Electronic Supplementary Information:

Posttranslational β-methylation and macrolactamidination in the biosynthesis of the bottromycin complex of ribosomal peptide antibiotics

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Experimental Procedures

Analytical methods

2µl of spent culture supernatant were analysed directly by LC-MS using a Dionex 3000RS UHPLC coupled with a Bruker MaXis Q-TOF mass spectrometer. An Agilent Zorbax Eclipse plus column (C18, 100x2.1mm, 1.8µm) was used. Mobile phases consisted of A (water with 0.1% formic acid) and B (methanol with 0.1% formic acid). After 5 minutes of an isocratic run at 10% B, a gradient of 10% B to 100% B in 12.3 minutes was used, followed by an isocratic run at 100% B for 5 minutes with a flow rate at 0.2ml/min. The UV detector was set at 240nm. The mass spectrometer was operated in electrospray positive mode with a scan range of 50-2,000 m/z. Source conditions were: end plate offset at -500V; capillary at -4500V; nebulizer gas (N_2) at 1.6bar; dry gas (N_2) at 8L/min; dry temperature 180 °C. Ion transfer conditions were: ion funnel RF at 200Vpp; multiple RF at 200Vpp; quadruple low mass set at 55m/z; collision energy at 5.0ev; collision RF at 600Vpp; ion cooler RF at 50-350Vpp; transfer time set at 121µs; pre-pulse storage time set at 1 µs. Calibration was done with sodium formate (10mM) through a loop injection of 20µL of standard solution at the beginning of each run.

Strains and growth conditions

Strains used and generated in this study are listed in Table S2. *Escherichia coli* strains were grown and manipulated following standard methods (Sambrook *et al*., 1989; Gust *et al*., 2003; Gust *et al*., 2004). *S. bottropensis* strains were grown on SFM agar (Kieser *et al*., 2000). Liquid cultures were grown in 50 ml of medium in 250 ml siliconized flasks with stainless springs and were incubated at 250 rpm at 30 ºC. To assess bottromycin production, the conditions used by Lerchen *et al.*, 2006 were adopted. Spores were inoculated into Seed Medium (tap water containing 4 g/l glucose, 4 g/l yeast extract and 1 g/l malt extract) and grown for 4 days at 28 C. 1 ml of the seed culture was used to inoculate 50 ml of Production Medium (distilled water containing 10 g/l glucose, 15 g/l soluble starch, 5 g/l yeast extract, 10 g/l soya meal (defatted), 5 g/l NaCl and 3 g/l $CaCO_3$) and incubated at 28 C for 7 days.

Nucleic acid manipulations

Nucleic acid manipulations were performed using standard methods (Sambrook *et al*., 1989) and according to the manufacturers of restriction enzymes and kits. *S. bottropensis* genomic DNA was isolated using the salting-out method of Kieser *et al*. (2000). Roche 454 sequencing was performed by The Genome Analysis Centre (www.tgac.ac.uk, Genome Enterprise Limited, Norwich Research Park, Norwich, UK). A quarter plate run resulted in 463 contigs of which 427 where larger than 500 bp; the average contig size was 20,702 bp, the largest contig was 183,403 bp, and a total of 8,840,013 bp of genome sequence were obtained. Contig sequences were analysed with Artemis (Rutherford *et al.*, 2000) using the GC Frame Plot utility (derived from Bibb *et al*., 1984) and BLASTP (Altschul *et al*., 1997) to identify and annotate PCSs, respectively. Adjacent contigs were identified by “bridge by BLAST” (see text).

Mutant construction, confirmation and complementation

S. bottropensis M1331. A 2 kb fragment containing most of *bmbB* was amplified by PCR using primers BTM01 and BTM02, cloned in pGEM-T Easy and confirmed by sequencing. The fragment was isolated after digestion with *Hin*dIII and *Xba*I and cloned into the same sites of pKC1132 to yield pIJ12601 which was introduced into *S. bottropensis* by conjugation (Gust *et al*., 2003; 2004). Selection for apramycin resistance resulted in strain M1331. To confirm integration of pIJ12601 by single cross-over recombination, marker rescue was performed. Total DNA was isolated

from M1331, digested with *Eco*RI (pIJ12601 contains a single *Eco*RI site), self-ligated with T4-DNA ligase and used to transform *E. coli* DH5α. Selection with apramycin led to the isolation of pIJ12604 (Fig. S1). Sequencing with universal primers M13F and M13R, which anneal on either side of the pKC1132 multi-cloning site, confirmed the desired disruption of the putative *bmbB-K* operon.

