Synthesis of Heteroaromatic Thyrotropin-releasing Hormone Analogues

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Novel thyrotropin-releasing hormone (TRH) analogues containing the unnatural and heteroaromatic L-pyrrolylalanine, D-pyrrolylalanine, and L-furylalanine amino acids in position 2 have been synthesised. L-Furylalanine was obtained by the stereospecific hydrolysis of the *N*-acetyl-D,L-amino acid with acylase I. A protected derivative of D,L-pyrrolylalanine was prepared, the racemate was incorporated into the tripeptide and the LLL and LDL diastereoisomers were separated at the end of the synthesis.

Thyrotropin-releasing hormone (TRH, pGlu-His-Pro-NH_2) is a hypothalamic peptide which governs the release of thyroid stimulating hormone (TSH) and prolactin from the pituitary. Modification of the central residue of TRH has previously been shown to lead to drastic effects on the biological activity. [(N^{3im}-Me-His)^2]-TRH (**2**)[1] is 8 times more active and [β-(pyrazolyl-1)-ala^2]-TRH (**4**)[2] is 1.5 times more active than TRH in releasing TSH although the isomeric [(N^{1im}-Me-His)^2]-TRH (**3**) and [β-(pyrazolyl-3)-ala^2]-TRH (**5**) analogues have only low activity (5% and 0.04%, respectively). pGlu-Thi-Pro-NH_2(Thi^2-TRH) (**6**)[3] is less active than the parent peptide.

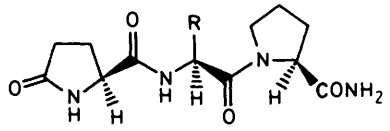
The overall activity of a molecule is a result of several factors and in TRH neither the aromaticity of the imidazole ring nor its basic character is exclusively responsible for overall activity.[4] The enhanced activity of the N^{3im} methyl analogue is thought to be due to a greater binding affinity for the TRH receptor possibly as a result of changes in the electron density on the nitrogen atoms and/or steric interactions.

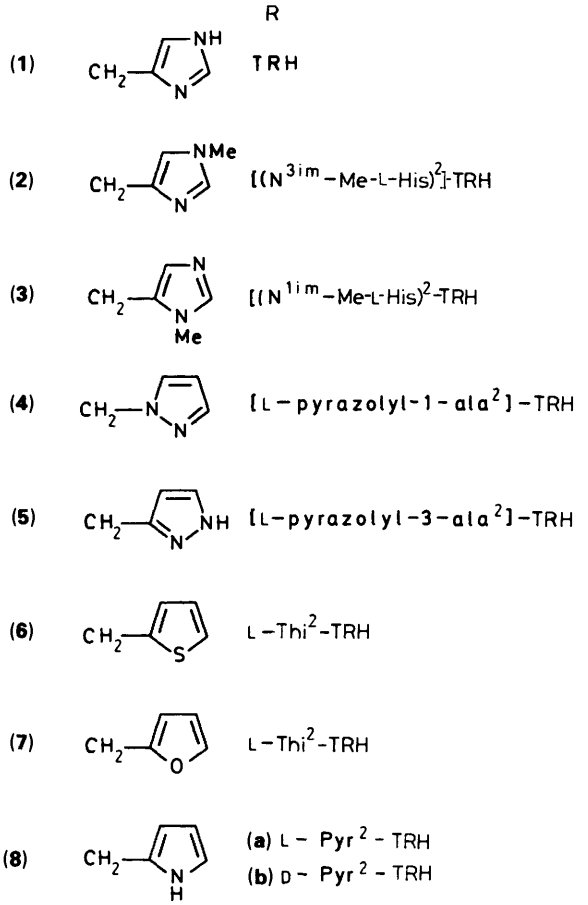
To further investigate the role of the central amino acid in maintaining TRH activity the L- and D-pyrrolyl tripeptide analogues (**8a, b**) have been synthesised for biological evaluation in addition to the L-furyl (**7**) and active L-thienyl (**6**) analogues.

The tripeptides were prepared using solution methods of synthesis with t-butoxycarbonyl (Boc) and benzyloxycarbonyl (Z) as *N*-protecting groups and pentafluorophenyl ester or dicyclohexylcarbodi-imide (DCC) activation of the carboxylic acid group. Thi^2-TRH (**6**) and Fur^2-TRH (**7**) were prepared using the optically active heteroaromatic amino acids (Schemes 1 and 2, respectively) but in the synthesis of Pyr^2-TRH (**8**) (Scheme 3) the racemic amino acid was incorporated and the LLL and LDL diastereoisomers separated at the end of the synthesis.

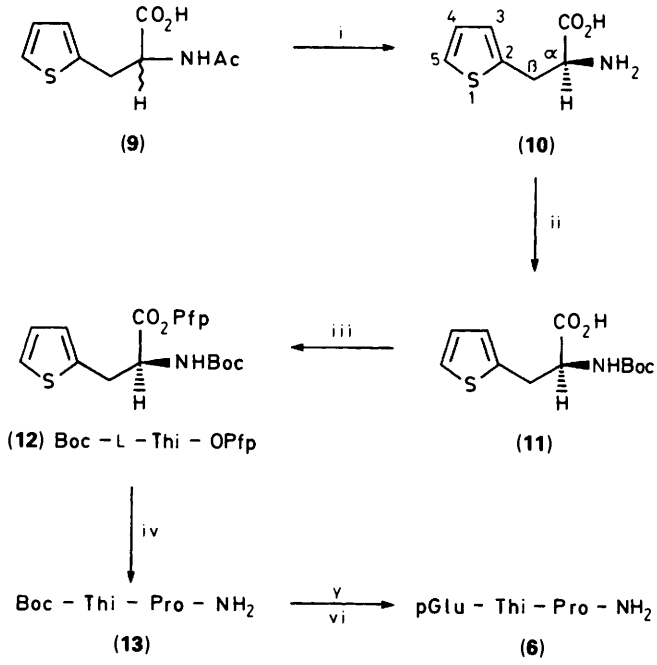
L-Thienylalanine (**10**) was prepared from the racemic *N*-acetyl amino acid (**9**) (Scheme 1) by treatment with the enzyme acylase I which selectively hydrolysed the L-acetamide. The optically active amino acid was then derivatised by first protecting the α-amine with the Boc group and then activating the carboxylic acid by formation of the pentafluorophenyl ester. Preparation of the Thi^2-TRH tripeptide (**6**) was straightforward. Reaction of the derivatised L-thienylalanine (**12**) and proline amide gave the protected dipeptide (**13**). Removal of the Boc group with trifluoroacetic acid, followed by addition of the pyroglutamic acid residue gave crystalline Thi^2-TRH (**6**) in 35% yield from (**12**).

