Mapping the Aspartic Acid Binding Site of *Escherichia coli* Asparagine Synthetase B Using Substrate Analogs

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Novel inhibitors of asparagine synthetase, that will lower circulating levels of blood asparagine, have considerable potential in developing new protocols for the treatment of acute lymphoblastic leukemia. We now report the indirect characterization of the aspartate binding site of *Escherichia coli* asparagine synthetase B (AS-B) using a number of stereochemically, and conformationally, defined aspartic acid analogs. Two compounds, prepared using novel reaction conditions for the stereospecific β-functionalization of aspartic acid diesters, have been found to be competitive inhibitors with respect to aspartate in kinetic studies on AS-B. Chemical modification experiments employing [(fluorosulfonyl)benzoyl]adenosine (FSBA), an ATP analog, demonstrate that both inhibitors bind to the aspartate binding site of AS-B. Our results reveal that large steric alterations in the substrate are not tolerated by the enzyme, consistent with the failure of previous efforts to develop AS inhibitors using random screening approaches, and that all of the ionizable groups are placed in close proximity in the bound conformation of aspartate.

Introduction

The enzyme L-asparaginase[1] is often employed in the treatment of acute lymphoblastic leukemia (ALL), its mechanism of action being to deplete the level of circulating asparagine synthesized by the liver.[2] Indeed, resistance of human neoplasms to protocols involving L-asparaginase arises from the synthesis of endogenous asparagine in these malignant cells.[3] There is also evidence to suggest that blood asparagine concentration may also play a role in T-cell response.[4] Inhibitors of asparagine synthetase (AS), the glutamine-dependent amidotransferase that converts aspartate to asparagine in the liver,[5] therefore represent targets with potential application in treating leukemia and in exploring cellular mechanisms of immunosuppression.[6] Extensive screening studies employing hundreds of analogs of aspartic acid, glutamine, and ATP have failed to yield any well-characterized, reversible AS inhibitors,[7,8] although several glutamine analogs capable of covalent attachment to the enzyme have been described.[9] These irreversible inhibitors, however, possess little selectivity, modifying all cellular glutamine-dependent amidotransferases. In efforts to develop AS inhibitors using rational methods, we have cloned, expressed, and purified the asparagine synthetase encoded by the *asnB* gene of *Escherichia coli* (AS-B).[10] This bacterial enzyme catalyzes the following reactions:

$$\text{L-Gln} + \text{L-Asp} + \text{L-ATP} \rightarrow \text{L-Asn} + \text{L-Glu} + \text{AMP} + \text{PP}_i \quad (1)$$

$$\text{NH}_3 + \text{L-Asp} + \text{L-ATP} \rightarrow \text{L-Asn} + \text{AMP} + \text{PP}_i \quad (2)$$

$$\text{L-Gln} \rightarrow \text{L-Glu} + \text{NH}_3 \quad (3)$$

In *E. coli*, *asnB* is one of two unlinked genes encoding asparagine synthetase for which nucleotide sequences have been reported.[11−13] AS-B is a member of the *purF* family of glutamine-dependent amidotransferases,[14] which also includes glutamine phosphoribosylpyrophosphate amidotransferase (GPA)[15] and glutamine fructose-6-phosphate amidotransferase (GFAT).[16] Although the crystal structure of an inactive form of GPA complexed with AMP has been reported,[17] no corresponding structural information has been obtained for any other *purF* enzyme. Functionalized aspartic acid analogs have proven useful tools in the elucidation of structure−function relationships associated with the NMDA[18] and glutamate[19] receptors and for the preparation of chiral intermediates in the synthesis of a number of complex natural products.[20] We have therefore prepared a series of β-functionalized aspartates as probes of the specificity and stereochemical preferences of the AS-B active site.[21] We now describe the first well-characterized, albeit weak, inhibitors of AS-B that are competitive with respect to L-aspartic acid and demonstrate their interaction with the AS-B aspartic acid binding in chemical modification experiments using [(fluorosulfonyl)benzoyl]-adenosine (FSBA).[22] Our results are consistent with a model of the bound conformation for aspartate in which all ionizable groups are placed on the same face of the substrate.

Chemistry

The N-protected diester **1** (Scheme 1) was readily prepared in large scale from (*S*)-aspartic acid in three steps.[23,24] Alkylation of the dianion of **1** had been shown to proceed without racemization at the chiral carbon using a wide variety of electrophiles.[23,25,26] Treatment of **1** with lithium hexamethyldisilazide (LiHMDS) in THF at −75 °C, followed by addition of neat methyl iodide, gave the desired methylated diester as a 3:1 mixture of diastereoisomers **2** and **3** after column chromatography. No N-methylated material was obtained, and the lack of stereoselectivity in this reaction was consistent with previous observations.[24−27] Recovered diester **1** had an unchanged optical rotation,

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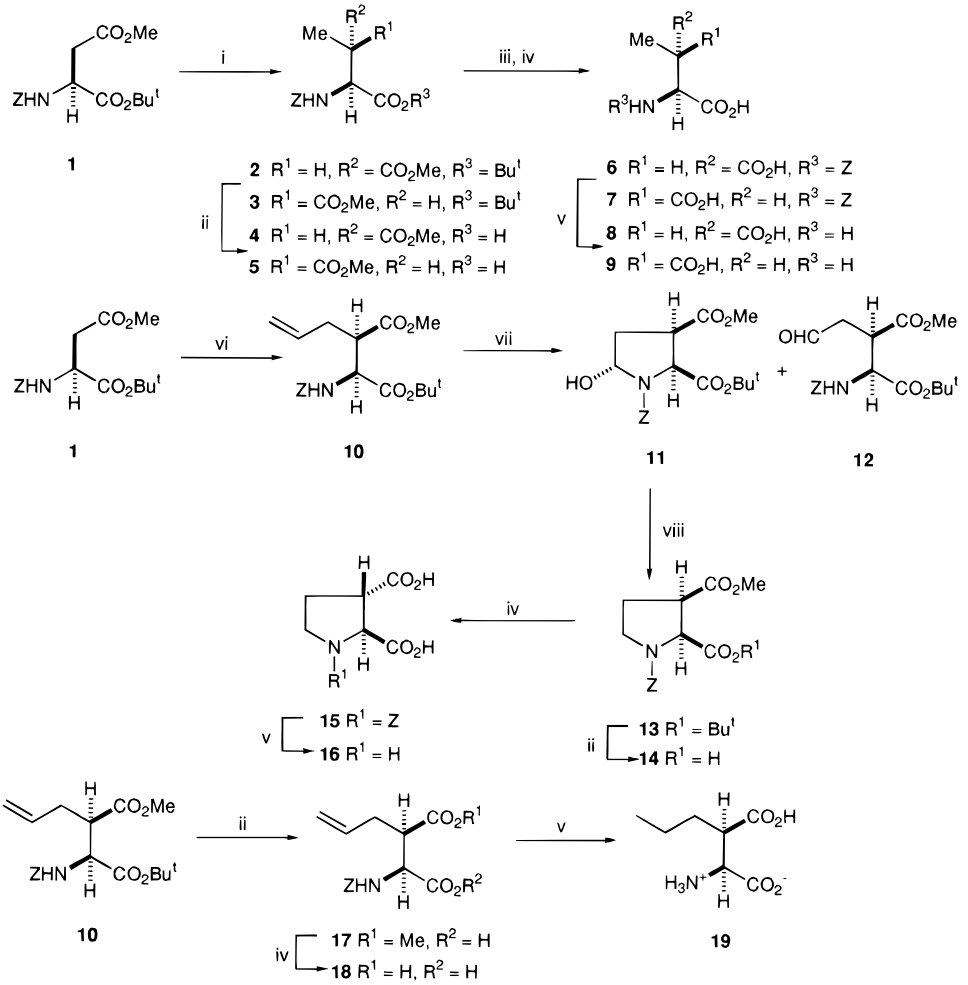
Scheme 1[a]



[a] Z = $PhCH_2OCO$. (i) LiHMDS, THF, −78 °C; MeI, THF, −78 °C; (ii) CF_3CO_2H, CH_2Cl_2, rt; (iii) C_{18} RP-HPLC separation of diastereoisomers; (iv) LiOH, MeOH, H_2O; (v) 10% Pd/C, C_6H_{10}, MeOH, reflux; (vi) LiHMDS, THF, −78 °C; $H_2C{=}CHCH_2Br$, THF, −78 °C; (vii) cat. OsO_4, $NaIO_4$, MeOH, H_2O, rt; (viii) CF_3CO_2H, Et_3SiH, $CHCl_3$, reflux.

suggesting deprotonation at C-2 had not occurred under these reaction conditions. In addition, the dianion of **1** was quenched with D_2O and the product examined by NMR. Deuterium was incorporated at C-3 but not at the α-carbon of diester **1**. At this stage in the synthesis we were unable to separate diesters **2** and **3**, and so the mixture was reacted with anhydrous trifluoroacetic acid (TFA)[28] giving mono-acids **4** and **5**. Further treatment with lithium hydroxide (LiOH) in aqueous MeOH[29] yielded two diacids **6** and **7**, that could be separated by reverse-phase HPLC on a C_{18} column. No epimerization at either chiral center was assumed to occur in these deprotection steps given that the ratio of **6** to **7** was identical to that determined for the mixture of monomethylated aspartates **2** and **3**. Deprotection of the major product **6** was effected using catalytic hydrogen transfer,[30] giving only (2*S*,3*S*)-2-amino-3-methylsuccinate (**8**) by examination of the behavior of its specific rotation as a function of solvent.[31−33] Thus, in agreement with previous findings,[32] the specific rotation associated with **8** was −12° in aqueous solution and +13.6° in 1 M HCl. Likewise, diacid **7** was deprotected to yield only (2*S*,3*R*)-2-amino-3-methylsuccinate (**9**). These assignments for **8** and **9** imply that the relative stereochemistry of compounds **2**−**7** is as shown (Scheme 1).

