Folding of the polyketide chain is not dictated by minimal polyketide synthase in the biosynthesis of mithramycin and anthracycline

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Background: Mithramycin, nogalamycin and aclacinomycins are aromatic polyketide antibiotics that exhibit antitumour activity. The precursors of these antibiotics are formed via a polyketide biosynthetic pathway in which acetate (for mithramycinone and nogalamycinone) or propionate (for aklavinone) is used as a starter unit and nine acetates are used as extender units. The assembly of building blocks is catalyzed by the minimal polyketide synthase (PKS). Further steps include regiospecific reductions (if any) and cyclization. In the biosynthesis of mithramycin, however, ketoreduction is omitted and the regiospecificity of the first cyclization differs from that of anthracycline antibiotics (e.g. nogalamycin and aclacinomycins). These significant differences provide a convenient means to analyze the determinants for the regiospecificity of the first cyclization step.

Results: In order to analyze a possible role of the minimal PKS in the regiospecificity of the first cyclization in polyketide biosynthesis, we expressed the *mtm* locus, which includes mithramycin minimal PKS genes, in *Streptomyces galilaeus*, which normally makes aclacinomycins, and the *sno* locus, which includes nogalamycin minimal PKS genes, in *Streptomyces argillaceus*, which normally makes mithramycin. The host strains are defective in the minimal PKS, but they express other antibiotic biosynthesis genes. Expression of the *sno* minimal PKS in the *S. argillaceus* polyketide-deficient strain generated mithramycin production. Auramycins, instead of aclacinomycins, accumulated in the recombinant *S. galilaeus* strains, suggesting that the mithramycin minimal PKS is responsible for the choice of starter unit. We also describe structural analysis of the compounds accumulated by a ketoreductase-deficient *S. galilaeus* mutant; spectroscopic studies on the major polyketide compound that accumulated revealed a first ring closure which is not typical of anthracyclines, suggesting an important role for the ketoreductase in the regiospecificity of the first cyclization.

Conclusions: These experiments clearly support the involvement of ketoreductase and a cyclase in the regiospecific cyclization of the biosynthetic pathway for aromatic polyketides.

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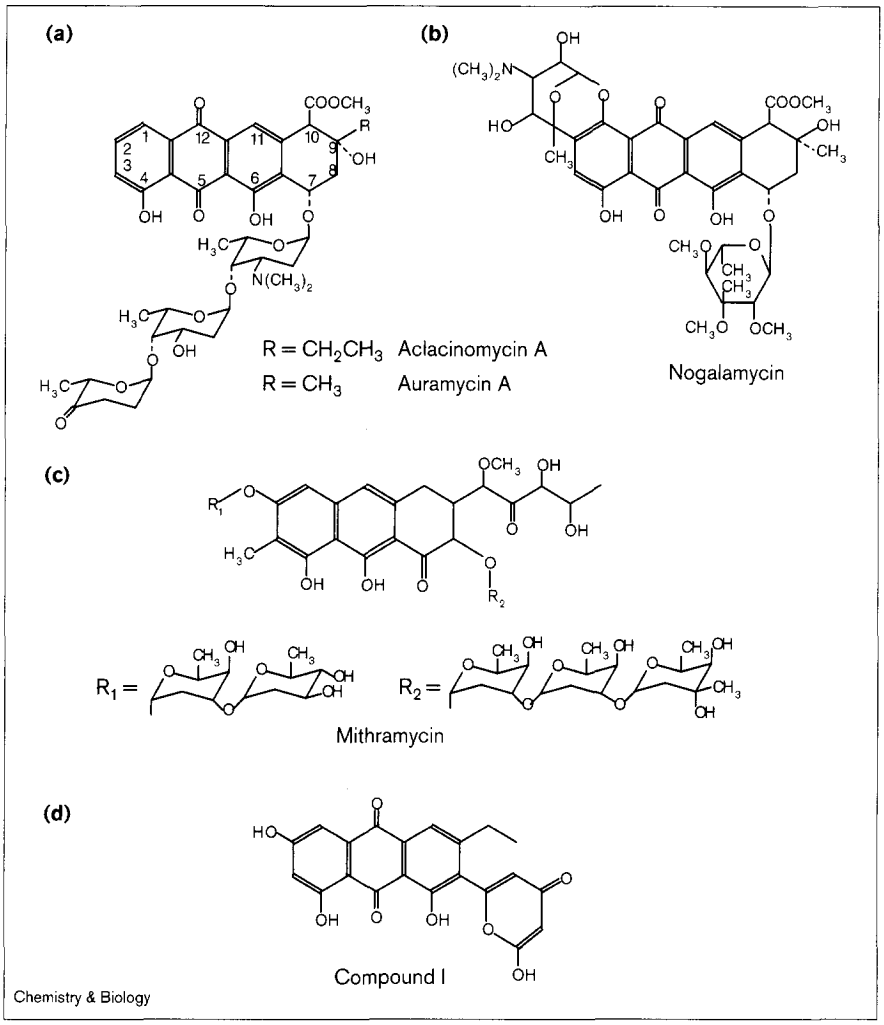
Introduction

