Synthesis of L-Glutamic Acid Labelled Stereospecifically at C-3 with Deuterium and Non-stereospecifically at C-4 with Tritium [1]

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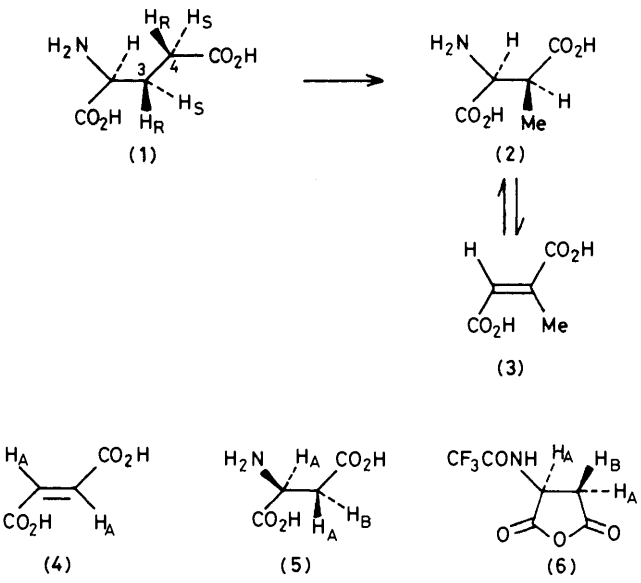
(2*S*,3*S*)-[3-$^2H_1$]-, (2*S*,3*R*)-[2,3-$^2H_2$]-, (2*S*,3*S*,4*RS*)-[3-$^2H_1$,4-$^3H_1$]-, and (2*S*,3*R*,4*RS*)-[2,3-$^2H_2$,4-$^3H_1$]-Glutamic acids have been synthesised from the corresponding labelled aspartic acids. The route involves a step where Wolff rearrangement occurs with retention of stereochemistry at a primary migrating chiral centre. The stereochemistry at C-3 of the glutamic acids has been verified by degradation to the corresponding stereospecifically labelled [$^2H_1$]succinic acids.

L-Glutamic acid (1) and its derivatives glutamine and glutathione play a central role in the metabolism of amino acids and ammonia.[2] One metabolic pathway which occurs in the bacterium *Clostridium tetanomorphum* involves the coenzyme-B_{12}-mediated rearrangement of glutamic acid (1) to β-methylaspartic acid (2). Subsequent elimination of ammonia yields mesaconic acid (3). The coenzyme-B_{12}-mediated rearrangement involves migration of the carbon C-2 of glutamic acid to C-4 and of the 4-*pro-S* hydrogen [3] to C-3 which becomes the methyl carbon in β-methylaspartic acid (2). If samples of L-glutamic acid were available which were stereospecifically labelled at C-3 with two of the isotopes of hydrogen and labelled in either the 4-*pro-S* position or non-chirally at C-4 with the third isotope of hydrogen, then the overall stereochemistry of the glutamate mutase-catalysed rearrangement could be assessed. We now report a synthesis of labelled glutamic acids which satisfy these criteria for studying the glutamate mutase-catalysed rearrangement and which should prove useful for other studies on stereochemical aspects of the metabolism of L-glutamic acid (1).

In earlier work on C-3-labelled amino acids, we prepared samples of L-cysteine stereospecifically labelled at C-3 with tritium.[4] These compounds were used to ascertain the stereochemistry of the cyclisations which give rise to the β-lactam rings in penicillins [4] and in cephalosporin C.[5] The synthesis of the labelled amino acids was lengthy and involved a time-consuming optical resolution. We were, therefore, attracted to the idea of employing a commercially available enzyme to generate useful amounts of an amino acid with (*S*)-stereochemistry at C-2 and a chiral label at C-3 and of using this as the starting point for the chemical synthesis of L-amino acids stereospecifically labelled at C-3.

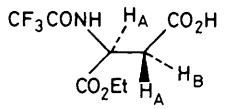
The enzyme L-aspartase (EC 4.3.1.1) has been known [6] for some time to add ammonia stereospecifically across the double bond of fumaric acid (4). The assignment [7] of the absolute stereochemistry to this process indicated that, in ^2H_2O, ammonia would add to fumaric acid to yield (2*S*,3*R*)-[3-$^2H_1$]aspartic acid (5; $H_B = {}^2H$). Since the enzyme L-aspartase is commercially available, it seemed that, if the reaction could yield useful amounts of L-aspartic acid, then we might have a synthon from which to prepare chirally labelled amino acids. In the event 25% yields † of L-aspartic acid were obtained from the incubations with L-aspartase. Use of unlabelled fumaric acid and 99.8% ^2H_2O in the incubation, even without prior equilibration of the buffers and the fumaric acid with ^2H_2O, gave (2*S*,3*R*)-[3-$^2H_1$]-aspartic acid (5; $H_B = {}^2H$) which was estimated to be *ca.* 77% monodeuteriated by mass spectrometry. Use of [2,3-$^2H_2$]fumaric acid (4; $H_A = {}^2H$), prepared by hydrolysis of the corresponding dimethyl ester [8] gave (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid (5; $H_A = {}^2H$), which was estimated to be *ca.* 90% dideuteriated by mass spectrometry. Samples with these incorporations have been used in the work reported in this publication but in later work [9] by changing the buffer and exchanging the medium and substrate with ^2H_2O prior to incubation, we have raised the incorporation in (2*S*,3*R*)-[3-$^2H_1$]aspartic acid (5; $H_B = {}^2H$) to 90%.



