Supporting information for:

Mechanistic insights into the C_{55}-P targeting lipopeptide antibiotics revealed by structure-activity studies and high-resolution crystal structures

Thomas M. Wood,[a,b,†] Matthieu R. Zeronian,[c,†] Ned Buijs,[a] Kristine Bertheussen,[a] Hanieh K. Abedian,[a] Aidan V. Johnson,[a] Nicholas M. Pearce,[c] Martin Lutz,[c] Johan Kemmink,[d] Tjalling Seirsma,[e] Leendert W. Hamoen,[e] Bert J. C. Janssen,[c,*] Nathaniel I. Martin[a,*]

[a]Biological Chemistry Group, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands. [b]Department of Chemical Biology & Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands. [c]Structural Biochemistry, Bijvoet Centre for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands. [d]Faculty of Science and Engineering, University of Groningen, Groningen, The Netherlands. [e]Bacterial Cell Biology and Physiology Group, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands.

[†]These authors contributed equally to this work

Corresponding Authors

b.j.c.janssen@uu.nl

n.i.martin@biology.leidenuniv.nl

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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. D-amino acids and 2-chlorotrityl resin was obtained from Iris Biotech GmbH, Egg PG and 0:6 PA was obtained from INstruchemie BV. C_{10}-P lithium salt was obtained from Sigma Aldrich and lyophilized from warm ^{t}BuOH:H_2O (1:1) to obtain a white powder with increased aqueous solubility.

Instrumentation for Compound Characterization

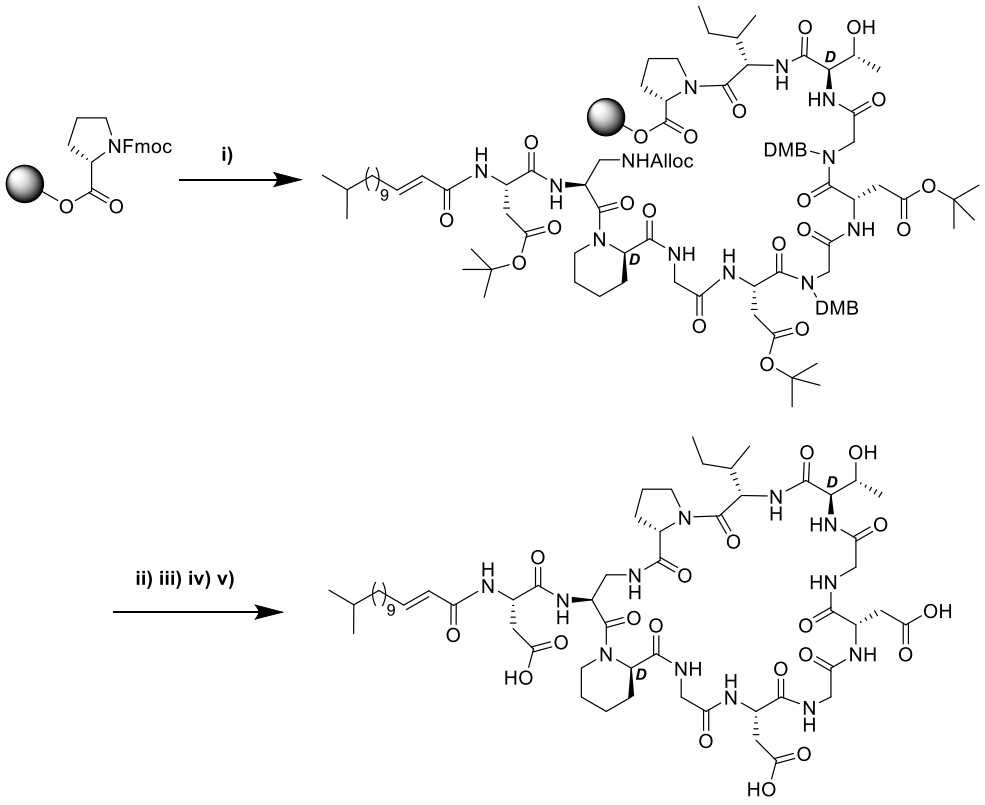
2D NMR experiments were performed on a 850 MHz instrument. HSQC, TOCSY and NOESY spectra were recorded for all peptides (5 mM in $DMSO_{d6}$) and the parent compound laspartomycin C matched pervious recorded spectra reported by our group.

HRMS analysis was performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionisation) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10000.

Purity of the peptides was confirmed to be ≥ 95% by analytical RP-HPLC using a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 µm) at 30 °C and equipped with a UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile, 95/5; solvent B, 0.1 % TFA in water/acetonitrile, 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 55 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

The compounds were purified via preparative HPLC using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 µm) and equipped with a ECOM Flash UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 55 min, 0:100 (A/B) for 2 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 2 min.

General Procedure for the Preparation of Laspartomycin C and Other Analogues



Scheme S1 (i) Fmoc SPPS; (ii) $Pd[(C_6H_5)_3P]_4$, $C_6H_5SiH_3$, CH_2Cl_2, 1 h; (iii) HFIP, CH_2Cl_2, 1 h; (iv) BOP, DIPEA, CH_2Cl_2, 16 h; (v) TFA, TIS, H_2O, 1 h

Solid Phase Peptide Synthesis

Chlorotrityl resin (5.0 g, 1.60 mmol/g) was loaded with Fmoc-Pro-OH. Resin loading was determined to be 0.41-0.62 $mmol.g^{-1}$. Linear peptide encompasing Pro11 to Asp1 were assembled manualy via standard Fmoc solid-phase peptide synthesis (SPPS) (resin bound AA:Fmoc-AA:BOP:DiPEA, 1:4:4:8 molar eq.) on a 0.1 mmol scale. DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4 v:v). Amino acid side chains were protected as follows: ^{t}Bu for Asp, Alloc for DAP, and DMB for Gly6 and Gly8. D-*allo*-Thr was introduced without side chain protection. Following coupling and Fmoc deprotection of Asp1, N-terminal acylation was achieved by coupling (*E*)-13-methyltetradec-2-enoic acid using the same coupling conditions used for SPPS. The resin-bound, Alloc protected intermediate was next washed with CH_2Cl_2 and treated with $Pd(PPh_3)_4$ (30mg, 0.03 mmol) and $PhSiH_3$

(0.30 mL, 3.0 mmol) in CH_2Cl_2 (ca. 7 mL) under argon for 1 hour. 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 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 (150 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 90 minutes. The reaction mixture was added to MTBE:hexanes (1:1) and the resulting precipitate washed once more with MTBE:hexanes (1:1). The crude cyclic peptide was lyophilized from ^{t}BuOH:H_2O (1:1) and purified with reverse phase HPLC. Pure fractions were pooled and lyophilized to yield the desired cyclic lipopeptide products in >95% purity as white powders, typically in 10-45 mg quantities (4.2-30 % yield based on resin loading).

Abbreviations:

AA	Amino acid
Alloc	Allyloxycarbonyl
^{t}Bu	tert-butyl
^{t}BuOH	tert-butanol
BOP	(benzotriazole-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
Dap	2,3-Diaminopropionic acid
DiPEA	*N,N*-diisopropylethylamine
DMB	2,4-dimethoxybenzyl
DMF	*N,N*-dimethylformamide
Fmoc	Fluorenylmethyloxycarbonyl
MTBE	Methyl tert-butyl ether
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane

Antibacterial Assays

Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to CLSI guidelines.[1] Blood agar plates were inoculated with glycerol stocks of MRSA 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 MRSA 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 three or more measurements. The following strains were obtained from BEI Resources, NIAID, NIH: *S. aureus* Strain 880 (BR-VRSA), NR-49120; *S. aureus* Strain LIM 2 (VISA), NR-45881.

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

	MRSA USA 300					***S. simulans 22***				
Compound	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**
1 (LaspC)	>128	8	4	4	2	>128	4	4	4	2
6	>128	8	4	2	1	>128	8	8	4	2
7	>128	16	8	4	2	>128	4	4	4	1
Friulimicin	>128	8	4	2	1-2	>128	2	2	1	1
Daptomycin	>128	0.5	0.25	0.25	0.125	>128	1	0.063	0.031	0.031

Table S2. MIC values (μg mL^{-1}) against VRSA and VISA at various Ca^{2+} concentrations.

	BR-VRSA					VISA LIM2				
Compound	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**
1 (LaspC)	>128	4	4	4	2	>128	8-16	4	4	2
6	>128	8	4	2	1	>128	8-16	4	2	1
7	>128	8	2	1	0.5	>128	8	4	2	2
Friulimicin	>128	2	2	2	1	>128	4	4	4	2
Daptomycin	>128	0.5	0.25	0.25	0.125	>128	1	0. 5	0.125	0.125

Table S3. MIC values (μg mL^{-1}) against *E. faeceum* E7128 (daptomycin resistant) and VRE 155 at various Ca^{2+} concentrations.

	E7128					VRE 155				
Compound	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**	**0 mM**	**1.0 mM**	**2.5 mM**	**5 mM**	**10 mM**
1 (LaspC)	>128	32	16	8	8	>128	8	4	4	2
6	>128	8	4	4	2	>128	8	2	1	0.5
7	>128	8	2	2	2	>128	8	1	1	0.5
Friulimicin	>128	4	4	4	2	>128	4	2	1	0.5
Daptomycin	>128	8	4	4	2	>128	0.5	0.25	0.25	0.125

UDP-MurNAc-pentapeptide Accumulation Assay

MRSA USA 300 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 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).

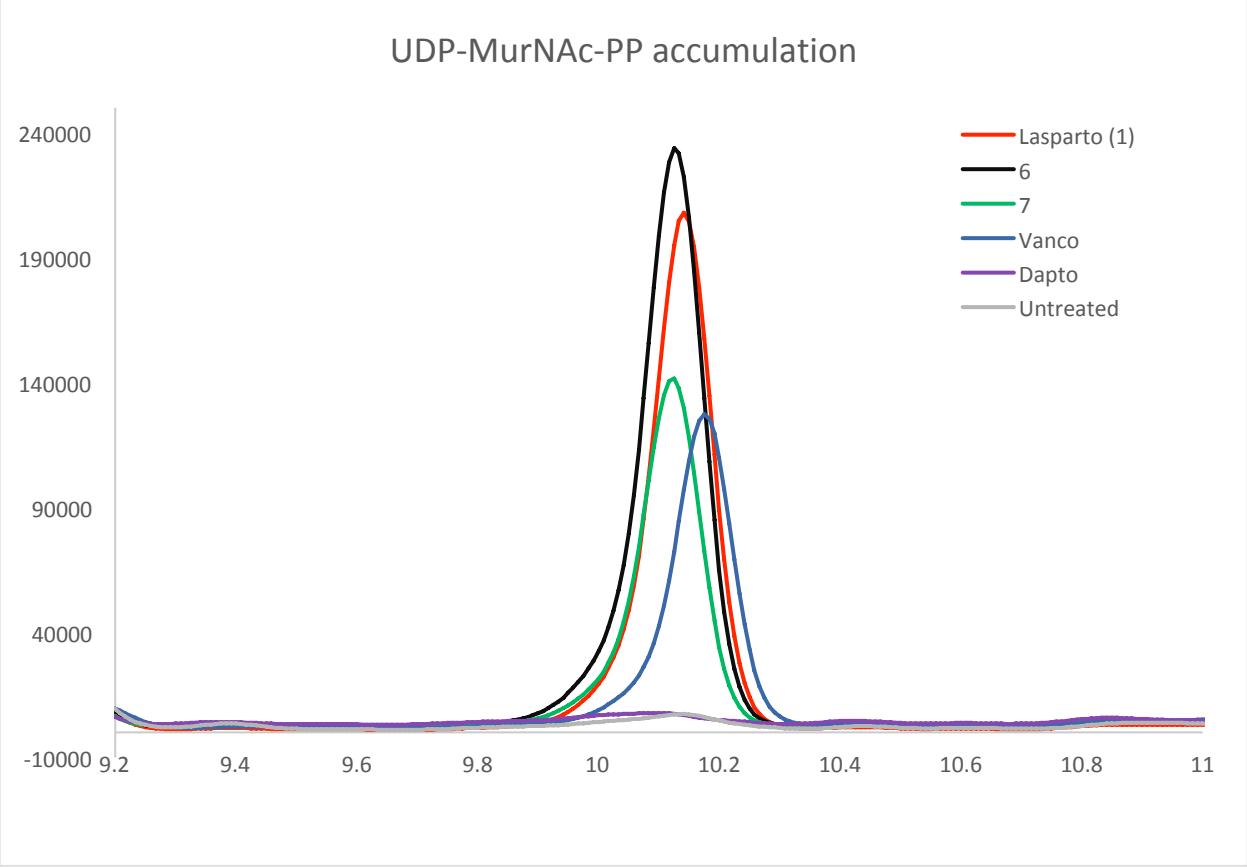
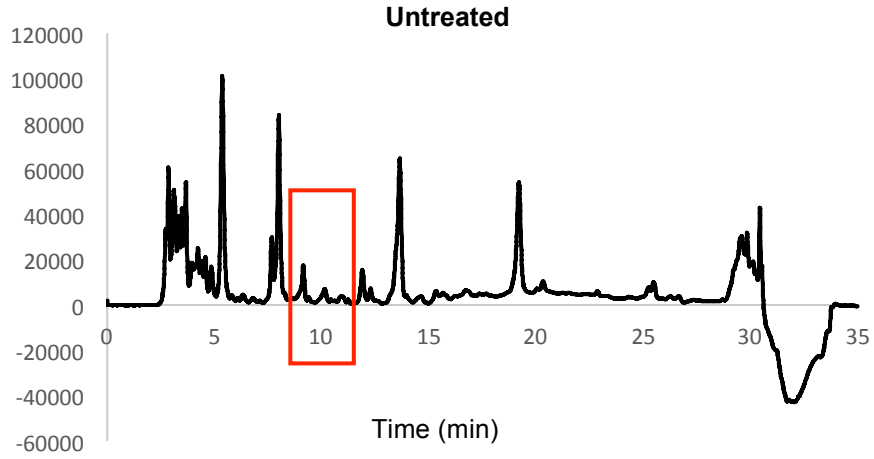
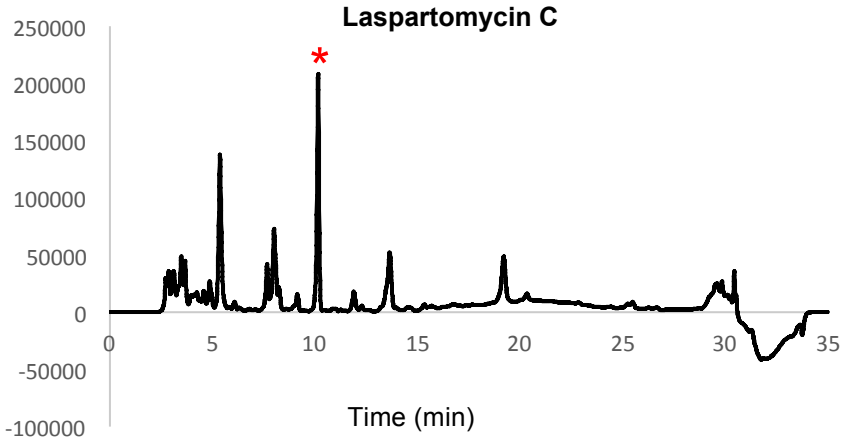
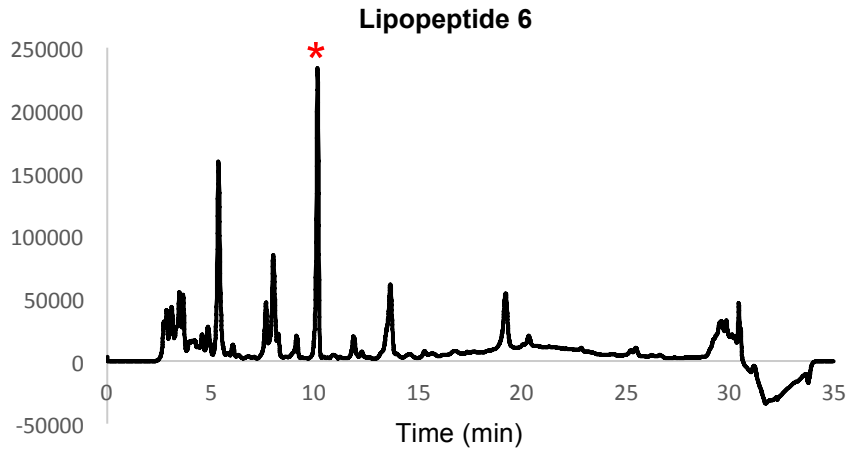


Figure S1. Analytical HPLC trace (zoom) for UDP-MurNAc-pentapeptide accumulation assay. Treatment of MRSA USA 300 with laspartomycin C (**1**), and lipopepeptides **6** and **7** results in accumulation of UDP-MurNAc-pentapeptide, an effect not observed with daptomycin. Vancomycin included as positive control.

