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Actinoperylone, a novel perylenequinone-type shunt product, from a deletion mutant of the *act*VA-ORF5 and ORF6 genes for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2)

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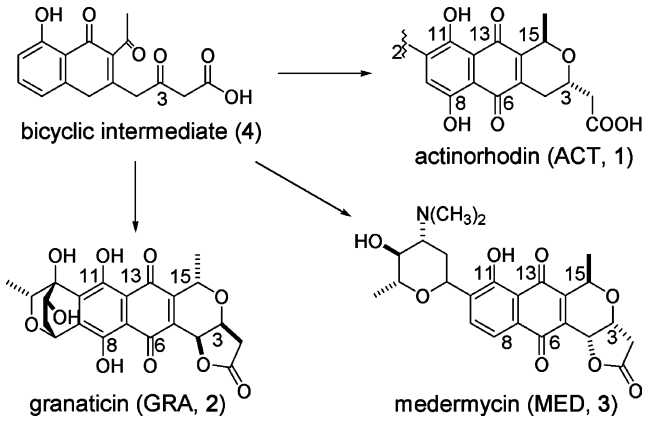
Abstract

A novel shunt product, actinoperylone, has been isolated from a deletion mutant of the *act*VA-ORF5 and ORF6 genes involved in the biosynthesis of a benzoisochromanequinone (BIQ) antibiotic actinorhodin (ACT) in *Streptomyces coelicolor* A3(2). Spectroscopic analysis revealed its perylenequinone-type skeleton with the four chiral centers, obviously derived from the dimerization of an ACT intermediate. The structure of actinoperylone indicates the essential role of ActVA-ORF5 in the oxygen introduction at C-6, which is common to the formation of BIQ chromophore. The present results also agree with the distribution of the *act*VA-ORF5 homologues in all known BIQ biosynthetic clusters in streptomycetes.

Keywords: Actinorhodin; Biosynthesis; Monooxygenase; Hydroxylase

Actinorhodin (ACT, **1**), produced by the well characterized *Streptomyces coelicolor* A3(2),[1] is a member of benzoisochromanequinone (BIQ) antibiotics, a class of aromatic polyketides. In the biosynthesis of BIQs, the basic carbon skeleton assembled by the type II minimal polyketide synthase is converted to the bicyclic intermediate (**4**) by ketoreductase (KR), aromatase (ARO), and cyclase (CYC). The intermediate undergoes further modifications including stereospecific ketoreduction, enoylreduction, and oxygenation in the later 'tailoring' steps.[2] One of the key and common modifications is the oxygenation at C-6 position (Scheme 1). In the ACT biosynthetic gene (*act*) cluster, *act*VA-ORF6 protein was proposed[3] (but not proved) to encode an enzyme to oxidize C-6. Using analogous substrates, biochemical[4] and crystallographic[5] studies of the ActVA-ORF6 protein were performed, and the analyses demonstrated its catalytic activity as an unusual monooxygenase



Scheme 1. Structures of BIQs derived from the bicyclic intermediate. Numbering of positions is based on the biosynthetic order.

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without any prosthetic group, metal ion or cofactor. Subsequent comparative analysis[6] of the complete biosynthetic gene clusters for the other BIQ examples, granaticin (GRA, **2**: the *gra* cluster) from *S. violaceoruber* Tü22 and medermycin (MED, **3**: the *med* cluster) from *Streptomyces* sp. AM-7161, revealed the unexpected lack of an *act*VA-ORF6 homologue in the *gra* and *med* clusters.

Instead, the *gra* and *med* clusters include the common oxygenase genes (*gra*-ORF21 and *med*-ORF7, respectively) homologous to *act*VA-ORF5, which was initially deduced to encode a hydroxylase at C-8 position,[7] although no hydroxylation occurs at the C-8 position of **3**. These findings raise questions about the contributions of the *act*VA-ORF5 and ORF6 genes to ACT biosynthesis. This Letter deals with the characterization of a novel shunt product, actinoperylone, from a deletion mutant of *act*VA-ORF5 and 6 genes.

