

# 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  $\mu$ g mL<sup>-1</sup>, apramycin at 50  $\mu$ g mL<sup>-1</sup>, carbenicillin at 30  $\mu$ g mL<sup>-1</sup>, chloramphenicol at 25  $\mu$ g mL<sup>-1</sup> and nalidizic acid at 25  $\mu$ g mL<sup>-1</sup>.

#### 1.2 Bacterial strains

Spores of *Streptomyces scabies* DSM 41658 were prepared from cultures on instant potato mash agar (20 g L<sup>-1</sup> Smash (Premier Foods), 20 g L<sup>-1</sup> 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<sup>[1]</sup> 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*  $\Delta btmC$ ,  $\Delta btmE$ ,  $\Delta btmF$ ,  $\Delta btmG$ ,  $\Delta btmI$  and  $\Delta btmJ$  has been previously described<sup>[2]</sup>.

#### 1.3 Complementation plasmids

Complementation plasmids were generated using the integrative vector pIB139, which contains the strong constitutive *ermE*\* promoter. For  $\Delta btmD$  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<sup>[2]</sup>.

#### 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<sub>3</sub>, 25 µg mL<sup>-1</sup>CoCl<sub>2</sub> 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 *AbtmD* 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<sup>[3,4]</sup>. 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  $\mu$ L) were injected onto a Phenomenex Kinetex 2.6  $\mu$ m 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<sup>-1</sup>. Positive mode mass spectrometry data was collected between *m*/*z* 200 and 2000, and MS<sup>2</sup> 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  $\Delta btmD$  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<sup>2</sup> 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 ( $\sqrt{area} = 0, 20$  points;  $\sqrt{area} = maximum$  (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  $\mu$ L) were injected onto an Acquity BEH C18 reversed phase column (1.7  $\mu$ m, 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<sub>6</sub>-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<sub>600</sub> reached approximately 0.6. Isopropyl-b-Dthiogalactopyranoside (IPTG) was then added to a final concentration of 15  $\mu$ M 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<sub>6</sub> 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. N2 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<sub>6</sub>-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  $\mu$ M 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  $\mu$ M ZnCl<sub>2</sub>, (iii) 32  $\mu$ M CoCl<sub>2</sub>, (iv) 320  $\mu$ M ZnCl<sub>2</sub>, (v) 5 mM TCEP and 32  $\mu$ M ZnCl<sub>2</sub>. 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  $\mu$ m 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<sup>-1</sup>.

#### 1.9 Production of O-desmethyl bottromycin

The methyl ester of bottromycin was hydrolyzed using LiOH, according to the protocol of Kobayashi *et al.*<sup>[5]</sup>. THF (4.5 mL) was added to bottromycin A<sub>2</sub> purified from a *S. scabies* culture (175 mL fermentation volume). 500  $\mu$ L of this was mixed with 500  $\mu$ L of 1 M LiOH, and the mixture stirred at room temperature for 4 h. This was quenched with 500  $\mu$ L of 1 M HCl, and the THF was removed by evaporation under reduced pressure. The remaining solution (500  $\mu$ L) 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<sub>2</sub>O (500  $\mu$ L). Preparative HPLC was carried out using a Phenomenex Luna 10  $\mu$ m 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<sup>-1</sup>.

#### 1.10 BtmB assays

*O*-desmethyl bottromycin A<sub>2</sub> (25  $\mu$ L) was mixed with BtmB (5  $\mu$ M), *S*-adenosyl methionine (SAM) (2 mM) in a final volume of 100  $\mu$ L 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.*<sup>[2]</sup>. 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 AbtmJ

A 5-day production culture of *S. scabies*  $\Delta btmJ$  (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  $\mu$ L 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<sup>-1</sup> on a Phenomenex Luna 5 $\mu$ m 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  $\mu$ m 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<sub>2</sub>O (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<sub>2</sub>O 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-MS<sup>2</sup> using a Shimadzu IT-TOF as described in section 1.6. The results are shown in Figures S12 and S13.

## 2. Supplementary Tables

Primer Name	Primer Sequence (5'→3')	<b>Restriction site</b>
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	GTCCGGTTCCGCGGGGGAATTCCGC	EcoRI
btmJ-start	GGCCCGCTGCCATATGGACTTCGAC	NdeI
btmJ-end	GTTTCGGAATTCGCGCCCTCACCAG	EcoRI
btmM-start	GCGTATGACCATATGACCCGGGTCGT	NdeI
btmM-end	CGCGGCGGAATTCCCGGGCCTAC	EcoRI
btmD_end_vector	ATAAAGCTTGGAACCGCGTGGCACCAGTGAGGTGGCT	HindIII
htmD RBS		Asel Ndel
ound_RD5	TAGTCG	Aser, Nder
$RT_hrdB_fw$	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 S1**Primers 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 $\Delta btmC$
pIB139-RBS-btmD	This study	Apramycin	Complementation of <i>AbtmD</i>
pIB139-RBS-btmE	This study	Apramycin	Complementation of <i>AbtmE</i>
pIB139-RBS-btmF	This study	Apramycin	Complementation of $\Delta btmF$
pIB139-RBS-btmI	This study	Apramycin	Complementation of <i>AbtmI</i>
pIB139-RBS-btmJ	This study	Apramycin	Complementation of $\Delta btmJ$
pET28-btmM	This study	Kanamycin	Expression of N-terminally His6-tagged BtmM
pET29-btmD	This study	Kanamycin	Expression of C-terminally His6-tagged BtmD
pET28-btmB	This study	Kanamycin	Expression of N-terminally His6-tagged BtmB

**Table S2**Plasmids used in this study.

Table S3	MS data for bottromycin-like metabolites.	All data from wild type S. scabies unless
otherwise stated	1.	

