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Molecular Basis for Autocatalytic Backbone *N*-Methylation in RiPP Natural Product Biosynthesis

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Abstract

N-Methylation of nucleic acids, proteins and peptides is a chemical modification with significant impact on biological regulation. Despite the simplicity of the structural change, *N*-methylation can influence diverse functions including epigenetics, protein complex formation, and microtubule stability. While there are limited examples of *N*-methylation of the α -amino group of bacterial and eukaryotic proteins, there are no examples of catalysts that carry out the post-translation methylation of backbone amides in proteins or peptides. Recent studies have identified enzymes that catalyze backbone *N*-methylation on a peptide substrate, a reaction with little biochemical precedent, in a family of ribosomally synthesized natural products produced in basidiomycetes. Here, we describe the crystal structures of *Dendrothele bispora* dbOphMA, a methyltransferase that catalyzes multiple *N*-methylation on the peptide backbone. We further carry out biochemical studies of this catalyst to determine the molecular details that promote this unusual chemical transformation. The structural and biochemical framework described here could facilitate biotechnological applications of catalysts for the rapid production of backbone *N*-methylated peptides.

INTRODUCTION

N-methylation of biological molecules is one of the simplest chemical modifications, but has wide-ranging impact on the modulation of diverse processes including epigenetics,¹ chromosomal segregation,² and cell cycle progression,³ among others. In the modification of protein substrates, *N*-methylation most commonly occurs on side chains,^{4–6} although there are some examples of bacterial and eukaryotic proteins that are modified at the α -amino terminus.^{7, 8} Examples of the latter include the Ran nucleotide-exchange factor RCC1.⁹ Deficiencies in methylation can alter the regulatory activities of eukaryotic proteins¹⁰ which may be correlated with mitotic defects, as observed in the transcription factor TAF-I, and the

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Supporting Information

For experimental details regarding efforts to establish in trans and in vitro activity, and the sequence similarity network analysis please refer to the Supporting Information. The structure factors and coordinates have been deposited in the Protein Data Bank with the accession code (XXX).

retinoblastoma protein Rb.^{11, 12} Each of these proteins is α -*N*-methylated by the NRMT methyltransferase, and knockdown of this enzyme recapitulates the multi-spindle phenotype of the desmethyl RCC1 mutants.¹³ To date, there are no known examples of mammalian proteins that contain *N*-methylated amide bonds.

While protein backbone amide *N*-methylation is not a known posttranslational modification in mammalian systems, there are multiple examples of both linear and cyclic naturally occurring peptide secondary metabolites with *N*-methylated amide linkages (Figure 1A). Examples include the bioactive *N*-methylated cyclic peptide cyclosporine¹⁴ (an immunosuppressant that blocks the protein phosphatase calcineurin), bouvardin¹⁵ (an antitumour drug that blocks the 80S ribosome), the depsipeptide enniatins¹⁶ (ionophores that disrupt membrane potential) and the cyclic peptide omphalotin¹⁷ (a potent nematocidal agent). Linear peptides with amide *N*-methylation include the tubulin polymerization inhibitors hemiasterlin,^{18, 19} and dolastatin 10.²⁰ Each of these peptides is synthesized by multifunctional nonribosomal peptide synthetases (NRPSs), by the action of an *N*-methylation domain embedded within an adenylation domain that functions on an activated amino acid substrate prior to condensation.²¹ Until recently, there were no examples of enzymes that carry out post-translational amide *N*-methylation on peptide (as opposed to amino acid) substrates.

Efforts in the field of medicinal chemistry have long focused on strategies for *N*-methylation to improve both the pharmacological profiles of peptides, as well as to limit enzymatic degradation by non-specific proteases. For instance, although cyclosporine violates almost all of the Lipinski rules, it is administered as an oral solution (trade name Neoral). Likewise, *N*-methylated derivatives of bioactive somatostatin analogs show improved oral bioavailability.²² These, and other studies highlight a role for amide *N*-methylation in imparting drug-like properties to both linear and cyclic peptidic drugs.²³

Although most natural *N*-methylated peptides are presumed to originate from NRPS-like pathways, recent studies have identified a catalyst that modifies a peptide substrate in the biosynthesis of omphalotin and its congeners.^{24, 25} Biochemical characterization of the corresponding methyltransferase, OphMA from *Omphalotus olearius*, suggests that these *N*-methylated and N-C macrocyclized products are a type of fungal ribosomally synthesized and post-translationally modified peptides (RiPPs).²⁶ The biosynthesis of omphalotin and its related class of natural products (termed borosins) require only two gene products: a methyltransferase, and a prolyl oligopeptidase (POP), which liberates and cyclizes the fused peptide to afford the final product. Notably, unlike other RiPP pathways, the precursor peptide is not an independently transcribed gene product but rather is a sequence fused to the C-terminus of the methyltransferase. Herein, we describe the structural and biochemical characterization of a borosin methyltransferase, dbOphMA from the corticioid fungus *Dendrothele bispora*. We have determined crystal structures of the enzyme in the absence and presence of substrate peptide, and have carried out structure-function analysis of residues that may be responsible for activating the backbone nitrogen of the core peptide. We also endeavor to understand the substrate scope of this enzyme, and have characterized the specificity for the methyl transfer reaction.