In contrast to the methylation of **1**, we were able to develop conditions for reaction of allyl bromide with the dianion of **1** that gave allylated diester **10** as a *single* diastereoisomer.[34] By analogy to the major methylation product, functionalized diester **10** was tentatively assigned to be the (2*S*,3*R*) diastereoisomer. The transformation of **10** into the functionalized pyrrolidine **11** was carried out by double-bond cleavage using sodium periodate and a catalytic amount of osmium tetraoxide (Scheme 1).[35] Pyrrolidine **11** was formed in 83% yield as a single diastereoisomer, suggesting that cyclization proceeded with complete stereocontrol at C-5. The aldehyde **12**, presumably an intermediate in this cyclization reaction, was also isolated in 6% yield. The relative stereochemistry of **11** was established using NOE difference spectroscopy[36] and by examination of scalar coupling constants.[37] Thus, the *cis*-arrangement of the ester substituents was suggested by the scalar coupling, $J_{23} = 8.4$ Hz,[37] and supported by a strong NOE between the protons at C-2 and C-3 (Figure 1A). Assuming no epimerization at C-3, other NOEs were consistent with **11** being the (2*S*,3*R*,5*R*) diastereoisomer. So as to confirm that the conformational properties of **11** had not led to a misassignment of the relative stereochemistry at C-2 and C-3, we prepared a mixture of **10** and its epimer at C-3 by β-functionalization using

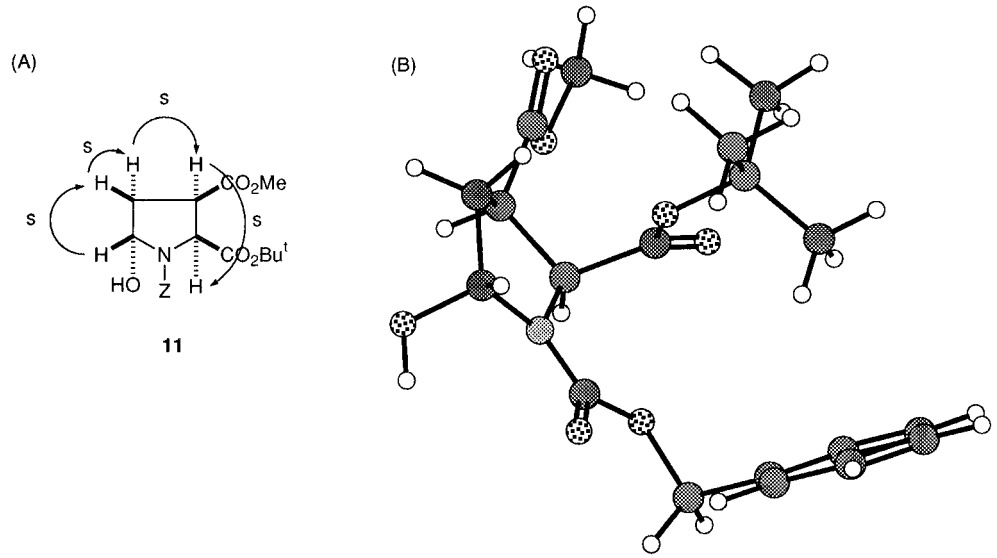


Figure 1. (A) Schematic representation of interproton NOEs observed for **11**. (B) Global energy minimum for **11** obtained using the AMBER* parameters and potential energy functions. Shading represents atom type. C: fully shaded; H: white; N: high density dots; O: low density dots.

literature conditions.[23,25,26] Cyclization of this mixture using osmium tetraoxide/sodium periodate then gave **11** and its C-3 epimer. Although these compounds were not separated, the relevant C-2 proton was identified using ^{1}H NMR and the key coupling constant found to be $J_{23} = 3.9$ Hz, confirming our assignment of **11**. Given that the stereospecific formation of **11** as the (2*S*,3*R*,5*R*) diastereoisomer, a complete conformational analysis of **11** and its C-5 epimer was carried out using a stochastic search algorithm[38] and the AMBER* force-field[39] as implemented in MacroModel V5.0 (Figure 1B).[40] This study indicated that **11** was more stable than its C-5 epimer by 7 kJ/mol, a result consistent with the hypothesis that the cyclization proceeds under thermodynamic control. Removal of the hydroxyl group from **11** was accomplished using triethylsilane to give **13**,[41] and subsequent deprotection of the *tert*-butyl ester proceeded smoothly in anhydrous TFA, yielding the acid **14** in good overall yield.[28] Unfortunately, removal of the methyl ester under basic conditions gave the (2*S*,3*S*)-diacid **15**, as the predominant product due to epimerization at C-3. Removal of the N-protecting group by catalytic hydrogen transfer gave conformationally constrained aspartate analog **16** in excellent yield.[42]

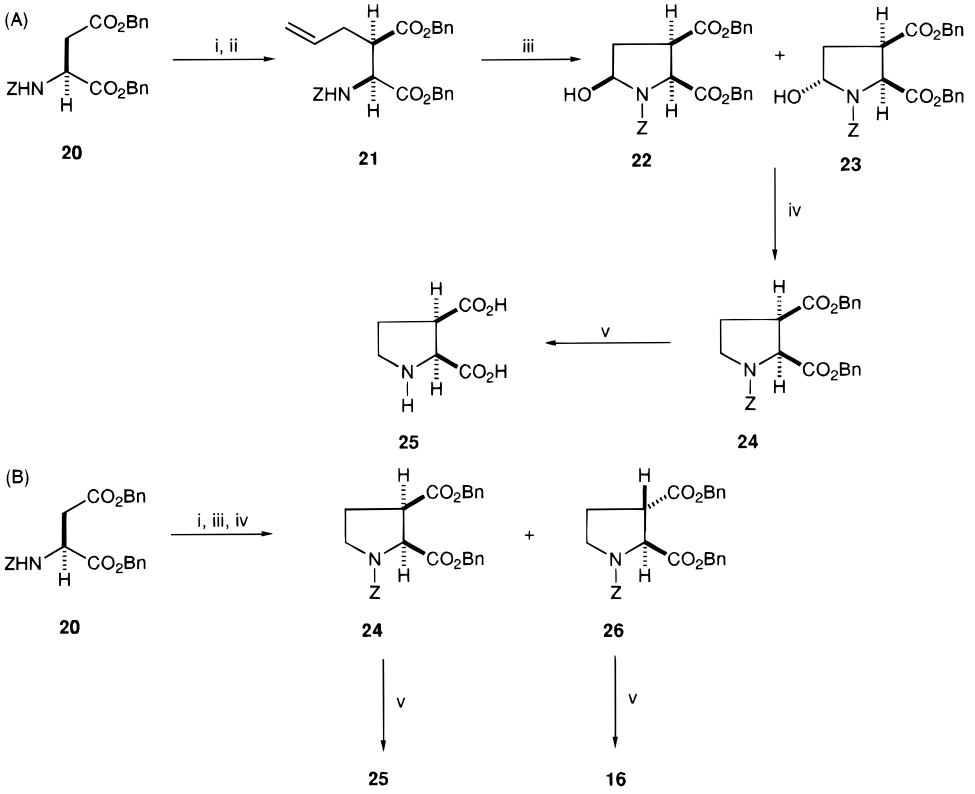
Having defined the stereochemistry of allylated aspartate **10** unambiguously, we converted it into diacid **18** by sequential reaction with TFA and then LiOH in aqueous methanol. Unfortunately, all efforts to remove the N-protecting group, such as reaction with 48% HBr in acetic acid,[43] failed to give products that were easily separable, presumably due to side-reactions involving the double bond. Removal of the *N*-carbobenzoxy protecting group, however, under standard catalytic hydrogen transfer conditions, proceeded with concomitant reduction of the double bond to give 3-*n*-propyl aspartate **19** (Scheme 1).[30]

In order to obtain large amounts of material, we next examined an alternate preparation of the diacid **25** from allylated aspartate derivative **21** in which the key step was β-functionalization of dibenzyl ester **20** (Scheme 2A). Standard reaction conditions for the alkylation of the dianion of **20** with allyl bromide gave the desired β-functionalized diester as a 6:1 mixture of diastereoisomers. The major product **21** could be obtained, however, by chromatography and fractional recrystallization. The relative stereochemistry of **21** was tentatively assigned by analogy to alkylation products **2** and **10**. Cleavage of the double bond and subsequent cyclization was accomplished using sodium periodate and catalytic OsO_4, giving substituted pyrrolidines **22** and **23** in a 9:1 ratio. As these diastereoisomers proved difficult to separate, the mixture was reduced by reaction with triethylsilane forming the desired *cis*-diester **24** as a single product. Removal of the benzyl protecting groups to yield the target proline analog **25** was successfully accomplished using catalytic hydrogen transfer in reasonable overall yield.[30,42] As the usefulness of this route was limited, however, by difficulties in purifying large quantities of **21** from the β-allylation reaction product mixture, we investigated purification of the diastereoisomeric products at a later stage in the synthesis (Scheme 2B). Hence, cyclization of a mixture of **21** and its C-3 epimer, followed by sequential treatment with $OsO_4/NaIO_4$ and Et_3SiH/TFA gave a 2.5:1 mixture of the pyrrolidines **24** and **26**. These could be separated by column chromatography and independently converted to the desired aspartic acid analogs **25** and **16**, respectively.

Biological Results and Discussion

The inhibitory effects of a series of aspartic acid analogs on glutamine-dependent asparagine synthesis were determined using recombinant, wild-type AS-B.[9] Our initial studies focused upon the commercially available compounds **27**−**32** (Figure 2). Standard reaction solutions in these experiments contained 1 mM glutamine, 1 mM ATP, 1 mM aspartic acid, and 10 mM $MgCl_2$ in TrisHCl, pH = 8.[9] Asparagine production was assayed by measuring the amount of pyrophosphate formed under steady state conditions.[9] The sulfinate **27** proved to be an effective, competitive inhibitor of aspartate ($K_{IS} = 1.45$ mM), consistent with the results

Scheme 2[a]



[a] Z = $PhCH_2OCO$. (i) LiHMDS, THF, −78 °C; $H_2C{=}CHCH_2Br$, THF, −78 °C; (ii) chromatography followed by fractional recrystallization; (iii) cat. OsO_4, $NaIO_4$, MeOH, H_2O, rt; (iv) CF_3CO_2H, Et_3SiH, $CHCl_3$, reflux; (v) 10% Pd/C, C_6H_{10}, MeOH, reflux.

of previous studies on aspartate transcarbamoylase.[44] On the other hand, compounds **28**−**32** exhibited only low levels of inhibition even at 10 mM concentration. The inability of malic acid to inhibit asparagine synthesis clearly shows that the hydroxyl cannot act as an isostere of the charged amino group, consistent with results reported for the asparagine synthetase present in RADA1, a murine leukemia.[45] It therefore appears that AS-B requires the presence of all of the ionizable groups in aspartate for substrate binding. The significance of the β-carboxylate in recognition is underscored by the failure of L-proline to inhibit AS-B, within the limits of the assay, at concentrations up to 50 mM.

