ELECTRONIC SUPPLEMENTARY INFORMATION

A COMBINATION OF POLYUNSATURATED FATTY ACID, NONRIBOSOMAL PEPTIDE AND POLYKETIDE BIOSYNTHETIC MACHINERY IS USED TO ASSEMBLE THE ZEAMINE ANTIBIOTICS

Joleen Masschelein[a,b], Charlien Clauwers[a,b], Ufedo R. Awodi[c], Karen Stalmans[a], Wesley Vermaelen[a], Eveline Lescrinier[d], Abram Aertsen[b], Chris Michiels[b], Gregory L. Challis[c,*], Rob Lavigne[a,*]

[a] Laboratory of Gene Technology, KU Leuven, Kasteelpark Arenberg 21 - box 2462, B-3001 Heverlee, Belgium

[b] Laboratory of Food Microbiology, KU Leuven, Kasteelpark Arenberg 22, B-3001 Heverlee, Belgium

[c] Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK

[d] Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

* Corresponding authors:
Professor GL Challis. Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK. E-mail: g.l.challis@warwick.ac.uk; Fax: +44 (0)2476 524112; Tel: +44 (0)2476 574024

Professor R Lavigne. Division of Gene Technology, KU Leuven, Kasteelpark Arenberg 21 - box 2462, B-3001 Heverlee, Belgium. E-mail: rob.lavigne@biw.kuleuven.be; Fax: +32 (0)16 32 19 65; Tel: +32 (0)16 37 95 24

EXPERIMENTAL SECTION

Bacterial strains, plasmids and culture conditions

Serratia plymuthica RVH1 served as the wild type zeamine producer[1] whereas the RVH1Δ*zmn22* and the RVH1Δ*zmn18* mutants were used for production and isolation of the prezeamines and zeamine II respectively. *Escherichia coli* S17-1 was used for conjugative transfer of DNA to *S. plymuthica* RVH1. The commercially available *E. coli* TOP10 and *E. coli* BL21 (DE3) pLysS (Life Technologies) were used for routine cloning purposes and protein expression respectively. *Staphylococcus aureus* ATCC27661 and *Escherichia coli* MG1655 were used in antimicrobial activity assays. Plasmids and bacterial strains used or constructed during this work are listed in Table S6.

S. plymuthica RVH1 was grown at 30°C in Lysogeny Broth (LB) for liquid cultivation and on LB agar (LB broth supplemented with 1.5% w/v agar) for solid cultivation. *E. coli* and *S. aureus* strains were grown at 37°C in LB broth and on LB-agar. When appropriate, media were supplemented with antibiotics at the following concentrations: kanamycin (50 µg/ml), ampicillin (100 µg/ml), carbenicillin (50 µg/ml).

Gene deletion and complementation in *S. plymuthica* RVH1

In-frame deletion of target genes in *Serratia plymuthica* RVH1 was accomplished via double homologous recombination. The chromosomal fragments (approx. 500 bp) that flank the genes were amplified and cloned into the suicide vector pSF100[2] and introduced into *E. coli* S17-1 λ-pir by electroporation. The construct was transferred from *E. coli* S17-1 λ-pir to *Serratia plymuthica* RVH1 via biparental mating. More specifically, late exponential cultures of both strains (0.5 ml) were mixed on a 0.45 µm filter and placed on an LB agar plate for overnight incubation at 30°C. The mixture was resuspended in 1 ml of sterile saline solution and spread on LB agar plates supplemented with kanamycin. Subsequent overnight incubation at 16°C allowed the selective growth of *Serratia* clones carrying the suicide plasmid while restricting *E. coli* growth. To select for vector excision, co-integrant clones were replica plated on LB agar plates and LB agar plates supplemented with kanamycin. Knock-out mutagenesis was confirmed by PCR and sequencing.

Genetic complementations were made by amplifying the ORF of interest by PCR and cloning it into the pTrc99A expression vector under the control of a trc promoter.[3] The resulting construct was introduced via electroporation to the corresponding *S. plymuthica* RVH1 deletion mutant. Transformants were selected on LB agar plates supplemented with carbenicillin.

For an overview of all gene deletion and complementation experiments, see table S1.

Antibacterial activity assays

Minimal inhibitory concentrations (MIC) of purified zeamine compounds were determined by the broth microdilution method (National Committee for Clinical Laboratory Standards, document M7-A5). In a 96-well microtiter plate, 50 µl of serial twofold dilutions of the antibiotics in LB broth were mixed with 50 µl of bacterial suspension (10^6 CFU/ml in LB broth). After 18h incubation at 37°C, MICs were defined as the lowest concentrations that visibly inhibited bacterial growth.

The antimicrobial activity of knockout mutants and complementation strains was monitored by plate lawn assays. An LB agar plate was overlaid with a lawn of stationary phase *S. aureus* ATCC27661 cells, diluted to OD_{600nm} = 0,03. 10 µl samples of stationary phase test strains were spotted on top of the lawn. After incubation for 48h at 16°C and 24h at 30°C, the size of the inhibition zones was measured. Complementation strains were grown to stationary phase in LB medium supplemented with carbenicillin to maintain the plasmid. Prior to spotting, the cells were washed with LB-medium to remove traces of the antibiotic. IPTG (0 – 0.1 – 0.5 mM) was added to the agar plates to induce expression.

LC-MS analysis of zeamine production

Supernatant from 50 mL cultures of wild type *S. plymuthica* RVH1 and deletion mutants grown in minimal medium were analyzed by ultra-high resolution LC-MS using a Dionex UltiMate 3000 UHPLC connected to a Zorbax Eclipse Plus column (C_{18}, 100 × 2.1 mm, 1.8 µm) coupled to a Bruker MaXis IMPACT ESI-QTOF mass spectrometer. Mobile phases consisted of water (A) and acetonitrile (B), each supplemented with 0.1% formic acid. A gradient of 5% B to 100% B in 20 minutes was employed at a flow rate of 0.1 mL/min. The mass spectrometer was operated in positive ion mode with a scan range of 50–3000 m/z. Source conditions were: end plate offset at −500 V; capillary at −4500 V; nebulizer gas (N_2) at 1.6 bar; dry gas (N_2) at 8 L min−1; dry temperature at 180 °C. Ion transfer conditions were: ion funnel RF at 200 Vpp; multiple RF at 200 Vpp; quadrupole low mass at 55 m/z; collision energy at 5.0 eV; collision RF at 600 Vpp; ion cooler RF at 50–350 Vpp; transfer time at 121 µs; pre-pulse storage time at 1 µs. Calibration was performed with 10 mM sodium formate through a loop injection of 20 µL at the start of each run.

Purification of zeamine, zeamine I and zeamine II

For production and purification of zeamine and zeamine I, *Serratia plymuthica* RVH1 *w*as grown in minimal medium[4] (10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 2 g/l mannitol, 0.2 g/l $MgSO_4.7H_2O$, 5 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ and 2 mg/l $MnCl_2$; pH 7.0) supplemented with 5 µM synthetic N-(3-oxo-hexanoyl)-C_6-homoserine lactone (Sigma) at 16°C for 72 hours on a rotary shaker (80 rpm). Cells were removed by centrifugation (4600g, 45 min), after which the supernatant was adjusted to pH 10.0 with 10M NaOH and extracted (1:1) with n-butanol twice. The extract was concentrated *in vacuo* and the resulting residue was redissolved in MeOH-CH_2Cl_2 (1-8). This solution was acidified (pH 3) with formic acid and subjected to silica gel column chromatography as described previously[5]. Zeamine and zeamine I eluted with a slightly increased retention time compared to zeamine II. Fractions of interest were pooled and dried by rotary evaporation *in vacuo* and lyophilization. Residual NaCl was removed by redissolving the solids in $CHCl_3$ and extracting (1:1) the resulting solution with 1% NaOH. The organic phase was then dried over $MgSO_4$, filtered and concentrated to dryness *in vacuo* to give the purified zeamines as a colourless solid.

For purification of zeamine II, *Serratia plymuthica* RVH1Δ*zmn18 w*as used as the producing strain. Fermentation conditions and extraction procedures were identical to those employed for the isolation of zeamine and zeamine I. The extract was dried by rotary evaporation *in vacuo* and lyophilization. Solids were dissolved in MeOH supplemented with 5% triethylamine, mixed with three times the amount of silica and allowed to dry overnight. The silica powder was then added to the top of a silica column and washed sequentially with MeOH-CH_2Cl_2 (1-8), MeOH-CH_2Cl_2 (1-4), MeOH-CH_2Cl_2 (1-2), MeOH-CH_2Cl_2 (1-1) and 100% MeOH. Zeamine II eluted when 1% formic acid was added to the MeOH solvent.

Purification and structure elucidation of the prezeamines

Serratia plymuthica RVH1 Δ*zmn22* was grown in minimal medium[4] (10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 2 g/l mannitol, 0.2 g/l $MgSO_4.7H_2O$, 5 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ and 2 mg/l $MnCl_2$; pH 7.0) supplemented with 5 µM synthetic N-(3-oxo-hexanoyl)-C_6-homoserine lactone (Sigma) at 16°C for 72 hours on a rotary shaker (80 rpm). Cells were pelleted by centrifugation (4600 rpm, 45 min, 4°C) and the culture supernatant was added to Diaion HP-20 resin (20 g/L, Sigma). The resin was washed sequentially with 10%, 30%, 50% and 100% acetonitrile (MeCN) in water. The prezeamines eluted with 30% MeCN as detected by LC-MS. This solution was dried by rotary evaporation *in vacuo* and subsequent lyophilization. Solids were washed with MeOH and finally dissolved in H_2O.

High resolution LC-ESI-MS/MS analysis was performed with a Dionex UHPLC Ultimate 3000 equipped with a Dionex Acclaim Pepmap 100 column (C_{18}, 75 µm x 15 cm, 3 µm, 100 Å) coupled to a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer. Mobile phases consisted of water (A) and 80% acetonitrile (B), each

supplemented with 0.1% formic acid. Samples were eluted at a flow rate of 0.300 µL/min using a gradient of 4% to 65% B in 40 min. The mass spectrometer was operated in nano-online positive ion mode using the following parameters: scan range: 200-1600 m/z, nanospray voltage: 2.2kV, source temperature: 180°C, normalized collision energy: 30 eV, isolation window: ± 3 Da. ProteoMAss LTQ/FT-Hybrid ESI Pos. Mode CalMix (MSCAL5-1EA SUPELCO, Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. The Thermo RaW files were converted to mzXML format using MassMatrix v3.9 and further analyzed with MZmine 2.

