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Molecules and Cells

Angucyclines Sch 47554 and Sch 47555 from *Streptomyces* sp. SCC-2136: Cloning, Sequencing, and Characterization

Devi Bahadur Basnet, Tae-Jin Oh, Thi Thu Hang Vu, Basundhara Sthapit, Kwangkyoung Liou, Hei Chan Lee, Jin-Cheol Yoo[1], and Jae Kyung Sohng*

Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical Engineering, Sun Moon University, Asan 336-708, Korea.
[1] Department of Pharmacy, Chosun University, Gwangju 501-759, Korea.

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The entire gene cluster involved in the biosynthesis of angucyclines Sch 47554 and Sch 47555 was cloned, sequenced, and characterized. Analysis of the nucleotide sequence of genomic DNA spanning 77.5-kb revealed a total of 55 open reading frames, and the deduced products exhibited strong sequence similarities to type II polyketide synthases, deoxysugar biosynthetic enzymes, and a variety of accessory enzymes. The involvement of this gene cluster in the pathway of Sch 47554 and Sch 47555 was confirmed by genetic inactivation of the aromatase, including a portion of the ketoreductase, which was disrupted by inserting the thiostrepton gene.

Keywords: Aculose; Amicetose; Angucycline; Polyketide; Sch 47554; Sch 47555; *Streptomyces*.

Introduction

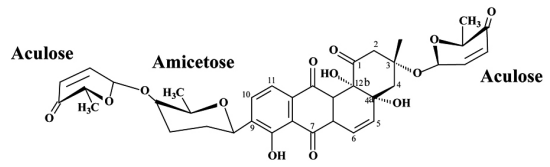
Polyketides, a large, structurally diverse family of pharmaceutically important natural products, are generated by polyketide synthase (PKS) complexes, via a sequence of reactions. Polyketide synthase type II (PKS II) produces aromatic carbon backbones by the asymmetrical assembly of acetate units which are iteratively assembled into a carbon chain *via* the polyketide pathway, which comprises a set of discrete mono- or bi-functional proteins (Rawlings, 1999). The angucycline group of antibiotics is a family of biologically active microbial natural products, and is classified within the type II polyketides (Rohr and Thiericke, 1992). These compounds share many features, including a tetracyclic benz(a)nthracene aglycone system (Rohr, 2000), but the folding pattern of the nascent β-polyketide chains differs from that observed in the well-analyzed compounds, actinorhodin and tetracinomycin C (type II polyketide) (Rohr and Thiericke, 1992).

Over the past decade gene clusters encoding many structurally related and biologically active angucyclines have been cloned, sequenced, and identified including the following: jadomycin B (Han *et al.*, 1994), urdamycin A (Decker and Haag, 1995), and landomycin A (Westrich *et al.*, 1999), as well as several recent additions, such as oviedomycin (Lombo *et al.*, 2004). Sch 47554 and Sch 47555 are antifungal polyketides that are active against several fungi, such as *Candida albicans*, *C. tropicalis* and *C. stellatoidea.* They are produced by *Streptomyces* sp. SCC-2136, and were initially isolated and characterized as shown Fig. 1 (Chu *et al.*, 1993). Structurally, they are characterized by multiaromatic angucycline aglycones, which are appended with deoxysugar (DOH) moieties. They differ from the aforementioned molecules in that the *O*-glycoside (monosugar) is located on C-3 instead of on C-12b as in urdamycin A.

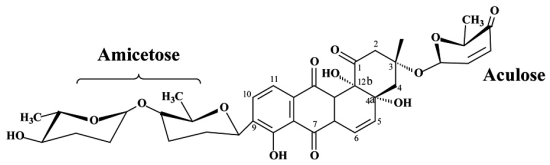
The isolation of genes responsible for the production of an angucycline would expand the number of genes currently available for the combinatorial design of hybrid compounds. The possibility of combining individual polyketide synthase domains from different clusters has been examined, and studies of the active enzyme complexes and expression of hybrid PKSs in a heterologous host have also been conducted, resulting in the production of several novel aromatic polypeptides (Katz and Donadio, 1993; McDaniel *et al.*, 1993). However, intense efforts are currently focused on altering the structures of the deoxysugars as they appear to have a profound affect on biological activity (Hutchinson and Fujii, 1995; Perez *et al.*, 2005; Salas and Mendez, 2005).

* To whom correspondence should be addressed.
Tel: 82-41-530-2246; Fax: 82-41-544-2919
E-mail: sohng@sunmoon.ac.kr

Abbreviation: PKS, polyketide synthase.



Sch-47554



Sch-47555

Fig. 1. Structures of Sch 47554 and Sch 47555.

We describe here the cloning of the biosynthetic gene cluster for Sch 47554 and Sch 47555 from *Streptomyces* sp SCC-2136. This provides not only an insight into the biosynthesis of these compounds, but also extends our current knowledge regarding genetic backgrounds for further combinatorial biosynthesis, both in terms of hybrid polyketides and deoxysugars. We also propose a pathway for the biosynthesis of Sch 47554 and Sch 47555.

Materials and Methods

Bacterial strains, vectors and culture conditions *Streptomyces* sp. SCC-2136 ATCC 55186 was maintained on R2YE plates at 28°C, and grown in R2YE liquid medium for isolating genomic DNA. A different medium was used for product isolation, as described elsewhere (Chu *et al.*, 1993). *E. coli* XL1 Blue MRF (Stratagene), grown in LB (Luria-Bertani) medium at 37°C and supplemented with antibiotics when necessary (ampicillin, 100 μg/ml and apramycin, 100 μg/ml), was employed for gene manipulations. Vector pOJ446 (Invitrogen) was used in library construction, and pGEM-T Easy vector (Promega) was used for cloning PCR fragments (Park *et al*., 2001).

