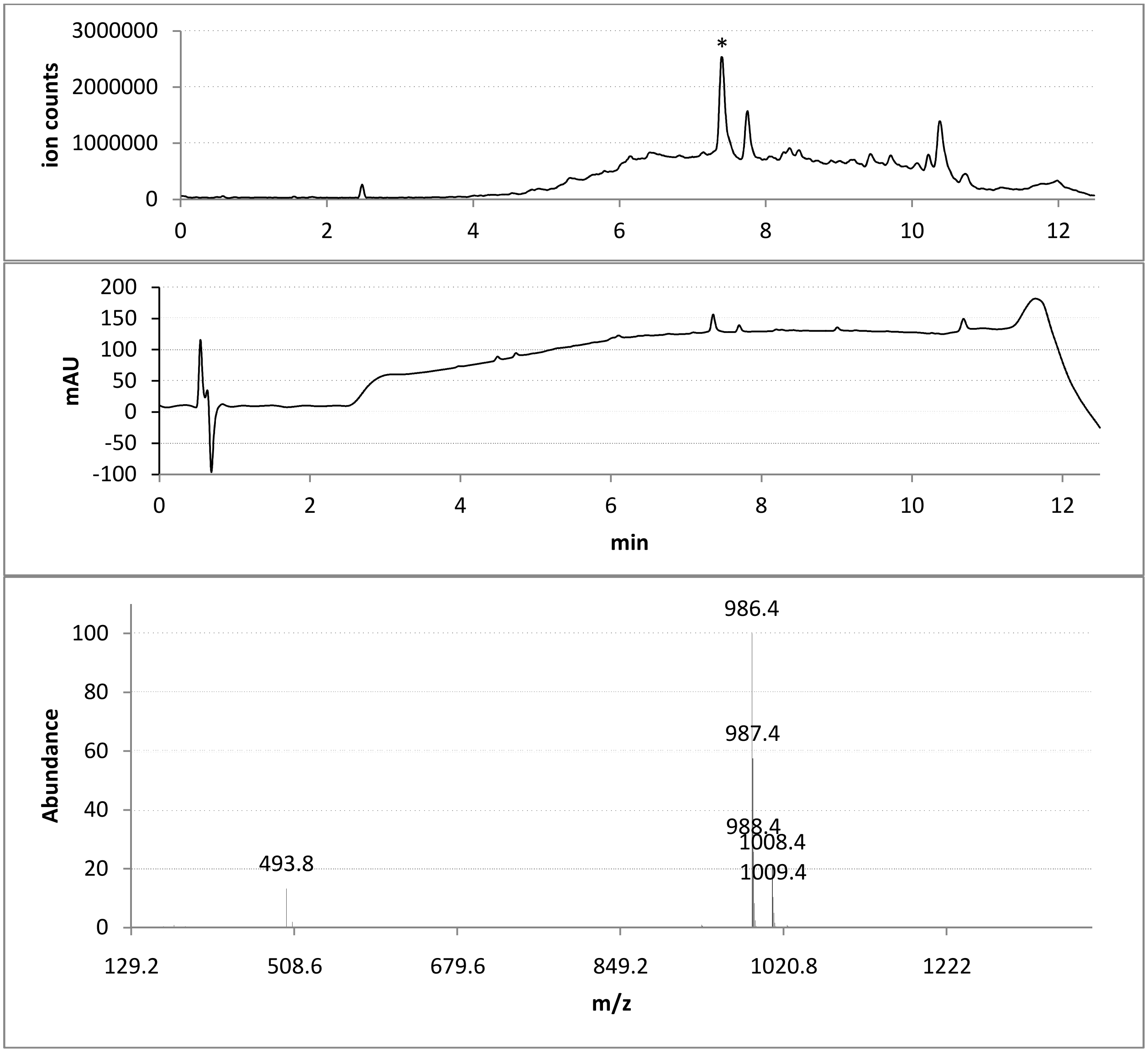
$m/z + H^+ = 873.4$, $m/z + Na^+ = 895.2$, $\frac{m/z\ + 2H^+}{2} = 437.0$ observed

LC/MS, UV, and MS Traces of Crude Compound 3 Intermediate after Aziridine Ring-Opening with Sodium Azide



$m/z + H^+ = 1130.4$, $m/z + Na^+ = 1152.4$, $\frac{m/z\ +2H^+}{2} = 565.8$ observed

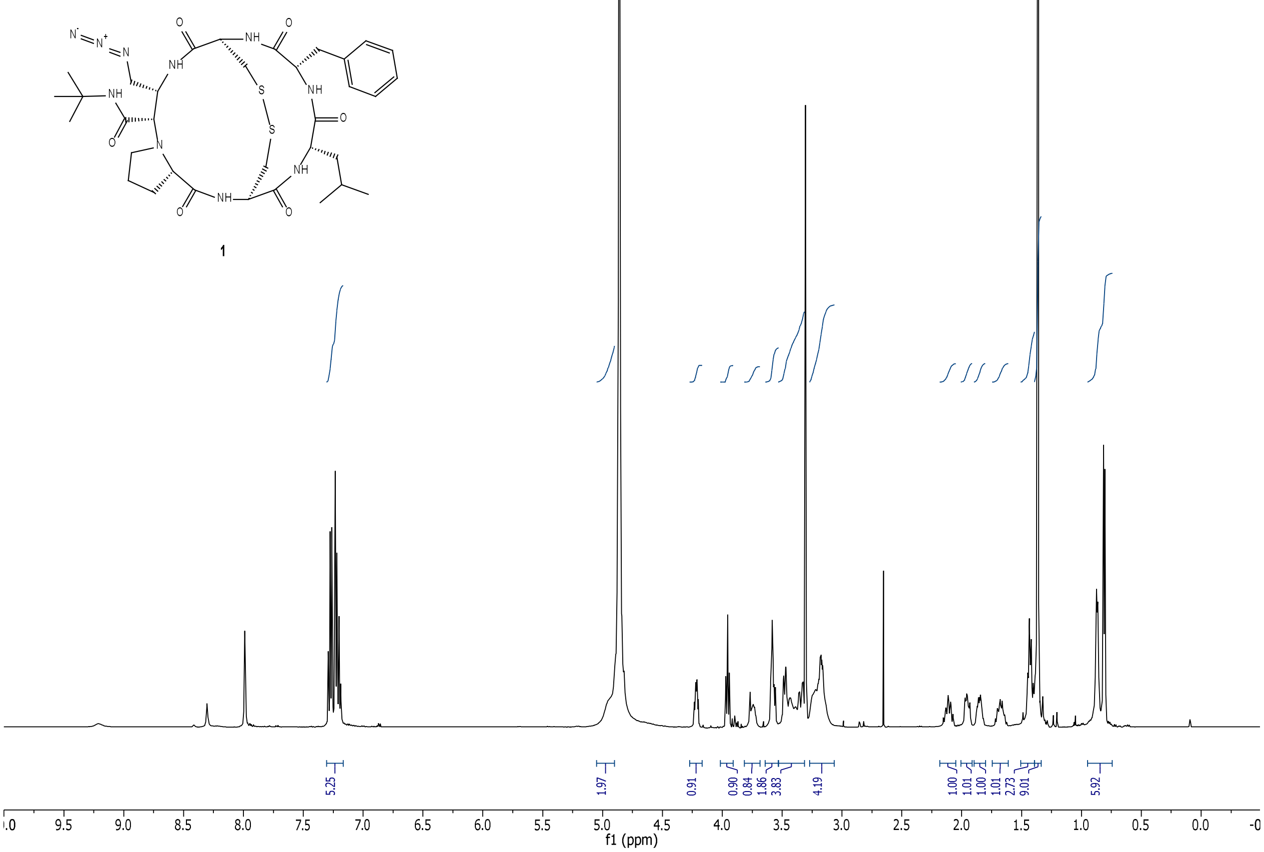
LC/MS, UV, and MS Traces of Crude Compound 3 after Oxidative Deprotection with Thallium Trifluoroacetate



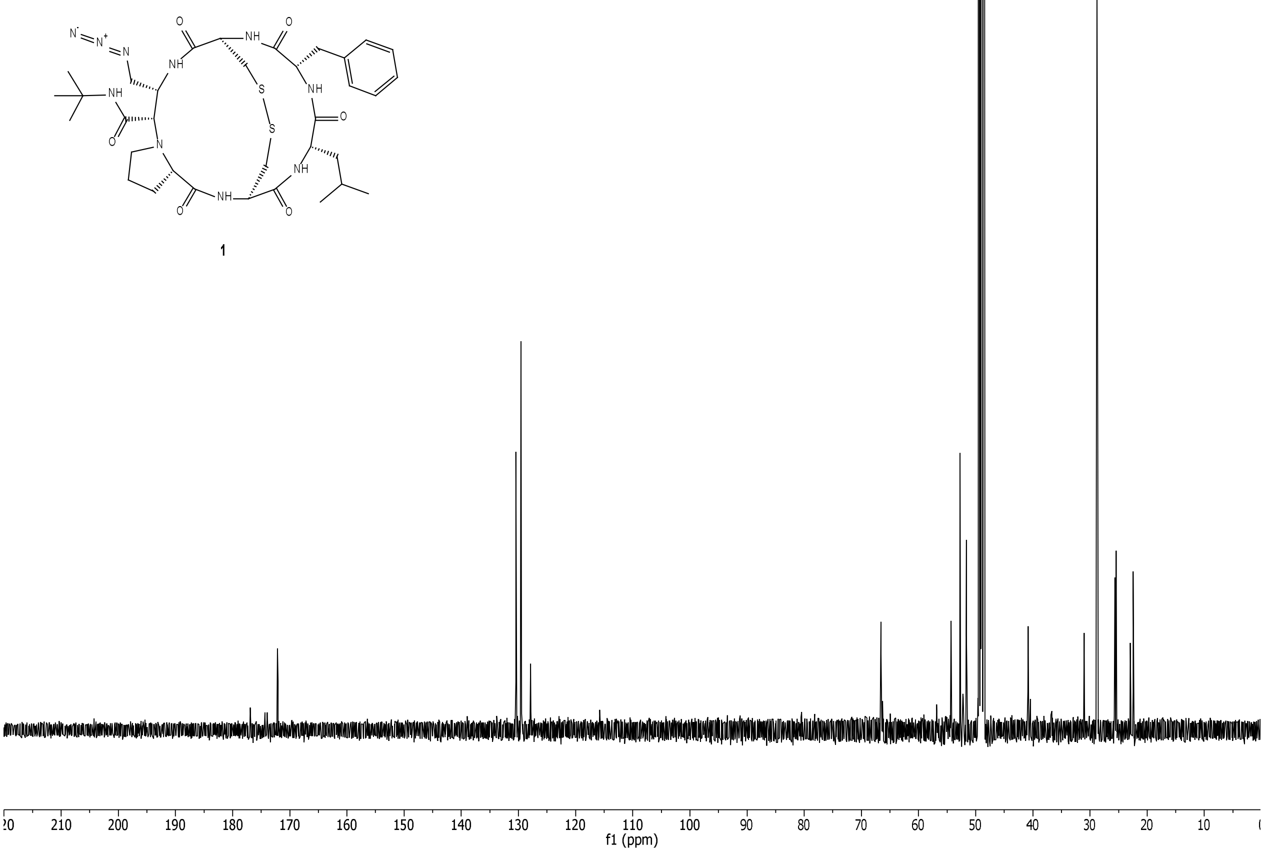
m/z + H^+ = 986.4, m/z + Na^+ = 1008.4, $\frac{m/z\ +2H^+}{2}$ = 493.8 observed