S. bottropensis M1332. A 2 kb fragment containing *bmbD* and the N-terminal portion of *bmbE* was amplified by PCR using primers BTM03 and BTM04, cloned in pGEM-T Easy and confirmed by sequencing. The fragment was isolated after digestion with *Xba*I and *Eco*RI and cloned into the same sites of pKC1132 to yield pIJ12602 which was introduced into *S. bottropensis* by conjugation as for pIJ12601. Selection for apramycin resistance yielded strain M1332. To confirm integration of pIJ12602 by single cross-over recombination, a similar marker rescue experiment was carried out. Sequencing with universal primers M13F and M13R confirmed the desired disruption of the putative *bmbB-K* operon.

S. bottropensis M1333 (ΔbmbC::neo). The 2 kb *Xba*I-*Eco*RI *bmbDE* fragment of pIJ12602 was cloned in *Xba*I-*Eco*RI cleaved pIJ12601 to yield pIJ12613. The kanamycin-resistance gene *neo* of pTC192km (Rodríguez-García *et al.*, 2006) was extracted with *Xba*I and cloned in the *Xba*I site of pIJ12613 to yield pIJ12603. pIJ12603, which contains *neo* flanked by *bmbB* and *bmbDE* and which cannot replicate autonomously in *Streptomyces*, was introduced into *S. bottropensis* by conjugation as for pIJ12601. Selection for kanamycin resistance resulted in chromosomal integration of pIJ12603 through single crossover homologous recombination. After one round of sporulation in the presence of kanamycin, but absence of apramycin (the pIJ12601 vector backbone carries the apramycin resistance gene), kanamycin-resistant apramycin-sensitive segregants were obtained. Replacement of the *bmbC* with the *neo* gene was confirmed by PCR using primers BTM05 and BTM06 and the mutant strain was named M1333.

For complementation of the mutants, the following plasmids were constructed.

pIJ12604 (containing *bmbC-bmbI*): The plasmid was constructed by marker rescue – for details, see construction of the *S. bottropensis M1331* mutant.

pIJ12605 (containing *bmbC-bmbI*): the *Bgl*II-*Xho*I fragment of the pKC1132 backbone of pIJ12064 that contains the origin of transfer for conjugation, *oriT*, was replaced with the *Bam*HI-*Xho*I fragment of pSET152 that contains the same *oriT* and also the ϕC31 integration functions.

pIJ12606 (containing *bmbA-bmbI*): A 3.8 kb fragment spanning from *bmbA* to *bmbC* was PCR-amplified using primers BTM06 and BTM07, cloned in pBluescript II KS+ digested with *Eco*RV and sequenced; the fragment was extracted by digestion with *Bgl*II and *Bsp*HI and coligated with the *Bsp*HI-*Eco*RI insert from pIJ12604 with pKC1132 digested with *Bgl*II and *Eco*RI.

pIJ12608 (pSET152 containing *bmbA-bmbK*): A 4.6 kb fragment containing the C-terminal half of *bmbJ* plus *bmbRK* was PCR-amplified using primers BTM08 and BTM09 and cloned in pBluescript II KS+ digested with *Sma*I; the fragment was extracted with *Eco*RI (one site located in *bmbJ* and another in the pBluescript multi cloning site) and ligated with pIJ12606 digested with *Eco*RI. A clone with the *Eco*RI fragment inserted in the correct orientation was identified by restriction analysis and designated pIJ12607. The 17.6 kb insert of pIJ12607 was isolated with *Bgl*II and cloned in *Bam*HI-digested pSET152. Selected clones were sequenced with the primers listed in Supplementary Table 3 to confirm the correct construction of pIJ12608.

pIJ12609 (containing *bmbC*): *bmbC* and 292 bp of upstream sequence were PCR-amplified using primers BTM05 and BTM06, the resulting fragment cloned in pGEM-T Easy and sequenced; the fragment was extracted with *Eco*RI (sites flank the site of insertion in pGEM-Teasy) and cloned in the *Eco*RI site of pSET152.

