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Transketolase Activity in the Formation of the Azinomycin Azabicycle Moiety

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Supporting Information

ABSTRACT: The biosynthesis of the azinomycins involves the conversion of glutamic acid to an aziridino-[1,2-*a*]pyrrolidine moiety, which together with the epoxide moiety imparts anticancer activity to these agents. The mechanism of azabicycle formation is complex and involves at least 14 enzymatic steps. Previous research has identified *N*-acetyl-glutamate 5-semialdehyde as a key intermediate, which originates from protection of the amino terminus of glutamic acid and subsequent reduction of the γ-carboxylate. This study reports on the seminal discovery of a thiamin-dependent transketolase responsible for the formation of 2-acetamido-5,6-dihydroxy-6-oxoheptanoic acid, which accounts for the two-carbon extension needed to complete the carbon framework of the azabicycle moiety.

The azinomycins (Figure 1) represent a family of natural products produced by *Streptomyces sahachiroi*, a soil

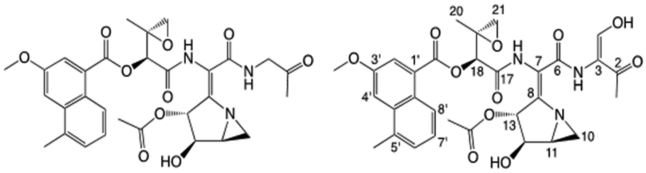
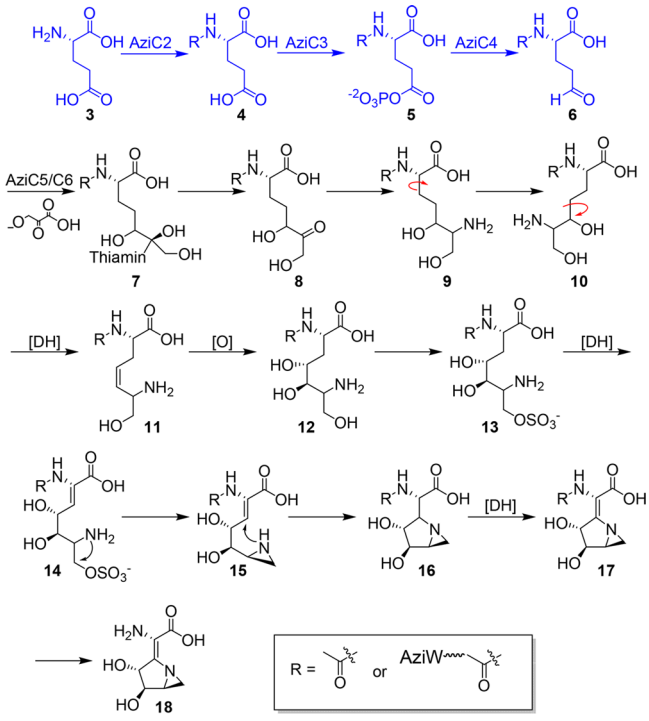


Figure 1. Chemical structures of the azinomycins.

microorganism.[1] They have demonstrated potent anticancer activity based on their ability to form covalent interstrand cross-links in the major groove of double-stranded DNA. These covalent linkages occur between the N7 position of purine bases of DNA and the electrophilic C10 and C21 atoms of the azinomycins.[2−4] Time course analysis of the cross-linking ability of the azinomycins reveals the aziridine C10−DNA adduct forms first prior to the formation of the epoxide C21−DNA adduct.[5] Their activity manifests as cytotoxic effects *in vitro* and antitumor activity *in vivo*. *In vitro* cytotoxicity evaluated against L5178Y cells results in an IC_{50} of azinomycin A and B of 0.07 and 0.11 μg/mL, respectively. Against P388 murine transplantable tumors *in vivo*, azinomycin B resulted in an increased life span of 193% at 32 μg/kg. While azinomycin A is more cytotoxic *in vitro*, it is not as potent as azinomycin B *in vivo*, resulting in an increased life span of 76% against P388 tumors.[6,7] Azinomycin B also showed promising activity in 36 cases of malignant neoplasms.[8]

The biosynthesis of the aziridino[1,2-*a*]pyrrolidine moiety of the azinomycins has proven to be particularly elusive. Scheme 1 shows a proposed biosynthetic route for formation

Scheme 1. Proposed Biosynthetic Route to Azabicycle Formation



of the aziridino[1,2-*a*]pyrrolidine moiety, with previously validated colored blue. Whole cell feeding studies revealed glutamic acid as the starting precursor.[9] Additionally, studies have shown AziC2 carries out a protection step on the amino group giving *N*-acetyl-glutamic acid. Bioinformatics suggest this protection might also be facilitated with an amino group carrier protein, AziW.[9,10] Protection is followed by AziC3 and

