Deciphering the biosynthetic origin of the aglycone of the aureolic acid group of anti-tumor agents

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Background: Mithramycin, chromomycin, and olivomycin belong to the aureolic acid family of clinically important anti-tumor agents. These natural products share a common aromatic aglycone. Although isotope labeling studies have firmly established the polyketide origin of this aglycone, they do not distinguish between alternative biosynthetic models in which the aglycone is derived from one, two or three distinct polyketide moieties. We set out to determine the biosynthetic origin of this moiety using a recombinant approach in which the ketosynthase and chain-length factor proteins from the antibiotic-producer strain, which determine the chain length of a polyketide, are produced in a heterologous bacterial host.

Results: The ketosynthase and chain-length factor genes from the polyketide synthase gene cluster from the mithramycin producer, *Streptomyces argillaceus* ATCC12956, and the acyl carrier protein and ketoreductase genes from the actinorhodin polyketide synthase were expressed in *Streptomyces coelicolor* CH999. The recombinant strain produced a 20-carbon polyketide, comprising the complete backbone of the aglycone of mithramycin.

Conclusions: The aglycone moieties of mithramycin, chromomycin, and olivomycin are derived from a single polyketide backbone. The nascent polyketide backbone must undergo a series of regiospecific cyclizations to form a tetracenomycin-like tetracyclic intermediate. The final steps in the aglycone biosynthetic pathway presumably involve decarboxylation and oxidative cleavage between C-18 and C-19, followed by additional oxidation, reduction, and methylation reactions.

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Introduction

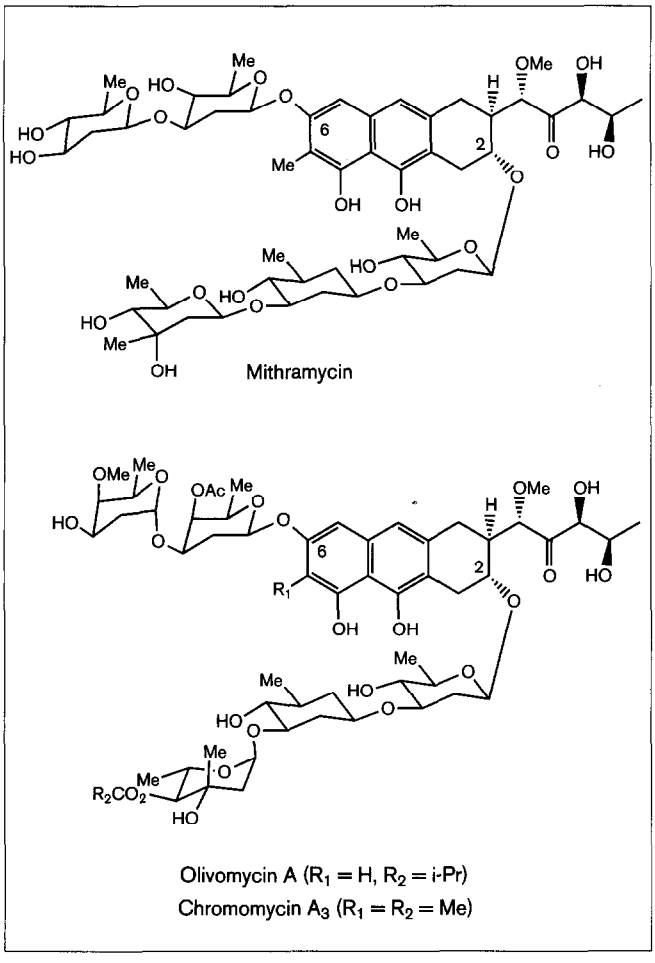
Mithramycin, chromomycin, and olivomycin (Fig. 1) comprise the aureolic acid group of anti-tumor agents [1–3]. Although their mode of action is not completely understood, it is well-known that they interact with GC-rich DNA regions in a non-intercalative manner, requiring Mg^{2+} ions for activity [4]. They also possess antibiotic activity against gram-positive, but not gram-negative, bacteria. Mithramycin is used clinically in the treatment of some tumor diseases [5].

The aglycones of these three natural products are very similar, and consist of a tricyclic chromophore (designated as mithramycinone in the case of mithramycin). A disaccharide (D-olivose and D-olivose in mithramycin) and a trisaccharide (D-olivose, D-olivose and L-mycarose in mithramycin) are attached to this chromophore at positions 6 and 2, respectively. Isotope labeling studies (Fig. 2) have established that the entire carbon skeleton of the chromomycin aglycone is derived from acetate [6,7], suggesting a polyketide biosynthetic mechanism [8]. The unusual labeling pattern observed in these studies in the aliphatic moiety of the aglycone, however, has precluded unambiguous determination of its biosynthetic pathway. Indeed, it has been argued that the aglycone could be derived from a condensation of two or even three independently synthesized polyketide intermediates [6,7]. But it is also possible that the characteristic structure of chromomycinone (and, by analogy, mithramycinone) could be derived from a tetracenomycin-like tetracyclic intermediate that undergoes cleavage of a C–C bond [9].

