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In Vitro Biosynthesis of the Antitumor Agent Azinomycin B

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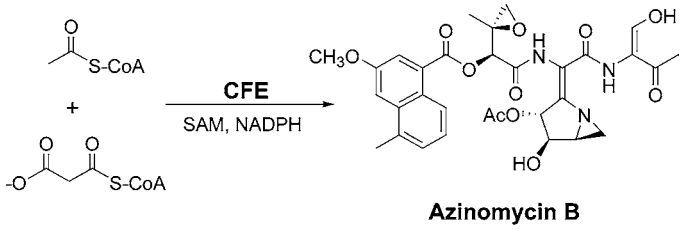
ABSTRACT



CFE = cell-free extract

Azinomycins have potential therapeutic value as antitumor agents; however, their biosynthesis is poorly understood. Here, we provide the first demonstration of a protein cell-free system capable of supporting complete in vitro biosynthesis of the antitumor agent azinomycin B. The cell-free system is utilized to probe the cofactor dependence and substrate requirements of the pathway en route to azinomycin.

Azinomycins A (1a) and B (1b) (Figure 1) are antitumor antibiotics isolated from two *Streptomyces* species, *S. sahachiroi*[1] and *S. griseofuscus*,[2] respectively. Both compounds exhibit in vitro cytotoxic activity at submicromolar levels and demonstrate antitumor activities comparable to that of mitomycin C in vivo.[3] Unique to this class of natural products is the presence of an aziridino[1,2-*a*]pyrrolidine (1-azabicyclo-[3.1.0]hexane) ring system. Coupled with an epoxide moiety, these structural functionalities impart the ability to form interstrand cross-links with DNA via the electrophilic C10 and C21 carbons of azinomycin and the N7 positions of suitably disposed purine bases.[4]

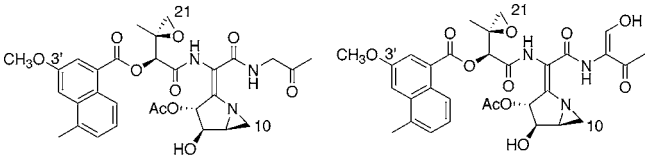


Figure 1. Structures of azinomycins A and B.

The novel architecture, intricate functionalization, and compelling mode of action of the azinomycins have made these agents attractive targets from both a synthetic and a biosynthetic perspective. Synthetic routes to the azabicyclic system have been reported including total synthesis of azinomycin A.[5] A variety of synthetic analogues have also been generated.[6] Considerably less is known about the biosynthetic origin of these compounds.

(1) Hata, T.; Koga, F.; Sano, Y.; Kanamori, K.; Matsumae, A.; Sugawara, R.; Hoshi, T.; Shimi, T.; Ito, S.; Tomizawa, S *J. Antibiot.* **1954**, *A7*, 107−112.

(2) (a) Nagaoka, K.; Matsumoto, M.; Oono, J.; Yokoi, K.; Ishizeki, S.; Nakashima, T. *J. Antibiot.* **1986**, *39*, 1527. (b) Yokoi, K.; Ishizeki, S.; Nakashima, T. *Chem. Pharm. Bull.* **1986**, *34*, 4554.

(3) Ishizeki, S.; Ohtsuka, M.; Irinoda, K.; Kukita, K.; Nagaoka, K.; Nakashima, T. *J. Antibiot.* **1987**, *40*, 60.