For NMR analysis, the sample was prepared by dissolving 10 mg prezeamine (**4**) in 600 µl D_2O. All spectra were recorded at 288 K using a 5 mm TCI HCN Z gradient cryoprobe on a Bruker Avance II 600 operating at a proton frequency of 600.13 MHz and ^{13}C-frequency of 150.92 MHz. The Bruker Topspin 2.1 software was used to process the spectra. The signal of residual solvent was suppressed by presaturation in the 1D proton spectrum. ^{1}H-chemical shifts are referenced to the HOD signal (4.87 ppm) while ^{13}C-chemical shifts were indirectly referenced using $SR_{13C}=0.25144954*SR_{1H}$ according to IUPAC recommendations.[6] To obtain the assignment of non-exchangeable proton and carbon resonances (listed in table S4) various 2-D techniques were used. HSQC and HSQC-TOCSY spectra were recorded using 64 scans and 1024/1024 complex data points and 100/11 ppm spectral widths in t1 and t2, respectively. The pulse sequence for the 2-D gradient enhanced ^{1}H-^{13}C HSQC was as described by Sattler et al.[7] The delays Δ1 and Δ2 in the inept transfer were set to 1.67 ms (1/4JCH). The 2-D HSQC–TOCSY consisted of an HSQC building block[8] (Δ1 = Δ2 = 1.67 ms) followed by a clean MLEV17 TOCSY transfer step[9] during a mixing time of 60 ms prior to detection. Decoupling during the acquisition of the HSQC and HSQC–TOCSY spectra was achieved by using the garp sequence.[10] In the HMBC[11] spectrum a delay of 62.5 ms was used to allow long-range couplings to evolve. It was recorded using 64 scans and 1024/1024 complex data points and 200/11 ppm spectral widths in t1 and t2, respectively. Sine-bell shaped gradients were applied along the z-axis during the sequences to obtain coherence selection and sensitivity enhancement. Prior to Fourier transformation, a squared sine-bell function was applied in both dimensions of 2-D spectra.

Feeding experiments

Overnight cultures of *Serratia plymuthica* RVH1Δ*zmn10,* Δ*zmn11 and* Δ*zmn12* in LB-medium were inoculated 1:1000 in minimal medium (10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 2 g/l mannitol, 0.2 g/l $MgSO_4.7H_2O$, 5 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ and 2 mg/l $MnCl_2$; pH 7.0) and incubated at 16°C on a rotary shaker (80 rpm). After 16h, fermentation cultures were supplemented with 5 µM synthetic N-(3-oxo-hexanoyl)-C_6-homoserine lactone and 0.9 mM zeamine II (75 mM stock solution in MeOH) and allowed to grow for an additional 72h. Supernatant from these cultures was subsequently analyzed by ultra-high resolution LC-MS as described above.

Cloning, overexpression and purification of Zmn14

zmn14 (1233 bp) was amplified from purified genomic DNA of *S. plymuthica* RVH1 with a final extension step to add a single deoxyadenosine to the 3' ends of the PCR product (Pfu polymerase; Fermentas). The purified PCR product was subsequently ligated in linearized pEXP5-CT/TOPO® vector with single overhanging 3' deoxythymidine (T) residues using the pEXP5-CT/TOPO® TA Expression Kit (Invitrogen). This ligation mixture was used to transform One Shot® chemically competent *E. coli* TOP10 cells. Transformants were selected on LB plates supplemented with ampicillin and the correct orientation of the construct was confirmed by sequence analysis (ABI 3130 Sequencer, BigDye chemistry, Applied Biosystems). Correct constructs were used to transform chemically competent *E. coli* BL21 Star™ (DE3) pLysS cells (Invitrogen) for expression of *zmn14* as a His_6-tag fusion protein. Recombinant Zmn14 was overproduced overnight at 16°C (180 rpm) after induction of exponentially growing cells ($0D_{600nm}$ = 0.6) with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in 500 ml LB-medium. Cells were harvested by centrifugation (3000 rpm, 20 min, 4°C) and cell pellets were resuspended in 10 ml lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 10% glycerol, pH 8.5) Cells were lysed using a high pressure cell disruptor (20000 psi, Constant Systems, LA Biosystems) and the lysate was subsequently centrifuged (11000 rpm, 30 min, 4°C) to remove cellular debris.

Protein purification was carried out on a GE Healthcare Akta Purifier using a HisTrap™ HP 1 ml column (GE Healthcare). After column equilibration with washing buffer (50 mM imidazole, 20 mM NaH_2PO_4, 0.5 M NaCl, 10% glycerol, pH 8.5, 2 column volumes) the lysate was loaded and washed with 20 column volumes of washing buffer. The protein was eluted in elution buffer (500 mM imidazole, 20 mM NaH_2PO_4, 0.5 M NaCl, 10% glycerol, pH 8.5, 15 column volumes) and applied to a Amicon Ultra centrifugal filtration device (10000 MWCO, Millipore) for concentration and buffer exchange. The protein was shown to have a purity of over 95% as assessed by SDS-PAGE.

Enzymatic assays

NAD(P)H consumption and substrate specificity of Zmn14 were examined as described by Chhabra et al.[12] Purified enzyme (20 µM) was mixed with NAD(P)H (200 µM), substrate (200 µM) and buffer (10mM NaH_2PO_4, 0.25M NaCl, pH 7.4) and the change in NAD(P)H absorption was monitored during a period of 30 min using a Perkin Elmer LS55 fluorescence spectrometer (25°C, λ_{ex} = 340 nm, λ_{em} = 462 nm, 5 nm slits, 0.1 sec data interval). Test substrates used were lauroyl-CoA, butyryl-coA, octanoyl-CoA, butanal, octanal, 2-butanone and 2-octanone (Sigma).

To detect octanal conversion to octanol, a reaction was set up consisting of 100 µM purified Zmn14, 20 mM octanal, 20 mM NADH and 25 mM NH_4HCO_3 buffer in a total volume of 500 µl. The reaction mixture was incubated overnight at room temperature and products were extracted twice with chloroform (1:1). The organic phase was subsequently dried with $MgSO_4$ and filtered using a cotton plug. One drop of N,O-Bis-(trimethylsilyl)acetamide was added, after which samples were analyzed by GC-MS (Varian 400). 5 µl samples were injected using a CP8400 sample injector in splitless mode and passed through a VF-5ms column (30m length, 0.25 mm internal diameter) using helium as a carrier gas. The temperature was held at 50 °C for 1 minute before being ramped up to 300 °C at a rate of 25 °C/min. The temperature was held at 300 °C for 9 minutes before being ramped back down. The sample was ionized using electron ionization with 70 eV electrons and detected with an ion trap mass analyzer. Two independent assays were performed.

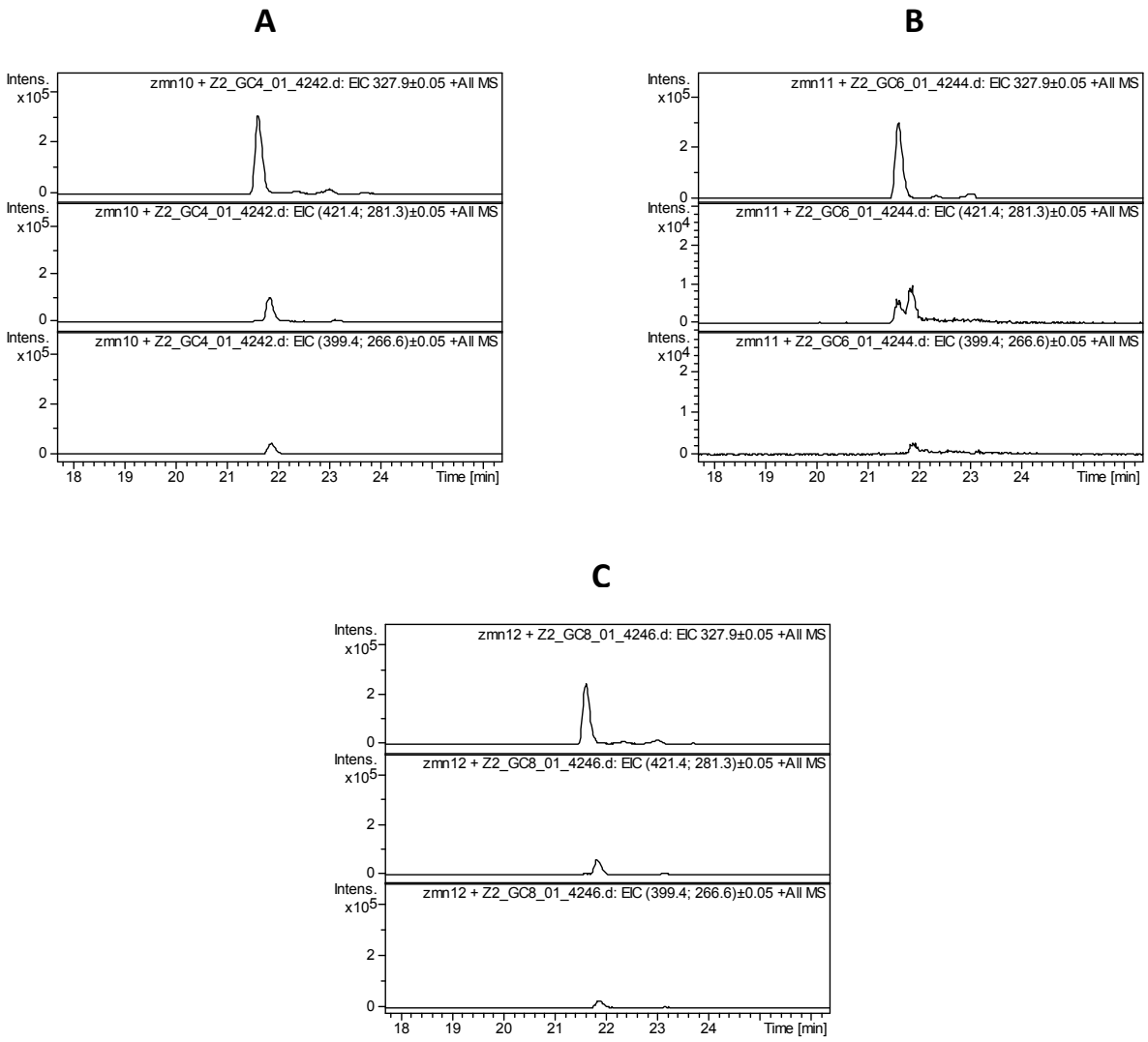

Fig. S1 Extracted ion chromatograms at m/z 327.9 ± 0.05 (top), 421.4 and 281.3 ± 0.05 (middle), and 399.4 and 266.6 ± 0.05 (bottom) from LC-ESI-MS analyses of culture supernatants from small scale feedings of zeamine II ($[M+2H]^{2+}$ = 327.8533) (**1**) to the *zmn10* (A), *zmn11* (B) and *zmn12* (C) mutants. Production of zeamine ($[M+2H]^{2+}$ = 421.4138, $[M+3H]^{3+}$ = 281.2784) (**2**) and zeamine I ($[M+2H]^{2+}$ = 399.4006, $[M+3H]^{3+}$ = 266.6030) (**3**) can be observed when **1** is added to the cultivation medium, implicating the PUFA synthase-like multienzyme complex in the biosynthesis of **1**.

