Angewandte
Eine Zeitschrift der Gesellschaft Deutscher Chemiker
Chemie

Supporting Information

Dissecting Bottromycin Biosynthesis Using Comparative Untargeted Metabolomics

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SUPPORTING INFORMATION

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1. Supplementary Methods

1.1 Materials

All chemicals were analytical grade and obtained from Sigma Aldrich unless otherwise specified. Antibiotics were used at final concentrations as follows: kanamycin was used at 50 μg mL^{-1}, apramycin at 50 μg mL^{-1}, carbenicillin at 30 μg mL^{-1}, chloramphenicol at 25 μg mL^{-1} and nalidixic acid at 25 μg mL^{-1}.

1.2 Bacterial strains

Spores of *Streptomyces scabies* DSM 41658 were prepared from cultures on instant potato mash agar (20 g L^{-1} Smash (Premier Foods), 20 g L^{-1} agar) and were stored as spore suspensions in 20% glycerol at -20 °C. *E. coli* NovaBlue competent cells (Novagen) were used for genetic manipulation, and the methylation deficient strain of *E. coli,* ET12657 was used[1] for transfer of genetic material to *S. scabies* by conjugation. *E. coli* cells transformed with plasmids were stored in a 30% glycerol stock at -80 °C. The preparation of *S. scabies ΔbtmC*, *ΔbtmD*, *ΔbtmE*, *ΔbtmF*, *ΔbtmG*, *ΔbtmI* and *ΔbtmJ* has been previously described[2].

1.3 Complementation plasmids

Complementation plasmids were generated using the integrative vector pIB139, which contains the strong constitutive *ermE** promoter. For *ΔbtmD* complementation, the *btmD* gene was amplified using primers bottro-rev (EcoRI) and btmD-RBS (AseI), which adds a ribosome binding site (RBS) and an NdeI site to the 5' end of *btmD*. This was digested with AseI/EcoRI and ligated into NdeI/EcoRI-treated pIB139 to generate pIB139-RBS-*btmD*. All other pIB139-RBS vectors were generated from this construct by digestion with NdeI/EcoRI to remove the *btmD* gene and replacing this with *btm* genes amplified using the primers in Table S1. Plasmids were transformed into *E. coli* ET12657 containing the pUZ8002 plasmid, which was then used for conjugation into *S. scabies* as previously described[2].

1.4 *Streptomyces scabies* fermentation conditions

Triplicate cultures were prepared by inoculating 25 mL GYM medium (0.4% glucose, 0.4% yeast extract, 1.0% malt extract, in Milli-Q (MQ) water) with 50 μL of a *S. scabies* spore suspension in a 250 mL flask and incubating at 30 °C, 250 rpm for 48 hours. Seed culture (200 μL) was then used to inoculate 10 mL production medium (PM: 1% glucose, 1.5% starch, 0.5% yeast extract, 1.0% soy flour, 0.5% NaCl, 0.3% $CaCO_3$, 25 μg mL^{-1} $CoCl_2$ in MQ water) in 50 mL conical centrifuge tubes with the caps replaced with foam bungs. These were incubated at 28 °C, 230 rpm for 72 hours. An equal volume of acetonitrile (MeCN) was added to each sample and the mixture was vortexed then centrifuged for 5 minutes at 13,000 rpm. The time course experiment was carried out in triplicate by transferring 1 mL seed culture to 50 mL PM in 250 mL flasks with springs and incubating at 28 °C with shaking at 230 rpm. Samples were taken at 24 hour intervals

during 10 days and stored frozen until their analysis. Co-cultures involved transferring 0.5 mL seed culture from each mutant strain into 50 mL PM and fermenting the culture as described above for 6 days.

1.5 RT-PCR analysis

Both WT *S. scabies* and *ΔbtmD* were grown in bottromycin production medium following the procedure described above. After 72 h, 2 mL culture samples of each strain were collected, washed with an equal volume of RNA*later*® (ThermoFisher Scientific) and cell pellets were stored at -80 °C until processing. RNA was then extracted according to published procedures[3,4]. The mycelium was resuspended in 1 ml of RLT buffer from the RNeasy kit (Qiagen) and then transferred to lysing matrix B tubes (MP Biomedicals) to undergo mechanical lysis using a FastPrep instrument (Bio101). The lysis program consisted of 3 pulses of 30 s at 6.0 m/s with 1 min cooling intervals on ice. After centrifuging the lysed samples for 10 min at 13,000 rpm the supernatants were transferred to fresh tubes and treated following the instructions of the RNeasy Kit. Possible DNA contaminating the samples was removed twice, once with the on-column DNAseI treatment from Qiagen and a second time after elution with TURBO DNA-*free* Kit (Ambion, Invitrogen). The resulting RNA was quantified using a NanoDrop, and approximately 250 ng of each sample were used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen), following manufacturer's instructions. The obtained cDNA was used as a template in PCRs with Taq polymerase and specific primers used to assess the expression of relevant genes in the different operons of the *btm* gene cluster (Table S1).

1.6 LC-MS analysis of extracts

Spectra were obtained using a Shimadzu Nexera X2 UHPLC coupled to a Shimadzu IT-TOF mass spectrometer. Samples (5 µL) were injected onto a Phenomenex Kinetex 2.6 µm XB-C18 column (50 mm x 2.1 mm, 100 Å) set at a temperature of 40 °C, eluting with a linear gradient of 5 to 95% acetonitrile in water + 0.1% formic acid (FA) over 6 minutes with a flow-rate of 0.6 mL min^{-1}. Positive mode mass spectrometry data was collected between *m/z* 200 and 2000, and MS^2 data was collected in a data-dependent manner using collision-induced dissociation of the most abundant singly charged species in a scan, with an exclusion time of 0.8 seconds.

Untargeted comparative metabolomics was carried out on triplicate data using Profiling Solution 1.1 (Shimadzu) with an ion m/z tolerance of 100 mDa, a retention time (RT) tolerance of 0.1 min and an ion intensity tolerance of 100,000 units. The data was filtered by removing all species that appeared in either *ΔbtmD* or unmodified production medium. Any species with a p-value >0.002 were omitted and the matrix only displays data from 0.5 – 4 min (Figure S5). Triplicate LC-MS^2 data was used to construct mass spectral networks using the Global Natural Products Social Molecular Networking server (GNPS, http://gnps.ucsd.edu). The following settings were used for analysis: parent mass tolerance = 1 Da, ion tolerance = 0.5 Da, minimum pair cosine = 0.6, minimum matched peaks = 3, minimum cluster size = 2, minimum peak intensity = 25. Network data was visualized and manually cropped in Cytoscape 2.8.3.

Manual cropping consisted of removal of nodes with an RT < 30 s, removal of $[M+Na]^+$ nodes when a corresponding $[M+H]^+$ node is observed, removal of any nodes erroneously duplicated by GNPS, and removal of any nodes that resulted from fewer than 3 spectra. Edges between nodes were sized according to similarity (a linear gradient of 1 to 20 points between cosine 0.6 and 1). The peak areas of extracted ion chromatograms for each node related to bottromycin were quantified in triplicate and imported into Cytoscape (areas were only reported if they appeared in every triplicate sample for a given mutant). The square root of these areas was used to define node diameter (√area = 0, 20 points; √area = maximum (7613), 120 points). A node for 406.27 (identified by Profiling Solution) was manually inputted into the network as an unconnected node.

Additional high-resolution mass spectra were acquired on a Synapt G2-Si mass spectrometer (Waters) operated in positive mode with a scan time of 1.5 s in the mass range of m/z 100-1200. Samples (5 µL) were injected onto an Acquity BEH C18 reversed phase column (1.7 µm, 2.1 x 50 mm, Waters) and eluted with a gradient of 0-90% acetonitrile in water (+ 0.1% formic acid) over a time of 11 min with a flow rate of 0.4 mL/min. Capillary voltage was 0.5 V, cone voltage 30 V, source temperature 100°C, desolvation temperature 250°C. Leu-enkephalin peptide was used to generate a dual lock-mass calibration with *m/z*= 278.1135 and *m/z*=556.2766 measured every 10 s during the run.

1.7 Protein expression

The *btmM* gene was amplified from genomic DNA using primers btmM-start and btmM-end. Following digestion with NdeI and EcoRI, the gene was ligated into NdeI/EcoRI digested pET28(a)+ (Novagen) to generate a construct for the expression of N-terminally His_6-tagged BtmM. pET28-*btmM* was transformed into *E. coli* BL21(DE3) (Novagen), which was were grown in LB medium (1 L) containing kanamycin (50 mg/mL) with shaking at 37 °C until the OD_{600} reached approximately 0.6. Isopropyl-b-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 15 µM and cell growth was continued with shaking at 25 °C for 48 h at 200 rpm. The cells were then harvested by centrifugation and the resulting cell pellet was resuspended in 30 mL binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9, 10% glycerol) and lysed by sonication. The resulting cell lysate was clarified by centrifugation and BtmM-His_6 was purified by immobilized nickel-affinity chromatography at 4 °C. The protein eluate was desalted using an Amicon Ultra centrifugal filter (Millipore, 30 kDa MWCO) and the buffer was exchanged to 50 mM Tris-HCl pH 8, 500 mM NaCl. Glycerol was added to 40% and aliquots were frozen in liq. N_2 and stored at -80 °C. Protein identity was confirmed by LC-MS analysis using a Hewlett-Packard HPLC 1100 series instrument coupled to a Thermo Finnigan LCQ ion trap mass spectrometer: BtmM observed mass = 51,360 (predicted mass after loss of N-formyl-methionine = 51,369 Da). BtmB was cloned and expressed in an analogous way to BtmM using primers btmB-start and btmB-end. The procedure was modified by inducing expression using 200 µM IPTG and then shaking at 16 °C for 16 h at 220 rpm. Additionally, a 10 kDa MWCO Amicon filter was used for buffer exchange.

The *btmD* gene was amplified from genomic DNA using primers bottro-for and btmD_end_vector. The btmD_end_vector primer was designed to remove the *btmD* stop codon and introduce sequence to code for a thrombin cleavage site before a HindIII site. Following digestion with NdeI and HindIII, the gene was ligated into NdeI/HindIII digested pET29 (Novagen) to generate a construct for the expression of *C*-terminally His_6-tagged BtmD with the following sequence:
MGPVVVFDCMTADFLNDDPNNAELSALEMEELESWGAWDGEATSLVPRGSKLAAALEHHHHHH.

pET29-*btmD* was transformed into *E. coli* BL21(DE3) (Novagen), which was then grown in LB medium (1 L) containing kanamycin (50 mg/mL) with shaking at 37 °C until the OD_{600} reached approximately 0.6. IPTG was then added to a final concentration of 0.5 mM and cell growth was continued with shaking at 37 °C for 3 h at 250 rpm. The protein was then purified as described for BtmM, but instead used a 3 kDa MWCO Amicon filter. Protein identity was confirmed by LC-MS (Figure S24).

1.8 BtmM assays with BtmD

BtmM was incubated with BtmD using a variety of concentrations and conditions. For a comparison of conditions for activity. 6.4 μM BtmM was tested in 50 mM Tricine pH 9 at 30 °C for 3 hours with the following variables: (i) no added divalent metals, (ii) 32 μM $ZnCl_2$, (iii) 32 μM $CoCl_2$, (iv) 320 μM $ZnCl_2$, (v) 5 mM TCEP and 32 μM $ZnCl_2$. Assays were analyzed using a Thermo Finnigan Surveyor HPLC system coupled to a Thermo Finnigan LCQ Deca ion trap mass spectrometer. Samples were injected onto a Phenomenex Aeris WIDEPORE 3.6 μm C4 column (100 mm x 2.1 mm, 200 Å) set at a temperature of 30 °C, eluting with a linear gradient of 10 to 90% acetonitrile (+ 0.1% trifluoroacetic acid, TFA) in water (+ 0.1% TFA) over 14 minutes with a flow-rate of 0.35 mL min^{-1}.

1.9 Production of *O*-desmethyl bottromycin

The methyl ester of bottromycin was hydrolyzed using LiOH, according to the protocol of Kobayashi *et al.*[5]. THF (4.5 mL) was added to bottromycin A_2 purified from a *S. scabies* culture (175 mL fermentation volume). 500 μL of this was mixed with 500 μL of 1 M LiOH, and the mixture stirred at room temperature for 4 h. This was quenched with 500 μL of 1 M HCl, and the THF was removed by evaporation under reduced pressure. The remaining solution (500 μL) was subjected to purification by preparative HPLC in order to separate hydrolyzed and intact bottromycin, which separate by ~1.5 min in the HPLC conditions (below). The fractions containing the hydrolyzed bottromycin were pooled, freeze-dried and dissolved in H_2O (500 μL). Preparative HPLC was carried out using a Phenomenex Luna 10 μm C18(2) 250 mm x 21.2 mm column connected to an Agilent 1200 series HPLC eluting with a linear gradient of 5 to 95% acetonitrile in water over 40 minutes with a flow-rate of 20 mL min^{-1}.

1.10 BtmB assays

O-desmethyl bottromycin A_2 (25 μL) was mixed with BtmB (5 μM), *S*-adenosyl methionine (SAM) (2 mM) in a final volume of 100 μL in 5 mM Tris-HCl pH 7.5. The full reaction, along with control reactions without either hydrolyzed bottromycin, SAM, or BtmB were incubated at 30 °C for 30 min. The reactions were quenched with an equal volume of methanol, and the samples were analyzed by LC-MS using the same method as described in Crone *et al*.[2]. This LC-MS method provides different ionization parameters and retention times to the other data reported, and is shown in Figure S27.

1.11 Isomerisation of 13 produced by *S. scabies ΔbtmJ*

A 5-day production culture of *S. scabies ΔbtmJ* (400 mL PM) was mixed with MeOH (400 mL) for 20 minutes. The resulting methanolic supernatant was concentrated to 50 mL by rotary evaporation and 50 μL of this solution was purified using a Shimadzu LCMS-2020 eluting with a linear gradient of 5 to 40% acetonitrile in water (+ 0.1% FA) over 20 minutes with a flow-rate of 3.5 mL min^{-1} on a Phenomenex Luna 5μm C18 column (250 x 10 mm, 100 Å). To avoid rapid thiazoline hydrolysis of **13**, fractions were collected in tubes containing Tris-HCl to provide a final concentration of 100 mM Tris-HCl with pH 7.5. This buffered solution was then incubated at room temp. and then analysed on the LCMS-2020 using a Phenomenex Kinetex 2.6 μm XB-C18 column (50 mm x 2.1 mm, 100 Å) eluting with a linear gradient of 5 to 95% acetonitrile in water (+ 0.1% FA) over 6 minutes. This result is shown in Figure S11.

