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Supporting Information

Production of a photohexapeptide library from entomopathogenic *Photorhabdus asymbiotica* PB68.1

Lei Zhao[a,b] and Helge B. Bode*[,a,c]

[a]Molecular Biotechnology, Department of Biosciences, Goethe University Frankfurt, 60438 Frankfurt am Main, Germany

[b]Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, 210014 Nanjing, China

[c]Buchmann Institute for Molecular Life Sciences (BMLS), Goethe University Frankfurt, 60438 Frankfurt am Main, Germany

*Corresponding author: Tel: +49 69 798 29557. Fax: +49 69 798 29527. E-mail: h.bode@bio.uni-frankfurt.de

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Experimental Procedures

Strain construction

Construction of promoter exchange mutant of *Photorhabdus asymbiotica* PB68.1 was carried out as described previously.[1] Briefly, pCEP (cluster expression plasmid, pLZ39) carrying the first 696 bp of *phpS* gene (*ppb6_04101*) from *P. asymbiotica* PB68.1 was constructed by using Gibson cloning and was transformed into *E. coli* S17-λpir. For the conjugation, *P. asymbiotica* PB68.1 was mated with *E. coli* S17-λpir carrying pLZ39. Both strains were grown in lysogeny broth (LB) medium with 50 μg/mL kanamycin added to *E. coli* S17-λpir. The cells were harvested and washed three times with fresh LB medium after OD_{600} of 0.5–0.7. Subsequently, *E. coli* S17-λpir and *P. asymbiotica* PB68.1 strains were mixed on a LB agar plate in a ratio of 1:3 and incubated at 37°C for 3 hours followed by incubation at 30°C overnight. The next day, the bacterial cell layer was harvested and resuspended in fresh LB medium. Serial dilutions were spread out on selective LB agar plates with kanamycin and ampicillin (100 μg/mL), and incubated at 30°C for 2–3 days. The genotype of individual clones was verified by PCR.

For construction of heterologous *E. coli* strains expressing *phpS*, functional plasmid (pLZ51) was achieved by using yeast cloning[2] and verified by enzyme digestion, and transformed into *E. coli* DH10B MtaA and *E. coli* DH10B MtaA Δ*penta*, respectively. Individual clones were analyzed by HPLC-MS for the photohexapeptide production.

Strain cultivation and culture extraction

100 μL of overnight culture of photohexapeptide production strains were inoculated into 10 mL liquid LB medium containing appropriate antibiotics, and 0.1% L-arabinose (from a 25% stock solution) and 2% Amberlite XAD-16 resin were added. The cultures were grown at 30 °C on a rotary shaker. After 72 h, the cultures were harvested, and XAD-16 beads were separated and extracted with 10 mL methanol for 1 h. Subsequently, the extracts were analyzed by HPLC-MS.

HPLC-MS analysis

HPLC-MS analysis was performed on a Dionex UltiMate 3000 system coupled to a Bruker Impact II QTOF mass spectrometer. Crude extracts and synthetic compounds in 5 μL injection volume were eluted on an ACQUITY UPLC BEH C_{18} column (130 Å, 2.1 mm × 50 mm, 1.7 µm) using a gradient from 5% to 95% (or 5% to 60%) acetonitrile water solution containing 0.1% formic acid

at a flow rate of 0.4 mL/min for 16 min. Positive mode with scan range from 100 to 1200 *m/z* was used to detect photohexapeptides.

Labelling experiments

The strain cultivation, culture extraction, and HPLC-MS analysis for labelling experiments were carried out as described above. The production culture was additionally fed with L-[D_{10}]leucine and L-[D_8]valine, respectively, in LB medium.

Compound isolation

Compound **1**-OMe was isolated from 1 L culture of promoter exchange mutant of *P. asymbiotica* PB68.1. The XAD extract was washed with EtOAc and fractionated by Sephadex LH-20 chromatography using MeOH as the eluent. The enriched fractions containing **1**-OMe were collected and used for further purification. The purification was carried out on an Agilent semi-preparative HPLC system. The compounds were eluted on a Cholester column (10 mm ID × 250 mm) by using an isocratic 35% acetonitrile water solution containing 0.1% formic acid for 40 min at a flow rate of 3 mL/min to yield **1**-OMe (2.5 mg).

Chemical synthesis

The selected photohexapeptides were synthesized by using a solid-phase peptide synthesis method as described previously.[3] For a schematic overview see Fig. S2 showing the synthesis of **1** as an example. Step **I** was loading of the isoleucine on the 2-chlorotrityl chloride (2-CTC) resin. A solution of Fmoc-Ile-OH (212 mg, 0.6 mmol, 3 eq.) and *N*,*N*-diisopropylethylamine (DIPEA, 0.31 mL, 1.8 mmol, 9 eq.) in 4 mL dry dichloromethane (DCM) was placed in a plastic reactor vessel filled with 2-CTC resin (125 mg, 0.2 mmol, 1.0 eq.). The resulting mixture was incubated at room temperature overnight. The remaining free binding sites were capped upon incubating twice with DCM/CH_3OH/DIPEA (80:15:5) for 10 min at room temperature. The resin was washed several times with dimethylformamide (DMF), CH_3OH, and DCM and treated with 20% piperidine in DMF (3 × 10 min) to remove the Fmoc-protecting group. The combined filtrates were used to determine the actual loading of the resin at $\lambda_{301\ nm}$. Afterwards, the resin was washed with DCM and dried. Step **II** is solid-phase peptide synthesis. The linear sequence was synthesized on the preloaded Ile-2-CTC resin on a 25 μmol scale with a Syro Wave peptide synthesizer by using standard Fmoc chemistry. The resin was placed in a plastic reactor vessel

with a Teflon frit and an amount of 6 eq. of amino acid derivatives (Fmoc-D-Leu-OH, Fmoc-Ile-OH, Fmoc-D-*allo*-Ile-OH, 0.2 M) were activated in situ at room temperature with 6 eq. of *O*-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*Ń*,*Ń*-tetramethyluronium hexafluorophosphate (HCTU, 0.6 M) in DMF in the presence of 12 eq. DIPEA (2.4 M) in NMP for 50 min. Fmoc-protecting groups were removed with a solution of 40% piperidine in DMF for 5 min and the deprotection step was repeated for another 10 min with 20% piperidine in DMF. After each coupling and deprotection step, the resin was washed with NMP. After the addition of the final residue, the resin was washed with NMP, DMF, and DCM and dried. Step **III** is cleavage of peptide from the resin. A total of 1 mL 95% trifluoroacetic acid and 2.5% triisopropylsilane in water were added to the peptidyl resin (25 μmol) and the mixture was agitated for at least 2 h at room temperature. The resin was removed by filtration and washed twice with TFA. The solution was concentrated *in vacuo*. The peptide was purified by Agilent HPLC system to give **1**. The structure of **1** was confirmed by HR-MS and NMR.

Bioactivity testing

Bioactivity of **1** and **1**-OMe against *Micrococcus luteus* and *Saccharomyces cerevisiae* was tested using filter paper discs loaded with MeOH-dissolved compounds (1 mg/mL) on the prepared plates which were incubated at 30 °C for 2 days.

Quantitative analysis

The amount of all produced photohexapeptides in promoter exchange mutant of *P*. *asymbiotica* PB68.1 was deduced from quantitative analysis using the synthesized **1** as standard as described previously.[4]

Supplementary Tables

Table S1 A domain specificity prediction of PhpS

A domain	Stachelhaus sequence	most likely amino acid predicted	amino acid detected
A1	DAFWIGATFK	Val	Val/Ile
A2	DAFWIGATFK	Val	Val/Ile
A3	DAFWIGATFK	Val	Val/Ile
A4	DAFWIGATFK	Val	Val/Ile
A5	DAWCIGAVCK	Phe	Leu
A6	DAFWIGATFK	Val	Val/Ile

Table S2 Bacterial strains used in this study

strain	genotype	reference
E. coli S17-λpir	Tp^r Sm^r *recA thi hsdR* RP4-2-Tc::Mu-Km::Tn7, λ*pir*	[5]
E. coli DH10B MtaA	F-*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *end*A1, *ara*D139, Δ(*ara leu*)7697, *gal*U, *gal*K, *rps*L, *nup*G, λ–, *ent*D::*mtaA*	[2]
E. coli DH10B MtaA Δ*penta*	F-*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *end*A1, *ara*D139, Δ(*ara leu*)7697, *gal*U, *gal*K, *rps*L, *nup*G, λ–, *mtaA*, Δ*penta* (Δ*aspC* Δ*ilvE* Δ*tyrB* Δ*avtA* Δ*ybfQ*), Cm^R	[6]
P. asymbiotica PB68.1	wild type	[7]
E. coli S17-λpir pLZ39	*E. coli* S17-λpir pLZ39, Km^R	this work
PB68.1 pCEP-*phpS*	PB68.1 pCEP-*phpS*, Km^R	this work
E. coli pFF1-*phpS* (pLZ51)	*E. coli* DH10B MtaA, pFF1-*phpS*, Km^R	this work
E. coli Δ*penta* pFF1-*phpS* (pLZ51)	*E. coli* DH10B MtaA Δ*penta*, pFF1-*phpS*, Cm^R, Km^R	this work

Table S3 Plasmids used in this study

plasmid	Genotype/description	reference
pCEP	pDS132 based vector, R6Kγ ori, oriT, *ara*C, *ara*BAD promoter, Km^R	[1]
pFF1	2μ ori, $G418^R$, $_P$BAD promoter, pCOLA ori, MCS, Ypet-Flag, kan^R	[8]
pLZ39	5489 bp, first 696 bp of *phpS* gene from PB68.1 genomic DNA assembled into pCEP, Km^R	this work
pLZ51	27505 bp, *phpS* gene from PB68.1 genomic DNA assembled into pFF1, Km^R	this work

