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Bottromycins - biosynthesis, synthesis and activity

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Covering: 1950s up to the end of 2020

Bottromycins are a class of macrocyclic peptide natural products that are produced by several *Streptomyces* species and possess promising antibacterial activity against clinically relevant multidrug-resistant pathogens. They belong to the ribosomally synthesised and post-translationally modified peptide (RiPP) superfamily of natural products. The structure contains a unique four-amino acid macrocycle formed *via* a rare amidine linkage, C-methylation and a D-amino acid. This review covers all aspects of bottromycin research with a focus on recent years (2009–2020), in which major advances in total synthesis and understanding of bottromycin biosynthesis were achieved.

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1 Introduction

Bottromycins were originally isolated from the fermentation broth of *Streptomyces bottropensis* in the late 1950s and described as peptidic natural products with antibacterial activity against Gram-positive pathogens.[1,2] A series of mechanism of action (MoA) studies revealed that bottromycins inhibit protein synthesis by binding to the aminoacyl-tRNA binding site (A-site) of the prokaryotic 50S ribosome.[3–5] The bottromycin target site is currently not addressed by any antibiotic used in the clinic, which makes cross-resistance unlikely. As a result, bottromycins are effective against the problematic human pathogens methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE).

The chemical structure of bottromycins proved very difficult to elucidate and consequently underwent several revisions that ultimately led to the assignment of **1**. It showed that bottromycins are highly modified heptapeptides that are comprised of an N-terminal, four-amino acid macrocycle formed *via* a unique amidine linkage, several C-methylated residues, D-aspartate and a C-terminal thiazole (Fig. 1A). This structure was confirmed by total synthesis in 2009.[10] Shortly after the successful total synthesis, several groups reported the discovery of the bottromycin biosynthetic gene cluster (BGC), which revealed that they are unusual ribosomally synthesized and post-translationally modified peptides (RiPPs).[6,8,11,12]

The emerging RiPP superfamily encompasses highly diverse molecules with interesting bioactivities.[13] The unifying feature is their biosynthetic logic: a short structural gene is expressed and yields the precursor peptide, which consists of one or more core peptide(s) (the eventual natural product(s)) and an N-terminal leader or C-terminal follower peptide that is important for recognition of the precursor peptide by parts of the

biosynthetic machinery. The post-translational modifications introduced in the core peptide have been reviewed extensively elsewhere and expand the chemical and structural features far beyond the 20 canonical amino acids.[13,15] These modifications include, but are not limited to, heterocyclisation of Ser/Thr and Cys residues to oxazolines and thiazolines, oxidation of these heterocycles to the corresponding azoles, epimerisation of amino acids to give D-stereocentres, methylation, Ser/Thr/Tyr prenylation, dehydration, hydroxylation, macrocycle formation and the formation of new C–C bonds through different chemistries. In fact, in some RiPPs such as pyrroloquinoline quinone, the final product does not contain any peptide bonds.[15] Bottromycins are the only RiPP of bacterial origin that utilises a follower peptide rather than the canonical leader peptide for biosynthesis.[6,8,11,12]

This review aims to cover all aspects of bottromycin research with a focus on the recent years (2009–2020). We will place particular emphasis on the total synthesis of bottromycins, studies conducted to investigate the biosynthesis and produce derivatives *in vivo* and very recent progress on the enzymology of individual steps involved in bottromycin biosynthesis.

2 Discovery, structure elucidation and activity

In 1955, a new *Streptomyces* species, *Streptomyces bottropensis*, isolated from a soil sample taken in the German town Bottrop, was reported.[16] Its fermentation broth displayed antibiotic activity and led to the discovery of a new antibiotic, named bottromycin.[1,16] Waisvisz and coworkers were the first group to investigate the bottromycin structure and reported that although bottromycin itself gave a negative ninhydrin test, hydrolysis of bottromycins gave 7 ninhydrin positive compounds. Four remained unspecified, but two that could be identified as glycine and valine.[1,17–19] Acetylation of bottromycin yielded two degradation products, which were further analysed. This resulted in the identification of two ninhydrin-positive compounds, α-amino-β-phenylbutyric (MePhe) acid and β-(2-

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Andrew Truman obtained his PhD at the University of Cambridge in 2008, where he remained to conduct postdoctoral research. In 2013, he established his research group at the John Innes Centre as a Royal Society University Research Fellow. His laboratory use genomics to guide the discovery of new natural products from bacteria, with a major focus on ribosomally synthesised and post-translationally modified peptides. His group aim to understand the biosynthesis and natural functions of these specialised metabolites.

Uli Kazmaier studied chemistry at the University of Stuttgart where he obtained his PhD 1990 while working with U. Schmidt. Afterwards he joined the groups of M. T. Reetz (Marburg) and B. M. Trost (Stanford) as postdoctoral fellow. In 1992, he moved to Heidelberg, starting his own scientific work under the mentorship of G. Helmchen. In 2000, he received a Novartis Chemistry Lectureship and in 2001 an offer for a full professorship at Saarland University. His current research interests focuses on new organometallic reagents and reactions especially for amino acid and peptide synthesis, and their application to natural product synthesis.

Jesko Koehnke studied Biochemistry at Leibniz University Hannover. After graduating in 2005, he obtained his PhD at Columbia University under the supervision of Prof. L. Shapiro. After graduating in 2010, he joined Prof. J. H. Naismith at the University of St Andrews as a postdoctoral researcher. In 2015 he established his independent junior group leader at the Helmholtz Institute for Pharmaceutical Research Saarland, where his work focused on the structural biology and biochemistry of RiPPs biosynthesis. In 2020 he moved to the University of Glasgow as a Reader to continue his research centered around RiPPs.

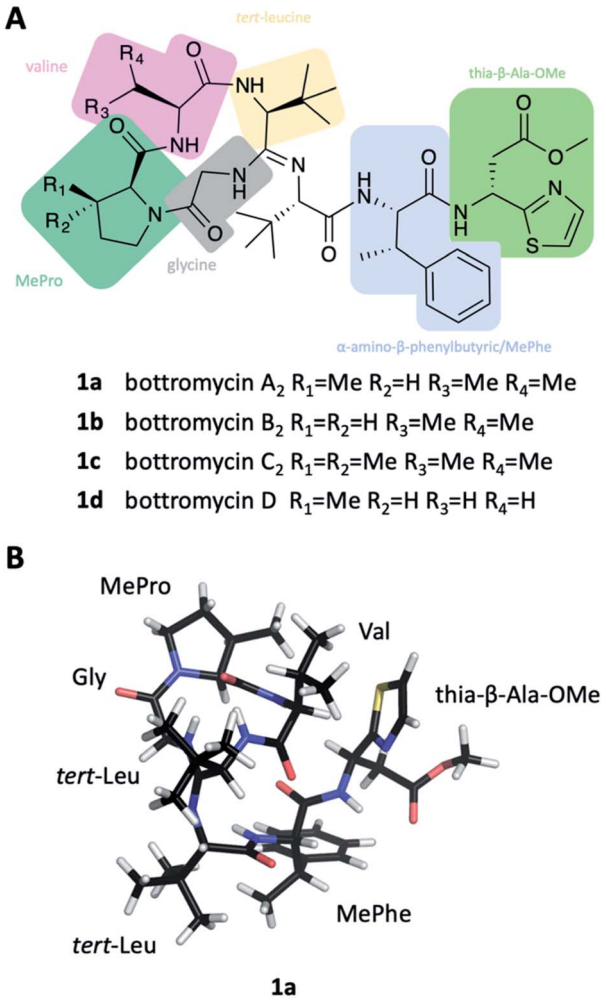


Fig. 1 Structures of bottromycins. (A) Structural formular of bottromycin A_2, B_2, C_2 and D. (B) NMR structure of bottromycin A_2 in $CDCl_3$. Coloured fragements in (A) represent the identified ninhydrin positive compounds after hydrolysis of bottromycin during the structure elucidation process of bottromycin.

thiazole)-β-alanine methyl ester (thia-β-Ala-OMe)[18] as well as the previously identified glycine and valine.[19] Thus, only two of the unknown hydrolysis products remained obscure. As part of these studies, a methyl ester moiety was identified and further analysed: mild alkaline hydrolysis yielded a biologically inactive bottromycin,[17] while re-esterification in methanolic HCl solution yielded a biologically active compound identical with bottromycin. Esterification with ethanol or *n*-butyl alcohol produced less active bottromycin derivatives.[17]

In 1965, Nakamura and colleagues reported the isolation of bottromycin from *Streptomyces* No. 3668-L2.[20] They were able to identify the two remaining unknown ninhydrin-positive substances from acid hydrolysis as L-β,β-dimethyl-alpha-aminobutyric acid (*tert*-leucine, *t*-Leu) and L-*cis*-3-methylproline (MePro).[21] Analysis of the isolated antibiotic by thin-layer chromatography revealed it to contain a major component, which was identical to the previously studied bottromycin and designated as bottromycin A, and two minor components, designated as

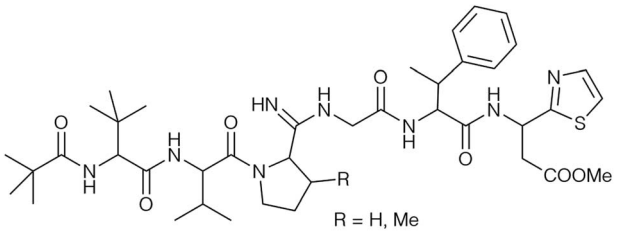
Table 1 MIC values for bottromycins A_2–C_2. MIC values from Nakamura *et al.*[22a]

	MIC (μg ml^{-1})		
Strain	A_2	B_2	C_2
Staphylococcus aureus (Smith)	0.2	0.8	0.1
Staphylococcus aureus (209 P)	0.1	0.8	0.1
Staphylococcus aureus (BR4)	0.4	0.8	0.4
Staphylococcus aureus (R1)	0.4	1.5	0.4
Staphylococcus aureus (R5)	0.4	1.5	0.4
Staphylococcus aureus (R6)	0.4	1.5	0.2
Micrococcus flavus	0.4	1.5	1.5
Bacillus subtilis (PCI 219)	0.06	0.2	0.06
Bacillus cereus (IAM 1729)	0.4	0.8	0.25
Corynebacterium xerosis	0.06	0.2	0.06
Mycobacterium phlei	0.1	1.5	0.1
Mycobacterium 607	25	(25)	(12)
Shigella dysenteria	12	25	25
Shigella sonnei	25	>100	50
Salmonella typhosa	50	>100	>100
Salmonella paratyphi	25	6	6
Escherichia coli B	3	25	6
Escherichia coli K12	25	100	50
Klebsiella pneumoniae 602	50	>100	>100
Pseudomonas aeruginosa A3	50	>100	100
Sarcina lutea	0.4		0.8
Proteus vulgaris OX-19	12	100	50

[a] *S. aureus* BR4, R1, R5 and R6 are clinical isolates resistant to antibiotics: BR4 is erythromycin–carbomycin resistant, R1 and R6 are penicillin-tetracycline resistant.

bottromycin B and C.[21,22] Bottromycin B and C are almost identical to bottromycin A, but contain L-proline (bottromycin B) and L-3,3-dimethylproline (Me_2Pro) (bottromycin C) instead of MePro. Bottromycins B and C are biologically active, but bottromycin B displayed 3–4 times less potency than bottromycin A and C (see Tables 1 and 5).[22] Nakamura and colleagues also reported the recovery of pivalic acid after hydrolysis of bottromycin beside the 6 previously described compounds.[21] Different tests (*i.e.* van Slyke test) also suggested the existence of an amidine group in bottromycin in the tetrapeptide moiety.[21] It was concluded that the N-terminus of bottromycins must not be free, because they are negative in ninhydrin reactions, Edman degradation and Sanger decomposition.[21] The structure (**2**) that was proposed based on these data harboured pivalic acid at the N-terminus of the tetrapeptide (*t*-Leu, Val, MePro, Gly) (Fig. 2). A subsequent revision of the structure postulated 1-Δ^1-caproic acid instead of pivalic acid at the N-terminus.[23] Bottromycin with pivalic acid was designated as bottromycin A_1, and bottromycin containing 1-Δ^1-caproic acid was designated as bottromycin A_2, but synthetic attempts by Yamada *et al.* indicated that the proposed structure was incorrect.[24]

Ten years later, Takita and colleagues proposed a new structure for bottromycin, based on mass spectrometry and ^{1}H NMR data.[25] This cyclic structure of the tetrapeptide was revised by Shipper in 1983,[26] who demonstrated that an unusual amidine moiety links the cyclic tetrapeptide and the linear chain and is formed by condensation of N-terminal amino group and the backbone amide carbonyl. In spite of these iterative revisions, and the fact that



2

Fig. 2 Structure of bottromycin (2) according to Nakamura *et al.*[21]

bottromycin A_1 and A_2 were actually identical, the designation bottromycin A_2 (as well as B_2 and C_2) for bottromycin A, B and C was retained. It took until 2009 before the correct structure of bottromycin A_2 (**1a**) (see Fig. 1A) was determined by Shimamura *et al.* through the total synthesis of bottromycin A_2, demonstrating the D-configuration of the thia-β-Ala-OMe.[10] In 2012, Gouda *et al.* determined the three-dimensional structure of bottromycin A_2 in $CDCl_3$ based on NMR data.[27] In this structure (Fig. 1B) the C-terminal residues fold back on the macrocycle made by the four N-terminal amino acids. Hence, the MePro and the thia-β-Ala-OMe, which are essential for activity, are on one side of the three-dimensional structure, which suggests an involvement of this region in target engagement.

