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Exploration of the Molecular Origin of the Azinomycin Epoxide: Timing of the Biosynthesis Revealed

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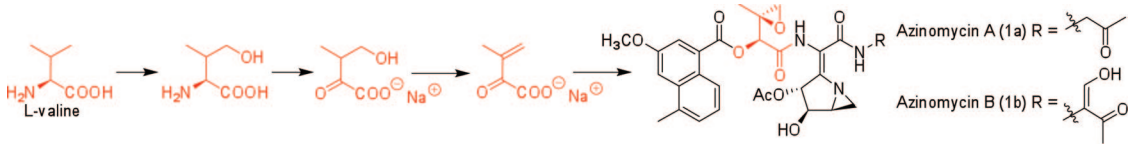
ABSTRACT



Streptomyces sahachiroi **whole cell feeding experiments, utilizing putative precursors labeled with stable isotopes, established that the epoxide unit of the DNA cross-linked agents, azinomycin A and B, proceeds via a valine-dependent pathway and that hydroxylation and dehydration precedes formation of the terminal epoxide. Sodium 3-methyl-2-oxobutenoate, formed through a transimination reaction, was shown to be the penultimate precursor incorporated into the azinomycin epoxide.**

Azinomycin B is a potent antitumor agent produced by certain soil-dwelling *Streptomyces* species.[1] The metabolite interacts with DNA, forming interstrand cross-links within the major groove without prior activation. In vitro studies reveal that the agent interacts with the duplex DNA sequence 5′-d(PuNPy)-3′, forming interstrand covalent linkages between the electrophilic C10 and C21 carbons of azinomycin and the N7 positions of suitably disposed purine bases. [2]

Fluorescence imaging with the natural product revealed localization within the nuclei of yeast and experiments with DNA microarrays resulted in transcriptional effects that were closely associated with DNA damage and repair responses providing a direct correlation of in vitro DNA cross-linking with an in vivo cellular response.[3]

The overall structure of azinomycin B is suggestive of a mixed biosynthetic origin based upon a polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) skeleton (Figure 1). Formation of the naphthoate ring system can be

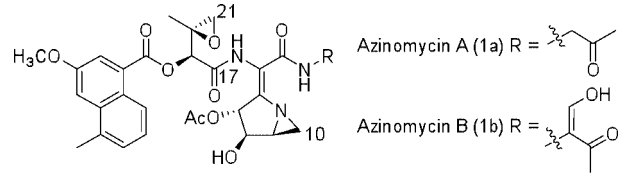


Figure 1. Structure of azinomycins A and B.

rationalized by the successive condensation of acetate and malonate units by a PKS. Further functionalization of the natural product by the action of an NRPS and various tailoring enzymes would give the epoxide, enol, and azabicycle. Data available on the biosynthesis has been limited, largely due to difficulties with the culture method and

(1) (a) Nagaoka, K.; Matsumoto, M.; Oono, J.; Yokoi, K.; Ishizeki, S.; Nakashima, T. *J. Antibiot.* **1986**, *39*, 1527–32. (b) Yokoi, K.; Nagaoka, K.; Nakashima, T. *Chem. Pharm. Bull.* **1986**, *34*, 4554–61. (c) Hata, F.; Koga, Y.; Sano, K.; Kanamori, A.; Matsumae, R.; Sugawara, T.; Hoshi, T.; Shimi, S.; Ito, S.; Tomizawa, S. *J. Antibiot.* **1954**, *7*, 107–112.

(2) (a) Lown, J. W.; Majumdar, K. C. *Can. J. Biochem.* **1977**, *55*, 630–635. (b) Coleman, R. S.; Perez, R. J.; Burk, C. H.; Navarro, A. *J. Am. Chem. Soc.* **2002**, *124*, 13008–13017. (c) Lepla, R. C.; Landreau, A. S.; Shipman, M.; Jones, G. D. D. *Org. Biomol. Chem.* **2005**, *3*, 1174–1175. (d) Armstrong, R. W.; Salvati, M. E.; Nguyen, M. *J. Am. Chem. Soc.* **1992**, *14*, 3144–3145. (e) Zang, H.; Gates, K. S. *Biochemistry* **2000**, *39*, 14968–14975. (f) Fujiwara, T.; Saito, I.; Sugiyama, H. *Tetrahedron Lett.* **1999**, *40*, 315–318.