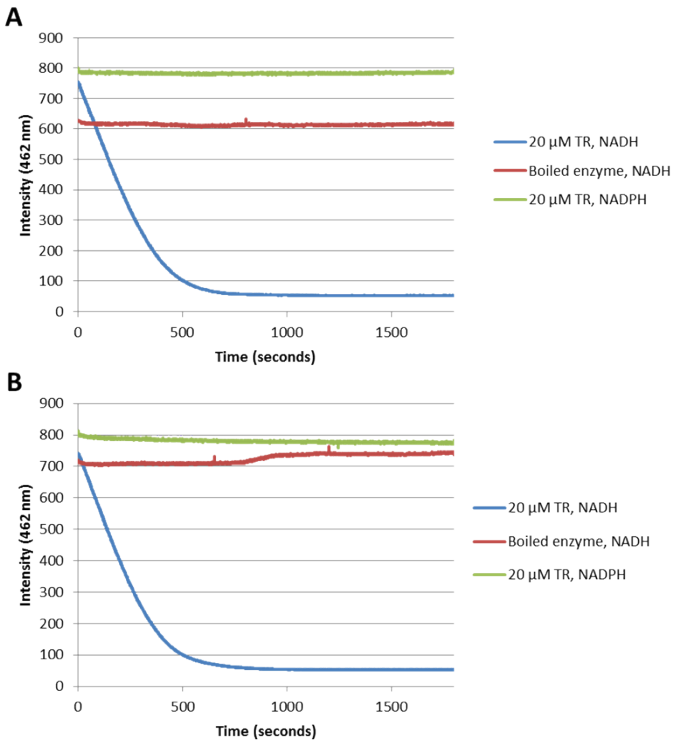

Fig. S2 Consumption of NADH when Zmn14 is incubated with either butanoyl-CoA (A) or octanoyl-CoA (B). The change in fluorescence emission at 462 nm was monitored.

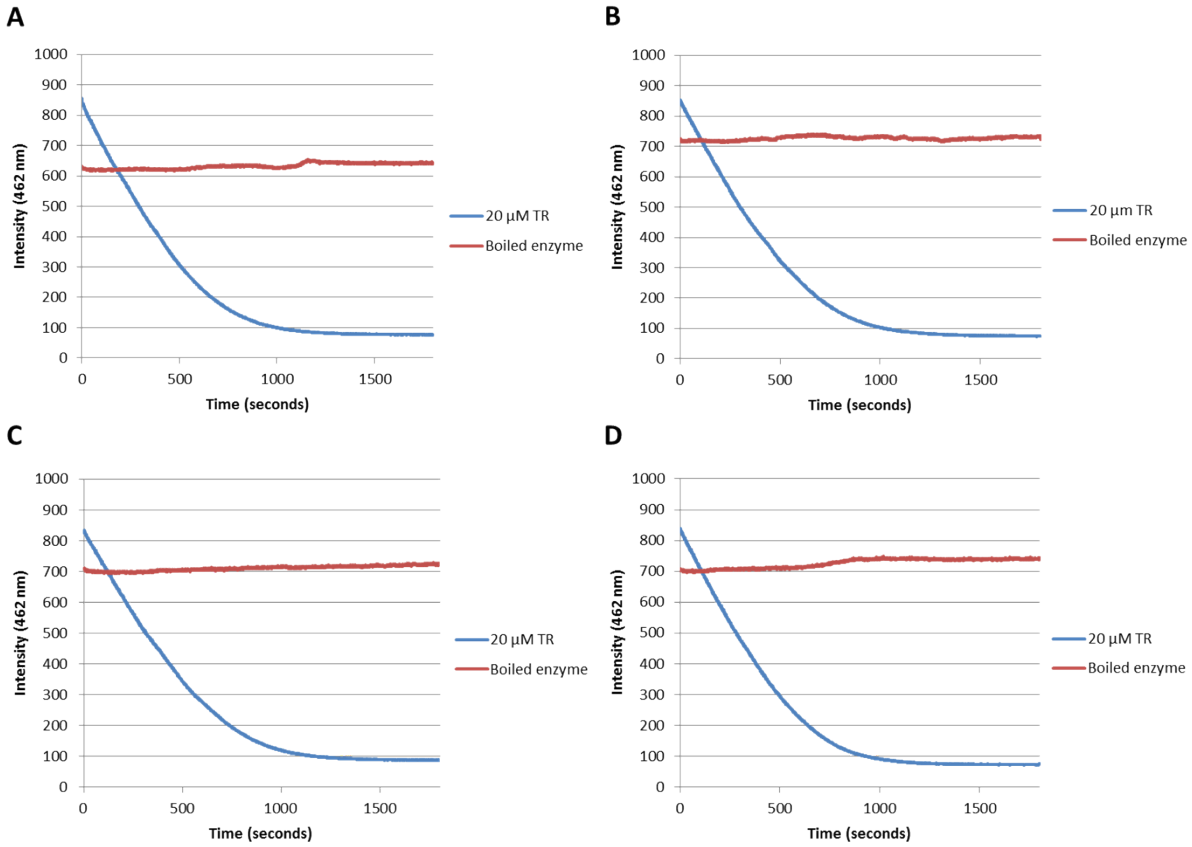

Fig. S3 Consumption of NADH when Zmn14 is incubated with butanal (A), octanal (B), butanone (C) or octanone (D). The change in fluorescence emission at 462 nm was monitored.

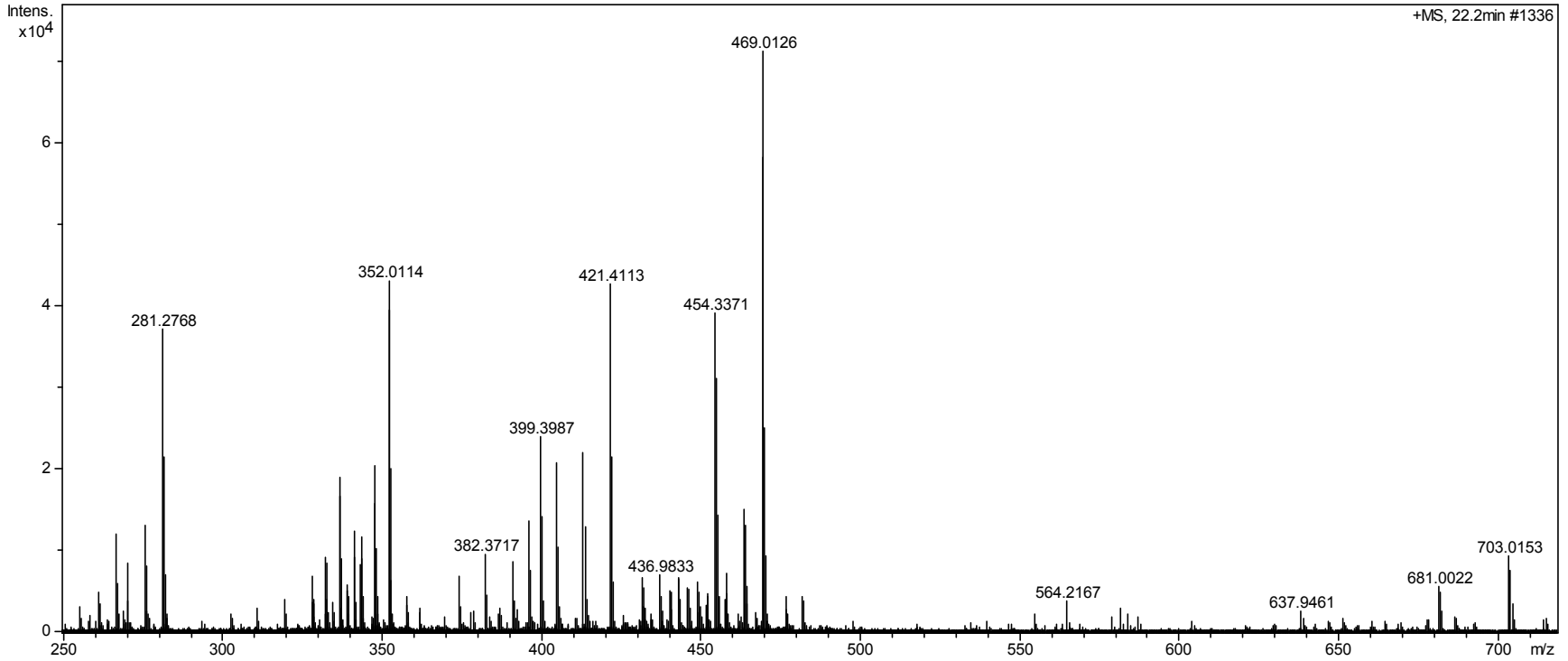

Fig. S4 High resolution mass spectrum of zeamines detected in wild type *S. plymuthica* RVH1 culture supernatant. Multiply-charged ions derived from zeamine (*m/z* = 841.8191), zeamine I (*m/z* = 797.7929), zeamine II (*m/z* = 654.6983), prezeamine (*m/z* = 1405.0286) and prezeamine I (*m/z* = 1361.0023) can be detected.

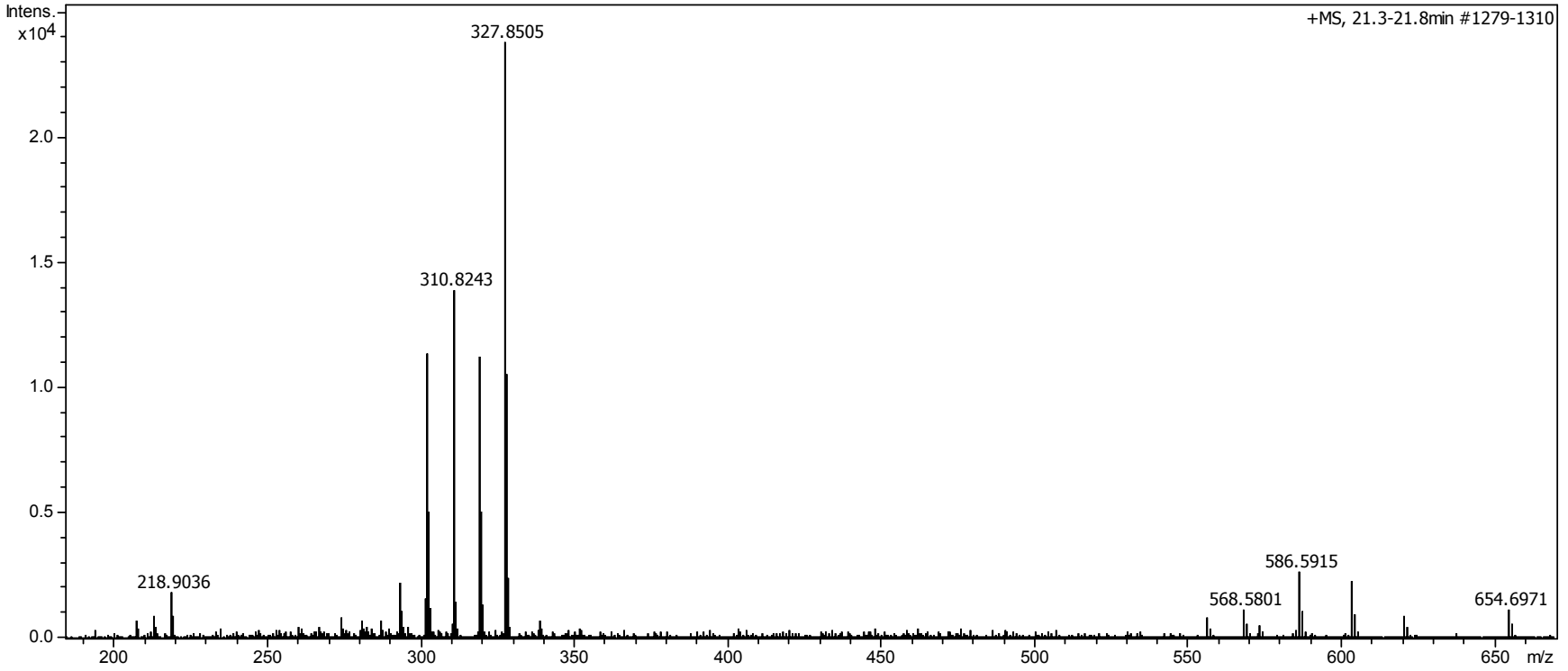

Fig. S5 High resolution mass spectrum of zeamine antibiotics accumulated in the culture supernatant of the *zmn17* deletion mutant. Only singly and multiply charged ions derived from zeamine II (*m/z* = 654.6971) were observed. Analogous results were observed for the Δ*zmn16*, Δ*zmn18* and Δ*zmn19* strains.

