Supporting Information for:

Total Synthesis of Laspartomycin C and Characterization of its Antibacterial Mechanism of Action

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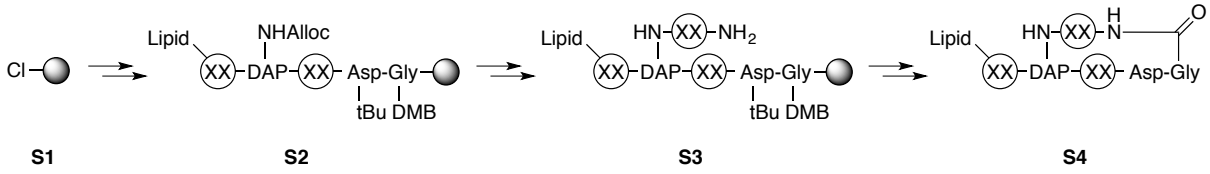
Reagents and general methods All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Fmoc-(Dmb)Gly-OH and Fmoc-Kyn-OH were obtained via previously published procedures.[1,2] Fmoc-*L*-Dap(Aloc)-OH, Fmoc-*D*-allo-Thr and 2-chlorotrityl resin were obtained from Iris Biotech GmbH and the latter was used without protection of the side chain hydroxyl moiety. All known compounds prepared had NMR spectra, mass spectra, and optical rotation values consistent with the assigned structures. All reactions and fractions from column chromatography were monitored by thin layer chromatography (TLC) using plates with a UV fluorescent indicator (normal SiO_2, Merck 60 F254). One or more of the following methods were used for visualization: UV absorption by fluorescence quenching; iodine staining; phosphomolybdic acid:ceric sulfate:sulfuric acid:H_2O (10 g:1.25 g:12 mL:238 mL) spray; and ninhydrin staining. Flash chromatography was performed using Merck type 60, 230–400 mesh silica gel.

Instrumentation for compound characterization NMR spectra were recorded at 400 or 500 MHz with chemical shifts reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS). 2D NMR experiments (TOCSY, HSQC and NOESY) were performed on a 500 MHz instrument. High-resolution mass spectrometry (HRMS) analysis was performed using an ESI instrument. Circular Dichroism spectra were recorded on a Jasco J-810 CD-spectrometer and ITC experiments were carried out using a MicroCal Auto-ITC_{200}. Automated peptide synthesis was performed on a CS Bio CS336x peptide synthesizer. TLC plates were spotted using a CAMAG LINOMAT5 and developed with a CAMAG ADC2 Automatic Development Chamber.

General procedure for the preparation of Laspartomycin C and analogs of Daptomycin and Laspartomycin C



Scheme S1. Solid- and solution phase synthesis of the cyclic lipopeptides.

2-Chlorotrityl resin **S1** (5.0 g, 1.60 mmol/g) was loaded with DMB-Fmoc-Gly-OH. Resin loading was determined after coupling of the second amino acid because complete Fmoc deprotection of resin bound DMB-Fmoc-Gly required nonstandard conditions: DMB-Fmoc-Gly 2-chlorotrityl resin (6.0 g) was thus treated with ethanolamine:DMF (1:4 v:v, 1x30 min, 1x90 min) followed by washing with DMF. Overnight coupling of Fmoc-Asp(^{t}Bu)-OH (3.7 g, 9.0 mmol), BOP (4.0 g, 9.0 mmol) and DiPEA (3.1 mL, 18.0 mmol) in DMF followed by end capping with Ac_2O:DiPEA:DMF (0.5:0.5:9 v:v:v, 20 mL) yielded Fmoc-Asp(tBu)-(DMB)-Gly 2-chlorotrityl resin (0.52 mmol.g^{-1} as determined spectrophotometrically).

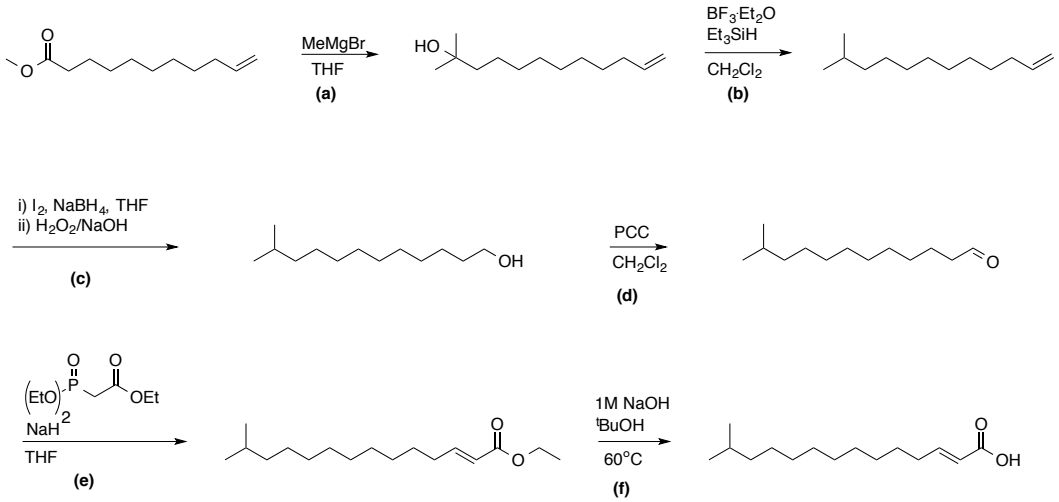
Linear precursor peptides encompassing Gly_8 to Asp_1 were assembled via standard Fmoc solid-phase peptide synthesis (SPPS) either via manual synthesis (resin bound AA:Fmoc-AA:BOP:DiPEA, 1:4:4:8 molar eq.) or automated synthesis (resin bound AA:Fmoc-AA:HBTU:HOBt:DiPEA, 1:4:3.75:3.75:8 molar eq.) typically on 0.25 mmol scale. NMP or DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF or piperidine:NMP (1:4 v:v). Amino acid side chains were protected as follows: Boc for Orn and Trp, Trt for *D*-Asn, Aloc for DAP, ^{t}Bu for Asp, Glu and *D*-Ser, DMB for Gly in Asp-Gly sequences. Kyn and *D-allo*-Thr were introduced without side chain protection. Following coupling and Fmoc deprotection of Asp_1, N-terminal acylation was achieved by coupling (*E*)-13-methyltetradec-2-enoic acid using the same coupling conditions used for the SPPS.

The resin-bound, Alloc protected intermediate **S2** was next washed with CH_2Cl_2 and treated with $Pd(PPh_3)_4$ (74 mg, 0.06 mmol) and $PhSiH_3$ (0.74 mL, 6.0 mmol) in CH_2Cl_2 (ca. 10 mL) under argon for 1 hour.[3] The resin was subsequently washed with CH_2Cl_2 (5x10 mL), followed by a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5x10 mL), and DMF (5x10 mL). The remaining three amino acids where added via standard Fmoc SPPS with removal of the final Fmoc protecting group to yield the complete linear resin-bound peptide **S3** with a free N-terminal amine. The resin was treated with $(CF_3)_2CHOH$:CH_2Cl_2 (1:4, 10 mL) for 1 hour and rinsed with additional $(CF_3)_2CHOH$:CH_2Cl_2 and CH_2Cl_2. The combined washings were then evaporated to yield the linear protected peptide with free C- and N-termini. The residue was dissolved in CH_2Cl_2 (250 mL) and treated with BOP (0.22 g, 0.5 mmol) and DiPEA (0.17 mL, 1.0 mmol) and the solution was stirred overnight after which TLC indicated complete cyclization. The reaction mixture was concentrated and directly treated with TFA:TIS:H_2O (95:2.5:2.5, 10 mL) for 60-90 minutes. The reaction mixture was added to Et_2O:hexanes (1:1) and the resulting precipitate washed once more with Et_2O:hexanes (1:1). The crude cyclic peptide was lyophilized from ^{t}BuOH:H_2O (1:1) and purified with reverse phase HPLC by applying a gradient of 25% to 65% buffer B (buffer A: H_2O:MeCN:TFA, 95:5:0.1 v:v:v; buffer B: H_2O:MeCN:TFA, 5:95:0.1 v:v:v) over 1 hour with a flow rate of 12 mL min^{-1} on a C_{18} Maisch 250x22 mm column. Pure fractions were pooled and lyophilized to yield the desired cyclic lipopeptide products **S4** in >95% purity as white powders, typically in 10-20 mg quantity (4.2-9.3 % yield based on resin loading).

Abbreviations:

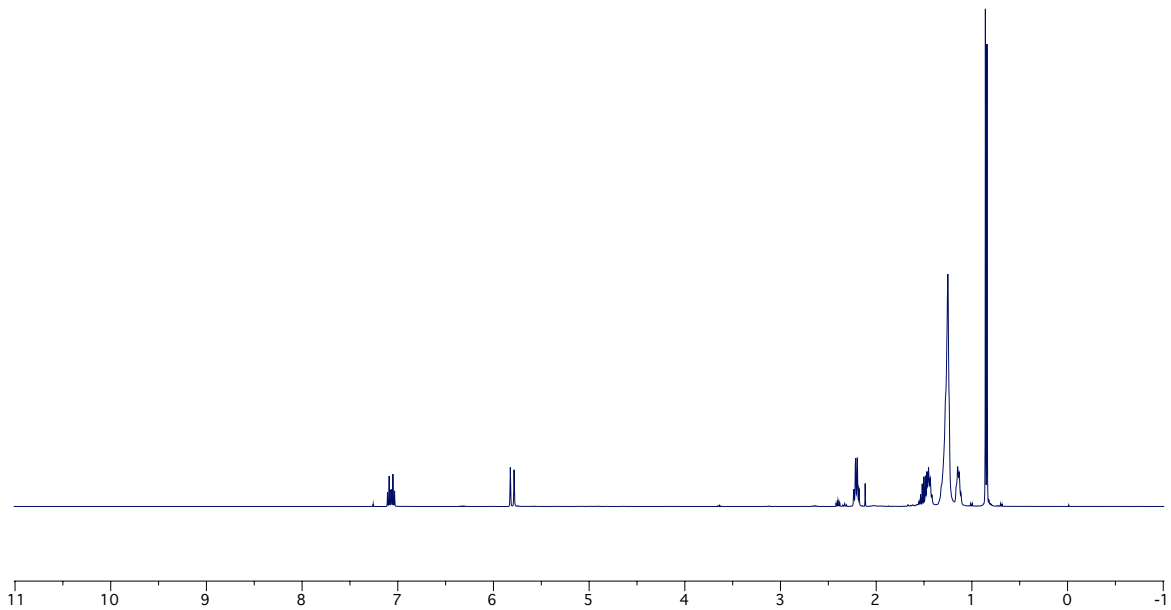
AA	amino acid
Alloc	allyloxycarbonyl
Boc	tert-butyloxycarbonyl
^{t}Bu	tert-butyl
^{t}BuOH	*tert*-butanol
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
DiPEA	N,N-diisopropylethylamine
DMB	2,4-dimethoxybenzyl
DMF	*N,N*-dimethylformamide
Fmoc	Fluorenylmethyloxycarbonyl
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HBTU	N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
Kyn	kynurenine
NMP	1-Methyl-2-pyrrolidinone
TIS	triisopropylsilane
Trt	trityl

Synthesis of lipid (*E*)-13-methyltetradec-2-enoic acid

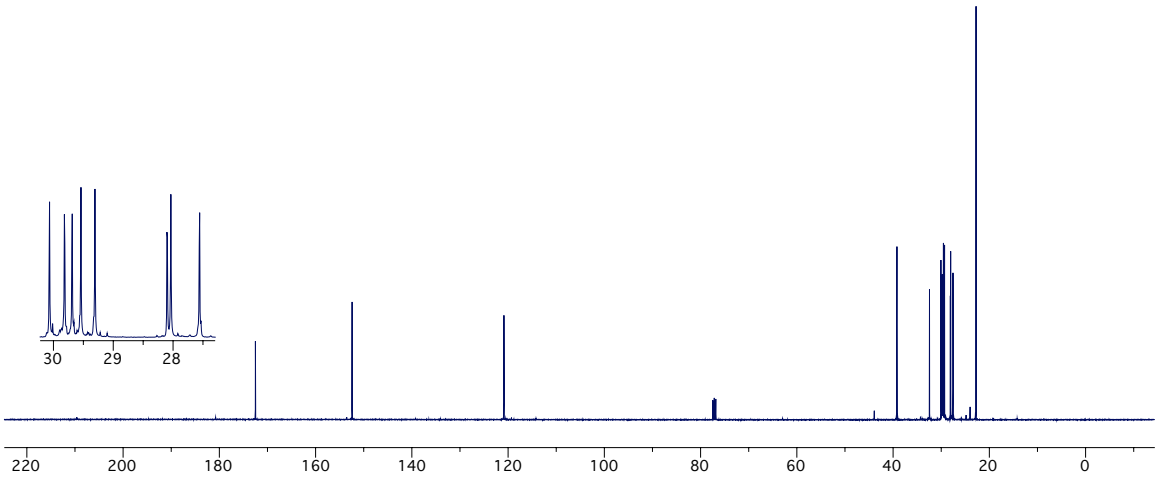


Scheme S2. Synthesis of (*E*)-13-methyltetradec-2-enoic acid. Steps **a-c**[4] and steps **d-e**[5] were carried out according to literature procedures. Step **f** was carried out in accordance with the published synthesis of the same compound from a similar ester precursor.[6] 1H NMR ($CDCl_3$): δ 12.03 (br, 1H), 7.07 (td, J_1 = 15.6 Hz, J_2 = 7.2 Hz, 1H), 5.81 (d, J = 15.6 Hz, 1H), 2.21 (q, J = 7.2 Hz, 2H), 1.56-1.42 (m, 3H), 1.25 (m, 12H), 1.13 (m, 2H), 0.85 (d, J = 6.4 Hz, 6H); 13C NMR ($CDCl_3$): δ 172.5, 152.4, 120.9, 39.2, 32.4, 30.1, 29.8, 29.7, 29.5, 29.3, 28.1, 28.0, 27.6, 22.8; HR-MS [M-H+]: Calc. 239.2017, found 239.2023.

