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Hydroxyquinone O-Methylation in Mitomycin Biosynthesis

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Abstract: Mitomycins are bioreductively activated DNA-alkylating agents. One member of this family, mitomycin C, is in clinical use as part of combination therapy for certain solid tumors. The cytotoxicity displayed by mitomycins is dependent on their electrochemical potential which, in turn, is governed in part by the substituents of the quinone moiety. In this paper we describe studies on the biogenesis of the quinone methoxy group present in mitomycins A and B. An engineered *Streptomyces lavendulae* strain in which the *mmcR* methyltransferase gene had been deleted failed to produce the three mitomycins (A, B, and C) that are typically isolated from the wild type organism. Analysis of the culture extracts from the *mmcR*-deletion mutant strain revealed that two new metabolites, 7-demethylmitomycin A and 7-demethylmitomycin B, had accumulated instead. Production of mitomycins A and C or mitomycin B was selectively restored upon supplementing the culture medium of a *S. lavendulae* strain unable to produce the key precursor 3-amino-5-hydroxybenzoate with either 7-demethylmitomycin A or 7-demethylmitomycin B, respectively. MmcR methyltransferase obtained by cloning and overexpression of the corresponding *mmcR* gene was shown to catalyze the 7-O-methylation of both C9β- and C9α-configured 7-hydroxymitomycins *in vitro*. This study provides direct evidence for the catalytic role of MmcR in formation of the 7-OMe group that is characteristic of mitomycins A and B and demonstrates the prerequisite of 7-O-methylation for the production of the clinical agent mitomycin C.

Introduction

The natural product mitomycin C (**1**, Figure 1) has been in clinical use as an anticancer agent for over five decades and is still part of combination therapy of certain solid tumors.[1] Mitomycins are selectively activated in hypoxic cells because of their requirement for reductive activation to exert their full potential.[2] Numerous biological agents such as diaphorase or glutathione have been identified as participants in performing one- or two-electron reduction of the quinone portion of mitomycin to give the highly reactive semiquinone or hydroquinone species, respectively. The hydroquinone is a very short-lived molecule ($T_{1/2}$ < 1 s) that rapidly loses the C9a substituent.[3,4] The mitosene derivative formed in this way subsequently acts as a DNA alkylating agent with concomitant opening of the aziridine ring and displacement of the carbamoyl substituent. It is now well-established that the cytotoxic activity displayed by mitomycins correlates with the electrochemical potential of the quinone ring. Thus, the reactivity of mitomycins is governed in part through functional group modulation at the quinone moiety. Indeed, substitutions at the C6 and C7 positions, achieved through derivatization of naturally occurring mitomycins or through directed precursor feeding in the producing organism, provide further support for this notion.[5−8] Given the influence of the quinone substituents on the activity of mitomycins, we focused our attention on the biogenesis of the C7-methoxy group. In this article, we show that methylation of the C7-hydroxy group of α- and β-mitomycins is performed by the MmcR methyltransferase whose gene resides within the mitomycin biosynthetic gene cluster.[9]

The actinomycete *Streptomyces lavendulae* NRRL 2564 produces mitomycin C (**1**), mitomycin A (**2**), and mitomycin B (**3**, Figure 1). These compounds differ in the stereochemistry of the C9-substituent, in the nature of the C7-functional group, and finally in their methylation pattern. Mitomycins with C9β-configuration, **1** and **2**, constitute the major components of the metabolite profile, whereas the C9α-mitomycin **3** is only produced as a minor constituent. Investigations into the biosynthesis of mitomycins started in the early 1960s, and by 1980 D-glucosamine and 3-amino-5-hydroxybenzoic acid (AHBA) were established as the biosynthetic precursors that comprise the mitosane skeleton (Scheme 1).[10,11] AHBA is also a precursor

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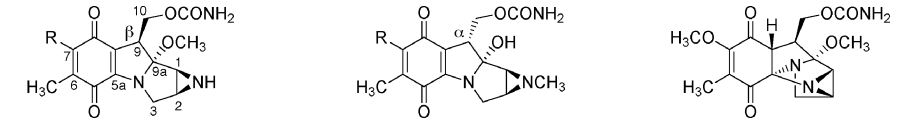


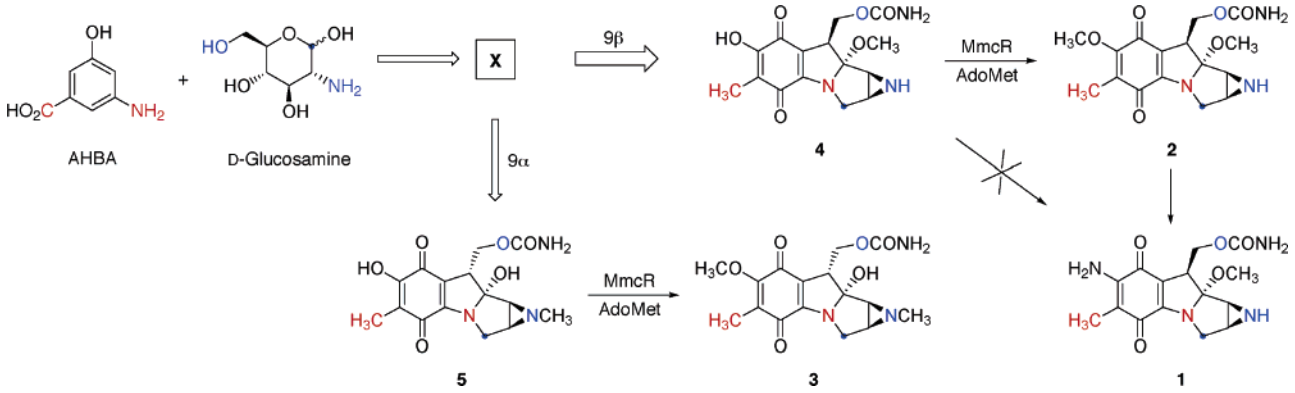
R = NH_2: Mitomycin C (**1**)
R = OCH_3: Mitomycin A (**2**)
R = OH: 7-Demethylmitomycin A (**4**)

