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Accepted Article

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This manuscript has been accepted and appears as an Accepted Article online.

This work may now be cited as: *Chin. J. Chem.* **2019**, *37,* 10.1002/cjoc.201900399.

The final Version of Record (VoR) of it with formal page numbers will soon be published online in Early View: http://dx.doi.org/10.1002/cjoc.201900399.

WILEY-VCH SIOC CCS

ISSN 1001-604X • CN 31-1547/O6
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Radical SAM-dependent adenosylation involved in bacteriohopanepolyol biosynthesis

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Abstract
Bacteriohopanepolyols are a group of triterpenoids that play important role in regulating bacterial cell membrane function. As an intermediate in bacteriohopanepolyol biosynthesis, adenosylhopane production is related to a putative Fe-S protein HpnH, but the exact role of this enzyme remains unsolved. Here we report characterization of HpnH as a novel radical S-adenosylmethionine (SAM) superfamily enzyme. In contrast to almost all the members in the superfamily, HpnH does not initiate the reaction by a hydrogen atom abstraction process. Instead, it catalyzes the adenosylation of hopene via a radical addition reaction to produce adenosylhopane, representing the second example of radical SAM-dependent adenosylation involved in natural product biosynthesis.

The radical S-adenosylmethionine (SAM) superfamily is thus far the largest known enzyme superfamily with more than 220 000 members found in all three domains of life.[1-4] These enzymes typically have a CX3CX2C motif that binds a [4Fe–4S] cluster via the three Cys thiols. The fourth iron of the [4Fe–4S] cluster is vacant and can bind a SAM molecule in a bidentate fashion via the Met moiety of SAM. Such a binding allows an inner-sphere electron transfer from the [4Fe-4S] cluster to SAM to generate a 5′-deoxyadenosyl (dAdo) radical, which can form a transient organometallic intermediate Ω with the [4Fe–4S] cluster.[5,6] Homolysis of the C-Fe bind of Ω would regenerate the dAdo radical, which leads to a highly diverse array of reactions.

For almost all the known radical SAM enzymes, the reactions are initiated by a dAdo radical-mediated hydrogen atom abstraction process, which produces a substrate radical intermediate as well as 5′-deoxyadenosine (dAdoH) as a byproduct (Figure 1A). Due to the high energetic barrier of hydrogen abstraction from an *sp2* carbon and the propensity of radical addition to double bonds, the canonical radical SAM chemistry can be easily switched to an adenosylation reaction to result in a nucleoside-containing product. Such a strategy was early used in the study

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cjoc.201900399

of pyruvate formate-lyase activase (PFL) to capture the dAdo radical[7] and has been recently developed for several radical SAM enzymes, including L-Trp lyase NosL,[8-10] the class C radical SAM methyltransferase NosN,[11] 7-carboxy-7-deazaguanine synthase QueE,[12] and three L-Tyr lyases ThiH, HydG and FibC[13] (Figure 1B). To date, the menaquinone biosynthesis enzyme MqnE represents the only biochemically characterized enzymes that naturally catalyzes a radical SAM-dependent adenosylation reaction (Figure 1C).[14,15] Because of the high efficiency of dAdo radical addition, it is expected that such an adenosylation strategy should also be involved in other biosynthesis systems.

