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Research Article

Synthesis of isotopically labelled glycyl-L-prolyl-L-glutamic acid (Glypromate®) and derivatives

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Summary

The related tripeptides glycyl-L-prolyl-L-glutamic acid (GPE) and glycyl-L-2-methylprolyl-L-glutamic acid (G-2-MePE) were labelled with commercially available [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]-L-glutamic acid in 3 steps in excellent overall yield with high isotope incorporation. A related cyclic dipeptide was labelled with [2,2-$^{2}H_2$, 2-$^{15}N_1$]glycine giving a mixture of compounds resulting from deuterium scrambling. Copyright © 2006 John Wiley & Sons, Ltd.

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Key Words: GPE; neuroprotective; ^{2}H-,^{13}C-,^{15}N-amino acids; isotope labelling

Introduction

The syntheses of several analogues of the neuroprotective tripeptide glycyl-L-prolyl-L-glutamic acid (GPE) have been reported by this research group and others.[1–6] Currently we required isotopically labelled internal standards of GPE and glycyl-L-2-methylprolyl-L-glutamic acid (G-2-MePE) for several bioassay applications carried out using LC–MS analysis. The internal standards needed to be of sufficiently different molecular weight to GPE and G-2-MePE (and related structures). Our previous strategy for the preparation of GPE and its analogues relied on the use of a urethane-protected glycyl-L-proline (or derivative). Direct deprotection and cyclization afforded cyclic diketopiperazines whilst coupling with suitably protected glutamic acid and subsequent deprotection yielded a tripeptide (Scheme 1).

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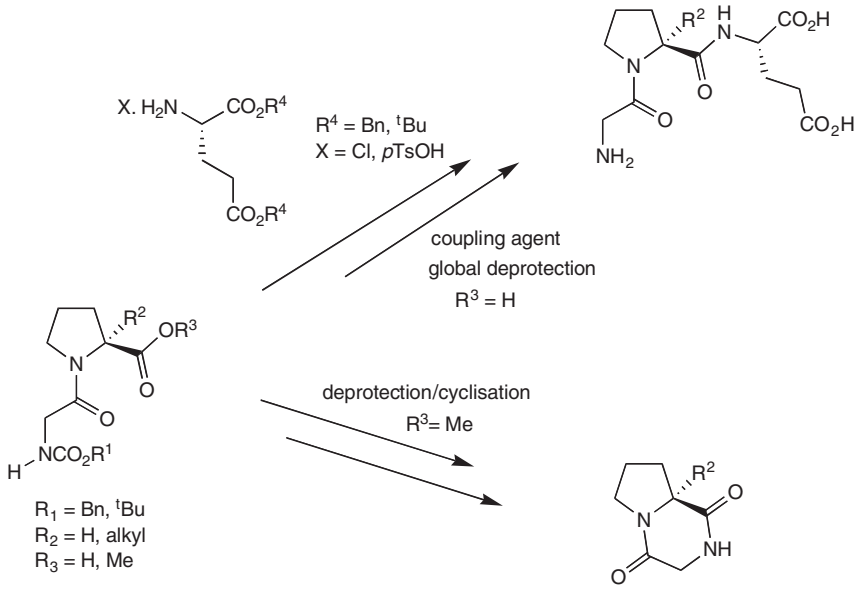

Scheme 1. Convergent synthesis of GPE analogues

It was envisaged that employing a similar strategy starting from commercially available ^{13}C-, ^{15}N-, and ^{2}H-labelled amino acids of high isotope purity, would allow the preparation of isotopically labelled analogues.

Results and discussion

For the synthesis of isotopically labelled GPE and G-2-MePE commercially available [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]-L-glutamic acid provides an additional 6 atomic mass units and was easily protected as the novel dibenzyl ester salt **3** in excellent yield. For our previously published synthesis of unlabelled GPE[2] and G-2-MePE,[1] we used the mixed anhydride method or bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BoP-Cl) as coupling reagents, respectively, to install the amide bond between Z-GlyPro-OH and the protected glutamate residue, affording the tripeptides in $>90\%$ yields. However, in the present work coupling Z-GlyPro-OH **1** with isotopically labelled dibenzyl glutamate **3** using ethyl chloroformate only afforded tripeptide **4** in 60% yield. This yield was unsatisfactory given the use of an expensive isotopically labelled reagent. Fortunately use of the more powerful coupling agent HBTU afforded tripeptide **4** in a superior 87% yield (Scheme 2).

Hydrogenolysis of the benzyl groups in tripeptide **4** yielded the expected tripeptide **6** in good yield showing the correct mass analysis. The reaction required use of dioxane as solvent (cf. methanol for the unlabelled tripeptide) to fully solubilize the protected tripeptide during the deprotection step.