R = OCH_3: Mitomycin B (**3**)
R = OH: 7-Demethylmitomycin B (**5**)

Albomitomycin A (**6**)

Figure 1. Structures of mitomycins produced by *S. lavendulae* NRRL 2564 and *S. lavendulae* Δ*mmcR*.

Scheme 1



in the biosynthesis of the ansamycin and ansamitocin antibiotics,[12−14] and its biosynthesis via the amino-shikimate pathway has been extensively studied.[12,15−19] The aminosugar D-glucosamine is incorporated intact, providing the nitrogen atom of the aziridine ring (Scheme 1). AHBA is hydroxylated and further oxidized to the quinone while its carboxy group is reduced to the C6-methyl group after condensation with D-glucosamine.[20] Meanwhile, all the N- and O-linked methyl groups are derived from *S*-adenosylmethionine (AdoMet),[21,22] and the carbamoyl group is derived from carbamoyl phosphate.[23,24] Mitomycin A has been suggested to be the direct precursor of mitomycin C (Scheme 1) based on the initial accumulation of mitomycin A in the culture broth and its subsequent decrease in concentration with a corresponding increase in the levels of mitomycin C.[25] Few details are currently available regarding enzymatic assembly of mitomycins. This is partially due to the unique structural composition of the metabolites, the relatively low levels of mitomycins produced by wild type *S. lavendulae*, and the inherent lability of biosynthetic intermediates.

A complete genetic characterization of the mitomycin biosynthetic gene cluster was reported several years ago.[9] This information provided considerable insight into the regulation and cellular self-resistance of these important DNA alkylating agents.[26−29] Subsequent work on dissecting the enzymatic steps responsible for the assembly of the molecule has focused on late stage functional group modification.[30] In this paper, we describe the identification of two new biosynthetic intermediates that accumulate in an *mmcR* gene deletion mutant strain of *S. lavendulae*. These compounds, 7-demethylmitomycin A (**4**) and 7-demethylmitomycin B, (**5**) were shown by *in vivo* and *in vitro* studies to be converted to the C9*β*-configured **2** and the C9α-configured **3**, respectively, by MmcR, the 7-*O*-methyltransferase responsible for assembly of mitomycin A and mitomycin B.

Experimental Section

Materials. Mitomycins were obtained as a kind gift from Kyowa Hakko Kogyo Ltd. (Tokyo, Japan). Buffer components and reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific and used without further purification. *S*-[Methyl-^{14}C]-adenosyl-L-methionine was obtained from ARC Inc. (St. Louis, MO) at 0.1 mCi/mmol.

Analytical Methods. A ThermoFinnigan LTQ linear ion-trap instrument equipped with electrospray source and Surveyor HPLC system was routinely used for LC-MS analysis. For liquid chromatography, a 10 mM ammonium acetate in water (solvent A)/methanol (solvent B) system was used. Separations were carried out with a Waters XBridge C18 (3.5 μm, 2.1 × 150 mm) column at a flow rate of 208 μL/min and a linear gradient of 5−100% solvent B in 24.2 min starting 1.2 min after sample injection. For mass spectrometry, the capillary temperature was set to 200 °C with the source voltage at 4.5 kV, the capillary voltage at 45 V, and the tube lens at 85 V. The normalized collision energy for fragmentation was 25% (arbitrary unit). Spectra

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were recorded in positive ion mode. Varian Mercury 300 MHz or Bruker DRX 500 MHz instruments were used to record NMR spectra. Spectra were calibrated to residual solvent at 1.93 ppm for acetonitrile or 2.52 ppm for DMSO. A Beckman-Coulter DU 640 spectrophotometer was used for the determination of extinction coefficients. Absorption bands below 230 nm were not considered.

Bacterial Strains, Culture Conditions, and DNA Manipulation. *E. coli* XL1-Blue and BL21(DE3) (Novagen, Madison, WI) were grown at 37 °C in Luria−Bertani broth or on Luria−Bertani agar supplemented with 50 μg/mL of kanamycin. Strains of *S. lavendulae* were cultured in R2YE broth without antibiotics unless otherwise stated. For mitomycin production, *S. lavendulae* was cultured in Nishikohri medium as previously described.[9,31] Standard methods for DNA isolation and manipulation were performed as described by Sambrook et al.[32] *S. lavendulae* genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. PCR was carried out in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA) with *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's recommendations. DNA sequencing was performed by the University of Michigan DNA Sequencing Core facility using an ABI Model 3730 sequencer.