Synthesis of the furylalanyl peptide (**7**) (Scheme 2) was similar and used Boc-L-Fur-OPfp (**20**). Racemic furylalanine was obtained *via* the diethylacetamidomalonate route, similar to the ethylcyanoacetamidomalonate method of Herz *et al.*[5] Furfuryl





chloride was condensed with the sodium salt of diethyl acetamidomalonate to give (**14**). Partial hydrolysis with potassium hydroxide gave the monoacid–monoester (**15**) which was decarboxylated by boiling in dioxane. Further treatment with potassium hydroxide to hydrolyse the ethyl ester, followed by enzymic hydrolysis of the acetamide, gave L-furylalanine (**18**). Derivatisation of the optically active amino acid and the

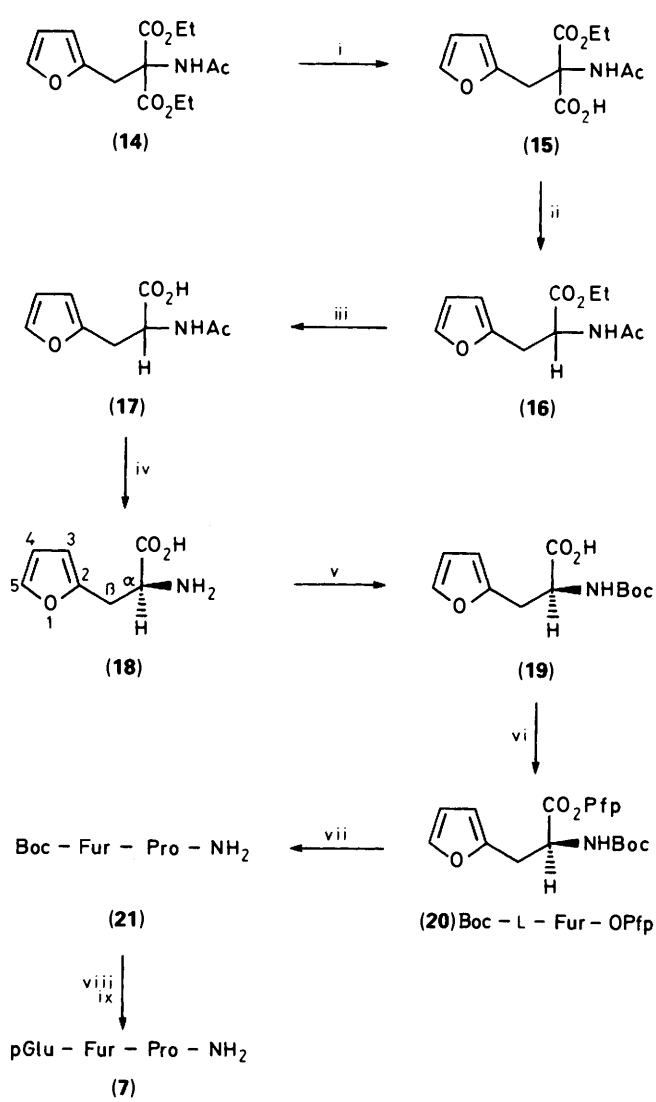


Scheme 1. *Reagents and conditions:* i, acylase I, 38 °C; ii, $(Boc)_2O$, dioxane–H_2O; iii, C_6F_5OH, DCC/EtOH; iv, $ProNH_2$, DMF; v, TFA; vi, pGlu-OPfp, NEt_3, DMF.

synthesis of Fur²-TRH was identical to that described above for thienylalanine and Fur²-TRH was isolated as a white powder (54%) yield from (**20**).

Investigations into the synthesis of β-2-pyrrolylalanine *via* the malonate pathway have previously been undertaken. Herz and co-workers[6] reported a product which was believed to contain pyrrolylalanine whilst Hanck and Kutscher[7] synthesised the amino acid in low yield from diethylaminoethylpyrrole and diethylformamidomalonate. The problem with the diethylacetamidomalonate route lay in the basic hydrolysis of the ethyl ester. This route was reinvestigated (Scheme 4) and it was found that if the pyrrole nitrogen was protected with, for example, the Boc group, the hydrolysis of the ethyl ester of (**24**) gave a reasonable yield of the crystalline monoacid (**25**). It proved impossible, however, to remove the acetyl group; Ac-Pyr(Boc)-OH (**25**) was either too insoluble to react in aqueous solution with acyclase or a complex mixture of products was obtained if subjected to acid hydrolysis. Removal of the *N*-acetyl group from the dipeptide (**26**) also failed. To overcome the problems associated with removal of the acetyl group the *N*(α)-benzyloxycarbonyl derivative (**31**) (Scheme 3) was prepared and this compound proved to be a useful intermediate and led to a successful synthesis of the pyrrolylalanyl tripeptide.

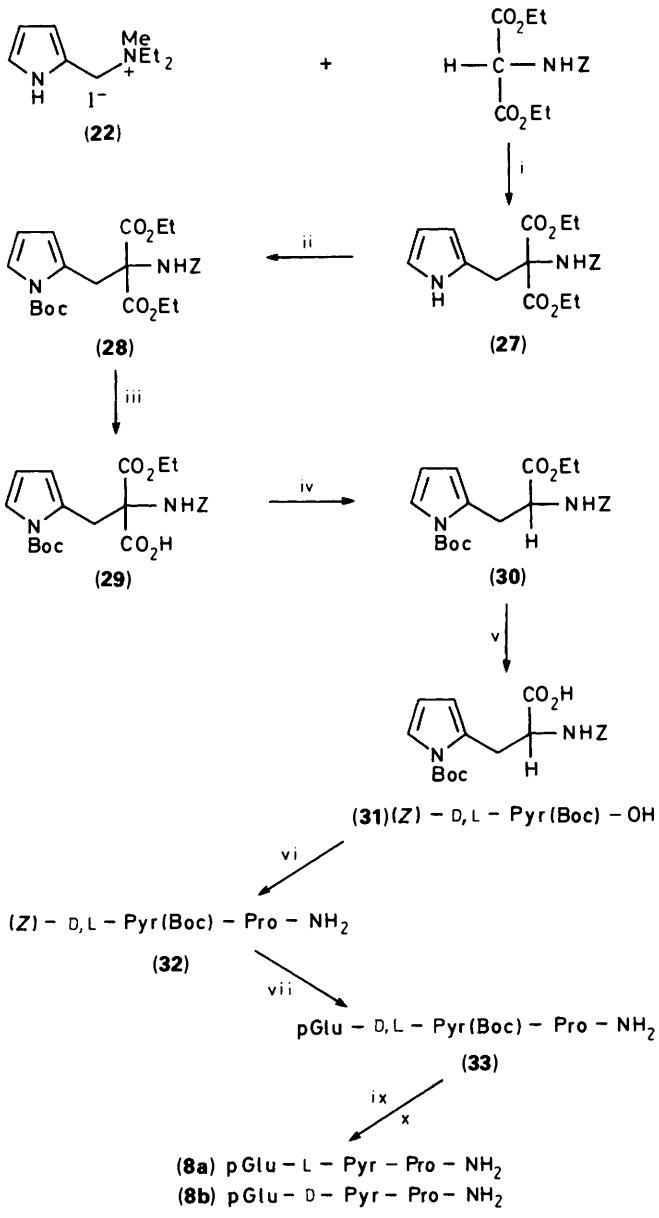
Diethylaminoethylpyrrole methiodide (**22**) (Scheme 3) was condensed with diethyl benzyloxycarbonylaminomalonate to give the diethyl ester (**27**) (*cf.* Scheme 4 in which the monoester (**23**) was isolated directly from the condensation reaction). Protection of the pyrrole nitrogen of (**27**) with the Boc group, followed by a similar series of reactions as with furylalanine, *viz a viz* partial hydrolysis, decarboxylation and a second hydrolysis gave the pyrrolylalanine derivative (**31**). This compound was used in peptide synthesis as the racemate since proteolytic enzyme hydrolysis of the ethyl ester (**30**) failed.[8] The benzyloxycarbonylpyrrole compounds were all isolated as oils and were purified, as appropriate, by silica chromatography. The pyrrolylalanine (**31**) and proline amide were coupled in the presence of DCC to give, after purification, the dipeptide (**32**) (71%). Hydrogenolysis removed the benzyloxycarboxyl group and the deprotected dipeptide reacted with pyroglutamic acid



