Expanding the Structural Diversity of Drimentines by Exploring the Promiscuity of Two N-methyltransferases

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HIGHLIGHTS
The methylation steps during drimentines biosynthesis were unraveled
Two N-MTs with different regioselectivities were identified
The substrate promiscuities of DmtMT1 and DmtMT2-1 were probed
Combinatorial biosynthesis expanded the chemical space of drimentines
Expanding the Structural Diversity of Drimentines by Exploring the Promiscuity of Two N-methyltransferases

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SUMMARY
Methylation is envisioned as a promising way to rationally improve key pharmacokinetic characteristics of lead compounds. Although diverse tailoring enzymes are found to be clustered with cyclodipeptide synthases (CDPSs) to perform further modification reactions on the diketopiperazine (DKP) rings generating complex DKP-containing compounds, so far, a limited number of methyltransferases (MTs) co-occurring with CDPS have been experimentally characterized. Herein, we deciphered the methylation steps during drimentines (DMTs) biosynthesis with identification and characterization of DmtMT2-1 (from Streptomyces sp. NRRL F-5123) and DmtMT1 (from Streptomyces youssoufiensis OUC6819). DmtMT2-1 catalyzes N4-methylation of both pre-DMTs and DMTs; conversely, DmtMT1 recognizes the DKP rings, functioning before the assembly of the terpene moiety. Notably, both MTs display broad substrate promiscuity. Their combinatorial expression with the dmt1 genes in different Streptomyces strains successfully generated eight unnatural DMT analogs. Our results enriched the MT tool-box, setting the stage for exploring the structural diversity of DKP derivatives for drug development.

INTRODUCTION
Natural products with 2,5-diketopiperazine (DKP) scaffolds are a large class of specialized metabolites with structural diversity and notable bioactivities (Borthwick, 2012). The DKP rings confer structural stability and rigidity against proteolysis, making them attractive in pharmaceutical development (Borthwick, 2012). From the biosynthetic point of view, the DKP ring is assembled via a traditional non-ribosomal peptide synthetase (NRPS) or a recently characterized cyclodipeptide synthase (CDPS) machinery (Belin et al., 2012). Notably, diverse tailoring enzymes are often found to be clustered with CDPSs to perform further modification reactions on the DKP rings such as oxidation (Cryle et al., 2010; Meng et al., 2018; Patteson et al., 2018), methylation (Giessen et al., 2013a, 2013b; Li et al., 2019; Liu et al., 2019; Shi et al., 2019), prenylation (Yao et al., 2018), as well as cyclization (Yao et al., 2018), generating complex DKP-containing compounds. Although relatively few tailoring enzymes co-occurring with CDPS clusters have been experimentally characterized, most of them exhibit broad substrate scopes (Giessen et al., 2013a, 2013b; Li et al., 2019; Liu et al., 2019; Shi et al., 2019). The small sizes and substrate promiscuities of the CDPSs and their associated tailoring enzymes highlight the CDPS pathway enzymes as potential powerful tools for the generation of structurally unique DKP compounds by combinatorial biosynthetic approaches. The use of CDPSs as “biosynthetic hooks” is thus considered an effective strategy for identifying genes modifying 2,5-DKP rings to expand the chemical space of DKPs (Borgman et al., 2019; Canu et al., 2020).

Methylation of O-, C-, N-, and S-centered nucleophiles are ubiquitous tailoring reactions during the biosynthesis of small molecules, which increases the lipophilicity and membrane permeability of small-molecule scaffolds, enhancing their membrane transport, oral bioavailability, absorption, and excretion (Barreiro et al., 2011; Liscombe et al., 2012). N-methylation is envisioned as a promising way to rationally improve key pharmacokinetic characteristics of cyclic peptides (Chatterjee et al., 2008). Although bioinformatics analyses indicate the presence of a large number of methyltransferases (MTs) genetically associated with CDPSs (Skinnider et al., 2018), most of them remain unexplored. Up to now, only five MTs...
from CDPS-dependent pathways have been functionally characterized: O-methyltransferase Ndas_1149 from the nocazine pathway methylating phenolic hydroxyl groups of cyclo(L-Phe-L-Tyr) (cFY) (D3), cFY(D3, D6) and cyclo(L-Tyr-L-Tyr) (cYY) (D3, D6) (Giessen et al., 2013a), N-methyltransferase Amir_4628 catalyzing two successive N-methylations at the cWW ring to generate Me2-cWW (Giessen et al., 2013b), GutE/PcmE from guanitrypmycin pathway transferring a methyl group onto the guaninyl residue (Liu et al., 2019; Shi et al., 2019), and C-methyltransferase StspM1 mediating C3-methylation of indole ring and cyclization between the indole C2 of the Trp residue and the α-nitrogen (Li et al., 2019). These facts drive us to explore novel MTs from CDPS-dependent pathways for the generation of diverse DKP derivatives. We previously identified three homologous CDPS loci dmt1-3 encoding drimentines (DMTs) (Figure 1, Yao et al., 2018), which are a family of terpenylated diketopiperazine alkaloids with antibacterial, antifungal, and anthelmintic activities (Che et al., 2012; Lacey et al., 1998). The CDPS DmtBs synthesize