Preparation of *mmcR* Deletion Mutant. *S. lavendulae* DHS9505 was prepared in a two-step procedure *via* an insertional mutation in the *mmcR* gene. The target gene and flanking sequences were subcloned into pUC119 and a 1.4 kb *Apa*L1-*Hin*dIII fragment from pFD666[33] containing the *aphII* gene for kanamycin resistance was inserted into an *Eco*NI site within *mmcR*. The insert was transferred into pKC1139[34] and conjugated into wild type *S. lavendulae*.[35] Transconjugants were selected on AS1 plates,[36] overlaid with apramycin, kanamycin, and nalidixic acid, and followed by propagation on R5T[37] plates at 37 °C for several generations. Insertional disruption mutants were selected based on the phenotype changing from apramycin- and kanamycin-resistant to apramycin-sensitive and kanamycin-resistant. For the preparation of the *mmcR* deletion construct, flanking DNA was PCR-amplified using the following primers: 5′-gcgaattcgtaggggtgccctc-3′ introducing an *Eco*RI site and 5′-gctctagaggtcatacgggggttcct-3′ introducing an *Xba*I site for the 5′-flanking region, and 5′-gctctagatgaaaccgccctcctga-3′ introducing an *Xba*I site and 5′-gcaagcttctcgagcaccgcttggctctcctccctgcc-3′ introducing a *Hin*dIII site for the 3′-flanking region. After digestion with the appropriate restriction enzymes, the inserts were ligated into pUC119 and transferred into pKC1139 for conjugation into the *S. lavendulae mmcR::aphII* strain as described above. Deletion mutants were selected based on the phenotype changing from apramycin and kanamycin resistant to apramycin- and kanamycin-sensitive. The in-frame deletion of *mmcR* from the chromosome was verified by Southern blot hybridization (see Supporting Information).

Analysis of *mmcR* Deletion Mutant and Chemical Complementation. *S. lavendulae* NRRL 2564 and DHS9505 were cultured in R2YE for 3 days. This starter culture was used to inoculate 50 mL of Nishikohri medium, and the main culture was incubated at 30 °C for 4 days. The pH of the fermentation broth was adjusted through addition of an equal volume of 1 M sodium citrate (pH 6) before extraction with ethyl acetate. The organic solvent was removed, and the residue was dissolved in methanol/10 mM ammonium acetate 1:1 to give a final concentration of 1 mg/mL. Aliquots of the crude extract (20 μL) were analyzed by LC-MS as described above.

S. lavendulae strain DHS5349 (*mitA*::*tsr*) was used for complementation with **4** and **5**. For mitomycin production, 10 mL of Nishikohri medium was inoculated with 2.5% starter culture and incubated with shaking at 30 °C. Individual cultures were supplemented with 0.1 M solutions of either **4** or **5** in DMSO, or with DMSO alone after 72, 96, 100 h (20 μL each). Cells were removed after 115 h of culture time, and the broth was extracted with ethyl acetate without adjusting the pH. Analysis of the extracts was performed as described above.

Cloning, Protein Expression, and Purification. The *mmcR* gene was PCR-amplified from *S. lavendulae* NRRL 2564 genomic DNA using the primers 5′-atcatctcatatgaccgtggagcagacccc-3′ and 5′-attcaagcttcaggccctgcggatctcg-3′ to introduce *Nde*I and *Hin*dIII restriction sites, respectively. The digested PCR product was ligated into pET-28b(+) (Novagen, Madison, WI) to allow for expression of MmcR as a His-tagged fusion protein. Sequencing of the construct revealed the following deviations from the reported DNA sequence: deletion of bases 411−415 and of base 460 of the *mmcR* gene (Pro^{138}-Arg^{153} to Asp^{138}-Glu^{151}), and transition of base 1013 (Cys^{338} to Tyr^{336}). Repeated cloning of the gene confirmed the deletions but revealed the transition to be a PCR error. The *mmcR* gene sequence has been revised in the original database entry (AF127374). Expression of the Cys338Tyr mutant resulted in soluble but inactive enzyme. The expression construct containing the wild type *mmcR* sequence was obtained through PCR amplification from genomic DNA using *Pfu* as the DNA polymerase. His-tagged MmcR was expressed in *E. coli* BL21(DE3) and purified to apparent homogeneity in one step using Ni-NTA resin. The enzyme was transferred into storage buffer containing 20% glycerol using PD-10 columns (Amersham Biosciences). Protein concentrations were determined by the Bradford method using BSA as the standard. Approximately 20 mg of His_6-MmcR was isolated from a 0.5 L culture.

Enzyme Assays and Kinetics. For conversion assays, the reaction mixture contained 5 μM MmcR, 1.5 mM substrate (**4** or **5**), and 0.25 mM *S*-adenosylmethionine (AdoMet) in 75 mM sodium phosphate buffer at pH 7.4. As a negative control, the enzyme was omitted. After 2 h of incubation at 30 °C, an equal volume of 1 M sodium citrate (pH 6) was added and the product was extracted with ethyl acetate. The products were analyzed by LC-MS as described above.