	Calc.	Shima	dzu IT-TOF	Synapt G2-Si				
Compound	[ <b>M+H</b> ] <sup>+</sup>	Obs. $m/z$	Error (ppm)	Obs. <i>m/z</i>	Error (ppm)			
1 (bottromycin A <sub>2</sub> )	823.4535	823.4523	-1.46	823.4525	-1.21			
<b>2</b> (bottromycin $B_2$ )	809.4378	809.4365	-1.61	809.4384	0.74			
3	795.4222	795.4216	-0.75	795.4221	-0.13			
<b>4</b> ( <i>O</i> -desmethyl bottromycin A)	809.4378	809.4377	-0.12	809.4380	0.25			
<b>5</b> ( <i>O</i> -desmethyl bottromycin B)	795.4222	795.4196	-3.27	795.4219	-0.38			
<b>6</b> (from $\Delta btmC$ )	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			
<b>10</b> (from $\Delta btmF$ )	873.4539	873.4500	-4.47	873.4551	1.37			
<b>11</b> (from $\Delta btmF$ )	887.4695	887.4696	0.11	887.4671	-2.70			
<b>12</b> (from $\Delta btmJ$ )	841.4277	841.4247	-3.57	841.4288	1.31			
<b>13</b> (from $\Delta btmJ$ )	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			
<b>18</b> (from $\Delta btmC$ )	378.2387	378.2381	-1.59	378.2388	0.26			
<b>19</b> (from $\Delta btmC$ )	344.2544	344.2529	-4.36	344.2547	0.87			
<b>20a/b</b> (from $\Delta btmC$ )	330.2387	330.2377	-3.03	330.2391	1.21			

	Revised bottronnyeni gene eruster data.								
Gene name	<i>btm</i> 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	<i>btmG</i>	Class B radical SAM methyltransferase	$\beta$ -methylation of Val4 and Val5						
SCAB_56641	btmH	$\alpha/\beta$ 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 <i>N</i> -terminal methionine of BtmD						

