Chemical Characterisation of Disruptants of the *Streptomyces coelicolor* A3(2)

*act*VI Genes Involved in Actinorhodin Biosynthesis

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The *act*VI genetic region of *Streptomyces coelicolor* A3(2) is part of the biosynthetic gene cluster of actinorhodin (ACT), the *act* cluster, consisting of six ORFs: ORFB, ORFA, ORF1, ORF2, ORF3, ORF4. A newly devised method of ACT detection with a combination of HPLC and LC/MS was applied to the analysis of the disruptants of each ORF. ACT was produced by those of ORFB, ORFA, ORF3, and ORF4. Instead of ACT, the ORF1 disruptant produced 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC) and aloesaponarin II as shunt products. The ORF2 disruptant gave 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-*c*]-pyran-3-(*S*)-acetic acid, (*S*)-DNPA. These results support our previous proposal for stereospecific pyran ring formation in the biosynthesis of ACT, most importantly suggesting that the *act*VI-ORF2 product would recognize (*S*)-DNPA as a substrate for stereospecific reduction at C-15. The disruptant of ORFA produced (*S*)-DNPA together with ACT, suggesting that *act*VI-ORFA might play a role such as stabilising the multicomponent, type II PKS complex.

Actinorhodin[1] (ACT, **1**) produced by *Streptomyces coelicolor* A3(2) has a unique dimeric structure with connection of two benzoisochromanequinone (BIQ) chromophores by a C–C bond. Its pH indicator properties (red under acidic conditions and blue under basic ones) have provided a convenient method of ACT detection simply by blue pigmentation, resulting in the successful characterization[2] of mutants in ACT biosynthesis, followed by cloning[3] of the whole biosynthetic gene cluster (the *act* cluster) from the *S. coelicolor* chromosome. The carbon skeleton of ACT is produced from a linear polyketide chain (an octaketide) condensed by a polyketide synthase (PKS) from an acetate starter unit with seven malonyl CoAs.

Sequencing of the region of the *act* cluster[4] encoding the PKS, and of homologous clusters from the producers of granaticin[5] and tetracenomycin[6], revealed the architecture of these enzymes as type II PKSs, with a minimal PKS[7,8] consisting of three subunits: ketosynthase (KS), chain length factor (CLF), and acyl carrier protein (ACP).

Besides the minimal PKS genes, the *act* cluster, spanning over 22 kb, contains other genes encoding proteins relevant to ACT biosynthesis. The early biosynthetic enzymes closely associated with the minimal PKS include a ketoreductase[9] (KR, encoded by *act*III) functioning at C-9, an aromatase[4] (ARO, encoded by *act*VII) involved in aromatisation of the first ring by dehydration, and a