For kinetic analysis, the reaction mixture contained 1 μM MmcR and 0.3 mM *S*-[methyl-^{14}C]-adenosylmethionine (26 μCi/mL) in 75 mM sodium phosphate (pH 7.4). The enzyme solution was equilibrated at 30 °C, and the reaction was started through the addition of 0.05−5 mM **4**, or 0.1−10 mM **5**. For the determination of kinetic parameters for the demethylation of AdoMet, a fixed concentration of **4** was used (5 mM) and the concentration of AdoMet was varied from 1.5 to 300 μM. For the investigation of reaction conditions, additives were introduced from a 10X stock solution and with saturating substrate concentrations. The reactions were terminated by the addition of 1 M sodium citrate (pH 6) within the range of linear product formation, and the products were extracted as described above. Products were resolved on TLC (silica 60 F_{254}, 15% MeOH in dichloromethane). The TLC plate was exposed to a phosphoimager screen overnight alongside standards. Radiolabeled products were detected using a Typhoon 9410 variable mode imager and quantified using ImageQuant TL software (both Amersham Biosciences). Curves were fitted using Prism 4.0a software.

Preparation of 7-Demethylmitomycin A (4) and 7-Demethylmitomycin B (5). The syntheses of **4** and **5** were adapted from Garrett[38] and Beijnen et al.[39] as described below.

7-Demethylmitomycin A (**4**): A solution of 10 mg/mL mitomycin C (**1**, 51 μmol) in 50 mM aqueous NaOH was stirred at room temperature for 4 h. Unreacted **1** was removed by extraction with two

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volumes of ethyl acetate (five times). The reaction mixture was then adjusted to pH 5−6 by addition of 0.1 M aqueous citric acid, the product was extracted four times with two volumes of ethyl acetate each, and the solvent was removed under a stream of nitrogen gas to give 84% of the title compound **4**. ^{1}H NMR (300 MHz, CD_3CN) δ 1.93 (s, 3H, 6-CH_3), 2.77 (dd, J = 1.9, 4.4, 1 H, H2), 2.88 (d, J = 4.4, 1H, H1), 3.16 (s, 3H, 9a-OCH_3), 3.43 (dd, J = 1.6, 12.9, 1H, H3′), 3.51 (dd, J = 4.4, 10.7, 1H, H9), 3.98 (d, J = 12.9, 1H, H3), 4.19 (t, J = 10.7, 1H, H10′), 4.64 (dd, J = 4.4, 10.7, 1H, H10), 5.28 (br. s, 2H, $CONH_2$). ^{13}C NMR (DEPT135, HMBC; 500/125 MHz, DMSO-d_6) δ 7.1 (6-CH_3), 31.5 (*C*2), 35.3 (C1), 42.8 (C9), 49.0 (OCH_3), 49.5 (C3), 60.6 (C10), 106.2 (C9a), 110.5 (C8a), 111.3 (C6), 153.4 (C5a), 156.4 ($CONH_2$), 156.5 (C7), 181.4 (C5). TLC (silica 60, 15% methanol in dichloromethane) R_f 0.20. LC-MS t_{Ret} 7.7 min; m/z 336.0 (M + H$^+$); MS/MS m/z 243.1 (100%). HRMS (ESI+) m/z 358.1012 (M + Na$^+$, $C_{15}H_{17}N_3O_6Na$ requires m/z 358.1015). UV−vis (MeOH) λ_{max} 330 nm (ϵ 8217 M^{-1} cm^{-1}), 530 nm (ϵ 523 M^{-1} cm^{-1}).

7-Demethylmitomycin B (**5**): A solution of 10 mg/mL mitomycin B (**3**, 40 μmol) in 50 mM aqueous NaOH was stirred at room temperature for 2 h. Unreacted **3** was removed by extraction with two volumes of ethyl acetate (four times). The pH of the reaction mixture was then adjusted to pH 6 by addition of 0.1 M aqueous citric acid, the product was extracted four times with two volumes of ethyl acetate each, and the solvent was removed under a stream of nitrogen gas to give 72% of the title compound **5**. ^{1}H NMR (500 MHz, CD_3CN) δ 1.68 (s, 3H, 6-CH_3), 2.27 (s, 3H, NCH_3), 2.34 (d, J = 4.8, 1H, H1), 2.43 (dd, J = 2.0, 4.7, 1H, H2), 3.43 (dd, J = 2.0, 13.0, 1H, H3′), 3.64 (dd, J = 3.1, 8.5, 1H, H9), 3.91 (d, J = 13.0, 1H, H3), 4.34 (dd, J = 8.5, 11.0, 1H, H10′), 4.57 (dd, J = 3.0 10.9, 1H, H10), 5.17 (br s, 2H, $CONH_2$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 7.3 (6-CH_3), 43.0 (NCH_3), 43.6 (*C*2), 43.9 (C9), 48.2 (C1), 48.3 (C3), 59.5 (C10), 100.1 (C9a), 110.3 (C8a), 110.9 (C6), 152.5 (C5a), 156.8 ($CONH_2$), 157.0 (C7), 174.9 (C8), 181.9 (C5). TLC (silica 60, 15% methanol in dichloromethane) R_f 0.08. LC-MS t_{Ret} 3.8 min; m/z 336.0 (M + H$^+$); MS/MS m/z 247.1 (30%), 257.1 (30%), 275.2 (100%). HRMS (ESI+) m/z 358.1011 (M + Na$^+$, $C_{15}H_{17}N_3O_6Na$ requires m/z 358.1015). UV−vis (MeOH) λ_{max} = 332 nm (ϵ = 14290 M^{-1} cm^{-1}), 550 nm (ϵ = 1207 M^{-1} cm^{-1}).