Full analytical HPLC traces for UDP-MurNAc-pentapeptide accumulation assays



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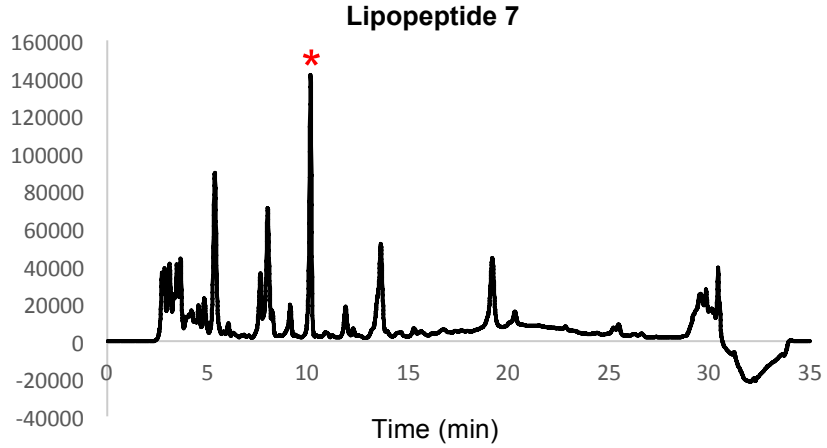
5
10
15
20
25
30
35
Time (min)
*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30
35
Time (min)
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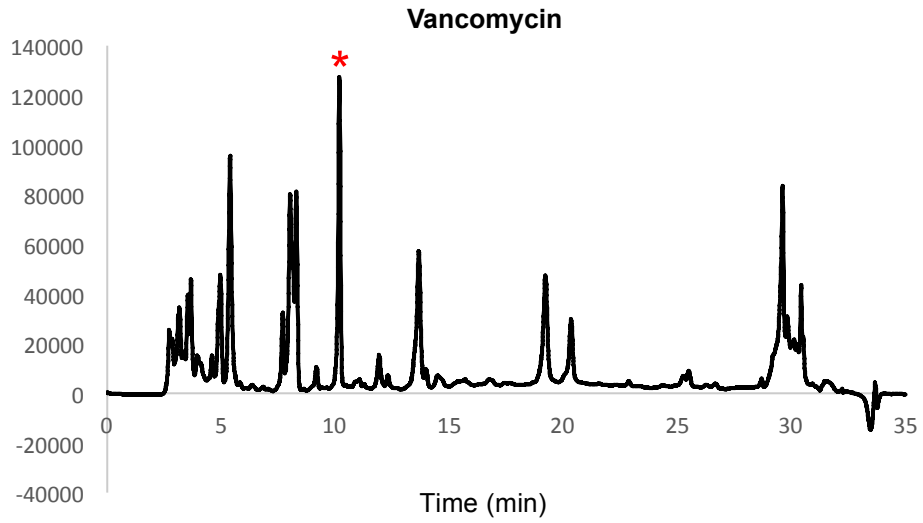
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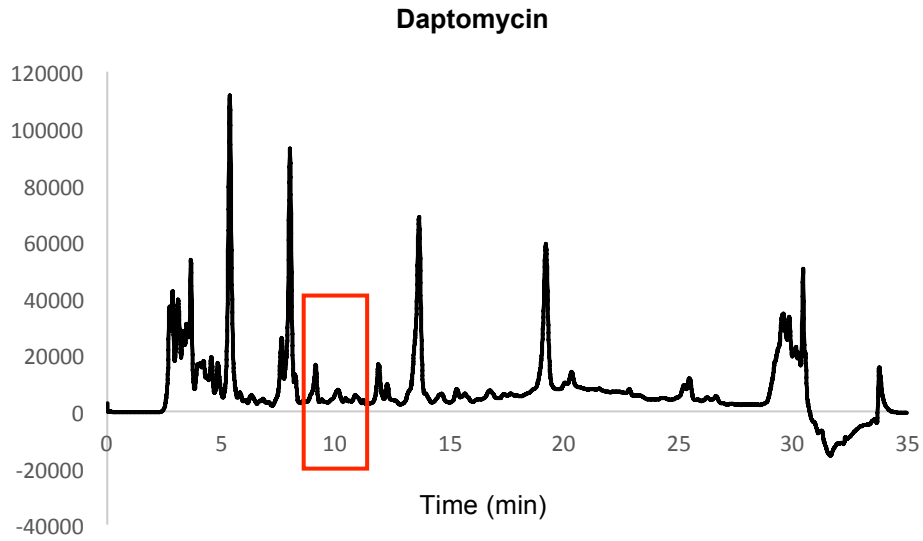
Time (min)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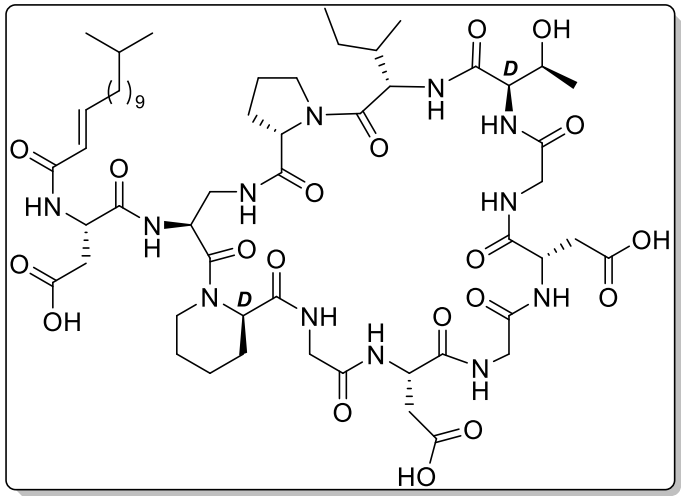
Time (min)

Characterization of Synthetic Peptides 1-15

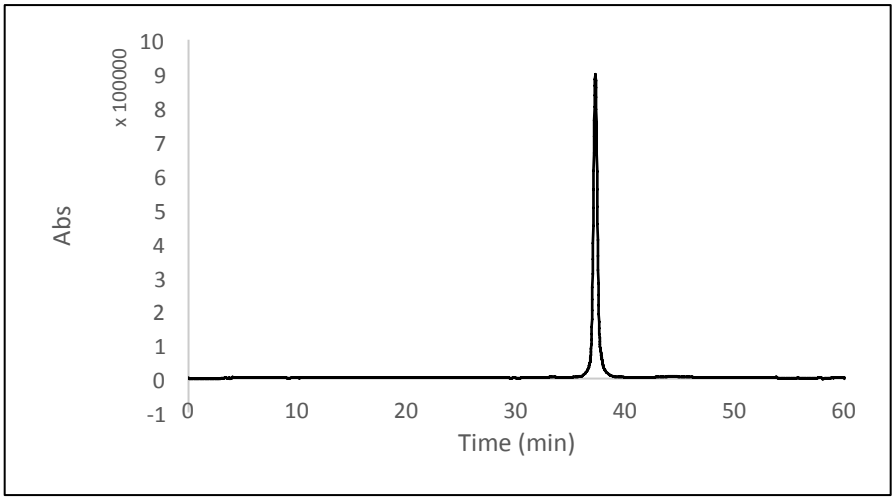
Laspartomycin C (1)



Yield: 47.3 mg (18.7 umol, 15.3%)

HR-MS [M+H$^+$]: Calc.: 1247.6479, found: 1247.6522

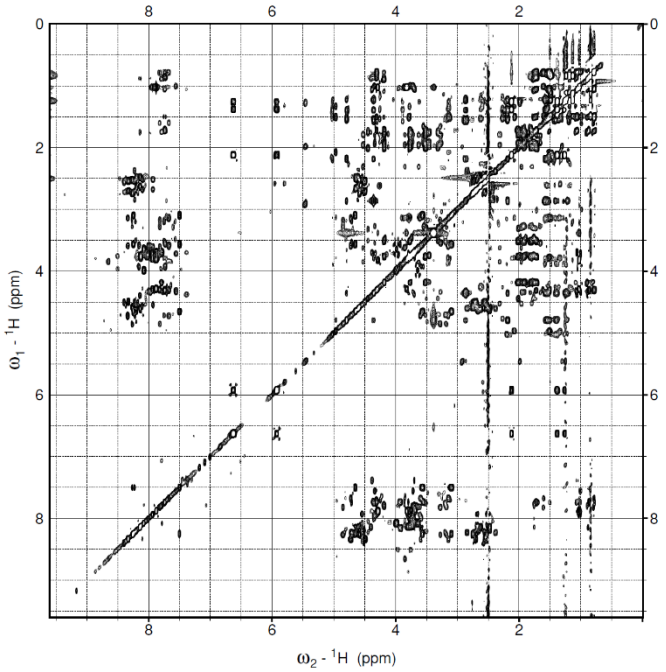
Analytical HPLC



Laspartomycin C (1) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.92 (123.7)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.1), $C_\delta H_2$ (1.37, 27.5), $C_\varepsilon H_2$-$C_\iota H_2$ (1.23-1.27, 28.7), $C_\kappa H_2$ (1.23, 26.5), $C_\lambda H_2$ (1.12, 38.0), $C_\mu H$ (1.49, 27.1), $2C_\nu H_3$ (0.84, 22.4)
Asp-1	8.14	4.61 (48.9)	$C_\beta H_2$ (2.63/2.50, 35.9)
Dap-2	8.25	4.66 (48.5)	$C_\beta H_2$ (3.56/3.10, 39.5)
D-Pip-3	-	4.80 (55.9)	$C_\beta H_2$ (2.18/1.53, 26.4), $C_\gamma H_2$ (1.55/1.39, 20.1), $C_\delta H_2$ (1.51/1.22, 24.1), $C_\varepsilon H_2$ (4.35/2.86, 39.6)
Gly-4	8.08	4.00/3.65 (41.9)	-
Asp-5	8.25	4.61 (49.7)	$C_\beta H_2$ (2.74/2.52, 35.8)
Gly-6	8.13	3.76 (41.9)	-
Asp-7	8.33	4.50 (49.8)	$C_\beta H_2$ (2.70/2.54, 35.6)
Gly-8	7.87	3.80/3.67 (41.9)	-
D-*allo*-Thr-9	7.88	4.28 (58.1)	$C_\beta H$ (3.81, 66.6), $C_\gamma H_3$ (1.02, 19.3)
Ile-10	7.74	4.30 (54.0)	$C_\beta H$ (1.73, 35.8), $C_\gamma H_2$ (1.50/1.07, 24.0), $C_\gamma H_3$ (0.86, 14.5), $C_\delta H_2$ (0.78, 10.3)
Pro-11	-	4.18 (59.4)	$C_\beta H_2$ (2.00/1.74, 29.3), $C_\gamma H_2$ (1.92/1.80, 24.3), $C_\delta H_2$ (3.77/3.50, 46.9)

Laspartomycin C (1) 2D NMR Spectra



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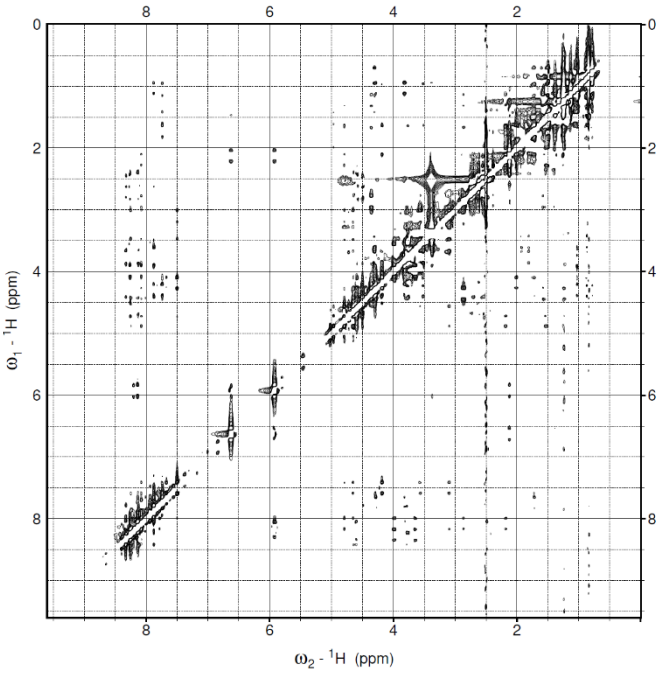

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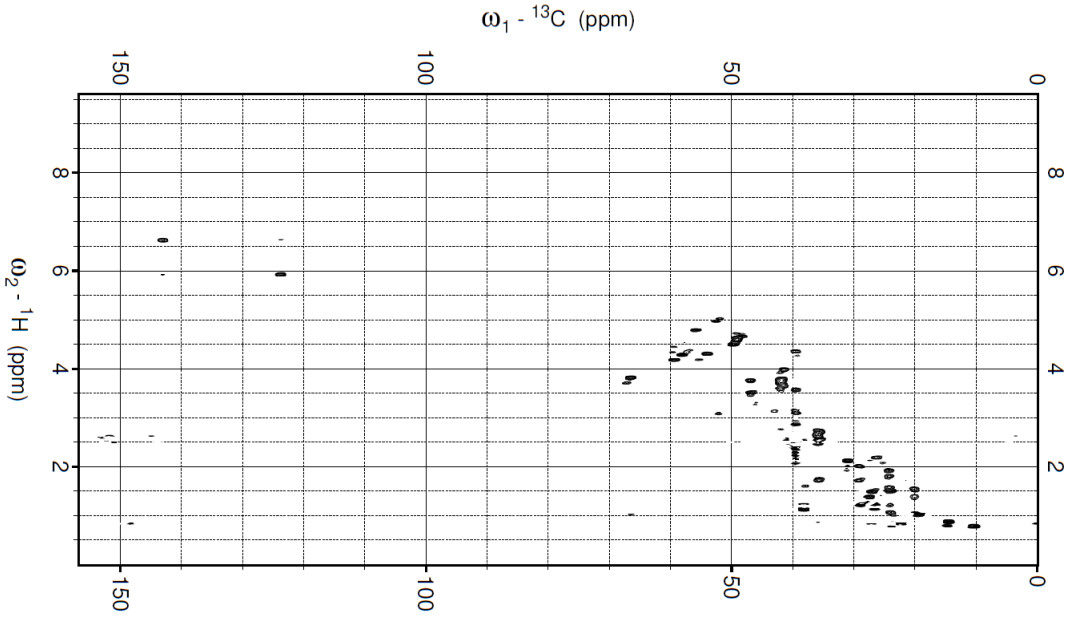
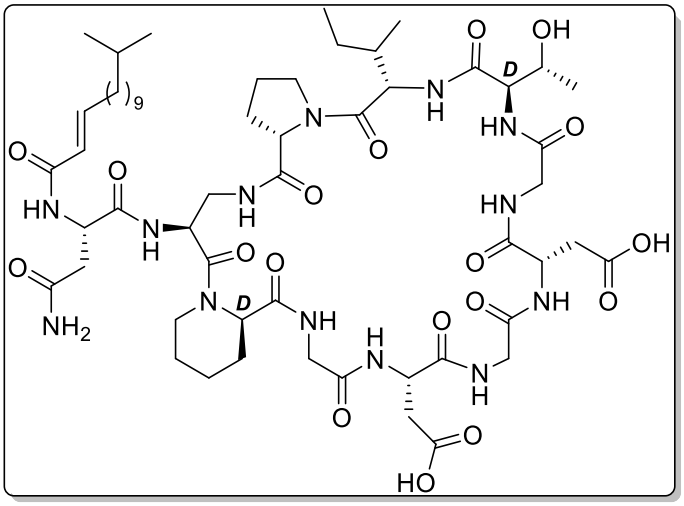


HSQC

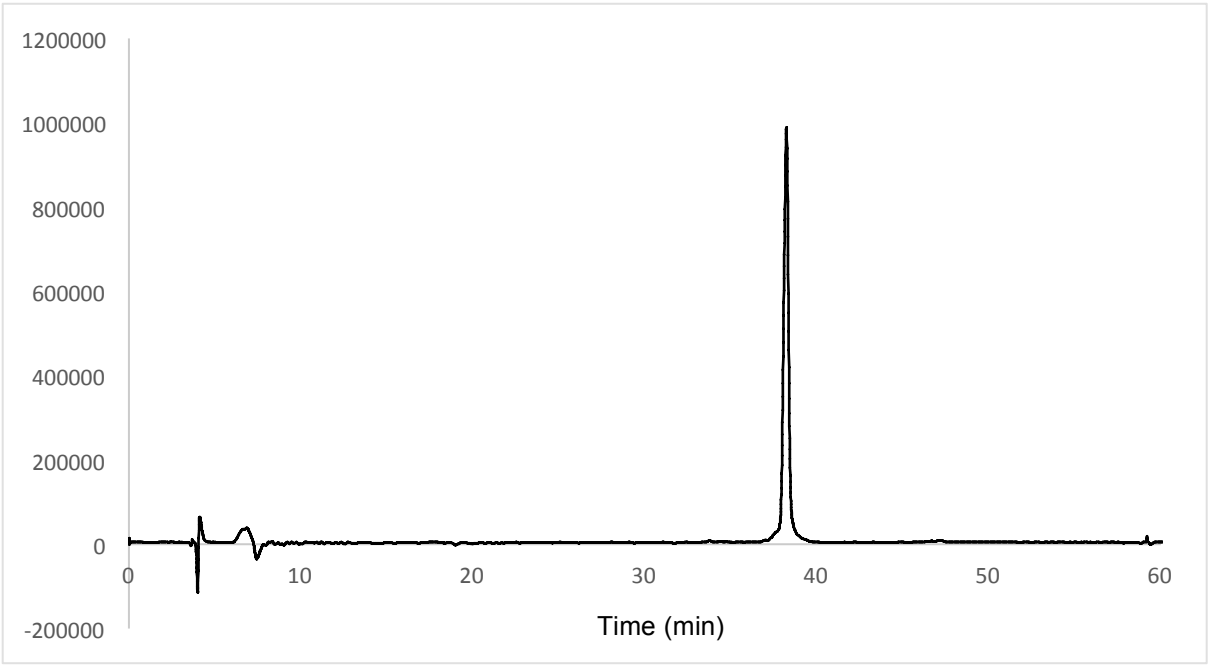
Asn_1 containing lipopeptide (2)



Yield: 10.3 mg (8.3 umol, 3.3%)

HR-MS [M+H$^+$]: Calc.: 1246.6639, found: 1246.6605

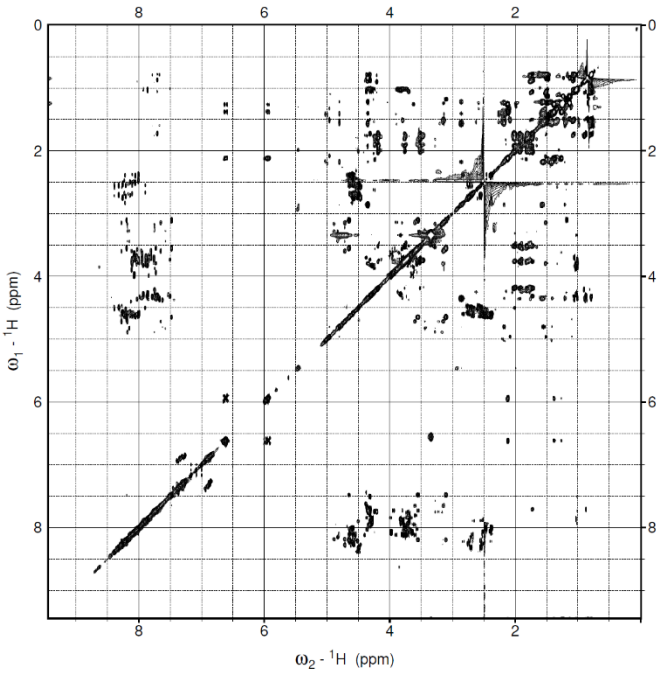
Analytical HPLC



Asn_1 containing lipopeptide (2) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.95 (124.6)	$C_\beta H$ (6.63, 143.6), $C_\gamma H_2$ (2.12, 31.8), $C_\delta H_2$ (1.39, 28.3), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.5), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.14, 39.0), $C_\mu H$ (1.50, 28.0), $2C_\nu H_3$ (0.85, 22.9)
Asn-1	8.00	4.60 (50.1)	$C_\beta H_2$ (2.48/2.40, 37.7)
Dap-2	8.19	4.66 (48.9)	$C_\beta H_2$ (3.57/3.10, 40.3)
D-Pip-3	-	4.81 (56.6)	$C_\beta H_2$ (2.19/1.50, 27.3), $C_\gamma H_2$ (1.56/1.42, 20.8), $C_\delta H_2$ (1.57/1.23, 24.8), $C_\varepsilon H_2$ (4.37/2.87, 40.3)
Gly-4	8.07	3.98/3.66 (42.4)	-
Asp-5	8.26	4.58 (50.0)	$C_\beta H_2$ (2.75/2.53, 36.4)
Gly-6	8.11	3.77 (42.6)	-
Asp-7	8.32	4.50 (50.7)	$C_\beta H_2$ (2.70/2.55, 36.4)
Gly-8	7.89	3.79/3.70 (42.6)	-
D-*allo*-Thr-9	7.86	4.28 (58.9)	$C_\beta H$ (3.83, 67.3), $C_\gamma H_3$ (1.03, 20.5)
Ile-10	7.71	4.32 (54.8)	$C_\beta H$ (1.76, 36.5), $C_\gamma H_2$ (1.52/1.08, 24.8), $C_\gamma H_3$ (0.88, 15.2), $C_\delta H_2$ (0.79, 11.1)
Pro-11	-	4.20 (60.3)	$C_\beta H_2$ (2.02/1.74, 29.8), $C_\gamma H_2$ (1.93/1.83, 25.0), $C_\delta H_2$ (3.77/3.53, 47.7)

