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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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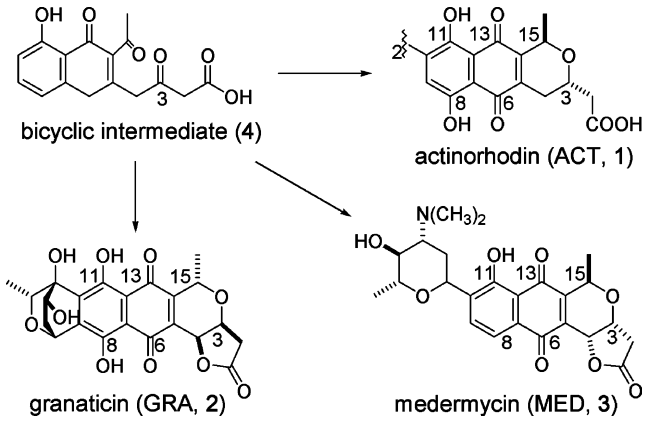
Abstract

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.

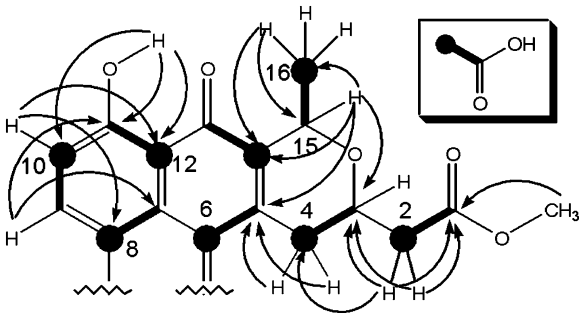


Fig. 1. The monomer structure of the target product as the dimethyl ester. HMBC correlations are shown by arrows and incorporation of [1,2-$^{13}C_2$] acetic acid is indicated by bold lines. The filled circles indicate the carbons derived from [2-^{13}C] sodium acetate.

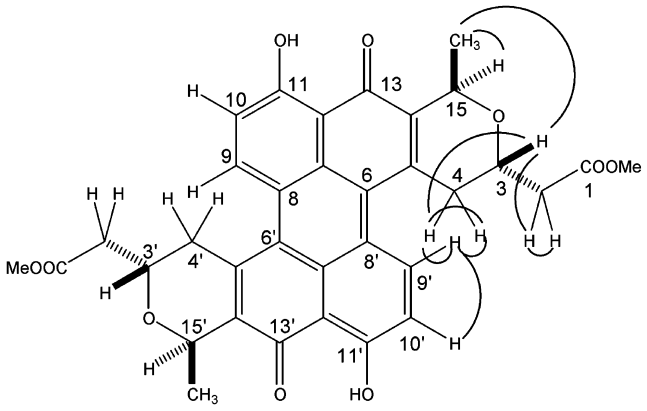


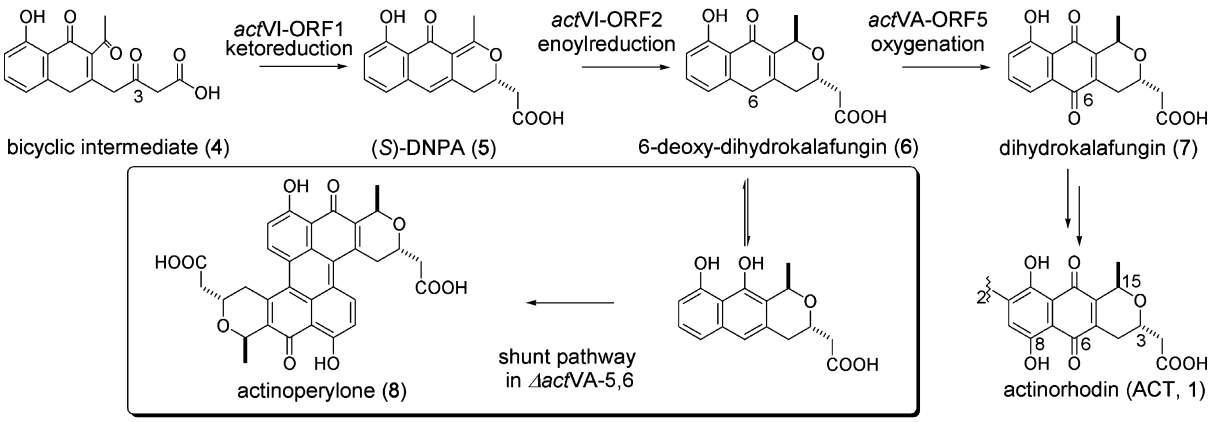
Fig. 2. The structure of dimethyl ester of **8**. Selected NOEs are shown by curved lines.

cated that the compound has a symmetrical dimer structure derived from the same octaketide molecules. The correlation data of COSY, HMQC and HMBC spectra indicated a tricyclic monomer structure consisting of the same pyran ring as that of ACT and a tetrasubstituted benzene ring.

To obtain further information, feeding experiments of [2-^{13}C] sodium acetate and [1,2-$^{13}C_2$] acetic acid were carried out.[16] A labeled target compound in each experiment was isolated as the dimethyl ester. Its ^{13}C NMR analysis revealed the ^{13}C -^{13}C coupling constants and the pairs of the coupled signals were confirmed by 2D-INADEQUATE spectrum (Table 1). Additionally, ^{13}C NMR spectrum of the sample from the feeding of [2-^{13}C] sodium acetate clearly indicated the incorporation pattern of acetate units. Based on these data, the monomer structure was elucidated as shown in Figure 1.