Results

Identification of the Mitomycin 7-*O*-Methyltransferase. The mitomycin gene cluster contains three genes encoding AdoMet-dependent methyltransferases.[9] Two of these genes, *mitM* and *mitN*, are located adjacent to one another. They share significant homology to each other and to other methyltransferases involved in secondary metabolism. A functional copy of *mitM* is required for the production of mitomycin C,[9] but so far, *in vitro* experiments with heterologously expressed enzyme have only revealed an aziridine *N*-methyltransferase function.[30] Given the absence of a methyl group on the aziridine ring of **1**, this finding was surprising and the function of MitM continues to be under investigation in our laboratory. The third putative methyltransferase gene, *mmcR*, was also shown to be required for the production of **1**.[9] Its deduced amino acid sequence shows significant overall similarity to methyltransferases involved in the biosynthesis of numerous bacterial natural products such as enediyne and type II polyketide synthase-derived antibiotics, as well as to plant methyltransferases involved in the biosynthesis of flavonoids. The common structural element to all of these natural products is the presence of methylated phenols or methoxy-substituted quinone moieties. This made MmcR a very good candidate for the installment of the 7-*O*-methyl group of mitomycin A that is thought to be the direct precursor of mitomycin C. A more detailed sequence analysis of MmcR is given in the Supporting Information.

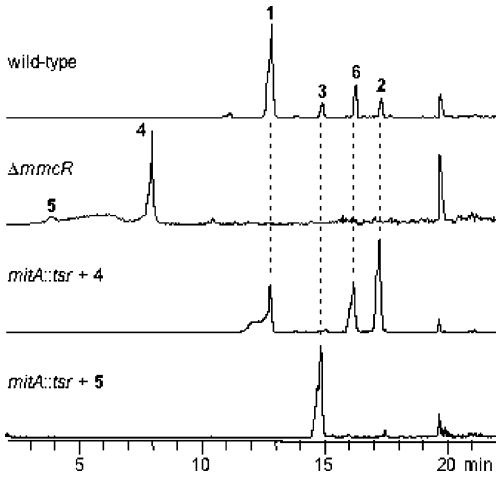

Figure 2. Analysis of *S. lavendulae* wild type, mutant, and complemented extracts. The extracted ion chromatograms for m/z 335, 336, and 350 are shown.

***In Vivo* Experiments.** An *mmcR* in-frame deletion mutant in *S. lavendulae* (DHS9505) was prepared by first inserting a kanamycin resistant marker into the gene and subsequently removing the resistance cassette together with the whole *mmcR* gene by a second round of homologous recombination. Extracts from the resulting mutant strain DHS9505 were analyzed by LC-MS and found to be devoid of mitomycins **1**, **2**, and **3**, produced by wild type *S. lavendulae*. However, upon closer examination of the DHS9505 metabolite profile, two new compounds were identified to be accumulated in the *mmcR* deletion mutant but were absent in the wild type strain (Figure 2). The major compound (**4**) had a retention time of 8.0 min, and the minor compound (**5**) eluted at 3.9 min. In good agreement with the expected function of MmcR as the 7-*O*-methyltransferase, both compounds exhibited a molecular ion peak of m/z 336 corresponding to mitomycins **2** or **3** with the loss of a methyl group. For further examination of the structures of **4** and **5**, MS/MS experiments were performed. Unlike the molecular ion peaks, the product ions obtained from **4** and **5** were distinct from each other. Fragmentation of the molecular ion peak of **4** yielded a single product ion with m/z 243 corresponding to loss of carbamate and methanol from the from the C10 and C9a positions, respectively. Analogous product ions were also observed for mitomycin A (**2**) and mitomycin C (**1**) that both possess the 9a-methoxy substituent. Hence, the analytical data were consistent with **4** corresponding to 7-demethylmitomycin A (Figure 1). Fragmentation of the molecular ion peak of **5**, on the other hand, gave rise to three product ions with m/z 247, 257, and 275 corresponding to loss of carbamate, loss of carbamate and water, and loss of carbamate and carbon monoxide, respectively. The observed series of product ions is typical for mitomycins with a 9a-hydroxy substituent, and an analogous fragmentation pattern was also observed for mitomycin B (**3**). The minor metabolite **5** was therefore assigned as 7-demethylmitomycin B (Figure 1). The identity of both compounds was further substantiated through comparison to semisynthetic standards of **4** and **5** (see above) that matched the LC-MS characteristics of the mitomycin intermediates obtained from the Δ*mmcR* mutant strain DHS9505 (Supporting Information). It is also noteworthy that the ratio of

4 to **5** is approximately the same as the ratio for the C9β-mitomycins **1** and **2** to the C9α-mitomycin **3**. The overall production yields of **4** and **5** in *S. lavendulae* DHS9505 are estimated to be in the same range as those of mitomycins in the wild type organism (between 1 and 4 mg/L culture).

