Supporting Information

Activity and Predicted Nephrotoxicity of Synthetic Antibiotics Based on Polymyxin B

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1) Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072 Australia. E-mail: m.cooper@uq.edu.au, m.blaskovich@uq.edu.au, Fax +61-7-3346-2090, Phone +61-7-3346-2044

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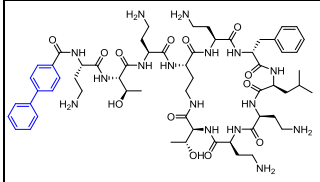
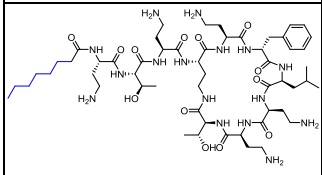
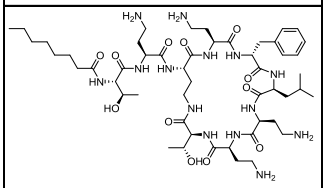
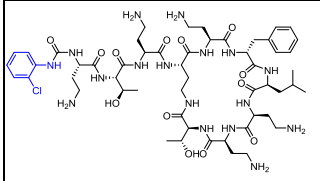
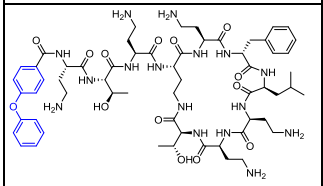
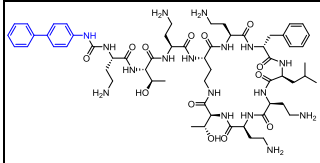
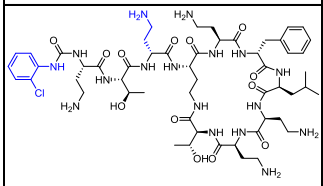
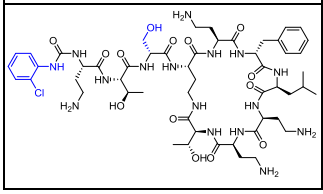
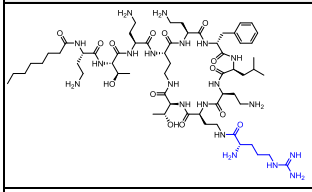
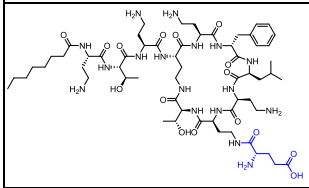
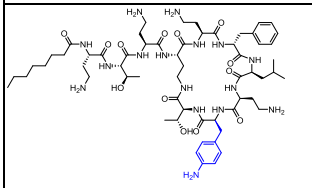
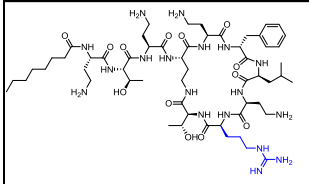
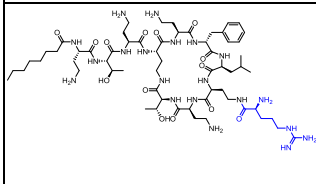
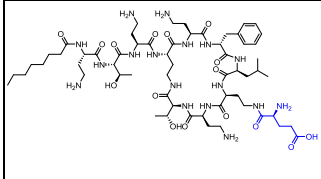
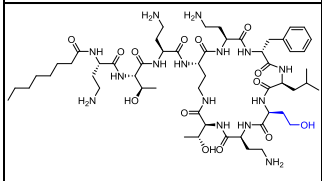
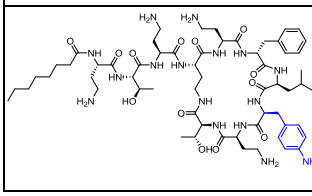
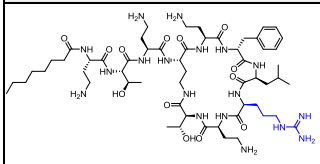
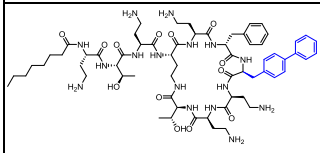
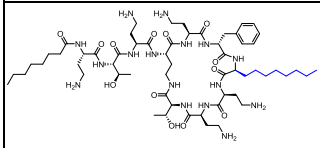
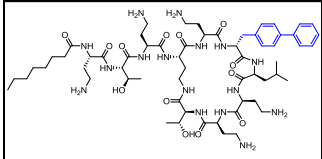
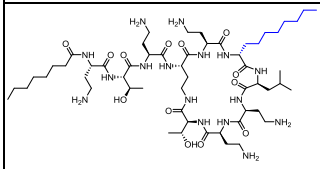
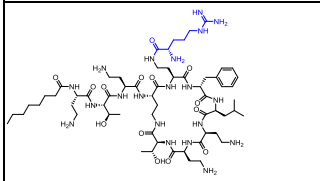
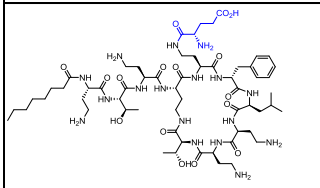
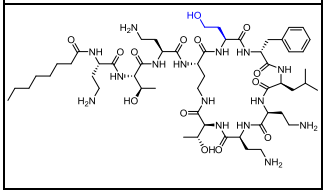
Table of Contents

Table S1. Compound Structures and Summary of Characterization.

Structure	Cmpd	Molecular Formula	Exact Mass Parent	Calculated M+2	Meassured M+2	Err (ppm)	% Purity by ELSD	% Purity by UV 210nm
	**10**	C60H90N16O13	1242.6873	622.3509	622.3512	-0.40	>95	>95
	**11**	C55H96N16O13	1188.7343	595.3744	595.3757	-2.10	>95	>95
	**12**	C51H88N14O12	1088.6700	545.3350	545.3430	-0.71	>95	>95
	**13**	C54H86N17O13Cl	1216.8200	608.8213	608.8227	-2.32	>95	>95
	**14**	C60H90N16O14	1258.6822	630.3484	630.3487	-0.44	>95	>95
	**15**	C60H91N17O13	1257.6982	629.8564	629.8594	-4.86	>95	>95
	**16**	C54H86N17O13Cl	1215.6280	608.8213	608.8230	-2.90	>95	>95
	**17**	C53H83N16O14Cl	1202.5963	602.3054	602.3063	-1.47	>95	>95

Structure	Cmpd	Molecular Formula	Exact Mass Parent	Calculated M+2	Meassured M+2	Err (ppm)	% Purity by ELSD	% Purity by UV 210nm
	**18**	C61H108N20O14	1344.8354	673.4250	673.4280	-4.42	>95	>95
	**19**	C60H103N17O16	1317.7769	659.8957	659.8959	-0.22	>95	>95
	**20**	C60H98N16O13	1250.7499	626.3822	626.3850	-4.35	>95	>95
	**21**	C57H100N18O13	1244.7717	623.3931	623.3957	-4.09	>95	>95
	**22**	C61H108N20O14	1344.8354	673.4250	673.4256	-0.91	>95	>95
	**23**	C60H103N17O16	1317.7769	659.8957	659.8983	-3.94	>95	>95
	**24**	C55H95N15O14	1189.7183	595.8664	595.8685	-3.56	>95	>95
	**25**	C60H98N16O13	1250.7499	626.3822	626.3847	-3.86	>95	>95

Structure	Cmpd	Molecular Formula	Exact Mass Parent	Calculated M+2	Meassured M+2	Err (ppm)	% Purity by ELSD	% Purity by UV 210nm
	**26**	C57H100N18O13	1244.7717	623.3931	623.3955	-3.81	>95	>95
	**27**	C64H98N16O13	1298.7499	650.3822	650.3848	-3.90	>95	>95
	**28**	C59H104N16O13	1244.7969	623.4057	623.4084	-4.26	>95	>95
	**29**	C61H100N16O13	1264.7656	633.3901	633.3907	-0.93	>95	>95
	**30**	C56H106N16O13	1210.8125	606.4135	606.4152	-2.72	>95	>95
	**31**	C61H108N20O14	1344.8354	673.4250	673.4247	0.47	>95	>95
	**32**	C60H103N17O16	1317.7769	659.8957	659.8984	-4.09	>95	>95
	**33**	C55H95N15O14	1189.7183	595.8664	595.8689	-4.09	>95	>95

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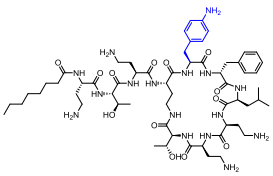
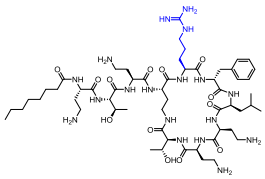
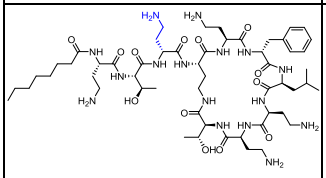
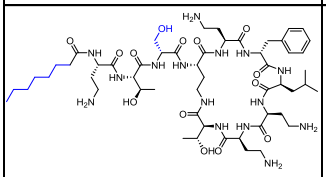
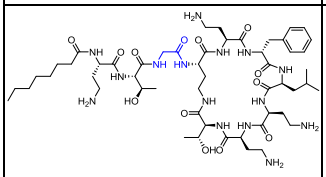
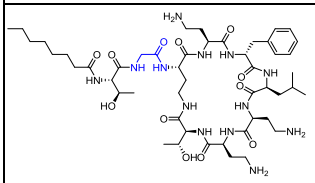
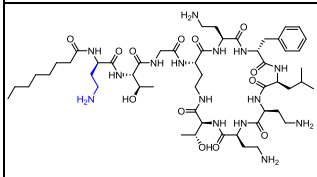
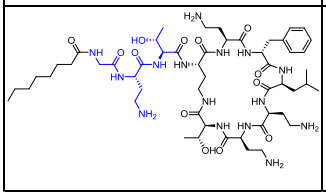
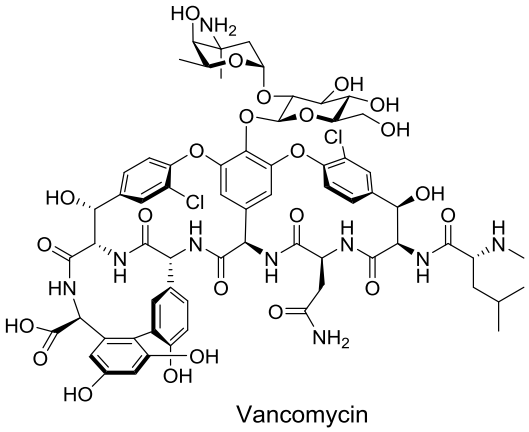
	**34**	C60H98N16O13	1250.7499	626.3822	626.3849	-4.30	>95	>95
	**35**	C57H100N18O13	1244.7717	623.3931	623.3958	-4.25	>95	>95
	**36**	C55H96N16O13	1188.7343	595.3744	595.3731	2.26	>95	>95
	**37**	C54H93N15O14	1175.7026	588.8586	588.858	1.05	>95	>95
	**38**	C53H91N15O13	1145.6921	573.8533	573.8547	-2.43	>95	>95
	**39**	C49H83N13O12	1045.6300	523.8150	523.8231	-3.06	>95	>95
	**40**	C53H91N15O13	1145.6921	573.8533	573.8541	-1.39	>95	>95
	**41**	C53H91N15O13	1145.6921	573.8533	573.8517	2.88	>95	>95

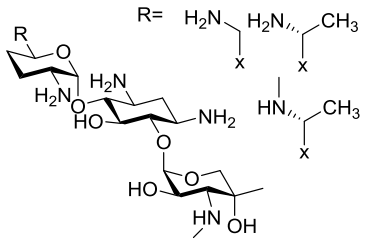
Table S2. Bacterial strains used for Minimum Inhibitory Concentration (MIC) determinations.

Organism	Strain	Strain description	Strain Source
Escherichia coli	ATCC 25922	FDA strain Seattle 1946	ATCC
Klebsiella pneumoniae	ATCC 13883	Control strain	ATCC
Klebsiella pneumoniae	ATCC 700603	Multi-drug resistant	ATCC
Klebsiella pneumoniae	BAA-2146	NDM-1 (New Delhi Metallo-beta-lactamase-1) positive	ATCC
Acinetobacter baumannii	ATCC 19606	Type strain	ATCC
Pseudomonas aeruginosa	ATCC 27853	Type strain	ATCC
Pseudomonas aeruginosa	FADDI-PA070	Clinical isolate, polymyxin resistant	Nation and Li labs, Monash University
Pseudomonas aeruginosa	PA9704	Clinical isolate, polymyxin resistant	Wang Hengzhuang, University Hospital of Copenhagen, Denmark
Acinetobacter baumannii	Ptyela 100734512:2	Clinical isolate, Carbapenem & polymyxin resistant	Ilias Karaiskos and Helen Giamarellou (6th Dept. of Internal Medicine, Hygeia General Hospital, Athens, Greece
Klebsiella pneumoniae	Koprana 100650661:1	Clinical isolate, Carbapenem, & polymyxin resistant	
Klebsiella pneumoniae	138-16357-20362	Clinical isolate, Carbapenem, & polymyxin resistant	Cely S. Abboud (Instituto Dante Pazzanese de Cardiologia, São Paulo, Brazil
Staphylococcus aureus	ATCC 25923	MSSA (methicillin Susceptible *S. aureus*)	ATCC

Figure S1. Structures of Vancomycin and Gentamicin

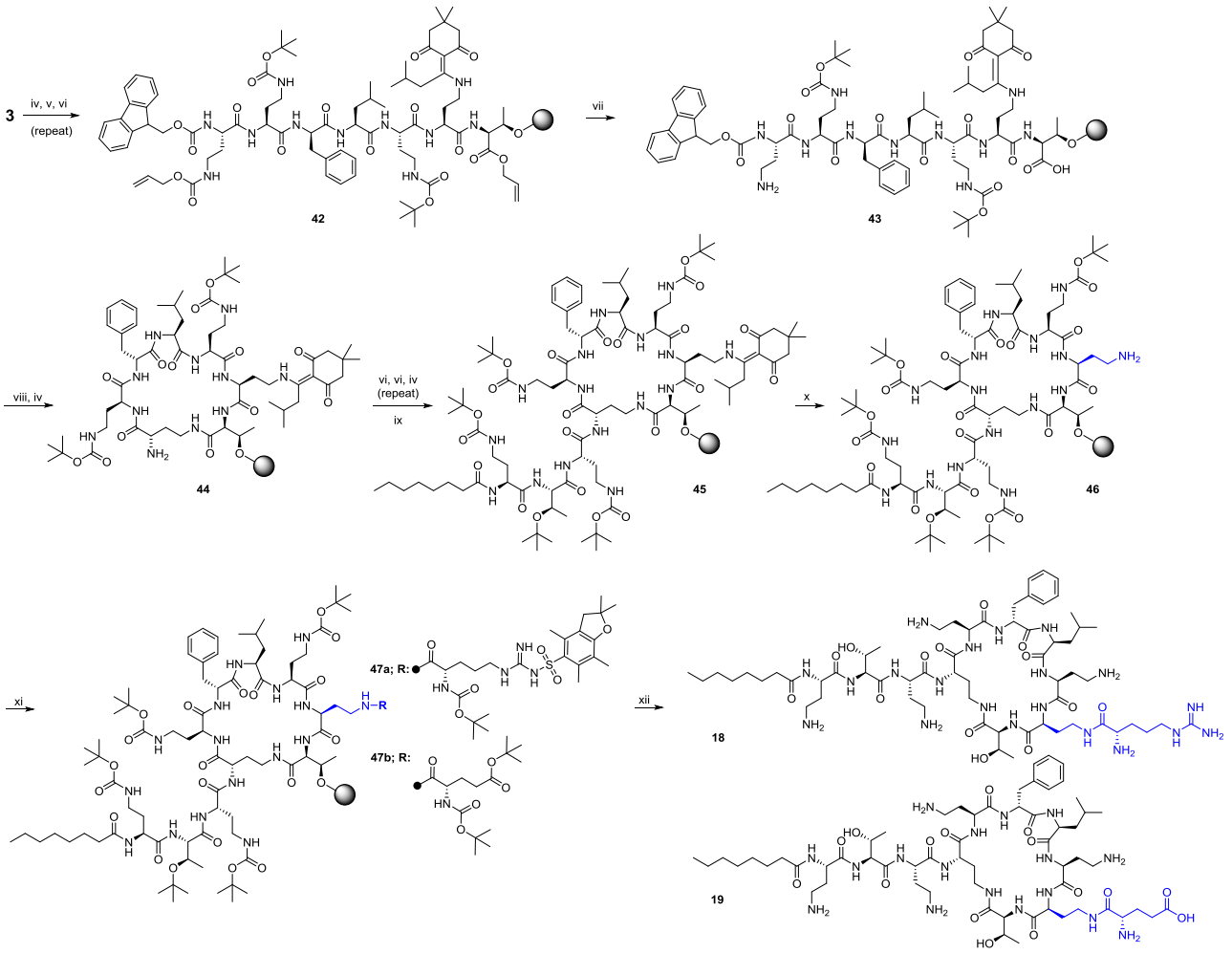


Vancomycin



Gentamicin

Scheme S1. Synthesis example for compounds 18 and 19.



