Production of Aromatic Minimal Polyketides by the Daunorubicin Polyketide Synthase Genes Reveals the Incompatibility of the Heterologous DpsY and JadI Cyclases

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Our investigations into whether the biosynthesis of a linearly fused ring system of an aromatic polyketide (jadomycin) could be modified to produce an angularly fused system (daunorubicin) and vice versa showed that introduction of the respective cyclases did not have the desired effect. Genes from the daunorubicin pathway produced a novel 21-carbon polyketide.

Polyketides represent a large and structurally diverse group of secondary metabolites that are often of high pharmaceutical interest.[1] The name polyketide defines a class of molecules that are built by repeated decarboxylative condensation of fatty acids by the polyketide synthase enzymes, in a manner similar to the biosynthesis of long chain fatty acids catalyzed by fatty acid synthases.[2] On the basis of the composition of these enzymes, bacterial polyketides, as well as their respective synthases, are divided into Type I and Type II classes.[3,4] Type I polyketide synthases, which mainly produce aliphatic compounds as typified by macrolide antibiotics such as erythromycin, consist of large, modular, multifunctional enzymes with different active sites for each catalytic step. This results in the modification of the polyketide chain by reduction and dehydration as it is still being extended. Type II polyketide synthases, which largely have a separate polypeptide for each activity, are thought to generate the entire carbon-chain backbone before modifying it by oxidation, aromatization, etc. They are often called iterative or aromatic polyketide synthases because their main products are usually multicyclic aromatic compounds. Fungi host a class of iterative Type I polyketide synthases that can make aromatic and aliphatic products.[5]

Jadomyin B (**5**) is a Type II decapolyketide produced by *Streptomyces venezuelae* and belongs to the group of angucyclines or benz[*a*]anthracenes having the characteristic angular orientation of its fourth ring.[6−8] The jadomycin biosynthetic genes (*jad*) have been studied during the past few years,[7,9−12] and it has been shown that in order to produce the first isolable pathway intermediate, UWM6 (**3**), six genes in the cluster are required: the minimal polyketide synthase (PKS) genes (*jadABC*), a cyclase (*jadD*), a C-9 ketoreductase (*jadE*), and a second cyclase (*jadI*).[7] The latter gene is believed to be involved in the closure of the third and fourth ring for production of UWM6 (**3**) and the intermediate rabelomycin (**4**), as shown in Figure 1. Earlier experimental results implied that JadI directs formation of the angucyclic characteristic of the jadomycin structure.[7] SEK43 (**1**) and UWM4 (**2**) are the lone products observed when only the minimal jadomycin PKS genes *jadD* and *jadE* are expressed.[11]

In the present study we wanted to investigate if the biosynthesis of a linear polyketide could be altered to generate an angucycline and vice versa. The daunorubicin (*dps*) pathway seemed to be well suited as a model system. As in the case of the jadomycins, the first cyclization in this pathway takes place between C-7 and C-12 of the putative decaketide intermediate (Figure 2). Daunorubicin (**9**) and its derivative doxorubicin (**10**) are the final products of this pathway in *Streptomyces peucetius*, and the biosynthetic genes have been intensively studied.[13−22] The *dpsA* and *dpsB* genes encode the ketosynthase subunits, *dpsG* encodes the acyl carrier protein, *dpsE* encodes the ketoreductase responsible for the reduction of the C-9 keto group,[13] and *dpsF* and *dpsY* encode cyclases that are believed to be responsible for the first as well as the second and third ring closures, respectively (Figure 2).[11,16,17,20] The *dpsC* gene encodes a starter unit-specifying enzyme that uses propionyl-coenzyme A instead of acetyl-coenzyme A, resulting in a 21-carbon backbone for daunorubicin (**9**) and doxorubicin (**10**). The absence of *dpsD* seems to have no effect on the biosynthesis of daunorubicin (**9**).[20,22] The expression of *dpsABCDEFG* in combination with the second cyclase *dpsY* and the C-12 oxygenase *dnrG* results in the production of aklanonic acid (**8**).[13]