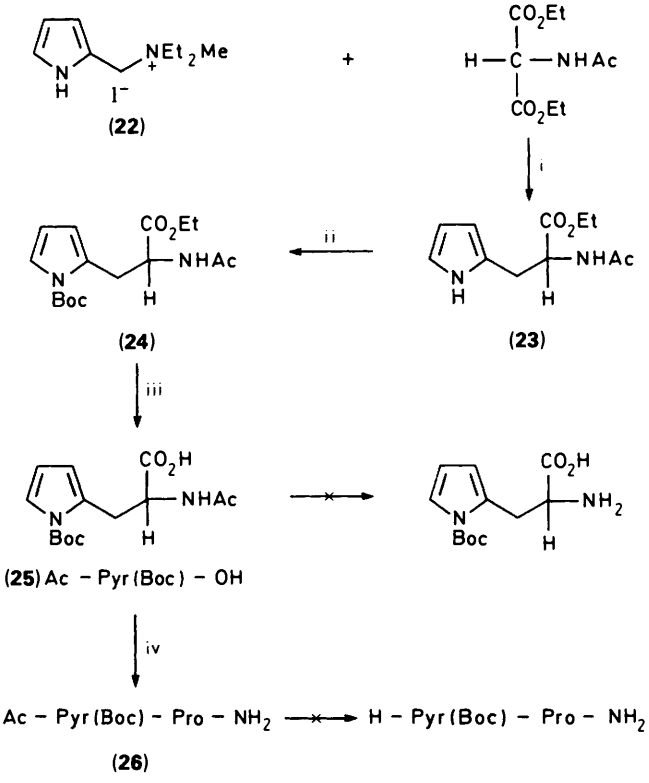
Scheme 2. *Reagents and conditions:* i, KOH, EtOH; ii, dioxane, 101 °C; iii, KOH, EtOH; iv, acylase I, 38 °C; v, $(Boc)_2O$, dioxane–H_2O; vi, C_6F_5OH, DCC, EtOH; vii, Pro-NH_2, DMF; viii, TFA; ix, pGlu-OPfp, NEt_3, DMF.

pentachlorophenyl ester to give the protected tripeptide (**33**). Removal of the Boc group from the pyrrole nitrogen with trifluoroacetic acid gave the pyrrolylalanyl tripeptide (**8**) as a diastereoisomeric LLL and LDL mixture. The isomers were separated by semi-preparative reverse-phase HPLC and, after lyophilisation, were obtained as an off-white powder and as a pale yellow powder (**8a**, **b**). Separation of the diastereoisomers at the earlier dipeptide stage (**32**) was attempted by preparative TLC but a satisfactory separation was not achieved although on analytical plates two distinct bands had been observed.

The four TRH analogues (**6**), (**7**), and (**8a**, **b**) were analysed by ^1H and ^{13}C NMR spectroscopy and assignments, based on the published data available for TRH[9–11] and Thi²-TRH,[12] are given in Tables 1 and 2. The overall appearance of the proton spectra for TRH, Thi²-TRH, Fur²-TRH and the off-white Pyr²-TRH were very similar. This suggested that the off-white Pyr²-TRH compound was the LLL diastereoisomer and was adopting a conformation, in D_2O, similar to that of TRH. The pale yellow Pyr²-TRH compound, assigned by default to possess LDL stereochemistry, produced a proton spectrum which was substantially different. One of the δ-protons of proline resonates at a higher field (upfield shift of *ca.* 0.4 ppm) and overlaps with the



Scheme 3. *Reagents and conditions:* i, NaOH, EtOH, 78 °C; ii, $(Boc)_2O$, CH_3CN; iii, NaOH, EtOH; iv, dioxane, 101 °C; v, NaOH, EtOH; vi, Pro-NH_2, DCC, CH_2Cl_2; vii, H_2, Pd/C, EtOH; viii, pGlu-OPcp, NEt_3, DMF; ix, TFA; x, HPLC separation.



Scheme 4. *Reagents and conditions:* i, NaOH, EtOH, 78 °C; ii, $(Boc)_2O$, CH_3CN; iii, KOH, EtOH; iv, Pro-NH_2, DCC, CH_2Cl_2.

β-proton resonances of the pyrrolylalanine residue. Furthermore, the α-proton resonances of the proline and pyroglutamic acid residues were no longer separate but overlapped. In TRH the histidine side chain is preferentially folded back over the prolinamide residue and the imidazole ring significantly influences the δ-proline protons. Clearly, as the D-pyrrolylalanyl residue of Pyr^2-TRH adopts a position different to that in the L isomer the chemical shifts of the δ-proline protons will be markedly altered. This type of effect was indeed observed in the spectrum of (**8b**) supporting the assumption that the pale yellow compound was the LDL Pyr^2-TRH diastereoisomer.

The ^{13}C NMR spectra of the TRH analogues is presented in Table 2. In each of the spectra the carbon resonances were accompanied by a weaker peak resulting from the *cis* proline isomer. The level of *cis* isomer is known to be solvent dependent and in D_2O approximately 5–15% of each tripeptide existed in the *cis* form.

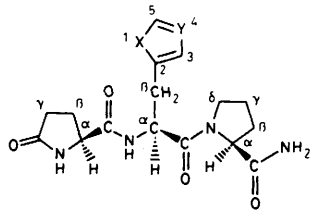
The TRH analogues (**6**), (**7**), and (**8a**, **b**) are currently being evaluated for their TSH and prolactin releasing activity and the results will be reported elsewhere.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured on an Optical Activity polarimeter using a 1 dm cell.* ^1H NMR spectra were recorded on a Perkin-Elmer R32 (90 MHz) or a Bruker WM 250 (250 MHz) as indicated, and ^{13}C NMR spectra were recorded on the Bruker WM 250 operating at 62.9 MHz. Fast atom bombardment mass spectra were recorded on a Kratos MS 50TC instrument. Microanalyses were carried out by Butterworth Laboratories. HPLC was performed on a Gilson system using 10 micron Brownlee Aquapore RP300 columns and linear gradient elution with 0.1% aqueous TFA (solvent A) and 90% CH_3CN–10% solvent A (solvent B). The eluant from the column was monitored at 230 nm. Samples for amino acid analysis were prepared by sealed tube hydrolysis with constant boiling HCl at 110 °C for 18 h and analysed on a LKB 4150 instrument. Analytical TLC was carried out on Merck Kieselgel 60 F254 plates using CH_2Cl_2–MeOH (9:1), preparative purifications were carried out on silica columns using either Kieselgel 60 or 60–120 mesh grade; and visualisation of the compounds was achieved with either iodine vapour or short wave UV absorption. Most solvents were distilled before use.

