*Tetrahedron: Asymmetry* Vol. 4, No. 6, pp. 1141-1152, 1993
Printed in Great Britain

0957-4166/93 $6.00+.00

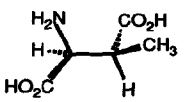

# Syntheses of (2S,3R)- and (2S,3R)[3-$^2$H]- 3-Methylaspartic acid: Slow Substrates for a *syn*-Elimination Reaction catalysed by Methylaspartase.

**Catherine H. Archer, Neil R. Thomas and David Gani***
Chemistry Department, The Purdie Building, The University, St. Andrews, Fife, KY16 9ST, UK.

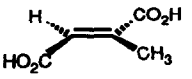
(*Received* 17 *February* 1993; *accepted* 18 *March* 1993)

**Abstract** : Methylaspartase catalyses the slow *syn*-elimination of ammonia from the (2S,3R)-[L-*erythro*]-diastereomer of the natural substrate, (2S,3S)-3-methylaspartic acid, to give mesaconic acid. To provide material of sufficient stereochemical purity to probe the mechanism of the reaction, two synthetic routes to (2S,3R)- and (2S,3R)[3-$^2$H]- 3-methylaspartic acid were devised. The use of these (2S,3R)-3-methylaspartic acids revealed that the enzymic reaction does not involve C-3 epimerisation followed by normal *anti*-elimination, ruling-out the possibility of a carbanion intermediate. Conversely, the substrate displayed very large primary deuterium isotope effects indicating rate-limiting C-H bond cleavage.

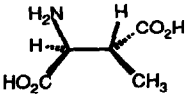
3-Methylaspartase (EC. 4.3.1.2) catalyses the second step in the catabolism of (2S)-glutamic acid in *Clostridia* and several other bacteria, the *anti*-elimination of ammonia from (2S,3S)-3-methylaspartic acid (**1**) to give mesaconic acid (**2**).




(1)



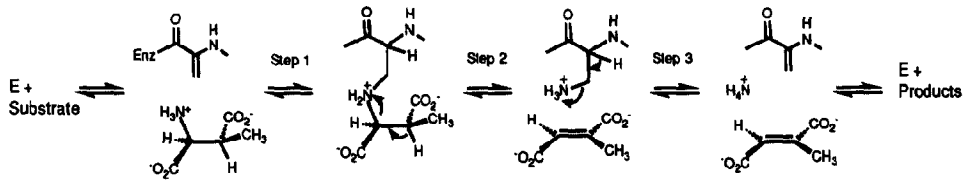

(2)




(3)

Early reports by Barker claimed that methylaspartase was also able to catalyse the *syn*-deamination of the L-*erythro*-isomer, (2S,3R)-3-methylaspartic acid (**3**), at *ca.* 1% of the rate for the natural substrate, to give mesaconic acid.[1] Based on this and other observations, including the finding that solvent hydrogen exchange into the C-3 position of (2S,3S)-3-methylaspartic acid (**1**) occurred at a rate faster than the natural deamination reaction, Bright suggested that each of the deamination reactions might proceed *via* a C-3 carbanion intermediate.[2] However, it was later shown that the natural substrate (**1**) displayed significant primary deuterium isotope effects for both the deamination[3] and the C-3 hydrogen exchange reaction.[4] The existence of

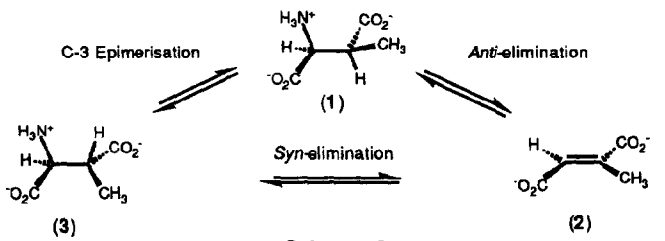
a carbanion was finally discounted for the L-*threo*-substrate when subsequent work, using double $^{15}N/^{14}N$-$^{2}H/^{1}H$-isotope fractionation measurements, showed that the elimination reaction was concerted[5] and was followed by a slow step (step 3) which could account for the rapidity of C-3 hydrogen exchange, Scheme 1.[4,6]




**Scheme 1**

In the absence of a carbanion intermediate derived from the L-*threo*-substrate (**1**), it was difficult to understand why methylaspartase should process the L-*erythro*-diastereomer. Nevertheless, it was possible to verify that the L-*erythro*-diastereomer (**3**) was a substrate, both for the homogeneous enzyme isolated from *Clostridium tetanomorphum* H1 and for a pure preparation of the recombinant wild-type enzyme over-expressed in *E. coli*.[6] In each case the deamination product was the *trans*-dioic acid (**2**). These results indicated unequivocally that L-*erythro*-3-methylaspartase activity (a direct *syn*-elimination), or, C-3 L-*erythro*-L-*threo*-3-methylaspartic acid epimerase activity (conversion of the L-*erythro*- to the L-*threo*- diastereomer prior to normal *anti*-elimination) is an inherent activity of L-*threo*-3-methylaspartase.

In order to distinguish between C-3 epimerase and L-*erythro*-3-methylaspartate ammonia-lyase activities, access to stereochemically pure (2S,3R)-3-methylaspartic acid and (2S,3R)[3-$^{2}$H]-3-methylaspartic acid was required. It was envisaged that these materials would allow the rate of the elimination reaction to be compared with the rate of epimerisation, if such a reaction occurred, by measuring solvent hydrogen exchange at C-3, Scheme 2.

