Oxidative cleavage of premithramycin B is one of the last steps in the biosynthesis of the antitumor drug mithramycin

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Background: Mithramycin is a member of the clinically important aureolic acid group of antitumor drugs that interact with GC-rich regions of DNA nonintercalatively. These drugs contain a chromophore aglycon that is derived from condensation of ten acetate units (catalyzed by a type II polyketide synthase). The aglycones are glycosylated at two positions with different chain length deoxyoligosaccharides, which are essential for the antitumor activity. During the early stages of mithramycin biosynthesis, tetracyclic intermediates of the tetracycline-type occur, which must be converted at later stages into the tricyclic glycosylated molecule, presumably through oxidative breakage of the fourth ring.

Results: Two intermediates in the mithramycin biosynthetic pathway, 4-demethyl-premithramycinone and premithramycin B, were identified in a mutant lacking the mithramycin glycosyltransferase and methyltransferase genes and in the same mutant complemented with the deleted genes, respectively. Premithramycin B contains five deoxysugars moieties (like mithramycin), but contains a tetracyclic aglycon moiety instead of a tricyclic aglycon. We hypothesized that transcription of *mtmOIV* (encoding an oxygenase) was impaired in this strain, preventing oxidative breakage of the fourth ring of premithramycin B. Inactivating *mtmOIV* generated a mithramycin nonproducing mutant that accumulated premithramycin B instead of mithramycin. *In vitro* assays demonstrated that MtmOIV converted premithramycin B into a tricyclic compound.

Conclusions: In the late stages of mithramycin biosynthesis by *Strepyomyces argillaceus*, a fully glycosylated tetracyclic tetracycline-like intermediate (premithramycin B) is converted into a tricyclic compound by the oxygenase MtmOIV. This oxygenase inserts an oxygen (Baeyer–Villiger oxidation) and opens the resulting lactone. The following decarboxylation and ketoreduction steps lead to mithramycin. Opening of the fourth ring represents one of the last steps in mithramycin biosynthesis.

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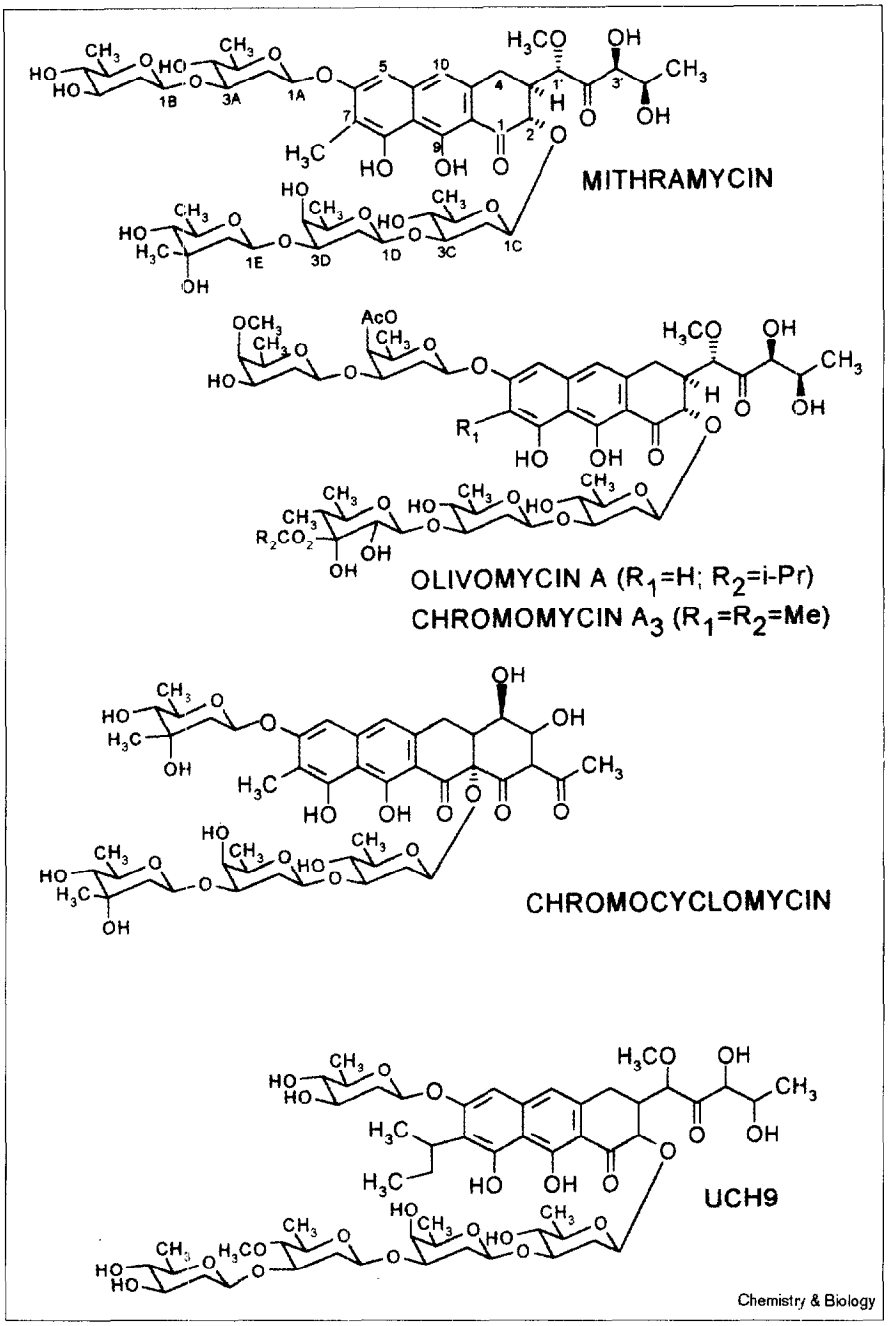
Introduction

The aureolic acid group of antitumor agents is comprised of mithramycin, chromomycin, olivomycins, chromocyclomycin and UCH9 [1–6]. They interact with GC-rich regions of DNA in a non-intercalative way in the presence of Mg^{2+}, which is essential for activity [7,8]. Mithramycin is the only aureolic acid drug used clinically. It shows a remarkable cytotoxicity against a variety of tumor cell lines and has been used in the treatment of certain tumors, including disseminated embryonal cell carcinoma and Paget's bone disease. It is also used to control hypercalcemia in patients with malignant diseases [4,5].

