Stereospecific synthesis of (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine[1]

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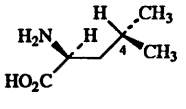
The first stereospecific chemical synthesis of a sample of the amino acid (2*S*)-leucine labelled in one of the diastereotopic methyl groups has been achieved using (2*S*)-pyroglutamic acid as a chiral template. This has been used to develop a method for assigning resonances to the diastereotopic methyl groups of the leucine residues in the ^1H NMR spectra of proteins.

Introduction

The amino acid L-leucine **1** has two diastereotopic methyl groups, and the stereochemistry of various metabolic reactions involving these methyl groups has been studied extensively.[2] The amino acid is also important in proteins, conferring tertiary structure by its involvement in hydrophobic interactions. At the start of this current study, assignment of the resonances of the diastereotopic methyl groups in leucine residues in protein NMR spectra was not possible and so an important area of molecular recognition could not be addressed.

We first became interested in this problem through our long standing interest in the enzyme dihydrofolate reductase (EC 1.5.1.3) which is a target both for the anti-cancer drug methotrexate **2** and for the anti-bacterial drug trimethoprim **3**. We had shown[3] that this enzyme catalysed the reduction of the vitamin folic acid **4** to the coenzyme 5,6,7,8-tetrahydrofolic acid **5** by transfer of the 4-*pro*-*R*-hydrogen of NADPH to the same face (the *re*-face at C-6 and the *si*-face at C-7) of the vitamin **4**. This implied that the substrate folic acid **4** bound to the enzyme



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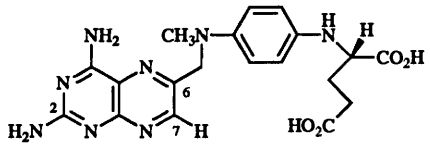

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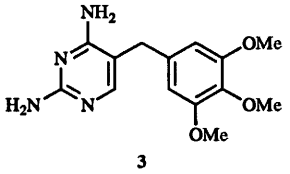

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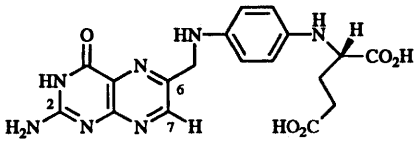

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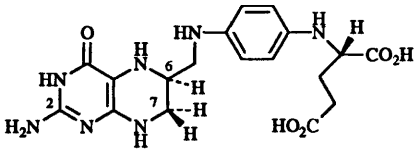

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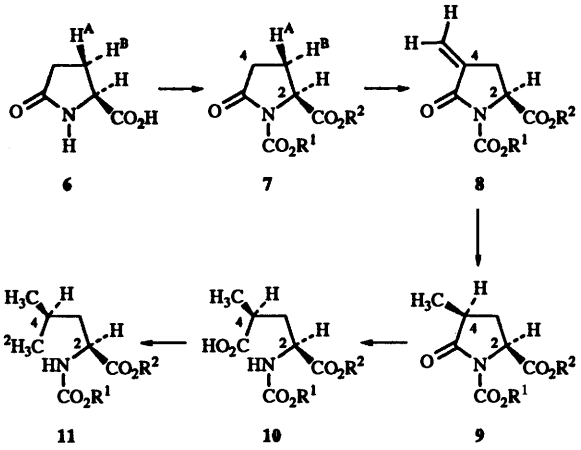
with the pteridine ring aligned in the opposite sense to that shown in the binding of the pteridine ring of the drug methotrexate **2** to the enzyme. More recent work by Feeney and co-workers[4] has shown that, in fact, there are three conformations in which folic acid **4** binds to the enzyme and that, in two of these, methotrexate **2** and folic acid **4** bind in a similar manner. In the third conformation, which is unique to folic acid and which is the sole catalytically competent conformation, the pteridine ring binds in a different manner from the two unproductive conformations, thus accounting for our results.

In the ^1H NMR spectroscopic studies which defined the three conformations,[4] it was noted that one of the methyl groups from each of the residues leucine 19 and leucine 27 in the protein was within nuclear Overhauser enhancement (NOE) distance from 7-H of methotrexate **2**. The inability to assign the resonances due to the diastereotopic methyl groups directly, however, prevented further refinement of our understanding of how the residues were aligned with respect to the bound compound. We therefore determined to address this important problem and reasoned that, if we could synthesize a sample of L-leucine in which one of the diastereotopic methyl groups is replaced by a trideuteriomethyl group, then incorporation of this into the protein would allow us to identify deletions in the ^1H NMR spectrum and hence assign the diastereotopic methyl groups of all leucine residues in the protein. This method is, in principle, applicable to all proteins and so we would have developed a general method.

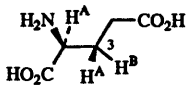
The first step was to synthesize a sample of L-leucine labelled with deuterium in only one of the diastereotopic methyl groups. Specifically [5-^{13}C]-, [5-^{14}C]- and [5-^3H]-labelled samples of leucine have been synthesized by non-stereospecific synthesis involving resolution,[5-7] by homologation of labelled valine[8] and by biosynthetic methods,[9,10] but a fully stereospecific chemical synthesis had not yet been achieved. We decided, therefore, to develop a stereospecific synthesis and opted to use (2*S*)-pyroglutamic acid **6** as the starting point. This compound not only had the desired stereochemistry at C-2, but the 1,3-relationship of C-2 and C-4 in the ring offered the possibility of inducing chirality at C-4. The presence of the ring would also allow stereochemical assignments to be made unambiguously by NOE techniques before ring-opening to acyclic synthetic precursors of leucine. Our overall plan is shown in Scheme 1. Protection of L-pyroglutamic acid **6** as the urethane ester **7** should allow functionalisation at C-4 by an electrophile. Attack at the α-face should be favoured by the bulky ester on the β-face at C-2. Alternatively reaction at C-4 to yield an exomethylene derivative **8** would allow reduction from the α-face to yield the *cis*-4-methyl derivative **9**. Ring opening to the acid **10** and reduction of the acid with deuteriated reagents would then lead to the compound **11** which would afford (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine on deprotection. The synthesis required that we



Scheme 1

effect the ring-opening reaction, **9** ⟶ **10**, without also saponifying the ester at C-2 and so the *tert*-butyl ester was chosen to avoid the subsequent problems of regiospecific control between C-1 and C-4 that would result from a diacid intermediate.

When we began our synthesis, the method of preparing N-protected esters of pyroglutamic acid was by a five-step sequence, starting from glutamic acid **12**.[11] We used this laborious sequence in our initial studies, since we had already prepared (2*S*,3*S*)-[3-$^2H_1$]- and (2*S*,3*R*)-[2,3-$^2H_2$]-glutamic acids, **12**; H_B = ^2H and **12**; H_A = ^2H, respectively,[12] and we concluded that, should our synthesis of leucine be successful, then we would be in a position to prepare samples of leucine labelled stereospecifically both at C-3 and in the diastereotopic methyl groups. When the labelled benzyl *N*-(benzyloxycarbonyl)pyroglutamates were prepared, however, the ^1H NMR spectrum of the (2*S*,3*S*)-[3-$^2H_1$] compound **7**; R^1 = R^2 = CH_2Ph, H_B = ^2H, exhibited resonances at both δ 2.33 and 2.04 for 3*S*-H and 3*R*-H, respectively, in an integrated ratio of ~0.3 H:0.7 H and the ^1H NMR spectrum of the (2*S*,3*R*)-[2,3-$^2H_2$] compound had an absorption integrating as 0.16 H at δ 4.70 for 2-H. These results were consistent with racemisation having occurred at the α-centre during the synthesis from glutamic acid **12**.