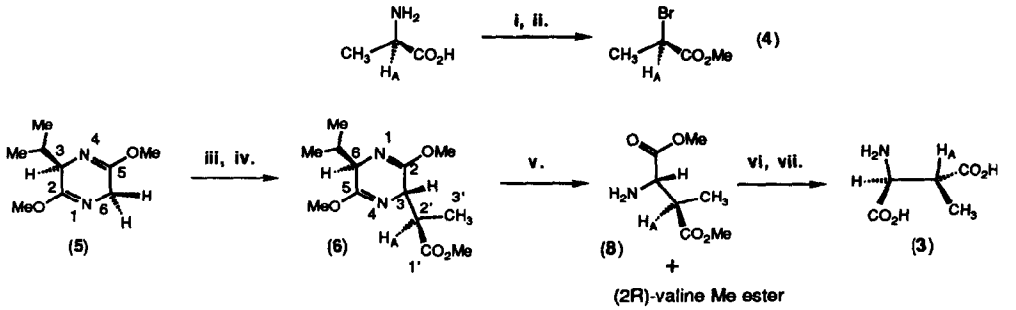



**Scheme 2**

(2R)-2-Bromopropanoic acid (**4**) can be prepared in good yield from (2R)-alanine.[7] The C-2 deuteriated material (**4**, $H_A = {}^{2}H$) is available from (2R)[2-$^{2}$H]-alanine which itself can be prepared either *via* the C-2 deuteriation of N-acetylalanine azlactone, followed by lactone hydrolysis, acylase resolution and then amino

group deprotection, or, *via* the methylation of Schollkopf's (3S)-2,5-dialkoxy-3-isopropyl-3,6-dihydropyrazines dideuteriated at C-6.[8]

Alkylation of (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (**5**) with the methyl esters of (2R)-2-bromopropanoic acid should then give the protected (2S,3R)-3-methylaspartic acid precursors with inversion of stereochemistry (and retention of absolute configuration) at C-2 of the electrophile.



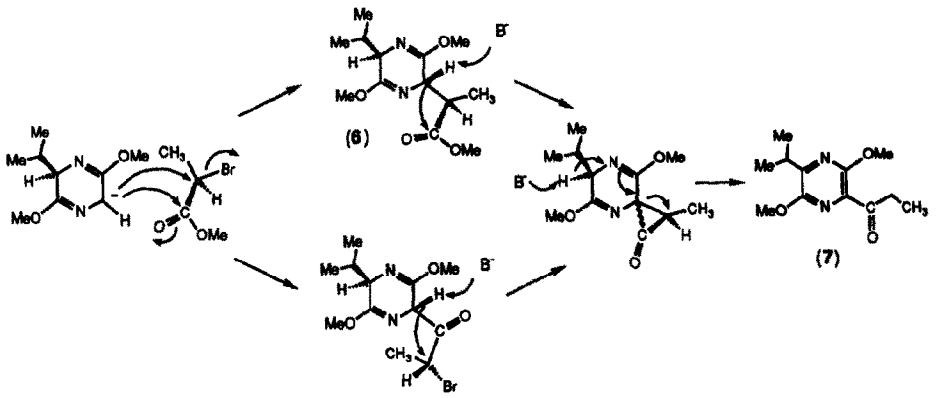

**Scheme 3**

I. KBr, HBr, $NaNO_2$, 0°C; II. $CH_2N_2$, $Et_2O$, distil; III. 1.1 eq. n-BuLi, THF -80 °C; IV. 1.5 eq. (4) added at -65°C, flash chromatography on silica; V. 0.07 M HCl in 2.5:1 water: MeCN; VI. 1.0 M NaOH, 70° C, 15 min. then HCl to pH 7.0; VII. Ion exchange chromatography on Biorad AG1-X8.

In the event, the C-6 alkylation of the n-BuLi generated anion of commercially available (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (**5**) with methyl (2R)-2-bromopropanoate (**4**), Scheme 3, was successful and gave mainly the required (3S,6R,2'R)-methyl ester (**6**), as judged by $^1$H-NMR spectroscopic examination of the crude product. Small amounts of other stereoisomers of unknown absolute configuration [total < 10% of compound (**6**)] were also detected but, the major side product, up to 32% of the crude product, proved to be the ketone (**7**). This material can arise either through an internal Claisen condensation of ester (**6**), or, *via* initial attack of the dihydropyrazine C-6 carbanion on C-1 of the methyl (2R)-2-bromopropanoate, Scheme 4.

All attempts to prevent the formation of the ketone (**7**), by reducing the amount of base used in generating the anion of (**5**), by increasing the amount of the electrophile (**4**) and/or, changing the temperature for the reaction, or, even by using the more sterically shielded isopropyl (2R)-2-bromopropanoate ester, were unsuccessful.

Under the best conditions the ester (**6**) was obtained in 56% yield after chromatographic purification on flash silica and contained less than 5% of other stereoisomers. Cleavage of the dihydropyrazine ring was effected using 0.07 M HCl to give a mixture of methyl (2S)-valinate and (2S,3R)-3-methylaspartic acid dimethyl ester (**8**). These were saponified and the amino acids were separated by ion exchange chromatography to give to required 3-methylaspartic acid (**3**) in 76% yield from the ester (**6**) after recrystallisation from water/ethanol.

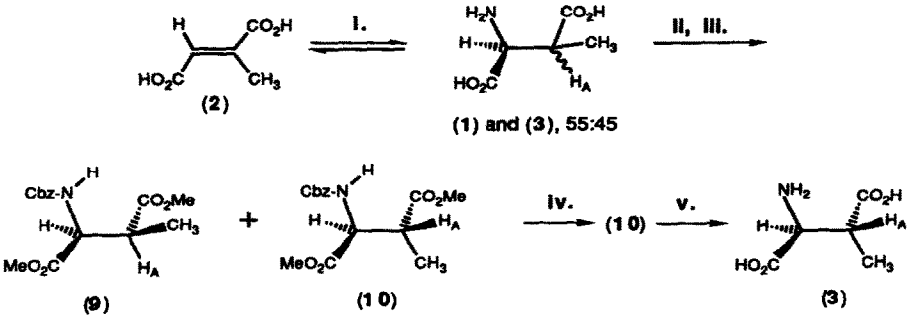


**Scheme 4**

The (2S,3S)-diastereomer of 3-methylaspartic acid can be easily distinguished from the (2S,3R)-diastereomer in $^1$H-NMR spectra by examining the chemical shifts for the signals due to the methyl groups. These occur at ~1.05 and 1.20 ppm respectively. By this assessment the synthesis provided material containing 95% of the required (2S,3R)-diastereomer and 5% of the unwanted (2S,3S)-diastereomer. The material could not be purified by further recrystallisation and the chiral integrity at C-3 was too low to be of utility in several of the planned investigations with methylaspartase. Indeed, the contaminant, (2S,3S)-3-methylaspartic acid (**1**), the natural substrate for the enzyme, was selectively processed by the enzyme, in a burst, at a rate much faster than for the (2S,3R)-diastereomer. This finding precluded the use of the material in any kinetic experiments. Nevertheless it was possible to use the material to verify that the product of the deamination of (2S,3R)-3-methylaspartic acid was the *trans*-enedioic acid, mesaconic acid, *vide supra*.

