Preparation of N-substituted aspartic acids *via* enantiospecific conjugate addition of N-nucleophiles to fumaric acids using methylaspartase: synthetic utility and mechanistic implications[1]

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A range of new N-substituted aspartic and 3-alkylaspartic acids are prepared *via* the enantiospecific conjugate addition of substituted amines to fumaric acids using the enzyme methylaspartase. The stereochemical courses of the additions to mesaconic acid are determined for hydrazine and hydroxylamine. Each addition is found to follow the same course as that for ammonia, the natural nucleophile for the enzyme; *anti*-addition to the 3-*si*-face of mesaconic acid gives the (2*S*,3*S*)-3-methylaspartic acid derivative. The addition of hydrazine, methylamine, hydroxylamine and methoxylamine occurs in excellent conversion and the resulting hydrazino- and methylamino-succinic acids can be isolated for full characterisation. The corresponding hydroxyamino- and methoxyamino-succinic acids are recovered as unstable oils. The size of the substituent on the N-nucleophile tolerated by the enzyme displays a profound dependence on the size of the substituent on the acceptor fumaric acid in a mutually exclusive manner. This finding indicates that the two substituents are able to access close regions in three-dimensional space at the active site of the enzyme.

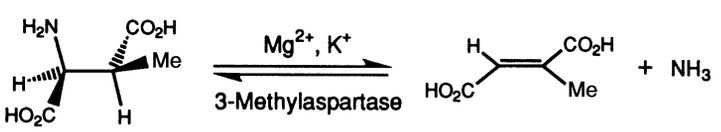
Introduction

3-Methylaspartate ammonia-lyase (methylaspartase: EC 4.3.1.2) catalyses the reversible α,β-elimination of ammonia from (2*S*,3*S*)-3-methylaspartic acid **1** to give mesaconic acid (≡ methylfumaric acid) **2**[2] in clostrida and other bacteria (Scheme 1).[3]

Previously it has been demonstrated that the enzyme is useful in providing access to various 3-substituted aspartic acids *via* the direct amination of substituted fumaric acids.[4,5] These studies led us to investigate the mechanism of the reaction catalysed by the enzyme and to discover that, contrary to previous claims,[6,7] a solvent-accessible carbanion intermediate does not exist.[8,9,10] Indeed, evidence was obtained from $^{15}N/^{14}N$ isotope fractionation and $^{15}N/^{14}N–H/^{2}H$ double isotope fractionation experiments to indicate that the elimination reaction occurred *via* a concerted process.[9] Further work showed that the deamination of the *erythro*-isomer of the natural substrate (2*S*,3*R*)-3-methylaspartic acid[11] displayed a large primary deuterium isotope effect on *V* and *V*/*K* and occurred without the exchange of labelled hydrogen between C-3 and the solvent.[12] In order to account for many of the kinetic properties of this enzyme-catalysed reaction that were not consistent with a simple single step deamination process,[10] we proposed that the substrate might form a covalent link to the enzyme, *via* a conjugate addition to a dehydroalanine residue, prior to the key deamination step.[13] Further support for this notion was obtained from two studies. First it was shown that hydrazines and hydroxylamines inhibit the enzyme, essentially irreversibly, but that protection is afforded in the presence of substrates. Second, when the enzyme was sequenced[13] it was discovered that the hydroxy group of Ser-173, after pretreatment of the enzyme with substrate, behaved more like an amino group in undergoing a conjugate addition reaction with *N*-alkylmaleimide.[14] Supporting model chemical studies showed that amines but not alcohols undergo facile addition to *N*-alkylmaleimides and suitably N-protected dehydroalanine esters and amides.[15] Thus, in common with some other ammonia-lyases, including phenylalanine and histidine ammonia-lyase,[16,17] the active site of methylaspartase is now believed to contain a dehydroalanine residue to which the amino group of amino acid substrates adds [to form covalent C–N enzyme–substrate adducts (see the reverse of Scheme 2)] prior to the deamination of these substrates. Note that all three ammonia-lyases for methylaspartic acid,[13] phenylalanine and histidine share the active-site motif Ser-Gly-Asp, where the Ser residue is later dehydrated to give the electrophilic dehydroalanine residue.[18,19]