* Insufficient material precluded accurate optical rotations being recorded for the peptides (**6**), (**7**), (**8a**, **b**).

Table 1. ^{1}H NMR spectra (D_2O, ref. Bu^{t}OH) of TRH analogues (**6**), (**7**), and (**8a, b**).[a]



	X	Y
TRH	NH	N
Thi^2-TRH	S	CH
Fur^2-TRH	O	CH
Pyr^2-TRH	NH	CH

	TRH	Thi^2-TRH (**6**)	Fur^2-TRH (**7**)	L-Pyr^2-TRH (**8a**)	D-Pyr^2-TRH (**8b**)
pGlu	H_α 4.29 (dd) *J* 8.9, 4.8 Hz	H_α 4.29 (dd) *J* 9.0, 4.7 Hz	H_α 4.29 (dd) *J* 9.0, 4.7 Hz	H_α 4.29 (dd) *J* 9.1, 4.6 Hz	H_α 4.29–4.36 (2 H, m)
Xaa	5-H 7.70 (s) 3-H 7.01 (s)	5-H 7.33 (dd) 4-H, 3-H 7.00 (m) *J* 4.8, 1.5 Hz	5-H 7.45 (d) 4-H 6.39 (dd) 3-H, 6.25 (d) *J* 3.1, 1.8 Hz	5-H 6.79 (m) 4-H 6.12 (m) 3-H 6.05 (m)	5-H 6.78 (dd) 4-H 6.12 (t) 3-H 5.99 (m) *J* 3.2, 1.5 Hz
	H_α 4.90 His ABX system β, β′, H: 3.08, 2.99 *J* 7.2 Hz	H_α 4.94 Thi ABX system β, β′, H: 3.42, 3.22 *J* 15.0, 9.1, 5.4 Hz	H_α 4.38 Fur ABX system β, β′, H: 3.20, 3.05 *J* 15.2, 8.9, 5.5 Hz	H_α 4.90 Pyr ABX system β, β′, H: 3.13, 3.04 *J* 15.4, 7.6, 7.3 Hz	H_α *ca.* 4.9 Pyr ABX system β, β′, H: 2.99–3.12 (3 H, m)
Pro	H_α 4.38 (dd) *J* 8.8, 4.9 Hz δ, δ′, H: 3.80 (m), 3.54 (m)	H_α 4.39 (dd) *J* 8.5, 5.0 Hz δ, δ′, H: 3.83 (m), 3.70 (m)	H_α 4.38 (dd) *J* 8.6, 5.1 Hz δ, δ′, H: 3.82 (m), 3.65 (m)	H_α 4.38 (dd) *J* 8.5, 5.4 Hz δ, δ′, H: 3.78 (m), 3.44 (m)	H_α 4.29–4.36 (2 H, m) δ, δ′, H: 3.74 (1 H, m)

[a] The ββ′ and γγ′ Pro and pGlu resonances occur as complex multiplets between 1.5 and 2.5 ppm.

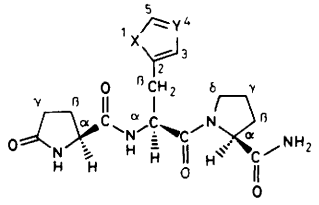
D,L-N-*Acetyl*-β-2-*thienylalanine* (**9**).—D,L-Thienylalanine (Fluka, 1.5 g, 8.8 mmol) was dissolved with warming in glacial acetic acid (75 ml) and acetic anhydride (1.64 ml; 7.5 mmol) was added. The solution was stirred at 60 °C for 45 min, evaporated and the crystalline residue recrystallised from ethyl acetate to afford the acetyl amino acid (**9**) (1.74 g, 93%); m.p. 129–130 °C (lit.,[13] 130 °C). δ_H([$^2H_6$]DMSO, 250 MHz) 1.82 (3 H, s, CH_3), 3.07 (1 H, dd, $J_{\beta,\beta'}$ 14.9 Hz, $J_{\alpha,\beta}$ 9.0 Hz, CH_β), 3.26 (1 H, dd, $J_{\beta,\beta'}$ 14.9 Hz, $J_{\alpha,\beta'}$ 4.8 Hz, $CH_{\beta'}$), 4.38 (1 H, ddd, $J_{\alpha,\beta}$ 9.0 Hz, $J_{\alpha,\beta'}$ 4.8 Hz, $J_{CH,NH}$ 8.2 Hz, CH_α), 6.88 (1 H, dd, $J_{3,4}$ 3.5 Hz, $J_{3,5}$ 1.3 Hz, 3-H), 6.93 (1 H, dd, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 5.1 Hz, 4-H), 7.33 (1 H, dd, $J_{3,5}$ 1.3 Hz, $J_{4,5}$ 5.1 Hz, 5-H), and 8.22 (1 H, d, $J_{CH,NH}$ 8.2 Hz, NH).

L-β-2-*Thienylalanine* (**10**).—The procedure used for the enantioselective hydrolysis is that followed by Leukart *et al.*[14] Racemic *N*-acetyl-β-2-thienylalanine (**9**) (1.49 g, 7 mmol) was dissolved in water (75 ml) and the pH was adjusted to 7–8 with 25% aq. ammonium hydroxide. Acylase I (Sigma, 1.54 mg) and cobalt acetate (6.05 mg) were added and the solution was heated at 38 °C for 18 h. A further portion of acylase I (1.62 mg) was added and the hydrolysis was continued for another 4 h. Trifluorocetic acid (0.5 ml) was added and the solution was heated at 50 °C for 10 min to denature the enzyme. After addition of charcoal, the mixture was filtered and the pH of the filtrate adjusted to 7 with 25% aq. ammonium hydroxide. Most of the solvent was evaporated and the residue was treated with acetone to precipitate L-thienylalanine (**10**) which was filtered off and recrystallised from water (384.2 mg, 64%); m.p. 224–229 °C (decomp.) (lit.,[15] 238–244 °C); $[\alpha]_D^{26}$ −30° (*c* 0.5 in water) (lit.,[15] $[\alpha]_D^{21}$ −31.4°) (Found: C, 49.12; H, 5.26; N, 8.27; S, 18.40. $C_7H_9NO_2S$ requires C, 49.11; H, 5.30; N, 8.18; S, 18.73%); δ_H(D_2O, 250 MHz) 3.45 (2 H, ABX with H_α, $J_{\beta,\beta'}$ 15.0 Hz, $J_{\alpha,\beta}$ 6.4 Hz, $J_{\alpha,\beta'}$ 5.3 Hz, CH_β,$H_{\beta'}$), 3.98 (1 H, ABX with $H_{\beta,\beta'}$, CH_α), 7.00–7.06 (2 H, m, 3-H and 4-H), and 7.37 (1 H, dd, $J_{3,5}$ 1.3 Hz, $J_{4,5}$ 4.9 Hz, 5-H).