1.12 Deuterium labelling of hydrolysed 13

100 uL of a crude extract of **13** was dried *in vacuo* (GeneVac miVac DUO), resuspended in D_2O (100 uL) and incubated at room temperature for 48 hours. 2 uL of DCl (2M in D_2O) was then added and the acidified sample was incubated at room temperature for 2 hours. This was dried *in vacuo* again, resuspended in H_2O (100 uL) and incubated at room temperature for a further 2 hours to facilitate proton back exchange. An equivalent non-deuterium labelled compound was prepared in H_2O using 0.1% formic acid (hydrolysis occurred spontaneously following HPLC purification containing 0.1% FA when the acid was not neutralised). These samples were then analysed by $LC\text{-}MS^2$ using a Shimadzu IT-TOF as described in section 1.6. The results are shown in Figures S12 and S13.

2. Supplementary Tables

Table S1 Primers used in this study.

Primer Name	Primer Sequence (5'→3')	Restriction site
btmB-start	GAGGCGAGAGGTCATATGAAGATTTCCC	NdeI
btmB-end	CTGGCGCCTCGGAATTCCTGGCG	EcoRI
btmC-start	CGGGGGTTGATCATATGCCTACCGCCA	NdeI
btmC-end	GCGCTCTTCTGAATTCCGGCGCTC	EcoRI
bottro-for	GACCACCATATGGGACCCGTAGTCG	NdeI
bottro-rev	CGGCGAATTCTCATGAGGTGGCTTC	EcoRI
btmE-start	GGGAGAGGGCATATGCGCGAAGCG	NdeI
btmE-end	CTGCCGGAATTCCGTCTCGCGCTC	EcoRI
btmF-start	AGGAGGACATATGACCCGTCCCGC	NdeI
btmF-end	ACAGGGAATTCCAGGGCGGTGCCAC	EcoRI
btmG-start	CGGGGGTGCGCGCATATGAAGCTC	NdeI
btmG-end	ACCACCTCGAAGCTTTCCGTCGCAC	HindIII
btmI-start	ACGGCGGGGGCCATATGACGGCCA	NdeI
btmI-end	GTCCGGTTCCGCGGGGAATTCCGC	EcoRI
btmJ-start	GGCCCGCTGCCATATGGACTTCGAC	NdeI
btmJ-end	GTTTCGGAATTCGCGCCCTCACCAG	EcoRI
btmM-start	GCGTATGACCATATGACCCGGGTCGT	NdeI
btmM-end	CGCGGCGGAATTCCCGGGCCTAC	EcoRI
btmD_end_vector	ATAAAGCTTGGAACCGCGTGGCACCAGTGAGGTGGCT TCACCGTCCCAGGC	HindIII
btmD_RBS	CAGAATTAATAAAGGAGGACCACCATATGGGACCCG TAGTCG	AseI, NdeI
RT_hrdB_fw	GGACCTTGCCGATCTGCTTGA	
RT_hrdB_rv	GGGGAAAGGCTGAGGGGCA	
qRT-A_fw	GATGTCCGGTGCGAGGGCT	
qRT-A_rv	CCGTCGTCTGGTTCGTCGTCC	
qRT-B_fw	CGATCAGCCGCAACCCG	
qRT-B_rv	ACCCGCTCCCTGACCCTCTT	
qRT-C_fw2	GCAACTGCGAGGGTTCCATG	
qRT-C_rv2	GTGGAGGAGGGTGACGAAGG	
qRT-D_fw2	GTATTCGACTGCATGACCGC	
qRT-D_rv2	CCAGGACTCCAGCTCCTCCA	
qRT-E_fw	ACCCGTCTCTGCTTCGCCC	
qRT-E_rv	GGTCCTCCTCACTTCTCCCGT	
qRT-L_fw2	CAGAGGCCAAGGTCAACGC	
qRT-L_rv2	CAGCAGCTCCTCGTCCAC	

Table S2 Plasmids used in this study.

Name	Source	Resistance Marker	Function
pUZ8002	[6]	Kanamycin	Facilitates transfer of genetic material by conjugation
pIB139-RBS-*btmC*	This study	Apramycin	Complementation of *ΔbtmC*
pIB139-RBS-*btmD*	This study	Apramycin	Complementation of *ΔbtmD*
pIB139-RBS-*btmE*	This study	Apramycin	Complementation of *ΔbtmE*
pIB139-RBS-*btmF*	This study	Apramycin	Complementation of *ΔbtmF*
pIB139-RBS-*btmI*	This study	Apramycin	Complementation of *ΔbtmI*
pIB139-RBS-*btmJ*	This study	Apramycin	Complementation of *ΔbtmJ*
pET28-*btmM*	This study	Kanamycin	Expression of *N*-terminally His_6-tagged BtmM
pET29-*btmD*	This study	Kanamycin	Expression of *C*-terminally His_6-tagged BtmD
pET28-*btmB*	This study	Kanamycin	Expression of *N*-terminally His_6-tagged BtmB

Table S3 MS data for bottromycin-like metabolites. All data from wild type *S. scabies* unless otherwise stated.

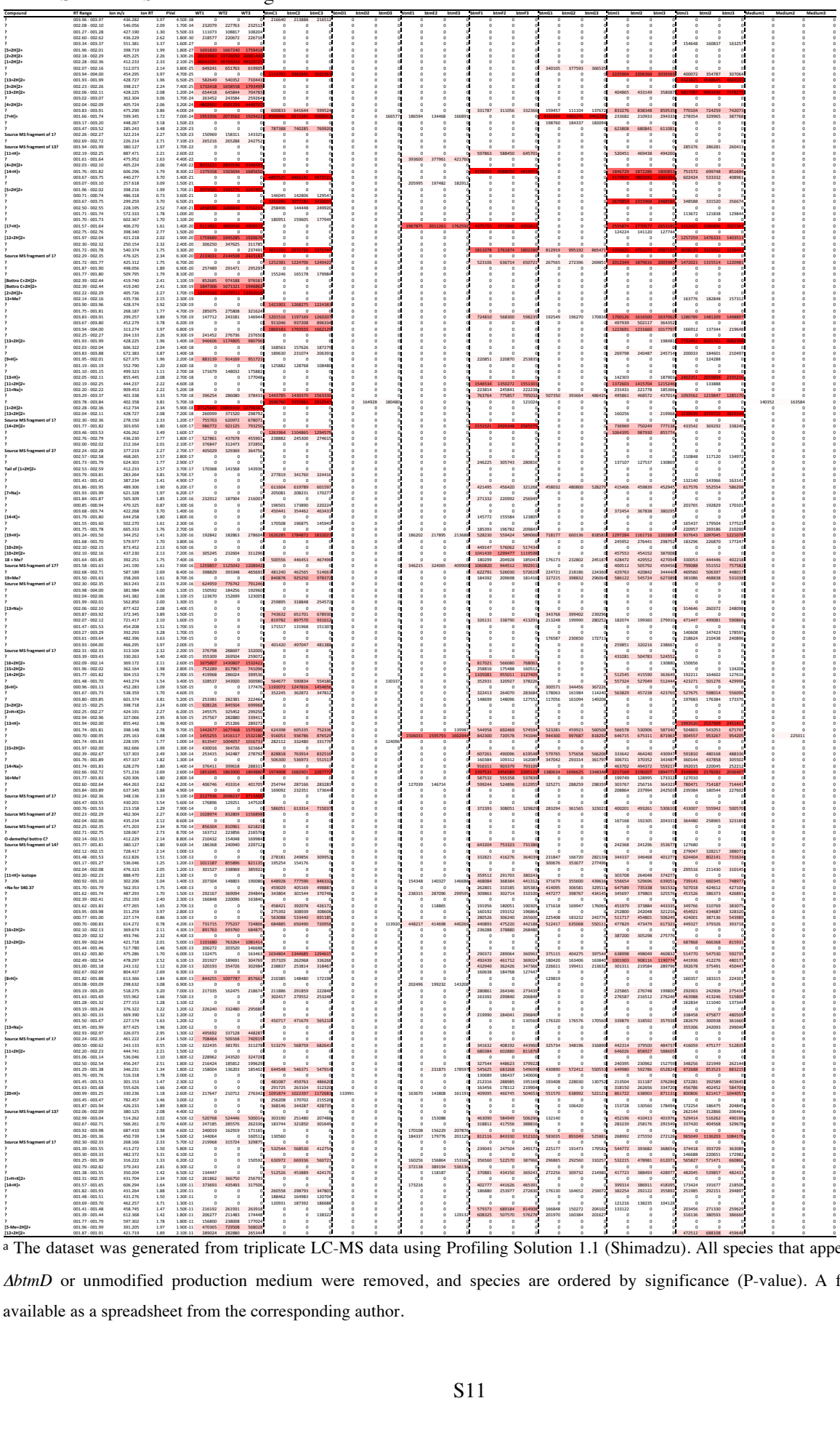
Compound	Calc. $[M+H]^+$	Shimadzu IT-TOF		Synapt G2-Si	
		Obs. *m/z*	Error (ppm)	Obs. *m/z*	Error (ppm)
1 (bottromycin A_2)	823.4535	823.4523	-1.46	823.4525	-1.21
2 (bottromycin B_2)	809.4378	809.4365	-1.61	809.4384	0.74
3	795.4222	795.4216	-0.75	795.4221	-0.13
4 (*O*-desmethyl bottromycin A)	809.4378	809.4377	-0.12	809.4380	0.25
5 (*O*-desmethyl bottromycin B)	795.4222	795.4196	-3.27	795.4219	-0.38
6 (from *ΔbtmC*)	452.2867	452.2862	-1.11	452.2864	-0.66
7	599.3552	599.3546	-1.00	599.3544	-1.33
8	613.3708	613.3686	-3.59	613.3691	-2.77
9	627.3865	627.3857	-1.28	627.3845	-3.19
10 (from *ΔbtmF*)	873.4539	873.4500	-4.47	873.4551	1.37
11 (from *ΔbtmF*)	887.4695	887.4696	0.11	887.4671	-2.70
12 (from *ΔbtmJ*)	841.4277	841.4247	-3.57	841.4288	1.31
13 (from *ΔbtmJ*)	855.4433	855.4411	-2.57	855.4441	0.94
14	606.2956	606.2960	0.66	606.2966	1.65
15	723.3204	723.3229	3.46	723.3195	-1.24
16	737.3361	737.3365	0.54	737.3375	1.90
17	406.2699	406.2700	0.25	406.2697	-0.49
18 (from *ΔbtmC*)	378.2387	378.2381	-1.59	378.2388	0.26
19 (from *ΔbtmC*)	344.2544	344.2529	-4.36	344.2547	0.87
20a/b (from *ΔbtmC*)	330.2387	330.2377	-3.03	330.2391	1.21

Table S4 Revised bottromycin gene cluster data.

Gene name	*btm* name	Protein family classification	Proposed function
SCAB_56711*	*btmA*	Major facilitator superfamily	Host immunity by export of mature bottromycin
SCAB_56701*	*btmB*	Methyltransferase	*O*-methylation of Asp7
SCAB_56691	*btmC*	Class B radical SAM methyltransferase	β-methylation of Phe6
SCAB_56681	*btmD*	-	Precursor peptide
SCAB_56671	*btmE*	YcaO domain protein	Role in thiazoline formation
SCAB_56661	*btmF*	YcaO domain protein	Role in macrocyclic amidine formation
SCAB_56651	*btmG*	Class B radical SAM methyltransferase	β-methylation of Val4 and Val5
SCAB_56641	*btmH*	α/β hydrolase	Hydrolysis of the follower peptide
SCAB_56631	*btmI*	Metal-dependent hydrolase	Role in macrocyclic amidine formation
SCAB_56621	*btmJ*	Cytochrome P450	Oxidative decarboxylation to convert thiazoline into thiazole
SCAB_56611	*btmK*	Class B radical SAM methyltransferase	β-methylation of Pro2
SCAB_56601	*btmL*	DUF2087	Transcriptional regulator
SCAB_56591	*btmM*	M17 aminopeptidase	Hydrolysis of the *N*-terminal methionine of BtmD

* Genes on opposite strand

Table S5 Selected untargeted metabolomic data across all strains[a].



[a] The dataset was generated from triplicate LC-MS data using Profiling Solution 1.1 (Shimadzu). All species that appeared in either *ΔbtmD* or unmodified production medium were removed, and species are ordered by significance (P-value). A full dataset is available as a spreadsheet from the corresponding author.

2. Supplementary Figures

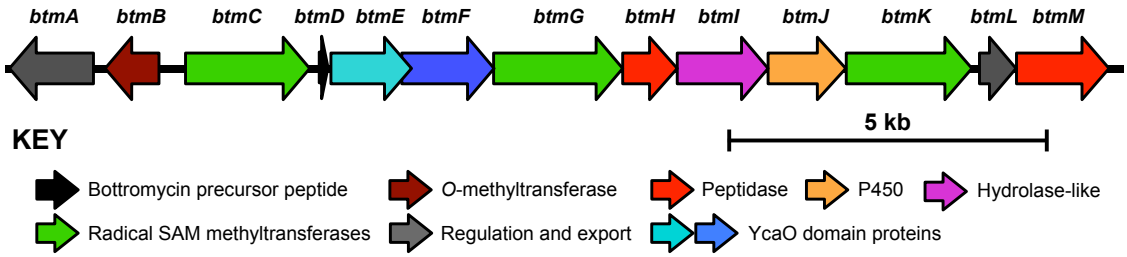


Figure S1 Bottromycin gene cluster in *S. scabies*.

