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Sungeidines from a non-canonical enediyne biosynthetic pathway

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Supporting Information Placeholder

ABSTRACT: We report the genome-guided discovery of sungeidines, a class of microbial secondary metabolites with unique structural features. Despite evolutionary relationships with dynemicin-type enediynes, the sungeidines are produced by a biosynthetic gene cluster (BGC) that exhibits distinct differences from known enediyne BGCs. Our studies suggest that the sungeidines are assembled from two octaketide chains that are processed differently than those of the dynemicin-type enediynes. The biosynthesis also involves a unique activating sulfotransferase that promotes a dehydration reaction. The loss of genes, including a putative epoxidase gene, is likely to be the main cause of the divergence of the sungeidine pathway from other canonical enediyne pathways. The findings disclose the surprising evolvability of enediyne pathways and set the stage for characterizing the intriguing enzymatic steps in sungeidine biosynthesis.

Naturally occurring enediynes are a family of microbial secondary metabolites that contain a highly distinct 1, 5-diyne-3-ene core embedded in a ten- or nine-membered carbon skeleton.¹⁻⁶ In the past three decades, enediyne natural products received considerable attention for their unprecedented molecular architecture and potent DNA-damaging bioactivity. In the presence of a chemical trigger, the 1, 5-diyne-3-ene core rearranges into a reactive diradical via the Bergman or Myers-Saito cyclization mechanism.⁷⁻⁹ The reactive diradical can abstract hydrogen atoms from the deoxyribose backbone of DNA to cause the cleavage of chromosomal DNA.¹⁰⁻¹³ Genome-mining has revealed a diversity of cryptic enediyne BGCs,¹⁴⁻¹⁶ suggesting that the structural diversity of enediyne natural products remains to be fully explored.

Here we report the discovery of sungeidines, a series of metabolites produced by a biosynthetic pathway evolutionarily related to the pathways that produce dynemicin-type enediynes. The dynemicins are characterized by a ten-membered enediyne core that is fused to an anthraquinone moiety.^{5,15,17-19} The remarkable structural differences between the sungeidines and dynemicins signal a divergence of the

enediayne biosynthetic pathways as a result of adaptive evolution.

Micromonospora sp. MD118, which was isolated from mangrove sediment in Singapore, contains a BGC that shares similarities with the BGCs of dynemicin-type enediynes (Figure 1A & 1B, Table S1).²⁰ The putative enediayne BGC, which is referred to as *sgd* BGC, possesses the characteristic ‘minimal enediayne polyketide synthase (PKS) gene cassette’ consisting of a polyketide synthase gene (*sgdE8*),²¹⁻²⁵ a thioesterase gene (*sgdE7*),²⁶⁻²⁷ and three unannotated genes (*sgdT3*, *U14*, and *U15*).²¹⁻²³

Apart from the minimal gene cassette, the *sgd* BGC exhibits notable differences from the other four dynemicin-type enediayne BGCs. The *sgd* BGC lacks a few genes that are conserved in the other four BGCs, including *dynA1*, *dynA2* and *dynE13* in the *dyn* BGC,²⁸ or the corresponding *N*, *O*, *P* genes from the *ypm*, *tnm* and *ucm* BGCs (Figure 1A).^{15,18,29} The genes *dynA1* and *dynA2* share homology with the AclR/SnoaL-like cyclases;^{30,31} whereas *dynE13* encodes a putative flavin-dependent epoxidase.²⁸ The *sgd* BGC also lacks a non-heme iron oxygenase gene (*ypmM*, *ucmM*, and *tnmM1/M2*), which is also absent in the *dyn* BGC.^{15,18,29} Meanwhile, the *sgd* BGC possesses five additional genes (*sgdX1-X5*) that are not present in the other four BGCs. The genes *sgdX2* and *X3* are predicted to encode a sulfotransferase and a metallo β -lactamase protein respectively; *sgdX5* is a transposase gene and *sgdX1* and *X4* are unannotated. The observations led us to speculate that the *sgd* BGC may produce an enediayne that is structurally distinct from the known dynemicin-type enediynes.

The inactivation of the BGC producing sioxanthin generated a strain (MD118A) that exhibited a colorless colony morphology and higher growth rate without affecting the metabolite profile. We used MD118A to reduce fermentation time and facilitate product detection. When cultured in the M5 medium supplemented with NaI, which is known to promote dynemicin production,³² MD118A produced a series of compounds (1-9) with the UV-Vis spectra reminiscent of anthraquinones (Figure 1C & 1D). We employed the CRISPR/Cas9^{33,34} method to delete

Figure 1. Discovery of sungeidines. (A) Comparison of the *sgd* BGC with those of dynemicin-type enediynes. The genes that are unique to the *sgd* BGC are colored blue; whereas the genes that are missing in *sgd* BGC but conserved in the canonical enediyne BGCs are colored red. (B) Structures of dynemicin-type enediynes. (C) HPLC analysis (314 nm wavelength) of the putative products of the *sgd* BGC. The 32 min peak is from a compound from the culture medium. (D-E) UV-Vis spectra and structures of **1**, **2** and **9**. The extra carbon atoms C30 and C31 are labeled red.

sgdU15, a gene from the minimal enediyne PKS gene cassette. The deletion of *sgdU15* abolished the production of **1-9** (Figure 1C), suggesting that the compounds are produced by the *sgd* BGC.

