Supporting Information for:

In vitro biosynthetic studies of bottromycin expand the enzymatic capabilities of the YcaO superfamily

Christopher J. Schwalen[†], Graham A. Hudson[†], Simone Kosol[§], Nilkamal Mahanta[†‡], Gregory L. Challis[§,#], Douglas A. Mitchell[†‡]*

†Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, USA. §Department of Chemistry and Warwick Integrative Synthetic Biology Center, University of Warwick, Coven-try CV4 7AL, UK. ‡Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, Illinois 61801, USA. # Department of Biochemistry and Molecular Biology and ARC Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, Victoria 3800, Australia

* Corresponding author:

Douglas A. Mitchell (douglasm@illinois.edu), phone: 1-217-333-1345, fax: 1-217-333-0508

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Experimental Methods

General materials and methods. Reagents used for molecular biology experiments were purchased from New England Biolabs (NEB, Ipswich, MA), Thermo Fisher Scientific (Waltham, MA) or Gold Biotechnology Inc. (St. Louis, MO). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *Escherichia coli* DH5α and BL21(DE3) strains were used for plasmid maintenance and protein overexpression, respectively. Plasmid inserts were sequenced at ACGT Inc. (Wheeling, IL). MALDI-TOF-MS analysis was performed using a Bruker UltrafleXtreme MALDI-TOF-TOF mass spectrometer (Bruker Daltonics) in reflector positive mode at the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory. ESI-MS/MS analyses were performed using a SYNAPT ESI quadrupole TOF Mass Spectrometry System (Waters) equipped with an ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Waters).

Molecular biology techniques. Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Genes optimized for recombinant expression in *E. coli* were synthesized by GenScript (Piscataway, NJ). Site-directed mutagenesis on BotA was performed using the QuikChange method (Agilent) as per the manufacturer's instructions using Platinum Taq (Thermo Fisher) or PfuTurbo (Agilent) DNA polymerase. The primers for each mutant are listed in Table 1. The mutations were verified by sequencing using the maltose-binding protein (MBP)-forward primer or the T7 reverse primer.

MBP-tagged enzyme overproduction and purification. *E. coli* BL21 (DE3) cells were transformed with a pET28 plasmid encoding the MBP-tagged enzyme of interest. Cells were grown for 24 h on LB agar plates containing 50 µg/mL kanamycin at 37 °C. Single colonies were picked to inoculate 10 mL of LB containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and grown at 37 °C for 16-18 h. This culture was used to inoculate 1 L of LB containing 50 µg/mL kanamycin and 34 µg/mL and grown to an optical density OD_{600} of 0.6 then placed on ice for 15 min. Protein expression was induced with the addition of 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG), 500 µM $ZnSO_4$ and 2 mM $MgSO_4$. Expression proceeded for 16 h at 16 °C. Cells were harvested by centrifugation at 3,000 × g for 20 min, washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) and centrifuged at 4,000 × g for 20 min. The harvested cells were stored at -80 °C for a maximum of three weeks before use.

Harvested cells were resuspended in lysis buffer [50 mM Tris-HCl pH 7.3, 150 mM NaCl, 2.5% glycerol (*v/v*)] containing 4 mg/mL lysozyme, 2 µM leupeptin, 2 µM benzamidine HCl, 2 µM E64 and 30 mM phenylmethane sulfonyl fluoride (PMSF). Cells were homogenized by sonication (3 × 40 s with 10 min equilibration periods at 4 °C with rocking). Insoluble cell debris was removed by centrifugation at 20,000 × g for 60 min at 4 °C. The supernatant was then applied to a pre-equilibrated amylose resin (NEB; 10 mL of resin per L of cells). The column was washed with 30 mL of lysis buffer [with 0.5 mM tris-(2-carboxyethyl)-phosphine (TCEP)] followed by 30 mL of wash buffer (lysis buffer with 400 mM NaCl, 0.5 mM TCEP). The MBP-tagged proteins were eluted using 30 mL elution buffer (lysis buffer with 300 mM NaCl, 10 mM maltose, 0.5 mM TCEP). Elution was concentrated using a 30 kDa MWCO Amicon Ultra centrifugal filter (EMD Millipore). A buffer exchange with 10 × volume of protein storage buffer (50 mM HEPES pH 7.3, 300 mM NaCl, 2.5% glycerol (*v/v*), 0.5 mM TCEP) was performed prior to final concentration and storage. Protein concentrations were assayed using both 280 nm absorbance (theoretical extinction coefficients were calculated using the ExPASy ProtParam tool; http://web.expasy.org/protparam/) and a Bradford colorimetric assay using bovine serum albumin (BSA) as a standard (Thermo Fisher Scientific). Purity and possible truncation were assessed by Coomassie-stained SDS-PAGE gel (**Figure S4**).

MBP-tagged precursor peptide overproduction and purification. Precursor peptide expression was performed as stated in the above section (**MBP-tagged enzyme overproduction and purification**), with the following modifications: cultures were grown to an optical density OD_{600} of 1.2 then placed on ice for 15 min and induced with the addition of 100 μM IPTG and 2 mM $MgSO_4$. Expression proceeded for 1 h at 30 °C for LB or 16 h at 16 °C for TB.

Harvested cells were resuspended in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5% glycerol (*v/v*)] containing 4 mg/mL lysozyme, 2 μM leupeptin, 2 μM benzamidine HCl, 2 μM E64 and 30 mM phenylmethane sulfonyl fluoride (PMSF). Elution was concentrated using a 30 kDa MWCO Amicon Ultra centrifugal filter (EMD Millipore). A buffer exchange with 10 × volume of protein storage buffer [50 mM HEPES pH 7.5, 300 mM NaCl, 2.5% glycerol (*v/v*), 0.5 mM TCEP] was performed prior to final concentration and storage.

Precursor peptide cleavage and HPLC purification. MBP-tagged precursor peptides at a concentration of 300 μM were cleaved with tobacco etch virus (TEV) protease at a concentration of 55 μM for 16 h at 25 °C. MBP and TEV were precipitated by equilibrating the reaction at 85 °C for 10 min and insoluble material was removed by centrifugation at 4,000 × g for 30 min. The soluble fraction was purified by either high-performance liquid chromatography (HPLC) or medium-pressure liquid chromatography (MPLC).

HPLC method: For LB expressions, soluble fractions were purified by HPLC using a Betasil C18 column (100 Å pore size, 250 × 4.6 mm, 5 μM particle size) (Thermo Scientific) with a mobile phase of 10 mM NH_4HCO_3/MeCN gradient from 15% to 30% aq. MeCN over 30 min, then held at 95% aq. MeCN for 10 minutes at 1 mL min^{-1}. Elution of the precursor peptide was monitored at 220 and 280 nm. Fractions were analyzed via MALDI-TOF and dried under reduced pressure. Peptides were finally dissolved in a minimal volume of 10 mM NH_4HCO_3, flash-frozen in liquid N_2 and lyophilized overnight to dryness.

MPLC method: For TB expressions, soluble fractions were dried under reduced pressure onto 5 g of Celite 545 adsorbent. Precursor peptide adsorbed onto Celite was purified by MPLC using a Teledyne Isco Combiflash EZprep equipped with a RediSep Rf C18 cartridge (43 g media, 100 Å pore size, 20–40 μm particle size, 400–632 mesh) using a mobile phase of 10 mM aq. NH_4HCO_3/MeCN at 30 mL min^{-1} over a gradient from 10–40% MeCN (20 column volumes), then 40–95% MeCN (5 column volumes). Elution of the precursor peptide was monitored at 220 and 280 nm. Fractions were analyzed via MALDI-TOF and dried under reduced pressure. Peptides were finally dissolved in a minimal volume of 10 mM NH_4HCO_3, flash-frozen in liquid N_2 and lyophilized overnight to dryness.

In vitro cyclodehydration of bottromycin precursor peptides. The following reaction conditions were used for cyclodehydration reactions. In synthetase buffer (50 mM Tris-HCl pH 8.5, 125 mM NaCl, 20 mM $MgCl_2$, 1 mM TCEP, 5 mM ATP): BotA precursor peptide (50 μM), MBP-BmbD (10 μM) or MBP-BmbE (10 μM) in a final volume of 50 μL. The mixture was allowed to react at 25 °C for 5 h, subjected to centrifugation to remove insoluble material (17,000 × g, 10 min, 25 °C), desalted using C18 ZipTips (EMD Millipore), eluted into 2 μL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target. Samples were analyzed by a Bruker UltrafleXtreme MALDI-TOF MS using manufacturer methods for reflective positive mode.

Chemical labeling of cyclodehydration products. Cyclodehydration reactions described above were subjected to either N-terminal dimethylation or cysteine thiol alkylation.[1,2] Reactions were performed in synthetase buffer for cysteine alkylation or a 3-(*N*-morpholino)propanesulfonic acid (MOPS) variant for dimethylation (identical to synthetase buffer with 50 mM MOPS pH 7.5 replacing Tris-HCl). For dimethylation, cyclodehydratase reactions were precipitated by adding equal volume of MeCN, removing insoluble proteins by centrifugation at 17,000 × g for 15 min, then drying the soluble fraction under reduced

pressure. Samples were re-dissolved in 80 µL of water and 30 mM BH_3-C_5H_5N (Sigma) and 20 mM aq. CH_2O (VWR) were added. Reactions were briefly sonicated and incubated at 25 °C for 16 h. Samples were desalted using C18 ZipTips, eluted into 2 µL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target. Samples were analyzed by MALDI-TOF MS.

For cysteine alkylation, cyclodehydratase reactions were precipitated by adding equal volume of MeCN, removing insoluble proteins by centrifugation at 17,000 × g for 15 min, then drying the soluble fraction under reduced pressure. Samples were re-dissolved in 80 µL of water and 10 mM $BrCH_2CH_2NH_2$ (Sigma) was added and reacted at 25 °C for 16 h. Samples were desalted using C18 ZipTips, eluted into 2 µL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target. Samples were analyzed by MALDI-TOF MS.

Tandem mass spectrometry characterization of cyclodehydrated products. In vitro cyclodehydrations were conducted as described in the above section (**In vitro cyclodehydration of bottromycin precursor peptides**) on a 100 µL scale. Insoluble material was removed by centrifugation (17,000 × g, 10 min, 25 °C) and 2 µg of GluC endoproteinase (Worthington Biochemical) or 0.25 µg of AspN and 5 mM $ZnSO_4$ was added and allowed to react for 60 min at 25 °C. Insoluble material was again removed by centrifugation (17,000 × g, 10 min, 25 °C), and desalted using C18 ZipTips, eluted into 20 µL of 75% aq. MeCN and were analyzed by ESI MS/MS.

Purification of the macrolactamidine product of the BmbE-catalyzed reaction. The following reaction conditions were used for cyclodehydration in synthetase buffer (50 mM Tris-HCl pH 8.5, 125 mM NaCl, 20 mM $MgCl_2$, 1 mM TCEP, 5 mM ATP): BmbC precursor peptide (50 µM), MBP-BmbE (5 µM) in a final volume of 10 mL. The reaction mixture was allowed to proceed at 25 °C for 5 h, and 1 volume equivalent of acetonitrile was added to precipitate the enzyme at 25 °C for 30 min, insoluble material was removed by centrifugation (17,000 × g, 15 min, 25 °C). The soluble fraction containing processed precursor peptide was dried under reduced pressure.

Dried samples were dissolved in 10% MeOH/10 mM NH_4HCO_3 and purified by preparative HPLC with a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (Thermo Scientific) reverse phase column (250 × 10 mm, 5 µm particle size, 100 Å pore size). A mobile phase of 10 mM aq. NH_4HCO_3/MeOH was utilized at a flow rate of 4 mL min^{-1} with a method of: 10% MeOH (isocratic, 5 min), 10%–30% MeOH (gradient, 35 min), 30%–95% MeOH (gradient, 5 min), 95% MeOH (isocratic, 3 min). Elution was monitored at 220 nm and fractions containing cyclodehydrated bottromycin precursor peptide were dried under reduced pressure to yield a white solid.

Dried bottromycin peptide was dissolved in 0.5 mL of 10 mM NH_4HCO_3 and cleaved with 100 µg of sequencing-grade trypsin (Promega) for 3 h at 37 °C, then transferred to 16 h at 25 °C. Reactions were then stored at -20 °C until further purification.

Final purification was performed by HPLC with a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (Thermo Scientific) reverse phase column (250 × 4.6 mm, 5 µm particle size, 100 Å pore size). A mobile phase of 10 mM aq. NH_4HCO_3/MeOH was utilized at a flow rate of 1 mL min^{-1}. Bottromycin N15K-macrolactamidine was injected as 1 mL solution with a method of: 40% MeOH (isocratic, 5 min), 40–75% MeCN (gradient, 40 min). Elution was monitored at 220 nm and spotted on 2 µL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target for confirmation by MALDI-TOF MS.

Purification of the thiazoline product of the BmbD-catalyzed reactions. The following reaction conditions were used for cyclodehydration in synthetase buffer (50 mM Tris-HCl pH 7.5, 125 mM NaCl,

20 mM $MgCl_2$, 1 mM TCEP, 5 mM ATP): BmbC precursor peptide (50 μM), MBP-BmbD (5 μM) in a final volume of 10 mL. The reaction was allowed to proceed at 25 °C for 5 h, and 1 volumetric equivalent of acetonitrile was added to precipitate enzyme at 25 °C for 30 min, then centrifuged to remove insoluble material (17,000 × g, 15 min, 25 °C). The soluble fraction containing processed precursor peptide was dried under reduced pressure.

Dried samples were dissolved in 10% MeOH/10 mM NH_4HCO_3 and purified by preparative HPLC with a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (Thermo Scientific) reverse phase column (250 × 10 mm, 5 μm particle size, 100 Å pore size). A mobile phase of 10 mM aq. NH_4HCO_3/MeOH was utilized at a flow rate of 4 mL min^{-1} with a method of: 10% MeOH (isocratic, 5 min), 10%–30% MeOH (gradient, 35 min), 30%–95% MeOH (gradient, 5 min), 95% MeOH (isocratic, 3 min). Elution was monitored at 220 nm and fractions containing cyclodehydrated bottromycin precursor peptide were dried under reduced pressure to yield a white solid.

Dried bottromycin peptide was dissolved in 0.5 mL of 10 mM NH_4HCO_3 and cleaved with 100 μg of sequencing-grade trypsin (Promega) for 3 h at 37 °C, then transferred to 16 h at 25 °C. Reactions were then stored at -20 °C until further purification.

Final purification was performed by HPLC with a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (Thermo Scientific) reverse phase column (250 × 4.6 mm, 5 μm particle size, 100 Å pore size). A mobile phase of 10 mM aq. NH_4HCO_3/MeOH was utilized at a flow rate of 1 mL min^{-1}. Bottromycin N15K-thiazoline was injected as 1 mL solution with a method of: 40% MeOH (isocratic, 5 min), 40–75% MeCN (gradient, 40 min). Elution was monitored at 220 nm and spotted on 2 μL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target for confirmation by MALDI-TOF MS.

Preparation and purification of fluorescein-labeled bottromycin precursor peptide. The double mutant, C-terminal His-tagged construct (C8S-S43C-His) was generated via site-directed mutagenesis and prepared and purified using above methods. Purified, lyophilized peptide was labeled with 6-(iodoacetamido)fluorescein (IAF, Sigma) by reducing 500 μM of precursor peptide in 10 mM NH_4HCO_3 buffer with 500 μM TCEP for 30 minutes at 25 °C. 5 molar equivalents of IAF was dissolved in 1 volumetric equivalent of *N,N*-dimethylformamide and added to the reduced peptide for 45 minutes at 25 °C away from light. Reaction was quenched with the addition of 20 mM dithiothreitol (DTT) and further incubation for 60 minutes at 25 °C away from light. The reaction conversion was monitored by desalted using C18 ZipTips, eluted into 2 μL of 75% aq. MeCN saturated with sinapinic acid and spotted on a stainless steel MALDI target and analyzed by MALDI-TOF MS.

Labeled peptide was purified by HPLC with a Perkin Elmer Flexar HPLC equipped with a Betasil C18 (Thermo Scientific) reverse phase column (250 × 4.6 mm, 5 μm particle size, 100 Å pore size). A mobile phase of 10 mM aq. NH_4HCO_3/MeOH was utilized at a flow rate of 1 mL min^{-1}. Bottromycin N15K-macrolactamidine was injected as 1 mL solution with a method of: 40% MeOH (isocratic, 5 min), 40–95% MeCN (gradient, 20 min). Elution was monitored at 220 nm, 280 nm and 495 nm.

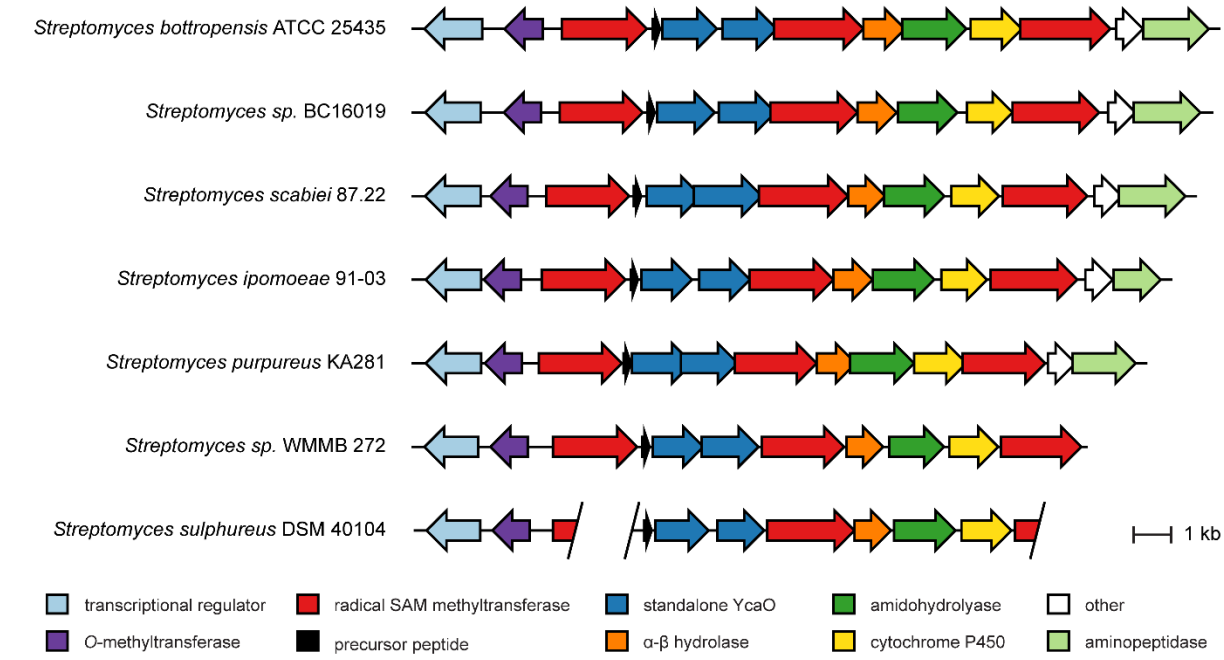
Mass spectrometric characterization of thiazoline and macrolactamidine intermediates. Peptides desalted using a ZipTip were eluted into 75% aq. MeCN. Samples were directly infused into a ThermoFisher Scientific Orbitrap Fusion ESI-MS using an Advion TriVersa Nanomate 100. The MS was calibrated and tuned with Pierce LTQ Velos ESI Positive Ion Calibration Solution (ThermoFisher). The MS was operated using the following parameters: resolution, 100,000; isolation width (MS/MS), 2 m/z; normalized collision energy (MS/MS), 35; activation q value (MS/MS), 0.4; activation time (MS/MS), 30 ms. Fragmentation was performing using collision-induced dissociation (CID) at 35%. Data analysis was conducted using the Qualbrowser application of Xcalibur software (Thermo-Fisher Scientific).

Nuclear magnetic resonance spectroscopic characterization of thiazoline and macrolactamidine intermediates. The three compounds were dissolved in D_6MSO. All solution NMR data was recorded at 305 K on a 700 MHz Bruker Avance spectrometer equipped with a cryoprobe. Standard COSY, TOCSY, and NOESY spectra with solvent suppression were acquired for each compound with ^{1}H spectral widths of 8400 Hz. FIDs for TOCSY and COSY spectra had 2406 and 256 points, and 2048 and 448 points for NOESY spectra. TOCSY spectra were recorded with mixing times between 70 and 100 ms and NOESY spectra with 150-200 ms. All spectra were processed in TopSpin 3.2 and analysed with CCPNMR.[3]

Fluorescence polarization binding studies of bottromycin follower peptide. The interactions between the fluorescently labeled precursor peptide and BmbD, BmbE, excised BmbB-RRE (residues 492-593) and excised BmbF-RRE (residues 526-670) were quantified using a fluorescence polarization (FP) assay. To maximize the polarization signal, all proteins remained MBP tagged. In general, protein was serially diluted into binding buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 2.5% (*v/v*) glycerol, 0.5 mM TCEP) and mixed with 25 nM of the appropriate fluorescein-labeled BmbC. Binding assays were carried out in nonbinding-surface, 384-black-well polystyrene microplates (Corning) and measured using a Synergy H4 Hybrid plate reader (BioTek) with λ_{ex} = 485 nm and λ_{em} = 538 nm and data were recorded with Gen5 software. Prior to measurement, the dilutions were equilibrated with shaking for 30 min at 25 °C. Dissociation constant (Kd) values were calculated from the 50% saturation point using a dose-response curve fit in Origin Pro 9.1 (OriginLab) with three independent titrations. Background fluorescence from the proteins alone was subtracted from the fluorescence polarization signal obtained with the fluorophore.

Figure S1: Bottromycin BGCs from sequenced organisms. (A) Additional bottromycin BGCs were identified by BLAST-P searches using BmbD and BmbE as the query sequences. Incomplete assembly of sequencing reads from *Streptomyces sulphureus* are indicated by the broken gene diagram. (B) Putative precursor peptides for bottromycin scaffolds aligned by Clustal Omega ("*" indicates identical residues, ":" indicates more similar residues, "." Indicates less similar residues). Divergent regions to the general bottromycin precursor consensus sequence are highlighted in red.

A



B

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Streptomyces bottropensis ATCC 25435  MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS--
Streptomyces sp. BC16019              MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS--
Streptomyces scabiei 87.22            MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS--
Streptomyces ipomoeae 91-03           MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGDWDGDVTS--
Streptomyces purpureus KA281          MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWSEGDSAV-
Streptomyces sp. WMMB 272             MGPAVVFDCMTADFLNDDPNNAELSSLEMEELESWGAWSDDTDQSV
Streptomyces sulphureus DSM 40104     MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWSEGDSAV-
                                      ***.********************:********** *.
                                      |--------|||-------------------------|
                                      core peptide  follower peptide
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Table S1: Similarity/identity comparison of bottromycin biosynthetic enzymes. NCBI accession numbers for bottromycin biosynthetic enzymes are shown below. The bottromycin biosynthetic enzymes were analyzed for sequence similarity using MatGAT 2.02 with a PAM250 scoring matrix.[4] Percent similarity values are yellow while percent identity values are blue. *S*-Adenosylmethionine methyltransferases are denoted "SAM MT". A separate table of gene nomenclature for other characterized bottromycin BGCs is provided at the bottom.

BmbA	1	2	3	4	5	6	7
1. CCM09441.1		90	91	86	75	67	66
2. AFV25479.1	94		90	87	77	68	68
3. CBG72696.1	96	94		87	75	66	66
4. WP_048819678.1	93	94	93		78	68	68
5. WP_019887073.1	86	87	86	87		68	68
6. AFU90406.1	80	81	80	81	81		99
7. WP_019549436.1	80	81	80	80	81	99	

BmbB	1	2	3	4	5	6	7
1. CCM09442.1		91	92	89	80	73	73
2. AFV25480.1	96		92	91	78	74	74
3. CBG72695.1	97	96		90	77	75	75
4. WP_048819673.1	95	96	97		79	76	75
5. WP_078513318.1	91	90	90	90		76	70
6. AFU90405.1	87	89	89	89	89		69
7. WP_013003257.1	87	87	89	87	84	85	

BmbD	1	2	3	4	5	6	7
1. CCM09444.1		83	86	80	66	60	60
2. AFV25482.1	89		79	77	64	57	57
3. CBG72693.1	92	86		76	64	57	57
4. WP_048819674.1	87	83	83		69	62	62
5. WP_019887077.1	80	77	77	85		66	65
6. AFU90403.1	79	74	75	79	84		99
7. WP_019549855.1	79	74	74	80	84	99	

BmbE	1	2	3	4	5	6	7
1. CCM09445.1		74	91	68	77	70	58
2. AFV25483.1	80		72	82	67	64	69
3. CBG72692.1	94	79		66	75	68	56
4. WP_009298727.1	74	88	72		64	61	75
5. WP_019887079.1	87	79	84	73		75	61
6. AFU90402.1	82	77	81	72	92		80
7. WP_019549857.1	66	81	65	87	73	81	

BmbF	1	2	3	4	5	6	7
1. CCM09446.1		91	93	85	76	73	73
2. AFV25484.1	96		90	87	78	75	75
3. CBG72691.1	97	94		84	77	74	74
4. WP_009298718.1	95	94	93		78	73	73
5. WP_019887080.1	86	87	85	86		79	79
6. AFU90401.1	82	84	82	83	91		100
7. WP_019549858.1	82	84	82	83	91	100	

BmbG	1	2	3	4	5	6	7
1. CCM09447.1		69	73	60	57	49	49
2. AFV25485.1	77		78	76	68	60	61
3. CBG72690.1	78	85		74	67	59	60
4. WP_048819675.1	74	88	84		69	58	58
5. WP_028797636.1	67	80	83	81		62	62
6. AFU90400.1	66	77	78	78	77		99
7. WP_019549859.1	66	77	79	78	77	100	

BmbH	1	2	3	4	5	6	7
1. CCM09448.1		78	80	71	61	64	58
2. AFV25486.1	86		87	85	70	61	66
3. CBG72689.1	85	95		81	70	60	65
4. WP_009298712.1	80	92	92		69	59	64
5. WP_019887083.1	76	87	85	86		62	67
6. AFU90399.1	79	76	74	74	78		91
7. WP_019549860.1	73	83	81	81	86	91	

BmbI	1	2	3	4	5	6	7
1. CCM09449.1		78	87	82	75	70	69
2. AFV25487.1	82		85	83	74	69	68
3. CBG72688.1	90	89		88	80	75	74
4. WP_048819679.1	88	87	95		81	73	73
5. WP_019887084.1	83	83	89	90		75	75
6. AFU90398.1	80	80	87	86	89		100
7. WP_026328876.1	80	80	87	86	88	100	

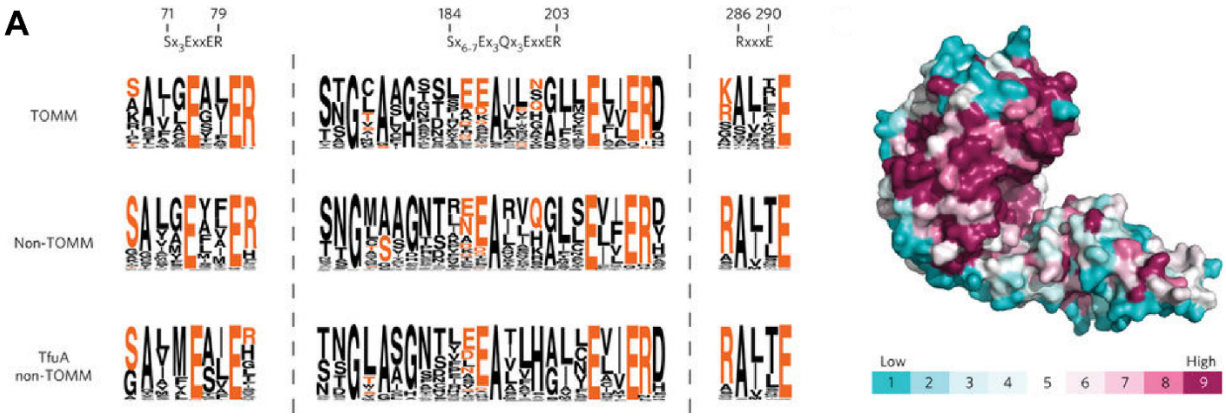
BmbJ	1	2	3	4	5	6
1. CCM09450.1		87	88	84	70	66
2. AFV25488.1	95		87	88	84	70
3. CBG72687.1	94	92		73	69	73
4. WP_009298729.1	91	92	92		69	73
5. WP_040875142.1	85	84	88	88		69
6. AFU90397.1	80	79	82	83	85	

BmbR	1	2	3	4	5	6
1. CCM09451.1		94	87	77	78	68
2. AFV25489.1	99		88	81	79	67
3. CBG72686.1	94	94		77	73	63
4. WP_009298734.1	87	87	87		69	60
5. WP_019887087.1	90	92	86	80		72
6. WP_019549673.1	85	86	82	77	89	

BmbK	1	2	3	4	5	6
1. CCM09452.1		82	88	58	68	64
2. AFV25490.1	92		86	62	68	65
3. CBG72685.1	91	91		61	69	65
4. WP_048819676.1	68	70	70		52	49
5. WP_019887089.1	82	83	85	66		69
6. WP_019549672.1	81	82	84	64	84	

Annotated Function	***Streptomyces bottropensis* DSM 40262**	***Streptomyces* sp. WMMB272**	***Streptomyces scabies***	***Streptomyces* sp. BC16019**
Major facilitator	*bmbT*	*bstK*	*btmA*	*botT*
O-methyltransferase	*bmbA*	*bstB*	*btmB*	*botO*
Radical SAM MT (Phe6 β-methylation)	*bmbB*	*bstC*	*btmC*	*botRMT1*
Precursor peptide	*bmbC*	*bstA*	*btmD*	*botA*
YcaO (thiazoline)	*bmbD*	*bstD*	*btmE*	*botC*
YcaO (macrolactamidine)	*bmbE*	*bstE*	*btmF*	*botCD*
Radical SAM MT (Val4 & Val5 β-methylation)	*bmbF*	*bstF*	*btmG*	*botRMT2*
α/β hydrolase	*bmbG*	*bstG*	*btmH*	*botH*
Metallo-hydrolase	*bmbH*	*bstH*	*btmI*	*botAH*
Cytochrome P450	*bmbI*	*bstI*	*btmJ*	*botCYP*
Radical SAM MT (Pro2 β-methylation)	*bmbJ*	*bstJ*	*btmK*	*botRMT3*
Transcriptional regulator	*bmbR*		*btmL*	*botR*
M17 aminopeptidase	*bmbK*		*btmM*	*botP*

Figure S2: Conservation of ATP-binding motifs in bottromycin cyclodehydratases. (A) The conserved ATP-binding pockets containing glutamate-rich motifs present in previously characterized YcaO cyclodehydratases. This figure has been reproduced from an earlier publication from our group[5]. (B) On the following page, Ala-substitutions of BmbD and BmbE at the corresponding positions were assayed for activity. As with the canonical ThiF/E1-dependent cyclodehydratases, these variants were inactive.



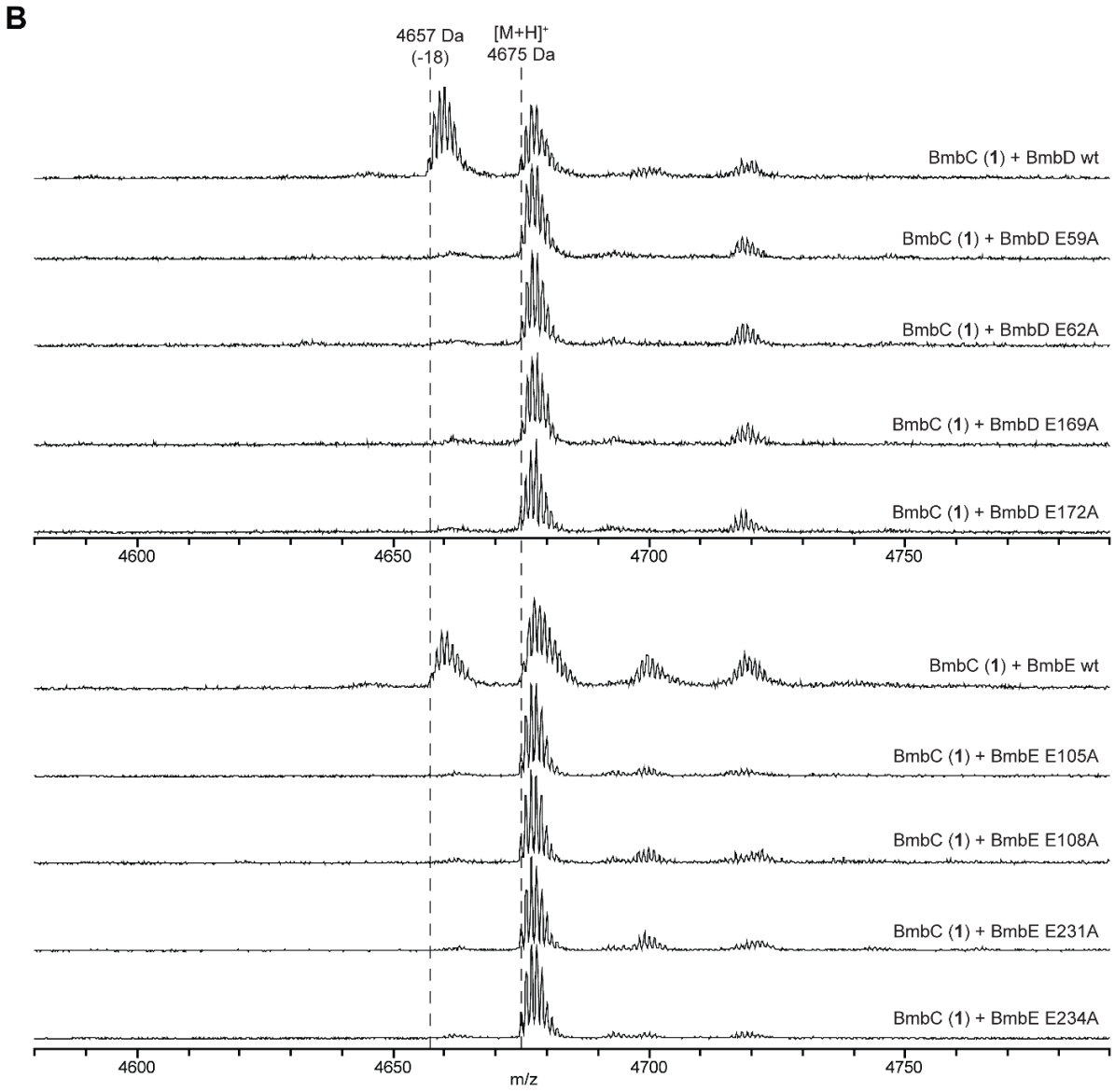

B
4657 Da
(-18)
[M+H]+
4675 Da
BmbC (1) + BmbD wt
BmbC (1) + BmbD E59A
BmbC (1) + BmbD E62A
BmbC (1) + BmbD E169A
BmbC (1) + BmbD E172A
4600
4650
4700
4750
BmbC (1) + BmbE wt
BmbC (1) + BmbE E105A
BmbC (1) + BmbE E108A
BmbC (1) + BmbE E231A
BmbC (1) + BmbE E234A
4600
4650
4700
4750
m/z

Table S2: Oligonucleotide primers used in this study. All sequences provided 5' to 3'. "fwd" indicates a forward primer while "rev" indicates reverse primer. "ins" indicates an insertion sequence at this position and "del" indicates a deletion sequence. Asterisks indicate a stop codon position.