^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (100 MHz, $CDCl_3$)



MIC determinations

Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to CLSI guidelines.[7] Blood agar plates were inoculated with glycerol stocks of *S. aureus* 29213 and *S. simulans* 22 followed by incubation for 16 hours at 37°C and 30°C respectively. Cation adjusted Mueller-Hinton broth (MHB) containing 10 mg L^{-1} Mg^{2+} was inoculated with individual colonies of *S. aureus* and *S. simulans,* and incubated for 16 hours at 220 RPM. The peptides were dissolved in MHB (10 mg L^{-1} Mg^{2+}) and serially diluted on polypropylene microtiter plates with a volume of 50 µL per well. Inoculated MHB ($2x10^5$ CFU.mL^{-1}) containing 10 mg L^{-1} Mg^{2+} and varying concentrations of Ca^{2+} was added to reach a total volume of 100 µL per well. The microtiter plates were sealed with an adhesive membrane and after 16 hours of incubation at 37°C or 30°C and 220 RPM the wells were visually inspected for bacterial growth. All reported MIC values result from two or more measurements.

Table S1. Complete table of MIC values (µg mL^{-1}) against *S. simulans* and *S. aureus* at various Ca^{2+} concentrations.

	***S. simulans* 22**				***S. aureus* 29213**			
Compound	**no Ca^{2+}**	**1.25 mM**	**5.0 mM**	**10 mM**	**no Ca^{2+}**	**1.25 mM**	**5.0 mM**	**10 mM**
Laspartomcyin C	>128	8	4	≤1	>256	8	4	2
6	>128	32	4	1	>256	160	16	4
7	>128	64	8	4	>256	320	64	64
8	>128	128	16	8	>256	>256	128	32
9	>128	64	4	4	>256	256	32	8
10	>128	>128	>128	>128	>256	>256	>256	>256
11	>128	>128	>128	>128	>256	>256	>256	>256
12	>128	>128	>128	>128	>256	>256	>256	>256
Daptomycin	32-64	0.063	0.031	0.016-0.031	>256	0.5	0.25	0.125

Circular Dichroism

CD spectra were recorded for 60 μM peptide solutions in 20 mM HEPES (pH=7.4) buffer with and without 5.0 mM $CaCl_2$. Data collection was limited to the 210-260 nm range because extensive scattering occurs below 210 nm under these conditions.[8] The experiments were performed using a 1.0 mm cuvet, a bandwith of 1 nm and a scan speed of 20 nm min^{-1}. The average of 10 scans was baseline corrected by subtracting the average elipticity over 255-260 nm and units were converted to mean residue molar elipticities (deg cm^2 $dmol^{-1}$).

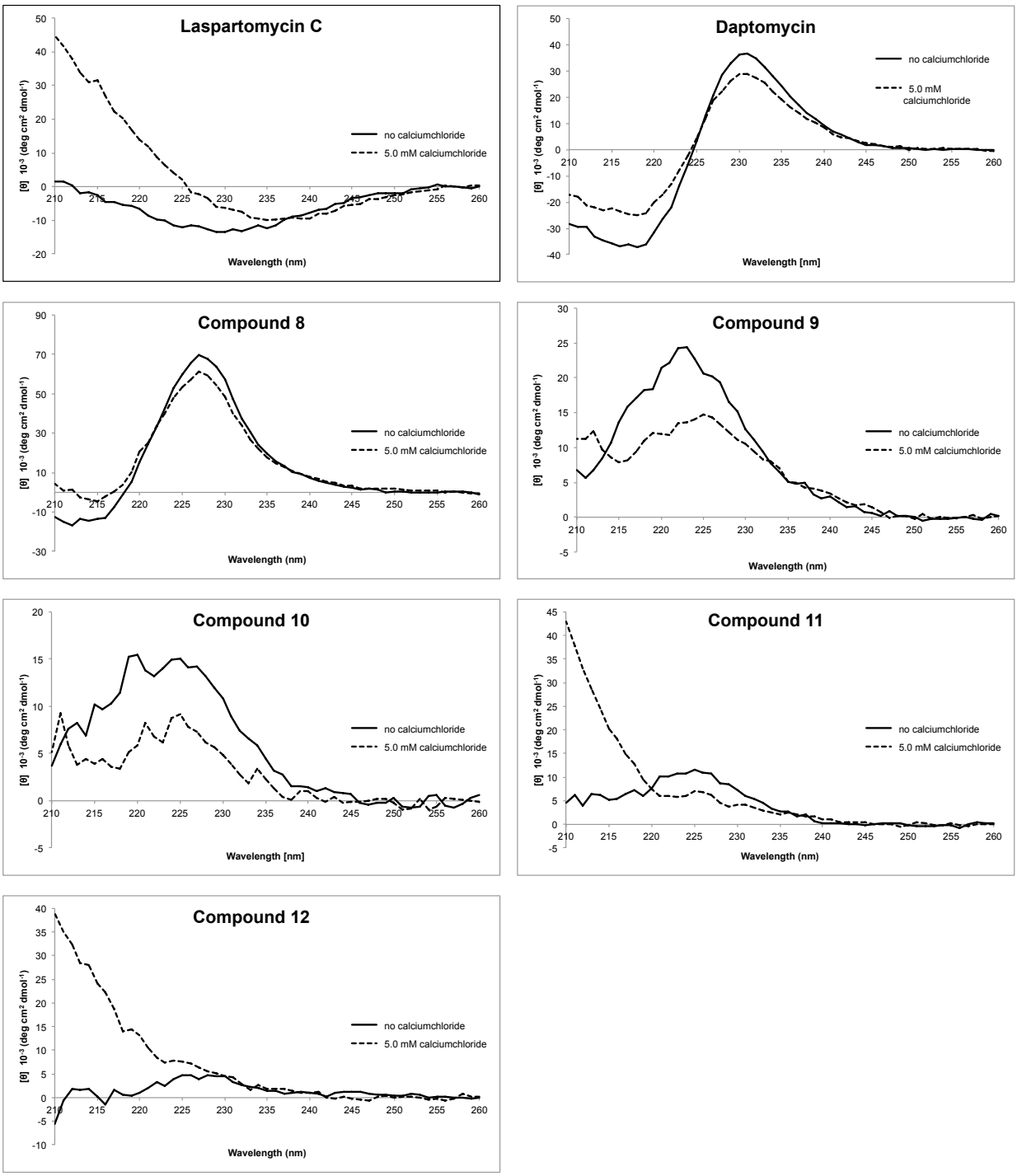


Figure S1. Circular dichroism: spectra of laspartomycin, daptomycin, and compounds **8**-**12** were recorded over 210-260 nm.

UDP-MurNAc-pentapeptide accumulation assay

S. aureus 29213 was grown until OD_{600} = 0.5 in TSB supplemented with $CaCl_2$ (5.0 mM). Chloramphenicol (130 µg mL^{-1}) was added and after incubation for 15 minutes at 37°C, the culture was divided in 5 mL aliquots. Antibiotics were added at 10xMIC (laspartomycin C, daptomycin) and one aliquot remained untreated. After 60 minutes, cells were separated from the medium and extracted with boiling d-H_2O (1 mL) for 15 minutes. The suspensions were spun down and the supernatant was lyophilized. The resulting material was analyzed by HPLC applying a gradient from 100% eluent A (50 mM $NaHCO_3$:5 mM Et_3N, pH = 8.3) to 75% eluent A over 15 minutes using a C18 column (eluent B: MeOH). Formation of UDP-MurNAc-pentapeptide was confirmed by comparison with authentic material by HPLC, and LC-MS analysis applying the same gradient with an adjusted eluent A (50 mM NH_4HCO_3:5 mM Et_3N, pH = 8.3).

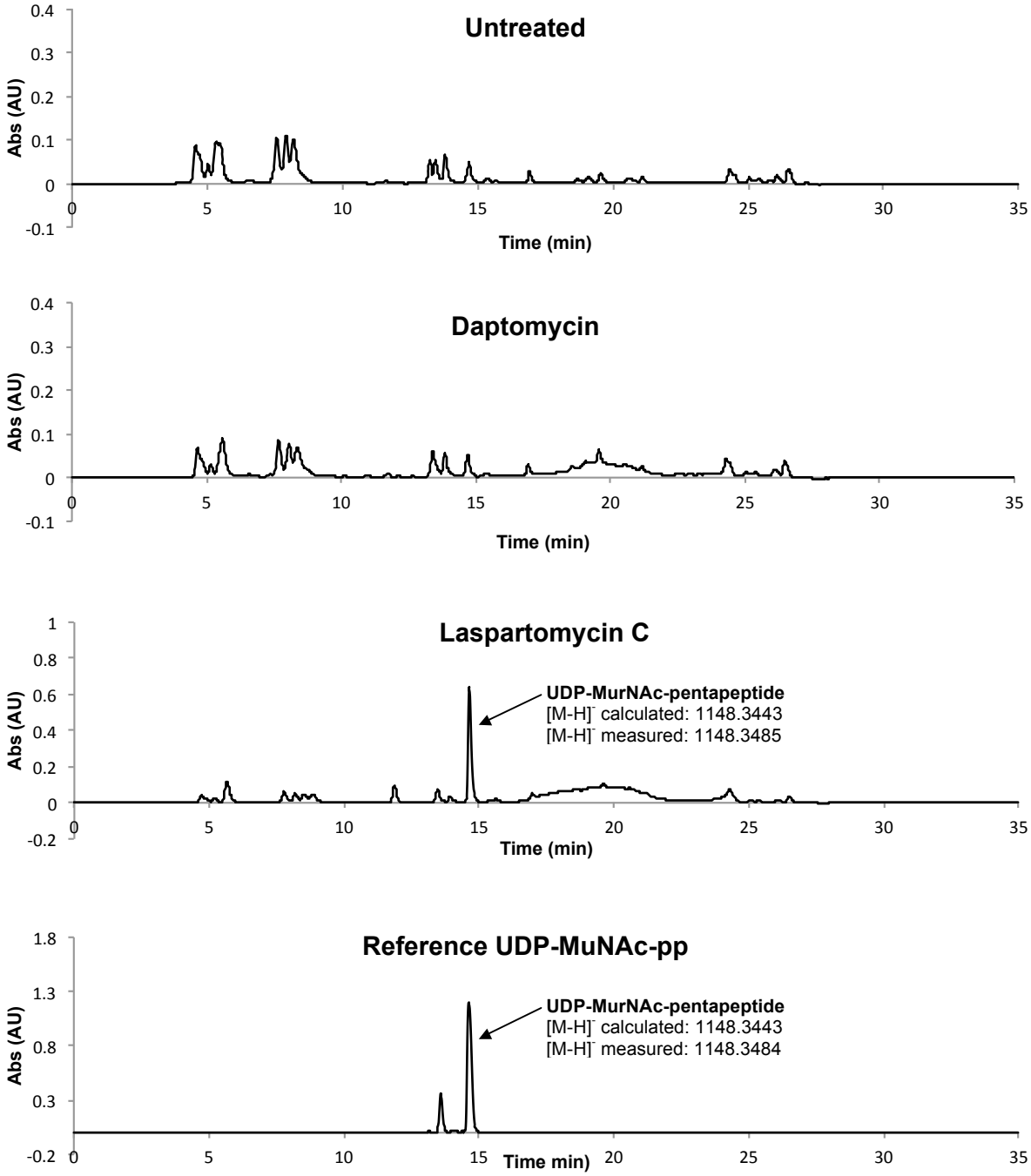


Figure S2. Full analytical HPLC traces for UDP-MurNAc-pentapeptide accumulation assay. Treatment of *S. aureus* 29213 with laspartomycin C results in accumulation of UDP-MurNAc-pentapeptide, an effect not observed with daptomycin.

Antagonization assay

Appropriate amounts of antagonists lipid I, lipid II, UDP-MurNAc-pentapeptide, UDP-GlcNAc, C_{55}-P, C_{55}-PP, C_{15}-P and C_{15}-PP in $CHCl_3$:MeOH (1:1) or MeOH were evaporated and redissolved in MHB or MQ-H_2O. Antagonists were added to the peptides in MHB to achieve a 5-fold excess of antagonist relative to the concentration of peptide antibiotic. These solutions were then added to bacterial cultures of *S. simulans* 22 in MHB (10^7 CFU mL^{-1}) to reach a final concentration of peptide antibiotic corresponding to 8 times the MIC. Each experiment was executed in triplicate and after incubation for 16 hours at 30°C and 220 RPM bacterial growth was inspected visually (Table S2).

