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Activation and Characterization of Bohemamine Biosynthetic Gene Cluster from *Streptomyces* sp. CB02009

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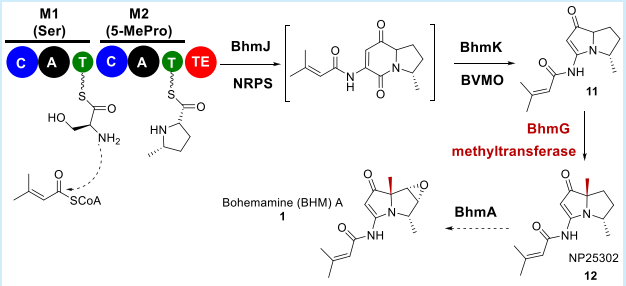
ABSTRACT: Bohemamines (BHMs) are bacterial alkaloids containing a pyrrolizidine core with two unusual methyl groups. Herein we report the activation of BHMs biosynthesis using a ribosome engineering approach. Characterization of the *bhm* gene cluster reveals that nonribosomal peptide synthetase BhmJ and Baeyer−Villiger monooxygenase BhmK are responsible for the formation of the pyrrolizidine core, which is further methylated on C-7 by methyltransferase BhmG. The 9-methyl group of BHMs is instead originated from a nonproteinogenic amino acid (2*S*,5*S*)-5-methyl-proline.



Bacterial pyrrolizidine alkaloids, as well as structurally related lipocyclocarbamates and azetidomonamides, are an emerging family of natural products with promising biological activities (Figures 1A, S1−2).[1−3] They are produced as quorum-sensing metabolites or used as self-defensive mechanisms against predation by amoebae.[4−6] Several *Streptomyces* species produced bohemamines (BHMs, **1**−**4**), featuring a pyrrolizidine core with two unusual methyl groups.[6−14] In contrast to plant pyrrolizidines originated from homospermidine,[2,15−17] the biosynthesis of bacterial alkaloids may involve nonribosomal peptide synthetases (NRPSs) for the formation of indolizidine intermediates, followed by consecutive reactions to generate pyrrolizidine scaffolds by Baeyer−Villiger monooxygenases (BVMOs).[1,6,18] The biosynthesis of BHM derivative spithioneines was instead proposed to utilize a peptide/polyketide logic.[11] However, the *bhm* biosynthetic gene cluster (BGC) has not been identified despite the isolation of **1**−**3** in 1980 and the recent genome sequencing of alternative BHMs producers.[8,13,19]

A typical actinomycete genome contains 20−40 BGCs, and most of them are silent under laboratory culture conditions.[20,21] Ribosome engineering is a rapid and cost-effective strategy to improve the yields of many secondary metabolites or activate silent BGCs.[22−28] Herein we report the activation of the *bhm* BGC in *Streptomyces* sp. CB02009 from our strain collection using this strategy. Using gene inactivation and heterologous expression, we discovered that BHMs are biosynthesized by BhmJ NRPS capable of incorporating a nonproteinogenic amino acid (2*S*,5*S*)-5-methyl-proline (5-Mepro). The tandem action of a BMVOs BhmK and a C-methyltransferase BhmG is responsible for the pyrrolizidine formation and C-7 methylation.

A total of 11 rifampicin-resistant colonies, named CB02009-R-1 to CB02009-R-11, were obtained from *Streptomyces* sp. CB02009 using ribosome engineering (Table S3, Figures S3−4). Compared to wild-type, two additional peaks were observed in the fermentation extracts of the mutant CB02009-R-5 with R440H mutation in RNA polymerase *β*-subunit (RpoB) (Figure 1B, I−II). HRESIMS and NMR analysis confirmed the production of BHMs in CB02009-R-5 and the presence of trace amounts of BHMs in several other mutants (Figures S5−7).[8,10]

