Synthesis, Resolution, and Assignment of Configuration of Potent Hypotensive Retro-inverso Bradykinin Potentiating Peptide 5a(BPP_{5a}) Analogues†

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The effect on the biological activity of an inverted amide group as an isosteric replacement for the Phe-Ala scissile bond of the BPP_{5a} analogue [Phe^3]BPP_{5a} has been studied. The synthesis of the retro-inverso pentapeptide [$gPhe^3$,(*R,S*)-$mAla^4$]BPP_{5a} has been carried out in solution by coupling the pseudopeptide epimer HO-(*R,S*)-mAla-Pro-OBu^t to Glp-Lys(Boc)-gPhe-H·HCl, separation and identification of the resulting diastereoisomers [Lys^2(Boc),$gPhe^3$,(*S*)-$mAla^4$,Pro^5-OBu^t]BPP_{5a} and [Lys^2(Boc),$gPhe^3$,(*R*)-$mAla^4$,Pro^5-OBu^t]BPP_{5a}, and finally acidolytic cleavage. [$gPhe^3$,(*S*)-$mAla^4$]BPP_{5a}, a moderate inhibitor of angiotensin converting enzyme (ACE) *in vitro* (I_{50} = 14 × 10^{-5}M), was obtained and proved to be more potent than BPP_{5a} as a hypotensive in normotensive rats without manifesting the bradykinin potentiating action of the natural peptide.

Modifications of peptide scissile bonds to give non-hydrolysable moieties have been carried out in simple synthetic peptide substrates and inhibitors of ACE to provide enzyme-resistant analogues.[1] Moderate inhibition has been observed with peptide analogues containing CH_2S, CH_2NH and *trans*-olefin groups as substitutes for the hydrolysable CONH bonds.[1,2] The 'keto methylene' modification ($COCH_2$ in place of CONH) of the scissile Phe-Ala bond in Bz-Phe-Ala-Pro-OH‡ has led, on the other hand, to the most potent ACE inhibitor prepared thus far.[3] An inverted amide group (NHCO in place of CONH) can also be incorporated into the peptide backbone at or adjacent to the scissile bond to prevent cleavage by ACE. Both planarity and configurational restriction of the CONH group are retained and the spatial orientation of the side chains remains closely related to that of the parent peptide.[4–7] Thus, a retro-inverso analogue of BPP_{5a} such as Glp-Lys-NH-$CH(CH_2Ph)$-NH-CO-$CH(CH_3)$-CO-Pro-OH which incorporates the geminal diamino analogue of phenylalanine and the 2-alkylmalonyl analogue of alanine at the Phe-Ala scissile bond should maintain the biological activity and acquire stability toward ACE degradation. Apart from the interchange of the carbonyl group and the secondary nitrogen, the retro-inverso pentapeptide retains all the crucial components for a strong interaction with the catalytic centre and with the auxiliary binding site of the postulated active site of the enzyme.[8]

The synthetic pathway for the preparation of the retro-inverso diastereoisomeric pentapeptides is shown in Scheme 1. Treatment of the peptide amide (**3**) with [bis(trifluoroacetoxy)-iodo]benzene followed by the addition of equimolar HCl (0.1M) in EtAc gave compound (**4**) as the hydrochloride salt in excellent yield and with retention of configuration at the chiral carbon of the geminal diamino residue.[9] Incorporation of the monoacyl geminal diaminoalkyl was achieved by the condensation of fragments (**4**) and (**6**) with DCC and HOBt as an additive. The resulting diastereoisomeric mixture was directly resolved into its components by reversed phase preparative h.p.l.c. using a water–acetonitrile solvent system (Figure 1). The diastereoisomers (**7A**) and (**7B**) were identified as shown in Scheme 2, through the following experiments: (*a*) separation of epimers (**6A**) and (**6B**) by preparative h.p.l.c.; (*b*) assignment of the absolute configuration of the 2-methylmalonyl residues in (**6A**) and (**6B**);[10] and (*c*) coupling of the epimers (**6A**) and (**6B**) to compound (**4**) followed by the analytical h.p.l.c. comparison of the resulting pentapeptide isomers to the mixture of diastereoisomers (**7**).