Construction of a genomic library and screening Cloning, recombinant plasmid transformation, and *in vitro* DNA manipulations were conducted in accordance with standard protocols (Sambrook and Russell, 2001). The chromosomal DNAs of wild-type *Streptomyces* sp. SCC-2136 and the ΔPKS mutant were isolated as described (Kieser *et al.*, 2000). Freshly isolated genomic DNA from *Streptomyces* sp. SCC-2136 was used in the construction of the library as described, with slight modifications (Kieser *et al.*, 2000). Cosmid vector pOJ446, *Mob*I restriction enzyme, and the Gigapack III XL system (Stratagene, USA) for packaging were used in the construction of the genomic library. For the initial screening, PCR products were obtained using the following set of primers: DW11 (upstream): 5′-CACTTCGGGGGCGAGTCGCACGT-3′ and DW32 (downstream): 5′-GGGCCGTAGTTGTTCGAGCA-3′, as described (Sohng *et al.*, 1998). For hybridization, the probe was labeled with ^{32}P using a random primer labeling kit (Stratagene, USA), and purified by gel filtration. Hybridization was conducted as described (Sohng *et al.*, 1998).

DNA manipulation, sequencing and computer analysis Cosmid and plasmid DNAs were extracted from *E. coli* by alkaline lysis (Sambrook and Russell, 2001). Nucleotide sequences were determined by the dideoxy chain termination method, using an automatic sequencer. Computer-aided sequence analysis was conducted with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), and ORFs were identified using the OFR finder provided by NCBI, and confirmed by a BLAST database search.

Generation of the ΔPKS mutant The disruption plasmid was generated in pKC1139. A 2.58-kb genomic fragment was amplified by PCR using the following primers encompassing *schP3*, *schP4*, and *schP5*: PKSF (5′-ACACTTCTAGATGTCCTCGCCGGTCACCCGC-3′, *Xba*I site added) and PKSB (5′-TCGTGAAGCTTCTGGACGGCGACATCCTCGAC-3, *Hin*dIII site added). The products were cloned into pWHM3 to yield pDPK01, in which the 776 bp *Pst*I/*Pst*I fragment encompassing aromatase (*schP4*) and the C-terminal region of ketoreductase (*schP5*) were replaced with the *tsr* marker gene with the same restriction site, in order to construct pDPK02. Finally, the recombinant fragment, including *tsr* as a marker, was inserted into the final disruption vector, pKC1139, using the *Eco*RI/*Hin*dIII sites, yielding pDPK03. The disrupted vector was then transferred into the demethylation host, *E. coli* ET12567, by protoplast transformation. *tsr*-sensitive colonies were selected and then screened to identify single and double crossover mutants.

Product analysis For product analysis, the wild type and ΔPKS mutant strains were cultured in production medium [Dextrin, 3%; pea flour, 1.5%; maltose, 0.5%; fructose, 0.5%; molasses (dark), 0.5%; yeast extract, 0.3%; and NZ-amine, 0.3%] after 90 h of incubation in seed medium at 28°C (Chu *et al.*, 1993). The products were obtained by extracting the culture broth with ethyl acetate, and purified by silica gel chromatography, followed by a biological activity test. TLC of the crude extracts was conducted on aluminum silica plates (25DC Alufolien, Keiselgel 90F254, and MERCK). The samples from the wild-type and ΔPKS mutant strains were compared by HPLC at 218 nm using water (0.1% TFA) and acetonitrile as eluents at a flow rate of 1 ml/min (20–60% gradient) through a Mightysil RP-18, GP 250-4.5 (5 μm) column. The materials acquired from the silica column were further subjected to ESI-MASS (Finnigan TSQ 7000 Mass Spectrometer) and LC/MS analysis. Antimicrobial activity was monitored by disc agar diffusion assays against *Stapylococcus aureus,* as described previously (Chu *et al*., 1993).

Nucleotide sequence accession number The nucleotide sequences determined in this report were deposited in the EMBL

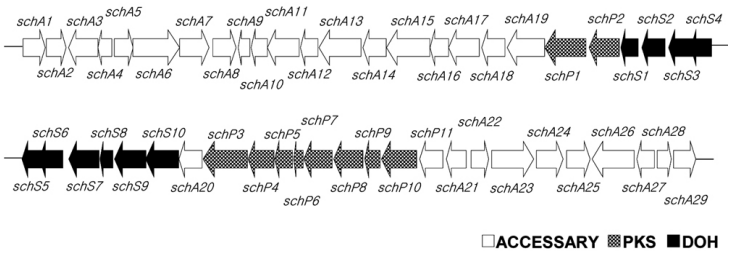


Fig. 2. Complete gene cluster map of Sch 47554 and Sch 47555. Genes are indicated by arrows oriented in the transcriptional direction. Genes for PKS, DOH and accessory genes are shown in different code.

nucleotide sequence database, under the accession number AJ628018.

Results

Cloning and sequencing of the biosynthetic gene cluster A genomic library was constructed for S*treptomyces* SCC-2136, using pOJ446 as cloning vector for the biosynthetic gene clusters of Sch 47554 and Sch 47555, both of which have deoxysugar as a structural constituent (Kharel *et al.*, 2004; Subba *et al.*, 2005). Adopting the general approach developed for cloning the biosynthetic gene cluster for deoxysugars (Sohng *et al.*, 1998), degenerate primers were derived from the conserved region of the known dTDP-glucose 4,6-dehydratase genes of different actinomycetes, as discussed previously. One of 12 overlapping clones isolated was designated pSCC-1, and sequencing revealed an insert of 42.9-kb harboring 30 ORFs. This included the DOH biosynthetic genes and two polyketide synthesis-related genes. In order to complete the expected gene cluster controlling synthesis of the angucyclines, a library fragment from the terminus of pSCC-1 towards the PKS gene was used to identify cosmids from the library containing the remainder of the PKS genes. The positive cosmids were subjected to restriction analysis, and pSCC-2, which expanded the upstream sequence by an additional 34.6-kb and 25 ORFs, was analyzed. Each of the ORFs was analyzed individually on the basis of its sequence similarity with known genes in the database. Analysis of their products revealed strong similarities to the previously validated type II PKS and deoxysugar biosynthetic gene clusters of various *Streptomyces* spp.

