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A critical electrostatic interaction mediates inhibitor recognition by human asparagine synthetase

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ABSTRACT

The first sulfoximine-based inhibitor of human asparagine synthetase (ASNS) with nanomolar potency has been shown to suppress proliferation of asparaginase-resistant MOLT-4 cells in the presence of L-asparaginase. This validates literature hypotheses concerning the viability of human ASNS as a target for new drugs against acute lymphoblastic leukemia and ovarian cancer. Developing structure–function relationships for this class of human ASNS inhibitors has proven difficult, however, primarily because of the absence of rapid synthetic procedures for constructing highly functionalized sulfoximines. We now report conditions for the efficient preparation of these compounds by coupling sulfoxides and sulfamides in the presence of a rhodium catalyst. Access to this methodology has permitted the construction of two new adenylated sulfoximines, which were expected to exhibit similar binding affinity and better bioavailability than the original human ASNS inhibitor. Steady-state kinetic characterization of these compounds, however, has revealed the importance of a localized negative charge on the inhibitor that mimics that of the phosphate group in a key acyl-adenylate reaction intermediate. These experiments place an important constraint on the design of sulfoximine libraries for screening experiments to obtain ASNS inhibitors with increased potency and bioavailability.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of childhood cancer, with an annual incidence in the USA of four cases per 100,000 children. A variety of clinical protocols have been developed and substantial success in treating the disease has been achieved, resulting in a cure rate of approximately 80%.[1] The enzyme L-asparagine amidohydrolase (ASNase), which catalyzes the hydrolysis of L-asparagine to yield L-aspartate and ammonia,[2] is a key component in the majority of the clinical treatments for ALL,[3] albeit that this drug has a relatively narrow therapeutic index and hypersensitivity reactions occur in up to two-thirds of patients receiving intensive ASNase therapy.[4,5] It has been shown that incubation of ASNase-sensitive cancer cell lines with ASNase leads to cell cycle arrest and subsequent apoptosis even though the enzyme is not taken up by the leukemic blasts.[6,7] Although the basis for the effectiveness of ASNase in treating childhood ALL remains the subject of some debate, one widely accepted explanation postulates that malignant lymphocytes must import L-asparagine from their surroundings.[8,9] Thus, in the presence of ASNase, which hydrolyzes L-asparagine, ASNase-sensitive leukemic blasts undergo cell death, perhaps from impaired protein synthesis.[8]

Further support for this hypothesis is provided by the observation that ASNase-resistance is correlated with the up-regulation of glutamine-dependent asparagine synthetase (ASNS), the enzyme responsible for de novo asparagine biosynthesis (Scheme 1),[10] in response to asparagine depletion.[11] One prediction of this model is that increased levels of cellular ASNS are the cause of ASNase-resistance in lymphoblasts because sufficient asparagine is made available as a result of increased intracellular asparagine biosynthesis.[8,12,13] Support for this hypothesis has come from studies employing transformed cell lines. For example, in vitro studies with U937 cells demonstrated that ASNase-sensitive cells can be made resistant by persistent exposure to increasing levels of sublethal doses of the drug, and constitutive expression of ASNS in a human ALL cell line (MOLT-4) conferred ASNase-resistance onto sensitive parental cells.[14] Additional evidence has been provided from recent work by our group, which demonstrated that incubation of ASNase-resistant MOLT-4 cells with ASNase and the adenylated sulfoximine derivative **1** (Fig. 1) suppresses cell proliferation.[15] Compound **1** is a potent inhibitor of human ASNS (hASNS),[15] and both classes of bacterial asparagine synthetase,[16,17] because it is stable analog of the transition state for the reaction of ammonia (released in the glutaminase site of ASNS) with an acyl-

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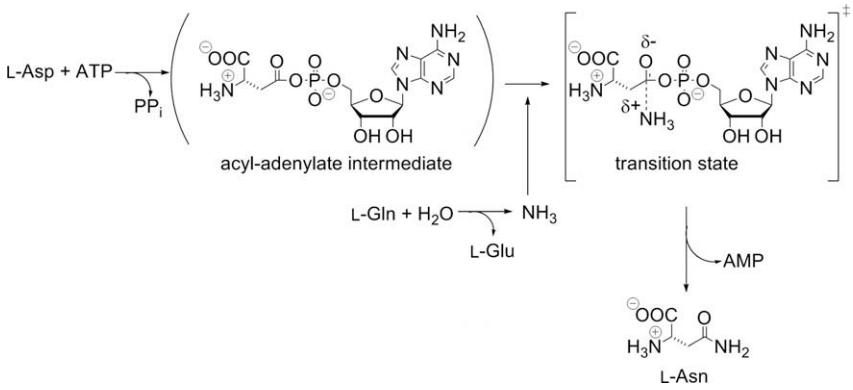


Scheme 1. Overview of the reaction catalyzed by human glutamine-dependent asparagine synthetase, showing the structure of the acyl-adenylate intermediate and the transition state for asparagine formation.

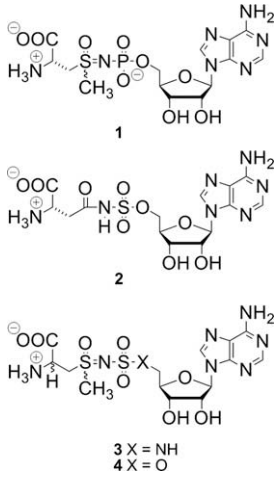


Figure 1. Chemical structures of the adenylated sulfoximine derivative **1** and the acylsulfamate **2**, which are nanomolar human ASNS inhibitors, together with the target sulfoximine derivatives **3** and **4**.

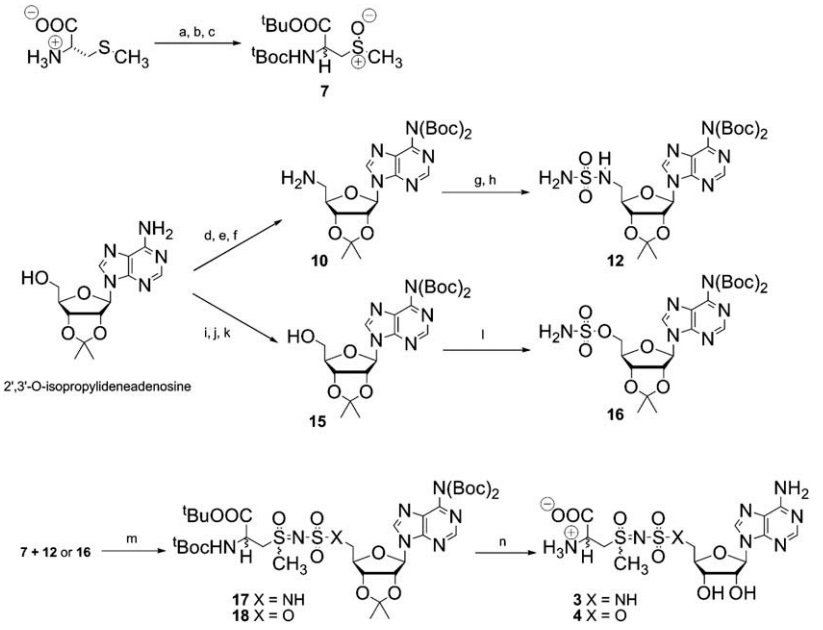
adenylate intermediate in the synthetase site of the enzyme (Scheme 1). Despite having a high affinity for human ASNS, the adenylated sulfoximine **1** must be incubated with ASNase-sensitive cells at 100–1000 μM concentration before it can exert its biological effects.[15] Although a number of factors may be responsible for the need to use high concentrations of sulfoximine to see its biological effects, our working hypothesis is that the ionized groups in **1** preclude its facile entry into cells. The preparation of modified 'pro-drug' variants of **1** containing phosphate protecting groups that are labile within cells represents one solution to this problem.[18] The modification of the relatively difficult synthetic sequence used to obtain **1** to prepare suitably functionalized analogs seemed a less attractive prospect to the development of novel compounds containing more drug-like functional groups that might substitute for the phosphate group.[19] Moreover, we anticipated that the preparation of such structures would permit the use of simpler synthetic transformations than those used in the original synthesis of **1**.[16] In previous work,[20] we had shown that replacing the phosphate by a sulfamate group gave an acyl-adenylate analog **2** (Fig. 1) that inhibited hASNS with micromolar potency, and limited structure–function studies showed the importance of the amino and carboxylate groups in recognition and binding to the synthetase active site. We therefore decided to prepare the functionalized sulfoximine **3** (Fig. 1) to determine the effects on hASNS inhibition. This target, in which an amine connects the sulfur to the ribose, was selected in preference to the related adenosylsulfamate derivative **4** because cyclization to the corresponding cycloadenosine can often occur due to the leaving group ability of the sulfamate moiety.[21,22]

In this paper we now report the synthesis of the adenylated sulfoximines **3** and **4**, and their ability to inhibit hASNS relative to sulfoximine **1**. Somewhat unexpectedly in light of our previous work with acylsulfamate **2**, the ability of **3** to inhibit the enzyme is reduced by several orders of magnitude while **4** is not a hASNS inhibitor. These results suggest that the negative charge on the phosphate is an essential recognition element in the adenylated sulfoximine **1**, which is consistent with computational models.

