A Potent Transition-State Analogue Inhibitor of *Escherichia coli* Asparagine Synthetase A

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L-Asparagine synthetase [L-aspartate: ammonia ligase (AMP-forming) EC 6.3.1.1] (AS-A) from *Escherichia coli*[1] is a typical member of ammonia-dependent asparagine synthetases[2] and catalyzes the formation of L-Asn from L-Asp and ammonia with concomitant hydrolysis of ATP to AMP and pyrophosphate. In asparagine biosynthesis, another family of asparagine synthetases (AS-B) which utilize glutamine as a nitrogen source is more ubiquitous for eukaryotes.[3] Asparagine synthetase is a potential target for cancer chemotherapy[3] because asparagine depletion caused by the administration of L-asparaginase is a currently implemented protocol for the treatment of acute lymphoblastic leukemia.[4] Although AS-A is not related structurally and evolutionarily to AS-B[3] and is more related to amino-acyl tRNA synthetases,[5] the reaction catalyzed by AS-A is prototypic of this class of enzyme from a mechanistic point of view: the substrate carboxyl group is activated by adenylation followed by substitution by amine nucleophile.[2a,b,6] It is therefore highly desirable to obtain good inhibitors of AS-A not only for use as a probe to define the detailed reaction mechanisms of asparagine synthetases, but also for generating a lead for chemotherapeutic agents targeted toward the inhibition of asparagine biosynthesis. For these purposes, transition-state analogues[7] are far more suitable than substrate analogues[8] or intermediate mimics.[9] We now describe the synthesis and characterization of a transition-state analogue, *N*-adenylated *S*-methyl-L-cysteine sulfoximine **1**. Compound **1** served as an extremely potent slow-binding inhibitor of *E. coli* AS-A with an overall inhibition constant of 67 nM. An X-ray crystal structure of the enzyme complexed with **1** revealed several key amino acid residues responsible for catalysis as well as those for substrate recognition.

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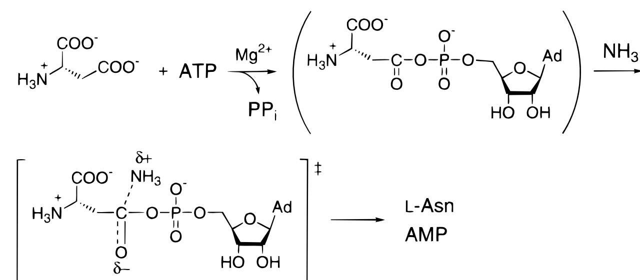
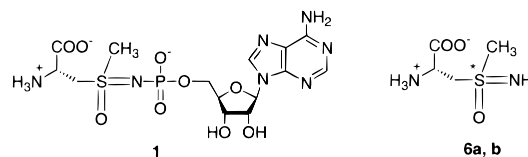
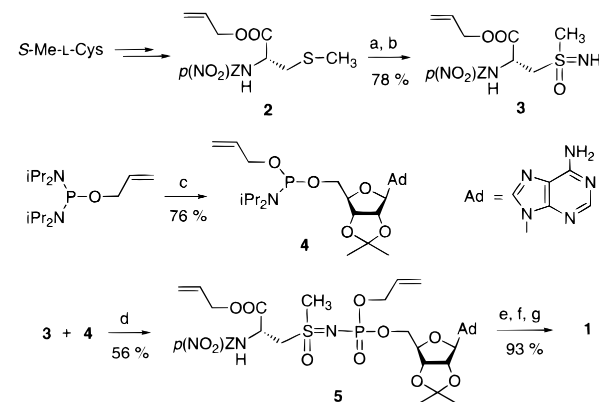
Scheme 1





Scheme 2[a]



[a] Reagents and conditions: (a) $NaIO_4$, MeOH−H_2O, 25 °C, 7 h; (b) *O*-mesitylsulfonylhydroxylamine (MSH), CH_3CN, 25 °C, 3 days; (c) 2′,3′-*O*-isopropylideneadenosine, diisopropylammonium 1*H*-tetrazolide, dry pyridine, 25 °C, 15 h; (d) 1*H*-tetrazole, dry CH_3CN, 25 °C, 4 h, then t-BuOOH; (e) TFA-H_2O (8:3), 25 °C, 11 h; (f) $Pd(PPh_3)_4$, PPh_3, HCOOH, NEt_3, dry THF, 25 °C, 9 h; (g) H_2, 10% Pd−C, CH_3CN−H_2O, 1 h.