Primer	Oligonucleotide Sequence
bmbC V3A fwd	CCTGTACTTCCAAGGACCCGCAGTCG
bmbC V3A rev	ATGCAGTCGAATACGACTGCGGGTC
bmbC D7N fwd	GGACCCGTAGTCGTATTCAACTGCATGAC
bmbC D7N rev	AGTCCGCGGTCATGCAGTTGAATACG
bmbC D7A fwd	GACCCGTAGTCGTATTCGCCTGCATG
bmbC D7A rev	GTCCGCGGTCATGCAGGCGAATAC
bmbC D7T fwd	AAGGACCCGTAGTCGTATTCACCTGCATG
bmbC D7T rev	GAAGTCCGCGGTCATGCAGGTGAATAC
bmbC C8P fwd	GACCCGTAGTCGTATTCGACCCCATGAC
bmbC C8P rev	GGAAGTCCGCGGTCATGGGGTCG
bmbC C8A fwd	GACCCGTAGTCGTATTCGACGCCATGAC
bmbC C8A rev	GGAAGTCCGCGGTCATGGCGTCG
bmbC N15K fwd	CGGACTTCCTCAAGGACGACCCCAACAACGCGGAGCTGAG
bmbC N15K rev	TTGGGGTCGTCCTTGAGGAAGTCCGCGGTCATGCAGTCG
bmbC ins2G fwd	ACTTCCAAGGAGGCCCCGTAGTCGTATTCGACTGCATGACC
bmbC ins2G rev	ACGACTACGGGGCCTCCTTGGAAGTACAGGTTCTCAGATCC
bmbC ins2A fwd	ACTTCCAAGGAGCGCCCGTAGTCGTATTCGACTGCATGACC
bmbC ins2A rev	ACGACTACGGGCGCTCCTTGGAAGTACAGGTTCTCAGATCC
bmbC ins2P fwd	ACTTCCAAGGACCGCCCGTAGTCGTATTCGACTGCATGACC
bmbC ins2P rev	ACGACTACGGGCGGTCCTTGGAAGTACAGGTTCTCAGATCC
bmbC delV3 fwd	TCCAAGGACCCGTCGTATTCGACTGCATGACCGCGGAC
bmbC delV3 rev	TCGAATACGACGGGTCCTTGGAAGTACAGGTTCTCAGA
bmbC delG1 fwd	TGTACTTCCAACCCGTAGTCGTATTCGACTGCATGACCGCG
bmbC delG1 rev	ACGACTACGGGTTGGAAGTACAGGTTCTCAGATCCACGCGG
bmbC delP2 fwd	ACTTCCAAGGAGTAGTCGTATTCGACTGCATGACCGCG
bmbC delP2 rev	AATACGACTACTCCTTGGAAGTACAGGTTCTCAGATCC
bmbC P2G fwd	ACTTCCAAGGAGGCGTAGTCGTATTCGACTGCATGACCGCG
bmbC P2G rev	AATACGACTACGCCTCCTTGGAAGTACAGGTTCTCAGATCC
bmbC F6A fwd	CCGTAGTCGTAGCGGACTGCATGACCGCGGACTTCCTCAAC
bmbC F6A rev	GTCATGCAGTCCGCTACGACTACGGGTCCTTGGAAGTACAG
bmbC ins9C fwd	TATTCGACTGCTGCATGACCGCGGACTTCCTCAACGACGAC
bmbC ins9C rev	TCCGCGGTCATGCAGCAGTCGAATACGACTACGGGTCCTTG
bmbC ins9T fwd	TATTCGACTGCACCATGACCGCGGACTTCCTCAACGACGAC
bmbC ins9T rev	TCCGCGGTCATGGTGCAGTCGAATACGACTACGGGTCCTTG
bmbC ins9S fwd	TATTCGACTGCAGCATGACCGCGGACTTCCTCAACGACGAC
bmbC ins9S rev	TCCGCGGTCATGCTGCAGTCGAATACGACTACGGGTCCTTG
bmbC ins5V fwd	GACCCGTAGTCGTGGTATTCGACTGCATGACCGCGGACTTC

bmbC ins5V rev	CAGTCGAATACCACGACTACGGGTCCTTGGAAGTACAGGTTC
bmbC V4A fwd	AACCTGTACTTCCAAGGACCCGTAGCCGTATTC
bmbC V4A rev	CGGTCATGCAGTCGAATACGGCTACGGG
bmbC V4G fwd	CTGTACTTCCAAGGACCCGTAGGCGTATTC
bmbC V4G rev	CGGTCATGCAGTCGAATACGCCTACGGG
bmbC V4I fwd	GAACCTGTACTTCCAAGGACCCGTAATCGTATTC
bmbC V4I rev	CGCGGTCATGCAGTCGAATACGATTACGGG
bmbC V5A fwd	GTACTTCCAAGGACCCGTAGTCGCATTCGAC
bmbC V5A rev	CCGCGGTCATGCAGTCGAATGCGACTAC
bmbC V5G fwd	CTGTACTTCCAAGGACCCGTAGTCGGATTCGAC
bmbC V5G rev	TCCGCGGTCATGCAGTCGAATCCGACTAC
bmbC V5L fwd	CCTGTACTTCCAAGGACCCGTAGTCCTATTCGAC
bmbC V5L rev	GTCCGCGGTCATGCAGTCGAATAGGACTAC
bmbC W34* fwd	GATGGAGGAGCTGGAGTCCTGGTGAGCCTGG
bmbC W34* rev	AGGTGGCTTCACCGTCCCAGGCTCACCAGGAC
bmbC S33* fwd	GGAGATGGAGGAGCTGGAGTCCTGAGGCGCC
bmbC S33* rev	TGGCTTCACCGTCCCAGGCGCCTCAGGACTC
bmbC E32* fwd	GCTGGAGATGGAGGAGCTGGAGTGATGGGGC
bmbC E32* rev	CTTCACCGTCCCAGGCGCCCCATCACTCCAG
bmbC L31* fwd	CGCGCTGGAGATGGAGGAGCTGTAGTCCTGG
bmbC L31* rev	CACCGTCCCAGGCGCCCCAGGACTACAGCTC
bmbC S43C fwd	CGCCTGGGACGGTGAAGCCACCTGTTGAGCG
bmbC S43C rev	GGTGCTCGAGTGCGGCCGCTCAACAGGTGGC
bmbC G35* fwd	AGTCCTGGGGCTGAGCCTGGGACGGTGAAGCCACCTCATG
bmbC G35* rev	CCGTCCCAGGCTCAGCCCCAGGACTCCAGCTCCTCCATCTC
bmbC E30* fwd	AGATGGAGGAGTGACTGGAGTCCTGGGGCGCCTGGGACGG
bmbC E30* rev	CAGGACTCCAGTCACTCCTCCATCTCCAGCGCGCTCAGCTC
bmbC A25* fwd	AGCTGAGCGCGTGACTGGAGATGGAGGAGCTGGAGTCCTGG
bmbC A25* rev	TCCATCTCCAGTCACGCGCTCAGCTCCGCGTTGTTGGGGTC
bmbC N20* fwd	ACCCCAACAACTGAGCGGAGCTGAGCGCGCTGGAGATGGAG
bmbC N20* rev	CTCAGCTCCGCTCAGTTGTTGGGGTCGTCGTTGAGGAAGTC
bmbC N15* fwd	ACTTCCTCAACTGAGACGACCCCAACAACGCGGAGCTGAGC
bmbC N15* rev	TTGGGGTCGTCTCAGTTGAGGAAGTCCGCGGTCATGCAGTC
bmbC S43C His fwd	GTGAAGCCACCTGTGCGGCCGCACTCGAGCACCACCACCAC
bmbC S43C His rev	AGTGCGGCCGCACAGGTGGCTTCACCGTCCCAGGCGCCCCAG
bmbC F13A fwd	TGACCGCGGACGCCCTCAACGACGACCCCAACAACGCGGAG
bmbC F13A rev	TCGTCGTTGAGGGCGTCCGCGGTCATGCAGTCGAATACGAC
bmbC L14A fwd	CCGCGGACTTCGCCAACGACGACCCCAACAACGCGGAGCTG
bmbC L14A rev	GGGTCGTCGTTGGCGAAGTCCGCGGTCATGCAGTCGAATAC
bmbC N15A fwd	CGGACTTCCTCGCCGACGACCCCAACAACGCGGAGCTGAGC
bmbC N15A rev	TTGGGGTCGTCGGCGAGGAAGTCCGCGGTCATGCAGTCGAA

bmbC D16A fwd	ACTTCCTCAACGCCGACCCCAACAACGCGGAGCTGAGCGCG
bmbC D16A rev	TTGTTGGGGTCGGCGTTGAGGAAGTCCGCGGTCATGCAGTC
bmbC D17A fwd	TCCTCAACGACGCCCCCAACAACGCGGAGCTGAGCGCGCT
bmbC D17A rev	GCGTTGTTGGGGGCGTCGTTGAGGAAGTCCGCGGTCATGC
bmbC E22A fwd	CCAACAACGCGGCGCTGAGCGCGCTGGAGATGGAGGAGCTG
bmbC E22A rev	AGCGCGCTCAGCGCCGCGTTGTTGGGGTCGTCGTTGAGGAA
bmbC L23A fwd	ACAACGCGGAGGCGAGCGCGCTGGAGATGGAGGAGCTGGAG
bmbC L23A rev	TCCAGCGCGCTCGCCTCCGCGTTGTTGGGGTCGTCGTTGAG
bmbC S24A fwd	ACGCGGAGCTGGCCGCGCTGGAGATGGAGGAGCTGGAGTCC
bmbC S24A rev	ATCTCCAGCGCGGCCAGCTCCGCGTTGTTGGGGTCGTCGTT
bmbC L26A fwd	AGCTGAGCGCGGCGGAGATGGAGGAGCTGGAGTCCTGGGGC
bmbC L26A rev	TCCTCCATCTCCGCCGCGCTCAGCTCCGCGTTGTTGGGGTC
bmbC E27A fwd	TGAGCGCGCTGGCGATGGAGGAGCTGGAGTCCTGGGGCGCC
bmbC E27A rev	AGCTCCTCCATCGCCAGCGCGCTCAGCTCCGCGTTGTTGGG
bmbC M28A fwd	GCGCGCTGGAGGCGGAGGAGCTGGAGTCCTGGGGCGCCTGG
bmbC M28A rev	TCCAGCTCCTCCGCCTCCAGCGCGCTCAGCTCCGCGTTGTT
bmbC E29A fwd	CGCTGGAGATGGCGGAGCTGGAGTCCTGGGGCGCCTGGGAC
bmbC E29A rev	GACTCCAGCTCCGCCATCTCCAGCGCGCTCAGCTCCGCGTT
bmbC E30A fwd	TGGAGATGGAGGCGCTGGAGTCCTGGGGCGCCTGGGACGGT
bmbC E30A rev	CAGGACTCCAGCGCCTCCATCTCCAGCGCGCTCAGCTCCGC
bmbC L31A fwd	AGATGGAGGAGGCGGAGTCCTGGGGCGCCTGGGACGGTGAA
bmbC L31A rev	CCCCAGGACTCCGCCTCCTCCATCTCCAGCGCGCTCAGCTC
bmbC E32A fwd	TGGAGGAGCTGGCGTCCTGGGGCGCCTGGGACGGTGAAGCC
bmbC E32A rev	GCGCCCCAGGACGCCAGCTCCTCCATCTCCAGCGCGCTCAG
bmbD E59A fwd	AAGCCCTGTCCGCAGCCGTGGAGCGGCTGGTGGCGTGCACC
bmbD E59A rev	CGCTCCACGGCTGCGGACAGGGCTTTCGCCCGTGCCACGGC
bmbD E62A fwd	CCGAAGCCGTGGCGCGGCTGGTGGCGTGCACCCCCTTCGCG
bmbD E62A rev	GCCACCAGCCGCGCCACGGCTTCGGACAGGGCTTTCGCCCG
bmbD E169A fwd	GCGCGCTGCTGGCACTGACCGAACTGATCGACTACGGAGTC
bmbD E169A rev	AGTTCGGTCAGTGCCAGCAGCGCGCCGCGCAGCGCTCCCTC
bmbD E172A fwd	TGGAACTGACCGCACTGATCGACTACGGAGTCTTCCTGCAC
bmbD E172A rev	TAGTCGATCAGTGCGGTCAGTTCCAGCAGCGCGCCGCGCA
bmbE E105A fwd	CTGCGCTGTTTGCAAGCGCGGAACATTATCACCTGGACTGG
bmbE E105A rev	TGTTCCGCGCTTGCAAACAGCGCAGAAGCCATGCTACGATG
bmbE E108A fwd	TTGAAAGCGCGGCACATTATCACCTGGACTGGCGTCGCG
bmbE E108A rev	GGTGATAATGTGCCGCGCTTTCAAACAGCGCAGAAGCCATG
bmbE E231A fwd	ACGCAGTGAATGCACTGATTGAACGCGATGCGTGGTCATATC
bmbE E231A rev	CGTTCAATCAGTGCATTCACTGCGTGGACCAGGGCTTCATG
bmbE E234A fwd	ATGAACTGATTGCACGCGATGCGTGGTCATATCAACTGGCC
bmbE E234A rev	CACGCATCGCGTGCAATCAGTTCATTCACTGCGTGGACCAG
bmbD R391* fwd	GCGGTGGCCATTGAGATCGTAGCGCAGACCGTGGTCCGGCA

bmbD R391* rev	GCGCTACGATCTCAATGGCCACCGCGGTCAGCCGGACGATC
bmbD D396* fwd	ATCGTAGCGCATAGCGTGGTCCGGCACGTCGCGGTCACGAAG
bmbD D396* rev	GCCGGACCACGCTATGCGCTACGATCACGATGGCCACCGCG
bmbD R401* fwd	GTGGTCCGGCATGACGCGGTCACGAAGGCCGTCGCTCTGCG
bmbD R401* rev	TCGTGACCGCGTCATGCCGGACCACGGTCTGCGCTACGATC
bmbD G406* fwd	GCGGTCACGAATGACGTCGCTCTGCGTAACTCGAGCACCAC
bmbD G406* rev	GCAGAGCGACGTCATTCGTGACCGCGACGTGCCGGACCACG
bmbE D434* fwd	TGGAAATGTTTTAGAAAGCCCGCGCAGGTTATCCGGTGCTG
bmbE D434* rev	GCGCGGGCTTTCTAAAACATTTCCAGGCCCGGTGCAACCAC
bmbE G439* fwd	AAGCCCGCGCATGATATCCGGTGCTGCCGACCGGCCGTCTG
bmbE G439* rev	AGCACCGGATATCATGCGCGGGCTTTATCAAACATTTCCAG
bmbE P444* fwd	ATCCGGTGCTGTAGACCGGCCGTCTGGGTGAACGTCTGCGTC
bmbE P444* rev	AGACGGCCGGTCTACAGCACCGGATAACCTGCGCGGGCTTTATC
bmbE G449* fwd	CCGGCCGTCTGTGAGAACGTCTGCGTCCGCGTGGTGATGGC
bmbE G449* rev	CGCAGACGTTCTCACAGACGGCCGGTCGGCAGCACCGGATAAC
bmbE P454* fwd	AACGTCTGCGTTAGCGTGGTGATGGCGGTGGCGCACGTCGC
bmbE P454* rev	CCATCACCACGCTAACGCAGACGTTCACCCAGACGGCCGGTC
bmbE G459* fwd	GTGGTGATGGCTGAGGCGCACGTCGCTAACTCGAGCACCAC
bmbE_G459* rev	CGACGTGCGCCTCAGCCATCACCACGCGGACGCAGACGTTC
bmbB RRE fwd	AAAGGATCCGCGAACGGTCCGGTGCG
bmbB RRE rev	AAAGCGGCCGCTTTGGTACCCTCAATGTAAACCCAA
bmbF RRE fwd	AAAGGATCCCTGCACGAGCAGAGCC
bmbF RRE rev	AAAGCGGCCGCACGACGGCCTTCCAG

Table S3: Sequences for *E. coli* expression. All sequences are provided 5' to 3'. Gene constructs for BmbD and BmbE were codon optimized and synthesized by GenScript (Piscataway, NJ, USA). The amino acid sequences for the two excised RRE domains of BmbB and BmbF are also given (these were not codon optimized). All proteins were expressed as MBP fusions (see methods for details).

BmbD

ATGCGTGAAGCCACCGCTACCGAATGCGAACTGCGTGAAGTCGTCCACCGCTCATACCCGAGTGAACGTACCGTTACCGTCCGCTG
CACCGTTCGTCCGGCAGAAGGTTCAGCGCAGGCCGATGGTTATGGCACGGCCACCACGGAAGCAGTGGCTCGTGCGAAAGCCCTGT
CGGAAGCAGTGGAACGCCTGGTTGCTTGCACCCCGTTTGCAACGGTTGCACGTCCGCCGACCGCATGTGCTGATCCGGGTACGGGC
AGCGGTCCGGTGCCGCCGTTCCCGGCAGCCGGCGTTCGCACCGCGCCGGAAGGTTGCGCAGGTCGTGTCTATCGTCCGCTGACGGG
CGGTGGCCCGCGTCGCGTGCCGCTGTACTGGAGCTCTCCGTGGACCGCAGGTGCAGAACTGCGTGCAGGTACCCTGACGGCAGCTG
AAGCACGTCTGAGTTCCACCGTCGGTTGGGCAGTGGCTCCGACGCCGGAAGGTGCACTGCGTGGTGCACTGCTGGAACTGACCGAA
CTGATTGACTATGGCGTGTTTCTGCATCGTCGCCTGGCAGGTCCGGCTCGTCGCCCGTCAGCAGGTGATCGTACCCTGGTGGTTCC
GCTGGGTGGCATCGGTCGTACGCCGGCAGTTCTGGCGGTCGCCTACGGTCGTGGTCGCCTGATGCCGGCAACCGGTCTGGGTTGCG
GTGCAACCCGTGGCGAAGCAACGGATCGCGCTCTGCTGGAACTGGCACAGGCTGAAACCATGTGGCGTTCCAACCCGACGGCAGAA
CCGGCAGAACGCTTTTTCCTGCGTCGCTTTGAACGTTGGCCGCTGCTGGCACGTTGCGCTACCCTGGATTTCGATCTGCCGGACCC
GCGTGATCCGCCGGCACGTAGTGGTCCGGCGGGTGGCCCGGGTCCGTACGATGACGGTCCGTGTCCGGCATCCCCGCTGGAAGAAC
TGGAAGCCGGTGGCATTCGTGTTTGGGCGGATTCAGGTGCCGTCGACATTTCGGGTCCGGATATCCCGCGTACCCGTCTGTGTTTT
GCGCACGTCGTGAGCGATCCGCAACCGCTGCTGGGTCTGGTTCGTGCAGGCATCCCGGTCTTCGACACCGGTGAAGTGCGTCGCAC
GCTGGACCCGAGCCGTCGTCCGGCAGATCGTCCGGCTGACCGCGGTGGCCATCGTGATCGTAGCGCAGACCGTGGTCCGGCACGTC
GCGGTCACGAAGGCCGTCGCTCTGCGTAA

BmbE

ATGACCCGCCCGGCAGACCCGCAAACCGCCCCGCAAACCGAAGCCGCCACCGAAACGGACCCGCTGACCGCTGTGCTGCTGGATGG
TGCGACCAAAGAAGACGTGCCGCCGGAACGCGAAACGGATATTCGTCAGGCACTGGCAAGCGTTGGTGACTGGCTGGCAGCAGAAG
GTCTGACCGCGGATATTCGCAAATATGGCATCGAAAGTGCCCCGACGTACGAAGTGCGTCTGTCAGAAGCTTCGGGTGCGCTGTGC
TCCCGCAGCTCTGGTAAAGGCCTGGGTCATCGTAGCATGGCTTCTGCGCTGTTTGAAAGCGCGGAACATTATCACCTGGACTGGCG
TCGCGATCCGCGCGCCTCTGAAGCAGAATTCCGCCCGGGCCGTGAAATTGCAGAACAGGCAACCGCACGTCGCAGCGCACTGCTGG
GCCGTCTGGGTCAAATCATGCCGGATGCACCGGTGCTGACCCGTACGTATCGTCCGGTTGAAGAAGCTCTGACCGGTCGTGCAGGT
GACGGTGAAGCAGATGGTCCGCATCGTCACCCGGTTTTTCTGCGCGATGGCGGTTACCGTAACTGGCCGCATCCGGATGACGATCG
CTCTTTCCAGCCGCTGTGGCACTATACCAGTTCCGCAGGCTACGCAGCTGGTGCTACGCGTCATGAAGCCCTGGTCCACGCAGTGA
ATGAACTGATTGAACGCGATGCGTGGTCATATCAACTGGCCCGTTCGTACTTTGGCCTGCCGGGTGAAGGTCCGGAACTGCGTGTG
GTTGAACATGGTACCCTGCCGGCAGAACTGCGCGAACTGACCCGTACGGTTGAAGAAGTCCGTGGCGCGCCGGTGCTGATTGTTGA
CATCACCTGCGATACGGGTGTTCCGGCATATGTCGTGTGTGACGCTCGTAGCCGCGAAGATGTCCGTCTGATCGGCAGTGGTGCAT
CCCCGGTCCGTACCTATGCTGTGCAGCGTGCGCTGCTGGAATACCTGCAAGTCCGTACGATGTTCGAAAACGGTCCGGTGGACGCA
GATACCGAAGCAGGCCAGATTGGTACGGCTCTGGCACGTTACCCGCGTCATCTGGCAGCAGCACGCTTTGATATCCATCGTCTGCC
GCACGAATCAACCGCTTTCGAAGCGGACGATGGTCTGCCGGCTGCAACGGGTCCGGAACGTCTGCTGCGTCACCTGGTGGATCGTC
TGCGCGAATCGGGCGTTGAAGCACATCACCGTGTCCTGACCCCGCCGGGTCCGGTTACGGTTGTCGACGTGGTTGCACCGGGCCTG
GAAATGTTTGATAAAGCCCGCGCAGGTTATCCGGTGCTGCCGACCGGCCGTCTGGGTGAACGTCTGCGTCCGCGTGGTGATGGCGG
TGGCGCACGTCGCTAA

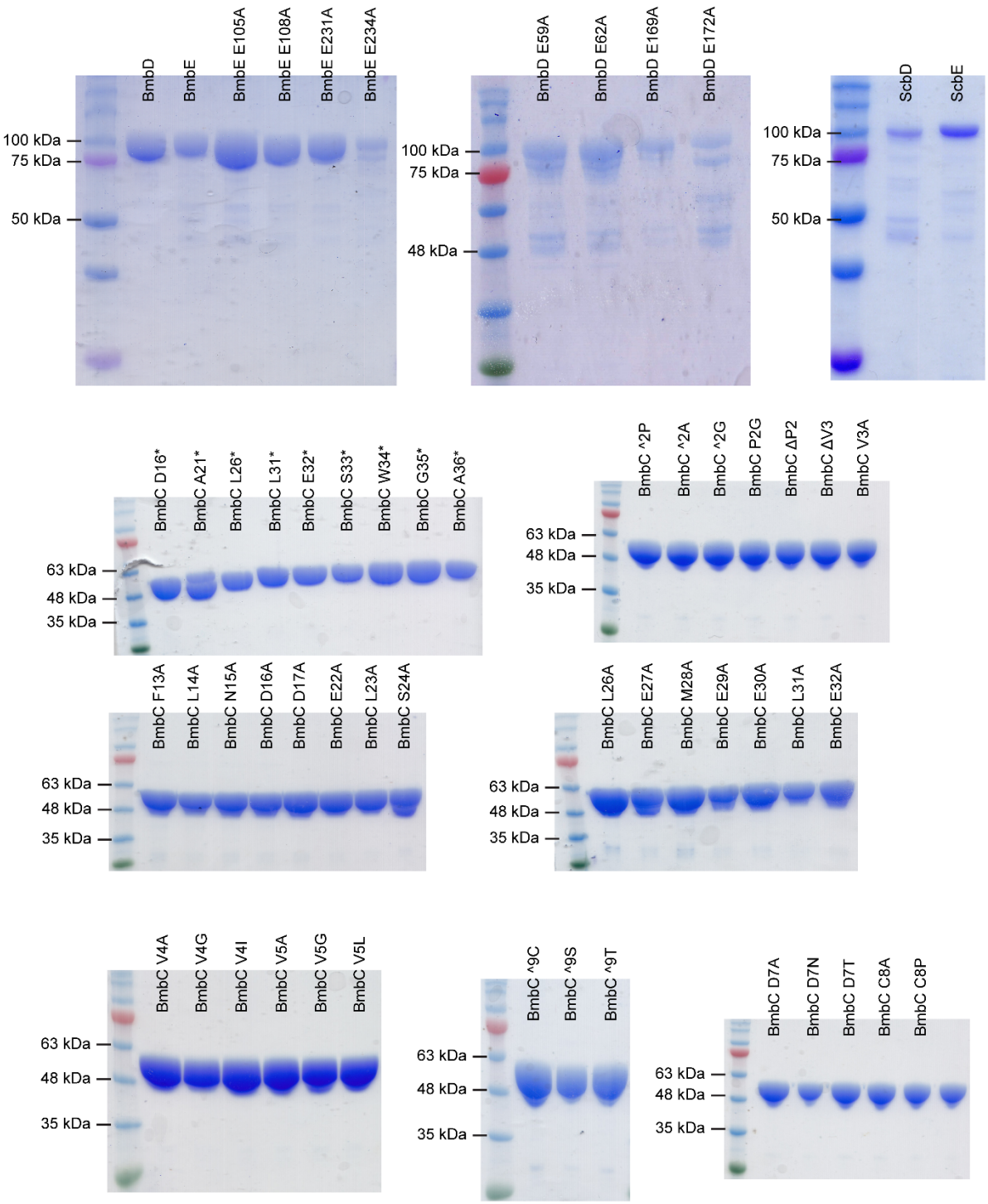
BmbB (residues 492-593, excised RRE domain)

ANGPVRRRLAARVRLWRAHHESARFGYRLGFDSLTLTDERPGLPPRTTTLRGEEARLFRAVIGGTRFRDLQGQEWQGERWDQALDT
LHAWRRKRWVYIEGTK

BmbF (residues 526-670, excised RRE domain)

LHEQSRFSFRFGPNMTVLHDGRPHVGTGRHVLRGGDDQLFRCLVHGGRLHQAVGRVAAMTGRPEDEVHATVREWAGRGWIHLEGRR
GLVLAVRDGMRDELGRAAELREAADGPERAAAGPERAVEPPQAVESERAGGSGRVGAGR

Figure S3: SDS-PAGE analysis of proteins used in this study. All protein were expressed as N-terminal maltose-binding protein (MBP) fusions. Minor impurities found at ~45 kDa are cleaved MBP. Bmb sequences from *S. bottropensis*, Scb sequences from *S. scabiei*, insertion, deletion and truncation variants are denoted by "^", "Δ" and "*", respectively.



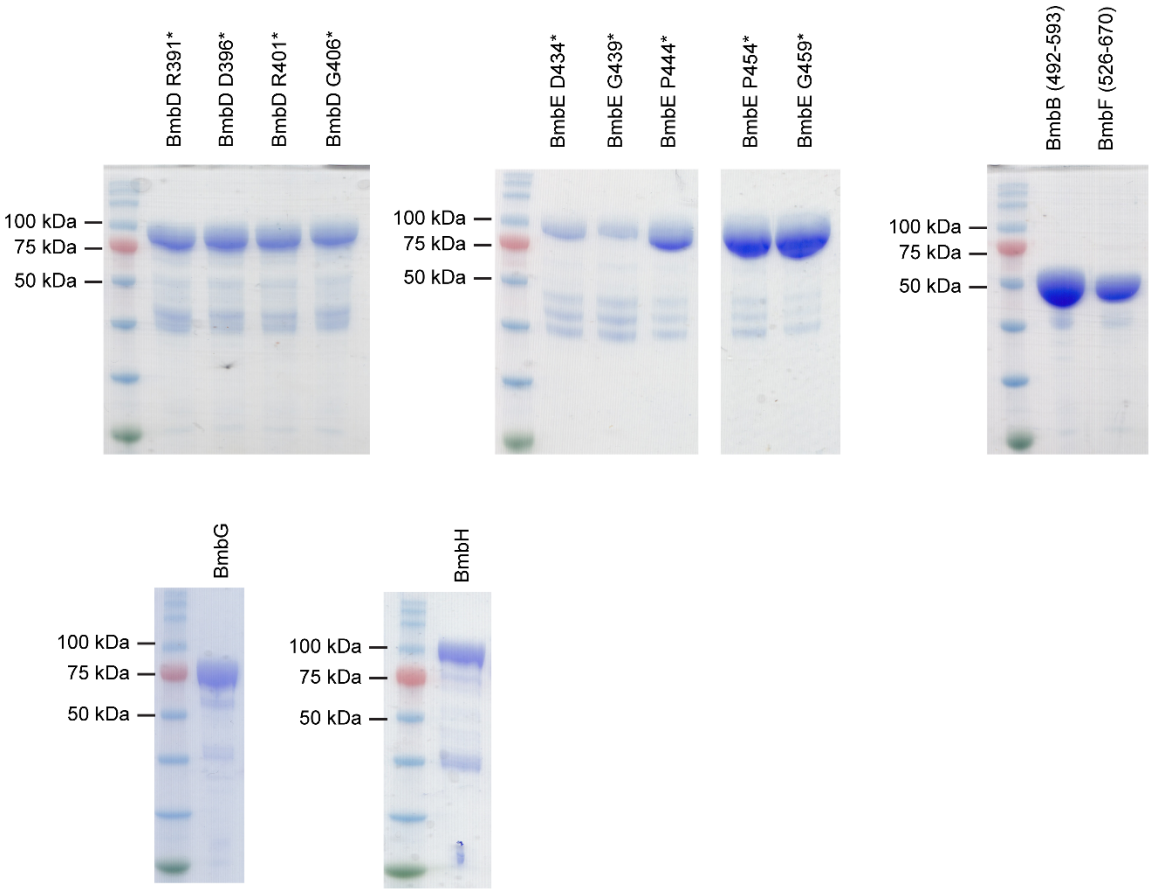

BmbD R391*
BmbD D396*
BmbD R401*
BmbD G406*
100 kDa
75 kDa
50 kDa
BmbE D434*
BmbE G439*
BmbE P444*
BmbE P454*
BmbE G459*
100 kDa
75 kDa
50 kDa
BmbB (492-593)
BmbF (526-670)
100 kDa
75 kDa
50 kDa
BmbG
100 kDa
75 kDa
50 kDa
BmbH
100 kDa
75 kDa
50 kDa

Figure S4: Multiple sequence alignment of BmbD and BmbE proteins. Multiple sequence alignment performed by Clustal Omega of BmbD and BmbE proteins from bottromycin BGCs. ATP-binding pocket residues (ExxE) are highlighted in red while the C-terminal truncations are highlighted in blue.

