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The Biosynthesis of Aureolic Acid Group Antibiotics

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Biosynthetic studies on mithramycin (**1**, aureolic acid) using to a great extent modern genetic methods revealed several novel aspects of the biosynthesis of this class of antitumor agent. It could be proven that the aglycon moiety of the aureolic acids is constructed by a type II polyketide synthase via a single decaketide chain which undergoes a folding, as seen in the tetracyclines, followed by an initial 7,12-cyclization. Finally, after three more cyclizations, a linear tetracyclic intermediate (premithramycinone **9**) arises, which is a tetracycline-like molecule. Premithramycinone (**9**) is consecutively methylated and glycosylated (via premithramycins A_1 **11**, A_1 **12**, A_2 **13**, and A_3 **14**) to form premithramycin B (**10**), the ultimate tetracyclic intermediate, whose fourth ring finally gets oxidatively opened through a Baeyer-Villiger type oxidation to yield mithramycin **1**. Most of the gene cluster coding for the mithramycin biosynthesis has been identified and sequenced and several gene functions were identified through insertional inactivation of specific genes and structure elucidation of accumulated products.

INTRODUCTION, HISTORY

Mithramycin (**1**, synonyma: aureolic acid, plicamycin, mithracin, LA-7017, PA-144), produced by *Streptomyces argillaceus* and some other streptomycetes, forms, along with the chromomycins (e.g., chromomycin A_3 **2**), olivomycin (e.g., olivomycin A **3**), chromocyclomycin (**4**), and UCH9 (**5**) the small, but distinct group of the aureolic acid antibiotics (Fig. 1; Refs. *1–9*). Mithramycin (**1**) itself is the most interesting compound because of its various biological activities: it shows a remarkable cytotoxicity against a variety of tumor cell culture lines, including brain tumors and experimental animal tumors, and has been clinically used for the treatment of certain tumors, such as disseminated embryonal cell carcinoma as well as for Paget's bone disease (*4,10,11*) It also finds use for control of hyperglycaemia in patients with malignant disease (*12*). As the suggested mechanism of action, mithramycin binds to the DNA, as a Mg^{2+} dimer, which cross-links the two strands (*13–15*).

Since the discovery of mithramycin in 1953 (*1*), various partially contradictive

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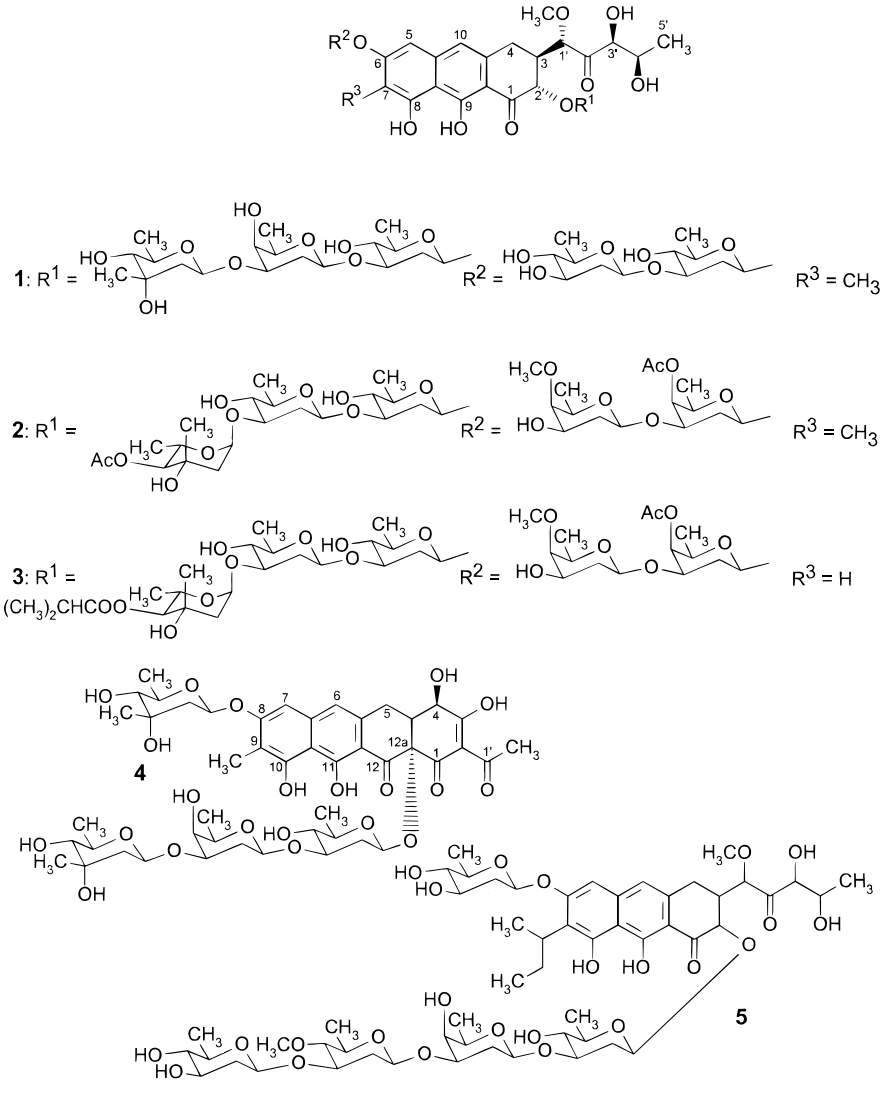