Figure 1. Genetic Organization of the dmt1-3 loci and Structures of Their Encoding Compounds
(A) dmt1-3 loci from S. youssoufensis OUC6819, Streptomyces sp. NRRL F-5123, and S. aidingensis CGMCC 4.5739. PT, prenyltransferase; CDPS, cyclodipeptide synthase; TC, terpene cyclase; MT, methyltransferase.
(B) Chemical structures of drimentines, pre-drimentines, and their methylated derivatives. Compounds 7–10 and 13–16 are generated in this study.
cyclo(L-Trp-L-Xaa) (cWX) (X = Val, Pro, Leu, Ile or Ala), followed by prenylation and cyclization, which are, respectively, accomplished by the phytoene-synthase-like (PSL) prenyltransferase DmtCs and the membrane terpene cyclase DmtAs to afford DMTs (Yao et al., 2018). Noticeably, three putative MT genes are located adjacent to the dmt2 locus (from Streptomyces sp. NRRL F-5123) and the dmt3 locus (from S. aidingensis CGMCC 4.5739), suggesting that these two clusters might encode more complicated methylated DMTs. In contrast, no putative MT gene is found around the dmt1 locus from S. youssoufensis OUC6819, although this strain is able to produce drimentine F (DMT F), which harbors N15-methyl group (Che et al., 2012). Herein, on one hand, we characterized the function of the dmt2- and dmt3-associated MT genes; on the other hand, we deciphered the N15-methylation step during the biosynthesis of DMT F; thereby, two S-adenosylmethionine (SAM)-dependent N-MTs (DmtMT2-1 and DmtMT1) with different regioselectivities were obtained. Their substrate spectra were probed, revealing that both of them exhibited considerable substrate promiscuities. Mixing and matching dmtMT2-1 and dmtMT1 with other DMT biosynthetic genes afforded unnatural methylated DMTs, providing novel effective CDPS-associated MTs for compound structural diversification.

RESULTS

Function of the MT Genes Adjacent to the dmt Loci

As indicated in Figure 1 and Table S3, DmtMT2-1 shows 36.7% identity/49.8% similarity to MitM (AAD28459.1), which functions as an aziridine N-methyltransferase during the biosynthesis of mitomycin (Varoglu et al., 2001); DmtMT2-2 shows 16.4% identity/25.3% similarity to UbiE (YP_026269.1), which catalyzes the carbon methylation reaction in the biosynthesis of ubiquinone and menaquinone (Lee et al., 1997); DmtMT3 shows 24.2% identity/34.6% similarity to PrmC (AY600244.1) of Chlamydia trachomatis involving in methylation of the class 1 peptide chain release factors (Pannekoek et al., 2005).

To investigate the function of these MT genes during the biosynthesis of DMTs, we solubly expressed them in E. coli (Figure S1) and tested their enzymatic activities in vitro. Given methylation may occur at different phases during DMTs biosynthesis, different substrates were subjected to assays based on the encoding products of dmt2/3 (Yao et al., 2018). HPLC analysis of the reactions showed: (1) DmtMT2-1 was able to recognize both DMT A (3)/C (4) (Figure 2Ai and iii) and pre-DMT A (5)/C (6) (Figure 2Bi and iii), generating compounds 7–10, but could not recognize cWL or cWP (Figure S2A), which indicated that a pyrroloindoline
ring is necessary for the activity of DmtMT2-1; (2) neither DmtMT2-2 (Figures S2B–S2D) nor DmtMT3 (Figures S2E and S2F) recognized the tested substrates.

Subsequently, large volume (30 mL) of reactions followed by chemical isolation was performed, leading to identification of compounds 7–10. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis indicated the molecular formula of 7 as C33H47N3O2 (m/z [M + H]+ 518.3776, calcd 518.3747, Data S1), 8 as C32H43N3O2 (m/z [M + H]+ 502.3434, calcd 502.3434, Data S1), 9 as C33H47N3O2 (m/z [M + H]+ 518.3776, calcd 518.3747, Data S1), and 10 as C32H43N3O2 (m/z [M + H]+ 502.3476, calcd 502.3434, Data S1), which are 14 mass units bigger than that of DMT A (3), DMT C (4), pre-DMT A (5), and pre-DMT C (6), respectively (Yao et al., 2018). Comparison of the 1H and 13C NMR data between 7 and 3 (Yao et al., 2018) revealed that they share similar structure except the presence of an additional methyl group (δH 2.87, δC 33.2) in 7 (Data S1). Based on HMBC correlation observed from the methyl proton H-36 (δH 2.87) to C3 (δC 83.6) and C5 (δC 150.6), we determined the methyl group is attached to the nitrogen of the dihydroindole system instead of the DKP ring (Data S1). Thus, compound 7 was determined as N4-methyl-DMT S. youssoufiiensis OUC6819 is able to produce DMT F, which harbors a methyl group at N15 position. This fact indicates that the responding MT gene is located in another place of the genome. With comparative liquid chromatography-mass spectrometry (LC-MS) analysis of the fermentation products from the OUC6819 strains, a compound peak with molecular weight of 14 Daltons bigger than that of cWV (m/z [M + H]+ 286.1556, Figure S3B) was observed in ΔdmtC1 (the prenyltransferase gene in charge of the assembly of farnesyl group onto cWV) (Figure S3Aii) and the dmtB1 overexpression strain (Figure S3Aiii) but not in the wild-type strain (Figure S3Aii). This phenotype led us to hypothesize that methylation may happen before the assembly of the terpene moiety. Therefore, Amir_4628, which conducts two successive N-methylations at the DKP ring (Giessen et al., 2013b), was subjected to BLAST against the OUC6819 genome, revealing a homologous gene 6880MT (renamed as dmtMT1; 33.5% identity/45.0% similarity to Amir_4628) at 0.56 Mb upstream of the dmt1 locus (Figure 1, Table S3).

To detect if dmtMT1 is involved in the biosynthesis of DMT F, in-frame deletion was performed, generating ΔdmtMT1 (Figure S4). HPLC analysis revealed that the production of DMT F was completely abolished; conversely, DMT G was still produced in ΔdmtMT1 albeit in small amount (Figure 3ii). Moreover, complementation of ΔdmtMT1 restored the production of DMT F (Figure 3iii); overexpression of dmtMT1 resulted in increased production of DMT F by about 2-fold (Figure 3iv). These results ambiguously demonstrated dmtMT1 to be responsible for the N15-methylation of DMT F.