In order to investigate whether **4** and **5** were chemically competent intermediates in the mitomycin biosynthetic pathway *in vivo*, we performed feeding experiments with a *S. lavendulae* strain deficient in mitomycin production. The strain selected for these experiments (DHS5349) carries an insertional mutation of the *mitA* gene.[9] MitA (AHBA synthase) is the final enzyme in the biosynthesis of AHBA and had been shown to be essential for mitomycin biosynthesis.[9,35] The insertional inactivation of *mitA* leads to a polar effect on downstream genes that act after assembly of AHBA. Hence, complementation with AHBA does not restore mitomycin production in this strain. However, *mmcR* should be expressed as part of a distinct transcript separate from *mitA* based on analysis of the gene cluster. As had been previously shown, mitomycins **1**, **2**, or **3** were absent from the culture medium of DHS5349.[9] However, when the strain was supplemented with the 9β-configured **4,** production of mitomycins **1** and **2** was fully restored. Conversely, supplementing the DHS5349 culture medium with the 9α-configured **5** resulted in production of **3** as the only mitomycin (Figure 2). These results demonstrate that **4** and **5** are able to serve as substrates *in vivo* for the biosynthesis of mitomycins with C9β- or C9α-stereochemistry, respectively. Rather unexpectedly, a fourth compound (**6**) was also dependent on AHBA production and its biosynthesis could only be restored upon addition of **4,** but not **5** to the fermentation medium. Both molecular ion peak and fragmentation pattern were identical to that of **2**, suggesting **6** to be an isomer of **2**. Two possible structures, 9-*epi*-mitomycin A and albomitomycin A (Figure 1), could be assigned based on these findings. The former compound has not been previously isolated; however, the latter product is a known mitomycin derivative.[40] Albomitomycin A forms from **2** in protic solvents through an intramolecular Michael addition of the aziridine nitrogen to C5a of the quinone moiety. Furthermore, this compound is reported to yield the same MS/MS spectrum as **2**,[40] providing a clear rationale for assignment of **6** as albomitomycin A.

***In Vitro* Assays.** In order to characterize MmcR further we cloned the corresponding *mmcR* gene into pET-28b for heterologous expression as a His_6-fusion protein. Upon closer inspection of the gene sequence, deviations from the previously reported sequence were apparent. As the same base pair changes occurred from two independent PCR reactions with genomic DNA as template and with two different DNA polymerases, the reported *mmcR* gene sequence requires minor corrections. The gene sequence of *mmcR* has been revised in the original database entry. His-tagged MmcR was expressed in *E. coli* and purified to near homogeneity using affinity chromatography. Compounds **4** and **5** were prepared as authentic standards from **1** and **3**, respectively, through base hydrolysis. The separation of products from starting material was facilitated through the presence of the enolic hydroxy group in **4** and **5** that is deprotonated at basic pH. Starting material could thus be removed through ethyl acetate extraction of the basic reaction mixture, and the products were recovered through extraction of the remaining mixture after neutralization. A semisynthetic method was chosen over isolation from fermentation extract due to the instability of **4** and **5** in solution[41] and the relatively low quantities obtained from *S. lavendulae* DHS9505 cultures.

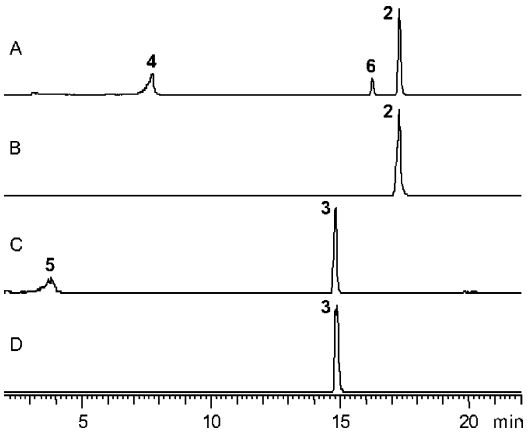

Figure 3. *In vitro* assays with heterologously expressed MmcR. Extracted ion chromatograms for *m*/*z* 336 and 350 are shown. A: **4** + AdoMet + MmcR; B: authentic standard of **2**; C: **5** + AdoMet + MmcR; D: authentic standard of **3**.

The proposed activity of MmcR as a 7-*O*-methyltransferase was confirmed by incubating **4** with the enzyme in the presence of AdoMet. Analysis of the reaction mixture revealed the presence of one major product that matched **2** in retention time, molecular mass, and MS/MS pattern (Figure 3), and the same minor product **6** that was also found in the *S. lavendulae* wild type extracts (Figure 2). Isolation of **6** from the reaction mixture initially yielded a colorless compound as expected for albomitomycin A.[40] Repeated dissolving of **6** in water or methanol resulted in the reappearance of the purple color characteristic for **2**. As **2** and **6** exist in equilibrium, our previous assignment of **6** as albomitomycin A was therefore further substantiated. With **5** as the substrate, a single product corresponding to **3** in retention time, molecular ion peak, and MS/MS pattern was observed (Figure 3). No products were detected when the enzyme was omitted (data not shown). Furthermore, none of the other two methyltransferases found in the mitomycin cluster (MitM and MitN) were capable of methylating **4** (data not shown).