Table S1. Genes and proteins involved in bottromycin biosynthesis and their putative functions (those not likely to be involved in bottromycin production are shaded).

Gene name	*S. scabies* homolog	PFAM motifs	Proposed function in bottromycin production
	SCAB_56731	RNase PH - 3' exoribonuclease family	Not involved
	SCAB_56721	Phosphotransferase system, glucose/sucrose specific IIB subunit	Not involved
bmbT	SCAB_56711	Bacterial protein of unknown function DUF894; putative integral membrane protein, Major Facilitator Superfamily transporter	Bottromycin export and immunity
bmbA	SCAB_56701	Leucine carboxyl methyltransferase	O-methyl transferase
bmbB	SCAB_56691	B12 binding/Radical SAM	C-methyl transferase
bmbC	SCAB_56681	Contains core peptide sequence GPVVVFDC	Precursor peptide
bmbD	SCAB_56671	YcaO-like family	Thiazoline biosynthesis
bmbE	SCAB_56661	YcaO-like family (same family as microcin B17-processing protein McbD)	Thiazoline biosynthesis
bmbF	SCAB_56651	B12 binding/Radical SAM	C-methyl transferase
bmbG	SCAB_56641	Abhydrolase 1 family, alpha/beta hydrolase fold domain	Hydrolase for cyclic tetrapeptide formation
bmbH	SCAB_56631	Metallo-dependent hydrolase	Hydrolase for cyclic tetrapeptide formation
bmbI	SCAB_56621	Cytochrome P450 domain	Cytochrome P450
bmbJ	SCAB_56611	B12 binding/Radical SAM	C-methyl transferase
bmbR	SCAB_56601	Uncharacterized protein conserved in bacteria (DUF2087); DNA-binding domain of ArsR	Putative transcriptional regulator
bmbK	SCAB_56591	Peptidase M17 cytosol aminopeptidase family	Aminopeptidase
	SCAB_56581	Phosphotransferase system, EIIC Family	Not involved
	SCAB_56571	Metallo-beta-lactamase superfamily	Not involved

Table S2. Strains used and constructed in this study

Strain	Genotype or Comments	Reference
E. coli DH5α	F^- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Hanahan, 1983
E. coli ET12567/pUZ8002	*dam dcm hsdM hsdS hsdR cat tet*; carrying plasmid pUZ8002	Gust *et al.*, 2003; 2004
S. bottropensis DSM 40262	Bottromycin producer	DSMZ
S. scabies 87-22	Wild-type strain	Lambert and Loria, 1989
S. bottropensis M1331	Disruption of putative *bmbB-K* operon	This work
S. bottropensis M1332	Disruption of putative *bmbB-K* operon	This work
S. bottropensis M1333	*ΔbmbC::neo*	This work

Table S3. Oligonucleotides used in this study (incorporated restriction sites are underlined)

Name	Sequence
BTM01	GAAGCTTGTGTCATCCGTCCTTCGATT
BTM02	GTCTAGATGTCTTCTCGTCCGTCTTCC
BTM03	GTCTAGAAGGTCGTCCACCGTTCGTAT
BTM04	TGAATTCCACACCACATAGGCCGGTA
BTM05	GAAAGCGGTGGGTCTACATC
BTM06	AGGGATACGAACGGTGGAC
BTM07	GGAGATCTCGTGATCCCGGTGA
BTM08	GGGATCGAAAGTCTCAGCAC
BTM09	CTCGTCCTCACCCACGTC
KCbgl	ATGCGCTCCATCAAGAAGAG
M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13F	TCACACAGGAAACAGCTATGAC

