Iterative type II polyketide synthases, cyclases and ketoreductases exhibit context-dependent behavior in the biosynthesis of linear and angular decapolyketides

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Background: Iterative type II polyketide synthases (PKSs) produce polyketide chains of variable but defined length from a specific starter unit and a number of extender units. They also specify the initial regiospecific folding and cyclization pattern of nascent polyketides either through the action of a cyclase (CYC) subunit or through the combined action of site-specific ketoreductase (KR) and CYC subunits. Additional CYCs and other modifications may be necessary to produce linear aromatic polyketides. The principles of the assembly of the linear aromatic polyketides, several of which are medically important, are well understood, but it is not clear whether the assembly of the angular aromatic (angucyclic) polyketides follows the same rules.

Results: We performed an *in vivo* evaluation of the subunits of the PKS responsible for the production of the angucyclic polyketide jadomycin (jad), in comparison with their counterparts from the daunorubicin (dps) and tetracenomycin (tcm) PKSs which produce linear aromatic polyketides. No matter which minimal PKS was used to produce the initial polyketide chain, the JadD and DpsF CYCs produced the same two polyketides, in the same ratio; neither product was angularly fused. The set of *jadABCED* PKS plus putative *jadI* CYC genes behaved similarly. Furthermore, no angular polyketides were isolated when the entire set of jad PKS enzymes and JadI or the jad minimal PKS, JadI and the TcmN CYC were present. The DpsE KR was able to reduce decaketides but not octaketides; in contrast, the KRs from the jad PKS (JadE) or the actinorhodin PKS (ActIII) could reduce octaketide chains, giving three distinct products.

Conclusions: It appears that the biosynthesis of angucyclic polyketides cannot be simply accomplished by expressing the known PKS subunits from artificial gene cassettes under the control of a non-native promoter. The characteristic structure of the angucycline ring system may arise from a kinked precursor during later cyclization reactions involving additional, but so far unknown, components of the extended decaketide PKS. Our results also suggest that some KRs have a minimal chain length requirement and that CYC enzymes may act aberrantly as first-ring aromatases that are unable to perform all of the sequential cyclization steps. Both of these characteristics may limit the widespread application of CYC or KR enzymes in the synthesis of novel polyketides.

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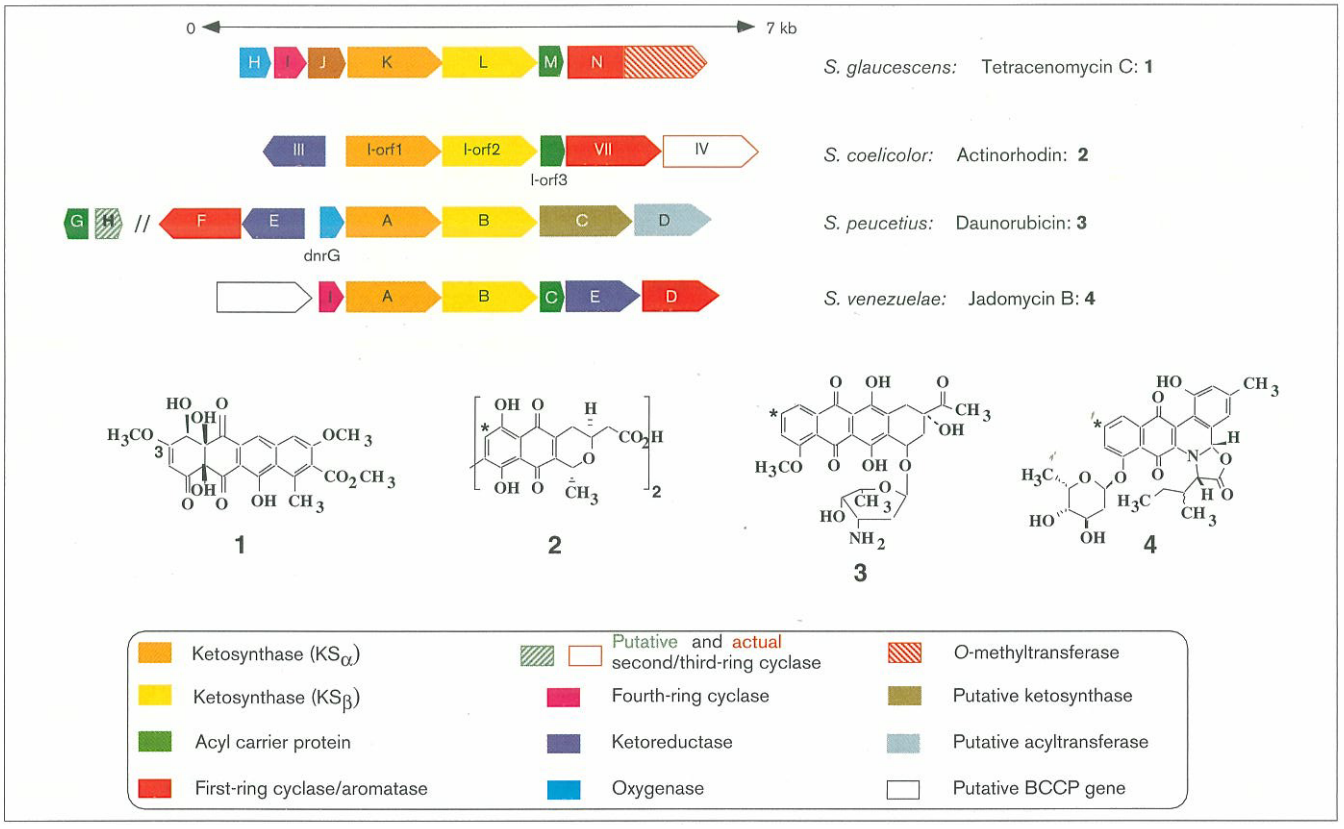
Introduction

Polyketides, a large and structurally diverse family of pharmaceutically important natural products, are produced by polyketide synthase (PKS) complexes through a specialized variation of fatty acid biosynthesis. Strong sequence and mechanistic similarities among many fatty acid and polyketide synthase enzymes led to PKSs being classified into type I and type II PKS systems, following the fatty acid synthase (FAS) classification system. Bacterial type I PKSs are usually large multifunctional enzyme complexes that process each of the initial or intermediate condensation products through ketoreduction, ketoreduction and dehydration, or ketoreduction, dehydration and enoyl reduction to produce highly reduced compounds from acetate, propionate and butyrate residues. In contrast, type II PKS complexes normally consist of three to seven monofunctional or bifunctional proteins in which none of the intermediate condensation products is subjected to processing until the full length polyketide chain is synthesized. Subsequent cyclizations and other modifications of the nascent polyketide introduce additional structural variability and typically yield aromatic compounds.