RESULTS AND DISCUSSION

Heterologous production and activity assays of dbOphMA

We used the JGI genome portal to identify homologs of OphMA from *Omphalotus olearius* for biochemical and structural studies. A candidate biosynthetic cluster (Figure 1B) containing both a methyltransferase and a POP macrocyclase was identified in the fungus *Dendrothele bispore* CBS 962.96 (JGI protein ID: 765760). The methyltransferase-precursor fusion protein from this organism (hereafter dbOphMA) shares 85% sequence identity with OphMA, and contains both the methyltransferase domain, as well as the C-terminal precursor fusion as first identified in OphMA (Figure 1C). The C-terminal fusion consists of a core sequence accompanied by a “follower” sequence that is excised from the final cyclic product. For our studies, we used a codon-optimized synthetic gene for dbOphMA for heterologous production in *E. coli*.

In order to test for in vivo methylation on the amide backbone of the precursor peptide, we treated purified dbOphMA with endoproteinase GluC to liberate the C-terminal precursor peptide and analyzed the peptide using MALDI-TOF mass spectrometry (Figure 2A). The non-methylated state is detected with a relatively low intensity. Interestingly, up to ten methylations are observed, even though purified omphalotin only contains nine residues in the final cyclic product. Hence, these data suggest that the final methylation likely occurs on the follower sequence on our omphalotin-like linear derivative.

To generate a minimal peptide for analysis, residues upstream of the core sequence were mutated to incorporate the tobacco etch virus (TEV) cleavage sequence to yield the dbOphM_{TEV}A construct. After fermentation and purification, dbOphM_{TEV}A was treated with TEV, and was subject to MALDI-TOF analysis; masses corresponding to methylated peptides were observed (Figure 2B). Masses corresponding to the non-methylated species were only detectable through LC-MS analysis (Figure 2C). These data confirmed that typically nine methylations (but sometimes a tenth) are observed upon liberation of the C-terminal peptide from dbOphM_{TEV}A. We attribute the heterogeneity of the methylated peptide samples to the fact that unmodified substrate, i.e. unmethylated core peptide, will be continuously produced as so long as dbOphM_{TEV}A is expressed in *E. coli*.

Overall Structure of dbOphMA

In order to elucidate the molecular mechanism for autocatalytic backbone *N*-methylation, we determined the structure of the catalytic component of dbOphMA to 2.2 Å resolution, and of the full-length fusion (residues Met1-Ala417) to 2.15 Å resolution, each with SAH bound in the active site. The latter fusion protein also contains the precursor peptide that is the substrate for the autocatalytic *N*-methylation reaction. Crystallographic phases were determined using single wavelength anomalous diffraction methods from a crystal soaked into a solution of 0.5 M NaBr prior to data collection at the Br absorption edge. Initial phases were further improved using non-crystallographic symmetry averaging, automated and manual chain building, interspersed with rounds of crystallographic refinement until convergence. Data collection and refinement statistics are provided in Table S1.

In each of these structures, dbOphMA is arranged as a homodimer, consistent with the solution behavior of the protein. The overall architecture may be demarcated into four domains that are linked by long loop regions (Figure 3A, B). The homodimeric assembly facilitates the spatial dispersal of the unconnected secondary structural motifs, which may be necessary for activity. The first 250 residues (through Pro249) make up the methyltransferase module, which is comprised of two α/β fold domains: domain I (Pro9 through Met124) consists of a five-stranded parallel β -sheet that is flanked by two sets of α -helices. The second domain (II; Glu131 through Ile248) is composed of a mixed five-stranded β -sheet flanked by four helices on one side and a single helix on the other. The homodimer assembly results in interactions across the central β -sheets of domain II. The methyltransferase active site is located at the interface between the two domains. The overall fold on the methyltransferase module resembles that of the catalytic domains of class III tetrapyrrole methylases such as CysG from *Salmonella typhimurium* strain LT2 (PDB ID code: 1PJQ; RMSD of 2.7 Å over 217 aligned C α atoms),²⁷ and NirE from *Pseudomonas aeruginosa* (PDB ID code: 2YBO; RMSD of 2.5 Å over 212 aligned C α atoms).²⁸ The methyltransferase module comprises the only similarity shared between dbOphMA and these other enzymes, as the remainder of the structure is unique.

Following the methyltransferase domain, residues Ala252 through Thr320 form an extended structure interrupted by two small helices. The helices wrap around the second protomer to buttress the homodimeric assembly. This largely unstructured region ends with a four-helical bundle composed of residues Lys322 through Glu384, which is further engaged through interactions with one of the helices of domain I. These extensive interactions between the extended region of one monomer, and the methyltransferase module of the adjacent monomer results in an enforcement of the homodimeric assembly, as well as a burial of 3074.8 Å², as calculated by Chimera, of surface area upon dimer formation.

