In vitro characterization of MitE and MitB: formation of *N*-acetylglucosaminyl-3-amino-5-hydroxybenzoyl-MmcB as a key intermediate in the biosynthesis of antitumor antibiotic mitomycins

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Abstract

Mitomycins, produced by several *Streptomyces* strains, are potent anticancer antibiotics that comprise an aziridine ring fused to a tricyclic mitosane core. Mitomycins have remarkable ability to crosslink DNA with high efficiency. Despite long clinical history of mitomycin C, the biosynthesis of mitomycins, especially mitosane core formation, remains unknown. Here, we report *in vitro* characterization of three proteins, MmcB (acyl carrier protein), MitE (acyl AMP ligase), and MitB (glycosyltransferase) involved in mitosane core formation. We show that 3-amino-5-hydroxybenzoic acid (AHBA) is first loaded onto MmcB by MitE at the expense of ATP. MitB then catalyzes glycosylation of AHBA-MmcB with uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to generate a key intermediate, GlcNAc-AHBA-MmcB, which contains all carbon and nitrogen atoms of the mitosane core. These results provide important insight into mitomycin biosynthesis.

Mitomycins are antitumor antibiotics isolated from several *Streptomyces* strains.[1, 2] The structures of mitomycins comprise an aziridine ring fused to a tricyclic mitosane core (Figure 1A).[3-7] Mitomycins inhibit DNA synthesis as a result of their ability to form covalent bonds with DNA molecules, with specificity favoring the 5′-CG-3′ sequence, and form both inter- and intra-strand DNA cross-links.[8-11] These DNA cross-linking processes rely on activation of mitomycin by either enzymatic or nonenzymatic reduction of the quinone moiety. Because of its promising activity, mitomycin C was approved in 1974 to treat stomach and pancreatic cancer and has since been used in several other cancer types such as lung, liver, breast, colon, and bladder cancer. After landmark total synthesis of mitomycins by Kishi *et al*., several other studies have reported total syntheses of mitomycins.[12-14]