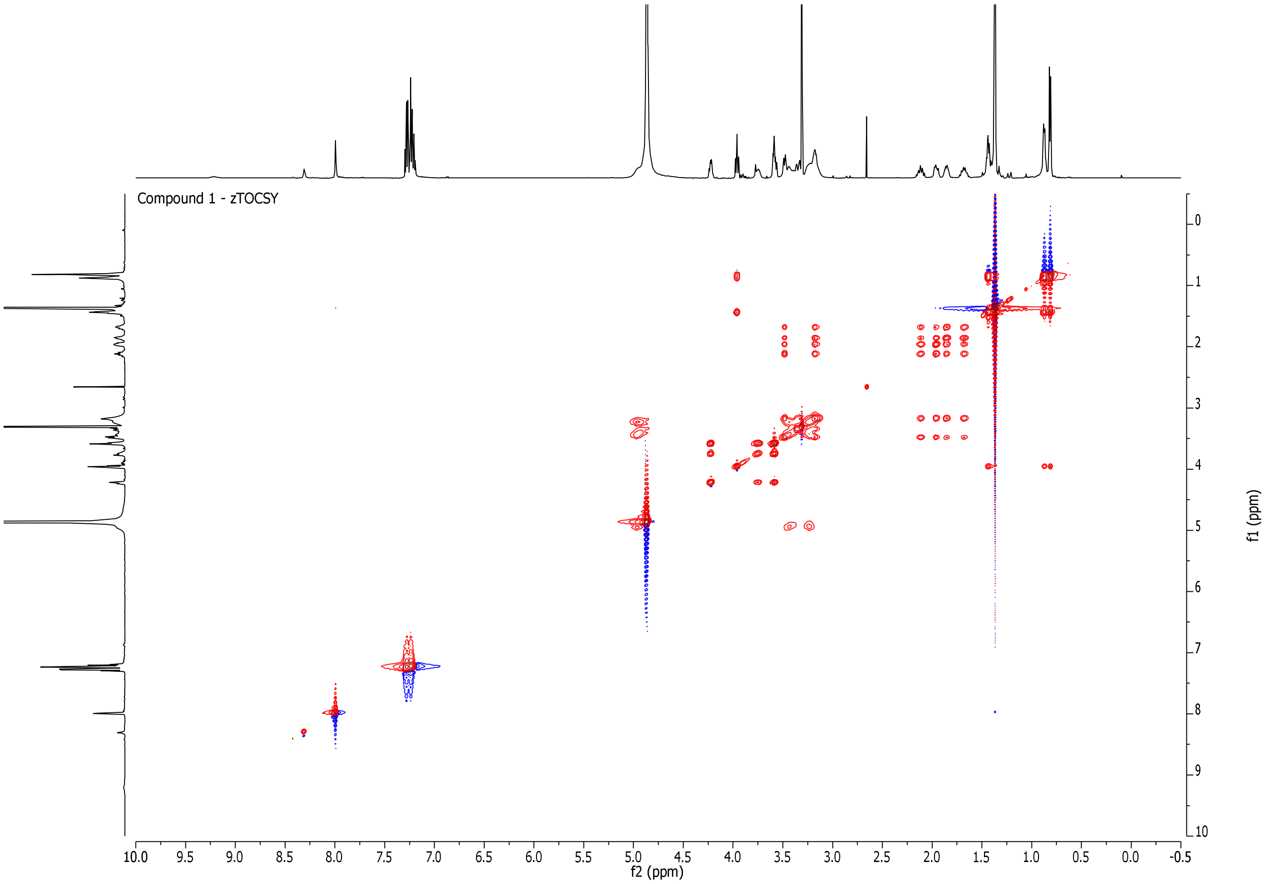
Section 7 – NMR Spectra

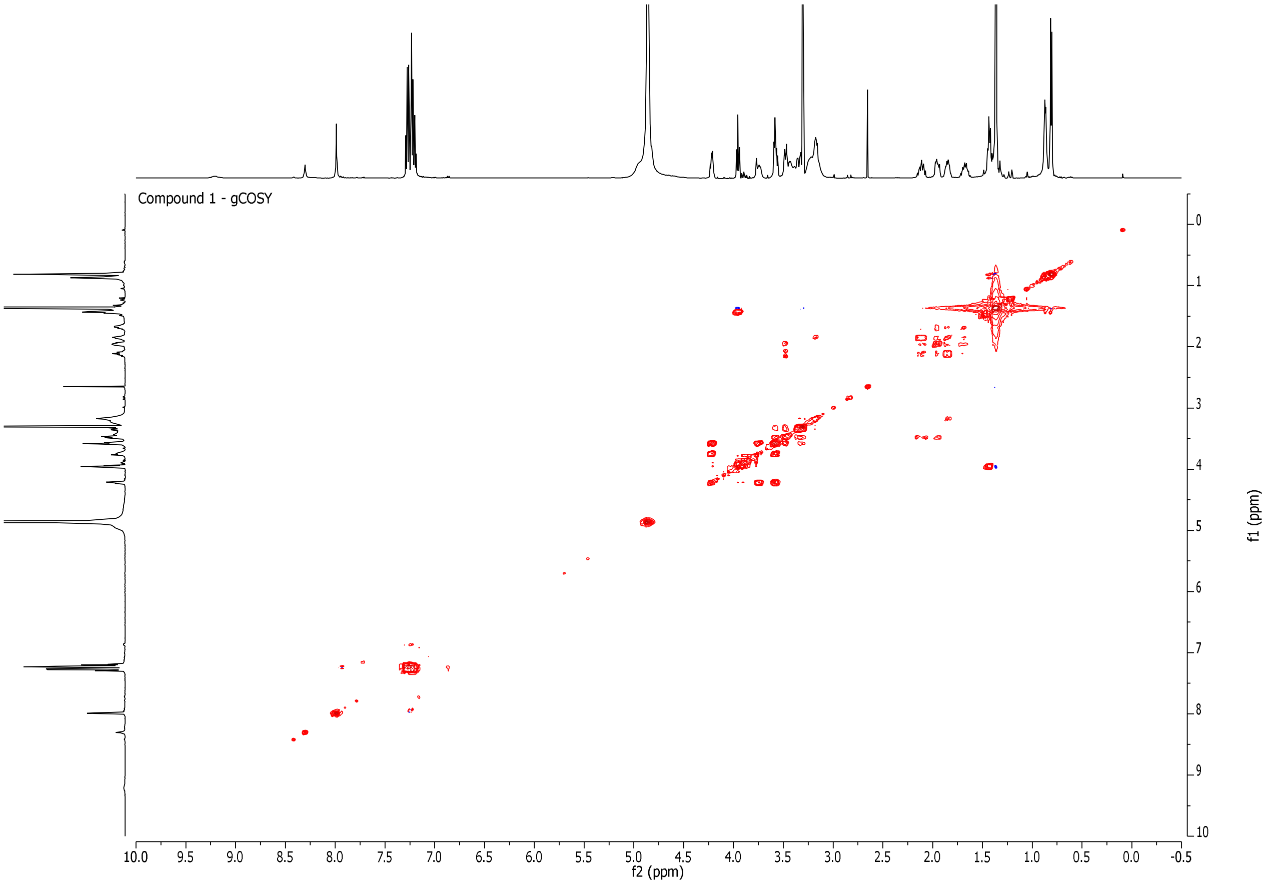
Compound 1 - 1H NMR

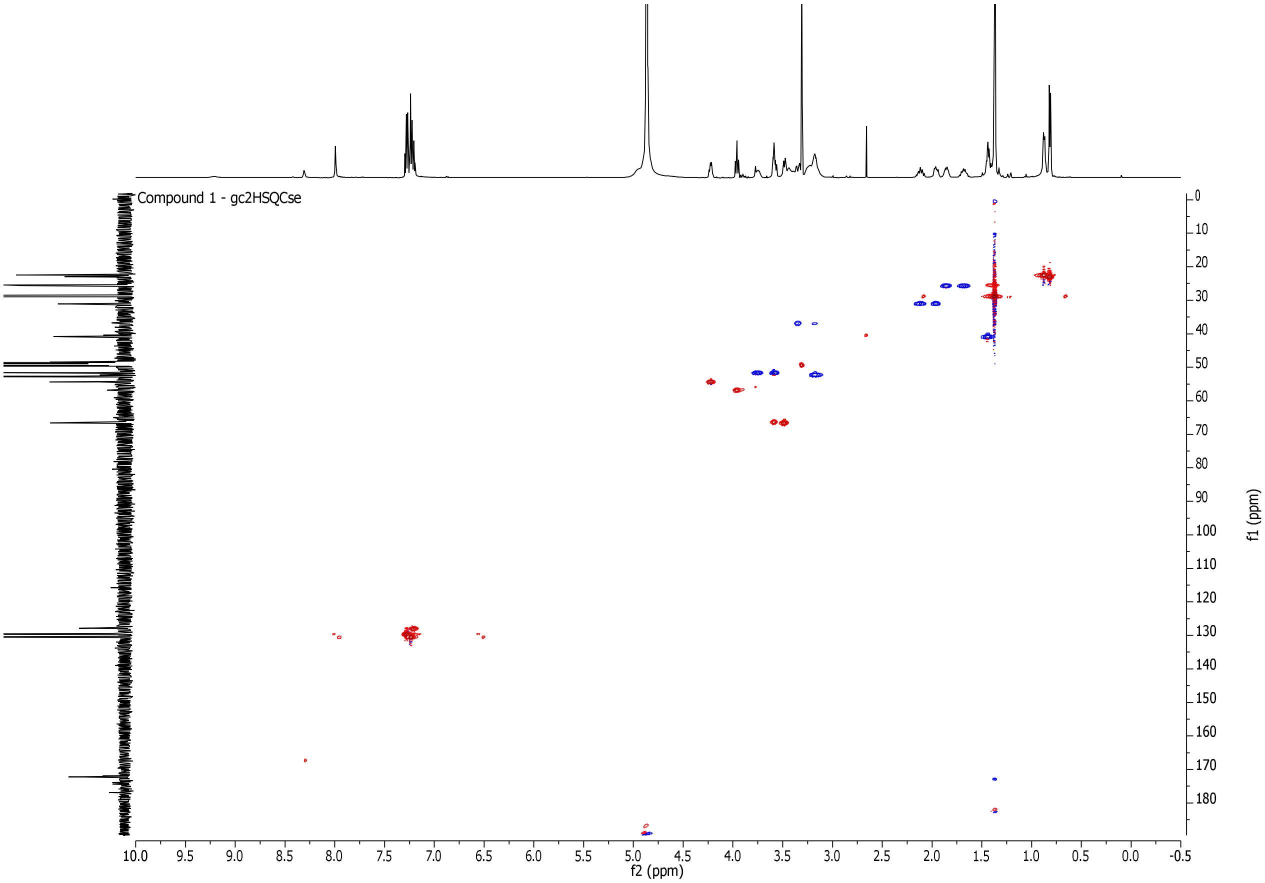


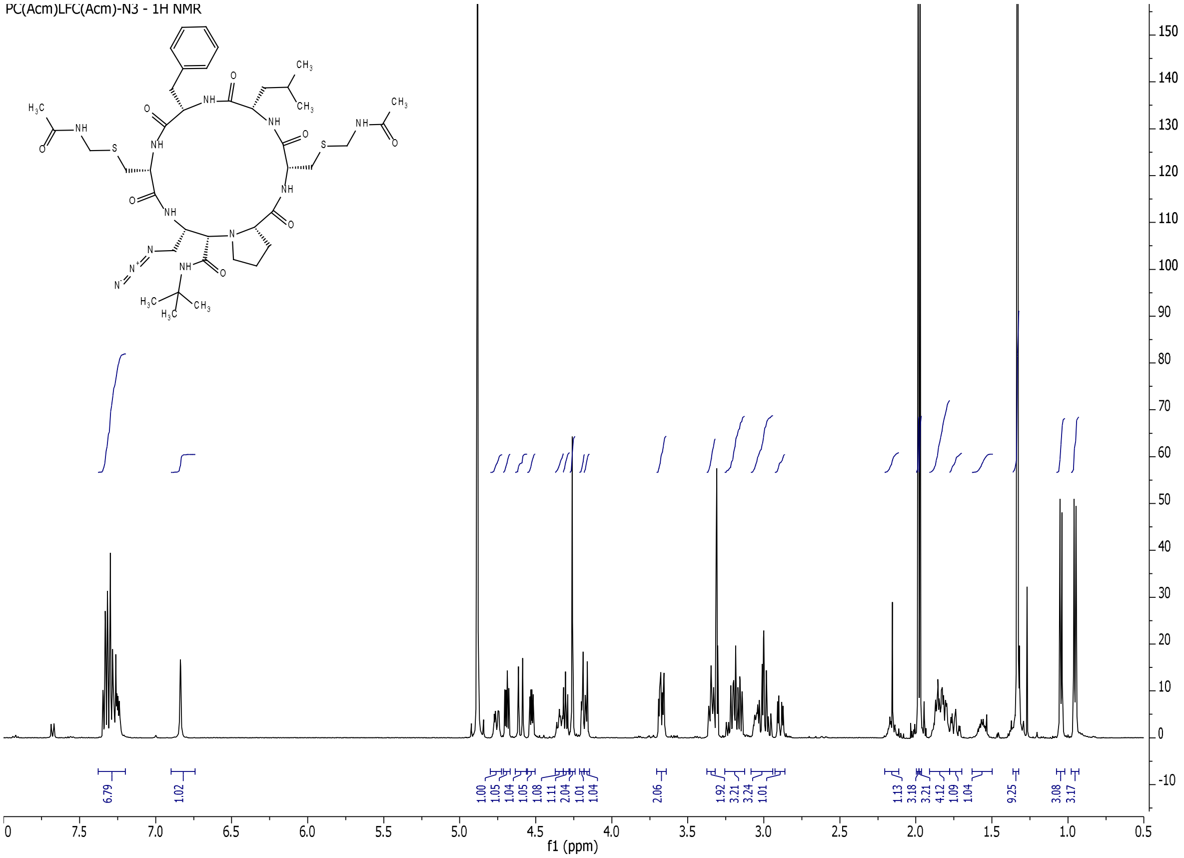
Compound 1 - 13C NMR

