Characterization of Two Polyketide Methyltransferases Involved in the Biosynthesis of the Antitumor Drug Mithramycin by Streptomyces argillaceus

(Received for publication, September 24, 1999, and in revised form, November 24, 1999)

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A DNA chromosomal region of Streptomyces argillaceus ATCC 12596, the producer organism of the antitumor polyketide drug mithramycin, was cloned. Sequence analysis of this DNA region, located between four mithramycin glycosyltransferase genes, showed the presence of two genes (mtmMI and mtmMI) whose deduced products resembled S-adenosylmethionine-dependent methyltransferases. By independent insertion inactivation of both genes nonproducing mutants were generated that accumulated different mithramycin biosynthetic intermediates. The M3ΔMI mutant (mtmMI-minus mutant) accumulated 4-demethylpremithramycinone (4-DPMC) which lacks the methyl groups at carbons 4 and 9. The M3ΔMI (mtmMI-minus mutant) accumulated 9-demethylpremithramycin A3 (9-DPMA3), premithramycin A1 (PMA1), and 7-demethylmithramycin, all of them containing the O-methyl group at C-4 and C-1′, respectively, but lacking the methyl group at the aromatic position. Both genes were expressed in Streptomyces lividans TK21 under the control of the erythromycin resistance promoter (ermEp) of Saccharopolyspora erythraea. Cell-free extracts of these clones were precipitated with ammonium sulfate (90% saturation) and assayed for methylation activity using different mithramycin intermediates as substrates. Extracts of strains MJM1 (expressing the mtmMI gene) and MJM2 (expressing the mtmMI gene) catalyzed efficient transfer of tritium from [3H]-S-adenosylmethionine into 4-DPMC and 9-DPMA3, respectively, being unable to methylate other intermediates at a detectable level. These results demonstrate that the mtmMI and mtmMI genes code for two S-adenosylmethionine-dependent methyltransferases responsible for the 4-O-methylation and 9-C-methylation steps of the biosynthetic precursors 4-DPMC and 9-DPMA3, respectively, of the antitumor drug mithramycin. A pathway is proposed for the last steps in the biosynthesis of mithramycin involving these methylation events.

Actinomycetes, and particularly streptomycetes, are producers of a great variety of bioactive compounds, many of which have clinical application as antibiotics, antifungal, antiparasites, or antitumor drugs. Many of these bioactive drugs contain methyl groups in their structures, which are introduced during biosynthesis. However, not all these methylations are due to the action of methyltransferases. In the case of the polyketides, some of these methyl groups derive from the carboxylic acids used as starter or extender units during the condensation reactions (1). In other cases, the action of specific methyltransferases is responsible for the methylation reactions. Most of them are O-methylations, as is the case of tetacenocymycin C (2, 3), daunorubicin (4–6), erythromycin (7), and tylosin (8). But also N-methylations (9) and C-methylations (10, 11) have been described. As far as they have been characterized, most of the methyltransferases participating in antibiotic biosynthesis use S-adenosylmethionine as cofactor, although biotin and tetrahydrofolate can be alternatively used by other methyltransferases.

Mithramycin (also designated as aureolic acid, plicamycin, mithracin, LA-7017, PA-144; Fig. 1A) is an aromatic polyketide which shows antibacterial activity against Gram-positive bacteria but not against Gram-negative bacteria because a permeability barrier. It also shows a remarkable cytotoxicity against a variety of tumor cell culture lines, including brain tumors and experimental animal tumors. Mithramycin has been clinically used for the treatment of certain tumors, such as disseminated embryonal cell carcinoma as well as for Paget’s bone disease (12, 13) and also finds use for control of hypercalcemia in patients with malignant disease (14). Mithramycin, together with the chromomycins, olivomycins, chromocyclomycin, and UCH9, constitutes the aureolic acid group of antibiotics. All they belong to the large and important family of the aromatic polyketides. Great progress has been made in the last few years on the understanding of the mithramycin biosynthetic pathway. It has been shown that the biosynthesis of the mithramycin aglycon derives from the condensation of 10 acetate units in a series of reactions catalyzed by a type II polyketide synthase thus generating a decaketide (15). After several aromatizations and cyclizations two nonglycosylated intermediates are synthesized, 4-demethylpremithramycinone (4-DPMC) and premithramycinone (PMC) (16, 17). This last compound is later glycosylated with (in this order) a trisaccharide (D-olivose-D-oliose-D-olivosamine).