Structurally, all the members of the group (with the exception of chromocyclomycin) consist of a tricyclic chromophore that is glycosylated at two different positions of the aglycon with saccharides of various chain lengths (Figure 1). Chromocyclomycin differs in having a tetracyclic instead of a tricyclic chromophore. The polyketide aglycon of these compounds is derived from the condensation of ten acetates in a series of condensation reactions catalysed by a type II polyketide synthase [9–11]. Early reports suggested that the aureolic acid aglycon could be derived from the condensation of two or even three independently synthesized polyketide chains [12,13]. This hypothesis was ruled out, however, on the basis of results of two heterologous expression experiments. Expression of the mithramycin ketoacylsynthase (*mtmP*) and chain length factor (*mtmK*) genes in *Streptomyces coelicolor* CH999, an appropriately engineered

Figure 1



Structures of the aureolic acid group of antitumor agents.

expression host, led to the formation of the 20-carbon polyketides RM20b,c [9], and expression of the mithramycin minimal polyketide synthase alone in *S. lividans* produced SEK15 [14]. Isolation of premithramycinone and its nonmethylated derivative 4-demethyl-premithramycinone from a mutant blocked at early steps in 6-deoxysugar biosynthesis showed that biosynthesis of mithramycin proceeds through tetracyclic tetracycline-like intermediates [15]. It was proposed that transformation of the tetracyclic into the tricyclic chromophore could occur through an oxidative cleavage of the fourth ring [9,15–17]. It was not clear when this oxidative cleavage should take place, however — before, during or after glycosylation. Recently, several glycosylated intermediates of the mithramycin pathway have been isolated through the insertional inactivation of two genes (*mtmGI* and *mtmGII*) that code for the two glycosyltransferases responsible for disaccharide formation and its transfer to the aglycon [18]. Three of these intermediates were tetracyclic compounds containing one sugar (premithramycin A1), two sugars (premithramycin A2) or three sugars (premithramycin A3) attached to the C-12a-O of the

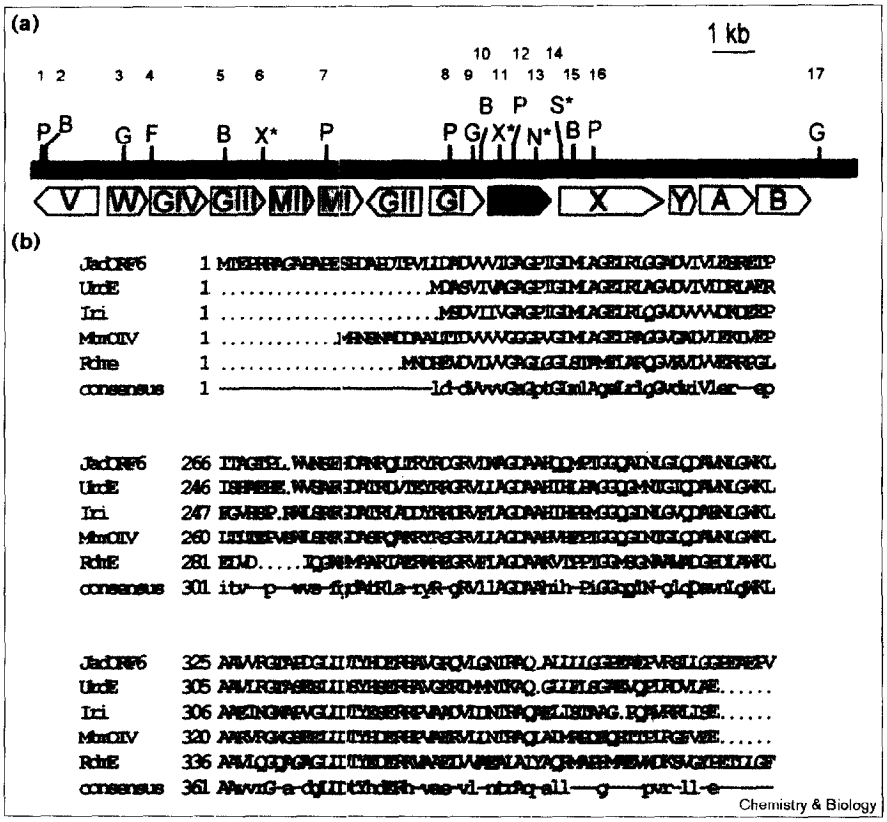
aglycon. The last compound (premithramycin A4) resembled premithramycin A3, except that the fourth ring was already open. Here we report the cloning, sequencing, expression and insertional inactivation of a gene, *mtmOIV*, from the mithramycin producer *S. argillaceus* that codes for an oxygenase. Several lines of experimental evidence show that action of this oxygenase leads to the breakage of the fourth ring of an inactive biosynthetic intermediate, premithramycin B, and that this event occurs after complete formation of both oligosaccharide chains.

Results and discussion

The *mtmOIV* gene encodes an oxygenase

The mithramycin biosynthetic gene cluster has been isolated within three overlapping cosmid clones of genomic DNA from the mithramycin producer *S. argillaceus*. It extends over ~50 kilobases (kb) and contains 34 genes [11]. At the right-hand side of the cluster two genes (*mtmGII* and *mtmGI*) that encode glycosyltransferases responsible for the transfer of the D-olivose disaccharide to the aglycon [18] and four genes (*mtrX*, *mtrY*, *mtrA* and *mtrB*) involved in resistance to and secretion of mithramycin [19] have been cloned and characterized. We have now sequenced a 2.1 kb *Bam*HI fragment located (and partially overlapping) between the *mtmGI* and *mtrX* genes (Figure 2a). Analysis of the sequence using the CODONPREFERENCE program [20] revealed an open reading frame (named *mtmOIV*) with the characteristic codon usage of *Streptomyces* genes. The *mtmOIV* gene is comprised of 1602 nucleotides and would code for a 534 amino acid polypeptide with an estimated molecular weight (M_r) of 57,128. There are two potential consecutive ATG starting codons for *mtmOIV* that overlap the stop codon of *mtmGI*. Between *mtmOIV* and *mtrX* there is an intergenic region of 70 nucleotides that, when analysed using the STEMLOOP program [20], has a long inverted repeat sequence capable of forming a stem–loop secondary structure that has a formation energy of −19.2 kcal/mol (calculated using the FOLD program [21]). This stem–loop probably functions as a transcriptional terminator, as has been described previously in *Streptomyces* species [22,23]. Therefore, *mtmOIV*, together with *mtmGI*, might form part of the same transcriptional unit transcribed independently of *mtrX*. Comparison of the deduced product of *mtmOIV* with databases using the BLAST program [24] revealed similarities with various oxygenases: 44% identity with the JadORF6 oxygenase of the jadomycin pathway from *S. venezuelae* [25], 44% identity with the Iri protein of *Rhodococcus equi* involved in resistance to rifampicin [26], 43% identity with the UrdE oxygenase of the urdamycin pathway from *S. fradiae* [27] and 33.1% identity with the RdmE oxygenase

Figure 2



(a) Schematic organization of the region of the mithramycin biosynthetic gene cluster that surrounds the *mtmOIV* gene. B, *Bam*HI; F, *Fsp*I; G, *Bgl*II; N, *Not*I; P, *Pst*I; S, *Sal*I; X, *Xho*I. The restriction sites indicated with an asterisk are not unique sites in the region shown. Designations for the genes are as follows: V and W represent the *mtmV* and *mtmW* genes, which are sugar biosynthetic genes; MI and MII represent the *mtmI* and *mtmII* genes that encode methyltransferases; GI, GII, GIII and GIV represent the *mtmGI*, *mtmGII*, *mtmGIII* and *mtmGIV* genes that encode glycosyltransferases; X, Y, A and B represent the *mtrX*, *mtrY*, *mtrA* and *mtrB* genes involved in mithramycin resistance and secretion.
(b) Pile-up comparison of the amino-acid sequences of different oxygenases showing the two motifs present in many FAD- and NADPH-dependent enzymes. JadORF6, jadomycin hydroxylase from *S. venezuelae* [25]; UrdE, urdamycin hydroxylase from *S. fradiae* [27]; Iri, oxygenase from *Rhodococcus equi* involved in resistance to rifampicin [26]; RdmE, rhodomycin hydroxylase from *S. purpurascens* [28]; MtmOIV, mithramycin oxygenase (this paper).