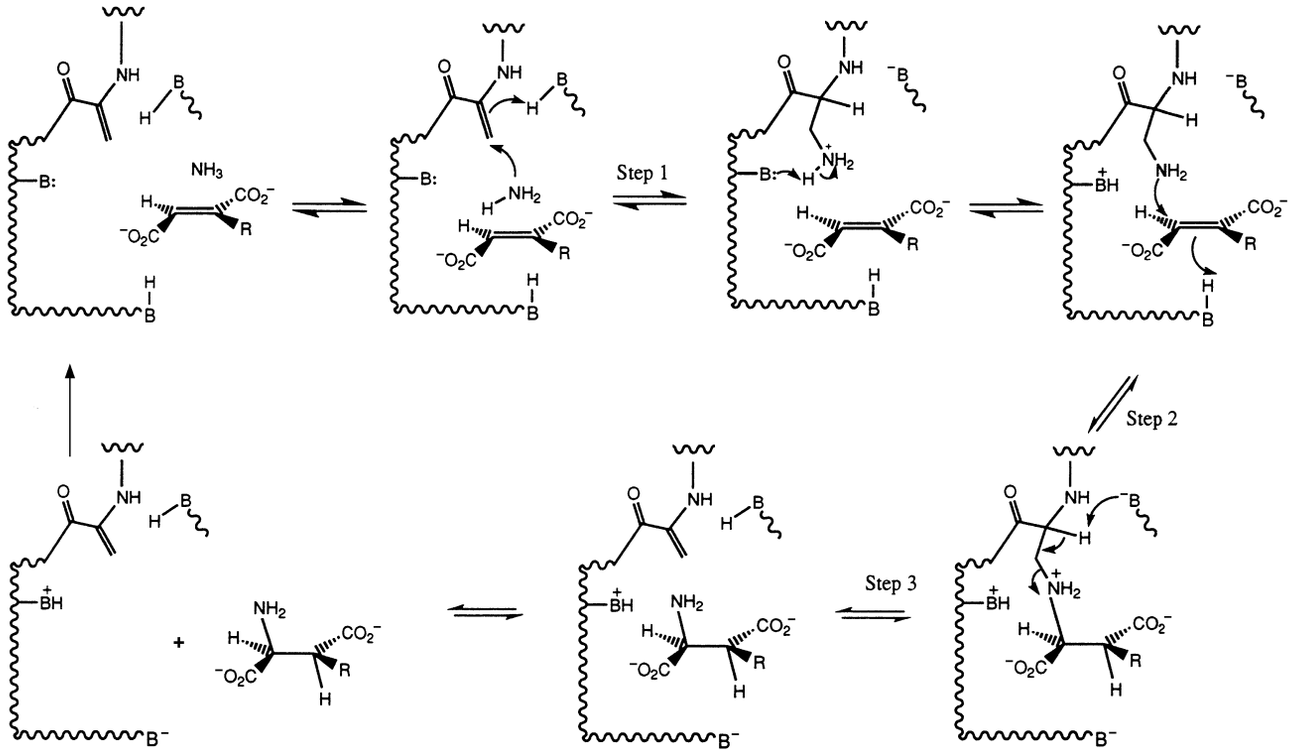
Results and discussion

The earlier finding that hydrazine, hydroxylamine and phenylhydrazines could also react with the dehydroalanine residue in methylaspartase to cause irreversible inhibition prompted us to attempt to isolate the adduct.[20] However, when ^{14}C-labelled *p*-nitrophenylhydrazine[1] was reacted with the enzyme, the radiolabelled fragment slowly dissociated from the protein during dialysis. This result indicated that the inhibition of the enzyme was not completely irreversible and that, for small hydrazines which would allow the simultaneous co-occupation of the active site by Michael acceptors, it might be possible to synthesise hydrazino acids.



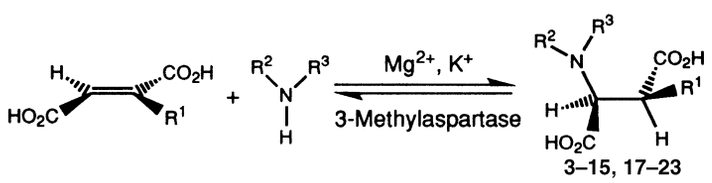
1 **2**

Scheme 1



Scheme 2

Table 1 Summary of enzyme catalysed addition reactions



3–15, 17–23

Compound	R^1	R^2	R^3	Conversion (%)	Yield[a] (%)
3	Me	NH_2	H	91	61
4	H	NH_2	H	89	42
5	Cl	NH_2	H	*b*	—
6	Et	NH_2	H	90	57
7	Pr^i	NH_2	H	90	33
8	Pr	NH_2	H	90	31
9	H	Me	H	55	45
10	Me	Me	H	54	40
11	Et	Me	H	60	35
12	Pr	Me	H	*c*	—
13	Pr^i	Me	H	*c*	—
14	H	Et	H	5	—
15	H	Me	Me	70	28
17	Me	Me	Me	*c*	—
18	H	OH	H	90	28
19	Me	OH	H	90	19
20	Et	OH	H	60	12
21	H	OMe	H	80	31
22	Me	OMe	H	70	34
23	Et	OMe	H	*c*	—

[a] See Experimental section for full details. [b] See text. [c] No reaction.

To test this idea, hydrazine and mesaconic acid were incubated at pH 9.0 with methylaspartase in the presence of the potassium and magnesium ions, and the reaction was monitored by the periodic removal of aliquots of the solution for analysis by ^{1}H NMR spectroscopy. After several hours new signals appeared at δ 3.55 (1 H, d, H^{α}), 2.62 (1 H, m, H^{β}) and 0.87 (3 H, d, β-CH_3). After 3 days, the reaction was essentially complete and *ca.* 90% of the starting material had been converted (see Table 1). ^{1}H NMR Spectroscopy of the crude reaction mixture showed that only unreacted starting material and a single diastereoisomer of a new product were present. The 2-hydrazino-3-methylsuccinic acid **3** was obtained as a crude white precipitate containing hydrazine salts after acidification of the concentrated incubation solution. Recrystallisation from water gave the required pure material in 61% yield {mp 169–172 °C; $[a]_D^{22}$ −18.6 (*c* 0.6 in 6 mol dm^{-3} HCl)} which displayed

all of the expected spectroscopic and analytical properties. Thus, the enzyme was able to catalyse the addition of hydrazines to fumaric acids.

Methylaspartase is able to reversibly deaminate both (2*S*,3*S*)-3-methylaspartic acid and (2*S*,3*R*)-3-methylaspartic acid, the latter at a 100-fold lower rate.[12] Since the addition of hydrazine to mesaconic acid was very slow compared to the rate of addition of ammonia, we wished to verify that the absolute stereochemistry at C-3 of the 2-hydrazino-3-methylsuccinic acid **3** was, indeed, the same as for the preferred diastereoisomer of the natural substrate, (2*S*,3*S*)-3-methylaspartic acid. To determine the stereochemical course of the conjugate addition reaction, compound **3** was subjected to catalytic reduction to cleave the N–N bond. The product was isolated and recrystallised and was found to be (2*S*,3*S*)-3-methylaspartic acid, identical in all respects to an authentic sample prepared by the direct addition of ammonia to mesaconic acid.[3] Therefore, the addition of hydrazine to mesaconic acid **2** follows the same stereochemical course as the addition of ammonia and occurs from the 3-*si*-face in an *anti*-fashion.

In order to determine the scope of the stereospecific addition reaction, the N-nucleophile and the structure of the Michael acceptor were varied. In a similar enzymic hydrazine addition experiment, performed using fumaric acid as the Michael acceptor, the known (2*S*)-2-hydrazinosuccinic acid **4** was formed in 89% yield and was isolated in 42% yield {mp 123–124 °C (lit.,[21,22] 109 and 116–118 °C); $[\alpha]_D^{22}$ −14.4 (*c* 1 in H_2O) [lit.,[21,22] −14.2 (*c* 1 in H_2O)]}. Note that the stereochemical course of the reaction, with respect to C-2 of the product, was identical to that for the methyl homologue described above.

The methylaspartase-catalysed addition of ammonia to chloro- and bromo-fumaric acid[4,5] had been shown to proceed *via* a similar stereochemical course to that for methylfumaric acid to give (2*R*,3*R*)-3-halogenoaspartic acids (note the stereochemical priority changes). Although the bromoaspartic acid could not be isolated intact due to rapid aziridine formation and inhibition of the enzyme, the chloroaspartic acid could be isolated (from incubations containing large amounts of enzyme) and fully characterised. When chlorofumaric acid was treated with hydrazine, the disappearance of the starting material and the formation of the expected 2-hydrazino-3-chlorosuccinic acid **5** was detected by ^{1}H NMR spectroscopy [δ 4.62 (1 H, d, *J* 4.9, H–2), 3.82 (1 H, d, *J* 4.9, H–3)]. However, due to the relatively low rates for the addition of hydrazine compared to those for ammonia and due to the presumably enhanced rates of *N*-aminoaziridine formation and the inherent instability of such compounds, many side products formed and no 2-hydrazino-3-chlorosuccinic acid **5** could be isolated from the incubation solution.