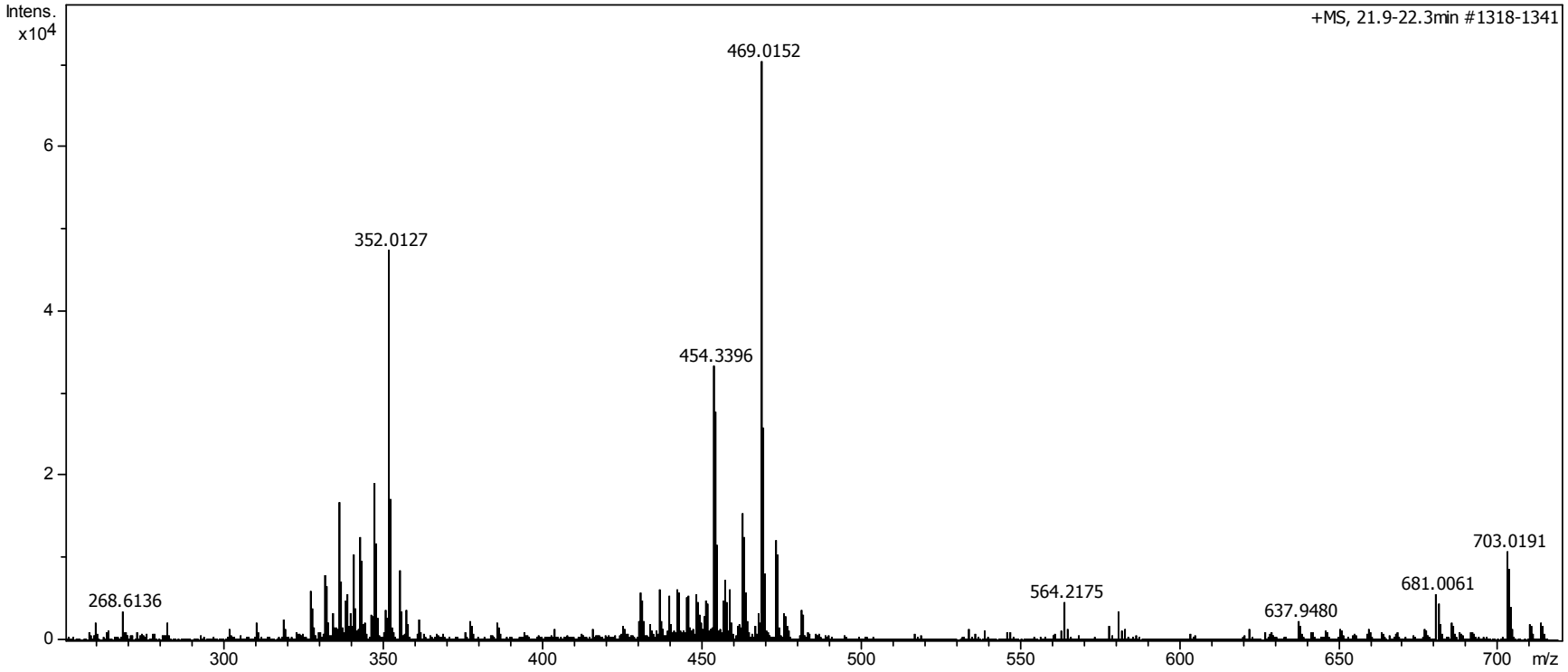

Fig. S6 High resolution mass spectrum of zeamines accumulated in the culture supernatant of the *zmn22* deletion mutant. Only singly and multiply charged ions derived from zeamine II (*m/z* = 654.6971), prezeamine (*m/z* = 1405.0286) and prezeamine I (*m/z* = 1361.0023) were observed.

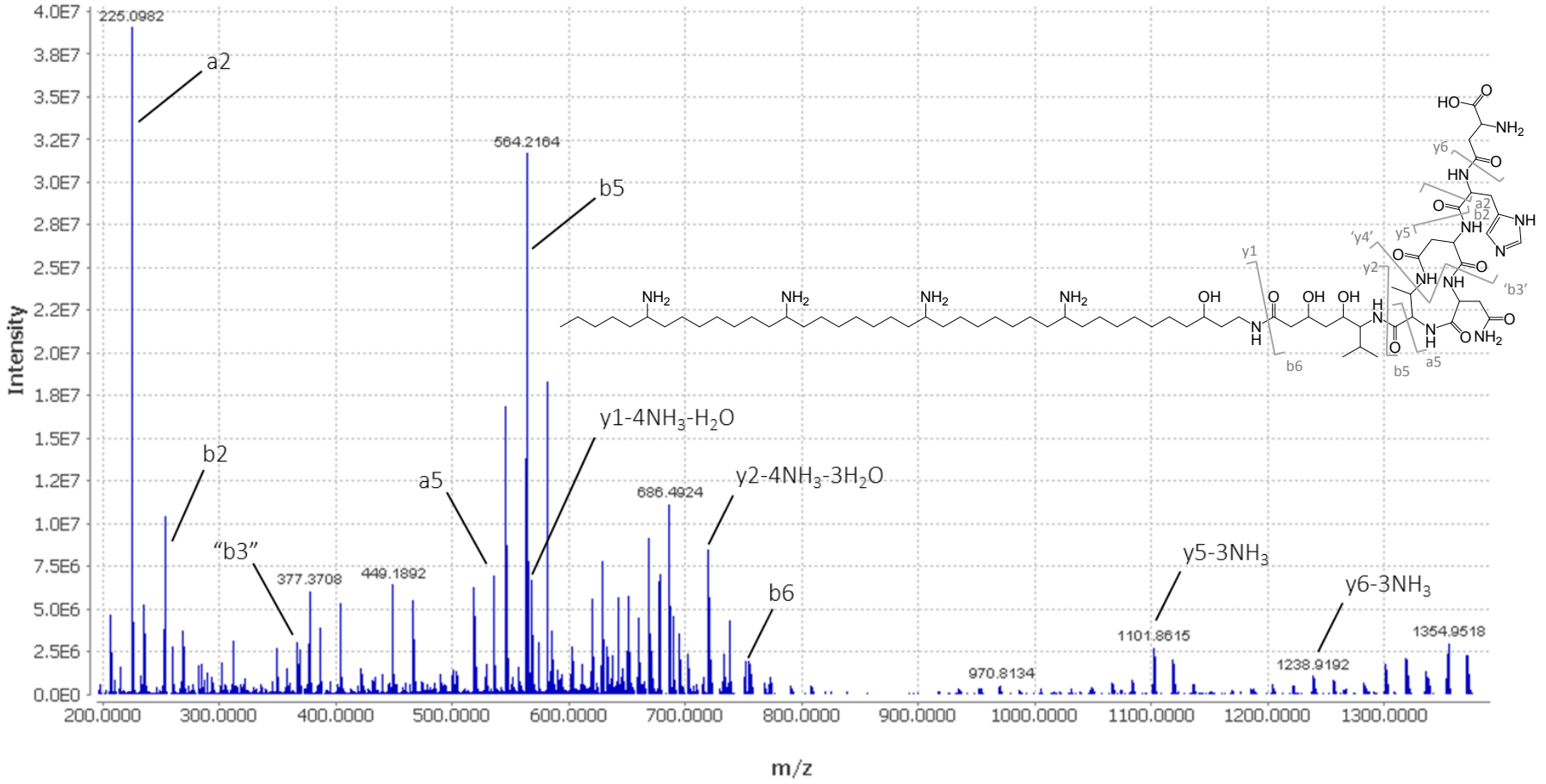

Fig. S7 High resolution LC-ESI-MS/MS spectrum of prezeamine (**4**) ([M+3H]$^{3+}$ = 469.0150 Da). Selected a, b and y-fragment ions are indicated. A comprehensive overview of the detected fragment ions and their molecular formulae, calculated *m/z* and observed *m/z* is shown in table S2. Inset: structure of prezeamine and detected fragment ions.