Reagent and conditions: (iv) 30% piperidine, DMF; (v) Solid-phase peptide synthesis (SPPS) with corresponding amino acid (See Table S3), HCTU, DIPEA; (vi) Acetic anhydride, pyridine (50/50, v/v); (vii) $Pd(PPh_3)$, $PhSiH_3$; (viii) DPPA, DIPEA, DMF; (ix) nC7CO, HCTU, DIPEA; (x) 2% H_2NNH_2 in DMF; (xi) Boc-Arg(Pbf)-OH or Boc-Glu(OtBu)-OH, HCTU, DIPEA; (xii) $TFA/Et_3SiH/H_2O$ (95:1:4).

Materials and Methods: Synthesis

All chemicals were obtained from commercial suppliers and used without further purification. All Fmoc α-amino acids including *N*-α-Fmoc-*N*-γ-Boc-L-2,4-diaminobutyric acid (Fmoc-L-Dab(Boc)-OH), *N*-α-(9-Fluorenylmethyloxycarbonyl)-*N*-γ-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl]-L-2,4-diaminobutyric acid (Fmoc-L-Dab(ivDde)-OH), *N*-alpha-(9-fluorenylmethyloxycarbonyl)-4-*t*-butyloxycarbonylamino-L-phenylalanine (Fmoc-L-Phe(4-NHBoc)-OH)), Fmoc-4-phenyl-L-phenylalanine (Fmoc-L-Bip(4,4')-OH, N-α-(9-fluorenylmethyloxycarbonyl)-DL-octylglycine (Fmoc-DL-OctGly-OH), N-α-Fmoc-N-γ-Alloc-L-2,4-diaminobutyric acid (Fmoc-L-Dab(Alloc)-OH), *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) were purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). 3,4-Dihydro-2H-pyran-2-yl-methoxymethyl polystyrene resin (DHP HM resin 100 – 200 mesh) was purchased from Novabiochem (Merck). Peptide grade trifluoroacetic acid (TFA), piperidine, methanol and *N,N*-dimethylformamide (DMF) were purchased from AusPep (Melbourne, Australia). 2-Chlorophenyl isocyanate (2-ClPhNCO), biphenyl-4-carboxylic acid (Ph-4-PhCO), 4-phenoxybenzoic acid (Ph-4-OPhCO), 4-biphenyl isocyanate (Ph-4-PhNCO), octanoic acid (nC7CO), pyridine, acetic anhydride, triethylsilane (Et_3SiH) and diisopropylethylamine (DIPEA), tetrakis(triphenylphosphine)palladium(0) ($Pd[PPh_3]_4$), diphenyl phosphoryl azide (DPPA) and phenylsilane ($PhSiH_3$), allyl bromide, hydrazine (H_2NNH_2), sodium diethyl dithiocarbamate trihydrate $(C_2H_5)_2NCSSNa \times 3H_2O$) and anhydrous 1,2-dichloroethane (DCE) were purchased from Sigma-Aldrich. Gentamicin sulfate (G1914), Polymyxin B sulfate (P0972) and vancomycin hydrochloride hydrate (861987) were purchased from Sigma-Aldrich (Sydney, Australia). Cesium carbonate and pyridinium *p*-toluensulfonate (PPTS) were purchased from AK Scientific (Union City, CA). All other solvents were HPLC grade and all chemicals were used without further purification. LC-MS analysis were conducted using Agilent Technologies 1200 Series Instrument with a G1316A variable wavelength detector set at λ= 210 nm, 1200 Series ELSD, 6110 quadrupole ESI-MS, using an Agilent Eclipse XDB-Phenyl column (3 × 100mm, 3.5 μm particle size, flow rate 1 mL/min, the mobile phases 0.05% formic acid in water and 0.05% formic acid in acetonitrile). Compound purification was done using an Agilent 1260 Infinity Preparative HPLC with a G1365D multiple wavelength detector set at λ= 210 nm and an Agilent Eclipse XDB-Phenyl column 21.2 x 100mm, 5 μm particle

size. Ultraviolet/visible spectra were recorded with a Varian Cary 50 Bio spectrophotometer. Identities of final products were confirmed by MS/MS spectra, obtained using an API QSTAR™ Pulsar Hybrid LC-MS/MS System, high resolution mass spectrometry (HRMS), performed on a Bruker Micro TOF mass spectrometer using (+)-ESI calibrated to sodium formate, and by ^{1}H (600 MHz) and 2D NMR spectra, obtained using a Bruker Avance-600 spectrometer equipped with a TXI cryoprobe in D_2O, referenced externally with NaOAc[66] (δ_H 1.90 and 8.44; 10 mg in 500 μL D_2O) and then internally with the HDO resonance at δ 4.77. Final purity of more than 95% for all compounds was confirmed by LCMS analysis using both ELSD and UV (210 nm) detection.

Fmoc-L-Thr-OAllyl *(**4**; Scheme 1):* A solution of Cs_2CO_3 in H_2O (2.5 g in 10 mL, 25% w/v) was added dropwise to a solution of commercially available Fmoc-L-Thr-OH (**3**) (2.0 g, MW 341.36, 5.86 mmol) in MeOH (300 mL) and H_2O (60 mL) to pH 7-8. The solution was concentrated under vacuum to evaporate most of the MeOH, the resulting mixture was diluted with H_2O and lyophilized to obtain the cesium salt as a white fluffy powder. The crude cesium salt was treated with a solution of allyl bromide (545 μL, d 1.43, MW 120.98, 6.44 mmol, 1.1 equiv) in dry DMF (15 mL) inside a glove box. The resulting suspension became thick and was stirred at rt under nitrogen overnight. The solvent was removed under vacuum and the residue was dissolved in ethyl acetate (50 mL) and washed with 10% aqueous citric acid (2 × 50 mL). The organic layer was separated, dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography (2.5-5% MeOH in DCM) to afford compound **4** (2.2 g, 98.6% yield) as a white solid. LCMS (ESI+): 382.43 $(M + H)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28 (d, *J* = 5.9 Hz, 3H) 4.26 (t, *J* = 6.9 Hz, 1H) 4.33-4.42 (m, 2H) 4.44 (d, *J* = 7.0 Hz, 2H) 4.70 (d, *J* = 5.6 Hz, 2H) 5.22-5.31 (m, 1H) 5.36 (d, *J* = 17.0 Hz, 1 H) 5.59 (d, *J* = 8.8 Hz, 1H) 5.93 (ddt, *J* = 16.9, 11.1, 5.6 Hz, 1H) 7.29-7.38 (m, 2H) 7.38-7.48 (m, 2H) 7.57-7.68 (m, 2H) 7.78 (d, *J* = 7.3 Hz, 2H).

DHP-Resin Fmoc-L-Thr-OAllyl *(**5**; Scheme 1):* A solution of PPTS (1.26 g, MW 251.30, 5.0 mmol, 0.5 eq) in dry DCE (80 mL) was added to compound **4** (15.26 g, MW 381.43, 40 mmol, 4 equiv) in a glove box. The resulting solution was added to DHP polystyrene resin (12.5 g, loading 0.8 mmol/g, 10 mmol) in a round bottom flask. The mixture was heated at 80 °C for four days without agitation, and then allowed to cool to rt. The reaction was quenched with dry pyridine (811 μL, 10 mmol, 1 eq.). The resin was filtered under vacuum, washed with DMF (× 3), MeOH (× 3) and DCM (× 3), and dried under high vacuum overnight to provide resin **5** with 0.62 mmol/g,

determined by Fmoc removal and subsequent UV detection of liberated dibenzofulvene-piperidine adduct. The bulk resin was divided in labeled polypropylene SPE tubes with PE frits (6 mL), 100 mg of resin (**5**) in each tube.

DHP-Resin NH-L-Thr-OAllyl *(**6**; Scheme 1)*: Fmoc protected resin **5** (100 mg, loading 0.62 mmol/g) was treated with a solution of 30% piperidine in DMF (2 mL) and shaken at rt for 15 min. The Fmoc deprotected resin **6** was drained and washed with DMF (× 3), MeOH (× 3) and DCM (× 3). The deprotection step was repeated.

General method for Peptide Coupling *(Compound 5 in Scheme 1 as example)*: A solution of the corresponding amino acid (see **Table S3** for amounts; 0.14 mmol, 2 eq.), DMF (0.29 mL), HCTU (0.5 M in DMF, 0.29 mL, 0.14 mmol, 2 eq.) and DIPEA (50 µL, 0.14 mmol, 4 eq.) was reacted for 1 min and then added to the deprotected resin **6** (100 mg; TL: 0.62 mmol/g). The resin was shaken for 30 min and then washed with DMF (× 3), MeOH (× 3) and DCM (× 3). The coupling step was repeated. An analytical sample was cleaved with TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm coupling with corresponding amino acid.

General method for O-Allyl/N-Alloc deprotection *(Compound **6** to **7** in Scheme 1 as example)*: The corresponding O-Allyl / N-Alloc protected heptapeptide DHP-resin **6** (100 mg, 0.62 mmol) was washed with dry DCM (× 3). A solution of $Pd(PPh_3)_4$ (MW 1155.6, 215 mg, 0.186 mmol, 0.25 equiv) in dry DCM (2 mL) was added to the resin followed by phenylsilane (MW 108.21, d 0.878, 2.19 mL, 17.76 mmol, 24 equiv). The resin was carefully shaken and the excess pressure was released several times until no increase in pressure was observed. The resin was then shaken at rt for 2 h, drained and washed with 0.5% DIPEA in DCM (× 5), 0.5% w/v sodium diethyl dithiocarbamate trihydrate in DMF (× 8), MeOH (× 3) and DCM (× 3) and dried under vacuum. An analytical sample was cleaved using TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm complete *bis*-deprotection.

General method for DHP-resin cyclization *(Compound **7** to **8** in Scheme 1 as example)*: The O-Allyl / N-Alloc deprotected resin **7** (100 mg) was washed with DCM (× 3) and DMF (× 3) and then treated with a solution of DPPA (MW 275.2, d 1.277, 5.0 equiv) and DIPEA (10 equivalents) in DMF (2 mL). The resin was shaken at room temperature overnight, drained and washed with DMF

(× 3), MeOH (× 3) and DCM (× 3). An analytical sample was cleaved using TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm complete cyclization.

General method for Fatty Acid Coupling*:* A solution of the corresponding fatty acid (see **Table S3** for amounts; 0.14 mmol, 2 equiv), DMF (0.29 mL), HCTU (0.5 M in DMF, 0.29 mL, 0.14 mmol, 2 eq.) and DIPEA (50 µL, 0.14 mmol, 4 equiv) was reacted for 1 min and then added to the deprotected resin **9** (100 mg; TL: 0.62 mmol/g). The resin was shaken for 30 min and then washed with DMF (× 3), MeOH (× 3) and DCM (× 3). An analytical sample was cleaved with TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm coupling.

DHP resin cleavage*:* A solution of TFA/Et_3SiH/H_2O (95:1:4) was added to the resin and the reaction vessel was shaken for 30 min at room temperature. The solvent was captured in a 20 mL vial, the resin was then washed with DCM (× 3), THF (× 3) and DCM (× 3) and all the fractions were combined to be evaporated under a stream of nitrogen. The crude peptides were dissolved in 5 mL of acetonitrile/water (50/50, v/v) and the samples were freeze dried.

General method for ivDde removal *(Scheme 2 as example)*: A solution of 2% hydrazine hydrate in DMF (2 mL) was added to the reaction vessel containing the corresponding resin for peptides **18d**, **19d**, **22d**, **23d**, **31d** and **32d** (100 mg each). The reaction vessel was shaken for 1 h at rt, the solvent was drained and fresh 2% hydrazine hydrate in DMF (2 mL) was added and the vessel shaken for an extra hour at rt. The solvent was drained, washed with DMF (× 3), MeOH (× 3), and DCM (× 3) and dried under vacuum. An analytical sample was cleaved using TFA/Et_3SiH/H_2O (95:1:4) followed by LCMS analysis to confirm complete deprotection.

General method for L-Arg and L-Glu coupling *(Scheme 2 as example)*: A solution of the corresponding amino acid (Boc-Arg(Pbf)-OH, Boc-Glu(OtBu)-OH; 0.14 mmol, 2 eq.), DMF (0.29 mL), HCTU (0.5 M in DMF, 0.29 mL, 0.14 mmol, 2 equiv) and DIPEA (50 µL, 0.14 mmol, 4 equiv) was reacted for 1 min and then added to the deprotected resin (100 mg; TL: 0.62 mmol/g). The resin was shaken for 30 min and then washed with DMF (× 3), MeOH (× 3) and DCM (× 3). The coupling step was repeated. An analytical sample was cleaved with TFA/Et_3SiH/H_2O (95:1:4) and the coupling was monitored by LCMS analysis to confirm product.

Compound **10** *(Scheme 1)*: The crude product (39 mg) was dissolved in 2 mL acetonitrile:H_2O (1:1) and separated using an Agilent 1260 Infinity Prep HPLC on an Agilent Eclipse XDB-Phenyl

21.2 × 100 mm 5 μm column (flow 20 mL/min, mobile phase A = 0.05% formic acid in water and B = 0.05% formic acid in acetonitrile, gradient 5→100% B over 20 min) and then lyophilized. The compound as a formate salt was converted to TFA by re-dissolving the compound with 0.1% TFA in acetonitrile:water (1:1) (concentration of 0.5 mg/mL) and lyophilized to give a white powder (6.5 mg, 17% yield). LC-UV purity: >95%. LC-ELSD purity: >95%. ^{1}H NMR (500 MHz, D_2O) δ 7.88 (d, *J* = 8.4 Hz, 2H), 7.80 (d, *J* = 8.4 Hz, 2H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.52 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.34 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.30 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.20 (d, *J* = 7.5Hz, 2H), 4.70 (dd, *J* = 5.3, 9.5 Hz, 1H), 4.53 (t, *J* = 8.2 Hz, 1H), 4.45 (dd, *J* = 5.2, 9.5 Hz, 1H), 4.42 (dd, *J* = 5.2, 9.0 Hz, 1H), 4.38 (d, *J* = 4.4 Hz, 1H), 4.25–4.16 (m, 6H), 4.14 (d, *J* = 4.6 Hz, 1H), 3.29 (ddd, *J* = 7.6, 7.6, 13.9 Hz, 1H), 3.20–3.00 (m, 13H), 2.82 (ddd, *J* = 4.9, 10.1, 12.7 Hz, 1H), 2.73 (ddd, *J* = 6.1, 9.8, 12.7 Hz, 1H), 2.32 (m, 1H), 2.26–1.79 (m, 11H), 1.45 (ddd, *J* = 4.0, 9.7, 14.0 Hz, 1H), 1.36 (ddd, *J* = 3.9, 11.1, 14.0 Hz, 1H), 1.19 (d, *J* = 6.5 Hz, 3H), 1.13 (d, *J* = 6.5 Hz, 3H), 0.79 (m, 1H), 0.72 (d, *J* = 6.5 Hz, 3H), 0.65 (d, *J* = 6.5 Hz, 3H). MS-ESI (*m/z*) 1243.8 $[M + H]^+$, 622.4 $[M + 2H]^{2+}$, 415.3 $[M + 3H]^{3+}$. HRMS-ESI (*m/z*): $[M + 2H]^{2+}$ calcd for $(C_{60}H_{90}N_{16}O_{13} + 2H)/2$, 622.3509; found 622.3512.

