Supplementary Information:

Direct modifications of the cyclic peptide Polymyxin B leading to analogues with enhanced *in vitro* antibacterial activity

Pamela Brown[a,b*], Omar Abdulle[a], Steven Boakes[a], Esther Duperchy[a], Stephen Moss[c], Mona Simonovic[a], Steven Stanway[c], Antoinette Wilson[c], Michael J Dawson[a,b]

[a]Cantab Anti-Infectives Ltd , Welwyn Garden City, AL7 3AX, UK,
[b]Spero Therapeutics, Inc, 675 Massachusetts Avenue, 14th Floor, Cambridge, MA 02139 USA,
[c]Eurofins Integrated Discovery UK Ltd, Fyfield Business & Research Park, Fyfield Road, Ongar, Essex, CM5 0GS, UK.

Table of contents:

Synthesis and characterisation of final compounds 3a, 5a, 5b, 9a, 9b, 10 and 13a-c

All reagents used for chemical synthesis were purchased from commercially available sources and used without further purification. Analytical HPLC was performed on all final compounds on an Agilent 1100 System with a Phenomenex Hyperclone C18 BDS 5 µm (4.6 mm x 150 mm) column, eluted with appropriate water/acetonitrile gradients containing 0.15% TFA, with detection at 210 and 254 nm. All final compounds had purity >90% by HPLC, using the method shown in Table S1. Preparative HPLC was performed on a Gilson preparative HPLC system using a Waters Sunfire C18 OBD 5 µm (19 mm x 150 mm) column eluted with appropriate water/acetonitrile gradients containing 0.15% TFA, with detection at 210 nm. A representative gradient system is given in Table S2. ^{1}H NMR spectra were

recorded at 400 MHz on a Mercury 400 NMR spectrometer (Agilent Technologies) or at 300 MHz on a DPX300 spectrometer (Bruker). Chemicals shifts (δ) are reported in ppm downfield from TMS. Coupling constants J are recorded in Hertz (Hz). Mass spectra were recorded on an LCQ DecaXP mass spectrometer with +ve ion electrospray ionisation. High resolution nanoelectrospray analysis (HRMS (+ve nESI)) was performed on the Thermo LTQ Orbitrap XL using the Advion TriVersa NanoMate.

Table S1: Analytical HPLC conditions

Column:	Phenomenex Hyperclone C18 BDS 5 µm × 4.6 mm × 150 mm	
Mobile phase:	A: water/acetonitrile 90/10, v/v, 0.15% TFA.	
	B: acetonitrile/water 90/10, v/v, 0.15% TFA	
Flow rate:	1 mL/min	
Gradient:	Time (mins)	% mobile phase A
	0	100%
	20	0%
	11	0%
	11.2	100%
Cycle time: 15 mins		
Detection:	210, 254 nm	
Injection volume: 20 µL		

Table S2: Representative Preparative HPLC conditions

Column:	Sunfire C18 OBD 5µm x 30mm x 150mm	
Mobile Phase A:	Acetonitrile + 0.15 %TFA	
Mobile Phase B:	water + 0.15 %TFA	
Flow rate:	25 mL/min	
Gradient:	Time (min)	%mobile phase A
	0	3%
	2	3%
	25	40%
	30	97%
Time	32	97%
Cycle time: 40 min		
Detection: 210 nm		

3a. [(6-(*S*)-methyl)octanoyl]-Dab-Thr-Dab-Cyclo[Dab-Dab-D-(4-Bromo)Phe-Leu-Dab-Dab-Thr] TFA salt

The bromination of Polymyxin B was carried out as described for Compound 5a Step 1 using 200 mg Polymyxin B as starting material. The crude product was purified by preparative HPLC using the method of Table B to afford the title compound (3a) as the TFA salt as a lyophilized solid in 16% yield. m/z 1282 ($[M+H]^+$, 10%), 642 ($[M+2H]^{2+}$, 100%). HRMS (+ve nESI): $[M + H]^+$ calcd for $C_{56}H_{98}BrN_{16}O_{13}$, 1281.6683; found,1281.6724. ^{1}H NMR (300 MHz, D_2O): 0.48-0.55 (4H, m), 0.62 – 0.74 (9H, m), 0.98 – 1.40 (15H, m), 1.40-1.50 (2H, m), 1.71-2.30 (14 H, m), 2.72 – 3.08 (13H, m), 3.20-3.28 (1H, m),4.00 (1H, dd), 4.10-4.22 (7H, m), 4.33-4.45 (4H,m), 7.05 (2H, d, J = 8 Hz), 7.45 (2H, d), J = 8 Hz).

(5a) [(6-(*S*)-methyl)octanoyl]-Dab-Thr-Dab-Cyclo[Dab-Dab-D-(4-phenyl)Phe-Leu-Dab-Dab-Thr] TFA salt

Step 1 . Compound 3a/3b

Polymyxin B sulphate (source: Biotika) (20.0 g, 15.4 mmol) and N-bromosuccinimide (4.2 g, 23.6 mmol) were charged to a 1 L 3-neck round-bottomed flask, fitted with an efficient overhead paddle stirrer and a N2 inlet. To the flask under N2 was added boron trifluoride dihydrate (200 mL) and the mixture was vigorously stirred at ambient temperature for 1h during which time all solids dissolved to give a frothy, orange solution. The solution was then poured over 5 minutes into a stirred mixture of ammonia 880 solution (400 mL) and ice (900 g) to give a white suspension. To the suspension (pH 9) was added water (300 mL) and the mixture was stirred at ambient temperature for 2h then filtered under suction through a large (20 cm diameter, porosity 2) glass sinter funnel. The solid was washed with water (200

mL) and sucked free of excessive moisture. The material was then suspended in methanol (1.5 L)and re-evaporated to a residue. This was repeated with more methanol (1.5 L) to afford a foam which was dried at ambient temperature in vacuo for 3h (22.4 g) and identified as the brominated material 3(a) and (b) m/z = 1282 $[M+ H]^+$, 642 $[M+2H]^{2+}$. The crude material was used without purification in the next stage.

Step 2 Compound 4a/4b

The crude product from step 1 (15.4 mmol nominal amount, based on Polymyxin B sulphate used) was charged to a flask and acetonitrile (400 mL) and water (200 mL) were added. To the stirred solution was added triethylamine (15 mL, 108 mmol), followed by a solution of di-tert-butyl-dicarbonate (23.5 g, 108 mmol) in acetonitrile (200 mL). The cloudy mixture was stirred at ambient temperature for 20 h. The reaction mixture was then concentrated in vacuo and the residue re-evaporated from methanol (1 L) and dried. The dry residue was stirred with a mixture of diethyl ether (75 mL) and iso-hexane (75 mL) for 0.5 h and the insoluble solid was filtered off under vacuum. The solid was partitioned between dichloromethane/methanol (9:1) (400 mL) and 10% brine (300 mL). To the organic extract was added methanol (40 mL) and the solution was washed with 10% brine (100 mL), dried (Na2SO4) and concentrated in vacuo to a foam residue. This material was suspended in 20 dichloromethane/methanol (95:5) (140 mL) and left to stand for 0.5h. The mixture was filtered under suction to remove unwanted gelatinous solid and the filtrate was purified by column chromatography over silica gel, eluting with a gradient of dichloromethane/methanol to afford the title compound as a colourless foam (5.1 g) m/z 1782 $[M+H]^+$. This partly purified material containing approx. 6:1 ratio of 4-bromo to 2-bromo isomers (See Compound 9a Step 1) was used directly in the next stage.

Step 3:

To a solution of the crude product from Step 2 (100mg, 0.056 mmol) in anhydrous DMF (4ml) was added phenylboronic acid (17 mg, 0.14 mmol), PdCl2(Ph3P)2 (10 mg, 0.014 mmol). The solution was purged with Nitrogen, and then treated with 2M aqueous sodium carbonate solution (which had been purged with Nitrogen) . The mixture was sonicated for 1 minute then heated to 120 deg C in a microwave with stirring for 30min. After cooling the mixture was diluted with DCM (15ml) , and washed with saturated sodium bicarbonate solution . The phases were separated and the aqueous layer was further extracted with DCM (15ml). The combined organics were dried (Na_2SO_4) and the solvent evaporated to give the crude product. This was dissolved in DCM (4 ml) and treated with TFA (1ml). The solution was stirred at room temperature for 5h. The reaction mixture was treated with toluene and evaporated to an orange oil. This crude material was chromatographed by preparative HPLC using the conditions of Table 2.

Product-containing fractions were combined and lyophilised to afford **Compound 5a** as the TFA salt (10.7 mg, 10%) . m/z 1279 ($[M+H]^+$, 10%), 640 ($[M+2H]^{2+}$, 100%). ^{1}H NMR (300 MHz, D_2O): 0.45-0.58 (7H, m), 0.62 – 0.68 (6H, m), 0.98 – 1.40 (15H, m), 1.50-1.58 (2H, m), 1.75-2.30 (14 H, m), 2.78 – 3.15 (13H, m), 3.22-3.34 (1H, m), 4.05 (1H, dd), 4.10 - 4.25 (6H, m) 4.28 (1H, d), 4.40-4.50 (4H,m), 7.28 (2H, d, J = 8 Hz), 7.38-7.43 (1H, m), 7.44 – 7.50 (2H, m), 7.62 – 7.68 (4H, m).

(5b) **[(6-(*S*)-methyl)octanoyl]-Dab-Thr-Dab-Cyclo[Dab-Dab-D-{4-(4-pyridyl)}Phe-Leu-Dab-Dab-Thr] TFA salt**

To a solution of the crude product from **compound 5a Step 2** (100mg, 0.056 mmol) in anhydrous DMF (4ml) was added 4-pyridyl boronic acid (17 mg, 0.14 mmol), $PdCl_2(Ph_3P)_2$ (10 mg, 0.014 mmol). The solution was purged with Nitrogen, and then treated with 2M aqueous sodium carbonate solution (which had been purged with Nitrogen) . The mixture was sonicated for 1 minute then heated to 120 deg C in a microwave with stirring for 30min. After cooling the mixture was filtered through Celite, the filter pad washed with DMF (2ml), MeOH (10ml), followed by DCM (10ml) , the filtrate diluted with toluene and evaporated under reduced pressure. The residue was re-dissolved in toluene, evaporated and left under vacuum for 3h. The resultant solid was triturated with DCM/MeOH (4:1) (2x 5ml) and filtered through celite. The filtrate was evaporated under reduced pressure to afford the crude product. This was dissolved in DCM (12 ml) and treated with TFA (3ml) . The solution was stirred at room temperature for 6h. The reaction mixture was evaporated and the crude product was chromatographed by preparative HPLC using the conditions of Table 2. Product-containing fractions were combined and concentrated, followed by lyophilization to afford the title compound as the TFA salt as a white solid (13mg, 7%).

m/z 1281 ($[MH]^+$, 10%), 641 ($[M+2H]^{2+}$, 100%). ^{1}H NMR (300 MHz, D_2O): 0.35-0.58 (7H, m), 0.70 – 0.78 (6H, m), 0.98 – 1.40 (15H, m), 1.48-1.55 (2H, m), 1.78 -2.28 (14 H, m), 2.78 – 3.15 (12H, m), 3.22-3.38 (2H, m), 4.05 (1H, dd), 4.12 - 4.28 (6H, m), 4.30 (1H, d), 4.38-4.58 (4H,m), 7.38 (2H, d, J = 8 Hz), 7.86 (2H, d, J = 8 Hz), 8.10-8.15 (2H, m), 8.62-8.66 (2H, m).

9a [(2*S*)-1-(2-Methylpropyl)piperazine-2-carbonyl]-Thr-Dap-Cyclo[Dab-Dab-D-(4-phenyl)Phe-Leu-Dab-Dab-Thr] TFA salt.

Step 1. Compound 6(a) and 6(b)

A suspension of partially purified **compound 4**, (8.3 g, 4.7 mmol) in 1,4-butanediol (160 mL) was stirred at 50°C for 1h until a thick solution was formed. Phosphate buffer solution (pH 8) (19 mL) was added and the stirred solution was cooled to 37°C. Savinase solution (Protease from Bacillus sp. Liquid >16U/g, from Sigma Aldrich) (7ml) was added and the viscous solution was stirred at 37°C for 5 days. The solution was poured into a mixture of ethyl acetate (400 mL) and water (400 mL) and the whole was shaken vigorously. The aqueous layer was re-extracted with ethyl acetate (150 mL) and the combined organic extracts were re-washed with water (2 x 200 mL), dried (Na_2SO_4) and evaporated in vacuo to afford a white foam (7.4 g). This material was dissolved in ethyl acetate/methanol (4:1) (30 mL) and the solution purified by column chromatography over silica gel eluting with a gradient of Solvent A/ethyl acetate (0-60%) where Solvent A = methanol/ammonia .880 solution (9:1). Relevant fractions were pooled and evaporated to a white solid (3.48g). This was subject to further purification by preparative HPLC (see Table S2). Fractions containing the major compound were combined and evaporated to yield **compound 6a,** the desired (4-bromo)phenylalanine polymyxin heptapeptide as a white solid (1.46g, 27%). m/z 1140 $[M+H]^+$. HPLC retention time (method of Table S1) 7.2 min. ^{1}Hnmr was consistent with the desired product (see spectrum on p S37).

Fractions containing the minor product were pooled and evaporated to yield **compound 6b** isomer assigned as the 2-bromo regioisomer based on ^{1}Hnmr signals in the aromatic region (see spectrum on p S39) (0.24g, 4.5%). m/z 1140 $[M+H]^+$. HPLC retention time (method of Table S1) 7.1 min.

Step 2 . Compound 7

The 4-bromo(Phenylalanine)heptapeptide from Step 1 (500mg, 0.438 mmol) and the dipeptide CBZThr(OtBu)Dap(BOC)-OH (217 mg, 0.438 mmol) were dissolved in DCM (10ml) and treated with diisopropylethylamine (0.229 ml, 1.314 mmol) followed by HATU (217 mg, 0.438 mmol). The reaction mixture was stirred at room temperature for 18h. The solvent was evaporated, and the residue stirred with water for 3h. The resultant solid was collected by filtration and dried in vacuo for 18h, to afford the desired coupled product as a white solid (765 mg, quant) m/z 1642 ($[M+ Na]^+$, 100%), 1618 ($[M+H]^+$, 50%).

Step 3. Compound 8a

To a solution of Compound 7 (646mg, 0.4mmol) was added phenylboronic acid (63 mg, 0.52 mmol), palladium (II) acetate (9 mg, 0.04 mmol), XPhos (39 mg, 0.08mmol) and potassium phosphate tribasic (169 mg, 0.8 mmol) in toluene (13 mL) and the stirred mixture was degassed with nitrogen for 2 minutes. The reaction was sealed and heated to 100°C for 21 hours. After cooling the mixture was diluted with EtOAc partitioned with 50:50 water:saturated brine. The mixture was filtered through Celite and washed through with ethyl acetate. The phases were separated and the aqueous layer was further extracted with EtOAc (60ml). The combined organics were dried ($MgSO_4$) and the solvent evaporated. The crude product was purified by chromatography: 40 g column, using a gradient of 0 to 5% MeOH in EtOAc to afford the desired compound as a colourless foam 320 mg, 50%, m/z 1616 ($[MH]^+$, 20%), 708 ($[M+2H]^{2+}$ - BOC, 100%, -ve ion ESI 1614 $[M-H]^-$

Step 4. Compound 8b

The CBZ protected compound 8a (306 mg, 0.189 mmol) was dissolved in methanol (12ml) and treated with ammonium formate (394 mg, 6.2 mmol) and 10% Pd/C (120mg). The

reaction mixture was stirred at room temperature under nitrogen for 5.5 h. The catalyst was removed by filtration, the filter pad washed with methanol and the filtrate evaporated. The residue was dissolved in ethyl acetate containing 20% methanol and washed with water. The aqueous layer was further extracted with 20% methanol in ethyl acetate . The organic extracts were dried, evaporated and the solvent evaporated to afford the desired product as a colourless foam (261 mg, 93%) . m/z 1482 $[MH]^+$

Step 5 Compound 9a, title compound.

Compounds 8b, the Boc protected nonapeptide from step 4 (80 mg, 0.054mmol) was dissolved in dichloromethane (4 mL) and DMF (0.8mL) , and treated with (*2S*)-4-(tert-butoxycarbonyl)-1-(2-methylpropyl)piperazine-2-carboxylic acid (17 mg, 1.1 equiv) and N,N-diisopropylethylamine (28 uL, 3.0 equiv.), followed by HATU (22 mg, 1.1 equivalent). After 16 h completion of the reaction was confirmed by LCMS and the reaction mixture was evaporated to dryness, the residue dissolved and the solution re-evaporated. The residue was dried in vacuo for 1h. Water (15mL) was added and the mixture stirred vigorously for 2 h. The resultant precipitate was collected by filtration and dried in vacuo overnight. This crude Boc-protected material was dissolved in dichloromethane (4 mL) and treated with TFA (1 mL). The reaction mixture was stirred at room temperature for 16h until LCMS confirmed complete deprotection. The solvent was evaporated and the residue chromatographed by preparative HPLC using the conditions of Table 2. Product-containing fractions were combined, evaporated to low volume, and lyophilised to afford **Compound 9a** as the TFA salt as a white solid (22 mg, 26%). m/z 1194 $[M+H]^+$, 598 $[M+2H]^{2+}$, 399 $[M+3H]^{3+}$. HRMS (+ve nESI): $[M + H]^+$ calcd for $C_{57}H_{93}N_{16}O_{12}$, 1193.7159; found, 1193.7183. ^{1}H NMR (300 MHz, D_2O): 0.48-0.55 (7H, m), 0.78 – 0.84 (6H, m), 1.10 – 1.18 (8H, m), 1.20-1.38 (2H, m),

1.71-2.20 (9 H, m), 2.72 – 2.86 (2H, m), 2.50 – 2.58 (1H, m), 2.84 – 3.28 (16H, m), 3.35 – 3.48 (3H, m), 4.00 (1H, dd), 4.10-4.20 (6H, m), 4.36 (1H, d), 4.40 – 4.50 (2H,m), 7.25 (2H, d, J = 8 Hz),), 7.34-7.38 (1H, m), 7.40 – 7.48 (2H, m), 7.58 – 7.65 (4H, m).