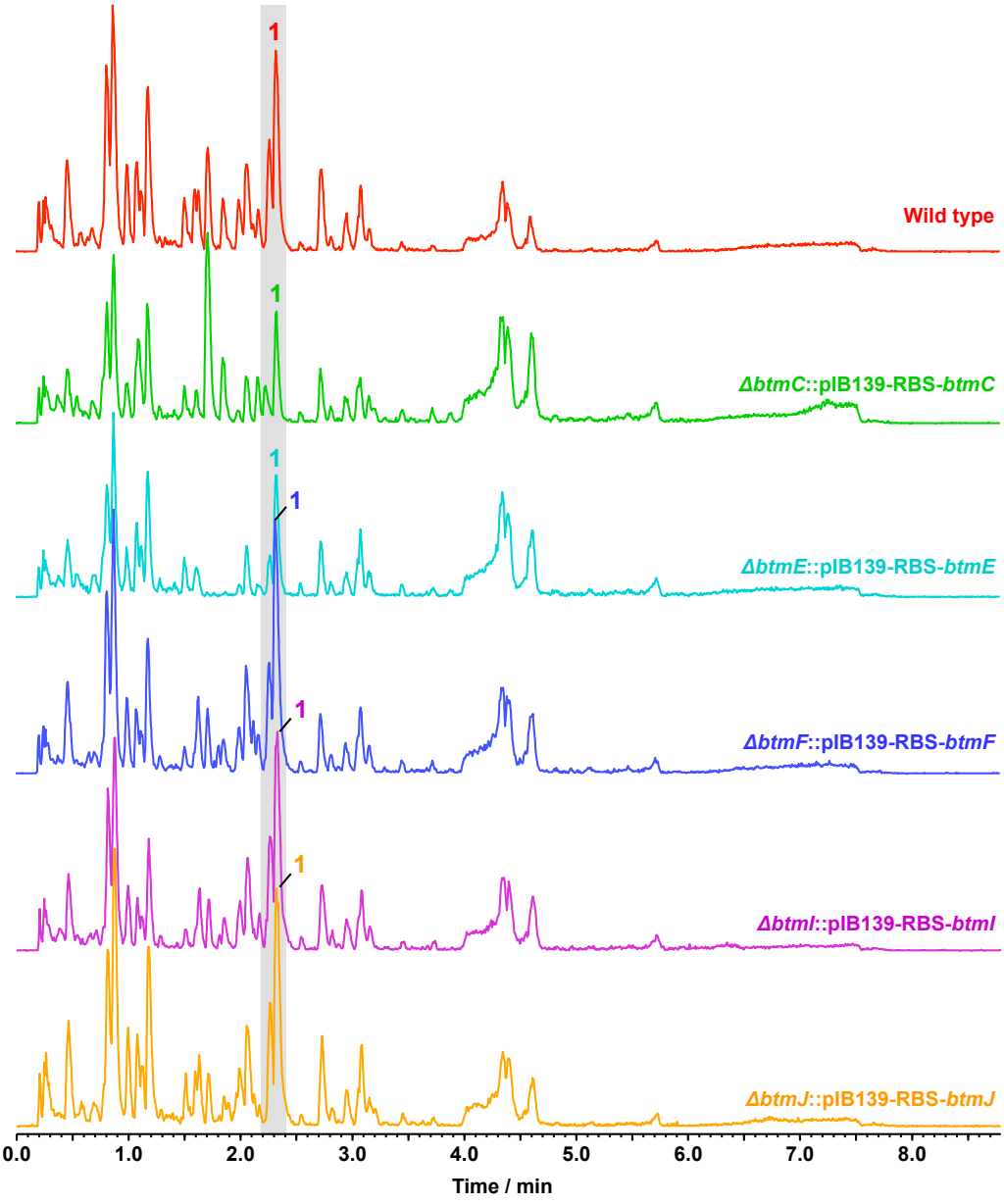


Figure S2 MS base peak chromatograms (BPCs) for all complemented strains compared to wild type *S. scabies* after 7 days in production medium. Bottromycin A_2 (**1**) production is highlighted.

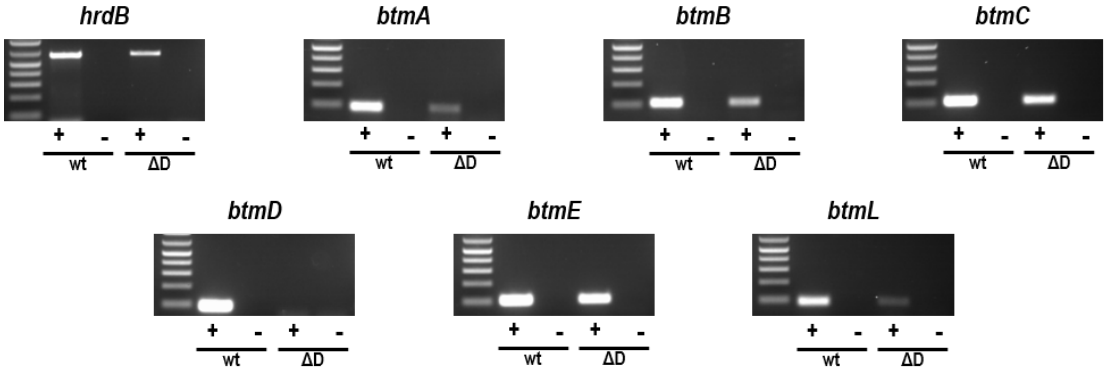


Figure S3 RT-PCR analysis of the wild type (WT) and *ΔbtmD* (ΔD) strains. Expression of several genes of the *btm* cluster (*A*, *B*, *C*, *D*, *E* and *L*) was assessed in 72h cultures of these strains, including negative controls (-) to check possible DNA contamination in the samples, and using *hrdB* as a positive control for gene expression.

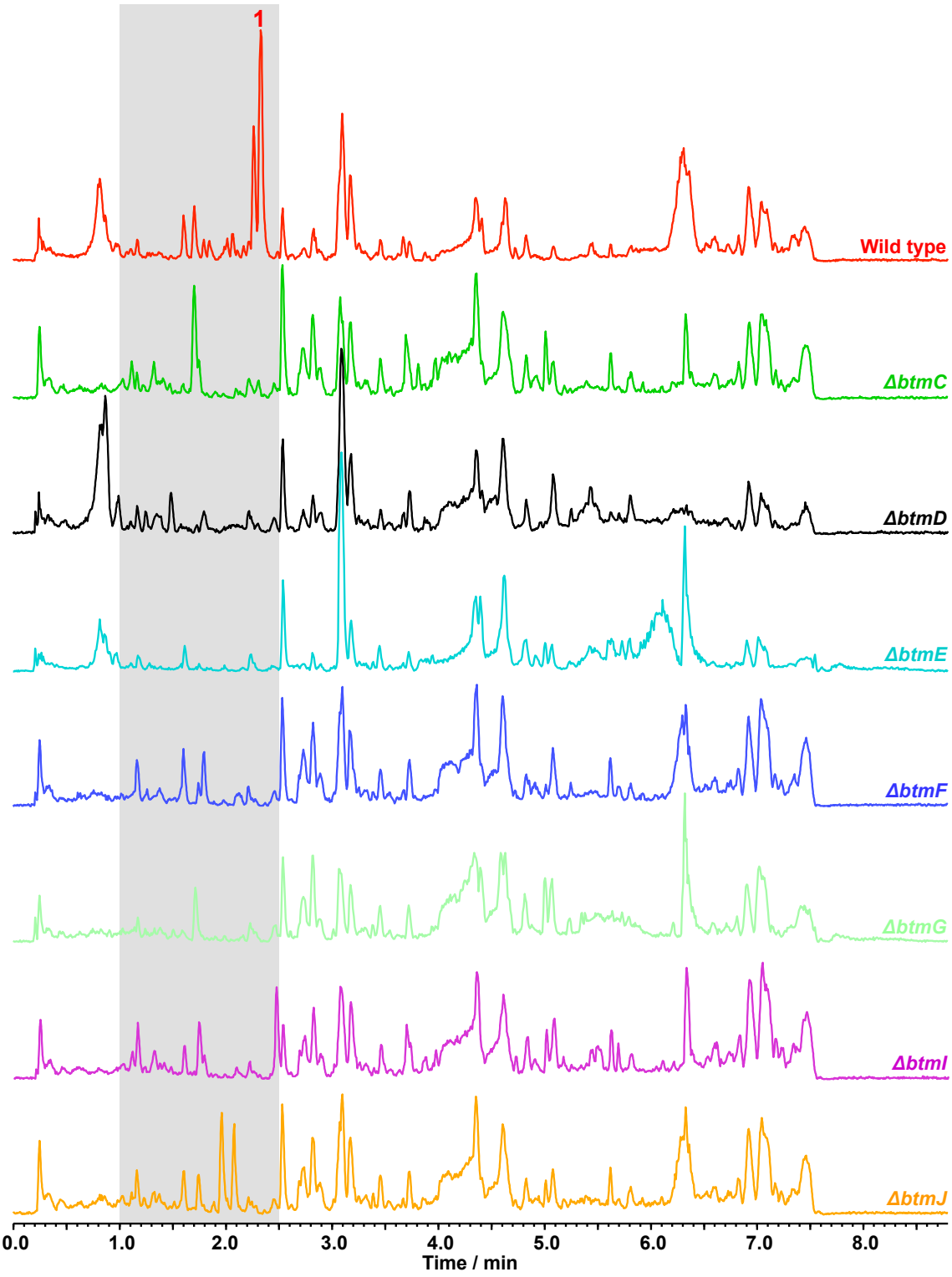


Figure S4 MS base peak chromatograms (BPCs) for all mutant strains compared to wild type *S. scabies* after 3 days in production medium. Bottromycin A_2 (**1**) production is indicated for the WT and the grey box indicates the region in which all bottromycin-like compounds eluted.

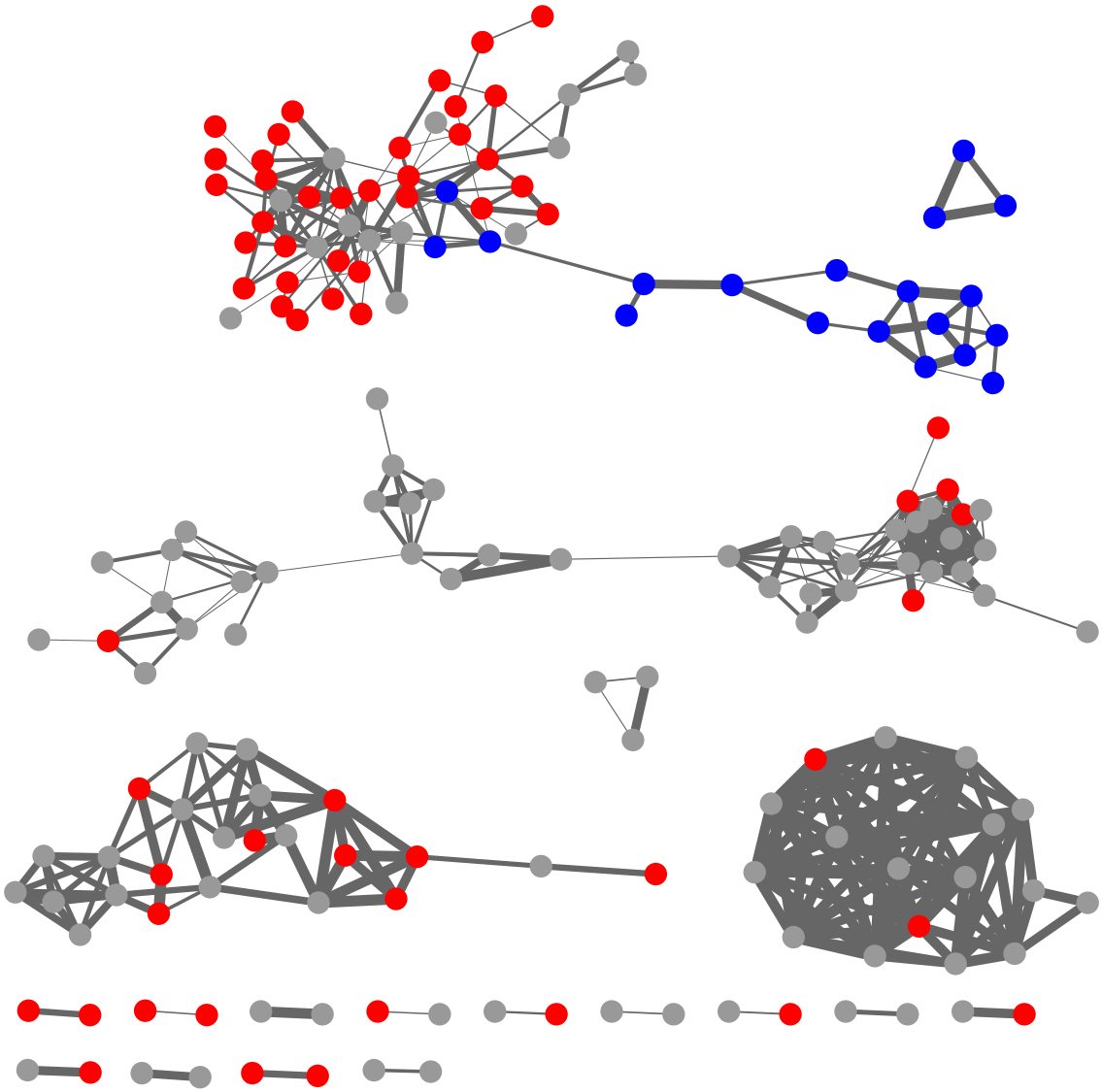


Figure S5 Mass spectral network view of the metabolomes of wild type (WT) *S. scabies* and *ΔbtmC*, *ΔbtmD*, *ΔbtmF*, *ΔbtmI* and *ΔbtmJ* mutant strains, generated using Cytoscape 2.8.3. All red nodes are species observed in one or more of the WT, *ΔbtmC*, *ΔbtmF*, *ΔbtmI* and *ΔbtmJ* strains, but not in *ΔbtmD*. The blue nodes refer to the bottromycin-related species identified in this study. Edge thickness reflects how closely related each node is (a linear gradient of 2 to 40 points between cosine 0.6 and 1). Nodes not found in *ΔbtmD* that are in other areas of the network could either reflect a degree of natural variation between MS data or as-yet-uncharacterized metabolites related to the bottromycin pathway.

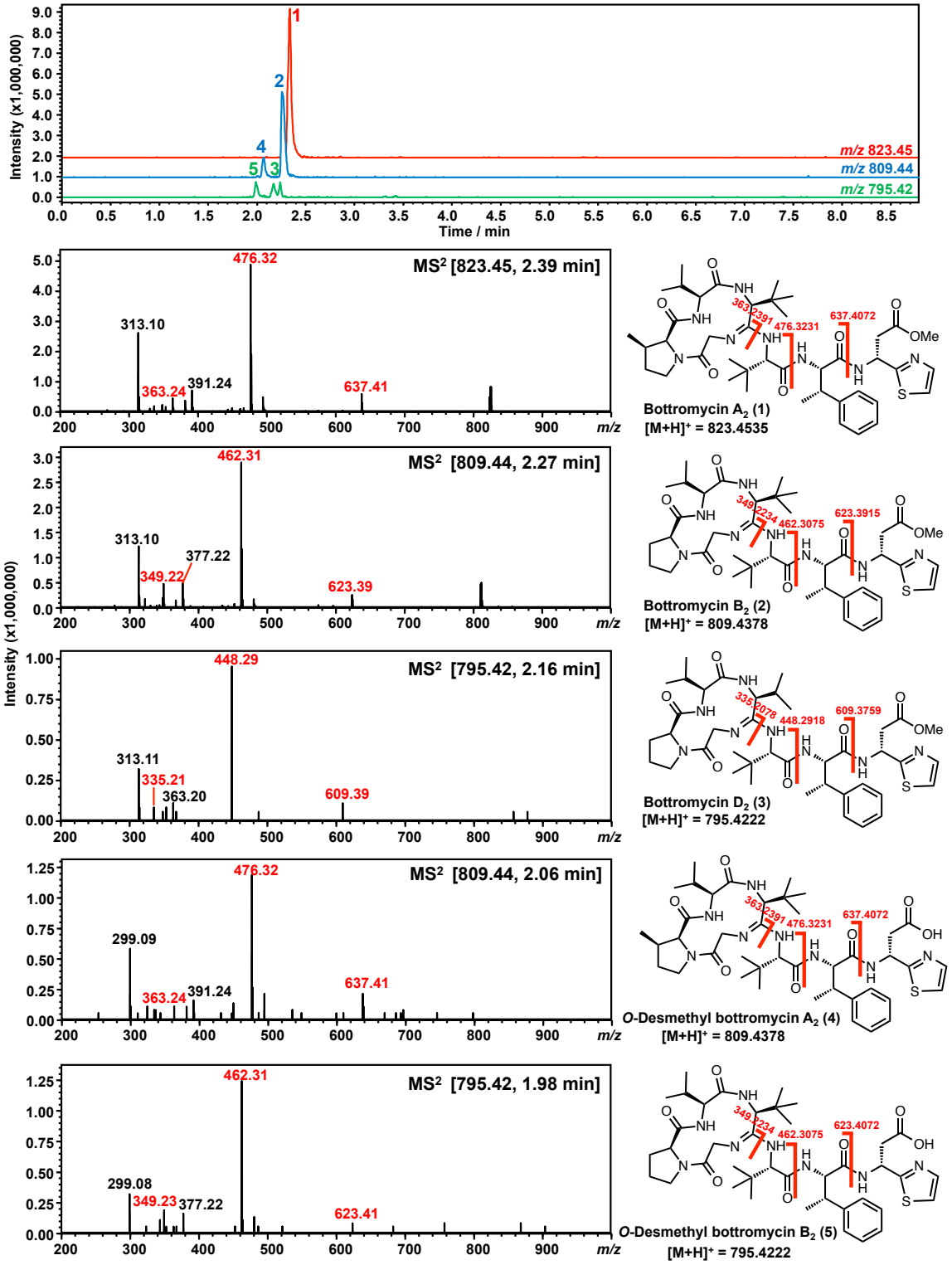


Figure S6 LC-MS^2 data for mature bottromycins **1**, **2** and **3**, and *O*-desmethyl bottromycins (DMBs) **4** and **5**. All data is from wild type *S. scabies*.