With this information as a guide, we set out to determine if substitution of *dpsY* by *jadI* or vice versa in cassettes carrying the *dps* or *jad* PKS genes would interchange linearly and angularly fused aromatic ring systems. Also, expression of the *dpsABCDG* genes in the course of this work was anticipated to produce a new polyketide having a predictable structure. Since our previous work had shown that SEK43 (**1**) and UWM5 (**7**) are produced when the *dpsABEFG* or *dpsABCDEFG* genes are expressed in a heterologous host,[20] we anticipated that the principal product made by the strain carrying the *dpsABCDG* genes would be a C-9 hydroxyl analogue of UWM5 (**7**). This prediction was validated by the isolation of UWM7 (**6**) from the *S. lividans* pWHM1010 strain, a novel polyketide that results from the spontaneous cyclization of the unreduced decaketide intemediate (Figure 2). The structure of UWM7 (**6**) was easily determined by 1D and 2D NMR and in comparison with the NMR data of UWM5 (**7**).[19]

For the second part of our investigation we constructed a number of plasmids with different combinations of the *dps* and *jad* PKS genes (Table 1) and transformed these into *S. lividans*. The metabolic production profiles of the resulting strains were compared to known standards and

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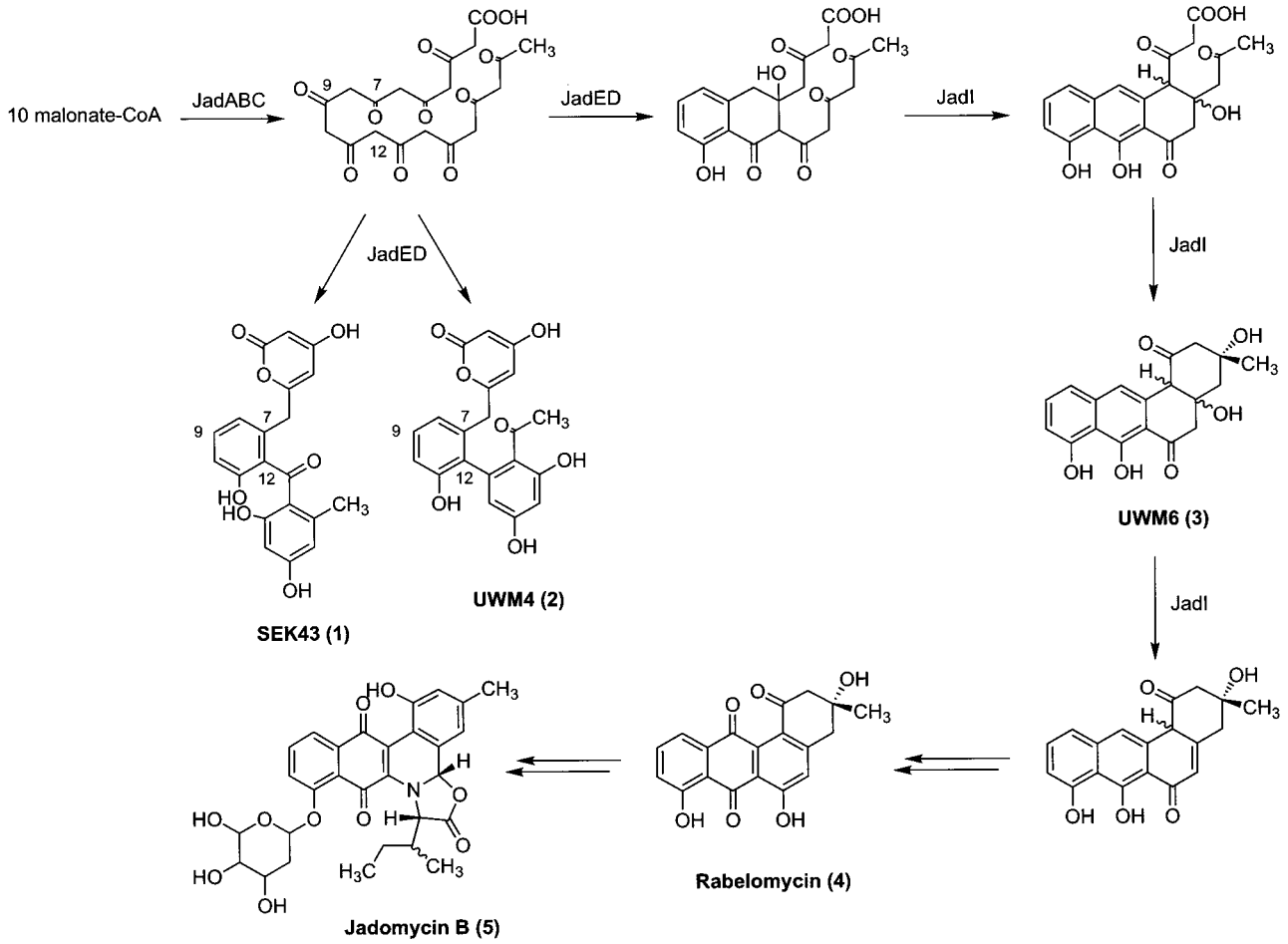

Figure 1. Polyketides produced from the jadomycin biosynthetic gene cluster.