Table S4. Vectors and constructs used in this study

Vector/Construct	Description	Reference
pBluescript II KS(+)	General cloning vector	Alting-Mees and Short, 1989
pGEM-T-easy	T-cloning vector for Taq-PCR products	Promega Corp.
pKC1132	Cloning vector, conjugative, non-integrative	Bierman *et al.*, 1992
pSET152	Cloning vector, conjugative, integrative (ϕC31 *attB*)	Bierman *et al.*, 1992
pTC192km	Source of *neo* (kanamycin resistance gene)	Rodríguez-García *et al.*, 2006
pIJ12601	pKC1132 containing most of *bmbB*	This work
pIJ12602	pKC1132 containing *bmbD* and the N-terminal portion of *bmbE*	This work
pIJ12603	pKC1132 containing *bmbB-neo-bmbDE*	This work
pIJ12604	pKC1132 containing *bmbC-bmbI*	This work
pIJ12605	pSET152 containing *bmbC-bmbI*	This work
pIJ12606	pKC1132 containing *bmbA-bmbI*	This work
pIJ12607	pKC1132 containing *bmbA-bmbK*	This work
pIJ12608	pSET152 containing *bmbA-bmbK*	This work
pIJ12609	pSET152 containing *bmbC*	This work

Figure S1. (**A**) Assembly of contigs from the draft genome sequence of *S. bottropensis* and organisation of PCSs within and flanking the bottromycin biosynthetic gene cluster. (**B**) Genetic organisation of the constructed mutants.