BmbD homologs:

```
AFU90403.1          ------------MADSSATDCVLREVAFRSYRAEHTVTVQCTVRPDSGPGQAEGYGTATT
WP_019549855.1      ------------MADSSATDCVLREVAFRSYRAEHTVTVQCTVRPDSGPGQAEGYGTATT
WP_019887077.1      ------------MLEASVADCALRDVVHRSYRSDRTVTVRCTVHPASGSPPADGYGTATT
EKX68557.1          MKSARRTTGRGSMLEATATECALREVVYRSYPSERTVTVRCTVRPAEGSARADGYGTAAT
AFV25482.1          ------------MLEATATECELREVVHRSYPSDRTVTVRCTVRPAEGTAQADGYGTATT
CCM09444.1          ------------MREATATECELREVVHRSYPSERTVTVRCTVRPAEGSAQADGYGTATT
CBG72693.1          ------------MREATATECELREVVHRSYPSEGTVTVRCTVRPAEGDARADGYGTATT
                                * :::.::* **:*..*** :: ****:***:* .*   *:*****:*

AFU90403.1          EAVARKKALSEAVERLLACTPFALIARRPTPRTDRSRGTDP---W-QRGLRSAPAGCQVR
WP_019549855.1      EAVARKKALSEAVERLLACTPFALIARRPTPRTDRSRGTDP---W-QRGLRSAPAGCQVR
WP_019887077.1      EAVARKKALSEAVERLLACTPFASVARPPTTHLTTTG--DDRNPW-PAGVRTAPEGCLTR
EKX68557.1          KAVARLKALSEAVERLVACTPFASVVRPPTT----SRGSGAVPPFPASGVRTVPDGCASR
AFV25482.1          EAVARAKALSEAVERLVACTPFATVARPPTT-PAPGSGSGPVPPFPAAGVRTAPDGCASR
CCM09444.1          EAVARAKALSEAVERLVACTPFATVARPPTACADPGTGSGPVPPFPAAGVRTAPEGCAGR
CBG72693.1          EAVARAKALSEAVERLVACTPFTAVARPPTAFADPGTGSGPVPPFPAAGVRTAPDGCADR
                    :**** **********:*****: :.* **         .    :   *:*:.* **  *

AFU90403.1          NYRSLSGE-QPRQVPLFWSSPWVAGEEMRSANLAPQAARLSSTVGWAVAPSPEAALQGAL
WP_019549855.1      NYRSLSGE-QPRQVPLFWSSPWVAGEEMRSANLAPQAARLSSTVGWAVAPSPEAALQGAL
WP_019887077.1      TYRPLTRGALPRQVPLFWSSPWIAGEELRSALLTAQTARLSSTIGWAVAPSPEAALRGAL
EKX68557.1          VYRPLTGG-GPRRVPLYWSSPWTAGEELRAAVLTAPEARLSSTVGWAVAPTPEAALHGAL
AFV25482.1          EYRPLSGD-GPRRVPLYWSSPWVAGEELRAGTLSAAEARLSSTVGWAVAPTPEAALRGAL
CCM09444.1          VYRPLTGG-GPRRVPLYWSSPWTAGAELRAGTLTAAEARLSSTVGWAVAPTPEGALRGAL
CBG72693.1          VYRPLTGG-GPRRVPLYWSSPWTAGAELRAGTLTAAQARLSSTVGWAVAPTPEAALRGAL
                     ** *:    **:***:***** ** *:*:. *:   ******:******:**.**:***

AFU90403.1          YELTELLNYGAFLYRCLAAPRERPAPGGGPEPREEGRIQTVPISWATRTPTILAVTRSSD
WP_019549855.1      YELTELLNYGAFLYRCLAAPRERPAPGGGPEPHEEGRTQTVPISWATRTPTILAVTRSSD
WP_019887077.1      FELTELINYGVFLFRALAGPPHDGRDS-------GPGPLILPIDGATRTPTVLAVARGGG
EKX68557.1          LELTELLNHGVFLHRSLAGPRR--PAA-------GDETLVLPLGGPVRTPTVLAVAYGRG
AFV25482.1          LELTELIDYGVFLHRRLAGPARP-RSA-------GDETLVVPLGGAVRTPTVLAVAYGRG
CCM09444.1          LELTELIDYGVFLHRRLAGPARR-PSA-------GDRTLVVPLGGIGRTPAVLAVAYGRG
CBG72693.1          LELTELIGYGVFLHRRLAGPPRR-PSG-------DDRTLVVPLEGVGRTPAVLAVAYGRG
                     *****:.:*.**.* **.* .    .             :*:    ***::***: . .

AFU90403.1          RLMPATGIGSGTTASEAAERAFLELAQAETLWRANSTAVPAERYFMRRFEKWPLLRRCAA
WP_019549855.1      RLMPATGIGSGTTASEAAERAFLELAQAETLWRANSTAVPAERYFMRRFEKWPLLRRCAA
WP_019887077.1      RRMPATGLGSGTTRADATDRALLELAQAVTLWRSNPTGIPAERHFMRRFDRWPLLTRCAT
EKX68557.1          RRMPATGLGCGATRAEATDRALLELAQAETMWRSNPTAVPAERFFLRRFERWPLLGRCAT
AFV25482.1          RLMPATGLGCGASRAEATDRALLELAQAETMWRSNPTAEPAERFFLRRFERWPLLTRCAT
CCM09444.1          RLMPATGLGCGATRGEATDRALLELAQAETMWRSNPTAEPAERFFLRRFERWPLLARCAT
CBG72693.1          RLMPATGLGCGETVAEATGRALLELAQAETMWRSNPTAEPAERFFLRRFERWPLLTRCAT
                    * *****:*.* : .:*: **:****** *:**:* *. ****.*:***::**** ***:

AFU90403.1          LDFDLGEHA----------------------------------GPFDDGPHP-SPLGALES
WP_019549855.1      LDFDLGEHA----------------------------------GPFDDGPHP-SPLGALES
WP_019887077.1      LDFDLTGYDA---------------------------------P-PDGVPRPSPLEELEA
EKX68557.1          LDFDLSG-----------------------------------HGTPYDDERSVASPLEELEA
AFV25482.1          LDFELSDLSGLDGLSGLDGLSRLDGLSRLDHPSDLNDPAGRPAPYDDGPHPASPLEELEA
CCM09444.1          LDFDLPDPRD---------------------PPARSGPAGGPGPYDDGPCPASPLEELEA
CBG72693.1          LDFDLKDADA---------------------PHGPH----DPAPGDDEPRPATPLEELEA
                    ***:*                                    *  *     :**  **:

AFU90403.1          RAIEVWADPGAVDISGADLRRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRKILVCPR
WP_019549855.1      RAIEVWADPGAVDISGADLRRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRKILVCPR
```

```
WP_019887077.1      RGIAVWADSGALDLSGPDLARTRLHFAQVVADPQPLLGLVRAGIPVFDTGEVRKILDSSR
EKX68557.1          GGISVWADSGAVDISGPDTPRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRRTLDPPR
AFV25482.1          GGITVWADSGAVDISGPDIPRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRRTLDPSR
CCM09444.1          GGIRVWADSGAVDISGPDIPRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRRTLDPSR
CBG72693.1          GGIRVWADSGALDISGPDIPRTRLCFAHVVSDPQPLLGLVRAGIPVFDTGEVRRTLDPSR
                     .* **** **:*:** *  **** **:**:**********************: *   *

AFU90403.1          RARSAD-----------------------RSPSGRVAHSGRPTRA
WP_019549855.1      RARSAD-----------------------LSPSGRVAHSGRPTRA
WP_019887077.1      RERPAD-----------------------RRPSGRNAQGGCPARA
EKX68557.1          RDR------------------PAHHARSADHRAAGRGRQGRRTA--
AFV25482.1          RDAHRDTPRGA--------PRDAARDRSADRGPAGRGRQGRRSA--
CCM09444.1          RPADR------------PADRGGHRDRSADRGPARRGHEGRRSA--
CBG72693.1          PSADPSDPAGRAVPADTRRERRDLRDRSADRGPARRGREGRRSA--
                                           : *  ..   :
```

BmbE homologs:

```
AFU90402.1          ------------------------MSVHAEHDLLTAALLDGSLTQDVPPERETPIGRALS
WP_019549857.1      ------------------------------------------------------------
WP_019887079.1      ------------------------MTRPAENDLLTAALLDGTLKEDVPPERETPLGKALA
CCM09445.1          MTRPADPQTAP------------QTEAATETDPLTAVLLDGATKEDVPPERETDIRQALA
CBG72692.1          MTRPAHPQTRPTRPAEPFPRTPAENAETSETDPLTAVLLDGAAKEDVPPERETDLRQALA
AFV25483.1          ------------------------------------------------------------
EKX68554.1          ------------------------------------------------------------


AFU90402.1          AASDWLSSEQLTPDIWKYGLDSAPTYEVHLKDAAGTLCSRSSGKGLGHRSMASALFESAE
WP_019549857.1      ----------------------------------------------------MASALFESAE
WP_019887079.1      AVGDWLAAERLTADIRKYGIDSAPTYEVQLKDADGALCSRSSGKGLGHRSLASALFESAE
CCM09445.1          SVGDWLAAEGLTADIRKYGIESAPTYEVRLSEASGALCSRSSGKGLGHRSMASALFESAE
CBG72692.1          SVGDWLAAEGLTADIRKYGIESAPTYEVRLSDAAGALCSRSSGKGLGHRSMASALFESAE
AFV25483.1          --------------------------MRLTDAEGALCSRSSGKGLGHRSMASALFESAE
EKX68554.1          ----------------------------------------------------MASALFESAE
                                                                        :*********

AFU90402.1          HYHLDWRRDPLADEAAFVPGREVAGQSTAAGSALLHRLGGILGDSPVLTREYRPVAEALG
WP_019549857.1      HYHLDWRRDPLADEAAFVPGREVAGQPTAAGSALLHRLGGILGDSPVLTREYRPVAEALG
WP_019887079.1      HYHLDWRRDPRASDTVFLSGREIARQPTARRSALLQRLGGIAADAPVLTREYRPVAEALA
CCM09445.1          HYHLDWRRDPRASEAEFRPGREIAEQATARRSALLGRLGQIMPDAPVLTRTYRPVEEALT
CBG72692.1          HYHLDWRRDPRASEAEFRPGREIAGQATARRSALLRRLGGIMPDAPVLTRTYRPVEEALT
AFV25483.1          HYHLDWRRDPRASEAEFLPGREIAGQDTARRSALLRRLGGIMPDAPVLTRTYRPVEETLT
EKX68554.1          HYHLDWRRDPRAAEAEFRPGRDIAGQATARRSALLRRLGGIMPDSPVLTRAYRPVEEALT
                    ********** * :: *  **::* * **  **** *** *  *:***** **** *:*

AFU90402.1          A-------TGGAAPPHRHPVFLRDGGYRNWPHPSDDGSFKPLWHYTTSAGYAAGTTLHEA
WP_019549857.1      A-------TGGAAPPHRHPVFLRDGGYRNWPHPSDDGSFKPLWHYTTSAGYAAGTTLHEA
WP_019887079.1      EPADG-------AAPHRQPVFLRDGGYRNWPHPADDGSFRTLWHYTSSAGYAAGATLHEA
CCM09445.1          GRAGDGE----ADGPHRHPVFLRDGGYRNWPHPDDDRSFQPLWHYTSSAGYAAGATRHEA
CBG72692.1          GRADDGGGAGPAGQPHRHPVFLRDGGYRNWPHPDDDRSFQPLWHYTSSAGYAAGATRHEA
AFV25483.1          GRADHG----PAGRPHRHPVFLRDGGYRNWPHPDDDRSFQPLWHYTSSAGYAAGATVHEA
EKX68554.1          GRASDA-------AGHRHPVFLRDGGYRNWPHPDDDRSFQPLWHYTSSAGYAAGATLHEA
                              **:*************** ** **: *****:*******:* ***

AFU90402.1          LVHAVNELIERDAWSYQLARSYFGLPDESGRALRVIDHESLPAELRALVDDVEASSGSPI
WP_019549857.1      LVHAVNELIERDAWSYQLARSYFGLPDESGRALRVIDHESLPAELRALVDDVEASSGSPI
WP_019887079.1      LVHAVNELVERDAWSYQLARSYFGLADG-GPALRVVDPATLPAELRQLARRVEAVRDAPV
CCM09445.1          LVHAVNELIERDAWSYQLARSYFGLPGE-GPELRVVEHGTLPAELRELTRTVEEVRGAPV
CBG72692.1          LVHAINEVIERDAWSYQLARSYFGLPGE-GPELRVVDHGTLPAELRELTRTVEEVRDSPV
AFV25483.1          LVHAVNELIERDAWSYQLARSYFGLPGE-GPELRVVDHDTLPAELRELTGTVEEVREAPV
EKX68554.1          LVHAVNELIERDAWSYQLARSYFGLPGE-GPELRVVDHDSLPAELRELTGTIEEVRRAPV
                    ****:**::**************** .  *  ***::  :****** *.  :*    :*:

AFU90402.1          LIVSIPCDTGVPAYVVCDAESDEDVRLIGSGASPVQAYALQRGLLEYLQVCTMYQHGPVD
```

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WP_019549857.1      LIVSIPCDTGVPAYVVCDAESDEDVRLIGSGASPVQAYALQRGLLEYLQVCTMYQHGPVD
WP_019887079.1      LVVDVTCDTDVPAYVVCDAESREDVRLIGSGASPVRTYALQRALLEYLQVRTMFEHGPVD
CCM09445.1          LIVDITCDTGVPAYVVCDARSREDVRLIGSGASPVRTYAVQRALLEYLQVRTMFENGPVD
CBG72692.1          LIVDITCDTGVPAYVVCDAVSREDVRLIGSGASPVRTYAVQRALLEYLQVRTMFENGPVD
AFV25483.1          LIVDITCDTGVPAYVVCDARSREEVRLIGSGASPVRTYALQRALLEYLQVRTMFEHGPVD
EKX68554.1          LIVSIACDTGVPAYVVCDAESREDVRLIGSGASPVRTYALQRALLEYLQVRTMFEHGPVD
                    *:*.: ***.********* * *:***********::**:**.******* **:::****

AFU90402.1          AEREAAQIATALARYPRHLAAAGFDVRQLPHERQPFDSDG----LPAAATPEELLRHLID
WP_019549857.1      AEREAAQIATALARYPRHLAAAGFDVRQLPHERQPFDSDG----LPAAATPEELLRHLID
WP_019887079.1      ADTEARQIGTALARYPRHLAAARFDIHELPHEQVAFASDGGLS---AAASPEDLLRHLVD
CCM09445.1          ADTEAGQIGTALARYPRHLAAARFDIHRLPHESTAFEADDGLP---AATGPERLLRHLVD
CBG72692.1          ADTEAGQIGSALARYPRHLAAARFDIHRLPHESRAFEADDGLP---AATGPERLLRHLVD
AFV25483.1          ADTEAGQIATALARYPRHLAAARFDVGQLPHERRAFDADDGLP---AAASPEQLLRHLVD
EKX68554.1          ADTEAGQIATALARYPRHLAAARFDIHRLPHEMREFDADDGLPLLPAAASPEELLRHLVE
                    *: ** **.:************ **: .****   * :*.      **: ** *****::

AFU90402.1          RLQGVGVDLHFRVLTRPGTITVVDVVAPGLEMLDKARAGYPVLPTGRLGERLRPQRREP-
WP_019549857.1      RLQGVGVDLHFRVLTRPGTITVVDVVAPGLEMLDKARAGYPVLPTGRLGERLRPQRREP-
WP_019887079.1      RLRAVRVDVHYRVLTRPGAVTVVDVVAPGLEMLDKARAGYPVLPTGRLAERLRSRGSERA
CCM09445.1          RLRESGVEAHHRVLTPPGPVTVVDVVAPGLEMFDKARAGYPVLPTGRLGERLRPRGDGGG
CBG72692.1          RLRESGIEAHHRVLTPPGPVTVVDVVAPGLEMFDKARAGYPVLPTGRLGERLRPRGDGGG
AFV25483.1          RLRDLGVDVHHRVLTPPGSVTVVDVVAPGLEMFDKARAGYPVLPTGRLGERLRPRKDHER
EKX68554.1          RLREVGVDVHYRVLTPPGAVTVVDVVAPGLEMFDKARAGYPVLPTGRLGERLRPGTSGG-
                    **:   :: *.**** ** :************:***************.****