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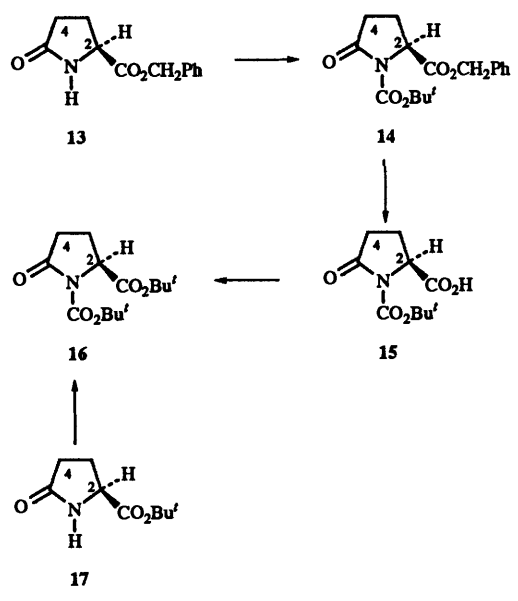
The length of the synthesis and our discovery that partial racemisation occurred at some step in it indicated that a more direct approach should be taken. We therefore investigated a shorter route to the *N*-*tert*-butoxycarbonyl *tert*-butyl ester. Initially we accessed large amounts of this compound by first preparing the benzyl ester **13** in 85% yield from pyroglutamic acid **6** using the method of Danishefsky *et al.*[13] This ester was converted into the urethane **14** by adapting Grieco's method[14] of functionalising lactams and amides but using di-*tert*-butyl dicarbonate and 4-(dimethylamino)pyridine (DMAP) in acetonitrile.[15] The desired product **14** was obtained in 75% yield. This exhibited an imide carbonyl absorption in the IR spectrum, and other spectra were in accord with the structure. Hydrogenolysis of the benzyl ester **14** in ethyl acetate using 10% palladium on carbon gave the acid **15** in 98% yield and this was esterified *via* the mixed anhydride by reaction with di-*tert*-butyl dicarbonate, DMAP and triethylamine in acetonitrile to yield the diprotected product **16** in 89% yield. More recently we have found that compound **16** can be prepared even more directly by esterification of pyroglutamic acid **6** using *tert*-butyl acetate and perchloric acid as in the method of Miller[16] (to give the ester **17**), followed by formation of the urethane **16** using di-



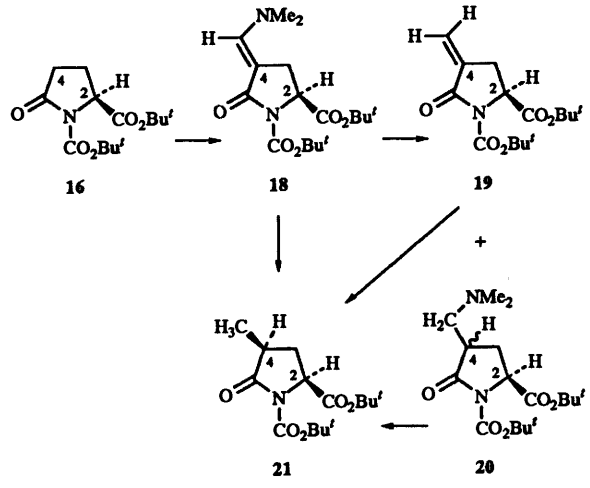
Scheme 2

tert-butyl dicarbonate and DMAP in acetonitrile. (see Scheme 2).

The protected pyroglutamate **16** was now converted into the enaminone **18** in 83% yield using Bredereck's reagent.[17] The product exhibited a characteristic λ_{max} in the UV spectrum at 313 nm and appeared to exist as only one of the two possible geometric isomers. When the olefinic proton, δ 7.12 in the ^1H NMR spectrum, was irradiated then the only observable NOE was in the NMe_2 singlet at δ 3.02. However, irradiation of this latter singlet caused not only a 15% NOE in the olefinic absorption, but a 3% NOE in the absorption due to the proton 3*S*-H (which showed an NOE to 2-H) at δ 3.25 and a 5% NOE in the absorption due to 3*R*-H at δ 2.80. These results were consistent with the enaminone **18** having the *E*-geometry shown.

Our strategy now required that we reduce the enaminone **18** to the exomethylene derivative **19** which we hoped would be hydrogenated with asymmetric induction due to the bulky ester at C-2 to yield the *cis*-methyl group at C-4 as in structure **21**. Although reduction with diisobutylaluminium hydride (DIBAL) usually occurs with 1,2-addition of hydride ion, the enaminone **18** is a vinylogous amide and Ziegler[18] has successfully reduced analogous compounds with concomitant elimination of the secondary amine to yield exomethylene derivatives. We therefore investigated the reduction of the enaminone **18** with DIBAL in tetrahydrofuran (THF). Eventually the exomethylene derivative **19** was obtained in excellent yield, as a solid $C_{15}H_{23}NO_5$, with λ_{max} 228 nm and a ^1H NMR spectrum which exhibited two olefinic triplets, at δ 5.50 and 6.22, and no absorption corresponding to that found for the NMe_2 singlet in the spectrum of the starting material. Yields were inconsistent, however, and a mixture of the diastereoisomeric Mannich bases **20** accompanied the product. These were quaternised and although Hofmann elimination did afford further amounts of the olefin **19** yields were low.

When the olefin was hydrogenated in methanol, using 10% palladium on carbon, *tert*-butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylpyroglutamate **21** was obtained as the sole product in 86% yield. The ^1H NMR spectrum of this compound in [$^2H_6$]benzene showed a new methyl doublet at δ 0.95 which, when irradiated, caused an NOE of 4% in the proton 3*R*-H signal at δ 1.19. This latter absorption had been assigned on the basis that irradiation of the proton 2-H at δ 4.16 led to an NOE of 9% in the absorption for 3*S*-H at δ 1.73. It was evident therefore that reduction had occurred stereospecifically and that it had led entirely to the *cis*-isomer **21** (Scheme 3).

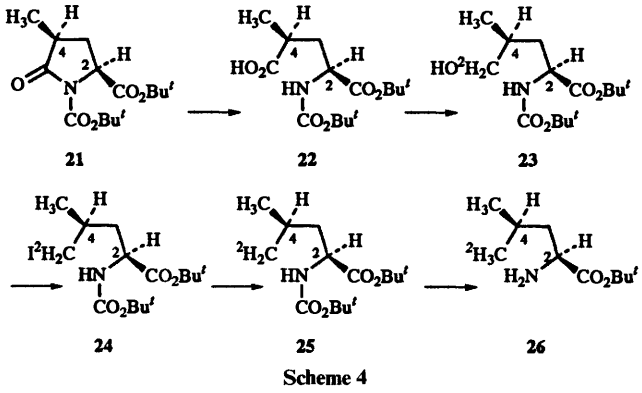


Scheme 3

Although, by achieving asymmetric induction, we had succeeded in the first objective of our synthesis, the variability of yield in the reduction leading to the exomethylene derivative **19** led us to consider an alternative approach to the problem. Reports that the enaminone of a butyrolactone could be catalytically hydrogenated to yield a Mannich base [19] and that a Mannich base could be catalytically hydrogenated with elimination of a secondary amine to yield a methyl group [20] suggested to us that, if these two steps could be induced to happen simultaneously, we might have a more direct synthesis of the 4-methylpyroglutamate **21**. In initial studies, reduction of the enaminone gave mixtures of the desired product and the Mannich bases **20**. Whereas the *trans*-isomer of the Mannich base had appeared to be the major component of the by-product of reduction of the enaminone **18** with DIBAL, the *cis*-isomer appeared to be the major isomer on catalytic hydrogenation. Eventually, hydrogenation of the enaminone **18** to the methyl derivative **21** was achieved in 78% yield when the reduction was performed in ethyl acetate, using 50% w/w of 10% palladium on carbon as catalyst. Hydrogenation of the Mannich bases **20** also gave the *cis*-methyl derivative **21**, in 42% yield.

The second chiral centre had now been installed stereospecifically and so the pyroglutamate template had served its purpose. The compound **21** was therefore subjected to ring-opening using 1 mol dm^{-3} aq. LiOH in THF. The resultant protected 4-methylglutamic acid **22**, obtained in 94% yield, exhibited a carboxylic acid carbonyl absorption in the IR spectrum and no longer showed the imide carbonyl absorption which had been present in the IR spectrum of the starting material. The ^1H NMR spectrum showed a new NH doublet and there was no evidence of epimerisation during the ring-opening reaction. The acid **22** was now treated with isobutyl chloroformate and triethylamine in THF at −40 °C and the intermediate mixed anhydride was reduced with NaB^2H_4. The labelled alcohol **23** was obtained as a solid in an overall yield of 75%. The alcohol **23** was now converted into the iodide **24** in hexamethylphosphoric triamide (HMPA) by using commercially available methyltriphenoxyphosphonium iodide, purified by the method of Verheyden and Moffatt.[21] The intermediate iodide **24** was reduced *in situ* with $NaB(CN)^2H_3$ at 70 °C to give the protected leucine **25** in 73% yield (Scheme 4). A sample of the intermediate iodide was purified by column chromatography on silica gel and its structure confirmed.

