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Supporting Information

Food-Poisoning Bacteria Employ a Citrate Synthase and a Type II NRPS To Synthesize Bolaamphiphilic Lipopeptide Antibiotics**

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Experimental procedures

Bacterial strains and media

Bacterial and fungal strains used in this study are listed in table S1. *Burkholderia gladioli* pv. *cocovenenans* HKI0521 was continuously cultured in MGY+M9, PDB or CYMG medium (Table S2). For long-term storage, the bacteria were frozen with 1:1 volume of 50% glycerol at –80 °C. *Burkholderia* gladioli pv. *cocovenenans* HKI0521 mutant strains were either cultured in MGY+M9 or PDB medium/agar with addition of kanamycin (50 µg mL^{-1}). *E. coli* strains were cultured in LB medium or on LB agar plates at 37 °C with appropriate antibiotic concentrations (kanamycin 50 µg mL^{-1}).

Table S1. Bacterial and fungal strains used in this study.

Bacterial strain	Additional information	Reference
B. gladioli pv. *cocovenenans*	HKI0521	-
B. gladioli DSM4285	ATCC 10248[1]	
Escherichia coli	One Shot™ TOP10 Electrocomp™ *E. coli*	ThermoFisher Scientific

Table S2. Composition of media used in this study.

Medium or *medium additive*	Composition (L^{-1})
MGY+M9 medium	10 g Glycerol, 1.25 g yeast extract (technical yeast extract, BD, Bacto®), 960 mL distilled water, sterilization at 120 °C for 20 min, then add: 20 mL M9 salt A, 20 mL M9 salt B
CYMG	10 g Glycerol, 8 g casein peptone, 4 g yeast extract (technical yeast extract, BD, Bacto®), 6.29 g $MgCl_2$*6 H_2O, sterilization at 120 °C for 20 min
PDB	Potato dextrose broth (BD, Bacto®)
PDA	PDB with addition of 1.5% agar (BD, Bacto®)
LB medium/agar	10 g Tryptone (BD, Bacto®), 5 g yeast extract (BD, Bacto®), 10 g NaCl, sterilization at 120 °C for 20 min; for agar: addition of 1.5% agar
NAG agar	34 g Standard I nutrient agar (Merk KGaA), 10 g glycerol, sterilization at 120 °C for 20 min
NA	Standard I nutrient agar (Merk KGaA)
M9 salt solution A	350 g K_2HPO_4, 100 g KH_2PO_4, sterilization at 120 °C for 20 min
M9 salt solution B	29.4 g Sodium citrate, 50 g $(NH_4)_2SO_4$, 5 g $MgSO_4$, sterilization at 120 °C for 20 min

General analytical methods

LC-MS analysis was performed using an Exactive Orbitrap High Performance Benchtop LC-MS (Thermo Fisher Scientific) with an electron spray ion source and an Accela HPLC System, C18 column (Betasil C18, 150 x 2.1 mm, Thermo Fisher Scientific, Germany), solvents: acetonitrile and water (both supplemented with 0.1% formic acid), flow rate: 0.2 mL min^{-1}; program: hold 1 min at 5% acetonitrile, 1–16 min 5–99% acetonitrile, hold 16–31 min 99% acetonitrile, 31-32 min 99–5%, 32–43 min to 5% acetonitrile. For MS-MS measurements a QExactive Orbitrap High Performance Benchtop LC-MS (Thermo Fisher Scientific) with an electron spray ion source and an Accela HPLC System was used (column: Accucore C18 2.6 µm, 100 x 2.1 mm, Thermo Fisher Scientific solvents: acetonitrile and water (both supplemented with 0.1% formic acid), flow rate: 0.2 mL min^{-1}; program: hold 1 min at 5% acetonitrile, 1–16 min 5–99% acetonitrile, hold 16-31 min 99% acetonitrile, 31–32 min 99–5 %, 32–43 min to 5% acetonitrile. NMR spectra were recorded with a Bruker 500 or 600 MHz Avance III Ultra Shield (Bruker BioSpin GmbH) in DMSO-d_6 ^{1}H 600 MHz; ^{13}C 150 MHz.

Identification, isolation and purification of bolagladin

For isolation of bolagladins, bacteria were cultured in PDB media. 50 mL of PDB medium was inoculated (OD_{600} ~ 0.4) and incubated at 30 °C overnight, at 120 rpm. Fourteen 1 L flasks with 250 mL of PDB each were inoculated with 4 mL of this bacterial preculture and incubated without shaking at 30 °C for 4 d. The cultures were thoroughly extracted twice with 1:1 volume of ethyl acetate. The organic extract was dried with sodium sulfate and concentrated under reduced pressure. The residue was dissolved in methanol and separated by size exclusion chromatography using Sephadex LH-20 (Pharmacia Fine Chemicals) with MeOH as eluent. Bolagladin-containing fractions were concentrated (minimum: 5 mL methanol). Final purification was achieved by preparative reversed-phase HPLC (Shimadzu LC-8A HPLC system) using either a Phenomenex Synergi 4 µm Fusion-RP 80 Å, 250 × 21.2 mm column with gradient MeCN/H_2O supplemented with 0.01% trifluoroacetic acid (*v/v*) (58/42 in 20 min to 80/20, 80/20 in 2 min to 100/0. MeCN 100% for 10 min, flow rate 12 mL min^{-1}).

Absolute configuration of amino acids

Bolagladins were hydrolyzed with 6 M HCl for 12 h at 105 °C and subsequently dried under reduced pressure. After the addition of 100 µL 1 M $NaHCO_3$ and 50 µL 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution (L-FDAA, Marfey reagent, 10 mg mL^{-1} acetone) the samples were heated for 1 h at 50 °C. 50 µl 2 M HCl was added as well as 200 µL 50% (*v/v*) MeCN. The samples were analyzed with an analytical HPLC and a Kinetex 5 µm XB-C18 100 Å 250 × 4.6 µm (Phenomenex) column and a flow rate of 0.5 mL min^{-1}. MeCN and H_2O both supplemented with 0.1% trifluoroacetic acid were used. The gradient can be found in Table S3.

Table S3. Gradient used for Marfey method.

Zeit (min)	dH_2O (%)	ACN (%)
5	70	30
30	50	50
35	0	100
37	70	30
42	70	30

The retention times of derivatized amino acids can be found in Table S4 and Table S5. To distinguish isoleucine and *allo*-isoleucine the samples were treated with L-FDAA 50 µl 1-fluoro-2,4-dinitrobenzol (Sanger reagent) (10 mg mL^{-1} acetone). A Eurospher C18 5 µm 100 Å 250 × 4.6 µm column and an isocratic flow of 40% MeCN in H_2O supplemented with 0.1% trifluoroacetic acid was used.

Table S4. Analysis of the absolute configuration of amino acids bolagladin A (**1**) and B (**2**) using Marfey method.

Amino acid	Configuration	Retention time of derivatized standard amino acid [min]	Retention time of derivatized amino acid in bolagladin A (**1**) [min]	Retention time of derivatized amino acid in bolagladin B (**2**) [min]
Serine	D	11.6	11.4 (L)	11.4 (L)
	L	11.4		
Valine	D	30.4	30.4 (D)	-
	L	25.3		
Homoserine	D L	12.0 and 12.4	12.0 (L)	12.0 (L)
	L	12.0		
Isoleucine	D	35.1	-	35.1 (D)
	L	29.8		

Table S5. Analysis of the configuration of Isoleucine in bolagladin B (**2**) using Sanger reagent.

Amino acid	Configuration	Retention time of standard amino acid [min]	Retention time of amino acid in bolagladin B (**2**)[min]
Isoleucine	D	25.5	25.5 (D)
	D-*allo*	26.5	

Comparison of the retention times of the standard amino acid and the samples allowed for identification of the amino acid building blocks of bolagladin A (L-serine, D-valine, L-

homoserine and L-serine) and bolagladin B (L-serine, D-*allo*-isoleucine, L-homoserine and L-serine). This is in accordance with the predictions based on the Stachelhaus code.

Sequencing and annotation of bolagladin gene cluster

The bolagladin gene cluster (*bol*) was uploaded to the NCBI database, accession number: (MT844061). The webtool antiSMASH 5.0[2] was used to for the identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in the genome of *Burkholderia gladioli* pv*. cocovenenans* HKI0521. The bolagladin gene cluster consists of a 13.6 kb NRPS gene that contains four modules (Table S6). The predicted amino acid and the Stachelhaus code were taken from the antiSMASH analysis.

Table S6. Prediction of the NRPS modules of BolH according to antiSMASH 5.0; M – Module; C – condensation, A – adenylation, T – peptidylcarrier domain, TE – thioesterase, X – no prediction available, Ser – serine, Hse – homoserine, Val - valine.

Modules of BolH	**Specificity code (antiSMASH)**	**Predicted amino acid (Stachelhaus code match)**	**Amino acid sequence of bolagladin A (1)**
M1 (C-A-T)	DVWHMSLVDK	Ser (100%)	Ser
M2 (C-A-T)	DALWMGGVFK	Val (80%)	Val
M3 (C-A-T)	DLKNVGSDVK	X	Hse
M4 (C-A-TE)	DVWHVSLIDK	Ser (100%)	Ser

NCBI Blast and HHpred[3] were used for sequence database searching to find homologous genes or proteins that allow for speculations on their functionalities. A promotor search was conducted using PromotorHunter (http://www.phisite.org/promoterhunter). Based on the calculated Gibbs energy and a position-specific weight matrix, a scoring system was used to predict putative promotor sites.

Matrix (–35 region):

A: 10 6 9 56 21 54

C: 10 7 12 17 54 13

G: 10 8 61 11 9 16

T: 70 79 18 16 16 1782

Matrix (–10 region):

A: 5 76 15 61 56 6

C: 10 6 11 13 20 7

G: 8 6 14 14 8 5

T: 77 12 60 12 15

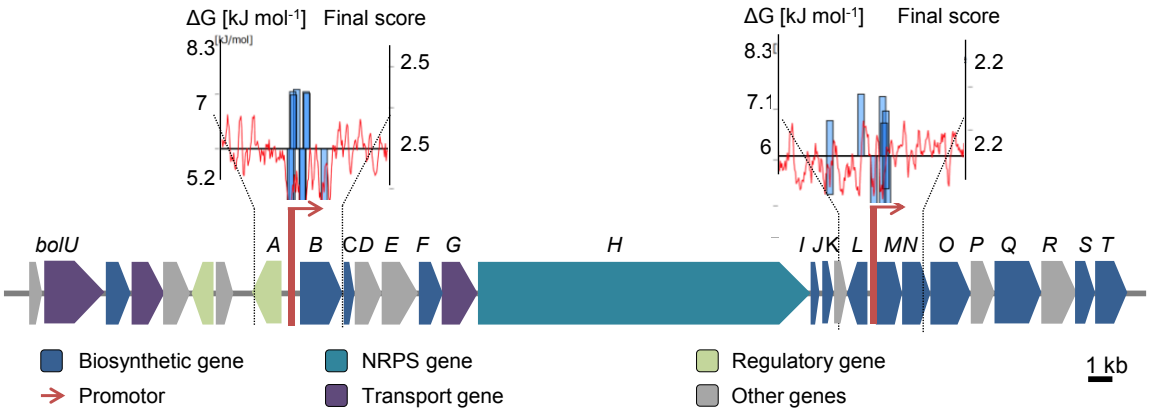

Figure S1: Bolagladin biosynthetic gene cluster (*bol*).

Further information on the genes respectively encoded proteins can be found in Table S7.

Table S7. Proteins encoded up- and downstream of *bolH*.

Gene	Length [AA]	Putatively encoded protein	Closest characterized orthologous protein and (organism)	Accession number	Identity/ similarity
bolU (+13)	804	TonB-dependent Rezeptor	Enterobactin-iron-transporter (*Pseudomonas aeruginosa*)	Q05098.1	22%/37%
bolV (+12)	333	Ribokinase	Ribokinase (*Homo sapiens*)	Q9H477.1	38%/54%
bolW (+11)	296	Transcriptional regulator	Deoxyribose operon repressor (*Escherichia coli* O157)	P0ACK7.1	43%/57%
bolX (+10)	226	/	Deoxyribose-phosphate aldolase (*Burkholderia lata*)	Q39NL8.1	85%/90%
bolY (+9)	140	Putative plasmid stability protein	VapC ribonuclease (*Sinorhizobium fredii*)	P55511.1	32%/48%
bolZ (+8)	54	/	/	/	/
bolA (+7)	54	HTH-type transcriptional regulator	Transcriptional activator (*Pseudomonas aeruginosa* PAO1)	P24734.3	36%/49%
bolB (+6)	583	Long-chain-fatty-acid-CoA ligase	Long-chain-fatty-acid ligase (*Mycobacterium tuberculosis*)	P0A4X9.1/	37%/48%
bolC (+5)	82	ACP	Acyl carrier protein (*Nitrosomonas europaea*)	2LKI_A	15%/18%
bolD (+4)	352	Oxygenase/ reductase	p-Aminobenzoate N-oxygenase (*Streptomyces thioluteus*)	3CHH_B	15%/4%
bolE (+3)	480	Dehydrogenase, putative oxidoreductase	D-2-hydroxyglutarate dehydrogenase (*Oryza sativa*)	Q7XI14.1	33%/50%
bolF (+2)	343	Desaturase	Sphingolipid Δ(4)-desaturase/C4-monooxygenase DES2 (*Homo sapiens*)	Q6QHC5.2	28%/41%
bolG (+1)	441	Major facilitator superfamily (MFS)	MFS-type transporter MT0042 (*Mycobacterium tuberculosis*)	P9WJY0.1	28%/28%
*bol*H (0)	4,547	NRPS	Tyrocidine synthase 3 (*Brevibacillus parabrevis*)	O30409.1	35%/51%

Table S8. Proteins encoded up- and downstream of *bolH* (continued).

Gene	Length [AA]	Putatively encoded protein	Closest characterized orthologous protein and (Organism)	Accession number	Identity/ similarity
bolI (-1)	69	Domain binding protein	MbtH-Homolog (*Geobacillus* sp. Y4.1MC1)	5U89_B	40%/90%
bolJ (–2)	140	/	/	/	/
bolK (-3)	162	/	/	/	/
bolL (–4)	281	Reductase/ desaturase	Possible acyl desaturase (*Mycobacterium tuberculosis*)	1ZA0_A	19%/20%
bolM (–5)	364	Transferase	Alkyldiketide–CoA synthase; polyketidesynthase (*Tetradium ruticarpum*)	5WX6_A	
bolN (–6)	392	Decarboxylase	Diaminopimelate decarboxylase (*Actinosynnema pretiosum* subsp. *auranticum*)	O69203.1	28%/36%
bolO (–7)	527	Ligase/A-domain	Long chain fatty acid-CoA ligase (*Bacillus subtilis*)	O07610.2	29%/44%
bolP (–8)	260	Fatty acid biosynthesis	3-Oxoacyl-acyl-carrier-protein synthase III (*Haemophilus influenzae*)	3IL3_A	17%/11%
bolQ (–9)	615	Acyl-CoA dehydrogenase	Dehydrogenation (*Roseovarius nubinhibens* ISM)	6IJC_B	29%/48%
bolR (–10)	459	Citrate synthase	Citrate synthase (*Burkholderia thailandensis*)	4XGH_A	20%/18%
bolS (–11)	220	Methyltransferase	SAM–dependent methyltransferase (*Thiobacillus denitrificans*)	5EPE_A	16%/16%
bolT (–12)	357	Dioxygenase/ Decarboxylase	Clavaminate synthase- Protein ScoE Protein (*Arabidopsis thaliana*)	2Q4A_A/	30%/54%

Phylogenetic analysis of C domains from the bolagladin gene cluster

C domain amino acid sequences of the bolagladin biosynthesis gene cluster as well as sequences acquired from the NaPDos website[4] were aligned using MAFFT[5] with default settings. Maximum likelihood phylogeny was constructed using IQ-tree[6]. Ultrafast bootstrapping (1000 iterations) analysis was performed. The neighbor-joining tree construction method showed similar tree topology. The phylogeny indicates the functionalities of the subjected C domains and allows a proposal for the biosynthetic logic of bolagladin.

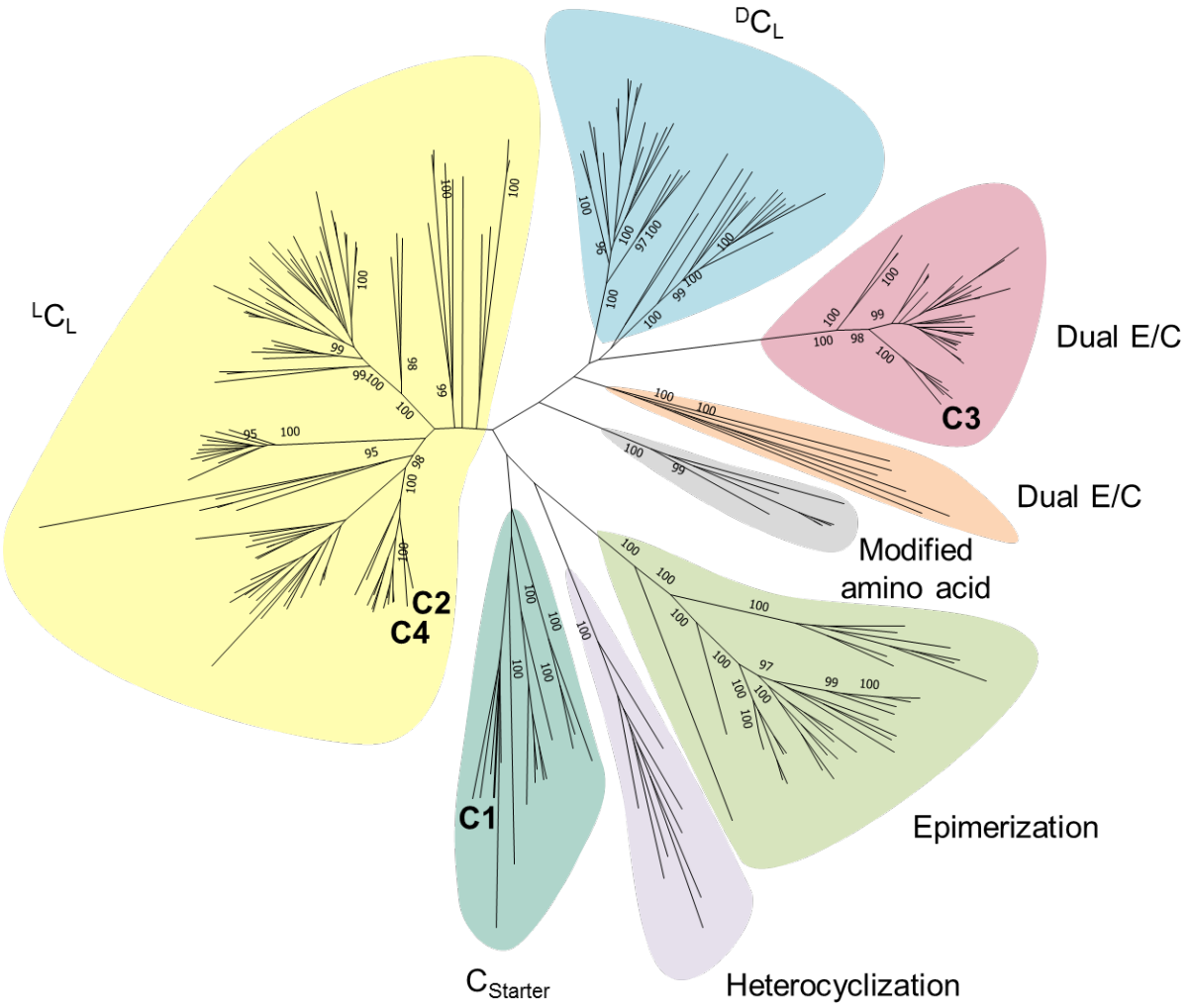

Figure S2. Phylogenetic analysis of C domains from BolH and from the NaPDos website.
C domains cluster into the canonical subfamilies ($^{L}C_{L}$, $^{D}C_{L}$, starter, dual E/C, epimerization, heterocyclization domains and modified amino acid domain).

Phylogenetic analysis of the citrate synthase BolR

Characterized (bold) and non-characterized *Re*- and *Si*-citrate synthases amino acid sequences were downloaded from Uniprot database. Additionally, three proteins annotated as citrate synthases encoded in the genome of *B. gladioli* pv. *cocovenenans* were added, one of which was encoded in the bolagladin biosynthesis gene cluster. Citrate synthase proteins from the phomoidride[7], maleidrides[8] and squalestatin[9] biosynthesis pathways were added. Malate synthesis amino acid sequences were added as outgroup. The

sequence alignment was performed using MAFFT 7 with L-INS-I, an iterative refinement method. Maximum likelihood phylogeny was constructed using IQ-tree. Ultrafast bootstrapping (1000 iterations) analysis was performed. The bolagladin citrate synthase (BolR), as well as the CS-phomoidride, CS-maleidrides and CS-squalestatin clusters with *Si*-citrate synthases. *Re*-CS cluster next to 2-isopropylmalate (EC 2.3.3.13), citramalate synthases (EC 2.3.3.14) and are sometimes wrongly annotated such because they have a high sequence similarity. Notable, citramalate synthase or 2-isopropylmalate synthases are *Re*-face stereospecific with respect to C-2 of their substrates, 2-oxoglutarate and 2-oxo-3-methylbutyrate.[10] Inspection of the multiple sequence alignment of BolR shows conservation of reported, catalytically active residues His302, His350 and Asp356 (Figure S4).