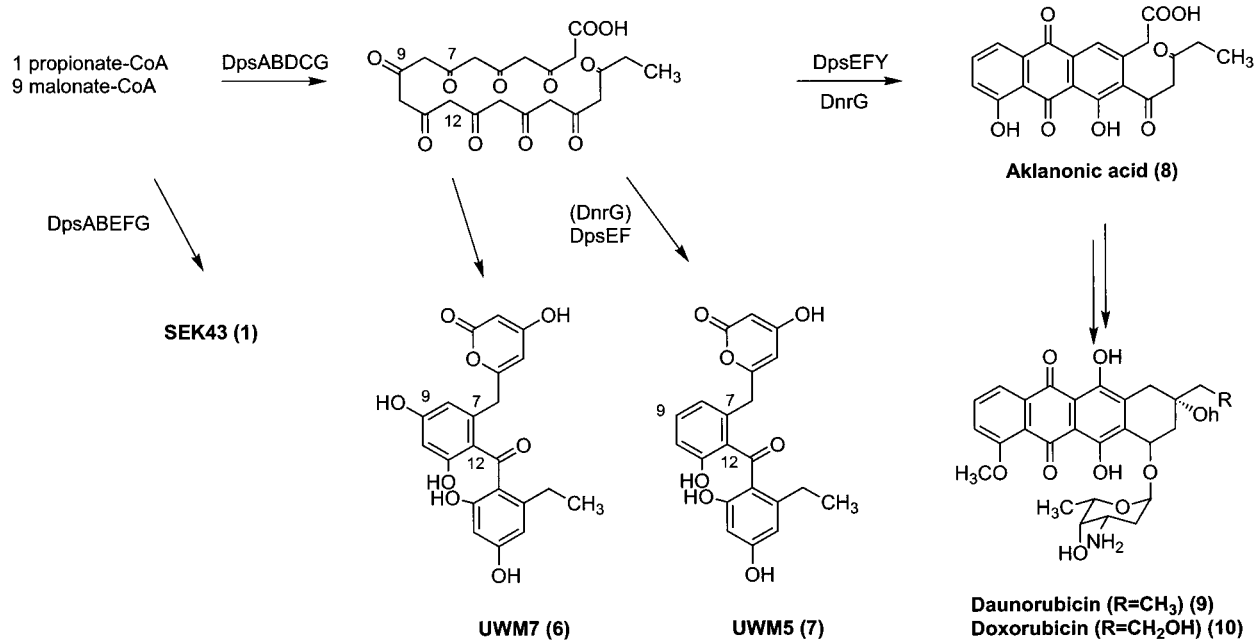

Figure 2. Metabolites produced by the daunorubicin (**9**) and doxorubicin (**10**) biosynthetic genes leading to aromatic polyketides with 20- and 21-carbon backbones.

analyzed for new compounds using HPLC and TLC, respectively. Those strains containing plasmids chosen to verify the activity of genes as well as production of known metabolites gave the expected results (pWHM3,[23] pWHM80,[14] pWHM1010,[20] pWHM1011,[20] pWHM1012,[20] pWHM1221,[11] pWHM1237;[7] Table 1 and Experimental Section), including production of UWM7 (**6**). By replacing cyclase *dpsY* as in pWHM1012 with *jadI* (pWHM1018), we intended to produce a 12-deoxyaklanonic acid-derived angucycline. Substitution of *jadI* as in pWHM1237 with *dpsY* (pWHM1019) was done in an attempt to create a "linear jadomycin" analogue. However, the metabolites

Table 1. Plasmid Constructs Used and Metabolites Made

plasmid	genes	metabolite[a]
pWHM3		
pWHM80	dnrG dpsABEFG	**1, 2**[c]
pWHM1010[b]	tcmJ dpsABCDG	**6**
pWHM1011[b]	tcmJ dpsABCDGEF	**1**, **7**, (**9**)
pWHM1012[b]	tcmJ dpsABCDGEFY	**1**, **7**, **9**
pWHM1018[b]	tcmJ dpsABCDGEF jadI	**1**, **7**
pWHM1019	jadABCDE dpsY	**1, 2**[c]
pWHM1221	jadABCDE	**1, 2**[c]
pWHM1237	jadABCDEI	**1**, **4**