**Probing the MT Gene Involved in N15-Methylation of DMT F**

Although no MT gene is found adjacent to dmt1, S. youssoufiiensis OUC6819 is able to produce DMT F, which harbors a methyl group at N15 position. This fact indicates that the responding MT gene is located in another place of the genome. With comparative liquid chromatography-mass spectrometry (LC-MS) analysis of the fermentation products from the OUC6819 strains, a compound peak with molecular weight of 14 Daltons bigger than that of cWV (m/z [M + H]+ 286.1556, Figure S3B) was observed in ΔdmtC1 (the prenyltransferase gene in charge of the assembly of farnesyl group onto cWV) (Figure S3Aii) and the dmtB1 overexpression strain (Figure S3Aiii) but not in the wild-type strain (Figure S3Aii). This phenotype led us to hypothesize that methylation may happen before the assembly of the terpene moiety. Therefore, Amir_4628, which conducts two successive N-methylations at the DKP ring (Giessen et al., 2013b), was subjected to BLAST against the OUC6819 genome, revealing a homologous gene 6880MT (renamed as dmtMT1; 33.5% identity/45.0% similarity to Amir_4628) at 0.56 Mb upstream of the dmt1 locus (Figure 1, Table S3).

**Timing of the N15-Methylation during DMT F Biosynthesis**

To identify the exact timing of the methylation step, in vitro biochemical reactions were carried out using cWV, pre-DMT G (11), and DMT G (1) as substrates. As indicated in Figure 4, DmtMT1 was capable of recognizing cWV to give a new peak (Figure 4A) but could not accept pre-DMT G or DMT G (Figure S5), supporting the methyl group is assembled right after the formation of cWV. Time course analysis showed that only a single methylation takes place, as indicated by the presence of the sole product with a molecular ion peak [M + H]+ at m/z 300.1726, which was 14 units bigger than that of cWV (m/z [M + H]+ 286.1556) (Figures 4A and 4B).

To clarify the position of the methylation, HR-ESI-MS analysis was conducted. As shown in Figure 4B, in addition to the characteristic fragmentation pattern of 2,5-DKP with neutral losses of 28 Da (-CO) and 45 Da (-HCONH2) (Guo et al., 2009), successive neutral loss of 59 Da corresponding to a HCONHCH3 fragment could also be detected, suggesting that the methylation takes place at the DKP ring; the protonated substituent ion at m/z 130 indicated the presence of tryptophan (Guo et al., 2009); the ions at m/z 169.0984, 183.1130 resulting from elimination of 3-methyl-1-H-indole and indole, respectively, combined with m/z 86.0964 from sequential losses of CO and Trp residue proved that the methyl group is attached to the
α-nitrogen of Val in cWV, which was further confirmed by the HMBC correlation of Me-cWV (12) from the methyl proton H-20 (δH 2.82) to C12 (δC 166.0) and C14 (δC 66.6) (Figure 4C and Data S1).

This fact means the PSL-family prenyltransferase DmtC1, which transfers farnesyl group onto cWV to generate pre-DMT G (Yao et al., 2018), should be able to recognize Me-cWV as well. Thus, we incubated DmtC1 with Me-cWV (12) in the presence of farnesyl diphosphate (FPP), and a new compound (13) appeared as expected (Figure S6), with a molecular ion peak [M + H]+ at m/z 504.3579, which was 14 units bigger than that of pre-DMT G (Data S1). The 1H and 13C NMR spectra of 13 provided a dataset similar to that of pre-DMT G, with the chemical shift difference observed for a methyl group (δH 2.81, δC 32.7) (Data S1). The absence of one-proton singlet at δH 7.92 in pre-DMT G indicated that the methyl group was attached to N15 as confirmed by the strong HMBC correlation from the methyl proton H-35 (δH 2.81) to C3 (δC 83.6) and C5 (δC 150.8) (Data S1). Considering the biosynthetic assembly line of DMT G, we proposed that compound 13 is the biosynthetic precursor of DMT F, and thus it was named as pre-DMT F, which would be further cyclized by the terpene cyclase DmtA1 to afford DMT F.

**PROBING SUBSTRATE PROMISCUITY OF DMTMT2-1 AND DMTMT1**

With the two methyltransferases (DmtMT2-1 and DmtMT1) in hand, we evaluated their potentials as tool enzymes to diversify structures of DMT compounds. For DmtMT2-1, another two substrates, DMT G (1) and pre-DMT G (11), were tested. As shown in Figure 5A, both of them were recognized by DmtMT2-1, transforming 1 into 14 and 11 into 15 as expected. All the products were isolated and their structures elucidated by HR-ESI-MS and NMR analyses (Data S1). Finally, 14 (m/z [M + H]+ 504.3630, calcd 504.3590) and 15 (m/z [M + H]+ 504.3647, calcd 504.3590) were determined to be N4-methyl-DMT G and N4-methyl-pre-DMT G as indicated by the HMBC correlations from the methyl proton H-35 (δH 2.86 for 14 and δH 2.91 for 15) to C3 (δC 83.6 for 14 and δC 84.2 for 15) and C5 (δC 150.8 for 14 and δC 150.9 for 15) (Data S1).

To probe the substrate promiscuity of DmtMT1, a series of DKPs were tested. Delightedly, DmtMT1 exhibited broad substrate flexibility and was able to recognize a bunch of DKPs, including cWL, cWI, cWA, cWT, cWF, cWY, cWW, and noticeably cyclo(o-Trp-L-Val) (cDWV) and cYV as well (the nomenclature of a cyclodipeptide is indicated here by the one-letter code for the two L-configured amino acids) (Figure SB and Data S2). The responding methylated DKPs were identified by HR-ESI-MS2 analysis (Data S2). Their fragmentation patterns were in agreement with that of Me-cWV (Figure 4B), supporting that each N-methylation regio-specifically occurs at the position derived from the second amino acid (from the biosynthetic point of view). Based on the relative catalytic efficiencies, we can clearly see DmtMT1 prefers cWXs with X being aliphatic over aromatic amino acids (Figure 5B). Delightedly, DmtMT1 was capable of transferring the methyl group onto cDWV and cYV, albeit at much lower efficiencies, implying it has certain flexibility toward the first amino acid as well.
The above results indicated that both DmtMT2-1 and DmtMT1 are N-methyltransferases with broad substrate promiscuity and stringent regiospecificity, which would potentially serve as efficient catalysts for structural diversification in drug development.