(4) (a) For a review of the azinomycins, see: Hodgkinson, T. J.; Shipman, M. *Tetrahedron* **2001**, *57*, 4467. (b) Lown, J. W.; Majumdar, K. C. *Can. J. Biochem.* **1977**, *55*, 630. (c) Coleman, R. S.; Perez, R. J.; Burk, C. H.; Navarro, A. *J. Am. Chem. Soc.* **2002**, *124*, 13008. (d) Armstrong, R. W.; Salvati, M. E.; Nguyen, M. *J. Am. Chem. Soc.* **1992**, *114*, 3144. (e) Zang, H.; Gates, K. S. *Biochemistry* **2000**, *39*, 14968. (f) Fujiwara, T.; Saito, I.; Sugiyama, H. *Tetrahedron Lett.* **1999**, *40*, 315. (g) Coleman, R. S.; Burk, C. H.; Navarro, A.; Brueggemeier, R. W.; Diaz-Cruz, E. S. *Org. Lett.* **2002**, *4*, 3545.

(5) (a) Coleman, R. S.; Li, J.; Navarro, A. *Angew. Chem., Int. Ed.* **2001**, *40*, 1736. (b) Hodgkinson, T. J.; Shipman, M. *Tetrahedron* **2001**, *57*, 4467. (c) Coleman, R. S. *Strategies Tactics Org. Synth.* **2004**, *5*, 51.

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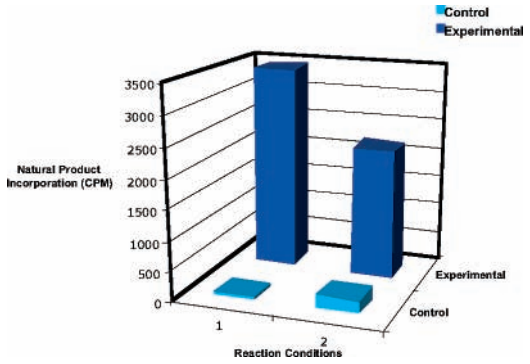
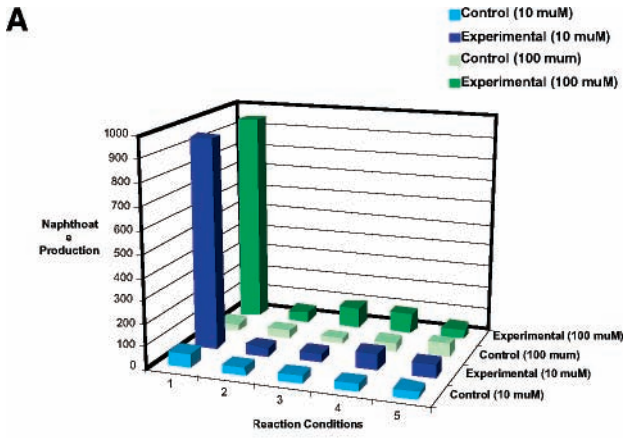


Figure 2. Demonstration of in vitro biosynthesis. Controls: cpm ± 10. Exptl: cpm ± 50. (1) Conversion to the naphthoate. (2) Conversion to azinomycin B. Reaction conditions: CFE + [1-^{14}C]-malonyl-CoA, acetyl-CoA, SAM, FAD, and NADPH.

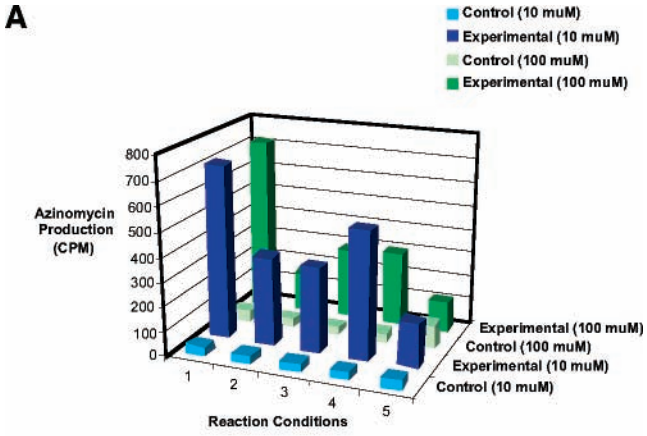
Preliminary evaluation has been performed by Lowden and co-workers establishing the polyketide origin of these molecules.[7] Here, we report details on the first cell-free system capable of supporting in vitro biosynthesis of azinomycin B. All steps from the initial construction of the naphthoate fragment of the molecule to the formation of the azabicyclo ring sytem have been achieved. The approach sets the stage for protein purification and examination of discrete