(3) Kelly, G. T.; Liu, C.; Smith, R., III; Coleman, R. S.; Watanabe, C. M. H. *Chem. Biol.* **2006**, *13*, 1–8.

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securing a consistent source of the natural product. Initially, some gains were made to establish the polyketide origin of the naphthoate moiety,[4] and a cell-free system was developed to support synthesis of azinomycin B in vitro.[5] Recent breakthroughs have led to optimization of culture conditions by nutrient limitation resulting in marked improvement in azinomycin production. This methodology enables a reliable culture method for isotopic feeding studies as illustrated by the definitive assignment of threonine as the most advanced intermediate accepted by the NRPS machinery in final processing and construction of the enol moiety.[6]

In this paper, we explore the biosynthetic route to the azinomycin epoxide, where exact timing of individual enzymatic transformations and intermediacy of metabolites were substantiated by whole cell feeding studies with isotopically labeled substrates. Structural evaluation and results from a recent cell-free study would suggest that L-valine serves as a logical precursor (Figure 2).[5] If this is indeed the case, several scenarios can be envisaged. One possibility is that valine or respective advanced precursors are converted to the epoxide while tethered to the NRPS (Figure 2, path A), where catalysis is achieved by modifying enzymes either contained within NRPS domains or elsewhere within the gene cluster. In cerulide biosynthesis, for example, a reductase module found within NRPS adenylation domains, reduces tethered α-keto acids (α-ketoisocaproic acid and α-ketoisovaleric acid) to α-hydroxy acids, while still bound to their respective PCP domains.[7] Alternatively, it is conceivable that the epoxide moiety is fully constructed by other biosynthetic pathway enzymes prior to activation by its respective adenylation module. For instance, the epoxide might be derived from radical cyclization of a terminal alcohol (Figure 2, path B).[8] In contrast, the epoxide might originate from reaction with molecular oxygen via oxygen insertion into an olefin.[9] This olefin might in turn be derived from a terminal alcohol (Figure 2, path C).[9] In either case (Figure 2, path B or C), the amino group of valine also requires biochemical conversion to give the α-hydroxy group of the epoxide fragment **9**, presumably arising by transimination and subsequent reduction of the resulting ketone to give the alcohol. The exact timing of these biosynthetic transformations and feasibility of the overall route can only be resolved through experimentation. Therefore, to test our hypotheses, we exogenously fed valine and a variety of

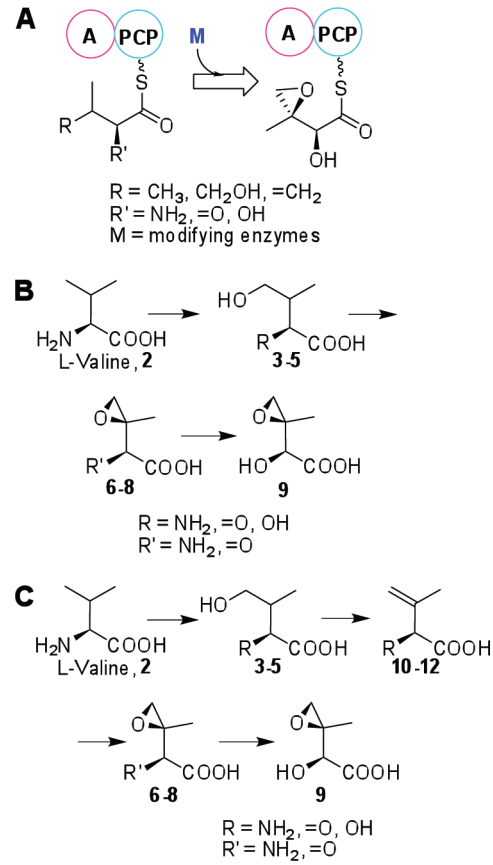


Figure 2. Possible biosynthetic routes to the epoxide moiety in the azinomycins.

synthesized advanced precursors in isotopically labeled form to whole cells (Table 1).

When [1-^{13}C]-valine was administered to cultures of *S. sahachiroi*, site-specific enrichment was observed at C-17 of the natural product, corroborating our cell-free experiments with [1-^{14}C]-valine. Analysis of the ^{13}C NMR spectrum revealed a 8.71% incorporation at C-17, 164.0 ppm (see Table 1 and Supporting Information). Encouraged by this result, we synthesized the advanced precursors shown in Table 1.