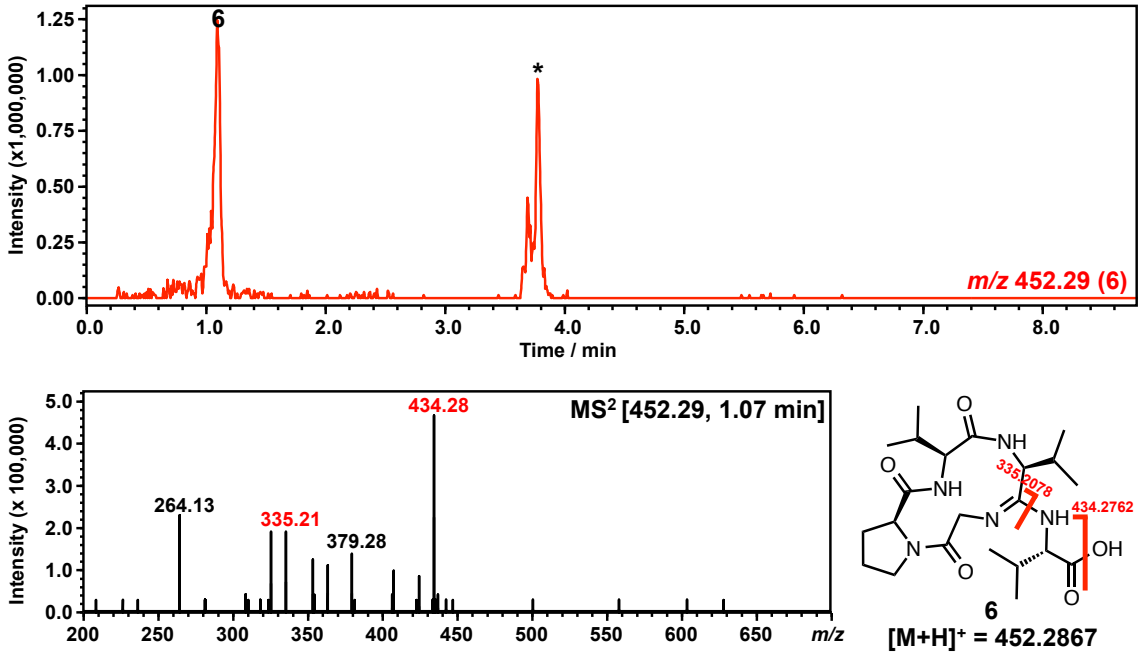


Figure S7 LC-MS^2 data for truncated cyclized metabolite **6**. The starred peak is an unrelated metabolite with the same mass as **6**. Data is from *S. scabies ΔbtmC*.

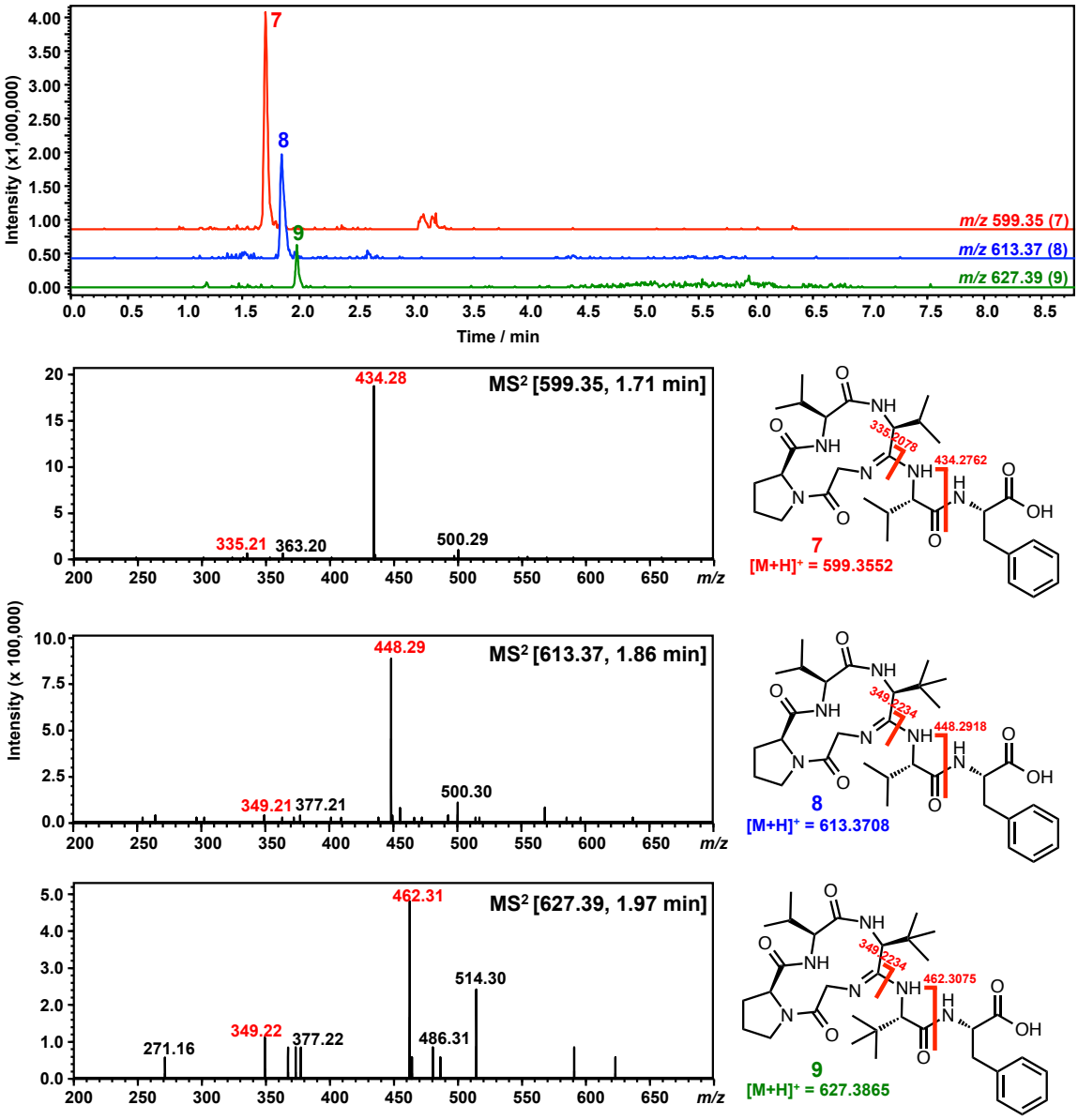


Figure S8 LC-MS^2 data for truncated cyclized metabolites **7**, **8** and **9**. All data is from wild type *S. scabies*.

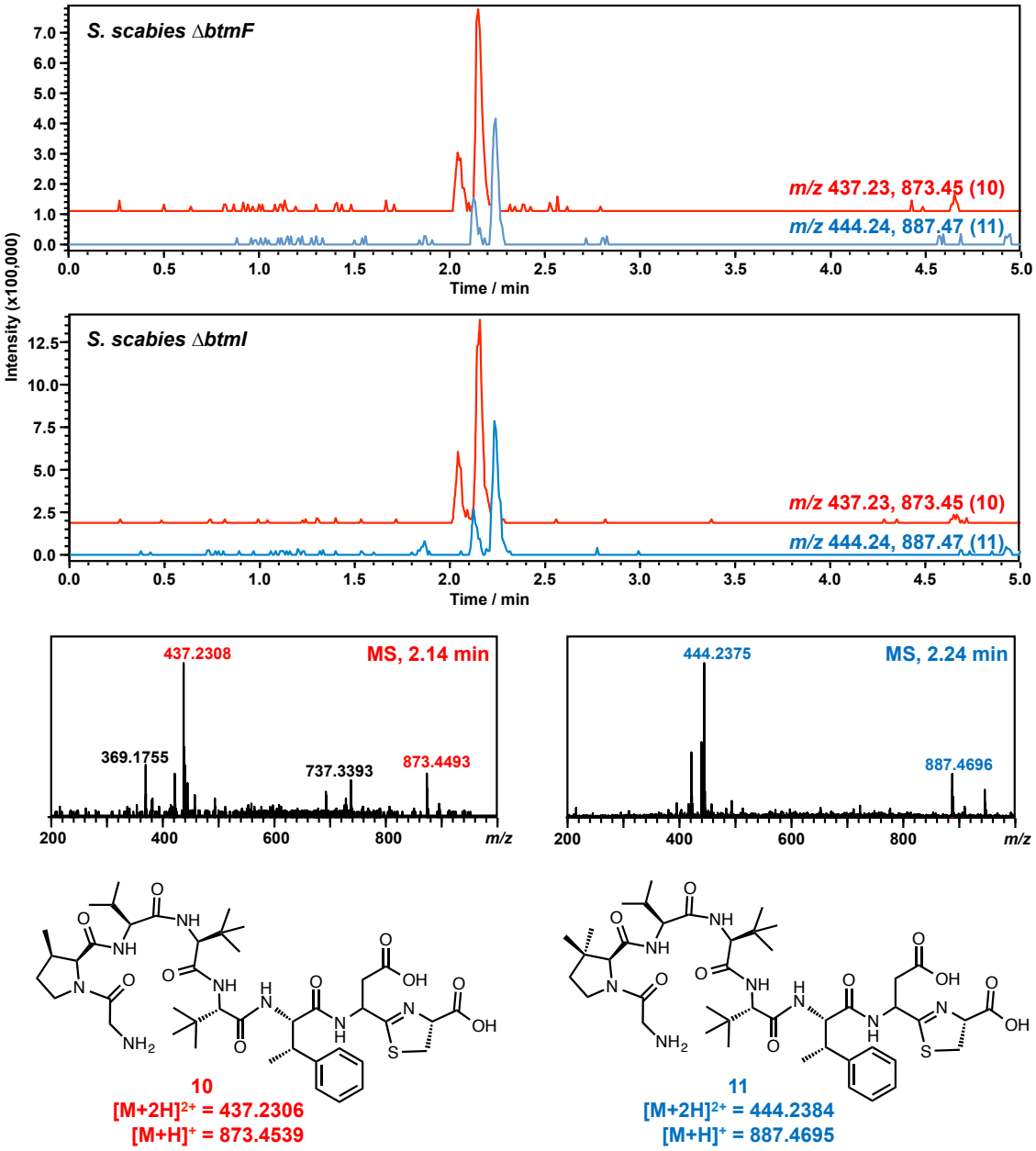


Figure S9 LC-MS data for linear metabolites **10** and **11** produced by *S. scabies ΔbtmF* and *ΔbtmI*.

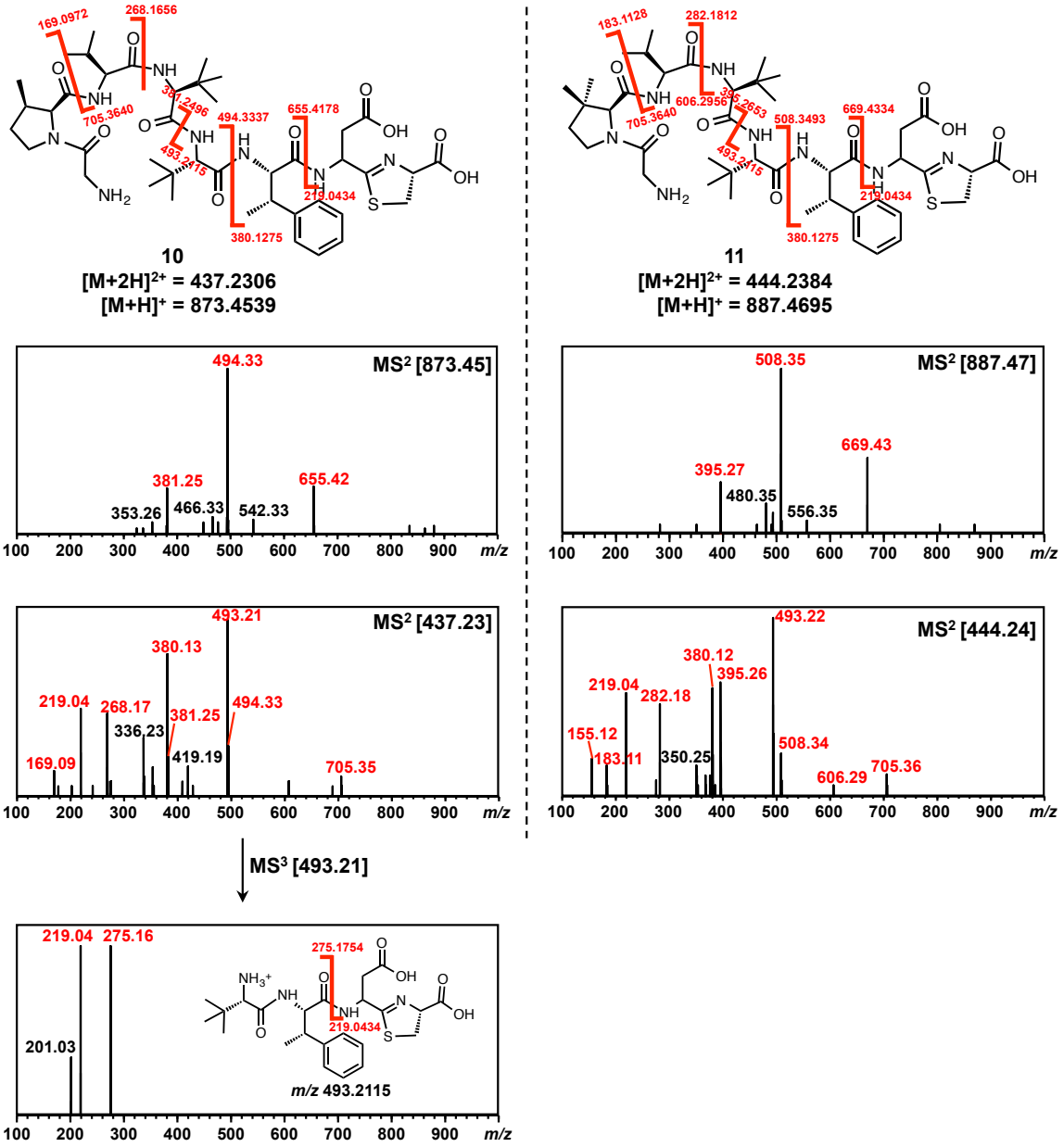


Figure S10 MS^2 and MS^3 data for **10** and **11** from *S. scabies ΔbtmI*.