Asn_1 containing lipopeptide (2) 2D NMR Ppectra



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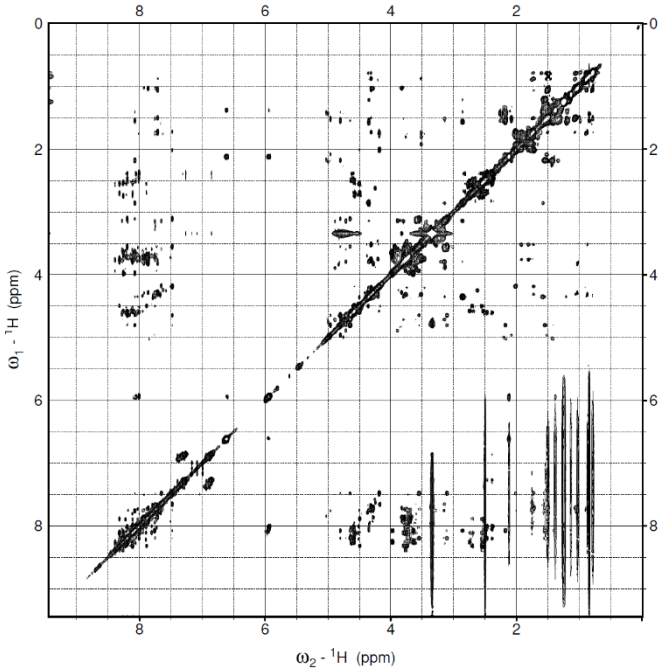

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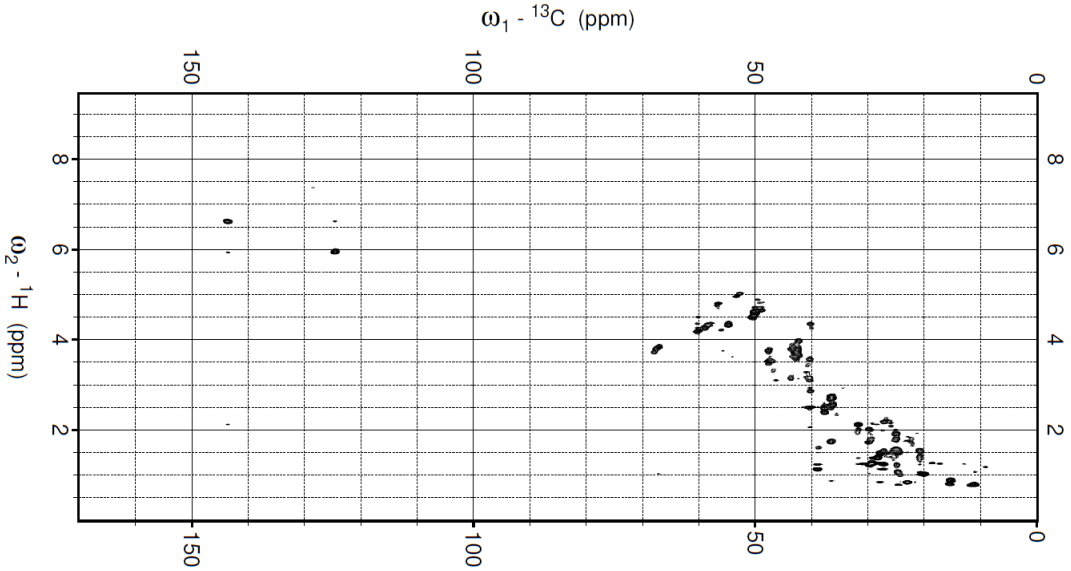
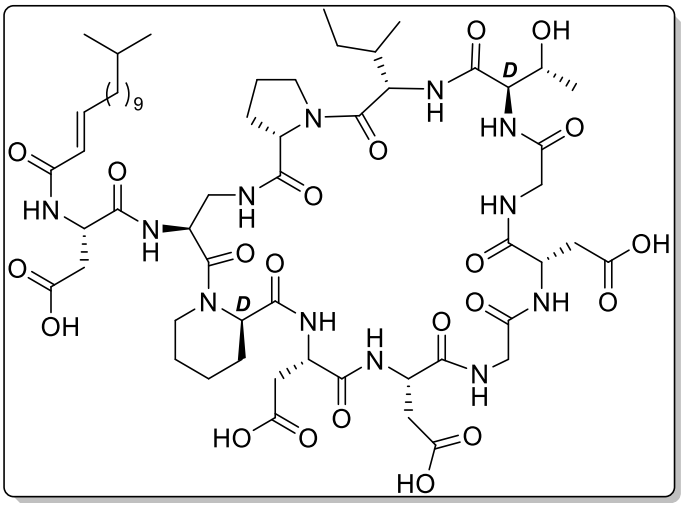


HSQC

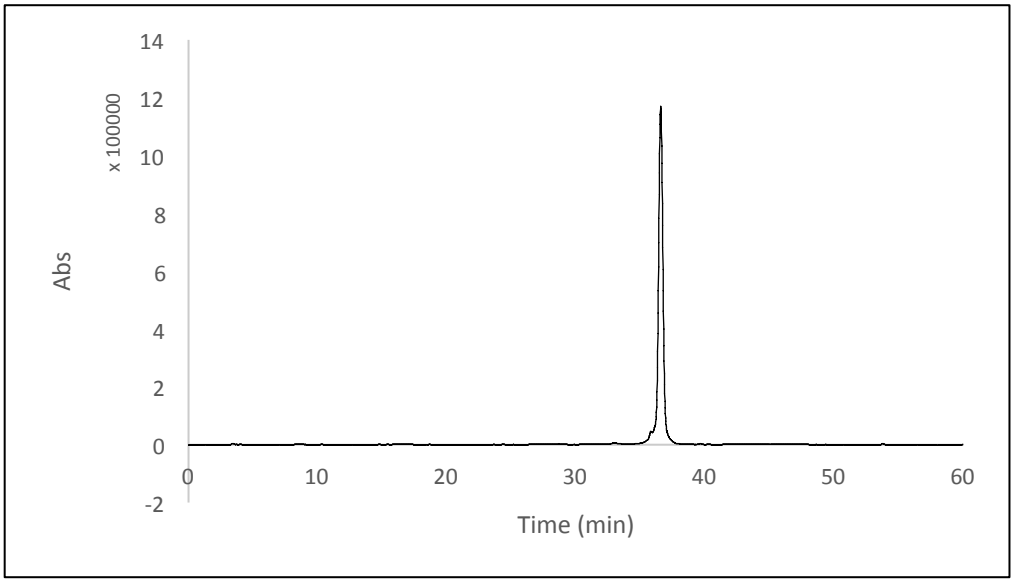
Asp_4 containing lipopeptide (3)



Yield: 20.0 mg (15.3 umol, 6.1%)

HR-MS [M+H$^+$]: Calc.: 1305.6578, found: 1305.6583

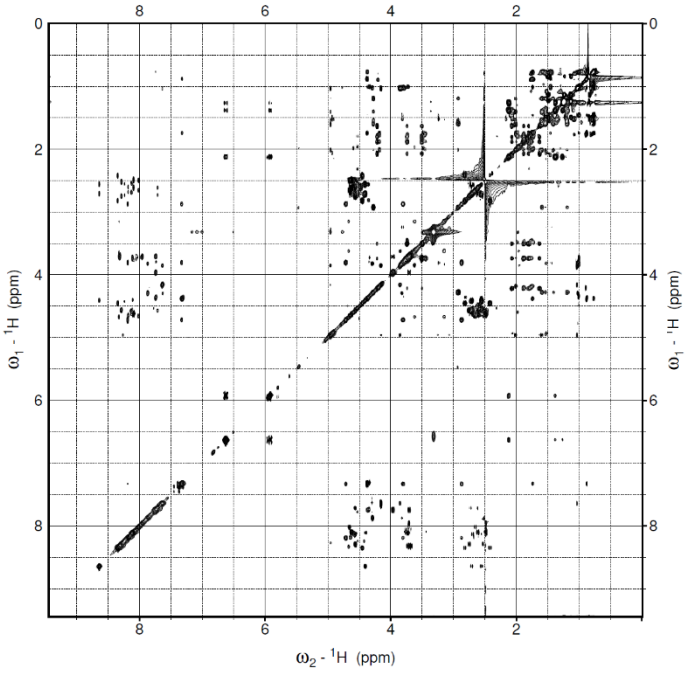
Analytical HPLC



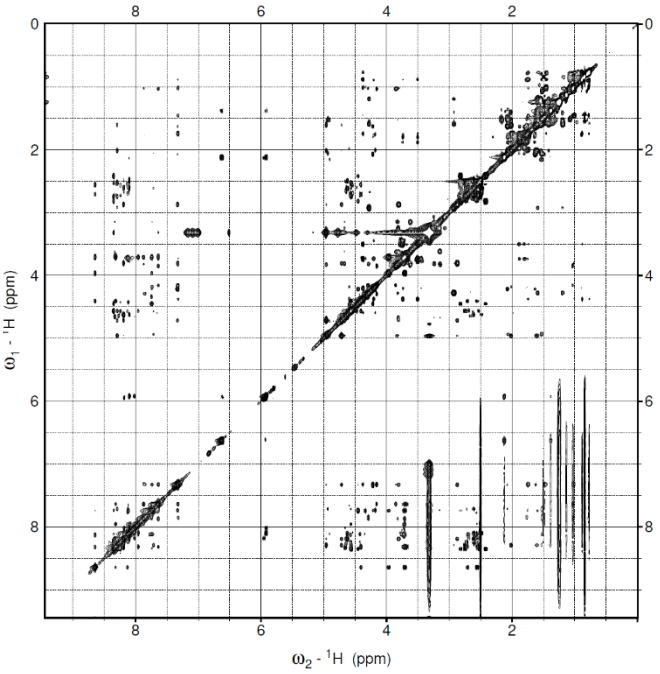
Asp_4 containing lipopeptide (3) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.92 (124.4)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.8), $C_\delta H_2$ (1.38, 28.3), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.5), $C_\kappa H_2$ (1.24, 27.2), $C_\lambda H_2$ (1.13, 39.0), $C_\mu H$ (1.49, 27.9), $2C_\nu H_3$ (0.84, 23.0)
Asp-1	8.10	4.62 (49.9)	$C_\beta H_2$ (2.63/2.50, 36.7)
Dap-2	8.19	4.72 (48.7)	$C_\beta H_2$ (3.80/2.89, 40.3)
D-Pip-3	-	4.95 (55.7)	$C_\beta H_2$ (2.06/1.52, 26.4), $C_\gamma H_2$ (1.53/1.39, 20.4), $C_\delta H_2$ (1.59/1.19, 24.8), $C_\varepsilon H_2$ (4.28/2.93, 39.9)
Asp-4	8.64	4.41 (51.2)	$C_\beta H_2$ (2.71/2.56, 36.3)
Asp-5	8.29	4.56 (50.2)	$C_\beta H_2$ (2.75/2.52, 36.3)
Gly-6	7.96	3.72 (42.7)	-
Asp-7	8.35	4.47 (50.6)	$C_\beta H_2$ (2.81/2.47, 36.1)
Gly-8	7.74	3.96/3.71 (42.3)	-
D-*allo*-Thr-9	7.87	4.29 (58.7)	$C_\beta H$ (3.83, 67.3), $C_\gamma H_3$ (1.03, 20.0)
Ile-10	7.74	4.38 (54.7)	$C_\beta H$ (1.75, 36.8), $C_\gamma H_2$ (1.47/1.07, 24.6), $C_\gamma H_3$ (0.88, 15.3), $C_\delta H_2$ (0.78, 11.1)
Pro-11	-	4.16 (59.3)	$C_\beta H_2$ (2.00/1.75, 29.5), $C_\gamma H_2$ (1.88/1.80, 24.9), $C_\delta H_2$ (3.74/3.50, 47.9)

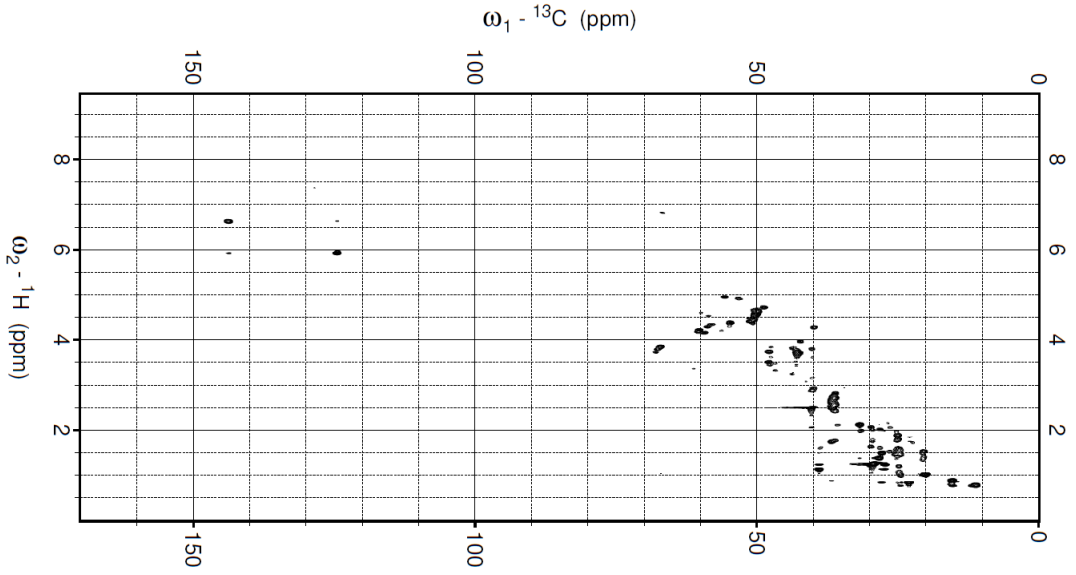
Asp$_4$ containing lipopeptide (3) 2D NMR Spectra



TOCSY

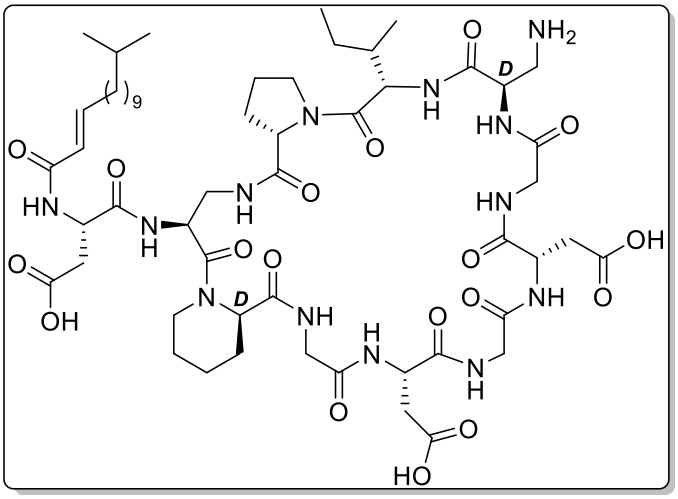


NOESY



HSQC

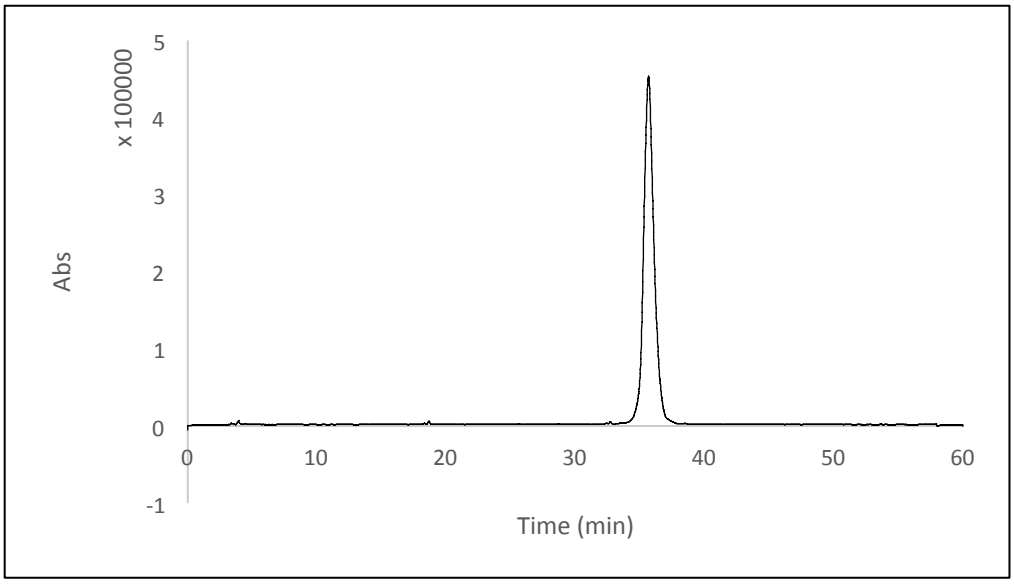
D-Dap$_9$ containing lipopeptide (4)



Yield: 11.3 mg (9.1 umol, 3.6%)

HR-MS [M+H$^+$]: Calc.: 1232.6527, found: 1232.6531

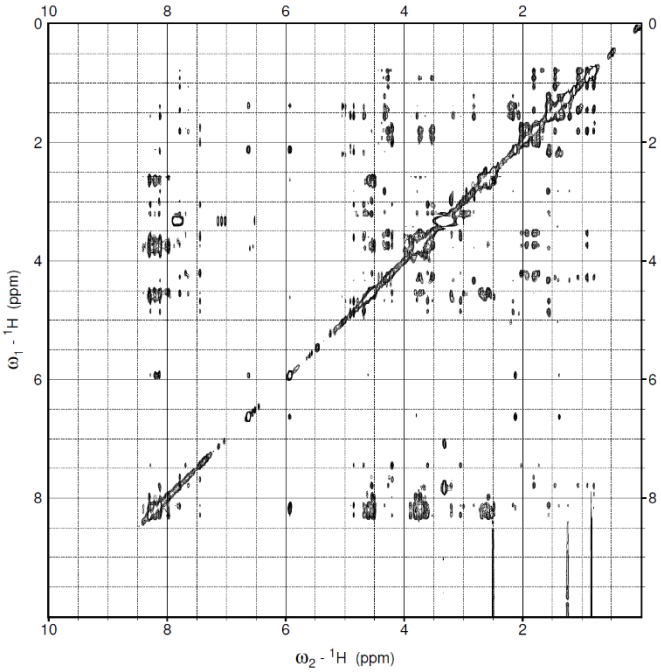
Analytical HPLC



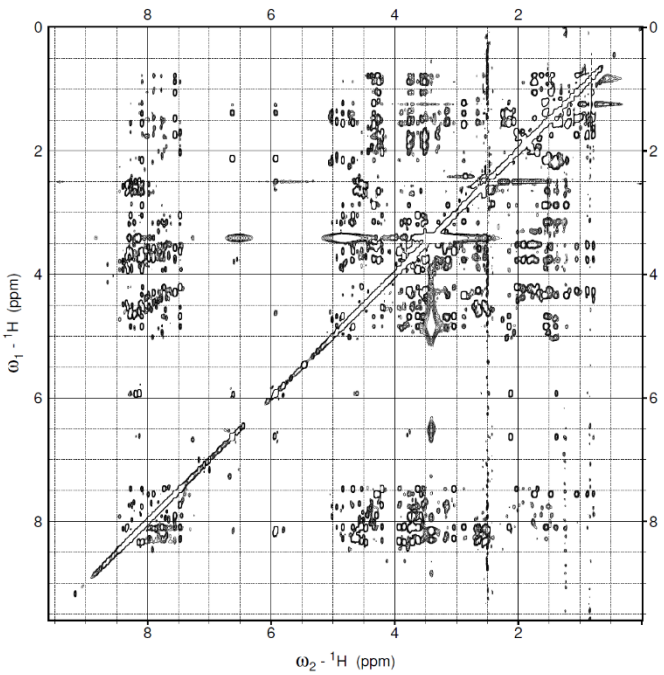
D-Dap$_9$ containing lipopeptide (4) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.93 (124.1)	$C_\beta H$ (6.63, 143.3), $C_\gamma H_2$ (2.12, 31.3), $C_\delta H_2$ (1.38, 27.8), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.0), $C_\kappa H_2$ (1.23, 26.8), $C_\lambda H_2$ (1.13, 38.5), $C_\mu H$ (1.49, 27.4), $2C_\nu H_3$ (0.84, 22.5)
Asp-1	8.13	4.64 (49.4)	$C_\beta H_2$ (2.63/2.51, 36.1)
Dap-2	8.29	4.67 (48.5)	$C_\beta H_2$ (3.54/3.03, 39.8)
D-Pip-3	-	4.85 (55.9)	$C_\beta H_2$ (2.16/1.56, 28.5), $C_\gamma H_2$ (1.56/1.40, 20.2), $C_\delta H_2$ (1.57/1.21, 24.3), $C_\varepsilon H_2$ (4.34/2.83, 39.6)
Gly-4	8.21	3.80/3.63 (42.0)	-
Asp-5	8.21	4.60 (49.5)	$C_\beta H_2$ (2.73/2.57, 35.9)
Gly-6	8.14	3.78 (42.1)	-
Asp-7	8.30	4.50 (50.0)	$C_\beta H_2$ (2.71/2.48, 35.9)
Gly-8	7.97	3.73 (42.1)	-
D-Dap-9	7.43	4.67 (48.5)	$C_\beta H$ (3.60/3.05, 39.7)
Ile-10	7.45	4.26 (54.8)	$C_\beta H$ (1.81, 35.8), $C_\gamma H_2$ (1.46/1.06, 24.2), $C_\gamma H_3$ (0.91, 14.7), $C_\delta H_2$ (0.80, 10.6)
Pro-11	-	4.20 (59.7)	$C_\beta H_2$ (2.02/1.72, 29.4), $C_\gamma H_2$ (1.93/1.81, 24.5), $C_\delta H_2$ (3.74/3.53, 47.3)

