Functional Characterization of the Cyclomarin/Cyclomarazine Prenyltransferase CymD Directs the Biosynthesis of Unnatural Cyclic Peptides[⊥]

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In vitro and in vivo characterization of the cyclomarin/cyclomarazine prenyltransferase CymD revealed its ability to prenylate tryptophan prior to incorporation into both cyclic peptides by the nonribosomal peptide synthetase CymA. This knowledge was utilized to bioengineer novel derivatives of these marine bacterial natural products by providing synthetic *N*-alkyl tryptophans to a prenyltransferase-deficient mutant of *Salinispora arenicola* CNS-205.

The anti-inflammatory cyclomarin A[1] (Figure 1, **8**) and antibacterial cyclomarazine A[2] (**5**) are cyclic hepta- and dipeptides produced by the marine actinobacterium *Salinispora arenicola* CNS-205.[3] Common to both peptides is a *N*-(1,1-dimethyl-1-allyl)tryptophan (**2**) residue, which is further oxidized in **8** and cyclomarin C (**9**). While prenylated indoles, such as **2**, are common structural features in fungal and plant natural products,[4] these tryptophan-derived moieties are rare in bacteria. We recently characterized the 47 477-bp cyclomarin/cyclomarazine gene cluster *cym*, which is dominated by the 23 358-bp *cymA* gene encoding a heptamodular nonribosomal peptide synthetase (NRPS) responsible for the dual assembly of **5** and **8**.[2] The discovery of the *cym* locus led to the in vivo characterization of the prenyltransferase (PTase) CymD, which in turn suggested its role in prenylating a common biosynthetic precursor to both peptides prior to assembly.[2] This observation contrasts peptidyl PTase reactions such as in the biosynthesis of the cyanobacterial toxin lyngbyatoxin A[5] and various fungal diketopiperazines (DKPs),[4a−c] in which prenylation occurs post-NRPS assembly of the peptide product, although it has been suggested that prenylation of aromatic amino acid residues precedes the assembly of an unknown *Aspergillus fumigatus* peptide[4d] as well as in sirodesmin PL.[4e] In this study, we established the biological function of CymD as **2** synthase, which provided a general strategy to readily generate unnatural *N*-alkylated tryptophan analogues of **5** and **8** by a unified mutasynthetic approach.

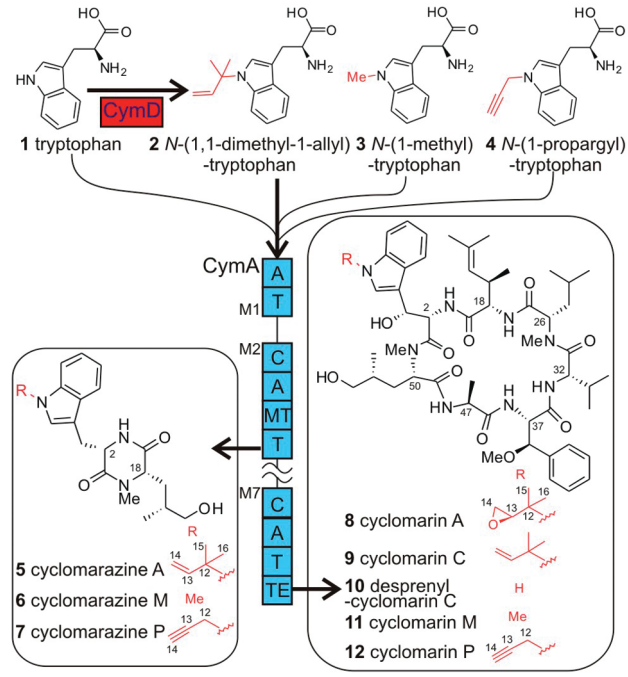

Figure 1. Structures and biosynthesis of natural and unnatural cyclomarin and cyclomarazine analogues. Supplementing cultures of the *S. arenicola cymD*$^-$ mutant with tryptophan analogues yields known and novel cyclomarins and cyclomarazines, thereby establishing **2** as the in vivo substrate for the heptamodular CymA NRPS. Thick arrows denote the pathway operative in wild-type *S. arenicola* CNS-205, while thin lines represent pathways accessible via the *cymD*-deficient mutant. Abbreviations: A, adenylation domain; C, condensation domain; M1−M7, modules 1−7; MT, methyltransferase; T, thiolation domain; and TE, thioesterase.