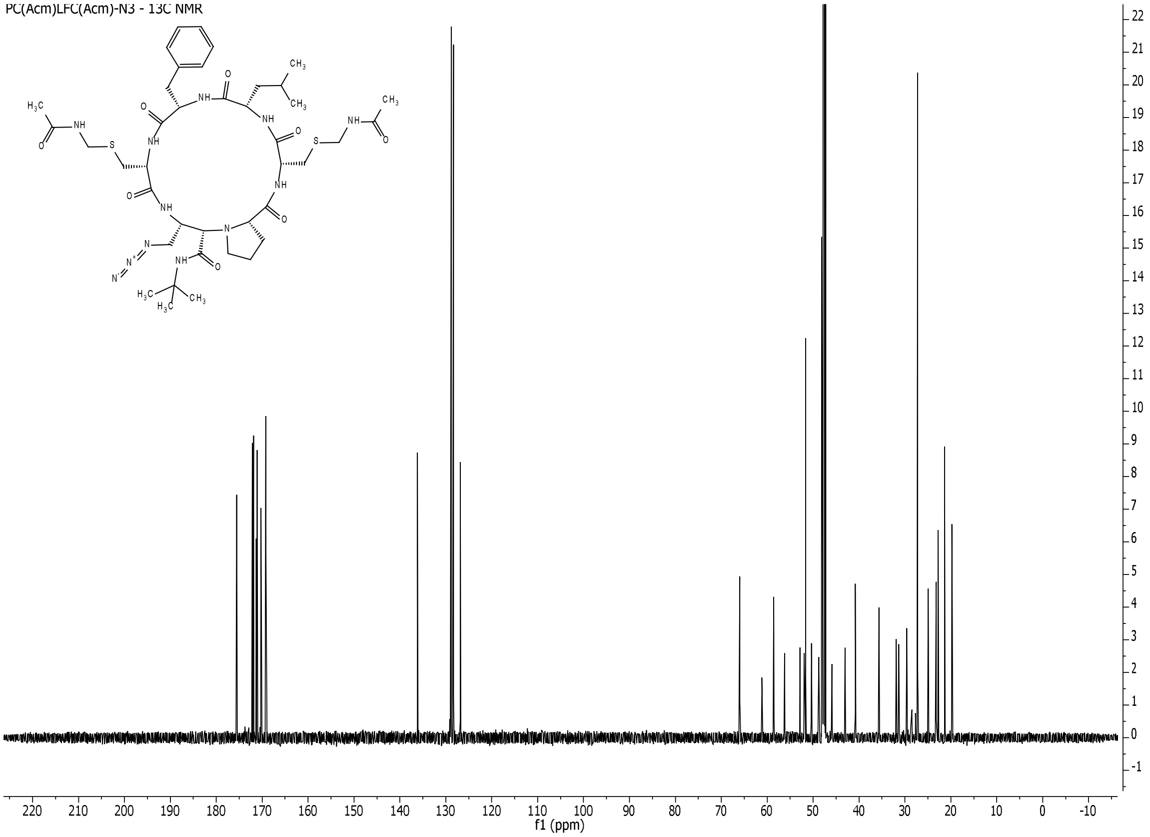


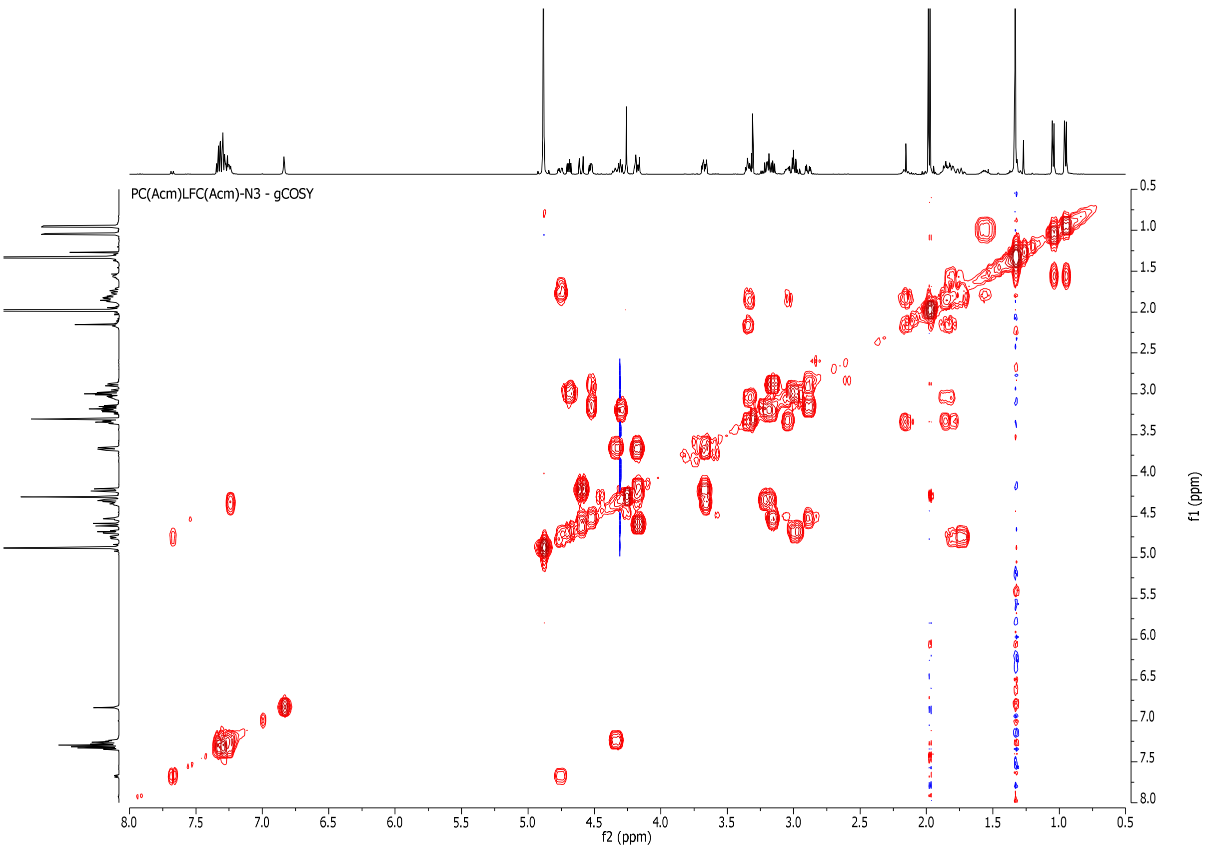

Compound 1 - zTOCSY
f1 (ppm)
f2 (ppm)
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
0 1 2 3 4 5 6 7 8 9 10

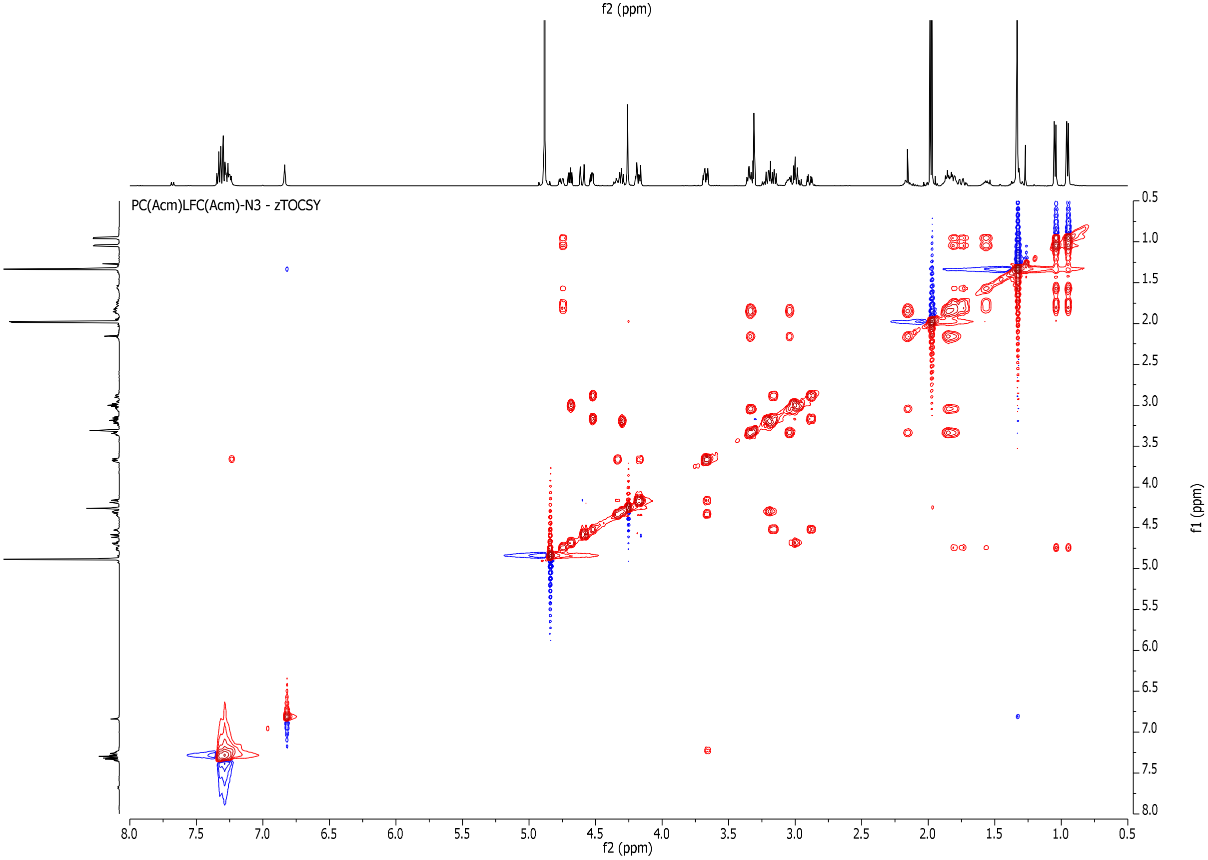