Biological data for bottromycins A_2–C_2 were reported by Nakamura *et al.*[22] They determined the minimal growth inhibitory concentrations (MIC) towards a wide range of bacterial strains (Table 1). In addition, bottromycin A_2 also showed strong inhibition against mycoplasma (0.001–0.01 μg mL^{-1}),[2,28] the multidrug-resistant human pathogens MRSA (1 μg mL^{-1}) and VRE (0.5 μg mL^{-1})[2] and *Xanthomonas oryzae pv. oryzae* KACC 10331, a pathovar that causes rice bacterial blight,[29] which makes bottromycins potentially interesting for agrochemical use.

Although highly active *in vitro*, the bottromycins showed no convincing *in vivo* efficiency because of their instability in oral and parenteral administration,[30] which is mainly the result of the lability of the methyl ester under physiological conditions.[2] Synthetic approaches to change the methyl ester moiety could increase plasma stability without decreasing the activity (see Tables 2–5). *In vivo* studies using bottromycin derivatised at the methyl ester moiety displayed *in vivo* activity against staphylococcal and streptococcal infection in animals,[30] and *Mycoplasma gallispetium* (pleuropneumonia-like organisms) in chicken using subcutaneous administration.[28,31,32]

3 Mechanism of action

In MoA studies, it was demonstrated that bottromycin A_2 inhibits protein synthesis,[33] which is a common target of antibiotics (*e.g.* aminoglycosides and macrolides). How bottromycin inhibits protein synthesis was investigated by different groups, who came to divergent conclusions.

Tanaka and co-workers were the first group that studied the MoA of bottromycin and demonstrated the inhibition of protein biosynthesis *in vivo* and *in vitro*.[33] The inhibition was reported to be highly dependent on the base composition.[33] They showed that bottromycin inhibits neither aminoacyl-tRNA synthesis nor its binding to the ribosome.[33] The puromycin reaction, which is regarded as an analogous reaction to the peptide bond formation, was not significantly affected by bottromycin A_2 in the absence of guanosine triphosphate (GTP) and G factor. This reaction does not require GTP or G factor. Addition of GTP and G factor stimulate translocation of the peptidyl-tRNA from the A- to the P-site. In the presence of GTP and G factor the puromycin reaction was inhibited by bottromycin A_2.[34,35] They concluded from these results that bottromycin A_2 interferes with the translocation of peptidyl-tRNA and movement of mRNA on the ribosomes.[35,36] In a cell-free system, it was determined on which subunit of the ribosome the antibiotic acts. Examining the inhibitory effect of bottromycin A_2 in a protein synthesising system containing excess of either 30S or 50S ribosomal subunit, the excess of 50S over 30S subunit decreased the inhibitory effect by bottromycin. This effect could not be observed using an excess of 30S over 50S subunit. From these results it was concluded that bottromycin interacts with the 50S subunit of the ribosome.[37]

Pestka and Brot also examined the effect of bottromycin on several steps of the protein synthesis.[38] They also determined an effect of bottromycin on the translocation process, using an oligophenylalanine formation assay. An inhibitory effect was observed in the absence and presence of G protein and GTP. In contrast to Tanaka *et al.*, they also observed an effect of bottromycin on peptide bond synthesis using an acetyl-phenylalanyl-puromycin formation assay.[38] As the degree of inhibition on oligophenylalanine synthesis and the puromycin reaction were comparable, they suggested that the inhibition of the peptide bond formation may be the primary action of bottromycin A_2.[38]

The latest studies examining the MoA of bottromycins were carried out in the early 1980s by Otaka and coworkers.[3–5] They reported that bottromycin interferes with the interaction of aminoacyl- or peptidyl-tRNA with the A (aminoacyl) site of ribosomes[3,5] and proposed the hypothesis that bottromycin binds to (or close to) the A site of the ribosome and lowers the affinity of aminoacyl-, peptidyl-tRNA or puromycin.[4,5]

The proposed MoA of bottromycin from Otaka and coworkers is similar to the mechanism of tetracyclines. Tetracyclines block the binding of aminoacyl-tRNAs to the A site of the ribosome,[39] but only bottromycins are able to release bound tRNA from the A site. While tetracyclines bind to the 30S ribosomal subunit,[39,40] bottromycins are reported to bind the 50S subunit of the ribosome.[37] The different MoAs and binding sites between tetracycline and bottromycin are also supported by the observation that no cross-resistance to the tetracycline-resistant strains *S. aureus* R1 and R6 (Table 1) is observed. Other antibiotics that act at the A-site can also have different functions, such as negamycin, which inhibits translocation and stimulates miscoding.[40–42] In the past decade, multiple structures of the 70S ribosome or its subunits in complex with antibiotics have been determined, which provided insights into their mechanism of action.[42,43] Unfortunately, no ribosome–bottromycin complex structure has been published yet, so our understanding of the MoA of bottromycin remains limited.

4 Synthetic approaches and total syntheses towards bottromycins

4.1 Synthesis of the unusual amino acids[44]

Based on the early structure proposals, the first syntheses of the unusual amino acid building blocks were reported from the mid 1970's on. A first stereospecific synthesis of (2*S*,3*R*)-3-methylproline (MePro) was reported by Titouani *et al.* in 1980 using a Hofmann–Löffler–Freitag reaction.[45] Herdeis *et al.* reported the syntheses of both, the (2*S*,3*S*)- and the (2*S*,3*R*)-isomer of MePro from a pyroglutaminol derivate,[46] while Karoyan and Chassaing used a 5-*exo* trig cyclisation approach to generated the five-membered ring.[47] Kamenecka *et al.* developed a protocol starting from commercially available 3-hydroxy-(*S*)-proline using Stille cross coupling chemistry,[48] and a stereoselective cuprate addition was the key step in the synthesis by Flamant-Robin *et al.*[49]

3,3-Dimethyl-(2*S*)-proline (Me_2Pro) is one of the unusual amino acids found in bottromycin C_2. A first enantioselective synthesis was reported by Sharma and Lubell in 1996.[50] A regioselective enolisation of a 4-oxo-proline derivative followed by alkylation with different alkyl halides allowed the synthesis of a variety of proline derivatives. Two approaches towards racemic Me_2Pro were described by Medina[51] and Bott *et al.*[52]

Most investigations focused on the synthesis of (2*S*,3*S*)-3-methylphenylalanine (MePhe) because it also appears in some other natural products, such as mannopeptimycin[53] and the isoleucyl-tRNA-synthetase inhibitor SB-203208.[54] In connection with one of the first synthetic studies towards bottromycins, Kataoka *et al.* described the synthesis and optical resolution of MePhe *via* condensation of racemic 1-bromo-1-phenyl-ethane with acetaminomalonate.[55] Many attempts have been undertaken to separate the stereoisomers more easily using modern chromatographic techniques.[56–64] Ogawa *et al.* reported an enzymatic approach to MePhe,[65] while Tsuchihashi *et al.* used the Michael addition of malonate onto a chiral vinyl sulfoxide as a key step.[66] Dharanipragada *et al.*[67,68] and Fioravanti *et al.*[69] described the asymmetric syntheses of MePhe using auxiliary-controlled enolate chemistry, while the groups of Pericas and Rieva developed a protocol using a Sharpless epoxidation as a stereo-controlling step.[70] O'Donnell *et al.* reported an acyclic stereoselective boron alkylation as a key step using a chiral boron reagent in the presence of cinchona alkaloids,[71] while the group of Turner developed a chemo-enzymatic route towards enantiomerically pure MePhe derivatives, based on an oxidation-reduction sequence.[72] Ooi *et al.* described a phase transfer-catalysed alkylation of a glycinate Schiff base with 1-bromo-1-phenylethane under the influence of chiral quaternary ammonium bromide and 18-crown-6.[73] And finally, Zhang *et al.* reported a palladium-catalysed C–H functionalisation of $C(sp^3)$–H bonds using 8-aminoquinoline (AQ) as a directing group, giving access to fully protected MePhe derivatives.

The unusual C-terminal thiazolyl amino acid thia-β-Ala was the last one whose configuration was determined. It required the total synthesis of the bottromycins to establish it definitively. The problem arose from the structure elucidation of bottromycin. By hydrolysis of the natural product with conc. HCl, Waisvisz *et al.* obtained a "sulfur-containing amino acid", which unfortunately showed no optical activity.[18] Umezawa's group subsequently obtained an optically active amino acid ($[\alpha]_D^{18}$: +9) by hydrolysing the antibiotic with acetic anhydride.[74] To determine the structure of the C-terminal amino acid Waisvisz prepared racemic thia-β-Ala by addition of hydroxylamine towards β-2-thiazolacrylic acid, unfortunately only with moderate yield.[75]

Seto *et al.* tried to obtain optically active (*S*)-thia-β-Ala starting from (*S*)-aspartic acid.[75] Thiazole formation was performed by condensation of the corresponding protected aspartic acid thioamide with bromoacetaldehyde, but unfortunately, these derivatives were also optically inactive. Obviously, complete epimerisation occurred in the thiazole formation step. The racemic amino acid, however, could be resolved into its enantiomers by treating the Phth-derivative with brucine.[49] After cleavage of the Phth-protecting group, the (+)-amino acid, the constituent of bottromycin, was isolated in pure form. It should be mentioned that the thia-β-Ala derivatives prepared also lost their optical activity after heating under reflux in 6 N HCl for 8 h, while the same compounds were stable at room temperature or under slightly basic conditions, illustrating the configurational lability of these compounds. The only enantioselective synthesis of enantiomerically pure (*S*)-and (*R*)-thia-β-Ala so far was reported by the groups of Sunazuka and Ōmura,[10] taking advantage of the chiral sulfinamide chemistry developed by Davis and Ellman,[76,77] which allowed the synthesis of both enantiomers in a highly stereoselective fashion.

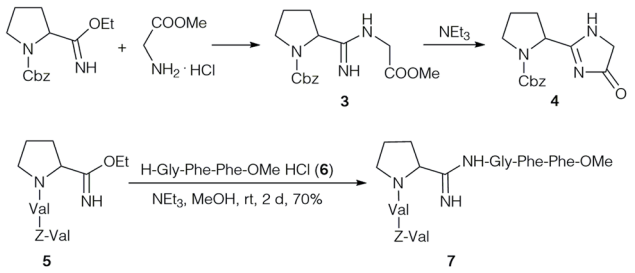
4.2 Synthetic studies towards bottromycins

The total synthesis of bottromycins was adversely affected by the long-standing erroneous structure proposals for the compounds, and so far only one total synthesis exists.[10,78] It confirmed that the C-terminal thia-β-Ala is (*R*)-configured and not (*S*)- as originally reported.

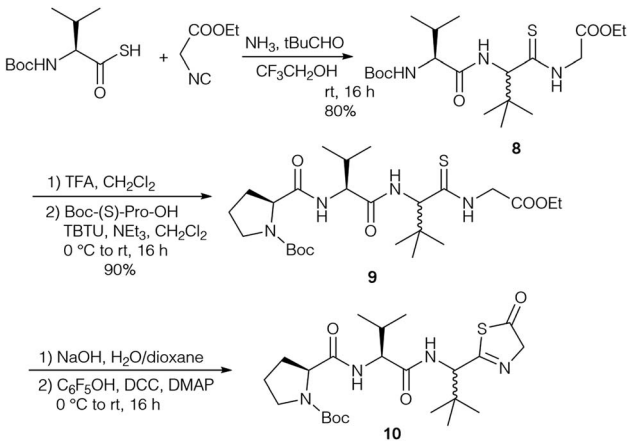
While the wrong structural assumptions meant that early synthetic work could only ever be unsuccessful, significant efforts were directed towards the synthesis of the partial structure of this rather unique peptide. The first investigations were already reported by Yamada *et al.* in 1997.[79] Their synthetic route was based on the linear hexapeptide **2** proposed by Nakamura *et al.* (Fig. 2).[22,74,80,81]

Yamada *et al.* focused on the synthesis and properties of the central amidine unit. Several amidines were prepared by condensation of Cbz-protected amino acid imido esters with amino acid esters (Scheme 1).[24] The desired amidine **3** could be obtained without problems, but it was impossible to extend the dipeptides at the C-terminus. On activation, or even on standing under basic conditions cyclisation occurred to the corresponding imidazolone **4**. Therefore, the authors decided to form the amidine unit of **7** by coupling two model tripeptide fragments, the tripeptide imido ester **5** and tripeptide **6**.[24]

Interestingly, the pK_a of all synthesised amidines ($pK_a \sim 9.3$) were around 1 pK_a higher than in the natural product (~8.2), a first indication that the structure proposal might not be



Scheme 1 Formation of amidine 7.



Scheme 2 Synthesis of endothiopeptides *via* Ugi reaction.

correct. The antimicrobial activities of these amidines were examined, but no activity was observed.

