Deciphering a Cyclodipeptide Synthase Pathway Encoding Prenylated Indole Alkaloids in *Streptomyces leeuwenhoekii*

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ABSTRACT Cyclodipeptide synthases (CDPSs) catalyze the formation of cyclodipeptides using aminoacylated tRNAs as the substrates and have great potential in the production of diverse 2,5-diketopiperazines (2,5-DKPs). Genome mining of *Streptomyces leeuwenhoekii* NRRL B-24963 revealed a two-gene locus, *saz*, encoding CDPS SazA and a unique fused enzyme (SazB) harboring two domains: phytoene synthase-like prenyltransferase (PT) and methyltransferase (MT). Heterologous expression of the *saz* gene(s) in *Streptomyces albus* J1074 led to the production of four prenylated indole alkaloids, among which streptoazines A to C (compounds 3 to 5) are new compounds. Expression of different gene combinations showed that the SazA catalyzes the formation of *cyclo*(L-Trp-L-Trp) (cWW; compound 1), followed by consecutive prenylation and methylation by SazB. Biochemical assays demonstrated that SazB is a bifunctional enzyme, catalyzing sequential C-3/C-3' prenylation(s) by SazB-PT and N-1/N-1' methylation(s) by SazB-MT. Of note, the substrate selectivity of SazB-PT and SazB-MT was probed, revealing the stringent specificity of SazB-PT but relative flexibility of SazB-MT.

IMPORTANCE Natural products with a 2,5-diketopiperazine (2,5-DKP) skeleton have long sparked interest in drug discovery and development. Recent advances in microbial genome sequencing have revealed that the potential of cyclodipeptide synthase (CDPS)-dependent pathways encoding new 2,5-DKPs are underexplored. In this study, we report the genome mining of a new CDPS-encoding two-gene operon and activation of this cryptic gene cluster through heterologous expression, leading to the discovery of four indole 2,5-DKP alkaloids. The *cyclo*(L-Trp-L-Trp) (cWW)-synthesizing CDPS SazA and the unusual prenyltransferase (PT)-methyltransferase (MT) fused enzyme SazB were characterized. Our results expand the repertoire of CDPSs and associated tailoring enzymes, setting the stage for accessing diverse prenylated alkaloids using synthetic biology strategies.

KEYWORDS cyclodipeptide synthase, streptoazine, heterologous expression, *Streptomyces*, methyltransferase, prenyltransferase

Actinomycetes are important contributors to drug discovery because of their valued production of diverse bioactive natural products (1). Microbial genome sequencing reveals abundant untapped secondary metabolite biosynthetic gene clusters (BGCs); however, most of them are silent or poorly expressed under conventional laboratory conditions, thus severely hampering novel compound discovery (2). With the wealth of genome sequences available, large numbers of secondary metabolite BGCs await to be linked to their encoded products.

2,5-Diketopiperazine (DKP)-containing natural products represent an intriguing class of secondary metabolites with notable biological activities (3). The DKP ring system can be biosynthesized via two machineries in nature: nonribosomal peptide

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synthetases (NRPSs) and cyclodipeptide synthases (CDPSs) (4). Unlike multimodular NRPSs, CDPSs are small sized and readily manipulated, hijacking aminoacyl-tRNAs from the ribosomal machinery; moreover, they display outstanding performance in generating chemically diverse 2,5-DKPs through the incorporation of proteinogenic or noncanonical amino acids (5). Noticeably, various tailoring enzymes are associated with CDPSs and introduce multifarious chemical modifications onto the 2,5-DKP skeletons, including dehydrogenation (6–8), methylation (7–10), prenylation (11), dimerization (12, 13), cyclization (11), and hydroxylation (14, 15), to further expand the chemical space of 2,5-DKP derivatives (5, 16). However, to date, only limited numbers of CDPS-encoding gene clusters have been functionally characterized, albeit bioinformatic analyses disclose their abundant presence, indicating great potential of mining complex 2,5-DKP derivatives by using CDPS as the “biosynthetic hooks” (5, 17).

Tryptophan-containing DKPs have received great attention due to the promising electron-rich indole ring in the tryptophanyl moiety, which can undergo various enzymatic or spontaneous modifications and rearrangement to increase structural and chemical complexity (3, 18). As the prenyl groups at the periphery of the molecules are able to increase lipophilicity to favor interactions with biological membranes, prenylation of indole DKPs is considered to be closely related to their bioactivities (19). Further modifications, such as oxidation (20), cyclization (21), and methylation (11), would generate a plethora of complex DKP derivatives with a wide range of bioactivities (18). Previously, we identified a CDPS pathway harboring a phytoene synthase-like (PSL) family prenyltransferase (PT) with relaxed substrate specificity, which is able to transfer the farnesyl group onto *cyclo*(L-Trp-L-X) (cWX) (X is Ala, Pro, Val, Leu, or Ile) (11). To enrich the functional parts of the CDPS-dependent biosynthetic pathways for further synthetic biology studies, herein, *in silico* analysis of the *Streptomyces* genomes in GenBank was carried out using cWX-synthesizing CDPS and PSL family PT as the hooks. A CDPS locus, *saz*, was thus discovered from *Streptomyces leeuwenhoekii* NRRL B-24963. Through heterologous expression and *in vitro* experiments, the biosynthetic pathway encoded by the *saz* locus was elucidated along with the discovery of three new prenylated indole alkaloids (Fig. 1), providing new opportunity for delivering bioactive 2,5-DKP derivatives via manipulation of the CDPS-dependent pathways.

