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ENANTIOSPECIFIC SYNTHESIS OF 3-SUBSTITUTED ASPARTIC ACIDS VIA ENZYMIC AMINATION OF SUBSTITUTED FUMARIC ACIDS

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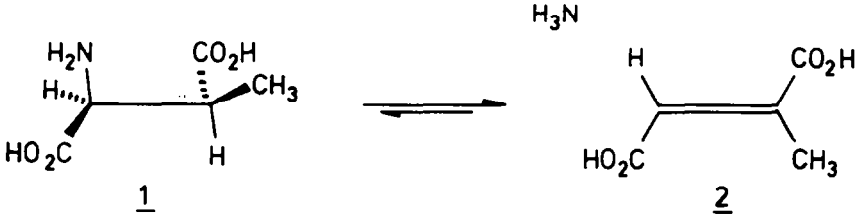
Abstract The use of the enzyme 3-methylaspartase in the synthesis of L-aspartic acids containing 3-halogeno- or 3-alkyl- substituents, in the (S)-configuration, and also some of the corresponding C-3 deuteriated isotopomers is described.

The enzyme 3-methylaspartate ammonia-lyase (EC 4.3.1.2) catalyses the reversible α,β-elimination of ammonia from (2S,3S)-3-methylaspartic acid (1) to give mesaconic acid (2), Scheme 1.[1]

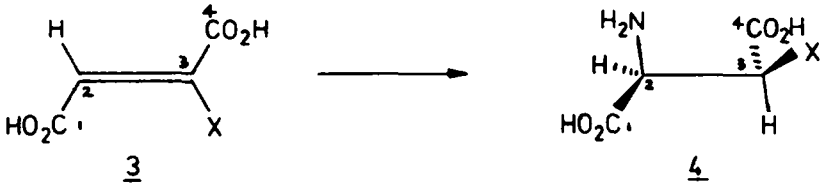


Scheme 1

The enzyme is also capable of catalysing the deamination of 3-alkylaspartic acids at low to moderate rates.[2]

In a preliminary study we showed that the enzyme from Clostridium tetanomorphum can be used efficiently, in a retro-physiological reaction direction, to synthesize 3-halogenoaspartic acids, Scheme 2.[3] We found that although fluorofumaric acid (3, X=F) was not a good substrate for the enzyme, both chloro- (3, X=Cl) and bromo- (3, X=Br) fumaric acid were converted efficiently to the corresponding (2R,3S)-3-halogenoaspartic acids (4,X=Cl and 4, X=Br respectively).† Iodofumaric acid was not a substrate for the enzyme.

†(2R,3S)-3-Bromoaspartic acid is a k_{cat} inhibitor for the enzyme hence in incubations with bromofumaric acid as substrate the initially high rate of amination falls-off rapidly due to enzyme inactivation.



3 4

Scheme 2

During these studies it was noted that the catalytic rates for the enzymic amination of fumaric and mesaconic acid were faster than the rates for the corresponding deamination reactions. In order to exploit the synthetic utility of the enzyme and also determine the maximum permissible size for substituents at C-3 of the substrate, a range of 3-alkylfumaric acids were prepared for incubation with 3-methylaspartase. Here we report on the results of these studies and describe the use of the enzyme in the enantiospecific synthesis of new 3-alkylaspartic acids and also the deuteriated isotopomers of aspartic, methylaspartic and ethylaspartic acid. We also present the experimental details of the syntheses of 3-halogenoaspartic acids.

RESULTS AND DISCUSSION

The physiological substrate for the enzyme 3-methylaspartase contains a methyl group at C-3, Scheme 1. We reasoned that it might be possible to utilize the complimentary pocket of the enzyme to bind other groups at C-3 of fumaric acid substrates. If the size of the substituent was not too big for binding, or the +M effect was not so large as to prevent reactions with ammonia at C-2, it was expected that stereospecific amination reactions to give L-aspartic acids would occur.

In a preliminary note we commented upon our initial findings with halogenofumaric acids as substrates.[3] We now present the results of the attempted amination of ten fumaric acids (3, X=F,Cl,Br,I,H,CH_3,Et,i-Pr,n-Pr and n-Bu), Table 1. From these results and from the results of a kinetic study using these substrates,[4] it is clear that C-3 substituents larger than bromine, isopropyl or n-propyl will not fit into the active-site methyl-binding pocket of the enzyme.

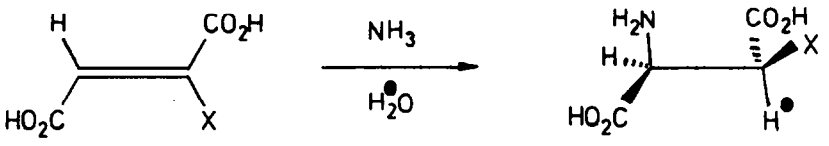
Fumaric acid and mesaconic acid were obtained commercially. Fluorofumaric acid was prepared according to the method of Raash *et al.*[5] in four steps from 1,1,2-trifluoro-2-chloroethene.

Chlorofumaric acid was prepared from (2S,3S)-tartaric acid according to the method of Perkin.[6] Bromofumaric acid was prepared through bromination of maleic acid[7] to give racemic 2,3-dibromosuccinic acid followed by dehydrohalogenation.[8] No bromomaleic acid was produced using this protocol.

Ethyl, iso-propyl-, n-propyl and n-butyl- fumaric acids were prepared from ethyl acetoacetate *via* alkylation followed by treatment of the products with bromine and then potassium hydroxide in ethanol to effect a Favorskii type rearrangement.[9] The alkylfumaric acids we obtained in good to moderate overall yield after an acidic work-up, Scheme 3.