We generated a sequence similarity network²⁹ sampling 3,000 members of the tetrapyrrole methylase protein family using the amino acid sequences of dbOphMA and OphMA as query seeds. The resultant network was analyzed using an alignment score of 45 (corresponding to the sequence similarity at which individual nodes are demarcated, Figure S1). The network reveals that the borosin methyltransferases are fairly unique and isolated in their protein sequence as compared to other annotated members of the tetrapyrrole methylase family. The amino acid sequences for borosin methyltransferases appear highly conserved as demonstrated in the alignment of dbOphMA with OphMA and LedMA, a third candidate methyltransferase from *Lentinula edodes* (Figure S2). This putative cluster also contains a putative prolyl oligopeptidase/macrocyclase that is situated one gene away from the methyltransferase.

Features of the dbOphMA Active Site

In order to garner molecular details on substrate binding, we also determined the structure of full-length dbOphMA, which consists of the precursor peptide fused to the C-terminus of the dimeric assembly as described above. This crystal form contains a monomer in the asymmetric unit, but the homodimer observed for the catalytic component is recapitulated by crystallographic symmetry. The structure generally resembles that of the catalytic

component. Electron density can be observed for residues Gly10 through Gln388, but no density could be observed for residues Ser388 through Val414, indicative of disorder at these regions of the polypeptide. However, electron density is evident for the last three residues of the core peptide (Val409-Gly411), and almost all of the follower peptide (Ser412 through Ser416).

Of note, each methyltransferase module's active site contains a bound substrate from the opposing monomer, indicative that each precursor is processed in trans. A molecule of *S*-adenosyl homocysteine (SAH) resides at the interface between domains I and II of the methyltransferase modules, and its location is indicative of where *S*-adenosyl methionine (SAM) would be positioned for productive methyl transfer onto the peptide backbone. The SAH/SAM binding pocket is fairly hydrophobic, and is composed of residues including the Ile19/Val243 pair that sandwich the adenine ring, and Tyr98/Phe171 pair that is in van der Waals contact with the Met moiety of SAH/SAM (Figure S3). Additional residues that interact with the ligand include Ser244 that is within hydrogen-bonding distance to the 2' OH of the ribose ring, the backbone carbonyl oxygen atoms of His100 and Val103 that stabilize the α -amine of SAH/SAM, and finally Ser129 that interacts with the nitrogen on the adenine ring.

The C-terminal peptide substrate is located in a tunnel roughly ~ 10 Å in diameter that runs adjacent to the SAH/SAM binding site. Residues 175–189 form a loop that serves as a clamp that appears to lock both substrates inside the active site. The substrate-binding tunnel is fairly hydrophobic, with a limited number of polar residues that engage mainly in interactions with the backbone amide and carbonyl oxygen atoms. The lack of specific interactions with side chains of the substrate is consistent with the sequence agnostic nature of dbOphMA in carrying out multiple *N*-methylations of the peptide substrate. Electron density corresponding to *N*-methylation can be observed for residues Val409, Ile410, Gly411 of the core peptide, as well as Val413 from the follower sequence. In the structure, the *N*-methyl moiety on Val413 is positioned roughly 3.1 Å adjacent to the sulfur atom from SAH, which likely represents a product complex. Putative catalytic residues reside in the vicinity of *N*-methylated Val413 (Figure 3D), and these include Tyr66, (2.6 Å away from the amide oxygen), Arg72 (3.2 Å away from the hydroxyl of Tyr76), and Tyr76 (3.5 Å away from the amide nitrogen, and 2.8 Å away from the amide oxygen). The function of these individual residues is interrogated through mutational analysis as described below.

The Follower Peptide is Not Essential for dbOphMA Activity

Most RiPP biosynthetic enzymes contain a recognition element (RRE) embedded in the protein fold that directs the enzymes to their cognate peptidic substrates.^{30, 31} Borosin methyltransferases do not contain an RRE, and therefore must utilize a different means of recognizing its substrates. Initial reports on OphMA found that it was able to function without its follower region, and can methylate residues in the follower as well,²⁴ as we have similarly observed for dbOphMA. However, our crystal structure of dbOphMA shows that the enzyme is poised to use the last four residues of the core peptide and the follower region as a handle to position the substrate peptide in the active site. Consequently, we sought to determine if the follower sequence affects the biochemical activity of dbOphMA. To this

end, we generated the dbOph_{TEV}NF variant that lacks the entire follower peptide from the C-terminus of the enzyme. Fermentation and processing of the resultant TEV-cleaved protein yielded the same methylation pattern as previously observed for the wild-type enzyme (Figure S4). The peptides liberated by TEV protease treatment also contain species that are unmodified, singly and doubly modified, as detected by MALDI-TOF spectrometry. This may be due to the fact that the peptides lacking the follower sequence are more readily ionized. Small-scale fermentation and TEV cleavage of the dbOph_{TEV}NF lysate in triplicate (data not shown) also supports our observations from the large-scale fermentation. These data suggest that the follower peptide does not significantly contribute to the autocatalytic methyltransferase activity of dbOphMA.