Aromatic polyketides are a large group of structurally diverse natural products that exhibit a broad range of biological activities. Nevertheless, they share a common biosynthetic pathway in which single carboxylic acid residues (often acetates) are used to build up a polyketide chain in a reaction catalyzed by polyketide synthase (PKS). The enzyme complex responsible for forming the polyketide chain is the minimal PKS, which probably controls the choice of the starter unit and the number of extenders used in the assembled polyketide chain, thereby allowing variability in the polyketide backbones. In addition, the degree of reduction and regiospecific cyclizations of the polyketide chain gives structural diversity to the aromatic polyketides formed.

Mithramycin [1], aclacinomycins [2] and nogalamycin [3] (Figure 1) are aromatic polyketide antibiotics that exhibit powerful antitumour properties. Nogalamycin and aclacinomycin belong to the anthracycline group and mithramycin belongs to the aureolic acid group of antibiotics. The structures of the aglycone moieties in these two antibiotic groups differ remarkably from each other (Figure 1).

Anthracyclinone, the aglycone moiety of anthracyclines, is formed from the starter unit propionate or acetate and nine acetate extender units. Aklavinone, the aglycone moiety of aclacinomycins and a precursor of many other anthracyclines, uses propionate as a starter unit, whereas in nogalamycin biosynthesis, acetate is chosen to be the

Figure 1



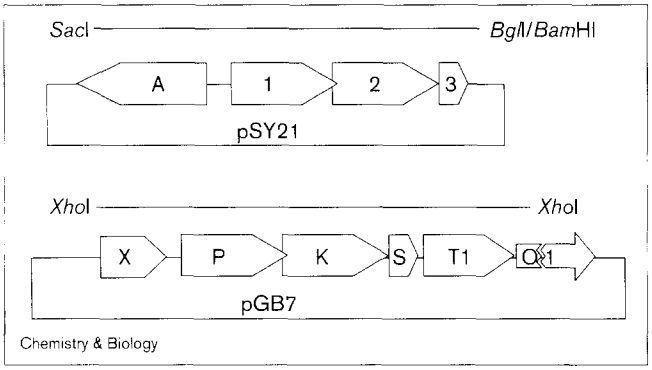
The structures of **(a)** aclacinomycin A and auramycin A, **(b)** nogalamycin, **(c)** mithramycin, and **(d)** compound I derived from H061. Nogalamycin and aclacinomycin belong to the anthracycline group of antibiotics and mithramycin belongs to the aureolic acid group.

starter unit. In both cases following the addition of nine acetate units to the primer, ketoreduction occurs at position nine (calculated from the carboxyl terminus; corresponding to C-2 in anthracyclines; Figure 1) and the intramolecular cyclization occurs at positions seven and 12 in the nascent polyketide chain (between C-7 and C-12 in anthracyclines). Similarly, mithramycinone is synthesized from ten acetates, but, in contrast to anthracyclinone biosynthesis, the ketoreduction does not occur at the polyketide stage and the first ring closure is formed between C-9 and C-14 in a similar manner to early tetracenomycin intermediates [4–7].