DNA sequence analysis Annotation of two cosmids of 42.9-kb and 34.6-kb was achieved using an ORF finder followed by sequence homology searches against the database, and our findings were consistent with the notion that the cluster contained genes involved in the biosynthesis of Sch 47554 and Sch 47555. Almost all of the deduced products of the identified ORFs had a high degree of similarity to other genes involved in type II PKS biosynthesis (Hopwood, 1997). The PKS and DOH genes appeared to be intact with short intergenic regions, and were oriented in the same transcriptional direction. The start and stop codons of *schS3-schS4*, *schS5-schS6*, *schS7-schS8*, and *schP7-schP8* overlap, which indicates the possibility of translational coupling. All of the genes use ATG and TGA as their start and stop codons, except for *schS1*, *schS4*, *schS7* and *schP8*, which harbor GTG start codons. The PKS and DOH gene clusters are separated by a transporter gene, and are flanked by various accessory genes. The orientation, gene map, and putative functions of the biosynthetic genes are given in Fig. 2 and Table 1. Although we sequenced and deposited up to 77.5-kb, only the genes necessary for the biosynthesis of angucycline derivatives are discussed.

Genes putatively involved in the formation of the angucycline moiety The PKS genes *schP3-schP10* strongly resemble their counterparts in the type II PKS systems of urdamycin A and landomycin A, with regard to both deduced amino acid sequences and genetic organization. The minimal PKS genes involved in the biosynthesis of angucycline antibiotics were identified as *schP8*, *schP7*, and *schP6*, and these genes exhibited strong sequence similarity to KS, chain length factor, and acyl carrier protein subunits, respectively, like the genes present in the urdamycin producer (Decker and Haag, 1995). SchP6 had the highest sequence similarity to PKS-ACP, and harbored an active ^{43}Ser residue in the highly conserved motif, ^{39}LGYE**S**LALLE^{49}, to which phosphopantethine binds in order to connect the incoming extender unit (Walsh *et al.*, 1997). The start codon of *schP7* overlaps with the stop codon of *schP8*, indicating the presence of a CLF/KS pair, a feature also observed in other type II PKS. The N-terminal catalytic domain of SchP8 harbors an active ^{172}Cys in the conserved region ^{166}TVVSTG**C**TSGLD^{177}, while its C-terminal region contains the conserved region, ^{348}GH**S**LG^{352} (GHSXG) which represents the AT domain with the active ^{350}Ser residue that binds to an acyl chain (Fernandez-Moreno *et al.*, 1992). A similar protein, also encoded by *schP7*, lacks this active CYS, which is replaced by GLU, thereby defining a characteristic enzyme with a potent influence on PKS chain length (Burson and

Table 1. Putative functions of genes in the gene cluster, assigned from the most similar sequence in the database. Only genes shown on the map (Fig. 2) are tabulated.

ORF (aa)	Putative function	Closest homology	% identity	Accession no.
SchA1 (327)	Putative transport system permease ABC transporter	*B*. sp. NRRL B-14911	61	EAR68640
SchA2 (295)	Putative oligogalacturonate transport system permease protein	*A. tumefaciens* str. C58	55	NP_357486
SchA3 (359)	Putative 3-(2-hydroxyphenyl) propionic acid transporter	*S. avermitilis*	85	NP_822955
SchA4 (221)	TetR-family transcriptional regulator	*S. avermitilis*	89	NP_8229
SchA5 (297)	Shikimate 5-dehydrogenase	*S. avermitilis*	73	NP_822953
SchA6 (626)	4-hydroxyphenyl pyruvate dioxygenase	*S. avermitilis*	80	NP_822952
SchA7 (493)	Amino acid transporter transmembrane protein	*R. solanacearum*	36	NP_522607
SchA8 (320)	Hypothetical protein	*S. coelicolor*	34	NP_631068
SchA9 (197)	Phosphate mutase	*S. avermitilis*	77	NP_822948
SchA10 (292)	6-phosphogluconate dehydrogenase	*S. avermitilis*	93	NP_822947
SchA11 (467)	Glucose-6-phosphate isomerase	*S. coelicolor*	79	O88015
SchA12 (311)	Hypothetical protein	*S. avermitilis*	72	NP_822945
SchA13 (558)	Glucose-6-phosphate-1-dehydrogenase	*S. avermitilis*	90	NP_822944
SchA14 (386)	Transaldolase	*S. avermitilis*	83	NP_822943
SchA15 (691)	Transketolase	*S. avermitilis*	90	NP_822942
SchA16 (297)	AraC-family transcriptional regulator	*S. avermitilis*	62	NP_822941
SchA17 (460)	Endo-1,3-beta-glucanase	*S. avermitilis*	69	NP_822940
SchA18 (335)	Oxidoreductase	*S. coelicolor*	87	NP_628756
SchA19 (596)	Oxidoreductase, alpha subunit	*S. coelicolor*	85	NP_630370
SchP1 (617)	Acyl-CoA carboxylase	JadJ (*S. venezuelae*)	84	AF126429
SchP2 (523)	Methyl malonyl Co-A decarboxylase	*S. avermitilis*	83	NP_823879
SchS1 (340)	dNDP-hexose 3-ketoreductase	LanT (*S. cyanogenus*)	65	AAD13550
SchS2 (465)	dNDP-hexose 2, 3-dehydratase	UrdS (*S. fradiae*)	80	AF269227
SchS3 (434)	NDP-hexose 3-dehydratase	UrdQ (*S. fradiae*)	88	AAF72550
SchS4 (319)	NDP-hexose 4-keto reductase	UrdZ3 (*S. fradiae*)	51	AAF72549
SchS5 (328)	dNDP-glucose 4, 6-dehydratase	UrdH (*S. fradiae*)	83	AF164960
SchS6 (355)	dNDP-glucose synthase	UrdG (*S. fradiae*)	74	AF164960
SchS7 (378)	C-glycosyltransferase	UrdGT2 (*S. fradiae*)	71	AAF00209
SchS8 (196)	NDP-hexose 3, 5-epimerase	UrdZ1 (*S. fradiae*)	71	AAF00208
SchS9 (390)	*O*-glycosyltransferase	UrdGT1c (*S. fradiae*)	53	AAF00217
SchS10 (430)	*O*-glycosyltransferase	UrdGT1a / b (*S. fradiae*)	48	AF164961
SchA20 (407)	Transporter	UrdJ2 (*S. fradiae*)	52	AAF00207
SchP3 (607)	Oxygenase-reductase	UrdM (*S. fradiae*)	64	AAF00206
SchP4 (312)	Aromatase	SimA5 (*S. antibioticus*)	73	AAK06788
SchP5 (266)	Ketoreductase	UrdD (*S. fradiae*)	88	S54815
SchP6 (89)	Acyl carrier protein	UrdC (*S. fradiae*)	82	S54814
SchP7 (408)	Chain length factor	UrdB (*S. fradiae*)	73	CAA60570
SchP8 (420)	Ketoacyl synthase	UrdA (*S. fradiae*)	83	CAA60569
SchP9 (108)	Cyclase	UrdF (*S. fradiae*)	87	CAA60568
SchP10 (494)	Oxygenase	UrdE (*S. fradiae*)	84	CAA60567
SchP11 (291)	Unknown	MedORF27 (*S.* sp. AM-7161)	42	BAC79023
SchA21 (251)	TetR-family regulatory protein	*S. coelicolor*	33	CAA16726
SchA22 (199)	Hypothetical protein	UrdO (*S. fradiae*)	62	CAC42480
SchA23 (527)	Transporter	UrdJ (*S. fradiae*)	33	AAF00219
SchA24 (372)	Oxidoreductase	*S. avermitilis*	60	NP_630744
SchA25 (283)	Repressor-response regulator	LanI (*S. cyanogenus*)	58	AA043177
SchA26 (532)	Oxidoreductase	AclO (*S. galilaeus*)	52	BAB72054
SchA27 (250)	Transcriptional regulator	*S. coelicolor*	78	NP_630744
SchA28 (202)	3-Oxoacyl carrier protein reductase	FabG1 (*C. glutamicum*)	47	CAF18856
SchA29 (294)	Hypothetical protein	*A. tumefaciens* str. C58	59	NP_354429

