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PROOF THAT THE *ACT*VI GENETIC REGION OF *STREPTOMYCES COELICOLOR* A3(2) IS INVOLVED IN STEREOSPECIFIC PYRAN RING FORMATION IN THE BIOSYNTHESIS OF ACTINORHODIN

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Abstract: Pyran ring formation in the biosynthesis of actinorhodin in *Streptomyces coelicolor* A3(2) was studied using the *act* cluster deficient strain, CH999, carrying pRM5-based plasmids harbouring combinations of the *act*VI genes. The strain, CH999/pIJ5660 (pRM5 + *act*VI-ORF1), produced a chiral intermediate, (*S*)-DNPA, suggesting that the *act*VI-ORF1 product is a reductase determining the C-3 stereochemical centre.

Polyketides (PKs) have attracted much interest in biosynthetic studies of secondary metabolism because of their crucial importance as therapeutic agents. This derives from their structural diversity, which gives rise to a wide variety of activity including antibiotic and anti-cancer properties. Earlier studies proved that a basic carbon skeleton for polyketides is derived from repeated Claisen condensations of thioesters of simple organic acids such as acetate, propionate, malonate, and methylmalonate.[1] The polyketide synthase (PKS) exerts selective control of starter unit, kinds and number of extending units, reductive processing of β-keto groups of the nascent polyketide chain, and its regiospecific cyclisation.

One of the most extensively studied examples is actinorhodin (**1**), a dimeric benzoisochromanequinone (BIQ) antibiotic produced by *Streptomyces coelicolor* A3(2), genetically the most characterised PK producer.[2] The early stages of the actinorhodin pathway, controlled by the *act* cluster, proceed as follows (Figure 1): (i) a type II minimal *act* PKS, with components ketosynthase (KS), chain length factor (CLF), and acyl carrier protein (ACP), builds an octaketide chain and may catalyse first ring cyclisation by aldol-type condensation between C-7 and C-12; (ii) keto-reduction occurs at C-9 by a ketoreductase (KR); (iii) an aromatase (ARO) catalyses aromatisation of the first ring by dehydration; and (iv) a cyclase (CYC) controls second ring closure between C-5 and C-14. The structure of the resultant intermediate (**2**) was deduced from its shunt products, 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC, **3**) and aloesaponarin II (**4**).[3, 4]

Further structural diversity is given to nascent PKS-derived backbones particularly by pathway-specific modifying (tailoring) steps including cyclisations, oxido-reductions, dehydrations, rearrangements, and group transfers, most of which occur in a regio- and/or stereo-specific manner. As part of the goal of understanding the whole biosynthetic pathway of actinorhodin, we report here functional assignment of the genes involved in the step immediately after the intermediate (**2**), stereospecific pyran ring formation.