Figure 1



Polyketide synthase, ketoreductase and cyclase genes from the *Streptomyces* gene clusters governing the biosynthesis of tetracenomycin (*tcm*) C, actinorhodin (*act*), daunorubicin (*dps* or *dnr*) and jadomycin (*jad*) B. The organization of each set of genes is shown by wedges that are oriented in the direction of transcription and the size of each is proportional to the size of the protein product. The role of individual genes is indicated by a color corresponding to the function specified in the key at the bottom (the functions of these genes are further described in the text, except for those of the *tcmH* and *dnrG* oxygenase genes, and the putative *dpsC* ketosynthase, *dpsD* acyltransferase, *dpsH* cyclase and biotin carboxy carrier protein (BCCP) genes); orf, open reading frame; // indicates that the adjacent *dpsG* and *dpsH* genes are located several kilobases from the other *dps* genes; kb, kilobase; the number 3 in **1** and * in **2–4** indicate the positions not reduced (3) or reduced (*) by the KR enzyme in each pathway.

Iterative polyketide synthases that form aromatic compounds have become the subject of considerable interest over the last few years, not least because the common enzymatic mechanisms and the high degree of conservation that exists among type II PKS gene clusters (summarized in Figure 1 [1]) make them amenable to use for generating novel polyketides through genetic engineering. Towards this end, many aromatic PKS recombinant gene cassettes have been constructed and tested to identify the proteins that are involved in determining chain length, the degree and regiospecificity of ketoreduction, and the regiospecificity of cyclizations and aromatizations [2–10]. It has been shown that chain length determination is an inherent property of the so-called 'minimal PKS', which contains the β-ketoacyl synthase subunits KS_α and KS_β (KS_β was previously known as chain length determination factor (CLF), but in fact both KS_α and KS_β affect chain length) and an acyl carrier protein (ACP; Figure 1) [6].

Two groups of initial polyketide cyclases (CYCs) have been identified so far. One group regiospecifically folds unreduced polyketides of various chain length at the C-11 position, directing the first cyclization to take place between C-9 and C-14 and also influences the formation of the second and third ring [8–10]. A typical member of this group is the bifunctional TcmN CYC/*O*-methyltransferase involved in the biosynthesis of tetracenomycin C (compound **1**, Figure 1) [11]. The second and larger group, most often represented by the ActVII enzyme involved in the biosynthesis of actinorhodin (compound **2**, Figure 1) [12], seems to promote only the regiospecific first-ring closure and subsequent aromatization of C-9 ketoreduced polyketides in hybrid PKS systems [5,13]. Consequently, the ActVII enzyme has been named an aromatase (ARO) [13]. (Some ActVII homologs can do more than just form and aromatize a single six-membered ring, however, and ARO is an inappropriate designation for these enzymes.)

Members of the ARO group of enzymes may exhibit an upper chain length limit, as was shown for ActVII [2,6] and the frenolicin (Fren) ARO [14], and more than one CYC may be necessary for the defined closure of additional aromatic rings to form linear tricyclic or tetracyclic compounds [14]. Recently, a set of predictive design rules for the generation of aromatic polyketides was proposed on the basis of these studies and tested through the engineered biosynthesis of three novel products [14,15]. Here, we test these design rules further by concentrating on ketoreductases (KRs) and CYCs that promote the biosynthesis of reduced decaketides, especially those that produce the group of angucyclic polyketides (angucyclines) [16] represented by jadomycin (jad) B (compound **4**, Figure 1) [17].

Results and discussion

The design rules for the biosynthesis of reduced aromatic polyketides [14] are based primarily on results obtained by co-expressing a variety of minimal PKS gene sets containing only the KS_α, KS_β and ACP genes with just a single KR gene (*actIII*), one of three ARO genes (*actVII*, *fren* ARO or *griseusin (gris)* ARO, depending on the expected chain length of the polyketide produced by the minimal PKS) and in some cases the *actIV* CYC gene [13] as well. Because all of these PKS genes come from well-known systems that produce linear aromatic polyketides, it was unclear whether angucycline-producing PKS systems [16] would follow these same rules.

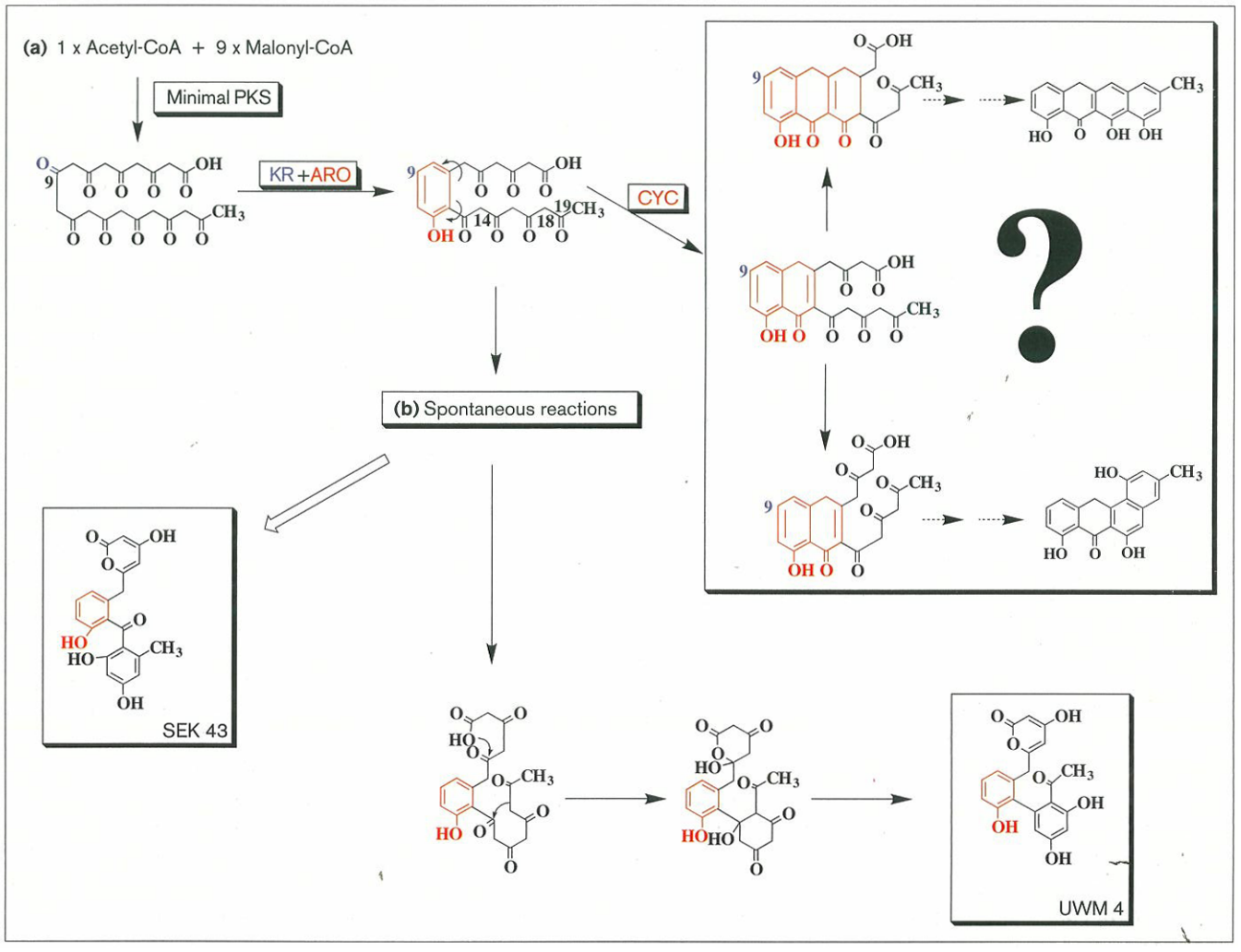
We therefore set out to examine the behavior of functional subunits of an angucycline PKS, choosing the PKS that forms the carbon skeleton of jadomycin B (compound **4**, Figure 1) [18,19]. The enzyme complex we studied consists of the putative minimal PKS (JadA, JadB and an ACP, either JadC or TcmM, a homolog of JadC) plus the JadE KR and the *jadD* and *jadI* gene products, proposed to have CYC activity. Although only limited information was available about the function of the *jad* PKS genes in angucycline biosynthesis [18,19], the daunorubicin (dps) PKS genes, *dpsABCDEFG*, which also govern the formation of a reduced decaketide and thence an aromatic product with three fused aromatic rings (compound **3**, Figure 1), were better understood [20,21]. From the results obtained by co-expressing the *tcm* minimal PKS (specifying the formation of a decaketide), *actIII* KR and *gris* ARO genes [14], which gave the major polyketide product SEK43 (Figure 2) we predicted that a similar expression construct using the *dpsF* CYC instead of the *gris* ARO gene, and the *dpsE* KR instead of the *actIII* KR gene should also yield SEK43 [14]. We therefore decided to use a set of functional expression cassettes containing the *dpsF* and *dpsE* genes, and a corresponding set of constructs containing the *jadD* CYC gene together with either the *dpsE* or *jadE* KR genes, to study the design rules for this class of polyketide products. Later, our observations on KR specificity, which suggested that some KRs are specific for substrates of defined chain length, led us to study the properties of polyketide KR enzymes in more detail. Previous work on the ActIII KR implied that this enzyme could be used for site-specific reduction of polyketides of any chain length [5,6,14].