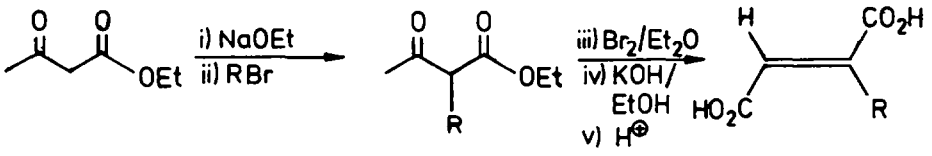
TABLE 1

Attempted amination of substituted fumaric acids using 3-methylaspartase in the presence of saturating ammonia. See experimental section for details of individual reactions.



Substrate	Amination Rate	Yield %	M.p. °C
X = F	v. slow*	-	-
X = Cl	mod. fast	60	168-71
X = Br	mod. fast	see ref 10	-
X = I	-	-	-
X = H	v. fast	90	> 300
X = H, H* = ^{2}H	-	66	290 dec
X = CH_3	fast	61	272-75
X = CH_3, H* = ^{2}H	-	60	292-94
X = CH_2CH_3	mod. fast	60	245-46
X = CH_2CH_3, H* = ^{2}H	-	58	250-52
X = $CH(CH_3)_2$	v. slow	54	239-42
X = $CH_2CH_2CH_3$	v. slow	49	235-37
X = $CH_2CH_2CH_2CH_3$	none	-	-
ACETYLENEDICARBOXYLIC ACID	none	-	-

* Reaction rate extremely slow; complications due to side reactions, see ref. 3



Scheme 3

In order to test the substituted fumarates as substrates for 3-methylaspartase a series of ^{13}C-nmr spectroscopic and u.v. spectrophotometric assays were devised.[cf. 3] These methods and the results of kinetic experiments will be reported elsewhere.[4] In order to prepare useful quantities of 3-substituted aspartic acids, each of the fumaric acids were incubated with the enzyme and the necessary metal ions in the presence of excess ammonia at pH 9. The reactions were followed by the periodic removal of aliquots of the incubation mixture for the determination of OD_{240}, corresponding to the absorbance due to the conjugated double bond of the substrate. For some incubations it was necessary to add additional amounts of enzyme during the course of the reaction to compensate for activity loss due to time-dependent protein denaturation. When the reactions were judged to have reached equilibrium the protein was denatured and the excess ammonia removed. The exact work-up depended on the substrate, see experimental section, but in general, acidification to pH 1-2 followed by extraction with an organic solvent allowed the removal of the excess substrate. Adjustment of the aqueous solution to pH 3-4 facilitated crystallisation of the amino acid. In difficult cases ion exchange or preparative celluluose thin layer chromatography was used prior to crystallisation.

The stereochemical courses of amination have been determined for fumaric, mesaconic, chlorofumaric and bromofumaric acid.[10] Ethylfumaric acid is also aminated *via si*-face nucleophilic attack of ammonia at C-2 in an *anti*-fashion, see experimental section. This has been determined by comparing the coupling constants for 2-H-3-H from the ^{1}H nmr spectra of the N-trifluoroacetyl aspartic anhydrides of 3-methyl- and 3-ethylaspartic acid (they are almost identical) and by degrading the ethyl derivative to (2S)-2-ethylsuccinic acid for which a specific rotation has been reported.[11] Based on the now established stereochemical mode for the amination of five substrates we expect that the absolute configuration of the higher alkylaspartic acids described here is also (2S,3S).†

EXPERIMENTAL

Melting points were determined using either a Kofler hot-stage or an electrothermal melting point apparatus. I.r. spectra were recorded using a Perkin Elmer 298 infra-red spectrophotometer. U.v.-visible spectra and optical density measurements were obtained using a Pye-Unicam SP8-500 spectrophotometer. Nmr spectra were recorded on Hitachi Perkin-Elmer R24(B), Jeol FX90Q, Jeol JNM-GX270 and Bruker AM 360 instruments. TMS was used as standard for ^{1}H-nmr and methanol or chloroform were used to reference ^{13}C-nmr spectra. Mass spectra were obtained using an AEI-MS30 spectrometer. Microanalysis facilities were provided on a service basis by University College London, UK. Specific rotations were determined on an Optical Activity Ltd. AA-100 polarimeter using 10 cm path-length cells. 3-Methylaspartase was partially purified from *Clostridium tetanomorphum* (ATCC 15920) using a modification of literature procedures.[1,4] The cells were grown by Prof. C. Greenwood and Mr. A. Thompson,

† Note the priority change in assigning the absolute configuration of 3-halogenoaspartic acids compared to 3-alkylaspartic acids.

Department of Biology, University of East Anglia, UK, using a fresh vial of the bacterium from the American Typed Culture Collection for each growth. All chemicals and solvents were of analytical grade or were purified by recrystallisation or redistillation before use.

Fluorofumaric acid (3,X=F) was prepared according to the method of Raasch et al.[5] as outlined below. 1,1,2-Trifluoro-2-chloroethene (20g, 0.17 mol), 1,1-dichloroethene (23.2g, 0.24 mol) and hydroquinone (100 mg) were heated in a 100 cm^3 pressure bomb at 180°C for 14 hours. The reaction was cooled and the contents were filtered to remove polymerised material. The filtrate was distilled at 760 mmHg, collecting fractions from 118-120°C, to give 1,1,2-Trichloro-2,3,3-trifluorocyclobutane as a colourless mobile oil (11.91 g, 32.6%); (lit[5] b.p. 120-21°C); ν_{max} (neat), 3050-2990 (CH), 805-620 cm^{-1} (C-Hal); δ_H (60 MHz, C^2HCl_3), 3.31 (dt, J 11 and 3 Hz).