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Fig. 1. Proposed biosynthetic pathway of actinorhodin (1) in *Streptomyces coelicolor* A3(2) and shunt pathway leading to aloesaponarin II (3).
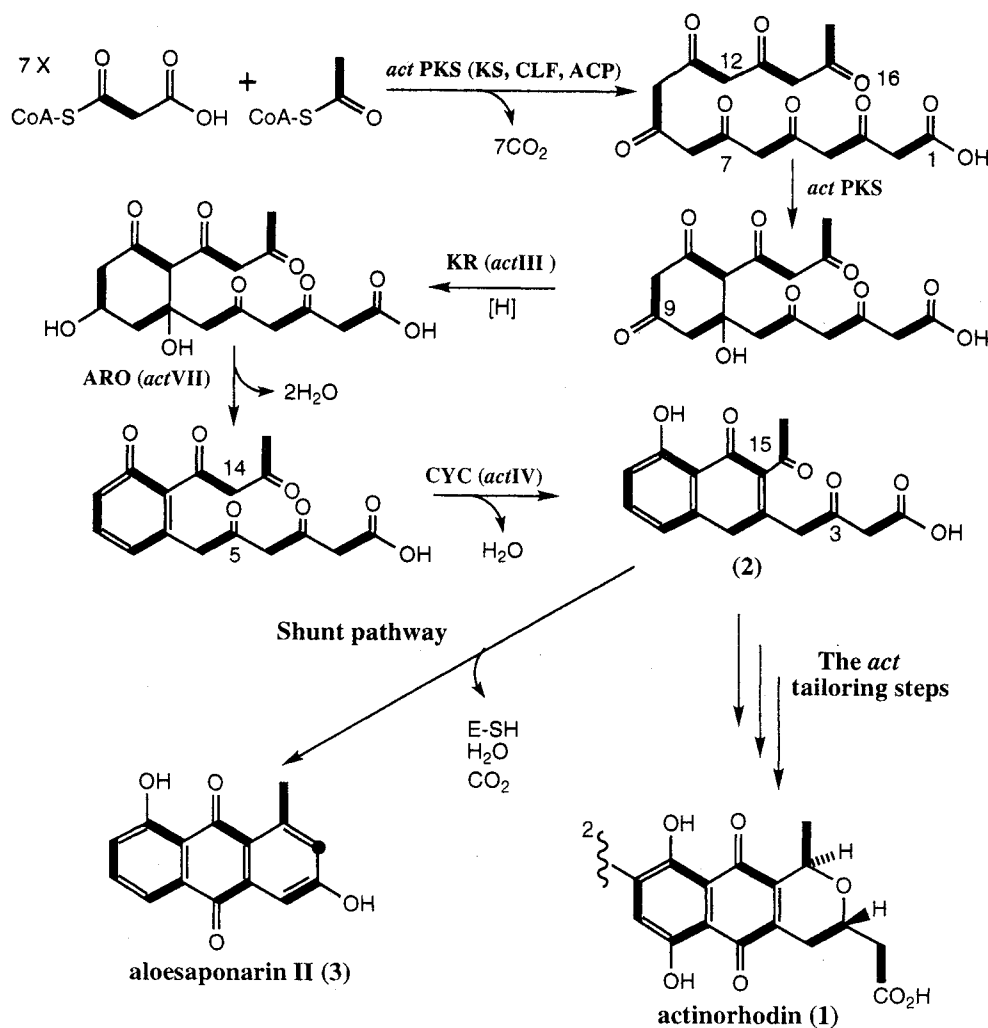

Numbering is based on the biosynthetic origin. The *act* PKS is the minimal PKS (see text). The bold lines/dot indicate the biosynthetic origins from acetate/malonate units. For simplicity, all the intermediates are shown as free acids, but some might exist enzyme-bound as thioesters: RCOS-Enz.

cyclase[4] (CYC, encoded by *act*IV) controlling second ring formation by aldol condensation between C-5 and C-14, all of which, together with the minimal PKS, afford the proposed bicyclic intermediate (2) (Figure 1). The intermediate (2) is subject to post-PKS modifying ('tailoring') steps[10] to produce the final structure, involving a number of interesting biosynthetic problems. One of the key issues is formation of the third (pyran) ring, with stereochemical control to provide the (3*S*, 15*R*) configuration.

The *act*VI region, located near the left-end of the *act* cluster (Figure 2), was deduced to be involved in pyran ring

formation based on the fact that the two *act*VI mutants[2] produce aloesaponarin II (3) as a shunt product[11] (Figure 1). DNA sequencing[12] of the *act*VI region (5.7 kb) revealed six ORFs: ORFB, ORFA, ORF1, ORF2, ORF3, ORF4, and subsequent gene disruption of each ORF gave the following phenotypes in respect of pigmentation: (1) disruption of ORFB and ORF4 did not prevent blue pigmentation at apparently wild type levels; (2) the ORFA-disruptant showed an obvious reduction in blue pigmentation; (3) brownish pigment was produced by the disruptants of ORF1 and ORF2; (4) the ORF3-disruptant produced a reddish pigment which became yellowish and later turned