**GENERATION OF UNNATURAL DMTS BY USING THE LOGIC OF SYNTHETIC BIOLOGY**

The availability of these two MTs drove us to further generate unnatural DMT analogs by using the logic of synthetic biology. Based on the biosynthetic machinery of DMTs, we introduced *dmtMT2-1* or/and *dmtMT1* into different cells, including DMTs producer *S. youssoufianus* OUC6819, *S. coelicolor* M1146/*dmtB1C1* (accumulating pre-DMTs), and *S. coelicolor* M1146/*dmtA1B1C1* (producing DMTs) (Yao et al., 2018).

As shown in Figure 6, expression of *dmtMT2-1* in *S. coelicolor* M1146/*dmtB1C1* led to the appearance of three small peaks, which were identified to be methylated pre-DMT compounds 10, 15, and 9, respectively (Figure 6iii and iv). When *dmtMT2-1* was introduced into *S. youssoufianus* OUC6819, in addition to 14, another compound peak 16 showed up (Figure 6vi and vii). Considering the regioselectivity of *dmtMT2-1*, we speculated 16 might be N4-methyl-DMT F, which was confirmed by HR-ESI-MS (Data S1) and NMR data with the presence of the methyl group (δH 2.85, δC 31.4), along with its strong HMBC correlations to C3 (δC 82.8) and C5 (δC 151.5) (Data S1).
Expression of dmtMT1 in *S. coelicolor* M1146/dmtB1C1 led to decreased amount of 11 and simultaneous accumulation of pre-DMT F (13) (Figure 6viii and ix). When introducing dmtMT1 into *S. coelicolor* M1146/dmtA1B1C1, DMT F (2) was accumulated as expected (Figure 6xi and xii). Unfortunately, no new product was observed after further introduction of dmtMT2-1 into *S. coelicolor* M1146/dmtB1C1+dmtMT1 (Figure 6xiii).

**DISCUSSION**

CDPSs are small enzymes that utilize two aminoacyl-tRNAs to catalyze the formation of a 2,5-DKP ring system (Gondry et al., 2009). To date, over 110 CDPSs have been functionally characterized and usually exhibit a certain degree of substrate promiscuity, making CDPSs intriguing members of Nature’s biosynthetic repertoire (Canu et al., 2020). The resulting cyclodipeptides are generally further modified by at least one tailoring enzyme adjacent to CDPS, including formation of C-C bond and C-O bond, and regioselective methylation and prenylation (Borgman et al., 2019). In this study, we identified and characterized two promiscuous N-methyltransferases and elucidated the methylation machinery during DMTs biosynthesis through *in vivo* and *in vitro* experiments, providing significant insights into generation of DKP derivatives by using synthetic biology approaches.

Although both DmtMT2-1 and DmtMT1 are involved in DMT biosynthesis, they act at different timings with different manners of function. As indicated in Figure 7, in OUC6819, the methyl group is assembled right after the formation of cWV instead of happening as the last step, and the following prenylation and cyclization steps occur in parallel, indicating both DmtC1 and DmtA1 display flexible substrate promiscuity, making CDPSs intriguing members of Nature’s biosynthetic repertoire (Canu et al., 2020). The resulting cyclodipeptides are generally further modified by at least one tailoring enzyme adjacent to CDPS, including formation of C-C bond and C-O bond, and regioselective methylation and prenylation (Borgman et al., 2019). In this study, we identified and characterized two promiscuous N-methyltransferases and elucidated the methylation machinery during DMTs biosynthesis through *in vivo* and *in vitro* experiments, providing significant insights into generation of DKP derivatives by using synthetic biology approaches.

In microbes, the genes responsible for production of a specialized metabolite are mostly found in close proximity to another in dedicated biosynthetic gene clusters. However, dmtMT1 is located at 0.56 Mb downstream of the dmt1 locus. In comparison with its homolog Amir_4628, which doubly methylates DKPs constituting two identical aromatic amino acids (Giessen et al., 2013b), DmtMT1 prefers cWX with X being aliphatic amino acids and strictly methylates nitrogen originating from the X residue. Notably, DmtMT1 displays remarkable
substrate promiscuity. It allows the second amino acid to be Phe and Tyr and even admits the first amino acid to be d-configured Trp and Tyr, making DmtMT1 a very promising tool enzyme for compound diversification.

With the advent of next-generation sequencing, massive microbial genome sequence data have been uploaded to the public domain. Simple BLAST-based searches reveal large numbers of MTs associated with CDPSs in prokaryotic genomes. Hence, we constructed a sequence similarity network from selected MT sequences and assigned them to known MTs. As indicated in Figure S7, three MT homologs (WP_055513754.1, WP_029387245.1, and WP_078513178.1) clustered with DmtMT2-1 are associated with a CDPS and a PSL-prenyltransferase, indicating that they might be involved in the biosynthesis of novel DMT-like compounds; notably, another four DmtMT2-1 homologs (StspM2/StflM2/5971M2/StalM2) are proposed to be involved in the N-methylation step during nocardioazine biosynthesis, but no in vivo nor in vitro results are provided (Li et al., 2019). Our results would enlighten deciphering of the methylation steps of nocardioazine-like natural products. Moreover, there are still several MTs that are not clustered with any known MTs, suggesting the untapped potentials of MTs as sources of new catalysts.