Khosla, 2000).

Three ORFs, *schP4*, *schP5*, and *schP9*, are most similar to angucycline gene clusters encoding aromatase, ketoreductase, and cyclase, respectively, and also appear to be involved in modification of the nascent polyketide chain. SchP4 showed profound sequence similarity to the aromatase-like protein from another gene cluster that plays an important role in the aromatization of the first ring (Hopwood, 1997). This had a high degree of similarity to UrdL (72% identity) of *S. fradae* and to SimA5 (73% identity) of *S. antibioticus* TU6040 (Decker and Haag, 1995; Trefzer *et al.*, 2002). SchP9 was more closely related to the angucycline cyclase (Kulowski *et al.*, 1999) than to the anthracyclines, as it was similar to UrdF (87% identity) and JadI (85% identity), both of which originate from the angucycline-producer gene cluster (Decker and Haag, 1995; Han *et al.*, 1994). Evidence of the incompatibility of cyclases from anthracyclines and angucyclines has been provided by the Hutchinson group (Wohlert *et al.*, 2001). SchP5 is very similar to UrdD (88% identity) as well as to several other ketoreductases from a variety of angucycline gene clusters, most notably oviedomycin and jadomycin. These ketoreductases are homologous to other aromatic PKS ketoreductases also involved in the region-specific reduction of the C-9 carbonyl group of the nascent polyketide chain (Hopwood, 1997).

Unlike modular PKS, the most important aspect of the angucycline gene clusters is the presence of a number of oxygenases. The Sch-gene cluster also harbors two oxygenases, encoded by *schP3* and *schP10*. SchP3 and SchP10 were most similar to UrdE (84%, identity) and UrdM (64%, identity), respectively. UrdE was identified as the oxygenase responsible for the oxidative opening of ring B within the angucyclinone backbone during biosynthesis (Faust *et al.*, 2000; Han *et al.*, 1994). We also identified a highly conserved N-terminal FAD-binding domain (aa 4-136, Pfam: 01494.11) and a C-terminal oxygen-binding domain (aa 144-335, Pfam: 01360.11), which it shares with oxygenase, in SchP10. A similar oxygenase was identified in the mithramycin producer, in which a Baeyer-Villiger type oxidation mechanism for ring opening was thought to exist (Rodríguez *et al.*, 2003). However, SchP3, an oxygenase-reductase, harbors two distinct domains: an N-terminal oxygen binding domain (aa 96-285, Pfam: 01360.11) and a C-terminal NAD (P) H binding domain (aa 363-604, Pfam: 00106.11). Thus, SchP3 appears likely to introduce a hydroxyl group at the C-12b position, as does UrdM during the biosynthesis of urdamycin A (Faust *et al.*, 2000).

Carboxylation of acetyl-CoA is the first step in producing malonyl-CoA, and it is catalyzed by SchP1, which has the highest sequence similarity to the acyl-CoA carboxylase of *S. avermitilis* (88% identity) and JadJ (84% identity) of *S. venezuelae* ISP5230. Proteins in this group are bi-functional (Samols *et al.*, 1988), and frequently share a high degree of sequence similarity to fatty acid synthases (FAS). However, it is evident that JadJ is functionally associated with PKS (Han *et al.*, 2000). An adjacent gene, *schP2*, is also related to a precursor-generating gene for polyketide biosynthesis, which is similar to JadN (78% identity) and LanP (76% identity), and probably encodes malonyl-CoA decarboxylase.

Genes putatively involved in the formation of deoxysugars The genes: *schS1-schS10* appear to encode deoxysugar biosynthetic gene clusters. SchS5 and SchS6 are most similar to dNDP-glucose-4,6-dehydratase (UrdH, 83% identity) and dNDP-glucose synthase (UrdG, 74% identity), respectively. These have been shown to catalyze the first two conserved steps in the formation of dNDP-4-keto-6-deoxy-D-glucose. SchS2 resembles UrdS (80% identity) and Gra-ORF27 (53% identity), which are involved in the biosynthesis of L-rhodinose and D-olivose in urdamycin A biosynthesis, and in C-2 deoxygenation with C2-H retention in granaticin biosynthesis, respectively (Draeger *et al.*, 1999; Spines *et al.*, 1979). Accordingly, we propose that the function of SchS2 as that of a 2, 3-dehydratase. A highly reactive product of Gra-ORF27 is trapped by Gra-ORF26 (3-ketoreductase), resulting in the formation of a stable product. Similarly, we speculate that SchS1, which is somewhat similar to the Gra-ORF26 3-ketoreductase (50%, identity), assists the C-2 deoxygenation facilitated by SchS2.