FIG. 1. Structures of aureolic acid group antibiotics and anticancer agents.

structures of the molecule have been published in context with reports on the structure elucidation (*2*,*5*,*16*,*17*), DNA interactions (*13*–*15*), syntheses of the saccharide chains (*6*,*7*,*17*–*20*), and genetic work (*21*–*26*). Consequently, wrong structures still can be found in original publications, textbooks, and important encyclopediae (*3*,*12*,*27*,*28*). Along with our biosynthetic studies, we also reinvestigated the structure of mithramycin using the most modern NMR methods and concluded that structure **1**, initially used by Patel *et al.* (*13*), is correct (*29*).

Several wrong hypotheses were suggested in context with the biosyntheses of aureolic acid antibiotics, by several authors including us (*12*,*22*,*24*,*30*–*32*; see below).

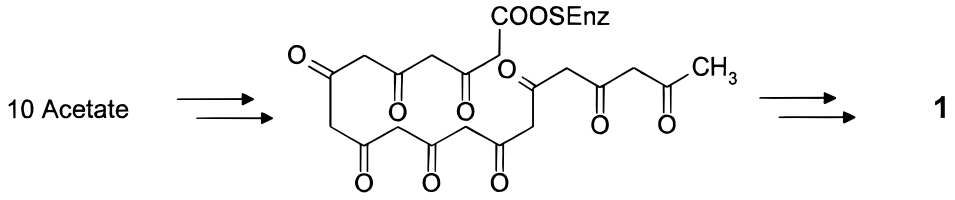


FIG. 2. Early (wrong) suggestion for the polyketide biosynthesis of the aureolic acid aglycon (see *12*).

Although our studies on the model compound mithramycin (**1**) are still incomplete, the results available until now justify a review on the biosynthesis of aureolic acid antibiotics.

All authors who discussed the biosynthesis of one of members of the aureolic acid group recognized correctly the polyketide origin of the aglycon. Initial hypotheses (e.g., cited in *12*) suggested a single polyketide chain which finally is located in the molecule starting from the end of the side chain at C-5′ and ending at C-4 (with decarboxylation, Fig. 2).

INCORPORATION EXPERIMENTS WITH STABLE ISOTOPE-LABELED PRECURSORS

First incorporation experiments on an aureolic acid group member were performed by Rosazza *et al.* (30), namely on chromomycin A_3 (**2**). Using ^{13}C- and ^{18}O-labeled acetate, Rosazza *et al.* concluded that at least two, or even three, independent polyketide chains form the aglycon backbone (Fig. 3; Ref. *30*).