The identification of epimers (**6A**) and (**6B**), separated by reverse phase preparative h.p.l.c., using an ammonium acetate–0.01M-acetonitrile solvent system, was achieved by (*a*) synthesis of the epimers (**9A**) and (**9B**) by condensation of (**6A**) and (**6B**) with ammonia, added as its HOBt salt, and DCC; (*b*) Hofmann rearrangement of the epimers (**9A**) and (**9B**) with TIB in acqueous acetonitrile[11] to give the diastereoisomers (**10A**) and (**10B**); (*c*) removal of the t-butyl groups with hydrochloric acid; and (*d*) comparison of the resulting diastereoisomers (**11A**) and (**11B**) with chirally pure alanylproline hydrochloride and D-alanylproline hydrochloride by analytical ion-pair h.p.l.c.

On a C_{18} column with the solvent system sodium heptanesulphonate–0.005M-TFA (0.1%), single and well retained peaks were obtained for each dipeptide diastereoisomer, thus avoiding the peak splitting due to (i) the presence of the *cis–trans* forms and (*ii*) the slow kinetics of the *cis-trans* interconversion[12] (Figure 2).

The diastereoisomers (**11A**) and (**11B**) emerged at the same elution time as alanylproline hydrochloride and D-alanylproline hydrochloride respectively and showed identical mass, n.m.r., and i.r. spectra and elemental analyses. Hence, the optical configurations of the asymmetric carbon atoms of the 2-methylmalonyl residues in the epimers (**6A**) and (**6B**) were *S* and *R*, respectively. The coupling of compound (**4**) to the epimers (**6A**) and (**6B**) with DCC and HOBt as an additive led to the diastereoisomers (**12A**) and (**12B**) which, after purification, were compared by analytical h.p.l.c. with the crude diastereoisomeric mixture (**7**) in order to identify both components. The earlier emerging diastereoisomer (**7A**) had the *S* configuration for the asymmetric carbon of the 2-methylmalonyl residue. Once identified, the diastereoisomers (**7A**) and (**7B**) were acidolytically deblocked to yield the diastereoisomeric pentapeptide hydrochlorides (**8A**) and (**8B**) in *ca.* 70% overall yield.

The activity of angiotensin converting enzyme in the presence of retro-inverso BPP_{5a} analogues was determined as described by Cushman and Cheung.[13] The two peptides proved to be long-lasting (*ca.* 3 h against *ca.* 15 min for the natural peptide) and moderate inhibitors of ACE: [$gPhe^3$,(*S*)-$mAla^4$]BPP_{5a}, I_{50} = 14.0 × 10^{-5}M and [$gPhe^3$,(*R*)-$mAla^4$]BPP_{5a}, I_{50} =