D-Dap$_9$ containing lipopeptide (4) 2D NMR Spectra



TOCSY



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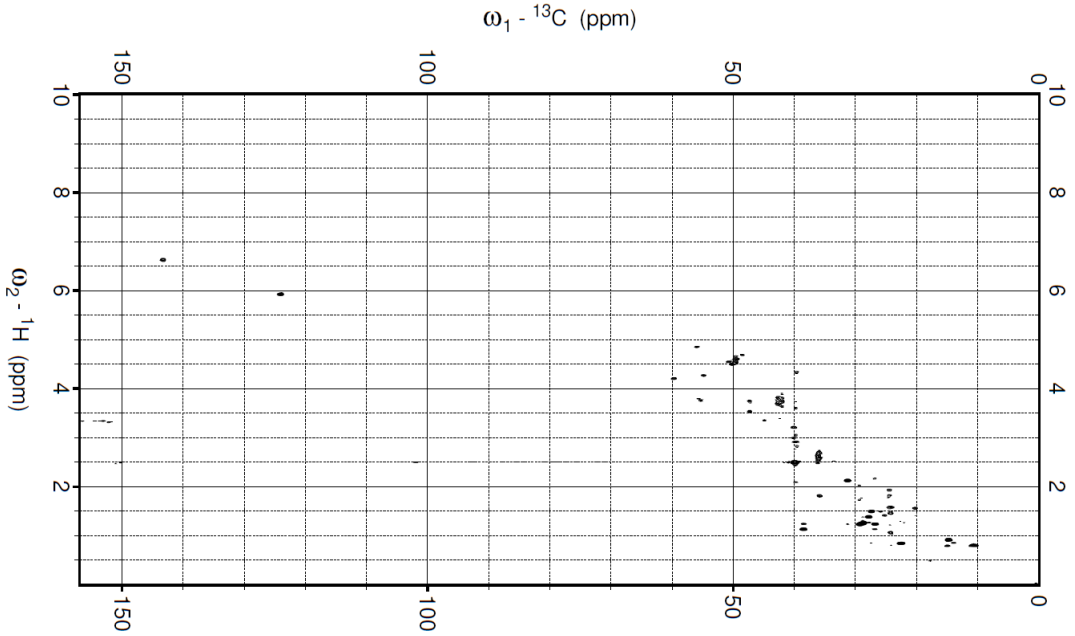
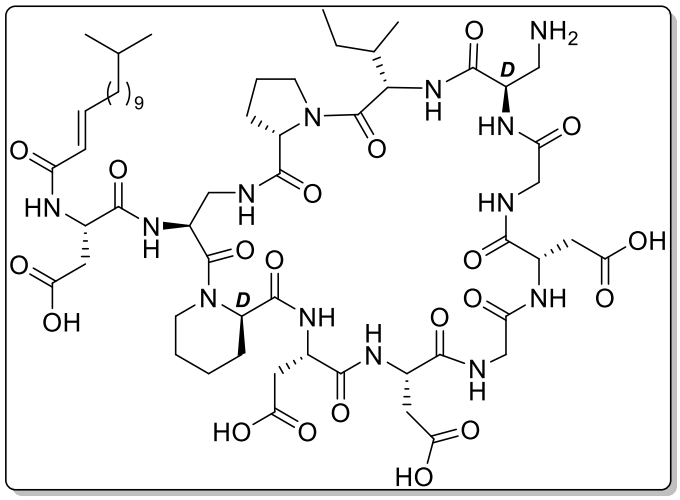


HSQC

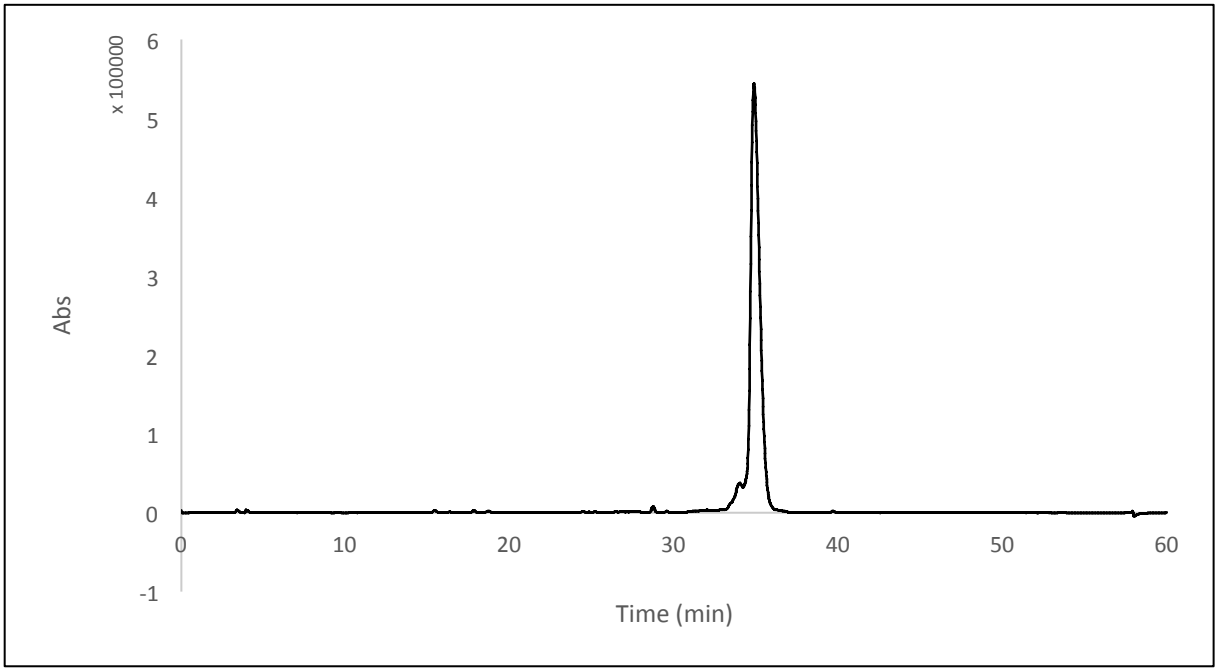
Asp_4, D-Dap_9 containing lipopeptide (5)



Yield: 12.3 mg (9.5 umol, 3.8%)

HR-MS [M+H^+]: Calc.: 1290.6582, found: 1290.6603

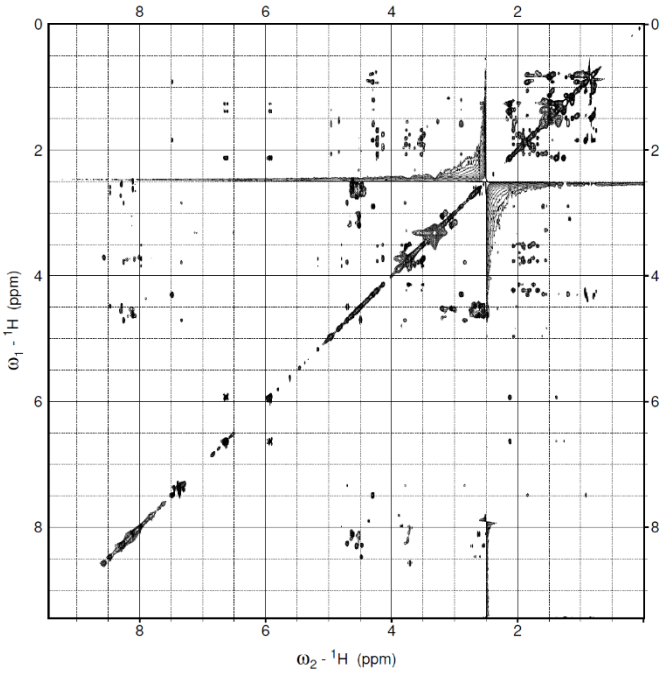
Analytical HPLC



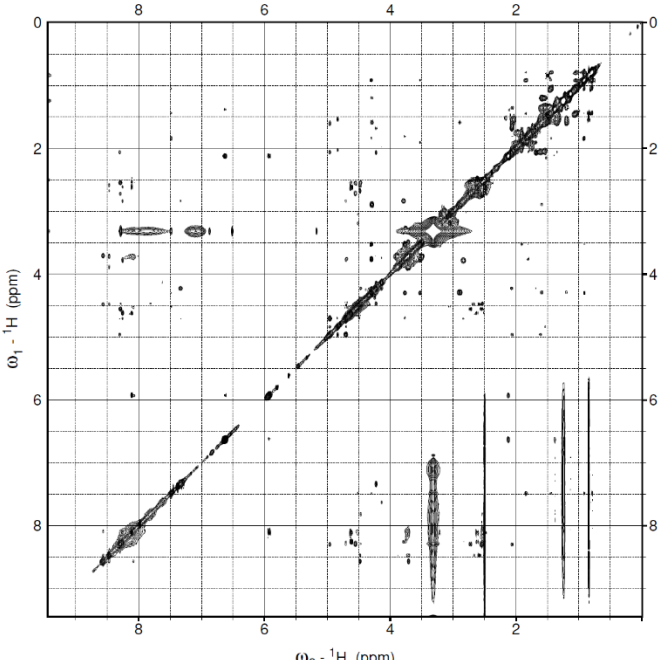
Asp_4, D-Dap_9 containing lipopeptide (5) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.94 (124.4)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.8), $C_\delta H_2$ (1.38, 28.2), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.4), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.13, 38.9), $C_\mu H$ (1.49, 27.8), $2C_\nu H_3$ (0.84, 23.0)
Asp-1	8.11	4.62 (50.1)	$C_\beta H_2$ (2.62/2.50, 36.6)
Dap-2	8.25	4.69 (48.9)	$C_\beta H_2$ (3.21/3.06, 40.4)
D-Pip-3	-	4.95 (55.8)	$C_\beta H_2$ (2.08/1.35, 28.2), $C_\gamma H_2$ (1.54/1.35, 20.4), $C_\delta H_2$ (1.58/1.20, 24.8), $C_\varepsilon H_2$ (4.29/2.94, 39.8)
Asp-4	8.47	4.48 (50.7)	$C_\beta H_2$ (2.68/2.60, 36.5)
Asp-5	8.28	4.49 (50.7)	$C_\beta H_2$ (2.53, 36.6)
Gly-6	8.09	3.71 (43.6)	-
Asp-7	8.30	4.56 (50.4)	$C_\beta H_2$ (2.72/2.55, 36.5)
Gly-8	8.20	3.74 (42.9)	-
D-Dap-9	7.34	4.74 (48.9)	$C_\beta H$ (3.19/3.05, 40.4)
Ile-10	7.49	4.30 (55.1)	$C_\beta H$ (1.83, 36.3), $C_\gamma H_2$ (1.46/1.05, 24.6), $C_\gamma H_3$ (0.92, 15.2), $C_\delta H_2$ (0.79, 11.0)
Pro-11	-	4.23 (60.3)	$C_\beta H_2$ (2.06/1.68, 29.8), $C_\gamma H_2$ (1.91/1.80, 25.0), $C_\delta H_2$ (3.76/3.52, 47.9)

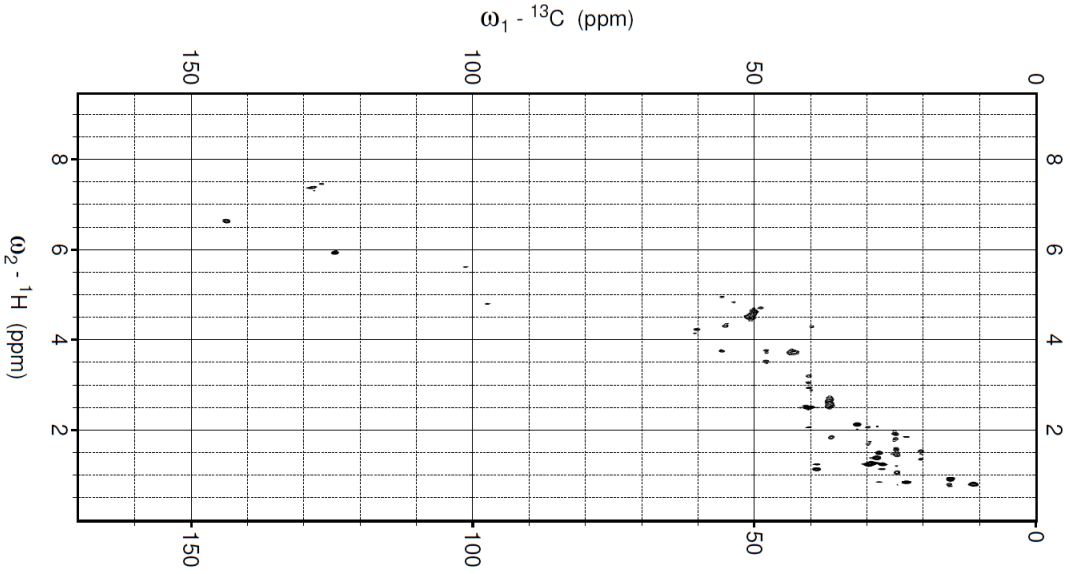
Asp_4, D-Dap_9 containing lipopeptide (5) 2D NMR Spectra



TOCSY

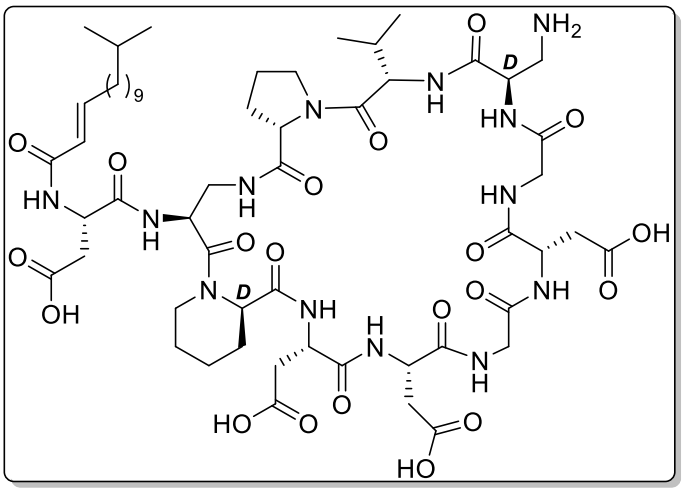


NOESY



HSQC

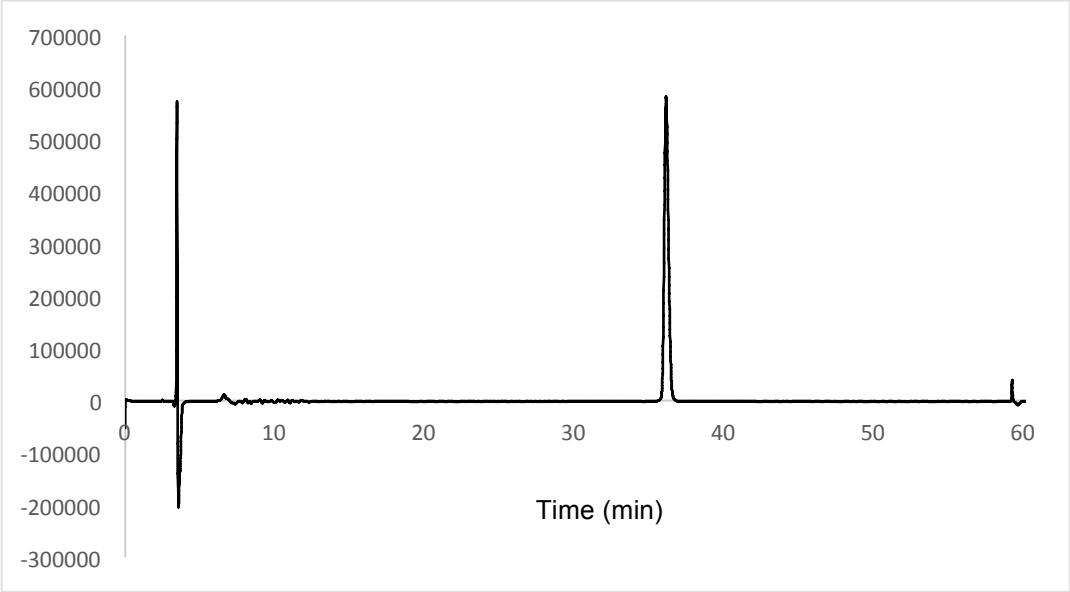
Asp_4, D-Dap_9, Val_{10} containing lipopeptide (6)



Yield: 45 mg (32.9 umol, 32%)