This paper is available on line at http://www.jbc.org

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The abbreviations used are: 4-DPMC, 4-demethylpremithramycinone; 9-DMPA3, 9-demethylpremithramycin A3; 7-DNTM, 7-demethylpremithramycinone; PMC, premithramycinone; kb, kilobase(s); PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; ORF, open reading frame; PMA1, premithramycin A1; PMA2, premithramycin A2; PMA3, premithramycin A3.

The Journal of Biological Chemistry Vol. 275, No. 5, Issue of February 4, pp. 3065–3074, 2000
Printed in U.S.A.
and d-mycarose) and a disaccharide (d-olivose-d-olivose) (18,19). Finally, as one of the last events in mithramycin biosynthesis, an oxidative breakage (followed by a keto reduction) of the fourth ring of the fully glycosylated intermediate premithramycin B takes place generating the final mithramycin molecule (20). The structure of mithramycin bears nine methyl groups, C-5', which is derived from the acetate starter unit, the O-methyl group at C-1'-O and the methyl side chain at C-7 of the polyketide-derived aglycon moiety. Furthermore, five methyl groups (always C-6 of each deoxysugar unit) are derived from the dehydration step affecting C-4 and C-6 of the corresponding sugars during an early step in the deoxysugar biosynthesis. Finally, another methyl side chain is attached at C-3 of the d-mycarose, the third sugar of the trisaccharide synthesis. For the corresponding sugars during an early step in the deoxysugar biosynthesis. For the corresponding sugars during an early step in the deoxysugar biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Microorganisms, Culture Conditions, and Vectors**

Streptomyces argillaceus ATCC 12596, 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 (21). For growth in liquid medium, the organisms were grown either on TSB medium (trypticase soy broth, Oxoid) or R5A medium (18). The structure of mithramycin bears nine methyl groups, C-5', which is derived from the acetate starter unit, the O-methyl group at C-1'-O and the methyl side chain at C-7 of the polyketide-derived aglycon moiety. Streptomyces argillaceus. These studies have allowed the identification of the possible substrates for the two methyltransferases and to propose a pathway for the methylation steps in mithramycin biosynthesis.

**DNA Manipulation**

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, DNA ligations, and other DNA manipulations were performed according to standard techniques for E. coli (26) and Streptomyces (27). Preparation of Streptomyces protoplasts, transformation, and selection of transformants was carried out as described in Ref. 27 with minor modifications for S. argillaceus (20). The structure of mithramycin bears nine methyl groups, C-5', which is derived from the acetate starter unit, the O-methyl group at C-1'-O and the methyl side chain at C-7 of the polyketide-derived aglycon moiety. Streptomyces argillaceus. These studies have allowed the identification of the possible substrates for the two methyltransferases and to propose a pathway for the methylation steps in mithramycin biosynthesis.

**DNA Sequencing**

Sequencing was performed on double-stranded DNA templates by using the dideoxynucleotide chain termination method (28) and the Cy5 AutoCycle Sequencing Kit (Amersham Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) using the F-Express automatic DNA sequencer (Amerham Pharmacia Biotech). Computer-aided data base searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package (UWGGC; Ref. 29) and the BLAST program (30).

**Insertional Inactivation**

The mtmMI and mtmMII genes were inactivated by gene replacement as follows.

**PCR Amplification**

PCR reaction conditions were as follows: 100 ng of template DNA were mixed with 30 pmol of each primer and 2 units of Vent DNA Polymerase (New England Biolabs) in a total reaction volume of 50 μl containing 2 mM of each dNTP, 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris·HCl (pH 8.8), 2 mM MgSO₄, and 0.1% Triton X-100. This reaction mixture was overlaid with 50 μl of mineral oil (Sigma). The polymerization reaction was performed in a thermocycler (MینyCycler, MJ Research) under the following conditions: an initial denaturation of 3 min at 98 °C; 30 cycles of 30 s at 98 °C, 1 min at 61.6 °C, and 1.2 min at 72 °C; after the 30 cycles, an extra extension step of 5 min at 72 °C was added.