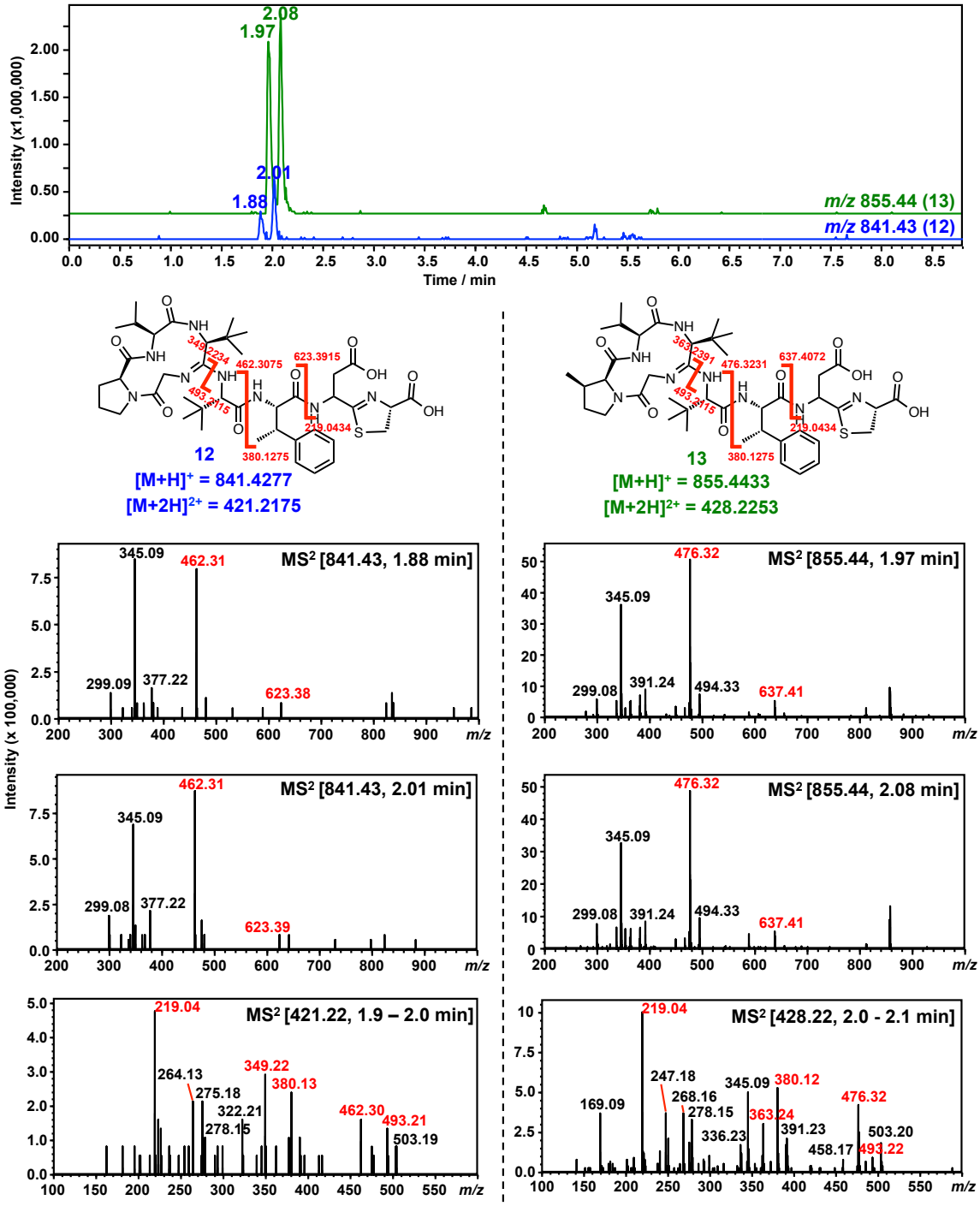


Figure S11 LC-MS^2 data for carboxylated bottromycins (CBs) **12** and **13**. All data is from *S. scabies ΔbtmJ*.

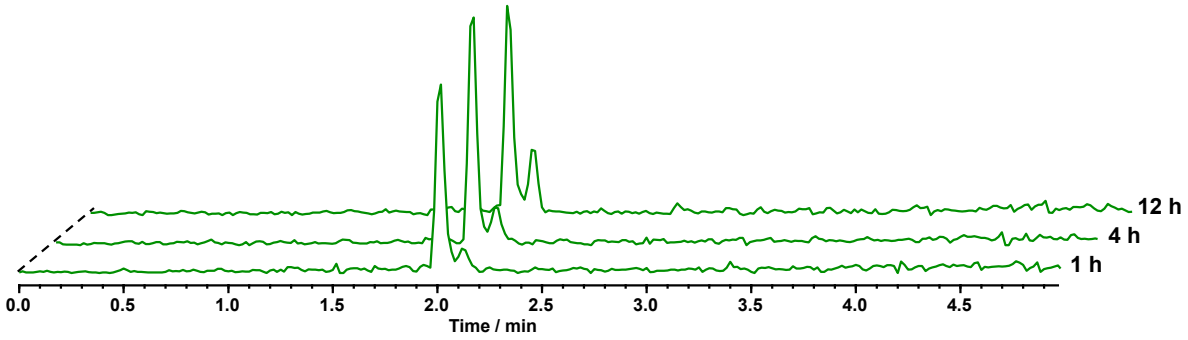


Figure S12 Time course showing the conversion of a single purified peak of **13** into two peaks with identical masses. Extracted ion chromatograms from LC-MS analysis are shown.

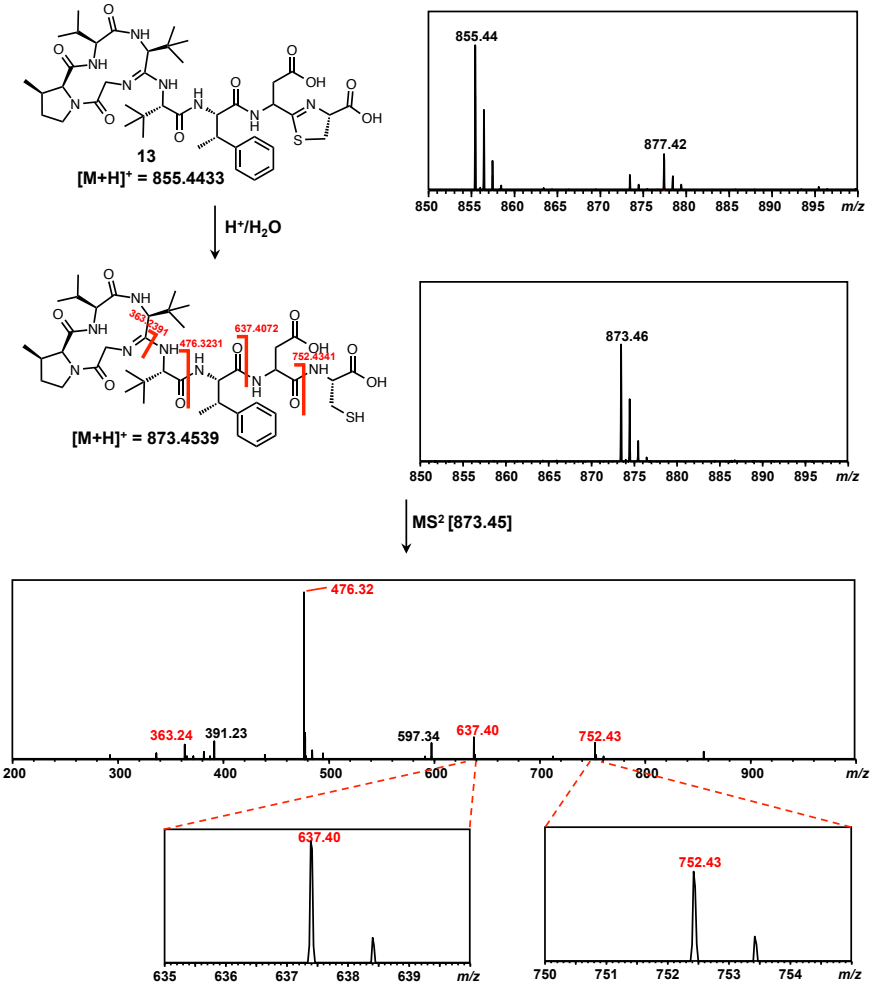


Figure S13 LC-MS^2 data for carboxylated bottromycin **13** treated with 0.1% aqueous formic acid, which hydrolyzes the thiazoline back to a cysteine residue. This conversion is characterized by an increase of 18.01 Da to the mass of the compound and the *m/z* 752.43 fragment in the MS^2 spectrum. This distinguishes this compound from **10**, which has an identical mass but retains the thiazoline and instead has no amidine.

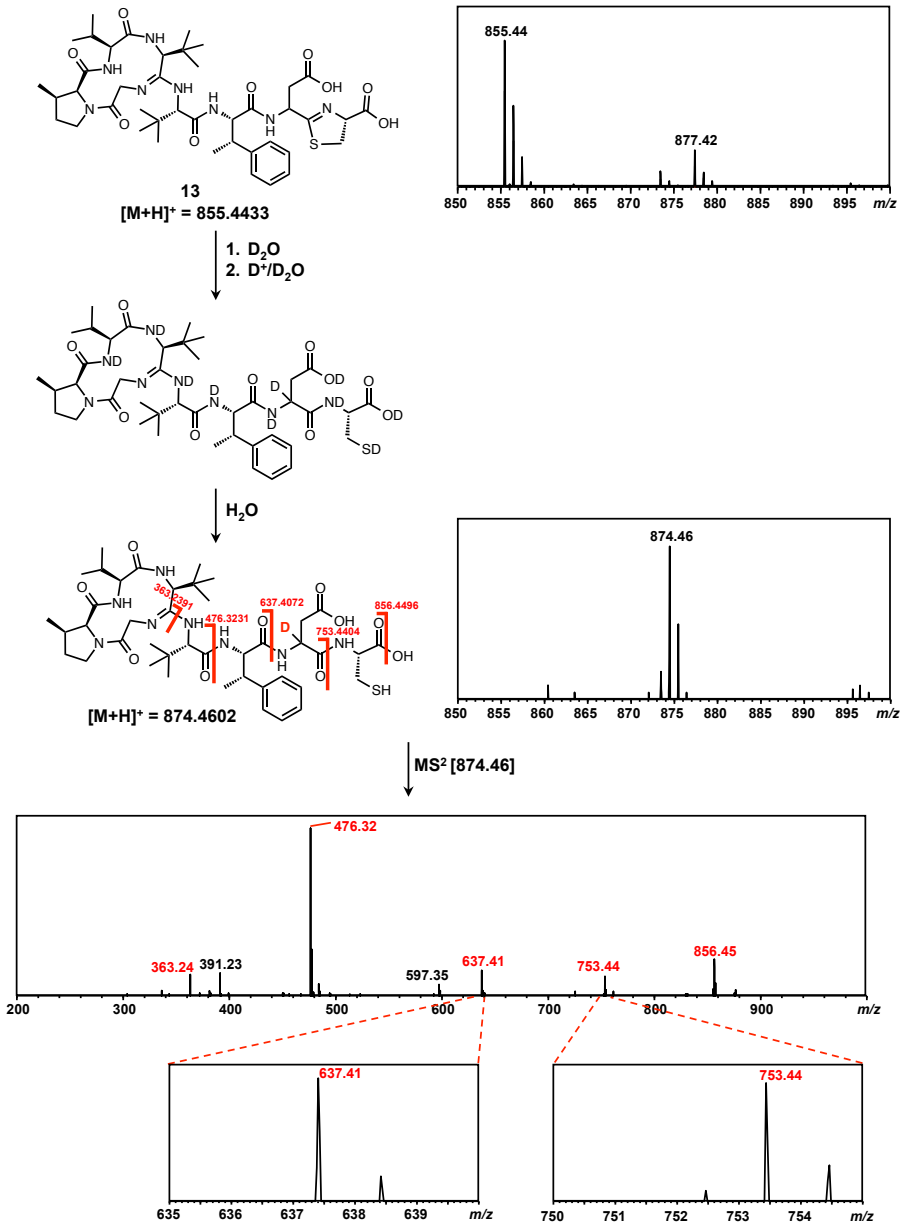


Figure S14 Deuterium exchange showing aspartate epimerization in carboxylated bottromycin **13**. LC-MS^2 data is shown for **13** treated sequentially with: (1) D_2O and (2) 40 mM DCl in D_2O; and then H_2O. The D_2O steps first lead to the replacement of protons with deuteriums in all readily exchangeable positions and acid treatment hydrolyses the thiazoline residue as shown in Fig. S13. Following hydrolysis, back exchange of all exchangeable deuteriums takes place in H_2O. However, the deuterium in the α-position of aspartate is trapped, as there is no longer an adjacent thiazoline to enable exchange. The specific incorporation of one deuterium is shown by the increase in one mass unit in the full molecule, while the location of this deuterium is demonstrated by the *m/z* 753.44 and *m/z* 637.41 fragments.