HR-MS [$M+H^+$]: Calc.: 1276.6380, found: 1276.6395

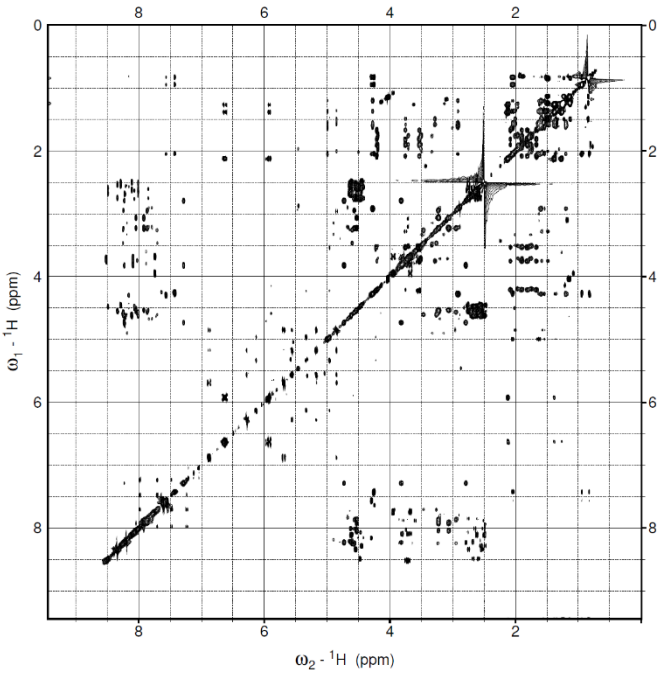
Analytical HPLC



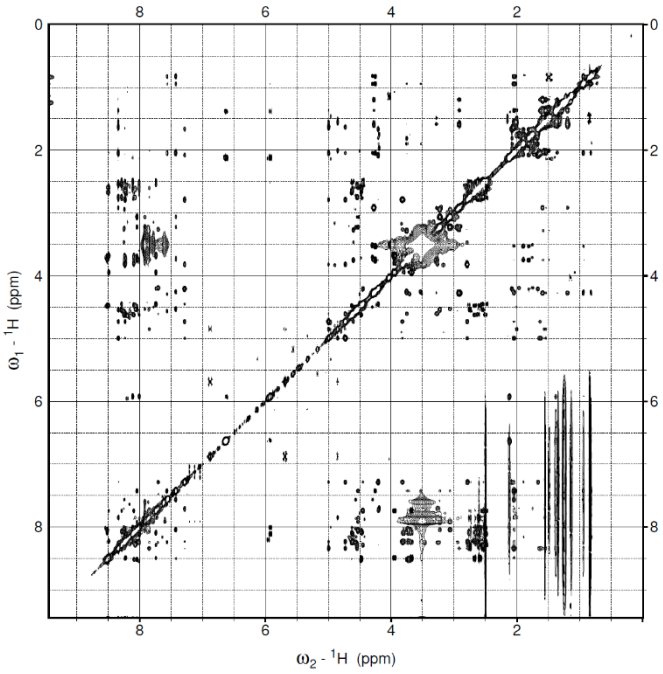
Asp_4, D-Dap_9, Val_{10} containing lipopeptide (6) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.94 (124.5)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.7), $C_\delta H_2$ (1.38, 28.3), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.4), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.13, 38.9), $C_\mu H$ (1.49, 27.8), $2C_\nu H_3$ (0.84, 23.0)
Asp-1	8.11	4.89 (49.8)	$C_\beta H_2$ (2.61/2.49, 36.5)
Dap-2	8.23	4.73 (48.8)	$C_\beta H_2$ (3.76/3.77, 40.3)
D-Pip-3	-	4.99 (55.7)	$C_\beta H_2$ (2.06/1.36, 28.4), $C_\gamma H_2$ (1.54/1.36, 20.3), $C_\delta H_2$ (1.60/1.20, 24.8), $C_\varepsilon H_2$ (4.28/2.92, 39.8)
Asp-4	8.34	4.55 (50.5)	$C_\beta H_2$ (2.76/2.54, 36.2)
Asp-5	8.22	4.61 (50.0)	$C_\beta H_2$ (2.68, 36.3)
Gly-6	8.09	3.76 (43.4)	-
Asp-7	8.29	4.47 (50.6)	$C_\beta H_2$ (2.51, 36.3)
Gly-8	7.74	3.94/3.67 (42.3)	-
D-Dap-9	7.28	4.72 (48.8)	$C_\beta H$ (3.81/2.80, 40.3)
Val-10	7.42	4.26 (56.6)	$C_\beta H$ (2.05, 30.4), $C\gamma 1H_3$ (0.94, 19.4), $C\gamma 2H_3$ (0.82, 18.9)
Pro-11	-	4.20 (60.3)	$C_\beta H_2$ (2.09/1.67, 29.8), $C_\gamma H_2$ (1.90/1.80, 24.9), $C_\delta H_2$ (3.75/3.53, 47.8)

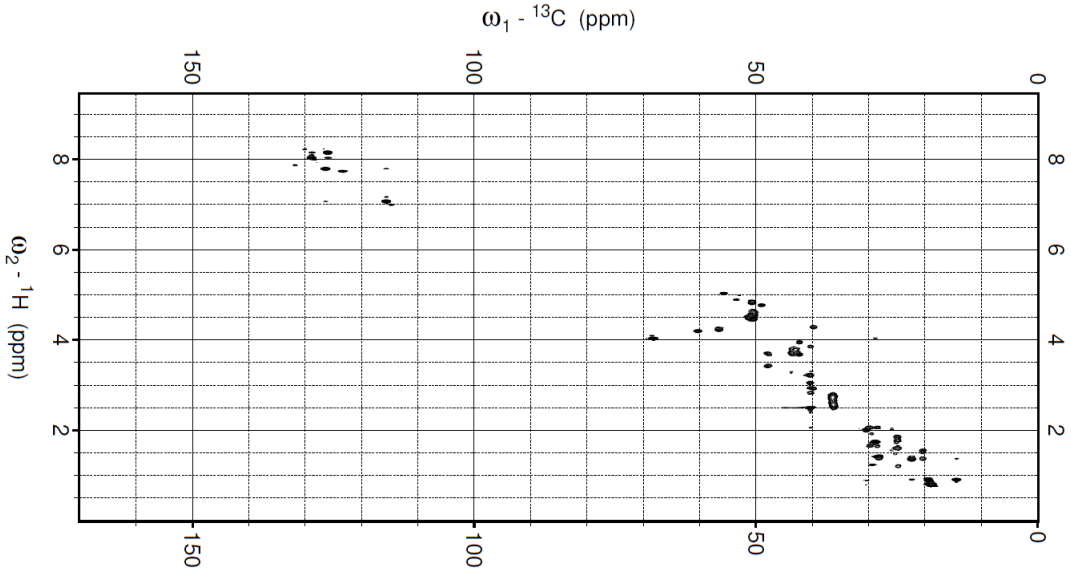
Asp_4, D-Dap_9, Val_{10} containing lipopeptide (6) 2D NMR Spectra



TOCSY

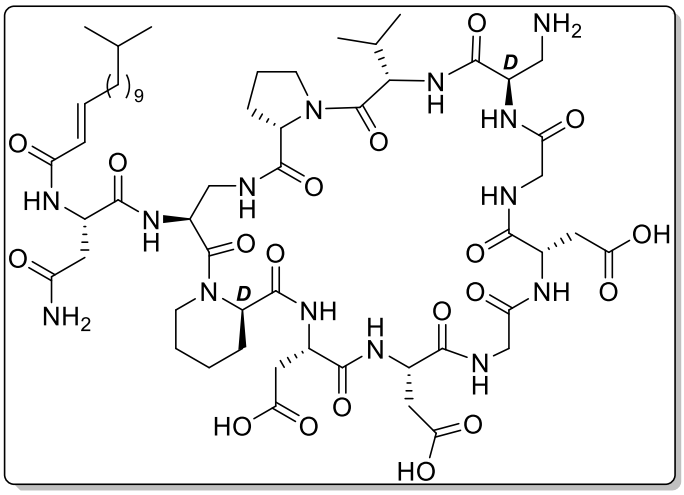


NOESY



HSQC

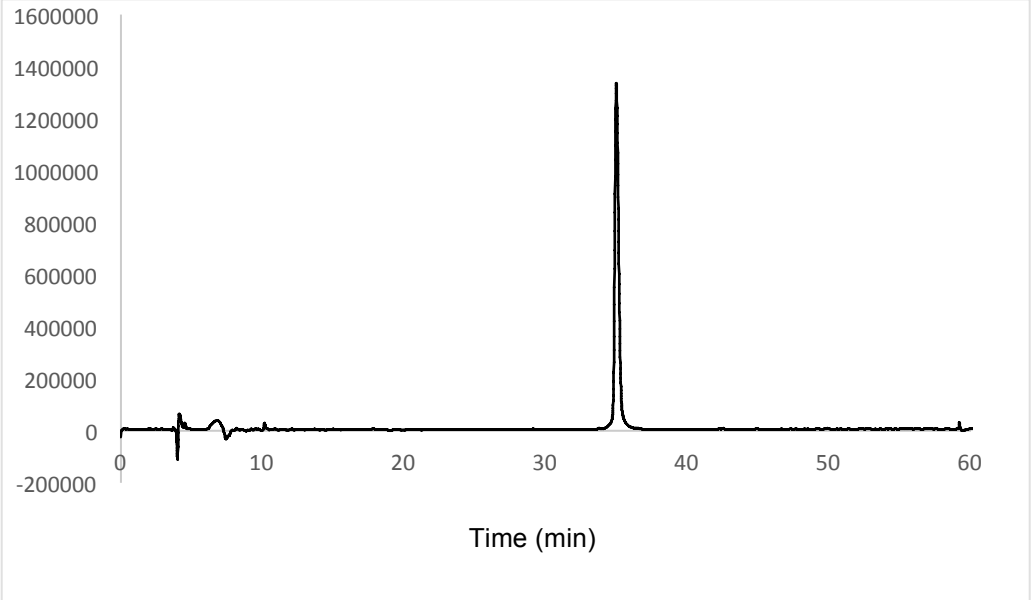
Asn_1, Asp_4, D-Dap_9, Val_{10} containing lipopeptide (7)



Yield: 12.5 mg (9.8 umol, 9.8%)

HR-MS [M+H$^+$]: Calc.: 1275.6585, found: 1275.6585

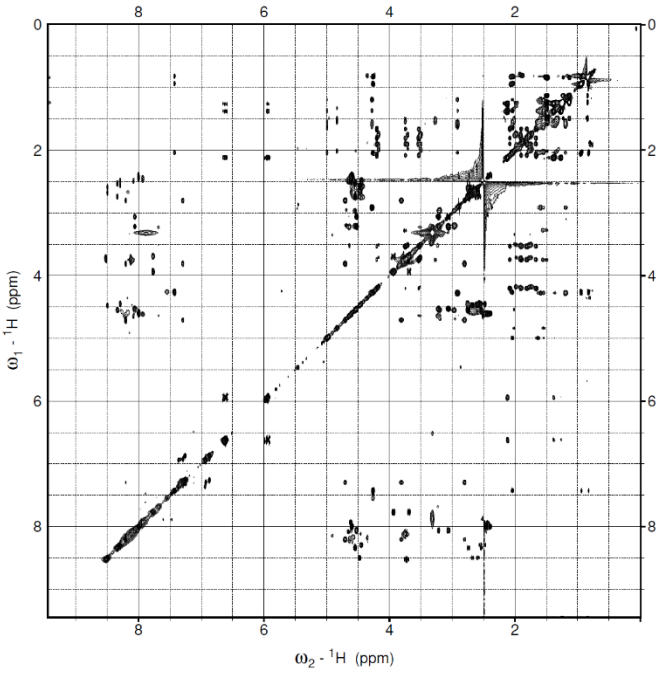
Analytical HPLC



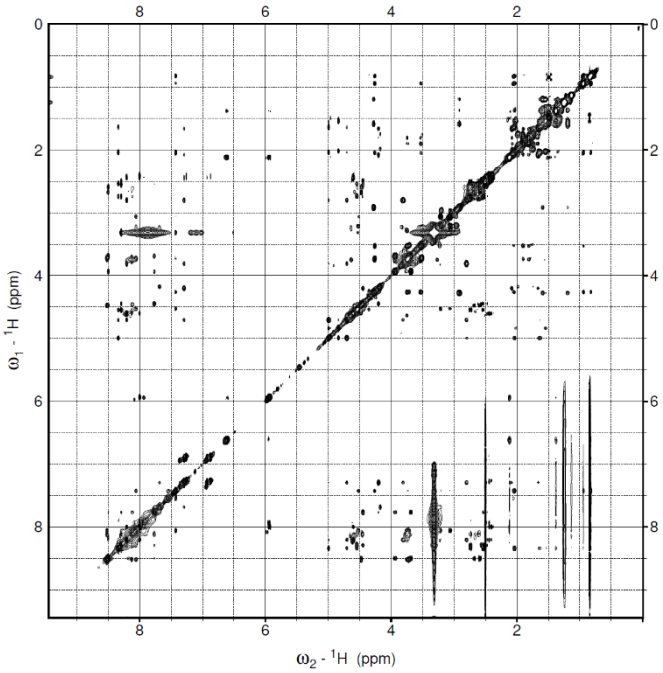
Asn_1, Asp_4, D-Dap_9, Val_{10} containing lipopeptide (7) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.93 (124.6)	$C_\beta H$ (6.62, 143.6), $C_\gamma H_2$ (2.12, 31.8), $C_\delta H_2$ (1.38, 28.4), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.5), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.13, 40.0), $C_\mu H$ (1.49, 27.9), $2C_\nu H_3$ (0.84, 23.0)
Asn-1	8.50	4.47 (50.9)	$C_\beta H_2$ (2.49/2.43, 37.7)
Dap-2	8.21	4.69 (48.7)	$C_\beta H_2$ (3.78/3.78, 40.3)
D-Pip-3	-	4.99 (55.7)	$C_\beta H_2$ (2.04/1.35, 28.3), $C_\gamma H_2$ (1.54/1.36, 20.4), $C_\delta H_2$ (1.58/1.20, 24.8), $C_\varepsilon H_2$ (4.28/2.91, 39.8)
Asp-4	8.34	4.55 (50.5)	$C_\beta H_2$ (2.68/2.60, 36.3)
Asp-5	8.00	4.60 (50.2)	$C_\beta H_2$ (2.68, 36.3)
Gly-6	8.12	3.73 (43.5)	-
Asp-7	8.29	4.45 (50.4)	$C_\beta H_2$ (2.51, 36.2)
Gly-8	7.77	3.93/3.69 (42.3)	-
D-Dap-9	7.29	4.71 (48.6)	$C_\beta H$ (3.81/2.81, 40.3)
Val-10	7.43	4.26 (56.7)	$C_\beta H$ (2.05, 30.4), $C\gamma 1H_3$ (0.94, 19.4), $C\gamma 2H_3$ (0.83, 19.1)
Pro-11	-	4.20 (60.4)	$C_\beta H_2$ (2.08/1.67, 29.8), $C_\gamma H_2$ (1.90/1.80, 25.0), $C_\delta H_2$ (3.74/3.53, 48.0)

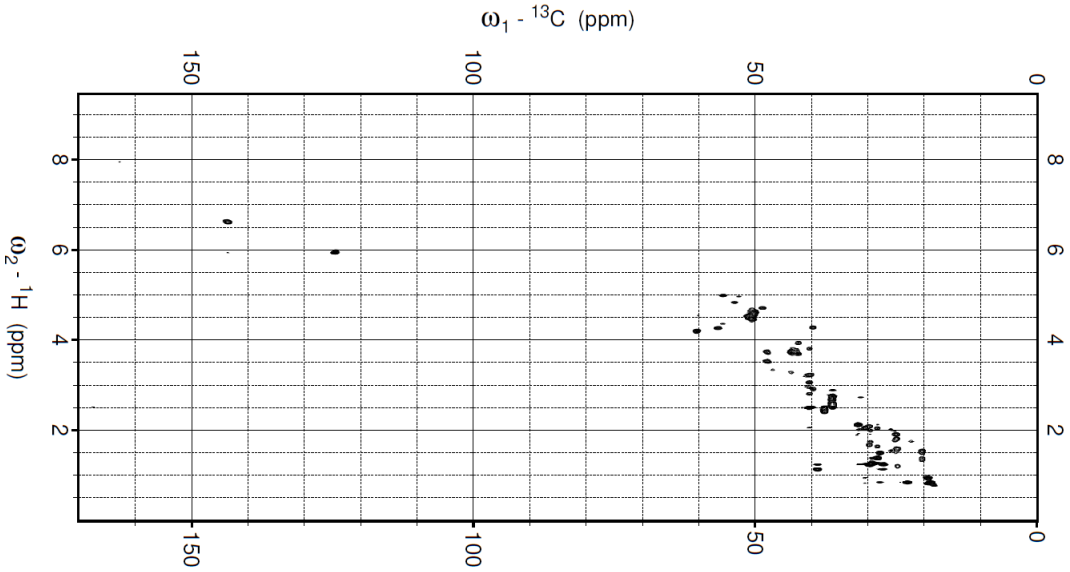
Asn_1, Asp_4, D-Dap_9, Val_{10} containing lipopeptide (7) 2D NMR Spectra



TOCSY

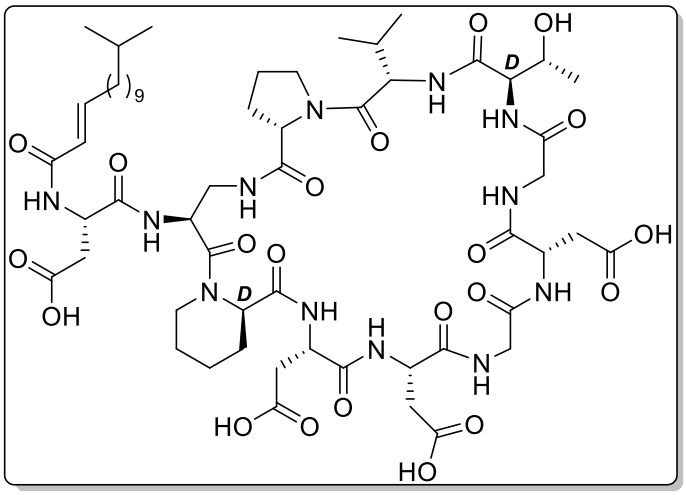


NOESY



HSQC

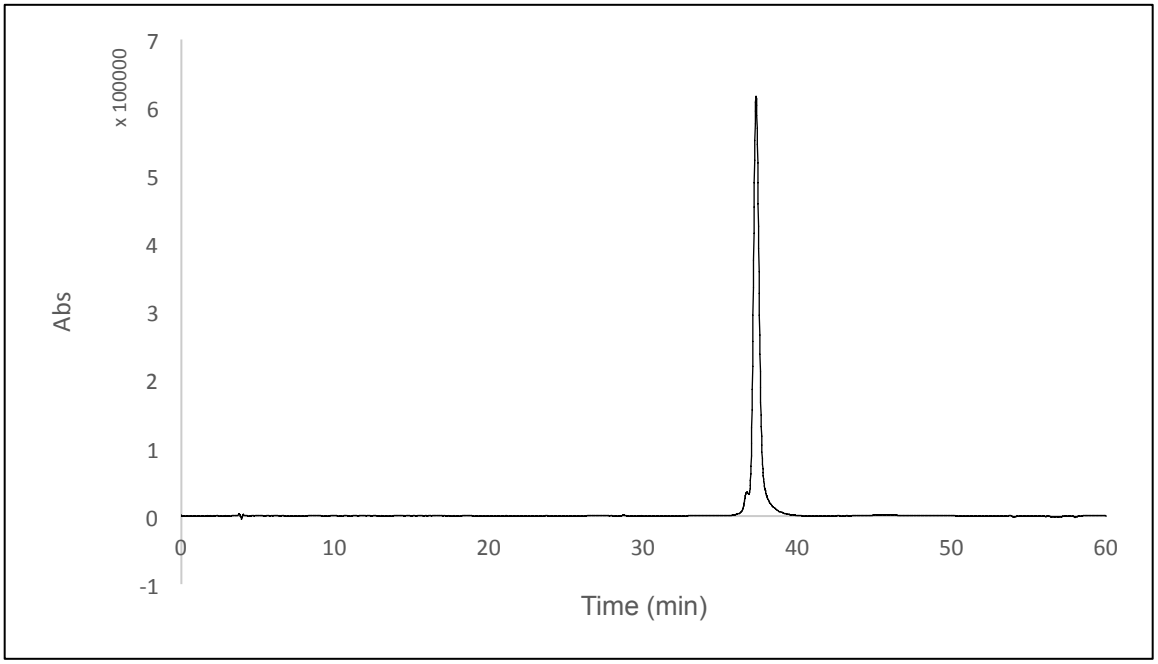
Asp_4, Val_{10} containing lipopeptide (8)