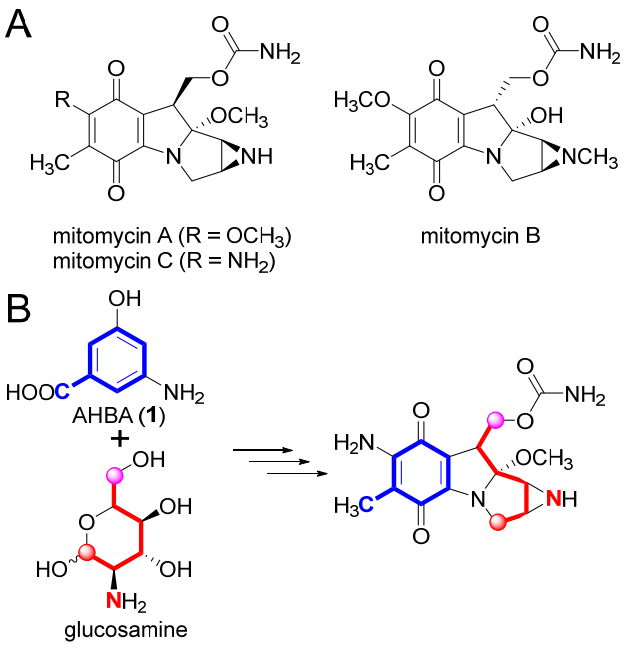


Figure 1. Structure (A) and biosynthetic origin (B) of mitomycins

While many studies on mitomycins have focused on the mechanism of action and chemical synthesis, the biosynthesis, especially the mechanism of mitosane core formation, remains elusive. Early isotope tracer experiments revealed that the *O*-methyl groups and a carbamate group in mitomycins are derived from methionine and citrulline, respectively. Later, the biosynthetic origins of the mitosane core were identified to be 3-amino-5-hydroxybenzoic acid (AHBA, **1**) and glucosamine (Figure 1B).[15] Identification and sequencing of the mitomycin biosynthetic gene cluster by Sherman's group further shed light on the biosynthesis.[16, 17] Besides genes for AHBA formation and tailoring enzymes, the cluster contains several genes putatively responsible for mitosane formation, which include *mmcB* (acyl carrier protein (ACP)), *mitB* (glycosyltransferase), *mitC* (deacetylase), *mitE* (acyl AMP ligase), *mitH* (reductase), and *mitF* (reductase). Among these, *mmcB, mitB, mitE*, and *mitH* were shown to be indispensable for mitomycin biosynthesis by gene knockout experiments. In addition, no detectable intermediate was accumulated in the culture of any disruptants. Consequently, it was suggested that biosynthetic intermediates are at all stages tethered to the acyl carrier protein, MmcB. The proposed reactions for the assembly of AHBA and glucosamine units are depicted in Figure 2. The carboxyl group of the AHBA intermediate is first activated and loaded onto MmcB by MitE. MitB then catalyzes the glycosylation between AHBA-MmcB and glucosamine. In the latter reaction, we speculated that uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc, **2**) is used as the glycosyl donor, because **2** but not UDP-glucosamine is available from the primary metabolism. Deacetylation of the GlcNAc moiety by MitC might be catalyzed in later stages in a similar manner to aminoglycoside and mycothiol biosyntheses.[18, 19] Here, we report the *in vitro* characterization of three proteins, MmcB, MitE, and MitB, and show that GlcNAc-AHBA-MmcB is a key intermediate in mitomycin biosynthesis.

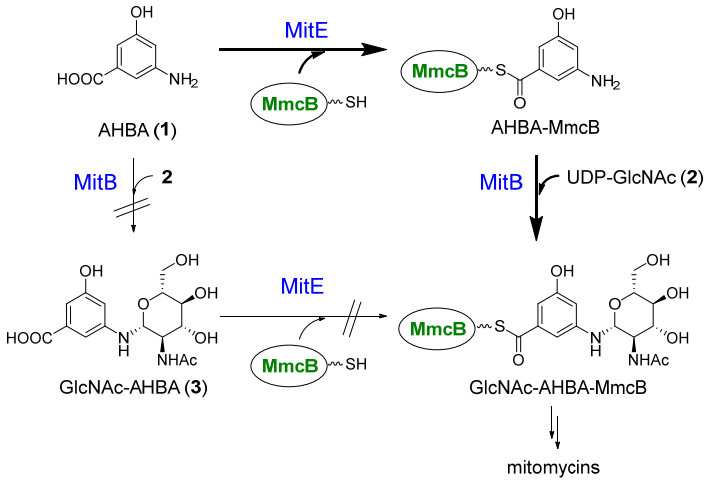


Figure 2. Proposed biosynthetic pathway of mitomycins

To investigate the proposed reactions, the three genes, *mitB*, *mitE*, and *mmcB* of *Streptomyces ardus* NBRC 13490 (=NRRL 2564)[16] were individually cloned into pET28b(+) expression vector and heterologously expressed in *Escherichia coli* BL21(DE3). MitB and MitE were designed to contain N-terminal His-tags and the His-tag was fused to both the N- and C-termini of MmcB. Because the expression of MitB with the original assigned start codon resulted in the production of insoluble protein, we reexamined the sequence of the *mitB* gene and the flanking regions and obtained soluble MitB using the alternative start codon 129 nucleotides upstream of the original start codon (Figure S1). Each protein was purified by affinity chromatography using Ni-NTA resin to near homogeneity. SDS-PAGE of the purified proteins clearly showed the production of recombinant proteins (Figure S2). Because the ACPs expressed in *E. coli* are generally apo forms, we incubated MmcB with CoA and recombinant phosphopantetheinyl transferase, Sfp, to generate the holo forms. As shown in Figure S3, efficient conversion from apo-MmcB (obs. 13783.06 Da, cal. 13783.09 Da) to holo-MmcB (obs. 14123.21 Da, cal. 14123.18 Da) was observed by LC-ESI-

MS analyses. The resulting holo form of MmcB was used for *in vitro* reactions after buffer exchange by ultrafiltration.

