The bottromycin epimerase BotH defines a group of atypical α/β-hydrolase-fold enzymes

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Abstract

D-amino acids endow peptides with diverse, desirable properties, but the post-translational and site-specific epimerization of L-amino acids into their D-counterparts is rare and very challenging. Bottromycins are ribosomally synthesized and post-translationally modified peptides that have overcome this challenge and feature a D-aspartate (D-Asp), which was proposed to arise spontaneously during biosynthesis. We have identified the highly unusual alpha/beta-hydrolase (ABH) fold enzyme BotH as a novel peptide epimerase responsible for the post-translational epimerization of L-Asp to D-Asp during bottromycin biosynthesis. The biochemical characterization of BotH combined with the structures of BotH and the BotH-substrate complex allowed us to propose a mechanism for this reaction. Bioinformatic analyses of BotH homologs show that similar ABH enzymes are found in diverse biosynthetic gene clusters. This places BotH as the founding member of a new group of atypical ABH enzymes that may be able to epimerize non-Asp stereocenters across different families of secondary metabolites.

Introduction

Enzymes belonging to the superfamily of alpha/beta-hydrolase-fold proteins (ABHs) are found in all domains of life[1-3]. Their catalytic roles are highly diverse and they participate in primary and secondary metabolism, where they are usually responsible for the hydrolysis of (thio)ester and peptide bonds[4]. In addition, ABHs have also been reported to function as dehalogenases, epoxide hydrolases, dioxygenases, decarboxylases and haloperoxidases[3,4]. Despite low overall sequence conservation, ABH enzymes share a remarkably conserved core fold[2,3] that has a V-shaped lid domain above the active-site as a frequent addition[5,6]. A majority of ABH family members possesses a canonical Ser, His, Asp catalytic triad at the active-site, but other catalytic residues have also been observed[3,4]. In addition to catalytic roles, a ABH family members have also been reported to fulfil several other important functions, including small-molecule receptors that are involved in signal transduction, cell-cell interaction and channel regulation[5-8]. The ABH fold is thus a prime example for the reappropriation of a conserved core fold during evolution to fulfill a myriad of functions.

An aberrant ABH protein, BotH, is encoded in the biosynthetic gene cluster (BGC) for bottromycins[9-12]. This natural product family was first isolated from the terrestrial bacterium *Streptomyces bottropensis* and originally described as peptidic antibiotics with potent activity against Gram-positive bacteria[13,14]. Bottromycins are active against problematic human pathogens, such as Methicillin-resistant *Staphylococcus aureus*[15,16], address a novel target (the A-site of the prokaryotic ribosome)[17-19] and belong to the fast growing superfamily of ribosomally synthesized and post-translationally modified peptides (RiPPs)[9-12]. As is typical for RiPPs, their biosynthesis begins with the expression of a small structural gene to yield the precursor peptide (PP)[20]. Uniquely amongst bacterial RiPPs, the bottromycin PP contains an N-terminal core peptide (the eventual natural product) and a C-terminal follower peptide[9-12], which is important for substrate recognition by several of the biosynthetic enzymes[21,22]. The order of biosynthetic steps (and responsible enzymes) has since been proposed based on an untargeted metabolomics approach using mass spectral networking[23]. Subsequent *in vitro* work has largely corroborated the metabolomics data[21,22,24,25]. In the first phase of bottromycin biosynthesis, the N-terminal methionine is removed, proline, valine and phenylalanine residues of the core peptide are C-methylated and a cysteine-derived thiazoline is installed in no particular order[23]. The hallmark

macroamidine linkage is then formed in this intermediate[21,22], which is followed by proteolytic removal of the follower peptide to yield **1** (Fig. 1a)[25]. To complete the biosynthesis of bottromycin A2 (**2**, Fig. 1a), **1** undergoes epimerization, oxidative decarboxylation and O-methylation. While the latter two modifications have been attributed to specific enzymes, epimerization of the L-Asp of **1** was observed to progress spontaneously, albeit very slowly[23].

Amino acid epimerization in non-ribosomal peptide synthesis is usually catalyzed by epimerization domains embedded within the assembly line that function on carrier protein-bound aminoacyl substrates[26]. Due to their ribosomal origin, RiPPs must undergo post-translational epimerization after the PP has been expressed as an all L-amino acid peptide. To date, only two enzymatic mechanisms for this process have been described in RiPPs, involving either a radical-SAM enzyme[27-30] or a two-step dehydration-hydrogenation process to generate D-alanine from L-serine (Fig. 1b)[31,32].

Here, we report the identification of BotH, an unusual ABH enzyme from the bottromycin BGC, as the epimerase of the biosynthetic pathway. This is the first reported instance of an ABH enzyme catalyzing peptide epimerization and thus expands the catalytic scope of this vast enzyme family. Biochemical data together with the structure of the BotH–substrate complex allowed us to propose a mechanism for this reaction. Interestingly, BotH is also able to bind bottromycins with high affinity, which hints at additional function(s) in the biosynthetic process. We show that all canonical ABH active-site residues required for hydrolase activity are absent in BotH, and bioinformatic analyses indicate that BotH homologs with comparable non-hydrolytic residues are widespread amongst BGCs and may catalyze similar biosynthetic steps.

Results

BotH is an unusual member of the ABH superfamily

In a search for an enzyme that may catalyze the epimerization in the bottromycin pathway, we noticed that the gene encoding for BotH had been annotated as an ABH, but that the predicted active-site residues (Ser/His/Asp) were not present and there were no prior experimental data on the role of this protein. We therefore expressed, purified and crystallized BotH (see Materials and Methods for details). A high-resolution (1.18 Å) native BotH dataset was collected from a crystal belonging to

space group I222, which was phased using seleno-methionine BotH data (data collection and refinement statistics for all structures can be found in Supplementary Table 1). The crystals contained one protomer in the asymmetric unit and the electron density for residues 10 – 262 was continuous in the refined model. BotH is comprised of the prototypical ABH core structure (Extended Data Fig. 1) and the putative active site was covered by a V-shaped loop consisting of four α-helices (Extended Data Fig. 1). A search for similar structures using the DALI server[33] revealed 3-oxoadipate-enol-lactonase (PDB 2xua)[34] as the closest structural homolog (Extended Data Fig. 1). A comparison of the two proteins revealed that in BotH, the active-site Ser has been mutated to a Phe, which is part of a Phe-Phe motif that spans a large, hydrophobic plane at the active site (Extended Data Fig. 1). The remaining residues of the catalytic triad are either mutated (His to Ile) or missing (Asp) (Extended Data Fig. 1). In spite of these mutations, the sizeable cavity found in this region of the structure appeared to be large enough for binding **1**.