Yield: 41 mg (32.9 umol, 32%)

HR-MS [$M+H^+$]: Calc.: 1291.6422, found: 1291.6483

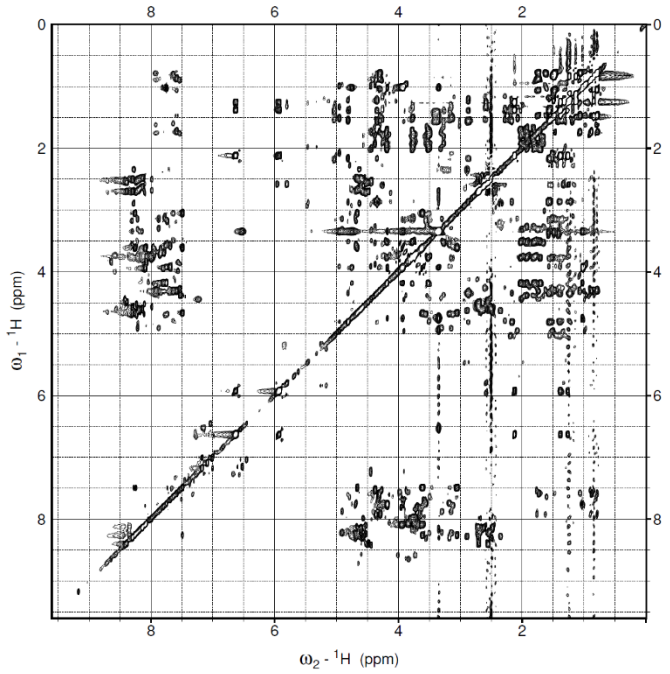
Analytical HPLC



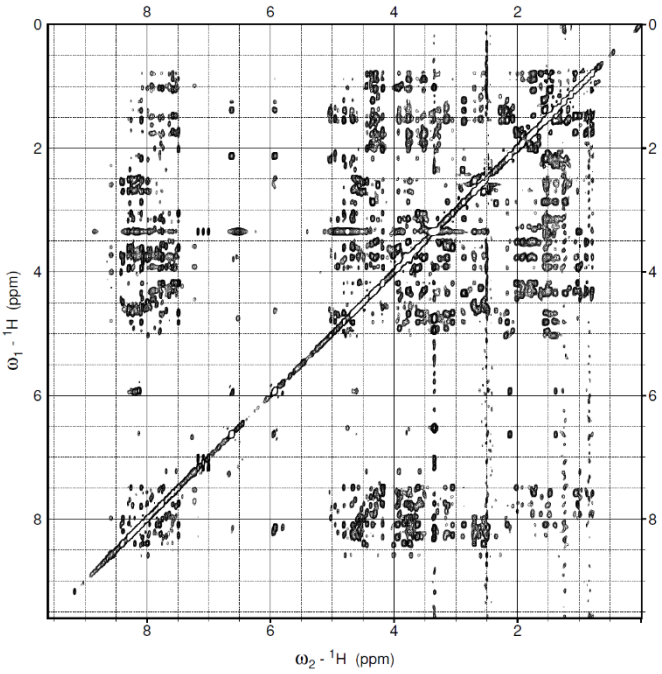
Asp_4, Val_{10} containing lipopeptide (8) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.94 (124.5)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.7), $C_\delta H_2$ (1.38, 28.3), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.4), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.13, 38.9), $C_\mu H$ (1.49, 27.8), $2C_\nu H_3$ (0.84, 23.0)
Asp-1	8.11	4.89 (49.8)	$C_\beta H_2$ (2.61/2.49, 36.5)
Dap-2	8.23	4.73 (48.8)	$C_\beta H_2$ (3.76/3.77, 40.3)
D-Pip-3	-	4.99 (55.7)	$C_\beta H_2$ (2.06/1.36, 28.4), $C_\gamma H_2$ (1.54/1.36, 20.3), $C_\delta H_2$ (1.60/1.20, 24.8), $C_\varepsilon H_2$ (4.28/2.92, 39.8)
Asp-4	8.34	4.55 (50.5)	$C_\beta H_2$ (2.76/2.54, 36.2)
Asp-5	8.22	4.61 (50.0)	$C_\beta H_2$ (2.68, 36.3)
Gly-6	8.09	3.76 (43.4)	-
Asp-7	8.29	4.47 (50.6)	$C_\beta H_2$ (2.51, 36.3)
Gly-8	7.74	3.94/3.67 (42.3)	-
D-*allo*-Thr-9	7.88	4.29 (58.7)	$C_\beta H$ (3.83, 67.2), $C_\gamma H_3$ (1.02, 20.1)
Val-10	7.43	4.26 (56.7)	$C_\beta H$ (2.03, 30.5), $C\gamma 1H_3$ (0.94, 19.4), $C\gamma 2H_3$ (0.82, 18.9)
Pro-11	-	4.20 (60.3)	$C_\beta H_2$ (2.09/1.67, 29.8), $C_\gamma H_2$ (1.90/1.80, 24.9), $C_\delta H_2$ (3.75/3.53, 47.8)

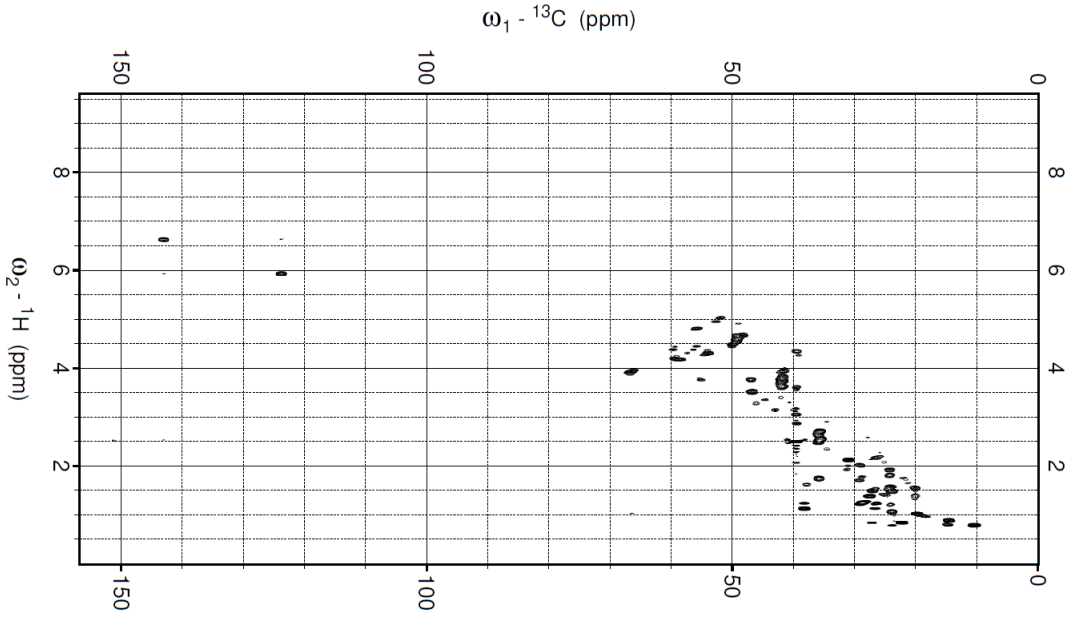
Asp_4, Val_{10} containing lipopeptide (8) 2D NMR Spectra



TOCSY

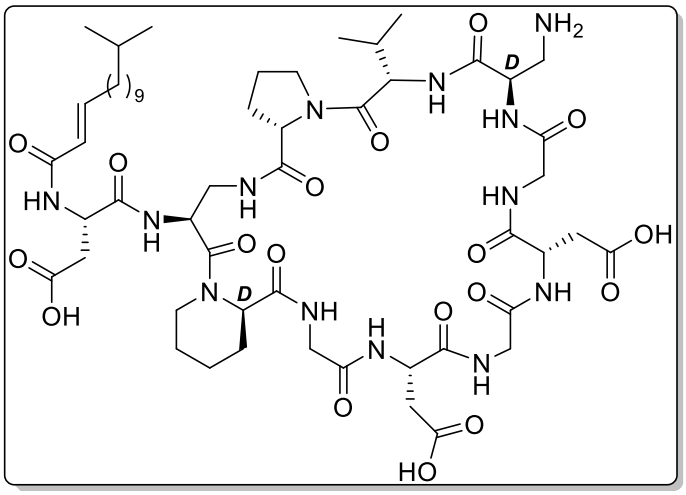


NOESY



HSQC

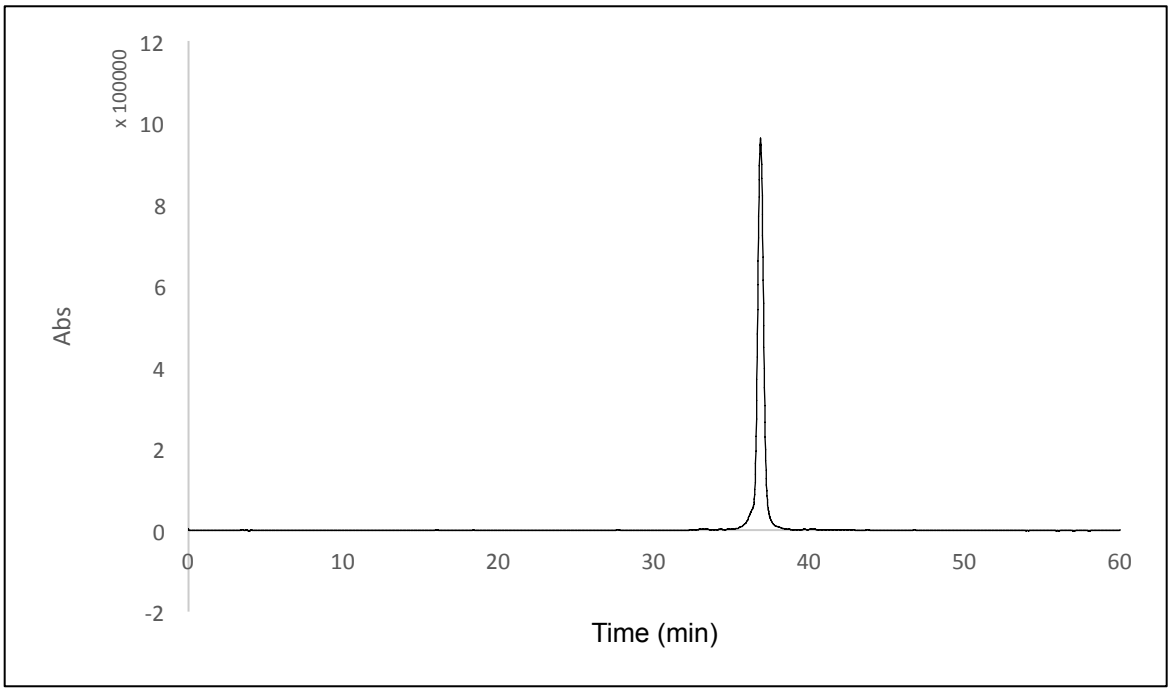
D-Dap$_9$, Val$_{10}$ containing lipopeptide (9)



Yield: 38 mg (30.9 umol, 32%)

HR-MS [M+H$^+$]: Calc.: 1218.6370, found: 1218.6385

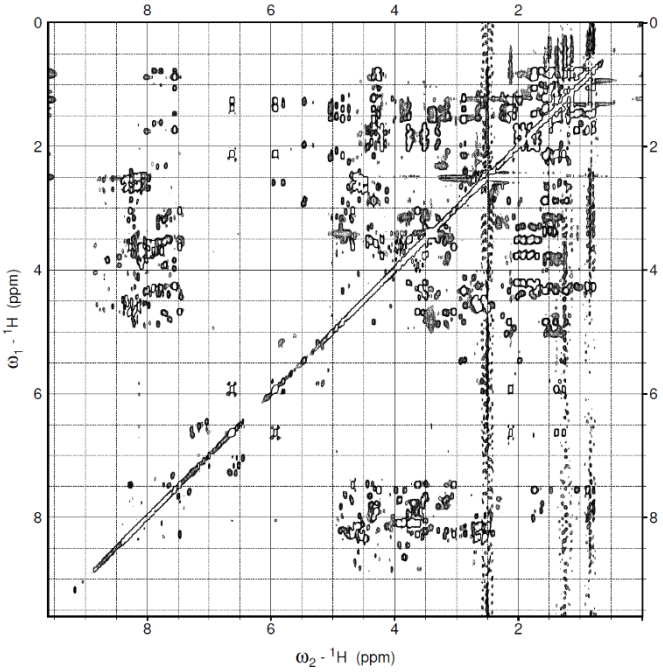
Analytical HPLC



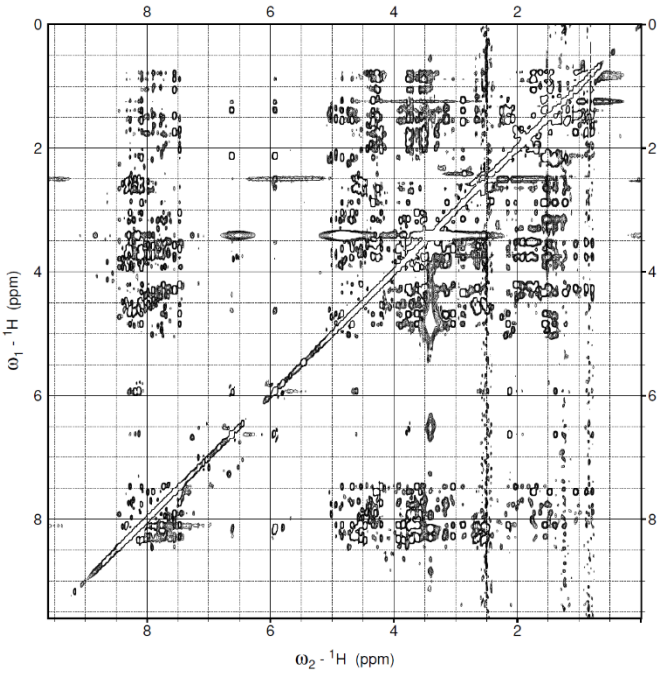
D-Dap$_9$, Val$_{10}$ containing lipopeptide (9) NMR Chemical Shifts

Residue	NH	$H_\alpha(C_\alpha)$	Sidechain
Tail	-	5.94 (124.5)	$C_\beta H$ (6.63, 143.8), $C_\gamma H_2$ (2.12, 31.7), $C_\delta H_2$ (1.38, 28.3), $C_\varepsilon H_2$-$C_\iota H_2$ (1.25, 29.4), $C_\kappa H_2$ (1.24, 27.3), $C_\lambda H_2$ (1.13, 38.9), $C_\mu H$ (1.49, 27.8), $2C_\nu H_3$ (0.84, 23.0)
Asp-1	8.11	4.89 (49.8)	$C_\beta H_2$ (2.61/2.49, 36.5)
Dap-2	8.23	4.73 (48.8)	$C_\beta H_2$ (3.76/3.77, 40.3)
D-Pip-3	-	4.99 (55.7)	$C_\beta H_2$ (2.06/1.36, 28.4), $C_\gamma H_2$ (1.54/1.36, 20.3), $C_\delta H_2$ (1.60/1.20, 24.8), $C_\varepsilon H_2$ (4.28/2.92, 39.8)
Gly-4	8.20	3.81/3.63 (42.0)	-
Asp-5	8.22	4.61 (50.0)	$C_\beta H_2$ (2.68, 36.3)
Gly-6	8.09	3.76 (43.4)	-
Asp-7	8.29	4.47 (50.6)	$C_\beta H_2$ (2.51, 36.3)
Gly-8	7.74	3.94/3.67 (42.3)	-
D-Dap-9	7.26	4.74 (48.9)	$C_\beta H$ (3.83/2.82, 40.1)
Val-10	7.42	4.25 (56.4)	$C_\beta H$ (2.04, 30.4), $C\gamma 1H_3$ (0.95, 19.3), $C\gamma 2H_3$ (0.81, 19.0)
Pro-11	-	4.20 (60.3)	$C_\beta H_2$ (2.09/1.67, 29.8), $C_\gamma H_2$ (1.90/1.80, 24.9), $C_\delta H_2$ (3.75/3.53, 47.8)

D-Dap$_9$, Val$_{10}$ containing lipopeptide (9) 2D NMR Spectra



TOCSY



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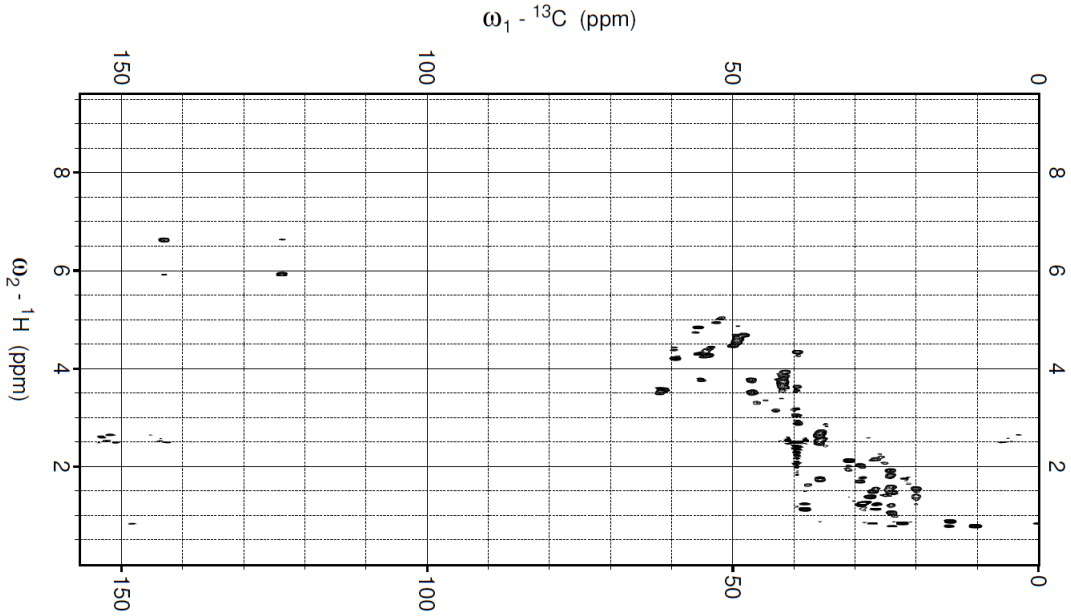
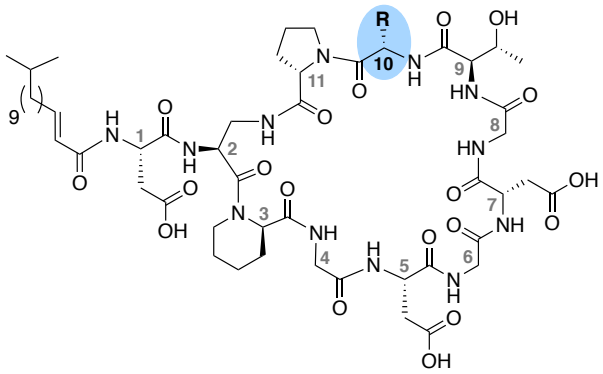
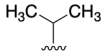


