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Probing the Role of *N*-Acetyl-glutamyl 5-Phosphate, an Acyl Phosphate, in the Construction of the Azabicycle Moiety of the Azinomycins

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Supporting Information

ABSTRACT: The azinomycins are potent antitumor agents produced by the soil bacterium *Streptomyces sahachiroi* and contain a novel aziridino[1,2-*a*]pyrrolidine core; its synthesis involves at least 14 steps. This study reports the first reconstitution of *N*-acetylglutamine semialdehyde formation by two enzymes encoded in the azinomycin biosynthetic gene cluster. The reaction proceeds through the formation of an acylphosphate and establishes *N*-acetyl-glutamyl 5-phosphate and *N*-acetyl-glutamine semialdehyde as intermediates in the complex biosynthesis of the aziridino[1,2-*a*]pyrrolidine moiety.

Carboxylic acids are typically derivatized to improve reactivity in acyl substitution reactions in organic synthesis, e.g., conversion to an acid chloride or anhydride. In enzymatic processes, the carboxylate group is equally unreactive and requires "activation". Use of protein-templated ester or thioester bond formation is a frequently used strategy for carboxylate activation, e.g., fatty acid/polyketide bond formation,[1−3] amide bond formation by nonribosomal peptide synthetases.[4] Acyl phosphates can also often serve this purpose and improve the susceptibility of carboxylic acids to nucleophilic attack. Glutamine synthetase, for example, catalyzing the conversion of glutamate to glutamine proceeds through formation of a glutamyl 5-phosphate intermediate.[5] Glycinamide ribonucleotide synthetase similarly converts glycine to glycinamyl phosphate as mediated by ATP in the formation of glycinamide ribonucleotide,[6] while asparagine synthetase proceeds by way of an aspartyl AMP intermediate.[7]

The azinomycins [A (**1**) and B (**2**) (Figure 1)] are antitumor agents produced by the soil bacterium *Streptomyces sahachiroi*.[8] The compounds exhibit potent *in vitro* cytotoxicity and *in vivo* antitumor effects.[9,10] Against the leukemic cell line L5178Y, azinomycin A exhibits an IC_{50} of 0.07 μg/mL and azinomycin B gives an IC_{50} of 0.11 μg/mL.[10] Azinomycin B is efficacious against murine transplantable tumors over a range of 2−32 μg/kg. An increased life span of 193% is observed at 32 μg/kg. Azinomycin A, on the other hand, exhibits lower potency and a lower antitumor spectrum in xenographic mice.[10] In an early phase clinical investigation, azinomycin B showed favorable results in 36 cases of malignant neoplasms, with the most promising effects against squamous cell carcinoma, a form of skin cancer.[11]

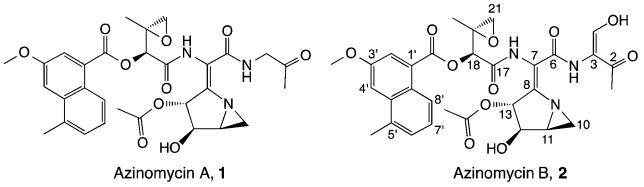
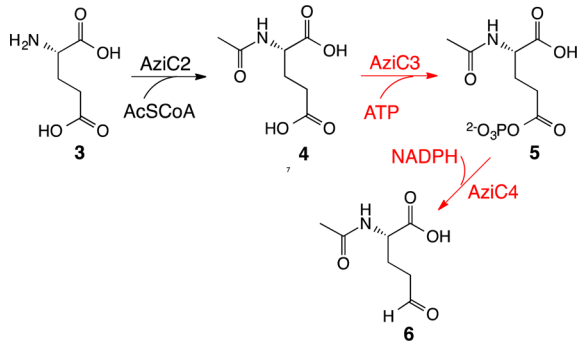


Figure 1. Chemical structures of the azinomycins.

Epoxide and aziridino[1,2-*a*]pyrrolidine moieties within the azinomycins impart their ability to form interstrand cross-links within the major groove of DNA.[12−14] Evaluation of the cross-link chemistry of azinomycin B by high-performance liquid chromatography time course analysis reveals that the azinomycin aziridine−DNA adduct is the first to form followed by cross-linking through the epoxide.[15]

The densely functionalized nature of the azinomycins poses an intriguing biosynthetic challenge with origins in polyketide, nonribosomal peptide, and alkaloid biosynthesis. Whole cell feeding experiments have shown that the principal precursor to the aziridino[1,2-*a*]pyrrolidine moiety of the natural product is glutamic acid **3** (Scheme 1).[16] A "protection" step of the glutamic acid amine initiates the process through the action of AziC2, which prevents cyclized intermediates and other adducts from forming throughout the biosynthesis of the azabicyclic ring system.[16] The proposed next step in the biochemical sequence of events is the formation of *N*-acetyl-glutamyl 5-phosphate **5**, an acyl phosphate, by the action of AziC3, which