Results and Discussion

The targeted disruption of the PTase gene *cymD* in *S. arenicola* CNS-205 previously led to a mutant deficient in the known cyclomarin and cyclomarazine chemistry and provided a novel analogue, desprenylcyclomarin C (**10**).[2,6] The >100-fold lower production of **10** in the *cymD* knockout mutant not only implied that prenylation occurs pre-NRPS assembly but also suggested that tryptophan is a poor substrate for CymA.[2] We thus set out to explore the in vitro function of CymD, which was prepared as a 42 kD octahistidyl-tagged recombinant protein in *Escherichia coli*. The affinity-purified protein was incubated with L-tryptophan (**1**) and dimethylallyl pyrophosphate (DMAPP) to yield **2** (Figure 2), which was identical chromatographically and spectroscopically to synthetic **2** prepared by direct prenylation[7] of Boc-Trp-OMe (**13**) followed by deprotection (Scheme 1). Reactions in which DMAPP was replaced by isoprenyl, geranyl, or farnesyl pyrophosphate yielded no detectable products, suggesting a high level of specificity for the isoprene donor. Moreover, addition of Ca^{2+} or Mg^{2+} to the reaction mixture did not significantly alter yield, nor did addition of the cation-chelating agent ethylenediaminetetraacetic acid, suggesting that CymD functions cation independently. Alignment of CymD with the fungal indole PTase FgaPT2 (Figure 3) revealed the presence of two conserved lysine residues at positions 146 and 207 consistent with cation-independent fungal PTases[8] that belong to an emerging group of divergent prenylating enzymes from bacteria and fungi that share a α/β barrel fold.[9]

[⊥] Dedicated to the late Richard E. Moore of the University of Hawaii at Manoa for his pioneering work on bioactive natural products.

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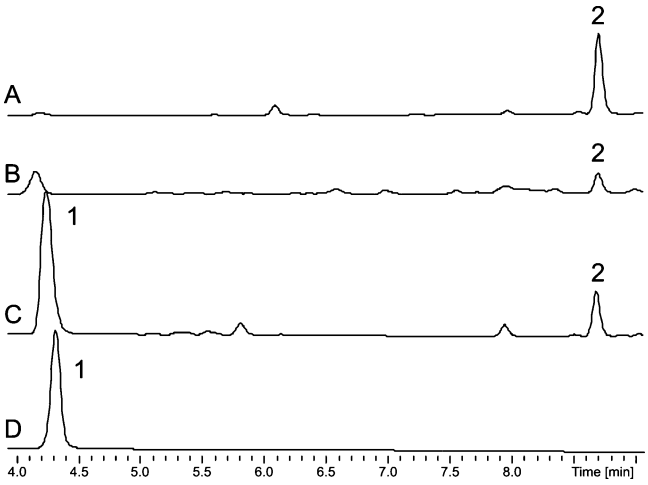
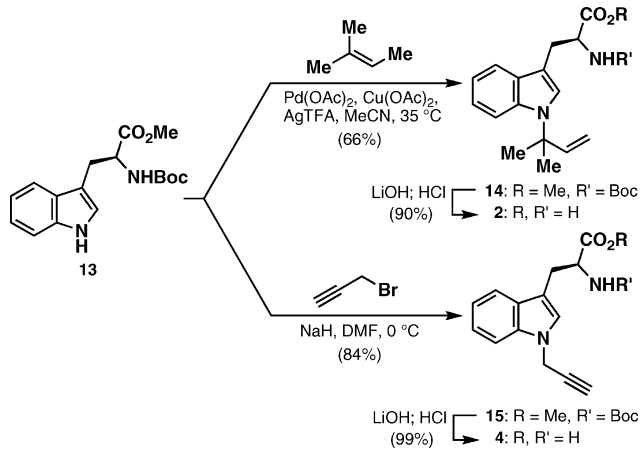


Figure 2. LC-DAD analysis of *N*-(1,1-dimethyl-1-allyl)tryptophan (**2**) at 210 nm. (A) Synthetic **2**; (B) natural **2** in the crude organic extract of *S. arenicola* CNS-205; (C) enzymatic **2** from the CymD-catalyzed reaction of DMAPP and tryptophan (**1**); and (D) the CymD boiled control of the enzymatic reaction in C.

Scheme 1. Synthesis of Tryptophan Substrates



Analysis of the *S. arenicola* CNS-205 fermentation broth revealed that **2** is indeed a natural product produced at 2 mg/L (Figure 2) and that its assembly ceases in the *cymD*$^-$ mutant. The structure of natural **2** was identical in all regards to enzymatic and synthetic **2** (Supporting Information, Figure S3). These data suggested that **2** is preformed and selected by the initiating NRPS module M1 on the heptamodular CymA synthetase to give a common biosynthetic intermediate that ultimately gives rise to natural cyclomarin and cyclomarazine cyclic peptides (Figure 1). We verified this conclusion by the chemical complementation of the *cymD*$^-$ mutant with synthetic **2** that restored the in vivo production of **5** and **8** to wild-type levels (Supporting Information, Figures S1 and S2), establishing **2** as the endogenous substrate of the CymA loading didomain M1.