Table S4 Primers used in this study

primer	sequence (5'-3')	targeting DNA fragment	plasmid
LZ_88	TTTGGGCTAACAGGAGGCTAGCATATGAAAGAT AGCATTGCTAACGC	first 696 bp of *phpS* gene from PB68.1 (696 bp)	pLZ39
LZ_89	TCTGCAGAGCTCGAGCATGCACATTTGCGAGAG ATCTTGTGGATG		
pCEP_fw_gib	ATGTGCATGCTCGAGCTC	pCEP vector backbone (4841 bp)	
pCEP_rv_gib	ATGCTAGCCTCCTGTTAGC		
V_pCEP_fw	GCTATGCCATAGCATTTTTATCCATAAG	insert verification from pLZ39 (882 bp)	
V_pCEP_rv	ACATGTGGAATTGTGAGCGG		
V_pCEP_fw	GCTATGCCATAGCATTTTTATCCATAAG	conjugation verification from PB68.1_pCEP-*phpS* (1342 bp)	
LZ_90	TCTGCAACAACATCTGCAATG		
LZ_126	ATCGCAACTCTCTACTGTTTCTCCATACCCGTTTT TTTGGGCTAACAGGAGGAATTCCATGAAAGATA GCATTGCTAACGC	fragment I of *phpS* gene from PB68.1 (7314 bp)	pLZ51
LZ_127	ATCAGTATGCTGACAGACCAGC		
LZ_128	AGTACCTGATGTGGAGAAACAAC	fragment II of *phpS* gene from PB68.1 (7240 bp)	
LZ_129	TGAGATGGTCAACCCAGGTAG		
LZ_130	ACAGGTGGTAGAAATTGTCCAAC	fragment III of *phpS* gene from PB68.1 (6272 bp)	
LZ_131	TCTTCACCTTTGCTCATGAACTCGCCAGAACCAG CAGCGGAGCCAGCGGATCCGGCGCGCCTCACTG AACTTCTCCATCACC		

Table S5 HR-MS data of isolated and synthetic compounds

compound	sum formula	calcd. $[M + H]^+$	found $[M + H]^+$	Δ ppm
isolated **1**-OMe	$C_{37}H_{71}N_6O_7$	711.5379	711.5366	1.7
synthetic **1**	$C_{36}H_{69}N_6O_7$	697.5222	697.5205	2.4
synthetic **2a**	$C_{35}H_{67}N_6O_7$	683.5066	683.5050	2.3
synthetic **2b**	$C_{35}H_{67}N_6O_7$	683.5066	683.5056	1.4
synthetic **2c**	$C_{35}H_{67}N_6O_7$	683.5066	683.5056	1.5
synthetic **2d**	$C_{35}H_{67}N_6O_7$	683.5066	683.5055	1.5
synthetic **2e**	$C_{35}H_{67}N_6O_7$	683.5066	683.5054	1.7
synthetic **6**	$C_{31}H_{59}N_6O_7$	627.4440	627.4426	2.2

Supplementary Figures

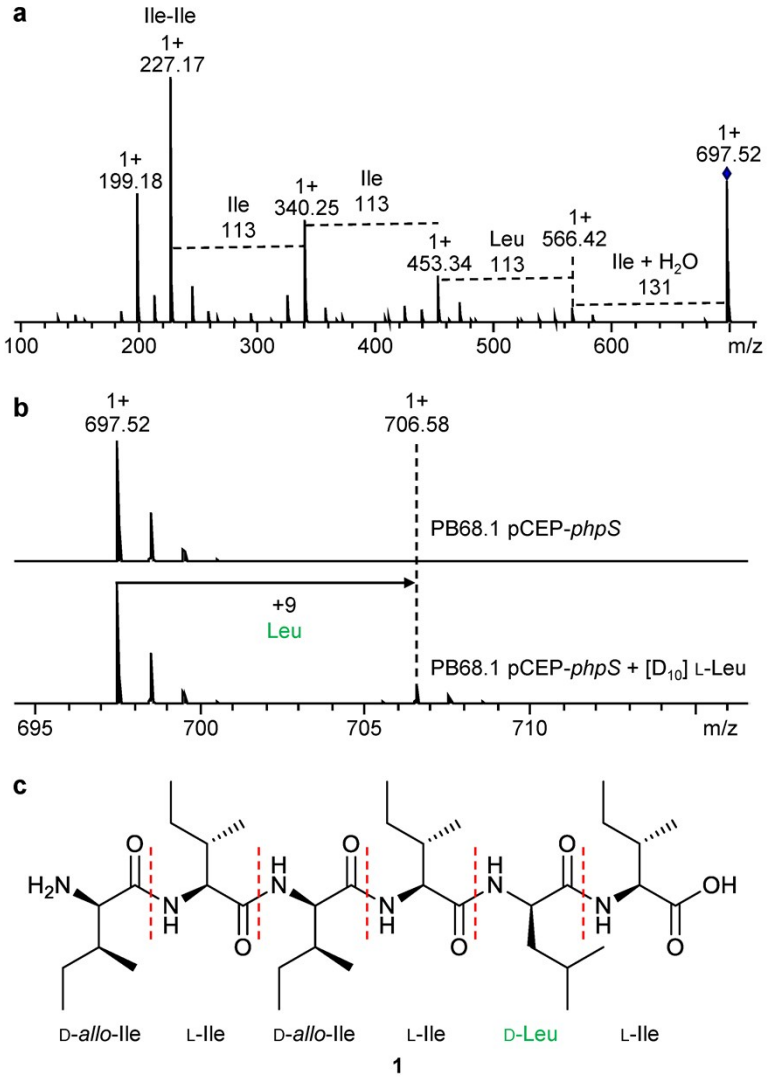


Fig. S1 Structure identification of **1** using labelling experiments combined with HPLC-MS analysis. (**a**) MS^2 fragmentation pattern of **1**; (**b**) MS data of **1** from labelling experiments with/without [D_{10}] L-Leu fed to *P. asymbiotica* PB68.1 pCEP-*phpS* in LB medium; (**c**) Structure of **1**.

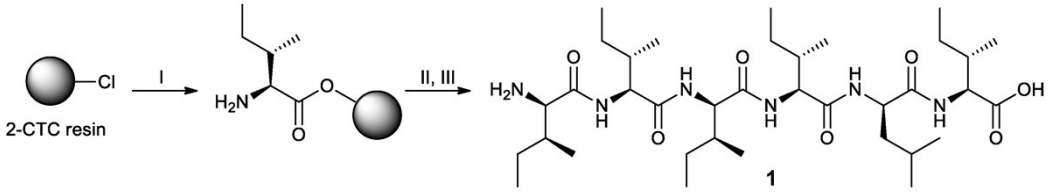


Fig. S2 Solid-phase peptide synthesis, shown for **1** as an example. **I**. Fmoc-Ile-OH, DIPEA, DCM, overnight, DCM/MeOH/DIPEA (80:15:5), then 20% piperidine/DMF; **II**. Fmoc-AA-OH, HCTU, DIPEA, DMF, NMP, 50 min, then 40% piperidine/DMF, 20% piperidine/DMF; **III**. TFA/TIS/Water (95:2.5:2.5), 2 h.

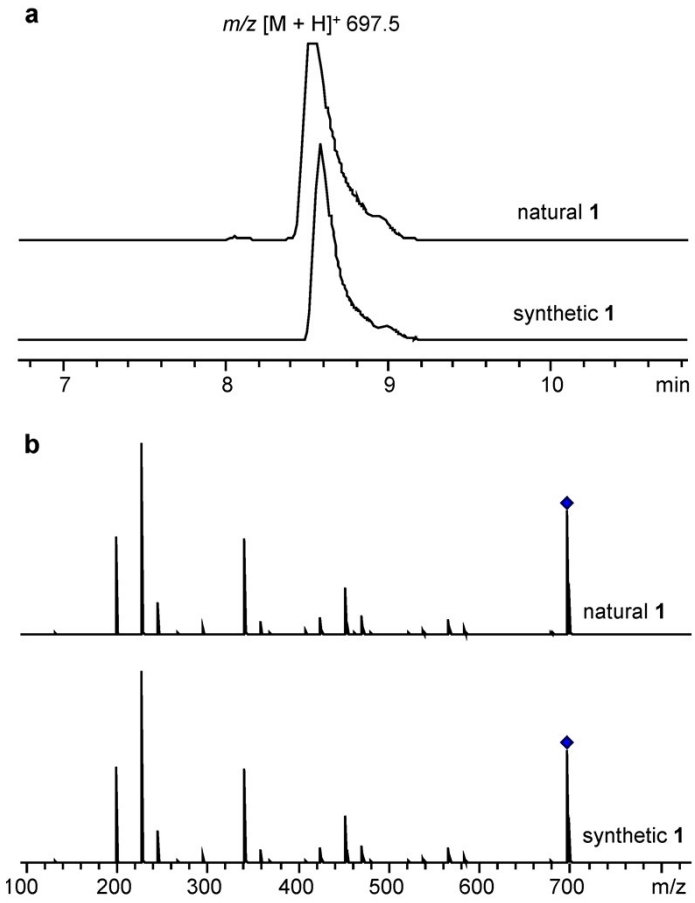


Fig. S3 Comparison of natural and synthetic **1** by HPLC-MS analysis. (**a**) Extracted ion chromatograms (EICs) of natural and synthetic **1**; (**b**) MS^2 fragmentation patterns of natural and synthetic **1**.

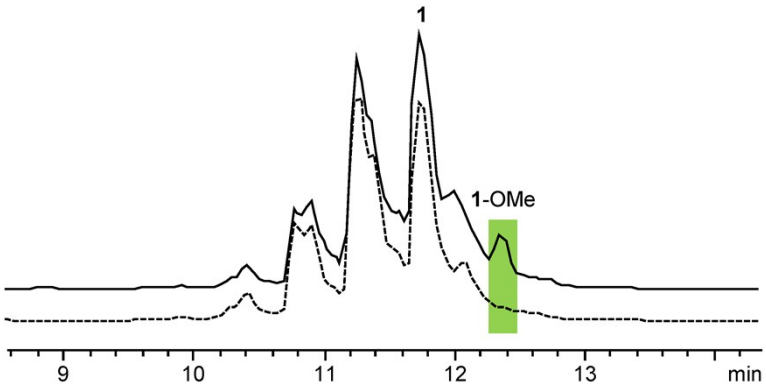
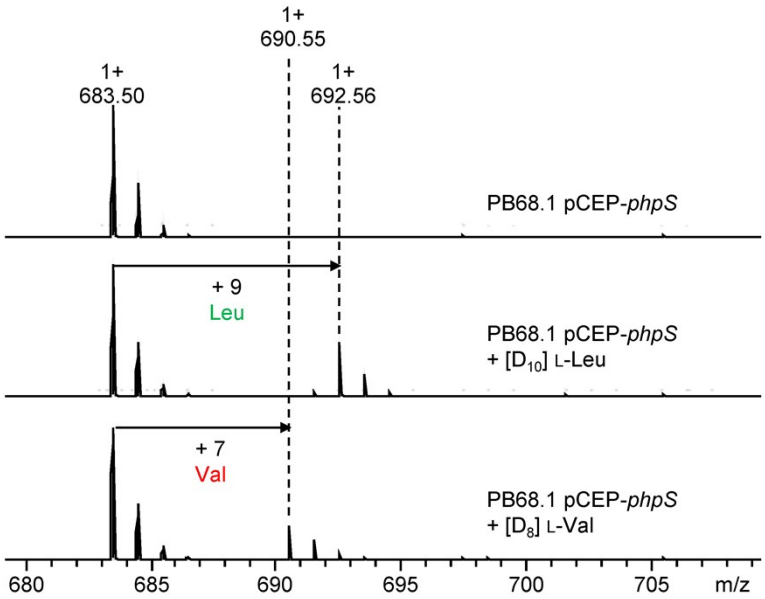
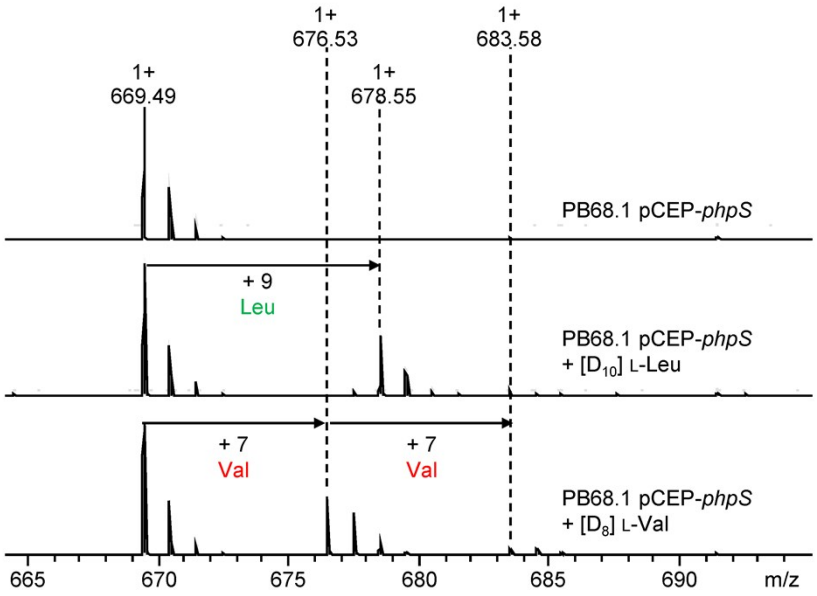