Compound **14:** The crude product (34 mg) was dissolved in 2 mL acetonitrile:water (1:1) and separated using a Agilent 1260 Infinity Prep HPLC on an Agilent Eclipse XDB-Phenyl 21.2 × 100 mm 5 μm column (flow 20 mL/min, mobile phases A = 0.05% formic acid in water and B = 0.05% formic acid in acetonitrile, gradient 5→100% B over 20 min) and then lyophilized. The pure compound 14 as a formate salt was converted to TFA salt by re-dissolving it with 0.1% TFA in ACN: H_2O (1:1) (concentration of 0.5 mg/mL) and lyophilized to give a white powder (7.5 mg, 22% yield). LC-UV purity: >95%. LC-ELSD purity: >95%. ^{1}H NMR (500 MHz, D_2O) δ 7.89 (d, *J* = 8.8 Hz, 2H), 7.44 (dd, *J* = 7.6, 8.8 Hz, 2H), 7.35 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.30 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.25 (dd, *J* = 7.6, 7.6 Hz, 1H), 7.22 (d, *J* = 7.5Hz, 2H), 7.12 (d, *J* = 7.6 Hz, 2H), 7.08 (d, *J* = 8.8 Hz, 2H), 4.66 (dd, *J* = 5.3, 9.5 Hz, 1H), 4.54 (t, *J* = 8.2 Hz, 1H), 4.45 (t, *J* = 5.5 Hz, 1H), 4.40 (t, *J* = 5.6 Hz, 1H), 4.35 (d, *J* = 4.4 Hz, 1H), 4.25–4.16 (m, 6H), 4.15 (d, *J* = 4.7 Hz, 1H), 3.28 (ddd, *J* = 7.6, 7.6, 13.9 Hz, 1H), 3.15–3.03 (m, 13H), 2.82 (ddd, *J* = 4.9, 10.1, 12.7 Hz, 1H), 2.73 (ddd, *J* = 6.1, 9.8, 12.7 Hz, 1H), 2.32–1.79 (m, 12H), 1.45 (ddd, *J* = 4.0, 9.7, 14.0 Hz, 1H), 1.37 (ddd, *J* = 3.9, 11.1, 14.0 Hz, 1H), 1.17 (d, *J* = 6.5 Hz, 3H), 1.14 (d, *J* = 6.4 Hz, 3H), 0.79 (m, 1H), 0.73 (d, *J* = 6.5 Hz, 3H), 0.66 (d, *J* = 6.4 Hz, 3H). MS-ESI (*m/z*) 1259.8 $[M + H]^+$, 630.4 $[M + 2H]^{2+}$, 420.5 [M

+ 3H]$^{3+}$. HRMS-ESI (*m/z*): [M + 2H]$^{2+}$ calcd for ($C_{60}H_{90}N_{16}O_{14}$ + 2H)/2, 630.3484; found 630.3487.

Compound **15**: The crude product (40 mg) was dissolved in 2 mL acetonitrile:water (1:1) and separated using a Agilent 1260 Infinity Prep HPLC on an Agilent Eclipse XDB-Phenyl 21.2 × 100 mm 5 μm column (flow 20 mL/min, mobile phases A = 0.05% formic acid in water and B = 0.05% formic acid in acetonitrile, gradient 5→100% B over 20 min) and then lyophilized. The final compound **15** as a formate salt was converted to TFA salt by re-dissolving it with 0.1% TFA in ACN: H_2O (1:1) (concentration of 0.5 mg/mL) and lyophilized to give a white powder (7.5 mg, 18% yield). LC-UV purity: >95%. LC-ELSD purity: >95%. ^{1}H NMR (500 MHz, D_2O) δ 7.66 (d, *J* = 8.5 Hz, 2H), 7.65 (d, *J* = 8.5 Hz, 2H), 7.48 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.39 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.34 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.29 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.20 (d, *J* = 7.5Hz, 2H), 4.53 (t, *J* = 8.2 Hz, 1H), 4.42 (dd, *J* = 5.2, 9.2 Hz, 2H), 4.41 (dd, *J* = 5.0, 9.7 Hz, 1H), 4.36 (d, *J* = 4.5 Hz, 1H), 4.27–4.16 (m, 6H), 4.14 (d, *J* = 4.7 Hz, 1H), 3.29 (ddd, *J* = 7.6, 7.6, 13.9 Hz, 1H), 3.15–2.99 (m, 13H), 2.80 (ddd, *J* = 4.9, 10.1, 12.7 Hz, 1H), 2.71 (ddd, *J* = 6.1, 9.8, 12.7 Hz, 1H), 2.29–1.76 (m, 12H), 1.45 (ddd, *J* = 4.0, 9.7, 14.0 Hz, 1H), 1.37 (ddd, *J* = 3.9, 11.1, 14.0 Hz, 1H), 1.20 (d, *J* = 6.6 Hz, 3H), 1.12 (d, *J* = 6.3 Hz, 3H), 0.80 (m, 1H), 0.73 (d, *J* = 6.5 Hz, 3H), 0.66 (d, *J* = 6.5 Hz, 3H). MS-ESI (*m/z*) 1258.9 [M + H]$^+$, 629.9 [M + 2H]$^{2+}$, 420.3 [M + 3H]$^{3+}$. HRMS-ESI (*m/z*): [M + 2H]$^{2+}$ calcd for ($C_{60}H_{91}N_{17}O_{13}$ + 2H)/2, 629.8564; found 629.8594.

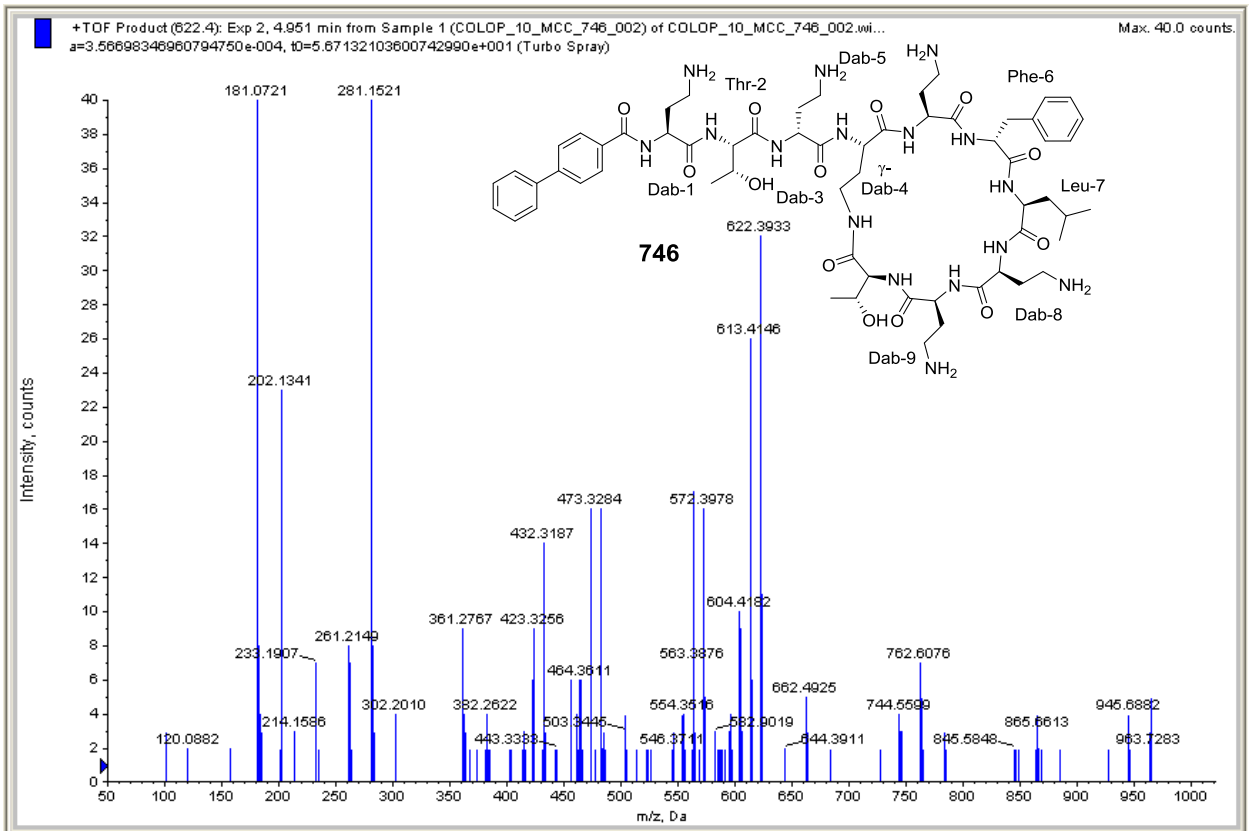
Compound **38**: The crude product (42 mg) was dissolved in 2 mL acetonitrile:H_2O (1:1) and separated using a Agilent 1260 Infinity Prep HPLC on an Agilent Eclipse XDB-Phenyl 21.2 × 100 mm 5 μm column (flow 20 mL/min, mobile phases A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, gradient 5→100% B over 20 min) and then lyophilized to give a white powder (6.1 mg, 15% yield). LC-UV purity: >95%. LC-ELSD purity: >95%. ^{1}H NMR (500 MHz, D_2O) δ 7.35 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.30 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.22 (d, *J* = 7.5Hz, 2H), 4.53 (t, *J* = 8.3 Hz, 1H), 4.47 (dd, *J* = 5.2, 9.3 Hz, 1H), 4.46 (dd, *J* = 5.3, 9.1 Hz, 1H), 4.32 (d, *J* = 4.0 Hz, 1H), 4.26–4.21 (m, 4H), 4.18 (dq, d, *J* = 6.2, 6.2 Hz, 1H), 4.15 (m, 1H), 4.14 (d, *J* = 5.0 Hz, 1H), 3.95 (s, 2H), 3.33 (ddd, *J* = 7.6, 7.6, 13.9 Hz, 1H), 3.13–2.99 (m, 13H), 2.85 (ddd, *J* = 4.9, 10.1, 12.7 Hz, 1H), 2.76 (ddd, *J* = 6.1, 9.8, 12.7 Hz, 1H), 2.28 (t, *J* = 7.4Hz, 2H), 2.28–1.81 (m, 12H), 1.56 (tt, *J* = 7.4, 7.4 Hz, 2H), 1.44 (ddd, *J* = 4.0, 9.7, 14.0 Hz, 1H), 1.37 (ddd, *J* = 3.9, 11.1, 14.0 Hz, 1H), 1.26–1.20 (m, 8H), 1.17 (d, *J* = 6.5 Hz, 3H), 1.15 (d, *J* = 6.5 Hz, 3H), 0.81 (t, *J* = 7.0 Hz, 3H), 0.77 (m, 1H), 0.72

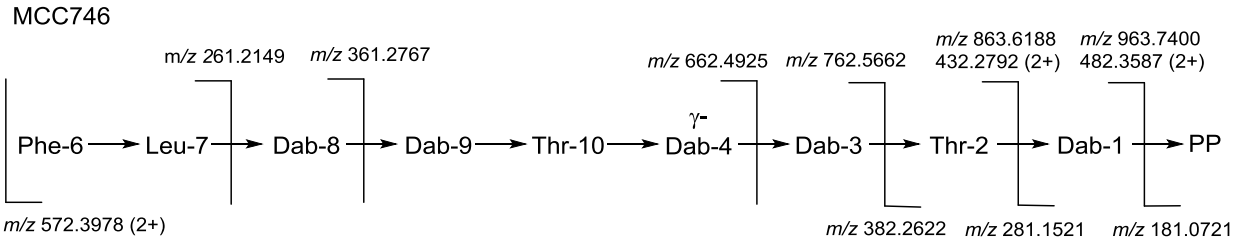
(d, J = 6.3 Hz, 3H), 0.65 (d, J = 6.2 Hz, 3H). MS-ESI (*m/z*) 1146.7 $[M + H]^+$, 573.8 $[M + 2H]^{2+}$, 382.9 $[M + 3H]^{3+}$. HRMS-ESI (*m/z*): $[M + 2H]^{2+}$ calcd for $(C_{53}H_{91}N_{15}O_{13} + 2H)/2$, 573.8533; found 573.8547.

Table S3. Summary of amino acids and the calculated amounts.

Position	Coupling Condition	Amino acid / Fatty acid	MW	eq	N	mmol	amount (mg)	DMF (µL)	HCTU (µL 0.5M)	DIPEA (µL)
P9	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-Dab(ivDde)-OH	546.65	2	1	0.14	79	288	288	50
	c	Fmoc-Phe(4-NHBoc)-OH	502.57	2	1	0.14	72	288	288	50
	d	Fmoc-Arg(Pfb)-OH	648.77	2	1	0.14	93	288	288	50
P8	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-Dab(ivDde)-OH	546.65	2	1	0.14	79	288	288	50
	c	Fmoc-Arg(Pfb)-OH	648.77	2	1	0.14	93	288	288	50
	d	Fmoc-HomoSer(Trt)-OH	583.68	2	1	0.14	84	288	288	50
	e	Fmoc-Phe(4-NHBoc)-OH	502.57	2	1	0.14	72	288	288	50
P7	**a**	Fmoc-Leu-OH	353.40	2	1	0.14	51	288	288	50
	b	Fmoc-Bip(4,4')-OH	463.53	2	1	0.14	67	288	288	50
	c	Fmoc-DL-OctGly-OH	409.52	2	1	0.14	59	288	288	50
P6	**a**	Fmoc-D-Phe-OH	387.43	2	1	0.14	56	288	288	50
	b	Fmoc-D-Bip(4,4')-OH	463.53	2	1	0.14	67	288	288	50
	c	Fmoc-DL-OctGly-OH	409.52	2	1	0.14	59	288	288	50
P5	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-Dab(ivDde)-OH	546.65	2	1	0.14	79	288	288	50
	c	Fmoc-HomoSer(Trt)-OH	583.68	2	1	0.14	84	288	288	50
	d	Fmoc-Phe(4-NHBoc)-OH	502.57	2	1	0.14	72	288	288	50
	e	Fmoc-Arg(Pfb)-OH	648.77	2	1	0.14	93	288	288	50
P4		Fmoc-Dab(Alloc)-OH	424.45	2	1	0.14	61	288	288	50
P3	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-D-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	c	Fmoc-D-Ser(tBu)-OH	383.4	2	1	0.14	55	288	288	50
	d	Fmoc-Gly-OH	297.3	2	1	0.14	43	288	288	50
	e	Fmoc-Thr(tBu)-OH	397.48	2	1	0.14	57	288	288	50
P2	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-Thr(tBu)-OH	397.48	2	1	0.14	57	288	288	50
P1	**a**	Fmoc-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	b	Fmoc-D-Dab(Boc)-OH	440.49	2	1	0.14	63	288	288	50
	c	Fmoc-Gly-OH	297.3	2	1	0.14	43	288	288	50
Fatty Acids		$nC7CO_2H$	144.21	2	1	0.14	21	288	0.288	50
		Ph-4-$PhCO_2H$	198.07	2	1	0.14	29	288	0.288	50
		PhO-4-$PhCO_2H$	214.06	2	1	0.14	31	288	0.288	50
		Ph-4-PhNCO	195.22	2	1	0.14	28	288	0.288	50
		2-ClPhNCO	153.57	2	1	0.14	22	288	0.288	50

All amino acids are L-isomers unless otherwise indicated.
Fmoc-Dab(Boc)-OH = *N*-α-Fmoc-*N*-γ-Boc-L-2,4-diaminobutyric acid
Fmoc-Dab(ivDde)-OH = *N*-α-Fmoc-*N*-γ-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl]-L-2,4-diaminobutyric acid
(Fmoc-Phe(4-NHBoc)-OH)) = *N*-α-Fmoc-4-*t*-butyloxycarbonylamino-L-phenylalanine
Fmoc-Bip(4,4')-OH = Fmoc-4-phenyl-L-phenylalanine
Fmoc-DL-OctGly-OH = *N*-α-Fmoc-DL-octylglycine
Fmoc-Dab(Alloc)-OH = *N*-α-Fmoc-*N*-γ-Alloc-L-2,4-diaminobutyric acid





Other fragments:
Dab-Leu-Thr *m/z* 302.2010
Dab-Thr *m/z* 202.1341

Figure S2a. (+)-ESI-TOF-MS/MS fragmentation assignments of compound 10 from fragmentation of the $[M+2H]^{2+}$ mass ion peak at *m/z* 622.4, which were consistent with those previously reported for other polymyxins.[1-3]