Compound 1 - gCOSY
f1 (ppm)
f2 (ppm)
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
0 1 2 3 4 5 6 7 8 9 10

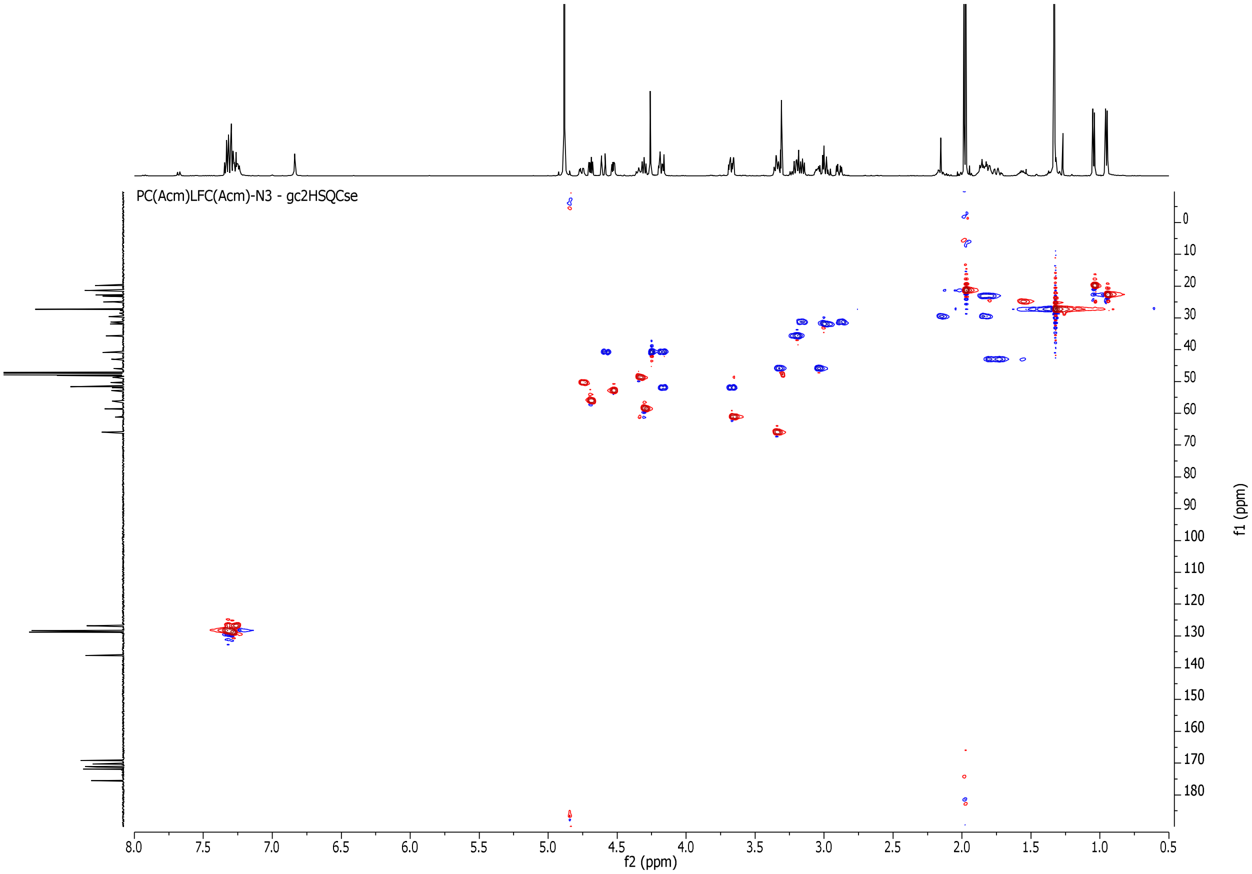

Compound 1 - gc2HSQCse
f2 (ppm)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180