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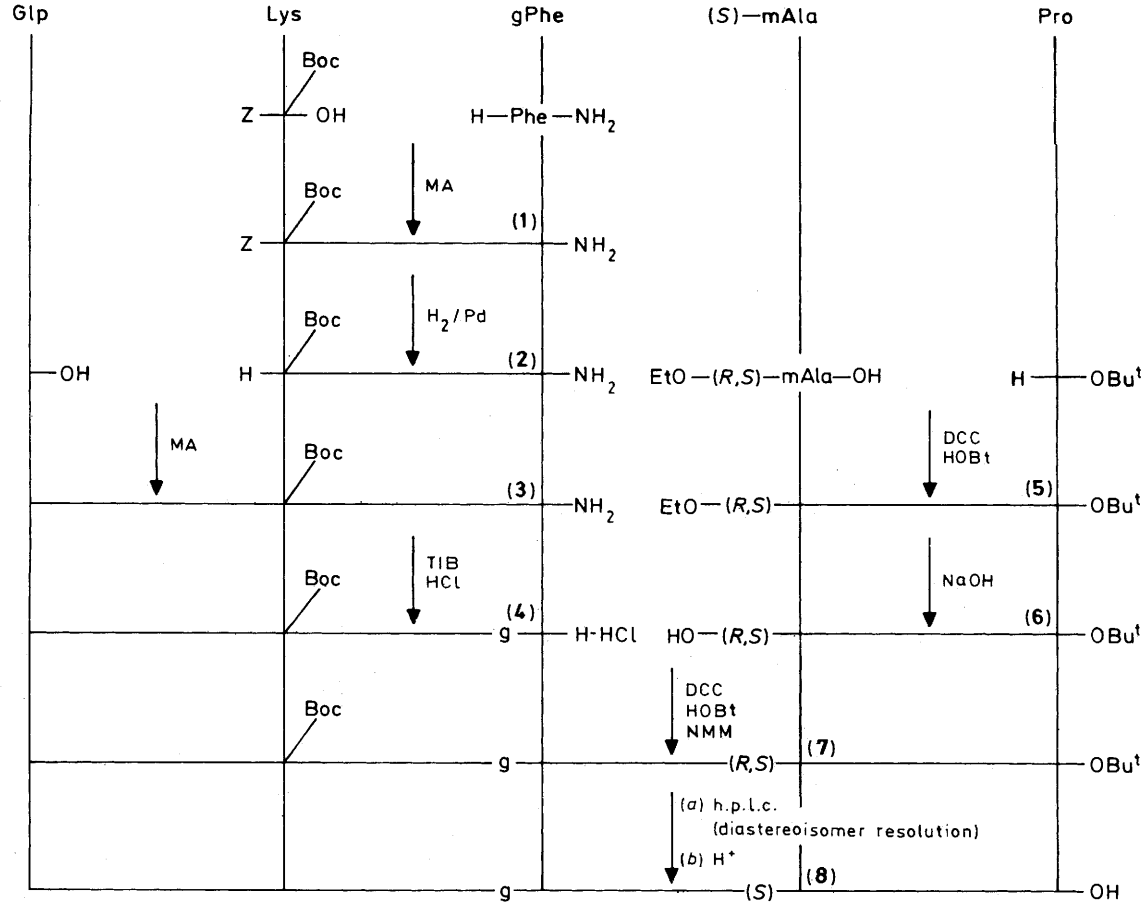
‡ Abbreviations used in the text: gPhe = $NHCH(CH_2Ph)NH$; mAla = $COCH(CH_3)CO$; TIB = [bis(trifluoroacetoxy)iodo]benzene; DCC = *N,N'*-dicyclohexylcarbodi-imide; HOBt = 1-hydroxybenzotriazole; DMF = *N,N'*-dimethylformamide; THF = tetrahydrofuran; MeOH = methanol; Et_2O = diethyl ether; EtAc = ethyl acetate; TFA = trifluoroacetic acid.



Scheme 1. Synthesis of [gPhe^3,(*S*)-mAla^4]BPP$_{5a}$

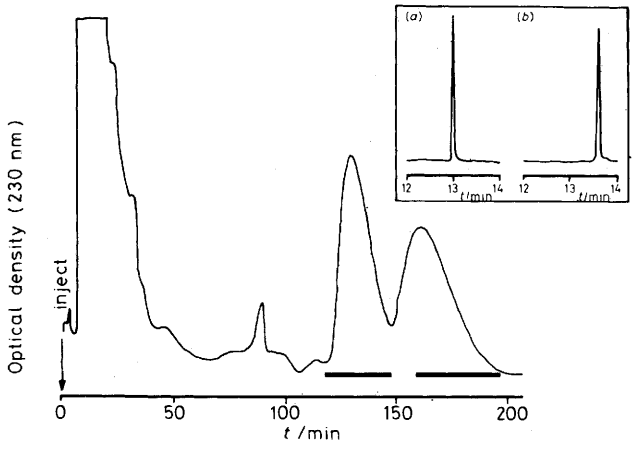


Figure 1. Preparative reverse-phase h.p.l.c. of [Lys^2(Boc),gPhe^3,(*R*,*S*)-mAla^4,Pro^5-OBu^t]BPP$_{5a}$. Chromatographic conditions are given in the Experimental section. Solid bars indicate the pooled fractions of each diastereoisomer analysed by h.p.l.c. (inset)