The discovery of BHMs in CB02009-R-5 provided an excellent opportunity to study their biosynthesis. In silico analysis of the draft genome of *Streptomyces* sp. CB02009 using antiSMASH 5.0 revealed a candidate *bhm* BGC based on its similarity with those of legonmycins and pyrrolizixenamide (Figure 1D, Table S4).[6,18,29] The *bhm* BGC consists of 17 genes encoding NRPS, tailoring enzymes, precursor biosynthetic protein complexes, regulatory, and resistance proteins.[30,31] Further genome mining revealed three additional streptomycetes with similar BGCs, among which *Streptomyces* sp. TPA0873 is a known BHMs producer (Tables S5−7).[19] The BhmJ NRPS contains two distinct modules predicted for serine and methyl-proline incorporation (Table S8).[6,18,32] To study if *bhmJ* is involved in the biosynthesis of BHMs we

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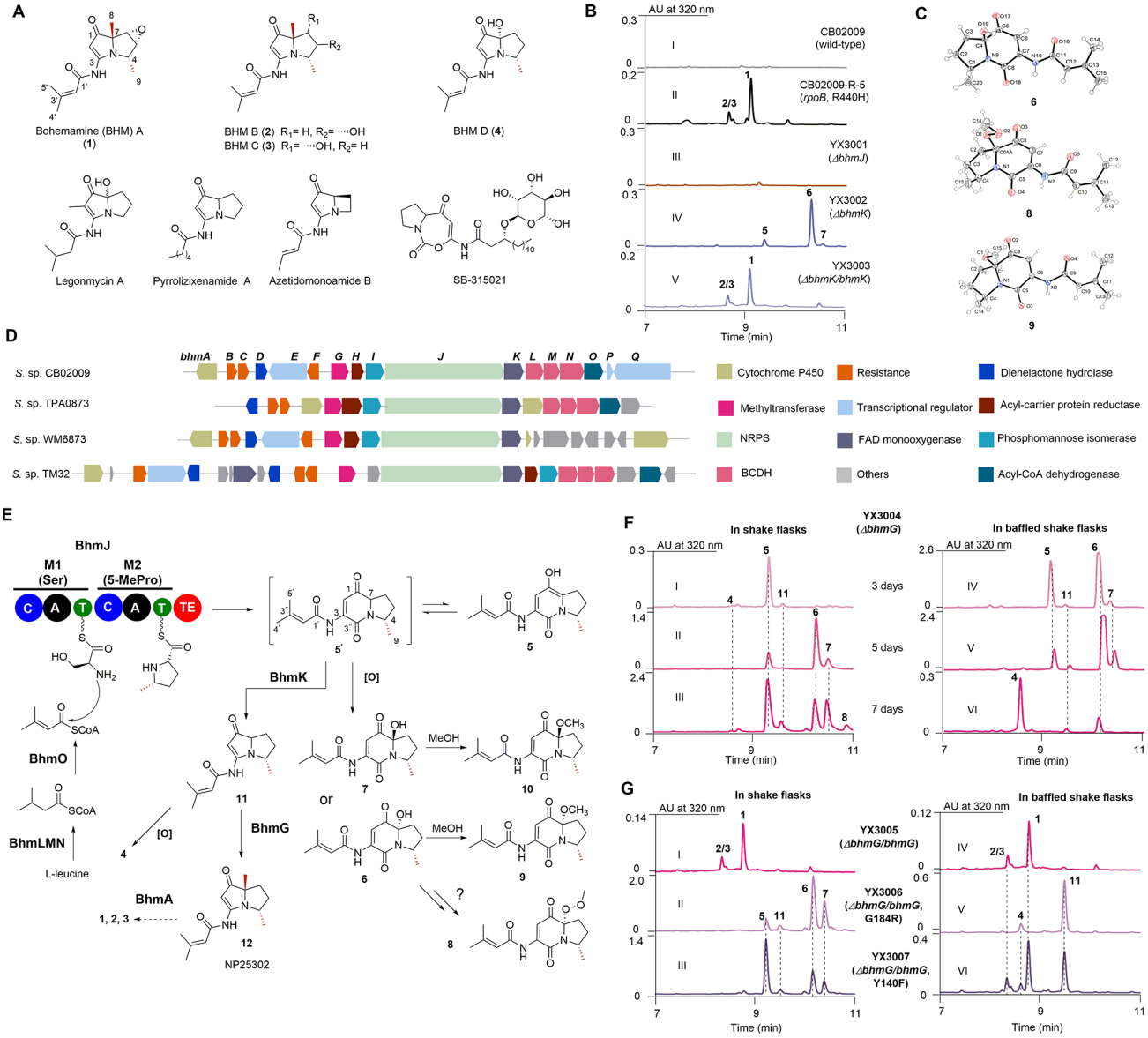