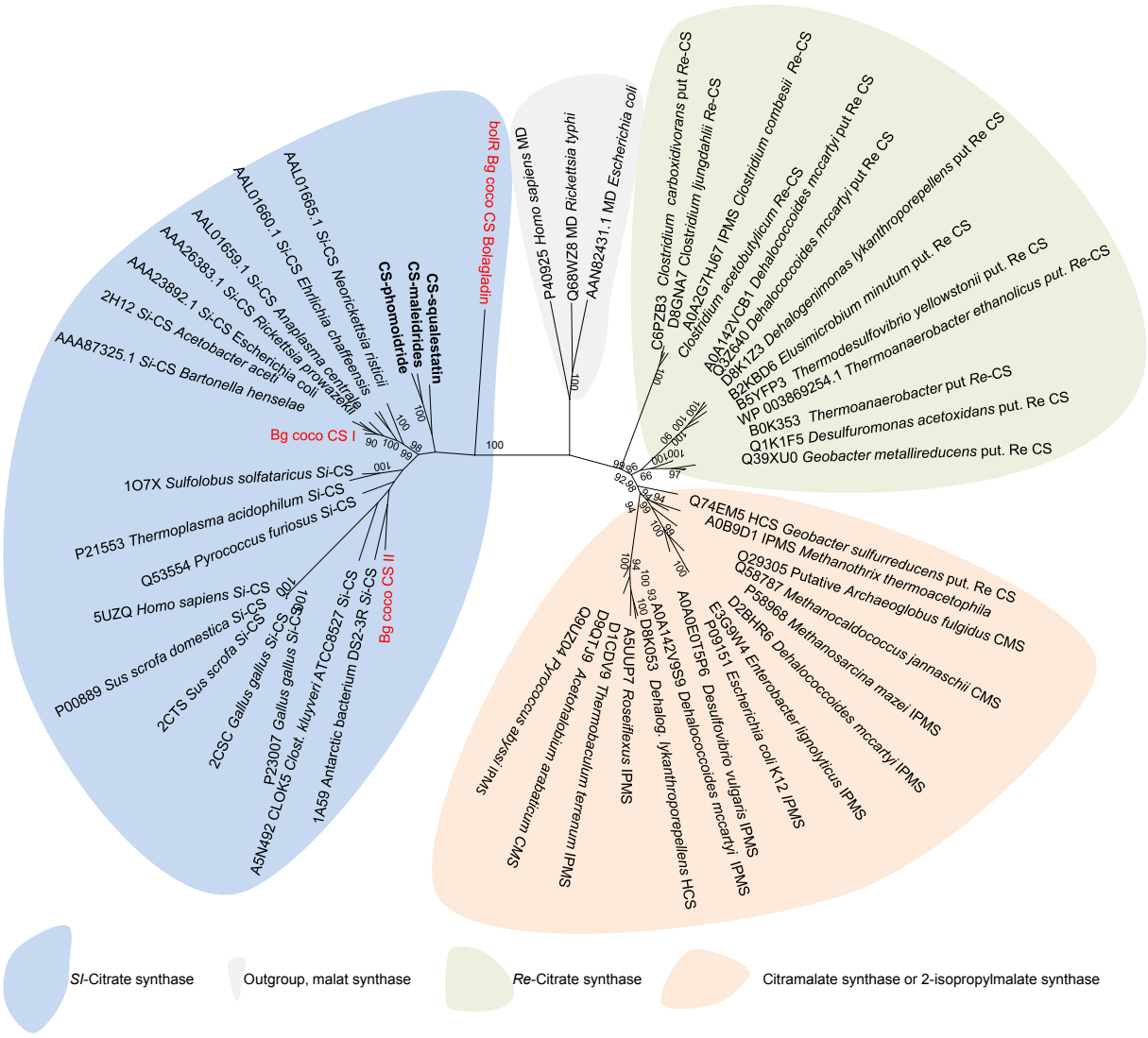

Figure S3. Phylogenetic analysis of *Si*- and *Re*-citrate synthase (CS) proteins.
Citramalate synthase (CMS), homocitrate synthase (HCS) and 2-siopropylmalate synthase (IPMS) proteins are included. Malate synthase are used as outgroup. Alignment and construction of the phylogenetic tree was performed using MAFFT 7 respectively IQ-Tree. Malate synthases were chosen as an out group. Ultrafast bootstrapping (1000 iterations) analysis was performed. Red characters, *B. gladioli* pv. *cocovenenans* CS; green characters CS from other secondary metabolite pathways, bold characters in vitro characterized proteins.

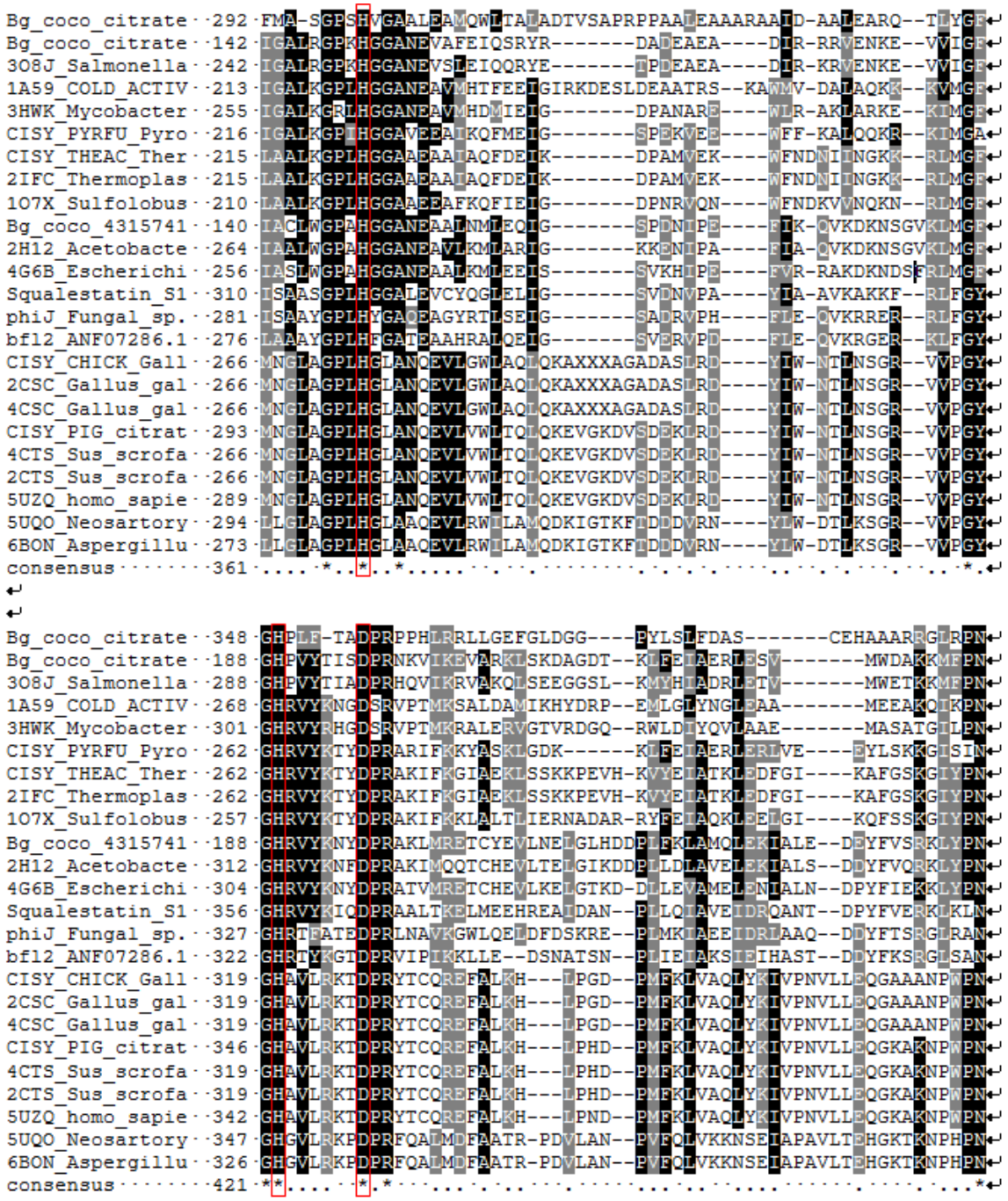

Figure S4. Extract of the multiple sequence alignment of citrate synthase amino acid sequences confirms conservation of catalytically active residues in BolR (marked with red boxes).

Strains containing the bolagladin gene cluster

Table S9. Genomes of *Burkholderia* spp. that contain the bolagladin NRPS gene cluster.

Organism	Origin	Accession number
B. gladioli pv. *cocovenenans* HKI0521	Contaminant in fungal culture in the fermentation of coconut patties	DSM11318
Burkholderia gladioli AU26456	Sputum, *Homo sapiens*	NZ_PVGZ01000021.1
Burkholderia gladioli AU30473	Sputum, *Homo sapiens*	NZ_PVHI01000143.1
Burkholderia gladioli AU29541	Sputum, *Homo sapiens*	NZ_PVHE01000070.1
Burkholderia gladioli 3723STDY6437372	*Homo sapiens*, cystic fibrosis, USA	NZ_UWYX01000001.1
Burkholderia gladioli BSR3	Diseased rice sheath (South Korea)	NC_015376.1
Burkholderia gladioli 3723STDY6437373	Clinical; Pathogen (United Kingdom)	NZ_UWYW01000003.1
Burkholderia gladioli Co14	Fermented corn meal (Heilongjiang Province)	NZ_CP033431.1
Burkholderia gladioli AU0032	Sputum, *Homo sapiens*	NZ_PVFC01000046.1
Burkholderia gladioli MSMB1756	Soil	NZ_LOZK01000085.1
Burkholderia BCC238	Clinical	NZ_UZVS01000001.1
Burkholderia gladioli pv. *gladioli* FDAARGOS_188	-	NZ_CP022210.1
Burkholderia gladioli NCTC12378	Plant (*Gladiolus* species)	NZ_UARB01000021.1
Burkholderia gladioli pv. *gladioli* KACC 11889	*Gladiolus*, (South Korea)	NZ_CP022006.1
Burkholderia gladioli ATCC 10248	Plant	NZ_CP009322.1
Burkholderia gladioli NBRC 13700	Free living	NZ_BBJG01000123.1
Burkholderia gladioli ATCC 25417	Leaves, *Gladiolus*	NZ_KN150850.1

Gene cluster comparison using EasyFig2.3

The sequence similarity of *bol* gene clusters is visualized using the tool Easyfig 2.3.[11] Color code represents the sequence similarity values as indicated (Figure S5).

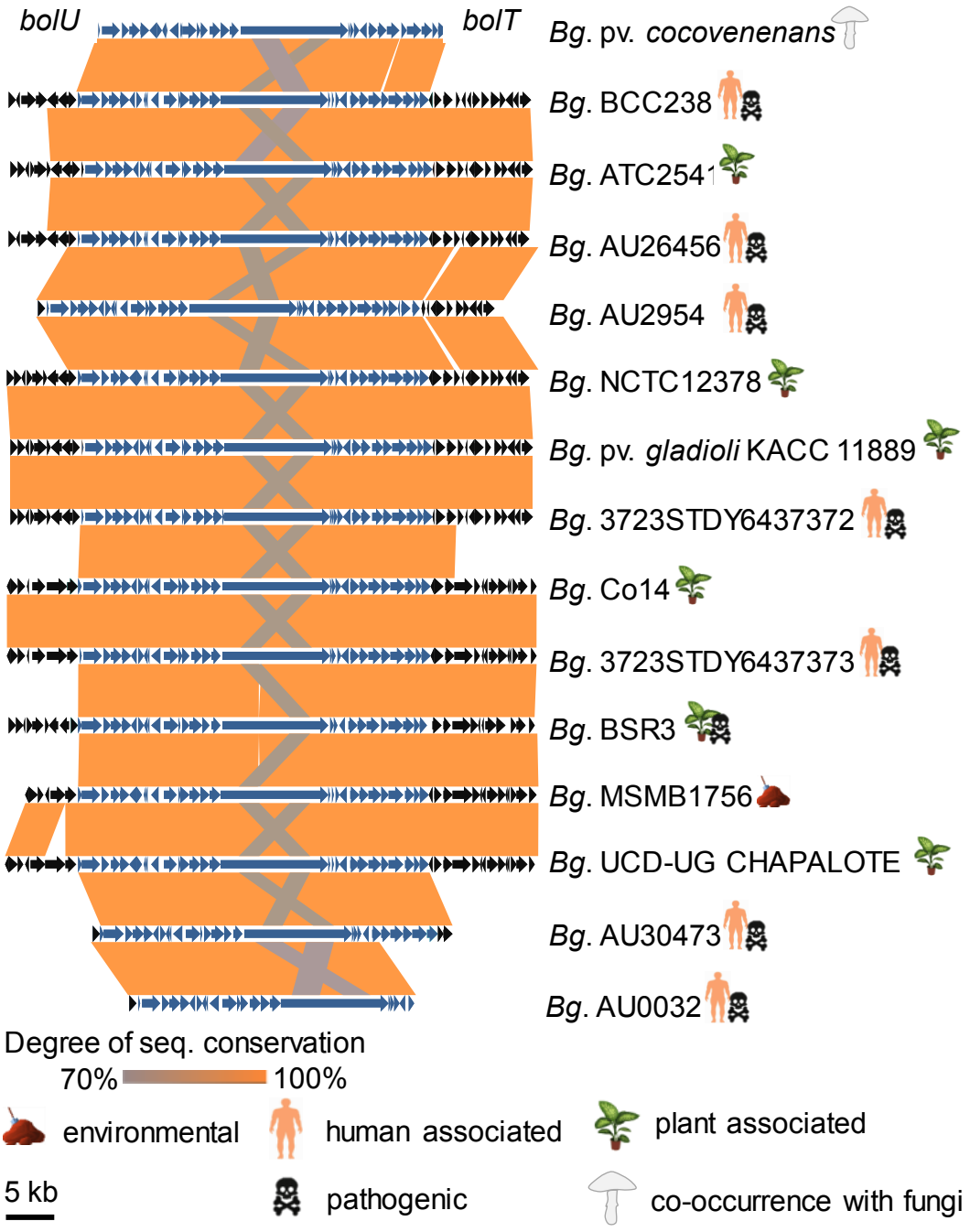

Figure S5. Comparison of *bol* gene clusters from *Burkholderia gladioli* (*Bg.*) spp. isolated from different sources. Genes shared by all listed *bol* gene clusters are represented by blue arrows, others are depicted in black.

Genetic manipulation of *Burkholderia gladioli* pv. *cocovenenans* HKI0521

Burkholderia gladioli was genetically manipulated by homologous recombination to inactivate genes putatively involved in the biosynthesis of bolagladin. Homologous regions encoded on a suicide plasmid were recombined with the genome of the bacteria leading to the integration of a kanamycin resistance cassette and thereby rendering the target gene inactive.

Construction of the knockout plasmids

All experiments have been performed according to manufacturer's recommendations if not stated otherwise. Genomic DNA isolation of bacteria was performed using PureTM DNA-Isolation kit (Epicentre Biotechnologies). PCRs have been performed as follows: *Burkholderia gladioli* HKI0521genomic DNA, 2x master mix of KAPA2G Robust HotStart ReadyMix PCR kit (Merck KGaA) and 35 cycles of 95 °C for 30 s, 60 °C for 30 s kb^{-1}, and a final extension time at 72 °C for 30 s kb^{-1}. The following cloning strategies have been used to construct the knockout plasmid:

I: Amplification of homologous regions and subsequently restriction to introduce a kanamycin resistance cassette, construction of pJB02, pJB04, pJB06 and pJB14. Primers listed in table S10 have been used in a PCR to amplify homologous regions from *Burkholderia gladioli* genomic DNA. The PCR product was blunt-end ligated into pJET1.2 linear vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific). The resulting plasmids as well as the kanamycin resistance cassettes amplified from pGEM-Kan[12] with the listed primers have been digested with the indicated restriction enzymes (Table S10).

Table S10. Detailed information on plasmids constructed using cloning strategy I.

Plasmid (target gene)	Name and sequence 5' → 3'	Product size and name	Digestion enzyme
pJB02 (NRPS gene, *bolH*)	JB01, ccagttcgtcctcgctgtag JB02, cctctacctgctggccttc	2,299 bp, homologous region	*Eco*RI
	JB03, cgtggaattcgtaagcttaggctgctgcc JB04, cgtggaattctcagaagaactcgtcaag	1,182 bp, KanR	-
pJB04 (fatty acid ligase gene, *bolO)*	JB07, cttctcgtcgagcttgttcc JB08, gcctcgactacgaggtgttc	2,371 bp, homologous region	*Sac*I
	BD247, cggacgagctcgtaagcttaggctg BD248, cgcgcgagctctcagaagaactcgtcaag	1,162 bp, KanR	-
pJB06 (desaturase gene, *bolF*)	JB11, cgcacatcgacatgaagc JB12, tctcaagcagctgttcatcg	2,071 bp homologous region	*Bam*HI
	BD357, ctcgggatccgtaagcttaggctgctgcc BD358, tcaaggatcctcagaagaactcgtcaag	1,162 bp KanR	-
pJB14 (fatty acid ligase gene, *bolB*)	BD406, gaacttgtggcggtgcag BD407, gcgatgttcgacggctatc	2,032 bp homologous region	*Sph*I
	BD408, gccggcatgctcagaagaactcgtcaag BD409, aagcgcatgctcagccaatcggatgaatg	1,088 bp, KanR	-

II: NEBuilder mediated fusion of two homologous regions with a resistance cassette and subsequent pJet1.2 cloning, construction of pJB10, pJB12, and pBD87.

Primers listed in table S11 were used to amplify homologous regions from genomic DNA of *Burkholderia gladioli* pv. *cocovenenans* respectively the kanamycin resistance cassette from pGEM-Kan. In a subsequent step a NEBuilder three-fragments ligation have been performed (New England Biolabs, Frankfurt am Main) according to the manufacture's

recommendations. Finally, the ligated DNA fragments have been blunt-end ligated into pJET1.2 linear vector yielding the final knockout plasmids.

Table S11. Detailed information plasmids constructed using cloning strategy II.

Plasmid (target gene)	Name and sequence of primers used 5' → 3'	Product size and name
pJB10 (Thiolation domain gene, *bolC*)	JB27, ctcctttggattcctgatcg JB28, ctaagcttacagatcaacctcacgcagg	1,241 bp, homologous region
	JB29, aggttgatctgtaagcttaggctgctgc JB30, tcgtgttctgtcagaagaactcgtcaagaag	1,182 bp, KanR
	JB31, gttcttctgacagaacacgatcagctcg JB32, acgtgctcgacaacagctac	1,471 bp, homologous region
pJB12 (Desaturase gene, *bolL*)	JB43, gtcgaagccggtctcgtag JB44, gatggacgagctcatgaaggcggagaagtc	1,296 bp, homologous region
	JB45, ccttcatgagctcgtccatcgtaagcttag JB46, atagcgattcccgatcgatgtcagaagaac	1,182 bp, KanR
	JB47, catcgatcgggaatcgctatcgcaaggaag JB48, aggacatccacctcgacaag	1,079 bp, homologous region
pBD87 (Ketosynthase III gene, *bolM*)	BD424, gcgatagccgtcgaacatc BD425, ctaagcttacctacgagaccggcttcgac	1,206 bp, homologous region
	BD426, ggtctcgtaggtaagcttaggctgctgc BD427, ccagccgttctcagaagaactcgtcaagaag	1,162 bp, KanR
	BD428, gttcttctgagaacggctgggtatgcac BD429, gacttctccgccttcatgag	946 bp, homologous region

III: Four fragment NEBuilder fusion of two homologous regions, a resistance cassette, and a vector backbone. Primers listed in table S12 were used to amplify two homologous regions from genomic DNA of *Burkholderia gladioli* pv. *cocovenenans* respectively the kanamycin resistance cassette from pGEM-Kan. The plasmid pGL42a_T251A[13] was digested with *Xho*I and *Xba*I. In a subsequent step a NEBuilder four-fragment ligation has been performed according to the manufacture's recommendations yielding the final knockout plasmids (Table S12).

Table S12. Detailed information on the plasmids constructed using cloning strategy III; hr, homologous region; KanR, Kanamycin resistance cassette.

