Effect of the Daunorubicin *dpsH* Gene on the Choice of Starter Unit and Cyclization Pattern Reveals That Type II Polyketide Synthases Can Be Unfaithful yet Intriguing

Martin Gerlitz,[†] Guido Meurer,[†,§] Evelyn Wendt-Pienkowski,[†] Krishnamurthy Madduri,[†,||] and C. Richard Hutchinson*,[†,‡]

School of Pharmacy and Department of Bacteriology University of Wisconsin, 425 North Charter Street Madison, Wisconsin 53706

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Anthracyclines, an important class of antitumor antibiotics produced by microorganisms,[1a] are most often represented by daunorubicin (DNR)[1b,c] and its C-14-hydroxylated derivative doxorubicin (DOX).[1d] Both antibiotics are made by *Streptomyces peucetius* ATCC 29050 through a pathway involving the formation of a decaketide from propionyl-Coenzyme A and malonyl-Coenzyme A as the respective chain starter and extender units (Figure 1).[2] Subsequent intramolecular aldol condensations of the nascent decaketide and C-12 oxidation form the tricyclic aromatic pigment, aklanonic acid (AA).[3] AA is converted by *O*-methylation, ring A cyclization, C-7 reduction, and C-11 hydroxylation to ϵ-rhodomycinone, which is glycosylated to rhodomycin D from which DNR and DOX ultimately result.[4]

AA biosynthesis is governed by the DNR/DOX polyketide synthase (*dps*) genes encoding the components of an iterative type II polyketide synthase (PKS).[5] Rajgarhia and Strohl[6] have recently reported that only the following seven *Streptomyces sp*. strain C5 genes[5b] (which have the same function as their *S. peucetius* homologs[5a]) are required to produce AA (the functions of the *dps* and *dnr* genes are given in Table 1, footnote b): *dpsG*, *dpsA*, *dpsB*, *dpsE*, *dpsF*, *dnrG* (syn, *dauG*), and *dnrI*. AA was also produced by a *Streptomyces* sp. strain C5 mutant that lacked the *dpsC* and *dpsD* genes previously proposed to specify propionate as the chain starter unit.[5a,b] Consequently, the PKS constituted from the products of the *dpsABEFG* genes appears to be responsible for the choice of starter unit and also the reduction, folding and cyclization of the nascent 21 carbon decaketide to form 12-deoxy-AA (Figure 1).

Studies of recombinant strains carrying PKS genes over the past few years have established the functions of individual PKS genes and enzymes[7] and resulted in the formation of many new natural products.[8a,b] A set of design rules has even been proposed for the biosynthesis of novel aromatic metabolites using type II PKS genes.[8c] We have reported that AA is produced by strains of *S. lividans* cotransformed with pWHM75 and pWHM77 or pWHM75 and pWHM79 (Table 1).[5a] In these plasmids, the *dps* genes are in their natural order and expressed from their four native promoters under control of the *dnrI* regulatory gene (Figure 1). Since type II PKS genes are transcribed in the same direction under control of one promoter in other cases,[9,10] we set out to study the effect of reorganizing the *dps* gene order as a prelude to the genetic engineering of novel anthracyclines.[8c,11] An additional aim was to test the predicted roles of the *dps* genes by examining the behavior of heterologous PKS gene sets, using *dps* homologs from the tetracenomycin (*tcm*) C biosynthesis pathway in *Streptomyces glaucescens*.[12,13] This work had several unexpected outcomes, leading to the belief that type II PKS genes can act aberrantly.

To test the effect of altering both gene order and promoter, *dnrG*, *dpsA*, *dpsB*, *dpsG*, *dpsE*, and *dpsF* were cloned in this order downstream of the *ermE** promoter[14] (Figure 2) as pWHM80 in the high-copy plasmid pWHM3.[15] *S. lividans* 1326[16](pWHM80) transformants produced similar amounts of AA and SEK43.[8c] Since SEK43 has an acetyl starter unit and,