	Motif I		Motif II	
	*		*	
FgaPT2	186	LKTYIYPALKAVVTGK 201	247	SCDLTSPAKSRIKIYL 262
CdpNPT	218	AKEYFFPGIKCAATGQ 223	274	CCDLVDPAHTRFKVYI 289
FtmPT2	191	AKAYFFPGMKSLATGL 206	254	GVDLCTPERSRLKFYV 269
CymD	145	FKAWFYLNVTGPDGAF 160	195	SLDLSDDPAARVKVYF 210
LtxC	187	MKTYLNLNIAGFESGP 202	238	ALDLAHSDHPRLKIYL 253
NphB	149	AELFARYGLDK----- 164	185	EAESVLALVRELGLHV 200
CloQ	148	LKDFLALGLAH----- 163	187	ARIHALSGSTPPAAHV 202

Figure 3. Portion of an alignment of CymD with the active site of FgaPT2 and other prenyltransferases. Fungal indole prenyltransferases (PTases) from *Aspergillus fumigatus* FgaPT2 (accession no. AAX08549), CdpNPT (ABR14712), and FtmPT2 (AFUA_8G00250) were aligned with bacterial indole PTases CymD from *Salinispora arenicola* CNS-205 (SARE_4565) and LtxC (*Lyngbya majuscula,* AAT12285) and bacterial aromatic PTases NphB (*Streptomyces* sp. CL190, AB187169) and CloQ (*Streptomyces roseochromogenes* AF329398). Li and co-workers[8] have identified the conserved residues in bold, two of which are lysines (*) responsible for the non-metal-dependent activity of fungal indole PTases.

Since prenylation in the cyclomarin/cyclomarazine series occurs on free tryptophan rather than on a peptide precursor as in precedent examples,[4a−c,5] it provided us an opportunity to employ a mutasynthesis approach[10] to explore whether *N*-alkyl tryptophan analogues could be simultaneously assimilated into both natural product classes to give rise to novel analogues. Commercially available *N*-(1-methyl)tryptophan (**3**) was first evaluated by administering it to a culture of the *cymD*$^-$ mutant. LC-MS analysis of the resulting organic extract revealed anticipated products that were subsequently isolated and fully characterized by NMR and high-resolution MS to give the novel *N*-methyl indoles cyclomarin M (Figure 1, **11**) and cyclomarazine M (**6**). NMR analyses (Tables 1 and 2) verified the loss of the respective prenyl groups of **8** and **5** while maintaining the remainder of the peptidic structures. HMBC correlations and chemical shift analyses further established the *N*-methyl substitution (C-12) on the tryptophan indole ring.

We next explored incorporating an *N*-propargyl group into these cyclic peptides in order to prepare analogues appropriate for biological evaluation via semisynthesis utilizing Click chemistry.[11] *N*-(1-Propargyl)tryptophan (**4**) was synthesized by alkylation of Boc-Trp-OMe (**13**) with propargyl bromide followed by deprotection (Scheme 1). Upon the addition of **4** to the *S. arenicola cymD* knockout mutant, new *cym* analogues cyclomarin P (**12**) and cyclomarazine P (**7**) carrying the anticipated propargyl side chains (C-12 through C-14) were similarly produced by fermentation. The yields of unnatural **11** and **12** were comparable to that of natural **8** in *S. arenicola* CNS-205 at 1−3 mg/L with a similar trend observed in the cyclomarazine series. These observations coupled with the inability of tryptophan to restore wild-type biosynthetic levels[2] suggest that the native CymA loading adenylation (A) domain in module 1 (M1) can accommodate varied N-1-substituted tryptophan substrates for assimilation into the *cym* hepta- and dipeptides.

Herein we report the characterization of CymD as a bacterial *N*-(1,1-dimethyl-1-allyl)tryptophan synthase, which alkylates tryptophan with DMAPP as the prenyl donor in a cation-independent manner prior to NRPS assembly. Although several biochemical studies have been performed on bacterial[5] and fungal indole PTases,[4] few studies have probed the in vivo timing of the prenyltransfer reaction. In vitro kinetic analysis suggests tryptophan and tyrosine are prenylated by 7-DMATS[4d] and SirD,[4e] respectively, prior to incorporation into nonribosomal peptides, although the final product of the 7-DMATS-containing pathway is unknown and in vivo radioisotope incorporation studies contradict the in vitro kinetic analysis of SirD.[12] Numerous studies have revealed the promiscuity of bacterial[9a] and fungal[4a] prenyltransferases toward related aromatic substrates, making it difficult to solely extend in vitro observations to in vivo biological relevance. *N*-(1,1-Dimethyl-1-allyl)tryptophan synthase activity was recently reported for the fungal PTase CdpNPT, which functions to reverse prenylate various tryptophan-containing DKPs at the indole nitrogen yet exhibits a high level of in vitro promiscuity toward indoles such as tryptophan.[4b] In contrast to CdpNPT's preference for preformed peptides, CymD prenylates free tryptophan, providing both in vitro and in vivo evidence that prenylation occurs prior to NRPS-mediated incorporation into the cyclomarin and cyclomarazine peptides. The preference of CymA module 1 for **2** over tryptophan provided us a unique opportunity to prepare novel *N*-alkylated cyclomarin and cyclomarazine analogues, which in turn supported the pre-NRPS timing of CymD-catalyzed prenyl transfer. This work demonstrates the amenability of cyclomarin/cyclomarazine to