Treatment of ethyl-, isopropyl- and propyl-fumaric acid with hydrazine in the presence of methylaspartase under similar conditions gave the expected hydrazino acid products **6**–**8** (Table 1) which were isolated and fully characterised. We assume, on the basis of the stereochemical course of the addition of hydrazine to fumaric and methylfumaric acid, that each of the products possessed (2*S*,3*S*) absolute stereochemistry. The 2-hydrazino-3-ethylsuccinic acid **6** was isolated in 57% yield.

Since hydrazine was an effective surrogate for ammonia, the possibility of using hydroxylamine, methoxylamine[23] and alkylamines as the nucleophiles in conjugate addition reactions was tested. Accordingly, fumaric acid was incubated with each of the nucleophiles and in each case reaction occurred to give the corresponding N-substituted aspartic acid derivative (Table 1). (2*S*)-*N*-Methylaspartic acid **9** was isolated in 45% yield from the reaction of methylamine with fumaric acid as the monohydrate {mp 189–191 °C; $[\alpha]_D^{22}$ +14.4 (*c* 2 in H_2O)}. All spectral and analytical data were consistent with the expected structure and the known properties of the D-antipode, better known as NMDA {(lit.,[24] mp 189–190 °C); lit.,[24] $[\alpha]_D^{22}$ −15.4 (*c* 0.5 in H_2O)}. The higher homologues, *N*,3-dimethylaspartic acid **10** and *N*-methyl-3-ethylaspartic acid **11** were formed similarly from methyl- and ethyl-fumaric acid but, interestingly, both propyl- and isopropyl-fumaric acid failed to react with methylamine to give the corresponding amino acids **12** and **13**. The enzyme was able, however, to catalyse the addition of ethylamine to fumaric acid. The reaction occurred very slowly, as expected, on account of the steric constraints of the active site. The product was not isolated, due to its low final concentration in the incubation solution, but was characterised by ^{1}H and ^{13}C NMR spectroscopy (see Experimental section).

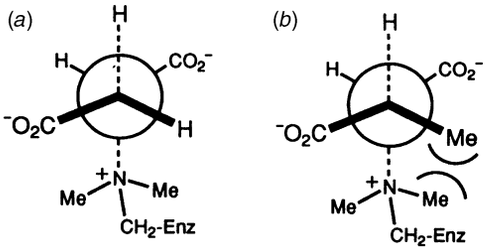


Fig. 1 Expected geometry for the transition states for the enzymic addition of dimethylamine to (*a*) fumaric acid and (*b*) mesaconic acid

Remarkably, the enzyme was also able to catalyse the addition of dimethylamine to fumaric acid to give the expected product, (2*S*)-*N*,*N*-dimethylaspartic acid **15**. This was isolated in 28% yield {mp 196–198 °C (lit.,[25] 198 °C); $[\alpha]_D^{22}$ −2.0 (*c* 0.6 in 6 mol dm^{-3} HCl)} and showed the expected spectral and analytical properties. Interestingly, while fumaric acid was able to support this reaction, the amination of methylfumaric acid with dimethylamine failed (see below).

The reactions of fumaric acid and methylfumaric acid with hydroxylamine and methoxylamine proceeded smoothly to give the expected addition products, as judged by NMR spectroscopy (see Experimental section), but in each case the compounds could not be crystallised for full characterisation and were isolated as unstable viscous oils. The stereochemical courses of the addition of hydroxylamine to methylfumaric acid and methoxylamine to fumaric acid were determined by reduction of the substituted aspartic acid products. In each case the expected stereochemical outcome was obtained. The enzymic addition of methoxylamine to ethylfumaric acid did not occur (Table 1).

The rate of the additions varied widely for different substrate pairs. However, in general, hydrazine and hydroxylamine were found to be the fastest substrates and dimethylamine the slowest. Curiously, we have found that for incubations involving hydrazine and hydroxylamine, little starting material remains relative to the situation for the addition of simple amines, including ammonia. These observations suggest that the reverse (elimination) reactions occur very slowly and that the equilibria favour the addition products.

The finding that the enzyme was able to catalyse the addition of dimethylamine to fumaric acid to give (2*S*)-*N*,*N*-dimethylaspartic acid **15** is quite remarkable [Fig. 1(*a*)]. Methylfumaric acid failed to serve as a co-substrate in this reaction. Insufficient space at the active site, and in particular unfavourable steric interactions between the *N*-methyl groups and the alkyl group of the substituted fumaric acid, probably account for the fact that the larger compounds were unable to act as Michael acceptors, [Fig. 1(*b*)]. Indeed, both propyl- and isopropylfumaric acid, which are known to react with ammonia to give the corresponding 3-alkylaspartic acids, failed to react with methylamine although the smaller co-substrate, ethylfumaric acid, did react with methylamine. Similarly, ethylfumaric acid, which is known to react with ammonia[5] and shown here to react with methylamine, failed to react with the slightly larger nucleophile, methoxylamine, although the reaction of methoxylamine with both fumaric acid and methylfumaric acid

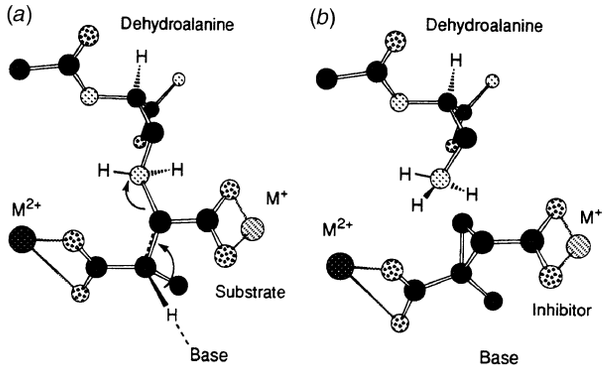


Fig. 2 (*a*) Expected transition state for C–N bond cleavage in the substrate. (*b*) Possible interaction of (1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid with the enzyme

was facile (Table 1). Thus, the active-site space seems to be quite accessible to each of the substrates in a mutually exclusive fashion.

These findings suggest that there are regions of the active-site-space that are accessible to the groups attached to each of the substrates; that is, the substituents on the N-nucleophile and the C-2 alkyl side-chains of fumaric acid Michael acceptors. This analysis is consistent with the active-site geometry expected for the transition state for the elimination of the amino-enzyme from the covalent aspartic acid derivative (reverse step 2, Scheme 2). Here, in order for the C-3 proton and the amino group nucleofuge to exist in an antiperiplanar arrangement, the N-nucleofuge and the alkyl side chain of the aspartic acid must reside on the same face of the plane of the double bond in the fumaric acid product [Fig. 2(*a*)].