Table S2. Inhibition of antibiotic activity by selected antagonists. (-) Antibiotic activity unaffected; (+) Antibiotic activity antagonized; (±) Antagonization unclear

Antagonist	Laspartomycin C (**1**)	Analogues **6**-**9**	Daptomycin (**2**)	Vancomycin
Lipid I	-	-	-	+
Lipid II	-	-	-	+
UDP-MurNAc-pp	-	-	-	+
UDP-GlcNAc	-	-	-	-
C_{55}-P	±	-	-	-
C_{55}-PP	±	-	-	-
C_{15}-P	+	-	-	-
C_{15}-PP	-	-	-	-

C_{55}-P and Lipid II binding experiment

C_{55}-P (5 nmol) or lipid II (5 nmol) were incubated for 2 hours with laspartomycin C or daptomycin (0.5-2 equivalents) in 75 µL 100 mM Tris-HCl (pH=7.5) containing 0.1% TX100, $MgCl_2$ (13 mM) and $CaCl_2$ (13 mM). Treatment with PyOAc buffer (pH=4.2) was followed by BuOH extraction. The BuOH phase was evaporated, the residue was dissolved in $CHCl_3$:MeOH (1:1) and subsequently analyzed by TLC with iodine visualization ($CHCl_3$:MeOH:H_2O:NH_4OH, 88:48:10:1). Figure 3 in the body of the main manuscript was adapted from the original Figure S3 below (the figure in the manuscript was cropped and converted to gray scale with brightness set to -75 and contrast to +100).

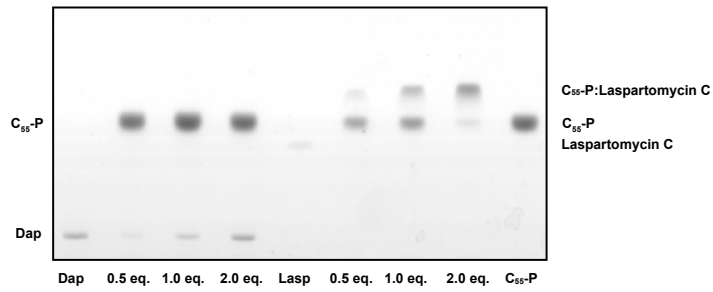


Figure S3. TLC analysis of C_{55}-P binding. Laspartomycin C and daptomycin were incubated with 0.5-2.0 eq. C_{55}-P. Laspartomycin C form a complex with C_{55}-P seemingly in 2:1 stoichiometry while daptomycin does not form a complex. The TX100 bands are not visible.

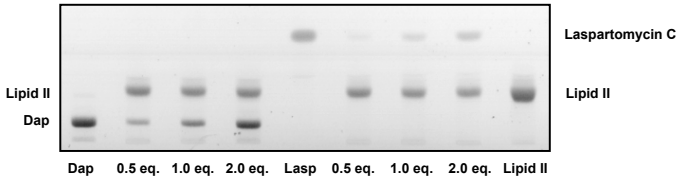


Figure S4. TLC analysis of lipid II binding. Laspartomycin C and daptomycin were incubated with 0.5-2.0 eq. lipid II. Neither Laspartomycin C nor daptomycin form a stable complex with lipid II. The TX100 bands are not visible.

Lipid II synthesis inhibition assay

C_{55}-P (5 nmol) was mixed with 0.5, 1 or 2 equivalents of peptide in 100mM Tris-HCl (pH=7.5) containing 0.1% TX100, $MgCl_2$ (13.3 mM) and $CaCl_2$ (13.3 mM). An excess of UDP-GlcNAc and UDP-MurNAc-pentapeptide was added followed by *M. flavus* membrane vesicles in Tris-HCl to reach a total volume of 75 µL. Quenching with 6 M PyOAc buffer (pH=4.2) after 2 hours was followed by extraction with BuOH. The BuOH phase was evaporated, the residue was dissolved in $CHCl_3$:MeOH (1:1) and subsequently analyzed by TLC with iodine visualization ($CHCl_3$:MeOH:H_2O:NH_4OH, 88:48:10:1).

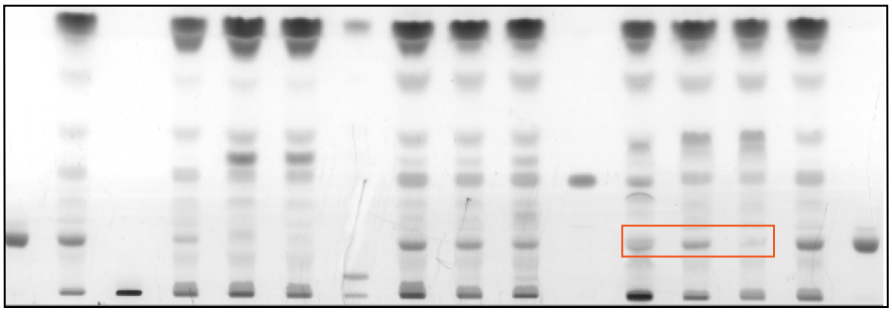


Figure S5. TLC analysis of the lipid II synthesis assay. The figure shows the result of supplementing the lipid II synthesis reaction with nisin, daptomycin and laspartomycin C in 0.5-2 eq. relative to C_{55}-P. Two reference bands are included: pure lipid II (lipid II) and the lipid II synthesis reaction mixture in the absence of antibiotics ("Control"). The presence of laspartomycin C blocks the formation of lipid II in a dose dependent manner (see red box) while daptomycin has no effect on lipid II production. The lantibiotic nisin is known to inhibit lipid II formation and was therefore used as positive control.

ITC experiments

DOPC (10 mM) and DOPC:C_{55}-P (10 mM:0.15 mM) vesicles were obtained after appropriate volumes of lipid stock solutions in $CHCl_3$:MeOH (1:1) were evaporated under vacuum and suspended in 20 mM HEPES buffer containing 5.0 mM $CaCl_2$. The vesicles were extruded using filters with a 0.2 µm pore size and degassed by stirring for 20 minutes under reduced pressure. A laspartomycin C solution (25 µM) in the same buffer was degassed using ultrasound. Vesicles were titrated into the laspatomycin C solution in triplicate at 25°C with a reference power of 2.0 µcal sec^{-1} over 26 titrations of 1.5 µL with exception of the first titration (0.5 µL). The observed titration peaks were integrated using NITPIC software.[9] Fitting of the C_{55}-P:laspartomycin C binding curves using SEDPHAT software excluding the first 13 data points provided the parameters listed in table *S*3a and *S*3b. The thermodynamic parameters reported are averages of three independent experiments and errors were estimated by Monte Carlo simulation using standard deviations of the individual experiments. The last 4 data points of the isotherms were zeroed to 0 kJ mol^{-1} and GraphPad Prism was used to generate figure 5.

Table S3a. Results of the individual ITC experiments for C_{55}-P:Laspartomycin C binding (association constant K_a, binding enthalpy ΔH and stoichiometry N).

Parameter	**Measurement #1**	**Measurement #2**	**Measurement #3**
K_a (M^{-1})	$1.27x10^8 \pm 0.83x10^8$	$1.81x10^8 \pm 1.23x10^8$	$1.04x10^8 \pm 0.69x10^8$
ΔH (kJ mol^{-1})	-9.54 ± 0.86	-9.76 ± 0.70	-10.05 ± 0.82
N	0.912	0.899	0.906

Table S3b. Binding constant (K_d) and thermodynamic parameters based on triplicate binding experiment.

Parameter	***Final values***
K_d (nM)	7.29 ± 3.75
ΔH (kJ mol^{-1})	-9.79 ± 0.58
N	0.906
ΔG (kJ mol^{-1})	-46.42 ± 1.28
ΔS (J mol^{-1} K^{-1})	122.94 ± 4.70

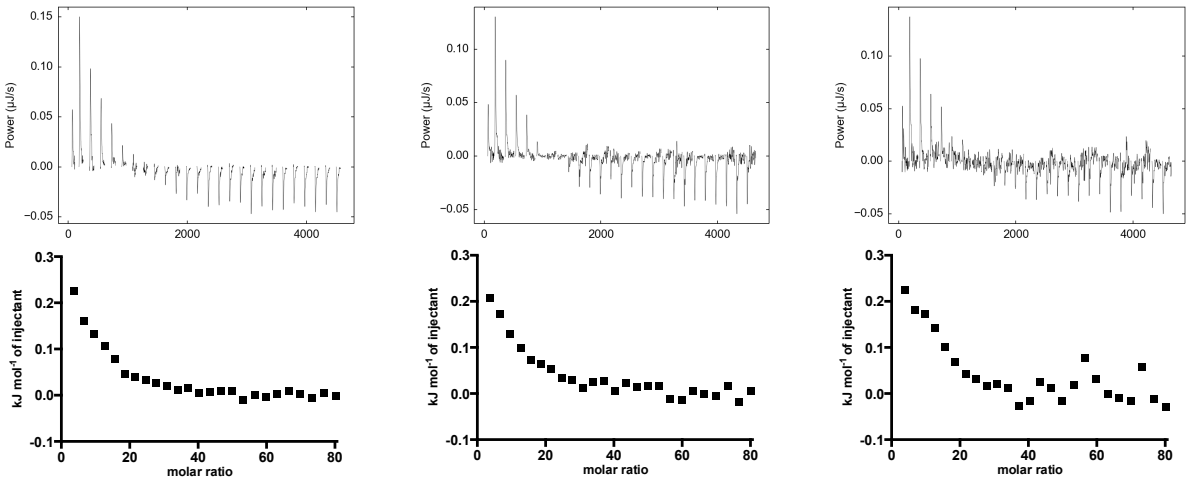


Figure S6. ITC: Titration 100% DOPC vesicles into laspartomycin C in triplicate

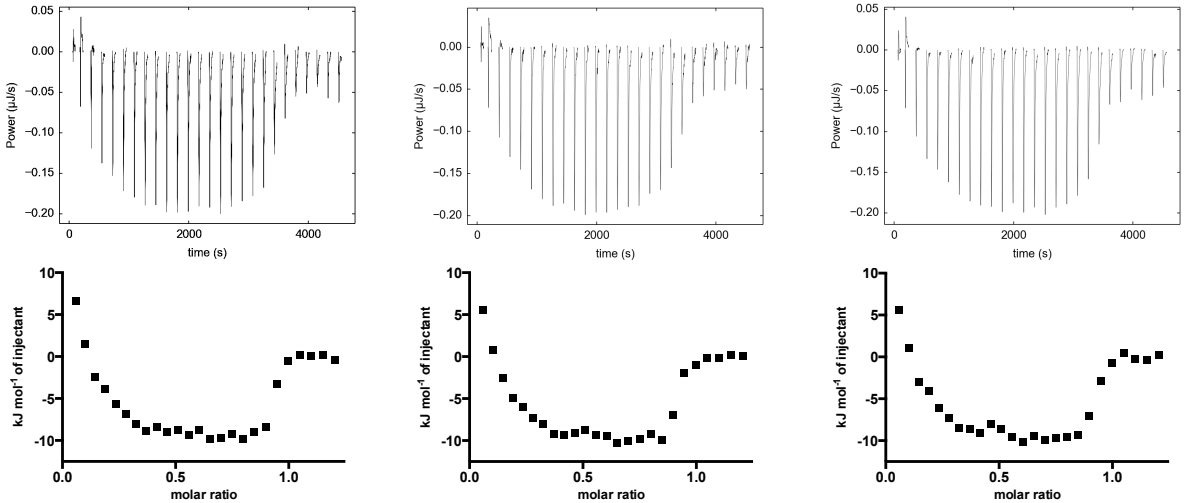


Figure S7. ITC: Titration of 1.5% C_{55}-P containing DOPC vesicles into laspartomycin C in triplicate

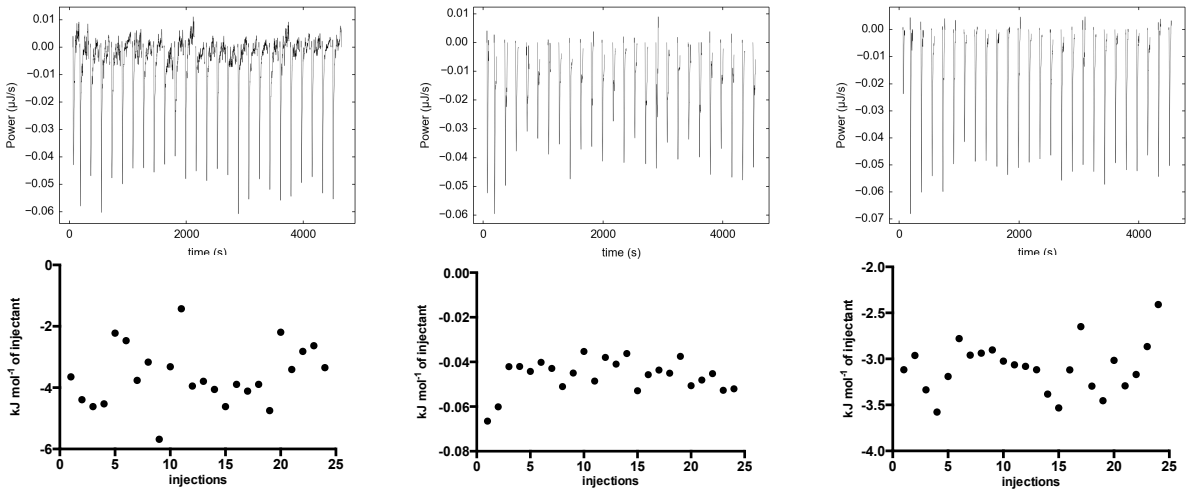
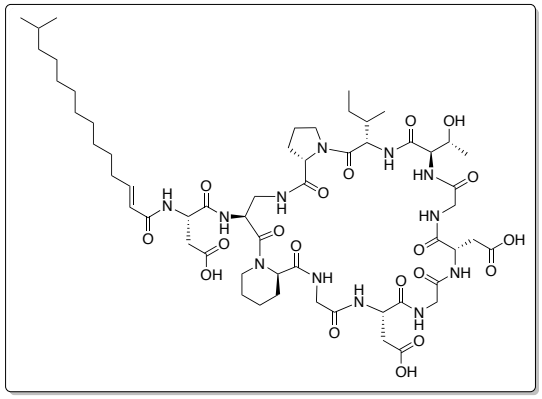


Figure S8. ITC control experiments: **Left**: Titration of C_{55}-P:DOPC vesicles (0.15 mM:10 mM) into 20 mM HEPES buffer. **Middle**: Titration of DOPC (10 mM) into 20 mM HEPES buffer. **Right**: Titration of 20 mM HEPES buffer into 25 µM Laspartomycin C.