AFU90402.1          ---------------
WP_019549857.1      ---------------
WP_019887079.1      ---------------
CCM09445.1          ARR------------
CBG72692.1          ARR------------
AFV25483.1          GHGVDHGKDRGGAPR
EKX68554.1          -------TGRGGAPK
```

Figure S5: Activity assessment of bottromycin cyclodehydratase C-terminal truncations. Four C-terminal truncations to BmbD and five C-terminal truncations to BmbE were prepared to assess the importance to catalytic activity. Refer to Figure S4 for a depiction of the truncation locations.

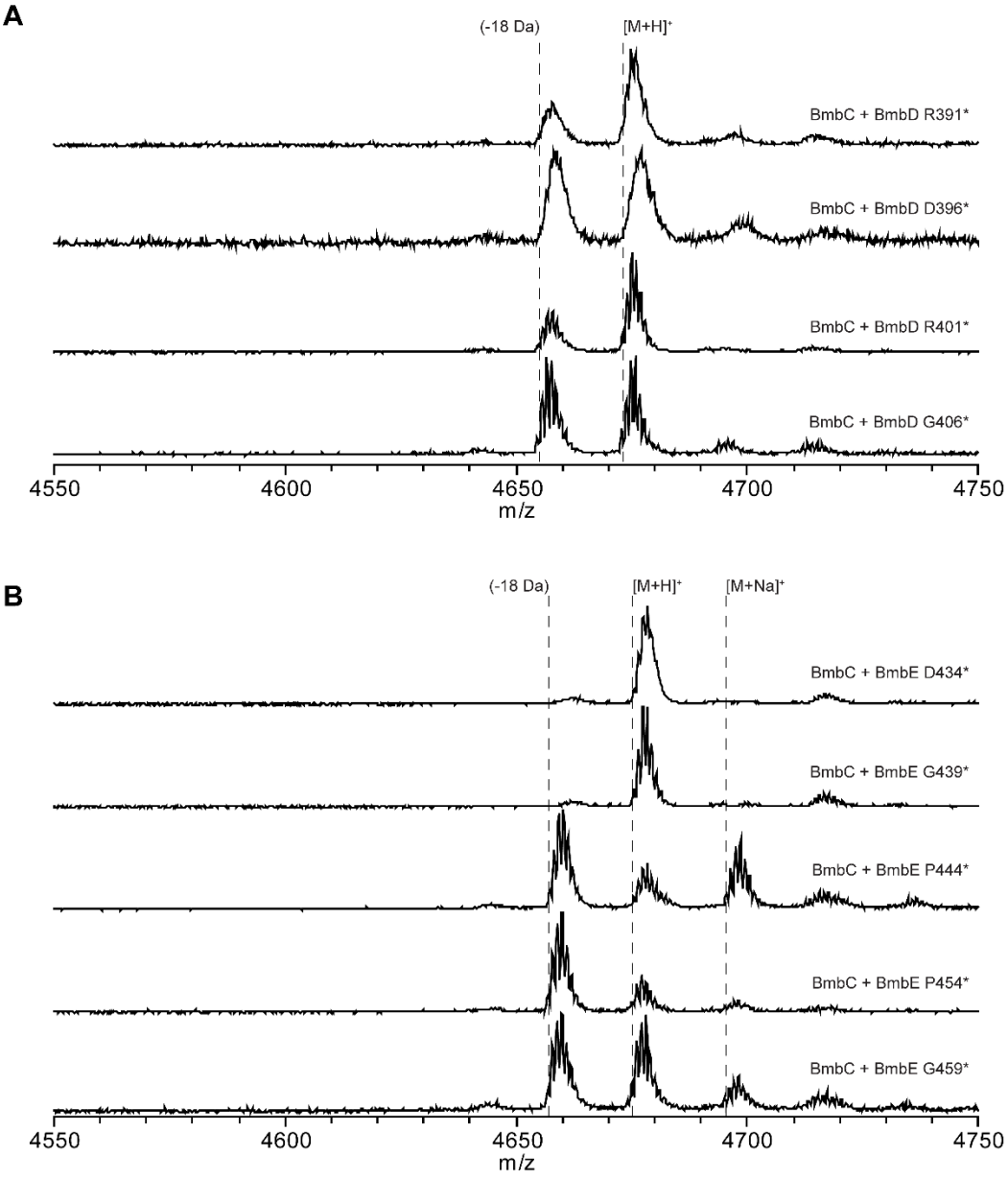


Figure S6: Chemical labeling evidence for thiazoline and macrolactamidine formation. Products of BmbD and BmbE cyclodehydratases are distinct, isobaric dehydrations of the bottromycin precursor peptide (BmbC). (A-B) Reaction products with BmbD or BmbE proteins were alkylated at the cysteine thiol with bromoethylamine or methylated at the N-terminus by reductive amination. Products of the reactions were visualized by MALDI-TOF MS as shifts of +28 Da for dimethylation of the thiazoline substrate and +43 Da for the ethylamine alkylated macrolactamidine substrate relative to the dehydrated product. (C) Precursor peptide mutant C8A was only processed by the macrolactamidine-forming enzyme.

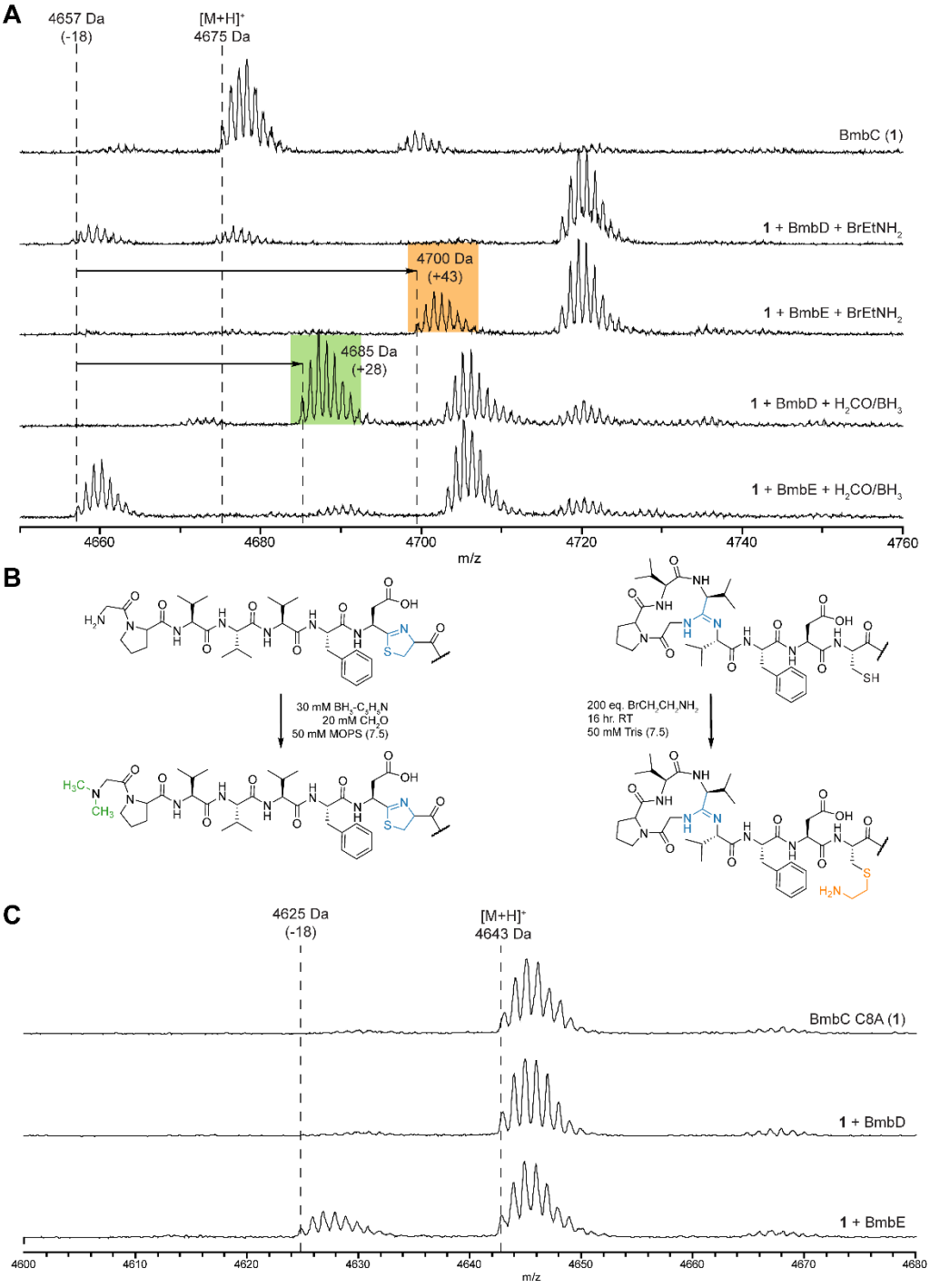


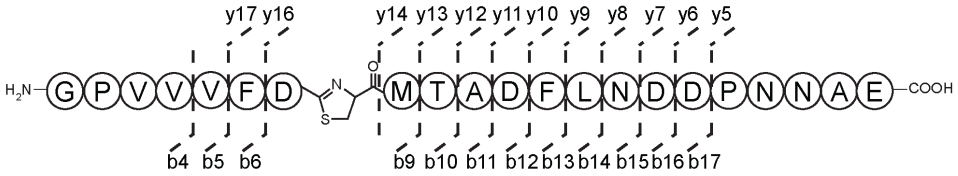
Table S4: Precursor peptide sequences and processing by BmbD and BmbE YcaOs. Processing was analyzed by endpoint MALDI-TOF MS for the presence of a dehydrated mass. Plus signs indicate the presence of a dehydrated mass while minus signs indicate only the parent peptide mass was detected. Sequence changes are highlighted in red. "N/A" indicates not applicable for processing *i.e.* no sulfhydryl side chain to generate a thiazoline. "*" indicates mutation to a stop codon, "^" indicates an insertion mutation and "Δ" indicates a deletion mutation.

Region varied	Variant	Sequence	BmbD processing	BmbE processing
Wild-type	-	GPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Follower	F13A	GPVVVFDCMTAD**A**LNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Follower	L14A	GPVVVFDCMTADF**A**NDDPNNAELSALEMEELESWGAWDGEATS	-	+
Follower	N15A	GPVVVFDCMTADFL**A**DDPNNAELSALEMEELESWGAWDGEATS	+	+
Follower	D16A	GPVVVFDCMTADFLN**A**DPNNAELSALEMEELESWGAWDGEATS	+	+
Follower	D17A	GPVVVFDCMTADFLND**A**PNNAELSALEMEELESWGAWDGEATS	+	-
Follower	E22A	GPVVVFDCMTADFLNDDPNNA**A**LSALEMEELESWGAWDGEATS	+	+
Follower	L23A	GPVVVFDCMTADFLNDDPNNAE**A**SALEMEELESWGAWDGEATS	-	+
Follower	S24A	GPVVVFDCMTADFLNDDPNNAEL**A**ALEMEELESWGAWDGEATS	-	+
Follower	L26A	GPVVVFDCMTADFLNDDPNNAELSA**A**EMEELESWGAWDGEATS	+	+
Follower	E27A	GPVVVFDCMTADFLNDDPNNAELSAL**A**MEELESWGAWDGEATS	-	-
Follower	M28A	GPVVVFDCMTADFLNDDPNNAELSALE**A**EELESWGAWDGEATS	+	+
Follower	E29A	GPVVVFDCMTADFLNDDPNNAELSALEM**A**ELESWGAWDGEATS	+	+
Follower	E30A	GPVVVFDCMTADFLNDDPNNAELSALEME**A**LESWGAWDGEATS	+	+
Follower	L31A	GPVVVFDCMTADFLNDDPNNAELSALEMEE**A**ESWGAWDGEATS	-	-
Follower	E32A	GPVVVFDCMTADFLNDDPNNAELSALEMEEL**A**SWGAWDGEATS	+	+
Follower	D16*	GPVVVFDCMTADFLN	-	-
Follower	A21*	GPVVVFDCMTADFLNDDPNN	-	-
Follower	L26*	GPVVVFDCMTADFLNDDPNNAELSA	-	-
Follower	L31*	GPVVVFDCMTADFLNDDPNNAELSALEMEE	-	-
Follower	E32*	GPVVVFDCMTADFLNDDPNNAELSALEMEEL	-	-
Follower	S33*	GPVVVFDCMTADFLNDDPNNAELSALEMEELE	-	-
Follower	W34*	GPVVVFDCMTADFLNDDPNNAELSALEMEELES	-	-
Follower	G35*	GPVVVFDCMTADFLNDDPNNAELSALEMEELESW	+	+
Follower	A36*	GPVVVFDCMTADFLNDDPNNAELSALEMEELESWG	+	+
Follower	N15K	GPVVVFDCMTADFL**K**DDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	^2P	G**P**PVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	^2A	G**A**PVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	^2G	G**G**PVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	P2G	G**G**VVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	ΔP2	GVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	-
Core	ΔV3	GPVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	V3A	GP**A**VVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	+
Core	V4A	GPV**A**VFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	+
Core	V4G	GPV**G**VFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	V4I	GPV**I**VFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	V5A	GPVV**A**FDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	+
Core	V5G	GPVV**G**FDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	+
Core	V5L	GPVV**L**FDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	D7A	GPVVVF**A**CMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	D7N	GPVVVF**N**CMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	D7T	GPVVVF**T**CMTADFLNDDPNNAELSALEMEELESWGAWDGEATS	+	+
Core	C8A	GPVVVFD**A**MTADFLNDDPNNAELSALEMEELESWGAWDGEATS	N/A	+
Core	C8P	GPVVVFD**P**MTADFLNDDPNNAELSALEMEELESWGAWDGEATS	N/A	-
Core	^9C	GPVVVFDC**C**MTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	^9S	GPVVVFDC**S**MTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-
Core	^9T	GPVVVFDC**T**MTADFLNDDPNNAELSALEMEELESWGAWDGEATS	-	-

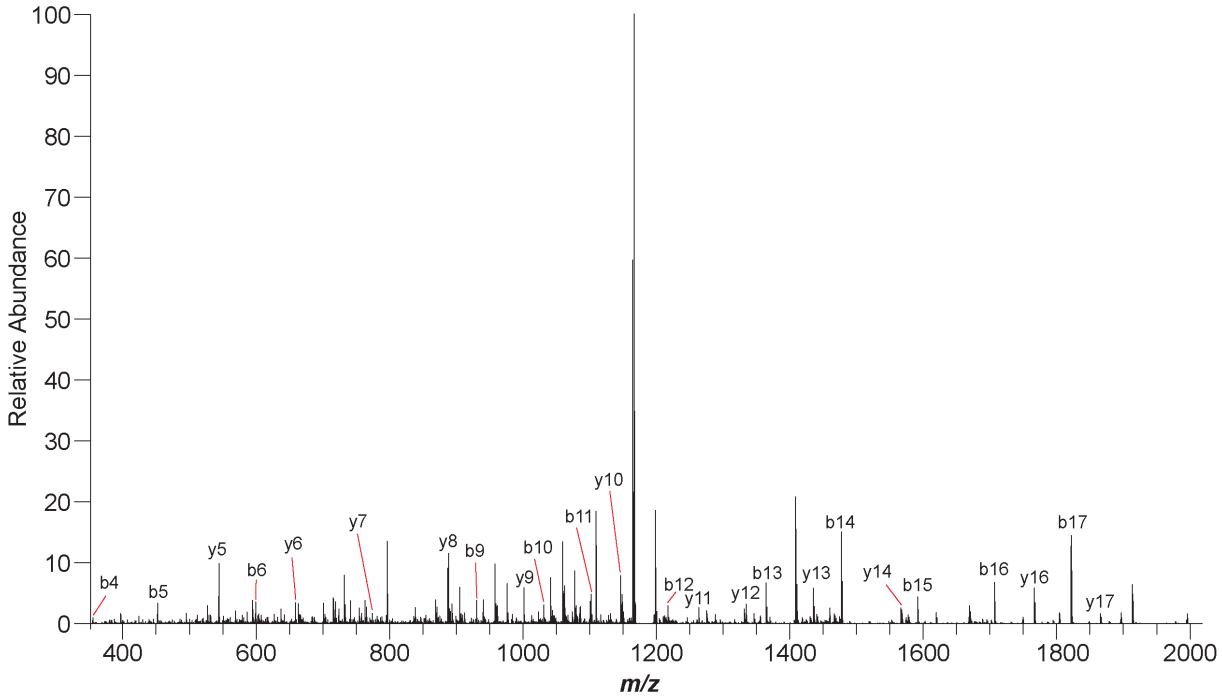
Figure S7 (i-xvi): Tandem MS fragmentation of bottromycin cyclodehydratase products. Thiazoline and macrolactamidine formation were confirmed by MS/MS fragmentation. (A) schematic representation of the modified products and (B) fragmentation data with assigned peaks corresponding to the ions of interest. Mutated residues are shown in red. Mass values are given in **Table S5**.

Figure S7i: BmbC + BmbD

A

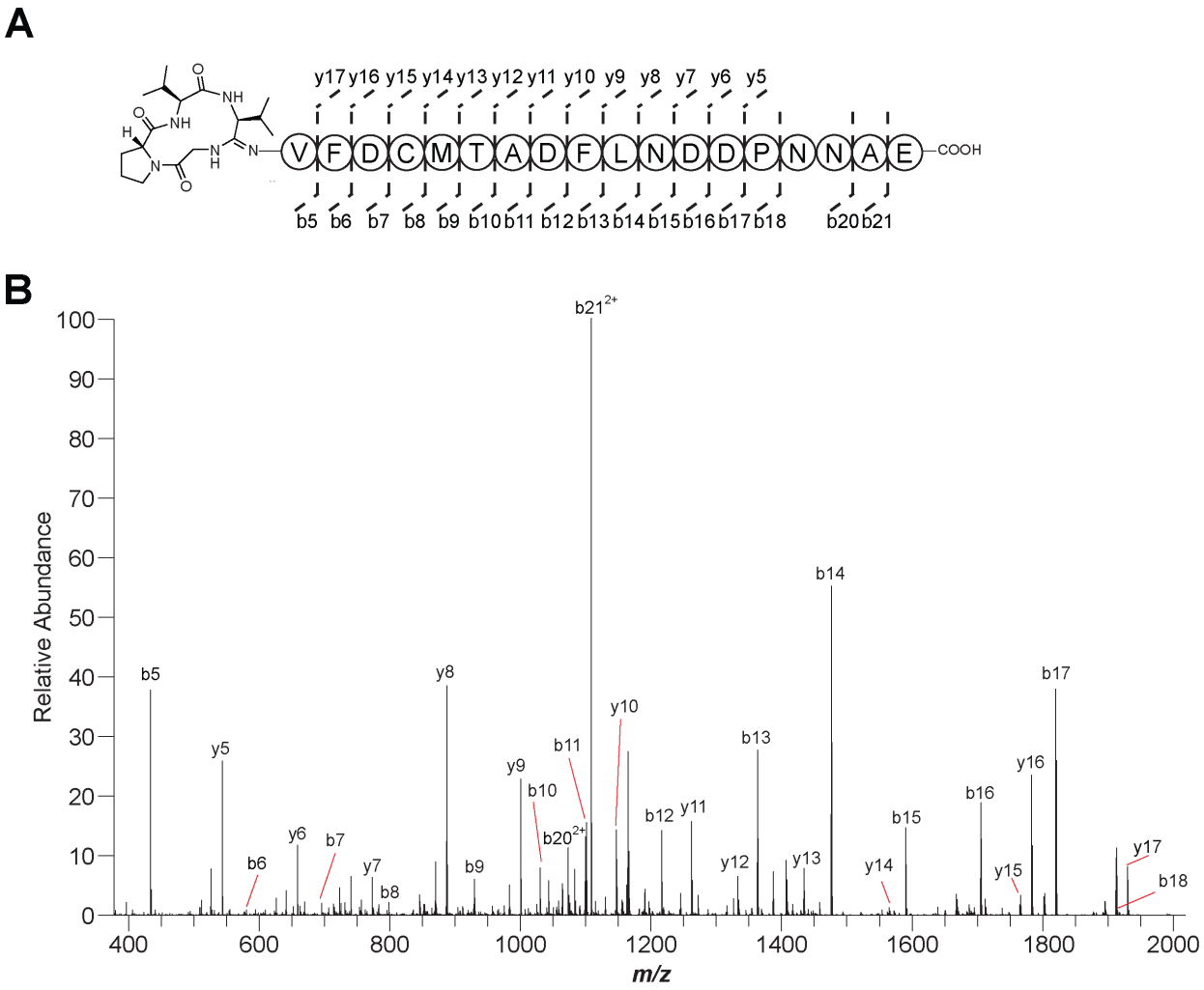


B



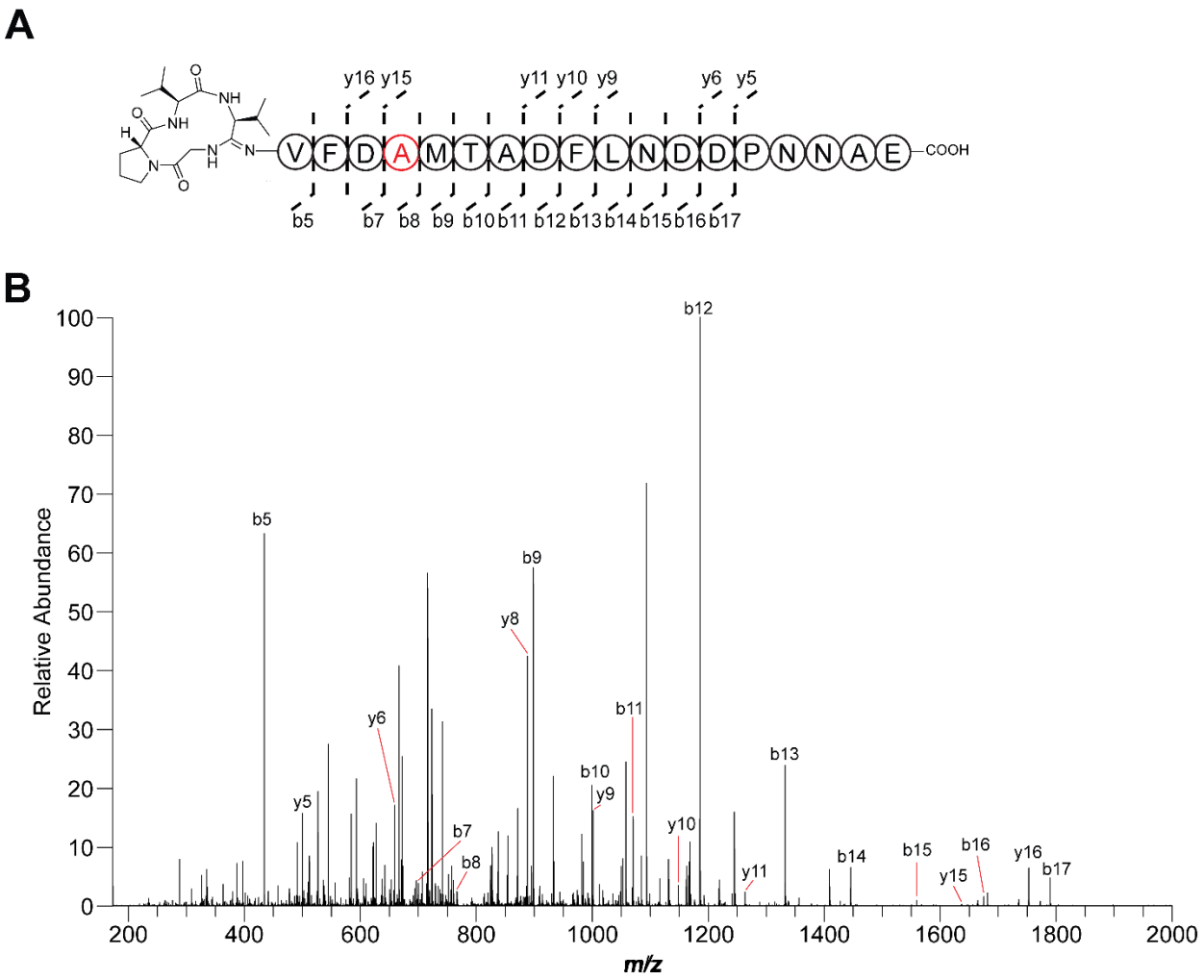
(MS fragment assignments in **Table S5i**)

Figure S7ii: BmbC + BmbE



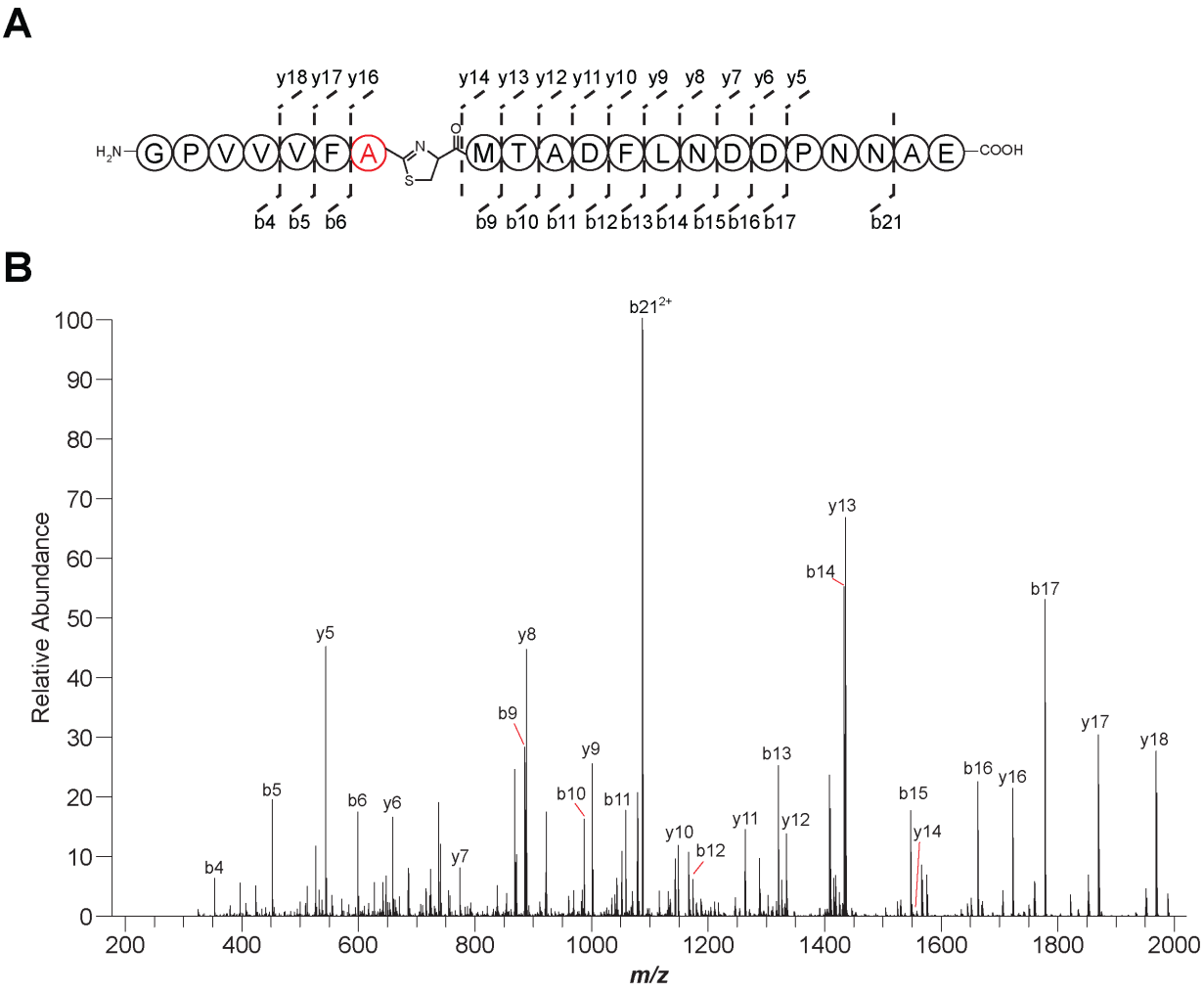
(MS fragment assignments in **Table S5ii**)

Figure S7iii: BmbC-C8A + BmbE



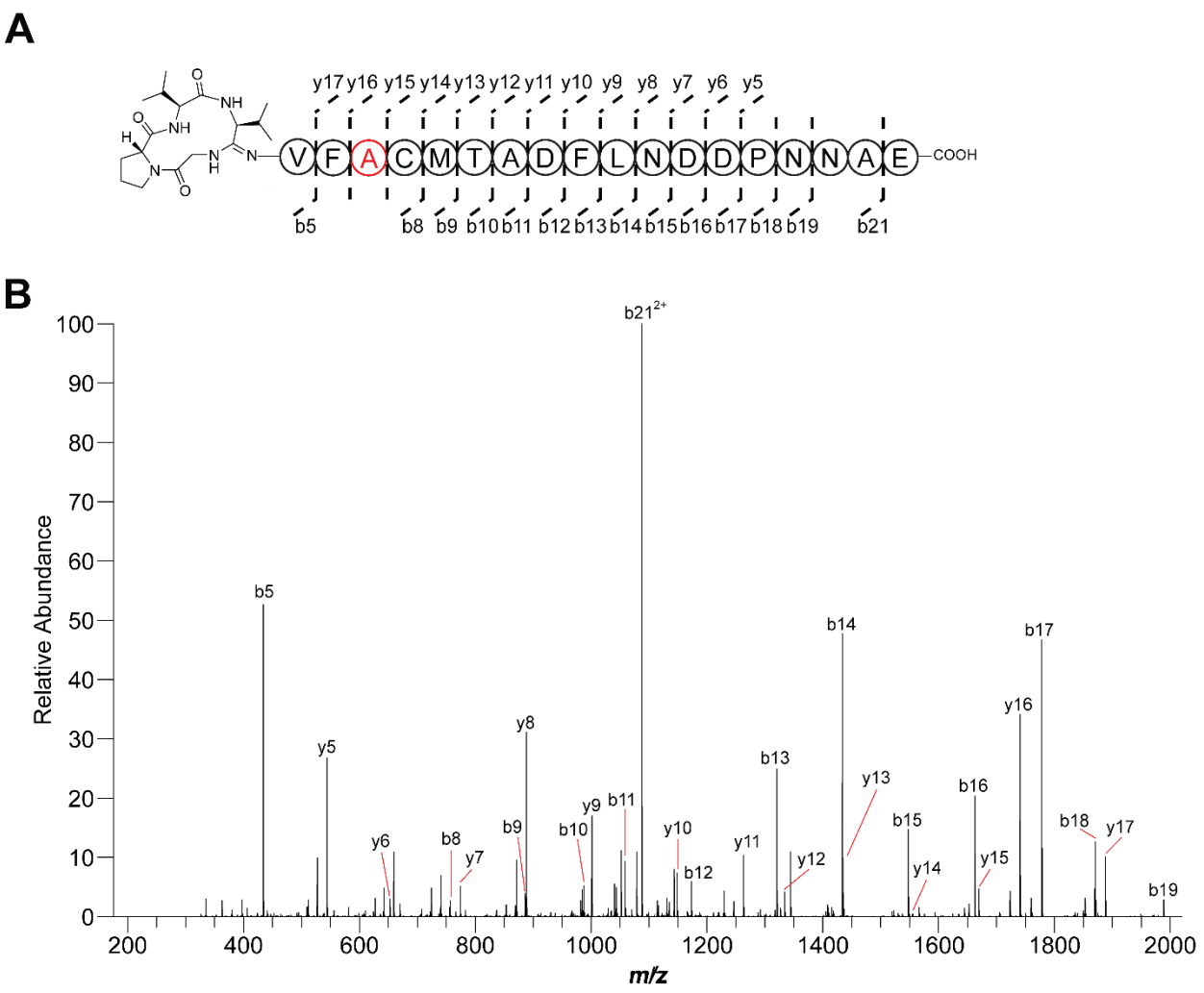
(MS fragment assignments in **Table S5iii**)

Figure S7iv: BmbC-D7A + BmbD



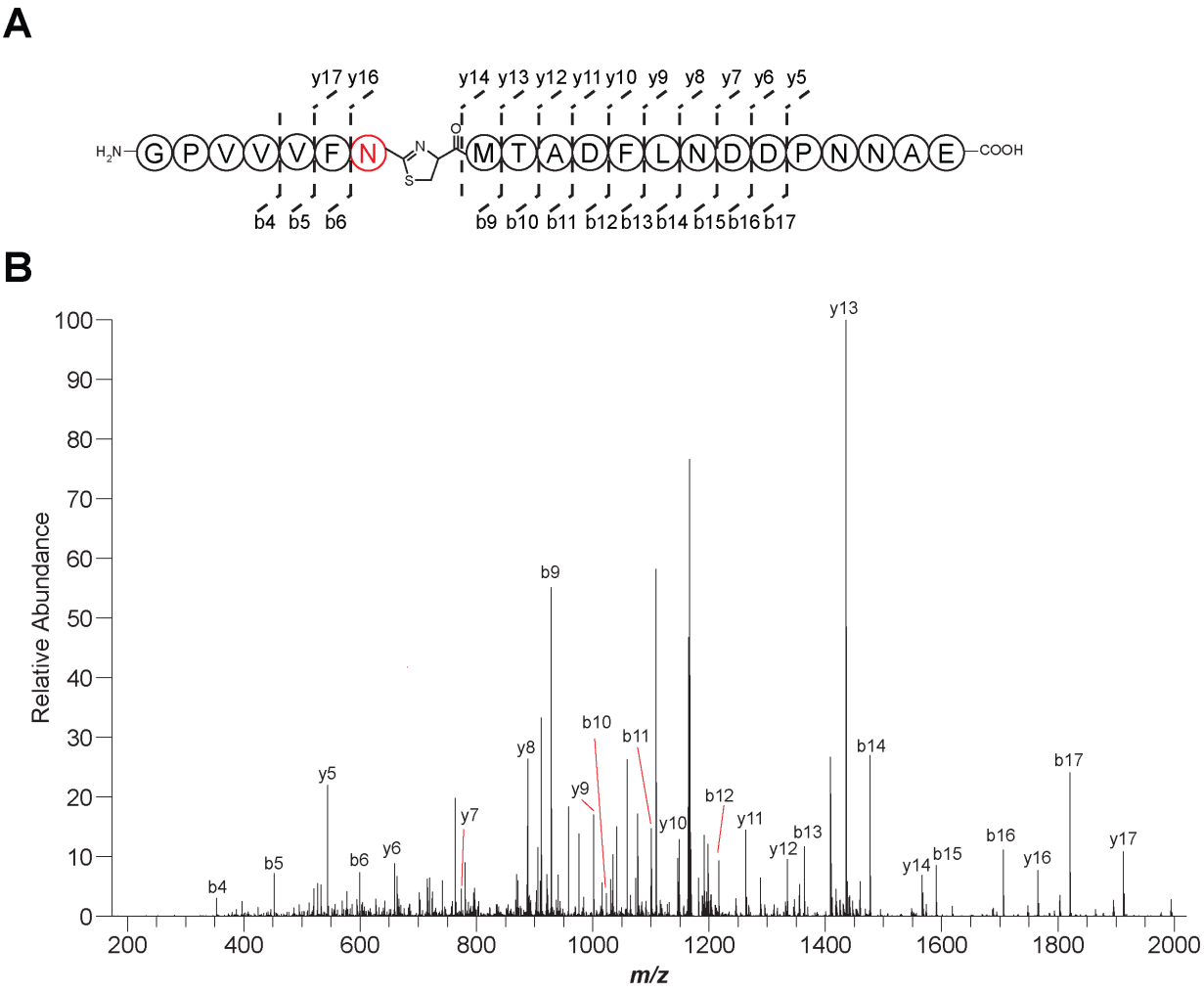
(MS fragment assignments in **Table S5iv**)

Figure S7v: BmbC-D7A + BmbE



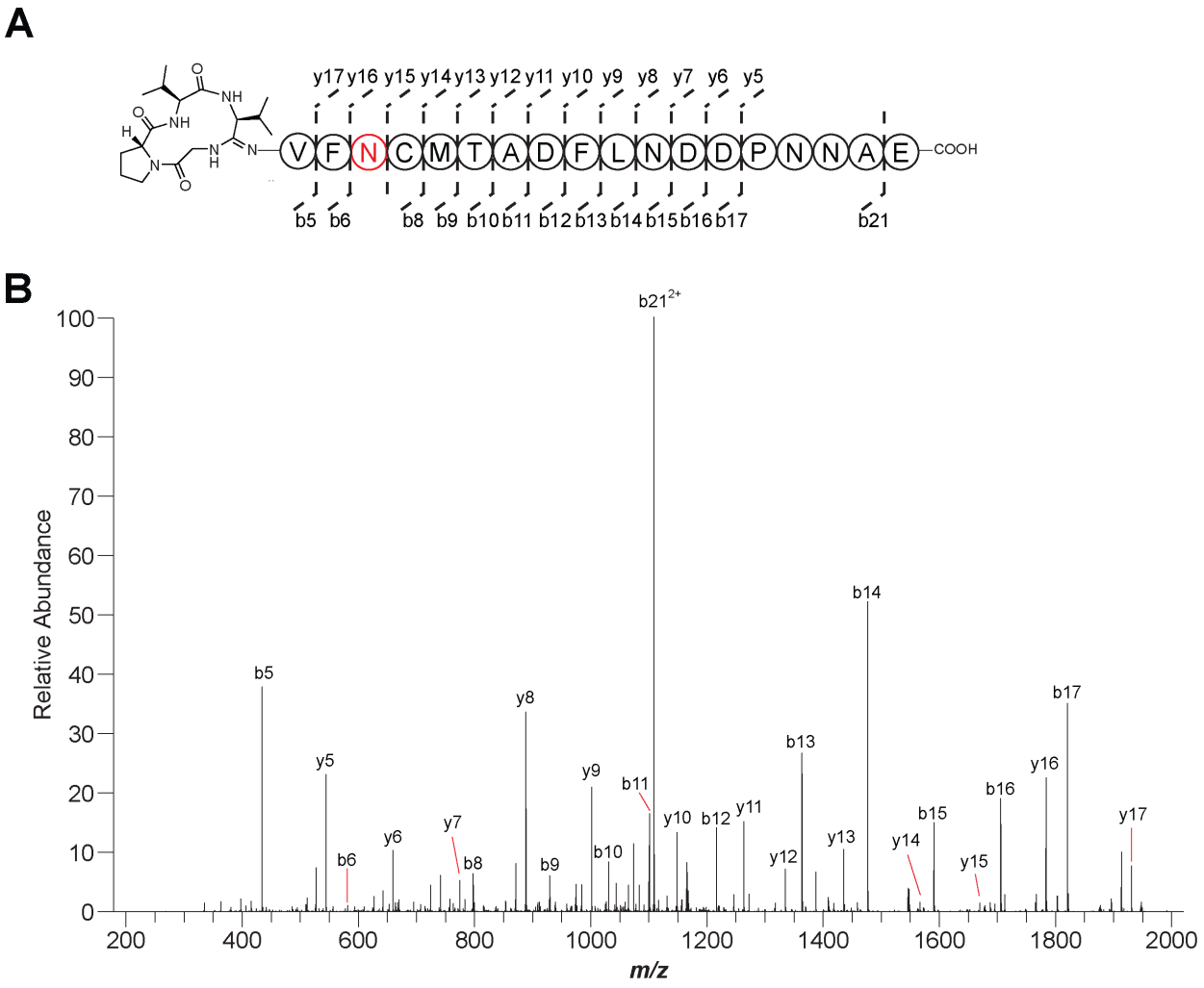
(MS fragment assignments in **Table S5v**)

Figure S7vi: BmbC-D7N + BmbD



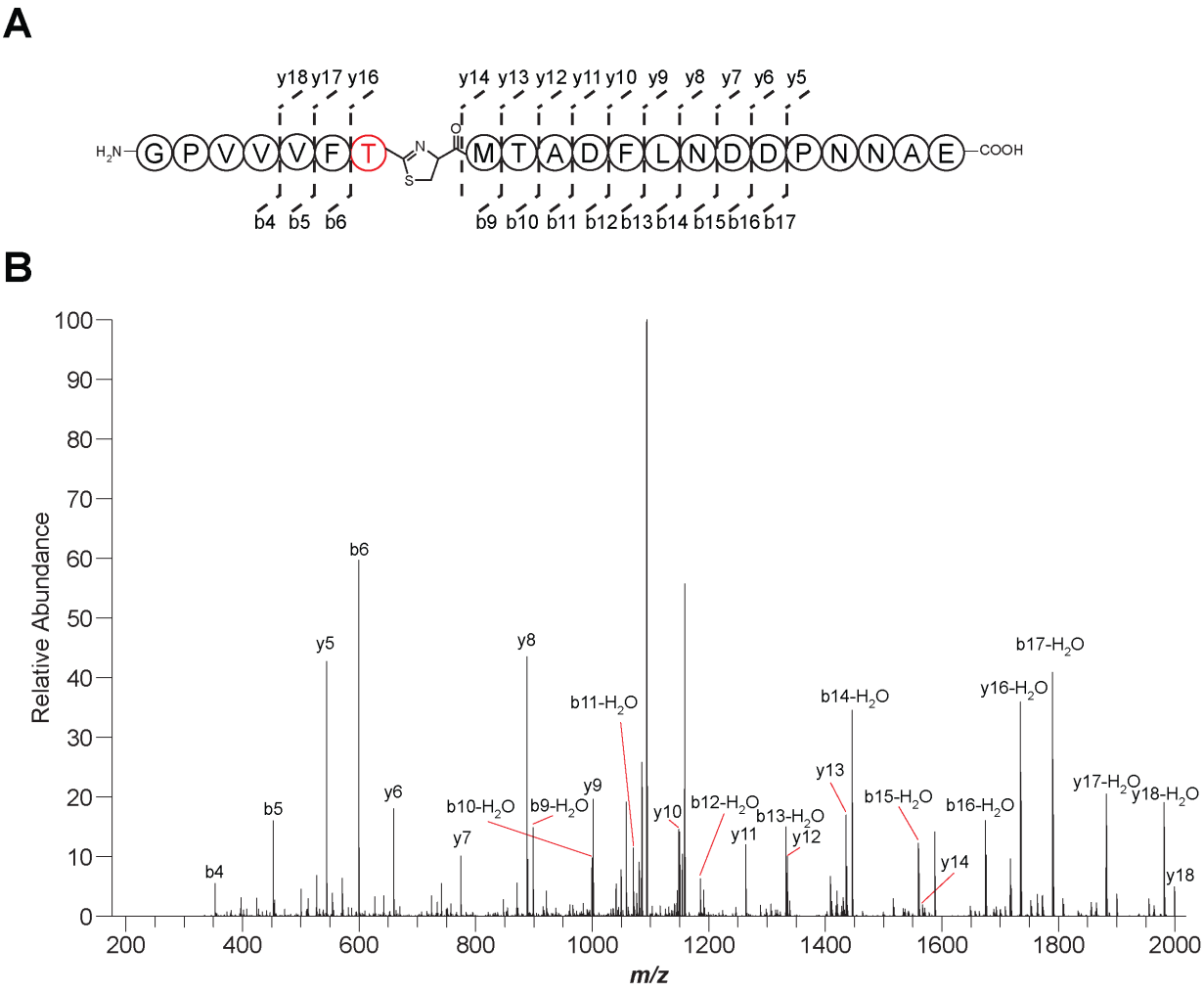
(MS fragment assignments in **Table S5vi)**

Figure S7vii: BmbC-D7N + BmbE



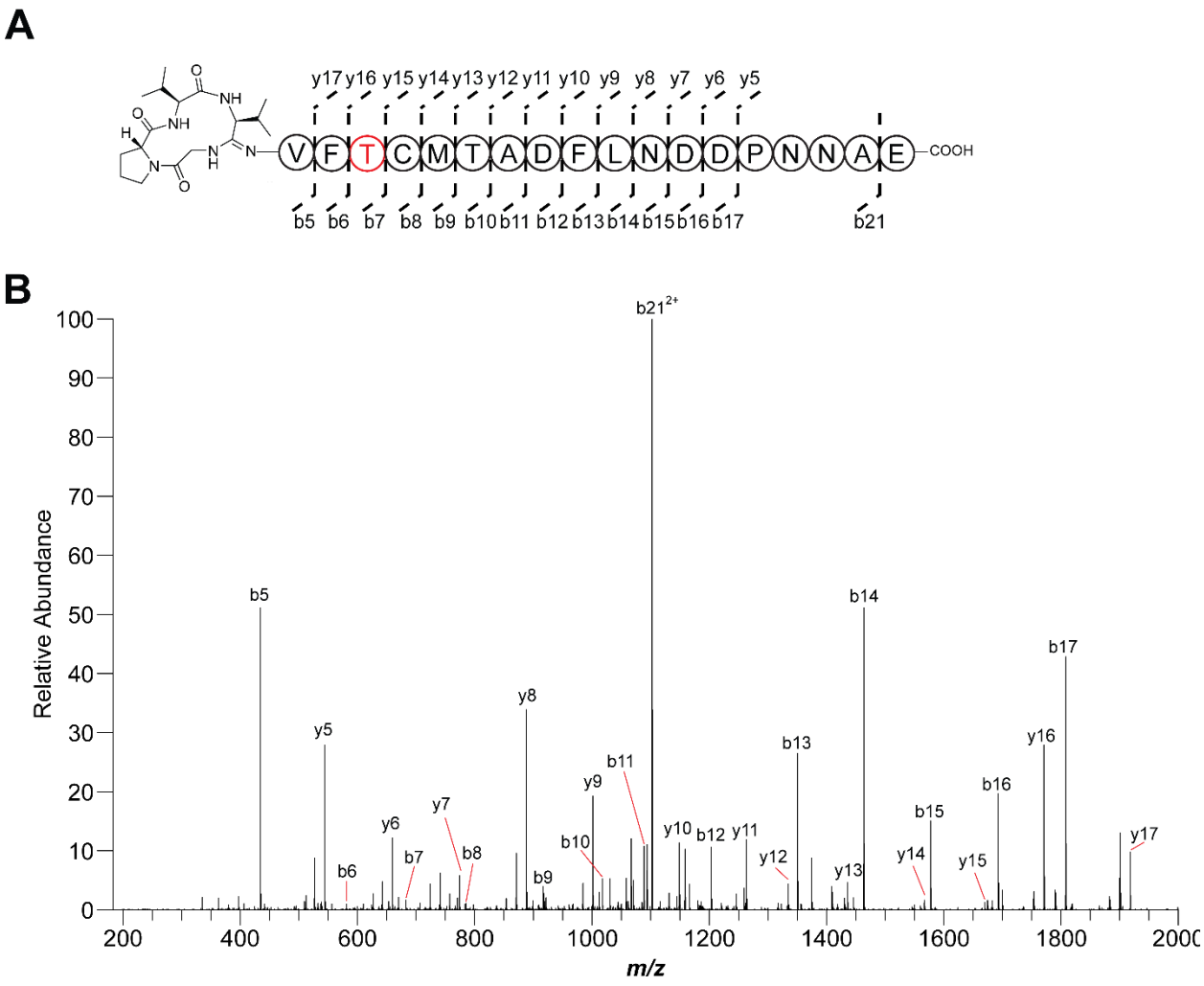
(MS fragment assignments in **Table S5vii**)

Figure S7viii: BmbC-D7T + BmbD



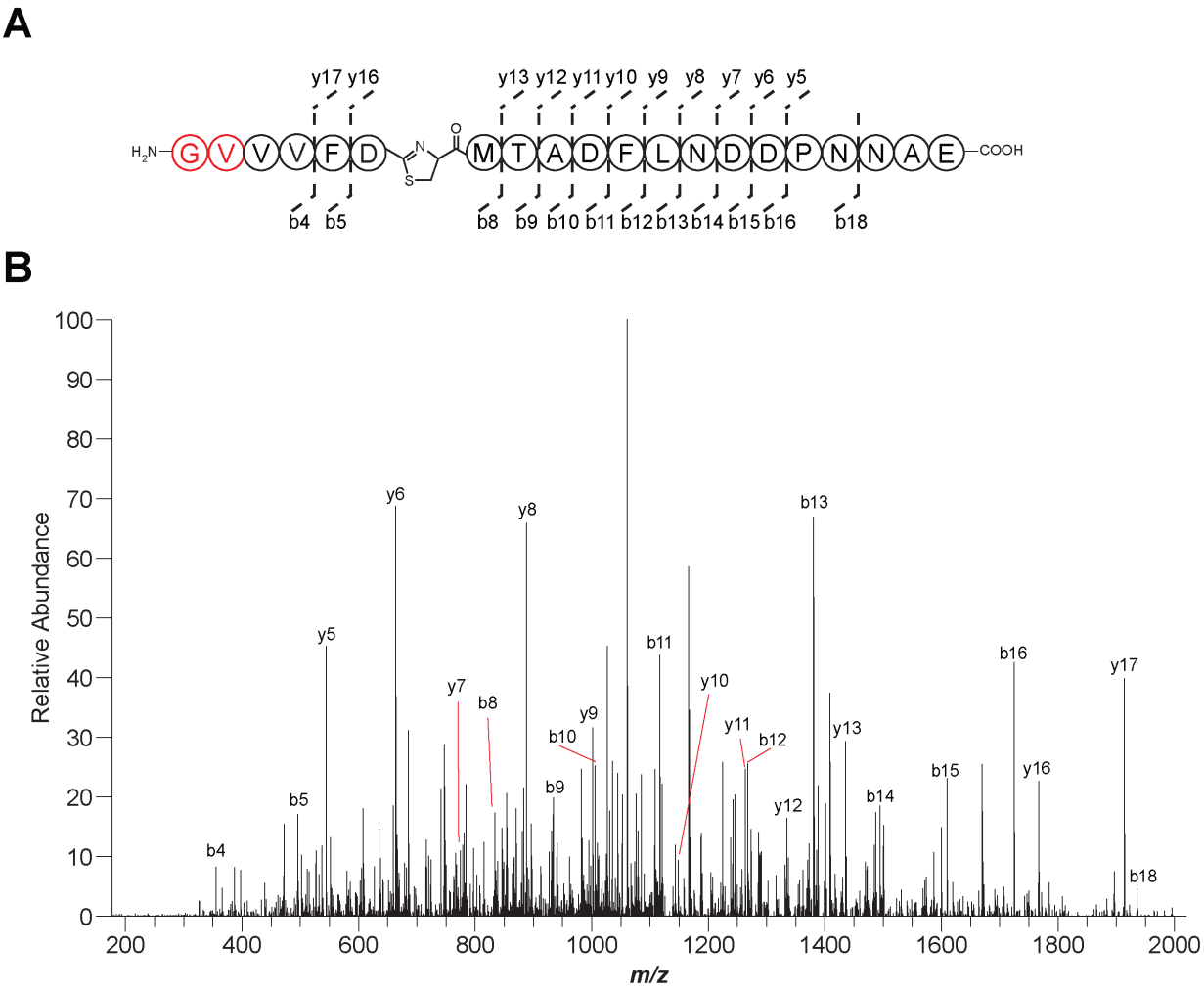
(MS fragment assignments in **Table S5viii**)

Figure S7ix: BmbC-D7T + BmbE



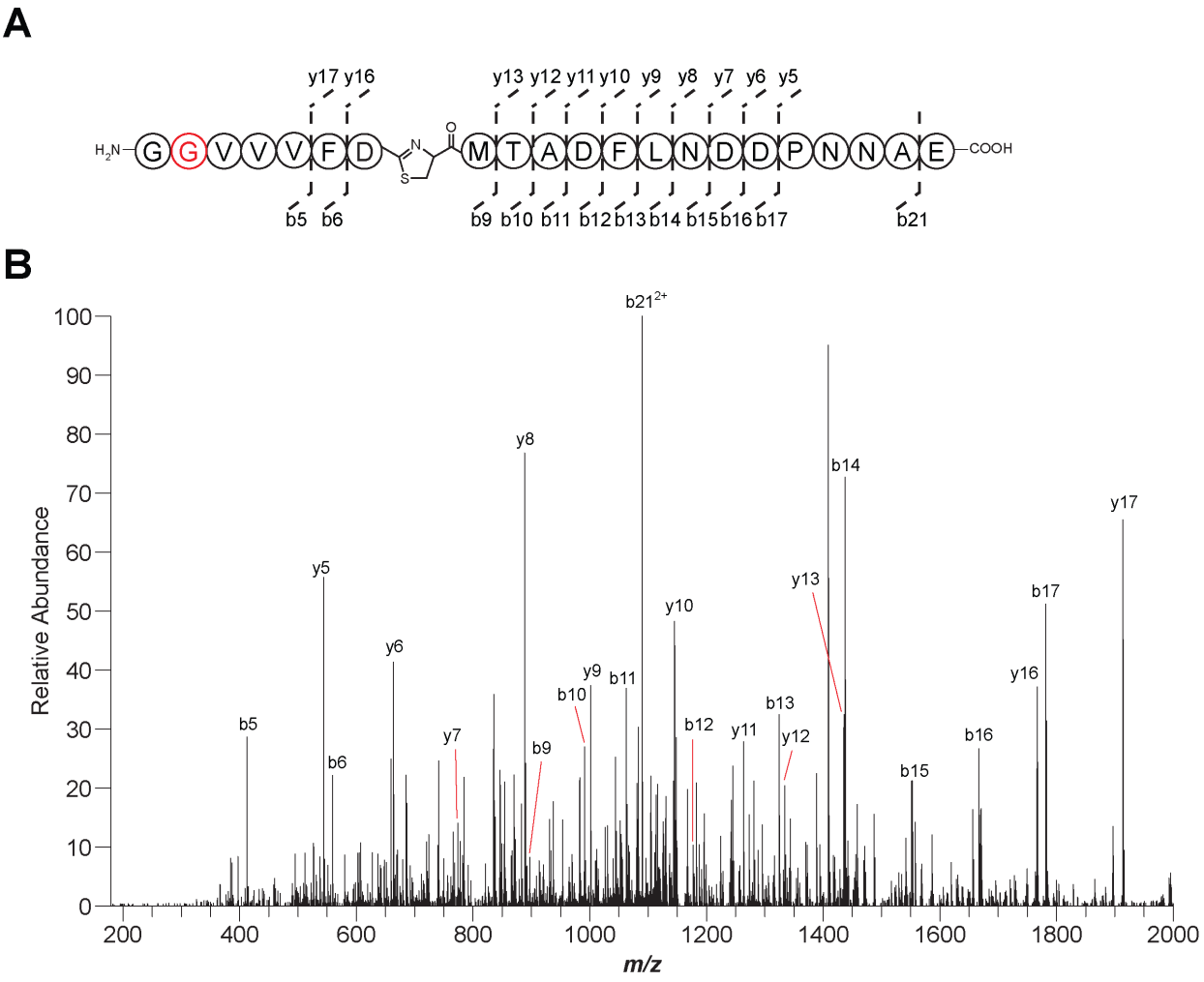
(MS fragment assignments in **Table S5ix**)

Figure S7x: BmbC-ΔP2 + BmbD



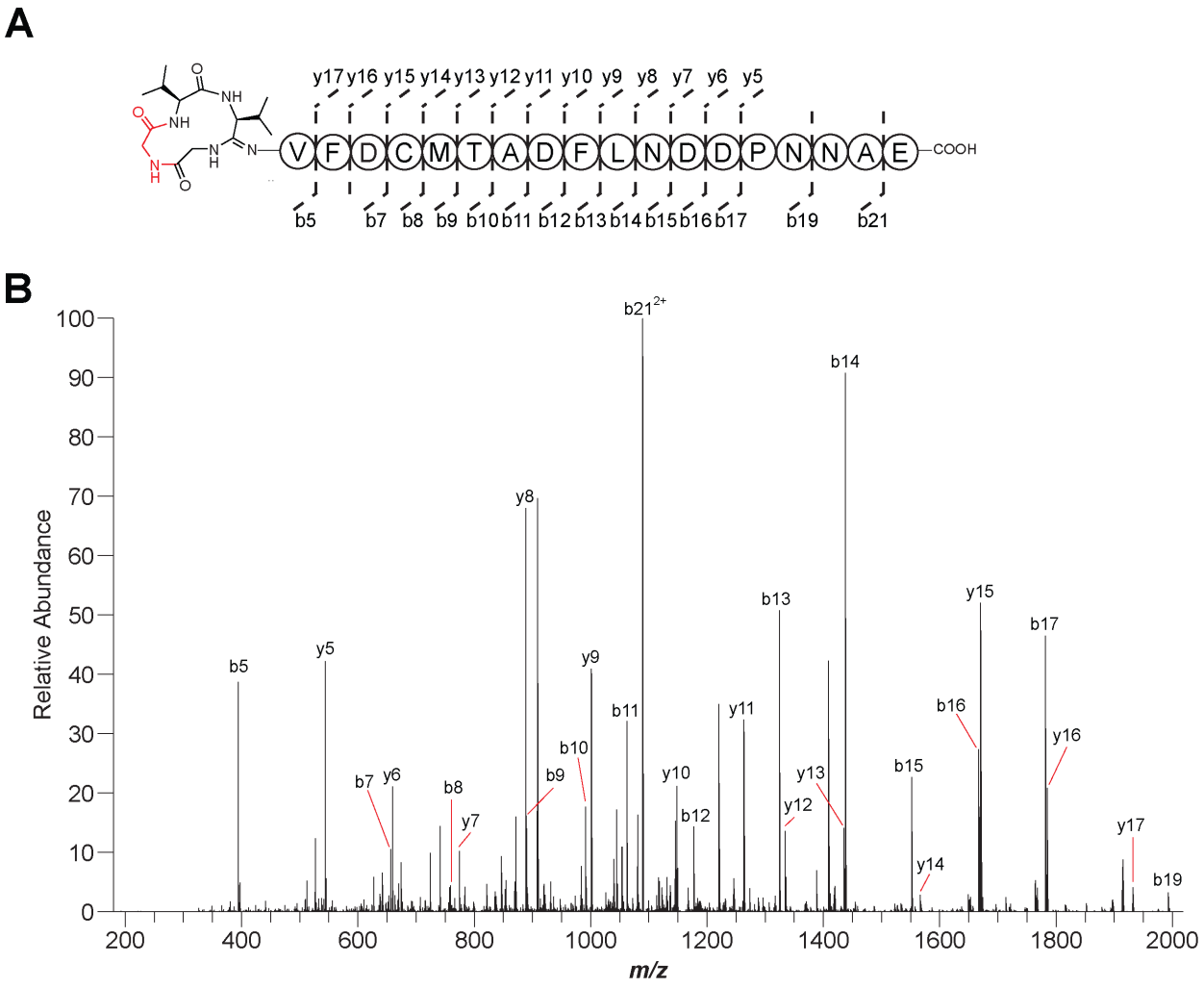
(MS fragment assignments in **Table S5x**)

Figure S7xi: BmbC-P2G + BmbD



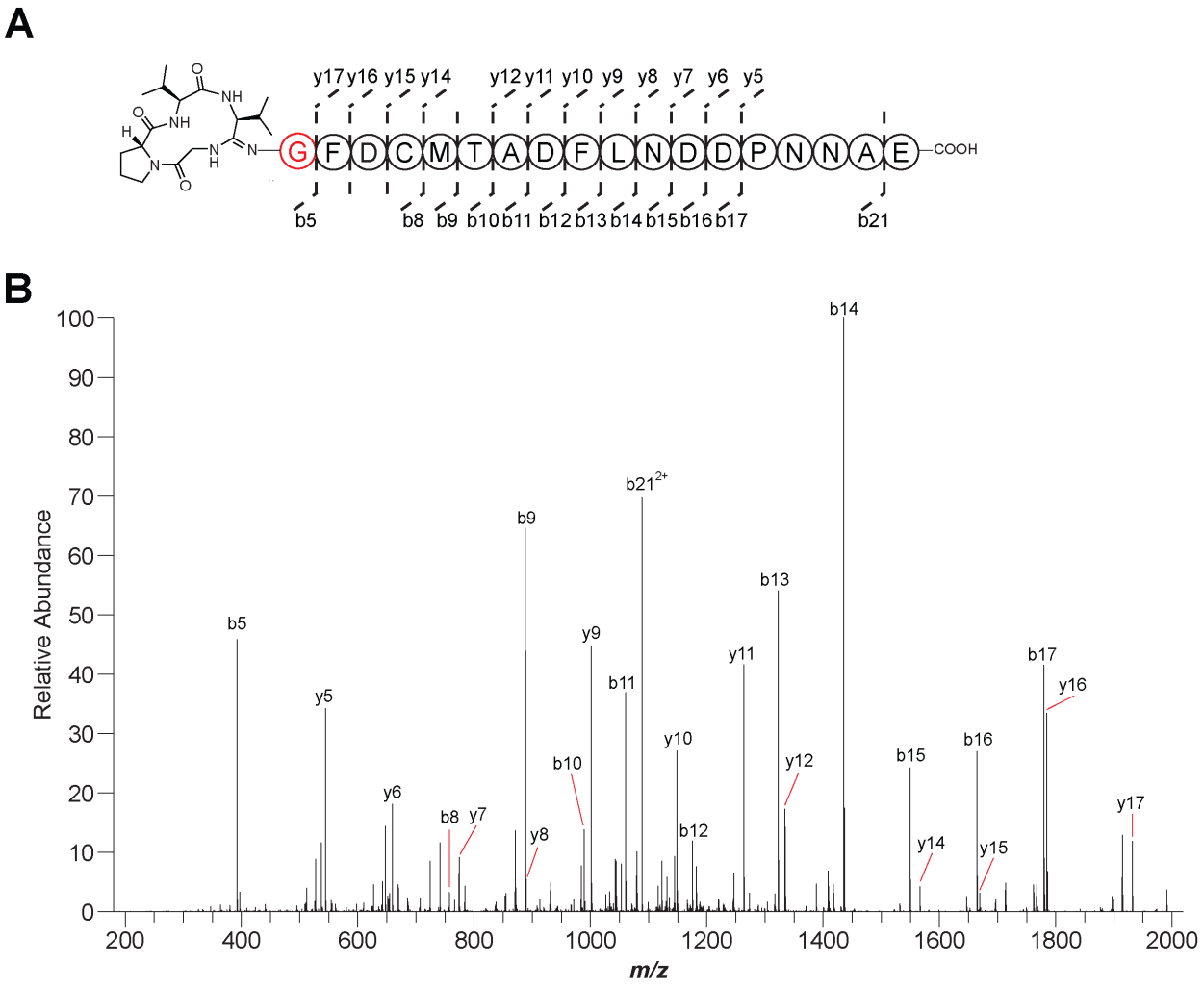
(MS fragment assignments in **Table S5xi**)

Figure S7xii: BmbC-P2G + BmbE



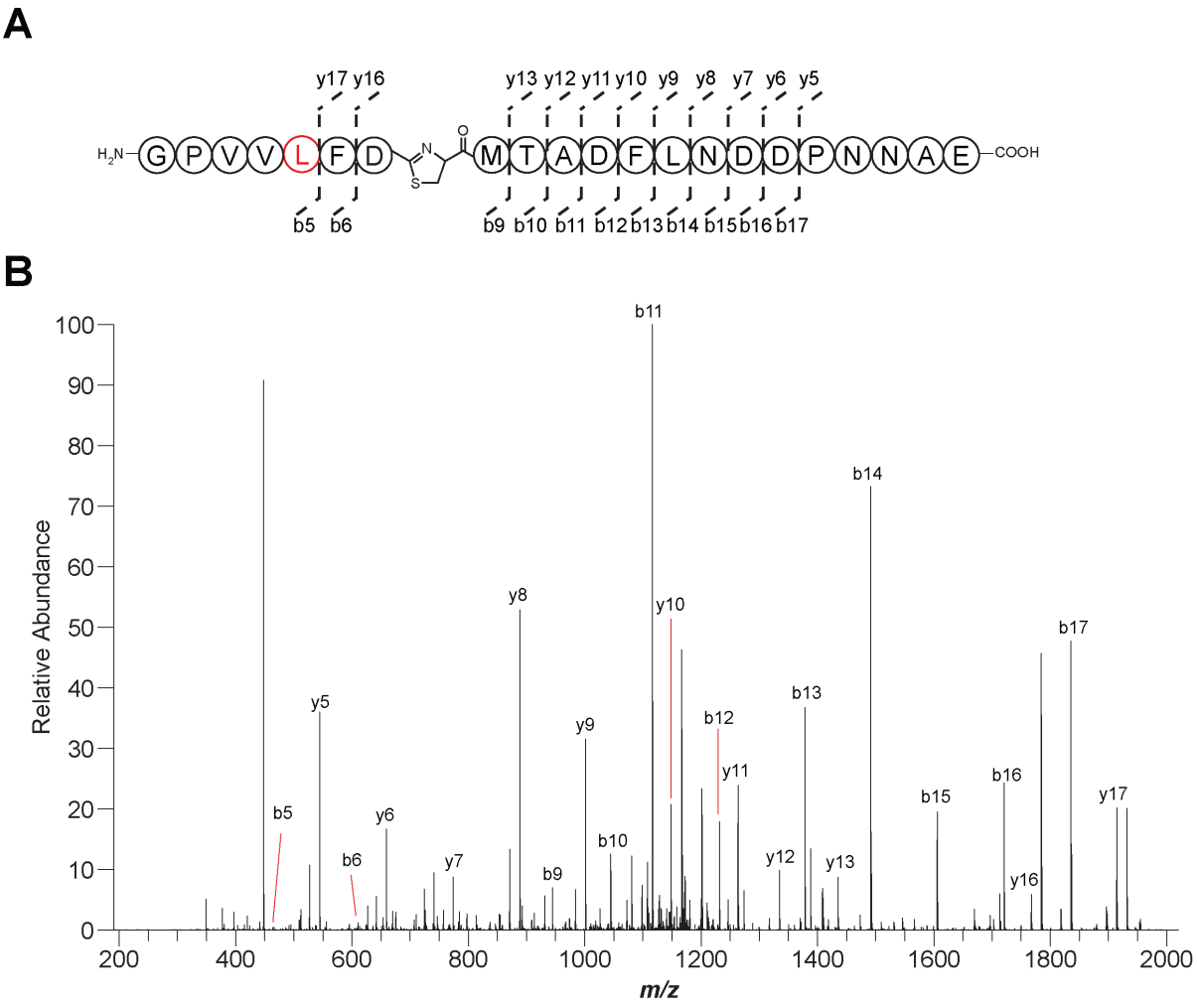
(MS fragment assignments in **Table S5xii**)

Figure S7xiii: BmbC-V5G + BmbE



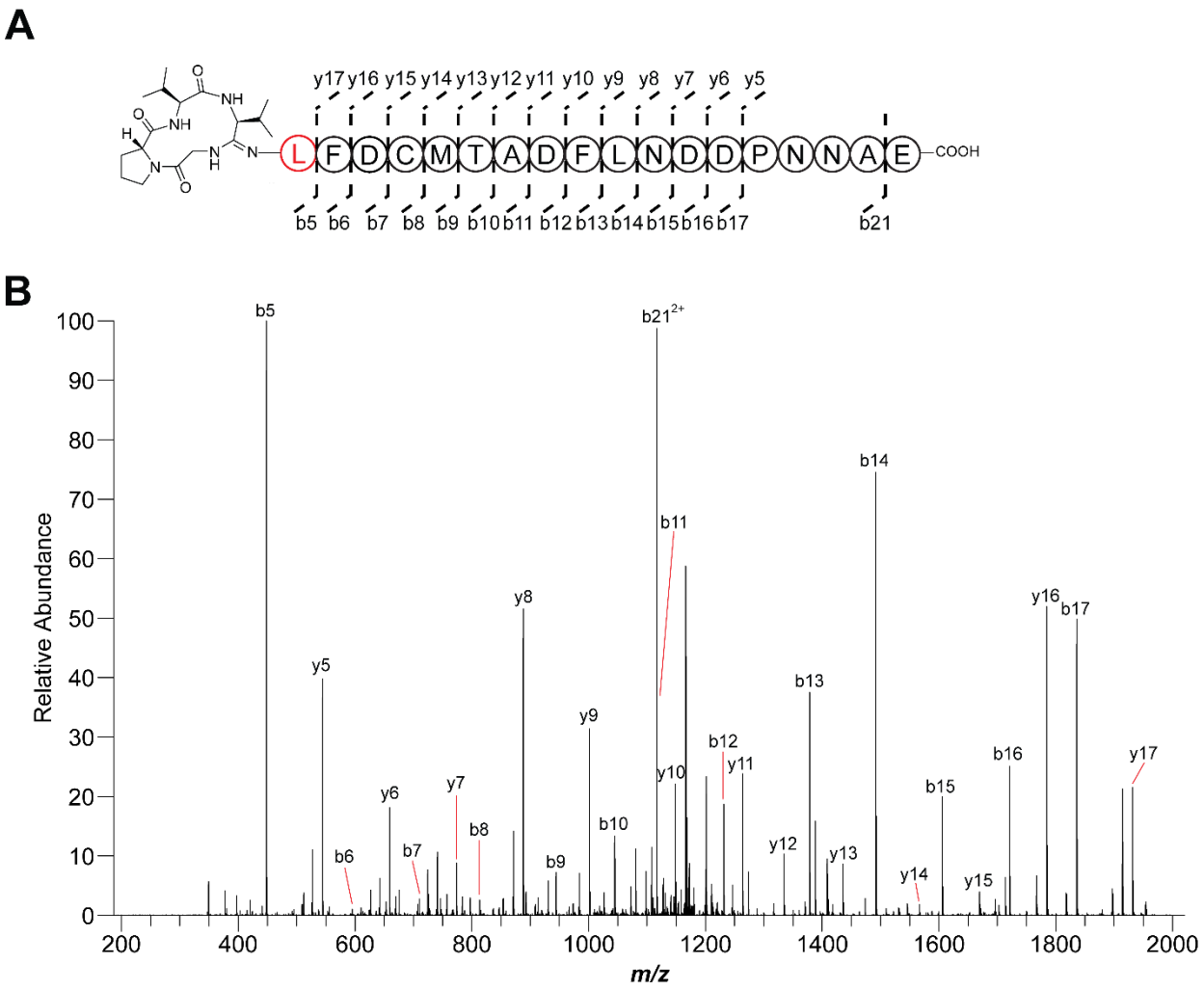
(MS fragment assignments in **Table S5xiii**)

Figure S7xiv: BmbC-V5L + BmbD



(MS fragment assignments in **Table S5xiv**)

Figure S7xv: BmbC-V5L + BmbE



(MS fragment assignments in **Table S5xv**)

Figure S7xvi: BmbC-V3A, V4A, V4I, V5A + BmbE.

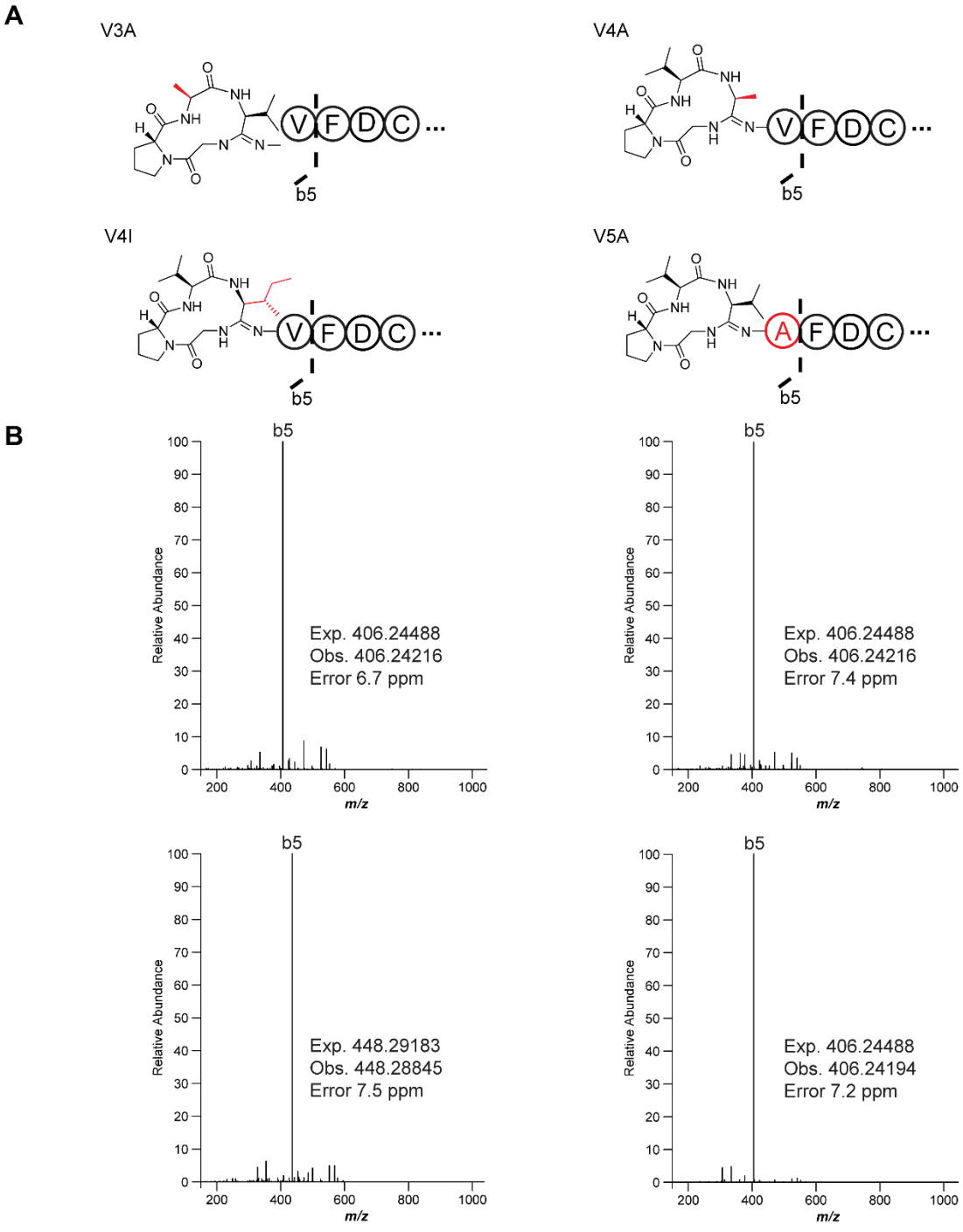


Table S5 (i-xv): MS fragment assignments.

Table S5i: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b4	353.21833	353.21868	1.0
b5	452.28675	452.28714	0.9
b6	599.35516	599.35572	0.9
b9	930.42121	930.42162	0.4
b10	1031.46889	1031.46966	0.7
b11	1102.50600	1102.50637	0.3
b12	1217.53294	1217.53380	0.7
b13	1364.60136	1364.60259	0.9