The stereochemical integrity of the label was evident from the ^1H n.m.r. spectra in 10% NaO^2H–^2H_2O since the ABX system for 3*R*-H, 3*S*-H, and 2-H of unlabelled aspartic acid became an AX system in the spectrum of (2*S*,3*R*)-[3-$^2H_1$]-aspartic acid (5; $H_B = {}^2H$) and a singlet in the spectrum of (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid (5; $H_A = {}^2H$). The 3-*pro-S*-hydrogen was to higher field than the 3-*pro-R*-hydrogen.

Having obtained the labelled starting material in synthetically useful amounts, it was now necessary to change the nature of the carbonyl group at C-3 to prepare other amino acids. This required differentiation of the two carboxylic acid functionalities. In 1957 Weygand had reported that when L-aspartic acid was treated with trifluoroacetic anhydride and the intermediate anhydride (6) was treated with ethanol, then the product was exclusively the α-ethyl ester (7).[10] Although the hydrogen bond between the side chain NH and the α-carbonyl group in the anhydride (6) may render the latter carbonyl group more electrophilic and thus account for this regiospecificity, it is interesting to note that the trifluoroacetate of the anhydride of malic acid shows identical regio-

† Subsequently these yields have been raised to *ca.* 40%.[9]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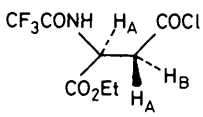
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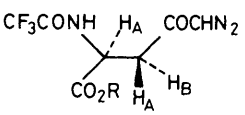
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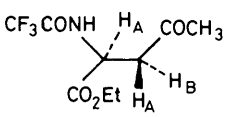
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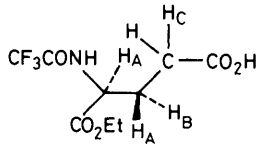
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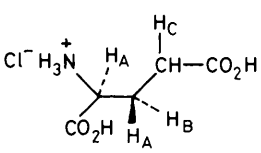
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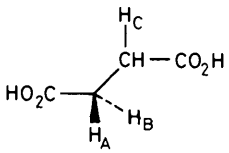
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specificity.[11] The electronegativity of the side chain must, therefore, be an important factor in determining the direction of attack on the anhydride.

When we repeated the trifluoroacetylation-ethanolysis sequence we obtained a solid,* the dicyclohexylammonium salt of which had the reported [10] melting point and optical rotation. Use of the stereospecifically labelled samples (5; $H_B = {}^2H$) and (5; $H_A = {}^2H$) of aspartic acid in this reaction sequence gave products with ^1H n.m.r. spectra which indicated that the stereochemical integrity of the labels had been preserved in the process. The 3-*pro*-*R*-hydrogen now absorbed to higher field than the 3-*pro*-*S*-hydrogen.

The esters (7) were treated with thionyl chloride to yield the acid chlorides (8) which could be converted into the diazoketones (9; R = Et) with diazomethane. To complete the synthesis of the glutamic acids, we now required to effect Wolff rearrangement and deprotection. Although 'normal' tertiary and quaternary chiral centres have been shown [12] to rearrange in the Wolff reaction with retention of stereochemistry, there had, as yet, been no example of such a rearrangement involving a migrating primary chiral centre. It was evident, therefore, that the absolute stereochemistries of the labelled glutamic acids would have to be assessed after the Wolff rearrangement if we were to be sure of our assignments.

The Wolff rearrangement was effected by photolysis of the diazoketones (9; R = Et) in dioxan containing a small amount of water, using a medium-pressure lamp and a quartz filter. Although photolysis in isopropyl alcohol using a Pyrex filter and a triplet sensitiser [9] had led to the methyl ketone (10), the photolysis in dioxan–water gave the protected glutamic acids (11), characterised as their dicyclohexylammonium salts. These salts were hydrolysed in 6M-hydrochloric acid to yield the corresponding labelled glutamic acid hydrochlorides (12). These had the appropriate optical rotations and the ^1H n.m.r. spectra [1] (see Figure for spectra obtained on other † samples) showed that the labelling was stereospecific. If the Wolff rearrangement had occurred with retention of configuration as expected, then the sample obtained from (2*S*,3*R*)-[3-$^2H_1$]aspartic acid (5; $H_B = {}^2H$) would be (2*S*,3*S*)-[3-$^2H_1$]glutamic acid (12; $H_B = {}^2H$) and the sample obtained from (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid (5; $H_A = {}^2H$) would be (2*S*,3*R*)-[2,3-$^2H_2$]glutamic acid (12; $H_A = {}^2H$).