Characterization of synthetic peptides

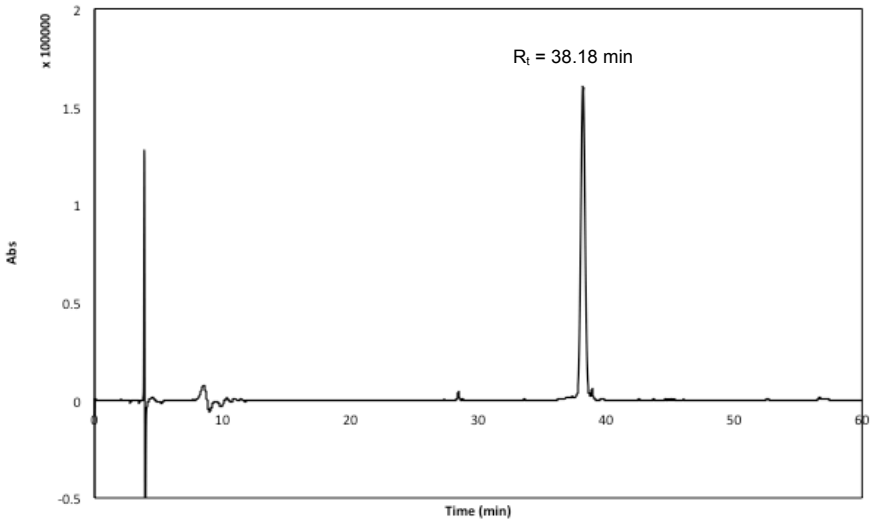


Laspartomycin C (1)

Yield: 10.3 mg (8.26 µmol, 6.6 %)

HR-MS [M+H$^+$]: Calc.: 1247.6518, found: 1247.6507

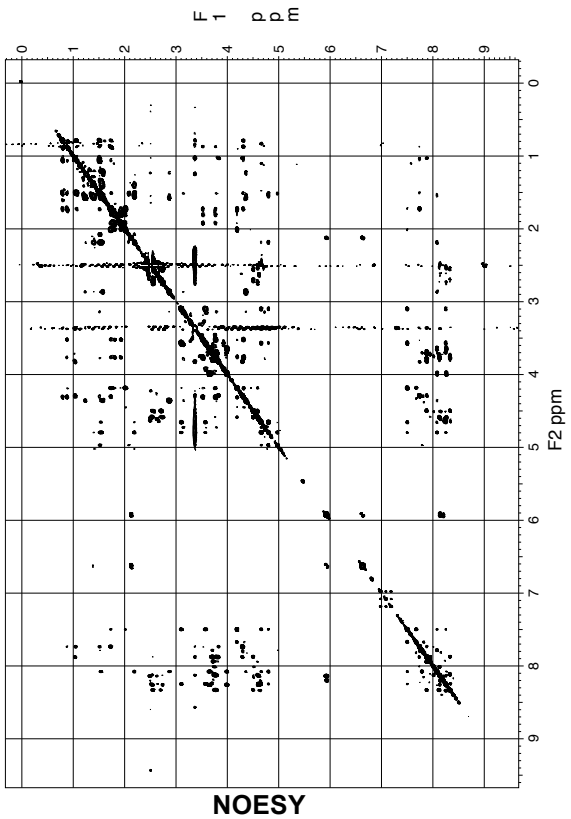
Analytical HPLC



NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^{1}H, ^{13}C)
C15	-	5.93 (123.7)	$C_\beta H$ (6.63, 142.9), $C_\gamma H_2$ (2.12, 31.0), $C_\delta H_2$ (1.38, 27.5), $C_\varepsilon H_2$ (1.26, 28.5), $C_\zeta H_2$-$C_\iota H_2$ (1.22-1.28, 28.2-29.3), $C_\kappa H_2$ (1.23, 26.5), $C_\lambda H_2$ (1.12, 38.2), $C_\mu H$ (1.38, 27.5), $2C_\nu H_3$ (0.84, 22.2)
Asp[1]	8.13	4.61 (49.1)	$C_\beta H_2$ (2.50/2.63, 35.9)
Dap[2]	8.24	4.66 (48.2)	$C_\beta H_2$ (3.10/3.57, 39.5), $N_\gamma H$ (7.50)
D-Pip[3]	-	4.80 (55.8)	$C_\beta H_2$ (1.54/2.18, 26.4), $C_\gamma H_2$ (1.40/1.51, 20.1), $C_\delta H_2$ (1.22/1.51, 24.2), $C_\varepsilon H_2$ (2.88/4.36, 39.5)
Gly[4]	8.08	3.65/4.00 (41.5)	-
Asp[5]	8.25	4.59 (49.2)	$C_\beta H_2$ (2.53/2.74, 35.8)
Gly[6]	8.13	3.76 (41.8)	-
Asp[7]	8.33	4.50 (49.7)	$C_\beta H_2$ (2.56/2.71, 35.6)
Gly[8]	7.88	3.68/3.74 (41.7)	-
D-allo-Thr[9]	7.88	4.29 (58.1)	$C_\beta H$ (3.82, 66.4), $C_\gamma H_3$ (1.03, 19.3)
Ile[10]	7.73	4.31 (54.0)	$C_\beta H$ (1.73, 35.7), $C_\gamma H_2$ (1.07/1.50, 24.1), $C_\gamma H_3$ (0.87, 14.5), $C_\delta H_3$ (0.78, 10.4)
Pro[11]	-	4.19 (59.3)	$C_\beta H_2$ (1.72/2.02, 29.1), $C_\gamma H_2$ (1.81/1.92, 24.2), $C_\delta H_2$ (3.52/3.77, 46.9)

Laspartomycin C 1



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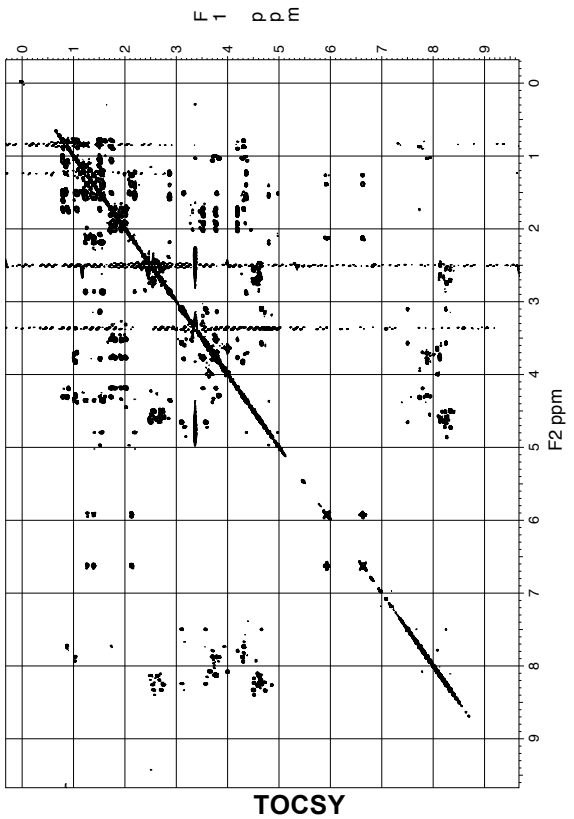
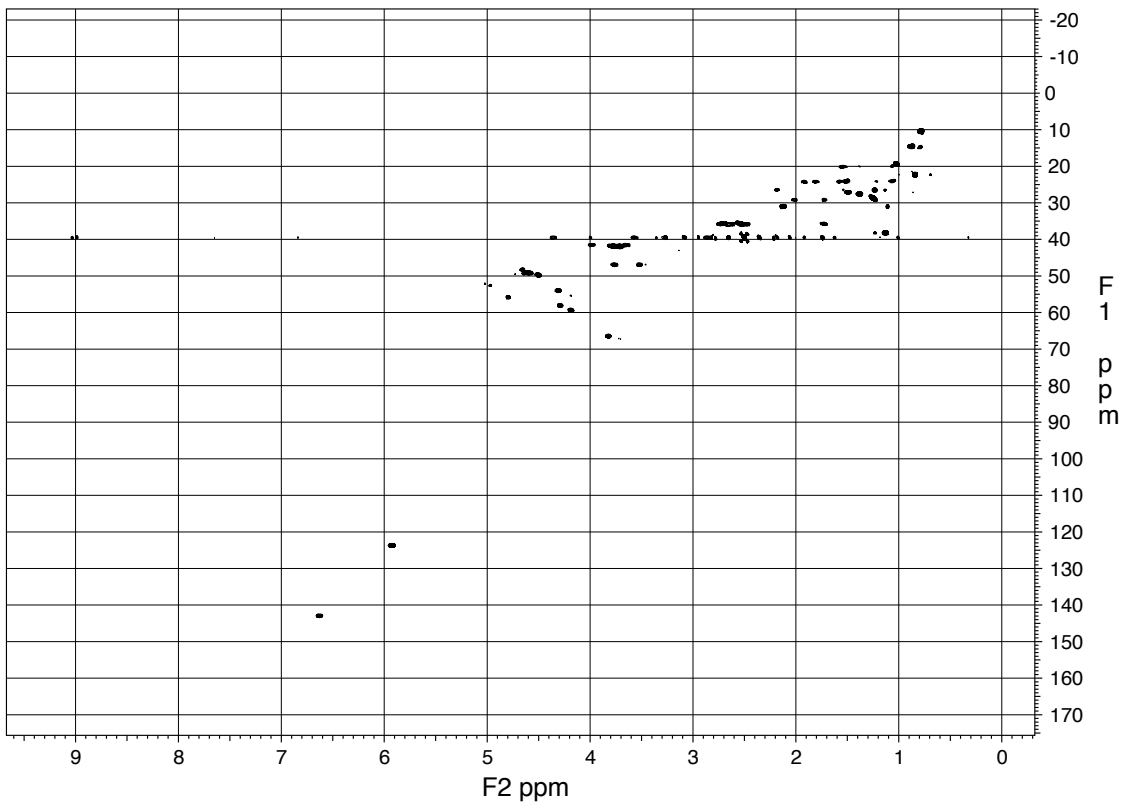
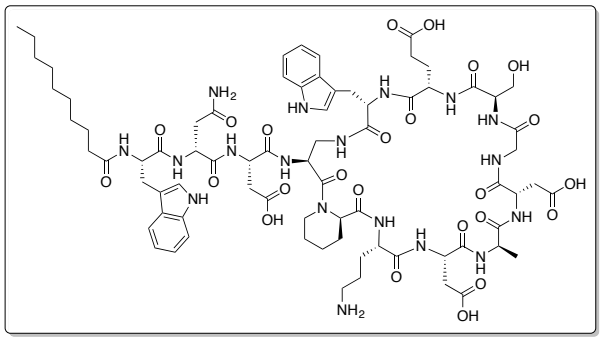