In order to obtain pure (2S,3R)-3-methylaspartic acid (**3**) alternative approaches incorporating resolution steps were considered. Since we had confirmed that the L-*erythro*-diastereomer (**3**) was a substrate for methylaspartase, it seemed likely that conditions could be found under which both L-diastereomers of 3-methylaspartic acid could be generated in significant amounts from mesaconic acid and ammonia. As the enzyme only operates on L-amino acids, the remaining problem would be to separate the 3-methylaspartic acids on the basis of their configurations at C-3. Such a strategy would also provide C-3 deuteriated material, simply by conducting the amination reactions in deuterium oxide.

Accordingly, (2S,3RS)-3-methylaspartic acid and [3-$^2$H](2S,3RS)-3-methylaspartic acid were prepared through the catalytic amination of mesaconic acid with methylaspartase, in the appropriately labelled solvents, by allowing the reaction to reach equilibrium over several weeks, Scheme 5.




**Scheme 5**

I. $NH_4Cl$ (0.2 M), $MgCl_2$ (20 mM), KCl (10 mM) in $H_2O$, pH 9.0 or $^2H_2O$, p$^2$H 8.6, methylaspartase (500 units [μmol $mg^{-1}$ $min^{-1}$] for 5 g of mesaconic acid), 30°C, ~25-30 days required to reach equilibrium, 86%; II. N-(benzyloxycarbonyloxy)-succinimide, $K_2CO_3$ (aq), 99%; III. $CH_2N_2$, $Et_2O$, 98%; Iv. Chromatography on flash silica, eluting with $Et_2O$/pet. ether (1:1), N-Cbz-(2S, 3R)-3-methylaspartate dimethyl ester is obtained in quantitative recovery, after recycling, and elutes first; v. AcOH / HCl (1:1), reflux, 55 %.

The unlabelled and deuteriated L-*erythro*-3-methylaspartic acids were obtained by resolving the (2S,3RS)-N-benzyloxycarbonyl-3-methylaspartic acid dimethyl esters (4 and 5 $H_A$ = H or $^2$H) as shown in Scheme 5. The (2S,3R)-3-methylaspartic acids (3, $H_A$ = H) and (3, $H_A$ = $^2$H) showed the expected spectral and analytical data and contained less than 2% of the (2S,3S)-diastereomer as judged by $^1$H-NMR spectroscopic and enzyme kinetic analyses.

To distinguish between epimerase and L-*erythro*-3-methylaspartase activities, (2S,3R)-3-methylaspartic acid (3, $H_A$ = H) was incubated with the enzyme in deuterium oxide in the presence of $K^+$ and $Mg^{2+}$ ions and the reaction was monitored by 200 MHz $^1$H-NMR spectroscopy. The deamination reaction proceeded smoothly to give mesaconic acid and then, much later, (2S,3S)[3- $^2$H]-3-methylaspartic acid. No solvent hydrogen incorporation into C-3 of the substrate, (2S,3R)-3-methylaspartic acid was detected over the time course of the reaction (24 h.). The estimated limit of detection by 200 MHz $^1$H-NMR is ~ 2 % of deuterium incorporation. Moreover, it was evident that the L-*threo*-isomer was not formed *via* direct epimerisation at C-3 since its formation depended on the amination of mesaconic acid generated during the initial phase of the reaction. Hence, methylaspartase is able to catalyse, directly, the *syn*-elimination of ammonia from the L-*erythro*-substrate.

Examination of the kinetic properties of the L-*erythro*-substrate (3) in the presence of 1 mM $K^+$and 20 mM $Mg^{2+}$ at pH 9.0 revealed that $V_{max}$ was 17.2 x $10^{-6}$ mol $dm^{-3}$ $min^{-1}$( $k_{cat}$ = 12.9 $s^{-1}$), 38 times slower, and $K_m$ was 40 mM, 17 times larger, than the corresponding parameters for the L-*threo*-diastereomer.[11] As with the L-*threo*-substrate, increasing the $K^+$ concentration from 1 to 50 mM increased the apparent value of $V_{max}$ and also decreased the apparent value of $K_m$. For the L-*erythro*-substrate, these factors were 2.4 and 7.6 respectively.

At 1 mM $K^+$, $^{D}V$ [or $V_H/V_D$] was 7.15 ± 2.74 and $^{D}(V/K)$ [or $(V_H/V_D)/(K_H/K_D)$] was 3.39 ± 1.6. These unusually large primary deuterium isotope effects clearly indicate that C-H bond cleavage is the rate determining step in the reaction and suggest that the elimination process might be concerted.[11] The use of (2S,3R)-3-methylaspartic acid and its C-3 deuteriated isotopomer in other experiments designed to probe the mechanism of the *syn*-elimination process will be described elsewhere.

## Experimental

All solvents and reagents were of analytical grade or were purified before use. Melting points were determined using an Electrothermal melting point apparatus and are uncorrected. Elemental analyses were performed in the departmental microanalytical laboratory or, on a service basis, by the microanalytical laboratory at University College, London. Optical rotations were measured at room temperature (21-25 °C) using Optical Activity Ltd AA 100 and AA 1000 polarimeters and 10 cm path-length cells. Infrared spectra were recorded on a Perkin-Elmer 1500 series FT IR spectrometer or on Perkin-Elmer 1310 and 1330 IR spectrometers. Absorption maxima are given in wavenumbers ($cm^{-1}$) relative to a polystyrene standard.

$^1$H-Nuclear magnetic resonance spectra were recorded at 200 MHz on a Varian Gemini-200, at 270 MHz on a Jeol JNM-GX270 or at 300 MHz on a Bruker AM-300. Chemical shifts are given in parts per million downfield from TMS or the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-$^2H_4$ (at 0.0 ppm) or are referenced to internal $CHCl_3$ or $HO^2H_2$ signals at 7.27 and 4.61 ppm respectively, as appropriate for the sample. $^{13}$C-Nuclear magnetic resonance spectra were recorded at 50.30 MHz on a Varian Gemini-200, at 67.8 MHz on a Jeol JNM-GX270 and at 75.47 MHz on a Bruker AM-300. Chloroform (at 77.20 ppm) or methanol (at 47.00 ppm) were used as internal reference signals. Mass spectra and accurate mass measurements were obtained on a VG-70 250 SE or a Kratos MS 50 spectrometer, or on a VG ZAB E at the Science and Engineering Research Council service at Swansea, UK. Major fragments are given as percentages of the base peak intensity (100 %).