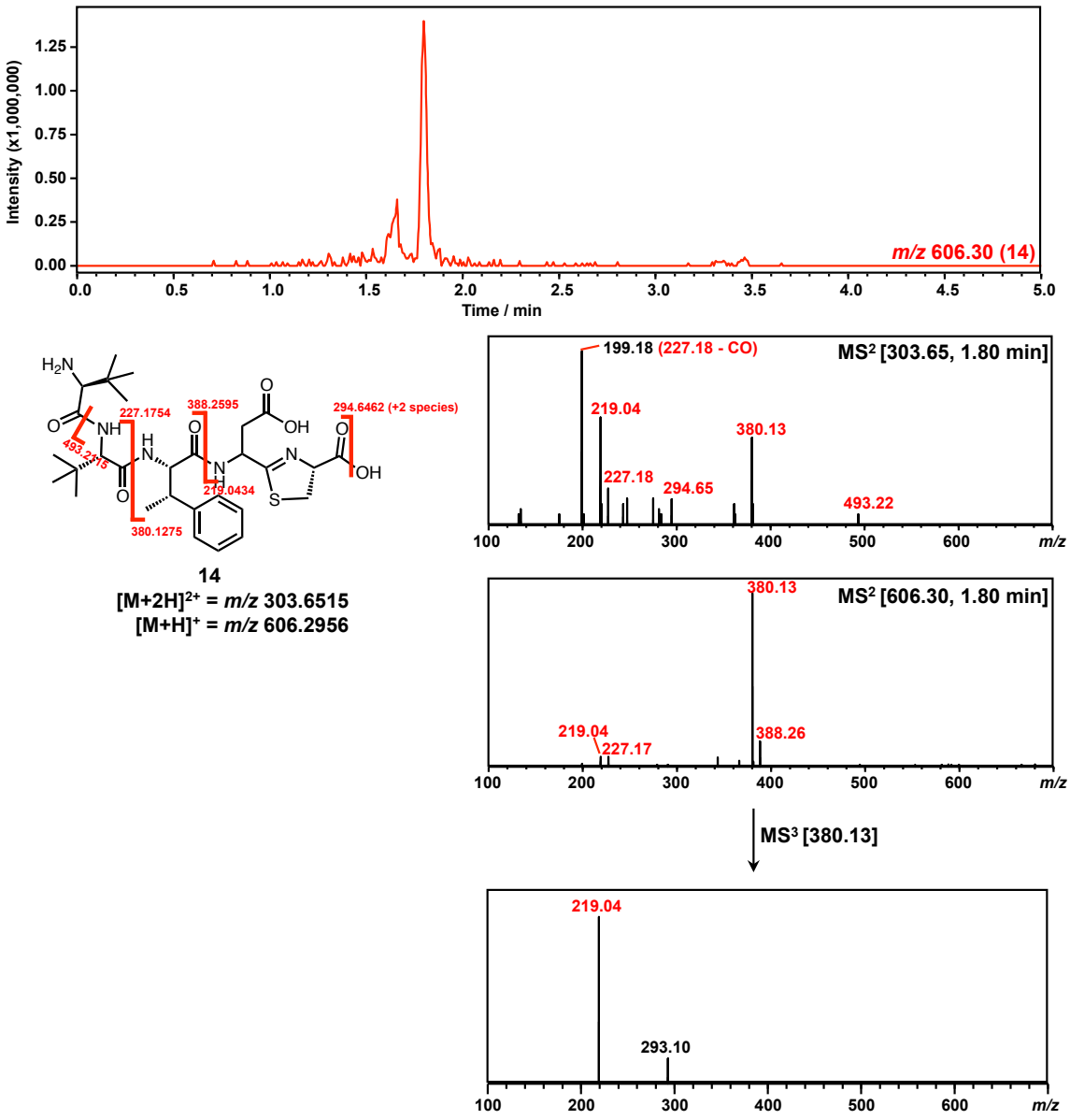


Figure S15 LC-MS^n data for **14** produced by wild type *S. scabies*.

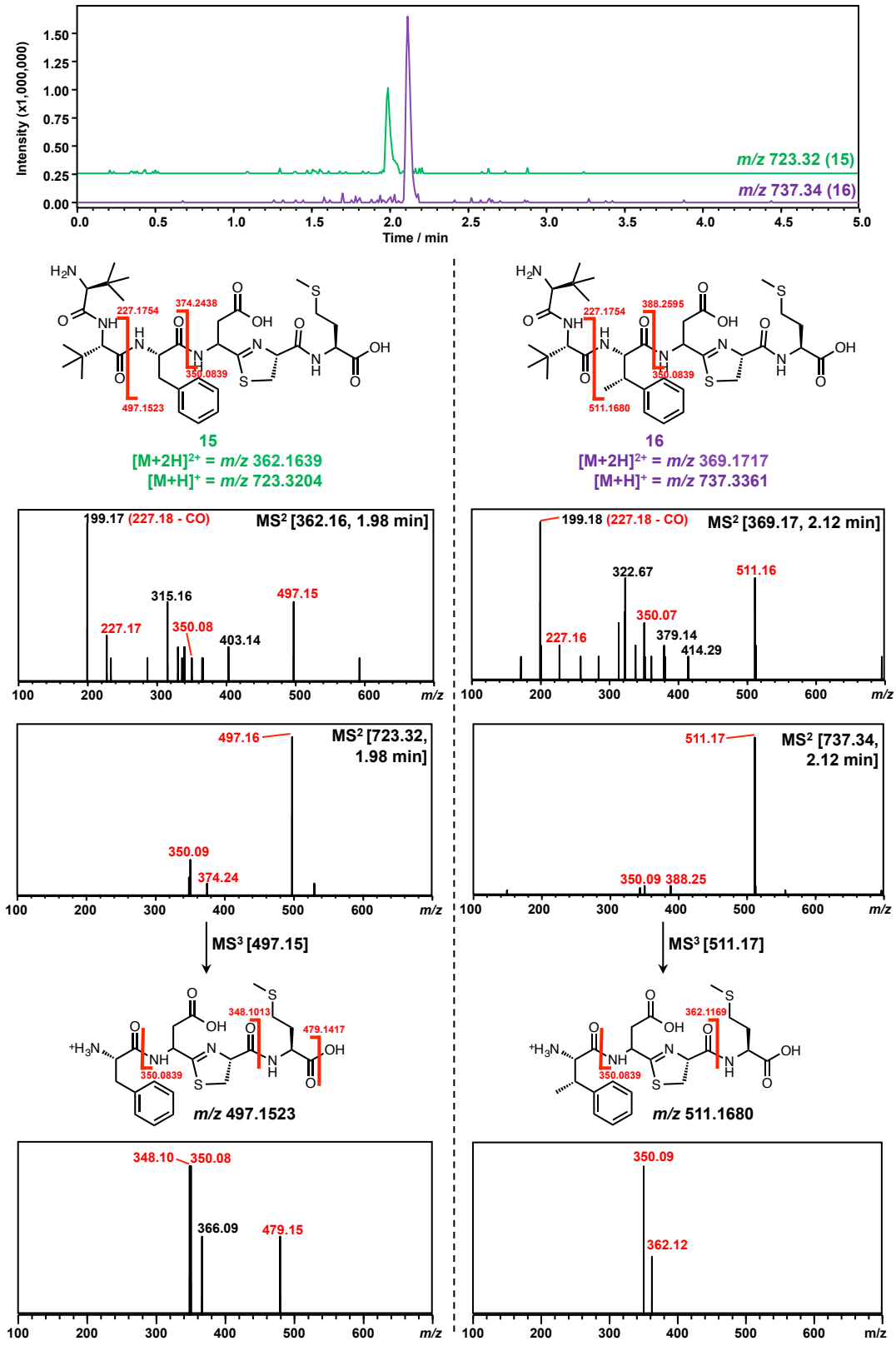


Figure S16 LC-MS^n data for **15** and **16** produced by wild type *S. scabies*.

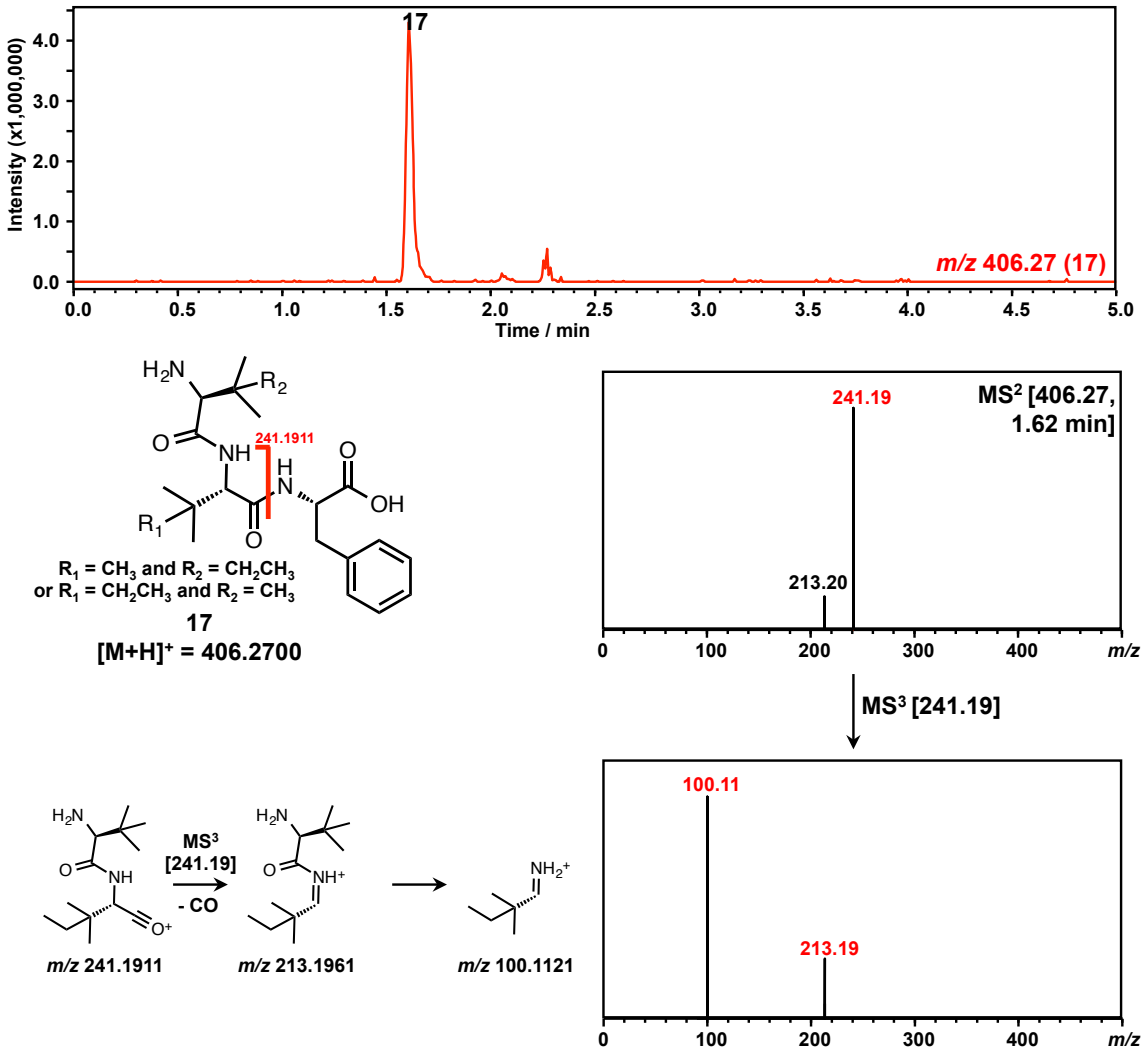


Figure S17 LC-MS^n data for **17** produced by *S. scabies ΔbtmC*. A fragmentation pathway for *m/z* 241.19 is proposed, although an alternative fragmentation route could place the additional methyl group on the other valine.

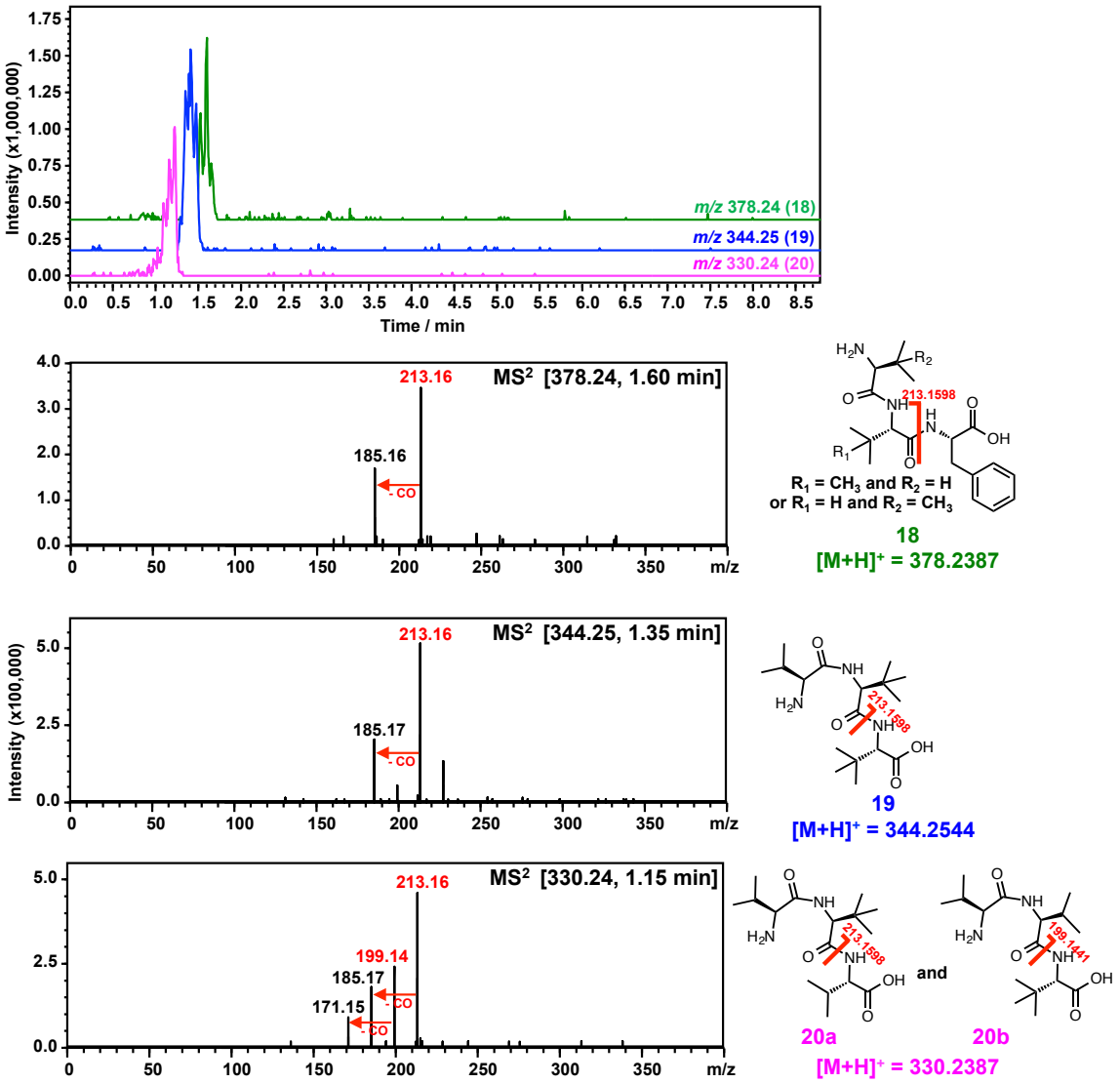


Figure S18 LC-MS^2 data for peptide metabolites deriving from the middle of the bottromycin core peptide. All data is from *S. scabies ΔbtmC*. Loss of CO from a peptide b ion results in a loss of 27.99 Da.