The transition state analogue (1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid [Fig. 2(*b*)] is by far the best competitive inhibitor known for methylaspartase ($K_i = 20$ μmol dm^{-3}). The efficacy of the inhibitor is markedly increased in the presence of ammonia, indicating that both species can bind to the enzyme simultaneously.[26] The expected mode of binding for this inhibitor [Fig. 2(*b*)] places the cyclopropyl methylene group very close to the amino group of the aminoenzyme. This arrangement is in accord with the findings reported here, which indicate that up to two extra heavy atoms can fit into the space available for the amino group. Compounds which can, within the same molecule, provide an N-nucleophile and the geometry of the cyclopropane-1,2-dicarboxylic acid are likely to be tight-binding inhibitors of utility in trapping the electrophilic dehydroalanine prothetic group.

The stereochemical and steric size information gained from this study also refines our three-dimensional working model of the active site of the enzyme. There is good evidence to suggest that the divalent metal ion (normally Mg^{2+}) utilised by methylaspartase binds to the β-carboxy group of the substrate in order to enhance the acidity of the C-3 proton for elimination.[27] In phenylalanine and histidine ammonia-lyase this C-3 proton is benzylic and no divalent metal ion cofactor is required. The antiperiplanar arrangement of the C-3 hydrogen and the N-atom of the covalently bound substrate in all three enzyme systems places tight constraints on possible geometries and all three enzymes follow the same stereochemical course.[3] Comparison of the active-site peptides for the three enzymes shows a common motif, Ser-Gly-Asp, where the Ser residue becomes a modified 2,3-diaminopropanoic acid residue after the conjugate addition of the substrate to the enzyme. The conserved Asp residue is likely to play an important catalytic role—it is completely conserved in over a dozen different ammonia-lyase sequences[18]—and it is extremely well positioned to interact with the N-atom of the covalent complex. If we assume this role, the Ser-Gly-Asp triad forms part of a reverse turn where following residues in methylaspartase,

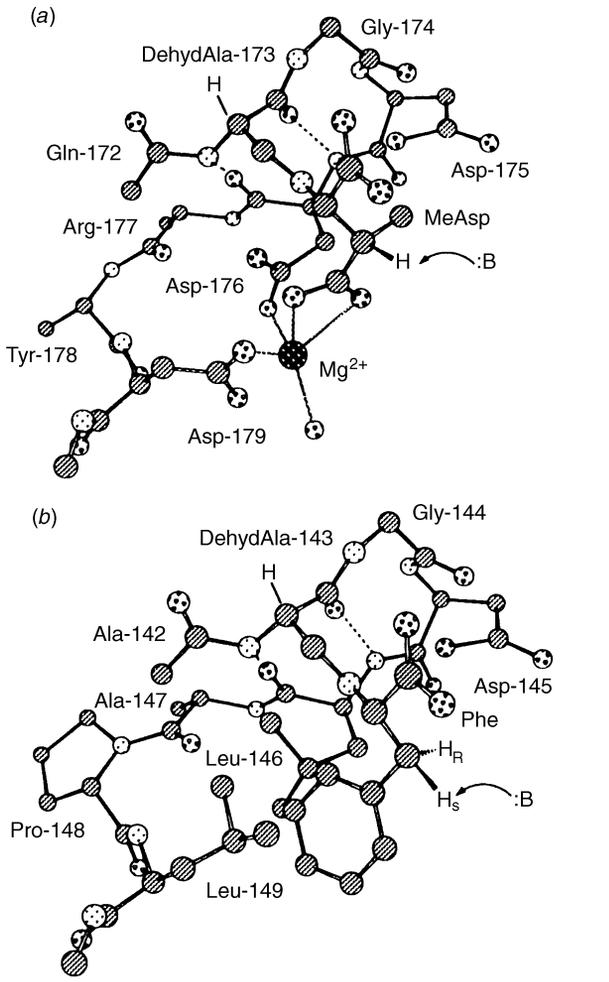


Fig. 3 Expected arrangement at the active site of (*a*) methylaspartase and (*b*) Phe ammonia-lyase showing the antiperiplanar arrangement of the C-3 hydrogen and N-atom of the substrate and a symmetrical arrangement for the aminated dehydroalanine moiety

phenylalanine and histidine ammonia-lyases are functionally compatible with the side-chain of the substrate (Fig. 3). For example, the sequence for methylaspartase[13] is Ser[173]-Gly-Asp-Asp-Arg-Tyr-Asp while the consensus sequence for thirteen different phenylalanine and histidine ammonia-lyases is Ser[143]-Gly-Asp-Leu-Xxx-Pro-Leu.[18] An aspartate residue is isosteric with a Leu residue and the size of the β-carboxy group of methylaspartic acid, together with the activating metal ion, is very similar to that for the β-phenyl group of phenylalanine and the β-imidazole group of histidine. Thus, a single conformation of the active-site peptides for each of the three enzymes would account for the known stereochemical and mechanistic features of each enzyme (Fig. 3). This speculative model, which is further constrained by Pro-148 in the case for phenylalanine ammonia-lyase, is a low energy structure for each of the active-site peptides and in the absence of X-ray crystal data is the only three-dimensional model. The arrangement is consistent with all available data and suggests that Asp-176 and Asp-179 form at least part of the divalent metal ion binding site in methylaspartase. Experiments to test these ideas are presently underway.

Experimental

Elemental microanalyses were performed in the departmental microanalytical laboratory. NMR Spectra were recorded on a Bruker AM-300 (^{1}H, 300 MHz; ^{13}C, 74.76 MHz) or a Varian gemini 200 (^{1}H, 200 MHz, ^{13}C, 50.3 MHz) spectrometer. Chemical shifts are described in parts per million relative to

$SiMe_4$ and are reported consecutively as position (δ_H or δ_C), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = double of doublets, sep = septet, m = multiplet and br = broad), coupling constant (Hz) and assignment (numbering according to the IUPAC nomenclature for the compound). ^{1}H NMR Spectra were referenced internally to ^{2}HOH (δ 4.68), C^2HCl_3 (δ 7.27) or $(C^2H_3)_2SO$ (δ 2.47). ^{13}C NMR Spectra were referenced on CH_3OH (δ 49.9), C^2HCl_3 (δ 77.5), or $(CH_3)_2SO$ (δ 39.70).