Figure 1. Biosynthesis of BHMs. (A) Representative bacterial pyrrolizidine alkaloid derivatives. (B) HPLC analysis of BHMs production in *Streptomyces* sp. CB02009 (I) and its mutants (II–V). (C) Single-crystal X-ray structures of **6**, **8**, and **9**. (D) Organization of *bhm* BGC in *Streptomyces* strains. (E) Proposed BHMs biosynthetic pathway. (F) HPLC profiles of YX3004 in shake flasks (I–III) and baffled shake flasks (IV–VI). (G) HPLC profiles of YX3005 (I, IV), YX3006 (II, V), and YX3007 (II, VI) in shake flasks and baffled shake flasks for 3 days.

inactivated *bhmJ*, resulting in a mutant YX3001(Δ*bhmJ*) without the production of **1**–**3** (Figure 1B, III; Figure S8).

The FAD-dependent monooxygenase gene *bhmK* is transcriptionally coupled to *bhmJ*, due to the overlapping of the start codon of *bhmK* with the stop codon of *bhmJ*. BhmK shares high sequence identity with PxaB, LgnC, AzeC, and LpiC, previously reported BMVOs involved in pyrrolizidine formation (Figure S9).[3,4,6,18] We next inactivated *bhmK* by replacing it with a mutant copy in which *bhmK* was disrupted by a thiostrepton-resistance gene with a *kas*Op* promoter (Figure S10).[33,34] The recombinants completely abolished the production of **1**–**3**, while they accumulated three new metabolites **5**–**7** (Figure 1B, IV). A *bhmK* complementation strain YX3003 restored BHMs production (Figure 1B, V). Large scale fermentation of YX3002(Δ*bhmK*) led to the isolation of **5**–**7**, and their structures were deducedby HRESIMS, CD spectra, and 1D and 2D NMR spectroscopy (Tables S9–10). The production of **5**–**7** in YX3002(Δ*bhmK*) suggests that BhmK is responsible for the formation of pyrrolizidine core in BHMs through a Bayer-Villiger type oxidation (Figure 1E). The storage of **6** or **7** in methanol afforded methylated products **9** or **10**, respectively (Tables S10, 15). The structures of **6** and **9** were further confirmed using single-crystal X-ray diffraction analysis (Figure 1C).

The methyltransferase BhmG resembles MdpB1 and PokMT-1, responsible for C-methylation on aryl-CoA substrates (Figure S11).[35] To ascertain if BhmG is responsible for C-7 methylation in BHMs, a mutant strain YX3004-(Δ*bhmG*) was similarly obtained (Figure S12). Compared to CB02009-R-5, YX3004(Δ*bhmG*) abolished the production of **1**–**3**, while also accumulating **5**–**7**, as well as **4**, **8**, and **11** (Figure 1F). Note that **8** is a novel indolizidine alkaloid containing a rare methylperoxyl moiety (Tables S11, 14).[36,37] Compound **11** has the characteristic BHM-type UV–vis