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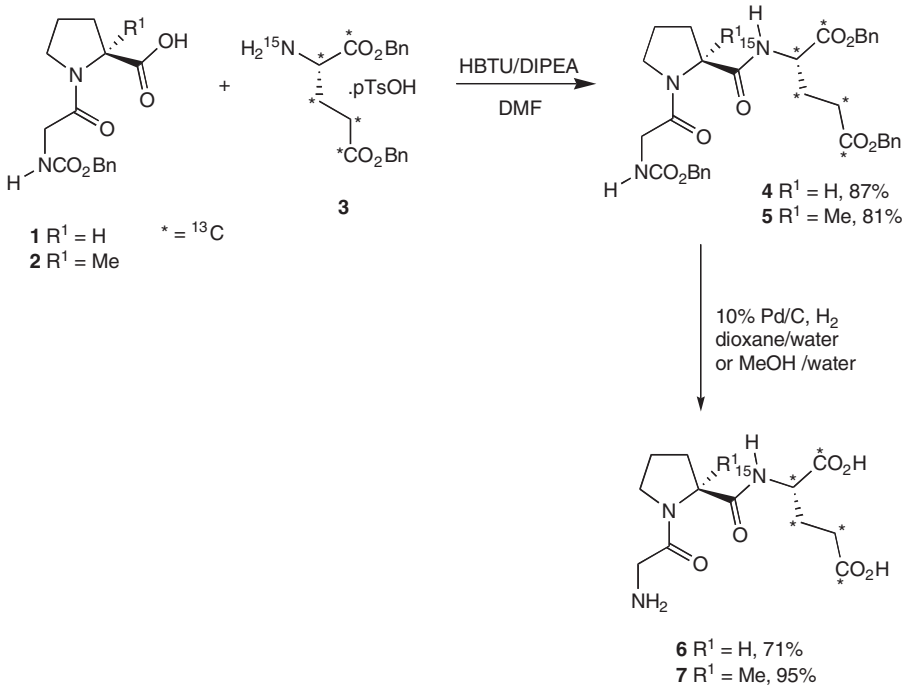

Scheme 2. Synthesis of [1,2,3,4,5-$^{13}C_5$,2-$^{15}N_1$] GPE and G-2-MePE

Coupling of the 2-methylproline derivative **2**[1] with dibenzyl glutamate **3** using the more powerful reagent HBTU afforded the protected tripeptide **5** in 81% yield and subsequent global deprotection afforded the 2-methyl GPE analogue **7** in excellent yield. No solubility problems were encountered which is reflected in the better yield obtained in the deprotection step for the labelled methylated tripeptide **7** (95%) compared to the non-methylated tripeptide **6** (71%).

With successful syntheses of isotopically labelled tripeptides in hand, attention next turned to the preparation of the labelled diketopiperazines **11a–c**. The minimum requirement for isotope labelling to avoid overlap with the mass spectrum umbrella of the non-labelled compound is 3 atomic mass units. In the preparation of the diketopiperazine isotope **11** we were restricted by the initial requirement to prepare a 2-alkylated proline which required 3 steps from L-proline. Use of appropriately labelled L-proline is expensive hence [2,2-$^{2}H_2$, 2-$^{15}N_1$]glycine was chosen as the labelled amino acid to provide the required additional 3 atomic mass units.

The preparation of [2,2-$^{2}H_2$, 2-$^{15}N_1$] *N-tert*-butyloxyglycine **9** has yet to be reported but was easily obtained in 96% yield as a 3:1 mixture of conformers about the carbamate bond.[7] No scrambling of the deuterium was observed under the reaction conditions used (pH > 8). Previously the coupling of **8** with

