Biosynthetic Studies of Daunorubicin and Tetracenomycin C

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C. Richard Hutchinson was born in Dayton, OH, and received his undergraduate degree at The Ohio State University (1966). He conducted graduate (PH.D. in 1970) and postdoctoral research (1971) on the biosynthesis of plant products. His initial academic appointment was as an assistant professor of pharmacognosy at the University of Connecticut, Storrs (1971–74); he then moved to the University of Wisconsin, Madison, where he has been an assistant (1974–77), associate (1977–82), and full (1982-present) professor in medicinal chemistry. He was appointed Professor of Bacteriology in 1986 by a joint appointment in the department of bacteriology, UW-Madison. He has held fellowships from the NIH (graduate research, 1966–69, and career development awardee, 1976–81), NSF (postdoctoral, 1971), and J. S. Guggenheim and Fulbright Foundations (1983). He became the Edward Leete Professor of Medicinal Chemistry in 1995.

I. Introduction

The anthracycline antibiotics were discovered almost 60 years ago,[1] but their chemistry was not investigated until the 1950s and 1960s (reviews: Brockmann[2] and Brockmann and Brockmann[3]). This class of natural products is defined by the presence of a four fused-ring aromatic system, based on a 7,8,9,10-tetrahydro-5,12-naphthacenequinone framework, which usually occurs as a glycoside with a deoxy(amino)sugar and is notable because of the widely used antitumor drugs, daunorubicin[4,5] (*syn.* daunomycin, rubidomycin) and doxorubicin[6] (*syn.* adriamycin), discovered in the 1960s.[3] We entered the field in 1986 with a study of the genetic basis of the biosynthesis of tetracenomycin C,[7,8] a cytotoxic antibiotic produced by *Streptomyces glaucescens* Tü 49(ETH22794) that is notable for its broad activity against actinomycetes.[9,10] In 1989 we initiated an investigation of the genetic analysis of daunorubicin and doxorubicin biosynthesis in *Streptomyces peucetius* ATCC 29050 and 27952[11] that has been continued through the generous support of Pharmacia and Upjohn (formerly Farmitalia Carlo Erba). Our research was motivated by the belief that the antibiotic production genes could be used to facilitate eventual studies of the biosynthetic enzymes, a hope that has born considerable fruit as shown by the results of our work over the past 10 years, reviewed here. Additional information about the mechanisms of self-resistance and regulation of daunorubicin and tetracenomycin C production was uncovered through our genetic approach; highlights of these matters are also reviewed because they reveal interesting features of bacterial secondary metabolism, especially the way that the bacteria determine when and how much antibiotic to produce.

A previous review of the genetics and biochemistry of daunorubicin and tetracenomycin C biosynthesis by myself was published in 1995,[12] and a comprehensive review by W. R. Strohl and co-workers, who have also made major contributions to the knowledge of daunorubicin and doxorubicin biosynthesis, has just appeared.[13] Aspects of our work pertaining to the production of novel natural products (hybrid antibiotics) using the daunorubicin and tetracenomycin genes was published jointly with the latter article.[14]

II. Early Biosynthetic Studies

A. Daunorubicin

By the mid-1980s it had been established through isotope-labeling experiments[12,13] that the polyketide-

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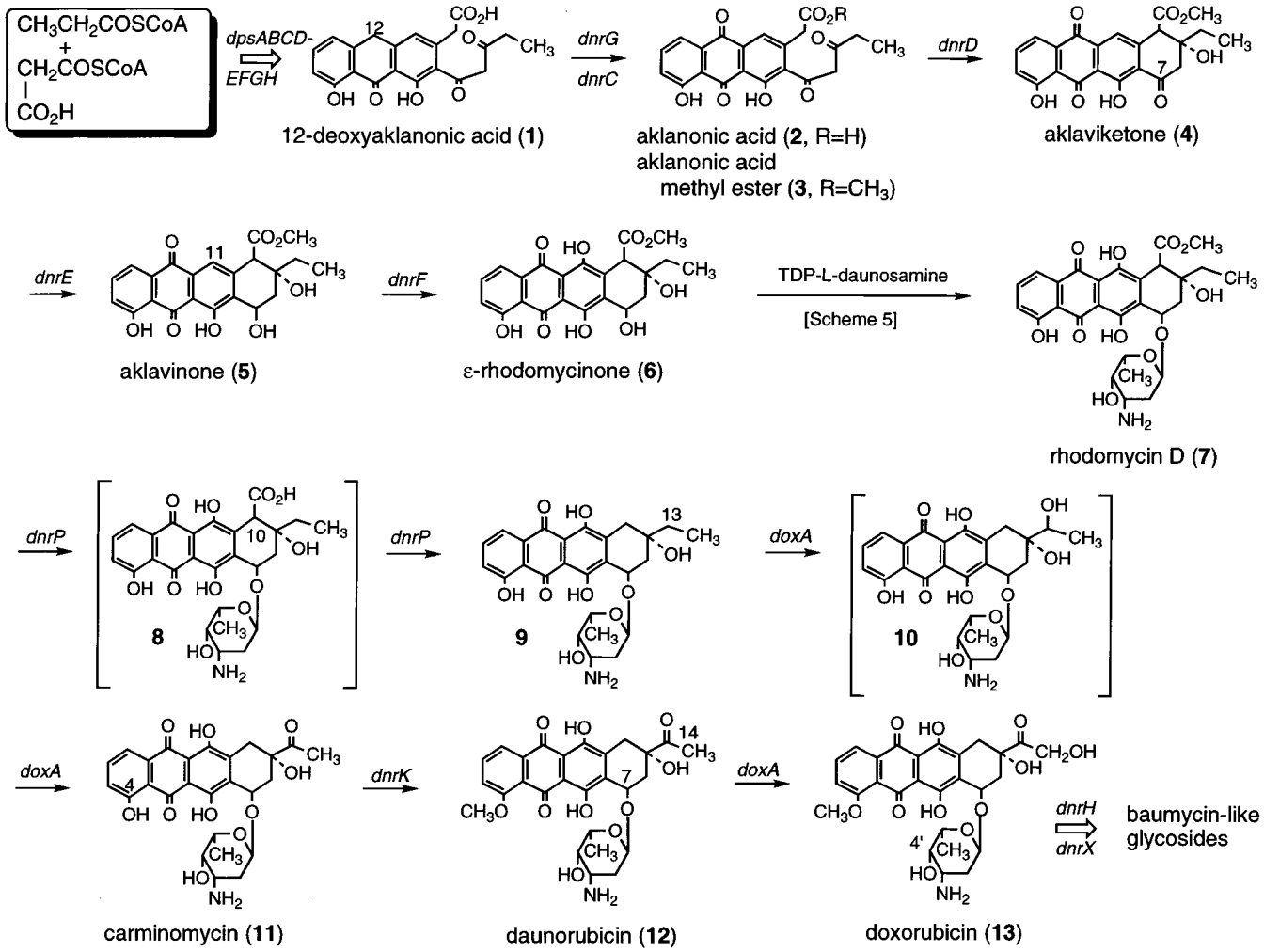
Scheme 1. Biosynthesis of Daunorubicin and Doxorubicin in *S. peucetius*[a]



