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Synthesis and neuroprotective activity of analogues of glycyl-L-prolyl-L-glutamic acid (GPE) modified at the α-carboxylic acid

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Abstract—The synthesis of nine GPE* analogues, wherein the α-carboxylic acid group of glutamic acid has been modified, is described by coupling readily accessible *N*-benzyloxycarbonyl-glycyl-L-proline **2** with various analogues of glutamic acid. Pharmacological evaluation of the novel compounds was undertaken to further understand the role of the glutamate residue on the observed neuroprotective properties of the endogenous tripeptide GPE.
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1. Introduction

Insulin-like growth factor-1 (IGF-1) is broadly distributed within the mammalian central nervous system[1] (CNS) and is a potent neurotrophic factor.[2,3] In experimental animal models, IGF-1, produced endogenously in damaged regions of the brain, has potent antiapoptotic effects on neurons and glial cells and is postulated to restrict progressive cell death.[4–9] When administered following hypoxic-ischemic (HI) brain injury, IGF-1 reduced neuronal loss and cortical infarction.[10–14]

Much of the endogenous IGF-1 in the CNS is thought to be proteolytically cleaved into des-*N*(1–3)-IGF-1, a truncated IGF-1 comprised of 67 amino acids, and the *N*-terminal tripeptide Gly-Pro-Glu-OH (GPE **1**) (Scheme 3).[15–21] There is, however, no direct evidence that GPE is naturally present or produced in the brain.[22] Although des-*N*(1–3)-IGF-1 is more neurotrophically potent than its precursor IGF-1,[23–25] and both exhibit similar neuroprotective actions in rats,[12] the former is a less potent protective agent in the treatment of post-HI brain injury. This reduced efficacy can be attributed to the significantly lower affinity of des-*N*(1–3)-IGF-1 for IGF binding proteins,[10,12,26,27] hypothesised to transport IGF-1 to the injured region for neuronal rescue.[12,28] Together with the neuroprotective actions of the aforementioned des-*N*(1–3)-IGF-1, binding proteins and the IGF-1 receptor,[24,27,29,30] IGF-1 is believed to function as a prohormone for GPE **1**, which acts on a yet unknown receptor.[31–33] GPE **1** is not known to modulate or cross-react with the brain's IGF-1 receptor.[15]

GPE **1** was observed to act as a neuronal rescue factor following transient HI brain injury for cerebral cortical, striatal and hippocampal neurons,[34] with peripheral activity, relative to local administration, producing wider neuroprotective effects.[33] Although the precise mechanism of action of GPE **1** remains obscure, it exhibits preferential binding to glia upon local injection, potentially representing a complementary conduit for central and systemic IGF-1's antiapoptotic effects.[33] In vitro studies by Sara and co-workers[15,31] indicated that GPE **1** potentiated the K^+-stimulated release of dopamine and acetylcholine through the *N*-methyl-D-aspartate (NMDA) receptor system and a nonelucidated mechanism, respectively.

In addition, some preliminary observations suggest that GPE **1** has a neuromodulatory role in the CNS, possibly through interaction with glutamate receptors.[15,16,21,31,32,34–37] L-Glutamate, being the main

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excitatory amino acid neurotransmitter, functions through a heterogeneous family of two major receptor types—the ionotropic (iGluRs)[38,39] and metabotropic (mGluRs)[40–42] glutamate receptors.[43–46] Malfunctioning of the glutaminergic system may be involved in psychiatric and neurodegenerative brain disorders, with glutamate accumulation after brain injury having a prominent role in developing excitotoxic neuronal death.[47–51] Thus the glutamate receptors provide an excellent target for rational design of neuroprotective agents.[52–54]

Based on these scientific findings, it has been proposed that GPE **1**, or analogues thereof, may provide a novel class of pharmaceutical agents for the treatment of CNS injuries; neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's Diseases; multiple sclerosis and general ageing-induced cognitive dysfunction.[55–58] Analogues of GPE should be low molecular weight and must be lipophilic to cross the blood–brain barrier. The ideal synthetic analogue of GPE **1** would result in an enhanced pharmacokinetic profile, and, depending on the nature of the modification, lead to improved metabolic stability and increased oral bioavailability. Ultimately, this would lead to less intrusive routes of administration.

We herein report the preparation and pharmacological evaluation of a variety of analogues of general structure Gly-Pro-Glu-OH (GPE*), in which the α-position of Glu (E*) has been modified. We also report the development of a mixed neuronal, astrocytic brain cell culture (modified method from Chen et al.[59]) deriving from the striatum, to gain an efficient first, in vitro screening tool of these GPE analogue compounds. This work is part of a structure–activity relationship study to determine the significance of the Glu residue of this tripeptide on neuroprotective activity, greatly aiding the selection of suitable candidates for continued drug development.

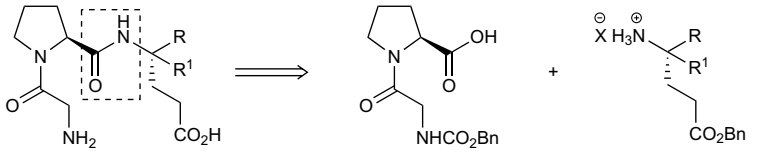
2. Results and discussion

In order to ascertain the importance of the α-carboxylic acid functionality on the glutamate residue of GPE, nine novel analogues were synthesised and evaluated for their neuroprotective effects. The synthetic strategy employed involved preparation of known *N*-benzyloxycarbonyl-glycyl-L-proline[60,61] **2**, and effecting peptide bond construction with several modified benzyl-protected glutamic acid residues (Scheme 1).

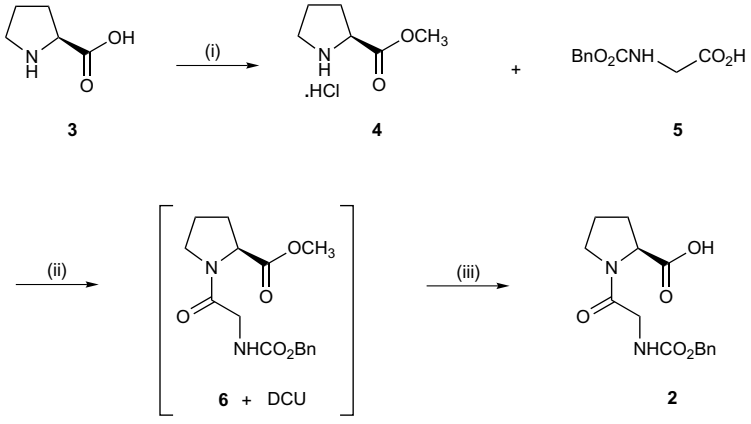
After assembly of the new pseudo-peptide bond, a simple deprotection procedure furnishes the desired GPE* analogues.

The synthesis of *N*-CBz peptide[60,61] **2** was achieved in 82% yield over three steps from readily available L-proline **3** (Scheme 2). Thus, treatment of **3** with thionyl chloride in methanol gave quantitatively hydrochloride salt[62,63] **4**, which was subsequently coupled with commercial *N*-benzyloxycarbonylglycine **5**, using 1,3-dicyclohexylcarbodiimide (DCC) to realise protected dipeptide[64] **6**. Finally, saponification of the methyl ester afforded acid **2**.

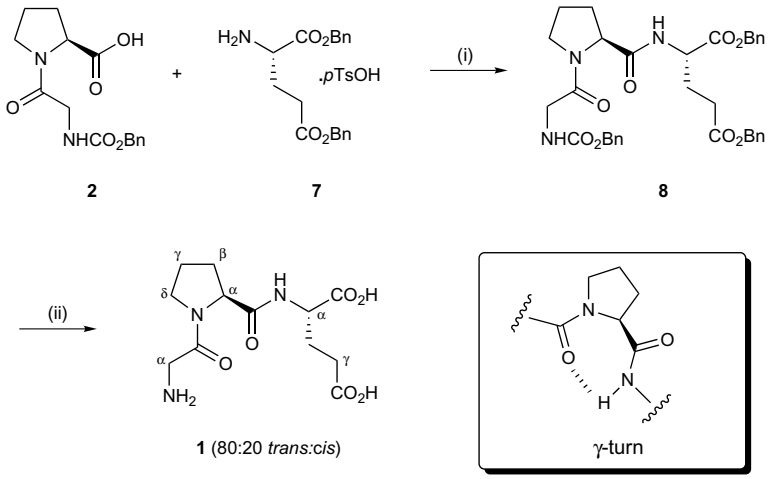
An initial model study was carried out to assess the reliability of the key proline–glutamate bond forming step



Scheme 1. General retrosynthesis for GPE* analogues.



Scheme 2. Reagents and conditions: (i) $SOCl_2$, MeOH, −5 °C to rt, N_2, 19 h (98%); (ii) Et_3N, 1,3-dicyclohexylcarbodiimide (DCC), CH_2Cl_2, 0 °C to rt, N_2, 18 h; (iii) 1 M NaOH (aq), 1,4-dioxane, rt, 21 h (82% in two steps from **4**).



Scheme 3. Reagents and conditions: (i) Et_3N, EtOCOCl, CH_2Cl_2, N_2, 0 °C to rt, 17 h (100%); (ii) H_2, 10% Pd/C, 80:20 MeOH–H_2O, rt, 18 h (94%).

by first preparing GPE **1** itself using a mixed anhydride protocol.[65–67] Reaction of acid **2** with ethyl chloroformate under basic conditions generated the mixed anhydride in situ, which was then treated with dibenzyl glutamate **7** to provide perbenzylated tripeptide **8** as a single optical isomer in 100% yield. The final step in the sequence involved hydrogenolysis of amide **8** over activated palladium on charcoal to afford glycyl-L-prolyl-L-glutamic acid[15] (GPE) **1** after trituration from diethyl ether (Scheme 3). The deprotection step was performed in a mixture of water and methanol in order to solubilise the product as it formed.

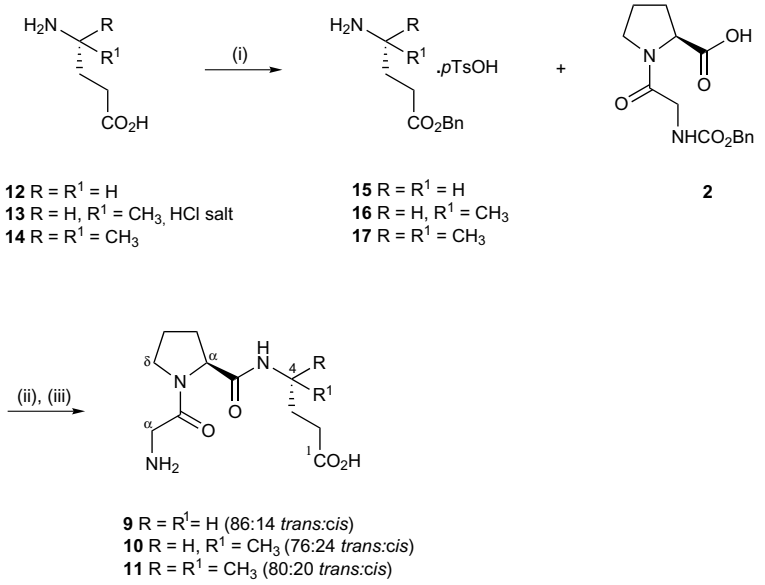
In a deuterium oxide solution, GPE **1** existed as an 80:20 *trans:cis* mixture of the two proline–glycine peptide bond-rotamers. The major conformer was assigned as having the *trans*-configuration, or γ-turn, by comparison of the differences in the ^{13}C NMR chemical shifts observed for the β and γ proline carbons of the major and minor isomers. With small acyclic or cyclic peptides the γ-turn functions to facilitate stabilising hydrogen bonding between the first and third amino acids in the sequence.[68] In a review by Kessler[69] on cyclic peptide conformations, it is stated that generally there is an approximate 10 ppm difference between the C-β and C-γ proline resonances for the *cis* conformation but only 5 ppm for the alternative *trans* form. In our case, the two major signals were separated by 5.1 ppm thereby establishing the *trans* conformation. This assignment was supported by a strong nuclear Overhauser effect (NOE) observed between the glycine-α and proline-δ protons.

Having successfully prepared GPE **1** our attention then focused on the synthesis of various GPE* analogues using a similar strategy. The first set of three analogues **9**, **10** and **11** involved replacement of the α-carboxylic acid group by a hydrogen, a methyl group and a geminal dimethyl group, starting from the modified glutamic acids **12**, **13**[70] and **14**,[71] respectively (Scheme 4). Elimination of the carboxylic acid in this fashion removes the amino acid character from the glutamate residue of GPE **1**, potentially suppressing the action of proteases, thereby imparting improved metabolic stability. Additionally, the overall polarity of the molecule is reduced, thus increasing lipophilicity and membrane permeability of the analogue.

The commercial amino acid **12** and variants **13**[70] and **14**,[71] as synthesised according to literature procedures, were initially protected as benzyl esters **15**, **16** and **17**, respectively, by heating with benzyl alcohol and *p*-toluenesulfonic acid in benzene. Coupling of the aforementioned acid **2** with amino ester **15** using the mixed anhydride method described above gave an amide that underwent hydrogenolysis to afford analogue **9** as an 86:14 *trans:cis* mixture of rotamers in 80% yield over the two steps. Similarly, subjection of the pseudo-glutamate residues **16** and **17** to a peptide coupling reaction with acid **2**, followed by deprotection, afforded GPE* analogues **10** and **11**, respectively.

Another strategy for reducing the overall charge in the GPE molecule whilst maintaining the steric bulk of the glutamate α-position involves replacement of the acid with an amide moiety. To this end, preparation of analogues **18**, **19** and **20,** with increasing degrees of amide substitution, were investigated (Scheme 5).