Having established that all three ionized functional groups were essential to the recognition of aspartate by AS-B, we then assayed the β-functionalized aspartic acid analogs **8**, **9**, **16**, **19**, and **25**. We note that in previous experiments on asparagine synthetase isolated from asparaginase-resistant Novikoff hepatomas,[8] 27% AS inhibition was observed when synthetase activity was assayed in the presence of the racemate of *erythro*-β-hydroxyaspartate **33** (Figure 2). While no reduction in AS-B activity was observed for 3-*n*-propyl aspartate **19**, the remaining analogs were weak AS-B inhibitors (Table 1). Furthermore, there was a significant effect of stereochemistry at C-3 upon the level of AS-B inhibition. Again, this observation is consistent with reports that *threo*-β-hydroxyaspartate **34** (Figure 2) cannot inhibit AS isolated from Novikoff hepatomas.[8] A Dixon plot of the reciprocal of the initial velocity, $1/v_o$, against inhibitor concentration, [I], for AS-B in the presence of analog **16**, gave an apparent K_I of 60 mM for this compound (data not shown). In contrast, **25**, differing only in its C-3 configuration, was competitive with respect to aspartate, having a K_{IS} of 2.65 mM. A

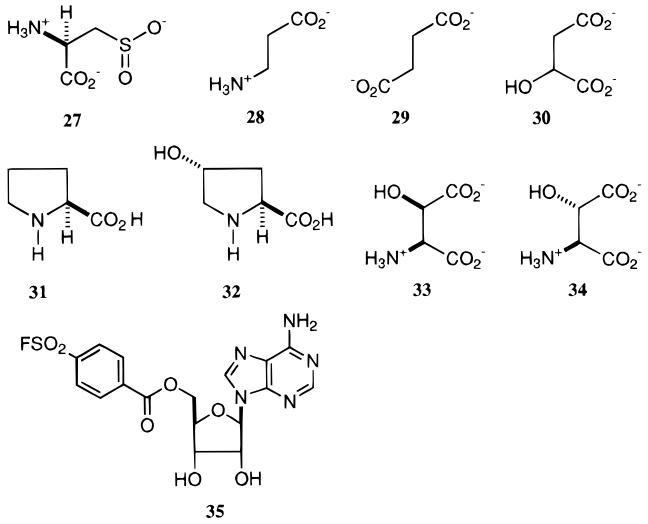


Figure 2. Aspartic acid analogs **27**−**34** used in these and previous inhibition studies, together with the irreversible inhibitor FSBA (**35**).

similar dependence of inhibitory properties upon C-3 stereochemistry was also observed for the methylated analogs **8** and **9** (Table 1). Detailed kinetic analysis confirmed that **9** and **25** were competitive only with respect to aspartate, suggesting that these compounds did indeed bind to the same form of the enzyme (probably the E·ATP complex[46]) as aspartic acid, preventing the natural substrate from binding and taking part in subsequent chemical transformations.[47]

Given the conformationally restricted nature of **16** and **25**, and the observation that these compounds are only competitive with respect to aspartate, it is reasonable to assume that **25** defines the bound conformation

Table 1. Inhibition Constants for Selected Aspartic Acid Analogs in the Glutamine-Dependent Synthetase Activity of AS-B

inhibitor	substrate varied	inhibition pattern	K_{is}[a] (mM)	K_{ii}[a] (mM)
8	aspartate	noncompetitive	18.0	>50.0
8	glutamine	noncompetitive	93.0	16.5
8	ATP	noncompetitive	8.0	15.0
9	aspartate	competitive	0.25	na
9	glutamine	noncompetitive	1.62	1.71
9	ATP	noncompetitive	0.45	3.28
25	aspartate	competitive	2.65	na
25	glutamine	uncompetitive	na	2.57
25	ATP	noncompetitive	2.65	4.80
27	aspartate	competitive	1.45	na

[a] K_{is} and K_{ii} are the inhibition constants computed from the intercepts and slopes, respectively, of the double reciprocal plots of $1/v_o$ versus 1/[aspartate] obtained at various concentrations of the inhibitor.[51] [b] na = not applicable.

of aspartic acid. In addition, the identical correlation between C-3 stereochemistry and the ability to inhibit AS-B observed for the diastereoisomers of β-methylaspartate suggests that **9** adopts a bound conformation on the enzyme similar to that of **25**. Both **9** and **25** possess a carboxyl group capable of undergoing reaction with ATP to form reactive intermediates cognate to β-aspartyl-AMP. The turnover of pyrophosphate by AS-B was not observed, however, in control experiments in which AS-B was incubated with either **9** or **25** in the presence of glutamine and ATP. We therefore became concerned about the possibility that these aspartate analogs were binding to a region of the enzyme other than the aspartate binding site. In order to address this question, the ability of β-methylaspartate **9** to behave in an identical manner to aspartic acid in chemical modification experiments was examined.

5′-*O*-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) (**35**) (Figure 2) is an ATP analog that covalently modifies proteins by reaction with hydroxyl or thiol groups[22] and has been widely used in studies of ATP-dependent enzymes.[48] When FSBA is incubated with AS-B in the absence of substrates or substrate analogs, the enzyme is covalently modified, resulting in a linear decrease of activity with respect to time when the incubated material is exposed to a mixture of glutamine, aspartate, and ATP. That FSBA indeed bound within the AS-B ATP binding site was demonstrated by a decreased rate of covalent modification when ATP was present in the incubation mixture (Figure 3). In contrast, when AS-B was incubated with FSBA in the presence of aspartate or analog **9**, there appeared to be little effect on the rate of covalent modification (Figure 3). The formation of β-aspartyl-AMP as a reaction intermediate in AS-catalyzed asparagine synthesis has been shown using isotopic labeling, implying that the ATP and aspartate binding sites are located close together in the enzyme. This is consistent with the observation that when ATP and aspartic acid were both present with AS-B and FSBA in the incubation mixture, the rate of enzyme inactivation was significantly decreased. Although this result might imply that β-aspartyl-AMP is tightly bound by AS-B, the presence of both substrates is also likely to enhance the stickiness of ATP with the free enzyme. In agreement with the latter proposal, similar decreases in the ability of FSBA to modify AS-B covalently were observed when either mixtures of aspartate/ATP or **9**/ATP were present during incubation. Hence, although

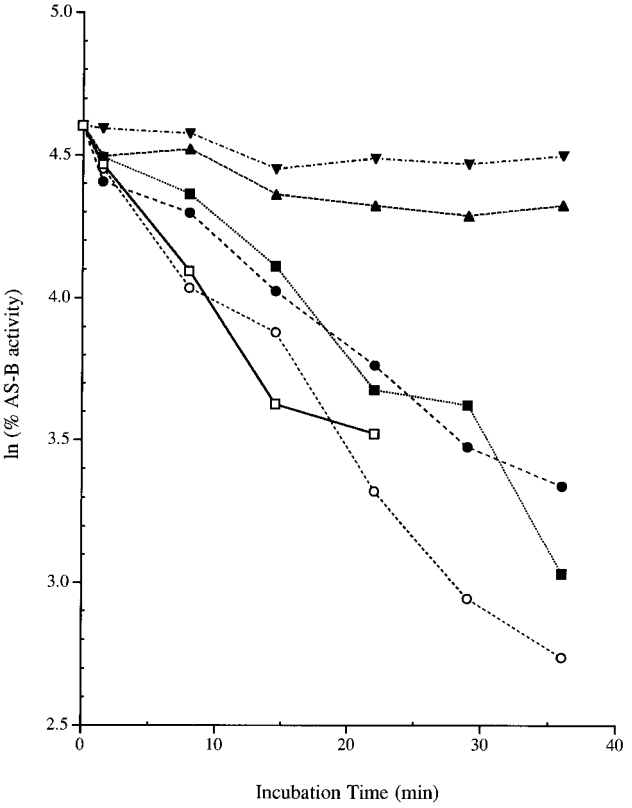


Figure 3. Effects of added substrates and aspartic acid analog **9** on the rate of AS-B inactivation by covalent modification by FSBA. At sufficiently high ATP concentrations, FSBA cannot inactivate AS-B. In these experiments, however, the ATP concentration employed was chosen so as to indicate any differential ability of aspartic acid and **9** to protect against FSBA inactivation. Enzyme activity was assayed by measuring the amount of pyophosphate released by the incubated enzyme in the presence of glutamine, aspartate, and ATP. Each experiment differed only in the compounds present in the incubation solution with AS-B. (○) FSBA only; (●) FSBA + ATP; (□) FSBA + Asp; (■) FSBA + **9**; (▲) FSBA + ATP + Asp; (▼) FSBA + ATP + **9**.

no reaction between ATP and **9** takes place to release pyrophosphate, analog **9** almost certainly occupies the same site on the enzyme as aspartate. In the absence of detailed structural data upon the complexes between AS-B and these aspartate analogs, we also propose that **25** binds within the same pocket as **9**, especially given the correlation between C-3 stereochemistry and inhibition for the aspartate analogs.

It is reasonable to suggest that aspartic acid binds, at least initially, to AS-B in a conformation that is identical to that of the rigid analog **25**. In this shape, all of the polar functionality is placed upon one face of the molecule, with the hydrogen atoms defining a hydrophobic surface that makes contact with the enzyme (Figure 4A). Given that the methylated analog **9** also interacts with this site, there is some flexibility in the protein that can be used to accommodate the larger substitutent. On the other hand, the inability of **19** to inhibit AS-B synthetase activity argues that this pocket in the enzyme cannot be distorted significantly.

There are now three examples of aspartate binding sites for which the X-ray structures have been reported.[49] In all cases, arginine residues interact with the carboxylate groups of aspartate, although the number employed is variable. Given that both carboxylates are placed in close proximity in our model of the bound

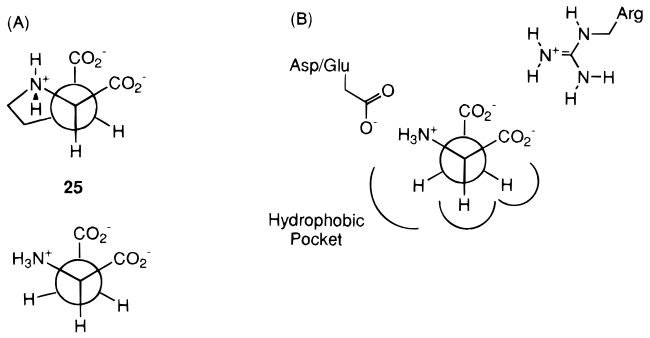


Figure 4. (A) Newman projection of the bound conformation of L-aspartate in the AS-B active site based on the constrained analog **25**. (B) Schematic model for the aspartic acid binding site in AS-B.

aspartate conformation, it is possible that only a single arginine residue will be present in the binding site of AS-B. Interactions that stabilize the protonated amine of L-aspartate, however, seem less predictable. For example, the amino group is placed over the face of a tryptophan ring in adenylosuccinate synthetase, while in the bacterial aspartate receptor, backbone carbonyl groups are used to bind the amine. Our data suggest that while two of the three protons in aspartate are placed within a well-defined pocket on the surface of the enzyme, there is room in the site to accommodate a small hydrophobic substituent in place of the pro-(*R*) hydrogen at C-3 (Figure 4B).