[a] The compounds shown in brackets may remain bound to an enzyme and be converted to the next metabolite shown. The thick arrow in the first step represents the process of decaketide assembly and cyclization and in the last step, formation of further glycosides like the baumycins. The gene(s) for each step is indicated above the arrow.

derived framework of daunorubicin (**12**, Scheme 1) was made from propionate and malonate as the chain starter and extender units, and that daunosamine was formed from glucose, presumably via its thymidylyl diphosphate derivative. Many of the pathway intermediates shown in Scheme 1 had also been identified, largely through work with blocked mutants in industrial labs, leaving the sequence of the steps between the formation of ϵ-rhodomycinone (**6**) and carminomycin (**11**) to be elucidated. Daunorubicin was shown to be hydroxylated to doxorubicin in *S. peucetius* and both metabolites were found to undergo facile reduction of the C-13 carbonyl or loss of daunosamine, or both events, upon bioconversion in other actinomycetes.[12,13]

B. Tetracenomycin C

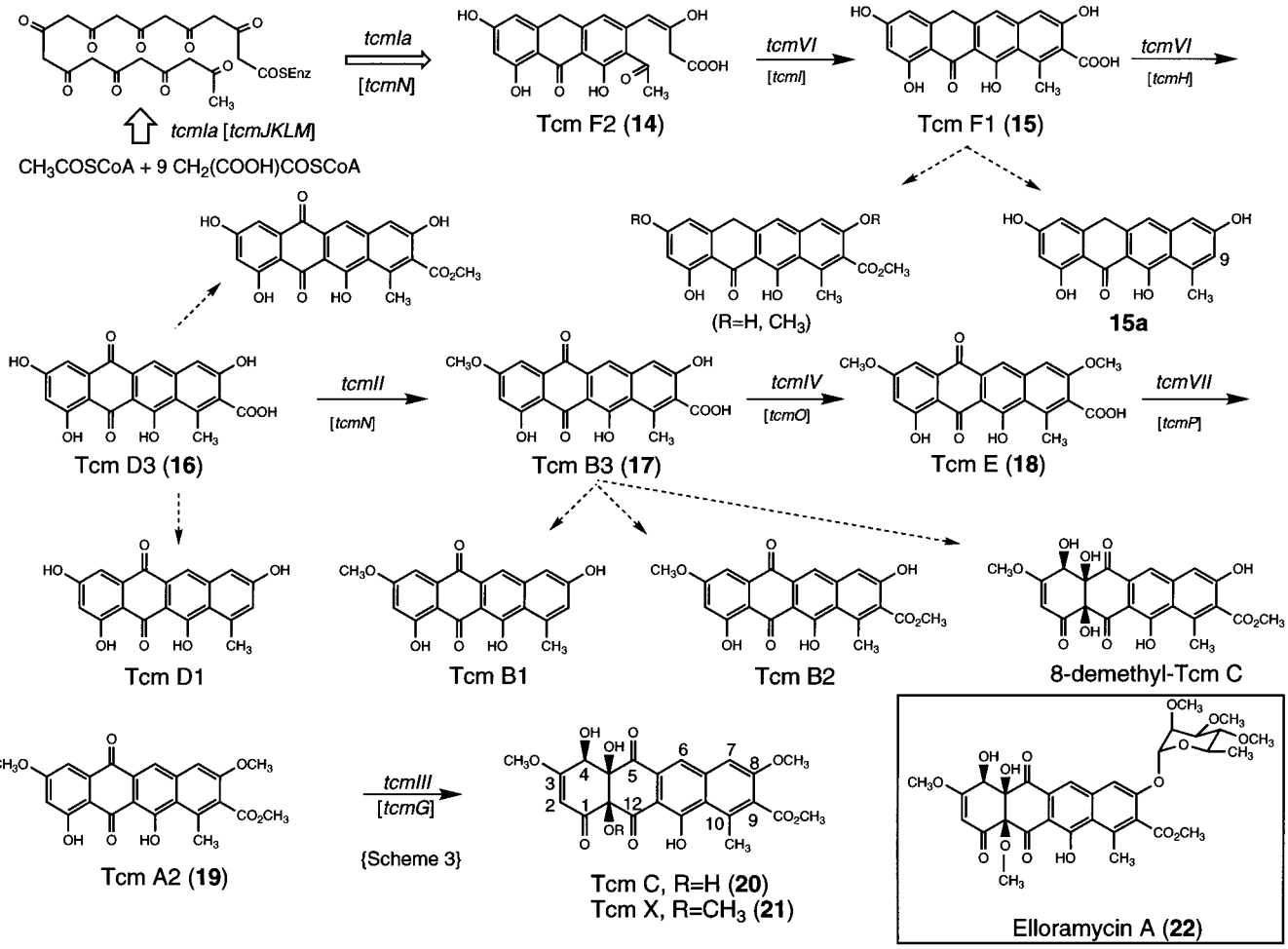
The biosynthesis of tetracenomycin C (**20**; Scheme 2) was investigated to a limited extent by Hans Zähner and co-workers at the time of its discovery, using [^{13}C]acetate-labeling experiments along with the isolation of several putative biosynthetic intermediates from *S. glaucescens*.[9,10,15] Subsequent work by this group with *Streptomyces olivaceus* Tü 2353, which produces the elloramycins A (**22**) to F,[16] provided further details of tetracenomycin C biosynthesis[17] and led to the pathway illustrated in Scheme 2. (The absolute configuration of tetracenomycin C was established by the same laboratory.[18]) Anderson *et al.*[19] reported that in the biosynthesis of tetracenomycin X (**21**) by *Nocardia mediterranea*, the three oxygen atoms at C-4, C-5, and C-12a of tetracenomycin X (and thus tetracenomycin C) are derived from molecular oxygen but none from the same molecule of O_2. The origin of the C-4a oxygen was later shown to come from H_2O,[20] perhaps by hydrolysis of an enzyme-bound intermediate (Scheme 3) that was invoked to explain the creation of the *cis* stereochemistry between the C-4a and C-12a hydroxyls.