Electronic adsorption spectra were obtained using a Pye-Unicam SP8-500 or SP8-100 spectrophotometer and flash chromatography was performed using Sorbsil C 60 (40 - 60 mm) silica gel or Macherey-Nagel silica gel N.

### Methyl (2R)-Bromopropanoate (4).

(2R)-Alanine (4.0 g, 45 mmol) was added to a saturated solution of potassium bromide (10 ml), followed by the dropwise addition of hydrogen bromide (15 ml of a 47% soln). The resulting mixture was then cooled to 0 °C and sodium nitrite (6.21 g, 90 mmol) was added over 1h. The reaction mixture was maintained below 5 °C for a further 1h. and then allowed to warm to room temperature. The resulting solution was then extracted with diethyl ether (3 x 25 ml), the combined ether extracts were then dried ($MgSO_4$) and concentrated *in vacuo* to give a pale yellow oil (6.54 g) in 95% yield. This was distilled under reduced pressure to give pure (2R)-

bromopropanoic acid. B.p. 68-70 °C/0.1 mm Hg; $[\alpha]_D$ +29.1 (c 2.0 in $CHCl_3$) (Lit.[7] 45.4 (neat)); $\upsilon_{max.}$ ($CHCl_3$) 3110 (O-H), 1730 $cm^{-1}$ (C=O); $\delta_H$ (270 MHz, C $^2H$ $Cl_3$) 1.85 (3H, d, *J* 7Hz, 3-$CH_3$), 4.41 (1H, q, *J* 7Hz, 2-CH), 10.75 (1H, s br, 1-$CO_2H$).

To a solution of (2R)-bromopropanoic acid (5.0 g, 33 mmol) in ether (10 ml) was slowly added an ethereal solution of diazomethane until the yellow colour persisted. The excess diazomethane was removed in a stream of nitrogen, and the solution was then concentrated *in vacuo*, to give a pale yellow oil which was distilled from $CaH_2$ to give methyl (2R)-2-bromopropanoate as a colourless oil (5.04 g, 93 %); B.p. 143-144 °C; $[\alpha]_D$ +69.1 (neat) (lit.[7] +68.6 ); $\upsilon_{max.}$ ($CHCl_3$) 1750 $cm^{-1}$ (C=O); $\delta_H$ (270 MHz, $C^2H$ $Cl_3$) 1.86 (3H, d, *J* 7 Hz, 3-$CH_3$), 3.83 (3H, s, 1-$CO_2CH_3$), 4.42 (1H, q, *J* 7 Hz, 2-CH).

**Alkylated Dihydropyrazine (6) {(3S,6R)-2,5-dimethoxy-3-((2'R)-methoxypropionyl)-6-isopropyl-3,6-dihydropyrazine}.**

To a solution of (3R)-2,5-dimethoxy-3-isopropyl-3,6-dihydropyrazine (**5**) (4.0 g, 21.7 mmol) in anhydrous THF (15 ml) under argon cooled to -80 °C was added n-butyl lithium (9.5 ml of a 2.5 M solution in hexane, 23.75 mmol). The solution was allowed to warm to -65 °C, when the yellow colour of the *bis*-lactim ether anion became apparent. The solution was cooled to -80 °C and a solution of methyl (2R)-bromopropanoate (**4**) (5.43 g, 33 mmol) in THF (10 ml), precooled to -80 °C, was added. The reaction was kept at -55 °C for at least 12 h. and was then allowed to warm to room temperature. Removal of the solvent *in vacuo* gave an oil which was partitioned between diethyl ether (25 ml) and potassium phosphate buffer (100 mM, pH 7.0; 25 ml). The ethereal phase was separated, and the aqueous phase was extracted with diethyl ether (2 x 25 ml). The pooled fractions were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil containing both the required alkylated *bis*-lactim ether (**6**) and the pyrazine ketone (**7**). These were separated by flash column chromatography on silica (15% ethyl acetate; pet. ether) to give 3.27 g, (55.8%) of the required alkylated *bis*-lactim ether (**6**) as a colourless oil and 1.62 g (31.2%) of the pyrazine ketone (**7**) as white crystals.

For compound (**6**): (Found: C, 57.85; H, 8.60; N, 10.00; $M^+$, 270.1581. $C_{13}H_{22}N_2O_4$ requires C, 57.80; H, 8.20; N, 10.35%; $M^+$, 270.1580); $[\alpha]_D$ +24.6 (c 1.0 in $CHCl_3$); $\upsilon_{max.}$ ($CHCl_3$) 2970 (CH), 1734 (C=O), 1696 $cm^{-1}$ (C=N); $\delta_H$ (270 MHz, $C^2HCl_3$) 0.73 and 1.04 (3H, d, *J* 7 Hz, 6-CH($C\underline{H}_3$)$_2$), 1.16 (3H, d, *J* 7 Hz, 3-(CH($C\underline{H}_3$)$CO_2CH_3$)), 2.24 (1H, dsp, *J* 3 and 7 Hz, 6-CH), 3.01 (1H, dq, *J* 3 and 7 Hz, 3-($C\underline{H}$($CH_3$)$CO_2CH_3$)), 3.66 (1H, s br, 2,5-$OCH_3$, and 3-(CH($CH_3$)$CO_2C\underline{H}_3$), 3.94 (1H, t, *J* 3 Hz, 6-H), 4.20 (1H, t, *J* 3 Hz, 3-H); $\delta_C$ (67.9 MHz, $C^2HCl_3$) 11.57, 16.84 (6-CH($\underline{C}H_3$)$_2$), 19.17 (3-(CH($\underline{C}H_3$)$CO_2CH_3$)), 32.10 (6-$\underline{C}$H($CH_3$)$_2$), 43.67 (3-($\underline{C}$H($CH_3$)$CO_2CH_3$), 51.83 (3-(CH($CH_3$)$CO_2\underline{C}H_3$), 52.59 52.68 (2,5-$OCH_3$), 57.96 (6-CH), 61.10 (3-CH), 162.08, 164.74 (2,5-O-C=N), 174.03 ppm (C=O); *m/z* (EI) 270 ($M^+$, 0.3%), 255 (0.2 %, [*M* - $CH_3$]$^+$), 239 (1.4, [*M* - $OCH_3$)$^+$), 227 (16.8, [*M* - CH($CH_3$)$_2$]$^+$).