Fig. 2. Restriction map of the whole *act* cluster (22 kb) and the organisations of the *act*VI, and the *act*III, I, VII, IV regions.
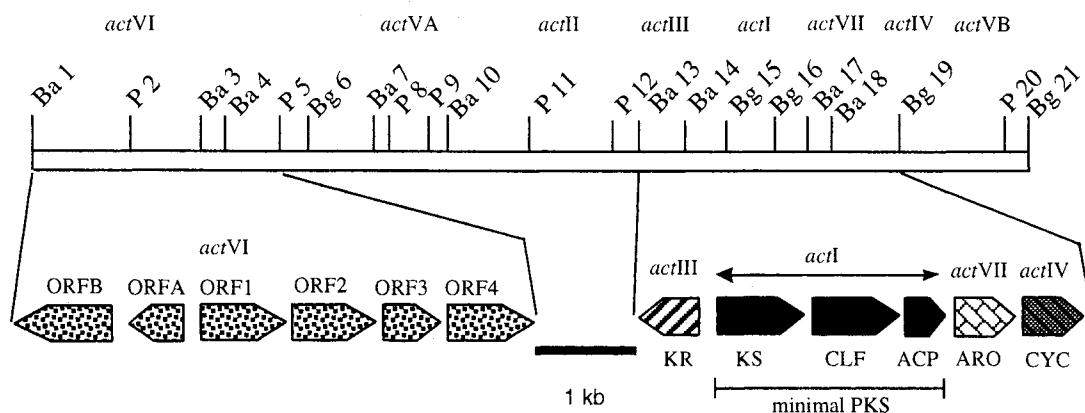
The sites are numbered as described in ref. 3: Ba *(Bam*HI), Bg *(Bgl*II), P *(Pst*I).

blue on ammonia fuming. These results suggested that ORFB and ORF4 are not essential for ACT production; further understanding of the gene functions would be gained by chemical characterisation of the metabolites of each disruptant. That is the aim of the work described here.

Materials and Methods

Bacterial Strains and Culture Conditions

The ACT-producing *S. coelicolor* A3(2) strains were M145[13] *(SCP1⁻, SCP2⁻)* and J1501[14] *(hisA1 uraA1 strA1 pgl SCP1⁻ SCP2⁻)*. The gene disruptants of *act*VI ORFs in J1501 were obtained by insert-directed recombination[15] using φC31-derived KC515[13] vectors carrying fragments internal to each ORF as described before[12]. *Streptomyces* strains and disruptants were grown using a modification of the method of reference 16. Seed cultures (10 ml) were grown in 50 ml culture tubes containing glucose 2%, peptone (DIFCO) 0.3%, beef extract (DIFCO) 0.3%, yeast extract (DIFCO) 0.1%, NaCl 0.3%, CaCO₃ 0.1%, pH 7.0, on a rotary shaker (220 rpm) at 30°C for 2 days. Aliquots of the culture were transferred to 50 ml of production medium (pH 7.0) consisting of glycerol 1.5%, soybean meal (Nissin Soya Flour F-T) 1%, and NaCl 0.3% in 500 ml Erlenmeyer flasks which were grown with shaking (200 rpm) at 28°C for 4 days.

Isolation and Purification of Actinorhodin (ACT)

The mycelium of M145 cultures (2 litres) was harvested by centrifugation (1,700×g, 10 minutes), washed with 800 ml of 1 N HCl, and stirred with 800 ml of 1 N NaOH. The solubilised blue pigment was separated from the mycelial residue by centrifugation (1,700×g, 10 minutes). The supernatant was acidified to pH 3 with conc. HCl, producing a red precipitate of crude ACT. The precipitate was collected by centrifugation (1,700×g, 10 minutes), and washed with acetone (600 ml) followed by filtration. The residual pigment was dried *in vacuo* and recrystallised from 1, 4-dioxane to give 233 mg of ACT as a reddish powder.