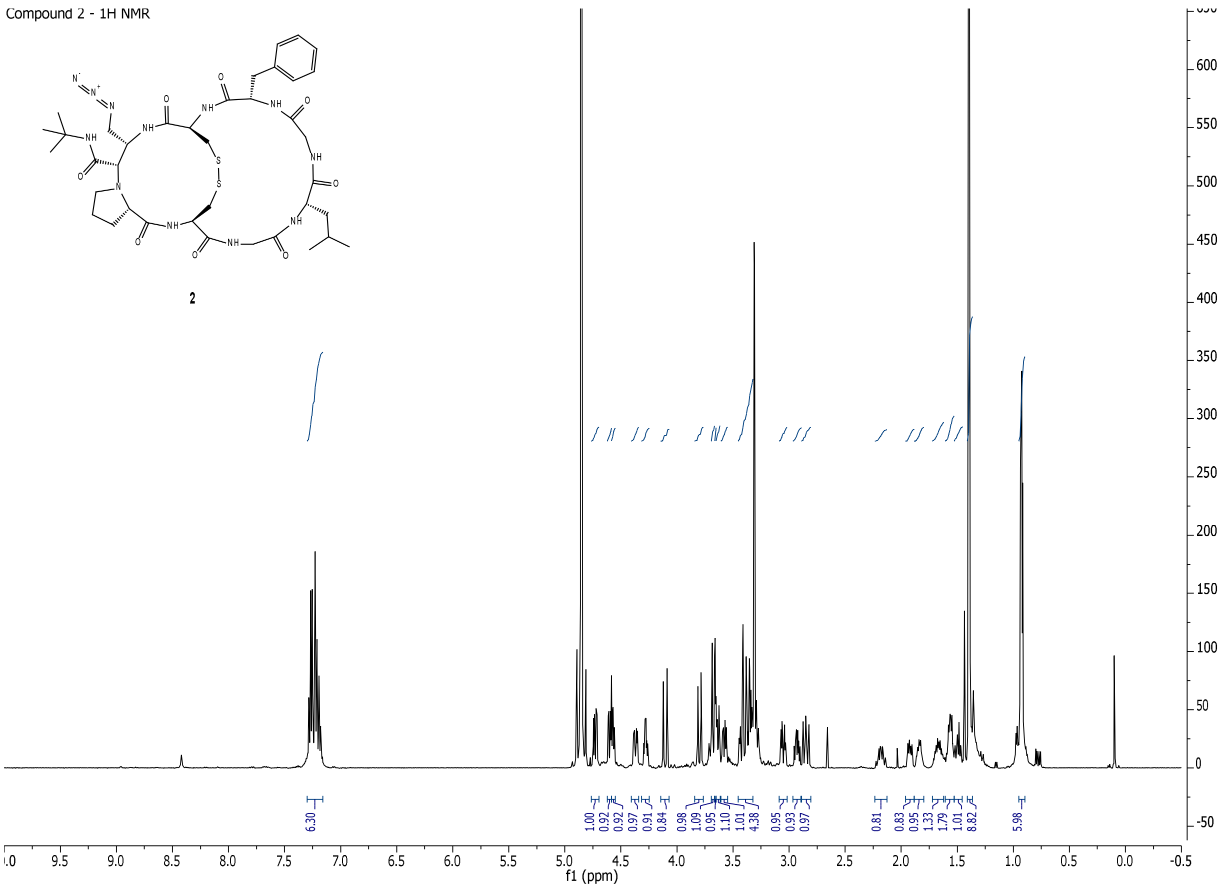

PC(Acm)LFC(Acm)-N3 - 1H NMR
6.79
1.02
1.00
1.05
1.04
1.05
1.08
1.11
2.04
1.01
1.04
2.06
1.92
3.21
3.24
1.01
1.13
3.18
3.21
4.12
1.09
1.04
9.25
3.08
3.17
f1 (ppm)

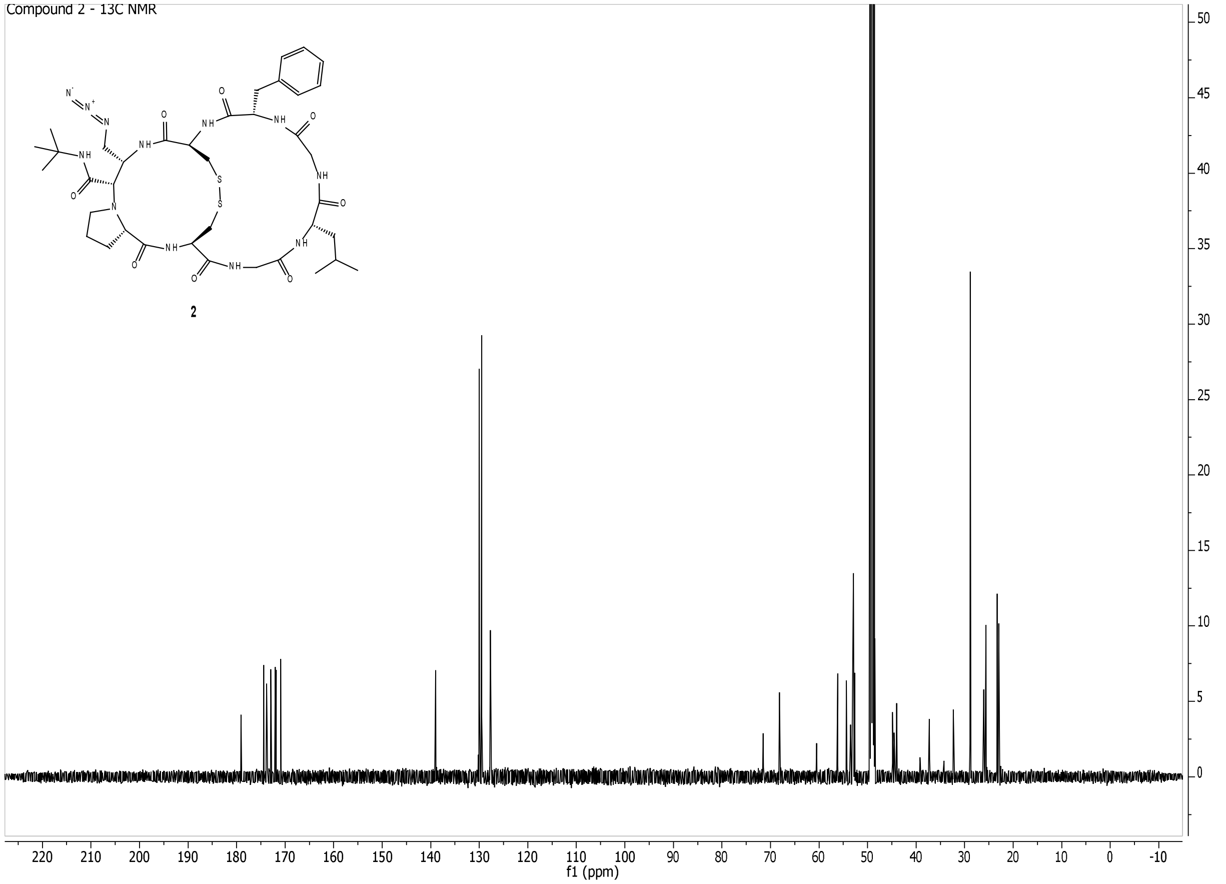

PC(Acm)LFC(Acm)-N3 - 13C NMR
f1 (ppm)

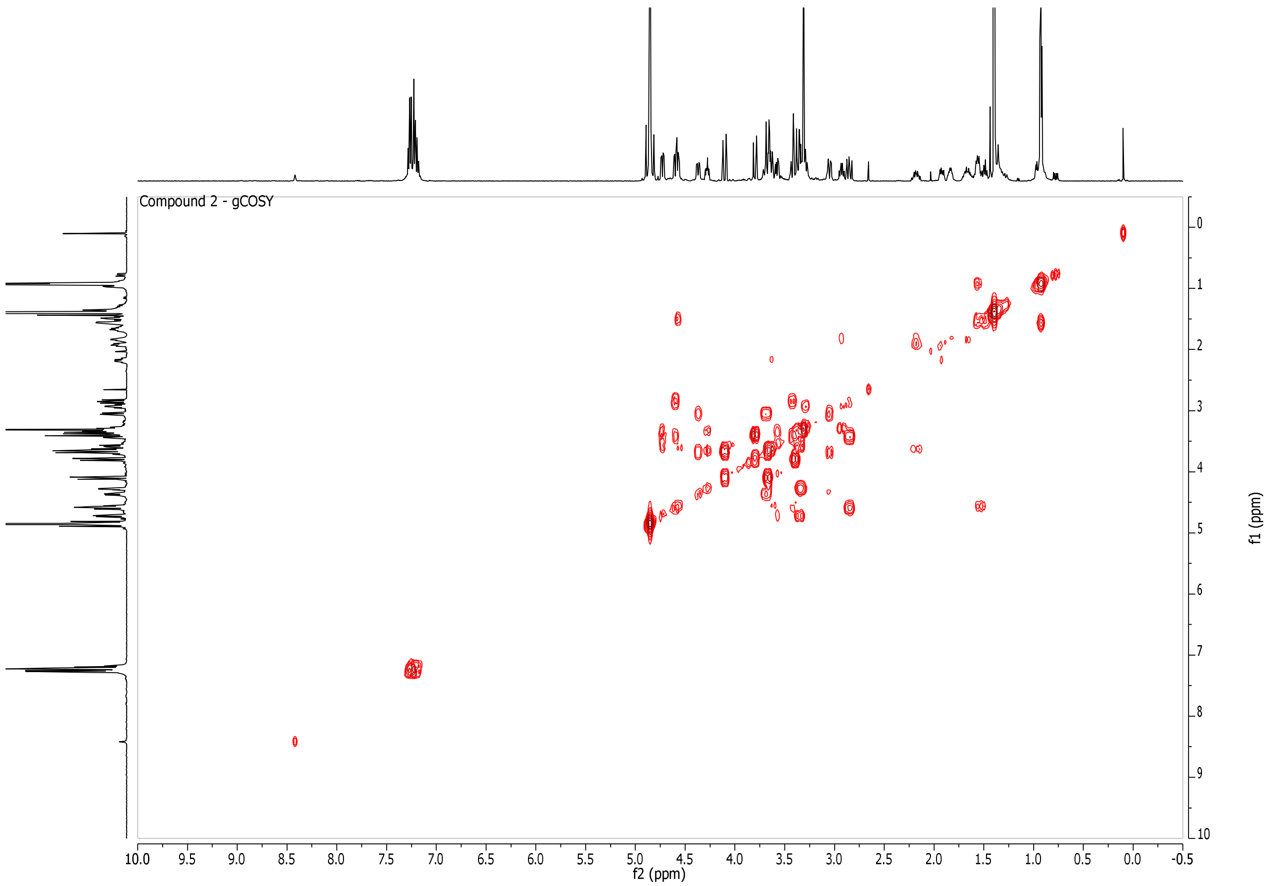

PC(Acm)LFC(Acm)-N3 - gCOSY
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
f1 (ppm)