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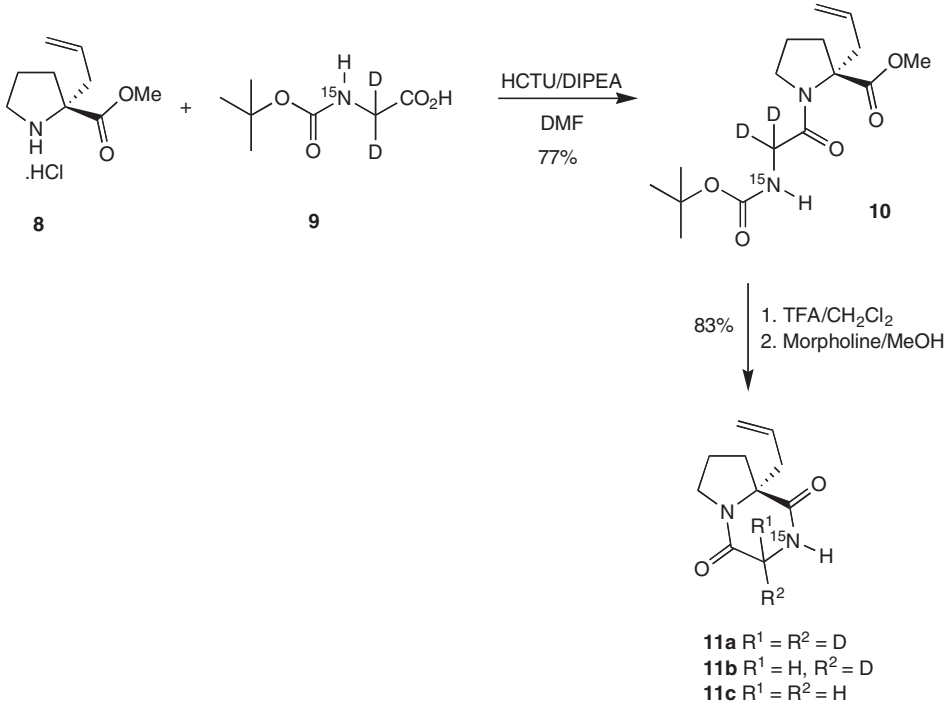
unlabelled *N*-*tert*-butyloxyglycine using BoP-Cl resulted in a low yield (45%) of the desired dipeptide.[1] HCTU[8] has recently been shown to be a superior reagent for hindered couplings; coupling of **8**[1] with **9** with HCTU gave the protected dipeptide **10** in a superior yield of 77% as a single conformer with high isotopic incorporation (Scheme 3).



Scheme 3. Synthesis of [2,2-$^{2}H_2$,2-$^{15}N_1$] cyclic dipeptide 11a

Removal of the Boc group with trifluoroacetic acid and subsequent neutralization with morpholine resulted in complete cyclization to the diketopiperazine **11a** in excellent yield with the correct mass $m/z = 198.1229$ (MH^+ $C_{10}H_{13}NO_2D_2^{15}N$ requires 198.1229). Morpholine was the base of choice for this transformation due to the high water solubility of its trifluoroacetate salt; the corresponding triethylamine salt was difficult to remove by either extraction or chromatography. Unfortunately under these conditions scrambling of the deuterium label occurred to the extent of 5–10% (^{1}H NMR analysis). The deuterium scrambling was clearly evident in the ^{1}H NMR spectrum with a doublet (^1J ^{15}N–H = 91 Hz) being observed for each of the di-deuterated, mono-deuterated and non-deuterated compounds. Mass spectrometric analysis of the mixture also supported this conclusion. For compound **11c** ($m/z = 196$, MH^+) which is ^{15}N labelled but does not contain deuterium, the MH^+ intensity was measured as 9%. The corresponding ^{13}C

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isotope ion should be observed at $m/z = 197$ with an intensity of 0.99% (based on 1.1% natural abundance). However, the actual ^{13}C isotope ion observed at $m/z = 197$ exhibited an intensity of 11% thus suggesting the presence of the mono-deuterated species **11b** ($m/z = 197$ expected for MH^+). This conclusion was confirmed by accurate mass measurements of the non-deuterated compound **11c** [196.1106 (MH^+ $C_{10}H_{15}NO_2^{15}N$ requires 196.1104)] and mono-deuterated compound **11b** [197.1165 (MH^+ $C_{10}H_{14}NO_2D^{15}N$ requires 197.1167)].

The observation of a small amount of isotope scrambling in the diketopiperazine (DKP) **11a** but not the uncyclized precursor **10** was not unexpected. In simple model systems exploring peptide racemization at 120°C and at pH = 8 Smith and Baum found that the α-CH of diketopiperazines racemized 2–7 times faster than the respective dipeptides[9] and the percentage of racemization was generally higher for the DKP (44% D isomer after 14 h) although this value was dependent on the exact nature of the amino acid sequence. In the present work mild experimental conditions were used perhaps accounting for the lower level of isotopic scrambling observed in our case.

Conclusion

In summary we have prepared ^{13}C and ^{15}N analogues of GPE **6** and G-2-MePE **7** in good overall yield by incorporation of dibenzyl protected [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]-L-glutamic acid using HBTU coupling followed by global deprotection. Preparation of a related cyclic diketopiperazine **11** via incorporation of *N*-Boc protected [2,2-$^{2}H_2$, 2-$^{15}N_1$]glycine and cyclization proceeded in excellent yield albeit with minor deuterium scrambling being observed (5–10%).