BotH catalyzes the epimerization of 3b and 3a

To confirm this hypothesis, a des-methyl analog of **1**, **3** (des-methyl Pro2, Val4, Val5 and Phe6), was enzymatically produced as reported previously (Fig. 2a and Extended Data Fig. 2)[25]. Careful analysis revealed **3** to exist as an epimeric mixture of **3a** (D-Asp) and **3b** (L-Asp) (Fig. 2a, Extended Data Fig. 3, Supplementary Figure 1 and Synthetic Procedure 1). When 20 µM **3a**/**b** was incubated with 5 µM BotH and the reactions were analyzed by high-resolution electrospray ionization liquid chromatography−mass spectrometry (HR-ESI-LCMS), we observed a change in **3a** : **3b** ratios (Fig. 2b), which was BotH-concentration dependent within the 2 h time-scale of the experiment (Fig. 2c): Increasing the BotH concentration resulted in a shift to **3a** (D-Asp), which is the required epimer to proceed with biosynthesis (S. Adam, L. Franz, M. Milhim, R. Bernhardt and J. Koehnke, unpublished). To probe if only **3b** or both epimers were substrates, we incubated **3a**/**b** with BotH in D_2O. This resulted in rapid (< 60 s) deuteron incorporation at the Asp7 position in both peaks, while very little deuteron incorporation was observed in the absence of BotH even after 24 hours (Fig. 2d, Extended Data Fig. 3 and Synthetic Procedure 2). In fact, BotH concentrations low enough to leave epimer ratios unchanged still resulted in accelerated deuteron incorporation (Synthetic Procedure 2).

Repeating this experiment using the deuterated sample in H_2O showed an equally rapid exchange with solvent protons back to **3a**/**b** (Synthetic Procedure 2). These data implied that BotH accepts both, **3a** and **3b**, as substrates in a reversible reaction, while favoring D-Asp (**3a**) as the product.

Structure of the BotH-3a complex

To better understand the mechanism of this intriguing enzyme, the high-resolution crystal structure of BotH in complex with its substrate **3a**/**b** was determined to 1.25 Å resolution. The overall structure of the complex was virtually unchanged when compared to the apo structure (C_α rmsd of 0.12 Å) (Fig. 3a and Extended Data Fig. 4). The substrate is curled into the active site in a way that places the thiazoline underneath the four amino acid macrocycle and the substrate engages in extensive hydrophobic interactions as well as inter- and intramolecular hydrogen bonds (Fig. 3b and Extended Data Fig. 4). Of particular note are hydrogen bonds of the thiazoline carboxy group with the backbone NH of Val41 and Phe110. These two BotH residues are in the position of the oxyanion hole found in many hydrolases. The carboxy group of the substrate's Asp7 is involved in a hydrogen bonding network with ordered water molecules that ultimately link this side-chain to BotH residues (Extended Data Fig. 4). An intramolecular hydrogen bond links the substrate's carbonyl of Val3 with the backbone NH of Asp7. The best fit to the electron density is achieved by choosing the amidine resonance structure that places the double-bond inside the macrocycle and a D-Asp in position 7, which indicates that the ligand observed in the complex structure is **3a** (Extended Data Fig. 4). Since the epimerization mechanism would, at least formally, involve proton abstraction and addition, we scanned the complex structure for potential catalytic residues within 4 Å of the C_α hydrogen of Asp7, the site of catalysis, but could not identify any. This left two possibilities: bulk solvent or the side chain of substrate Asp7; its carboxy group is within 2.2 Å of the C_α hydrogen of interest. We would like to note that the carboxy group of Asp7 is surrounded closely (O – O distances 2.5 – 3.1 Å) by four ordered water molecules (Extended Data Fig. 5), which appear well-positioned to facilitate the exchange of the abstracted proton/deuteron with bulk solvent during catalysis (Extended Data Fig. 5).

To probe the importance of the side chain identity in position seven for epimerization, we first tested the conservative mutant substrates Asp7Ala and Asp7Asn. While both substrates stabilized BotH in thermal shift assays comparable to **3a**/**b**, we observed no epimerization (Extended Data Fig. 6). The extension of the Asp7 side-chain by an additional methylene group (Asp7Glu), results in severely reduced turnover by BotH, but this substrate epimerizes (Extended Data Fig. 6). These data imply that a side-chain carboxy group in position 7 of the substrate is essential for catalysis and that the appropriate distance of this carboxy group relative to the residue's C_α proton has a significant impact on turnover, which suggests that the BotH reaction may be an example of substrate-assisted catalysis.

Bottromycins act as orthosteric inhibitors of BotH

Since **3a**/**b** are close structural homologs of bottromycin A2 (**2**), it seemed feasible that BotH may be able to bind to bottromycins. We thus performed microscale thermophoresis (MST) experiments using heterologously produced **2** and three closely related variants (**4** – **6**, Extended Data Fig. 7). BotH was able to bind all four bottromycins with K_D values in the high nM to low µM range but unable to epimerize oxidatively decarboxylated **3a** (Extended Data Fig. 7). To understand the mode of binding, we determined the crystal structures of three bottromycin–BotH complexes to 1.40 (**2**), 1.70 (**5**), and 1.48 (**6**) Å resolution, respectively (Fig. 3c, Extended Data Fig. 8 and Supplementary Figure 2). These also represent the first crystal structures of any bottromycin. As observed for the complex with **3a**, the overall structural change in BotH due to ligand binding was minimal (C_α RMSD < 0.2 Å) and the bottromycins bound in a similar manner as **3a** (Figs. 3b and 3c). Despite the high resolution, it is not obvious which way the thiazole is flipped, since the loss of the carboxy group due to thiazoline oxidation allows a fit of both rotamers without inducing a clash with BotH (Supplementary Figure 3). The bottromycins themselves are oriented in a twisted fashion that results in several tight, intramolecular hydrogen bonds (Supplementary Figure 2). Compared to the published NMR structure[35], bottromycin A2 experiences significant strain as a result of binding to BotH, as it is forced to adopt a horseshoe shape with Val5 and Phe6 at its apex and the C-terminal thiazole stacked parallel

under the macrocycle (Extended Data Fig. 9). As expected, bottromycin A2 acts as an orthosteric inhibitor of epimerization (Supplementary Figure 4).