1,1,2-Trichloro-2,3,3-trifluorocyclobutane (12g, 56.2 mmol) was added to a refluxing suspension of zinc dust (4.5g 68.8 mmol) in dry ethanol (9 ml) and reflux was continued for a further 4.5 h. The cooled reaction was diluted to a volume of 50 ml with water and 12M HCl (2.5 ml, 30 mmol) was added. The solid material was removed by filtration and the organic phase of the filtrate was collected and distilled to give 1-chloro-2,3,3-trifluorocyclobutene as a colourless oil (5.45 g, 68.3%); b.p.50-51°C, (Lit.[5] 51.5 - 52°C), δ_H (60 MHz, C^2HCl_3), 3.73 (dt, J 11 and 3 Hz).

To a cooled solution of potassium permanganate (314 mg, 1.89 mmol) and sodium hydroxide (160 mg, 4 mmol) in water (4 ml) was added 1-chloro-2,3,3-trifluorocyclobut-2-ene (214 mg, 1.5 mmol) so that the temperature of the reaction did not rise above 15°C. The reaction mixture was stirred for 3 hrs and then 30% hydrogen peroxide was added until the purple colour was discharged. The precipitated manganese dioxide was removed by filtration, washed with water (20 ml) and the filtrate was acidified with conc. H_2SO_4 and extracted with diethyl ether (3 x 50 ml). The organic solution was dried ($MgSO_4$) and reduced in volume in vacuo to give 2,2-difluorosuccinic acid as a white amorphous solid (105 mg, 39%); m.p. 129-31°C, (lit.[5], 154-5°C); m/z(EI) (Found: MH^+, 155.0407. Calc. for $C_4H_5F_2O_4$: 155.0156); ν_{max} (nujol), 3420 (OH), 1760 and 1720 cm^{-1} (C=O); δ_H (60 MHz, $(C^2H_3)_2SO$), 3.1 (2H, t, J_{HF} 15 Hz, 3-H_2), 10.2 (1H, s, -CO_2H).

2,2-Difluorosuccinic acid (0.8 g, 5.2 mmol) was added to a cold solution of sodium hydroxide (0.6 g, 15 mmol) in water (2.48 ml) and the reaction mixture was refluxed for 19 hrs. After cooling, water (0.7 ml) and 12M HCl (1.4 ml) were added and the solution was extracted with diethyl ether (5 x 20 ml). The ethereal solution was dried ($MgSO_4$) and reduced in volume in vacuo to give a white solid which was recrystallized from diethyl ether - light petroleum (540 mg, 77%); m.p. 206-208°C (with dec), (lit. 236-7°C); ν_{max} (nujol), 1710 (C=O), 1660 cm^{-1} (C=C); δ_H (60 MHz, $(C^2H_3)_2SO$), 6.28 (1H, d, J_{HF}, 36 H_3, 3-H); δ_C (90 MHz, $\{^1H\}$, containing 5% ^2H_2O, pH 9), 108.35 (1C, dd, J_{CH} 174.0, J_{CF} 11.7 Hz, 3-CH), 152.19 (1C, d, J_{CF} 270.1 Hz, 2-CF), and 169.50 (1C, s, 4-CO_2); m/z(EI) 134(M^+), 117([M-OH]$^+$). (This sample contained a small amount (ca. 5%) of the cis-isomer.)

Chlorofumaric acid (3,X=Cl) was prepared by the method of Perkin.[6] Phosphoruspentachloride (250 g, 1.2 mol) and (2S,3S)-tartaric acid (38 g, 0.25 mol) were mixed and warmed until the reaction became liquid. The reaction mixture was distilled to remove $POCl_3$. The residue was cooled and the liquid decanted from the resulting crystalline mass. Distillation at 66-68°C (10 mm Hg) gave chlorofumaryl diacid chloride as a pale yellow liquid in 49% yield. The entire sample was mixed with water (50 ml) with vigorous stirring and then extracted with diethyl ether. The solution was dried ($MgSO_4$) and reduced in volume in vacuo to give chlorofumaric acid as an off-white amorphous solid which was recrystallized from dilute HCl (5 g, 40% overall); m.p. 190-91°C, (lit.[6] 191-2°C); (Found: C, 31.65; H, 1.90; Cl, 23.30. Calc. for $C_4H_3ClO_4$, C, 31.90; H, 2.00; Cl, 23.55%); ν_{max} (nujol), 1700 (C=O), and 1630 cm^{-1} (C=C); δ_H (60 MHz, ^2H_2O), 7.25 (1H, s); δ_C (90 MHz, $\{^1H\}$, water containing 5% ^2H_2O, pH 9), 128.00 (1C, C=CH), 129.05 (1C, brd m, C=C-Cl), 166.70 and 170.70 (2xCO_2H); m/z(EI), 150 and 152 (M^+, Cl isotopes).

