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Facile Preparation of the 1-Hydroxybenzotriazolyl Ester of *N*-Tritylpyroglutamic Acid and its Application to the Synthesis of TRH, [D-His^2]TRH and Analogues Incorporating *cis*- and *trans*-4-Hydroxy-L-proline

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One-pot treatment of *N*-trityl-L-glutamic acid with DCC followed by DCC–HOBt provided a high yielding synthesis of the 1-hydroxybenzotriazolyl ester of *N*-trityl-L-pyroglutamic acid (Trt-Glp). Coupling of this active ester with the methyl esters of N^{im}-tritylated L- and D-histidine provided the corresponding dipeptides which upon saponification and coupling with the methyl esters of L-proline and *trans*-4-hydroxy-L-proline (Hyp) gave the protected tripeptides Trt-Glp-L and D-His-(N^{im}-Trt)-Pro-OMe and Trt-Glp-L and D-His(N^{im}-Trt)-Hyp-OMe. Some 10% of the epimeric (at the His residue) products were formed during this procedure. Sequential saponification and one-pot Mitsunobu-type intramolecular esterification of the latter tripeptides, followed by transesterification with MeOH, provided the corresponding tripeptides Trt-Glp-L and D-His(N^{im}-Trt)-cHyp-OMe with inversion of configuration at C-4 of the Hyp ring. The observed conformer ratios about the His-Pro amide for all of these tripeptides are discussed in terms of structural features. Detritylation with trifluoroacetic acid followed by ammonolysis completed the synthesis of TRH and its analogues Glp-D-His-Pro-NH_2, Glp-L- and D-His-Hyp-NH_2 and Glp-L- and -D-His-cHyp-NH_2.

As part of our attempts to develop experimentally simple procedures for the synthesis of a variety of γ-derivatives of glutamic acid (Glu) suitable for use in peptide synthesis, we decided to synthesize, as a key intermediate, the anhydride of *N*-triphenylmethylglutamic acid (Trt-Glu). It was anticipated that nucleophilic attack on the α-carbonyl group of the anhydride would be highly disfavoured owing to the bulk of the Trt group. In addition to this expected property, the N^{α}-Trt group, when used as an amino protecting group for chiral α-amino acids, confers excellent resistance to racemisation[1,2] and increased lipophilicity of intermediates. This in turn facilitates work-up and purification using flash column chromatography (FCC) procedures.[3,4] Nevertheless, this protecting group is easily introduced in excellent yields[5] and may finally be removed under mild acidic conditions.[6]

However, treatment of Trt-Glu (**1**) with *N,N'*-dicyclohexylcarbodiimide (DCC) initially at 0 °C for 30 min, followed by stirring at room temperature for 24 h, produced a mixture of two products which were UV active, when viewed on thin layer chromatographic (TLC) plates. The less polar, minor product (ca. 10%), was also ninhydrin active whereas the major one (ca. 90%) was ninhydrin inactive. When the mixture of these products was examined by IR spectroscopy it showed weak characteristic bands at 1827 and 1738 and a strong one at 1710 cm^{-1}. Furthermore, when the same mixture was treated with glacial (gl.) AcOH–H_2O (9:1) for 30 min at room temperature, the major component, which was identified as the pyroglutamic acid (Glp) derivative **2**, was isolated in 89% yield. The minor product, which streaks on TLC, is

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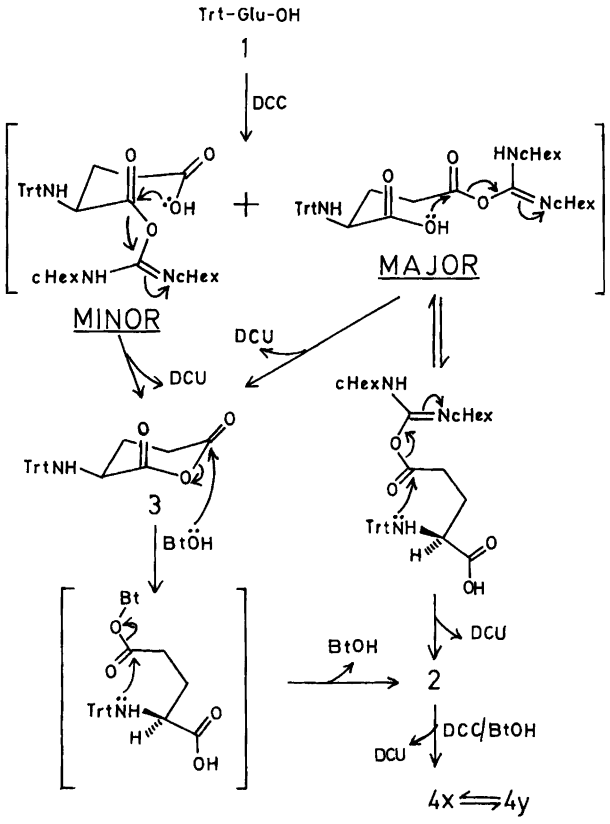
thought to be the expected product, namely the anhydride **3**. This is of course in accord with the DCC-1-hydroxybenzotriazole (HOBt)-mediated conversion of a crude mixture of **2** and **3** solely to the corresponding active ester **4**, which is described below. Since the TLC picture of the initial reaction mixture does not change even after 1 h at 60°C, this is taken to mean that the two products are formed through different routes as indicated in Scheme 1. When the same reaction was performed at −70°C with the dropwise addition (ca. 30 min) of a solution of DCC in dichloromethane (DCM), the ratio of **2**:**3** was almost reversed. However, the reaction was quite sluggish and when the reaction temperature was slowly raised to −10°C a mixture of almost equal quantities of these two products and remaining unchanged starting material was observed (TLC). This implies that as the reaction temperature is raised, five-membered ring formation competes favourably with anhydride ring formation (six-membered) despite the presence of the bulky Trt group on nitrogen. These results are in contrast with the well established, facile, DCC-mediated preparation of glutamic anhydride derivatives where the nitrogen bears an electron-withdrawing group, e.g., acetyl, benzyloxycarbonyl, formyl, phthalyl and 4-toluenesulphonyl.[7] It should be mentioned that such derivatives can be converted into the corresponding pyroglutamic acid derivatives by a procedure involving two separate steps, i.e., treatment with dicyclohexylamine followed by acidification with HCl.



Scheme 1. Mechanisms for the formation of the active ester **4** by DCC-mediated activation of Trt-Glu-OH (**1**) in the presence of HOBt.

Prompted by these results we turned our attention to the applicability of **2** to peptide synthesis, by considering its conversion into the corresponding, isolable, active ester **4**. It should be indicated that Glp constitutes the *N*-terminus of many biologically important peptides, such as thyrotropin-releasing hormone (TRH), gastrin-34, the adipokinetic hormones, some tachykinins and bombesin to name a few.[8] Thus, treatment of the above-mentioned 9:1 mixture of **2** and **3** with additional DCC and 1-hydroxybenzotriazole (HOBt) for 30 min at 0°C and 2 h at room temperature produced cleanly a 9:1 mixture of active ester **4x** and active amide **4y**. Infrared spectroscopy confirmed the identity of these compounds and thus that the situation was analogous to that earlier reported for a similar case.[9] Structures of these and other compounds encountered in this work may be found in Fig. 1: for compounds **9–23** only the structures **9a–23a** having the same stereochemistry as L-histidine, are shown, while their epimers with respect to this centre, **9b–23b**, are omitted for brevity. Aqueous work-up allowed isolation of this mixture in 95% overall yield based on Trt-Glu-OH.