Deprotection of the leucine derivative **25** was effected by hydrolysis in 6 mol dm^{-3} aq. hydrochloric acid at room temperature to give (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine hydrochloride **26** in 95% yield. The ^{13}C NMR spectrum of this compound is shown in Fig. 1 and, although the methyl absorptions in the ^1H NMR spectrum were too close for direct assignment, this was achieved by two-dimensional ^{13}C–^1H shift correlation as



Scheme 4

shown in Fig. 2. The signals due to the 4-*pro-R* methyl group are to lower field in both ^1H and ^{13}C NMR spectra and those due to the 4-*pro-S* methyl group are to higher field. The α-isotope shift in the ^{13}C NMR spectrum for ^{13}C–$^2H_3$ is as expected,[22] as is the β-shift for C-4.[22] The latter absorption was accompanied by a small absorption for ^{13}C–C–H^2H_2 and this small amount of dideuteriated compound was confirmed to be in the 4-*pro-R* methyl group by a distortionless enhancement by polarisation transfer (DEPT) experiment. The incorporation of protium was confirmed by integration of the ^1H NMR spectrum to represent 25% of one proton. Thus 8% of the C^2H_3 group consisted of ^1H, which was not considered sufficient to interfere with our protein NMR spectroscopic experiments. The source of the protium was evidently in the $NaB(CN)^2H_3$ reduction of the iodide **24**.

The sample of (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine hydrochloride **26** was used with a medium containing all other amino acids in a fermentation of an auxotrophic strain of *Lactobacillus casei* and the dihydrofolate reductase produced was purified.[23] The ^1H NMR spectrum of a 1:1 complex of the enzyme with methotrexate **2**, when compared with the spectrum of an unlabelled sample of the complex, then allowed the diastereotopic methyl resonances of twelve of the thirteen leucine residues present in the enzyme to be assigned.[23]

Our method for assigning the resonances associated with the diastereotopic methyl groups in proteins is therefore viable and should be generally applicable. During the course of our work, Wuthrich and his colleagues developed an extremely interesting alternative method to assign the resonances of the diastereotopic methyl groups of valine and leucine residues in the ^{13}C NMR spectra of proteins by 'biosynthetic fractional ^{13}C-labelling.' Thus they were able to assign the resonances of the diastereotopic methyl groups of valine and leucine residues in the ^{13}C and ^1H NMR spectra of cyclosporin A,[24] the DNA-binding domain of the 434 repressor[25] and other proteins.[26] The method relies on feeding a mixture of fully ^{13}C-labelled glucose and unlabelled glucose to an organism. The biosynthetic pathway then causes the 4-*pro-R* methyl and C-4 carbon atoms of leucine to be labelled contiguously so that they will show coupling, whereas the 4-*pro-S* methyl carbon, which arises by reductoisomerase-catalysed rearrangement, will not be coupled to the C-4 carbon.

Experimental

Mps were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations (given in units of 10^{-1} deg cm^2 g^{-1}) were measured on a Perkin-Elmer PE241 polarimeter, using a 1 dm pathlength micro cell. IR spectra were recorded on a Perkin-Elmer 1720 Fourier transform instrument, and UV spectra on a Philips PU8720 UV/VIS scanning spectrophotometer. ^1H NMR spectra were recorded on Bruker WM 360 (360 MHz) and AMX 500 (500 MHz) Fourier transform instruments. *J*-Values are given in Hz. ^{13}C NMR spectra (broad band ^1H decoupled) were recorded on Bruker WM 360 (90.6 MHz), AMX 500 (125.8 MHz) and AC-P 250 (62.9 MHz)

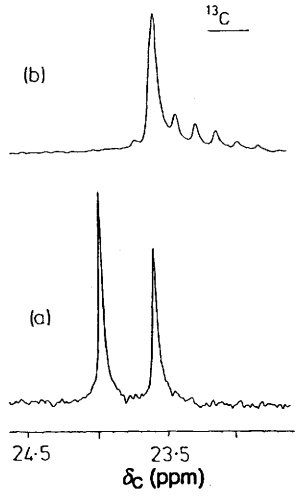


Fig. 1 Part of the ^1H broad band-decoupled ^{13}C NMR spectrum, taken in 20% ^2HCl–^2H_2O at 125.8 MHz, of (a) (2*S*)-leucine hydrochloride; (b) (2*S*,4*R*)-[5,5,5-$^2H_3$]leucine hydrochloride **26**

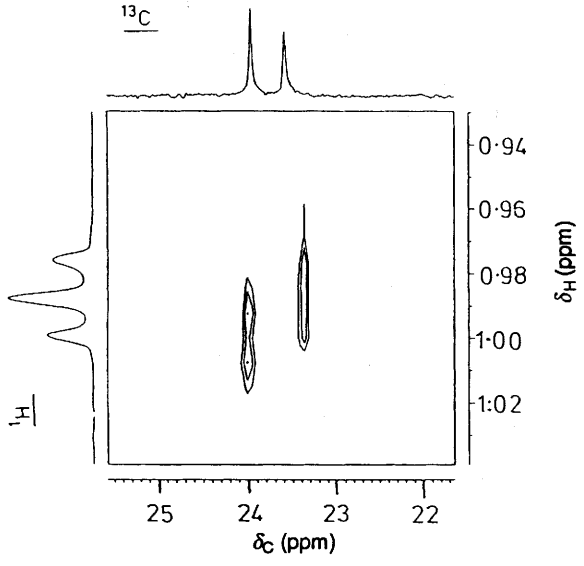


Fig. 2 Two-dimensional ^1H–^{13}C shift-correlation of leucine hydrochloride in 20% ^2HCl–^2H_2O

Fourier transform instruments. Insensitive nuclei enhanced by polarisation transfer (INEPT) experiments were used to help assign ^{13}C NMR resonances where necessary. ^2H NMR spectra were recorded on a Bruker AC-P 250 (38.4 MHz) Fourier transform instrument. Unless otherwise stated, residual solvent peaks were used as an internal reference in the NMR spectra. Mass spectra were recorded on Kratos MS80RF, MS50 and MS25 spectrometers and the accurate mass measurement (for compound **25**) was recorded on Kratos MS80RF and V67070 spectrometers by Dr S. Chotai (Wellcome Research Laboratories). NBA refers to *m*-nitrobenzyl alcohol. Microanalyses were performed by Mrs P. Firmin (Wellcome Research Laboratories), Mr B. Crook (Zeneca Pharmaceuticals Division), and Miss K. Plowman and Miss M. Patel (Sussex). TLC was performed using Merck Kieselgel 60 F_{254} pre-coated silica gel plates of thickness 0.2 mm (ART 5554), and column chromatography was performed using Merck Kieselgel 60 (230–400 mesh—ART 9385). Light petroleum refers to the 40–60 °C fraction except where indicated otherwise.

Benzyl (2*S*)-*N*-(benzyloxycarbonyl)pyroglutamate 7; R^1 = R^2 = $PhCH_2$

(2*S*)-*N*-(Benzyloxycarbonyl)pyroglutamic acid **7**; R^1 = $PhCH_2$, R^2 = H (30 g, 0.114 mol), prepared by the method of Gibian and Klieger,[11] was dissolved in dry acetone (200 cm^3), and redistilled triethylamine (12.7 g, 0.126 mol) was added. Benzyl chloride (15.8 g, 0.125 mol) was added and the solution was heated to reflux for 3 days. The supernatant was decanted from the resulting triethylamine hydrochloride and the solvent was removed under reduced pressure. The resulting oil was dissolved in a mixture of chloroform (50 cm^3) and saturated aq. sodium hydrogen carbonate (100 cm^3). The layers were separated and the aqueous layer was extracted with chloroform (3 × 50 cm^3). The combined organic phases were dried (Na_2SO_4) and the solvent was removed under reduced pressure. The resulting solid was recrystallised from benzene–light petroleum (60–80 °C) (34.2 g, 85%), mp 110.5–111.5 °C (lit.,[13] 107–108 °C); $[\alpha]_D^{23}$ −40.2 (*c* 0.972, EtOH) (lit.,[13] −40.7); ν_{max}(KBr)/cm^{-1} 1790, 1735 and 1705; δ_H(360 MHz; C^2HCl_3) 2.05 (1 H, m, $J_{3R,2}$ 2.6, $J_{3R,4R}$ 3.3, $J_{3R,4S}$ 9.4, $J_{3R,3S}$ 13.1, 3*R*-H), 2.34 (1 H, m, $J_{3S,4R}$ 9.3, $J_{3S,2}$ 9.6, $J_{3S,4S}$ 10.1, $J_{3S,3R}$ 13.1, 3*S*-H), 2.49 (1 H, ddd, $J_{4R,3R}$ 3.3, $J_{4R,3S}$ 9.3, $J_{4R,4S}$ 17.5, 4*R*-H), 2.63 (1 H, ddd, $J_{4S,3R}$ 9.4, $J_{4S,3S}$ 10.1, $J_{4S,4R}$ 17.5, 4*S*-H), 4.76 (1 H, dd, $J_{2,3R}$ 2.6, $J_{2,3S}$ 9.6, 2-H), 5.12 (2 H, s, $C_6H_5CH_2$), 5.28 (2 H, AB, J_{AB} 12.6, $C_6H_5CH_2$) and 7.34 (10 H, br s, 2 × Ph).