We first incubated AHBA (**1**) and MmcB with MitE in the presence of ATP and Mg^{2+} ions, and the reaction mixtures were analyzed by LC-ESI-MS equipped with a C8 column. A new product peak was observed in the reaction mixture (Figure 3 and S4). The deconvoluted mass spectrum of this new product was consistent with that of the proposed product, AHBA-MmcB (obs. 14258.19 Da, cal. 14258.21 Da). Formation of this product was not observed in assays lacking any one of the reaction components (Figure 3 and S4). These results indicated that MitE catalyzed the activation of **1** and loading onto MmcB. We next investigated the glycosylation step. When MitB and UDP-GlcNAc (**2**) were added into the MitE reaction mixture, we detected a new reaction product by LC-MS (Figure 4 and S5). The molecular mass of the product agreed with that of the proposed GlcNAc-AHBA-MmcB product (obs. 14461.33 Da, cal. 14461.29 Da) and this product was absent in the control reaction in which either MitB or **2** was omitted. To gain more support for the structural assignment of the MitB reaction product, the MmcB-bound products were subjected to alkaline hydrolysis in 0.2 M NaOH at 4°C for 16 h and were analyzed by LC-MS. A selected ion monitoring chromatogram (*m*/*z* 357 for GlcNAc-AHBA $[M+H]^+$) clearly revealed a peak that coeluted with synthetic GlcNAc-AHBA (**3**, Figure S6). These results fully established the identity of the MitB product as GlcNAc-AHBA-MmcB.

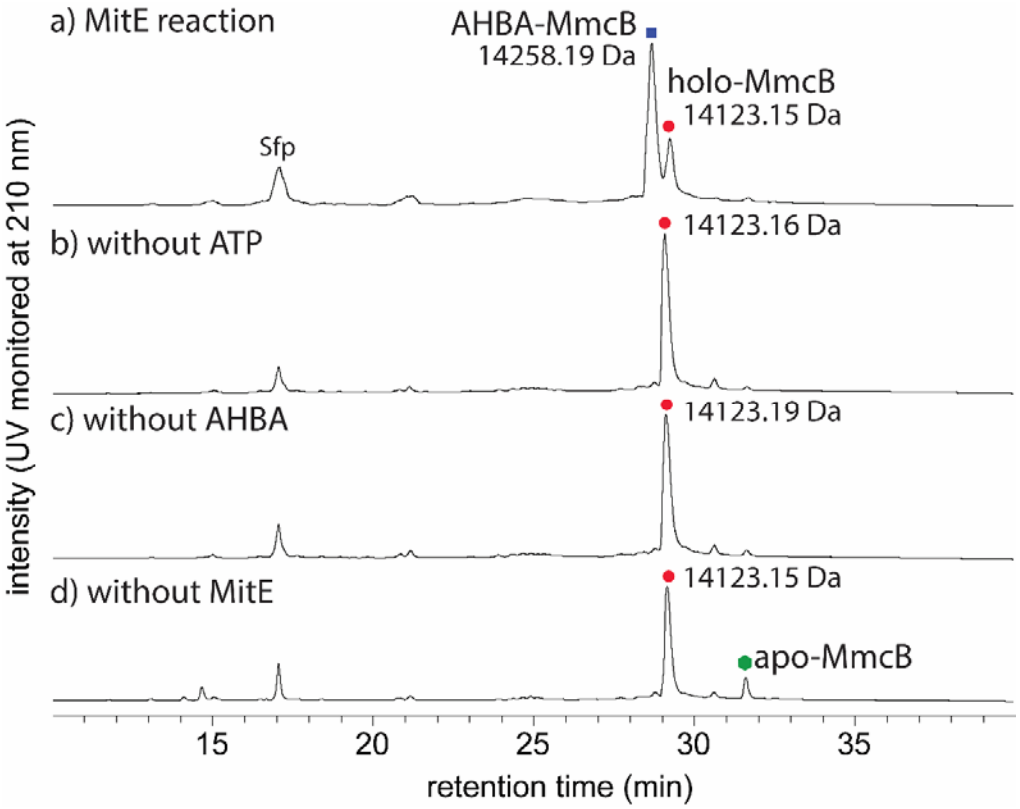
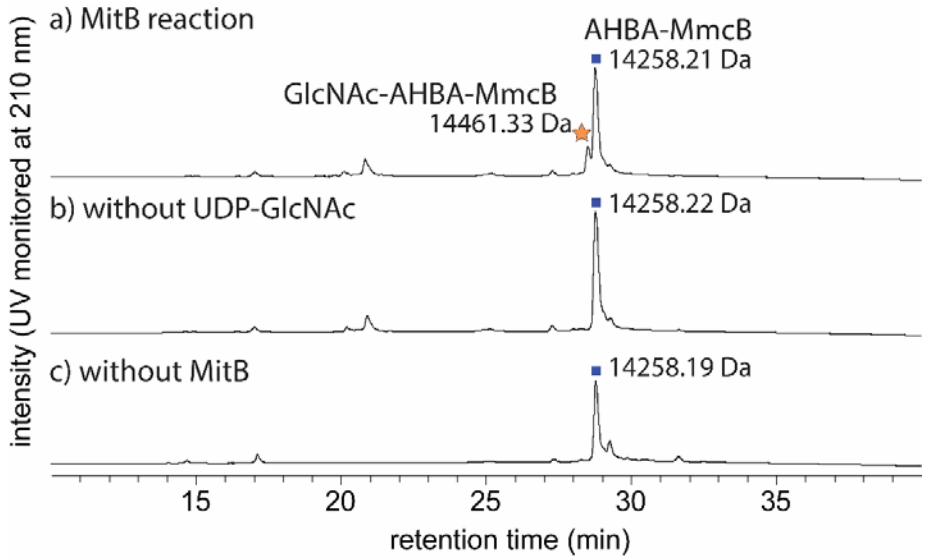