Having established the high-yielding 'one pot' synthesis of **4**, we decided to study its acylation potential and the conditions required for removing the Trt group using H-Ala-OMe as the amino component for model studies. Thus, coupling of **4** with H-Ala-OMe proceeded unexceptionally within 2 h at room temperature using a 9:1 mixture of **4x** and **4y**, to give cleanly a 90% yield of the dipeptide **5**. When however a 1:1 mixture (see the Experimental) of **4x** and **4y** was used the coupling required 12 h to go to completion, a result which is in accord with earlier results of our research group.[9] Attempts to cleave the Trt group from **5** by catalytic hydrogenolysis in gl. AcOH–H_2O (9:1) at room temperature for several hours in the presence of 10% Pd–C were unsuccessful, as were attempts to effect Trt cleavage by heating a solution of **5** in gl. AcOH–H_2O (9:1) at 60°C for 1 h. This last result parallels the established stability of the similar derivatives N^{ω}-Trt-Asn and N^{ω}-Trt-Gln to this system.[10] The unexpected resistance of the Trt group, in **5**, to cleavage by catalytic hydrogenolysis might be attributed to steric hindrance. However, treatment of **5** with 5%, 10%, 25% and 50% solutions of trifluoroacetic acid (TFA) in DCM for 5–45 min at room temperature showed (TLC) that 10 min treatment with the 10% solution or 30 min with the 5% solution is enough to remove the Trt group of **5** quantitatively.

We have recently shown that TRH analogues incorporating *trans*- and *cis*-4-hydroxy-L-proline, Hyp (**7**) and cHyp (**8**) respectively, can only be obtained in high yields by using solid-phase peptide synthesis.[11] Attempts to synthesize such peptides by solution-phase peptide synthesis using Z-protected or unprotected Glp were either unsuccessful or proceeded in a rather complex manner leading

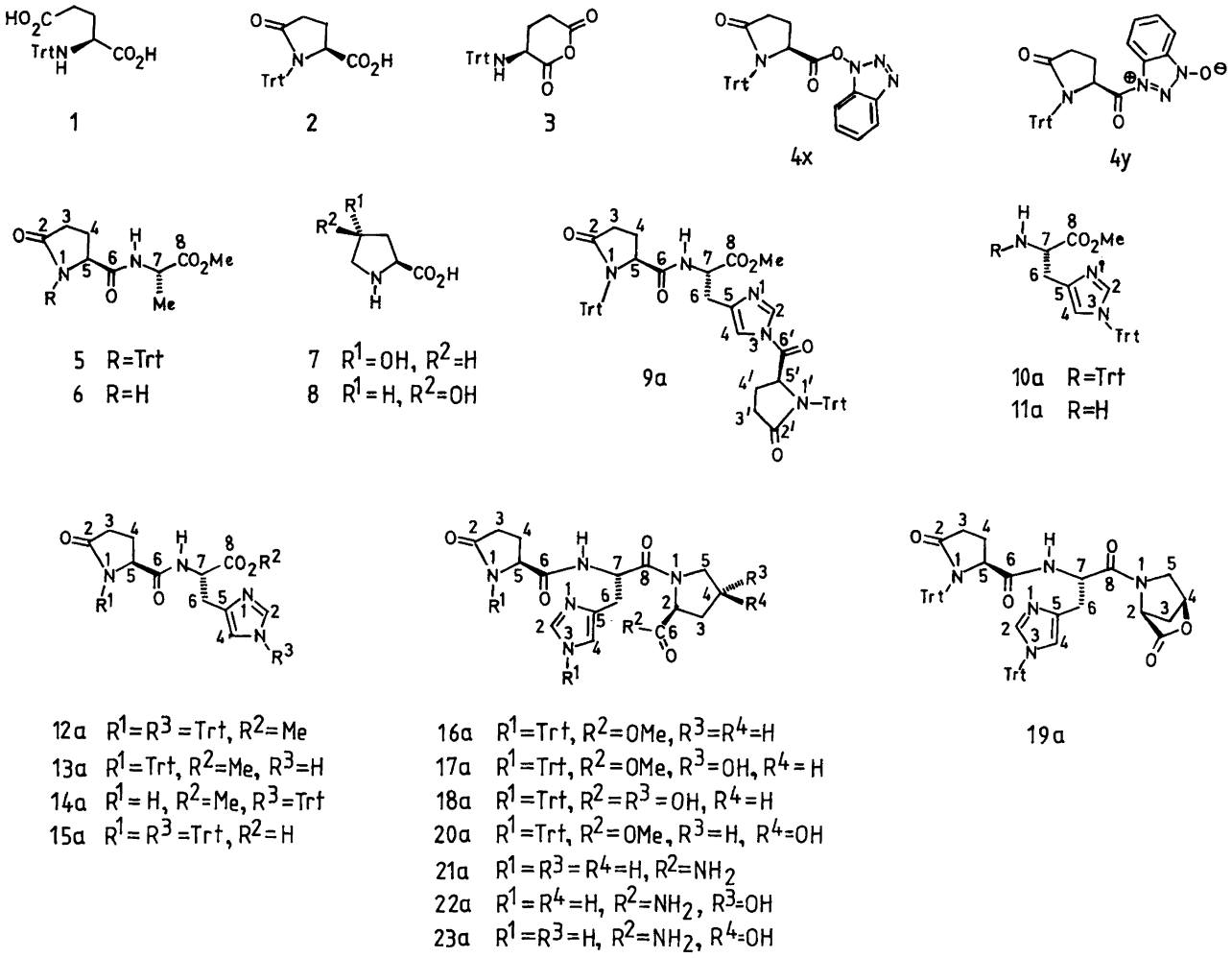
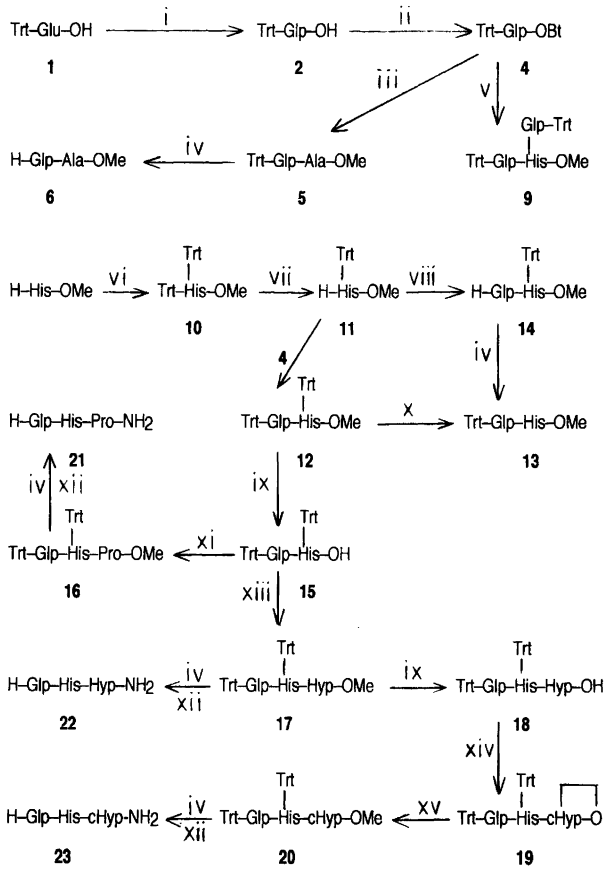


Fig. 1. Structures of the compounds encountered in this work. For the compounds **9**–**23** only the epimers **9a**–**23a** incorporating L-histidine are given, the structures **9b**–**23b**, which differ from these only in having the same stereochemistry as D-histidine, are omitted for brevity.

