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Structural and Functional Studies of a *gem*-Dimethylating Methyltransferase from a *trans*-Acyltransferase Assembly Line

Jessica L. Meinke[†], M. Rachel Mehaffey[‡], Drew T. Wagner[†], Ningze Sun[†], Zhicheng Zhang[‡], Jennifer S. Brodbelt[‡], and Adrian T. Keatinge-Clay^{*†}

[†]Department of Molecular Biosciences, The University of Texas at Austin, Austin, Texas 78712, United States

[‡]Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712, United States

Abstract

The methyl substituents in products of *trans*-acyltransferase assembly lines are usually incorporated by S-adenosyl-methionine (SAM)-dependent methyltransferase (MT) domains. The *gem*-dimethyl moieties within the polyketide disorazol are installed through the iterative action of an MT in the third module of its assembly line. The 1.75-Å-resolution crystal structure of this MT helps elucidate how it catalyzes the addition of two methyl groups. Activity assays of point mutants on β -ketoacyl chains linked to an acyl carrier protein and *N*-acetylcysteamine provide additional insights into the roles of active site residues. The replacement of an alanine with a phenylalanine at an apparent gatekeeping position resulted in more monomethylation than dimethylation. MTs may form an interface with ketoreductases (KRs) and even mediate the docking of *trans*-acyltransferase assembly line polypeptides through this association.

Graphical Abstract

*Corresponding Author: adriankc@utexas.edu.

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ASSOCIATED CONTENT

Supporting Information

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Figures S1–S5 and Tables S1 and S2 (PDF)

Accession Codes

The DisMT3 coordinates have been deposited in the Protein Data Bank under accession code 6CCA.

Polyketides are bioactive secondary metabolites produced by diverse bacteria through the action of molecular factories called modular polyketide synthases (PKSs).^{1–4} The chemistry of a polyketide is determined by the types of modules within its assembly line. In order for a module to catalyze a two-carbon elongation of a polyketide intermediate, an acyltransferase (AT) that selects an α -carboxyacyl extender unit, a ketosynthase (KS) that performs the decarboxylative condensation of the extender unit to a growing chain, and an acyl carrier protein (ACP) that covalently shuttles polyketide intermediates between domains are required.¹ Combinations of processing enzymes, such as a ketoreductase (KR), dehydratase (DH), and enoylreductase (ER), may also be present within the module to afford functionality such as a β -hydroxyl group, an α/β -double bond, or a β -methylene.

Assembly line installation of methyl groups on the α -carbon of polyketide intermediates is accomplished through two routes. In *cis*-AT assembly lines, which harbor embedded AT domains, it is primarily by condensation with a methylmalonyl extender unit.¹ In *trans*-AT assembly lines, which rely on separately encoded, malonyl-specific ATs, it is primarily by the transfer of methyl groups by C-methyltransferases (MTs) from S-adenosylmethionine (SAM) to β -ketoacyl-ACP substrates.² MT domains are present in ~2% of *cis*-AT assembly lines and ~80% of *trans*-AT assembly lines;^{2,3} they are also commonplace in iterative PKSs.

Four representatives of these MTs have been structurally elucidated—two catalytically inactive MTs from the porcine and human fatty acid synthases (FASs; PDBs 2VZ8, 4PIV),^{5,6} the MT from the citrinin iterative PKS (CitMT, PDB 5MPT),⁷ and the MT from the eighth module of the curacin *cis*-AT assembly line (CurMT8, PDB 5THZ; updated nomenclature is used throughout this manuscript).^{8–10} They are class I MTs that possess an N-terminal lid subdomain and a C-terminal SAM-binding, Rossmann-fold subdomain. The MTs from the mammalian FASs and the curacin assembly line are inserted into the structural subdomain of KR (KR_s) after its first β -strand. Domain–domain contact with KR_s is extensive within both the porcine and human FASs and thought to be similar within the curacin synthase.¹⁰ Within *trans*-AT assembly lines, MT domains are C-terminal to KR domains; however, KRs and MTs may associate here too as docking interactions seem to be mediated by KRs at the C-terminal ends of upstream polypeptides and MTs at the N-terminal ends of downstream polypeptides.¹¹