To confirm that the follower peptide recognition is not necessary for backbone *N*-methylation, we carried out fluorescence polarization (FP) studies. For these studies, we used the catalytic component of dbOphMA (Met1-Asn377), which lacks the precursor peptide, a construct we henceforth refer to as dbOphM. Two follower peptide constructs were tested: one that was acetylated at the N-terminus and labeled with fluorescein on the C-terminus via a GGK linker, and the other was labeled with fluorescein on the N-terminus. The two peptides were used to rule out interference from the fluorophore upon binding of the enzyme to the substrate. Additional FP assays were conducted in the presence of trace metals in order to test if a cationic species aids the enzyme in coordinating the peptide in the active site. Binding data for both constructs suggests that the enzyme has no measureable affinity for the follower peptide (Figure 4A, B). The enzyme most likely possesses the follower peptide in order to serve as a recognition motif for the prolyl oligopeptidase that subsequently cyclizes the methylated product, as is the case for GmPOPB in amanitin biosynthesis,³² and PCY1 in orbitide biosynthesis.^{33, 34}

Characterization of SAM and SAH binding to dbOphMA

We next sought to establish the binding affinities of dbOphMA for SAM and SAH. Prior work determined that the homolog OphMA is not active outside the *E. coli* cytosol,²⁴ and we likewise observed the same behavior with recombinantly expressed and purified dbOphMA. We attempted to address if the lack of activity may be due to the inability of the recombinant enzyme to bind the methyl donor SAM outside of *E. coli*. Using microscale thermophoresis, the binding affinity (K_D) of dbOphM to SAM was measured to be $80.7 \mu\text{M} \pm 14.7 \mu\text{M}$, and to SAH was measured to be $6.88 \mu\text{M} \pm 1.13 \mu\text{M}$, respectively (Figure 5A). The higher measured affinity for SAH is consistent with product inhibition, which has also been observed in other SAM-dependent methyltransferases.^{35, 36} The higher measured affinity for SAH is to be expected, as SAH is a competitive inhibitor of SAM-dependent methyltransferases. The K_D for SAM is also higher than other methyltransferases,^{37–39} suggesting that the enzyme requires an environment that is abundant in SAM as well as *S*-adenosylhomocysteine nucleosidase, to ensure productive turnover. As a control experiment, we measured the binding affinity of a dbOphMA variant (Y98A) that lacks a residue critical for binding SAM/SAH. As expected, this variant demonstrated no binding affinity for either of these ligands (Figure 5B). These data confirm that the ability of dbOphM to bind SAM/SAH is not impaired outside of the *E. coli* cytosol, and is not the cause for the lack of methyltransferase activity in vitro.

Examination of the Substrate Scope and Directionality of dbOphMA

We next set out to reconstitute dbOphMA activity *in vitro*, and to probe the substrate scope of dbOphMA. Despite numerous efforts (summarized in Table S2), we were unable to observe any methylation activity by purified dbOphMA or its variants on native peptide substrates of various lengths and composition. Our data suggest that either a) the proximity of the substrate to the enzyme active site created by the covalent linkage is necessary for the reactivity of dbOphMA, or b) the *in vitro* reconstitution efforts lacked a necessary co-factor that is present only within the native cells or *E. coli* cytoplasm. Consequently, we carried out co-expression of dbOphMA, along with numerous variants, concurrently with MBP-tagged substrate peptide. Even under the coexpression conditions, we could not observe any methylation of the substrate peptide that was produced *in trans* with the methyltransferase (Table S2). Methylation could only be observed for peptides that were covalently linked to dbOphMA, or its variants (data not shown). These results suggest that the enzyme has minimal affinity for the free peptide substrate. In conjunction with data that shows that the enzyme possesses no affinity for the follower region, which contains one methylated residue, we suggest that autocatalytic *N*-methylation likely occurs as the result of high local concentrations of the substrate produced by both the covalent linkage to the catalytic domain, and by proximity created by formation of the dbOphMA dimer.