Conclusion

β-Alkylation of the aspartate derivative **1** has allowed the preparation of a number of stereochemically defined aspartate analogs suitable for probing the molecular features of the aspartic acid binding site in AS-B. The enzyme appears to be extremely selective, being able to discriminate between metabolites that have similar structures to aspartic acid, which is clearly important in terms of cellular metabolism. On the other hand, the protein residues that are responsible for mediating this selectivity remain to be defined by site-directed mutagenesis or X-ray crystallography. Although it appears that AS-B can tolerate only relatively minor structural alterations in the aspartic acid substrate, these data suggest that functionalized aspartate analogs can be developed that are selective, tight-binding AS inhibitors.

Experimental Section

Melting points were recorded using a Fisher-Johns melting point apparatus, and are uncorrected. Optical rotations were measured using a Polyscience Model SR-6 polarimeter. ^{1}H and ^{13}C NMR spectra were obtained on General Electric QE-300, Bruker AM-360, or Varian Unity-500 spectrometers. Chemical shifts are reported in ppm (δ) downfield of tetramethylsilane as an internal reference (δ 0.0). Splitting patterns are abbreviated as follows: s, singlet, d, doublet, t, triplet, q, quartet, and m, multiplet. Infrared spectra were recorded on a Perkin-Elmer 1600 FT-IR instrument. Combustion analyses for C, H, and N were determined in the Microanalysis Facility in the Department of Chemistry, University of Florida. EI, CI, and FAB mass spectra were recorded upon a VG Analytical 7-250-SE (low resolution) or a Finnigan MAT 25Q (high resolution) spectrometer. Ammonia or isobutane were used in the CI measurements. Analytical thin layer chromatrography (TLC) was performed on silica gel 60F-245 plates. Flash chromatography was performed using standard methods[50] on Davisil grade 633 type 60A silica gel (200−425 mesh). Analytical and preparative HPLC was carried out on Dynamax C_{18} and C_8 reverse-phase columns (250 mm × 4.6 mm or 250 mm × 21.4 mm), monitoring at 226 nm with a Rainin Dynamax UV-1 absorbance detector. HPLC grade solvents were obtained from Fisher Scientific and were used without further purification. Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl. Aspartate analogs **27**−**32** and **35** were purchased from Sigma and used without further purification.

General Procedure for the Alkylation of Aspartate Diester 1. LiHMDS was prepared *in situ* by the dropwise addition of *n*-butyllithium (2.1 equiv as a solution in hexane) to hexamethyldisilazane (2.2 equiv) dissolved in dry THF (5 mL), under a stream of dry N_2 so as to maintain the reaction temperature at 0 °C. After 60−90 min at 0 °C, the resulting solution was cooled to −78 °C before the addition of **1** (1 equiv) as a solution in dry THF (5 mL) to give a pale-yellow reaction mixture. After stirring at −35 °C for a further 150 min to ensure dianion formation, the solution was recooled to −78 °C, and neat electrophile (5−6 equiv) added at a rate so as to maintain the temperature below −75 °C. After stirring at −75 °C, for between 6 to 16 h, depending on the electrophile, the reaction was quenched by pouring the cold solution directly into 1 M HCl (60 mL) at rt. The resulting mixture was extracted with Et_2O (4 × 20 mL), and the organic extracts were dried ($MgSO_4$) before evaporation of the solvent under reduced pressure. The crude material was purified by "flash" chromatography (eluant: 4:1 petroleum ether/EtOAc).

***tert*-Butyl (2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carbomethoxybutanoate (2) and *tert*-Butyl (2*S*,3*S*)-2-(Benzyloxycarbonylamino)-3-carbomethoxybutanoate (3).** The methylation of the diester **1** (100 mg, 0.3 mmol) was carried out according to the general procedure outlined above, using hexamethyldisilazane (0.16 mL, 0.74 mmol), *n*-butyllithium (0.25 mL of a 2.48 M solution in hexane, 0.86 mmol) and neat methyl iodide (0.12 mL, 1.8 mmol). After 17 h at −75 °C, followed by quenching, workup, and purification by chromatography, the methylated product was obtained as a 3:1 mixture of diastereoisomers **2**:**3** based on ^{1}H NMR, as a clear oil: 67 mg, 64%; IR (neat) ν 3431, 3359, 2980, 1730, 1504, 1456, 1370, 1219, 1157 cm^{-1}; ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 12.66 (q), 27.82 (q), 41.84 (d), 51.86 (q), 56.26 (d), 68.58 (t), 82.53 (s), 127.72 (d),128.01 (d),128.31 (d), 136.29 (s), 156.39 (s), 169.36 (s), 173.77 (s); MS (CI: CH_4) *m/e* (relative intensity) 352 (MH^+, 100), 296 (89), 252 (10), 206 (6), 91 (6); exact mass calcd for MH^+ $C_{18}H_{26}NO_6$ requires 352.1760, found 352.1705 (CI).

2: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.26 (3 H, d, J = 8.0 Hz), 1.46 (9 H, s), 2.96 (1 H, m), 3.69 (3 H, s), 4.51−4.61 (1 H, m), 5.10 (2 H, s), 5.50 (1 H, d, J = 9.0 Hz), 7.35−7.39 (5 H, m).

3: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.20 (3 H, d, J = 8.0 Hz), 1.42 (9 H, s), 3.20 (1 H, m), 3.69 (3 H, s), 4.51−4.61 (1 H, m), 5.12 (2 H, s), 5.63 (1 H, d, J = 9.0 Hz), 7.35−7.39 (5 H, m).

(2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carbomethoxybutanoic Acid (4) and (2*S*,3*S*)-2-(Benzyloxycarbonylamino)-3-carbomethoxybutanoic Acid (5). TFA (1.48 mL, 19.3 mmol) was added to a solution of diesters **2** and **3** (342 mg, 0.97 mmol) in CH_2Cl_2 (40 mL) and stirred at rt for 72 h. During this period, a further portion of TFA (1 mL, 13 mmol) was added. Upon complete consumption of starting diester, removal of the solvent under reduced pressure yielded an oily residue which was redissolved in EtOAc (30 mL). This solution was extracted using 10% (w/v) aqueous $NaHCO_3$ (3 × 15 mL). After removal of the organic phase, the aqueous solution was acidified to pH 1 using 1 M HCl and then reextracted with EtOAc (4 × 20 mL). The organic phases were combined and dried ($MgSO_4$), and the solvent was removed under reduced pressure to give the desired acids as a 3:1 mixture of diastereoisomers **4**:**5** in quantitative yield. This material was used without further purification in subsequent reactions: IR (neat) ν 3730−2696, 3331, 2602, 1732, 1713, 1519, 1456, 1408, 1384, 1092, 1061 cm^{-1}; ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 13.90 (q), 41.55 (d), 52.16 (q), 55.58 (d), 67.65 (t), 127.79 (d), 127.96 (d),128.36 (d), 135.96 (s), 156.93 (s), 174.41 (s), 175.11 (s); MS (EI) *m/e* (relative intensity) 295 (M^+, 8), 220 (3), 206 (8), 160 (8), 108 (35), 91 (100); exact mass calcd M^+ for $C_{14}H_{17}NO_6$ requires 295.1056, found 295.1053 (EI).

4: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 (3 H, d, J = 4.0 Hz), 3.05 (1 H, m), 3.69 (3 H, s), 4.75 (1 H, m), 5.13 (2 H, s), 5.75 (1 H, d, J = 9.0 Hz), 7.35−7.39 (5 H, m), 10.58 (1 H, s).

5: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.25 (3 H, d, J = 4.0 Hz), 3.30 (1 H, m), 3.69 (3 H, s), 4.59−4.65 (1 H, dd, J = 9.0, 3.5 Hz), 5.13 (2 H, s), 5.86 (1 H, d, J = 9.0 Hz), 7.35−7.39 (5 H, m), 10.58 (1 H, s).

(2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-methylsuccinic Acid (6) and (2*S*,3*S*)-2-(Benzyloxycarbonylamino)-3-methylsuccinic Acid (7). The mixture of diastereoisomeric acids **4** and **5** (285 mg, 0.97 mmol) was dissolved in 4:1 MeOH/H_2O so as to give an 0.1 M solution. After the addition of 4 equiv of $LiOH\cdot H_2O$, the reaction was stirred at room temperature until the starting esters were completely consumed. The reaction mixture was concentrated, under reduced pressure, to approximately 20% of its initial value, and poured into an excess of 1 M HCl. After adjustment of the solution pH to 1−2, the mixture was extracted with EtOAc. The combined organic phases were dried ($MgSO_4$), and the solvent was removed under reduced pressure. Purification of this crude mixture of diacids was accomplished using reverse phase HPLC with gradient elution (C_{18} column; solvent flow rate 20 mL/min; 80:20 H_2O/CH_3CN + 1% TFA to 66:34 H_2O/CH_3CN + 1% TFA over a period of 28 min), with monitoring at 226 nm. The minor product, diacid **7** (ret time: 19.6 min), eluted first and was finally obtained as a white, hygroscopic solids by freeze-drying overnight: 29 mg, 11%; IR ($CHCl_3$) ν 3264−2719, 1714, 1514, 1455, 1416, 1343, 1061 cm^{-1}; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.21 (3 H, d, J = 8.0 Hz), 3.12 (1 H, m), 4.49 (1 H, d, J = 5.0 Hz), 5.12 (2 H, s), 7.30−7.42 (5 H, m); ^{13}C NMR (CD_3OD, 75.4 MHz) δ 12.74 (q), 41.02 (d), 55.77 (d), 66.44 (t), 127.92 (d), 128.66 (d), 129.05 (d), 136.65 (s), 157.31 (s), 174.14 (s), 177.79 (s); MS (EI) *m/e* (relative intensity) 281 (M^+, 11), 263 (6), 192 (4), 146 (7), 108 (52), 91 (100); exact mass calcd for M^+ $C_{13}H_{15}NO_6$ requires 281.0899, found 281.0887 (EI).