Scheme 1



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facilitates reduction and formation of *N*-acetyl-glutamate 5-semialdehyde **6** as mediated by AziC4 (Scheme 1). The study of acyl phosphates in enzymatic reactions can be tricky, however, given their overall instability and/or lability. Here we provide the first evidence of the involvement of *N*-acetyl-glutamyl 5-phosphate **5** in the biosynthesis of the azabicycle moiety of the azinomycins; *N*-acetyl-glutamyl 5-phosphate **5** is subsequently shown to be reduced to the aldehyde, giving *N*-acetyl-glutamate 5-semialdehyde **6**.

On the basis of DNA annotation, *aziC3* encodes a putative *N*-acetyl glutamate kinase that is 55% similar and 36% identical to *N*-acetyl glutamate kinase from *Thermus thermophilus*.[17] AziC3 was successfully cloned, overexpressed, and purified from pET24b/*Escherichia coli* BL21(DE3). The formation of *N*-acetyl [γ-^{32}P]glutamyl phosphate was initially evaluated by use of an autoradiographic thin layer chromatography (TLC) assay, where [γ-^{32}P]ATP was utilized to label the carboxylate group of *N*-acetyl-glutamic acid **4** as mediated by AziC3. AziC3 was incubated with *N*-acetyl-glutamate **4** and [γ-^{32}P]ATP for 16 h. Following incubation, the protein was removed by filtration and the metabolites were analyzed by TLC/autoradiographic analysis (Figure 2). AziC3 incubated with [γ-^{32}P]ATP, without

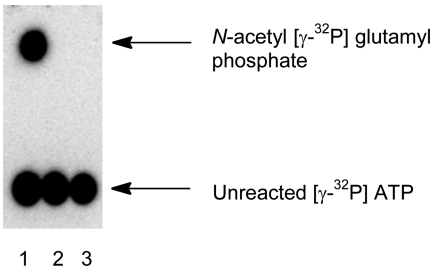
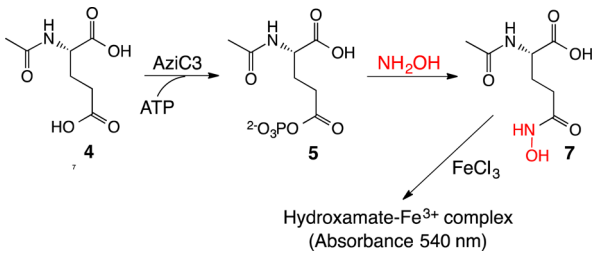


Figure 2. [γ-^{32}P]ATP radioactive labeling assay: lane 1, AziC3, *N*-acetyl-glutamic acid, [γ-^{32}P]ATP; lane 2, AziC3, [γ-^{32}P]ATP; lane 3, *N*-acetyl-glutamic acid, [γ-^{32}P]ATP.

substrate (lane 2), and with *N*-acetyl-glutamic acid incubated with [γ-^{32}P]ATP, without protein, served as controls (lane 3). In all three lanes, unreacted [γ-^{32}P]ATP was detected at the baseline. A higher running spot was detected, which was consistent with the formation of *N*-acetyl-[γ-^{32}P]glutamyl phosphate.

Given the inherent instability of acyl phosphate intermediates, to provide further evidence of the formation of *N*-acetyl-glutamyl-5 phosphate **5**, a colorimetric assay was performed (Scheme 2). Hydroxylamine hydrochloride was utilized to convert the acyl phosphate intermediate (as mediated by AziC3) into *N*-acetyl-glutamyl-γ-hydroxamate **7**. The hydroxamate (**7**) forms a complex with ferric chloride ($FeCl_3$) that can be detected spectrophotometrically at 540 nm.[18]

Scheme 2



One unit of AziC3 is defined as the amount of enzyme needed to catalyze the formation of 1 μmol of *N*-acetyl-glutamyl-γ-hydroxamate-Fe^{3+} complex per minute under the assay conditions. The molar absorption coefficient ε of this complex at 540 nm is 456 $\pm$ 4 M^{-1} cm^{-1}. On the basis of this information, the concentration of the *N*-acetyl-glutamyl-γ-hydroxamate-Fe^{3+} complex was calculated (in micromolar) and used to evaluate the kinetics of the reaction (Figure 3), giving k_{cat}, K_m, and k_{cat}/K_m values of 0.42 s^{-1}, 0.76 $\times$ 10^{-3} M, and 5.4 $\times$ 10^2 M^{-1} s^{-1}, respectively.