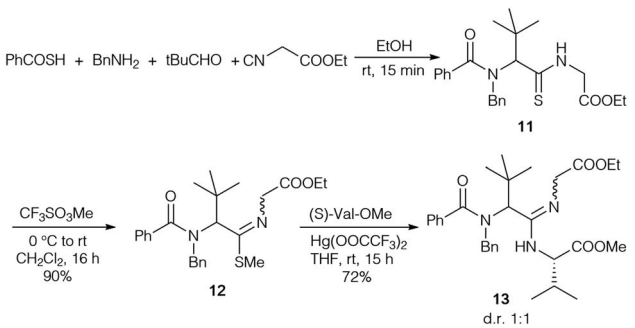
Based on the revised structures by Schipper[26] and Kaneda,[82] who proposed a cyclic tetrapeptide with a tripeptide chain connected *via* an unusual amidine moiety, Kazmaier *et al.* focused on the synthesis of the corresponding peptide ring and the highly substituted amidine.[83] A key step of their approach was an Ugi reaction using a protected thioamino acid and NH_3 as the amine compound (Scheme 2). Although Ugi reactions with NH_3 are often non-specific and yield a range of side products, good results were obtained with sterically demanding aldehydes.[84,85] With thiocarboxylic acids this approach allowed the synthesis of endothiopeptides.[86–88] With isocyanoacetate the linear tripeptide **8** was obtained, which could be extended to the desired tetrapeptide **9** under standard conditions. Attempts to cyclise **9** or to connect the side chain *via* peptide coupling failed, because the thioamide underwent cyclisation to the thiazolinone **10**, comparable to the imidazolone formation reported by Yamada (Scheme 1).[24]

To figure out if amidine formation is possible between sterically demanding amino acids, thiopeptide **11** was synthesised in an analogous fashion (Scheme 3). Attempts to couple **11** directly with amines failed, so the thioamide was converted into the corresponding thioimidoester **12**. In the presence of $Hg(OOCCF_3)_2$ **12** could be coupled with valine methyl ester to obtain amidine **13** in good yield.

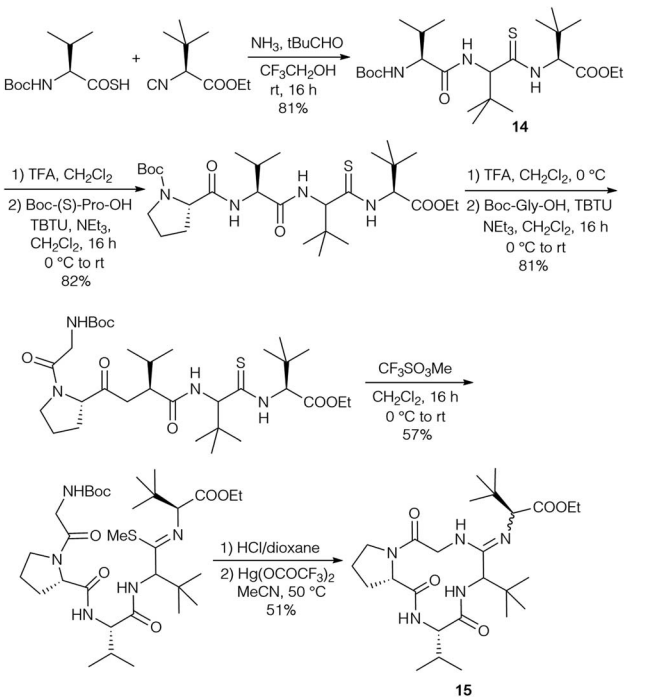
The diastereomers formed could be separated by flash chromatography, but unfortunately this protocol could not be carried out with endothiopeptide **8**. This resulted in a change in the strategy, replacing the intermolecular amidine formation with an intramolecular one by using the isocyanide of *t*Leu-OMe (Scheme 4). The endothiopeptide **14** was obtained in high yield and could be extended on the N-terminus. *S*-Methylation and cyclisation in the presence of $Hg(OOCCF_3)_2$ gave access to cyclic amidine **15**.

Amidine formation as the key step was also investigated in detail by Ōmura and Sunazuka *et al.* during their synthesis of bottromycin A_2 (**1a**) and B_2 (**1b**) (Scheme 5).[78] They investigated the reaction of thioamide **16** with the tripeptide side chain **17**. No reaction was observed in THF using NEt_3 as a base, while in the presence of $Hg(OAc)_2$ the desired amidine **18** was not obtained, and instead the amide **19** was produced. Better results were obtained using $HgCl_2$ and $Hg(OTf)_2$ as Lewis acids. Finally, 2,6-lutidine in acetonitrile was the method of choice to yield **19**.

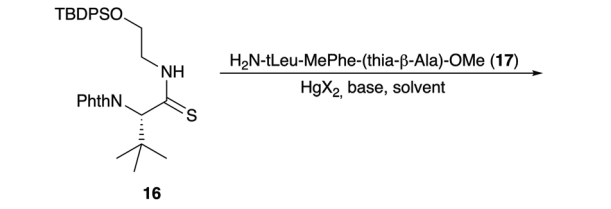
The same groups also performed degradation studies of bottromycin obtained by fermentation (Scheme 6).[89] They subjected **1a** to pyrolysis in MeOH in a sealed tube at 130 °C,



Scheme 3 Synthesis of amidine **13** *via* Ugi reaction.

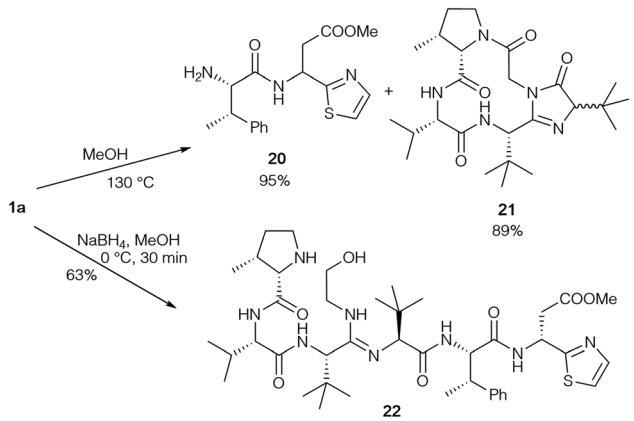


Scheme 4 Synthesis of cyclic amidine **15** *via* Ugi reaction.



HgX_2	Base	Solvent	Yield (%) 18	19
$Hg(OAc)_2$	NEt_3	THF	—	100
$Hg(OTf)_2$	2,6-lutidine	MeCN	96	—

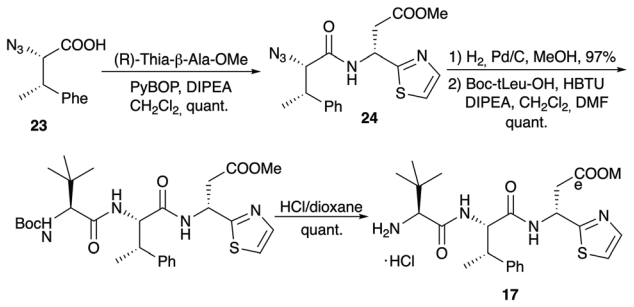
Scheme 5 Synthesis of linear amidine **18**.



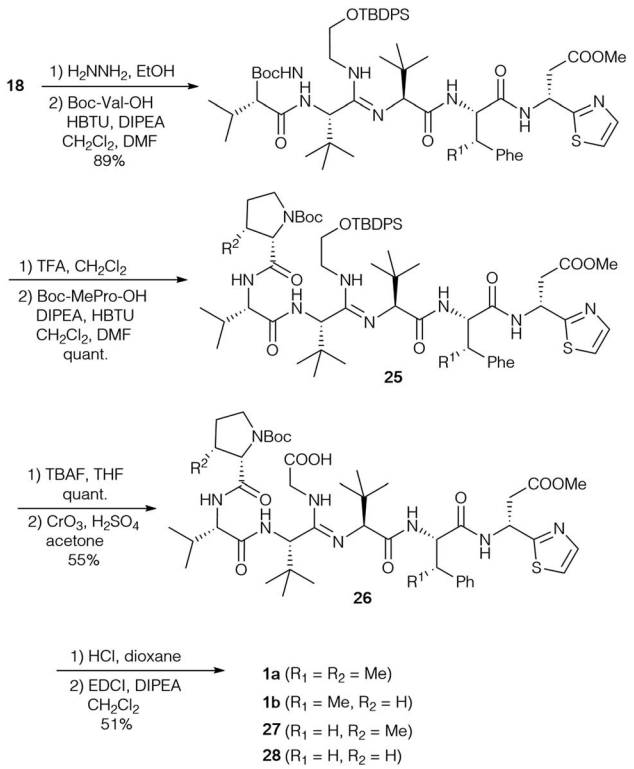
Scheme 6 Degradation of bottromycin A_2 (**1a**).

resulting in cleavage of the tripeptide side chain. Besides dipeptide **20**, cyclic product **21** was also obtained as a diastereomeric mixture. Obviously, the epimerisation of the *t*Leu in the side chain occurred *via* the enol-form of **21**. This could explain why the *t*Leu obtained by total hydrolysis of the bottromycins has a lower optical rotation than the synthetic enantiopure amino acid. Reduction under mild conditions converted the natural product into alcohol **22**, which could be used to investigate cyclisation conditions.

Dipeptide **20** was also used to determine the configuration of the thia-β-Ala, an amino acid that is rather configurational labile.[75] Both enantiomers of thia-β-Ala-OMe were synthesised *via* the sulfinamide protocol and subsequently coupled with azido-MePhe **23** (Scheme 7). Reduction of the azido functionality of **24** provided the two diastereomeric dipeptides **20**. Comparison of their ^{1}H NMR spectra with the spectrum of **20** obtained *via* pyrolysis clearly indicated that the (*R*)-isomer is incorporated into the bottromycins and that the original structure proposal (*S*) was incorrect. Coupling of **20** with Boc-(*S*)-*t*Leu and subsequent Boc-cleavage provided tripeptide **17**,



Scheme 7 Synthesis of tripeptide side chain **17**.

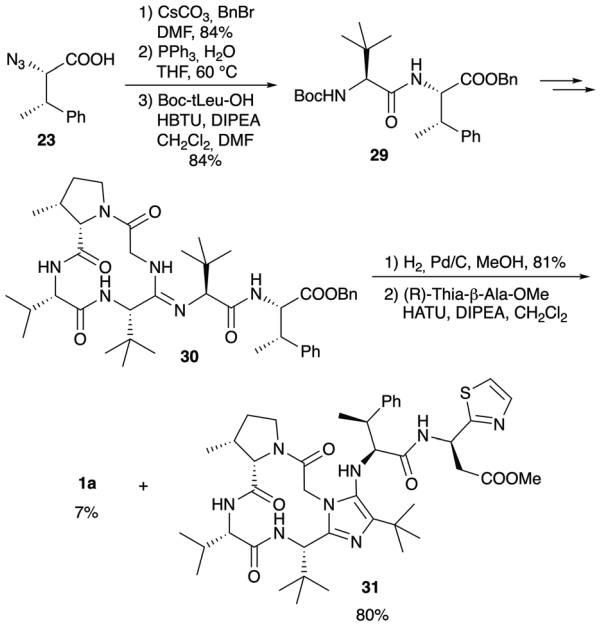


Scheme 8 Total synthesis of bottromycin A_2 (**1a**).

which was also used in the amidine formation experiments (Scheme 5).

4.3 Total synthesis of bottromycin and analogs

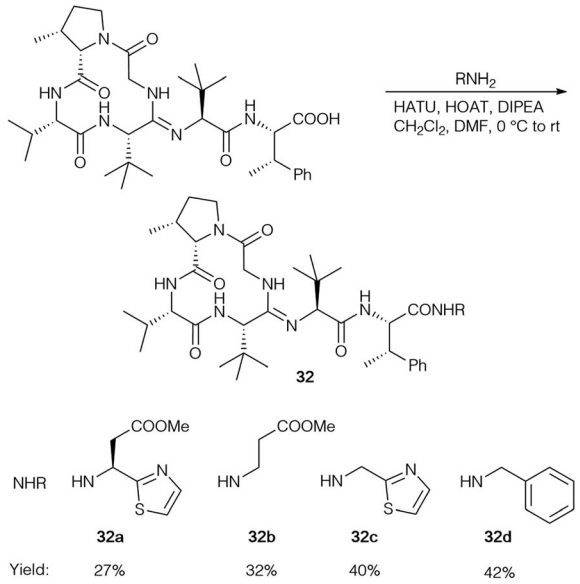
Based on their own synthetic studies and with all building blocks in hand, Sunazuka and Ōmura *et al.* developed the first and so far only complete total synthesis of bottromycin (Scheme 8).[10,78] To extend the peptide chain, the Phth-group of amidine **18** was removed and the free amine was coupled with Boc-(*S*)-Val. Further elongation gave rise to hexapeptide **25**, which was subjected to desilylation and oxidation. These last two steps had to be carried out on the stage of the hexapeptide, as attempts to oxidise tetrapeptide **18** resulted in the formation of a diketopiperazine. The oxidation was a very critical step due to the nucleophilicity of the internal amidine. Thus all oxidation methods proceeding *via* an aldehyde failed, because this



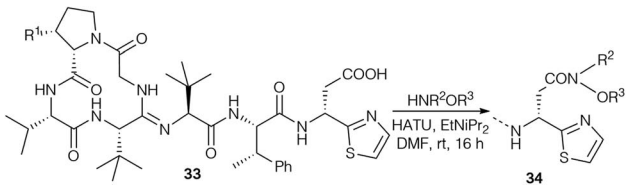
Scheme 9 Variable synthesis of **1a**.

aldehyde intermediate was trapped by the amidine forming an imidazole. Only Jones oxidation was successful, providing an acceptable yield of the desired carboxylic acid **26**. The amidine was also problematic in the final macrocyclisation step, and the best results were obtained using EDCI/DIPEA in CH_2Cl_2, although the yield of **1a** was only moderate.