Plasmid (target gene)	Name and sequence of primers used 5' → 3'	Product size and name
pBD92 (Decarboxylase gene, *bolN*)	BD459, tatagggagagcggccgccagatcttccggatggcagccggaggtgtagagcag BD460, ctgcggactggctttctacgtgttcaatcactagtgatggacgaggacttcatgc	1,498 bp, hr
	JK582, gaattcgattctggtcggtc JK583, actagtgattgaacacgtag	1,151 bp, KanR
	BD461, tctggggttcgaaatgaccgaccagaatcgaattccgggtcgtacacgtagcag BD462, tttccatggcagctgagaatattgtaggagatcttgcggattcgacaagatgattg	1,371 bp, hr
pBD93 (Ketosynthase III gene, *bolP*)	BD476, tatagggagagcggccgccagatcttccggatggctcggaagtgccgaaatac BD477, ctgcggactggctttctacgtgttcaatcactagtaagggatgcagccatttatc	1,272 bp, hr
	JK582, gaattcgattctggtcggtc JK583, actagtgattgaacacgtag	1,151 bp, KanR
	BD478, tctggggttcgaaatgaccgaccagaatcgaattccgacacgctcgactggtg BD479, tttccatggcagctgagaatattgtaggagatcttggctcttcatgtcgatcagc	1,421 bp, hr
pBD95 (Citrat synthase gene, *bolR*)	BD459, tatagggagagcggccgccagatcttccggatggctctgggaaggcaccaactac BD460, ctgcggactggctttctacgtgttcaatcactagtcacggtcggcgtgatatag	1,387 bp, hr
	JK582, gaattcgattctggtcggtc JK583, actagtgattgaacacgtag	1,151 bp, KanR
	BD461, tctggggttcgaaatgaccgaccagaatcgaattccctacctgagcctgttcg BD462, tttccatggcagctgagaatattgtaggagatcttatgtaggccatctcctgg	1,434 bp, hr
pBD107 (acyl-CoA-dehydrogenase, bolQ)	BD555, tatagggagagcggccgccagatcttccggatggccgcagggctacctctacttc BD556, ctgcggactggctttctacgtgttcaatcactagtgacatccgattgctggtag	1, 505 bp, hr
	JK582, gaattcgattctggtcggtc JK583, actagtgattgaacacgtag	1,151 bp, KanR
	BD557, tctggggttcgaaatgaccgaccagaatcgaattctcctgacccaggtgctctac BD558, tttccatggcagctgagaatattgtaggagatcttcgaggaagatctccagttcg	1,201 bp, hr

Gene knockout of *Burkholderia gladioli* pv*. cocovenenans* HKI0521

Burkholderia gladioli pv*. cocovenenans* HKI0521 cells were washed three times with one volume of 300 mM sucrose in dH_2O. Cells have been spun down at 6,000 x g for 5 min. Afterwards the cells have been suspended in 100 µL aliquots. 2 µL of a plasmid have been added to the cell solution and afterwards a pulse of 2.5 kV has been applied. Cells have been suspended in MGY media and recovered for 3–4 h at 30 °C and 120 rpm. Afterwards, cell solution was plated onto NAG agar containing 50 µg mL^{-1} kanamycin and incubated at 30 °C until colonies appeared. Colonies grown after transformation with knockout plasmids have been subjected to a colony PCR to detect mutations. The templates were colony material that have been picked with a pipette tip and transferred into 100 µL dH_2O, incubated for 5 min at 99 °C and centrifuged at 18,000 × g for 1 min. 5 µL of the template have been added to 5 µL of 2x master mix of KAPA2G Robust HotStart ReadyMix PCR kit (Merck KGaA) with the appropriate primers.

Three separate PCRs have been performed to confirm homologous recombination in each mutant that has been created. The PCR were named front arm (FA), back arm (BA) and wild type control (WT). The primer pairs and expected products are listed (Table S13 and Table S14). The PCRs FA and BA only yield a product if the recombination of the front and respectively back homologous region with the genome took place. The WT PCR was used to exclude that wild type cells were present in the tested colony. As an additional control all PCRs were run in parallel with wild type cell material as template.

Table S13. Primer pairs and expected product sizes for colony PCRs to test for homologous recombination.

Mutant strain	PCR	Name and sequence of used primers 5' → 3'	Product size
Bg_coco_pJB02 (Δ*bolH*)	FA	JB05, tatcgttccattcgctcaag TIISSD_B_fwd, cgttggctacccgtgatatt	1,360 bp
	BA	JB06, ctacaacctgccgaagatgc TIISS_A_rv, agtgacaacgtcgagcacag	2,224 bp
	WT	JB05, tatcgttccattcgctcaag JB06, ctacaacctgccgaagatgc	2,919 bp
Bg_coco_pJB04 (Δ*bolO*)	FA	JB09, gcgttaccaggtccttgatg TIISS_A_rv, agtgacaacgtcgagcacag	2,134 bp
	BA	JB10, tgtcacgtcatgggagattc TIISSD_B_fwd, cgttggctacccgtgatatt	1,505 bp
	WT	JB09, gcgttaccaggtccttgatg JB10, tgtcacgtcatgggagattc	2,880 bp
Bg_coco_pJB06 (Δ*bolF*)	FA	JB13, gaaggccagcaggtagagg TIISSD_B_fwd, cgttggctacccgtgatatt	1,038 bp
	BA	JB14, ctgctcaagtacggcgacac TIISS_A_rv, agtgacaacgtcgagcacag	2,180 bp
	WT	JB13, gaaggccagcaggtagagg JB14, ctgctcaagtacggcgacac	2,459 bp
Bg_coco_pJB10 (Δ*bolC*)	FA	JB33, gtgtcgccgtacttgagcag TIISS_A_rv, agtgacaacgtcgagcacag	2,363 bp
	BA	JB34, gcctcgactacgaggtgttc TIISSD_B_fwd, cgttggctacccgtgatatt	2,045 bp
	WT	JB33, gtgtcgccgtacttgagcag JB34, gcctcgactacgaggtgttc	4,818 bp
Bg_coco_BD87 (Δ*bolM*)	FA	BD430, gagtaggtgcggtgataggc TIISS_A_rv, agtgacaacgtcgagcacag	2,198 bp
	BA	BD431, tctgctcctcgaagaaccac TIISSD_B_fwd, cgttggctacccgtgatatt	1,090 bp
	WT	BD430, gagtaggtgcggtgataggc BD431, tctgctcctcgaagaaccac	2,876 bp
Bg_coco_pJB14 (Δ*bolB*)	FA	BD410, aggctcatgcgtgtgtctc TIISS_A_rv, agtgacaacgtcgagcacag	1,773 bp
	BA	BD411, gatggacgaggacttcatgc TIISSD_B_fwd, cgttggctacccgtgatatt	1,403 bp
	WT	BD410, aggctcatgcgtgtgtctc BD411, gatggacgaggacttcatgc	2,511 bp
Bg_coco_pBD92 (Δ*bolN*)	FA	BD463, tcagcccgtacatcaggtag TIISS_A_rv, agtgacaacgtcgagcacag	1,968 bp
	BA	BD462, ccctgacaaacgacttttcc TIISSD_B_fwd, cgttggctacccgtgatatt	1,558 bp
	WT	BD463, tcagcccgtacatcaggtag BD462, ccctgacaaacgacttttcc	3,483 bp
Bg_coco_pBD93 (Δ*bolP*)	FA	BD480, gatcaatccgcagttgaagg TIISS_A_rv, agtgacaacgtcgagcacag	2,225 bp
	BA	BD481, cgaggaagatctccagttcg TIISSD_B_fwd, cgttggctacccgtgatatt	1,902 bp
	WT	BD480, gatcaatccgcagttgaagg BD481, cgaggaagatctccagttcg	3,646 bp

Table S14. Primer pairs and expected product sizes for colony PCRs to test for homologous recombination. (continued)

Bg_coco_ pBD107 (Δ*bolQ*)	FA	BD559, tcggaagtgccgaaatactg TIISS_A_rv, agtgacaacgtcgagcacag	2,560 bp
	BA	BD560, tcgtctccatgtcattccac TIISSD_B_fwd, cgttggctacccgtgatatt	1,722 bp
	WT	BD559, tcggaagtgccgaaatactg BD560, tcgtctccatgtcattccac	3,610 bp
Bg_coco_pBD95 (Δ*bolR*)	FA	BD492, gcatgctgatcgacatgaag TIISS_A_rv, agtgacaacgtcgagcacag	2,174 bp
	BA	BD493, aagttgcgcaggtacatcac TIISSD_B_fwd, cgttggctacccgtgatatt	1,693 bp
	WT	BD492, gcatgctgatcgacatgaag BD493, aagttgcgcaggtacatcac	3,432 bp

All mutant strains were cultivated on 20 mL PDA agar overnight at 30 °C, extracted, analyzed using LC-MS and their metabolic profiles compared to the wild type.

Bioactivity assays

Antibiotic assays

Bolagladin B (2 mg mL $^{-1}$, in DMSO) was tested against *Bacillus subtilis*, *Escherichia co*li, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium vaccae*, *Candida albicans* and *Penicillium notatum* as described previously.[14] Ciprofloxacin (5 µg mL^{-1}, in dH_2O) or amphotericin B (10 µg mL^{-1}, in DMSO and MeOH) was used as a positive control for test against bacteria respectively fungi (Table S15).

Table S15. Inhibitory effects of bolagladin B (**2**) against selected strains.

Bacterial strains tested	**Zone of inhibition in mm**		
	Bolagladin B (2) (1 mg mL^{-1})	**Ciprofloxacin (5 µg mL^{-1})**	**DMSO**
Bacillus subtilis 6633 B1	13 p	28	11 p
Escherichia coli 458 B4	15 p	24/31p	13
Pseudomonas aeruginosa SG 137 B9	0	27/34 p	13 p
Staphylococcus aureus 134/94 R9 (MRSA)	14 p	0	11 p
Enterococcus faecalis 1528 R10 (VRE)	17	16	12 p
Mycobacterium vaccae 10670 M4	18	22 p	13 p
Fungal strains tested	**Bolagladin B (2) (1 mg mL^{-1})**	**Amphotericin B (10 mg mL^{-1})**	**DMSO**
Candida albicans H8	14 p	20	0
Penicillium notatum JP36 P1	15 p	18 p	12 p

p – partial inhibition

Swarming assays

MGY+M9 medium containing 1.5% agar was prepared, sterilized, and diluted with 1:1 water (0.7% MGY agar plates). 10 µL of culture with an OD_{600} of 0.4, that was inoculated from an overnight *B. gladioli* pv. *cocovenenans* wild type and Δ*bolH* culture was pipetted in the middle of a 0.7% MGY agar plate and incubated at 30 °C. Similar swarming behavior was observed within 14 days of incubation.

Drop-collapsing assay[15]

Droplets of water (20 µL) were placed on a hydrophobic surface (Parafilm 'M'). Bacterial cells of a single colony were transferred with a toothpick and resuspended in one droplet (Figure S6). The positive control, water *B. gladioli* HKI0739 (Bg739) droplets, resulted in a collapsed droplet. The control water, no additive and neither the *B. gladioli* pv. *cocovenenans* (*Bg*. pv. *coco*) nor the *B. gladioli* pv. *cocovenenans* Δ*bolO* lead to a collapse of the droplet. For visualization purposes 0.0025% crystal violet were added to the droplet. It had no influence on the shape of the droplet.

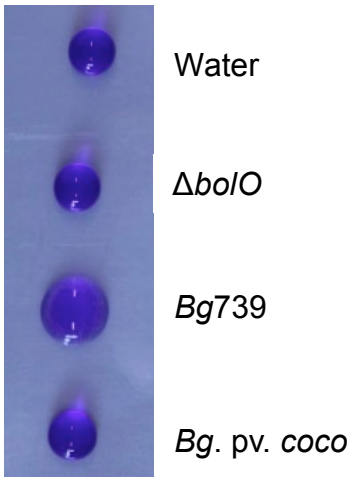

Figure S6. Droplet collapsing assay to test for surface activity of wild type *versus* mutant strains. *ΔbolO*, *B. gladioli* pv. *cocovenenans ΔbolO*; Bg739*, B. gladioli* HKI0739; *Bg*. pv. *coco*, *B. gladioli* pv. *cocovenenans*).

CAS agar test

To test for siderophore production of *B. gladioli* pv. *cocovenenans* we used CAS agar plates.[16] 100 µL of an overnight culture was pipetted onto the agar and incubated for a varying periods at 30 °C. A discoloration and halo formation colonies indicates siderophore activity. No or minor differences were observed when comparing the halo formation of *B. gladioli* pv. *cocovenenans* wild type, *B. gladioli* pv. *cocovenenans* Δ*bolH* or *B. gladioli* pv. *cocovenenans* Δ*bolR* (producer of **3** and **4**) colonies (Figure S7). The discoloration likely occurs due to the production of gladiobactin. Spotting of pure compound did not result in discoloration of the agar.

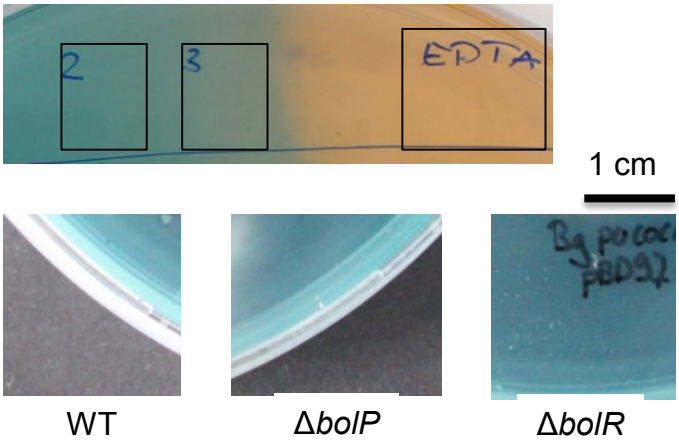

Figure S7. *B. gladioli* pv. *cocovenenans* or bolagladin incubated on CAS agar plates at 30 °C, respectively, 20 °C for 24 h. Spotting of 2 = bolagladin B (**2**) 0.1 mg ml^{-1}; 3 = bolagladin B (**2**) 0.5 mg ml^{-1}. EDTA was used as positive control. No noteworthy differences have been observed between B. gladioli pv. cocovenenans wild type (WT), *B. gladioli* pv. *cocovenenans* Δ*bolP* (no bolagladin production), *B. gladioli* pv. *cocovenenans* Δ*bolR* (producer of **3** and **4**).

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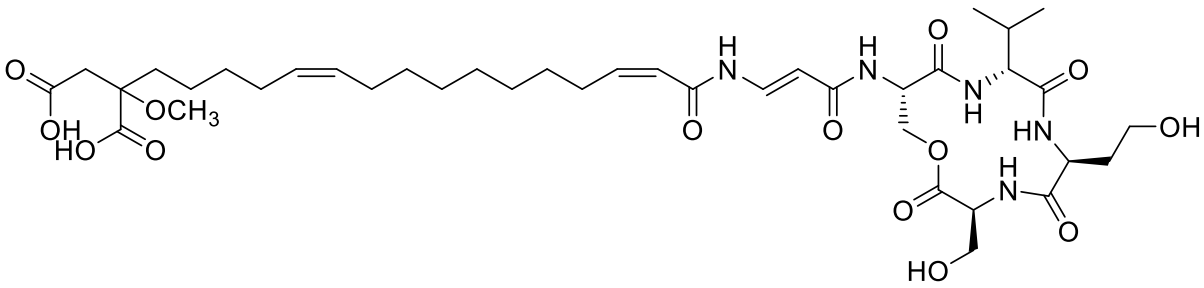
Physicochemical data

Bolagladin A (1)



(–)ESI HR-MS: *m/z* 822.4163 $[M–H]^-$ (calcd. $C_{39}H_{60}N_5O_{14}$ 822.4142)

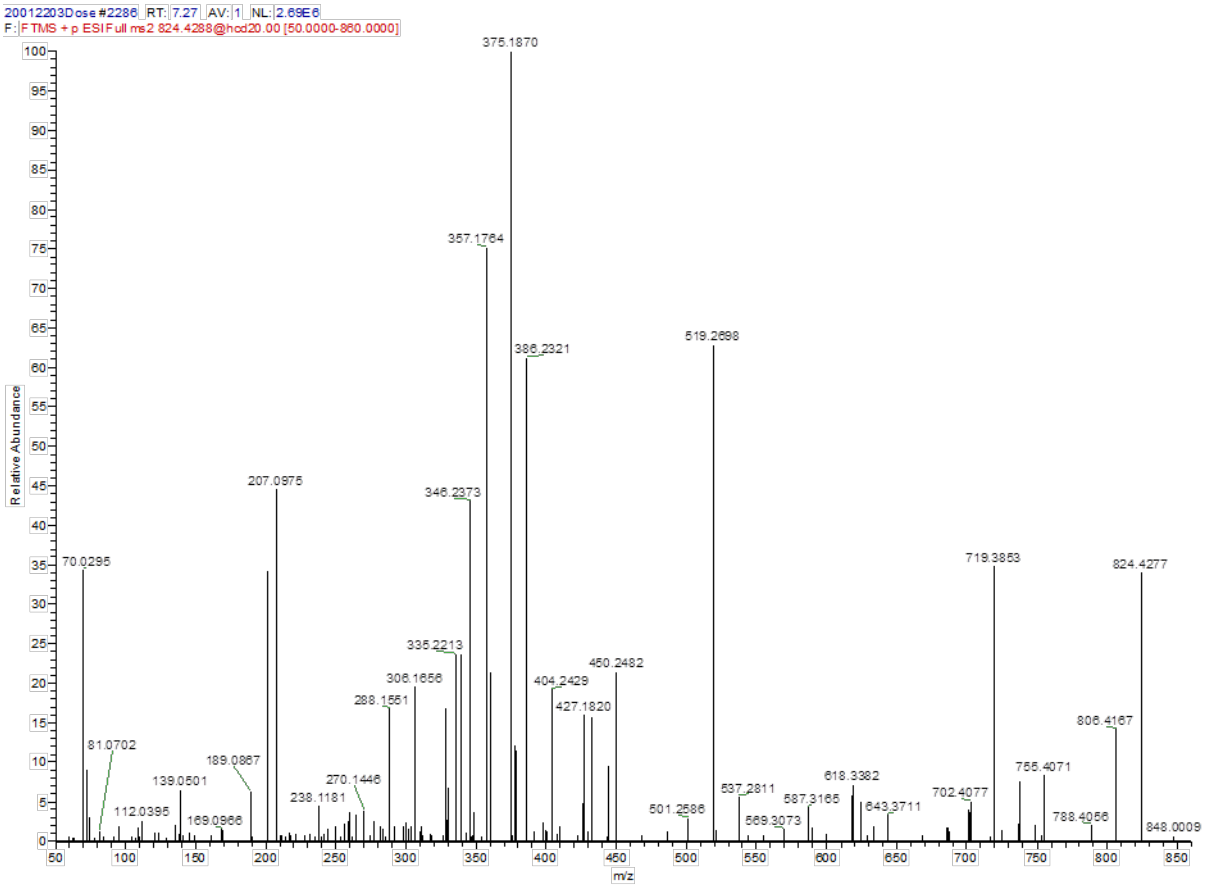


Figure S8. (+)ESI MS/MS spectrum of bolagladin A (**1**).

Table S16. NMR data of bolagladin A (**1**).