Our 1992 reinterpretation of Rosazzas results suggested an alternative (*31*): a single decaketide chain forms initially a tetracyclic, tetracenomycin-type intermediate. This

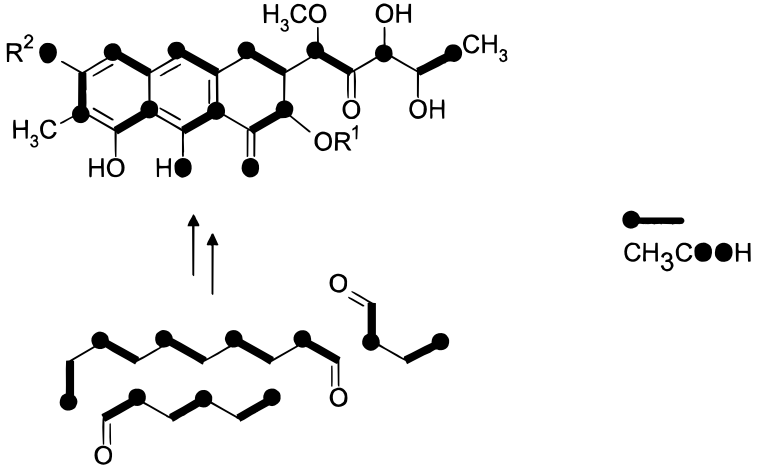


FIG. 3. Results of incorporation experiments on chromomycin A_3 (*30*) with various labeled acetates and one of the authors (wrong) "multiacetate chain" hypotheses suggested for explanation.

way, Rosazzas' incorporation pattern could also be explained if an oxidative cleavage at C-10/C-10a of the tetracenomycin intermediate (e.g., Tetracenomycin F_1 **6**; Ref. *33*) takes place, resulting in the characteristic tricyclic mithramycinone with its C_5-side chain (Fig. 4).

This hypothesis was initially of pure theoretical background, and ^{18}O incorporation experiments were intended to prove the oxidative cleavage. From incorporation experiments under $^{18}O_2$-enriched atmosphere, we expected ^{18}O-labels at C-2-O as well as at C-4′-O. Furthermore, also C-1′-O and C-3′-O should be labeled due to their position at a carbon which has been formerly a C-2 of an acetate unit. On the contrary, all other oxygens should be labeled with 1-^{13}C,$^{18}O_2$-acetate. Rosazzas' work on chromomycin already showed that the oxygens positioned at C-1, C-6, and C-9 derive from acetate (*30*). Our studies on mithramycin using 1-^{13}C-acetate, 2-^{13}C-acetate, and 1-^{13}C,$^{18}O_2$-acetate and a fermentation in an $^{18}O_2$-enriched atmosphere (investigated by ^{13}C NMR on mithramycin decaacetate for solubility reasons) resulted in the same incorporation pattern from labeled acetate as found for chromomycin A_3. Furthermore, the feedings revealed that the oxygens linked at C-1, C-6, C-8, C-9, C-2′, and C-4′ derive from the acetate building blocks, while the oxygens linked at C-2 and C-1′ derive from aerial oxygen (*34*). The results were not very clear always due to the tautomerism in the tricyclic ring system as well as broad C-signals in the side chain. For the oxygen at C-3′, no observable upfield shift could be detected (Fig. 5).

From these results, Rosazzas' hypothesis (Fig. 3) seemed to be supported, while our "tetracenomycin-hypothesis" (Fig. 4) was refuted. Later (see below), however, it could be demonstrated that it was principally correct to suggest a one-chain hypothesis leading to a tetracyclic intermediate of which the fourth ring is subsequently cleaved through an oxygenase. Further investigations on the mithramycin biosynthesis were performed using molecular biological techniques.