Mass Spectrum Molecular Formula Report

Analysis Info

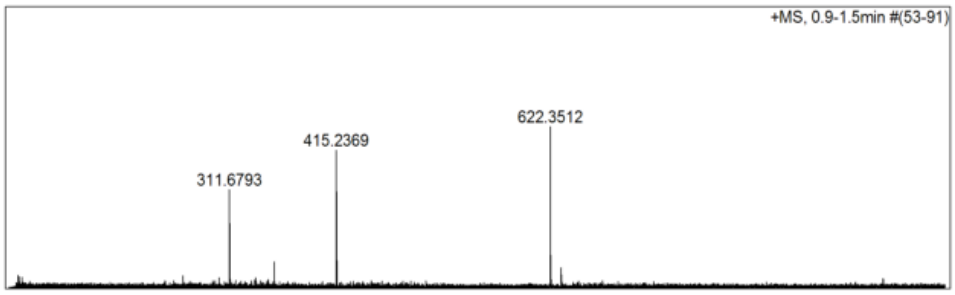
Analysis Name	D:\Data\cooper\MCC_000746_002_RA4_01_8660.d	Acquisition Date	9/19/2014 4:37:32 PM
Method	tune-wide_50ul_hystar_withcal_direct_medhighmass_2.m	Operator	a.piggott
Sample Name	MCC_000746_002	Instrument / Ser#	micrOTOF 232
Comment	Comments		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	100 m/z	Set Capillary	4500 V	Set Dry Gas	5.0 l/min
Scan End	1500 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C60H92N16O13						
Formula, max.	C60H92N16O13						
Measured m/z	622.351	Tolerance	10	mDa	Charge	2	
Check Valence	no	Minimum	0		Maximum	0	
Nirogen Rule	yes	Electron Configuration	both				
Filter H/C Ratio	yes	Minimum	0		Maximum	3	
Estimate Carbon	yes						



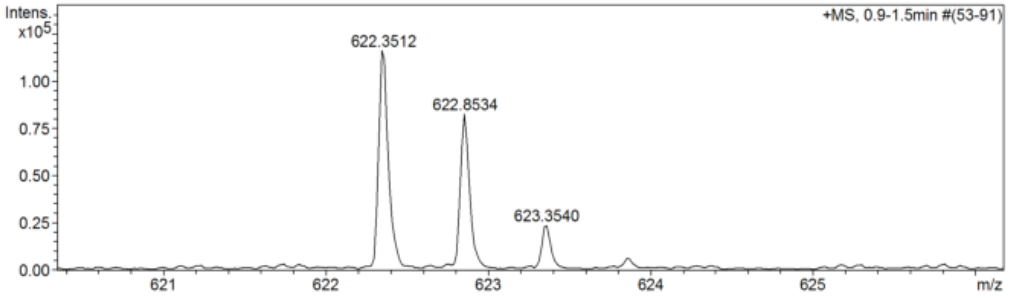


Figure S2b. HR-(+)-ESI-TOF-MS of the $[M+2H]^{2+}$ mass ion peak of compound 10.

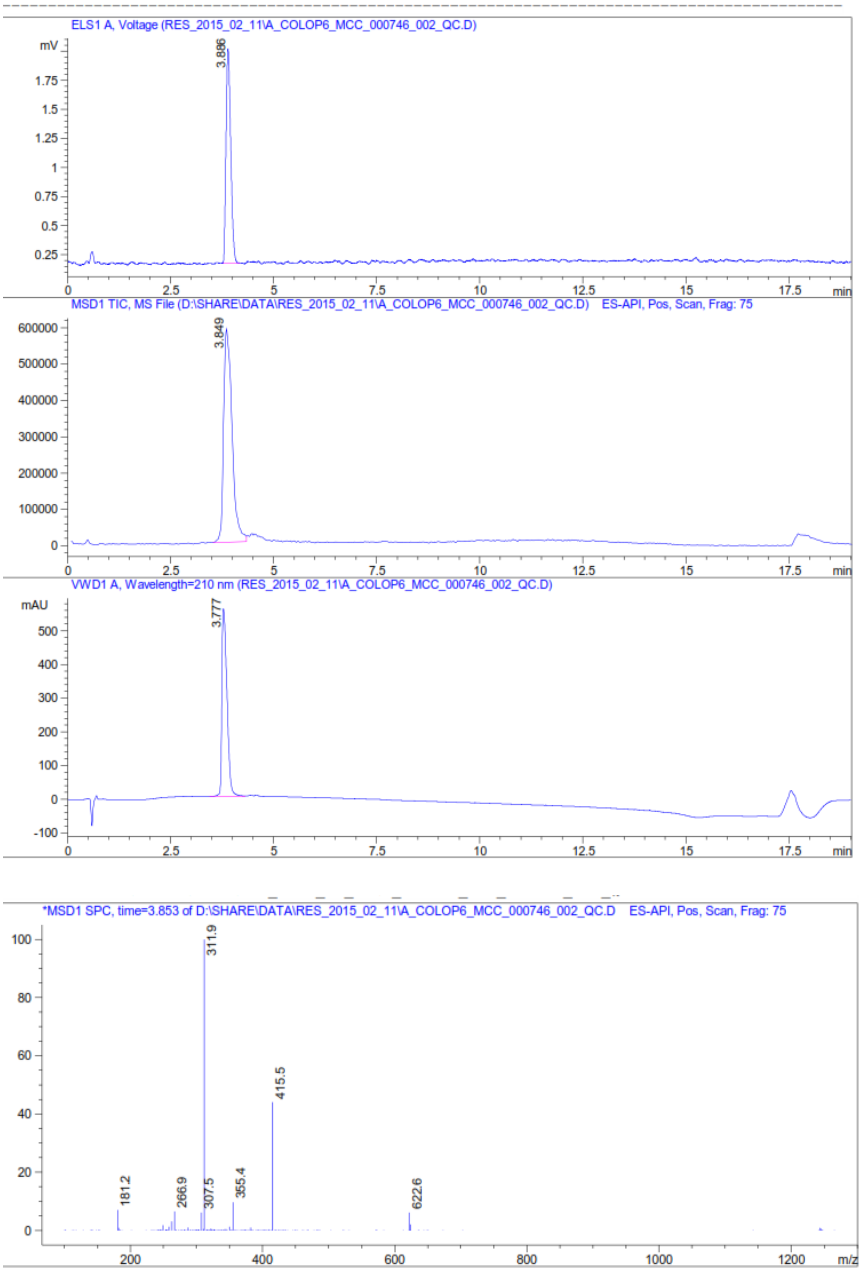


Figure S2c. LC-MS analysis of compound 10 showing ELSD, TIC, UV-vis absorption at 210 nm and ESI-MS.

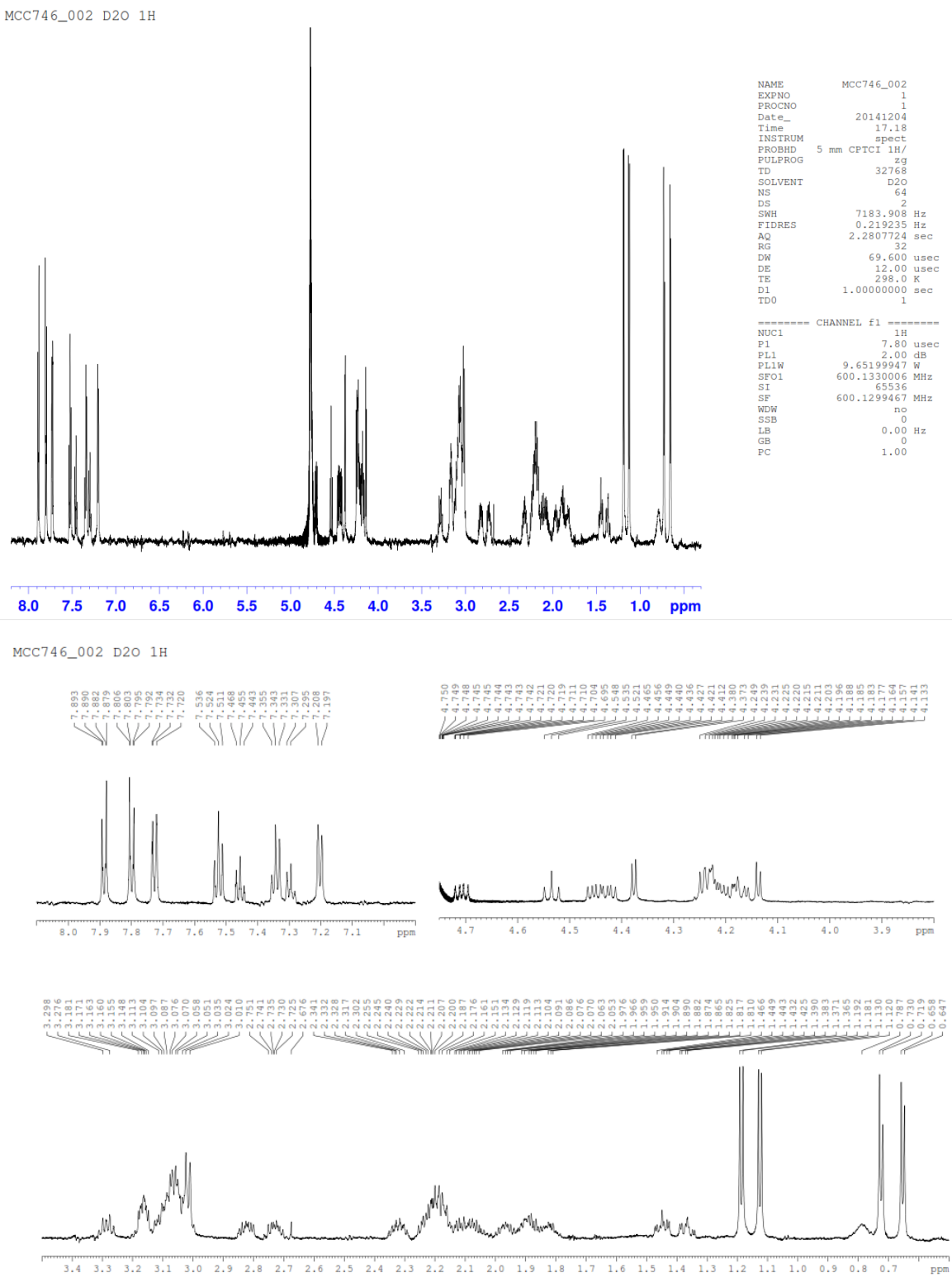


Figure S2d. ^{1}H NMR in D_2O of compound 10.

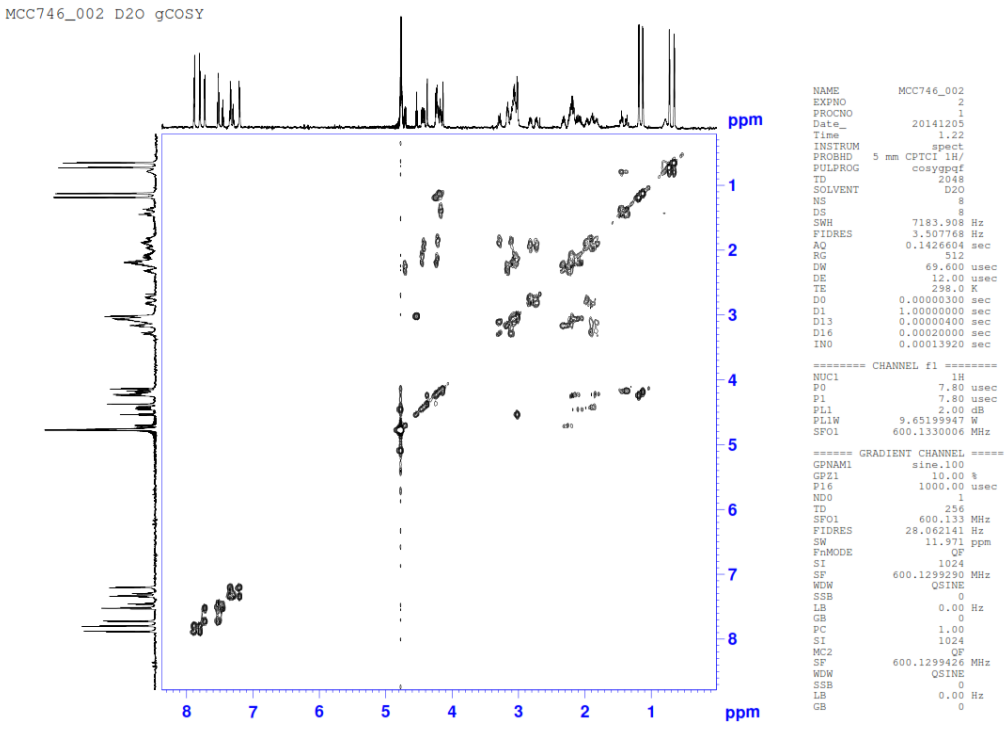


Figure S2e. gCOSY spectra in D2O of compound 10.

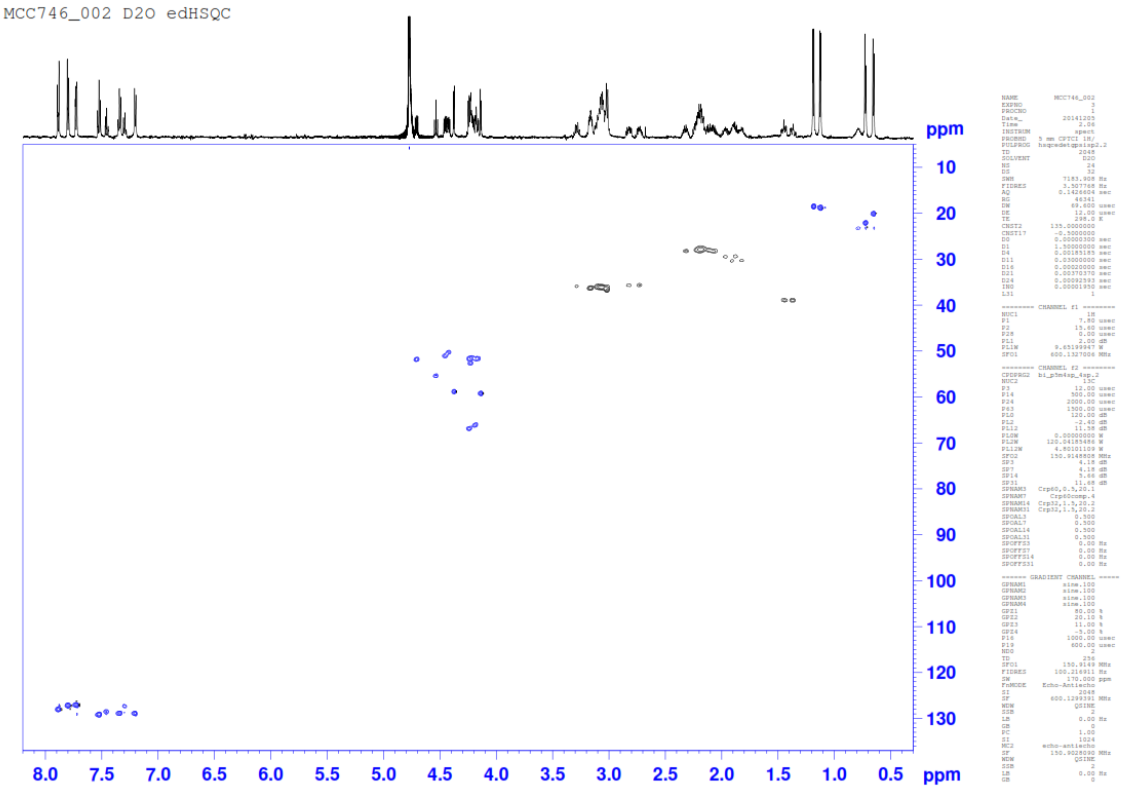
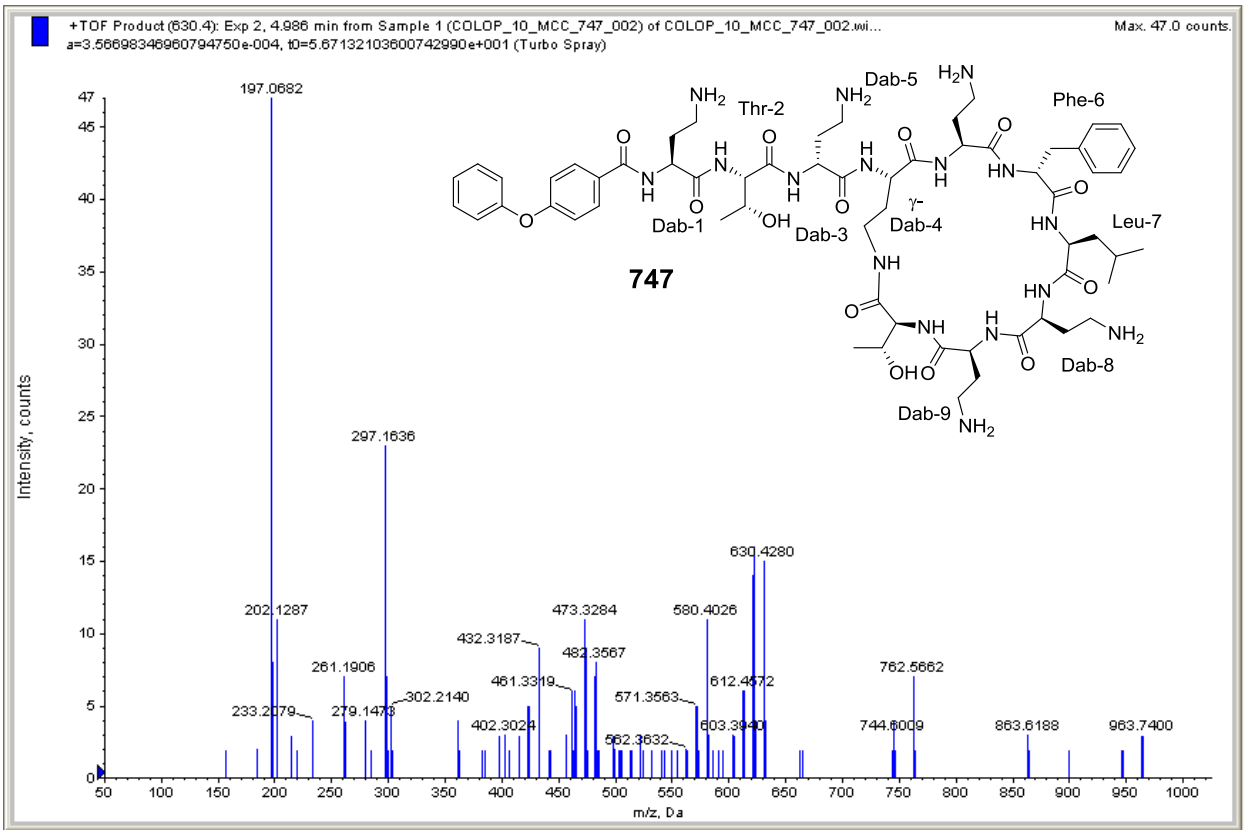