Studies of the biosynthesis of aromatic polyketides have been successful due to the structural analysis of several compounds synthesized by combinations of polyketide genes from different producers [8]. But because the compounds formed are derived from highly reactive early intermediates for biosynthesis, elucidation of the functions of the first enzymes participating in polyketide biosynthesis is complicated. Indeed, it is not clear which enzymes are really needed to determine the folding of a polyketide chain, and several possibilities have been suggested. A role for the minimal PKS in the first cyclization using individual genes was discussed by Fu *et al.* [9] in studies suggesting temporal control of cyclization. But studies of tetracenomycin biosynthesis, where a polyketide chain is not a substrate for a ketoreductase, suggest that a polyketide cyclase, rather than the minimal PKS, determines the first cyclization [10]. Furthermore, Kramer *et al.* [11] suggested that the auxiliary subunits of PKS determine the size and folding pattern of the polyketide chain.

Here, we report the expression of *mtm* mithramycin minimal PKS genes in *Streptomyces galilaeus* H028 and H039 mutants and of the *sno* nogalamycin minimal PKS genes in the *Streptomyces argillaceus* polyketide-deficient (PK^-) strain. The target strains H028 and PK^- were blocked in an early step of biosynthesis for aclacinomycins and mithramycin,

Figure 2



Expression constructs for nogalamycin PKS (pSY21) and mithramycin PKS (pGB7). A, *sno*A; 1, *sno1*; 2, *sno2*; 3, *sno3*; X, *mtmX*; P, *mtmP*; K, *mtmK*; S, *mtmS*; T1, *mtmT1*; O1, *mtmO1* (not complete). See Materials and methods section for gene functions.

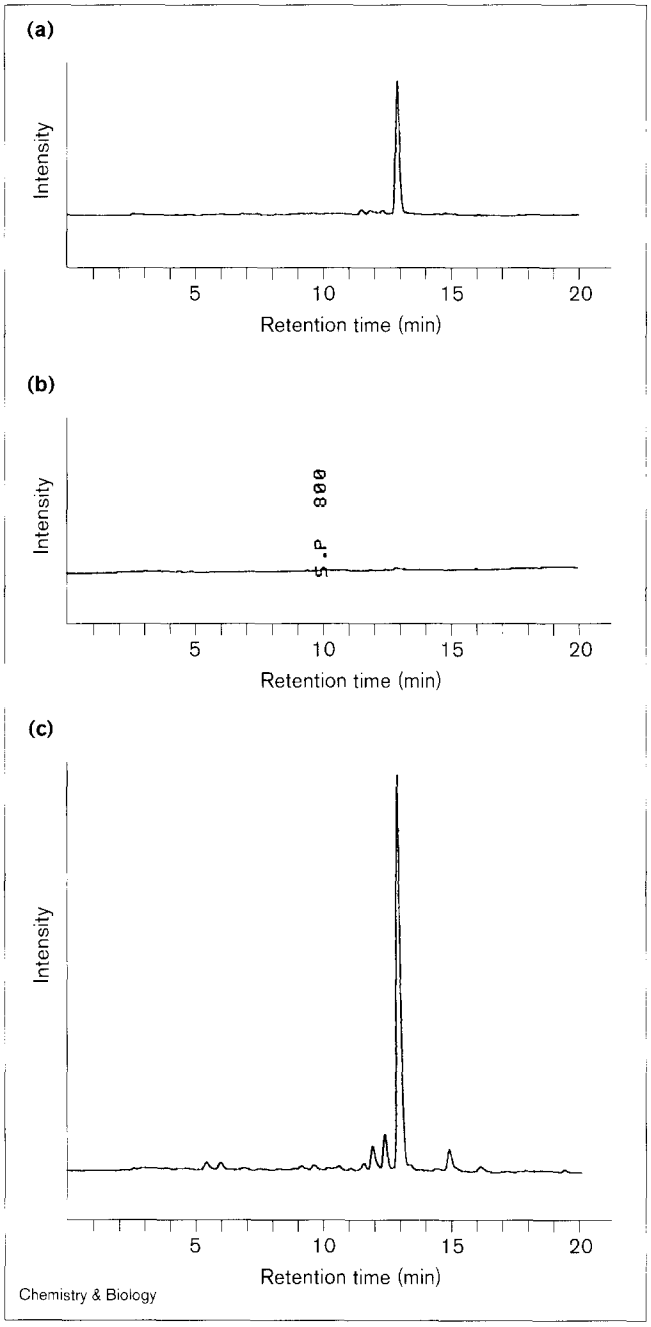
respectively; both strains failed to accumulate any coloured compounds in liquid cultures. Thus, this study supports the conclusion that the minimal PKS does not dictate the regiospecificity of the first cyclization.