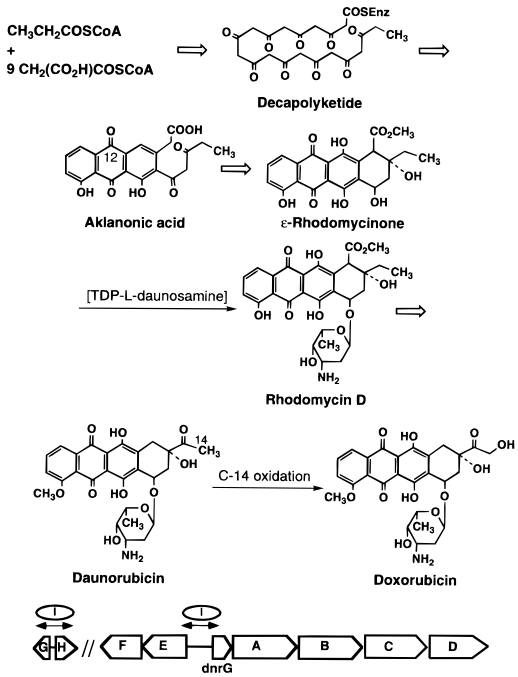

Figure 1. Biosynthesis of doxorubicin in *S. peucetius*. The DNR PKS and *dnrG* genes are shown beneath the pathway as wedges whose relative sizes are proportional to the sizes of the gene products. The genes are arranged in the order found in *S. peucetius*, with the direction of transcription indicated by the point of each wedge. The DnrI transcription factor required for expression of each *dps* and *dnr* gene or operon is indicated by an oval above the promoter regions (double headed arrows) it acts on. The "//" indicates that the *dpsG* and *dpsH* genes are located several kilobases from the *dpsF* gene.

* Author to whom correspondence should be addressed at the following: crhutchi@facstaff.wisc.edu.
† School of Pharmacy.
‡ Department of Bacteriology.
§ Present address: TerraGen Diversity, Vancouver, B.C., V6T 1Z3 Canada.
|| Present address: Henkel Corporation, Cincinnati, OH 45232.

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Table 1. Metabolite Production by Plasmids Used in This Work

construct[a]	SEK43	aklanonic acid	genes involved,[b] cloned in the order listed
pWHM75 + pWHM77[c]	trace	+	*dpsF*, *dpsE*, *dnrG*, *dpsA*, *dpsB*, *dpsC*, *dpsD* and truncated *dnrC* + *dnmJ*, *dnrI*, *doxA*, *dnrV*, *dnrU*, *dpsG*, *dpsH*, *dnmT* and *dnrH*
pWHM75 + pWHM79[c]	trace	+	*dpsF*, *dpsE*, *dnrG*, *dpsA*, *dpsB*, *dpsC*, *dpsD* and truncated *dnrC* + truncated *dnrU*, *dpsG*, tuncated *dpsH* and *dnrI*
pWHM76 + pWHM79	trace	trace	*dpsF*, *dpsE*, *dnrG*, *dpsA*, *dpsB*, *dpsC* + truncated *dnrU*, *dpsG*, truncated *dpsH* and *dnrI*
pWHM80	+	+	*dnrG*, *dpsA*, *dpsB*, *dpsG*, *dpsE* and *dpsF*
pWHM1222	+	trace	*dnrG*, *dpsA*, *dpsB*, *tcmM*, *dpsE* and *dpsF*
pWHM1210	+	−	*tcmJ*, *dpsA*, *dpsB*, *tcmM*, *dpsE* and *dpsF*
pWHM1210 + pWHM79	+	trace	*tcmJ*, *dpsA*, *dpsB*, *tcmM*, *dpsE*, *dpsF* + truncated *dnrU*, *dpsG*, truncated *dpsH* and *dnrI*
pWHM1223	trace	+	*tcmJ*, *dpsA*, *dpsB*, *tcmM*, *dpsH*, *dpsE* and *dpsF*

[a] The vector was pWHM3 unless specified otherwise. [b] Function of gene products: *dpsA* and *dpsB*, β-ketoacyl (KS_α and KS_β):acyl carrier protein (ACP) synthase; *dpsC*, putative KS; *dpsD*, propionyl-Coenzyme A:ACP acyltransferase; *dpsE*, ketoreductase (KR); *dpsF*, polyketide cyclase; *dpsG* and *tcmM*, ACPs; *dpsH* and *tcmJ*, putative polyketide cyclases; *dnrC*, aklanonic acid *O*-methyltransferase; *dnrG*, 12-deoxyaklanonic acid monooxygenase; *dnrI*, regulatory gene; *dnrH*, glycosyltransferase; *dnrV*, unknown; *dnrU*, KR; *dnmT* and *dnmJ*, daunosamine biosynthesis; *doxA*, daunorubicin C-14 hydroxylase. [c] The vector was pWHM601.