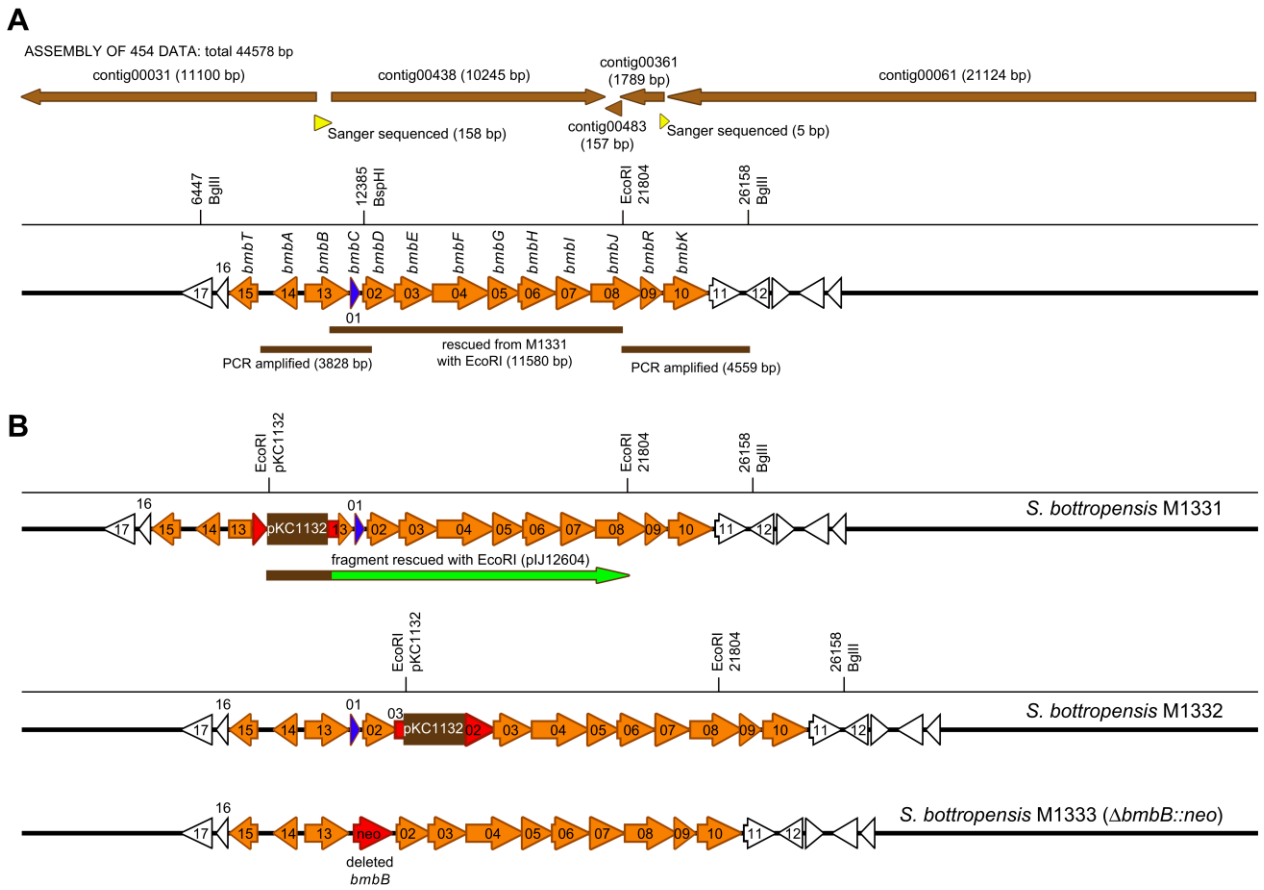


Figure S2. Bottromycin congeners produced by *S. bottropensis* and detected by LC-MS as $[M+2H]^{2+}$ ions with *m/z* = 405.2220, 398.2140 and 412.2300 for bottromycin A2, B2 and C3, respectively (Top, observed spectrum; bottom, calculated spectrum and molecular formulae).

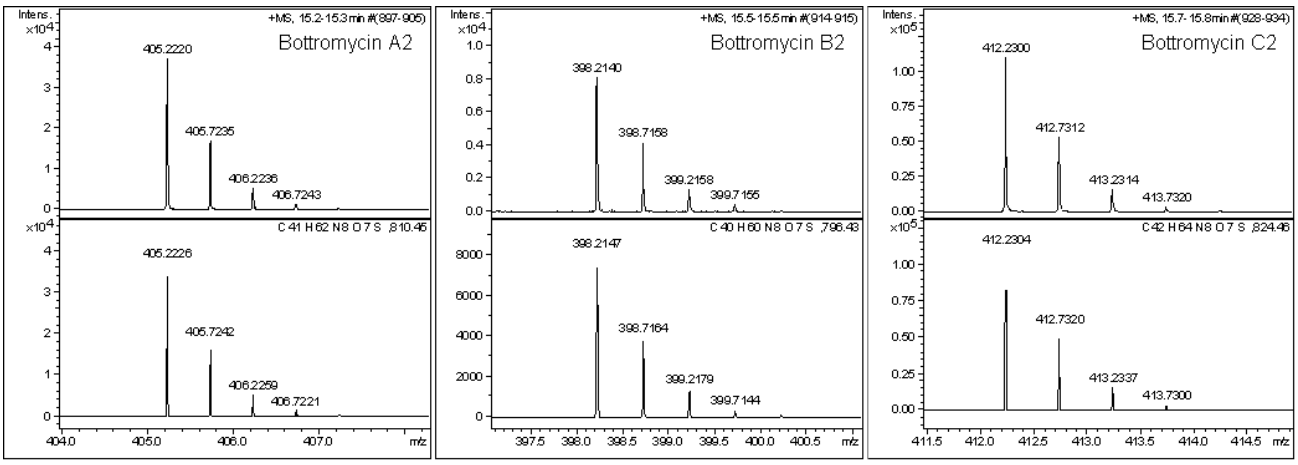


Figure S3. Analysis of production of bottromycins by mutant (left) and complemented (right) strains. Top panels show the extracted-ion chromatograms (EIC) for *m/z* = 405.2200 corresponding to the $[M+2H]^{2+}$ ion for bottromycin A2; middle panels show the EIC for *m/z* = 398.2100 corresponding to the $[M+2H]^{2+}$ ion for bottromycin B2; and bottom panels show the EIC for *m/z* = 412.2300 corresponding to the $[M+2H]^{2+}$ ion for bottromycin C2.