The structures of CitMT and CurMT8 have provided clues for how methyl groups are transferred to β -ketoacyl substrates. An invariant glutamate/histidine pair is prominent within both active sites. This histidine has been proposed to serve as a general base that enables the α -carbon to nucleophilically attack the SAM methyl group.^{7,10} Bound S-adenosylhomocysteine (SAH) molecules reveal how SAM is positioned during catalysis. Previous work from both *cis*- and *trans*-AT assembly lines demonstrated that MTs generally operate on β -ketoacyl-ACPs instead of malonyl-ACPs and that the methyl group is installed with a D-orientation.^{12–15}

The features that control whether zero, one, or two methyl groups are transferred to a substrate are also unknown. During citrinin biosynthesis, CitMT transfers a methyl group to the diketide, triketide, and tetraketide intermediates but not to the final pentaketide intermediate.⁷ Most characterized *trans*-AT PKS assembly lines contain monomethylating

MTs. The calyculin, diaphorin, disorazol, elansolid, kirromycin, onnamide, oxazolomycin, pederin, phormidolide, psymberin, and rhizopodin each contain one *gem*-dimethylating MT, while the bryostatin assembly line contains two *gem*-dimethylating MTs (Figure 1).^{2,3,16} Except in the bryostatin and calyculin assembly lines, *gem*-dimethylating MTs are associated with a KR domain that operates only after two methyl groups have been transferred. Recent bioinformatic analysis on *trans*-AT KRs revealed that KRs associated with MTs clade separately from KRs not associated with MTs, suggesting that an MT-associated KR may be more active on a β -ketoacyl intermediate after one or two methyl groups have been transferred to its α -carbon.⁹

Here, we report the 1.75 Å-resolution crystal structure of the *gem*-dimethylating MT from the third module of the *trans*-AT assembly line that *Sorangium cellulosum* So ce12 utilizes to biosynthesize the antimitotic polyketide disorazol (DisMT3; Figure 1).^{2,17} DisMT3 is the first MT from a *trans*-AT assembly line as well as the first *gem*-dimethylating MT to be structurally determined. Assays of active site point mutants toward β -ketoacyl chains bound to both ACP and *N*-acetylcysteamine (NAC) provide insight into how the iterative transfer of methyl groups is accomplished. Modeling of the substrate and monomethylated intermediate in the active site show the positioning required for each methylation. How MT and KR domains associate to dock adjacent assembly line polypeptides is also discussed.

RESULTS AND DISCUSSION

Domain Boundaries and Structure Solution.

In contrast to the MT domains of *cis*-AT assembly lines or mammalian FASs that are embedded within the KR domain, the MT domains of the *trans*-AT assembly line are completely C-terminal to KR domains.^{5,12,13} The boundaries of DisKR3, DisMT3, and DisACP3 were hypothesized through sequence alignments and known structures of KRs and ACPs (Figure S1).^{11,18–20} The DNA encoding the putative MT domain (His3097 to Ala3498 of DisA) was then amplified and cloned into the expression vector pET28b. DisMT3 was expressed in *E. coli* BL21(DE3) cells, purified by nickel affinity and size-exclusion chromatography, and crystallized. The 1.75-Å-resolution structure of DisMT3 (numbering beginning with the first visible residue, equivalent to Ala3099 in DisA) was solved by molecular replacement using CurMT8 (PDB 5THZ) as a search model (Table S1).^{10,21}

Overall Structure.

The model of DisMT3 contains 392 contiguous residues that form an N-terminal lid subdomain and a SAM-binding core subdomain like CurMT8 and CitMT (1.76 and 1.74 Å C_α RMSD, respectively; Figures 2 and S2). The lid (residues 23–173) is comprised of eight helices as well as a three-stranded, antiparallel β -sheet that has so far only been observed in MTs from assembly lines and iterative PKSs (the lid from the catalytically inactive MT of the mammalian FAS is half the size, and the lid of CitMT has one fewer helix than CurMT8 and DisMT3). The core subdomain (residues 174–392) possesses a Rossmann fold that contains motifs I–VI of class I MTs.^{22,23} Following its fifth β -strand, a large insertion termed the “core insertion” plays a significant role in forming the active site.¹⁰ The N-terminal 22 residues of DisMT3 form loops and a helix that interact with the core

subdomain; the interactions made by these first residues in DisMT3, CurMT8, and CitMT are distinct from one another.

