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Aminoacetone as the Penultimate Precursor to the Antitumor Agent Azinomycin A

Vasudha Sharma, Gilbert T. Kelly, Jennifer Foulke-Abel, and Coran M. H. Watanabe*

Department of Chemistry, Texas A&M University, College Station, Texas 77843

watanabe@mail.chem.tamu.edu

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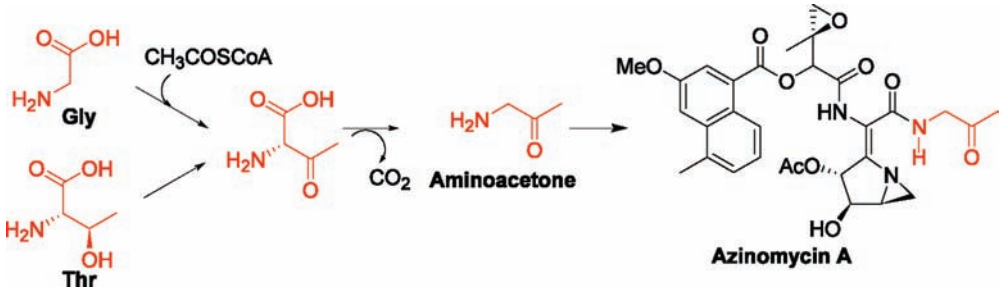
ABSTRACT



Experiments reveal that the metabolic precursor aminoacetone is a key intermediate in the production of the antitumor agent azinomycin A relative to the structurally and functionally related agent, azinomycin B. Azinomycin A and B arise through bifurcation of the biosynthetic pathway and competition between metabolic substrates. The availability of the biosynthetic precursors *in vivo*, aminoacetone for azinomycin A and threonine for azinomycin B, controls the overall ratio of azinomycin A to B produced.

Azinomycins A (**1**) and B (**2**) are potent antitumor agents produced by soil-dwelling *Streptomyces* species.[1−3] These natural products form interstrand cross-links with DNA, within the major groove without prior activation. *In vitro* interstrand covalent linkages arise between the electrophilic C10 and C21 carbons of azinomycin and the N7 positions of suitably disposed purine bases of DNA.[4−9] The azinomycins represent an architecturally unusual class of natural products where three distinct biosynthetic classes contribute to the overall assembly of these molecules. The skeletal framework of the molecule is generated through a polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS) skeleton while the aziridino[1,2-a]pyrollidine moiety is alkaloid derived. The presence or absence of the enol fragment at the terminal end of the molecule, distinguishes azinomycin A from B (Figure 1).

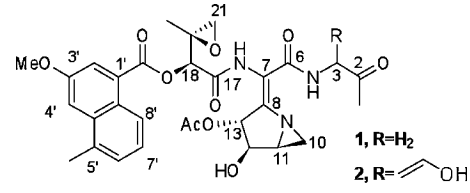


Figure 1. Structure of azinomycin A (**1**) and B (**2**).

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Initially, progress in elucidating the biosynthesis of these molecules was impeded by difficulties with the culture method and securing a consistent source of the natural product. Some gains had been made to establish the polyketide origin of the naphthoate moiety[10,11] and a cell-free system developed to support synthesis of azinomycin B *in vitro*.[12] Recently, we optimized culture conditions by nutrient limitation resulting in marked improvement in azinomycin production. The methodology provides 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 of azinomycin B.[13] This fermentation procedure has also enabled successful assignment of sodium 3-methyl-2-oxobutenoate, generated through a transimination reaction, as the final precursor incorporated into the epoxide moiety in these molecules.[14] In this report, we explore the biosynthetic origin of the terminal fragment of azinomycin A and evaluate its formation in relation to the biosynthesis of azinomycin B.

Several different biosynthetic scenarios can be envisioned to give the aminoacetone fragment of azinomycin A. One possibility is that a threonine fragment undergoes modification while tethered to the NRPS liberating azinomycin B, which is further modified to provide azinomycin A (Figure 2, path A). Alternatively, azinomycin A could arise by hydrolysis from PCP, oxidation, and a decarboxylation step analogous to the mechanism of acetoacetate decarboxylase (Figure 2, path B).[15]

Another possibility is that the terminal aminoacetone moiety is fully constructed prior to being incorporated in the natural product. For instance, aminoacetone might be derived from threonine where threonine dehydrogenase (Figure 2, path C) catalyzes the formation of a β-keto amino acid followed by subsequent decarboxylation to give aminoacetone.[16−18] In contrast, aminoacetone might also originate from glycine through action of 2-amino 3-ketobutyrate CoA ligase, facilitating a condensation reaction with acetyl CoA and loss of CO_2 (Figure 2, path D).[19−21] In either case (Figure 2, path C or D), the β-keto amino acid **4** must be formed *in situ* to give aminoacetone **5** by decarboxylation. To distinguish between these mechanistic hypotheses, we fed isotopically labeled (^{15}N and ^{13}C) aminoacetone, threonine, and glycine (Table 1, Supporting Information Table 1) exogenously to whole cells.

