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Note

The Discovery and Biosynthesis of Nicotinic Myxochelins from an *Archangium* sp. SDU34

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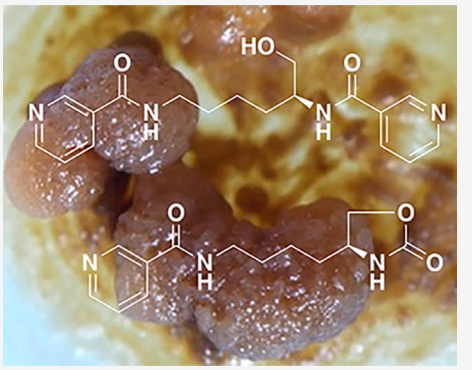
ABSTRACT: Myxobacteria are a prolific source of structurally diverse natural products, and one of the best-studied myxobacterial products is the siderophore myxochelin. Herein, we report two new compounds, myxochelins N (**1**) and O (**2**), that are nicotinic paralogs of myxochelin A, from the terrestrial myxobacterium *Archangium* sp. SDU34; **2** is functionalized with a rare 2-oxazolidinone. A precursor-feeding experiment implied that the biosynthesis of **1** or **2** was due to altered substrate specificity of the loading module of MxcE, which likely accepts nicotinic acid and benzoic acid instead of more conventional 2,3-dihydroxybenzoic acid. We also employed a phylogenomic approach to map the evolutionary relationships of the myxochelin biosynthetic gene clusters (BGCs) in all the available myxobacterial genomes, to pave the way for the future discovery of potentially hidden myxochelin derivatives. Although the biological function of **1** and **2** is unclear yet, this work underpins that even extensively studied BGCs in myxobacteria can still produce new chemistry.



Microbial natural products are of paramount importance to human beings, and have been a prolific source for drug development.[1] The Gram-negative myxobacteria have a complex social lifecycle, involving multicellular surface motility, fruiting body formation, sporulation, and wolf-like predatory behavior.[2] This branch of life domain has been recognized as one of the foremost producers of specialized metabolites, and a conspicuous number of diverse chemical topologies with pronounced bioactivities have been discovered.[3,4] One of the best examples is epothilone, a 16-membered antitumor polyketide isolated from *Sorangium cellulosum*, whose semi-synthetic analogue ixabepilone has been approved by the FDA for the treatment of cancer patients.[5] Genome mining has revealed that only a small fraction of the myxobacteria-specialized metabolites encrypted at the DNA level have been discovered.[3] In addition, compared to intensively studied microorganisms such as streptomycetes and bacilli, myxobacteria have been studied less, largely because of the difficulty in the cultivation and/or genetic manipulation. Therefore, myxobacteria are a largely underexploited resource for the discovery of novel natural products.

In our laboratory, a library of ~2000 myxobacterial strains have been isolated from diverse ecological niches, forming a resource for the discovery of novel drug leads. A terrestrial myxobacterium, *Archangium* sp. SDU34 (Figure S1) isolated from the Taklimakan desert (P. R. China), was prioritized for the systematic isolation of secondary metabolites based on HPLC-DAD profiling. This strain was grown in VY/2 liquid medium (30 L), and the culture broth was extracted with HP-20 resin after the cell biomass had been removed by filtration. The afforded crude extract was fractionated by ODS C_{18} flash chromatography eluted by increasing concentrations of methanol in water, followed by sieve chromatography with Sephadex LH-20 in methanol. Final purification was achieved on a reversed-phase C_{18} HPLC column, yielding **1** (1.2 mg) and **2** (0.4 mg).

Compound **1** was isolated as a yellowish amorphous solid. Its molecular formula was determined as $C_{18}H_{22}N_4O_3$ based on the HR-ESIMS analysis. The ^{13}C NMR spectrum of **1** (Table S1) clearly revealed 18 carbons, corroborating the molecular formula. In the region of δ_C 120−170 of the ^{13}C NMR spectrum, a total of 12 carbons could be classified into two sets of closely related signals, which indicated two similar aromatic rings in **1**. The downfield ^1H resonances including δ_C 8.99, 8.68, and 8.25 suggested a nitrogen atom was involved in the aromatization. The nature of the aromatic ring was determined to be nicotinic acid based on the analysis of the splitting pattern of protons, which was corroborated by HMBC and COSY correlations (Figure S2). The remaining six protonated aliphatic carbons, C-1−C-6, including one methine and five