Bromofumaric acid (3,X=Br) was prepared through bromination of maleic acid followed by dehydrobromination of the product according to the methods of McKenzie[7] and Michael[8] in 80% overall yield. M.p. 176-177°C, (lit.[8], m.p. 185-186°C); (Found: C, 24.35; H, 1.35; Br, 41.15, Calc. for $C_4H_3BrO_4$; C, 24.65; H, 1.55; Br, 41.00%); ν_{max} (nujol), 2640 (OH), 1720 (C=O), 1620 cm^{-1} (C=C); δ_H (60 MHz, $(C^2H_3)_2SO$), 7.45 (1H, s, 2-H), 11.35 (2H, brd s, 2x$CO_2\underline{H}$); δ_C (90 MHz, {^1H}, water containing 5% ^2H_2O, pH 9), 119.50 (C=$\underline{C}$-H), 132.75 (brd m, C=$\underline{C}$-Br), 167.00 and 171.35 (2x$\underline{C}O_2H$); m/z(EI) 194 and 196 (M^+, Br isotopes).

Iodofumaric acid (3,X=I) was prepared from monopotassium acetylenedicarboxylic acid by a modification of the method of Thiel,[12] in 85% yield. M.p. 190-193°C, (lit.[12] 193-4°C); (Found: C, 19.90; H, 1.20. Calc. for $C_4H_3IO_4$, C, 19.85; H, 1.25%); ν_{max} (nujol), 1700 (C=O), 1610 cm^{-1} (C=C); δ_H (60 MHz, ^2H_2O) 7.51 (1H, s); δ_C (90 MHz, {^1H}, water containing 5% ^2H_2O, pH 9), 99.25 (1C, s, C=$\underline{C}$H), 138.85 (1C, brd m, C=$\underline{C}$=I), 168.40 and 172.05 (2xCO_2H).

Ethylfumaric acid (3,X=Et) was prepared according to the method of Walden.[9] To a solution of sodium ethoxide prepared by dissolving sodium (9.6 g, 0.42 mol) in anhydrous ethanol (150 ml) was added ethyl acetoacetate (48.97 g, 0.37 mol) dropwise over 10 minutes. The solution was stirred for an additional 5 minutes and ethyl bromide (53.7 g, 0.49 mol) was added slowly dropwise. Following the addition, the reaction was heated at reflux for 2 hours and was then allowed to cool. The solution was poured into water (200 ml) and then extracted with diethyl ether (3 x 200 ml). The pooled organic extracts were washed with water and dried ($MgSO_4$). The solvent was removed *in vacuo* to give a pale yellow oil which was purified through distillation to yield 2-ethylacetoacetate ethyl ester as a colourless oil, 35 g, 61%. The product was used in the following reaction without further purification.

To a vigorously stirred solution of the ester (3 g, 18.9 mmol) in dry diethyl ether (25 ml) was slowly added bromine (6 g, 37.5 mmol) and the solution then refluxed for 3 hours. The solvent and HBr were removed *in vacuo* to give the dibromide as a pale yellow oil.
The dibromide was added slowly to a solution of ethanol (20 ml) containing powdered potassium hydroxide (6 g, 10.7 mmol) with rapid stirring. The mixture was refluxed for 30 mins and was then steam distilled until 100 ml of distillate had been collected. The acidified solution was extracted with diethyl ether (4 x 50 ml) and the pooled extracts were dried (Na_2SO_4) and reduced in volume *in vacuo* to give an amorphous off-white solid. The product was decolourized with active charcoal and recrystallized from diethyl ether - light petroleum to give ethylfumaric acid as white crystals 1.65 g (67% overall yield); m.p. 195°C, (lit.[9], 194-95°C); ν_{max} (nujol), 1700 cm^{-1} (C=O); δ_H (360 MHz, ^2H_2O, $NaHCO_3$, HO^2H at 4.6 ppm), 0.82 (3H, t, J 7 Hz, -$\underline{C}H_3$), 2.28 (2H, q, J 7 Hz, -$\underline{C}H_2CH_3$), 6.15 (1H, s, C=$\underline{C}$H); δ_C (90 MHz, {^1H}, ^2H_2O, $NaHCO_3$), 10.89 (CH_3), 19.88 (CH_2), 125.33 (3C), 143.70 (2C), 175.15 and 174.17 (2x$\underline{C}O_2H$); m/z(EI), 126 ([M - H_2O]$^+$, 29%), and 98 (100, [M - HO_2CH]$^+$).

n-Propylfumaric acid (3,X=n-Pr) was prepared from ethyl acetoacetate and n-propyl bromide, using the procedure described above, in 50% overall yield; m.p. 172-4°C (lit.[13] 175°C); ν_{max} (nujol) 1700 cm^{-1} (C=O); δ_H (360 MHz ^2H_2O, $NaHCO_3$, HO^2H at 4.6 ppm), 0.67 (3H, t, J 7.5 Hz, $\underline{C}H_3$), 1.19 (2H, m, J 7.5 Hz, -$\underline{C}H_2CH_3$), 2.24 (2H, t, J 7.5 Hz, -C=C-$\underline{C}H_2$), and 6.16 (1H, s, C=$\underline{C}$H); δ_C (90 MHz, {^1H}, ^2H_2O, $NaHCO_3$), 11.36 (CH_3), 19.76 (CH_2), 28.72 (CH_2), 126.24 (3C), 142.23 (2C), 175.41 and 174.31 (2xCO_2H); m/z(EI), 140 ([M - H_2O]$^+$, 18%), and 112 (100, [M - HO_2CH]$^+$).