It is still unclear why bottromycin A2 contains a D-Asp, as studies with synthetic derivatives have shown that both epimers display the same bioactivity[36]. Selectivity of the bottromycin exporter BotT appears possible, despite a very small fraction of bottromycin A2 in culture supernatant appearing to be the L-Asp epimer (Synthetic Procedure 3). The main benefit of epimerization may be providing increased resistance to proteolytic degradation of bottromycin, since D-amino acid containing peptides have longer half-lives[37,38]. It is of course also possible that the bottromycin target of organisms in a native setting requires a D-Asp for optimal target binding. Interestingly, the structure elucidation of novel bottromycin analogs via crystallization of their complex with BotH appears to require less compound and be more straightforward than NMR, due to the robust crystallization condition, high affinity and very well-diffracting crystals.

The ability of BotH to bind both the substrate and the mature natural product with high affinity may serve as a cautionary tale for attempting to identify the binding partners of "non-catalytic" ABHs. When using BotH to capture its ligand/substrate from either supernatant or lysate of a bottromycin producing strain, we were only able to detect bottromycin A2, but not the actual substrate **1** (Supplementary Figure 5). Since biosynthetic pathway products tend to be present at higher concentrations than pathway intermediates, careful analysis of the biosynthetic pathway supplying the ligand is required to exclude additional, non-canonical catalytic function(s) of the ABH under investigation.

BotH has relaxed substrate specificity

We had previously reported that the enzymes used to generate **3a**/**b** possess relaxed substrate specificities for core-peptide residues 2 – 7 (Supplementary Figure 6)[25]. Since substrate position 7 was restricted to Asp or Glu for epimerization, we wondered if BotH tolerates mutations in the remaining positions. To this end, an additional series of 13 BotA core-peptide mutants were used to produce the variant BotH substrates enzymatically. These substrates were then incubated with or without BotH and

analyzed by LC-MS (Fig. 4a and Supplementary Figures 7 - 8). All but two mutations in positions two to six were processed by BotH. The positions of Val5 and Phe6 are intimately connected and it appeared that the orientation of the Phe6 side-chain is critical for epimerization. In agreement with this hypothesis, Phe6Ala could not be epimerized by BotH while Phe6Tyr and Phe6Trp were substrates. The side-chain of position 5 (Val) is engaged in hydrophobic interactions with the side-chains of Val4 and Phe6. Accordingly, Val5Thr is not a BotH substrate, while Val5Ala, Val5Leu and Val5Glu can be epimerized by the enzyme. The ability of BotH to process Val5Glu may appear surprising, but the flexibility of the Glu side chain should allow the C_β methylene of Glu5 to engage in hydrophobic interactions with Val4 and Phe6, while the C_γ methylene and terminal carboxy group point towards bulk solvent. Our data demonstrate that BotH is able to process a variety of substrates, which will be invaluable for the production of bottromycin derivatives.

Evolution of BotH and its spread amongst bacterial BGCs

ABHs comprise one of the largest protein families (~500,000 protein matches in InterPro[39], ~400,000 of them in Bacteria), of which 192,602 belong to Pfam[40] family PF00561 (Abhydrolase_1). The Ser-His-Asp catalytic triad is surprisingly poorly conserved: in 101,123 bacterial proteins from the PF00561 family, at least one of the catalytic residues is mutated or missing. In most cases (73,116 sequences), His is missing; and in the majority of these proteins (64,852 sequences) Asp is missing as well. Ser is mutated in only 42,810 sequences, most frequently to an aspartic acid, and completely missing in 1,283 sequences. Using these data, we identified 1,530 proteins that are unlikely to have hydrolase activity (see Materials and Methods section for details), including BotH. Taxonomic analysis revealed that these non-functional hydrolases are widespread among both Gram-positive and Gram-negative bacteria.

From these sequences, we then selected those that were in or near (less than 1 kb away) BGCs, which resulted in 107 proteins that were used to build a sequence–similarity network (Fig. 4b and Extended Data Fig. 10). In this network, all BotH homologs from bottromycin BGCs cluster together. Among

the identified BGCs, two other major natural product superfamilies can be identified in addition to RiPPs: NRPS (non-ribosomal peptide synthetase) and PKS (polyketide synthase) clusters.

Discussion

We have identified the enzyme BotH as the epimerase of the bottromycin BGC, which is selective for Asp and Glu, but promiscuous with regards to mutations at other positions of the substrate. Based on the biochemical and structural data, we propose the following mechanism for epimerization in bottromycin biosynthesis (Fig. 5b): Cleavage of the follower peptide converts the 2-thiazoline residue into a 2-thiazoline-4-carboxy moiety at the carboxy terminus of **3b**, which is bound by BotH. Interestingly, the two BotH residues that form hydrogen bonds with the thiazoline carboxy group are in the position of the canonical oxyanion hole found in most ABHs. By binding, **3b** traps an ordered water molecule within hydrogen-bonding distance of the thiazoline's nitrogen and carboxy group. The side-chain of Asp7 is positioned such that it may serve as a base to abstract the C_α proton from itself, which triggers enamine formation and leads to proton transfer from the ordered water molecule to the thiazoline nitrogen. The resulting hydroxide is still hydrogen bonded to the thiazoline's carboxy group and the thiazoline nitrogen proton. Abstraction of this proton by the hydroxide triggers reprotonation of the enamine by the side chain of Asp7 and results in epimerization. The intermediate appears to be long-lived enough to allow exchange of the proton abstracted by Asp7 with bulk solvent (Extended Data Fig. 5). The inability of BotH to epimerize substrate with an Asp7Asn mutation suggests an essential role of the carboxylic acid side chain and thus substrate-assisted catalysis. As demonstrated by our hydrogen-deuterium-hydrogen exchange experiments, this reaction is fully reversible. It appears that lowering of the energetic barrier for epimerization by BotH is sufficient to supply the succeeding enzyme with sufficient substrate for complete turnover, even without changing the **3a** : **3b** ratios (S. Adam, L. Franz, M. Milhim, R. Bernhardt and J. Koehnke, unpublished). This situation is reminiscent of non-ribosomal peptide synthetases that can contain epimerase domains, which produce a mixture of D- and L-epimers of a particular amino acid. The succeeding enzyme (condensation domain) then provides the stereochemical resolution of the pathway through selective incorporation of the D-amino acid[41].