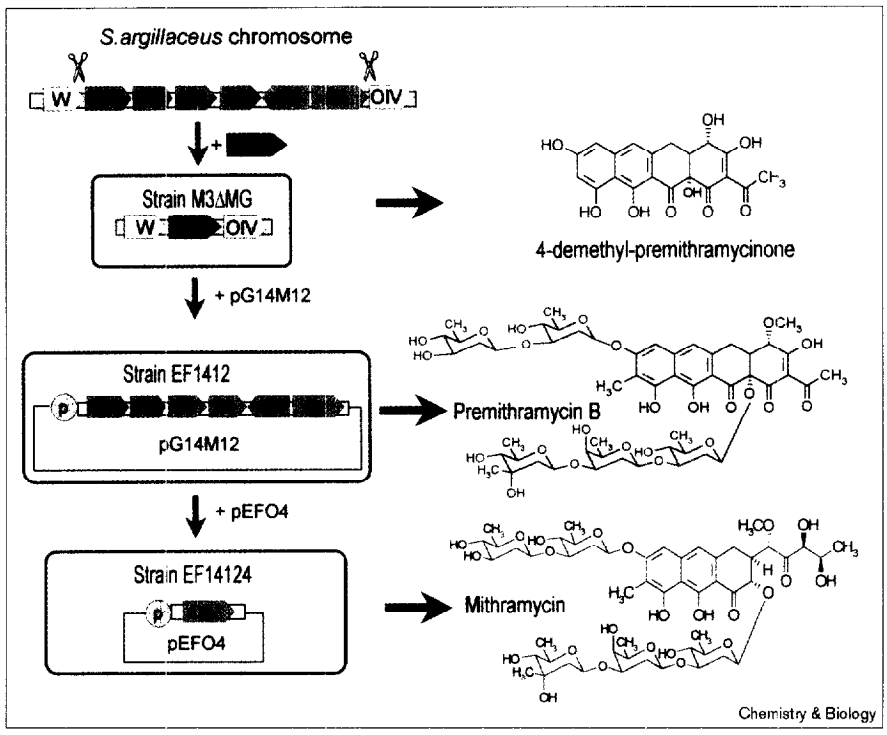
from the rhodomycin pathway in *S. purpurascens* [28]. All of these oxygenases contain two conserved motifs that are characteristic of flavin-type hydroxylases (Figure 2): a region very close to the amino terminus (Figure 2b), the so-called βαβ fold, that is involved in binding of the ADP moiety of FAD [29] and a second region that is believed to be involved in the binding of the ribityl chain of FAD [30,31].

According to isotope-labeling studies using 1-^{13}C, $^{18}O_2$-labeled acetate and fermentation under $^{18}O_2$, three of the oxygens in the aureolic acid aglycon are derived from molecular oxygen and the rest of them are derived from acetate oxygen atoms [11]. Consequently, we initially thought that the MtmOIV oxygenase might introduce some of these oxygens into the aglycon. Three other oxygenases have been identified in the mithramycin biosynthetic cluster, however. Two of the oxygenases (MtmOI and MtmOIII) do not seem to be essential in the pathway as their inactivation does not affect mithramycin biosynthesis [17]. The third oxygenase, MtmOII, is probably responsible for introducing two hydroxyl groups into 4-demethyl-premithramycinone [17]. On this basis, and to assign a role for the MtmOIV oxygenase in mithramycin biosynthesis, MtmOIV was tentatively proposed as a candidate for catalysing the oxidative cleavage of the fourth ring of the aglycon of the mithramycin precursor.

A mutant lacking the mithramycin glycosyltransferases and methyltransferases is impaired in *mtmOIV* expression

A clue to the possible involvement of MtmOIV oxygenase in fourth-ring breakage arose serendipitously during the course of insertional inactivation experiments in the mithramycin glycosyltransferase region. To determine the individual role of the four glycosyltransferase genes (*mtmGI*, *mtmGII*, *mtmGIII* and *mtmGIV*) in sugar transfer during mithramycin biosynthesis, a deletion mutant lacking the four glycosyltransferase genes (and the two methyltransferase genes located between *mtmGIII* and *mtmGII*; Figure 2a) was generated using the plasmid construct pEFΔMG. The construct consisted of an erythromycin resistance cassette flanked by *S. argillaceus* genomic DNA from just outside the glycosyltransferase and methyltransferase region (Figure 3). After transforming *S. argillaceus* protoplasts with this construct, several erythromycin-resistant thiostrepton-sensitive colonies were obtained (thiostrepton was the antibiotic marker in the plasmid and erythromycin was the marker used to select for the replacement). Southern blot hybridization verified that these recombinant strains had arisen as a consequence of a Campbell-type double crossover that deleted the wild-type genomic DNA region. High-performance liquid chromatography (HPLC) analyses of culture supernatants of these recombinant strains revealed that they did not produce mithramycin but instead accumulated 4-demethyl-premithramycinone (Figure 3).

Figure 3



Evidence for a role of MtmOIV in fourth ring breakage. The M3ΔMG mutant was generated by deleting four glycosyltransferase and two methyltransferase genes from the chromosome of *S. argillaceus*; this mutant accumulated 4-demethyl-premithramycinone instead of mithramycin. Complementation experiments with a plasmid expressing all these genes produced premithramycin B. Fourth-ring breakage and conversion of premithramycin B into mithramycin was enabled by further cloning and expression of *mtmOIV* gene. p, erythromycin resistance promoter.

This is a tetracyclic compound that lacks sugars and the two methyl groups at the C-4-O and C-9 of the aglycon [15]; the methyl groups are present at these positions in premithramycins A2, A3 and B, as well as in the corresponding positions of the mithramycin aglycon. These two methyl groups are introduced by two methyltransferases coded by the *mtmMI* (O-4-methylation) and *mtmMII* (C-9-methylation) genes (M.J. Fernández Lozano, C.M. and J.A.S., unpublished observations).