N-*t-Butoxycarbonyl*-L-β-2-*thienylalanine* (**11**).—L-Thienylalanine (257.0 mg, 1.50 mmol) was suspended in dioxane and water (2:1, 6 ml), cooled to *ca.* 5 °C, and 1M sodium hydroxide (2 ml) was added. Di-t-butyl dicarbonate (362.0 mg, 1.66 mmol) was added and the solution was stirred at 5 °C for 10 min and then at room temperature for 1 h. The solvent was evaporated, the residue was taken up in water and acidified to between pH 2 and 3 with 5% sodium hydrogen sulphate. The solution was extracted with ethyl acetate (3 × 10 ml) and the combined organic phases were washed with water (15 ml), dried (Na_2SO_4) and evaporated to yield the Boc-amino acid (**11**) (334.1 mg, 82%) as an oil which was used without purification. δ_H($CDCl_3$, 90 MHz) 1.40 (9 H, s, Bu^t), 3.35 (2 H, br, CH_2), 4.58 (1 H, br, CH), 6.80–7.30 (3 H, m, 3-H, 4-H, and 5-H), and 9.00 (1 H, br, NH).

N-*t-Butoxycarbonyl*-L-β-2-*thienylalanine Pentafluorophenyl Ester* (**12**).—The preceeding Boc amino acid (**11**) (334.1 mg, 1.23 mmol) and pentafluorophenol (251.3 mg, 1.36 mmol) were dissolved in ethyl acetate (7 ml) and the solution was stirred at 0 °C. DCC (279.2 mg, 1.35 mmol), dissolved in ethyl acetate (2 ml), was added and the resulting mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. The precipitated urea was filtered off, the filtrate was evaporated to dryness and the crystalline residue recrystallised from hexane to afford the pentafluorophenyl ester (**12**) (531.5 mg, 99%); m.p. 103–104 °C (Found: C, 49.58; H, 3.62; N, 3.27; F, 21.40; S, 7.25. $C_{18}H_{16}NO_4F_5S$ requires C, 49.43; H, 3.69; N, 3.20; F, 21.72; S, 7.33%) δ_H($CDCl_3$, 250 MHz) 1.46 (9 H, s, Bu^t), 3.51 (2 H, br, $J_{CH_2,CH}$ 5.5 Hz, CH_2), 4.94 (1 H, m, CH), 5.11 (1 H, br, NH), 6.93

Table 2. ^{13}C NMR spectra (D_2O, ref. Bu^{t}OH) of TRH analogues (**6**), (**7**), and (**8a**, **b**).[a]



	X	Y
TRH	NH	N
Thi^2-TRH	S	CH
Fur^2-TRH	O	CH
Pyr^2-TRH	NH	CH

		TRH[b]	Thi^2-TRH (**6**)	Fur^2-TRH (**7**)	L-Pry^2-TRH (**8a**)	D-Pyr^2-TRH (**8b**)
pGlu	αCH	57.84	56.65	56.65	58.20	58.21
	βCH_2	26.55	25.07	25.10	26.64	26.70
	γCH_2	30.36	na	na	na	na
ring	C=O	181.47	182.18	182.22	183.84	183.84
	C=O	174.56	174.52	174.39	176.04	176.15
Xaa	αCH	53.12	52.78	50.68	53.46	54.01
	βCH_2	29.92	na[c]	na	na	na
	C-5	136.61	127.28	142.60	*ca.* 120	119.77
	C-4	—	127.07	110.65	109.01	109.49
	C-3	nd[d]	125.30	108.21		108.58
	C-2	nd	138.13	150.20	127.63	127.46
	C=O	172.34	170.81	170.89	173.08	173.45
Pro	αCH	61.69	60.44	60.50	62.09	62.14
	βCH_2	30.80	na	na	na	na
	γCH_2	25.83	24.50	24.54	26.14	25.63
	δCH_2	47.84	48.02	47.90	49.54	49.21
	C=O	177.13	176.61	176.64	178.36	178.45
	pGlu γCH_2		30.39	29.42	31.10	30.98
	Xaa βCH_2		29.42	29.04	30.67	30.82
	Pro βCH_2		28.99	28.92	30.16	30.32

[a] *cis* Conformation resonances not included. [b] Run in CD_3OD. [c] na = not unambiguously assigned. [d] nd = not determined.

(1 H, br, $J_{3,4}$ 3.5 Hz, 3-H), 6.99 (1 H, dd, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 5.1 Hz, 4-H), and 7.23 (1 H, dd, $J_{3,5}$ 1.3 Hz, $J_{4,5}$ 5.1 Hz, 5-H).

L-*Pyroglutamyl*-L-*thienylalanyl*-L-*prolinamide* (**6**).—The procedure used for peptide synthesis is essentially that of Kisfaludy *et al.*[16] *N*-t-Butoxycarbonyl-L-β-thienylalanine pentafluorophenyl ester (**12**) (262.5 mg, 0.60 mmol) dissolved in dimethylformamide (DMF) (3 ml) was added to a stirred solution of L-proline amide (75.3 mg, 0.66 mmol) in DMF (5 ml) at 0 °C. The mixture was allowed to attain room temperature and after 30 min the ninhydrin test[17] was negative. Dimethylaminoethylamine (1 drop) was added to react with excess active ester and, after a further 10 min the solvent was evaporated. The residue was taken up in methylene dichloride (20 ml) and the solution was washed with 10% citric acid (2 × 20 ml), 5% sodium hydrogen carbonate (2 × 20 ml), and water (20 ml), and then dried (Na_2SO_4) and evaporated to afford *N*-t-butoxycarbonyl-L-thienylalanyl-L-prolinamide (**13**) as an oily foam (191.4 mg, 87%) which gave a single spot on TLC (R_f 0.48).

The foregoing protected dipeptide (191.4 mg, 0.52 mmol) was dissolved in trifluoroacetic acid–water (9:1, 25 ml) at room temperature. After 30 min the solvent was evaporated and the residue was re-evaporated with methanol (3 × 10 ml). Ether (20 ml) was added and the resulting precipitate, the dipeptide trifluoroacetate salt, was filtered off and dried (190.5 mg, 96%).

The foregoing dipeptide salt (190.5 mg, 0.50 mmol) was dissolved in DMF (5 ml) and triethylamine (0.14 ml, 1.0 mmol) was added, followed by pyroglutamic acid pentafluorophenyl ester* (162.6 mg, 0.55 mmol) in DMF (3 ml). The mixture was stirred at room temperature for 30 min after which time the ninhydrin test indicated that the reaction was complete. The solvent was evaporated and the residue was triturated with ethyl acetate and then recrystallised from water to afford L-pyroglutamyl-L-thienylalanyl-L-prolinamide (**6**) (80.5 mg, 43%, overall yield from Boc-Thi-OPfp was 35%); homogeneous on TLC (R_f 0.35) and HPLC (linear gradient of 5–50% solvent B over 30 min at 1 ml min^{-1}, t_R = 17.5 min); amino acid analysis $Glu_{1.00}$, $Thi_{1.02}$, $Pro_{0.98}$; found MH^+ 379. $C_{17}H_{23}N_4O_4S$ requires MH^+ 379.14; for δ_H and δ_C see Tables).