2. Chemistry

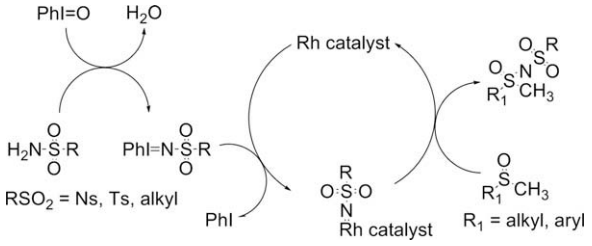
Although the original synthetic route to **1** was relatively efficient, proceeding in good overall yield from *S*-methylcysteine,[16] the construction of the sulfoximine functional group was accomplished using *O*-mesitylsulfonylhydroxylamine (MSH) or sodium azide as nitrogen donors, which are both potentially dangerous iminating agents when employed on a large scale.[23–25] We were therefore interested in exploring alternate strategies for the preparation of these highly functionalized molecules. In addition, we required an approach that might be amenable to making large libraries of functionalized sulfoximines for use in high-throughout screening assays, that is, one that was capable of yielding the desired sulfoximine moiety from simple, readily available precursors.

We therefore decided to exploit a recent method for coupling sulfoxides with sulfamides in the presence of iodosylbenzene and either a rhodium[26] or iron catalyst.[27] Our synthesis therefore began with (i) commercially available 2′,3′-*O*-isopropylideneadenosine,[28] and (ii) the protected sulfoxide **7**, which was prepared as a mixture of four diastereoisomers from *S*-methyl-L-cysteine (Scheme 2).[16] Although we expected to obtain a mixture of epimers at the sulfur center when the thioether was oxidized to the sulfoxide, complete racemization at C_α occurred under the conditions used to introduce the *t*-butyl ester. The desired sulfoxide **7** was therefore obtained as a mixture of four diastereoisomers, which was used without further purification in subsequent coupling steps. 2′,3′-*O*-Isopropylideneadenosine was converted in three steps to protected adenosylamine **10**, which was then reacted with *Z*-sulfamoyl chloride. Catalytic hydrogenation to remove the benzyl protecting group from the resulting adduct gave the desired adenosylsulfamide derivative **12** in 59% overall yield from 2′,3′-*O*-isopropylideneadenosine (Scheme 2). Attempts to couple intermediates **7** and **12** using published conditions, that is, using $Rh_2(OAc)_4$ as a catalyst with 1,4-dioxane as solvent, gave the protected sulfoximine **17** in only 4% yield (Table 1). Moreover, these reagents failed to yield any of the desired sulfoximine when $Fe(acac)_3$ was used as



Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, NaOH, Et_2O–H_2O, rt, 100%; (b) DCC, DMAP, ^tBuOH, CH_2Cl_2, 0 °C to rt, 90%; (c) $NaIO_4$, THF–H_2O, rt, 93%; (d) diphenylphosphoryl azide, DBU, 1,4-dioxane, rt, then NaN_3, 15-crown-5, 100 °C, 91%; (e) $(Boc)_2O$, DMAP, Et_3N, DMF, 0 °C to rt, 82%; (f) H_2, Pd/C, MeOH–H_2O, rt, 100%; (g) *Z*-sulfamoyl chloride, Et_3N, 0 °C to rt, 82%; (h) H_2, Pd/C, MeOH–H_2O, rt, 88%; (i) TBDMS-Cl, imidazole, 0 °C to rt, 100%; (j) $(Boc)_2O$, DMAP, Et_3N, DMF, 0 °C to rt, 91%; (k) TBAF, THF, rt, 100%; (l) H_2NSO_2Cl, Et_3N, DMF, 0 °C to rt, 87%; (m) iodosylbenzene, $Rh_2(esp)_2$, 4 Å molecular sieves, CH_3CN, 36 °C, 75% (X = NH) or 85% (X = O); (n) 5:4:1 TFA–CH_2Cl_2–H_2O rt, 74% (X = NH) or 71% (X = O).

the catalyst. By considering the details of the Rh-catalyzed imination mechanism (Scheme 3), we were able to identify several modifications to our initial coupling conditions that raised the yield of the target compound to 75% (Table 1). First, $Rh_2(esp)_2$ was used in place of $Rh_2(OAc)_4$ because the steric bulk of the α,α,α′,α′-tetramethyl-1,3-benzenedipropionate ligands would prevent coordination of the rhodium center (with concomitant inactivation of the catalyst) by nitrogen atoms in the adenine ring. Another important problem was rhodium-catalyzed intramolecular C–H bond amination of both sulfamide **12** and sulfamate **16** (Scheme 4),[29] which competed with intermolecular imination. The solution was to employ an excess of the sulfoxide component **7** in order to increase the rate of sulfoximine formation. The overall rate of coupling was also accelerated by the addition of 4 Å molecular sieves to remove water generated from iodosylbenzene, and increasing the reaction temperature gave improved yields of the sulfoximine targets.



Scheme 3. Schematic overview of the rhodium-catalyzed imination reaction.

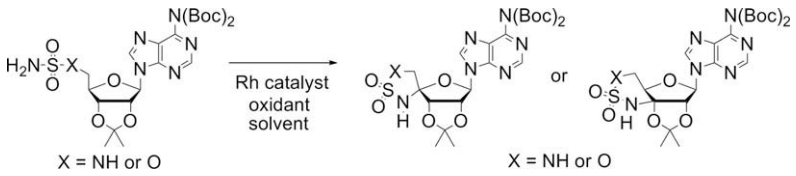
3. Enzyme assays

Having obtained the adenylated sulfoximine **3** (Fig. 1), we investigated its ability to inhibit human ASNS using well-established steady-state kinetic protocols.[17,30,31] The recombinant enzyme was obtained by expression in *Sf9* insect cells, and purified by metal-affinity chromatography, as reported previously.[32] The effect of sulfoximine **3** (as a mixture of four diastereoisomers) on glutamine-dependent asparagine synthesis was assayed by measuring the rate of inorganic pyrophosphate (PP_i) production.[33] Somewhat unexpectedly, these experiments showed that **3** was much less effective at inhibiting human ASNS than the adenylated sulfoximine **1**, even though it still exhibited slow-onset kinetics (Fig. 2).[34] Similar behavior was observed when ammonia was used in place of L-glutamine as the nitrogen source (data not shown). After establishing that **3** did not inhibit the coupling enzymes used

Table 1
Yield of adenylated sulfoximine **17** upon coupling reaction conditions

7 (equiv)	**12** (equiv)	Catalyst (20 mol %)[a]	Solvent	Temp (°C)	4 Å Sieves[b]	Yield (%)
1	1.5	$Fe(acac)_3$	CH_3CN	25	–	ND[c]
1	1.5	$Rh_2(OAc)_4$	1,4-Dioxane	25	–	4
1	1.5	$Rh_2(OAc)_4$	CH_3CN	25	–	14
1	1.5	$Rh_2(esp)_2$	CH_3CN	25	–	20
5	1	$Rh_2(esp)_2$	Dry CH_3CN	25	–	40
5	1	$Rh_2(esp)_2$	Dry CH_3CN	25	+	50
3	1	$Rh_2(esp)_2$	Dry CH_3CN	36	+	75

[a] Esp—α,α,α′,α′-tetramethyl-1,3-benzenedipropionic acid.
[b] Indicates whether sieves were present (+) or absent (–) in the reaction mixture.
[c] Not determined.



Scheme 4. By-products formed by the rhodium-catalyzed, intramolecular C–H insertion reaction.

to detect PP_i (data not shown), the observed kinetic behavior was analyzed using a standard kinetic model in which it is assumed that the inhibitor binds to the free enzyme, as demonstrated for the structurally related sulfoximine **1**.[16] When glutamine was employed as the nitrogen source, curve fitting gave values of 20 ± 10 μM and 10 ± 6 μM for K_I and K_I^*, respectively. As discussed elsewhere,[16,35,36] there is considerable evidence for a strong stereochemical dependence of tight-binding inhibition in the case of functionalized sulfoximines. For example, only a single enantiomer of a given sulfoximine derivative exhibits potent inhibitor activity in experiments where diastereoisomeric and/or enantiomeric mixtures of sulfoximines have been separated.[37,38] Additional support for this assumption is provided by experimental[39,40] and computational studies[41] showing that hydrogen bonding interactions stabilize a single epimer of the tetrahedral intermediate formed when nucleophiles react with activated carbonyl groups. Assuming that only one of the four diastereoisomers of **3** is active, then the K_I^* value demonstrates that the sulfamide derivative is three orders of magnitude less effective an inhibitor of human ASNS than the adenylated sulfoximine **1**. No chromatographic conditions were identified under which the diastereoisomers of the adenylated sulfoximines **3**, or **17** could be separated, and although it was possible to separate the diastereoisomers of sulfoxide derivative **7**, we decided to examine the ability of other sulfoximine analogs to inhibit human ASNS in light of the substantial decrease in the K_I^* value for **3** relative to that of **1**. Thus, given that there are numerous examples of potent inhibitors in which a phosphate moiety has been replaced by either sulfamide or sulfamate functional groups,[42–44] including acylsulfonamide **2**,[20] we prepared and assayed the sulfamate analog **4** from the protected sulfamate **16** (Scheme 2). To our surprise, kinetic experiments showed that the sulfamate **4** had essentially no effect on either the glutamine- or ammonia-dependent synthetase activity of human ASNS at concentrations up to 1 mM, at least on the basis of PP_i production. Control experiments were also undertaken, which established that neither **3** nor **4** perturbed the 1:1 Asn:PPi ratio by stimulating ATP hydrolysis (data not shown).