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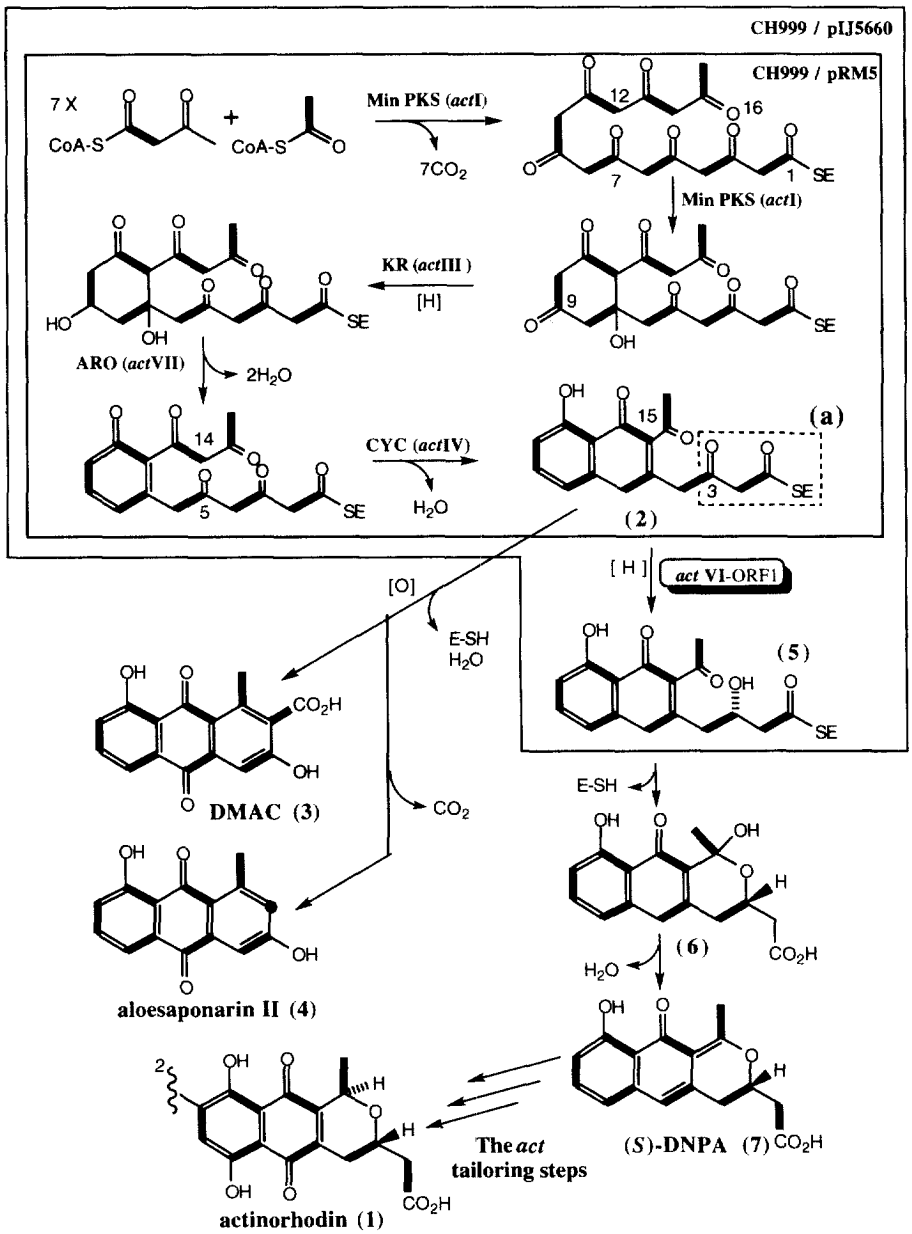

Figure 1. Proposed biosynthetic pathway of actinorhodin (**1**) in *Streptomyces coelicolor* A3(2): Numbering is based on the biosynthetic origin. Min PKS is minimal PKS in the *act* cluster (see text). The bold lines/dot indicate the biosynthetic origins from acetate/malonate units. Presumed enzyme-bound intermediates are shown as *RCO-SE*, and shunt products are tentatively shown to arise as free acids.

The *act*VI region, located near the left-end of the *act* cluster,[5] was initially postulated to be involved in pyran ring formation based on the fact that the two classical *act*VI mutants[6] produce aloesaponarin II (**4**) as a

shunt product[3]. DNA sequencing[7] of the *act*VI region (5.7 kb) identified six ORFs: ORFB, ORFA, ORF1, ORF2, ORF3, ORF4 (Figure 2a). By complementation the mutants were assigned to ORF1. The ORF1 protein resembles β-hydroxyacyl CoA dehydrogenases (HACD) of bacteria and mammals: the closest homologue (52% similarity) is β-hydroxybutyryl CoA dehydrogenase from *Clostridium acetobutylicum*,[8] which belongs to a family of short-chain alcohol dehydrogenases possessing a well-conserved nucleotide binding motif.[9]