Experimental

General

[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]-L-glutamic acid (> 98% ^{13}C, > 98% ^{15}N) and [2,2-$^{2}H_2$, 2-$^{15}N_1$]glycine (> 97% ^{2}H, > 98% ^{15}N) were purchased from Spectra Stable Isotopes. General experimental details are described in a previous paper.[1] HBTU was obtained from Peptides International. HCTU was obtained from Luxembourg Industries Ltd. Positional assignments for NMR spectroscopy for di- and tripeptides follow Greek locants, i.e. α for C-2, β for C-3, etc. for the amino acid in question. When two sets of peaks are observed in the NMR spectrum due to *cis*/*trans* isomerism about the C(O)–NH bond, the minor isomer is labelled with an asterisk (*).

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[1,2,3,4,5-$^{13}C5$, 2-$^{15}N_1$]Dibenzyl L-*glutamate p-toluenesulfonate* **3**

A mixture of [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]-L-glutamic acid (0.3 g, 1.96 mmol), *p*-toluenesulfonic acid (0.410 g, 2.51 mmol) and benzyl alcohol (5 ml) was refluxed in benzene (15 ml) for 24 h with the removal of water. The clear solution was cooled to room temperature, concentrated to half volume and dry ether (30 ml) was added resulting in a white precipitate. The mixture was stored at 4°C for 2 h, and the product collected by filtration, washed with ether and dried *in vacuo* to afford the title compound (0.825 g, 83%) as a white powder: mp 135–140°C; $[\alpha]_D$ −19.5 ($c = 0.077$ in dichloromethane); ^{1}H NMR (400 MHz; $CDCl_3$) $\delta = 2.05$ (1H, br s), 2.01–2.45 (5H, m), 2.50–2.75 (1H, br m), 4.17 (1H, d, $J = 147$ Hz, Gluα-H), 4.94 (1H, dd, $J = 12.4$ and 3.1 Hz, OCH_AH$_B$Ar), 4.98 (1H, dd, $J = 12.4$ and 3.0 Hz, OCH$_A H_B$Ar), 5.01 (1H, dd, $J = 12.2$ and 3.0 Hz, OCH_AH$_B$Ar), 5.08 (1H, dd, $J = 12.3$ and 3.0 Hz, OCH$_A H_B$Ar), 7.0 (2H, d, $J = 8.0$ Hz), 7.23–7.32 (10H, m) 7.75 (2H, d, $J = 8.1$ Hz), 8.40 (3H, br d, $J = 68$ Hz). ^{13}C NMR (100 MHz; $CDCl_3$) $\delta =$ 21.3 (CH_3, Ar-*C*H$_3$), 25.1 (CH_2, t, $J = 35$ Hz, Gluβ-C), 29.3 (CH_2, dd, $J =$ 59.6 and 35.7 Hz, Gluγ-C), 52.3 (CH, qq, $J = 63.8$, 33.9 and 3.8 Hz, Gluα-C), 66.4 (CH_2, O*C*H$_2$Ar), 67.9 (CH_2, O*C*H$_2$Ar), 126.1 (CH, Ar), 128.1 (CH, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 128.45 (CH, Ar), 128.29 (CH, Ar), 128.9 (CH, Ar), 134.6 (quat., d, $J = 1.71$ Hz, Ar), 135.7 (quat., d, $J = 2.31$ Hz, Ar), 140.3 (quat., Ar), 141.2 (quat., Ar), 168.8 (quat., d, $J = 61.3$ Hz, Glu-CO), 172.0 (quat., dd, $J = 57.3$ and 3.0 Hz, Glu-CO). m/z (FAB+) 506.1889 (MH^+ $C_{21}H_{30}O_7S^{13}C_5^{15}N$ requires 506.1881).

*Dibenzyl N-benzyloxycarbonylglycyl-*L*-prolyl-*L*-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]glutamate* **4**

To a solution of *N*-benzyloxycarbonyl-glycyl-L-proline (0.138 g, 0.451 mmol), [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]dibenzyl L-glutamate *p*-toluenesulfonate (0.228 g, 0.451 mmol) and HBTU (0.171 g, 0.451 mmol) in DMF (3 ml) were added DIPEA (0.174 g, 1.35 mmol) in one portion and stirred overnight. The solution was poured into 2 M HCl (25 ml), extracted with ethyl acetate (25 ml) and the organic layer washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by flash chromatography (silica gel, hexane:ethyl acetate, 2:3, 1:2, 1:3, 1:4) and trituration from ethyl acetate afforded the title compound (0.244 g, 87%): mp 122–123°C; $[\alpha]_D$ −51.9 ($c = 0.081$ in dichloromethane); ^{1}H NMR (400 MHz; $CDCl_3$) $\delta = 1.76$–2.7 (8H, m, Gluβ-H_2, Gluγ-H_2, Proβ-H_2, Proγ-H_2), 3.40 (0.82H, q, $J = 9.4$ Hz, Proδ-H_AH$_B$), 3.46–3.54 (0.82H, m, Proδ-H$_A H_B$), 3.59–3.66* (0.18H, m, Proδ-H_AH$_B$), 3.69–3.73* (0.18H, m, Proδ-H$_A H_B$), 3.86* (0.18H, dd, $J = 16.8$ and 4.2 Hz, Glyα-H_AH$_B$), 3.94 (1.09H, app. dd, $J = 17.1$ and 3.9 Hz, Glyα-H_AH$_B$, Glyα-H$_A H_B$*, 0.5 Gluα-H*), 4.12 (0.82H, dd, $J = 17.1$ and 3.9 Hz,

