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

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12 Keywords: acyl carrier protein, antibiotics, biosynthesis, glycosyltransferase, mitomycin.

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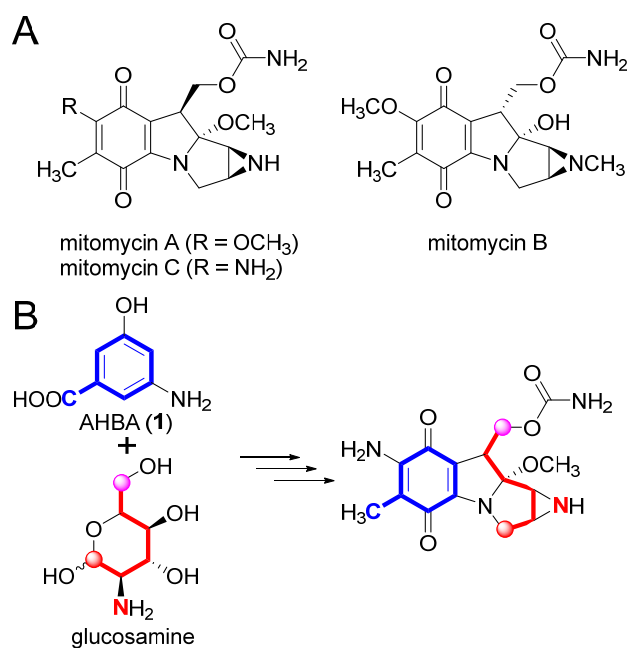
14 **Abstract**

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

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28 Mitomycins are antitumor antibiotics isolated from several *Streptomyces* strains.<sup>1, 2</sup> The  
 29 structures of mitomycins comprise an aziridine ring fused to a tricyclic mitosane core (Figure  
 30 1A).<sup>3-7</sup> Mitomycins inhibit DNA synthesis as a result of their ability to form covalent bonds with  
 31 DNA molecules, with specificity favoring the 5'-CG-3' sequence, and form both inter- and intra-  
 32 strand DNA cross-links.<sup>8-11</sup> These DNA cross-linking processes rely on activation of mitomycin  
 33 by either enzymatic or nonenzymatic reduction of the quinone moiety. Because of its promising  
 34 activity, mitomycin C was approved in 1974 to treat stomach and pancreatic cancer and has since  
 35 been used in several other cancer types such as lung, liver, breast, colon, and bladder cancer. After  
 36 landmark total synthesis of mitomycins by Kishi *et al.*, several other studies have reported total  
 37 syntheses of mitomycins.<sup>12-14</sup>