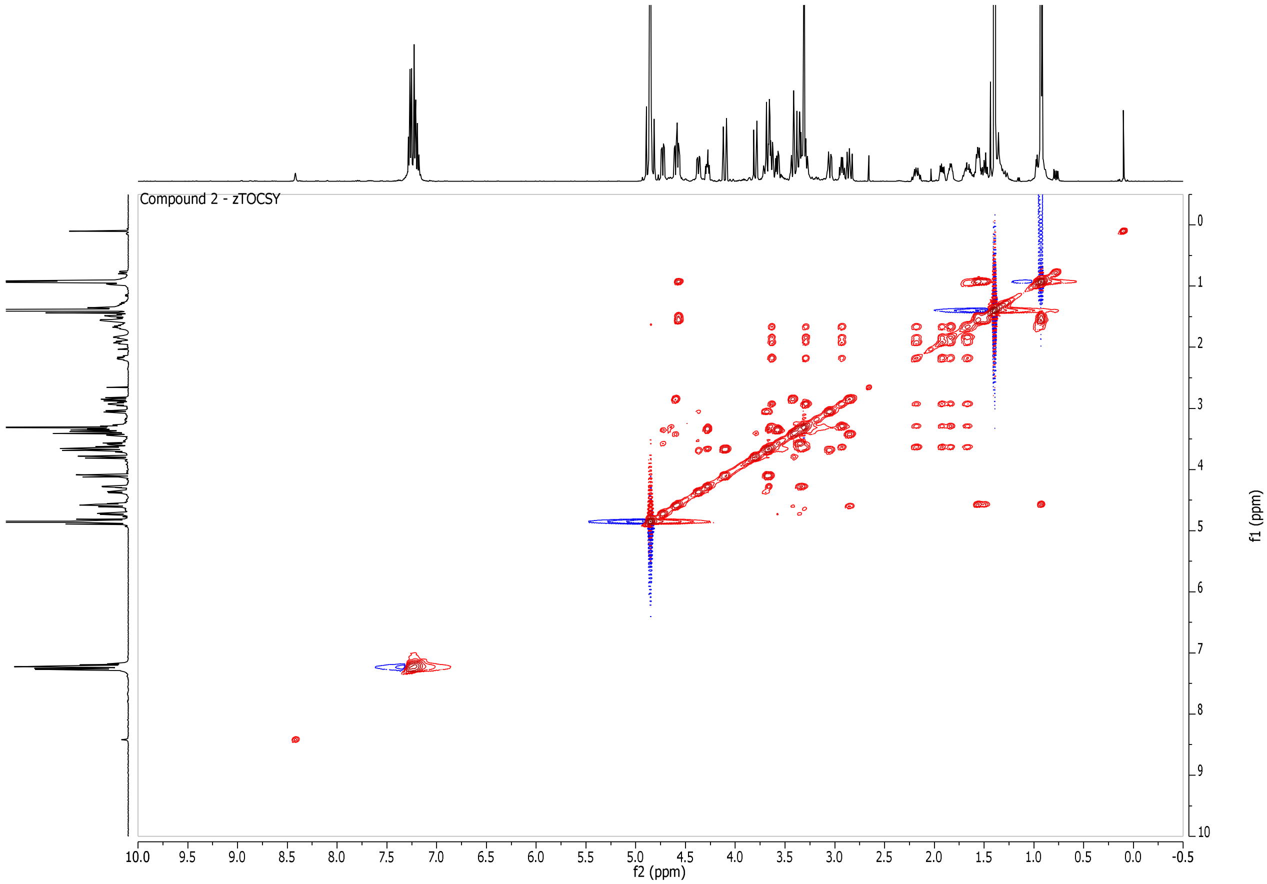

PC(Acm)LFC(Acm)-N3 - zTOCSY
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)
f1 (ppm)

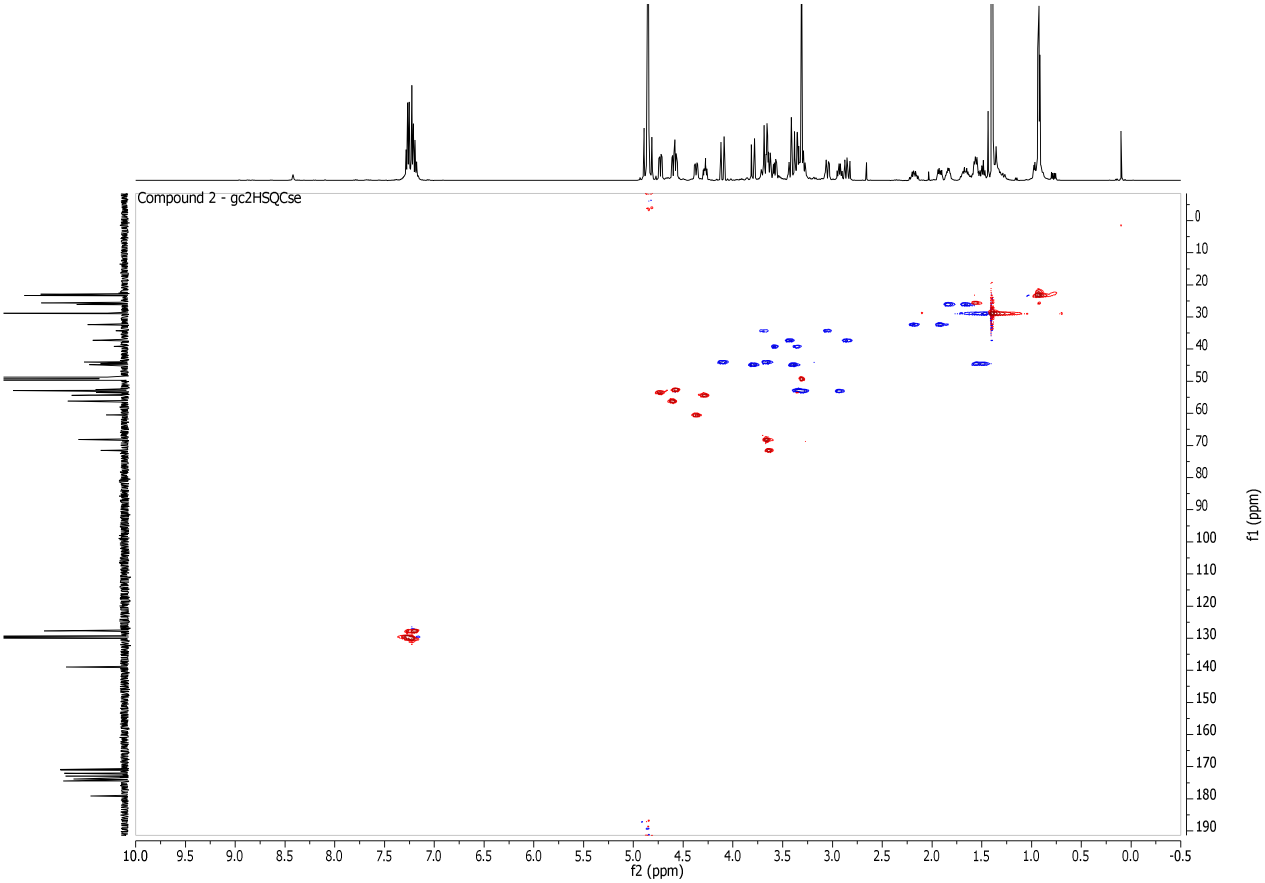

PC(Acm)LFC(Acm)-N3 - gc2HSQCse
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
f1 (ppm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f2 (ppm)

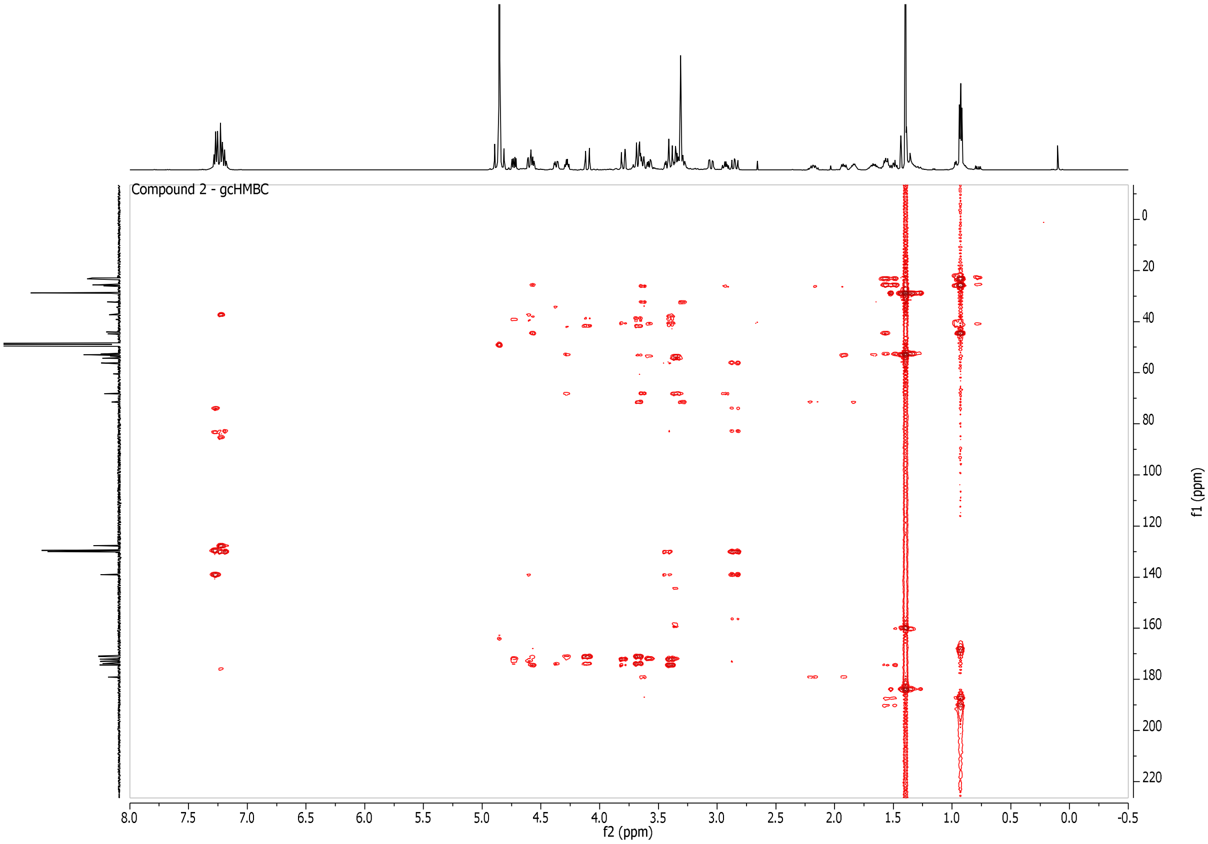

Compound 2 - 1H NMR
2
6.30
1.00
0.92
0.92
0.97
0.91
0.84
0.98
1.09
0.95
1.10
1.01
4.38
0.95
0.93
0.97
0.81
0.83
0.95
1.33
1.79
1.01
8.82
5.98
f1 (ppm)