HSQC

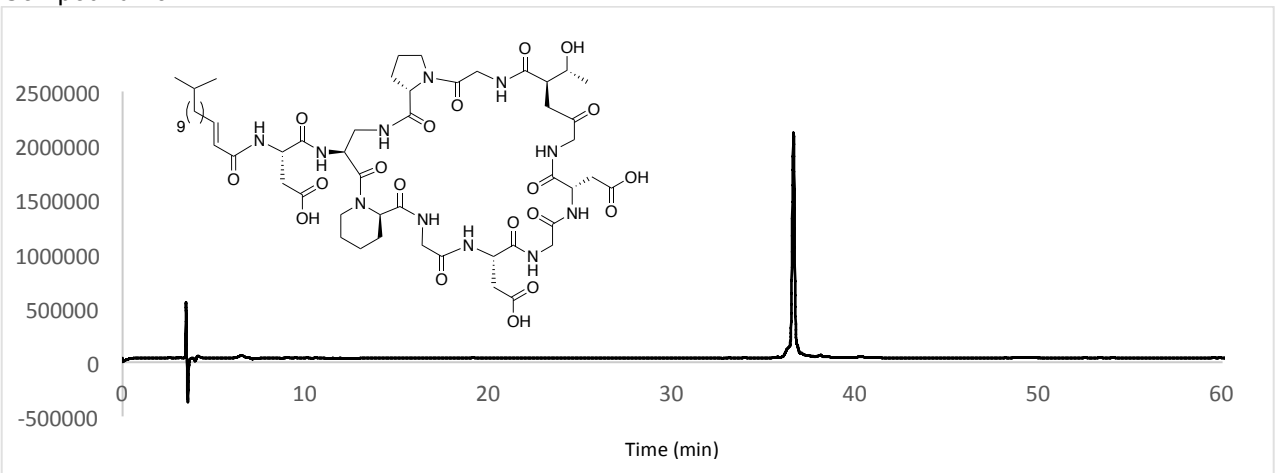
Analytical Data for Laspartomycin C position 10 variants



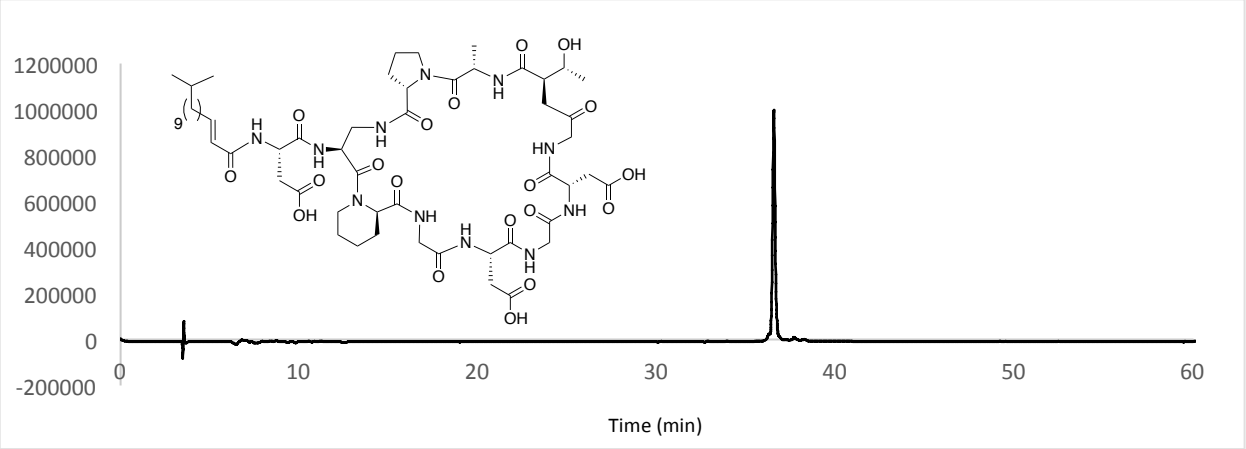
Compound	AA10	R	Chemical Formula	HR-MS [M+H$^+$]: Calc.	HR-MS [M+H$^+$]: Found
10	Gly		$C_{53}H_{82}N_{12}O_{19}$	1191.5892	1191.5886
11	L-Ala		$C_{54}H_{84}N_{12}O_{19}$	1205.6049	1205.6050
12	L-Abu		$C_{55}H_{86}N_{12}O_{19}$	1219.6205	1219.6198
13	L-Nval		$C_{56}H_{88}N_{12}O_{19}$	1233.6362	1233.6360
14	L-Val		$C_{56}H_{88}N_{12}O_{19}$	1233.6362	1233.6360
15	L-Phe		$C_{60}H_{88}N_{12}O_{19}$	1281.6362	1281.6360

Analytical HPLC traces

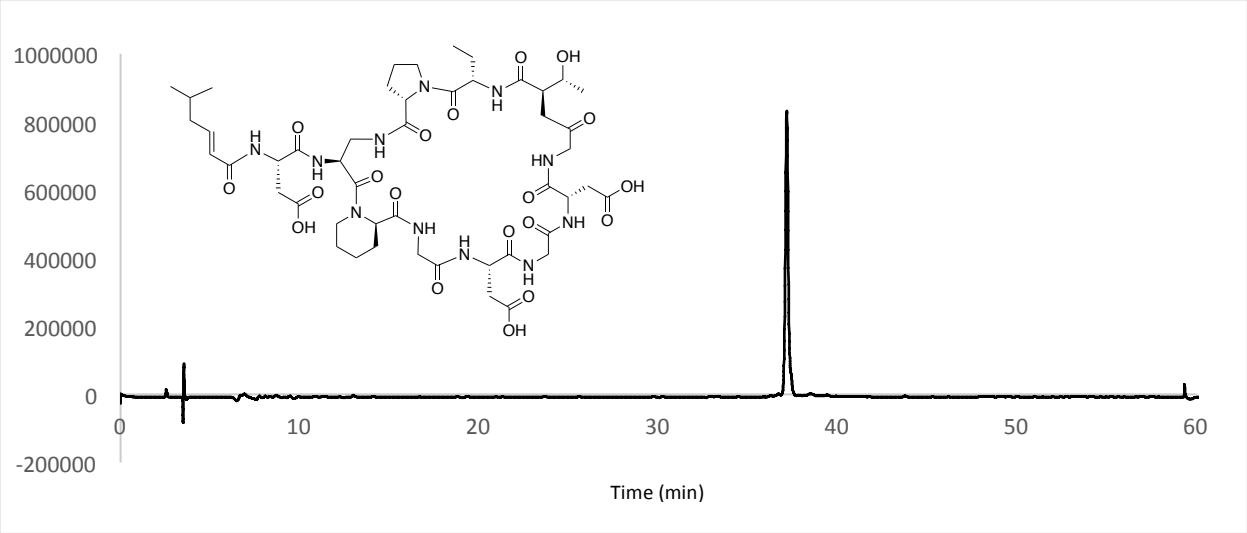
Compound **10**



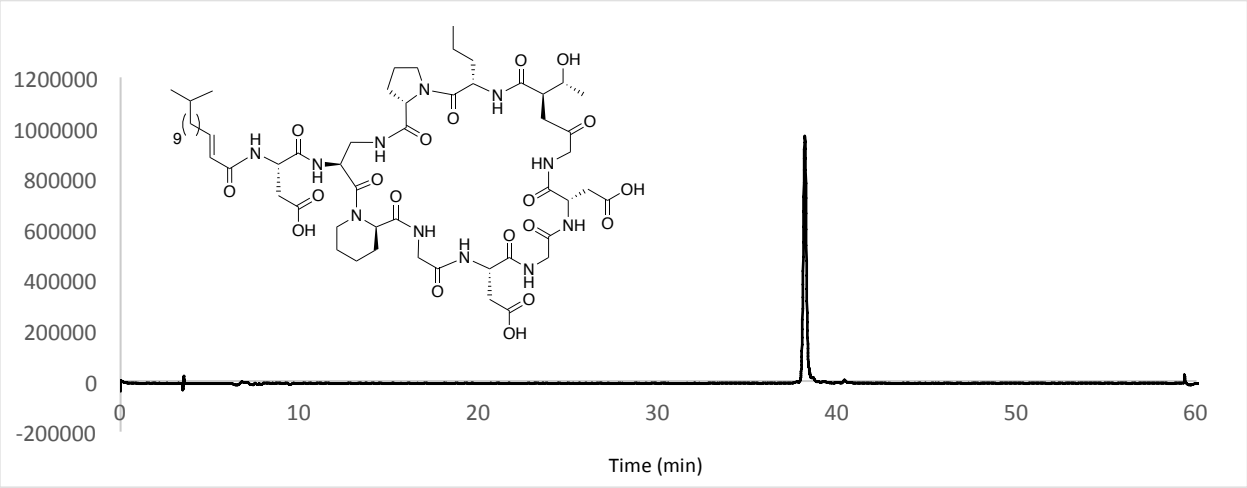
Compound **11**



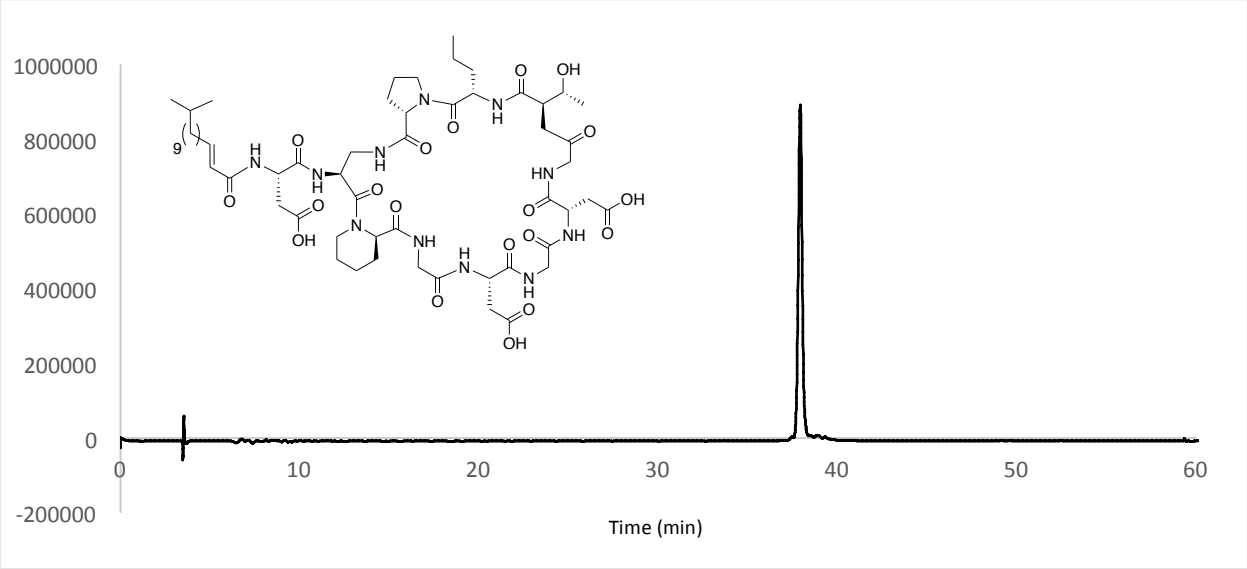
Compound **12**



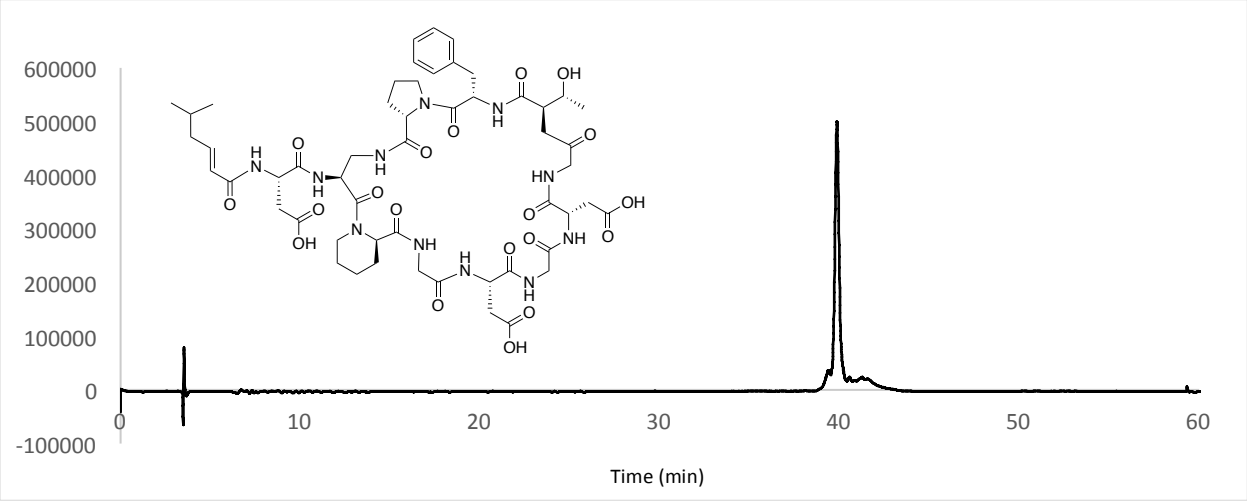
Compound **13**



Compound **14**



Compound **15**



Crystallization and data collection

Lipopeptide **5** or **7** was solubilized in 5 mM HEPES pH 7.5, 10 mM $CaCl_2$ and mixed 1 : 2 with C_{10}-P, to achieve a final concentration of 7.2 mM : 14.4 mM in presence of 10 % v/v PEG 200. Crystals were obtained by sitting drop vapour diffusion at 18 °C, by mixing 150 nL of the peptide solution with 150 nL of the reservoir solution, composed of 0.2 M sodium formate and 40 % v/v MPD for lipopeptide **5**, or 0.2 M cadmium chloride and 40 % v/v MPD for lipopeptide **7**, both supplemented by 10 % v/v PEG 200. Crystals were harvested without additional cryoprotectant and flash-cooled in liquid nitrogen. Datasets were collected at 100 K at the Diamond Light Source beamline I04-1 (lipopeptide **5**) or I04 (lipopeptide **7**).

Structure solution and refinement

The dataset of lipopeptide **5** was processed in the DIALS pipeline[2], whereas autoPROC[3] was used for lipopeptide **7**. The crystal of lipopeptide **7** was initially indexed in a hexagonal setting but based on the merging R-values the true symmetry appeared to be Primitive monoclinic with β = 120°. The reflection file was therefore re-indexed accordingly, and parameters for pseudo-merohedral twinning were included in the structure refinement. Additional anisotropic correction was done for the datasets of both analogues in STARANISO.[3] Structures were solved by molecular replacement using PHASER,[4] and one copy (lipopeptide **5**) or one dimer (lipopeptide **7**) of laspartomycin C in complex with geranyl phosphate (PDB: 5O0Z)[5] was used as a search model. Models were manually improved in *Coot*,[6] refinement was performed using REFMAC[7] and Molprobity[8] was used for validation. Structures of lipopeptides **5** and **7** in complex with Ca^{2+} and C_{10}-P were deposited to the Protein Data Bank under the accession codes 7AG5 and 7ANY, respectively.

Table S4. Data collection and refinement statistics. Highest resolution shell in parentheses.

	Lipopeptide 5 (PDB: 7AG5)	Lipopeptide 7 (PDB: 7ANY)
Data collection		
Space group	*P* 6 2 2	*P* 2_1
Cell dimensions		
a, *b*, *c* (Å)	40.43, 40.43, 31.03	40.13, 68.32, 40.13
α, *β*, *γ* (°)	90, 90, 120	90, 120, 90
Resolution (Å)	35.01 - 1.03 (1.12 - 1.03)	34.76 - 1.14 (1.27 - 1.14)
No. observed reflections	74114 (4481)	119460 (3464)
No. unique reflections	6321 (421)	36022 (1799)
R_{merge}	0.185 (1.584)	0.087 (0.373)
Mean I/σI	8.0 (1.5)	6.2 (2.8)
$CC_{1/2}$	0.997 (0.726)	0.995 (0.852)
Completeness (spherical, %)	80.9 (26.4)	52.2 (9.1)
Completeness (ellipsoidal, %)	92.2 (53.1)	85.3 (31.6)
Ellipsoidal resolution limits (Å) [direction]	1.03 [a*] 1.03 [b*] 1.19 [c*]	1.14 [a*] 1.81 [b*] 1.20 [c*]
Redundancy	11.7 (10.5)	3.3 (1.9)
Refinement		
Resolution (Å)	35.01 - 1.04	34.76 - 1.14
R_{work}/R_{free} (%)	12.04 / 14.32	15.96 / 19.21
Average *B*-factors (Å^2)		
Protein	12.3	12.6
Ligands/ions	26.5	18.3
Waters	26.7	21.4
R.M.S. deviations		
Bond lengths (Å)	0.019	0.017
Bond angles (°)	1.72	2.19
No. atoms		
Protein	178	1080
Ligands/ions	34	237
Waters	19	173

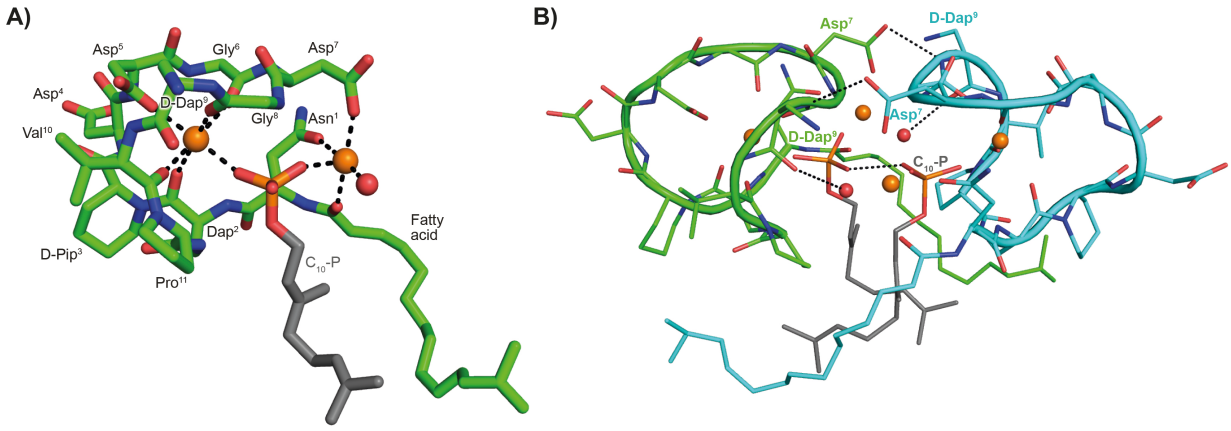
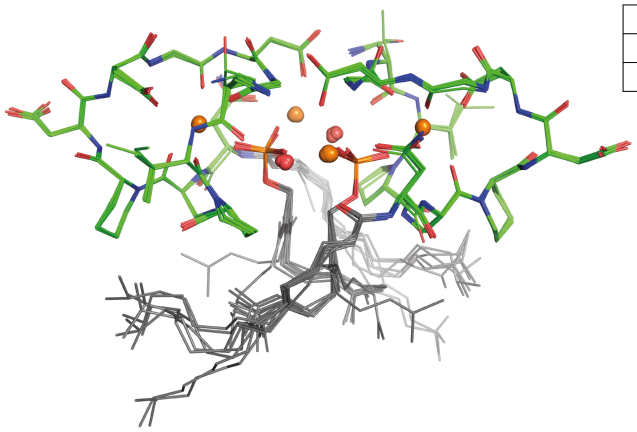


Figure S2. A) Structure of the ternary complex with lipopeptide **7** (green stick representation), two bound Ca^{2+} ions (orange spheres), a bound water molecule (red sphere), and the C_{10}-P ligand (lipid in grey). **B)** Lipopeptide **7** adopts a saddle-shaped conformation when complexed with two Ca^{2+} ions and C_{10}-P and forms a dimer in the crystal.