HPLC Analysis of ACT from *Streptomyces* Mycelium

The mycelium from a 50-ml liquid culture was harvested by centrifugation (1,700×g, 10 minutes), washed twice with 20 ml of 1 N HCl and with 20 ml of deionised H₂O, followed by lyophilization. The ground residue was extracted with 50 ml of 1,4-dioxane with shaking overnight. The mixture was centrifuged at 4,000×g for 5 minutes, and the supernatant was subjected to reversed-phase HPLC analysis by monitoring the absorbance at 520 nm using a Luna C18(2) (4.6 i.d. mm×15 cm, Phenomenex) column maintained at 40°C and isocratically eluted with CH₃CN- H₂O (1:1) containing 0.5% AcOH at a flow rate of 1.0 ml/minute on a TOSOH SC8010 system. The typical yield of ACT from the J1501 strain was 42 mg/liter. The amounts of ACT from the disruptants, relative to the J1501 control, were evaluated from the average of four

independent results obtained from separate culture flasks.

HPLC Analysis of Metabolites from *Streptomyces* Culture Medium

The medium separated from a *Streptomyces* production culture was subjected directly to reversed-phase HPLC analysis on a TOSOH 8020 system under the following conditions: column, TSK gel ODS-80Tm (4.6 i.d. mm× 150 mm, TOSOH); column temperature 40°C; gradient elution, solvent A (0.5% AcOH in CH_3CN) and solvent B (0.5% AcOH in deionised H_2O), gradient profile (0~5 minutes, 35% A; 5~30 minutes, 35~95% A; 30~35 minutes, 95% A; 35~40 minutes, 95~35% A); flow rate, 1.0 ml/minute; photo-diode array detector (PD-8020, TOSOH), 250~500 nm.

LC/MS Analysis of Metabolites

MS spectra were recorded on a Thermoquest LCQ equipped with a Hewlett Packard HP1100 series LC system under the same chromatographic conditions used for the HPLC analysis mentioned above. Atmospheric pressure chemical ionization (APCI) was applied to detect positive and negative ions of samples.

Results and Discussion

The previous method for chemical characterisation of ACT is based on its conversion to the dimethyl ester, then further to tetracetate[17,18]. This chemical derivatisation is made inefficient (overall yield is less than 10%) because of the chemical properties of ACT: it is almost insoluble in any standard organic solvent used for spectroscopic studies at neutral pH. 1,4-Dioxane was found to solubilise a reasonable amount of ACT from mycelium of *S. coelicolor* A3(2), allowing direct chracterisation of ACT. The highly conjugated chromophore of ACT gives an absorbance maximum in the visible region (520 nm), which turned out to be diagnostic for ACT detection on HPLC analysis. The peak corresponding to ACT detected by monitoring at 520 nm was evaluated by LC/MS analysis under the same chromatographic conditions used for HPLC (Figure 3). It gave a protonated molecular ion of ACT ($C_{32}H_{26}O_{14}$) at *m/z* 635, indicating the reliability of this method.

The HPLC method was applied to the chemotype analysis of the gene disruptants of the *act*VI region of *S. coelicolor* A3(2). ACT was detected from the disruptants of ORFB, ORFA, ORF3, and ORF4, whereas the disruptants of ORF1 and ORF2 were found to be ACT non-producers. This is consistent with the previous phenotypic

observation[12] based on blue pigmentation alone.

The culture medium of the disruptants was also analysed by reversed-phase HPLC (Figure 4). The ORF1-disruptant produced 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC, **8**) and aloesaponarin II (**3**) as shunt products. This result agrees with the fact that the *act*VI mutants[2] mentioned earlier were mapped[12] to ORF1 by complementation. The culture medium of the disruptant of ORF2 was more yellowish compared with the other disruptants, and it produced 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-*c*]-pyran-3-(*S*)-acetic acid, (*S*)-DNPA, characterised previously and shown to be a precursor of ACT[19]. Interestingly, (*S*)-DNPA was detected from the ORFA disruptant, together with an unknown compound X, at retention time 9.0 minutes. Compound X had a similar absorbance spectrum to those of DMAC and (*S*)-DNPA. It could be an intermediate or shunt product in ACT biosynthesis, whose structural elucidation is in progress. No significant difference was observed in the HPLC chromatographic profiles between the wild type strain (J1501) and the disruptants of ORFB, ORF3, and ORF4. Chemical analysis on the disruptants is summarised in Table 1.