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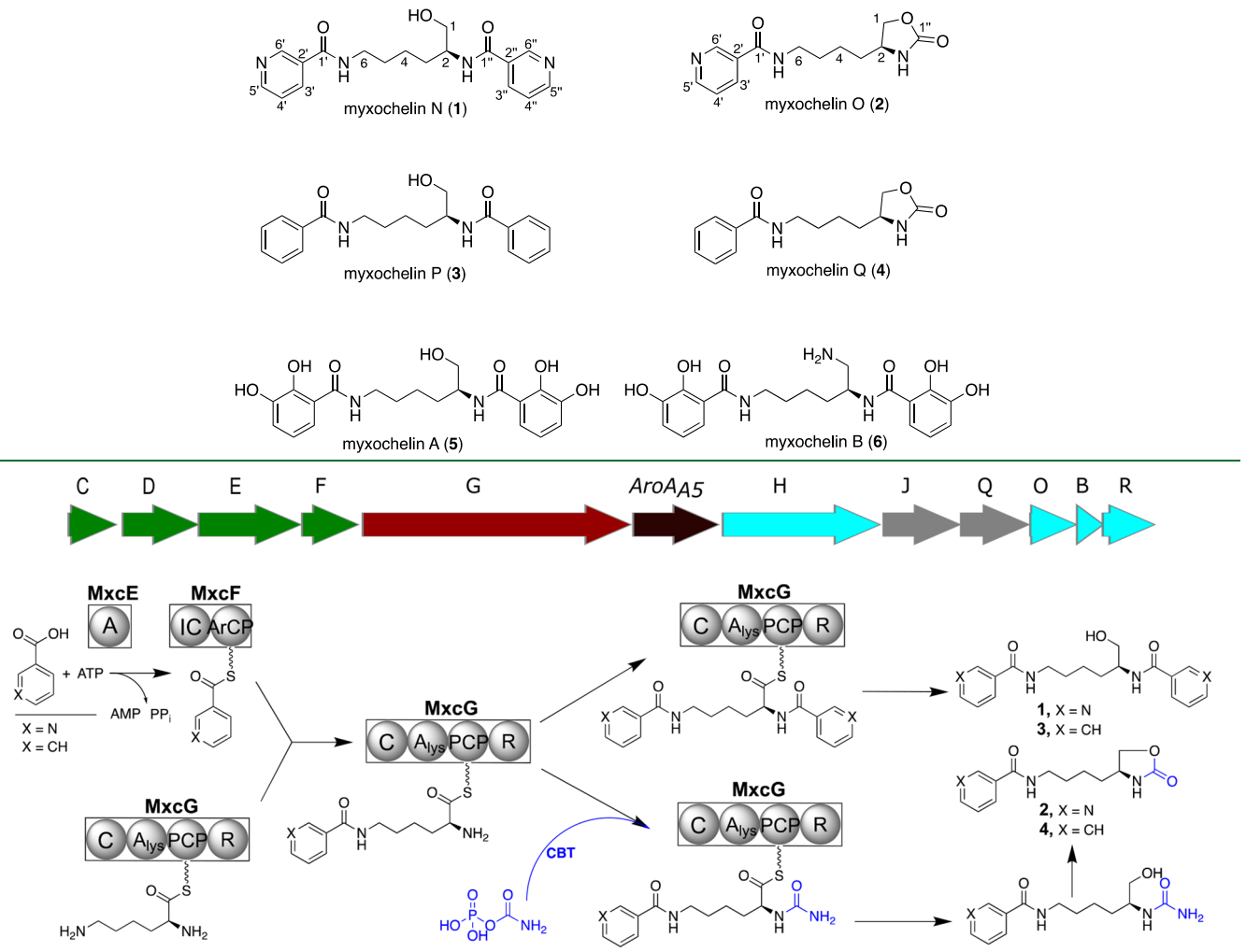

Figure 1. Plausible biosynthetic route to myxochelins **1**−**4** in *Archangium* sp. SDU34. *mxc*E, F, and G are responsible for the activation and condensation of nicotinic acid with L-lysine, in a similar fashion as previously described for the biosynthesis of myxochelin A (**5**).[9,10,13,23] The NRPS MxcG consists of an N-terminal condensation (C) domain, followed by an adenylation (A) that is specific for the recognition of L-lysine, a peptidyl carrier protein (PCP), and a C-terminal reductase (R) domain. The origin of 2-oxazolidinone in myxochelin O is the outcome of a putative carbamoyltransferase (CBT).