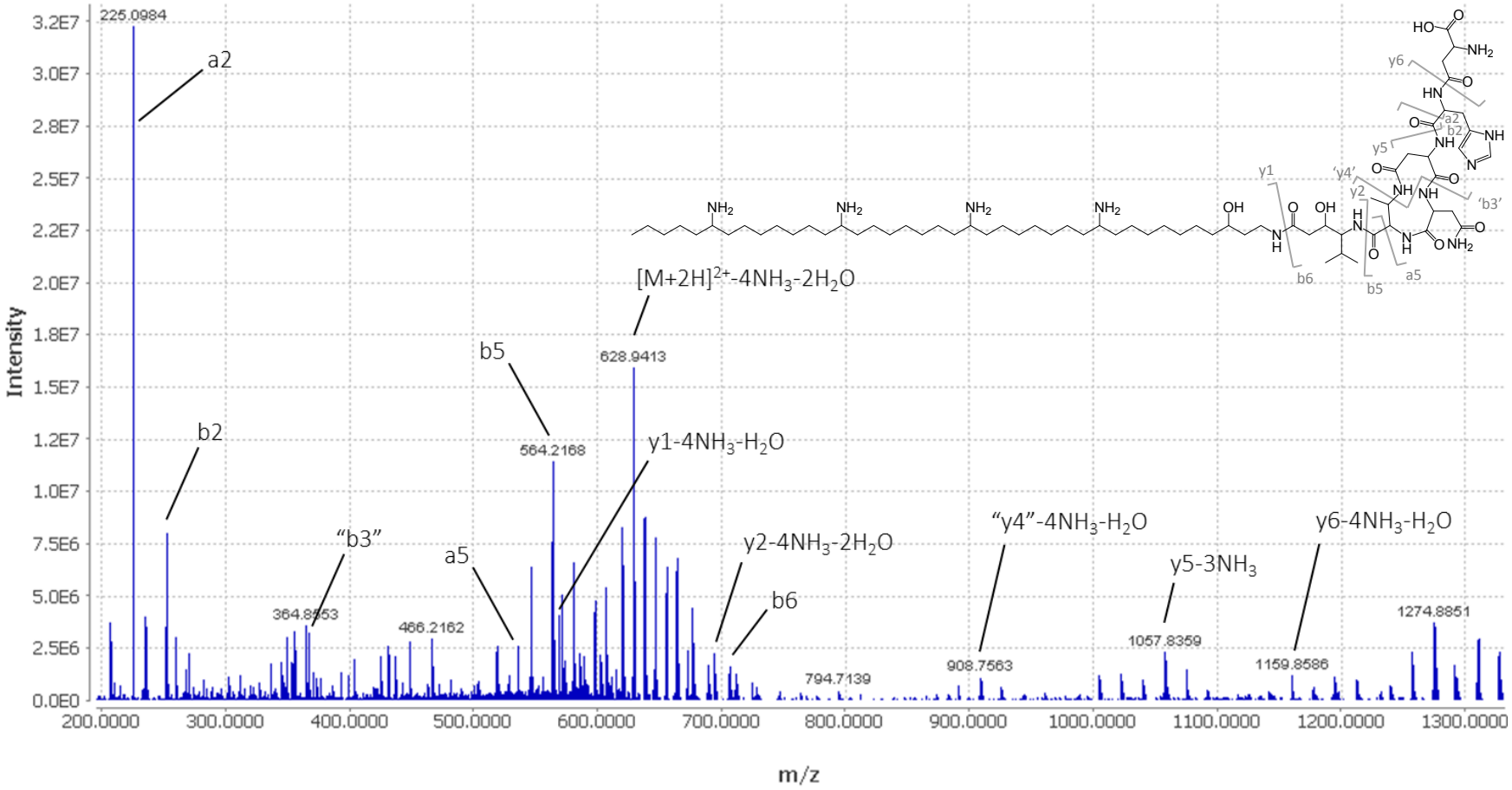

Fig. S8 High resolution LC-ESI-MS/MS spectrum of prezeamine I (**5**) ($[M+3H]^{3+}$ = 454.3400 Da). Selected a, b and y-fragment ions are indicated. A comprehensive overview of the detected fragment ions and their molecular formulae, calculated *m/z* and observed *m/z* is shown in table S3. Inset: structure of prezeamine I and detected fragment ions.

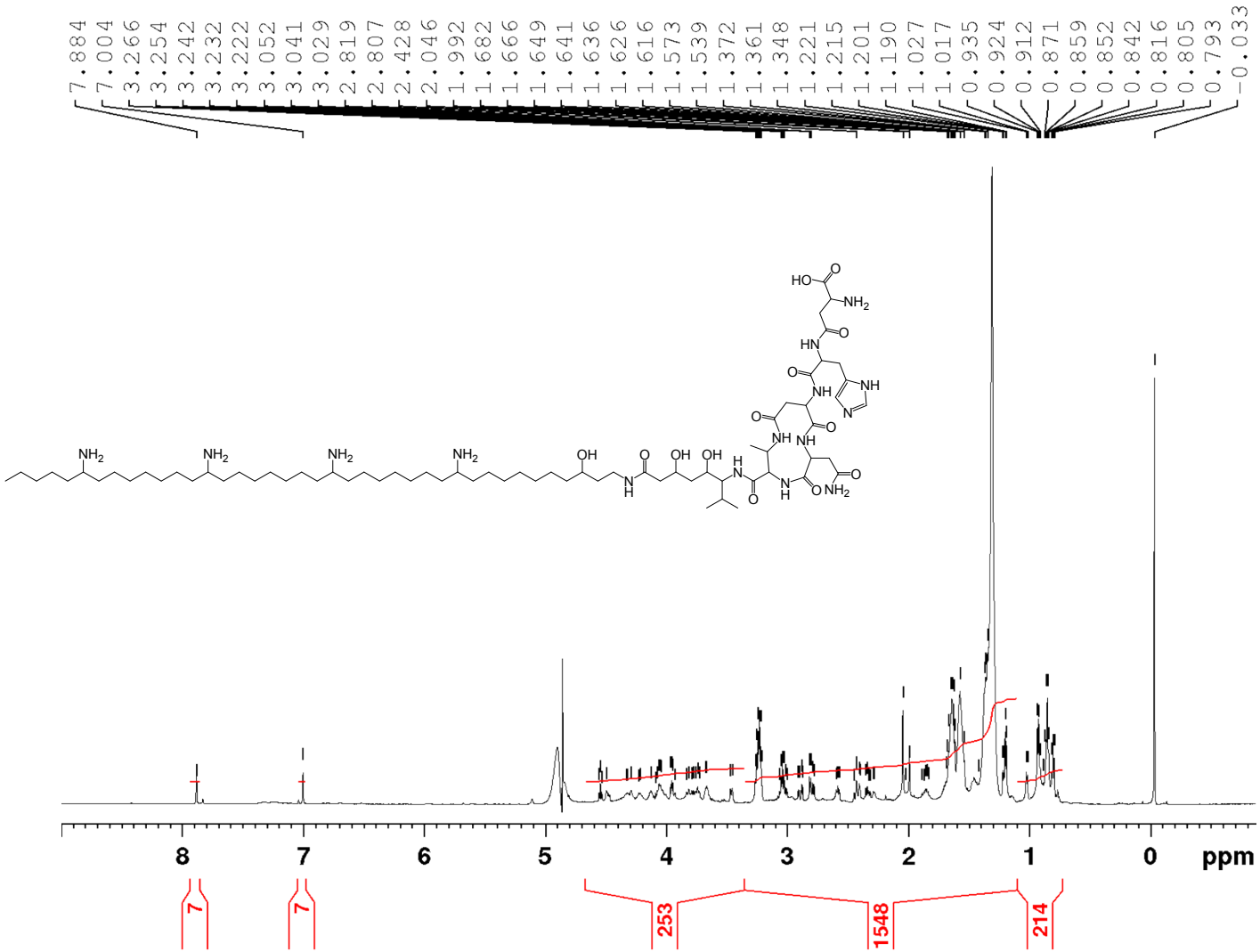

Fig. S9 ^{1}H NMR spectrum of prezeamine (**4**) in D_2O at 288K (600.13 MHz). Presaturation was used to reduce the HOD signal at 4.87ppm.

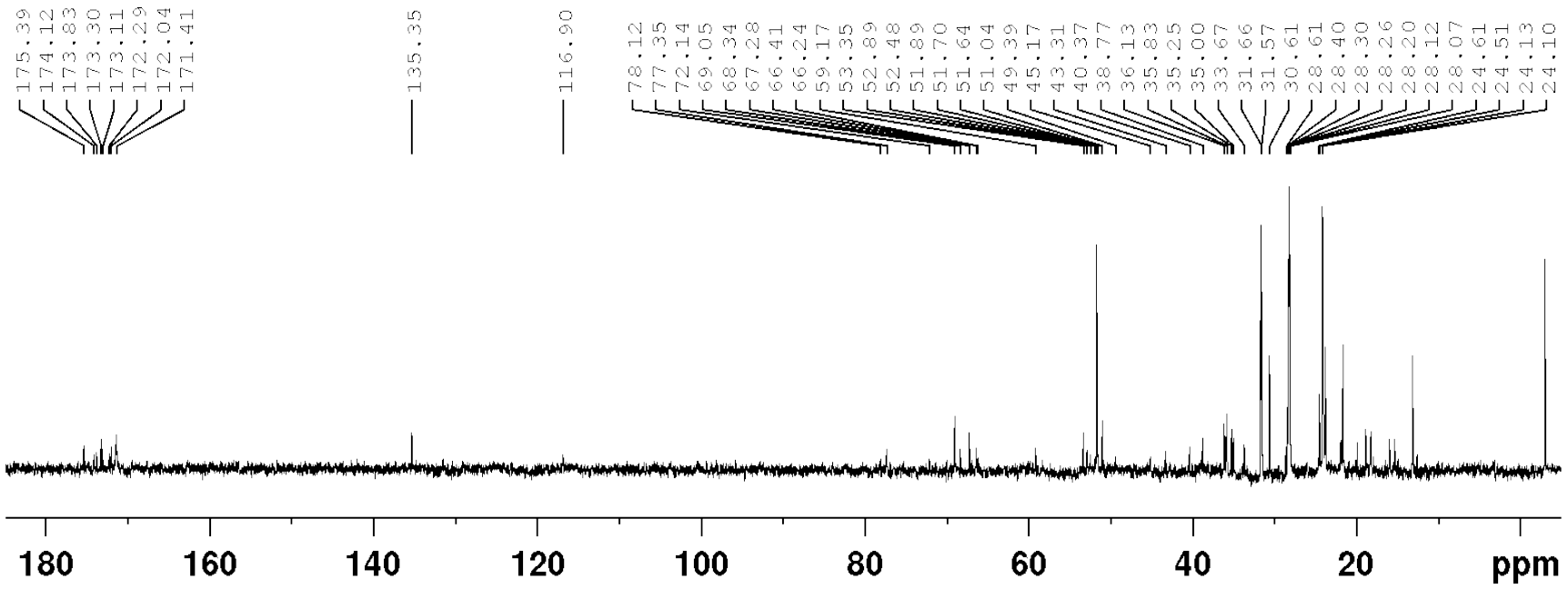

Fig. S10 Proton decoupled ^{13}C NMR spectrum of prezeamine (**4**) in D_2O at 288K (150.92 MHz).