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Glyα-H_AH_B) 4.31 (0.09H, m, 0.5 Gluα-H$*$), 5.0–5.22 (6H, m, $3 \times OCH_2Ar$), 5.60$*$ (0.18H, br s, GlyN–H), 5.73 (0.82H, br s, GlyN–H), 7.17 (0.5H, d, $J = 7.2$ Hz, Pro-NH, ^1J N–H obscured), 7.34–7.40 (15.5H, m, Ar, Pro-NH). ^{13}C NMR (100 MHz; $CDCl_3$) $\delta = 24.8$ (CH_2, Proγ-C), 25.9$*$ (CH_2, t, $J = 35.7$ Hz, Gluβ-C), 26.8 (CH_2, t, $J = 35.5$ Hz, Gluβ-C), 27.8 (CH_2, Proβ-C), 30.0 (CH_2, dd, $J = 59.3$ and 35.2 Hz, Gluγ-C), 43.4 (CH_2, Glyα-C), 46.3 (CH_2, Proγ-C), 51.8 (CH, dddd, $J = 63.0$, 34.9, 9.15, 2.5, Gluα-C), 60.1 (CH, d, $J = 8.9$ Hz, Proα-C), 66.5 (CH_2, OCH_2Ar), 66.9 (CH_2, OCH_2Ar), 67.3 (CH_2, OCH_2Ar), 128.03 (CH, Ar), 128.1 (CH, Ar), 128.18 (CH, Ar), 128.28 (CH, Ar), 128.45 (CH, Ar), 128.49 (CH, Ar), 128.54 (CH, Ar), 128.59 (CH, Ar), 135.18 (quat., Ar), 135.7, (quat., Ar), 136.3 (quat., Ar), 156.2 (quat., NCO_2), 168.1 (quat., Gly-CO), 171.1$*$ (quat., d, $J = 58$ Hz, Glu-CO), 171.3 (quat., d, $J = 60$ Hz, Glu-CO), 172.7 (quat., dd, $J = 57$ and 2 Hz, Glu-CO (minor isomer partially obscured). m/z (FAB+) 622.2820 (MH^+ $C_{29}H_{38}N_2O_8^{13}C_5^{15}N$ requires 622.2797).

*Glycyl-*L*-prolyl-*L*-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]glutamic acid* **6**

To a solution of dibenzyl *N*-benzyloxycarbonylglycyl-L-prolyl-L-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]glutamate (0.818 g, 1.31 mmol) in a mixture of dioxane:water (5:1, 60 ml) was added 10% Pd/C (140 mg) under an atmosphere of nitrogen. A balloon of hydrogen was fitted, the mixture stirred overnight, filtered through Celite and washed with 1:1 H_2O:dioxane (100 ml) then 1:1 H_2O:methanol (200 ml). Concentration of the solution *in vacuo* afforded the crude product (0.39 g) that was triturated from cold methanol, filtered, washed with dry ether and dried *in vacuo* to afford the title compound (0.287 g, 71%) as a white powder: mp 212–214°C (dec.); $[\alpha]_D$ −82.0 ($c = 0.075$ in water); ^{1}H NMR (400 MHz; D_2O) $\delta = 1.77$–2.32 (7H, m), 2.5–2.65 (1H, m), 3.52–3.64 (2H, m, Proδ-H_2), 3.47$*$ (0.15H, d, $J = 16.2$, Glyα-H_AH_B), 3.93–4.04 (2.28H, m, Glyα-H_AH_B*, Glyα-H_2, 0.5 Gluα-H), 4.32–4.42 (0.5H, Gluα-H), 4.47–4.50 (1H, m, Proα-H). ^{13}C NMR (100 MHz; D_2O) $\delta = 24.1$ (CH_2, Proγ-C), 26.4$*$ (CH_2, t, $J = 35.4$ Hz, Gluβ-C), 26.8 (CH_2, t, $J = 35.2$ Hz, Gluβ-C), 29.3 (CH_2, Proβ-C), 30.0 (CH_2, ddd, $J = 55.4$, 35.2 and 2.2 Hz, Gluγ-C), 31.8$*$ (CH_2, d, $J = 35.2$, Gluγ-C, minor isomer partially obscured/unresolved), 40.3 (CH_2, Glyα-C), 46.8 (CH_2, Proγ-C), 54.15 (CH, dddd, $J = 56.7$, 34.9, 10.6, 3.3, Gluα-C), 60.6 (CH, d, $J = 7.8$ Hz, Proα-C), 165.7 (quat., Gly-CO), 177.3 (quat., dd, $J = 55$ and 3.0 Hz, Glu-CO), 178.5 (quat., dd, $J = 53$ and 3 Hz, Glu-CO (minor isomer partially obscured/unresolved). m/z (FAB+) 308.1503 (MH^+ $C_7H_{20}N_2O_6^{13}C_5^{15}N$ requires 308.1490).