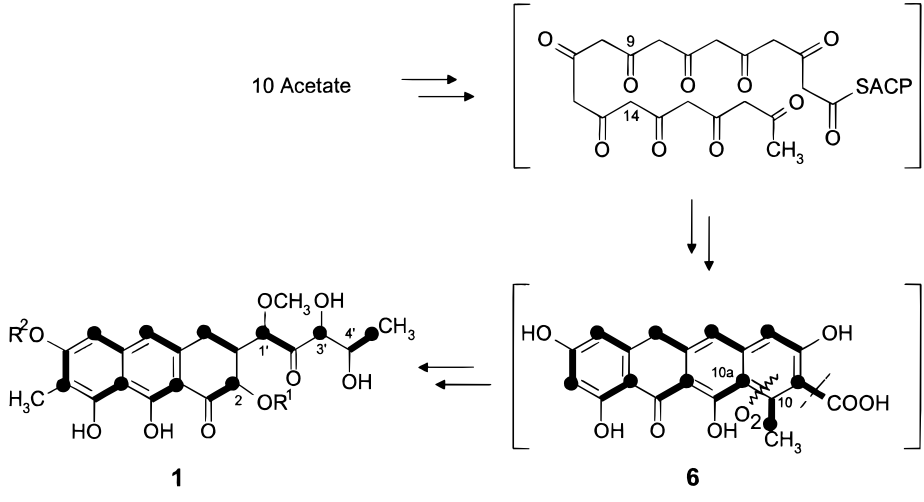


FIG. 4. Single decaketide chain hypothesis leading to a tetracyclic (tetracenomycin-type) intermediate (here tetracenomycin F_1 **6**) which is futher cleaved by an oxygenase (*31*). This hypothesis was stimulating, but proven to be wrong regarding the tetracenomycin intermediate.

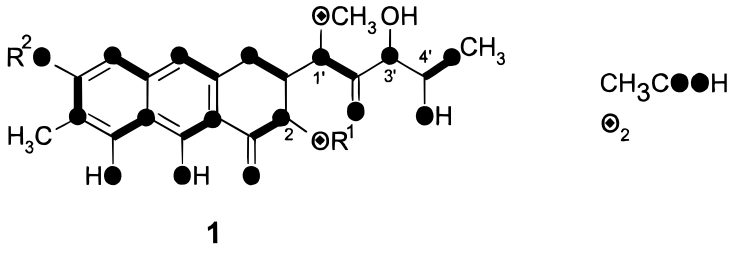


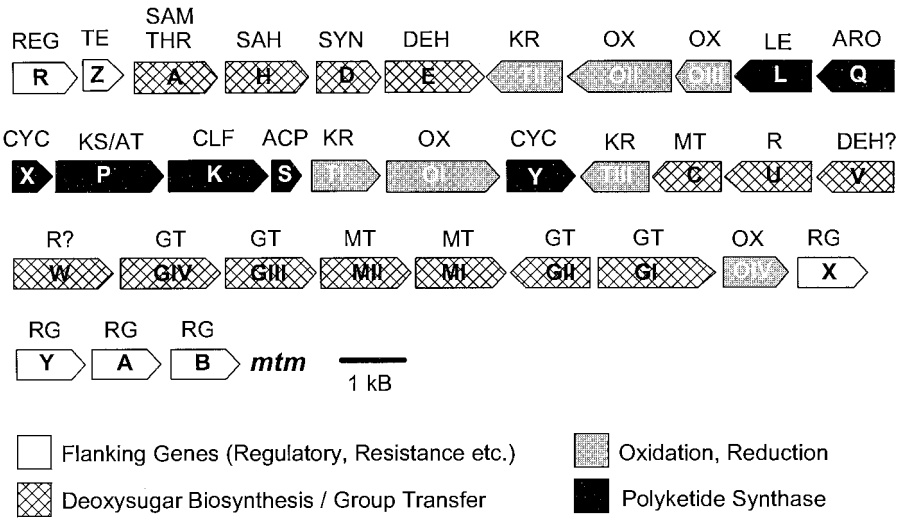
FIG. 5. Result of the incorporation experiments (with ^{13}C- and ^{13}C,^{18}O-labeled acetate and $^{18}O_2$) on mithramycin (*34*).