methylenes, were unambiguously resolved by an HSQC experiment. A $^{1}H-^{1}H$ COSY experiment confirmed the protons attached to these six carbons constituted a large continuous spin system, and the HMBC experiment established the connectivity of these carbons to be a C-1-reduced lysine that was found in the structure of myxochelin A (**5**). The amide linkages of the C-1-reduced lysine with two portions of nicotinic acid were demonstrated by the key HMBC correlations from H_2-6 (δ_H 3.44) to C-1′ (δ_C168.5) and from H-2 (δ_H 4.18) to C-1″ (δ_C 168.7). Therefore, compound **1** was distinguished from **5** by the appended two moieties of nicotinic acid instead of 2,3-dihydroxybenzoate (DHBA) and accordingly dubbed myxochelin N. The absolute configuration at C-2 of **1** was determined to be *S* based on the optical rotation measurement ($[\alpha]_D^{20}$ −26.3) in comparison with myxochelin A.[6]

Compound **2** was isolated as a yellowish oil. Its molecular formula $C_{13}H_{17}N_3O_3$ was established from the HR-ESIMS data. The ^{1}H NMR spectrum of **2** displayed characteristic signals for a moiety of nicotinic acid as well as C-1-reduced lysine, implying **2** was a congener of **1**. However, the existence of a single set of proton signals for nicotinic acid in the aromatic region indicated that only one primary amine group of the C-1-reduced lysine was amidated. The key HMBC correlation from H_2-6 (δ_H 3.45) to C-1′ (δ_C 168.6) confirmed nicotinic acid was linked with the ε-amine group of C-1-reduced lysine. The α-amine group of the C-1-reduced lysine in **2** was also amidated with a carbonyl (C-1″) based on the key HMBC correlation from H-2 (δ_H 3.92) to C-1″ (δ_C 163.2). The HMBC correlations from H_2-1 to C-1″ demonstrated a lactone functionality between C-1 and C-1″, forming a unique five-membered 2-oxazolidinone ring. In contrast to free rotation of the single bond between C-1 and C-2 in **1**, the rigidity of the heterocyclic ring system led to a chemical inequivalence of the two protons on C-1 of **2** (Table S1). The 2-oxazolidinone in **2** was remarkable because a very limited number of microbial natural products, such as pactamycin[7] and gelstriamine A,[8] contain this rare ring system. Likewise, the stereochemistry of **2** was deduced to be *S* in view of its biogenetic relatedness to **1** (see below), which was further demonstrated by the measured optical rotation data ($[\alpha]_D^{20}$ −8.0). Compound **2** was given the trivial name myxochelin O.

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The close structural relatedness to myxochelins A (**5**) implied **1** and **2** are encoded by *mxc*, an extensively studied model BGC in myxobacteria.[9,10] Genome sequencing followed by antiSMASH analysis[11] verified the existence of *mxc* in the genome of SDU34. The essential genes *mxc*CDEF for the *de novo* synthesis of DHBA along with the central NRPS gene *mxc*G for the assembly of myxochelin clearly exist (Table S2), as verified in a handful of myxobacterial strains known to produce myxochelins A (**5**) and B (**6**), including *Myxococcus xanthus* DK1622,[9] *Stigmatella aurantiaca* Sg a15,[10] *Pseudoalteromonas piscicida* S2040,[12] and *Sorangium cellulosum* So ce56[13] (Figure S3). As genetic diversity of BGCs normally correlates with structural differences between their synthesized products, and even small chemical modifications can lead to disparate biological activities, we systematically compared the *mxc* BGCs from different strains and genera of myxobacteria. AntiSMASH analysis[11] of all the 152 myxobacterial genomes available to us demonstrated 116 strains contain the alleged BGCs. The *mxc* BGCs are widely distributed in the genera *Corallococcus*, *Myxococcus*, *Archangium*, *Sorangium*, *Polyangium*, *Stigmatella*, *Cystobacter*, and *Hyalangium* (Figure S4), implicating its ecological importance for myxobacteria. Afterward, CORASON[14] was employed to generate a multilocus phylogeny of all the *mxc* BGCs to reveal their potential evolutionary relationships. Given the *mxc* from *S. aurantca Sg* a15 as a query input, CORASON identified homologues of the respective genes among all the 116 BGCs. Although architectural arrangements were variable in different genera, the central biosynthetic genes *mxcC*−*G* were uniformly present (Figure S5). The clustering pattern of *mxc* BGCs mostly seemed genus-dependent; for example, all the *mxc* BGCs from *Archangium* were devoid of *mxc*L, the gene encoding for an aminotransferase that is indispensable for the production of myxochelin B (**6**),[13] which could be explanatory for the absence of the variant of 1-aminated **1** in the LC-MS analysis of SDU34 crude extract. The *mxc* BGCs from *Myxococcus* constituted two different clades, differentiating in *mxc*H, which encodes a TonB-dependent siderophore receptor.