TOCSY



HSQC

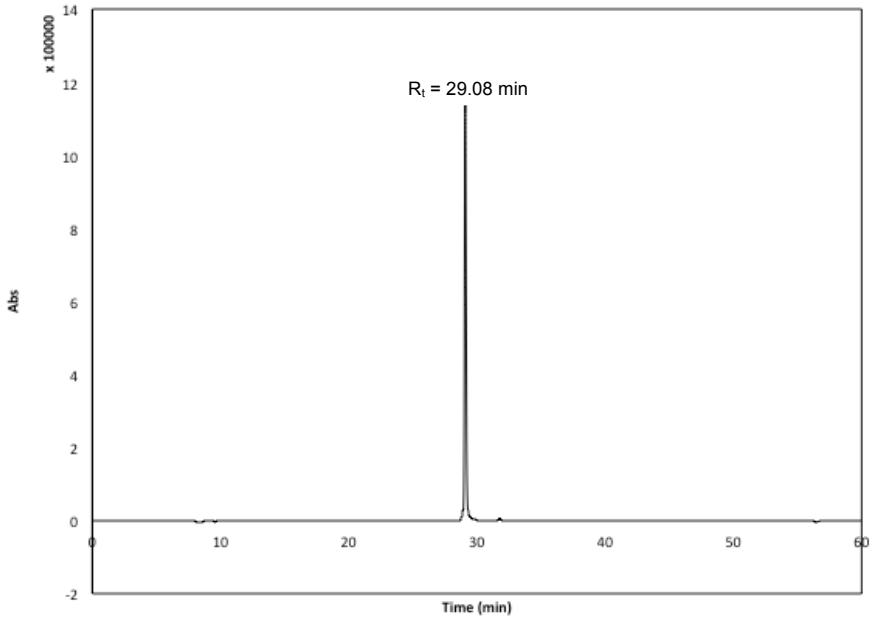
Compound 8



Yield: 10.5 mg (6.4 µmol, 7.7 %)

HRESI-MS [M+H$^+$]
Calculated 1641.7544
Found 1641.7580

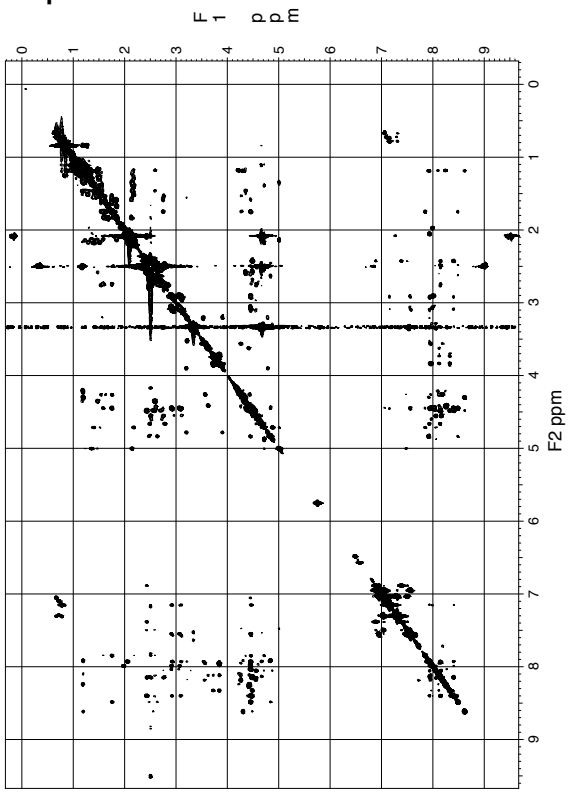
Analytical HPLC



NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^1H, ^{13}C)
C10	-	2.05 (34.80)	$C_\beta H_2$ (1.30-1.38, 24.7) $C_{\varepsilon-\iota}H_2$ (1.09-1.19, 28.5), $C_\kappa H_3$ (0.85, 13.7)
Trp[1]	7.93	4.46 (53.6)	$C_\beta H_2$ (2.92/3.10, 27.1), $CH_{\delta1}$ (7.15, 123.4), $NH_{\varepsilon1}$ (10.67), $CH_{\varepsilon3}$ (7.53-7.59, 118.1), $CH_{\zeta1}$ (7.28-7.32, 110.9), $CH_{\zeta2}$ (6.93-6.97, 117.8), CH_η (7.01-7.06, 120.5)
D-Asn[2]	8.40	4.49 (49.9)	$C_\beta H_2$ (2.43/2.58, 36.1)
Asp[3]	8.14	4.56 (49.2)	$C_\beta H_2$ (2.52/2.76, 35.7)
Dap[4]	8.18	4.79 (49.5)	$C_\beta H_2$ (3.21/3.55, 40.1) $N_\gamma H$ (7.94)
D-Pip[5]	-	4.71 (55.8)	$C_\beta H_2$ (1.40/2.18, 25.9), $C_\gamma H_2$ (1.30/1.52, 20.3), $C_\delta H_2$ (1.16/1.46, 24.1),
			$C_\varepsilon H_2$ (2.57/4.35, 39.5)
Orn[6]	7.85	4.27 (52.1)	$C_\beta H_2$ (1.75, 28.5), $C_\gamma H_2$ (1.58, 23.1), $C_\delta H_2$ (2.73, 38.3), $N_\varepsilon H_2$ (7.48)
Asp[7]	8.48	4.84 (48.7)	$C_\beta H_2$ (2.47/2.64, 36.1)
D-Ala[8]	7.92	4.30 (48.0)	$C_\beta H_3$ (1.18, 17.3)
Asp[9]	8.62	4.49 (49.8)	$C_\beta H_2$ (2.55/2.69, 35.7)
Gly[10]	8.33	3.72/3.84 (41.7)	-
D-Ser[11]	8.11	4.41 (54.9)	$C_\beta H_2$ (3.62, 61.3)
Glu[12]	8.26	4.47 (49.8)	$C_\beta H_2$ (1.68/1.85, 27.9), $C_\gamma H_2$ (2.17-2.22, 29.8)
Trp[13]	8.03	4.46 (53.6)	$C_\beta H_2$ (2.90-3.05, 27.2), $CH_{\delta1}$ (7.09, 123.3), $NH_{\varepsilon1}$ (10.77), $CH_{\varepsilon3}$ (7.53-7.59, 118.1), $CH_{\zeta1}$ (7.28-7.32, 110.9), $CH_{\zeta2}$ (6.93-6.97, 117.8), CH_η (7.01-7.06, 120.5)

Compound 8



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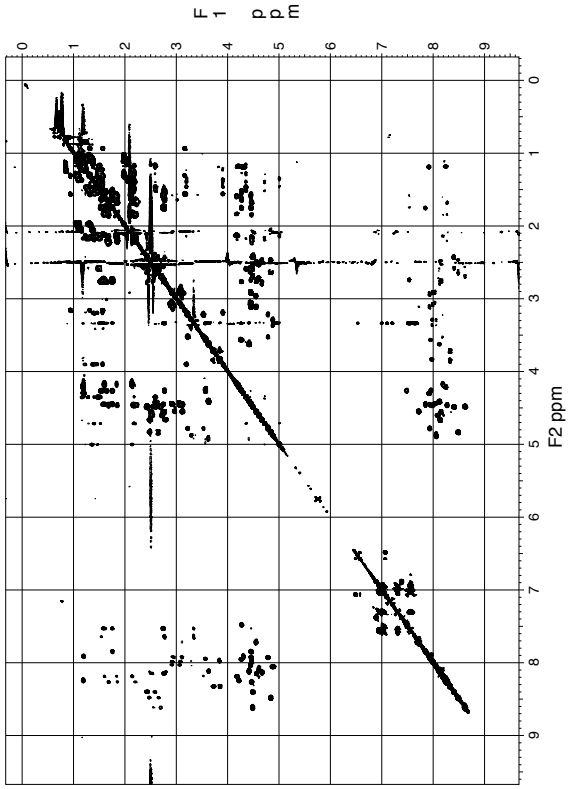
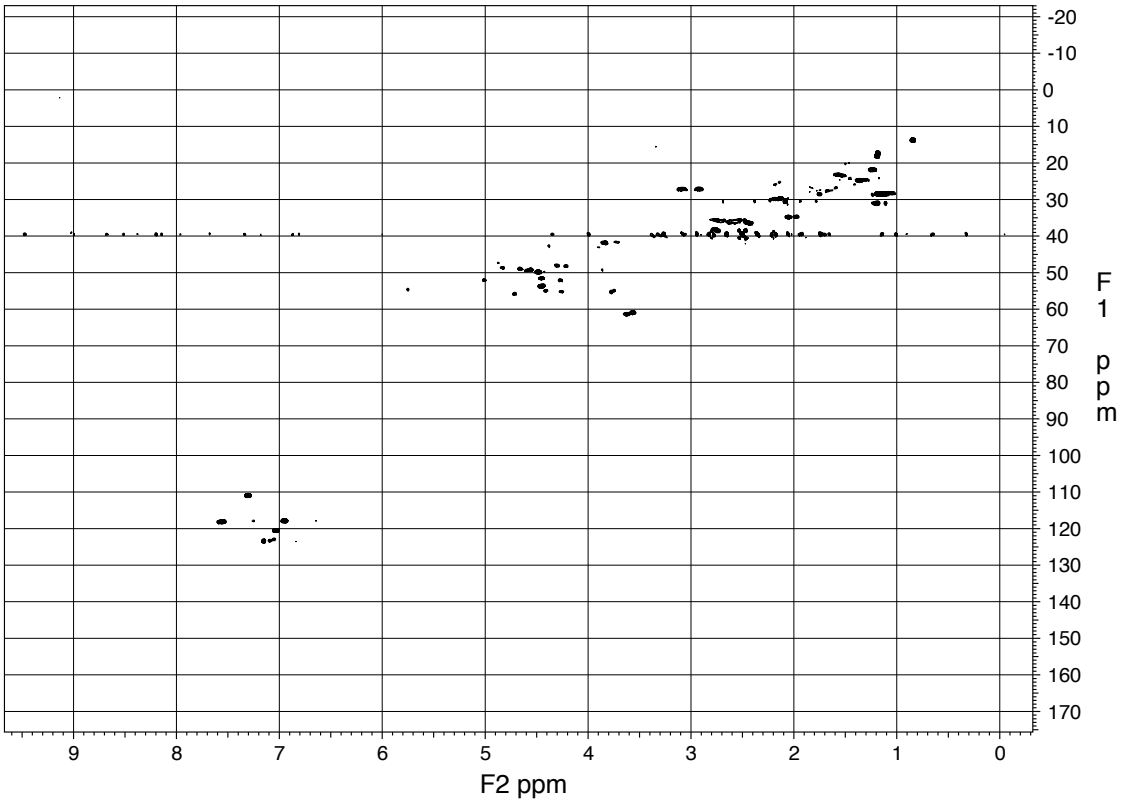
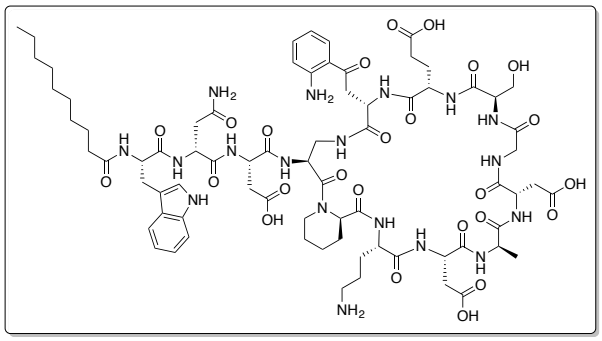


TOCSY



HSQC

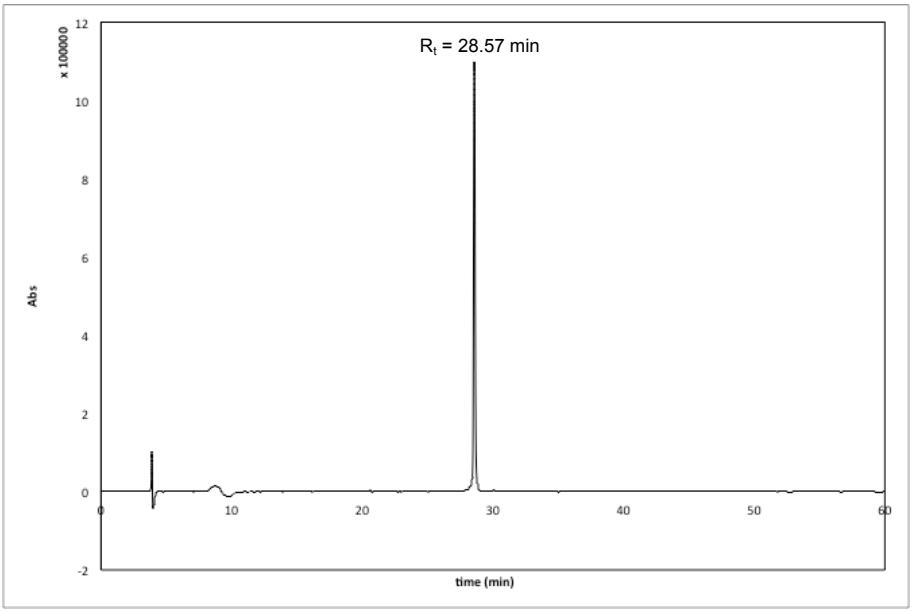
Compound 9:



Yield: 19.1 mg (11.6 µmol, 9.3 %)