Quantification of ACT in the HPLC analysis (Table 1) showed that the disruptions of ORFA, ORF3, and ORF4 caused significant reduction in ACT production, suggesting that they encode proteins possibly catalysing ACT biosynthetic steps that otherwise proceed spontaneously (see below). The result on the ORF4 disruptant contradicts our earlier observation that it gave blue pigmentation on agar medium at an apparently wild type level, and indicates the importance of quantifying ACT production in the present study. Disruption of ORFB gave comparable ACT productivity to the wild type, J1501. Apparently, this gene is not directly relevant to ACT biosynthesis, possibly being outside of the *act* cluster.

Significant similarity between the *act*VI-ORF1 protein and β-hydroxyacyl CoA dehydrogenases (HACD) belonging to a family of short-chain alcohol dehydrogenases led us to propose[12] (Figure 5) that the partial structure (**a**) of (**2**) can be recognised as the substrate motif of HACD: the *act*VI-ORF1 protein would reduce the keto-group at C-3 to give the chiral secondary alcohol (**4**), which would cyclise to the hemiketal (**5**), followed by dehydration to afford (*S*)-DNPA (**6**). This hypothesis was proved[20] when (**6**) was produced by *S. coelicolor* CH999,[21] a host strain deleted for the *act* cluster, carrying the recombinant plasmid, pIJ5660; this carries the *act* minimal PKS, KR, ARO and CYC, together with *act*VI-ORF1.

Fig. 3. LC/MS profiles of actinorhodin (1) from *S. coelicolor* A3(2).
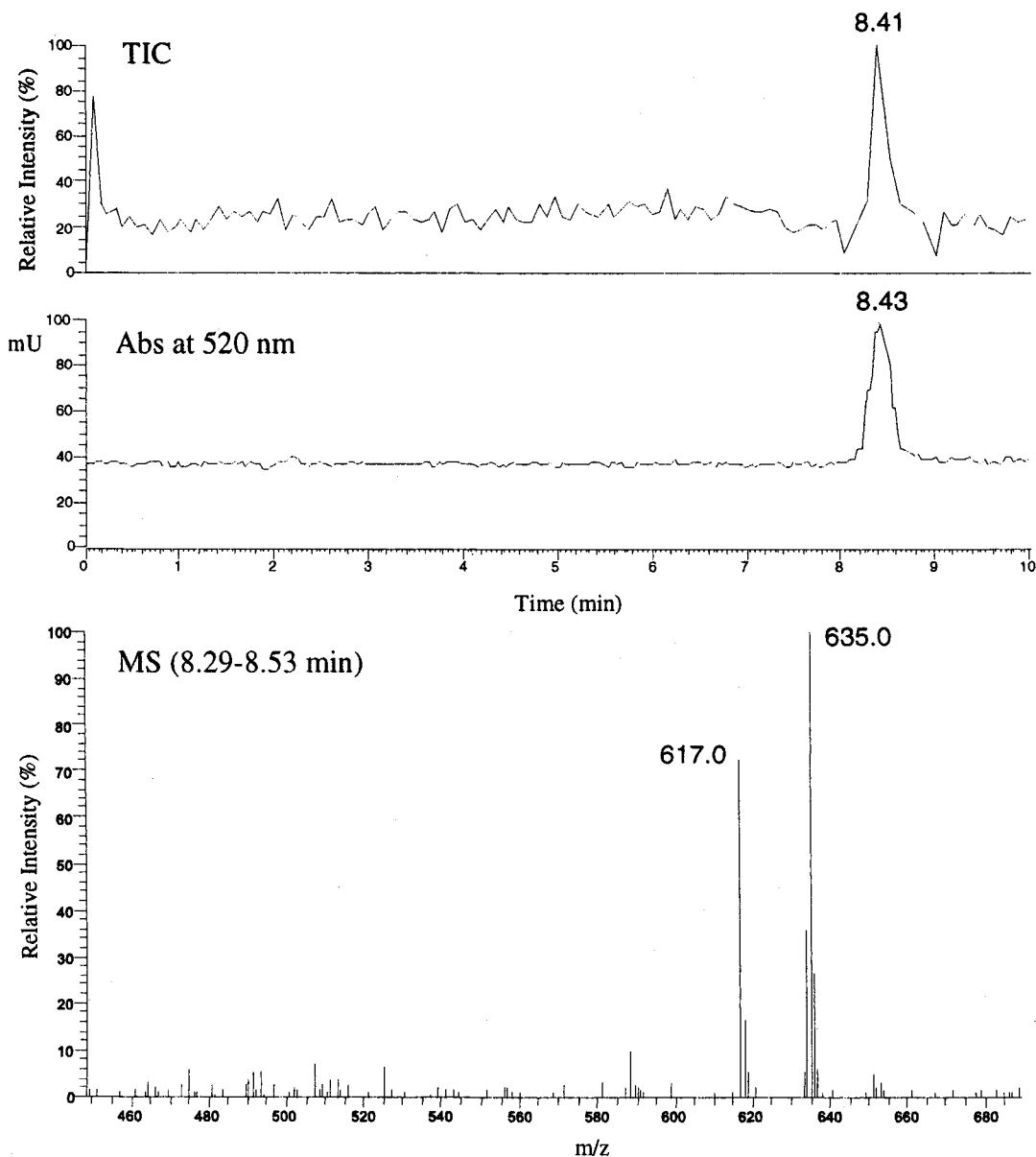
TIC: Total ion chromatogram for positive ions.