This protocol was also used to generate derivatives missing some β-methyl groups, such as bottromycin B_2 (**1b**) (Pro instead of MePro), or derivatives where β-MePhe was replaced by Phe [Phe-BotA_2 (**27**), PheBotB_2 (**28**)]. Their NMR spectra were rather complicated (existence of conformers), which suggests that the methyl group of the β-MePhe is important for the three-dimensional structure of the bottromycins.



Scheme 10 Synthesis of bottromycin A_2 analogs **32**.



Scheme 11 Synthesis of bottromycin A_2 analogs **34**.

Since it is known that the methyl ester of the thia-β-Ala has an effect on the biological activity of bottromycins *in vitro* and *in vivo*[2] Sunazuka and Ōmura considered the synthesis of a bottromycin derivative missing the C-terminal amino acid, so that this position can be varied in the last step by coupling a wide range of amines to the "shortened" hexapeptide. Although this is a highly interesting approach, it was not as trivial as hoped. Azido-MePhe **23** was converted into the corresponding benzyl ester and, after reduction of the azide, coupled to Boc-(*S*)-*t*Leu (Scheme 9). The dipeptide **29** was incorporated into bottromycin derivative **30** according to Scheme 9. The benzyl ester could be cleaved easily to the carboxylic acid, the key intermediate for the synthesis of analogs. To validate the concept, the acid was coupled with (*R*)-thia-β-Ala-OMe to the original natural product **1a**. The reaction proceeded smoothly, but **1a** was only a side product. The main product was derivative **31** containing an imidazole on the tetrapeptide ring.

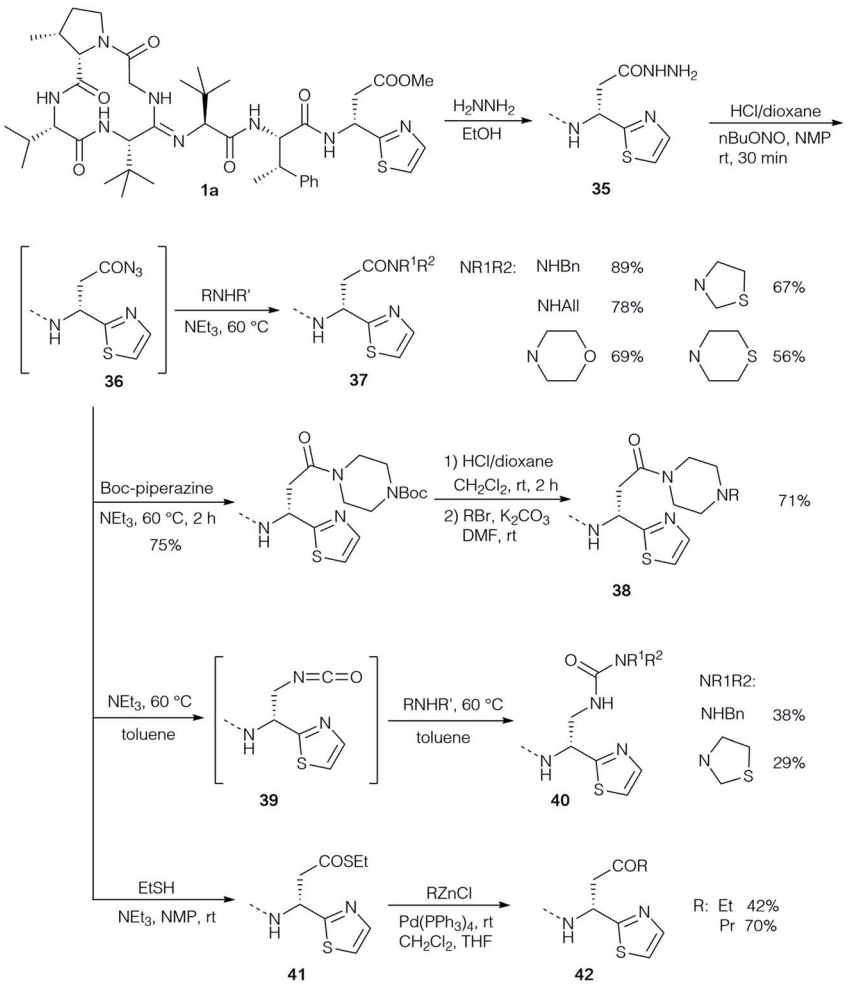
So far, HATU as the coupling reagent gave the best yields for bottromycin A_2 analogs (**32**) and was used to generate a range of amides (Scheme 10), but the corresponding imidazole was the main product in all cases.

Further bottromycin derivatives were obtained by saponification of the natural product **1a** at the C-terminus and coupling the free acid **33** with suitable nucleophiles (Scheme 11). Researchers at AiCuris used this approach for the synthesis of "Weinreb-amide"-type *N*-alkyl-*N*-alkoxyamides **34** by reaction with linear or cyclic *N*,*O*-dialkylhydroxylamines (Table 2).[90]

The groups of Ōmura and Sunazuka synthesised a range of different derivatives *via* the corresponding hydrazide **35** as a common intermediate (Scheme 12).[2] The hydrazide was easily obtained by heating the solution of bottromycin A_2 with hydrazine. Nitrosation gave rise to acyl azide **36** as an active intermediate, which could be coupled with a range of amines to the corresponding amides **37**. Application of mono Boc-protected piperazine allowed further modification by replacing the Boc-protecting group (**38**). On the other hand, heating the acyl azide to 60 °C resulted in a Curtius rearrangement that gave rise to an isocyanate **39**, which on treatment with amines provided ureas **40**. Reacting **36** with thiols gave rise to thioesters such as **41** which could be subjected to palladium-catalysed cross coupling reactions with organozinc reagents generating ketones **42**.

4.4 Biological activities and structure–activity relationship (SAR) studies of bottromycins and derivatives

Based on the importance of the methyl ester for bioactivity and its lability *in vivo* a range of amide derivatives **37** were prepared and their *in vitro* and *in vivo* activity towards *S. aureus* (Table 3)



Scheme 12 Synthesis of bottromycin A_2 analogs *via* hydrazine **35**.

was compared.[32] The tests were either carried out *in vitro* using a rapid tube dilution test, or in an *in vivo* mouse model, where compounds were administered intraperitoneally once. All compounds were active, but the primary and secondary amides were less active than the esters in the *in vitro* test, but more active *in vivo*. Aromatic amides (**37k–m**) and those with a basic substituent (**37n**) were less active to almost inactive *in vivo*, and

Table 2 Synthesis of bottromycin analogs **34**

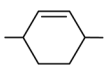
34	R^1	R^2	R^3	Yield (%)
34a	Me	Me	Me	70
34b	Me	$-(CH_2)_3-$		40
34c	Me	$-(CH_2)_4-$		35
34d	H	$-(CH_2)_4-$		14
34e	Me	Et	Et	50
34f	Me	Me	Et	47
34g	Me	$-CH_2-CHOH-CH_2-$		63
34h	Me	$-(CH_2)_3-CH(COOEt)-$		52
34i	Me	$-(CH_2)_3-CH(COOEt)-$		84
34k	Me			90

Table 3 Biological activities of bottromycins and C-terminal amide derivatives ($-NR^1R^2$). MIC values from Miller *et al.*[32]

Compound	R^1	R^2	*In vitro* MIC (μg ml^{-1})	*In vivo* ED_{50} (μg per dose)	Ratio *in vivo* : *in vitro*
1a			0.01	50	5000
1b			0.04	200	5000
37a	H	H	0.10	25	250
37b	Me	H	0.05	10	200
37c	Et	H	0.05	15	300
37d	*n*Pr	H	0.5	10	20
37e	*i*Pr	H	1.0	10	10
37f	*t*Bu	H	0.25	10	40
37g	Bn	H	0.5	18	36
37h	$CH_2CHOHCH_2OH$	H	1.0	25	25
37i	CH_2CH_2OH	H	0.5	18	36
37k	Ph	H		60	
37l	*p*-F-C_6H_4			>100	
37m	α-Naphthyl	H		>100	
37n	$NH(CH_2)_2NEt_3$	H		>100	
37o	iPr	*i*Pr		82	
37p	$-CH_2CH_2CH_2CH_2-$			95	
37q	NMe_2	H		35	
37r	Me	OH		28	
35	NH_2	H		46	

Table 4 Biological activities of bottromycin hydroxamate derivatives **34**. MIC values from Lerchen *et al.*[90]

	MIC (μM)			
34	*S. aureus* 133	*S. pneumoniae* G9a	*E. faecium* BM4147	*E. faecalis* ICB27159
34a	0.78	<0.05	0.39	0.78
34c	0.39	<0.05	0.1	0.39
34d	3.13	<0.05	0.39	1.56

tertiary amides (**37o,p**) were also significantly less active, while hydrazides (**35**, **37q**) and hydroxamates (**37r**) were relatively active *in vivo*.

In a patent, researchers at AiCuris described the synthesis of *N*,*O*-dialkylated bottromycin hydroxamates **34a–k** (Table 2) and their biological evaluation (Table 4).[90]

By far the most detailed SAR studies were reported by Ōmura and Sunazuka, who also investigated the desmethyl derivatives **27** and **28**, which were obtained by total synthesis (Scheme 8). A wide range of different derivatives such as amides **32** (Scheme 12), **37** and **38**, hydrazide **35**, ureas **40**, thioester **41** and ketones **42** were prepared from bottromycins obtained by fermentation (Scheme 12). Their activity was tested towards a panel of Gram-positive strains, using vancomycin (VCM) and linezolid (LZD) as references (Table 5).[2,78] The results of the SAR studies are summarised schematically in Fig. 3.

The unusual methylation pattern (cyan) has a significant effect on the bioactivity towards *S. aureus*. Bottromycin D (**1d**), where the valine is replaced by an alanine, and bottromycin B_2 (**1b**), which does not have the methyl group at the proline, were less active than bottromycin A_2 (**1a**) (Tables 2 and 5).[22] Bottromycin C_2 (**1c**), the analog dimethylated on proline, was roughly as active as bottromycin A_2. The β-methyl group on the Phe seems to be essential and its removal (**27**, **28**) causes a dramatic drop in activity (Table 5). It appears that this methyl group influences the conformation of the side chain and controls the three-dimensional structure of the whole molecule, an assumption which is supported by ^{1}H NMR.[10,78] Bicyclic derivatives such as **31** and linear peptides do not show significant activity, probably due to an undesired three dimensional conformation, which clearly indicates that the cyclic peptide ring (red) is essential.[78] No activity was observed for derivatives with either a COOH-group at the C-terminus, such as **33**, or if the thia-β-Ala is missing completely. This might be caused by a drop in the hydrophobicity. Interestingly, incorporating the opposite (*S*)-isomer of thia-β-Ala (**32a**) had no significant effect

Table 5 Biological activities of bottromycin derivatives towards Gram-positive strains. MIC values from Yamada *et al.*[78] and Kobayashi *et al.*[2]

			MIC (μg ml^{-1})						
Comp.	R^1	R^2	*S. aureus* FDA209P[a]	*S. aureus* Smith[a]	MRSA HH-1[b]	MRSA 92-1191[b]	VRE NCTC12201[c]	VRE NCTC12203[c]	Rates of residual anti-MRSA activity (%)
1a			1	1	1	2	1	0.5	0
1b			4	4		4	4		
27			32	>32		>32	32		
28			>32	>32		>32	>32		
30			>32	>32		>32	32		
31			>32	>32		>32	>32		
32a			2	2		2	2		
32b			8	8		8	8		
32c			4	4		4	2		
32d			2	4		2	2		
33	Me	H	64	64	64	128	128	32	—
35			16	16	16	32	8	4	86
37g	Bn	H	8	8	8	8	8	2	71
37s	CH_2CCH	H	8	8	8	16	4	2	100
37t	$-(CH_2)_2O(CH_2)_2-$		16	8	16	32	16	4	100
37u	$-(CH_2)_2SCH_2-$		4	4	8	8	8	2	100
37v	$-(CH_2)_2S(CH_2)_2-$		8	4	8	8	4	4	100
38a	Boc	H	8	4	8	8	8	4	42
38b	H		64	32	64	128	32	32	—
38c	CH_2CCH		16	16	16	32	16	16	67
38d	Bn		4	4	4	4	4	4	84
40a	$-(CH_2)_2SCH_2-$		4	4	4	4	4	2	100
40b	Bn		8	16	16	16	8	4	100
41			<0.25	0.5	<0.25	0.5	<0.25	<0.25	0
42a	Et		1	1	2	2	2	1	100
42b	*n*Pr		1	1	1	2	2	0.5	100
VCM[d]			1	1	0.5	1	>128	>128	—
LZD[e]			2	2	2	2	2	2	—

[a] *S. aureus* FDA209P and Smith: susceptible strains. [b] MRSA HH-1 and 92-1191: MRSA strains isolated from clinical patients. [c] Vancomycin resistant *Enterococcus faecalis* NCTC12201 and NCTC12203: encoded by *vanA* gene. [d] Vancomycin. [e] Linezolid.