B

reaction condition	inhibitor	cpm (10 μM)	cpm (100 μM)
1 (Expt.)	—	945	945
1 (Cont.)	—	59	59
2 (Expt.)	miconazole	38	43
2 (Cont.)	miconazole	37	37
3 (Expt.)	metyrapone	41	91
3 (Cont.)	metyrapone	34	34
4 (Expt.)	chloramphenicol	73	93
4 (Cont.)	chloramphenicol	32	26
5 (Expt.)	cerulenin	55	47
5 (Cont.)	cerulenin	33	38

Figure 3. Effect of enzyme inhibitors on naphthoate production. Reaction 1 exptl: cpm ± 50. Reactions 1−5: cpm ± 10. (A) Graphical representation of data. (B) Tabulated data.



B

reaction condition	inhibitor	cpm (10 μM)	cpm (100 μM)
1 (Expt.)	—	714	714
1 (Cont.)	—	40	40
2 (Expt.)	miconazole	360	164
2 (Cont.)	miconazole	32	32
3 (Expt.)	metyrapone	348	292
3 (Cont.)	metyrapone	36	30
4 (Expt.)	chloramphenicol	523	307
4 (Cont.)	chloramphenicol	36	30
5 (Expt.)	cerulenin	178	119
5 (Cont.)	cerulenin	38	95

Figure 4. Effect of protein inhibitors on azinomycin production. Controls: cpm ± 10. Exptl: cpm ± 50. (A) Graphical representation of data. (B) Tabulated data.

enzyme activities or the identification of genes by reverse-genetic techniques. In this report, we illustrate this method of cell-free enzyme (CFE) preparation to investigate the cofactor and substrate requirements of the pathway.

The fortified crude enzyme preparation was generated by culturing *S. sahachiroi* and flash freezing the cells in liquid nitrogen. The frozen material was transferred to a bead beater containing cell-free extract buffer (pH 7.5) and glass beads. The cells were pulverized (utilizing 10 intermittent cycles) and centrifuged to generate the crude cell-free extract.

Initially, we examined the cell-free preparation for its ability to support synthesis of the naphthoate core of azinomycin and azinomycin B. The enzyme assay was performed in duplicate by incubating the protein extract with acetyl-CoA, cofactors (NADPH, SAM, and FAD), and radiolabeled malonyl-CoA for 24 h. Following incubation, the reactions were quenched by vortexing with dichlo-

(6) (a) LePla, R. C.; Landreau, C. A. S.; Shipman, M. S.; Hartley, J. A.; Jones, G. D. D. *Bioorg. Med. Lett.* **2005**, *15*, 2861. (b) Hodgkinson, T. J.; Kelland, L. R.; Shipman, M. *Bioorg. Med. Lett.* **2000**, *10*, 239. (c) Casely-Hayford, M. A.; Pors, K.; Patterson, L. H.; Gerner, C.; Neidle, S.; Searcey, M. *Bioorg. Med. Lett.* **2005**, *15*, 653. (d) Hartley, J. A.; Hazrati, A.; Kelland, L. R.; Khanim, R.; Shipman, M.; Suzenet, F.; Walker, L. F. *Angew. Chem., Int. Ed.* **2000**, *39*, 3467. Casely-Hayford, M. A.; Pors, K.; James, C. H.; Patterson, L. H.; Hartley, J. A.; Searcey, M. *Org. Biomol. Chem.* **2005**, *3*, 3585.

(7) (a) Corre, C.; Lowden, P. A. S. *Chem. Commun.* **2004**, 990. (b) Corre, C.; Landreau, C. A. S.; Shipman, M.; Lowden, P. A. S. *Chem. Commun.* **2004**, 2600.