IR Spectra were recorded on a Perkin-Elmer 1710 FT-IR spectrometer. The samples were prepared as Nujol mulls or thin films between sodium chloride discs. The frequencies (ν) as absorption maxima are given in wavenumbers (cm^{-1}) relative to a polystyrene standard. Mass spectra and accurate mass measurements were recorded on a VG 70-250 SE, a Kratos MS-50 or by the SERC service at Swansea using a VG AZB-E. Fast atom bombardment spectra were recorded using glycerol as matrix. Major fragments are given as percentages of the base peak intensity (100%). UV Spectra were recorded on Pye-Unicam SP8-500 or SP8-100 spectrophotometers. $[\alpha]_D$ Values are given in units of 10^{-1} deg cm^2 g^{-1}.

Flash chromatography was performed according to the method of Still *et al.*[28] using Sorbsil C 60 (40–60 μm mesh) silica gel. Analytical thin layer chromatography (TLC) was carried out on 0.25 mm precoated silica gel plates (Macherey–Nagel SIL g/UV254) and compounds were visualised using UV fluorescence, iodine vapour, ethanolic phosphomolybdic acid or ninhydrin.

Ethyl-, propyl- and isopropyl-fumaric acids were prepared according to the method of Akhtar *et al.*[5]

Methylaspartase

Frozen cell paste (18.75 g), obtained from a recombinant *Escherichia coli* strain,[13] was thawed in 50 mol dm^{-3} potassium phosphate (37.5 cm^3, pH 7.6) containing 2-sulfanylethanol (1 mol dm^{-3}) and phenylmethanesulfonyl fluoride (0.2 cm^3 of a 34 mg cm^{-3} solution in propan-2-ol). After thawing, the solution was sonicated (140 W, 20 KHz) in 6 × 30 s bursts at 0–5 °C. Acetone (46 cm^3), precooled to −20 °C, was added with stirring over 5 min and stirring was continued at 4 °C for a further 5 min. The cell debris and precipitated protein were removed by centrifugation at 0 °C (37 000*g*, 30 min) to give the crude extract. A further portion of precooled acetone (55 cm^3) was added and, after stirring for an additional 10 min, the precipitated protein was collected by centrifugation at 0 °C (37 000*g*, 20 min). The protein pellet was resuspended in 50 mol dm^{-3} potassium phosphate (5.3 cm^3, pH 7.6) with swirling at 4 °C, and the insoluble material removed by centrifugation at 10 °C (37 000*g*, 2 min).

The protein was typically 1100 units cm^{-3} and was used in the enzyme incubations without any further purification.

(2*S*,3*S*)-2-Hydrazino-3-methylsuccinic acid 3

Aqueous hydrazine hydrate (70%) was added dropwise to a suspension of mesaconic acid (4.7 g, 36.2 mmol) in water (10 cm^3) until dissolution occurred. Water was then added until the total volume was 40 cm^3. The pH was adjusted to 9 with aqueous hydrazine hydrate, and magnesium chloride hexahydrate (80 mg), potassium chloride (14 mg) and finally 3-methylaspartase (120 units) were added. The reaction flask was then incubated at 30 °C in a water bath and aliquots (100 mm^3) of the incubation solution were removed periodically for analysis by ^{1}H NMR spectroscopy. After the reaction ceased (*ca.* 3 days), approximately 90% of the starting material had been consumed. The reaction mixture was concentrated to 10 cm^3 under reduced pressure to give a viscous oil which was acidified with concentrated hydrochloric acid to pH 4 to cause precipitation of the product. The precipitate was filtered and recrystallised from boiling water to give a white crystalline solid **3** (3.6 g, 61%), mp 169–172 °C (Found: C, 37.0; H, 6.45; N, 17.15. $C_5H_{10}N_2O_4$ requires C, 37.0; H, 6.25; N, 17.3%) (HRMS: found $[M + H]^+$, 163.0720. $C_5H_{11}N_2O_4$ requires 163.0719); $[\alpha]_D^{22}$ −18.6 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3261 (NH) and 1739 (CO); δ_H(200 MHz; ^2H_2O, p^{2}H 1) 0.84 (3 H, d, *J* 7.5, 3-CH_3), 2.85 (1 H, m, AB splitting, 3-H) and 3.83 (1 H, d, *J* 5.0 2-H); δ_C(50.3 MHz; ^2H_2O, p^{2}H 1) 12.30 (CH_3), 41.47 (3-CH), 62.74 (2-CH) and 174.46 and 177.92 (2 × CO_2H); *m*/*z* (CI) 163 (6%, $[M + H]^+$) and 145 (100, $[M - NH_3]^+$).

(2*S*)-2-Hydrazinosuccinic acid monohydrate 4

This compound was prepared in a manner identical to that described for (2*S*,3*S*)-2-hydrazino-3-methylsuccinic acid **3**, using fumaric acid (2.0 g, 17.2 mmol) to give the desired compound as a white crystalline solid (1.2 g, 42%), mp 123–124 °C (lit.,[21,22] 109 and 116–118 °C) (Found: C, 29.0; H, 6.35; N, 16.7. Calc. for $C_4H_{10}N_2O_5$: C, 28.9; H, 6.05; N, 16.85%); $[\alpha]_D^{22}$ −22.0 (*c* 0.6 in 6 mol dm^{-3} HCl) and −14.4 (*c* 1 in H_2O) [lit.,[21,22] −14.2 (*c* 1 in H_2O)]; ν_{max}(Nujol)/cm^{-1} 3285 and 3105 (NH) and 1705 (CO); δ_H(200 MHz; ^2H_2O, p^{2}H 1) 2.77 (2 H, d, *J* 7.5, 3-CH_2) and 3.92 (1 H, d, *J* 7.5, 2-H); δ_C(50.3 MHz; ^2H_2O, p^{2}H 1) 35.87 (3-CH_2), 57.68 (2-CH) and 174.75 and 175.04 (2 × CO_2H).

(2*S*,3*S*)-2-Hydrazino-3-ethylsuccinic acid 6

This compound was prepared in a manner identical to that described for (2*S*,3*S*)-2-hydrazino-3-methylsuccinic acid **3**, using ethylfumaric acid (2.0 g, 13.9 mmol) to give the desired compound. The product was further purified by a charcoal wash and recrystallisation to give an off-white powder (1.4 g, 57%), mp 189–192 °C (Found: C, 40.55; H, 6.75; N, 15.75. $C_6H_{12}N_2O_4$ requires C, 40.9; H, 6.9; N, 15.9%) (HRMS: found $[M + H]^+$, 177.0871. $C_6H_{13}N_2O_4$ requires 177.0875); $[\alpha]_D^{22}$ −13.9 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3344 and 3219 (NH) and 1700 (CO); δ_H(200 MHz; ^2H_2O, p^{2}H 1) 0.48 (3 H, t, *J* 8.3, CH_2CH_3), 1.5 (2 H, m, CH_2CH_3), 2.8 (1 H, m, 3-H) and 3.95 (1 H, d, *J* 4.2, 2-H); δ_C(50.3 MHz; ^2H_2O, p^{2}H 1) 12.25 (CH_3), 21.84 (CH_2), 48.79 (3-CH), 62.04 (2-CH) and 174.50 and 177.62 (2 × CO_2H); *m*/*z* (CI) 177 (98%, $[M + H]^+$) and 159 (93, $[M - NH_3]^+$).