Apart from methyltransferases, we note that several other ORFs are in close proximity to CDPSs in cluster dmt2/3, such as the cyclodipeptide oxidases (CDOs), which have been reported to catalyze Cα-Cβ dehydrogenations on diverse cyclodipeptides (Giessen et al., 2013a; Lautru et al., 2002). Thus, further experiments are required to completely characterize the functions of these proteins and gain a broader understanding of DMTs biosynthesis.

Limitations of the Study
The molecular mechanism underlying the promiscuity of DmtMT1 and DmtMT2-1 has not been elucidated in the present study. Future crystallographic studies and systematic structure-guided mutagenesis would shed light on these issues.

Resource Availability
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Dr. Wenli Li (email: liwenli@ouc.edu.cn).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact without restriction.
Data and Code Availability

The accession number for the dmtMT1 reported in this paper is GenBank: MK894429 (https://www.ncbi.nlm.nih.gov/nucore/MK894429.1). All relevant data supporting the findings of this study are available within the paper and its Supplemental Information files. Additional data are provided upon reasonable request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101323.

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AUTHOR CONTRIBUTIONS

Conceptualization, W.L.; Methodology, J.L. and Z.L.; Investigation, T.Y. and E.J.; Formal Analysis, H.L.; Writing – Original Draft, W.L. and T.Y.; Writing – Review & Editing, W.L. and T.Y.; Funding Acquisition, W.L. and T.Y.; Resources, Q.C., T.Z., and D.L.; Supervision, W.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.
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Supplemental Information

Expanding the Structural Diversity
of Drimentines by Exploring the Promiscuity
of Two $N$-methyltransferases

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Figure S1. SDS-PAGE gels of DmtMT2-1 (lane 1), DmtMT1 (lane 2), DmtMT2-2 (lane 3) and DmtMT3 (lane 4), Related to Figures 2, and 4-5.
Figure S2. *In vitro* assays of DmtMT2-1, DmtMT2-2 and DmtMT3, Related to Figure 2.
Figure S3. (A) HPLC traces of the fermentation products from (i) the wild-type OUC6819 strain; (ii) ΔdmtC1; (iii) overexpression of dmtB1 in *S. youssoufienensis* OUC6819; (iv) standard of cWV. (B) The HR-ESI-MS spectrum of the compound peak indicated by red arrow, Related to Figure 3.
**Figure S4.** Inactivation of dmtMT1. (A) Construction of the ΔdmtMT1 mutant. (B) PCR confirmation of the ΔdmtMT1 mutant. M: DNA marker; W: *S. youssoufiensis* OUC6819 wild-type strain; Mutant: the ΔdmtMT1 mutant, Related to Figure 3.

**Figure S5.** *In vitro* methyltransferase activity of DmtMT1 using DMT G (1) and pre-DMT G (11) as substrates, Related to Figure 4.
Figure S6. DmtC1-catalyzed reaction using Me-cWV and FPP as substrates, Related to Figure 4.

Figure S7. Sequence similarity networks (SSNs) of selected methyltransferases using an alignment score of 30. The details of the above proteins were shown in Data S3. Multiple MTs within this network are grouped with DmtMT1/Amir_4628 involved in the DKP-ring methylation. A couple of MTs clustered with known MTs are also included: four PcmE/GutE homologs (WP_078645497.1, WP_078897103.1, WP_078624486.1, and WP_023552492.1), two Ndas_1149/1145 homologs (WP_017534685.1 and WP_017534689.1), and one StspM1/StalM1 homolog (WP_027751607.1), Related to Figure 1.
Table S1. Bacteria and plasmids used in this study, Related to Figures 2-6.

| Strains or plasmids | Description | Reference or source |
|---------------------|-------------|---------------------|
| **Strains**         |             |                     |
| *E. coli* Top10     | Host strain of cosmid vector SuperCos1 | Invitrogen |
| *E. coli* DH5a      | Host strain for general cloning | Invitrogen |
| *E. coli* ET12567/pUZ8002 | Host strain for conjugation | (Gust et al., 2003) |
| *E. coli* BW25113/pJ790 | Host strain for PCR-targeting | (Gust et al., 2003) |
| *E. coli*           | Protein expression host | Stratagene |
| BL21-CodonPlus(DE3) | Protein expression host | Invitrogen |
| *E. coli* BL21(DE3) | Protein expression host | Stratagene |
| *Streptomyces* youssoufiensis OUC6819 | Strain harboring the dmt1 locus | (Che et al., 2012) |
| *Streptomyces* sp. NRRL F-5123 | Strain harboring the dmt2 locus | NRRL* |
| *Streptomyces* aidingensis CGMCC 4.5739 | Strain harboring the dmt3 locus | CGMCCb |
| ΔdmtMT1             | dmtMT1 inactivation mutant of *S. youssoufiensis* OUC6819 | This study |
| *Streptomyces* coelicolor M1146 | Host strain for heterologous expression | (Gomez - Escribano and Bibb, 2011) |
| **Plasmids**        |             |                     |
| SuperCosI           | AmpR, KanR, cosmid vector | Stratagene |
| pIJ773              | AprR, source of acc(3)I-oriT cassette | (Gust et al., 2003) |
| pIJ790              | CmR, λ RED recombination plasmid | (Gust et al., 2003) |
| pSET152C            | pSET152 derivative, with insertion of the neo gene from SuperCos1 at the sites of Apal and SgrAI | (Yao et al., 2018) |
| pIJ10500            | HygR, integrative plasmid containing the ϕBT1 integrase gene | (Kieser et al., 2000) |
| pET28a(+)           | KanR, expression vector | Novagen |
| pET32a(+)           | AmpR, expression vector | Novagen |
| pWLI628             | pET28a carrying dmtMT1 | This study |
| pWLI629             | pET28a carrying dmtMT2-1 | This study |
| pWLI630             | pET28a carrying dmtMT3 | This study |
| pWLI631             | pET32a carrying dmtMT2-2 | This study |
| pWLI632             | cosmid harboring dmtMT1 gene from *S. youssoufiensis* OUC6819 | This study |
| pWLI633             | pWLI632 derivative where dmtMT1 was replaced with acc(3)I-oriT cassette | This study |
| pWLI634             | pSET152C derivative harboring dmtMT1 under the control of P<sub>gapdh</sub> | This study |
| pWLI635             | pSET152C derivative harboring dmtMT1-2 under the control of P<sub>gapdh</sub> | This study |
| pWLI636             | pIJ10500 derivative harboring dmtMT1 under the control of P<sub>gapdh</sub> | This study |
| pWLI637             | pIJ10500 derivative harboring dmtMT2-1 under the control of P<sub>gapdh</sub> | This study |
| pWLI638             | pWLI636 derivative harboring dmtMT2-1 under the control of P<sub>gapdh</sub> | This study |