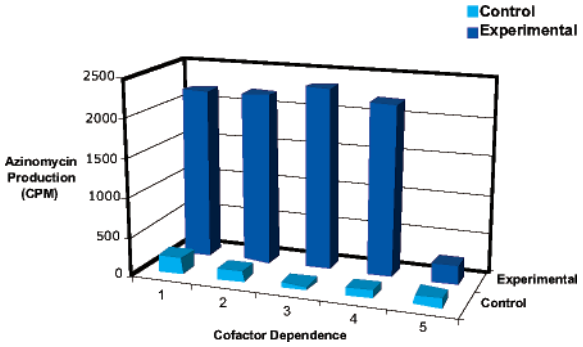


Figure 5. Cofactor requirements of the azinomycin biosynthetic pathway (conversion to azinomycin B). Controls: cpm ± 10. Exptl: cpm ± 50. (1) Reaction conditions: CFE + [1-^{14}C]malonyl-CoA, acetyl-CoA, SAM, FAD, and NADPH. (2) Reaction conditions: CFE + [1-^{14}C]malonyl-CoA, acetyl-CoA, SAM and NADPH. (3) Reaction conditions: CFE + [1-^{14}C]malonyl-CoA, acetyl-CoA, ATP, SAM, FAD, and NADPH. (4) Reaction conditions: CFE + [1-^{14}C]malonyl-CoA, acetyl-CoA, THF, SAM, FAD, NADPH. (5) Reaction conditions: CFE + [1-^{14}C]malonyl-CoA, acetyl-CoA, SAM and FAD preparation.

romethane and centrifuged. The dichloromethane fraction was transferred to new tubes and evaporated to dryness. The organic residue was resolubilized in a minimal volume of dichloromethane containing standards of azinomycin B or naphthoate. Regions of the TLC plate corresponding to products with appropriate R_f values (as observed by UV analysis) were scraped from the plate and analyzed by scintillation counting. The results of this initial experiment are depicted in Figure 2 (reaction 1, conversion to the naphthoate; reaction 2, conversion to azinomycin B). The control experiment for these reactions consisted of boiled cell-free extract (5 mL) incubated with tested substrates and cofactors as detailed above. These results were also confirmed by HPLC co-injection.

As an additional control for these experiments, we examined the effect of the FAS/PKS inhibitor cerulenin[8] on these reactions as well as that of P-450 inhibitors miconazole,[9] metyrapone,[10] and chloramphenicol.[11] Reactions were incubated with 10 and 100 μM of each inhibitor, respectively, and assayed for both naphthoate and azinomycin B production (Figures 3 and 4). As expected, cerulenin exhibited inhibition against formation of the naphthoate (the putative PKS product) and azinomycin B. Likewise, all P-450 inhibitors had a marked effect on both naphthoate and azinomycin production. This suggests minimally the involvement of a P-450 oxygenase in the first step of the biosynthesis to generate what becomes the 3′-hydroxyl of the naphthoate fragment (Figure 1), which is subsequently methylated, via a SAM-dependent process, to give the final product. Completion of the biosynthesis of azinomycin B requires a number of oxidative transformations and could also involve other P-450-dependent processes.

The cell-free system was utilized to probe the cofactor requirements of the azinomycin biosynthetic pathway. Enzyme assays were performed as previously described varying only the cofactor preparation. The results are shown in Figure 5 (reaction 1, SAM, FAD, and NADPH; reaction 2, SAM and NADPH; reaction 3, ATP, SAM, FAD, and NADPH; reaction 4, THF (tetrahydrofolate), SAM, FAD, and NADPH; reaction 5, SAM and FAD). Not surprisingly, elimination of NADPH from the reaction mixture abolished production of azinomycin B. Neither removal of THF nor FAD, on the other hand, appeared to have an effect. Azinomycin production thus necessitates the involvement of two cofactors, NADPH and SAM. The participation of SAM in the biosynthesis has been demonstrated by Lowden and coworkers through feeding experiments.[7] Both NADPH and