The synthetic routes were designed to allow easy and efficient preparation, in high yields and with minimal chromatographic separations, providing a cost-effective approach to these molecules in isotopically labeled form. In addition to the biosynthetic studies described here, the availability of synthetic routes to unnatural amino/α-hydroxy acid is of pharmaceutical importance as they can serve as peptidomimetic substrates[10] as well as building blocks in the synthesis of natural product pharmacophores.[11]

Biosynthetic precursors **11** and **12** (Scheme 1) were synthesized as follows. Commercially available 2-methyl-

(4) (a) Corre, C.; Lowden, P. A. S. *Chem. Commun.* **2004**, *n/a*, 990–991. (b) Corre, C.; Landreau, C. A. S.; Shipman, M.; Lowden, P. A. S. *Chem. Commun.* **2004**, *n/a*, 2600–2601.

(5) Liu, C.; Kelly, G. T.; Watanabe, C. M. H. *Org. Lett.* **2006**, *8*, 1065–1068.

(6) Kelly, G. T.; Sharma, V.; Watanabe, C. M. H. *Bioorganic Chem.* **2008**, *36*, 4–15.

(7) Magarvey, N. A.; Ehling-Schulz, M.; Walsh, C. T. *J. Am. Chem. Soc.* **2006**, *128*, 10698–10699.

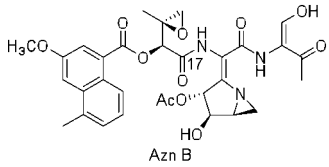
(8) (a) Yan, F.; Munos, J. W.; Liu, P.; Liu, H. W. *Biochem.* **2006**, *45*, 11473–11481. (b) Munos, J. W.; Moon, S. J.; Mansoorabadi, S. O.; Change, W.; Hong, L; Yan, F.; Liu, A.; Liu, H. W. *Biochemistry* **2008**, *47*, 8726–35.

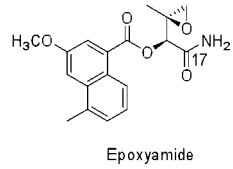
(9) (a) Grüschow, S.; Sherman, D. H. The Biosynthesis of Epoxides. In *Aziridines and Epoxides in Organic Synthesis*, Yudin, A. K., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinhem, 2006; pp 349−398 and references within. (b) Watanabe, C. M. H.; Townsend, C. A. *J. Org. Chem.* **1996**, *61*, 1990–1993.

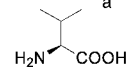
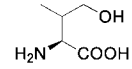
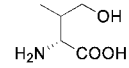
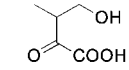
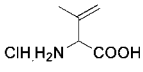
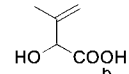
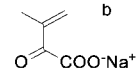
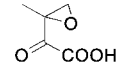
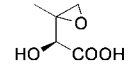
(10) (a) Hanessian, S.; Auzzas, L. *Acc. Chem. Res.* **2008**, *41*, 1241-1251.

(11) (a) Aina, O. H.; Liu, R.; Sutcliffe, J. L.; Marik, J.; Pan, C.-X.; Lam, K. S. *Mol. Pharm.* **2007**, *4*, 631–651.

Table 1. Percent Incorporation[c] at C-17 of Azinomycin B and the Epoxyamide (Samples Were Prepared As Detailed in Kelly et al.[6])

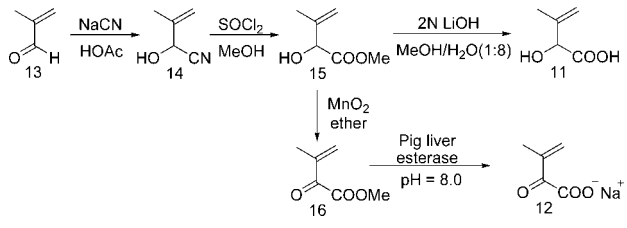




compound	no.	% incorporation azn B	% incorporation epoxyamide
 [a]	**2**	8.71	n.d.
	**3**	4.18	3.2
	**3***	n.d.	n.d.
	**5**	8.10	0.32
	**10**	n.d.	n.d.
 [b]	**11**	n.d.	n.d.
	**12**	6.03	2.95
	**8**	n.d.	n.d.
	**9**	n.d.	n.d.