For compound (7): m.p. 34-35 °C; (Found: C, 60.55; H, 7.60; N, 11.70; $M^+$, 238.1330. $C_{12}H_{18}N_2O_3$ requires: C, 60.50; H, 7.60; N, 11.75%; $M^+$, 238.1317); $\delta_H$ (270 MHz, $C^2HCl_3$) 1.16 (3H, t, *J* 7 Hz, 3-$COCH_2\underline{CH}_3$), 1.23 (6H, d, *J* 7 Hz, 6-$CH(\underline{CH}_3)_2$), 3.06 (2H, q, *J* 7 Hz, 3-$COCH_2\underline{CH}_3$), 3.34 (1H, sp, *J* 7Hz, 6-$\underline{CH}(CH_3)_2$), 3.94 and 4.01 ppm (6H, 2s, 2,5-$OCH_3$); *m/z* (EI) 238 ($M^+$,66.83%), 223 (13.46, $[M - CH_3]^+$), 209 (100, $[M - C_2H_5]^+$).

**(2S,3R)-3-Methylaspartic acid (3) from compound (6)**

The alkylated *bis*-lactim ether (6) (3.27 g, 12.0 mmol) was dissolved in 0.10 M hydrochloric acid (250 ml, 25 mmol) and acetonitrile (100 ml) and was stirred at room temperature for 12 h. The resulting solution was extracted with diethyl ether (2 x 50 ml) which was then discarded. The aqueous phase was concentrated *in vacuo* to give a 1:1 mixture of (2R)-valine methyl ester hydrochloride and (2S,3R)-dimethyl-3-methylaspartic acid hydrochloride as a white solid which was dissolved in 1.0 M sodium hydroxide solution (60 ml, 60 mmol). The solution heated to 70 °C for 15 min., cooled on ice and adjusted to pH 7.0 using 5.0 M hydrochloric acid. The solution was then concentrated *in vacuo* to *ca.* 4.0 ml and was applied to a column of Biorad AG1-X8 anion exchange resin (1 x 15 cm: OH form). The column was washed with water (500 ml) and then the pH of the elutant was gradually decreased using acetic acid until all of the (2R)-valine had been eluted. The column was then washed with 0.5 M acetic acid until all of the 3-methylaspartic acid had eluted. These fractions were combined and were concentrated *in vacuo* to give (2S,3R)-3-methylaspartic acid (3) as a white solid which was recrystallised from water/ethanol, (1.34 g, 76 %). m.p. 257-259 °C dec.; (Found: C, 40.95; H, 6.15; N, 9.50; $C_5H_9NO_4$ requires C, 40.80; H, 6.15; N, 9.50%); $[\alpha]_D$ + 37.9 (c 1.0 5 M HCl) (lit.[9] +38.7 (c 1.83 in 5 M HCl); $\delta_H$ (270 MHz, $^2H_2O$, pH 9.0; TMS(Na)) 1.29 (3H, d, *J* 7 Hz, 3-$CH_3$), 3.16 (1H, dq, *J* 4 and 7 Hz, 3-H), 3.98 (1H, d, *J* 4 Hz, 2-H); $\delta_C$ (67.9 MHz, $^2H_2O$, pH 9.0; ref. dioxane) 16.10 (3-$CH_3$), 42.62 3-C), 58.31 (2 -C), 174.98 and 182.07 ppm (1 and 4-C=O); *m/z* (CI - $NH_3$) 165 ($[M + NH_4]^+$, 10.4%), 148 (100, $[M + H]^+$). This material contained upto 5% of the (2S,33)-diastereomer.

**(2S,3RS)-3-methylaspartic acid, (1) and (3).**

Mesaconic acid (5 g, 38.5 mmol) was dissolved in water (5 ml) and 35 M ammonia solution was added to adjust the pH to 9.0. The water was removed *in vacuo* to give the ammonium salt. This was redissolved in buffer (50 ml) containing 0.2 M ammonium chloride, 20 mM magnesium chloride hexahydrate and 10 mM potassium chloride, and the pH readjusted to 9.0. 3-Methylaspartase (0.5 ml, 50 units) was added and the mixture incubated at 30 °C. The reaction was monitored by removing 0.5 ml aliquots at various time intervals, which were concentrated *in vacuo*, redissolved in deuterium oxide and examined by $^1$H-NMR spectroscopy. Further aliquots of enzyme were added as required, until approximately half of the 3-methylaspartate produced was in the L-*erythro* -form. At this time the enzyme was denaturated by heating at 80 °C for several minutes and was removed by filtration. The filtrate was acidified to pH 1.0 with 12 M hydrochloric acid and then extracted with

diethyl ether (2 x 30 ml). The aqueous layer was concentrated *in vacuo* to give 5 g of a 55:45 mixture of (2S,3S:3R)-3-methylaspartate containing ~20% by weight of buffer salts (3.98 g, 86%). $\delta_H$ (200 MHz, $^2H_2O$) 1.20 (3H, d, *J* 7.6 Hz, *threo* -3-$CH_3$), 1.31 (3H, d, *J* 7.6 Hz, *erythro* -3-$CH_3$), 3.01 (2H, m, *J* 7.6 & 5.0 Hz, 3-H), 3.81 (1H, d, *J* 5.0 Hz, *erythro*-2-H), 4.06 (1H, d, *J* 5.0 Hz, *threo*-2-H).