The *mtm* locus from *Streptomyces argillaceus* ATCC12956, the bacterium that produces mithramycin, has recently been cloned [10]. Sequence analysis revealed a set of genes with a high degree of similarity to other bacterial aromatic polyketide synthases ([11–13]; reviewed in [14]). Chromosomal disruption at this locus resulted in ablation of mithramycin production in *S. argillaceus*, confirming the notion that this polyketide synthase is involved in the mithramycin biosynthetic pathway [10].

Bacterial aromatic polyketide synthases are structurally and mechanistically related to each other and to the fatty acid synthases [8,14]. These multifunctional enzymes catalyze repeated decarboxylative condensations

Figure 1



Structures of mithramycin, chromomycin, and olivomycin.

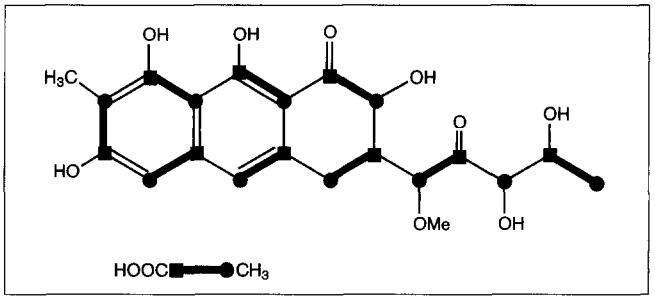
between acyl-CoA primers (usually acetyl-CoA) and malonyl-CoA extender units. After the carbon chain backbone has grown to its final chain length, it undergoes a series of regiospecific reduction, aromatization, and cyclization steps leading to the formation of a polycyclic aromatic polyketide. The chain length of the polyketide is exclusively controlled by two subunits of the minimal polyketide synthase, the ketosynthase and the chain-length factor [15–16]. Analysis of the chain-length specificity of the *mtm* polyketide synthase should help to determine the number of distinct polyketide moieties (and consequently, the number of polyketide synthases) involved in mithramycin biosynthesis. Here we report on the specificity of the *mtm* polyketide synthase for the chain length of its product. To isolate the ketosynthase and chain-length factor genes of the *mtm* polyketide synthase from other synthases that might be present in this gene cluster, we expressed them in a heterologous host, from which the entire polyketide locus has been deleted.

Results and discussion

To deduce the chain-length specificity of the *mtm* polyketide synthase, a hybrid polyketide synthase gene cluster was cloned into plasmid pRM5 [15]; recombinant polyketide synthase is produced from this vector at the onset of the stationary phase in *Streptomyces coelicolor* CH999 [15], an appropriately engineered expression host. This hybrid gene cluster included the *mtm* ketosynthase and the *mtm* chain-length factor, as well as the acyl carrier protein and the ketoreductase from the actinorhodin (*act*) gene cluster. The polyketide products of the resulting recombinant strain, CH999/pGB1, were purified and structurally analyzed by ^{1}H and ^{13}C NMR spectroscopy. The two most abundant products were found to be identical to two decaketides, RM20b and RM20c (Fig. 3), previously isolated from a recombinant strain, CH999/pRM20, that expresses the tetracenomycin (*tcm*) ketosynthase and chain-length factor, together with the *act* acyl carrier protein and ketoreductase [17].Thus, the synthesis of these 'hybrid' polyketides once again illustrates the potential for combinatorial biosynthesis using protein components from different polyketide synthases. The product yields for RM20b and RM20c were similar from both recombinant strains (90 mg l^{-1} and 12 mg l^{-1}, respectively). The recently cloned *mtm* polyketide synthase gene cluster thus specifies production of a 20-carbon polyketide chain.