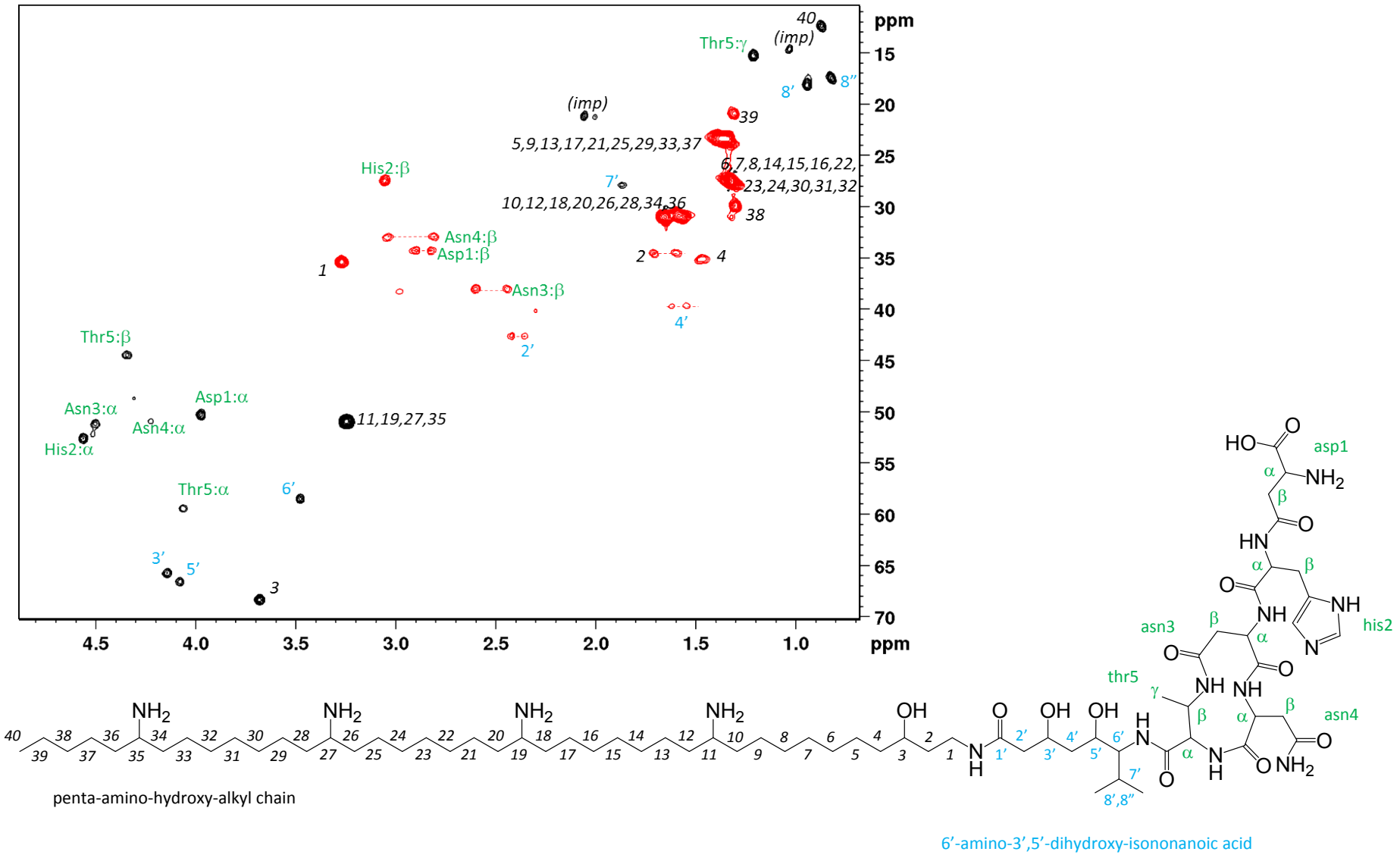

Fig. S11 Multiplicity edited HSQC NMR spectrum of prezeamine (**4**) in D_2O at 288K

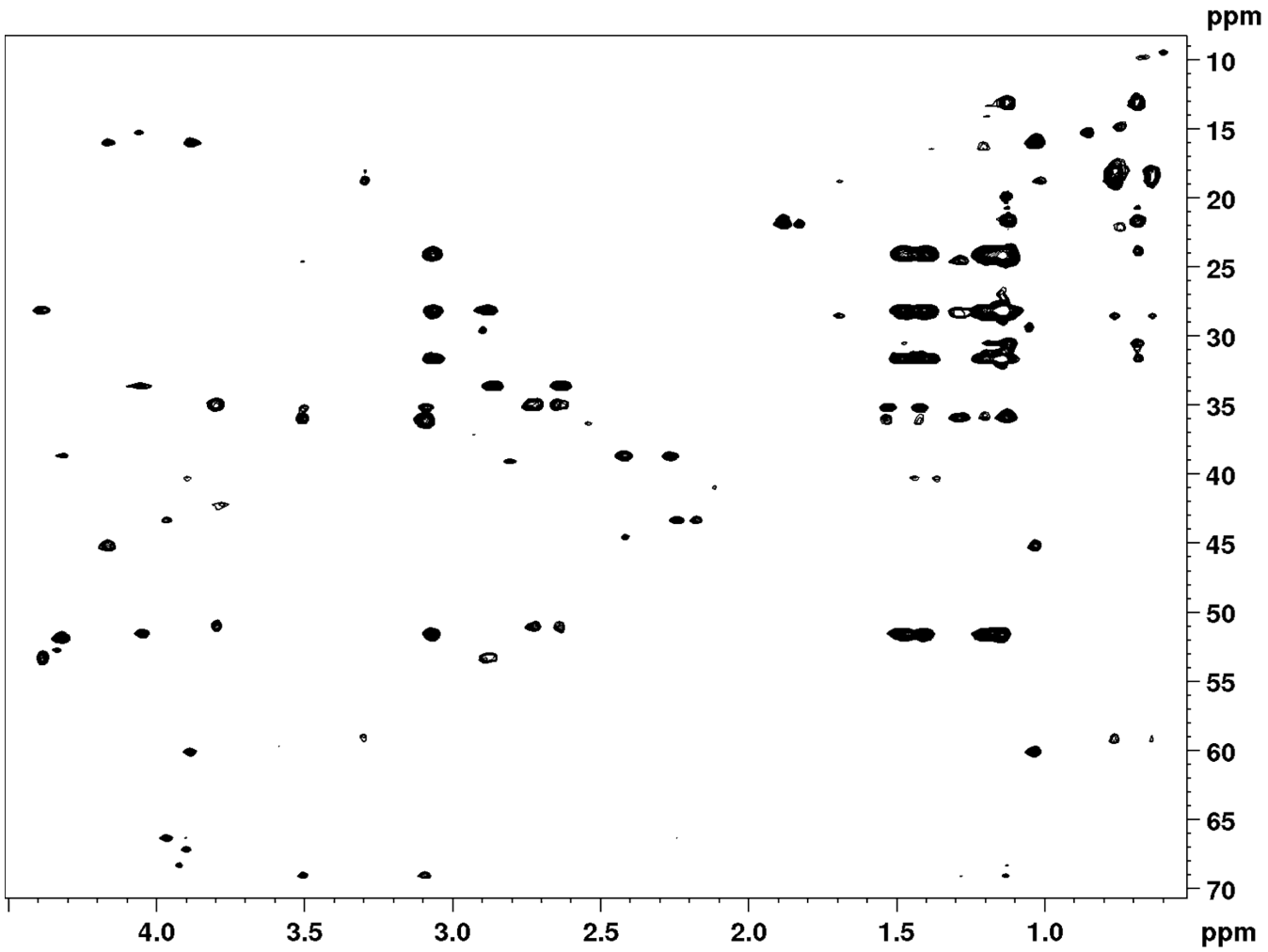

Fig. S12 HSQC-TOCSY NMR spectrum of prezeamine (**4**) in D_2O at 288K.

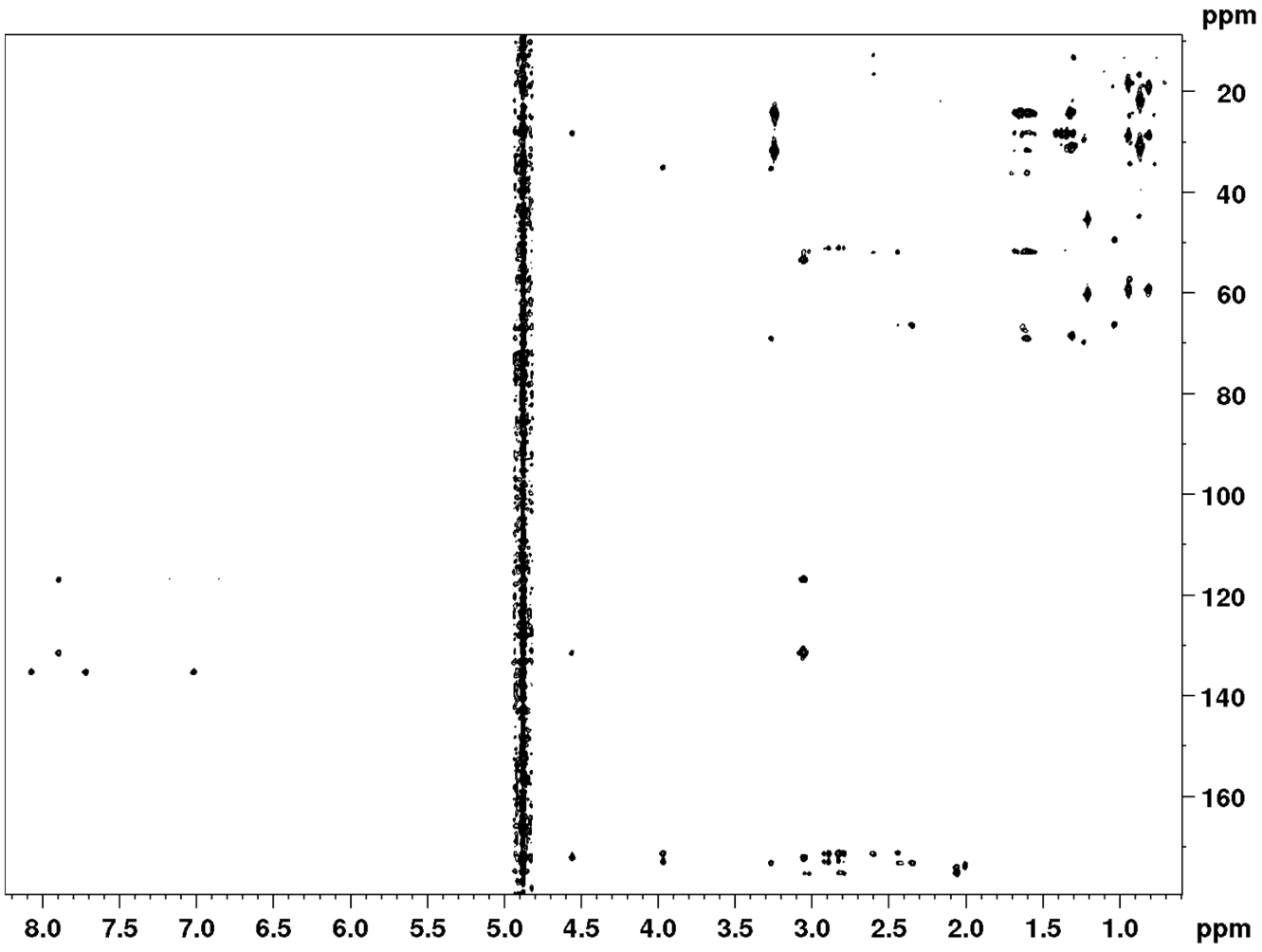

Fig. S13 HMBC NMR spectrum of prezeamine (**4**) in D_2O at 288K.