[a] L-Valine TFA salt showed an overall incorporation of 6.97%. [b] This α-keto carboxylic acid degraded rapidly. [c] The percent incorporation = [(A − B)/B] × 1.10 where A = intensity of labeled carbon normalized to the intensity of the 3′OCH_3 of azinomycin B and the C18 of the epoxyamide; B = intensity of normalized unlabeled carbon; 1.10 = natural abundance of ^{13}C. The table entry reads n.d. if incorporation was not detected by ^{13}C NMR.

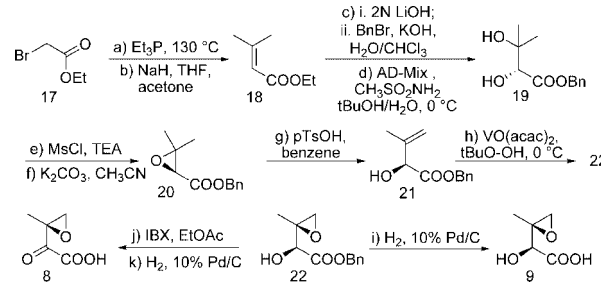
Scheme 1



2-propenal was treated with sodium cyanide and acetic acid to afford cyanohydrin **14**,[12] which upon refluxing with methanolic HCl generated the α-hydroxy methyl ester **15**. Additionally, hydrolysis with LiOH generated the α-hydroxy-3-methylbut-3-enoic acid **11**.

(12) Faulkner, D. J.; Petersen, M. R. *J. Am. Chem. Soc.* **1973**, *95*, 553–63.

Scheme 2



Allylic oxidation with activated MnO_2[13] in ether afforded the α,β-unsaturated ester **16** which upon hydrolysis with esterase at pH 8.0 gave sodium 3-methyl-2-oxobut-3-enoate **12**.

Epoxides **8** and **9** (Scheme 2[6]) were synthesized from ethyl bromoacetate **17** converting it to its corresponding phosphonate, which upon coupling under standard Horner−Wadsworth−Emmons conditions afforded the acrylic ester **18** in good yields. The ester was hydrolyzed and converted to the benzyl ester, which upon Sharpless asymmetric dihydroxylation with AD-mix α afforded a chiral diol system **19**.[14] Mesylation followed by reflux with anhydrous K_2CO_3 generated the chiral epoxide **20**, which was carefully opened with catalytic amounts of anhydrous pTsOH to generate a chiral allylic alcohol **21**. Treatment with $VO(acac)_2$ and *t*-butyl hydroperoxide gave the desired benzyl epoxy ester **22**, which upon hydrogenation in the presence of 10% Pd/C generated the precursor **9**. Mild oxidation of the allyic alcohol with 2-iodoxybenzoic acid (IBX) gave the corresponding α-ketoester, which upon hydrogenation facilitated debenzylation giving the desired product **8**.

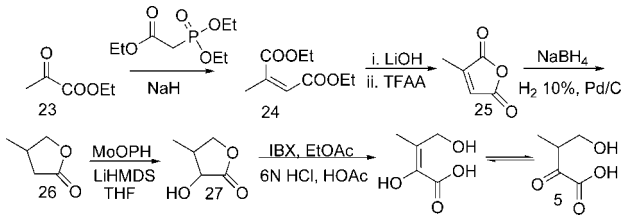
Isodehydrovaline **10** (Scheme 1 of the Supporting Information) was synthesized via a modified route detailed by Baldwin et al.,[15] and γ-hydroxyvaline **3** was generated by an extension of earlier work by Kazmaier et al. (Scheme 2 of the Supporting Information).[16]

The γ-hydroxy α-keto acid **5** (Scheme 3) was generated by condensing ethyl pyruvate with the ylide of triethylphosphonoacetate via a Horner−Wadsworth−Emmons condensation. Hydrolysis followed by dehydration, regioselective reduction of anhydride followed by hydrogenation afforded the lactone **26**. α-Hydroxylation using Vedejs' reagent, oxidation, and subsequent hydrolysis provided **5** in good yields.

Each of the modified valine derivatives were synthesized in ^{13}C-labeled form (at C-1) and fed separately to whole cell suspension cultures as detailed previously.[6] Table 1 provides

(13) Capps, N. K.; Davies, G. M.; Loakes, D.; Mecabe, R. W.; Young, D. W. *J. Chem. Soc., Perkin. Trans. 1* **1991**, *12*, 3077–3086.
(14) Bryant, H. J.; Dardonville, C. Y.; Hodgkinson, T. J.; Hursthouse, M. B.; Abdul Malik, K. M.; Shipman, M *J. Chem. Soc., Perkin Trans. 1* **1998**, 1249–1255.
(15) Baldwin, J. E.; Haber, S. B.; Hoskins, C.; Kruse, L. I. *J. Org. Chem.* **1977**, *42*, 1239–1241.
(16) (a) Kazmaier, U.; Krebs, A. *Tetrahedron Lett.* **1999**, *40*, 479–482. (b) Kazmaier, U.; Krebs, A. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2012.