Benzyl (2*S*,3*S*)-*N*-(benzyloxycarbonyl)-[3-$^2H_1$]pyroglutamate 7; R^1 = R^2 = $PhCH_2$, H^B = ^2H

This was prepared according to the method above from the *N*-(benzyloxycarbonyl)pyroglutamate **7**, R^1 = $PhCH_2$; H^B = ^2H (846 mg, 3.02 mmol) synthesized by the method of Gibian and Klieger[11] from (2*S*,3*S*)-[3-$^2H_1$]glutamic acid **12**; H^B = ^2H.[12] The product (381 mg, 34%) had δ_H(360 MHz; C^2HCl_3) 2.04 (0.68 H, m, 3*R*-H), 2.33 (0.32 H, m, 3*S*-H), 2.48 (1 H, md, $J_{4R,4S}$ 17.5, 4*R*-H), 2.62 (1 H, dd, $J_{4S,3R}$ 9.5, $J_{4S,4R}$ 17.5, 4*S*-H), 4.70 (1 H, m, 2-H), 5.12 (2 H, s, $C_6H_5CH_2$), 5.21 (2 H, s, $C_6H_5CH_2$) and 7.34 (10 H, s, 2 × Ph).

Benzyl (2*S*,3*R*)-*N*-(benzyloxycarbonyl)-[2,3-$^2H_2$]pyroglutamate 7; R^1 = R^2 = $PhCH_2$, H^A = 2-H = ^2H

This was prepared according to the method above from the *N*-(benzyloxycarbonyl)pyroglutamate **7**, R^1 = $PhCH_2$; H^A = 2-H = ^2H (122 mg, 0.46 mmol) synthesized by the method of Gibian and Klieger[11] from (2*S*,3*R*)-[2,3-$^2H_2$]-glutamic acid **12**; H^A = ^2H.[12] The product (105 mg, 65%) had δ_H(360 MHz; C^2HCl_3) 2.33 (0.94 H, t, $J_{3S,4}$ = $J_{2,3S}$ = 9.9, 3*S*-H), 2.48 (1 H, dd, $J_{4R,3S}$ 9.2, $J_{4R,4S}$ 17.5, 4*R*-H), 2.62 (1 H, dd, $J_{4S,3R}$ 10.2, $J_{4S,4R}$ 17.5, 4*S*-H), 4.70 (0.16 H, d, $J_{2,3S}$ 9.5, 2-H), 5.12 (2 H, s, $C_6H_5CH_2$), 5.21 (2 H, s, $C_6H_5CH_2$) and 7.34 (10 H, s, 2 × Ph).

Benzyl (2*S*)-pyroglutamate 13

This was prepared by the following variation of the method of Danishefsky *et al.*[13] (2*S*)-Pyroglutamic acid **6** (100 g, 0.775 mol) was dried by heating at 55 °C and 0.1 mmHg for 1 h and was then added to acetone (1 dm^3). Triethylamine (86.99 g, 0.861 mol) was added to the stirred slurry under nitrogen, and the mixture was stirred at room temperature until a colourless solution was obtained. Benzyl chloride (107.84 g, 0.852 mol) was added, and the reaction mixture was heated at reflux for 7 days. A yellow solution was obtained on cooling, containing a white crystalline deposit. The reaction mixture was filtered and the solvents were removed under reduced pressure to afford a brown oil, which was dissolved in ethyl acetate (300 cm^3). The organic layer was washed with saturated aq. sodium hydrogen carbonate (3 × 100 cm^3) and the aqueous layer was extracted with ethyl acetate (3 × 100 cm^3). The organic layers were combined, and washed successively with 1 mol dm^{-3} aq. hydrochloric acid (3 × 100 cm^3) and 10% aq. sodium chloride (200 cm^3). The organic layer was dried (Na_2SO_4) and filtered. The solvents were removed under reduced pressure to afford a pale yellow oil (144.75 g, 85%); *m*/*z* [+ve FAB, NBA] 220 $[M + H]^+$; ν_{max}(film)/cm^{-1} 3224br (NH), 1744 (ester) and 1703 (lactam); δ_H(360 MHz; C^2HCl_3) 2.15–2.50 (4 H, m, 3- and 4-H_2), 4.27 (1 H, dd, $J_{2,3S}$ = $J_{2,3R}$ = 5.0, 2-H), 5.18 (2 H, s, $C_6H_5CH_2$), 6.67 (1 H, br, NH) and 7.35 (5 H, s, Ph).

Benzyl (2*S*)-*N*-(*tert*-butoxycarbonyl)pyroglutamate 14

Benzyl (2*S*)-pyroglutamate **13** (144.75 g, 0.661 mol) was dissolved in stirred acetonitrile (900 cm^3) at 0 °C under nitrogen and DMAP (8.07 g, 0.066 mol) was added, followed by a solution of di-*tert*-butyl dicarbonate (187.43 g, 0.859 mol) in acetonitrile (100 cm^3). The solution effervesced and was stirred at 0 °C for 2 h and at room temperature overnight. A dark orange solution was obtained. The solvent was removed under reduced pressure, and the brown crude solid was purified by column chromatography on silica gel with diethyl ether–light petroleum as eluent. The resultant pale yellow solid was recrystallised from ethyl acetate–light petroleum (60–80 °C) to yield a *crystalline solid* (158.5 g, 75%), mp 72–74 °C; $[\alpha]_D^{25}$ −35.0 (*c* 0.98, $CHCl_3$) (Found: C, 63.9; H, 6.65; N, 4.3. $C_{17}H_{21}NO_5$ requires C, 63.9; H, 6.6; N, 4.4%); *m/z* [+ve FAB, NBA] 320 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1785, 1741 and 1703; δ_H(360 MHz; C^2HCl_3) 1.41 [9 H, s, $C(CH_3)_3$], 2.02 (1 H, m, $J_{3R,3S}$ 13.2, $J_{3R,2}$ 2.8, 3*R*-H), 2.32 (1 H, m, $J_{3S,3R}$ 13.2, $J_{3S,2}$ 9.5, 3*S*-H), 2.46 (1 H, m, $J_{4B,4A}$ 17.5, 4-H^B), 2.59 (1 H, m, $J_{4A,4B}$ 17.5, 4-H^A), 4.63 (1 H, dd, $J_{2,3S}$ 9.5, $J_{2,3R}$ 2.8, 2-H), 5.21 (2 H, AB, J_{AB} 12.1, $C_6H_5CH_2O$) and 7.35 (5 H, s, Ph).

(2*S*)-*N*-(*tert*-Butoxycarbonyl)pyroglutamic acid 15

Benzyl (2*S*)-*N*-(*tert*-butoxycarbonyl)pyroglutamate **14** (20 g, 0.063 mol) was dissolved in ethyl acetate (150 cm^3) and 10% palladium on carbon (2 g; 10% w/w) was added. The reaction mixture was stirred for 5 days at room temperature under hydrogen and filtered. The solvent was removed under reduced pressure to afford a pale yellow oil, which was crystallised from ethyl acetate–light petroleum (60–80 °C) to yield a crystalline solid (14.13 g, 98%), mp 118–119 °C [lit.,[27] 115–116 °C]; $[\alpha]_D^{29}$ −36.9 (*c* 1.11, HOAc) {lit.,[27] $[\alpha]_D^{25}$ −35.3 (*c* 1.0, HOAc)} (Found: C, 52.35; H, 6.7; N, 5.8. Calc. for $C_{10}H_{15}NO_5$: C, 52.4; H, 6.6; N, 6.1%); *m/z* [+ve FAB, NBA] 230 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1790, 1741 and 1690; δ_H(360 MHz; C^2HCl_3) 1.49 [9 H, s, $C(CH_3)_3$], 2.15 (1 H, m, $J_{3R,3S}$ 13.2, $J_{3R,2}$ 3.0, 3*R*-H), 2.37 (1 H, m, $J_{3S,3R}$ 13.2, $J_{3S,2}$ 9.5, 3*S*-H), 2.55 (1 H, m, $J_{4B,4A}$ 17.5, 4-H^B), 2.65 (1 H, m, $J_{4A,4B}$ 17.5, 4-H^A), 4.65 (1 H, dd, $J_{2,3S}$ 9.5, $J_{2,3R}$ 3.0, 2-H) and 9.20 (1 H, br, OH).

***tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)pyroglutamate 16**

Method A. (2*S*)-*N*-(*tert*-Butoxycarbonyl)pyroglutamic acid **15** (40 g, 0.175 mol) was dissolved in acetonitrile (500 cm^3), and DMAP (2.14 g, 0.018 mol) and triethylamine (26.51 g, 0.262 mol) were added at 0 °C to the stirred solution under nitrogen. Di-*tert*-butyl dicarbonate (57.18 g, 0.262 mol) as a solution in acetonitrile (100 cm^3) was added dropwise at 0 °C over a period of 45 min. Effervescence occurred, and the resultant light-brown solution was stirred at room temperature overnight. The solvent was removed under reduced pressure to afford a red-brown oil, which was purified by column chromatography on silica gel with diethyl ether as eluent. The resultant pale yellow oil was crystallised from diethyl ether–light petroleum to yield a *solid* (44.30 g, 89%), mp 54–56 °C (lit.,[28] oil); $[\alpha]_D^{25}$ −36.6 (*c* 0.93, $CHCl_3$) {lit.,[28] $[\alpha]_D^{22}$ −35.1 (*c* 0.9, $CHCl_3$)} (Found: C, 58.7; H, 8.2; N, 4.8. $C_{14}H_{23}NO_5$ requires C, 58.9; H, 8.1; N, 4.9%); *m/z* [+ve FAB, NBA] 286 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1775 (imide) and 1719 (ester); δ_H(360 MHz, C^2HCl_3) 1.47 [9 H, s, $C(CH_3)_3$], 1.49 [9 H, s, $C(CH_3)_3$], 1.95 (1 H, m, $J_{3R,2}$ 2.6, $J_{3R,3S}$ 13.3, 3*R*-H), 2.25 (1 H, m, $J_{3S,2}$ 9.4, $J_{3S,3R}$ 13.3, 3*S*-H), 2.45 (1 H, m, $J_{4B,4A}$ 17.5, 4-H^B), 2.60 (1 H, m, $J_{4A,4B}$ 17.5, 4-H^A) and 4.46 (1 H, dd, $J_{2,3S}$ 9.4, $J_{2,3R}$ 2.6, 2-H); δ_C(90.6 MHz; C^2HCl_3) 173.2 (CON), 170.3 (C-1), 150.0 (NCO_2), 83.2 [$OC(CH_3)_3$] 82.2 [$OC(CH_3)_3$], 59.6 (C-2), 31.1 (C-4), 27.9 [$C(CH_3)_3$] and 21.7 (C-3).

Method B. *tert*-Butyl (2*S*)-pyroglutamate **17**[16] (5.0 g, 0.027 mol) was dissolved in acetonitrile (100 cm^3) and the reaction mixture was cooled to 0 °C. DMAP (0.33 g, 2.70 mmol) was added to the stirred mixture, followed by a solution of di-*tert*-butyl dicarbonate (8.85 g, 0.041 mol) in acetonitrile (30 cm^3). The solution effervesced and was stirred at 0 °C for 2 h and for 12 h at room temperature. A dark orange solution was obtained. The solvent was removed under reduced pressure to afford a brown solid, which was purified by column chromatography on silica gel with diethyl ether–light petroleum as eluent to afford a pale yellow solid, which was recrystallised from diethyl ether–light petroleum to yield a solid (5.50 g, 72%), mp 54–56 °C; $[\alpha]_D^{25}$ −36.6 (*c* 0.93, $CHCl_3$) {lit.,[28] $[\alpha]_D^{22}$ −35.1 (*c* 0.9, $CHCl_3$)}. Spectroscopic data were identical with those found for the sample prepared by method A above.

***tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-(dimethylamino-methylene)pyroglutamate 18**

tert-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)pyroglutamate **16** (81.94 g, 0.288 mol) was dissolved in 1,2-dimethoxyethane (700 cm^3) and *tert*-butoxybis(dimethylamino)methane (Bredereck's reagent) (75.08 g, 0.431 mol) was added. The reaction mixture was heated under nitrogen at a constant temperature of 75 °C for 16 h. The solvent was removed under reduced pressure to afford a red-brown oil, which was crystallised from diethyl ether–light petroleum to yield a *pale yellow solid* (80.94 g, 83%), mp 126–127 °C; $[\alpha]_D^{26}$ −47.5 (*c* 1.31, $CHCl_3$) (Found: C, 59.85; H, 8.5; N, 8.1. $C_{17}H_{28}N_2O_5$ requires C, 60.0; H, 8.2; N, 8.2%); *m/z* [+ve FAB, NBA] 341 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1759, 1738, 1687 and 1631; λ_{max}(MeOH)/nm 313 (ε 31 700 dm^3 mol^{-1} cm^{-1}); δ_H(500 MHz, C^2HCl_3) 1.47 [9 H, s, $C(CH_3)_3$]; 1.50 [9 H, s, $C(CH_3)_3$], 2.80 (1 H, dd, $J_{3R,2}$ 3.8, $J_{3R,3S}$ 14.8, 3*R*-H), 3.02 [6 H, s, $N(CH_3)_2$], 3.25 (1 H, dd, $J_{3S,2}$ 10.7, $J_{3S,3R}$ 14.8, 3*S*-H), 4.40 (2 H, dd, $J_{2,3S}$ 10.7, $J_{2,3R}$ 3.8, 2-H) and 7.12 (1 H, t, $J_{6,3A}$, $J_{6,3B}$ 1.7, =CH); δ_C(90.6 MHz; C^2HCl_3) 171.1 (CON), 169.4 (C-1), 150.7 (NCO_2), 146.1 (*C*HNMe$_2$), 91.8 [*C*=CHN$(CH_3)_2$], 81.9 [$OC(CH_3)_3$], 81.6 [$OC(CH_3)_3$], 56.7 (C-2), 41.9 [$N(CH_3)_2$], 28.2 [$C(CH_3)_3$], 28.0 [$C(CH_3)_3$] and 26.4 (C-3).

***tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylenepyro-glutamate 19**

Method A—by reduction of the enaminone 18. *tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-(dimethylaminomethylene)pyroglutamate **18** (10.0 g, 29.4 mmol) was dissolved in THF (300 cm^3) and the solution was cooled to −78 °C. A 1 molar solution of DIBAL in THF (44 cm^3, 44.0 mmol) was added dropwise to the stirred mixture during 10 min, and the mixture was stirred at − 78 °C for 1 h. The reaction mixture was allowed to warm to room temperature and was stirred for a further 2 h. The mixture was quenched with saturated aq. ammonium chloride (~50 cm^3) at room temperature and stirred overnight at room temperature to afford a pale yellow solution containing a white slurry. The mixture was decanted, and the slurry was washed with ethyl acetate (5 × 40 cm^3). The organic layer was washed in turn with 10% aq. citric acid (40 cm^3), 10% aq. sodium chloride (40 cm^3), saturated aq. sodium hydrogen carbonate (40 cm^3) and 10% aq. sodium chloride (40 cm^3) and dried (Na_2SO_4). The solvent was removed under reduced pressure to afford a pale yellow oil (6.45 g, 74% crude olefin **19**), which was repeatedly crystallised from diethyl ether–light petroleum to yield the major product, tert-*butyl* (2S)-N-(tert-*butoxycarbonyl*)-4-*methylenepyroglutamate* **19** as a crystalline solid (6.01 g, 69%), mp 108–110 °C; $[\alpha]_D^{28}$ − 3.5 (*c* 1.37, $CHCl_3$) (Found: C, 60.3; H, 8.1; N, 4.3. $C_{15}H_{23}NO_5$ requires C, 60.6; H, 7.7; N, 4.7%); *m/z* [+ve FAB, NBA] 298 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1777, 1736, 1698 and 1662; λ_{max}(MeOH)/nm 228 (ε 8900); δ_H(500 MHz; C^2HCl_3) 1.46 [9 H, s, $C(CH_3)_3$], 1.52 [9 H, s, $C(CH_3)_3$], 2.65 (1 H, qq, $J_{3R,3S}$ 17.5, $J_{3R,2}$ 3.1, $J_{3R,6}$ 3, 3*R*-H), 3.03 (1 H, tttt, $J_{3S,3R}$ 17.5, $J_{3S,2}$ 10.1, $J_{3S,6}$ 2.5, 3*S*-H), 4.47 (1 H, dd, $J_{2,3S}$ 10.1, $J_{2,3R}$ 3.1, 2-H), 5.50 (1 H, t, $J_{6B,3A}$ = $J_{6B,3B}$ 2.5, =CH) and 6.22 (1 H, t, $J_{6A,3A}$ = $J_{6A,3B}$ = 3.0, =CH); δ_C(125.8 MHz, C^2HCl_3) 170.0 (CON), 165.5 (C-1), 149.9 (NCO_2), 136.9 (*C*=CH_2), 120.3 (C=*C*H_2), 83.5 [$OC(CH_3)_3$], 82.3 [$OC(CH_3)_3$], 56.4 (C-2), 28.0 (C-3), 27.9 [$C(CH_3)_3$] and 27.9 [$C(CH_3)_3$].