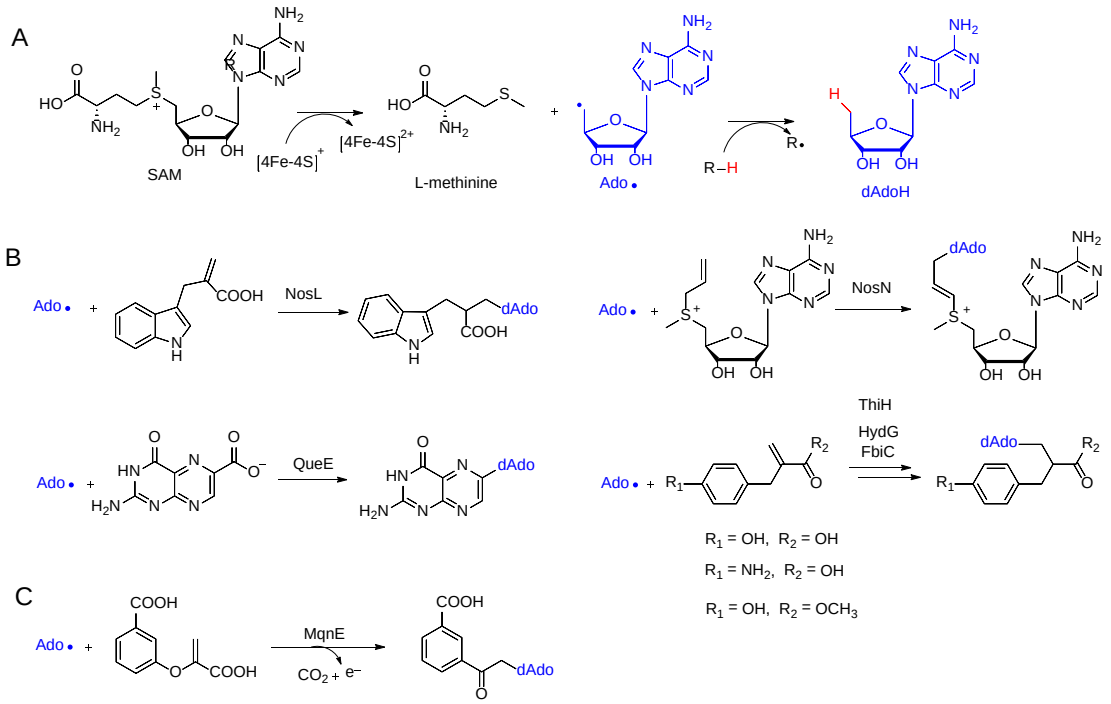

Figure 1. Reactions catalyzed by radical SAM enzymes. (A) Canonical radical SAM-dependent reactions involving a dAdo radical-mediated hydrogen abstraction. (B) Radical SAM-dependent adenosyltion involving unnatural substrate analogues. (C) MqnE-catalyzed reaction, which prior to this study represents the only known naturally occurred radical SAM-dependent adenosylation reaction.

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Bacteriohopanepolyols are a group of pentacyclic triterpenoids biosynthesized by many bacteria to perform a regulating and rigidifying function in membranes, in a way analogous to that of the sterols in eukaryotes.[16,17] These compounds are ubiquitous in sedimentary deposits,

and the diverse range and degree of taxonomic specificity enables their use as molecular biomarkers in organic geochemistry.[18] The model actinomycete strain *Streptomyces coelicolor* A(3)2 produces aminobacteriohopanetriol (ATBH) as the only detected elongated hopanoid, and a gene cluster was identified to be responsible for ATBH biosynthesis (Figure 2A).[19] It has been proposed that HpnH, a putative radical SAM enzyme, catalyzes the coupling of hopene and SAM to produce adenosylhopane, which is subsequently converted to ribosylhopane by a nuclesidase HpnG, and the latter compound is then converted to ATBH by a putative aminotransferase HpnO (Figure 2B).[20,21] Deletion of *hpnG* in *Streptomyces coelicolor* resulted in the exclusive production of adenosylhopane,[20] whereas deletion of *hpnH* in various strains resulted in the production of the C30 hopanoids such as hopene, but no C35 hopanoids such as ATBH.[22-24] These results are consistent with the proposed role of HpnH in catalyzing the adenosylaiton of hopene to produce adenosylhopane, but to date the exact role of HpnH in ATBH biosynthesis remains unclear.