Fig. S4 HPLC-MS analysis of extracts from *P. asymbiotica* PB68.1 pCEP-*phpS.* XAD extract was treated with methanol (continuous line) and supernatant was treated with acetonitrile (1:1) (dotted line), respectively. Base-peak chromatograms (BPCs) are shown.

Cluster **2**: *m/z* [M + H]$^+$ 683.50, 1 Leu + 1 Val + 4 Ile



Cluster **3**: *m/z* [M + H]$^+$ 669.49, 1 Leu + 2 Val + 3 Ile



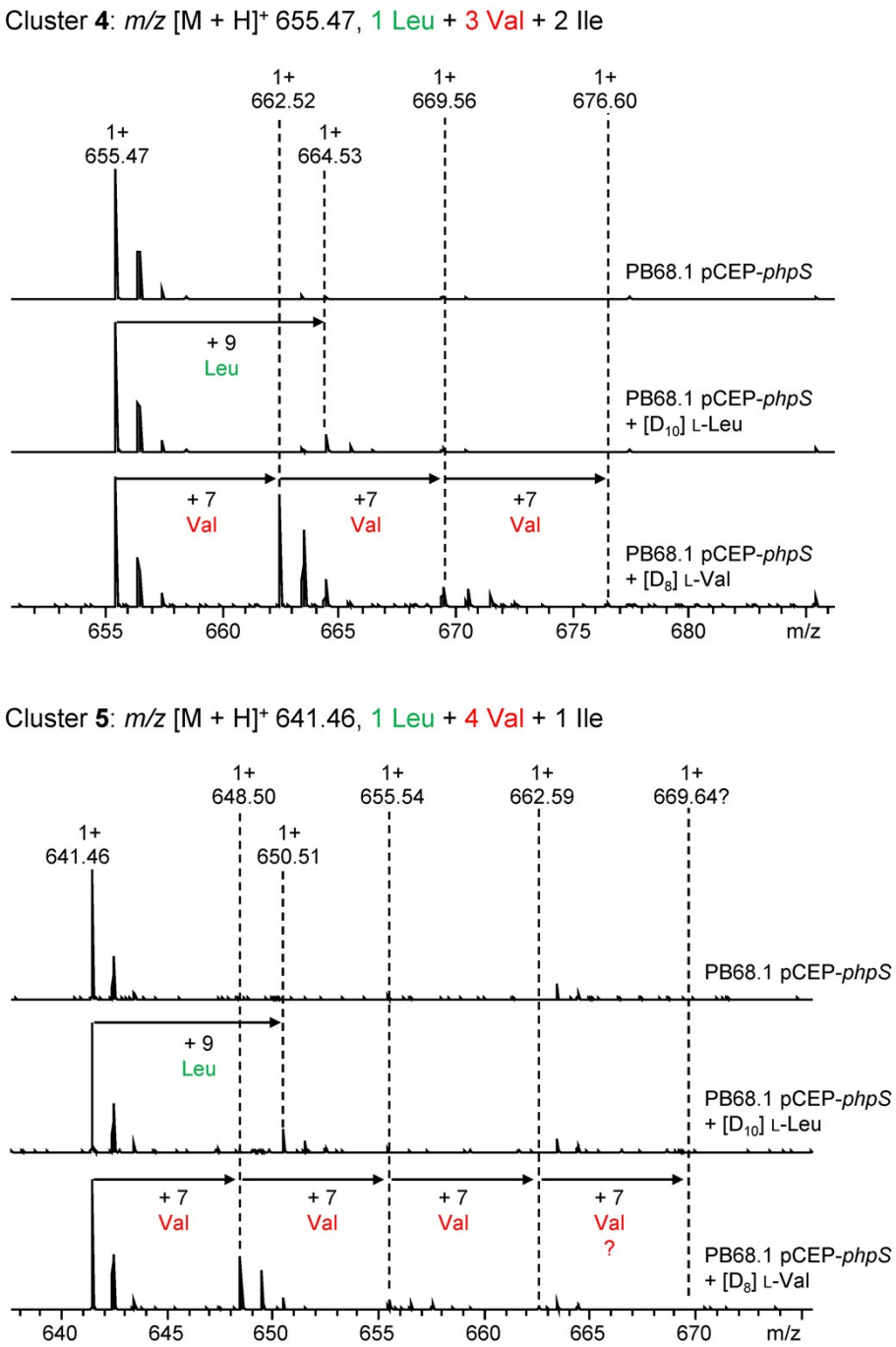


Fig. S5 MS data of compounds in clusters **2**–**5** from labelling experiments with/without [D_{10}] L-Leu or [D_8] L-Val fed to *P. asymbiotica* PB68.1 pCEP-*phpS* in LB medium.

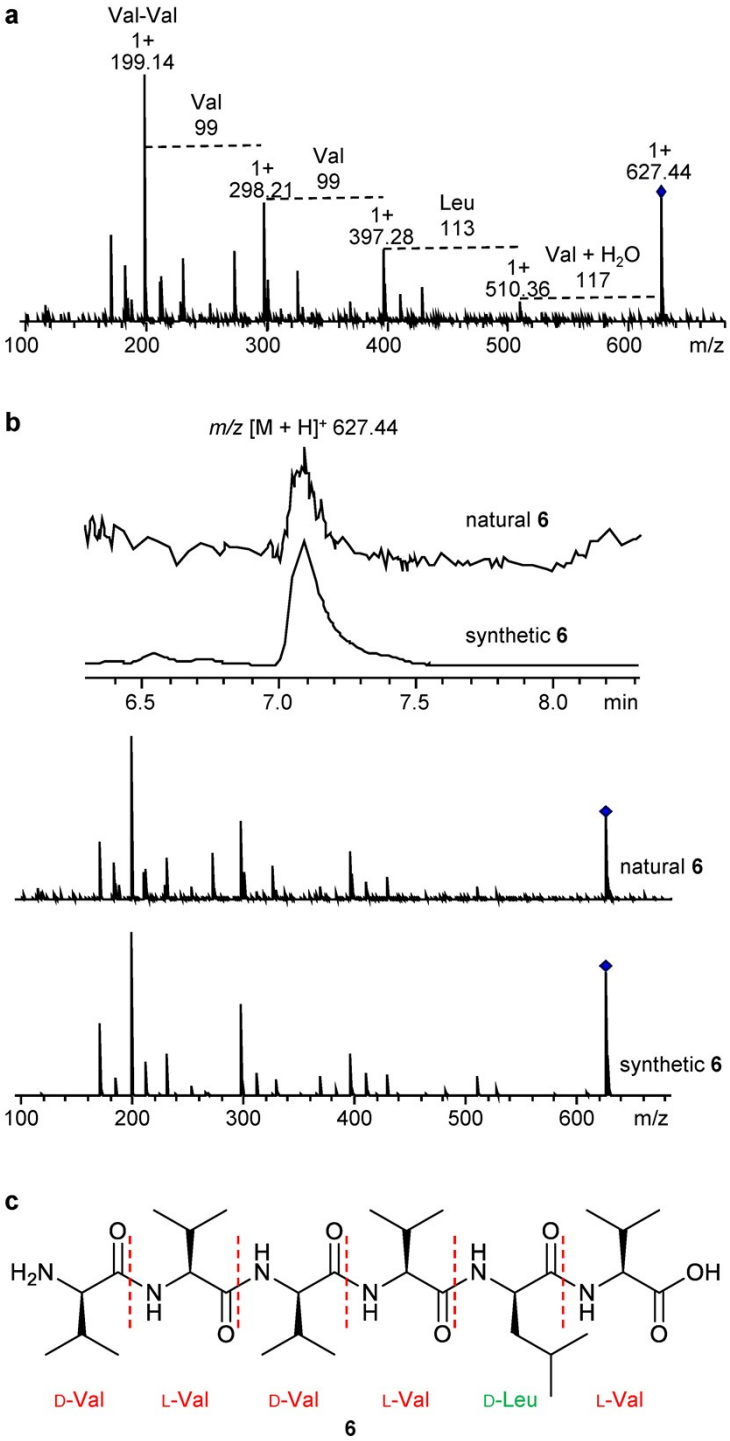
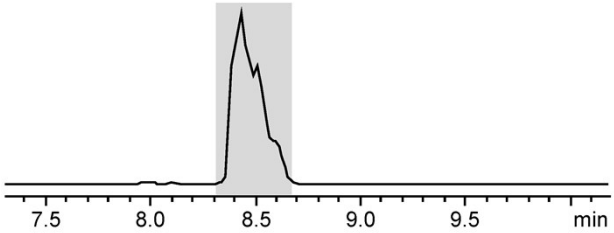


Fig. S6 Structure identification of **6** by HPLC-MS analysis and chemical synthesis. (**a**) MS^2 fragmentation pattern of **6**; (**b**) Comparison of natural and synthetic **6**; (**c**) Structure of **6**.