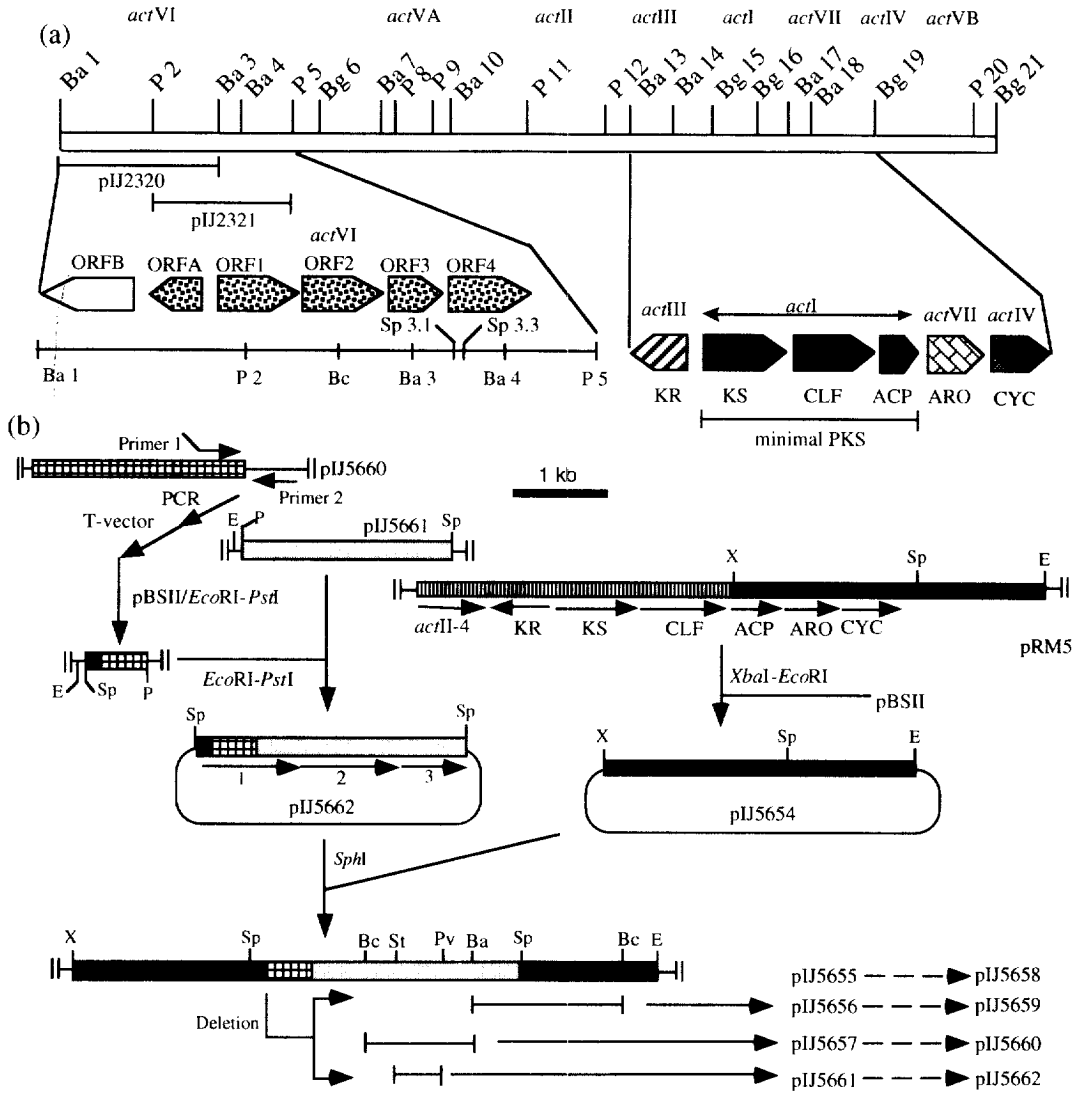

Figure 2. (a) Restriction map of the whole *act* cluster (22 kb) and the organisations of the *act*VI, and the III, I, VII, IV regions. (b) Scheme for construction of the recombinant plasmids (see note 12). The sites are numbered as described in ref. 5: Ba (*Bam*HI), Bc (*Bcl*I), Bg (*Bgl*II), E (*Eco*RI), P (*Pst*I), Pv (*Pvu*II), Sp (*Sph*I), St (*Stu*I), X (*Xba*I).

We previously proposed[7] the following possible mechanism of pyran ring formation in the actinorhodin pathway based on the idea that the partial structure (**a**) of (**2**) can be recognised as the substrate motif of HACD: the *act*VI-ORF1 product was postulated to reduce the keto-group at C-3 to give the chiral secondary alcohol (**5**), which would cyclise to the hemiketal (**6**), followed by dehydration to afford 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-*c*]-pyran-3-(*S*)-acetic acid, (*S*)-DNPA (**7**), shown previously to be a precursor of actinorhodin.[10] The recombinant strain consisting of the host, CH999,[11] carrying the *Streptomyces* expression vector, pRM5,[4] produces DMAC (**3**) and aloesaponarin II (**4**) by the combined actions of the *act* minimal PKS together with the *act* KR, ARO and CYC; these metabolites are believed to arise as shunt products (Figire 1) from a true bicyclic intermediate (**2**) which would be the *in vivo* substrate for a proposed stereospecific reduction.

Various combinations of the *act*VI genes (ORFs 1, 2, and 3) were introduced (Figure 2b)[12] downstream of the *act* CYC gene under the control of the *act*I promoter, at the *Sph*I site of pRM5 to give pIJ5658 (pRM5 + *act*VI-ORF1, 2, 3), pIJ5659 (pRM5 + *act*VI-ORF1, 2), pIJ5660 (pRM5 + *act*VI-ORF1) and pIJ5662 (pRM5 + *act*VI-ORFs 1, 3). These plasmids were constructed in *Escherichia coli*.

