ORGANIC LETTERS

2003
Vol. 5, No. 12
2033−2036

Synthesis and Characterization of an *N*-Acylsulfonamide Inhibitor of Human Asparagine Synthetase†

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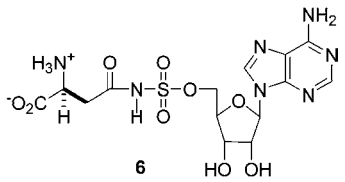
Received February 5, 2003

ABSTRACT



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The synthesis of *N*-acylsulfonamide 6, which is an analogue of *β*-aspartyl-AMP, is described. This compound appears to be the first *and only* potent inhibitor of human asparagine synthetase that has been described to date. The *N*-acylsulfonamide 6 exhibits slow-onset inhibition kinetics, with a K_i* of 728 nM. Preparation and characterization of two additional *N*-acylsulfonamide analogues has also demonstrated the importance of hydrogen-bonding interactions in the recognition of the AS inhibitor with the enzyme. These observations provide the basis for the discovery of new compounds with application in the treatment of drug-resistant leukemia.

In humans, asparagine is biosynthesized from aspartic acid by asparagine synthetase (AS), a glutamine-dependent Ntn amidotransferase,[1] in an ATP-dependent reaction for which the cellular nitrogen source is glutamine.[2] Several lines of evidence suggest that overexpression of asparagine synthetase (AS) in human T-cells results in metabolic changes that underpin the appearance of drug-resistant forms of acute lymphoblastic leukemia (ALL).[3] Hence, it is widely believed that, in combination with other drugs, potent AS inhibitors will provide new clinical approaches for the treatment of ALL and other solid tumors. The failure of large-scale screening studies to identify compounds that act as potent and selective AS inhibitors,[4] and a lack of routine access to large amounts of the human enzyme, have significantly hindered efforts to solve this important problem in medicinal chemistry. Our recent determination of the three-dimensional structure of the glutamine-dependent AS present in *Escherichia coli* (AS-B)[5] and demonstration that adenylated sulfoximine **1** (Figure 1)[6] inhibits AS-B,[7] however, have set the stage for rational strategies for discovering AS inhibitors.

The likely synthetic difficulty of routinely obtaining simplified analogues of **1** in large amounts prompted us to explore whether stable analogues of *β*-aspartyl-AMP (*β*AspAMP) **2** (Figure 1), which is an intermediate in the AS-catalyzed reaction,[8] would function as inhibitors of the human enzyme. Our initial work focused on the *β*AspAMP analogue **3** (Figure 1) that could be obtained using a novel

† Portions of the work described in this paper were presented at the 223rd National Meeting of the American Chemical Society, Orlando, FL, April 2002 (MEDI-143).

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10.1021/ol034212n CCC: $25.00
Published on Web 05/09/2003

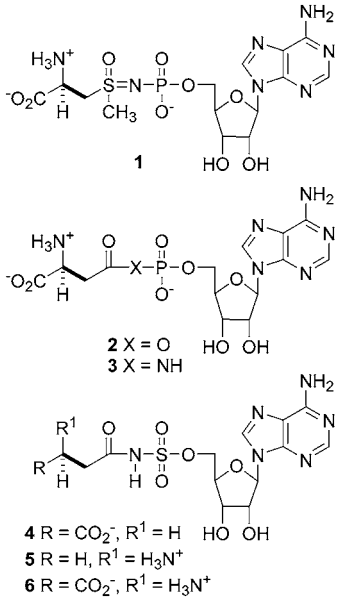
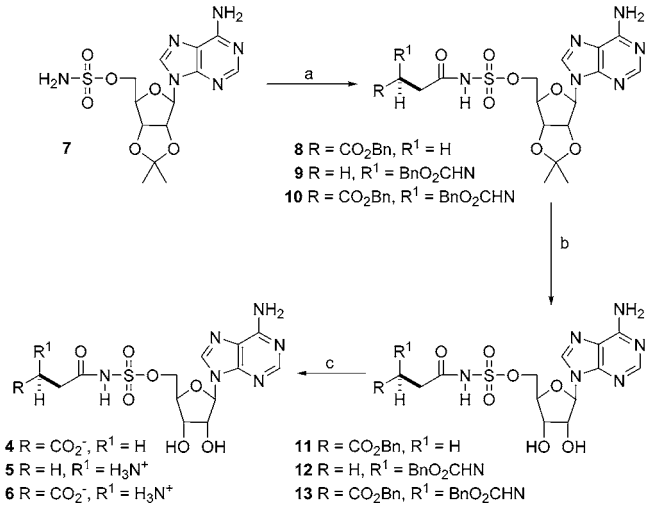