In order to verify that in the generation of this mutant (named M3ΔMG) only the deleted glycosyltransferase and methyltransferase genes were affected, we carried out a complementation experiment. An 8 kb *Bgl*II–*Xho*I DNA fragment (sites 3–11 in Figure 2a) containing the four glycosyltransferase and two methyltransferase genes was subcloned into the expression vector pEM4. In this construct, expression of the *mtmGIV*, *mtmGIII*, *mtmMII* and *mtmMI* genes was controlled by the erythromycin resistance promoter (ermE*) and the *mtmGII* and *mtmGI* genes were controlled by their own divergent promoters. This plasmid construct (pG14M12; Figure 3) was used to transform protoplasts of the M3ΔMG mutant strain and several transformants were analysed for the production of mithramycin using HPLC. Most of them produced mithramycin but normal levels of drug production were not restored, indicating that transcription of other gene(s) was probably impaired in these clones. Some of the transformants showed a new HPLC peak with a higher retention time than most of the mithramycin intermediates isolated thus far. In one of the transformants (strain EF1412) the new peak was also the major peak. After purification of the material present in this peak its structure was elucidated using nuclear magnetic resonance (NMR) and mass spectroscopy (MS; see below) and found to be a tetracyclic compound containing both methyl groups at C-4-O and C-9 and both oligosaccharide chains attached at the appropriate positions in the aglycon (Figure 3). This compound, designated premithramycin B, differs from mithramycin only in the aglycon moiety, which is tetracyclic in contrast to the tricyclic aglycon of mithramycin, and in the 4′-OH group present in mithramycin. The corresponding 1′-carbonyl group in premithramycin B is unreduced. This fact, together with the genomic location of *mtmOIV*, immediately downstream of *mtmGI*, prompted us to consider that perhaps expression of the gene encoding the enzyme responsible for breaking the fourth ring was impaired in the recombinant strain EF1412, which in turn suggested that the MtmOIV oxygenase might oxidatively cleave the fourth ring. To test this hypothesis we subcloned the *mtmOIV* gene alone under the control of the *ermE** promoter (pEFOIV) and transformed protoplasts of strain EF1412 (strain M3ΔMG, which contained pG14M12 with the glycosyltransferase and methyltransferase genes). All the transformants recovered the normal levels of mithramycin production (see strain EF14124 in Figure 3), suggesting that the MtmOIV oxygenase was impaired in the EF1412 strain, probably because its normal transcription was affected. The conclusion from all these experiments is that the *mtmOIV* gene codes an oxygenase that presumably is responsible for the breakage of the fourth ring of premithramycin B as one of the last steps in the mithramycin biosynthesis.

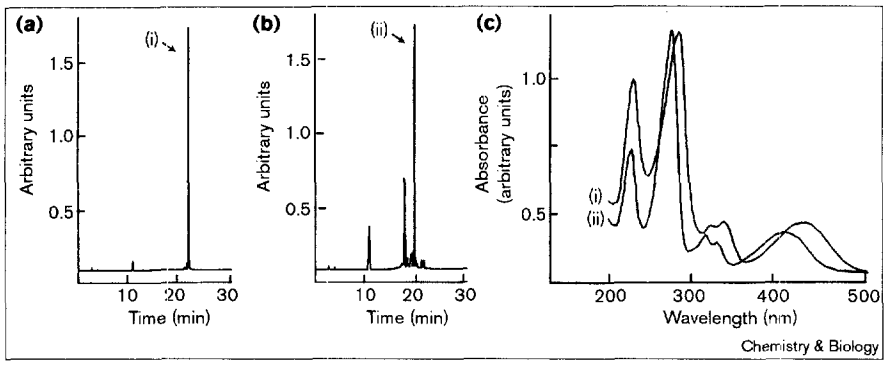
Insertional inactivation of *mtmOIV* gene precludes fourth ring breakage

To obtain further and definitive evidence for the involvement of MtmOIV in fourth ring breakage, the M3ΔO4 mutant was generated by deleting an internal fragment of *mtmOIV* and replacing it with an apramycin resistance cassette (see the Materials and methods section). After transforming *S. argillaceus* protoplasts with the appropriate construct (pLPΔO4) 11 apramycin-resistant transformants were obtained, two of which were simultaneously thiostrepton-sensitive, suggesting that they were the consequence of a double crossover. This was verified by Southern blot hybridization. HPLC analysis of culture supernatant of one of these mutants (M3ΔO4) showed that premithramycin B accumulated instead of mithramycin. To clearly determine that the gene replacement event only affected the *mtmOIV* gene and not other genes, *mtmOIV* was subcloned into the shuttle vector pIAGO under the control of the erythromycin resistance promoter (pLPO4) and introduced by transformation into M3ΔO4. In all the transformants obtained production of mithramycin was restored, indicating that the accumulation of premithramycin B by this mutant was the consequence of inactivation of the *mtmOIV* gene.

The MtmOIV oxygenase causes *in vitro* oxidative breakage of the fourth ring of premithramycin B

Biochemical confirmation of the fourth-ring cleavage of premithramycin B by the MtmOIV oxygenase was obtained after expressing the *mtmOIV* gene and using it in *in vitro* enzymatic assays. pLPO4 was transformed into *S. albus* protoplasts and cell-free extracts of one of the transformants (strain LPO4) were fractionated by ammonium sulphate precipitation. Breakage of the fourth ring of premithramycin B was then assayed as described in the Materials and methods section. Premithramycin B (Figure 4a) was converted into a new compound with an HPLC retention time very close to that of mithramycin (Figure 4b). The absorption spectrum of this new compound (ii in Figure 4c) was identical to that of mithramycin but differed from premithramycin B (i in Figure 4c) in being displaced to shorter wavelengths, suggesting that, in the new compound, fourth-ring breakage had occurred. When premithramycin B was incubated with extracts from the control strain (strain IAGO, which contained only the vector), no modification of premithramycin B was observed (data not shown). Most of the activity was detected in the 25–40% protein fraction of

Figure 4



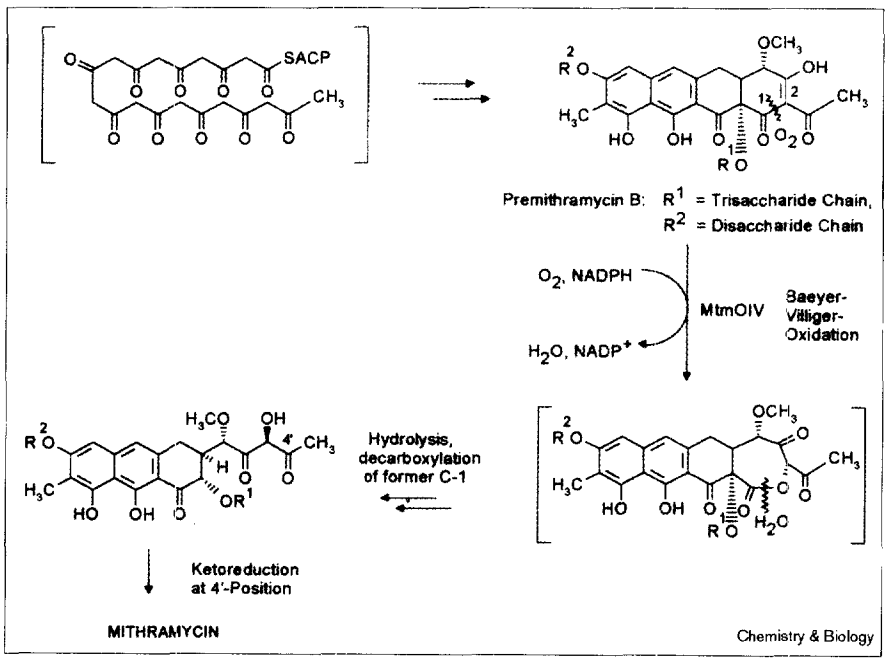
HPLC analysis of the reaction catalysed by the MtmOIV oxygenase. **(a)** Chromatogram of premithramycin B. **(b)** Chromatogram of the reaction mixture after 2 h of incubation. **(c)** Absorption spectra of peaks (i) and (ii).

strain LPO4. The addition of either NADH or NADPH was absolutely necessary for activity, with the requirement for NADPH much higher than that for NADH (data not shown). Breakage of the fourth ring of premithramycin B did not render the final molecule mithramycin. In addition to the oxidative ring cleavage, loss of one carbon unit, presumably as CO_2, must occur, and this might take place spontaneously. Furthermore, a reductive step is required to form the 4′-OH group of mithramycin (Figure 5). Consequently, the action of the MtmOIV oxygenase would render an intermediate compound between premithramycin B and mithramycin, probably the substrate for a ketoreductase that would catalyse the final step in the mithramycin biosynthesis. Candidates for this ketoreduction step could be the products of *mtmTI* [10], *mtmTII* or *mtmTIII* [17] ketoreductase genes, for which clear and definitive functions have not yet been assigned in mithramycin biosynthesis.