We next tested the general utility of dbOphM_{TEV}A as a catalyst for construction of *N*-methylated substrate. Firstly, we generated a number of Pro variants along the sequence of the core peptide. A Pro residue would be a structural mimic of *N*-methylated amide,⁴⁰ and can introduce a kink, or bend, to the peptide to be modified. The variants were generated in the context of dbOphM_{TEV}A protein, and each was analyzed using mass spectrometry following cleavage of the precursor peptide using TEV protease (Table 1). Methylation is entirely abolished in the V407P variant, and limited to only one methylation for the V403P variant (Table 1, Figure S5). This suggests that dbOphM_{TEV}A is likely not able to accept substrates that may prove conformationally challenging for the enzyme to bind. Additionally, we replaced the core peptide region of dbOphM_{TEV}A with sequences resembling that of the immunosuppressant cyclosporine, to yield two constructs, dbOphM_{TEV}PC (core peptide: LVLAALLVTAG) and dbOphM_{TEV}PC-reverse (TVLLALVLGA). Fermentation and analysis of these variants under the previously established conditions showed that the methylation pattern of the native peptide is not preserved in dbOphM_{TEV}PC nor dbOphM_{TEV}PC; the two constructs also possessed different methylation patterns (Figure S6). We reasoned that the lack of methylation may be due to the presence of the larger Leu residues in the core sequence of these variants. Altogether, these results suggest that certain residues may be tolerated and accepted over the others within the core peptide.

Dissecting the Catalytic Residues of dbOphMA

In order to further probe the role of specific residues in autocatalytic *N*-methylation, we generated site-specific variants of dbOphM_{TEV}A and examined the methylation pattern of the released precursor peptide after TEV cleavage. In the crystal structure, both Tyr98 and Ser129 are poised to interact with SAM. The S129A variant was largely insoluble and the soluble fraction consisted of various truncations of the full-length enzyme. In contrast,

Y98A dbOphM_{TEV}A variant was soluble and well behaved. Upon TEV-cleavage of the purified protein we did not observe masses corresponding to the expected methylated species. (Figure S7). DbOphMA Y98A was also fermented, purified, and treated with GluC in order to confirm these results. Only the unmethylated peptide was detected in both MALDI-TOF and LC-MS analysis (Figure S8, 9). In conjunction with the lack of affinity that dbOphMA Y98A has for SAM and SAH as demonstrated by microscale thermophoresis, these results further demonstrates that Tyr98 plays a role in engaging SAM, as suggested by the structure.

We next sought to determine the role of active site residues identified in the structure, which may play a role in acid-base catalysis. Residues along the inner-ridge of the methyltransferase closest to the methylated Val413 are Tyr66, Arg72, and Tyr76 (Figure 6A). The Y66F mutant was completely insoluble, while the R72A and Y76F variants were poorly soluble. We then analyzed the methylation pattern on the peptides released following treatment with GluC for the latter two mutants. Notably, no methylations were observed in either of these variants, as determined by MALDI-TOF and LC-MS analysis (Figure S8, 9). Disruption of these two residues was sufficient to impair methylation of the substrate, highlighting their importance in catalysis.

CONCLUSIONS

Most synthetic processes towards *N*-methylated peptides are based on either the use of *N*-methylated amino acid building blocks⁴¹ or via the use of isonitrile coupling reactions.⁴² Likewise, enzymatic synthesis via the multi-modular non-ribosomal peptide synthetases (NRPSs) that carry out the enzymatic production of *N*-methylated molecules, such as cyclosporine, utilize an embedded methyltransferase domain that modifies ACP-tethered constituent amino acids prior to formation of the peptide bond.^{43, 44} There are numerous challenges involved in the post-facto *N*-methylation of peptides as resonance delocalization significantly reduces the nucleophilicity of the amide nitrogen. Other hurdles include the difficulty in removal of the amide proton ($pK_a \sim 20$ in water), steric hindrance at the nitrogen caused by neighboring amino acid side chains, and the propensity for spontaneous epimerization of the C α via base-catalyzed enolization, due to the lack of an amide proton.⁴⁵ Catalysts that carry out backbone amide *N*-methylation on peptide substrates must overcome each of these hurdles.

Conceptually, these hurdles can be overcome in an enzymatic context through one of several strategies utilized by methyltransferase: (1) proximity and desolvation,⁴⁶ (2) metal-ion assisted catalysis,^{47, 48} and/or (3) general acid-base catalysis.⁴⁹ Metal aided catalysis may be ruled out based on our observation that addition of trace metals does not enhance the extent of *N*-methylation on the precursor peptide, regardless of whether it is fused to the catalytic domain or provided in trans. Our combined structural and biochemical data suggest that dbOphMA and, likely other related borosin methyltransferases, can catalyze this difficult transformation by exploiting substrate proximity, and acid-base catalysis. The extended region and helical bundles that are C-terminal to the catalytic domains in these enzymes affect proximity through dimer formation, which results in the disposition of the precursor peptide from one monomer near the catalytic domain active site of the other. We proposed

that Tyr76, which is suitably poised for this function, carries out deprotonation of the amide nitrogen, and the basicity of this residue may be enhanced by the proximity of Arg72 (Figure 6A). Tyr66 and Tyr76 may stabilize the negative charge on the oxygen atom of the resulting enolate. The proximity of a reactive SAM molecule to this species advances the alkylation reaction.