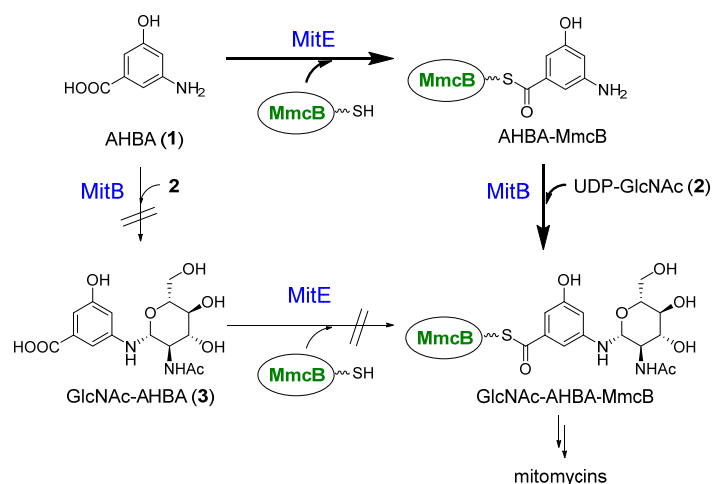
38

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

40

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

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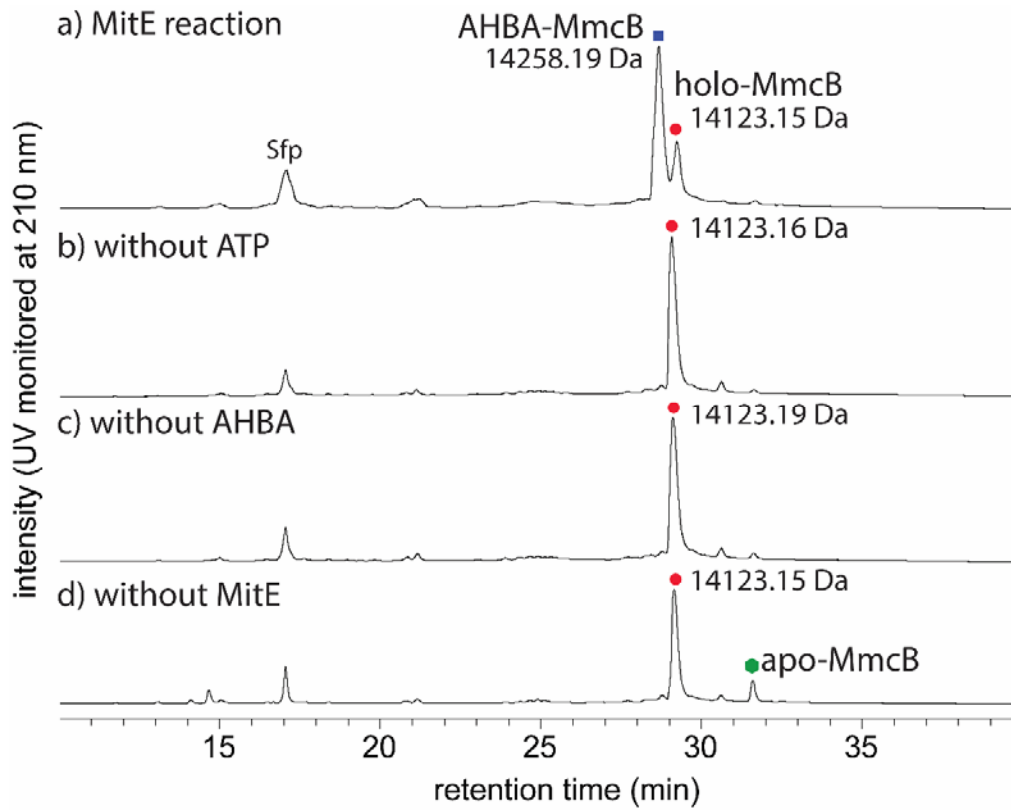
65 **Figure 2.** Proposed biosynthetic pathway of mitomycins

66

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

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

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

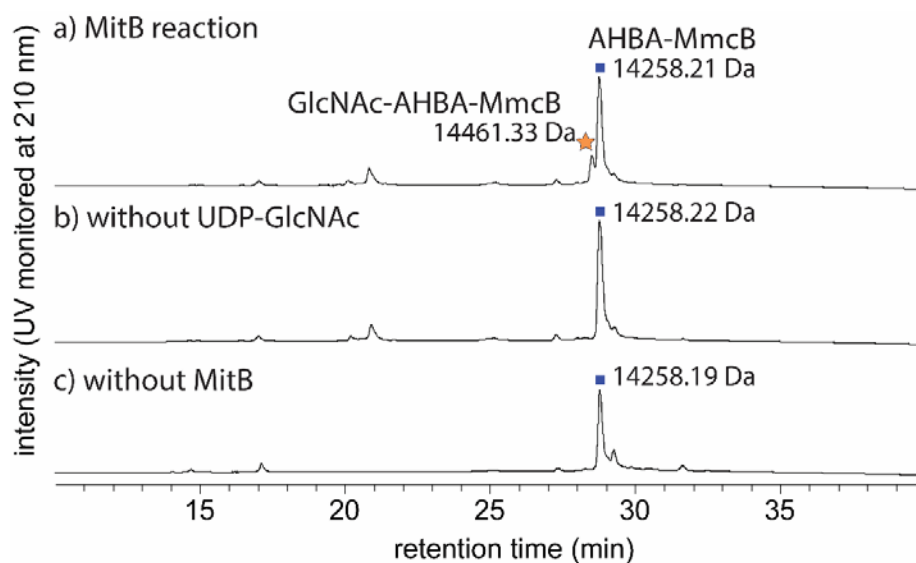


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99 **Figure 3.** LC-MS analysis of the MitE assays. a) MitE reaction, b) control reaction without ATP,  
 100 c) control reaction without AHBA, d) control reaction without MitE. UV chromatograms  
 101 monitored at 210 nm and the molecular weights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

103





104  
 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.<sup>20</sup>  
 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

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

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

131

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133 The authors declare no competing financial interest.

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