Structure elucidation of premithramycin B

The structure of premithramycin B was elucidated using physicochemical methods. The negative fast atom bombardment (FAB) MS (m/z 1092, $M\text{-}H^-$) in combination with the ^{1}H- and the ^{13}C-NMR spectra indicated the molecular formula $C_{53}H_{72}O_{24}$. The ^{1}H- and ^{13}C-NMR spectra also revealed the presence of five sugar moieties (recognizable in particular from the anomeric carbon signals at δ 101.4, 100.7, 98.4, 98.3, 96.9) and the 9-methyl group (δ_H 2.15 s; δ_C 8.4 q). The ultraviolet spectrum, as well as further NMR data of the aglycon moiety, was almost identical to those of

Figure 5



Proposed mechanisms for the MtmOIV oxygenase reaction and the following steps. This Baeyer–Villiger monooxygenase mechanism is consistent with the *in vitro* studies of MtmOIV, which require NADPH as a cofactor.

Table 1

^{1}H NMR data of premithramycin B comparison to premithramycin A3 and mithramycin in d_6-acetone, δ in ppm relative to TMS (J in Hz).

	Premithramycin B*	Premithramycin A3*	Mithramycin†
2-H	–	–	4.75 d (11.5)
3-H	–	–	2.76 dddd (11.5, 11, 3, 1.5)
4-H	4.21 br	4.04 s br‡	a:2.84-2.88§ e:2.63 dd (16, 3)
4-OMe	3.53 s#	3.52 s	–
4a-H	3.16 m	3.12 ddd (11, 4, 3)	–
5-H	a: 3.98 br d (17) e: 3.13 br d (17)	a: 3.79 ddd (16.5, 4, 1) e: 3.05 dd (16.5 , 3)	6.82 s
6-H	6.96 s	6.93 d (1)	–
7-H	6.63 s	6.71 s	–
7-Me	–	–	2.11 s
9-H	–	–	–
9-CH_3	2.13 s	2.16 s	–
10-H	–	–	6.80 s
1′-H	–	–	4.85 d (1.5)
1′-OMe	–	–	3.44 s
2′-H_3	2.65 br s	2.59 s	–
3′-H	–	–	4.23 d (2.5)
4′-H	–	–	4.21 dq (6.5, 2.5)
5′-H_3	–	–	1.26 d (6.5)
1A-H	5.42 dd (10, 2)	–	5.21 dd (10, 2)
2A-H*	1.88 ddd (12, 12, 10)	–	1.79 ddd (12, 12, 10)
2A-H#	2.50 ddd (12, 5, 2)	–	2.29 ddd (12, 5, 2)
3A-H	3.87 ddd (12, 8.5, 5)		3.60-3.75¶
4A-H	3.09 dd (9, 8.5)		3.04 dd (9, 9)
5A-H	3.77 dq (9, 6)		3.49 dq (9, 6)
6A-H_3	1.35 d (6)	–	1.32 d (6)
1B-H	4.77 dd (10, 2)	–	4.68 dd (10, 2)
2B-H*	1.58 ddd (12.5, 12, 10)	–	1.54 ddd (12, 12, 10)
2B-H#	2.21 ddd (12.5, 5, 2)	–	2.18 ddd (12, 5, 2)
3B-H	3.60 ddd (12, 9, 5)	–	3.58 ddd (12, 9, 5)
4B-H	3.01 dd (9, 9)	–	2.94 dd (9, 9)
5B-H	3.42 dq (9, 6)c	–	3.40 dq (9, 6)‡
6B-H_3	1.35 d (6)	–	1.29 d (6)
1C-H	5.00 br d (10)	4.88 dd (9.5, 2)	5.10 dd (10, 2)
2C-H*	1.80 ddd (12, 12, 10)	1.55 ddd (12, 12, 9.5)	1.61 ddd (12, 12, 10)
2C-H#	2.79 m br	2.55 m br	2.56 ddd (12, 5, 2)
3C-H	3.67 md	3.55 ddd (12, 8.5, 5)§	3.60 m§
4C-H	3.03 dd (9, 9)	2.90 dd (9, 8.5)#	2.94 dd (9, 9)
5C-H	3.43 dq (9, 6)‡	3.29 dq (9, 6)	3.31 dq (9, 6)‡
6C-H_3	1.39 d (6)	1.24 d (6)	1.29 d (6)
1D-H	4.69 dd (10, 2)f	4.62 dd (10, 2)	4.67 dd (10, 2)
2D-H*	1.75 ddd (12.5, 12, 10)	1.74 ddd (12, 12, 10)	1.78 ddd (12, 12, 10)
2D-H#	1.92 ddd (12, 4.5, 2)	1.90 ddd (12, 4.5, 2)	1.94 ddd (12, 4.5, 2)
3D-H	3.90 ddd (12.5, 4.5, 3)	3.86 ddd (12, 4.5, 3)	3.88 ddd (12, 4.5, 3)
4D-H	3.71 s br	3.69 s	3.72 s br
5D-H	3.70 q (6.5)§	3.67 q (6.5)	3.70 q (6.5)§
6D-H_3	1.28 d (6.5)	1.26 d (6.5)	1.29 d (6.5)
1E-H	4.95 dd (9.5, 2)	4.94 dd (9.5, 2)	4.96 dd (9.5, 2)
2E-H*	1.52 dd (13.5, 9.5)	1.52 dd (13.5, 9.5)	1.54 dd (13.5, 9.5)
2E-H#	1.87 dd (13.5, 2)	1.86 dd (13.5, 2)	1.89 dd (13.5, 2)
3E-Me	1.20 s	1.20 s	1.22 s
4E-H	2.93 d (9.5)	2.91 d (9.5)#	2.94 d (9.5)#¶
5E-H	3.63 dq (9.5, 6.5)	3.62 dq (9.5, 6)	3.64 dq (9.5, 6)
6E-H_3	1.21 d (6.5)	1.20 d (6)	1.22 d (6)

OH signals for premithramycin B: δ 3.26, 4.70, 4.70 and 9.41 (all broad s). OH signals for premithraycin A3: δ 4.59, 9.50, 9.90, 14.9 (all broad s); OH signals for mithramycin: δ 3.23, 3.75, 3.82, 3.91, 3.92, 4.10, 4.11, 4.20, 4.64, 9.80 and 15.9 (all broad s); br = broad; *at 500 MHz; †at 300 MHz; ‡assignments interchangeable; §complex, partially obscured; #broad, partially obscured; ¶complex, overlapping signals.