Table 1. NMR Spectroscopic Data for Cyclomarins M (**11**) and P (**12**) in $CDC1_3$

	cyclomarin M (**11**)			cyclomarin P (**12**)		
position[a]	δ_C[b]	δ_H[c], (*J* in Hz)	HMBC	δ_C[b]	δ_H[c] (*J* in Hz)	HMBC
1	170.8			170.5		
2	52.9	4.57, t (3.9)	1, 3, 5, 17	53.3	4.58, t (4.1)	1, 3, 5, 17
NH-2		6.70, d (2.9)	3, 17		6.75, d (1.9)	2, 3, 17
3	67.9	5.29, d (3.9)	1, 4, 5, 6	68.3	5.28, d (4.4)	1, 4, 5, 6
OH-3		4.13, s	2, 3		4.04[d], m	2, 3, 5
4	126.9	7.08[d], m	3, 5, 6, 7, 10, 11	125.4	7.21, s	2, 3, 5, 10, 11, 12
5	112.3			113.5		
6	125.3			127.9		
7	118.8	7.50, d (8.0)	5, 6, 8, 9, 10, 11	119.4	7.58, d (8.0)	4, 5, 8, 9, 10, 11
8	119.7	7.08[d], m	3, 5, 6, 7, 10, 11	120.4	7.11[d], m	4, 9, 10, 11
9	122.5	7.22, m	8, 10, 11	122.9	7.24, m	6, 7, 11
10	109.9	7.28, d (8.3)	6, 8	110.0	7.36, d (8.3)	4, 8, 9
11	137.0			136.2		
12	32.8	3.72, s	4, 11	35.8	4.79, d (2.4)	4, 11, 13, 14
13				77.1		
14				74.2	2.4, t (2.5)	12
17	172.4			172.6		
18	58.1	4.04, m	17, 19, 20, 24, 25	58.2	4.05[d], m	17, 19, 20, 24, 25
NH-18		8.00, d (9.4)	17, 18, 25		8.00, d (9.4)	17, 18, 25
19	35.5	1.60, s	18, 20, 21, 24	35.6	1.61, m	18, 20, 21, 23
20	124.7	4.71[d], m	22, 23, 24	124.8	4.72, d (9.9)	19, 22, 23
21	134.6			134.6		
22	25.7	1.23, s	18, 20, 21, 24	25.8	1.24, s	18, 20, 21, 24
23	19.5	1.70, s	18, 20, 21, 22, 24	18.9	1.72, s	18, 20, 21, 22, 24
24	18.6	0.62, d (6.5)	18, 19, 20	18.5	0.62, d (6.5)	18, 19, 20
25	168.5			168.5		
26	58.6	4.82, m	NMe-26	58.6	4.85, m	25, 27, 28, 31, NMe-26
NMe-26	29.5	2.82, s	26, 31	29.5	2.82, s	26, 31
27a	38.8	2.28, ddd (13.3, 10.9, 4.4)	25, 26, 28	38.9	2.25, m	25, 26, 28, 29
27b	38.8	1.02, m	25, 26, 28	38.9	1.03, m	25, 26. 28, 29, 30
28	25.2	1.43, m		25.0	1.45, m	27, 30
29	23.3	0.83, d (6.6)	27, 28	22.8	0.85, d (6.6)	27, 28
30	22.4	0.85, d (6.6)	27, 28	23.4	0.88, d (6.6)	27, 28, 29
31	170.4			171.4		
32	55.3	4.34, t (8.8)	31, 33, 34	55.3	4.35, t (8.7)	33, 34, 35, 36
NH-32		7.93, d (8.0)	32, 33, 36		7.93, d (8.0)	32, 33, 36
33	30.7	2.21, m	32	30.8	2.21, m	32, 34, 35
34	19.3	1.04, d (6.7)	32, 33, 35	19.2	1.04, d (6.7)	32, 33, 35
35	19.9	0.92, d (6.6)	32, 33, 34	20.5	0.92, d (6.7)	32, 33, 34
36	169.6			169.6		
37	55.9	4.88, m	36, 38, 39, 46	55.9	4.88, t (4.9)	36, 38, 39, 46
NH-37		7.14, d (4.7)	36, 37, 46		7.13[d], m	36, 37, 46
38	79.8	5.04, d (5.3)	37, 39, 45, 40−44	79.9	5.05, d (5.3)	36, 37, 39, 40−44, 45
39	135.1			135.2		38
40−44	127−128	7.23−7.25, m	38, 43	127−128	7.23−7.25, m	38, 39
45	57.8	3.34, s	38	57.8	3.34, s	38
46	171.7			171.8		
47	50.7	4.86, m	46, 48	50.6	4.86, m	46, 48
NH-47		8.19, d (10.3)	47, 49		8.17, d (10.2)	47, 49
48	20.8	1.29, d (7.3)	46, 47	20.8	1.28, d (7.3)	46, 47
49	169.7			169.1		
50	59.3	4.72[d], m	51, 52	59.3	4.68, dd (10.5, 3.0)	1, 49, 52, NMe-8
NMe-50	29.4	2.66, s	1, 50	29.3	2.64, s	1,50
51a	32.9	2.24, m	49, 52, 53, 54	32.8	2.16, m	49, 52, 53, 54
51b	32.9	0.58, ddd (13.9, 5.7, 3.2)	49, 52, 53, 54	32.8	0.41, m	49, 52, 53, 54
52	33.3	1.32, m	51, 54	33.2	1.26, m	50, 51, 53, 54
53a	66.1	3.23, m		66.3	3.12, m	51, 54
53b	66.1	3.16, m		66.3	3.18, m	51, 54
54	17.9	0.74, d (6.8)	51, 52, 53	17.7	0.65, d	52, 53