iso-Propylfumaric acid (3,X=i-Pr) was prepared from ethylacetoacetate and iso-Propyl bromide, using the procedure described above, in 42% overall yield; m.p. 180-81°C, (lit[14] 183-184°C); ν_{max} (nujol), 1700 (C=O), 1640 cm^{-1} (C=C). δ_H (360 MHz, ^2H_2O, $NaHCO_3$, HO^2H at 4.6 ppm), 0.91 (6H, d, J 7 Hz, -CH($\underline{C}H_3$)$_2$), 2.96 (1H, m, J 7 Hz, -$\underline{C}$HMe$_2$), and 5.72 (1H, s, C=$\underline{C}$H); δ_C (90 MHz, {^1H}, ^2H_2O, $NaHCO_3$), 19.05 (2xCH_3), 28.29 (3'-CH), 121.02 (3C), 149.02 (2C), 176.12 and 174.62 (2xCO_2H); m/z(EI), 140 ([M - H_2O]$^+$, 33%), and 112 (100, [M - HO_2CH]$^+$).

n-Butylfumaric acid (3,X=n-Bu) was prepared from ethylacetoacetate and n-butyl iodide, using the procedure described above, in 25% overall yield; m.p. 170-71°C, (lit.[15] 169-170°C);

ν_{max} (nujol), 3400-2800 (OH), 1700 (C=O), and 1650 cm^{-1} (C=C); δ_H (360 MHz, ^2H_2O, $NaHCO_3$, HOD at 4.6 ppm), 0.66 (3H, t, J 6.5 Hz, $-CH_3$), 1.11 (4H, m, $CH_2(CH_2)_2Me$), 2.26 (2H, t, J 7 Hz, $-CH_2$ -nPr), and 6.12 (1H, s, C=CH); δ_C (90 MHz, {^1H}, ^2H_2O, $NaHCO_3$), 11.27 (CH_3), 19.96 (CH_2), 26.39 (CH_2), 28.57 (CH_2), 125.81 (3C), 142.69 (2C), 175.67 and 174.45 (2xCO_2H); m/z(EI), 154 ($[M - H_2O]^+$, 25%), and 126 (36,$[M - HO_2CH]^+$).

(2R,3S)-3-Fluoroaspartic (4,X=F) Incubations conducted using fluorofumaric acid as substrate did not give isolatable products. Using a vast excess of enzyme and prolonged incubation times it was possible to detect small quantities (ca. 5%) of (2R,3S)-3-fluoroaspartic acid in the lyophilized incubation mixture by ^1H-nmr spectroscopy. δ_H (360 MHz ^2H_2O, pH 7), 4.04 (1H, dd J 32 and 1.7 Hz, 2-H), 5.17 (1H, dd, J 46.8 and 1.7 Hz, 3-H).

(2R,3S)-3-Chloroaspartic Acid (4,X=Cl) 3-Chlorofumaric acid (1.5 g, 9.96 mmols) was dissolved in water (15 ml) containing 10 mM $MgCl_2$ and 4.5 mM NaCl, and the pH adjusted to 9.5 using concentrated aqueous ammonia. To this was added 240 units of 3-methylaspartase and the reaction mixture was stirred at room temperature for two hours. The solution was boiled for 2 minutes to denature the enzyme, and the protein removed by filtration. The solvent was removed in vacuo to give a white solid. The solid was dissolved in water (10 ml) and was subjected to cation exchange chromatography on an Amberlite 1R - 120(H) resin (50 ml) prewashed with water at pH 3. The column was eluted with water at pH 3 (flow rate 40 ml hr^{-1}). The elutant was reduced in volume in vacuo and the crude (2R,3S)-3-chloroaspartic acid (1.12 g, 67%) was recrystallized from water/methanol to give white crystals(1.0 g, 60%); m.p. 168-171°C; (Found: C, 28.65; H, 3.70; N, 8.30. $C_4H_6ClNO_4$ requires C, 28.65; H, 3.60; N, 8.35%); $[\alpha]_D^{20}$ -38.8° (c 0.5 in 0.1 M HCl), $[\alpha]_D^{20}$ -37.5° (c 0.4 in H_2O); ν_{max} (nujol), 3200-2900 (NH_3^+), and 1600 cm^{-1} (CO_2^-); δ_H (360 MHz, ^2H_2O) 4.25 (1H, d, J 2.3 Hz, 3-H), and 4.76 (1H, d, J 2.3 Hz, 2-H); δ_C (90 MHz, {^1H}, $^2H_2O/K_2CO_3$), 55.4 (CH), 57.2 (CH), 170.7 and 169.1 (2xCO_2H); m/z(FAB, +ve ion) 167 and 169 (M^+), and 131 ($[M - HCl]^+$).

(2R,3S)-3-Bromoaspartic acid (4,X=Br) 3-Bromofumaric acid is a good substrate for 3-methylaspartase. Although enzymic amination occurs rapidly cf.[4] the product is unstable under the incubation conditions and cyclizes to form 2,3-aziridinedicarboxylic acid and also alkylates the enzyme. (2R,3S)-3-Bromoaspartic acid can be detected transiently in on-going enzymic amination reactions by {^1H} ^{13}C-nmr spectroscopy [(incubation buffer in water containing 5% ^2H_2O, pH 9), δ, 55 (C-3), 56.4 (C-2), 176 and 172 (2xCO_2H)] or can be trapped in acidic media after partially complete amination reactions. The absolute configuration at both chiral centres of the compound have been determined using this latter approach.[10]

Reaction with Iodofumaric acid and acetylenedicarboxylic acid. Neither of the diammonium salts of iodofumaric or acetylenedicarboxylic acid acted as substrates for the enzyme.