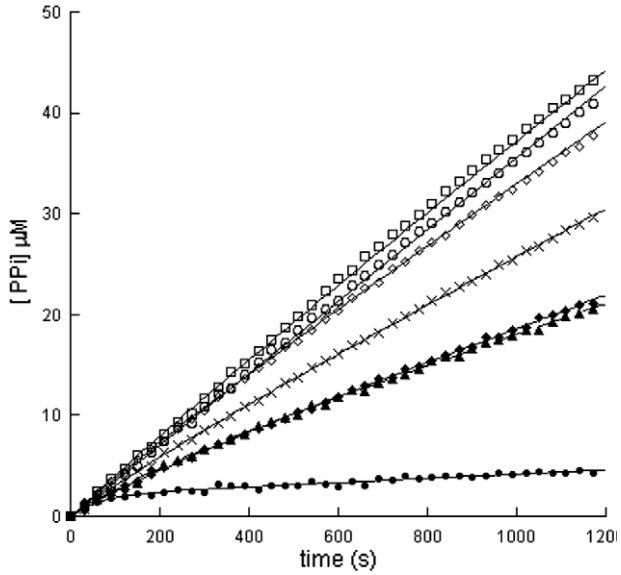


Figure 2. Glutamine-dependent production of PP_i in the presence of increasing amounts of the functionalized sulfoximine **3**: open circles, 0 μM; open squares, 10 μM; open diamonds, 50 μM; crosses, 100 μM; closed diamonds, 500 μM; closed triangles, 1 mM; closed circles, 10 μM N-adenylated sulfoximine **1**. Solid lines represent the best fit lines using Eq. 1 (see Section 5).

Assuming that diastereoisomers of compounds **1** and **3** having the same relative stereochemistry bind in the same fashion to the synthetase site of ASNS, these observations appear consistent with a model in which the negative charge on the phosphate group of the acyl-adenylate intermediate is an essential element in ligand recognition by the synthetase site of human ASNS. There are several reasons, in addition to those discussed above, that support the idea that the active forms of **1** and **3** bind in similar, if not identical, orientations. For example, it seems certain that the adenosyl moieties of **1** and **3** bind within the ATP pocket, which is very tightly defined in the AS-B crystal structure because of the need to form hydrogen bonds between the purine and the backbone amides of a conserved valine, thereby ensuring that the enzyme uses ATP rather than GTP as a substrate.[45] Prior work has also shown that ASNS binds the amino and carboxylate groups of aspartate in order to recognize this substrate with high specificity.[46] If the negative charge on the phosphate group of the acyl-adenylate intermediate is essential for ligand recognition, then the adenylated sulfoximine **1** can bind to the enzyme with high affinity because it contains an ionized phosphoramidate moiety. The acylsulfamate and sulfamide derivatives **2** and **3**, can only form this electrostatic interaction within the synthetase site, however, if they bind as their negatively charged conjugate bases (Fig. 3). As a result, if the pK_a of the acylated sulfamate NH in **2** is lower than that of the sulfamide NH in **3**, the former compound would be expected to be a more potent human ASNS inhibitor. That this is the case seems evident from qualitative considerations of resonance stabilization of the conjugate bases of **2** and **3**, but the likely differences in pK_a can be placed on a more quantitative footing through recent studies of sulfamate inhibitors for the adenylating enzyme MtbA,[47] which is present in *Mycobacterium tuberculosis*.[48] Thus, the pK_a of the *N*-benzoyl derivative **19** (Fig. 5) is reported to be 2.8,[47] suggesting that the pK_a of the *N*-acylsulfamate **2** is probably in the range of 4–5 as the carbonyl group of the latter compound is less electron-

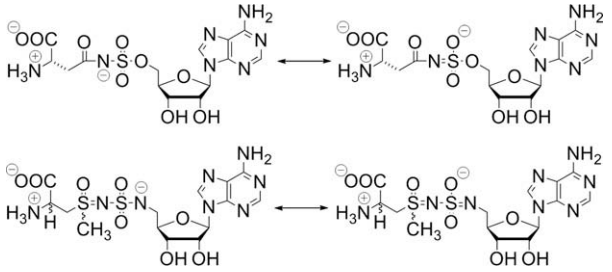


Figure 3. Conjugate bases of the functionalized acylsulfamate **2** (top) and sulfamide **3** (bottom).

deficient. Similarly, the NH pK_a of the electron-deficient, functionalized sulfamate **3** is probably in the range of 9–10 given that those of sulfanilamide and methanesulfonamide are 10.43 and 10.87. Finally, our model also explains the inability of functionalized sulfoximine **4** to exhibit significant inhibitory activity against human ASNS. Hence, the absence of suitably positioned ionizable groups in **4** meaning that this compound can only interact with the enzyme in its neutral form at the solution pH values employed in our assays.

Our hypothesis is reinforced by considering a computational model in which the acyl-adenylate intermediate (Scheme 1) is bound within the synthetase site of *Escherichia coli* AS-B (Fig. 4). Although this model was built from the crystal structure of AS-B (Ding, unpublished results), which is an ortholog of human ASNS, all residues within the active site are strictly conserved in the bacterial and human enzymes. In this model, the phosphate moiety interacts with the side chain of Lys-449 (AS-B numbering), which corresponds to Lys-466 of the human enzyme. This residue is invariant in known glutamine-dependent asparagine synthetases, suggesting that this electrostatic interaction is critical for ATP binding and/or catalysis. The importance of this active site lysine is underscored by the fact that efforts to obtain site-directed AS-B mutants at this position have only given enzymes lacking any synthetase activity (Meyer, unpublished data). Thus, we conclude that potent ASNS inhibitors must possess functional groups that can mimic the negative charge of the phosphate group when bound within the synthetase site so as to maintain this interaction with the lysine side chain. This is an important finding for (i) the design of sulfoximine-based libraries of small molecules with which to explore the extent to which the adenylate moiety can be replaced by other heterocycles, and (ii) the development of ASNS inhibitors that can easily be taken up into leukemia cells.

4. Conclusions

Conditions have been developed that will permit the facile construction of highly functionalized sulfoximines from readily available sulfoxide and sulfamide precursors. Such compounds are proven inhibitors of several biologically important classes of enzyme,[36] including glutathione synthetase,[49] γ-glutamylcysteine synthetase,[50] γ-glutamyl transpeptidase,[51] and asparagine synthetase.[15,16] This protocol also enables the creation of sulfoximine libraries that can be screened for ASNS inhibition using high-throughput methods and optimized by rational discovery strategies.[52] Perhaps more importantly, we conclude that potent ASNS inhibitors must possess functionality that can mimic the negative charge of the phosphate group so as to form a favorable electrostatic interaction with the conserved lysine when bound within the synthetase site. On this point, we note that previous work using modified acylsulfamates has already shown that similar electrostatic interactions involving the charged amino and carboxylate moieties of aspartic acid contribute a considerable amount of binding energy.[20] The importance of electrostatics in inhibitor recognition is therefore established, which is significant finding for the development of highly cell-permeable ASNS inhibitors that can be used clinically in the treatment of leukemia and ovarian cancer.[53,54]

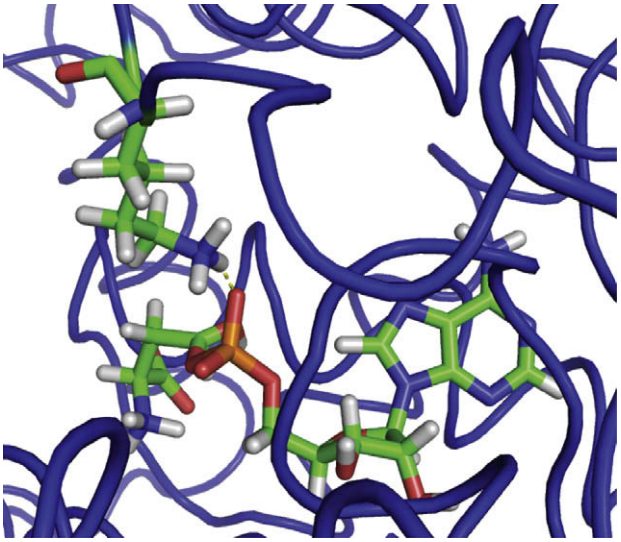
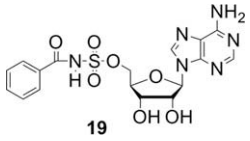