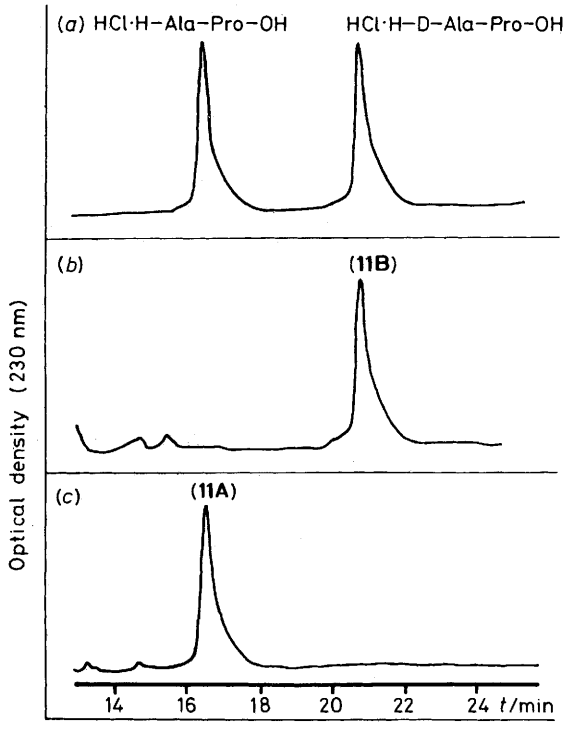
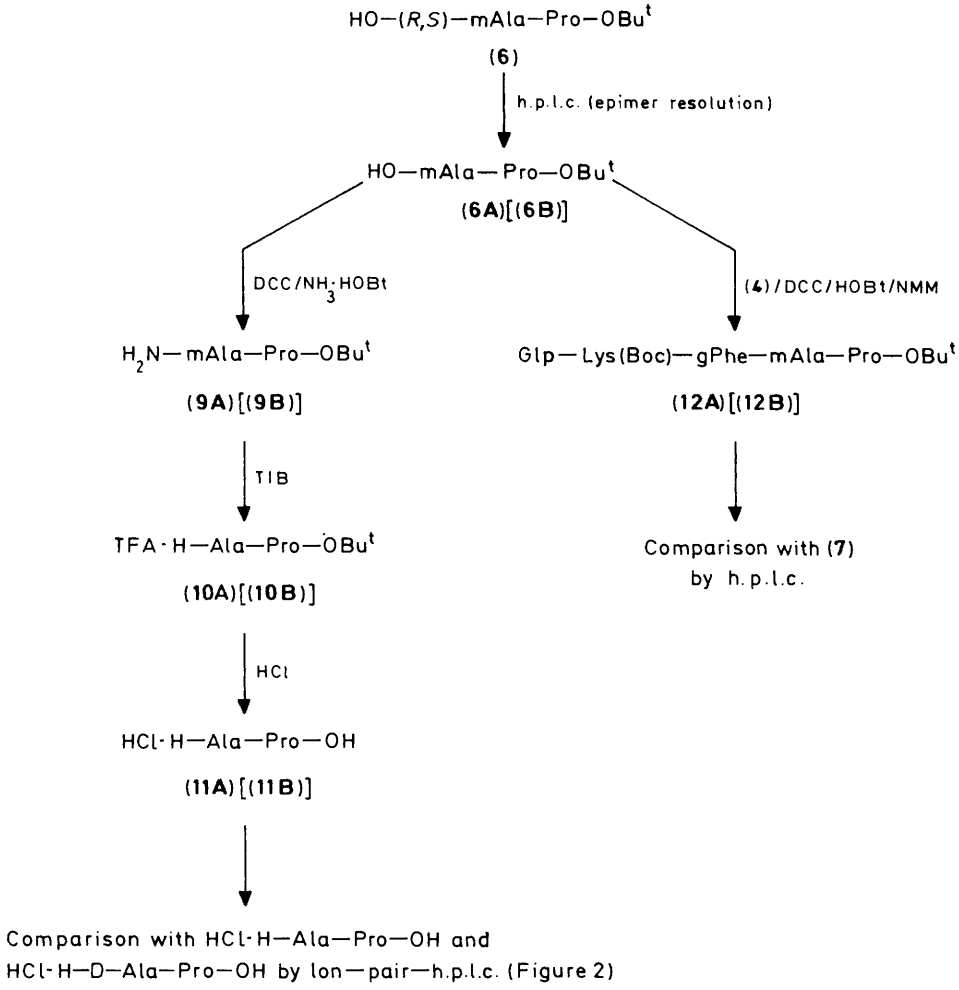


Figure 2. Identification of the absolute configuration of the 2-methylmalonyl residues in HO-mAla-Pro-OBu^t epimers

