Mapping the Mitomycin Biosynthetic Pathway by Functional Analysis of the MitM Aziridine *N*-Methyltransferase

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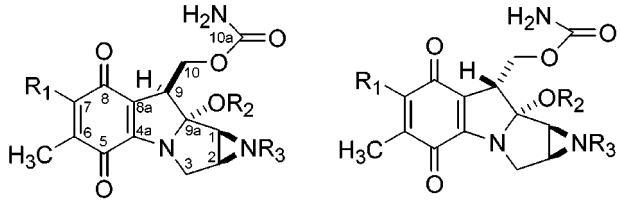
The antitumor antibiotic mitomycin C (MC) was first identified from cultures of *Streptomyces lavendulae* in the 1950s[2] and has been used clinically for treatment of a variety of soft tumors for over 30 years. Since its original discovery, several dozen naturally occurring mitomycins with varying biological properties have been identified with diverse methylation and amination patterns.[3] Intensive synthetic efforts have also been mounted to generate analogues of mitomycin C in a search for improved anti-cancer agents with enhanced therapeutic potential. Studies of mitomycin biosynthesis during the 1970s and 1980s[3] revealed that 3-amino-5-hydroxybenzoic acid,[4] D-glucosamine,[5] carbamoyl phosphate,[6] and *S*-adenosyl methionine[7] are involved in the convergent assembly of these important natural products. Although the basic building blocks have been known for some time, the specific order of natural product assembly has remained undefined. Recently, we identified and characterized the complete set of genes for mitomycin biosynthesis[8] and have developed a versatile genetic system for investigating the genes and enzymes responsible for cellular self-resistance, regulation, and assembly.[9] As the architecture of the mitomycin biosynthetic gene cluster does not provide sufficient information on the course of assembly of these molecules we have adopted a strategy of creating mutant strains of *S. lavendulae* that allows the isolation of biosynthetic intermediates. Application of this strategy has led to the isolation of a novel mitomycin, the first functional characterization of an aziridine *N*-methyltransferase, and the molecular examination of the late methylation steps responsible for the production of mitomycins with diverse structural and biological properties.

The varying activity and toxicity of the antitumor mitomycin family of compounds is largely determined by the methoxy- and amino-substitution patterns present on the mitosane skeleton.[10] Three decades ago, the role of *S*-adenosyl methionine (SAM) was shown through labeling of the mitomycin aziridine ring system, C7, and C9a *O*- and *N*-methyl groups.[7] Although there has been no reported analysis of enzymes that catalyze methyltransfer in mitomycin assembly, our recent investigation of the MC gene cluster allowed preliminary assignment[11] to the products of the *mitM*, *mitN*, and *mmcR* genes through recognized sequence similarity to other known secondary metabolite methyltransferases.[12] A detailed understanding of the sequence of reactions carried out by these enzymes is likely to provide important information on the basis of the diversity of mitomycin natural products.



Compound	R1	R2	R3	Compound	R1	R2	R3
1 9a-demethyl mitomycin A	OCH_3	H	H	**8** mitomycin B	OCH_3	H	CH_3
2 9-epi mitomycin B	OCH_3	H	CH_3	**9** mitomycin D	NH_2	CH_3	CH_3
3 mitomycin A	OCH_3	CH_3	H				
4 mitomycin F	OCH_3	CH_3	CH_3				
5 mitomycin C	NH_2	CH_3	H				
6 porfiromycin	NH_2	CH_3	CH_3				

Mitomycin C production was completely abrogated in the *S. lavendulae* MM107 mutant strain,[13] containing a chromosomal in-frame *mitM* gene deletion. However, a low level of antibiotic activity against *Bacillus subtilis* was observed in culture supernatant extracts that allowed bioassay-guided isolation of a new intermediate, 9a-demethyl mitomycin A (**1**). The isolation method consisted of eluting the crude ethyl acetate extract of the culture broth with MeOH on a Sephadex LH-20 column followed by preparative and analytical reversed-phase HPLC (C_{18}) to provide the compound in a yield of 10−30 μg per L of culture.