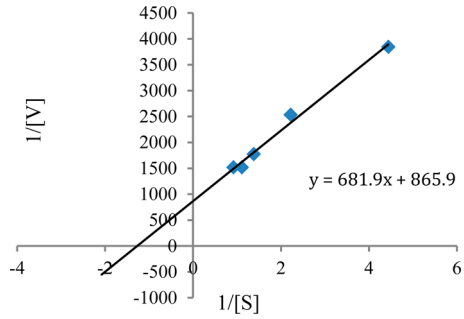
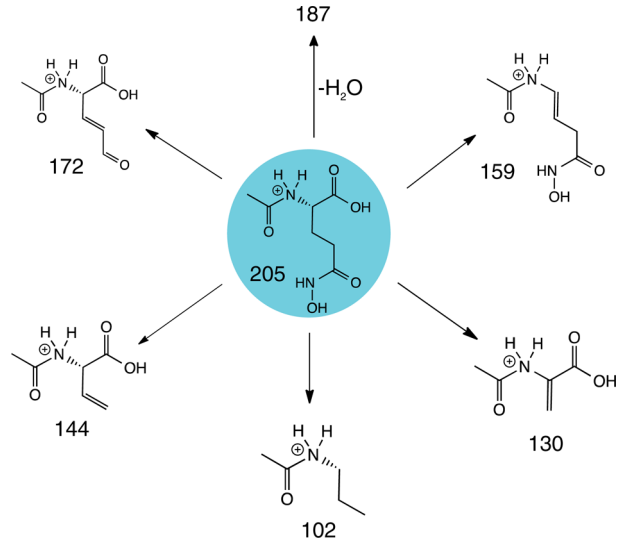


Figure 3. Kinetic analysis of the AziC3 reaction as monitored by the formation of the *N*-acetyl-glutamyl-γ-hydroxamate-Fe^{3+} complex.

The formation of *N*-acetyl-glutamyl-γ-hydroxamate **7** was detected by electrospray ionization mass spectrometry (ESI-MS), giving an observed mass of 205 [M + H]. Fragmentation of the M^+ peak gave expected daughter ions as shown in Scheme 3.

Scheme 3



AziC4 is 55% similar and 45% identical to *N*-acetyl-γ-glutamate phosphate reductase from *Deinococcus geothermalis*.[19] Soluble expression and purification of AziC4 could be achieved only when it was co-expressed and copurified with AziC3. The *aziC4* gene was cloned in pET21a and co-expressed with *aziC3*-pET24b in *E. coli* BL21(DE3). The activity of AziC4 was assessed spectrophotometrically in a coupled enzyme reaction with AziC3. *N*-Acetyl-glutamic acid **4** was first converted into *N*-acetyl-glutamyl 5-phosphate **5** by AziC3 and subsequently reduced to *N*-acetyl-glutamate-5-semialdehyde **6** by AziC4 with

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concomitant production of NAD(P) as generated from NAD(P)H. The rate of NAD(P)H oxidation was monitored as a change in absorbance at 340 nm, with the rate of NAD(P) formation reflecting the rate of product formation.[20]

The kinetics of the reaction were evaluated as shown in Figure 4. AziC4 accepts both NADPH and NADH as a cofactor