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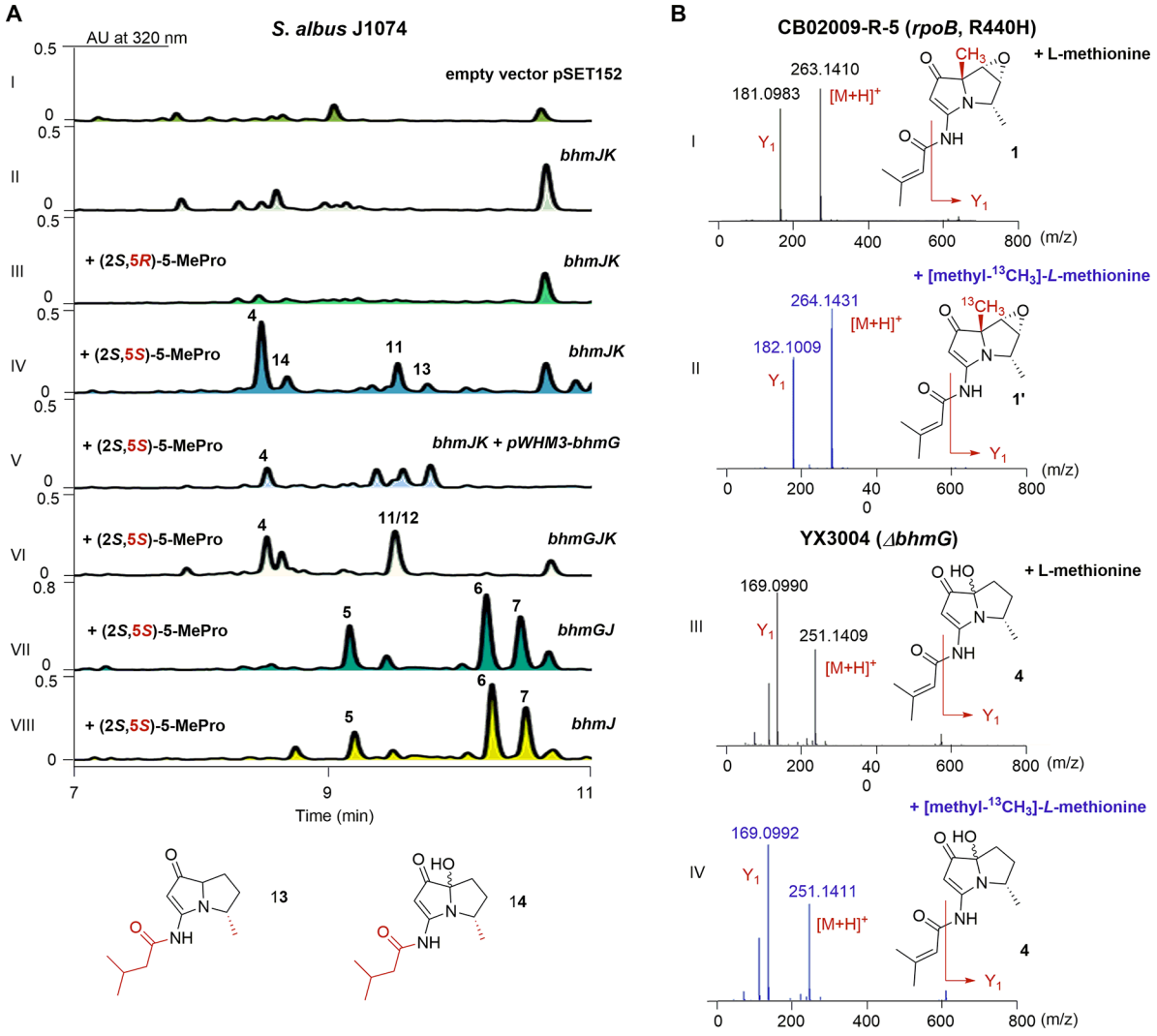

Figure 2. Heterologous expression of *bhmGJK* and the biosynthetic origin of the C-9 methyl group in BHMs. (A) Heterologous expression of *bhm* genes in *S. albus* J1074. I–II: Expression of pSET152 or *bhmJK* without (2*S*,5*R*)-5-MePro or (2*S*,5*S*)-5-MePro; III: Expression of *bhmJK* with (2*S*,5*R*)-5-MePro (2.5 mM); IV–VIII: Expression of various combinations of *bhmJ, bhmK,* and *bhmG* with (2*S*,5*S*)-5-MePro (2.5 mM). The proposed structures of **13** and **14** were based on LCMS. (B) HRMS analysis of ^{13}C-incorporation into BHMs in *Streptomyces* sp. CB02009-R-5 (I–II) and YX3004 (III–VI) by feeding L-methionine or [methyl-$^{13}CH_3$]-L-methionine.

adsorption and a distinctive $[M + H]^+$ ion (m/z 235.1446), which differed from **4** by one oxygen. Further NMR characterization of **11** was hindered due to its intrinsic instability. The production of **4**–**8**, **11** in YX3004(Δ*bhmG*) suggested that BhmG is only involved in C-7 methylation (Figure 1E).