[a] Numbering based on ref 2. [b] Assignment by HMQC and HMBC correlations. [c] 600 MHz. [d] Overlapping signals.

diversification via mutasynthesis, providing novel analogues of these di- and heptapeptides and furthering our general understanding of NPRS specificity and amenability to engineering.

Experimental Section

General Experimental Procedures. Chemicals were acquired from Sigma-Aldrich or Fisher Scientific unless noted otherwise. NMR spectroscopic data were obtained on a Bruker 600 MHz spectrometer equipped with a 1.7 mm cryoprobe. LC analysis was performed on a Agilent 1200 series LC or a HP series 1100 LC-MS system utilizing electrospray ionization in positive mode with a linear gradient of 10−90% acetonitrile at 0.7 mL/min over 24 min utilizing a Luna 4.6 × 100 mm, 5 μm $C_{18}(2)$ column (Phenomenex). High-resolution mass spectra were collected by ESI-HR-FTMS at the Chemistry & Biochemistry Mass Spectrometry Facility, University of California San Diego, and HR-ESI-TOFMS at the Scripps Research Institute, La Jolla, CA.

Bacterial Strains and Culture Conditions. Wild-type *S. arenicola* CNS-205 and the prenyltransferase-deficient mutant *S. arenicola cymD*$^-$ were grown in A1+BFe media (10 g of starch, 4 g of yeast extract, 2 g of peptone, 40 mg of $Fe_2(SO_4)_3 \cdot 4H_20$, 100 mg of KBr, 1 L of seawater) at 28 °C and 200 rpm.[2] Production cultures were grown for

Table 2. NMR Spectroscopic Data for Cyclomarazines M (**6**) and P (**7**) in DMSO-d_6

	cyclomarazine M (**6**)			cyclomarazine P (**7**)		
position[a]	δ_C[b]	δ_H[c], (*J* in Hz)	HMBC	δ_C[b]	δ_H[c] (*J* in Hz)	HMBC
1	166.2			165.5		
2	55.7	4.10, dd (7.6, 4.3)	1, 3, 5, 17	55.6	4.12, dd (7.7, 4.3)	3, 5
NH-2		8.19, d (2.4)	1, 2, 18		8.20, d (2.5)	
3a	29.7	3.19, dd (14.4, 4.9)	1, 2, 4, 5, 6	29.7	3.18, dd (14.4, 4.9)	1, 2, 4, 5, 6
3b	29.7	3.00, dd (14.4, 4.5)	1, 2, 4, 5, 6	29.7	3.02, dd (14.4, 4.4)	1, 2, 4, 5, 6
4	128.7	7.02, s	3, 5, 6, 7, 11, 12	127.2	7.11, s	2, 3, 5, 6, 11, 12
5	108.4			109.3		
6	128.6			128.4		
7	118.9	7.50, d (7.9)	5, 6, 8, 11	119.1	7.53, d (7.9)	5, 6, 8, 9, 11
8	118.5	6.98, dd (7.5, 7.5)	4, 10	118.9	7.02, dd (7.3, 7.3)	6, 7, 10
9	121.0	7.11, dd (7.6, 7.6)	6, 8, 10, 11	121.3	7.14, dd (7.6, 7.6)	7, 11
10	109.4	7.34, d (8.2)	6, 8, 9	109.7	7.44, d (8.2)	8, 6, 9, 11
11	136.9			135.5		
12	32.1	3.72, s	4, 11	34.6	5.01, m	4, 11, 13, 14
13				79.1		
14				75.4	3.39, t (2.4)	12, 13
17	167.5			166.7		
18	58.6	3.57, dd (9.0, 3.2)	1, 17, 19, NMe-18	58.6	3.57, dd (9.0, 3.1)	1, 17, 19, 20
NMe-18	31.7	2.66, s	1, 18	31.6	2.68, s	1, 17, 18
19a	35.7	0.52, ddd (13.1, 9.5, 3.3)	17, 18, 20, 21, 22	35.0	0.55[d], m	17, 18, 20, 21, 22
19b	35.7	0.38, ddd (13.7, 9.1, 4.2)	17, 18, 20, 21, 22	35.0	0.41, ddd (13.7, 9.0, 4.3)	17, 18, 20, 21, 22
20	32.0	1.32, m	19, 21, 22	32.2	1.34, m	
21a	66.0	2.83, dd (10.3, 5.3)	19, 20, 22	65.9	2.81, m	19, 20
21b	66.0	2.72, dd (10.1, 7.0)	19, 20, 22	65.9	2.74, m	19, 20
OH-21		4.34, m			4.28, m	
22	15.9	0.57, d (6.6)	19, 20	15.9	0.58[d], d (6.6)	17, 19, 20, 21

[a] Numbering based on ref 2. [b] Assignment by HSQC and HMBC. [c] 600 MHz. [d] Overlapping signals.