**Table S4**Revised bottromycin gene cluster data.

\* Genes on opposite strand

Compound	RT Range lon m/z	lon RT I	PVal WT1 WT2 WT3	btmC1 btmC2 btmC3	btmD1 btmD2	btmD3	btmE1 btm	mE2 btmE3	btm#1	btm#2 bt	tmf3 t	stmG1 btm	G2 btmG3	btml1 btml	t btml3	btmJ1	btmJ2	btmJ3 M	ledium1	Medium2	Medium3
2	003.96 - 003.97 436 002.08 - 002.10 546	5.282 3.97 5.056 2.09	4.50E-38 0 0 0 1.70E-34 232079 227763 232512	216640 213888 216512 0 0 0	0 0	0 0	0	0 0		0 0	0.0	0	0 0	0 0	0	0 0	0 0	0	0 0	0 0	0
2	001.27 - 001.28 427 002.60 - 002.62 436	7.190 1.30 6.229 2.62	5.50E-33 111073 108817 108204 1.80E-30 218577 220672 226716	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 0	0 0	0	0	0	0
? [5+2H]2+	003.34 - 003.37 551 001.96 - 002.01 398	1.381 3.37 8.719 1.99	1.60E-27 0 0 0 1.80E-27 1691820 1667240 1759418	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 154648	160837	163257	0	0	0
[2+2H]2+ [1+2H]2+	002.18-002.29 405 002.28-002.36 412	5.225 2.26 2.233 2.33	1.30E-26 20193993 19726090 20981440 2.10E-25 46043334 46765043 49329721		0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 0		0			0
2	002.07-002.16 512	2.073 2.14	3.805-25 649241 651763 619905		0 0	0	0	0 0		0	0	340105	177593 366535	2225904 22	0 20101	0 0	0 0	302064	0	0	0
[13+2H]2+ [3-2H]2+	001.93 - 001.99 428	8.727 1.96	6.508-25 582649 540352 710443	0 0 0	0 0	0	0				0	0	0 0	0	0	0 4322825	4541645	4503083		0	0
[13+2H]2+	002.05 - 002.11 428	1.225 2.08	1.205-24 654418 645844 704783	0 0 0	0 0	0	0	0 0			0	0	0 0	404865 4	13149 3580	87 6857087	6963438	7378275			0
[4+2H]2+	002.04 - 002.09 405	5.724 2.05	3.205-24 4808902 4501293 4448701	0 0 0	0 0	0	0			0	0	0	0 0	0	0	0 0	0	0		0	0
[7+H]+	001.66 - 001.74 599	9.345 1.72	4.00E-24 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	8596046 9272341 9000012	0 0	166577	186594	134468 166891	331/8/	0 0	332300	4161924 4	4402185	233682 2	10933 2943	33 278354	329965	387768	0	0	0
;	003.17-003.20 448 003.47-003.52 285	5.243 3.18	1.50E-23 0 0 0 2.20E-23 0 0 0	787388 740285 769920	0 0	0	0	0 0		0 0	0	198760	0 0	623808 6	0 00841 6110	81 0		0			0
Source MS fragment of 1?	002.26 - 002.27 322 002.69 - 002.72 226	2.214 2.27 5.214 2.71	5.50E-23 150969 158311 143325 7.10E-23 265216 265288 242752	0 0 0	0 0	0	0	0 0		o 0	0	0	0 0	0	0	0 0		0			0
Source MS fragment of 13? [11+H]+	001.94 - 001.99 380 002.19 - 002.25 887	0.127 1.97 7.471 2.21	1.70E-22 0 0 0 2.60E-22 0 0 0	0 0 0	0 0	0	0	0 0	597863	588450	645701	0	0 0	520451 4	0 9438 4942	0 285376 60 0	286281	260413	0	0	0
? [4+2H]2+	001.61 - 001.64 475 002.03 - 002.10 405	5.952 1.63 5.224 2.05	4.40E-22 0 0 0 0 7.40E-22 8333223 8893546 9348495	0 0 0	0 0	0	393600	377961 421760		0 0	0	0	0 0	0	0	0 0	0 0	0	0	0	0
[14+H]+ ?	001.76 - 001.82 605 003.67 - 003.75 440	6.296 1.79 0.277 3.70	8.30E-22 1379358 1503694 1685650 1.40E-21 0 0 0	0 0 0	0 0	0	0	0 0	4158355	4018050	4419473	0	0 0	1846729 18 4174024 38	72285 18008 32042 42043	53 751572 602424	699748 533332	851694 408961	0	0	0
? [5+2H]2+	003.07 - 003.10 257 001.96 - 002.02 398	7.618 3.09 8.216 1.99	1.50E-21 0 0 0 1.70E-21 2974505 3343775 324740		0 0	0	205995	197482 182912			0	0	0 0	0 0	0	0 0	0	0	0	0	0
1	000.71-000.74 486 003.67-003.75 299	5.318 0.73 9.259 3.70	3.60E-21 0 0 0 6.50E-21 0 0 0	146045 142805 129547	0 0	0	0	0 0		0 0	0	0	0 0	2570854 23	0	0 0	331520	356674			0
2	002.50-002.55 228 001.71-001.74 572	8.195 2.52 2.333 1.78	7.40E-21 3438598 3288848 3716211 1.00E-20 0 0 0	258496 14448 249920 0 0 0 0	0 0	0	0	0 0			0	0	0 0	0 0	0	0 0	0 0	0		0	0
? [17+H]+	001.70 - 001.73 602 001.57 - 001.64 406	2.367 1.70 5.270 1.61	1.10E-20 0 0 0 1.40E-20 5113933 4830936 4909572	180951 159605 177949 0 0 0 0	0 0	0	1967875 2	0 0	4375755	3772846	4292611	0	0 0	2555874 27	0	0 0	1100005	3527367	0	0	0
? [12+2H]2+	002.75-002.76 398 001.97-002.04 421	1.340 2.77 1.218 2.02	1.508-20 0 0 0		0 0	ŝ	0	0 0		0 0	0	0	0 0	124224 1	0 1277	44 0 0 1257259	1476333	1403513			0
2	002.30 - 002.32 250	0.154 2.32	2.40E-20 306250 347625 311785 3.30E-20 0 0 237493	0 0 0	0 0	0	0	0 0	1811078	0 0	1812280	817010	0 0	4154030 47	0	0 0	0	0	0	0	0
Source MS fragment of 1?	002.29 - 002.35 476	6.325 2.34	6.30E-20 2133031 2144508 2423181	0 0 0	0 0	0	0		E 222106	0 0	6507711	0	0 0	0	0	0 0	0	11100001		0	0
2	001.87 - 001.90 498	1.056 1.89	6.90E-20 257489 291471 295293		0 0	ò	0	0 0		0 0	0	0	0 0		0	0 0	0	0			0
