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Identification of a novel shunt product produced by a disruptant of the *act*VI-ORFA gene involved in the biosynthesis of actinorhodin in *Streptomyces coelicolor* A3(2)

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Abstract

NMR and MS spectroscopy elucidated the structure of a novel shunt product isolated from a disruptant of the *act*VI-ORFA gene involved in the biosynthesis of actinorhodin (ACT) in *Streptomyces coelicolor* A3(2). The product, 1,4-naphthoquinone-8-hydroxy-3-[(3*S*)-acetoxybutyric acid], was found without complete abolition of ACT production, implying that the ORFA product might stabilise the multicomponent, type II PKS complex and perhaps assist the chemically spontaneous dehydration of a hemiketal intermediate for efficient pyran ring formation.

Keywords: aromatic polyketides; actinorhodin; biosynthesis; *Streptomyces*.

Streptomyces coelicolor A3(2) is the most genetically characterised streptomycete, and produces at least four metabolically distinct antibiotics, allowing for studies on the biosynthesis of each antibiotic as well as on metabolic crosstalk between secondary metabolism and other physiological aspects such as primary metabolism and morphological differentiation.[1] One of the antibiotics, actinorhodin (ACT, **1**), has served[2] as one of the pioneering model compounds for understanding the genetic programming of aromatic polyketides whose basic carbon skeleton is formed by a type II polyketide synthase (PKS)[3] followed by post-PKS modification ('tailoring') steps.[4] The *act*VI genetic region[5] (ORFA, 1, 2, 3, 4), part of the ACT biosynthetic gene cluster (the *act* cluster), is largely related to stereospecific pyran ring formation. Our recent chemical characterisation[6,7] of disruptants of the *act*VI region revealed that disruption of ORFA led not only to significant reduction in ACT production, but also to accumulation of an unknown compound X, together with the known intermediate, 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-*c*]-pyran-(3*S*)-acetic acid, (*S*)-DNPA (**4**).[8]

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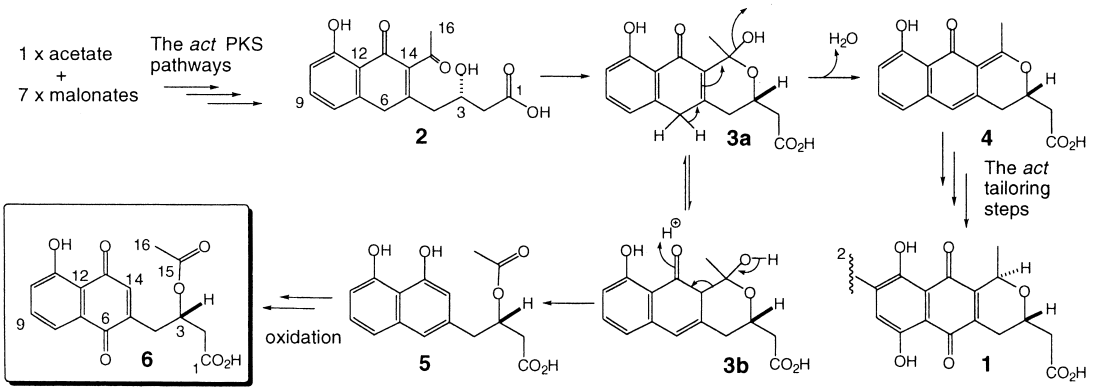
It had already been shown[7] by HPLC analysis that compound X has a UV–vis spectrum similar to that of **4** but with the peak (410 nm) in the visible region shifted ca. 20 nm to a shorter wavelength. The production medium from a large-scale culture (4 litres) of the *act*VI-ORFA disruptant was extracted with chloroform. The residue from the concentrated extracts was subjected to repeated column chromatography on oxalic acid-treated silica gel in chloroform–ethyl acetate to purify compound X (ca. 8 mg) which was used for extensive spectroscopic studies (Scheme 1). High resolution EI-MS measurement indicated its composition to be $C_{16}H_{14}O_7$ (found: 318.0785; requires: 318.0740). The major fragment peaks, beside the molecular ion peak (*m*/*z* 318), in the EI-MS spectrum were *m*/*z* 214 (base peak) and *m*/*z* 258. The ^{1}H NMR and ^{13}C NMR spectra showed similar signal patterns to those of **4**, suggesting the presence of fused bicyclic A–B rings. The characteristic ^{1}H NMR signal appearing for a terminal methyl signal of C-16[9] at 2.01 ppm is shifted to upper-field by ca. 0.6 ppm from that of **4**, indicating that a major structural difference is present involving C-15. Further extensive 2D NMR measurements using HMQC and HMBC unambiguously established full assignments of the signals of ^{1}H NMR and ^{13}C NMR spectra and their key correlations (Table 1). The C–C bond between C-14 and C-15 is disconnected to provide an acetoxy moiety derived from C-15 and C-16, thus being in agreement with the shift of the methyl ^{1}H NMR signal mentioned above. The elucidated structure perfectly explains the foregoing fragmentation peaks of the EI-MS: the elimination of the acetoxy moiety at C-3 and the decarboxylation at C-1 afford the naphthoquinone fragment at *m*/*z* 214; the remaining fragments also clearly reflect the proposed structure. Compound X should possess the (*S*)-configuration at the C-3 chiral centre, assuming the same stereochemical control,[10] and is described as 1,4-naphthoquinone-8-hydroxy-3-[(3*S*)-acetoxy-butyric acid], (*S*)-NHAB (**6**).