Properties of the JadD and DpsF cyclases in hybrid polyketide synthases

We wished to examine the ability of the putative JadD and DpsF CYCs to act on either unreduced or C-9 reduced octaketide or decaketide chains, with the aim of determining whether the proposed JadD CYC would be able to direct the formation of an angularly fused three-ring aromatic structure. We therefore constructed a series of expression cassettes (Table 1) containing the minimal PKS genes from either the tetracenomycin C producer *Streptomyces glaucescens* (*tcmJKLM* [9]), the daunorubicin producer *Streptomyces peucetius* (*tcmJ*, *dpsA*, *dpsB* [20] and *tcmM* [9]) or the actinorhodin producer *Streptomyces coelicolor* (*tcmJ*, *actI-orf1*, *actI-orf2* [12] and *tcmM*; Figure 1). The *jadC* and *tcmM* ACP genes were used interchangeably with the third open reading frame (orf) in the *actI* gene cluster (*actI-orf3*) or *dpsG*; several precedents indicated that this should not have an adverse effect on the hybrid PKSs [2,6,10,22]. To these cassettes, we added the *jadD* or *dpsF* CYC genes and the *dpsE* KR gene [20], to determine the product(s) resulting from the action of the CYC and KR enzymes on the octaketide or decaketide made by the minimal PKS. For expression in either *Streptomyces lividans* 1326 or the down promoter mutant *S. glaucescens* WMH1077 described previously [23,24], all gene cassettes were cloned under the control of the strong constitutive *ermE** promoter [25,26] in the *Escherichia coli–Streptomyces* shuttle vector pWHM3 [27].

The putative JadD and DpsF CYCs, when expressed together with *dpsE* and either the *tcm* or the *dps* minimal PKS genes, produced the same polyketides, SEK43 [14] and UWM4 (Figure 2; Table 1, entries 7,8,12,13). They failed to act on unreduced decaketides provided by the same minimal PKSs in the absence of the *dpsE* gene product, however, yielding SEK15 and SEK15b [5,6] (Figure 3; Table 1, entries 6,9,11). Moreover, both CYCs also failed to act on octaketides produced by the modified *act* minimal PKS whether or not the *dpsE* KR gene was present, so that compounds SEK4 and SEK4b ([5,7]; Figure 3) were formed (Table 1, entries 18,19,21). The failure of both DpsF and JadD to act on octaketides both in the presence and absence of the DpsE KR represents either a new feature of these CYCs or a minimal chain length requirement of DpsE (so far no minimal chain length requirement has been demonstrated for AROs, CYCs or KRs). The results indicate that, in the contexts tested, both the *jadD* and the *dpsF* gene products act like the ActVII ARO [12] as regiospecific first-ring cyclase/dehydrases of C-9 reduced decaketides (Figures 2,3). In addition, they demonstrate that the JadD CYC is not able to direct the formation of an angucyclic compound by itself,

Figure 2



Current concept for the roles of the minimal PKS, KR, ARO and CYC enzymes (colored according to Figure 1) that assemble and process a decaketide via the putative intermediates shown. **(a)** The decaketide is assembled from acetate and malonate, reduced and the first ring cyclized. Additional cyclization takes place, possibly stepwise in an unknown fashion as signified by the **?**, to form either the linearly fused (for daunorubicin) or angularly fused (for jadomycin B) tetracyclic structures. **(b)** The monocyclic intermediate cyclizes spontaneously in two alternative fashions, to form SEK43 or UWM4.

nor is the DpsF CYC able to form more than one six-membered aromatic ring. As DpsF acts as a multiring-forming CYC in other contexts (M.G., G.M., E.W.-P., K. Madduri and C.R.H., unpublished observations), JadD might do so as well (but see below). Moreover, Rajgarhia and Strohl [28] have found that DpsF is the only CYC needed in cooperation with DpsA, DpsB, DpsE and the DpsG ACP to form aklanonic acid, a tricyclic aromatic compound and daunorubicin precursor.