**Preparation of Cell-free Extracts**

50 ml of TSB medium were incoated with a spore suspension of the different S. lividans clones in the presence of 5 μg/ml thiostrepton. After 2 days of incubation at 30 °C, the mycelia was collected by centrifugation and washed 3 times with 50 ml Tris·HCl (pH 8.0) containing 1 mM dithiothreitol and 1 mM EDTA. The mycelia were suspended in a small volume of the same buffer and disrupted by sonication (10 pulses 20 s each with intermittent cooling in a MSE-ultrasonic disintegrator at 150 W and 20 kHz). After removal of unbroken cells and cellular debris by centrifugation, the soluble fraction was precipitated with ammonium sulfate (90% saturation) and after resuspension in a small volume of the buffer mentioned above extensively dialyzed against the same buffer.

## Footnotes

1. I. Aguirrezabalaga, C. Olano, N. Allende, L. Rodriguez, A. F. Braña, C. Méndez, and J. A. Salas, submitted for publication.
Methylation Assays

Methylation reactions consisted of the following components (500 μl final volume): 10 μl of S-adenosyl-L-[methyl-3H]methionine (1 μCi/ml; specific activity 68 Ci/mmol), 10 μl of the correspondant mithramycin intermediate (0.2 μM final concentration) and a variable volume of extract and buffer (total 500 μl) depending on the protein concentration of the extracts. After incubation at 37 °C for 1 h, the reactions were stopped by adding one-tenth of their volume 0.2 N HCl and extracted twice with 50 μl of methanol and the samples analyzed by HPLC. Fractions were collected and, after addition of scintillation mixture, the radioactivity in the samples was determined.

HPLC Analysis

Detection of mithramycin-related compounds was performed in a reversed phase column (Symmetry C18, 4.6 × 250 mm, Waters), with acetonitrile and 0.1% trifluoroacetic acid in water as solvents. A linear gradient from 10 to 100% acetonitrile was used. Detection and spectral characterization of peaks were made with a photodiode array detector and Millennium software (Waters), extracting bidimensional chromatograms at 280 nm.

Isolation of Mithramycin Intermediates

Spores of mutant M3AII were initially grown in TSB medium during 24 h at 30 °C and 250 rpm. This seed culture was used to inoculate (at 2.5%, v/v) eight 2-liter Erlenmeyer flasks containing 400 ml of R5A medium. After incubation for 4 days in the above conditions, the cultures were centrifuged, filtered, and extracted as described (18). Mithramycin-related compounds (identified according to their spectral characteristics) were purified by preparative HPLC in a μBondapak C18 radial compression cartridge (Prep Pak Cartridge, 25 × 100 mm, Waters). Short gradients using 0.1% trifluoroacetic acid in water and either methanol or acetonitrile, at 10 ml/min, were optimized for resolution of individual peaks. The purified material collected in each case was diluted 4-fold with water, applied to a solid-phase extraction column (Lichrolut RP-18, Merck), washed with water to eliminate trifluoroacetic acid, and finally eluted with methanol and dried in vacuo.

Physicochemical Data of 7-Deoxymethythymycin and 9-Deoxymethythymycin A3

7-Deoxymethythymycin—pH NMR (400 MHz, acetone-d6) δ 1.19 (s, 3C-Me), 1.20 (d, 6H-H, J = 6.0 Hz), 1.22 (d, 5'H, J = 6.5), 1.24–1.31 (d, 4J, J = 6, 6A-H, 6B-H, 6C-H, 6D-H), 1.54 (ddd, 2B-H, J = 12, 12, 10), 1.54 (dd, 2E-H, J = 13.5, 9.5), 1.60 (dd, 2C-H, J = 12, 12, 10), 1.73 (ddd, 2D-H, J = 12, 12, 10), 1.78 (ddd, 2A-H, J = 12, 10, 10), 1.88 (dd, 2E-H, J = 13.5, 2), 1.93 (ddd, 2D-H, J = 12, 4.5, 2), 2.16 (ddd, 2B-H, J = 12, 5, 2), 2.38 (ddd, 2A-H, J = 12, 5, 2), 2.54 (ddd, 2C-H, J = 12, 5, 2), 2.64 (dd, 4-H, J = 16, 3), 2.82 (bt, 3-H, J = 12, 2), 2.9–2.95 (complex, 4-H), 2.9–3.0 (overlapping signals, 4A-H, 4B-H, 4C-H, 4D-H, J = 6, 3), 3.32 (ddd, 1A-H, J = 8, 5, 2), 3.67 (s, 5-H, J = 1), 3.8 (s, 1-OCH3), 3.84–3.96 (overlapping signals, 3A-H, 3B-H, 3C-H, 3.54 (dq, 5A-H, J = 9, 6), 3.63 (dq, 5E-H, J = 9, 6), 3.71 (3H), 3.71 (bs, 4-H), 3.88 (ddd, 3D-H, J = 12, 5, 3), 4.23 (m, 3'-H, 4'-H), 4.70 (dd, 1D-H, J = 10, 2), 4.71 (dd, 1B-H, J = 10, 2), 4.78 (2H, J = 11.5), 4.83 (d, 1'-H, J = 1.5), 4.95 (dd, 1E-H, J = 9.5, 2.5), 5.06 (dd, 1C-H, J = 10, 2), 5.35 (dd, 1A-H, J = 10, 2), 6.44 (d, 7-H, J = 6.7), 6.76 (d, 5-H, J = 2.4), 6.87 (bs, 10-H) ppm.