L-Aspartic acid. Fumaric acid (2g, 17.2 mmol) was suspended in water (20 ml) and the pH adjusted to 9 with concentrated ammonia solution. The solution was then concentrated in vacuo. The diammonium fumarate was redissolved in water (20 ml),and magnesium chloride hexahydrate (40 mg, 10 mmol) and potassium chloride (7 mg, 4.5 mmol) was added. The pH was again adjusted to 9 with concentrated NH_3. The enzyme 3-Methylaspartase (90 units) was added and the reaction was incubated at 30°C until no further decrease in absorbance at 240 nm occurred (3 days).

The protein was denatured at 100°C for 2 minutes and was then removed by filtration through a celite pad. The filtrate was acidified to pH 1 with 12 M HCl, and was extracted with ether. The aqueous layer adjusted to pH 4 and the L-aspartic acid crystallized out on the addition of ethanol. The product was recrystallized from hot water/ethanol to give 2.06 g (90%); m.p. 300°C (dec); $[\alpha]_D^{20}$ +24.2° (c 0.5 in 6M HCl), (lit.[15a], $[\alpha]_D$ +24.6° (6M HCl)); δ_H (360 MHz, $^2H_2O/^2HCl$), 3.17 (2H, t, J 5.6 and 3.5 Hz), and 4.44 (1H, t, J 5.3 and 5.0 Hz).

(2S,3S)-3-Methylaspartic Acid (4,X=CH_3). Diammonium mesaconate (mesaconic acid (13 g, 0.1 mol) and NH_3 to pH 7.0). The required inorganic salts and 320 units of 3-methylaspartate ammonia lyase were incubated as described above for the preparation of L-aspartic acid.

Aliquots (20 µl) were taken at intervals, and diluted into water, and the absorbance at 240 nm measured. When no further decrease in absorbance was observed (ca. 40 hrs), the protein was denatured at 100°C, and then removed by filtration through a celite pad. The filtrate was evaporated to dryness, the residue redissolved in water (30 ml), the pH adjusted to 3.0 by addition of 12 M HCl, and the product crystallized with ethanol (60 ml). The solid was recrystallized twice from water/ethanol to give pure product (9.2 g, 61%); m.p. 272-275°C, (lit.[16], 276-278°C); (Found: C, 40.55; H, 6.20; N. 9.45. Calc. for $C_5H_9NO_4$; C, 40.38; H, 6.15; N, 9.50); $[\alpha]_D^{20}$ +13.4° (c 0.6 in 5M HCl), $[\alpha]_D^{20}$ -10.3° (c 0.6 in H_2O), (lit.[16], $[\alpha]_D^{20}$ -10±2° (c 0.42 in H_2O)); δ_H (360 MHz, ^2H_2O, pH 1), 1.38 (3H, d, J 7.6 Hz, 3-Me), 3.34 (1H, m, (AB splitting), 3-H), 4.50 (1H, d, J 3.2 Hz, 2-H); δ_C (90 MHz, {^{1}H}, ^2H_2O, pH 1), 12.93 (CH_3), 39.48 (CH), 54.62 (CH), 175.8 and 169.9 ($2xCO_2H$).

(2S,3S)-3-Ethylaspartic Acid (4,X=Et) was prepared as described above for (2S,3S)-3-methylaspartic acid using 2-ethylfumaric acid (2 g, 13.9 mmol) as the starting material. The product was obtained as white crystals (1.34 g, 60%); m.p. 245-246°C; (Found: C, 44.35; H, 6.90; N, 8.50. $C_6H_{11}NO_4$ requires C, 44.70; H, 6.90; N, 8.70%); $[\alpha]_D^{20}$ +15.0° (c 0.6 in 6M HCl); δ_H (360 MHz, ^2H_2O, pH 1), 1.02 (3H, t, J 7.4 Hz, $-CH_2\underline{CH_3}$), 1.82 (2H, m, $-\underline{CH_2}Me$), 3.06 (1H, m, 3-H), 4.43 (1H, d, J 4.2 Hz, 2-H); δ_C (90 MHz, {^{1}H}, ^2H_2O, pH 1), 8.93 (CH_3), 18.87 (CH_2), 44.16 (CH), 50.93 (CH), 172.6 and 167.3 ($2xCO_2H$).

(2S,3R)[3-^{2}H]-aspartic acid Fumaric acid (2 g, 17.2 mmol) was dissolved in deuterium oxide (20 ml) and concentrated aqueous ammonia was added to adjust the pH to 9.0. The solvent was removed at reduced pressure. The ammonium salt, magnesium chloride hexahydrate (40 mg, 0.2 mmol), and potassium chloride (7 mg, 0.09 mmol) were dissolved in deuterium oxide (20 ml) and the pH adjusted to 9.0 with further concentrated aqueous ammonia solution. To this was added 90 units of 3-methylaspartase and the solution incubated at 30°C. The absorbance at 240 nm was monitored by withdrawing aliquots (20 µl) which were then diluted into water. When no further decrease in absorbance was observed (ca. 48 hours) the enzyme was denatured by boiling the solution for 2 minutes. The protein was filtered off, and the pH of the solution adjusted to 1 with 12 M HCl, and washed with ether (2 x 30 ml). The pH was then adjusted to 3.0 with ammonium hydroxide, and the crude product crystallized out on the addition of ethanol. Recrystallized from water/ethanol gave (2S,3R) [3 - $^2H_1$] - aspartic acid as white crystals (1.53 g, 66%); m.p. 290°C (dec.); $[\alpha]_D^{20}$ +23.9° (c 0.6 in 6M HCl); δ_H (360 MHz, ^2H_2O, pH 1), 3.17 (1H, d, J 5.9 Hz, 3-H), 4.44 (1H, d, J 6.3 Hz, 2-H); δ_C (90 MHz, {^{1}H}, ^2H_2O, pH 1), 31.22 (triplet 3-C), 47.2 (2-C), 170.38 and 167.92 ($2xCO_2H$). Compare with preparation using L-aspartase.[17]