**Dimethyl N-carbobenzoxy-(2S,3RS)-3-methylaspartate (9) and (10).**

(2S,3S/R)-3-Methylaspartate (3.98 g, 27 mmol) was dissolved in potassium carbonate solution (10%, 100 ml). N-(Benzyloxycarbonyloxy)-succinimide (13.5g, 54 mmol) was added, and the reaction was stirred for 16 hours. The reaction mixture was extracted with dichloromethane (2 x 100 ml), the aqueous layer was acidified to pH 2.0 and then extracted with ethyl acetate (5 x 100 ml). The pooled fractions were dried ($MgSO_4$) and concentrated *in vacuo* to give N-carbobenzoxy-(2S,3RS)-3-methylaspartic acid a yellow oil (7.53 g, 99%). (Found: $(M + NH_4 - H_2O)^+$, 281.1137. $C_{13}H_{15}NO_6 + NH_4 - H_2O$ requires 281.1137); $\upsilon_{max}$ ($CHCl_3$) 3100 (O-H) & 1720 (C=O) $cm^{-1}$; $\delta_H$ (200 MHz, $C^2HCl_3$) 1.22 & 1.28 (6 H, 2d, *J* 7.6 Hz, 3-$CH_3$), 3.00 (1H, m, *J* 5.0 Hz, *threo* -3-H), 3.33 (1H, m, *J* 5.0 Hz, *erythro* -3-H), 4.58 (2H, m, *J* 3.6 Hz, 2-H), 5.07 & 5.10 (4H, 2s, $CH_2$) 6.71 (2H, 2 br d, *J* 10.0 Hz, NHs) & 7.30 (10H, s, Phs); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 13.77 & 14.03 (3-$CH_3$s), 41.65 (*threo* -3-C), 42.52 (*erythro* -3-C), 55.87 & 56.04 (2-Cs), 67.98 (Ph<u>C</u>$H_2$s), 128.54, 128.67 & 129.04 (Aromatics), 136.40 (*Phenyl* C-1), 157.12 & 157.71 (0C0NHs), 175.09, 175.66 & 178.73, 179.19 ($CO_2$Hs); *m/z* (EI) 263 ( $[M-H_2O]^+$, 7%), 108 (47, $PhCH_2OH$), 107 (51, $PhCH_2O$), 91 (100, $PhCH_2$), 44 (37, $CO_2$); *m/z* (CI) 281 ($[M + NH_4 - H_2O]^+$, 60%), 263 (14, $MH^+ - H_2O$), 192 (16, $M - C_2O_4H$) and 57 (6, $MH^+ - PhC_4H_4O_6$).

The material from the preceding experiment (7.53 g, 27 mmoles) was dissolved in diethyl ether (50 ml) and an ethereal solution of diazomethane was added slowly dropwise, until the yellow colour persisted. The excess diazomethane was removed in a stream of nitrogen and the solvent was removed *in vacuo* to give a pale yellow oil (8.12 g, 98%). (Found: $M^+$ 309.1212. $C_{15}H_{19}NO_6$ requires 309.1212); $\upsilon_{max}$ ($CHCl_3$) 3420 (N-H) & 1730 (C=O) $cm^{-1}$; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 (3H, d, *J* 7.5 Hz, *threo*-3-$CH_3$), 1.27 (3H, d, *J* 7.5 Hz, *erythro*-3-$CH_3$), 3.00 (1H, m,*J* 4.0 Hz, *threo*-3-H), 3.30 (1H, m, *J* 4.0 Hz, *erythro*-3-H), 3.67 & 3.74 (2 x 6H, 2s, $CO_2CH_3$s), 4.58 (1H, m, *J* 4 Hz, *erythro*-2-H), 4.70 (1H, m, *J* 4 & 5 Hz, *threo*-2-H), 5.12 & 5.18 (2 x2H, 2s, Ph<u>C</u>$H_2$), 5.55 (1H, br d, *J* 7.6 Hz, *threo*–NH), 5.70 (1H, br d, *J* 10.0 Hz, *erythro*-NH) & 7.35 (10H, s, Ar); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 12.29 ((3S)-3-$CH_3$), 14.19 ((3R)-3-$CH_3$), 41.62 ((3S)-C), 41.80 ((3R)-C), 51.76 & 52.22 (2 x *threo*-$CO_2$<u>C</u>$H_3$), 52.62 & 53.15 (2 x *erythro*-$CO_2$<u>C</u>$H_3$), 55.44 (*threo*-2-C), 56.34 (*erythro*-2-C), 66.70 (*threo*--Ph<u>C</u>$H_2$), 67.67 (*erythro*-Ph<u>C</u>$H_2$), 127.70, 127.79, 128.12 & 135.83 (*threo*–Ar), 128.53, 128.69, 129.02 & 136.66 (*erythro*-Ar), 155.56 (*threo*-OCONH), 157.13 (*erythro*-OCONH), 170.67 & 173.08 (2 x *threo*-$CO_2CH_3$), 171.70 & 174.67 (2 x *erythro*-$CO_2CH_3$); *m/z* (EI) 309 ($M^+$, 8%), 250 (19, $M - CO_2Me$), 206 (31, $M - C_3H_3O_4$) 174 (7, $M - PhCH_2CO_2$), 108 (41, $PhCH_2OH$) & 91 (100, $PhCH_2$).

**Dimethyl N-carbobenzoxy-(2S,3R)-3-methylaspartate (10).**

N-Carbobezoxy-(2S, 3RS)-3-methylaspartate dimethyl ester (1 g, 3.4 mmoles) was dissolved in diethyl ether and adsorbed onto silica (TLC grade), this was applied to a silica chromatgraphy column and the column was eluted with diethyl ether/petroleum ether (50:50). The (2S,3R)-3-methylaspartate ester eluted first and the pooled fractions were concentrated *in vacuo* to give compound (**10**) as a colourless oil (0.35 g, 35%). (Found: $M^+$ 309.1212. $C_{15}H_{19}NO_6$ requires 309.1212); $\upsilon_{max}$ ($CHCl_3$) 3420 (N-H) & 1730 (C=O) $cm^{-1}$; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.27 (3H, d, *J* 7.5 Hz, 3-$CH_3$), 3.30 (1H, m, *J* 4.0 Hz, 3-H), 3.67 & 3.74 (2 x 3H, 2s, $CO_2CH_3$s), 4.58 (1H, m, *J* 4 Hz, 2-H), 5.18 (2H, s, PhC$\underline{H}_2$), 5.70 (1H, br d, *J* 10.0 Hz, NH) & 7.35 (5H, s, Ar); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 14.19 (3-$CH_3$), 41.80 (3-C), 52.62 & 53.15 ($CO_2CH_3$), 56.34 (2-C), 67.67 (Ph$\underline{C}H_2$), 128.53, 128.69, 129.02 & 136.66 (Ar), 157.13 (OCONH), 171.70 & 174.67 ($CO_2CH_3$s); *m/z* (EI) 309 ($M^+$, 8%), 250 (19, *M* - $CO_2Me$), 206 (31, *M* - $C_3H_3O_4$) 174 (7, *M* - $PhCH_2CO_2$), 108 (41, $PhCH_2OH$) & 91 (100, $PhCH_2$).