(2*S*,3*S*)-2-Hydrazino-3-isopropylsuccinic acid 7

This compound was prepared in a manner identical to that described for (2*S*,3*S*)-2-hydrazino-3-methylsuccinic acid **3**, using isopropylfumaric acid (2.0 g, 12.7 mmol) to give the desired compound as an off-white powder (0.79 g, 33%), mp 193–195 °C (Found: C, 44.2; H, 7.4; N, 14.5. $C_7H_{14}N_2O_4$ requires C, 44.2; H, 7.4; N, 14.75%) (HRMS: found $[M + H]^+$, 191.1026. $C_7H_{15}N_2O_4$ requires 191.1032); $[\alpha]_D^{22}$ −42.0 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3347 and 3227 (NH) and 1703 (CO); δ_H(200 MHz; ^2H_2O, p^{2}H 1) 0.66 [3 H, d, *J* 6.6, 3-CH(CH_3)$_2$], 0.76 [3 H, d, *J* 6.6, 3-CH(CH_3)$_2$], 1.94 [1 H, m, 3-C*H*(CH_3)$_2$], 2.42 (1 H, t, *J* 6.6, 3-H) and 3.81 (1 H, d, *J* 6.4, 2-H); δ_C(50.3 MHz; ^2H_2O, p^{2}H 1) 19.82 [3-CH(CH_3)$_2$], 21.15 [3-CH(CH_3)$_2$], 27.81 [3-CH(CH_3)$_2$], 54.06 (3-CH), 60.74 (2-CH) and 174.51 and 177.12 (2 × CO_2H); *m*/*z* (CI) 191 (30%, $[M + H]^+$) and 173 (100, $[M - NH_3]^+$).

(2*S*,3*S*)-2-Hydrazino-3-propylsuccinic acid 8

This compound was prepared in a manner identical to that described for (2*S*,3*S*)-2-hydrazino-3-methylsuccinic acid **3**, using propylfumaric acid (0.9 g, 5.7 mmol) to give the desired compound as an off-white powder (0.34 g, 31%), mp 173–174 °C (Found: C, 44.1; H, 7.55; N, 14.7. $C_7H_{14}N_2O_4$ requires C, 44.2; H, 7.4; N, 14.75%) (HRMS: found $[M + H]^+$, 191.1027. $C_7H_{15}N_2O_4$ requires 191.1032); $[\alpha]_D^{22}$ −37.1 (*c* 0.41 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3346 and 3219 (NH) and 1699 (CO); δ_H(200 MHz; ^2H_2O, p^{2}H 1) 0.66 [3 H, d, *J* 6.6, 3-CH(CH_3)$_2$], 0.76 [3 H, d, *J* 6.6, 3-CH(CH_3)$_2$], 1.94 [1 H, m, 3-C*H*(CH_3)$_2$], 2.42 (1 H, t, *J* 6.6, 3-H) and 3.81 (1 H, d, *J* 6.4, 2-H); δ_C(50 MHz; ^2H_2O, p^{2}H 1) 14.04 (3-$CH_2CH_2CH_3$), 21.16

(3-$CH_2CH_2CH_3$), 30.37 (3-$CH_2CH_2CH_3$), 46.94 (3-CH), 62.31 (2-CH) and 174.47 and 177.71 (2 × CO_2H); *m*/*z* (CI) 191 (5%, $[M + H]^+$) and 173 (100, $[M - NH_4]^+$).

(2*S*)-*N*-Methylaspartic acid monohydrate 9

Fumaric acid (2.0 g, 17.2 mmol) was dissolved in aqueous methylamine solution (40% w/v; 20 cm^3). The resulting solution was concentrated under reduced pressure to give the dimethyl ammonium salt which was then redissolved in water (25 cm^3). Magnesium chloride hexahydrate (0.38 g) and potassium chloride (0.45 g) were added and the pH was adjusted to 9 with aqueous methylamine (40% w/v). 3-Methylaspartase (120 units) was added and the reaction flask was then incubated at 30 °C. Aliquots (100 mm^3) were then taken out and the reaction was followed by ^{1}H NMR spectroscopy. More enzyme was added every 24 h. After the reaction had ceased (*ca.* 7 days), the protein was denatured at 100 °C for 2 min and removed by filtration through a Celite pad. The filtrate was concentrated under reduced pressure and the resulting residue redissolved in water (10 cm^3). The solution was acidified to pH 1 with 12 mol dm^{-3} hydrochloric acid and was then extracted with diethyl ether (2 × 40 cm^3). The aqueous phase was adjusted to pH 4 with concentrated aqueous ammonia and was concentrated under reduced pressure. The resulting crude product was recrystallised from boiling water to give a white crystalline solid (1.28 g, 45%), mp 189–191 °C (lit.,[24] 189–190 °C) (Found: C, 36.65; H, 6.95; N, 8.3. Calc. for $C_5H_9NO_4 \cdot H_2O$: C, 36.35; H, 6.7; N, 8.5%) (HRMS: found $[M + H]^+$, 148.0610. Calc. for $C_5H_{10}NO_4$: 148.0609); $[\alpha]_D^{22}$ +14.4 (*c* 2 in H_2O) [lit.,[24] −15.4 (*c* 0.5 in H_2O) at 18 °C for D-antipode]; ν_{max}(Nujol)/cm^{-1} 3210 (NH) and 1637 (CO); δ_H(200 MHz; ^2H_2O) 2.77 (3 H, s, NCH_3), 3.02 (2 H, d, *J* 5.1, 3-CH_2) and 3.90 (1 H, t, *J* 5.2, 2-H); δ_C(50 MHz; ^2H_2O) 33.10 (NCH_3), 34.35 (C-3), 60.14 (C-2), 173.01 (C-4) and 175.09 (C-1); *m*/*z* (CI) 148 (100%, M^+) and 102 (32, $[M - CO_2H - H]^+$).