Our initial effort to isolate the compounds for structural elucidation was unsuccessful because of the low fermentation titer. The *sgd* BGC contains *sgdR2* and *sgdR7*, two genes predicted to encode transcriptional activators. We created the MD118AE strain by overexpressing the two activator genes in MD118A.^{35,36} With MD118AE, a three to tenfold increase in fermentation titer was observed for **1-9** (Figure 1C). Scaled-up fermentation of MD118AE led to the isolation of **1**, **2** and **9**. The structures of **1**, **2** and **9** were established using HRMS and NMR spectroscopy (Figures 1E, S1, S2, S9, Schemes S2, S3, Tables S4, S5), with the absolute

configuration determined by ECD calculations (Figures S12-S15). The novel compounds **1** and **2**, named as sungeidines A and B respectively, feature a pyrrolidine ring and a sterically congested tetracyclic backbone fused to an anthrathiophenone moiety. Notably, **1** and **2** contain two extra carbon (C30, C31) comparing to dynemicins. A *trans*-7,8-dihydrodiol iodoanthracene thiolactone (**9**) that co-produced with **1** and **2** was also isolated. An iodoanthracene thiolactone that lacks the two hydroxyl groups was recently reported by Townsend and coworkers to be an on-path intermediate in dynemicin A biosynthesis.³⁷ In contrast, **9** is likely a dead-end product in sungeidine biosynthesis, considering that the feeding of **9** to the culture of MD118A did not result in the production of sungeidines (Figure S16).

Figure 2. Sungeidines C-H (3-8) and the function of SgdX2. (A) HPLC analysis of the production of sungeidines by MD118A and three mutants at 314 nm. (B) Sungeidines C-H produced by the Δ *sgdX2* mutant. (C) In vitro assays demonstrating that SgdX2 catalyzes the conversion of sungeidine F (6) to sungeidine A (1). The control is the 20-hr reaction without adding SgdX2. (D) Regio- and stereo-selective dehydration of 6 promoted by the sulfotransferase SgdX2.

The five genes *sgdX1-X5* are unique to the *sgd* BGC, with *sgdX1*, *X2* and *X3* predicted to encode enzymes. We deleted the three genes individually to investigate their roles in sungeidine biosynthesis. The deletion of *sgdX1* or *sgdX3* had little effect on the production of the metabolites (Figure 2A); while the disruption of *sgdX2* eliminated the production of 1 and 2 and increased the production of 3-8, which were only produced in a minute or negligible amount by MD118AE. Large-scale fermentation of the Δ *sgdX2* strain with overexpressed *sgdR2* and *R7* led to the isolation of sungeidines C-H (3-8, Figure 2B). Comparing to 1 and 2, sungeidines C-H all contain a trans-vicinal diol at the C15/C16 position. Notably, 3 and 4 feature a carbonyl group at C2 position that is masked in other sungeidines, as a likely result of facile reductive amination of the carbonyl group.

The inability of the Δ *sgdX2* mutant to produce 1 and 2 suggested that SgdX2 is required for the synthesis of 1 and 2, likely by catalyzing the conversion of 5/6 to 2/1. The conversion would involve a 1, 2-elimination-based dehydration of 5 and 6; and it was not immediately clear how the putative sulfotransferase SgdX2 can catalyze such a reaction. To validate its enzymatic activity, we produced recombinant SgdX2 by *E. coli* overexpression. Incubation of SgdX2 with 6 and the sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) resulted in the formation of 1 and a new species

(Figure 2C). The new species, which is chemically labile and could only be obtained as a mixture containing 1, was determined to be the sulfated adduct of 6 based on the HRESIMS data ($[M+H]^+$, $C_{30}H_{22}NO_7S_2$, m/z 572.0805, calcd 572.0832) (Figure 2D). The observations suggest that the sulfonated 6 undergoes non-enzymatic dehydration to generate 1, likely driven by the thermodynamically favorable aromatization process. SgdX2 represents a rare sulfotransferase that promotes the dehydration of alcohol, reminiscent of the sulfotransferase domain in curacin A biosynthesis.³⁸

Based on the structures, the sungeidines are most likely to be assembled from two 15-carbon skeletons. In contrast, the dynemicins consist of two 14-carbon skeletons of polyketide origin.^{25,39} To test whether the two 15-carbon skeletons of sungeidines, in particular, the two extra carbon atoms, also originate from similar polyketide pathways, we performed isotope feeding experiments using $[1-^{13}C]$, $[2-^{13}C]$, and $[1,2-^{13}C_2]$ -labelled sodium acetate. ^{13}C -NMR analysis suggested that all the carbon atoms in sungeidines originate from acetate, and that the two 15-carbon skeletons are likely to derive from two head-to-tail octaketide chains with the loss of C1 (carboxyl) of a terminal acetate unit, as supported by the incorporation pattern of $[1,2-^{13}C_2]$ -acetate and matching $^1J_{CC}$ values (Figure 3, Scheme S4, Table S6). Hence, the sungeidines and dynemicins are likely to share the same octaketide origin (Scheme S5), but there must

Figure 3. Single- and double- ^{13}C -labelled acetate feeding experiments suggesting that the sungeidines consist of two 15-carbon skeletons that are likely to derive from the octaketides synthesized by SgdE8.

be a divergence in the processing of the octaketide to generate the 14- or 15-carbon skeletons.