Figure 3. LC-MS analysis of the MitE assays. a) MitE reaction, b) control reaction without ATP, c) control reaction without AHBA, d) control reaction without MitE. UV chromatograms monitored at 210 nm and the molecular weights obtained from the deconvoluted mass spectra are shown.



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105 **Figure 4**. LC-MS analysis of the MitB assays. a) MitB reaction, b) control reaction without **2**, c)
106 control reaction without MitB. UV chromatograms monitored at 210 nm and the molecular weights
107 obtained from the deconvoluted mass spectra are shown.

108

109 Because the conversion from AHBA-MmcB to GlcNAc-AHBA-MmcB was not efficient, a
110 reversal of the sequence of these two reactions is also plausible for the biosynthesis of GlcNAc-
111 AHBA-MmcB. To test this possibility, a mixture containing **1** and **2** was incubated with MitB. As
112 shown in Figure S7, the formation of **3** was not observed. In addition, **3** was not a substrate of the
113 MitE reaction (Figure S8). Overall, we unequivocally established the pathway to assemble AHBA
114 and glucosamine units in mitomycin biosynthesis.

115 Recently, Kudo *et al*. showed that a similar pathway is operating in pactamycin biosynthesis.[20]
116 In this pathway, the adenylation enzyme, PctU (29% identity to MitE) activates 3-amino benzoic
117 acid (ABA) and loads it onto holo-form ACP (PctK, 32% identity to MmcB) to yield 3-ABA-PctK.
118 Glycosyltransferase (PctL, 39% identity to MitB) then catalyzes *N*-glycosylation of 3-ABA-PctK

with UDP-GlcNAc to generate GlcNAc-3-ABA-PctK. Although the two pathways employ similar strategies in the early stages, the structures of mitomycins and pactamycin are quite different. Efforts are currently underway to further elucidate the biosynthesis of mitomycins.