The different fermentation profiles of YX3004(Δ*bhmG*) in either shake flasks (Erlenmeyer) or baffled flasks provide an opportunity to understand the detailed catalytic activity and mechanism of BhmK BMVO in vivo. First, compound **4** was accumulated as the main product in baffled shake flasks for 7 days, while only trace amounts of **4** and **11** were present in shake flasks (Figure 1F, VI vs III). These data suggested that the catalytic activity of BhmK is dependent on the level of dissolved oxygen during fermentation, consistent with previous reports of other BMVOs.[38,39]

Second, BhmK might only catalyze the ring expansion and contraction from the putative intermediate **5′**, because (1) compound **4** was produced with longer cultivation in YX3004(Δ*bhmG*) and (2) **11** is more likely to be the substrate for BhmG-catalyzed C-7 methylation than **4**.[6,18] In fact, no hydroxyl congeners were reported for pyrrolixenamides, lipocyclocarbamates, azetidomonamides, or azabicyclene.[3,4,40] Taken together, the BMVOs involved in bacterial pyrrolizidine biosynthesis might only execute a remarkable three-step cascade reaction, and the final hydroxylation reaction catalyzed by LgnC may be an exception.[18] Compounds **6** and **7** would likely be produced by air-oxidation of **5′**. The enolization of **5′** may generate **5**, since similar indolizidines legonindolizidine A and B were also observed in the Δ*lgnC* mutant.[18]

Third, the production of **5**–**7** in YX3004(Δ*bhmG*) suggests either the disruption of certain regulatory mechanisms for the productions of BHMs and the intermediates or the compromise of the catalytic activity of BhmK, due to the gene inactivation of *bhmG*. Interestingly, the presence of a functional BhmJ NRPS in YX3004(Δ*bhmG*) rules out any polar effect resulting from *bhmG* gene replacement. Instead, YX3004(Δ*bhmG*) produced at least >10-fold of **5**–**7** than YX3002(Δ*bhmK*) (Figure 1B, IV vs Figure 1F, IV).

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Therefore, in order to further study the function of BhmJ, BhmK, and BmhG, we first prepared three constructs in integrative vector pSET152 containing genes encoding BhmG, and functionally impaired BhmG(G184R) and BhmG(Y140F), based on the comparison with the amino acids in the active site of PokMT-1.[41] The constructs were introduced into YX3004-(Δ*bhmG*), and the resultant strains YX3005−YX3007 were fermented for 3 days. In baffled shake flasks, there was no accumulation of **5**−**7** in all three strains, suggesting that BhmK would be fully functional in the presence of BhmG and its variants, as well as high levels of dissolved oxygen (Figure 1G, IV−VI). Although the methylation activity of BhmG(G184R) was completely shut off, abundant amounts of **4** and **11** were still produced, further suggesting that the physical presence of BhmG(G184R) is also critical for the activity of BhmK. Due to the highly unstable nature of **11**, the coordinated action of BhmJ, BhmK, and BmhG might be one of Nature's strategies to avoid the accumulation of shunt metabolites **5**−**8** in CB02009-R-5. Similar enzyme pairs have previously been identified in the biosynthesis of bacillaenes,[42] microcin B17,[43] rebecamycin,[44] and coenzyme Q.[45] The identification of 12 known and 35 orphan NRPS/BVMOs pairs also suggests their potential evolutionary advantage for bacterial pyrrolizidine formation (Table S8, Figure S9).[46,47]

Next, we established an expression platform in *Streptomyces albus* J1074 for the expression of BhmJ, BhmK, and BmhG.[48,49] Various methylated prolines are incorporated into peptides through NRPS logic, including polyoxypeptin, nostopeptolide, and griselimycins (Figures S13−14).[50−52] However, 5-Mepro present in dozens of actinomycin congeners was suggested to derive from a post NRPS-logic.[53−55] Unique to the previously isolated BHMs analogs is the presence of a 9-methyl group. We hypothesized that an unusual (2*S*,5*S*)-5-Mepro might be incorporated in BHMs through M2-A adenylation domain in BhmJ *S. albus* strain containing *bhmJK* in pSET152 with the constitutive promoter *erm*Ep* produced no BHMs (Figure 2A, I−II). Since *S. albus* J1074 may be incapable to produce this nonproteinogenic proline, (2*S*,5*S*)-5-Mepro was then fed into *S. albus* J1074(*bhmJK*), which produced **4**, **11**, **13**, and **14** (Figure 2A, IV). Compounds **13** and **14** were identified to be the analogs of **4** and **11** with a 3-methyl-2-butylamide moiety (Figure S15). BhmO could catalyze the dehydrogenation of the 3-methyl-2-butylamide moiety synthesized by BhmLMN (Figure 1E). These compounds could be similarly produced when *Streptomyces coelicolor* M145 and *Streptomyces lividians* TK24 were used as hosts for *bhmJK* expression by supplementing (2*S*,5*S*)-5-Mepro (Figure S16). In contrast, no BHM analogs were produced when (2*S*,5*R*)-5-Mepro or other proline analogs were used (Figure 2A, III; Figure S17).