The syntheses of all three analogues **18**, **19** and **20** started from Boc-protected benzyl glutamic acid **21**.[72] Reaction of acid **21** with di-*tert*-butyl dicarbonate and ammonolysis of the intermediate anhydride with ammonium hydrogen carbonate under basic conditions yielded primary amide **22**[73] in 98% yield. Subsequent trifluoroacetic acid-mediated carbamate cleavage quantitatively afforded trifluoroacetate salt **25**. Treatment of this salt **25** with acid **2** was carried out using bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BoPCl) to provide amide **28** in good yield. The successful employment of BoPCl as the coupling agent to effect peptide bond formation, without concomitant isomerisation of either of the two stereogenic centres, provided an alternative to the previously used mixed anhydride protocol. Hydrogenation of the fully protected pseudo-tripeptide **28** under standard conditions gave analogue **18**[56] (65%),



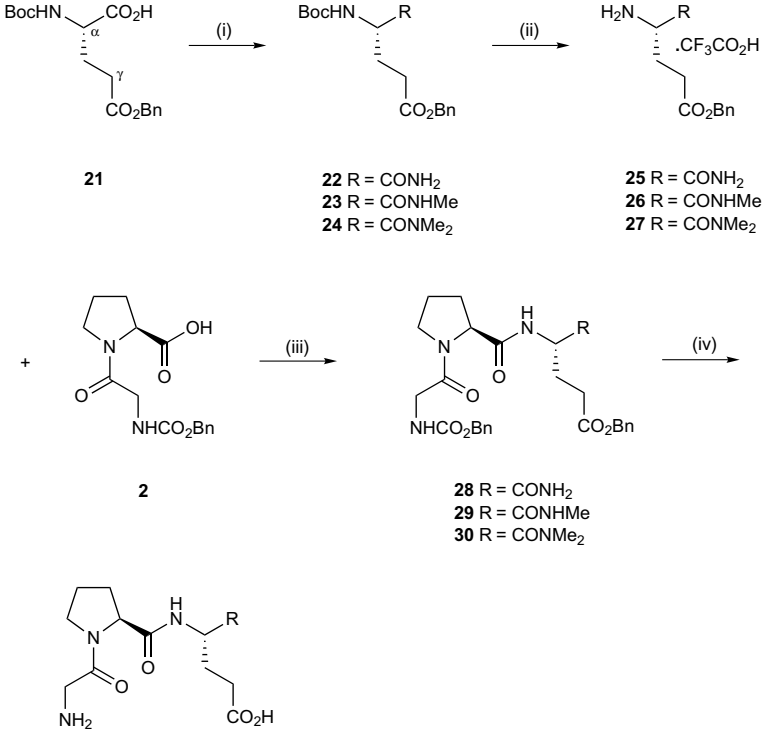
12 R = R^1 = H
13 R = H, R^1 = CH_3, HCl salt
14 R = R^1 = CH_3

15 R = R^1 = H
16 R = H, R^1 = CH_3
17 R = R^1 = CH_3

2

9 R = R^1= H (86:14 *trans*:*cis*)
10 R = H, R^1 = CH_3 (76:24 *trans*:*cis*)
11 R = R^1 = CH_3 (80:20 *trans*:*cis*)

Scheme 4. Reagents and conditions: For **9**: (i) *p*TsOH·H_2O, benzene, 80 °C, 68 h (86%); (ii) Et_3N, EtOCOCl, CH_2Cl_2, 0 °C to rt, N_2, 17 h (86%); (iii) H_2, 10% Pd/C, 80:20 MeOH–H_2O, 16 h (97%). For **10**: (i) ion exchange resin then benzyl alcohol, *p*TsOH·H_2O, benzene, 80 °C, 19 h (86%); (ii) Et_3N, EtOCOCl, CH_2Cl_2, 0 °C to rt, N_2, 17 h (80%); (iii) H_2, 10% Pd/C, MeOH, 20 h (93%). For **11**: (i) benzyl alcohol, *p*TsOH·H_2O, benzene, 80 °C, 43 h (65%); (ii) Et_3N, EtOCOCl, CH_2Cl_2, 0 °C to rt, N_2, 18 h (93%); (iii) H_2, 10% Pd/C, 1.6:1.3:1.0 THF–MeOH–H_2O, rt, 20 h (89%).



21

22 R = $CONH_2$
23 R = CONHMe
24 R = $CONMe_2$

25 R = $CONH_2$
26 R = CONHMe
27 R = $CONMe_2$

2

28 R = $CONH_2$
29 R = CONHMe
30 R = $CONMe_2$

18 R = $CONH_2$ (87:13 *trans*:*cis*)
19 R = CONHMe (83:17 *trans*:*cis*)
20 R = $CONMe_2$ (84:16 *trans*:*cis*)

Scheme 5. Reagents and conditions: For **18**: (i) $[(CH_3)_3COC(O)]_2O$, NH_4HCO_3, pyridine, 1,4-dioxane, N_2, rt, 17 h (98%); (ii) CF_3CO_2H, CH_2CH_2, 0 °C, 1.5 h (ca. 100%); (iii) BoPCl, *i*-Pr_2NEt, CH_2Cl_2, N_2, 0 °C to rt, 21.5 h (69%); (iv) H_2, 10% Pd/C, MeOH, rt, 20 h (65%). For **19**: (i) PyBoP, MeNH·HCl, Et_3N, CH_2Cl_2, N_2, rt, 3 days (75%); (ii) CF_3CO_2H, CH_2CH_2, 0 °C, 1.5 h (ca. 100%); (iii) BoPCl, *i*-Pr_2NEt, CH_2Cl_2, N_2, 0 °C to rt, 18.5 h (55%); (iv) H_2, 10% Pd/C, MeOH, rt, 20 h (97%). For **20**: (i) EtOCOCl, Et_3N, Me_2NH·HCl, CH_2Cl_2, N_2, 0 °C to rt, 20.5 h (100%); (ii) CF_3CO_2H, CH_2CH_2, 0 °C, 1.5 h (ca. 100%); (iii) BoPCl, *i*-Pr_2NEt, CH_2Cl_2, N_2, 0 °C to rt, 18.5 h (25%); (iv) H_2, 10% Pd/C, MeOH, rt, 20 h (78%).

which existed predominantly as the *trans* proline–glycine amide bond conformer.

The next target in this series was analogue **19**. Using literature precedence, amino acid **21** was transformed into

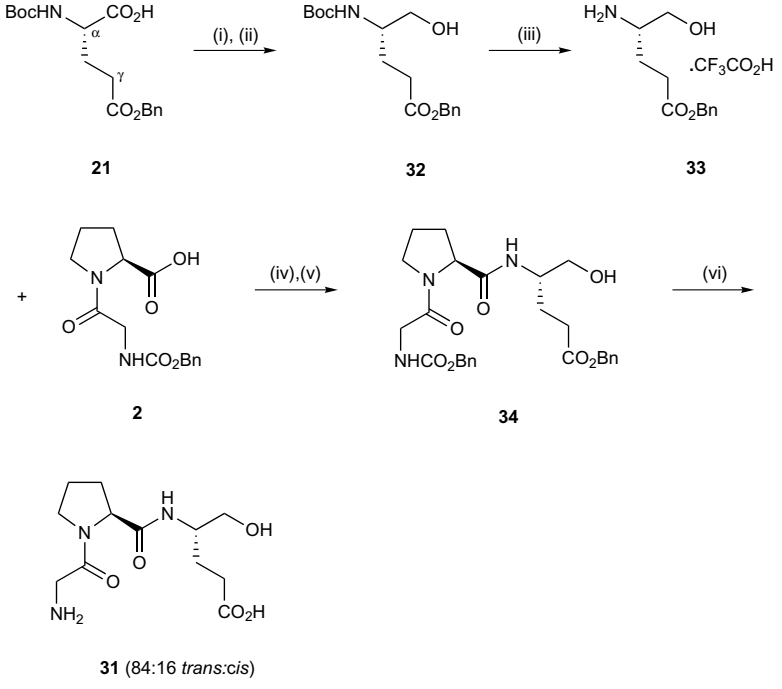
secondary amide[74] **23** by reaction with *N*-methylamine using the coupling reagent (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBoP) in methylene chloride. Amide **23** was next treated with TFA and the resultant salt **26** coupled to acid **2** with BoPCl under basic conditions to afford amide **29** that underwent deprotection to give analogue **19**. The third analogue **20** in this series was prepared from tertiary amide **24** following a similar sequence. Inexplicably, the BoPCl-promoted coupling reaction of **2** with **27** gave compound **30** in low yield (25%). The precursor amide **24** was efficiently synthesised by formation of a mixed anhydride of acid **21** with ethyl chloroformate and triethylamine, and subsequent amidation with *N*,*N*-dimethylamine.

Acid **21** was next used for the production of the GPE* analogue **31**, in which the α-carboxylic acid was replaced with an hydroxylmethyl group. *N*-Hydroxysuccinimide (NHS) 'active esters' were used for the reduction of the α-carboxylic acid functionality of glutamic acid and for the subsequent coupling to the GP unit (Scheme 6). The requisite stable NHS esters,[75,76] were easily prepared following minor modifications of the original literature procedure,[60] from the reaction of corresponding acids **21** and **2** with NHS and DCC in EtOAc and DME, respectively. Rapid sodium borohydride reduction of the intermediate NHS ester[75] of **21** in methanolic THF gave the desired *N*-Boc amino alcohol[77] **32** (72%), with typical *N*-Boc deprotection using TFA successfully furnishing the amine salt **33**. In spite of the acidic environment, no intramolecular condensation of the resultant alcohol with the γ-benzyl ester to afford a δ-lactone was observed. Coupling of amine **33** to the succinimide ester[76] of acid **2** in the presence of diisopropylethylamine gave pure amide **34**, albeit in 31% yield after purification by flash column chromatography. The sequence was completed by hydrogenolysis of amide **34** under standard conditions affording analogue **31** in 93% yield as a white solid.

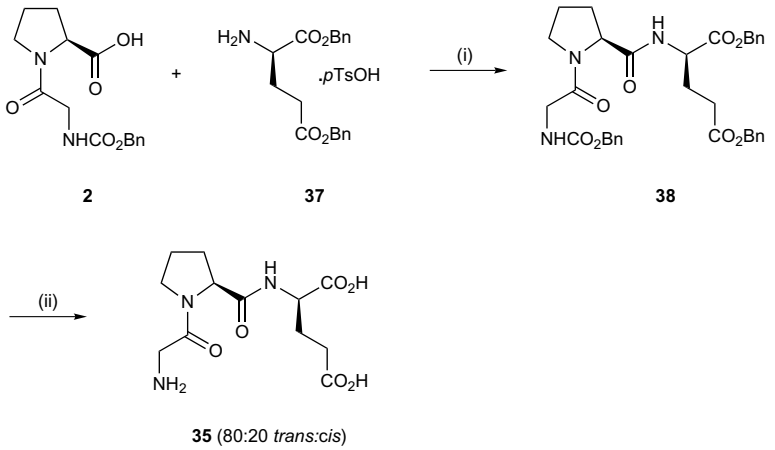
The final two GPE* analogues **35** and **36** reported herein differ from the seven described above in that the α-carboxylic acid functionality of the glutamic acid residue was left intact, however the nature of the α-carbon itself was modified (Schemes 7 and 8). In the first case, the naturally occurring L-glutamic acid was replaced by its optical antipode (analogue **35**) and in the second, an additional methyl group is introduced at the α-position (analogue **36**).

Following the detailed synthesis of the putative neuroprotective agent GPE **1**, protected D-glutamic acid **37** was coupled with acid **2** to give amide **38** which was subsequently deprotected in a 70:30 mixture of THF and water to afford GP-(D)-E **35**, a diastereoisomer of GPE **1** (Scheme 7). It was rationalised that analogue **35**, in which an unnatural D-amino acid residue had been incorporated, would not be recognised by proteases.[78] Similarly, it was envisaged that analogue **36**, which bears an additional alkyl group at the α-position, thereby increasing steric hindrance about the proline–glutamic acid amide bond, would also be resistant to proteolytic cleavage (Scheme 8).

Dibenzylation of racemic α-methylglutamic acid **39** afforded α-methylglutamate **40** in 55% yield. Condensation of glutamate **40** with acid **2** and subsequent



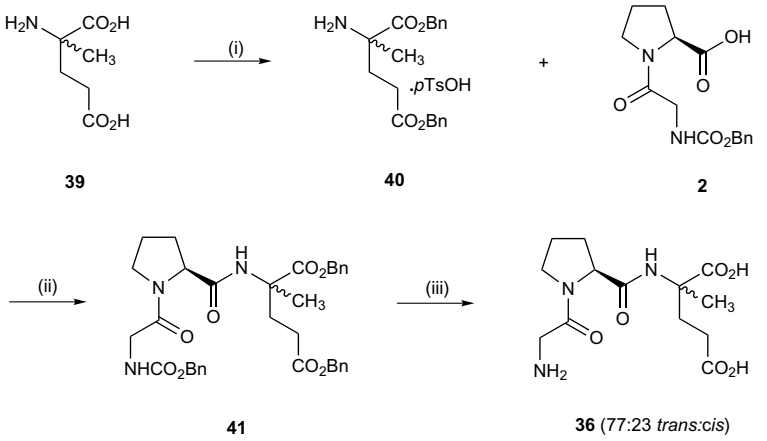
Scheme 6. Reagents and conditions: (i) *N*-hydroxysuccinimide, DCC, EtOAc, N_2, 0 °C to rt, 18 h (ca. 100%); (ii) $NaBH_4$, THF, MeOH, 0 °C, 1 h (72% in two steps from **21**); (iii) CF_3CO_2H, CH_2Cl_2, 0 °C, 1.5 h (ca. 100%); (iv) *N*-hydroxysuccinimide, DCC, DME, N_2, 0 °C to rt, 20 h (69%); (v) *i*-Pr_2NEt, CH_2Cl_2, N_2, 0 °C to rt, 2.5 h (31%); (vi) H_2, 10% Pd/C, MeOH, rt, 20 h (93%).



2 37 38

35 (80:20 *trans:cis*)

Scheme 7. Reagents and conditions: (i) Et_3N, EtOCOCl, CH_2Cl_2, N_2, 0 °C to rt, 18.5 h (64%); (ii) H_2, 10% Pd/C, 70:30 THF–H_2O, rt, 18 h (68%).



39 40 2

41 36 (77:23 *trans:cis*)

Scheme 8. Reagents and conditions: (i) benzyl alcohol, *p*TsOH·H_2O, benzene, 80 °C, 54.5 h (55%); (ii) Et_3N, EtOCOCl, CH_2Cl_2, N_2, 0 °C to rt, 22 h (97%); (iii) H_2, 10% Pd/C, 80:20 MeOH–H_2O, 21 h (81%).

hydrogenolysis of the product **41** afforded analogue **36** in excellent yield as a 77:23 *trans:cis* mixture of rotamers. Unfortunately however, the respective esters **41** and acids **36** were found to be inseparable mixtures of two diastereoisomers due to the lack of control of the stereochemistry of the α-methyl group. Reverse-phase HPLC analysis, showed that analogue **36** eluted as an equimolar set of two diastereoisomers at similar retention times.

The nine GPE* analogues synthesised were subjected to in vitro evaluation for prevention of neuronal cell death. Of the nine analogues, only the *N*,*N*-dimethylamide, **20,** showed neuroprotective activity at 1–100 μM with recovery values ranging from 20% to 40%. Amide **20** exhibits similar efficacy as the best dose of GPE (1 mM). However, analogue **20** may not be more effective than GPE **1** because of the low significance values (best recovery at 58% evaluated against the injured vehicle—see Fig. 1). In repeated experiments the recovery values vary from 20% to 40% whereas GPE **1** recovery values vary from 25% to 40%. Lower neuroprotective values were obtained with 1 mM of analogue **10** where the recovery value was around 20%. None of the other compounds exhibited neuroprotective activity.