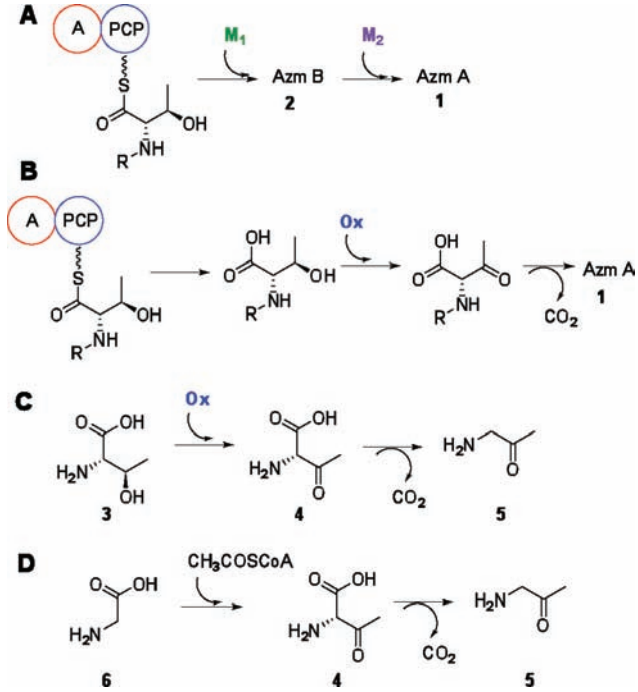

Figure 2. Proposed biosynthetic routes for production of the aminoacetone moiety of azinomycin A. M_1, M_2 = modifying enzymes; Ox = Oxidase/Dehydrogenase; R = azinomycin core.

Table 1. Percent Incorporation at C-2 of Azinomycin A and B

entry	compound	% incorporation in azinomycin A (**1**)	% incorporation in azinomycin B (**2**)
1	[U−^{13}C] -**3**	1.9	12
2	[2-^{13}C]-**5**	26.3	n.d.
3	[2-^{13}C]-**6**[1]	<1	1.8
4	[1-^{13}C]- **6**[2]	n.d.	n.d

[1] Incorporation at C-3 of azinomycin A, label scattered throughout the molecule. [2] Incorporation at C-4 of azinomycin B. % incorporation = [(A − B)/B] × 1.10 where A, intensity of labeled carbon; B, intensity of unlabeled carbon; 1.10, natural abundance of ^{13}C. n.d. = not detected by APCI Mass Spec or ^{13}C NMR. The low incorporation levels of labeled glycine and threonine into azinomycin A relative to aminoacetone in azinomycin A, is likely a reflection of differences in feeding regimen and the instability of aminoacetone generated in culture. Labeled aminoacetone (300 mg) was supplied to the *S. sahachiroi* culture in many aliquots (72 additions made over a 48 h period). Labeled threonine and glycine were provided to cells in two aliquots. If aminoacetone was supplied in this fashion, incorporation was not observed (see Supporting Information).

Isotopically labeled [2-^{13}C] aminoacetone was synthesized as its hydrochloride salt from [3-^{13}C] ethylacetoacetate as depicted in Scheme 1. Briefly, ethyl acetoacetate was treated with SO_2Cl_2 giving ethyl 2-chloroacetoacetate (**7**),[26] which when hydrolyzed with conc. H_2SO_4 gave chloroacetone (**8**) upon decarboxylation. Chloroacetone was treated with potassium phthalimide to give N-acetonyl phthalimide (**9**), which

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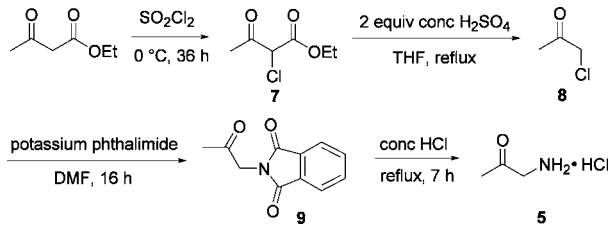
Scheme 1. Synthesis of Aminoacetone Hydrochloride (**5**)



upon acidic hydrolysis afforded [2-^{13}C]aminoacetone hydrochloride (**5**) in four steps with an overall ~75% yield.