Figure 1.

Scheme 1 [a]



[a] Key: (a) $R^1R^2CHCH_2CO_2H$, EDCI, HOBt, DMAP, CH_3CN, 0 °C to rt; (b) TFA/H_2O, rt; (c) Pd black, H_2, 5% TFA/MeOH, rt.

synthetic procedure for the "one-pot" preparation of *N*-acylphosphoramidates.[9] Although **3** was found to be an inhibitor of AS-B, technical limitations in preparing the compound caused us to undertake the synthesis and characterization of alternate βAspAMP analogues so as to identify inhibitors containing functional moieties that were more "drug-like" in structure.[10] As a consequence of our efforts in this area, we now report the preparation of a series of *N*-acylsulfonamides **4**−**6** together with their ability to inhibit the synthetase activity of recombinant, wild-type, human AS. These experiments clearly show that sulfonamide derivative **6** is the first inhibitor of the human enzyme to be described and reveal the importance of the α-carboxyl and α-amino groups in the recognition and/or binding of this compound by AS.

Our synthesis and purification of *N*-acylsulfonamides **4**−**6** (Scheme 1) was based on literature procedures for the preparation of isoleucyl- and tyrosyl-tRNA synthetase inhibitors.[11] Thus, our route employed the 2′,3′-protected adenosinesulfamate **7** as starting material, which could be readily prepared from 2′,3′-isopropylideneadenosine and sulfamyl chloride[12] by slight modification of literature conditions.[13] The adenosinesulfamate derivative **7** was then acylated with appropriately protected carboxylic acid derivatives to give the *N*-acylsulfamate derivatives **8**−**10** in 63−80% isolated yield after chromatographic purification. Removal of the acetonide protecting groups was then accomplished by treatment with aqueous TFA, giving the diols **11**−**13** in 80−89% isolated yield. Removal of the benzyl protecting groups, however, proved particularly problematic for diols **12** and **13**, with many standard methods leading to extensive decomposition of the product *N*-acylsulfonamides. We therefore employed short reaction times and determined that 5% TFA in MeOH increased the rate of Pd-catalyzed hydrogenolysis of the *N*-benzyloxycarbonyl protecting group. This procedure reproducibly gave the target *N*-acylsulfonamides **4**−**6** as their hygroscopic trifluoroacetate salts in 85−90% yield. Prior to their use in our enzyme assays, all of these compounds were subjected to chromatography on Mitsubishi HPSS20 resin[11a] and their purity checked using reversed-phase HPLC.[14]

Since our goal was to obtain clinically useful AS inhibitors, we also developed a baculovirus-based expression system so as to gain access to large quantities of human AS.[15] This procedure gave wild-type enzyme in multi-milligram amounts, which exhibited ammonia-dependent synthetase activity[16] comparable to that reported previously for recombinant, human AS obtained using a yeast-based expression system.[17] It is therefore unlikely that the additional C-terminal residues

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(14) All compounds prepared in this study exhibited spectroscopic properties consistent with their proposed structures. Full experimental details are included in the Supporting Information.

have any significant impact on the activity of the enzyme relative to that of wild-type human AS. We also note that this baculovirus-based expression system offers significant advantages for the obtaining human AS for the assay of clinically useful inhibitors, including simple scale-up for large-scale protein production.