The Active Site.

Neither SAM nor SAH were observed in the DisMT3 active site even though crystals were grown in 1 mM SAM (crystals soaked in 3 mM sinefungin were also unbound). Thus, the uncomplexed structure of DisMT3 was compared with the binary complex of CurMT8 (bound with SAH) and the ternary complex of CitMT (bound with SAH and an unknown substrate mimic).^{7,10} Through a superposition with the CitMT structure, SAM was modeled into the DisMT3 active site (Figures 2a and S2a). In the CitMT structure, a highly conserved threonine in Motif I forms a hydrogen bond with the SAH carboxylate, while in CurMT8 structures the residue does not consistently form the same interaction. The equivalent threonine of DisMT3 (Thr213) is not positioned to form a hydrogen bond with the SAM carboxylate. A highly conserved active site tyrosine (Tyr170 in DisMT3) possesses high B-factors in both unbound DisMT3 and SAH-bound CurMT8 yet is well-ordered in CitMT. Thus, the active site of an MT may not be ordered for catalysis until both SAM and a substrate are bound.

The histidine/glutamate dyad of DisMT3 (His288/Glu314) is structurally equivalent to the dyads of CurMT8 and CitMT, with the glutamate likely perturbing the pK_a of the histidine (Figure 2). A similar dyad is thought to help prepare the enolate of acetyl-coenzyme A (CoA) for an attack on α -ketoglutarate in the *Saccharomyces cerevisiae* homocitrate synthase.²⁴ In serine proteases, the pK_a of the active site histidine is 7.5 due to a neighboring aspartate and is elevated to 10–12 as an oxyanion develops during catalysis.²⁵ Thus, a positively charged histidine may bind the enolate of the β -ketoacyl substrate and help position it for an attack on the SAM methyl group. As seen in the ternary complex of CitMT, Tyr170 is likely to be positioned next to one of the CH bonds of the SAM methyl group to stabilize the transition state that forms as the methyl group migrates to the α -carbon of the substrate.²⁶

A large tunnel in DisMT3 can accommodate the tail of the polyketide intermediate. Hydrophobic side chains lining it include Tyr132, Met166, Leu169, and Tyr170 from the lid; Met315 from the core; as well as Phe321, Leu322, Ala326, and Trp322 from the core insertion (Figure 2a). While the DisMT3 substrate can fit into this cavity, many substrates for assembly line MTs are significantly longer and would likely need to double-back toward the phosphopantetheinyl arm.

DisMT3 Activity Assays.

The activity of the isolated DisMT3 domain was evaluated through *in vitro* assays. β -Ketobutyryl-DisACP3, generated using β -ketobutyryl-CoA and Sfp (the surfactin phosphopantetheinyl transferase²⁷), was incubated with DisMT3 and SAM. Combined liquid chromatography and ultraviolet photodissociation tandem mass spectrometry (UVPD) was then used to quantify and locate transferred methyl groups (Figure 3).²⁸ The reaction with wild-type DisMT3 yielded 77% of the anticipated *gem*-dimethylated product (as well as 4% monomethylated and 2% unmethylated products and 17% hydrolyzed byproduct).

Reactions with malonyl-DisACP3, generated using malonyl-CoA and Sfp, did not yield any *gem*-dimethylated product (although 4% monomethylated product was apparently formed), reaffirming that the preferred substrates of MTs from *trans*-AT assembly lines are β -ketoacyl-ACPs (Figure S3).^{12–15} UVPD analysis revealed that all modifications are attached to the phosphopantetheinylated serine (Figure S4).^{1,9}

The contributions of active site residues toward catalysis were evaluated with the point mutants Y170F, N285A, H288A, H288N, E314A, and E314Q (Figure 3). While the Y170F, N285A, E314A, and E314Q mutants yielded amounts of *gem*-dimethylated product similar to wild-type DisMT3, the His288 mutants were significantly impaired. The H288A mutant yielded 12% *gem*-dimethylated and 5% monomethylated product, and the H288N mutant gave just 2% *gem*-dimethylated and 4% monomethylated product. The CurMT8 and CitMT point mutants that showed the greatest abrogation of methylation activity were those with substitutions to the active site histidine.