9b [(trans)-5-(2-Methylpropyl)piperidine-3-carbonyl]-Thr-Dap-Cyclo[Dab-Dab- D-(4-phenyl)Phe -Leu-Dab-Dab-Thr] diastereomer 2

The Boc protected nonapeptide **8b** (120 mg, 0.081mmol) was coupled to *trans*- 1-(tert-butoxycarbonyl)-5-(2-methylpropyl)piperidine-3-carboxylic acid under the conditions described for **9a Step 5**. The crude coupled product was deprotected with TFA/DCM as previous described. Preparative HPLC using the conditions of Table 2 served to separate the diastereomers. Fractions containing the slower-eluting diastereomer were combined and lyophilized to a white solid (11.5 mg). m/z 1193 $[M+H]^+$, 597 $[M+2H]^{2+}$, 398 $[M+3H]^{3+}$. HRMS (+ve nESI): $[M + H]^+$ calcd for $C_{58}H_{94}N_{15}O_{12}$, 1192.7235; found, 1192.7206. ^{1}H NMR (300 MHz, D_2O): 0.43-0.55 (7H, m), 0.74 – 0.78 (6H, m), 1.05 – 1.12 (8H, m), 1.20-1.35 (2H, m), 1.50 – 1.65 (2H, m), 1.75 -2.20 (10 H, m), 2.60 (1H, t), 2.82 – 3.32 (14H, m), 3.40 – 3.48 (2H, m), 4.04 (1H, dd), 4.08-4.28 (6H, m), 4.36 (1H, d), 4.42 – 4.50 (2H,m), 7.25 (2H, d, J = 8 Hz), 7.30-7.36 (1H, m), 7.40 – 7.48 (2H, m), 7.58 – 7.65 (4H, m).

(10) . [(6-(*S*)-methyl)octanoyl]-Dab-Thr-Dab-Cyclo[Dab-Dab-DCha-Leu-Dab-Dab-Thr] TFA salt

A suspension of platinum oxide (20 mg, 0.088 mmol) in acetic acid (2 mL) was added to a stirred solution of polymyxin B sulphate (ex Biotika, 200 mg, 0.142 mmol) in acetic acid (20 mL). The reaction was hydrogenated for 24 h at ambient temperature and atmospheric pressure. A further 180 mg platinum oxide was added portionwise during the course of the

reaction. After 24h, the reaction mixture was filtered through Celite and washed with water (100 mL). The filtrate was evaporated at reduced pressure to leave a beige solid. The solid was dissolved in water (2 mL) and purified by preparative HPLC using the method of Table 2. Product containing fractions were combined and lyophilised to afford the title compound as a white fluffy solid as the penta-TFA salt (72 mg, 29%). m/z 1209.8 $[M+H]^+$. HRMS (+ve nESI): $[M + H]^+$ calcd for $C_{56}H_{105}N_{16}O_{13}$, 1209.8047; found,1209.8080. ^{1}H NMR (400 MHz, D_2O): 0.63-0.82 (14H, m), 0.90 – 1.16 (17H, m), 1.35 -1.56 (12H, m), 1.64 -2.16 (14 H, m), 2.78 – 3.06 (11H, m), 3.10 – 3.21 (1H, m), 4.03-4.19 (8H, m), 4.30 – 4.33 (2H, m), 4.35 – 4.40 (1H, m)

13a [4-Amino-3-cyclohexylbutanoyl]-Thr- Dap-Cyclo[Dab-Dab-DCha-Leu-Dab-Dab-Thr] diastereomer 1, TFA salt

Step 1 (compound 12)

A mixture of Thr(O-tBu)-Dap(BOC)-Cyclo[Dab-Dab(BOC)-DPhe-Leu-Dab(BOC)-Dab(BOC)-Thr] (BOC protected Dap-3 polymyxin nonapeptide) (our ref) (1.0g, 0.70 mmol) and platinum oxide (333mg) in glacial acetic acid (30mL) was hydrogenated for 48 h at ambient temperature and atmospheric pressure. After this time, the reaction mixture was filtered through Celite and washed with glacial acetic acid (20 mL). The filtrate was diluted with toluene (100ml) and evaporated at reduced pressure. The residue was azeotroped with toluene to leave a gum which was dried in vacuo at room temperature. The residue was dissolved in ethyl acetate (50 mL) and the solution stirred with Ambersep 900 (OH) (5g) resin for 0.5h to regenerate the free amine, then filtered and the resin washed with ethyl acetate (15ml). The filtrate was evaporated and dried in vacuo for 18h to leave the desired product as a colourless foam (970mg, 97%) m/z 1411.5 $[M+H]^+$.

Step 2

Coupling the nonapeptide from Step 1 to the N-terminal acid 4-[(tert-Butoxycarbonyl)amino]-3-cyclohexylbutanoic was carried out as described for Compound 9a step 5. followed by TFA deprotection . The diastereomers of the final product were separated by preparative HPLC. Fractions containing the faster-eluting diastereomer were combined and lyophilized to a white solid. m/z 1122.7 ($[M+H]^+$,10%), 562 ($[M+2H]^{2+}$, 100%). ^{1}H NMR (400 MHz, D_2O): 0.69 – 0.81 (8H, m), 0.83 – 1.10 (15H, m), 1.21-1.31 (1H, m), 1.38 – 1.59 (15H, m), 1.59 – 1.75 (1H, m), 1.76 – 2.16 (8H, m), 2.26 (1H, dd, J = 8, 16Hz), 2.41 (1H, dd, J = 4, 16 Hz), 2.70 – 3.03 (10H, m), 3.07 – 3.22 (2H, m), 3.27 – 3.35 (1H, m), 4.01 – 4.15 (7H, m), 4.19-4.25 (2H, m), 4.36 (1H, dd, J = 4, 8 Hz)

13b (4-Amino-3-benzylbutanoyl)-Thr- Dap-Cyclo[Dab-Dab-DCha-Leu-Dab-Dab-Thr] diastereomer 1, TFA salt.

Coupling of the nonapeptide 12 from 13a step 1 (170 mg) to 3-{[(tert-butoxycarbonyl)amino]methyl}-4-phenylbutanoic acid was carried out under the conditions of 9a step 5, followed by deprotection with TFA. The diastereomers of the final product were separated by preparative HPLC. Fractions containing the faster-eluting diastereomer were combined and lyophilized to a white solid (26 mg). m/z 1131 $[M+H]^+$, 566 $[M+2H]^{2+}$. ^{1}H NMR (300 MHz, D_2O): 0.79 – 0.90 (8H, m), 1.05 – 1.12 (10H, m), 1.50 – 1.65 (8H, m), 1.75 -2.22 (10 H, m), 2.35 - 2.48 (3H,m), 2.61 (1H, dd), 2.78 (1H, dd), 2.85 – 3.28 (12H, m), 3.42 (1H, dd), 4.12-4.28 (8H, m), 4.30 – 4.38 (1H, m), 4.45 (1H,dd), 7.18 – 7.32 (5H,m).

13c (4-Amino-3-benzylbutanoyl)-Thr- Dab-Cyclo[Dab-Dab-DCha-Leu-Dab-Dab-Thr] diastereomer 1, TFA salt.

Step 1.

A mixture of Thr(O-tBu)-Dab(BOC)-Cyclo[Dab-Dab(BOC)-DPhe-Leu-Dab(BOC)-Dab(BOC)-Thr] (1.71g, 1.2 mmol) and platinum oxide (300mg, 1.32 mmol) in acetic acid (60ml) was stirred under an atmosphere of hydrogen for 17h. The rection mixture was filtered through Celite, washing with acetic acid (40 ml). The resulting filtrate was evaporated to dryness. Toluene (30ml) was added and the mixture evaporated to dryness, and the process repeated . The residue was dissolved in ethyl acetate (100ml) and treated with Ambersep 900 (OH) resin (15g) to remove residual acetate. The resin was removed by filtration and the resulting solution evaporated to dryness to afford the reduced material as a white solid (1.28g, 75%). m/z 1426 $[M+H]^+$.

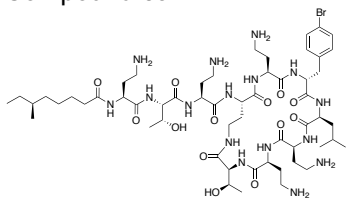
Step 2. **Title compound**

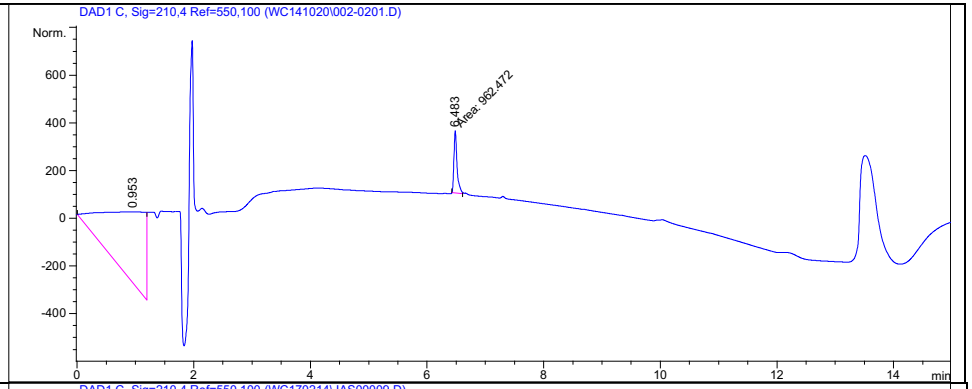
The nonapeptide from Step 1 (620 mg) and 3-{[(tert-Butoxycarbonyl)amino]methyl}-4-phenylbutanoic acid were coupled using the conditions of 9a Step 5, using dichloromethane as solvent. After workup, and precipitation from water, the crude BOC protected material was deprotected with TFA in DCM as described in 9a Step 5. The reaction mixture was evaporated to dryness. The diastereomers of the final product were separated by preparative HPLC. Fractions containing the faster-eluting diastereomer were combined and lyophilized to a white solid (207 mg, 41%). m/z 1146 $[M+H]^+$, 573$[M+2H]^{2+}$. ^{1}H NMR (400 MHz, D_2O): 0.72 – 0.89 (8H, m), 0.96 – 1.18 (10H, m), 1.45 – 1.61 (10H, m), 1.72 -2.22 (10 H, m), 2.27 - 2.46 (3H,m), 2.53 (1H, dd, J = 7.3, 14 Hz), 2.69 (1H, dd, J = 5.8, 14 Hz), 2.86 – 3.03

(12H, m), 3.08 – 3.20 (1H, m), 4.03-4.21 (7H, m), 4.25 – 4.33 (1H, m), 4.35 (1H,dd, J = 5.8, 8.6 Hz), 4.41(1H, dd, J 5.4, 8.6 Hz), 7.15– 7.31 (5H,m).

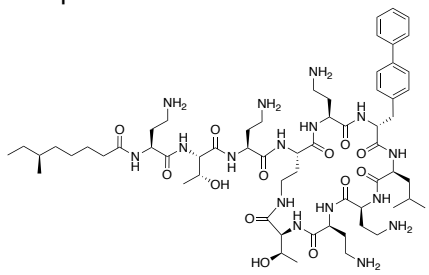
HPLC data for final compounds 3a, 5a, 5b, 9a, 9b, 10 and 13a-c:

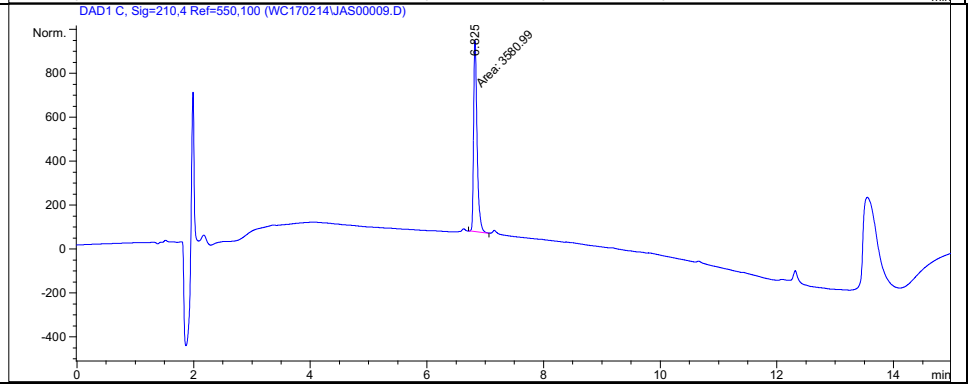
Compound 3a



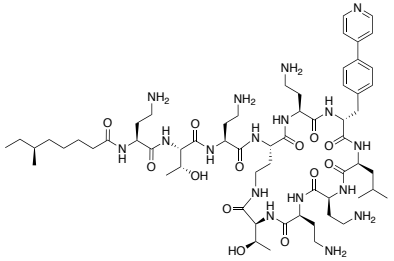


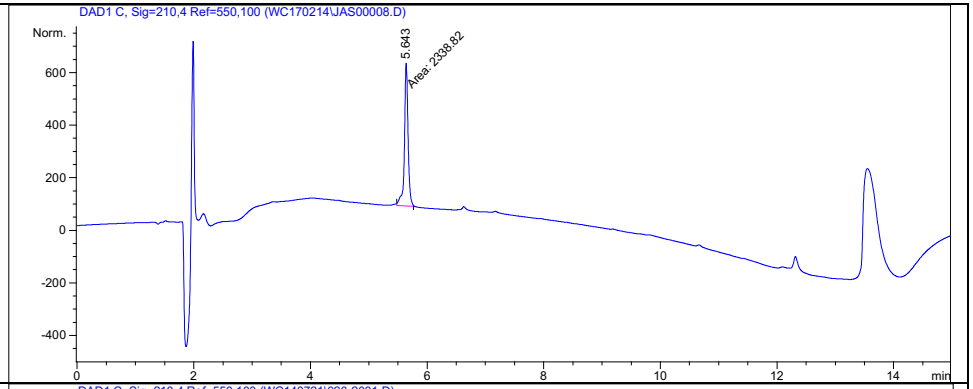
Compound 5a



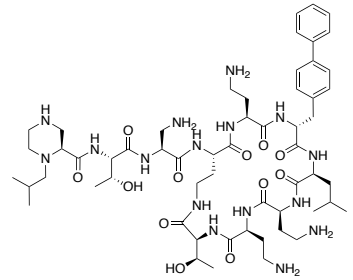


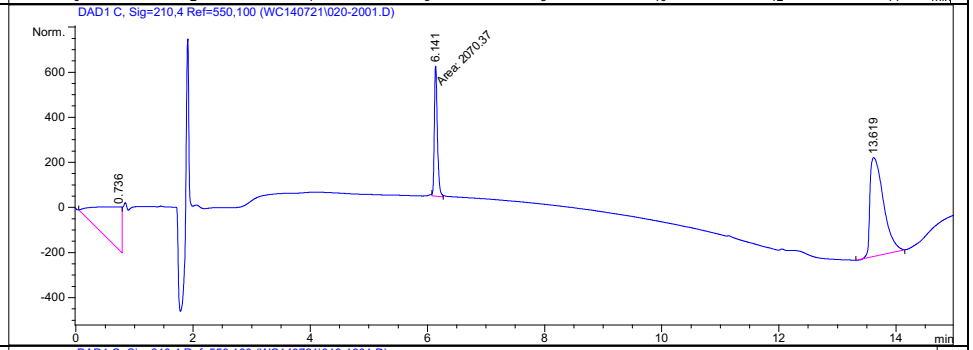
Compound 5b



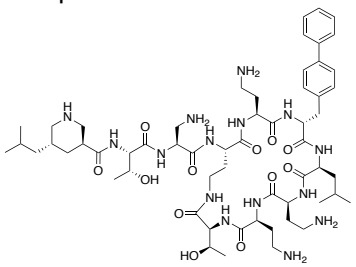


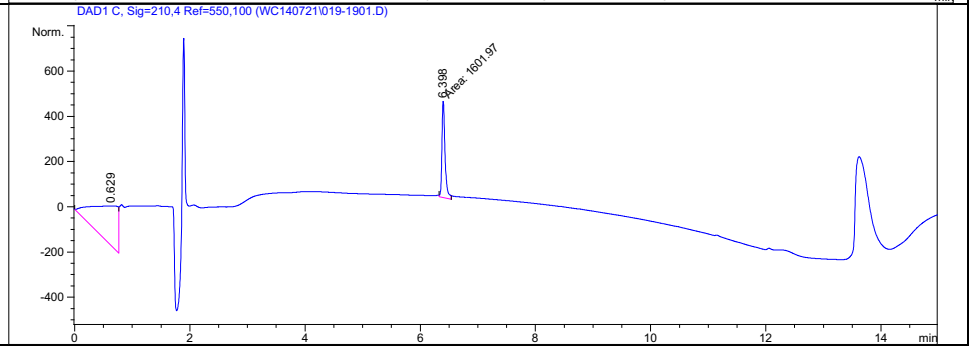
Compound9a



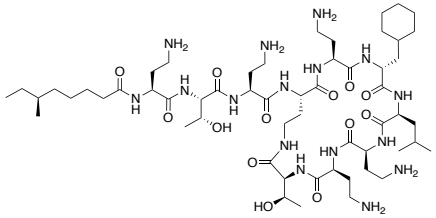


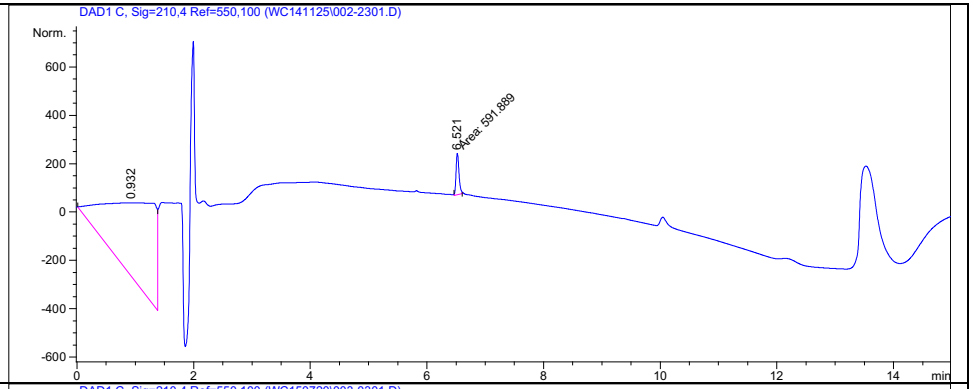
Compound 9b



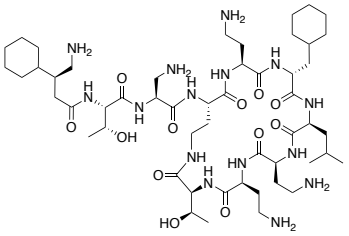


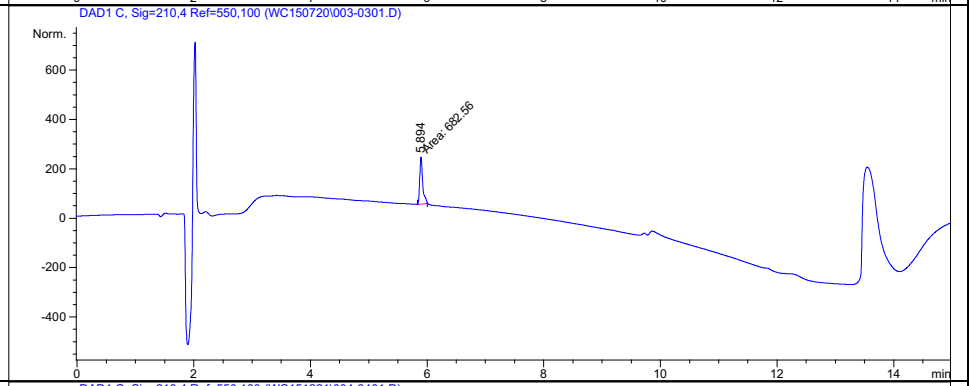
Compound 10



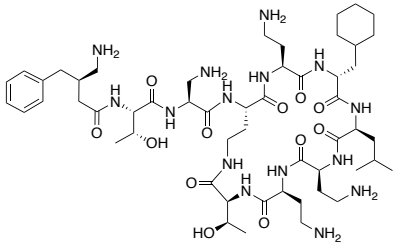


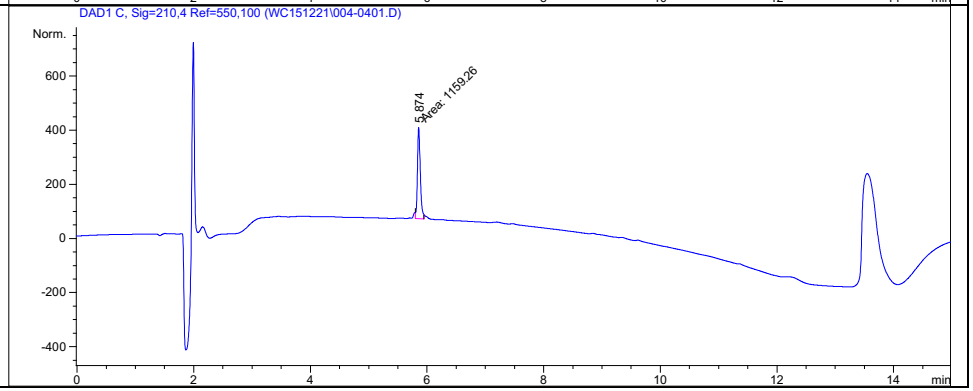
Compound 13a



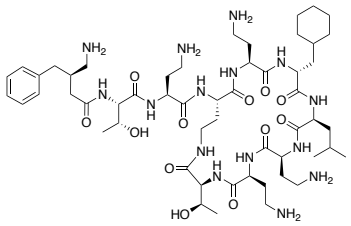


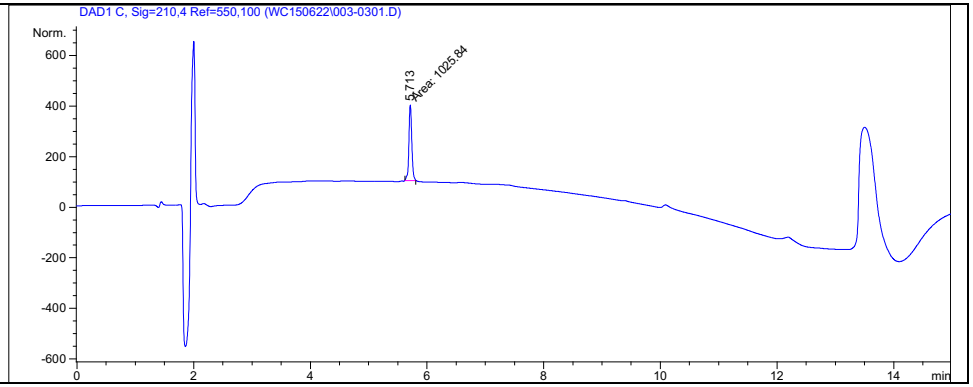
Compound 13b





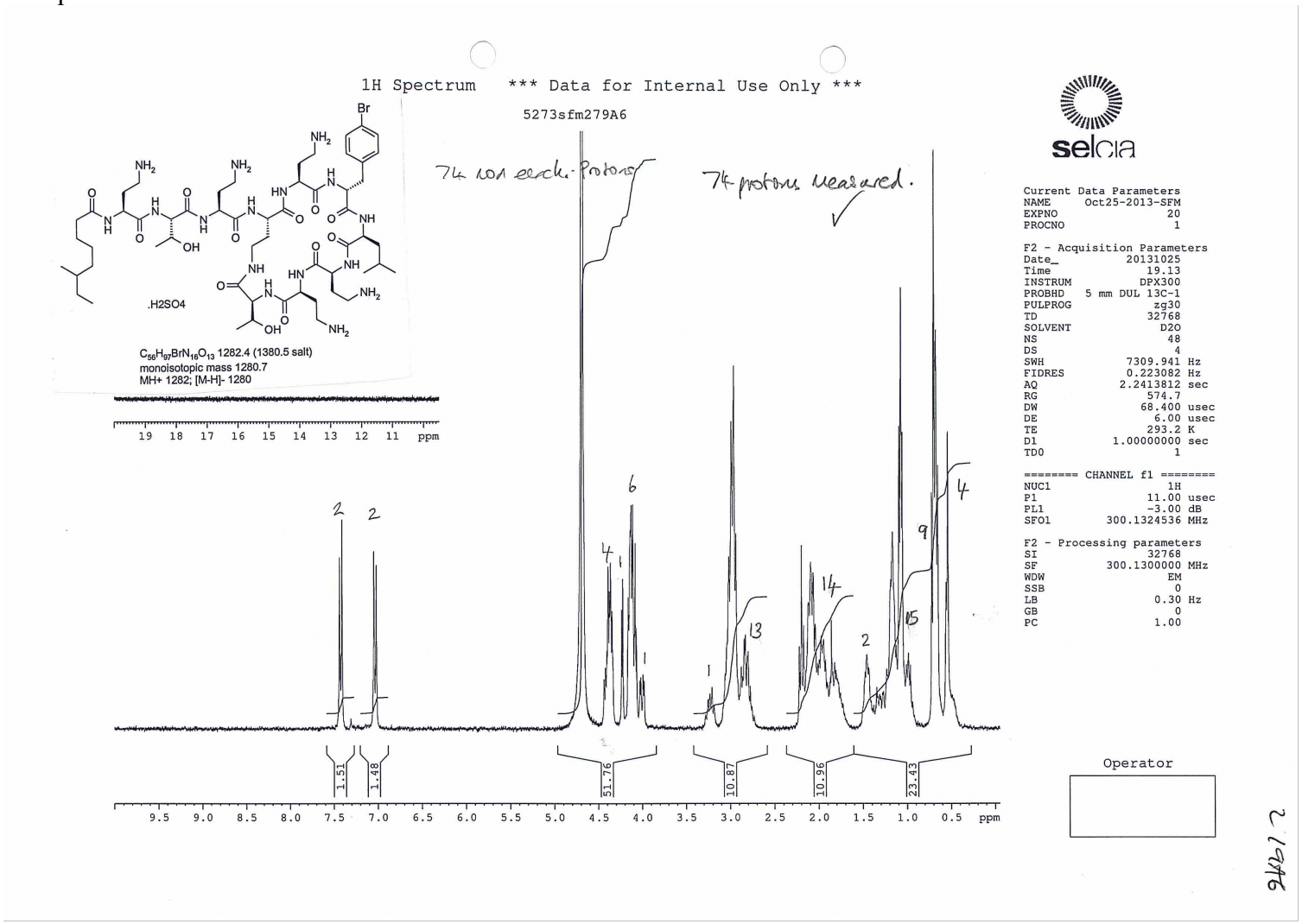
Compound 13c



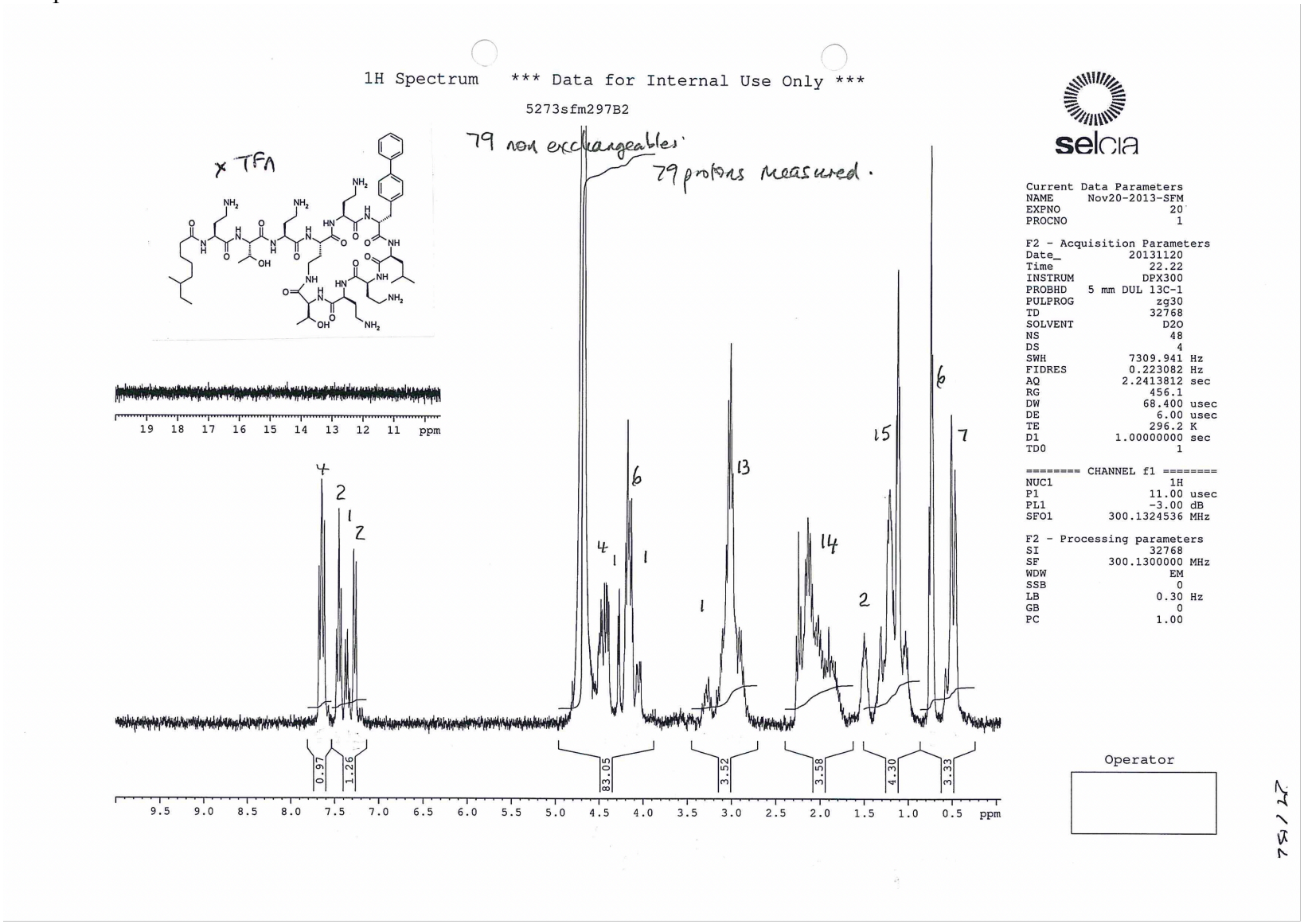


^{1}H Nmr data for final compounds 3a, 5a, 5b, 9a, 9b, 10 and 13a-c:

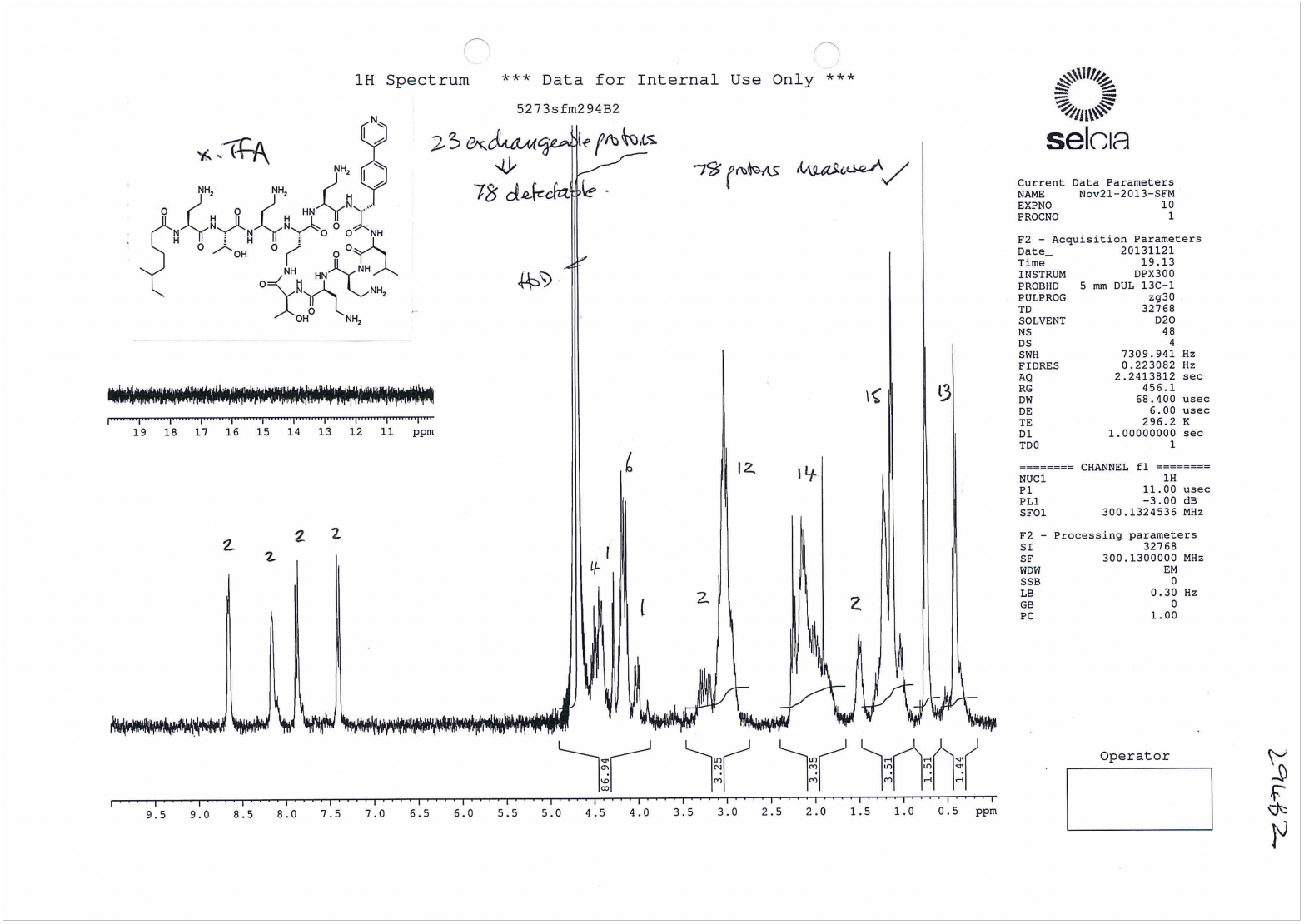
Compound 3a:



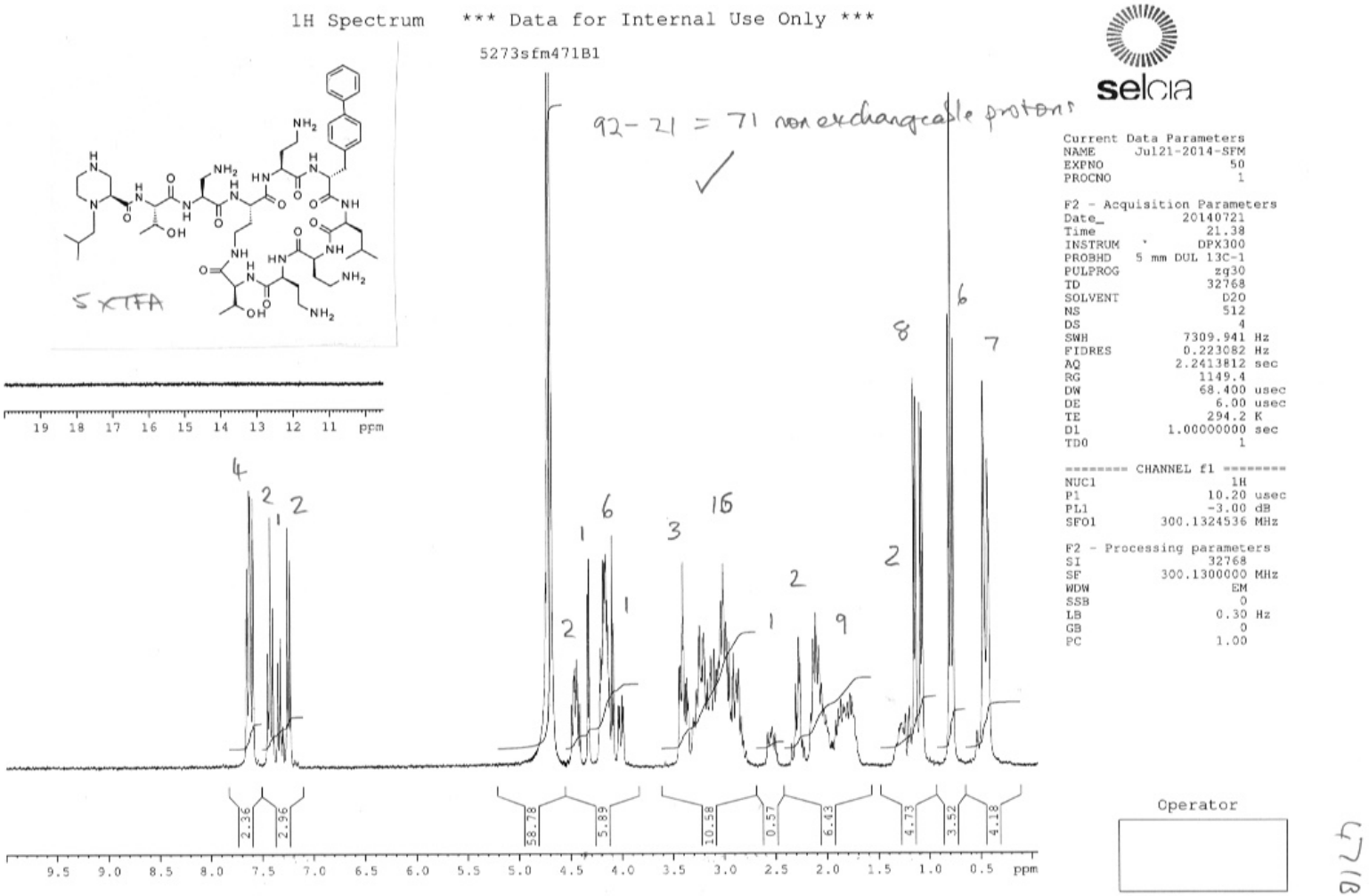
Compound5a:


1H Spectrum *** Data for Internal Use Only ***
5273sfm297B2
79 non exchangeables.
79 protons measured.
x TFA
selcia
Current Data Parameters
NAME Nov20-2013-SFM
EXPNO 20
PROCNO 1
F2 - Acquisition Parameters
Date_ 20131120
Time 22.22
INSTRUM DPX300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 32768
SOLVENT D2O
NS 48
DS 4
SWH 7309.941 Hz
FIDRES 0.223082 Hz
AQ 2.2413812 sec
RG 456.1
DW 68.400 usec
DE 6.00 usec
TE 296.2 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.00 usec
PL1 -3.00 dB
SFO1 300.1324536 MHz
F2 - Processing parameters
SI 32768
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
Operator
0.97
1.26
83.05
3.52
3.58
4.30
3.33
ppm

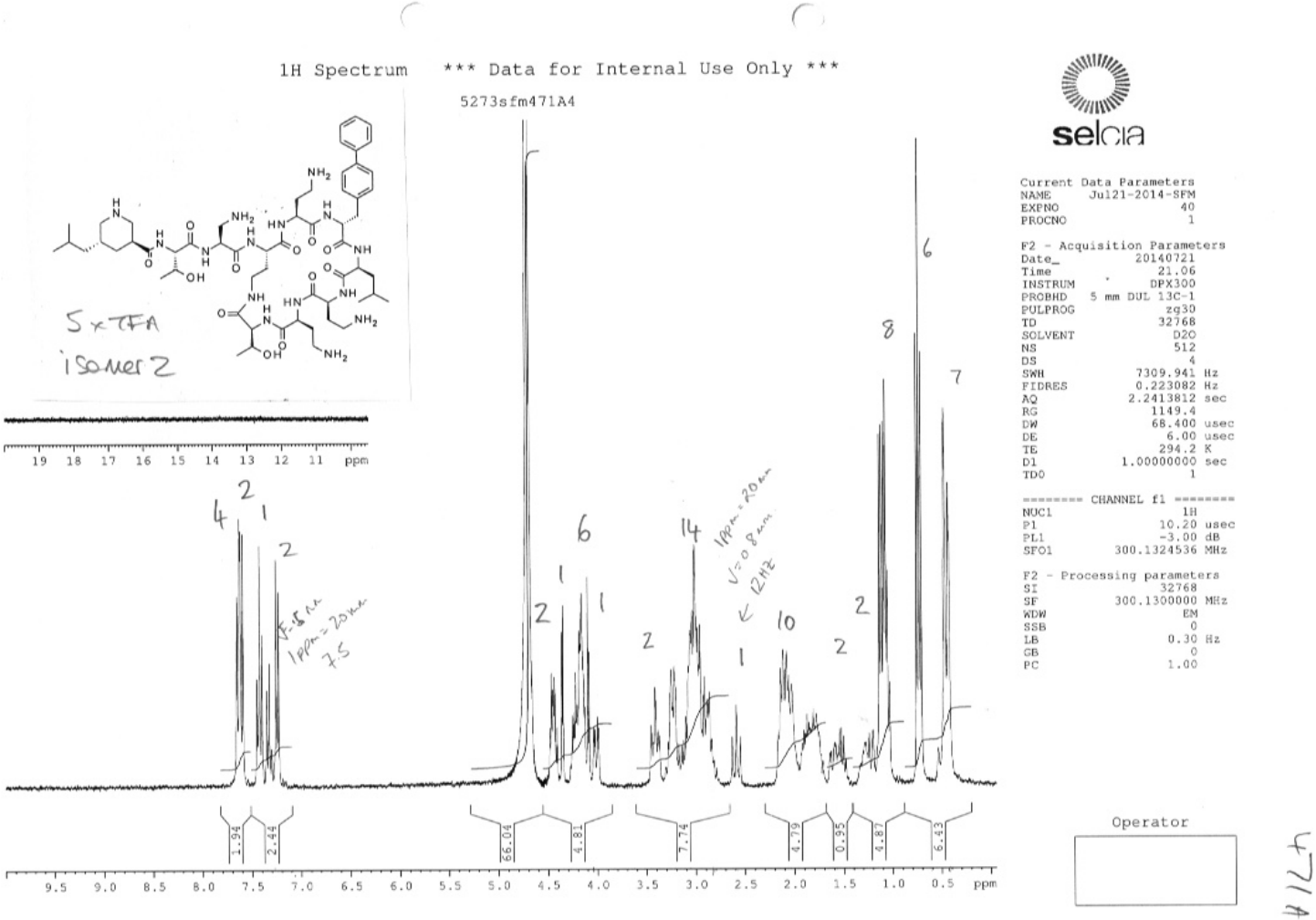
Compound 5b:


1H Spectrum *** Data for Internal Use Only ***
5273sfm294B2
x TFA
23 exchangeable protons
78 detectable
78 protons measured
HDO
selcia
Current Data Parameters
NAME Nov21-2013-SFM
EXPNO 10
PROCNO 1
F2 - Acquisition Parameters
Date_ 20131121
Time 19.13
INSTRUM DPX300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 32768
SOLVENT D2O
NS 48
DS 4
SWH 7309.941 Hz
FIDRES 0.223082 Hz
AQ 2.2413812 sec
RG 456.1
DW 68.400 usec
DE 6.00 usec
TE 296.2 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.00 usec
PL1 -3.00 dB
SFO1 300.1324536 MHz
F2 - Processing parameters
SI 32768
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
Operator
ppm

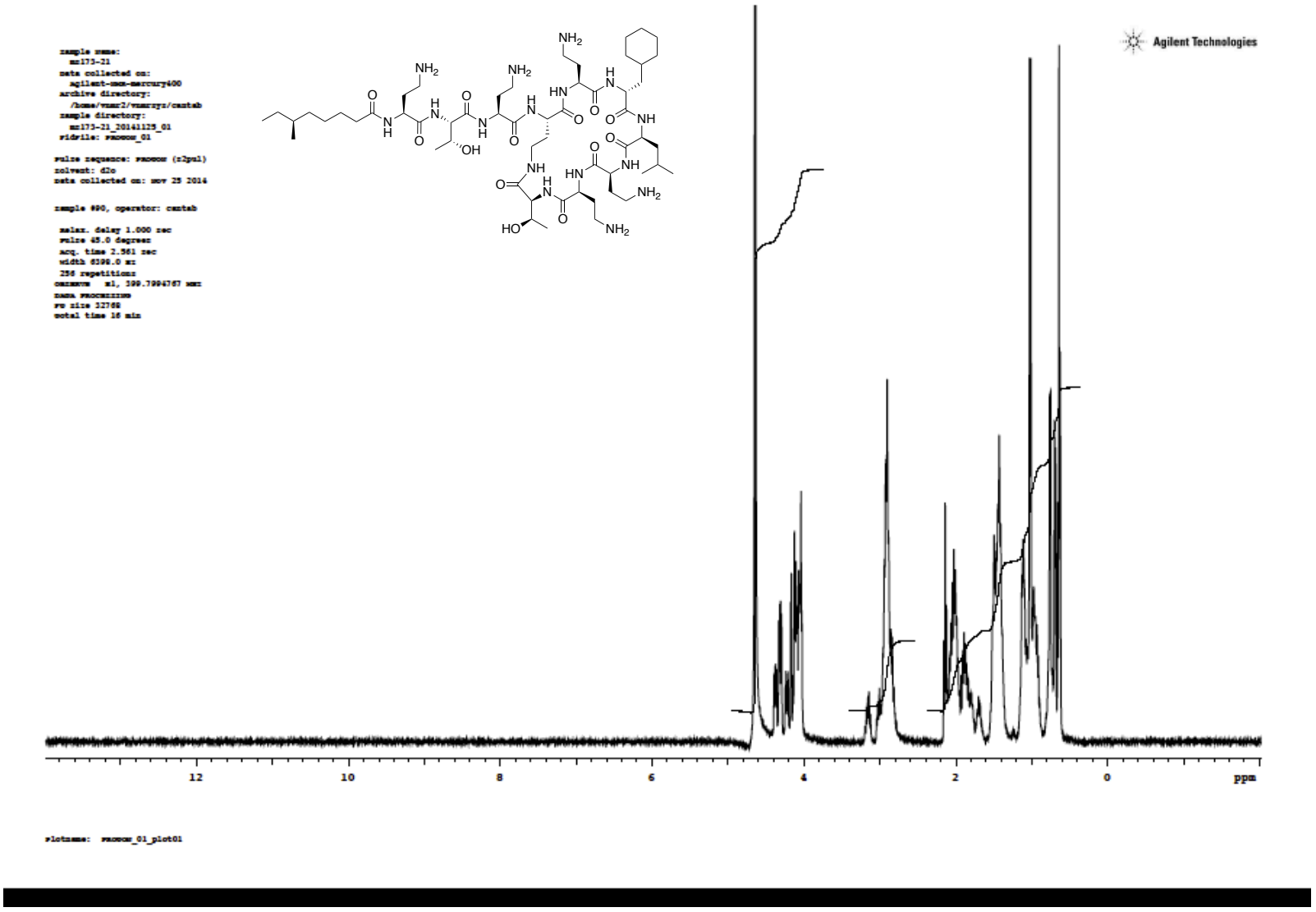
Compound 9a:


1H Spectrum *** Data for Internal Use Only ***
5273sfm471B1
92 - 21 = 71 non exchangeable protons
5 x TFA
selcia
Current Data Parameters
NAME Jul21-2014-SFM
EXPNO 50
PROCNO 1
F2 - Acquisition Parameters
Date_ 20140721
Time 21.38
INSTRUM DPX300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 32768
SOLVENT D2O
NS 512
DS 4
SWH 7309.941 Hz
FIDRES 0.223082 Hz
AQ 2.2413812 sec
RG 1149.4
DW 68.400 usec
DE 6.00 usec
TE 294.2 K
D1 1.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 10.20 usec
PL1 -3.00 dB
SFO1 300.1324536 MHz
F2 - Processing parameters
SI 32768
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
Operator
ppm

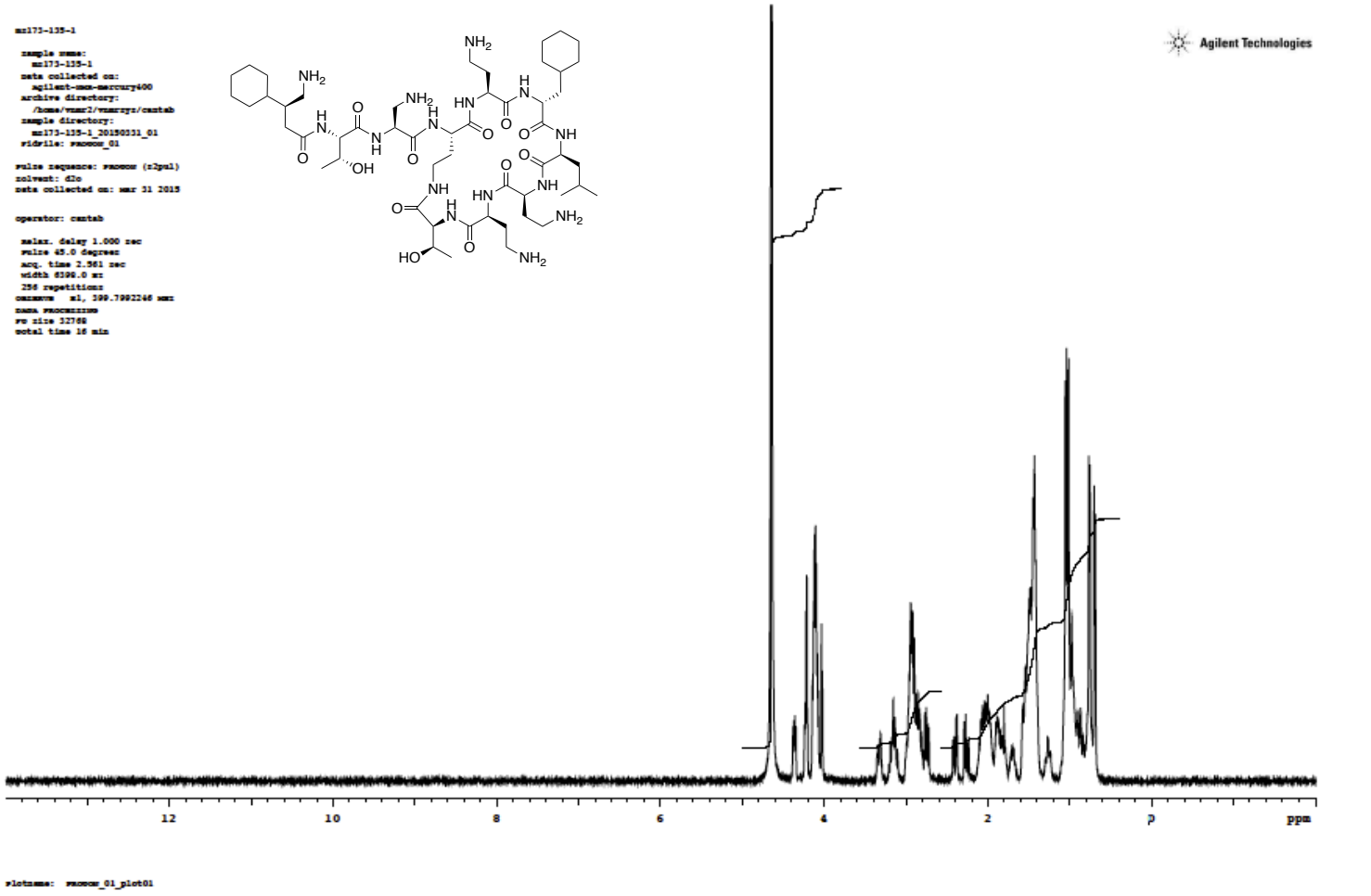
Compound 9b:


1H Spectrum *** Data for Internal Use Only ***
5273sfm471A4
5 x TFA
isomer 2
selcia
Current Data Parameters
NAME Jul21-2014-SFM
EXPNO 40
PROCNO 1
F2 - Acquisition Parameters
Date_ 20140721
Time 21.06
INSTRUM DPX300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 32768
SOLVENT D2O
NS 512
DS 4
SWH 7309.941 Hz
FIDRES 0.223082 Hz
AQ 2.2413812 sec
RG 1149.4
DW 68.400 usec
DE 6.00 usec
TE 294.2 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 10.20 usec
PL1 -3.00 dB
SFO1 300.1324536 MHz
F2 - Processing parameters
SI 32768
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
Operator
ppm