Results and discussion

We recently cloned the genes for nogalamycin PKS and reported their expression in *S. galilaeus* [12]. The minimal PKS was cloned in plasmid pIJ486 to give pSY21 which, in addition to the minimal PKS open reading frames (ORFs; *sno1–sno3*), contains *sno*A, an activator promoting the expression of the minimal PKS genes (Figure 2). Auramycins were produced by pSY21 in *S. galilaeus* strains [12]. When pSY21 was introduced into the *S. argillaceus* PK$^-$ mutant, transformants produced only mithramycin. This was verified by purifying mithramycin from the wild-type strain and analyzing by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy; the resonances detected for the compound isolated from the recombinant strain PK$^-$/pSY21 corresponded to the spectroscopic data of mithramycin found in the literature. The products accumulated by the recombinant strain were compared with those of the wild-type strain and it was found that a higher yield of mithramycin was obtained by the PK$^-$ mutant carrying pSY21 even though all the compounds were accumulated by the wild type as well. In Figure 3, the high performance liquid chromatography (HPLC) traces for the samples are shown. The recombinant strain PK$^-$/pSY21 accumulated mithramycin, but not the compounds sharing the folding pathway of anthracyclines (Figure 4), suggesting that the regiospecificity of the first ring formation in this case is not dictated by minimal PKS.

A gene cluster encoding the mithramycin PKS (Figure 2) was previously isolated and characterized [5,13]. The genes for the minimal PKS (Figure 2) were cloned into *Streptomyces lividans* TK24 and transferred into the *S. galilaeus*