b14	1477.68542	1477.68786	1.7
b15	1591.72835	1591.73062	1.4
b16	1706.75529	1706.75764	1.4
b17	1821.78223	1821.78512	1.6
y5	544.23617	544.23669	1.0
y6	659.26311	659.26379	1.0
y7	774.29005	774.29058	0.7
y8	888.33298	888.33414	1.3
y9	1001.41704	1001.41641	0.6
y10	1148.48546	1148.48990	3.9
y11	1263.51240	1263.51402	1.3
y12	1334.54951	1334.55133	1.4
y13	1435.59719	1435.59928	1.5
y14	1566.63768	1566.64026	1.6
y16	1766.66324	1766.67467	6.5
y17	1913.73165	1913.73419	1.3

Table S5ii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	434.27618	434.27555	1.5
b6	581.34460	581.34381	1.4
b7	696.37154	696.37068	1.2
b8	799.38072	799.37968	1.3
b9	930.42121	930.41991	1.4
b10	1031.46889	1031.46764	1.2
b11	1102.50600	1102.50472	1.2
b12	1217.53294	1217.53139	1.3
b13	1364.60136	1364.59980	1.1
b14	1477.68542	1477.68371	1.2
b15	1591.72835	1591.72693	0.9
b16	1706.75529	1706.75315	1.3
b17	1821.78223	1821.77975	1.4
b18	1918.83500	1918.83296	1.1
$b20^{2+}$	1073.96406	1073.96278	1.2
$b21^{2+}$	1109.48262	1109.48116	1.3
y5	544.23617	544.23563	1.0
y6	659.26311	659.26252	0.9
y7	774.29005	774.28908	1.3
y8	888.33298	888.33201	1.1
y9	1001.41704	1001.41541	1.6
y10	1148.48546	1148.48413	1.2
y11	1263.51240	1263.51050	1.5
y12	1334.54951	1334.54765	1.4
y13	1435.59719	1435.59599	0.8
y14	1566.63768	1566.63602	1.1
y15	1669.64686	1669.64441	1.5
y16	1784.67381	1784.68727	7.5
y17	1931.74222	1931.73938	1.5

Table S5iii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-C8A + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	434.27618	434.27582	0.8
b6	696.37154	696.37122	0.5
b7	767.40865	767.40771	1.2
b8	898.44914	898.44855	0.7
b9	999.49681	999.49628	0.5
b10	1070.53393	1070.53333	0.6
b11	1185.56087	1185.56006	0.7
b12	1332.62929	1332.62830	0.7
b13	1445.71335	1445.71289	0.3
b14	1559.75628	1559.75452	1.1
b15	1674.78322	1674.78296	0.2
b16	1789.81016	1789.80908	0.6
b17	544.23617	544.23578	0.7
b18	659.26311	659.26282	0.4
$b20^{2+}$	888.33298	888.33246	0.6
$b21^{2+}$	1001.41704	1001.41467	2.4
y5	1148.48546	1148.48474	0.6
y6	1263.51240	1263.51184	0.4
y7	1637.67479	1637.67358	0.7
y8	1752.70173	1752.71648	8.4

Table S5iv: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7A + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b4	452.28675	452.28659	0.4
b5	599.35516	599.35504	0.2
b6	696.37154	696.37068	1.2
b9	886.43138	886.43825	7.8
b10	987.47906	987.47867	0.4
b11	1058.51617	1058.51548	0.7
b12	1173.54311	1173.54219	0.8
b13	1320.61153	1320.61073	0.6
b14	1433.69559	1433.69538	0.1
b15	1547.73852	1547.73787	0.4
b16	1662.76548	1662.76455	0.6
b17	1777.79240	1777.79163	0.4
$b21^{2+}$	1087.48770	1087.48716	0.5
y5	544.23617	544.23607	0.2
y6	659.26311	659.26305	0.1
y7	774.29005	774.28992	0.2
y8	888.33298	888.33253	0.5
y9	1001.41704	1001.41543	1.6
y10	1148.48546	1148.48520	0.2
y11	1263.51240	1263.51177	0.5
y12	1334.54951	1334.54906	0.3
y13	1435.59719	1435.59682	0.3
y14	1566.63768	1566.63772	0.0
y16	1722.67341	1722.67843	2.9
y17	1869.74183	1869.74139	0.2
y18	1968.81024	1968.80952	0.4

Table S5v: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7A + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	434.27618	434.27591	0.6
b8	755.39089	755.39060	0.4
b9	886.43138	886.43069	0.8
b10	987.47906	987.47861	0.5
b11	1058.51617	1058.51559	0.5
b12	1173.54311	1173.54205	0.9
b13	1320.61153	1320.61072	0.6
b14	1433.69559	1433.69514	0.3
b15	1547.73852	1547.73779	0.5
b16	1662.76546	1662.76433	0.7
b17	1777.79240	1777.79140	0.6
b18	1874.84517	1874.84436	0.4
b19	1988.88810	1988.88696	0.6
$b21^{2+}$	1087.48770	1087.48698	0.7
y5	544.23617	544.23597	0.4
y6	659.26311	659.26297	0.2
y7	774.29005	774.28971	0.4
y8	888.33298	888.33240	0.7
y9	1001.41704	1001.41587	1.2
y10	1148.48546	1148.48490	0.5
y11	1263.51240	1263.51166	0.6
y12	1334.54951	1334.54890	0.5
y13	1435.59719	1435.59692	0.2
y14	1566.63768	1566.63757	0.1
y15	1669.64686	1669.65699	6.1
y16	1740.68398	1740.69794	8.0
y17	1887.75239	1887.75140	0.5

Table S5vi: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7N + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b4	353.21833	353.21823	0.3
b5	452.28675	452.28664	0.2
b6	599.35516	599.35510	0.1
b9	929.43719	929.43697	0.2
b10	1030.48487	1030.48410	0.7
b11	1101.52198	1101.52049	1.4
b12	1216.54893	1216.54781	0.9
b13	1363.61734	1363.61640	0.7
b14	1476.70140	1476.70107	0.2
b15	1590.74433	1590.74371	0.4
b16	1705.77128	1705.77066	0.4
b17	1820.79822	1820.79851	0.2
y5	544.23617	544.23613	0.1
y6	659.26311	659.26301	0.2
y7	774.29005	774.28952	0.7
y8	888.33298	888.33265	0.4
y9	1001.41704	1001.41590	1.1
y10	1148.48546	1148.48602	0.5
y11	1263.51240	1263.51190	0.4
y12	1334.54951	1334.54917	0.3
y13	1435.59719	1435.59687	0.2
y14	1566.63768	1566.63791	0.1
y16	1765.67923	1765.68169	1.4
y17	1912.74764	1912.74756	0.0

Table S5vii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7N + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	434.27618	434.27593	0.6
b6	581.34460	581.34425	0.6
b8	798.39671	798.39663	0.1
b9	929.43719	929.43666	0.6
b10	1030.48487	1030.48428	0.6
b11	1101.52198	1101.52143	0.5
b12	1216.54893	1216.54810	0.7
b13	1363.61734	1363.61660	0.5
b14	1476.70140	1476.70055	0.6
b15	1590.74433	1590.74334	0.6
b16	1705.77128	1705.77012	0.7
b17	1820.79822	1820.79712	0.6
y5	544.23617	544.23591	0.5
y6	659.26311	659.26294	0.3
y7	774.29005	774.28964	0.5
y8	888.33298	888.33250	0.5
y9	1001.41704	1001.41609	0.9
y10	1148.48546	1148.48458	0.8
y11	1263.51240	1263.51164	0.6
y12	1334.54951	1334.54884	0.5
y13	1435.59719	1435.59649	0.5
y14	1566.63768	1566.63714	0.3
y15	1669.64686	1669.64551	0.8
y16	1783.68979	1783.70364	7.8
y17	1930.75820	1930.75686	0.7

Table S5viii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7T + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b4	353.21833	353.21815	0.5
b5	452.28675	452.28662	0.3
b6	599.35516	599.35509	0.1
[b9-H_2O]	898.43138	898.43046	1.0
[b10-H_2O]	999.47906	999.47862	0.4
[b11-H_2O]	1070.51617	1070.51565	0.5
[b12-H_2O]	1185.54311	1185.54266	0.4
[b13-H_2O]	1332.61153	1332.61058	0.7
[b14-H_2O]	1445.69559	1445.69514	0.3
[b15-H_2O]	1559.73852	1559.73833	0.1
[b16-H_2O]	1674.76546	1674.76549	0.0
[b17-H_2O]	1789.79240	1789.79228	0.1
y5	544.23617	544.23611	0.1
y6	659.26311	659.26308	0.0
y7	774.29005	774.28995	0.1
y8	888.33298	888.33266	0.4
y9	1001.41704	1001.41649	0.5
y10	1148.48546	1148.48599	0.5
y11	1263.51240	1263.51185	0.4
y12	1334.54951	1334.54919	0.2
y13	1435.59719	1435.59686	0.2
y14	1566.63768	1566.63821	0.3
[y16-H_2O]	1734.67342	1734.67362	0.1
[y17-H_2O]	1881.74183	1881.74071	0.6
[y18-H_2O]	1980.81024	1980.80978	0.2

Table S5ix: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-D7T BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	434.27618	434.27594	0.6
b6	581.34460	581.34424	0.6
b7	682.39227	682.39221	0.1
b8	785.40146	785.40131	0.2
b9	916.44194	916.44147	0.5
b10	1017.48962	1017.48914	0.5
b11	1088.52673	1088.52612	0.6
b12	1203.55368	1203.55298	0.6
b13	1350.62209	1350.62146	0.5
b14	1463.70616	1463.70559	0.4
b15	1577.74908	1577.74878	0.2
b16	1692.77603	1692.77551	0.3
b17	1807.80297	1807.80237	0.3
$b21^{2+}$	1102.49299	1102.49255	0.4
y5	544.23617	544.23596	0.4
y6	659.26311	659.26288	0.3
y7	774.29005	774.28979	0.3
y8	888.33298	888.33264	0.4
y9	1001.41704	1001.41614	0.9
y10	1148.48546	1148.48584	0.3
y11	1263.51240	1263.51196	0.3
y12	1334.54951	1334.54895	0.4
y13	1435.59719	1435.59680	0.3
y14	1566.63768	1566.63696	0.5
y15	1669.64686	1669.64612	0.4
y16	1770.69454	1770.70911	8.2
y17	1917.76295	1917.76208	0.5

Table S5x: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-ΔP2 + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b4	355.23398	355.23377	0.6
b5	502.30240	502.30223	0.3