finally to very low total yields. We were therefore interested to see to what extent the active ester **4** might be applied to this difficult problem. The extent of the investigations eventually carried out can be seen in Scheme 2. Since we were aware of the extremely facile diketopiperazine formation when the synthesis starts from the *C*-terminus,[11] we turned our attention to coupling **4** initially with (L)-2HCl·H-His-OMe. Although coupling was cleaner and more efficient than using either Z-Glp or Glp and DCC–HOBt activation, the main product was isolated in only 42% yield. This product was shown by NMR analysis to be the expected dipeptide, carrying an additional Trt-Glp residue on the His side-chain (**9a**). This result is of course the result of the poor solubility of the bishydrochloride of H-His-OMe in the reaction medium. It was thus evident that protection of the N^{im}-atom was mandatory. We therefore decided to use the N^{im}-Trt protected His-OMe as the amino component, in the hope that coupling efficiency would be greatly improved. The required amino component was obtained by treating (L)-2HCl·H-His-OMe with TrtCl and triethylamine (TEA) in $CHCl_3$ to obtain a 97% yield of His derivative **10a**. Treatment of **10a** with 1% TFA in DCM for 1 min at 0°C and evaporation at low temperature and reduced pressure produced **11a** as a white powder.[12] Coupling of **11a** with **4**, in the presence of TEA in DMF produced an 85% yield of the expected dipeptide **12a**. When, however, **4** was used, as a 1:1 mixture of **4x** and **4y**, the reaction took place extremely sluggishly and required flash column chromatography (FCC) to separate the product from unchanged **4y**. This behaviour of compounds like **4**, i.e., very fast coupling of **4x** followed by slower consumption of **4y**, has already been documented.[9] Of course, in the present case the presence of the bulky Trt group on both components further reduced the coupling potential of **4**. It should be noted that in some couplings a more polar by-product than **12a** was formed in minute quantities. This by-product was identified as the peracylated His de-



Scheme 2. Synthetic pathways leading to TRH and analogues. Reagents and conditions are indicated by roman numerals as follows: (i) DCC; (ii) DCC–HOBt; (iii) H-Ala-OMe; (iv) 20% TFA–DCM; (v) H-His-OMe; (vi) TrtCl–TEA; (vii) 1% TFA–DCM; (viii) H-Glp-OH–BOP; (ix) NaOH; (x) AcOH–H_2O; (xi) H-Pro-OMe; (xii) NH_3; (xiii) H-Hyp-OMe; (xiv) TPP–DEAD; (xv) TPP–DEAD-MeOH.

rivative **9a**, by comparison with an authentic sample. It appears that in cases such as this, **9a** is formed due to complete detritylation of **10a** by the TFA solution used, which produces along with **11a**, small quantities of (L)-2TFA·H-His-OMe. Thus, careful monodetritylation of **10a** (see the Experimental) is required to avoid formation of this side product.

Under identical coupling conditions, **4** was condensed with TFA·(D)-H-His(Trt)-OMe (**11b**) to give the diastereomeric dipeptide **12b** as a foam in 88% yield. Comparison of the ^{1}H NMR spectra of **12a** and **12b** revealed that the H(H)-6 protons in the former compound appear as a doublet of doublets whereas in the latter as a doublet. This is taken to mean that in **12a** there is restricted rotation of the His side-chain, possibly due to a hydrogen bond between the N^{π}-atom of the imidazole ring and the H-atom of the peptide bond. Examination of Orbit Molecular Building System (OMBS) models (Cochranes of Oxford Ltd., 1972) of these two diastereomers showed that such a hydrogen bond in **12b** would force the bulky Trt group on the Glp ring into an unfavourable steric interaction with the methoxycarbonyl function at the His asymmetric centre. On the other hand, a hydrogen bonding between the same H-atom and the carbonyl *O*-atom of the ester function can be nicely accommodated in structure **12b**, which accordingly leaves the His side-chain free to rotate.

At this stage it became of interest to us to examine the behaviour of the Trt protecting groups of **12a** under the influence of the acidic media used for deprotection of the model dipeptide **5**. Thus, treatment of **12a** with gl. AcOH–H_2O (9:1) at 60°C, conditions known to cleave the N^{im}-Trt group,[13] led to the clean formation (73% isolated yield) of a polar product, that is the expected N^{im}-detritylated dipeptide **13a**, within 30 min. This product was, as expected, different (TLC) from the alternative isomer **14a**, which was obtained for comparison by coupling Glp with ester **11a**. Comparison of the ^{1}H NMR spectra of dipeptide derivatives **9a**, **12a**, **13a** and **14a** revealed that the appearance of the multiplet, at ca. δ 4.2, for the proton H(G)-5 is diagnostically significant for the presence of the Trt group on the *N*-atom of the Glp ring. Thus with the exception of **14a** for which this multiplet appears as a doublet of doublets, the same multiplet in all other compounds has the form of a single doublet, apparently because one of the coupling constants of H(G)-5 to the adjacent H(G)-4 protons is zero. It should be noted that in the case of compound **9a** a second doublet at δ 4.74 was assigned to the proton H(G)-5′ which is shifted further downfield due to the imidazole ring.

Treatment of **12a** with 10% TFA in DCM at room temperature led to complete detritylation within 1 h. TLC analysis of the reaction mixture showed an intermediate to be the monotritylated compound **13a**. In separate experiments, monotritylated dipeptide **14a** was treated with 10% TFA in DCM at room temperature and the deprotection reaction followed by TLC. These experiments showed that **14a** is first converted into **13a** (disappearance of starting material within 15 min), obviously through Trt migration from the N^{im} to the *N*(G)-1 position and that detritylation required a further 15 min for completion. Attempted catalytic hydrogenolysis of **12a**, under identical conditions with those unsuccessfully used for **5**, left both Trt-groups untouched.

Saponification of **12a** with dimethyl sulphoxide (DMSO) as a cosolvent[4] at room temperature was complete in 18 h. The expected **15a** was isolated in 81% yield after FCC. The small amount of the epimer **15b** also found, arose from racemisation during saponification. Coupling of acid **15a** with HCl·H-Pro-OMe mediated by DCC and HOBt in the presence of *N*-methylmorpholine (NMM) produced the tripeptide **16a** in only 48% yield. Significant quantities (10%) of the epimeric tripeptide **16b** were also obtained through FCC of the reaction mixture. When, however, this coupling was performed using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the condensing agent[14] a 75% yield of **16a** was secured along with 10% of **16b**. Cou-

pling of **15a** with H-Hyp-OMe, under identical reaction conditions with those used for the preparation of **16a**, was shown to proceed sluggishly and finally the expected product **17a** was isolated in 59% yield, after FCC in order to remove unchanged **15a** and small quantities of **17b**. BOP-mediated coupling produced, however, a much better yield (83%) of **17a** along with small quantities of **17b**. Saponification of ester **17a** proceeded unexceptionally to give the tripeptide acid **18a** in 96% yield. Treatment of this acid with triphenylphosphine (TPP)–diethyl azodicarboxylate (DEAD)[11] produced the lactone **19a** which, upon addition of excess MeOH in a one-pot experiment, gave 59% of the expected ester **20a**, with inversion of configuration at C-4 of the Hyp ring.