To confirm these assignments the samples of labelled glutamic acid were degraded by reaction with chloramine T followed by acidic hydrolysis to yield samples of labelled succinic acid. (2*R*)- and (2*S*)-[2-$^2H_1$]Succinic acids have been prepared [13] and their optical rotatory dispersion,[13b] and circular dichroism [14] spectra recorded. The samples of [$^2H_1$]-succinic acid (13) from the degradation of the labelled glutamic acids were exhaustively purified *via* their anhydrides and the o.r.d. and c.d. spectra were recorded by Dr. George Ryback, Shell Biosciences Laboratory, Sittingbourne. The percentage optical purity corresponded, within experimental error, to the percentage of deuterium in the sample and both o.r.d. and c.d. showed that the sample assumed to be (2*S*,3*S*)-[3-$^2H_1$]-glutamic acid (12; $H_B = {}^2H$) had given (2*S*)-[2-$^2H_1$]succinic acid (13; $H_B = {}^2H$) and the sample assumed to be (2*S*,3*R*)-[2,3-$^2H_2$]glutamic acid (12; $H_A = {}^2H$) had given (2*R*)-[2-$^2H_1$]succinic acid (13; $H_A = {}^2H$). This confirmed the stereochemical assignments of the samples of labelled glutamic acid. The Wolff rearrangement had therefore taken place with retention of configuration at the migrating primary centre.

To achieve non-chiral labelling at C-4, ^2H_2O was used instead of H_2O to quench the intermediate ketene in the photolytic Wolff rearrangement of the unlabelled diazoketone (9; R = Et). After hydrolysis the ^1H n.m.r. spectrum of the resultant glutamic acid, indicated that one of the protons at C-4 had been replaced with deuterium. The reaction was therefore repeated using the deuteriated diazoketones (9; R = Et, $H_B = {}^2H$) and (9; R = Et, $H_A = {}^2H$) in the presence of ^3H_2O to yield (2*S*,3*S*,4*RS*)-[3-$^2H_1$,4-$^3H_1$]glutamic acid (12; $H_B = {}^2H$, $H_C = {}^3H$) and (2*S*,3*R*,4*RS*)-[3-$^2H_1$,4-$^3H_1$]glutamic acid (12; $H_A = {}^2H$, $H_C = {}^3H$) respectively.

Experimental

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Specific rotations were obtained using a Perkin-Elmer 241 polarimeter and a 1 dm path-length cell. Optical rotatory dispersion (o.r.d.) and circular dichroism (c.d.) data were obtained by Dr. George Ryback, Shell Biosciences Laboratory, Sittingbourne, Kent, U.K., using Bellingham and Stanley Bendix Polarmatic 62 (o.r.d.) and Cary 61 (c.d.) instruments. I.r. spectra were recorded on Perkin-Elmer 257 and 477 spectrometers. ^1H N.m.r. spectra were obtained using Perkin-Elmer R12 (60 MHz) and R32 (90 MHz) instruments and a Bruker WM 360 instrument (360 MHz). 220 MHz Spectra were obtained from P.C.M.U., Harwell. Mass spectra were recorded on an AEI-MS 30 instrument with silylation, where necessary, using 50% *N*,*O*-bis(trimethylsilyl)trifluoroacetamide in pyridine at room temperature overnight and subsequent use of g.c./mass spec. on 5 ft of 4% OV-1. Radioactive samples

* Although we have assumed regiospecificity in this reaction, it is evident from later work [9] that the reaction is only 70—80% regiospecific. Purification is, however, effected in the course of the synthesis.

† Obtained by D. Gani using the diazoketones (9; R = Me) prepared as in the following paper.[9]