Table 2

^{13}C NMR data of premithramycin B compared to premithramycin A3 and mithramycin, in d_6-acetone at 125.7 MHz, δ in ppm relative to internal TMS, multiplicities and assignments from the APT, HMQC and HMBC experiments.

	Premithramycin B	Premithramycin A3	Mithramycin*
C-1	196.7 s†	196.2 s†	203.1 s
C-2	113.2 s	113.2 s	75.9 d
C-3	189.1 s†	189.0 s†	41.7 d
C-4	78.5 d	78.0 d	26.4 t
C-4a	42.8 d	43.0 d	135.5 s
4-OCH_3	61.9 q	61.4 q	–
C-5	26.4 t	26.6 t	100.7 d
C-5a	134.6 s	134.6 s	–
C-6	118.8 d	117.8 d	158.8 s
C-6a	139.8 s	140.1 s	–
C-7	101.4 d	102.8 d	110.0 s
7-CH_3	–	–	7.0 q
C-8	160.6 s	162.1 s	155.2 s
C-8a	–	–	107.0 s
C-9	111.4 s	110.7 s	164.2 s
C-9a	–	–	107.6 s
9-CH_3	8.4 q	8.2 q	–
C-10	156.6 s	157.5 s	116.2 d
C-10a	107.9 s	107.1 s	138.0 s
C-11	166.6 s	168.0 s	–
C-11a	109.0 s	108.0 s	–
C-12	193.5 s†	193.0 s†	–
C-12a	86.1 s	85.5 s	–
C-1′	204.3 s	204.3 s	81.1 d
1′-OCH_3	–	–	57.8 q
C-2′	28.6 q	28.4 q	210.6 s
C-3′	–	–	78.4 d
C-4′	–	–	67.5 d
C-5′	–	–	18.7 q
C-1A	96.9 d	–	95.7 d
C-2A	38.1 t	–	36.5 t
C-3A	82.2 d	–	80.6 d
C-4A	76.0 d	–	74.5 d
C-5A	72.9 d	–	71.5 d
C-6A	18.7 q‡	–	17.2 q
C-1B	100.7 d	–	99.1 d
C-2B	40.4 t	–	39.1 t
C-3B	71.8 d	–	70.4 d
C-4B	78.0 d	–	76.6 d
C-5B	73.2 d	–	71.7 d
C-6B	18.2 q	–	16.8 q
C-1C	98.5 d§	98.3 d#	100.0 d
C-2C	39.3 t	38.5 t	37.0 t
C-3C	82.1 d	81.9 d	80.8 d
C-4C	76.0 d	75.6 d	74.8 d
C-5C	73.0 d	72.8 d	71.6 d
C-6C	18.8 q‡	18.5 q	17.1 q
C-1D	100.7 d	100.5 d	99.3 d
C-2D	32.7 t	32.8 t	31.6 t
C-3D	77.1 d	77.1 d	75.8 d
C-4D	69.3 d	69.2 d	68.0 d
C-5D	71.5 d	71.2 d	70.0 d
C-6D	17.0 q	16.9 q	15.6 q
C-1E	98.3 d§	98.2 d#	97.1 d
C-2E	44.7 t	44.6 t	43.4 t
C-3E	71.1 s	71.1 s	69.8 s
3E-CH_3	27.5 q	27.7 q	26.1 q
C-4E	77.2 d	77.1 d	76.1 d
C-5E	71.4 d	71.3 d	70.1 d
C-6E	18.7 q§	18.7 q	17.4 q

Table 2 footnote

*Assignments could not be further confirmed, because the sample precipitated after the broad-band decoupled ^{13}C-NMR spectrum was determined; thus many assignments of similar chemical shift may be interchangeable; †,‡,§,#assignments interchangeable.

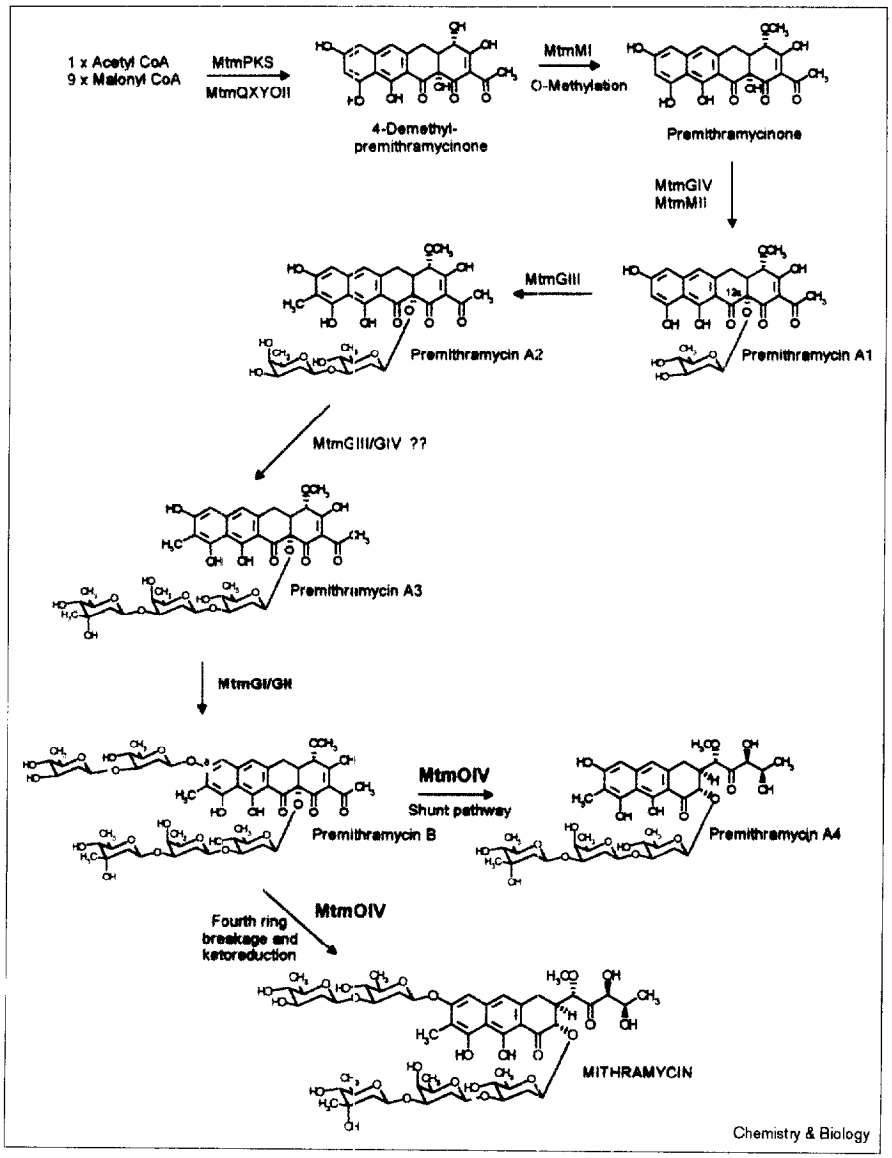
the premithramycins A2 and A3 [18], indicating the same tetracyclic aglycon. Typical for this aglycon is the acetyl group (C-1′, C-2′), the OCH_3 group at C-4, two aromatic protons of the C and D rings, one aromatic bound methyl group (δ_H 2.15; δ_C 8.4). and six sp^2-carbons attached to an oxygen atom, in addition to the carbonyl of the acetyl sidechain. As a result of this arrangement, an intensive tautomerism can be observed. As a consequence some of the carbons (C-1, C-3, C-11, C-12) typically show broad signals or variation of their chemical shifts and cannot be assigned unambiguously due to the lack of observable long-range C–H couplings. The NMR data of these saccharide moieties are identical to the corresponding ones of mithramycin; for example, the ^{1}H NMR signals of the anomeric protons indicate β-glycosidic linkages (all five 1-H signals show a 10 Hz transaxial coupling to the neighboring 2-H_a). Typical signals indicate the mycarose (e.g. the 3E-Me signal at δ 1.20; 4E-H at δ 2.93), the oliose (e.g. the broad singlet of 4D-H at δ 3.71) and the three olivose moieties (e.g. 2-H_e signals at δ 2.21, 2.5 and 2.79 or the 4-H 'triplets' at δ 3.01, 3.03 and 3.09). The interglycosidic links are clear from the heteronuclear multiple bond connectivity (HMBC) spectrum, which proves that they are arranged as one trisaccharide (β-D-olivose-3-1-β-D-oliose-3-1-β-D-mycarose) and one disaccharide (β-D-olivose-3-1-β-D-olivose) chain. The linkage positions of these saccharide moieties to the aglycon are also indicated from the HMBC spectrum. The most important $^3J_{C-H}$-couplings are: 1A-H→C-8, 3A-H→C-1B, 1B-H→C-3A; 1C-H→C-12a, 3C-H→C-1D, 1D-H→C-3C, 3E-H→C-3D. The complete NMR signals of premithramycin B in comparison with premithramycin A3, as well as mithramycin are shown in Tables 1 and 2.