HRESI-MS [M+H$^+$]
Calculated 1645.7493
Found 1645.7442

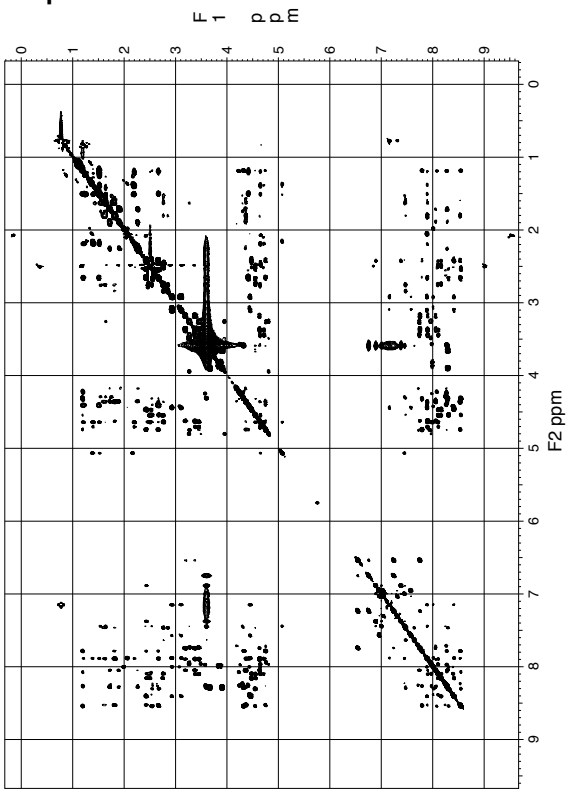
Analytical HPLC



NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^{1}H, ^{13}C)
C10	-	2.06 (34.8)	$C_\beta H_2$ (1.31/1.36, 24.7) $C_{\varepsilon\text{-}i}H_2$ (1.06-1.15, 28.5), $C_\kappa H_3$ (0.84, 13.7)
Trp[1]	7.88	4.45 (53.7)	$C_\beta H_2$ (2.92/3.11, 27.0), $C_{\delta 1}H$ (7.15, 123.4), $N_{\varepsilon 1}H$ (10.77), $C_{\varepsilon 3}H$ (7.58, 118.1), $C_{\zeta 1}H$ (7.31, 110.9), $C_{\zeta 2}H$(6.96, 117.8), $C_\eta H$ (7.04, 120.5)
D-Asn[2]	8.40	4.48 (49.8)	$C_\beta H_2$ (2.41/2.46, 35.5)
Asp[3]	8.16	4.55 (49.5)	$C_\beta H_2$ (2.50/2.75, 35.6)
Dap[4]	8.09	4.64 (49.1)	$C_\beta H_2$ (2.77/3.45, 39.7), $N_\gamma H$ (7.89)
D-Pip[5]	-	4.64 (55.7)	$C_\beta H_2$ (1.40/2.20, 25.8), $C_\gamma H_2$ (1.25/1.53, 20.2), $C_\delta H_2$ (1.21/1.59, 24.0), $C_\varepsilon H_2$ (2.65/4.42, 39.6)
Orn[6]	7.89	4.35 (52.0)	$C_\beta H_2$ (1.82, 28.2), $C_\gamma H_2$ (1.63, 23.4), $C_\delta H_2$ (2.75/2.85, 38.9), $N_\varepsilon H_2$ (7.46)
Asp[7]	8.53	4.75 (48.7)	$C_\beta H_2$ (2.43/2.67, 36.0)
D-Ala[8]	7.79	4.32 (47.9)	$C_\beta H_3$ (1.19, 18.4)
Asp[9]	8.54	4.55 (49.5)	$C_\beta H_2$ (2.55/2.68, 35.9)
Gly[10]	8.31	3.67/3.92 (41.2)	-
D-Ser[11]	8.27	4.25 (55.2)	$C_\beta H_2$ (3.59, 61.0)
Glu[12]	8.28	4.36 (51.1)	$C_\beta H_2$ (1.72/1.90, 27.9), $C_\gamma H_2$ (2.28, 29.8)
kyn[13]	8.05	4.63 (49.3)	$C_\beta H_2$ (3.17/3.39, 40.2), $C_{\varepsilon 1}H$ (7.75, 130.8), $C_{\zeta 1}H$ (6.55, 114.1), $C_{\zeta 2}H$ (6.76, 116.5), $C_\eta H$ (7.14, 133.9)

Compound 9



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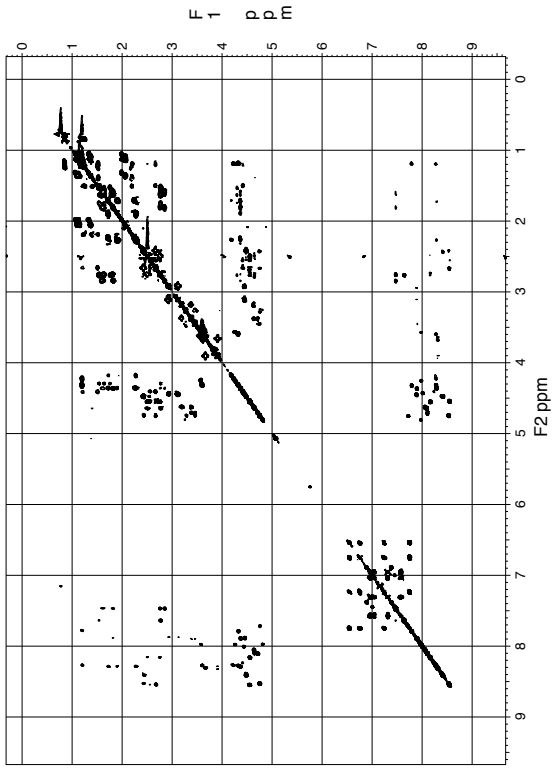
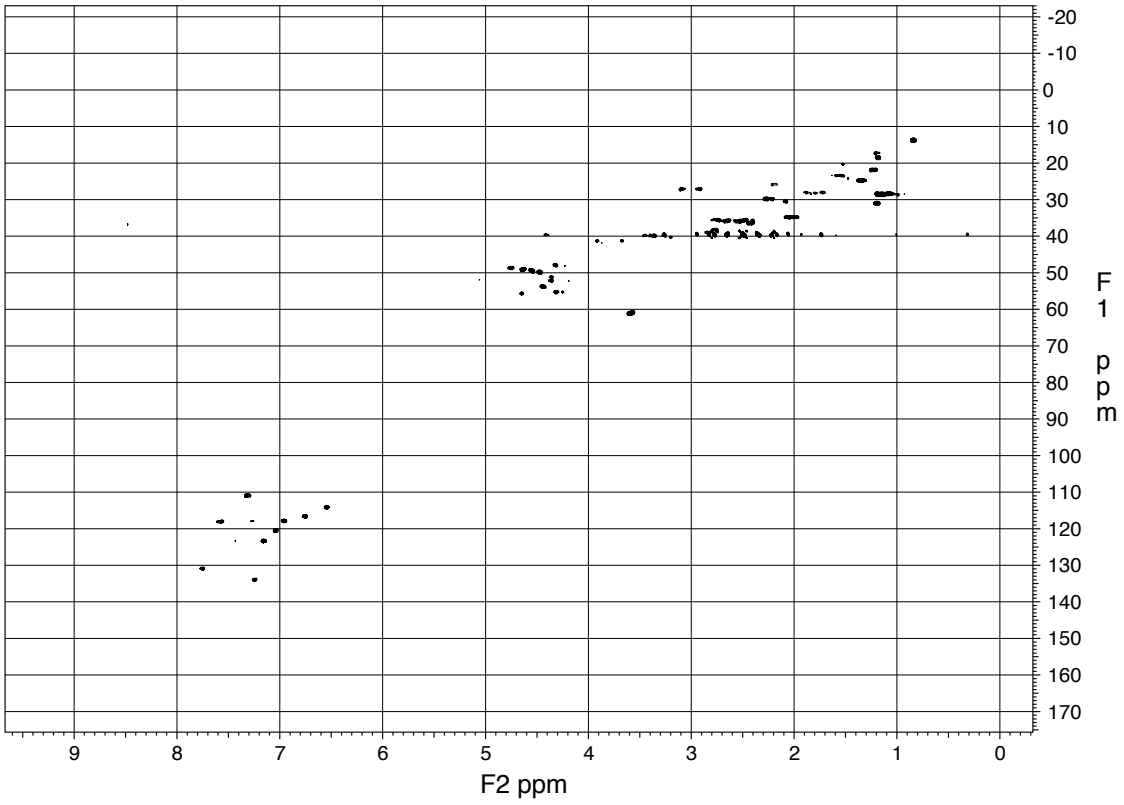
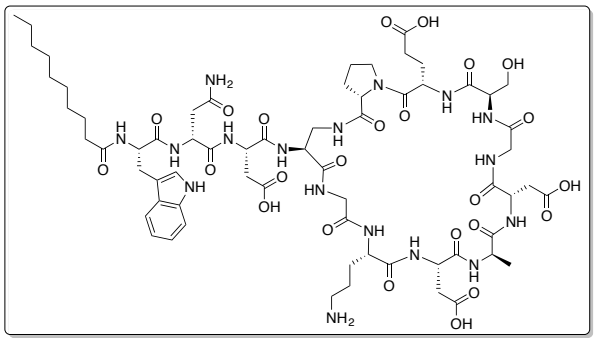


TOCSY



HSQC

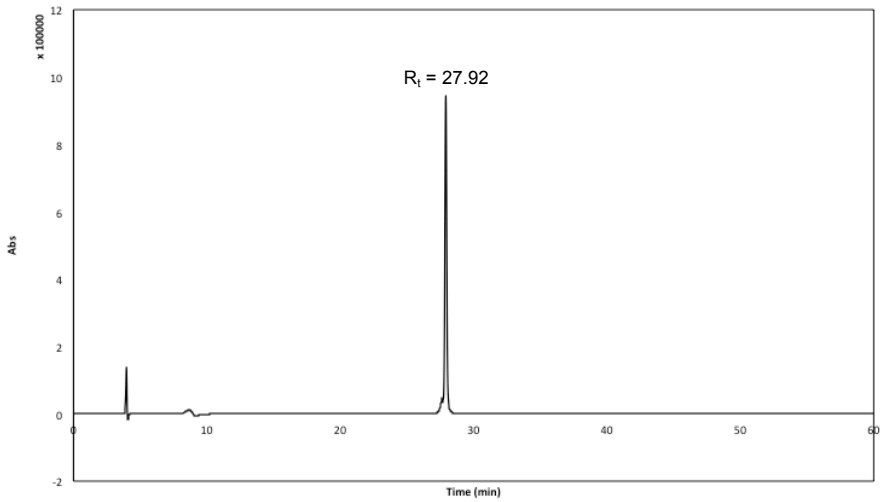
Compound 10



Yield: 9.7 mg (6.5 µmol, 4.2 %)