The inhibition of BotH by the mature natural product (Supplementary Figure 4) which suggests that BotH may be involved in a biosynthetic feedback mechanism to prevent self-poisoning of the producing strain (Figure 5b). In this model, an increase in the intracellular bottromycin concentrations offers a direct and faster means to reduce bottromycin production than altered gene expression since epimerization of Asp7 is highly important for the activity of the succeeding enzyme BotCYP (S. Adam, L. Franz, M. Milhim, R. Bernhardt and J. Koehnke, unpublished). It is of course also possible that BotH sequesters mature bottromycin to aid self-immunity.

The presence of BotH-like ABH protein encoding genes in over 100 BGCs encoding for the biosynthesis of diverse secondary metabolites places BotH as the founding member of a new subfamily of non-classical ABH enzymes. It will be fascinating to explore the functions of selected homologs, which may be able to epimerize non-Asp stereocenters across families of secondary metabolites via the unprecedented mechanism we have identified for bottromycin.

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Author Contributions

A.S. and J.K. established the production and purification of BotH and performed MST experiments. A.S. designed and performed crystallization experiments, determined the reported crystal structures, produced and purified Bottromycin A2 and performed pull-down experiments.. L.F. established BotH activity, carried out the biochemical experiments, produced BotH substrates, carried out Marfey's analysis and performed the mass spectrometry. S.A. established the purification of the BotH substrate. J.S.-A. and A.W.T. aided bioinformatic analyses. L.H. produced, purified and analyzed Bottromycin

A2 and derivatives under the guidance of A.L.. O.V.K. designed and performed the bioinformatic analyses and wrote the bioinformatics section. J.K. analyzed and visualized the crystal structures for publication and wrote the paper with contributions from all authors. The full program was carried out under the guidance and direction of J.K.

Competing Financial Interests Statement

The authors declare no competing interests.

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Figure Legends

Figure 1: Bottromycin biosynthesis and epimerization in RiPPs. **a** Bottromycin BGC found in *S. sp.* BC16019. After expression of the precursor peptide BotA, its N-terminal methionine is removed by BotP, three radical methyl transferases (RMT) perform four C-methylations and BotC installs a cysteine-derive thiazoline. These initial steps appear to follow no particular order. Next, BotCD catalyzes formation of the macroamidine, after which BotAH removes the follower peptide to yield **1**. Oxidative decarboxylation (BotCYP) and O-methylation complete the biosynthesis of bottromycin A2 (**2**). Epimerization was proposed to occur spontaneously. Genes, enzymes and modifications have matching colors. White arrows represent genes for which no or a regulatory function have been proposed. The unusual ABH protein encoded in the pathway, BotH, is highlighted in pink. **b** Epimerization in RiPP biosynthesis reported to date. In proteusins, including polytheonamides, and epipeptides a radical SAM enzyme epimerizes a range of amino acids. In lanthipeptide biosynthesis, l-Ser can be converted to d-Ala in a two-step process.

Figure 2: BotH acts as an epimerase. **a** Spontaneous epimerization of the Asp in position 7 after proteolytic removal of the follower peptide. Marfey's reagent was used to assign the stereochemistry of the Asp for both peaks (Supplementary Figure 1). Extracted ion chromatograms (EIC) of **3** ($[M+H]^+_{calc.}$=799.3807; ± 5 ppm) and mass spectra at 3.00 and 3.15 min are shown. Missing methyl groups are highlighted by dashed ovals. **b** Incubation of **3a**/**b** with BotH results in a change of **3a** : **3b** ratios with **3a** (d-Asp) now the more abundant species. EICs of **3** ($[M+H]^+_{calc.}$=799.3807; ± 5 ppm) are shown. **c** Incubating 20 µM **3a**/**b** with increasing concentrations of BotH led to a shift of the

equilibrium towards **3a**. Increasing BotH concentrations beyond 10 µM did not lead to a further shift of epimer ratios. EICs of **3** ($[M+H]^+_{calc.}$=799.3807; ± 5 ppm) are shown. **d** Deuteron incorporation into **3a/b** by BotH in D_2O buffer. EICs for **3** ($[M+H]^+_{calc.}$=799.3807; ± 5 ppm) (black) and deuterium incorporated **3** ($[M+H]^+_{calc.}$=800.3870; ± 5 ppm) (red), as well as mass spectra at the EIC maxima are shown. Representative experiments were repeated independently three times with similar results.

Figure 3: BotH-3a and -bottromycin A2 complexe structures. a Cartoon representation of the BotH-**3a** complex structure highlighting the V-shaped loop (magenta) positioned above the active site. The bound substrate is shown as sticks (grey (carbon), blue (nitrogen), red (oxygen) and yellow (sulfur). **b** The Close-up of **3a** (light gray sticks, labeled. Tzn = Thiazoline) and bottromycin A2 (dark gray stick. mPhe bound in the BotH active site (cartoon, yellow/magenta with semi-transparent surface representation). The ordered water molecule trapped between substrate and protein is shown as a red sphere. The Asp7 C_α hydrogen is shown as a white stick for clarity. **c** Same as **b**, but bottromycin A2 (dark gray sticks, labeled) bound to the active site of BotH. m = methylated residue, Om = O-methylated residue, Thz = Thiazole.

Figure 4: BotH substrate specificity and distribution of BotH-like proteins in BGCs. a Summary of BotA point mutants tested as BotH substrates. Purple positions cannot be varied (pentagon represents thiazoline). Accepted mutations are highlighted in green, orange indicates a poor substrate and red mutations cannot be processed by BotH. The accompanying HR-LCMS data can be found in Supplementary Figures 7 - 8. **b** Sequence similarity network of ABHs homologous to BotH (marked with an asterix). Of the 107 genes, 76 could be assigned to BGCs representing the three large bacterial natural product superfamilies (darker edges represent higher sequence similarity, NRPS = non-ribosomal peptide synthetase; PKS = polyketide synthase).