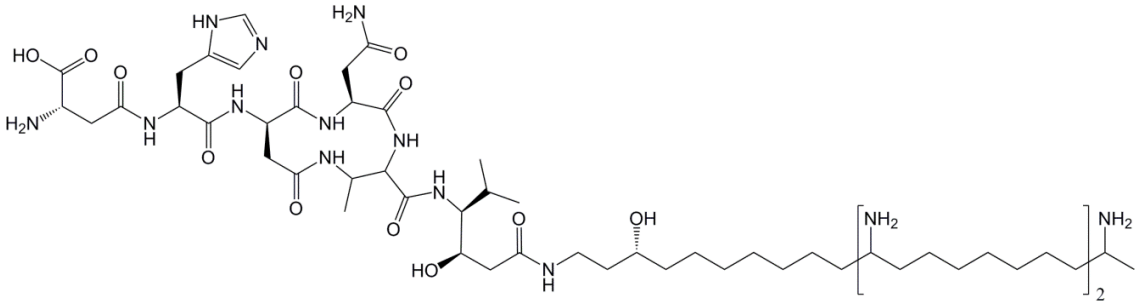

Fig. S14 Postulated structure of fabclavine IVa from from *Xenorhabdus szentirmaii.*[13]

Table S1. Overview of gene deletions and complementations.

Strain	Size (aa)[a]	Function	Bioassay[b]	Produced zeamines	Complementation bioassay[b]
Δzmn10	2259	PKS	0	none	
Δzmn11	1436	FAS/PKS	0	none	
Δzmn12	1010	PfaD family protein	0	none	
Δzmn13	255	3-oxoacyl-ACP reductase	0	none	100 (0.5 mM IPTG)
Δzmn14	412	Thioester reductase	0	none	89 (0.1 mM IPTG)
Δzmn15	259	Carbon-nitrogen hydrolase	36	(pre)zeamine (I) zeamine II	86 (0.1 mM IPTG)
Δzmn16	4169	NRPS	48	zeamine II	
Δzmn17	2180	NRPS	48	zeamine II	
Δzmn18	1531	PKS	48	zeamine II	
Δzmn19	439	Condensation domain-containing protein	48	zeamine II	100 (0.5 mM IPTG)
Δzmn22	345	Acylpeptide hydrolase	48	prezeamine (I), zeamine II	115 (0mM IPTG)

[a] Size of the translated CDSs prior to *in frame* deletion

[b] Average inhibition zone area minus colony area, expressed as percentage of WT

Table S2. Fragment ions, molecular formulae, calculated *m/z* and observed *m/z* from high resolution LC-ESI-MS/MS analysis of prezeamine (**4**). The corresponding spectrum is shown in Fig. S6.

Fragment-ion	Molecular formula	Calculated *m/z*	Observed *m/z*	Error (Da)	Error (ppm)
a2	$C_9H_{13}N_4O_3$	225.0988	225.0982	0.0006	2.67
a5	$C_{21}H_{30}N_9O_8$	536.2217	536.2214	0.0003	0.56
a5-H_2O	$C_{21}H_{28}N_9O_7$	518.2112	518.2110	0.0002	0.39
b2	$C_{10}H_{13}N_4O_4$	253.0937	253.0931	0.0006	2.37
"b3"	$C_{14}H_{19}N_6O_6$	367.1366	367.1361	0.0005	1.36
b5	$C_{22}H_{30}N_9O_9$	564.2166	564.2164	0.0002	0.35
b5-H_2O	$C_{22}H_{28}N_9O_8$	546.2061	546.2057	0.0004	0.73
b6	$C_{31}H_{47}N_{10}O_{12}$	751.3375	751.3366	0.0009	1.20
b6-H_2O	$C_{31}H_{45}N_{10}O_{11}$	733.3269	733.3265	0.0004	0.55
y1	$C_{40}H_{88}N_5O$	654.6989	n.d.		
y1-2NH_3-H_2O	$C_{40}H_{80}N_3$	602.6352	602.6348	0.0004	0.66
y1-3NH_3-H_2O	$C_{40}H_{77}N_2$	585.6086	585.6083	0.0003	0.51
y1-4NH_3-H_2O	$C_{40}H_{74}N$	568.5821	568.5818	0.0003	0.53
y2	$C_{49}H_{105}N_6O_4$	841.8197	n.d.		
y2-2NH_3	$C_{49}H_{99}N_4O_4$	807.7666	807.7667	0.0001	0.12
y2-3NH_3	$C_{49}H_{96}N_3O_4$	790.7400	790.7399	0.0001	0.13
y2-3NH_3-H_2O	$C_{49}H_{94}N_3O_3$	772.7295	772.7285	0.0010	1.29
y2-3NH_3-2H_2O	$C_{49}H_{92}N_3O_2$	754.7189	754.7186	0.0003	0.40
y2-4NH_3-2H_2O	$C_{49}H_{89}N_2O_2$	737.6924	737.6918	0.0006	0.81
y2-4NH_3-3H_2O	$C_{49}H_{87}N_2O$	719.6818	719.6813	0.0005	0.69
"y4"	$C_{57}H_{116}N_9O_7$	1038.8998	n.d.		
"y4"-NH_3	$C_{57}H_{113}N_8O_7$	1004.8466	1004.8452	0.0014	1.39
"y4"-2NH_3-H_2O	$C_{57}H_{108}N_7O_6$	986.8361	986.8343	0.0018	1.82
"y4"-3NH_3-H_2O	$C_{57}H_{105}N_6O_6$	969.8095	969.8077	0.0018	1.86
"y4"-3NH_3-2H_2O	$C_{57}H_{103}N_6O_5$	951.7989	951.7990	0.0001	0.11
"y4"-4NH_3-2H_2O	$C_{57}H_{100}N_5O_5$	934.7724	934.7710	0.0014	1.50

Fragment-ion	Molecular formula	Calculated *m/z*	Observed *m/z*	Error (Da)	Error (ppm)
y5	$C_{61}H_{122}N_{11}O_9$	1152.9427	n.d.		
y5-NH_3	$C_{61}H_{119}N_{10}O_9$	1135.9161	1135.9156	0.0005	0.44
y5-2NH_3	$C_{61}H_{116}N_9O_9$	1118.8895	1118.8889	0.0006	0.54
y5-3NH_3	$C_{61}H_{113}N_8O_9$	1101.8631	1101.8615	0.0016	1.45
y5-3NH_3-H_2O	$C_{61}H_{111}N_8O_8$	1083.8524	1083.8512	0.0012	1.11
y6	$C_{67}H_{129}N_{14}O_{10}$	1290.0016	n.d.		
y6-2NH_3	$C_{67}H_{123}N_{12}O_{10}$	1255.9485	1255.9482	0.0003	0.24
y6-3NH_3	$C_{67}H_{120}N_{11}O_{10}$	1238.9219	1238.9192	0.0027	2.18
y6-4NH_3	$C_{67}H_{117}N_{10}O_{10}$	1221.8954	1221.8920	0.0034	2.78
$y6^{2+}$-NH_3	$C_{67}H_{127}N_{13}O_{10}$	636.9914	636.9910	0.0004	0.63
$y6^{2+}$-2NH_3	$C_{67}H_{124}N_{12}O_{10}$	628.4781	628.4775	0.0006	0.95
$y6^{2+}$-3NH_3	$C_{67}H_{121}N_{11}O_{10}$	619.9649	619.9640	0.0009	1.45
$y6^{2+}$-3NH_3-H_2O	$C_{67}H_{119}N_{11}O_9$	610.9596	610.9585	0.0011	1.80
$[M+H]^+$-2NH_3	$C_{71}H_{128}N_{13}O_{13}$	1370.9754	1370.9763	0.0009	0.66
$[M+H]^+$-3NH_3	$C_{71}H_{125}N_{12}O_{13}$	1353.9488	1353.9467	0.0021	1.55
$[M+H]^+$-3NH_3-H_2O	$C_{71}H_{123}N_{12}O_{12}$	1335.9383	1335.9375	0.0008	0.60
$[M+H]^+$-4NH_3-H_2O	$C_{71}H_{120}N_{11}O_{12}$	1318.9117	1318.9094	0.0023	1.74
$[M+H]^+$-4NH_3-2H_2O	$C_{71}H_{118}N_{11}O_{11}$	1300.9012	1300.9010	0.0002	0.15
$[M+H]^+$-4NH_3-3H_2O	$C_{71}H_{116}N_{11}O_{10}$	1282.8906	1282.8910	0.0001	0.08
$[M+2H]^{2+}$-NH_3	$C_{71}H_{132}N_{14}O_{13}$	694.5049	694.5042	0.0007	1.01
$[M+2H]^{2+}$-2NH_3	$C_{71}H_{129}N_{13}O_{13}$	685.9916	685.9904	0.0012	1.75
$[M+2H]^{2+}$-2NH_3-H_2O	$C_{71}H_{127}N_{13}O_{12}$	676.9863	676.9856	0.0007	1.03
$[M+2H]^{2+}$-3NH_3-H_2O	$C_{71}H_{124}N_{12}O_{12}$	668.4731	668.4717	0.0014	2.09
$[M+2H]^{2+}$-3NH_3-2H_2O	$C_{71}H_{122}N_{12}O_{11}$	659.4678	659.4664	0.0014	2.12
$[M+2H]^{2+}$-3NH_3-3H_2O	$C_{71}H_{120}N_{12}O_{10}$	650.4625	650.4619	0.0006	0.92

Table S3. Fragment ions, molecular formulae, calculated m/z and observed m/z from high resolution LC-ESI-MS/MS analysis of prezeamine I (**5**). The corresponding spectrum is shown in Fig. S7.