HRESI-MS [M+H$^+$]
Calculated 1498.6809
Found 1498.6820

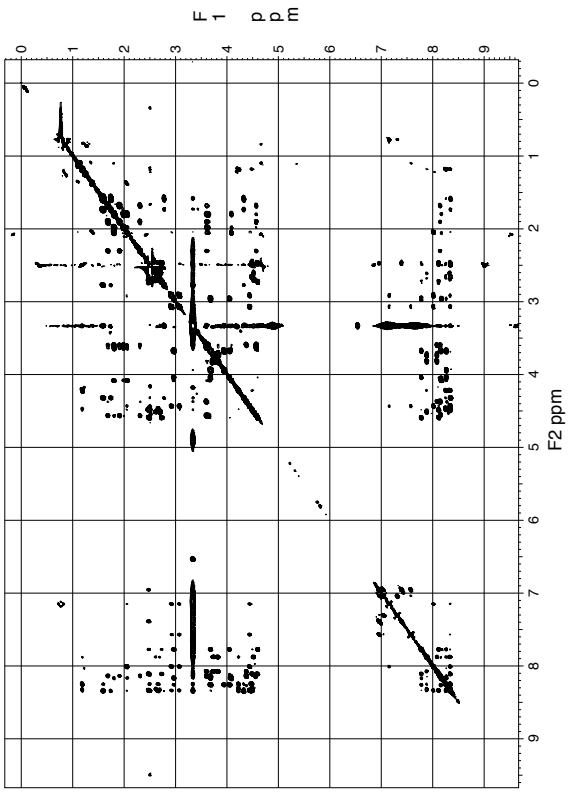
Analytical HPLC



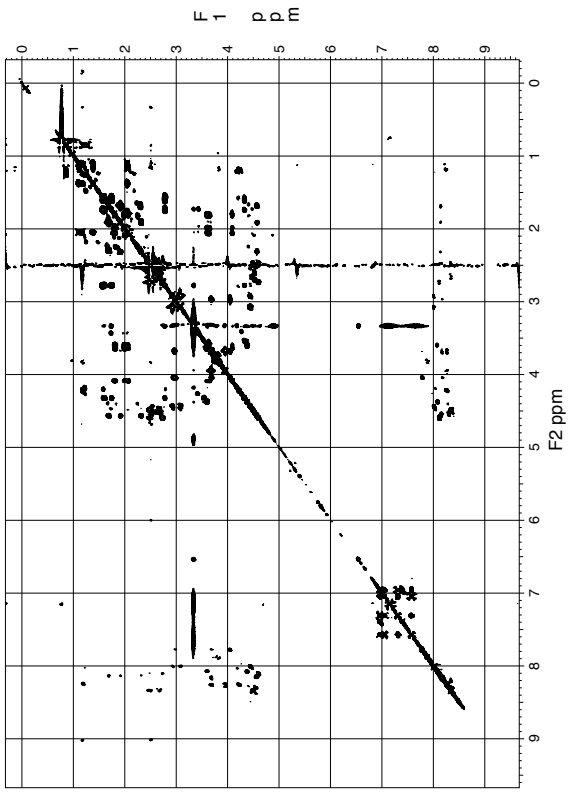
NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^{1}H, ^{13}C)
C10	-	2.05 (34.8)	$C_\beta H_2$ (1.37, 24.8), $C_{\varepsilon\text{-}\iota} H_2$ (1.08-1.20, 28.3-28.5), $C_\kappa H_3$ (0.84, 13.7)
Trp[1]	8.01	4.44 (53.8)	$C_\beta H_2$ (2.92/3.07, 26.9), $CH_{\delta 1}$ (7.16, 123.4), $NH_{\varepsilon 1}$ (10.77), $CH_{\varepsilon 3}$ (7.58, 118.0), $CH_{\zeta 1}$ (7.31, 110.9), $CH_{\zeta 2}$ (6.96, 117.8), CH_η (7.05, 120.5)
D-Asn[2]	8.34	4.49 (49.7)	$C_\beta H_2$ (2.51/2.66, 35.7)
Asp[3]	8.10	4.60 (49.0)	$C_\beta H_2$ (2.47/2.71, 35.6)
Dap[4]	7.77	4.05 (52.0)	$C_\beta H_2$ (2.94/3.68, 39.7), $N_\gamma H$ (8.17)
Gly[5]	8.25	3.67/3.94 (42.2)	-
Orn[6]	8.34	4.32 (51.5)	$C_\beta H_2$ (1.59/1.74, 28.2), $C_\gamma H_2$ (1.57, 23.0), $C_\delta H_2$ (2.77, 39.6)
Asp[7]	8.34	4.49 (49.7)	$C_\beta H_2$ (2.51/2.66, 35.7)
D-Ala[8]	8.25	4.22 (48.1)	$C_\beta H_3$ (1.18, 17.2)
Asp[9]	8.32	4.49 (49.7)	$C_\beta H_2$ (2.59/2.70, 36.3)
Gly[10]	7.88	3.73/3.81 (41.6)	-
D-Ser[11]	8.07	4.38 (54.9)	$C_\beta H_2$ (3.59, 61.6)
Glu[12]	8.14	4.56 (49.5)	$C_\beta H_2$ (1.69/1.91, 26.1), $C_\gamma H_2$ (2.30, 29.0)
Pro[13]	-	4.09 (60.1)	$C_\beta H_2$ (1.79/2.06, 28.7), $C_\gamma H_2$ (1.79/1.98, 24.5), $C_\delta H_2$ (3.60/3.65, 46.7)

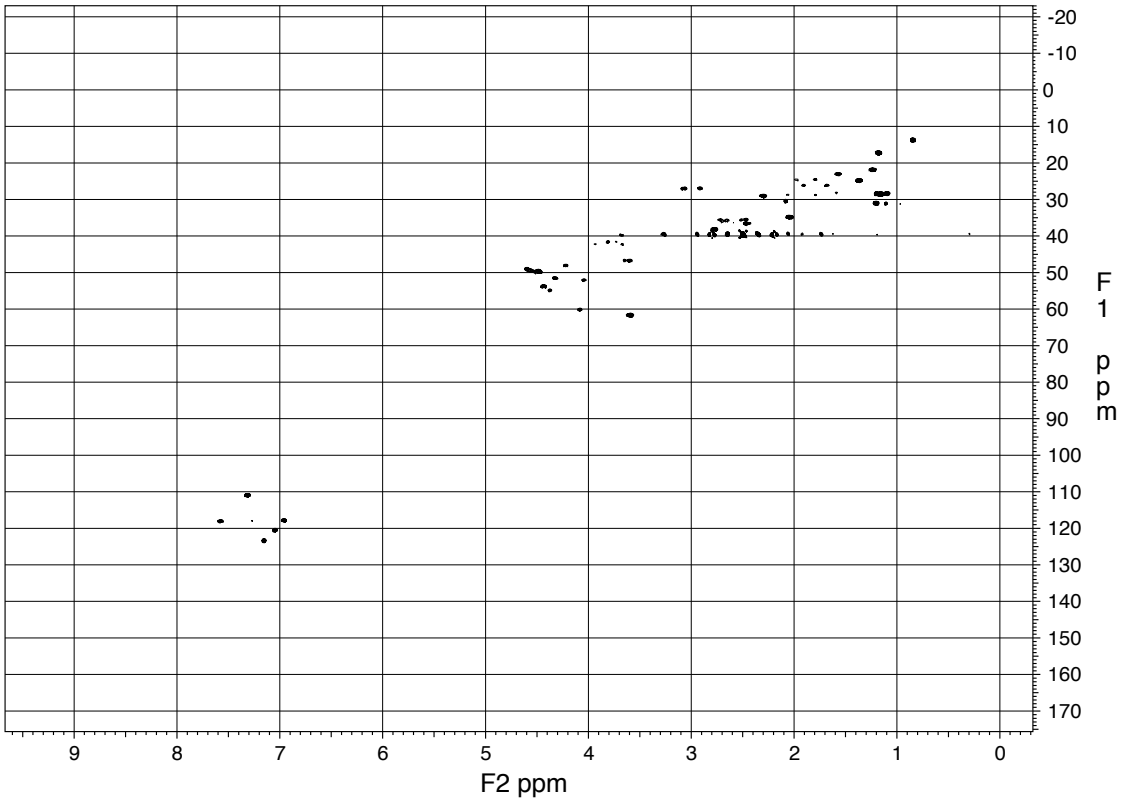
Compound 10



NOESY



TOCSY

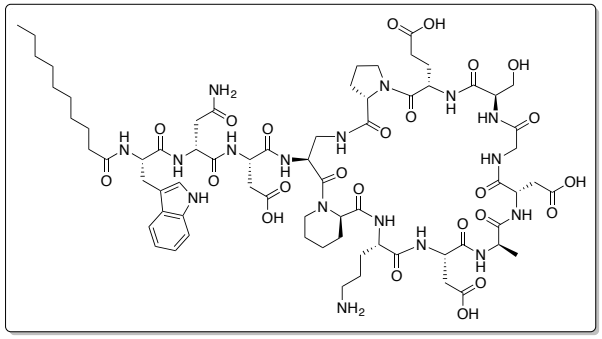


HSQC

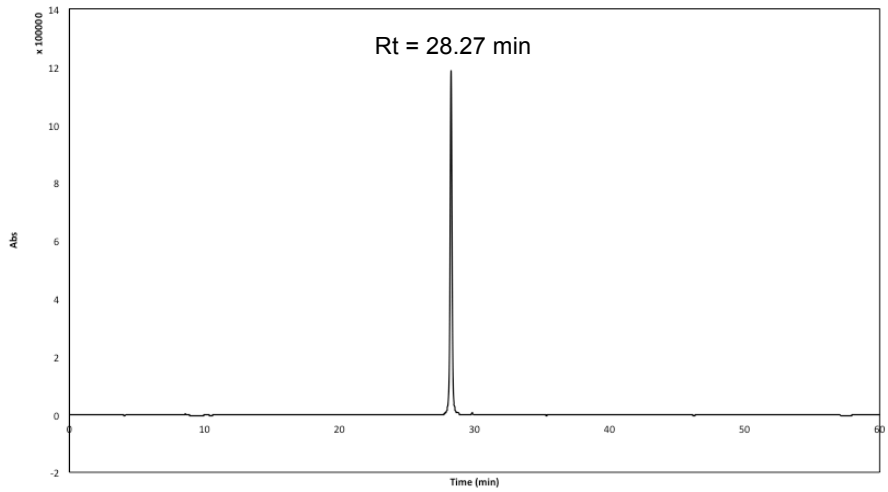
Compound 11

Yield: 15.7 mg (10.1 µmol, 8.1 %)

HRESI-MS [M+H$^+$]
Calculated 1552.7278
Found 1552.7290



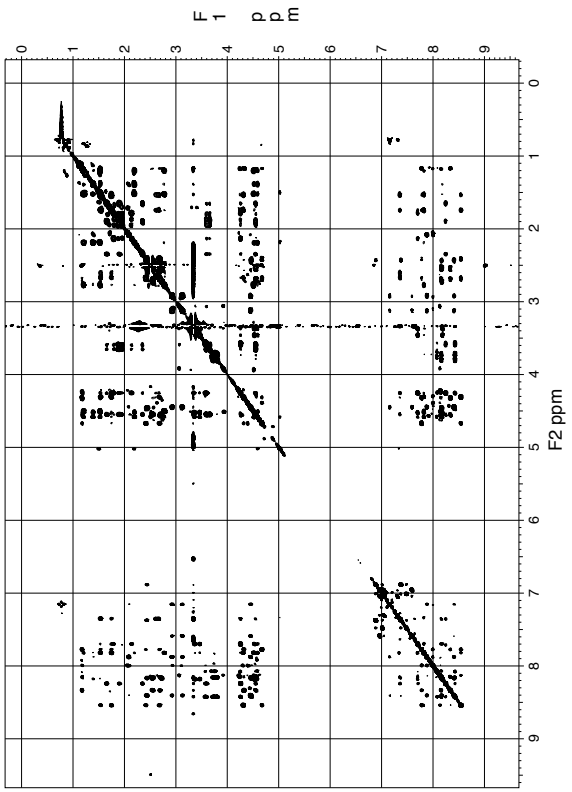
Analytical HPLC



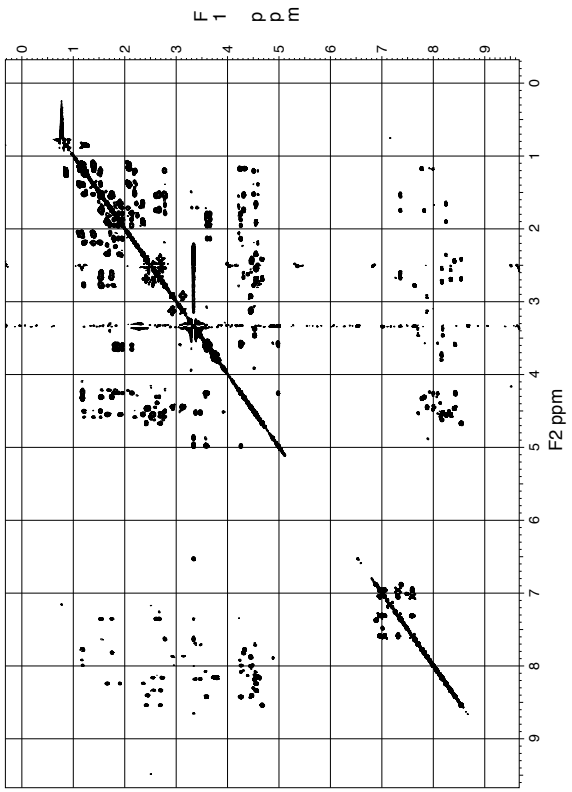
NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^1H, ^{13}C)
C10	-	2.05-2.09 (34.8)	$C_\beta H_2$ (1.37-1.40, 24.7) $C_{\varepsilon-\iota}H_2$ (1.08-1.18, 28.3-28.6), $C_\kappa H_3$ (0.84, 13.7)
Trp[1]	7.87	4.45 (53.7)	$C_\beta H_2$ (2.92/3.12, 27.1), $CH_{\delta1}$ (7.16, 123.4), $NH_{\varepsilon1}$ (10.77), $CH_{\varepsilon3}$ (7.59, 118.1), $CH_{\zeta1}$ (7.31, 110.9), $CH_{\zeta2}$ (6.96, 117.8), CH_η (7.05, 120.5).
D-Asn[2]	8.41	4.46 (49.8)	$C_\beta H_2$ (2.56/2.69, 35.8)
Asp[3]	8.16	4.53 (49.4)	$C_\beta H_2$ (2.54/2.73, 35.6)
Dap[4]	8.17	4.53 (49.4)	$C_\beta H_2$ (3.37/3.46, 39.0), $N_\gamma H$ (7.70)
D-Pip[5]	-	4.58 (55.7)	$C_\beta H_2$ (1.42/2.19, 25.6), $C_\gamma H_2$ (1.20/1.52, 20.4), $C_\delta H_2$ (1.18/1.52, 24.1), $C_\varepsilon H_2$ (2.78/4.50, 39.9)
Orn[6]	7.82	4.31 (52.0)	$C_\beta H_2$ (1.75, 27.8), $C_\gamma H_2$ (1.53, 23.3), $C_\delta H_2$ (2.60/2.67, 38.7), $N_\varepsilon H_2$ (7.35)
Asp[7]	8.54	4.67 (48.6)	$C_\beta H_2$ (2.41/2.68, 36.3)
D-Ala[8]	7.77	4.32 (47.7)	$C_\beta H_3$ (1.17, 18.6)
Asp[9]	8.33	4.55 (49.5)	$C_\beta H_2$ (2.56/2.69, 35.8)
Gly[10]	8.16	3.73/3.79 (40.9)	-
D-Ser[11]	8.42	4.25 (55.3)	$C_\beta H_2$ (3.56/3.59, 61.0)
Glu[12]	8.25	4.54 (49.3)	$C_\beta H_2$ (1.65/1.90, 26.4), $C_\gamma H_2$ (2.35, 29.0)
Pro[13]	-	4.25 (58.9)	$C_\beta H_2$ (1.78/2.13, 29.3), $C_\gamma H_2$ (1.87/1.95, 24.2), $C_\delta H_2$ (3.59/3.65, 46.5)