Cluster **2**: *m/z* [M + H]$^+$ 683.50, 1 Leu + 1 Val + 4 Ile



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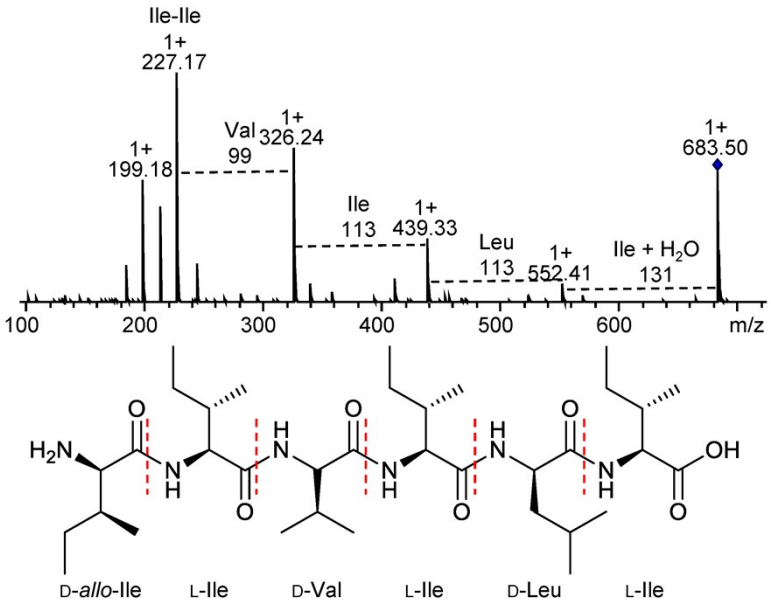

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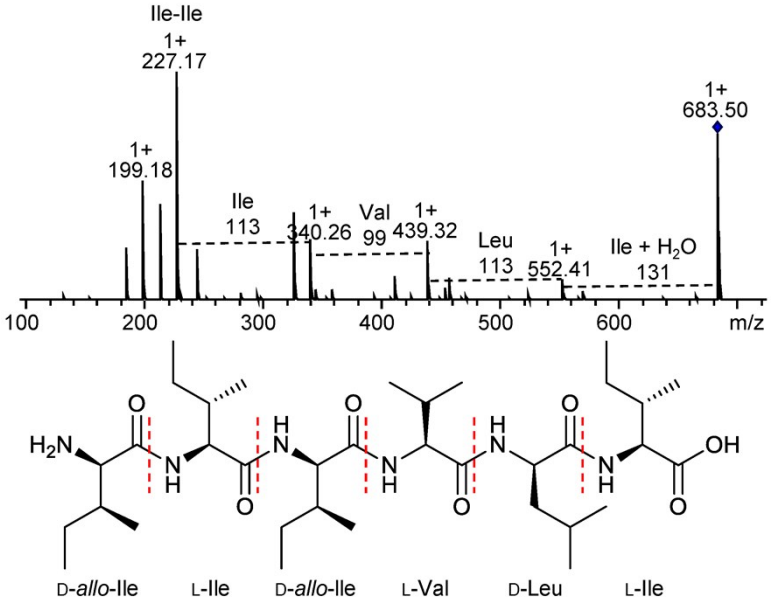

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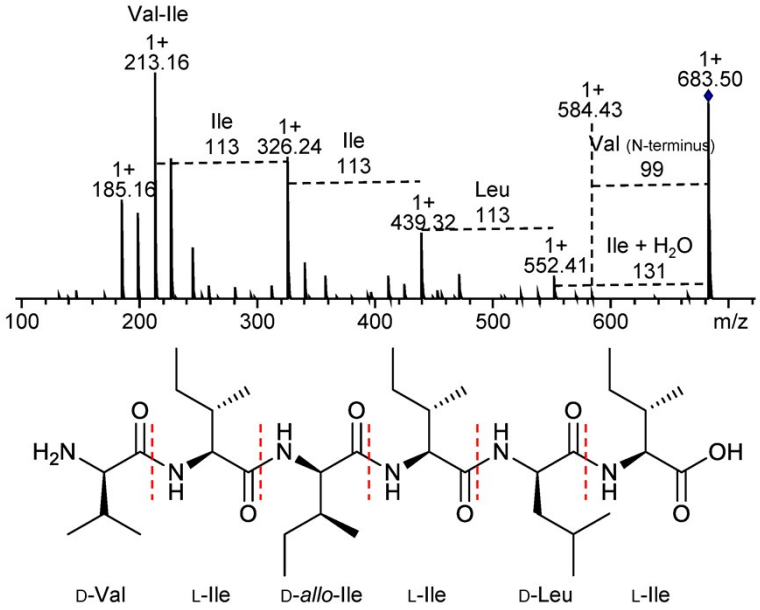
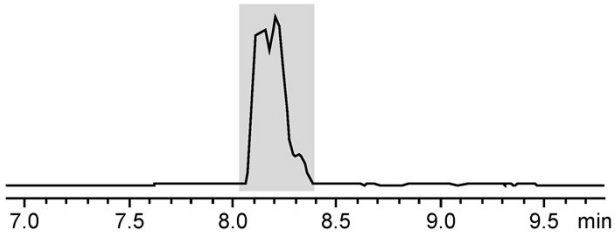


2e

Cluster **3**: *m/z* [M + H]$^+$ 669.49, 1 Leu + 2 Val + 3 Ile



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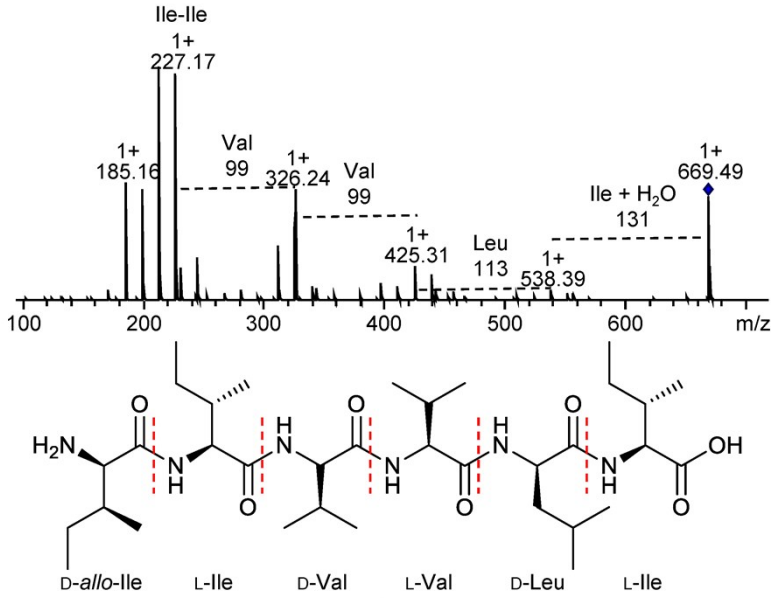

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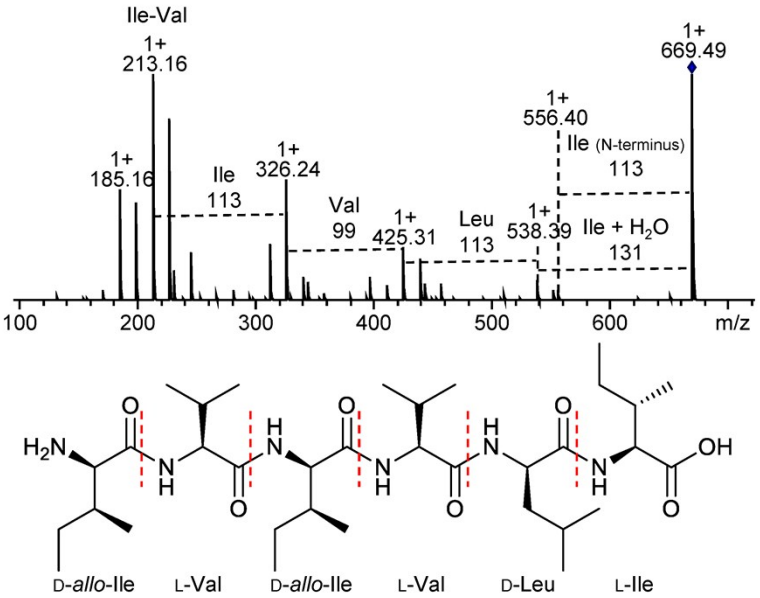

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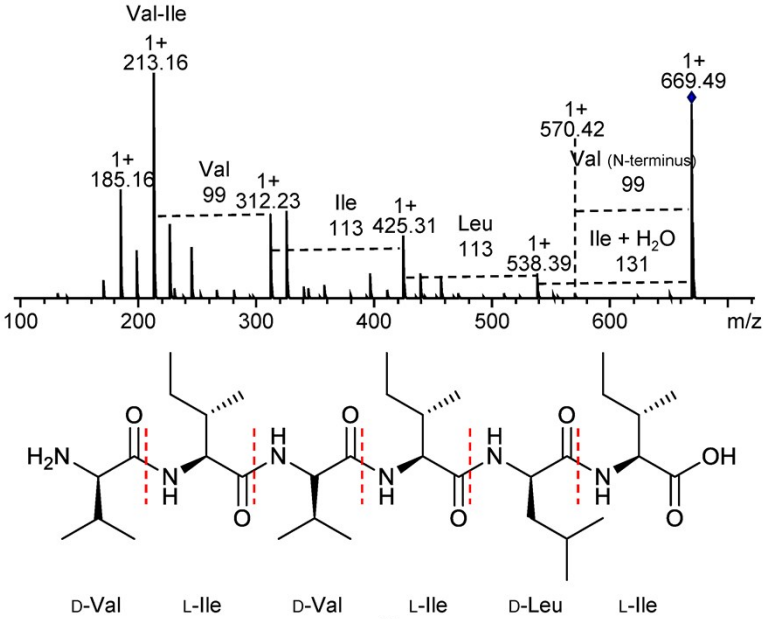