The MtmOIV oxygenase catalyses one of the last steps in mithramycin biosynthesis

Most of the intermediates in mithramycin biosynthesis are tetracyclic compounds (some of which are glycosylated); premithramycin B is the last tetracyclic compound in the pathway (Figure 6). Conversion of premithramycin B into mithramycin occurs in the last steps of mithramycin biosynthesis. The MtmOIV oxygenase that catalyses this reaction can also accept at least one other substrate. Premithramycin A4 (Figure 6) is a tricyclic compound in which the fourth ring breakage has already occurred. It contains the trisaccharide at C-2-O but lacks the disaccharide at C-6-O. This compound was isolated as a minor compound from two mutants in which the *mtmGI* and *mtmGII* genes were independently inactivated [18]. Premithramycin B must be the preferred

Figure 6

Proposed biosynthetic pathway for the biosynthesis of mithramycin.



substrate for this oxygenase as it accumulates dramatically when the *mtmOIV* gene is inactivated.

Premithramycin B does not have detectable antibiotic activity (50 μg did not cause inhibitory effect on *Micrococcus luteus* in a bioassay) in contrast to the very potent inhibitory effect of mithramycin (0.1 μg caused a big inhibition zone of *M. luteus* growth). Survival of *S. argillaceus* during mithramycin biosynthesis depends on an ABC transporter system, coded by the *mtrA* and *mtrB* genes, that participates in the export of the drug through the cell membrane (inactivation of *mtrA* produces a mithramycin-sensitive mutant) [19]. This transport system is very specific for mithramycin as it does not confer resistance to the structurally closely related aureolic acid drugs olivomycin and chromomycin [19]. The oxidative cleavage of premithramycin B by the MtmOIV oxygenase followed by decarboxylation and ketoreduction steps releases the toxic lethal drug mithramycin into the cytoplasm of *S. argillaceus*. To ensure its survival, the producer organism probably has to channel the active drug into the ABC transporter secretion system. This would probably require the MtmOIV oxygenase to be proximal to the cell membrane. It is worth mentioning that immediately upstream of the *mtrA* gene is another gene, *mtrY*, to which function has not yet been assigned. Its deduced product showed similarity with a family of proteins designated as ankyrins (30.6% identity in a 36 amino-acid overlapping region with a human erythrocyte ankyrin) [32]. The ankyrins are a family of proteins found widely in eukaryotes that include

structural proteins that participate in linking the spectrin skeleton of human erythrocytes to the cytoplasmic surface of the cell membrane [33]. It is possible that the MtrY protein participates in establishing a physical linkage between the MtmOIV oxygenase (and a ketoreductase) and the ABC secretion system, which might facilitate the channeling of some intermediates in the final steps of mithramycin biosynthesis.

Significance

The biosynthesis of the aureolic acid group of antitumor drugs proceeds at early stages through tetracyclic intermediates of the tetracycline-type that must be converted at later stages into the final tricyclic glycosylated molecule. We have shown here that one of the last intermediates in mithramycin biosynthesis, premithramycin B, a fully glycosylated tetracyclic compound, accumulates in the culture supernatant of mutants in which expression of the MtmOIV oxygenase gene has been affected. Because MtmOIV oxidatively cleaves the fourth ring in premithramycin B, it is therefore important in determining the final shape of mithramycin (and possibly the shape of the related drugs chromomycin and olivomycin). In addition, the oxygenase participates in the conversion of the biologically inactive premithramycin B into the active final molecule mithramycin in one of the last steps of the mithramycin biosynthesis. The MtmOIV oxygenase could be a tool potentially valuable for modifying the architecture of other tetracyclic compounds that could be used clinically, helping, therefore, to generate novel lead compounds.

Materials and methods

Microorganisms, culture conditions and plasmids

S. argillaceus ATCC 12956, a mithramycin producer, was used as donor of chromosomal DNA. For sporulation on solid medium, it was grown at 30°C on plates containing A medium [18]. *S. albus* G was used as host for transformation. For protoplast transformation *S. argillaceus* and *S. albus* were grown in YEME medium containing 17% sucrose. For growth in liquid medium, the organism was grown in TSB medium (trypticase soya broth, Oxoid). *Escherichia coli* TG1 recO1504::Tn5 [34] was used as host for subcloning. When plasmid-containing clones were grown, the medium was supplemented with appropriate antibiotics: 100 μg/ml for ampicillin, 25 μg/ml apramycin, 20 μg/ml erythromycin, 50 μg/ml thiostrepton or 20 μg/ml tobramycin. pUC18 and pBSK vectors were used for sequencing and subcloning. Plasmid pBSKT is a pBluescript derivative containing a thiostrepton resistance cassette [35]. pIAGO [35] and pEM4 [36] are bifunctional plasmids (*Streptomyces–E. coli*) containing the erythromycin resistance promoter (ermEp) but with different *Streptomyces* replicons. pUK21 [37] and pKC1218 [38] were used for subcloning.