III. Biosynthetic Studies with Blocked Mutants

A. Daunorubicin

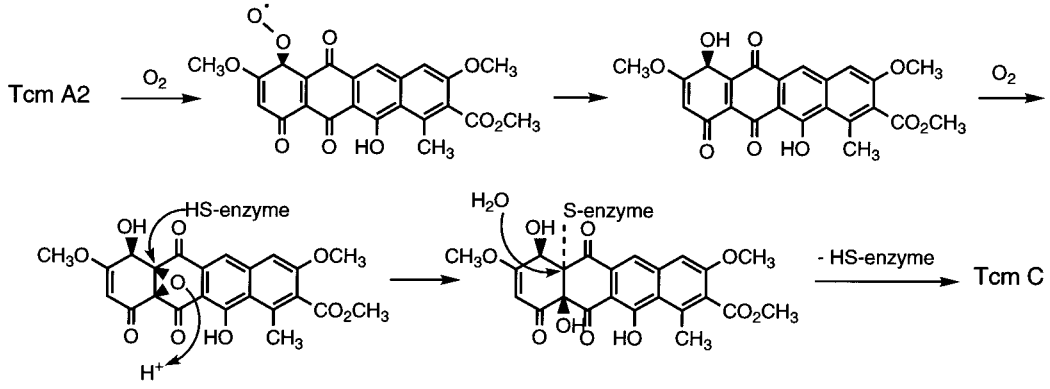
An interest in providing high daunorubicin-producing strains of *S. peucetius* and other streptomycetes that make this commercially important drug led several laboratories to pursue mutational studies of these bacteria. Grien[21] has summarized the results of work done in Italy on *S. peucetius*, and an overview of the work carried out in Germany and Japan can be found in the reviews by the author[12] and by Strohl *et al.*[13] Blocked mutants were isolated that ac-

Scheme 2. Biosynthesis of Tetracenomycin C in *S. glaucescens*[a]



[a] The class of tetracenomycin C⁻ mutation and *tcm* gene for each step are indicated above and below the arrow, respectively. Shunt products are indicated by dashed arrows. Elloramycin A is made by *S. olivaceus*.

Scheme 3. Hypothesis for the Oxidation of Tetracenomycin A2 to Tetracenomycin C by the TcmG Monooxygenase



cumulated aklaviketone (**4**),[22] aklavinone (**5**),[21] ϵ-rhodomycinone,[21,23,24] or rhodomycin D (**7**) and its 10-demethyl (**8**) and 10-decarbomethoxy (**9**;13-deoxycarminomycin [feudomycin A]) derivatives.[25−27] The conversion of daunorubicin to doxorubicin (**13**) and 13(*R*)-dihydrodaunorubicin in *S. peucetius* mutants was reported by two groups.[28−30] Bartel *et al.*[31] isolated daunorubicin nonproducing mutants of *Streptomyces* sp. strain C5[23] and classified them into nine phenotypically distinct groups on the basis of their cosynthesis behavior and the accumulation of aklanonic acid (**2**),[32] aklanonic acid methyl ester (**3**), aklaviketone (or its C-11 hydroxylation product, maggiemycin[33]), aklavinone, or ϵ-rhodomycinone. *Streptomyces griseus* IMET JA3933 mutants unable to produce the leukaemomycins that are closely related to daunorubicin were isolated by Wagner *et al.*[34] Several of the mutants were later used to identify functions of daunorubicin biosynthesis genes cloned from *S. peucetius*, *Streptomyces* sp. strain C5, and *S. griseus*. Finally, the isolation of *Streptomyces coeruleorubidus* mutants that produce feudomycins made from an acetate or butyrate starter unit instead of propionate[35] (or isobutyrate instead of propionate

in the case of the aclacinomycins produced by *Streptomyces galilaeus*,[36] which are anthracycline antibiotics made from aklavinone) suggests that the choice of starter unit is genetically determined and not just a function of precursor supply.

B. Tetracenomycin C

As a preface to cloning the biosynthetic genes, we isolated a set of *S. glaucescens* mutants blocked in tetracenomycin C production and characterized their metabolite accumulation profile and cosynthesis behavior.[7,8] The steps blocked in each of six mutant classes are indicated in Scheme 2 along with the structures of pathway intermediates and shunt products accumulated, including the ones found in the wild-type strain.[9,10,16] Tetracenomycin D3 (**16**) and tetracenomycin B3 (**17**) were also found in a mutant of *S. olivaceus* because they are intermediates of elloramycin as well as tetracenomycin C biosynthesis.[17] Most of the tetracenomycin C$^-$ mutants isolated were in the *tcmIa* class and did not accumulate any detectable metabolites but could bioconvert the mainstream intermediates accumulated by all other classes to tetracenomycin C. This property was later found to be the consequence of mutations in the type II polyketide synthase (PKS) genes that encompass almost half of the DNA commited to tetracenomycin C biosynthesis. PKSs use enzyme-bound intermediates, as do fatty acid synthases (FASs), which do not diffuse away from the enzyme and become excreted from the cells. Some mutant classes were affected in more than one step (*tcmVI*) or behaved as if nearly all of the steps had been blocked (*tcmIc*). Three of the intermediates, tetracenomycin F1 (**15**), **16**, and **17**, were converted to shunt products reflecting either loss of the C-9 carboxyl or the lack of certain *O*-methylations or C-5 oxidation (Scheme 2), as a consequence of the loose substrate specifity of secondary metabolism enzymes. Among the accumulated intermediates and shunt products, only 8-demethyltetracenomycin C and tetracenomycin C have antibiotic activity;[7] however, the molecular mechanism of their activity is unknown, despite a preliminary indication that tetracenomycin C binds to calf thymus DNA and inhibits the incorporation of [^{14}C]-uracil into acid-precipitable material in *Bacillus subtilis* but does not affect protein synthesis significantly.[10]