[Bottro C+2H]2+	002.39 - 002.44 419	9.740 2.41	1.102-19 852685 974188 976581	0 0 0	0 0		0			0	0	0	0 0		0	0 0		0		0	0
[2+2H]2+	002.22 - 002.29 405	5.726 2.27	1.705-19 12433168 11379711 13302029	0 0 0	0 0	0	0	0 0		0	0	0	0 0	0	0	0 0	0	0	0	0	0
13+Me? ?	002.14 - 002.16 435 003.90 - 003.96 428	5.736 2.15 8.374 3.92	2.50E-19 0 0 0 2.50E-19 0 0 0	1421901 1268275 1224383	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 163776	182848	157312		0	0
2	001.75 - 001.81 268 003.83 - 003.91 299	8.187 1.77 9.257 3.89	4.70E-19 285075 275808 321624 5.70E-19 147712 243181 146944	0 0 0 0	0 0	0	0	0 0	724810	568300	598235	192549 :	0 0	1700126 16	0 16500 16370	62 1280789	1481209	1498897	0	0	0
2	003.67 - 003.80 452 003.94 - 004.00 313	2.279 3.78 3.274 3.97	6.20E-19 0 0 0 6.80E-19 0 0 0	911046 937208 896334 1869183 1793555 1662129	0 0	0	0	0 0		0 0	0	0	0 0	497939 5 1223691 12	02117 3643 81660 10177	52 0 97 166912	137344	219648	0	0	0
? [13+2H]2+	002.25-002.27 264 001.93-001.99 428	4.133 2.26 8.225 1.96	9.30E-19 241452 276736 237650 1.40E-18 946606 1174805 980790	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0 1984	0 0 81 7723451	8101721	9182198	0	0	0
2	002.03 - 002.04 606 003.83 - 003.88 672	6.322 2.04 2.383 3.87	1.40E-18 0 0 0 1.40E-18 0 0 0	168563 157626 187279 189630 231074 206391	0 0	0	0	0 0		0 0	0	0	0 0 0 0	0 269798 2	0 10487 2457	0 0 14 200033	184601	0 210497	0	0	0
(9+H)+ ?	001.95 - 002.01 627 001.19 - 001.19 552	7.375 1.96 2.790 1.20	2.20E-18 883139 914169 95172 2.60E-18 0 0 0	0 0 0 0	0 0	0	0	0 0	220853	220870	253835	0	0 0 0 0	0	0	0 0	124288	0	0	0	0
? [13+H]+	001.10-001.15 499 002.05-002.11 855	9.323 1.11 5.445 2.08	2.70E-18 171679 148032 17588 2.70E-18 0 0 177048	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0 142303	0 1879	03 2402351	2653834	2335216	0	0	0
[11+2H]2+ [11+N#]+	002.19 - 002.25 444 002.20 - 002.22 901	4.237 2.22 9.453 2.22	4.60E-18 0 0 0 5.20E-18 0 0 0		0 0	0	0	0 0	223814	1350272 245841	1551301 222238	0	0 0	1372603 14 231431 2	15704 12152 1778 1853	46 0 66 0	133888	0	0	0	0
2	003.29 - 003.37 401 003.78 - 003.84 401	1.338 3.33	5.70E-18 396254 286080 378433 5.70E-18 0 0	1443785 1430370 1563330 2006760 1115582 7083	0 0	0	0	0 0	763764	775857	795032	507350 :	0 0	495861 4	0 0	14 1093562	1219847	1285170	140353	163584	0
[1+2H]2+ [13+2H]2+	002.28 - 002.36 412 002.04 - 002.11 428	2.734 2.34 8.727 2.08	5.90E-18 27125669 29809169 32798181 7.20E-18 260099 371520 998793	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0 160256	0 2100	63 3250010	137071	0	0	0	0
Source MS fragment of 1? [14+2H]2+	002.30 - 002.36 278 001.77 - 001.82	1.08 1.150 2.33 1.650 * ***	1.20E-17 755703 620972 678071 1.60E-17 986772 01113	0 0 0	0 0	0	0	0 0		24363	21610	0	0 0	736060	0	0 0 38 47***	160100	338240	0	0	0
;	003.46-003.53 426	1.40 1.262 3.49	1.60E-17 0 0 0 1.80E-17 5378E	1261964 1104865 1294579 216887 247100 2711	0 0	9	0	0 0		0 0	°,	0	- 0	1064395 9	87930 8557	74 0	0	0	0	0	0
2	002.00 - 002.02 212	2.164 2.01	2.10E-17 376847 312473 372850	0 0 0	0 0	0	0	ě č		0	0	0	0 0	0	0	0 0		0		0	0
source MS fragment of 2?	002.24 - 002.28 377 002.57 - 002.58 468	2.27 1.265 2.57	2.70E-17 405029 329369 364756 2.80E-17 0 0 0	0 0 0	0 0	0	0	0 0		0	0	0	0 0	0	0	0 110848	117120	0 134972	0	0	0
7 Tail of [1+2H]2+	001.73 - 001.79 624 002.53 - 002.59 412	4.303 1.77 2.233 2.57	2.90E-17 0 0 0 3.70E-17 170368 141568 143936	0 0 0 0 0 0	0 0	0	0	0 0	246225	305743	280816	0	0 0	137107 1	0 1308	69 0 0 0		0	0	0	0
2	003.79 - 003.81 283 001.41 - 001.42 387	3.264 3.81 7.234 1.41	3.70E-17 0 0 0 4.90E-17 0 0 0	277819 341760 324416 0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 132140	143966	163141	0	0	0
? [7+Na]+	001.86 - 001.95 489 001.93 - 001.99 621	9.305 1.90 1.328 1.97	6.20E-17 0 0 0 6.20E-17 0 0 0	611604 619789 601597 205081 208231 170277	0 0	0	0	0 0	421495	456420	321268	458032 4	0 0	415406 4	0 0	45 617576 0 0	552554	586298	0	0	0
2	001.84 - 001.87 565 000.85 - 000.94 470	5.309 1.85 0.325 0.87	1.20E-16 232912 187904 216003 1.30E-16 0 0 0	0 0 0 0 196501 173890 220224	0 0	0	0	0 0	271332	220992	256949	0	0 0	0	0	0 203765	0 192829	170101	0	0	0
? [14+K]+	003.68 - 003.74 422 001.79 - 001.80 644	2.268 3.70 4.258 1.80	1.40E-16 0 0 0	450441 354462 463431 0 0 0 0	0 0		0	0 0	145772	0 0	123805	0	0 0	372454 3	57838 3802 0	0 0	0	0			0