Figure 5: Role(s) of BotH in bottromycin biosynthesis. **a** Proposed mechanism for the epimerization of **3b** to yield **3a**. BotH residues are labeled in black, substrate residues in blue. Hydrogen bonds are shown as dashed lines. Ordered water molecules surrounding the Asp7 carboxy group are not shown

for clarity (see Extended Data Fig. 5 for details). **b** Proposed role of BotH in self-resistance. A rise of intracellular bottromycin concentrations leads to an inhibition of BotH epimerase activity, which may in turn prevent self-poisoning of the producing strain and act as an intracellular buffer to store bottromycins.

Online Methods

Protein expression and purification

The BotH coding sequence was amplified from genomic DNA isolated from *Streptomyces* sp. BC16019 and cloned into the pET-28b plasmid (Novagen). The resulting construct was verified by enzymatic restriction digestion and DNA sequencing before being transformed into *E. coli* BL21(DE3) competent cells.

A single colony was selected and grown in LB liquid medium supplemented with kanamycin (50 µg / mL) to make an overnight culture. This culture was used at a dilution of 1 to 100 to inoculate fresh LB medium containing the appropriate antibiotic, and the culture was grown at 37 °C, 180 rpm. Upon reaching an optical density (OD_{600}) of 0.6, the cultures were transferred to a precooled shaker at 16 °C, and protein expression was induced by addition of 1 mM IPTG. The cells were grown at 16 °C and 180 rpm over night before being harvested by centrifugation. Cell pellets were stored at -80 °C until further use.

The cell pellets were resuspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 20 mM Imidazole, and 3 mM DTT) supplemented with 0.4 mg DNAse per gram of wet cell pellet and cOmplete EDTA-free protease inhibitor tablets (Roche). The cell suspension was lysed via passage through a cell disrupter (Constant Systems) at 30,000 psi, and cell debris was removed by centrifugation (40,000 x g, 4 °C, 15 min). The supernatant was collected and directly loaded onto to a 5 mL Histrap HP column (GE healthcare) preequilibrated with lysis buffer. The column was washed extensively with lysis buffer (30 CV) before the protein was eluted with lysis buffer supplemented with 250 mM imidazole. Fractions containing BotH were directly loaded onto a gel filtration column (HiLoad 16/600 Superdex 200 pg, GE healthcare) preequilibrated in gel filtration buffer A (20 mM

HEPES pH 7.4, 200 mM NaCl, 1 mM TCEP). The fractions of the highest purity as judged by SDS-PAGE were pooled and concentrated to 5 mg / mL.

Crystallization and structure determination

For crystallization, BotH was treated with thermolysin (1 : 100) on ice for 2 h, after which the protein was passed over a HiLoad 16/600 Superdex 200 pg gel filtration column as described above and concentrated to 5 mg / mL. Crystals of apo BotH and BotH in complex with ligands were obtained at 18 °C in 1.2 - 1.8 M ammonium sulfate and 0.1 M Tris-HCl using the hanging drop vapor diffusion method. For the complex crystallization, the thermolysin-treated protein was incubated with excess ligand (1 - 2 mM) on ice overnight before setting up crystallization drops. Crystals appeared after a few days and were allowed to grow for an additional week. The crystals were cryoprotected in mother liquor supplemented with 30% glycerol and 0.5 mM ligand. Data was collected at ESRF (Beamlines ID23-1 and ID23-2), DESY (Beamline P11) and SLS (Beamline X06DA). To solve the apo structure, a single wavelength anomalous dispersion (SAD) data set was collected at the Se K absorption edge. Data were processed using Xia2[42], the structure was solved using PHENIX *AutoSol*[43] and the initial model used to obtain a molecular replacement solution for the high-resolution native data set using Phaser[44]. The solution was manually rebuilt in COOT[45] and refined using PHENIX Refine[43]. This apo structure was then used as a search model for structure determination of the complex crystal structures by molecular replacement (Phaser). Data for all complex crystal structures were processed using XDS[46] and POINTLESS[47], AIMLESS[48] and Ctruncate[49] implemented in ccp4[50]. All structures were validated using MolProbity. The images presented were created using PyMOL (Schrödinger, LLC) and LigPlot+[51].

Enzymatic reaction of BotH with **3**

To investigate the effect of BotH on **3a**/**b**, 20 µM **3a**/**b** were incubated with and without the addition of 5 µM BotH in GF buffer for 30 min. Reactions were stopped by the addition of 2 volumes ACN and were frozen at -80 °C until analysis. Reactions were set up and analyzed in at least triplicates.

To test the effect of different BotH concentrations on the **3a** : **3b** ratio, 20 µM **3a**/**b** were incubated

with 0, 0.37, 1.11, 3.33 or 10 µM BotH at 30 °C for 2 h in GF buffer. Reactions were stopped by the addition of 2 volumes ACN and were frozen at -80 °C until analysis. Higher concentrations than 10 µM or longer incubation times were also tested, but did not lead to a further shift of epimer ratios. Reactions were set up and analyzed in triplicates.

To produce a roughly racemic mixture of **3a** : **3b**, 20 µM **3a**/**b** were incubated with 4 µM BotH for 30 min at 30 °C in GF buffer. BotH was denatured at 98 °C, 10 min, pelleted by centrifugation (15 min, 15,000 rpm) and the supernatant was lyophilized to remove all solvent.

Production of seleno-methionine (SeMet) BotH

L-Selenomethionine-labeled (SeMet) protein was expressed in *E. coli* BL21 cells grown in minimal medium supplemented with glucose-free nutrient mix (Molecular Dimensions), kanamycin (50 µg / mL) and glycerol (5% w / v). After 15 min growth at 37 °C and 180 rpm, 60 mg / mL selonomethionine was added. Upon reaching an OD_{600} of 0.6, amino acids (lysine, phenylalanine, threonine (100 mg / L each) and isoleucine and valine (50 mg / L each)) were added and the culture was grown for an additional 30 min at 37 °C, 180 rpm. Afterwards the cultures were transferred to 16 °C, 180 rpm, and protein expression was induced by addition of 1 mM IPTG. The cells were harvested the next day and protein purified as described above.