The clarification of the genetic basis enabled us to propose the biosynthesis pathway of the newly characterized myxochelins N (**1**) and O (**2**), as depicted in Figure 1. In general, **1** follows the same biosynthetic logic as myxochelin A (**5**),[9,10,13] but differentiating in the MxcE-mediated priming of nicotinic acid instead of DHBA. The 2-oxazolidinone in myxochelin O (**2**) probably involves carbamoylation of the α-amine of L-lysine by a yet unidentified carbamoyltransferase, and the intermediate further goes through a reductive offloading, followed by a lactonization reaction. Carbamoylation-driven 2-oxazolidinone formation is also found in the biosynthesis of pactamycin in *Streptomyces pactum* ATCC 27456[7] and 5-methyl-2-oxazolidinone-4-carboxylate in *Bacillus subtilis*.[15] To test our assumption, nicotinic acid added to a SDU34 culture resulted in a more than 30-fold yield improvement of **1** and **2** (Figure 2). Though substrate promiscuity seems a peculiarity of the adenylating enzyme MxcE, which accepts a range of aryl carboxylic acids to produce disparate myxochelin derivatives,[13,16,17] there is no precedent example of using nicotinic acid as a precursor. Other catecholate-activating adenylation enzymes that also use nicotinic acid as a substrate include DhbE,[18] PyrA,[19] and SgvD1[20] in the catechol-siderophore BGCs governing the production of bacillibactin E,[18] pyridomycin,[19] and viridogrisein,[20] respectively. Interestingly, LC-MS analysis confirmed

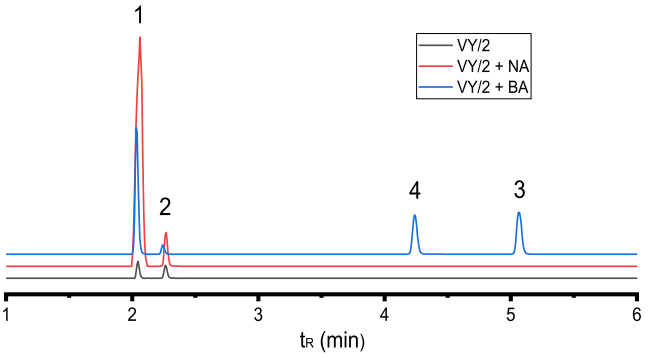

Figure 2. LC-MS analysis of the precursor-feeding experiment for *Archangium* sp. SDU34. Nicotinic acid (NA) greatly improved the production of **1** and **2** to 30-fold, whereas benzoic acid (BA) induced compounds **3** and **4**, which were not produced in the control group.

that SDU34 did not produce myxochelin A (**5**), even if fed with DHBA. Further precursor-directed biosynthesis with benzoic acid (BA) and salicylic acid (SA) showed the former rather than the latter is recognized by MxcE to produce the compounds **3**[21] (myxochelin P) and **4** (myxochelin Q), whose structural identifications were done on the basis of MS/MS analysis (Figure S6). These feeding experiments indicated that SDU34-MxcE has seemingly evolved a substrate specificity to accept aryl carboxylic acids with lower steric hindrance. Attempting to understand the underlying genetic basis, we phylogenetically compared the manually gleaned catecholate-activating adenylation enzymes encoded in catechol-siderophore BGCs that have been functionally characterized. However, we failed to correlate the grouping pattern with the substrate specificity (Figure S7). In addition, the A domain specificity code of PLPAQGVVNK in SDU34-MxcE was identical to its orthologs such as MxcE and EntE, which recognize DHBA, but instead considerably different from that of TWPSQGVLTK in PyrA, which recognizes nicotinic acid. Therefore, no obvious sequence hints could be concluded for the substrate preference of MxcE.