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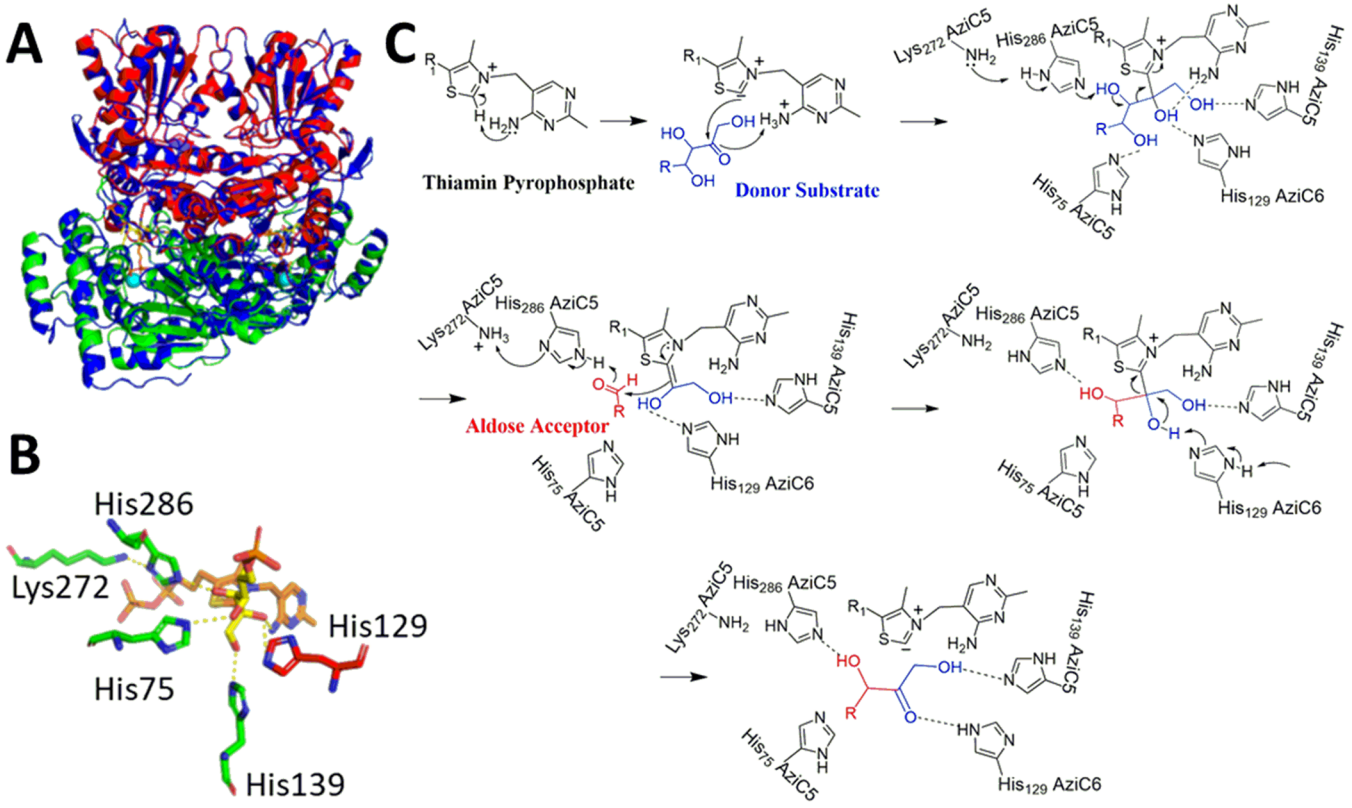

Figure 2. (A) Overlay of the AziC5/C6 model with human transketolase (Protein Data Bank entry 4KXU): blue for 4KXU, green for AziC5, red for AziC6, orange for thiamin pyrophosphate, yellow for D-fructose 6-phosphate, and cyan for Mg^{2+}. (B) Close-up of one catalytic site with conserved residues involved in substrate binding and enzymatic activity shown. (C) Predicted mechanism based on the homology model and conserved residues.

AziC4 catalyzing the formation of *N*-acetyl-glutamate 5-semialdehyde **6** via an acyl phosphate intermediate.[11] Here, we report on the next step in this pathway, a transketolase-mediated two-carbon extension to produce 2-acetamido-5,7-dihydroxy-6-oxoheptanoic acid **8** utilizing hydroxypyruvate as the two-carbon donor molecule.

