NOTE

Biosynthetic origin of anthracimycin: a tricyclic macrolide from *Streptomyces* sp.

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Anthracimycin (**1**) is a decalin-fused tricyclic macrolide isolated from terrestrial and marine *Streptomyces* species (Figure 1).^{1–3} **1** shows strong activity against Gram-stain-positive bacteria including *Bacillus anthracis* and methicillin-resistant *Staphylococcus aureus* (MRSA).^{3,4} The most intriguing feature of this molecule is its enantiomerism to chlorotonil A (**2**), a secondary metabolite of a myxobacterium *Sorangium cellulosum* (Figure 1).⁵ Except for a methyl substitution at C-8 in **2**, the absolute configurations of the remaining chiral centers at C-2, C-6, C-7, C-12, C-15 and C-16 are opposite between **1** and **2** although the dichlorination at C-4 in **2** is an additional difference between these compounds. In nature, such enantiomeric natural products can be seen in various organisms from bacteria to plants.⁶ Simple examples include monoterpenes such as (+)-limonene and (–)-limonene produced from a common linear precursor geranylgeranyl pyrophosphate by the catalysis of (+)-limonene and (–)-limonene synthases, respectively (Figure 2). One of the more complex examples is a fungal alkaloid notoamide. (–)-Notoamide B is produced by a marine *Aspergillus* species, whereas its enantiomer (+)-notoamide B is produced by a terrestrial *Aspergillus* strain.⁶ The enantiomeric biosynthesis of notoamides is proposed to include a common achiral precursor, which is enantiomerically cyclized to a pair of enantiomers through intramolecular Diels–Alder reaction (Figure 2). In these cases, a common single achiral precursor cyclizes into respective enantiomeric products: no chiral centers exist in precursors. Meanwhile, in the biosynthesis of **1** and **2** it is likely that a pair of chiral precursors independently produced in actinobacterial and myxobacterial cells undergo the intramolecular Diels–Alder reaction (Figure 3). To the best of our knowledge, there exist no such natural products that are generated from a pair of enantiomeric precursors into highly modified structures. In order to understand this unusual enantiomeric biosynthesis, we investigated the biosynthetic origins of **1** for further genetic and/or enzymatic analysis. Herein, we describe the results from the ¹³C-labeling experiment of **1**.

As the carbon skeleton of **1** suggested its generation through the malonate pathway, [1,2-¹³C₂]acetate was fed to the culture to ensure the alignment of extender units. In the ¹³C NMR spectrum, split

signals arising from the ¹³C–¹³C coupling were clearly observed for all the carbons except for three methyl groups (C-23, C-24 and C-25) and C-11 and C-12 (Supplementary Figure S1). The outer split peaks were invisible owing to the roof effect⁷ caused by the close chemical shifts between C-11 and C-12 (Δ 0.11 p.p.m.) although these carbons are likely derived from a single acetate unit. In the 2D-INADEQUATE spectrum measured with a parameter set optimized for ¹J_{CC} 45 Hz, cross peaks derived from the intact ¹³C₂ acetate units were observed for C-1/C-2, C-3/C-4, C-5/C-6, C-7/C-8, C-9/C-10, C-13/C-14, C-15/C-16, C-17/C-18, C-19/C-20 and C-21/C-22 (Table 1, Supplementary Figure S3). The INADEQUATE correlation for C-11/C-12 could not be observed owing to the strong coupling also caused by their close chemical shift. The peak separation for C-11 and C-12 was then compared in different solvents and the best separation (Δ 0.31 p.p.m.) was obtained in benzene-*d*₆ (Supplementary Figure S2). The 2D-INADEQUATE spectrum in this solvent, however, still did not give a cross peak for C-11/C-12. It is theoretically predicted that the conventional parameter setting for ¹³C–¹³C coupling detection is not appropriate for carbons with such a small chemical shift difference.⁸ According to this prediction, instead of a normal setting $\tau = 1/4^1J_{CC}$ for delay, τ was set to be $3/4^1J_{CC}$, which successfully allowed detection of a coupling between C-11 and C-12 (Supplementary Figure S4).

The labeling experiment with the doubly labeled acetate indicated the methyl groups (C-23, C-24, and C-25) were not derived from methylmalonate. To clarify the origin of these methyl carbons, a feeding experiment of a plausible precursor L-[methyl-¹³C]methionine was carried out. High levels of enrichments of the methyl carbons were observed in the ¹³C NMR spectrum, confirming that the methyl branching is created by C-methylation of the polyketide chain (Table 1, Figure 4, Supplementary Figure S5).