Chemically spontaneous formation of the hemiketal (5) and its dehydration might be enzymatically assisted for efficient pyran ring formation. Such potential assisting proteins could be encoded by *act*VI-ORF3 and -ORFA, as indicated by the phenotypes of the corresponding disruptants: disruption of ORF3 resulted in delayed pigmentation on ammonia fuming; and the ORFA-disruptant gave lower productivity of ACT compared with the wild type strain.

Of the two genes, *act*VI-ORFA has known homologues in the biosynthetic gene clusters for five other actinomycete aromatic polyketides: granaticin (*gra*-ORF31[22]), frenolicin (*fren*X[23]), mithramycin (*mtm*X[24]), doxorubicin/dauno-rubicin (*dps*H[25]; *dau*Z[26]), and an unknown compound (ORF1[27]). In no example has its role been definitively deduced. GERLITZ *et al.* suggested[25] that *dps*H might encode some kind of polyketide cyclase, based on its ability to favour formation of a tricyclic intermediate of

Fig. 4.
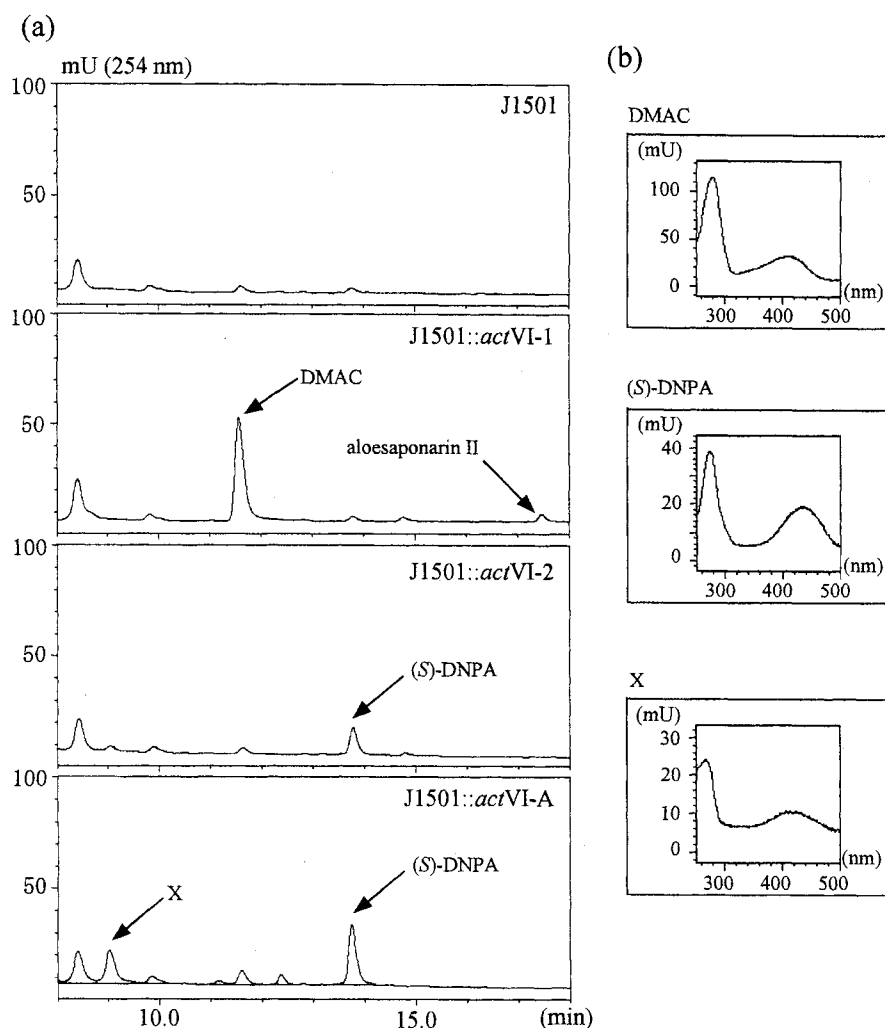
(a) HPLC chromatograms of the metabolites from culture medium of *S. coelicolor* J1501and its disruptants of *act*VI-ORF 1, 2, and A. (b) UV/VIS spectra of the peaks corresponding to DMAC, (*S*)-DNPA, and compound X as indicated by arrows.

doxorubicin biosynthesis rather than a monocyclic shunt product, but this was not confirmed in later work[28]. However, because of the presence of such genes in gene clusters for the biosynthesis of compounds that contain exclusively carbocyclic, not pyran ring systems, they are more likely to play a general role in stabilising the multicomponent, type