Diethyl 2-*Acetylamino*-2-(2-*furylmethyl*)*malonate* (**14**).—This was prepared by the method of Herz *et al.*[5] and was recrystallised from ethyl acetate–hexane, m.p. 81–82 °C (lit.,[5] 84 °C) δ_H($CDCl_3$, 250 MHz) 1.29 (6 H, t, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 2.01 (3 H, s, $COCH_3$), 3.73 (2 H, s, CH_2), 4.27 (2 H, q, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 4.28 (2 H, q, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 6.03 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{3,5}$ 0.9 Hz, 3-H), 6.26 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{4,5}$ 1.9 Hz, 4-H), 6.65 (1 H, brs, NH), and 7.27 (1 H, dd, $J_{3,5}$ 0.9 Hz, $J_{4,5}$ 1.9 Hz, 5-H).

* The pentafluorophenyl ester was initially used in the peptide syntheses. After some time however, the compound started to decompose and in later experiments the pentachlorophenyl ester was used.

Ethyl 2-Acetylamino-2-(2-furylmethyl)malonate (**15**).—A solution of potassium hydroxide (1.41 g, 25.2 mmol) in ethanol–water (9:1, 30 ml) was added slowly over 10 min to a stirred solution of the diethyl ester (**14**) (5 g, 16.8 mmol) in ethanol (40 ml). The resulting solution was stirred at room temperature for 3 h and then excess alkali was neutralised with 2M HCl. The solution was stirred at 0 °C overnight, the precipitated potassium chloride was filtered off and the filtrate was evaporated. The residue was dissolved in water (10 ml) and acidified to pH 2 with 2M HCl to precipitate the monoacid–monoester (**15**) as a white powder which was recrystallised from ethyl acetate–hexane (3.71 g, 82%); m.p. 120–121 °C δ_H([$^2H_6$]DMSO, 250 MHz) 1.14 (3 H, t, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 1.89 (3 H, s, $COCH_3$), 3.50 (2 H, q, $J_{\beta,\beta'}$ 15.1 Hz, $CH_\beta H_{\beta'}$), 4.12 (2 H, q, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 6.06 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{3,5}$ 0.8 Hz, 3-H), 6.33 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{4,5}$ 1.9 Hz, 4-H), 7.49 (1 H, dd, $J_{3,5}$ 0.8 Hz, $J_{4,5}$ 1.9 Hz, 5-H), and 7.98 (1 H, s, NH).

D,L-N-*Acetyl*-β-2-*furylalanine Ethyl Ester* (**16**).—The preceding monoacid–monoester (**15**) (3.5 g, 13 mmol) was dissolved/suspended in dioxane (70 ml) and the solution was refluxed for 39 h. The solvent was evaporated to yield a pale yellow oil which crystallised. Recrystallisation from ether afforded the ester (**16**) (2.34 g, 80%); m.p. 54–55 °C (Found: C, 58.65; H, 6.70; N, 6.32. $C_{11}H_{15}NO_4$ requires C, 58.66; H, 6.71; N, 6.22%); δ_H($CDCl_3$, 250 MHz) 1.27 (3 H, t, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 2.01 (3 H, s, $COCH_3$), 3.19 (2 H, d, $J_{CH_2,CH}$ 5.3 Hz, CH_2CH), 4.21 (2 H, q, J_{CH_2,CH_3} 7.1 Hz, CH_2CH_3), 4.83 (1 H, dt, J_{CH,CH_2} 5.3 Hz, $J_{CH,NH}$ 7.9 Hz, CH_2CH), 6.07 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{3,5}$ 0.8 Hz, 3-H), 6.12 (1 H, br, NH), 6.28 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{4,5}$ 1.9 Hz, 4-H), and 7.31 (1 H, dd, $J_{3,5}$ 0.8 Hz, $J_{4,5}$ 1.9 Hz, 5-H).

D,L-N-*Acetyl*-β-2-*furylalanine* (**17**).—The preceding ester (**15**) (2.25 g, 10 mmol) was dissolved in ethanol (20 ml) and was hydrolysed with potassium hydroxide (0.67 g, 12 mmol) in ethanol–water (10:1, 17.5 ml) for 17 h at room temperature. The ethanol was evaporated, water was added to the residue and the resulting solution was acidified to pH 3 with 1M HCl. The aqueous solution was extracted with ethyl acetate (3 × 25 ml) and the combined extracts were washed with water (25 ml), dried (Na_2SO_4), and evaporated. The crystalline residue was recrystallised from ethyl acetate–hexane to afford racemic *N*-acetylfurylalanine (**17**) (1.06 g, 54%); m.p. 153.5–154.5 °C (Found: C, 54.95; H, 5.50; N, 6.89; $C_9H_{11}NO_4$ requires C, 54.82; H, 5.62; N, 7.10%); δ_H([$^2H_6$]DMSO, 250 MHz) 1.80 (3 H, s, CH_3), 2.91 (1 H, dd, $J_{\beta,\beta'}$ 15.3 Hz, $J_{\alpha,\beta}$ 8.6 Hz, CH_β), 3.05 (1 H, dd, $J_{\beta,\beta'}$ 15.3 Hz, $J_{\alpha,\beta'}$ 5.2 Hz, $CH_{\beta'}$), 4.44 (1 H, ddd, $J_{\alpha,\beta}$ 8.6 Hz, $J_{\alpha,\beta'}$ 5.2 Hz, $J_{CH,NH}$ 8.2 Hz, CH_α), 6.13 (1 H, dd, $J_{3,4}$ 3.1 Hz, $J_{3,5}$ 0.9 Hz, 3-H), 6.33 (1 H, dd, $J_{3,4}$ 3.1 Hz, $J_{4,5}$ 1.9 Hz, 4-H), 7.51 (1 H, dd, $J_{3,5}$ 0.9 Hz, $J_{4,5}$ 1.9 Hz, 5-H), and 8.17 (1 H, d, $J_{CH,NH}$ 8.0 Hz, NH).

L-β-2-*Furylalanine* (**18**).—The enantioselective hydrolysis reaction was carried out as for the thienyl analogue described above, D,L-*N*-acetylfurylalanine (**17**) (985 mg, 5 mmol) gave, after recrystallisation from water–acetone, L-β-2-furylalanine (**18**) (244 mg, 63%), m.p. 232–235 °C (decomp.); $[\alpha]_D^{26}$ −45° (*c* 0.99 in water) (Found: C, 54.12; H, 5.74; N, 9.07. $C_7H_9NO_3$ requires C, 54.19; H, 5.85; N, 9.03%); δ_H(D_2O, 250 MHz) 3.25 (2 H, ABX with H_α, $J_{\beta,\beta'}$ 15.0 Hz, $J_{\alpha,\beta'}$ 5.1 Hz, CH_β,$H_{\beta'}$), 3.98 (1 H, ABX with H_β,$H_{\beta'}$, H_α), 6.28 (1 H, br, $J_{3,4}$ 3.2 Hz, 3-H), 6.40 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{4,5}$ 1.9 Hz, 4-H), and 7.46 (1 H, dd, $J_{3,5}$ 0.8 Hz, $J_{4,5}$ 1.9 Hz, 5-H).