Further elution gave the diastereoisomeric diacid **6** (ret time: 22.0 min), which was also obtained as a white, hygroscopic solid following lyophilization: 123 mg, 45%; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.17 (3 H, d, J = 8.0 Hz), 2.98 (1 H, m), 4.60 (1 H, d, J = 6.5 Hz), 5.11 (2 H, s), 7.28−7.39 (5 H, m); ^{13}C NMR (CD_3OD, 75.4 MHz) δ 12.47 (q), 41.35 (d), 56.02 (d), 67.58 (t), 127.92 (d), 128.66 (d), 129.05 (d), 136.65 (s), 157.31 (s), 174.14 (s), 177.79 (s); MS (EI) *m/e* (relative intensity) 281 (M^+, 2), 263 (3), 146 (4), 108 (39), 91 (100); exact mass calcd for M^+ $C_{13}H_{15}NO_6$ requires 281.0899, found 281.0887 (EI).

(2*S*,3*R*)-2-Amino-3-methylsuccinic Acid (9). The N-protected aspartic acid derivative **7** (780 mg, 2.77 mmol) was dissolved in MeOH (50 mL) together with freshly distilled cyclohexene (5.18 mL). After the addition of Pd/C (240 mg) the suspension was refluxed for 2.5 h, allowed to cool, and then poured into water (300 mL) and stirred for 1 h. Filtration through through Celite, followed by reduction of the solvent under reduced pressure, and freeze-drying yielded the aspartic acid analogue **9** as a hygroscopic solid: 310 mg, 76%; $[\alpha]^{23}_D$ +5.8° (c = 3.1, H_2O); IR (nujol) ν 3645−2692, 1712, 1632, 1514, 1416, 1096 cm^{-1}; ^{1}H NMR (D_2O, 300 MHz) δ 1.29 (3 H, d, J = 7.4 Hz), 3.18 (1 H, dq, J = 7.4, 4.3 Hz), 4.01 (1 H, d, J = 4.2 Hz); ^{13}C NMR (D_2O, 75.4 MHz) δ 12.87 (q), 39.95 (d), 56.04 (d), 171.95 (s), 176.92 (s); MS (FAB: glycerol + 5% TFA) *m/e* (relative intensity) 148 (100), 115 (67), 74 (40), 63 (40); exact mass calcd MH^+ for $C_5H_{10}NO_4$ requires 148.061, found 148.060 (FAB).

(2*S*,3*S*)-2-Amino-3-methylsuccinic Acid (8). The N-protected aspartic acid derivative **6** (360 mg, 1.28 mmol) was treated as for the corresponding diastereoisomer **7** for 1 h. Filtration through Celite, followed by reduction of the solvent under reduced pressure, and freeze-drying yielded the desired aspartic acid analogue **8** as a white hygroscopic solid: 184 mg, 98%; decomp at 275 °C; IR ($CHCl_3$) ν 3264−2766, 3119, 1710, 1610, 1494 cm^{-1}; $[\alpha]^{23}_D$ −12.0° (c = 0.5, H_2O); $[\alpha]^{23}_D$ +13.6° (c = 0.46, 1 M HCl); ^{1}H NMR (D_2O, 300 MHz) δ 1.20 (3 H, d, J = 7.4 Hz), 3.18 (1 H, dq, J = 7.4, 3.5 Hz), 4.01 (1 H, d, J = 3.5 Hz); ^{13}C NMR (D_2O, 75.4 MHz) δ 11.63 (q), 39.93 (d), 55.97 (d), 172.45 (s), 177.93 (s); MS (FAB: glycerol + 5% TFA) *m/e* (relative intensity) 148 (46), 115 (100); exact mass calcd for MH^+ $C_5H_{10}NO_4$ requires 148.0610, found 148.0620 (FAB).

***tert*-Butyl (2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carbomethoxyhex-5-enoate (10).** The allylated diester **10** was prepared by the general alkylation procedure using hexamethyldisilazane (1.41 mL, 6.67 mol), *n*-butyllithium (2.24 mL of a 2.48 M solution in hexane, 5.6 mmol), diester **1** (900 mg, 2.6 mmol), and allyl bromide (1.35 mL, 15.6 mmol). After 18 h at −75 °C, the standard workup procedure gave the allylated diester **10** as a clear oil: 600 mg, 60%; $[\alpha]^{23}_D$ +15.4° (c = 1.3, $CHCl_3$); IR (CH_2Cl_2) ν 3421, 3056, 2978, 1728, 1641, 1506, 1264, 1156, 922 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (9 H, s), 2.23−2.34 (1 H, m), 2.43−2.54 (1 H, m), 3.09−3.17 (1 H, m), 3.64 (3 H, s), 4.51−4.56 (1 H, dd, J = 10.1, 4.1 Hz), 5.04−5.09 (2 H, m), 5.12 (2 H, s), 5.70−5.85 (1 H, m), 5.78 (1 H, d, J = 10.1 Hz), 7.25−7.36 (5 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 27.04 (q), 31.83 (t), 45.94 (d), 51.04 (q), 53.85 (t), 66.17 (d), 81.54 (s), 117.13 (t), 127.28 (d),127.32 (d), 127.71 (d), 133.72 (d), 135.71 (s), 155.70 (s), 168.95 (s), 172.32 (s); MS (CI: H^+) *m/e* (relative intensity) 378 (MH^+, 41), 322 (100), 278 (41), 232 (9); exact mass calcd for MH^+ $C_{20}H_{28}NO_6$ requires 378.1917, found 378.1911 (CI).

***tert*-Butyl (2*S*,3*R*,5*S*)-1-(Benzyloxycarbonyl)-3-carbomethoxy-5-hydroxypyrrolidine-2-carboxylate (11).** OsO_4 (1.7 mL of a 2.5% solution in 2-propanol, 0.14 mmol) and $NaIO_4$ (1.28 g, 6.0 mmol) were added to a solution of diester **10** (750 mg, 2.0 mmol) in MeOH (44 mL) and water (22 mL). The resulting reaction mixture was stirred at rt for 75 min. After halving the solvent volume under reduced pressure, the solution was poured into water (140 mL) and extracted with EtOAc (6 × 50 mL). The organic extracts were dried ($MgSO_4$), and the solvent was removed under reduced pressure to yield the crude product as a brown oil. This material was purified by flash chromatography (eluant: 30% EtOAc/petroleum ether) to yield, as the first material from the column, the aldehyde **12** as a clear oil: 46 mgmg, 6%; IR ($CHCl_3$) ν 3433, 3020, 1724, 1508, 1455, 1439, 1395, 1369, 1342, 1296,1155 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (9 H, s), 2.60 (1 H, m), 2.93 (1 H, m), 3.69 (3 H, s), 3.72 (1 H, m), 4.55−4.61 (1 H, dd, J = 9.0, 3.5 Hz), 5.12 (2 H, s), 5.56 (1 H, d, J = 8.0 Hz), 7.32−7.39 (5 H, m), 9.77 (1 H, s); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 27.81 (q), 41.43 (d), 41.61 (t), 52.17 (q), 54.73 (d), 67.27 (t), 128.12 (d), 128.25 (d), 128.53 (d).

Continued elution then gave the desired pyrrolidine derivative **11** as an oil which solidified upon standing: 625 mg, 83%; mp 56−58 °C; IR ($CHCl_3$) ν 3464, 3009, 2980, 1744, 1708, 1498, 1410, 1367, 1345, 1306, 1128, 1067, 1031 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.36 (9 H, s), 2.05 (1 H, m), 2.50 (1 H, m), 3.65 (1 H, m), 3.72 (3 H, s), 4.20 (1 H, br s), 4.55 (1 H, d, J = 8.5 Hz), 5.14 (2 H, m), 5.70 (1 H, d, J = 6.0 Hz), 7.3−7.4 (5 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 27.72 (q), 34,46 (t), 44.27 (d), 52.06 (q), 61.50 (d), 67.38 (t), 81.24 (d), 82.31 (s), 128.28 (d), 128.02 (d),128.58 (d), 135.67 (s), 154.46 (s), 168.47 (s), 170.17 (s); MS (FAB) *m/e* (relative intensity) 379 (MH^+, 5), 362 (21), 262 (34), 155 (24), 133 (100), 91 (33); exact mass calcd for MH^+ $C_{19}H_{26}NO_7$ requires 380.171, found 380.166 (FAB). Anal. ($C_{19}H_{25}NO_7$) C, H, N, requires C 60.15, H 6.64, N 3.69, found C 60.12, H 6.68, N 3.64.

***tert*-Butyl (2*S*,3*R*)-1-(Benzyloxycarbonyl)-3-carbomethoxypyrrolidine-2-carboxylate (13).** Alcohol **11** (150 mg, 0.39 mmol) and Et_3SiH (0.1 mL, 0.59 mmol) were dissolved in dry $CHCl_3$ (2 mL). Neat TFA (0.3 mL, 3.9 mmol) was then added dropwise to this solution with vigorous stirring. Upon completion of the addition, the reaction mixture was stirred at rt for a further 40 min before the solvent volume was reduced under reduced pressure. This gave an oily residue which was redissolved in EtOAc (20 mL). After washing with 5% aqueous $NaHCO_3$ (3 × 5 mL), the organic layer was dried ($MgSO_4$) and the solvent removed to yield **13** as an oil which solidified on standing: 107 mg, 74%; mp 81−82 °C; $[\alpha]^{20}_D$ +17.5° (c = 0.71, $CHCl_3$); IR ($CHCl_3$) ν 3019, 2982, 1741, 1703, 1498, 1455, 1420, 1369, 1347, 1215, 1175, 1155 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.38 (9 H, s), 2.10 (1 H, m), 2.40 (1 H, m), 3.25 (1 H, m), 3.46 (1 H, m), 3.72 (3 H, s), 3.77 (1 H, m), 4.52 (1 H, d, J = 8.3 Hz), 5.10−5.16 (2 H, m), 7.30−7.40 (5 H, br m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 25.56 (t), 27.80 (q), 45.50

(t), 46.52 (d), 52.00 (q), 61.19 (d), 67.07 (t), 82.05 (s), 127.83 (d), 127.95 (d), 128.38 (d), 135.30 (s), 154.09 (s), 168.89 (s), 170.29 (s); MS (EI) *m/e* (relative intensity) 363 (M^+, 3), 307 (21), 262 (39), 218 (49), 91 (100); exact mass calcd for M^+ $C_{19}H_{25}NO_6$ requires 363.1682, found 363.1686 (EI). Anal. ($C_{19}H_{25}NO_6$) C, H, N, requires C 62.80, H 6.93, N 3.85, found C 63.06, H 7.03, N 3.77.