3. Conclusions

To summarise, we described herein the synthesis of nine analogues of GPE and their biological evaluation against striatal cell survival post apoptosis-induced injury. Compound **20** exhibited similar efficacy as GPE while **10** showed activity but was not as potent as **20**. The structure and spectra of the new analogues were determined by full analysis of the NMR and mass spectral data.

4. Experimental

All reagents were used as supplied. Solvents were purified by standard methods.[79] Analytical thin layer chromatography (TLC) was carried out on 0.20 mm pre-coated silica gel plates (ALUGRAM® SIL G/UV_{254}). Products were visualised by UV fluorescence

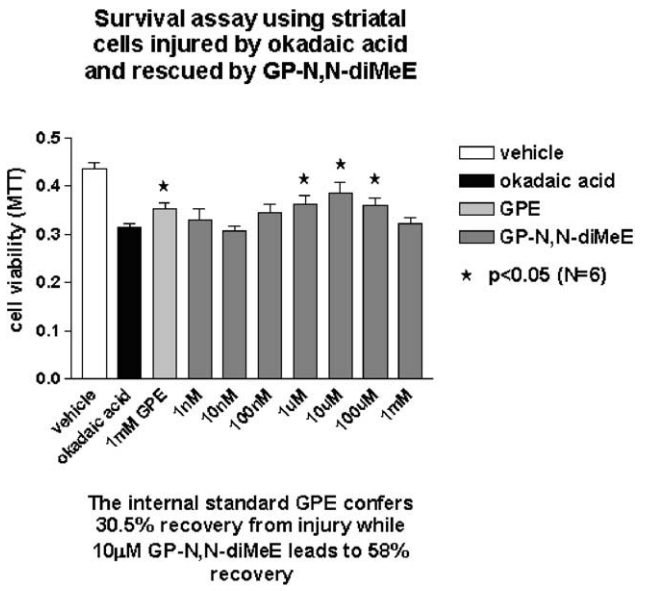


Figure 1. Cellular survival bioassay. MTT-values were taken after 24 h when apoptotic nuclei become apparent within the cell culture. The neuroprotective dose-response for GP-*N*,*N*-diMeE lies between 1 and 100 μM.

and heating of plates dipped in anisaldehyde in ethanolic sulfuric acid or alkaline potassium permanganate solution. Flash chromatography was performed using Scharlau 60 (40–60 μm mesh) silica gel. Melting points in degrees Celsius (°C) were measured on an Electrothermal® melting point apparatus and are uncorrected. Optical rotations were measured at the sodium D line (589 nm), at 20 °C, with a Perkin Elmer 341 polarimeter using a 1 dm path length cell and are given in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer and the samples were prepared as thin films between sodium chloride plates. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AVANCE DRX400 (^{1}H, 400 MHz; ^{13}C, 100 MHz), a Bruker AVANCE 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) or a Bruker AC200 (^{1}H, 200 MHz; ^{13}C, 50 MHz) spectrometer at 298 K. For ^{1}H NMR data, chemical shifts are described in parts per million (ppm) relative to tetramethylsilane (δ 0.00), DOH (δ 4.75), CHD_2OD (δ 3.30) or $CHD_2S(O)CD_3$ (δ 2.50) and are reported consecutively as position (δ_H), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, q = quintet, s = sextet, dd = doublet of doublets, m = multiplet and where br = broad), coupling constant (*J*/Hz) and assignment. For ^{13}C NMR data, chemical shifts (ppm) are referenced internally to $CDCl_3$ (δ 77.0), CD_3OD (δ 49.1) and $(CD_3)_2S(O)$ (δ 39.4) or externally to 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) and are reported consecutively as position (δ_C), degree of hybridisation and assignment. Assignments were aided by DEPT135, COSY, NOESY, HSQC and HMBC experiments. When two sets of peaks arise in the NMR spectra due to *cis*/*trans* isomerisation about the glycine–proline amide bond, the chemical shift for the minor *cis* conformer is asterisked (*). Assignment of the respective isomers is made according to Kessler et al.[69] and NOESY experiments. Mass spectra were recorded on a VG-70SE mass spectrometer (EI, CI and FAB). High-resolution mass spectra were recorded at a nominal resolution of 5000. Analytical HPLC studies were performed on a Waters 600 system with a 2487 dual λ absorbance detector using an Altech Econosphere C_{18}Si column (150 × 4.6 mm; 5 μm), with a 5 min H_2O flush (0.05% TFA) then steady gradient over 25 min to CH_3CN as eluent at a flow rate of 1 mL/min. All chemicals utilised in the bioassay were purchased from Invitrogen.

4.1. Striatal cell culture

Two pregnant Wistar rats (gestational day 18) underwent Caesarean section according to approved procedures from the animal ethics committee of the University of Auckland. The Wistar dams were anaesthetised in a CO_2-enriched atmosphere and sacrificed by subsequent spinal cord dislocation. The embryos were removed from their embryonic sacs and decapitated. After incisions into the skull with fine scissors, the embryonic brain was removed with a fine spatula. Striatal tissue was separated from neocortical tissue under the microscope and placed into serum-free DMEM/F-12 medium supplemented with penicillin/streptomycin (100 u/mL). Tissue underwent 15 trituration steps using a P1000 pipettor to obtain dissociated cells, which were centrifuged for 5 min at 250 G (4 °C) and the supernatant was discarded. Cells were re-suspended into 1 mL DMEM/F-12 medium and were kept on ice. Cell suspension (400,000 cells/cm^2) was applied to 0.1 mg/mL poly-L-lysine pre-coated 96-well plates (3 h at 37 °C) and the volume was increased up to 100 μL with DMEM/F-12 + 5% FBS. Cells were cultivated at 37 °C in 100% humidity and saturated 5% CO_2 atmosphere. After 24 h the medium was changed to serum-free Neurobasal/B27. Cells were fed every 3 days and maintained until 8 days in vitro (DIV).

4.2. Cellular injury, drug administration and cellular survival analysis

Striatal cells were injured after 7 DIV. The injury paradigm involved 30 nM okadaic acid treatment with simultaneous drug administration (GPE analogues) for 24 h. Afterward, 1 μL of 3 μM okadaic acid stock solution together with 1 μL of GPE analogue dissolved in PBS (vehicle received 2 μL of PBS) were incubated for 24 h with the striatal cells. Subsequently, 20 μL MTT (5 mg/mL in PBS) was added for 4 h. The reaction was terminated by addition of 100 μL 4% sodium dodecyl sulfate (SDS) solution. After 16–24 h incubation time, extinction values are read at 595 nm.

The difference between the vehicle and the okadaic acid injury condition was calculated. This value is set as the theoretical 100% recovery value. Measured values for the tested compounds are expressed in percentage of the 100% value. The unpaired Student's *t*-test is used for statistical analysis.

4.3. L-Proline methyl ester hydrochloride[62,63] (4)

Thionyl chloride (34 mL, 0.47 mol) was cautiously added dropwise to a stirred solution of L-proline **3** (13.49 g, 0.18 mol) in anhydrous methanol (180 mL) at −5 °C under an atmosphere of nitrogen. The reaction mixture was warmed to room temperature, stirred for 19 h and concentrated to dryness under reduced pressure. The resultant oil was dissolved in toluene (200 mL) and concentrated to dryness to remove residual thionyl chloride and methanol to yield *hydrochloride*[62,63] **4** (18.95 g, 98%) as a colourless oil. Cooling of the crude oil to 0 °C for 12 h afforded an hygroscopic white solid: δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.98–2.28 (3H, br m, Proβ-H_AH_B and Proγ-H_2), 2.32–2.53 (1H, br m, Proβ-H_AH_B), 3.43–3.71 (2H, br m, Proδ-H_2), 3.85 (3H, s, OCH_3), 4.41–4.60 (1H, br m, Proα-H), 9.28 (1H, br s, NH) and 10.73 (1H, br s, HCl); δ_C (75 MHz; $CDCl_3$; Me_4Si) 23.1 (CH_2, Proγ-C), 28.0 (CH_2, Proβ-C), 45.4 (CH_2, Proδ-C), 52.9 (CH_3, OCH_3), 58.7 (CH, Proα-C) and 168.7 (quat., CO); *m/z* (EI+) 129.0788 ($M^+-HCl{\cdot}C_6H_{11}NO_2$ requires 129.0790).

4.4. *N*-Benzyloxycarbonyl-glycyl-L-proline[60,61] (2)

Anhydrous triethylamine (0.44 mL, 3.16 mmol) was added dropwise to a stirred solution of hydrochloride **4** (0.51 g, 3.08 mmol) and acid **5** (0.63 g, 3.01 mmol) in methylene chloride under an atmosphere of nitrogen. The colourless solution was cooled to 0 °C, a solution of 1,3-dicyclohexylcarbodiimide (0.67 g, 3.25 mmol) in methylene chloride (9 mL) at 0 °C added dropwise and the reaction mixture warmed to room temperature and stirred for 18 h. The resultant white mixture was filtered through a Celite™ pad to partially remove 1,3-dicyclohexylurea, and the pad washed with methylene chloride (50 mL). The filtrate was washed successively with 10% aqueous hydrochloric acid (50 mL) and saturated aqueous sodium hydrogen carbonate (50 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. Further purification of the residue by flash column chromatography (SiO_2; 30–80% ethyl acetate–hexane; gradient elution) afforded *N-benzyloxycarbonyl-glycyl-L-proline methyl ester*[64] **6** (0.88 g), containing residual 1,3-dicyclohexylurea, as a white semi-solid: R_f 0.50 (EtOAc); *m/z* (EI+) 320.1371 ($M^+{\cdot}C_{16}H_{20}N_2O_5$ requires 320.1372).

To a solution of methyl ester **6** (0.88 g, ca. 2.75 mmol) in 1,4-dioxane (30 mL) was added dropwise 1 M aqueous sodium hydroxide (14 mL, 14 mmol) and the mixture was stirred for 21 h at room temperature. Methylene chloride (100 mL) was then added and the organic layer extracted with saturated aqueous sodium hydrogen carbonate (3 × 100 mL). Careful acidification of the combined aqueous layers with hydrochloric acid (32%), extraction with methylene chloride (3 × 100 mL), drying of the combined organic layers ($MgSO_4$), filtration and concentration to dryness gave *acid*[60,61] **2** (0.74 g, 82% in two steps from **4**). Addition of carbon tetrachloride and its removal in vacuo allowed for removal of residual 1,4-dioxane to give acid **2** as a white solid. Compound **2** was shown to be a 75:25 *trans:cis*[80] mixture of conformers: mp 154–157 °C (lit.,[60] 157–159 °C); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.75–2.35 (4H, m, Proβ-H_2 and Proγ-H_2), 3.25–3.70 (2H, m, Proδ-H_2), 3.78–4.17 (2H, m, Glyα-H_2), 4.39* (0.25H, t, *J* 5.7, Proα-H), 4.53 (0.75H, t, *J* 5.5, Proα-H), 5.10 (2H, s, OCH_2Ph), 5.80–6.02 (0.75H, m, Gly-NH), 6.02–6.15* (0.25H, m, Gly-NH), 7.20–7.45 (5H, m, Ph) and 8.06 (1H, br s, COOH); δ_C (75 MHz; $CDCl_3$; Me_4Si) 22.2* (CH_2, Proγ-C), 24.5 (CH_2, Proγ-C), 28.5 (CH_2, Proβ-C), 31.2* (CH_2, Proβ-C), 43.1* (CH_2, Glyα-C), 43.2 (CH_2, Glyα-C), 46.2 (CH_2, Proδ-C), 46.8* (CH_2, Proδ-C), 58.5* (CH, Proα-C), 59.2 (CH, Proα-C), 66.9 (CH_2, OCH_2Ph), 67.1* (CH_2, OCH_2Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.4 (CH, Ph), 136.2* (quat., Ph), 136.3 (quat., Ph), 156.5 (quat., NCO_2), 156.8* (quat., NCO_2), 167.8* (quat., Gly-CO), 168.4 (quat., Gly-CO), 173.8* (quat., CO_2H) and 174.0 (quat., CO_2H); *m/z* (EI+) 306.1213 ($M^+{\cdot}C_{15}H_{18}N_2O_5$ requires 306.1216).

Representative procedure A for the preparation of protected tripeptides and analogues from acid **2** is as follows:

4.5. Dibenzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-L-glutamate (8)

To a solution of acid **2** (0.12 g, 0.39 mmol) in methylene chloride (8 mL), cooled to 0 °C under nitrogen, were added dropwise triethylamine (0.07 mL, 0.50 mmol) and ethyl chloroformate (0.05 mL, 0.52 mmol) and the resultant mixture stirred at 0 °C for 40 min to form the mixed anhydride. Triethylamine (0.075 mL, 0.54 mmol) was added to a suspension of *p*-toluenesulfonate **7** (0.26 g, 0.52 mmol) in methylene chloride (8 mL) under nitrogen at 0 °C and the resulting mixture was added dropwise to the above mixed anhydride. The reaction mixture was warmed to room temperature, stirred for 17 h then successively washed with 10% aqueous hydrochloric acid (3 × 50 mL) and saturated aqueous sodium hydrogen carbonate (50 mL), dried ($MgSO_4$), filtered and evaporated to dryness in vacuo. Purification of the ensuing residue by flash column chromatography (SiO_2; 20–80% ethyl acetate–hexane; gradient elution) yielded *amide* **8** (0.24 g, 100%) as a colourless oil, which was cooled to 0 °C to give a white solid. Compound **8** was shown to be an 86:14 *trans:cis* mixture of conformers: R_f 0.55 (EtOAc); mp 116–118 °C; $[\alpha]_D$ −55.9 (*c* 0.37, CH_2Cl_2); ν_{max} (film)/cm^{-1} 3316 br (NH), 1732 (ester CO), 1650 (amide CO), 1532, 1453, 1257, 1168, 747 and 698; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.75–2.50 (8H, m, Proβ-H_2, Proγ-H_2, Gluβ-H_2 and Gluγ-H_2), 3.28–3.54 (1.72H, m, Proδ-H_2), 3.25–3.74* (0.28H, m, Proδ-H_2), 3.90 (1H, dd, *J* 17.0 and 3.7, Glyα-H_AH_B), 4.02 (1H, dd, *J* 17.2 and 4.7, Glyα-H_AH_B), 4.23–4.30* (0.14H, m, Proα-H), 4.44–4.63 (1.86H, m, Proα-H and Gluα-H), 5.06 (2H, s, OCH_2Ph), 5.10 (2H, s, OCH_2Ph), 5.14 (2H, s, OCH_2Ph), 5.60* (0.14H, br s, Gly-NH), 5.71 (0.86H, br s, Gly-NH) and 7.15–7.45 (16H, m, 3 × Ph and Glu-NH); δ_C (75 MHz; $CDCl_3$; Me_4Si) 22.2* (CH_2, Proγ-C), 24.6 (CH_2, Proγ-C), 25.9* (CH_2, Gluβ-C), 26.8 (CH_2, Gluβ-C), 27.8 (CH_2, Proβ-C), 30.0 (CH_2, Gluγ-C), 30.3* (CH_2, Gluγ-C), 31.8* (CH_2, Proβ-C), 43.3 (CH_2, Glyα-C), 46.1 (CH_2, Proδ-C),

47.0* (CH_2, Proδ-C), 51.7 (CH, Gluα-C), 52.0* (CH, Gluα-C), 60.0 (CH, Proα-C), 60.1* (CH, Proα-C), 66.3 (CH_2, OCH_2Ph), 66.6* (CH_2, OCH_2Ph), 66.7 (CH_2, OCH_2Ph), 67.0 (CH_2, OCH_2Ph), 67.2* (CH_2, OCH_2Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.2 (CH, Ph), 128.3 (CH, Ph), 128.4 (CH, Ph), 135.2 (quat., Ph), 135.7 (quat., Ph), 136.3 (quat., Ph), 156.2 (quat., NCO_2), 167.9 (quat., Gly-CO), 168.2* (quat., Gly-CO), 170.9 (quat., Pro-CON), 171.0* (quat., Pro-CON), 171.2 (quat., Gluα-CO), 171.5* (quat., Gluα-CO), 172.5 (quat., Glu-CO) and 172.8* (quat., Gluγ-CO); *m/z* (EI+) 615.2588 ($M^+ \cdot C_{34}H_{37}N_3O_8$ requires 615.2581).