MOLECULAR BIOLOGY AND THE ELUCIDATION OF THE MITHRAMYCIN BIOSYNTHETIC PATHWAY

Molecular biology can provide important tools for the elucidation of antibiotic and antitumor biosynthetic pathways. An initial approach comes through the cloning of the antibiotic/antitumor gene cluster. Sequencing of the correspondent genes and searching for similarities with proteins in databases can give preliminary information about possible roles for the different gene products. However, this is not sufficient to assign a role to the different proteins. Insertional inactivation of specific and selected genes can provide further clues to their function through the generation of nonproducing mutants that accumulate different intermediates in the pathway. The isolation and structure elucidation of these intermediates may shed light on the specific functions of the different gene products. Furthermore, deletion mutants can be also produced by removing one or more genes from the cluster through homologous recombination. Again, potential functions can be assigned after structure elucidation of accumulated intermediates. Expression of some genes in another heterologous host may also provide interesting information on gene functions. All these molecular biology techniques have been applied to the elucidation of the mithramycin pathway. The following text is easier to understand with the mithramycin biosynthetic gene cluster shown (*35*; Fig. 6).

A. Polyketide Biosynthetic Steps

The polyketide backbone of the aureolic acid group is synthesized through the condensation of 10 acetate units (*30*,*34*). As a characteristic of aromatic polyketides these condensation reactions are carried out by a type II polyketide synthase (PKS; see reviews *36*–*39*). Several genes encoding a type II PKS from a mithramycin producer, *S. argillaceus* ATCC 12596, have been sequenced (*21*). The *mtmP* (β-ketoacylsynthase), *mtmK* (chain length factor), and *mtmS* (acyl carrier protein) genes constitute the so-called "minimal PKS." Expression of these genes, together with *mtmTI* (ketoreductase) and *mtmX* (cyclase), into different streptomycetes led to the production of three different compounds: SEK15 (**7**) in *Streptomyces lividans*, tetracenomycin M (**8**) in *Streptomyces glaucescens* (tetracenomycin producer; Ref. *24*), and auramycinone in *Streptomyces galilaeus* (aclacinomycin producer; Ref. *32*).



REG =Regulatory Gene

TE = Thioesterase

SAM = S-Adenosylmethionine Synthase

THF = Tetrahydofolate Reductase

SAH = S-Adenosylhomocysteine Hydrolase

SYN = dTDP-Glu-Synthase

DEH = Deoxysugar Biosynthesis Dehydratase (mtmE = dTDP-Glu-4,6-DEH)

KR = Ketoreductase

OX = Oxygenase

LE = AcylCoA Ligase

ARO = Aromatase

CYC = Cyclase

KS = β-Ketosynthase

AT = Acyltransferase

CLF = Chain Length Factor

ACP = Acyl Carrier Protein

R = Reductase (Deoxysugar Biosynthesis)

MT = Methyltransferase

GT = Glycosyltransferase

RG = Resistance Genes

FIG. 6. The mithramycin biosynthetic gene cluster (*35*).

Tetracenomycin M (**8**) is a novel hybrid compound arising from combinatorial biosynthesis of the mithramycin and tetracenomycin gene clusters (*24*). Formation of tetracenomycin M gave a clue to the possible role of the *mtmX* gene in mithramycin biosynthesis as a fourth ring cyclase. It had been previously shown that the MtmX protein showed similarity with the ActVI–orfA protein of the actinorhodin pathway and another protein from the frenolicin pathway (*21*). However, it was not possible to assign a function to these proteins in the corresponding pathways. Tetracenomycin M (**8**) is cyclized through aldol addition in a manner that cannot be catalyzed by the cyclases from the tetracenomycin producer host. The corresponding gene product

TcmI catalyzes an aldol condensation step instead, which leads to the formation of tetracenomycin F_1 (**6**; Ref. *33*). Therefore, a protein encoded by one of the mithramycin genes was suggested to be responsible for this cyclization of the fourth ring. From the knowledge of the function for *mtmQ*, *mtmP*, *mtmK*, and *mtmS*, the candidate gene for catalyzing this fourth ring cyclization is *mtmX* (Fig. 7).