Despite our extensive effort to detect the initially anticipated enediyne product under dark and cold conditions, the enediyne product was never detected. It is plausible that the lack of the homologs of *dynA1*, *dynA2*, and *dynE13* genes renders the pathway completely defective in synthesizing the enediyne moiety. It is also plausible that the lack of the genes may result in an enediyne that is prone to Bergman cyclization due to the lack of a stabilizing epoxide or exocyclic alkene group. The presence of an epoxide or exocyclic alkene group is known to be crucial for preventing the spontaneous cycloaromatization of ten-membered enediynes.^{17,40,41} In dynemicins, C3-C8 epoxidation stabilizes the enediyne core by introducing steric strain to maintain a C23-C28 distance of 3.54 Å¹⁷ (Figure 1B); and opening of the epoxide shortens the C23-C28 distance to 3.2 Å to trigger the Bergman cyclization.^{42,43} The epoxidation is putatively catalyzed by the flavin monooxygenase DynE13 in dynemicin biosynthesis²⁸,

and the lack of the *dynE13* homolog in the *sgd* BGC may explain why the *sgd* BGC cannot produce a stabilized enediyne.

Based on the structure of sungeidines, ^{13}C -isotope labeling experiments, and the presence of the minimal enediyne PKS gene cassette in *sgd* BGC, the sungeidines are likely to be assembled from two octaketide chains synthesized by the iterative PKS SgdE8. Also, in light of the recent insights on enediyne biosynthesis,^{25,44-46} we propose that SgdE8 first synthesizes two octaketide-derived polyene chains, which are subsequently processed by separate oxygenases/oxidases and cyclases to furnish a SgdE8-tethered intermediate and an anthrathiophenone fragment that is structurally related to **9**. The SgdE8-tethered polyene could be oxidized and cyclized to generate an intermediate that resembles the right-side structure of sungeidines, which is subsequently

Figure 4. Biogenesis of sungeidines. (A) Biogenesis of sungeidines via a polyketide pathway. (B) Feeding of **3** to the Δ *sgdU15* strain in the M5 medium that does not contain NaI resulted in the production of **2**, supporting that **3** is an on-route intermediate to **2**.

joined with the anthrathiophenone fragment via a C-N linkage to yield the heterodimeric sungeidines. In this scenario, no enediyne intermediate is formed presumably due to the lack of essential genes and enzymes. Alternatively, the SgdE8-tethered polyene is transformed into an enediyne bicyclic core, which is joined with the anthrathiophenone fragment to yield an enediyne precursor. The hypothetical enediyne precursor undergoes facile Bergman cyclization to generate a diradical species to abstract hydrogen atoms from elsewhere to generate **3**, which undergoes reductive amination to yield **5** (Figure 4A). The diradical species can also

abstract hydrogen atom from C20 of the anthracene moiety to entail C-C radical coupling and reductive amination to produce **6**. Dehydration of **5** and **6** results in the formation of **2** and **1** respectively; whereas compounds **4**, **7** and **8** are likely generated by auto- or enzymatic oxidation. The proposal that **3** is an en-route intermediate to **2** is supported by the feeding experiment that demonstrated the conversion of **3** to **2** by the Δ *sgdU15* strain (Figure 4B).

We surmise that the *sgd* BGC evolved originally from a canonical enediyne BGC that produces a dymecicin-type enediyne. The loss of genes that generated a pathway that is either not able to assemble a bicyclic enediyne structure, or not able to produce a stable enediyne intermediate to preclude the C8-C9 coupling event that is crucial for generating a dymecicin-type enediyne. The Sgd pathway is likely the result of an adaptive evolution process, either driven by the survival advantage gained by the production of sungeidines, or by the lack of evolutionary pressure on the microorganism to maintain the canonical enediyne pathway. The presence of transposase genes in and near the *sgd* BGC supports such a view and suggests that this genomic region is prone to transposon-mediated modification. Considering the relatively compact *sgd* BGC and the genetic tractability of the host, the sungeidine biosynthetic pathway can serve as a model system to address some of the outstanding questions in enediyne biosynthesis.

ASSOCIATED CONTENT

Supporting Information

The supporting information is available free of charge on the ACS publications website. Experimental methods, spectroscopy and DNA data are included.

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Notes

The authors declare no competing financial interests.

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