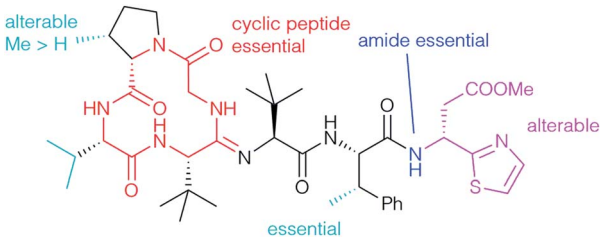


Fig. 3 Summary of SAR for bottromycin derivatives.

on activity (2 μg mL^{-1}). The thia-β-Ala (purple) was not essential at all for activity – derivatives missing the acetate side chain (**32c**) or the thiazole unit (**32b**) were only slightly less active.

Surprisingly benzyl amide **32d** is almost as active as **1a**, while the corresponding benzyl ester **30** is not very effective. The data showed that the amide functionality (blue) is necessary for good activities. Benzyl amide **32d** is more active than the dethiazolyl analog **32b**, which indicates that an (hetero)aromatic substituent at the C-terminus has a positive effect on activity. The moderate *in vivo* activity of the methyl ester in the natural products probably results from its low hydrolytic stability under physiological conditions and its cleavage towards the almost inactive carboxylic acid **33**. Although significantly less active *in vitro*, better *in vivo* stabilities were observed for secondary aliphatic amides (Table 3, **37a-i**), while aromatic (**37j–m**) and tertiary amides (**37o,p**) as well as those with basic side chains (**37n**) were almost inactive.[32] Piperazino derivatives **38** and ureas **40** exhibited 4- to 32-fold weaker activity *in vitro*, but better stability.[2] Thioesters such as **41** were significantly more active than **1a**, but due to their great reactivity completely unstable in mouse plasma. Ketones **42**, which cannot undergo hydrolysis, are perfectly stable and showed activities comparable to **1a** and

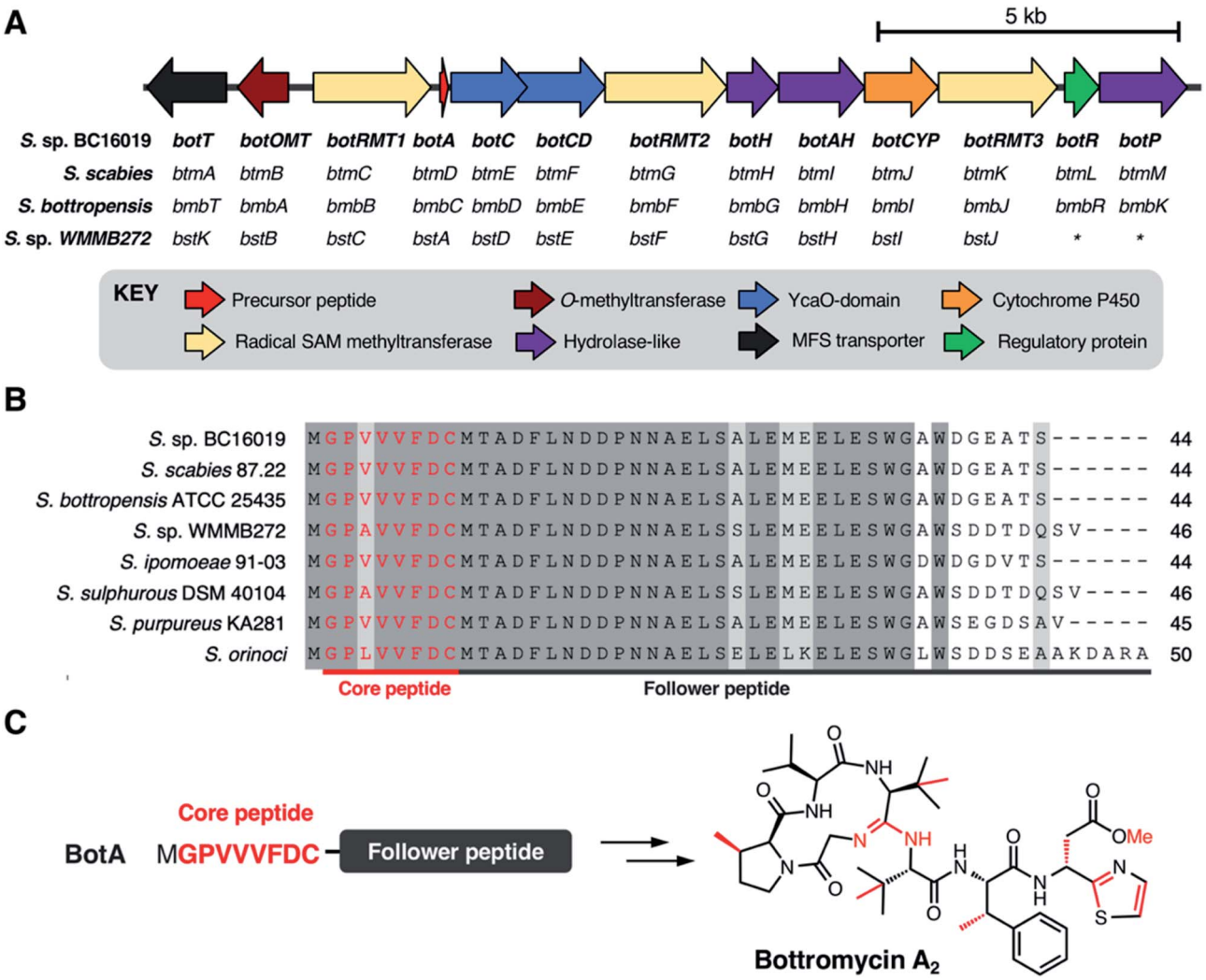


Fig. 4 Identification of the bottromycin BGC. (A) Organisation of the BGC. Gene nomenclature from each strain is shown (* = genes not identified in the *S.* sp. WMMB272 study as this BGC was at the end of a contig). (B) Sequence alignment of precursor peptides from every bottromycin BGC identified bioinformatically (all are *Streptomyces* species). Identical residues are shown with a dark grey background, similar residues are shown with a light grey background (Risler matrix score >0.7) and the core peptide is highlighted in red text. (C) Schematic showing the conversion of the BotA precursor peptide into bottromycin A_2, where all post-translational modifications are coloured red in the final product.

vancomycin, but importantly were also active against vancomycin-resistant strains. Subsequent biological evaluation using MRSA-infected mice showed that propyl ketone **42b** might be a good candidate for drug development. 100 mg kg^{-1} given to mice orally resulted in survival for at least five days after administration, while all non-treated animals died in the same time frame. Hydroxamates **34** (Table 4) might also be suitable for this purpose.[90]

5 Biosynthetic gene cluster and biosynthesis

The biosynthetic origin of the bottromycins was unknown for decades following their discovery. They could feasibly be synthesised *via* either ribosomal or non-ribosomal pathways, as all residues could theoretically be produced from proteinogenic amino acids. Early studies of bottromycin biosynthesis involved elegant isotope labelling experiments from Arigoni and colleagues, where it was shown that the methyl groups at the β-positions of proline, two valines and phenylalanine, along with the thia-β-Ala-OMe, were derived from methionine.[91] They also showed that β-methylated valine and phenylalanine derive from L-valine and L-phenylalanine. Feeding *S. bottropensis* with methionine featuring an isotopically labelled chiral methyl group ([methyl-(^2H,^3H)]-(2*S*,methyl-*R*)-methionine) showed that the β-methylation occurs with a retention of configuration. This was consistent with double inversion of configuration *via* a radical SAM mechanism.[92] Additional experiments using isotopically labelled amino acids were also consistent with a radical SAM mechanism.[91]

In 2012, four independent research teams identified bottromycin biosynthetic gene clusters (BGCs) in four different *Streptomyces* species: the known producers *S. bottropenesis*[11] and *Streptomyces* sp. BC16019,[8] the plant pathogen *Streptomyces scabies*[6] and the marine ascidian-derived *Streptomyces* sp. WMMB272 [12] (Fig. 4A). These reports corroborated the earlier feeding studies by showing that bottromycins are ribosomally synthesised and post-translationally modified peptides (RiPPs)[15] and that the BGCs encode three radical SAM methyltransferases. In each study, the BGC was identified by BLAST searches for genes that could encode a putative bottromycin core peptide, GPVVVFDC (or GPAVVFDC for bottromycin D in *S.* sp. WMMB272) (Fig. 4B). RiPPs originate from a larger ribosomally synthesised precursor peptide that usually consists of a leader peptide and a core peptide that is post-translationally modified by tailoring enzymes. However, the discovery of the bottromycin BGC provided the first (and still only) example of a bacterial RiPP that derives from an N-terminal core peptide that has no leader peptide and is attached to a "follower" peptide (Fig. 4C).

The genetic organisation of these BGCs is effectively identical, and while there are significant differences in protein sequence identity between each BGC, *S.* sp. BC16019 nomenclature will be used here onwards for clarity. The bottromycin BGC encodes 13 proteins (Fig. 4A): one precursor peptide (BotA), two YcaO-domain proteins (BotC and BotCD), three radical SAM methyltransferases (BotRMT1-3), three putative hydrolases (BotH, BotAH, BotP), one cytochrome P450 (BotCYP), one *O*-methyltransferase (BotOMT), one putative regulatory protein (BotR) and one major facilitator superfamily transporter (BotT). These initial studies revealed a number of key details relating to bottromycin biosynthesis. Gene inactivation experiments in *S. bottropenesis*,[11] *S. scabies*[6] and *S.* sp. BC16019 [8] confirmed the identity of the BGC, which was further validated by heterologous expression of the *S.* sp. BC16019 BGC. The identity of the BGC in *S.* sp. WMMB272 was demonstrated by the production of bottromycin A_2 upon expression of a mutant precursor peptide gene that encoded the bottromycin A_2 core peptide instead of the natural bottromycin D core peptide.[12]

Notably, gene deletions in the *S. scabies* BGC[6] and insertional inactivation of genes in the *S.* sp. BC16019 BGC[8] demonstrated the roles of the radical SAM methyltransferases BotRMT1-3 *via* the production of differentially methylated bottromycin derivatives by each mutant, thereby validating the earlier isotopic labelling studies.[91] BotRMT1 catalyses radical C-methylation of Phe6, BotRMT2 catalyses radical C-methylation of both Val4 and Val5, and BotRMT3 catalyses radical C-methylation of Pro2. At the time, this represented one of the first examples of radical β-methylation of amino acid residues, along with the polytheonamides, 49-amino acid RiPPs produced by '*Candidatus* Entotheonella factor', a member of a marine sponge microbiome.[93] Multiple non-ribosomal peptides contain β-methylated amino acids, but these are generated *via* conventional methylation of a precursor keto acid.[94] Inactivation of *botOMT* in the *S.* sp. BC16019 BGC confirmed its role in *O*-methylation of Asp7[8]. However, little else was known about the biosynthetic steps required to convert BotA into mature bottromycin, although plausible routes were initially proposed based on the predicted catalytic roles of *bot* proteins. In-frame gene deletions in the *S. scabies* BGC had demonstrated the essentiality of numerous putative biosynthetic genes,[6] including *botC* and *botCD* (encoding the two YcaO-domain proteins), and *botCYP* (encoding a P450), but no bottromycin related metabolites could be initially identified from these mutants. The challenge with identifying molecules related to RiPPs following gene deletions is that a pathway may "stall" if a key step is disrupted, with the core peptide still attached to the leader/follower peptide. This therefore is likely to undergo further degradation into a very short modified peptide that may be distantly related to the final product and therefore difficult to detect.