[a] Minor metabolites are shown in parentheses. [b]The *tcmJ* gene was carried along solely for convenience and has been reported to increase production of tetracenomycins in the case of its native cluster.[13] [c]Verified only by TLC.

produced by *S. lividans* pWHM1018 and *S. lividans* pWHM1019 were SEK43 (**1**) and UWM5 (**7**) or SEK43 (**1**) and UWM4 (**2**), respectively (Table 1). No traces of new compounds were detected. The metabolic profiles therefore mirror those of strains lacking the *dpsY* or *jadI* cyclases (Table 1). Similar experiments, using the fourth ring cyclase *tcmI* of the tetracenomycin gene cluster, had also failed to produce the desired metabolites.[7] These results lead us to conclude that heterologous cyclase subunit proteins of the jadomycin and daunorubicin PKSs cannot productively interact with one another and that any acyclic, mono-, or bicyclic intermediates possibly produced (Figures 1 and 2) are not substrates for the JadI and DpsY cyclases when they are paired with the heterologous components we tested. Hence, the presence of either heterologous cyclase alone was not able to specify formation of either a linearly or angularly fused aromatic ring system.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in methanol-d_4 on a Bruker AMX 500 NMR instrument (NMRFAM, University of Wisconsin) at 499.89 MHz for ^{1}H and 125.69 MHz for ^{13}C, using 1D spectra and 2D homo- and heteronuclear correlation experiments (^{1}H, ^{13}C, DEPT, H,H-COSY, HMQC, HMBC). Electrospray ionization mass spectrometry (ESIMS; University of Wisconsin Biotechnology Center) was used to determine the mass of purified compounds. HPLC was performed on a Waters HPLC system with two pumps (models 510 and 515), pump control module, and photodiode array detector (model 996), using a Nova-Pak C_{18} 60A 4 μm (150 × 3.9 mm) column and a Sentry Guard Columns Nova-Pak C_{18} 60A 4 mm (20 × 3.9 mm).

Bacterial Strains and Plasmids. *Streptomyces lividans* 1326[23] and *E. coli* DH5a[24] are described elsewhere, as are plasmids pWHM3,[23] pWHM80,[14] pWHM1010,[20] pWHM1011,[20] pWHM1012,[20] pWHM1221,[11] and pWHM1237.[7] Plasmid pIJ4070, containing the *ermEp** promoter, was obtained from Mervyn Bibb, John Innes Centre, Norwich, UK. pANT841 is pUC19 derivative with additional cloning sites (C. DeSanti, Ohio State University). pGEM-3Zf(−), pGEM-5Zf(+), and pGEM-7Zf(−) were purchased from Promega (Madison, WI). Plasmid pWHM1018 was constructed by first subcloning a 1.85 kb *Xba*I/*Pst*I fragment containing *dpsEF* from pWHM1015[20] into Litmus 28 (New England Biolabs, Beverly, MA). For earlier experiments a 0.42 kb *Eco*NI/*Bam*HI fragment containing *jadI* was excised from pJV58 and, along with a 0.3 kb *Eco*RI/*Hinc*II fragment containing *ermE** from pIJ4070, ligated into the *Eco*RI and *Bam*HI sites of pGEM-3Zf(−).[7] The *jadI* gene was cut out of this plasmid with *Xba*I and *Bam*HI, and the resulting fragment was inserted into pGEM-7Zf(−). From there, *jadI* was moved as a 0.42 kb *Xba*I/*Nsi*I fragment into the *Pst*I/*Spe*I sites of pANT841. Subsequently, an *Xba*I/*Xho*I fragment containing *dpsEF* from the above-described Litmus 28 plasmid was inserted into the *Xho*I/*Xba*I sites of the altered pANT841 plasmid. The *dpsEF* and *jadI* genes were then moved as an *Xba*I/*Hin*dIII fragment to replace *dpsEF* in pWHM1011, creating pWHM1018. Plasmid pWHM1019 was constructed by moving a 0.97 kb *Xho*I/*Eco*RV fragment containing *dpsY* from pWHM346[17] into pGEM-5Zf(+). An *Nsi*I/*Sph*I fragment was then removed from the resulting plasmid and combined with a 4.7 kb *Xmn*I/*Nsi*I fragment containing *jadABCDE* from pWHM1221 in a three-way ligation into the *Bam*HI (blunt end; treated with Klenow fragment, GIBCO BRL, Gaithersburg, MD) and *Sph*I sites of pWHM1250.[25]