IV. Gene Cloning and Analysis

A. Doxorubicin Production Genes

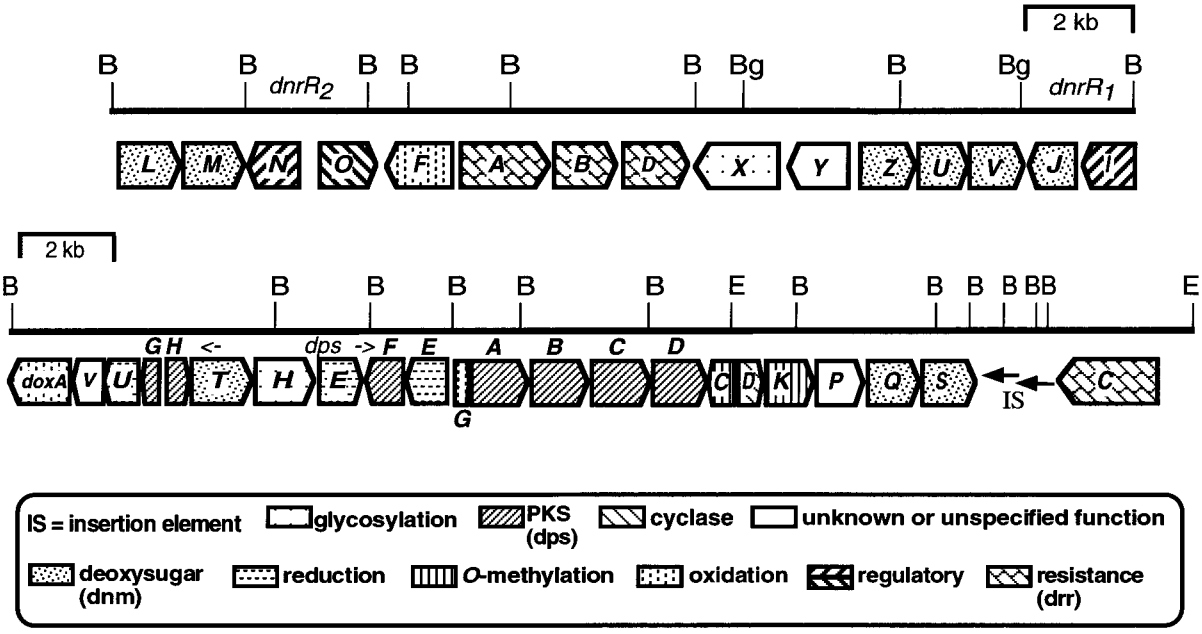
1. Structural Genes

By 1987, it seemed on the basis of DNA hybridization studies[37] that the PKS genes responsible for the biosynthesis of aromatic polyketides from bacteria were likely to be homologs of the *actI-ORF1* and *actI-ORF2* or *tcmK* and *tcmL* genes for the biosynthesis of actinorhodin and tetracenomycin, respectively (the *tcmK* and *-L* genes are discussed in section IV.B). Consequently, we sought the daunorubicin production genes by testing *S. peucetius* ATCC 29050[4] and 27952[6] DNA for hybridization to the *tcmK* and *tcmL* or *actI-ORF1* and *-ORF2* genes because they were the first type II PKS genes to be extensively studied, and because the aromatic framework of ϵ-rhodomycinone was known to be made from polyketide-derived precursors. Four hybridizing regions were identified[11] but only one was found to produce ϵ-rhodomycinone and its precursors and confer resistance to daunorubicin when genes cloned from it were introduced into *Streptomyces lividans*, which normally does not produce such metabolites and is daunorubicin sensitive.[11,38] The role of the three other hybridizing regions in *S. peucetius* is still unknown, although there were strong indications that they contain genes that can act on intermediates of daunorubicin biosynthesis, confer resistance to daunorubicin, or influence the amount of daunorubicin produced.[11] The doxorubicin structural, i.e., biosynthetic, genes were characterized by a series of subcloning, sequencing, expression, and mutational experiments over a seven year period to obtain the physical and functional map of the cluster of daunorubicin and doxorubicin production genes shown in Scheme 4. Although *Streptomyces* sp. strain C5 contains a cluster of daunorubicin production genes that is organized in the same way as the one in *S. peucetius*, there are enough differences in the DNA and deduced protein sequences to prove that the two species are not identical.[11,13]

Formation of the polyketide-derived portion of daunorubicin (**12**, Scheme 1) is governed by the type II daunorubicin/doxorubicin polyketide synthase (dps) genes specified in Scheme 1 and 4. The *dpsA*, *dpsB*, and *dpsG* genes are homologs of the *S. glaucescens tcmK*, *tcmL*, and *tcmM* genes, respectively, (discussed in section IV.B) and are the components of the PKS that create the (uncyclized) poly-β-carbonyl intermediate: *dpsA* and *dpsB* provide the subunits of the β-ketoacyl:acyl carrier protein (ACP) synthase or ketosynthase (KS) enzymes and *dpsG* encodes the ACP.[39−41] The latter gene has an unusual location since the ACP genes normally are adjacent to the KS genes in type II PKS systems. Once the linear decaketide has been assembled from propionyl-CoA and malonyl-CoA by the DpsA, DpsB, and DpsG enzymes, it is reduced at C-9 by the DpsE ketoreductase (KR) and cyclized by DpsF to **1**.[39,40,42,43] Although the *dpsF* cyclase (CYC) gene is sufficient for the biosynthesis of **1** in *Streptomyces* sp. C5,[42] *S. coelicolor*,[42] and *S. lividans*,[42,43] inclusion of *dpsH* along with *dpsF* in heterologous sets of *dps* PKS genes ensures that the proper tricyclic fused-ring system is formed.[43] Hence, *dpsH* and *dpsF* may both be CYC genes like *tcmN* and its homologs discussed below. DpsC, a KS homolog that lacks the expected active-site cysteine,[39,40] and DpsD, a presumed malonyl-CoA:ACP acyltransferase,[39,40] may ensure that propionyl-CoA instead of acetyl-CoA is used by the DpsA, DpsB, and DpsG enzymes. Although 12-deoxyaklanonic acid (**1**) is made with a propionate starter in the absence of the *dpsC* and *dpsD* genes,[13,42,43] the acetate-derived compound is also formed to some extent, suggesting that the starter unit specificity is due to the concerted activity of the DpsA, DpsB, DpsC, and DpsD enzymes.

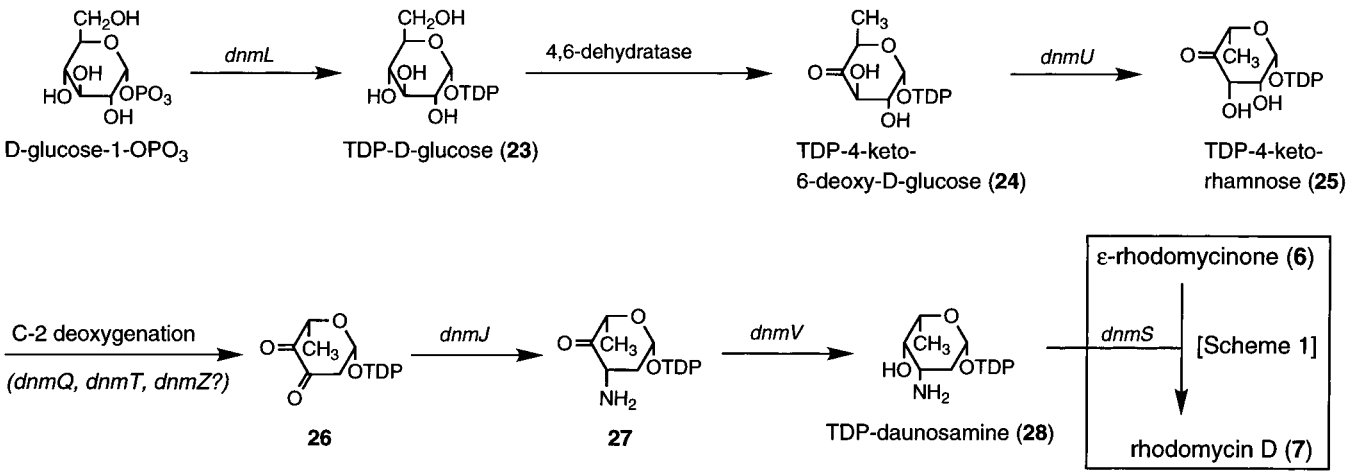
The remaining steps to the aglycon, ϵ-rhodomycinone (**6**), are catalyzed by products of *dnr* genes (or

Scheme 4. Physical Map of the Doxorubicin Gene Cluster from *S. peucetius*[a]



[a] The wedges are oriented in the direction of gene transcription and are proportional to the size of the gene product. Each wedge is shaded according to the symbols for gene function shown below the map. Restriction sites are indicated above the solid line according to the following abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI.