13C NMR (100.6 MHz, acetone-d6) δ 17.0 (C-6D), 18.0 (C-6B), 18.4 (C-6C), 18.5 (C-6A), 18.7 (C-6E), 20.1 (C-5'), 27.5 (3C-CH3), 27.8 (C-4'), 32.9 (C-2D), 37.9 (C-2A), 38.4 (C-2C), 40.4 (C-2B), 43.1 (C-3), 44.8 (C-2E), 59.1 (1-OCH3), 68.9 (C-4'), 69.3 (C-1D), 71.1 (C-3E), 71.4 (C-5D), 71.8 (C-3B), 73.0 (C-5A, C-5C), 73.1 (C-5B), 75.8 (C-4A), 76.1 (C-4C), 77.2 (C-3D), 77.3 (C-4E), 78.0 (C-4B), 78.6 (C-3C), 81.7 (C-3A), 82.3 (C-3C, C-1'), 97.1 (C-1A), 98.4 (C-1E), 100.4 (C-1B), 100.7 (C-1D), 101.3 (C-1C), 102.3 (C-5), 103.4 (C-7), 108.7 (C-8a), 109.1 (C-9a), 117.6 (C-10), 138.2 (C-4a), 141.8 (C-10a), 160.2 (C-8), 161.7 (C-6), 165.9 (C-9), 204.6 (C-1), 211.7 (C-2) ppm.

FAB MS m/z (relative intensity) [M + Na]+ 841 (100), [M + H]+ 819 (4), [M − H]− 817 (51); HRMS FAB MS 841.2908 (C46H62O15Na, calculated 841.2895).

Protein Determination

Protein estimation was carried out by measurement of absorbance at 540 nm by a protein-dye binding assay using bovine serum albumin as standard (31).

RESULTS

Cloning and Sequencing of the mtmMI and mtmMII Genes—Previous research in our laboratory on the mithramycin biosynthetic gene cluster allowed the isolation and characterization of a series of overlapping cosmids clones containing the mithramycin gene cluster. Through sequencing analysis in one of these cosmids, clone cosAR3, genes encoding two glycosyltransferases (18), an oxygenase (20), and an ABC transporter system (21) were identified. A region of approximately 2.2 kb located immediately upstream of the two glycosyltransferase genes mtmGIII and mtmGI (Fig. 1B) has been now characterized. We determined the nucleotide sequence of the DNA region (Fig. 2) and analyzed for the presence of potential coding regions using the CODONPREFERENCE program (29) and two open reading frames (ORFs) transcribed in the same direction were found. The first ORF (designated mtmMI) starts in a ATG codon, comprises 978 nucleotides and ends in a TGA stop codon; it would code for a polypeptide of 329 amino acids and an estimated M, of 35,529. The starting codon (ATG) of the second ORF (designated mtmMII) is separated 62 base pairs from the stop codon of mtmMI. It comprises 1,035 nucleotides and ends in a TGA codon; it would code for a polypeptide of 345 amino acids with an estimated M, of 37,863. Both genes show a high GC content characteristic of Streptomycetes genes and the bias in the third codon position characteristic of the genes of this genus. Downstream of mtmMI there is a DNA region with two long inverted repeats that could form stem-loop structures that might represent transcriptional terminators.