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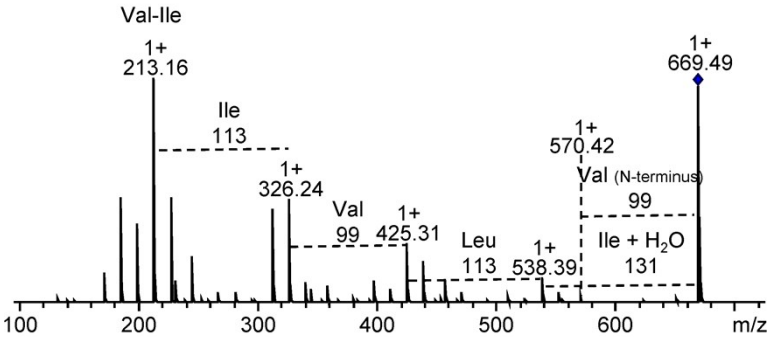
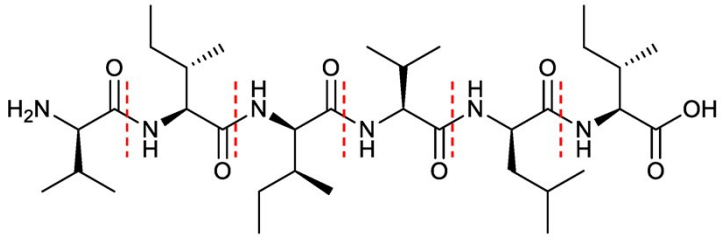
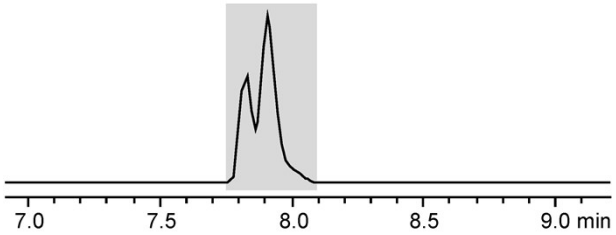




3h

Cluster **4**: *m/z* $[M + H]^+$ 655.47, 1 Leu + 3 Val + 2 Ile



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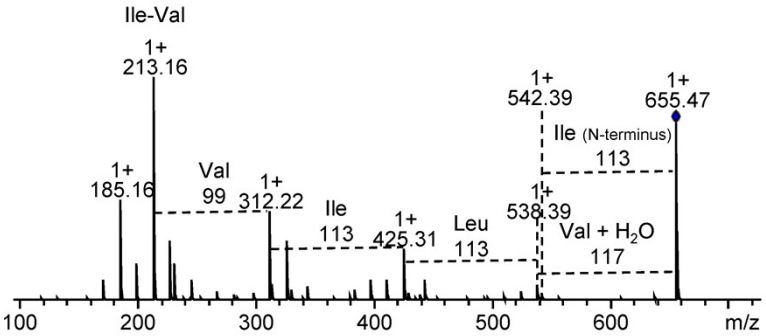

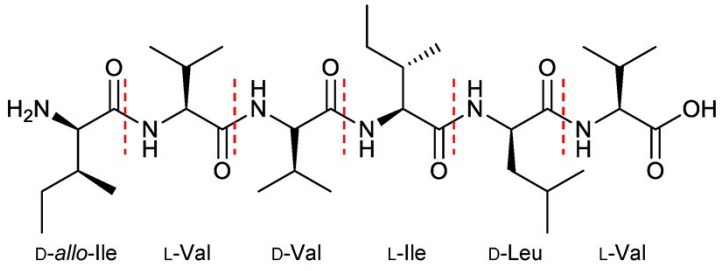

4c

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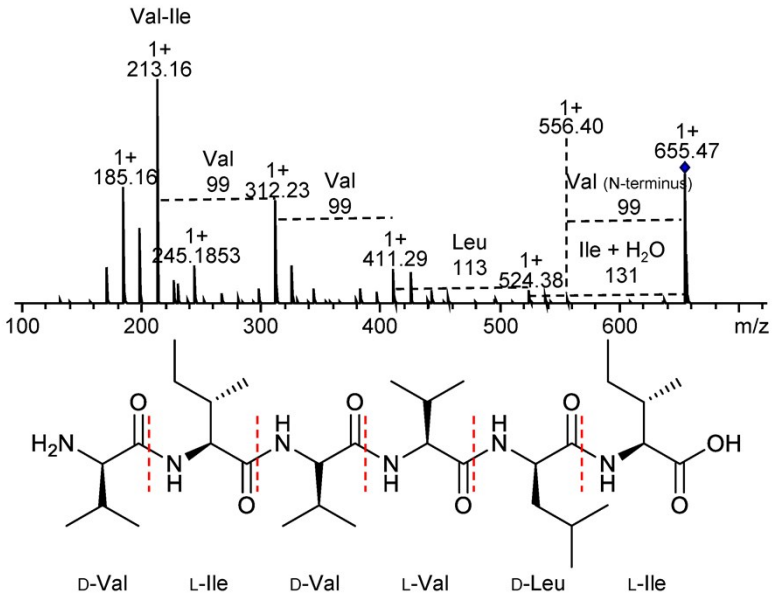
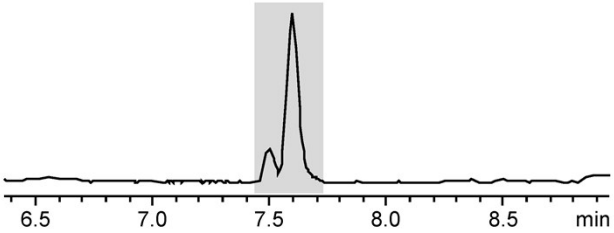
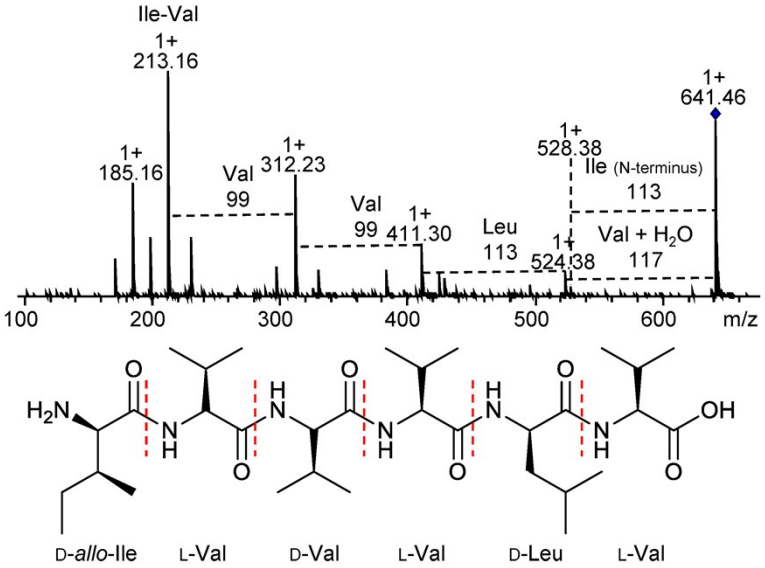


4g

Cluster **5**: *m/z* [M + H]$^+$ 641.46, 1 Leu + 4 Val + 1 Ile

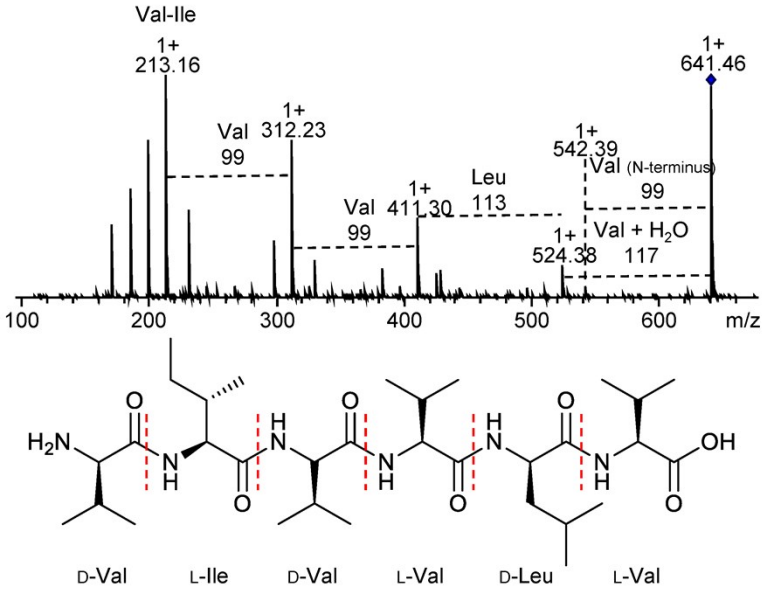


RT = 7.51 min



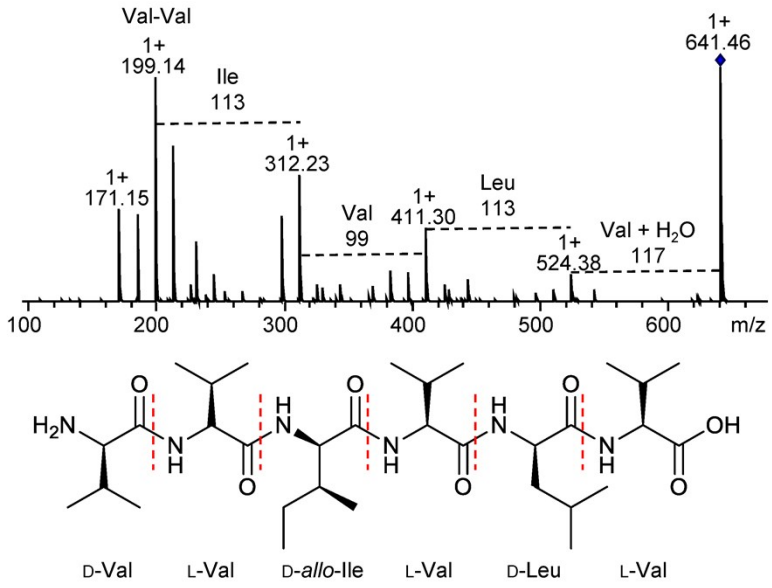
5a

RT = 7.57 min



5b

RT = 7.59 min



5c

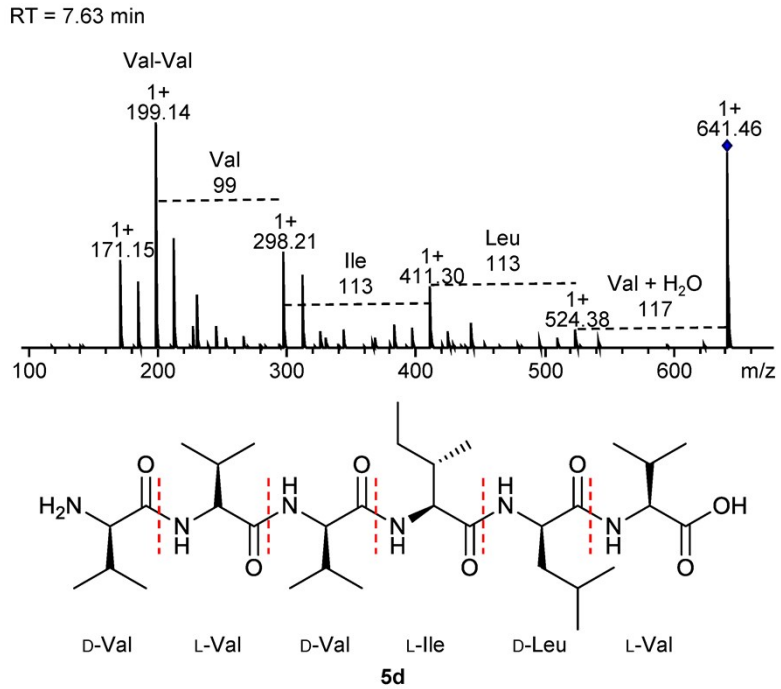


Fig. S7 Structure identification of thirteen compounds in cluster **2**–**5** by HPLC-MS analysis.