The lack of tolerance of dbOphMA to substrates that contain amino acids larger than Val/Thr, as observed in our studies with the fused cyclosporine core peptide, suggests that steric hindrance at the nitrogen atom may be a mitigating concern with regards to substrate tolerance. While conceptually appealing, it is unlikely that increasing the size of the substrate binding substrates through site-directed mutagenesis will provide a general, more promiscuous catalyst for backbone *N*-methylation. Nonetheless, it may be possible to carry out more discrete engineering experiments to accommodate non-cognate substrates. These, and future studies, should enable efforts to utilize dbOphMA as a general catalyst for the backbone *N*-methylation of other peptidic substrates.

MATERIALS AND METHOD

Cloning, Protein Expression, and Purification

Primers used for PCR-based gene amplification are listed in Table S2. The gene sequence for dbOphMA was obtained from the Joint Genomic Institute (JGI) database, and a corresponding codon optimized synthetic gene was ordered from Integrated DNA Technologies, Inc. Initially, dbOphMA was cloned into a pET His₆ TEV LIC cloning vector, while the isolated precursor peptide and variants were cloned into the pET MBP His₆ TEV LIC cloning vectors (gifts from Scott Gradia, Addgene plasmid #29666, and #29656, respectively). The integrity of all expression plasmids were verified by sequencing, and each plasmid was transformed into *E. coli* BL21(DE3) cells for protein expression. All derivatives of dbOphMA were grown in LB media at 37 °C to cell densities corresponding to an OD₆₀₀ of 0.6–0.8. Cultures were cooled in an ice bath for 15 minutes, and protein expression was induced by the addition of 0.4 mM IPTG, followed by incubation while shaking for another 18 hours at 18°C. MBP-tagged precursor peptides and variants were grown at 37 °C to an OD₆₀₀ of 1.0, before induction with 0.4mM IPTG, followed by incubation at 37 °C for three hours. Cells were harvested via centrifugation at 3,500 rpm, and were resuspended in 30 mL of buffer containing 500 mM NaCl, 25 mM Tris pH 8.0, 10% glycerol.

Cells were lysed by sonication, with 40 second on-cycles at 20% amplitude, with one minute resting intervals, for a total of five cycles. Lysates were centrifuged at 14,000 rpm for 45 minutes to pellet insoluble cell debris. The resultant supernatants were passed through 5 mL HisTrap HP columns (GE Healthcare Life Sciences) pre-equilibrated with 40 mL of the aforementioned resuspension buffer. Columns were washed with 40mL of wash buffer containing 1 M NaCl, 25 mM Tris pH 8.0, and 25 mM imidazole. Proteins were eluted on an ÄKTAprime system (GE Healthcare Life Sciences), using a linear gradient over 40 mL, from 25 mM to 250 mM imidazole, at a flow rate of 2 mL/min. All variants of dbOphMA were purified in a similar manner.

The purest fractions, as judged by SDS-PAGE, were combined for dbOphMA and dbOphM and used for crystallography. TEV was added to a final ratio of 1:100 (w/w) to cleave off the His-tag during overnight dialysis of the proteins against storage buffer containing 300 mM NaCl, 25 mM Tris pH 7.5, 10% glycerol. Subtractive Ni-NTA purification was used to exclude protein that still retained the His-tag. Cleaved samples were concentrated in a 30 kDa Amicon (Millipore Sigma), and then further purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare Lifesciences) equilibrated with 300 mM KCl, 20 mM HEPES pH 7.5. Sample purity was analyzed by SDS-PAGE, and the purest fractions were combined and concentrated in a 30 kDa Amicon. dbOphMA and dbOphM were concentrated to 20 mg/mL and 70 mg/mL, respectively as determined by a Bradford standard curve. dbOphM_{TEV}A and the other derivatives were purified in a similar manner, except that TEV was not added to the protein during dialysis. Each dbOphMA variant was concentrated and flash frozen in liquid nitrogen prior to usage.

Crystallization, Data Collection, Phasing and Refinement

Initial crystallization conditions for dbOphMA were established using sitting drop sparse matrix screening. Diffraction quality crystals were obtained by the hanging drop method. Briefly, protein samples (concentration of 10 mg/mL), was incubated with 2 mM SAH, and mixed with an 1:1 (v/v) solution containing 0.24 or 0.26 M NaF, 20–26% PEG3350, and 0.1 M Bis-tris propane pH 6.5, and equilibrated against the same solution. The resultant crystals were soaked in precipitant solution supplemented with 15% ethylene glycol immediately prior to vitrification by direct immersion into liquid nitrogen. All crystallographic measurements were collected on a CCD device at an insertion device synchrotron source at Sector 21 ID (LS-CAT, Advanced Photon Source, Argonne National Labs, IL). Data were indexed, integrated and scaled using XDS,⁵⁰ as implemented in the autoPROC suite.⁵¹ Crystallographic phases were determined by single wavelength anomalous diffraction methods from data collected at the Br absorption edge on crystals of dbOphMA soaked in 0.5 M NaBr for two minutes prior to vitrification. Heavy atoms sites were located using the SHELX⁵² and atom parameters were refined in autoSHARP⁵³, resulting in a figure of merit of 0.18. Following density modification, the initial electron density map was of sufficient quality to allow automated building of most secondary structural elements using BUCCANEER.⁵⁴ The model was subsequently improved manually using COOT,⁵⁵ interspersed with cycles of crystallographic refinement using REFMAC⁵⁶ until convergence. Final data collection, phasing, and refinement statistics may be found in Table S1.