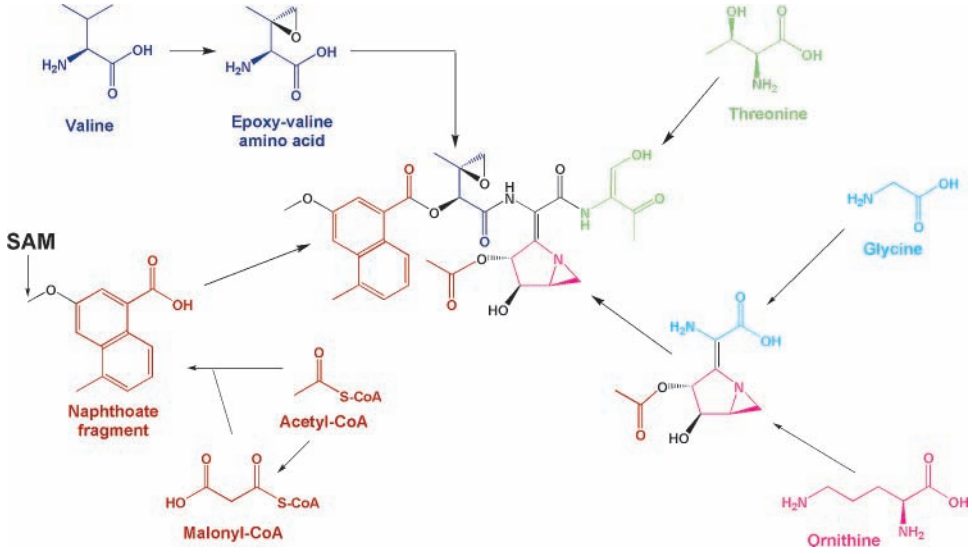
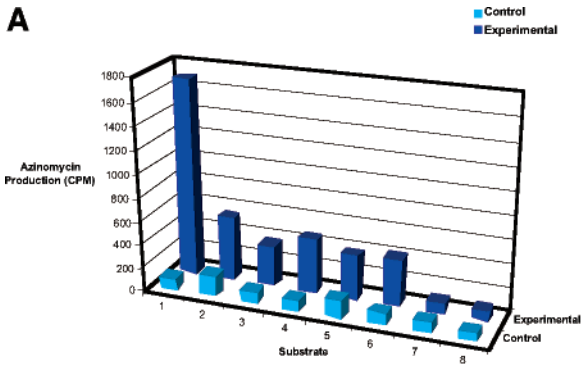


Figure 6. Schematic diagram depicting possible substrate requirements of the azinomycin B biosynthetic pathway.

A



B

reaction condition	amino acid	cpm
1 (Expt.)	[2-^{14}C]malonylCoA	1694
1 (Cont.)	[2-^{14}C]malonylCoA	103
2 (Expt.)	DL-[1-^{14}C]ornithine	548
2 (Cont.)	DL-[1-^{14}C]ornithine	182
3 (Expt.)	[U-^{14}C]glycine	352
3 (Cont.)	[U-^{14}C]glycine	92
4 (Expt.)	L[U-^{14}C]valine	460
4 (Cont.)	L[U-^{14}C]valine	92
5 (Expt.)	L[U-^{14}C]ornithine	378
5 (Cont.)	L[U-^{14}C]ornithine	144
6 (Expt.)	L[U-^{14}C]threonine	403
6 (Cont.)	L[U-^{14}C]threonine	109
7 (Expt.)	L[U-^{14}C]lysine	94
7 (Cont.)	L[U-^{14}C]lysine	97
8 (Expt.)	L[U-^{14}C]tyrosine	81
8 (Cont.)	L[U-^{14}C]tyrosine	72

Figure 7. Investigation of the principle building blocks of the pathway. Controls: cpm ± 10. Exptl: cpm ± 50. (A) Graphical representation. (B) Tabulated data.