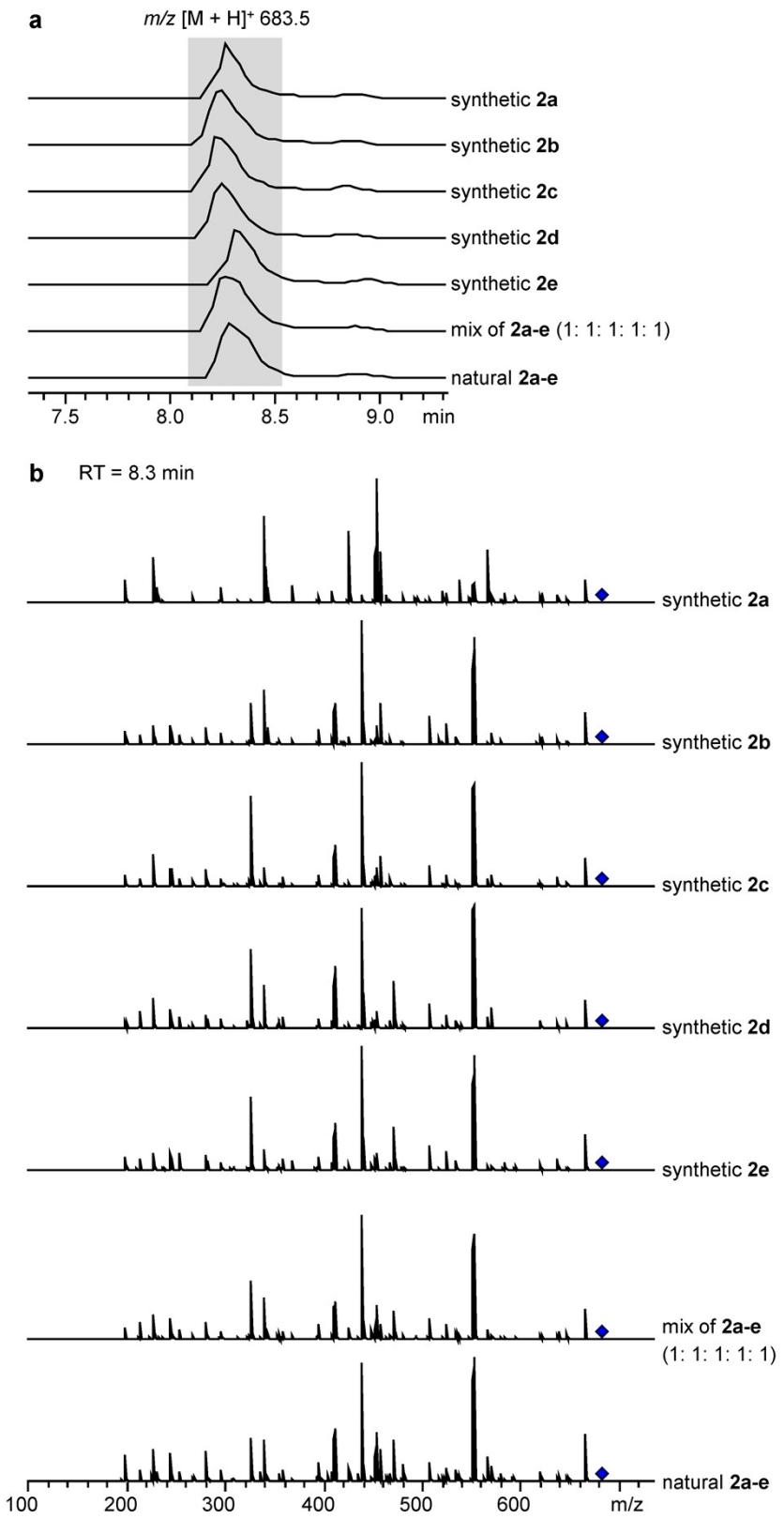


Fig. S8 Comparison of natural and synthetic **2a-e** by HPLC-MS analysis. (**a**) Extracted ion chromatograms (EICs) of natural and synthetic **2a-e**; (**b**) MS^2 fragmentation patterns of natural and synthetic **2a-e** at 8.3 min.

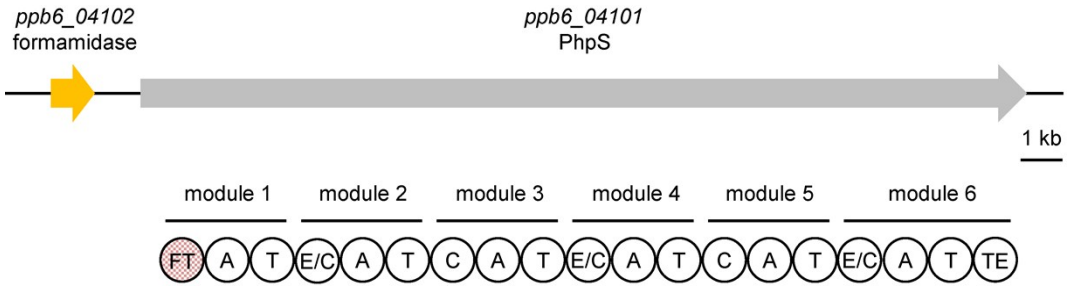


Fig. S9 Formamidase and PhpS NRPS encoding genes with domain organization. Domains: FT: formyl transferase, A: adenylation, T: thiolation, E/C: dual epimerization/condensation, C: condensation, TE: thioesterase.

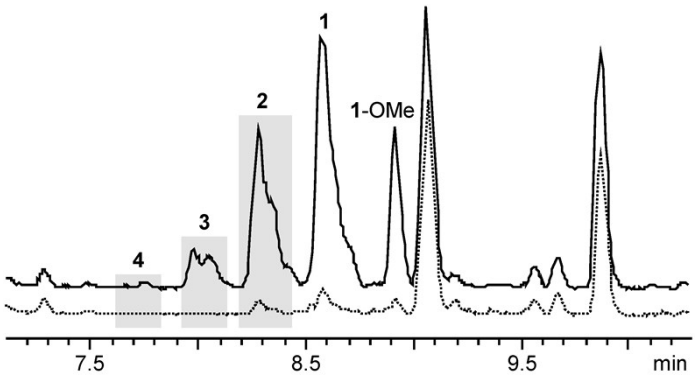


Fig. S10 HPLC-MS analysis of methanol extracts from *E. coli* expressing *phpS* with (continuous line) and without (dotted line) arabinose induction, respectively. Base-peak chromatograms (BPCs) are shown.