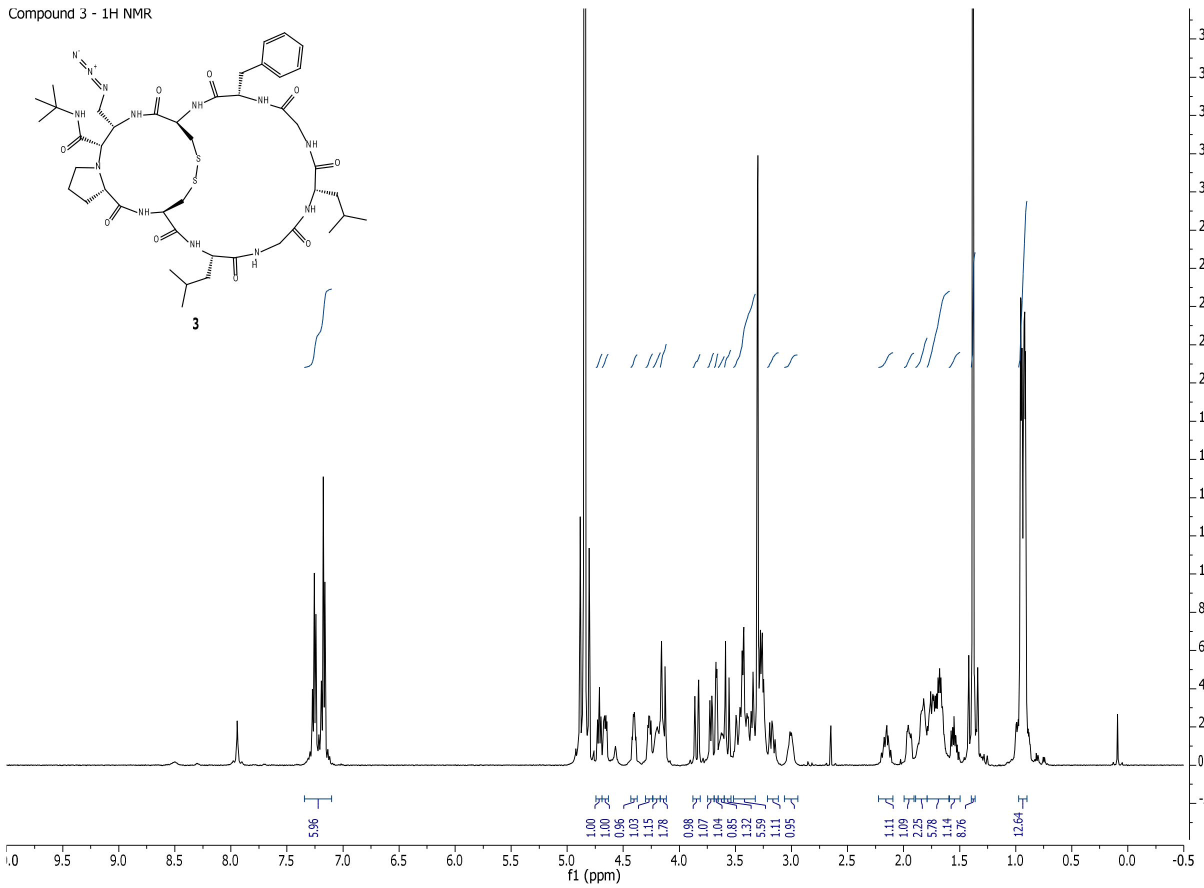

Compound 2 - 13C NMR
2
f1 (ppm)

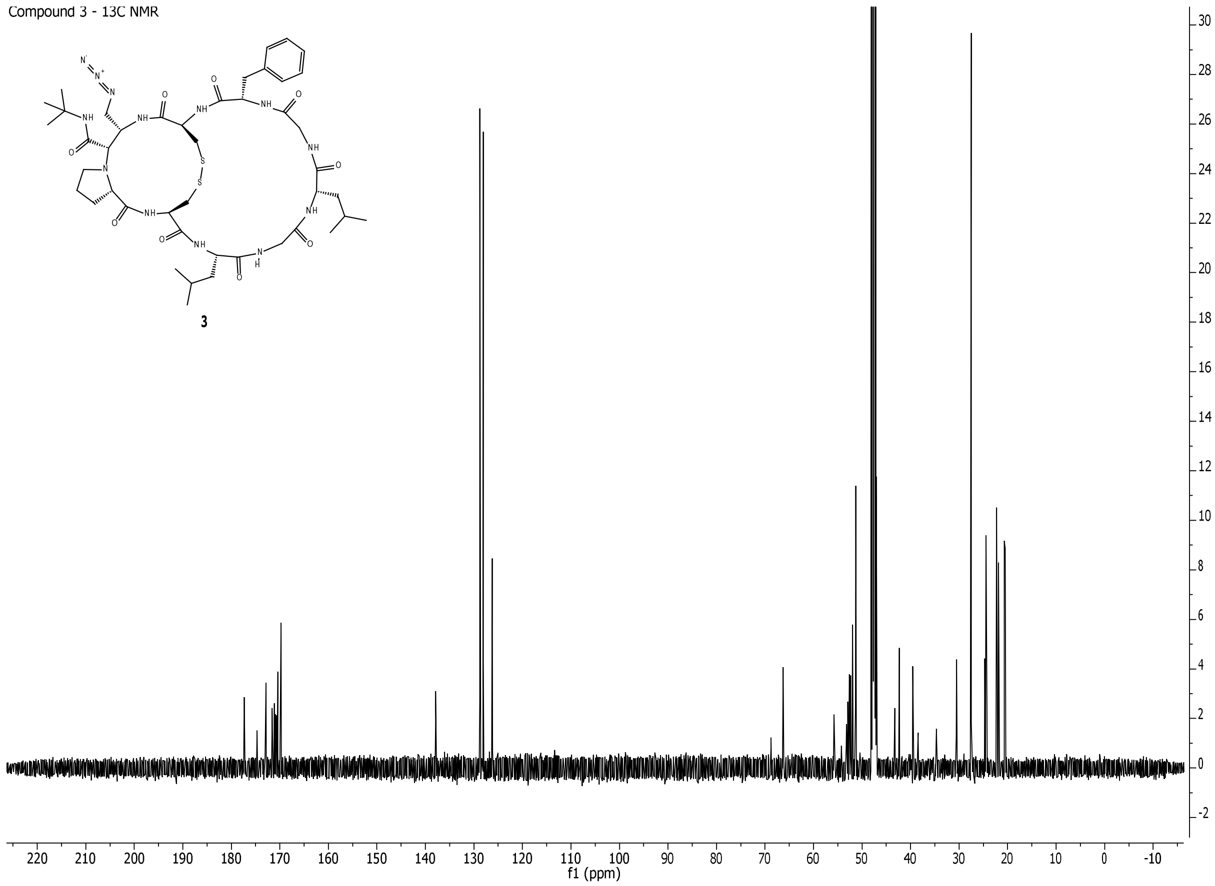

Compound 2 - gCOSY
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)
0
1
2
3
4
5
6
7
8
9
10
f1 (ppm)