Figure 4. The interaction between the Lys-449 side chain and the phosphate group of the acyl-adenylate intermediate (both shown in 'stick' representations) in a computational model of the AS-B/Gln/acyl-adenylate complex (Ding, unpublished). The protein backbone is shown by the blue ribbon. Color scheme: C, green; H, white; N, blue; O, red; P, orange. This image was generated in PYMOL.[65]



19

Figure 5. Structure of *N*-benzoylsulfamate **19** for which the pK_a of the sulfamate NH proton has been established.[47]

5. Experimental

5.1. General

All chemicals were obtained commercially and used without further purification unless otherwise stated. *N*,*N*-dimethylformamide (DMF), 1,4-dioxane, acetonitrile and CH_2Cl_2, triethylamine (Et_3N) were purchased from Wako Pure Chemical Industries (Osaka, Japan), dried by distillation from CaH_2 and stored over 4 Å molecular sieves. Melting points (mp) were recorded using a Mettler FP62 melting point apparatus and are corrected. Optical rotations were measured using a Horiba automatic polarimeter SEPA-200. ^{1}H NMR spectra were recorded on a JEOL JNM-AL 300 at 300 MHz. In $CDCl_3$, DMSO-d_6 and acetone-d_6, ^{1}H chemical shifts are reported in ppm (δ) downfield of tetramethylsilane as an internal reference (δ 0.0), except for measurements in D_2O for which sodium 3-(trimethylsilyl)propanesulfonate was used as an internal standard (δ 0.0). Splitting patterns are abbreviated as follows: s, singlet, d, doublet, t, triplet, q, quartet, and m, multiplet. Infrared spectra were determined using a Horiba FT-720 spectrophotometer, and mass spectra were recorded on a JEOL JMS 700 (high resolution) spectrometer. Elemental analyses were performed on a Yanaco MT-5 apparatus. Reactions were monitored with analytical thin layer chromatography (TLC) on Silica Gel 60-F_{254} plates (Merck). Compounds were purified by flash column chromatography on silica gel 60 N (Kanto Kagaku, 40–50 μm), or by medium-pressure, reverse-phase column chromatography on Diaion HP20SS resin (Mitsubishi Chemical Corporation) using a Yamazen YFLC System (Yamazen Co., Osaka, Japan).

5.1.1. 2-(*N*-*tert*-Butoxycarbonyl)amino-*S*-methylcysteine (5)

S-Methyl-L-cysteine (5.1 g, 37.7 mmol) was protected by a known procedure (Boc_2O, NaOH, *tert*-BuOH-H_2O) to give partially racemized **5** as a colorless oil (8.8 g, quant.): $[\alpha]_D^{17}$ −14.6 (*c* 1.05, AcOH) [lit.[55] −24.6 (*c* 0.705, AcOH)]; IR (NaCl, neat) ν_{max} 3600–3318 (br), 2978, 2925, 1720, 1693, 1666, 1511, 1394, 1369, 1336, 1299, 1249, 1164, 1056, 1025, 960, 914, 860, 777, 744, 669, 572,

478, 443 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.46 [s, 9H, $(CH_3)_3CO$-CON], 2.16 (s, 3H, SCH_3), 2.94–3.10 (m, 2H, CH_2S), 4.57 (m, 1H, α-proton), 5.42 (br d, J = 6.9 Hz, 1H, NH), 8.53 (br s, 1H, COOH); Anal. Calcd for $C_9H_{17}NO_4S$: C, 45.94; H, 7.28; N, 5.95. Found: C, 45.82; H, 7.43; N, 5.81. HRMS (FAB, glycerol) calcd for $C_9H_{18}NO_4S$ (MH^+) 236.0957, found 236.0954.

5.1.2. *tert*-Butyl 2-(*N*-*tert*-butoxycarbonyl)amino-3-methylsulfanylpropanoate (6)

A mixture of 2-(*N*-*tert*-butoxycarbonyl)amino-*S*-methylcysteine (**5**) (8.88 g, 37.7 mmol), 4-(*N*,*N*-dimethylamino)pyridine (1.38 g, 11.3 mmol), and 2-methyl-2-propanol (3.36 g, 45.3 mmol) in dry CH_2Cl_2 (50 mL) was cooled at 0 °C under an Ar atmosphere. *N*,*N*′-Dicyclohexylcarbodiimide (8.72 g, 42.3 mmol) was added to the solution, and the resulting reaction mixture was stirred initially at 0 °C for 2 h and then overnight at room temperature. After filtration to remove dicyclohexylurea, the filtrate was evaporated and the residue dissolved in EtOAc (100 mL). This solution was filtrated again, and the filtrate washed successively with 5% aq $KHSO_4$ (30 mL), water (30 mL) and satd NaCl (30 mL). After drying over Na_2SO_4, filtration and evaporation of the solvent under reduced pressure gave an oil that was purified by flash column chromatography on silica gel (EtOAc/hexane, 1:5) to afford the racemic sulfide **6** as a colorless oil (9.9 g, 90%): IR (NaCl, neat) ν_{max} 3372, 2977, 2931, 1716, 1498, 1455, 1367, 1309, 1249, 1222, 1155, 1054, 1010, 943, 846, 777, 669, 632, 557, 503, 464, 435 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.45 [s, 9H, $(CH_3)_3COCON$], 1.48 [s, 9H, $(CH_3)_3CO$], 2.15 (s, 3H, SCH_3), 2.89 and 2.92 (2 × dd, 2H, J = 5.4 and 13.1 Hz, CH_2S), 4.41 (m, 1H, α-proton), 5.34 (br d, J = 7.2 Hz, 1H, NH); Anal. Calcd for $C_{13}H_{25}NO_4S$: C, 53.58; H, 8.65; N, 4.81. Found: C, 53.48; H, 8.58; N, 4.94. HRMS (FAB, glycerol) calcd for $C_{13}H_{26}NO_4S$ (MH^+) 292.1588, found 292.1583.

5.1.3. (*R*,*S*)-*tert*-Butyl 2-(*N*-*tert*-butoxycarbonyl)amino-3-(*R*,*S*)-methylsulfinylpropanoate (7)

The sulfide **6** (10 g, 34.3 mmol) was dissolved in THF–H_2O (5:2, 70 mL) and was cooled at 0 °C before the addition of an aqueous solution (30 mL) of $NaIO_4$ (8.8 g, 41.1 mmol). The mixture was stirred at ambient temperature overnight and then filtered. After removal of THF under reduced pressure, EtOAc (100 mL) was added to the resulting aqueous solution, which was then washed successively with water (30 mL) and sat. NaCl (30 mL). After drying over Na_2SO_4 and filtration, the filtrate was evaporated to give the sulfoxide **7** as a 5:4 mixture of two diastereomers (9.79 g, 93%, colorless oil): IR (NaCl, neat) ν_{max} 3400–3200 (br), 2979, 2933, 2815, 1716, 1523, 1455, 1392, 1367, 1278, 1251, 1155, 1049, 1024, 971, 943, 846, 813, 750, 686, 576, 565, 497, 458, 431 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ_H 1.39 and 1.40 [2 × s, 9H, $(CH_3)_3CO$-CON], 1.405 and 1.413 [2 × s, 9H, $(CH_3)_3CO$], 2.577 and 2.584 (2 × s, 3H, SCH_3), 2.93 (dd, J = 7.8 and 13.2 Hz), 2.98 (m) and 3.19 (dd, J = 5.9 and 13.1 Hz) (2H, $CH_2S{=}O$), 4.15 (dt, J = 5.7 and 8.7 Hz) and 4.23 (dt, J = 6.0 and 7.8 Hz) (1H, α-proton), 7.37 and 7.46 (2 × br d, J = 8.1 Hz, 1H, NH); Anal. Calcd for $C_{13}H_{25}NO_5S$: C, 50.79; H, 8.20; N, 4.56. Found: C, 50.57; H, 8.28; N, 4.48. HRMS (FAB, glycerol) calcd for $C_{13}H_{26}NO_5S$ (MH^+) 308.1533, found 308.1532.