28.0 × 10^{-5}M. The hypotensive effect was demonstrated in standard pharmacological tests conducted in normotensive Sprague–Dawley male rats.[8]

[gPhe^3,(*S*)-mAla^4]BPP$_{5a}$ caused a decrease of 15 mmHg in the mean arterial blood pressure, at a dose of 0.185 mg/kg of body weight and no potentiation of bradykinin response. These findings, complemented by additional results from experimental



Scheme 2. Synthesis of HCl·H-Ala-Pro-OH, HCl·H-D-Ala-Pro-OH, and Glp-Lys(Boc)-gPhe-mAla-Pro-OBu^t diastereoisomers

animals,[14] suggest that, in contrast to what has been observed with the natural peptide, inhibition of ACE is a minor factor mediating the hypotensive action of [gPhe^3,(*S*)-mAla^4]BPP$_{5a}$.

Experimental

Analytical chromatography was performed with a high performance liquid chromatograph (Waters Associates), consisting of two Model 6000 A pumps, a WISP 710 B automated injector, and a System Controller and Data Module, coupled to a LC-85 Perkin-Elmer UV monitor; the chromatograph was equipped with a stainless-steel column (LiChrosorb RP-18, particle size 10 μm, Merck).

The preparative chromatographies were carried out on a liquid chromatograph (Miniprep, Jobin Yvon) equipped with a M45 Waters pump. The column was prepared by packing with LiChroprep RP-18 resin (35 g) particle size 25—40 μm (Merck).

A polarimeter model 141 M (Perkin-Elmer) was used for the measurements of the optical activity.

The ^{1}H n.m.r. spectra were recorded on a Bruker WP 80 DS 80 MHz spectrometer with tetramethylsilane as internal standard. The amino acid analyses were performed on a 119 CL Beckman amino acid analyser.

The products were identified on the basis of the H n.m.r. spectra and amino acid analyses. The purity of the products were checked by h.p.l.c. (eluant 0.01M-ammonium acetate–acetonitrile) and t.l.c. (eluant, butan-1-ol–acetic acid–water, 4:1:1; chloroform–methanol–acetic acid, 85:10:5). M.p.s were determined in open capillaries and are uncorrected.

The amino acid derivatives were purchased from Fluka or synthesized in our laboratory.

N$^\alpha$-Benzyloxycarbonyl-lysyl(N$^\varepsilon$-t-butyloxycarbonyl)phenylalaninamide, Z-Lys(Boc)-Phe-NH$_2$ (**1**).—To Z-Lys(Boc)-OH (4.50 g, 11.84 mmol) dissolved in dry THF (20 ml), cooled to −15 °C and kept under nitrogen, NMM (1.44 ml, 13.04 mmol) and isobutylchlorocarbonate (1.70 ml, 13.04 mmol) was added with vigorous stirring. After 2 min, H-Phe-NH$_2$ (1.96 g, 11.84 mmol) (obtained by hydrogenolysis of Z-Phe-NH$_2$ with 10% Pd on C), dissolved in dry DMF (10 ml), was added. During every addition the temperature never exceeded −10 °C. After equilibration at room temperature (*ca.* 1 h) the solvents were removed under reduced pressure, the residue dissolved in EtAc, and the solution washed with 5% aqueous ammonium hydrogen carbonate (twice), water, 5% aqueous citric acid (twice), and water. The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. Crystallization from MeOH–Et_2O yielded pure *compound* (**1**) (5.30 g, 85%), m.p. 182—184 °C, $[\alpha]_D^{22}$ −20.9° (*c* 1 in DMF) (Found: C, 63.6; H, 7.2; N, 10.6. $C_{28}H_{38}N_4O_6$ requires C, 63.8; H, 7.3; N, 10.6%).