Generation of dbOphMA Variants and Mutagenesis

Primers for variant construction and mutagenesis are listed in Table S3. dbOphM_{TEV}A, dbOphM, dbOph_{TEV}NF, dbOphM_{TEV}PC, and dbOphM_{TEV}PC-reverse were all cloned using LIC-NHis₆ dbOphMA as a template. dbOphM_{TEV}A, and dbOphM_{TEV}PC-reverse were generated by site-directed, ligase-independent mutagenesis.⁵⁷ Mutants of dbOphMA, or dbOphM_{TEV}A were generated using QuikChange mutagenesis.

Protease Cleavage and Detection of Methylated Species

Endoproteinase GluC (Roche) was added to 50 μ M purified protein in a 1:100 (w/w) ratio prior to incubation at room temperature for an hour, followed by inactivation by heating to 95 °C for 10 minutes. Variants containing the TEV site was diluted to 50 μ M in storage buffer and 1:100 (w/w) TEV was added to each reaction, followed by incubation at 25 °C for four hours. Following protease treatment, peptide samples were clarified by centrifugation and desalted using C₁₈ ZipTips (Millipore Sigma) and eluted with a solution containing 75% acetonitrile and 0.1% formic acid prior to analysis by MALDI-TOF/TOF (Bruker Daltronics). Samples that required LC-MS/MS analysis were precipitated and clarified in the same manner, and submitted to the University of Illinois Mass Spectrometry Facility to be analyzed on a Waters Synapt G2-Si in positive ion mode. The sample was fractionated using a linear gradient from 3% to 97% solvent B (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) over 12 minutes at flow rate of 0.150 mL/min, with the PDA detector set to 220 nm.

Labeling (and Acetylation) of follower peptide with fluorescein isothiocyanate (FITC)

Peptide containing the follower sequence with a GGK linker was ordered from Genscript at 90% purity. To generate the C-terminus FITC labeled peptide, the peptide was dissolved in 100 mM sodium phosphate buffer (pH 6.5) to a final concentration of 50 μ M. Sulfo-NHS-acetate (Pierce™ Sulfo-NHS-Acetate; ThermoScientific™) was dissolved in ultrapure water to 10 mM, and a 25-fold molar excess was added to the dissolved peptide dropwise. Acetylation of the N-terminus of the peptide was allowed to proceed overnight at room temperature. The reaction mixture was lyophilized, and the dried sample was resuspended in distilled water. Insoluble material was removed by centrifugation at 13,000 rpm for 10 minutes, prior to HPLC purification using an YMC 250 mm x 4.6 mm, 5 μ m C18 column on a Shimadzu LC-20AD HPLC system. Chromatographic separations utilized a linear gradient (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile) starting at 5% B and going to 60% B over 30 minutes. The identity of the N-terminally acetylated follower peptide was confirmed by MALDI-TOF/TOF analysis, and by LC-MS/MS analysis.

The N-terminally acetylated follower peptide and the unmodified follower peptide were treated with fluorescein isothiocyanate (FITC) in order to label the free amine on the C-terminus, and N-terminus respectively. Both peptides were dissolved in 100 mM sodium phosphate buffer (pH 8.0) to a final concentration of 50 μ M. FITC (Sigma-Aldrich) was dissolved to 10 mg/mL in DMSO and added drop wise to the peptide solution to a final concentration of 250 μ M. Following overnight incubation, the reaction mixtures were lyophilized, and the two FITC-labeled peptides were HPLC purified using the method detailed above.

Fluorescence polarization (FP) binding assay

DbOphM was serially diluted into binding buffer (300 mM KCl, 20 mM HEPES pH 7.5) and mixed with FITC-labeled peptides to a final peptide concentration of 10 nM. Binding assays were carried out using nonbinding-surface, 384-black-well polystyrene microplates (Corning) and measurements were conducted using a Synergy H4 Hybrid plate reader (BioTek) with λ_{ex} = 485 nm and λ_{em} = 538 nm. Prior to measurement, the dilutions were

equilibrated at 25 °C for 20 minutes. Binding assays conducted in the presence of trace metals utilized a 1000X trace metals stock solution containing: 50 mM FeCl₃, 20mM CaCl₂, 10 mM MnCl₂, 10 mM ZnSO₄, 2 mM CoCl₂, 2 mM CuCl₂, 2 mM Na₂MnO₄, and 5 mM MgCl₂. Data were recorded with Gen5 software and processed using the GraphPad Prism software.