SchS3, SchS4, and SchS8 are most similar to the amino acid sequences of a variety of deoxysugar biosynthetic genes for dNDP-glucose 3-dehydratase (UrdQ, 88%, identity), dNDP-glucose 4-ketoreductase (UrdZ3, 51%, identity), and NDP-glucose 3,5-epimerase (UrdZ, 71%, identity), respectively. SchS7 rsembles UrdGT2 (71%, identity), which is involved in C-9 glycosylation of D-olivose *via* the C-C bond in urdamycin A (Faust *et al.*, 2000; Künzel *et al.*, 1999). Therefore, we assume that SchS7 is a *C*-glycosyltransferase that affects the transfer of D-amicetose to C-9 in Sch 47554 and Sch 47555. The *O*-glycosyltransferase encoded for by *schS9* was most similar to UrdGT1b/c (~53% identity), whereas another *O*-glycosyltransferase, SchS10, had homology to UrdGT1a (48% identity).

Genes with other functions The Sch-gene cluster contains five putative regulatory genes, six putative transport genes, and four putative redox function genes. *schA4* and *schA21* belong to the TetR-family of regulatory genes detected in several *Streptomyces* PKS clusters. Another regulator, *schA25*, exhibits similarity to *lanI* (58%, identity), which is responsible for positive regulation in the landomycin A pathway (Rebets *et al.*, 2003; 2005). Another regulatory gene, *schA27*, is similar to a transcriptional regulator (78%, identity) found in the *S. coelicolor* genome. Five genes, *schA1*, *schA2*, *schA3*, *schA7*, *schA20*,

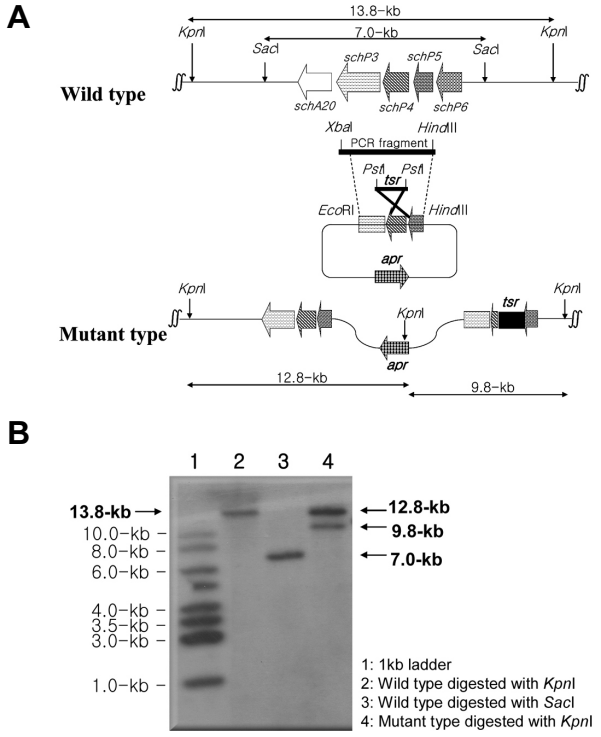


Fig. 3. Generation of a chromosomal mutant of ΔPKS (aromatase and ketoreductase) from *Streptomyces* sp. SCC-2136. **A.** Construction of a recombinant plasmid lacking a *Pst*I/*Pst*I fragment, and homologous recombination to generate a single crossover mutant. **B.** Southern hybridization of wild-type and mutant digested with *Kpn*I. The internal fragment obtained from pDPK02 with *Eco*RI/*Hin*dIII digestion was used as a probe.

and *schA23*, may be related to resistance. The first two genes belong to a family of permeases in bacteria, whereas *schA3* and *schA7* are hypothetically designated (not included in Table 1) as amino acid transporters. SchA20 has sequence similarity to UrdJ2 (52%, identity), and occupies the same position in the urdamycin A gene cluster. *schA23*, another transport gene, has sequence similarity to UrdJ/LanJ (33%, identity), which are known to be proton-dependent transporters of various drugs (Kieser *et al.*, 2000; Westrich *et al.*, 1999). During the biosynthesis of Sch 47554 and Sch 47555, SchA20 may confer resistance to its own secondary product by transporting it out of the cell. Although the *schP11* gene is adjacent to the PKS gene cluster, the deduced protein does not exhibit significant sequence similarity to any of the genes involved in angucycline biosynthesis. Rather, it has homology to med-ORF27 (42%, identity) from the medermycin producer, *S.* sp. AM-7161, the function of which has yet to be determined (Ichinose *et al.*, 2003).

Inactivation of genes of the PKS gene cluster In order to identify the Sch-gene cluster, chromosomal copies of *schP4* and *schP5* were replaced by *tsr*, thereby inactivating the aromatase. The pDPK01 plasmid was constructed as described above, yielding the disruption plasmid, pDPK03. This plasmid was then transferred into *Streptomyces* sp. SCC-2136 via protoplast transformation. Colonies resistant to *tsr* were selected, and integration was confirmed by Southern hybridization. Total DNAs from the ΔPKS mutant and wild-type strains of SCC-2136 were then separately digested with *Kpn*I and hybridized with the *Eco*RI/*Hin*dIII fragment of the pDPK02 plasmid as a probe. We detected a band of 13.8-kb in the wild-type and bands of 12.8-kb and 9.8-kb in the mutant, indicating that the latter resulted from a single crossover (Fig. 3). The mutant exhibited morphological characteristics different from the original strains. It grew at a rate of up to 100 micrograms per ml, and displayed no red (brown) pigmentation, a characteristic feature of wild-type *S.* sp. SCC-2136.

Confirmation of Sch47554 and Sch47555 production by HPLC/LC-MS HPLC analysis of *S.* sp. SCC-2136 wild type and the ΔPKS mutant was carried out by both mass spectrometry and by measuring absorbance (218 nm) (Fig. 4). The two major prodiginine products were eluted at 32.75 and 38.11 min from wild-type (Fig. 4A). Mass spectrometry analysis demonstrated that the first peak was Sch47555 observed at *m/z* 712.6 $[M + NH_4]^+$ in agreement with the $C_{37}H_{42}O_{13}$ molecular formula, and the second peak (38.11 min retention time) was Sch47554 observed at *m/z* 708.6 $[M+NH_4]^+$ in agreement with the $C_{37}H_{38}O_{13}$ molecular formula (Fig. 4C). These compounds were consistently observed in the *S.* sp SCC-2136 wild-type and were not detected in the ΔPKS mutant (Fig. 4B). We also analyzed ΔPKS for angucycline production using a *Staphylococcus aureus* bioassay. No zone of growth inhibition was formed (data not shown).