Pyroglutamyl-lysyl(N$^\varepsilon$-t-butyloxycarbonyl)phenylalaninamide, Glp-Lys(Boc)-Phe-NH$_2$ (**3**).—A solution of pyroglutamic acid (1.50 g, 11.62 mmol) in dry THF (30 ml) was cooled to

−15 °C and kept under nitrogen, and NMM (1.40 ml, 12.78 mmol) and isobutylchlorocarbonate (1.67 ml, 12.78 mmol) were then added with vigorous stirring. After 2 min, H-Lys(Boc)Phe-NH_2 (**2**) (4.62 g, 11.62 mmol) [obtained by hydrogenolysis of (**1**) with 10% *Pd on C*], dissolved in dry DMF (10 ml), was added. After equilibration at room temperature (*ca.* 1 h) the solvents were removed under reduced pressure, the residue dissolved in EtAc, and the solution washed with 5% aqueous sodium hydrogen carbonate (twice), water, 5% aqueous citric acid (twice), and water. The organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The precipitate was washed with Et_2O and dried to yield pure *compound* (**3**) (5.03 g, 86%), m.p. 180—182 °C; $[\alpha]_D^{22}$ −19.68° (*c* 0.56 in DMF) (Found: C, 59.5; H, 7.35; N, 13.7. $C_{25}H_{37}N_5O_6$ requires C, 59.6; H, 7.4; N, 13.8%).

Pyroglutamyl-lysyl(N-*t-butyloxycarbonyl*)-gem-*diamino-benzylmethane Hydrochloride*, Glp-Lys(Boc)-gPhe-H·HCl (**4**).—Compound (**3**) (3.0 g, 5.96 mmol) suspended in MeCN–water (3:2 v/v; 20 ml) was treated with TIB (2.63 g, 6.11 mmol) dissolved in MeCN (5 ml). A nitrogen flux was maintained during the reaction to remove the CO_2 produced. After 3 h the reaction mixture was evaporated under reduced pressure and the residue dissolved in EtAc and treated with 0.45M-HCl in EtAc (13.24 ml, 5.9 mmol). A white solid was obtained by addition of Et_2O. After filtration, the product was washed several times with Et_2O and dried to yield pure compound (**4**) (2.44 g, 80%), m.p. 130—133 °C (decomp.), $[\alpha]_D^{22}$ −25.41° (*c* 1.22 in DMF) (Found: C, 56.2; H, 7.4; N, 13.6. $C_{24}H_{38}N_5O_5Cl$ requires C, 56.3; H, 7.5; N, 13.6%).

Ethyl Ester of 2-*Methyl*-(R,S)-*malonylproline t-Butyl Ester*, EtO-(*R,S*)-mAla-Pro-OBu^t (**5**).—To the monoethyl ester of 2-methyl-(*R,S*)-malonic acid (2.0 g, 13.70 mmol), dissolved in CH_2Cl_2 (10 ml), HOBt (2.02 g, 15.07 mmol), dissolved in DMF (5 ml), was added at room temperature. The mixture was cooled to 0 °C and then treated with DCC (3.11 g, 15.07 mmol). After 20 min, H-Pro-OBu^t (2.58 g, 15.07 mmol) dissolved in CH_2Cl_2 (10 ml) was added to the mixture. After being warmed to room temperature the mixture was allowed to react overnight. The reaction mixture was then filtered, the solvent evaporated under reduced pressure and the residue, dissolved in EtAc, was washed with 5% aqueous sodium hydrogen carbonate (twice), water, 5% aqueous citric acid (twice), and water. The organic layer was dried ($MgSO_4$) and, after evaporation under reduced pressure, the pure *product* (**5**) was obtained as a colourless oil (2.87 g, 70%), $[\alpha]_D^{22}$ −21.18° (*c* 1 in DMF) (Found: C, 60.0; H, 8.3; N, 4.6. $C_{15}H_{25}NO_5$ requires C, 60.2; H, 8.4; N, 4.7%).

2-*Methyl*-(R,S)-*malonylproline t-Butyl Ester*, HO-(*R,S*)-mAla-Pro-OBu^t (**6**).—Compound (**5**) (2.69 g, 9.00 mmol), dissolved in MeOH (30 ml), was allowed to react with a solution of 1N-sodium hydroxide (20 ml) for 20 min. Then water (40 ml) was added and the bulk of the methanol removed by evaporation. After washing with Et_2O (30 ml, twice), the aqueous solution was acidified with sulphuric acid to pH 2 and extracted several times with EtAc. The organic layers were dried ($MgSO_4$) and, after evaporation, the pure *product* (**6**) was obtained as a colourless oil (2.20 g, 90%), $[\alpha]_D^{22}$ −53.16° (*c* 1.25 in DMF) (Found: C, 57.5; H, 7.75; N, 5.1. $C_{13}H_{21}NO_5$ requires C, 57.55; H, 7.8; N, 5.2%).