With the target *N*-acylsulfonamide analogues **4**−**6** in hand, we examined their ability to inhibit the ammonia-dependent activity of the recombinant, C-terminally tagged human AS using similar conditions to those previously used in characterizing inhibition of *E. coli* AS-B by the adenylated sulfoximine **1**.[7] Hence, progress curves were generated by incubating the purified enzyme (4 μg) in reaction mixtures containing 100 mM NH_4Cl, 0.5 mM ATP, 10 mM aspartate, and 10 mM $MgCl_2$ in 100 mM EPPS, pH 8, together with the inhibitor (0−50 μM) (1 mL total volume). Since asparagine and PPi are formed in a 1:1 stoichiometric ratio in the enzyme-catalyzed reaction, the synthetase activity of human AS under these conditions was determined by spectrophotometric monitoring of PP_i production.[18] The results showed that compound **6** did inhibit the recombinant human enzyme (Figure 2), presumably by binding within

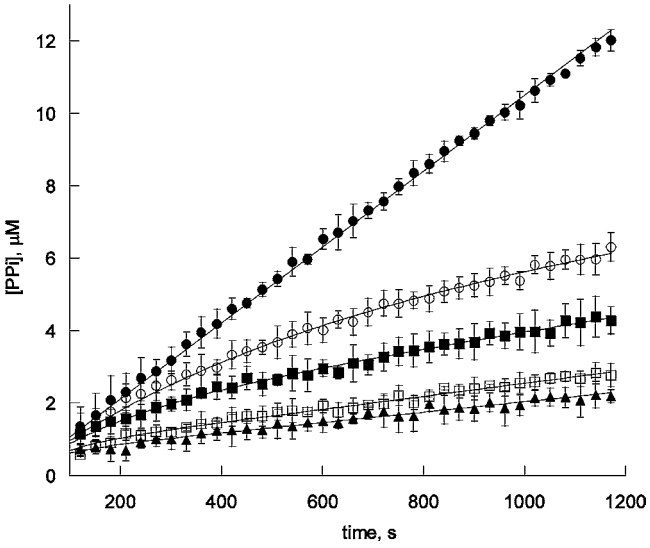


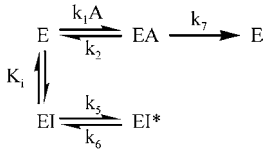
Figure 2. Progress curves showing the effect of *N*-acylsulfonamide **6** on PP_i production in the ammonia-dependent synthetase reaction catalyzed by recombinant, C-terminally tagged human AS. Key: [**6**] = 0 μM, filled circles; [**6**] = 5 μM, open circles; [**6**] = 10 μM, filled squares; [**6**] = 25 μM, open squares; [**6**] = 50 μM, filled triangles. Solid lines represent the theoretical curve computed from eq 1 that best fit the experimental data, and error bars correspond to the standard deviation of the [PP_i] concentration measured at a given time point.

the C-terminal, synthetase site of human AS in a fashion similar to the βAspAMP intermediate **2** that is formed in the catalytic mechanism of asparagine production. Under similar experimental conditions, we observed that sulfonamides **4** and **5** were only capable of inhibiting the recombinant human AS at 100-fold greater concentrations (data not shown). These results therefore show the importance of the ionized α-amino- and α-carboxylate groups to the interaction of *N*-acylsulfonamide **6** with the enzyme. Importantly, control experiments established that the pyrophosphate reagent is not affected by the presence of *N*-acylsulfonamides **4**−**6**.