Scheme 5. Hypothesis for the Biosynthesis of l-Daunosamine[a]



[a] The genes governing each step are shown above the arrows, but the order and nature of the steps beyond the formation of **24** are conjectural.

"*dau*", the acronym used for the homologous *Streptomyces* sp. C5 genes). DnrG is assumed to be responsible for the C-12 oxidation of **1** to **2**[39,40,42,43] (Scheme 1), which resembles the conversion of **15** to **16** in the tetracyclic case (Scheme 2). The 47% sequence similarity between DnrG and its TcmH homolog (see below) implies that the two monooxygenases also act through a similar mechanism, even though the two substrates have rather different structures. DnrC catalyzes the *O*-methylation of the carboxyl in **2**,[44,45] after which DnrD catalyzes an intramolecular Claisen cyclization of **3** to **4**.[44,45] Reduction of the C-7 carbonyl by the product of *dnrE*, a KR with a uniquely different specificity than that of the DpsE KR,[41] gives **5** which DnrF oxidizes at C-11 to give **6**. DnrF is a flavoprotein with considerable sequence similarity to the TcmG enzyme that is involved in the formation of tetracenomycin C from tetracenomycin A2 (Scheme 2).

ϵ-Rhodomycinone undergoes glycosylation with the thymidinediphospho (TDP) derivative of L-daunosamine (**28**), resulting in rhodomycin D (**7**).[42] This 3-amino-3,4,6-trideoxy sugar is thought to be made from TDP-glucose (**23**) by the pathway drawn in Scheme 5. The first two steps would be expected to involve *dnmL* and *dnmM* (daunosamine biosynthesis genes are designated by "*dnm*") because these genes encode proteins very similar to known glucose-1-phosphate thymidylyltransferases and TDP-glucose 4,6-dehydratases, respectively. Surprisingly, the wild-type *dnmM* gene contains a frameshift resulting in the formation of an inactive truncated protein, which accounts for the fact that disruption of *dnmM* had no effect on daunorubicin production.[46] Another TDP-glucose 4,6-dehydratase found in *S. peucetius* by Strohl and co-workers[47] (and not located in the daunorubicin gene cluster[46]) must provide the needed activity. The order of the steps in Scheme 5 following

the formation of **24** are conjectural since none of the substrates and products shown have been verified; quite reasonable functions can be suggested for *dnmJ*[48], *dnmU*,[49] *dnmV*,[49] and *dnmS*[51] on the basis of the similarity of their deduced products to well characterized enzymes (homologs of *dnmU*, *dnmV*, and *dnmJ* are present in *S. griseus*[52]), but the mechanism of C-2 deoxygenation and the roles of *dnmQ*,[50] *dnmT*,[51] and *dnmZ*[49] that are putatively responsible for this conversion remain unknown.

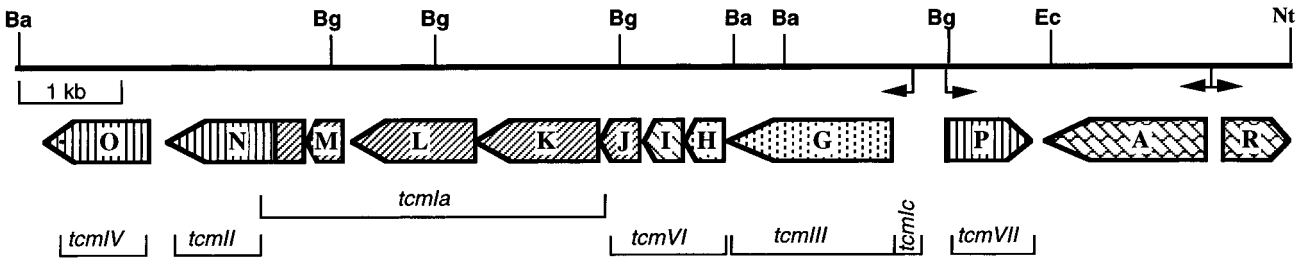
Only three genes are required to convert rhodomycin D to daunorubicin and doxorubicin.[53,54] The DnrP enzyme hydrolyzes the methyl ester of **7** to give **9** via **8** (the latter compound was discovered in a mutant of *S. coeruleorubidus*[25]),[45,54] DoxA oxidizes C-13 of **9** to give **11** (**10** may be an intermediate[54]), and DnrK methylates the C-4 phenolic group of **11** to give **12**.[54−56] DoxA, a cytochrome P450 protein, is thought to have evolved to oxidize **9** but is also able to convert **12** to doxorubicin, the more active antitumor drug, by further hydroxylation.[53,54,57] Both daunorubicin and doxorubicin are converted to the baumycin glycosides[58] and other uncharacterized acid-sensitive metabolites by the products of the *dnrH*[51] and *dnrX* (N. Lomovskaya, L. Fonstein, S. Filippini, A. L. Colombo, and C. R. H., unpublished results) genes, from which **12** and **13** can be recovered by acid-treatment of fermentation broths. It is not known whether the additional glycosylation steps represent a self-resistance or excretion mechanism or just incidental activity (baumycin derivatives of **12** have significant antitumor activity[58] but are not substrates for C-14 hydroxylation[29]).

2. Resistance and Regulatory Genes

Daunorubicin and doxorubicin production requires a means of self-resistance and, as typical of bacterial secondary metabolism,[59] is regulated by dedicated genes encoding transcription factors and other proteins required for expression of the structural and resistance genes. We identified the first set of resistance genes, *drrA* and *drrB* (Scheme 4), in a 1991 report[60] suggesting that the DrrA protein, whose deduced sequence reveals the presence of an ATP binding site, and DrrB with several potential transmembrane regions form a membrane-associated complex that actively exports daunorubicin and doxorubicin, analogous to the structure and mechanism of the Mdr1 doxorubicin resistance protein from humans. Kaur[61] has recently confirmed the soundness of this idea by demonstrating, through expression of the *drrAB* genes in *E. coli* (which conferred a significant level of doxorubicin resistance to an *acr* mutant), that DrrA[121] bound ATP or GTP in the presence of Mg^{2+} ions and that DrrB was localized in the membrane fraction. The *drrC* gene (Scheme 4) is another resistance gene but its mechanism of action is not yet clear: Although daunorubicin production requires a functional *drrC* gene and *S. lividans* (*drrC*) transformants exhibit significant daunorubicin resistance,[62] we could only provide tantalizing but not definitive support for the idea that the strong sequence similarity between DrrC and the bacterial UvrA excision repair protein means that DrrC also is somehow involved in repairing the DNA damage resulting from the nicking and alkylating properties of daunorubicin and doxorubicin once they undergo one- and two-electron reduction *in vivo*. Finally, a notable resemblance between the sequences of the deduced products of *drrD* and the McrA mitomycin resistance determinant that reoxidizes reduced mitomycin C before it can initiate the events leading to DNA cross-linking[63,64] implies that DrrD acts in a similar fashion to counteract the effects of the reduced antitumor drugs in *S. peucetius*.