The catalytic reaction of AS-A is thought to proceed by a two-step mechanism involving an intermediate β-aspartyl adenylate;[2a,b] the first step is the formation of the intermediate, and the adenylated β-carboxyl is attacked by ammonia in the second step to form L-Asn and AMP (Scheme 1). As a stable analogue of the transition state in the latter step, we designed *N*-adenylated *S*-methyl-L-cysteine sulfoximine **1** where the carbonyl to be attacked by ammonia is replaced by a tetrahedral sulfoximine sulfur atom with a methyl group mimicking ammonia. The synthesis of **1** is shown in Scheme 2. The sulfoximine nitrogen was adenylated by a conventional phosphoramidite method,[10] and the resulting P−N bond was hydrolytically stable even under acidic conditions. Compound **1** was obtained as a 1:1 mixture of diastereomers with respect to the chiral sulfur atom.

The *N*-adenylated sulfoximine **1** was found to be a potent slow-binding inhibitor that caused time-dependent inactivation of AS-A (Figure 1). The enzyme, for example, was totally inactivated in 15 min when 2.5 μM of **1** was present. The inhibition was virtually irreversible, and no regain of enzyme activity was observed after gel filtration.[11] Since the inhibition was time-dependent, the onset

(10) The amino group of adenine ring was unreactive under tetrazole- or tetrazolium salt-catalyzed phosphinylation conditions, and no protection was necessary.

(11) The enzyme did not regain activity for 10 days after gel filtration, but a very slow regain of enzyme activity was observed in the absence of Mg^{2+}.

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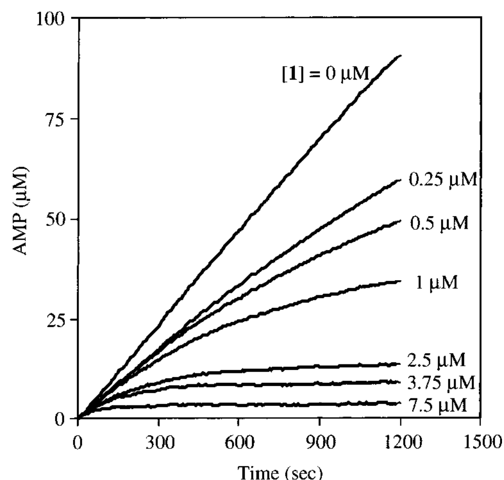

Figure 1. Progress curves for the inactivation of AS-A by transition-state analogue **1**. The reaction was initiated by adding the enzyme to an assay mixture containing L-Asp (1.5 mM), NH_4Cl (20 mM), ATP (3 mM) and the inhibitor **1** (0−7.5 μM) in 100 mM Tris-HCl (pH 7.8) at 37 °C.

rate of inactivation (k_{on})[12] was calculated from the progress curves and was found to be 3.27 s^{-1} mM^{-1}.[13] The overall inhibition constant (K_i*)[12] was calculated as 67 nM by measuring the inhibited enzyme activity after slow-binding inhibition equilibrium was reached. Considering that the kinetic constants for AS-A were K_m (Asp) = 1.69 mM, K_m (ATP) = 1.24 mM, and k_0/K_m (Asp) = 28.5 s^{-1} mM^{-1}, the inhibitor **1** binds to the enzyme at a rate 9 times smaller than that of the enzymatic reaction, but with 25000-fold higher affinity than its substrates.

We first attempted to inhibit AS-A with diastereomeric *S*-methyl-L-cysteine sulfoximine **6a**,**b**[14] and ATP in the hope that a mechanism-based inactivation of AS-A might result by in situ adenylation of the sulfoximine nitrogen by ATP within the enzyme active site. However, each diastereomer **6a** and **6b** was a poor and reversible inhibitor of AS-A[15] with K_i = 3.27 and 0.76 mM, respectively, and no enzyme inactivation was observed after a prolonged incubation of the enzyme with **6a** or **6b** in the presence of ATP. Since chemically synthesized **1** served as an extremely potent inhibitor of AS-A, the enzyme was not capable of adenylation of the sulfoximine **6**. This result is in sharp contrast to the inhibition of another class of ATP-dependent ligases such as glutamine synthetase[16] and γ-glutamylcysteine synthetase[17] by sulfoximine-based transition-state analogues, where the sulfoximine nitrogen was phosphorylated in a mechanism-based manner to cause time-dependent inactivation of the enzyme.