When [2-^{13}C] aminoacetone (entry 2, Table 1 and Supporting Information Figure 5) was supplied to whole cells, unambiguous incorporation (26%) was observed in azinomycin A but not in azinomycin B. These results negate Figure 2, path A, where azinomycin A is proposed to proceed through formation of azinomycin B. Moroever, as the data demonstrate intermediacy of aminoacetone in the biosynthesis of azinomycin A, these findings exclude the route depicted in Figure 2 path B. To evaluate the involvement of proposed biosynthetic routes C and D, [U−^{13}C] threonine was administered to cultures of *S. sahachiroi*. "End-to-end" enrichment was observed at C-1 through C-4 of azinomycin B, corroborating our earlier cell-free experiments with [1-^{14}C] threonine.[12,13] Interestingly, incorporation of [U−^{13}C] threonine was also observed at C-1 through C-3 of azinomycin A (*ca.* 1.9%), as detected by ^{13}C NMR (see Supporting Information Figure 4). This data lends credence for intermediacy of threonine in the biosynthesis of aminoacetone and the terminal end of azinomycin A, (path C). To test the possible role of glycine (path D, Figure 2) in the generation of aminoacetone and azinomycin A, we evaluated a series of precursors as shown in Table 1. For example, when [2-^{13}C]glycine was provided in cell culture, enrichment of signal was observed at C-3 of azinomycin A, albeit low (<1%) suggesting a possible role for glycine in the formation of aminoacetone via α-C-acylation followed by decarboxylation (Figure 2, path D). Azinomycin B was also labeled at C-3 (1.8%) as threonine can be biosynthesized from glycine.[13,22−26] As expected, metabolic scattering was observed as exhibited by the incorporation of label at other sites within the molecule and contributing to the low levels of incorporation at C-3(<1%).

These results were further substantiated by the lack of any measurable incorporation seen in azinomycin A when [1-^{13}C] glycine was supplied to whole cells. This is presumably due to the loss of labeled carbon as CO_2. Moreover, feeding of 2, 2-D, D-glycine to S. *sahachiroi* led to an observed decrease in the production of azinomycin A by 66% as compared to the unlabeled control. This is suggestive that deprotonation at α-C of glycine is kinetically significant in the production of **4**. The crystal structure of *E. coli* 2-amino-3-ketobutyrate CoA ligase complexed with the L-2-amino-3-ketobutyrate and pyridoxal 5′-phosphate (PLP) in the active site of the enzyme suggests a reaction mechanism in which PLP-activated glycine is deprotonated by a lysine residue.[27] Conceivably, the rate of deprotonation could be kinetically controlled, but such studies in the enzyme have not been undertaken. Since 2-amino-3-ketobutyrate CoA ligase is evolutionarily conserved, a similar mechanism would likely be at work in *S. sahachiroi* aminoacetone biosynthesis.

These investigations suggest that the most advanced putative precursor in the biosynthesis of azinomycin A is aminoacetone (**5**), as derived from both glycine (**6**) (Figure 2, path D) and threonine (**3**) (Figure 2, path C) through the intermediate L-2-amino-3-ketobutyrate (**4**). Metabolic enzymes mediating the biosynthesis of L-2-amino-3-ketobutyrate **4** from both threonine **3** and glycine **6** have been reported in other *Streptomyces* species, *S. coelicolor*, *S. griseus*, and *S. avermitilis* (Figure 3), where the aminoacetone generated (via spontaneous decarboxylation)[17,28,29] is utilized in porphyrin biosynthesis and pyruvate metabolism.[22−26] Moreover, our genomic sequencing efforts of

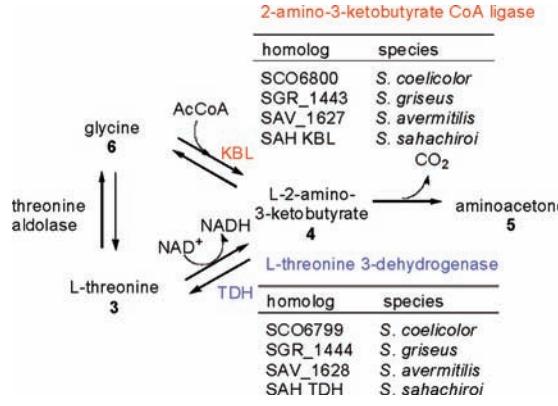


Figure 3. Biosynthetic pathways facilitating conversion of glycine and threonine to L-2-amino-3-ketobutyrate, which undergoes decarboxylation to give aminoacetone.

