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Biosynthesis of a Thiamin Antivitamin in *Clostridium botulinum*

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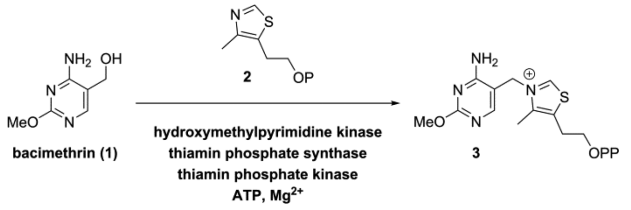
Supporting Information

ABSTRACT: Bacimethrin-derived 2′-methoxythiamin pyrophosphate inhibits microbial growth by disrupting metabolic pathways dependent on thiamin-utilizing enzymes. This study describes the discovery of the bacimethrin biosynthetic gene cluster of *Clostridium botulinum* A ATCC 19397 and *in vitro* reconstitution of bacimethrin biosynthesis from cytidine 5′-monophosphate.

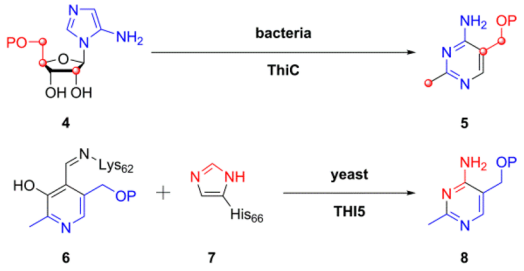
Bacimethrin [**1** (Scheme 1)] is an analogue of the thiamin pyrimidine that exhibits antibiotic activity against a variety of yeast and bacteria.[1] The toxicity of **1** involves its conversion, by the endogenous thiamin biosynthetic enzymes, to 2′-methoxythiamin pyrophosphate (**3**), which inhibits a subset of the thiamin-dependent enzymes in *Escherichia coli* (α-ketoglutarate dehydrogenase, transketolase, and deoxy-D-xylulose-5-phosphate synthase).[2,3]

Scheme 1. Enzymatic Conversion of 1 by the Late Thiamin Pyrophosphate Biosynthetic Enzymes to the Antivitamin 3



The biosynthesis of the thiamin pyrimidine in bacteria and in yeast is remarkably complex, as illustrated by the labeling patterns shown in Scheme 2. The bacterial pathway involves a unique rearrangement of 5-aminoimidazole ribonucleotide (AIR, **4**), while the yeast pathway involves a Diels–Alder addition of enzyme-bound pyridoxal phosphate (PLP, **6**) to an active site histidine followed by a complex rearrangement.[4,5]

Scheme 2. Complex and Unprecedented Chemistry of Thiamin Pyrimidine Biosynthesis in Bacteria and Yeast



Given the mechanistic complexity of this chemistry, the potentially "simpler" biosynthesis of bacimethrin was of interest and is the focus of this paper.

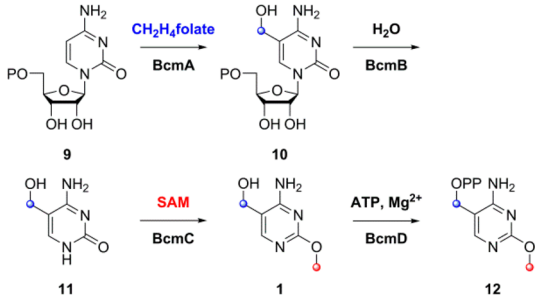
Our starting hypothesis for the biosynthesis of bacimethrin involved hydroxymethylation of cytosine and pyrimidine methylation (Figure S1 of the Supporting Information). This hypothesis led us to an operon in *Clostridium botulinum* A ATCC 19397 containing genes annotated as a thymidylate synthase, a putative glycosyltransferase, an *S*-adenosylmethionine-dependent methyltransferase, and a phosphomethylpyrimidine kinase (Figure 1). Additionally, the cluster contained

Figure 1. Genetic organization and homology-based annotation of the bacimethrin biosynthetic cluster (red) of *C. botulinum* A ATCC 19397: (1) ABC transporter, (2) ABC transporter, (3) glycosyltransferase (*bcmB*), (4) thymidylate synthase (*bcmA*), (5) methyltransferase (*bcmC*), (6) thiaminase-I (*bcmE*), (7) pyrimidine kinase (*bcmD*), and (8) hypothetical.

genes proposed to encode an ABC transporter and a putative thiaminase-I. The biosynthetic genes and the thiaminase were cloned and heterologously expressed in *E. coli*. The polyhistidine-tagged recombinant proteins were purified by nickel affinity chromatography, and then the catalytic activity of each enzyme was determined.

BcmA catalyzes the methylenetetrahydrofolate-dependent hydroxymethylation of cytidine 5′-monophosphate (CMP, **9**) to give 5-hydroxymethylcytidine 5′-monophosphate [**10** (Scheme 3)]. BcmB catalyzes the *N*-glycosyl bond cleavage of **10** to give 5-hydroxymethylcytosine (**11**).[6] BcmC catalyzes the methylation of **11** to give **1**. Bacimethrin is then pyrophos-

Scheme 3. Pathway for the Biosynthesis of Bacimethrin Pyrophosphate 12 Catalyzed by BcmABCD



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phorylated by BcmD to produce **12**. Each enzymatic reaction was analyzed by reverse-phase high-performance liquid chromatography (HPLC), and the identities of the products were verified by co-elution with authentic standards (Figure 2).