Since compound **1** was most likely to mimic the putative transition state, the enzyme complexed with **1** was crystallized and subjected to X-ray diffraction analysis. The inhibitor **1** gave a clear electron density in the enzyme active site, and several specific interactions were identified between the active-site amino acid residues and the inhibitor **1** (Figure 2). Of particular interest is the interaction regarding the sulfoximine moiety. First, the sulfoximine oxygen (S═O) is hydrogen bonded to Arg 100 and Gln 116. Since S═O represents the oxyanion generated by nucleophilic attack of ammonia, these residues are most likely to be key catalytic residues responsible for stabilizing the tetrahedral oxyanion transition state or intermediate. Another point is the methyl attached to the sulfur atom. This methyl, which is supposed to mimic the -NH^{3+} moiety of the zwitterionic tetrahedral transition state or intermediate, was found to be 3.1 Å distant from Asp 46. This negatively charged residue probably plays a key role in electrostatic stabilization of the -NH^{3+} part of the transition state and also acts as a base to abstract a proton from the tetrahedral intermediate completing the formation of the product asparagine.[18]

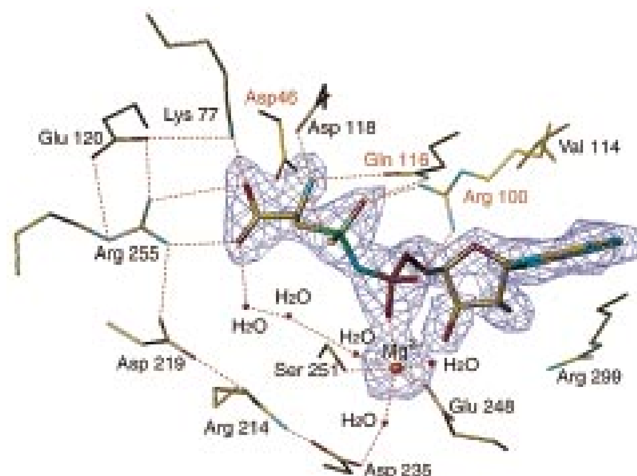

Figure 2. X-ray crystal structure of *E. coli* AS-A active site complexed with the inhibitor **1**, showing the SIGMAA weighted $F_o - F_c$ omit electron density map (blue), superimposed with the refined structure of the inhibitor **1** and the active site amino acid residues. The contour level is 3.0 σ.

In conclusion, a potent slow-binding inhibitor **1** of *E. coli* asparagine synthetase A was prepared. The crystal structure of the enzyme complexed with **1** gave a structural basis for the transition-state stabilization as well as for substrate recognition by the enzyme. Since this enzyme shares the same chemistry in carboxylate activation with glutamine-dependent asparagine synthetases (AS-B), and all AS-B from eukaryote can employ ammonia as an alternative nitrogen source in asparagine synthesis,[3] compound **1** should also formulate a basis for future inhibitor design of asparagine synthetase B.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Morrison, J. F.; Walsh, C. T. In *Advances in Enzymology*; Meister, A., Ed.; John Wiley: New York, 1988; Vol. 61, pp 201−301. The k_{on} value was calculated from a plot of pseudo-first-order inhibition rates (k_{obs}) vs [**1**] by regression to $k_{obs} = k_{on}$ [**1**] since no saturation was observed. K_i* was obtained by steady-state inhibition kinetics after the enzyme was preincubated with various concentrations of **1** (0−7.5 μM) at 37 °C for 30 min to establish the binding equilibrium. The substrate concentrations are [Asp] = 1.5 mM, [ATP] = 3 mM and [$NH_4^+Cl^-$] = 20 mM. The initial inhibition constant (K_i) was not obtained because the plot of k_{obs} vs [**1**] showed no saturation.

(13) We examined the effect of pyrophosphate (PP_i) to see if the generation and binding of PP_i would be a possible cause for time-dependent inactivation of the enzyme, but no effect was observed on the rate or the extent of enzyme inactivation up to 10 mM of PP_i.

(14) The sulfoximine **6** was synthesized from *S*-methyl-L-cysteine by stepwise oxidation with $NaIO_4$ and HN_3/H_2SO_4 by modifying the reported procedure (Griffith, O. W. *Methods Enzymol.* **1981**, *77*, 59−64). The diastereomers **6a** and **6b** with respect to the chiral sulfur were separated by fractional crystallization from EtOH−H_2O.

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