II PKS complex, rather than in catalysing pyran ring formation itself. This possibility agrees with the present results that the disruption of ORFA did not abolish ACT production, but resulted in accumulation of possible biosynthetic intermediates, leading to gross reduction in ACT biosynthesis. In contrast, the only homologue of *act*VI-ORF3 so far discovered[22] in a complete biosynthetic gene cluster (or elsewhere) is in

the granaticin cluster (ORF18), and granaticin, like actinorhodin, contains a pyran ring as part of the BIQ structure. ORF3 is therefore the most likely candidate to facilitate the closure of the pyran ring of actinorhodin, which is presumed to be capable of spontaneous chemical closure in the absence of this gene[22]. The hemiketal (5) was not detected from any disruptant described here under the present analytical conditions. Perhaps it was too unstable to be chemically characterised as in the case of the production of (*S*)-DNPA by CH999 carrying pIJ5660, mentioned earlier.

Based on the similarity of the partial structure (**b**) (Figure 5) of (**6**) with the substrate motif of enoyl reductases, with which the products of ORF2 and ORF4 are

Table 1. Summary of chemical analysis of metabolites of *S. coelicolor* A3(2) and disruptants of the *act*VI ORFs.

Strain / Disruptant	ACT production[a] (relative amount)	Other product(s)[a] relevant to ACT
J1501	+ (100)	–
J1501:: *act*VI-B	+ (97)	–
J1501:: *act*VI-A	+ (21)	(S)-DNPA
J1501:: *act*VI-1	–	DMAC, aloesaponarin II
J1501:: *act*VI-2	–	(S)-DNPA
J1501:: *act*VI-3	+ (55)	–
J1501:: *act*VI-4	+ (18)	–