*Dibenzyl N-benzyloxycarbonylglycyl-*L*-2-methylprolyl-*L*-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$] glutamate* **5**

To a solution of *N*-benzyloxycarbonyl-glycyl-L-2-methylproline (0.425 g, 1.33 mmol), [1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]dibenzyl L-glutamate *p*-toluenesulfonate

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(0.670 g, 1.33 mmol) and HBTU (0.503 g, 1.33 mmol) in DMF (5 ml) was added DIPEA (0.514 g, 3.98 mmol) in one portion and stirred overnight. The solution was poured into 2 M HCl (25 ml), extracted with ethyl acetate (25 ml) and the organic layer washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by flash chromatography (silica gel, hexane:ethyl acetate, 1:1, 2:3, 1:2) afforded the title compound (0.683 g, 81%) as a yellow oil: $[\alpha]_D$ −43.5 ($c = 0.77$ in dichloromethane); ^{1}H NMR (400 MHz; $CDCl_3$) $\delta = 1.66$ (3H, s, Proα-CH_3), 1.72–1.79 (1H, m), 1.91–1.95 (2H, m), 2.28–2.46 (4H, m), 2.62 (1H, br s), 3.45–3.59 (2H, m, Proδ-H_2), 3.94 (dd, $J = 17.3$ and 3.7 Hz, Glyα-H_AH_B), 4.01 (dd, $J = 17.2$ and 5.1 Hz, Glyα-H_AH_B), 4.57 (1H, d, $J = 142$ Hz, Gluα-H), 5.09–5.21 (6H, m, $3 \times OCH_2Ar$), 5.71 (br s, 0.12H, GlyN–H), 5.84 (br s, 0.88H, GlyN–H), 7.25–7.45 (15.5H, m, Ar and ProN–H), 7.59 (0.5H, d, $J = 6.7$ Hz, ProN–H). ^{13}C NMR (100 MHz; $CDCl_3$) $\delta = 21.8$ (CH_3, Proα-CH_3), 23.5 (CH_2, Proγ-C), 26.5 (CH_2, t, $J = 35.4$ Hz, Gluβ-C), 30.0 (CH_2, ddd, $J = 57.5$, 35.9 and 2.21 Hz, Gluγ-C), 38.4 (CH_2, Proβ-C), 43.9 (CH_2, Glyα-C), 47.7 (CH_2, Proγ-C), 51.8 (CH, dddd, $J = 61$, 35, 9.2, and 2.7, Gluα-C), 66.5 (CH_2, d, $J = 2.1$, OCH_2Ar), 66.8 (CH_2, OCH_2Ar), 67.3 (CH_2, OCH_2Ar), 68.2 (CH, d, $J = 7.9$ Hz, Proα-C), 127.98 (CH, Ar), 128.5 (CH, Ar), 128.17 (CH, Ar), 128.26 (CH, Ar), 128.42 (CH, Ar), 128.47 (CH, Ar), 128.55 (CH, Ar), 128.57 (CH, Ar), 135.2 (quat., d, $J = 2.0$ Hz, Ar), 135.6, (quat., d, $J = 2$ Hz, Ar), 136.4 (quat., Ar), 156.3 (quat., NCO_2), 167.6 (quat., Gly-CO), 171.6 (quat., dd, $J = 61$ and 2.0 Hz, Glu-CO), 173.1 (quat., dd, $J = 57$ and 2 Hz, Glu-CO). m/z (FAB+) 636.2971 (MH^+ $C_{30}H_{40}N_2O_8^{13}C_5^{15}N$ requires 636.2953.