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S. sahachiroi have identified genes for both L-threonine 3-dehydrogenase and 2-amino-3-ketobutyrate CoA ligase (Supporting Information), substantiating operation of these pathways in *S. sahachiroi*, as well.

Intrigued by these results, we reasoned that the available aminoacetone pool in the cell might compete with threonine influencing the ratio of azinomycin A to B produced in *S. sahachiroi*. We thus examined the effects of supplying aminoacetone exogenously to *S. sahachiroi* cultures. When aminoacetone was supplied to cells at high concentrations (entry 5, Figure 4A), azinomycin production was not observed. Aminoacetone has been reported to produce oxy radicals and cytotoxic oxidative stress via production of methyl glyoxal in various species of bacteria.[28−32] *S. sahachiroi* was no exception. Therefore, to prevent toxicity in bacterial cell culture, we aliquoted the aminoacetone to control the amount available at any given time. Consequently, unlabeled aminoacetone was provided to suspension cells in eight aliquots, which was supplied over a period of 2 days (Supporting Information Figure 7). The percentage of azinomycin A produced relative to B is reported in Figure 4 as a function of the amount of aminoacetone supplied in culture (Figure 4).

A

entry	amount of aminoacetone (**5**) fed (mg)	% azn A(**1**) produced$^{\nabla}$
1	0	28
2	300	52
3	700	80
4	1000	79
5	1250*	No production

* feeding performed in two aliquots of 0.625 g.

B

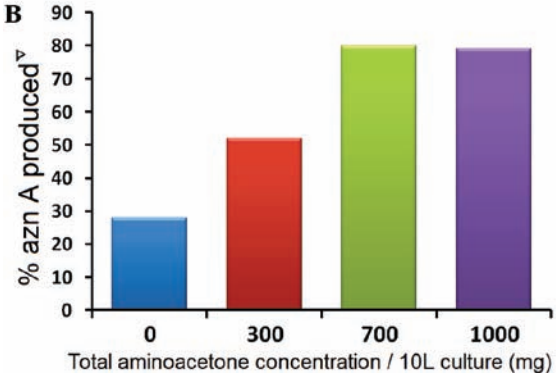

Figure 4. Production of azinomycin A as a function of the amount of aminoacetone, **5** fed per 10 L culture. ∇ % azn A (**1**) = [azn A/(azn A + azn B)].

Under native conditions (no supplied aminoacetone), azinomycin A comprises about 28% of the total mixture (A and B) isolated from the organism. Increasing the amount of supplied aminoacetone resulted in enhanced production of azinomycin A, which is suggestive of a competitive role between aminoacetone and threonine for the NRPS module. Saturation occurs at higher concentrations (Figure 4, panel B), which might reflect saturation of enzyme activities.

In this investigation, we unequivocally show that part of the structure of the antitumor agent azinomycin A is biosynthesized from the metabolic precursor, aminoacetone. The action of the enzymes L-threonine 3-dehydrogenase and 2-amino-3-ketobutyrate CoA ligase provides a convergent route to this precursor (Figure 5). We were intrigued to find that aminoacetone can compete directly with the threonine pool, where exogenous supplementation of the culture with aminoacetone can lead to the overproduction of azinomycin A relative to B.

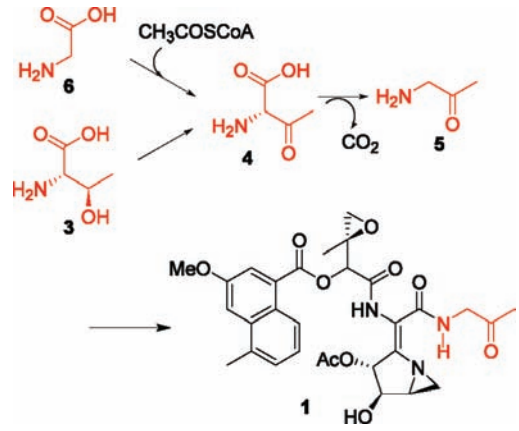

Figure 5. Proposed biosynthetic route for the formation of aminoacetone and its subsequent site-specific incorporation into azinomycin A, where positional incorporation of threonine and glycine in azinomycin A suggests a convergent synthetic route.

Results of a multitude of isotopic labeling experiments[10−14] taken together with the recent discovery of the azinomycin biosynthetic cluster,[33] will now make it possible to functionally characterize specific genes involved in the construction of these azabicycle containing antitumor agents.

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Supporting Information Available: Frameplot and alignment details, feeding conditions, spectral overlays and NMR spectra of the synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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