		Bolagladin A (1)	
	Position	**^{13}C**	**^{1}H (*J* in Hz)**
L-Serine 1	1	169.4	-
	2	55.0	4.32 m
	3	61.0	3.68 m
			3.57 m
	NH	-	7.22 d (*8.0*)
L-Homoserine	1	1	-
	2	51.5	4.22 m
	3	n. d.	1.80 m
	4	57.8	3.45 m
			3.37 m
	NH	-	8.56 d (*7.7*)
D-Valine	1	171.7	-
	2	59.6	4.07 t (*10.0*)
	3	29.0	1.23 m
	4	19.0	0.81 d (*6.5*)
	5	19.5	0.81 d (*6.5*)
	NH	-	7.29 d (*8.9*)
L-Serine 2	1	169.9	-
	2	53.1	4.54 m
	3	65.3	4.23 m
	NH	-	8.33 d (*7.3*)
Dehydro-*β*-alanine	1	166.7	-
	2	103.6	5.72 d (*14.0*)
	3	135.2	7.72 dd (*13.8; 11.3*)
	NH	-	10.53 d (*11.1*)
Fatty acid	1	164.4	-
	2	121.7	5.87 d (*11.4*)
	3	149.8	6.21 dt (*11.3; 7.5*)

4	28.7	2.63 m
		1.35 m
5-9	Not assigned	Not assigned
10	27.1	1.96 m
11	130.2	5.30 m
12	129.9	5.30 m
13	27.1	1.96 m
14	Not assigned	Not assigned
15	Not assigned	Not assigned
16	33.9	1.70 m
17	80.2	-
18	39.0	2.63
19	172.3	-
20	174.0	-
17-OCH_3	51.3	3.13 s

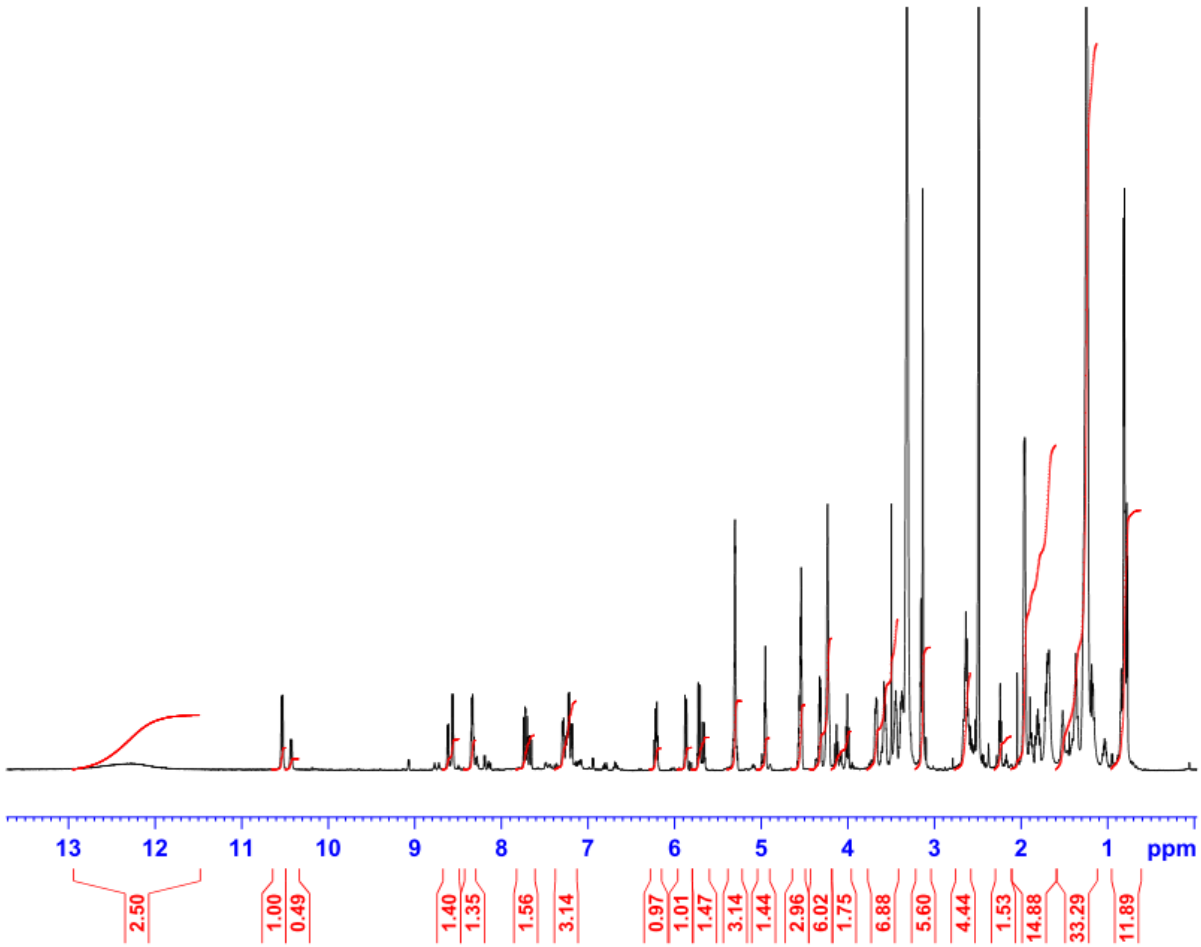


Figure S9. ^{1}H NMR spectrum of bolagladin A (**1**).

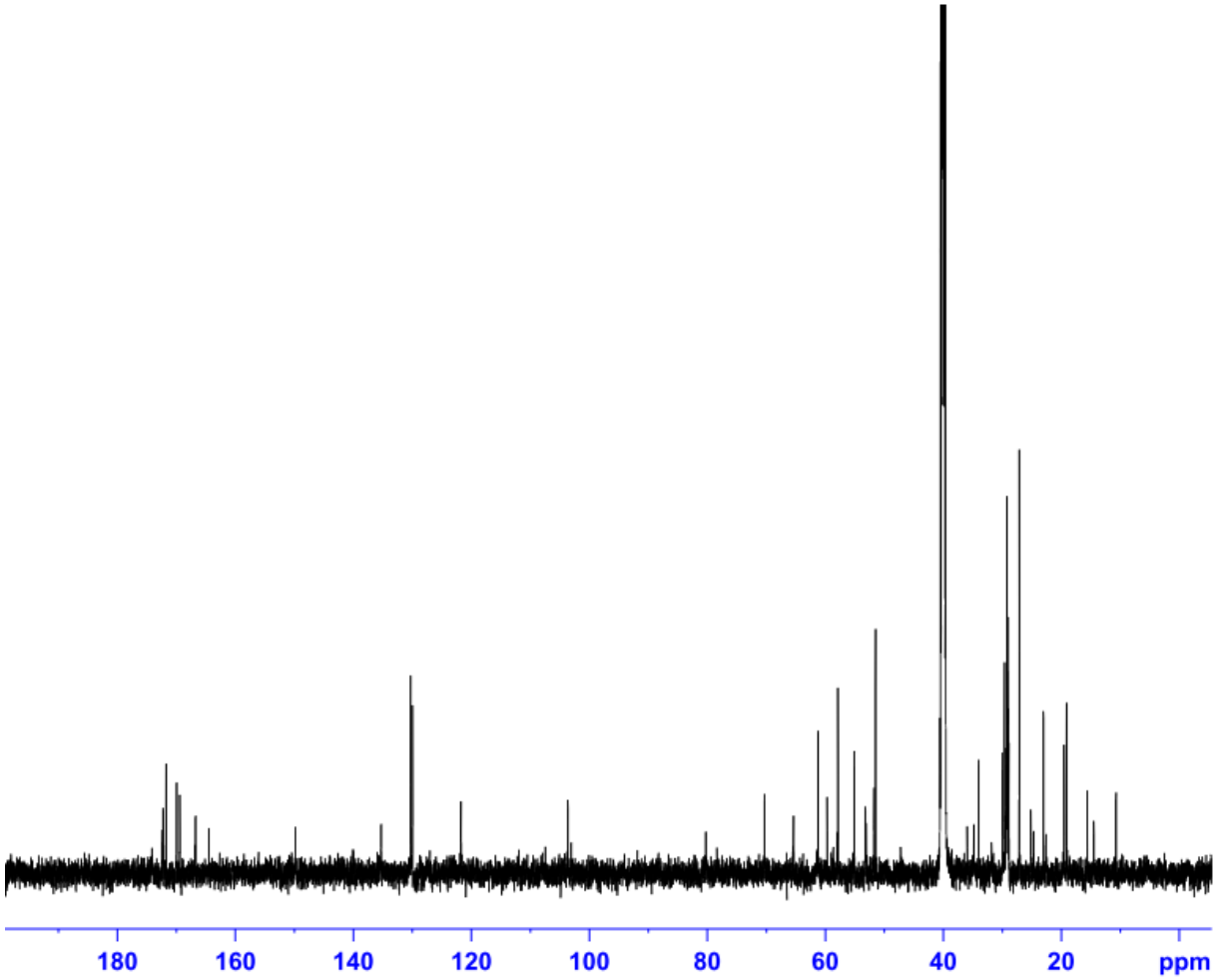


Figure S10. ^{13}C NMR spectrum of bolagladin A (**1**).

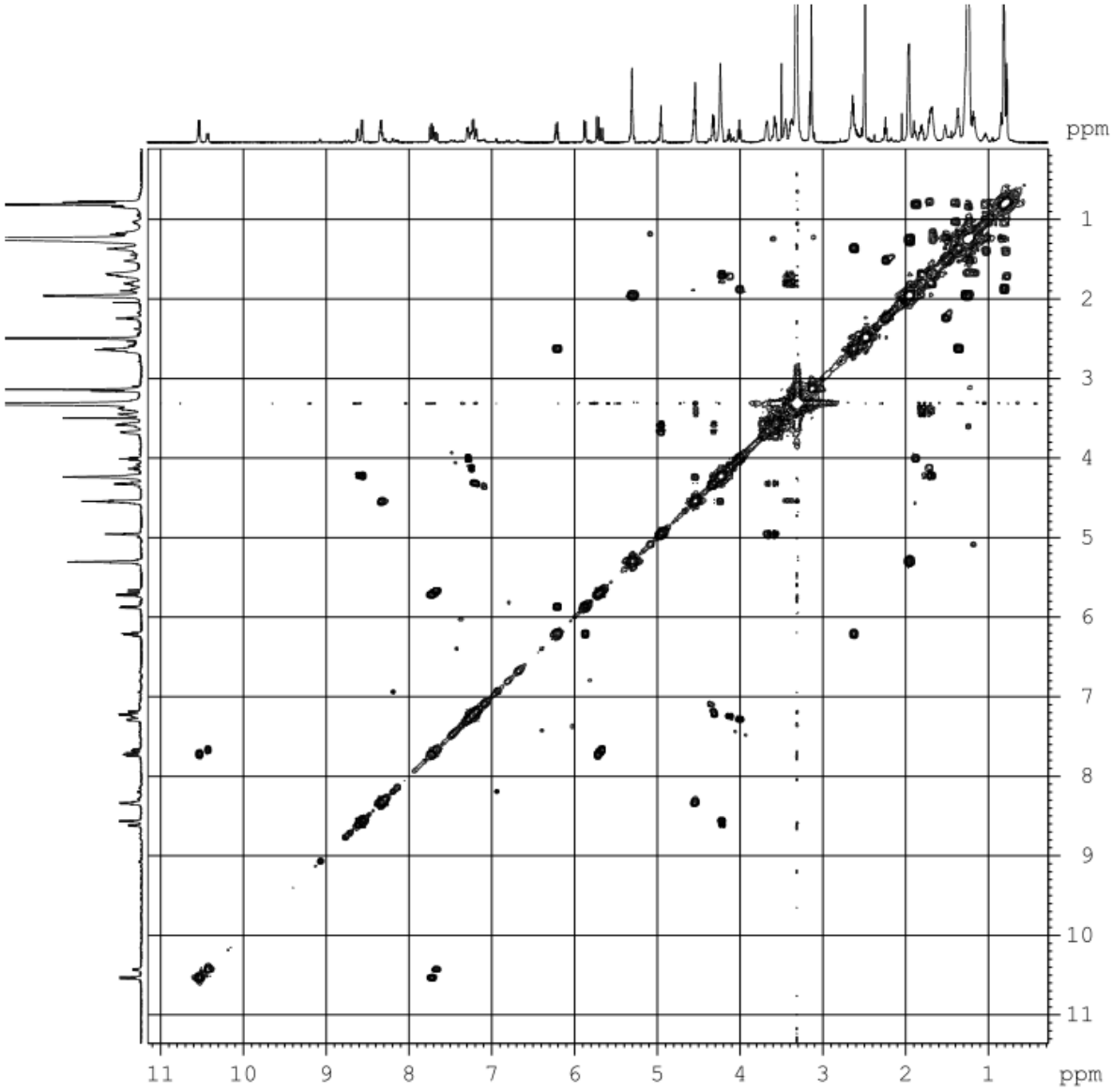


Figure S11. H,H-COSY spectrum of bolagladin A (**1**).

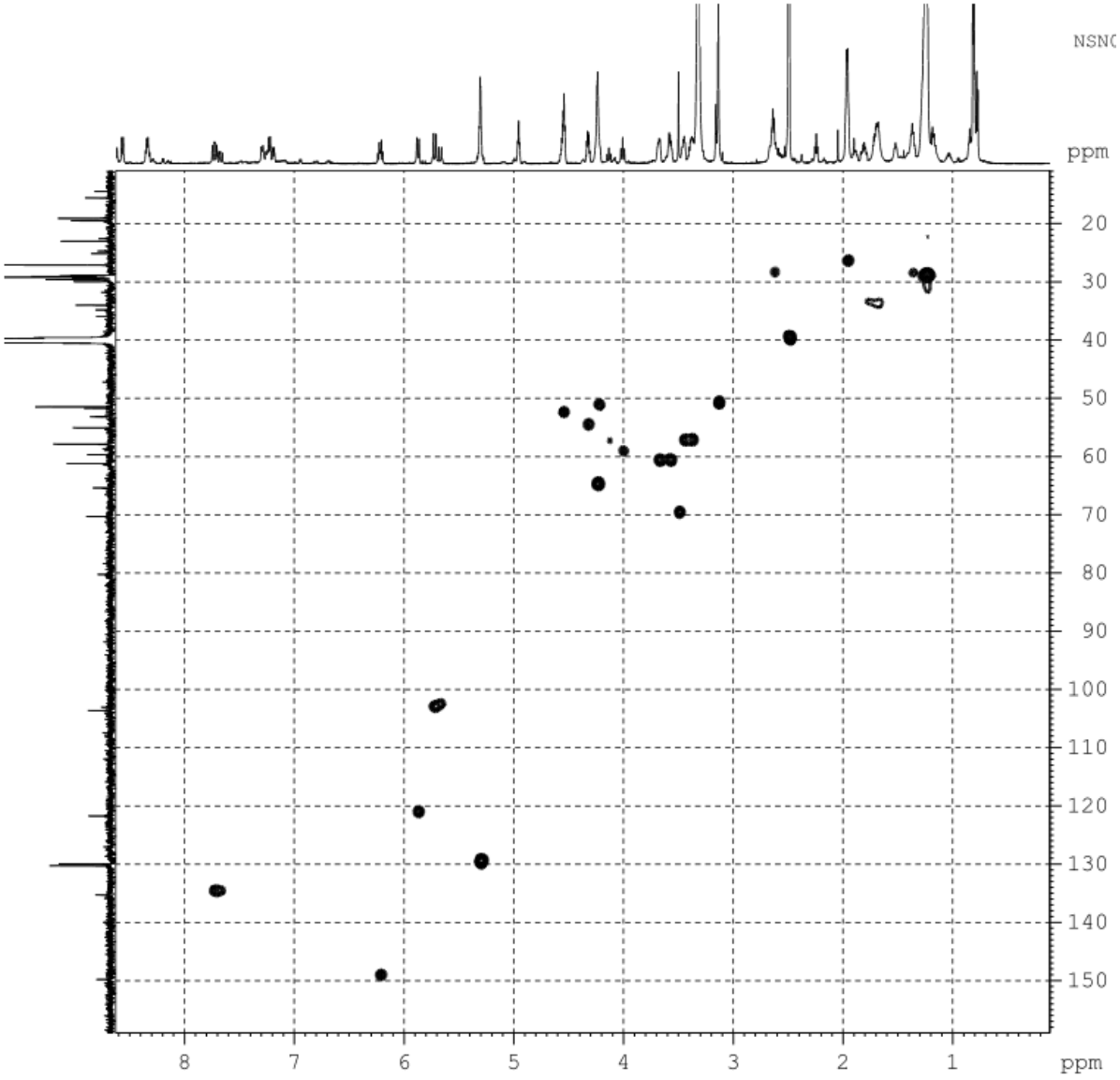


Figure S12. HSQC spectrum of bolagladin A (**1**).

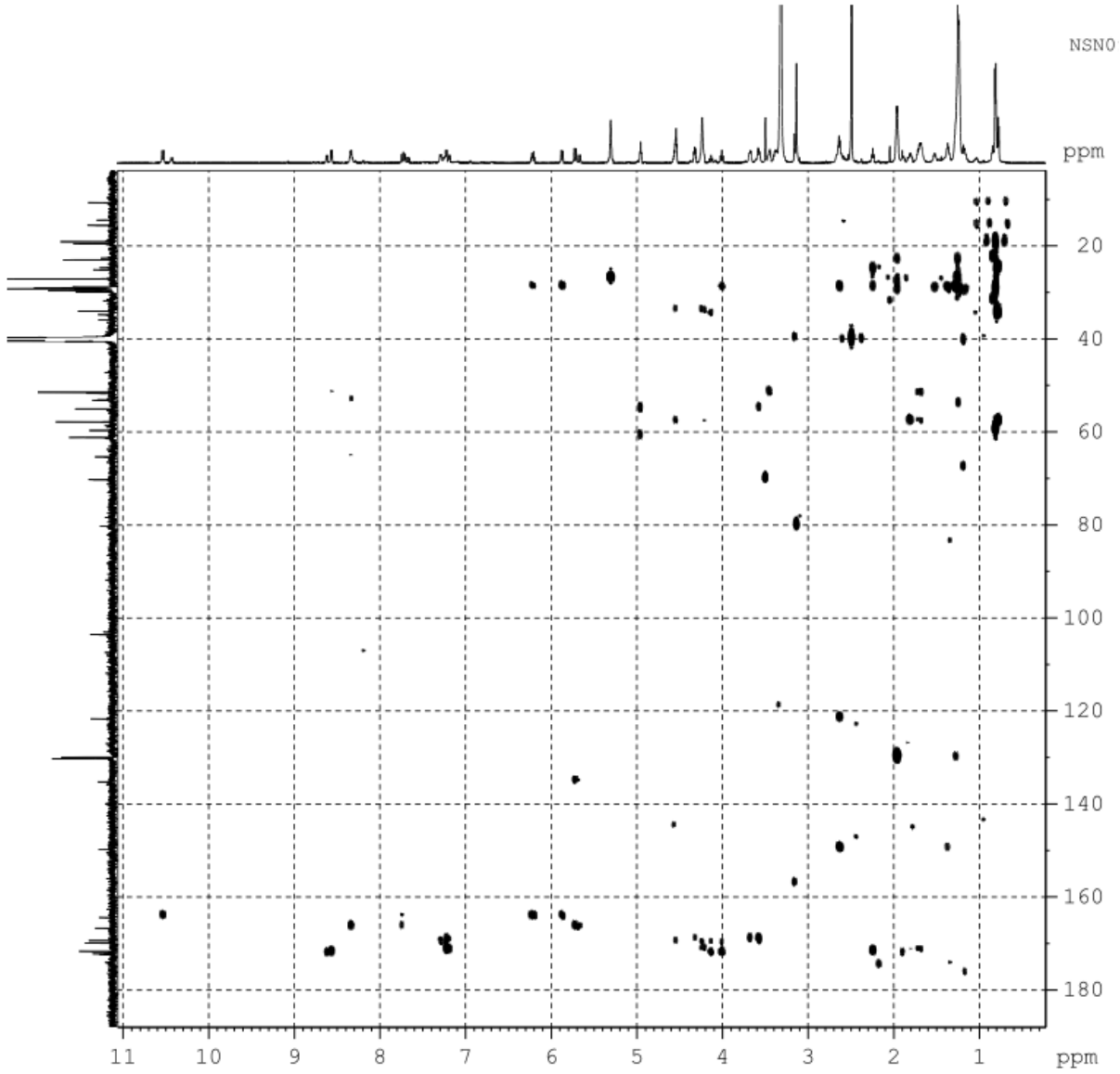
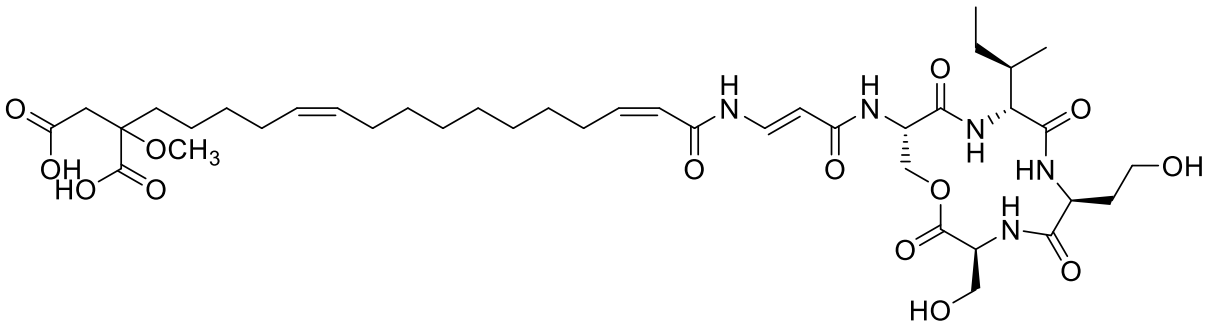


Figure S13. H,C-HMBC spectrum of bolagladin A (**1**).