2	001.55 - 001.60 502	2.270 1.61	2.305-16 0 0 0	170508 196875 145943	0 0	0	0	0 0	185393	0 0	200843	0	0 0	0	0	0 165437	179504	177523	0	0	0
[19+H]+	001.24 - 001.50 344	4.252 1.41	3.205-16 192842 182863 278604	1626285 1784872 1832023	0 0	0	186202	217895 213688	528230	559424	589066	718177 (	560136 818581	1297284 11	51716 12028	937643	1097045	1221078		0	0
[10+2H]+ [10-2H]2+	002.10 - 002.15 873	1.452 2.13	6.50E-16 0 0 0	0 0 0	0 0	0	0	0 0	449147	576062	517434	0	0 0	0	0	0 0	0 0	0			0
18 + Me?	001.64 - 001.85 392	2.251 1.75	7.405-16 0 0 0	500556 446453 467496	0 0	0	0	0 0	180299	204928	185041	176173	132802 245187	428472 4	19552 4270	94 330053	444445	402218	0	0	0
2	002.68 - 002.71 587	7.189 2.69	8.40E-16 398829 393348 465693	481240 462565 514663	0 0	0	346215	0 0	622791	526030	572619	224721 :	18186 224360	400512 5	0842 3444	40 469560	506397	448017		0	0
19+Me? Source MS fragment of 1?	001.50 - 001.63 358 002.30 - 002.35 363	1.269 1.61 3.243 2.33	8.70E-16 0 0 0 9.20E-16 624959 776742 791246	840878 925250 978372 0 0 0	0 0	0	0	0 0	184392	209698	181416	327215 :	0 0	586122 5	0	0 0	468838	531038		0	0
2	003.98 - 004.00 383 002.04 - 002.05 643	1.984 4.00 1.382 2.05	1.10E-15 150592 184256 192960 1.10E-15 123670 152699 123005	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0		0	0 0		0			0
7 [13+Na]+	001.99 - 002.01 562 002.06 - 002.10 877	2.850 2.00 7.422 2.08	1.40E-15 0 0 0	259895 318848 254572 0 0 0	0 0	0	0	0 0		o o	0	0	0 0	0	0	0 314646	260372	248098	0	0	0
2	003.87 - 003.92 372 002.07 - 002.12 721	2.345 3.89 1.417 2.10	1.50E-15 0 0 0 1.60E-15 0 0 0	743632 651701 678930 819782 897570 931612	0 0	0	0	0 0	326131	338790	413291	343768 213248 2	1999402 239296 199990 280252	182074 2	0 2799	0 0 10 471447	499081	59086/7	0	0	0
2	001.47 - 001.53 454 003.27 - 003.29 392	4.208 1.51 2.293 3.28	1.70E-15 0 0 0 1.70E-15 0 0 0	171517 131968 151307 0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 140608	0 0	178597	0	0	0
2	003.61 - 003.64 482 003.93 - 004.00 466	2.395 3.63 5.295 3.97	1.70E-15 0 0 0 2.00E-15 0 0 0	0 0 0 401420 497047 481388	0 0	0	0	0 0		0 0	0	176587 2	0 0	0 259851 3	0 2386	0 218624 67 0	210438	240896	0	0	0
Source MS fragment of 1?	002.31-002.33 313 003.39-003.43 330	3.104 2.32 0.263 3.40	2.20E-15 376798 288697 332000 2.40E-15 355309 269504 259072		0 0	0	0	0 0		0 0	0	0	0 0	0 433281 5	0	o 0 52 0		0			0
[16+2H]2+ [15+2H]2+	002.09-002.14 369 001.96-002.02 362	9.172 2.11 2.164 1.98	2.60E-15 1675807 1430807 1532422 2.80E-15 752289 817967 741096		0 0	0	0	0 0	817021	175488	768063	0	0 0	0	0 1308	80 150656 0 0	0	0		0	0
[14+2H]2+ 2	001.77-001.82 304	4.153 1.79	2.90E-15 419968 286024 399530 3.40E-15 328537 343920 300980	0 0 0	0 0	0	0	0 0	1105081	955011	1127409	0	0 0	512545 4	15590 3636	49 192211	164602	127616	0	0	0
[6+H]+	000.96 - 001.13 452	2.283 1.09	3.502-15 0 0 177470	1192072 1247816 1454656	0 0	0	0	0 0	0	0 0	0	300571	144456 367226	0	0	0 0	0 0	0			0
2	003.80 - 003.85 601	1.374 3.81	5.208-15 253381 282381 222464		0 0	0	0	0 0	148699	148096	127552	117056	161094 149208	0	0	0 197683	176384	173376	0	0	0
[2+01+K]2+	002.25 - 002.25 398	4.191 2.27	6.20E-15 928126 845504 699968 6.20E-15 245575 325452 299250	0 0 0	0 0	0	0	0 0			0	0	0 0	0	0	0 0		0	0	0	0
7 [13+H]+	002.94 - 002.96 327 001.94 - 002.00 855	7.066 2.95 5.442 1.96	8.50E-15 257567 282880 339411 9.40E-15 0 251266 289272	0 0 0	0 0	ő	0	0 0		0 0	ê	0	0 0	0	0	0 1993520	2537900	2451411		0	0
2	001.74 - 001.81 398 000.70 - 000.95 295	5.163 0.88	9.70E-15 1442677 1677468 1579380 1.00E-14 1455255 1416117 1532180	624398 605335 752336 916053 936786 876529	0 0	°	1506033 1	0 139383	544958 842300	092469 720578	574594	944300 1	159923 560508 297687 818292	566578 5 646715 6	0906 5873 75311 8719	40 504803 63 904557	953267	671747 954205		225011	0
? [15+2H]2+	001.74 - 002.83 228 001.97 - 002.00 362	1.195 1.77 2.666 1.99	1.00E-14 813547 1004057 1016733 1.30E-14 430016 364726 321664	282112 332480 331776 0 0 0	0 0	124096	0	0 0		0 0	0	0	0 0	0	0	0 0	0 0	0	0	0	0
2	002.39-002.67 537 001.76-001.89 457	7.303 2.49 7.337 1.82	1.30E-14 253415 342487 278792 1.30E-14 0 0 0	828818 763914 832516 506300 536973 551513	0 0	0	0	0 0	607261 160384	490096	619548 162085	579765 5	575658 566206 193314 361797	333642 4 306731 3	54240 4309 70352 3434	47 591810 87 360144	480168	488336 305502	0	0	0