Figure S2f. edHSQC spectra in D_2O of compound 10.



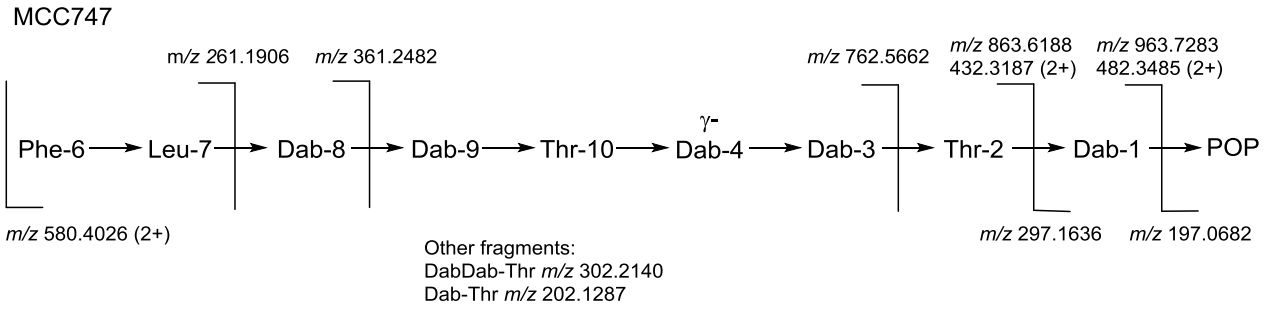


Figure S3a. (+)-ESI-TOF-MS/MS fragmentation assignments of compound 14 from fragmentation of the $[M+2H]^{2+}$ mass ion peak at *m/z* 630.4, which were consistent with those previously reported for other polymyxins.[1-3]

Mass Spectrum Molecular Formula Report

Analysis Info

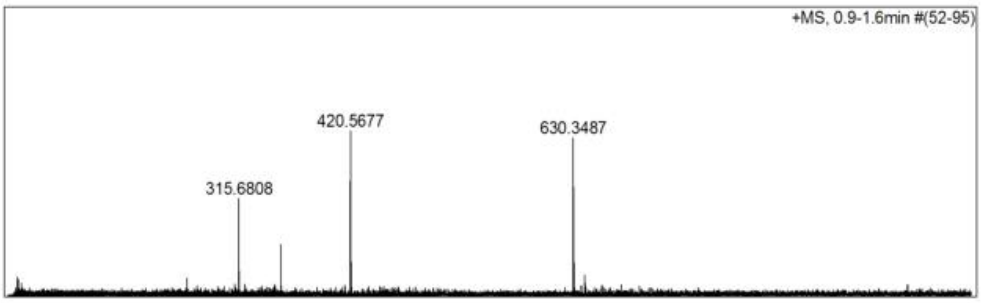
Analysis Name	D:\Data\cooper\MCC_000747_002_RA5_01_8662.d	Acquisition Date	9/19/2014 4:48:33 PM
Method	tune-wide_50ul_hystar_withcal_direct_medhighmass_2.m	Operator	a.piggott
Sample Name	MCC_000747_002	Instrument / Ser#	micrOTOF 232
Comment	Comments		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	100 m/z	Set Capillary	4500 V	Set Dry Gas	5.0 l/min
Scan End	1500 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C60H92N16O14				
Formula, max.	C60H92N16O14				
Measured m/z	630.349	Tolerance	10 mDa	Charge	2
Check Valence	no	Minimum	0	Maximum	0
Nirogen Rule	yes	Electron Configuration	both		
Filter H/C Ratio	yes	Minimum	0	Maximum	3
Estimate Carbon	yes				



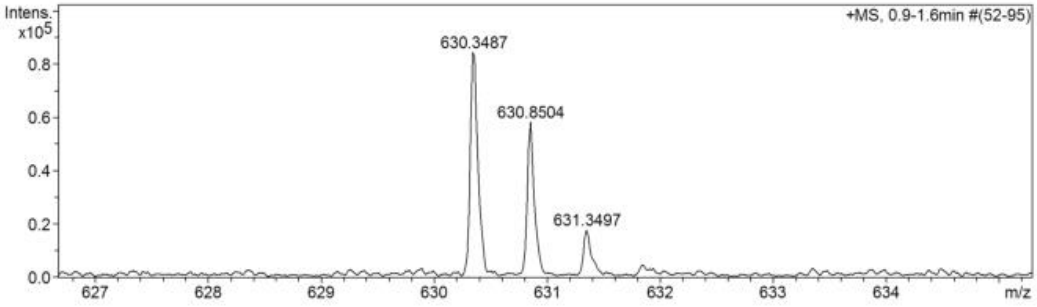


Figure S3b. HR-(+)-ESI-TOF-MS of the $[M+2H]^{2+}$ mass ion peak of compound 14.

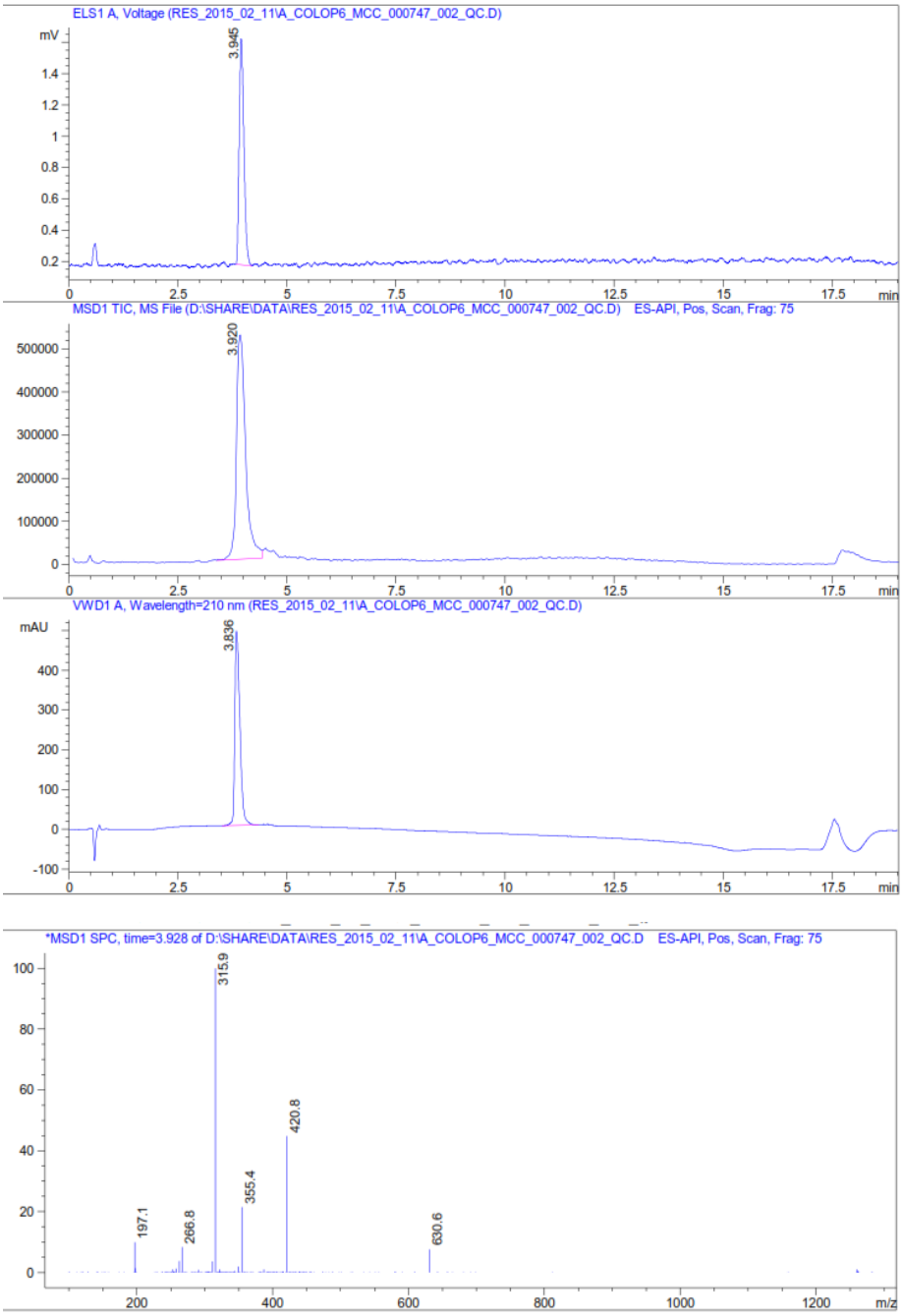


Figure S3c. LC-MS analysis of compound 14 showing ELSD, TIC, UV-vis absorption at 210 nm and ESI-MS.

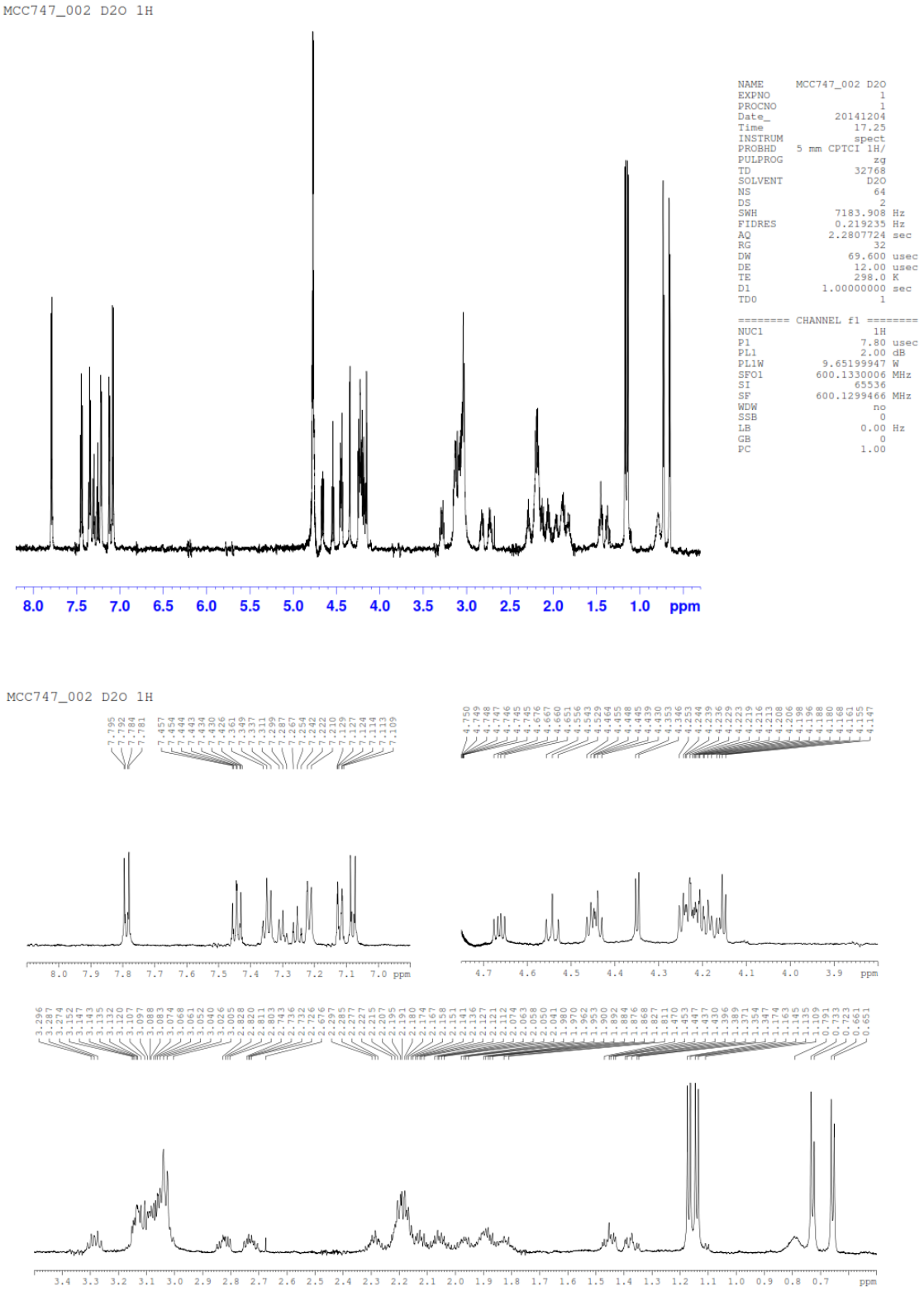


Figure S3d. ^{1}H NMR in D_2O of compound 14.