The acidic aqueous layer was treated with saturated aq. sodium hydrogen carbonate until basic (pH 8–9 by pH paper), separated, and extracted with ethyl acetate (3 × 60 cm^3). The organic layers were combined and dried (Na_2SO_4), and the solvent was removed under reduced pressure to yield the minor products (2S,4RS)-tert-*butyl* N-(tert-*butoxycarbonyl*)-4-(*dimethylaminomethyl*)*pyroglutamate* **20** as a pale yellow crystalline solid (1.96 g, 19%), mp 76–78 °C, and as a 3:1 mixture of the *trans*- and *cis*-amines **20** which was not separated (Found: C, 59.2; H, 8.8; N, 8.0. $C_{17}H_{30}N_2O_5$ requires C, 59.6; H, 8.8; N, 8.2%); *m/z* [+ve FAB, NBA] 343 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1779, 1735 and 1703; δ_H(*trans*-amine; 500 MHz; C^2HCl_3) 1.47 [9 H, s, $C(CH_3)_3$], 1.50 [9 H, s, $C(CH_3)_3$], 2.11 (1 H, m, $J_{3R,2}$ 1.5 $J_{3R,3S}$ 13.5, $J_{3R,4}$ 9.8, 3*R*-H), 2.23 [6 H, s, $N(CH_3)_2$], 2.80–2.30 (4 H, overlapping, 4-H, CH_2N, and 3*S*-H) and 4.44 (1 H, dd, $J_{2,3S}$ 9.6, $J_{2,3R}$ 1.5, 2-H); δ_H[*cis* (2*S*,4*R*) amine; 500 MHz; C^2HCl_3] 1.48 [9 H, s, $C(CH_3)_3$], 1.50 [9 H, s, $C(CH_3)_3$] 1.99 (1 H, m, $J_{3R,2}$ 5.4, $J_{3R,3S}$ 13.5, $J_{3R,4}$ 6.0, 3*R*-H), 2.21 [6 H, s, $N(CH_3)_2$], 2.60–2.35 (3 H, overlapping, 3*S*-H and CH_2N), 2.70 (1 H, m, 4-H) and 4.42 (1 H, dd, $J_{2,3S}$ 9.5, $J_{2,3R}$ 5.4, 2-H); δ_C(*trans*-amine; 125.8 MHz; C^2HCl_3) 174.1 (CON), 170.3 (C-1), 149.3 (NCO_2), 83.2 [$OC(CH_3)_3$], 82.1 [$OC(CH_3)_3$], 59.9 (CH_2NMe_2), 57.8 (C-2), 45.6 [$N(CH_3)_2$], 40.7 (C-4), 27.8 [$C(CH_3)_3$] and 25.9 (C-3); δ_C(*cis*-amine; 125.8 MHz; C^2HCl_3) 174.3 (CON), 170.6 (C-1), 149.3 (NCO_2), 83.2 [$OC(CH_3)_3$], 81.9 [$OC(CH_3)_3$], 60.1 (CH_2NMe_2), 58.1 (C-2), 45.3 [$N(CH_3)_2$], 41.6 (C-4), 27.8 [$C(CH_3)_3$] and 25.9 (C-3).

Method B—from the Mannich bases 20 by Hofmann elimination. *tert*-Butyl (2*S*,4*RS*)-*N*-(*tert*-butoxycarbonyl)-4-(dimethylaminomethyl)pyroglutamate **20** (3:1 mixture of the *trans*-and *cis*-isomers) (1.0 g, 2.92 mmol) was dissolved in methanol (50 cm^3) and methyl iodide (0.46 g, 3.24 mmol) was added. The reaction mixture was stirred in the absence of light at room temperature overnight. The solvent was removed under reduced pressure to yield *tert*-butyl (2*S*,4*RS*)-*N*-(*tert*-butoxycarbonyl)-4-(trimethylammoniomethyl)pyroglutamate iodide as a solid (1.45 g, 100%), mp 143–145 °C; *m/z* [+ve FAB, NBA] 357 $[M]^+$. Tetrahydrofuran (20 cm^3) and saturated aq. sodium hydrogen carbonate (30 cm^3) were added to the salt and the reaction mixture was stirred vigorously for 1 h at room temperature to yield a pale yellow solution and a white precipitate. The solution was decanted, ethyl acetate (40 cm^3) was added, and the solution was washed successively with saturated aq. sodium hydrogen carbonate (40 cm^3) and 10% aq. citric acid (40 cm^3). The orange organic layer was dried (Na_2SO_4) and the solvent was removed under reduced pressure to afford an orange oil (556 mg) which turned colourless upon further drying at high vacuum. The oil was purified by column chromatography on silica gel with ethyl acetate–light petroleum (60–80 °C) as eluent to afford tert-*butyl* (2S)-N-(tert-*butoxycarbonyl*)-4-*methylenepyroglutamate* **19** as a solid (94 mg, 11%) with an identical ^1H NMR spectrum with that of the above sample.

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylpyroglutamate 21

Method A—from the exomethylene derivative 19. *tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylenepyroglutamate **19** (1.90 g, 6.34 mmol) was dissolved in methanol (50 cm^3) and 10% palladium on carbon (0.2 g; 10% w/w) was added. The reaction mixture was stirred under hydrogen for 24 h at room temperature, and filtered. The solvents were removed under reduced pressure to afford an oil (95% recovery), which was crystallised from diethyl ether–light petroleum to yield tert-*butyl* (2S,4S)-N-(tert-*butoxycarbonyl*)-4-*methylpyroglutamate* **21** as a crystalline solid (1.64 g, 86%), mp 54–56 °C; $[\alpha]_D^{27}$ −6.1 (*c* 1.12, $CHCl_3$) (Found: C, 60.15; H, 8.6; N, 4.6. $C_{15}H_{25}NO_5$ requires C, 60.2; H, 8.4; N, 4.7%) *m/z* [+ve FAB, NBA] 300 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 1783 (imide) and 1738 (ester); δ_H(500 MHz; $C_6{}^2H_6$) 0.95 (3 H, d, $J_{CH3,4}$ 7.3, CH_3), 1.19 (1 H, ddd, $J_{3R,3S}$ 12.9, $J_{3R,4}$ 7.7, $J_{3R,2}$ 6.6, 3*R*-H), 1.35 [9 H, s, $C(CH_3)_3$], 1.45 [9 H, s, $C(CH_3)_3$], 1.73 (1 H, ddd, $J_{3S,3R}$ 12.9, $J_{3S,4}$ 9.3, $J_{3S,2}$ 8.7, 3*S*-H), 1.88 (1 H, m, $J_{4,CH3}$ 7.3, $J_{4,3S}$ 9.3, $J_{4,3R}$ 7.7, 4-H) and 4.16 (1 H, dd, $J_{2,3S}$ 8.7, $J_{2,3R}$ 6.6, 2-H); δ_H(500 MHz; C^2HCl_3) 1.26 (3 H, d, $J_{CH3,4}$ 7.0, CH_3), 1.48 [9 H, s, $C(CH_3)_3$], 1.51 [9 H, s, $C(CH_3)_3$], 1.58 (1 H, m, 3*R*-H), 2.57 (2 H, m, 3*S*-H and 4-H) and 4.38 (1 H, m, 2-H); δ_C(125.8 MHz; $C_6{}^2H_6$) 173.9 (CON), 170.9 (C-1), 150.9 (NCO_2), 82.5 [$OC(CH_3)_3$], 81.3 [$OC(CH_3)_3$], 58.0 (C-2), 37.5 (C-4), 29.6 (C-3), 28.0 [$C(CH_3)_3$], 27.8 [$C(CH_3)_3$] and 16.3 (CH_3).