*Glycyl-*L*-2-methylprolyl-*L*-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]glutamic acid* **7**

To a solution of dibenzyl *N*-benzyloxycarbonylglycyl-L-2-methylprolyl-L-[1,2,3,4,5-$^{13}C_5$, 2-$^{15}N_1$]glutamate (0.683 g, 1.08 mmol) in a mixture of methanol:water (9:2, 11 ml) was added 10% Pd/C (115 mg) under an atmosphere of nitrogen. A balloon of hydrogen was fitted, the mixture stirred overnight, filtered through Celite and washed with 1:4 H_2O:methanol (200 ml). Concentration of the solution *in vacuo* afforded the crude product (0.4 g) that was triturated with dry ether and dried *in vacuo* to afford the title compound (0.33 g, 95%) as a white powder†: $[\alpha]_D$ −60.5 ($c = 0.063$ in water); ^{1}H NMR (400 MHz; D_2O) $\delta = 1.59$ (3H, s, Proα-CH_3), 1.81 (0.5H, br s), 2.0–2.31 (6.5H, m), 2.52–2.62 (1H, m), 3.65 (2H, m, Proδ-H_2), 3.93 (1H, d, $J = 16.5$, Glyα-H_AH_B), 3.98 (1H, d, $J = 16.5$, Glyα-H_AH_B), 4.05–4.1 (0.5H, m, Gluα-H), 4.38–4.45 (0.5H, m, Gluα-H). ^{13}C NMR (100 MHz; D_2O) $\delta = 19.8$ (CH_3, Proα-CH_3), 23.0 (CH_2, Proγ-C), 26.8 (CH_2, t, $J = 35.3$ Hz, Gluβ-C), 30.0

†The hygroscopic nature of this compound precluded the recording of a true melting point.

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(CH_2, ddd, $J = 53.7$, 35.6 and 2.1 Hz, Gluγ-C), 38.8 (CH_2, Proβ-C), 40.7 (CH_2, Glyα-C), 47.5 (CH_2, Proγ-C), 54.3 (CH, dddd, $J = 55.0$, 34.9, 11.0, 2.9, Gluα-C), 60.6 (CH, d, $J = 7.2$ Hz, Proα-C), 164.6 (quat., Gly-CO), 177.3 (quat., dd, $J = 55$ and 3.1 Hz, Glu-CO), 178.5 (quat., dd, $J = 53$ and 3 Hz, Glu-CO. m/z (FAB+) 322.1647 (MH^+ $C_8H_{22}N_2O_6^{13}C_5^{15}N$ requires 322.1647).

[2,2-$^2H_2$, 2-$^{15}N_1$] N-tert-Butyloxycarbonylglycine **9**

Prepared according to the procedure as described in Keller *et al.*[7] to afford a white crystalline solid (96%): mp 82–85°C; ^{1}H NMR (400 MHz; $CDCl_3$) $\delta =$ 1.44 [9H, s, C(CH_3)$_3$], 5.33 (0.64H, d, $J = 94$ Hz, N–H), 6.62* (0.36H, d, $J = 93$ Hz, N–H), 10.37 (br s, OH). ^{13}C NMR (150 MHz, $CDCl_3$) 28.08* [CH_3, C(CH_3)$_3$], 28.19 [CH_3, C(CH_3)$_3$], 41.2–42.0 (CD_2, m), 42.4–43.0* (CD_2, m), 80.3 [quat., C(CH_3)$_3$],), 81.7* [quat., C(CH_3)$_3$], 156.12 (quat., d, $J = 27$ Hz, NCO_2), 157.28* (quat., d, $J = 27$ Hz, NCO_2), 173.9 (quat., CO), 174.3* (quat., CO). m/z (FAB+) 179.1019 (MH^+ $C_7H_{12}O_4D_2^{15}N$ requires 179.1019).

*[2,2-$^2H_2$, 2-$^{15}N_1$] Methyl N-tert-Butyloxycarbonylglycyl-*L*-2-allylprolinate* **10**

To a solution of methyl L-2-allylprolinate (0.286 g, 1.61 mmol) [2,2-$^2H_2$, 2-$^{15}N_1$] *N-tert*-butyloxycarbonylglycine (0.328 g, 1.61 mmol) and HCTU (0.664 g, 1.61 mmol) in DMF (8 ml) was added DIPEA (0.623 g, 4.82 mmol) in one portion and stirred overnight. The solution was poured into 2 M HCl (20 ml), extracted with ethyl acetate (20 ml) and the organic layer washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), filtered and concentrated *in vacuo*. Purification by flash chromatography (silica gel, hexane:ethyl acetate, 2:1, 1:1) afforded the title compound (0.401 g, 77%) as a yellow oil: $[\alpha]_D + 45.7$ ($c = 0.29$ in dichloromethane); ^{1}H NMR (400 MHz; $CDCl_3$) $\delta = 1.46$ [9H, s, C(CH_3)$_3$], 1.95–2.17 (4H, m, Proβ-H_2 and Proγ-H_2), 2.67 (1H, dd, $J = 14.1$ and 8.0 Hz, $CH_ACH_BCH=CH_2$), 3.16 (1H, dd, $J = 14.1$ and 6.8 Hz, $CH_ACH_BCH=CH_2$), 3.40–3.46 (1H, m, Proδ-H_AH_B), 3.59–3.64 (1H, m, Proδ-H_AH_B), 3.78 (3H, s, OCH_3), 5.10–5.14 (2H, m, CH=CH_2), 5.43 (1H, d, $J = 92$ Hz, N–H), 5.63–5.73 (1H, m, CH=CH_2). ^{13}C NMR (150 MHz; $CDCl_3$) 23.5 (CH_2, Proγ-C), 28.1 [CH_3, C(CH_3)$_3$], 34.8 (CH_2, Proβ-C), 37.5 (CH_2, CH_2CH=CH_2), 42.2–43.1 (CH_2, m, Glyα-CD_2), 47.4 (CH_2, Proδ-C), 52.3 (CH_3, OCH_3), 68.6 (quat., Proα-C), 79.3 [quat., C(CH_3)$_3$], 119.3 (CH_2, CH=CH_2), 132.7 (CH, CH=CH_2), 155.6 (quat., d, $J = 25.6$ Hz, NCO_2), 166.8 (quat., Gly-CO) and 173.7 (quat., Pro-CO). m/z (EI+) 329.1934 (M^+ $C_{16}H_{24}NO_5D_2^{15}N$ requires 329.1937).