Structure and chemical properties of UWM4

The polyketide UWM4 is the minor product of several decaketide-producing expression constructs involving combinations of the DpsF or JadD CYCs with the DpsE or JadE KRs (Table 1, entries 7,8,12,13,24). When separated by thin layer chromatography (TLC) UWM4 changed from colorless to yellow in visible light (and from light-absorbing to a pale yellow fluorescence under 310 nm UV) which, in addition to a slightly higher R_f value, enabled UWM4 to be distinguished from SEK43. The structure of UWM4 was characterized using nuclear magnetic resonance (NMR), mass spectroscopy (MS) and formation of the tetra-*O*-methyl derivative. The fast atom bombardment (FAB) mass spectrum (m/z: 367.1; negative ions) in combination with the NMR experiments led to a molecular mass of 368.34 g/mol and the formula $C_{20}H_{16}O_7$ for UWM4. The similarities between the ^{1}H-NMR and ^{13}C-NMR chemical shifts of UWM4 and those of SEK43 indicated a close relationship (Table 2). Nevertheless, the backbone structure of UWM4 was shown to be different from that of SEK43

Table 1

Composition of plasmids containing the type II polyketide synthase genes referred to in this paper.

Entry	Plasmid*,†	KS_α	KS_β‡	ACP	KR	CYC	Metabolites produced§	Reference¶
1	pWHM1200	*tcmK*	*tcmL*	*tcmM*	–#	–	TcmF2, SEK15, SEK15b	[9]
2	pWHM1201	*tcmK*	*tcmL*	*tcmM*	*dpsE*	–	RM20b,c	-
3	pWHM1202	*tcmK*	*tcmL*	*tcmM*	*jadE*	–	RM20b,c	-
4	pWHM862	*tcmK*	*tcmL*	*tcmM*	–	*tcmN*	TcmF2	[9]
5	pWHM1203	*tcmK*	*tcmL*	*tcmM*	*dpsE*	*tcmN*	TcmF2, RM20b	-
6	pWHM1204	*tcmK*	*tcmL*	*tcmM*	–	*jadD*	SEK15, SEK15b	-
7	pWHM1205	*tcmK*	*tcmL*	*tcmM*	*dpsE*	*jadD*	SEK43, UWM4, SEK15	-
8	pWHM1206	*tcmK*	*tcmL*	*tcmM*	*dpsE*	*dpsF*	SEK43, UWM4, SEK15	-
9	pWHM885	*dpsA*	*dpsB*	*tcmM*	–	–	SEK15, SEK15b	[8]
10	pWHM1207	*dpsA*	*dpsB*	*tcmM*	*dpsE*	–	RM20b,c	-
11	pWHM1208	*dpsA*	*dpsB*	*tcmM*	–	*jadD*	SEK15, SEK15b	-
12	pWHM1209	*dpsA*	*dpsB*	*tcmM*	*dpsE*	*jadD*	SEK43, UWM4	-
13	pWHM1210	*dpsA*	*dpsB*	*tcmM*	*dpsE*	*dpsF*	SEK43, UWM4	-
14	pWHM1211	*actI-orf1*	*actI-orf2*	*tcmM*	–	–	SEK4, SEK4b	[9]
15	pWHM1212	*actI-orf1*	*actI-orf2*	*tcmM*	*dpsE*	–	SEK4, SEK4b	-
16	pWHM1213	*actI-orf1*	*actI-orf2*	*tcmM*	*actIII*	–	Mutactin, dehydromutactin	[6]
17	pWHM1214	*actI-orf1*	*actI-orf2*	*tcmM*	*jadE*	–	Mutactin, dehydromutactin	-
18	pWHM1215	*actI-orf1*	*actI-orf2*	*tcmM*	–	*jadD*	SEK4, SEK4b	-
19	pWHM1216	*actI-orf1*	*actI-orf2*	*tcmM*	*dpsE*	*jadD*	SEK4, SEK4b	-
20	pWHM1217	*actI-orf1*	*actI-orf2*	*tcmM*	*actIII*	*jadD*	SEK34	-
21	pWHM1218	*actI-orf1*	*actI-orf2*	*tcmM*	*dpsE*	*dpsF*	SEK4, SEK4b	-
22	pWHM1219	*actI-orf1*	*actI-orf2*	*tcmM*	*actIII*	*dpsF*	SEK34	-
23	pWHM1220**	*jadA*	*jadB*	*tcmM*	–	*tcmN*	TcmF2	-
24	pWHM1221**	*jadA*	*jadB*	*jadC*	*jadE*	*jadD*	SEK43, UWM4	-

*The *tcmJ* gene is present in all of the plasmids unless indicated otherwise. †The first three genes were cloned in the order shown in the table, preceded by *ermE*::tcmJ*; the order of the KR and CYC genes varied, although the KR gene was most often last. ‡Synonymous with a CLF gene. §The same metabolites were produced in *S. lividans* and *S. glaucescens*. ¶If no reference is given, the data referred to are from this work. #Indicates the lack of such a gene. ****tcmJ* replaced with *jadI*.

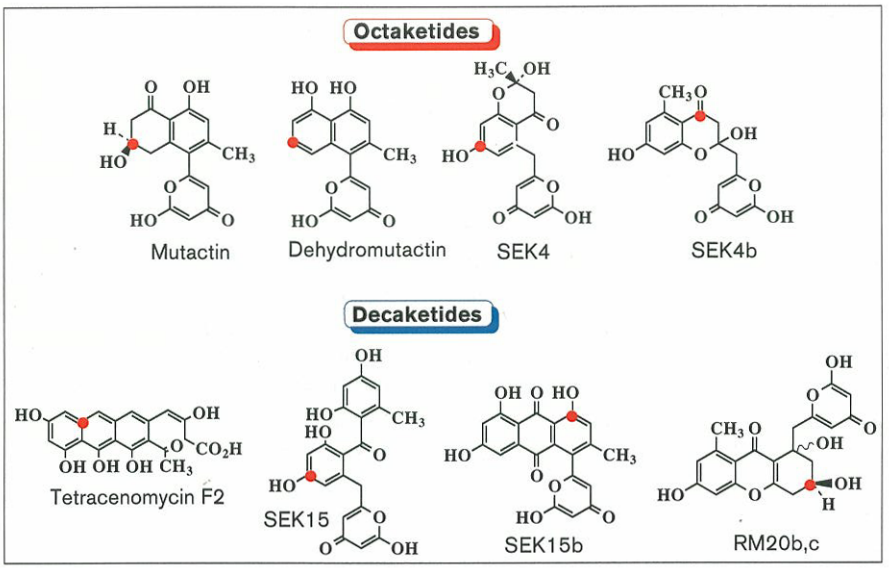
(Figure 2) by recording heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC) spectra of their tetra-*O*-methyl derivatives. These data also suggested that, although ring A had previously been assumed to form a pyrone structure in this kind of compound [6], in fact a δ-lactone is preferred. The long-range correlations in the HMBC spectrum (Figure 4a), the X-ray crystal structure (Figure 4b) and the ^{13}C-NMR data of UWM4 and SEK43 proved that the 3-methoxy and not the 1-methoxy isomer was formed upon methylation, from which it was possible to assign the chemical shifts of C-3 to an enol group and C-1 to a lactone. The conversion of UWM4 to a yellow compound under acidic conditions is likely to have involved an aldol condensation between the methylene group at C-6 and the keto group at C-19 (Figure 4c) because the resulting tetracyclic product should be a new chromophore.