Bottromycin extraction and purification

For bottromycin production, the *S. sp.* DG2-kmP41hyg strain[52] was cultivated in TSB seed medium at 28 °C. After 2 - 3 days the seed culture was used to inoculated SG production medium (Glucose: 20 g; Peptone: 10 g and $CaCO_3$: 2 g per liter) and left to grow for 7 days at 28 °C. To the pooled cultures an equal volume of ethyl acetate was added and mixed for 1 hour. The organic layer was dried and resuspended in methanol before being loaded onto a sephadex LH-20 1-meter long column. The fractions containing bottromycin were pooled together and purified further by RP-HPLC (XBridge® Peptide BEH^{TM} CSH C_{18} OBD Prep Column, 130 Å, 5 µm, 10 mm x 250 mm, 1 / pkg) using a linear gradient from 95% A (H_2O, 0.1% formic acid) to 95% B (acetonitrile, 0.1 % formic acid) over 40

min. The fractions containing bottromycin of the highest purity as judged by LC-MS were pooled and dried on a rotary evaporator. The resulting white amorphous solid was resuspend in methanol and dried under nitrogen to yield pure bottromycin A2.

In vitro production and purification of **3**

To produce **3,** bottromycin precursor peptide without the N-terminal methionine ($BotA^P$) was enzymatically processed by IpoC, PurCD and PurAH, to introduce the thiazoline, the macroamidine and cleave off the follower peptide, respectively (see Extended Data Fig. 2).

$BotA^P$, IpoC, PurCD and PurAH were expressed and purified as described previously [21,25]. Large scale IpoC reactions were carried out on a 9 mL scale in GF buffer (150 mM NaCl, 10 mM HEPES, 0.5 mM TCEP, pH 7.4) using the reaction setup 50 µM $BotA^P$, 5 µM IpoC, 5 mM ATP as well as 5 mM $MgCl_2$ and incubating the reaction mixture for 16 h at 37 °C. The reaction mixture was filtered through a 0.22 µm filter and loaded onto a Superdex 30 16/60 size exclusion chromatography column pre-equilibrated in GF buffer. Heterocyclized-peptide-containing fractions were pooled, analyzed by MS and concentrated using a 5 kDa cutoff filter. Next, large scale reactions with PurCD and PurAH were carried out on a 9 mL scale by incubating 50 µM $BotA^{PC}$, 5 µM PurCD, 1 µM PurAH, 10 mM ATP, 10 mM $MgCl_2$ and 100 µM $CoCl_2$ for 12 h at 37 °C in reaction buffer (200 mM NaCl, 50 mM Tris, 10% glycerol, pH 8.5). The reaction process was monitored by LC-MS. The analysis under acidic LC conditions, which leads to partial (about 50%) opening of the thiazoline ring, revealed the presence of two peaks (**3a**/**b**) with identical mass and fragmentation patterns, with a ratio of **3a** : **3b** of ~ 1 : 9.

After completion of the reaction, the mixture was filtered through a 0.22 µm filter and applied to a Superdex 30 16/60 size exclusion chromatography column pre-equilibrated in ultrapure water. Every fraction was analyzed by mass spectrometry and the pure **3a**/**b**-containing fractions were pooled.

For further purification, **3a/b** was purified in H_2O and ACN containing 0.1% ammonium acetate pH 8.5. Separation was carried out on a Kinetex XB-C18 2.6 µm, 4.6 x 100 mm column (Phenomenex) at 60 °C using a 8 min gradient from 0 to 80% ACN. Pure fractions were lyophilized and the remaining **3a**/**b** powder was weighed using a precision scale. The yield of purified product was approximately 70%. A large-scale purification was carried out by Peptide Protein Research Ltd.

Microscale Thermophoresis (MST)

Microscale thermophoresis experiments were carried out on a Monolith NT.115 (NanoTemper) in MST buffer (PBS with 0.05% Tween 20) using BotH labelled with the RED-tris-NTA fluorescent dye NT-647 following manufacture's protocol. The concentration of His-labelled BotH was 200 nM. Ligands were diluted in MST buffer to a starting concentration of 62.5 µM and then used in serial dilutions. Instrument settings were 40% excitation power and 40% MST power. Data fitting and evaluation was performed using *MO.Affinity* analysis software (Nanotemper).

Thermal Shift Assays

Ligands used for thermal shift assays were prepared as described for the in vitro production and purification of **3** (see above) with the following modification: After incubation with PurCD/AH, enzymes were removed by passing the reactions through a 3 kDa cut-off filter and used for thermal shift assays. Protein melting temperatures were determined by monitoring protein unfolding using SYPRO orange as a fluorescence probe. BotH was diluted to 5 µM in buffer containing 5x SYPRO orange (Sigma). For the determination of stabilizing effects of the substrates on BotH, buffer containing 150 mM NaCl and 10 mM HEPES (pH 7.4) was used. Final substrate concentrations of 3.0, 5.0 and 12 µM were used. Samples (20 µL/well) were analyzed in 96 well plates (Microamp™ Reaction Plates) that were sealed (MicroAmp™ Adhesive Film). Measurements were carried out with a realtime PCR machine (StepOnePlus™ Real-Time PCR System) using manufacture's guidelines and a temperature gradient from 25 to 95 °C with stepwise increments of 1 °C and 1 min hold. After each temperature step the fluorescence intensity was measured. The melting temperatures were obtained using the derivative method (Protein Thermal Shift™ Software v1.4). All conditions were tested in triplicates and mean values were calculated for the graphic presentation.

Epimerization under single-turnover conditions

To conduct an epimerization reaction under single-turnover conditions, 5 µM **3a**/**b** was added to 20 µM BotH in D_2O and the reaction quenched after 2 seconds by adding 2 reaction volumes of ACN and

flash-freezing the samples immediately. Selection of an appropriate time-point was critical because BotH catalyzes the forward and backward reactions, and the substrate exists as an epimeric mixture. At the start of the reaction, the substrate consisted of approximately 35% D-Asp and 65% L-Asp, while the end-point presented as a roughly equimolar mixture of epimers. This difference is not the actual end-point of the reaction with excess BotH, but was chosen because it resulted in a shift of epimer ratios that was clearly distinguishable from experimental error while minimizing the amount of D-Asp to L-Asp conversion. We then used these data to calculate the proportion of hydrogen-containing D-Asp and deuteron-containing D-Asp in the total Asp-pool at the start and finish of the experiment. The data are presented in Extended Data Fig. 5.