Next, we determined the kinetic parameters for the MmcR-catalyzed methylation of **4** and **5** using [^{14}C]-AdoMet. The conversion of **4** to **2** followed Michaelis−Menten kinetics with a K_m value of 170 ± 25 μM and a k_{cat} value of 0.103 ± 0.004 min^{-1} with saturating AdoMet (Figure 4A). Using **5** as the substrate, no saturation was observed up to 10 mM substrate (Figure 4C). Instead, a biphasic linear increase in the rate of product formation with increasing substrate concentration was observed. The specificity constant k_{cat}/K_m for the conversion of **5** to **3** was calculated from the slope of the linear regression up to 0.6 mM substrate concentration and determined to be 0.458 ± 0.002 M^{-1} s^{-1}. This is approximately 20-fold lower than the specificity constant for the conversion of **4** to **2**. In order to investigate the integrity of the active site, the kinetic parameters for AdoMet were determined under saturating conditions of **4** (Figure 4B). A K_m value of 3.8 ± 0.5 μM for AdoMet was

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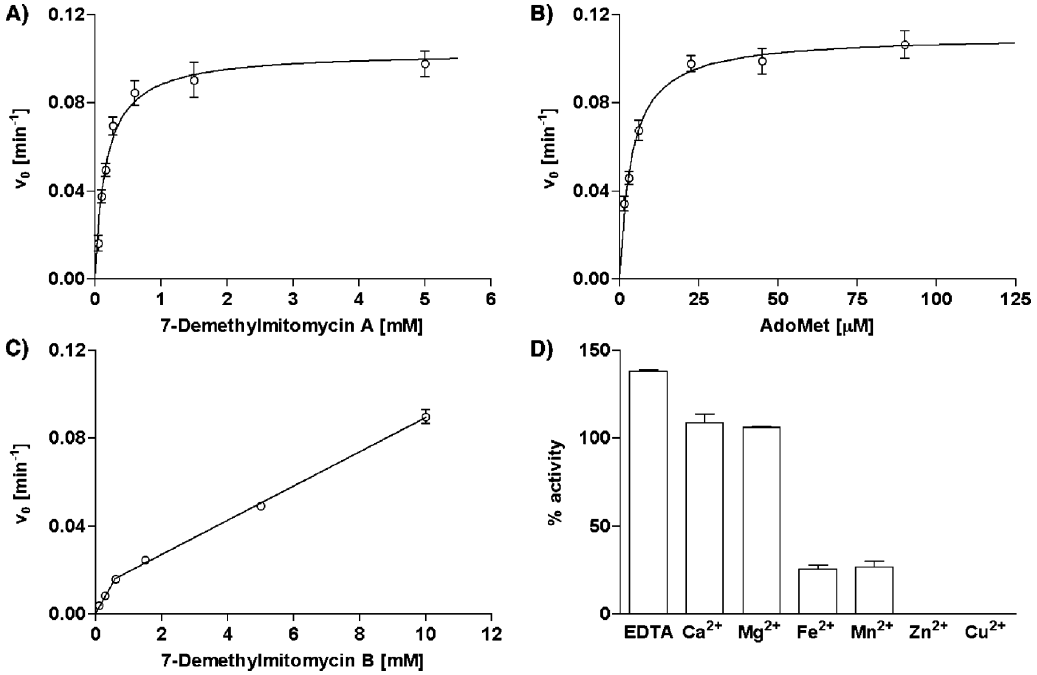

Figure 4. Activity of the MmcR-catalyzed methylation reactions. (A) Conversion of **4** to **2** with saturating concentration of AdoMet. (B) Conversion of **4** to **2** with saturating concentration of **4**. (C) Conversion of **5** to **3** with saturating concentration of AdoMet. (D) Effects of divalent cations on MmcR activity.

obtained, suggesting that the ability of MmcR to bind its cofactor was not impaired, and hence the integrity of the active site was unlikely to be affected by the presence of the His_6-fusion tag. The k_{cat} value obtained for AdoMet was 0.110 ± 0.003 min^{-1} and is in agreement with the value obtained for **4** with saturating AdoMet.

We further investigated the effect of different reaction conditions on the efficiency of MmcR using **4** as the substrate. MmcR has a relatively broad pH optimum between 7 and 7.5 (data not shown). Increasing the salt concentration from 50 to 500 mM resulted in a gradual decrease in activity to 60% of the initial value, whereas the addition of nonionic detergent did not significantly affect enzymatic activity (data not shown). As some methyltransferases depend on the presence of divalent cations for optimal activity,[42−44] a series of metals were tested. Notably, the presence of 1 mM EDTA resulted in a slight but significantly higher activity, whereas Mg^{2+} or Ca^{2+} did not alter the reactivity of MmcR. Interestingly, both Mn^{2+} and Fe^{2+} resulted in a 4-fold decrease of enzyme activity, and the presence of Zn^{2+} or Cu^{2+} completely inhibited the methylation reaction (Figure 4D). A very similar pattern for the influence of metal ions on enzyme activity was found for the rifamycin 27-*O*-methyltransferase.[45] However, no reaction conditions were found that substantially improved the methylation reaction catalyzed by MmcR with **4** as the substrate.