In conclusion, we carried out *in vitro* functional characterization of three proteins, MmcB, MitE, and MitB, and established the pathway to assemble AHBA and glucosamine units in mitomycin biosynthesis. Our data indicated that MitE activated AHBA at the expense of ATP and loaded it onto MmcB. MitB then catalyzed glycosylation of AHBA-MmcB with UDP-GlcNAc to biosynthesize GlcNAc-AHBA-MmcB. While this paper was in revision, Nguyen *et al*. also reported the functional characterization of MitB.[21] Overall, these results lay the foundation for further biochemical and mechanistic studies of mitosane formation in mitomycin biosynthesis and will be useful in modifying these pathways to produce new unnatural natural products with improved biomedical properties.

The authors declare no competing financial interest.

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References

1. Hata, T.; Hoshi, T.; Kanamori, K.; Matsumae, A.; Sano, Y.; Shima, T.; Sugawara, R. J Antibiot (Tokyo) 1956, 9, 141.
2. Wakaki, S.; Marumo, H.; Tomioka, K.; Shimizu, G.; Kato, E.; Kamada, H.; Kudo, S.; Fujimoto, Y. Antibiot Chemother (Northfield) 1958, 8, 228.
3. Lefemine, D. V.; Dann, M.; Barbatschi, F.; Hausmann, W. K.; Zbinovsky, V.; Monnikendam, P.; Adam, J.; Bohonos, N. Journal of the American Chemical Society 1962, 84, 3184.
4. Webb, J. S.; Cosulich, D. B.; Mowat, J. H.; Patrick, J. B.; Broschard, R. W.; Meyer, W. E.; Williams, R. P.; Wolf, C. F.; Fulmor, W.; Pidacks, C.; Lancaster, J. E. Journal of the American Chemical Society 1962, 84, 3185.
5. Webb, J. S.; Cosulich, D. B.; Mowat, J. H.; Patrick, J. B.; Lancaster, J. E.; Broschard, R. W.; Meyer, W. E.; Williams, R. P.; Wolf, C. F.; Fulmor, W.; Pidackas, C. Journal of the American Chemical Society 1962, 84, 3187.
6. Tulinsky, A. Journal of the American Chemical Society 1962, 84, 3188.
7. Tulinsky, A.; Van den Hende, J. H. Journal of the American Chemical Society 1967, 89, 2905.
8. Bizanek, R.; McGuinness, B. F.; Nakanishi, K.; Tomasz, M. Biochemistry 1992, 31, 3084.
9. Iyer, V. N.; Szybalski, W. Proc Natl Acad Sci U S A 1963, 50, 355.
10. Iyer, V. N.; Szybalski, W. Science 1964, 145, 55.
11. Tomasz, M. Chem Biol 1995, 2, 575.
12. Nakatsubo, F.; Fukuyama, T.; Cocuzza, A. J.; Kishi, Y. Journal of the American Chemical Society 1977, 99, 8115.
13. Fukuyama, T.; Yang, L. Journal of the American Chemical Society 1987, 109, 7881.
14. Fukuyama, T.; Yang, L. Journal of the American Chemical Society 1989, 111, 8303.
15. Anderson, M. G.; Kibby, J. J.; Rickards, R. W.; Rothschild, J. M. Journal of the Chemical Society, Chemical Communications 1980, 1277.
16. Mao, Y.; Varoglu, M.; Sherman, D. H. Chem Biol 1999, 6, 251.
17. Mao, Y.; Varoglu, M.; Sherman, D. H. J Bacteriol 1999, 181, 2199.
18. Truman, A. W.; Huang, F.; Llewellyn, N. M.; Spencer, J. B. Angew Chem Int Ed Engl 2007, 46, 1462.
19. Newton, G. L.; Ko, M.; Ta, P.; Av-Gay, Y.; Fahey, R. C. Protein Expr Purif 2006, 47, 542.
20. Kudo, F.; Zhang, J.; Sato, S.; Hirayama, A.; Eguchi, T. Chembiochem, in press (doi: 10.1002/cbic.201900239).
21. Nguyen, H. P.; Yokoyama, K. Biochemistry in press (doi: 10.1021/acs.biochem.9b00379).