RESULTS

Bioinformatic analysis of the *saz* genes. Through genome mining, a two-gene CDPS genetic locus, *sazA-sazB*, was detected from *S. leeuwenhoekii* NRRL B-24963 (Fig. 2A). Phylogenetic analysis revealed that SazA belongs to the NYH subfamily and is clustered with the cWX-synthesizing CDPSs (see Fig. S1 in the supplemental material). Comparison of the sequence logos of SazA in pockets P1 and P2 with those of known CDPSs indicated SazA is possibly responsible for the formation of *cyclo*(L-Trp-L-Trp) (cWW) (see Table S1). Interestingly, SazB harbors a PSL family PT domain (from amino acids [aa] 1 to 314, named SazB-PT) as well as a methyltransferase (MT) domain (378 to 653 aa, named SazB-MT). SazB-PT displays 33.6% identity/47.3% similarity to DmtC1 (AVP32202), which is involved in the prenylation step during the biosynthesis of drimentines (DMTs) (11); SazB-MT displays 40.4% identity/51.2% similarity to DmtMT2-1 (WP_052397356.1), which catalyzes *N*-methylation of pre-DMTs and DMTs (10) (Table 1). Therefore, we speculated that this locus possibly encodes indole alkaloids with modifications of prenylation and methylation.

Heterologous expression of the *saz* genes. To understand the function of this locus, we first cloned the CDPS gene *sazA* into pET28a to generate pWLI640, which was subsequently overexpressed in *Escherichia coli* BL21(DE3). High-pressure liquid chromatography (HPLC) analysis of the culture supernatants showed that only one compound (compound 1) showed up in *E. coli*/pWLI640 (Fig. 2Bii), which was identified as *cyclo*(L-Trp-L-Trp) (cWW) based on the retention time (Fig. 2Biii) and the high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data (see Fig. S2). This result demonstrated that SazA is a cWW-synthesizing CDPS, consistent with the bioinformatic analysis result (Fig. S1 and Table S1).

FIG 1 Chemical structures of compounds 1 to 5.

To figure out the function of *sazB*, we then constructed two versions of expression vectors, pWLI644 and pWLI645, respectively containing *sazA-sazB-PT* (where *PT* is the gene encoding prenyltransferase) and *sazA-sazB* under the control of the constitutive promoter P_{gapdh} . Considering the influences caused by different genetic contexts, pWLI644 and pWLI645 were introduced into both *Streptomyces coelicolor* M1146 and *Streptomyces albus* J1074. HPLC analysis of the fermentation products of the heterologous expression strains showed (i) no product when using *S. coelicolor* M1146 as the heterologous expression host (see Fig. S3), which might be due to low expression levels of the *saz* genes in M1146, and (ii) accumulation of two compounds (compounds 2 and 3) when pWLI644, carrying *sazA* and *sazB-PT*, was expressed in J1074 (Fig. 2Cii); another two compounds (compounds 4 and 5) appeared when pWLI645, harboring *sazA* and the entire *sazB*, was expressed in J1074 (Fig. 2Ciii).

Structure elucidation of compounds 2 to 5. Subsequently, large-scale fermentation of J1074/pWLI645 was carried out, followed by the chemical isolation as described in Materials and Methods, which led to the identification of compounds 2 to 5. Their structures were elucidated on the basis of the HR-ESI-MS (see Fig. S4) and nuclear magnetic resonance (NMR) analysis (^1H NMR, ^{13}C NMR, correlation spectroscopy [COSY], heteronuclear single quantum coherence [HSQC], heteronuclear multiple-bond correlation [HMBC], and nuclear Overhauser effect spectroscopy [NOESY]) (Table 2, Fig. 3, and Fig. S5 to S8).

FIG 2 Heterologous expression of the *saz* gene(s). (A) The *saz* genes from *S. leeuwenhoekii* NRRL B-24963. CDPS, cyclodipeptide synthase; PT, prenyltransferase; MT, methyltransferase. (B) HPLC traces of the culture supernatants of *E. coli* cells expressing SazA. Chromatograms show the absorbance at 280 nm. (C) HPLC analysis of the fermentation products of *S. albus* J1074 harboring *saz* genes. Chromatograms show the absorbance at 300 nm.

The HR-ESI-MS data for compounds 2 to 5 established the molecular formulae of compound 2 as $C_{27}H_{28}N_4O_2$ (m/z $[M+H]^+$ 441.2287, calculated [calcd] 441.2246) (Fig. S4A), compound 3 as $C_{32}H_{36}N_4O_2$ (m/z $[M+H]^+$ 509.2918, calcd 509.2872) (Fig. S4B), compound 4 as $C_{33}H_{38}N_4O_2$ (m/z $[M+H]^+$ 523.3073, calcd 523.3028) (Fig. S4C), and compound 5 as $C_{34}H_{40}N_4O_2$ (m/z $[M+H]^+$ 537.3234, calcd 537.3185) (Fig. S4D). The molecular weights of compounds 2 and 3 are 68 and 134 mass units larger, respectively, than that of cWW, indicating the presence of one C_5 unit in compound 2, and two C_5 units or one C_{10} unit in compound 3. The further increased molecular weights of compounds 4 and 5 by 14 and 28 mass units, respectively, compared to that of compound 3 suggested subsequent methyl substitution(s).