Bolagladin B (2)



(–)ESI HR-MS: *m/z* 836.4315 $[M–H]^-$ (calcd. $C_{40}H_{62}N_5O_{14}$ 836.4299)

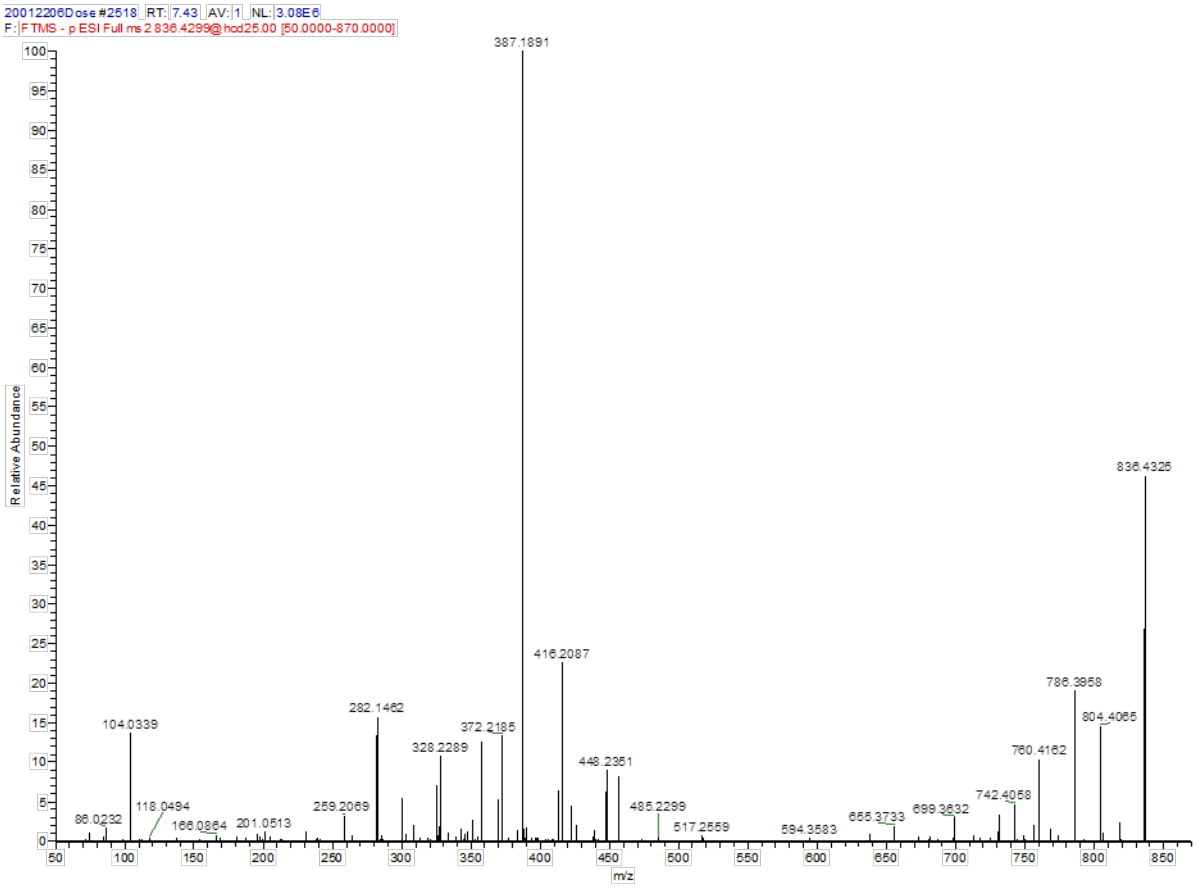


Figure S14. (–)ESI MS/MS spectrum of bolagladin B (**2**).

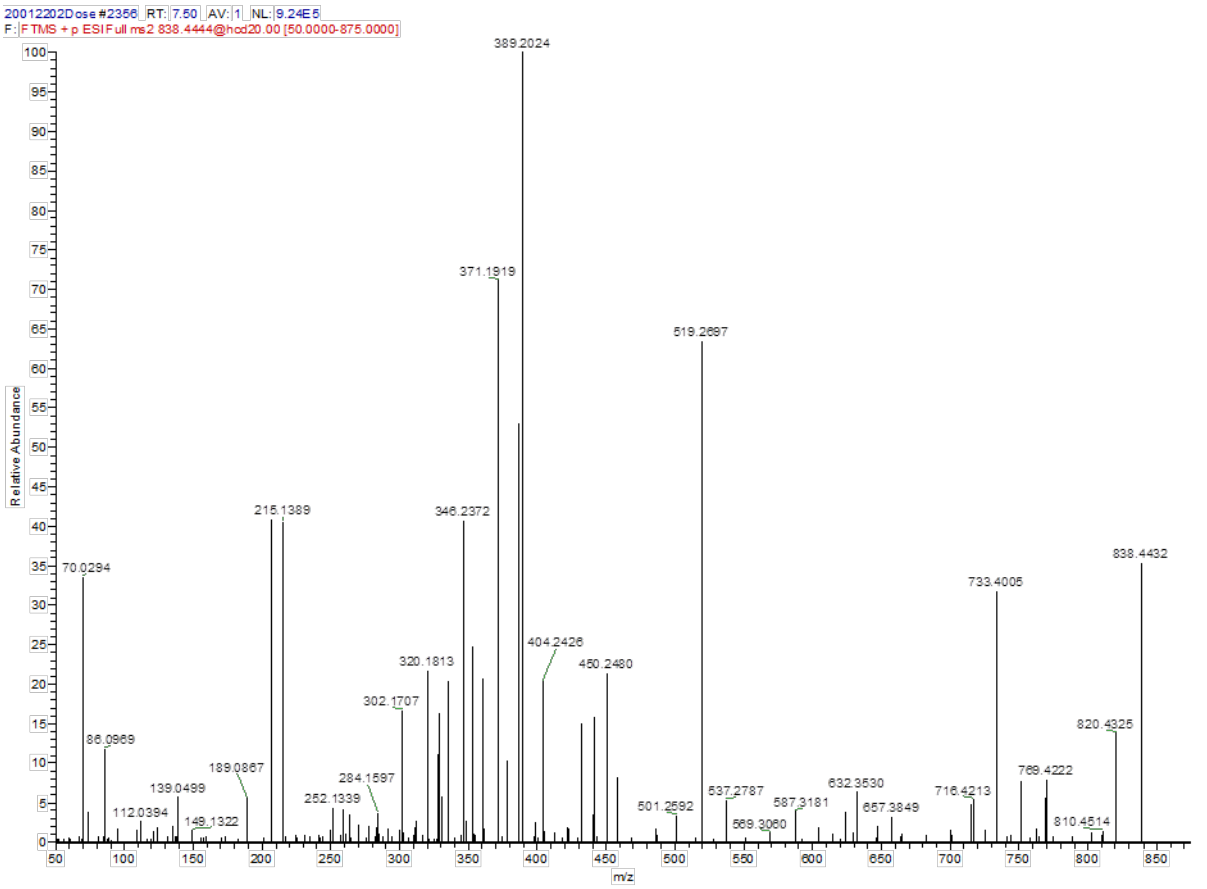


Figure S15. (+)ESI MS/MS spectrum of bolagladin B (**2**).

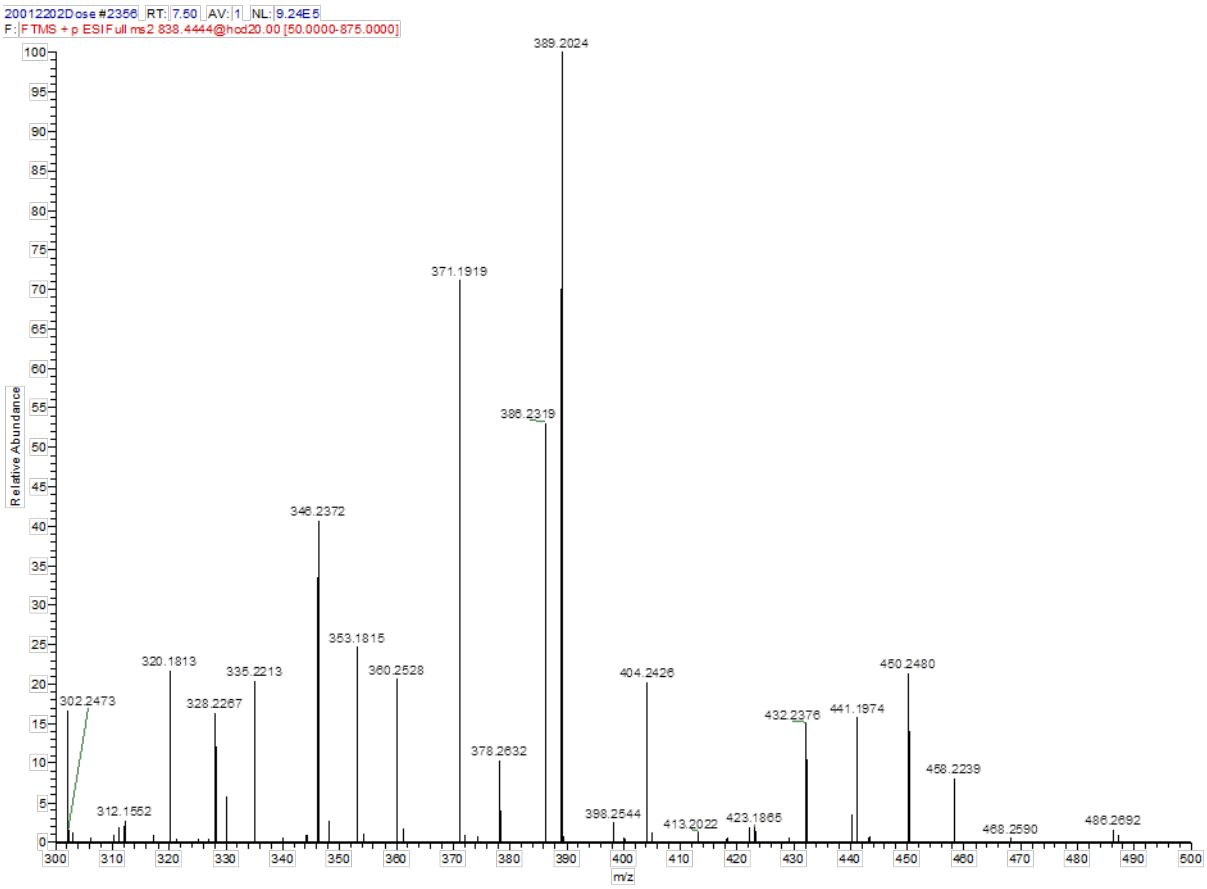


Figure S16. (+)ESI MS/MS spectrum of bolagladin B (**2**), mass range *m/z* 300–500.

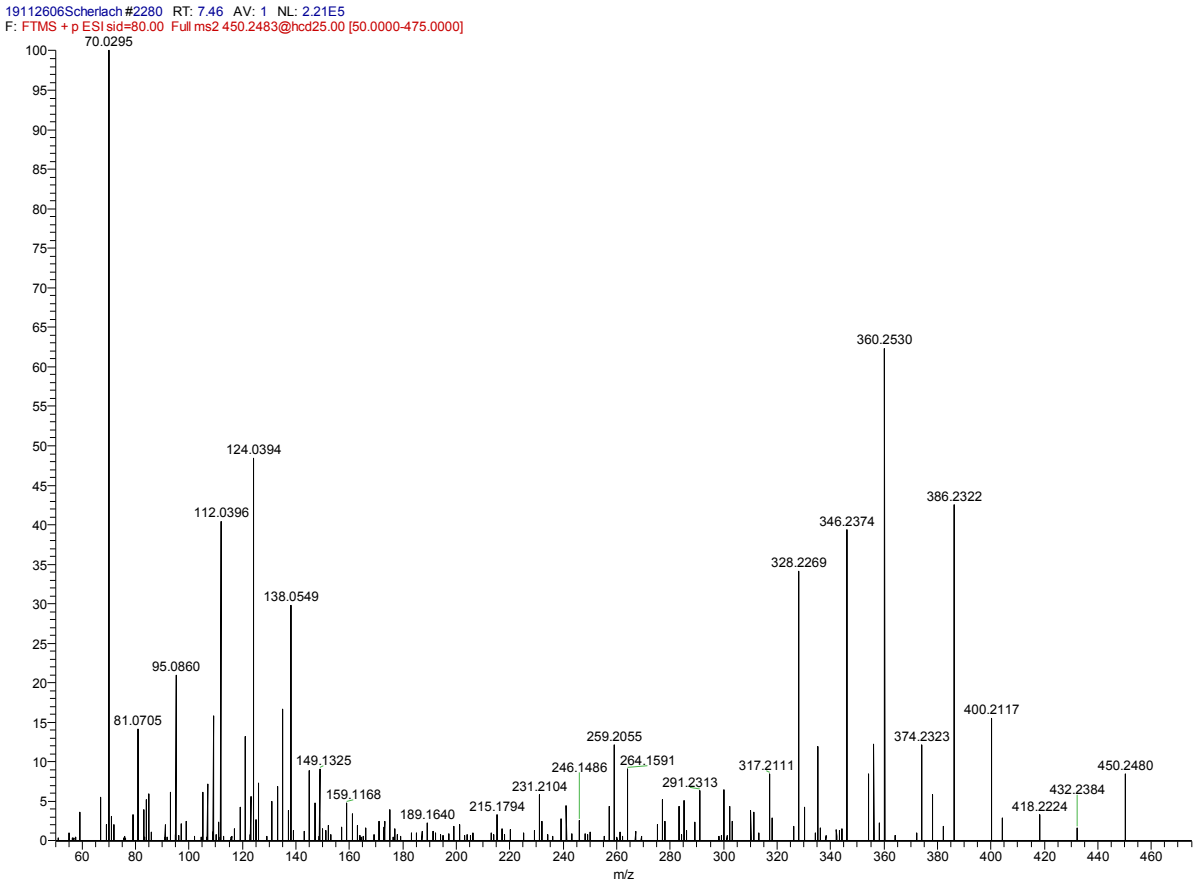


Figure S17. (+)ESI MS/MS spectrum of the fragment *m/z* 450 amu derived from bolagladin B (**2**).

Table S17. NMR data of bolagladin B (**2**).

		Bolagladin B (**2**)	
	Position	^{13}C	^{1}H (*J* in Hz)
L-Serine 1	1	168.8	-
	2	54.4	4.33 m
	3	60.7	3.67 m
			3.57 m
	NH	-	7.20 d (*7.8*)
L-Homoserine	1	171.0	-
	2	51.2	4.22 m
	3	33.5	1.78 m
	4	57.3	3.45 m
			3.37 m
	NH	-	8.62 d (*7.7*)
D-Isoleucine	1	171.8	-
	2	57.5	4.11 t (*10.1*)
	3	34.1	1.73 m
	4	24.2	1.42 m
			1.04 m
	5	10.2	0.80 t (*7.4*)
	6	15.0	0.77 d (*6.7*)
	NH	-	7.38 brd
L-Serine 2	1	169.5	-
	2	52.5	4.56 m
	3	64.8	4.22 m
	NH	-	8.45 brd
Dehydro-*β*-alanine	1	166.2	-
	2	103.1	5.76 d (*14.0*)
	3	134.7	7.72 dd (*14.0; 11.2*)
	NH	-	10.73 d (*10.8*)

Fatty acid	1	163.9	-
	2	121.3	5.92 d (*11.5*)
	3	149.1	6.20 dt (*11.5;7.5*)
	4	28.3	2.63 m
			1.35 m
	5-9	Not assigned	Not assigned
	10	26.6	1.92 m
	11	129.8	5.30 m (*10; 6*)
	12	129.3	5.30 m (*10; 6*)
	13	26.5	1.92 m
	14	29.2	1.25 m
	15	22.4	1.23 m
			1.17 m
	16	33.9	1.73 m
	17	79.8	-
	18	39.0	2.63
	19	171.2	-
	20	173.2	-
	17-OCH_3	50.9	3.16 s

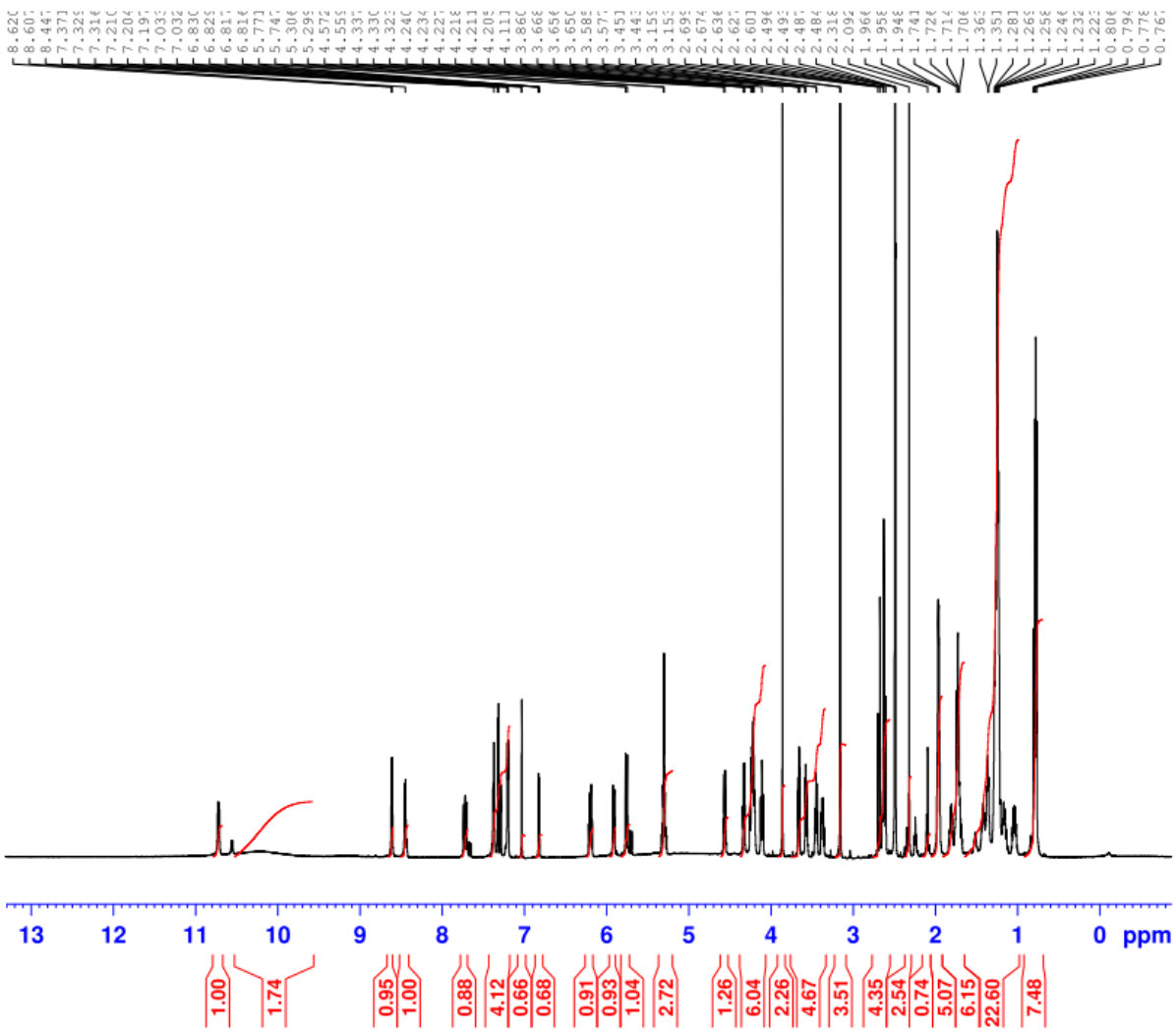


Figure S18. ^{1}H NMR spectrum of bolagladin B (**2**).