Scheme 3



the feeding results for all of the amino acid precursors. In addition to valine **2**, only substrates **3**, **5**, and **12** resulted in site-specific incorporation above background at 164.0 ppm. Interestingly, incorporation was also observed at 168.7 ppm corresponding to the epoxyamide (Table 1), a metabolite that frequently accompanies production of the azinomycins.[1b] We were gratified to find that L-γ-hydroxyvaline **3** was unambiguously incorporated, substantiating its involvement in either forming the epoxide directly (Figure 2, path B) or generating an olefin where subsequent oxygen insertion would give the epoxide (Figure 2, path C). As expected, only the L-isomer **3** served as a substrate over its corresponding D-isomer **3***, confirming the stereospecific nature of these reactions. The site-specific incorporation of α-keto hydroxy acid **5** was also observed and is suggestive that hydroxylation of valine (to **3**) precedes transimination. Reconstitution of the enzymes involved in these transformations will, however, be needed to rigorously establish this notion. The most advanced putative precursor shown to be processed by the azinomycin biosynthetic machinery was 3-methyl-2-oxobutenoic acid **12** negating direct formation of the epoxide from the alcohol (Figure 2, path B). In contrast, isodehydrovaline **10** failed to incorporate, further substantiating the order of biosynthetic steps (favoring Figure 2, path C), where dehydration of the γ-alcohol to the double bond is suggested to follow transimination.

Interestingly, neither of the epoxides showed incorporation into the natural product. This is likely attributed to their instability in aqueous medium, which increased dramatically over time (Figure 1 of Supporting Information), with ring-opened products and considerable lactonization occurring over a 24 h period at room temperature (data not shown). Notwithstanding the instability of epoxides **8** and **9**, the lifetimes of the other amino acid derivatives in aqueous media were sufficient, under the conditions of our feeding regimen (two separate and equal aliquots fed 24 h apart pH 7.1−7.5, 30 °C), to yield reliable incorporation data.[6]

The azinomycins are a structurally unique class of soil-derived antitumor antibiotics that have shown promising activity against carcinomas, reticulosarcoma, and terablastomas in clinical trials.[17] While DNA modifying agents (e.g., mitomycin C, calicheamin)[18] are often a first line of defense against many cancers, compound toxicity and selectivity is an issue. The azinomycins are no exception, and significant effort has already been made to synthesize chemical analogues of these compounds.[19] In this investigation, we demonstrate timing and intermediacy of several metabolites along the biosynthetic route to the epoxide moiety (Figure 3). We were intrigued to find that the majority if not all of the enzymatic steps required to generate the epoxide fragment occur prior to loading onto the NRPS machinery (invalidating Figure 2, path A).

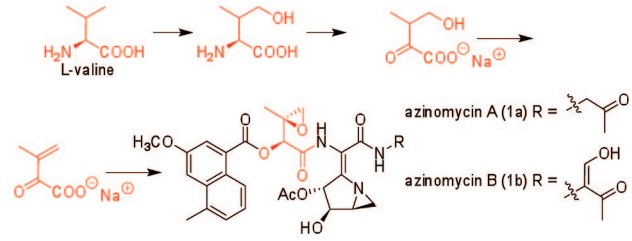


Figure 3. Biosynthetic route to the epoxide moiety in the azinomycins where failure to incorporate **11** suggests that 3-methyl-2-oxobutenoic acid **12** is epoxidized to **8** which is then reduced to **9**.

With the recent discovery of the azinomycin biosynthetic cluster,[20] it will now be possible to assign specific genes to these and other enzymatic activities of the pathway. Such experiments will pave the way for future genetic engineering and/or chemoenzymatic manipulation of the biosynthetic operon in the design of more effective anticancer agents.

Acknowledgment. We thank Dr. Chaomin Liu (former postdoctoral researcher in the Watanabe laboratory) for preliminary work on the synthesis and the American Cancer Society and the Robert A. Welch Foundation (A-1587) for financial support.

Supporting Information Available: Experimental details and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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