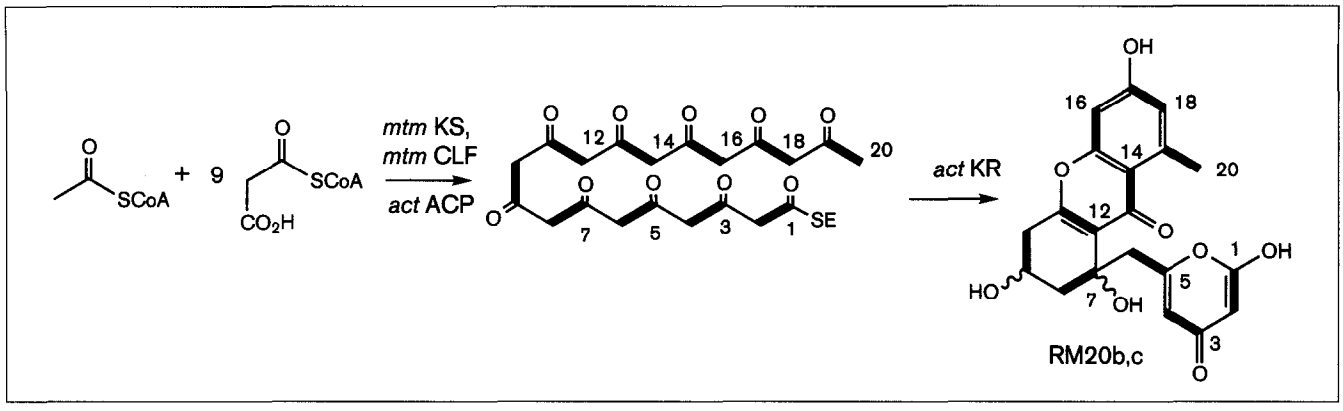
Of the various alternative models for the biosynthesis of the carbon skeleton of mithramycinone, the only one consistent with the above result is shown in Fig. 4. Here, the *mtm* minimal polyketide synthase (comprising ketosynthase, chain-length factor, and acyl carrier protein) uses an acetyl CoA primer unit and nine malonyl CoA extender units to form a decaketide backbone, which then undergoes four successive intramolecular cyclization and dehydration steps, leading to the formation of tetracenomycin F1 (compound **1**) [18]. Decarboxylation yields compound **2**, which then

Figure 2



The mithramycin aglycone is probably made by a polyketide synthase. The acetate incorporation pattern as deduced from isotope labeling experiments is shown; such patterns are diagnostic of compounds made by polyketide synthases .