Identical reaction sequences were used in order to synthesize in comparable yields (see the Experimental) the TRH derivative **16b**, with D-His in place of L-His, as well as the analogues **17b** and **20b** with Hyp and cHyp in place of Pro, respectively. It should be noted that the dipeptide ester **12b** readily underwent saponification with aq. NaOH–MeOH–DMSO within 1.5 h at room temperature and in that case only trace amounts of the epimeric dipeptide acid **15a** could be detected along with the expected **15b**. Racemisation during the coupling of **15b** with either HCl·H-Pro-OMe or HCl·H-Hyp-OMe was also rather low, in the order of 10%, and main products were separated from minor epimeric products by FCC. Comparison of the ^{1}H NMR spectra of the tripeptide esters **16a/b**, **17a/b** and **20a/b** revealed that all derivatives, with the exception of **17b**, which appeared as a single conformer, showed the presence of the two possible conformers around the His-Pro amide bond in the approximate ratios of 3:1 (**16a**), 8:1 (**16b**), 1:1 (**17a**), 3:1 (**20a**) and 5.5:1 (**20b**). It therefore appears that replacement of L-His by D-His greatly favours the formation of one of the conformers whereas the presence of a hydroxy group on C-4 of the Pro ring affects the ratio of the two conformers mainly when this hydroxy group is *trans* to the methoxycarbonyl function, that is when Pro is replaced by Hyp. Moreover, as it is the case with the dipeptide esters **12a** and **12b**, special conformational significance can be attributed to the H(H)-6 protons. Thus, in all three peptides incorporating L-His these protons appear as a doublet, indicating free rotation of the His side chain. However, in each of the three peptides incorporating D-His, the same protons appear as a doublet of doublets and this is taken to mean that the imidazole ring of His side chain is probably involved in a hydrogen bond with the Glp-His amide bond. Examination of the OMBS models of both *cis* and *trans* conformers, around the His-Pro amide bond, of the tripeptides **16a** and **16b** revealed that in the case of both putative conformers of **16a** the requirement to avoid steric congestion between the two Trt groups would, on introduction of the proposed hydrogen bond, place the *N*(G)-Trt group in an unfavourable steric interaction with the Pro residue of the tripeptide. Moreover, these models indicated that in **16a** the *cis* conformation might be stabilized by a hydrogen bond between the H-atom of the Glp-His amide bond and the carbonyl O-atom of the ester function. Such a hydrogen bond is not possible in the alternative *trans* arrangement. It is thus reasonable to propose that, in the tripeptides incorporating L-His, the *cis* conformer predominates, with the exception of course of Hyp-incorporating tripeptides, e.g., **17a**, where the two arrangements exist in almost equal amounts. In that case it is assumed, and this possibility can be shown by the appropriate model, that there might also be a stabilization in the *trans* arrangement through a hydrogen bond which involves different structural components, i.e., the hydroxy group as the donor and the carbonyl *O*-atom of the Glp-His amide bond as the acceptor. On the other hand in the D-His incorporating tripeptides, when the Glp-His amide functions as the donor and the N^{π}-atom acts as acceptor in a hydrogen bond, steric crowding can be minimized only when the His-Pro amide bond has the *trans* arrangement. In the *cis* arrangement, such a hydrogen bond would involve significant unfavourable steric interaction between the *N*(G)-Trt group and the methoxycarbonyl group of the Pro ring. Thus, in the cases of the D-His incorporating tripeptides, the *trans* conformer appears to be the main conformer and in particular in the case of the tripeptide **17b** is the exclusive one.

Complete deprotection of esters **16a/b**, **17a/b** and **20a/b** was routinely performed with a 20% solution of TFA in DCM–trifluoroethanol (TFE) (6:1). The trifluoroacetate salts thus obtained were, without any further purification, subsequently subjected to ammonolysis for 4–5 days at room temperature to produce TRH (**21a**) and analogues **21b**, **22a/b** and **23a/b** in good yields (see the Experimental). TRH and analogues thus produced were routinely purified by reversed-phase FCC (RP-FCC)[15] and their purity was tested by HPLC (Fig. 2). It should be noted that alternative syntheses of TRH,[16] and its analogues **21b**,[17] **22a**[18] and **23a**[11] have been disclosed. Biological evaluation and NMR studies of the TRH analogues as well as research on further applications of the active ester **4** in synthesis are now in progress.

Experimental

General. Capillary melting points were taken on a Büchi SMP-20 apparatus and are uncorrected. Optical rotations were determined with a Carl-Zeiss precision polarimeter. IR spectra were recorded for KBr pellets on a Perkin Elmer 16PC FT-IR spectrophotometer. All tritylated derivatives of amino acids and peptides showed characteristic single sharp bands (of the Trt-group) at 700–704 and 740–750 cm^{-1}. In most cases an additional single sharp band at 753–764 cm^{-1} was observed.

^{1}H NMR spectra were obtained at 200.132 and 400.13 MHz and ^{13}C NMR at 50.32 and 100.62 MHz on Bruker AC 200F and AM 400WB spectrometers. $CDCl_3$ and tetramethylsilane (TMS) were used as the solvent and the internal standard, respectively. Chemical shifts

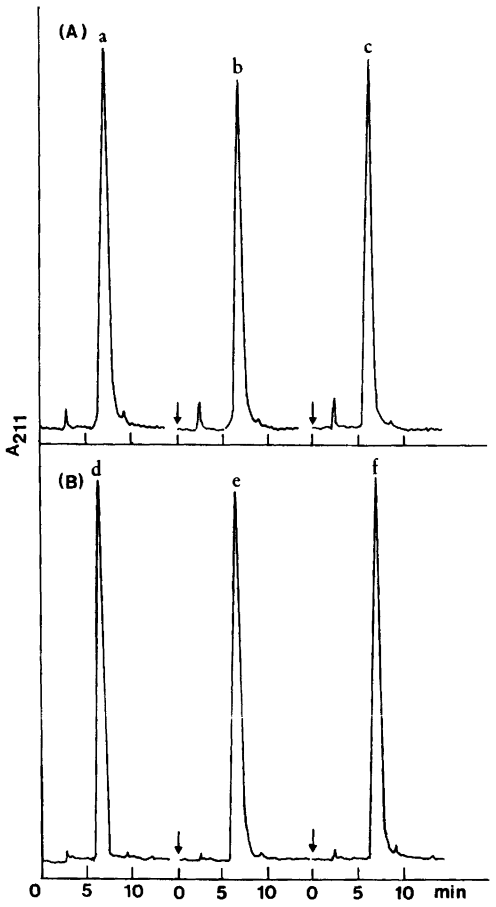


Fig. 2. HPLC of TRH and analogues incorporating L-His (**A**) and D-His (**B**). (a) H-Glp-His-Pro-NH_2, (b) H-Glp-His-Hyp-NH_2, (c) H-Glp-His-cHyp-NH_2, (d) H-Glp-His-cHyp-NH_2, (e) H-Glp-His-Pro-NH_2, (f) H-Glp-His-Hyp-NH_2.

are reported in δ units, parts per million (ppm) downfield from TMS. The assignments of the ^1H spectra are based on chemical shift arguments, signal intensities, analysis of coupling patterns and consideration of information from homo- and hetero-nuclear 2D experiments. The ^{13}C spectra were assigned using the *J*-modulated spin-echo technique,[19] DEPT and, if necessary, 2D-heteronuclear chemical shift correlations.

FAB spectra were recorded on a Fisons-VG ZAB 2f instrument, operated at 8 keV accelerating potential, with an ion gun of M-SCAN operated at 20 μA and 9 keV Xenon beam. Matrix: *m*-nitrobenzyl alcohol unless otherwise noted.