a: Agricultural Research Service Culture Collection, NRRL
b: China General Microbiological Collection Center, CGMCC
Table S2. Primers used in this study. Related to Figures 2-6.

| Name | Sequence (5'-3') |
|------|------------------|
| For DmtMT1, DmtMT2-1, DmtMT2-2 and DmtMT3 protein expression |
| DmtMT2-1-FP | GGAATTC**CATATG**CAGCACGAGCACACGGCG |
| DmtMT2-1-RP | CGG**CTCAG**TACATTTGCCGCGCGAGAG |
| DmtMT2-2-RP | CGG**CTCAG**TACATTTGCCGCGCGAGAG |
| DmtMT3-FP | GGAATTC**CATATG**TACATTTGCCGCGCGAGAG |
| DmtMT3-RP | CGG**CTCAG**TACATTTGCCGCGCGAGAG |
| DmtMT1-FP | GGAATTC**CATATG**TACATTTGCCGCGCGAGAG |
| DmtMT1-RP | CGG**CTCAG**TACATTTGCCGCGCGAGAG |

For PCR-targeted mutagenesis of *dmtMT1*

| dmtMT1MF | GCGTAAAGAGACGATTCGCGCAAGGCAGCGCGAGGCACCGTCGACGatcgggagtcc |
| dmtMT1MR | TGTCACTTCACCGCCTACCTACTGACCTGACGCGGCGACGtaggtcggagtcc |
| dmtMT1CF | CGTAGAAGAGCAGCTGCGGAG |
| dmtMT1CR | TCACCGCCTACTGACCG |
| dmtMT1EF | GTGGAAAGTAGAAGCTGAGCAG |
| dmtMT1ER | CTGACTGACCTTACCCGCGGCTCCAGAG |

For heterologous expression of *dmtMT1* and *dmtMT2-1*

| dmtMT2-1-FP | ATGCACGACGACGACGAC |
| dmtMT2-1-RP | CATACGTCAGATTGCCGCGCGAGACG |
| dmtMT2-1-RP2 | CGG**CTCAG**TACATTTGCCGCGCGAGAG |
| dmtMT1-FP | GGAATTC**CATATG**TACATTTGCCGCGCGAGAG |
| dmtMT1-RP | CCAGTAGTTACCTTACCCGCGGCTCCAGAG |
| pHFP | GGTACCTTAGACGGCCTCCTCCGCGGCG |
| pHRP | GAACACGCTCTCCGGAACGTTTG |
| pGFP | CCAATGCATCGTGCCGAGGAACTGTTG |
| pGRP | GAACCGATCTCCTGGTG |

Underlined red letters represent restriction sites. The primer pair of DmtMT1-FP/RP was also used for genomic library screening. The 3'-OH of pGRP and pHRP was phosphorylated. pHFP/pHRP and pGFP/pGRP were used for amplification of promoter P<sub>hrdB</sub> and P<sub>gapdh</sub>, respectively.

Table S3. Predicted functions of the four MT genes, Related to Figure 1.

| Strain | Protein | Size (aa) | Proposed function | Homologs | Accession no. (Identity/Similarity %) |
|--------|---------|-----------|-------------------|----------|--------------------------------------|
| 6819   | DmtMT1  | 249       | methyltransferase | Amir<sub>4628</sub>/Actinosynnema mirum | ACU38461.1 (33.5/45.0) |
| 4.5739 | DmtMT3  | 275       | N5-glutamine methyltransferase | PrmC/Chlamydia trachomatis | AY600244.1 (24.2/34.6) |
| F-5123 | DmtMT2-1 | 294       | methyltransferase | MitM/ Streptomyces lavendulae | AAD28459.1 (36.7/49.8) |
|       | DmtMT2-2 | 275       | methyltransferase | UbiE/Escherichia coli | YP_026269.1 (16.4/25.3) |
Transparent Methods

Strains

Streptomyces Strains

S. youssoufensis OUC6819 strains were grown at 30 °C on R2YE agar medium. MS agar medium (3% soya flour, 2% mannitol, 2% agar powder) was used for the cultivation of Streptomyces sp. NRRL F-5123, S. aidingensis CGMCC 4.5739, and S. coelicolor M1146 strains. All the above Streptomyces strains were cultured in liquid TSBY medium (3% tryptic soya broth, 10.3% sucrose, 0.1% tryptone, 0.05% yeast extract) at 30 °C for genomic DNA extraction. For the DMTs production, the strains were incubated in the production medium (1% soluble starch, 2% glucose, 4% corn syrup, 1% yeast extract, 0.3% beef extract, 0.05% MgSO_4·7H_2O, 0.05% KH_2PO_4, 0.2% CaCO_3, and 3% bay salt, pH = 7.0), followed by incubation at 30 °C, 220 rpm for 7 days.