To improve the detection of bottromycin-related metabolites from pathway mutants, Truman and co-workers used mass spectrometry-based molecular networking[95] and untargeted metabolomics to study in-frame deletions of orthologues of *botA*, *botC*, *botCD*, *botAH*, *botRMT1*, *botRMT2* and *botCYP* in *S. scabies*.[96] This analysis identified a series of bottromycin-related molecules (intermediates or shunt metabolites) associated with each mutant strain, which were then used to propose a feasible pathway based on where the pathway stalled (Fig. 5). This indicated that radical methylation by BotRMT1 and BotRMT2 were early steps in the pathway in *S. scabies*, as was heterocyclisation of Cys8 by the standalone YcaO-domain protein

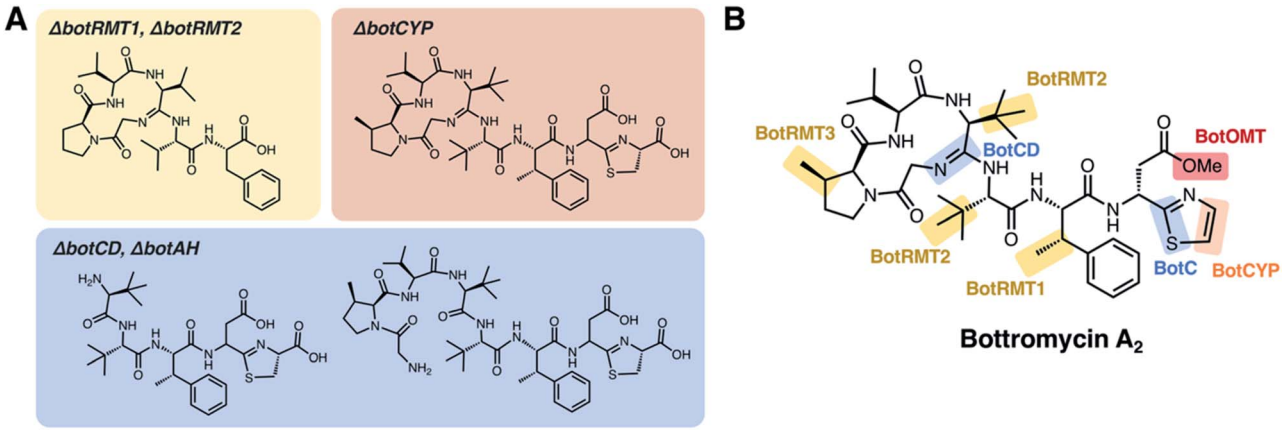


Fig. 5 Key metabolites identified by mutational analysis of *bot* biosynthetic genes. (A) Structures of metabolites based on tandem MS data from Crone *et al.* (B) Proposed roles of biosynthetic proteins based on the studies of Crone *et al.*[6] and Huo *et al.*[8]

BotC. Additionally, this study showed that the M17-family leucine aminopeptidase BotP removes methionine from the N-terminus of BotA.

Following removal of the N-terminal methionine by BotP, the unique macrocyclic amidine of bottromycin was proposed to be formed by BotCD (YcaO-domain protein) and BotAH (hydrolase), based on the production of linear bottromycin-related peptides by each mutant (Fig. 5). Deletion of *botCYP* led to the accumulation of *O*-desmethyl bottromycins A_2 and B_2 carboxylated at their C-termini. This was consistent with BotCYP catalysing late-stage oxidative decarboxylation of the thiazoline moiety to generate a terminal thiazole. Each compound mass appeared as twin peaks *via* liquid chromatography -mass spectrometry (LC-MS), suggesting a mixture of aspartate epimers, which was supported by deuterium labelling and therefore provided a potential route to D-aspartate in mature bottromycin. Epimerisation was shown to happen spontaneously, but it was not clear whether other proteins were involved in accelerating this key step. No mutant strains produced metabolites that are *O*-methylated on Asp7. This suggested that *O*-methylation is the final biosynthetic step, and it was shown that purified BotOMT could methylate *O*-desmethyl bottromycin A_2. This was in agreement with the earlier gene inactivation work by Huo *et al.*[8]

6 Biosynthetic enzymes

The following paragraphs will be dedicated to the enzymes involved in the biosynthesis of the bottromycin core scaffold (desmethyl bottromycin): BotP, BotC, BotCD, BotAH, BotH, and BotCYP. The activity of these enzymes has been reconstituted *in vitro*, and structural information is available for several of them. Although the enzymes were studied using enzyme homologues from different BGC containing *Streptomyces* species (see Fig. 4B), the *S.* sp. BC16019 nomenclature will be used for clarity.

6.1 Aminopeptidase BotP

The aminopeptidase BotP belongs to the family of hexameric M17 leucine aminopeptidases (LAP). LAPs are metallo-exopeptidases found in all kingdoms of life and cleave N-terminal residues from proteins and peptides. They do not only hydrolyse N-terminal leucine residues, but often are promiscuous. The function of LAPs is diverse and goes beyond the function of recycling amino acids and includes the processing of bioactive peptides and peptides for major histocompatibility complex (MHC) class I antigen presentation, gene regulation and vesicular trafficking.[97,98]

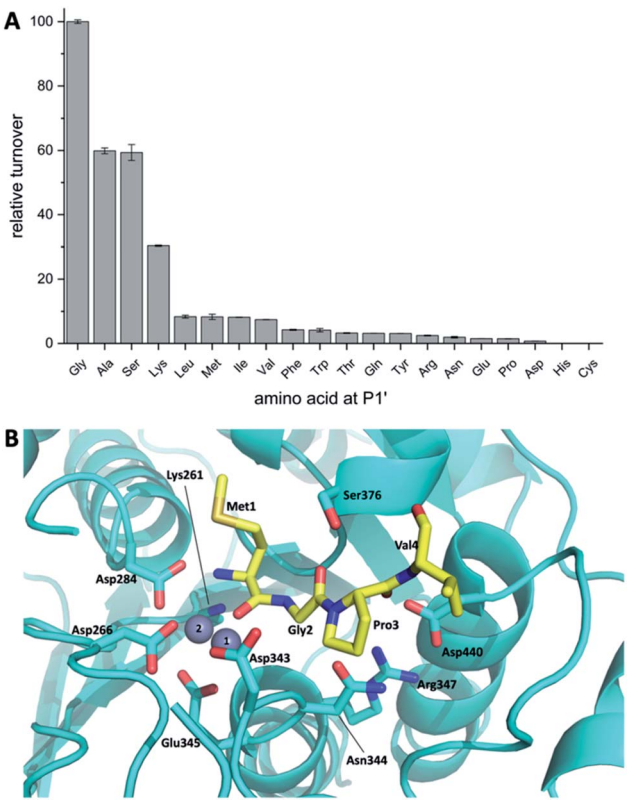


Fig. 6 (A) BotP substrate promiscuity using pentapeptide mimics of BotA with amino acid changes in P1′position. (B) Model of BotP-Mn^{2+} (cyan) with the peptide MGPV (yellow).

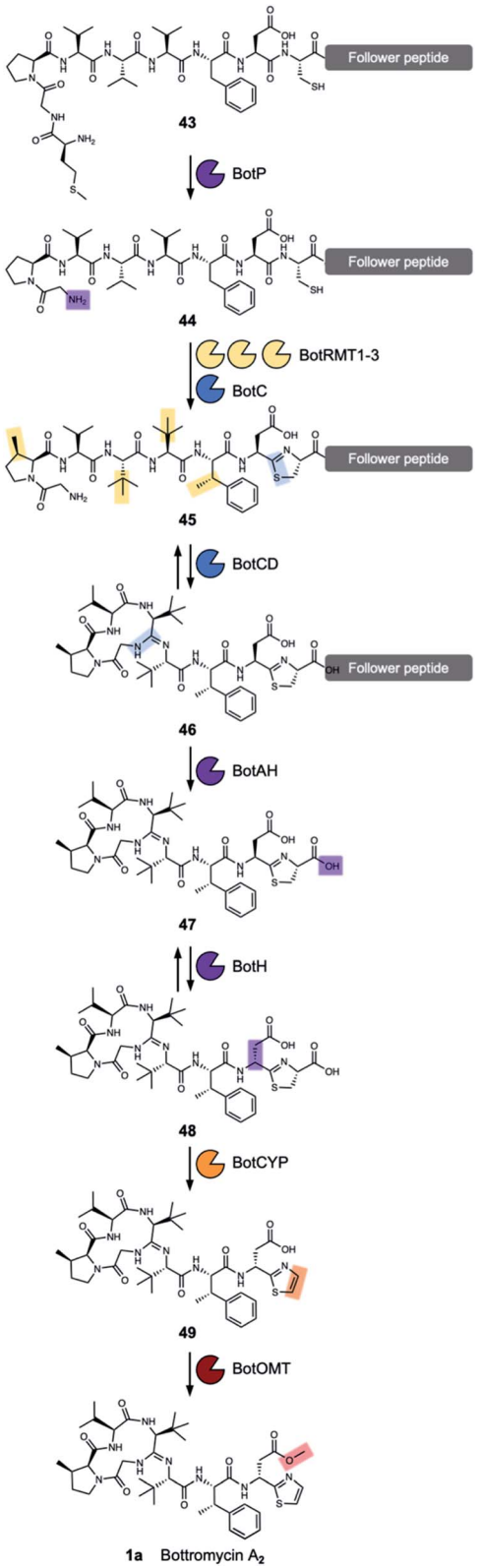


Fig. 7 Biosynthetic pathway for bottromycin A_2.

Usually, N-terminal methionine is hydrolysed by endogenous aminopeptidases, but these do not function efficiently with the MGP sequence found at the N-terminus of BotA.[99] The aminopeptidase BotP was predicted[6,8] and confirmed[96,100] to remove the N-terminal methionine from the precursor peptide BotA (**43**), which generates the free glycine amino group (**44**) (Fig. 7) that is necessary for the cyclisation onto an internal amide carbonyl to generate the unique amidine macrocycle found in bottromycins.

Koehnke and coworkers determined the crystal structure of BotP (Fig. 6B) and assessed the substrate promiscuity of BotP using pentapeptide mimics of BotA (Fig. 6A).[100] BotP showed a hexameric structure typical for M17 LAPs and the activity of recombinant BotP, isolated from *E. coli*, could be reconstituted in presence of Co^{2+} (or Mn^{2+}) ions. RiPP enzymes catalysing the initial biosynthetic steps often bind to the follower (or for other RiPPs leader) peptide to aid substrate recognition and enzyme activity.[101] For BotP modelling suggests that only the first 3–4 amino acids contribute to substrate binding, but an *in vitro* assay using pentapeptides showed that these truncated substrates were processed slower than full-length BotA. The reasons for this discrepancy remain to be determined. BotP tolerates several amino acid changes in P1-P3′, but processing is reduced drastically.

6.2 YcaO domain enzymes BotC and BotCD

The bottromycin biosynthetic gene cluster contains two genes encoding for YcaO-domain enzymes, BotC and BotCD.[6,8,11,12] YcaO proteins can be found in bacteria and archaea. Their function is best characterised in RiPPs such as linear azol(in)e-containing peptides (LAPs), thiopeptides and cyanobactins, where the YcaO enzymes catalyse the ATP dependent cyclodehydration reaction of Cys, Ser or Thr side chains onto the preceding backbone amide carbonyl to form azoline heterocycles.[102–104] They can however also be used to generate non-natural heterocycles.[105,106]

From an untargeted metabolomic approach it was predicted that BotC catalyses the heterocyclisation of Cys to thiazoline and BotCD, together with BotAH, catalyses the formation of the unique macroamidine linkage.[96] The function of the two bottromycin YcaO enzymes were independently studied in *in vitro* approaches by the Mitchell and Koehnke groups.[107,108] It was demonstrated that BotC catalyses the heterocyclisation reaction that converts the core peptide's Cys residue to a thiazoline. The second YcaO enzyme, BotCD, was sufficient to catalyse macroamidine formation. Both proteins bind to the follower peptide, but with low affinity.[107] BotC and BotCD were quite tolerant to changes in the core peptide sequence but recalcitrant to changes of the nucleophile.[107,108] BotC was unable to utilise Ser or Thr instead of Cys to generate oxazolines.[107] While the turnover of all bottromycin biosynthetic enzymes has been shown to be relatively fast, heterocyclisation by BotC was shown to be slow and could be the rate limiting step of the pathway, since BotCD strongly prefers a heterocyclised substrate for macrocyclisation.[108] In contrast to all other YcaO enzymes studied to date, the BotCD reaction was shown to be reversible: the enzyme catalysed amidine formation and ring opening, both in an ATP and Mg^{2+}-dependent fashion.[108] Thus BotCD expanded the catalytic scope of YcaO enzymes in RiPP pathways

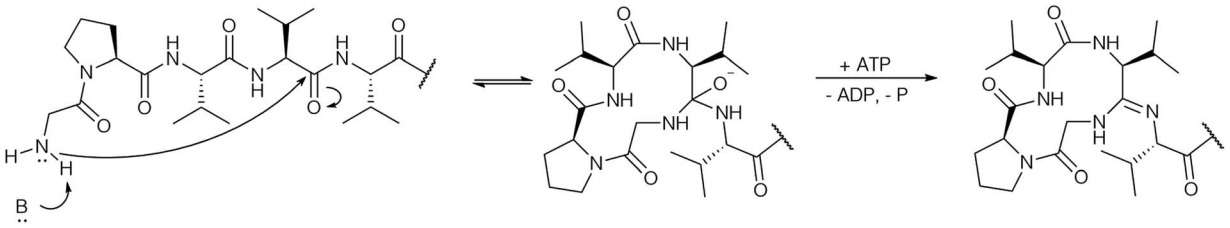


Fig. 8 Proposed mechanism for macroamidine formation by the YcaO domain enzyme BotCD.

to amidine formation, but also raised questions as to possible partner proteins for BotCD, which may prevent ring opening.

Based on the biochemical data it was proposed that macroamidine formation proceeds analogously to heterocyclization: after nucleophilic attack of the N-terminal amino group onto the amide carbon a hemiorthoamide intermediate is formed, which is then ATP-dependent *O*-phosphorylated, followed by subsequent phosphate elimination to form the macroamidine (Fig. 8).