10 days in 2.8 L Fernbach flasks containing 1 L of media unless stated otherwise. *Escherichia coli* DH5α was used for cloning and expression experiments as described.[13] DNA purification and manipulation were performed according to standard procedures.[13,14]

CymD Purification. The *cymD* gene (GenBank protein accession SARE_4565, genome accession CP00850) was amplified from genomic *S. arenicola* CNS-205 DNA via PCR utilizing the forward primer cymDF1 5′-CGTGGTTCGAGCTCTTGACCGAGGAGTTGACG-ACGGTCCG (SacI site underlined) and the reverse primer cymDR1 5′-GCTCGAATTCAAGCTTTCATTCGGTTCTCCCTCTCG (*Hin*dIII site underlined). Ligation into pHIS8[15] yielded plasmid pHIS8-*cymD*, which was transformed into *E. coli* BL21(DE3) (Invitrogen) for expression. Transformants were grown in ZYP-5052 autoinduction media[16] containing 50 μg/mL kanamycin at 16 °C for two days. Cells were harvested and lysed by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol) with the addition of 0.5 g/L lysozyme. The lysate was cleared by centrifugation at 20 000 rpm for 50 min, and the resulting supernatant was loaded onto a polypropylene column containing 6 mL of Ni-NTA agarose (Qiagen). The column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol) and eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 10% glycerol, 2 mM β-mercaptoethanol). The resulting eluate was placed in a 10 kD cutoff dialysis tube and dialyzed overnight against 2 L of phosphate buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 2 mM DTT). Octahistidyl-tagged CymD was further purified by FPLC (GE Healthcare) utilizing a Superdex 200 26/60 size exclusion column with phosphate buffer as the mobile phase, and fractions containing CymD were pooled and concentrated to a final concentration of 2.34 mg/mL using a Centriprep Ultracel YM-10 (10 kD cutoff, Millipore).

CymD Prenyltransferase Assay. His_8-tagged CymD (1 μg, 0.2 μM) was incubated with 1 mM L-tryptophan and 1 mM dimethylallyl pyrophosphate (DMAPP) in 100 μL of 50 mM imidazole buffer, pH 6.8, and incubated for 2 h at 37 °C. The reaction was quenched with 10 μL of 1.5 M TCA and centrifuged for 10 min at 13 000 rpm. The supernate was then analyzed by LC or LC-MS. To assay for isoprene pyrophosphate specificity, DMAPP was replaced with 1 mM isopentenyl pyrophosphate, geranyl pyrophosphate, or farnesyl pyrophosphate. Metal dependence was interrogated by including 10 mM Ca^{2+}, 10 mM Mg^{2+}, or no metal added with or without 50 mM EDTA. To characterize the product of of CymD-catalyzed tryptophan conversion, 3.25 mmol of L-tryptophan, 3.25 mmol of DMAPP, and 380 μg of enzyme were incubated in 3.25 mL of 50 mM imidazole buffer, pH 6.8, overnight at 37 °C. The reaction was dried in vacuo, redissolved in a minimal volume of MeOH, and fractionated by preparative HPLC utilizing an Onyx C_{18} 100 × 10 mm column (Phenomenex) in isocratic 40% MeOH at 5 mL/min, with *N*-(1,1-dimethyl-1-allyl)tryptophan eluting at 9.5 min, yielding 2.3 mg of product after removing the mobile phase in vacuo.

Purification of *N*-(1,1-Dimethyl-1-allyl)tryptophan from *S. arenicola* CNS-205. A 4 L production culture of *S. arenicola* CNS-205 was incubated with XAD-7HP resin for 2 h. The resin and cells were collected by filtration and extracted in acetone, which in turn was evaporated in vacuo. The pH of the remaining aqueous residue was adjusted to ∼14 with 10 N NaOH and washed with EtOAc. The aqueous phase was then adjusted to ∼pH 2 with concentrated HCl and extracted with EtOAc. The EtOAc fraction was dried over $MgSO_4$ and evaporated in vacuo. The resulting residue was fractionated using YMC ODS-A resin (60 mm × 100 mm) with a step gradient of aqueous 20% MeOH followed by 50% MeOH. Fractions containing *N*-(1,1-dimethyl-1-allyl)tryptophan (**2**) were concentrated in vacuo, and the remaining aqueous residue was acidified with glacial acetic acid to pH ∼4. Compound **2** was absorbed onto an equilibrated 1 g SCX cation-exchange SPE cartridge (Phenomenex) from the acidified fractions, which in turn was eluted with 3 M NH_4OH. The eluate was desalted using a 1 g C_{18} SPE cartridge (Thermo), yielding 2.3 mg of **2**: HRMS (ESI^+) *m/z* 273.1599 [M + H^+] (calcd for $C_{16}H_{21}N_2O_2$, 273.1597).