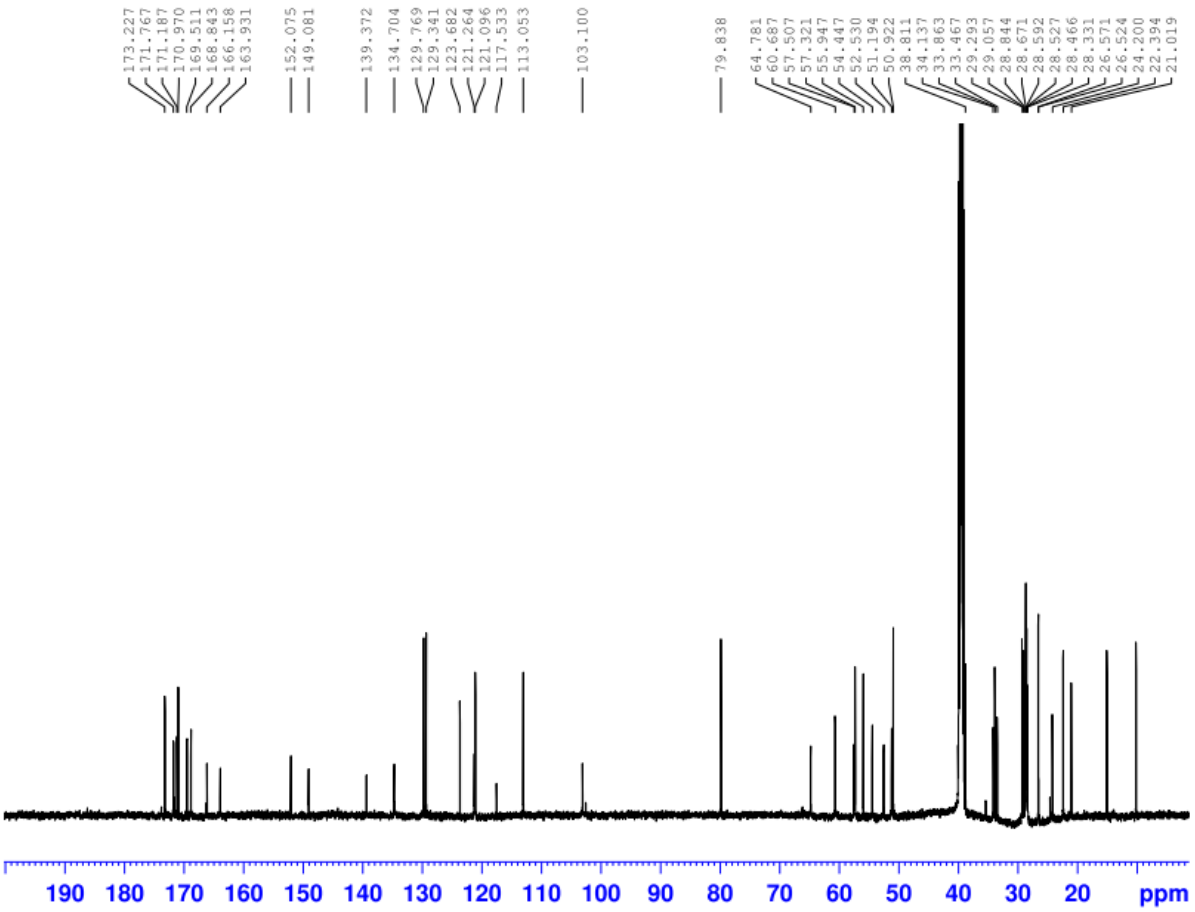


Figure S19. ^{13}C NMR spectrum of bolagladin B (**2**).

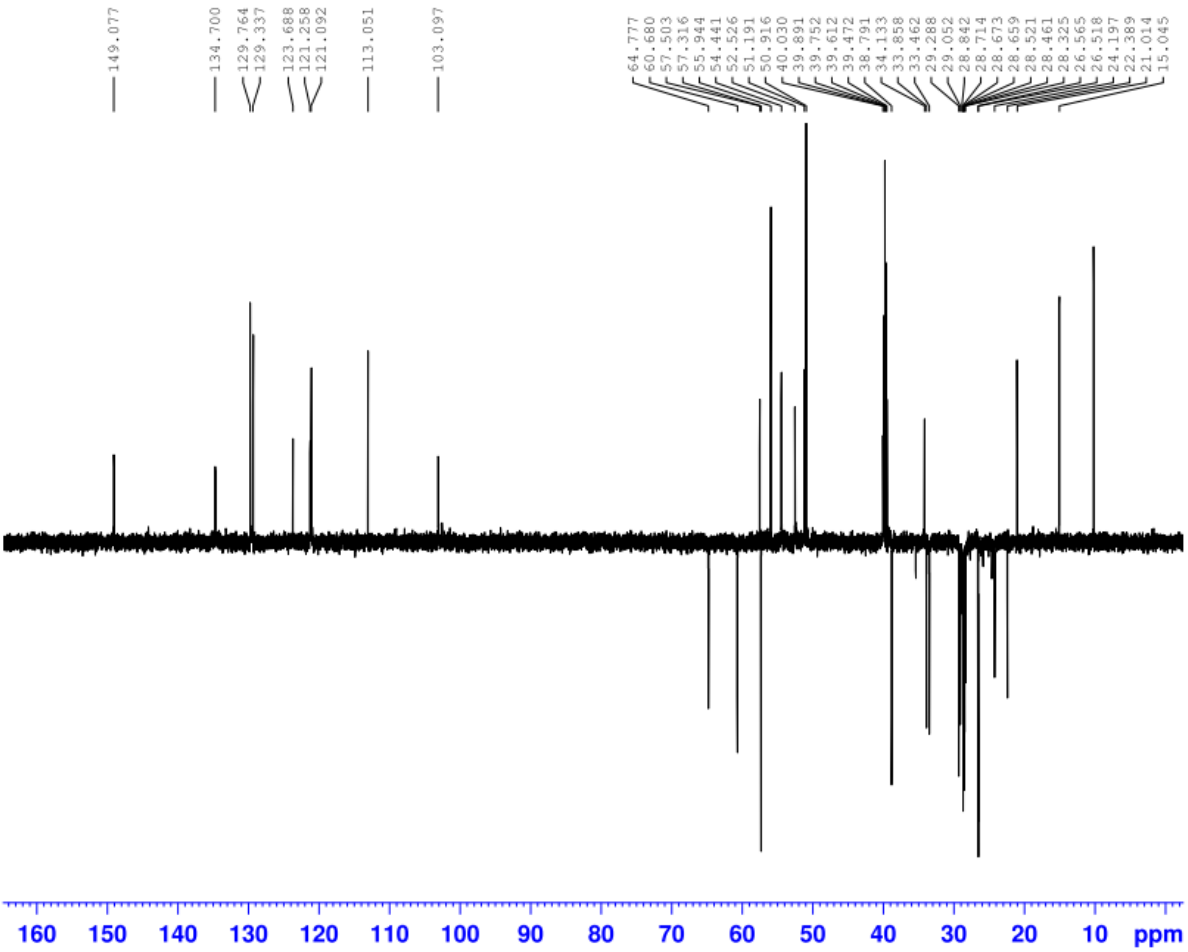


Figure S20. DEPT-135 NMR spectrum of bolagladin B (**2**).

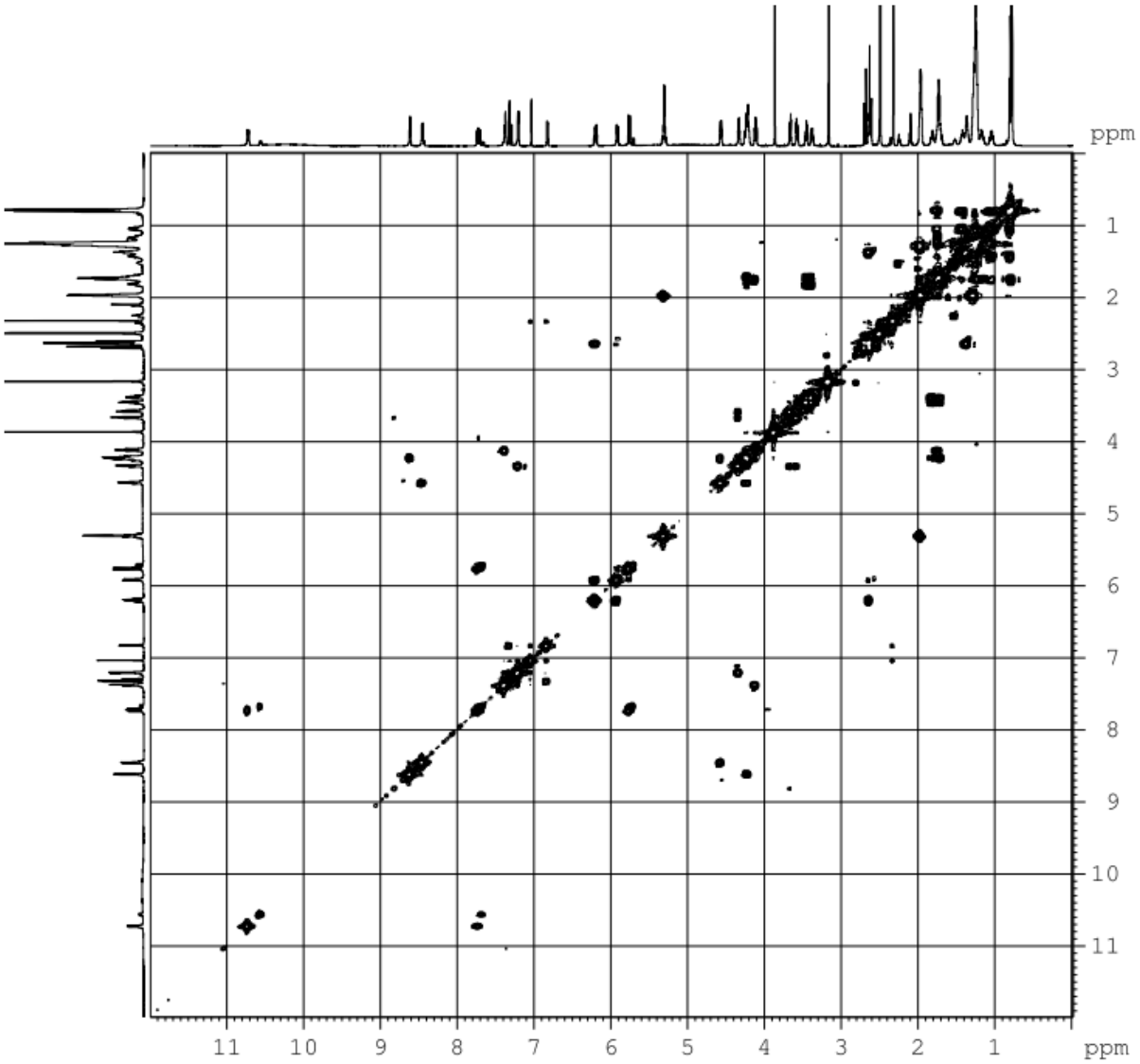


Figure S21. H,H-COSY spectrum of bolagladin B (**2**).

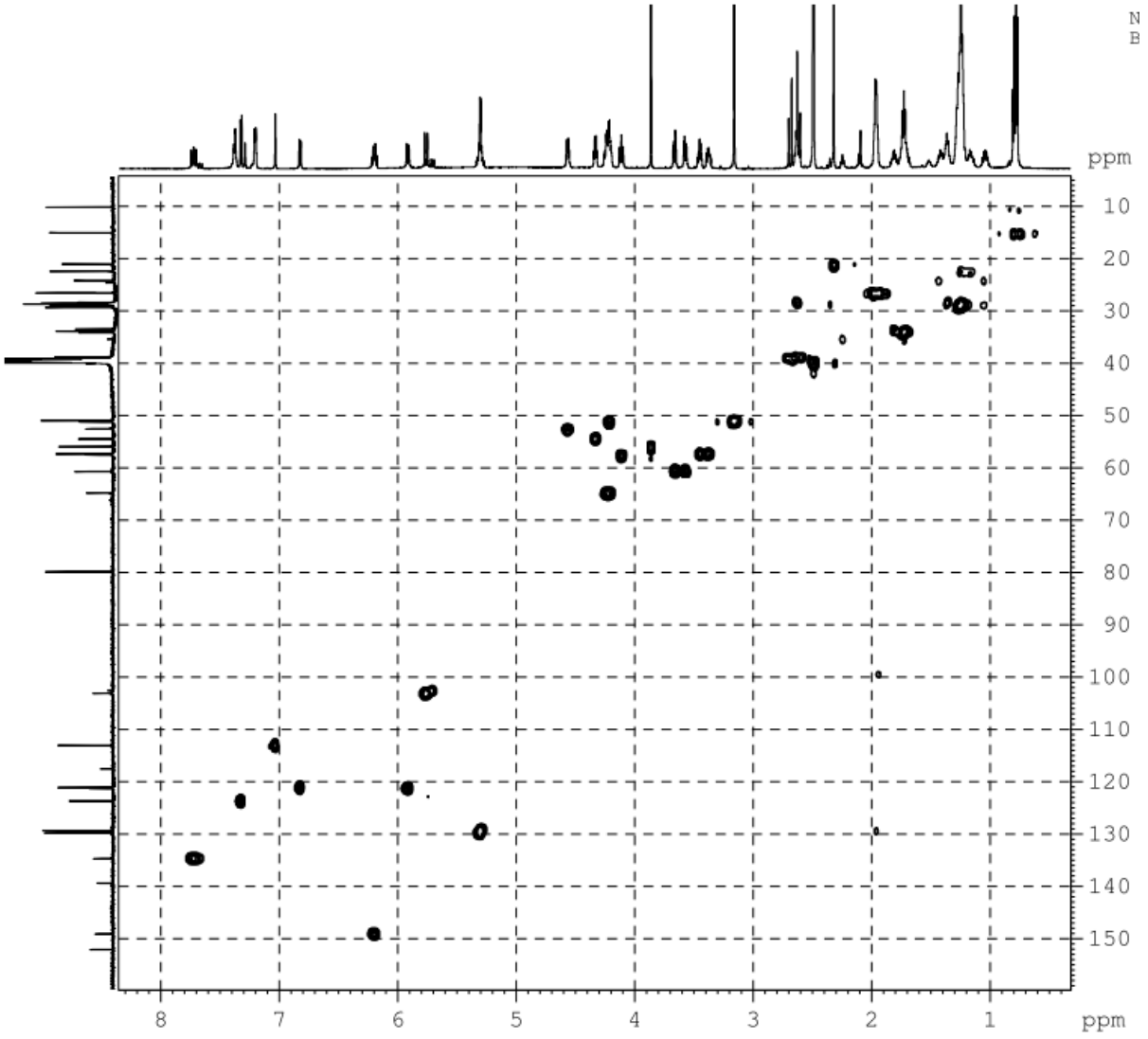


Figure S22. HSQC spectrum of bolagladin B (**2**).

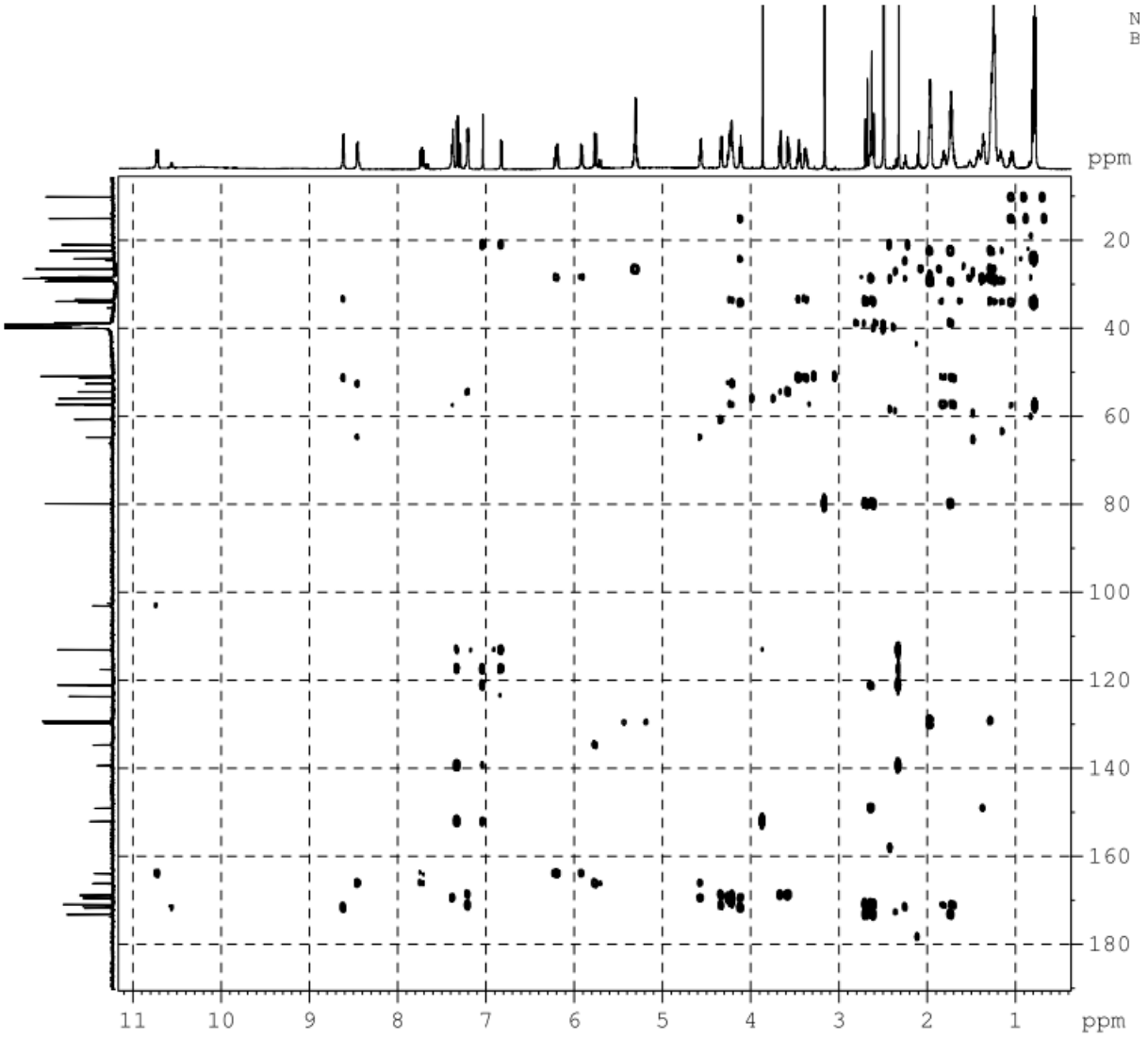
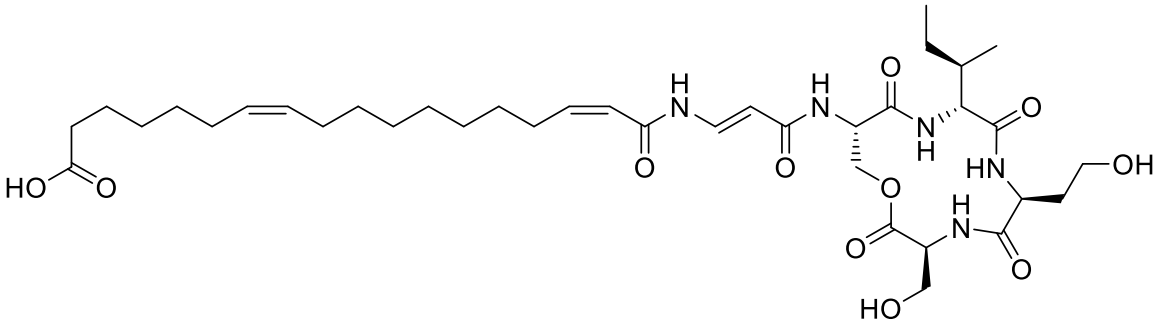


Figure S23. H,C-HMBC spectrum of bolagladin B (**2**).

Bolagladin M749 (3)



(–)ESI HR-MS: *m/z* 748.4157 $[M–H]^-$ (calcd. $C_{37}H_{58}N_5O_{11}$ 748.4138)

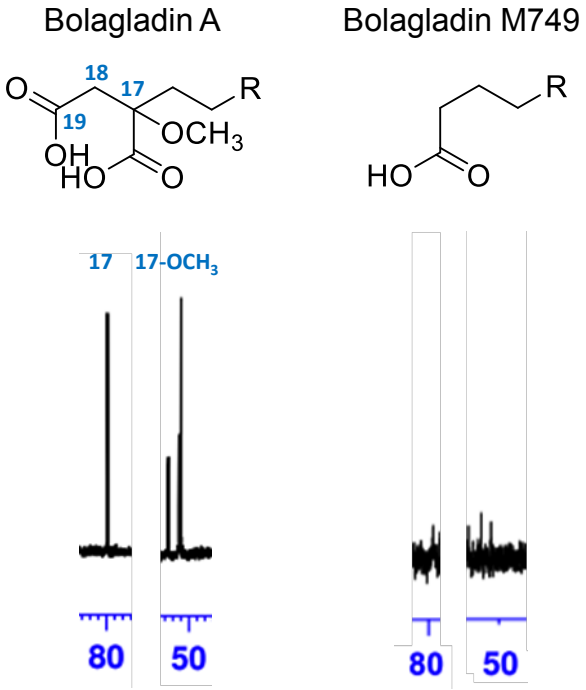


Figure S24. Selected diagnostic ^{13}C NMR signals of bolagladin A (**1**) and bolagladin M749 (**3**).

It should be noted that the signals for C18 and C19 in bolagladin A **(**1**)** are overlapping with either the solvent signal (C18) or amide carbon signals (C19).