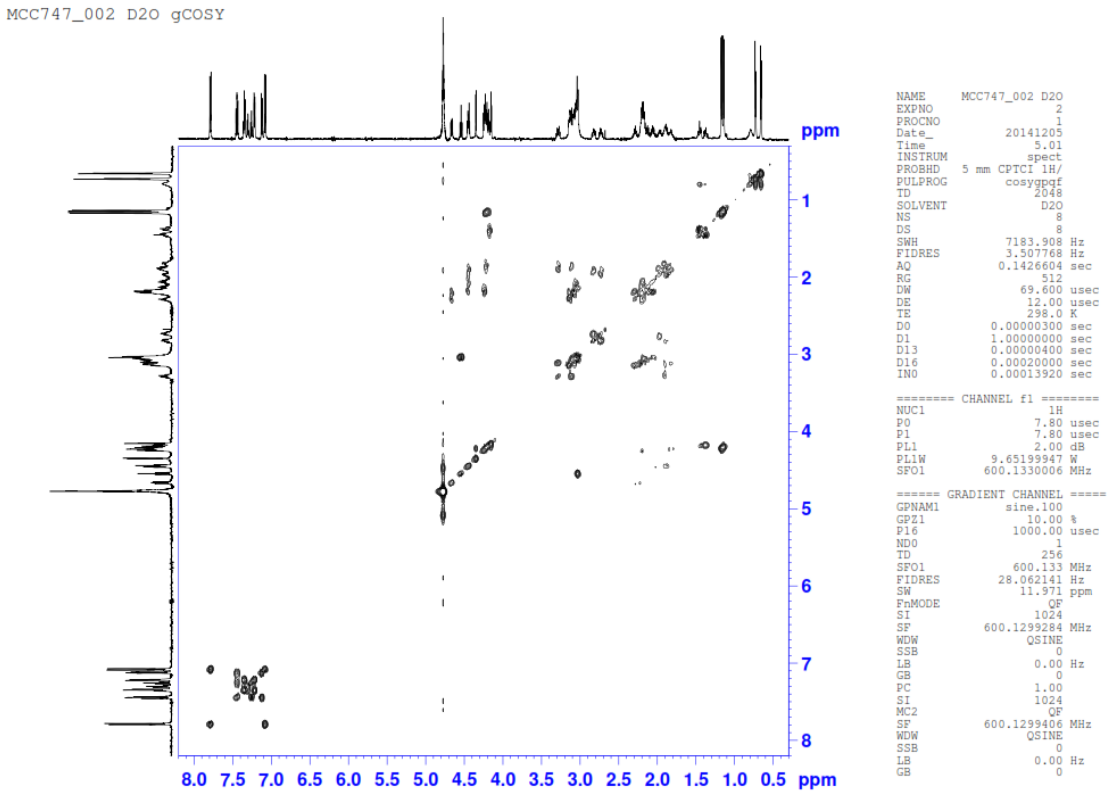


Figure S3e. gCOSY spectra in D_2O of compound 14.

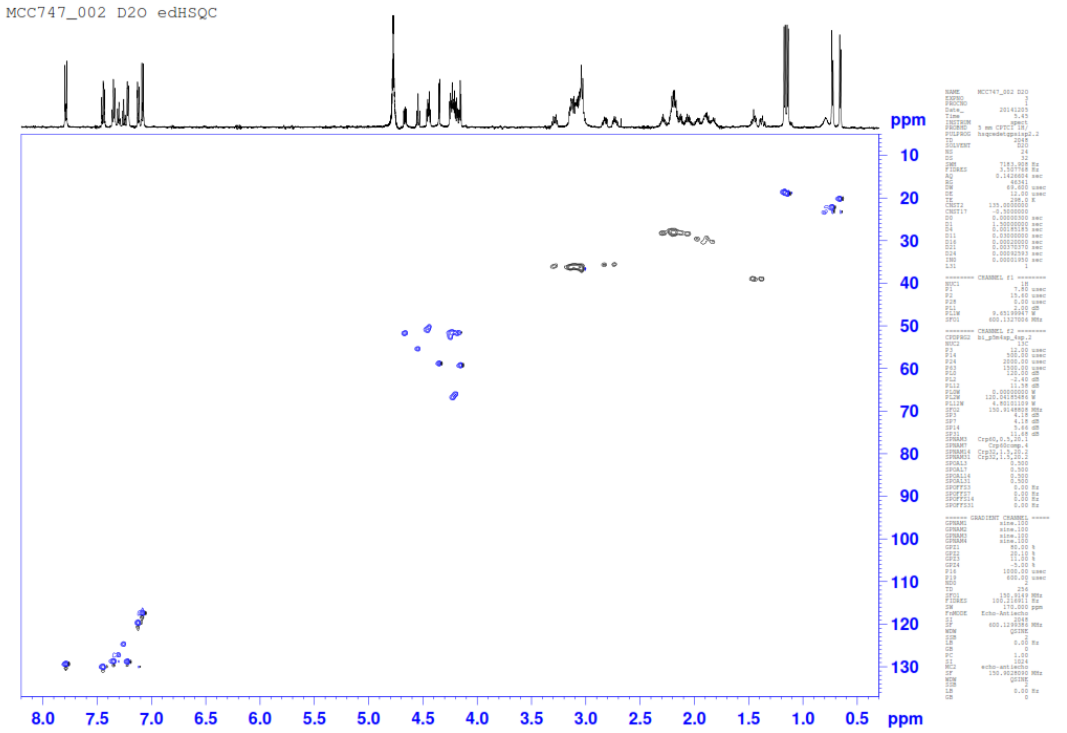


Figure S3f. edHSQC spectra in D2O of compound 14.

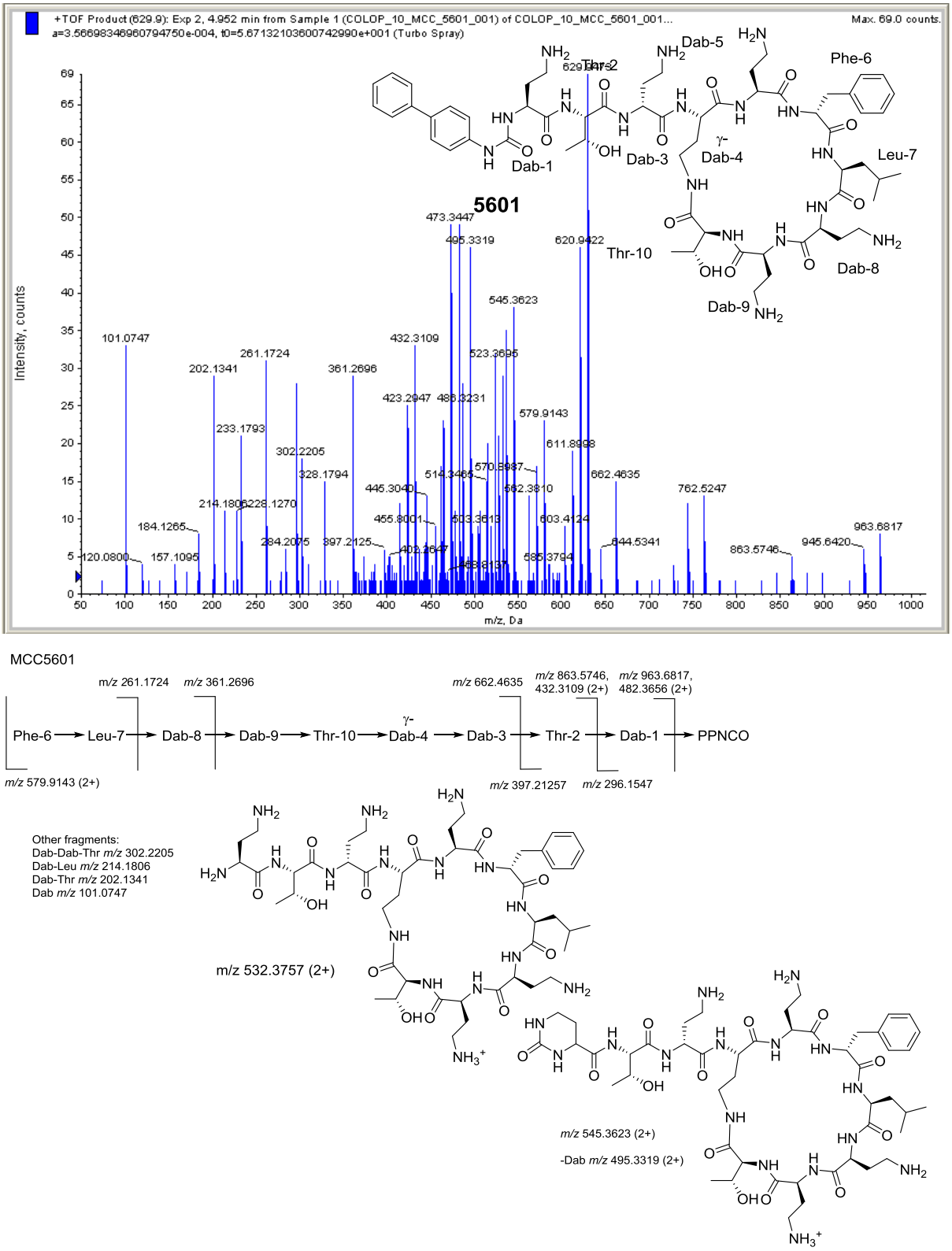


Figure S4a. (+)-ESI-TOF-MS/MS fragmentation assignments of compound 15 from fragmentation of the $[M+2H]^{2+}$ mass ion peak at *m/z* 629.9, which were consistent with those previously reported for other polymyxins.[1-3]

Mass Spectrum Molecular Formula Report

Analysis Info

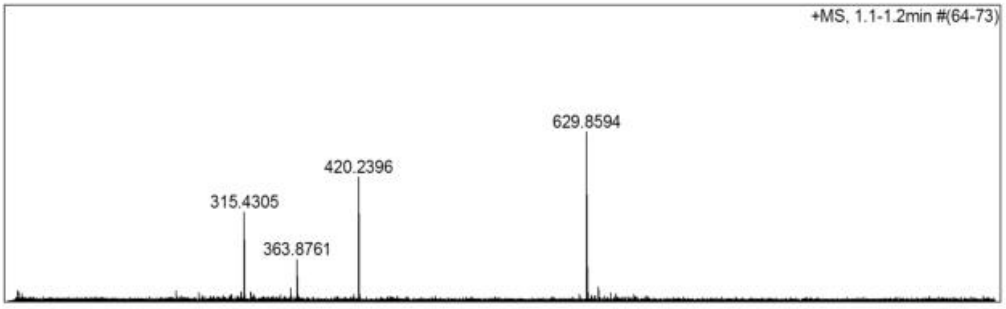
Analysis Name	D:\Data\cooper\MCC_005601_001_RD6_01_8629.d	Acquisition Date	9/18/2014 10:13:42 PM
Method	tune-wide_50ul_hystar_withcal_direct_medhighmass_2.m	Operator	a.piggott
Sample Name	MCC_005601_001	Instrument / Ser#	micrOTOF 232
Comment	Comments		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	100 m/z	Set Capillary	4500 V	Set Dry Gas	5.0 l/min
Scan End	1500 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C60H93N17O13						
Formula, max.	C60H93N17O13						
Measured m/z	629.859	Tolerance	10	mDa	Charge	2	
Check Valence	no	Minimum	0		Maximum	0	
Nirogen Rule	yes	Electron Configuration	both				
Filter H/C Ratio	yes	Minimum	0		Maximum	3	
Estimate Carbon	yes						



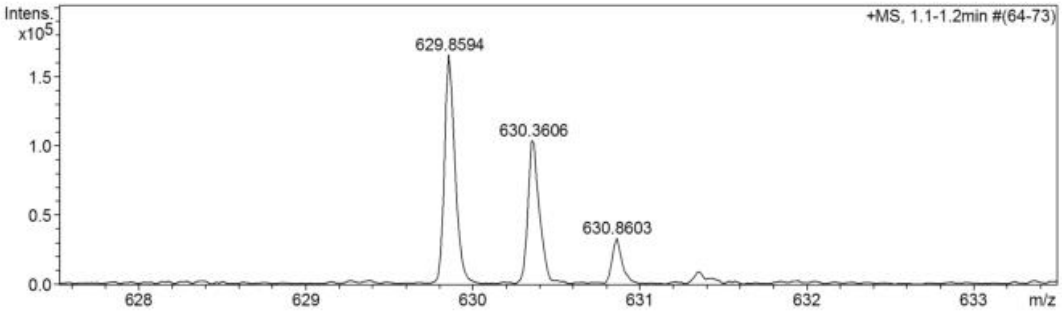


Figure S4b. HR-(+)-ESI-TOF-MS of the $[M+2H]^{2+}$ mass ion peak of compound 15.

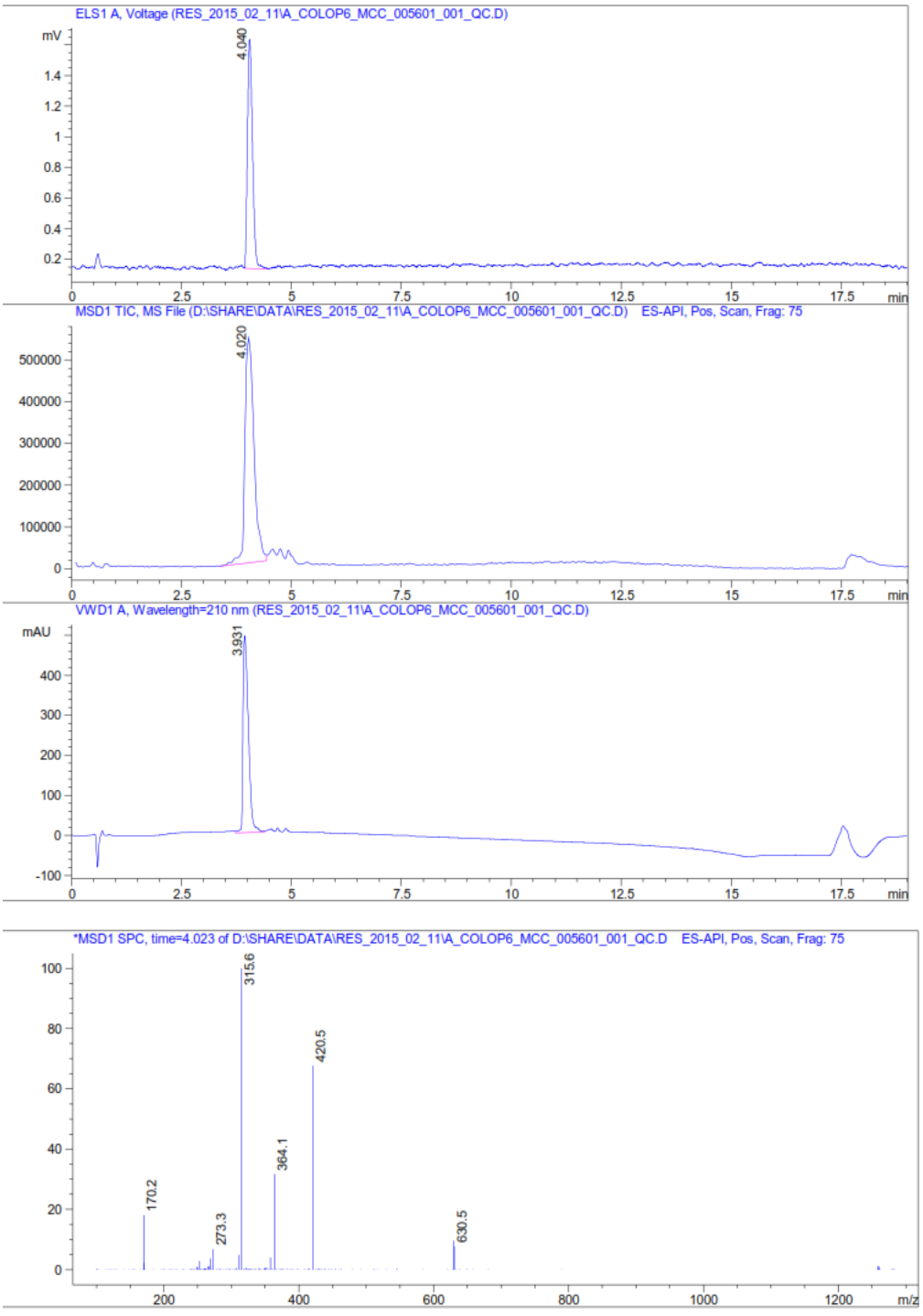


Figure S4c. LC-MS analysis of compound 15 showing ELSD, TIC, UV-vis absorption at 210 nm and ESI-MS.