The one-dimensional (1D) and two-dimensional (2D) NMR (dimethyl sulfoxide $[DMSO]-d_6$) data from compounds 2 to 5 indicated a bis-indole diketopiperazine scaffold, inclusive of one prenyl moiety (δ_H 5.01, δ_C 119.7, δ_H 2.45, δ_C 34.2, δ_H 1.63, δ_C 25.7, δ_H 1.58, δ_C 18.0, δ_C 134.0) in compound 2, and two prenyl moieties (δ_H 4.97 and 5.05, δ_C 119.7 and 120.2, δ_H 2.48 and 2.56, δ_C 32.8 and 34.3, δ_H 1.62 and 1.65, δ_C 25.6 and 26.2, δ_H 1.59 and 1.61, δ_C 18.0 and 18.5, δ_C 134.1 and 134.6) in compounds 3 to 5. Based on the HMBC correlations from H-12 (δ_H 2.45) to C-2 (δ_C 79.8), C-3 (δ_C 55.1) and C-3a (δ_C 132.6) (Table 2; Fig. S5E), we determined a regular C-3 prenylation in compound 2. Similarly, two regular prenyl moieties were symmetrically attached to C-3 and C-3' in compounds 3 to 5. Comparison of the 1H and ^{13}C NMR data for compounds 4 and 5 with those for compound 3 revealed that they share similar structures, except

TABLE 1 Predicted functions of *saz* genes

Protein	Size (aa)	Proposed function	Homolog	
			Protein/organism	Accession no. (identity/similarity %)
SazA	253	Cyclodipeptide synthase	AlbC/ <i>Streptomyces griseocarneus</i>	RLV08931.1 (25.8/37.3)
SazB				
SazB-PT	314	Phytoene synthase	DmtC1/ <i>Streptomyces youssoufiensis</i>	AVP32202 (33.6/47.3)
SazB-MT	276	Methyltransferase	DmtMT2-1/ <i>Streptomyces</i> sp. NRRL F-5123	WP_052397356.1 (40.4/51.2)

