Daunorubicin Type II Polyketide Synthase Enzymes DpsA and DpsB Determine Neither the Choice of Starter Unit nor the Cyclization Pattern of Aromatic Polyketides

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Recent advances in understanding the biosynthesis of aromatic polyketides have led to the concept of a minimal type II polyketide synthase complex[1a,b] that performs the condensations of a specific acyl coenzyme A (CoA) starter unit with a varying, but polyketide synthase-specific, number of malonyl-CoA extender units. The result of these condensations is presumably a linear polyketide precursor [Figure 1 (**I** and **II**)], which is further elaborated by enzyme-catalyzed cyclization, oxidation, methylation, etc. reactions. This minimal polyketide synthase is proposed to consist of a β-ketoacyl:acyl carrier protein synthase, a chain-length-determining factor,[2] and an acyl carrier protein, but may have additional acyltransferase or thioesterase activities.[3,4] These proteins and many of their genes are highly homologous among the different polyketide synthase systems studied,[5−7] and it is generally accepted that the acyl carrier protein components can be freely interchanged without affecting the product(s) of a given polyketide synthase system.[8−11] The tetracenomycin (tcm) C minimal polyketide synthase consists of the TcmK β-ketoacyl:acyl carrier protein synthase, TcmL chain-length-determining factor, and TcmM acyl carrier protein proteins, but requires the presence of at least TcmN, a bifunctional polyketide cyclase/*O*-methyltransferase,[12] to produce TCM F2 [Figure 1 (**1**)] efficiently, an early intermediate of TCM C biosynthesis in *Streptomyces glaucescens*.[13] In contrast, the minimal requirements of the daunorubicin polyketide synthase[14,15] needed to produce 12-deoxyaklanonic acid [Figure 1 (**2**)], an early intermediate of daunorubicin biosynthesis in *Streptomyces peucetius*, are as yet uncertain and might involve as many as four additional proteins besides the DpsA, DpsB, and DpsG[14] proteins that correspond to TcmK, TcmL, and TcmM, respectively.

Three questions need to be answered to understand the function of the daunorubicin polyketide synthase: (i) Do the DpsA and DpsB proteins function as β-ketoacyl:acyl carrier protein synthase and chain-length-determining factor subunits, respectively, as predicted?[14,15] (ii) Is the selection of propionyl-CoA over acetyl-CoA as primer for daunorubicin biosynthesis an intrinsic property of the minimal daunorubicin polyketide synthase, or is the choice made by an additional, daunorubicin-specific propionyl-CoA acyltransferase (DpsD[14]) and a condensing enzyme (DpsC[14]) that together specify the correct starter unit? (iii) What determines the regiospecific folding and cyclization pattern of the linear decaketide precursor **II**, i.e., how is this polyketide caused to undergo an initial aldol condensation at C-7/C-12, leading to **2**, vs an alternative cyclization at C-9/C-14 leading to **1**, for instance?

To address these questions, we investigated how the DpsA and DpsB enzymes would perform when they replaced their corresponding TcmK and TcmL homologues in the tcm polyketide synthase. We constructed three different gene cassettes (whose expression was under control of the strong constitutive *ermE** promoter[16]) consisting of the *dpsA*, *dpsB*, and *tcmM* genes and either or both of the putative *tcmJ*[17] or *tcmN*[12] polyketide cyclase genes in the plasmid vector pWHM3.[18] The cassettes were introduced into *Streptomyces lividans* 1326[19] or the *S. glaucescens* WMH1077 mutant strain in which the tcm polyketide synthase genes were not expressed.[20,21] While we were not able to detect the production of any compounds by the *dpsABtcmMN* genes (cloned as pWHM881), expression of the *tcmJdpsABtcmMN* genes (pWHM883) yielded large amounts of a TCM F2-like compound according to TLC analysis in $CHCl_3$/MeOH/HOAc (80:20:2.5). This compound was subsequently isolated as described earlier,[13] yielding ca. 2 mg of pure **1**, as determined by HPLC analysis [an 8 mm × 10 cm C_{18} reversed-phase column developed with a linear gradient from MeCN/H_2O/HOAc (80:20:0.1) to MeCN in 12 min at a flow rate of 2 mL/min, with UV detection at 280 nm]. The structure of this compound was identified by subjecting it to a bioconversion assay[13] using purified TcmI cyclase,[22,23] which converts **1** to **5** (Figure 1), and by high-resolution fast atom bombardment mass spectroscopy (M^+ = 384.0842 and $[M + H]^+$ = 385.0922), which indicated a molecular formula of $C_{20}H_{16}O_8$ (calcd 384.0845), identical to **1**. Expression of the *tcmJdpsABtcmM* genes in pWHM885 in both host strains produced significant amounts of SEK15[1b] (**3**), SEK15b[24] (**4**), and traces of **1** (Figure 1) upon TLC and HPLC analysis, when compared to authentic SEK15, SEK15b, and TCM F2 reference standards (data not shown).

These results confirm the proposed function of DpsA and DpsB[14] because of their ability to replace TcmK and TcmL in

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(1) (a) Glossary: *tcm* and *dps* designate genes encoding polyketide synthase or cyclase enzymes, Tcm or Dps designate the protein products of the corresponding genes, and TCM indicates the intermediates or products of tetracenomycin biosynthesis. For instance, the *tcmJKLMN* genes encode the TcmJ, TcmK, TcmL, TcmM, and TcmN proteins. Plasmid vectors for expression of genes are designated by pWHM and bacterial strains by WMH prefixes, respectively. (b) Fu, H.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *J. Am. Chem. Soc.* **1994**, *116*, 4166−4170.