b8	833.36844	833.36789	0.7
b9	934.41612	934.41487	1.3
b10	1005.45324	1005.45210	1.1
b11	1120.48018	1120.47874	1.3
b12	1267.54859	1267.54820	0.3
b13	1380.63266	1380.63127	1.0
b14	1494.67558	1494.67542	0.1
b15	1609.70253	1609.70232	0.1
b16	1724.72947	1724.72922	0.1
b18	1935.82516	1935.82460	0.3
y5	544.23617	544.23597	0.4
y6 (?)	659.26311	659.28690	36.1
y7	774.29005	774.28979	0.3
y8	888.33298	888.33228	0.8
y9	1001.41704	1001.41427	2.8
y10	1148.48546	1148.48429	1.0
y11	1263.51240	1263.51153	0.7
y12	1334.54951	1334.54905	0.3
y13	1435.59719	1435.59671	0.3
y16	1766.66324	1766.66769	2.5
y17	1913.73165	1913.73074	0.5

Table S5xi: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-P2G + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	412.25545	412.25519	0.6
b6	559.32386	559.32355	0.6
b9	890.38991	890.38904	1.0
b10	991.43759	991.43689	0.7
b11	1062.47470	1062.47339	1.2
b12	1177.50164	1177.50049	1.0
b13	1324.57006	1324.56921	0.6
b14	1437.65420	1437.65344	0.5
b15	1551.69705	1551.69653	0.3
b16	1666.72399	1666.72339	0.4
b17	1781.75093	1781.75024	0.4
$b21^{2+}$	1089.46697	1089.46643	0.5
y5	544.23614	544.23590	0.4
y6	659.26311	659.26282	0.4
y7	774.29005	774.28986	0.2
y8	888.33298	888.33258	0.5
y9	1001.41704	1001.41449	2.5
y10	1148.48546	1148.48523	0.2
y11	1263.51240	1263.51208	0.3
y12	1334.54951	1334.54883	0.5
y13	1435.59719	1435.59668	0.4
y16	1766.66324	1766.66565	1.4
y17	1913.73165	1913.73059	0.6

Table S5xii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-P2G + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	394.24488	394.24457	0.8
b7	656.34024	656.34011	0.2
b8	759.34942	759.34917	0.3
b9	890.38991	890.38948	0.5
b10	991.43759	991.43736	0.2
b11	1062.47470	1062.47420	0.5
b12	1177.50164	1177.50096	0.6
b13	1324.57006	1324.56928	0.6
b14	1437.65412	1437.65383	0.2
b15	1551.69708	1551.69636	0.5
b16	1666.72399	1666.72312	0.5
b17	1781.75093	1781.75059	0.2
b19	1992.84662	1992.84525	0.7
$b21^{2+}$	1089.46697	1089.46669	0.3
y5	544.23617	544.23604	0.2
y6	659.26311	659.26301	0.2
y7	774.29005	774.28983	0.3
y8	888.33298	888.33259	0.4
y9	1001.41704	1001.41584	1.2
y10	1148.48546	1148.48496	0.4
y11	1263.51240	1263.51182	0.5
y12	1334.54951	1334.54893	0.4
y13	1435.59719	1435.59659	0.4
y14	1566.63768	1566.63664	0.7
y15	1669.64686	1669.64613	0.4
y16	1784.67381	1784.68814	8.0
y17	1931.74222	1931.74063	0.8

Table S5xiii: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-V5G + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	392.22923	392.22897	0.7
b8	757.33377	757.33364	0.2
b9	888.37426	888.37064	4.1
b10	989.42194	989.42161	0.3
b11	1060.45905	1060.45863	0.4
b12	1175.48599	1175.48527	0.6
b13	1322.55441	1322.55348	0.7
b14	1435.63847	1435.63844	0.0
b15	1549.68140	1549.68114	0.2
b16	1664.70834	1664.70774	0.4
b17	1779.73582	1779.73465	0.7
$b21^{2+}$	1088.45915	1088.45859	0.5
y5	544.23617	544.23601	0.3
y6	659.26311	659.26298	0.2
y7	774.29005	774.28973	0.4
y8	888.33298	888.33248	0.6
y9	1001.41704	1001.41612	0.9
y10	1148.48546	1148.48490	0.5
y11	1263.51240	1263.51162	0.6
y12	1334.54951	1334.54887	0.5
y14	1435.59719	1435.59628	0.6
y15	1566.63768	1566.63754	0.1
y16	1669.64686	1669.64601	0.5
y17	1784.67381	1784.68254	4.9

Table S5xiv: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-V5L + BmbD:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	466.30240	466.30226	0.3
b6	613.37081	613.37060	0.3
b9	944.43686	944.43649	0.4
b10	1045.48454	1045.48432	0.2
b11	1116.52165	1116.52200	0.3
b12	1231.54859	1231.54868	0.1
b13	1378.61701	1378.61667	0.2
b14	1491.70107	1491.70063	0.3
b15	1605.74400	1605.74297	0.6
b16	1720.77094	1720.76939	0.9
b17	1835.79788	1835.79882	0.5
y5	544.23617	544.23609	0.1
y6	659.26311	659.26312	0.0
y7	774.29005	774.28988	0.2
y8	888.33298	888.33267	0.3
y9	1001.41704	1001.41628	0.8
y10	1148.48546	1148.48644	0.9
y11	1263.51240	1263.51186	0.4
y12	1334.54951	1334.54901	0.4
y13	1435.59719	1435.59739	0.1
y16	1766.66324	1766.66739	2.3
y17	1913.73165	1913.73284	0.6

Table S5xv: Tandem MS assignments. Mass values assigned to the cyclodehydration products with calculated and observed values and associated error.

BmbC-V5L + BmbE:

Species	Predicted mass (Da)	Observed mass (Da)	Error (ppm)
b5	448.29183	448.29164	0.4
b6	595.36025	595.35983	0.7
b7	710.38719	710.38693	0.4
b8	813.39637	813.39602	0.4
b9	944.43686	944.43636	0.5
b10	1045.48454	1045.48405	0.5
b11	1116.52165	1116.52168	0.0
b12	1231.54859	1231.54830	0.2
b13	1378.61701	1378.61602	0.7
b14	1491.70107	1491.70041	0.4
b15	1605.74400	1605.74345	0.3
b16	1720.77094	1720.77004	0.5
b17	1835.79788	1835.79707	0.4
$b21^{2+}$	1116.49045	1116.49012	0.3
y5	544.23617	544.23600	0.3
y6	659.26311	659.26296	0.2
y7	774.29005	774.28977	0.4
y8	888.33298	888.33238	0.7
y9	1001.41704	1001.41602	1.0
y10	1148.48546	1148.48518	0.2
y11	1263.51240	1263.51154	0.7
y12	1334.54951	1334.54905	0.3
y13	1435.59719	1435.59658	0.4
y14	1566.63768	1566.63669	0.6
y15	1669.64686	1669.64652	0.2
y16	1784.67381	1784.68874	8.4
y17	1931.74222	1931.74117	0.5

Figure S8 (i-x): NMR spectroscopic characterization of cyclodehydration products. BmbC-N15K unmodified peptide and cyclodehydration products were subjected to trypsin cleavage for NMR analysis.

Figure S8i: Overlays of BmbC-N15K trypsin-cleaved cyclodehydration products. (A) COSY spectra of the unmodified bottromycin precursor peptide (black) and the thiazoline-bearing form after treatment of BmbC with BmbD (blue). (B) COSY spectra of the unmodified bottromycin precursor peptide (black) and the macrolactamidine-bearing form after treatment with BmbE (green). Insets highlight the key changes between the spectra.

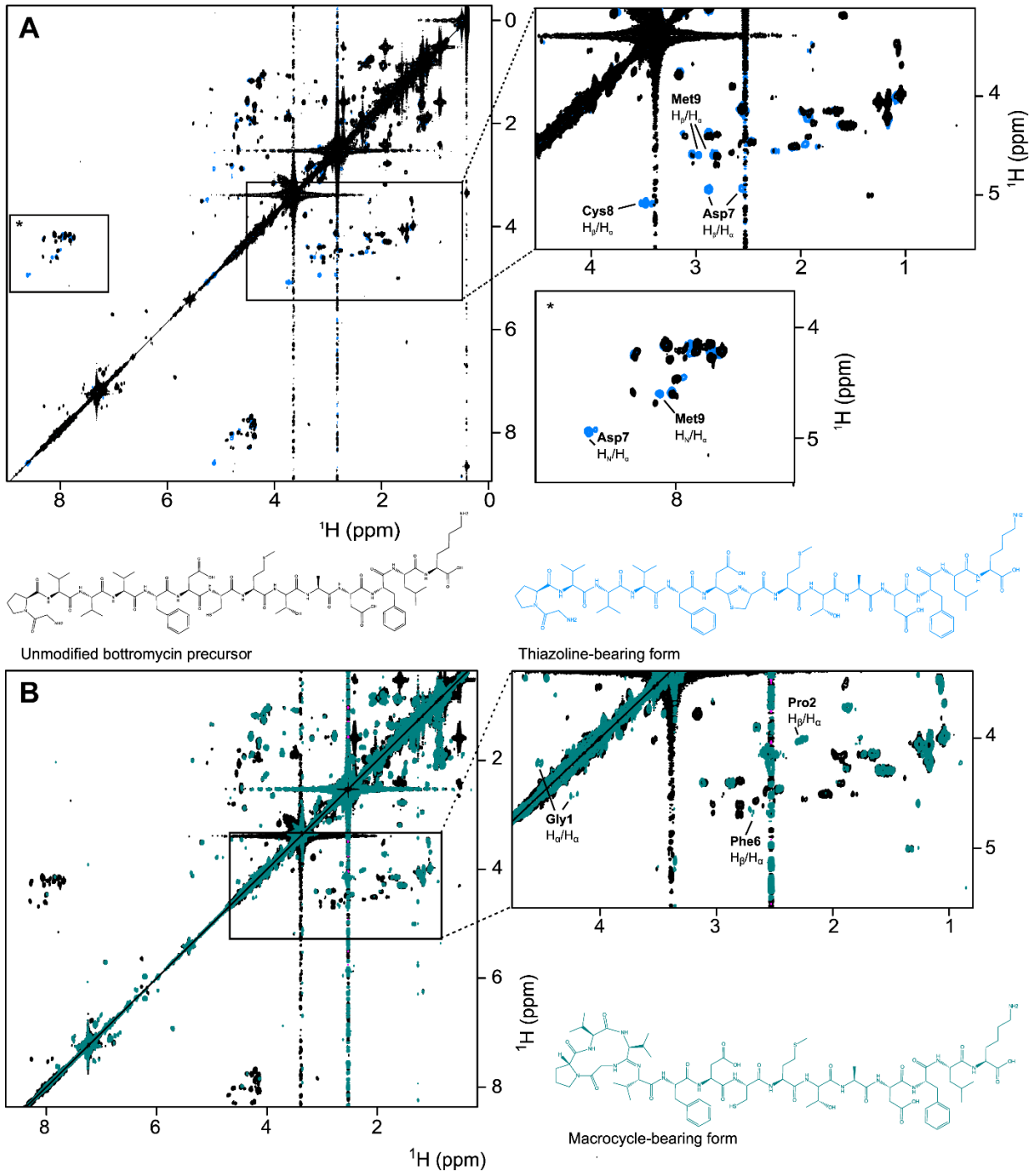


Figure S8ii: ^{1}H-^{1}H COSY (DMSO-d_6) of BmbC-N15K-trypsinized

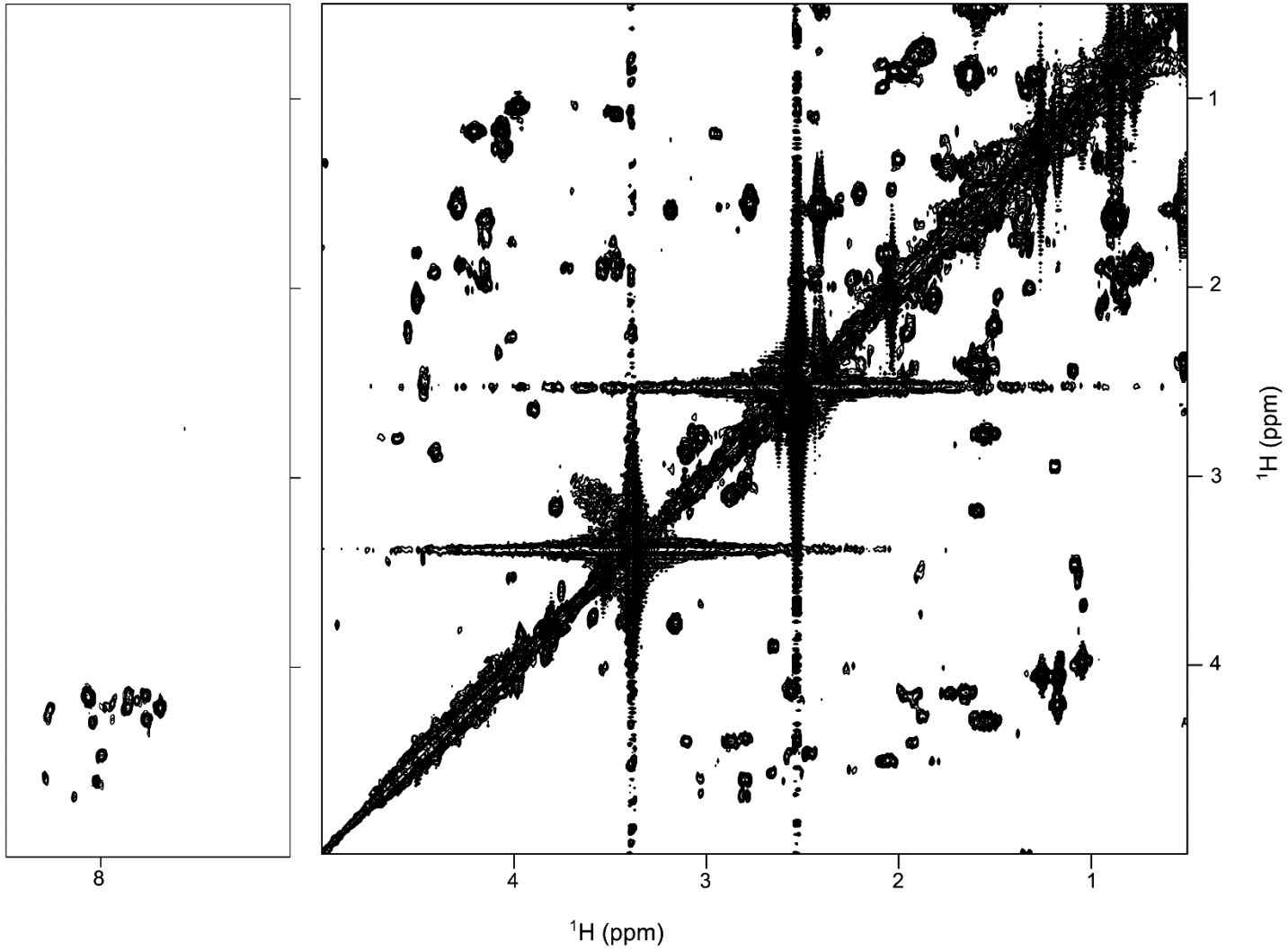


Figure S8iii: ^{1}H-^{1}H TOCSY (DMSO-d_6) of BmbC-N15K-trypsinized

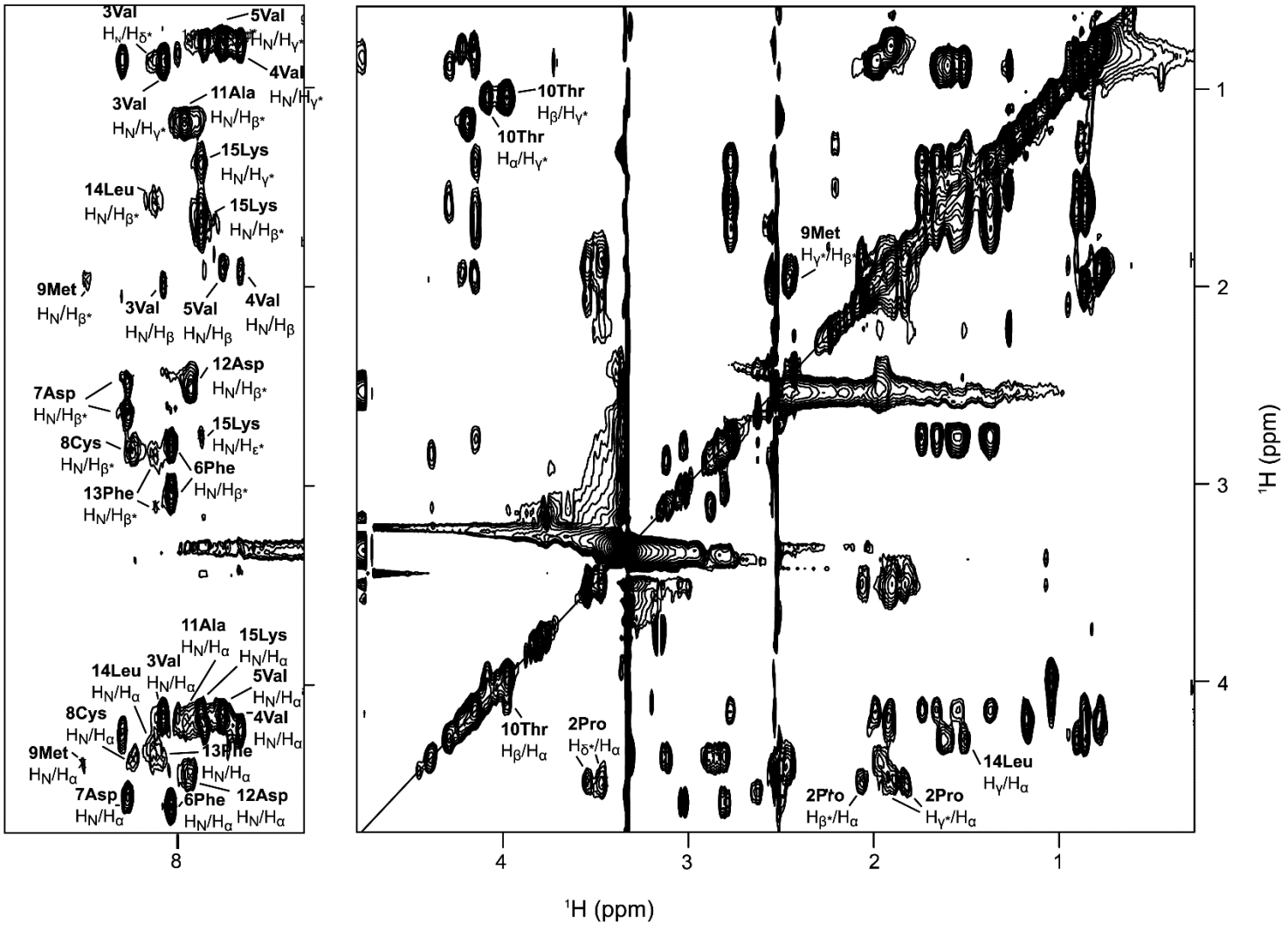


Figure S8iv: ^{1}H-^{1}H NOESY (DMSO-*d*$_6$) of BmbC-N15K-trypsinized.

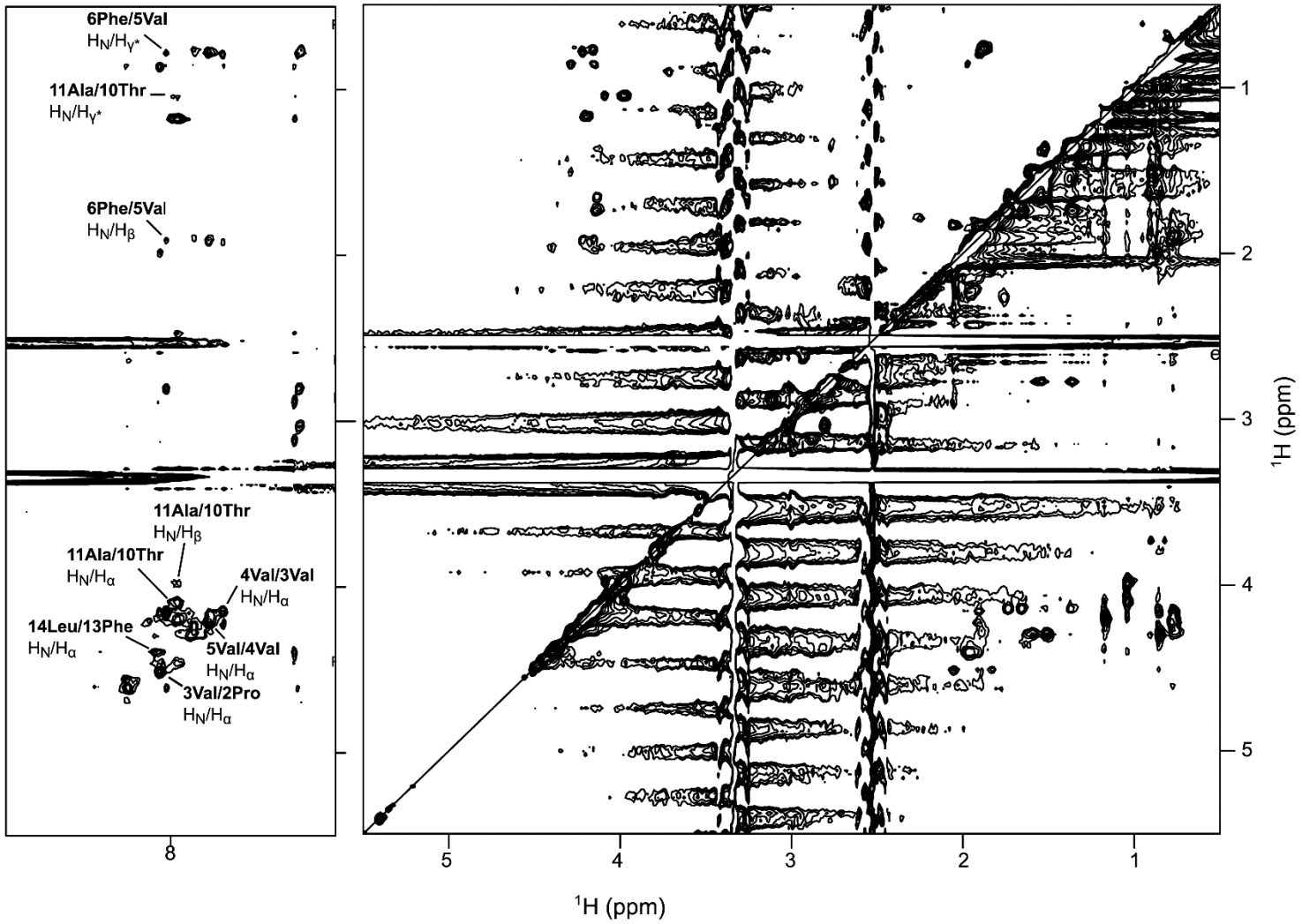


Figure S8v: ^{1}H-^{1}H COSY (DMSO-*d*$_6$) of BmbC-N15K-trypsinized thiazoline

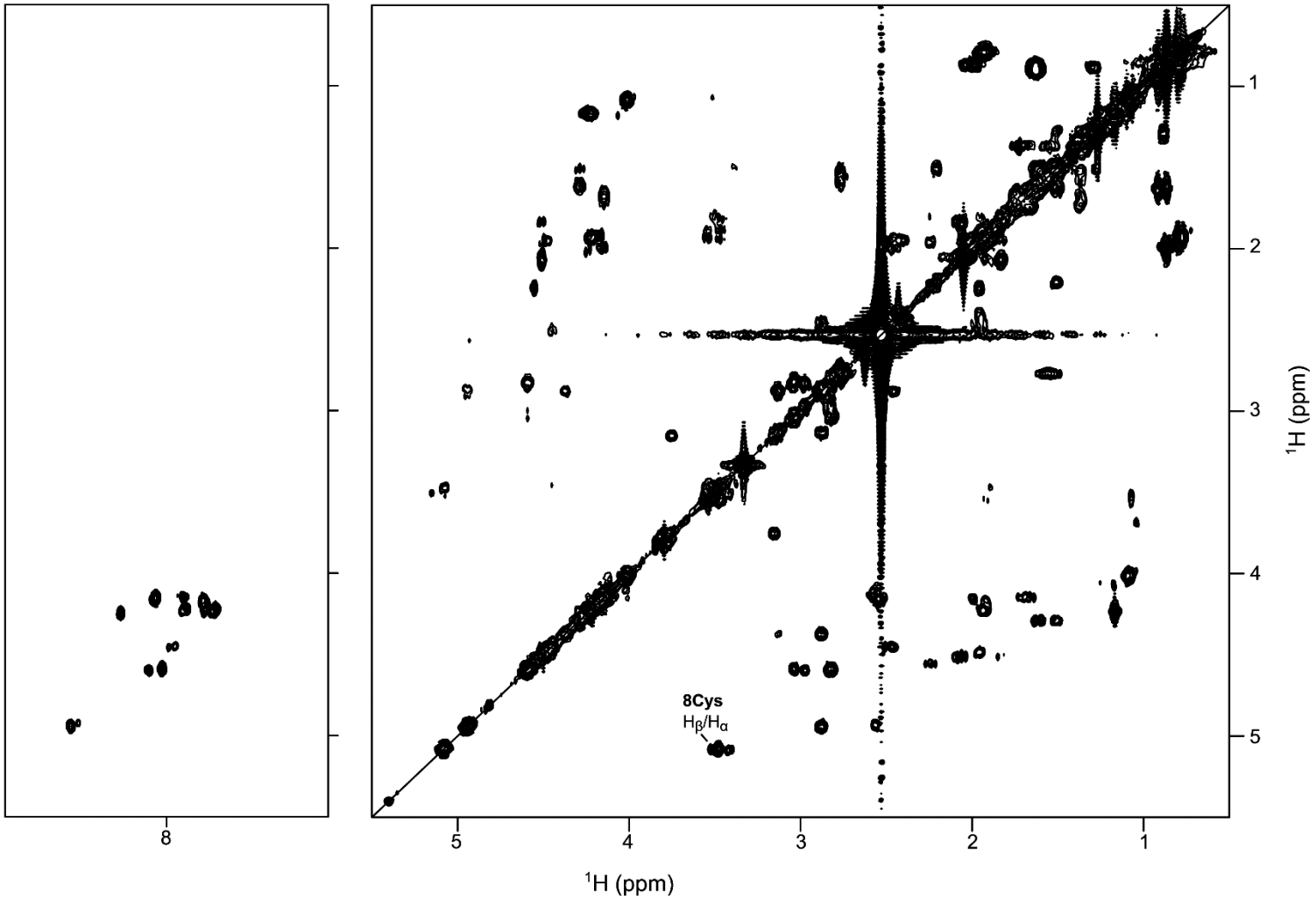


Figure S8vi: ^{1}H-^{1}H TOCSY (DMSO-*d*$_6$) of BmbC-N15K-trypsinized thiazoline

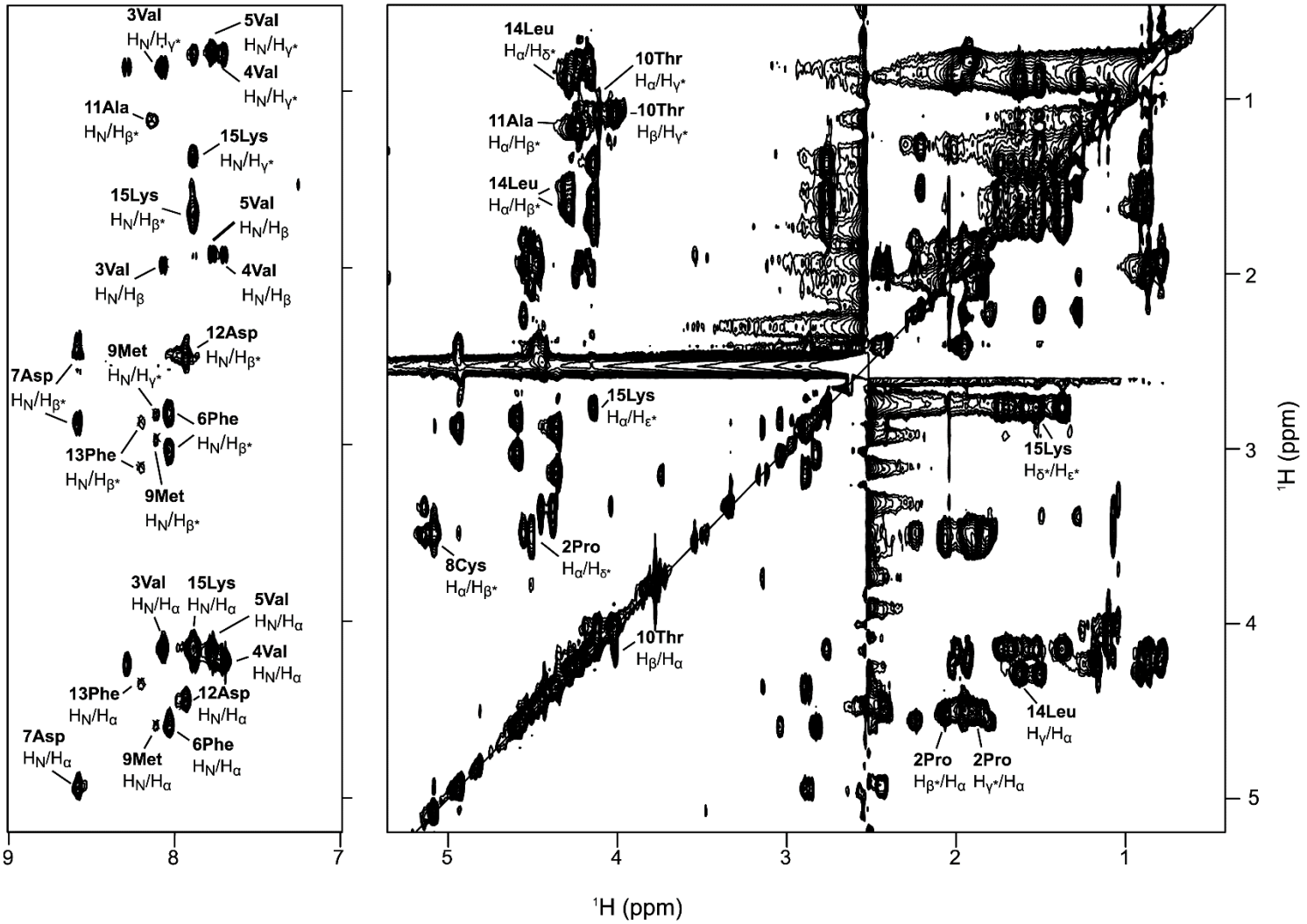


Figure S8vii: ^{1}H-^{1}H NOESY (DMSO-d_6) of BmbC-N15K-trypsinized thiazoline

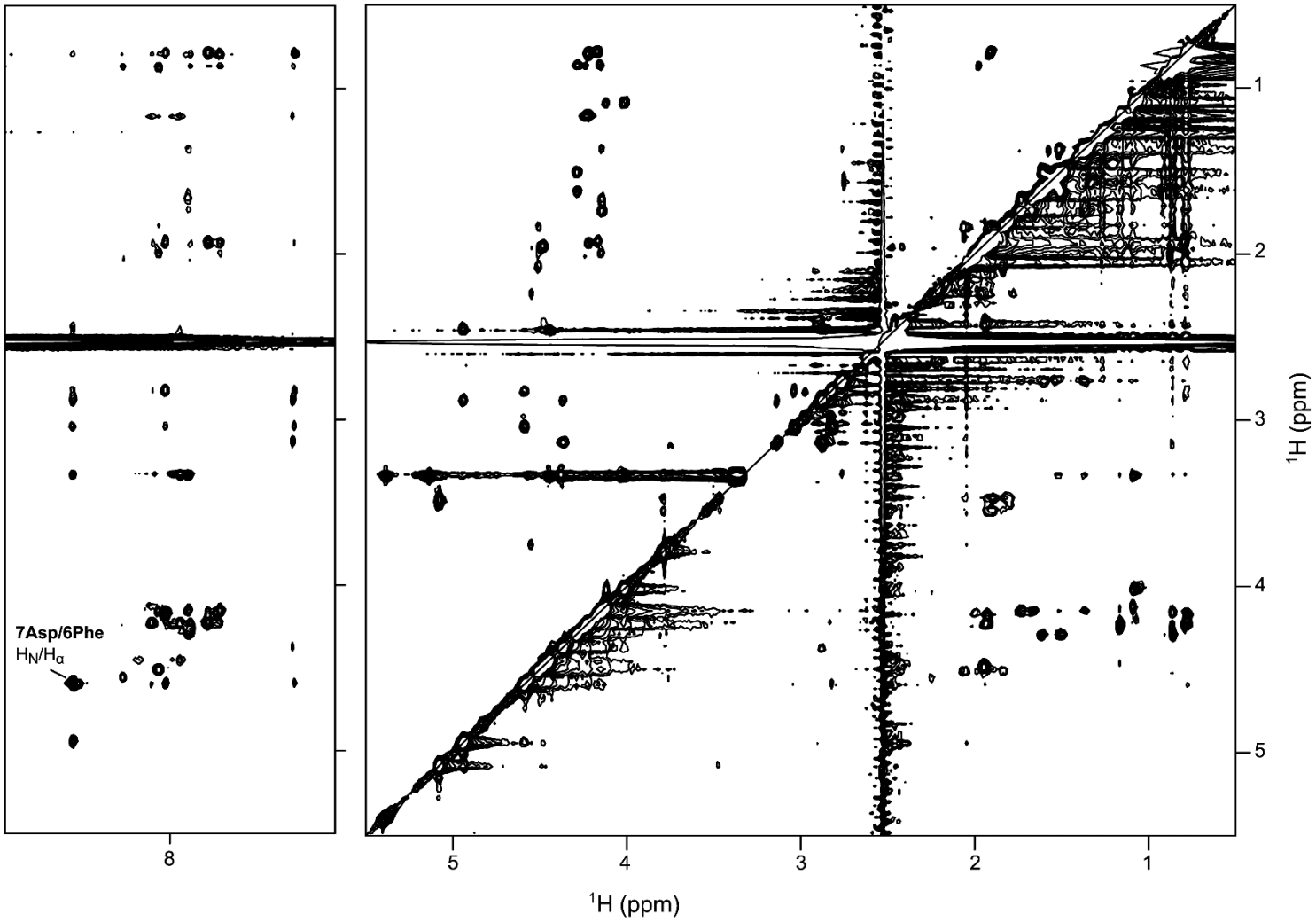


Figure S8viii: ^{1}H-^{1}H COSY (DMSO-d_6) of BmbC-N15K-trypsinized macrolactamidine

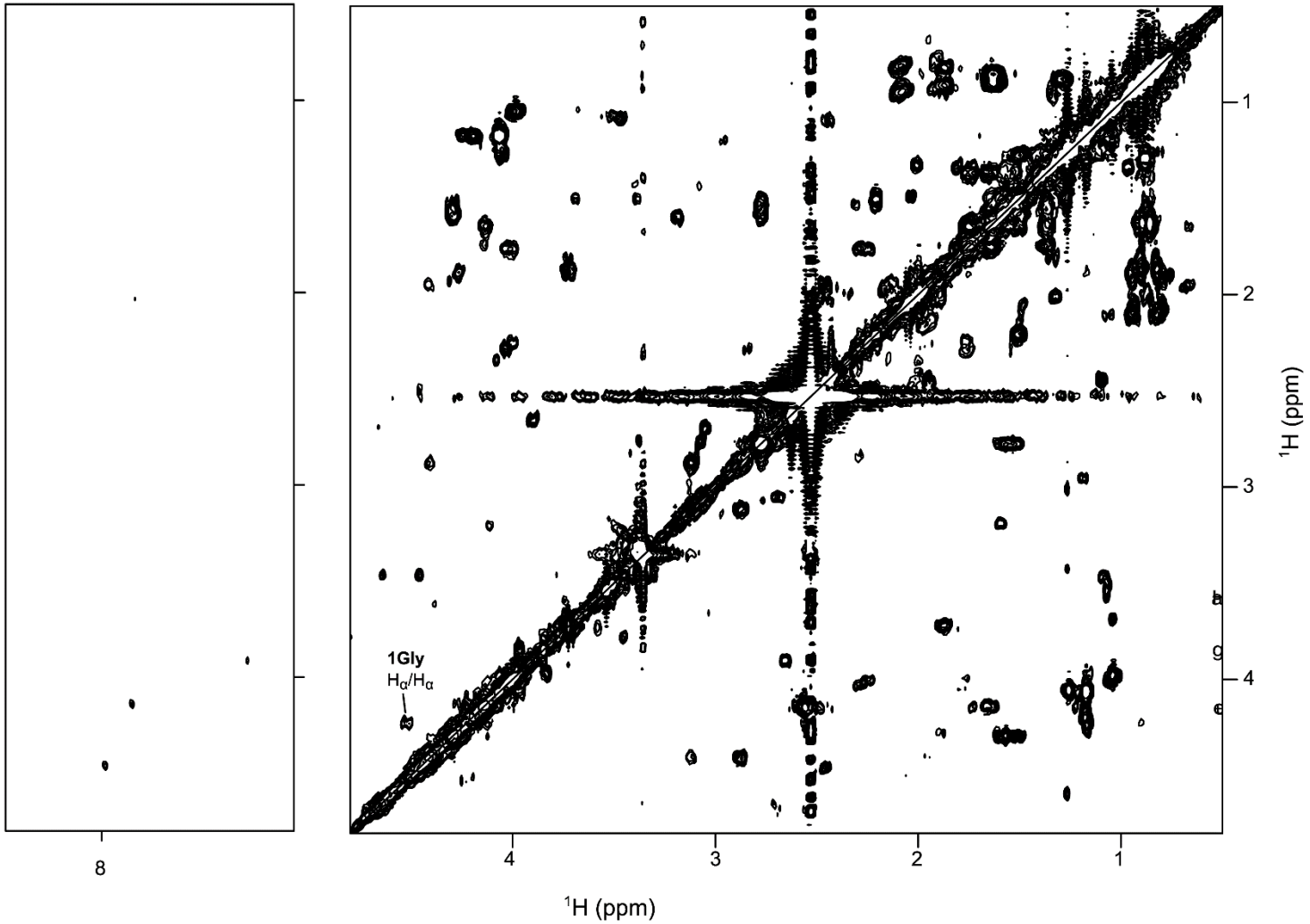


Figure S8ix: ^{1}H-^{1}H TOCSY (DMSO-*d*$_6$) of BmbC-N15K-trypsinized macrolactamidine

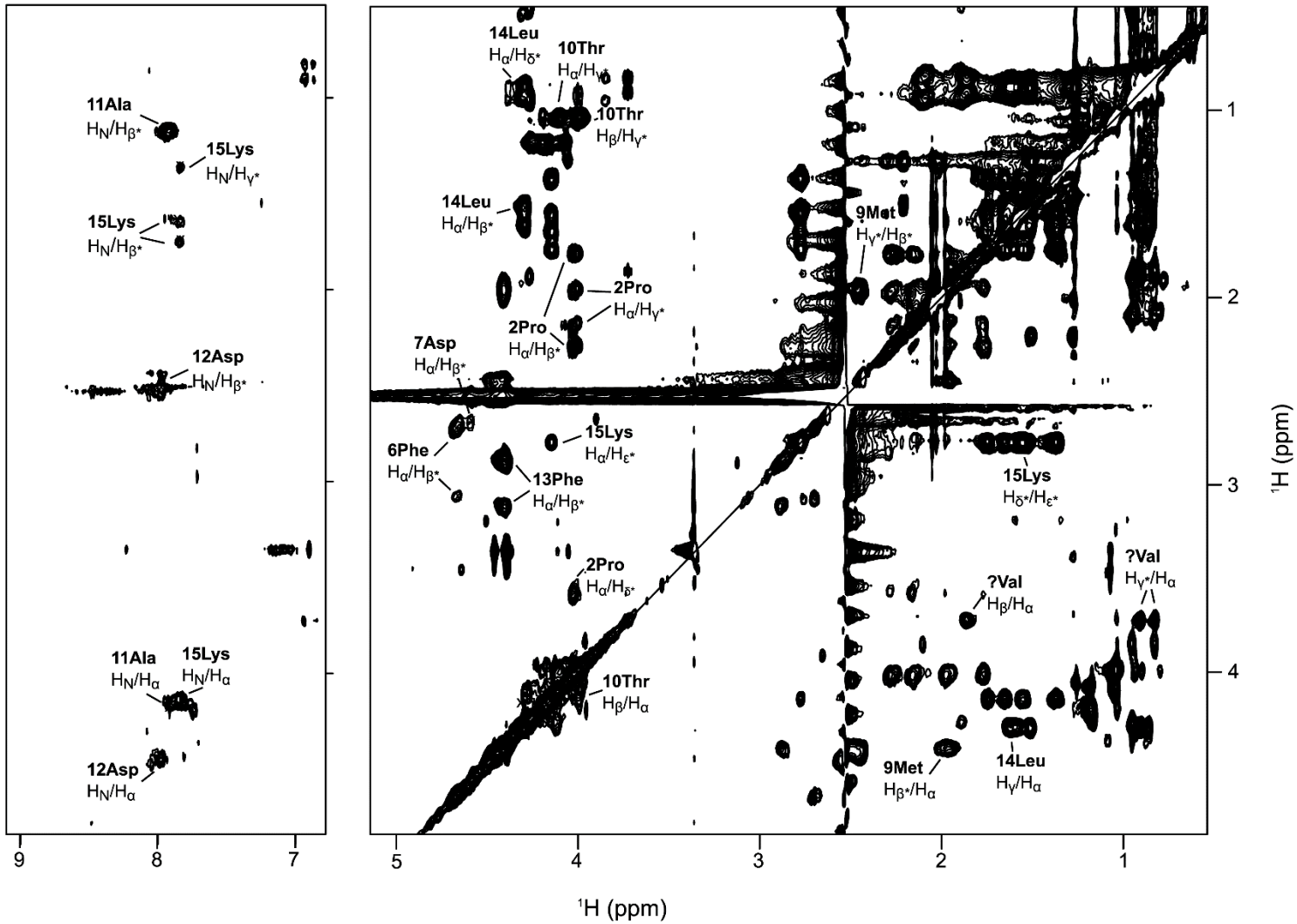


Figure S8x: ^{1}H-^{1}H NOESY (DMSO-d_6) of BmbC-N15K-trypsinized macrolactamidine

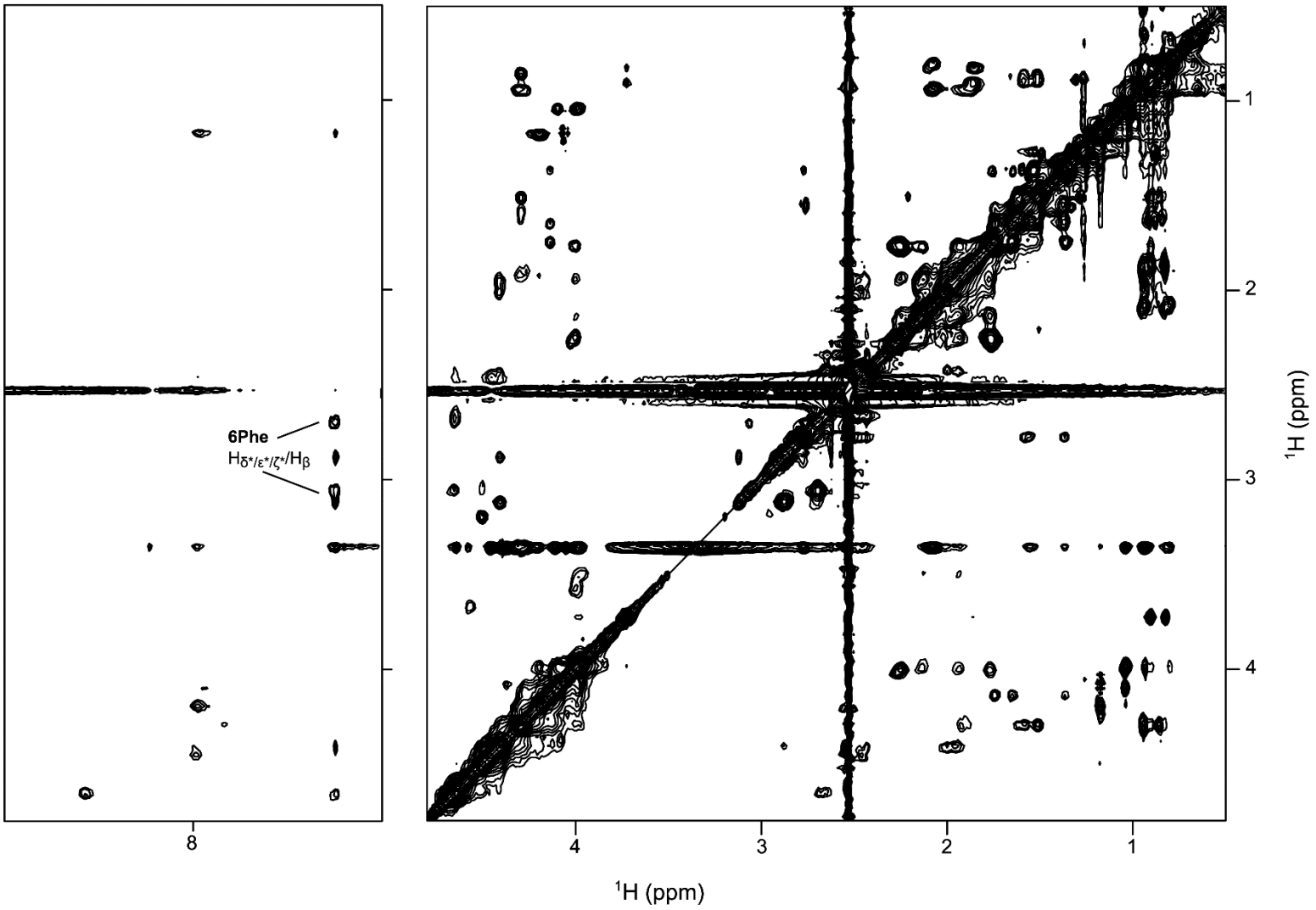


Table S6: Assignments for signals due to protons in NMR spectra of cyclodehydrated products. Chemical shift values in ppm are given for the products of the BmbD and BmbE –catalyzed reactions and compared to the corresponding values for the substrate. *na* unambiguous assignment not possible from spectra; * resonances with the same shift. Protons with different shifts are highlighted in gray.

	Proton Shifts Unprocessed δ (ppm)							Proton Shifts Macrolactamidine δ (ppm)							Proton Shifts Thiazoline δ (ppm)						
	H_N	H_α	H_β	H_γ	H_δ	H_ε	H_ζ	H_N	H_α	H_β	H_γ	H_δ	H_ε	H_ζ	H_N	H_α	H_β	H_γ	H_δ	H_ε	H_ζ
Gly1	*na*	*na*	-	-	-	-	-	*na*	4.52/4.23	-	-	-	-	-	*na*	*na*	-	-	-	-	-
Pro2	-	4.51	2.06*	1.91*	3.52	-	-	-	4.01	2.28/1.76	1.98/2.17	3.58	-	-	-	4.51	2.07*	1.91*	3.52	-	-
Val3	8.07	4.16	2	0.86*	-	-	-	*na*	*na*	*na*	*na*	-	-	-	8.06	4.15	1.99	0.86*	-	-	-
Val4	7.69	4.22	1.92	0.79*	-	-	-	*na*	*na*	*na*	*na*	-	-	-	7.7	4.22	1.93	0.78*	-	-	-