TABLE 2 ^1H and ^{13}C NMR data of compounds 2 to 5 in $\text{DMSO}-d_6^a$

Position	NMR finding for compound:							
	2		3		4		5	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
N-1	6.56 (1H, d, 3.0)		6.48 (1H, d, 3.6)					
2	5.28 (1H, d, 3.0)	79.8	5.26 (1H, d, 3.6)	79.9	5.08 (1H, s)	86.2	5.10 (1H, s)	86.5
3		55.1		56.1		55.3		55.4
3a		132.6		132.9		132.6		132.7
4	7.05 (1H, d, 7.2)	122.4	7.07 (1H, d, 7.2)	122.6	7.09 (1H, m)	121.7	7.11 (1H, d, 7.0)	121.8
5	6.58 (1H, td, 7.2, 1.2)	117.5	6.59 (1H, t, 7.2)	118.1	6.67 (1H, td, 7.8, 1.2)	118.1	6.70 (1H, t, 7.5)	118.3
6	6.95 (1H, td, 7.2, 1.2)	127.8	6.93 (1H, td, 7.8)	128.3	7.06 (1H, td, 7.8, 1.2)	128.2	7.08 (1H, t, 7.5)	128.2
7	6.49 (1H, d, 7.2)	108.8	6.43 (1H, d, 7.8)	109.4	6.41 (1H, d, 7.8)	107.7	6.47 (1H, d, 8.0)	107.9
7a		148.6		148.9		149.8		150.0
8	2.32 (1H, dd, 13.2, 7.8)	39.2	2.40 (1H, dd, 13.2, 7.2)	39.8	2.32 (1H, dd, 13.2, 7.2)	38.4	2.38 (1H, dd, 13.0, 7.5)	38.6
	1.97 (1H, dd, 12.6, 10.2)		1.95 (1H, dd, 13.2, 10.8)		1.89 (1H, dd, 13.2, 11.4)		1.94 (1H, t, 12.0)	
9	4.50 (1H, t, 9.0)	57.4	4.66 (1H, dd, 10.8, 7.2)	59.5	4.73 (1H, m)	59.9	4.76 (1H, dd, 11.0, 7.0)	60.5
10		169.8		168.2		167.8		169.1
11								
12	2.45 (2H, d, 7.2)	34.2	2.51 (2H, m)	34.3	2.52 (2H, m)	32.5	2.56 (2H, m)	32.8
13	5.01 (1H, m)	119.7	4.99 (1H, t, 7.2)	120.2	4.97 (1H, m)	119.7	4.99 (1H, t, 7.0)	119.7
14		134.0		134.6		134.1		134.2
15	1.63 (3H, s)	25.7	1.62 (3H, s)	26.2	1.62 (3H, s)	25.7	1.63 (3H, s)	25.6
16	1.58 (3H, s)	18.0	1.59 (3H, s)	18.5	1.60 (3H, s)	18.0	1.61 (3H, s)	18.0
17					2.71 (3H, s)	35.7	2.87 (3H, s)	36.2
1'	10.85 (1H, s)		6.48 (1H, d, 3.6)		6.53 (1H, d, 3.0)			
2'	7.22 (1H, d, 2.4)	124.2	5.26 (1H, d, 3.6)	79.9	5.26 (1H, d, 3.0)	79.5	5.10 (1H, s)	86.5
3'		109.5		56.1		56.0		55.4
3a'		127.3		132.9		132.3		132.7
4'	7.57 (1H, d, 7.8)	118.4	7.07 (1H, d, 7.2)	122.6	7.09 (1H, m)	122.5	7.11 (1H, d, 7.0)	121.8
5'	6.98 (1H, td, 7.8, 1.2)	118.3	6.59 (1H, t, 7.2)	118.1	6.60 (1H, td, 7.8, 1.2)	117.6	6.70 (1H, t, 7.5)	118.3
6'	7.07 (1H, td, 7.8, 1.2)	120.9	6.93 (1H, td, 7.8)	128.3	6.95 (1H, td, 7.8, 1.2)	127.9	7.08 (1H, t, 7.5)	128.2
7'	7.32 (1H, d, 7.8)	111.3	6.43 (1H, d, 7.8)	109.4	6.47 (1H, d, 7.8)	109.0	6.47 (1H, d, 8.0)	107.9
7a'		136.0		148.9		148.5		150.0
8'	3.37 (1H, m)	24.6	2.40 (1H, dd, 13.2, 7.2)	39.8	2.43 (1H, dd, 13.2, 7.8)	38.5	2.38 (1H, dd, 13.0, 7.5)	38.6
	3.00 (1H, dd, 15.10, 6.6)		1.95 (1H, dd, 13.2, 10.8)		2.13 (1H, dd, 13.2, 9.0)		1.94 (1H, t, 12.0)	
9'	4.36 (1H, t, 5.4)	55.2	4.66 (1H, dd, 10.8, 7.2)	59.5	4.66 (1H, t, 8.4)	59.7	4.76 (1H, dd, 11.0, 7.0)	60.5
10'		167.8		168.2		169.1		169.1
11'	7.64 (1H, s)							
12'			2.51 (2H, m)	34.3	2.48 (2H, m)	34.0	2.56 (2H, m)	32.8
13'			4.99 (1H, t, 7.2)	120.2	5.05 (1H, m)	119.7	4.99 (1H, t, 7.0)	119.7
14'				134.6		134.1		134.2
15'			1.62 (3H, s)	26.2	1.65 (3H, s)	25.7	1.63 (3H, s)	25.6
16'			1.59 (3H, s)	18.5	1.59 (3H, s)	18.0	1.61 (3H, s)	18.0
17'							2.87 (3H, s)	36.2

^a ^1H NMR data were collected at 500 MHz for compound 5 and at 600 MHz for compounds 2 to 4. ^{13}C NMR data were collected at 125 MHz for compound 5 and at 150 MHz for compounds 2 to 4.

for an *N*-methyl group (δ_{H} 2.71, δ_{C} 35.7) in compound 4 and two *N*-methyl groups (δ_{H} 2.87, δ_{C} 36.2) in compound 5.

In the NOESY spectrum of compound 3 (Fig. 3 and Fig. S6F), the strong correlations of H-12/H-9 and H-9/H-2 proved that H-2, H-9, and H-12 are located on the same side. Given the fact that the CDPS-synthesized cWW, being derived from proteinogenic acids, has an *S*-configuration, both C-2 and C-3 were assigned to an *R*-configuration. The positive cotton effects at 296 nm in the circular dichroism (CD) spectra of compounds 4 and 5 (22) further supported the *R*-configured C-2 and C-3 (see Fig. S9). Thus, compounds 3 to 5 were finally identified as new prenylated cyclopeptides, named streptoazines A to C. The ^1H and ^{13}C NMR chemical shifts of compounds 2 to 5 are listed in Table 2.

Biochemical characterization of SazB. Based on the above-described results, SazB should be a bifunctional enzyme, in charge of the attachments of the dimethylallyl group to C-3/C-3' and the methyl group to N-1/N-1' of the indole moiety. To validate the function of SazB, the entire SazB as well as the PT domain (SazB-PT) and the MT

FIG 3 Selected key ^1H - ^1H COSY (bold bonds, black), HMBC (red arrows), and NOESY (dashed blue double-headed arrows) correlations of compound 3.

domain (SazB-MT) were expressed in *E. coli* (see Fig. S10). Biochemical reactions were set up followed by HPLC analysis. As shown in Fig. 4A, when using cWW (compound 1) as the substrate, SazB (trace ii) and SazB-PT (trace iii) transfer the prenyl group onto compound 1 in a regular manner to generate monoprenylated (compound 2) and bis-prenylated product, streptoazine A (compound 3), in the presence of dimethylallyl pyrophosphate (DMAPP). With further addition of *S*-adenosylmethionine (SAM), SazB (trace iv) as well as the discrete SazB-PT and SazB-MT (trace v) catalyze the formation of bis-methylated compound streptoazine C (compound 5), together with the disappearance of compound 3; conversely, with the presence of SAM only, no product was generated (traces vi and vii). These results suggested that prenylation happens prior to methylation. Compounds 2 and 3 were then tested as the substrate for SazB-MT. HPLC analysis (Fig. 4B) showed time-dependent conversion of compound 3 to the monomethylated compound streptoazine B (compound 4) and the bis-methylated compound streptoazine C (compound 5) (traces iv to vii); in contrast, no product peak was observed with compound 2 under the same reaction conditions (trace ii). These results undoubtedly demonstrate that SazB is responsible for the sequential assembly of a prenyl group and methyl group during streptoazine biosynthesis.