(2*S*,3*S*)-1-(Benzyloxycarbonyl)-3-carbomethoxypyrrolidine-2-carboxylic Acid (14). Neat TFA (1.1 mL, 1.4 mmol) was added to a solution of diester **13** (230 mg, 0.7 mmol) dissolved in CH_2Cl_2 (10 mL), and the resulting solution was stirred at −5 °C for 30 min. After warming to rt, the reaction was stirred for a further 20 h. Removal of the solvent under reduced pressure then yielded the desired mono-acid **14** as an oil of sufficient purity for use in subsequent reactions, in quantitative yield: $[\alpha]^{20}_D$ −11.2° (*c* = 1.34, $CHCl_3$); IR (neat) *ν* 3557−2736, 1738, 1704, 1670, 1498, 1434, 1360, 1305, 1269, 1133, 1090, 1030 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) *δ* 2.15 (1 H, m), 2.40 (1 H, m), 3.31 (1 H, m), 3.50 (1 H, m), 3.68 (3 H, s), 3.75 (1 H, m), 4.67 (1 H, d, *J* = 8.5 Hz), 5.10−5.20 (2 H, m), 7.25−7.38 (5 H, m), 10.00−10.25 (1 H, s); ^{13}C NMR ($CDCl_3$, 75.4 MHz) *δ* 26.32 (t), 45.76 (t), 46.65 (d), 52.32 (q), 60.54 (d), 67.65 (t), 127.82 (d), 128.11 (d), 128.47 (d), 136.03 (s), 155.50 (s), 170.39 (s), 170.50 (s); MS (EI:) *m/e* (relative intensity) 307 (M^+, 8), 262 (7), 218 (29), 172 (35), 128 (21), 91 (100); exact mass calcd for M^+ $C_{15}H_{17}NO_6$ requires 307.1056, found 307.1062 (EI).

(2*S*,3*S*)-1-(Benzyloxycarbonyl)-pyrrolidine-2,3-dicarboxylic Acid (15) and (2*S*,3*R*)-1-(Benzyloxycarbonyl)-pyrrolidine-2,3-dicarboxylic Acid (36). The methyl ester **14** (196 mg, 0.6 mmol) was dissolved in 4:1 MeOH/H_2O so as to give an 0.1 M solution. After the addition of 4 equiv of $LiOH·H_2O$, the reaction was stirred at room temperature until the starting esters were completely consumed. The reaction mixture was concentrated, under reduced pressure, to approximately 20% of its initial value and poured into an excess of 1 M HCl. After adjustment of the solution pH to 1−2, the mixture was extracted with EtOAc. The combined organic phases were dried ($MgSO_4$), and the solvent was removed under reduced pressure to give the crude diacid as a buff-colored solid. Purification by reverse phase HPLC with gradient elution (C_{18} column; solvent flow rate 20 mL/min; from 80:20 H_2O:CH_3CN + 1% TFA to 65:35 H_2O:CH_3CN + 1% TFA over a period of 30 min), with monitoring at 226 nm, gave diacid **15** (ret time: 18.7 min) as a clear oil after freeze-drying overnight: 95 mg, 51%; IR (neat) *ν* 3742−2719, 3413, 3019, 2590, 1715, 1499, 1424, 1360, 1129 cm^{-1}; ^1H NMR (CD_3CN, 300 MHz) *δ* 2.12−2.24 (2 H, m), 3.18−3.26 (1 H, m), 3.46−3.58 (2 H, m), 4.56 (1 H, d, *J* = 3.6 Hz), 5.15−5.24 (2 H, m), 7.30−7.42 (5 H, m), 7.80−8.30 (2 H, br s); ^{13}C NMR (CD_3CN, 75.4 MHz) *δ* 27.03 (t), 45.49 (t), 46.24 (d), 60.90 (d), 66.63 (t), 127.32 (d), 127.66 (d), 128.14 (d), 136.53 (s), 154.76 (s), 172.05 (s), 172.64 (s); MS (LSIMS: glycerol + 5% TFA) *m/e* (relative intensity) 294 (MH^+, 3), 284 (18), 264 (20), 133 (100) 93 (92); exact mass calcd for MH^+ $C_{14}H_{16}NO_6$ requires 294.0978, found 294.1350 (LSIMS: glycerol + 5% TFA).

Continued elution also yielded the diastereoisomeric diacid **36** (ret time: 21.0 min) as a clear oil after lyophilization: 52 mg, 28%; IR (neat) *ν* 3542−2531, 1742, 1710, 1651, 1499, 1428, 1361, 1147 cm^{-1}; ^1H NMR (CD_3CN, 300 MHz) *δ* 2.12 (1 H, m), 2.22 (1 H, m), 3.32 (1 H, m), 3.40 (1 H, m), 3.63 (1 H, m), 4.54 (1 H, d, *J* = 9.2 Hz), 5.06−5.15 (2 H, m), 6.30−6.60 (2 H, s), 7.30−7.40 (5 H, m); ^{13}C NMR (CD_3CN, 75.4 MHz) *δ* 26.41 (t), 45.53 (t), 46.30 (d), 60.38 (d), 66.74 (t), 127.56 (d), 127.90 (d), 128.37 (d), 136.83 (s), 171.14 (s); MS (LSIMS: glycerol + 5% TFA) *m/e* (relative intensity) 294 (MH^+, 100), 250 (60), 158 (7) 91 (89); exact mass calcd for MH^+ $C_{14}H_{16}NO_6$ requires 294.0978, found 294.1060 (LSIMS: glycerol + 5% TFA).

(2*S*,3*S*)-Pyrrolidine-2,3-dicarboxylic Acid (16). The N-protected diacid **15** (64 mg, 0.22 mmol) was dissolved in MeOH (10 mL) together with dry cyclohexene (5 mL). Pd/C (10%) (37 mg) was added and the reaction mixture refluxed for 1 h. Filtration through Celite, followed by removal of the solvent under reduced pressure, yielded the crude aspartic acid analog **16**. This hygroscopic solid was redissolved in a minimum amount of water and purified using Bio Rad Ag-1-X2 anion exchange resin (5 g, wet weight) by elution with 1% AcOH.[42] Ninhydrin positive fractions were collected and freeze-dried to give **16** as a white solid: 26 mg, 76%; mp > 300 °C dec (lit.[42] 333 °C dec); IR (KBr) *ν* 3600−2800, 1702, 1618, 1460, 1102 cm^{-1}; ^1H NMR (D_2O, 300 MHz) *δ* 2.19−2.40 (2 H, m), 3.32−3.51 (3 H, m), 4.46 (1 H, d, *J* = 5.6 Hz); ^{13}C NMR (CD_3CN, 75.4 MHz) *δ* 28.11 (t), 45.33 (t), 47.34 (d), 62.91 (d), 172.64 (s), 176.06 (s); MS (FAB: glycerol + 5% TFA) *m/e* (relative intensity) 160 (MH^+, 10), 154 (15), 133 (100), 93 (41); exact mass calcd for MH^+ $C_6H_{10}NO_4$ requires 160.0610, found 160.044 (FAB). Anal. ($C_6H_9NO_4$) C, H, N, requires C 45.28, H 5.70, N 8.80, found C 45.25, H 6.02, N 8.57.

(2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carbomethoxyhex-5-enoic Acid (17). A solution of the diester **10** (1.01 g, 2.7 mmol) dissolved in CH_2Cl_2 (20 mL) was cooled to −8 °C before the dropwise addition of TFA (4.13 mL, 53.7 mmol) with vigorous stirring. After 70 h at this temperature, the reaction mixture was warmed to 15 °C over a period of 24 h, before being extracted with 5% (w/v) aqueous $NaHCO_3$ (3 × 100 mL). The aqueous phases were combined, acidified to pH 1 using 1 M HCl, and extracted using EtOAc (5 × 100 mL). The organic extracts were dried ($MgSO_4$), and removal of the solvent under reduced pressure yielded a clear oil which solidified on standing. The desired ester **17** was obtained as a white solid after recrystallization from EtOAc/petroleum ether: 763 mg, 89%; mp 93−94 °C; $[\alpha]^{20}_D$ +72.6° (*c* = 0.31, $CHCl_3$); IR (CH_2Cl_2) *ν* 3672−2732, 1728, 1512, 1266, 1223, 738 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) *δ* 2.30 (1 H, m), 2.45 (1 H, m), 3.16 (1 H, m), 3.54 (3 H, s), 4.52 (1 H, m), 5.04 (2 H, m), 5.10 (2 H, m), 5.70−5.86 (1 H, m), 7.25−7.38 (5 H, m); ^{13}C NMR (CD_3OD, 75.4 MHz) *δ* 32.55 (t), 46.53 (d), 51.12 (q), 53.86 (d), 66.50 (t), 116.93 (t), 127.48 (d), 127.66 (d), 128.07 (d), 134.38 (d), 136.61 (s), 157.11 (s), 172.61 (s), 173.13 (s); MS (CI: H^+) *m/e* (relative intensity) 322 (MH^+, 37), 278 (40), 219 (44), 91 (100); exact mass calcd for MH^+ $C_{16}H_{20}NO_6$ requires 322.1291, found 322.1292 (CI).

(2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carboxyhex-5-enoic Acid (18). Methyl ester **12** (200 mg, 0.33 mmol) was dissolved in MeOH (30 mL) and water (30 mL) together with NaOH (400 mg, 10.2 mmol) and the resulting solution refluxed for 1.5 h. After cooling to rt, the reaction mixture was poured into 1 M HCl (60 mL) and extracted using EtOAc (4 × 40 mL). The combined organic phases were dried ($MgSO_4$), and the solvent was removed under reduced pressure. The crude diacid was then purified using reverse phase HPLC with gradient elution (C_8 column; solvent flow rate 12 mL/min; 50:50 H_2O/CH_3CN + 1% TFA to 10:90 H_2O/CH_3CN + 1% TFA over a period of 20 min), with monitoring at 226 nm. Diacid **18** (ret time: 8.1 min) was obtained as a clear gum: 58%; $[\alpha]^{20}_D$ +52.5° (*c* = 0.67, $CHCl_3$); IR ($CHCl_3$) *ν* 3660−2308, 3418, 3056, 2980, 1722, 1514, 1419, 1226 cm^{-1}; ^1H NMR (CD_3CN, 300 MHz) *δ* 2.3 (1 H, m), 2.5 (1 H, m), 3.14 (1 H, m), 4.51 (1 H, dd, *J* = 10.0, 4.0 Hz), 4.98 (2 H m), 5.12 (2 H, s), 5.70−5.80 (1 H, m), 6.10 (1 H, d, *J* = 10.0 Hz), 7.20−7.30 (5 H, m), 8.00−8.50 (2 H, br s); ^{13}C NMR (CD_3CN, 75.4 MHz) *δ* 32.55 (t), 45.81 (d), 53.45 (d), 66.45 (t), 117.29 (t), 127.67 (d), 127.94 (d), 128.43 (d), 134.70 (d), 135.95 (s), 156.45 (s), 171.81 (s), 173.73 (s); MS (EI) *m/e* (relative intensity) 307 (M^+, 2), 289 (6), 262 (3) 218 (9), 172 (6), 108 (22), 91 (100); exact mass calcd for MH^+ $C_{15}H_{18}NO_6$ requires 308.1134, found 308.1133 (CI, C_4H_8).