Transketolase enzymes catalyze the transfer of a dihydroxyethyl group from a ketose donor molecule to an aldose acceptor resulting in an α,α-dihydroxyketone molecule.[12] Transketolases are prevalent in the pentose phosphate pathway and Calvin cycle as well as numerous biosynthetic pathways. Thiamin and a divalent cation M^{2+} are necessary cofactors for transketolase activity.[13] DNA annotation suggests that *aziC5* and *aziC6* encode C- and N-terminal subunits of a transketolase enzyme, respectively. On the basis of their 54% similarity to the transketolase in vazabitide biosynthesis, it was proposed that AziC5 and AziC6 use thiamin pyrophosphate (TPP) and magnesium(II) as cofactors to catalyze the transfer of a two-carbon unit onto *N*-acetyl-glutamate 5-semialdehyde **6**.[10]

A homology model based on the structure of human transketolase was generated, and the binding pocket identified with highly conserved histidine residues, including the Lys272-His286 catalytic dyad (Figure 2).[14] The predicted structure is a tetramer with a homodimer of AziC5 and a homodimer of AziC6. The first step in the proposed transketolase catalytic cycle of AziC5/C6 involves the formation of the thiamin ylide, which attacks the carbonyl carbon of the keto donor substrate. Deprotonation by His286 of AziC5 leads to the dihydroxyethyl-thiamin enamine intermediate, which reacts with the aldose acceptor forming a new carbon−carbon bond. Subsequent deprotonation by His129 of AziC6 generates the final α,α-dihydroxyketone product as well as regenerated thiamin ylide for another catalytic cycle.[12,15] His75 and His139 of AziC5 are also highly conserved and are proposed to function in H-bonding of the substrate during the catalytic cycle. This reaction is reversible *in vivo*. Hydroxypyruvate is often employed as the donor molecule *in vitro*, resulting in the loss of carbon dioxide, which constitutes an irreversible reaction.[12,16,17]

aziC5 and *aziC6* were successfully cloned, overexpressed, and purified in *Escherichia coli* BL21 (DE3) to evaluate this step of the pathway. To demonstrate the thiamin dependency and binding capabilities of AziC5/C6, a thiochrome assay was performed. Oxidation of thiamin produces a highly fluorescent thiochrome that can be characterized by high-performance liquid chromatography (HPLC).[18] Exposure of purified AziC5/C6 to the oxidant resulted in the production of thiochrome as characterized by HPLC. The thiochrome produced from AziC5/C6 was compared to a synthetic thiochrome standard (Figure S14).

To observe formation of 2-acetamido-5,7-dihydroxy-6-oxoheptanoic acid **8**, two routes were taken. Route A utilized enzymatic reconstitution of intermediates in the azinomycin biosynthetic pathway to generate the *N*-acetyl-glutamate 5-semialdehyde **6** substrate needed for the AziC5/C6 reaction. Alternatively, intermediate **6** was synthesized as the racemate and supplied with hydroxypyruvate to AziC5/C6. Both routes showed product formation as detected by electrospray ionization mass spectrometry (ESI-MS), giving masses of

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233 [M + H] and 256 [M + Na], respectively. Synthetic semialdehyde **6** (route B) was used to obtain ESI-MS/MS data, giving the fragmentation seen in Figure S12. ESI-MS data can be found in Figures S11−S13. To evaluate the necessity of both subunits in the reaction, controls were performed with one transketolase subunit at a time (AziC5 or AziC6). Minimal product formation is observed in the control reactions, likely catalyzed by TPP in solution, confirming the need for both subunits in the enzymatic transketolase reaction (Figure 3D and Figure S13). Transketolase activity was also probed using a tetrazolium red colorimetric assay in which the α-hydroxy ketone product produced by the transketolase reaction reduces a colorless tetrazolium to a red formazan precipitate (Figure 3A−C and Figure S15).[17] A red precipitate formed only when the transketolase was incubated with *N*-acetyl-glutamate 5-semialdehyde, thiamin pyrophosphate, and the hydroxypyruvate donor molecule. This suggests the product forms only in the presence of the AziC5/C6 enzyme and cofactors.