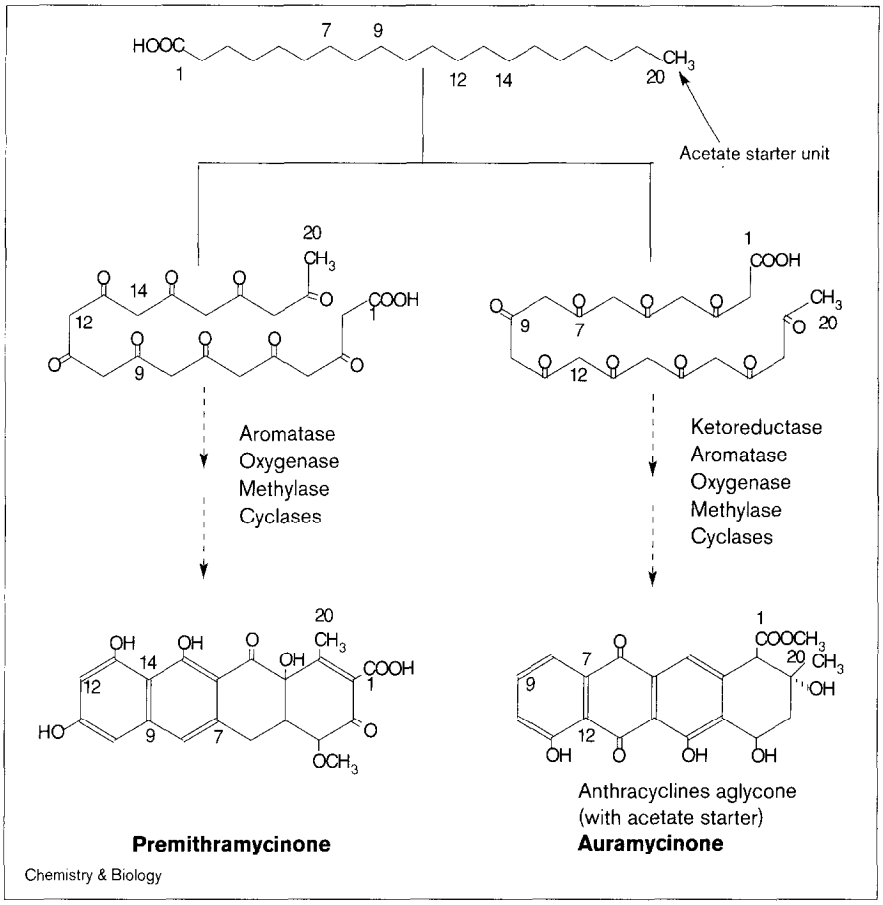
Figure 3



High performance liquid chromatography (HPLC) traces of the samples from **(a)** wild-type *Streptomyces argillaceus*, **(b)** PK$^-$, and **(c)** PK$^-$/pSY21. The major peak (retention time = 13 min) corresponds to mithramycin. Minor products were not identified.

mutants, H039 and H028. H039 is a mutant blocked in rhodosamine and deoxyfucose biosynthesis, but was used here because of a higher transformation frequency than in the other mutants or the wild-type *S. galilaeus*. The non-producing mutant chosen, H028, was previously shown to be complemented by the genes for the nogalamycin PKS, resulting in the production of auramycins. Similarly,

Figure 4



Two different folding patterns in polyketide assembly. The polyketide (top) is derived from an acetate starter unit (indicated) and nine acetate extender units. The two folding pathways are distinct and result in two different products. The key difference is that the left-hand pathway does not involve ketoreduction.

expression of the *mtm* genes in H028 and H039 resulted in the production of auramycins. Nevertheless, the compound with the largest fraction of the total products was found to be auramycinone, by comparing to a standard by thin layer chromatography (TLC) and HPLC and by confirming the structure of a purified major product from H039/pGB7 by mass spectroscopy. The main signals detected were at *m/z* (%): 398 M+ (12), 380 (15), 362 (100), 347 (16) 331 (30), 321 (35) and 303 (13). The accumulation of auramycins in the *S. galilaeus* mutants expressing the mithramycin minimal PKS suggests that the host strain determines the folding of a polyketide chain, whereas the use of acetate as the starter unit in biosynthesis is dictated by *mtm* genes.

H061 is a polyketide ketoreductase deficient mutant derived from the aclacinomycin biosynthetic pathway (as shown by complementation by a gene encoding a nogalamycin polyketide ketoreductase, KR [12]). The structural analysis of the main product compound I, 60% of the whole extract obtained from the culture, revealed a structure in which the first intramolecular condensation had occurred between C-10 and C-15 (see Figure 1a); this is different from the condensation seen between C-7 and C-12 in anthracyclines (e.g. auramycinone, Figure 4). Thus, the structure of the main product seen here suggests that the folding of a linear polyketide chain in PKSs that contain KR is influenced by the KR subunit.

Significance

Nogalamycin, mithramycin and aclacinomycins are aromatic polyketide antibiotics that exhibit antitumour activity. Using genes encoding the minimal polyketide synthase (PKS) for nogalamycin and mithramycin biosynthesis (which have different patterns of ketoreduction and first cyclization) in *Streptomyces* strains that lack endogenous minimal PKS activity, we have demonstrated that the folding of a polyketide chain is not dictated by the minimal PKS. This study also demonstrates the usefulness of the blocked mutants in analyzing the functions of the genes, because the unwanted shunt products (formed by spontaneous reactions and not used as intermediates for biosynthesis) derived from highly reactive intermediates that appear in genetic engineering systems are avoided. The concomitant action of the PKS genes influences the structure formed, making it difficult to analyze the functions of individual genes in the system missing the structural PKS components. It is possible that the enzyme complex binds the nascent polyketide

chain, keeping it in the correct orientation to promote the catalytic activities of the proteins. Mutants that have intact enzymes, but missing catalytic activities, might be suitable tools for determining the functions of the genes. In this study, PKS components other than the minimal PKS were expressed in the host strains, thereby demonstrating that the minimal PKS is not essential for regiospecific cyclization.