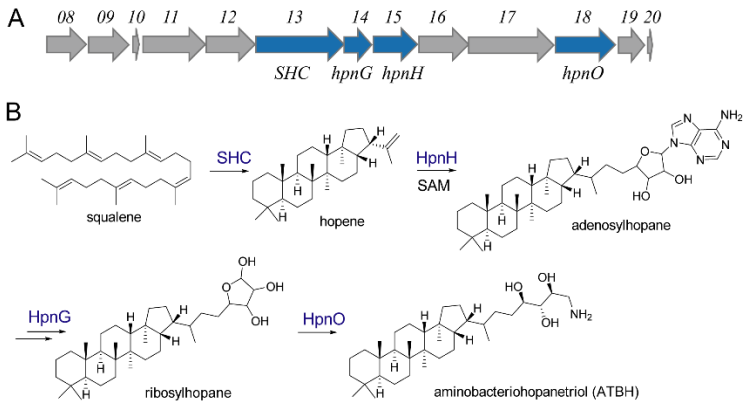

Figure 2. Biosynthesis of bacteriohopanepolyol. (A) The biosynthetic gene cluster in *S. coelicolor*. Annotation of the genes is according to the SCO numbers and as described by Poralla et al.[19] The key enzymes in the pathway (shown in B) are highlighted in blue. orf08/SCO6759, phytoene or squalene synthase; orf09/SCO6760, phytoene or squalene synthase; orf10/SCO6761, unknown function; orf11/SCO6762, phytoene dehydrogenase; orf12/SCO6763, polyprenyl diphosphate synthase or farnesyl diphosphate synthase; orf13/SCO6764, squalene-hopene cyclases (*SHC*); orf14/SCO6765, nucleosidase (*hpnG*); orf15/SCO6766, adenosylhopane synthase (*hpnH*); orf16/SCO6767, putative 4- hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; orf17/SCO6768, putative 1-deoxy-d-xylulose 5-phosphate synthase; orf18/SCO6769, putative aminotransferase (*hpnO*); orf19/SCO6770, DNA-binding protein; orf20/SCO677, unknown function. (B) The proposed biosynthetic

pathway of ATBH in *S. coelicolor* A(3)2.

To investigate the catalytic activity of HpnH, *hpnH* gene was amplified from the genomic DNA of *S. coelicolor* and expressed in *Escherichia coli* with an N-terminal hexa-histidine tag, and the protein was purified by Ni^{2+} affinity chromatography under strictly anaerobic conditions. Analysis of the reaction mixture containing SAM, sodium dithionite, and the reconstituted HpnH showed that dAdoH was produced in the assay mixture (Figure 3A, trace ii), suggesting that HpnH is a radical SAM enzyme.

We next synthesized hopene from squalene by using the cell lysate of the *E. coli* cells that overexpress the squalene-hopene cyclase SHC from *S. coelicolor*.[25] Hopene was then fed to the *E. coli* cells that overexpresses *hpnH*. Liquid chromatography (LC)-high resolution (HR)-mass spectrometry (MS) analysis of the resulting cell extract revealed a compound exhibited a positive charged molecular ion at m/z = 662.5002 (0.3 ppm for a molecular formula of $C_{40}H_{63}N_5O_3$, $[M+H]^+$ = 662.5004) (Figure 3B, trace ii, and Figure S3A), and this compound was not observed with the control *E. coli* cells carrying an empty vector (Figure 3B, trace i). HR-MS/MS analysis of this compound showed a major fragment ion at m/z = 136.06, which is characteristic of the adenosine-containing compounds (Figure 3C and Figure S3B). To further validate the identity of the compound, we followed the procedure of Rohmer et al by deleting *hpnG* in *S. coelicolor*.[20] The *hpnG*-knockout mutant was grown using R2YE solid culture and adenosylhopane was obtained by extracting the cell medium. LC-HRMS analysis showed that the compound obtained in the feeding experiment coeluted with adenosylhopane produced from the *hpnG*-knockout mutant, supporting the compound produced in feeding experiment is adenosylhopane (Figure 3B, trace iii).

We next performed the *in vitro* reaction of HpnH with hopene, SAM and sodium dithionite. To increase the solubility of hopene, the nonionic surfactant triton X-100 was added in the reaction to a final concentration of 1%. LC-HRMS analysis of the reaction mixture showed that adneosylhopane was indeed produced in the reaction as expected (Figure 3B, trace v), which was absent in the control assays either with the supernatant of boiled enzyme (Figure 3B, trace vi). Although dAdoH was also produced in the reaction, the yield decreased 3-4 folds compared to that without hopene (Figure 3A), supporting that the newly generated dAdo radical was

trapped by hopene. Moreover, SAM consumption also increased apparently in the assay with hopene (Figure 3A, trace i). These results strongly support that HpnH catalyzes a radical SAM-dependent adenosylation of hopene to produce adenosylhopane.