HPLC was performed on an LDC system consisting of an LDC III pump, a UV–VIS detector LDC 1204A with an 8 μl flow cell and a 100 μl loop injector. Solvents used for the HPLC experiments were of HPLC grade (Merck). FCC was performed on Merck silica gel 60 (230–400 mesh) and TLC on Merck $60F_{254}$ films (0.2 mm) precoated on aluminium foil. The solvent systems used were: (A) $CHCl_3$–MeOH (95:5), (B) BuOH–AcOH–H_2O (4:1:1), (C) $CHCl_3$–MeOH (9:1), (D) BuOH–AcOH–Py–H_2O (30:6:20:24), (E) MeCN–H_2O (5:1), (F) MeOH–MeCN–H_2O–Et_3N (60:34:1:5) and (G) $CHCl_3$–MeOH (97:3). Spots were visualized with ninhydrin where applicable, UV light at 254 nm and the charring agent $(NH_4)_2SO_4$–conc. H_2SO_4–H_2O (20 g–4 ml–100 ml). All solvents (Merck) were dried and/or purified according to standard procedures[20] prior to use. Anhydrous Na_2SO_4 was used for drying organic solutions and subsequently solvents were routinely evaporated at ca. 30°C under reduced pressure (water aspirator).

All reagents used in the present work were purchased from Aldrich and used without any further purification. L- and D-histidine methyl ester dihydrochlorides were prepared according to a published procedure.[21] Amino acid analyses were performed on a Beckmann Model 120C amino acid analyser using a three-buffer column system.

N-*Trityl-L-glutamic acid* (**1**). Procedure C, as described in Ref. 5, was used to prepare **1**, with the following modifications: (a) selective N–Si bond splitting was performed at −10°C by the dropwise addition of anhydrous 2-propanol in DCM within 30 min, and (b) slow acidification at 0°C to pH 5 with 5% aq. citric acid to effect complete precipitation of **1**. The thus precipitated product was immediately extracted twice into EtOAc and the combined organic layers were washed several times with brine. Drying and evaporation of the solvent produced a residue which, upon trituration with petroleum ether b.p. 40–60°C (PE), gave **1** as a white powder. Thus, from 5.9 g (40 mmol) of Glu there was obtained 14.7 g (95%) of **1**.

N-*Trityl-L-pyroglutamic acid* (**2**). Trt-Glu (1.95 g, 5 mmol) was dissolved in THF (20 ml) and treated with DCC (1.13 g, 5.5 mmol) at 0°C for 30 min and at room temperature for 24 h. Dicyclohexylurea (DCU) was filtered off and washed with EtOAc, and the combined filtrates were evaporated under reduced pressure. The residue was dissolved in 20 ml gl. AcOH–H_2O (9:1) and kept at room temperature for 30 min. The resulting solution was evaporated at reduced pressure and the residue was partitioned between diethyl ether (DE) and 1 M NaOH. The aqueous layer was brought to pH 3 by the dropwise addition of 5% aq. HCl and extracted twice with EtOAc. Drying and evaporation of the combined organic layers afforded a foam which upon trituration with cold DE provided 1.65 g (89%) of product as a white powder. Acid **2** had m.p. 168–172°C, $[\alpha]_D^{25}$ +19.2° (*c* 1, MeOH), R_f (A) 0.23, R_f (B) 0.76 and R_f (E) 0.6 2. Anal. $C_{24}H_{21}NO_3$: C, H. IR: 3434, 3290–2330 (br), 1724, 1699 and 1666 cm^{-1}. ^1H NMR (400 MHz): δ 7.40–7.10 (15 H, m, Ph-H), 4.22 (1 H, d, *J* 8.7 Hz, H-5), 2.79 (1 H, dd, *J* 4.6 and 11.6 Hz, H-3a), 2.58–2.36 (2 H, m, H-4b and H-3b), 2.11 (1 H, d, *J* 12.8 Hz, H-4a).

N-*Trityl-L-pyroglutamic acid 1-hydroxybenzotriazolyl ester* (**4**). Trt-Glu (7.8 g, 20 mmol) was treated with DCC (4.5 g, 22 mmol) as described above. The resulting re-

action mixture was cooled to 0°C and treated sequentially with HOBt (4.05 g, 30 mmol) and DCC (4.5 g, 22 mmol) for 20 min at 0°C and 2 h at room temperature when reaction was found to be complete (TLC, solvent system $CHCl_3$–MeOH 9:1). The resulting reaction mixture was filtered to remove DCU, diluted with EtOAc and washed sequentially with 5% aq. citric acid, water, 5% $NaHCO_3$ and finally brine, dried and evaporated to leave 9.4 g (96%) of **4**, as a foamy mixture of **4x** and **4y** in the approximate ratio of 9:1 (by charring on a TLC plate). When activation of Trt-Glp was left to carry on for 12 h, in a separate small-scale experiment, a mixture of approximately 1:1 **4x** and **4y** was obtained. It should be noted that direct synthesis of **4** from **1**, using 2 equiv. DCC and 1.5 equiv. HOBt from the beginning of the reaction also produced **4** but in a less clean reaction. R_f (PhMe–EtOAc 1:1) 0.39 and 0.24, respectively IR: 1838 (s), 1824 (w) and 1720 cm^{-1}.

N-*Trityl-L-pyroglutamyl-L-alanine methyl ester (***5***).* HCl·H-Ala-OMe (0.77 g, 5.5 mmol) was taken up in DMF (5 ml), cooled to 0°C and neutralized by the dropwise addition of TEA. To the resulting reaction mixture, active ester **4** (2.45 g, 5 mmol) was added and after 20 min at 0°C it was allowed to attain room temperature and then left for 2 h. During this period the pH of the solution was kept at ca. 8 by the dropwise addition of further TEA. The resulting reaction mixture was diluted with ice-cold 5% aq. citric acid and the precipitated product was extracted twice into ethyl acetate (EtOAc). The combined organic layers were sequentially washed with water, 5% aq. $NaHCO_3$ and brine, dried and evaporated to afford 2.17 g (95%) of pure product upon trituration with DE. Dipeptide **5** had m.p. 238°C (decomp.), $[\alpha]_D^{25}$ −65.8° (*c* 1, $CHCl_3$), R_f (A) 0.49, R_f (B) 0.77 and R_f (E) 0.73. Anal. $C_{28}H_{28}N_2O_4$: C, H. IR: 3292, 1742 and 1680 cm^{-1}. ^1H NMR: 7.35–7.20 (15 H, m, Ph-H), 5.71 (1 H, d, *J* 7.2 Hz, CONH), 4.19 [1 H, quintet, *J* 7.2 Hz, H(A)-7], 4.11 [1 H, d, *J* 8.5 Hz, H(G)-5], 3.67 (3 H, s, CO_2Me), 2.91–2.77 [1 H, m, H(G)-3a], 2.36–2.22 [2 H, m, H(G)-3b, H(G)-4a], 2.05–1.96 [1 H, m, H(G)-4b], 1.096 (3 H, d, *J* 7.3 Hz, Me). ^{13}C NMR: 176.4, 173.0 and 172.0 [C(G)-2, C(G)-6 and C(A)-8], 142.6 (PhC-1), 130.6 (PhC-2/6), 127.5 (PhC-3/5), 127.1 (PhC-4), 74.8 (Ph_3C), 62.9 [C(G)-5], 52.4 (OMe), 47.7 [C(A)-7], 31.5 [C(G)-3], 25.9 [C(G)-4] and 18.8 (Me).