Additional enzymatic assays were performed on the small molecule mimic β -ketopentanoyl-*S*-*N*-acetylcysteamine (SNAC). Wild-type DisMT3 did not yield any *gem*-dimethylated product but did produce some monomethylated product, α -methyl- β -ketopentanoyl-SNAC (Figure S5). Only the Y170F point mutant generated detectable levels of the monomethylated product. The absence of *gem*-dimethylation in these assays suggests that the interaction between these small molecule mimics and DisMT3 provides insufficient binding energy to catalyze the second methylation reaction. Thus, protein–protein interactions between the MT and ACP domains may greatly aid in completing the transfer of both methyl groups. Perhaps because small molecules rely more on interactions with active site residues, alterations to the active site through the N285A, E314A, and E314Q substitutions resulted in a greater loss of activity toward the SNAC-bound substrate than the ACP-bound substrate.

Association of MTs and KRs.

Assembly lines are split into several polypeptides that noncovalently associate. To accomplish this, the polypeptides of *trans*-AT assembly lines commonly utilize split domains located partially on the upstream polypeptide and partially on the downstream polypeptide. Such docking interactions include a four-helix bundle formed by two helices on each polypeptide and a DH domain formed by ~10 residues on the upstream polypeptide with the remainder on the downstream polypeptide.^{11,19,29,30} Since many *trans*-AT assembly lines possess polypeptide disconnections between KR and MT domains, a similar docking interaction is likely to exist between the KR of upstream polypeptides and the MTs of downstream polypeptides.

In both the porcine and human FASs, the KR domain and the catalytically inactive MT domain form a large interface (Figure 4). The first helix of the core subdomain of MT is inserted into a large groove on the KR surface, and its side chains make ionic and polar contacts with complementary KR side chains (Figure 4a). The same interface has been proposed for the MT/KR interactions within *cis*-AT assembly lines because of the similarity of CurMT8 with the catalytically inactive MTs of mammalian FASs and its equivalent insertion into the KR domain immediately following its first β -strand (Figure 4a and b).^{4,10}

Because the MT domains of *trans*-AT assembly lines are C-terminal to KR domains, one might expect a docking interface that is distinct from the mammalian FAS and *cis*-AT assembly lines. However, modeling reveals that the end of the KR domain is close to the beginning of the MT domain.^{5,6} In *trans*-AT assembly lines, the number of residues between KR and MT is the same whether the domains are embedded in the same or separate polypeptides (Figure 4c). The C-terminus of an upstream polypeptide may often provide the N-terminal residues of the downstream MT domain. However, the seventh module of the difficidin assembly line and the 11th module of the elansolid assembly line reveal this is not always the case. Thus, domain–domain interactions may make the major contribution to KR/MT docking and facilitate the noncovalent assembly of split modules (Figure 4d).^{5,6,10}

KRs from *trans*-AT modules containing an MT form a clade separate from KRs from modules not containing an MT⁹ [fingerprint differences include YAAG(C/I) vs YAAAN and WG(F/Y)W_x₅ vs WPxW_{xx}GGM].^{18,19} These KRs may contain structural elements that interface with the MT domain. They may also react faster on α -substituted β -ketoacyl intermediates compared with α -unsubstituted β -ketoacyl intermediates, thus promoting methylation prior to reduction. Within *cis*-AT assembly lines MT-associated KRs often contain histidine in place of the catalytic tyrosine.⁴

Model for Mono- and Dimethylation.