Although it is not known if daunorubicin or doxorubicin are beneficial to *S. peucetius* in its natural environment, we have found that production of these antibiotics is controlled transcriptionally by the *dnrI*, *dnrN*, and *dnrO* genes. Presumably, these genes coordinate expression of the structural and resistance genes to regulate the timing of antibiotic production in relation to other cellular processes or to environmental changes. The DnrI protein is required for expression of many, if not all, of the *dnr*, *dnm*, and *dps* structural and *drr* resistance genes,[65,66] and purified DnrI binds to specific sites in the −35 regions of the promoters of the *dnrGdpsABCD*, *dpsEF*, and *dnrDKPdnmQS* operons.[67] Overexpression of the *dnrI* gene results in a major increase in ϵ-rhodomycinone production along with a lesser increase in daunorubicin production,[66] similar to the effects of the *S. coelicolor actII-ORF4*[68] and *redD*[69,70] genes (whose products share a close sequence relationship with DnrI[66]) on production of the actinorhodin and undecylprodigiosin pigments, respectively. These facts suggest that the level of DnrI is one factor limiting the amount of antibiotic produced. Since the *dnrN* gene is required for *dnrI* expression[71,72] and DnrN, another transcription factor, binds to the *dnrI* promoter but not to those of the *dnrGdpsABCD* and *dpsEF* operons,[72] one can speculate that *dnrN* regulates the timing of antibiotic production. Initially, we had assumed that the close relationship between the sequences of DnrN and other two-component response regulators found in bacteria, which enable them to transduce changes in the environment into cellular and metabolic responses, meant that a key aspartate in DnrN was phosphorylated by a protein kinase that itself underwent autophosphorylation in reponse to some external stimulus. However, alteration of the conserved aspartic acid residue to asparagine or glutamic acid considerably decreased but did not abolish the ability of DnrN to stimulate daunorubicin production[71] and did not affect its binding to the *dnrI* promoter.[70] Interestingly, the DnrN binding site strongly resembles the canonical DNA sequence for dauno- and doxorubicin intercalation; since these two antibiotics inhibited the binding of DnrN to the *dnrI* promoter, they may feedback regulate their biosynthesis at this point.[72] The adjacent and divergently transcribed *dnrN* and *dnrO* genes (Scheme 4) are set up for autoregulation of *dnrN* expression by DnrO, whose sequence contains a likely DNA binding site resembling those of bacterial repressor proteins. Yet disruption of *dnrO* resulted in a daunorubicin nonproducer instead of a precocious or overproducer (extra copies of the *dnrN* gene stimulate daunorubicin production consider-

Scheme 6. Physical Map of the Tetracenomycin C Gene Cluster from *S. glaucescens*[a]



[a] The wedges are oriented in the direction of gene transcription and are proportional to the size of the gene product. Each wedge is shaded according to the symbols for gene function shown in Scheme 4. The brackets beneath the map indicate which genes belong to the different classes of Tcm C⁻ mutations. Restriction sites are indicated above the solid line according to the following abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; Nt, *Not*I. The bent arrows beneath the solid line show the locations of the four known promoters.

ably[71]), and restoration of antibiotic production resulted from introduction of *dnrO* and *dnrN*, while restoration to the wild-type level required that *dnrNO* be introduced together into the *dnrO* mutant (S. L. Otten and C. R. H., unpublished work). Therefore, normal *dnrN* and *dnrO* function appears to require a *cis* arrangement of the genes.

B. Tetracenomycin Production Genes

1. Structural Genes

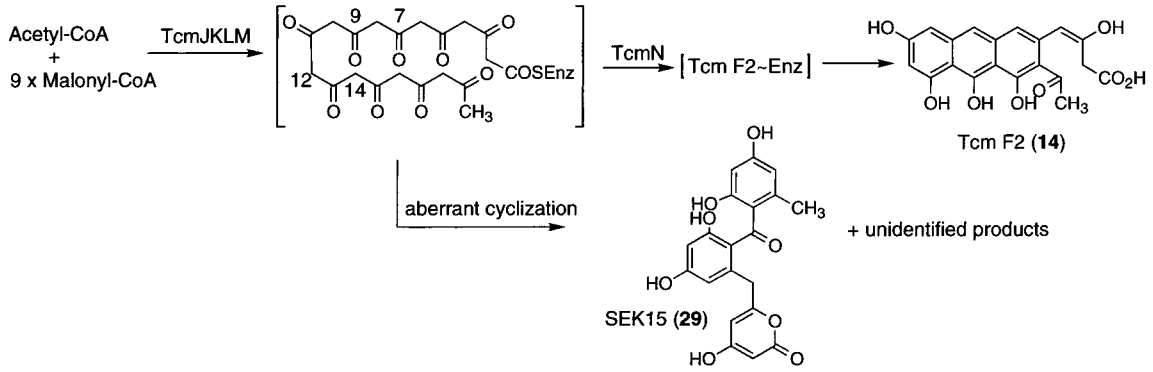
The hint of a comparatively straightforward biosynthetic pathway to tetracenomycin C provided by Zähner and co-workers[9,10,15] and an interest in *S. glaucescens* genetics developed by the Hütter group[73] led us in 1985−86 to attempt to clone the tetracenomycin C production genes, using the strategy established by Hopwood and co-workers that had resulted in their pioneering 1984 feat—cloning of the entire actinorhodin gene cluster.[74] We had a distinct tetracenomycin C⁻ mutant for all but two steps in the biosynthetic pathway (Scheme 2) and using pIJ702, one of the earliest cloning vectors developed in the Hopwood laboratory,[75] we were able by 1987 to clone all of the *tcm* genes as two large, overlapping DNA segments that together complemented a representative of each *tcm* mutant.[76] One of the segments also conferred tetracenomycin C resistance when introduced into *S. lividans*. Although technically challenging, we were also able to introduce a pIJ702 clone containing each large segment into *S. lividans* simultaneously and observe transient tetracenomycin C production.[76] These results set the stage for a detailed analysis of the *tcm* gene cluster (Scheme 6) that was carried out over the following six years. Highlights of this work are summarized below.