Comparison of the structures of UWM4 and SEK43 suggests that DpsF or JadD catalyzed cyclization of the first ring between C-7 and C-12 in both compounds, but that folding and cyclization of the two other rings was somewhat random and directed more by kinetic or thermodynamic factors than by enzymatic catalysis. The ratio of SEK43 to UWM4 in the isolated material (17:1) suggests that the aldol condensation involving the terminal carbonyl group at C-19 and the C-14 methylene of the decaketide intermediate is favored over that involving the C-18 methylene and C-13 carbonyl. This outcome is consistent with earlier work of Harris and Harris [29] on the solution chemistry of poly-carbonyl compounds and observations by Khosla and coworkers [5,7] on the cyclization of an octaketide to SEK4 and SEK4b.

Properties of the DpsE ketoreductase compared to the ActIII and JadE ketoreductases

To investigate further whether the activities of the JadD CYC, DpsF CYC or DpsE KR require polyketides of a certain chain length, the *dpsE* gene was replaced by the *actIII* gene in all relevant constructs involving the modified *act* minimal PKS genes. The compounds produced from the expression of these new cassettes were analysed as

Figure 3



Structures of some of the octaketide and decaketide metabolites discussed in the text. The position corresponding to the C-9 carbonyl of the polyketide precursor, which may or may not have undergone reduction by a KR, is marked with a red dot in each structure. The decaketides RM20b and RM20c are diastereomers.

before. All of the new constructs produced either mutactin [30], dehydromutactin [6] or SEK34 [13] (Figures 3,5; Table 1, entries 16,17,20,21), indicating that DpsE requires a polyketide chain length of more than 16 carbons to be able to catalyze carbonyl reduction, whereas both the JadD and the DpsF CYCs work properly with reduced octaketides. We then made two additional expression constructs by adding the *jadE* KR gene to the *tcm* and modified *act* minimal PKS genes, respectively. As expected, the combination of the tcm minimal PKS and JadE KR produced the decaketides RM20b and RM20c (Figure 3; Table 1, entry 3); the JadE KR was also able to reduce the octaketides produced by the modified act minimal PKS, yielding mutactin and dehydromutactin (Table 1, entry 17). These results reveal an unexpected feature of the DpsE KR and suggest that KR subunits in general might exhibit different specificities for different sized polyketide intermediates unless there is some other reason for the apparent lack of DpsE function, such as critical protein–protein interactions. Cloning artifacts are unlikely, since the same restriction fragment was used in all constructs. The specificity of KR enzymes could be context dependent; if so, this may explain why the *dpsE* homolog in *Streptomyces galilaeus* apparently can act on the octaketide produced by the introduction of the *actI-orf1* and *actI-orf2* PKS genes into this organism [31] but cannot act on the substrate produced when these genes are introduced into *Streptomyces sp.* strain C5 (W.R. Strohl, personal communication).

The DpsE ketoreductase and TcmN cyclase: competition for the nascent decaketide chain

McDaniel *et al.* [10] had reported earlier that a combination of the tcm minimal PKS, the ActIII KR and the TcmN CYC produced the C-9 reduced decaketides RM20b and RM20c. They concluded that ketoreduction must occur prior to cyclization during the biosynthesis of reduced polyketides, as suggested earlier by Bartel *et al.* [31], and that TcmN is unable to act on C-9 reduced polyketides. In view of the order of events in the biosynthesis of reduced and unreduced decaketides, it would seem that the nascent decaketide chain produced by the minimal PKS could serve as substrate for both a KR and the TcmN CYC. The expected outcome would be a mixture of tetracenomycin [32] and RM20b and RM20c as a result of the competition between the two enzymes for the same substrate. We therefore constructed an expression cassette containing the *tcmJKLM* PKS genes, the *tcmN* CYC gene and the *dpsE* KR gene. The production of secondary metabolites during cultivation of *S. lividans* 1326 transformants in liquid medium was followed using TLC and established that tetracenomycin F2 as well as RM20b and RM20c were formed initially (Table 1, entry 5), but the amount of the unstable tetracenomycin F2 diminished to the point of undetectability after one week. This explains the observations of McDaniel *et al.* [10] and clearly demonstrates that the DpsE KR and TcmN CYC, although competing for the same substrate, perform mutually exclusive reactions.

The jad minimal polyketide synthase does not make angucyclic polyketides in artificial polyketide synthase systems

The above results reveal that the JadD CYC itself cannot influence the heterologous sets of PKS genes studied to form an angucyclic polyketide structure. A second gene product from the *jad* gene cluster, JadI, is predicted to be a CYC enzyme on the basis of its close sequence similarity to TcmI, the CYC that converts the tricyclic tetracenomycin

Table 2

^{1}H and ^{13}C NMR data for SEK43 and UWM4, tetra-*O*-methyl-SEK43 and tetra-*O*-methyl-UWM4.