A chrome azurol S (CAS) assay[22] confirmed myxochelins N (**1**) and O (**2**) were devoid of iron-chelation (or siderophore) activity, because the functional catechol residues of myxochelin A are replaced by nicotinic acid. **1** and **2** were inactive against *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Candida albicans* at 20 μg/disc and the human prostate carcinoma cell line PC3, human lung carcinoma cell line A549, leukemia cell line K562, and human hepatocarcinoma cell line HepG-2 at 50 μM. The biological and/or ecological relevance of myxochelins N (**1**) and O (**2**) awaits further investigations.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded in CD_3OD on a Bruker AVNEO 600 MHz calibrated to a residual CD_3OD (3.31 ppm). The HR-Q-TOF ESIMS analyses were performed on a rapid separation liquid chromatography system (Dionex, UltiMate3000, UHPLC) coupled with an ESI-Q-TOF mass spectrometer (Bruker Daltonics, Impact HD). HPLC analysis was performed with an Agilent 1260 series HPLC apparatus (Agilent Technologies Inc., Santa Clara, CA, USA), using a 250 × 4.6 mm Luna 5 μm C_{18} (2) 100 Å column equipped with a guard column containing C_{18} 4 × 3 mm cartridges (Phenomenex Inc., Torrance, CA, USA). Semipreparative HPLC separation was performed on a reversed-phase column (Phenomenex Luna 5 μm C_{18} (2) 100 Å column, 250 × 10 mm). HR-Q-TOF ESIMS instruments equipped with a C_{18} column (Thermo Fisher Scientific, C_{18}, 250 × 4.6 mm, 5

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μm) were used. Samples were detected by the UV absorbance at 210, 254, 280, and 365 nm. According to specific experiments, all organic solvents and chemicals were of analytical or HPLC grade.

Fermentation and Compound Isolation. *Archangium* sp. SDU34 (Figure S1) was activated on VY/2 medium containing (L^{-1}) 5 g of baker's yeast, 1 g of $CaCl_2$, 0.5 mg of vitamin B_{12}, 15 g of agar, and 1.97 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), at pH 7.2 for 3 days at 30 °C. The colony cells were inoculated into 100 mL of sterile liquid VY/2 medium in Erlenmeyer flasks (300 mL), which were then continuously shaken at 200 rpm at 30 °C for 3 days. The 100 mL of seed culture was equally divided into three portions and transferred into fresh liquid VY/2 medium in Erlenmeyer flasks (300 mL), and a total of 300 individual flasks were inoculated. After 7 days of fermentation, the cultures (30 L) were combined and the cell mass was removed by centrifugation. Around 0.6 kg of Diaion HP-20 resin was added to the supernatant, and this slurry was shaken overnight. The column-packed resin was twice washed with 2 L of 1% methanol in H_2O and then eluted with 3 L of methanol. After evaporation of organic solvent under a vacuum at 36 °C, the resulting residue was dissolved in MeOH and applied to reverse-phase preparative medium-pressure liquid chromatography (Buchi, Flawil, Japan) to give four fractions (FrA−FrD). FrA was separated by Sephadex LH-20 (GE Healthcare Bio-Sciences Inc., Uppsala, Sweden) eluted with MeOH to generate six subfractions (FrA1−FrA6). FrA2 was purified by semipreparative HPLC [column: Phenomenex Luna 5 μm C_{18} (2) 100 Å, 250 × 10 mm; solvent A: H_2O (60%); solvent B: MeOH (40%); flow rate 1.8 mL/min; 30 °C; UV detection at 210 nm], to afford compounds **1** (1.0 mg) and **2** (0.4 mg).

*Myxochelin N (**1**):* yellowish powder; $[\alpha]_D^{20}$ −26.3 (*c* 0.087, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.16), (log ε) 256 (2.80) nm; IR ν_{max} 3291, 2931, 2861, 1641, 1594, 1547, 1475, 1419, 1316, 1028, 828, 707 cm^{-1}; ^1H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) data are shown in Table S1; HRESIMS (positive mode) *m*/*z* 343.1773 $[M + H]^+$ for **1** (calcd for $C_{18}H_{23}N_4O_3$, 343.1765).