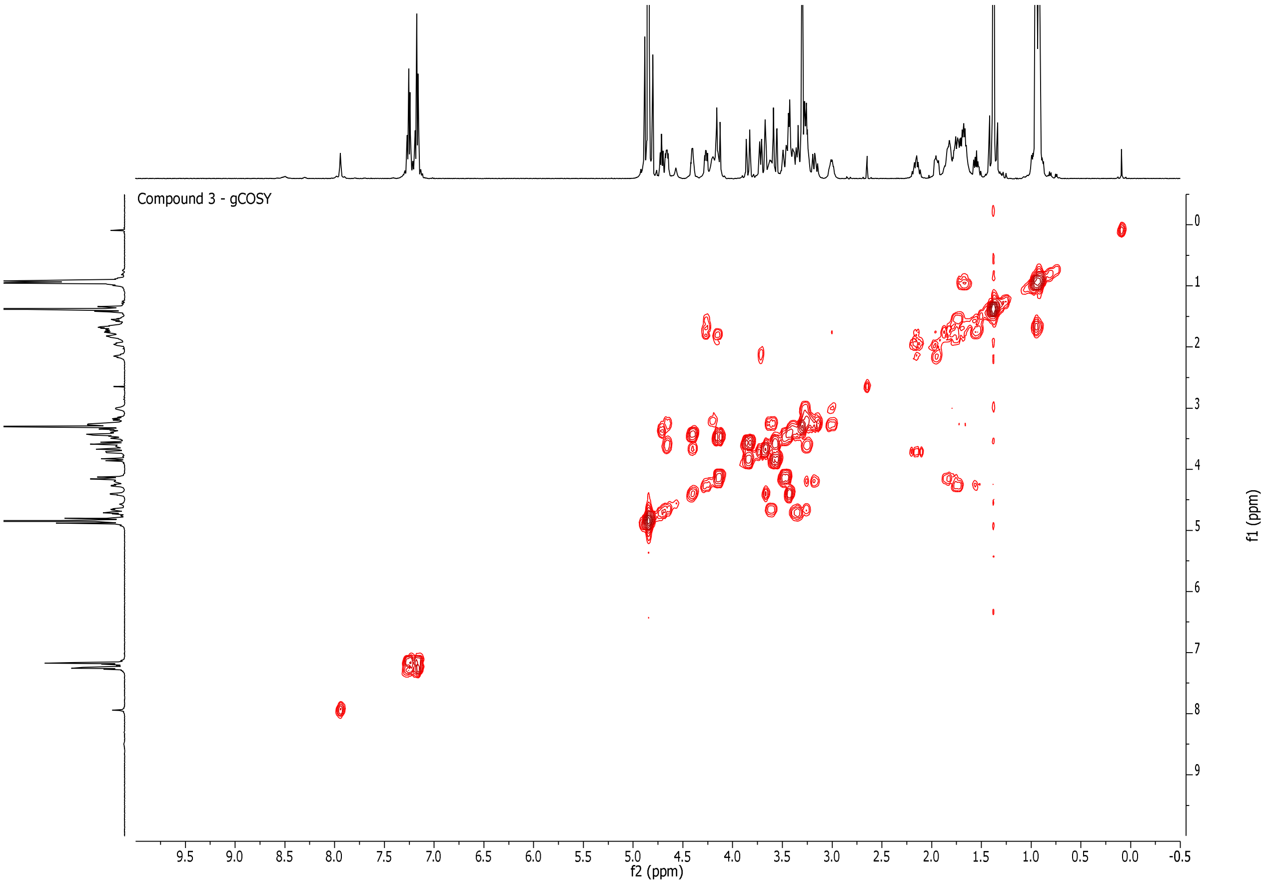

Compound 2 - zTOCSY
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)
0
1
2
3
4
5
6
7
8
9
10
f1 (ppm)

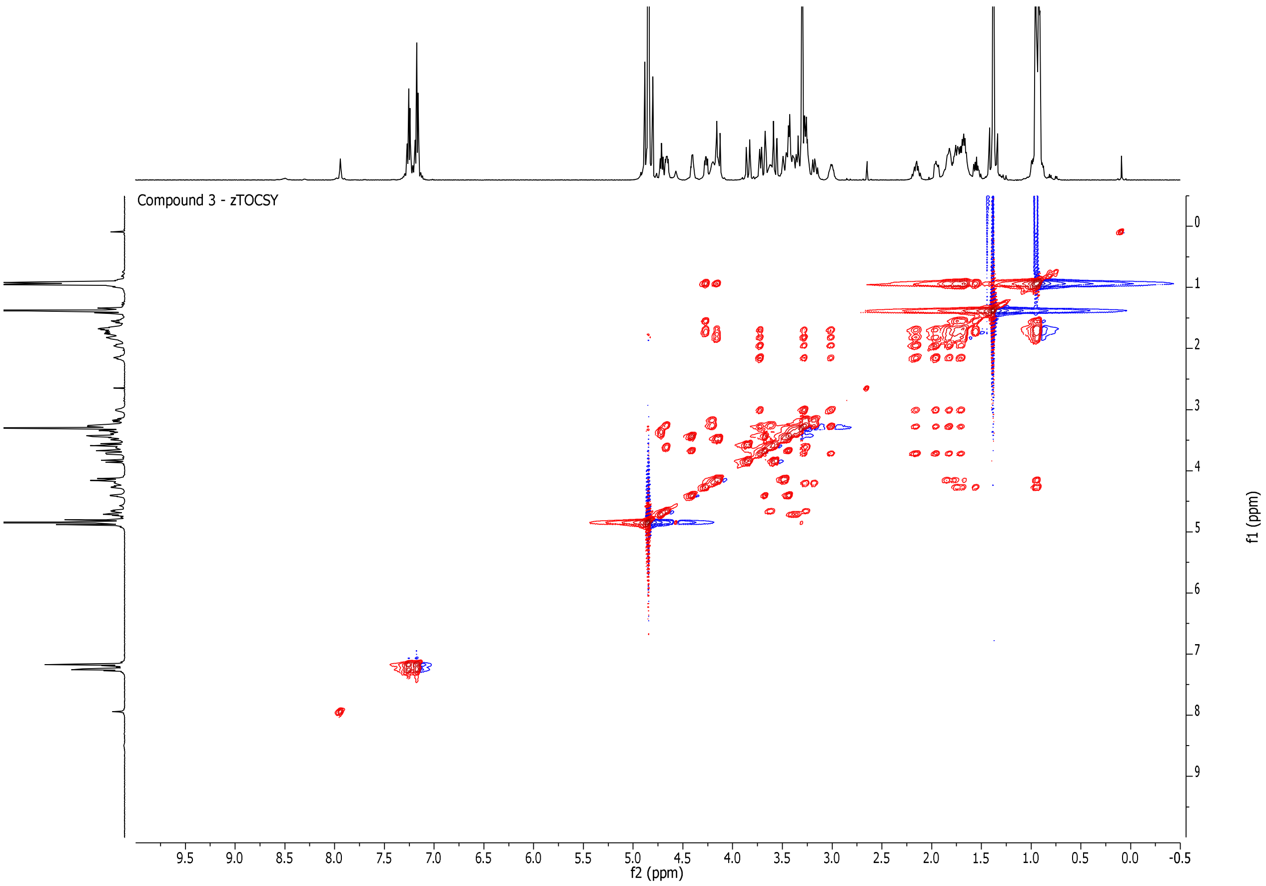

Compound 2 - gc2HSQCse
f1 (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)

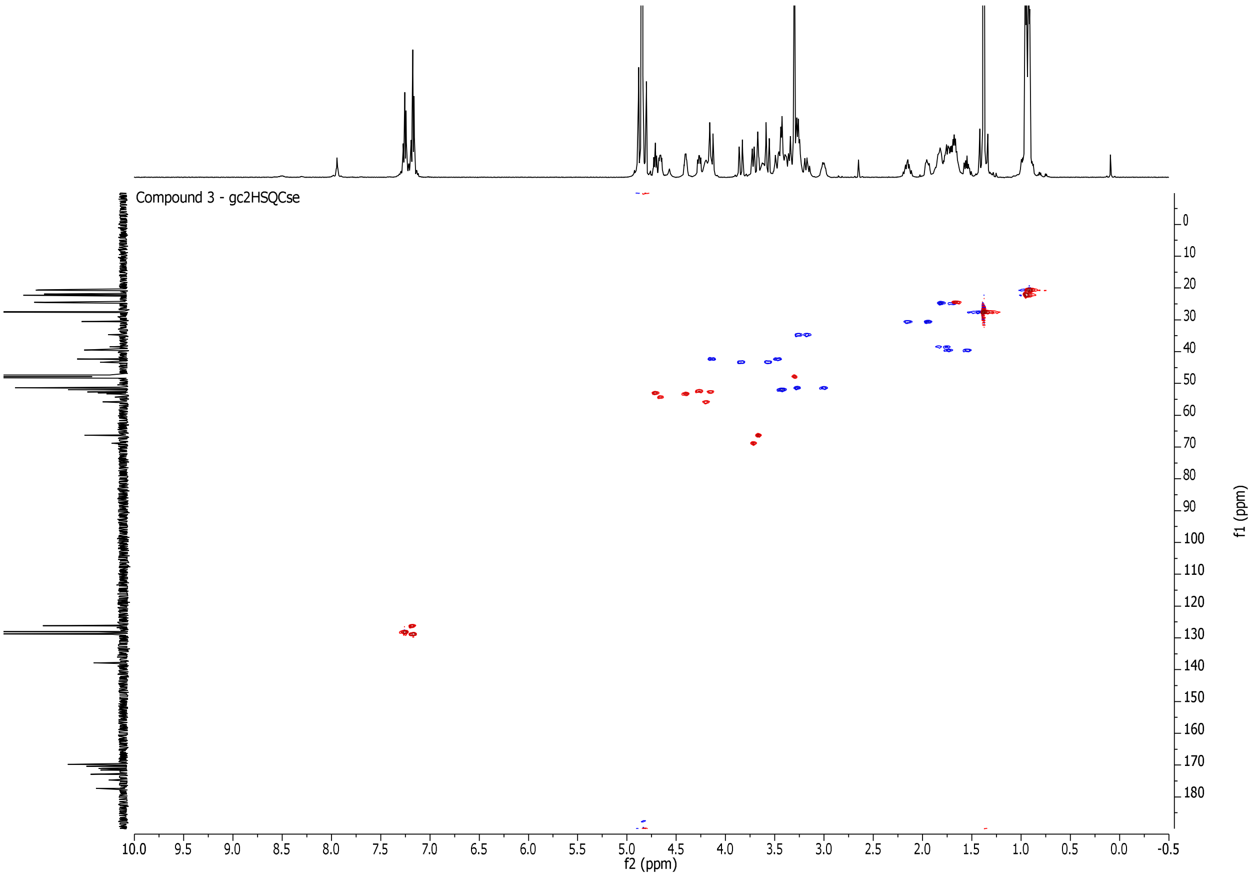

Compound 2 - gcHMBC
f1 (ppm)
0
20
40
60
80
100
120
140
160
180
200
220
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)


Compound 3 - 1H NMR
3
5.96
1.00
1.00
0.96
1.03
1.15
1.78
0.98
1.07
1.04
0.85
1.32
5.59
1.11
0.95
1.11
1.09
2.25
5.78
1.14
8.76
12.64
10.0 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 -0.5
f1 (ppm)
380 360 340 320 300 280 260 240 220 200 180 160 140 120 100 80 60 40 20 0 -20


Compound 3 - 13C NMR
3
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2


Compound 3 - gCOSY
f1 (ppm)
0
1
2
3
4
5
6
7
8
9
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
-0.5
f2 (ppm)


Compound 3 - zTOCSY
f1 (ppm)
0
1
2
3
4
5
6
7
8
9
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
-0.5
f2 (ppm)


Compound 3 - gc2HSQCse
f2 (ppm)
f1 (ppm)
10.0
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
-0.5
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180