Marfey derivatization

For stereochemical assignment of the aspartate α-carbon in **3**, Marfey derivatization was performed. For the assay, 300 µg **3** (ratio of **3a : 3b** of about 1 : 9) was used. Solvent was evaporated at 110 °C and the compound was hydrolyzed by addition of 100 µl 6 N HCl and incubation at 110 °C for 35 min in a closed vial with nitrogen. The vial was opened and incubated for further 15 min at 110 °C to dry the contents. Residues were dissolved in 110 µl H_2O.

For derivatization of the amino acids in the hydrolyzed compound **3** or the amino acid standards (2 mg / mL D- or L- aspartic acid), 50 µL solution were mixed with 20 µl 1 N $NaHCO_3$ and 20 µl of 1% D-FDLA (*Nα*-(2,4-dinitro-5-fluorophenyl)-D-leucinylamide) in acetone. The mixture was incubated for 1.5 h at 40 °C, 700 rpm. To stop the reaction, 10 µl of 2 N HCl and 300 µl ACN were added. The derivatized amino acids were separated by RP-HPLC-MS and the stereochemistry of the aspartate in the compounds **3a** and **3b** were assigned by comparing retentions times of the amino acid standards.

Spontaneous epimerization of **3**

To determine the propensity for spontaneous (non-enzymatic) epimerization of the Asp Cα in **3a**/**b**, a lyophilized, racemic mixture of **3a** : **3b** was dissolved in D_2O. Samples were taken after 2 h, 1 day, 4 days and 6 days and analyzed by LC-MS. Three independent experiment were performed.

Pull-down of bottromycin A2 and **3a**/**b** using BotH

S. sp. DG2-KMp41hyg strain was cultivated as described above. 50 mL cultures were centrifuged for 10 min at room temperature to separate cells from the medium. The cell pellet was subsequently washed twice with PBS buffer before being resuspend in fresh 50 mL PBS buffer. The cell suspension was then lysed by sonication, centrifuged and the supernatant was decanted. His_6-BotH (75 µg) was subsequently incubated with medium (1 mL), supernatant (1 mL) and **3a**/**b** (50 µg dissolved PBS) for 30 min before being purified using a KingFisher^{TM}mL Purification System. The protein was precipitated using ice cold ACN to liberate bound compounds and the supernatant was analyzed by HRLC-MS.

Incorporation of a deuteron at Asp Cα of **3**

Deuteron incorporation at the Asp Cα was achieved by performing a BotH reaction in D_2O GF buffer. Lyophilized **3a**/**b** was dissolved in D_2O GF buffer and incubated for 30 min at 30 °C with (or without as control) 5 µM BotH. The reactions were stopped by addition of 2 volumes ACN and stored at -80 °C until analysis. Reactions were set up in triplicates and analyzed by LC-MS and MS-fragmentation.

Back exchange of deuterated **3a**/**b** with solvent protons was shown by performing a BotH reaction using deuterated **3a**/**b** in H_2O GF buffer: A reaction of **3a**/**b** with 5 µM BotH in D_2O GF was stopped after 30 min at 30 °C by denaturation of BotH at 98 °C for 10 min. Denatured BotH was pelleted by centrifugation (15 min, 15,000 rpm). The supernatant was lyophilized to remove the D_2O and re-dissolved in the same volume H_2O. Samples were split up and incubated 30 min at 30 °C with or without the addition of 5 µM BotH. Reactions were stopped by the addition of 2 volumes ACN and analyzed by LC-MS.

Reaction of BotH with derivatives of **3**

To generate derivatives of **3,** bottromycin precursor peptides ($BotA^P$) with singe amino acid changes in the core peptide were cloned, expressed and purified as described previously[21,25]. Shortly, *botA* was mutated by using mutation carrying primers and cloned into a pHisSUMOTEV vector, which was a

gift from Dr. Huanting Liu, St. Andrews University[53]. To produce the native N-terminus of the bottromycin precursor peptide $BotA^P$ (after cleavage of the N-terminal methionine by BotP) (GPVVV….) without cloning artefacts, primers which introduce a lysine residue were used (...GAMAGKGPVVV...). Peptides were expressed in *E. coli* Lemo21(DE3) cells carrying the respective expression vector and purified by Ni^{2+}-NTA-chromatography and, after cleavage with Trypsin, by gel filtration[21,25].

To produce the **3** derivatives, 50 µM $BotA^P$ derivative was incubated with 5 µM IpoC, 10 mM ATP as well as 5 mM $MgCl_2$ for 12 h at 37 °C in reaction buffer (200 mM NaCl, 50 mM Tris, 10 % glycerol, pH 8.5). Then, 5 µM PurCD, 1 µM PurAH and 100 µM $CoCl_2$ were added and incubated for a further 4 h at 37 °C. Each reaction was then divided and incubated with or without the addition of 5 µM BotH for 2 h at 30 °C. For the $BotA^P$ Asp7-mutants, deuteron incorporation by BotH was tested additionally: After incubation with IpoC, PurCD and PurAH, reactions were lyophilized and redissolved in D_2O. Afterwards the samples were divided and incubated with and without the addition of BotH (as described above).

Reactions were stopped by the addition of 2 volumes ACN and frozen at -80 °C until analysis. Reactions were set up and analyzed in triplicates.

Orthosteric inhibition of BotH epimerization by bottromycin A2

0.2 µM BotH in D_2O GF buffer was pre-incubated with and without 50 µM bottromycin A2 for 30 min at 30 °C. **3a/b** in D_2O GF buffer was added (final concentration 10 µM) and the solution was incubated for 7 min at 30 °C. The reactions were stopped by the addition of 2 volumes ACN and the samples were frozen at -80 °C until analysis by LC-MS. As a control for non-enzymatic deuteron incorporation, samples without enzyme were set up. Reactions were performed in triplicates and analyzed by LC-MS. For analysis of the LC-MS data, the EICs (± 5 ppm) for the 1st and the 2nd isotope peak of the doubly charged ion of **3** (1st isotope peak $[M+2H]^{2+}$ = 400.1940; 2nd isotope peak $[M+2H]^{2+}$ = 400.6955) were generated. The respective areas for **3a** (2.96 - 3.10 min) were calculated using the Bruker Compass DataAnalysis 4.2. software. The ratio of the area of the 1st isotope peak to the 2nd

isotope peak was used to quantify the deuteron incorporation. The significance (*p*-value) of the ratio differences was calculated using an unpaired two-tailed t-test.