Method B—by reduction of the enaminone 18. *tert*-Butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-(dimethylaminomethylene)pyroglutamate **18** (20 g, 0.059 mol) was dissolved in ethyl acetate (300 cm^3) and 10% palladium on carbon (10 g; 50% w/w) was added. The reaction mixture was stirred under hydrogen for 4 days at room temperature, filtered, and washed successively with ice-cold 10% aq. citric acid (150 cm^3), 10% aq. sodium chloride (100 cm^3), saturated aq. sodium hydrogen carbonate (200 cm^3) and 10% aq. sodium chloride (100 cm^3). The organic layer was dried (Na_2SO_4) and the solvent was removed under reduced pressure to afford an oil, which was purified by column chromatography on silica gel with diethyl ether as eluent. The resultant major product, (2S,4S)-tert-*butyl* N-(tert-*butoxycarbonyl*)-4-*methylpyroglutamate* **21** was obtained as a solid (13.79 g, 78%), mp 68–69 °C, with identical spectra with those of the sample prepared by method A.

The acidic aqueous layer was treated with saturated aq. sodium hydrogen carbonate until basic (pH 8), and ethyl acetate (100 cm^3) was added. The organic layer was then separated, and the basic aqueous layer was extracted with ethyl acetate (2 × 100 cm^3). The organic layers were combined, dried (Na_2SO_4) and the solvent was removed under reduced pressure to yield the minor product, tert-*butyl* (2S,4RS)-N-tert-*butoxycarbonyl*-4-(*dimethylaminomethyl*)*pyroglutamate* **20** as a solid (2.29 g, 11%). The *cis*- and *trans*-amines **20** were identified by comparison of their spectra with those of the previous sample and were obtained as a diastereoisomeric, inseparable mixture in the ratio 5:1.

Methyl C—by reduction of the Mannich bases 20. *tert*-Butyl (2*S*,4*RS*)-*N*-(*tert*-butoxycarbonyl)-4-(dimethylaminomethyl)-pyroglumate **20** as a 3:1 mixture of the *trans*- and *cis*-isomers (1.0 g, 2.92 mmol) was dissolved in propan-2-ol (25 cm^3) and 10% palladium on carbon (0.5 g, 50% w/w) was added. The reaction mixture was stirred under hydrogen for 3 days at room temperature, was then filtered, and the solvent was removed under reduced pressure to yield an oil, which was dissolved in diethyl ether. The organic layer was washed successively with 10% aq. citric acid (30 cm^3), 10% aq. sodium chloride (30 cm^3), saturated aq. sodium hydrogen carbonate (30 cm^3), and 10% aq. sodium chloride (30 cm^3) and dried (Na_2SO_4). The solvent was removed under reduced pressure to yield tert-*butyl* (2S,4S)-N-(tert-*butoxycarbonyl*)-4-*methylpyroglutamate* **21** as an oil (370 mg, 42%) with identical spectra with those of the sample prepared by method A.

The acidic aqueous layer was treated with saturated aq. sodium hydrogen carbonate until basic (pH 8–9 by pH paper), and extracted with diethyl ether (3 × 20 cm^3). The organic layers were combined, dried (Na_2SO_4), and the solvent was removed under reduced pressure to yield starting material containing traces of *tert*-butyl (2*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylenepyroglutamate **19** as an oil (250 mg, 29%).

1-*tert*-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylglutamic acid 22

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylpyroglutamate **21** (4 g, 13.4 mmol) was dissolved in THF (67 cm^3) and 1 mol dm^{-3} aq. lithium hydroxide (16.1 cm^3) was added dropwise at 0 °C to the vigorously stirred mixture over a period of 15 min.

The mixture was stirred for a further 15 min at 0 °C. Ethyl acetate (100 cm^3) and saturated aq. sodium hydrogen carbonate (100 cm^3) were added to the reaction mixture and the organic layer was separated, and extracted with saturated aq. sodium hydrogen carbonate (100 cm^3). The aqueous layers were combined and carefully acidified to pH 4–4.5 (by pH paper), while being stirred at 0 °C, by the dropwise addition of 10% aq. citric acid. The aqueous layer was extracted with ethyl acetate (5 × 100 cm^3), the organic layers were combined and dried (Na_2SO_4) and the solvent was removed under reduced pressure to afford a foamy solid, which crystallised as a solid on long storage (3.99 g, 94%). A sample of *acid* **22** was recrystallised from ethyl acetate–light petroleum as a solid, mp 97–99 °C; $[\alpha]_D^{22}$ −12.0 (*c* 1.9, MeOH) (Found: C, 56.9; H, 8.8; N, 4.3. $C_{15}H_{27}NO_6$ requires C, 56.8; H, 8.6; N, 4.4%); *m/z* [+ve FAB, NBA] 318 $[M + H]^+$; ν_{max}(KBr)/cm^{-1} 3300br (NH) and 1719 (acid); δ_H(500 MHz; $C^2H_3O^2H$) 1.17 (3 H, d, $J_{CH3,4}$ 7.0, CH_3), 1.43 [9 H, s, $C(CH_3)_3$], 1.46 [9 H, s, $C(CH_3)_3$], 1.75 (1 H, ddd, J_{3B3A} 13.9, $J_{3B,2}$ 5.4, $J_{3B,4}$ 8.0, 3*B*-H), 1.95 (1 H, ddd, $J_{3A,3B}$ 13.9, $J_{3A,2}$ 9.9, $J_{3A,4}$ 6.5, 3A-H), 2.50 (1 H, m, $J_{4,CH3}$ 7.0, 4-H) and 4.03 (1 H, dd, $J_{2,3A}$ 9.9, $J_{2,3B}$ 5.4, 2-H); δ_H(360 MHz; C^2HCl_3) showed NH (δ 5.0, d, $J_{NH,2}$ 7.9) which disappeared upon addition of ^2H_2O, and 2-H at δ 4.20; δ_C(125.8 MHz; $C^2H_3O^2H$) 179.6 (CO_2), 173.6 (CO_2), 158.0 (CON), 82.8 [$OC(CH_3)_3$, 80.6 [$OC(CH_3)_3$], 54.0 (C-2), 37.5 (C-4), 36.1 (C-3), 28.7 [$C(CH_3)_3$], 28.3 [$C(CH_3)_3$] and 17.3 (CH_3).

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-5-hydroxy-[5,5-$^2H_2$]leucine 23

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-4-methylglutamic acid **22** (1 g, 3.15 mmol) was dissolved in THF (15 cm^3) and the solution was cooled to −40 °C under nitrogen. Triethylamine (0.41 g, 4.05 mmol) was added followed by dropwise addition of isobutyl chloroformate (0.51 g, 3.72 mmol). A pale yellow colour was observed and a white sediment was formed in the reaction mixture, which was stirred for 1 h at −40 °C and filtered under nitrogen. A mixture of sodium tetradeuterioboranuide (0.40 g, 9.57 mmol) in THF (10 cm^3) and ^2H_2O (2 cm^3) was added dropwise to the stirred filtrate at 0 °C. Effervescence was observed and a sediment was obtained in the reaction mixture, which was stirred at room temperature for 1.5 h and cooled to 0 °C. Ethyl acetate (20 cm^3) and 10% aq. sodium chloride (20 cm^3) were added, and the organic layer was separated, washed successively with ice-cold 10% aq. citric acid (20 cm^3) and 10% aq. sodium chloride (20 cm^3) and dried (Na_2SO_4). The solvents were removed under reduced pressure to afford a foam, which was purified by column chromatography on silica gel with 1:1 light petroleum–ethyl acetate as eluent to afford an oil (0.72 g, 75%). This crystallised on long storage and a sample was recrystallised from ethyl acetate–light petroleum to give compound **23** mp 73–75 °C; $[\alpha]_D^{28}$ −1.1 (*c* 2.16, $CHCl_3$) (Found: C, 59.3; H, 9.8; N, 4.5. $C_{15}H_{27}{}^2H_2NO_5$ requires C, 59.0; H, 10.2; N, 4.6%); *m/z* [+ve FAB, NBA] 306 $[M + H]^+$; ν_{max}(film)/cm^{-1} 3300 (NH, OH) and 1713 (ester); δ_H(500 MHz; $C_6{}^2H_6$) 0.88 (3 H, d, $J_{CH3,4}$ 6.6, CH_3), 1.41 [9 H, s, $C(CH_3)_3$], 1.31 [9 H, $C(CH_3)_3$], 1.55 (1 H, m, 3-H^B), 1.6–1.85 (2 H, m, 3-H^A and 4-H), 4.45 (1 H, m, 2-H) and 5.40 (1 H, d, $J_{NH,2}$ 8.2, NH); selective irradiation of the multiplet at δ 4.45 (2-H) caused the doublet at δ 5.40 (NH) to collapse to a singlet and a change in the splitting pattern of the multiplet at δ 1.60–1.85 (3-H^A and 4-H); selective irradiation of the doublet at δ 5.40 (NH) caused a change in the splitting pattern of the multiplet at δ 4.45 (2-H); selective irradiation of the methyl doublet at δ 0.88 (CH_3) caused a change in the splitting pattern of the multiplet at δ 1.6–1.85 (3-H^A and 4-H); δ_D(38.4 MHz; $CHCl_3$) 3.40 and 3.53 (2 × s, C^2H_2OH); δ_C(125.8 MHz; $C_6{}^2H_6$) 172.8 (C-1), 156.0 (CON), 81.1 [$OC(CH_3)_3$], 79.3 [$OC(CH_3)_3$], 66.6 (m, C^2H_2OH), 53.0 (C-2), 36.8 (C-3), 32.8 (C-4), 28.4 [$C(CH_3)_3$], 27.9 [$C(CH_3)_3$] and 16.6 (CH_3).