Representative procedure B for the hydrogenation of protected tripeptides and analogues is as follows:

4.6. Glycyl-L-prolyl-L-glutamic acid[15] (1)

A mixture of protected tripeptide **8** (0.13 g, 0.21 mmol) and 10 wt% palladium on activated carbon (0.07 g, 0.07 mmol) in 80:20 methanol–water (24 mL; v/v) was stirred under an atmosphere of hydrogen at room temperature, protected from light, for 18 h. The reaction mixture was filtered through a Celite™ pad and the pad washed with 50:50 methanol–water (100 mL; v/v). The filtrate was concentrated to dryness under reduced pressure and the residue triturated with anhydrous diethyl ether to afford *tripeptide*[15] **1** (0.06 g, 94%) as a white solid. Compound **1** was shown to be an 80:20 *trans:cis* mixture of conformers: mp 209–211 °C (lit.,[81] 216 °C); (Found: C, 47.57; H, 6.29; N, 13.68. $C_{12}H_{19}N_3O_6$ requires C, 47.84; H, 6.36; N, 13.95); $[\alpha]_D$ −79.1 (*c* 0.07, H_2O) [lit.,[81] −84.8 (*c* 0.034, H_2O)]; ν_{max} (KBr disc)/cm^{-1} 3467 br, 3317 br, 3170 br, 2932, 1960, 1646 (amide CO), 1595, 1538, 1310, 1263, 639 and 470; δ_H (400 MHz; D_2O) 1.80–2.07 (4H, m, Gluβ-$H_A$$H_B$, Proβ-$H_A$$H_B$ and Proγ-H_2), 2.07–2.23 (1H, m, Gluβ-$H_A$$H_B$), 2.23–2.32 (1H, m, Proβ-$H_A$$H_B$), 2.40 (2H, t, *J* 7.6, Gluγ-H_2), 3.47–3.67 (2H, m, Proδ-H_2), 3.72* (0.20H, d, *J* 16.2, Glyα-$H_A$$H_B$), 3.94* (0.20H, d, *J* 16.4, Glyα-$H_A$$H_B$), 3.96 (0.80H, d, *J* 16.6, Glyα-$H_A$$H_B$), 4.00 (0.80H, d, *J* 16.6, Glyα-$H_A$$H_B$), 4.21 (1H, dd, *J* 8.7 and 4.9, Gluα-H) and 4.47 (1H, dd, *J* 8.3 and 4.0, Proα-H); δ_C (100 MHz; D_2O) 24.8* (CH_2, Proγ-C), 27.0 (CH_2, Proγ-C), 29.3* (CH_2, Gluβ-C), 29.8 (CH_2, Gluβ-C), 32.1 (CH_2, Proβ-C), 33.9 (CH_2, Gluγ-C), 34.5* (CH_2, Gluγ-C), 43.0* (CH_2, Glyα-C), 43.2 (CH_2, Glyα-C), 49.6 (CH_2, Proδ-C), 50.3* (CH_2, Proδ-C), 57.0 (CH, Gluα-C), 57.4* (CH, Gluα-C), 62.9* (CH, Proα-C), 63.3 (CH, Proα-C), 168.4 (quat., Gly-CO), 168.8* (quat., Gly-CO), 175.7* (quat., Pro-CON), 176.2 (quat., Pro-CON), 180.1 (quat., Gluα-CO) and 181.4 (quat., Gluγ-CO); *m/z* (FAB+) 302.1355 ($MH^+ \cdot C_{12}H_{20}N_3O_6$ requires 302.1352).

Representative procedure C for the benzylation of carboxylic acids is as follows:

4.7. Benzyl 4-aminobutanoate *p*-toluenesulfonate (15)

A mixture of 4-aminobutanoic acid **12** (1.00 g, 9.70 mmol), *p*-toluenesulfonic acid monohydrate (2.02 g, 10.5 mmol) and benzyl alcohol (1.30 g, 12.00 mmol) in benzene (20 mL) was heated under reflux, using a Dean and Stark distilling receiver, for 68 h. The reaction mixture was cooled to room temperature and anhydrous diethyl ether (20 mL) was added to afford *p-toluenesulfonate* **15** (3.04 g, 86%) as a crystalline white solid: mp 103–106 °C; ν_{max} (film)/cm^{-1} 3579, 3039 br, 2942 br, 1732, 1643, 1532, 1450, 1416, 1393, 1344, 1296, 1181, 1127, 1037, 1011, 996, 948, 819, 773, 732 and 687; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.87 (2H, quintet, *J* 7.3, 3-H_2), 2.30 and 2.32 (5H, overlapping s and t, *J* 7.4, CH_3 and 2-H_2), 2.80–2.97 (2H, m, 4-H_2), 5.02 (2H, s, OCH_2Ph), 7.09 (2H, d, *J* 8.0, 2 × 3′-H), 7.23–7.37 (5H, m, Ph) and 7.67–7.84 and 7.73 (5H, overlapping br m and d, *J* 8.1, N^+H_3 and 2 × 2′-H); δ_C (75 MHz; $CDCl_3$) 21.3 (CH_3, CH_3), 22.4 (CH_2, 3-C), 30.8 (CH_2, 2-C), 39.2 (CH_2, 4-C), 66.3 (CH_2, OCH_2Ph), 125.9 (CH, Ar), 128.1 (CH, Ar), 128.2 (CH, Ar), 128.5 (CH, Ar), 129.0 (CH, Ar), 135.8 (quat., Ph), 140.6 (quat., Ar), 141.2 (quat., Ar) and 172.2 (quat., CO); *m/z* (FAB+) 559.2452 [M_2·*p*TsOH·H^+: $(C_{11}H_{15}NO_2)_2 \cdot C_7H_8O_3S \cdot H$ requires 559.2478].

4.8. Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-4-aminobutanoate (42)

Following procedure A, coupling of acid **2** with *p*-toluenesulfonate **15** yielded *amide* **42** (1.12 g, 86%) as a slightly opaque oil. Compound **42** was shown to be an 88:12 *trans:cis* mixture of conformers: R_f 0.15 (EtOAc); $[\alpha]_D$ −66.1 (*c* 0.39, CH_2Cl_2); ν_{max} (film)/cm^{-1} 3314 br, 2950, 1727, 1649, 1535, 1452, 1255, 1167, 1054, 985, 740 and 698; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.65–2.27 (3H, m, Proβ-$H_A$$H_B$ and Proγ-H_2), 1.83 (2H, quintet, *J* 7.0, 3-H_2), 2.28–2.47 (1H, m, Proβ-$H_A$$H_B$), 2.39 (2H, t, *J* 7.2, 2-H_2), 3.14–3.70 (4H, m, 4-H_2 and Proδ-H_2), 3.72–4.07 (2H, m, Glyα-H_2), 4.19–4.27* (0.12H, m, Proα-H), 4.50 (0.88H, dd, *J* 8.0 and 1.5, Proα-H), 5.10 (2H, s, OCH_2Ph), 5.11 (2H, s, OCH_2Ph), 5.58* (0.12H, br s, Gly-NH), 5.68 (0.88H, br s, Gly-NH), 6.70* (0.12H, br s, 4-NH), 6.95 (0.88H, br s, 4-NH) and 7.24–7.43 (10H, m, 2 × Ph); δ_C (75 MHz; $CDCl_3$) 22.1* (CH_2, Proγ-C), 24.1* (CH_2, 3-C), 24.2 (CH_2, 3-C), 24.5 (CH_2, Proγ-C), 28.0 (CH_2, Proβ-C), 31.4 (CH_2, 2-C), 31.8* (CH_2, Proβ-C), 38.6 (CH_2, 4-C), 38.9* (CH_2, 4-C), 43.0* (CH_2, Glyα-C), 43.2 (CH_2, Glyα-C), 46.1 (CH_2, Proδ-C), 46.8* (CH_2, Proδ-C), 60.1 (CH, Proα-C), 66.0 (CH_2, OCH_2Ph), 66.1* (CH_2, OCH_2Ph), 66.6 (CH_2, OCH_2Ph), 127.7* (CH, Ph), 127.7 (CH, Ph), 127.9 (CH, Ph), 127.9 (CH, Ph), 128.2 (CH, Ph), 128.3 (CH, Ph), 135.6* (quat., Ph), 135.7 (quat., Ph), 136.2 (quat., Ph), 156.2 (quat., NCO_2), 156.4* (quat., NCO_2), 167.9* (quat., Gly-CO), 168.0 (quat., Gly-CO), 170.9 (quat., Pro-CON), 171.0* (quat., Pro-CON) and 173.0 (quat., 1-CO); *m/z* (EI+) 481.2201 ($M^+ \cdot C_{26}H_{31}N_3O_6$ requires 481.2213).

4.9. Glycyl-L-prolyl-4-aminobutanoic acid (9)

Following procedure B, hydrogenation of amide **42** yielded *analogue* **9** (0.26 g, 97%) as an extremely hygroscopic, white amorphous solid. Compound **9** was shown to be an 86:14 *trans:cis* mixture of conformers: $[\alpha]_D$

−92.0 (*c* 0.19, H_2O); ν_{max} (film)/cm^{-1} 3262 br, 2952 br, 2644, 1659, 1558, 1457, 1429, 1401, 1355, 1302, 1245, 1191, 1092 and 919; δ_H (400 MHz; D_2O) 1.79 (2H, quintet, *J* 7.0, 3-H_2), 1.87–2.14 (3H, m, Proβ-H_AH_B and Proγ-H_2), 2.24 (2H, t, *J* 7.4, 2-H_2), 2.14–2.48 (1H, m, Proβ-H_AH_B), 3.17–3.33 (2H, m, 4-H_2), 3.53–3.72 (2H, m, Proδ-H_2), 3.92–4.12 (2H, m, Glyα-H_2), 4.44 (0.86H, dd, *J* 8.3 and 4.0, Proα-H) and 4.40–4.53* (0.14H, m, Proα-H); δ_C (100 MHz; D_2O) 23.9* (CH_2, Proγ-C), 26.0 (CH_2, Proγ-C), 26.9 (CH_2, 3-C), 27.1* (CH_2, 3-C), 31.5 (CH_2, Proβ-C), 33.8* (CH_2, Proβ-C), 36.6 (CH_2, 2-C), 41.2 (CH_2, 4-C), 41.3* (CH_2, 4-C), 42.2* (CH_2, Glyα-C), 42.4 (CH_2, Glyα-C), 48.7 (CH_2, Proδ-C), 49.4* (CH_2, Proδ-C), 62.0* (CH, Proα-C), 62.7 (CH, Proα-C), 168.0 (quat., Gly-CO), 168.3* (quat., Gly-CO), 175.0* (quat., Pro-CON), 175.6 (quat., Pro-CON), 184.2* (quat., 1-CO) and 184.5 (quat., 1-CO); *m/z* (FAB+) 258.1470 ($MH^+ \cdot C_{11}H_{20}N_3O_4$ requires 258.1454).

4.10. (4*R*)-Benzyl 4-aminopentanoate *p*-toluenesulfonate (16)

Hydrochloride[70] **13** (0.50 g, 3.25 mmol) was dissolved in water and added to an Amberlite IR-120(H) ion exchange column (2 cm × 15 cm). The column was eluted with water (200 mL) then 2 M ammonia solution. Ninhydrin-positive fractions (TLC, 1:1, methanol–methylene chloride, 3–4 drops ammonia solution) were collected, combined and the solvent removed to afford an oil (0.45 g). A solution of this oil, benzyl alcohol (5 mL) and *p*-toluenesulfonic acid (0.62 g, 3.25 mmol) in benzene (20 mL), were heated under reflux, using a Dean and Stark distilling receiver, for 19 h. The solution was cooled to room temperature and anhydrous diethyl ether added to give *p-toluenesulfonate* **16** (1.06 g, 86%) as an off-white solid: mp 134–137 °C; $[\alpha]_D$ +9.5 (*c* 0.20, CH_3OH); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.20 (3H, d, *J* 6.6, 5-H_3), 1.75–2.06, (2H, m, 3-H_2), 2.32 (3H, s, Ar-CH_3), 2.41 (2H, t, *J* 7.6, 2-H_2), 3.24–3.27 (1H, m, 4-H), 5.00–5.09 (2H, m, OCH_2Ph), 7.09 (2H, d, *J* 8.0, 3′-H); 7.27–7.38 (5H, m, Ph), 7.76 (2H, d, *J* 8.0, 2′-H) and 7.82 (2H, br s, NH_2); δ_C (75 MHz; $CDCl_3$) 18.3 (CH_3, 5-C), 21.2 (CH_3, Ar-*C*H_3) 29.4 (CH_2), 29.8 (CH_2), 47.5 (CH, 4-C), 66.3 (CH_2, O*C*H_2Ph), 125.9 (CH, Ph), 128.1 (CH, Ph), 128.15 (CH, Ph), 128.5 (CH, Ph), 128.9 (CH, Ph), 135.8 (quat., Ph), 140.6 (quat., Ph), 141.2 (quat., Ph) and 172.3, (quat., 1-CO); *m/z* (FAB+) 587.2781 [M_2·*p*TsOH·H^+: $(C_{12}H_{17}NO_2)_2$·$C_7H_8O_3S$·H requires 587.2791].