(2*S*,3*R*)-2-Amino-3-*n*-propylsuccinic Acid (19). The N-protected diacid **18** (88 mg, 0.29 mmol) was dissolved in MeOH (5 mL) together with dry cyclohexene (2.5 mL). Pd/C (10%) (38 mg) was added and the reaction mixture refluxed for 75 min. Filtration through Celite, followed by removal of the solvent under reduced pressure, yielded the desired aspartic acid analogue **19** as an off-white gum: 21 mg, 42%; IR ($CHCl_3$) *ν* 3668−2698, 3408, 3017, 2625, 1714, 1634, 1516, 1505, 1410 cm^{-1}; ^1H NMR (D_2O, 300 MHz) *δ* 0.93 (3 H, t, *J* = 7.2 Hz), 1.35−1.48 (2 H, m), 1.52−1.65 (1 H, m), 1.68−1.70 (1 H, m), 3.03−3.11 (1 H, m), 3.94 (1 H, d, *J* = 4.3 Hz); ^{13}C NMR (D_2O, 75.4 MHz) *δ* 12.93 (t), 12.95 (t), 19.93 (d), 19.95 (d), 30.61 (q), 177.55 (s), 182.52 (s); MS (FAB: glycerol + 5% TFA) *m/e* (relative intensity) 176 (MH^+, 100), 155 (21), 130 (9), 93 (19); FAB HRMS exact mass calcd for MH^+ $C_7H_{14}NO_4$ requires 176.0923, found 176.0920 (FAB, glycerol + 5% TFA).

Dibenzyl (2*S*)-2-(Benzyloxycarbonylamino)succinate (20). The dibenzyl ester of L-aspartic acid, as its *p*-toluenesulfonate salt (1 g, 2 mmol) was added to a solution of dioxane (16 mL) and water (32 mL) containing Na_2CO_3 (1.24 g, 10 mmol). After cooling the reaction mixture to 0 °C, neat benzyl chloroformate (1.43 mL, 10 mmol) was added. The reaction was warmed to rt and stirred for 18 h. Water (30 mL) was added, and the resulting solution was extracted with Et_2O (5 × 15 mL). The aqueous portion was then acidified to pH 1 with HCl and extracted using EtOAc (5 × 25 mL). The organic extracts were then combined and dried, and the solvent was removed under reduced pressure to give an oil. Excess benzyl alcohol was then removed by vacuum distillation to give the desired N-protected diester **20** as a clear oil which solidified to a white solid on cooling: 871 mg; 95%; mp 61−63 °C (lit.[52] 61−63 °C); $[\alpha]^{22}_D$ +14.1° (*c* = 6.9, $CHCl_3$); IR ($CHCl_3$) *v* 3429, 3357, 3033, 1733, 1506, 1456, 1338 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.82−3.15 (2 H, ABX q, J_{AB} = 17.1 Hz, J_{AX} = 4.5 Hz, J_{BX} = 4.5 Hz), 4.69 (1 H, m), 5.05 (2 H, s), 5.11 (2 H, s), 5.13 (2 H, s), 5.80 (1 H, d, *J* = 8.4 Hz), 7.3−7.36 (15 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 36.76 (t), 50.57 (d), 66.83 (t), 67.12 (t), 67.54 (t), 126.97 (d), 127.56 (d), 128.06 (d), 128.19 (d), 128.25 (d), 128.33 (d), 128.44 (d), 128.52 (d), 128.59 (d), 131.27 (s), 135.15 (s), 135.32 (s), 140.99 (s), 156.00 (s), 170.50 (s); MS (FAB NBA) *m/e* (relative intensity) 448 (MH^+, 11), 404 (7), 181 (7), 136 (11), 91 (100); exact mass calcd MH^+ for $C_{26}H_{26}NO_6$ requires 448.1760, found 448.1700 (FAB, NBA). Anal. ($C_{26}H_{25}NO_6$) C, H, N, requires C 69.79, H 5.63, N 3.13, found C 69.70, H 5.64, N 3.03.

Benzyl (2*S*,3*R*)-2-(Benzyloxycarbonylamino)-3-carbobenzoxyhex-5-enoate (21). A solution of LiHMDS (3.8 mL of a 1.0 M solution in THF, 3.80 mmol) dissolved in dry THF (10 mL) was cooled to −80 °C before the addition of diester **20** (0.77 g, 1.72 mmol) dissolved in dry THF (30 mL) to give a pale-yellow solution. The reaction mixture was stirred at −78 °C for 30 min and then warmed slowly to −30 °C and stirred for 2 h to ensure formation of the dianion. After recooling the resulting orange solution to −78 °C, neat allyl bromide (0.75 mL, 8.6 mmol) was added at such a rate that the solution temperature did not exceed −75 °C. Stirring was continued for 16 h, at −78 °C, before the reaction was quenched by pouring it into aqueous 1 M HCl (120 mL) at rt. After extraction using Et_2O (4 × 50 mL), the organic phases were dried ($MgSO_4$), and the solvent was evaporated under reduced pressure. The crude material was purified by flash chromatography (eluant: 70:30 40−60 °C petroleum ether/EtOAc) to yield a clear oil, which was a mixture of epimers at C-3, followed by starting diester **20** (236 mg, 31%). However, the oil solidified on standing and careful recrystallization from petroleum ether/EtOAc gave the desired allylated diester **21** as a single diastereoisomer: 476 mg, 57%; mp 82−83 °C; $[\alpha]^{20}_D$ +29.4° (*c* = 2.3, $CHCl_3$); IR ($CHCl_3$) *v* 3428, 3019, 1733, 1507, 1456 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.26−2.38 (1 H, m), 2.47−2.58 (1 H, m), 3.21−3.29 (1 H, m), 4.68 (1 H, dd, *J* = 9.9, 3.7 Hz), 5.04−5.14 (8 H, m), 5.68−5.84 (2 H, m), 7.25−7.36 (15 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 32.86 (t), 46.56 (d), 54.12 (d), 66.93 (t), 67.14 (t), 67.39 (t), 118.35 (t), 128.00 (d), 128.06 (d), 128.13 (d), 128.22 (d), 128.40 (d), 128.46 (d), 128.51 (d), 128.55 (d), 128.60 (d), 133.99 (d), 135.29 (s), 135.33 (s), 136.34 (s), 156.55 (s), 170.76 (s), 172.76 (s); MS (FAB) *m/e* (relative intensity) 488 (MH^+, 100), 444 (58), 308 (19), 181 (96); exact mass calcd for MH^+ $C_{29}H_{30}NO_6$ requires 488.2073, found 488.2082 (FAB). Anal. ($C_{29}H_{29}NO_6$) C, H, N requires C 71.44, H 6.00, N 2.87, found C 71.20, H 5.98, N 2.82.

Dibenzyl (2*S*,3*R*,5*S*)-1-(Benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate (22) and Dibenzyl (2*S*,3*R*,5*R*)-1-(Benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate (23). OsO_4 (1.8 mL of a 2.5% solution in 2-propanol, 0.144 mmol) and $NaIO_4$ (1.39 g, 6.5 mmol) were added to a solution of diester **21** (960 mg, 2.16 mmol) in MeOH (40 mL) and water (20 mL). The resulting reaction mixture was stirred at rt for 75 min. After halving the solvent volume under reduced pressure, the solution was poured into water (140 mL) and extracted with EtOAc (6 × 50 mL). The organic extracts were dried ($MgSO_4$), and the solvent was removed under reduced pressure to yield the crude product as a brown oil. This material was purified by flash chromatography (eluant: 30% EtOAc/petroleum ether) to yield a mixture of the diastereoisomers **22** and **23**, which solidified on standing (214 mg, 22%). ^1H NMR analysis showed that this material was a 9:1 ratio of **23**:**22**, which was used in the subsequent reduction reaction. Careful column chromatography gave a small amount of pure diester **23**: mp 71−72 °C; $[\alpha]^{20}_D$ +31.3° (*c* = 0.64, $CHCl_3$); IR ($CHCl_3$) *v* 3446, 3019, 1749, 1734, 1715, 1699, 1558, 1540, 1507, 1457, 1419 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.05−2.15 (1 H, m), 2.48−2.62 (1 H, m), 3.65−3.80 (1 H, m), 3.83 (1 H, br s), 4.68−4.78 (1 H, m), 4.80−5.20 (6 H, m), 5.72 (1 H, d, *J* = 5.6 Hz), 7.16−7.22 (5 H, m), 7.25−7.36 (10 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 34.73 (t), 44.43 (d), 60.96 (d), 67.18 (t), 67.41 (t), 67.62 (t), 81.43 (d), 127.76 (d), 128.06 (d), 128.14 (d), 128.26 (d), 128.35 (d), 128.47 (d), 128.50 (d), 128.68 (d), 128.78 (d), 135.13 (s), 135.18 (s), 135.74 (s), 154.31 (s), 169.54 (s), 169.69 (s); MS (FAB NBA) *m/e* (relative intensity) 490 (MH^+, 2), 472 (30), 428 (100), 338 (15); HRMS exact mass calcd for MH^+ $C_{28}H_{28}NO_7$ requires 490.1866, found 490.1858.