Val5	7.77	4.17	1.91	0.78*	-	-	-	*na*	*na*	*na*	*na*	-	-	-	7.77	4.17	1.93	0.78*	-	-	-
Phe6	8.03	4.6	3.03/2.81	-	7.23*	7.23*	7.23*	*na*	4.67	3.06/2.70	-	7.26*	7.26*	7.26*	8.03	4.58	3.04/2.82	-	*na*	*na*	*na*
Asp7	8.26	4.56	2.48/2.63	-	-	-	-	*na*	4.59	2.66/2.49	-	-	-	-	8.58	4.94	2.88/2.46	-	-	-	-
Cys8	8.23	4.39	2.82*	-	-	-	-	*na*	*na*	*na*	-	-	-	-	-	5.08	3.49/3.42	-	-	-	-
Met9	8.46	4.41	1.96/1.96	2.45*	-	-	-	8.47	4.41	1.98*	2.46	-	-	-	8.11	4.6	2.97/2.97	2.84*	-	-	-
Thr10	8.22	4.09	3.98	1.04	-	-	-	7.74	4.1	4	1.04	-	-	-	8.4	4.13	4.01	1.09	-	-	-
Ala11	7.97	4.19	1.18	-	-	-	-	7.93	4.19	1.18	-	-	-	-	8.14	4.22	1.16	-	-	-	-
Asp12	7.94	4.45	2.48*	-	-	-	-	7.94	4.42	2.47*	-	-	-	-	7.94	4.45	2.47*	-	-	-	-
Phe13	8.12	4.39	3.12/2.87	-	7.25*	7.25*	7.25*	7.97	4.41	3.12/2.88	-	7.24*	7.24*	7.24*	8.2	4.35	3.14/2.88	-	*na*	*na*	*na*
Leu14	8.11	4.3	1.54*	1.6	0.88*	-	-	8.08	4.29	1.52*	1.62	0.88*	-	-	8.26	4.29	1.62/1.51	1.62	0.91/0.86	-	-
Lys15	7.88	4.15	1.70*	1.37*	1.55	2.77*	-	7.85	4.14	1.65/1.74	1.37	1.55*	2.77*	-	7.89	4.15	1.71*	1.37*	1.59	2.76*	-

Figure S9: In vitro processing is not enhanced by BmbG or BmbH. Putative partner proteins BmbG and BmbH were assessed for enhancement of processing by BmbD and BmbE in 16 h endpoint MALDI-TOF-MS assay comparing conversion of the BmbC precursor to the cyclodehydrated species.

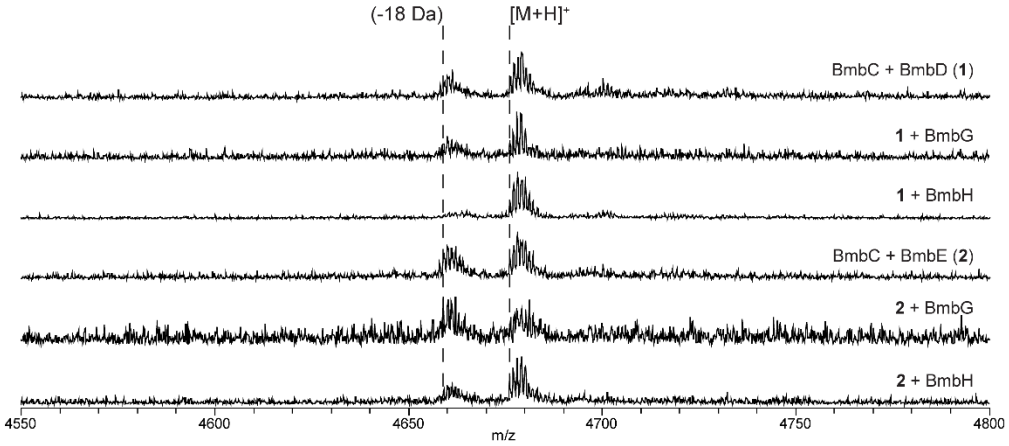


Figure S10: Minimal follower peptide for bottromycin cyclodehydratase processing. (A) C-terminal truncations of the bottromycin precursor peptide (BmbC) were prepared by introducing stop codons at evenly spaced intervals. The BmbC core peptide region is shown in green. Truncations tested as in vitro substrates of BmbD and BmbE and labeled below the sequence. Variants which were not processed are labeled in purple. Truncated precursor which was processed and denoted as the minimal follower peptide is labeled in blue. (B) Additional stop codons were installed adjacent to the G35* variant, indicating an identical minimal follower motif for both bottromycin cyclodehydratases.

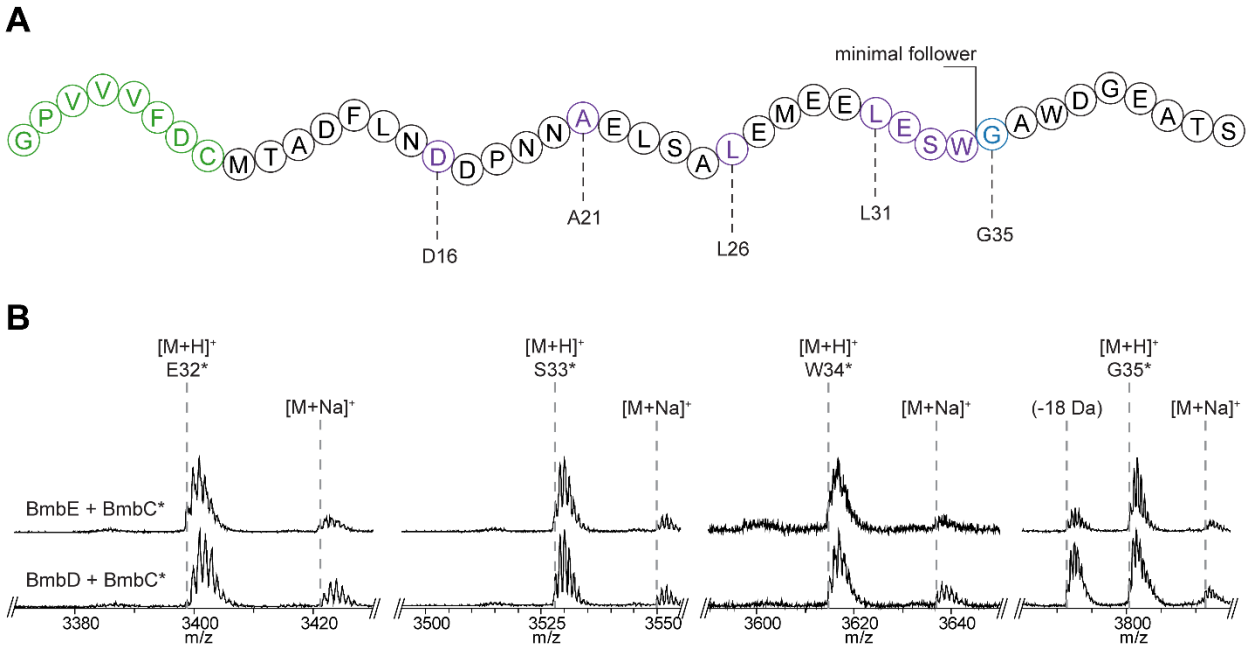
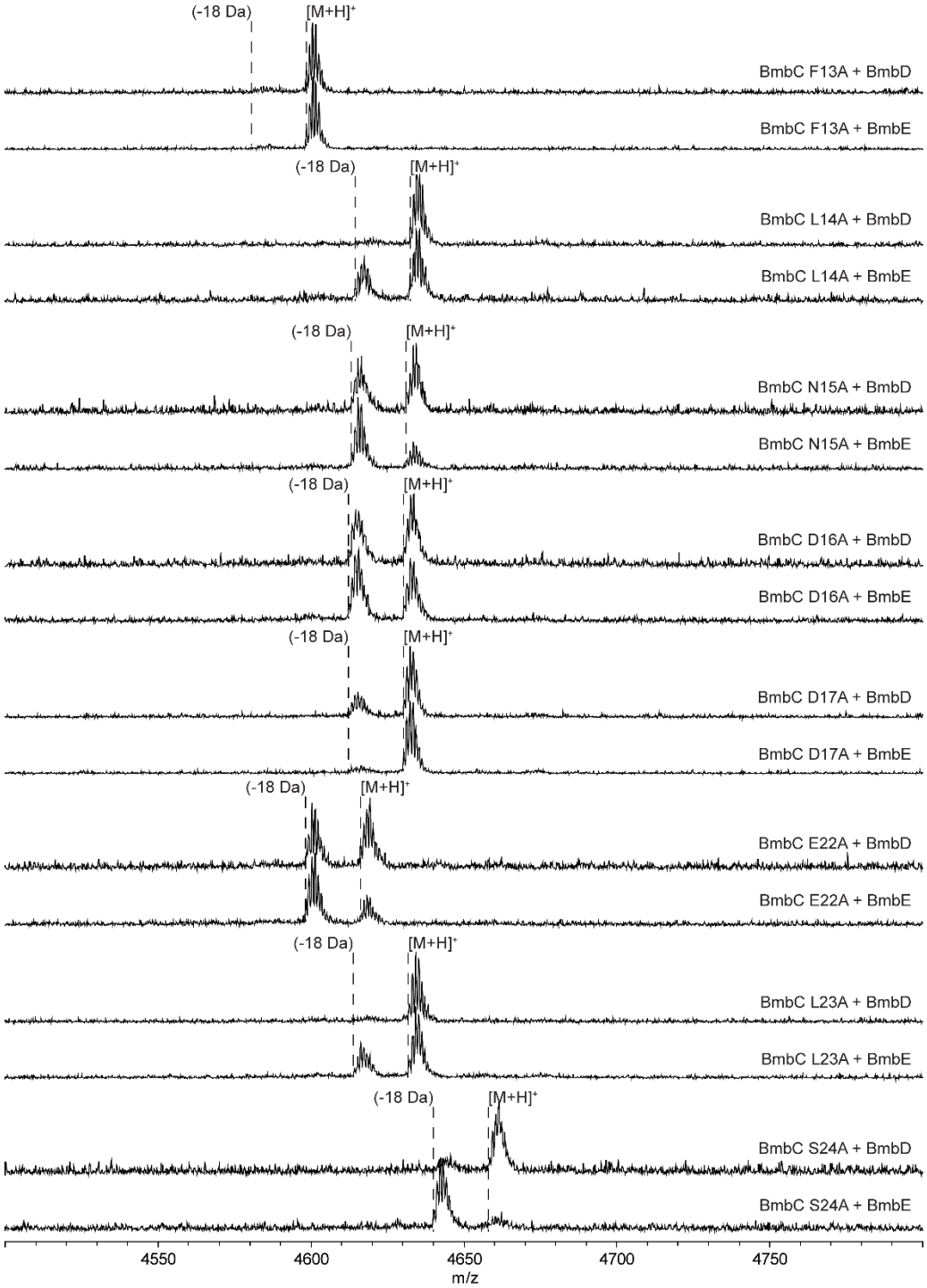


Figure S11: Follower peptide residues important for thiazoline and macrolactamidine formation. Single alanine variants were made in the bottromycin follower peptide in regions similar to lanthipeptide and cyanobactin recognition sequences. Dehydration was monitored by MALDI-TOF-MS.



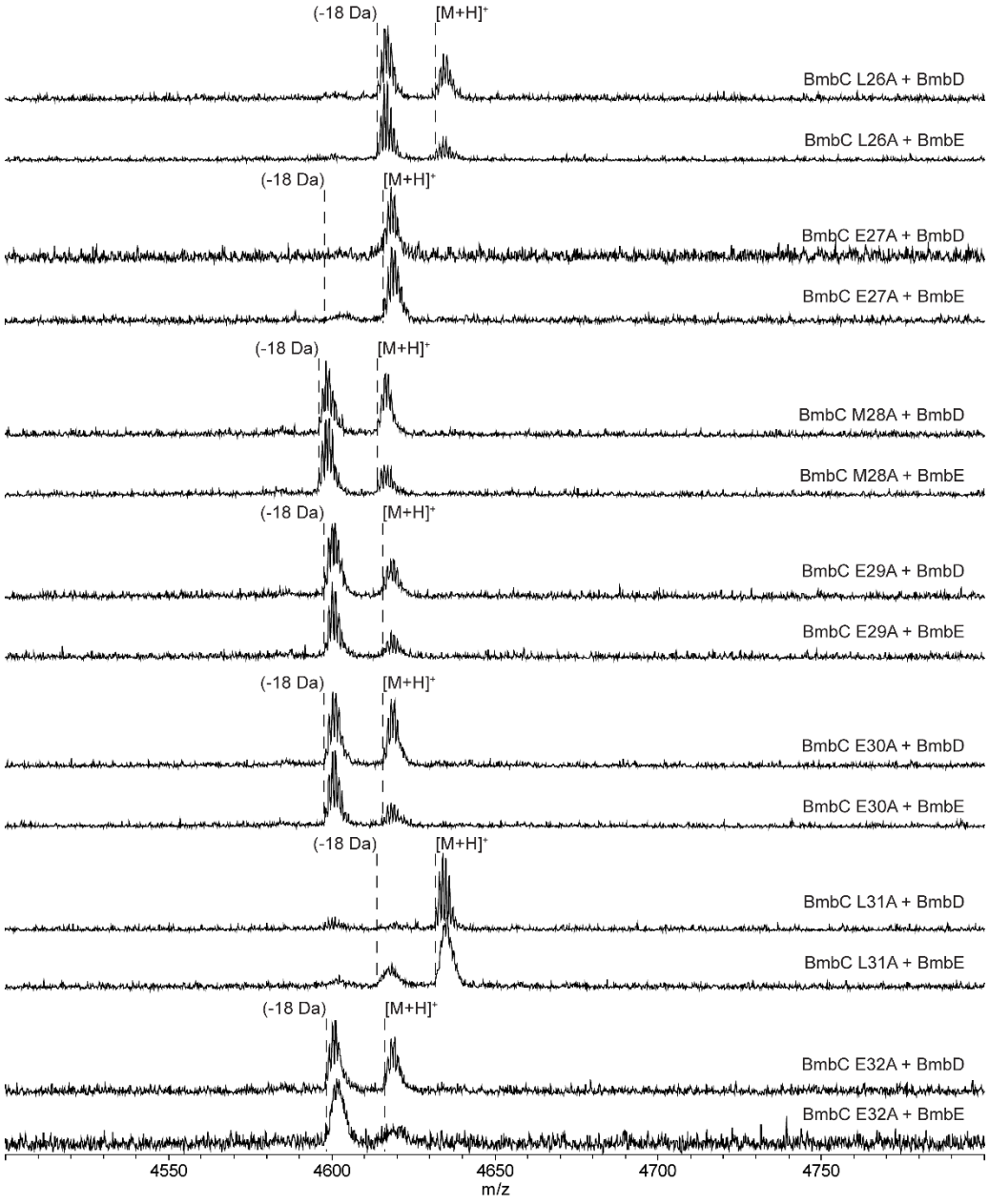

(-18 Da)
[M+H]+
BmbC L26A + BmbD
BmbC L26A + BmbE
BmbC E27A + BmbD
BmbC E27A + BmbE
BmbC M28A + BmbD
BmbC M28A + BmbE
BmbC E29A + BmbD
BmbC E29A + BmbE
BmbC E30A + BmbD
BmbC E30A + BmbE
BmbC L31A + BmbD
BmbC L31A + BmbE
BmbC E32A + BmbD
BmbC E32A + BmbE
4550
4600
4650
4700
4750
m/z

Figure S12: Generation of a fluorescently-labeled bottromycin precursor peptide. Expressed double mutant BmbC-C8S-S43C-His and labeled with 6-(iodoacetamido)fluorescein (IAF). MALDI-TOF-MS was used to assess reaction progress.

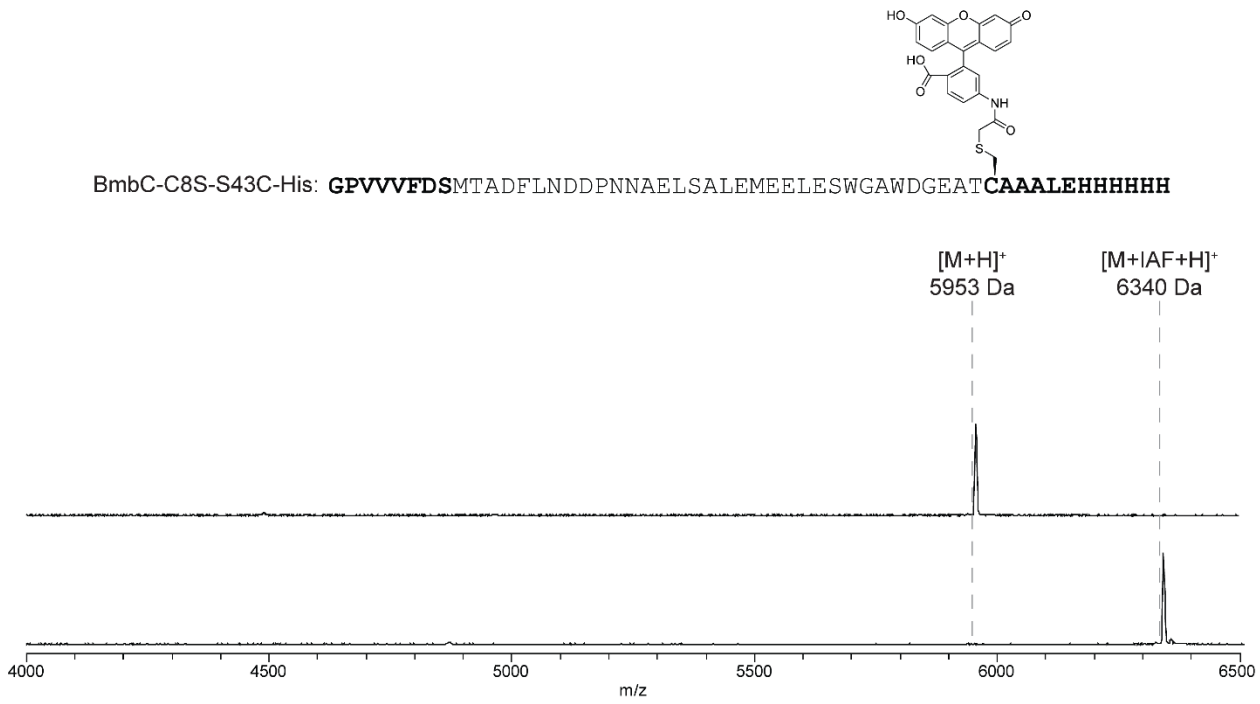
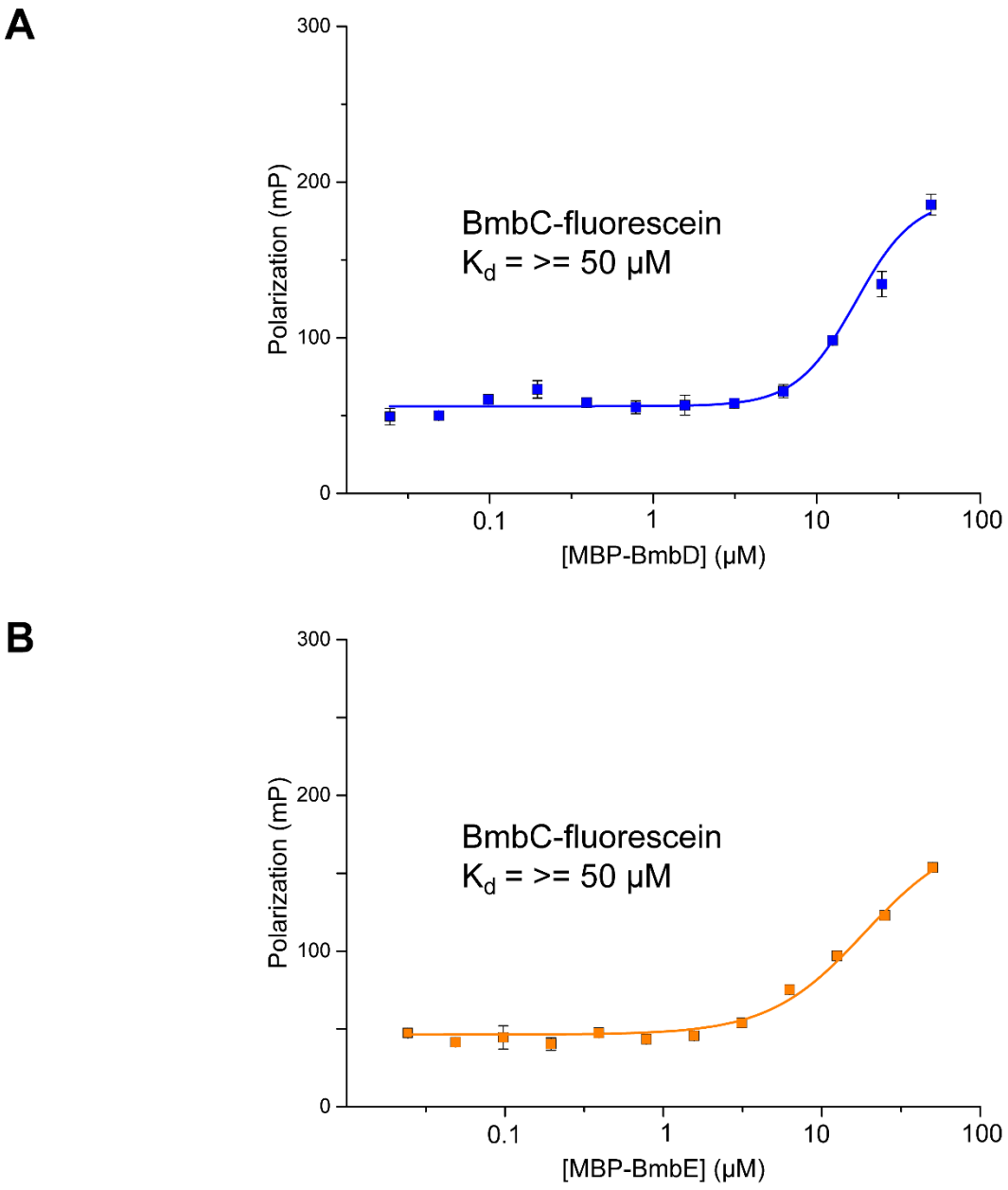
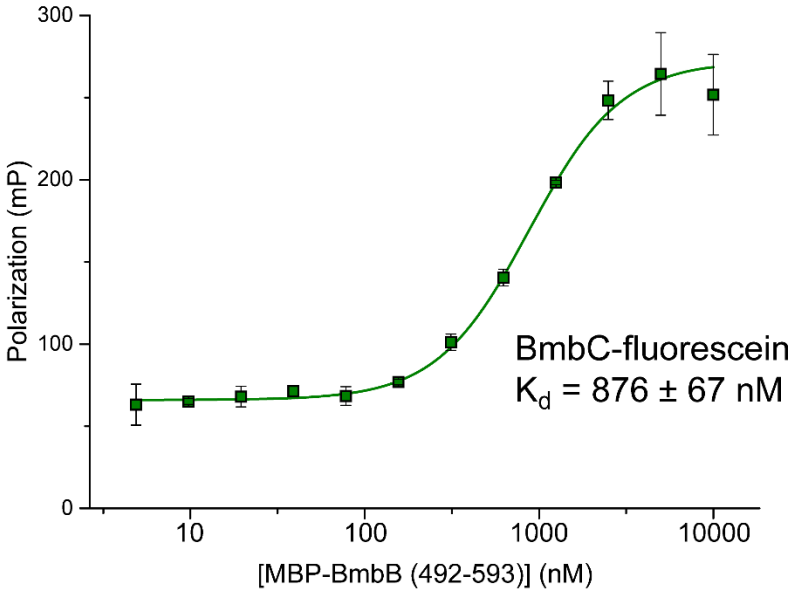


Figure S13: Fluorescence polarization analysis of BmbC binding. BmbD, BmbE and excised RiPP recognition element (RRE) domains from BmbB and BmbF were analyzed for binding affinity to BmbC. (A) Binding of fluorescently tagged BmbC to BmbD. (B) Binding of fluorescently-tagged BmbC to BmbE. (C) Binding of fluorescently tagged BmbC to BmbB (residues 492-593). (D) Binding of fluorescently-tagged BmbC to BmbF (residues 526-670). Error bars show s.e.m. from three independent trials.



C


300
200
100
0
Polarization (mP)
10
100
1000
10000
[MBP-BmbB (492-593)] (nM)
BmbC-fluorescein
K_d = 876 ± 67 nM

D

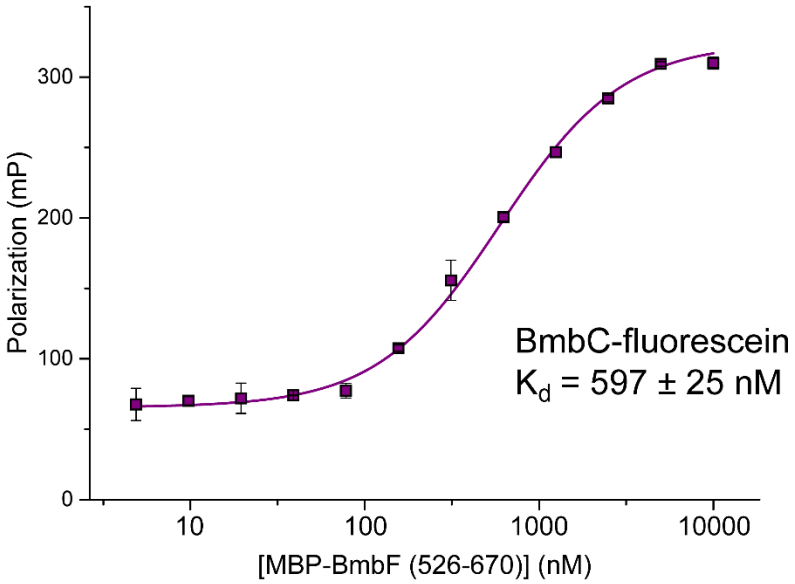
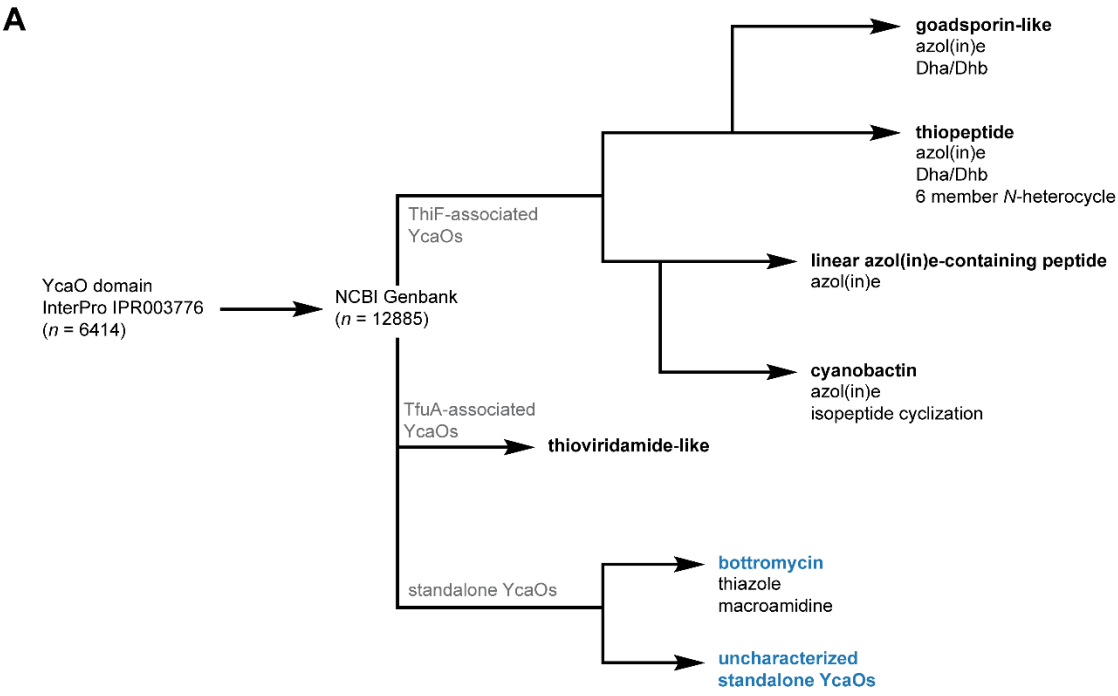

300
200
100
0
Polarization (mP)
10
100
1000
10000
[MBP-BmbF (526-670)] (nM)
BmbC-fluorescein
K_d = 597 ± 25 nM

Figure S14: RODEO prospecting of YcaO superfamily classes. (A) Flow chart describing the classification methods used for grouping YcaOs based on contexts. Classifications were made to generate a conservative estimate of standalone YcaOs (*i.e.* any Ocin-ThiF-like protein would disqualify any YcaO in the cluster from qualifying as a "standalone", E1-like domains are conflated with ThiF-like domains, dehydroalanine = "Dha" dehydrobutyrine = "Dhb"). (B) Distribution of YcaOs based on the classification presented in panel A.



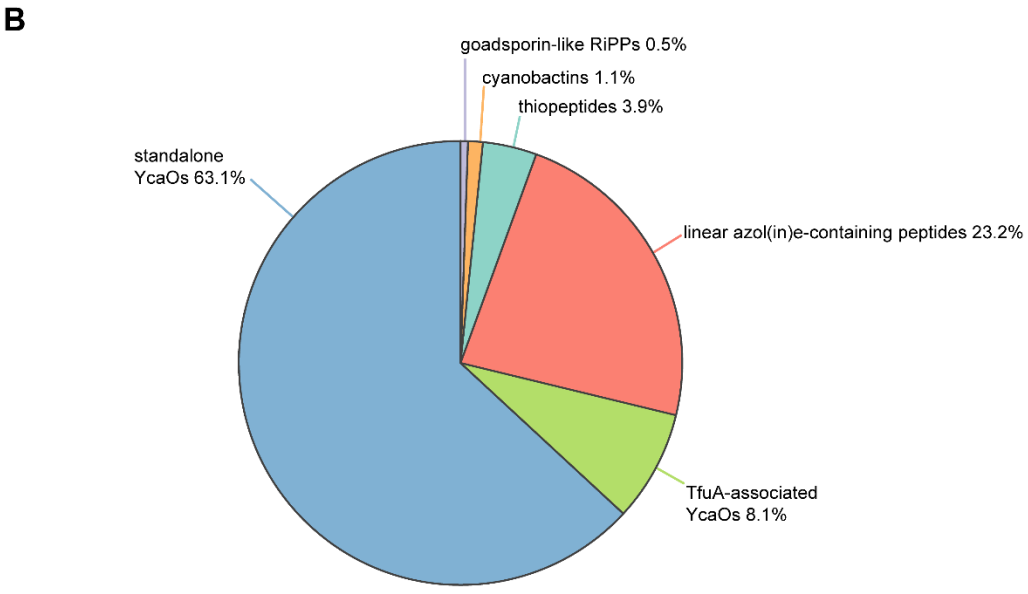


Table S7: Co-Occurrence of genes with non-canonical YcaOs. RODEO was used to inspect BGC contexts for genes co-occurring with YcaOs that lacked a nearby gene encoding for either an E1 ubiquitin-activating enzyme or ocin-ThiF-like protein. The top 100 protein families are shown, organized by frequency of occurrence. Genes predicted to be involved in transport are red, regulation are yellow, ATPase-like are green, putative RiPP biosynthetic genes are in blue and domains of unknown function (DUF) are in purple.

Pfam Title	Description	Count
YcaO	YcaO-like family	**13811**
ABC_tran	ABC transporter	10786
AAA_21	AAA domain putative AbiEii toxin Type IV TA system	6337
MFS_1	Major Facilitator Superfamily	6093
SMC_N	RecF/RecN/SMC N terminal domain	5990
ABC_membrane	ABC transporter transmembrane region	5768
Radical_SAM	Radical SAM superfamily	4665
Sugar_tr	Sugar (and other) transporter	4407
Aminotran_5	Aminotransferase class-V	4286
Fer4_12	4Fe-4S single cluster domain	3872
AAA_17	AAA domain	3814
S1	S1 RNA binding domain	3753
Lactamase_B	Metallo-beta-lactamase superfamily	3730
Fer4_14	4Fe-4S single cluster domain	3692
MFS_4	Uncharacterised MFS-type transporter YbfB	3648
Form_Nir_trans	Formate/nitrite transporter	3505
Gly_radical	Glycine radical	3491
EPSP_synthase	EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase)	3478
NTP_transf_3	MobA-like NTP transferase domain	3442
Pyr_redox_2	Pyridine nucleotide-disulphide oxidoreductase	3432
DUF4131	Domain of unknown function (DUF4131)	3370
HTH_1	Bacterial regulatory helix-turn-helix protein lysR family	3273
Bac_DNA_binding	Bacterial DNA-binding protein	3269
Cytidylate_kin	Cytidylate kinase	3254
PCB_OB	Penicillin-binding protein OB-like domain	3240
DUF4496	Domain of unknown function (DUF4496)	3237
Nitroreductase	Nitroreductase family	3231
APH	Phosphotransferase enzyme family	3213
S1_2	S1 domain	3210
LysR_substrate	LysR substrate binding domain	3182
Cytidylate_kin2	Cytidylate kinase-like family	3120
LpxK	Tetraacyldisaccharide-1-P 4'-kinase	3104
CTP_transf_3	Cytidylyltransferase	3102
Trm112p	Trm112p-like protein	3074
Competence	Competence protein	3034
AAA	ATPase family associated with various cellular activities (AAA)	3016
DUF421	Protein of unknown function (DUF421)	2925
DUF2064	Uncharacterized protein conserved in bacteria (DUF2064)	2898
MobB	Molybdopterin guanine dinucleotide synthesis protein B	2872
tRNA-synt_2b	tRNA synthetase class II core domain (G H P S and T)	2856
Keratin_assoc	Keratinocyte-associated protein 2	2852
HTH_42	Winged helix DNA-binding domain	2823
HTH_24	Winged helix-turn-helix DNA-binding	2820
HU-DNA_bdg	DNA-binding domain	2800
DUF218	DUF218 domain	2783
Methyltransf_11	Methyltransferase domain	2757
Methyltransf_31	Methyltransferase domain	2736
DUF2318	Predicted membrane protein (DUF2318)	2639
Acetyltransf_1	Acetyltransferase (GNAT) family	2601
DmsC	DMSO reductase anchor subunit (DmsC)	2581

Seryl_tRNA_N	Seryl-tRNA synthetase N-terminal domain	2563
Fer4	4Fe-4S binding domain	2557
Abhydrolase_5	Alpha/beta hydrolase family	2525
AAA_assoc_2	AAA C-terminal domain	2521