Deduced Functions of the MtmMI and MtmMII Proteins—The deduced products of the mtmMI and mtmMII genes were compared with other proteins in data bases using the BLAST program (30). Both proteins showed similarities with methyltransferases from different sources. The MtmMI protein showed the highest similarity with a methylase (ORF3) possibly involved in tetracycline biosynthesis in S. aureofaciens, 47.2% identity (32), the TcmO O-methyltransferase from S. glaucescens involved in the biosynthesis of tetracyclomycin C, 42.2% identity (2), the DnrK carminomycin 4-O-methyltransferase from S. pescuecides involved in the conversion of carminomycin to daunorubicin, 26.4% identity (6), the DauK methyltransferase involved in daunorubicin biosynthesis, 26.4% identity (6), the DauK methyltransferase involved in daunorubicin biosynthesis, 26.4% identity (6), the DauK methyltransferase involved in daunorubicin biosynthesis, 26.4% identity (6). In addition it showed significant similarity to several O-methyltransferases involved in hydroxindol and catechol methylation. The MtmMI protein showed the highest similarities with the RdmB methyltrans-
ferase from the rhodomycin pathway in _S. purpurascens_, 26.9% identity (35), the TcmN multifunctional cyclase-dehydratase-3-O-methyltransferase involved in methylation in the biosynthesis of tetracenomycin C by _S. glaucescens_, 25.8% identity (2), the TcmO O-methyltransferase, 25.1% identity (2), the DnrK carminomycin 4-O-methyltransferase, 25.3% identity (6) and the DauK methyltransferase, 25% identity (33). MtMII also resembled catechol and hydroxyindol O-methyltransferases but the BLAST scores were smaller than those for the MtMI BLAST searches. All these methyltransferases show three conserved motifs (Fig. 3) that are supposed to be involved in the binding of the S-adenosylmethionine cofactor and/or one of the products of the methylation reaction (S-adenosylhomocysteine) (36). Motif I, a glycine-rich region, is the most conserved of these three motifs in the different methyltransferases. It is usually separated from motif II by approximately 36–90 amino acid residues. Motif III is normally located between 12 and 38 amino acids from the end of motif II. In some methyltransferases not all these motifs are clearly identified. In the MtMI and MtMII proteins, the three motifs are clearly recognized (Fig. 3). On the basis of the profiles deduced from these data base comparisons it was assumed that _mtmMI_ and _mtmMII_ could code S-adenosylmethionine-dependent methyltransferases.

**Inactivation of the mtmMI and mtmMII Genes**—To assign a role to MtMI and MtMII in the mithramycin biosynthesis, both corresponding genes were independently inactivated, by deleting an internal DNA fragment and its replacement by an apramycin resistance cassette. In the case of the _mtmMI_ gene, one of two apramycin-resistant thiostrepton-sensitive colonies obtained was selected for further characterization (M3ΔMII mutant). Southern hybridization showed that the wild type region in the chromosome of this mutant was replaced by the _in vitro_ mutated one (Fig. 4, A and B). Using as probe a 1.2-kb NcoI-StuI fragment (sites 9–12 in Fig. 1B), two _BamHI_ hybridizing bands (3.6 and 2.8 kb) were observed in the M3ΔMII mutant in comparison to the unique 5.3-kb _BamHI_ band of the wild type strain (Fig. 4B). HPLC analysis of cultures of this mutant showed the presence of three major peaks. Peak b corresponded to another previously isolated mithramycin intermediate, PMA1 (18). To further characterize the compounds present in peaks a and c, they were purified by preparative HPLC and their structures elucidated.

**Structural Elucidaedation of the Accumulated Compounds**—The negative ion FAB MS spectra of the two new compounds support the following molecular formulae: _mlz_ 1069.4, C_{40}H_{74}O_{24} for 7-demethylmithramycin (peak a), and _mlz_ 817, C_{40}H_{50}O_{18} for 9-demethylpremithramycin A3 (peak c). The former compound was elucidated to be 7-demethylmithramycin by comparison of the _1H_ and _13C_ NMR data with that of mithramycin. In the _1H_ NMR spectrum, the 7-CH_3_ singlet at 2.11 ppm is missing, and an aromatic proton at 6.44 ppm can be observed instead. The _13C_ NMR spectrum supports the presence of a proton at the 7-position by the loss of the 7-CH_3_ signal at 7.0 ppm and a shift in the 7-C signal from the characteristic quarternary carbon at 6110.0 ppm to a dublet at δ 103.4 ppm (comparable to premithramycin A1). All other _1H_ and _13C_ signals appeared to be like mithramycin. The _1H_ and _13C_ NMR spectra for the latter compound were similar to that of premithramycin A3. All premithramycin A3 signals were present, except the 9-CH_3_ signals at 2.16 and 8.2 ppm (_1H_ and _13C_, respectively). Instead, an aromatic proton signal was observed at δ 6.41 and the 9-C signal was shifted from δ 110.7 (quarternary carbon) to 102.4 (doublet). Based on these observations, the compound was identified as 9-demethylpremithramycin A3.