E. coli Strains

E. coli strains including DH5α, BL21, BW25113/pIJ790 and ET12567/pUZ8002 were cultivated at 37 °C in Luria–Bertani (LB) liquid medium or on LB agar. When necessary, the medium was supplemented with 50 μg/mL of apramycin, 25 μg/mL of chloramphenicol, 100 μg/mL of kanamycin, 100 μg/mL of hygromycin, or 50 μg/mL of ampicillin.

DNA Manipulation and Plasmid Constructions

Plasmid extractions and DNA purification were carried out using commercial kits (OMEGA, BIO-TEK). Chromosomal DNA isolation, restriction endonuclease digestion, ligation, and transformation were performed according to standard procedures (Sambrook et al., 1989) or manufacturer’s instructions.

For the expressions of dmtMT1, dmtMT2-1/2 and dmtMT3 in E. coli, the responding genes were amplified by polymerase chain reaction (PCR) using primer pairs listed in Table S2. dmtMT1, dmtMT2-1, and dmtMT3 were digested with Ndel and Xhol, ligated into the pET28a(+) resulting in pWLI628-630; while dmtMT2-2 was cloned into the EcoRI and Xhol sites of pET32a(+) resulting in pWLI631. After confirmation by sequencing, the resulting constructs pWLI628-630 were transformed into E. coli BL21 (DE3), and pWLI631 was transformed into E. coli BL21-CodonPlus (DE3).

For the combinatorial expressions of dmtMT1 and dmtMT2-1 in Streptomyces strains, the corresponding gene was put under the control of the constitutive promoter P_gapdh or P_hrdB. P_gapdh was amplified using primer pair of pGFP/3' phosphorylated pGRP (Table S2) and digested with NsiI; P_hrdB was amplified using primer pair of pHFP/3' phosphorylated pHRP (Table S2) and digested with KpnI. For expressions in OUC6819, dmtMT1 and dmtMT2-1 were respectively amplified with the primer pairs of dmtMT1EF/dmtMT1ER and dmtMT2-1-FP/dmtMT2-1-RP (Table S2) followed by digestion with XbaI; and then they were respectively ligated with P_gapdh, and cloned into the NsiI and XbaI sites of pSET152C to give pWLI634-635. For expressions in M1146, dmtMT1 was ligated with P_hrdB followed by insertion into the KpnI and XbaI sites of pIJ10500 to give pWLI636; the P_gapdh-dmtMT2-1 fragment was cloned into the Ndel and Xhol sites of pIJ10500 and pWLI636 to yield pWLI637-638. After confirmation by sequencing, the resulting plasmids pWLI636-638 were passed through E. coli ET12567/pUZ8002, and then introduced into S. coelicolor M1146/dmtB1C1 or S. coelicolor M1146/dmtA1B1C1 via conjugation (Kieser et al., 2000).

Protein Expression and Purification
The expressions of dmtMT1, dmtMT2-1, dmtMT2-2 and dmtMT3 followed the same protocol and were detailed as follows. Overnight culture of E. coli harboring the expression plasmid (10 mL) was inoculated into 1 L of LB medium (containing 50 μg/mL of kanamycin, or 25 μg/mL of chloramphenicol and 50 μg/mL of ampicillin) and grown at 37 °C, 220 rpm. Expression was induced at an OD600 of approximately 0.6 by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (with final concentration of 0.05 mM), and cultivation was continued for additional 16 hrs at 16 °C.

The cells were pelleted by centrifugation (15 min at 8,000 x g) and resuspended in 30 mL of binding buffer A (0.05 M Tris-HCl, 0.5 M NaCl, 5.0% glycerol (v/v), pH 7.5, containing cOmplete™ protease inhibitor cocktail). The resuspended cells were lysed by sonication in an ice-water bath with an ultrasonic processors VCX750 (Sonics & Materials Inc, PA, USA), and were centrifuged at 10,000 x g for 30 min at 4 °C. The resulting supernatant was applied to a HisTrap HP column (1 mL, GE Healthcare) and the His-tagged protein was eluted with a linear gradient of imidazole (30–500 mM) in the binding buffer using an ÄKTA Purifier system. After SDS–PAGE analysis, fractions containing pure protein were pooled, concentrated and exchanged to Tris buffer (0.025 M Tris-HCl, 0.02 M NaCl, and 10.0% glycerol, pH 7.5) by using Amicon Ultra-15 30-kDa cutoff centrifugal concentrator (Millipore).

In Vitro Assays

For in vitro experiments, each of the recombinant DmtMT1, DmtMT2-1/2, and DmtMT3 (10 μM) was incubated with 0.5 mM DKPs/pre-DMTs/DMTs (Sun et al., 2013) and 0.5 mM SAM in Tris buffer [50 mM Tris (pH 8.0) and 0.1 mM DTT] at 30 °C for 12 hrs. Reactions were stopped by the addition of equal volume of methanol and mixed by vortexing. For the detection of pre-DMTs/DMTs, the mixtures were subjected to HPLC analysis, using a YMC-Pack ODS-AQ C18 column (150 mm × 4.6 mm, particle size of 5 μm, pore size of 120 Å) under the program: phase A consisting of 0.1% (v/v) formic acid and ddH2O, phase B consisting of 0.1% (v/v) formic acid and acetonitrile; 50% B (0–5 min), 50% to 100% B (5–30 min), 100% B (30–45 min), at a flow rate of 1 mL min⁻¹ and UV detection at 300 nm. For the detection of DKPs, the program was set as follows: 10% B (0–5 min), 10% to 50% B (5–25 min), 100% B (25–35 min), at a flow rate of 1 mL min⁻¹ and UV detection at 280 nm. For probing substrate promiscuity of DmtMT1, different DKPs (0.5 mM) were tested as described above and were analyzed with HPLC-MS²; the enzymatic reactions were performed in triplicate, and all rates were calculated with their peak areas at 280 nm.