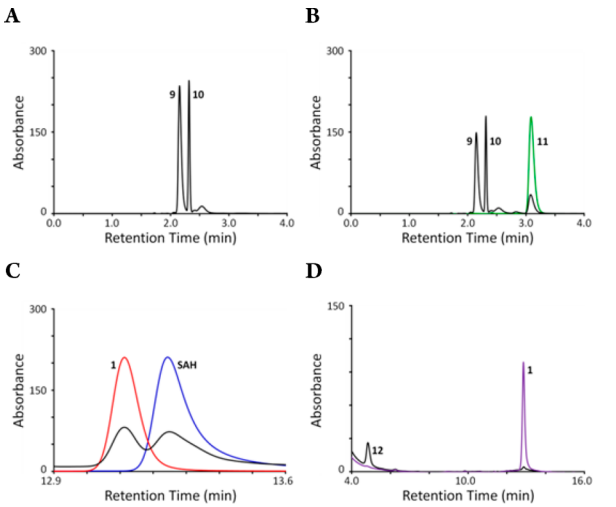

Figure 2. HPLC chromatograms for the reactions catalyzed by BcmA, BcmB, BcmC, and BcmD. (A) BcmA-catalyzed reaction showing the partial conversion of **9** to **10**. (B) BcmB-catalyzed reaction (black trace) of the BcmA reaction mixture showing conversion of **10** to **11** (the authentic standard of **11** is colored green). (C) BcmABC-catalyzed reaction (black trace) showing the conversion of **9** to **1**. The red and blue traces are for authentic standards of **1** and *S*-adenosylhomocysteine (SAH), respectively. (D) BcmD-catalyzed reaction (black trace) showing the conversion of **1** to **12**. The purple trace is for an authentic standard of **1**.

Formation of **1** from **11**, catalyzed by BcmC, was also confirmed by positive ion mode electrospray ionization time-of-flight mass spectrometry analysis. The observed mass for **1** was 156.078 Da [theoretical exact mass $[M + H]^+$ = 156.0773 Da (Figure S4 of the Supporting Information)]. Fragmentation of this ion by collision-induced dissociation resulted in the accumulation of daughter ions at 138 Da (loss of water) and 81 Da (pyrimidine fragmentation), consistent with the bacimethrin structure.

A putative thiaminase-I gene, *bcmE*, is located in the bacimethrin biosynthetic gene cluster.[7] Thiaminases catalyze degradation of thiamin by replacement of the thiazole moiety with a wide range of nucleophiles (type I) or water (type II).[7,8] Its presence suggested that BcmE might function as a resistance protein catalyzing the degradation of methoxythiamin. To test this hypothesis, BcmE was incubated with thiamin (**13**) or 2′-methoxythiamin (**16**) as a possible substrate. The reaction was monitored by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and under conditions where BcmE efficiently cleaved **13**, no reaction of **16** was observed (Figure 3). Thus, *bcmE* encodes a thiamin-specific degradation enzyme and is not involved in protecting *C. botulinum* from the toxicity of methoxythiamin.

The identification of the bacimethrin biosynthetic cluster led us to explore the prevalence of this pathway in other bacteria. Sequence analysis using the Microbes Online Database reveals the presence of the pathway in several strains of *C. botulinum* and *Burkholderia pseudomallei* as well as *Clostridium sporogenes*, *Burkholderia thailandensis*, and *Burkholderia glumae*. In addition, bacimethrin was previously isolated from *Bacillus megaterium*

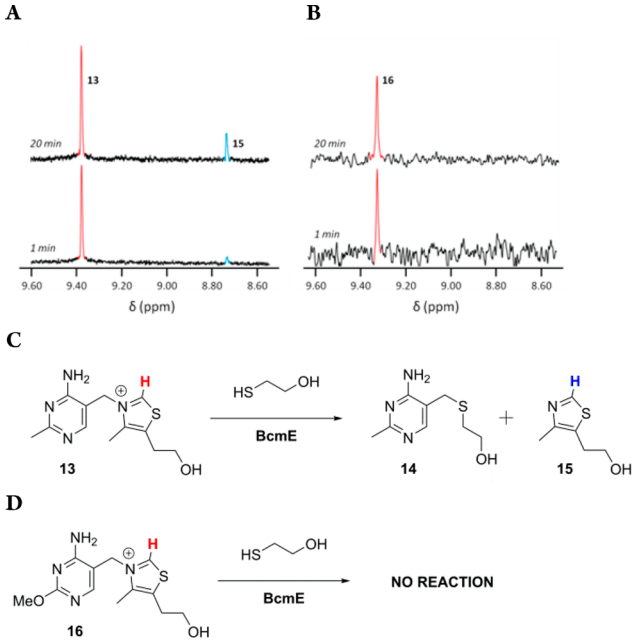

Figure 3. Analysis of the BcmE-catalyzed reaction. (A) ^{1}H NMR for the reaction shown in panel C. (B) ^{1}H NMR for the reaction shown in panel D. δ_H for the thiazolium C2 proton in **13** shifts from 9.38 (red) to 8.73 ppm in thiazole **15** (blue). Exchange with water was not observed under the reaction conditions.

and *Streptomyces albus*.[9,10] Interestingly, in all of the sequenced genomes, the thiaminase-I gene is found in the bacimethrin biosynthetic cluster. This observation suggests that bacimethrin and thiaminase-I are elements of a coordinated antibiotic strategy in which degradation of environmental thiamin by secreted thiaminase-I makes target bacteria more sensitive to the toxicity of bacimethrin. Such a strategy highlights the complex chemical logic underlying the dynamics of microbial communities.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

L.E.C. and S.E.O. contributed equally to this work. L.E.C., S.E.O., and T.P.B. were responsible for experimental design and preparation of the manuscript. L.E.C. and S.E.O. were responsible for execution of the biochemical studies.

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Notes

The authors declare no competing financial interest.

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