6.3 Amidohydrolase BotAH

Analysis of the metabolomic network from *S. scabies* Δ*btmI* (*botAH* orthologue) and Δ*btmF* (*botCD* orthologue) revealed two new bottromycin related molecules, which could not be found in *S. scabies* WT and that were not macrocyclised. From this observation it was proposed that BotCD and BotAH are involved in macrocyclisation.[96] However, *in vitro* data showed that BotCD is sufficient to catalyse macrocyclisation. Pfam-analysis classifies the protein encoded by the gene *botAH* as a putative metallo-dependent amidohydrolase. From its putative function it was assumed that either BotAH, or the putative α/β hydrolase BotH, catalyses the liberation of the modified core from the follower peptide for the final biosynthetic steps. The crystal structure of the BotAH homologue PurAH confirmed the Pfam prediction. When the role of BotAH was probed *in vitro* it was discovered that it cleaved the follower peptide off the hetero- and macrocyclised core peptide, and a mechanism was proposed (Fig. 9).[109] As observed for BotP, the highest activity of BotAH was observed after addition of Co^{2+}. This may explain increasing bottromycin production levels in *S. scabies* by supplementation of production medium with Co^{2+}.[7]

The biosynthetic role of BotAH raised the question of whether it could aid macrocyclisation by influencing the equilibrium of the BotCD reaction. After all, the activity of BotCD was dependent on the follower peptide (attached to the modified core peptide). Indeed, when added to macrocyclisation reactions, BotAH's activity precluded ring opening and pulled the macroamidine formation equilibrium to the side of macrocyclised product.[109] As a result, BotAH can be viewed as a YcaO accessory protein, the first hydrolase for which such a function has been reported. These observations also rationalise why the amidohydrolase is essential for macroamidine formation *in vivo*.

6.4 Atypical α/β hydrolase BotH

The function of the α/β hydrolase (ABH) BotH was not studied *in vivo* by gene deletion experiments and remained unclear until very recently. Enzymes of the α/β hydrolase family catalyse the hydrolysis of (thio)ester and peptide bonds, but some members have diverse functions, such as dehalogenases, dioxygenases and decarboxylases.[110,111]

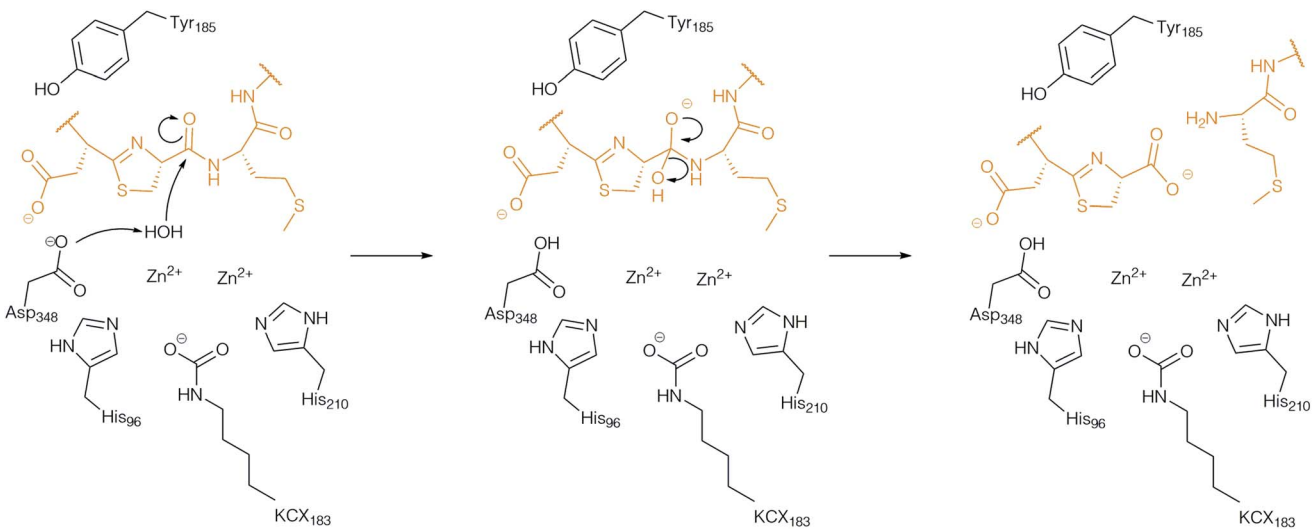


Fig. 9 Proposed mechanism of the follower peptide cleavage by the amidohydrolase AH. BotAH active side residues are shown in back, the peptide substrate is shown in orange.

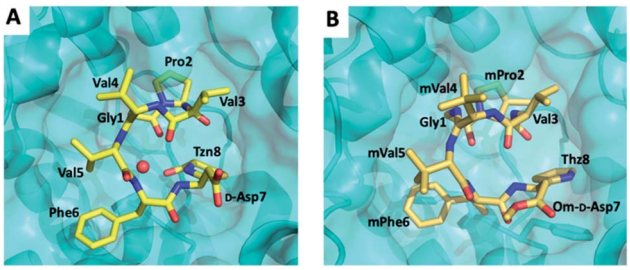


Fig. 10 BotH-**48** (A) and BotH-bottromycin A_2 (B) complex structures.

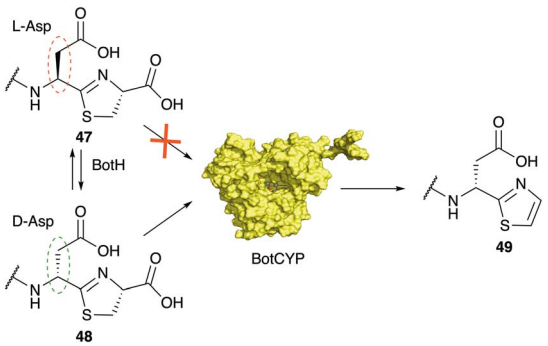


Fig. 11 BotCYP catalyses the oxidative decarboxylation reaction of the thiazoline in **48** to the thiazole found in the bottromycin core structure. The cytochrome P450 enzyme is stereoselective for the D-Asp containing intermediate and thus provides stereochemical resolution for the pathway.

BotH is annotated as an ABH, and its crystal structure revealed a typical ABH fold, but the canonical Ser–His–Asp catalytic triad residues, which the majority of the ABH family members possess, is mutated (Ser to Phe, His to Ile) or missing (Asp).[112] Accordingly, no hydrolytic activity was detected for BotH. Instead, the follower-cleaved intermediate **47** binds to the large cavity of the designated BotH active site and *in vitro* biochemical assays determined BotH to function, unexpectedly, as the epimerase of the Asp residue found in bottromycins (**1**).[112] Deuteron labelling experiments revealed, that the enzyme catalyses the rapid epimerisation of L-Asp to D-Asp, and its back-reaction (Fig. 7). The action of BotH leads to a mixture of **47** and **48**, but provides a much greater abundance of the D-Asp containing intermediate **48** than the spontaneous epimerization, which proceeds a glacial speeds and favours **47**. In the complex crystal structure of BotH with **48**, no potential catalytic residues of BotH were in a reasonable distance of the substrate Asp Cα proton. In addition, mutation of the core peptide Asp residue to Ala or Asn prevented epimerisation, but not binding. Substitution of Asp with Glu still allowed epimerization, which led the suggestion of substrate-assisted catalysis. BotH is the first reported ABH to catalyse peptide epimerisation.

In other RiPP pathways, epimerisation usually involves radical SAM enzymes or a two-step dehydration–hydrogenation process to generate D-alanine from L-serine.[113–116] BotH is thus the founding member of a group of atypical ABH enzymes that may be able to epimerise amino acids post-translationally and but also other secondary metabolites. Further, BotH binds the pathway product bottromycin A_2, but is not able to epimerise it. The complex crystal structure of BotH and bottromycin A_2 was the first crystal structure of any bottromycin (Fig. 10). The resulting orthosteric inhibition of BotH by bottromycin A_2 may results in a biosynthetic feedback mechanism to prevent self-poisoning.[112]

6.5 Cytochrome P450 enzyme BotCYP

Thiazoles (and oxazoles) as heterocycles derived from cysteine (or serine/threonine) are a common motif in RiPPs and are associated with linear azol(in)e-containing peptides (LAPs), cyanobactins, thiopeptides, and bottromycins. Thiazoline oxidation is more frequently observed than oxazoline oxidation and usually involves an FMN-dependent dehydrogenase.[13,15] In bottromycin biosynthesis the oxidative carboxylation of the C-terminal thiazoline is catalysed by the P450 enzyme found in the gene cluster.[96] P450 enzymes are not very common in RiPP pathways, although a recent large-scale survey of the RiPP landscape identified ~1800 P450 genes associated with putative RiPP BGCs.[117] The handful of P450 enzymes that had been characterized from RiPP biosynthetic pathways predominantly catalysed amino acids hydroxylation,[118–121] but other functions had also been reported.[120,122,123] The P450-catalyzed oxidative decarboxylation of a heterocycle in RiPP biosynthesis had not been investigated *in vitro* but recent work by the Koehnke group demonstrated that BotCYP selectively acts on D-Asp containing intermediate **48** (Fig. 11).[117] Given the substrate-assisted mechanism of BotH, this observation rationalizes why all attempts to produce bottromycin analogs with residues other than Asp/Glu were unsuccessful (Fig. 13). Without a D-amino acid in core peptide position 7 the pathway stalls at the point of oxidative decarboxylation. Unfortunately the crystal structure of a close BotCYP homologue did not provide an answer as to how this enzyme may provide stereochemical resolution for the pathway. The reconstitution of this step has nevertheless enabled the production of the bottromycin core scaffold, which may aid the production of bottromycin analogs for bioactivity testing in the future.

7 Heterologous production of bottromycins and derivatives

Early studies on bottromycin benefitted from large-scale fermentation to aid with product isolation and characterisation, such as a 3000 litre fermentation used by Waisvisz and colleagues in 1957 to first characterise bottromycin.[1] However, more recently, numerous groups have reported very low bottromycin yields with laboratory-scale fermentations,[8,9,96] which has presented challenges for understanding bottromycin biosynthesis and engineering the pathway to generate derivatives.

7.1 Regulation of the bottromycin biosynthetic gene cluster

To identify the transcriptional organisation of the bottromycin BGC, Vior *et al.*[7] used 5′-tag-RNA-seq in *S. scabies*.

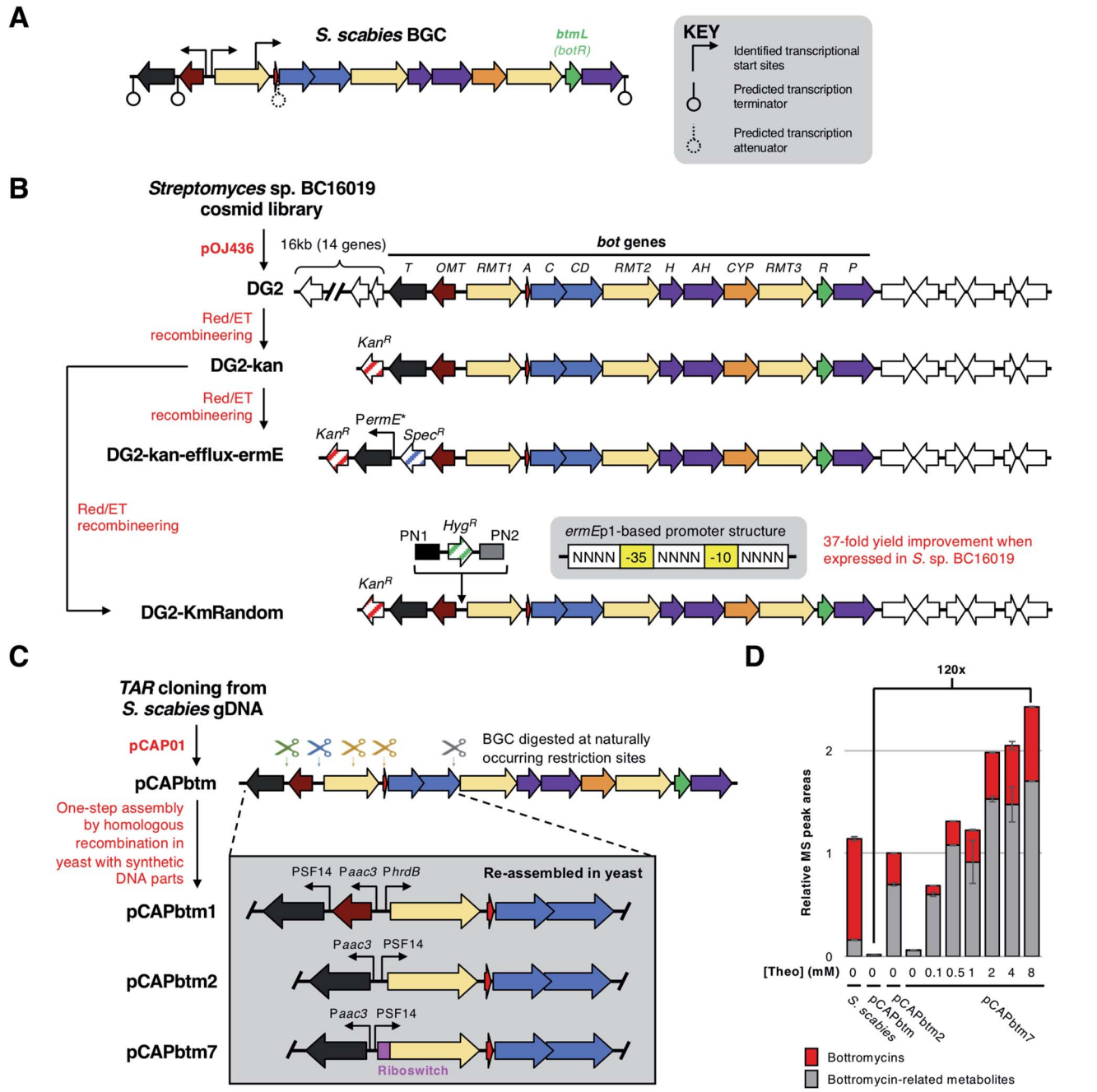


Fig. 12 Overview of bottromycin BGC regulation and methods used genetically engineer the BGC. (A) Natural transcriptional start sites and predicted terminators determined by Vior *et al.*[7] The gene encoding the pathway-situated transcriptional modulator BtmL (BotR) is highlighted. (B) Overview of cloning and engineering the *Streptomyces* sp. BC16019 BGC by Huo *et al.*[8] and Horbal *et al.*[9] (C) Overview of cloning and engineering the *Streptomyces scabies* BGC by Eyles *et al.*[14] showing selected examples of engineered BGCs. (D) Bottromycin production chart adapted from Eyles *et al.*[14] [Theo] = theophylline induction concentration.