*Pyroglutamyl-L-alanine methyl ester (***6***).* Dipeptide **5** (0.91 g, 2 mmol) was subjected to TFA treatment, as described for the tripeptide **16a**. Evaporation of the solution afforded an oily residue which upon FCC, using $CHCl_3$–MeOH (93:7) as the eluant, gave compound **6** as an oil which solidified on treatment with DE. Yield: 0.38 g (88%), m.p. 60–63°C, $[\alpha]_D^{25}$ −43.4° (*c* 0.5, MeOH), R_f (B) 0.43, R_f (D) 0.68 and R_f (E) 0.27. IR: 3429, 3304, 3219, 1752, 1696 and 1668 cm^{-1}.

N^{α},N^{im}*-Di(*N*-trityl-L-pyroglutamyl)-L-histidine methyl ester (***9a***).* To an ice-cold solution of 2HCl·H-His-OMe (0.12 g, 0.5 mmol) in DMF (1 ml), 1,3-diazabicyclo[4,5]-undec-2-ene (DBU) (0.15 ml) was added followed by active ester **4** (0.24 g, 0.49 mmol). The reaction mixture was allowed to reach room temperature, the pH was adjusted to ca. 8 by addition of DBU and stirring was continued for an additional 30 min at room temperature after which time the reaction was found to be complete. Work-up as for the dipeptide **12a** provided the main product dipeptide **9a** (90 mg, 42% yield) after FCC with system G. Similar results were obtained when TEA was used as the base instead of DBU. Compound **9a** had m.p. 173–176°C, $[\alpha]_D^{25}$ −86.8° (*c* 0.5, MeOH), R_f (A) 0.38, R_f (B) 0.74 and R_f (E) 0.88. Anal. $C_{55}H_{49}N_5O_6$: C, H. IR: 3470, 3328, 1742 and 1690 cm^{-1}. ^1H NMR: 7.50–7.14 (30 H, m, Ph-H), 7.44 [1 H, s, H(H)-2], 6.94 (1 H, d, *J* 7.6 Hz, CONH), 6.86 [1 H, s, H(H)-4], 4.74 [1 H, d, *J* 9.4 Hz, H(G)-5′], 4.55 [1 H, ddd, *J* 4.6, 4.9 and 7.6 Hz, H(H)-7], 4.19 [1 H, d, *J* 9.1 Hz, H(G)-5], 3.59 (3 H, s, OMe), 2.94 [1 H, dd, *J* 4.9 and 15.0 Hz, H(H)-6a], 2.84 [1 H, ddd, *J* 15.8, 12.0 and 8.3 Hz, H(G)-3′a], 2.82 [1 H, ddd, *J* 16.1, 12.5 and 8.8, H(G)-3a], 2.62 [1 H, dd, *J* 4.6 and 15.0 Hz, H(H)-6b], 2.58–2.38 [2 H, m, H(G)-4b and H(G)-4′b], 2.45 [1 H, dd, *J* 15.8 and 9.2 Hz, H(G)-3′b], 2.30 [1 H, dd, *J* 16.1 and 8.9 Hz, H(G)-3b], 2.14 [1 H, dd, *J* 12.3 and 8.8 Hz, H(G)-4a], 2.01 [1 H, dd, *J* 12.4 and 8.8 Hz, H(G)-4′a]. ^{13}C NMR: 176.6, 175.1, 172.0, 170.6 and 169.0 [C(G)-2, C(G)-6, C(G)-2′, C(G)-6′ and C(H)-8], 142.6 and 141.7 (PhC-1 and PhC-1′), 139.6 [C(H)-5], 134.9 [C(H)-2], 130.4 and 130.1 (PhC-2/6 and PhC-2′/6′), 127.6 and 127.4 (PhC-3/5 and PhC-3′/5′), 127.3 and 127.0 (PhC-4 and PhC-4′), 112.8 [C(H)-4], 75.1 and 75.0 (Ph_3C and Ph_3C'), 63.5 [C(G)-5], 61.5 [C(G)-5′], 52.2 (OCH_3), 51.2 [C(H)-7], 31.4 [C(G)-3], 30.8 [C(G)-3′], 28.5 [C(H)-6], 25.9 [C(G)-4] and 25.0 [C(G)-4′].

N^{α},N^{im}*-Ditrityl-L- and -D-histidine methyl ester (***10a***) and (***10b***).* To an ice-cold suspension of 2HCl·H-His-OMe (4.84 g, 20 mmol) and TrtCl (11.15 g, 40 mmol) in dry $CHCl_3$ (100 ml), anhydrous TEA (11.15 ml, 80 mmol) was added dropwise over 30 min. The resulting reaction mixture was stirred at 0°C for 15 min and 30 min at room temperature, then concentrated and the residue taken up in EtOAc and washed sequentially with 5% citric acid, water, 5% $NaHCO_3$ and brine, dried and evaporated to leave pure products.

*(L)-Trt-His(Trt)-OMe (***10a***).* Powder from DE–petroleum ether 40–60°C (PE). Yield: 12.84 g (98%), m.p. 76–78°C, $[\alpha]_D^{25}$ +13.7° (*c* 1, MeOH), R_f (A) 0.82, R_f (B) 0.85, R_f (EtOAc) 0.47. IR: 3300 and 1736 cm^{-1}. ^1H NMR: 7.50–7.05 (30 H, m, Ph-H), 7.36 [1 H, d, *J* 1.4 Hz, H(H)-2], 6.63 [1 H, d, *J* 1.4 Hz, H(H)-4], 3.67 [1 H, ddd, *J* 10.7, 6.2 and 6.9 Hz, H(H)-7], 2.96 [1 H, dd, *J* 14.1 and 6.2 Hz, H(H)-6a], 2.79 [1 H, dd, *J* 14.1 and 6.9 Hz, H(H)-6b], 2.72 (1 H, d, *J* 10.7 Hz, TrtNH),

3.04 (3 H, s, OCH_3). ^{13}C NMR: 175.1 [C(H)-8], 146.1 and 142.5 (PhC-1 and PhC-1′), 138.4 [C(H)-2], 137.0 [C(H)-5], 129.8 and 128.8 (PhC-2/6 and PhC-2′/6′), 128.0 and 127.6 (PhC-3/5 and PhC-3′/5′), 128.0 and 126.2 (PhC-4 and PhC-4′), 119.7 [C(H)-4], 75.1 and 71.0 (Ph_3C and Ph_3C'), 56.6 [C(H)-7], 51.3 (OCH_3) and 34.8 [C(H)-6].

*(D)-Trt-His(Trt)-OMe (***10b***).* Crystalline from EtOAc–hexane. Yield: 12.32 g (94%), m.p. 79–81°C, $[\alpha]_D^{25}$ −13.3° (*c* 1, MeOH), R_f (A) 0.82, R_f (B) 0.85, R_f (EtOAc) 0.47. Anal. $C_{45}H_{39}N_3O_2$: C, H.

N-Trityl-L-pyroglutamyl-L- and -D-histidine (N^{im}*-trityl) methyl ester (***12a***) and (***12b***).* Trt-His(Trt)-OMe (5.23 g, 8 mmol) was added to an ice-cold 1% solution (180 ml) of TFA in anhydrous DCM. The resulting yellow solution was kept at 0°C for 1 min and immediately concentrated at 0°C under high vacuum (0.01 mmHg) to leave a residue. This residue was treated with DE followed by an excess of PE. The supernatant liquor was decanted off and the residue was treated sequentially once more with DE and PE and the resulting white powder was filtered and washed with ice-cold anhydrous DE to give the corresponding trifluoroacetate salts (**11**). TFA·(L)-H-His(Trt)-OMe: yield 3.92 g (93%). TFA·(D)-H-His(Trt)-OMe: yield 3.75 g (89%).