Pyroglutamyl-lysyl(N$^\varepsilon$-*t-butyloxycarbonyl*)-gem-*phenyl-alanyl*-2-*methyl*-(R,S)-*malonylproline t-Butyl Ester*, Glp-Lys-(Boc)-gPhe-(*R,S*)-mAla-Pro-OBu^t (**7**).—Compounds (**6**) (1.50 g, 5.53 mmol) and (**4**) (2.83 g, 5.53 mmol) and HOBt (0.82 g, 6.08 mmol) were dissolved in DMF (10 ml). After the mixture had been cooled to 0 °C, DCC (1.54 g, 6.08 mmol), dissolved in DMF (3 ml) and NMM (0.67 ml, 6.08 mmol, were added and the mixture stirred for 1 h at 0 °C and for a night at room temperature. After elimination of the precipitate by filtration, the solution was evaporated under reduced pressure, the residue dissolved in EtAc and washed with 5% aqueous sodium hydrogen carbonate (twice), water, 5% aqueous citric acid (twice), and water. After drying ($MgSO_4$), the pure product (2.74 g, 68%) was obtained by careful precipitation with light petroleum of a dilute solution of the crude material in ethanol. The diastereoisomers were resolved by preparative RP-h.p.l.c. (water–MeCN 33.5%, flow rate 5 ml/min) of the product (**7**) (0.3 g) dissolved in MeOH–H_2O (1:1 v/v; 4 ml). The pure diastereoisomers (**7A**) (first eluting peak, 0.140 g) and (**7B**) (second peak, 0.100 g) were isolated by lyophilization after removal of acetonitrile under reduced pressure. *Diastereoisomers* (**7A**) first peak: m.p. 173—175 °C, $[\alpha]_D^{22}$ −28.84° (*c* 1.1 in MeOH) (Found: C, 60.8; H, 7.7; N, 11.5. $C_{37}H_{56}N_6O_9$ requires 60.9; H, 7.75; N, 11.55%). *Diastereoisomers* (**7B**), second peak: m.p. 135—130 °C, $[\alpha]_D^{22}$ −38.63° (*c* 0.55 in MeOH) (Found: C, 60.8; H, 7.7; N, 11.5. $C_{37}H_{56}N_5O_9$ requires C, 61.0; H, 7.75; N, 11.5%).

Resolution of the Epimers (**6**) *and Identification of the Absolute Configuration of the* 2-*Methylmalonyl Residue in the Epimers* (**6A**) *and* (**6B**).—(i) Resolution of the epimeric mixture (**6**) was carried out by preparative RP-h.p.l.c. [0.01M-ammonium acetate–MeCN (5%), 5ml/min] of (**6**) (0.60 g) dissolved in MeCN–H_2O (2:1 v/v; 2 ml). The two peaks were separately collected and the acetonitrile removed under reduced pressure. The pure diastereoisomers (**6A**) (first peak, 0.222 g) and (**6B**) (second peak, 0.180 g) were obtained by lyophilization. *Epimer* (**6A**) first eluting peak: $[\alpha]_D^{22}$ −96.36° (*c* 1.3 in MeOH) (Found: C, 57.5; H, 7.75; N, 5.1. $C_{13}H_{21}NO_5$ requires C, 57.55; H, 7.80; N, 5.1%). *Epimer* (**6B**) second eluting peak: $[\alpha]_D^{22}$ −152.3° (*c* 1.2 in MeOH) (Found: C, 57.45; H, 7.7; N, 5.1%).

(ii) Compound (**6A**) (0.10 g, 0.37 mmol) was dissolved in CH_2Cl_2 (5 ml) and cooled to 0 °C; then NH_3·HOBt (0.62 g, 0.41 mmol) dissolved in DMF (3 ml), and DCC (0.846 g, 0.41 mmol) dissolved in CH_2Cl_2 (3 ml), were added. The mixture was stirred for 1 h at 0 °C and for a night at room temperature. The solvents were removed under reduced pressure, and the residue, dissolved in MeOH–H_2O (50:50 v/v; 3 ml), was purified by a preparative RP-h.p.l.c. (0.01M-ammonium acetate–MeCN 13%, flow rate 5 ml/min). In these chromatographic conditions the diastereoisomers of H_2N-(*R,S*)-mAla-Pro-OBu^t were not resolved as demonstrated by separate experiments. After removal of the organic solvent, pure compound (**9A**) was recovered by lyophilization.