5.1.4. 5′-Azido-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (8)

2′,3′-*O*-Isopropylideneadenosine (5.0 g, 16.3 mmol) was suspended in dry 1,4-dioxane (50 mL) at room temperature under an Ar atmosphere. Diphenylphosphoryl azide (9.45 g, 7.4 mL, 32.5 mmol) and DBU (7.64 g, 7.5 mL, 48.8 mmol) were added dropwise to the suspension, and the reaction mixture stirred at ambient temperature for 4 h. Sodium azide (5.29 g, 81.4 mmol) and 15-crown-5 (0.359 g, 0.323 mL, 1.63 mmol) were added, and the mixture heated at 110 °C for 4 h under an Ar atmosphere. Filtration and evaporation of the solvent gave a residue that was re-dissolved in $CHCl_3$ (150 mL). The $CHCl_3$ solution was washed successively with water (50 mL) and sat. NaCl (50 mL), and then dried over Na_2SO_4. After filtration, and $CHCl_3$ was evaporated to give a brown oil, which was purified by flash column chromatography on silica gel (EtOH/$CHCl_3$, 1:9) to afford the azide **8** as an amorphous, yellow solid that could be used in subsequent steps without further purification (5.4 g, 100%): IR (KBr) ν_{max} 3309, 3154, 3133, 2989, 2935, 2105 (N_3), 1675, 1646, 1600, 1473, 1417, 1375, 1328, 1301, 1249, 1213, 1155, 1118, 1078, 1010, 931, 869, 798, 715, 690, 649, 595, 538, 511, 412 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.40 (s, 3H, isopropylidene), 1.62 (s, 3H, isopropylidene), 3.56 (dd, J = 5.4 and 12.9 Hz, 1H) and 3.61 (dd, J = 6.3 and 12.9 Hz, 1H) (5′-CH_2), 4.39 (dt, J = 3.6 and 5.9 Hz, 1H, 4′-H), 5.07 (dd, J = 3.6 and 6.3 Hz, 1H, 3′-H), 5.47 (dd, J = 2.4 and 6.3 Hz, 1H, 2′-H), 5.65 (br s, 2H, adenyl NH_2), 6.11 (d, J = 2.4 Hz, 1H, 1′-H), 7.92 (s, 1H, adenyl 2-H), 8.37 (s, 1H, adenyl 8-H); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{13}H_{17}N_8O_3$ (MH^+) 333.1426, found 333.1424.

5.1.5. 5′-Azido-N^6,N^6-bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (9)

4-(*N*,*N*-Dimethylamino)pyridine (349 mg, 2.85 mmol) and dry Et_3N (4.33 g, 5.96 mL, 42.8 mmol) were added to a solution of azide **8** (4.74 g, 14.3 mmol) in dry DMF (100 mL) at 0 °C under an Ar atmosphere. Boc_2O (12.45 g, 57.1 mmol) was then added to this solution, and the mixture stirred at 0 °C for 1 h and at room temperature for 4 h. The reaction mixture was filtered and evaporated to give a residual oil that was purified by flash column chromatography on silica gel (EtOAc/hexane, 1:1). The desired azide **9** was obtained as an amorphous solid (6.24 g, 82%): IR (KBr) ν_{max} 3324, 3170, 2983, 2937, 2103 (N_3), 1789, 1758, 1731, 1600, 1577, 1498, 1454, 1419, 1371, 1336, 1278, 1253, 1211, 1106, 1039, 914, 850, 811, 775, 734, 646, 590, 559. 509, 462, 433 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.41 (s, 3H, isopropylidene), 1.46 [s, 18H, 2 × $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 3.57–3.63 (m, 2H, 5′-CH_2), 4.41 (q, J = 3.6 Hz, 1H, 4′-H), 5.06 (dd, J = 3.6 and 6.3 Hz, 1H, 3′-H), 5.44 (dd, J = 2.4 and 6.3 Hz, 1H, 2′-H), 6.19 (d, J = 2.4 Hz, 1H, 1′-H), 8.21 (s, 1H, adenyl 2-H), 8.89 (s, 1H, adenyl 8-H); Anal. Calcd for $C_{23}H_{32}N_8O_7$: C, 51.87; H, 6.06; N, 21.04. Found: C, 51.75; H, 6.05; N, 20.98. HRMS (FAB, glycerol) calcd for $C_{23}H_{33}N_8O_7$ (MH^+) 533.2475, found 533.2471.

5.1.6. 5′-Amino-N^6,N^6-bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (10)

5′-Azido-N^6,N^6-bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (**9**) (9.4 g, 17.7 mmol) and 10% Pd on carbon (wet, Degussa type E101 NE/W, Aldrich) were suspended in 9:1 MeOH–H_2O (250 mL). Hydrogen gas was passed through the mixture at ambient temperature for 5 h, and completion of reaction, as monitored by TLC (eluant: $CHCl_3$/MeOH, 10:1), the mixture was filtered through Hyflo Super-Cel, and the filtrate was evaporated to give crude amine **10** as an amorphous solid (8.94 g), which was contaminated with approximately 10% of 5′-*N*-Boc-N^6-Boc-adenosine. This material **10** was used in the next step without further purification (8.94 g, 100%): ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.40 (s, 3H, isopropylidene), 1.47 [s, 18H, 2 × $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 2.92–3.08 (m, 2H, 5′-CH_2), 4.26–4.30 (m, 1H, 4′-H), 5.02 (dd, J = 3.6 and 6.6 Hz, 1H, 3′-H), 5.44 (dd, J = 3.0 and 6.6 Hz, 1H, 2′-H), 6.13 (d, J = 3.0 Hz, 1H, 1′-H), 8.23 (s, 1H, adenyl 2-H), 8.87 (s, 1H, adenyl 8-H); HRMS (FAB, *p*-nitrobenzylalcohol) calcd for $C_{23}H_{35}N_6O_7$ (MH^+) 507.2570, found 507.2567.

5.1.7. 5′-[(*N*-Benzyloxycarbonyl)sulfamoylamino]-N^6,N^6-bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylideneadenosine (11)

Benzyl alcohol (1.39 g, 1.33 mL, 12.9 mmol) was added dropwise to a stirred solution of chlorosulfonyl isocyanate (1.82 g,

1.12 mL, 12.9 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C, and the mixture was stirred at 0 °C for 1 h under an Ar atmosphere. After completion of the reaction, as judged by ^{1}HNMR (δ_H 4.615 and 5.319 for PhC*H*$_2$O proton for benzyl alcohol and *N*-benzyloxycarbonylsulfamoyl chloride, respectively), the resulting solution was added via cannula to an ice-cold solution of the crude amine **10** (5.93 g, 11.7 mmol) and dry Et_3N (2.48 g, 3.42 mL, 23.4 mmol) in dry CH_2Cl_2 (60 mL). The mixture was stirred at ambient temperature overnight, before being evaporated under reduced pressure to give a residue that was dissolved in EtOAc (100 mL). This solution was washed successively with 5% $KHSO_4$ (40 mL), water (30 mL) and sat. NaCl (30 mL). After drying over Na_2SO_4 and filtration, evaporation of the solvent gave an amorphous solid, which was purified by flash column chromatography on silica gel (EtOAc/hexane, 3:2) to afford **11** as an amorphous solid (6.94 g, 82%): ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 1.37 (s, 3H, isopropylidene), 1.48 [s, 18H, 2 × $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 3.41–3.57 (m, 2H, 5′-CH_2), 4.58 (m, 1H, 4′-H), 5.03 (d, *J* = 12 Hz, 1H, PhC*H*$_2$O), 5.05 (dd, *J* = 1.5 and 6.3 Hz, 1H, 3′-H), 5.14 (d, *J* = 12 Hz, 1H, PhC*H*$_2$O), 5.23 (dd, *J* = 4.8 and 6.3 Hz, 1H, 2′-H), 5.86 (d, *J* = 4.8 Hz, 1H, 1′-H), 7,28–7.36 (m, 6H, Ph and SO_2NHCH_2), 8.06 (s, 1H, adenyl 2-H), 9.06 (s, 1H, adenyl 8-H), 9.28 (d, *J* = 9.6 Hz, 1H, $CONHSO_2$); Anal. Calcd for $C_{31}H_{41}N_7O_{11}S$: C, 51.73; H, 5.74; N, 13.62. Found: C, 51.70; H, 5.84; N, 13.56. HRMS (FAB, glycerol) calcd for $C_{31}H_{42}N_7O_{11}S$ (MH^+) 720.2666, found 720.2654.