[14+Na]+ ?	001.74 - 001.83 628 002.66 - 002.72 571	8.279 1.80 1.216 2.69	1.40E-14 376411 399618 288311 2.60E-14 1851045 1863900 1869880	0 0 0	0 0	0	0	0 0	916311 2397531	903379 2456589	793109 2395119	1380634 1	0 0	463702 4 2217169 21	M372 5592 0207 18947	17 392015 71 2549049	220045	252212 2036407	0	0	0
16+Me? ?	001.77-001.83 620 002.60-002.64 464	0.306 1.80 4.263 2.62	2.80E-14 0 0 0 4.20E-14 406746 433314 401705	0 0 0 0 0 254744 287166 283281	0 0	0	0	0 0	587532	555358 524896	537839 612955	325271 2	0 0	199749 1 303767 2	18995 1733 56716 3643	12 127010 23 780471	714187	714441	0	0	0
? Source MS fragment of 1?	003.84 - 003.89 637 002.24 - 002.36 348	7.345 3.88 8.136 2.33	4.90E-14 0 0 0 5.10E-14 2127936 2048237 2713408	169092 232351 173644 0 0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	208864 2	0 2425	03 239384	180544	227602	0	0	0
1	003.47 - 003.55 430 000.76 - 001.53 213	0.201 3.54 3.158 1.29	5.60E-14 176896 129251 147520 7.90E-14 0 0 0	0 0 0 0 586051 613314 715037	0 0	0	0	0 0	372393	0 0	329829	0 283294 2	0 0	400201 4	0 3261 5306	0 0	555942	500570		0	0
Source MS fragment of 2?	002.23-002.29 462	2.304 2.27	8.002-14 1028974 832809 1156898	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0 22305 2043	0 0	258965	323189		0	0
Source MS fragment of 1?	002.25 - 002.35 471	1.203 2.34	8.702-14 856304 810961 62182	0 0 0	0 0	0	0	0 0			0	0	0 0	0	0	0 0	0	0			0
O-demethyl bottro C? Source MS frammer of 141	002.14 - 002.15 412 001.77 - 001.81	2.229 2.14	8.805-14 210432 154048 169984 9.605-14 186368 340040 1001	0 0 0	0 0	0	0	0 0	6.014	75577	731594	0	0 0	242369	0	0 0		0	0	0	0
2	002.12 - 002.15 728	1417 2.14 2.826 1.71	1.005-13 0 0 0	0 0 0	0 0	0	0	0 0	30101	0 0	0	0	0 0	0	0	0 279047	328217	388071	0	0	0
?	001.17 - 001.27 536	1.016 1.25 6.026 1.25	1.205-13 1011187 855896 821135	195254 154176 0	0 0	9	0	0 0		0 0	0	300576	153677 277496	0	0	0 0	0	0		0	0
[11+H]+ isotope	002.20-002.23 888	1470 2.22 2.205 1.01	1.30E-13 0 0 0	0 0 0	0 0	0	0	0 0	359512	291703	380243	0	0 0	303708 2	54048 3742	77 0	0 0	0	0	0	0
+Na for 540.37	001.70 - 001.79 562	2.353 1.75	1.40E-13 0 0 0	459029 405169 498887	0 0	0	0	0 0	262801	310185	305383	414095	439016 106581 329511	647589 7	15338 5615	32 507018	424612	427104	0	0	0
2	002.39 - 002.41 252	2.193 2.40	2.30E-13 166848 220096 163840 2.70E-13 0	0 0 0	0 0	0	0	0 0	10100	0 0	0	0	0 0	0	0	451526	0	0		0	0
2	003.95 - 003.98 311	1.259 3.97	2.80E-13 0 0 0	275392 308939 308608 553088 572440 681172	0 0	0	0	0 0	160192	193152	196864	0	0 0	252800 2	12048 3212 14801 601	16 454921	434687	328320	0	0	0
? [16+2H]2+	000.70-000.83 614	4.272 0.78	4.205-13 731715 775237 72480	684885 650490 720958	0 0	113920	448217	414698 440265	440893	475220	442188	512417	335068 550111	477829 4	73479 6173	23 449327	379326	393718		0	0
2.00%2012* ?	002.10 - 002.13 369 002.29 - 002.32 493	1.746 2.11 1.746 2.32	4.40E-13 0 0 0		0 0	0	0	0 0	236288	3/6550	206430	0	0 0	387200 3	05298 2757	76 0	ŝ	0	0	0	0
[12+2H]2+ ?	001.99 - 002.04 421 001.44 - 001.46 517	1./18 2.01 7.780 1.46	5.00E-13 1101680 763264 1081414 5.60E-13 206272 203520 146688	0 0 0	0 0	0	0	0 0		0	0	0	0 0	0	0	0 687868	006368	815931	0	0	0
2	u31.62 - 001.80 475 002.49 - 002.54 478	1.70 1.297 2.52	6.10E-13 132475 0 163463 6.10E-13 201927 189691 304765	1034804 1364685 1294615 357329 262968 336268	0 0	0	0	0 0	492439	289064	360963 369024	375115 4	163406 163840	538998 4 1001903 9	4608 38116 11907	34 514770 74 441936	547530 412276	592735 480173	0	0	0
;	ud1.00 - 001.18 243 002.67 - 002.69 804	1.152 1.12 4.437 2.69	6.30E-13 320193 354726 302984 6.30E-13 0 0 0	248837 253814 318467 0 0 0	0 0	0	0	0 0	432940	362426	347366 127447	225611 :	0 0	301311 2 0	0	0 0	375491	450447	0	0	0
5 [8+94]+	001.82 - 001.88 613 003.08 - 003.09 298	1.366 1.84 8.632 3.08	6.80E-13 844255 1097787 857663 6.90E-13 0 0 0	210385 148480 172198 0 0 0	0 0	0	0 202496	0 0		0 0	0	129819 0	0 0 0 0	0	0	0 160357	183315	224301 0	0	0	0
2	003.19-003.20 518 001.63-001.69 555	8.275 3.20 5.962 1.66	7.00E-13 217335 162475 218674 7.50E-13 0 0 0	211886 291859 222848 302417 279552 253248	0 0	0	0	0 0	280861	264346 299840	273439 206848	0	0 0 0 0	225865 2 276587 2	16748 1998 16512 2762	00 292903 46 463988	242906 413246	275434 515800	0	0	0
2	001.28 - 001.32 277 003.19 - 003.24 376	7.153 1.28 6.322 3.22	1.10E-12 0 0 0 1.20E-12 226240 332480 295680	0 0 0	0 0	0	0	0 0		0 0	0	0	0 0 0 0	0	0	0 162834	111040	137344	0	0	0