The following mechanism is proposed for how a *trans*-AT assembly line MT adds a methyl group to the *re* face of an α -unsubstituted, β -ketoacyl substrate to generate a D-oriented α -methyl substituent (Figure 5).¹⁴ Within the active site the substrate tautomerizes between its keto and enol forms (similar dicarbonyl compounds enolize to a greater extent in less polar environments).^{31,32} The active site histidine deprotonates the enol form of the substrate to generate the enolate. With the enolate and the imidazolium of the histidine forming a salt bridge (the decreased activity of the H288A and H288N mutants highlight the importance of this interaction), the *re* face of the α -carbon is positioned for an attack on the methyl group of SAM to generate a monomethylated D- α -methyl- β -ketoacyl product (Figure 5a). Transfer of the methyl group is facilitated by a CH–O bond between a conserved active site tyrosine (Tyr170 in DisMT3) and the SAM methyl group; the backbone carbonyl of an active site asparagine (Asn285 in DisMT3) may also form CH–O bonds with the methylenes neighboring the SAM sulfonium.²⁶ The methylated acyl chain and SAH then exit the active site.

gem-Dimethylating MTs like DisMT3 bind another molecule of SAM and the monomethylated product of the first reaction to transfer a second methyl group (Figure 5a). The enol form of the α -methyl- β -ketoacyl substrate is deprotonated by the active site histidine (the methyl group of the keto form would clash with the SAM methyl group). The increased pK_a of the monomethylated substrate compared to the unmethylated substrate (10.6 vs 9.0) may have an impact on kinetics.³³ Within monomethylating MTs, sterics may be the most significant factor inhibiting a second methylation, as a clash would form between the methyl group of an α -methyl, β -ketoacyl enolate, and an invariant phenylalanine in the core insertion of these MTs (CurMT8 and CitMT also possess this residue). This predicted clash would not occur in DisMT3 as the equivalent residue is an

alanine (Ala326 in DisMT3). *gem*-Dimethylating MTs contain significant differences from core insertion residues that are invariant in monomethylating MTs (Figure 5b). The most obvious fingerprints are substitutions within the core insertion of the aforementioned phenylalanine, the threonine immediately upstream, and the tryptophan seven residues downstream. To test the hypothesis that a phenylalanine in place of Ala326 can gatekeep to prevent a second methylation, the A326F point mutant was generated and assayed (Figure 3). Reactions with β -ketobutyryl-DisACP3 yielded 60% greater monomethylation than dimethylation, indicating that in monomethylating MTs this phenylalanine residue helps prevent iterative methylation.

CONCLUSION

The 1.75-Å-resolution structure of DisMT3 reveals the architecture of MTs from *trans*-AT assembly lines as well as *gem*-dimethylating MTs for the first time. Accompanying assays of point mutants demonstrate the importance of active site residues toward catalysis and specificity. Binding modes for β -ketoacyl substrates and roles of residues during catalysis are proposed. Fingerprint residues of *gem*-dimethylating MTs are appropriately positioned on the core insertion to enable binding of the singly methylated intermediate. KRs that accompany MTs belong to a unique clade of KRs that may be catalytically delayed relative to methyltransfer. A significant interface may be formed between these KRs and the downstream MTs, even across polypeptide disconnections to mediate docking.

METHODS

Cloning, Expression, and Purification.

DNA encoding the DisMT3 domain was amplified from *S. cellulorum* So ce12 genomic DNA with primers DW490 and DW495, gel extracted, and Gibson-assembled (New England Biolabs) into the pET28b expression vector (all primer sequences are in Table S2). N-terminally His₆-tagged DisMT3 was expressed in *E. coli* BL21(DE3) (6 L of LB media), grown to an OD₆₀₀ of 0.6 at 37 °C, induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside, and shaken overnight at 15 °C. Protein was purified from cell lysate using affinity chromatography (HisPur nickel-NTA resin, Thermo Scientific) and gel filtration chromatography (GE Superdex 200 Increase 10/300 column equilibrated in 150 mM NaCl and 15 mM HEPES at pH 7.5). DisMT3 was concentrated to 5 mg mL⁻¹ and flash-frozen in 100 μ L aliquots. Point mutations of DisMT3 were generated following standard site-directed mutagenesis techniques. DNA encoding DisACP3 was amplified as described for DisMT3 using primers DisACP-FWD and DisACP-REV. DisACP3 and DisMT3 mutants were expressed and purified in the same fashion as wild-type DisMT3.

Crystallization.