5.1.8. N^6,N^6-Bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylidene-5′-sulfamoylamino adenosine (12)

The protected sulfamide **11** (6.43 g, 8.94 mmol) and 10% Pd on carbon (wet, Degussa type E101 NE/W, Aldrich) were suspended in 9:1 MeOH–H_2O (250 mL). Hydrogen gas was passed through the mixture at ambient temperature for 2 h, and on completion of the reaction, as monitored by TLC (eluant: EtOAc/hexane, 3:2), the mixture was filtered through Hyflo Super-Cel, and the filtrate evaporated to give crude **12** as an amorphous solid. The crude product was purified by flash column chromatography on silica gel (EtOAc/hexane, 2:1) to afford sulfamide **12** as an amorphous solid (4.59 g, 88%): IR (KBr) ν_{max} 1798, 1758, 1735, 1602, 1581, 1500, 1455, 1421, 1371, 1336, 1276, 1253, 1214, 1159, 1106, 983, 941, 852, 775, 696, 644, 590, 549, 464 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 1.38 (s, 3H, isopropylidene), 1.48 [s, 18H, 2 × $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 3.50 (dd, *J* = 3.0 and 5.9 Hz, 2H, 5′-CH_2), 4.55 (br s, 1H, SO_2NH_2), 4.58 (m, 1H, 4′-H), 5.13 (dd, *J* = 2.4 and 6.5 Hz, 1H, 3′-H), 5.34 (dd, *J* = 4.8 and 6.3 Hz, 1H, 2′-H), 5.90 (d, *J* = 4.5 Hz, 1H, 1′-H), 7.74 (br t, *J* = 5.7 and 5.6 Hz, 1H, SO_2NHCH_2), 8.11 (s, 1H, adenyl 2-H), 8.96 (s, 1H, adenyl 8-H); Anal. Calcd for $C_{23}H_{35}N_7O_9S$·0.3 EtOAc: C, 47.49; H, 6.15; N, 16.01. Found: C, 47.29; H, 5.97; N, 16.01. HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{23}H_{36}N_7O_9S$ (MH^+) 586.2297, found 586.2295.

5.1.9. 5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylideneadenosine (13)

Imidazole (2.83 g, 40.7 mmol) and *tert*-butyldimethylsilyl chloride (2.95 g, 19.5 mmol) were added to a solution of 2′,3′-*O*-isopropylideneadenosine (5.00 g, 16.3 mmol) in dry DMF (50 mL) at 0 °C under an Ar atmosphere. The mixture was stirred at 0 °C for 2 h and then at room temperature overnight before being filtered and evaporated under a high vacuum (1 mmHg). 2:1 EtOAc/H_2O (300 mL) was added to the resulting residue. The organic layer was separated, washed successively with water (100 mL) and satd NaCl (50 mL), and dried over Na_2SO_4. Filtration and evaporation of the solvent under reduced pressure yielded **13** as a colorless solid (6.81 g, 100%): IR (KBr) ν_{max} 3363, 3273, 3120, 3050, 2985, 2935, 2857, 2749, 2682, 1918, 1679, 1646, 1604, 1571, 1508, 1473, 1415, 1373, 1330, 1303, 1255, 1207, 1132, 1081, 1008, 964, 906, 860, 838, 773, 717, 659, 638, 592, 541, 511 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 0.014 (s, 3H, CH_3Si), 0.02 (s, 3H, CH_3Si), 0.845 (s, 9H, $(CH_3)_3CSi$), 1.41 (s, 3H, isopropylidene), 1.64 (s, 3H, isopropylidene), 3.76 (dd, *J* = 4.2 and 11.1 Hz, 1H, 5′-CH_2), 3.88 (dd, *J* = 3.9 and 11.1 Hz, 1H, 5′-CH_2), 4.26 (q, *J* = 2.4 Hz, 1H, 4′-H), 4.96 (dd, *J* = 2.4 and 6.0 Hz, 1H, 3′-H), 5.28 (dd, *J* = 2.4 and 6.0 Hz, 1H, 2′-H), 5.50 (br s, 2H, adenyl NH_2), 6.17 (d, *J* = 2.4 Hz, 1H, 1′-H), 8.05 (s, 1H, adenyl 2-H), 8.39 (s, 1H, adenyl 8-H); Anal. Calcd for $C_{19}H_{31}N_5O_4Si$: C, 54.13; H, 7.41; N, 16.01. Found: C, 54.04; H, 7.51; N, 16.21. HRMS (FAB, glycerol) calcd for $C_{19}H_{32}N_5O_4Si$ (MH^+) 422.2225, found 422.2216.

5.1.10. N^6,N^6-Bis(*tert*-butoxycarbonyl)-5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylidene-adenosine (14)

5′-*O*-*tert*-Butyldimethylsilyl-2′,3′-*O*-isopropylideneadenosine (**13**) (7 g, 16.6 mmol) was dissolved in dry DMF (150 mL) together with 4-(*N*,*N*-dimethylamino)pyridine (0.41 g, 3.49 mmol) and dry Et_3N (3.53 g, 4.86 mL, 34.9 mmol) under an Ar atmosphere. Boc_2O (7.61 g, 34.9 mmol) was added to this solution at 0 °C, and the mixture was stirred initially at 0 °C for 1 h and then at room temperature for 3 h. The reaction mixture was filtered and evaporated under a high vacuum (1 mmHg) to give an oily residue, which could be purified by flash column chromatography on silica gel (EtOAc/hexane, 1:2) to give **14** as a colorless solid (9.4 g, 91%): ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 0.031 [s, 6H, $(CH_3)_2Si$], 0.86 [s, 9H, $(CH_3)_3CSi$], 1.42 (s, 3H, isopropylidene), 1.44 (s, 18H, 2 × $(CH_3)_3$-CO), 1.66 (s, 3H, isopropylidene), 3.79 (dd, *J* = 3.9 and 11.1 Hz, 1H, 5′-CH_2), 3.90 (dd, *J* = 3.6 and 11.4 Hz, 1H, 5′-CH_2), 4.46 (q, *J* = 3.2 Hz, 1H, 4′-H), 4.96 (dd, *J* = 2.7 and 6.0 Hz, 1H, 3′-H), 5.23 (dd, *J* = 2.7 and 6.0 Hz, 1H, 2′-H), 6.26 (d, *J* = 2.4 Hz, 1H, 1′-H), 8.35 (s, 1H, adenyl 2-H), 8.89 (s, 1H, adenyl 8-H); Anal. Calcd for $C_{29}H_{47}N_5O_8Si$: C, 56.02; H, 7.62; N, 11.26. Found: C, 56.22; H, 7.61; N, 11.21. HRMS (FAB, glycerol) calcd for $C_{29}H_{48}N_5O_8Si$ (MH^+) 622.3266, found 622.3280.

5.1.11. N^6,N^6-Bis(*tert*-butoxycarbonyl)-2′,3′-*O*-isopropylideneadenosine (15)

N^6,N^6-Bis(*tert*-butoxycarbonyl)-5′-*O*-*tert*-butyldimethylsilyl-2′,3′-*O*-isopropylideneadenosine (**14**) (9.00 g, 14.5 mmol) was dissolved in dry THF (100 mL), and tetrabutylammonium fluoride (TBAF, 1.0 M solution in THF, 21 mL, 21.7 mmol) added to the solution at room temperature under an Ar atmosphere. The mixture was stirred for 3 h at room temperature before being evaporated to give a colorless oil, which was purified by flash column chromatography on silica gel (acetone/hexane, 1:2) to give **15** as an amorphous solid (7.33 g, 100%): IR (KBr) ν_{max} 3509–3369 (br), 3116, 3089, 2983, 2937, 2881, 1789, 1758, 1735, 1602, 1579, 1498, 1455, 1419, 1371, 1336, 1276, 1253, 1213, 1141, 1110, 1083, 950, 850, 775, 646, 588, 559, 511, 462 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 1.39 (s, 3H, isopropylidene), 1.47 [s, 18H, 2 × $(CH_3)_3CO$], 1.66 (s, 3H, isopropylidene), 3.80 (dd, *J* = 1.8 and 11.1 Hz), 3.84 (dd, *J* = 2.1 and 11.4 Hz) and 4.00 (dt, *J* = 1.5 and 12.9 Hz) (2H, 5′-CH_2), 4.56 (s, 1H, OH), 5.13 (dd, *J* = 1.2 and 6.0 Hz, 1H, 4′-H), 5.22 (dd, *J* = 5.1 and 5.4 Hz, 1H, 2′-H), 5.40 (dd, *J* = 2.4 and 11.1 Hz, 1H, 3′-H), 5.94 (d, *J* = 4.8 Hz, 1H, 1′-H), 8.12 (s, 1H, adenyl 2-H), 8.84 (s, 1H, adenyl 8-H); Anal. Calcd for $C_{23}H_{33}N_5O_8$: C, 54.43; H, 6.55; N, 13.80. Found: C, 54.42; H, 6.64; N, 13.51. HRMS (FAB, glycerol) calcd for $C_{23}H_{34}N_5O_8$ (MH^+) 508.2409, found 508.2399.

5.1.12. N^6,N^6-Bis(*tert*-butoxycarbonyl)-2′,3′-*O*-isopropylidene-5′-*O*-sulfamoyladenosine (16)

N^6,N^6-Bis(*tert*-butoxycarbonyl)-2′,3′-*O*-isopropylideneadenosine (**15**) (2.4 g, 4.73 mmol) and dry Et_3N (0.493 g, 0.679 ml, 4.87 mmol) were dissolved in dry DMF (10 mL) and the solution cooled to 0 °C under an Ar atmosphere. Sulfamoyl chloride (**20**)[56] (1.31 g, 11.3 mmol) was added to the solution, and the reaction mixture was stirred at 0 °C for 2 h and then at room temperature overnight.