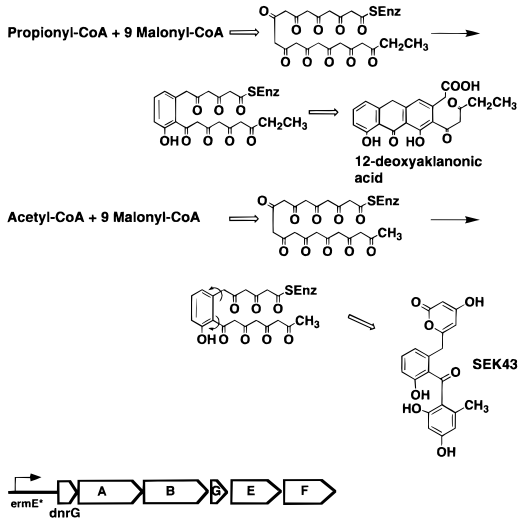

Figure 2. Hypothesis for the formation of 12-deoxy-AA and SEK43 from their respective decaketide precursors. The arrangement of the *dps* and *dnrG* genes in pWHM80 (Table 1) is shown below the pathways (see Figure 1 for an explanation of the symbols).

except for formation of the first ring, cyclizes in a different manner than that leading to AA (Figure 2), the change in gene order and promoter resulted in a *dps* PKS with a relaxed starter unit choice and cyclization behavior. (The *tcmJ*, *dpsA*, *dpsB*, *tcmM*, and *tcmN* cassette also forms a decaketide with an acetyl starter unit.[17]) When the *dpsG* gene in pWHM80 was replaced with the *tcmM* ACP gene (pWHM1222, Table 1), AA production was drastically reduced and SEK43 was detected as the major product. In addition, SEK43 became the only product when the *dnrG* monooxygenase gene in pWHM1222 was replaced with *tcmJ* (pWHM1210). Since *tcmJ* enhances the behavior of the *tcmKLMN* PKS genes,[12a,b] we assume that it is not the reason for the absence of AA. Note that oxidation of 12-deoxy-AA to AA in *S. lividans* does not require *dnrG*.[6] Therefore, the Dps PKS constituted around the TcmM ACP appears to be malfunctional, consistent with the inability of the *actI-ORF3* ACP gene to complement a *dpsG* mutation,[6] and forms a product of derailed cyclase activity. We cannot exclude a possible effect of limited substrate supply on the choice of starter unit since expression of the *eryA* PKS genes in *S. lividans* has produced macrolides from both acetate and propionate-derived starter units.[18]

The expected behavior of the Dps PKS was restored in two ways. Cotransformation of *S. lividans* with pWHM1210 and pWHM79 containing the *dpsG* gene led to AA as the minor product and SEK43 as the major one. In this case, we presume that a functionally normal *dps* PKS was formed with DpsG and an abnormal one with TcmM.

The fact that homologs of the *S. peucetius dpsH* gene adjacent to *dpsG* are present in other clusters of type II PKS genes (*actVI-orfA*,[19a] *frenX*,[19b] *hir-orf1*,[19c] and *mtmX*;[19d] a function has not been assigned to any of these genes) led us to examine a possible role of *dpsH* in AA production, although Rajgarhia and Strohl[6] reported that *dpsH* is not required. Nonetheless, in *S. lividans* the *tcmJ*, *dpsA*, *dpsB*, *tcmM*, *dpsH*, *dpsE*, and *dpsF* cassette (pWHM1223) led to the production of AA and SEK43 in an 11:1 ratio (the amount of AA was similar to that seen for pWHM75 + pWHM79 and much more than the amount obtained with pWHM1222). It is not clear whether this outcome is due to catalysis by DpsH of the second and third ring cyclizations leading to AA or to inexplicable but positive effects on DpsF function and selection of propionate as the starter unit. The negative effect of *tcmM* in pWHM1210 was also overridden by *dpsH*.

The behavior of the heterologous PKSs described above is likely to stem from two effects. Different levels of gene expression and/or mRNA stability, as a consequence of cloning the *dps* PKS genes in an unnatural order under control of a single promoter and/or inserting the heterologous *tcmM* gene in the midst of *dps* genes. This could result in an altered stoichiometry of the PKS proteins that, together with suboptimal interactions among heterologous PKS proteins, could result in the observed aberrations. Addition of DpsH somehow restored much of the normal functionality to this hybrid PKS. Regardless, our results clearly show that iterative type II PKSs can function abnormally, which will have to be taken into account in future efforts to manufacture novel natural products using such genes.

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Supporting Information Available: The experimental details for this paper (3 pages). See any current masthead page for ordering and Internet access instructions.

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