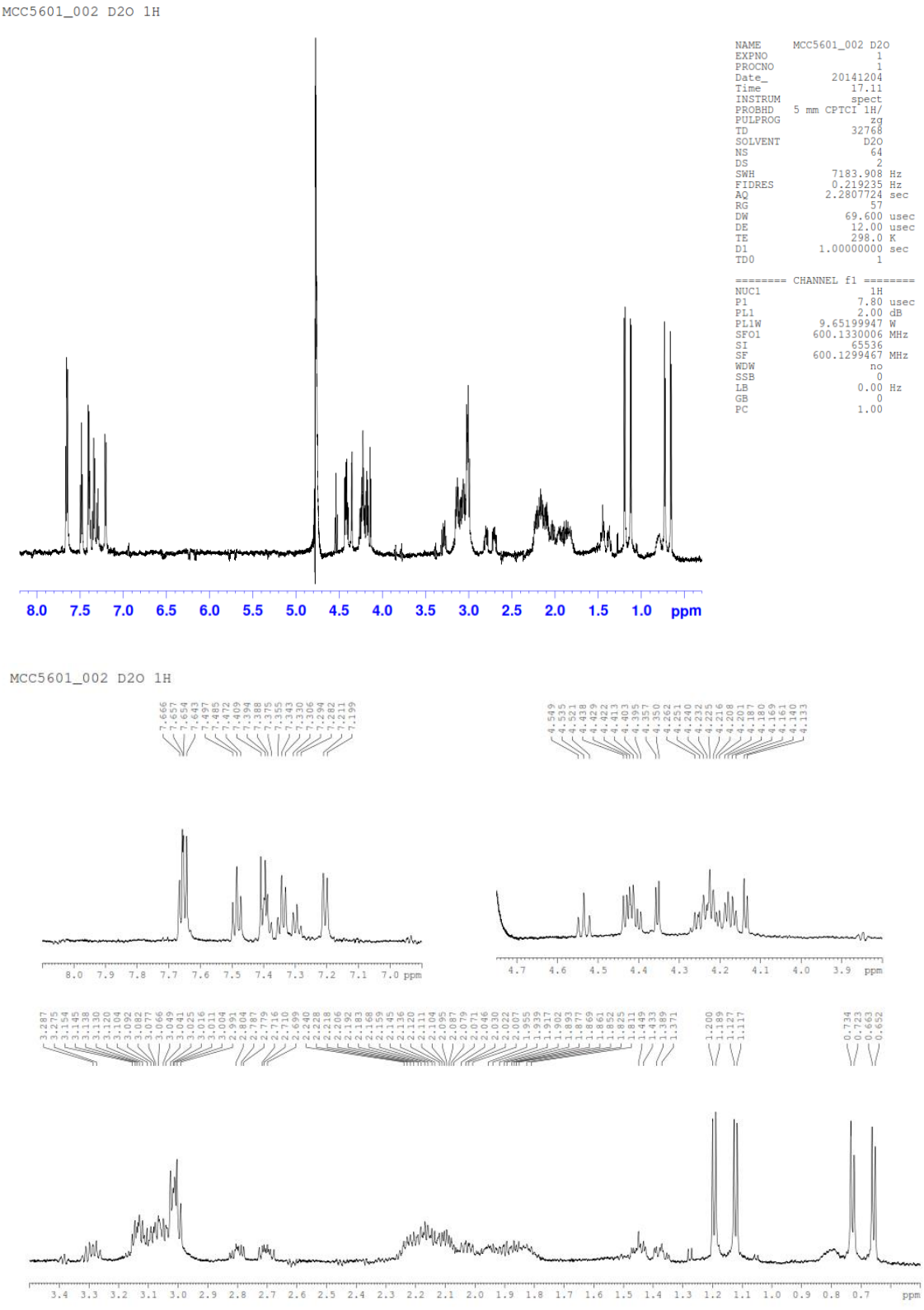


Figure S4d. 1H NMR in D2O of compound 15.

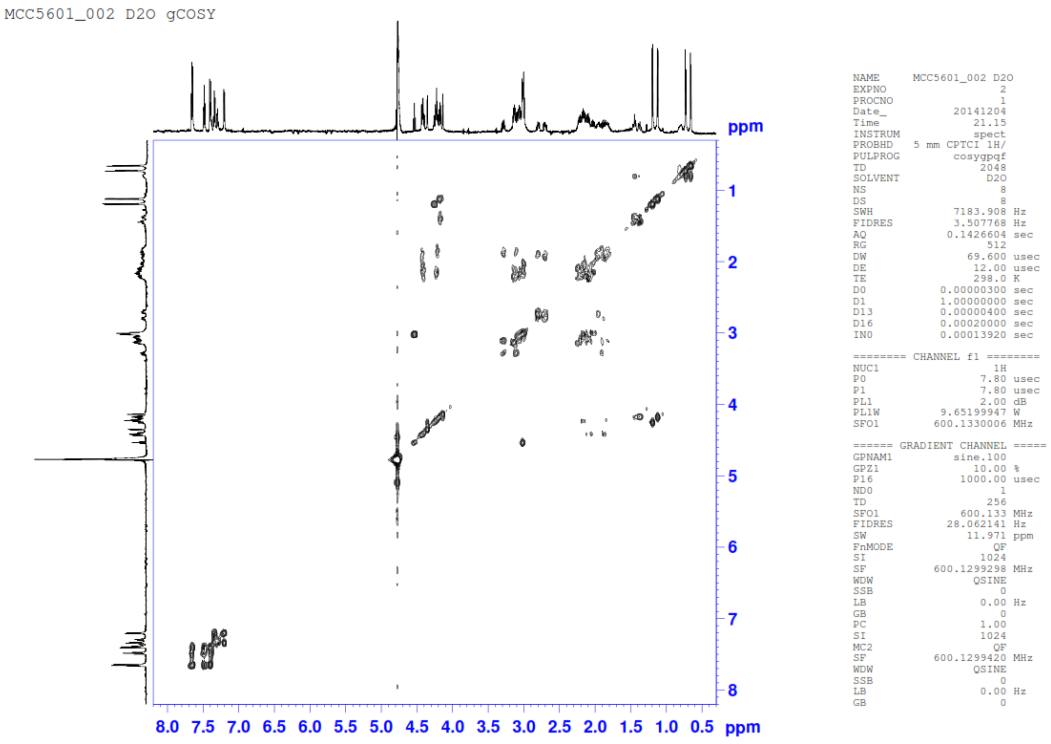


Figure S4e. gCOSY spectra in D_2O of compound 15.

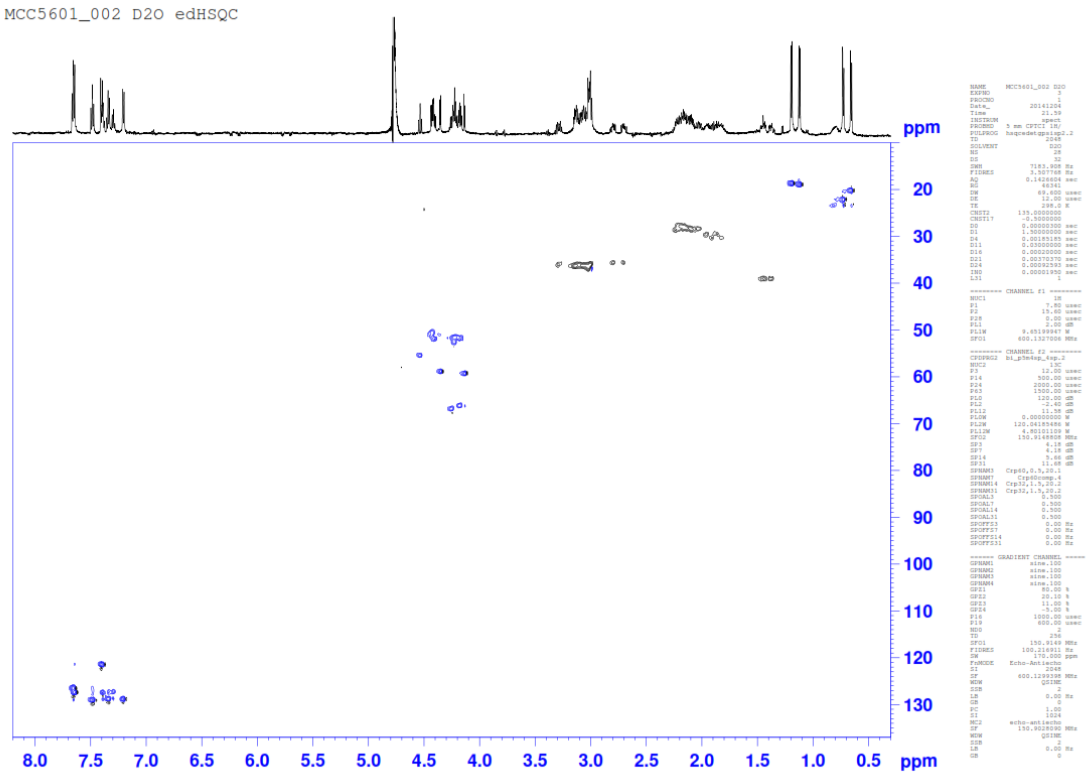


Figure S4f. edHSQC spectra in D_2O of compound 15.

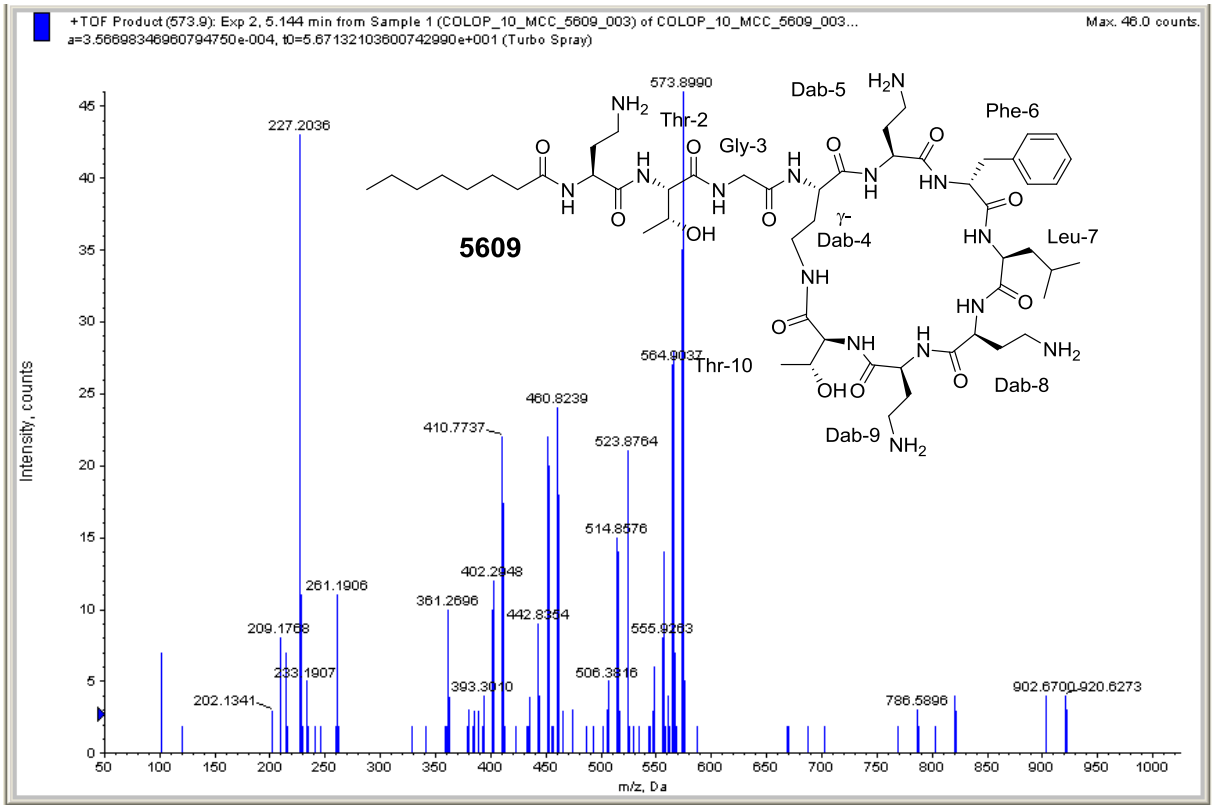


Figure S5a. (+)-ESI-TOF-MS/MS fragmentation assignments of compound 38 from fragmentation of the $[M+2H]^{2+}$ mass ion peak at *m/z* 573.8, which were consistent with those previously reported for other polymyxins. [1-3]

Mass Spectrum Molecular Formula Report

Analysis Info

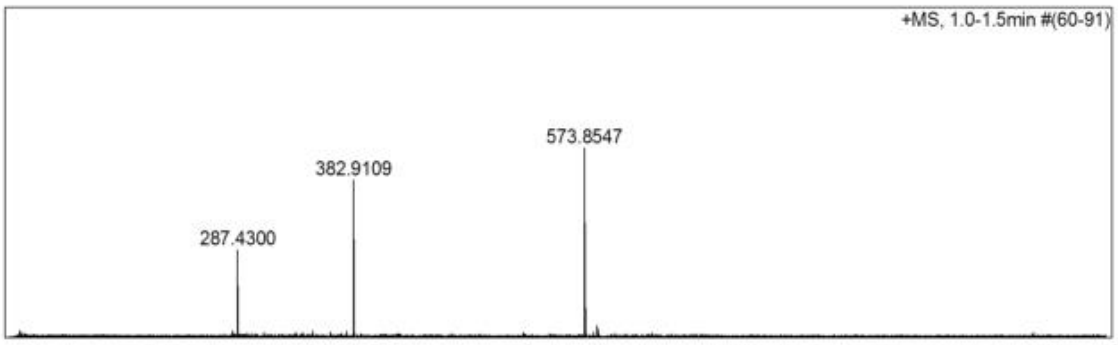
Analysis Name	D:\Data\cooper\MCC_005609_003_RD7_01_8630.d	Acquisition Date	9/18/2014 10:19:12 PM
Method	tune-wide_50ul_hystar_withcal_direct_medhighmass_2.m	Operator	a.piggott
Sample Name	MCC_005609_003	Instrument / Ser#	micrOTOF 232
Comment	Comments		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.8 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	100 m/z	Set Capillary	4500 V	Set Dry Gas	5.0 l/min
Scan End	1500 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Source

Generate Molecular Formula Parameter

Formula, min.	C53H93N15O13						
Formula, max.	C53H93N15O13						
Measured m/z	573.855	Tolerance	10	mDa	Charge	2	
Check Valence	no	Minimum	0		Maximum	0	
Nirogen Rule	yes	Electron Configuration	both				
Filter H/C Ratio	yes	Minimum	0		Maximum	3	
Estimate Carbon	yes						



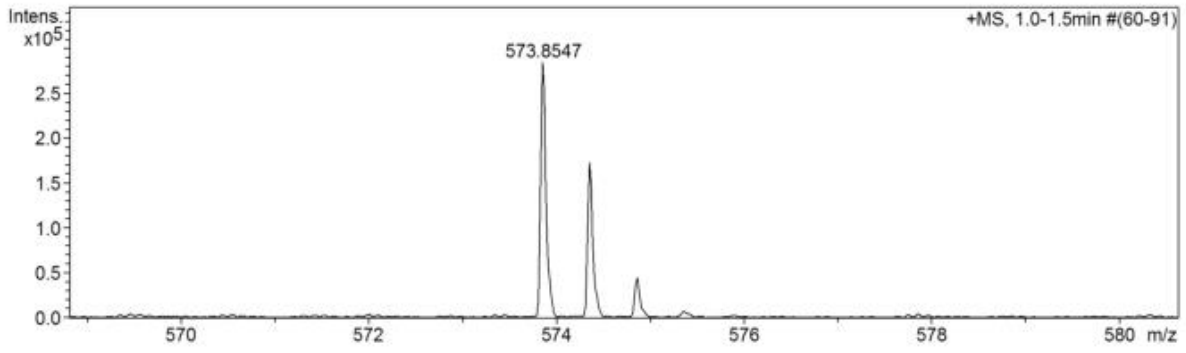


Figure S5b. HR-(+)-ESI-TOF-MS of the $[M+2H]^{2+}$ mass ion peak of compound 38.