4.11. (4*R*)-Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-4-aminopentanoate (43)

Following procedure A, coupling of acid 2 with *p*-toluenesulfonate **16** yielded amide **43** (0.39 g, 80%) as a colourless oil. Compound **43** was shown to be an 88:12 *trans:cis* mixture of conformers: $[\alpha]_D$ −58.2 (*c* 0.59, CH_2Cl_2); δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.12 (3H, d, *J* 6.0, 5-H_3), 1.72–2.29 (6H, m, 3-H_2, Proβ-H_2 and Proγ-H_2), 2.37 (2H, t, *J* 7.6, 2-H_2), 3.30–3.38 (0.88H, m, Proδ-H_AH_B), 3.37–3.56 (0.88H, m, Proδ-H_AH_B), 3.59–3.64* (0.24H, m, Proδ-H_2), 3.72–3.79* (0.24H, m, Glyα-H_2), 3.95 (3H, s, Glyα-H_2 and 4-H), 4.24* (0.12H, br s, Proα-H), 4.46 (0.88H, d, *J* 7.5, Proα-H), 5.09 (4H, d, *J* 6.9, 2 × OCH_2Ph), 5.80 (1H, br s, Gly-NH), 6.64* (0.12H, d, *J* 8.3, 4-NH), 6.76 (0.88H, d, *J* 7.9, 4-NH) and 7.32–7.34 (10H, m, 2 × Ph); δ_C (75 MHz; $CDCl_3$) 20.4* (CH_3, 5-C), 20.6 (CH_3, 5-C), 22.3* (CH_2, Proγ-C), 24.7 (CH_2, Proγ-C), 27.7 (CH_2, Proβ-C), 29.6* (CH_2, 3-C), 30.8 (CH_2, 3-C), 31.3 (CH_2, 2-C), 31.9* (CH_2, 2-C), 43.3 (CH_2, Glyα-C), 44.9 (CH, 4-C), 45.4* (CH, 4-C), 46.2 (CH, Proδ-C), 47.1* (CH, Proδ-C), 60.4 (CH, Proα-C), 66.2 (CH_2, O*C*H_2Ph), 66.4* (CH_2, O*C*H_2Ph), 66.8 (CH_2, O*C*H_2Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.4 (CH, Ph), 128.44 (CH, Ph), 135.8 (quat., Ph), 136.6 (quat., Ph), 156.3 (quat., NCO_2), 168.2 (quat., Gly-CO), 170.2 (quat., Pro-CON) and 173.4 (quat., 1-CO); *m/z* (FAB+) 495.2373 ($MH^+ \cdot C_{27}H_{33}N_3O_6$ requires 495.2370).

4.12. (4*R*)-Glycyl-L-prolyl-4-aminopentanoic acid (10)

Following procedure B, hydrogenation of amide **43** in methanol yielded *analogue* **10** (0.13 g, 93%) as a white foam. Compound **10** was shown to be a 76:24 *trans:cis* mixture of conformers: mp 65–75 °C; $[\alpha]_D$ −57.1 (*c* 0.11, CH_3OH); δ_H (300 MHz; D_2O) 1.19 (3H, d, *J* 6.5, 5-H_3), 1.69–2.39 (8H, m, 3-H_2, Proβ-H_2, Proγ-H_2 and 2-H_2), 3.30–3.38 (1H, m, Proδ-H_AH_B), 3.57–2.67 (1.24H, m, Proδ-H_AH_B and Glyα-H_AH_B*), 3.88–4.13 (3.24H, m, Glyα-H_2, 4-H and Glyα-H_AH_B*), 4.44 (0.76H, dd, *J* 8.8 and 4.8, Proα-H) and 4.50* (0.24H, dd, *J* 8.8 and 3.2, Proα-H); δ_C (75 MHz; D_2O) 19.5 (CH_3, 5-C), 19.5* (CH_3, 5-C), 21.9* (CH_2, Proγ-C), 24.1 (CH_2, Proγ-C), 29.5 (CH_2, Proβ-C), 31.2* (CH_2, 3-C), 31.4 (CH_2, 3-C), 32.0* (CH_2, 2-C), 32.4 (CH_2, 2-C), 40.0* (CH_2, Glyα-C), 40.3 (CH_2, Glyα-C), 45.5 (CH, 4-C), 45.8* (CH, 4-C), 46.8 (CH_2, Proδ-C), 47.5* (CH_2, Proδ-C), 59.8* (CH, Proα-C), 60.7 (CH, Proα-C), 165.4 (quat., Gly-CO), 173.0 (quat., Pro-CON) and 180.7 (quat., 1-CO); *m/z* (FAB+) 272.1617 ($MH^+ \cdot C_{12}H_{22}N_3O_4$ requires 272.1610).

4.13. Benzyl 4-amino-4-methylpentanoate *p*-toluenesulfonate (17)

Following procedure C, benzylation of acid[71] **14** yielded *p-toluenesulfonate* **17** (1.29 g, 65%) as a crystalline white solid: mp 113–115 °C; ν_{max} (film)/cm^{-1} 3582, 2913 br, 2623 br, 2326 br, 2028 br, 1729, 1631, 1530, 1447, 1400, 1378, 1313, 1205, 1183, 1125, 1035, 1017, 811, 724 and 681; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.24 (6H, s, 4-CCH_3 and 5-H_3), 1.95 (2H, br t, *J* 8.0, 3-H_2), 2.30 (3H, s, Ar-CH_3), 2.45 (2H, br t, *J* 8.0, 2-H_2), 5.05 (2H, s, OCH_2Ph), 7.01 (2H, d, *J* 7.5, 2 × 3′-H), 7.16–7.40 (5H, m, Ph), 7.70 (2H, d, *J* 7.8, 2 × 2′-H) and 7.84 (3H, br s, N^+H_3); δ_C (100 MHz; $CDCl_3$) 21.3 (CH_3, Ar-*C*H_3), 25.1 (CH_3, 4-C*C*H_3 and 5-C), 28.6 (CH_2, 3-C), 34.7 (CH_2, 2-C), 54.0 (quat., 4-C), 66.4 (CH_2, O*C*H_2Ph), 125.9 (CH, Ar), 128.1 (CH, Ar), 128.5 (CH, Ar), 128.9 (CH, Ar), 135.8 (quat., Ph), 140.3 (quat., Ar), 141.1 (quat., Ar) and 172.6 (quat., 1-CO); *m/z* (FAB+) 615.3123 [M_2·*p*TsOH·H^+: $(C_{13}H_{19}NO_2)_2$·$C_7H_8O_3S$·H requires 615.3104].

4.14. Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-4-amino-4-methylpentanoate (44)

Following procedure A, coupling of acid **2** with *p*-toluenesulfonate **17** yielded amide **44** (0.91 g, 93%) as a crystalline white solid. Compound **44** was shown to be a 92:8 *trans:cis* mixture of conformers: mp 140–142 °C; R_f 0.50 (EtOAc); $[\alpha]_D$ −71.3 (*c* 0.49, CH_2Cl_2); ν_{max} (film)/cm^{-1} 3305 br, 2971 br, 1724, 1650, 1545, 1452, 1284, 1253, 1162, 1043, 984, 735 and 697; δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.27 [2.76H, s, 4-C(CH_3)$_A$(CH$_3$)$_B$], 1.30 [2.76H, s, 4-C(CH$_3$)$_A$(CH_3)$_B$], 1.36* [0.24H, s, 4-C(CH_3)$_A$(CH$_3$)$_B$], 1.37* [0.24H, s, 4-C(CH$_3$)$_A$(CH_3)$_B$], 1.77–2.22 (5H, m, Proβ-H_AH$_B$, Proγ-H$_2$ and 3-H$_2$), 2.24–2.47 (3H, m, Proβ-H$_A$$H_B$ and 2-H$_2$), 3.35 (1H, dd, *J* 16.7 and 8.9, Proδ-H_AH$_B$), 3.43–3.72 (1H, m, Proδ-H$_A$$H_B$), 3.79* (0.08H, dd, *J* 17.0 and 4.5, Glyα-H_AH$_B$), 3.87* (0.08H, dd, *J* 17.0 and 5.2, Glyα-H$_A$$H_B$), 3.95 (0.92H, dd, *J* 17.1 and 4.2, Glyα-H_AH$_B$), 4.02 (0.92H, dd, *J* 17.3 and 4.8, Glyα-H$_A$$H_B$), 4.07–4.13* (0.08H, m, Proα-H), 4.42 (0.92H, dd, *J* 8.0 and 1.7, Proα-H), 5.09 (2H, s, OCH_2Ph), 5.11 (2H, s, OCH_2Ph), 5.62* (0.08H, br s, Gly-NH), 5.68 (0.92H, br s, Gly-NH), 6.52* (0.08H, br s, 4-NH), 6.70 (0.92H, br s, 4-NH) and 7.21–7.41 (10H, m, 2 × Ph); δ_C (100 MHz; $CDCl_3$) 22.4* (CH_2, Proγ-C), 24.7 (CH_2, Proγ-C), 25.9* [CH_3, 4-C(*C*H$_3$)$_A$(CH$_3$)$_B$], 26.3* [CH_3, 4-C(CH$_3$)$_A$(*C*H$_3$)$_B$], 26.6 [CH_3, 4-C(*C*H$_3$)$_A$(CH$_3$)$_B$], 26.6 [CH_3, 4-C(CH$_3$)$_A$(*C*H$_3$)$_B$], 27.6 (CH_2, Proβ-C), 29.1* (CH_2, 3-C), 29.4 (CH_2, 3-C), 32.2* (CH_2, Proβ-C), 34.7 (CH_2, 2-C), 35.2* (CH_2, 2-C), 43.2* (CH_2, Glyα-C), 43.4 (CH_2, Glyα-C), 46.2 (CH_2, Proδ-C), 47.1* (CH_2, Proδ-C), 53.0 (quat., 4-C), 53.6* (quat., 4-C), 60.2* (CH, Proα-C), 60.8 (CH, Proα-C), 66.3 (CH_2, O*C*H$_2$Ph), 66.7* (CH_2, O*C*H$_2$Ph), 66.8 (CH_2, O*C*H$_2$Ph), 127.2* (CH, Ph), 127.4* (CH, Ph), 128.0 (CH, Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.2* (CH, Ph), 128.4 (CH, Ph), 128.5 (CH, Ph), 135.6* (quat., Ph), 135.8 (quat., Ph), 136.3 (quat., Ph), 156.2 (quat., NCO_2), 167.8* (quat., Gly-CO), 168.1 (quat., Gly-CO), 170.0 (quat., Pro-CON), 170.4* (quat., Pro-CON) 173.7 (quat., 1-CO) and 174.2* (quat., 1-CO); *m/z* (FAB+) 510.2599 ($MH^+ \cdot C_{28}H_{36}N_3O_6$ requires 510.2604).

4.15. Glycyl-L-prolyl-4-amino-4-methylpentanoic acid (11)

Following procedure B, hydrogenation of amide **44** in 1.6:1.3:1.0 tetrahydrofuran–methanol–water yielded *analogue* **11** (0.23 g, 89%) as an hygroscopic, off-white amorphous solid following purification by reverse-phase flash column chromatography (10 g $C_{18}SiO_2$; 0–20% methanol–water; gradient elution). Compound **11** was shown to be an 80:20 *trans:cis* mixture of conformers: R_f 0.20 (MeOH) and 0.20 ($C_{18}SiO_2$, 30% MeOH/H_2O); mp 144–172 °C (decomp.); $[\alpha]_D$ −82.9 (*c* 0.23, H_2O), $[\alpha]_D$ −112.0 (*c* 0.19, MeOH); ν_{max} (film)/cm^{-1} 3266 br, 3068 br, 2969, 2652, 1658, 1560, 1457, 1398, 1360, 1307, 1266, 1203, 1169, 1139, 1035, 921 and 845; δ_H (400 MHz; D_2O) 1.28 and 1.31* (6H, overlapping s and s, 2 × 4-CCH$_3$), 1.77–2.08 (5H, m, Proβ-H_AH$_B$, Proγ-H$_2$ and 3-H$_2$), 2.09–2.42 (3H, m, Proβ-H$_A$$H_B$ and 2-H$_2$), 3.42* (0.20H, d, *J* 16.3, Glyα-H_AH$_B$), 3.46–3.62 (2H, m, Proδ-H$_2$), 3.83* (0.20H, d, *J* 16.6, Glyα-H$_A$$H_B$), 3.87 (0.80H, d, *J* 16.8, Glyα-H_AH$_B$), 3.94 (0.80H, d, *J* 16.5, Glyα-H$_A$$H_B$), 4.31 (0.80H, dd, *J* 8.3 and 4.5, Proα-H) and 4.36* (0.20H, dd, *J* 8.4 and 3.2, Proα-H); δ_C (100 MHz; D_2O) 23.9* (CH_2, Proγ-C), 26.0 (CH_2, Proγ-C), 27.3 [CH_3, 4-C(*C*H$_3$)$_A$(CH$_3$)$_B$], 27.5* [CH_3, 4-C(*C*H$_3$)$_A$(CH$_3$)$_B$], 27.6* [CH_3, 4-C(CH$_3$)$_A$(*C*H$_3$)$_B$], 27.8 [CH_3, 4-C(CH$_3$)$_A$(*C*H$_3$)$_B$], 31.6 (CH_2, Proβ-C), 34.1* (CH_2, Proβ-C), 34.2 (CH_2, 3-C), 34.5* (CH_2, 3-C), 37.7* (CH_2, 2-C), 38.1 (CH_2, 2-C), 42.3* (CH_2, Glyα-C), 42.5 (CH_2, Glyα-C), 48.7 (CH_2, Proδ-C), 49.5* (CH_2, Proδ-C), 55.6 (quat., 4-C), 56.0* (quat., 4-C), 62.1* (CH, Proα-C), 63.0 (CH, Proα-C), 168.1 (quat., Gly-CO), 168.8* (quat., Gly-CO), 174.1* (quat., Pro-CON), 174.9 (quat., Pro-CON), 184.8* (quat., 1-CO) and 185.1 (quat., 1-CO); *m/z* (FAB+) 286.1775 ($MH^+ \cdot C_{13}H_{24}N_3O_4$ requires 286.1767).