Table S18. NMR data of bolagladin M749 (**3**).[1]

		Bolagladin M749 (3)	
	Position	**^{13}C**	**^{1}H (*J* in Hz)**
L-Serine 1	1	169.3	-
	2	54.4	4.33 m
	3	60.7	3.67 m
			3.57 m
	NH	-	7.19
L-Homoserine	1	171.2	-
	2	51.2	4.24 m
	3	33.5	1.73 m
	4	57.3	3.45 m
			3.37 m
	NH	-	8.62 d (*7.7*)
D-Isoleucine	1	171.8	-
	2	57.3	4.12 t (*10.0*)
	3	33.9	1.73 m
	4	24.2	1.46 m
			1.04 m
	5	10.2	0.80 t
	6	15.0	0.77 d
	NH	-	7.23 brd
L-Serine 2	1	169.4	-
	2	52.5	4.57 m
	3	64.9	4.23 m
	NH	-	8.34 d
Dehydro-*β*-alanine	1	166.2	-.
	2	103.2	5.73 d
	3	134.7	7.72 dd (*14.0*; *11.2*)
	NH	-	10.53 d (*10.8*)
Fatty acid	1	163.9	-

2	121.2	5.87 d (*11.5*)
3	149.1	6.20 dt (*11.5;7.5*)
4	28.4	2.63 m
		1.35 m
5-9	Not assigned	Not assigned
10	26.6	1.95 m
11	129.7	5.30 m
12	129.5	5.30 m
13	26.6	1.95 m
14	29.2	1.25 m
15	22.0	1.12-1.25 m
16	24.2[a]	1.46 m[a]
17	31.6[a]	2.10-2.30 m[a]
18	-	-
19	-	-
20	174.4[a]	-
17-OCH_3	-	-

[1] Data deduced from the NMR spectra of a bolagladin M749-enriched fraction

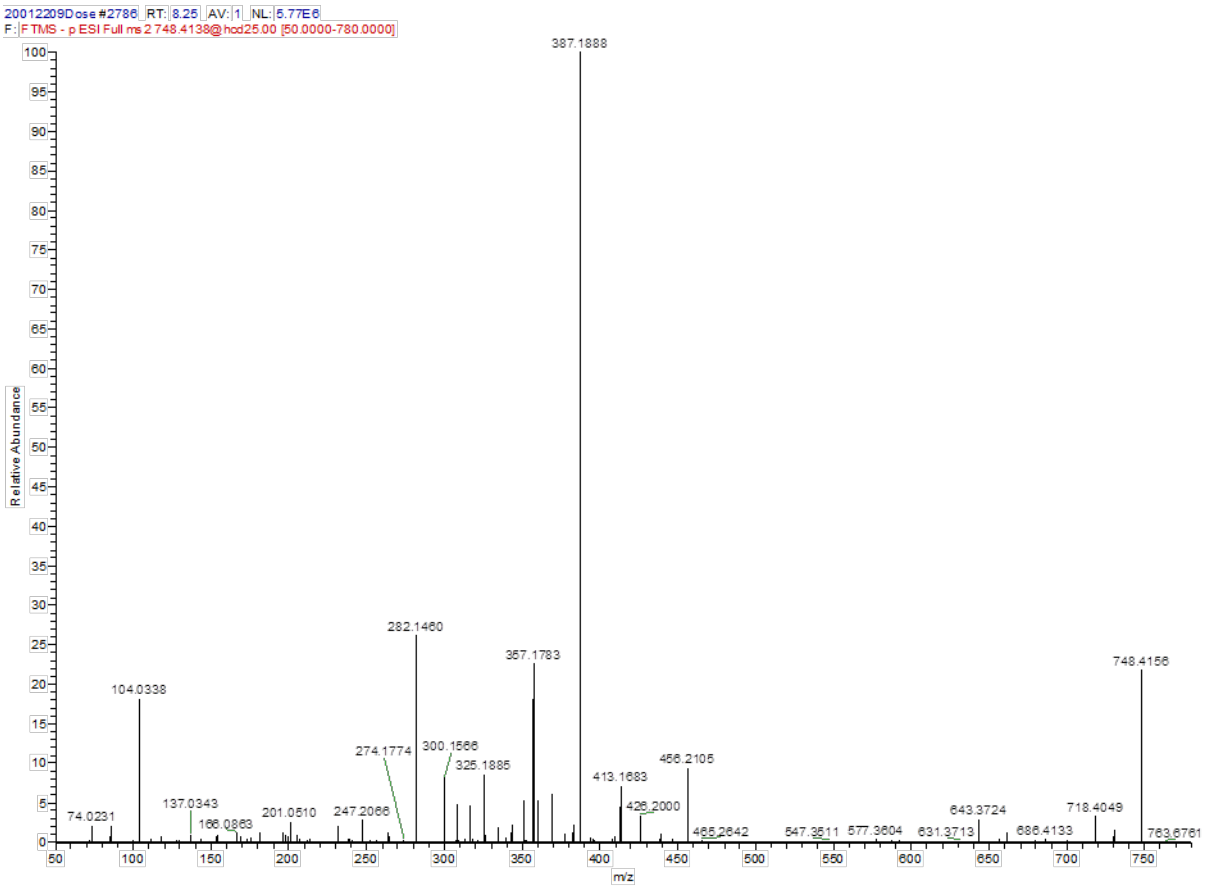


Figure S25. (–)ESI MS/MS spectrum of bolagladin M749 (**3**).

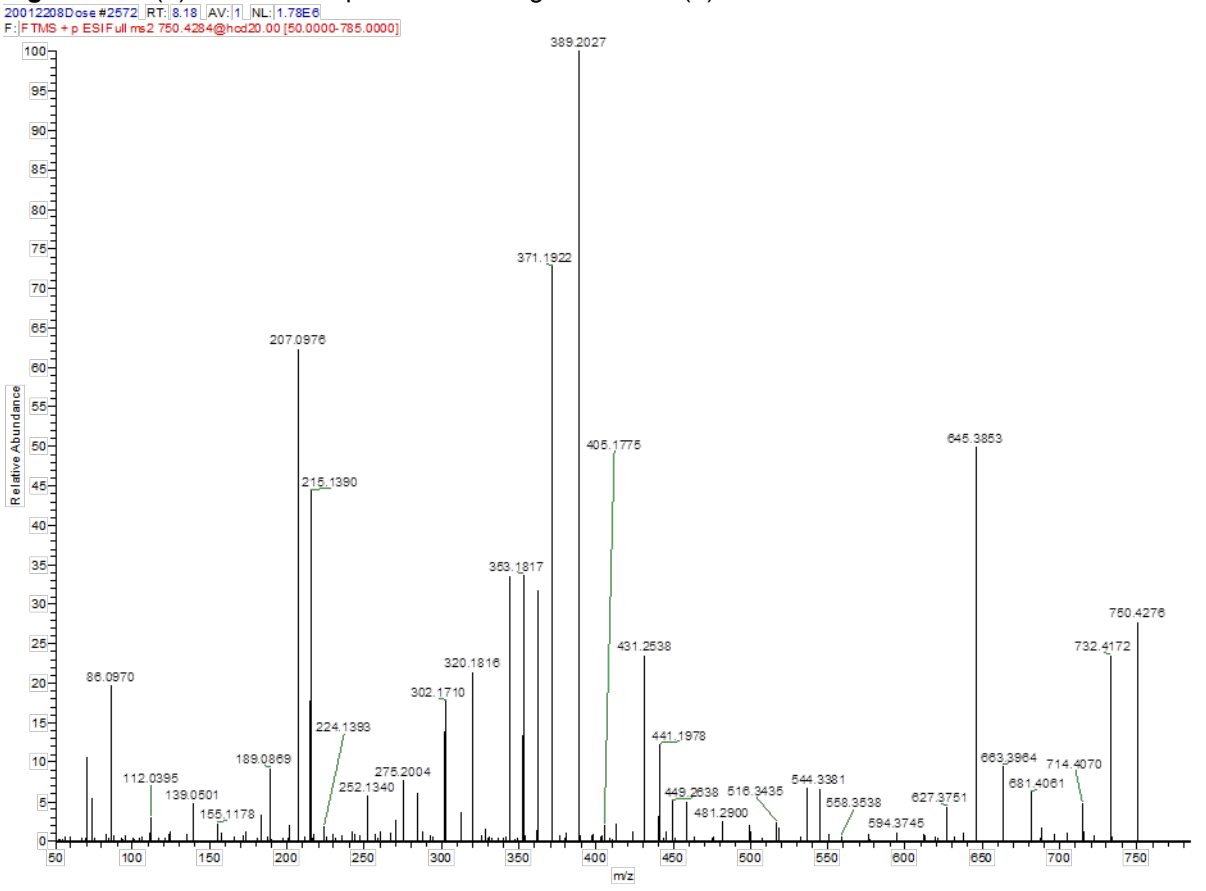


Figure S26. (+)ESI MS/MS spectrum of bolagladin M749 (**3**).

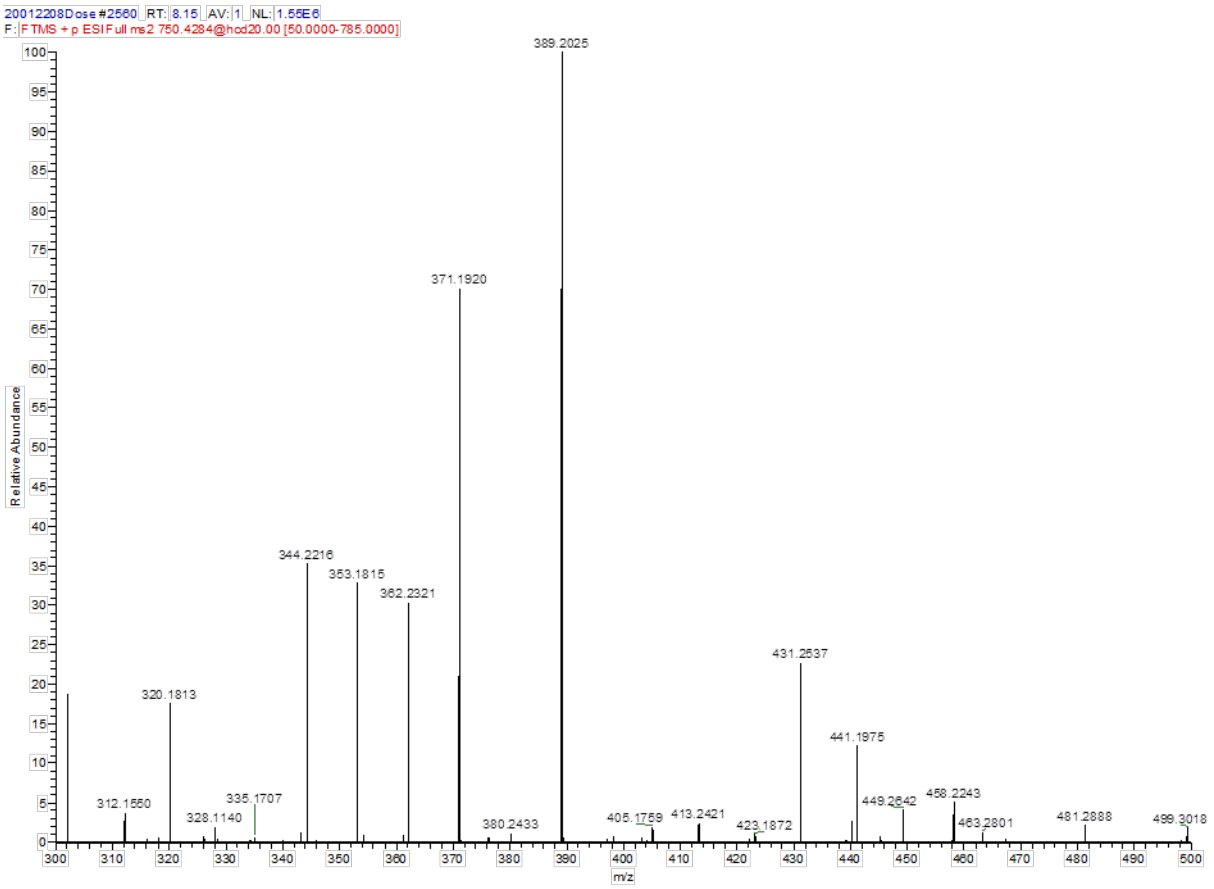


Figure S27. (+)ESI MS/MS spectrum of bolagladin M749 (**3**) mass range m/z 300–500.

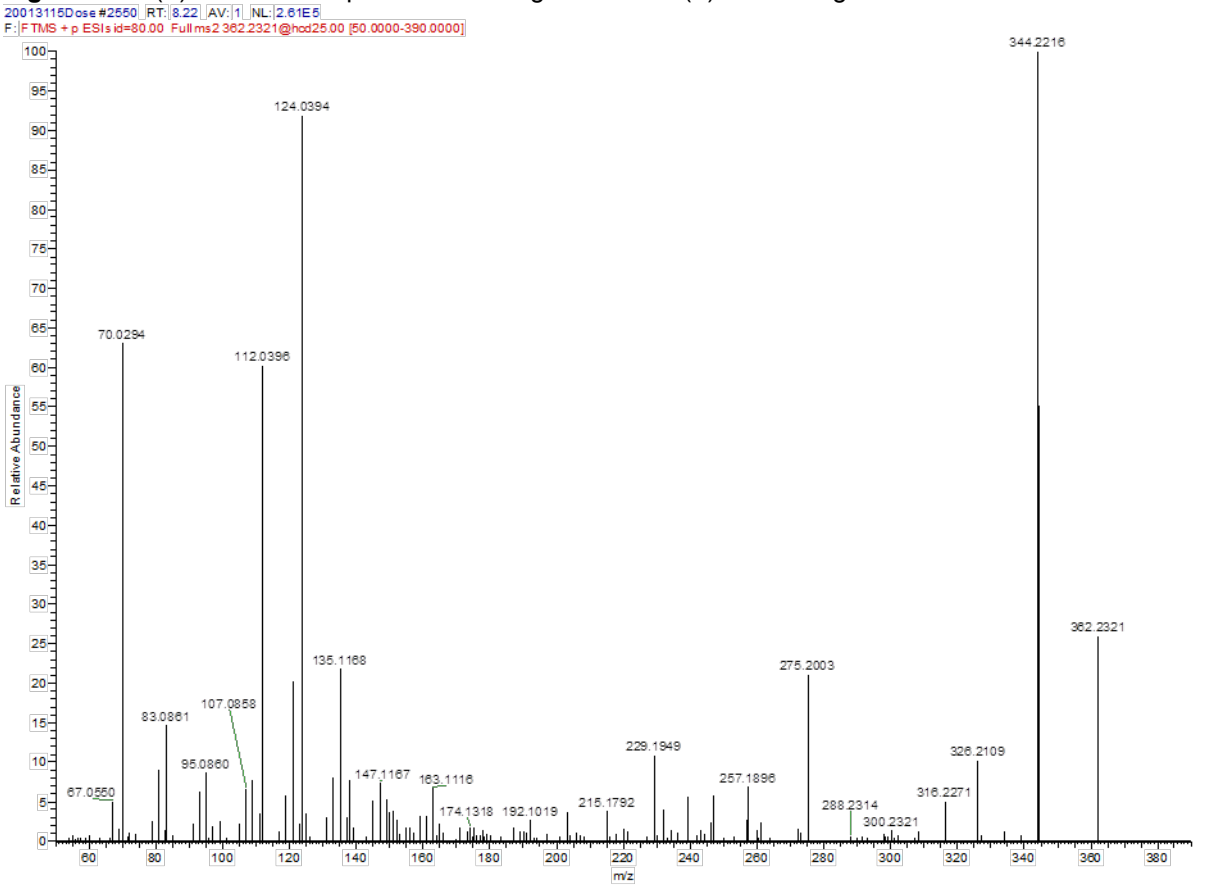
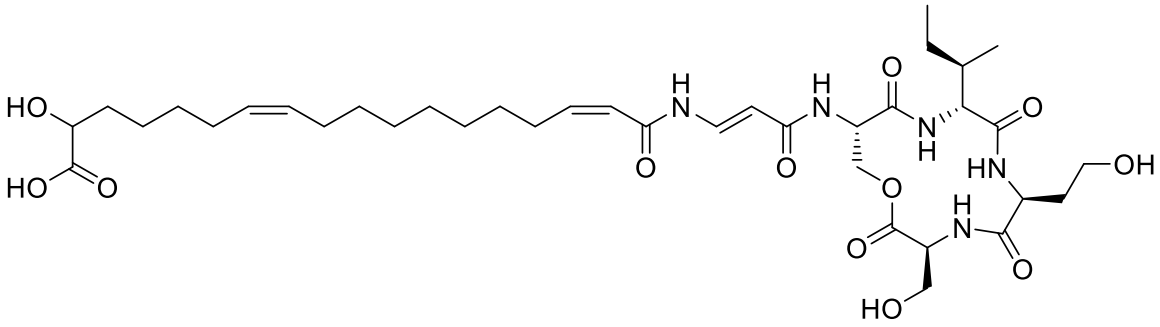


Figure S28. (+)ESI MS/MS spectrum of the fragment *m/z* 362 amu derived from bolagladin M749 (**3**).

Bolagladin M765 (4)



(–)ESI HR-MS: *m/z* 764.4099 $[M–H]^-$ (calcd. $C_{37}H_{58}N_5O_{12}$ 764.4087)

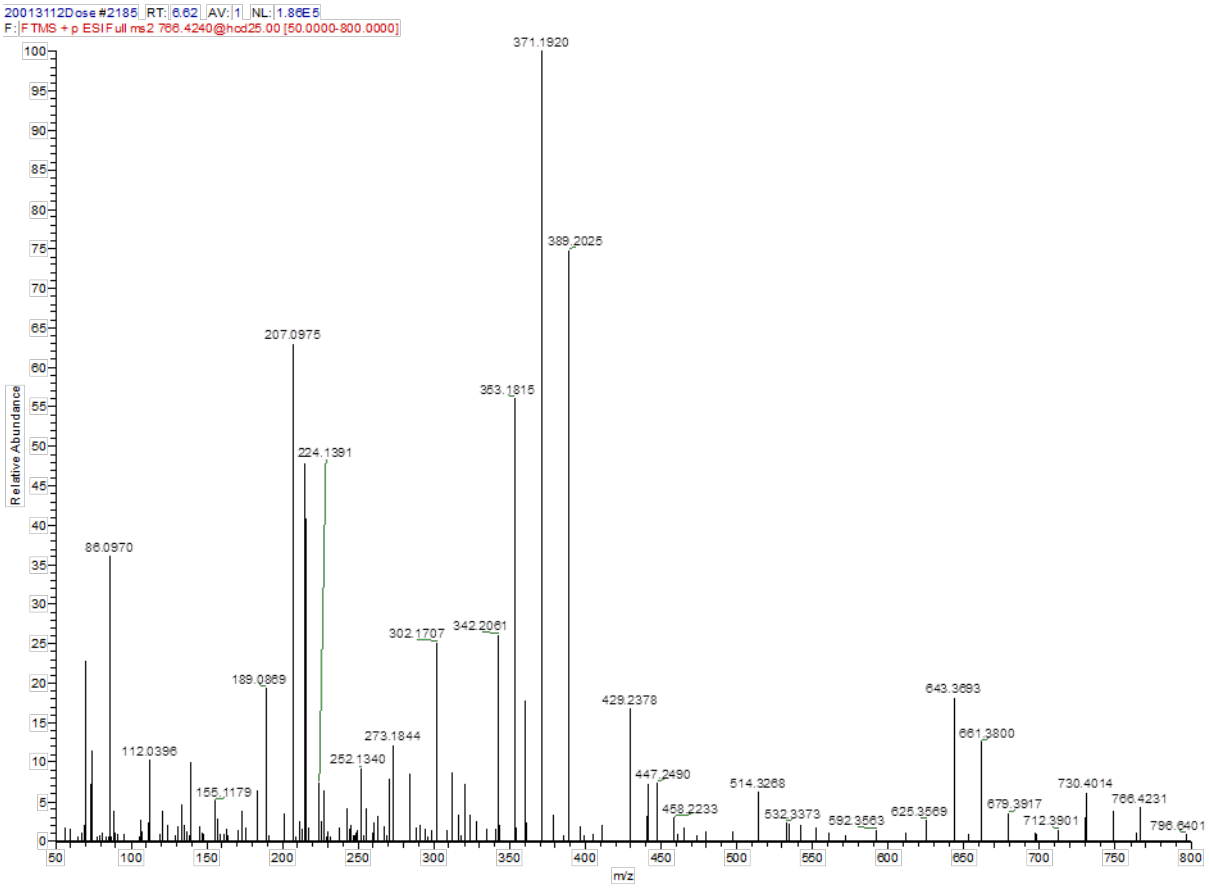


Figure S29. (+)ESI MS/MS spectrum of bolagladin M765 (**4**).