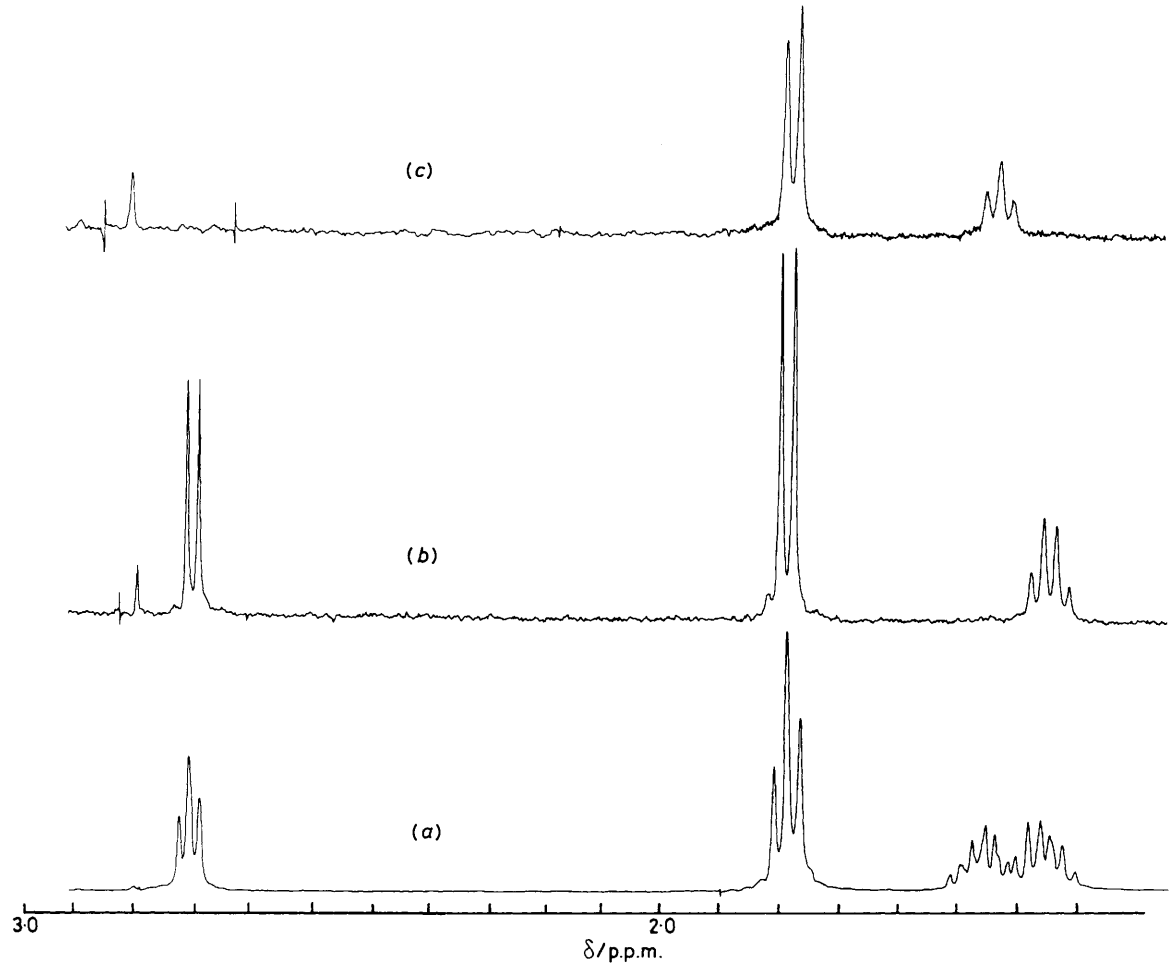

Figure. 360 MHz ^{1}H N.m.r. spectra in 10% NaO^2H–^2H_2O of (*a*) (2*S*)-glutamic acid; (*b*) (2*S*,3*S*)-[3-$^2H_1$]glutamic acid; (*c*) (2*S*,3*R*)-[2,3-$^2H_2$]glutamic acid. The chemical shifts are referenced to internal NaO^2H at δ 4.60 p.p.m. The 220 MHz spectra in the text and in ref. 1 are referenced to sodium 3-trimethylsilylpropionate

were counted using a Beckman LS 100 liquid scintillation counter with Nuclear Enterprises Ltd. NE 260 scintillant.

[2,3-$^2H_2$]*Fumaric Acid* (4; H_A = ^2H).—Dimethyl [2,3-$^2H_2$]fumarate [8] (5 g, 34.2 mmol) was stirred for 72 h at room temperature in 12% aqueous sodium hydroxide (30 ml). The mixture was acidified with concentrated hydrochloric acid to precipitate the diacid (4; H_A = ^2H) (3.6 g, 90%), *m/z* 118 (M^+), indicating incorporation of *ca.* 99% $^2H_2$; $\nu_{max.}$ (Nujol) 2 500br (CO*OH*), and 1 670 cm^{-1} (acid).

(2S,3R)-[3-$^2H_1$]*Aspartic Acid* (5; H_B = ^2H).—Fumaric acid (2.3 g, 20 mmol) was dissolved in ^2H_2O (99.8%, 30 ml) containing AnalaR sodium hydroxide (1.6 g, 40 mmol), AnalaR ammonium chloride (1.34 g, 25 mmol), AnalaR potassium dihydrogen phosphate (38.8 mg), and dipotassium hydrogen phosphate (172 mg). Careful addition of 10% NaO^2H–^2H_2O brought the pH to *ca.* 9. L-Aspartase (Sigma; 2 units) was added and the mixture was incubated at 30 °C for 10 days. The enzyme was denatured by boiling for 30 min. Excess of saturated aqueous copper sulphate was added and, on standing overnight at room temperature, a blue precipitate of copper aspartate was obtained. This was suspended in water (75 ml) and hydrogen sulphide was passed through the suspension until no blue colour remained. The black precipitate of copper sulphide was removed by filtration through Celite. Addition of ethanol to the filtrate caused the slow formation of white crystals of (2*S*,3*R*)-[3-$^2H_1$]aspartic acid which were dried *in vacuo* (660 mg, 25%); $[\alpha]_D$ +19.4° (*c* 0.918, 1M-HCl) (lit.,[15] for L-aspartic acid +24.6° in 6M-HCl); *m/z* of tris(trimethylsilyl) derivative 233 ($M^+ - CO_2 - SiMe_3$) indicated *ca.* 77% $^2H_1$ (Found: C, 35.9; H, 5.4; N, 10.3. $C_4H_6{}^2HNO_4$ requires C, 35.8; H, 5.9; N, 10.45%); $\nu_{max.}$ (Nujol) 2 700br (CO*OH*) and 1 685 cm^{-1} (acid); δ (10% NaO^2H–^2H_2O) 2.25br (1 H, d, *J* 9 Hz, 3*S*-H) and 3.48 (1 H, d, *J* 9 Hz, 2-H).