4.16. Benzyl *N*-*tert*-butyloxycarbonyl-L-γ-glutamate[72] (21)

To a solution of benzyl L-γ-glutamate **45** (2.00 g, 8.40 mmol) in 1:1 dioxane–water (30 mL, v/v) at 0 °C was successively added di-*tert*-butyl dicarbonate (1.95 g, 8.90 mmol) in one portion, and triethylamine (1.40 mL, 10.00 mmol) dropwise. The solution was stirred for 1 h, warmed to room temperature and stirred for a further 20 h then washed with diethyl ether (20 mL). The aqueous layer was acidified with 5 M HCl solution (2 mL) to pH 1 then extracted with ethyl acetate (2 × 50 mL) and the combined organic fractions washed with brine (20 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. The resultant oil was purified by flash column chromatography (ethyl acetate) to afford acid **21** (2.75 g, 97%) as a light green oil: δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.25 [9H, s, C(CH$_3$)$_3$], 1.95–2.00 (1H, m, Gluβ-H$_A$H$_B$), 2.10–2.20 (1H, m, Gluβ-H$_A$H$_B$), 2.37–2.46 (2H, m, Gluγ-H$_2$), 4.00–4.05 (1H, m, Gluα-H), 5.03 (2H, s, OCH$_2$Ph), 5.10–5.20 (1H, m, Gluα-NH), 7.25 (5H, s, Ph) and 9.63 (1H, br s, CO_2H); δ_C (50 MHz; $CDCl_3$) 27.4 (CH_2, Gluβ-C), 28.2 [CH_3, C(CH$_3$)$_3$], 30.3 (CH_2, Gluγ-C), 52.7 (CH, Gluα-C), 66.5 (CH_2, OCH$_2$Ph), 80.3 [quat., C(CH$_3$)$_3$], 128.2 (CH, Ph), 128.3 (CH, Ph), 128.5 (CH, Ph), 135.7 (quat., Ph), 155.6 (quat., NCO_2), 172.7 (quat., Gluγ-CO) and 176.0 (quat., Gluα-CO); *m/z* (EI+) 338 (MH^+, 20 %).

4.17. Benzyl *N*-*tert*-butyloxycarbonyl-L-glutamate α-amide[73] (22)

To a solution of acid **21** (0.87 g, 2.57 mmol) in 1,4-dioxane (25 mL), at room temperature under nitrogen, was successively added di-*tert*-butyl dicarbonate (0.73 g, 3.33 mmol), ammonium hydrogen carbonate (0.27 g, 3.35 mmol) and pyridine (0.27 mL, 3.33 mmol) dropwise. The resultant solution was stirred for 17 h, the solvent removed in vacuo and the residue partitioned between water (20 mL) and ethyl acetate (20 mL). The organic layer was dried ($MgSO_4$), filtered and concentrated to dryness to give a solid which was washed with hexane (2 × 10 mL) to afford *amide*[73] **22** (0.85 g, 98%) as a white

solid: mp 123–125 °C (lit.,[73] 122–123 °C); $[\alpha]_D$ +4.7 (*c* 0.06, MeOH) [lit.,[73] +4.1 (*c* 1.00, EtOH)]; δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.42 [9H, s, $C(CH_3)_3$], 1.87–2.00 (1H, m, Gluβ-H_AH_B), 2.11–2.21 (1H, m, Gluβ-H_AH_B), 2.45–2.57 (2H, m, Gluγ-H_2), 4.21–4.23 (1H, m, Gluα-H), 5.12 (2H, s, OCH_2Ph), 5.40–5.42 (1H, m, Gluα-NH), 5.82 (1H, br s, $CONH_AH_B$), 6.42 (1H, br s, $CONH_AH_B$) and 7.35 (5H, s, Ph); δ_C (50 MHz; $CDCl_3$) 27.8 (CH_2, Gluβ-C), 28.2 [CH_3, $C(CH_3)_3$], 30.4 (CH_2, Gluγ-C), 53.2 (CH, Gluα-C), 66.5 (CH_2, OCH_2Ph), 80.1 [quat., $C(CH_3)_3$], 128.2 (CH, Ph), 128.3 (CH, Ph), 128.5 (CH, Ph), 135.6 (quat., Ph), 155.7 (quat., NCO_2), 173.1 (quat., Gluγ-CO) and 174.0 (quat., Gluα-CO); *m/z* (FAB+) 337 (MH^+, 37%).

Representative procedure D for amine deprotection is as follows:

4.18. Benzyl L-glutamate α-amide trifluoroacetate (25)

Trifluoroacetic acid (1 mL) was added dropwise to a solution of amide **22** (0.10 g, 0.31 mmol) in methylene chloride (4 mL), under nitrogen at 0 °C, and the reaction mixture stirred for 1.5 h then concentrated to dryness in vacuo. The resultant residue was repeatedly dissolved in toluene and the solvent removed (2 × 3 mL) to give *trifluoroacetate* **25** (0.10 g) as an orange oil.

Representative procedure E for the preparation of protected tripeptide analogues from acid **2** is as follows:

4.19. Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-L-glutamate α-amide (28)

Diisopropylethylamine (0.16 mL, 0.92 mmol) was added dropwise to a solution of acid **2** (0.10 g, 0.31 mmol), crude trifluoroacetate **25** (0.07 g, 0.31 mmol) and bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BoPCl) (0.08 g, 0.31 mmol) in methylene chloride (5 mL) under nitrogen at 0 °C. The solution was stirred for 1.5 h, warmed to room temperature and stirred for a further 20 h then washed with saturated sodium hydrogen carbonate (10 mL), aqueous 2 M citric acid (10 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. The resultant residue was purified by flash column chromatography (10% methanol–ethyl acetate) to yield *amide* **28** (0.11 g, 69%) as a colourless oil, which solidified on standing to a white solid: mp 118–120 °C; $[\alpha]_D$ −58.1 (*c* 0.05, MeOH); δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.88–2.04 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.42–2.44 (2H, m, Gluγ-H_2), 3.40–3.50 (1H, m, Proδ-H_AH_B), 3.50–3.60 (1H, m, Proδ-H_AH_B), 3.89–3.92 (2H, m, Glyα-H_2), 4.30–4.40 (2H, m, Proα-H and Gluα-H), 5.00–5.09 (4H, m, 2 × OCH_2Ph), 6.20–6.29 (2H, m, Pro-NH and $CONH_AH_B$), 6.89 (1H, br s, $CONH_AH_B$), 7.27–7.29 (10H, m, 2 × Ph) and 7.67 (1H, d, *J* 8.0, Glu-NH); δ_C (50 MHz; $CDCl_3$) 24.6 (CH_2, Proγ-C), 25.7 (CH_2, Gluβ-C), 29.1 (CH_2, Proβ-C), 30.5 (CH_2, Gluγ-C), 43.4 (CH_2, Glyα-C), 46.7 (CH_2, Proδ-C), 52.9 (CH, Gluα-C), 61.3 (CH, Proα-C), 66.6 (CH_2, OCH_2Ph), 66.9 (CH_2, OCH_2Ph), 127.9 (CH, Ph), 128.2 (CH, Ph), 128.4 (CH, Ph), 128.5 (CH, Ph), 135.5 (quat., Ph), 136.2 (quat., Ph), 156.9 (quat., NCO_2), 169.8 (quat., Gly-CO), 172.1 (quat., Gluγ-CO), 174.2 (quat., Gluα-CO) and 174.4 (quat., Pro-CON); *m/z* (FAB+) 525.2381 ($MH^+ \cdot C_{27}H_{33}N_4O_7$ requires 525.2349).

4.20. Glycyl-L-prolyl-L-glutamic acid α-amide[56] (18)

Following procedure B, hydrogenation of amide **28** in methanol yielded *analogue*[56] **18** (0.19 g, 65%) as a white solid. Compound **18** was shown to be an 87:13 *trans:cis* mixture of conformers: mp 112–114 °C; $[\alpha]_D$ −68.5 (*c* 0.05, MeOH); δ_H (200 MHz; D_2O) 1.85–2.03 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.20–2.29 (2H, m, Gluγ-H_2), 3.48–3.61 (2H, m, Proδ-H_2), 3.94–3.96 (2H, m, Glyα-H_2), 4.14 (1H, m, Gluα-H) and 4.39–4.43 (1H, m, Proα-H); δ_C (50 MHz; D_2O) 24.9 (CH_2, Proγ-C), 27.6 (CH_2, Gluβ-C), 30.0 (CH_2, Proβ-C), 33.9 (CH_2, Gluγ-C), 41.1 (CH_2, Glyα-C), 47.6 (CH_2, Proδ-C), 54.5 (CH, Gluα-C), 61.0* (CH, Proα-C), 61.3 (CH, Proα-C), 166.8 (quat., Gly-CO), 174.7 (quat., Pro-CON), 176.9 (quat., Gluα-CO) and 182.2 (quat., Gluγ-CO); *m/z* (FAB+) 301.1507 ($MH^+ \cdot C_{12}H_{21}N_4O_5$ requires 301.1512).

4.21. Benzyl *N-tert*-butyloxycarbonyl-L-glutamate α-*N*-methylamide[74] (23)

To a solution of acid **21** (0.80 g, 2.40 mmol) and (benzotriazole-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBoP) (1.25 g, 2.40 mmol) in methylene chloride (40 mL), at room temperature under nitrogen, was successively added methylamine hydrochloride (0.20 g, 2.80 mmol), and triethylamine (0.92 mL, 2.40 mmol) dropwise. The reaction mixture was stirred for 3 days, washed with saturated sodium hydrogen carbonate (25 mL) and aqueous 2 M citric acid (25 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. The resultant oil was purified by flash column chromatography (ethyl acetate) and trituration with diethyl ether–10% hexane (10 mL) to give *amide*[74] **23** (0.63 g, 75%) as a white solid: mp 120–122 °C (lit.,[74] 120–123 °C); $[\alpha]_D$ +4.9 (*c* 0.06, MeOH) [lit.,[74] +6.0 (*c* 0.10, $CDCl_3$)]; δ_H [200 MHz, $(CD_3)_2SO$] 1.37 [9H, s, $C(CH_3)_3$], 1.74–1.93 (2H, m, Gluβ-H_2), 2.36 (2H, t, *J* 7.4, Gluγ-H_2), 2.58 (3H, d, *J* 4.7, $NHCH_3$), 3.89–3.92 (1H, m, Gluα-H), 5.09 (2H, s, OCH_2Ph), 6.88 (1H, d, *J* 8.1 Gluα-NH), 7.36 (5H, s, Ph) and 7.71–7.76 (1H, m, $NHCH_3$); δ_C [50 MHz, $(CD_3)_2SO$] 25.5 (CH_3, NCH_3), 27.1 (CH_2, Gluβ-C), 28.1 [CH_3, $C(CH_3)_3$], 30.1 (CH_2, Gluγ-C), 53.4 (CH, Gluα-C), 65.3 (CH_2, OCH_2Ph), 78.0 [quat., $C(CH_3)_3$], 127.8 (CH, Ph), 127.9 (CH, Ph), 128.3 (CH, Ph), 136.1 (quat., Ph), 155.2 (quat., NCO_2), 171.7 (quat., Gluα-CO) and 172.1 (quat., Gluγ-CO); *m/z* (FAB+) 351 (MH^+, 40 %).

4.22. Benzyl L-glutamate α-*N*-methylamide trifluoroacetate (26)

Following procedure D, treatment of amide **23** with trifluoroacetic acid yielded *trifluoroacetate* **26** (0.63 g) as an orange oil.

4.23. Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-L-glutamate α-*N*-methylamide (29)

Following procedure E, coupling of acid **2** with trifluoroacetate **26** yielded *amide* **29** (0.52 g, 55%) as a colourless oil: $[\alpha]_D$ −43.8 (*c* 0.05, MeOH); δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.93–2.25 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.46–2.54 (2H, m, Gluγ-H_2), 2.69–2.75 (3H, d, *J* 7.5, NCH_3), 3.42–3.47 (1H, m, Proδ-$H_A$$H_B$), 3.60–3.65 (1H, m, Proδ-$H_A$$H_B$), 3.93–4.17 (2H, m, Glyα-H_2), 4.42–4.45 (2H, m, Proα-H and Gluα-H), 4.98 (4H, s, OCH_2Ph), 5.10 (2H, s, OCH_2Ph), 5.77 (1H, br s, Gly-NH), 6.85 (1H, br s, N*H*CH_3), 7.32 (5H, s, Ph), 7.45 (5H, s, Ph) and 7.63–7.67 (1H, m, Glu-NH); δ_C (50 MHz, $CDCl_3$) 24.7 (CH_2, Proγ-C), 25.7 (CH_3, NCH_3), 26.2 (CH_2, Gluβ-C), 28.9 (CH_2, Proβ-C), 30.6 (CH_2, Gluγ-C), 43.5 (CH_2, Glyα-C), 46.7 (CH_2, Proδ-C), 53.0 (CH, Gluα-C), 61.2 (CH, Proα-C), 66.7 (CH_2, O*C*H_2Ph), 67.1 (CH_2, O*C*H_2Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.4 (CH, Ph), 128.5 (CH, Ph), 135.4 (quat., Ph), 138.0 (quat., Ph), 156.5 (quat., NCO_2), 169.1 (quat., Gly-CO), 171.2 (quat., Gluα-CO), 171.3 (quat., Gluγ-CO) and 174.7 (quat., Pro-CON); *m/z* (FAB+) 539.2504 ($MH^+ \cdot C_{28}H_{35}N_4O_7$ requires 539.2506).

4.24. Glycyl-L-prolyl-L-glutamic acid α-*N*-methylamide (19)

Following procedure B, hydrogenation of amide **29** in methanol yielded *analogue* **19** (0.19 g, 97%) as a white solid. Compound **19** was shown to be an 83:17 *trans:cis* mixture of conformers: mp 42–44 °C; $[\alpha]_D$ −58.2 (*c* 0.08, MeOH); δ_H (200 MHz; D_2O) 1.92–2.40 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.46 (2H, t, *J* 7.5, Gluγ-H_2), 2.72 (3H, s, NHCH_3), 3.55–3.65 (2H, m, Proδ-H_2), 3.99 (2H, s, Glyα-H_2), 4.26–4.27 (1H, m, Gluα-H), 4.47–4.50 (1H, m, Proα-H); δ_C (50 MHz; D_2O) 25.2 (CH_2, Proγ-C), 26.7 (CH_3, NCH_3), 27.0 (CH_2, Gluβ-C), 30.4 (CH_2, Proβ-C), 31.1 (CH_2, Gluγ-C), 41.0* (CH_2, Glyα-C), 41.3 (CH_2, Glyα-C), 47.8 (CH_2, Proδ-C), 54.3 (CH, Gluα-C), 61.3 (CH, Proα-C), 166.6 (quat., Gly-CO), 174.4 (quat., Gluα-CO), 175.0 (quat., Gluγ-C) and 178.2 (quat., Pro-CON); *m/z* (FAB+) 315.1668 ($MH^+ \cdot C_{13}H_{23}N_4O_5$ requires 315.1669).