2	001.30 - 001.33 669 001.50 - 001.67 227	9.390 1.32 7.174 1.63	1.20E-12 0 0 0 1.20E-12 0 0 0	0 0 0 450717 471679 565210	0 0	0	0	0 0	219990	284041	296840 130560	0 176320	0 0	0 339879 3	0 18592 3579	0 338458 38 282679	475877 300938	480509 361660	0	0	0
[13+Na]+ ?	001.95 - 001.99 877 002.93 - 002.97 326	7.425 1.96 6.073 2.95	1.20E-12 0 0 0 1.30E-12 495692 337128 448287	0 0 0 0 0 0	0 0	0	0	0 0		0 0	0	0	0 0	0	0	0 355306	242093	299040	0	0	0
Source MS fragment of 1? ?	002.24 - 002.35 461 000.50 - 000.62 241	1.222 2.34 3.133 0.55	1.50E-12 708464 509168 740915 1.50E-12 322435 381701 311276	0 0 0 0 513279 568759 687641	0 0	0	0	0 0	341632	408192	443960	325734	0 0	0 442314 3	0 4847	0 0 37 4160×0	475177	512835	0	0	0
[11+2H]2+ ?	002.20 - 002.23 444 001.05 - 001.14 516	4.741 2.21 6.046 1.10	1.50E-12 0 0 0 1.80E-12 228962 243520 374779		0 0	0	0	0 0	680384	602880	811876	0	0 0	646026 8	0 5886	05 0	0	0	0	0	0
2	002.50 - 002.54 456 001.29 - 001.18 ***	6.247 2.51 6.231 1.54	1.80E-12 216424 185812 199625 1.80E-12 158004 136203 198640	0 0 0	0 0	0	0	231873 178****	327544	448623 683248	379922	430890	0 0	240395 2	10962 1527	59 348256 24 9726**	321949	262144	0	0	0
?	001.76 - 001.76 516	6.318 1.78 1.153 1.78	2.005-12 0 0 0	0 0 0	0 0	0	0	0 0	130689	186437	140606	0	0 0	0	0	0 0	0 0	0	0	0	0
? [70#H]a	001.63 - 001.53 301	1.47 5.626 1.66 1.236	2.40E-12 0 0 0	291725 263104 312320 1091824 102240	0 0	0	0	0 0	163456	178112	219904	0	0 0	318150 2 861733	3762	3/2281 20 456786 31 8004	402452	584704	0	0	0
5 Immetile	003.45 - 003.47 782	2.457 3.46	3.00E-12 0 0 0	256208 170702 215520	133881 0	0	103670	0 0	409095	460745	0	0	0 0	0	0	C 0	6/1417	1044057	0	0	0
f Source MS fragment of 13?	002.05 - 002.09 380	0.253 1.89 0.125 2.08	3.802-12 0 0 0 4.402-12 0 0 0	3d8146 344287 428735 0 0 0	0 0	0	0	0 0		0 0	0	0 :	0 0	153728 1	0 1784	0 262144	186475	204845 205464	0	0	0
2	002.99 - 003.04 514 002.67 - 002.71 566	4.252 3.02 5.261 2.70	<.501-12 520768 524446 500014 4.601-12 247185 285576 262336	303190 251480 207488 183744 321850 301649	0 0	0	0	153088 C	463090	584949 417556	506290 388816	132140 0	0 0	452196 4 283239 2	10413 4019 18176 2915	49 337420	516262 404568	490196 329678	0	0	0
2	003.92 - 003.98 687 001.26 - 001.36 450	7.410 3.98 0.739 1.34	4.60E-12 240019 162919 171165 5.60E-12 144064 0 160512	0 0 0 130560 0 0	0 0	0	170108 184337	156229 207876 179776 201125	812116	843330	912102	0 583035	0 0	268992 2	0 2721	28 965049	1136203	1084176	0	0	0
Source MS fragment of 1? ?	002.30 - 002.33 268 001.39 - 001.55 413	1.165 2.33 3.272 1.50	5.70E-12 219968 315724 32987 5.80E-12 0 0 0	0 0 0 0 532544 568530 412754	0 0 0 0	0	0	0 0	239043	0 0 1 247504	0 249173	0 225177 :	0 0	0 544772 3	0 3682 3686	0 0 54 374418	393729	363089	0	0	0
2	003.30 - 003.33 482 001.25 - 001.39 316	2.372 3.31 5.222 1.33	6.10E-12 0 0 0 6.20E-12 0 0 150592	0 0 0 630972 669336 560723	0 0	0	0	0 0	356560	0 522570	387966	0 296865	0 0	532215 4	0 78981 6120	0 146688	220651	172982 660866	0	0	0
2	002.79 - 002.82 379 001.38 - 001.55 350	9.243 2.81 0.204 1.42	6.30E-12 0 0 0 6.50E-12 134447 0 0	0 0 0 512526 453889 424170	0 0	0	372138 0	389194 536136 118187 0	370683	0 0 434150	0 369243	0 272256	0 0	0 417723 3	0 18493 4289	0 0 77 482045	539857	0 482433	0	0	0
[1+H+K]2+ [14+H]+	002.31-002.35 431 001.57-001.65 606	1.704 2.34 6.294 1./4	7.30E-12 261862 366750 256793 1.00E-11 373693 415493 317504	0 0 0 0 0 0	0 0	0	0	0 0	402777	441626	465393	0	0 0	0 399314 3	0	0 0	0 0	0 218506	0	0	0
2	001.82 - 001.93 431 001.48 - 001.51 433	1.264 1.88 1.276 1.50	1.20E-11 0 0 0 1.30E-11 0 0 0	260558 298793 347803 188462 164983 120704	0 0	0	0	0 0	186880	253977	272830	176130	164652 259072	382254 2	0 3558	92 251985	292151	294897	0	0	0
2	003.69 - 003.70 462 001.41 - 001.48 444	2.257 3.71 8.745 1.47	1.30E-11 0 0 0 1.50E-11 216192 261931 26383	120931 187392 186688 0 0	0 0	0	0	0	57937	689184	814918	0	0 0	121216 1 133122	0 1041	28 0 0 2014**	271830	259679	0	0	0
?	001.39 - 001.44 612	2.368 1.42	1.80E-11 206277 211483 174448 1.80E-11 156800 116008	0 0 138322	0 0	0	0	0 129133	608325	507570	576278	201970	160384 201627	0	0	0 316136	380593	386660	0	0	0
[5-Me+2H]2+ [12+2H]2+	001.96 - 001.99 391 001.87 - 001.91 421	1.205 1.97 1.719 1.89	1.90E-11 470365 729508 568028 2.10E-11 289024 282880 265344		0 0	0	0	0 0				0	0 0	0	0	0 472512	688108	459648	0	0	0