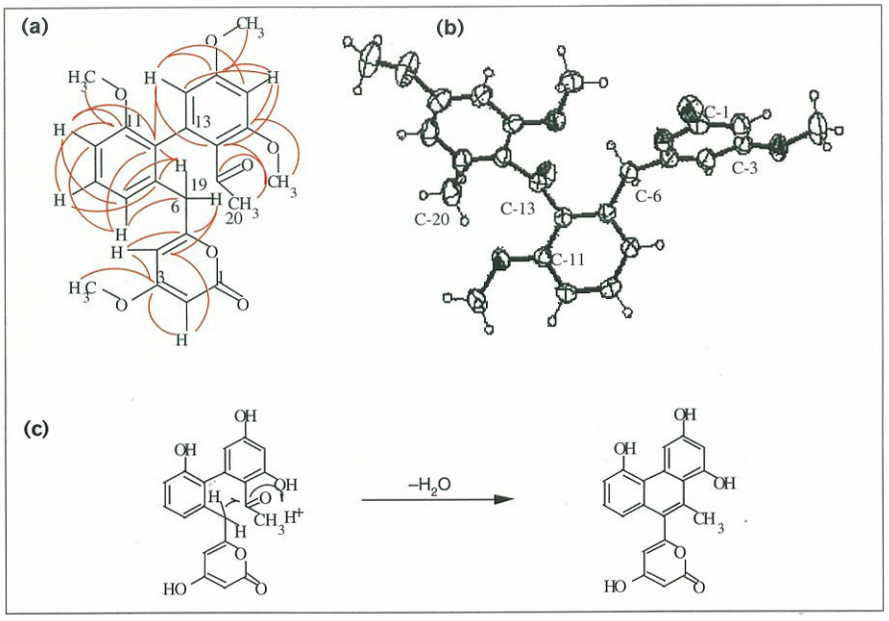
	SEK43		UWM4		Tetra-O-methyl-SEK43		Tetra-O-methyl-UWM4	
Carbon*	$^{13}C^{\dagger}$ δ (ppm)	$^{1}H^{\ddagger}$ δ (ppm) (m, J_{HH}[Hz], area)	$^{13}C^{\S}$ δ (ppm)	$^{1}H^{\ddagger}$ δ (ppm) (m, J_{HH}[Hz], area)	$^{13}C^{\S}$ δ (ppm)	$H^{\ddagger}$ δ (ppm) (m, J_{HH}[Hz], area)	$^{13}C^{\P}$ δ (ppm)	$^{1}H^{\#}$ δ (ppm) (m, J_{HH}[Hz], area)
1	168.1		166.5		161.0		164.4	
2	89.0	5.20 (d, 2.0, 1H)	89.6	5.19 (s, 1H)	87.9	5.39 (d, 2.2, 1H)	87.7	5.3** (1H)
3	173.3		171.7		172.0		171.7	
4	103.4	5.72 (d, 2.0, 1H)	103.5	5.72 (s, 1H)	101.5	5.69 (d, 2.2, 1H)	101.2	5.55 (d, 2.0, 1H)
5	165.0		164.1		164.2		164.3	
6	38.1	3.66 (s, 2H)	38.1	3.63 (d, 15.5, 1H) 3.55 (d, 15.5, 1H)	37.4	3.78 (s, 2H)	37.9	3.51 (d, 16.0, 1H) 3.61 (d, 16.0, 1H)
7	133.4		135.8		135.2		135.6	
8	123.2	6.91 (d, 8.0, 1H)	122.5	6.95 (d, 8.0, 1H)	123.5	6.94 (d, 8.0, 1H)	122.6	6.91 (d, 7.7, 1H)
9	123.2	7.29 (t, 8.0, 1H)	121.7	7.26 (t, 8.0, 1H)	130.7	7.33 (t, 8.0, 1H)	129.1	7.30 (t, 7.7, 1H)
10	116.1	6.94 (d, 8.0, 1H)	115.4	6.93 (d, 8.0, 1H)	111.5	6.94 (d, 8.0, 1H)	109.7	6.86 (d, 7.7, 1H)
11	155.2		155.2		158.3		156.5	
12	132.3		130.8		134.6		129.8	
13	202.2		142.6		198.8		136.6	
14	116.5		113.0	6.26 (d, 2.6, 1H)	141.6		107.6	6.23 (d, 2.2, 1H)
15	168.3		165.0		162.9		158.2	
16	102.1	6.19 (d, 3.0, 1H)	102.3	6.33 (d, 2.6, 1H)	96.7	6.33 (d, 2.2, 1H)	98.3	6.50 (d, 2.2, 1H)
17	165.7		166.5		165.0		161.5	
18	113.3	6.25 (d, 3.0, 1H)	115.4		109.3	6.45 (d, 2.2, 1H)	124.9	
19	145.3		202.9		124.8		202.6	
20	22.3	1.86 (s, 3H)	29**	1.87 (s, 3H)	20.9	2.37 (s, 3H)	30.1	2.23 (s, 3H)
3-CH_3					56.5	3.82 (s, 3H)	56.1	3.74 (s, 3H)
11-CH_3					56.0	3.60 (s, 3H)	55.9	3.73 (s, 3H)
15-CH_3					55.7	3.48 (s, 3H)	56.3	3.86 (s, 3H)
17-CH_3					56.3	3.81 (s, 3H)	55.9	3.70 (s, 3H)

*Carbons are labeled according to their number in the polyketide backbone. †(75.5 MHz, methanol-d_4); ‡(300 MHz, acetone-d_6); §(75.5 MHz, acetone-d_6); ¶(75.5 MHz, CD_2Cl_2); #(300 MHz, CD_2Cl_2). ** Indicates overlapping with solvent.

F2 to the tetracyclic tetracenomycin F1 (K. Yang, L. Han and L.C.V., unpublished observations.) We therefore investigated whether the jad minimal PKS or the putative JadI CYC could instead perform this transformation. To this end we constructed two expression cassettes both containing the *jad* minimal PKS genes (*jadA*, *jadB* and *jadC* or *jadA*, *jadB* and *tcmM*) and the *jadI* gene together with either the *tcmN* gene or the *jadE* KR and *jadD* CYC genes. If either one of the constructs produced an angucycline, we would then have had to determine the functions of JadI and the jad minimal PKS separately. The expression cassette comprising the *jadIABCED* genes again produced SEK43 and UWM4, however, in the same ratio seen with comparable but heterologous cassettes containing the *jadD* or *dpsF* genes, whereas the *jadIAB* plus *tcmMN* genes produced tetracenomycin F2 (Table 1, entries 23,24). Because the *jadIABCED* genes, which were cloned in the native order (Figure 1), did not produce an angular polyketide, the *jadABC* or *jadIABC* genes alone would also have been unable to produce such a backbone. These results indicate that the jad minimal PKS does not determine the formation of an angular polyketide backbone, that the intermediate with a single aromatic ring produced by JadABCED is not a substrate for the putative JadI CYC (explaining the formation of SEK43 and UWM4), and that tetracenomycin F2 is not a substrate for JadI either, even though we believe that JadI is a fourth ring CYC.

The data presented here, together with the results obtained from expression cassettes containing the *actIV* second/third ring CYC gene [2,13], imply that another CYC besides JadI might be required to form the second and third aromatic rings during the biosynthesis of jadomycin and other angucyclines. Many of the clusters of PKS genes that govern the biosynthesis of polycyclic reduced aromatic polyketides, such as the daunorubicin [20,21] or the urdamycin [33] (A. Bechthold and J. Rohr, personal communication) clusters, however, lack an homolog of *actIV* or

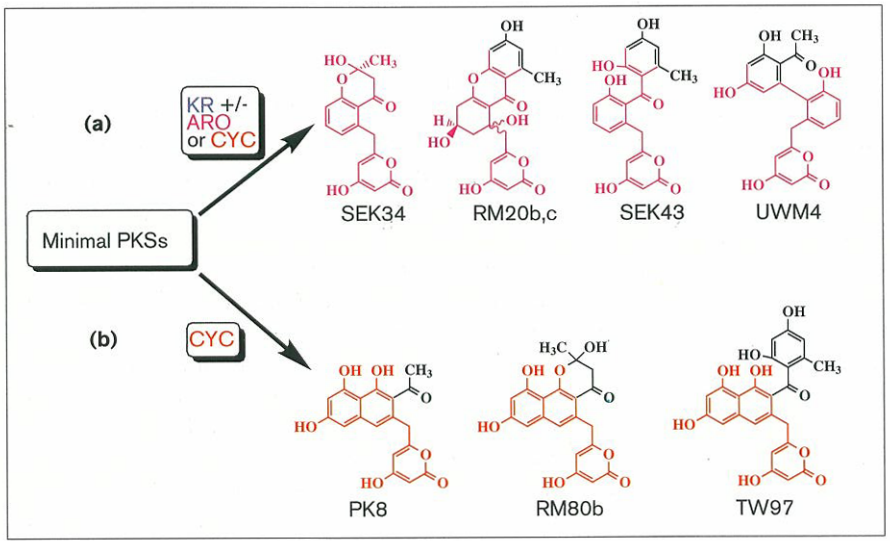
Figure 4



Properties of UWM4 and SEK43. **(a)** The long-range correlations observed in the HMBC spectrum of 3,11,15,17-tetra-O-methyl-UWM4. **(b)** The structure of 3,11,15,17-tetra-O-methyl-SEK43 determined by X-ray crystallography. **(c)** Postulated mechanism for the reaction of UWM4 with acid.