Substrate selectivity of SazB-PT and SazB-MT. The substrate selectivity of SazB-PT was further investigated. Different tryptophan-containing DKPs (i.e., cWA, cWD, cWF, cWP, and cWY) (see Fig. S11) were tested as the prenyl acceptors. As shown in Fig. 5A, SazB-PT specifically recognized cWW, and alteration of the second Trp led to detrimental effects. Then, DMAPP, geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}) were probed as the prenyl donors (Fig. 5B). The result revealed that SazB-PT can only accept DMAPP, suggesting that SazB-PT has strict substrate specificity toward the prenyl donor as well.

Given that SazB-MT displays considerable homology to DmtMT-1 from the drimentine pathway (12), pre-DMTs (pre-DMT G, pre-DMT A, and pre-DMT C) and DMTs (DMT G, DMT A, and DMT C) were used to probe the substrate flexibility of SazB-MT. As shown in Fig. 6A, SazB-MT was able to catalyze *N*-4 methylation of all the pre-DMTs tested to generate *N*-4-methyl-pre-DMTs; in contrast, SazB-MT did not recognize DMTs (Fig. 6B). These results indicate that SazB-MT has certain flexibility toward the DKP ring, but the linear farnesyl chain is necessary for its activity.

DISCUSSION

The abundant untapped CDPS-encoding gene clusters represent great potent biosynthetic machineries to produce novel 2,5-DKP derivatives. Recently, diverse 2,5-DKP derivatives from CDPS-encoding BGCs have been discovered in various *Actinobacteria* strains, such as BGCs encoding 1-(8-guaninyl)-cyclo-L-Trp-L-Trp (23), purincyclamide (8), guanitryptmycin, mono/bimethylated ditryptophan (9), naseseazines (12, 13), and drimnetines (10, 11). In this study, we identified a CDPS-encoding gene locus (*saz*) in *S. leeuwenhoekii* NRRL B-24963 and disclosed its products to be indole 2,5-DKP alkaloids

FIG 4 HPLC analysis of the SazB-catalyzed biochemical reactions. (A) *In vitro* reactions of SazB with cWW (compound 1). (B) *In vitro* reactions of SazB-MT with 3-dimethylallyl-cyclo (L-Trp-L-Trp) (compound 2) and streptozine A (compound 3). Chromatograms show the absorbance at 300 nm.

streptozines (Fig. 1). The biosynthetic machinery of streptozines was elucidated through *in vivo* and *in vitro* studies, opening up new opportunities for accessing diverse pyrroloindole alkaloids using synthetic biology strategies.

Here, for heterologous expression of *saz* genes, two different *Streptomyces* strains were used, but only *S. albus* J1074 worked, which is not uncommon due to varied genetic contexts between different hosts (24). Through *in vivo* expression and *in vitro* biochemical assays, the biosynthetic machinery encoded by *saz* was disclosed as indicated in Fig. 7. SazA synthesizes a sole DKP product, cWW, and then SazB-PT catalyzes

FIG 5 Substrate selectivity of SazB-PT. (A) *In vitro* reactions of SazB-PT with different prenyl acceptors. (B) *In vitro* reactions of SazB-PT with different prenyl donors. Chromatograms show the absorbance at 300 nm.

FIG 6 Substrate selectivity of SazB-MT. (A) *In vitro* reactions of SazB-MT with pre-DMTs. (B) *In vitro* reactions of SazB-MT with DMTs. In the presence of SAM, SazB-MT converted pre-DMTs to *N*-4-methyl-pre-DMTs. Chromatograms show the absorbance at 300 nm.

the coupling of cWW with the first dimethylallyl group in a regular manner resulting in compound 2, followed by symmetrical introduction of the second dimethylallyl moiety to give streptoazine A (compound 3); further methylation by SazB-MT occurs on the N-1/N-1' position(s) of streptoazine A, generating streptoazine B (compound 4) and C (compound 5).