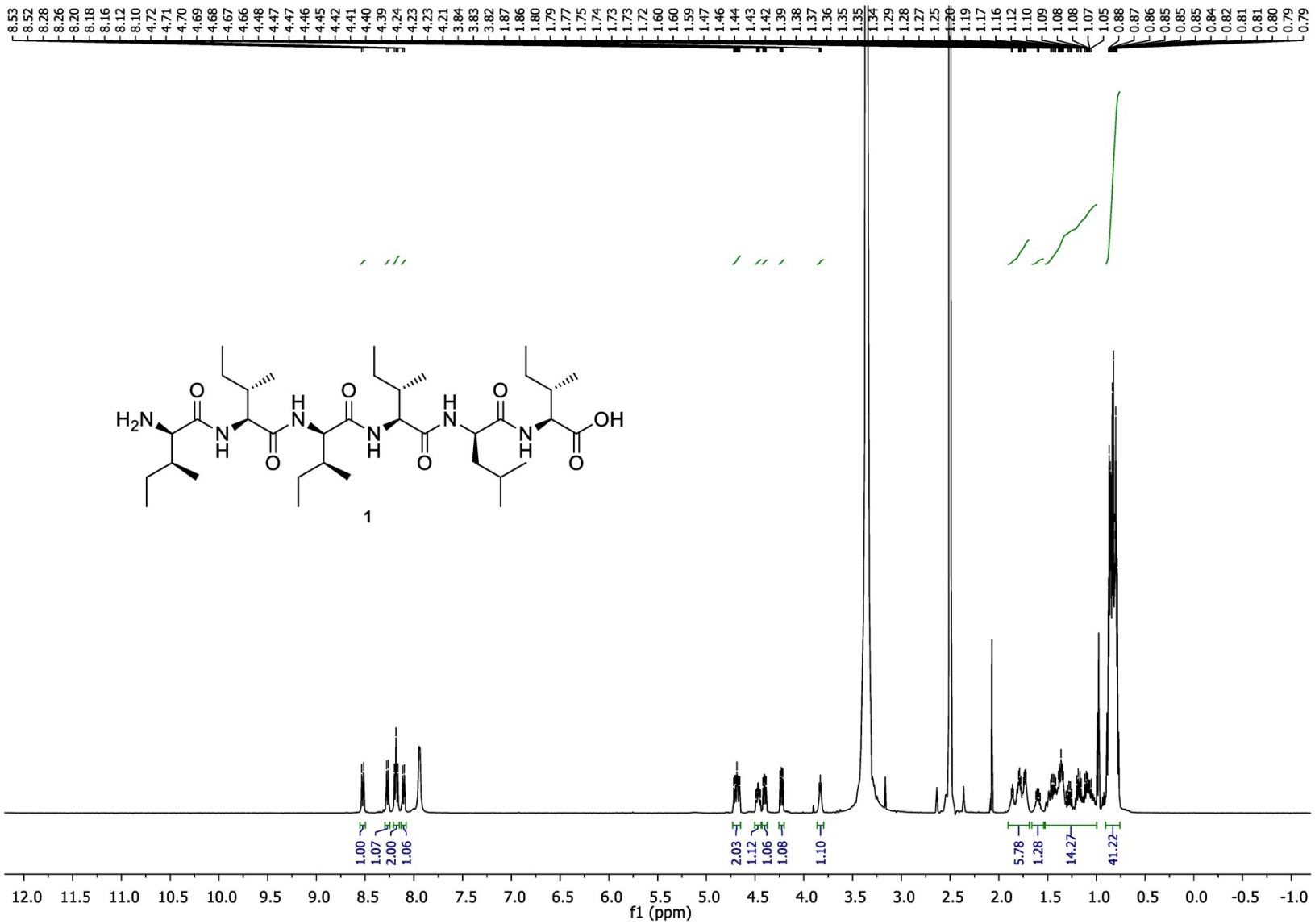


Fig. S11 ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of synthetic **1**

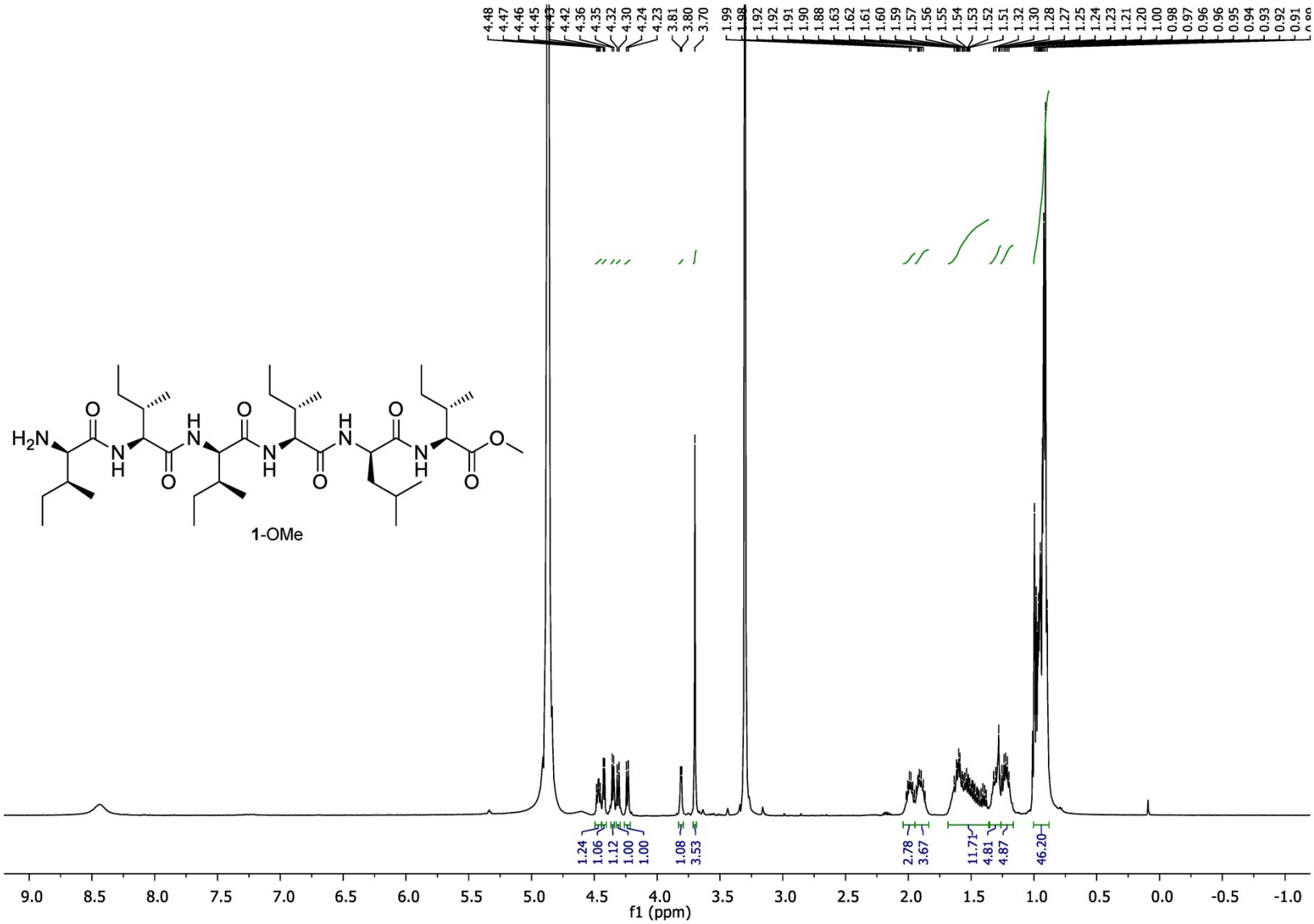


Fig. S12 ^{1}H NMR (500 MHz, methanol-d_4) spectrum of isolated **1**-OMe

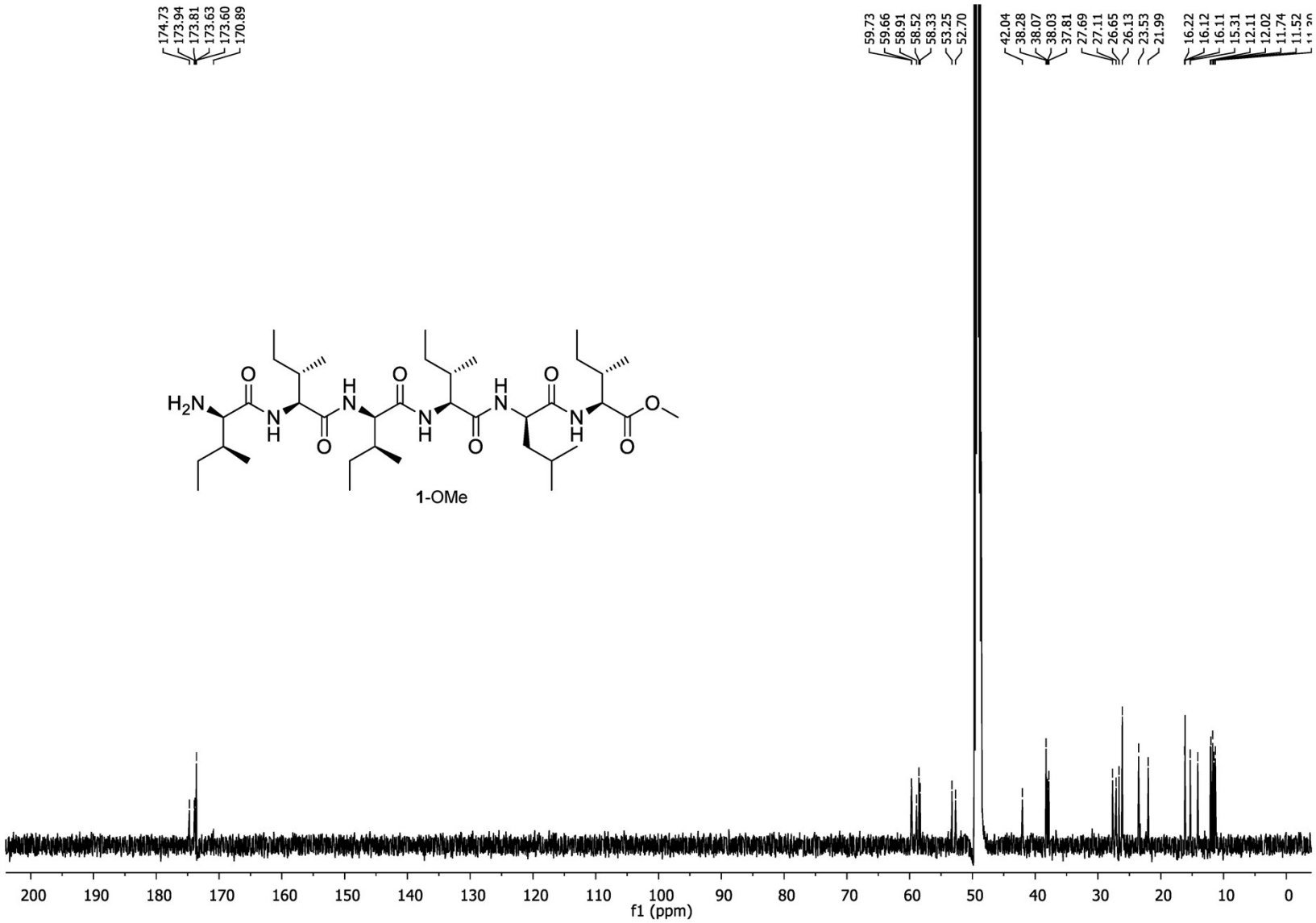


Fig. S13 ^{13}C NMR (125 MHz, methanol-d_4) spectrum of isolated **1**-OMe

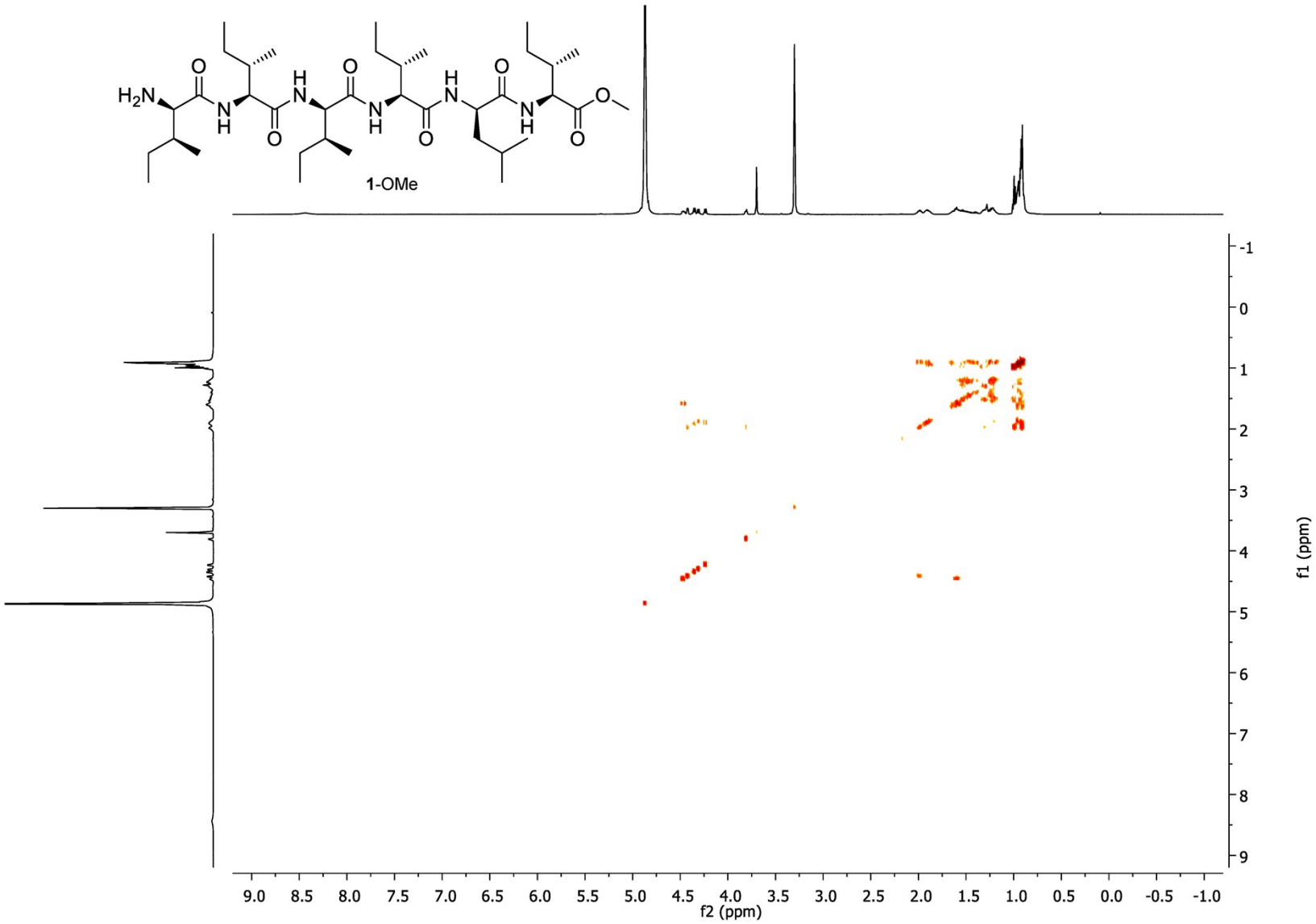


Fig. S14 COSY (methanol-d_4) spectrum of isolated **1**-OMe

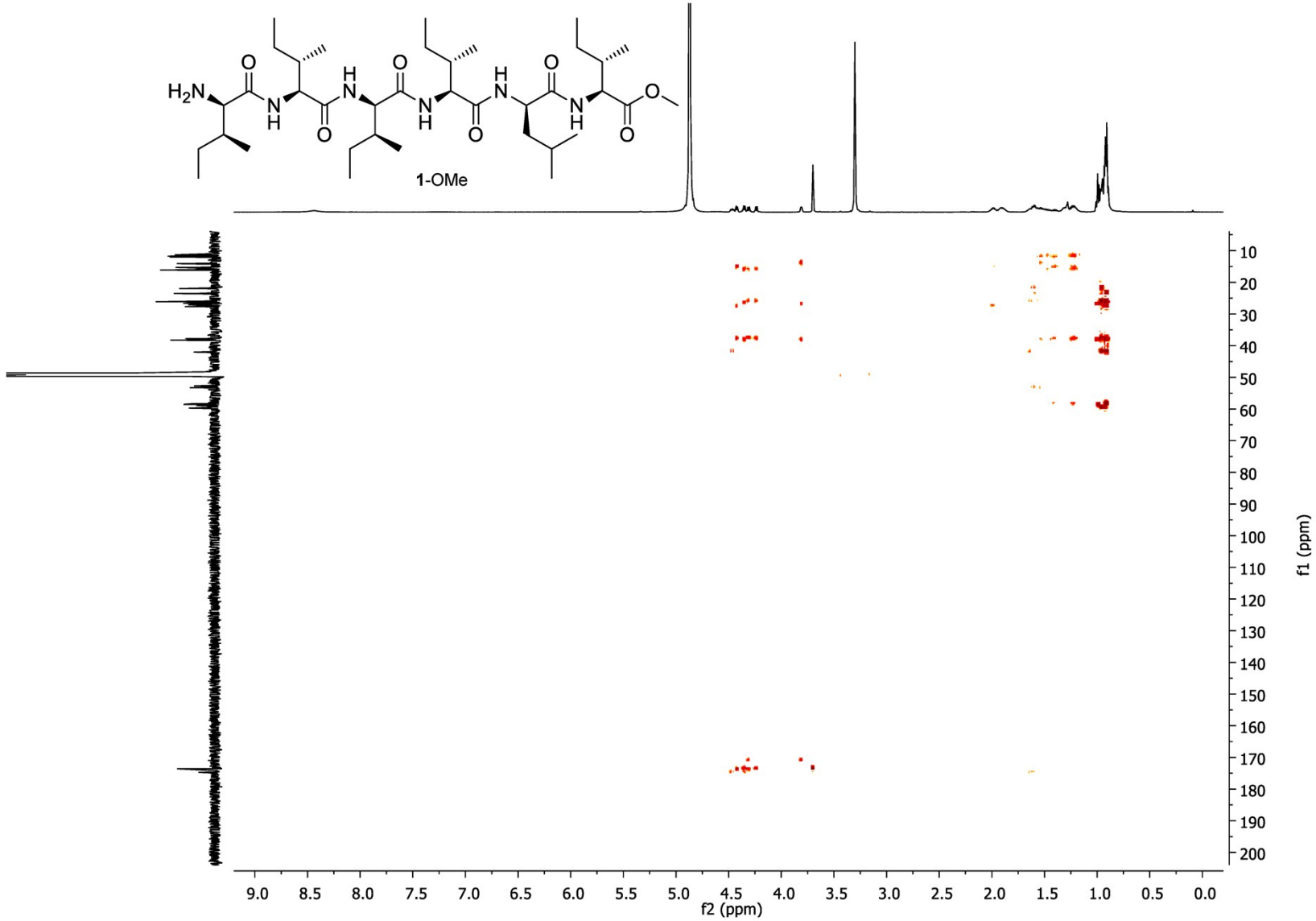


Fig. S15 HMBC (methanol-d_4) spectrum of isolated **1**-OMe

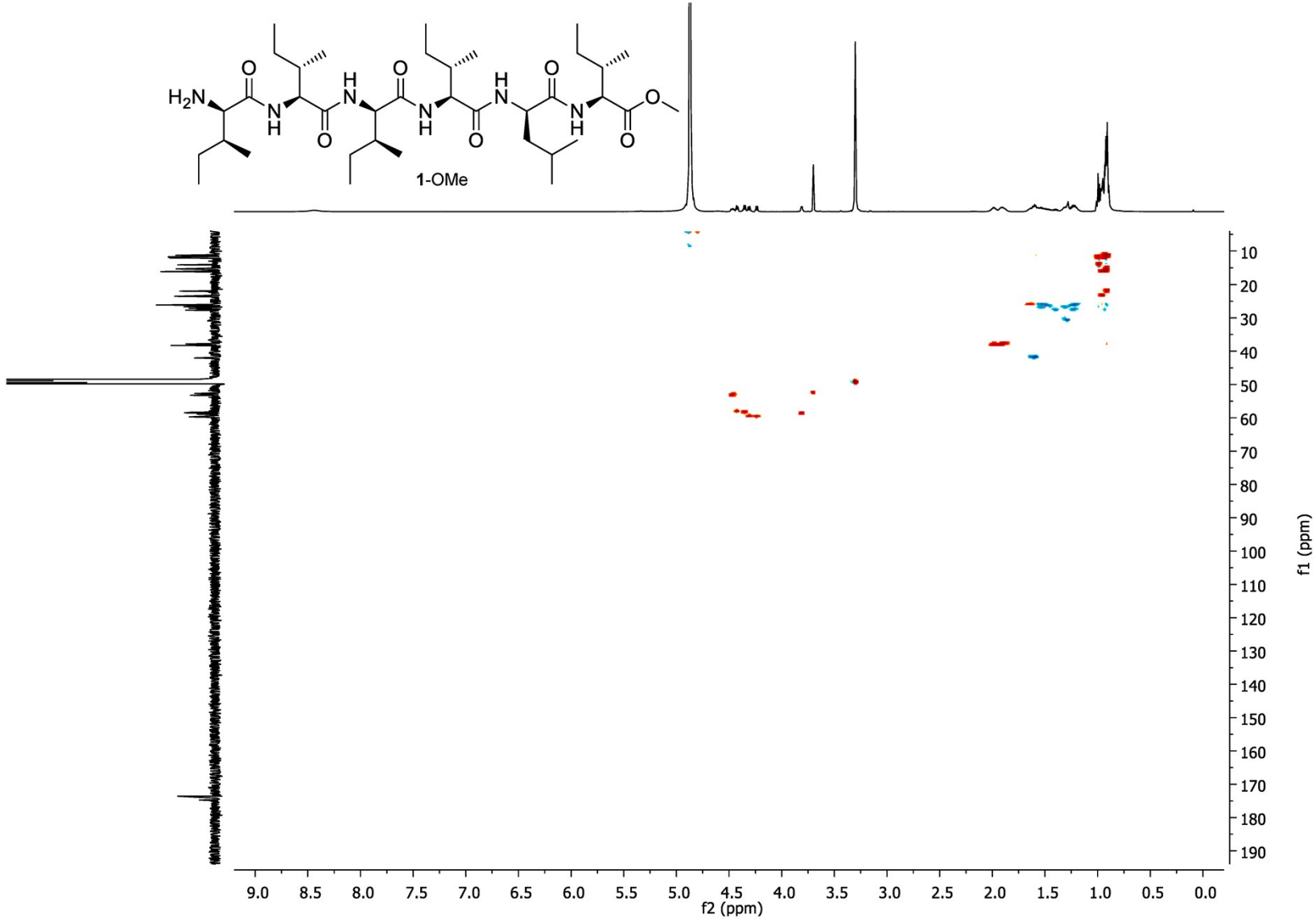


Fig. S16 HSQC (methanol-d_4) spectrum of isolated **1**-OMe

References

1 E. Bode, A. O. Brachmann, C. Kegler, R. Simsek, C. Dauth, Q. Zhou, M. Kaiser, P. Klemmt and H. B. Bode, *ChemBioChem*, 2015, **16**, 1115.