4.25. Benzyl *N*-*tert*-butyloxycarbonyl-L-glutamate α-*N*,*N*-dimethylamide (24)

To a solution of acid **21** (1.00 g, 2.97 mmol) in methylene chloride (20 mL), cooled to 0 °C under nitrogen, were successively added dropwise triethylamine (0.50 mL, 3.90 mmol) and ethyl chloroformate (0.37 mL, 3.90 mmol), and the resultant mixture stirred at 0 °C for 45 min to form the mixed anhydride. Triethylamine (0.90 mL, 6.46 mmol) was added to a suspension of dimethylamine hydrochloride (0.53 g, 6.50 mmol) in methylene chloride (20 mL) under nitrogen at 0 °C and the resulting solution added dropwise to the above mixed anhydride. The reaction mixture was stirred for 30 min, warmed to room temperature and stirred for a further 20 h then washed with saturated sodium hydrogen carbonate (10 mL), aqueous 2 M citric acid (10 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. The resultant residue was purified by flash column chromatography (10% diethyl ether–methylene chloride) to yield *amide* **24** (1.08 g, 100%) as a green oil: δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.42 [9H, s, $C(CH_3)_3$], 1.73–1.74 (1H, m, Gluβ-$H_A$$H_B$), 2.00–2.10 (1H, m, Gluβ-$H_A$$H_B$), 2.44–2.54 (2H, m, Gluγ-H_2), 2.90 (3H, s, NCH_3), 3.10 (3H, s, NCH_3), 4.70–4.71 (1H, m, Gluα-H), 5.13 (2H, s, OCH_2Ph), 5.48 (1H, d, *J* 8.1, Gluα-NH) and 7.36 (5H, s, Ph); δ_C (50 MHz, $CDCl_3$) 28.1 (CH_2, Gluβ-C), 28.2 [CH_3, C(*C*H_3)$_3$], 29.5 (CH_2, Gluγ-C), 35.6 (CH_3, NCH_3), 36.9 (CH_3, NCH_3), 49.2 (CH, Gluα-C), 66.3 (CH_2, OCH_2Ph), 79.5 [quat., *C*$(CH_3)_3$], 128.2 (CH, Ph), 128.5 (CH, Ph), 135.8 (quat., Ph), 155.6 (quat., NCO_2), 171.6 (quat., Gluα-CO) and 172.8 (quat., Gluγ-CO); *m/z* (FAB+) 364.1999 ($M^+ \cdot C_{19}H_{28}N_2O_5$ requires 364.1998).

4.26. Benzyl-L-glutamate α-*N*,*N*-dimethylamide trifluoroacetate (27)

Following procedure D, treatment of amide **24** with trifluoroacetic acid yielded *trifluoroacetate* **27** (0.98 g) as an orange oil.

4.27. Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-L-glutamate α-*N*,*N*-dimethylamide (30)

Following procedure E, coupling of acid **2** with trifluoroacetate **27** yielded *amide* **30** (0.36 g, 25%) as a colourless oil: Compound **30** was shown to be an 84:16 *trans:cis* mixture of conformers: $[\alpha]_D$ −67.0 (*c* 0.06, MeOH); δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.73–2.13 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.14–2.53 (2H, m, Gluγ-H_2), 2.84* (0.48H, s, NCH_3), 2.91 (2.52H, s, NCH_3), 3.03* (0.48 H, s, NCH_3), 3.07 (2.52 H, s, NCH_3), 3.30–3.40 (1H, m, Proδ-$H_A$$H_B$), 3.40–3.50 (1H, m, Proδ-$H_A$$H_B$), 3.92–4.13 (2H, m, Glyα-H_2), 4.46–4.50 (1H, m, Proα-H), 4.95–5.00 (1H, m, Gluα-H), 5.04 (2H, m, OCH_2Ph), 5.09 (2H, m, OCH_2Ph), 5.89 (1H, br s, Gly-NH) and 7.27–7.41 (10H, m, 2 × Ph); δ_C (50 MHz, $CDCl_3$) 24.7 (CH_2, Proγ-C), 27.6 (CH_2, Gluβ-C), 28.6 (CH_2, Proβ-C), 29.3 (CH_2, Gluγ-C), 35.7 (CH_3, NCH_3), 36.9 (CH_3, NCH_3), 43.4 (CH_2, Glyα-C), 46.3 (CH_2, Proδ-C), 48.2 (CH, Gluα-C), 60.4 (CH, Proα-C), 66.3 (CH_2, O*C*H_2Ph), 66.8 (CH_2, O*C*H_2Ph), 127.9 (CH, Ph), 128.4 (CH, Ph), 135.8 (quat., Ph), 136.0 (quat., Ph) 156.2 (quat., NCO_2), 167.7 (quat., Gly-CO), 171.0 (quat., Gluα-CO), 171.1 (quat., Gluγ-CO) and 172.9 (quat., Pro-CON); *m/z* (FAB+) 553.2661 ($MH^+ \cdot C_{29}H_{37}N_4O_7$ requires 553.2662).

4.28. Glycyl-L-prolyl-L-glutamic acid α-*N*,*N*-dimethylamide (20)

Following procedure B, hydrogenation of amide **30** in methanol yielded *analogue* **20** (0.22 g, 78%) as a white solid. Compound **20** was shown to be an 83:17 *trans:cis* mixture of conformers: mp 28–30 °C; $[\alpha]_D$ −56.9 (*c* 0.05, MeOH); δ_H (200 MHz; CD_3OD) 1.69–1.90 (6H, m, Proβ-H_2, Proγ-H_2 and Gluβ-H_2), 2.33 (2H, t, *J* 7.5,

Gluγ-H_2), 2.82 (3H, s, NCH_3), 3.05 (3H, s, NCH_3), 3.34–3.50 (2H, m, Proδ-H_2), 3.78 (2H, m, Glyα-H_2), 4.34–4.37 (1H, m, Gluα-H) and 4.70–4.80 (1H, m, Proα-H); δ_C (50 MHz, CD_3OD) 25.7 (CH_2, Proγ-C), 28.1 (CH_2, Gluβ-C), 30.6 (CH_2, Proβ-C), 30.7 (CH_2, Gluγ-C), 31.2* (CH_2, Gluγ-C), 36.2 (CH_3, NCH_3), 37.6 (CH_3, NCH_3), 41.6 (CH_2, Glyα-C), 47.7 (CH_2, Proδ-C), 48.5* (CH_2, Proδ-C), 50.1 (CH, Gluα-C), 50.8* (CH, Gluα-C), 61.2* (CH, Proα-C), 61.6 (CH, Proα-C), 166.2 (quat., Gly-CO), 173.3 (quat., Gluγ-CO), 174.1 (quat., Gluα-CO) and 176.8 (quat., Pro-CON); *m/z* (FAB+) 329.1818 ($MH^+ \cdot C_{14}H_{25}N_4O_5$ requires 329.1825).

4.29. Benzyl *N-tert*-butyloxycarbonyl-L-α-succinimidoglutamate[75] (46)

To a solution of acid **21** (0.20 g, 0.60 mmol) in ethyl acetate (8 mL) at 0 °C under nitrogen, was successively added *N*-hydroxysuccinimide (0.08 g, 0.71 mmol) and 1,3-dicyclohexylcarbodiimide (0.15 g, 0.71 mmol). The reaction mixture was stirred for 1 h, warmed to room temperature and stirred for a further 17 h then washed with saturated aqueous sodium hydrogen carbonate (10 mL) and brine (10 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. The resultant colourless oil solidified to give succinimide ester[75] **46** (0.30 g) as a white solid.

4.30. (4*S*)-Benzyl *N-tert*-butyloxycarbonyl-4-amino-5-hydroxypentanoate[77] (32)

Sodium borohydride (0.03 g, 0.66 mmol) was added portionwise over 15 min to a solution of crude succinimide ester **46** (0.30 g, 0.60 mmol) in tetrahydrofuran (5 mL) at 0 °C. Methanol (2.5 mL) was added dropwise over 30 min and the solution stirred for a further 15 min then quenched with saturated aqueous ammonium chloride (10 mL). The reaction mixture was extracted with ethyl acetate (2 × 30 mL) and the combined organic fractions were washed with brine (10 mL), dried ($MgSO_4$), filtered and concentrated to dryness in vacuo. Purification of resultant residue by flash column chromatography (1:1 ethyl acetate–hexane) afforded *alcohol*[77] **32** (0.14 g, 72% in two steps from **21**) as a white semi-solid: $[\alpha]_D$ −19.6 (*c* 0.06, MeOH); δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.43 [9H, s, $C(CH_3)_3$], 1.75–1.95 (2H, m, 3-H_2), 2.46 (2H, t, *J* 7.3, 2-H_2), 3.55–3.65 (3H, m, 4-H and 5-H_2), 4.86–4.89 (1H, m, NH), 5.12 (2H, s, OCH_2Ph) and 7.35 (5H, s, Ph); δ_C (50 MHz, $CDCl_3$) 26.3 (CH_2, 3-C), 28.3 [CH_3, C(CH_3)$_3$], 30.8 (CH_2, 2-C), 52.2 (CH, 4-C), 65.0 (CH_2, OCH_2Ph), 66.4 (CH_2, 5-C), 79.6 [quat., $C(CH_3)_3$], 128.2 (CH, Ph), 128.5 (CH, Ph), 135.7 (quat., Ph), 156.2 (quat., NCO_2) and 173.5 (quat., 1-CO); *m/z* (FAB+) 324.1817 ($M^+ \cdot C_{17}H_{26}NO_5$ requires 324.1811).

4.31. (4*S*)-Benzyl 4-amino-5-hydroxypentanoate trifluoroacetate (33)

Following procedure D, treatment of alcohol **32** with trifluoroacetic acid yielded *trifluoroacetate* **33** (1.00 g) as an orange oil.

4.32. *N*-Benzyloxycarbonyl-glycyl-L-proline *N*-hydroxysuccinimide ester[76] (47)

1,3-Dicyclohexylcarbodiimide (1.48 g, 7.17 mmol) and *N*-hydroxysuccinimide (0.75 g, 6.50 mmol) were successively added to a solution of acid **2** (2.00 g, 6.50 mmol) in dimethoxyethane (25 mL) at 0 °C under nitrogen. The reaction mixture was stirred for 3 h, warmed to room temperature and stirred for a further 17 h. The resultant solid was filtered, washed with diethyl ether (150 mL) and the filtrate concentrated to dryness in vacuo to give succinimide ester[76] **47** (1.81 g, 69%) as a white solid.

4.33. (4*S*)-Benzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-4-amino-5-hydroxypentanoate (34)

Diisopropylethylamine (0.67 mL, 3.84 mmol) was added dropwise to a solution of trifluoroacetate **33** (1.04 g, 3.08 mmol) and succinimide ester **47** (1.35 g, 3.38 mmol) in methylene chloride (40 mL) at 0 °C under nitrogen. The reaction mixture was stirred for 30 min, warmed to room temperature and stirred for a further 2 h, then washed with saturated aqueous hydrogen carbonate (25 mL) and 2 M citric acid (25 mL), dried ($MgSO_4$), filtered and the solvent removed in vacuo. The resultant residue was purified by flash column chromatography (10% methanol–ethyl acetate) to afford *amide* **34** (0.49 g, 31%) as a colourless oil: $[\alpha]_D$ −43.0 (*c* 0.05, MeOH); δ_H (200 MHz; $CDCl_3$; Me_4Si) 1.83–2.16 (6H, m, Proβ-H_2, Proγ-H_2 and 3-H_2), 2.41 (2H, t, *J* 7.3, 2-H_2), 3.40–3.66 (5H, m, Proδ-H_2, Glyα-H_2 and OH), 3.90–3.95 (3H, 5-H_2 and 4-H), 4.40–4.50 (1H, m, Proα-H), 5.08 (2H, s, OCH_2Ph), 5.09 (2H, s, OCH_2Ph), 5.90 (1H, m, Gly-NH), 6.90 (1H, m, 4-NH) and 7.26–7.32 (10H, m, 2 × Ph); δ_C (50 MHz, $CDCl_3$) 24.7 (CH_2, Proγ-C), 25.7 (CH_2, 3-C), 28.5 (CH_2, Proβ-C), 30.8 (CH_2, 2-C), 43.4 (CH_2, Glyα-C), 46.5 (CH_2, Proδ-C), 51.4 (CH, 4-C), 60.7 (CH, Proα-C), 64.3 (CH_2, 5-C), 66.4 (CH_2, OCH_2Ph), 67.0 (CH_2, OCH_2Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.5 (CH, Ph), 135.7 (quat., Ph), 136.2 (quat., Ph), 156.6 (quat., NCO_2), 168.6 (quat., Gly-CO), 171.5 (quat., 1-CO) and 173.7 (quat., Pro-CON); *m/z* (EI+) 512.2398 ($MH^+ \cdot C_{27}H_{34}N_3O_7$ requires 512.2397).

4.34. (4*S*)-Glycyl-L-prolyl-4-amino-5-hydroxypentanoic acid (31)

Following procedure B, hydrogenation of amide **34** in methanol yielded *analogue* **31** (0.27 g, 93%) as a white solid. Compound **31** was shown to be an 84:16 *trans:cis* mixture of conformers: mp 63–65 °C; $[\alpha]_D$ −56.1 (*c* 0.05, MeOH); δ_H (200 MHz; CD_3OD) 1.94–2.03 (4H, m, Proβ-H_2 and 3-H_2), 2.15–2.25 (4H, m, Proγ-H_2 and 2-H_2), 3.51–3.56 (4H, m, Proδ-H_2 and 5-H_2), 3.84–3.87 (1H, m, 4-H), 3.95 (2H, s, Glyα-H_2) and 4.40–4.50 (1H, m, Proα-H); δ_C (50 MHz, CD_3OD) 23.1* (CH_2, Proγ-C), 25.1 (CH_2, Proγ-C), 25.3 (CH_2, 3-C), 30.7 (CH_2, Proβ-C), 33.3* (CH_2, 2-C), 34.8 (CH_2, 2-C), 41.5 (CH_2, Glyα-C), 47.9 (CH_2, Proδ-C), 52.6 (CH, 4-C), 60.5* (CH, Proα-C), 61.8 (CH, Proα-C), 64.3

(CH_2, 5-C), 166.8 (quat., Gly-CO), 175.1 (quat., Pro-CON) and 183.5 (quat., 1-CO); *m/z* (FAB+) 288.1551 ($M^+ \cdot C_{12}H_{22}N_3O_5$ requires 288.1559).