(iii) Compound (**9A**) (0.65 g, 0.24 mmol), dissolved in MeCN–water (3:2 v/v; 10 ml) was treated with TIB (0.124 g. 0.29 mmol), dissolved in MeCN (3 ml). After 5 h the reaction mixture was evaporated under reduced pressure, and the residue (**10A**) dissolved in 4.5M-HCl in EtAc (10 ml). After 30 min the solvent was evaporated, the oily residue (**11A**) washed with Et_2O, dissolved in MeOH and compared by RP-h.p.l.c. with HCl·H-Ala-Pro-OH and HCl·H-D-Ala-Pro-OH synthesized through an independent chemical route. The eluant was prepared by dissolving PIC B-7 reagent (Waters) and TFA (1 ml) in water (1 000 ml).

The identification of the absolute configuration of the 2-methylmalonyl residue in (**6B**) was carried out by the same procedure.

Pyroglutamyl-lysyl(N$^\varepsilon$-*t-butyloxycarbonyl*)-gem-*phenyl-alanyl*-2-*methyl*-(S)-*malonylproline t-Butyl Ester*, Glp-Lys-(Boc)-gPhe-(S)-mAla-Pro-OBu^t (**12A**).—Compounds (**6A**) (0.12 g, 0.44 mmol), and (**4**) (0.23 g, 0.44 mmol) and HOBt (0.65 g, 0.48 mmol) were dissolved in DMF (10 ml). After being cooled to

0 °C, DCC (0.95 g, 0.48 mmol) dissolved in DMF (5 ml) and NMM (52.8 ml, 0.48 mmol) were added to the mixture. Stirring was continued for 1 h at 0 °C and overnight at room temperature. The precipitate was filtered, the filtrate evaporated under reduced pressure, and the residue dissolved in EtAc. After being washed with water, 5% aqueous sodium hydrogen carbonate, water, 5% aqueous citric acid, and water, the organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The resulting product was dissolved in MeOH and used as a chromatographic standard for the assignment of the absolute configuration of the 2-methylmalonyl residue in the diastereoisomeric mixture of (**7A**) and (**7B**).

The synthesis of pyroglutamyl-lysyl (N^{ε}-t-butyloxycarbonyl)-*gem*-phenylalanyl-2-methyl-(*R*)-malonylproline t-butyl ester (**12B**) was carried out by the same procedure. Compound (**12B**) was used as a chromatographic standard for the assignment of the absolute configuration of the 2-methylmalonyl residue in the diastereoisomeric mixture of compounds (**7A**) and (**7B**).

Pyroglutamyl-lysyl-gem-*phenylalanyl-2-methyl*-(S)-*malonyl-proline*, Glp-Lys-gPhe-(S)-mAla-Pro-OH (**8A**).—Compound (**7A**) (0.10 g, 0.14 mmol) was treated with 4.5M-HCl in EtAc (10 ml), for 30 min. The mixture was then evaporated under reduced pressure and the residue purified by preparative RP-h.p.l.c. [0.01M-ammonium acetate–MeCN (9%), flow rate 5 ml/min]. The desired peak was collected, and the pure *product* (**8A**) (0.62 g, 73%) isolated by lyophilization after evaporation of acetonitrile; $[\alpha]_D^{22}$ −29.08° (*c* 0.85 in MeOH) (Found: C, 55.2; H, 6.7; N, 13.8. $C_{28}H_{41}N_6O_7Cl$ requires C, 55.2; H, 6.8; N, 13.8%).

Pyroglutamyl-lysyl-gem-*phenylalanyl-2-methyl*-(R)-*malonyl-proline*, Glp-Lys-gPhe-(R)-mAla-Pro-OH (**8B**).—The synthesis of this diastereoisomer was carried out as described for compound (**8A**). The pure *product* (**8B**) had $[\alpha]_D^{22}$ −44.43° (*c* 1.3 in MeOH) (Found: 55.1; H, 6.6; N, 13.7%).

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