**Table S5**Selected untargeted metabolomic data across all strains<sup>a</sup>.

<sup>a</sup> The dataset was generated from triplicate LC-MS data using Profiling Solution 1.1 (Shimadzu). All species that appeared in either  $\Delta btmD$  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 S2MS 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  $\Delta btmD$  ( $\Delta D$ ) 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  $\Delta btmC$ ,  $\Delta btmD$ ,  $\Delta btmF$ ,  $\Delta btmI$  and  $\Delta btmJ$  mutant strains, generated using Cytoscape 2.8.3. All red nodes are species observed in one or more of the WT,  $\Delta btmC$ ,  $\Delta btmF$ ,  $\Delta btmI$  and  $\Delta btmJ$  strains, but not in  $\Delta btmD$ . 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  $\Delta btmD$  that are in other areas of the network could either reflect a degree of natural variation between MS data or asyst-uncharacterized metabolites related to the bottromycin pathway.



**Figure S6** LC-MS<sup>2</sup> 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<sup>2</sup> data for truncated cyclized metabolite **6**. The starred peak is an unrelated metabolite with the same mass as **6**. Data is from *S. scabies*  $\Delta btmC$ .



**Figure S8** LC-MS<sup>2</sup> 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 Δ*btmI*.



**Figure S10**  $MS^2$  and  $MS^3$  data for **10** and **11** from *S. scabies*  $\Delta btmI$ .



**Figure S11** LC-MS<sup>2</sup> data for carboxylated bottromycins (CBs) **12** and **13**. All data is from *S. scabies*  $\Delta btmJ$ .



**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<sup>2</sup> 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<sup>2</sup> 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<sub>2</sub>O and (2) 40 mM DCl in D<sub>2</sub>O; and then H<sub>2</sub>O. The D<sub>2</sub>O 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<sub>2</sub>O. However, the deuterium in the  $\alpha$ -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 S15LC-MS<sup>n</sup> data for 14 produced by wild type S. scabies.



Figure S16 LC-MS<sup>n</sup> data for 15 and 16 produced by wild type *S. scabies*.



**Figure S17** LC-MS<sup>n</sup> data for **17** produced by *S. scabies*  $\Delta btmC$ . A fragmentation pathway for m/z 241.19 is proposed, although an alternative fragmentation route could place the additional methyl group on the other value.



**Figure S18** LC-MS<sup>2</sup> data for peptide metabolites deriving from the middle of the bottromycin core peptide. All data is from *S. scabies*  $\Delta btmC$ . Loss of CO from a peptide b ion results in a loss of 27.99 Da.



**Figure S19** LC-MS<sup>n</sup> 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<sup>n</sup> pattern is characteristic for metabolites that feature the macrocyclic amidine.



**Figure S20** LC-MS<sup>n</sup> data for molecules featuring the same macrocyclic amidine as bottromycin  $A_2$  (1). These all provide an MS<sup>3</sup> pattern that is consistent with the fragmentation pathway detailed in Fig. S19.



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



**Figure S22** LC-MS<sup>n</sup> data for molecules featuring the same macrocyclic amidine as bottromycin  $D_2$  (3). These all provide an MS<sup>3</sup> 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<sub>6</sub>-tagged BtmD is catalyzed by BtmM. MS data from 7 - 8 min is shown. The molecular weight of BtmD-His<sub>6</sub> is 6,937.6 Da (calc.  $[M+4H]^{4+}$  m/z = 1,735.4; calc.  $[M-Met+4H]^{4+}$  m/z = 1,702.6).



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



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



**Figure S28** In vitro BtmB activity with 4 (*O*-desmethyl bottromycin A<sub>2</sub>).

### 4. Supplementary References

- [1] D. J. MacNeil, K. M. Gewain, C. L. Ruby, G. Dezeny, P. H. Gibbons, T. MacNeil, *Gene* 1992, 111, 61–68.
- [2] W. Crone, F. J. Leeper, A. W. Truman, *Chem. Sci.* **2012**, *3*, 3516-3521.
- [3] N. M. Vior, C. Olano, I. García, C. Méndez, J. A. Salas, *Microbiology* **2014**, *160*, 467–478.
- [4] L. T. Fernández-Martínez, J. P. Gomez-Escribano, M. J. Bibb, *Mol. Microbiol.* 2015, 97, 502–514.
- Y. Kobayashi, M. Ichioka, T. Hirose, K. Nagai, A. Matsumoto, H. Matsui, H. Hanaki, R. Masuma, Y. Takahashi, S. Ömura, et al., *Bioorg. Med. Chem. Lett.* 2010, 20, 6116–6120.
- [6] M. S. Paget, L. Chamberlin, A. Atrih, S. J. Foster, M. J. Buttner, J. Bacteriol. 1999, 181, 204–211.