Materials and methods

Bacterial strains, plasmids, and culture conditions

S. argillaceus ATCC 12596 is a mithramycin producer and *S. galilaeus* ATCC 31615 is an aclacinomycin producer strain. A non-producing *S. galilaeus* mutant H028 was obtained by NTG (N-methyl-N′-nitro-N-nitrosoguanide) mutagenization of the wild type [14]. H061 is an *S. galilaeus* mutant deficient in polyketide ketoreductase [14]. *S. galilaeus* H039 is blocked in rhodosamine and deoxyfucose biosynthesis [14], but the relevant characteristic here is a better transformation of H039 than that of the wild type or other mutants. The *S. argillaceus* PK$^-$ mutant has a deletion affecting the *mtm*P and *mtm*K genes of the mithramycin minimal PKS [13]. Plasmid pSY21 contains the genes for the minimal PKS of nogalamycin biosynthesis and the regulator gene *snoA* encoding an activator that promotes the expression of the minimal PKS genes [12]. Plasmid pGB7 is a pWHM3 derivative that contains the genes for the mithramycin minimal PKS (*mtm*P, *mtm*K and *mtm*S) in addition to three other genes (*mtm*X encodes presumably the last cyclase in mithramycinone biosynthesis; *mtm*T1 encodes a putative ketoreductase and an incomplete *mtm*O1; [7,13]). pSY21 was introduced into the *S. argillaceus* wild type and into the PK$^-$ mutant by protoplast transformation. In the same manner, pGB7 was propagated in *S. lividans* and subsequently introduced into H039 by transformation. Plasmids isolated from H039 were then introduced into the *S. galilaeus* blocked mutant, H028. Transformants were selected for thiostrepton resistance.

Production and identification of compounds

E1 medium containing glucose (20 g/l), starch (20 g/l), Pharmamedia (Trader's protein; 5 g/l), yeast extract (2.5 g/l), $CaCO_3$ (3 g/l), NaCl (1 g/l), $MgSO_4{\cdot}7H_2O$ (1 g/l) and K_2HPO_4 (1 g/l) in tap water (pH 7.5) was used to produce anthracyclines and mithramycin. Cultivation was carried out in 250 ml Erlenmayer flasks containing 60 ml of E1 medium at 30°C for 4–7 days in a rotary shaker at 300 rpm. Culture samples, adjusted to pH 7, were extracted with methanol-chloroform (1:2). Chloroform extracts were dried and the production profiles were determined by HPLC on a Merck–Hitachi instrument (L-6200A/L-4250) using a Merck LiChrocart RP-18 column. The eluent used was a gradient of acetonitrile: potassium dihydrogen phosphate buffer (60 mM; pH 3.0 adjusted with citric acid). The flow-rate was 1 ml/min and detection was at 420 nm. The purification of the main product from H039/pGB7 was done by preparative thin-layer chromatography using Silica Gel $60F_{254}$ (Merck & Co.). Development was done with toluene:ethyl acetate:methanol:formic acid (50:50:15:10, by vol.). Mass spectroscopy was performed on a Varian VG707E spectrometer.

Isolation of the H061 product

Strain H061 was grown in a 10 l fermenter (E1-medium, 28°C, 500 rpm, 20 l/min) for 6 days. Compounds were extracted separately from mycelia and supernatant with dichloromethane/methanol at pH 3.0. Solvents were removed under vacuum and viscous residue was flashed through a polyamide 11 column, eluent being water:methanol (1:9 to 0:10). Fractions were further purified on a Merck–Hitachi HPLC using preparative reversed phase column C-18 (mobile phase; acetonitrile:phosphate buffer 1:1).

Nuclear magnetic resonance spectroscopy

^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer using DMSO-d_6 as a solvent. NMR-analysis also included nuclear Overhauser enhancement (NOE) difference, DEPT and HMBC techniques. Spectra were internally referenced to tetramethylsilane.

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