The presence of a demethylated mitomycin from the *S. lavendulae* MM107 mutant strain was anticipated from assignment of methyltransferase activity to MitM based on homology to other secondary and primary metabolite methyltransferases. However, database comparisons lead us to the initial hypothesis that MitM would likely function as a C-9 *O*-methyltransferase, which was consistent with the direct conversion of 9a-demethyl mitomycin A (**1**) to MA. We were, therefore, surprised to find that MitM converts 9a-demethyl mitomycin A (**1**) to 9-*epi*-mitomycin B (**2**), demonstrating that this enzyme functions as an aziridine *N*-methyltransferase.

Observation of the signals for protons H1, H2, H3α, H3β, Me6, H9, H10, and H10′ in the ^{1}H NMR spectrum of **1** indicated that the mitomycin skeleton was intact.[14] The presence of the methoxy singlet at 3.95 ppm and the absence of other methoxy or aziridine *N*-methyl signals at 3.3 and 2.2 ppm, respectively, led to a preliminary structure consistent with that of **1**. A *m*/*z* of 374.0774 (5.1 ppm of calculated) from HRESIMS of $[M + K]^+$ analyzed for a molecular formula of $C_{15}H_{17}N_3O_6$ further supported this structure. The ^{13}C NMR signals for the atoms of the carbon skeleton were observed by HMQC and HMBC experiments due to difficulties in obtaining sufficient signal from directly detected ^{13}C NMR experiments. In addition to providing the ^{13}C NMR spectrum the heteronuclear correlation experiments demonstrated the presence of the quinone moiety, the connectivities for the

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(14) 9a-Demethyl mitomycin A: ^{1}H NMR (CD_3CN, 800 MHz) δ 1.77 (s, C6-methyl), 2.74 (m, H2), 2.81 (m, H1), 3.31 (dd, J = 4.3, 11.3 Hz, H9), 3.39 (d, J = 12.8 Hz, H3α), 3.84 (d, J = 12.8, H3β), 3.95 (s, C7-OCH_3), 4.16 (dd, J = 10.4, 11.3 H10′), 4.71 (dd, J = 4.3, 10.4, H10).

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7-methoxy and C6 methyl groups to the aminoquinone group, and the presence of the carbamoyl carbon from an H10 to C10a correlation. Determination of the stereochemistry at C9 was first attempted by difference nOe, NOESY, and ROESY experiments; however, the lack of a clear nOe signal between the protons on C10 and the H1, H2, and H3 protons prevented this assignment.

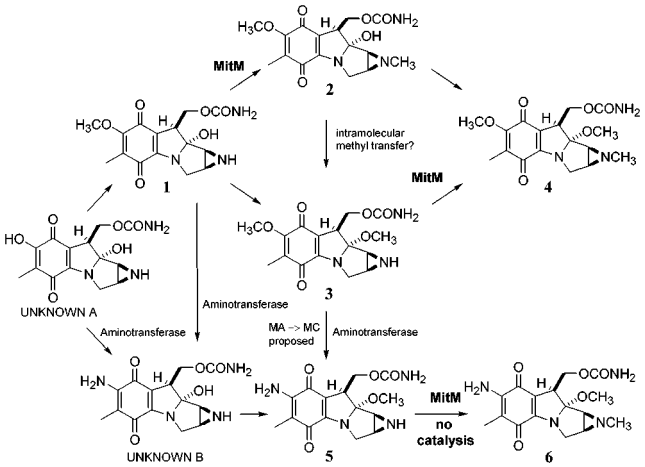
Overexpression of MitM from *Escherichia coli* was used to convert 9a-demethyl mitomycin A (**1**) to the aziridine *N*-methylated product **2**.[15] HRESIMS of **2** provided a *m*/*z* of 372.1186 ($[M + Na]^+$, 3.7 ppm of of calculated), consistent with the molecular formula of $C_{16}H_{19}N_3O_6$, which confirmed addition of a methyl group. The methyl signal at 2.22 ppm and the upfield shift of the H1 and H2 signals from 2.81 and 2.74 ppm to merge at 2.31 ppm is consistent with an aziridine *N*-methyl group in the mitomycin series of compounds.[16] By thin-layer chromatography (silica gel, 9:1 $CHCl_3-CH_3OH$) it was apparent that the product ($R_f = 0.21$) was not mitomycin B (**8**) ($R_f = 0.26$), and thus suggested 9-*epi*-mitomycin B (**2**) with a C9−C10 bond in the β (or (*S*)-C9) configuration. The stereochemistry of **1** and **2** at the C9a, C1, and C2 carbons was thus assigned as depicted based on biosynthetic correlation with previously isolated mitomycins.[17]

A further survey of MitM *N*-methyl (or 9a *O*-methyl) transferase activity was carried out with **3**, **5**, and **8**. Only mitomycin A (**3**) served as a substrate, and was methylated at the aziridine nitrogen to produce mitomycin F (**4**). This analysis suggests that MitM accepts mitomycins with a C7-methoxy group such as **1** and **3**, but not those with a C7-amino group such as mitomycin C (**5**). Clearly, a more complete analysis of the substrate specificity of MitM will be required to understand further these structure−activity relationships.