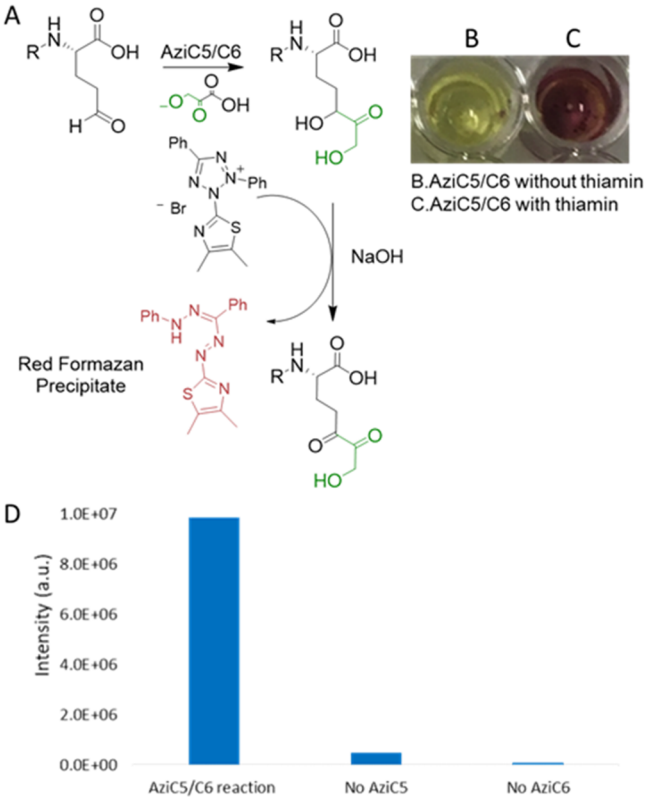
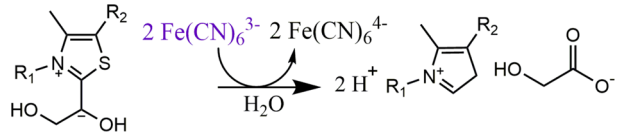


Figure 3. Transketolase activity assays. (A) Colorimetric assay. (B) Incubation of colorless tetrazolium with AziC5/C6 protein in the absence of thiamin. (C) Incubation of AziC5/C6 protein, substrates, cofactors, and colorless tetrazolium. Reduction of the colorless tetrazolium occurs only when all necessary cofactors and substrates are present. (D) Mass spectrometry ion count for compound **8**. Significant product formation is observed in the reaction mixture containing both subunits compared to the controls lacking one subunit.

The activity of AziC5/C6 toward dihydroxyethyl donor compounds was measured spectrophotometrically using potassium ferricyanide absorbance at 420 nm. The α-carbanion intermediate is oxidized in the presence of ferricyanide, resulting in a decrease in absorbance at 420 nm as the ferricyanide is reduced.[13,16,19,20] Fructose 6-phosphate (F6P), xylulose 5-phosphate (X5P), and hydroxypyruvate (HP) were used as the dihydroxyethyl group donor molecules. The data are listed in Table 1 and shown as a Michaelis−Menten plot in Figure S16. F6P showed the highest activity with a k_{cat}/K_m of 54.35 mM^{-1} s^{-1}, followed by X5P with a k_{cat}/K_m of 28.72 mM^{-1} s^{-1} and HP with a k_{cat}/K_m of 13.81 mM^{-1} s^{-1}. The cellular concentration of these types of metabolites and their K_m values suggest that enzyme activity might fluctuate with substrate availability.[21]

Table 1. Kinetic Values for Donor Substrates



	F6P	X5P	HP
K_m (mM)	2.0 ± 0.4	3.0 ± 0.5	4.0 ± 0.9
k_{cat} (s^{-1})	91 ± 5.4	76 ± 3.6	61. ± 3.8
k_{cat}/K_m (mM^{-1} s^{-1})	54 ± 13	29 ± 6.3	14 ± 3.6

This study demonstrates the key two-carbon extension required in the generation of the carbon framework of the aziridino[1,2-*a*]pyrrolidine moiety. *N*-Acetyl-glutamate 5-semialdehyde **6** is converted to 2-acetamido-5,7-dihydroxy-6-oxoheptanoic acid **8** by AziC5/C6, a thiamin-dependent transketolase. AziC5 and AziC6 encode the N- and C-terminal subunits of the enzyme, catalyzing the two-carbon extension with a suitable donor, e.g., hydroxypyruvate. Scheme 1 depicts the overall proposed route to the formation of 2-acetamido-5,7-dihydroxy-6-oxoheptanoic acid **8** and its role in the formation of the aziridino[1,2-*a*]pyrrolidine based upon previous work and bioinformatic analysis of the azinomycin gene cluster.[9,11,22,23] In the long term, understanding the mechanism of formation of the aziridino[1,2-*a*]pyrrolidine moiety might enable genetic engineering of novel azabicycle-containing agents and its application to synthetic biology methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.9b00477.

Experimental procedures, primers and plasmids used in this study, homology model, azinomycin gene cluster and proposed biosynthetic pathway, nuclear magnetic resonance and ESI-MS spectra, thiochrome HPLC results, colorimetric activity assay results, kinetic values, and a Michaelis−Menten plot for donor substrates (PDF)

Accession Codes

AziC6, B4XYB0; AziC5, B4XYB1; AziC3, B4XYB4; AziC4, B4XYB3.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

TPP, thiamin pyrophosphate; F6P, fructose 6-phosphate; X5P, xylulose 5-phosphate; HP, hydroxypyruvate.

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