SAM can be envisioned to play critical roles in the construction of the naphthoate fragment of the molecule. Surprisingly, ATP (a necessary cofactor in NRPS biosynthesis) did not appear to have much of an effect on azinomycin B production. Presumably, ATP levels in the cell are near saturation. Alternatively, although we suspect that the azinomycins are constructed by a PKS/NRPS system, without elucidation of the gene cluster, we cannot exclude the possibility of another mechanism.

The cell-free enzyme system was further exploited to investigate the substrate requirements of the azinomycin biosynthetic pathway. Proposed intermediate building blocks are diagrammatically represented in Figure 6. Enzyme assays were performed as previously described with the following modification. With the exception of the CFE enzyme control reaction (reaction 1), to each protein extract was added unlabeled malonyl-CoA, acetyl-CoA, and radiolabeled substrate. The results are depicted in Figure 7. All suspected intermediates (as shown in Figure 6) showed positive incorporation, except for tyrosine (an amino acid with no involvement in the pathway) and lysine which were included as negative controls. Although lysine could be envisioned to give rise to the azabicyclic ring system of azinomycin B (Figure 8), lack of incorporation of *S*-adenosylmethionine

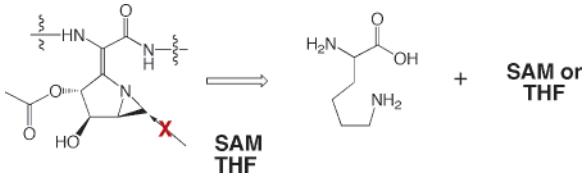


Figure 8. Schematic diagram depicting the formation of the azabicyclic ring system from lysine.

(by way of ^{13}C-methylmethionine)[7] and background level counts observed in vitro with radiolabeled lysine contradict such a mechanism. Moreover, THF, a cofactor that can also facilitate methyl transfer, did not exhibit an effect in vitro. The higher levels of incorporation observed with malonyl-CoA vs other amino acid substrates are a reflection of the multiple malonyl units (five) incorporated into the naphthoate fragment of the molecule. All other substrates are represented only once.

Our results provide the first demonstration of a cell-free enzyme system that supports biosynthesis of the interstrand DNA cross-linker azinomycin B. Investigations with this enzyme preparation impart important information regarding the substrate and cofactor requirements of the pathway.

These studies pave the way for isotopic labeling studies and mechanistic investigations that will ultimately provide definitive proof for the intermediacy of proposed biosynthetic precursors and the involvement of specific cofactors.

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Supporting Information Available: Experimental details on the preparation of the cell-free extract, enzyme assays, and synthesis of the naphthoate fragment (modification of Coleman et al.).[5] This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) (a) Omura, S. *Bacteriol. Rev.* **1976**, *40*, 681. (b) Omura, S. *Methods Enzymol.* **1981**, 520. (c) Hiltunen, M.; Soderhall, K. *Appl. Environ. Microbiol.* **1992**, *58*, 1043.

(9) (a) Rodrigues, A. D.; Gibson, G. G.; Ioannides, C.; Parke, D. V. *Biochem. Pharmacol.* **1987**, *36*, 4277. (b) Rodrigues, A. D.; Lewis, D. F.; Ionnides, C.; Parke, D. V. *Xenobiotica* **1987**, *17*, 1315.

(10) Williams, P. A.; Cosme, J.; Vinkovic, D. M.; Ward, A.; Angove, H. C.; Day, P. J. *Science* **2004**, *305*, 683.

(11) Park, J. Y.; Kim, K. A.; Kim, S. L. *Antimicrob. Agents Chemother.* **2003**, *47*, 3464.