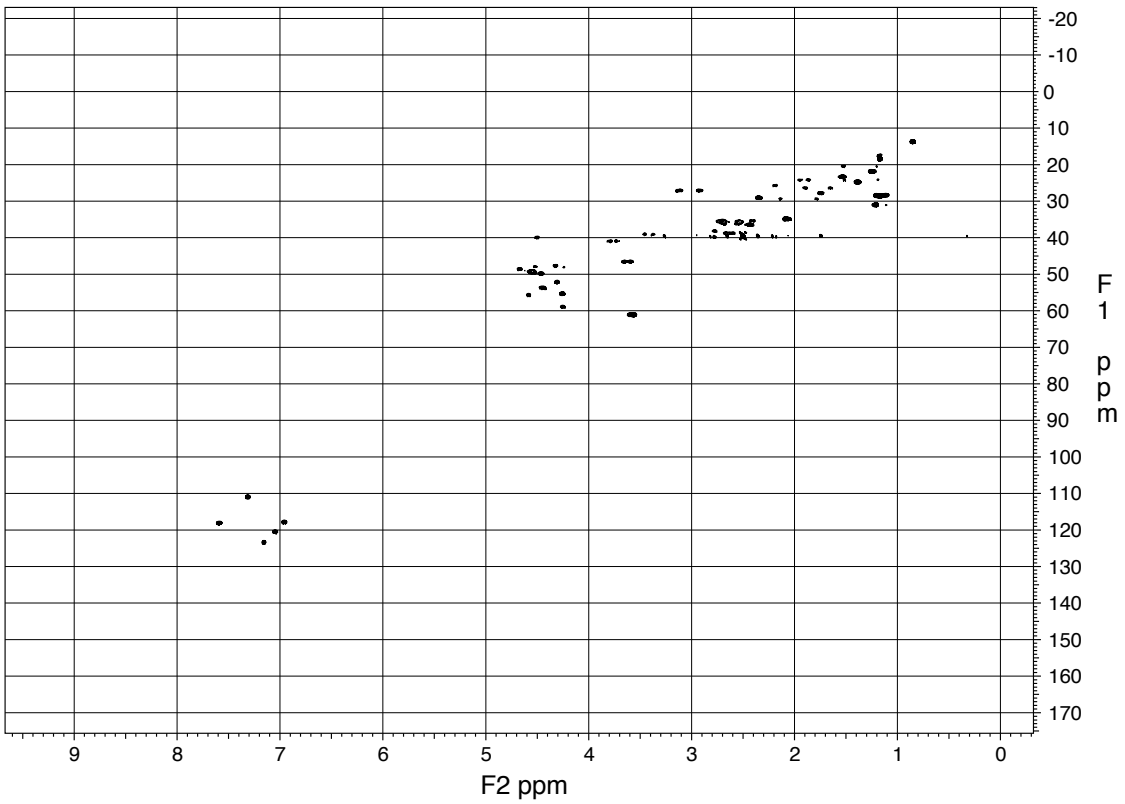
Compound 11



NOESY

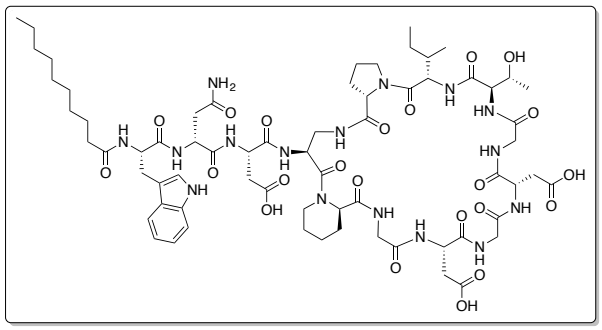


TOCSY



HSQC

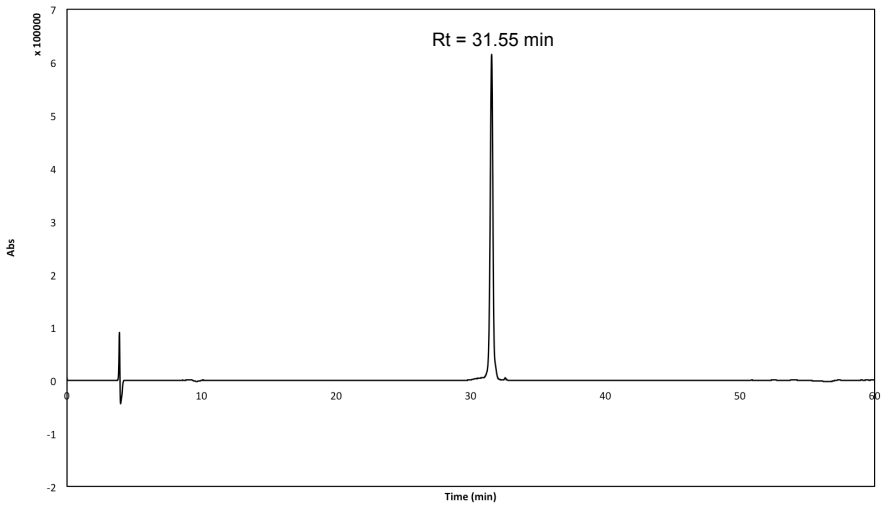
Compound 12



Yield: 11.6 mg (7.8 µmol, 6.3 %)

HRESI-MS [M+H$^+$]
Calculated 1479.7114
Found 1479.7117

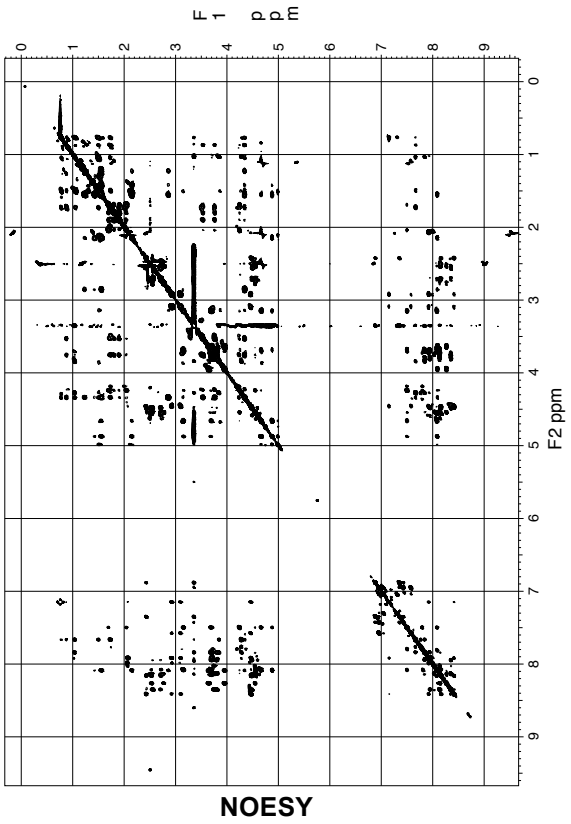
Analytical HPLC



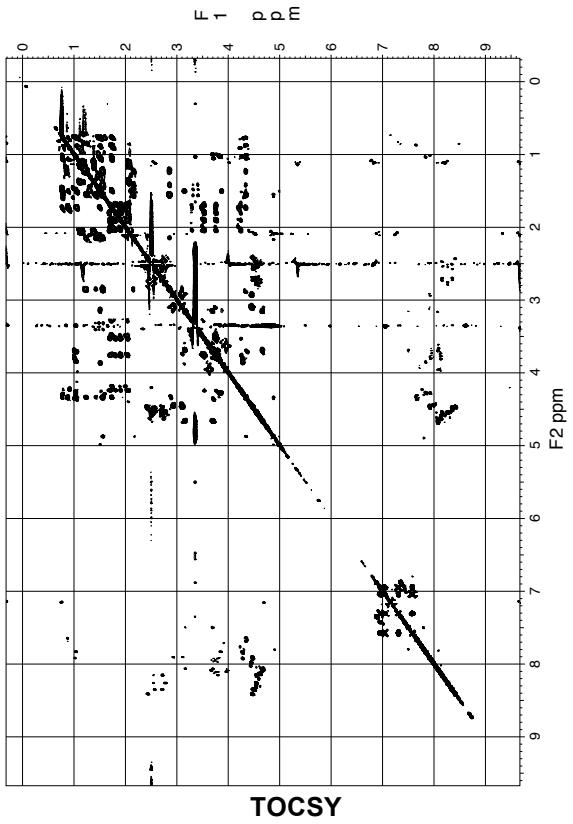
NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Additional (^1H, ^{13}C)
C10	-	2.05-2.09 (34.8)	$C_\beta H_2$ (1.37, 24.7) $C_{\varepsilon\text{-}l}H_2$ (1.09-1.19, 28.2-28.6), $C_\kappa H_3$ (0.85, 13.7)
Trp[1]	7.92	4.46 (53.6)	$C_\beta H_2$ (2.92/3.09, 27.1), $CH_{\delta 1}$ (7.15, 123.4), $NH_{\varepsilon 1}$ (10.76), $CH_{\varepsilon 3}$ (7.58, 118.1), $CH_{\zeta 1}$ (7.30, 110.9), $CH_{\zeta 2}$ (6.96, 117.8), CH_η (7.05, 120.5)
D-Asn[2]	8.40	4.47 (49.9)	$C_\beta H_2$ (2.43, 36.5)
Asp[3]	8.16	4.55 (49.2)	$C_\beta H_2$ (2.54/2.72, 35.6)
Dap[4]	8.06	4.67 (47.5)	$C_\beta H_2$ (3.16/3.69, 39.6), $N_\gamma H$ (7.50)
D-Pip[5]	-	4.88 (55.5)	$C_\beta H_2$ (1.55/2.16, 26.3), $C_\gamma H_2$ (1.41/1.55 19.9), $C_\delta H_2$ (1.22/1.51, 24.2), $C_\varepsilon H_2$ (2.88/4.34, 39.4)
Gly[6]	8.08	3.63/3.94 (41.6)	-
Asp[7]	8.26	4.55 (49.3)	$C_\beta H_2$ (2.54/2.77, 35.6)
Gly[8]	8.13	3.80 (41.8)	-
Asp[9]	8.35	4.50 (49.9)	$C_\beta H_2$ (2.55/2.71, 35.6)
Gly[10]	7.94	3.68/3.79 (41.8)	-
D-allo-Thr[11]	7.84	4.28 (58.2)	$C_\beta H$ (3.84, 66.5), $C_\gamma H_3$ (1.02, 19.3)
Ile[12]	7.66	4.34 (53.9)	$C_\beta H$ (1.72, 35.9), $C_\gamma H_2$ (1.04/1.50, 24.0), $C_\gamma H_3$ (0.87, 14.6), $C_\delta H_3$ (0.77, 10.4)
Pro[13]	-	4.23 (59.1)	$C_\beta H_2$ (1.70/2.04, 29.2), $C_\gamma H_2$ (1.80/1.89, 24.2), $C_\delta H_2$ (3.53/3.77, 46.9)

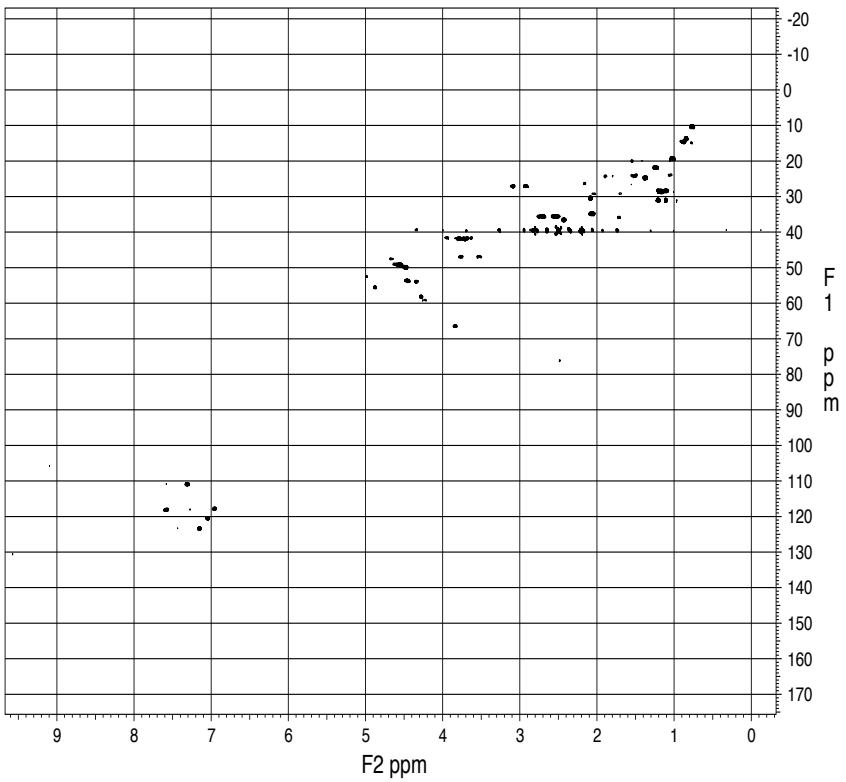
Compound 12



NOESY



TOCSY



HSQC

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