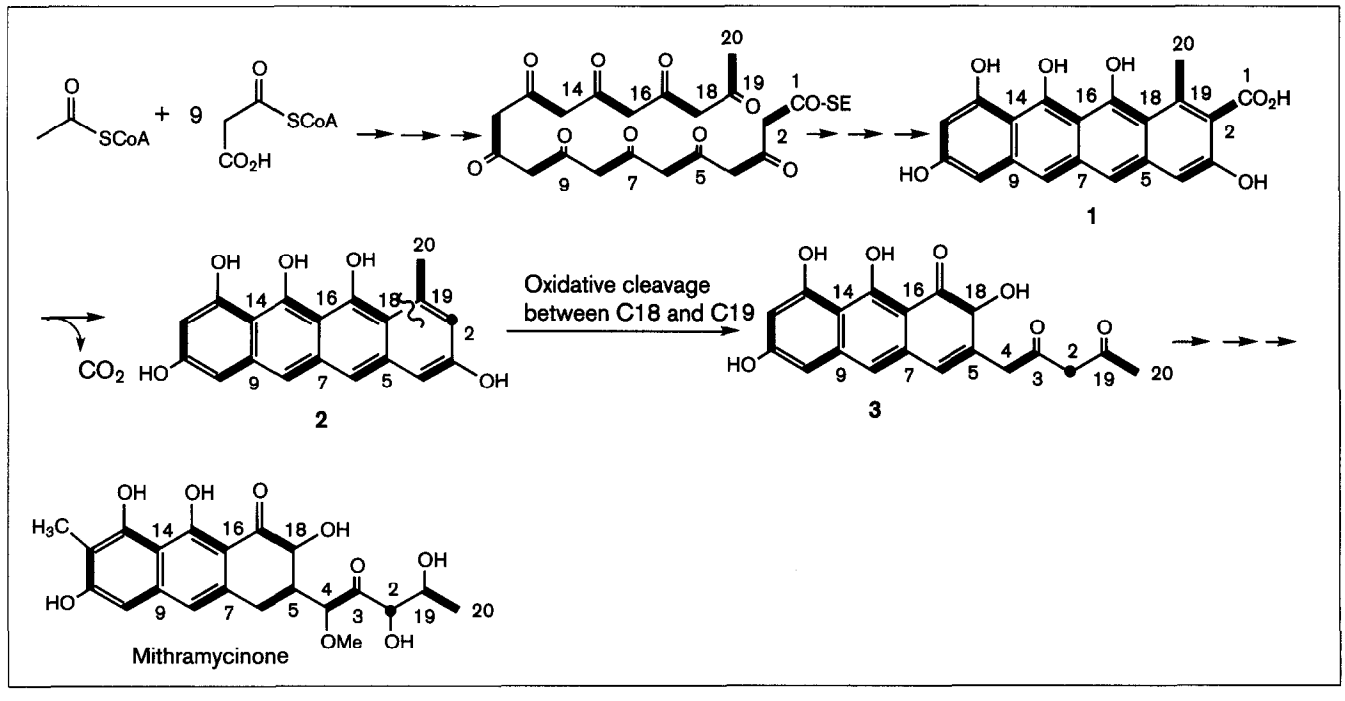
Figure 3



The aglycone of mithramycin is derived from a single polyketide backbone. Isomers RM20b and RM20c of the reduced polyketide (right) are the primary products of *S. coelicolor* CH999 expressing *mtm* ketosynthase (KS), *mtm* chain-length factor (CLF), *act* acyl carrier protein (ACP) and *act* ketoreductase (KR).

undergoes oxidative cleavage between C18 and C19, giving rise to compound 3. Subsequent oxidation, reduction, and methylation reactions result in mithramycinone formation. The biosynthetic pathways leading to the formation of the aglycone moieties of chromomycin and olivomycin are presumably very similar. Thus, the gene cluster for biosynthesis of members of this interesting class of anti-tumor agents probably encode one and only one polyketide synthase. Further genetic and chemical analysis of the *mtm* gene cluster will shed light on the precise functions and mechanisms of the remarkable post-polyketide synthase enzymes involved in the biosynthesis of this structurally interesting and medicinally important natural product.

Figure 4



Deduced biosynthetic pathway of mithramycinone, the mithramycin aglycone. The decaketide backbone synthesized by the *mtm* minimal PKS undergoes four successive intramolecular cyclization and dehydration steps, leading to the formation of tetracenomycin F1 (compound **1**) [18]. Decarboxylation follows to yield compound **2**, which then undergoes oxidative cleavage between C18 and C19, giving rise to compound **3**. Subsequent oxidation, reduction, and methylation reactions result in mithramycinone formation.

Significance

We show here that a hybrid polyketide synthase, which includes the mithramycin ketosynthase and chain-length factor together with the acyl carrier protein and ketoreductase from the actinorhodin polyketide synthase, results in the biosynthesis of a 20-carbon polyketide that undergoes a C-9 ketoreduction. Thus, the aglycones of mithramycin, chromomycin, and olivomycin must be derived from a single polyketide backbone, instead of two or three. The nascent polyketide backbone must undergo a series of regiospecific cyclizations to form a tetracenomycin-like tetracyclic intermediate. The final steps in the aglycone biosynthetic pathway presumably involve decarboxylation and oxidative cleavage between C-18 and C-19, followed by additional oxidation, reduction, and methylation reactions.

Our results rule out the possible involvement of a second polyketide synthase in the biosynthesis of the aureolic acid family of natural products. They also emphasize the value of a versatile host–vector system for heterologous expression of recombinant natural product gene clusters. Finally, the synthesis of a 'hybrid' polyketide by subunits from the mithramycin and the actinorhodin polyketide synthases once again highlights the potential for combinatorial biosynthesis of 'unnatural' natural products.

Materials and methods

Bacterial strains, plasmid construction, and culture conditions

The *mtm ketosynthase* and chain-length factor genes were engineered by PCR amplification as a *PacI–XbaI* cassette using the following primer sequences: (5') TTTTAAGCTTAATTAAGGAGGACCATCATGAACCGT-CGCGTCGTC; (3') TTTTGAATTCTAGATTAGGACAAGGCCCGCACGA. Plasmid pGB1 was constructed by replacing the *act* ketosynthase and *act* chain-length factor genes in pRM5 [15] with the corresponding *mtm* genes engineered as above.

Purification of polyketides from CH999/pGB1

S. coelicolor CH999/pGB1 was grown on R2YE agar medium (~500 ml) containing 50 mg l^{-1} thiostrepton at 30 °C for 6 days. The agar was chopped and extracted with 3 x 500 ml ethyl acetate:methanol (5:1) containing 1 % acetic acid. The solvent was removed under vacuum. The residue was applied to a Florisil column (Fisher Scientific) and eluted with ethyl acetate:ethanol:acetic acid (17:2:1). Eluted fractions were further purified on a Beckman HPLC using a preparative C-18 reverse-phase column (mobile phase, acetonitrile:water = 1:9 to 3:2 over a period of 53 min). The yields of the two most abundant products thus purified, RM20b and RM20c [17], were 45 mg and 6 mg, respectively.

NMR spectroscopy

^{13}C and ^{1}H NMR spectra were recorded on a Varian XL-400 using DMSO-d_6 as solvent. Spectra were referenced internally to the solvent for ^{13}C NMR and to TMS for ^{1}H NMR.

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