[2,2-$^2H_2$, 2-$^{15}N_1$] (8aR)-Allylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **11a**

[2,2-$^2H_2$, 2-$^{15}N_1$] Methyl *N-tert*-butyloxycarbonylglycyl-L-2-allylprolinate (0.4 g, 1.21 mmol) was stirred in a mixture of trifluoroacetic acid:

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dichloromethane (1:1, 8 ml) for 2 h and concentrated *in vacuo*. The oily residue was suspended in benzene and concentrated to remove traces of trifluoroacetic acid, re-dissolved in methanol (4 ml) and morpholine (0.423 g, 4.86 mmol) was added with the generation of white fumes. After stirring overnight and removal of the volatiles *in vacuo* the residue was dissolved in chloroform (10 ml), and washed with 0.2 M HCl. The aqueous layer was extracted with chloroform (10 ml portions) until no product remained in the aqueous layer by tlc analysis (4 ×). The combined organic layers were dried, filtered, concentrated and purified by flash chromatography (silica gel, 5% MeOH–EtOAc then 10% MeOH–EtOAc) to afford the title compound (0.198 g, 83%) as an off-white solid: mp 116–118°C; $[\alpha]_D + 117.3$ ($c = 0.13$ in dichloromethane); ^{1}H NMR (400 MHz; $CDCl_3$) 1.95–2.05 (2H, m, Proγ-H_2), 2.15–2.20 (2H, m, Proβ-H_2), 2.45 (1H, dd, $J = 13.8$ and 7.6 Hz, $CH_ACH_BCH=CH_2$), 2.57 (1H, dd, $J = 13.8$ and 7.5 Hz, $CH_ACH_BCH=CH_2$), 3.49–3.56 (1H, m, Proδ-H_AH_B), 3.81–3.88 (1.12H, m, Proδ-H_AH_B and §Gluα-H_AH_B), 4.11§ (0.12H, d, $J = 17$ Hz, Gluα-H_AH_B), 5.19–5.23 (2H, m, $CH=CH_2$), 5.76–5.87 (1H, m, $CH=CH_2$), 6.98 (1H, d, $J = 90$ Hz N–H), 6.99§ (d, $J = 91$ Hz, N–H), 7.0¶ (d, $J = 91$ Hz, N–H). ^{13}C NMR (150 MHz; $CDCl_3$) 20.1 (CH_2, Proγ-C), 20.2§ (CH_2, Proγ-C), 34.1 (CH_2, Proβ-C), 41.7 (CH_2, $CH_2CH=CH_2$), 44.9 (CH_2, Proδ-C), 44.5–46.4 (CD_2, m, Glyα-CD_2), 46.45§ (CH_2, d, $J = 9$ Hz, Glyα-CH_2), 67.25 (quat., d, $J = 7.5$ Hz, Proα-C), 120.9 (CH_2, $CH=CH_2$), 131.0 (CH, $CH=CH_2$), 163.4 (quat., Gly-CO) and 173.7 (quat., d, $J = 15$ Hz, Pro-CO). m/z (FAB+) 198.1229 (MH^+ $C_{10}H_{13}NO_2D_2^{15}N$ requires 198.1229).

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§Assigned as non-deuterated **11c** m/z (FAB+) 196.1106 (MH^+ $C_{10}H_{15}NO_2^{15}N$ requires 196.1104).
¶Assigned as mono-deuterated **11b** m/z (FAB+) 197.1165 (MH^+ $C_{10}H_{14}NO_2D^{15}N$ requires 197.1167).

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