(2S,3S)-[2,3-$^2H_2$]*Aspartic Acid* (5; H_A = ^2H).—This was prepared as above using [2,3-$^2H_2$]fumaric acid in water (27% yield); $[\alpha]_D$ +22.7° (*c* 0.74, 1M-HCl), *m/z* of tris(trimethylsilyl) derivative *m/z* 234 indicating *ca.* 90% $^2H_2$; δ(10% NaO^2H–^2H_2O) 2.60 (s, 3*R*-H).

α-*Ethyl* N-*Trifluoroacetyl*-L-*aspartate* (7).—L-Aspartic acid (200 mg, 1.5 mmol) was suspended in dry distilled tetrahydrofuran (5 ml) in an ice-bath. The suspension was stirred under nitrogen and trifluoroacetic anhydride (2 ml) was added during 10 min. The suspension was allowed to warm to room temperature and stirring was continued for 40 min when the solution became homogeneous. After the mixture had been stirred at room temperature for a further 2 h, the solvents were removed under reduced pressure to yield

a white solid, m.p. 125—129 °C; $\nu_{max.}$ (Nujol) 1 850, 1 820, 1 795 (anhydride), and 1 705 cm^{-1} (trifluoroacetamide). The crude anhydride (6) was heated to reflux in absolute ethanol (10 ml) under nitrogen for 20 min. The solvent was removed under reduced pressure to yield an off-white solid (340 mg, 88%), m.p. 78—85 °C; $[\alpha]_D$ −14.7° (*c* 0.54, THF) {lit.,[10] of sample obtained by freeing from the dicyclohexylammonium salt, m.p. 96—97 °C, $[\alpha]_D$ −10.2° (THF)}; *m/z* 212 ($M^+ - CO_2H$); $\nu_{max.}$ (Nujol) 3 300 (NH), 1 734 (ester), and 1 710 cm^{-1} (acid and trifluoroacetamide); δ(C^2HCl_3) 1.27 (3 H, t, *J* 6.5 Hz, CH_2CH_3), 3.02 (2 H, m, 3-H), 4.22 (2 H, q, *J* 6.5 Hz, CH_2CH_3), 4.82 (1 H, m, 2-H), 7.59br (1 H, d, NH), and 10.42br (1 H, s, CO_2H). The latter two absorptions were exchangeable on shaking with ^2H_2O. A sample was dissolved in benzene and one equivalent of dicyclohexylamine was added. The salt was precipitated on standing and could be recrystallised from water, m.p. 168.5—171 °C (lit.,[10] 169—171 °C), $[\alpha]_D$ −6.9° (*c*, 2.0, $CHCl_3$) (lit.,[10] −6.9°, MeOH).

α-Ethyl (2S,3R)-N-*Trifluoroacetyl*[3-$^2H_1$]*aspartate* (7; H_B = ^2H).—This was prepared from (2*S*,3*R*)-[3-$^2H_1$]aspartic acid (5; H_B = ^2H) as outlined above, m.p. 89—93 °C, $[\alpha]_D$ −11.3° (*c* 0.97, THF), *m/z* 213 ($M^+ - CO_2H$) indicating *ca.* 79% $^2H_1$. Other spectra were similar to those of the unlabelled material except that 3*S*-H appeared as a doublet (*J* 4 Hz) at δ 3.08 in the ^1H n.m.r. spectrum (C^2HCl_3).

α-Ethyl (2S,3S)-N-*Trifluoroacetyl*[2,3-$^2H_2$]*aspartate* (7; H_A = ^2H).—This was prepared from (2*S*,3*S*)-[2,3-$^2H_2$]aspartic acid (5; H_A = ^2H) as outlined above, m.p. 90—94 °C, $[\alpha]_D$ −12.2° (*c* 1.03, THF), *m/z* 214 ($M^+ - CO_2H$) indicating *ca.* 88% $^2H_2$. Other spectra were similar to those of the unlabelled material except that 3*R*-H appeared as a broad singlet, δ 2.91, in the ^1H n.m.r. spectrum (C^2HCl_3).