Synthesis of *N*-(1,1-Dimethyl-1-allyl)tryptophan (2). Literature procedures were utilized for the synthesis of *N*-(1,1-dimethyl-1-allyl)-Boc-Trp-OMe (**14**).[7] Compound **14** (190.0 mg, 0.492 mmol) was dissolved in THF (5 mL) and MeOH (0.5 mL). LiOH (250 μL, 1 M) was then added and the reaction monitored by TLC. After completion of hydrolysis, the reaction was acidified with 1 M HCl and repeatedly extracted with CH_2Cl_2. The organic extracts were combined, dried with brine, and then further dried over $MgSO_4$. The reaction mixture was concentrated and then deprotected with HCl·dioxane (4 mL, 4 M). After complete deprotection, the reaction mixture was concentrated to afford an off-white product (135.8 mg, 90% over two steps).

***N*-(1,1-Dimethyl-1-allyl)tryptophan (2):** mp 135−138 °C; $[\alpha]^{20.0}$ +21.1 (*c* 0.5, pyridine); IR (film) ν_{max} 2978, 2930, 1715, 1589, 1515, 1457, 1366, 1315, 1215, 1161, 919 cm^{-1}; ^1H NMR (D_2O, 600 MHz) δ 7.68 (1 H, d, *J* = 7.8 Hz), 7.66 (1 H, d, *J* = 8.4 Hz), 7.48 (1 H, s), 7.21 (1 H, t, *J* = 7.2 Hz), 7.17 (1 H, t, *J* = 7.8 Hz), 6.13 (1 H, dd, *J* = 17.4, 10.8 Hz), 5.22 (1 H, d, *J* = 10.8 Hz), 5.12 (1 H, d, *J* = 17.4 Hz), 4.28 (1 H, t, *J* = 6.0 Hz), 3.48 (1 H, dd, *J* = 15.6, 5.4 Hz), 3.39 (1 H, dd, *J* = 15.6, 7.2 Hz), 1.73 (H6, s); ^{13}C NMR (D_2O, 150 MHz) δ 171.2, 142.4, 134.1, 127.3, 125.1, 120.1, 118.4, 117.4, 113.5, 112.4,

104.0, 58.1, 52.6, 25.9, 24.7; HRMS (ESI$^+$) *m/z* 273.1600 [M + H$^+$] (calcd for $C_{16}H_{21}N_2O_2$, 273.1597).

Synthesis of *N*-(1-Propargyl)tryptophan. Boc-Trp-OMe (**13**, 150.0 mg, 0.471 mmol) was dissolved in DMF (3 mL), and NaH (60% dispersion in oil, 24.0 mg) was then added slowly at 0 °C. Propargyl bromide (63 μL, 80 wt % solution in toluene) was added dropwise over 5 min. After 1 h at 0 °C, TLC showed complete consumption of starting material and the reaction was quenched with aqueous NH_4Cl. The reaction was extracted three times with EtOAc, and the combined EtOAc extracts were then washed with water and brine three times. The crude mixture was dried over $MgSO_4$ and passed through a silica gel plug (1:3 EtOAc/hexanes v/v) to afford a colorless oil (141.8 mg, 84%). The alkylated material (**15**) was then dissolved in THF (5 mL) and hydrolyzed with LiOH (1.0 mL, 0.5 M). The reaction was complete by TLC after 2 h and was acidified with 1 M HCl. The reaction was then extracted several times with CH_2Cl_2, and the organic layer was sequentially dried with brine and $MgSO_4$. The crude acid was deprotected with HCl·dioxane (4 mL, 4 M) and monitored by LC-MS. After complete conversion to product, the reaction was then concentrated to provide a yellow solid (110.1 mg, 99% over two steps).

***N*-(1-Propargyl)-Boc-Trp-OMe (15):** $[\alpha]^{20.0}$ +30.3 (*c* 1.0, $CHCl_3$); IR (film) ν_{max} 3284, 2977, 1742, 1709, 1500, 1467, 1438, 1366, 1250, 1165, 1060, 1015 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.58 (1 H, d, J = 7.8 Hz), 7.35 (1 H, d, J = 8.4 Hz), 7.26 (1 H, t, J = 6.6 Hz), 7.16 (1 H, t, J = 7.2 Hz), 7.02 (1 H, s), 5.17 (1 H, d, J = 7.8 Hz), 4.78 (2 H, d, J = 2.4 Hz), 4.67 (1 H, br q, J = 7.8 Hz), 3.69 (3 H, s), 3.31 (1 H, dd, J = 15.0, 5.4 Hz), 3.26 (1 H, dd, J = 15.0, 6.0 Hz), 2.41 (1 H, t, J = 2.4 Hz), 1.46 (9 H, s); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 172.7, 155.2, 136.0, 128.6, 125.8, 122.1, 119.7, 119.1, 109.8, 109.4, 79.7, 77.7, 73.6, 54.2, 52.2, 35.6, 28.3, 27.9; HRMS (ESI$^+$) *m/z* 357.1814 [M + H$^+$] (calcd for $C_{20}H_{25}N_2O_4$, 357.1809); TLC R_f 0.59 [EtOAc/hexanes (3:7)].