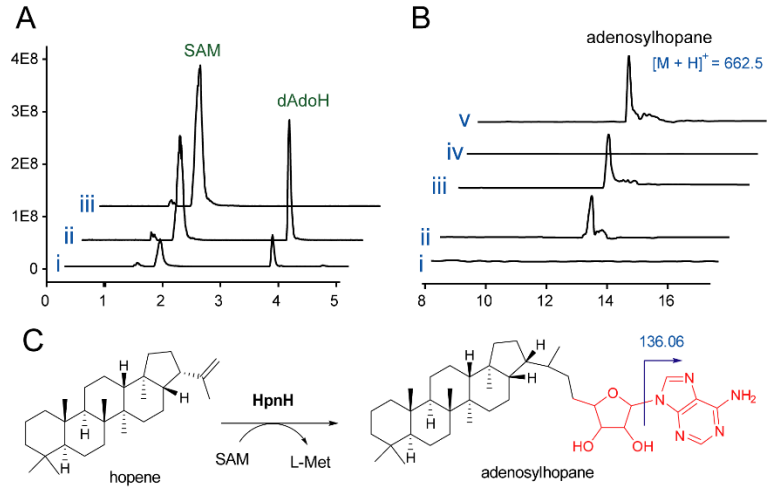

Figure 3. Characterization of HpnH reaction. (A) LC-MS analysis of SAM cleavage assays, the multiple selected ion monitoring (SIM) mode includes $[M+H]^+$ = 399.1 (corresponding to SAM), and 252.1 (corresponding to dAdoH), showing i) the reaction with the supernatant of boiled HpnH, ii) the HpnH reaction in which hopene was omitted, and iii) HpnH with all the required components. (B) LC-MS analysis of adenosylhopane, showing the extracted ion chromatograms (EICs) of $[M+H]^+$= 662.5 for i) control experiments with *E. coli* cells carrying an empty vector, ii) the extract of cell culture in which hopene was fed to the *hpnH*-expressing *E. coli* cells, iii) the authentic adenosylhopane produced in the *hpnG*-knockout mutant of *Streptomyces coelicolor*, iv) control reaction with the supernatant of boiled HpnH, and vi) in vitro assay of HpnH with all the required components. (C) The Hpn-catalyzed reaction.

We previously showed that S-guanosylmethionine (SGM) and S-cytidinylmethionine (SCM), two SAM analogues containing a guanine and a cytosine moiety, respectively, were able to support the catalysis of the tryptophan lyase NosL, allowing for the generation of different nucleoside adducts in NosL reaction with a tryptophan structural analogue with an olefin moiety.[8,9] Aiming to produce novel hoponoids with a different nucleoside moiety, we ran the HpnH assay with SGM and SCM, respectively, and similar reactions with NosL were also performed as positive control assays. However, although significant amounts of 5′-

deoxyguanosine (dGuoH) and 5′-deoxycytidine (dCydH) were produced in NosL reaction, neither of them was produced in HpnH reaction. These observations suggested that HpnH has a more stringent substrate specificity, which can only recognize SAM, but not SGM and SCM.

In summary, we have shown that HpnH in the biosynthetic pathway of bacteriohopanepolyols is a novel radical SAM enzyme. In contrast to most members of the radical SAM superfamily that initiates the reaction by a hydrogen abstraction mediated by the dAdo radical, the dAdo radical generated in HpnH catalysis adds to the double bond of hopene to produce adenosylhopane, representing the second example of naturally-occurred radical SAM-dependent adenosylation reaction. Owing to the enormous number of the radical SAM superfamily and the fact that most of the members are functionally unknown, we expect that such a type of adenosylation reaction could be more general and awaits characterization by future biochemical studies.

Acknowledgements

This work is supported in part by grants from the National Key Research and Development Program (2016 Y F A0501302), and from National Natural Science Foundation of China (31670060 to Q.Z.).

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