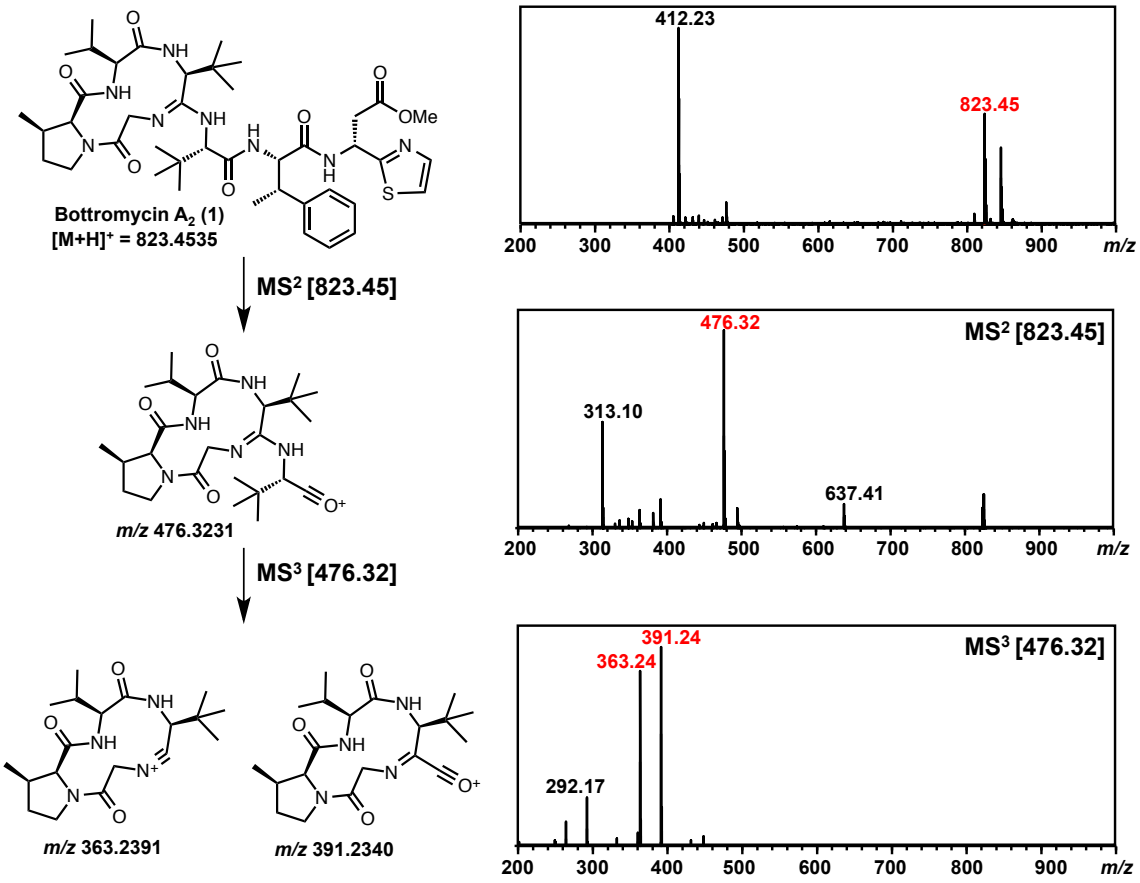


Figure S19 LC-MS^n data for bottromycin A_2 (**1**) showing the production of a fragment of *m/z* 476.32 that is consistent with a CO adduct of the nitrilium ion (*m/z* 391.2340). This could form by fragmentation to give CO, an imine and the nitrilium ion followed by recombination of the CO and nitrilium ion before they separate. This MS^n pattern is characteristic for metabolites that feature the macrocyclic amidine.

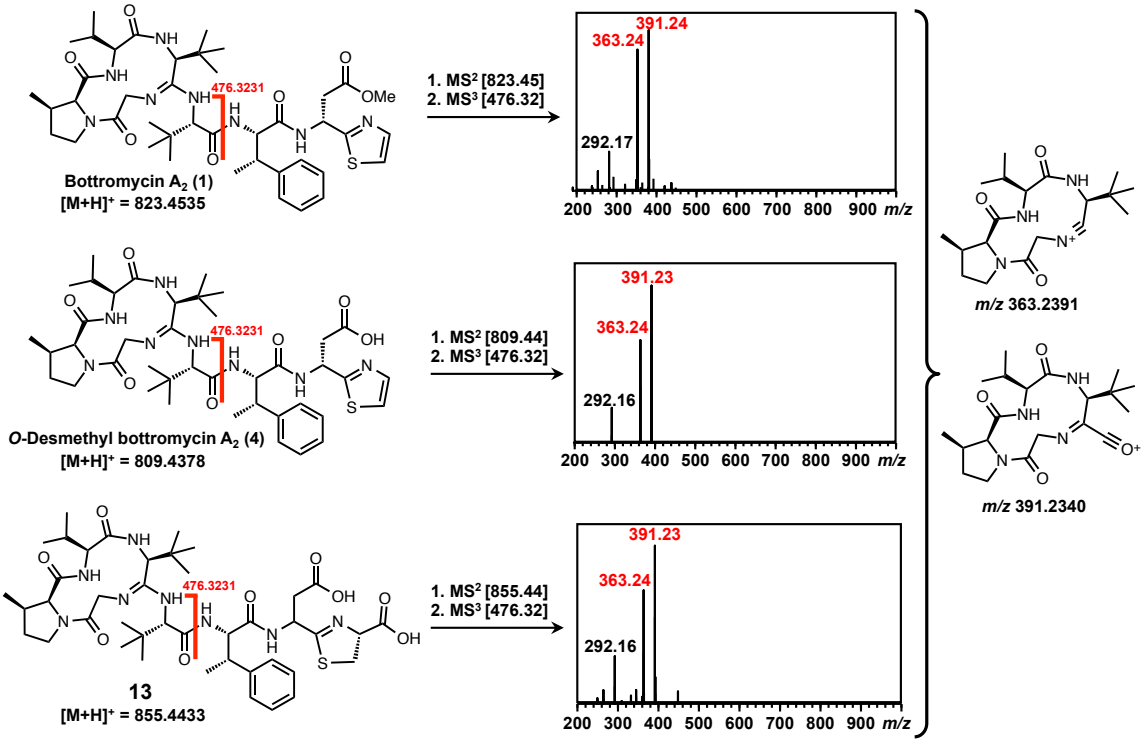


Figure S20 LC-MS^n data for molecules featuring the same macrocyclic amidine as bottromycin A$_2$ (**1**). These all provide an MS^3 pattern that is consistent with the fragmentation pathway detailed in Fig. S19.

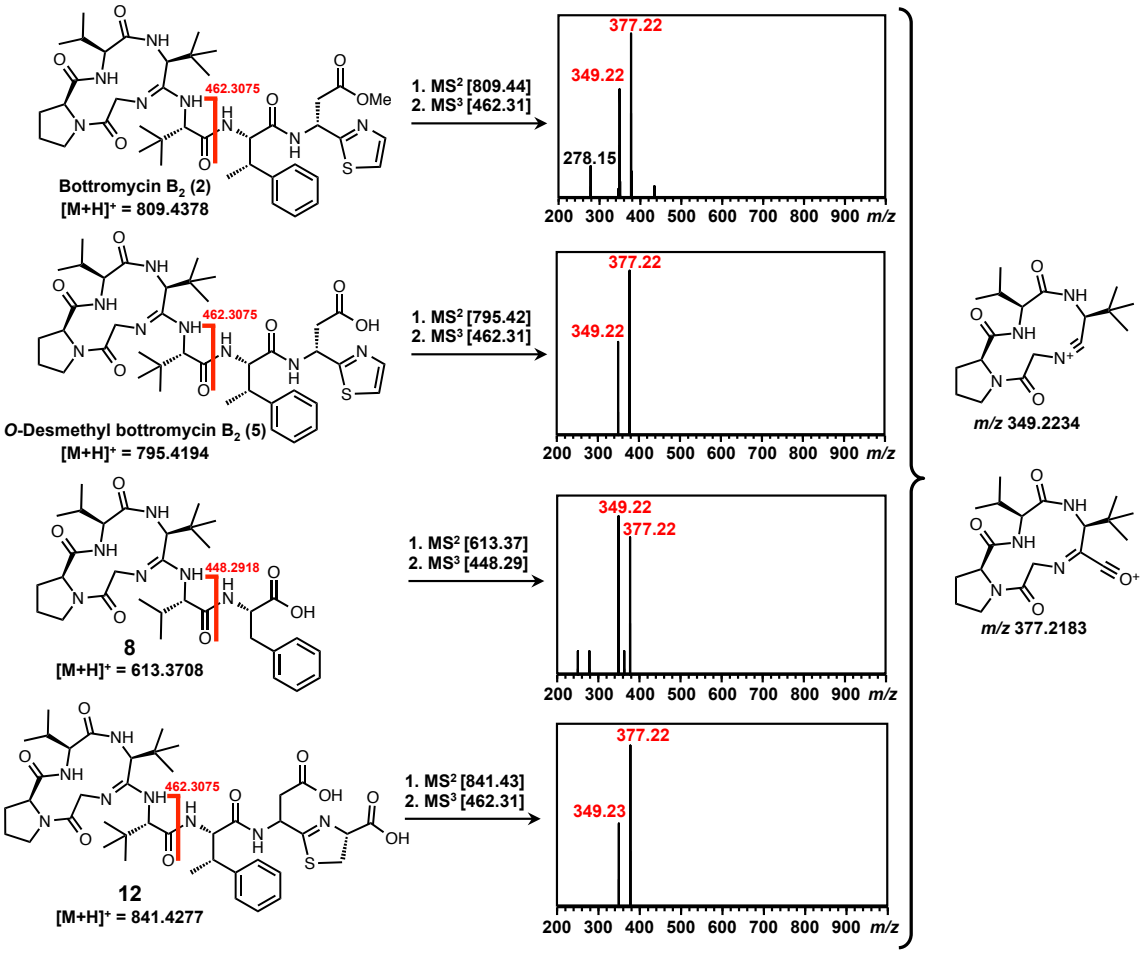


Figure S21 LC-MS^n data for molecules featuring the same macrocyclic amidine as bottromycin B_2 (**2**). These all provide an MS^3 pattern that is consistent with the fragmentation pathway detailed in Fig. S19.

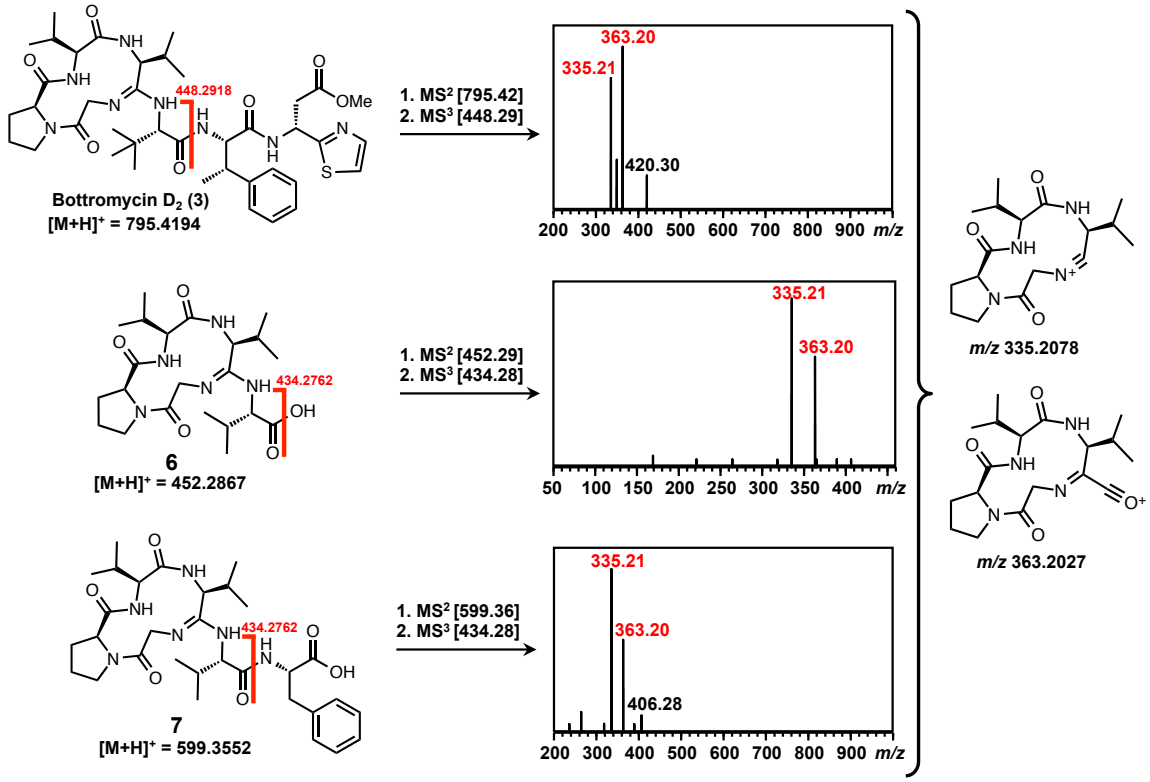


Figure S22 LC-MS^n data for molecules featuring the same macrocyclic amidine as bottromycin D_2 (**3**). These all provide an MS^3 pattern that is consistent with the fragmentation pathway detailed in Fig. S19.