(2*S*,3*S*)-*N*,3-Dimethylaspartic acid 10

This compound was prepared in a manner identical to that described for (2*S*)-*N*-methylaspartic acid monohydrate **9**, using mesaconic acid (2.0 g, 15.4 mmol), and was obtained as a white crystalline solid (1.0 g, 40%), mp 195–197 °C (Found: C, 44.75; H, 6.95; N, 8.65. $C_6H_{11}NO_4$ requires C, 44.75; H, 6.9; N, 8.7%) (HRMS: found $[M + H]^+$ 162.0766. $C_6H_{12}NO_4$ requires 162.0765); $[\alpha]_D^{22}$ +17.1 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3227 (NH) and 1696 (CO); δ_H(300 MHz; ^2H_2O) 1.18 (3 H, d, *J* 7.5, 3-CH_3), 2.69 (3 H, s, NCH_3), 3.11 (1 H, m, AB splitting, *J* 7.5 and 3.1, 3-H) and 3.84 (1 H, d, *J* 3.1, 2-H); δ_C(50 MHz; ^2H_2O) 14.25 (3-CH_3), 35.88 (NCH_3), 42.39 (C-3), 67.40 (C-2), 174.17 (C-4) and 180.00 (C-1); *m*/*z* (CI) 162 (100%, $[M + H]^+$), 144 (15, $[M + H - OH_2]^+$) and 116 (57, $[M - CO_2H]^+$).

(2*S*,3*S*)-*N*-Methyl-3-ethylaspartic acid 11

This compound was prepared in a manner identical to that described for (2*S*)-*N*-methylaspartic acid monohydrate **9**, using ethylfumaric acid (2.0 g, 13.9 mmol), and was obtained as a white crystalline solid (0.85 g, 35%), mp 225–227 °C (Found: C, 47.95; H, 7.4; N, 6.85. $C_7H_{13}NO_4$ requires C, 48.0; H, 7.5; N, 7.0%) (HRMS: found $[M + H]^+$, 176.0923. $C_7H_{14}NO_4$ requires 176.0921); $[\alpha]_D^{22}$ +20.2 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 3230 (NH) and 1694 (CO); δ_H(200 MHz; ^2H_2O, NaO^{2}H) 0.88 (3 H, t, *J* 5.9, 3-CH_2CH_3), 1.42 (2 H, m, 3-CH_2CH_3), 2.60 (1 H, m, 3-H), 2.65 (3 H, s, NCH_3) and 3.63 (1 H, d, *J* 1.7, 2-H); δ_C(50 MHz; ^2H_2O, NaO^{2}H) 12.85 (CH_3), 21.44 (CH_2), 34.30 (NCH_3), 49.69 (C-3), 66.36 (C-2), 173.29 (C-4) and 181.93 (C-1); *m*/*z* (CI) 176 (100%, $[M + H]^+$) and 158 (32, $[M + H - H_2O]^+$).

(2*S*)-*N*-Ethylaspartic acid 14

This compound was prepared in a manner identical to that described for (2*S*)-*N*-methylaspartic acid monohydrate **9**, using fumaric acid (0.2 g, 1.4 mmol) and aqueous ethylamine (70%). After 2 weeks, the protein was denatured at 100 °C for 2 min and removed by filtration through a Celite pad. The filtrate was concentrated under reduced pressure, and due to the low final concentration of **14**, the compound could not be isolated in the usual manner. The compound was, therefore, characterised on the basis of the ^{1}H and ^{13}C NMR spectra for the crude material; δ_H(200 MHz; ^2H_2O, p^{2}H 9) 1.22 (3 H, t, *J* 7.0, NCH_2CH_3), 2.68 (2 H, dd, ABX, *J* 7.0 and 4.8, 3-CH_2), 3.04 (2 H, q, *J* 7.0, NCH_2CH_3), 3.73 (1 H, dd, *J* 4.8, 2-CH); δ_C(50 MHz; ^2H_2O, p^{2}H 9) 13.43 (NCH_2CH_3), 36.25 (NCH_2CH_3), 45.48 (3-C), 59.25 (2-C), 174.09 (4-C) and 176.15 (1-C).

(2*S*)-*N*,*N*-Dimethylaspartic acid 15

This compound was prepared in a manner identical to that described for (2*S*)-*N*-methylaspartic acid monohydrate **9**, using fumaric acid (2.0 g, 172 mmol) and aqueous dimethylamine (40%), and was obtained as a white crystalline solid (0.70 g, 28%), mp 196–198 °C (lit.,[25] 198 °C) (Found: C, 44.85; H, 6.85; N, 8.75. $C_6H_{11}NO_4$ requires C, 44.7; H, 6.9; N, 8.7%) (HRMS: found $[M + H]^+$, 162.0766. $C_6H_{12}NO_4$ requires 162.0765); $[\alpha]_D^{22}$ −2.0 (*c* 0.6 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 1699 (CO); δ_H(200 MHz; ^2H_2O) 2.86 [6 H, s, $N(CH_3)_2$], 2.98 (2 H, d, *J* 6.4, 3-CH_2) and 4.05 (1 H, t, *J* 6.2, 2-H); δ_C(50 MHz; ^2H_2O) 34.74 (C-3), 62.19 (C-2), 68.76 [$N(CH_3)_2$], 175.0 (C-4) and 176.67 (C-1); *m*/*z* (CI) 162 (100%, $[M + H]^+$), 144 (22, $[M + H - OH_2]^+$) and 116 (30, $[M - H - CO_2H]^+$).

(2*S*,3*S*)-*N*,3-Dimethyl[3-^{2}H]aspartic acid 16

This compound was prepared in a manner identical to that described for (2*S*,3*S*)-*N*,3-dimethylaspartic acid **10**, using mesaconic acid (2.0 g, 15.4 mmol) in ^2H_2O, and was obtained as a white crystalline solid (0.98 g, 40%), mp 209–211 °C (HRMS: found $[M + H]^+$, 163.0821. $C_6H_{11}{}^2HNO_4$ requires 163.0829); $[\alpha]_D^{22}$ +18.7 (*c* 0.63 in 6 mol dm^{-3} HCl); ν_{max}(Nujol)/cm^{-1} 2853 (NCH_3) and 1688 (CO); δ_H(200 MHz; ^2H_2O) 1.14 (3 H, s, 3-CH_3), 2.68 (3 H, s, NCH_3) and 3.83 (1 H, s, 2-H); δ_C(50 MHz; ^2H_2O) 12.50 (3-CH_3), 34.18 (NCH_3), 40.10 (C-3), 65.61 (C-2) and 172.50 and 178.29 (2 × CO_2H); *m*/*z* (CI) 163 (100%, $[M + H]^+$) and 145 (21, $[M + H - OH_2]^+$).