*Myxochelin O (**2**):* yellowish oil; $[\alpha]_D^{20}$ −8.0 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ε) 257 (2.07) nm; IR ν_{max} 3352, 2924, 2854, 1742, 1561, 1377, 1259, 1161, 1102, 803, 698 cm^{-1}; ^1H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) data are shown in Table S1; HRESIMS (positive mode) *m*/*z* 264.1345 $[M + H]^+$ for **2** (calcd for $C_{13}H_{18}N_3O_3$, 264.1343).

Genome Sequencing, Assembly, and Annotation. Genomic DNA of *Archangium* sp. SDU34 was extracted by using the whole genome DNA sequencing kit (Oxford Nanopore Technologies Inc., Oxford, UK). Genome sequencing was conducted by Benagen (Wuhan, China), by joining the strength of Illumina and Nanopore technologies. Illumina sequencing produced a total of 2.22 G of raw data, which were filtered to generate 2.21 G of clean data. After passing the data measured by the third-generation Nanopore sequencer through the filter to remove connector, short fragments and low-quality data, a total of 1 000 038 128 bp clean data was obtained for assembly. This sequencing was based on the sequence of pass reads (*Q* > 7), extracting the longest sequence of reads, and the total valid data are about 1 G. Unicycler (0.4.8) software (https://github.com/rrwick/Unicycler) was used to assemble the filtered reads. The genes were annotated based on BLAST against databases COG (https://www.ncbi.nlm.nih.gov/COG/), KEGG (https://www.kegg.jp/kegg/), Refseq (https://www.ncbi.nlm.nih.gov/refseq/), and Uniprot (https://www.uniprot.org/). The genome has been deposited at GenBank under the accession number CP069338.

Construction of CORASON Phylogeny of *mxc* BGCs from Myxobacteria. The 145 sequenced genomes of *Myxococcales* deposited in the NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/) were downloaded, along with our seven in-house myxobacterial genomes. All 152 myxobacterial genomes were subjected to the offline antiSMASH 6.0 analysis.[11] The .gbk file of the biosynthetic gene cluster (*mxc* BGC) annotated to encode "myxochelin A/myxochelin B" was retrieved from the antiSMASH 6.0 analysis result of each strain if applicable. CORASON[14] was employed to generate a multilocus phylogeny of all the *mxc* BGCs. The .fasta file of the gene *mxc*G was used as the query gene, and the *mxc* from *Stigmatella aurantca* Sg a15 was used as the reference BGC. The e_value of "minimal for a gene to be considered a hit" was set to $3e^{-174}$, while all the other parameters of CORASON were set as default. The MEGA 7.0 software was used to the visualize the phylogenetic tree generated by CORASON.

Precursor Feeding Experiments. Seed culture of *Archangium* sp. SDU34 (10 mL) was added into a 250 mL Erlenmeyer flask containing 100 mL of liquid VY/2 medium. After 3 days of growth at 30 °C and 200 rpm, cultures were fed with 1 mM nicotinic acid (NA), BA, SA, and DHBA, respectively. The control group was done by supplementing 200 μL of DMSO. Further incubation lasted for another 5.5 days. The cultures were harvested by centrifugation at 5500*g* for 5 min, and the supernatant was extracted with 1 g of HP-20 resin. The resin was first rinsed with H_2O, and then the adsorbed compounds were recovered with methanol. After evaporation of organic solvent under vacuum, the afforded crude extracts were subjected to LC-MS metabolic analysis. Each experiment was done in duplicate.

LC-MS Analysis. LC-MS analysis was performed on an UHPLC system (Thermo Scientific Vanquish) coupled with a Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive HFX, ThermoFisher Scientific) equipped with an ESI source. The chromatographic separation was done using a C_{18} column packed with solid-core particles (Acquity UPLC HSS T3 (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, MA, USA). The elution solvent system was water and acetonitrile. The optimized elution gradient was a linear gradient from 5% to 100% acetonitrile in water in 0−10 min, at a constant flow rate of 0.3 mL/min. ESI source parameters were as follows: heater temperature 425 °C, sheath gas flow rate 50 arb, Aux gas flow rate 13 arb, spray voltage ±3500 V, and normalized collision energy 20, 40, and 60 eV.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00524.

NMR, HRESIMS, IR, and UV spectra for **1** and **2**; experimental details for bioassay, gene cluster annotation, MS/MS fragmentation of **1**−**4**; and other supporting data (PDF)

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Notes

The authors declare no competing financial interest.

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