N-*t-Butoxycarbonyl*-L-β-2-*furylalanine Pentafluorophenyl Ester* (**20**).—The two-step derivatisation procedure was as that described above for the thienylalanine analogue. L-β-2-Furylalanine (**18**) (201.3 mg, 1.3 mmol) gave, after recrystallisation from hexane, the derivative (**20**) (444.2 mg, 81%); m.p. 91–92 °C (Found: C, 51.56; H, 3.83; N, 3.36; F, 22.53. $C_{18}H_{16}NO_5F_5$ requires C, 51.31; H, 3.83; N, 3.32; F, 22.55%); δ_H($CDCl_3$, 250 MHz) 1.46 (9 H, s, Bu^t), 3.33 (2 H, m, CH_2), 4.94 (1 H, m, CH), 5.21 (1 H, br, NH), 6.20 (1 H, d, $J_{3,4}$ 3.2 Hz, 3-H), 6.33 (1 H, dd, $J_{3,4}$ 3.2 Hz, $J_{4,5}$ 1.9 Hz, 4-H), and 7.38 (1 H, dd, $J_{3,5}$ 0.8 Hz, $J_{4,5}$ 1.9 Hz, 5-H).

L-*Pyroglutamyl*-L-*furylalanyl*-L-*prolinamide* (**7**).—The tripeptide was prepared in a similar procedure to that described above for the thienylalanyl analogue, and was isolated as a white powder (117.9 mg) in 54% yield from the derivative (**20**) (252.5 mg, 0.60 mmol). The peptide was homogeneous on TLC (R_f 0.33) and HPLC [conditions as for compound (**16**), t_R = 15.5 min]. Amino acid analysis: $Glu_{0.98}$, $Pro_{1.02}$ (furylalanine destroyed by hydrolysis conditions) (Found: MH^+ 363. $C_{17}H_{23}N_4O_5$ requires MH^+ 363.17); for δ_H and δ_C see Tables.

Diethyl N-*Benzyloxycarbonylaminomalonate*.—*N*-(Benzyloxycarbonyl)succinimide (11.2 g, 44.9 mmol) was dissolved in methylene dichloride (50 ml) and the solution was added to a cooled (0 °C) and stirred solution of diethyl aminomalonate hydrochloride (10 g, 47.3 mmol) and triethylamine (26.5 ml, 190 mmol) in methylene dichloride (250 ml). The resulting solution was stirred at 0 °C for 10 min and then at room temperature for 1 h. The solution was washed with 10% citric acid (2 × 100 ml), 10% sodium hydrogen carbonate (2 × 100 ml), and water (100 ml) and was then dried (Na_2SO_4) and evaporated to afford diethyl *N*-benzyloxycarbonylaminomalonate as a colourless oil which crystallised upon standing at 0 °C (14.5 g, 99%); m.p. 31–32 °C (lit.,[8] 32–33 °C); δ_H($CDCl_3$, 90 MHz) 1.25 (6 H, t, CH_2CH_3), 4.25 (4 H, q, CH_2CH_3), 5.13 (H, s, CH_2Ph), 5.95 (1 H, br, CH), and 7.33 (5 H, s, arom.).

Diethyl 2-Benzyloxycarbonylamino-2-pyrrol-2-ylmethylmalonate (**27**).—Diethylaminomethylpyrrole[7] (4.95 g, 32.6 mmol) was dissolved in absolute ethanol (35 ml) and methyl iodide (2.2 ml, 35.8 mmol) was added. The resulting solution was stirred in the dark for 2 h and then ether (200 ml) was added. The supernatant was decanted off and the residue was triturated with ether (2 × 50 ml) to afford the salt (**22**) as a brown oil (7.15 g, 75%).

Diethyl *N*-benzyloxycarbonylaminomalonate (7.51 g, 24.3 mmol) was added to a solution of sodium (561 mg, 24.4 mmol) in absolute alcohol (35 ml). The foregoing oily methiodide (**22**) (7.15 g, 24.3 mmol) was dissolved in absolute ethanol (35 ml), added to the above solution and the resulting orange mixture was refluxed for 2 h. The reaction mixture was filtered, the filtrate was evaporated and the residue was partitioned between methylene dichloride (50 ml) and water (50 ml). The layers were separated and the aqueous layer was re-extracted with methylene dichloride (2 × 50 ml). The combined organic layers were washed with water (50 ml), dried (Na_2SO_4) and evaporated. The oily residue was purified by column chromatography on silica (60–120 mesh, 100 g) using methylene dichloride as eluant. The appropriate fractions were combined and evaporated to afford (**27**) as a pale brown oil (6.85 g, 73%, overall yield 54% from diethylaminomethylpyrrole); δ_H($CDCl_3$, 90 MHz) 1.16 (6 H, t, CH_2CH_3), 3.58 (2 H, s, CH_2-pyrrole), 4.16 (4 H, q, CH_2CH_3), 5.10 (2 H, s, CH_2Ph), 5.82 (1 H, m, 3-H), 6.05 (2 H, m, 4-H) and NH), 6.55 (1 H, m, 5-H), 7.33 (5 H, s, arom.), and 8.05 (1 H, br, NH).

Diethyl 2-Benzyloxycarbonylamino-2-[N-t-butoxycarbonyl)-pyrrol-2-ylmethyl]malonate (**28**).—The procedure of Grehn and Ragnarsson[18] was followed. The pyrrole derivative (**27**)

(3.9 g, 10.05 mmol) was dissolved in acetonitrile (25 ml) and di-t-butyl dicarbonate (2.62 g, 12.02 mmol) in acetonitrile (10 ml) was added dropwise over 5 min followed by dimethylaminopyridine (123.3 mg, 1.01 mmol) in acetonitrile (3 ml). The resulting solution was stirred at room temperature for 16 h and then partitioned between 10% citric acid (50 ml) and ethyl acetate (50 ml). The layers were separated and the organic layer was washed with 10% citric acid (50 ml), 10% sodium hydrogen carbonate (2 × 50 ml), and water (50 ml) and then dried (Na_2SO_4) and evaporated to dryness to yield the t-butoxycarbonylpyrrole derivative (**28**) as a brown oil (4.75 g, 97%) which would not crystallise and was used without purification, δ_H($CDCl_3$, 90 MHz) 1.18 (6 H, t, CH_2CH_3), 1.52 (9 H, s, Bu^t), 4.01 (2 H, s, CH_2-pyrrole), 4.20 (4 H, q, CH_2CH_3), 5.08 (2 H, s, CH_2Ph), 5.80–6.10 (2 H, m, 3-H and 4-H), 7.13 (1 H, m, 5-H), and 7.31 (5 H, s, arom.).