Discussion

The Sch-gene cluster not only has a high degree of similarity to the gene cluster for urdamycin A, but also has the same organization. It is also similar to that of the other angucyclines, landomycin A, and oviedomycin. The genes encoding PKS in Sch 47554 and Sch 47555 (*schP 10P9P8P7P6P5P4P3*), as well as those for the other angucycline antibiotics, are strictly conserved in the order: *urdEFABCDLM*, *ovmOICPKSTAOII*, and *lanEFABCDLM2* (data not shown).

We propose the following model, not yet supported experimentally, based on analogy with other systems. With an angucycline backbone, the core structure is formed via chain initiation with acetyl-CoA, followed by condensation with nine other malonyl-CoAs, yielding a decaketide, a common intermediate for the majority of the aromatic PKS II. The condensation reaction is then catalyzed by a set of minimal PKSs: SchP6, SchP7, and SchP8. The

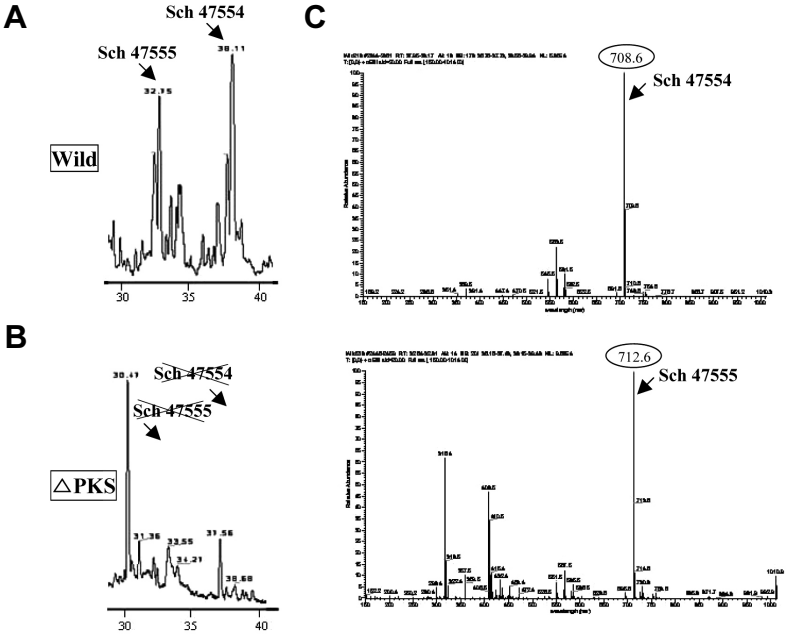


Fig. 4. HPLC and LC/MS analysis of Sch47554 and Sch47555 production. (A) and (B) are HPLC spectra from *S.* sp. SCC-2136 wild-type and the ΔPKS mutant, respectively. Prodiginines were detected by 218 nm absorbance as well as mass spectral analysis of Sch47554 and Sch47555 (C). The corresponding MS spectra of Sch47554 and Sch47555 with their mass numbers (circled) are shown.

SchP5 protein, encoded by the upstream genes of the minimal set of PKS genes (Fig. 2), exhibits a high degree of sequence similarity to ketoreductases in the aromatic PKS gene clusters; these are involved in the region-specific reduction of the C-9 carbonyl group of the nascent poly-β-ketone chain (Hopwood, 1997). The next step in angucycline biosynthesis is performed by a protein encoded by *schP4* (aromatase), which also has an important role in the formation of the first aromatic ring of the aromatic polyketide in type II PKS (Hopwood, 1997). Therefore, we propose that SchP4 is involved in aromatization of the first ring (first cyclization followed by aromatization). The Sch-gene cluster also harbors another cyclase gene, SchP9, immediately downstream of the minimal PKS, that is similar in sequence to UrdF of the urdamycin gene cluster and JadI of the jadomycin gene cluster, both of which are putative cyclases catalyzing the cyclization of the fourth ring (the final ring in jadomycin). Post-PKS tailoring enzymes are responsible for conversion of the aromatic aglycone to a biologically active metabolite (Motamedi *et al.*, 1996), and both oxygenases and glycosyltransferases are crucial for the angucyclines. Generally, the Type II PKS clusters harbor genes which encode post-PKS modifying enzymes including oxygenases, methyl transferase, and glycosyltransferases, which then help to tailor the polyketide backbone into its final, bioactive form.

Two oxygenases, SchP3 and SchP10, are encoded by their respective genes. SchP3, with greatest similarity to UrdM, has been proposed to take part in hydroxylation at C-12b, as seen in the urdamycin biosynthesis pathway (Faust *et al.*, 2000). Faust *et al.* previously isolated the shunt product, a seven-membered lactone ring compound, from an *UrdM* deletion mutant, and postulated that the native UrdM protein catalyzed two distinct steps: the Baeyer-Villiger oxygenation to a lactone in ring A, and a subsequent base-assisted rearrangement of ring A, which re-establishes the six-membered carbocycle. However, the actual mechanism of SchP3 in angucycline PKS remains a matter of investigation. SchP10 exhibits homology to UrdE, a FAD-dependent aromatic hydroxylase, and has been identified as an oxygenase responsible for the oxidative opening of the aromatic ring (Faust *et al.*, 2000; Yang *et al.*, 1996).