CH999/pIJ5660[15] produced (*S*)-DNPA together with a trace amount of aloesaponarin II which is a major product of CH999/pRM5.[4] Spectral data[16] (NMR, MS) including circular dichroism (CD) (Figure 3) of the isolated sample agreed with those of the "yellow pigment" previously characterised from the *S. coelicolor* A3(2) mutant strain B1.[10] This finding clearly proves that *act*VI-ORF1 indeed encodes a reductase determining the C-3 stereochemical centre.

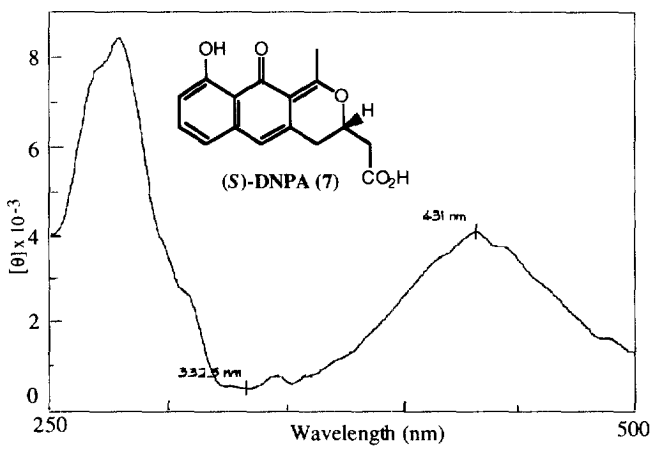

Figure 3. Circular Dichroism (CD) spectrum of (*S*)-DNPA isolated from *Streptomyces coelicolor* A3(2) CH999 transformed with pIJ5660.

Although formation of the hemiketal (**6**), as well as its dehydration, are considered to be chemically spontaneous, they might be under enzymatic control *in vivo*. Our previous gene disruption[7] of the *act*VI ORFs indicated that disruption of ORF3 resulted in a strain that initially failed to give the blue pigmentation that is characteristic of actinorhodin (it was yellowish instead) but later turned blue on ammonia fuming. A plausible explanation is that the *act*VI-ORF3 product would assist cyclisation-dehydration of the alcohol (**5**), which otherwise proceeds spontaneously but ineffciently. In order to investigate this possibility chemical analysis of the disruptant[17] of *act*VI-ORF3 was performed to test the requirement of *act*VI-ORF3 for actinorhodin production. The production of actinorhodin in the *act*VI-ORF3 disruptant was confirmed by chemical identification[18] from

the mycelium, suggesting that the *act*VI-ORF3 is involved in the actinorhodin pathway, most likely in a step, that would proceed spontaneously by a change of conditions such as a rise in pH (ammonia fuming). The most plausible explanation is that the product of *act*VI-ORF3 is a cyclase to form the pyran ring.

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11. The *S.coelicolor* A3(2) strains were CH999 (*proA1 argA1 SCP1⁻ SCP2⁻ redE60 ermE::Δact*)[4] for recombinant expression, and J1501[13] (*hisA1 uraA1 strA1 pgl SCP1⁻ SCP2⁻*) for gene disruption.[14]
12. Plasmid constructions were performed in *E. coli.* as follows. pIJ2320 and pIJ2321 are pBR325 derivatives carrying *act* DNA restriction fragments from partial digestion of pIJ2301[5].They contain, respectively, a *Bam*HI fragment (sites 1-3)[5] and a *Pst*I fragment (sites 2-5)[5]. A 2.6 kb *Bam*HI-*Pst*I fragment containing the N-terminal region of *act*VI-ORF1 of pIJ2320 was subcloned into pUC118 to afford pIJ5650 which was used as template for PCR amplification with the engineering primer (Primer 1: 5' GCATGCggaggTCGCC **ATG**AGCACCGTGACAGTG-3': *Sph*I site, underlined; rbs, small letters; the ATG translation start codon, bold) and M13 forward primer (Primer 2: 23-mer). The PCR was performed in a final volume of 50 μl for 30 cycles of amplification with *Taq* DNA polymerase (Boehringer) using a step program (30 sec at 95 °C, 1 min at 69 °C, 1 min at 72 °C) under standard conditions except for the presence of 8 % (final concentration) of