In vitro production of oxidatively decarboxylated **3a**, with deuteron incorporation at the Asp Cα

To investigate if BotH can epimerize decarboxylated **3a,** we produced decarboxylated **3a** with deuteron incorporation at the Asp Cα. 20 µM BotCYP substrate analogue **3a**/**b** with deuteron incorporated (see above) was incubated with 5 µM BotCYP, 5 µM BmCPR, 50 µM Fdx2, 1 µM BotH and 2.5 mM NADPH at 30 °C for 2 h in H_2O GF buffer. Enzymes were denatured at 98 °C for 10 min and pelleted by centrifugation (15.000 x g, 20 min, 4 °C). The supernatant was incubated with and without 5 µM BotH for 30 min at 30 °C. Reactions were stopped and enzymes were precipitated by addition of two volumes of ACN. The samples were frozen at -80 °C and precipitated enzymes were pelleted by centrifugation at 15.000 x g for 20 min. The turnover was analyzed by LC-MS measurements. Reactions were set up and analyzed in triplicates.

LC-MS and MS^2 analysis

Measurements were performed on a Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific) using a flow rate of 600 µL min^{-1} and column oven temperature of 45 °C. Standard measurements were performed using a BEH C18, 50 x 2.1 mm, 1.7 µm dp column equipped with a C18 precolumn (Waters). Samples were separated by a gradient from (A) H_2O + 0.1% formic acid to (B) ACN + 0.1% formic acid. The linear gradient was initiated by a 1 min isocratic step at 5% B, followed by an increase to 95% B in 9 min to end up with a 1.5 min plateau step at 95% B before re-equilibration under the initial conditions. UV spectra were recorded by a DAD in the range from 200 to 600 nm.

For Marfey's analysis, samples were separated on a BEH C18, 100 x 2.1 mm, 1.7 µm dp column equipped with a C18 precolumn (Waters). A multistep gradient using (A) H_2O + 0.1% formic acid and (B) ACN + 0.1% formic acid was applied for sample preparation. The gradient was initiated by a 1 min isocratic step at 5% B, an increase to 10% B in 1 min, an increase to 35% B in 14 min, followed by an increase to 55% B in 7 min and an increase to 80% B in 3 min to end up with a 1 min plateau step at 80% B before re-equilibration to the initial conditions.

For MS measurements on maXis-4 hr-qToF mass spectrometer (Bruker Daltonics), the LC flow was split 1:8 before entering the mass spectrometer using the Apollo II ESI source. In the source region, the temperature was set to 200 °C, the capillary voltage was 4000 V, the dry-gas flow was 5.0 L/min and the nebulizer was set to 1 bar. Ion transfer settings were set to Funnel 1 RF 350 Vpp and Multipole RF 400 Vpp, quadrupole settings were set to an ion energy of 5.0 eV and a low mass cut of 120 *m/z*. The collision cell was set to an energy of 5.0 eV and the pulse storage time was 5 µs. Data were recorded in centroid mode ranging from 150 to 2500 *m/z* at a 2 Hz scan rate. Calibration of the maXis4G qTOF spectrometer was achieved with sodium formate clusters before every injection to avoid mass drifts. All MS analyses were acquired in the presence of the lock masses $C_{12}H_{19}F_{12}N_3O_6P_3$, $C_{18}H_{19}O_6N_3P_3F_2$ and $C_{24}H_{19}F_{36}N_3O_6P_3$ which generate the $[M+H]^+$ Ions of 622.028960, 922.009798 and 1221.990638.

LC-MS^2 fragmentation spectra were recorded using a scheduled precursor list (SPL). Separation was achieved using a BEH C18, 100 x 2.1 mm, 1.7 µm dp column equipped with a C18 precolumn (Waters) and a linear gradient from (A) H_2O + 0.1% formic acid to (B) ACN + 0.1% formic acid. The gradient was initiated by a 1 min isocratic step at 5% B, followed by an increase to 95% B in 18 min to end up with a 2 min plateau step at 95% ACN before re-equilibration to the initial conditions. SPL entries and parameters were set to fragment only the ions of interest. SPL tolerance parameters for precursor ion selection were 0.17 min and 0.05 *m/z*. The CID energy was ramped from 35 eV for 500 *m/z* to 45 eV for 1000 *m/z*.

Data were displayed and analyzed using the Bruker Compass DataAnalysis software (Version 4.2). Shown MS spectra are in general single spectra (taken at the EIC maximum), except for Extended Data Figs. 3 and 6b where spectra are averaged. Signals in the MS-spectra are labelled with the observed monoisotopic mass. Extracted-ion chromatograms were generated using the calculated monoisotopic mass with a range of 5 ppm. All EICs (except Extended Data Figs. 3 and 10 and Synthetic Procedure 4 Figure 1A) and MS-spectra are scaled to a relative intensity of 1.

Bioinformatics analysis

We downloaded bacterial sequences of the InterPro[54] family IPR000073 and aligned them with HMMer (http://hmmer.org/)[55] using the HMM for the Pfam[40] family PF00561 (Abhydrolase_1), retaining only those from this Pfam family. Then sequences containing the following mutations at the catalytic site positions were selected with a custom Perl script: Ser mutated to either Val, Ile, Leu, Met, Ala, Phe, Tyr, or Trp; Asp mutated to anything except Asn or Glu or missing; His mutated to any amino acid or missing.

The corresponding genomes were identified by parsing UniProt[56] data files and retrieved from NCBI using the batch download option. Biosynthetic gene clusters (BGCs) were predicted using antiSMASH[57]. All BotH homologs whose genomic coordinates fall within the predicted boundaries of BGCs or lie outside them separated by no more than 1000 nt were considered to be associated with these BGCs.

The distance matrix between all proteins that are associated with BGCs was calculated using the R package seqinr[58]. The sequence similarity networks were visualized in Cytoscape[59], all edges connecting pairs of proteins that shared less than 15% sequence identity were omitted.

Data availability

Atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with accession codes 6T6H (BotH apo), 6T6X (BotH-**3a** complex), 6T6Y (BotH-**2** complex), 6T6Z (BotH-**5** complex), and 6T70 (BotH-**6** complex). Other relevant data supporting the findings of this study are available in this published article, its Supplementary Information files or from the corresponding author upon request.

Code availability

Custom scripts used by the authors are available from O. V. Kalinina upon request.

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