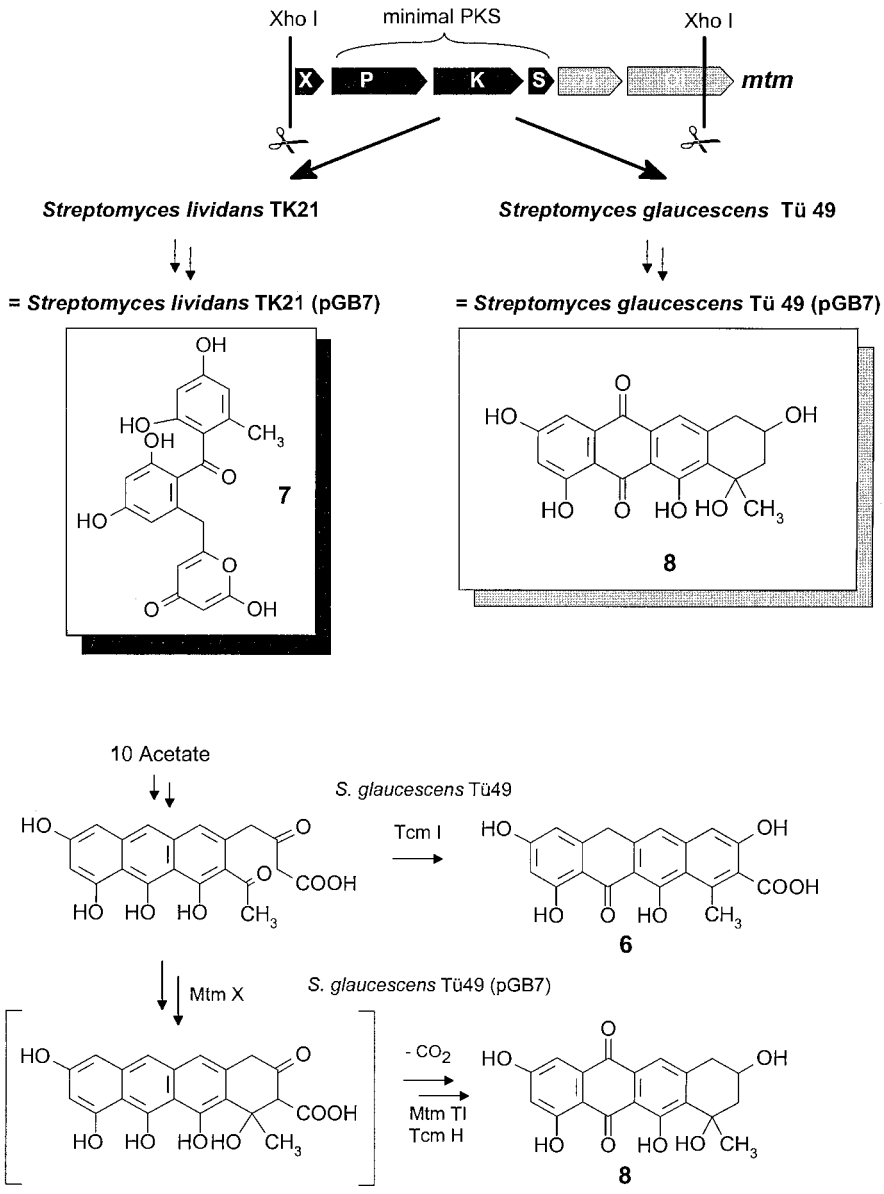


FIG. 7. Recombination of the *mtmXhol* fragment (*mtmXPKSTIOI*) in two different hosts. (Bottom) Hybrid product tetracenomycin M (**8**) is a result of the combined *tcm* and *mtm* gene products.