**(2S,3R)-3-Methylaspartic acid (3)**

N-Carbobenzoxy-(2S,3R)-3-methylaspartate dimethyl ester (0.72 g, 2.33 mmol) was refluxed in concentrated hydrochloric acid and glacial acetic acid (50:50) (20 ml) for 2 h. The solvent was removed *in vacuo* and the residue was redissolved in water (20 ml) and then lyophilised to give the dry hydrochloride salt. The residue was dissolved in the minimum volume of water and 20 M ammonia solution (0.11 ml) was added. Addition of ethanol gave (2S, 3R)-3-methylaspartic acid as white crystals (0.19 g, 55%). m.p. 257 - 259 °C (decomp.) (Found: C, 40.60; H, 6.45; N, 9.65. $C_5H_9NO_4$ requires C, 40.80; H, 6.15; N, 9.50.) (Found: $(M + H)^+$, 148.0610. $C_5H_9NO_4$ requires 148.0610); $[\alpha]_D$ +36.3 (c 1.0, 5M HCl), lit[9] +38.7 (c 1.83, 5M HCl), lit[10] +32.9° (c 0.8, 5M HCl); $\delta_H$ (200 MHz; $^2H_2O$) 1.26 (3H, d, *J* 7.5 Hz, 3-$CH_3$), 3.13 (1H, m, *J* 7.5 & 5.0 Hz, 3-H), 3.95 (1H, d, *J* 5.0 Hz, 2-H); $\delta_C$ (50.3 MHz; $^2H_2O$) 15.72 ($CH_3$), 42.78 (3-C), 58.99 (2-C), 175.25 & 180.13 ($CO_2$Hs); *m/z* (CI) 148 ($MH^+$, 100%), 102 (20, *M* - $CO_2H$), 58 (53, *M* - $C_2O_4H$). This material contained less than 2% of the (2S,3S)-diastereomer as judged by $^1$H-NMR spectroscopic and also enzyme kinetic analysis where the end-point for (fast) L-*threo*-substrate processing was measured by following ΔOD at 240 nm.[4]

**(2S,3RS)[3-$^2$H]-3-Methylaspartic acid (1, $H_A$ = $^2$H) and (3, $H_A$ = $^2$H).**

Mesaconic acid (5 g, 38.5 mmol) was dissolved in deuterium oxide (5 ml) and ammonia solution added to adjust the pD to 8.6. The deuterium oxide was removed *in vacuo* to give the ammonium salt. The buffer salts (ammonium chloride (0.535 g, 10 mmol), magnesium chloride hexahydrate (0.203 g, 1 mmol) and potassium chloride (37 mg, 0.5 mmol) were dissolved in deuterium oxide and concentrated *in vacuo* twice before dissolved in deuterium oxide (50 ml). The mesaconate diammonium salt was redissolved in the deuteriated buffer (50 ml, 0.2 M ammonium chloride, 20 mM magnesium chloride hexahydrate and 10 mM potassium chloride) and the pH readjusted to 8.6 with sodium deutoxide. A portion of 3-methylaspartase enzyme solution

(0.5 ml, 50 units) was diluted with deuterium oxide (2 ml) and lyophilysed. The residue was dissolved in deuterium oxide (0.5 ml) and added to the buffered substrate solution. The mixture was incubated at 30 °C and the reaction was monitored as described previously until approximately one third the [3-$^2$H]-3-methylaspartic acid produced was the L-*erythro* -form. The reaction was terminated and worked-up as described previously to give a 2:1 mixture of (2S,3S/3R)[3-$^2$H]-3-methylaspartic acid (97 % deuteriated) (4.61 g, 81.1 %) and buffer salts. $\delta_H$ (200 MHz; $^2H_2O$) 1.20 (3H, s, *threo*-3-$CH_3$), 1.31 (3H, s, *erythro*-3-$CH_3$), 3.81 (1H, s, *erythro*-2-H), 4.06 (1H, s, *threo*-2-H).

**(2S,3R)[3-$^2$H]-3-Methylaspartic acid (3, $H_A$ = $^2$H)**

The material from the previous experiment (4.61 g, 31 mmol) was dissolved in potassium carbonate solution (10%, 100 ml) and was treated with N-(benzyloxycarbonyloxy)-succinimide (15.5 g, 62 mmol) as described previously for the labelled compound to give N-carbobenzoxy-(2S,3RS)[3-$^2$H]-3-methylaspartic acid as a yellow oil (8.75 g, 99.6 %), (Found: (*M* + $NH_4$ - $H_2O$)$^+$, 282.1200. $C_{13}H_{15}NO_6$ + $NH_4$ - $H_2O$ requires 282.1200); $\upsilon_{max}$ ($CHCl_3$) 3100 (O-H) & 1720 (C=O) cm$^{-1}$; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 & 1.28 (6 H, 2s,2 x 3-$CH_3$), 4.60 & 4.65 (2H, 2d, *J* 5.0 Hz, 2 x 2-H), 5.08 & 5.11 (4H, 2s, 2 x Ph$\underline{C}H_2$) 6.91 & 6.98 (2H, 2d, *J* 5.0 Hz, 2 x NH) & 7.30 (10H, s, 2 x Ph); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 13.71 & 13.96 (2 x 3-$CH_3$), 56.00 (2 x 2-C), 67.98 (2 x Ph$\underline{C}H_2$), 128.54, 128.77 & 129.04 (Aromatics), 136.32 (*phenyl* C-1), 157.01 & 157.70 (OCONH), 175.02 & 175.65, 178.71 & 179.21 ($CO_2$Hs); *m/z* (CI) 282 ([*M* + $NH_4$ - $H_2O$]$^+$, 10%), 264 (20, $MH^+$- $H_2O$), 220 (12, $MH^+$- $CH_2O_3$), 108 (100, $PhCH_2OH$), 91 (83, $PhCH_2$), 58 (6, $MH^+$ - $PhC_4H_5O_6$).