Cultivation and Fermentation. *S. lividans* was grown in liquid R2YENG medium (pH 6)[26] for preparation of protoplasts, on R2YENG agar plates for protoplast regeneration, and in R2YENG liquid medium for production of secondary metabolites. Spores were isolated after growth on R2YE agar.[27] Transformed *S. lividans* strains were selected with thiostrepton (10 μg/mL in liquid and 50 μg/mL on solid media). Recombinant *E. coli* DH5α strains were grown in Luria-Bertani medium[24] containing ampicillin (150 μg/mL). DNA fragments containing the PKS expression cassettes under control of the *ermE** promoter were cloned into pWHM3 to give the plasmids described in Table 1. These were then introduced by transformation into protoplasts of *S. lividans*.[27] Selection of the transformants was done by overlaying the plates with thiostrepton (final concentration of 10 μg/mL). Transformants were initially grown in culture tubes containing 5 mL of R2YENG medium plus thiostrepton for 1−2 days before being transferred into 250 mL baffled flasks containing 15 mL of R2YENG with thiostrepton. These cultures were then grown for 5 days. For the final analyses, cultures of the *S. lividans* strains hosting the different constructs were grown for 48 h.

Identification of Produced Metabolites. Samples of the different fermentation broths (1 mL) were taken after 1, 3, and 5 days, acidified with 100 μL of 1 M KH_2PO_4 (pH 4.8), extracted with 0.25 mL of ethyl acetate, and concentrated under a stream of nitrogen. The extracts were analyzed by HPLC and on TLC plates (silica gel 60 F_{254}, Merck, Darmstadt, Germany; metabolites were detected under UV light of 254 or 310 nm) by comparison of their chromatographic and spectral properties with known standards. HPLC and TLC systems are described elsewhere.[7,14,19,22,28] SEK43 (**1**), UWM7 (**6**), and UMW5 (**7**) were also identified by TLC plates developed in chloroform−methanol−acetic acid (100:10:0.25). ESIMS data were obtained from analytical amounts of SEK43 (**4**), UWM7 (**6**), UWM5 (**7**), rabelomycin (**4**), and aklanonic acid (**8**), isolated out of the different extracts by HPLC and dried under nitrogen.

Isolation and Characterization of UWM7 (6). Forty flasks of *S. lividans* pWHM1010 were grown as described above for 48 h. The combined broth was acidified with 4 mL of 1 M KH_2PO_4 (pH 4.8) and extracted with 200 mL of ethyl acetate three times. The combined organic phases were evaporated to dryness under vacuum. The residue was subjected to silica gel column chromatography (Aldrich Chemicals, Milwaukee, WI; solvent system chloroform−methanol−acetic acid 9:1:0.1), and the resulting fraction was finally purified by gel filtration on a Sephadex LH-20 column (Pharmacia; 2.5 by 30 cm) by elution with methanol: ^{1}H NMR (CD_3OD, 500 MHz) δ 6.15 (1H, d, J = 2.0 Hz, H-18), 6.10 (1H, d, J = 2.0 Hz, H-16), 6.08 (2H, s, H-8, H-10), 5.40 (1H, s, H-4), 3.40 (2H, s, H_2-6), 2.35 (2H, q, J = 7.0 Hz, H_2-20), 0.95 (3H, t, J = 7.0 Hz, H_3-21); ^{13}C NMR (CD_3OD, 75.5 MHz) δ 200.3 (s, C-13), 183.6 (s, C-1), 171.5 (s, C-5), 164.0 (s, C-11), 164.0 (s, C-9), 163.9 (s, C-3), 159.5 (s, C-15), 146.2 (s, C-19), 140.3 (s, C-7), 120.7 (s, C-14), 116.7 (s, C-12), 115.6 (d, C-8), 110.0 (d, C-18), 109.7 (d, C-4), 103.9 (d, C-10), 102.1 (d, C-16), 87 (d, C-2[a]), 38.7 (t, C-6), 27.6 (t, C-20), 15.7 (q, C-21), [a]observed as HMBC coupling; ESIMS m/z 421 [M + Na]; UV (MeOH) λ_{max} 294, 340 nm.

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