The stereochemistry of the pyran ring was unambiguously determined to be a *trans* configuration based on the NOE of 3-H with 16-H, but not with 15-H. Its obvious biosynthetic relationship with **1** suggests the *trans* (3*S*,15*R*) stereochemistry. In addition, the NOESY crosspeak was detected between 4-H and 9-H although these hydrogens on a monomer are not close enough to each other. However, the anti-parallel connection of the two half units reasonably explains the detection of the NOE between 4-H and 9′-H (4′-H and 9-H) (Fig. 2). Consequently, the novel shunt product was elucidated as 7*H*,16*H*-perylo[2,1*c*:8,7-*c*′]dipyran-7,16-dione,1,3,4,9,11,12-hexahydro-8,15-dihydroxy-1*R*,9*R*-dimethyl-3*S*,11*S*-diacetic acid, and was designated as actinoperylone (**8**) (Scheme 2). Similar perylenequinone derivatives were isolated as natural products of plant[17] and fungal[18] origin. Especially, squitaquinone B[17] has the similar pyran ring moiety fused to perylenequinone and its NMR data ensured the structure of **8**.

The formation of **8** is reasonably explained based on our previous proposal[3] as follows (Scheme 2). The stereospecific 1,4-reduction of 4,10-dihydro-9-hydroxy-1-methyl-10-oxo-3*H*-naphtho[2,3-*c*]pyran-3-(*S*)-acetic acid, (*S*)-DNPA (**5**), by ActVI-ORF2 occurs to afford the probable reduced product, 6-deoxy-dihydrokalafungin (**6**), which readily undergoes the oxygenation at C-6 to lead to **1** via dihydrokalafungin (**7**). The interruption of the oxidation would result in the possible tautomerization to the dihydroxynaphthalene derivative followed by oxidative dimerization to afford **8**. In *Streptomyces griseus*, a cytochrome P-450 enzyme, P-450mel, was reported to catalyze the oxidative biaryl coupling of 1,3,6,8-tetrahydroxynaphthalene to yield 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone.[19] There is a possibility that a similar P-450[20] outside the *act* cluster



Scheme 2. The proposed biosynthetic pathway of ACT and the novel shunt product, **8**. Intermediate **6** and its tautomer are not isolated and hypothetical.

is related to the formation of **8**. Thus, the structure of **8** strongly indicates ActVA-ORF5 to be involved in the C-6 oxygenation.

Recently, ActVA-ORF5 was demonstrated to function as a FMN-dependent monooxygenase coupled with a NADH:flavin oxidoreductase encoded by *act*VB, which is an essential gene for ACT biosynthesis.[21] Under the reported assay conditions, the ActVA–ActVB system catalyzed the oxygenation of **7** to produce a product, DHK-OH.[22] The structural assignment of DHK-OH to 8-hydroxylated dihydrokalufungin would agree with the fact that ACT itself has a hydroxyl group at C-8. Interestingly, the *gra* and *med* clusters also carry *act*VB homologues,[6] indicating a critical enzymatic function in BIQ biosynthesis. The oxygen introduction at the C-6 position is the only shared oxygenation step among the biosynthesis of ACT, GRA and MED. The ActVA–ActVB system is reasonably deduced to play the essential role for the C-6 oxygenation step as well as the hydroxylation at C-8 of BIQ biosynthesis. This is a new idea, consistent with the so-far common presence of *act*VA-ORF5 homologues in the BIQ clusters, and is currently studied by biochemical characterization of ActVA-ORF5.

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Supplementary data

Comparison of genetic organizations, phenotypes on R4 agar medium, and HPLC analyses among wild type and the deletion mutants are provided. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.12.033.

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12. Centrifuged supernatant was subjected to analytical HPLC with TOSOH TSK-gel ODS-100S (4.6 mm i.d. × 150 mm; solvent A, acetonitrile containing 0.5% acetic acid; solvent B, 0.5% aq acetic acid; gradient profile, 0–5 min 35% A, 5–30 min 35–95% A, 30–35 min 95% A, 35–40 min 95–35%A; flow rate, 1.0 mL/min; temperature, 40 °C). The LC condition for LC/ESI-MS was the same as that of analytical HPLC.
13. To the crude extract dissolved in anhydrous MeOH, concd H_2SO_4 (1/20 vol) was added dropwise. The reaction mixture was heated under reflux for 2 h. Water (1 vol) was added and the mixture was concentrated in vacuo. The resultant suspension was extracted with EtOAc, and EtOAc removed in vacuo. The residue was subjected to a silica gel column chromatography.
14. Preparative HPLC was carried out with Waters SunFire (10 mm i.d. × 250 mm; eluent, 90% aqueous methanol; flow rate, 2.5 mL/min; temperature, 40 °C).
15. Physico-chemical properties of actinoperylone dimethyl ester: $[\alpha]_D$ −50.0 (*c* 0.02, MeOH), UV–vis (90% MeOH): λ_{max} (nm) 248, 344, 440 (sh), 463, 540, 585.
16. The seed culture was transferred to 100 mL each of R4 medium in 500 mL Erlenmyer flasks (total 2.0 L), which were grown shaking (200 rpm) at 28 °C. An aqueous solution of the labeled acetic acid (200 mg/mL) was sterilized by 0.22 μm pore filter. Aliquots (100 μL) of the solution (containing 20 mg of acetic acid) were added to each flask of a 2-day culture followed by fermentation for further 3 days.
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