While remarkable progress has been made in identification and characterization of indole PTs involved in prenylated DKP alkaloids biosynthesis, most of them are members of the soluble indole PT superfamily and prenylate at all possible positions of the indole ring (N-1, C-2, C-3, C-4, C-5, C-6, and C-7) (25, 26). Recently, we characterized the PSL-type indole PT DmtCs responsible for C-3-regular prenylation, which showed no homology to the identified bacterial or fungal indole PTs (11). As a new member of the PSL-type indole PTs, SazB-PT is highly specific toward both the prenyl donor and the prenyl acceptor (Fig. 5); in contrast, its homolog DmtCs from the DMT pathway exhibit substrate flexibilities (11) regardless of their considerable similarity and identity (33.2%

FIG 7 The proposed biosynthetic pathway encoded by the *saz* locus.

to 36.8% and 45.3% to 49.4%, respectively). The other PSL-type PT NzsG from the neo-carazostatin pathway, which shows much lower similarity and identity (25.5% and 37.3%, respectively) to SazB-PT, was also reported to display strict substrate specificity, transferring only DMAPP onto precarazostatin (27). The underlining molecular basis for substrate selectivity of PSL-PTs needs further investigation.

Until now, seven MTs from CDPS-dependent pathways have been functionally characterized: O-MT Ndas_1149 (nocazine biosynthesis) (6), C-MT StspM1 (nocardioazine biosynthesis) (25), and four N-MTs, Amir_4628 (cWW-Me/Me₂) (9), GutE/PcmE (guanitrypmycin/purincyclamide biosynthesis) (7, 8), and DmtMT1 and DmtMT2-1 (DMT biosynthesis) (12). GutE/PcmE transfers a methyl group onto the guaninyl residue (7, 8), Amir_4628 (9) and DmtMT1 (12) conduct methylation at the DKP ring nitrogens, and DmtMT2-1 catalyzes methylation at the dihydroindole nitrogen of pre-DMTs and DMTs (12). Similar to DmtMT2-1, SazB-MT is also a dihydroindole N-MT, symmetrically introducing methyl groups onto the N-1/N-1' position(s) of compound 3 to give compounds 4 and 5 (Fig. 4). It is worth mentioning that SazB-MT displays certain substrate flexibility and also recognized pre-DMTs but not DMTs (Fig. 6); in contrast, DmtMT2-1 catalyzes N-4 methylation of both pre-DMTs and DMTs. The substrate bias between SazB-MT and DmtMT2-1 would open up new opportunities for the rational synthesis of methylated 2,5-DKP derivatives.

Nocardioazines, originally isolated from *Nocardioopsis* sp. strain CMB-M0232, feature a 2,5-DKP core functionalized with indole C-3-isoprenyl and indoline N-methyl as well as an unusual C-3-methyl group (28). A recent study demonstrated that StspM1 mediates C-3 methylation of the indole ring and pyrroloindoline cyclization. However, the N methylation and C-3 isoprenylation steps remain uncharacterized (29). Hence, our results would enlighten deciphering the integral biosynthetic machinery of nocardioazine-like natural products.

In summary, we identified an uncharacterized CDPS locus (*saz*) from *S. leeuwenhoe-kii* NRRL B-24963. Heterologous expression illustrated the encoding products of *saz*,

TABLE 3 Bacteria and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> DH5 α	Host strain for general cloning	Stratagene
<i>E. coli</i> ET12567/pUZ8002	Host strain for conjugation	35
<i>E. coli</i> BL21(DE3)	Protein expression host	Invitrogen
<i>E. coli</i> BL21(DE3)-groELS	Protein expression host	Washington State University
<i>E. coli</i> CodonPlus	Protein expression host	Invitrogen
<i>Streptomyces leeuwenhoekii</i> NRRL B-24963	Wild-type strain	NRRL
<i>Streptomyces coelicolor</i> M1146	Host strain for heterologous expression	36
<i>Streptomyces albus</i> J1074	Host strain for heterologous expression	37
Plasmids		
pSET152C	pSET152 derivative, with insertion of the <i>neo</i> gene from SuperCos1 at the sites of <i>Apal</i> and <i>SgrAI</i>	Our laboratory
pET28a	Kan ^r , expression vector	Novagen
2CT	Amp ^r , expression vector	Institute of Oceanology, CAS
pWLI640	pET28a carrying <i>sazA</i>	This study
pWLI641	2CT carrying <i>sazB</i>	This study
pWLI642	pET28a carrying <i>sazB-MT</i>	This study
pWLI643	2CT carrying <i>sazB-PT</i>	This study
pWLI644	pSET152C derivative harboring <i>sazA</i> and <i>sazB-PT</i> under the control of P _{gapdh}	This study
pWLI645	pSET152C derivative harboring <i>sazAB</i> under the control of P _{gapdh}	This study

leading to the discovery of new compounds streptoazines A to C (compounds 3 to 5). Through *in vivo* and *in vitro* experiments, *saz* genes were functionally characterized, demonstrating a biosynthetic machinery assembling prenylated indole alkaloids. These results expand the repertoire of CDPs and associated tailoring enzymes, setting the stage for accessing diverse prenylated alkaloids using synthetic biology strategies.

MATERIALS AND METHODS

General materials and methods. Bacterial strains and plasmids used in this study are listed in Table 3, and primers are listed in Table 4. *S. leeuwenhoekii* NRRL B-24963 was obtained from Agricultural Research Service Culture Collection (NRRL). General methods employed in this work were performed according to standard procedures (30) or manufacturer's instructions. Plasmid extraction and DNA purification were carried out using commercial kits (Omega, Bio-Tek). Primer synthesis and DNA sequencing were performed by TSINGKE Biotech Co. Ltd. (Qingdao, China). HR-ESI-MS was carried out on a Thermo LTQ Orbitrap XL mass spectrometer. All NMR spectra were taken on an Agilent-DD2-500 or Bruker Avance III 600 spectrometer and were processed with MestReNova 12.0.0 (Mestrelab). CD spectra were taken on a Jasco J-810 CD spectrometer.