DNA manipulation techniques

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were according to standard procedures for *E. coli* [39] and for *Streptomyces* [40]. Southern blot hybridization was according to standard procedures [40]. DNA sequencing was carried out using the dideoxynucleotide chain-termination method [41] with Taq polymerase and an ALF-express automatic DNA sequencer (Pharmacia). To overcome band compression artifacts, 7-deaza-dGTP was routinely used instead of dGTP [42]. Both DNA strands were sequenced using universal primers or internal oligoprimers. Computer-aided database searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package [20] and the BLAST program [24].

Generation of deletion and insertional inactivation mutants

For the generation of a deletion mutant lacking the four glycosyltransferase and the two methyltransferase genes (mutant M3ΔMG), a construct was generated in pBSKT containing a 11.7 kb *PstI–SalI* DNA fragment (sites 1–14 in Figure 2a) and then the internal 7 kb *FspI-BglII* DNA fragment (sites 4–9 in Figure 2a) was replaced by an erythromycin resistance gene (*ermE*) orientated in the direction of transcription. This construct was named pEFΔMG.

For insertional inactivation of *mtmOIV*, a plasmid construct was generated in pBSKT containing a 12 kb *XhoI-BglII* fragment (sites 6–17 in Figure 2a) and then an internal 0.4 kb *XhoI-NotI* fragment (sites 11–13 in Figure 2a) was replaced by an apramycin resistance cassette orientated in the same direction of transcription than *mtmOIV*. This construct was named pLPΔO4.

Both constructs (pEFΔMG and pLPΔO4) were used to transform protoplasts of *S. argillaceus* and transformants selected for resistance to erythromycin (pEFΔMG) or apramycin (pLPΔO4) and then tested for their susceptibility to thiostrepton.

Gene expression

Plasmid pG14M12 harboring the four mithramycin glycosyltransferases and the two methyltransferases was constructed as follows. A 8 kb *BglII-XhoI* DNA fragment (sites 3–11 in Figure 2a) containing the 3′ end of *mtmW*, *mtmGIV*, *mtmGIII*, *mtmMII*, *mtmMI*, *mtmGII*, *mtmGI* and the 5′ end of *mtmOIV* was subcloned into pEM4, generating pG14M12. In this construct transcription of *mtmGIV*, *mtmGIII*, *mtmMII* and *mtmMI* genes is under the control of the erythromycin resistance promoter (ermE*). The divergent *mtmGII* and *mtmGI* genes are transcribed from their own promoters.

For expression of *mtmOIV*, a 2.1 kb *BamHI* fragment (sites 10–15 in Figure 2a) was subcloned under the control of the erythromycin resistance promoter in the low-copy number vector pKC1218, generating pEFO4. A similar construct was also made in pIAGO (pLPO4).

Enzymatic assays

S. albus strains IAGO and LPO4 were grown at 30°C for 48 h in TSB medium containing 5 μg/ml thiostrepton. The mycelia were collected by centrifugation and washed in 50 mM Tris-HCl (pH 8.0) containing 1 mM ethylenediaminotetracetic acid (EDTA) and 1 mM dithiothreitol (DTT). Mycelia was broken by ultrasound (10 pulses 10 s each with intermittent cooling on ice water) in a MSE-ultrasonic disintegrator at 150 W and 20 kHz. Cell debris were removed by centrifugation at 30,000 × g for 15 min. The supernatant was fractionated by ammonium sulphate precipitation and the precipitates obtained at different salt concentrations dissolved in a small volume of the buffer and extensively dialysed against the same buffer. The fractions obtained were used as source for oxygenase activity. *In vitro* activity of the MtmOIV oxygenase was performed in a 1 ml reaction volume containing 225 μg protein extract, 400 μM premithramycin B and 1 mM NADH or NADPH. The reaction was carried out at 30°C for 2 h. Then, the reaction was extracted twice with one volume of ethyl acetate after decreasing the pH by adding 100 μl HCl 0.2 N. After evaporation of the solvent, the residue was dissolved in 50 μl methanol and analysed by HPLC as described below.

HPLC analysis and isolation of mithramycin intermediates

Spores of strains M3ΔMG, EF1412 and EF14124 were inoculated in TSB medium and incubated for 24 h at 30°C and 250 rpm. The seed cultures obtained were used to inoculate (at 2.5% v/v) eight 2 l Erlenmeyer flasks each containing 400 ml of modified R5 medium [18].

Media for strains EF1412 and EF14124 contained 2.5 μg/ml thiostrepton (and 2.5 μg/ml apramycin in the case of EF14124). These cultures were incubated as above and formation of mithramycin-related compounds were monitored by analytical HPLC. After 3 days (strain M3ΔMG) or 4 days (strains EF1412 and EF14124) of incubation, the cultures were centrifuged, the supernatants were filtered and applied to a solid phase extraction cartridge. Details concerning HPLC and extraction were as described previously [18]. The retained compounds were eluted with a mixture of methanol and 0.1% trifluoroacetic acid (TFA) in water. A linear gradient from 0% to 100% methanol in 60 min, at 10 ml/min, was used. Fractions were taken every 5 min and subsequently analyzed by HPLC. Fractions containing the main products accumulated by each strain were evaporated, redissolved in a small volume of methanol and chromatographed in a (μBondapak C18 radial compression cartridge (PrepPak Cartridge 25 × 100 mm, Waters). Gradients with acetonitrile and 0.1% TFA in water, at a flow rate of 10 ml/min, were optimized for resolution of each peak. The purified material collected was applied to a solid phase extraction cartridge (Lichrolut RP-18, 500 mg, Merck), washed with water, eluted with methanol and dried *in vacuo*.

Determination of antibiotic activity

Antibacterial activity was determined by bioassay against *Micrococcus luteus* ATCC 10240 as described [43].

Physicochemical properties of premithramycin B

Rf 0.26 ($CHCl_3$/MeOH/acetic acid/H_2O = 8.34:1:0.5:0.16); IR (KBr): ν = 3425, 2927, 1634, 1376, 1165, 1067, 613 cm^{-1}; negative FAB MS: m/z 1091 ($[M-H]^-$); positive FAB MS: m/z 1115 ($[M+Na]^+$); UV (MeOH) λ_{max} (ε) 426.0 (8,200), 335.2 (7,300), 318.7 (8,900), 283.0 (44,100), 230.0 (26,200), 202.0 (19,600) nm; (MeOH/NaOH) λ_{max} (ε) 426.0 (11,700), 280.0 (42,100), 228.0 (21,000), 205.0 (25,400) nm; (MeOH/HCl) λ_{max} (ε) 431.0 (7,900), 335.4 (7,800), 321.0 (7,700), 284.0 (38,900), 230.0 (31,700), 201.0 (19,300) nm; CD (c = 2.36 · 10^{-5} mol/L, MeOH): $\lambda_{extr.}$ ($[\Theta]^{24}$) 431.4 (−9,000), 342.2 (17,400), 328.4 (13,600), 286.8 (97,100), 264.6 (-29,000), 243.8 (3,300), 228.6 (−13,700) nm. NMR data: see Tables 1 and 2.

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