glycerol. The PCR product was subcloned into a T-vector, pCR®2.1 (Invitrogen), and the insert was further subcloned into pBluescript®II (pBSII: Stratagene)-SK(-) as an *Eco*RI-*Pst*I fragment (sequence checked). A *Eco*RI-*Pst*I fragment from the foregoing plasmid was subcloned into the *Eco*RI-*Pst*I sites of pUC118-based pIJ5651 which carries 2.1 kb *Pst*I-*Sph*I fragment of pIJ2321 extending from the internal *Pst*I site of *act*VI-ORF1 to the end of *act*VI-ORF3. The resultant plasmid, pIJ5652, harbours the *Sph*I gene cassette carrying full-length of *act*VI-ORFs 1, 2, and 3 (ORF 2 and 3 have their own native rbs sequences[7]). The cassette was cloned into a unique *Sph*I site of pIJ5654 which is a pBSII-SK(+) derivative carrying *Xba*I-*Eco*RI fragment of pRM5 to give pIJ5655. Three internal deletions were made on pIJ5655 to give rise to pIJ5657 (*Bcl*I-*Bam*HI fragment deleted, carrying ORF1 only), pIJ5656 (*Bam*HI-*Bcl*I fragment deleted, carrying ORFs 1 and 2), pIJ5661(*Stu*I-*Pvu*II fragment deleted, carrying ORFs 1 and 3). The *Xba*I-*Eco*RI fragments from pIJ5655, pIJ5656, pIJ5657, pIJ5661 were replaced with the original *Xba*I-*Eco*RI fragment of pRM5 to afford pIJ5658, pIJ5659, pIJ5660 and pIJ5662, respectively.

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15. Plasmids were introduced into *S. coelicolor* CH999 by standard protoplast transformation[2] with selection for thiostrepton resistance. Production culture was performed on R5[2] agar plates (30 °C for 1 week). The agar was mashed into a paste and adjusted to pH 3 with 2N HCl. The mixture was extracted with ethyl acetate, dried, and concentrated under reduced pressure. The crude extracts were subjected to flash chromatography (1:1; ethyl acetate/PE) followed by further purification by preparative TLC (ethyl acetate) to afford (*S*)-DNPA (the typical yield was 180mg/l).
16. Spectral data of (*S*)-DNPA (the numbering given is based on the biosynthetic origin). δ^1H NMR (*J* in Hz, 270 MHz/$CDCl_3$): 7.38 (1H, dd, *J*=8.2, 7.6, 9-H), 6.79 (1H, dd, *J*=8.2, 0.9, 10-H), 6.75 (1H, dd, *J*=7.6, 0.9, 9-H), 6.26 (1H, br. s, 6-H), 4.75 (1H, m, 3-H), 2.95 (1H, dd, *J*=16.4, 7.4, 2-H), 2.93 (1H, ddd, *J*=16.4, 3.4, 1.0, 4-H), 2.79 (1H, ddd, *J*=16.0, 10.5, 1.7, 4-H), 2.79 (1H, dd, *J*=16.4, 6.0, 2-H), 2.65 (3H, s, 16-H); δ^{13}C NMR (67.5 MHz/$CDCl_3$): 188.5 (C-13), 176.7 (C-1), 163.2 (C-11), 138.3 (C-7, 15), 134.9 (C-6), 128.2 (C-12, 14), 117.0, 116.1, 114.5 (Ph), 111.3 (C-5), 39.9 (C-3), 33.3 (C-2), 26.5 (C-4), 23.3 (C-16); CI-MS (m/z): 286 (M+); CD (MeCN, $[\theta]_{\lambda nm} \times 10^{-3}$): $[\theta]_{\lambda nm332.5}$ +0.4895, $[\theta]_{\lambda nm431}$ +4.0973. Data were obtained on a JEOL EX-270 (NMR) and a Jasco J-600 (CD).
17. The *act*VI-ORF3 gene disruptant (J1501::*act*VI-3) was derived from insert-directed recombination[14] on the J1501[11] strain using ϕC31-derived KC515 vector, ϕAB24, which carries a *BssH*II-*Apa*I fragment internal to ORF3.[7]
18. Production culture of J1501::*act*VI-ORF3 was performed in AM7161 liquid medium as described.[19] A similar phenotype (delayed pigmentation after exposure to ammonia fumes on agar media compared with the wild type strain) was also observed. The cultures were filtered to separate mycelium and medium. The mycelium was used for identification of actinorhodin as described.[20] The sample was derivatised to the methyl ester, then further to a tetraacetate.[20] The product was identified as actinorhodin dimethyl ester tetraacetate by comparing its properties with those in the literature.[21]
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