α-Ethyl N-*Trifluoroacetylaspartyl* β-*Chloride* (8) *and the* (2S,3R)-[3-$^2H_1$]- (8; H_B = ^2H) *and* (2S,3S)-[2,3-$^2H_2$]-*Compounds* (8; H_A = ^2H).—These were prepared using Weygand's method,[10] $\nu_{max.}$ 1 805 cm^{-1} (COCl) and used directly to prepare the diazoketones (9; R = Et) as outlined below.

Ethyl 5-*Diazo*-4-*oxo*-N-*trifluoroacetyl*-(2S)-*norvalinate* (9; R = Et).—Freshly prepared acid chloride (8) (1 g, 3.6 mmol) was dissolved in dry ether (30 ml) and the solution was added slowly with shaking to an excess of ice-cold ethereal diazomethane.[16] The solution was stirred overnight at room temperature and the solvent was removed in a stream of nitrogen to yield a solid which crystallised from diethyl ether–light petroleum (b.p. 60—80 °C) as rosettes (775 mg, 76%), m.p. 103—106 °C, $[\alpha]_D$ +7.7° (*c* 1.236, THF) (lit.,[10] m.p. 104—106 °C, $[\alpha]_D$ +7.33°); *m/z* 212 ($M^+ - COCHN_2$); $\nu_{max.}$ (Nujol) 3 300 (NH), 2 110 (N≡N), 1 745 (ester), and 1 710 and 1 620 cm^{-1}; δ(C^2HCl_3) 1.27 (3 H, t, *J* 7 Hz, CH_2CH_3), 3.00 (2 H, *ABX*, J_{AB} 18 Hz, $J_{AX} = J_{BX}$ = 4 Hz, 3-H), 4.2 (2 H, q, *J* 7 Hz, CH_2CH_3), 4.84 (1 H, m, 2-H), and 5.3 (<1 H, s, CHN_2).

Ethyl (2S,3R)-5-*Diazo*-4-*oxo*-N-*trifluoroacetyl*[3-$^2H_1$]-*norvalinate* (9; R = Et, H_B = ^2H).—This was prepared in the same way as the unlabelled compound, and had m.p. 102—105 °C, $[\alpha]_D$ +7.9° (*c* 1.13, THF). Spectra were similar to those of the unlabelled compound except that the proton 3*S*-H appeared at δ 3.1br (C^2HCl_3) in the ^1H n.m.r. spectrum and the mass spectral fragment ion was one mass number higher at *m/z* 213, showing incorporation of *ca.* 82%.

Ethyl (2S,3S)-5-*Diazo*-4-*oxo*-N-*trifluoroacetyl*[2,3-$^2H_2$]-*norvalinate* (9; R = Et, H_A = ^2H).—This was prepared in the same way as the unlabelled compound above, m.p. 103—106 °C, $[\alpha]_D$ +7.4° (*c* 1.43, THF). Spectra were similar to those of the starting material except that there was no proton 2-H in the ^1H n.m.r. spectrum and the proton 3*R*-H appeared at δ 2.9br (C^2HCl_3). The mass spectral fragment ion, *m/z* 214, was two mass units higher than in the unlabelled compound and incorporation was estimated to be *ca.* 90% $^2H_2$.

1-*Ethyl* 5-*Dicyclohexylammonio*-N-*trifluoroacetyl*-(2S)-*glutamate*.—The diazoketone (9; R = Et) (200 mg, 0.7 mmol) was dissolved in dioxan (400 ml) containing water (5 ml). The solution was degassed by vigorous passage of nitrogen for 30 min and then photolysed for 2.75 h using a 125-W medium-pressure lamp with a quartz filter. The solvent was removed under reduced pressure to yield a gum which was dissolved in benzene (3 ml). Dicyclohexylamine (126 mg, 0.7 mmol) was added and the solution was left for several hours at room temperature when the crystalline salt was obtained. This recrystallised as needles from methanol (186 mg, 58%), m.p. 185—188 °C (lit.,[17] 188 °C), $[\alpha]_D$ −21.4° (*c* 1.95, MeOH); $\nu_{max.}$ 1 740 (ester), 1 715 (trifluoroacetamide), and 1 635 cm^{-1} (carboxylate); δ($C^2H_3O^2H$) 1.1—2.4br (*ca.* 27 H, m, CH_2, CH_3, 3-H and 4-H), 3.0—3.4 (solvent + CHNH), and 4.20 (2 H, q, *J* 7 Hz, CH_2CH_3).

1-*Ethyl* (2S,3S)-5-*Dicyclohexylammonio*-N-*trifluoroacetyl*-[3-$^2H_1$]*glutamate*.—This was prepared from the diazoketone (9; R = Et, H_B = ^2H) as outlined above for the unlabelled compound, m.p. 186—188 °C, $[\alpha]_D$ −21.9° (*c* 1.32, MeOH). Spectra were in keeping with those of the unlabelled compound.