There are three major features that make anthracimycin (**1**) unusual as an actinomycete metabolite. First, three methyl groups in the polyketide chain of **1** are derived from methionine. In polyketide biosynthesis, actinobacteria mostly utilize methylmalonyl CoA for generation of methyl branching in the carbon chain, whereas the fungal polyketide synthase (PKS) exclusively use methionine as a

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substrate for C-methylation to create methyl branching.⁹ It is uncommon for actinobacteria to generate the methyl substitution in the polyketide chain by C-methylation in type I polyketides. Examples are limited to several secondary metabolites: aplasmomycin,¹⁰ boromycin,¹¹ aurodox¹² and lankacidin C.¹³ Second, anthracimycin (1) has the same carbon framework as a myxobacterial metabolite chlorotonil A (2) except for an extra methyl substitution and halogenation modification. Actinobacteria and myxobacteria are phylogenetically distinct: the former are Gram-stain-positive and the latter are Gram-stain-negative. It is known that distantly related microbes can engage in horizontal transfer of secondary metabolite biosynthetic genes. However, it is very rare that actinomycetes and myxobacteria are producing secondary metabolites that share a common carbon framework. One such example only includes actinofuranones from *Streptomyces* and aurafurones from a *Stigmatella* strain although the structural similarity is lower than the case of 1 and 2.¹⁴ Third, anthracimycin (1) and chlorotonil A (2) have the opposite absolute configuration. There is no other example of enantiomeric compounds with polycyclic, complex structures among the type I PKS-derived secondary metabolites.

Recently, we reported the draft genome sequencing of the producing strain *Streptomyces* sp. TP-A0875.¹⁵ Two type I PKS gene clusters exist in the genome and one of them is assigned to the biosynthetic gene cluster of 1 on the basis of PKS gene function prediction. Future

work will be directed toward biochemical analysis to elucidate the molecular basis of enantiomeric biosynthesis.

GENERAL PROCEDURE

Sodium [1,2-¹³C₂]acetate was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA), Inc. L-[Methyl-¹³C]methionine was purchased from ISOTEC Inc. (Miami, OH, USA). NMR spectra were obtained on a Bruker AVANCE 500 spectrometer (Bruker BioSpin KK Kanagawa, Japan) in CDCl₃ and benzene-*d*₆. ¹³C chemical shifts were referenced to the residual solvent signals (CDCl₃: δ_C 77.0; benzene-*d*₆: δ_C 127.7).

Microorganism

Streptomyces sp. strain TP-A0875 was isolated from compost material collected in Ishikawa, Japan. The strain was identified as a member of the genus *Streptomyces* on the basis of 99.4% 16S rRNA gene sequence identity (1453 nucleotides; DDBJ accession number AB451554) with *Streptomyces collinus* NBRC 12759^T (accession number AB184123).

FERMENTATION

Strain TP-A0875 growing on a plate culture was inoculated into a 500-ml K-1 flask containing 100 ml of the V-22 seed medium consisting of soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, yeast extract, 0.2%, Tryptone (Difco Laboratories, Detroit, MI, USA) 0.5%, K₂HPO₄ 0.1%, MgSO₄·7H₂O 0.05% and CaCO₃ 0.3% (pH 7.0). The flask was placed on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. Then, the seed culture (3 ml) was transferred into 500-ml K-1 flasks each containing 100 ml of the A-3M production medium consisting of soluble starch 2.0%, glycerol 2.0%, glucose 0.5%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3% and Diaion HP-20 resin (Mitsubishi Chemical Co., Kanagawa, Japan) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 6 days.

Figure 1 Structures of anthracimycin (1) and chlorotonil A (2).

Figure 2 Enantiomeric natural products from achiral precursors.

Figure 3 Plausible biogenesis of anthracimycin (1) and chlorotonil A (2) from chiral precursors.