In the *act* cluster, *act*VA-ORF5 is located in the immediate upstream of *act*VA-ORF6 and they should be transcriptionally coupled. First, we inactivated both genes simultaneously by deleting the genes and replacing with a spectinomycin–streptomycin resistant (*aadA*) cassette (Fig. S1[8]).[9] The *Δact*VA-5,6 mutant produced a yellowish brown pigment instead of ACT on R4 agar medium, demonstrating the involvement of *act*VA-ORF5 and/or ORF6 in ACT biosynthesis (Fig. S2[8]). Next, we constructed an *act*VA-ORF6 deletion mutant but no obvious effect of the mutation on ACT production was observed (Fig. S3[8]). In addition, ectopic expression of *act*VA-ORF5 alone in the *Δact*VA-5,6 mutant restored ACT production to wild-type levels (Okamoto et al., manuscript in preparation). The genomic sequence analysis of *S. ceolicolor* A3(2) revealed no any other homologues of *act*VA-ORF5 and ORF6 genes on its genome.[1] These observations and facts indicated that *act*VA-ORF5 but not *act*VA-ORF6 is essential for ACT biosynthesis. To elucidate the biosynthetic step(s) governed by *act*VA-ORF5, we performed the characterization of a yellowish brown pigment produced by the *Δact*VA-5,6 mutant.

The *Δact*VA-5,6 mutant was inoculated into TSB medium for seed culture[10] and grown on a rotary shaker at 200 rpm, 28 °C, for 2 days. Aliquots of the seed culture were transferred to R4 liquid culture and grown as previously described.[11] The supernatant of the culture was directly subjected into HPLC and LC/ESIMS analyses.[12] A compound detected at the retention time of 19.8 min showed the unique UV–vis spectrum different from that of ACT. LC/HRESIMS gave a molecular formula of $C_{32}H_{26}O_{10}$ (*m*/*z* $[M+H]^+$ calcd for $C_{32}H_{27}O_{10}$, 571.1604. Found, 571.1649). This formula indicated that this compound has a dimer structure derived from two octaketides.

The crude extract obtained from 1.5 L of R4 culture was methylated under acidic conditions.[13] HPLC analysis detected the peak with the same UV–vis spectrum at the retention time of 29.0 min. LC/HRESIMS analysis gave a molecular formula of $C_{34}H_{30}O_{10}$ (*m*/*z* $[M+H]^+$ calcd for $C_{34}H_{31}O_{10}$, 599.1917. Found, 599.1932). The increase of molecular weight suggested the existence of two carboxylic groups. Silica gel column chromatography eluting with $CHCl_3$ and preparative HPLC[14] gave a pure compound (3 mg),[15] which was subjected to NMR analysis in $CDCl_3$.

NMR data suggested the existence of seventeen carbons and fifteen protons (Table 1), although the molecular formula was suggested as $C_{34}H_{30}O_{10}$. These results indi-

Table 1
Summary of NMR data

Position	^{13}C (100 MHz)		^{1}H (400 MHz)				Correlations	
	δ (ppm)	$^{1}J_{C-C}$ (Hz)[b]	δ (ppm)	Integral	Multiplicity	*J* (Hz)	NOESY	HMBC
1	171.0	58.5						
2	40.5[a]	58.5	2.70	1H	dd	15.6, 5.0	2-Hb	C-1, 3
			2.80	1H	dd	15.6, 7.6	2-Ha, 3-H	C-1, 3, 4
3	64.4	37.4	4.20	1H	m		2-Hb, 4-Ha, 16-H	
4	38.7[a]	37.4	3.21	1H	dd	15.6, 2.4	3-H, 4-Hb, 9′-H[c]	C-5
			3.53	1H	dd	15.6, 9.2	4-Ha, 9′-H[c]	C-5
5	141.5	57.5						
6	127.1[a]	57.5						
7	126.5	55.6						
8	124.7[a]	55.6						
9	137.6	61.3	8.68	1H	d	9.6	4′-Ha,[c] 4′-Hb,[c] 10-H	C-7, 11
10	123.4[a]	61.3	7.25	1H	d	9.6	9-H	C-8, 12
11	176.9	59.4	16.19	1H (OH)	s			C-10, 11, 12
12	109.3[a]	59.4						
13	176.5	58.5						
14	136.7[a]	58.5						
15	69.3	37.4	5.47	1H	q	6.4	16-H	C-3, 5, 14, 16
16	19.4[a]	37.4	1.64	3H	d	6.4	3-H, 15-H	C-14, 15
O–Me	51.9		3.73	3H	s			C-1

[a] Intensity increased by feeding of [2-^{13}C] sodium acetate.
[b] Coupling constant was based on feeding of [1,2-$^{13}C_2$] acetic acid.
[c] NOEs between counterpart molecules were detected.