MgsA_C	MgsA AAA+ ATPase C terminal	2520
LolA	Outer membrane lipoprotein carrier protein LolA	2486
Molydop_binding	Molydopterin dinucleotide binding domain	2417
DUF2092	Predicted periplasmic protein (DUF2092)	2411
Molybdopterin	Molybdopterin oxidoreductase	2395
FtsK_SpoIIIE	FtsK/SpoIIIE family	2383
Response_reg	Response regulator receiver domain	2347
AAA_26	AAA domain	2344
Ftsk_gamma	Ftsk gamma domain	2321
Fer4_11	4Fe-4S dicluster domain	2315
Molybdop_Fe4S4	Molybdopterin oxidoreductase Fe4S4 domain	2288
Pyr_redox	Pyridine nucleotide-disulphide oxidoreductase	2217
Fer4_2	4Fe-4S binding domain	2194
Filament	Intermediate filament protein	2143
Peptidase_M48	Peptidase family M48	2131
A2L_zn_ribbon	A2L zinc ribbon domain	2121
HATPase_c	Histidine kinase- DNA gyrase B- and HSP90-like ATPase	2120
FtsK_4TM	4TM region of DNA translocase FtsK/SpoIIIE	2114
HTH_AsnC-type	AsnC-type helix-turn-helix domain	2023
adh_short	short chain dehydrogenase	1951
AsnC_trans_reg	AsnC family	1942
Acetyltransf_7	Acetyltransferase (GNAT) domain	1929
XK-related	XK-related protein	1916
HTH_3	Helix-turn-helix	1911
MarR_2	MarR family	1903
AA_permease_2	Amino acid permease	1886
BPD_transp_1	Binding-protein-dependent transport system inner membrane component	1876
adh_short_C2	Enoyl-(Acyl carrier protein) reductase	1866
ABC2_membrane_3	ABC-2 family transporter protein	1856
AAA_16	AAA ATPase domain	1847
AA_permease	Amino acid permease	1839
Pyr_redox_3	Pyridine nucleotide-disulphide oxidoreductase	1829
Methyltransf_23	Methyltransferase domain	1826
DUF4345	Domain of unknown function (DUF4345)	1817
Acetyltransf_10	Acetyltransferase (GNAT) domain	1797
OsmC	OsmC-like protein	1761
HTH_19	Helix-turn-helix domain	1754
HTH_31	Helix-turn-helix domain	1719
KR	KR domain	1713
Isochorismatase	Isochorismatase family	1644
ABC2_membrane	ABC-2 type transporter	1594
Sigma70_r4_2	Sigma-70 region 4	1568
TetR_N	Bacterial regulatory proteins tetR family	1460
HTH_18	Helix-turn-helix domain	1443
HTH_AraC	Bacterial regulatory helix-turn-helix proteins AraC family	1395
TfuA	TfuA-like protein	1350

Figure S15: Sequence similarity network (SSN) of non-canonical YcaOs. The Enzyme Function Initiative Enzyme Similarity Tool (EFI-EST) was used to generate a SSN for YcaOs belonging to BGCs that did not contain an E1 ubiquitin-activating like protein (previously denoted "C" protein) or an ocin-ThiF-like protein (previously denoted "F" protein), sequences obtained from RODEO were analyzed with a cutoff e-value of 10^{-50} and members with >85% identity were conflated to a single node.[6-8] Visualization of the SSN was performed with Cytoscape 3.0.[9]

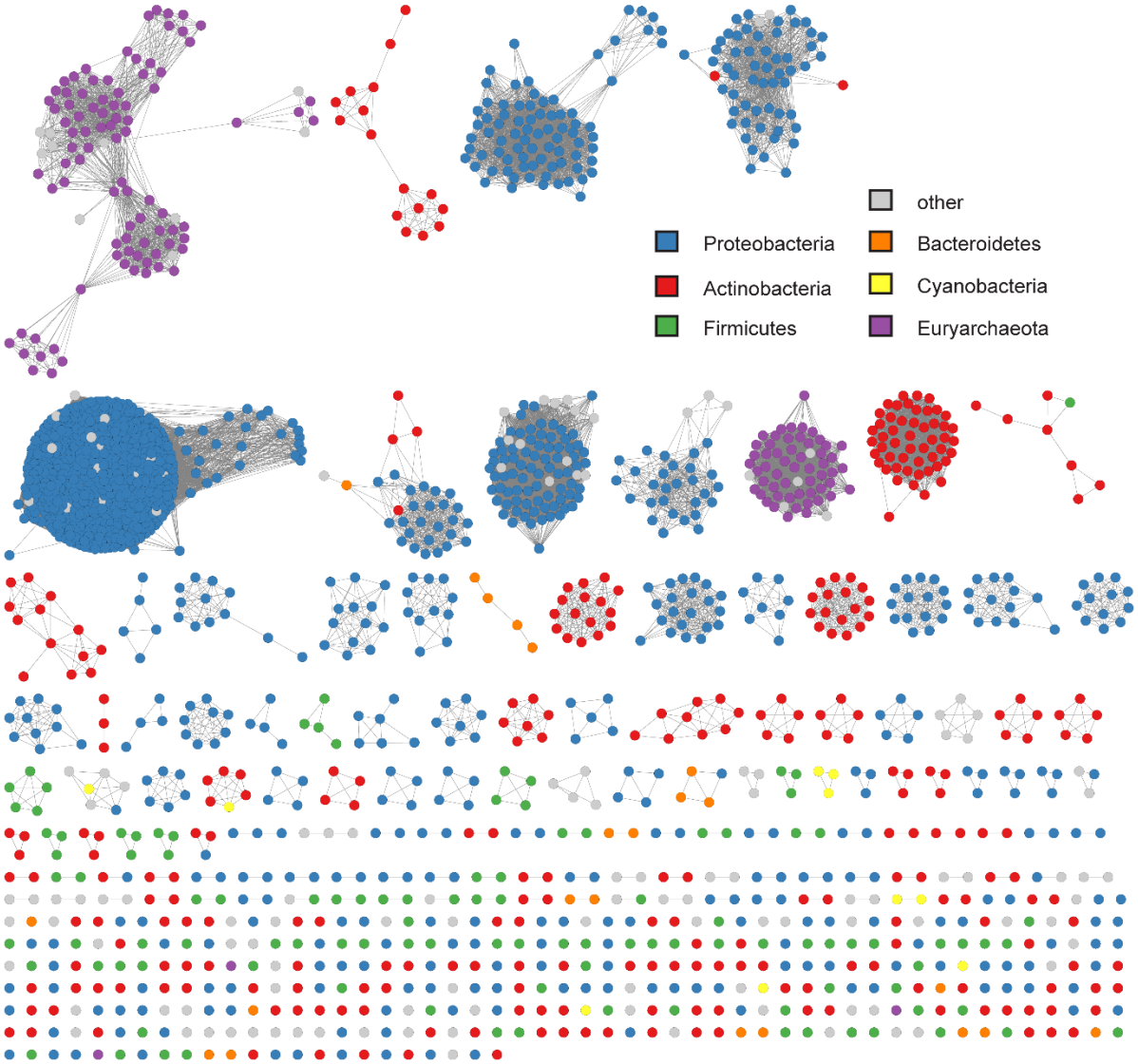


Figure S16: SSN of non-canonical YcaOs related to bottromycin BmbD. The SSN from **Figure S15** recolored to represent similarity to BmbD assessed by HMMER 3.1b1 hmmscan utility against a profile hidden Markov model (pHMM) of bioinformatically-identifiable BmbD sequences.[10] Scores were binned into three groups and mapped to the SSN. The bottromycin BmbD cluster is boxed.

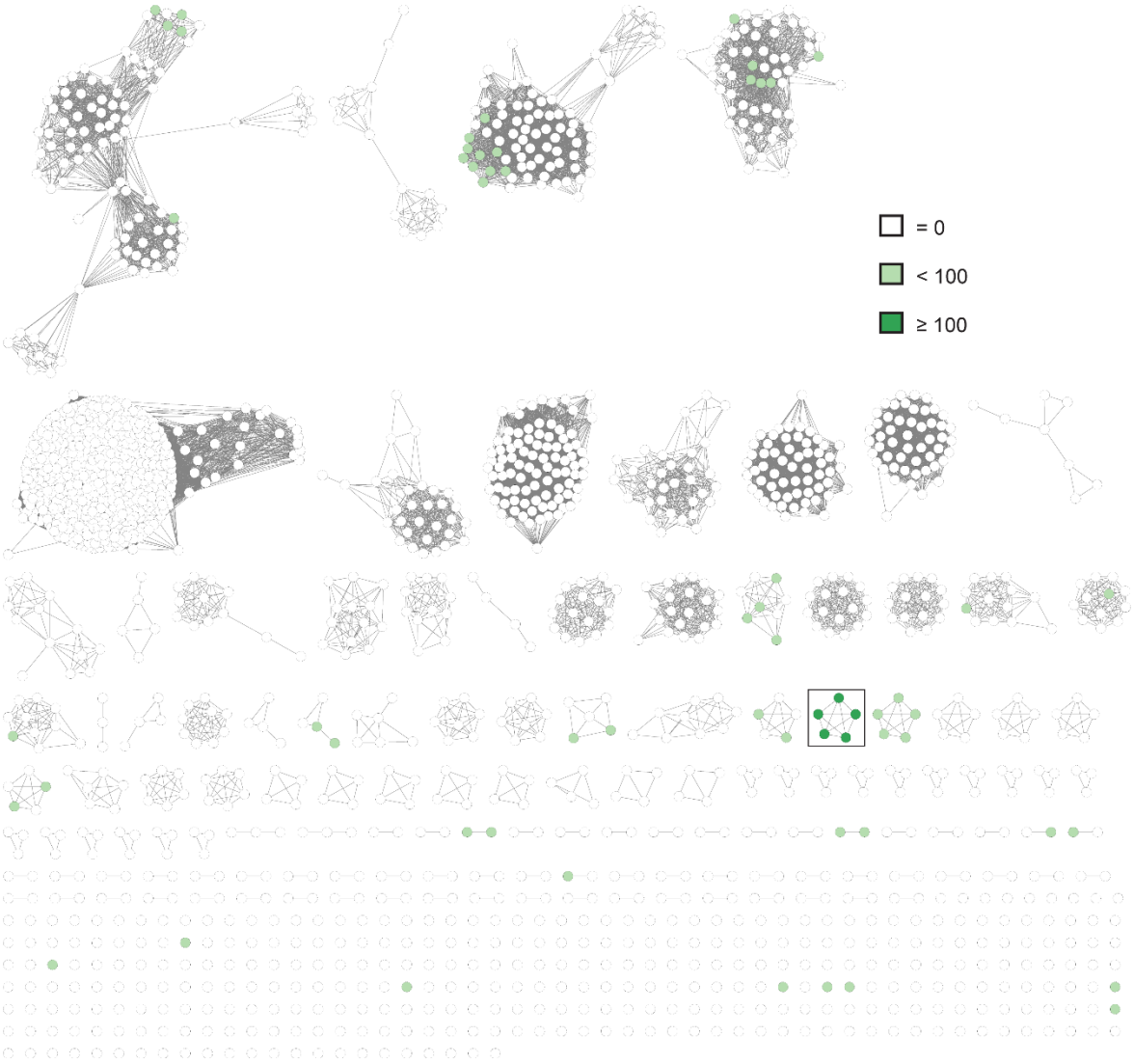


Figure S17: SSN of non-canonical YcaOs related to bottromycin BmbE. The SSN from **Figure S15** recolored to represent similarity to BmbE assessed by HMMER 3.1b1 hmmscan utility against a profile hidden Markov model (pHMM) of bioinformatically-identifiable BmbE sequences.[10] Scores were binned into three groups and mapped to the SSN. The bottromycin BmbE cluster is boxed.

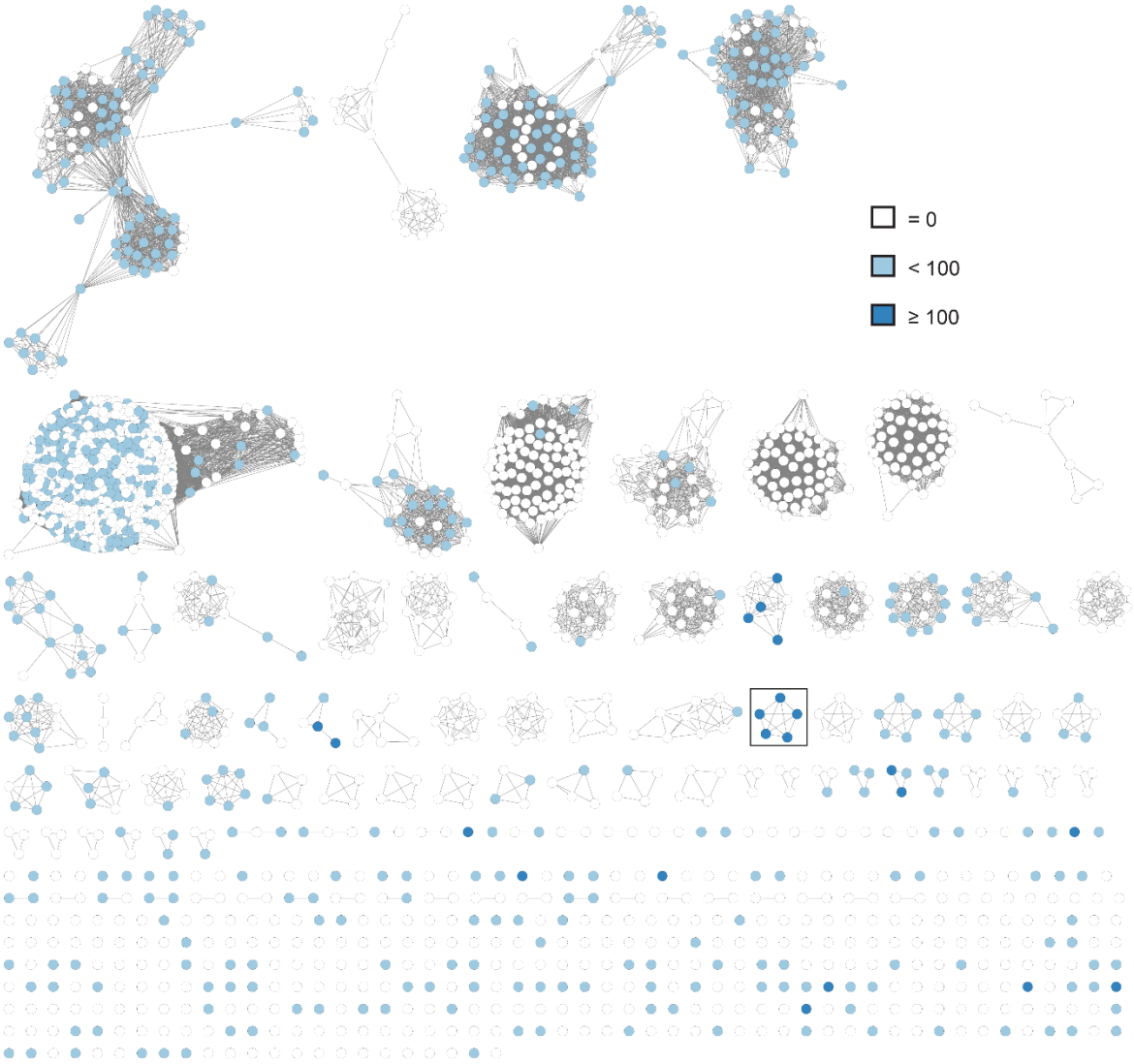
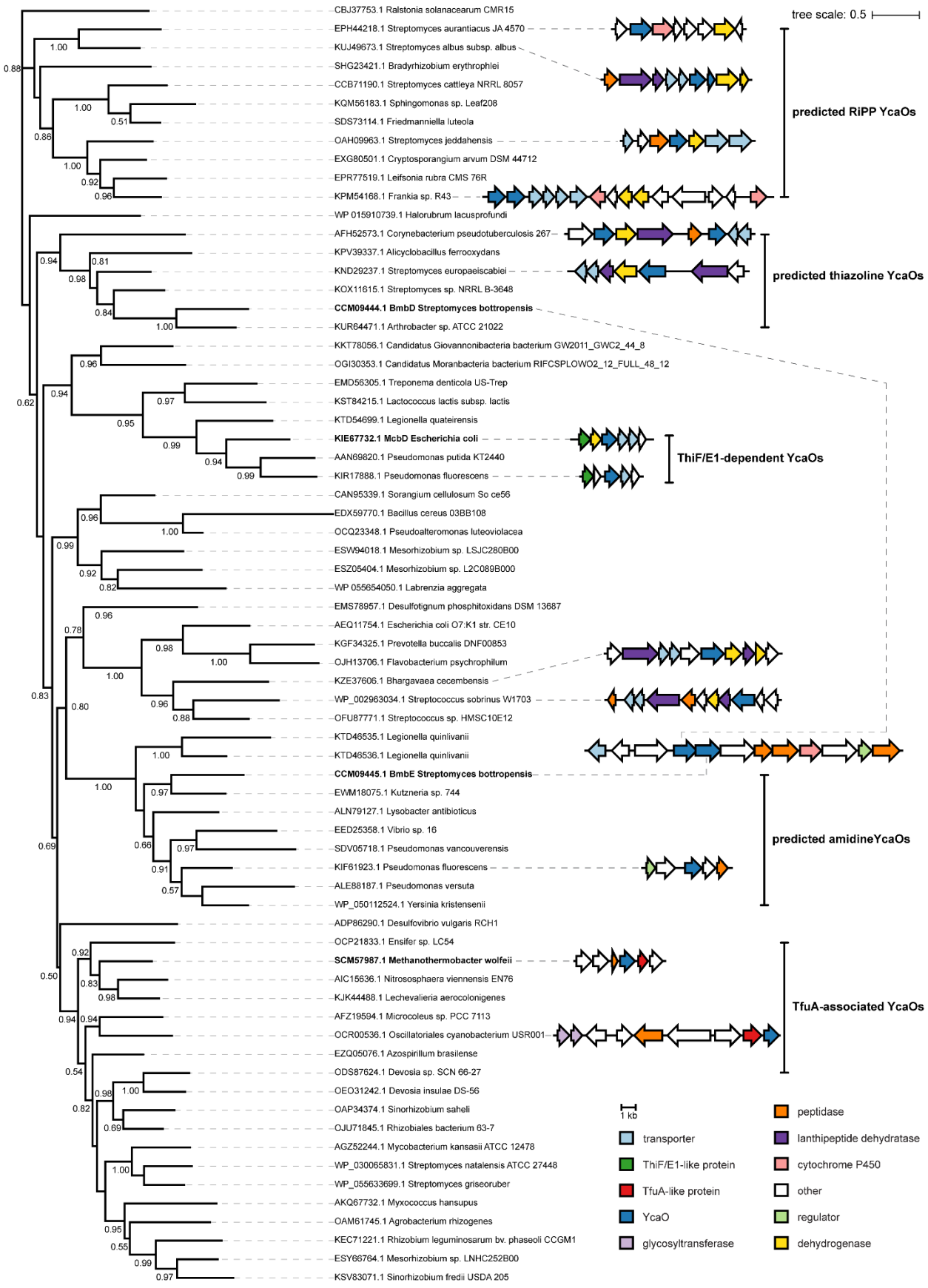


Figure S18: Phylogenetic tree of YcaO subfamilies with representative BGCs. (next page) Unrooted maximum likelihood phylogeny of representative YcaO sequences chosen to represent a diversity of YcaO sequence space and BGC contexts. Representative BGCs likely associated with RiPPs are shown as callouts. Alignments were made using MAFFT v7.222 using the G-INS-I strategy. The phylogenetic tree was created using FastTree v2.1.10 and visualized with iTOL v3 (http://itol.embl.de/).[11,12]


tree scale: 0.5
CBJ37753.1 Ralstonia solanacearum CMR15
EPH44218.1 Streptomyces aurantiacus JA 4570
KUJ49673.1 Streptomyces albus subsp. albus
SHG23421.1 Bradyrhizobium erythrophlei
CCB71190.1 Streptomyces cattleya NRRL 8057
KQM56183.1 Sphingomonas sp. Leaf208
SDS73114.1 Friedmanniella luteola
OAH09963.1 Streptomyces jeddahensis
EXG80501.1 Cryptosporangium arvum DSM 44712
EPR77519.1 Leifsonia rubra CMS 76R
KPM54168.1 Frankia sp. R43
predicted RiPP YcaOs
WP 015910739.1 Halorubrum lacusprofundi
AFH52573.1 Corynebacterium pseudotuberculosis 267
KPV39337.1 Alicyclobacillus ferrooxydans
KND29237.1 Streptomyces europaeiscabiei
KOX11615.1 Streptomyces sp. NRRL B-3648
predicted thiazoline YcaOs
CCM09444.1 BmbD Streptomyces bottropensis
KUR64471.1 Arthrobacter sp. ATCC 21022
KKT78056.1 Candidatus Giovannonibacteria bacterium GW2011_GWC2_44_8
OGI30353.1 Candidatus Moranbacteria bacterium RIFCSPLOWO2_12_FULL_48_12
EMD56305.1 Treponema denticola US-Trep
KST84215.1 Lactococcus lactis subsp. lactis
KTD54699.1 Legionella quateirensis
KIE67732.1 McbD Escherichia coli
AAN69820.1 Pseudomonas putida KT2440
KIR17888.1 Pseudomonas fluorescens
ThiF/E1-dependent YcaOs
CAN95339.1 Sorangium cellulosum So ce56
EDX59770.1 Bacillus cereus 03BB108
OCQ23348.1 Pseudoalteromonas luteoviolacea
ESW94018.1 Mesorhizobium sp. LSJC280B00
ESZ05404.1 Mesorhizobium sp. L2C089B000
WP 055654050.1 Labrenzia aggregata
EMS78957.1 Desulfotignum phosphitoxidans DSM 13687
AEQ11754.1 Escherichia coli O7:K1 str. CE10
KGF34325.1 Prevotella buccalis DNF00853
OJH13706.1 Flavobacterium psychrophilum
KZE37606.1 Bhargavaea cecembensis
WP_002963034.1 Streptococcus sobrinus W1703
OFU87771.1 Streptococcus sp. HMSC10E12
KTD46535.1 Legionella quinlivanii
KTD46536.1 Legionella quinlivanii
CCM09445.1 BmbE Streptomyces bottropensis
EWM18075.1 Kutzneria sp. 744
ALN79127.1 Lysobacter antibioticus
EED25358.1 Vibrio sp. 16
SDV05718.1 Pseudomonas vancouverensis
KIF61923.1 Pseudomonas fluorescens
ALE88187.1 Pseudomonas versuta
WP_050112524.1 Yersinia kristensenii
predicted amidineYcaOs
ADP86290.1 Desulfovibrio vulgaris RCH1
OCP21833.1 Ensifer sp. LC54
SCM57987.1 Methanothermobacter wolfeii
AIC15636.1 Nitrososphaera viennensis EN76
KJK44488.1 Lechevalieria aerocolonigenes
AFZ19594.1 Microcoleus sp. PCC 7113
OCR00536.1 Oscillatoriales cyanobacterium USR001
EZQ05076.1 Azospirillum brasilense
ODS87624.1 Devosia sp. SCN 66-27
OEO31242.1 Devosia insulae DS-56
OAP34374.1 Sinorhizobium saheli
OJU71845.1 Rhizobiales bacterium 63-7
AGZ52244.1 Mycobacterium kansasii ATCC 12478
WP_030065831.1 Streptomyces natalensis ATCC 27448
WP_055633699.1 Streptomyces griseoruber
AKQ67732.1 Myxococcus hansupus
OAM61745.1 Agrobacterium rhizogenes
KEC71221.1 Rhizobium leguminosarum bv. phaseoli CCGM1
ESY66764.1 Mesorhizobium sp. LNHC252B00
KSV83071.1 Sinorhizobium fredii USDA 205
TfuA-associated YcaOs
1 kb
transporter
ThiF/E1-like protein
TfuA-like protein
YcaO
glycosyltransferase
peptidase
lanthipeptide dehydratase
cytochrome P450
other
regulator
dehydrogenase

Supplemental References

Full citation for reference 1: Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K.-D.; Fischbach, M. A.; Garavelli, J. S.; Goeransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Mueller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Rebuffat, S.; Ross, R. P.; Sahl, H.-G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Suessmuth, R. D.; Tagg, J. R.; Tang, G.-L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. *Nat. Prod. Rep.* **2013**, 30, 108.

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