The reaction mixture was evaporated, and the residue was diluted with EtOAc (50 mL). This solution was then washed successively with water (30 mL) and satd NaCl (30 mL) before being dried over Na_2SO_4. The EtOAc solution was filtered, and the filtrate was evaporated to give sulfamate (**16**) as an amorphous solid that was used without further purification (2.4 g, 87%): IR (KBr) ν_{max} 2981, 2938, 1789, 1731, 1604, 1581, 1496, 1455, 1373, 1338, 1278, 1253, 1213, 1186, 1139, 1110, 998, 850, 809, 775, 646, 593, 553, 514, 466 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.43 (s, 3H, isopropylidene), 1.50 [s, 18H, 2 × $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 4.22 (dd, J = 4.5 and 10.8 Hz, 1H, 5′-CH_2), 4.30 (dd, J = 3.3 and 11.1 Hz, 1H, 5′-CH_2), 4.6–4.7 (m, 1H, 4′-H), 4.83 (br s, 2H, SO_2NH_2), 5.08 (dd, J = 2.1 and 6.0 Hz, 1H, 3′-H), 5.72 (dd, J = 1.5 and 6.0 Hz, 1H, 2′-H), 6.26 (d, J = 1.5 Hz, 1H, 1′-H), 8.26 (s, 1H, adenyl 2-H), 8.90 (s, 1H, adenyl 8-H); HRMS (FAB, glycerol) calcd for $C_{23}H_{35}N_6O_{10}S$ (MH^+) 587.2134, found 587.2124.

5.1.13. (*R,S*)-*tert*-Butyl 2-(*N*-*tert*-butoxycarbonyl)amino-3-(*N*-{[N^6,N^6-bis(*tert*-butoxycarbonyl)-5′-deoxy-2′,3′-*O*-isopropylideneadenosin-5′-yl]aminosulfonyl}-*S*-methyl-(*R,S*)-sulfonimidoyl) propanoate (17)

The protected adenosine (**12**) (1.5 g, 2.56 mmol) and (*R,S*)-*tert*-butyl 2-(*N*-*tert*-butoxycarbonyl)amino-3-(*R,S*)-methylsulfinylpropanoate (**7**) (2.11 g, 6.86 mmol) were dissolved in dry CH_3CN (30 mL) before the addition of 4 Å molecular sieves (1 g), $Rh_2(esp)_2$ (389 mg, 0.512 mmol) and iodosylbenzene (1.18 g, 5.38 mmol). The reaction mixture was stirred overnight at 36 °C under an Ar atmosphere before being filtered and evaporated to yield a brown oil. Purification of this material using flash column chromatography on silica gel (CH_3CN/toluene, 1:4) gave the desired sulfoximine (**17**) as a mixture of diastereomers (1.71 g, 75%, amorphous solid): IR (KBr) ν_{max} 3600–3386 (br), 2981, 2937, 1798, 1727, 1602, 1500, 1455, 1371, 1328, 1276, 1253, 1214 (O═S═N), 1151, 1103, 1079 (S═O), 977, 941 (S═N), 850, 775, 646, 588, 509, 468, 437 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.38 (s, 3H, isopropylidene), 1.43–1.47 [m, 36H, 3 × $(CH_3)_3COCON$ and $(CH_3)_3CO$], 1.64 (s, 3H, isopropylidene), 3.293 and 3.317 and 3.349 (3 × s, 3H, SCH_3), 3.50–3.56 (m, 2H, 5′-CH_2), 3.80–4.30 (m, 2H CH_2S), 4.38–4.53 (m, 1H, α-proton), 4.60 (m, 1H, 4′-H), 5.18–5.20 (m, 1H, 3′-H), 5.29–5.40 (m, 1H, 2′-H), 5.60 (m, 1H, CONH), 5.89 (2 × d, J = 4.2 and 4.1 Hz, 1H, 1′-H), 7.60–7.82 (m, 1H, SO_2NHCH_2), 8.08 (s, 1H, adenyl 2-H), 8.980 and 8.988 (2 × s, 1H, adenyl 8-H); Anal. Calcd for $C_{36}H_{58}N_8O_{14}S_2$: C, 48.53; H, 6.56; N, 12.58. Found: C, 48.64; H, 6.51; N, 12.51. HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{36}H_{59}N_8O_{14}S_2$ (MH^+) 891.3596, found 891.3597.

5.1.14. *tert*-Butyl (*R,S*)-2-(*N*-*tert*-butoxycarbonyl)amino-3-{*N*-[(N^6,N^6-bis(*tert*-butoxycarbonyl)-2′,3′-*O*-isopropylideneadenosin-5′-yl)-5′-*O*-sulfonyl]-*S*-methyl-(*R,S*)-sulfonimidoyl}propanoate (18)

The functionalized sulfamate (**16**) (1.23 g, 2.10 mmol) was dissolved in dry CH_3CN (30 mL) together with the diastereomeric mixture of sulfoxide (**7**) (2.00 g, 6.50 mmol). 4 Å Molecular sieves (1 g), $Rh_2(esp)_2$ (319 mg, 0.42 mmol) and iodosylbenzene (693 mg, 3.2 mmol) were then added, and the resulting mixture stirred overnight at 36 °C under an Ar atmosphere. Filtration and evaporation of the solvent under reduced pressure gave a brown oil, which was purified by flash column chromatography on silica gel (CH_3CN/toluene, 1:3) to give the sulfoximine (**18**) as a mixture of diastereomers (1.59 g, 85%, amorphous solid): IR (KBr) ν_{max} 3415–3384 (br), 2981, 2937, 1789, 1724, 1600, 1575, 1500, 1455, 1394, 1371, 1338, 1213 (O═S═N), 1164, 1106, 989, 948 (S═N), 850, 825, 775, 680, 646, 592, 512, 468 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ_H 1.40–1.50 [m, 39H, isopropylidene, 3 × $(CH_3)_3COCON$ and $(CH_3)_3CO$], 1.65 (s, 3H, isopropylidene), 3.29, 3.311 and 3.338 (3 × s, 3H, SCH_3), 3.83–4.48 (m, 5H CH_2S, 5′-CH_2, α-proton), 4.62 (m, 1H, 4′-H), 5.13–5.16 (m, 1H, 3′-H), 5.31–5.40 (m, 1H, 2′-H), 5.64 (br, 1H, CONH), 6.27 (d, J = 2.7 Hz, 1H, 1′-H), 8.323 and 8.331 (2 × s, 1H, adenyl 2-H), 8.880 and 8.888 (2 × s, 1H, adenyl 8-H); Anal. Calcd for $C_{36}H_{57}N_7O_{15}S_2$: C, 48.47; H, 6.44; N, 10.99. Found: C, 48.69; H, 6.54; N, 10.77. HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{36}H_{58}N_7O_{15}S_2$ (MH^+) 892.3435, found 892.3430.

5.1.15. (*R,S*)-2-Amino-3-{*N*-[(5′-deoxyadenosin-5′-yl)aminosulfonyl]-*S*-methyl-(*R,S*)-sulfonimidoyl}propanoic acid (3)

A suspension of the fully protected sulfoximine (**17**) (910 mg, 1.02 mmol) in 5:1:4 TFA–H_2O–CH_2Cl_2 (10 mL) was stirred at ambient temperature for 5 h, and then evaporated to dryness to give a residual syrup that was purified by reversed phase, medium-pressure column chromatography on Diaion HP20SS eluting with a linear gradient of THF in H_2O (0–99%). The eluant was monitored with UV (254 nm), and the desired functionalized sulfoximine eluted at 79:21 H_2O/THF. UV positive-fractions were then combined, and lyophilization afforded (**3**) as a mixture of diastereomers (374 mg, 74%, colorless solid): IR (KBr) ν_{max} 3600–3000 (br), 1648, 1604, 1508, 1477, 1419, 1378, 303, 1216 (O═S═N), 1139, 1079 (S═O), 997, 962 (S═N), 898, 848, 798, 728, 680, 647, 599, 586, 539, 462 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ_H 3.30–3.40 (m, 2H, 5′-CH_2), 3.487, 3.496, 3.513 and 3.522 (4 × s, 3H, SCH_3), 3.80–4.40 (m, 4H CH_2S, α-proton, 4′-H), 4.54–4.81 (m, 2H, 3′-H and 2′-H), 5.92 (dd, J = 3.0 and 6.2 Hz, 1H, 1′-H), 8.14 (s, 1H, adenyl 2-H), 8.170 and 8.176 (2 × s, 1H, adenyl 8-H); Anal. Calcd for $C_{14}H_{22}N_8O_8S_2{\cdot}1.4H_2O$: C, 32.36; H, 4.81; N, 21.56. Found: C, 32.36; H, 4.77; N, 21.88. HRMS (FAB, glycerol) calcd for $C_{14}H_{23}N_8O_8S_2$ (MH^+) 495.1083, found 495.1088.