To a solution of freshly prepared Trt-Glp-OBt (3.42 g, 7 mmol) in anhydrous DMF (6 ml), methyl ester **11a** (3.92 g, 7.45 mmol) was added and the resulting solution was cooled to 0°C and set to pH 8 by the dropwise addition of TEA (ca. 1.2 ml). The resulting mixture was allowed to attain room temperature, its pH was readjusted to 8 by the dropwise addition of further TEA and stirring was continued at room temperature for 1 h after which reaction was found to be complete (TLC). The precipitated product was filtered off and washed with DE to give 3.49 g (65%) of pure **12a**. The combined filtrates were diluted with EtOAc and sequentially washed with 5% citric acid, water, 5% $NaHCO_3$ and brine. The filtrate was then dried and evaporated to leave the crude product which was subjected to FCC with EtOAc as the eluant to give an additional 1.07 g (20%) of pure **12a**.

When the isomeric methyl ester **11b** was used, the coupling step was also complete within 1 h at room temperature and the reaction mixture was then diluted with EtOAc and further processed, as described for **12a**, to give the dipeptide **12b** as a foam.

*Trt-Glp-(L)-His(Trt)-OMe (***12a***).* Yield: 4.56 g (85%), m.p. 242°C (decomp.), $[\alpha]_D^{25}$ −42.1° (*c* 1, $CHCl_3$), R_f (A) 0.63, R_f (B) 0.79 and R_f (E) 0.73. Anal. $C_{50}H_{44}N_4O_4$: C, H. IR: 3430, 3218, 1746, 1698 and 1682 cm^{-1}. ^1H NMR (400 MHz): δ 7.48–7.08 [32 H, m, Ph-H, CON*H* and H(H)-2], 6.52 [1 H, s, H(H)-4], 4.56 [1 H, ddd, *J* 4.39, 4.52 and 7.74 Hz, H(H)-7], 4.21 [1 H, d, *J* 9.14 Hz, H(G)-5], 3.51 (3 H, s, OMe), 3.00 [1 H, dd, *J* 4.52 and 14.57 Hz, H(H)-6a], 2.95 [1 H, ddd, *J* 9.20, 12.51 and 15.94 Hz, H(G)-3a], 2.66 [1 H, dd, *J* 4.39 and 14.57 Hz, H(H)-6b], 2.44 [1 H, m, *J* 8.95, 9.14, 12.46 and 12.51, H(G)-4b], 2.31 [1 H, dd, *J* 8.95 Hz and 15.94 Hz, H(G)-3b], 2.18 [1 H, dd, *J* 12.46 and 9.20 Hz, H(G)-4a].

*Trt-Glp-(D)-His(Trt)-OMe (***12b***).* Yield: 4.71 g (88%), m.p. 136–138°C, $[\alpha]_D^{25}$ −53.3° (*c* 1, $CHCl_3$), R_f (A) 0.57, R_f (B) 0.81, R_f (E) 0.72 and R_f (EtOAc) 0.23. ^1H NMR: δ 7.39–7.00 [31 H, m, Ph-H and H(H)-2], 6.87 [1 H, d, *J* 6.9 Hz, CONH], 6.51 [1 H, d, *J* 1.2 Hz, H(H)-4], 4.50 [1 H, dt, *J* 4.3 and 6.9 Hz, H(H)-7], 4.01 [1 H, d, *J* 8.6 Hz, H(G)-5], 3.57 (3 H, s, OMe), 2.97 [2 H, d, *J* 4.3 Hz, H(H)-6], 2.82 [1 H, m, H(G)-3a], 2.38–2.10 [2 H, m, H(G)-4b and H(G)-3b], 2.03 [1 H, m, H(G)-4a].

Glacial acetic acid treatment of dipeptide **12a**. *Synthesis of N-trityl-L-pyroglutamyl-L-histidine methyl ester (***13a***).* A solution of dipeptide **12a** (0.38 g, 0.5 mmol) in 4 ml gl. AcOH–H_2O (9:1) was heated at 60°C for 1 h, and then diluted with H_2O (20 ml) at room temperature. The resulting mixture was extracted twice with DE to remove tritylcarbinol. The aqueous layer was then extracted twice with $CHCl_3$ and the combined organic layers were dried and evaporated to leave an oily residue which upon trituration with hexane provided 0.19 g (73%) of **13a** as a white powder. Dipeptide **13a** had m.p. 226°C (decomp.), $[\alpha]_D^{25}$ −75.5° (*c* 0.5, MeOH), R_f (A) 0.16, R_f (B) 0.47 and R_f (E) 0.60. Anal. $C_{31}H_{30}N_4O_4$: C, H. IR: 3368, 3246, 1748 and 1686 cm^{-1}. ^1H NMR (based on 200 MHz data): δ 7.46–7.08 [17 H, m, Ph-H, H(H)-2 and CONH], 6.58 [1 H, s, H(H)-4], 4.46 [1 H, dt, *J* 4.9 and 7.3 Hz, H(H)-7], 4.18 [1 H, d, *J* 8.7 Hz, H(G)-5], 3.54 (3 H, s, OMe), 2.99 [1 H, dd, *J* 5.4 and 15.0 Hz, H(H)-6a], 2.84 [1 H, m, H(G)-3a], 2.70 [1 H, dd, *J* 4.6 and 15.0 Hz, H(H)-6b], 2.54–1.98 [3 H, m, H(G)-3b and H(G)-4]. ^{13}C NMR: δ 176.9, 172.1 and 171.3 [C(G)-2, C(G)-6 and C(H)-8], 142.5 (PhC-1), 135.5 [C(H)-5], 130.4 [PhC-2/6 and C(H)-2], 127.4 (PhC-3/5), 127.0 [PhC-4 and C(H)-4], 75.0 (Ph_3C), 63.5 [C(G)-5], 52.0 [OCH_3 and C(H)-7], 31.5 [C(G)-3], 29.6 [C(H)-6], 27.0 [C(G)-4].

L-Pyroglutamyl-L-histidine (N^{im}*-trityl) methyl ester (***14a***).* Trt-L-His(Trt)-OMe (0.65 g, 1 mmol) was converted into the trifluoroacetate salt **11a** as described above. This salt was dissolved in anhydrous DMF (0.3 ml) and treated sequentially with H-Glp-OH (0.12 g, 0.93 mmol), BOP reagent (0.41 g, 0.93 mmol), anhydrous DCM (0.3 ml) and diisopropylethylamine (DIPEA) (0.51 ml, 3 mmol). The resulting reaction mixture was stirred at room temperature for 1 h, when reaction was found to be complete (TLC). Solvents were then evaporated off and the residue diluted with 5% aq. citric acid and extracted twice with EtOAc. The combined organic phases were washed sequentially with 5% aq. citric acid, water, 5% aq. $NaHCO_3$ and brine, dried and evaporated to give an oily residue which upon trituration with PE gave 0.32 g (65%)

of **14a** as a white powder, m.p. 68°C (decomp., softens at 55°C), $[\alpha]_D^{25}$ +13.7° (*c* 0.5, MeOH), R_f (A) 0.25, R_f (B) 0.59 and R_f (E) 0.62. Anal. $C_{31}H_{30}N_4O_4$: C, H. IR: 3266, 1742 and 1682 cm^{-1}. ^1H NMR: δ 8.01 (1 H, d, *J* 7.8 Hz, CONH), 7.41 [1 H, d, *J* 1.3 Hz, H(H)-2], 7.36–7.04 (15 H, m, Ph-H), 6.55 [1 H, d, *J* 1.3 Hz, H(H)-4], 6.33 [1 H, br s, H(G)-1], 4.79 [1 H, dt, *J* 4.8 and 8.0 Hz, H(H)-7], 4.18 [1 H, dd, *J* 4.9 and 7.9 Hz, H(G)-5], 3.58 (3 H, s, OMe), 3.05 [1 H, dd, *J* 5.2 and 14.7 Hz, H(H)-6a], 2.98 [1 H, dd, *J* 4.5 and 14.7 Hz, H(H)-6b], 2.58–2.09 [4 H, m, H(G)-3 and H(G)-4]. ^{13}C NMR: δ 178.4, 171.8 and 171.2 [C(G)-2, C(G)-6 and C(H)-8], 142.0 (PhC-1), 138.6 [C(H)-2], 136.0 [C(H)-5], 129.6 (PhC-2/6), 128.1 (PhC-4), 128.0 (PhC-3/5), 119.6 [C(H)-4], 56.7 [C(G)-5], 52.5 [C(H)-7], 52.1 (OMe), 29.2 and 29.1 [C(G)-3 and C(H)-6], and 25.5 [C(G)-4].