other genes that can be deduced to produce such CYCs, although *dpsH* is one candidate (Figure 1). Furthermore, in *S. lividans* expression of the *dps* PKS genes in their natural order (←*dpsG* and ←*dpsFdpsE*//*dnrGdpsAdpsB*→, where the arrows indicate the direction of transcription), under control of the native promoters, leads to the production of aklanonic acid (M.G., G.M., E.W.-P., K. Madduri and C.R.H., unpublished observations) [28], whereas the same genes, when expressed from an artificial operon (*dnrGdps-AdpsBdpsEdpsFdpsG*→) under control of the *ermE** promoter, produce mainly SEK43 and only traces of aklanonic acid (M.G., G.M., E.W.-P., K. Madduri and C.R.H., unpublished observations). These observations reveal that PKS subunits, which function normally in the native context, can function aberrantly when they are produced *in vivo* from an artificially constructed gene cassette. It is an attractive possibility that this difference in function may be due to a change in the ratio of PKS subunits. It seems likely that the *jad* PKS genes studied here can form an angucycline-producing PKS complex if expressed in the native setting but cannot do so in the context of currently available hybrid or otherwise artificial PKS expression systems. This belief has

Figure 5



Representative polyketides produced by type II PKSs. **(a)** The octaketide or decaketide is reduced at C-9 by the ActIII or JadE KR and the first (central) ring is formed by the ActVII ARO, or DpsF or JadD CYC enzyme. The octaketide framework is colored pink. **(b)** The octaketide, decaketide or dodecaketide is not reduced before the bicyclic ring system is formed by the TcmN CYC enzyme for PK8 [15] and RM80b [10] and the WhiE CYC for TW97 [40] (C. Khosla, personal communication). The octaketide framework is colored red.

an important corollary: heterologous sets of type II PKS genes may have functional limitations that have previously been unappreciated in the quest for the production of hybrid antibiotics by recombinant bacteria [14].

Significance

Combinatorial expression of genes that encode the subunits of *Streptomyces* polyketide synthase (PKS) complexes has been proven to be supremely successful in probing the function of these enzymes. The resulting increase in the understanding of the inner workings of a type II PKS and, consequently, the improved predictability of metabolite production patterns led to the establishment of design rules for the engineered biosynthesis of novel polyketides. These design rules are also a direct consequence of an emerging set of basic, re-occurring compound structures — produced by hybrid PKS systems — that vary in their peripheral functionality only because of differences in chain length of the nascent polyketides (Figure 5). By incorporating secondary polyketide cyclases into hybrid PKS expression systems a more diverse range of polyketide backbone will be produced which can be still further modified at later stages.

Here we investigated whether it was possible to achieve an additional degree of polyketide structural diversity through the early introduction of a kink into the nascent polyketide chain. We attempted to do this by expressing PKS genes from an angucycline producer together with PKS genes from other systems that have been better defined. The products we expected were not formed, however. Instead, we found that the biosynthesis of angular aromatic polyketides superficially seems to adhere to the same rules established for the production of linear aromatic decaketides, in which the initial polyketide undergoes one carbonyl reduction step prior to ring formation. Although it can be inferred from this observation that a subsequent cyclization step (and perhaps an extra enzyme) is responsible for the introduction of the kink, our results favor the notion that certain PKS complexes function abnormally when formed from artificial gene sets, resulting in single rather than multiple enzymatic cyclizations of the nascent polyketide. In the course of this work we also established a specific chain length requirement for the DpsE polyketide ketoreductase that distinguishes it from ActIII and JadE, the other ketoreductase enzymes tested to date.

Materials and methods

Bacterial strains and plasmids

The *S. glaucescens* type strains [24], *S. lividans* 1326 [34], the bacteriophages M13mp18, M13mp19 [35] and the plasmid pWHM3 [27] are described elsewhere. The *ermE** promoter was isolated as reported earlier [24,25]. The vectors pGEM3,5,7,9,11Zf(+) were purchased from Promega (Madison, WI, USA), pUC19 from New England Biolabs (Beverly, MA, USA) and pBluescript SK+ from Stratagene (La Jolla, CA, USA). The *E. coli* strains used were DH5α, TG1 and DH10. The plasmids used in this study are described in Table 1.

Culture conditions, chemicals and other materials

R2YENG liquid medium (pH 6) and agar plates [36] were used for production of secondary metabolites. *S. glaucescens* spores were isolated after growth on HT medium [24] and *S. lividans* spores were isolated after growth on R2YE agar [36]. *S. glaucescens* and *S. lividans* were grown in CRM medium [37] for preparation of protoplasts, and protoplasts were regenerated on R2YE agar. Thiostrepton concentrations of 50 μg/ml on solid media and 10 μg/ml in liquid media were used for selective growth of all *Streptomyces sp.* Recombinant *E. coli* DH5α and DH10 were grown in LB or TB [38] medium containing 100 μg of ampicillin per ml and M13mp18, M13mp19 were propagated in TG1 cells grown in 2xYT medium [38]. Thiostrepton was obtained from Sal Lucania at the Squibb Institute for Medical Research (Princeton, NJ, USA) and all chemicals used in this study were analytical grade. Silica gel (Kieselgel 60; 0.040–0.063 μm) and TLC plates (silica gel 60 F_{254}) were obtained from E. Merck (Darmstadt, Germany). Restriction enzymes, DNA ligase and other molecular biology materials were purchased from standard commercial sources.