Analysis of the steady-state kinetics of AS inhibition by *N*-acylsulfonamide **6** was undertaken to determine the mechanism by which the inhibitor exerted its effects. The data were best fit (Figure 2) using the following equation, corresponding to slow-onset inhibition[19]

$$[PP_i] = v_{ss}t + [(v_o - v_{ss})/k][1 - \exp(-kt)] \qquad (1)$$

where v_o is the initial velocity of the reaction, v_{ss} is the velocity at large t, and k is a parameter that depends on the inhibitor concentration. Further experiments demonstrated that elevated levels of ATP decreased the ability of **6** to inhibit recombinant human AS (see the Supporting Information). This implies that ATP and **6** compete for the free enzyme, an observation consistent with the assumption that **6** binds within the C-terminal, synthetase site of AS. The inhibition mechanism is consistent with the following model



for which the variation of k with inhibitor concentration is given by (see the Supporting Information):

$$k = k_6 + k_5(I/K_i)/[1 + [ATP]/K_a + I/K_i] \qquad (2)$$

Analysis of the progress curves using eq 1 then gave values of k as a function of the concentration of **6**, and a replot of

(15) Briefly, the gene encoding human AS was obtained and modified so that the expressed enzyme would contain additional residues at the C-terminus corresponding to a *c-myc* tag. The resulting construct was then cloned into a pBAC-1 transfer vector (Novagen), which includes a C-terminal histidine tag for protein purification, so that expression of human AS is under control of the *polh* promoter allowing high levels of protein expression during late phases of viral infection. Recombination of pBAC-1 with the BacVector-3000 construct (Novagen) was then used successfully to obtain a baculovirus capable of replicating and infecting conditioned *Sf9* cells, which also contained the gene encoding human AS. Using standard procedures, we expressed human AS in conditioned *Sf9* cells, the recombinant enzyme being purified by chromatography on a preequilibrated Ni-Agarose column (see the Supporting Information). Additional details of this expression system can be found in the following references: (a) Farrell, P. J.; Lu, M. L.; Prevost, J.; Brown, C.; Behie, L.; Iatrou, K. *Biotechnol. Bioeng.* **1998**, *60*, 656−663. (b) Lenhard, T.; Reilander, H. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 823−830.

(16) An important limitation of the baculovirus-based expression protocol is that oxidation of the N-terminal cysteine residue can take place during protein isolation and purification. Since the thiolate side chain of Cys-1 is required for glutamine-dependent activity, our samples of wild-type human AS cannot usually employ glutamine as a nitrogen source. The active site that catalyzes the formation of the βAspAMP intermediate **2** and its subsequent reaction with ammonia is unaffected by oxidation of Cys-1, and therefore, recombinant human AS can be used in assaying for AS inhibitors with clinical utility.

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k versus [**6**] then yielded values for K_i, k_5, and k_6 of 21 μM, 3.6×10^{-2} s^{-1}, and 1.3×10^{-3} s^{-1}, respectively. The overall dissociation constant (K^*_i) for the slow-onset inhibitor **6** is therefore 728 nM.[19] While still not sub-nanomolar in potency, we note that **6** inhibits human AS at a level that is 1000-fold greater than for any compound yet reported, and hence, this *N*-acylsulfonamide represents a useful lead compound for future studies in this area.

In summary, we have synthesized and characterized the first inhibitor of human AS, and have obtained important information for the rational discovery of more potent compounds that can be employed clinically to treat drug-resistant leukemia. Efforts to discover nonionizable moieties that can be substituted for the amino and carboxylate substituents in **6** are underway in our laboratory, and the results will be reported in due course.

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Acknowledgment. We thank the Chiles Endowment Biomedical Research Program of the Florida Department of Health for funding. Useful discussions with W. Wallace Cleland (University of Wisconsin—Madison) and Pamela Brown (GlaxoSmithKline) concerning details of slow-onset inhibition kinetics and selected synthetic procedures, respectively, are gratefully acknowledged. We also thank Professor Michael Kilberg (University of Florida) who generously provided the gene encoding human AS.

Supporting Information Available: Synthetic procedures and spectroscopic characterization of compounds **4**−**6** and **8**−**13**, a brief description of procedures used to obtain recombinant, wild-type human AS, and progress curves for the kinetics of AS inhibition by *N*-acylsulfonamide **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL034212N