(2*S*)-*N*-Hydroxyaspartic acid 18

Fumaric acid (0.3 g, 2.6 mmol) was dissolved in ethylamine and the solution was concentrated under reduced pressure to give the salt. The bis(ethylammonium) salt was dissolved in water (2 cm^3) and the pH was adjusted with aqueous hydroxylamine (40% w/v, pH 11 in 70% w/v aqueous ethylamine) to 9. Magnesium chloride hexahydrate (64 mg) and potassium chloride (76 mg) were added and the solution volume was made up to 4 cm^3 with water. 3-Methylaspartase (20 units) was added and the reaction was incubated at 30 °C. After 3 days the solution was concentrated under reduced pressure to give crude bis(ethylammonium) (2*S*,3*S*)-*N*-hydroxyaspartate. This material was redissolved in the minimum amount of water and was acidified to pH 1 with dilute HCl and was then purified by ion exchange chromatography (Dowex, 50W-X8, H^+ form). After washing the column with water, the product was eluted with aqueous ammonia (0.5%). The eluate was concentrated under reduced pressure to give (2*S*)-*N*-hydroxyaspartic acid **18** as an off-white solid, which gradually formed a sticky gum (0.11 g, 28%); $[\alpha]_D^{22}$ +5.3 (*c* 0.19 in H_2O); δ_H(200 MHz; ^2H_2O) 2.93 (2 H, m, ABX splitting, *J* 4.8, 3-CH_2) and 4.13 (1 H, t, *J* 5.0, 2-H); δ_C(50 MHz; ^2H_2O) 34.90 (3-CH_2), 64.18 (2-CH) and 174.31 and 177.59 (2 × CO_2H).

(2*S*,3*S*)-*N*-Hydroxy-3-methylaspartic acid 19

This compound was prepared in a manner identical to that described for (2*S*)-*N*-hydroxyaspartic acid **18**, using mesaconic acid (2.0 g, 15.4 mmol), to give (2*S*,3*S*)-*N*-hydroxy-3-methylaspartic acid **19** (0.48 g, 19%); $[\alpha]_D^{22}$ −23.3 (*c* 0.25 in

H_2O); δ_H(200 MHz; ^2H_2O) 1.07 (3 H, d, *J* 7.3, 3-CH_3), 3.01 (1 H, m, AB splitting, 3-H) and 4.11 (1 H, d, *J* 3.2, 2-H); δ_C(50 MHz; ^2H_2O, p^{2}H 1) 14.20 (3-CH_3), 41.42 (3-CH), 69.49 (2-CH) and 174.12 and 181.12 (2 × CO_2H).

(2*S*,3*S*)-*N*-Hydroxy-3-ethylaspartic acid 20

This compound was prepared in a manner identical to that described for (2*S*)-*N*-hydroxyaspartic acid **19**, using ethylfumaric acid (0.2 g, 1.4 mmol) to give the crude compound (30 mg, 12%); δ_H(200 MHz, ^2H_2O, p^{2}H 9) 0.69 (3 H, t, *J* 7.0, CH_2CH_3), 1.42 (2 H, m, CH_2CH_3), 2.33 (1 H, m, 3-H) and 3.48 (1 H, d, *J* 4.2, 2-H).

(2*S*)-*N*-Methoxyaspartic acid 21

Fumaric acid (2.0 g, 17.2 mmol) was dissolved in concentrated (40%) aqueous ethylamine. The resulting solution was concentrated under reduced pressure to give the bis(ethylammonium) salt which was then redissolved in water (15 cm^3). Aqueous methoxylamine (40%) was added (10 cm^3) and the pH adjusted to 9 using aqueous ethylamine. Magnesium chloride hexahydrate (0.38 g), potassium chloride (0.45 g) and finally 3-methylaspartase (120 units) were added. The reaction was then incubated at 30 °C and aliquots (100 mm^3) were removed periodically to follow the reaction by ^{1}H NMR spectroscopy. More enzyme was added every 24 h. After the reaction had ceased (*ca*. 3 days), the protein was denatured by heating to 100 °C for 2 min and was removed by filtration through a Celite pad. The filtrate was concentrated under reduced pressure and the resulting residue was redissolved in water (10 cm^3). The solution was acidified to pH 1 with 12 mol dm^{-3} hydrochloric acid and was extracted with diethyl ether (2 × 40 cm^3). The aqueous phase was then concentrated under reduced pressure to give a yellow oil, which was purified using ion exchange chromatography (Dowex, 50W-X8, H^+ form). After washing the column with water, the product was eluted with aqueous ammonia (0.5%). The eluate was concentrated under reduced pressure to give (2*S*)-*N*-methoxyaspartic acid **21** as a yellow viscous oil (0.87 g, 31%); $[a]_D^{22}$ −7.2 (*c* 0.35 in H_2O); δ_H(200 MHz; ^2H_2O, pH 9) 2.20 (2 H, dd, *J* 7.5, 3-CH_2), 3.64 (3 H, s, OCH_3) and 3.92 (1 H, d, *J* 7.5, 2-H); δ_C(50 MHz; ^2H_2O, p^{2}H 1) 39.31 (3-CH_2), 55.01 (2-CH), 63.79 (OCH_3) and 180.0 and 181.22 (2 × CO_2H).

(2*S*,3*S*)-*N*-Methoxy-3-methylaspartic acid 22

This compound was prepared in a manner identical to that described for (2*S*)-*N*-methoxyaspartic acid **21**, using mesaconic acid (0.3 g, 2.3 mmol). Compound **22** was obtained as a light brown viscous oil (0.14 g, 34%); δ_H(200 MHz; ^2H_2O, p^{2}H 9) 0.90 (3 H, d, *J* 7.3, 3-CH_3), 2.58 (1 H, m, AB splitting, 3-H), 3.36 (3 H, s, OCH_3) and 3.72 (1 H, d, *J* 4.2, 2-H); δ_C(50 MHz; ^2H_2O, p^{2}H 1) 15.26 (CH_3), 43.02 (3-CH), 58.8 (2-CH), 63.88 (OCH_3) and 181.0 and 182.3 (2 × CO_2H).

Absolute stereochemistry of N-substituted aspartic acids

General procedure. N-Substituted aspartic acid (3 mmol) was dissolved in 2 mol dm^{-3} potassium hydroxide (10 cm^3) and water (10 cm^3) was added. Nickel–aluminium alloy (6.2 g) was added in small portions and the mixture left stirring for 24 h. The mixture was then filtered through a Celite pad and the pH of the filtrate was adjusted to 7. The nickel–aluminium salts were filtered and the filtrate was acidified to pH 3–4 and then concentrated under reduced pressure to give a solid residue. Recrystallisation from boiling water gave the product as white crystals in moderate to good yields.

Using the above procedure, 2-hydrazino-3-methylsuccinic acid and *N*-hydroxy-3-methylaspartic acid each gave (2*S*,3*S*)-3-methylaspartic acid, and *N*-methoxyaspartic acid gave (2*S*)-aspartic acid, as determined by comparison with authentic samples.

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