Fragment-ion	Molecular formula	Calculated *m/z*	Observed *m/z*	Error (Da)	Error (ppm)
a2	$C_9H_{13}N_4O_3$	225.0988	225.0984	0.0004	1.78
a5	$C_{21}H_{30}N_9O_8$	536.2217	536.2218	0.0001	0.19
a5-H_2O	$C_{21}H_{28}N_9O_7$	518.2112	518.2114	0.0002	0.39
b2	$C_{10}H_{13}N_4O_4$	253.0937	253.0932	0.0005	1.98
"b3"	$C_{14}H_{19}N_6O_6$	367.1366	367.1363	0.0003	0.82
b5	$C_{22}H_{30}N_9O_9$	564.2166	564.2168	0.0002	0.35
b5-H_2O	$C_{22}H_{28}N_9O_8$	546.2061	546.2062	0.0001	0.18
b6	$C_{29}H_{43}N_{10}O_{11}$	707.3113	707.3108	0.0005	0.71
b6-H_2O	$C_{29}H_{41}N_{10}O_{10}$	689.3007	689.3005	0.0002	0.29
y1	$C_{40}H_{88}N_5O$	654.6989	n.d.		
y1-2NH_3-H_2O	$C_{40}H_{80}N_3$	602.6352	602.6352	0	0.00
y1-3NH_3-H_2O	$C_{40}H_{77}N_2$	585.6086	585.6088	0.0002	0.34
y1-4NH_3-H_2O	$C_{40}H_{74}N$	568.5821	568.5822	0.0001	0.18
y2	$C_{47}H_{101}N_6O_3$	797.7935	n.d.		
y2-2NH_3	$C_{47}H_{95}N_4O_3$	763.7404	763.7406	0.0001	0.13
y2-2NH_3-H_2O	$C_{47}H_{93}N_4O_2$	745.7298	745.7299	0.0001	0.13
y2-3NH_3-H_2O	$C_{47}H_{90}N_3O_2$	728.7033	728.7033	0	0.00
y2-4NH_3-H_2O	$C_{47}H87N_2O_2$	711.6767	711.6765	0.0002	0.28
y2-4NH_3-2H_2O	$C_{47}H85N_2O$	693.6662	693.6661	0.0001	0.14
"y4"	$C_{55}H_{112}N_9O_6$	994.8736	n.d.		
"y4"-2NH_3-H_2O	$C_{55}H_{104}N_7O_5$	942.8098	942.8084	0.0014	1.48
"y4"-3NH_3-H_2O	$C_{55}H_{101}N_6O_5$	925.7833	925.7829	0.0004	0.43
"y4"-4NH_3-H_2O	$C_{55}H_{98}N_5O_5$	908.7568	908.7563	0.0005	0.55
"y4"-4NH_3-2H_2O	$C_{55}H_{96}N_5O_4$	890.7462	890.7465	0.0003	0.34
y5	$C_{59}H_{118}N_{11}O_8$	1108.9165	n.d.		
y5-NH_3	$C_{59}H_{115}N_{10}O_8$	1091.8899	1091.8904	0.0005	0.46
y5-2NH_3	$C_{59}H_{112}N_9O_8$	1074.8633	1074.8624	0.0009	0.84
y5-3NH_3	$C_{59}H_{109}N_8O_8$	1057.8368	1057.8359	0.0009	0.85

Fragment-ion	Molecular formula	Calculated *m/z*	Observed *m/z*	Error (Da)	Error (ppm)
y5-3NH_3-H_2O	$C_{59}H_{107}N_8O_7$	1039.8262	1039.8262	0	0.00
y5-4NH_3-H_2O	$C_{59}H_{104}N_7O_7$	1022.7997	1022.7994	0.0003	0.29
y5-4NH_3-2H_2O	$C_{59}H_{102}N_7O_6$	1004.7891	1004.7891	0	0.00
y6	$C_{65}H_{125}N_{14}O_9$	1245.9754	n.d.		
y6-2NH_3	$C_{65}H_{119}N_{12}O_9$	1211.9222	1211.9233	0.0011	0.91
y6-3NH_3	$C_{65}H_{116}N_{11}O_9$	1194.8957	1194.8949	0.0008	0.67
y6-3NH_3-H_2O	$C_{65}H_{114}N_{11}O_8$	1176.8851	1176.8859	0.0008	0.68
y6-4NH_3-H_2O	$C_{65}H_{111}N_{10}O_8$	1159.8586	1159.8586	0	0.00
$y6^{2+}$-2NH_3	$C_{65}H_{120}N_{12}O_9$	606.4650	606.4650	0	0.00
$y6^{2+}$-3NH_3	$C_{65}H_{117}N_{11}O_9$	597.9518	597.9516	0.0002	0.33
$y6^{2+}$-3NH_3-H_2O	$C_{65}H_{115}N_{11}O_8$	588.9465	588.9464	0.0001	0.17
$[M+H]^+$-2NH_3	$C_{69}H_{124}N_{13}O_{12}$	1326.9492	1326.9506	0.0014	1.06
$[M+H]^+$-3NH_3	$C_{69}H_{121}N_{12}O_{12}$	1309.9226	1309.9221	0.0005	0.38
$[M+H]^+$-3NH_3-H_2O	$C_{69}H_{119}N_{12}O_{11}$	1291.9121	1291.9124	0.0003	0.23
$[M+H]^+$-4NH_3-H_2O	$C_{69}H_{116}N_{11}O_{11}$	1274.8855	1274.8851	0.0004	0.31
$[M+H]^+$-4NH_3-2H_2O	$C_{69}H_{114}N_{11}O_{10}$	1256.8750	1256.8756	0.0006	0.48
$[M+H]^+$-4NH_3-3H_2O	$C_{69}H_{112}N_{11}O_9$	1238.8644	1238.8663	0.0001	0.08
$[M+2H]^{2+}$-NH_3	$C_{69}H_{128}N_{14}O_{12}$	672.4918	672.4915	0.0003	0.45
$[M+2H]^{2+}$-2NH_3	$C_{69}H_{125}N_{13}O_{12}$	663.9785	663.9776	0.0009	1.36
$[M+2H]^{2+}$-2NH_3-H_2O	$C_{69}H_{123}N_{13}O_{11}$	654.9732	654.9728	0.0004	0.61
$[M+2H]^{2+}$-3NH_3-H_2O	$C_{69}H_{120}N_{12}O_{11}$	646.4600	646.4592	0.0008	1.24
$[M+2H]^{2+}$-4NH_3-H_2O	$C_{69}H_{117}N_{11}O_{11}$	637.9467	637.9467	0	0.00
$[M+2H]^{2+}$-4NH_3-2H_2O	$C_{69}H_{115}N_{11}O_{10}$	628.9414	628.9413	0.0001	0.16
$[M+2H]^{2+}$-4NH_3-3H_2O	$C_{69}H_{113}N_{11}O_9$	619.9361	619.9361	0	0.00
$[M+3H]^{3+}$-NH_3-H_2O	$C_{69}H_{127}N_{14}O_{11}$	442.6603	442.6603	0	0.00
$[M+3H]^{3+}$-2NH_3-H_2O	$C_{69}H_{124}N_{13}O_{11}$	436.9848	436.9844	0.0004	0.92
$[M+3H]^{3+}$-2NH_3-2H_2O	$C_{69}H_{122}N_{13}O_{10}$	430.9812	430.9812	0	0.00
$[M+3H]^{3+}$-2NH_3-3H_2O	$C_{69}H_{120}N_{13}O_9$	424.9777	424.9775	0.0002	0.47

Table S4. Assignments for signals in the ^{1}H and ^{13}C NMR spectra of prezeamine (**4**) in D_2O at 288K.

		^{1}H	^{13}C
Asp-1	α	3.96	20.2
	β	2.89, 2.81	34.2
	γ	-	173.1
	C	-	171.5
His-2	α	4.55	52.5
	β	3.04	27.3
	γ	-	131.4
	δ2	7.01	116.8
	ε1	7.9	135.1
	C	-	172.3
Asn-3	α	4.49	51.01
	β	2.59, 2.43	37.9
	γ	-	171.4
	C	-	172
Asn-4	α	4.21	50.8
	β	3.03, 2.80	32.8
	γ	-	175.4
	C	-	172
Thr-5	α	4.05	59.3
	β	4.33	44.3
	γ	1.2	15.1
	C	-	171.4
6'-amino-3',5'-dihydroxy-isononanoic acid	1	-	173.3
	2	2.41, 2.34	42.5
	3	4.13	65.6
	4	1.61, 1.54	39.5
	5	4.07	66.4
	6	3.47	58.4
	7	1.86	27.7
	8	0.93	17.9
	9	0.81	17.3
penta-amino-hydroxy-alkyl chain	1	2.97	38.2
	2	1.70, 1.59	34.4
	3	3.67	68.2
	4	1.46	35
	5,9,13,17,21,25,29,33,37	1.35	23.2
	6,7,8,14,15,16,22,23,24,30,31,32	1.31	27.4
	10,12,18,20,26,28,34,36	1.61	30.8
	11,19,27,35	3.23	50.8
	38	1.3	29.8
	39	1.29	20.8
	40	0.86	12.2

Table S5. Overview of the specificity-conferring codes and the predicted and observed specificities of the adenylation domains from the Zmn16-17 NRPS from *S. plymuthica* RVH1 and the FclI-FclJ NRPS from *Xenorhabdus szentirmaii*.[5,13] Predictions were made using NRPSpredictor2.[14]

	Adenylation domain	Specificity-conferring code	Predicted specificity	Observed specificity
Zmn16-17 NRPS	A1	DPRHLALLAK	hydrophobic-aliphatic	γ-Asp
	A2	DTWTIASVSK	hydrophobic-aromatic, Phe	His
	A3	DATKVGEVGK	Asn	Asn
	A4	DATKVGEVGK	Asn	Asn
	A5	DFWNIGMVHK	Thr	Thr
	A6	DALFIGGTFK	Val	Val
FclI-J NRPS	A1	DPRHVSLLAK	hydrophobic-aliphatic	γ-Asp
	A2	DVWTMSAVGK	Phe	His
	A3	DATKVGEVGK	Asn	Asn
	A4	DATKVGEVGK	Asn	Asn
	A5	DFWNIGMVHK	Thr	Thr
	A6	DALKFIGGTKF	Val	Val

Table S6. Overview of bacterial strains and plasmids used or constructed in this study.

Strain or plasmid	Relevant Genotype or Description	Source or reference
Escherichia coli TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZΔM15* Δ*lacX74 recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL endA1 nupG*	Life Technologies
Escherichia coli BL21 (DE3) pLysS	F^- *omp* T *hsd* SB(rB^-, mB^-) *gal dcm* (DE3) pLysS (CamR)	Life Technologies
Escherichia coli S17-1	*recA*, *thi*, *pro*, *hsd* R^-M^+RP4: 2-Tc::Mu- Km::Tn7 λpir, Sm^R, Tp^R	[15]
Escherichia coli MG1655	Wild type	Lab collection
Staphylococcus aureus ATCC27661	Wild type	Lab collection
Serratia plymuthica RVH1	Wild type	[1]
pSF100	4955 bp, *oriR6K*, Ap^R, Km^R	[2]
pSFzmn10	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ6741 bp in *zmn10*)	This study
pSFzmn11	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ4242 bp in *zmn11*)	This study
pSFzmn12	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ3000 bp in *zmn12*)	This study
pSFzmn13	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ720 bp in *zmn13*)	This study
pSFzmn14	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ1185 bp in *zmn14*)	This study
pSFzmn15	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ774 bp in *zmn15*)	This study
pSFzmn16	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ12432 bp in *zmn16*)	This study
pSFzmn17	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ6474 bp in *zmn17*)	This study
pSFzmn18	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ4380 bp in *zmn18*)	This study
pSFzmn19	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ1188 bp in *zmn19*)	This study
pSFzmn22	pSF100 + *Kpn* I-*Eco* RI PCR fragment (Δ1032 bp in *zmn22*)	This study
pTrc99A	4176 bp, *Ptrc*, $lacI^q$, *pBR322* ori, Ap^R	[3]
pTrczmn13	pTrc99A + *zmn13* coding sequence	This study
pTrczmn14	pTrc99A + *zmn14* coding sequence	This study
pTrczmn15	pTrc99A + *zmn15* coding sequence	This study
pTrczmn19	pTrc99A + *zmn19* coding sequence	This study
pTrczmn22	pTrc99A + *zmn22* coding sequence	This study

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