After adding 1 mM SAM to 5 mg mL⁻¹ DisMT3 for 15 min, 2 μ L of this mixture was added to 1 μ L of 20% (w/v) PEG 6000 and 0.1 M Bicine (pH 8.5). Crystals grew between 2 days and 4 months by sitting drop diffusion. They were cryoprotected in 20% (w/v) PEG 6000, 20% (v/v) glycerol, and 0.1 M Bicine (pH 8.5) prior to flash freezing in liquid nitrogen. Diffraction was obtained at ALS Beamline 5.0.3. The structure was solved using molecular

replacement in Phaser with CurMT8 (PDB 5THZ) as a search model and refined using Refmac, ARP/wARP, and Phenix Refine.^{21,34–36} DisMT3 crystallized in P2₁ with one molecule per asymmetric unit. A resolution cutoff of 1.75 Å was selected to keep CC1/2 above 0.70 in the highest resolution bin.

Activity Assays.

Acyl-*S*-phosphopantetheinylation of DisACP3 was conducted with 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 2 nM Sfp, 300 μM DisACP3, and either 500 μM acetoacetyl-CoA or malonyl-CoA at 22 °C for 2 h. Following the acylation reaction, 50 μM acylated DisACP3 was incubated with 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 10 mM SAM, and 50 μM DisMT3 for 18 h at 22 °C. Protein solutions were desalted and concentrated to 10 μM in 50% (v/v) acetonitrile with 0.1% formic acid using a 3 kDa molecular weight cutoff filter device (MilliporeSigma, Burlington, MA). Solutions were loaded into Aucoated borosilicate emitters and infused with a nano-ESI source or introduced by chromatographic separation using a Dionex UltiMate 3000 RPLC nanoLC system integrated with a nano-ESI source. A solvent gradient of 15% to 55% B over 45 min at a flow rate of 300 nL min⁻¹ using water (mobile phase A) and acetonitrile (mobile phase B), each containing 0.1% formic acid, was used for chromatographic separations. Agilent PLRP-S resin (5 μm, 1000 Å pore size) was used to pack in-house a trap column (3 cm × 100 μm) and analytical column with an integrated emitter (20 cm × 75 μm). A Thermo Fisher Scientific Orbitrap Elite mass spectrometer (Bremen, Germany) modified to perform UVPD in the higher-energy collisional dissociation cell with a Coherent Excistar 193 nm ArF excimer laser (Santa Cruz, CA) was used for all mass spectrometry experiments. UV photoactivation was achieved using one pulse at 2 mJ per pulse. MS1 and MS/MS spectra (60–500 scans) were acquired at 240 K resolving power (at *m/z* 400). Xtract (Thermo Fisher Scientific) and ProSight Lite v1.4 were used to process MS/MS spectra and identify fragment ions.³⁷ Extracted ion chromatogram peak areas were divided by the sum of the observed areas for the species of interest and reported as the normalized abundance.

Small molecule activity assays with 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM β-ketopentanoyl-SNAC, 30 mM SAM, 5% (v/v) DMSO, and 50 μM DisMT3 were incubated for 18 h at 22 °C. Post-incubation, reactions were thrice extracted with 100 μL of ethyl acetate, evaporated, and resuspended in 50 μL of methanol. From this solution, 20 μL was diluted into 1.5 mL of 5% methanol for LCMS analysis (Agilent 1260 HPLC with an Agilent 6120 Quadrupole APCI/ ESI instrument in positive mode; ZORBAX Eclipse Plus 95 Å C18 column, 2.1 mm × 50 mm, 5 μm; column temperature of 40 °C; flow rate of 0.7 mL min⁻¹; gradient from 0 to 12 min of 5 to 100% methanol and from 12 to 15 min of 100% methanol). β-Ketopentanoyl-SNAC and the α-methyl-β-ketopentanoyl-SNAC standard were synthesized as previously reported.³⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *gem*-Dimethylation by MTs in *trans*-AT assembly lines. (A) DisMT3 from the third module of the disorazol assembly line twice methylates the α -carbon of a triketide intermediate before the accompanying KR reduces its β -keto group. (B) Products of *trans*-AT assembly lines show the methyl groups, marked with red circles, incorporated by MTs. Some MTs operate iteratively to install *gem*-dimethyl moieties indicated with “2x.”

dyad as well as a highly conserved tyrosine are indicated with circles, while residues that help form the substrate tunnel are indicated with squares. DisMT3, disorazol, *Sorangium cellulosum* So ce12, CAI43932; BaeMT10, bacillaene, *Bacillus amyloliquefaciens* FZB42, CAG23959; DifMT2, difficidin, *Bacillus amyloliquefaciens* FZB42, CAG23977; CurMT8, curacin, *Moorea producents* 3L, AEE88280; GphMT4, gephronic acid, *Archangium violaceum* Cb vi76, AHA38200; CitMT, citrinin, *Monascus purpureus*, BAD44749.