Like daunorubicin, the aromatic framework of tetracenomycin C is built by a type II PKS. In conjunction with the Hopwood group, we characterized a segment containing *tcmK* and part of the *tcmL* gene as a homolog of the corresponding *actI-ORF1* and *-ORF2* region.[37] Since many other actinomycetes contain DNA that hybridized to these genes,[37] we presumed at the time that they would be useful genetic markers for polyketides in general, although the distinction subsequently made between type I and type II PKSs revealed that *tcmK/tcmL* and *actI-ORF1/-ORF2* are only useful probes for type II PKS genes. Nonetheless, such genes have proven invaluable for cloning the latter type of polyketide biosynthesis genes, as witnessed by the work on daunorubicin described above. When the sequence of the *tcmK*, *tcmL*, and *tcmM* genes[77] as well as homologs of the *actI*, *actIII*, *actVII*, and *actIV* genes from the granaticin producer[78] were analyzed, the reason for the highly conserved DNA similarity became obvious from the deduced functions of each PKS gene. TcmK/ActI-Orf1, TcmL/ActI-Orf2, and TcmM/ActI-Orf3 and all their homologs subsequently discovered[79] represent the core enzymes of the type II PKSs (named on the basis of the established nomenclature for bacterial and plant FAS): The two KS subunits and the ACP, respectively. Although ActI-Orf2 and TcmL are also known as chain-length factors, we prefer to call them the KS_b subunits and ActI-Orf1 and TcmK, the KS_a subunits because the length of the polyketide chain is determined by both proteins, not just ActI-Orf2 or TcmL. ActIII represents the KR component (such an enzyme is not required for tetracenomycin C biosynthesis) and ActVII, the N-terminal third of TcmN[80] and ActIV are aromatase (ARO) and/or cyclase (CYC) enzymes.[81] The two KS subunits, ACP, KR, and CYC enzymes constitute a typical type II PKS. TcmJ is peculiar because homologs of it are not widely distributed among other clusters of type II PKS genes, and its absence in *S. glaucescens* or presence in the cloned *tcm* PKS genes has only a quantitative effect on polyketide biosynthesis.[82] The *tcmJ*, *tcmK*, *tcmL*, *tcmM*, and *tcmN* genes govern the formation of tetracenomycin F2 (Scheme 2); *tcmI* determines how the fourth ring is made,[82] as in tetracenomycin F1 (**15**), to complete the carbon framework of tetracenomycin C. All six genes are juxtaposed in the *tcm* gene cluster between the *tcmP*,[83] *tcmO*,[81] and [remaining portion of] *tcmN* *O*-methyltransferase genes, and the *tcmH* [81] and *tcmG* [83] monooxygenase genes (Scheme 6).

2. Resistance Genes and Regulation

The potentially deleterious effect of an antibiotically active substance made in the final step of tetracenomycin C biosynthesis is overcome by the action of the *tcmA* and *tcmR* gene products.[84] TcmA appears to have several transmembrane loops that may enable the protein to slip into the cell membrane and act as a tetracenomycin C exporter. Expression of the *tcmA* gene is controlled by the TcmR repressor that we postulated binds to operator sites in the *tcmA* promoter until tetracenomycin C binds to TcmR, releasing it from the DNA and initiating *tcmA* expression;[85] once the level of tetracenomycin C falls below some threshold, TcmR rebinds and represses

Scheme 7. Depiction of the Role of TcmN in the Biosynthesis of Tetracenomycin F2[a]



[a] The brackets indicate intermediates that are enzyme-bound either covalently (-COSEnz) or physically (F2~Enz).

tcmA. This model is based on the well-studied mechanism of tetracycline resistance mediated by the *tetAR* genes.[85]

Unlike daunorubicin, tetracenomycin C biosynthesis does not seem to be controlled by dedicated regulatory genes.[86] We mapped the location of the four promoters shown in Scheme 6,[85,86] and on the basis of the results of S1 nuclease protection experiments and the effect of the *tcmIc* mutation (a T → G transition in the −10 region of the *tcmG* promoter[83,86]), it appears that only one promoter controls expression of the *tcmGHIJKLMNO* operon. Sequence analysis of the DNA flanking the *tcm* gene cluster did not reveal putative regulatory genes[86] (Evelyn Wendt-Pienkowski and C. R. H., unpublished work), but this conclusion has not been tested by gene disruption.

V. Biosynthetic Enzymes

A. Doxorubicin

Several enzymes of doxorubicin biosynthesis have been studied in cell-free systems,[13] but only two, carminomycin-*O*-methyltransferase[56,87] and the DnrD cyclase[88] have been purified to homogeneity. Activities of the DnrC aklanonic acid *O*-methyltransferase,[89] DauE aklaviketone reductase,[41,89] DauP esterase,[54] and DoxA P450 hydroxylase[53,54] have been reported in cell-free systems. Only three enzymes, DnrP, DnrK, and DoxA, are needed to catalyze the conversion of rhodomycin D (**7**) to doxorubicin (**13**), and their substrate specificities are not tight since there appears to be more than a single route to **13** and several shunt products are also formed.[54] The DoxA hydroxylase is especially interesting because it has two substrates, 13-deoxycarminomycin (**9**) and daunorubicin (**12**), and involves an unusual $-CH_2-CH_3$ to $-C(=O)CH_3$ conversion followed by an α-hydroxylation of the $-COCH_3$ group.[54] The latter activity is low and does not take place with C-4 hydroxy or baumycin derivatives, which explains why the baumycin-producing *Streptomyces* sp. C5 strain does not produce doxorubicin even though it has the *doxA* gene.[53] Nevertheless, a large-scale industrial process for the conversion of daunorubicin to doxorubicin has been developed by using the *S. peucetius doxA* gene.[57]

B. Tetracenomycin

Several of the enzymes of the tetracenomycin C pathway have been the subject of detailed studies involving their purification and mechanistic analysis. The TcmM ACP[90] contains a 4′-phosphopantetheine group attached to S_{41} to which malonate is transfered by malonyl-CoA:ACP transferases from *S. glaucescens*[90,91] and *E. coli*.[91] However, TcmM is produced mostly as the apoprotein when the *tcmM* gene is expressed in *E. coli*;[70] some other type II PKS ACPs behave similarly.[92] The inability of the *E. coli* AcpS holo-ACP synthase (4′-phosphopantetheinyl transferase) to modify such ACPs can be overcome by introduction of *acpS* homologs from *E. coli* and other bacteria into the ACP-producing strain.[93] It is believed that the *S. glaucescens* FabD malonyl-CoA:ACP transferase presumably used by a type II FAS also catalyzes the charging of TcmM with malonate, although, as in *S. coelicolor*, this idea could not be substantiated because of an inability to inactivate the *fabD* gene to determine a possible negative effect this would have had on tetracenomycin F2 production.[91]