Four *S. albus* strains containing *bhmJ*, *bhmGJ*, or *bhmGJK* were also obtained and fermented. Intriguingly, the efficient production of BHM analogs in these heterologous hosts suggests that BhmJ, BhmK, and BhmG may function independently (Figure 2A, V−VIII; Figure S18). Compounds **5**−**7** were produced in the two *S. albus* strains when *bhmJ* or *bhmGJ* were overexpressed. Only *S. albus*(*bhmGJK*) produced a small amount of NP25302 (**12**), the methylation product of **11**, suggesting the attenuated activity of BhmG. Higher gene dosage of *bhmG* seemed detrimental to the activity of BhmG, while several new metabolites unrelated to BHMs were produced, suggesting the activation of certain cryptic BGCs from *S. albus* (Figure 2A, V). The disruption of the interdependency of BhmJ/BhmK/BhmG in these heterologous strains could be attributed to variable factors, such as lack of pathway-specific transcriptional regulators, different protein expression levels, substrate availability, and differences among the metabolome and proteome of the hosts.[56]

Finally, to study the effect of (2*S*,5*S*)-5-Mepro on BHMs production in their native producer, it was fed into CB02009-R-5 and resulted in 2-fold increase of BHMs production (Figure S19). Feeding of other amino acids, including D-proline and L-proline, resulted in no obvious change of BHMs production. This result also suggests that (2*S*,5*S*)-5-Mepro is incorporated into BHMs scaffold in the native producer. To further reveal the biosynthetic origin of the methyl group in (2*S*,5*S*)-5-Mepro, [methyl-$^{13}CH_3$]-L-methionine was next fed into the culture of both CB02009-R-5 and YX3004(Δ*bhmG*) (Figure 2B, II, IV; Figure S20). HRESIMS analysis showed that only 8-methyl group, not the 9-methyl group in **1**−**3**, was originated from the ^{13}C-labeled L-methionine. Both **4** and **11** were not labeled in YX3004(Δ*bhmG*), suggesting an unidentified pathway for the biosynthesis of (2*S*,5*S*)-5-MePro in CB02009-R-5. There are several conserved proteins, such as BlmD, BlmH, and BhmI among the identified *bhm* BGCs, and their role in (2*S*,5*S*)-5-MePro or BHM biosynthesis remains to be determined.

In conclusion, the *bhm* BGC was discovered in CB02009-R-5, assisted by a rapid ribosome engineering approach. The biosynthetic mechanisms for two unusual methyl groups in BHMs were also revealed, including (1) BhmG-catalyzed C-methylation and (2) incorporation of a rare amino acid (2*S*,5*S*)-5-MePro. Intriguingly, BhmJ NRPS or BhmK BMVO are functionally competent enzymes in heterologous *Streptomyces* hosts, while it seemed that unique regulatory mechanisms or certain protein−protein interactions among BhmJ, BhmK, and BhmG are operational in the native strain (Figure 1). The reasons could be that the native strain CB02009-R-5 may need to develop intricate mechanisms to enable the biosynthesis of proper amounts of **1**−**3**, when ample (2*S*,5*S*)-5-Mepro could be provided (Figure S21). Further study of BHM biosynthesis, such as in vitro characterization of BhmGJK would provide more insights for this observation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c01224.

Experimental procedures and additional data and figures including bioinformatic analysis of *bhm* BGCs, gene inactivation, heterologous expression, NMR and ESIHRMS data of BHM congeners (PDF)

Accession Codes

CCDC 1978350 and 1978354−1978355 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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