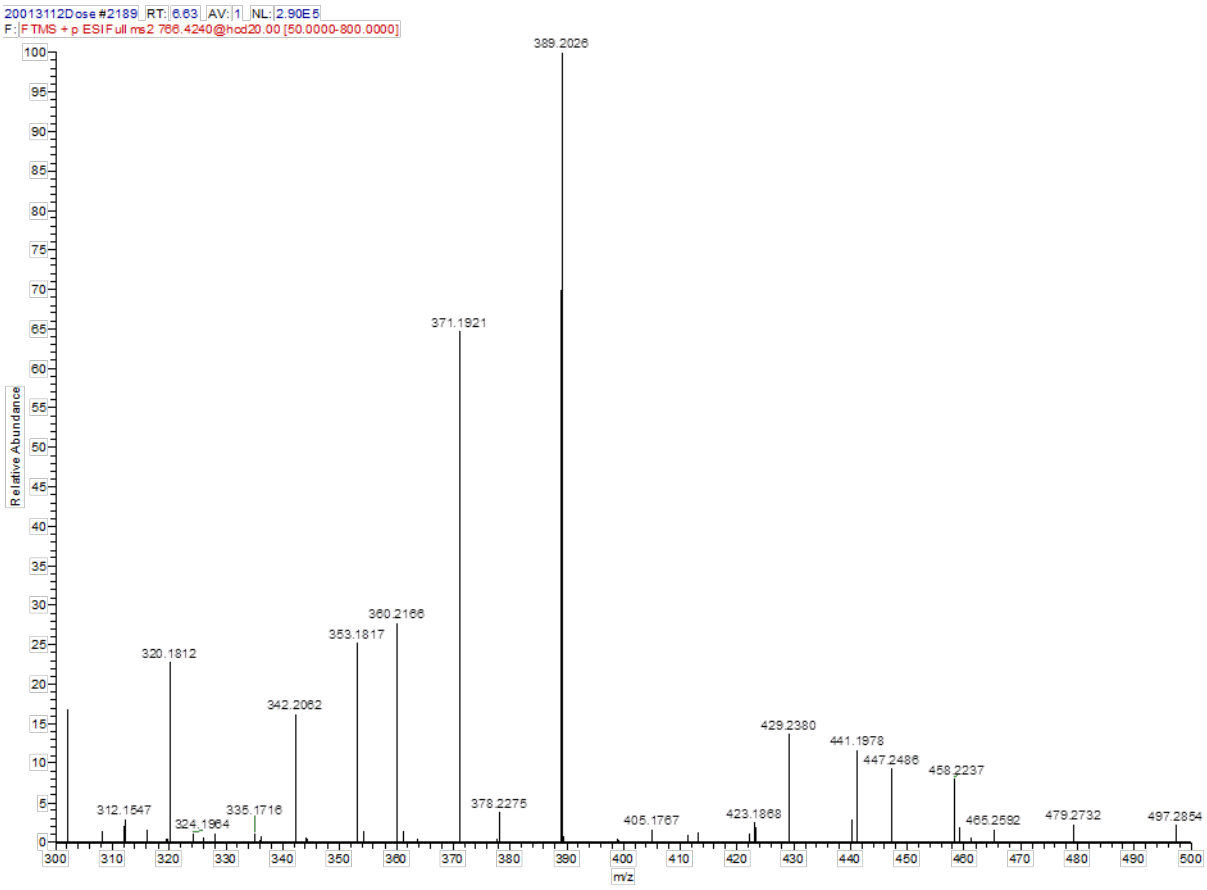


Figure S30. (+)ESI MS/MS spectrum of bolagladin M765 (**4**) mass range m/z 300–500.

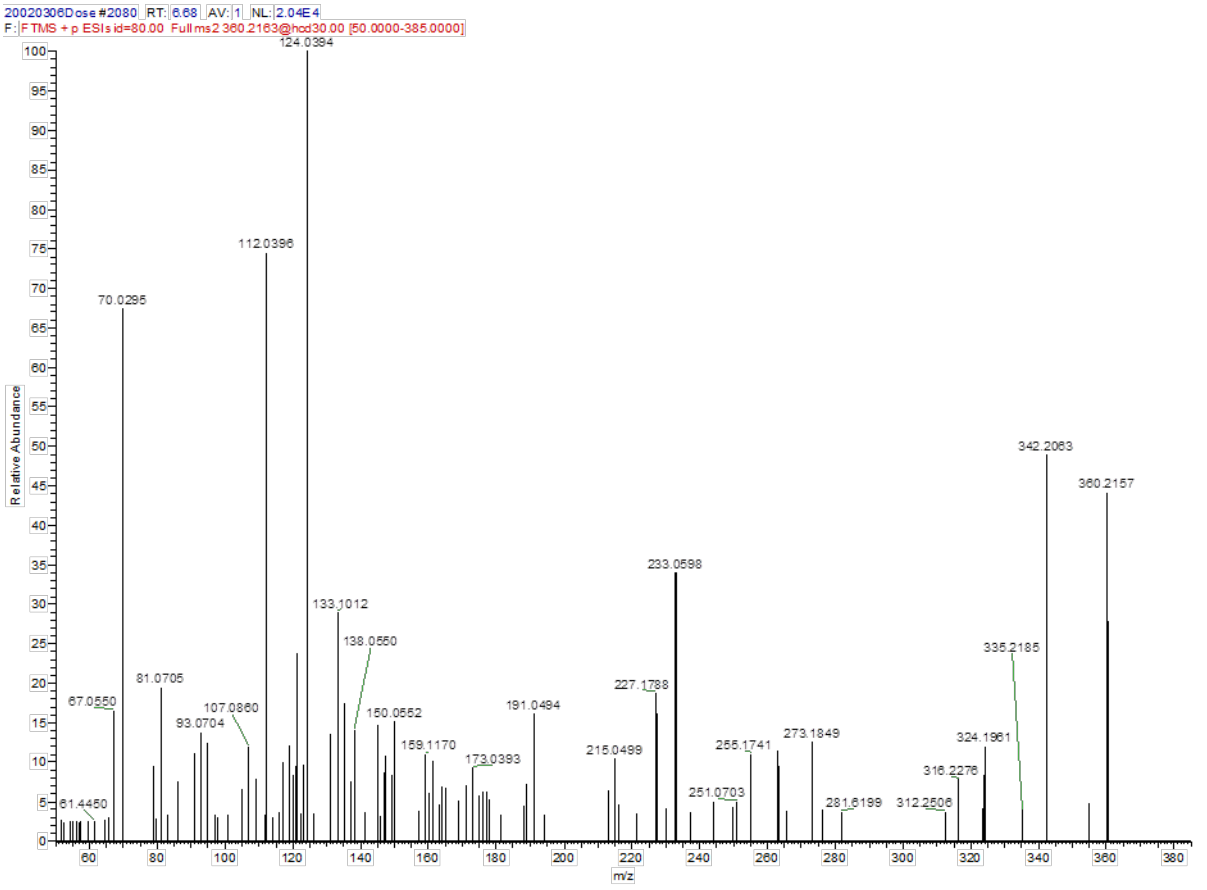
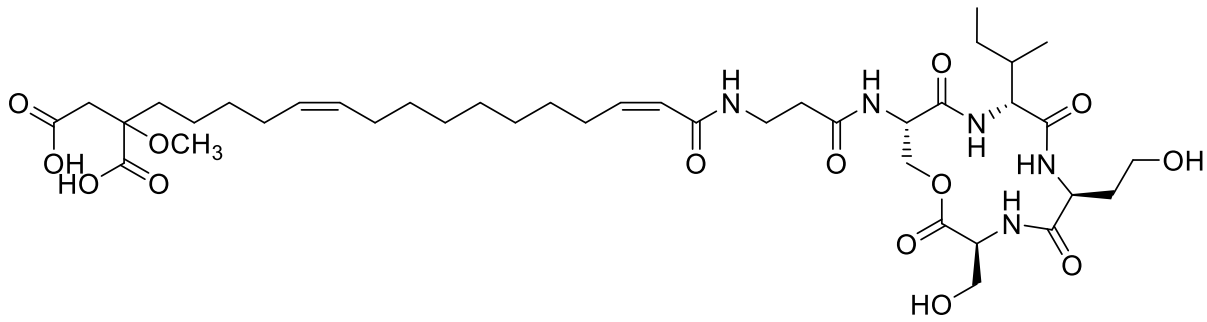


Figure S31. (+)ESI MS/MS spectrum of the fragment *m/z* 360 amu derived from bolagladin M765 (**4**).

Bolagladin M839 (5)

(–)ESI HR-MS: *m/z* 838.4479 $[M–H]^-$ (calcd. $C_{40}H_{64}N_5O_{14}$, 838.4455)



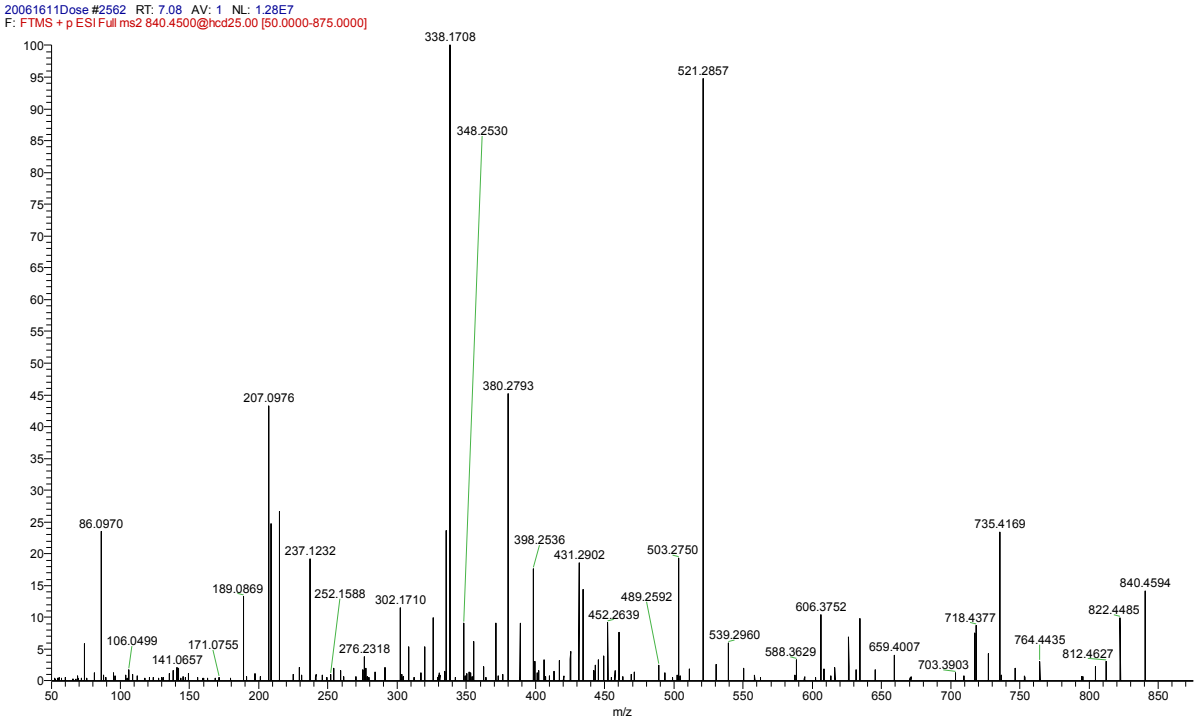


Figure S32. (+)ESI MS/MS spectrum of bolagladin M839 (**5**).

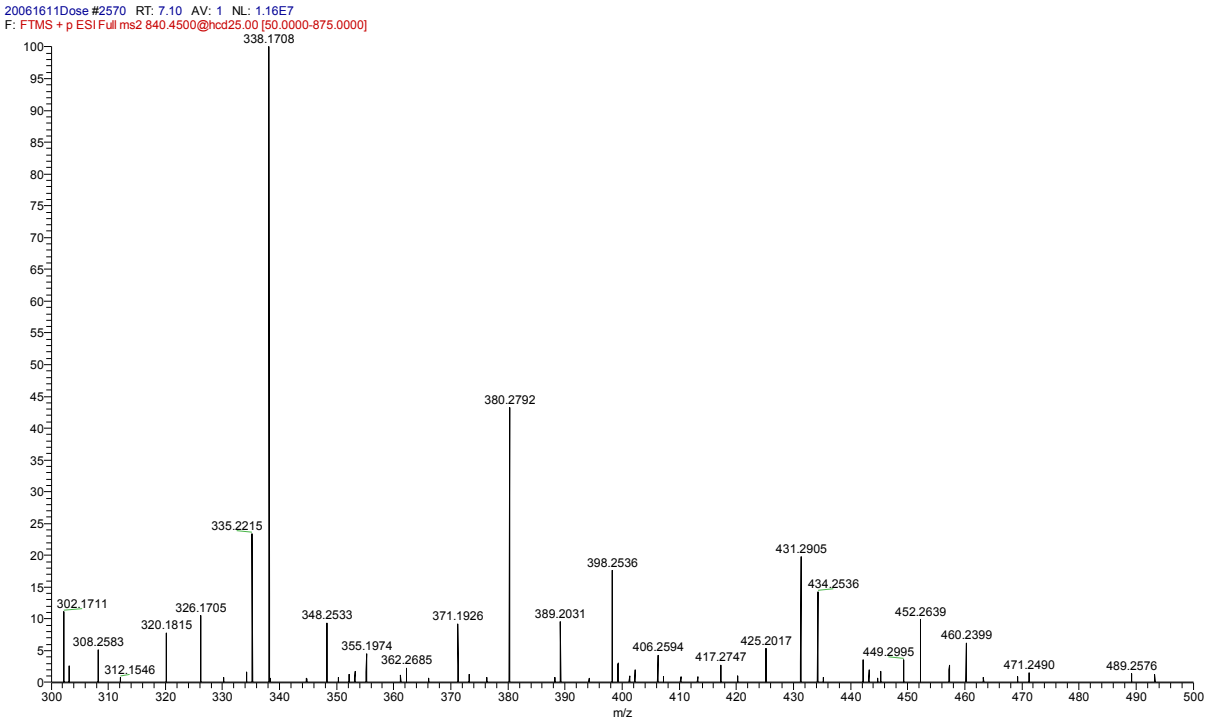
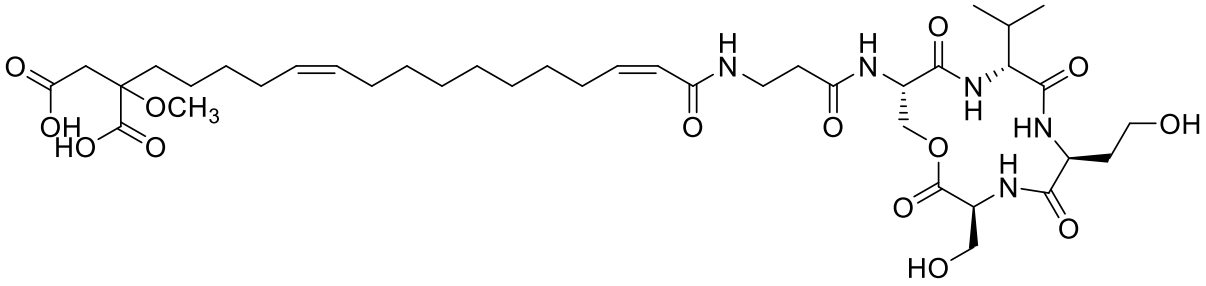


Figure S33. (+)ESI MS/MS spectrum of bolagladin M839 (**5**) mass range *m/z* 300–500.

Bolagladin M824 (6)

(–)ESI HR-MS: *m/z* 824.4322 $[M–H]^-$ (calcd. $C_{39}H_{62}N_5O_{14}$, 824.4299)



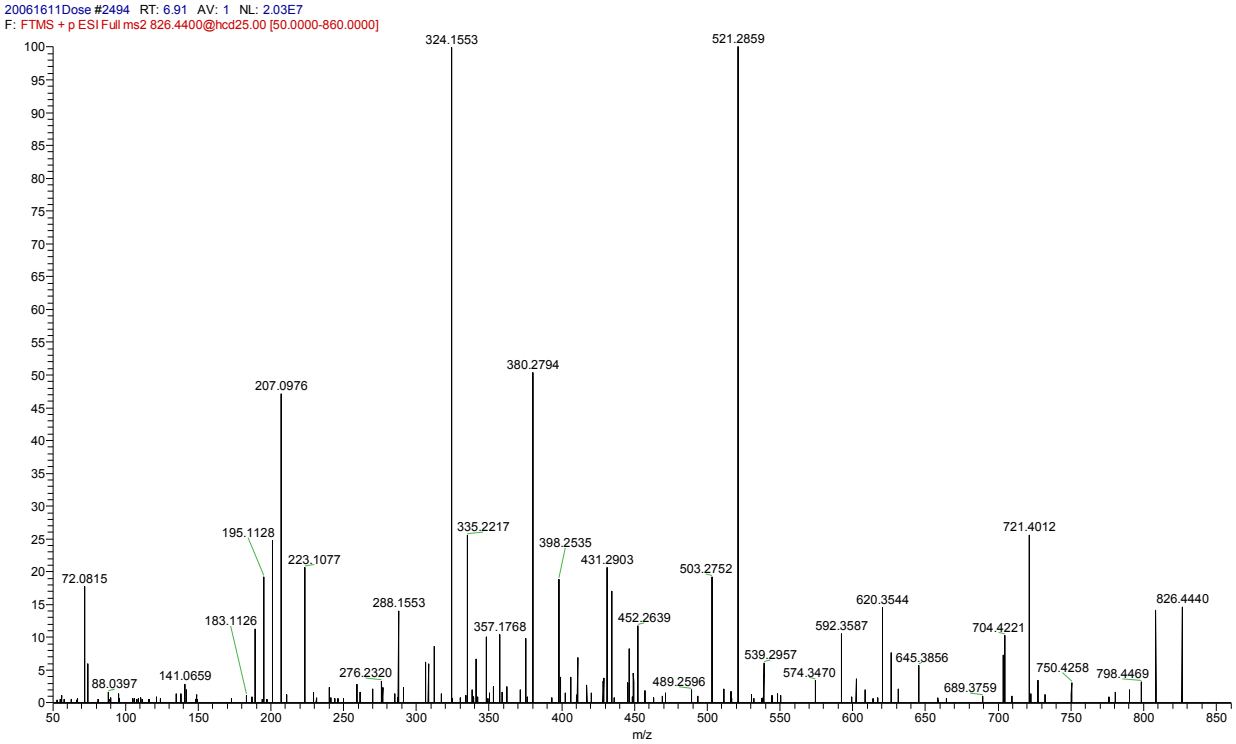


Figure S34. (+)ESI MS/MS spectrum of bolagladin M824 (**6**).

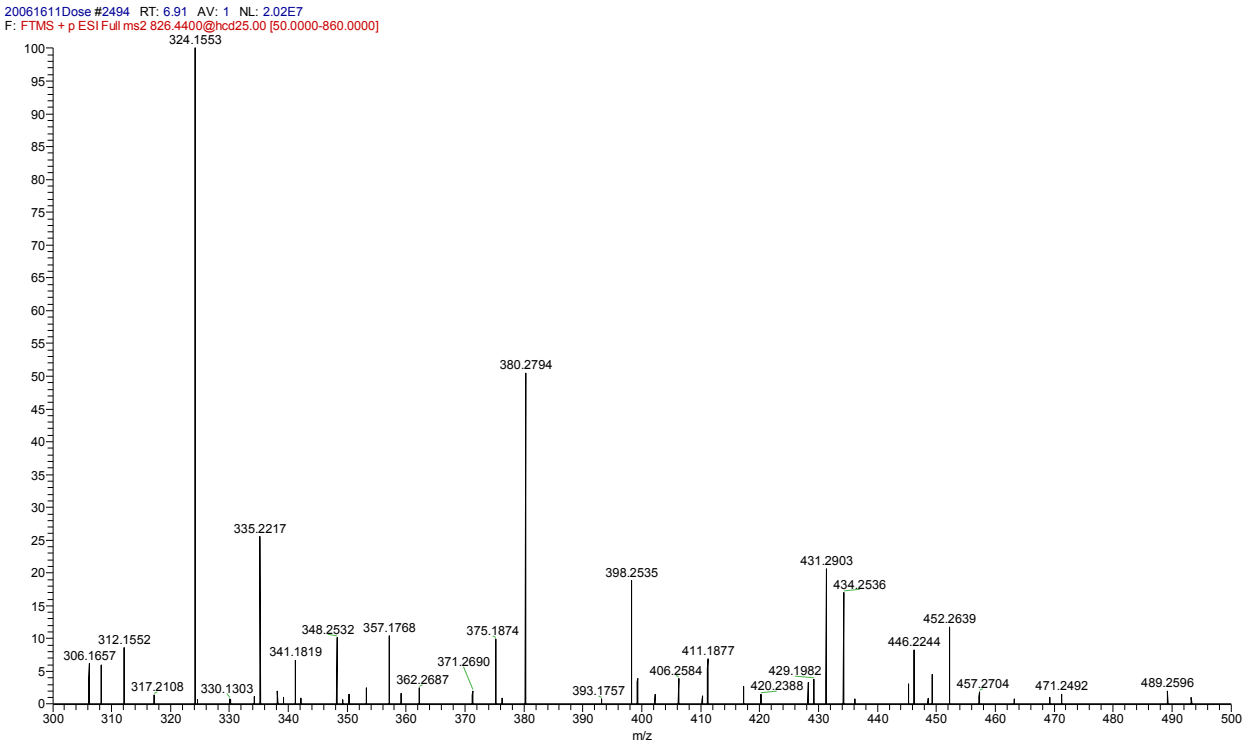


Figure S35. (+)ESI MS/MS spectrum of bolagladin M824 (**6**) mass range *m/z* 300–500.