Treatment of the diacid (8.65 g, 30.7 mmoles) with an ethereal solution of diazomethane as described previously gave dimethyl N-carbobenzoxy-(2S,3RS)-3-methylaspartate (9, $H_A$ = $^2$H) and (10, $H_A$ = $^2$H) as a pale yellow (7.42 g, 78%). (Found: $MH^+$ 311.1353. $C_{15}H_{19}{}^2H\,NO_6$ requires 311.1353); $\upsilon_{max}$ ($CHCl_3$) 3420 (N-H) & 1730 (C=O) cm$^{-1}$; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.22 (3H, s, *threo*-3-$CH_3$), 1.27 (3H, s, *erythro*-3-$CH_3$), 3.70 & 3.76 (2 x 6H, 2s, $CO_2CH_3$s), 4.58 (1H, d, *J* 10 Hz, *erythro*-2-H), 4.70 (1H, d, *J* 10 Hz, (*threo*-2-H), 5.12 & 5.17 (2 x 2H, 2s, Ph$\underline{C}H_2$), 5.53 (1H, br d, *J* 10 Hz, *threo*-NH), 5.71 (1H, br d, *J* 10 Hz,*erythro*-NH) & 7.35 (10H, s, Ar); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 13.06 (*threo*-3-$CH_3$), 14.12 (*erythro*-3-$CH_3$), 52.66 & 53.14 ((2S, 3S/R)-$CO_2\underline{C}H_3$s), 56.27 (2-Cs), 65.15 (*threo*-Ph$\underline{C}H_2$), 67.66 (*erythro*-Ph$\underline{C}H_2$), 128.58, 128.70, 129.02 & 136.59 (Aromatics), 156.29 (*threo*-OCONH), 157.13 (*erythro*-OCONH), 171.43 & 171.71 (-$\underline{C}O_2CH_3$s); *m/z* (EI) 311 ($MH^+$, 8%), 267 (9, MH - $CO_2$), 251 (5, M - $CO_2$Me), 207 (15, M - $C_3H_3O_4$) 174 (9, M - $PhCH_2CO_2$), 108 (11, $PhCH_2OH$) & 91 (100, $PhCH_2$); *m/z* (CI) 311 ($MH^+$, 100%), 267 (59, *M*H - $CO_2$), 251 (7, *M* - $CO_2CH_3$), 207 (27, *M* -$C_3H_3O_4$), 108 (20, $PhCH_2OH$) & 91 (77, $PhCH_2$).

The two diastereomers were separated on silica as described previously to give dimethyl N-carbobenzoxy-(2S,3RS)-3-methylaspartate (9, $H_A$ = $^2$H), (0.40 g, 40%), (Found: $MH^+$ 311.1353. $C_{15}H_{19}{}^2HNO_6$ requires 311.1353); $\upsilon_{max}$ ($CHCl_3$) 3420 (N-H) & 1730 (C=O) cm$^{-1}$; $\delta_H$ (200 MHz; $C^2HCl_3$) 1.27 (3H, s, 3-$CH_3$), 3.70 & 3.76 (2 x 3H, 2s, $CO_2CH_3$s), 4.58 (1H, d, *J* 10 Hz, 2-H), 5.17 (2H, s, Ph$\underline{C}H_2$), 5.71 (1H, br d, *J* 10 Hz, NH) & 7.35 (5H, s, Ar); $\delta_C$ (50.3 MHz; $C^2HCl_3$) 14.12 (3-$CH_3$), 52.66 & 53.14 ($CO_2\underline{C}H_3$s), 56.27

(2-C), 67.66 ($PhCH_2$), 128.58, 128.70, 129.02 & 136.59 (Ar), 157.13 (OCONH), 171.43 & 171.71 ($CO_2CH_3$s); *m/z* (EI) 311 ($MH^+$, 8%), 267 (9, *MH* - $CO_2$), 251 (5, *M* - $CO_2CH_3$), 207 (15, *M* - $C_3H_3O_4$) 174 (9, *M* - $PhCH_2CO_2$), 108 (11, $PhCH_2OH$) & 91 (100, $PhCH_2$); *m/z* (CI) 311 ($MH^+$, 100%), 267 (59, MH - $CO_2$), 251 (7, *M* - $CO_2CH_3$), 207 (27, *M* -$C_3H_3O_4$), 108 (20, $PhCH_2OH$) & 91 (77, $PhCH_2$).

The N-protected aspartate dimethyl ester (2.04 g, 6.58 mmol) was hydrolysed in acid as described for the unlabelled compound to give (2S,3R)[3-$^2$H]-3-methylaspartic acid (3, $H_A = {}^2H$) as white crystals (0.47 g, 32%). Further crops of less pure material were also obtained.

m.p. 258 - 259 °C (decomp.) (Found C, 40.10; H, 6.45; N, 9.75. $C_5H_8{}^2HNO_4$ requires C, 40.55; H, 6.80; N, 9.45); (Found: $MH^+$, 149.0673. $C_5H_9{}^2HNO_4$ requires 149.0670); $[\alpha]_D$ +30.5 (c 1.0, 5 M HCl); $\delta_H$ (200 MHz; $^2H_2O$) 1.25 (3H, s, 3-$CH_3$), 3.92 (1H, s, 2-H); $\delta_C$ (50.3 MHz; $^2H_2O$) 15.63 ($CH_3$), 58.91 (2-C), 175.27 & 180.15 (2 x $CO_2H$); *m/z* (CI) 149 ($MH^+$, 100%), 131 (6, *M* - OH), 103 (9, *M* - $CO_2H$), 59 (21, *M* - $C_2HO_4$).

## Acknowledgements.

We thank the SERC for a studentship to C.H.A. and for grants GR/D-63000 and GR/E-44673 which supported earlier work in this area.

## References

1. H. A. Barker, R. D. Smyth, R. M. Wilson and H. Weissbach, *J. Biol. Chem.*, 1959, **234**, 320.
2. H. Bright, *J. Biol. Chem.*, 1964, **239**, 2307.
3. N. P. Botting, M. A. Cohen, M. Akhtar and D. Gani, *Biochemistry*, 1988, **27**, 2956.
4. N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 1509.
5. N. P. Botting, A. A. Jackson and D. Gani, *J. Chem. Soc., Chem. Commun.*, 1989, 1583.
6. S. Goda, N. P. Minton, N. P. Botting and D. Gani, *Biochemistry*, 1992, **31**, 10 747.
7. W. A. Cowdrey, E. D. Hughes and C. K. Ingold, *J. Amer. Chem. Soc.*, 1937, **59**, 1208.
8. J. E. Rose, P. D. Leeson and D. Gani, *J. Chem. Soc. Perkin Trans. I*, 1992, 1563.
9. D. Seebach and D. Wasmuth, *Angew. Chem. Int. Ed. Engl.*, 1981, **20**, 971.
10. S. Hannessian, K. Sumi and B. Vanasse, *Syn. Lett.*, 1992, 33.
11. C. H. Archer and D. Gani, *J. Chem. Soc., Chem. Comm.*, 1993, 140.