Figure 3.

Functional assays of DisMT3 and point mutants. Deconvoluted MS1 spectra of DisACP3 constructs were obtained by averaging over each LCMS elution profile. Roles of active site residues were analyzed with point mutants Y170F, N285A, H288A, H288N, E314A, E314Q, and A326F. The mutation of His288 to either alanine or asparagine resulted in a large decrease in *gem*-dimethylation, indicating that this residue plays a key role. The A326F mutant yielded a greater percentage of monomethylation compared to *gem*-

dimethylation. The bar graph shows the normalized abundance of each species (error bars from duplicate experiments).

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Figure 4.

Association of MT and KR domains. (A) Domain/domain interactions may be similar for each of the discussed synthases. Stereodiagrams show the structure of the MT+KR fragment of the human FAS (PDB 4PIV) and models for the association of MT and KR within *cis*-AT and *trans*-AT assembly lines based on the mammalian FAS. Dotted lines indicate the expected connectivity of linkers between the two domains. (B) While the MT domain is completely C-terminal to the KR domain in *trans*-AT assembly lines, it is embedded within the KR domain (after its $\beta 1$) in *cis*-AT assembly lines and mammalian FASs. The location that the ER domain inserts into the FAS KR domain is shown. (C) The KR and MT domains of *trans*-AT assembly lines occur either in the same polypeptide (“together”) or in adjacent polypeptides (“split”). Initiating methionines are highlighted green to mark polypeptide disconnections. Split modules often also contain DH and ER domains. (D) A stereodiagram suggests how KR and MT make contact in the context of a module, either on the same polypeptide or split between polypeptides. Spheres indicate cofactors of KR and MT, the phosphopantetheinyl arm of ACP, and the reactive cysteine of KS. BaeMT15, bacillaene, *Bacillus amyloliquefaciens* FZB42, CAG23961; BasMT2/BasMT3, basiliskamide, *Brevibacillus laterosporus* PE36, ERM18798/ERM18797; BonMT3/BonMT10, bongkrekiec

acid, *Burkholderia gladioli*, AFN27480/AFN27483; CalMT24/CalMT25, calyculin, *Candidatus Entotheonella*, BAP05595; DifMT7/DifMT14, difficidin, *Bacillus amyloliquefaciens* FZB42, CAJ57409/CAG23983; DisMT3, disorazol, *Sorangium cellulosum* So ce12, CAI43932; ElaMT11, elansolid, *Chitinophaga sancti*, AEC04363; EtnMT8, etnangien, *Sorangium cellulosum* So ce56, CAN93349; LumMT7, luminaolide, *Planktothrix paucivesiculata* PCC 9631, AKQ22682; OocMT13, oocydin, *Serratia marcescens*, AFX60318; PedMT8, pederin, symbiont bacterium of *Paederus fuscipes*, AAS47564; PhmMT4, phormidolide, *Leptolyngbya* sp. ISBN3-Nov-94-8, AMH40421; RhiMT7, rhizoxin, *Paraburkholderia rhizoxinica*, CAL69890; SorMT15, sorangicin, *Sorangium cellulosum* So ce12, ADN68480.

Figure 5.