5.1.16. (*R,S*)-2-Amino-3-[*N*-(adenosin-5′-yl)-5′-*O*-sulfonyl-*S*-methyl-(*R,S*)-sulfonimidoyl]propanoic acid (4)

A suspension of the fully protected sulfoximine (**18**) (220 mg, 0.247 mmol) in TFA–H_2O–CH_2Cl_2 (5:1:4, 10 mL) was stirred at ambient temperature for 6 h. The resulting mixture was evaporated to dryness. The residual syrup was purified by reversed phase medium-pressure column chromatography on Diaion HP20SS eluting with a linear gradient of THF in H_2O (0–99%). The eluant was monitored with UV (254 nm). The final compound was eluted at H_2O/THF, 79:21. The UV positive-fractions were combined and lyophilized to afford the final product (**4**) as a mixture of diastereomers (394 mg, 71%): IR (KBr) ν_{max} 3465–3349 (br), 2929, 1698, 1644, 1606, 1506, 1481, 1417, 1384, 1338, 1220 (O═S═N), 1164, 1095 (S═O), 1024, 978, 948 (S═N), 889, 823, 723, 676, 647, 592, 538, 472, 410 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ_H 3.441 and 3.470 (2 × s, 3H, SCH_3), 3.80–4.40 (m, 8H, 5′-CH_2, CH_2S, α-proton, 4′-H, 3′-H, 2′-H), 5.97 (d, J = 3.9 Hz, 1H, 1′-H), 8.10 (s, 1H, adenyl 2-H), 8.18 (s, 1H, adenyl 8-H); HRMS (FAB, glycerol) calcd for $C_{14}H_{22}N_7O_9S_2$ (MH^+) 496.0923, found 496.0912.

5.1.17. Sulfamoyl chloride (20)[56]

Chlorosulfonyl isocyanate (14.7 g, 104 mmol) was placed in a dry three-necked flask equipped with a $CaCl_2$ drying tube, and formic acid (3.96 mL, 104 mmol) added dropwise via syringe with cooling in an ice bath (CAUTION: This reaction is vigorous and effervescence is observed). After the addition was complete, the mixture was stirred at room temperature until no more gas was evolved. After 1 h, colorless crystals accumulated in the flask, and dry benzene (30 mL) was added before the removal of insoluble material by filtration under an Ar atmosphere. Evaporation of the solution under reduced pressure gave **20** as colorless crystals (11.2 g, 93%): mp 36.2–37.1 °C (corrected), lit.[56] mp 40 °C.

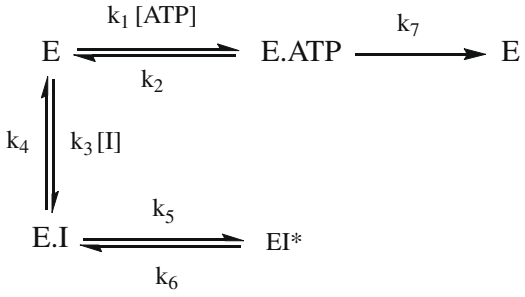
5.2. Steady-state kinetic assays

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) and were of the highest purity available. Protein concentrations were determined using the Bradford Assay1 (Pierce, Rockford, IL),[57] and are corrected as previously described.[31] L-Glutamine was recrystallized prior to use in all kinetic assays.[31] The ability of **3** and **4** to inhibit human ASNS was measured by determining their effect on PP_i production, under steady-state conditions, using a continuous assay in which product formation is coupled to NADH consumption (340 nm) (Sigma technical bulletin BI-100). In experiments employing the sulfamide derivative **3**, the compound at a number of different concentrations (0, 10, 50, 100, 500, and 1000 μM) was incubated with 0.5 mM ATP, 25 mM L-Gln and the pyrophosphate reagent (350 μL) in 100 mM EPPS, pH 8, containing 10 mM $MgCl_2$ (1 mL final volume). The reaction was initiated by the addition of ASNS (2 μg), and the production of pyrophosphate monitored spectrophotometrically at 37 °C for 20 min. Identical conditions were employed for the sulfamate derivative **4** except that progress curves were generated at 25 °C because this compound underwent cyclization to form the cyclo-adenosine at higher temperatures. Control experiments using known amounts of PP_i demonstrated that the assay reagent was not affected by the presence of either functionalized sulfoximine.

The sets of progress curves were analyzed by fitting the data, using the Kaleidagraph v3.5 software package (Synergy, Reading, PA), to Eq. 1:[34]

$$[PP_i] = v_{ss}t + \frac{(v_o - v_{ss})}{k}(1 - e^{-kt}) \quad (1)$$

where $[PP_i]$ is the concentration of inorganic pyrophosphate formed at time t, v_o and v_{ss} are the initial and steady-state rates, respectively, and k is the apparent first-order rate constant for isomerization of EI to EI*. By analogy to the known behavior of the adenylated sulfoximine **1**, we assumed that the compounds bound to free enzyme and were competitive with respect to ATP, as shown in the following kinetic model:



Having obtained values for k, v_o and v_{ss} at each concentration of the functionalized sulfoximine **3**, this information was then used to compute an estimate of k_6, using Eq. 2:

$$K_6 = k\frac{v_{ss}}{v_0} \quad (2)$$

With an estimate of k_6, values of K_i and k_5 could be determined by fitting the variation of k with the concentration of **3** using Eq. 3:

$$k = k_6 + k_5\left[\frac{I/K_i}{(1 + [ATP]/K_a + I/K_i)}\right] \quad (3)$$

where I is the concentration of the inhibitor, K_a is taken to be 0.1 mM,[30] and [ATP] = 0.5 mM. The overall inhibition constant, K_i^* was then computed using Eq. 4:

$$K_i^* = \frac{K_i k_6}{k_5 + k_6} \quad (4)$$

Experiments employing an HPLC-based assay[31] were performed to measure the amount of L-asparagine produced under the conditions of the inhibitor assay, which confirmed that neither **3** nor **4** was affecting the 1:1 stoichiometry of human ASNS-catalyzed Asn:PPi production. Briefly, recombinant human ASNS (2 μg) was incubated at 37 °C for 20 min in 100 mM EPPS, pH 8.0, buffer containing 0.5 mM ATP, 100 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM aspartic acid, and 1 mM of either sulfoximine **3** or **4** (1 mL total volume). The reaction was then quenched with TCA to a final concentration of 4%. Following enzyme precipitation, the solution was neutralized using 10 M NaOH, and a portion of the reaction mixture (40 μL) was taken for derivatization in a mixture of 800 μM Na_2CO_3 buffer, pH 9 (80 μL), DMSO (20 μL), and a solution of DNFB dissolved in absolute EtOH (60 μL). This reaction was incubated at 50 °C for 50 min before the addition of glacial AcOH and separation by reverse-phase HPLC on a Varian C_{18} Microscorb column using a step gradient of 40 mM formic acid, pH 3.6, and CH_3CN. DNP-L-asparagine was detected at 365 nm and quantified by comparison to a commercially available standard at known concentration.

5.3. Molecular modeling

Full details of the model for ASNS complexed to various reaction intermediate and inhibitors will be published elsewhere, although coordinates for the AS-B/glutamine/acyl-adenylate complex are available from the authors on request. Briefly, homology modeling tools in SYBYL 6.2 (Tripos) were used to add two missing loop segments (Ala-251 to Gln-266, and Gly-423 to Gly-425) to one of the monomers in the high-resolution crystal structure of *E. coli* AS-B (1CT9).[45] This gave an initial model of the bacterial enzyme (residues 1–516) bound to glutamine and AMP. Of the three uranyl ions in the original crystal structure, the one coordinated with the side chains of Asp-238 and Asp-351 was modified to Mg^{2+} because site-directed mutagenesis studies had shown that Asp-238 mediates metal binding (Boehlein, unpublished results). The remaining uranyl ions were deleted before the addition of hydrogen atoms and energy minimization using the CHARM software package[58] (version 22 parameter set).[59] All calculations employed a Generalized-Born continuum solvation potential.[60]

The bound AMP was graphically modified to ATP in a conformation that mimicked that observed in the active site of *Streptomyces clavuligerus* β-lactam synthetase (BLS),[61] an enzyme that is clearly evolutionarily related to ASNS.[62] In this model, the β- and γ-phosphate groups of ATP interacted with the 'pyrophosphate loop' motif,[63] as seen in structures of both BLS[54] and GMP synthetase.[64] After energy refinement of this AS-B/Gln/MgATP complex using CHARMM, aspartate was built into the structure at the cognate position to that at which substrate binds in BLS,[61] in the conformation delineated in previous studies employing constrained analogs of L-aspartic acid.[46] In this structure, the side chain carboxylate was also positioned for in-line attack on the γ-phosphate of ATP. Subsequent energy minimization then yielded a model for the AS-B/Gln/Asp/MgATP complex, and graphical manipulation then yielded a model of the acyl-adenylate bound in the synthetase active site together with $MgPP_i$.

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