PCR cloning of the jadD cyclase and jadE ketoreductase genes

The *jadD* gene was amplified using plasmid pJV69A [18], linearized with *Eco*RI, as template and the following oligonucleotides, synthesized by the University of Wisconsin Biotechnology Center, as PCR primers: *JadDf:* 5′ GCG GCG GAA TTC GAG CTC GAG GAG TTT CAC GAT GAC GAC CCG TGA GG 3′ and *JadDr:* 5′ CCG CGC ATG CAT TCA TCA GCG CTT GCC CTC GGC GTA GGC 3′. For the amplification of the *jadE* gene, a subclone of pJV69A was precut with *Eco*RI and used as template together with the PCR primers *JadEf:* 5′ ACG TCA TCT AGA GAA GAG ACG ACA CCG CAT GTC CCA GC 3′ and *JadEr:* 5′ ACG TCA AAG CTT TCA GAA GTT GCC CAG GCC GCC GC 3′ in the PCR assay. The following conditions were used to perform the PCR reactions: 100 μl of reaction mix contained 100 ng of precut template DNA, 0.5 μg of each of the custom made PCR primers, 2.5 mM $MgCl_2$, 100 μg BSA, 0.06 mM dCTP+dGTP, 0.04 mM dATP+dTTP and 10 U of *Taq*- polymerase (Fisher Scientific, Pittsburgh, PA, USA) in 1x assay buffer. The reaction conditions were chosen as follows: an initial denaturation for 5 min at 100°C was followed by 3 min at 71°C during which time the dNTPs and 5 U Taq-polymerase were added. Then 24 cycles of 1.5 min denaturation at 97°C and 3 min extension at 70°C during which an additional 5 U of *Taq*-polymerase was added. The program was concluded by a final denaturation and a 10 min extension cycle after which the reaction mix was cooled down to 4°C. The PCR mix was purified from contaminating oil and free dNTPs using an Ultrafree®-MC filter unit (Millipore Corporation, Bedford, MA, USA) following the manufacturer's instructions. The amplified *jadD* and *jadE* genes were used for further cloning of expression cassettes as *Eco*RI–*Nsi*I and *Xba*I–*Hin*dIII fragments, respectively.

DNA sequence analysis

The amplified *jadD* and *jadE* genes were sequenced by the chain termination method with Sequenase 2.0 (United States Biochemical, Cleveland, OH, USA) using [α-^{35}S]dCTP following the manufacturer's instructions and 7-deaza-dGTP to reduce the number of sequencing artifacts. The sequencing samples were run on 60 cm wedged 8 M urea-10% formamide-6% polyacrylamide gels. DNA and protein sequence analysis was done with the Genetics Computer Group software package version 8.1 [39].

While sequence analysis did confirm the sequence of the amplified *jadE* gene to be identical with the published sequence data [18], several differences were detected for the *jadD* PCR product. Sequence analysis of the template DNA used (pJV69A) and subsequently of a piece of genomic DNA isolated from *S. venezuelae* ISP5230 [18], using the *jadD* PCR product as hybridization probe, revealed the following errors in the 4864 bp *jadD* sequence published in [18]: position 4119: 5′ GGGCG-GCA 3′ instead of 5′ GGCGA 3′; position 4155: 5′ GTCCCG 3′ instead

of 5′GTCCG3′; position 4166: 5-CCTG3′ instead of 5′CCT*T*G3′ and position 4216: 5′TGG*A*TC3′ instead of 5′TGGGTC3′.

Metabolite production and purification

DNA fragments containing the hybrid PKS expression cassettes under control of the *ermE** promoter were cloned into pWHM3 to give the plasmids described in Table 1 and were introduced by transformation into protoplasts of *S. lividans* and *S. glaucescens* strains. Thiostrepton resistant transformants were selected by overlaying the plates with thiostrepton to give a final concentration of 20 μg/ml. Transformants were initially grown in 5 ml R2YENG medium plus thiostrepton for 4 days and subsequently transferred to 20–50 ml cultures and grown for another 40 h. A portion of each culture was extracted with ethyl acetate and the extract was analyzed by TLC on silica gel (E. Merck, Darmstadt, Germany) in chloroform:methanol:acetic acid (40:10:1.25) to detect the production of secondary metabolites by illuminating the solvent-damp plates with UV light (310 nm). For large scale production of metabolites 3 l of R2YENG (50 ml portions in 250 ml baffled Erlmeyer flasks) were inoculated with 5 x 5 mm slices of a well sporulated culture on HT or R2YE agar (+ thiostrepton). These were incubated at 30°C with shaking (350 rev/min) for 3 days. The culture broth was then mixed with 14 g/l KH_2PO_4 and the mycelia were removed by filtration. The filtrate was divided into 4 portions and each portion was then extracted 5 times with a total of 500 ml ethyl acetate. The mycelia was extracted 5 times with a total of 250 ml acetone. The combined organic layers were evaporated to dryness *in vacuo*, redissolved in 5 ml of chloroform:methanol (85:15) and subjected to chromatography on a silica gel column (35 x 3 cm) using the same solvent mixture for separation. Fractions containing SEK43 and UWM4 (analyzed by TLC: R_f=0.72 and 0.74 in chloroform:methanol:acetic acid [80:20:0.25]) were evaporated as before and further purified on a silica gel column (15 x 2 cm, chloroform:methanol [9:1]) to give 500 mg SEK43 and 30 mg UWM4 in a ratio of 17:1. RM20b, RM20c, SEK4, SEK4b, mutactin, dehydromutaction, and SEK34 were identified by comparison of their chromatographic and spectral properties with known standards.

Derivatization of UWM4 and SEK43

In a 10 ml round flask, 2.9 mg UWM4 (0.008 mmol) was dissolved in 5 ml acetone, 1.5 g K_2CO_3 and 1 ml methyl iodide. After 10 h the reaction was quenched by adding 5 ml of water, stirring for 3 h until homogeneous and extracted 3 times with 10 ml of ethyl acetate. The combined organic layers were washed with 5 ml of saturated aq. $NaHCO_3$ and 5 ml water and the organic fraction was dried *in vacuo*. The remaining residue was dissolved in a mixture of cyclohexane:ethyl acetate (6:4) and chromatographed in the same solvent system on 10 g of silica gel to give 3.6 mg of crude product (R_f=0.28). This product was purified by high performance liquid chromatography (HPLC) using a solvent system of acetonitrile:water (1:1) on a Waters RCM 25 x 10 mm RP-C_{18} preparative column to yield 2.0 mg (0.005 mmol, 63%) of 3,11,15,17-tetra-O-methyl-UWM4. The same procedure was done using 27.0 mg (0.08 mmol) SEK43 with doubled amounts of reagents and solvents to give 16.5 mg (0.04 mmol, 50%) 3,11,15,17-tetra-O-methyl-SEK43 (R_f=0.20 in cyclohexane:ethyl acetate [6:4]). Crystals of the latter product were obtained by dissolving 16.5 mg in 1 ml ethyl acetate and keeping the mixture uncovered in a 10 ml flask at room temperature to evaporate the solvent slowly.

MS, NMR and X-ray analysis

Electron ionization (EI)-MS was carried out on a Kratos MS-80RFA spectrometer and FAB-MS on a Finnigan MAT 8230 (matrix:*o*-nitrobenzylalcohol). The X-ray analysis was done using a Siemens Analytical X-ray Instrument and NMR spectra were recorded on a Bruker AM3000 spectrometer with exception of the HMBC and HMQC spectra, which were recorded on a Bruker DMX500. For recording NMR spectra, the compounds were dissolved in CD_3OD (Aldrich, 99.5 atom % D), acetone-d_6 (Aldrich, 99.5 atom % D), CD_2Cl_2 (Merck Sharp & Dohme, 99 atom % D) or, for direct comparison with the literature, DMSO-d_6 (Aldrich, 100.0% D).

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