tert-Butyl (2*S*,4*R*)-*N*-*tert*-butoxycarbonyl-[5,5,5-$^2H_3$]leucine 25

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-5-hydroxy-[5,5-$^2H_2$]-leucine **23** (2.50 g, 8.20 mmol) was dissolved in HMPA (25 cm^3). Methyltriphenoxyphosphonium iodide (5.56 g, 12 mmol), purified by the method of Verheyden and Moffatt,[21] as a solution in HMPA (25 cm^3) was added dropwise at room temperature to the stirred mixture under nitrogen. The reaction mixture was stirred for 40 min at room temperature, sodium cyanotrideuterioboranuide (2.70 g, 41 mmol) was added, and the reaction mixture was heated to 70 °C for 16 h under nitrogen. The orange solution was cooled and diethyl ether (250 cm^3) was added. The organic layer was washed successively with 10% aq. sodium chloride (2 × 150 cm^3), saturated aq. sodium hydrogen carbonate (2 × 150 cm^3), saturated aq. sodium thiosulfate (2 × 150 cm^3) and saturated aq. sodium chloride (200 cm^3) and dried (Na_2SO_4). The solvents were removed under reduced pressure to afford an oil, which was purified by column chromatography on silica gel, using 1:4 diethyl ether–light petroleum (60–80 °C) as eluent, to yield the *product* **25** as an oil which crystallised as a waxy solid on long storage (1.73 g, 73%), mp 30–35 °C; $[\alpha]_D^{22}$ −3.8 (*c* 1.2, $CHCl_3$) (Found: M^+, [EI] 290.2279. $C_{15}H_{26}{}^2H_3NO_4$ requires M, 290.2281); *m/z* [+ve FAB, NBA] 291 $[M + H]^+$; δ_H(360 MHz; $C_6{}^2H_6$) 0.83 (3 H, d, $J_{CH3,4}$ 7.0, CH_3), 1.30 [10 H, s, $C(CH_3)_3$ and m, 3-H^B], 1.41 [9 H, s, $C(CH_3)_3$], 1.53 (1 H, m, 3-H^A), 1.59 (1 H, m, 4-H), 4.47 (1 H, ddd, $J_{2,3A}$ 9.0, $J_{2,NH}$ 8.5, $J_{2,3B}$ 5.2, 2-H) and 4.90 (1 H, d, $J_{NH,2}$ 8.5, NH); selective irradiation of the methyl doublet at δ 0.83 led to a change in the splitting pattern of the multiplet at δ 1.59 (4-H); and selective irradiation of the multiplet at δ 4.47 (2-H) led to a change in the splitting pattern of the multiplets at δ 1.53 (3-H^A) and δ 1.30 (3-H^B); δ_C(125.8 MHz; $C_6{}^2H_6$) 172.9 (C-1), 155.7 (CON), 80.9 [$OC(CH_3)_3$], 79.1 [$OC(CH_3)_3$], 53.1 (C-2), 42.2 (C-3), 28.4 [$C(CH_3)_3$], 27.9 [$C(CH_3)_3$], 24.8 (C-4), 22.3 (m, C^2H_3) and 21.9 (CH_3).

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-5-iodo-[5,5-$^2H_2$]-leucine 24

tert-Butyl (2*S*,4*S*)-*N*-(*tert*-butoxycarbonyl)-5-hydroxy-[5,5-$^2H_2$]leucine **23** (118 mg, 0.387 mmol) was dissolved in HMPA (2 cm^3). Methyltriphenoxyphosphonium iodide (262 mg, 0.579 mmol), purified by the method of Verheyden and Moffatt,[21] was added and the reaction mixture was stirred at room temperature for 1 h to give an orange colour. Methanol (2 cm^3) was added and the mixture was stirred for a further 10 min, after which time diethyl ether (60 cm^3) was added. The organic layer was washed with 10% aq. sodium chloride (6 × 50 cm^3) and dried (Na_2SO_4), and the solvents were removed under reduced pressure to afford an oil, which was purified by column chromatography on silica gel with diethyl ether–light petroleum as eluent, to yield the iodide **24** as an oil (48 mg, 30%). The iodide **24** turned brown when left under nitrogen in the dark; *m/z* [+ve FAB, NBA] 416 $[M + H]^+$; δ_H(500 MHz; $C_6{}^2H_6$) 0.79 (3 H, d, $J_{CH3,4}$ 6.3, CH_3), 1.28 [9 H, $C(CH_3)_3$], 1.40 [10 H, s, $C(CH_3)_3$ and m, 3-H^B], 1.41–1.50 (2 H, m, 4-H and 3-H^A), 4.38 (1 H, m, 2-H) and 4.82 (1 H, d, $J_{NH,2}$ 8.8, NH); selective irradiation of the methyl doublet at δ 0.79 (CH_3) did not lead to observable changes in the ^1H NMR spectrum, but irradiation of the multiplet at δ 4.38 (2-H) caused the doublet at δ 4.82 (NH) to collapse to a singlet; δ_C(125.8 MHz; $C_6{}^2H_6$) 172.1 (C-1), 155.6 (CON), 81.4 [$OC(CH_3)_3$], 79.4 [$OC(CH_3)_3$], 52.8 (C-2), 39.9 (C-3), 31.9 (C-4), 28.4 [$C(CH_3)_3$], 27.9 [$C(CH_3)_3$], 19.9 (CH_3) and 16.2 (m, C^2H_2I).

(2*S*,4*R*)-[5,5,5-$^2H_3$]Leucine hydrochloride 26

tert-Butyl (2*S*,4*R*)-*N*-(*tert*-butoxycarbonyl-[5,5,5-$^2H_3$]leucine **25** (1.73 g, 5.95 mmol) was dissolved in THF (10 cm^3), and the solution was treated with 6 mol dm^{-3} aq. hydrochloric acid (20 cm^3) and stirred at room temperature for 2 days. The solvent was removed under reduced pressure and traces of residual

hydrochloric acid were removed by azeotropic distillation with diethyl ether to afford compound **26** as a solid (0.96 g, 95%), mp > 300 °C; $[\alpha]_D^{20}$ +6.8 (*c* 1, 5 mol dm^{-3} HCl); *m/z* [+ve FAB, glycerol/1 mol dm^{-3} HCl (aq.)] 227 $[M + glycerol]^+$ and 135 $[M + H]^+$; δ_H(500 MHz; 20% $^2HCl/^2H_2O$) 0.97 (3 H, d, $J_{4,CH3}$ 6.1, CH_3), 1.90–1.78 (3 H, m, 3-H_2 and 4-H) and 4.17 (1 H, t, $J_{2,3A}$ 8.1, $J_{2,3B}$ 6.0, 2-H); δ_C(125.8 MHz; 20% ^2HCl–^2H_2O) 174.0 (CO), 54.0 (C-2), 40.9 (C-3), 26.1 (C-4), 23.6 (CH_3) and 23.4 (m, 4-C^2H_3).

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