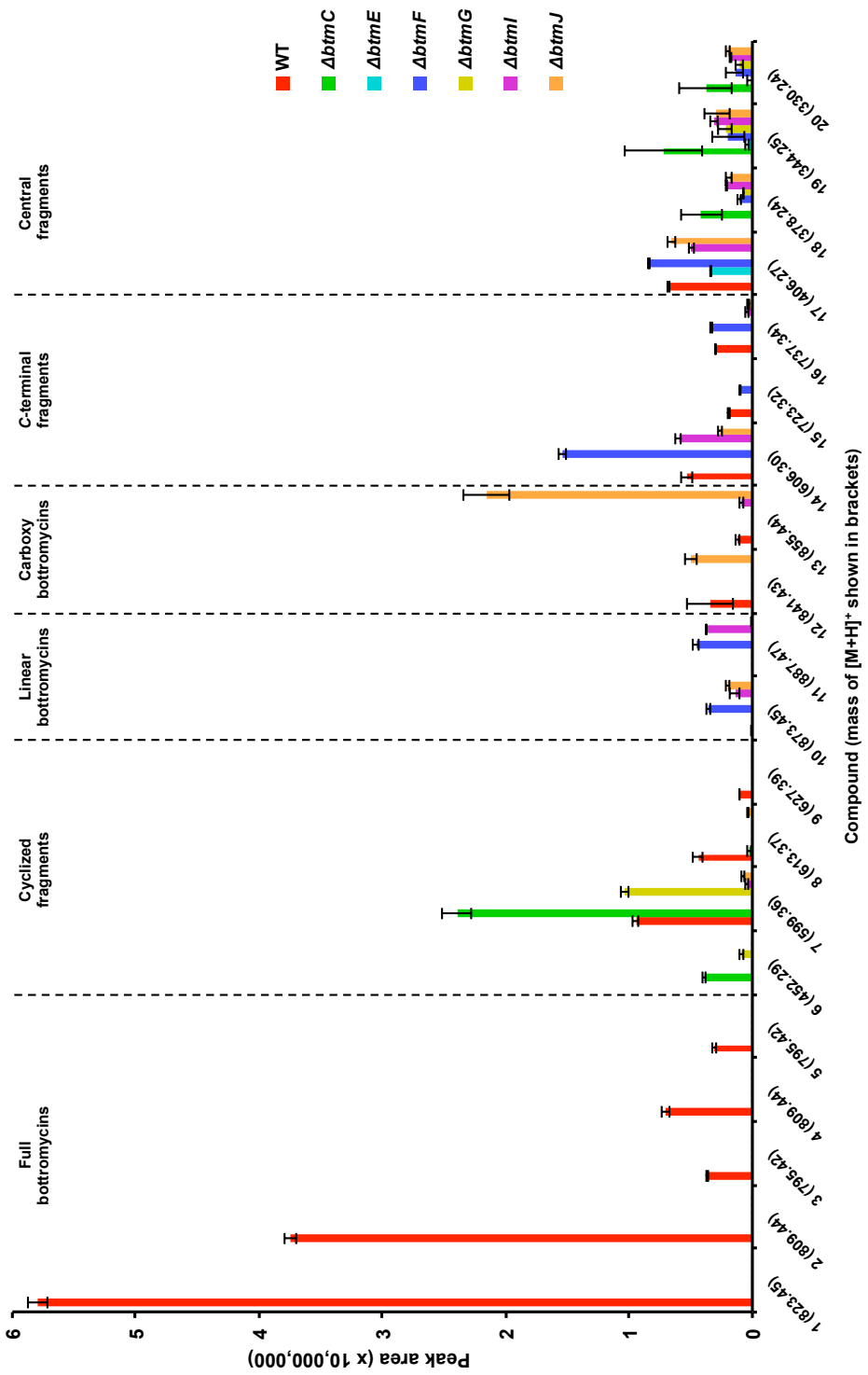


Figure S23 Chart showing the relative proportion of metabolites produced across all mutants after three days in production medium. The quantities represent the average of triplicate datasets and reflect the MS peak areas of the summed $[M+H]^+$ and $[M+2H]^{2+}$ signals for each compound. Error bars represent the standard deviation of triplicate data.

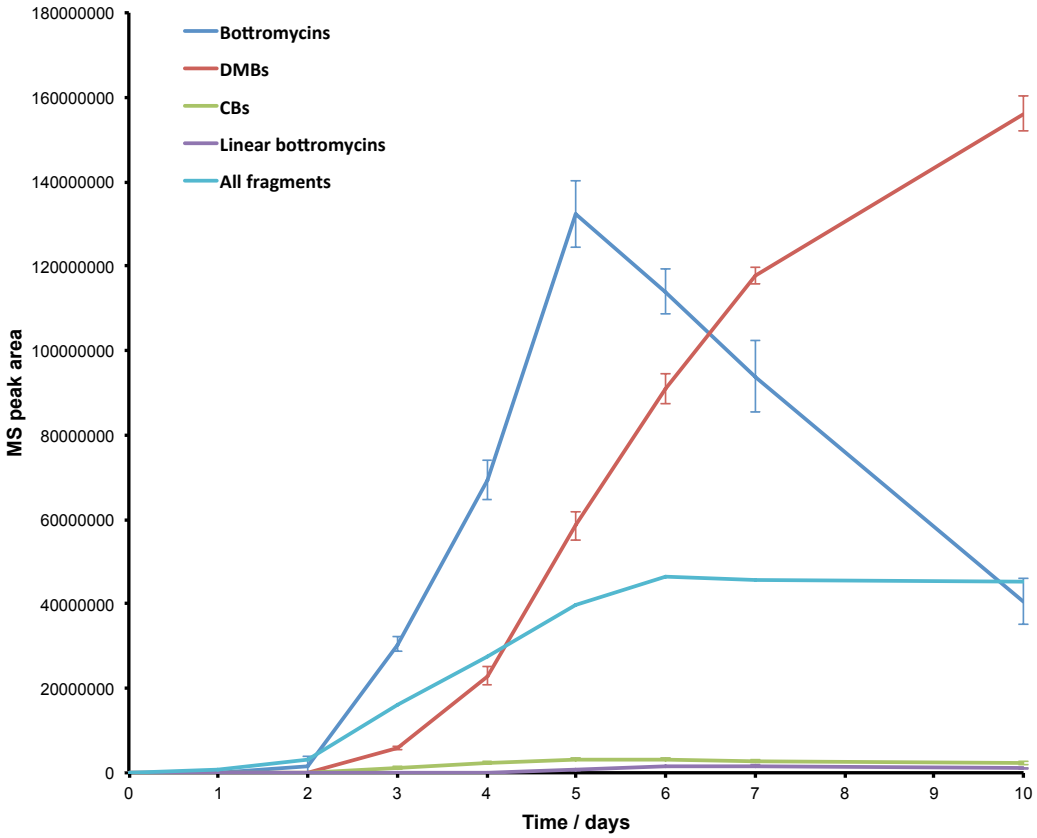


Figure S24 Time-course analysis of bottromycins produced by wild type *S. scabies*. Error bars represent the standard deviation of triplicate data. Molecules have been grouped as: (i) bottromycins (**1**, **2** and **3**), (ii) DMBs (*O*-desmethyl bottromycins, **4** and **5**), (iii) CBs (carboxy bottromycins, **12** and **13**), (iv) Linear bottromycins (**10** and **11**), (v) truncated bottromycin fragments (**7**, **8**, **9**, **14**, **15** and **16**). Small amounts of linear bottromycins only appear after 6 days, so do not feature as wild-type metabolites in Figures 1 and S23, which are based on data collected after 3 days.

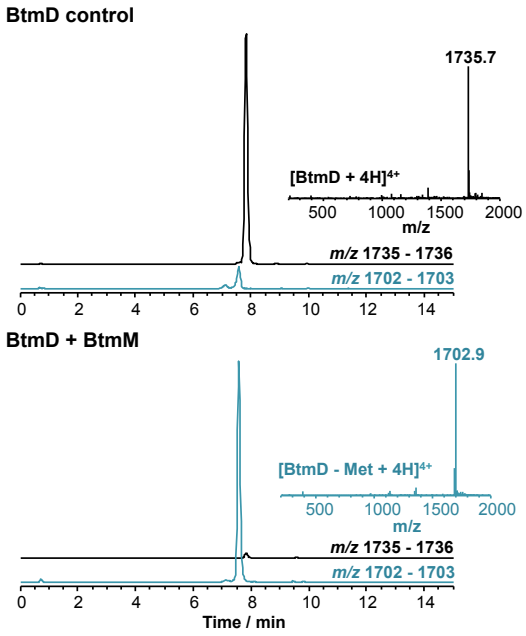


Figure S25 BtmM activity with BtmD. *N*-terminal methionine cleavage from *C*-terminally His_6-tagged BtmD is catalyzed by BtmM. MS data from 7 - 8 min is shown. The molecular weight of BtmD-His_6 is 6,937.6 Da (calc. $[M+4H]^{4+}$ m/z = 1,735.4; calc. $[M\text{-}Met+4H]^{4+}$ m/z = 1,702.6).

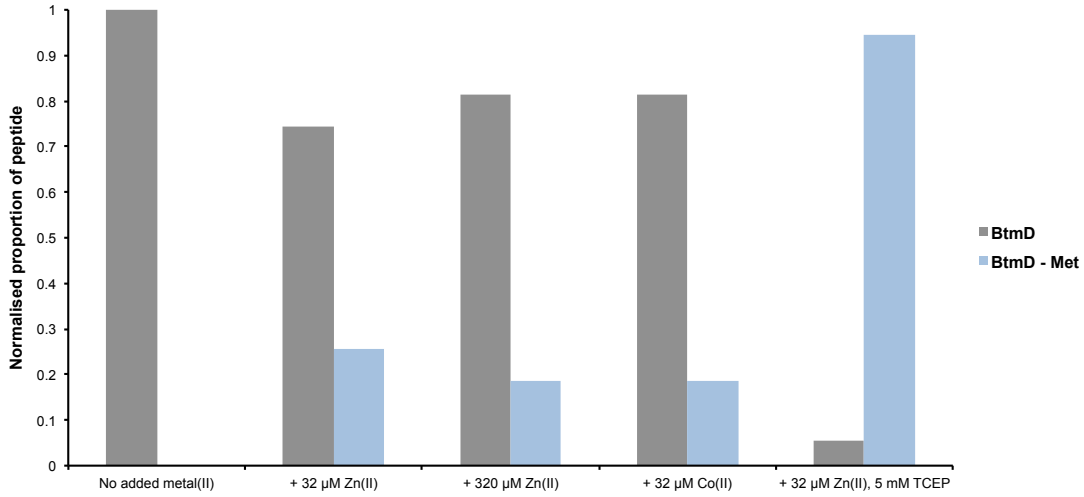


Figure S26 The efficiency of BtmD methionine cleavage by BtmM with various additives. Standard reaction conditions were 6.4 µM BtmM, 50 mM Tricine pH 9, 30 °C for 3 hours.

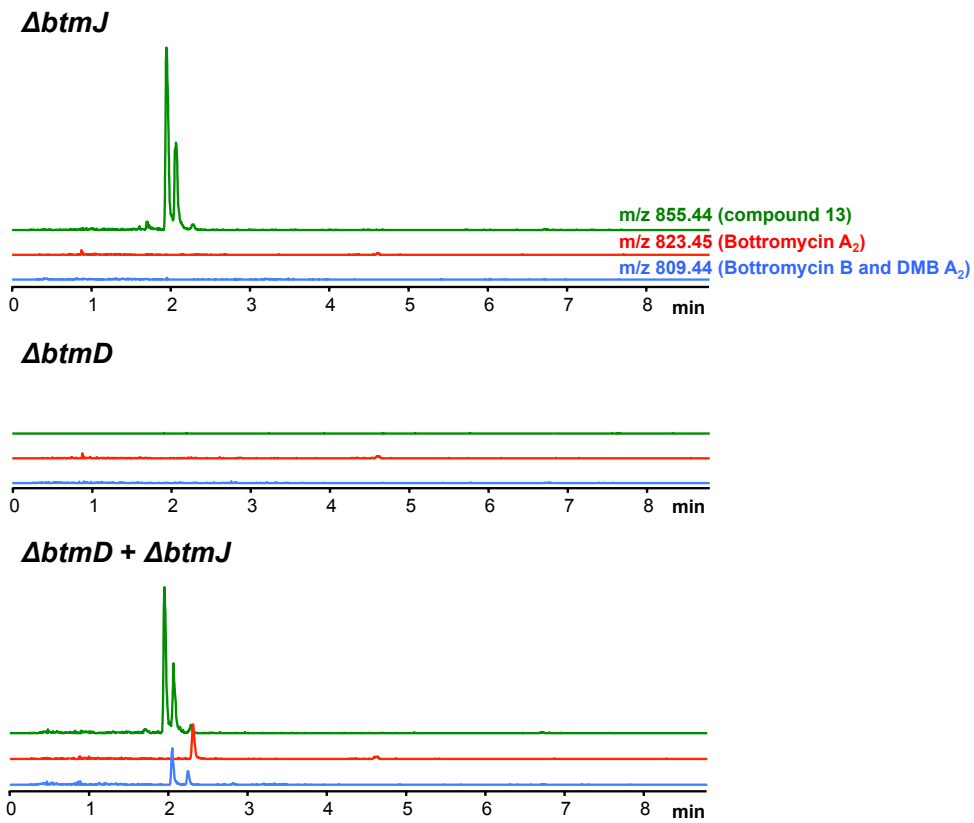


Figure S27 LC-MS analysis of *S. scabies ΔbtmJ* + *ΔbtmD* co-culture. Traces show extracted ion chromatograms for *m/z* 855.44, 823.45 and 809.44 in *ΔbtmJ* only, *ΔbtmD* only and in the co-culture.

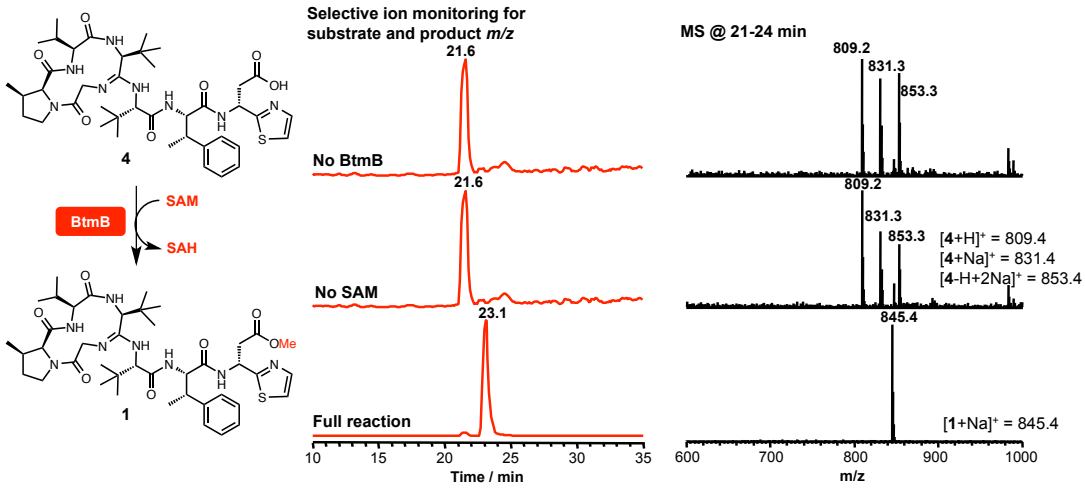


Figure S28 *In vitro* BtmB activity with **4** (*O*-desmethyl bottromycin A_2).

4. Supplementary References

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