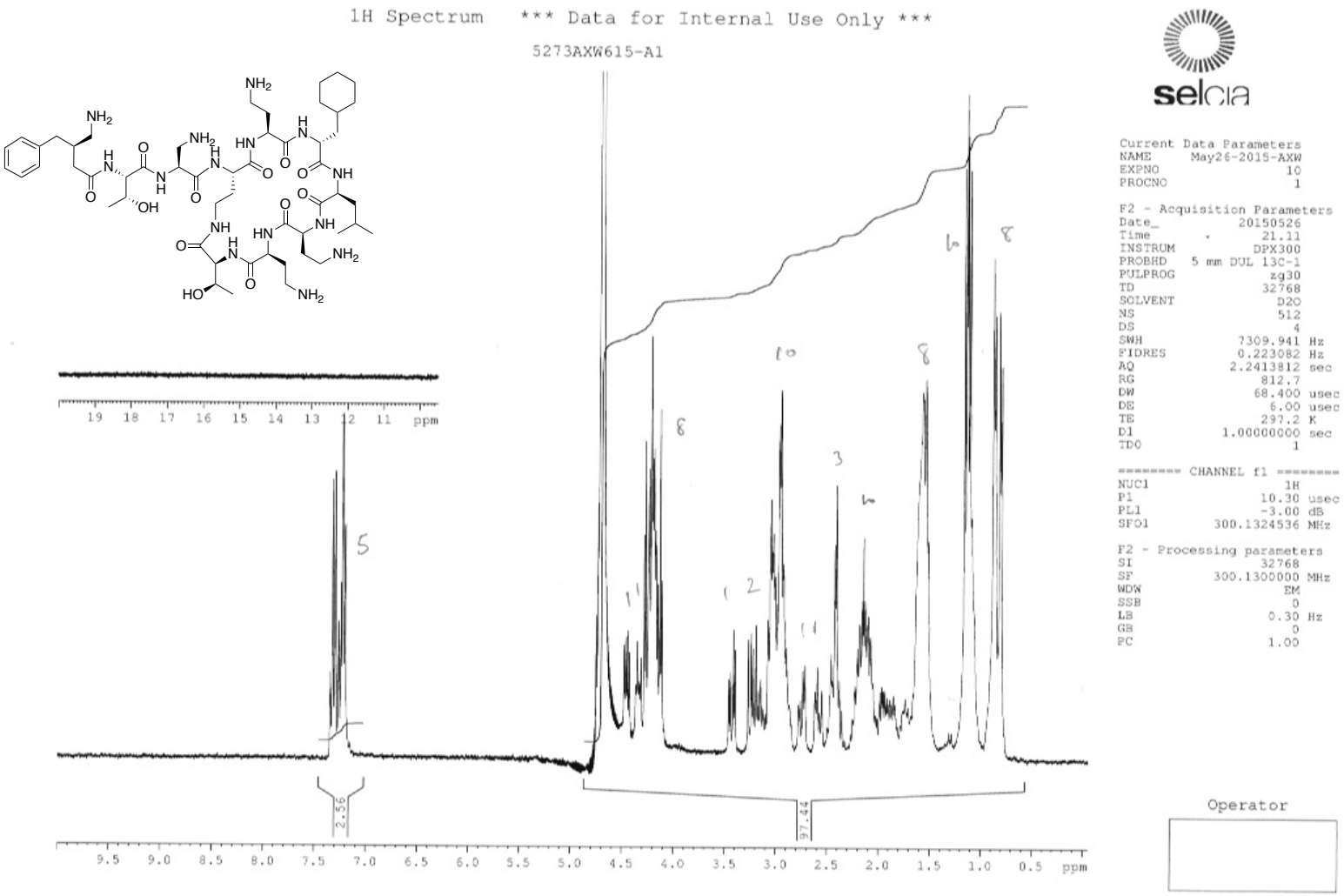
Compound 10:


sample name:
mr173-21
data collected on:
agilent-mrn-mercury400
archive directory:
/home/vnmr2/vnmrsys/cmtab
sample directory:
mr173-21_20141125_01
fidfile: rmonow_01
pulse sequence: rmonow (s2pul)
solvent: d2o
data collected on: nov 25 2014
sample #90, operator: cmtab
relax. delay 1.000 sec
pulse 45.0 degrees
acq. time 2.561 sec
width 6398.0 hz
256 repetitions
observe nl, 399.7994767 mhz
data processing
fid size 32768
total time 16 min
Agilent Technologies
12
10
8
6
4
2
0
ppm
plotname: rmonow_01_plot01

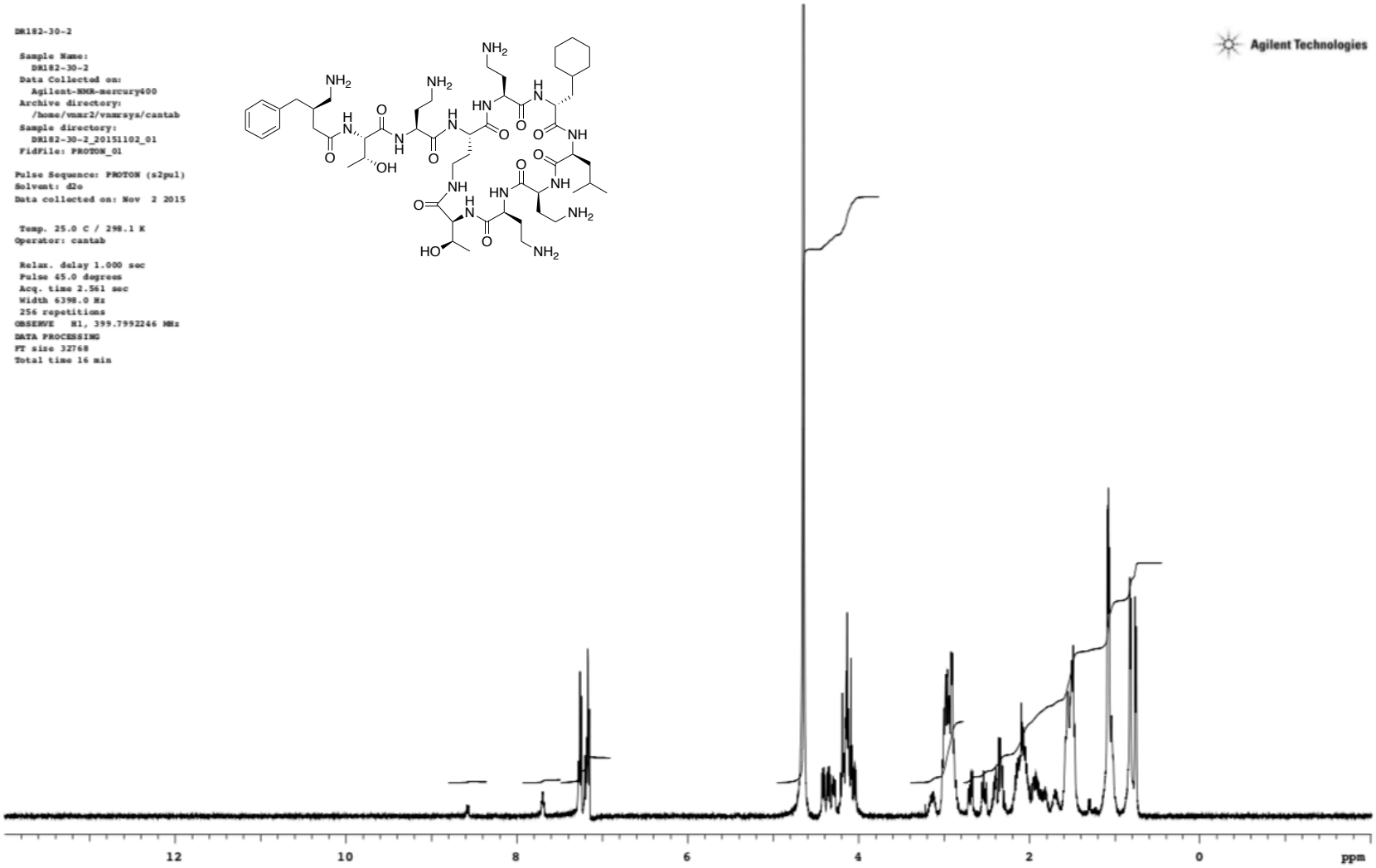
Compound 13a:



Compound 13b:

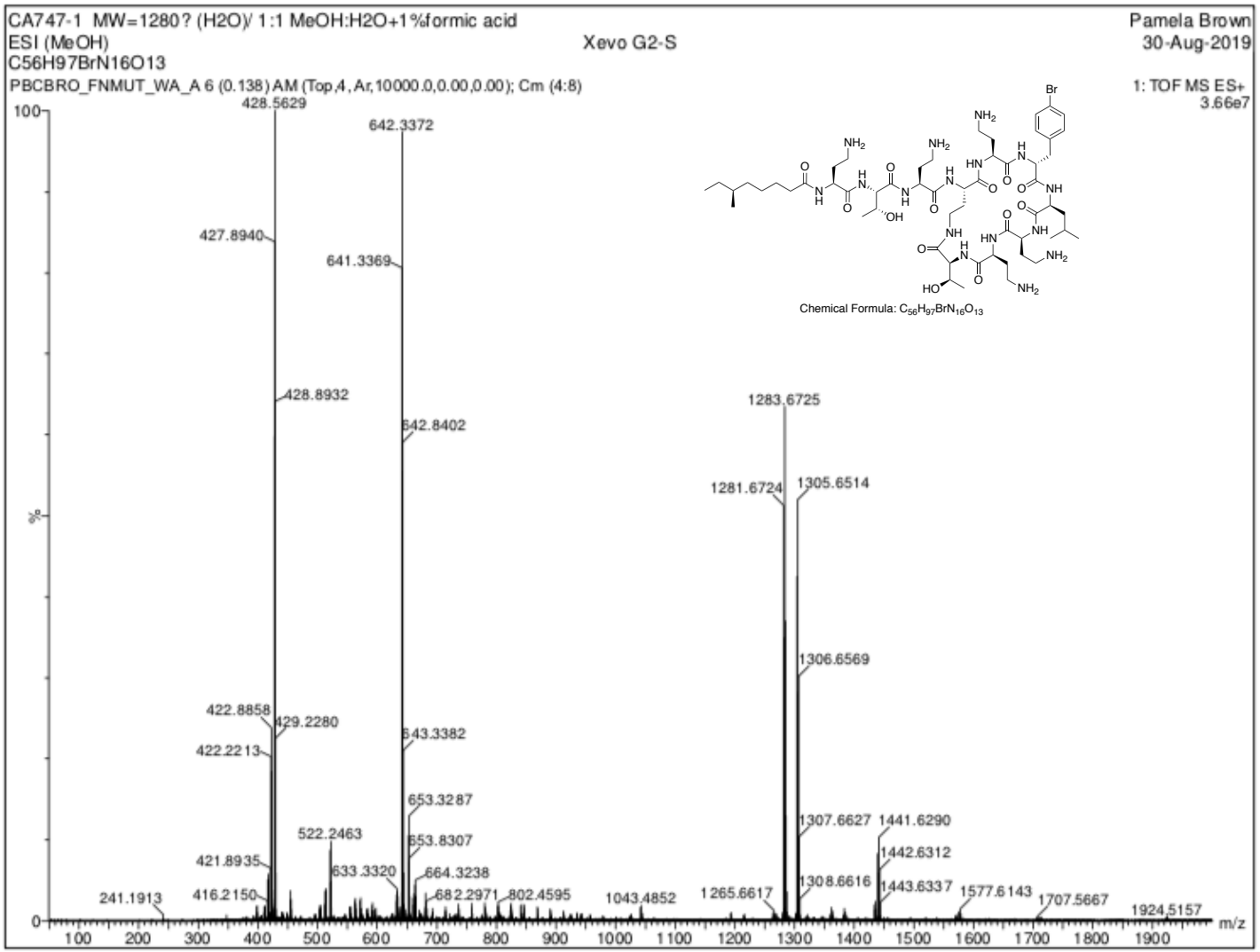


Compound 13c:

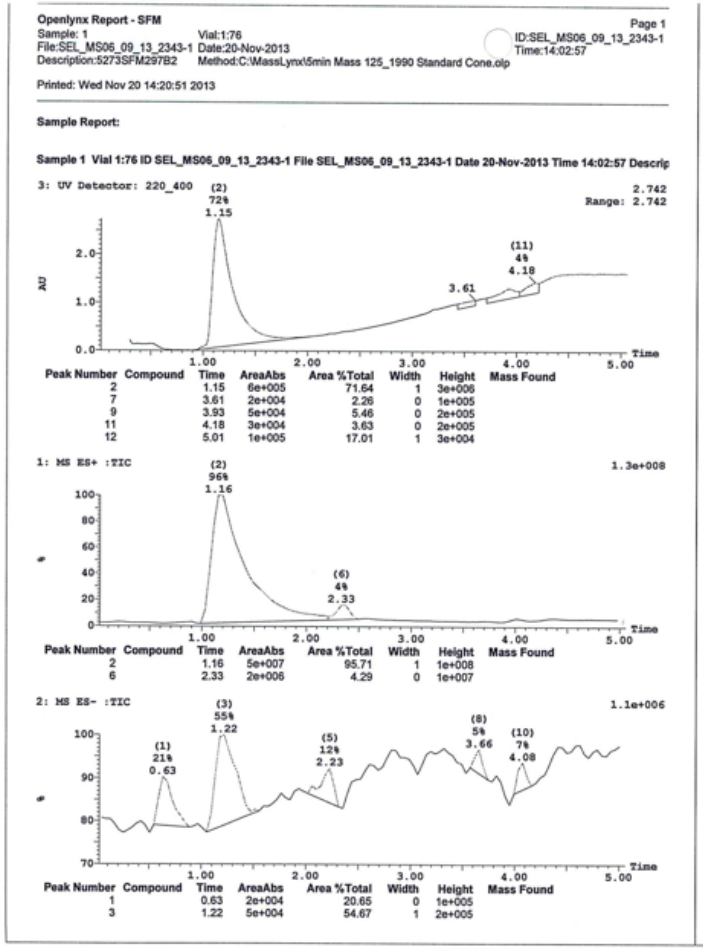


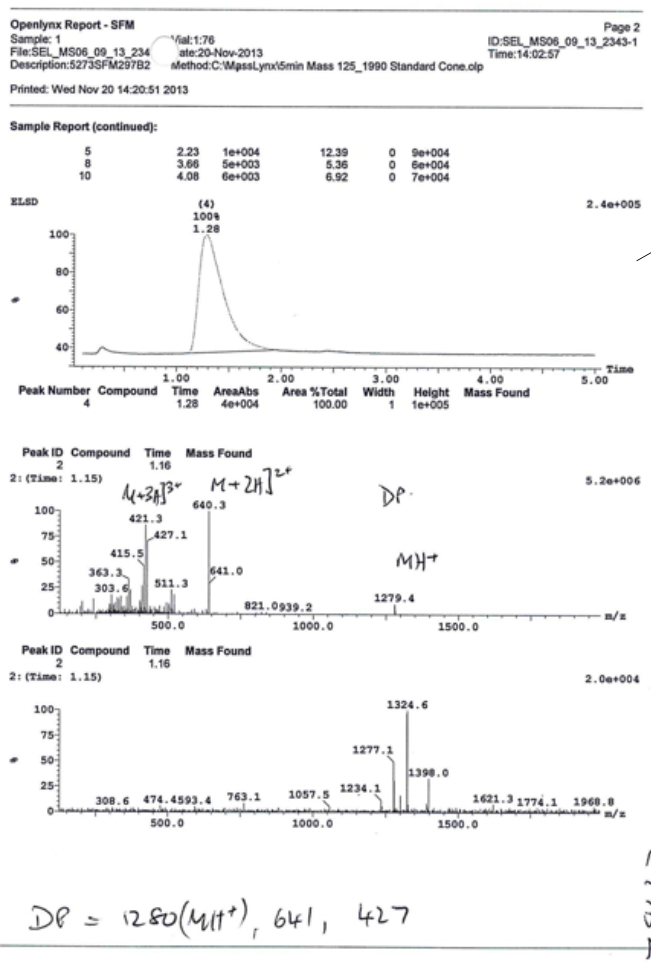
LCMS and HRMS data for 3a, 5a, 5b, 9a, 9b, 10 and 13a-c:

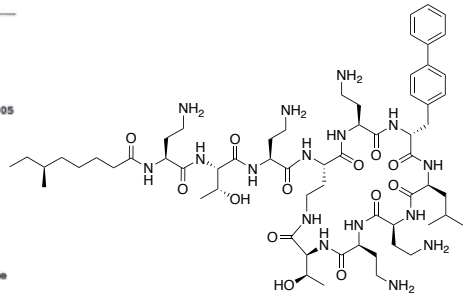
Compound 3a:



Compound 5a:

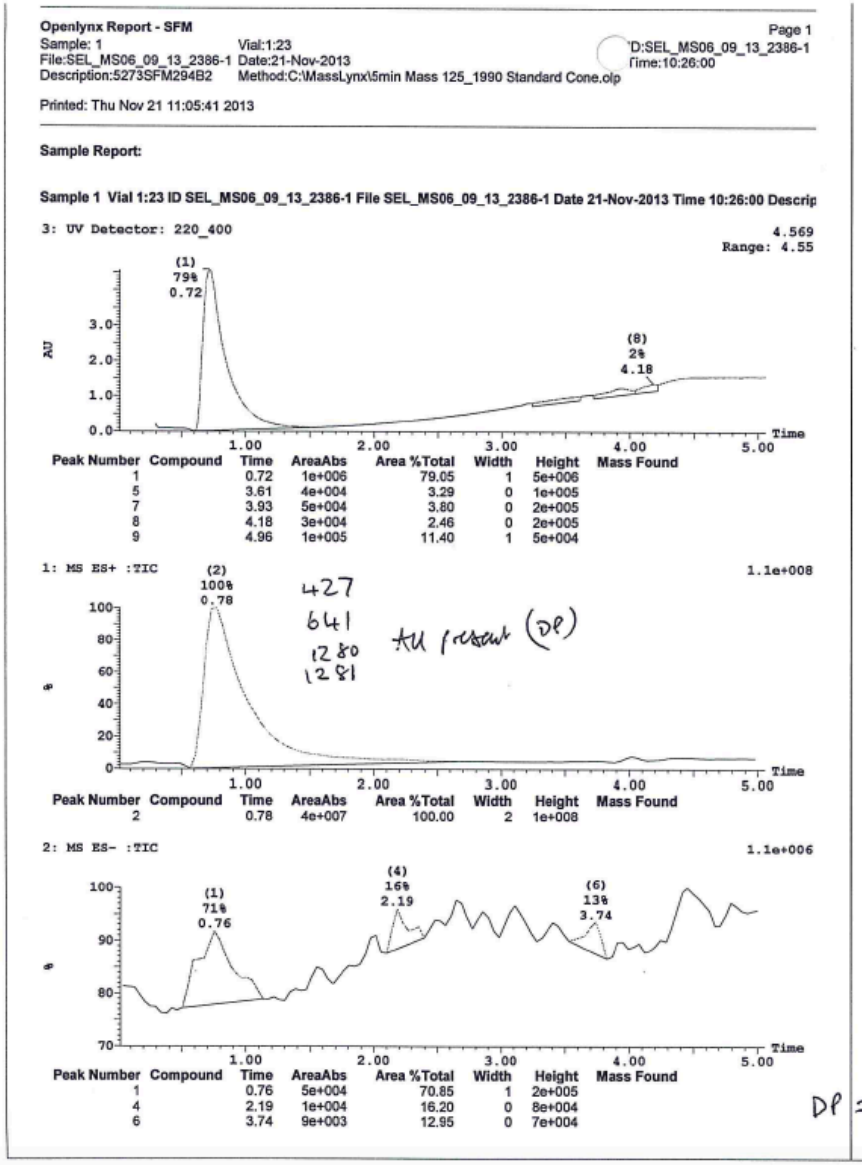


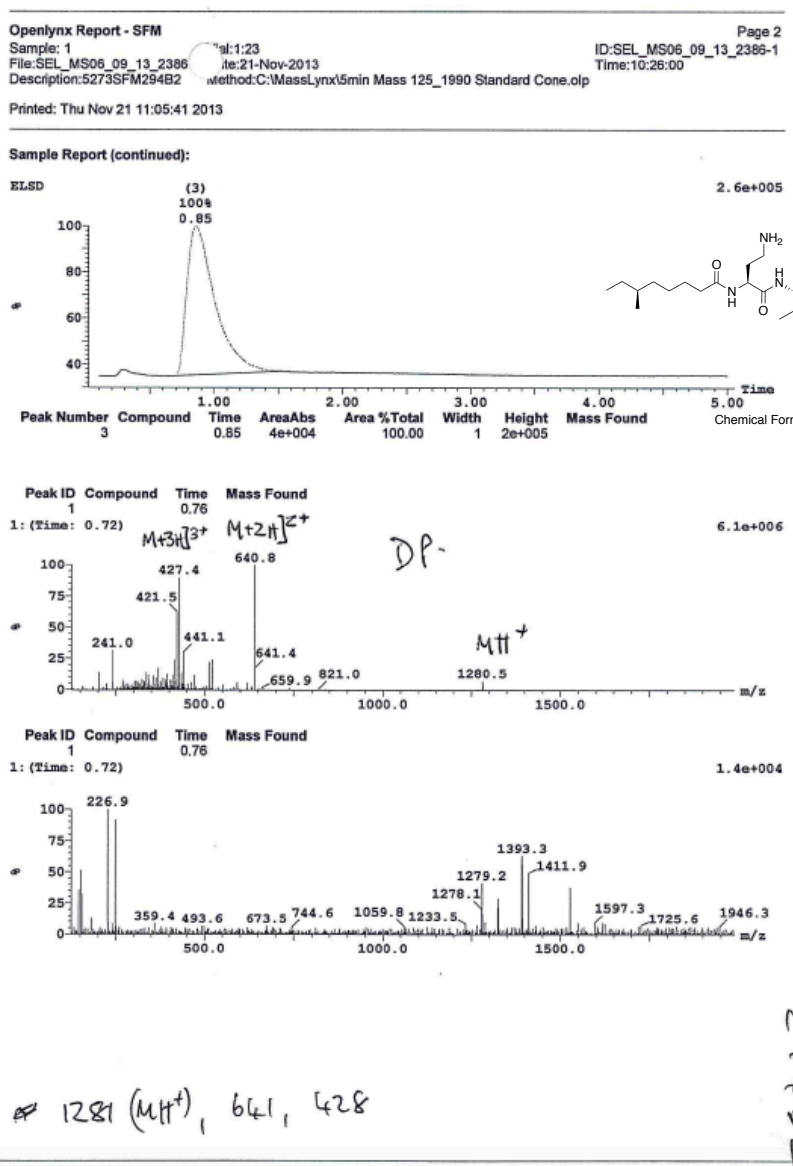




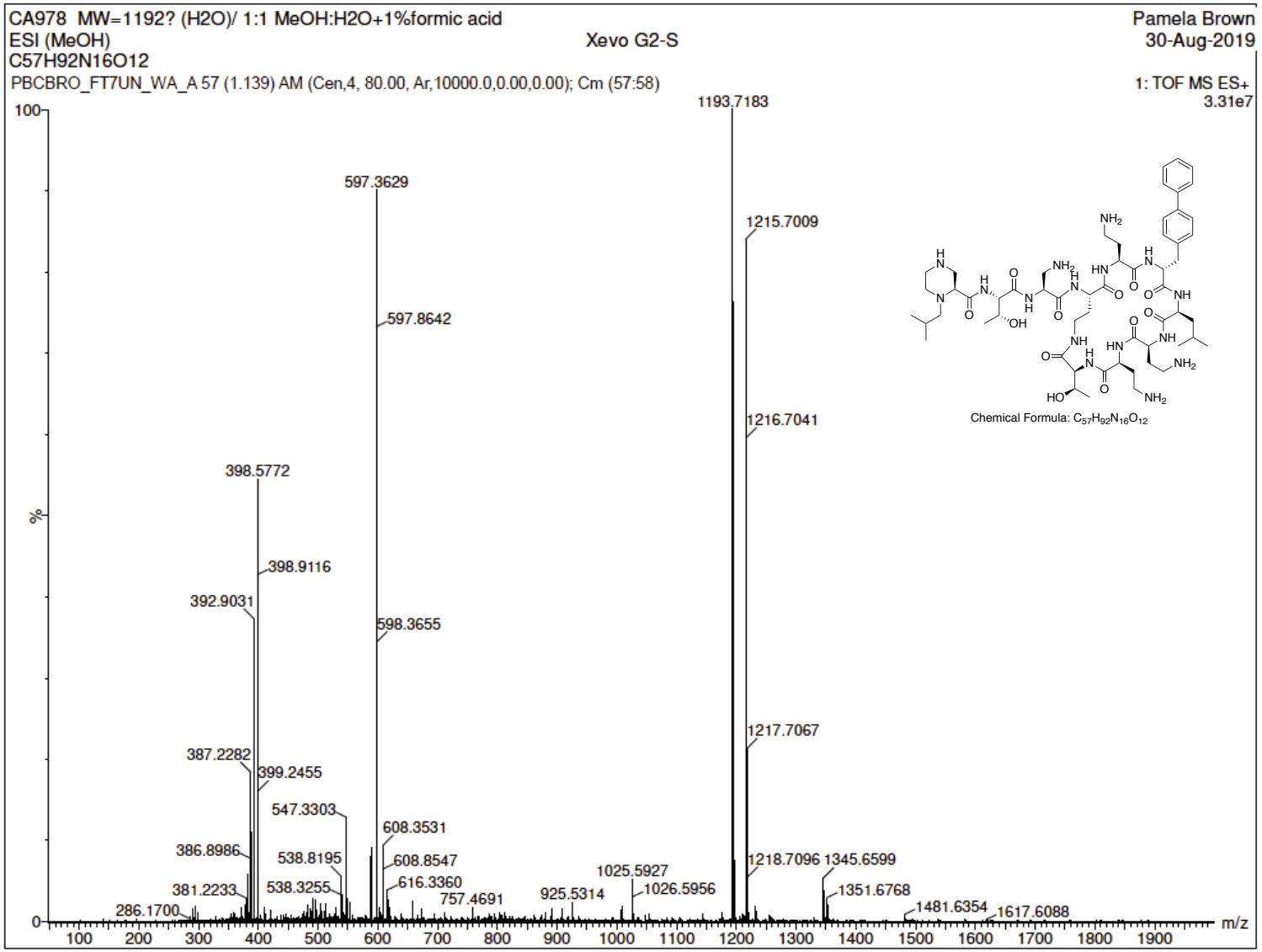
Chemical Formula: $C_{62}H_{102}N_{16}O_{13}$

Compound 5b:

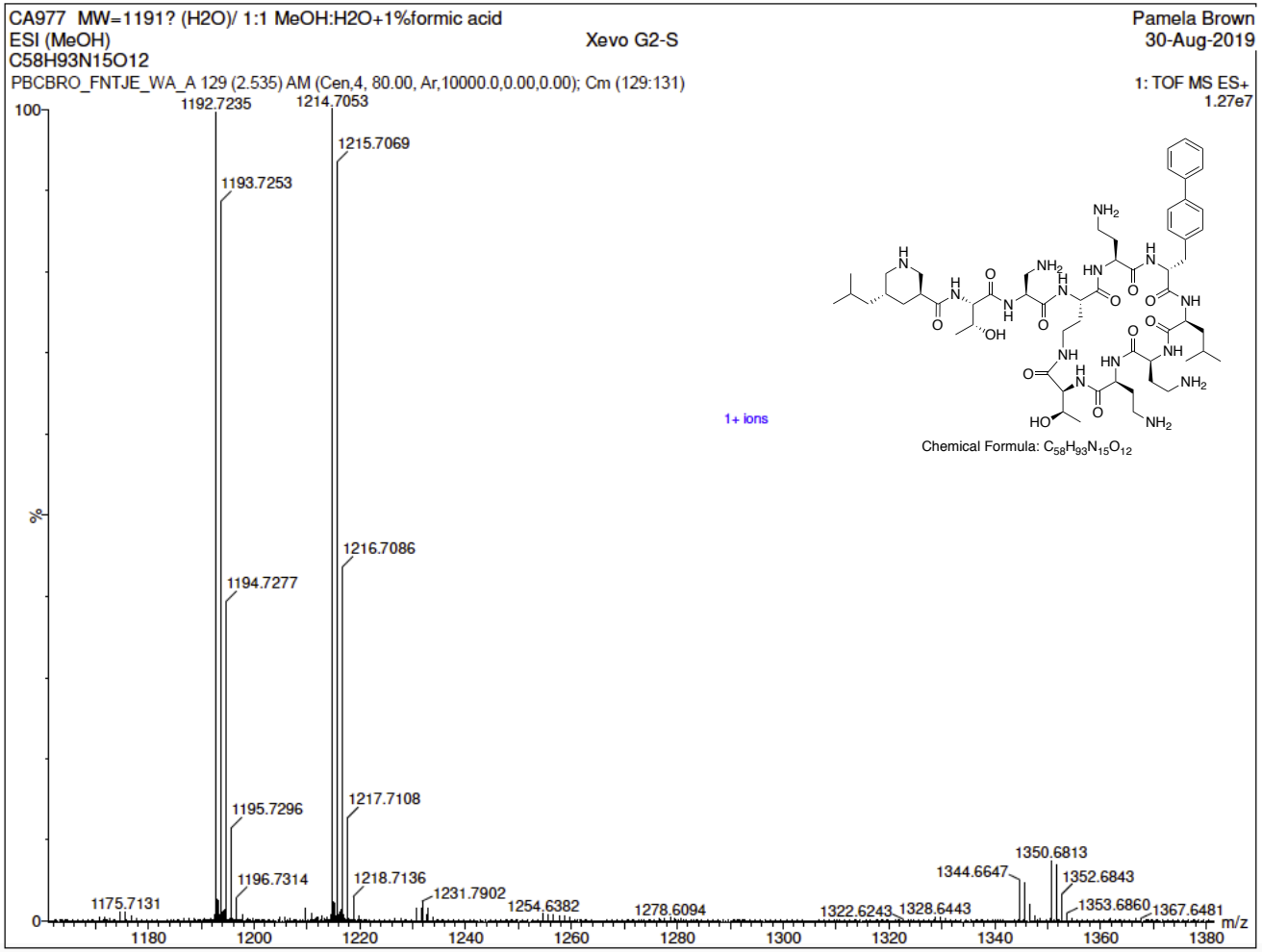

Openlynx Report - SFM
Page 1
Sample: 1
Vial:1:23
File:SEL_MS06_09_13_2386-1
Date:21-Nov-2013
Description:5273SFM294B2
Method:C:\MassLynx\5min Mass 125_1990 Standard Cone.olp
Printed: Thu Nov 21 11:05:41 2013
Sample Report:
Sample 1 Vial 1:23 ID SEL_MS06_09_13_2386-1 File SEL_MS06_09_13_2386-1 Date 21-Nov-2013 Time 10:26:00 Descrip
3: UV Detector: 220_400
4.569
Range: 4.55
(1) 79% 0.72
(8) 2% 4.18
AU
Time
Peak Number Compound Time AreaAbs Area %Total Width Height Mass Found
1 0.72 1e+006 79.05 1 5e+006
5 3.61 4e+004 3.29 0 1e+005
7 3.93 5e+004 3.80 0 2e+005
8 4.18 3e+004 2.46 0 2e+005
9 4.96 1e+005 11.40 1 5e+004
1: MS ES+ :TIC
1.1e+008
(2) 100% 0.78
427
641
1280
1281
M+ present (DP)
Peak Number Compound Time AreaAbs Area %Total Width Height Mass Found
2 0.78 4e+007 100.00 2 1e+008
2: MS ES- :TIC
1.1e+006
(1) 71% 0.76
(4) 16% 2.19
(6) 13% 3.74
Peak Number Compound Time AreaAbs Area %Total Width Height Mass Found
1 0.76 5e+004 70.85 1 2e+005
4 2.19 1e+004 16.20 0 8e+004
6 3.74 9e+003 12.95 0 7e+004
DP = 1281 (MH+), 641, 428


Openlynx Report - SFM
Page 2
Sample: 1
ID:SEL_MS06_09_13_2386-1
Time:10:26:00
Printed: Thu Nov 21 11:05:41 2013
Sample Report (continued):
ELSD
2.6e+005
(3) 100% 0.85
Time
Peak Number Compound Time AreaAbs Area %Total Width Height Mass Found
3 0.85 4e+004 100.00 1 2e+005
Chemical Formula: C61H101N17O13
Peak ID Compound Time Mass Found
1 0.76
1: (Time: 0.72)
6.1e+006
M+3H]3+
M+2H]2+
DP-
427.4
421.5
640.8
241.0
441.1
641.4
659.9
821.0
1280.5
MH+
m/z
Peak ID Compound Time Mass Found
1 0.76
1: (Time: 0.72)
1.4e+004
226.9
1393.3
1411.9
1279.2
1278.1
359.4
493.6
673.5
744.6
1059.8
1233.5
1597.3
1725.6
1946.3
m/z

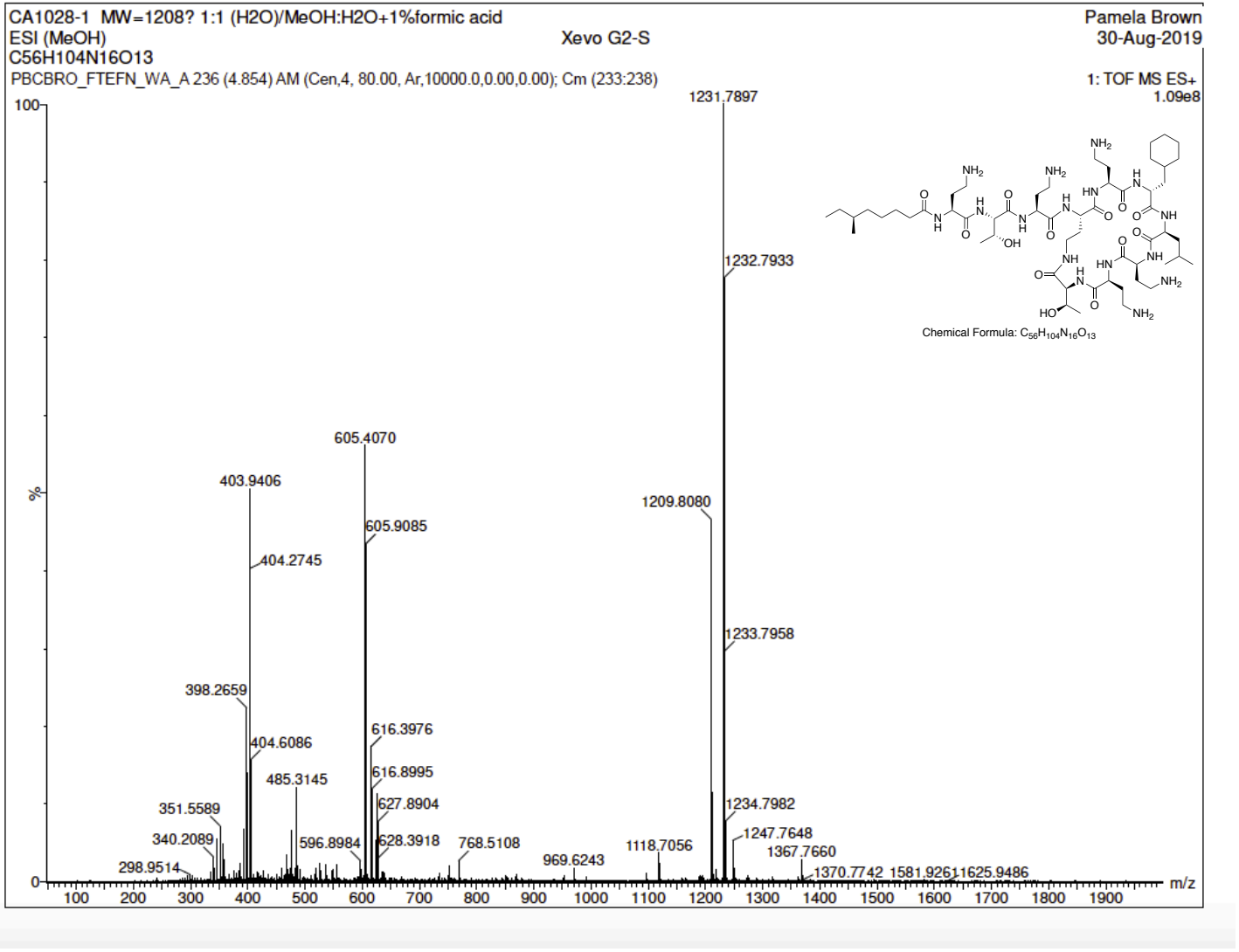
Compound 9a:


CA978 MW=1192? (H2O)/ 1:1 MeOH:H2O+1%formic acid
ESI (MeOH)
C57H92N16O12
Xevo G2-S
Pamela Brown
30-Aug-2019
PBCBRO_FT7UN_WA_A 57 (1.139) AM (Cen,4, 80.00, Ar,10000.0,0.00,0.00); Cm (57:58)
1: TOF MS ES+
3.31e7
Chemical Formula: C57H92N16O12
%
m/z

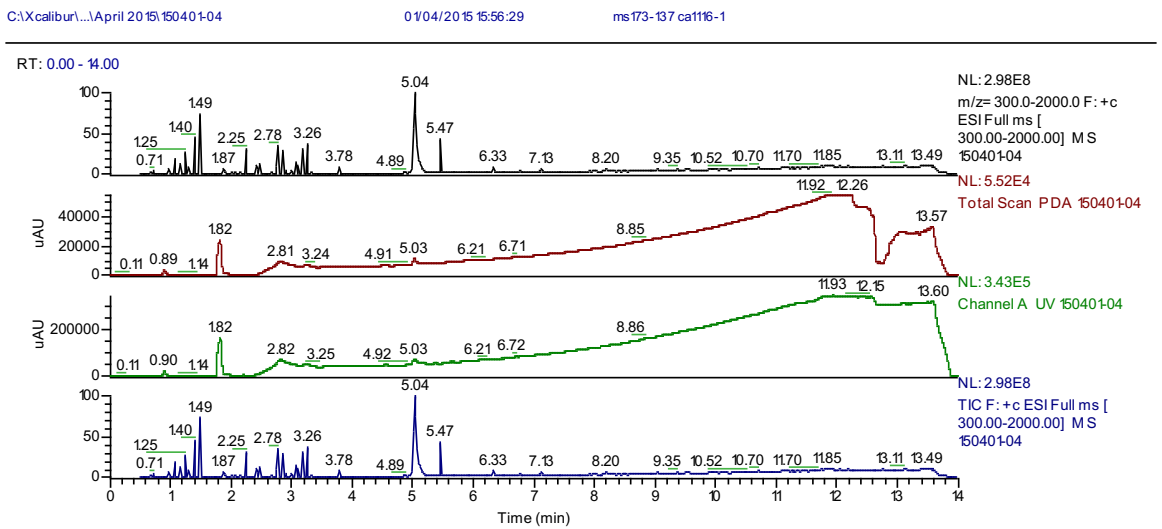
Compound 9b:

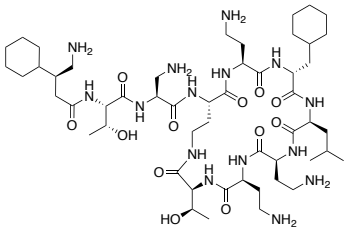

CA977 MW=1191? (H2O)/ 1:1 MeOH:H2O+1%formic acid
ESI (MeOH)
C58H93N15O12
Xevo G2-S
Pamela Brown
30-Aug-2019
PBCBRO_FNTJE_WA_A 129 (2.535) AM (Cen,4, 80.00, Ar,10000.0,0.00,0.00); Cm (129:131)
1: TOF MS ES+
1.27e7
1+ ions
Chemical Formula: C58H93N15O12
1192.7235
1214.7053
1215.7069
1193.7253
1216.7086
1194.7277
1195.7296
1217.7108
1196.7314
1218.7136
1175.7131
1231.7902
1254.6382
1278.6094
1322.6243
1328.6443
1344.6647
1350.6813
1352.6843
1353.6860
1367.6481
%
m/z

Compound 10:

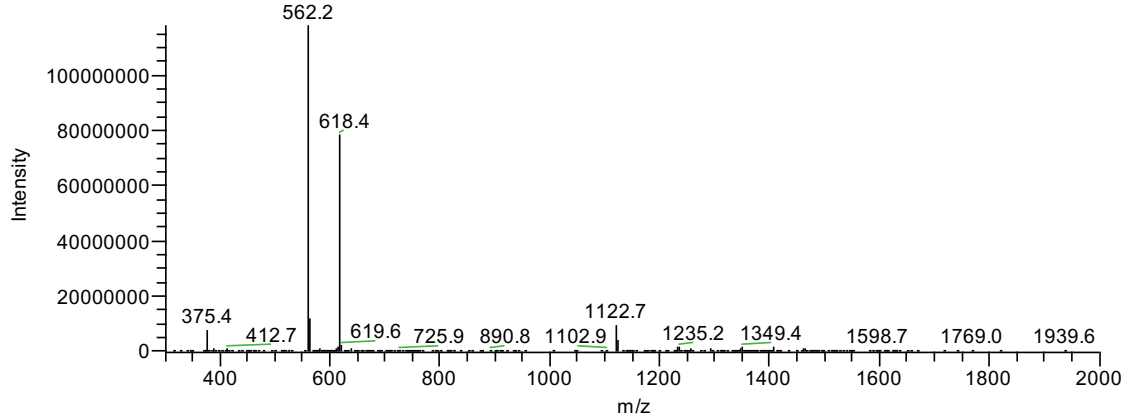

CA1028-1 MW=1208? 1:1 (H2O)/MeOH:H2O+1%formic acid
ESI (MeOH)
C56H104N16O13
Xevo G2-S
Pamela Brown
30-Aug-2019
PBCBRO_FTEFN_WA_A 236 (4.854) AM (Cen,4, 80.00, Ar,10000.0,0.00,0.00); Cm (233:238)
1: TOF MS ES+
1.09e8
Chemical Formula: C56H104N16O13
1231.7897
1232.7933
605.4070
403.9406
1209.8080
605.9085
404.2745
1233.7958
398.2659
616.3976
404.6086
616.8995
485.3145
627.8904
1234.7982
351.5589
1247.7648
340.2089
596.8984
628.3918
768.5108
1118.7056
1367.7660
969.6243
298.9514
1370.7742
1581.9261
1625.9486
%
m/z

Compound 13a:

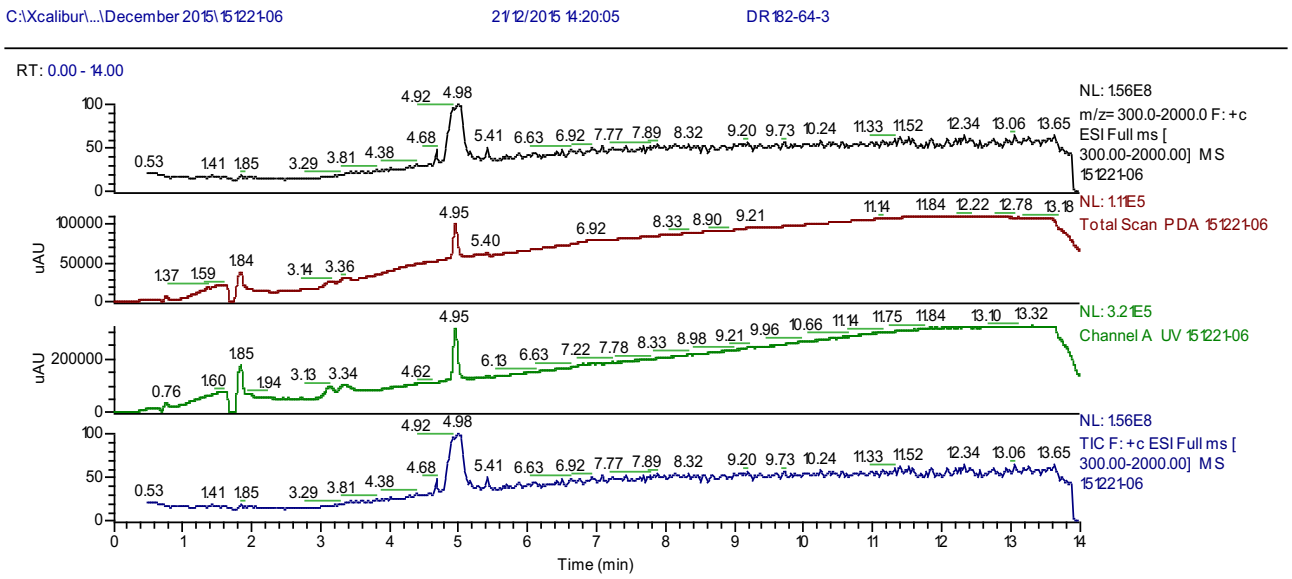


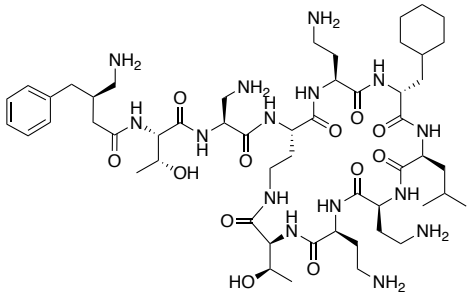


Chemical Formula: $C_{52}H_{95}N_{15}O_{12}$

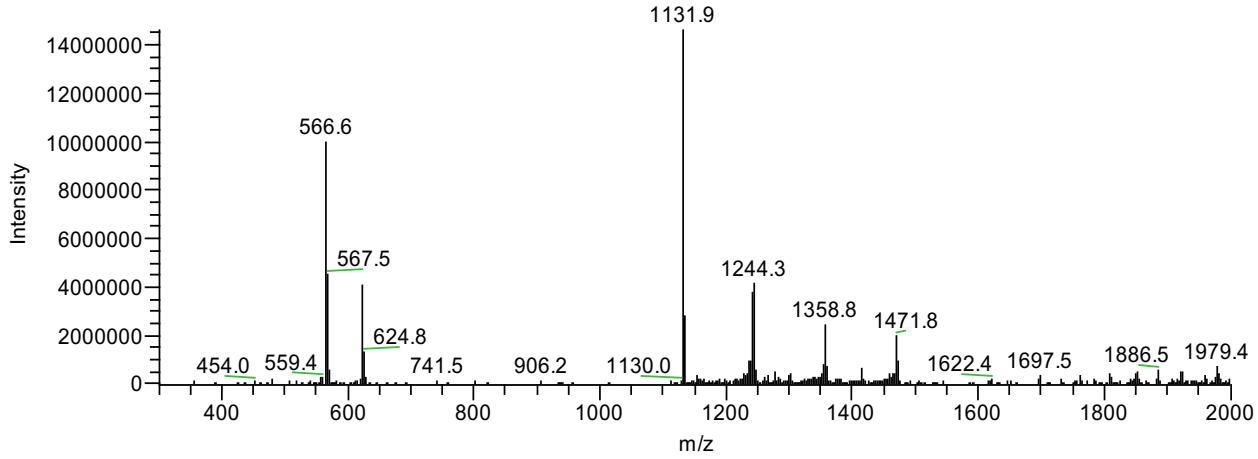


Compound 13b:

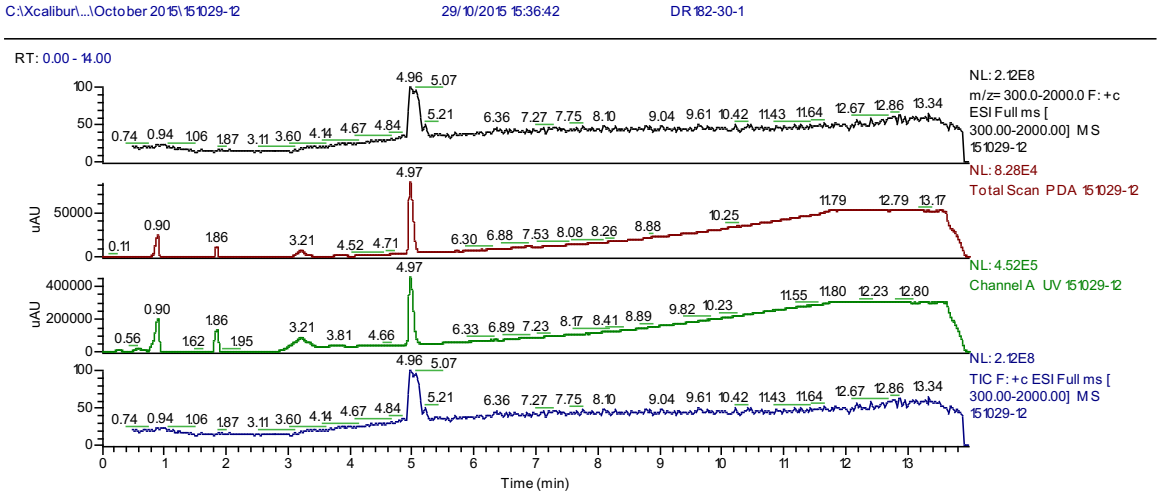


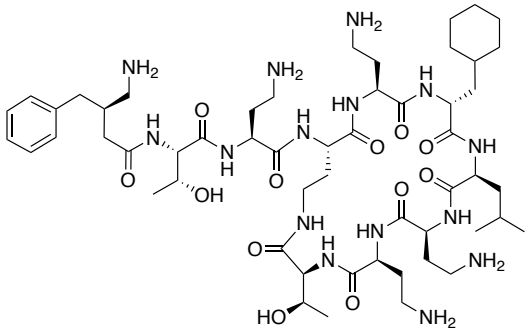


Chemical Formula: $C_{53}H_{91}N_{15}O_{12}$

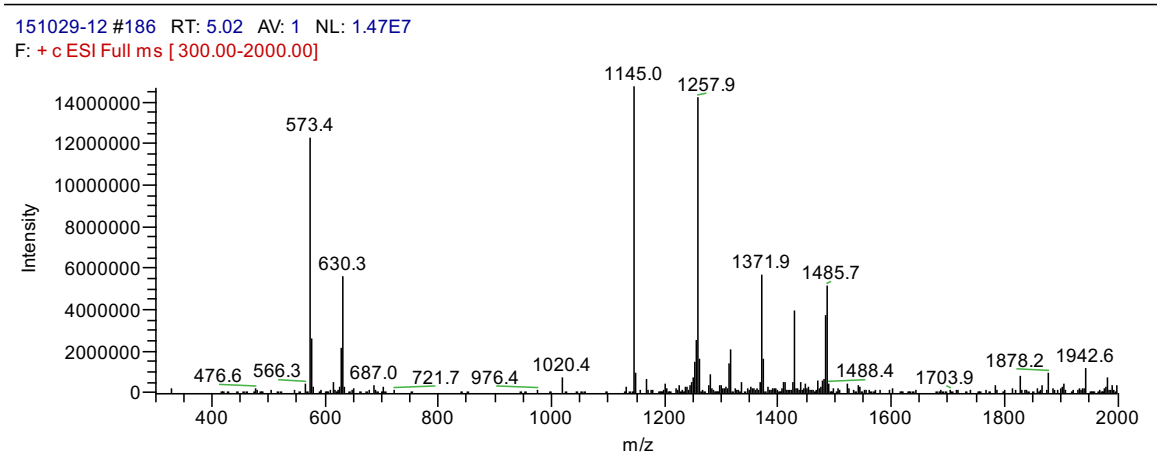


Compound 13c:



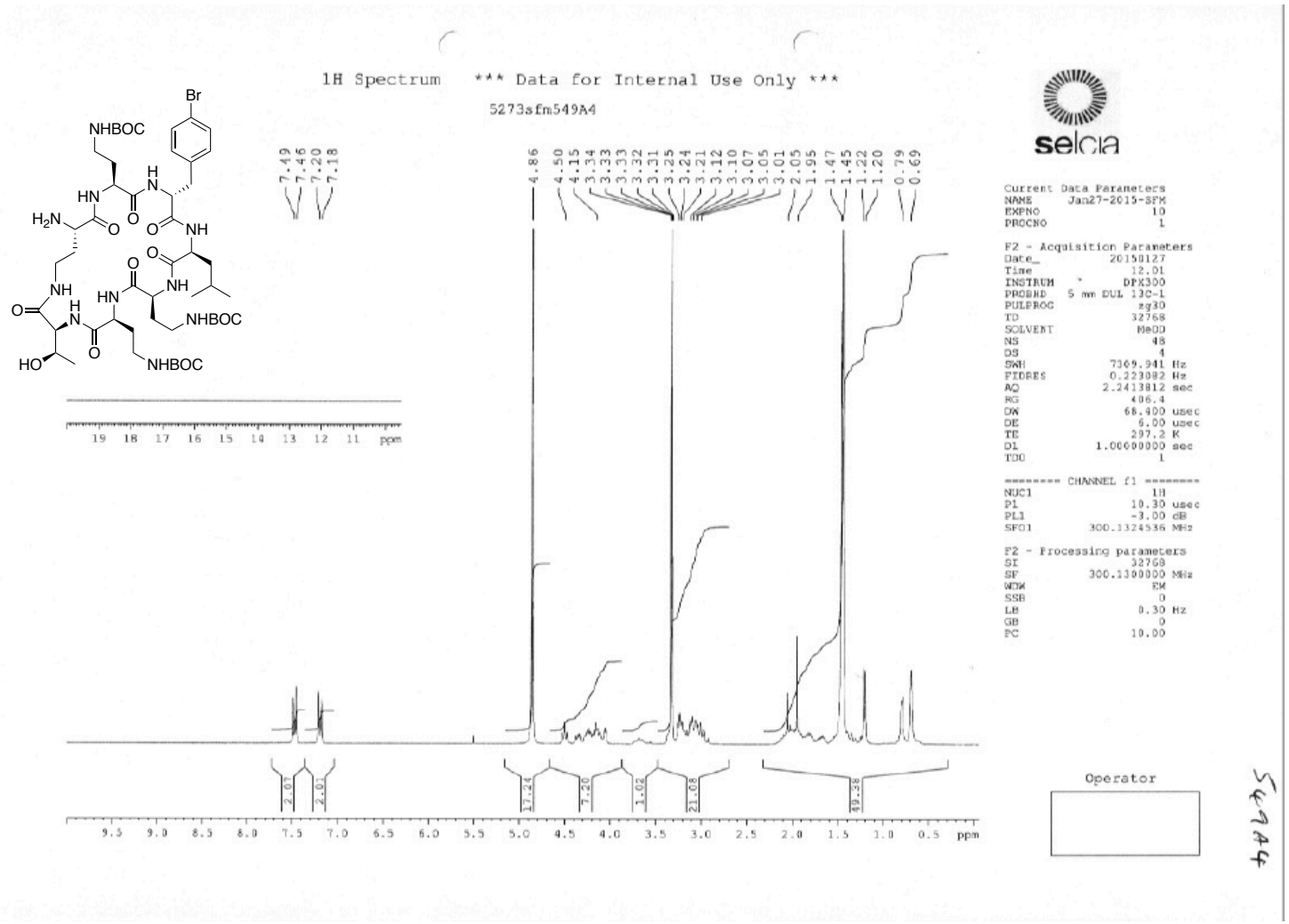


Chemical Formula: $C_{54}H_{93}N_{15}O_{12}$



[1]Hnmr and LCMS data for compounds 6a and 6b

6a:



Openlynx Report - SFN
Sample: 1
File:SEL_MS06_01_15_659-1
Description:5273SFM549A4

Vial:1:21
Date:27-Jan-2015

ID:SEL_MS06_01_15_659-1
Time:11:49:28

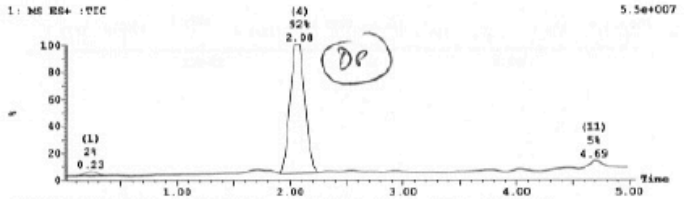
Page 1

Printed: Tue Jan 27 12:18:26 2015

Sample Report:

Sample 1 Vial 1:21 ID SEL_MS06_01_15_659-1 File SEL_MS06_01_15_659-1 Date 27-Jan-2015 Time 11:49:28 Description 5273SI

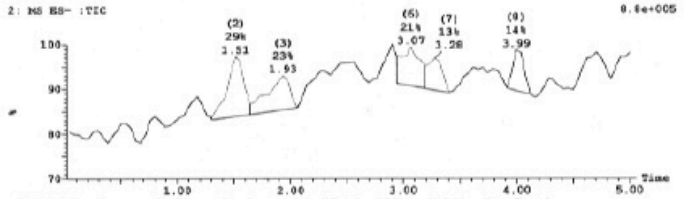
1: MS ES+ :TIC



5.5e+007

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		0.23	2e+005	2.43	0	2e+006	
4		2.08	8e+006	92.37	0	5e+007	
11		4.69	5e+005	5.20	0	3e+006	

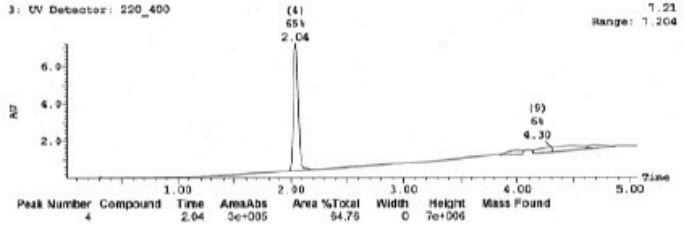
2: MS ES- :TIC



8.6e+005

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
2		1.51	2e+004	28.87	0	1e+005	
3		1.93	2e+004	23.11	0	7e+004	
6		3.67	1e+004	21.21	0	8e+004	
7		3.28	9e+003	13.28	0	6e+004	
8		3.99	9e+003	13.53	0	6e+004	

3: UV Detector: 220_400



7.21
Range: 7.204

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
4		2.04	3e+005	64.75	0	7e+006	

Openlynx Report - SFM
Sample: 1
File:SEL_MS06_01_15_659-1
Description:5273SFM549A4

Vial:1:21
Date:27-Jan-2015

ID:SEL_MS06_01_15_659-1
Time:11:49:28

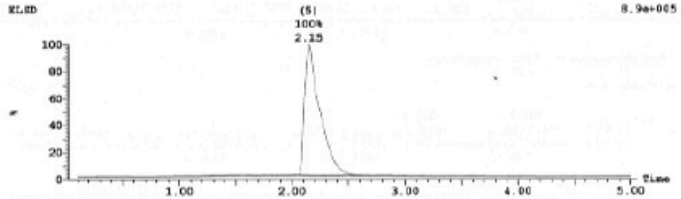
Page 2

Printed: Tue Jan 27 12:18:26 2015

Sample Report (continued):

8	3.99	4e+004	7.03	0	2e+005	
9	4.30	3e+004	5.24	0	2e+005	
10	4.47	8e+004	14.88	0	3e+005	
11	4.68	4e+004	7.10	0	2e+005	

RLSD

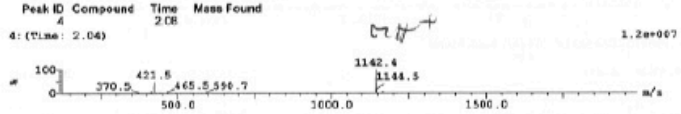


8.9e+005

Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
5		2.15	1e+005	100.00	1	9e+005	

Peak ID	Compound	Time	Mass Found
4		2.08	

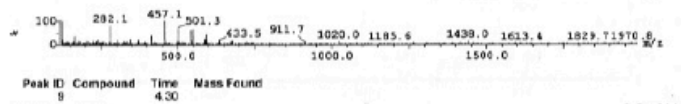
4: (Time: 2.04)



1.2e+007

Peak ID	Compound	Time	Mass Found
8		3.99	

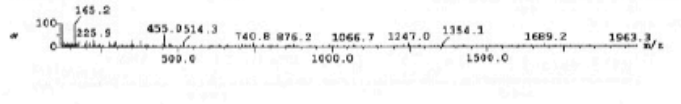
8: (Time: 3.99)



8.1e+004

Peak ID	Compound	Time	Mass Found
9		4.30	

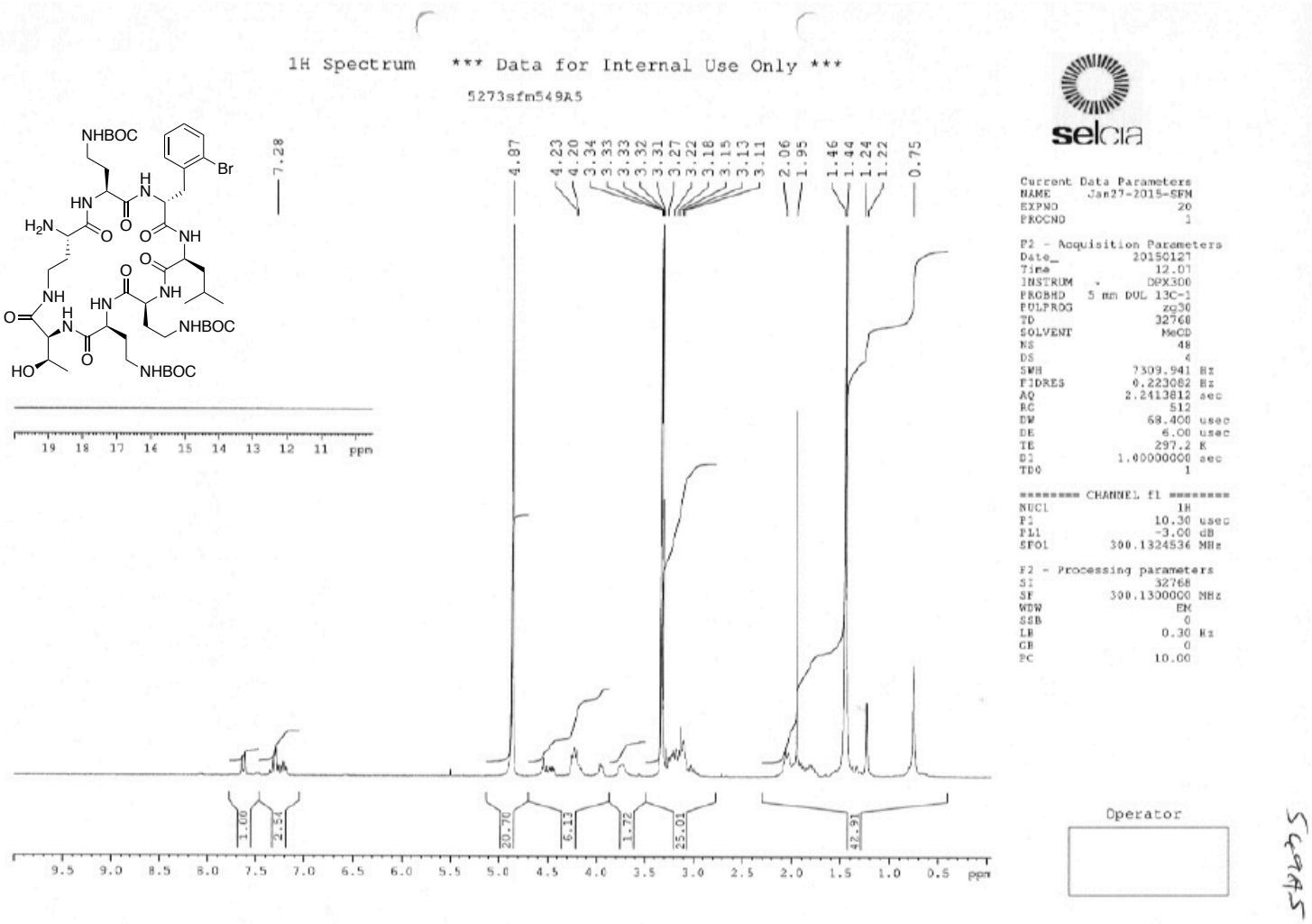
9: (Time: 4.30)



6.9e+004

5yaA4

6b:


1H Spectrum *** Data for Internal Use Only ***
5273sfm549A5
NHBOC
Br
NHBOC
NHBOC
HO
7.28
4.87
4.23
4.20
3.34
3.33
3.32
3.31
3.27
3.22
3.18
3.15
3.13
3.11
2.06
1.95
1.46
1.44
1.24
1.22
0.75
ppm
1.00
2.34
20.70
6.13
1.72
25.01
42.91
Current Data Parameters
NAME Jan27-2015-SFM
EXPNO 20
PROCNO 1
F2 - Acquisition Parameters
Date_ 20150127
Time 12.07
INSTRUM DPX300
PROBHD 5 mm DUL 13C-1
PULPROG zg30
TD 32768
SOLVENT MeOD
NS 48
DS 4
SWH 7309.941 Hz
FIDRES 0.223082 Hz
AQ 2.2413812 sec
RG 512
DW 68.400 usec
DE 6.00 usec
TE 297.2 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 10.30 usec
PL1 -3.00 dB
SFO1 300.1324536 MHz
F2 - Processing parameters
SI 32768
SF 300.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 10.00
Operator

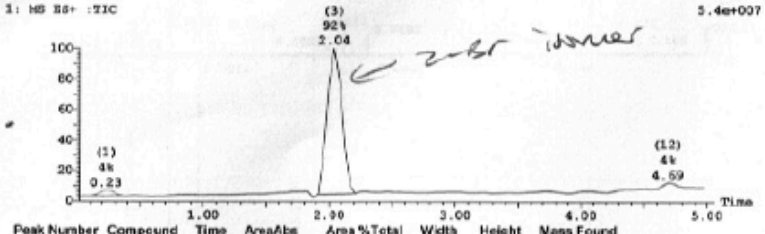
Openlynx Report - SFM
Sample: 2
File:SEL_MS06_01_15_659-2
Description:5273SFM649A5
Vial:1:22
Date:27-Jan-2015
ID:SEL_MS06_01_15_659-2
Time:11:56:17
Page 1

Printed: Tue Jan 27 12:18:33 2015

Sample Report:

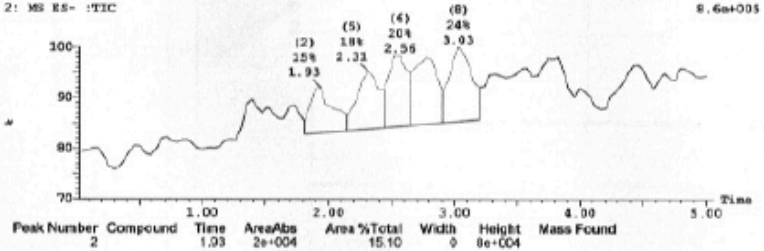
Sample 2 Vial 1:22 ID SEL_MS06_01_15_659-2 File SEL_MS06_01_15_659-2 Date 27-Jan-2015 Time 11:56:17 Description 5273SF

1: MS ES+ :TIC 5.4e+007



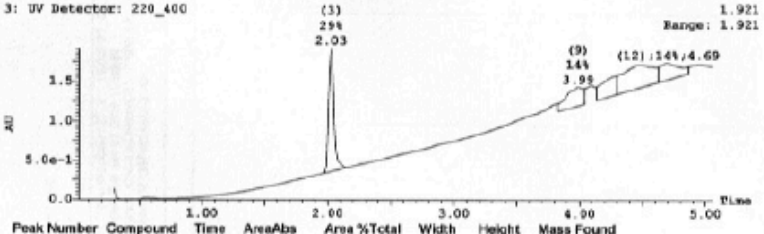
Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
1		0.23	3e+005	3.53	0	2e+006	
3		2.04	7e+006	92.08	0	5e+007	
12		4.60	3e+005	4.39	0	2e+006	

2: MS ES- :TIC 8.6e+005



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
2		1.93	2e+004	15.10	0	8e+004	
5		2.31	2e+004	18.43	0	1e+005	
6		2.56	2e+004	19.69	0	1e+005	
7		2.77	2e+004	22.53	0	1e+005	
8		3.03	3e+004	24.25	0	1e+005	

3: UV Detector: 220_400 1.921
Range: 1.921



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
3		2.03	8e+004	29.08	0	2e+006	

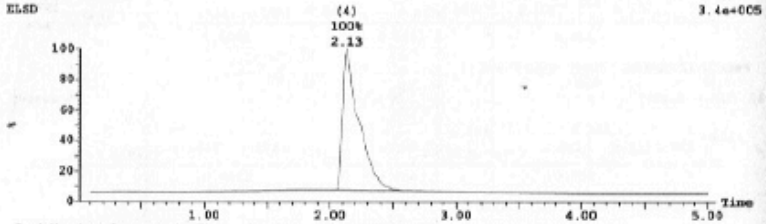
Openlynx Report - SFM
Sample: 2
File:SEL_MS06_01_15_659-2
Description:5273SFM649A5
Vial:1:22
Date:27-Jan-2015
ID:SEL_MS06_01_15_659-2
Time:11:56:17
Page 2

Printed: Tue Jan 27 12:18:33 2015

Sample Report (continued):

9	3.99	4e+004	14.31	0	2e+006
10	4.29	3e+004	12.48	0	2e+006
11	4.47	6e+004	30.23	0	3e+006
12	4.69	4e+004	13.90	0	2e+006

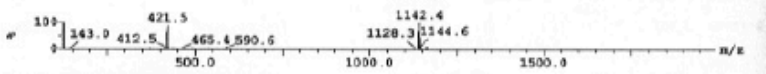
ELSD 3.4e+005



Peak Number	Compound	Time	AreaAbs	Area %Total	Width	Height	Mass Found
4		2.13	5e+004	100.00	1	3e+005	

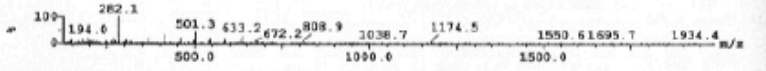
Peak ID	Compound	Time	Mass Found
3		2.04	

3: (Time: 2.03) 8.8e+006



Peak ID	Compound	Time	Mass Found
9		3.99	

9: (Time: 3.99) 1.2e+005



Peak ID	Compound	Time	Mass Found
10		4.29	

10: (Time: 4.25) 4.0e+004