The presence of three glycosyltransferases in the cluster is consistent with the transfer of three different deoxysugar moieties. Obviously, the similarity of SchS7 to other *C*-glycosyltransferases suggests that the glycosyltransferases may transfer 2,6-dideoxy or 2,3,6-trideoxysugars to the aglycone via the formation of C-C bonds. The lower degree of homology of SchS9 and SchS10 with urdamycin *O*-glycosyltransferases suggests that they transfer structurally different deoxysugar moieties than urdamycin *O*-glycosyltransferase. With regard to the transfer positions of the sugar moieties, SchS9 is a probable candidate for the D-amicetosyl transfer at C-9 in Sch 47555, whereas SchS10 may transfer L-aculosyl to both C-3 and C-9 in Sch 47554 and Sch 47555. However, the assignment of SchS9 and SchS10 to a particular DOH moiety transfer will require verification in further experi-

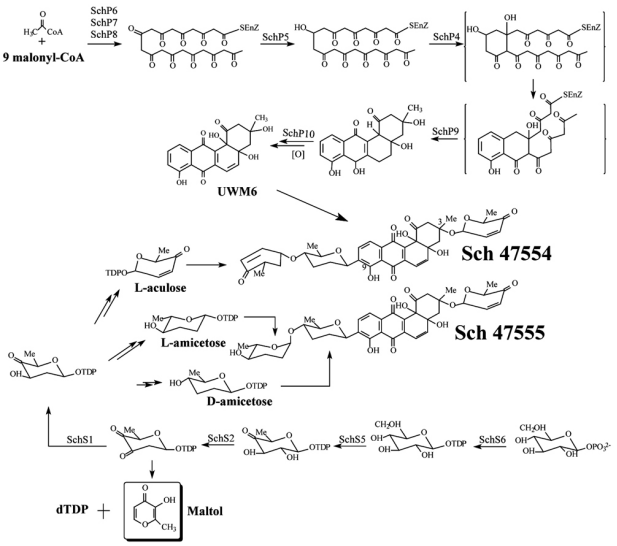


Fig. 5. Hypothetical biosynthetic pathway of Sch 47554 and Sch 47555.

ments. Moreover, the sequence by which sugars are glycosylated remains to be elucidated.

SchS4 may be involved in reduction of the 4-keto group rather than the 4-keto-2,3,6-trideoxy sugar, and the lower degree of sequence identity of SchS4 with UrdZ3 may be due to a difference in function. This may also be evidence for a difference in the stereospecificities of UrdZ3 and SchS4. Ketoreduction during the formation of D- and L-amicetose may be attributable to a single SchS4 within the cluster.

Finally, with regard to the biosynthesis of deoxysugars in Sch 47554 and Sch 47555, we surmise that the first two steps are straightforward, and are catalyzed by SchS6 and SchS5, thereby yielding dTDP-4-keto-6-deoxy-D-glucose (Fig. 5). The third step involves C-2 deoxygenation catalyzed by dTDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase. As in granaticin biosynthesis, this reaction presumably proceeds through a highly reactive intermediate, dTDP-3,4-diketo-2,6-dideoxy-D-glucose, which undergoes facile elimination of dTDP, resulting in the formation of a stable product (maltol) (unpublished data). Thus, it appears to operate in conjunction with another enzyme, thereby trapping the product. This trapping most likely involves the adjacent 3-ketoreductase (SchS1), yielding 4-keto-2,6-dideoxy-D-glucose. Such trapping by aminotransferases in the biosynthesis of L-epivancosamine has been reported in a coupled *in vitro* enzyme assay (Chen *et al.*, 2000). Thus, we have proposed that the first four steps are straightforward, and the pathway is bifurcated. This indicates that dTDP-4-keto-2,6-dideoxy-D-glucose may constitute an intermediate in the biosynthesis of all of the sugar moieties of Sch 47554 and Sch 47555. The remaining steps include C-3 deoxygenation, epimerization, and 4-ketoreduction. Finally, nucleotidyl-activated sugars are probably transferred to the aglycone by both *C*- and *O*-glycosyltransferases, yielding the final glycosylated products.

We confirmed that the cloned gene cluster was responsible for the production of Sch47554 and Sch47555 using a mutant in which the gene encoding the PKS (*SchP4 and SchP5*) was disrupted by insertion of resistant genes. Together, our findings may permit the synthesis of novel Sch47554 and Sch47555 analogs via genetic engineering of *sch* PKS and other sugar genes.

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References

Burson, K. K. and Khosla, C. (2000) Dissecting the chain length specificity in bacterial aromatic Polyketide synthases using chimeric genes. *Tetrahedron* **56**, 9401–9408.

Chen, H., Thomas, M. G., Hubbard, B. K., Losey, H. C., Walsh, C. T., *et al*. (2000) Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. *Proc. Natl. Acad. Sci. USA* **97**, 11942–11947.

Chu, M., Yarborough, R., Schwartz, J., Patel, M. G., Horan, A. C., *et al*. (1993) Sch 47554 and Sch 47555, two novel antifungal antibiotics produced from a *Streptomyces* sp. *J. Antibiot.* **46**, 861–865.

Decker, H. and Haag, S. (1995) Cloning and characterization of a polyketide synthase gene from *Streptomyces fradiae* Tu2717, which carries the genes for biosynthesis of the angucycline antibiotic urdamycin A and a gene probably involved in its oxygenation. *J. Bacteriol.* **177**, 6126–6136.

Draeger, G., Park, S. H., and Floss, H. G. (1999) Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin. *J. Am. Chem. Soc.* **121**, 2611–2612.

Faust, B., Hoffmeister, D., Weitnauer, G., Westrich, L., Haag, S., *et al*. (2000) Two new tailoring enzymes, a glycosyltransferase and an oxygenase, involved in biosynthesis of the angucycline antibiotic urdamycin A in *Streptomyces fradiae* Tu2717. *Microbiology* **146**, 147–154.

Fernandez-Moreno, M. A., Martinez, E., Boto, L., Hopwood, D. A., and Malpartida, F. (1992) Nucleotide sequence and deduced functions of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. *J. Biol. Chem.* **267**, 19278–19290.

Han, L., Yang, K., Ramalingam, E., Mosher, R. H., and Vining, L. C. (1994) Cloning and characterization of polyketide synthase genes for jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230. *Microbiology* **140**, 3379–3389.

Han, L., Yang, K., Kulowski, K., Wendt-Pienkowski, E., Hutchinson, C. R., *et al*. (2000) An acyl-coenzyme A carboxylase encoding gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230. *Microbiology* **146**,

903–910.

Hopwood, D. A. (1997) Genetic contributions to understanding polyketide synthases. *Chem. Rev.* **97**, 2465–2498.

Hutchinson, C. R. and Fujii, I. (1995) Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* **49**, 201–238.

Ichinose, K., Ozawa, M., Itou, K., Kunieda, K., and Ebizuka, Y. (2003) Cloning, sequencing and heterologous expression of the medermycin biosynthetic gene cluster of *Streptomyces* sp. AM-7161: towards comparative analysis of the benzoisochromanequinone gene clusters. *Microbiology* **149**, 1633–1645.