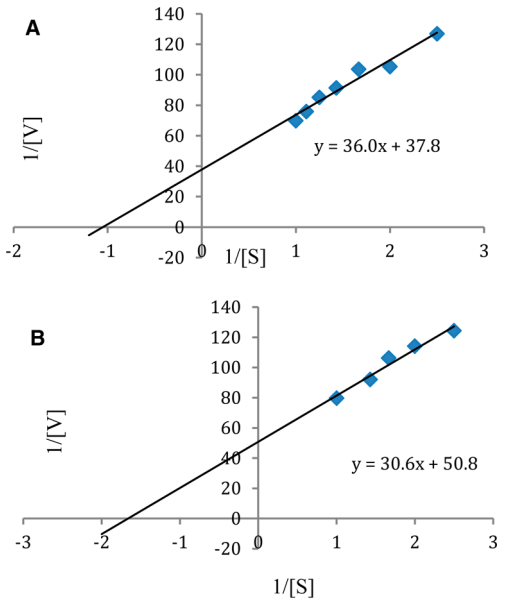


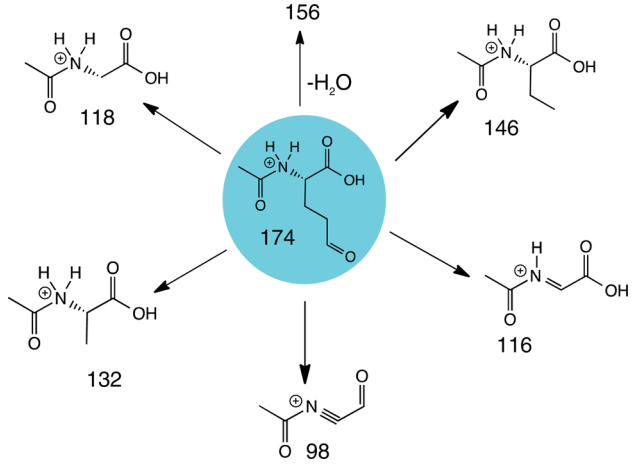
Figure 4. Kinetic analysis of the AziC4 reaction as monitored by UV assay [the consumption of NAD(P)H at 340 nm]: (A) NADPH used as a cofactor and (B) NADH used as a cofactor.

and did not show a significant preference for either. AziC4 gave k_{cat}/K_m values of 7.5 mM^{-1} min^{-1} (NADPH) and 5.1 mM^{-1} min^{-1} (NADH). The requirement of AziC3 for overexpression of AziC4 suggests the possibility of physical association of the proteins and channeling of the reactive acyl phosphate intermediate from the AziC3 active site to the AziC4 active site.

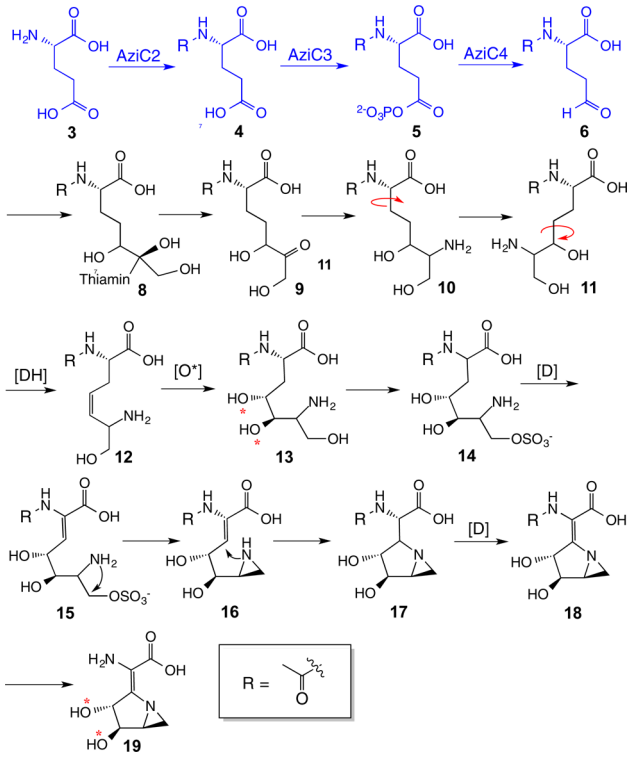
The formation of *N*-acetyl-glutamate-5-semialdehyde was also confirmed by ESI/MS, giving an ion at m/z 174 [M + H]. ESI-MS/MS analysis of the M^+ peak by ESI-MS gave the expected fragmentation ions as shown in Scheme 4.

A proposed route for the formation of the aziridino[1,2-*a*]pyrrolidine moiety is provided in Scheme 5 (highlighted in

Scheme 4



Scheme 5



blue are steps validated by experimentation). Our previous studies, including *in vitro* reconstitution and genetic knockout of *aziC2*, have shown that N-acetylation of glutamic acid **3** by AziC2 serves as a "protection step" in the biosynthesis of the 1-azabicyclo[3.1.0]hexane ring system.[16] The modification prevents cyclization of the amino acid and formation of the Δ^1-piperidine carboxylate, analogous to that observed in bacterial arginine biosynthesis. N-Acetylation reflects at least one mechanism by which azabicycle biosynthesis can be primed.[16] Taken together with our previous work with AziC2, our studies here are fully consistent with the involvement of AziC3, an *N*-acetylglutamate kinase, giving an acyl phosphate **5** that facilitates reduction to aldehyde **6** via *N*-acetyl-γ-glutamyl-phosphate reductase, AziC4. A transketolase would give a two-carbon extension, where subsequent transamination would generate an amine diol **10**. Dehydration would allow oxidation to give a diol **13**.[16] A sulfotransferase could facilitate aziridine ring formation through sulfation. Dehydrogenation and deprotection of the amine would generate the aziridinopyrrolidine moiety **19**. Experiments are underway to evaluate the remaining biosynthetic steps.

■ ASSOCIATED CONTENT

Supporting Information

Additional supplemental material. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00711.

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Notes

The authors declare no competing financial interest.

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