**Expression of the mtmMI and mtmMII Genes and Assays of Methylation**—In order to determine the putative substrates of the MtMI and MtMII methyltransferases, we decided to establish an _in vitro_ methylation assay to test the activity of both methyltransferases. Both genes were independently expressed in _S. lividans_ TK21. Plasmids pMJM1 and pMJM2 expressing the _mtmMI_ or _mtmMII_ gene, respectively, were introduced into _S. lividans_ TK21 by protoplast transformation. One transformant (thiostrepton-resistant) was selected from each transformation (strains MJM1 and MJM2, respectively) and, after cultivation in TSB medium for 3 days at 30 °C, methylation activity against different mithramycin intermediates was tested using extracts obtained as described under “Experimental Procedures.” As potential substrates for methylation several mithramycin intermediates were tested (Fig. 7):
4-DPMC (17, 20) and PMC (17, 37), premithramycin A1 (PMA1) (18), 9-demethylpremithramycin A3 (9-DPMA3; isolated in this work) and 7-demethylmithramycin (7-DMTM) (isolated in this work). 4-DPMC is a tetracyclic nonglycosylated intermediate that lacks both, the O-methyl group at C-4 and the methyl side chain at C-9 (20). PMC is the C-4 methylated derivative of 4-DPMC. PMA1 is a tetracyclic compound containing a D-olivose sugar unit attached to C-12a-O and lacking
the C-methyl group at C-9. For TLC and HPLC comparison, we used premithramycin A2 (PMA2) and premithramycin A3 (PMA3) (18) as potential products of the methylation reactions. PMA2 is similar to PMA1, but contains a disaccharide (D-olivose-D-oliose) and possesses already the C-methyl group at C-9. The latter is also true for PMA3, which has a structure like PMA2 plus a D-mycarose unit attached to the disaccharide D-olivose-D-oliose of the latter. Extracts of strain MJM1 were very active in the transfer of tritium from \(^{3} \text{H}-\text{adenosylmethionine} \) into 4-DPMC (Fig. 6A) but not to PMC and the other substrates tested (data not shown). Tritium transfer was dependent on the presence of 4-DPMC since no transfer of radioactivity was detected in the absence of 4-DPMC (Fig. 6A). The products of the reaction were analyzed by HPLC (Fig. 6B) and radioactivity of the different peaks counted (Fig. 6C). Most of the radioactivity was associated with a fraction, which corre-
sponded to PMC (Fig. 6C), indicating that the MtmMI methyltransferase converts 4-DPMC into PMC. When using extracts of the MJM2 strain only 9-DPMA3 was efficiently methylated (Fig. 7A). Again HPLC analysis of the reaction products was performed (Fig. 7B) and the radioactivity in the different fractions measured (Fig. 7C). Radioactivity was associated to a fraction corresponding to the mobility of PMA3, indicating that the MtmMII methyltransferase converts 9-DPMA3 into PMA3.

**DISCUSSION**

During biosynthesis of the antitumor drug mithramycin three methylation events occur. Two of them will modify the architecture of the polyketide moiety and involve a 4-O-methylation and a 9-C-methylation of the premithramycinone and premithramycin precursors, respectively. The third one is a C-methylation during the biosynthesis of D-mycarose, the third deoxysugar present in the trisaccharide chain. Experiments shown in this paper demonstrate that the products of the mtmMI and mtmMII genes are responsible for catalyzing the first two methylation reactions. The MtmMI methyltransferase would convert 4-DPMC into PMC. This assertion is based on two lines of experimental evidence: (i) the major compound accumulated by the M3D MI mutant was 4-DPMC and (ii) 4-DPMC was efficiently used as substrate by the MtmMI methyltransferase and was converted into PMC. This reaction is an important event in the mithramycin biosynthesis since the introduction of a methyl group into the 4-position of the aglycon is essential in order to proceed further on through the glycosylation steps and other methylation and oxygenation events. This conclusion can be deduced from the analysis of compounds accumulated by M3ΔMI mutant, which only accumulates 4-DPMC. In this mutant, genes involved in deoxysugar biosynthesis and sugar transfer to the aglycon are not affected. Therefore, it can be inferred that the introduction of the first D-olivose moiety of the trisaccharide at C-12a-O (PMC to PMA1), catalyzed by glycosyltransferase MtmGIV, requires the presence of the 4-O-methyl group.