***N*-(1-Propargyl)tryptophan (4):** mp dec > 175 °C; $[\alpha]^{20.0}$ +44.0 (*c* 0.5, pyridine); IR (film, cm^{-1}) 3265, 2853, 1731, 1584, 1485, 1464, 1329, 1204, 1181, 1058; ^{1}H NMR (D_2O, 600 MHz) δ 7.70 (1 H, d, J = 7.8 Hz), 7.55 (1 H, d, J = 8.4 Hz), 7.35 (1 H, t, J = 7.2 Hz), 7.32 (1 H, s), 7.23 (1 H, t, J = 7.2 Hz), 4.98 (1 H, d, J = 1.8 Hz), 4.27 (1 H, dd, J = 7.2, 5.4 Hz), 3.48 (1 H, dd, J = 15.0, 4.8 Hz), 3.38 (1 H, dd, J = 15.6, 7.2 Hz), 2.75 (1 H, s); ^{13}C NMR (D_2O, 150 MHz) δ 171.5, 135.2, 127.4, 126.8, 121.8, 119.4, 118.1, 109.6, 106.2, 78.0, 73.0, 52.8, 34.6, 25.0; HRMS (ESI$^+$) *m/z* 243.1130, [M + H$^+$] (calcd for $C_{14}H_{15}N_2O_2$, 243.1128).

Chemical Complementation of the *cymD*$^-$ Mutant and the Production of Novel Cyclomarin and Cyclomarazine Analogues via Mutasynthesis. Duplicate 50 mL cultures of *S. arenicola* CNS-205 and the *cymD*$^-$ mutant strain were grown in 250 mL Erlenmeyer flasks containing stainless steel springs. To duplicate mutant cultures, 4 mg of tryptophan, *N*-(1,1-dimethyl-1-allyl)tryptophan (**2**), *N*-(1-methyl)-tryptophan (**3**), or *N*-(1-propargyl)tryptophan (**4**) was added as 1 mg aliquots daily from day 6 through and including day 9. On day 10, the cultures were extracted with EtOAc, and the crude residues analyzed by analytical LC-MS as described above. For the production of analogues, 1 L cultures of the *cymD*$^-$ mutant were administered compound **3** (1 g) or **4** (50 mg) dissolved in H_2O in four equal daily aliquots starting on the sixth day of growth. The cultures were extracted by EtOAc partitioning to yield approximately 130 mg of crude organic extracts, which were fractionated utilizing Sephadex LH-20 resin (GE Healthcare, 35 g of resin in a 30 mm wide column) with MeOH as the mobile phase. Fractions containing the target compound, as judged on the basis of ESIMS, were pooled, dried in vacuo, and subjected to purification via preparative HPLC utilizing a Luna C_{18}(2) 250 × 10 mm column (Phenomenex) under isocratic conditions with a flow rate of 2.5 mL/min and monitored at 210 nm. Methyl indole derivatives cyclomarazine **6** (0.4 mg) eluted at 33 min with 21% MeCN and cyclomarin **11** (2.6 mg) eluted at 36 min with 52% MeCN. Propargyl derivatives cyclomarazine **7** (1.5 mg) eluted at 33 min with 24% MeCN, while cyclomarin **12** (1.0 mg) eluted at 29 min with 56% MeCN.

Cyclomarazine M (6): NMR data, see Table 2; HRMS (ESI$^+$) *m/z* 366.1790 [M + Na]$^+$ (calcd for $C_{19}H_{25}N_3O_3Na$, 366.1788).

Cyclomarazine P (7): NMR data, see Table 2; HRMS (ESI$^+$) 390.1790 *m/z* [M + Na]$^+$ (calcd for $C_{21}H_{25}N_3O_3Na$, 390.1788).

Cyclomarin M (11): NMR data, see Table 1; HRMS (ESI$^+$) 995.5587 *m/z* [M + Na]$^+$ (calcd for $C_{52}H_{76}N_8O_{10}Na$, 995.5577).

Cyclomarin P (12): NMR data, see Table 1; HRMS (ESI$^+$) 1019.5582 *m/z* [M + Na]$^+$ (calcd for $C_{54}H_{76}N_8O_{10}Na$, 1019.5577).

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Supporting Information Available: Additional materials and methods, bioinformatic analysis, and spectrophotometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

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