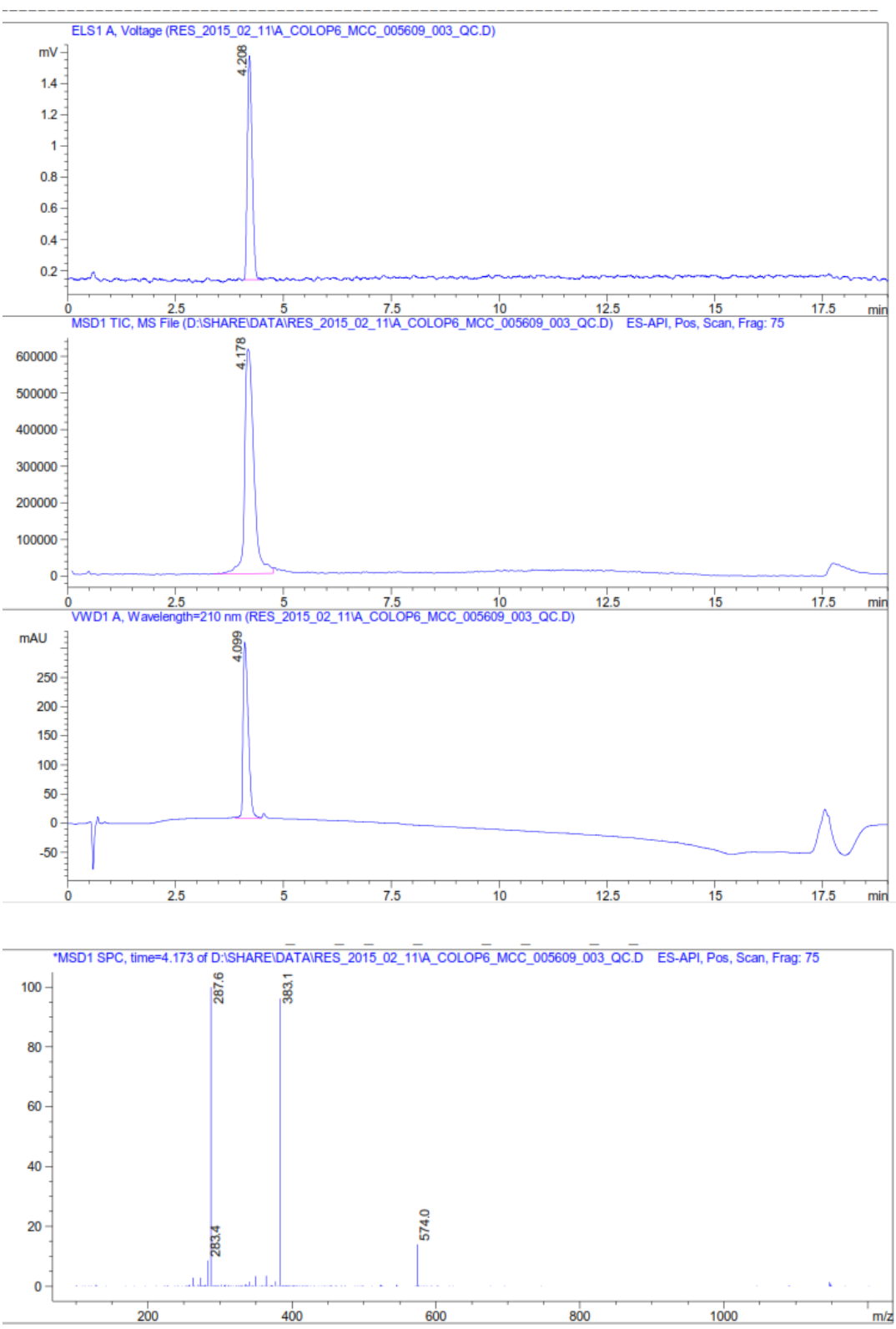


Figure S5c. LC-MS analysis of compound 38 showing ELSD, TIC, UV-vis absorption at 210 nm and ESI-MS.

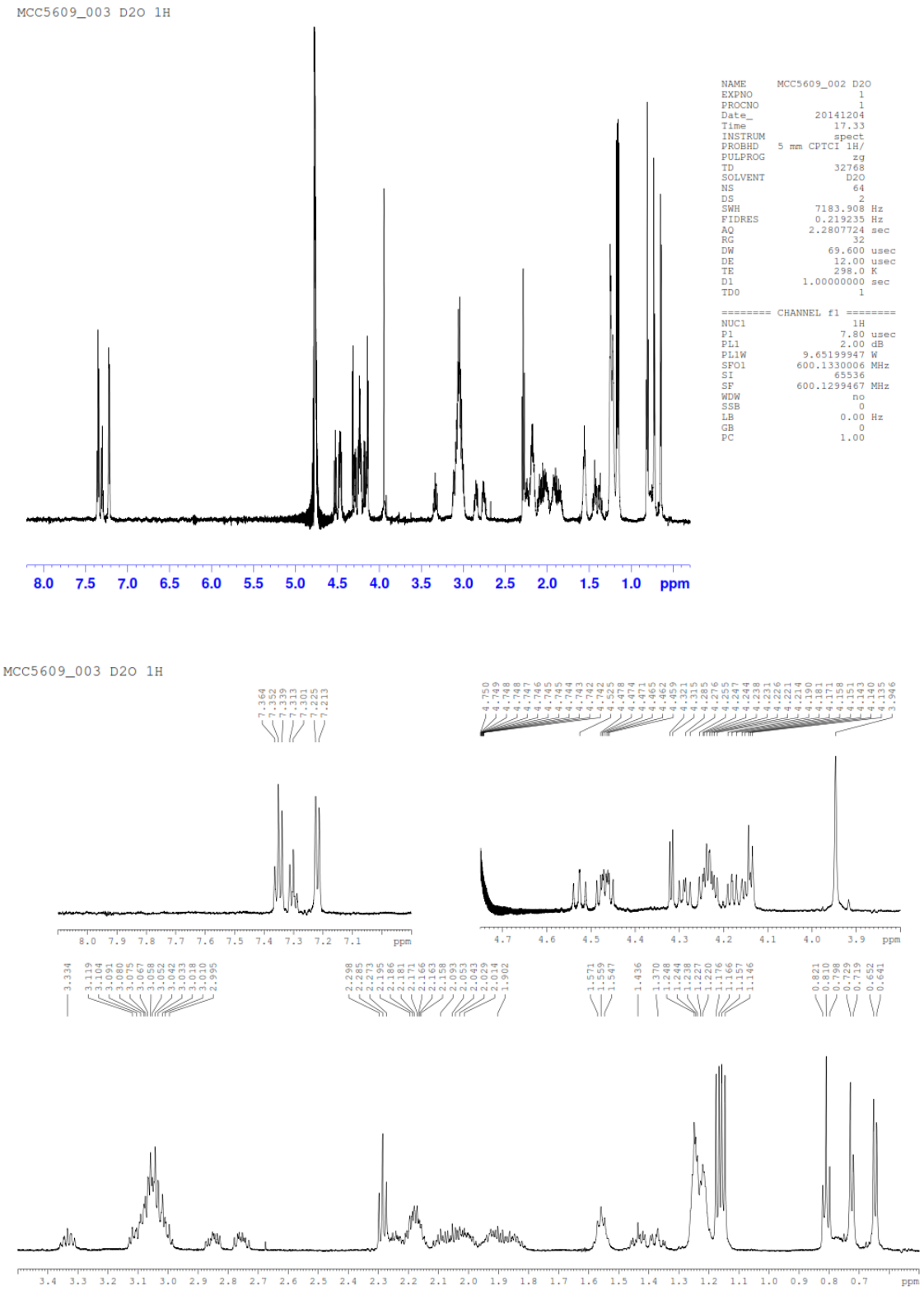


Figure S5d. ^{1}H NMR in D_2O of compound 38.

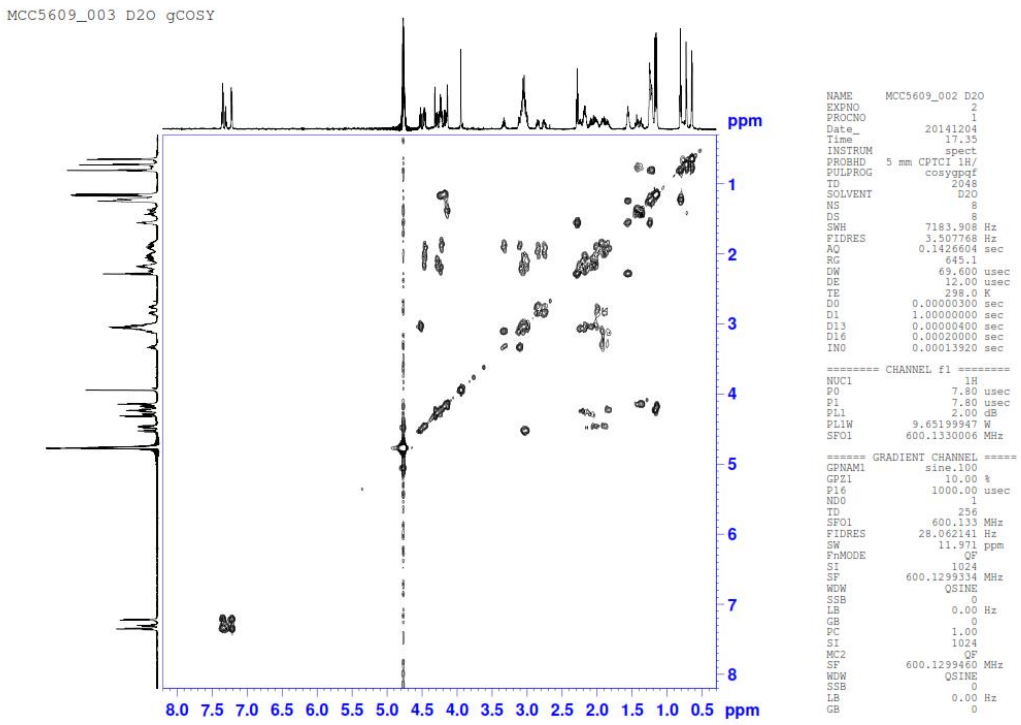


Figure S5e. gCOSY in D_2O of compound 38.

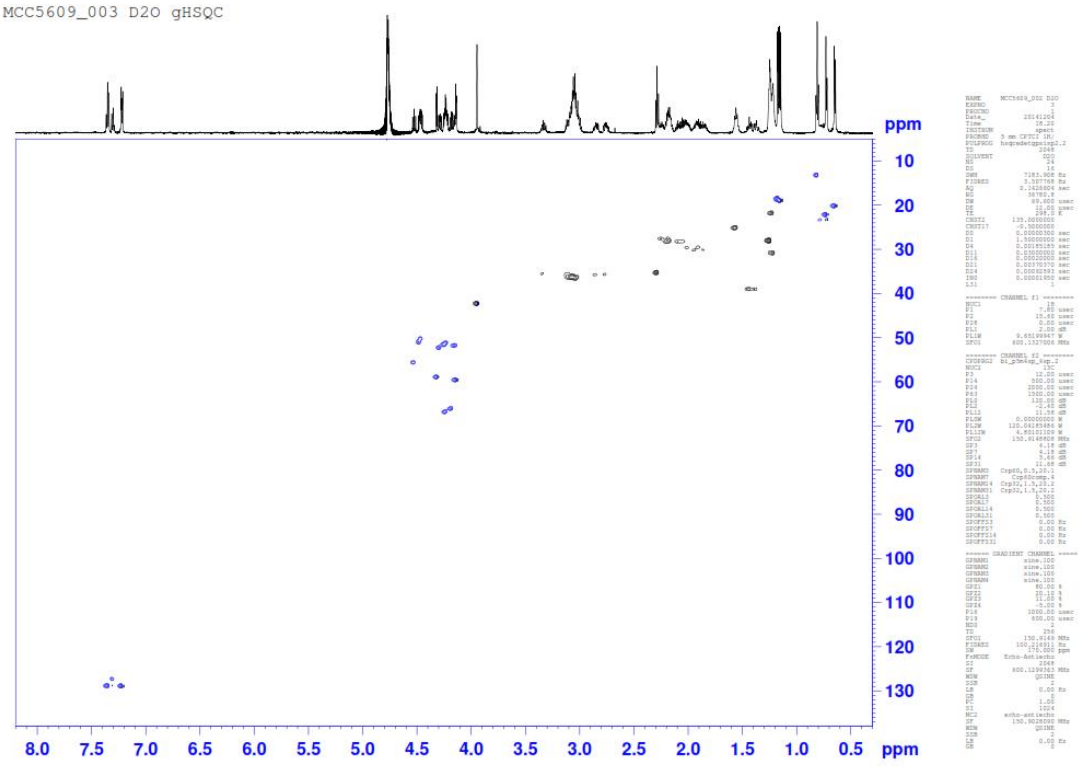


Figure S5f. edHSQC in D_2O of compound 38.

21/05/2013 10:13:05 AM

Formula $C_{22}H_{23}NO_6$	FW	381.4217					
Acquisition Time (sec)	3.4079	Comment	AGG_210513_FmocThrOAlyl			Date	May 21 2013
Date Stamp	May 21 2013						
File Name	\\dataonline.imb.uq.edu.au\a.gallardogodoy\Colistin Project\NMR\AGG_210513_FmocThrOAlyl\AGG_210513_FmocThrOAlyl_PROTON_cdcl3_20130521_02.fid\fid						
Frequency (MHz)	399.78	Nucleus	1H	Number of Transients	32	Original Points Count	16384
Points Count	16384	Pulse Sequence	s2pul	Receiver Gain	60.00	Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	2009.4801	Spectrum Type	STANDARD	Sweep Width (Hz)	4807.69	Temperature (degree C)	25.000

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.28 (d, *J*=5.87 Hz, 3 H) 4.26 (t, *J*=6.90 Hz, 1 H) 4.33 - 4.42 (m, 2 H) 4.44 (d, *J*=7.04 Hz, 2 H) 4.70 (d, *J*=5.58 Hz, 2 H) 5.22 - 5.31 (m, 1 H) 5.36 (d, *J*=17.02 Hz, 1 H) 5.59 (d, *J*=8.80 Hz, 1 H) 5.93 (ddt, *J*=16.87, 11.08, 5.54, 5.54 Hz, 1 H) 7.29 - 7.38 (m, 2 H) 7.38 - 7.48 (m, 2 H) 7.57 - 7.68 (m, 2 H) 7.78 (d, *J*=7.34 Hz, 2 H)

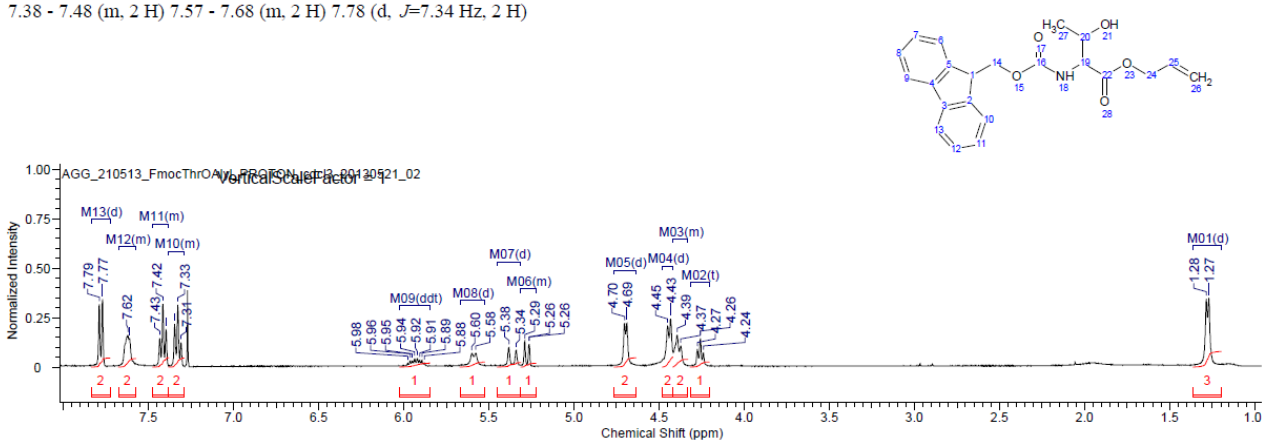


Figure S6. ^{1}H NMR in $CDCl_3$ of intermediate Fmoc-L-Thr-OAllyl (4; Scheme 1)

REFERENCES

1. Govaerts, C.; Rozenski, J.; Orwa, J.; Roets, E.; Van Schepdael, A.; Hoogmartens, J. Mass spectrometric fragmentation of cyclic peptides belonging to the polymyxin and colistin antibiotics studied by ion trap and quadrupole/orthogonal-acceleration time-of-flight technology. *Rapid Commun. Mass Spectrom.* **2002,** *16*, 823–833.
2. Deng, Y.; Lu, Z.; Bi, H.; Lu, F.; Zhang, C.; Bie, X. Isolation and characterization of peptide antibiotics LI-F04 and polymyxin B6 produced by *Paenibacillus polymyxa* strain JSa-9. *Peptides* **2011,** *32*, 1917–1923.
3. Qian, C. D.; Wu, X. C.; Teng, Y.; Zhao, W. P.; Li, O.; Fang, S. G.; Huang, Z. H.; Gao, H. C. Battacin (Octapeptin B5), a new cyclic lipopeptide antibiotic from Paenibacillus tianmuensis active against multidrug-resistant Gram-negative bacteria. *Antimicrob. Agents Chemother.* **2012,** *56*, 1458-1465.