Microscale Thermophoresis (MST) Measurements of SAH/SAM binding affinity

SAH and SAM (Sigma-Aldrich) were used to measure the binding affinity to dbOphM and dbOphMA Y97A using microscale thermophoresis. Both ligands were dissolved in binding buffer (300 mM KCl, 20 mM HEPES pH 7.5), and were serially diluted for use in the assays. Protein was diluted in binding buffer to a final concentration of 800 nM and mixed with the different concentrations of the ligand in a 1:1 ratio, so that the final protein concentration was 400 nM. Prior to measurement, the samples equilibrated at 25 °C for 20 minutes before being drawn into capillary tubes (Monolith™ NT.LabelFree Capillaries, Nanotemper, cat# MO-Z022). Data was collected on an Monolith™ NT LabelFree model (Nanotemper) using the MO Control software. Data was processed on the MO Affinity Analysis and GraphPad Prism platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Structure of N-methylated cyclic compounds cyclosporine A, omphalotin A, bouvardin, and enniatin A. Methylations occurring on amide bond nitrogen atoms are circled in pale green. (B) Putative gene cluster producing an omphalotin-like compound from *Dendrothele bispora* CBS 962.96. (C) Putative precursor peptides of dbOphMA and OphMA. Core peptides are highlighted in green, and follower peptides are highlighted in yellow.

Figure 2.

DbOphMA methylated peptides. (A) Representative MALDI-TOF trace of dbOphMA treated with GluC. Masses corresponding to the non-methylated peptide, and its methylation states have been annotated. Peaks that are not labeled indicate nonspecific masses due to GluC treatment of the entire protein. (B) Representative MALDI-TOF trace of dbOphM_{TEV}A treated with TEV protease. (C) De-convoluted MS/MS spectra of dbOphM_{TEV}A TEV treated peptide with five methylations to confirm the presence of the peptide (core residues in green, follower residues in yellow). Inset depicts spectra of mass

corresponding to the unmethylated peptide at the $z = +2$ charge state (theoretical $[M+2H]^{2+}$: 1022.5775). This peptide was not detectable on MALDI-TOF.

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Figure 3.

Overall structure of dbOphMA. (A) Schematic of the structural organization of dbOphM without the precursor peptide. (B) Structure of the dbOphM monomer. The coloring scheme adheres to the domain organization detailed in Fig. 3A. SAH (tan spheres) lies in the cavity between domain I and domain II. (C) Structure of dbOphMA homodimer. One monomer is shown in ribbons, in the same orientation and in panel B and the other is shown as a surface model in gray. (D) The terminal three residues of the core (green), and five residues in the follower (yellow) are bound in the active site in the structure of dbOphMA. Simulated annealing difference Fourier maps ($F_o - F_c$), calculated with the peptide omitted for one round of refinement prior to map calculation, is superimposed (blue).

Figure 4.

Fluorescence polarization binding curves of dbOphM and follower peptide constructs, indicating dbOphM does not bind the follower peptide. (A) Binding curves without the presence of trace metals. (B) Binding curves in the presence of trace metals for both peptide variants. The increase in polarization signal in (A) and (B) are due to increased protein aggregation at the highest concentration for the fluorescence polarization assay. All experiments were conducted three times and the mean average value was plotted. Error bars indicate standard deviation of each data point.

Figure 5.

(A) Binding curves depicting the affinity of dbOphM for SAM and SAH. The K_D was determined to be $80.7 \mu\text{M} \pm 14.7 \mu\text{M}$, and $6.88 \mu\text{M} \pm 1.13 \mu\text{M}$ respectively. (B) Binding curves showing that the dbOphMA-Y98A mutant does not bind SAM or SAH. All experiments were conducted four times and the mean average value was plotted. Data points that fell outside the $\pm 15\%$ average fluorescence intensity range as determined by a capillary scan was excluded. Error bars indicate standard deviance of each data point.

Figure 6. Structure-based mechanism for methylations catalyzed by dbOphMA. (A) Residues in the active site of dbOphMA that are necessary for methylation of the substrate. The core peptide is shown in green, the follower peptide in yellow, and the methylated amide nitrogens in orange. (B) Proposed mechanism for methylation. The nitrogen of the amide bond is deprotonated by Tyr76. Tyr66 and Tyr76 coordinate and stabilize the developing negative charge on the carbonyl on the peptide chain. Ultimately, this allows the nitrogen to function as a nucleophile to sequester the methyl group from SAM.

Table 1.

Proline scanning variants and number of methylations observed peptides when each construct was fermented at 18 °C for 18 hours. Masses of released peptides were detected by MALDI-TOF.

Construct Name	Peptide Sequence after TEV Cleavage	Number of Methylations Observed
dbOphM _{TEV} A W400P	GFP P VIVTGIVGVIGSVVSSA	0 – 6
dbOphM _{TEV} A V403P	GFPW V IP T GIVGVIGSVVSSA	0 – 1
dbOphM _{TEV} A V407P	GFPWVIVTG I PGVIGSVVSSA	0
dbOphM _{TEV} A G411P	GFPWVIVTGIVGV I PSVVSSA	0 – 7