1-*Ethyl* (2S,3R)-5-*Dicyclohexylammonio*-N-*trifluoroacetyl*-[2,3-$^2H_2$]*glutamate*.—This was prepared from the diazoketone (9; R = Et, H_A = ^2H) as outlined above for the unlabelled compound, m.p. 186—189 °C, $[\alpha]_D$ −22.1° (*c* 1.76, MeOH). Spectra were in keeping with those of the unlabelled compound.

1-*Ethyl* (2S,4RS)-5-*Dicyclohexylammonio*-N-*trifluoroacetyl*[4-$^2H_1$]*glutamate*.—This was prepared using ^2H_2O (99.8%; 5 ml) in the photolysis of the unlabelled diazoketone (9; R = Et) as outlined above, m.p. 185—187 °C, $[\alpha]_D$ −21.9° (*c* 1.24, MeOH). Spectra were in keeping with those of the unlabelled compound.

1-*Ethyl* (2S,3S,4RS)-5-*Dicylohexylammonio*-N-*trifluoroacetyl*[3-$^2H_1$,4-$^3H_1$]*glutamate*.—This was prepared using ^3H_2O (1.5 Ci in 3 ml) in the photolysis of (9; R = Et, H_B = ^2H) as described above. After three recrystallisations to constant activity, the specific activity was 5.027 × 10^8 d.p.m./mmol.

1-*Ethyl* (2S,3R,4RS)-5-*Dicylohexylammonio*-N-*trifluoroacetyl*[2,3-$^2H_2$,4-$^3H_1$]*glutamate*.—This was prepared using ^3H_2O (1.5 Ci in 3 ml) in the photolysis of (9; R = Et, H_A = $^2H_1$) as described above. After three recrystallisations from methanol to constant activity, the specific activity was 2.793 × 10^8 d.p.m./mmol.

L-*Glutamic Acid Hydrochloride* (12).—The above salt (100 mg, 0.2 mmol) was heated to reflux with 6M-aqueous hydrochloric acid (5 ml) for 2 h. The solution was allowed to cool to room temperature and cooled in ice. Dicyclohexylammonium chloride was filtered off and the filtrate was lyophilised to give a solid which was crystallised from 20% aqueous hydrochloric acid (36 mg, 90%), $[\alpha]_D$ +24.7° (*c* 0.84, 1M-HCl) (lit.,[18] +24.4°), *m/z* of tris-$SiMe_3$ derivative

363 (M^+) and 348 ($M^+ - CH_3$), $\nu_{max.}$ (Nujol) 3 300—2 500 (NH_3^+ and COOH), 1 725 (COOH) and 1 680 cm^{-1} (COOH); δ(220 MHz; 10% NaO^2H–^2H_2O), 1.80 (2 H, m, 3-H), 2.24 (2 H, t, *J* 8 Hz, 4-H), and 3.25 (1 H, d × d, *J* 7.5, 6 Hz, 2-H); see Figure (*a*) for 360 MHz spectrum.

(2S,3S)-[3-$^2H_1$]*Glutamic Acid Hydrochloride* (12; H_B = ^2H).—This was prepared as described above using the salt of (11; H_B = ^2H), $[\alpha]_D$ +22.8° (*c* 0.86, 1M-HCl). The spectra were similar to those of the unlabelled compound except that 3*R*-H appeared at δ 1.76br (q), 2-H at δ 3.23 (d, *J* 7 Hz) and 4-H at δ 2.20 (d, *J* 8 Hz) in the ^1H n.m.r. spectrum (220 MHz; 10% NaO^2H–^2H_2O); see Figure (*b*) for 360 MHz spectrum of sample with higher incorporation. The mass spectral fragment ion of the tris-$SiMe_3$ derivative, *m/z* 349, was one mass unit higher than in the unlabelled compound and incorporation was estimated to be *ca.* 78% $^2H_1$.

(2S,3R)-[2,3-$^2H_2$]*Glutamic Acid Hydrochloride* (12; H_A = ^2H).—This was prepared as described above using the salt of (11; H_A = ^2H), $[\alpha]_D$ +25.2° (*c* 0.97, 1M-HCl). The spectra were similar to those of the unlabelled compound except that 3*S*-H appeared at δ 1.83 as a broad triplet (*J* 8 Hz) and there was no absorption for 2-H in the ^1H n.m.r. spectrum; 220 MHz; see Figure (*c*) for 360 MHz spectrum. The fragment ion, *m/z* 350, was two mass numbers higher than in the unlabelled compound and incorporation was estimated to be *ca.* 91%.

(2S,4RS)-[4-$^2H_1$]*Glutamic Acid Hydrochloride* (12; H_C = ^2H).—This was prepared as described above using the salt of (11; H_C = ^2H), $[\alpha]_D$ +24.4° (*c* 0.73, 1M-HCl). The spectra were similar to those of the unlabelled compound except that 4-H at δ 2.2 integrated as one proton.