Models for mono- and dimethylation. (A) Stereodiagrams and a reaction scheme suggest how DisMT3 catalyzes the first and second methylations of its triketide substrate. His288 may be positively charged and form a salt bridge with the enolate of the substrate. The *re* face of the α -carbon can acquire the methyl group of SAM to generate a D-oriented methyl group in the product. The side chain of Tyr170 and the backbone carbonyl of Asn285 may form CH–O hydrogen bonds with SAM to help catalyze methyltransfer. Residues from the CitMT ternary complex structure are shown in transparent sticks—the Thr213 equivalent shifts to contact the SAM carboxylate, the Tyr170 equivalent shifts to contact the SAM methyl group, and the Ala326 equivalent is a phenylalanine that may sterically prevent a second methyltransfer. If MTs catalyze enolization, it may be through the cooperation of water molecules and His288. (B) Residues in the core insertion, especially those in the positions of Ser325, Ala326, and Trp333, are often different in dimethylating MTs compared to monomethylating MTs. SmdMT6, 9-methylstreptimidone, *Streptomyces himastatinicus* ATCC 53653, CCC21123; AtcMT8, anthracimycin, *Streptomyces* sp. T676, CTQ34882; BaeMT15, bacillaene, *Bacillus amyloliquefaciens* FZB42, CAG23961; BasMT2/BasMT3, basiliskamide, *Brevibacillus laterosporus* PE36, ERM18797/ERM18798; BatMT2, batumin, *Pseudomonas fluorescens*, ADD82939; BryMT6/BryMT11, bryostatin, *Candidatus Endobugula sertula*, ABM63527/ABM63528; CalMT18/CalMT25, calyculin, *Candidatus Entotheonella*, BAP05593/BAP05595; ChiMT3/ChiMT4/ChiMT9/ChiMT14/ChiMT15/ChiMT18, chivosazol, *Sorangium cellulosum* So ce56, AAY89049/AAY89050/AAY89052/AAY89053; CorMT13, coralloporynin, *Corallococcus coralloides*, ADI59534; ChxMT6, cycloheximide, *Streptomyces* sp. YIM 56141, AFO59866; DipMT2/DipMT8, diaphorin, *Candidatus Proffittella armatura*, AGS06823/AGS06887; DifMT2/DifMT7, diffidicin,

Bacillus amyloliquefaciens FZB42, CAG23977/CAJ57409; DisMT3, disorazol, *Sorangicin cellulosum* So ce12, CAI43932; ElaMT3/ElaMT4/ElaMT11, elansolid, *Chitinophaga sancti*, AEC04357/AEC04363; EtnMT8/EtnMT19, etnangien, *Sorangium cellulosum* So ce56, CAN93349/CAN93352; KirMT4/KirMT9, kirromycin, *Streptomyces collinus* Tu 365, CAN89632/CAN89634; LtmMT6, lactimidomycin, *Streptomyces amphibiosporus*, ACY01401; MgsMT6, migrastatin, *Streptomyces platensis* subsp. rosaceus, ACY01391; MisMT18, misakinolide, *Candidatus Entotheonella sarta*, AKQ22696; MmpMT2/MmpMT4, mupirocin, *Pseudomonas fluorescens* NCIMB 10586, AAM12913; NspMT2/NspMT7, nosperin, *Nostoc* sp. ‘*Peltigera membranacea cyanobiont*,’ ADA69237/ADA69239; OnnMT2/OnnMT8, onnamide, symbiont bacterium of *Theonella swinhoei*, AAV97870/AAV97877; OzmMT6/OzmMT7, oxazolomycin, *Streptomyces albus*, ABS90475/ABS90470; PtzMT12/PtzMT14/PtzMT18, patellazole, *Candidatus Endolissoclinum faulkneri* L5, AFK83819/AHC73995; PedMT8, pederin, symbiont bacterium of *Paederus fuscipes*, AAS47564; PhmMT4/PhmMT6, phormidolide, *Leptolyngbya* sp. ISBN3-Nov-94–8, AMH40421; RizMT3/RizMT9, rhizopodin, *Stigmatella aurantiaca* Sg a15, CCA89326; RhiMT5/RhiMT7, rhizoxin, *Paraburkholderia rhizoxinica*, CAL69889/CAL69890; SorMT4/Sor15/SorMT18, sorangicin, *Sorangium cellulosum* So ce12, ADN68476/ADN68480/ADN68482; Fr9MT9, spliceostatin, *Burkholderia* sp. FERM BP-3421, AIC32693; VirMT2, virginiamycin, *Streptomyces virginiae*, BAF50727.