Katz, L. and Donadio, S. (1993) Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* **47**, 875–912.

Kharel, M. K., Basnet, D. B., Lee, H. C., Liou, K. K., Moon, Y. H., *et al.* (2004) Molecular cloning and characterization of a 2-deoxystreptamine biosynthetic gene cluster in gentamicin-producing *Micromonospora echinospora* ATCC15835. *Mol. Cells* **18**, 71–78.

Kieser, T., Mervyn, J. B., Mark, B. J., Keith, C. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, John Innes Foundation Norwich, UK.

Kulowski, K., Wendt-Pienkowski, E., Han, L., Yang, K., Vining, L. C., *et al*. (1999) Functional Characterization of the *jadI* Gene As a Cyclase Forming Angucyclinones. *J. Am. Chem. Soc.* **121**, 1786–1794.

Künzel, E., Faust, B., Oelkers, C., Weissbach, U., Bearden, D. W., *et al*. (1999) Inactivation of the urdGT2 gene, which encodes a glycosyltransferase responsible for the C-glycosyltransferase of activated D-olivose, leads to formation of the novel urdamycins I, J, and K. *J. Am. Chem. Soc.* **121**, 11058–11062.

Lombo, F., Brana, A. F., Salas, J. A., and Mendez, C. (2004) Genetic organization of the biosynthetic gene cluster for the antitumor angucycline oviedomycin in *Streptomyces antibioticus* ATCC 11891. *Chembiochem* **5**, 1181–1187.

McDaniel, R., Ebert-Khosla, S., Hopwood, D. A., and Khosla, C. (1993) Engineered biosynthesis of novel polyketides. *Science* **262**, 1546–1550.

Motamedi, H., Shafiee, A., Cai, S. J., Streicher, S. L., Arison, B. H., *et al*. (1996) Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK506 and FK520. *J. Bacteriol.* **178**, 5243–5248.

Park, C.-H., Lee, D.-W., Kang, T.-B., Lee, K.-H., Yoon, T.-J., *et al.* (2001) cDNA cloning and sequence analysis of the lectin genes of the Korean Mistletoe (*Viscum album coloratum*). *Mol. Cells* **12**, 215–220.

Perez, M., Lombo, F., Zhu, L., Gibson, M., Brana, A. F., *et al*. (2005) Combining sugar biosynthesis genes for the generation of L- and D-amicetose and formation of two novel antitumor tetracenomycins. *Chem. Commun.* (*Camb*), 1604–1606.

Rawlings, B. J. (1999) Biosynthesis of polyketides (other than actinomycete macrolides). *Nat. Prod. Rep.* **16**, 425–484.

Rebets, Y., Ostash, B., Luzhetskyy, A., Hoffmeister, D., Brana, A., *et al*. (2003) Production of landomycins in *Streptomyces globisporus* 1912 and *S. cyanogenus* S136 is regulated by genes encoding putative transcriptional activators. *FEMS Microbiol. Lett.* **222**, 149–153.

Rebets, Y., Ostash, B., Luzhetskyy, A., Kushnir, S., Fukuhara, M., *et al*. (2005) DNA-binding activity of LndI protein and temporal expression of the gene that upregulates landomycin E production in *Streptomyces globisporus* 1912. *Microbiology* **151**, 281–290.

Rodríguez, D., Quirós, L. M., Braña, A. F., and Salas, J. A. (2003) Purification and characterization of a monooxygenase involved in the biosynthetic pathway of the antitumor drug mithramycin. *J. Bacteriol.* **185**, 3962–3965.

Rohr, J. (2000) *Bioorganic Chemistry, Deoxysugars, polyketides and related classes: Synthesis, Biosynthesis*, *Enzymes*, Springer. Germany.

Rohr, J. and Thiericke, R. (1992) Angucycline group antibiotics. *Nat. Prod. Rep.* **9**, 103–137.

Salas, J. A. and Mendez, C. (2005) Biosynthesis pathways for deoxysugars in antibiotic-producing actinomycetes: isolation, characterization and generation of novel glycosylated derivatives. *J. Mol. Microbiol. Biotechnol.* **9**, 77–85.

Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning*, *A Laboratory Manual*, third ed. edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C., *et al*. (1988) Evolutionary conservation among biotin enzymes. *J. Biol. Chem.* **263**, 6461–6464.

Sohng, J. K., Oh, T. J., and Kim, C. H. (1998) Method for cloning biosynthetic genes for secondary metabolites including deoxysugars from actinomycetes. *J. Biochem. Mol. Biol.* **31**, 484–491.

Spines, C. E., Chang, C. J., and Floss, H. G. (1979) Biosynthesis of the antibiotic granaticin. *J. Am. Chem. Soc.* **101**, 701–706.

Subba, B., Kharel, M. K., Lee, H. C., Liou, K. K., Kim, B.-G., *et al.* (2005) The ribostamycin biosynthetic gene cluster in *Streptomyces ribosidificus*: comparison with butirosin biosynthesis. *Mol. Cells* **20**, 90–96.

Trefzer, A., Pelzer, S., Schimana, J., Stockert, S., Bihlmaier, C., *et al*. (2002) Biosynthetic gene cluster of simocyclinone, a natural multihybrid antibiotic. *Antimicrob. Agents Chemother.* **46**, 1174–1182.

Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E., and Flugel, R. S. (1997) Post-translational modification of polyketide and nonribosomal peptide synthases. *Curr. Opin. Chem. Biol.* **1**, 309–315.

Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, D. A., *et al*. (1999) Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin biosynthesis. *FEMS Microbiol. Lett.* **170**, 381–387.

Wohlert, S. E., Wendt-Pienkowski, E., Bao, W., and Hutchinson, C. R. (2001) Production of aromatic minimal polyketides by the daunorubicin polyketide synthase genes reveals the incompatibility of the heterologous DpsY and JadI cyclases. *J. Nat. Prod.* **64**, 1077–1080.

Yang, K., Han, L., Ayer, S. W., and Vining, L. C. (1996) Accumulation of the angucycline antibiotic rabelomycin after disruption of an oxygenase gene in the jadomycin B biosynthetic gene cluster of *Streptomyces venezuelae*. *Microbiology* **142**, 123–132.