2 O. Schimming, F. Fleischhacker, F. I. Nollmann and H. B. Bode, *Chembiochem*, 2014, **15**, 1290.

3 C. Hacker, X. Cai, C. Kegler, L. Zhao, A. K. Weickhmann, J. P. Wurm, H. B. Bode and J. Wöhnert, *Nat. Commun.*, 2018, **9**, 4366.

4 X. Cai, V. L. Challinor, L. Zhao, D. Reimer, H. Adihou, P. Grün, M. Kaiser and H. B. Bode, *Org. Lett.*, 2017, **19**, 806.

5 S. R, P. U and Puhler A, *Nat. Biotechnol.*, 1983, **1**, 784.

6 C. Kegler, F. I. Nollmann, T. Ahrendt, F. Fleischhacker, E. Bode and H. B. Bode, *Chembiochem*, 2014, **15**, 826.

7 A. Thanwisai, S. Tandhavanant, N. Saiprom, N. R. Waterfield, P. K. Long, H. B. Bode, S. J. Peacock and N. Chantratita, *PLoS One*, 2012, **7**, e43835.

8 K. A. J. Bozhüyük, F. Fleischhacker, A. Linck, F. Wesche, A. Tietze, C.-P. Niesert and H. B. Bode, *Nat. Chem.*, 2018, **10**, 275.