Enzymes, chemicals, and reagents. The standards of cyclodipeptides were purchased from GL Biochem Ltd. (Shanghai, China). The prenyl donors, dimethylallyl, geranyl, farnesyl, and geranylgeranyl pyrophosphates (DMAPP, GPP, FPP, and GGPP, respectively), were purchased from Sigma-Aldrich. Common biochemicals and chemicals were purchased from standard sources for laboratory use.

Bioinformatic analysis. Sequence comparisons and database searches were accomplished with BLAST programs (31) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was generated using PhyML (v3.0) program (32) integrated into Seaview (33) based on the maximum likelihood method with an LG substitution model and NNI tree searching operation. The iTOL software (34) was used for graphical representation and editing of the phylogenetic tree.

Construction of the heterologous expression strains. For the heterologous expression in *Streptomyces* strains, the corresponding gene was put under the control of the constitutive promoter P_{gapdh} from *Streptomyces youssoufiensis* OUC6819 (11). Construction was performed as follows: taking the *sazAB* expression plasmid as an example, P_{gapdh} was amplified using the primer pair pGFP/pGRP (Table 4) and was digested with *NsiI*; the *sazAB* fragment was amplified with the primer pair *sazAB-FP/sazAB-RP* (Table 4) and was digested with *XbaI*. Then, these two fragments were ligated into the *NsiI* and *XbaI* sites of pSET152C to give pWLI645 (Table 3). After confirmation by sequencing, the expression plasmid was passed through *E. coli* ET12567/pUZ8002 and was then introduced into *S. coelicolor* M1146 or *S. albus* J1074 via conjugation.

Production and analysis of fermentation products. The heterologous expression strains were incubated on a rotatory shaker at 30°C in 250-ml Erlenmeyer flasks with each containing 50 ml of the fermentation medium (1% soluble starch, 2% glucose, 4% corn syrup, 1% yeast extract, 0.3% beef extract, 0.05% MgSO₄·7H₂O, 0.05% KH₂PO₄, 0.2% CaCO₃, pH 7.0). After 7 days of cultivation, the fermentation

TABLE 4 PCR primers used in this study

Primer	Sequence (5'→3') ^a
SazA-FP	GGAATTCCATATGATGCCGTACGTCTCTTCCTT
SazA-RP	CCGCTCGAGTCATGTGCGCCGACTTCC
SazB-FP	GGAATTCCATATGATGAGCCAGCGAGAACTCAC
SazB-MT-FP	GGAATTCCATATGATGTACCAGTCCGGCATCCG
SazB-MT-RP	CCGCTCGAGTCAGTGACGTTGGCGGTCA
SazB-PT-FP	CGCGGATCCGCTGGAGTTCTGTTCCAGGGGCCATGAGCCAGCGAGAACTCAC
SazB-PT-RP	CGGGGTACCTCAGGTCTCCGCAGGTATCC
sazAB-FP	ATGCCGTACGTCTCTTCCTT
sazAB-RP	GCTCTAGATAAGTGACGTTGGCGGTCA
sazAB-PT-RP	GCTCTAGATAAGGTCTTCCGCAGGTATCC
pGFP	CCAATGCATCCGTCGCGGAAAGCTGGC
pGRP	GAACCGATCTCCTCGTTGGTG

^aRestriction sites used for assembly are underlined.

cultures were harvested by centrifugation. The supernatants were extracted twice with equal volumes of ethyl acetate (EtOAc), and the combined EtOAc extracts were concentrated *in vacuo* to afford residue A. The precipitated mycelia were extracted with acetone twice followed by evaporation *in vacuo* to yield residue B. The residues were dissolved in methanol and were combined, followed by analysis using an Agilent 1260 HPLC system equipped with a YMC-Pack ODS-AQ C₁₈ column with UV detection at 300 nm under the following program: solvent (phase A, 0.1% formic acid in double-distilled water [ddH₂O]; phase B, 100% acetonitrile supplemented with 0.1% formic acid); 20% B (0 to 5 min), 20% to 100% B (5 to 45 min), 100% B (45 to 55 min), at a flow rate of 1 ml·min⁻¹.

Isolation of compounds 2 to 5. For isolation of compounds 2 to 5, *S. albus* J1074 expressing *sazAB* was fermented in a total volume of 30 liters. The fermentation cultures were treated as described above. The residues were applied to a reversed-phase C₁₈ open column, eluting with gradients of 40% to 100% methanol to give 10 fractions (Fr.1 to Fr.10). Compound 2 was obtained by further separation of Fr.4, eluting with a constant gradient of 56% B/A (phase A, ddH₂O; phase B, acetonitrile; 1.5 ml·min⁻¹, UV detection at 300 nm) in 30 min using a semipreparative HPLC column (YMC-Pack ODS-AA C₁₈ column, 120 Å, 250 by 10 mm, 5 μm). Compound 3 was obtained by separation of Fr.5, eluting with a constant gradient of 62% B/A in 70 min. Compound 4 was obtained by separation of Fr.6, eluting with a constant gradient of 70% B/A in 40 min. Compound 5 was obtained by further separation of Fr.7, eluting with a constant gradient of 90% B/A in 30 min. The structures of the compounds were characterized by HR-ESI-MS and NMR spectroscopy.