Ethyl 2-Benzyloxycarbonylamino-2-[(N-t-butoxycarbonyl)-pyrrol-2-ylmethyl]malonate (**29**).—The diethyl ester (**28**) (4.26 g, 8.73 mmol) was dissolved in absolute ethanol (45 ml) and 1M sodium hydroxide was added to the stirred solution until TLC showed that the hydrolysis was complete (35 ml, 4 equiv. added). After 2 h the solution was neutralised with 5M HCl and then concentrated. The aqueous residue was partitioned between 10% citric acid (50 ml) and methylene dichloride (50 ml). The layers were separated and the aqueous layer was again extracted with methylene dichloride (50 ml). The combined organic extracts were washed with water (30 ml), dried (Na_2SO_4), and evaporated to yield the monoacid–monoether (**29**) as a brown oil (2.97 g, 74%) which was used without purification. δ_H($CDCl_3$, 90 MHz) 1.18 (3 H, t, CH_2CH_3), 1.42 (9 H, s, Bu^t), 3.93 (2 H, s, CH_2-pyrrole), 4.18 (2 H, q, CH_2CH_3), 5.11 (2 H, s, CH_2Ph), 6.00 (2 H, m, 3-H and 4-H), 7.15 (1 H, m, 5-H), 7.30 (5 H, s, arom.), and 8.58 (1 H, br, NH).

N(α)-*Benzyloxycarbonyl*-β-2-(N-*t-butoxycarbonyl*)*pyrrolylalanine Ethyl Ester* (**30**).—The preceding monoacid–monoester (**29**) (2.97 g, 6.46 mmol) was dissolved in dioxane (100 ml) and the solution was refluxed for 16 h. The dioxane was evaporated and the brown oily residue was purified by column chromatography on silica (60–120 mesh, 50 g) using methylene dichloride as eluant. The appropriate fractions were combined and evaporated to afford (**30**) as a pale orange oil (2.67 g, 99%). δ_H($CDCl_3$, 90 MHz) 1.14 (3 H, s, CH_2CH_3), 1.52 (9 H, s, Bu^t), 3.05–3.40 (2 H, br, CH_2CH) 4.28 (2 H, q, CH_2CH_3), 5.02 (3 H, m, CH_2Ph and CH_2CH), 6.01 (2 H, m, 3-H and 4-H), 7.15 (1 H, m, 5-H), and 7.28 (5 H, s, arom.).

N(α)-*Benzyloxycarbonyl*-β-2-(N-*t-butoxycarbonyl*)*pyrrolylalanine* (**31**).—The preceding ester (**30**) (2.67 g, 6.4 mmol) was dissolved in absolute ethanol (30 ml) and 2M sodium hydroxide (6.4 ml, 12.8 mmol) was added. The resulting mixture was stirred at room temperature for 1.75 h by which time TLC indicated that hydrolysis was complete. The solution was neutralised and concentrated and then the aqueous residue was partitioned between 10% citric acid (30 ml) and methylene dichloride (30 ml). The layers were separated and the aqueous layer was again extracted with methylene dichloride (2 × 30 ml). The combined organic extracts were washed with water (20 ml), dried (Na_2SO_4), and evaporated to afford the racemic amino acid derivative (**31**) as a brown oily foam (2.09 g, 84%) which was used without further purification. δ_H($CDCl_3$, 90 MHz) 1.55 (9 H, s, Bu^t), 3.05–3.55 (2 H, br m, CH_2CH), 4.65 (1 H, br m, $CHCH_2$), 5.05 (2 H, s, CH_2Ph), 6.15 (2 H, m, 3-H and 4-H), 7.15–7.50 (6 H, m, 5-H and arom.), and 8.65 (1 H, br, NH).

L-*Pyroglutamyl*-D,L-*pyrrolylalanyl*-L-*prolinamide* (**8**).—The pyrrolylalanine derivative (**31**) (390.3 mg, 1.01 mmol) and proline amide (105.3 mg, 0.92 mmol) were dissolved in methylene dichloride (15 ml) and the resulting solution was cooled in an ice-bath. DCC (210.5 mg, 1.02 mmol) dissolved in methylene dichloride was added and the resulting mixture was stirred at 0 °C for 1 h and then at room temperature for 45 min. The precipitated urea was filtered off and the filtrate was evaporated. The brown oily residue was chromatographed on silica (Kieselgel 60, 9 g) using methylene dichloride–methanol (19:1) as eluant. The appropriate fractions were combined and evaporated to afford the fully protected dipeptide (**32**) (347.5 mg, 71%) as a yellow oily foam.

The preceding protected dipeptide (347.5 mg, 0.72 mmol) was dissolved in absolute ethanol (30 ml), Pd/C (5%, 73.4 mg) was added and the suspension was stirred vigorously under hydrogen at atmospheric pressure for 5 h. The mixture was filtered through Celite and the filtrate was evaporated to give an oily foam. The crude product was chromatographed on silica (Kieselgel 60, 4 g) with methylene dichloride–methanol (9:1) as eluant to afford the N(α)-deprotected dipeptide (191.3 mg, 76%) as a pale yellow oily foam.

A solution of the preceding dipeptide (191.3 mg, 0.55 mmol) in methylene dichloride (7 ml) was cooled and stirred in an ice-bath and pyroglutamic acid pentachlorophenyl ester (251.3 mg, 0.67 mmol) dissolved in methylene dichloride (13 ml) and triethylamine (0.1 ml, 0.72 mmol) were added. The resulting solution was stirred at 0 °C for 20 min and then at room temperature for 3 h by which time TLC indicated that all the dipeptide had been consumed. The solvent was evaporated and the residue was chromatographed on silica (Kieselgel 60, 4 g) with methylene dichloride–methanol (19:1) as eluant. The appropriate fractions were pooled and evaporated to give the protected tripeptide (**33**) (192.3 mg, 76%) as an oil which solidified upon trituration with ether (Found: MH^+, 462. $C_{22}H_{32}N_5O_6$ requires, MH^+ 462.24).

The preceding protected tripeptide (178.7 mg, 0.39 mmol) was dissolved in trifluoroacetic acid (10 ml) at room temperature. After 20 min the solvent was evaporated and the solid residue was filtered off and washed well with ether to give the tripeptide (**8**) (128.5 mg, 92%) as a mixture of diastereoisomers. (Found: MH^+, 362. $C_{17}H_{24}N_5O_4$ requires, MH^+ 362.18.) A portion of the diastereoisomeric mixture was subjected to semi-preparative reverse-phase HPLC to separate the two tripeptides (linear gradient 0–50% solvent B over 30 min at 3 ml min^{-1}, collected peaks between 12–13 min and 13–14 min). After lyophilisation, the earlier eluting peak [analytical HPLC conditions as for compounds (**6**) and (**7**), t_R = 15 min] afforded the LLL tripeptide (**8a**) as an off-white powder and the later eluting peak (t_R = 17.5 min) afforded the LDL tripeptide (**8b**) as a pale yellow powder. ^1H and ^{13}C NMR data are presented in the Tables.

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