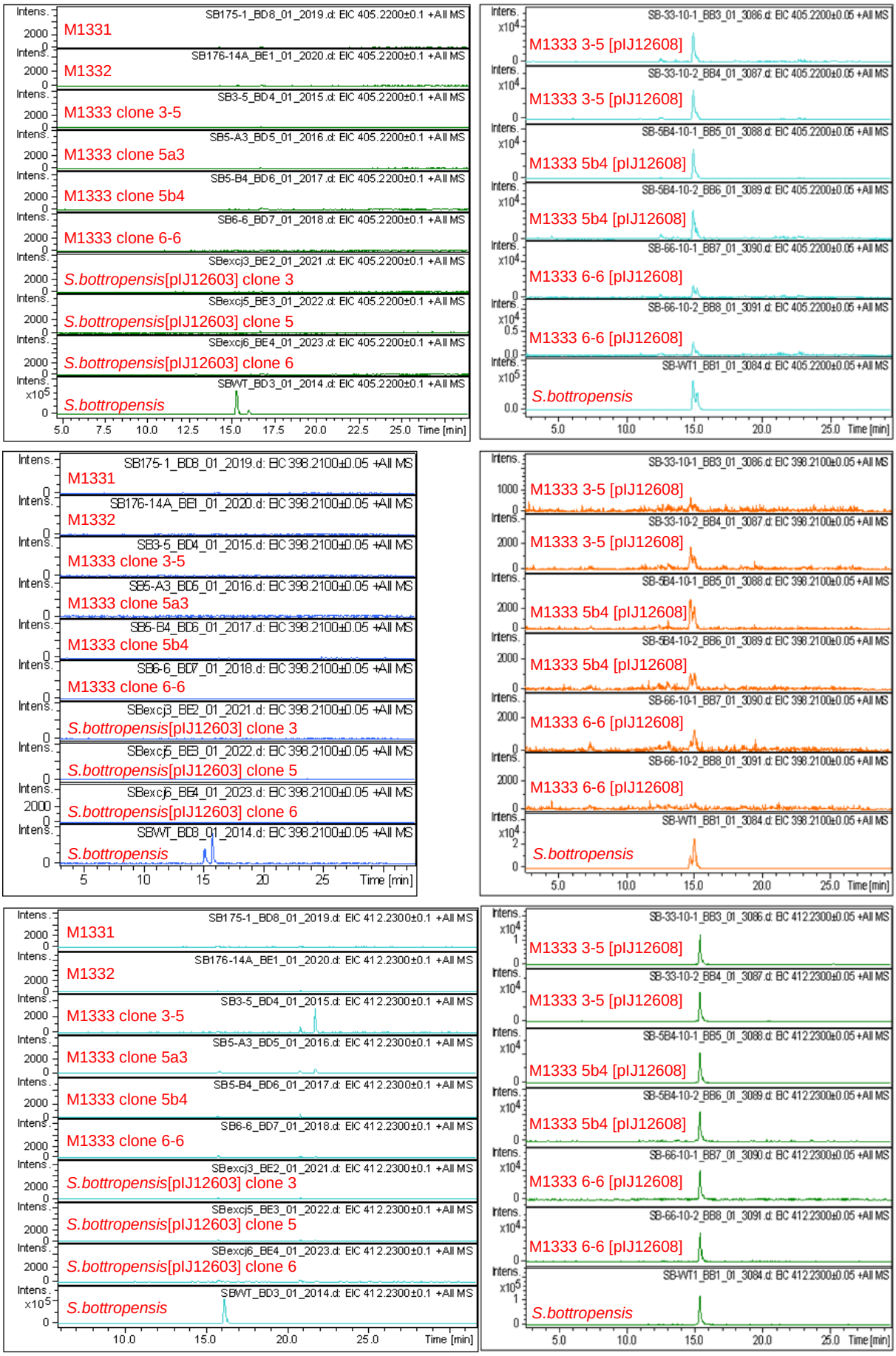
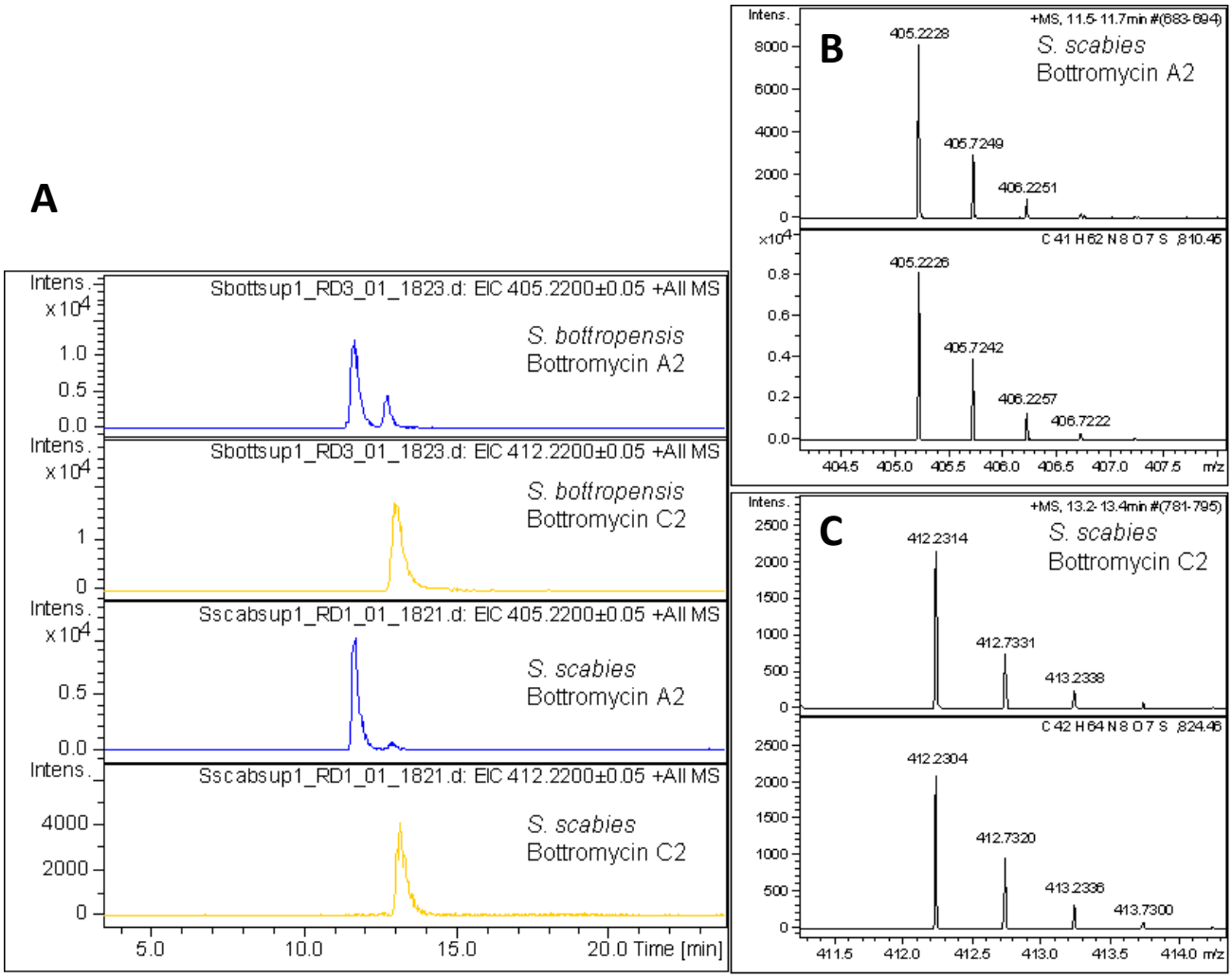


Figure S4. Bottromycin congeners produced by *S. scabies* detected by LC-MS analysis of culture supernatants. (**A**) Comparison of extracted-ion chromatograms (EIC) for *m/z* = 405.2200 and 412.2300 corresponding to the $[M+2H]^{2+}$ ions for bottromycin A2 and C2, respectively. Similar retention times and profiles were obtained for samples from *S. bottropensis* and *S. scabies*. Spectra for bottromycin A2 (**B**) and C2 (**C**) observed in samples from *S. scabies* (top panels) compared with the calculated spectrum from the expected molecular formulae (bottom panels).



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