4.35. Dibenzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-D-glutamate (38)

Following procedure A, coupling of acid **2** with *p*-toluenesulfonate **37** yielded *amide* **38** (1.62 g, 64%) as a white solid. Compound **38** was shown to be a 92:8 *trans:cis* mixture of conformers: R_f 0.50 (EtOAc); $[\alpha]_D$ −54.8 (*c* 0.29, CH_2Cl_2); ν_{max} (film)/cm^{-1} 3583 wk, 3317 br, 3063, 3033, 2953, 2881, 1732, 1658, 1528, 1454, 1255, 1213, 1167, 1055, 985, 915, 739 and 698; δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.78–2.11 (4H, m, Proβ-H_AH_B, Proγ-H_2 and Glu-H_AH_B), 2.13–2.49 (4H, m, Gluβ-H_AH_B, Proβ-H_AH_B and Gluγ-H_2), 3.33 (0.92H, br dd, *J* 16.6 and 9.3, Proδ-H_AH_B), 3.36–3.46 (0.92H, m, Proδ-H_AH_B), 3.52–3.71* (0.16H, m, Pro-H_2), 3.79* (0.08H, dd, *J* 16.8 and 4.7, Glyα-H_AH_B), 3.89 (1H, dd, *J* 17.1 and 4.0, Glyα-H_AH_B and Glyα-H_AH_B*), 4.00 (0.92H, dd, *J* 17.2 and 4.9, Glyα-H_AH_B), 4.27* (0.08H, br t, *J* 5.3, Proα-H), 4.52–4.64 (1.92H, m, Proα-H and Gluα-H), 5.02–5.21 (6H, m, 3 × OCH_2Ph), 5.58–5.63* (0.08H, br m, Gly-NH), 5.63–5.71 (0.92H, br m, Gly-NH), 7.28–7.38 (15H, m, 3 × Ph) and 7.52 (1H, d, *J* 7.7, Glu-NH); δ_C (100 MHz; $CDCl_3$) 22.0* (CH_2, Proγ-C), 24.4 (CH_2, Proγ-C), 25.8* (CH_2, Gluβ-C), 26.4 (CH_2, Gluβ-C), 27.9 (CH_2, Proβ-C), 30.1 (CH_2, Gluγ-C), 30.3* (CH_2, Gluγ-C), 32.0* (CH_2, Proβ-C), 43.3 (CH_2, Glyα-C), 46.1 (CH_2, Proδ-C), 46.9* (CH_2, Proδ-C), 51.8 (CH, Gluα-C), 52.2* (CH, Gluα-C), 60.0 (CH, Proα-C), 60.1* (CH, Proα-C), 66.4 (CH_2, OCH_2Ph), 66.6* (CH_2, OCH_2Ph), 66.7 (CH_2, OCH_2Ph), 67.0 (CH_2, OCH_2Ph), 67.2* (CH_2, OCH_2Ph), 127.9 (CH, Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.1 (CH, Ph), 128.2* (CH, Ph), 128.3 (CH, Ph), 128.4 (CH, Ph), 128.4 (CH, Ph), 135.0* (quat., Ph), 135.1 (quat., Ph), 135.4* (quat., Ph), 135.5 (quat., Ph), 136.2 (quat., Ph), 156.2 (quat., NCO_2), 156.3* (quat., NCO_2), 168.0* (quat., Gly-CO), 168.3 (quat., Gly-CO), 170.9 (quat., Pro-CON), 171.0* (quat., Pro-CON), 171.1 (quat., Gluα-CO), 171.5* (quat., Gluα-CO), 172.9 (quat., Gluγ-CO) and 173.1* (quat., Gluγ-CO); *m/z* (FAB+) 616.2653 ($MH^+ \cdot C_{34}H_{38}N_3O_8$ requires 616.2659).

4.36. Glycyl-L-prolyl-D-glutamic acid (35)

Following procedure B, hydrogenation of amide **38** in 70:30 tetrahydrofuran–water yielded *tripeptide* **35** (0.22 g, 68%) as an off-white solid. Compound **35** was shown to be an 80:20 *trans:cis* mixture of conformers: mp 198–200 °C; $[\alpha]_D$ −70.6 (*c* 0.27, H_2O); δ_H (400 MHz; D_2O) 1.83–2.18 (5H, m, Gluβ-H_2, Proβ-H_AH_B and Proγ-H_2), 2.21–2.45 (3H, m, Proβ-H_AH_B and Gluγ-H_2), 3.49–3.68 (2.20H, m, Proδ-H_2 and Glyα-H_AH_B*), 3.92* (0.20H, d, *J* 16.3, Glyα-H_AH_B), 3.95 (0.80H, d, *J* 16.4, Glyα-H_AH_B), 4.03 (0.80H, d, *J* 16.5, Glyα-H_AH_B), 4.17* (0.20H, dd, *J* 9.3 and 4.7, Gluα-H), 4.20 (0.80H, dd, *J* 9.1 and 4.5, Gluα-H), 4.42–4.53 (0.80H, m, Proα-H) and 4.52* (0.20H, dd, *J* 8.7 and 3.1, Proα-H); δ_C (100 MHz; D_2O) 24.6* (CH_2, Proγ-C), 26.6 (CH_2, Proγ-C), 29.3* (CH_2, Gluβ-C), 29.6 (CH_2, Gluβ-C), 32.2 (CH_2, Proβ-C), 34.2 (CH_2, Gluγ-C), 34.4* (CH_2, Gluγ-C), 34.6* (CH_2, Proβ-C), 42.7* (CH_2, Glyα-C), 43.0 (CH_2, Glyα-C), 49.4 (CH_2, Proδ-C), 50.0* (CH_2, Proδ-C), 57.1 br (CH, Gluα-C), 57.5* br (CH, Gluα-C), 62.3* (CH, Proα-C), 63.2 (CH, Proα-C), 168.3 (quat., Gly-CO), 168.4* (quat., Gly-CO), 175.6* (quat., Pro-CON), 175.8 (quat., Pro-CON), 180.2* br (quat., Gluα-CO), 180.2 br (quat., Gluα-CO), 181.5* br (quat., Gluγ-CO) and 181.9 br (quat., Gluγ-CO); *m/z* (FAB+) 302.1346 ($MH^+ \cdot C_{12}H_{20}N_3O_6$ requires 302.1352).

4.37. Dibenzyl (DL)-2-methylglutamate *p*-toluenesulfonate (40)

Following procedure C, benzylation of acid **39** yielded *p-toluenesulfonate* **40** (0.82 g, 55%) as a crystalline white solid: mp 112–116 °C; ν_{max} (film)/cm^{-1} 3579, 3028 br, 2949 br, 2166 br, 1739, 1649, 1602, 1537, 1494, 1450, 1393, 1313, 1212, 1181, 1125, 1031, 1009, 966, 814, 749, 739 and 677; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.56 (3H, s, Gluα-CCH_3), 2.13–2.38 (6H, m, Ar-CH_3, Gluβ-H_2 and Gluγ-H_AH_B), 2.46–2.68 (1H, m, Gluγ-H_AH_B), 4.94 [1H, d, *J* 12.4, $(OCH_AH_BPh)_A$], 4.99 [1H, d, *J* 12.3, $(OCH_AH_BPh)_A$], 5.05 [1H, d, *J* 12.2, $(OCH_AH_BPh)_B$], 5.11 [1H, d, *J* 12.2, $(OCH_AH_BPh)_B$], 6.98 (2H, d, *J* 7.9, 2 × 3-H), 7.16–7.40 (10H, m, 2 × Ph), 7.70 (2H, d, *J* 8.1, 2 × 2-H) and 8.53 (3H, br s, N^+H_3); δ_C (75 MHz; $CDCl_3$) 21.3 (CH_3, Ar-CH_3), 22.0 (CH_3, Gluα-CCH_3), 28.3 (CH_2, Gluβ-C), 32.0 (CH_2, Gluγ-C), 59.7 (quat., Gluα-C), 66.4 (CH_2, OCH_2Ph), 68.1 (CH_2, OCH_2Ph), 126.1 (CH, Ar), 128.1 (CH, Ar), 128.1 (CH, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 128.5 (CH, Ar), 128.5 (CH, Ar), 128.8 (CH, Ar), 134.7 (quat., Ph), 135.7 (quat., Ph), 140.2 (quat., Ar), 141.3 (quat., Ar), 170.3 (quat., CO) and 171.9 (quat., CO); *m/z* (FAB+) 855.3555 [$M_2 \cdot p$TsOH·H^+: $(C_{20}H_{23}NO_4)_2 \cdot C_7H_8O_3S \cdot H$ requires 855.3527].

4.38. Dibenzyl *N*-benzyloxycarbonyl-glycyl-L-prolyl-(DL)-2-methylglutamate (41)

Following procedure A, coupling of acid **2** with *p*-toluenesulfonate **40** yielded amide **41** (0.40 g, 97%) as a slightly opaque oil. Compound **41** was shown to be an 87:13 *trans:cis* mixture of conformers: R_f 0.70 (EtOAc); $[\alpha]_D$ −59.0 (*c* 0.26, CH_2Cl_2); ν_{max} (film)/cm^{-1} 3319 br, 3063, 3033, 2951, 1730, 1655, 1527, 1454, 1382, 1258, 1170, 1119, 1055, 985, 913, 736 and 698; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.53 (2.61H, s, Gluα-CCH_3), 1.57 (2.61H, s, Gluα-CCH_3), 1.61* (0.39H, s, Glu-CCH_3), 1.62* (0.39H, s, Gluα-CCH_3), 1.69–2.47 (16H, m, 2 × Proβ-H_2, 2 × Proγ-H_2, 2 × Gluβ-H_2 and 2 × Gluγ-H_2), 3.15–3.40 (3.48H, m, 2 × Proδ-H_2), 3.45–3.75* (0.52H, m, 2 × Proδ-H_2), 3.75–4.06 (4H, m, 2 × Glyα-H_2), 4.16* (0.26H, br d, *J* 6.7, 2 × Proα-H), 4.47 (1.74H, br t, *J* 6.6, 2 × Proα-H), 4.98–5.21 (12H, m, 6 × OCH_2 × Ph), 5.50* (0.26H, br s, 2 × Gly-NH), 5.65 (1.74H, br s, 2 × Gly-NH), 7.27–7.38 (30H, m, 6Ph), 7.41 (1H, br s, Glu-NH) and 7.52 (1H, br s, Glu-NH);

δ_C (75 MHz; $CDCl_3$) 21.9* (CH_3, Gluα-C*C*H_3), 22.0* (CH_2, Proγ-C), 22.2* (CH_2, Proγ-C), 22.5 (CH_3, Gluα-C*C*H_3), 22.6* (CH_3, Gluα-C*C*H_3), 22.7 (CH_3, Gluα-C*C*H_3), 24.4 (CH_2, Proγ-C), 24.5 (CH_2, Proγ-C), 27.5 (CH_2, Proβ-C), 27.7 (CH_2, Proβ-C), 28.7* (CH_2, Gluβ-C), 28.8 (CH_2, Gluβ-C), 28.9 (CH_2, Gluβ-C), 29.5* (CH_2, Gluβ-C), 31.3 (CH_2, Gluγ-C), 31.8 (CH_2, Gluγ-C), 43.2 (CH_2, Glyα-C), 45.9 (CH_2, Proδ-C), 46.0 (CH_2, Proδ-C), 46.9* (CH_2, Proδ-C), 46.9* (CH_2, Proδ-C), 58.9 (quat., Gluα-C), 58.9 (quat., Gluα-C), 59.1* (quat., Gluα-C), 59.1* (quat., Gluα-C), 60.1 (CH, Proα-C), 60.1 (CH, Proα-C), 66.2 (CH_2, O*C*H_2Ph), 66.3 (CH_2, O*C*H_2Ph), 66.7 (CH_2, O*C*H_2Ph), 67.1 (CH_2, O*C*H_2Ph), 67.3* (CH_2, O*C*H_2Ph), 127.8 (CH, Ph), 127.8 (CH, Ph), 127.9 (CH, Ph), 128.0 (CH, Ph), 128.0 (CH, Ph), 128.0 (CH, Ph), 128.1 (CH, Ph), 128.1 (CH, Ph), 128.2 (CH, Ph), 128.3 (CH, Ph), 128.3 (CH, Ph), 128.4 (CH, Ph), 135.2* (quat., Ph), 135.4 (quat., Ph), 135.4 (quat., Ph), 135.5 (quat., Ph), 135.6 (quat., Ph), 136.3 (quat., Ph), 156.1 (quat., NCO_2), 167.8 (quat., Gly-CO), 168.0 (quat., Gly-CO), 170.0 (quat., Pro-CON), 170.6* (quat., Pro-CON), 170.6* (quat., Pro-CON), 172.6* (quat., Glu-CO), 172.8 (quat., Glu-CO), 172.9 (quat., Glu-CO), 173.0 (quat., Glu-CO), 173.1 (quat., Glu-CO), 173.5* (quat., Glu-CO) and 173.6* (quat., Glu-CO); *m/z* (FAB+) 630.2811 ($MH^+ \cdot C_{35}H_{40}N_3O_8$ requires 630.2815).

4.39. Glycyl-L-prolyl-(DL)-2-methylglutamic acid (36)

Following procedure B, hydrogenation of amide **41** yielded *analogue* **36** (0.11 g, 81%) as a white solid. Compound **36** was shown to be a 77:23 *trans:cis* mixture of conformers of two equimolar diastereoisomers. Analytical reverse-phase HPLC studies indicated that **36** was a 1:1 mixture of *SS* and *SR* diastereoisomers with two peaks of approximately equal integration eluting with retention times of 13.52 and 13.95 min at 207 and 205 nm, respectively. Analogue **36**: mp 164–180 °C (decomp.); $[\alpha]_D$ −71.3 (*c* 0.15, H_2O); ν_{max} (Nujol mull)/cm^{-1} 3583, 3318 br, 2725, 1651, 1562, 1546, 1299, 1168, 1114 and 918; δ_H (300 MHz; D_2O) 1.17 (2H, apparent t, *J* 7.1, 2 × Proγ-H_AH_B), 1.44 (3H, s, Gluα-CCH_3), 1.45 (3H, s, Gluα-CCH_3), 1.77–2.48 (14H, m, 2 × Proβ-H_2, 2 × Proγ-H_AH_B, 2 × Gluβ-H_2 and 2 × Gluγ-H_2), 3.47–3.75 (4.46H, m, 2 × Proδ-H_2 and 2 × Glyα-H_AH_B*), 3.87–4.13 (3.54H, m, 2 × Glyα-H_AH_B and 2 × Glyα-H_AH_B) and 4.38–4.52 (2H, m, 2 × Proα-H); δ_C (75 MHz; D_2O) 23.7* (CH_3, Gluα-C*C*H_3), 23.9* (CH_2, Proγ-C), 24.2 (CH_3, Gluα-C*C*H_3), 24.3 (CH_3, Gluα-C*C*H_3), 26.0 (CH_2, Proγ-C), 26.0 (CH_2, Proγ-C), 31.2 (CH_2, Proβ-C), 33.7* (CH_2, Proβ-C), 33.8* (CH_2, Proβ-C), 33.9 (CH_2, Gluγ-C), 34.2 (CH_2, Gluγ-C), 34.8* (CH_2, Gluγ-C), 42.1* (CH_2, Glyα-C), 42.3 (CH_2, Glyα-C), 48.7 (CH_2, Proδ-C), 49.4* (CH_2, Proδ-C), 61.9* (CH, Proα-C), 62.0* (CH, Proα-C), 62.7(quat., Gluα-C), 62.9 (CH, Proα-C), 63.0 (CH, Proα-C), 167.5 (quat., Gly-CO), 167.8* (quat., Gly-CO), 173.5* (quat., Pro-CON), 173.5* (quat., Pro-CON), 174.0 (quat., Pro-CON), 181.5 br (quat., Glu-CO), 181.8 br (quat., Glu-CO) and 182.9 br (quat., Glu-CO); *m/z* (FAB+) 316.1517 ($MH^+ \cdot C_{13}H_{22}N_3O_6$ requires 316.1509).

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