a) +: detected; -: not detected

Fig. 5. Proposed pathway of the bicyclic intermediate (2) leading either to pyran ring formation in actinorhodin (1) biosynthesis, or to shunt products, (3) and (8).
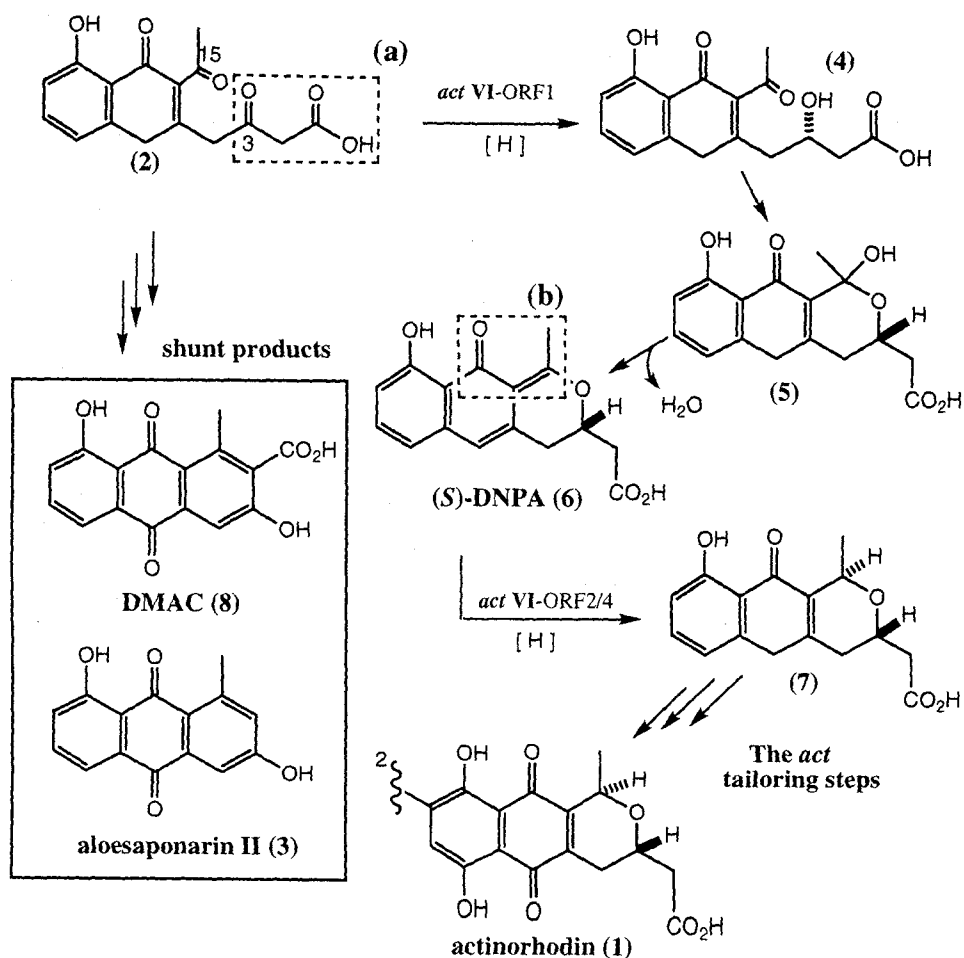
homologous, we proposed[12] that (6) would undergo stereospecific reduction at C-15 by the ORF2 protein with 1,4-reduction to facilitate (7) with the correct configuration found in ACT. This reduction might proceed in two coupled steps, with reduction of the double bond between C-14 and C-15 followed by isomerisation to afford the α,β-unsaturated carbonyl structure of (6). Despite the mutual similarity (52%) of ORF2 and ORF4, the ORF4 disruptant did produce ACT in substantially less quantity than the wild-type without accumulation of (S)-DNPA, favouring the idea that the ORF2 protein would function as the essential reductase at C-15, with the ORF4 product assisting in the chemically favoured isomerisation. Taken together with the fact that the ORF2 disruptant produced (6), which is a postulated substrate for a probable reductase encoded by ORF2, the results strongly reinforce the proposed biosynthetic scheme.

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