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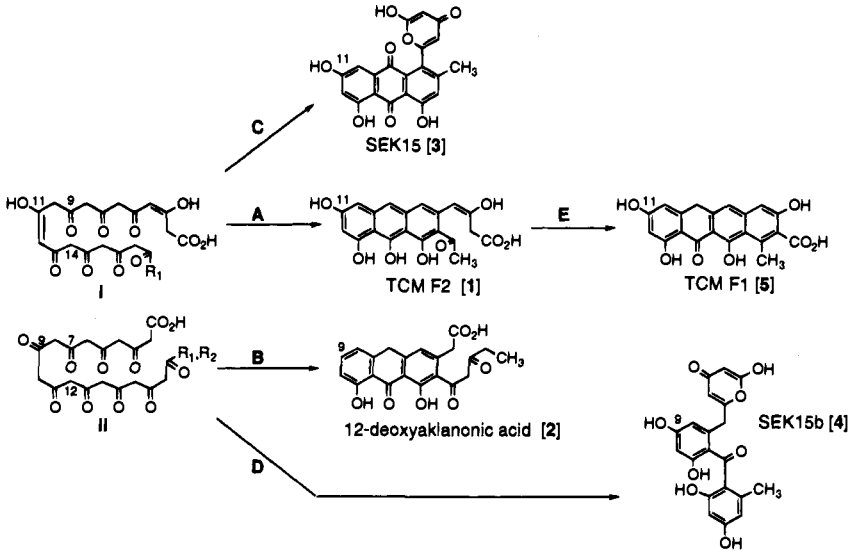

Figure 1. Structures of the naturally occurring aromatic polyketide intermediates TCM F2 (**1**), 12-deoxyaklanonic acid (**2**), and TCM F1 (**5**) and those of the previously characterized, genetically engineered polyketides SEK15 (**3**) and SEK15b (**4**), synthesized by recombinant polyketide synthases. Folding and cyclization of the linear decaketides: (A) under the influence of the TcmN cyclase to give **1** and (E) conversion with the TcmI cyclase to **5**; (B) under the influence of the DpsE ketoreductase and DpsF cyclase[14] to give **2**; and (C, D) spontaneous folding and cyclizations without directing cyclases. $R_1 = CH_3$; $R_2 = CH_2CH_3$.

a hybrid polyketide synthase complex.[25] They also demonstrate the inability of a presumed minimal daunorubicin polyketide synthase (DpsA, DpsB, and an acyl carrier protein) to enable the natural intramolecular aldol condensation at C-7/C-12 of a 21-carbon decaketide, such as that leading to **2**, in the presence of the TcmN cyclase. Instead, the 20-carbon decaketide precursor is cyclized at C-9/C-14 as in the tcm polyketide synthase. Similar results were recently obtained from expression of an actinorhodin (*act*) polyketide synthase-*tcmN* cassette.[25,26] Expression of a *tcm* polyketide synthase-*actVII* cyclase cassette yielded both C-9/C-14 and C-7/C-12 cyclized products.[1b,24-26] Moreover, it has been shown that both the minimal act and tcm polyketide synthases alone produce at least two major compounds each, featuring different polyketide folding and cyclization patterns,[1b,24-26] results that are consistent with the formation of **3** and **4** by the *tcmJdnrABtcmM* genes. Hence, we conclude that the minimal polyketide synthase is not sufficient to determine the correct folding and regiospecific cyclization pattern of the nascent polyketide chain,[27,28] and thus we believe that polyketides produced by minimal polyketide synthases may be subject to spontaneous aldol condensations according to their kinetic and/or thermodynamic properties.[29] The data presented here and elsewhere also lead us to believe that the regiospecific cyclizations of the linear polyketides are functions predominantly performed by the specific polyketide synthase cyclases like TcmN and ActVII. Furthermore, the biosynthesis of **1** by the presumed minimal daunorubicin polyketide synthase in *S. lividans* and *S. glaucescens* reveals a relaxed starter unit specificity for acetyl-CoA instead of propionyl-CoA, the primer employed normally.[14,15] Relaxed starter unit specificity was observed earlier with the oxytetracycline polyketide synthase.[30] Consequently, these findings implicate additional enzymes like DpsC and DpsD[14,15] to specify starter units other than acetyl-CoA. Although expression of the genes cloned as pWHM881 failed to produce any metabolites, we noted earlier that the addition of the *tcmJ* gene to groups of *tcm* or *act/tcm* genes boosted production of polyketide intermediates[17,25] as in the case of pWHM883 and pWHM885. This suggests that TcmJ may either stabilize the polyketide synthase complex or enhance the contact between downstream processing enzymes and the minimal polyketide synthase.

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Supplementary Material Available: Figure 2 depicting construction of the polyketide synthase gene cassettes in plasmids pWHM881, pWHM883, and pWHM885 and Figure 3 depicting HPLC chromatogram of a **1** → **5** bioconversion assay (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(27) This idea differs from the proposal of Khosla, Hopwood, and coworkers,[1b,24,28] who favor the possibility that the minimal polyketide synthase determines the regiospecificity of the initial aldol reaction that creates the first aromatic ring.

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