(2S,3S) [3-^{2}H]-3-Methylaspartic Acid This was prepared from mesaconic acid (2 g, 15.4 mmol) as described above using deuterium oxide as the solvent. (2S,3S)[3 - ^{2}H]-3-Methylaspartic acid was obtained as white crystals (1.37 g, 60%); m.p. 292-94°C; $[\alpha]_D^{20}$+12.0° (c 0.6 in 6M HCl); δ_H (360 MHz, ^2H_2O, pH 1), 1.36 (3H, s, 3-Me), 4.46 (1H, s, 2-H); δ_C (90 MHz, {^{1}H}, ^2H_2O, pH 1), 10.0 (CH_3), 36.88 (triplet, 3-C), 52.16 (2-C), 173.14 and 167.42 ($2xCO_2H$).

(2S,3S)[3-^{2}H]-3-Ethylaspartic Acid This was prepared as described above, from 3-ethylfumaric acid (2 g, 13.9 mmol) using deuterium oxide as the solvent. (2S,3S)[3 - ^{2}H]-3-ethylaspartic acid was obtained as white crystals (1.30 g, 58%); m.p. 250-2°C; $[\alpha]_D^{20}$+14.5° (c 0.6 in 6M HCl); δ_H (360 MHz, ^2H_2O, pH 1), 1.03 (3H, t, J 7.4 Hz, $-CH_2\underline{CH_3}$), 1.84 (2H, m, $-\underline{CH_2}CH_3$), 4.42 (1H, s, 2-H); δ_C (90 MHz, {^{1}H}, ^2H_2O, pH 1), 8.93 (CH_3), 18.35 (CH_2), 44.12 (triplet, 3-C), 51.18 (2-C), 172.61 and 167.38 ($2xCO_2H$).

N-Trifluoroacetyl-(2S,3S)-3-methylaspartic acid anhydride To 3-methylaspartic acid (100 mg, 0.68 mmol) was added excess trifluoroacetic anhydride (3 ml) in tetrahydrofuran (5 ml) at 0°C. The reaction mixture was then allowed to stir at room temperature until dissolution was complete (ca. 2 h). The solvent was removed *in vacuo* to give a white solid in quantitative recovery; ν_{max} (nujol), 3395 w (NH of amide), 1860 and 1800 cm^{-1} (cyclic anhydride carbonyls); δ_H (270 MHz, $(C^2H_3)_2SO$), 1.32 (3H, d, J 7 Hz, 3-$\underline{CH_3}$), 3.44 (1H, m, J 8.5 Hz,

3-C<u>H</u>), 4.90 (1H, t, J 8.5 Hz, 2-C<u>H</u>), and 10.17 (1H, d, J 8.5 Hz, -N<u>H</u>); m/z(EI) 198 ([MH - CO]$^+$, 6.6%), 153 (100, [M - C_2O_3H]$^+$), and 69 (48.6, CF_3).

<u>N-Trifluoroacetyl-(2S,3S)-3-ethylaspartic acid anhydride</u> was prepared as described above using 3-ethylaspartic acid; ν_{max} (nujol), 3405 w (NH of amide), 1855 and 1795 cm^{-1} st, (cyclic anhydride carbonyls); δ_H (270 MHz ($C^2H_3)_2SO$), 0.96 (3H, t, J 7.3 Hz, 3-$CH_2C\underline{H}_3$). 1.82 (2H, m, 3-$C\underline{H}_2CH_3$), 3.30 (1H, m, J 8.0 Hz, 3-C<u>H</u>), 4.92 (1H, dd, J 8.1 Hz, 2-C<u>H</u>), and 10.27 (1H, d, J 7.6 Hz, -N<u>H</u>); m/z(EI) 212 ([MH - CO]$^+$, 11.3%), and 167 (100, [M - C_2O_3H]$^+$).

<u>(2S,3S)-2-Bromo-3-ethylsuccinic acid</u> To water (10 ml) saturated with potassium bromide was added 3-ethyl-aspartic acid (120 mg, 0.74 mmol). Hydrogen bromide (0.11 ml, 2.2 mmol) was slowly added at 0°C, and then sodium nitrite (103 mg, 1.49 mmol) was added over a period of 20 min. The reaction was stoppered between additions to prevent the escape of nitrogen oxides. After 1 h the solution was extracted with diethyl ether (4 x 10 ml) and the pooled extracts were dried (Na_2SO_3) and reduced <u>in vacuo</u> to give a white solid. The product recrystallised from ether/light petroleum to give 80 mg, 50% yield; m.p. 130-32°C; (Found: C, 32.40; H, 4.10. $C_6H_9BrO_4$ requires C, 33.00; H, 4.00%); $[\alpha]_D^{20}$-97° (c 1 in EtOAc); ν_{max} (nujol), 3600-2500 (OH of acid), 1720-1700 (C=O of acid), 1300-1100 cm^{-1} (C-O of acid); δ_H (90 MHz, $^2H_2O/HCO_3^-$, HO^{2}H at 4.6 ppm), 0.72 (3H, t, J 7.7 Hz, 3-$CH_2C\underline{H}_3$), 1.67 (2H, m, 3-$C\underline{H}_2CH_3$), 2.82 (1H, m, 3-C<u>H</u>), and 4.33 (1H, d, J 10.3 Hz, 2-C<u>H</u>); m/z(EI) 209 and 207 ([M - OH]$^+$, bromine isotopes, 3.4 and 3.2%), 194 and 192 (6.0 and 5.9, bromine isotopes, [M - CH_4O]$^+$), and 99 (100, [M - CO_2H_2Br]$^+$).