Table 1 Incorporation of ^{13}C -labeled precursors into **1**

Position ^a	$[1,2-^{13}\text{C}_2]\text{acetate}$ (benzene- d_6)			$L\text{-}[methyl-^{13}\text{C}]\text{methionine}$ (CDCl_3)	
	δ_{C}	$^1J_{\text{CC}}$ (Hz)	2D-INADEQUATE	δ_{C}	Relative enrichment ^b
1	168.1	53	2	168.7	0.74
2	48.9	53	1	49.1	0.94
3	190.4	63	4	190.7	0.66
4	102.8	63	3	102.9	0.88
5	194.0	45	6	194.0	0.75
6	52.5	45	5	52.5	0.94
7	32.9	35	8	32.9	0.90
8	31.3	35	7	31.2	0.93
9	121.2	33	10	120.9	0.91
10	133.2	33	9	133.8	0.64
11	37.5	73	12	37.4	0.97
12	37.2	73	11	37.3	1.05
13	132.8	69	14	132.9	0.97
14	124.9	69	13	124.8	0.99
15	45.9	32	16	45.9	0.93
16	32.7	32	15	32.7	0.92
17	138.6	68	18	138.9	0.82
18	126.3	68	17	125.9	1.01
19	123.6	73	20	123.6	0.78
20	131.7	73	19	131.5	0.90
21	69.4	39	22	69.8	0.83
22	20.4	39	21	20.8	1.00
23	11.6			11.7	19.75
24	23.2			23.4	20.21
25	16.3			16.3	20.75

^aThree carbons C-7, C-11, and C-12 are misassigned in the previous report.³ Carbon chemical shifts were corrected to be 37.4 p.p.m. for C-11, 37.5 p.p.m. for C-12, and 33.0 p.p.m. for C-7 on the basis of ^{13}C - ^{13}C coupling constants and 2D-INADEQUATE analysis.

^b ^{13}C signal intensity of each peak in the labeled **1** divided by that of the corresponding signal in the unlabeled **1**, respectively, normalized to give an enrichment ratio of **1** for the unenriched C-22 methyl peak. The numbers in bold type indicate ^{13}C -enriched atoms from ^{13}C -labeled precursors.

Figure 4 ^{13}C -labeling pattern of anthracimycin (**1**).

EXTRACTION AND ISOLATION

After incubation, 100 ml of 1-butanol was added to each flask, and the flasks were allowed to shake for an hour. The mixture was centrifuged at 6000 r.p.m. for 10 min and the organic layer was separated from the aqueous layer. The solvent was removed by evaporation to give 2.0 g of a crude extract from 1 l of culture. This crude extract was fractionated using silica gel column chromatography with a step gradient of CHCl_3 –MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). Fraction 3 (10:1) containing **1** was concentrated to give 81 mg of pale yellow oil, which was further fractionated by reversed-phase ODS column chromatography with a step gradient of MeCN/0.1% HCO_2H (5:5, 6:4, 7:3, 8:2, 9:2, and 10:0 v/v). The fraction eluted with 90% MeCN was pooled and evaporated *in vacuo*, and the major part of the solvent was evaporated *in vacuo*. The remaining aqueous solution was extracted twice with EtOAc to give colorless oil (8.1 mg). The final

purification was achieved by preparative HPLC (Cosmosil 5C18-AR-II, 10×250 mm, 4 ml min^{-1}) using a gradient of MeCN/0.1% HCO_2H (MeCN concentration: 80–100–100% for 0–10–20 min) at 4 ml min^{-1} , yielding **1** (5.6 mg) with a retention time of 13.3 min.

INCORPORATION OF ^{13}C -LABELED PRECURSORS

Feeding experiments were performed using $[1,2-^{13}\text{C}_2]\text{acetate}$ and $L\text{-}[methyl-^{13}\text{C}]\text{methionine}$. The incubation, cultivation and purification were performed in the same manner as described above. Addition of ^{13}C -labeled precursors (1 ml solution per flask) was initiated at 48 h after inoculation and periodically carried out every 24 h for four times. After further incubation for 24 h the cultures were extracted with 1-butanol.

1. Sodium $[1,2-^{13}\text{C}_2]\text{acetate}$: After feeding of sodium $[1,2-^{13}\text{C}_2]\text{acetate}$ (total 800 mg; $20 \text{ mg} \times 10$ flasks $\times 4$ days), 5.6 mg of ^{13}C -labeled **1** was obtained from 1 l of culture as described above.
2. $L\text{-}[methyl-^{13}\text{C}]\text{methionine}$: After feeding of $L\text{-}[methyl-^{13}\text{C}]\text{methionine}$ (total 100 mg; $2.5 \text{ mg} \times 10$ flasks $\times 4$ days), 3.6 mg of ^{13}C -labeled **1** was obtained from 1 l of culture.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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