(2S,3S,4RS)-[3-$^2H_1$,4-$^3H_1$,U-^{14}C]*Glutamic Acid Hydrochloride* (12; H_B = ^2H,H_C = ^3H).—This was prepared from the salt of (11; H_B = ^2H, H_C = ^3H) as described above. The product was diluted with unlabelled L-glutamic acid and L-[U-^{14}C]glutamic acid and recrystallised five times from methanol to a specific activity ^3H = 1.505 × 10^7 d.p.m./mmol; ^{14}C = 2.488 × 10^6 d.p.m./mol (^3H : ^{14}C = 6.05 : 1).

(2S,3R,4RS)-[2,3-$^2H_2$,4-$^3H_1$,U-^{14}C]*Glutamic Acid Hydrochloride* (12; H_A = ^2H, H_C = ^3H).—This was prepared from the salt of (11; H_A = ^2H, H_C = ^3H) as described above. The product was diluted with unlabelled L-glutamic acid and L-[U-^{14}C]glutamic acid and recrystallised eight times from methanol to a specific activity of ^3H = 7.185 × 10^6 d.p.m./mmol; ^{14}C = 1.914 × 10^6 d.p.m./mmol (^3H : ^{14}C = 3.75 : 1).

Degradation of the [^2H]*Glutamic Acids* (12).—Glutamic acid hydrochloride (80 mg, 0.43 mmol) was dissolved in water (2 ml) and the pH was adjusted to 4.5 with 1M-aqueous sodium hydroxide. The solution was cooled in an ice-bath and treated with a freshly prepared 10% aqueous solution of chloramine T (1.7 ml). The pH was readjusted to 4.5 with 1M-aqueous sodium hydroxide and the solution heated at 40 °C for 10 min. The mixture was cooled in ice and toluene-*p*-sulphonamide was filtered off. Concentrated hydrochloric acid (2 ml) was added and the solution was heated in a boiling water-bath for 15 min. The solution was cooled, 6M-aqueous sodium hydroxide (2 ml) was added, and the solvent was removed by freeze drying. The resulting solid was dissolved in an excess of acetic anhydride and heated to 90 °C for 30 min. The solvent was carefully removed and succinic anhydride was sublimed at 50 °C and 0.5 mmHg, m.p. 115—117 °C (lit.,[19] 119.6 °C). The anhydride was dissolved in water and the solvent was removed by freeze drying to yield succinic acid which was recrystallised from water (13 mg, 25%), m.p. 182—184 °C (lit.,[19] 185—187 °C). The ^1H n.m.r. spectrum, i.r. spectrum and mass spectrum, *m/z* of bis-$SiMe_3$ derivative 247 ($M^+ - CH_3$), were identical with those of an authentic sample.

(2R)-[2-$^2H_1$]*Succinic Acid* (13; H_A = ^2H).—This was prepared from (2*S*,3*R*)-[2,3-$^2H_2$]glutamic acid hydrochloride (12; H_A = ^2H) (40 mg, 0.22 mmol) by the above method except that after the initial freeze drying (see above), the solid was freed from inorganic material by acetone extraction. The final (2*R*)-[2-$^2H_1$]succinic acid was crystallised once from water and twice from ethyl acetate, m.p. 184—185 °C. The mass spectral fragment ion (bis-$SiMe_3$ derivative) at *m/z* 248 was one mass unit higher than in the undeuteriated sample, indicating *ca.* 95% $^2H_1$. A negative o.r.d. of a solution of 2.98 mg in water (251 mg) in a 10 mm microcell at 20 °C showed $[\alpha]_{238}^{20}$ −32.2°, $[\alpha]_{244}^{20}$ −22.9°, $[\alpha]_{250}^{20}$ −16.2°, $[\alpha]_{263}^{20}$ −9.19°, $[\alpha]_{278}^{20}$ −5.67° indicating a mean 91.6% (2*R*)-[2-$^2H_1$]succinic acid. The c.d. spectrum had $\lambda_{max.}$ 207 nm.

(2S)-[2-$^2H_1$]*Succinic Acid.*—This was prepared in the same manner as the (2*R*)-isomer above from (2*S*,3*S*)-[3-$^2H_1$]-glutamic acid hydrochloride (40 mg, 0.22 mmol), m.p. 184—186 °C. The mass spectral fragment ion (bis-$SiMe_3$ derivative) at *m/z* 248 was one mass unit higher than in the unlabelled sample and indicated *ca.* 67.8% $^2H_1$. A positive o.r.d. of a solution of 3.01 mg in 258 mg water in a 10 mm microcell at 20 °C showed $[\alpha]_{238}^{20}$ +24.1°, $[\alpha]_{244}^{20}$ +17.9°, $[\alpha]_{250}^{20}$ +12.4°, $[\alpha]_{263}^{20}$ +6.7°, and $[\alpha]_{278}^{20}$ +4.4° indicating a mean 69.6% (2*S*)-[2-$^2H_1$]succinic acid. The c.d. spectrum had $\lambda_{max.}$ 207 nm.

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