<u>(2S)-2-Ethylsuccinic acid</u> (2S,3S)-2-Bromo-3-ethylsuccinic acid (100 mg, 0.44 mmol) was dissolved in methanol (10 ml) and then 20 mg of 5% Pd/C was added. The mixture was then hydrogenated, and the reaction followed by TLC. On completion of reaction (ca. 2 days) the solution was filtered through prewashed (with methanol) celite, and then the celite pad washed with methanol (50 ml). The effluent was concentrated <u>in vacuo</u> to yield a slightly off-white residue (50 mg, 78% recovery); $[\alpha]_D^{20}$ - 18.8°C (c 0.6 in acetone); (Lit.[15b], $[\alpha]_D^{24}$ - 20.8° (c 4.6 in acetone)); δ_H (90 MHz, ^2H_2O), 0.72 (3H, t, 2-$CH_2C\underline{H}_3$), 1.49 (2H, m, 2-$C\underline{H}_2CH_3$), 2.56 (1H, m, 2-C<u>H</u>) and 3.54 (2H, d, J 6 Hz, 3-C<u>H</u>); m/z(EI), 132 ([M - CH_2]$^+$, 18.3%), 129 (45, [M - OH]$^+$), 114 (64.4, [M - CH_4OH]$^+$), and 56 (100, $C_4H_8^+$).

<u>(2S,3S)-3-n-Propylaspartic acid (4,X=nPr)</u> was prepared as described above for (2S,3S)-3-methylaspartic acid using 3-<u>n</u>-Propylfumaric acid (1.5 g, 9.48 mmol) as the starting material. As this was a poor substrate an extended incubation time was required. Time-dependent enzyme activity loss therefore became significant, so fresh aliquots of enzyme were added at 48 h intervals. When no further decrease in absorbance was observed (<u>ca</u>. 2 weeks) the incubation mixture was heated to denature the enzyme. The solution was subjected to cation exchange chromatography on Amerlite IR-120(H), as escribed above, eluting with water and then 0.5 M aqueous ammonia. Fractions containing the amino acid were pooled and concentrated <u>in vacuo</u>. The residue was recrystallised from aqueous methanol (820 mg, 49%); m.p. 235-237°C; (Found: C, 47.80; H, 7.35; N, 8.15. $C_7H_{13}NO_4$ requires C, 48.00; H, 7.50; N, 8.00%); $[\alpha]_D^{20}$+7.6±0.2° (c 0.5 in 6M HCl); δ_H (270 MHz, ^2H_2O, pH 1) 0.93 (3H, t, J 7.2 Hz, 3'-C<u>H</u>$_3$), 1.43 (2H, m, 3'-C<u>H</u>$_2$CH$_3$), 1.66 (1H, m, 3'-C<u>H</u>-CH$_2$CH$_3$), 3.15 (1H, m, J 4.40, 5.86 and 5.79 Hz, 3-C<u>H</u>), and 4.43 (1H, d, J 4.40 Hz, 2-C<u>H</u>); δ_C (67.8 MHz, {^{1}H}, ^2H_2O, pH 1), 10.98 (CH$_3$), 18.09 (CH$_2$), 27.76 (CH$_2$), 42.76 (3-C), 51.26 (2-C), 173.74 and 168.25 (2xCO$_2$H); m/z (FAB, +ve) 176 (MH$^+$, 100%), 132 (25, [M - C_3H_7]$^+$),130 (20, [M - CO_2H]$^+$), and 75 (18, [M - $C_5H_8O_2$]$^+$).

<u>(2S,3S)-3-i-Propylaspartic acid (4,X=iPr)</u> was prepared as described above for (2S,3S)-3-<u>n</u>-propylaspartic acid using 3-<u>i</u>-propylfumaric acid (1.5 g, 9.48 mmol) as the starting material. The product was obtained as white crystals after recrystallisation from methanol (900 mg, 54%); m.p. 239-242°C; (Found: C, 47.60; H, 7.15; N, 7.90. $C_7H_{13}NO_4$ requires C, 48.00; H, 7.50; N, 8.00%); $[\alpha]_D^{20}$+7.35±0.2° (c 0.4 in 6M HCl); δ_H (270 MHz, ^2H_2O, Ph 1), 1.05 (6H, dd, J 6.34 and 22.46 Hz, 3'-CH(C<u>H</u>$_3$)$_2$), 2.14 (1H, m, 3'-C<u>H</u>(CH$_3$)$_2$), 2.85 (1H, dd, J 5.13 and 8.06 Hz, 3-C<u>H</u>), and 4.46 (1H, d, J 5.13 Hz, 2-C<u>H</u>); δ_C (67.8 MHz, {^{1}H}, ^2H_2O, pH 1), 17.34 (CH$_3$), 18.26 (CH$_3$), 25.00 (3'-CH), 50.34 (CH), 50.49 (CH), 172.99 and

168.41 ($2xCO_2H$); m/z(FAB, +ve) 176 (MH^+, 100%), 130 (18, $[M - CO_2H]^+$), and 75 (26, $[M - C_5H_8O_2]^+$).

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