The assay of DmtMT1 with cWV was scaled up and subsequently subjected to a semi-preparative HPLC column (YMC-Pack ODS-AA C18 column, 120 Å, 250×10 mm, 5 μm) for purification. The resulting Me-cWV was stored at -20 °C until use. The enzymatic assay of DmtC1 was carried out in 50 mM Tris-HCl buffer (pH 8.0) with 2.5 mM MgCl₂, containing 10 μM DmtC1, 1 mM Me-cWV, and 0.2 mM FPP at 30 °C. After 1 hr, the reaction was quenched by the addition of equal volume of methanol and mixed by vortexing. The mixture was centrifuged at 17,000 x g for 20 min to remove proteins. The supernatant was then applied to YMC-Pack ODS-AQ C18 column with UV detection at 300 nm under the program: 10% B (0–5 min), 10% to 50% B (5–15 min), 80% to 100% B (15–25 min), at a flow rate of 1 mL min⁻¹ (phase A, 0.1% formic acid in ddH₂O; phase B, 100% acetonitrile supplemented with 0.1% formic acid).

Gene Inactivation, Complementation, and Overexpression in S. youssoufiensis OUC6819

Inactivation of dmtMT1 in S. youssoufiensis OUC6819 was performed using the REDIRECT Technology according to the literature protocol (Yao et al., 2018). The genomic library was screened using the primer pair of DmtMT1-FP/ DmtMT1-RP, giving positive cosmid pWL632. The aac(3)Iv-orT resistance cassette was amplified with primer dmtMT1MF/ dmtMT1MR (Table S2) using pJ773 (Gust et al., 2003) as template and was transformed into E. coli BW25113/pIJ790 containing pWL632 to replace an internal region of dmtMT1, resulting in
mutant cosmid pWL633. pWL633 was passed through E. coli ET12567/pUZ8002 and was then introduced into S. youssoufiensis OUC6819 by intergenic conjugation using mycelia as recipients. The mutant ΔdmtMT1 was selected by the apramycin-resistant and kanamycin-sensitive phenotype and was further confirmed by PCR using the primer pair of dmtMT1CF/dmtMT1CR (Table S2). For overexpression, pWL634-635, containing intact dmtMT1 and dmtMT2-1, were respectively passed through E. coli ET12567/pUZ8002 and introduced into the wild-type S. youssoufiensis OUC6819 via conjugation. pWL634 was further introduced into ΔdmtMT1 for genetic complementation.

Production and Analyses of DMTs

Spores of Streptomyces strains were incubated into 50 mL of production medium in 250 mL Erlenmeyer flasks fitted with glass beads, at 30 °C and 220 rpm for 7 days. The supernatants were extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were concentrated in vacuo to afford residue A. The precipitated mycelia were extracted twice with acetone. The extracts were combined, and acetone was evaporated in vacuo to yield residue B. The combined residues were dissolved in methanol and filtered through a 0.2 μm filter. The resulting fermentation products derived from ΔdmtMT1, genetic complementation and overexpression strains were subjected to HPLC analysis, eluting with a linear gradient of B/A in 40 min (phase A, 0.1% formic acid in ddH2O; phase B, 100% acetonitrile supplemented with 0.1% formic acid; flow rate: 1 mL min⁻¹; wavelength: 300 nm) using a YMC-Pack ODS-AQ C18 column. The fermentation products of heterologous expression strains were detected under the identical conditions used for analyzing the DmtMT2-1-catalyzed reactions using pre-DMTs/DMTs as substrates.

Isolation and Characterization of the Methylated pre-DMTs and DMTs

For isolation of DmtMT2-1 enzymatic products, the in vitro assays contained pre-DMTs/DMTs (0.5 mM) and DmtMT2-1 (30 μM) were scaled up to 30 mL. The reactions were extracted three times with an equal volume of ethyl acetate and concentrated in vacuo. The extracts were further purified by eluting with linear gradient from 80 to 100% B/A (phase A: ddH2O; phase B: acetonitrile, 1.5 mL min⁻¹; UV detection at 300 nm) in 40 min using a semi-preparative HPLC column (YMC-Pack ODS-AA C18 column, 120 Å, 250×10 mm, 5 μm). For isolation of pre-DMT F (13), the reaction consisted of cWV (0.5 mM), SAM (1 mM), FPP (0.5 mM), DmtMT1 (50 μM), DmtC1 (30 μM), and MgCl2 (2.5 mM) in Tris-HCl buffer (50 mM, pH 8.0) was performed, the mixture was treated in the same way as that of the DmtMT2-1-catalyzed reactions.

To isolate compound 16, The S. youssoufiensis OUC6819 expressing dmtMT2-1 was fermented in a total volume of 15 L. The fermentation cultures were treated as described above. The residues were applied to reversed-phase C18 open column, eluting with a gradient eluent of 20%–100% methanol to give five fractions (Fr.1~Fr.5) for each fermentation culture. Compound 16 was obtained by further separation of the Fr.4, eluting with the identical program used for isolation of DmtMT2-1 enzymatic products. The structures of the above compounds were characterized by HR-ESI-MS carried out on Thermo LTQ Orbitrap XL mass spectrometer, and NMR spectroscopy recorded with Bruker Avance III 600 spectrometers. All spectra were processed with MestReNova.6.1.0 (Metrelab), and chemical shifts were referenced to those of the solvent DMSO-d6 signals.

Bioinformatic Analysis

ORF assignments and their proposed functions were accomplished by using FramePlot4.0 beta (Ishikawa and Hotta, 1999) (http://nocardia.nih.go.jp/fp4). Sequence comparisons and database searches were accomplished with BLAST programs (McGinnis and Madden, 2004) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 71 CDPS-associated MTs listed in Data S3 were extracted from the NCBI database (https://www.ncbi.nlm.nih.gov/protein); and Enzyme Function
Initiative-Enzyme Similarity Tool (EFI-EST) (Gerlt et al., 2015) was used to construct the sequence similarity network using an alignment score of 30. The network was visualized in Cytoscape (Shannon et al., 2003).

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