Scheme 1. Proposed biosynthetic pathway of actinorhodin and shunt pathway leading to (*S*)-NHAB (**6**)

Our previous experiments[6,7] suggested that **4** is formed by dehydration of the hemiketal **3a** which is derived from the secondary alcohol **2**. Interruption of this concerted mechanism would lead to an isomerisation of **3a** to **3b** followed by cleavage of the C–C bond between C-14 and C-15 to afford the dihydroxynaphthalene **5** that would easily undergo (spontaneous) oxidation[11] to **6**.[12] Thus, identification of **6** as a novel shunt product rigorously supports the hypothesis that pyran ring formation occurs via **3a**.

Disruption of *act*VI-ORFA caused accumulation of the intermediate **4** as well as the shunt product **6** without complete abolition of ACT production, implying that the ORFA product

Table 1
NMR data for **6** (in $CDCl_3$ 500 MHz for ^1H; 125 MHz for ^{13}C)

	^{13}C	^1H				Carbon(s) correlated
Position	δ (ppm)	δ (ppm)	intensity	multiplicity	J (Hz)	in HMBC spectrum
1	174.1					
2	38.5	2.74	2H	d d - like	J = 6.0, 7.0	C-1, 3, 4
3	68.4	5.47	1H	m		
4	34.3	2.78	1H	ddd	J = 14.0, 8.5, 1.0	C-2, 3, 5, 6, 14
		3.07	1H	ddd	J = 14.0, 4.5, 1.0	C-2, 3, 5, 6, 14
5	147.9					
6	183.9					
7	131.9					
8	119.6	7.66	1H	dd	J = 8.0, 1.5	C-6, 10, 12
9	136.4	7.62	1H	t	J = 8.0	C-7, 11
10	124.4	7.27	1H	dd	J = 8.0, 1.5	C-8, 12
11	161.4	11.87	1H (OH)	s		C-10, 11, 12
12	115.1					
13	187.8					
14	136.8	6.81	1H	s		C-4, 6, 12
15	170.2					
16	20.8	2.01	3H	s		C-15

might enzymatically assist the chemically spontaneous dehydration of **3a** for efficient pyran ring formation.

The *act*VI-ORFA gene is a member of a growing family of genes found in the biosynthetic gene clusters for actinomycete aromatic polyketides: granaticin (*gra*-ORF31[13]), frenolicin (*fren*X[14]), mithramycin (*mtm*X[15]), doxorubicin/daunorubicin (*dps*H[16]; *dau*Z[17]), and an unknown compound (ORF1[18]). Disruption of *dps*H abolished[16] production of daunorubicin and doxorubicin in *Streptomyces peucetius* to result in accumulation of an aglycone intermediate, ε-rhodomycinone, indicating the essential function of the *dps*H product. Although no definite role can be deduced from the available data, the products of the *act*VI-ORFA family of genes certainly play a crucial role in the biosynthesis of aromatic polyketides. The most likely current possibility is that they stabilise the multicomponent, type II PKS complex and perhaps assist the coupled enzymatic reactions involved in post-PKS tailoring steps.

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12. The structure of **3a** in the figure represents one of the possible tautomers. An alternative mechanism for the formation of **6** involves hydration of **4** at C-14 and C-15 to afford **3b**. The fact that **6** is detected exclusively from the *act*VI-ORFA disruptant not from the other producers of **4**[4,6] disfavours the possibility that **6** is spontaneously derived from **4**.
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