The MtmMII methyltransferase would be responsible for the C-methylation at C-9 position of the precursor aglycon. Analysis of the compounds accumulated by M3ΔMII mutant showed that all of them lacked this methyl group. However, in contrast to the methylation step catalyzed by MtmMI, C-9 methylation is not essential to complete mithramycin biosynthesis. The major compound isolated through insertional inactivation of the mtmMII gene (M3ΔMII mutant; compound a) corresponded to the final molecule mithramycin lacking the methyl group at position 7, 7-DMTM. This implies that in the absence of this methylation all glycosylation steps leading to the transfer of the disaccharide and trisaccharide chains can take place. Moreover, it also implies that the MtmOIV oxygenase responsible for opening the fourth ring of premithramycin B (20) can also act on a 9-demethylated substrate.

Previous studies on the compounds accumulated by mutants in the mtmGI and mtmGII genes (18) suggested that C-9 methylation could probably occur after the attachment of the first D-olivose unit to the aglycon. Accordingly, the substrate for the MtmMII methyltransferase would be PMA1. However, the in vitro enzymatic assays shown in this paper strongly suggest that MtmMII must act at a later stage of the mithramycin biosynthesis, namely by methylating 9-DPMA3.

Taking together previous work on mithramycin biosynthesis (18, 20) and the results shown here, a pathway is proposed for the methylation steps during mithramycin biosynthesis (Fig. 5).
4-DPMC is the first stable intermediate of the mithramycin biosynthesis. This is converted into PMC by methylation at C-4-O through the MtmMI methyltransferase. Then two consecutive glycosylation steps occur generating PMA1 (18) and 9-DPMA2. The latter is a hypothetical compound that has not yet been isolated and should be accumulated by a mutant.
affected in the glycosyltransferase gene responsible for the incorporation of D-mycarose as the last sugar in the trisaccharide chain. This gene has not been identified so far. After incorporation of D-mycarose, 9-DPMA3 is produced, the compound isolated in this work that serves as the substrate for the MtmMII methyltransferase, generating PMA3. Finally transfer of the disaccharide (18), fourth ring breakage (20), and a ketoreduction step takes place rendering the final compound mithramycin. In addition to this main pathway some side reactions can take place regarding methylation and glycosylation.

FIG. 8. Proposed pathway for the methylation steps in mithramycin biosynthesis (for details on the assignation of functions to the glycosyltransferase genes (see Refs. 18 and 23). All the compounds shown have been isolated except 9-demethylpremithramycin A2.
Methylation during Mithramycin Biosynthesis

steps. On the one hand, C-9 methylation can occur before the completion of the entire trisaccharide chain. This is suggested from the isolation of 9-C-methylpremithramycin A13 and PMA2 (18), compounds obtained from the M3G1 and M3G2 mutants (affected in the mtmGI and mtmGII genes, respectively). Conversion of PMA1 into PMA2 (Fig. 8) would require the participation of the MtmMII methyltransferase and a glycosyltransferase (probably the MtmGIII glycosyltransferase; Ref. 23). On the other hand, the C-9 methylation can be bypassed leading to the formation of 7-demethylmithramycin, a novel compound isolated in this work. All the studies on the mithramycin biosynthetic pathway so far suggest that enzymes acting on late biosynthetic steps, i.e., after the formation of the last nonglycosylated intermediate PMC (methyltransfer, glycosyltransfer, and oxygenation steps), show a certain substrate flexibility. This opens up the possibility of using these enzymes for generating molecular biodiversity that could produce novel potentially useful bioactive compounds.

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\textit{J. Biol. Chem.} 2000, 275:3065-3074.  
doi: 10.1074/jbc.275.5.3065 

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