The cell-free system developed for studying the tetracenomycin PKS[94] allowed us to establish some of its basic characteristics. The most important findings were the inability of the *E. coli* FAS ACP (AcpP) to substitute for TcmM, and propionyl-, butyryl-, or isobutyryl-CoA to substitute for acetyl-CoA as the starter unit. This system can also be used to purify the individual components by reconstitution of the PKS activity; for instance, TcmN activity was assayed by addition of TcmN [obtained from expression of the *tcmN* gene in *E. coli* and purified on the basis of its tetracenomycin D3 *O*-methyltransferase activity (Scheme 2)] to the cell-free extract obtained by expression of the plasmid-borne *tcmJKLM* genes in the *S. glaucescens tcmGHIJKLMNO* null mutant.[95] When this was done, SEK15 (**29**, Scheme 7) and other uncharacterized compounds, made along with tetracenomycin F2 (**14**) by the TcmJ, TcmK, TcmL, and TcmM enzymes, disappeared and **14** became almost the sole product. Since **29** is formed by aberrant cyclization of the decaketide between the C-7 carbonyl and C-12 methylene (Scheme 7), TcmN reinforced the normal cyclization regiochemistry between C-9 and C-14 or suppressed the abnormal chemistry leading to **29** and the other compounds. This result indicates that the normal behavior of a type II PKS requires the proper interaction of its

several components: the properties of *tcmK*, *tcmL*, and *tcmM* mutants (R. G. Summers, E. Wendt-Pienkowski, and C. R. H., unpublished work) and different combinations of the *tcm* PKS genes[94] show that no activity is seen without TcmK, TcmL, or TcmM; aberrant cyclization occurs without TcmN, while the lack of TcmJ just lowers the level of activity without affecting tetracenomycin F2 formation.[82,94]

Formation of tetracenomycin F2 in the absence of TcmN reveals the uncertainty about the timing of the intramolecular aldol reactions involved in the conversion of the decaketide to **14**. Can these take place spontaneously such that **14** is only one of several possible products (and TcmN blocks the formation of the latter compounds), or do the TcmK and TcmL enzymes have partial cyclase activity? Furthermore, is the decaketide released from TcmM before cyclization by TcmN or are the incompletely cyclized intermediates released and not acted upon by TcmN? The timing of release was addressed in two ways. Recovery of the protein fraction from the cell-free system by membrane ultrafiltration or gel filtration followed by solvent extraction of supernatant and protein fractions showed that **14** was bound tightly to the protein fraction.[95] Since **14** could be isolated by ethyl acetate extraction of this fraction but not the supernatant, it is not likely to be covalently bound; yet, the fact that it did not freely diffuse into the reaction medium implies that something must cause it to be released *in vivo*. Secondly, a putative thioesterase active site ($_{349}$GHSKGA$_{354}$) in TcmK, possibly involved in product release, was inactivated by making the S351A and H350L/S351A mutants and determining how each of these changes affected the ability of the *tcmJKLMN* genes to make tetracenomycin F2 in vivo.[96] Since the S351A mutation was silent, S_{351} does not play a role in tetracenomycin F2 formation even though it is the critical active-site residue in FAS thioesterases. Interestingly, the H350L, S351A mutant was unable to make tetracenomycin F2, which suggests that H_{350} may be an important base in the condensation reaction between $RCH_2COSEnz$ and $CH_2(COOH)COS$-TcmM substrates or for dehydration of the $RCH(OH)(CH_2CO)_nSEnz$ intermediate instead of working with S_{351} to catalyze product release. Although each of these results is interesting, they did not answer the initial question about the timing of aldol cyclizations and how the cyclized product is released.

In the biosynthesis of tetracenomycin C (Scheme 2), tetracenomycin F2 is cyclized to tetracenomycin F1 (**15**) and tetracenomycin F1 is oxidized to tetracenomycin D3 (**16**). The TcmI CYC enzyme catalyzes the former reaction,[97] whose mechanism must be analogous to the aldol reactions involved in tetracenomycin F2 formation that are catalyzed by TcmN.[95] We found with purified TcmI that at pH $\geq$ 8.0 tetracenomycin F1 was the favored product but at pH $\leq$ 6.5, its C-9 decarboxylation product (**15a**) was predominant (the ratio of **15:15a** changed from 10:1 to 1:10). This result was suggested to be due to a preference for dehydration of the aldol intermediate to **15** when the carboxyl group is deprotonated *vs* decarboxylation and loss of H_2O when it is protonated. Be that as it may, this property of TcmI explains how the decarboxylated shunt products shown in Scheme 2 could be formed *in vivo*. The mechanism of the C-5 oxidation of **15** catalyzed by the TcmH monooxygenase is unusual because we could not uncover evidence for the involvement of a cofactor or metal ion to aid activation of the O_2 or substrate.[98] We therefore suggested that a residue in the enzyme might react with **15** to form a radical intermediate and Enz$^{\bullet}$-H, which then would react with O_2 to form the HOO$^{\bullet}$ peroxy radical that could combine with the substrate radical to generate the C-5 peroxy precursor of **16**. This idea has not been followed up; e.g., by testing for the presence of radical intermediates by EPR spectroscopy of the enzyme/substrate complex.

The final step in tetracenomycin C biosynthesis is the oxidation of tetracenomycin A2 (**19**) to **20** and this is catalyzed by the TcmG flavoprotein to which one equivalent of FAD is bound (NADPH and O_2 are also required).[99] Scheme 3 shows a likely mechanism for the introduction of three oxygen atoms into **19** that is consistent with the stereochemical constraints, the properties of purified TcmG[99] and the results of labeling studies with [^{18}O]-labeled O_2 and H_2O.[19,20]

VI. Behavior of the Daunorubicin and Tetracenomycin Genes in Novel Situations

Considerable insight into the properties of the PKSs produced by the *tcmKLMN* and *dpsABEFGH* genes has also been obtained through investigations of their behavior in novel situations. By studying the metabolites produced in *S. lividans* and *S. glaucescens* from different combinations of the actinorhodin, daunorubicin, jadomycin, and tetracenomycin PKS genes introduced on a plasmid vector, we have helped lay some boundary conditions on the parameters that control the length of the polyketide chain (size) and cyclization pattern (shape).[100] In collaboration with Jürgen Rohr, Jose Salas, and their co-workers, we are currently taking a different approach that involves the characterization of new metabolites produced by the introduction of the tetracenomycin or elloramycin biosynthesis genes into other bacteria and the mithramycin biosynthesis genes into *S. glaucescens*, following up work initiated by the groups headed by Heinrich Decker, one of my former co-workers, and Jürgen Rohr.[101,102] Since the results of the published research by us and others in this area have been reviewed elsewhere recently,[14,79] interested readers can consult these sources for further information.

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