All methyltransferases involved in the methylation of aromatic substrates reported to date act on phenolic compounds. As such MmcR is the first methyltransferase to accept a hydroxyquinone as substrate. This also raises the possibility that MmcR could potentially catalyze methytransfer to a C7 hydroxyl group on the reduced, dihydroquinone variants of **4** or **5**. However, these compounds are not available for *in vitro* assays because of their very limited half-life.[3,4]

Discussion

Over the past several years, we have been dissecting the catalytic function of individual gene products involved in mitomycin biosynthesis in an effort to assign a specific role to the more than 40 enzymes believed to be involved in its assembly, resistance, and regulation.[9] The timing and sequence of biosynthetic steps from AHBA and D-glucosamine to mitomycins **1**, **2**, and **3** remain largely unknown and even difficult to predict due to the unique structure of the mitosane skeleton. An unusual feature in the biosynthesis of mitomycins is the presence of two series of products that differ in the stereochemistry of the C9-substituent. It is not known to what extent the biosynthetic pathways leading to mitomycins with C9β-configuration and C9α-configuration are shared or at what stage the two pathways might diverge. Interestingly, the difference in C9-configuration coincides with individual methylation patterns. We have identified from *in vivo* studies two methyltransferase genes, *mitN* and *mitM*, that are required for the aziridine-*N*-methylation of C9α-mitomycins and the 9a-O-methylation of C9β-mitomycins, respectively (N. Sitachitta et al., unpublished). This work suggests that methylation of the right side of the mitosane skeleton is performed by enzymes dedicated to a single branch of the biosynthetic pathway.

The third methyltransferase gene, *mmcR*, is required for the formation of mitomycins bearing C9α- or C9β-configurations. An *mmcR* deletion mutant of *S. lavendulae* was devoid of the three major mitomycins usually found in the wild type organism (**1**, **2**, and **3**). However, two new natural product metabolites, 7-demethylmitomycin A (**4**) and 7-demethylmitomycin B (**5**), were found to be accumulated in that strain. Mitomycin C (**1**), the only mitomycin with a 7-amino substituent, was not

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identified in *S. lavendulae* Δ*mmcR* extracts, indicating that the 7-methoxy group is required for formation of **1**. The production of C9*β*-configured (**1**, **2**) and C9α-configured (**3**) mitomycins could be selectively restored by supplementing the culture medium of an AHBA-deficient strain with either **4** or **5**, respectively. These results conclusively demonstrated that MmcR is responsible for the 7-O-methylation of both C9*β*- and C9α-mitomycins, and also that the partitioning of mitomycins into the C9*β*- and C9α-branches is largely independent of the function of this methyltransferase. However, the question remained whether the main metabolic flux in mitomycin biosynthesis proceeded through **4** and **5**.

In order to address this question, *in vitro* experiments with heterologously expressed MmcR were conducted. The 7-O-demethylated compounds **4** and **5** were converted to the expected 7-O-methylated products **2** and **3**, respectively. Metal ions were not required for activity and, in fact, tended to decrease enzyme efficiency. The conversion of **4** to **2** followed Michaelis−Menten behavior, whereas no saturation was observed for the analogous reaction with the C9α-configured **5**. The specificity constant obtained for the latter reaction was 20 times lower than for the former, indicating a significant preference of MmcR for C9*β*-substituted mitomycins. Methyltransferases typically have similar K_m values for both the methyl acceptor and the methyl donor, AdoMet. This is not the case for the MmcR-catalyzed conversion of **4** to **2**. The K_m value for the substrate **4** is 45 times higher than that obtained for AdoMet and approximately 3−20 times higher than what is typical for methyltransferases involved in secondary metabolism.[45−49] Moreover, the rate of methylation is relatively slow. Similar rates have been reported for other methyltransferases,[46,47] but typical rates of methylation are significantly faster.[45,48,49]

Thus, the late stage biosynthetic scheme for mitomycin assembly as shown in Scheme 1 is consistent with the results obtained from *mmcR* gene deletion and chemical complementation experiments. However, the kinetic evaluation of MmcR suggests that optimal enzyme conditions have yet to be established, another component of the methyltransferase is required for optimal catalysis, or an alternative mitomycin precursor is the preferred physiological substrate for MmcR. In this latter respect, it can be envisaged that methylation of the hydroxyquinone moiety might occur at an earlier stage in mitomycin biosynthesis, possibly before branching to the C9α- and C9*β*-mitomycins from a common precursor X (Scheme 1). This hypothesis would be supported by the presence of only one 7-*O*-methyltransferase gene in the cluster and the preference toward substrates with C9*β*-configuration displayed by heterologously expressed MmcR. Further investigation of additional biosynthetic steps will shed more light on the assembly of mitomycin natural products, and on the possible role of earlier stage intermediates as substrates for MmcR.

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Supporting Information Available: Mass spectrometry data for **1**−**6**, Southern blot analysis, MmcR sequence comparison. This material is available free of charge via the Internet at http://pubs.acs.org.

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