Although as many as four potential methyltransferases were predicted from the mitomycin pathway sequence analysis,[8] dissecting the function of MitM became our initial objective because the *S. lavendulae mitM* gene deletion mutant is completely blocked in MC biosynthesis.

While the deletion of *mitM* does not directly explain the loss of mitomycin C production, the possible existence of other enzymes in the putative biosynthetic route from **1** to mitomycin C (**5**) is suggested by this result (Scheme 1). An early observation that MA titers first increase and then decrease as MC titers rise[3] led to the notion that MC is produced directly from MA, although biochemical evidence for this conversion was never reported. When the late-stage methylations for mitomycins with β stereochemistry at C9 are examined, an early branch from a putative nonmethylated precursor (Unknown A) can be hypothesized. This putative precursor can be methylated at the C7 position to form **1** and enter into the MA series of compounds, or an amino group may be added at C7 (to form Unknown B) to initiate the MC group of metabolites. If the C7 position of Unknown A is methylated to generate **1** and MA (**3**) is one of the precursors of MC (**5**) then the formation of MA from **2** is critical for the production of **5**. An enzyme(s) that performs the formal methyl group shift from the aziridine nitrogen to the 9a oxygen (to form **3**) could then lead to production of **5**. Notably, although MA (**3**) is produced in wild type *S. lavendulae*, it is not detected in the *mitM* deletion mutant. Thus, abrogation of mitomycin C production in the absence of MitM appears to occur by blocking the formation of **2** from **1**.

Scheme 1



Our results have effectively dissected the *N*-methyltransferase activity of MitM; however, further studies are needed to understand the role of the C7 methoxy mitomycins in the production of the C7 amino group series of compounds. While the exact order and timing of methylation reactions leading to mitomycin C remains unclear, the results above infer that **1** and **2** are precursors of mitomycin C. Further study to establish which methyltransferase converts the C9a oxygen to form MA (**3**) from **1** or **2** and how the C7 amino and C7 methoxy series of compounds are formed in the mitomycin family should reveal the late steps involved in the generation of the diverse structures and activities of the mitomycin family of compounds.

In this work, we have provided an initial demonstration that molecular genetic manipulation of the mitomycin pathway can elucidate the sequence of reactions involved in mitomycin biosynthesis, as well as provide access to novel mitomycin natural products. Compound **1** has not been previously synthesized or isolated[18] and **2** has been obtained only through semi-synthesis using mitomycin B (**8**) as starting material.[19] Moreover, this study describes for the first time, the functional characteristics of an aziridine *N*-methyltransferase. As we gain a better understanding of the genetic programming of the mitomycin biosynthetic system and the role of the structural, resistance, and regulatory proteins, we expect this information will provide the means to generate novel mitomycin compounds using combinatorial biosynthetic strategies.

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Supporting Information Available: Figures S1−S9 provide NMR, mass, and UV spectra supporting the structures of **1** and **2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) MitM has an optimal activity at pH 8 and was found to have a K_m of 263 μM and a k_{cat} of 0.11 s^{-1} toward **1**. Limited amounts of **1** prevented determination of kinetic data for SAM.

(16) 9-*epi*-Mitomycin B (**2**): ^{1}H NMR (CD_3CN, 800 MHz) δ 1.78, (s, C6-methyl), 2.22 (s, N-aziridine Me), 2.31 (m, overlapped H1, H2), 3.31 (dd, J = 4.9, 9.8 Hz, H9), 3.38 (d, J = 12.2 Hz, H3α), 3.84 (d, J = 12.2, H3β), 3.96 (s, C7−OMe), 4.06 (dd, J = 9.8, 12.2 Hz, H10′), 4.67 (dd, J = 4.9, 12.2 Hz, H10).

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