RMSD (Å)	**Lipopeptide 5**	**Lipopeptide 7**
Lipopeptide 7	0.100	X
Laspartomycin C	0.165	0.192

Figure S3: Lipopeptide 5 and Lipopeptide 7 dimers are similar to the Laspartomycin C dimer. Superposition of the dimer structures of lipopeptides **5** and **7** with that of laspartomycin C (PDB: 5O0Z).[5] Asymmetric units are composed of one monomer for lipopeptide **5** (the dimer shown for lipopeptide **5** generated by applying two-fold crystallographic symmetry), six dimers for lipopeptide **7** and one dimer for laspartomycin C. RMSD between dimers is indicated in the table. Ca^{2+} ions are represented by orange spheres and water molecules are represented by red spheres, C_{10}-P is also indicated.

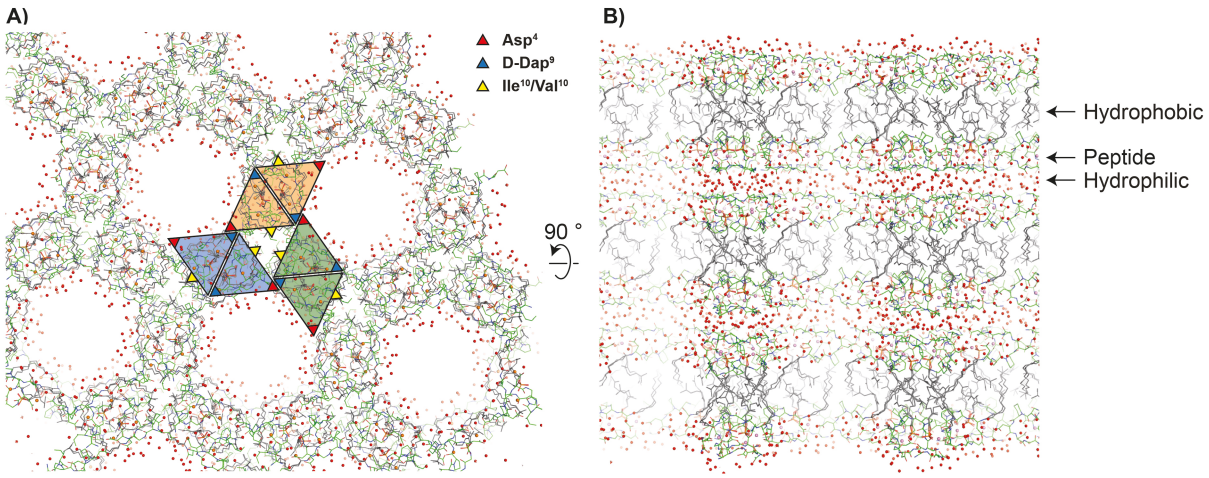


Figure S4. In the crystal state lipopeptide **5** forms a higher-ordered assembly when complexed with Ca^{2+} and C_{10}-P consisting of alternating hydrophobic (grey), peptidic (green), and hydrophilic (red) layers. A similar lattice is also observed for lipopeptide **7**. Notably, this higher ordered assembly is not seen for laspartomycin C.

Bacterial cytological profiling

B. subtilis reporter strains were aerobically grown at 30 °C in LB supplemented with 2mM $CaCl_2$ and antibiotic (5 µg/ml chloramphenicol or 100 µg/ml spectinomycin). Overnight cultures were diluted 100x without antibiotics and GFP-fusion protein expression induced with xylose (% in Table S5). At an OD_{600} of approximately 0.4 the cultures were diluted 10x in the same medium. At OD600 0.2-0.3 150 µl cells were incubated with 12.5 µg/ml laspartomycin C, 5 µg/ml lipopeptide **6,** or 2 µg/ml lipopeptide **6**. After 10 and 30 minutes 0.5 µl cells were immobilized on microscope slides covered with a 1% agarose film and imaged immediately.

Fluorescence microscopy was carried out using a Zeiss Axiovert 200M equipped with a Zeiss Neofluar 100x/1.30 Oil Ph3 objective, a Lambda S light source (Shutter Instruments), a Photometrics Coolnap HQ2 camera, and Metamorph 6 software (Molecular Devices). Images were analyzed using ImageJ (National Institutes of Health) v.1.52a.

Table S5: *B. subtilis* strains used in this study ref PMID: 27791134.

Strain	genotype	induction
1049	*amyE::spc Pxyl-rpsB-gfp*	1% xylose
1048	*cat rpoC-gfp Pxyl-rpoC*	1% xylose
YK405	*amyE::spc Pxyl-gfp-mreB*	0.3% xylose
4056	*amyE::spec Pxyl-gfp-pmut1-ftsZ*	0.1% xylose
TB35	*amyE::spc Pxyl-gfp-minD*	0.25% xylose
BS23	*atpA-gfp Pxyl-atpA cat*	0.1% xylose
TNVS91	*∆amyE::specR-PxylR-PolC-4GS-msfGFP*	0.03% xylose
TNVS175	*amyE::spc-Pxyl-murG-msfgfp*	0.05% xylose

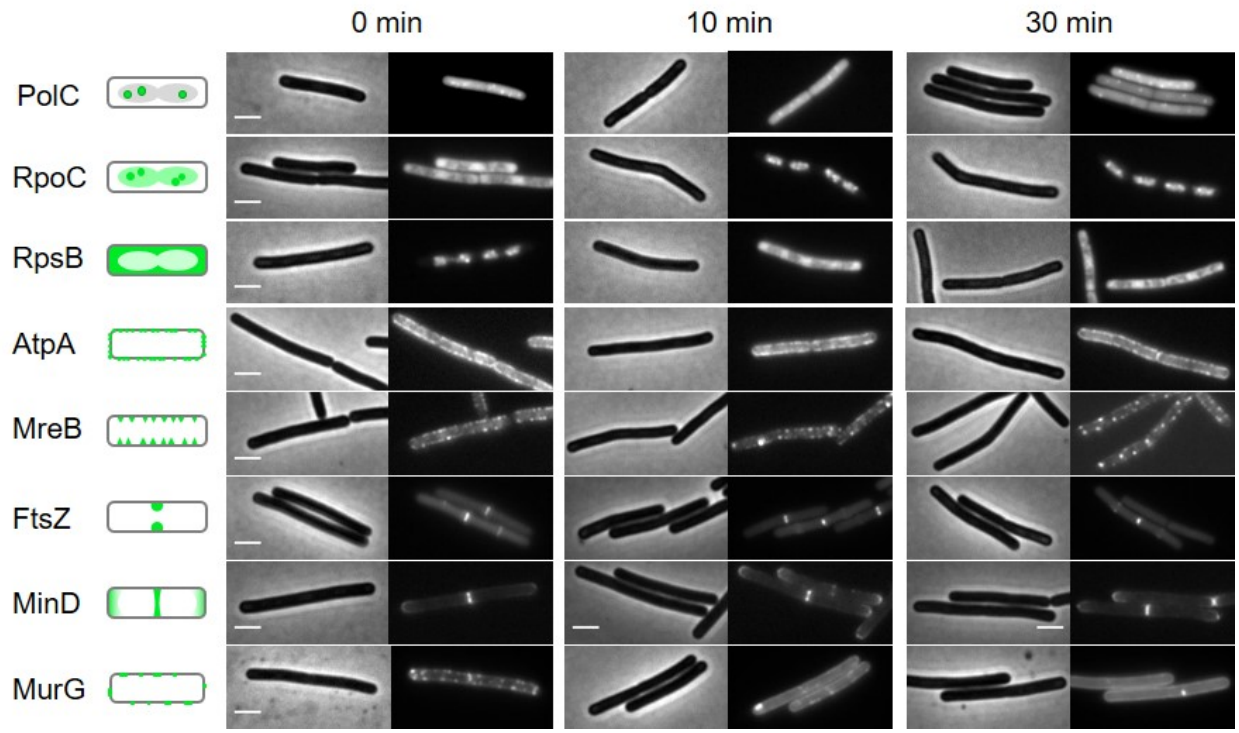


Figure S5. Bacterial cytological profiling analysis of lipopeptide **6**. The GFP-tagged marker proteins represents the following cellular activities: DNA polymerization (PolC), RNA polymerization (RpoC), protein synthesis (RpsB), F0F1 ATPase (AtpA), lateral cell wall synthesis regulation (MreB), cell division (FtsZ), cell division regulation (MinD) and peptidoglycan precursor synthesis (MurG). Left panels schematically show the normal localization patterns of the different GFP fusions. Strains were grown in LB medium supplemented with 2 mM $CaCl_2$ at 30 °C. 2x MIC concentration was added (0 min) and samples for microscopy were taken after 10- and 30-min incubation, respectively. Scale bars indicate 2 μm.

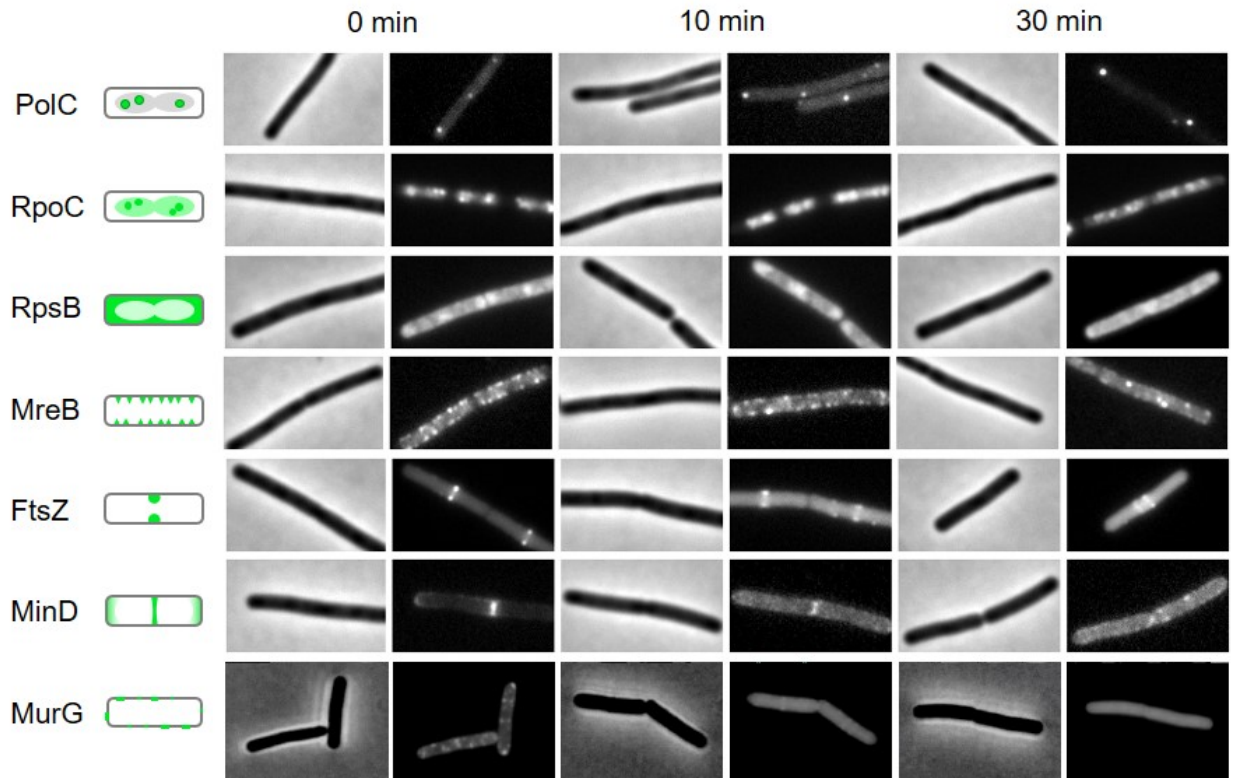


Figure S6. Daptomycin reference for the bacterial cytological profiling analyses. The figure was adapted from Müller et al.[9] The GFP-tagged marker proteins represents the following cellular activities: DNA polymerization (PolC), RNA polymerization (RpoC), protein synthesis (RpsB), lateral cell wall synthesis regulation (MreB), cell division (FtsZ), cell division regulation (MinD) and peptidoglycan precursor synthesis (MurG). Left panels schematically show the normal localization patterns of the different GFP fusions. Strains were grown in LB medium supplemented with 1.25 mM $CaCl_2$ and treated with 2 µg/mL daptomycin at 30 °C. Samples for microscopy were taken before (0 min) and after 10 and 30 min incubation.

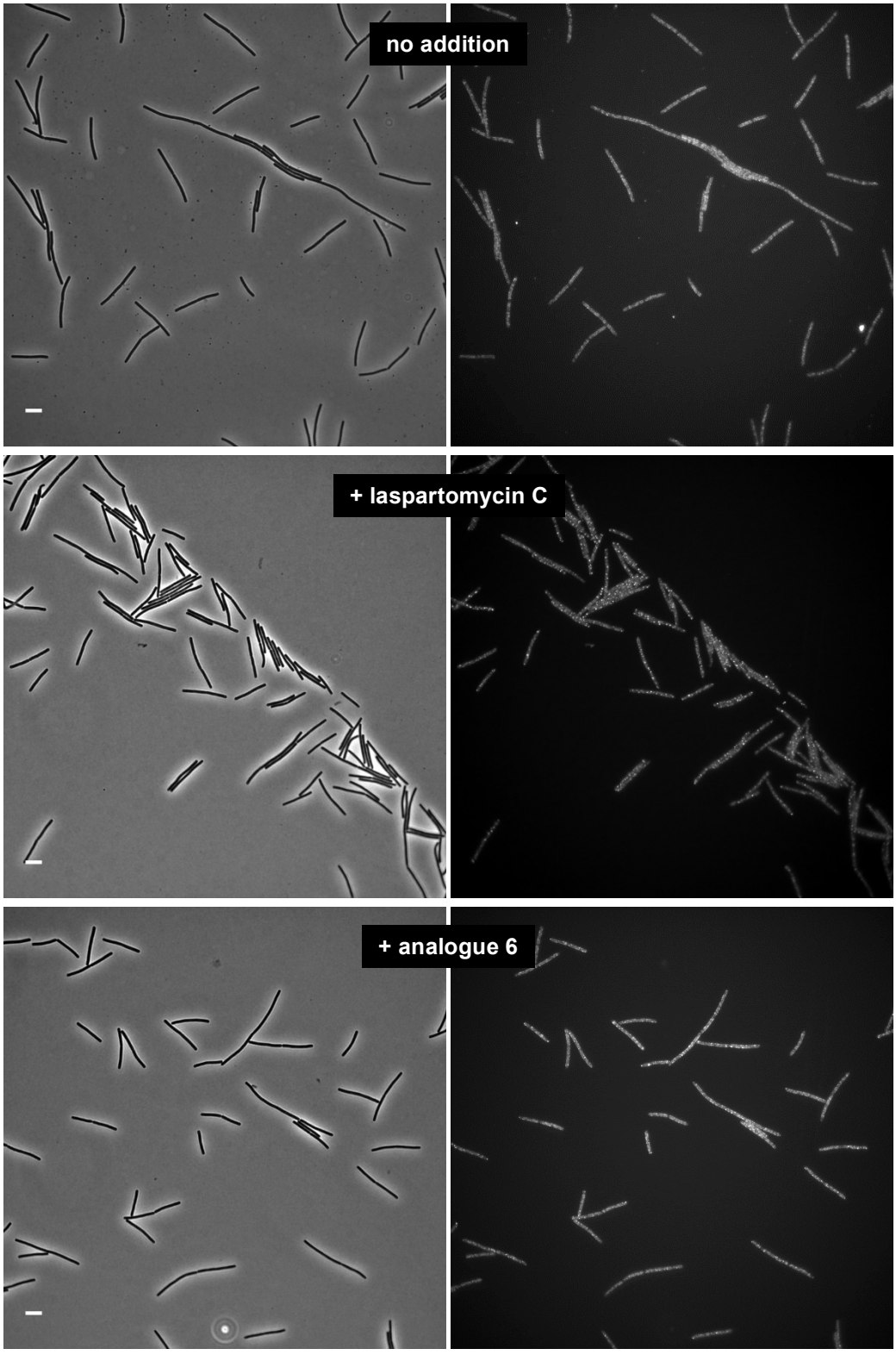


Figure S7. Large field phase contrast and fluorescent images showing the effect of 10 min incubation with 12.5 µg/ml laspartomycin C or with 5 µg/ml lipopeptide **6** on the localization of GFP-MreB. Strains were grown in medium supplemented with 2 mM $CaCl_2$ at 30 °C. Scale bars indicate 5 µm.

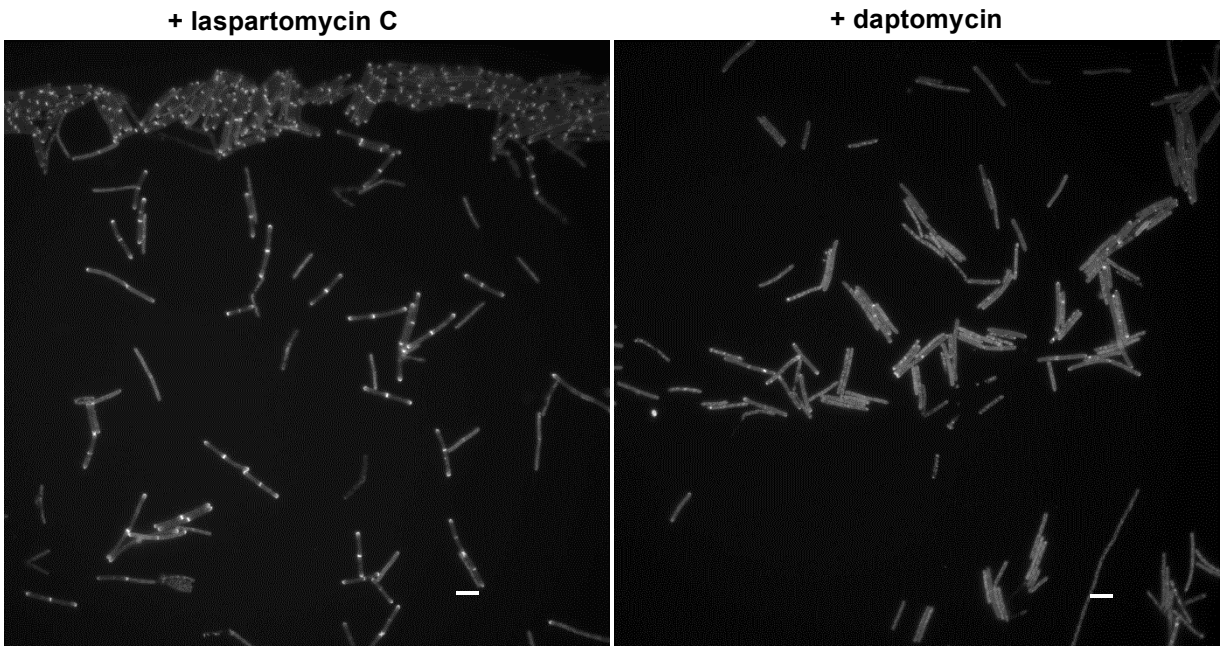


Figure S8. large field images showing the effect of laspartomycin C (12.5 µg/ml) or daptomycin (2 µg/ml) on the localization of GFP-MinD after 30 min incubation with the antibiotics. Localization of MinD is unaffected by laspartomycin C, whereas this protein detaches from the membrane when treated with daptomycin Strains were grown in medium supplemented with 2 mM $CaCl_2$ at 30 °C. Scale bars indicate 5 µm.

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