This revealed transcriptional start sites preceding *botRMT1* and *botOMT* (Fig. 12A), as well as an internal transcriptional start site within *botRMT1*, which precedes the precursor peptide gene, *botA*. This is hypothesised to increase expression of the precursor peptide in relation to the tailoring enzymes. RT-PCR (reverse transcription - polymerase chain reaction) and 5′-RACE (rapid amplification of cDNA ends) experiments were consistent with the 5′-tag-RNA-seq analysis, although inferred a possible additional transcriptional start site before *botT* that was not detected by 5′-tag-RNA-seq. This study showed that the bottromycin BGC does not encode a pathway-specific master regulator, and instead encodes a regulatory protein, BotR, that specifically modulates the expression of *botA* but not other genes in the BGC. The precise mechanism of BotR-mediated modulation remains enigmatic, especially as no strong promoter activity

could be detected from the internal transcriptional start site that precedes *botA*.

7.2 Heterologous expression of the *bot* BGC

Huo *et al.* were the first to express a bottromycin BGC in a heterologous host.[8] Here, a pOJ436-based[124] cosmid library from *Streptomyces* sp. BC16019 was screened for the intact *bot* BGC. The resulting DG2 construct was then expressed in *Streptomyces albus* J1074 and *Streptomyces coelicolor* A3(2) to successfully produce bottromycin A_2, although estimated production levels (1–4 μg L^{-1}) were much lower than from the native producer. This large construct was simplified by removing a 16 kb fragment of non-*bot* genes from the 5′-end of the BGC. This was replaced with a kanamycin resistance gene *via* Red/ET recombineering[125] to generate cosmid DG2-kan (Fig. 12B).

To increase production levels from DG2-kan, an approach pioneered by Ochi and colleagues[126,127] was employed. This strategy involved obtaining rifampicin-resistant isolates that contain mutations in the *rpoB* gene, which encodes the RNA polymerase β-subunit. These *rpoB* mutations can enhance levels of antibiotic production in *Streptomyces* without affecting growth. Accordingly, bottromycin production was increased by about 10-fold in rifampicin-resistant mutants of *S. coelicolor*-DG2-kan. Huo *et al.* hypothesised that a limiting factor preventing higher yields was a lack of bottromycin resistance in *S. coelicolor*. The most likely self-immunity gene in the cluster is *botT*, which encodes a major facilitator superfamily (MFS) transporter.[128] Therefore, to increase *botT* expression, the region preceding the *botT* gene was replaced with the strong P*ermE** promoter using Red/ET recombineering. This further increased bottromycin production levels two-fold compared to the rifampicin-resistant mutants.

Given that 11 *bot* biosynthetic genes (*botRMT1-botP*) are present on a polycistronic operon,[7] it should be possible to engineer pathway regulation by simply changing the promoter preceding *botRMT1* and optionally modifying the promoter(s) for *botOMT* and *botT*. Truman and colleagues used transformation-associated recombination (TAR) cloning[129,130] in *Saccharomyces cerevisiae* (yeast) to directly capture the bottromycin BGC from the genomic DNA of *S. scabies* DSM 41658.[14] This used the yeast/*E. coli* shuttle vector pCAP01,[129] which can also integrate into actinobacterial genomes *via* the ϕC31 attachment site. The resulting vector, pCAPbtm, was introduced into *S. coelicolor* M1146,[131] but bottromycin was produced in negligible amounts. Therefore, it was hypothesised that the bottromycin BGC could be efficiently engineered to improve productivity by use of homologous recombination in yeast. This strategy involved introducing double-strand breaks in pCAPbtm *via* restriction sites naturally found in the bottromycin BGC. This fragmented BGC was then repaired using a combination of double- and single-stranded DNA fragments to introduce new genetic features in a marker-free way (Fig. 12C).[14]

This approach was used to generate a series of modified pCAPbtm-derived plasmids that contained a variety of strong promoters (PSF14, P*hrdB*, P*aac3*, P*ermE**) in front of *botRMT1* and *botT*, as well as rearranged the *botA* and *botRMT1* genes. In an effort to limit the potential toxicity of bottromycin overproduction, the *botOMT* gene was removed, as previous work had shown that BotOMT-catalysed *O*-methylation is important for bottromycin activity.[2] To fully understand the metabolic consequences of engineering regulation, the total productivity of the pathway was assessed using LC-MS-based metabolomics to detect multiple peptides derived from the bottromycin pathway that likely resulted from incomplete biosynthesis and subsequent hydrolysis of modified BotA. One surprising challenge was that BotRMT1 was inactive in all conditions tested, which resulted in all molecules lacking a β-methyl group on phenylalanine. Sequencing revealed no mutations to the gene cluster in any of the constructed vectors, so to further control expression levels, an inducible theophylline-dependent riboswitch[132] was incorporated upstream of *botRMT1*. While this did not lead to active BotRMT1, theophylline induction did lead to the most productive heterologous expression system tested in this study, which was 120 times more productive than heterologous expression of the wild type BGC, as well as producing higher levels of bottromycin-related metabolites than the native *S. scabies* producer (Fig. 12D).

The DG2-kan cosmid generated by Huo *et al.*[8] was used as the basis for BGC engineering by Luzhetskyy and colleagues.[9] DG2-kan contains the *bot* BGC from *Streptomyces* sp. BC16019, and this cosmid was expressed in *Streptomyces lividans* TK24 to yield 0.23 mg L^{-1} bottromycin A_2. To improve pathway productivity, the BGC was initially engineered to replace the native promoters of *botOMT* and *botRMT1* with strong promoters from a promoter library previously generated by the Luzhetskyy group.[133] The selection of strong promoters initially led to *S. lividans* growth problems, potentially caused by toxicity of the pathway to the host. This was overcome by selecting for bottromycin-resistant *S. lividans* mutants, which led to bottromycin A_2 production levels of up to 3-fold higher than a control strain.

As it can be difficult to predict the precise relationship between promoter strength and pathway productivity, a 'random rational strategy' was employed to further boost yields from DG2-kan. Here, a library of *bot* BGCs was generated that featured random synthetic promoters inserted between *botOMT* and *botRMT1*. These random promoters were created based on the consensus −35 and −10 sequences of the *ermE*p1 [134] promoter (Fig. 12B). Degenerate primers were then used to randomise the sequences upstream, between and downstream of these consensus sequences. These were then introduced into the BGC using Red/ET cloning, and the mutated cosmids were conjugated into *S. lividans* TK24. Screening of 100 randomly selected strains harbouring mutated BGCs (DG2-KmRandom, Fig. 12B) revealed that 10% produced 5–50 fold more bottromycin A_2 than a control strain harbouring unmodified DG2-kan. Quantitative RT-PCR (RT-qPCR) revealed that the transcription from both promoters had increased in the best producer, but that the strength for each promoter was very different (1 : 59 ratio), which emphasises the benefit of screening a promoter library. Pathway productivity was further increased by introducing this mutated BGC into the native producer, *Streptomyces* sp. BC16019, which therefore contained

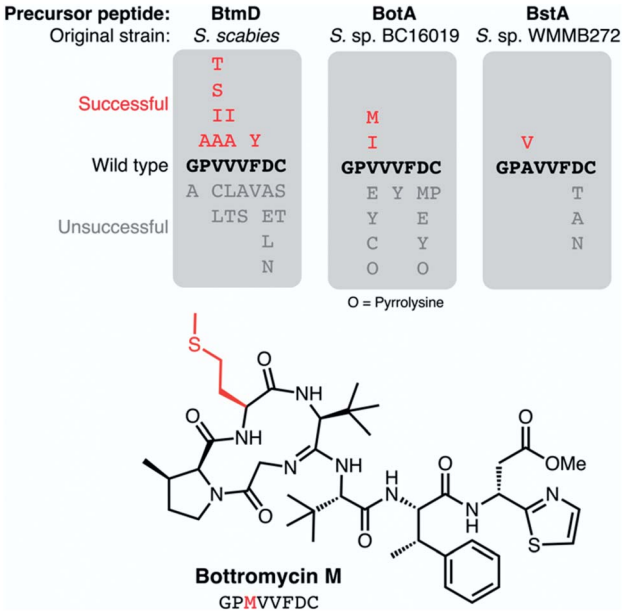


Fig. 13 Mutations made to precursor peptide BotA and homologues in other bottromycin BGCs.[2,9,136] The characterised structure of bottromycin M is shown.

one copy each of the wild type and mutated *bot* BGCs. This led to a 37-fold increase in bottromycin production in relation to wild type *Streptomyces* sp. BC16019.

There is a lack of natural diversity within the core peptides of known bottromycins (Fig. 4B), which is in contrast to most other RiPP families.[15] RiPPs are uniquely suited to pathway engineering to generate derivatives, as mutations to the core peptide lead to predictable changes to the final chemical structure. The lack of diversity amongst natural bottromycins means that there is a lot of chemical space to explore for bottromycin-like molecules, which could be important in the context of antibiotic discovery. Therefore, Luzhetskyy and colleagues generated DG2-kan cosmids with mutated *botA* genes,[9] and then introduced these into *Streptomyces* sp. BC16019 Δ*botA*, which is unable to make the wild type BotA precursor peptide. 12 mutants were generated, although most led to no detectable bottromycin-like metabolites, indicating a lack of pathway tolerance to these unnatural substrates, which is unlike many other RiPP pathways.[135] Based on these data, the position most tolerant of modifications was Val3 of the core peptide (Fig. 13). Peptides with isoleucine and methionine residues at this position were successfully converted into bottromycin derivatives containing all expected post-translational modifications. Fig. 13 shows methionine-containing bottromycin M. Position 3 of the core peptide is effectively the only amino acid that is not post-translationally modified during biosynthesis and is the only position where natural variation has been observed, as bottromycin D from *Streptomyces* sp. WMMB272 features an alanine at this position.[12] In this strain, an Ala3Val mutant was tolerated and therefore generated bottromycin A_2. In contrast, no products were detected from three different mutants of Asp7 in this strain (Fig. 13).

Comparable mutation results were obtained by Crone *et al.*[136] Here, 22 *botA* mutants were generated using the precursor peptide complementation strategy previously reported for *S. scabies* Δ*btmD*[6] (the *botA* orthologue in this strain). As with *Streptomyces* sp. BC16019, Val3 of the core peptide was most tolerant of mutations, where mutations to Ala, Ile, Ser and Thr residues all led to bottromycin analogs with expected masses and tandem MS fragmentation patterns (Fig. 13). As with the other mutant studies, no bottromycin analogs were produced by mutations to Asp7, which was targeted due to the reported importance of this residue for activity.

8 Conclusions

Over 60 years after their original discovery bottromycins continue to capture the attention of research groups and provide riddles to be solved. Given the developing antibiotic crisis and the effectiveness of bottromycins against (multi) drug-resistant pathogens, the recent advances in bottromycin research should be capitalized on: through chemical synthesis and a combination of biochemistry/biotechnology new chemical space has become available to explore a more comprehensive SAR and address the *in vivo* instability of the compounds. In addition, the actual target within the A-site of the prokaryotic 50S ribosome has remained elusive and the MICs of some derivatives suggest an alternative, unknown target. We therefore think that despite its long history, the story of bottromycins may be just beginning.

9 Conflicts of interest

There are no conflicts to declare.

10 Acknowledgements

We thank Prof. Shuichi Hirono and Prof. Hiroaki Gouda for providing the coordinate file of the NMR structure of bottromycin A_2 in $CDCl_3$.

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