Overexpression and purification of the target proteins. For the expression of SazA, SazB, SazB-PT, and SazB-MT in *E. coli*, the corresponding genes were amplified by PCR using primer pairs listed in Table 4 from the genome of *S. leeuwenhoekii* NRRL B-24963. Taking *sazB-MT* as an example, it was amplified using the primer pair SazB-MT-FP/SazB-MT-RP, and the PCR products were digested with NdeI and XhoI, followed by ligation into pET28a, resulting in pWLI642. After confirmation by sequencing, pWLI642 was transformed into *E. coli* BL21(DE3) Condon-plus. Ten-milliliter overnight culture of the transformant was inoculated into 1 liter of LB medium (containing 50 μg·ml⁻¹ kanamycin) and was grown at 37°C with shaking. Expression was induced at an optical density at 600 nm (OD₆₀₀) of approximately 0.6 by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG; 0.05 mM final concentration), and cultivation was continued overnight at 16°C. The cells were pelleted by centrifugation (15 min at 8,000 × g) and resuspended in 30 ml of binding buffer A (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.5, containing cComplete protease inhibitor cocktail). The resuspended cells were lysed by sonication in an ice-water bath with ultrasonic processor VCX750 (Sonics and Materials Inc., PA, USA), and centrifuged at 10,000 × g for 30 min at 4°C. The supernatants were then filtered and loaded on a nickel nitrilotriacetic acid (Ni-NTA) gravity flow column at 4°C and eluted with a linear gradient of imidazole (20 to 500 mM) in 50 mM Tris-HCl, 500 mM NaCl buffer (pH 7.5) using an ÄKTA purifier system. After SDS-PAGE analysis, fractions containing pure SazB-MT were pooled, concentrated, and exchanged to Tris buffer (25 mM Tris-HCl, 20 mM NaCl, pH 7.5) by using an Amicon Ultra-15 30-kDa cutoff centrifugal concentrator (Millipore).

Identification of DKP compound formed by SazA. Overnight culture of *E. coli* BL21(DE3) containing pWLI640 was used to inoculate (1:50 [vol/vol]) 50 ml of M9 minimal medium (9) containing 50 μg·ml⁻¹ kanamycin. Expression of SazA was induced at an OD₆₀₀ of approximately 0.8 by addition of IPTG (0.1 mM final concentration), and cultivation was continued for additional 20 h at 16°C. The culture was harvested by centrifugation, and the supernatant was extracted twice with equal volumes of ethyl acetate. The combined extracts were concentrated *in vacuo* and dissolved in 1 ml methanol. HPLC analysis was conducted using a YMC-Pack ODS-AQ C₁₈ column (150 mm by 4.6 mm, particle size of 5 μm, pore size of 120 Å) under the following program: solvent A consisting of 0.1% (vol/vol) formic acid and ddH₂O, solvent B consisting of 0.1% (vol/vol) formic acid and acetonitrile; 10% B (0 to 5 min), 10% to 50% B (5 to 25 min), 100% B (25 to 35 min), at a flow rate of 1 ml·min⁻¹ and UV detection at 280 nm.

In vitro assays. The enzymatic assay of SazB was carried out in 50 mM Tris-HCl buffer (pH 7.5) with 2.5 mM MgCl₂ and containing 10 μM SazB, 1 mM cWW, 0.2 mM DMAPP, and 0.2 mM 5'-adenosylmethionine. The enzymatic assay of SazB-PT was carried out in 50 mM Tris-HCl buffer (pH 8.0) with 2.5 mM MgCl₂ and containing 10 μM SazB-PT, 0.5 mM DKP, and 0.5 mM prenyl donor. The enzymatic assay of

SazB-MT was carried out in 50 mM Tris-HCl buffer (pH 8.0) containing 10 μ M SazB-MT, 0.3 mM substrate, and 0.5 mM SAM. All the reaction mixtures were incubated at 30°C for 2 h and were then quenched by the addition of equal volumes of methanol. After centrifugation, the supernatants were subjected to HPLC analysis. For the detection of streptozazines, HPLC was carried out under the conditions used for analyzing the fermentation products of the heterologous expression strains. For the detection of pre-DMTs/DMTs, HPLC was performed using a YMC-Pack ODS-AQ C₁₈ column (150 mm by 4.6 mm, particle size of 5 μ m, pore size of 120 Å) under the following program: phase A consisting of 0.1% (vol/vol) formic acid and ddH₂O, phase B consisting of 0.1% (vol/vol) formic acid and acetonitrile; 50% B (0 to 5 min), 50% to 100% B (5 to 30 min), 100% B (30 to 45 min), at a flow rate of 1 ml·min⁻¹ and UV detection at 300 nm.

Data availability. The nucleotide sequences of *sazA* and *sazB* were previously deposited in the NCBI database under accession numbers [CQR65852.1](#) and [CQR65853.1](#), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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