Dibenzyl (2*S*,3*R*)-1-(Benzyloxycarbonylamino)pyrrolidine-2,3-dicarboxylate (24). The mixture of diastereoisomeric diesters **22** and **23** (200 mg, 0.4 mmol) and Et_3SiH (0.1 mL, 0.59 mmol) were dissolved in dry $CHCl_3$ (5 mL). Neat TFA (0.31 mL, 4.0 mmol) was then added dropwise to this solution with vigorous stirring. Upon completion of the addition, the reaction mixture was stirred at rt for a further 40 min before the solvent volume was reduced under reduced pressure. This gave an oily residue which was redissolved in EtOAc (20 mL). After washing with 5% aqueous $NaHCO_3$ (3 × 5 mL), the organic layer was dried ($MgSO_4$) and the solvent removed to yield **24** as a pale-yellow oil: 171 mg, 89%; $[\alpha]^{20}_D$ +14.7° (*c* = 4.0, $CHCl_3$); IR ($CHCl_3$) *v* 3065, 3034, 2957, 2894, 1748, 1716, 1700, 1498, 1456, 1418, 1361, 1188 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 2.08−2.20 (1 H, m), 2.46−2.52 (1 H, m), 3.24−3.40 (1 H, m), 3.40−3.54 (1 H, m), 3.74−3.86 (1 H, m), 4.71 (1 H, d, *J* = 8.4 Hz), 4.75−5.15 (6 H, m), 7.18−7.35 (15 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 26.55 (t), 45.68 (t), 46.83 (d), 60.73 (d), 66.99 (t), 67.13 (t), 67.22 (t), 127.83 (d), 127.98 (d), 128.39 (d), 128.45 (d), 128.51 (d), 135.24 (s), 136.74 (s), 136.87 (s), 154.11 (s), 169.77 (s), 169.89 (s); MS (FAB NBA) *m/e* (relative intensity) 474 (MH^+, 100), 430 (67), 338 (33), 294 (35), 181 (45); exact mass calcd for MH^+ $C_{28}H_{28}NO_6$ requires 474.1917, found 474.1942 (FAB, NBA).

(2*S*,3*R*)-Pyrrolidine-2,3-dicarboxylic Acid (25). The N-protected diester **24** (160 mg, 0.34 mmol) was dissolved in a mixture of MeOH (10 mL) and cyclohexene (1 mL) together with Pd/C (50 mg). After refluxing for 3 h the reaction was cooled to rt, poured into water (100 mL), and stirred vigorously for a further hour. The resulting reaction mixture was then filtered through Celite. After repeated washing with water, the filtrate was collected and its volume reduced to 30 mL before lyophilization. This procedure yielded the crude diacid **25** as a hygroscopic white solid. This material was redissolved in a minimum amount of water and purified by chromatography on Bio Rad Ag-1-X2 anion-exchange resin (10 g wet weight) by elution with 1% AcOH.[42] Ninhydrin positive fractions were collected and lyophilized to give the desired diacid **25** as a white solid: mp > 300 °C dec (lit.[42] 332 °C dec); IR (KBr) v 3600−2800, 1702, 1637, 1416, 1101 cm^{-1}; ^1H NMR (D_2O, 500 MHz) δ 2.10−2.20 (1 H, m), 2.25−2.30 (1 H, m), 3.30−3.45 (3 H, m), 4.20 (1 H, d, *J* = 7.3 Hz); ^{13}C NMR (D_2O, 125.6 MHz) δ 26.38 (t), 42.09 (t), 43.80 (d), 61.95 (d), 169.70 (s), 174.62 (s); MS (FAB glycerol + TFA 5%) *m/e* (relative intensity) 160 (MH^+, 66), 130(17), 115 (100), 104 (25), 102 (37); exact mass calcd for MH^+ $C_6H_{10}NO_4$ requires 160.061, found 160.058 (FAB, NBA).

Dibenzyl (2*S*,3*R*)-1-(Benzyloxycarbonyl)pyrrolidine-2,3-dicarboxylate (24) and Dibenzyl (2*S*,3*R*)-1-(Benzyloxycarbonyl)pyrrolidine-2,3-dicarboxylate (26). A mixture of dibenzyl (2*S*,3*R*,5*R*)-1-(benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate, dibenzyl (2*S*,3*R*,5*S*)-1-(benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate, dibenzyl (2*S*,3*S*,5*R*)-1-(benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate, dibenzyl (2*S*,3*S*,5*S*)-1-(benzyloxycarbonyl)-5-hydroxypyrrolidine-2,3-dicarboxylate (872 mg), and Et_3SiH (0.45 mL, 2.82 mmol) were dissolved in dry $CHCl_3$ (25 mL) at rt.

Neat TFA (1.4 mL, 18.2 mmol) was then added dropwise to this solution with vigorous stirring. Upon completion of the addition, the reaction mixture was stirred at rt for a further 90 min before the solvent volume was reduced under reduced pressure. This gave an oily residue which was redissolved in EtOAc (20 mL). After washing with 5% aqueous $NaHCO_3$ (2 × 5 mL), the organic layer was dried ($MgSO_4$) and the solvent removed to give a mixture of the diastereoisomeric pyrrolidines **24** and **26** as a pale-yellow oil. These compounds could be separated by chromatogrpahy on silica using a gravity column (eluant: 3:1 40−60 °C petroleum ether/EtOAc). Diester **24** was obtained first from the column (401 mg, 47%) as a clear oil, with identical properties to the material prepared from purified **21**. Further elution then gave the diastereoisomeric diester **26** as a clear oil: 177 mg, 20%; $[\alpha]^{20}_D$ +28.8° (c = 1.7, $CHCl_3$); IR (neat) υ 3065, 3034, 2958, 2893, 1715, 1608, 1587, 1497, 1456, 984 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 3.20−3.32 (1 H, m), 3.66−3.74 (2 H, m), 4.85−4.92 (1 H, dd, J = 18.8, 3.4 Hz), 5.1−5.3 (7 H, m), 7.15−7.25 (15 H, m); ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ 27.1 (t), 45.4 (t), 47.9 (d), 61.5 (d), 66.9 (t), 127.5 (d), 126.6 (d), 127.7 (d), 127.8 (d), 127.9 (d), 128.0 (d), 128.05 (d), 128.2 (d), 128.25 (d), 128.3 (d), 128.4 (d), 135.0 (s), 135.2 (s), 136.1 (s), 136.2 (s), 153.7 (s), 154.4 (s), 170.5 (s), 171.1 (s), 171.2 (s); MS (FAB NBA) *m/e* (relative intensity) 474 (MH^+, 12), 430 (10), 338 (2), 294 (1), 181 (8); exact mass calcd for MH^+ $C_{28}H_{28}NO_6$ requires 474.1917, found 474.1918 (FAB, NBA).

(2*S*,3*S*)-Pyrrolidine-2,3-dicarboxylic Acid (16). Pd/C (10%) (50 mg) was suspended in a solution of the N-protected diester **26** (175 mg, 0.32 mmol) and NH_4OCHO (560 mg, 8.9 mmol) dissolved in MeOH (11 mL). After stirring at rt for 210 min, the suspension was filtered through Celite and the solvent removed under reduced pressure to give an oily residue. Lyophilization then gave the crude diacid **25**, which was purified using anion-exchange chromatography as described above.

General Procedure for Inhibitor Assays. Inhibition constants for **8**, **9**, **25**, and **26** were determined using standard methods. All assays were performed using 2.3−5 μg of wild-type AS-B in a reaction buffer composed of 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 8. In experiments to determine the extent of AS-B inhibition with respect to aspartate, ATP, and glutamine were fixed at 1 mM and the concentration of aspartic acid varied (0.1−1.1 mM). Inhibitor concentrations were the following: **8**, 0, 2, 6, 10, 15, and 20 mM; **9**, 0, 0.5, 1, 1.5, and 3 mM; **25**, 0, 1, 2.5, and 5 mM; **26**, 0, 0.5, 1, and 1.5 mM. In experiments to determine the extent of AS-B inhibition with respect to ATP, aspartate and glutamine were fixed at 1 mM and the concentration of ATP varied (0.075−0.3 mM). Inhibitor concentrations were the following: **8**, 0, 2.5, 5, and 7.5 mM; **9**, 0, 0.5, 1, and 1.5 mM; **25**, 0, 1, 2.5, and 5 mM. In experiments to determine the extent of AS-B inhibition with respect to glutamine, ATP and aspartate were fixed at 1 mM and the concentration of glutamine varied (0.1−0.85 mM). Inhibitor concentrations were the following: **8**, 0, 10, 15, and 20 mM; **9**, 0, 0.5, 1, and 1.5 mM; **25**, 0, 2.5, 5, and 7.5 mM. Inhibition constants were obtained from double-reciprocal plots using initial velocities that were the average of those measured in duplicate assays. The rate of asparagine synthesis was determined using a coupled assay for the detection of pyrophosphate (Sigma Bulletin No. B1-100).

FSBA Inactivation of AS-B. A stock solution of 10 mM FSBA in 1:1 EtOH/DMSO was used in all inactivation experiments. The concentration was verified by measuring the absorption of this solution at 259 nm (ϵ 1.35 × 10^4 M^{-1} cm^{-1}). Enzyme inactivation was carried out by incubating AS-B (28 μg) in 100 mM Tris, pH 8, with FSBA at rt in solutions (total volume 140 μL) containing substrate(s) and/or the aspartate analogs (Table 2). Incubations proceeded in solutions containing 1 mM FSBA, 0.2 μg/μL AS-B, 8 mM $MgCl_2$, and 100 MM Tris-HCl, pH 8, together with 5% EtOH and 5% DMSO. Control solutions had an identical composition except that FSBA was omitted. At a given time-point, 20 μL aliquots were withdrawn from each of the incubations and diluted into a reaction buffer (10 mM L-aspartate, 10 mM glutamine, 10 mM ATP, 8 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8) containing a coupling reagent for the detection of pyrophosphate (Sigma Bulletin No. B1-100) to a final volume of 140 μL. The reaction buffer (10 mM Gln, 10 mM Asp, 10 mM ATP, 15 mM was prewarmed to 37 °C. The ability of the FSBA-treated AS-B to catalyze asparagine synthesis was then determined by measuring the amount of pyrophosphate produced in 5 min by monitoring the reduction of NAD^+ to NADH by glutamate dehydrogenase. In control solutions from which FSBA was omitted, AS-B retained greater than 95% of its original activity over the entire duration of the incubation experiments.

Table 2. Substrate Concentrations Employed in Experiments To Determine the Ability of AS-B-Selected Substrates and the Aspartate Analog **7** To Protect the Enzyme against Covalent Modification by FSBA

	substrate/analog concentration (mM)			
experiment no.	ATP	Asp	**7**	FSBA
1	0.0	0.0	0.0	1.0
2	0.05	0.0	0.0	1.0
3	0.05	0.0	5.0	1.0
4	0.0	0.0	5.0	1.0
5	0.0	1.0	0.0	1.0
6	0.05	1.0	0.0	1.0

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