*N-Trityl-L-pyroglutamyl-L- and -D-(N^{im}-trityl)histidine (**15a**) and (**15b**) from saponification of dipeptides **12a** and **12b**, respectively.* To an ice-cold suspension of dipeptide **12a** (2.17 g, 2.84 mmol) in a mixture of DMSO (30 ml) and MeOH (7 ml) was added 1 M NaOH (3.26 ml) and the resulting reaction mixture was vigorously stirred at 0°C for 30 min and at room temperature for 18 h whereupon saponification was complete. The resulting solution was diluted, at 0°C, with an ice-cold 5% aq. solution of citric acid and the precipitated product was extracted twice into EtOAc. The combined organic layers were washed with water, dried and evaporated to afford the crude product which upon FCC, with the solvent system C as the eluant, afforded 1.72 g (81%) of the dipeptide acid **15a** and 0.15 g of the corresponding isomeric acid **15b**.

*Trt-Glp-L-His(Trt)-OH (**15a**).* M.p. 220°C, $[\alpha]_D^{25}$ −48.8° (*c* 0.5, MeOH), R_f (B) 0.70, R_f (C) 0.15 and R_f (E) 0.50. IR: 3406, 3250–2360 (br), 1709 and 1686 cm^{-1}. Dipeptide **12b** (3.83 g, 5 mmol) was saponified under reaction conditions identical with those described above for **12a** to provide 3.30 g (88%) of pure **15b** which was solidified by trituration with hexane. Saponification in this case was complete within 1.5 h at room temperature and only traces of isomeric acid **15a** could be detected by TLC.

*Trt-Glp-D-His(Trt)-OH (**15b**).* M.p. 130–132°C, $[\alpha]_D^{25}$ −41.5° (*c* 0.5, MeOH), R_f (B) 0.74, R_f (C) 0.28 and R_f (E) 0.55.

*N-Trityl-L-pyroglutamyl-L- and -D-histidyl(N^{im}-trityl)-L-proline methyl ester (**16a**) and (**16b**), respectively.* Fully protected tripeptides **16a** and **16b** were prepared by coupling dipeptide acids **15a** and **15b**, respectively, with HCl·H-Pro-OMe using the coupling agent BOP in exactly the same way as that described below for the synthesis of tripeptide derivative **17a**. Racemisation was observed in both couplings to the extent of ca. 10%. Both compounds were obtained pure, in 75% and 78% yields, respectively, after FCC using as the eluant the system G for the former and $CHCl_3$–MeOH (98:2) for the latter. When DCC–BtOH was used as the coupling system, both yields were substantially lower (48 and 55%, respectively) and increased amounts (ca. 10%) of the alternative diastereomeric tripeptides from racemisation were formed.

Tripeptide derivative **16a** had m.p. 123–126°C, $[\alpha]_D^{25}$ −107.7° (*c* 0.5, MeOH), R_f (A) 0.39, R_f (B) 0.77 and R_f (E) 0.75. Anal. $C_{55}H_{51}N_5O_5$: C, H. IR: 3404, 3293, 1746, 1702, 1691 and 1652 cm^{-1}. ^1H NMR of the major conformer: δ 7.45–7.00 [31 H, m, Ph-H and H(H)-2], 6.80 and 5.55 (minor conformer) (1 H, two d, *J* 5.3 and 8.0 Hz, respectively, CONH), 6.73 and 6.58 (minor conformer) [1 H, two s, H(H)-4], 4.56 [1 H, q, *J* 5.3 Hz, H(H)-7], 4.52 [1 H, dd, *J* 4.3 and 8.0 Hz, H(P)-2], 4.10 [1 H, d, *J* 8.0 Hz, H(G)-5], 3.74 (minor conformer) and 3.40 (3 H, two s, OMe), 3.64 [1 H, m, H(P)-5a], 3.52 [1 H, m, H(P)-5b], 2.75 [1 H, d, *J* 5.3 Hz, H(H)-6], 2.50–1.80 [8 H, m, H(G)-3, H(G)-4, H(P)-3 and H(P)-4].

The tripeptide derivative **16b** had m.p. 153–155°C, $[\alpha]_D^{25}$ −47.1° (*c* 0.5, MeOH), R_f (A) 0.39, R_f (B) 0.80 and R_f (E) 0.76. Anal. $C_{55}H_{51}N_5O_5$: C, H. IR: 3424, 3308, 1744, 1702 and 1656 cm^{-1}. ^1H NMR of the major conformer: δ 7.42–7.00 [32 H, m, Ph-H, CONH and H(H)-2], 6.56 (minor conformer) and 6.52 [1 H, two s, H(H)-4], 5.96 (minor conformer) (d, *J* 6.9 Hz, CONH), 4.62 [1 H, t, *J* 5.2 Hz, H(H)-7], 4.32 [1 H, dd, *J* 3.5 and 6.9 Hz, H(P)-2], 3.94 [1 H, d, *J* 8.6 Hz, H(G)-5], 3.69 and 3.60 (minor conformer) (3 H, two s, OMe), 3.39 [1 H, m, H(P)-5a], 2.92 [1 H, dd, *J* 5.2 and 13.8 Hz, H(H)-6a], 2.78 [1 H, dd, *J* 5.2 and 13.8 Hz, H(H)-6b], 2.80 [1 H, m, H(P)-5b], 2.20–1.70 [8 H, m, H(G)-3, H(G)-4, H(P)-3 and H(P)-4].

*N-Trityl-L-pyroglutamyl-L-histidyl(N^{im}-trityl)-trans-4-hydroxy-L-proline methyl ester (**17a**).* To a solution of acid **15a** (0.75 g, 1 mmol) in anhydrous DCM (1.5 ml) was added HCl·H-Hyp-OMe (0.2 g, 1.1 mmol) followed by BOP reagent (0.44 g, 1 mmol) and the dropwise addition of DIPEA (0.51 ml, 3 mmol). The resulting mixture was stirred at room temperature for 30 min when reaction was found to be complete. The solvent was evaporated off and the residue was diluted with 5% aq. citric acid and extracted twice with EtOAc. The combined organic phases were washed sequentially with 5% aq. citric acid, water, 5% aq. $NaHCO_3$ and brine and dried. Evaporation of the solvents afforded the crude product which, upon FCC with the system G as the eluant, gave 0.73 g (83%) of fully protected TRH (**17a**) and 0.06 g of the epimeric tripeptide **17b**.

Tripeptide derivative **17a** had m.p. 155–158°C, $[\alpha]_D^{25}$ −110.3° (*c* 0.5, MeOH), R_f (A) 0.30, R_f (B) 0.76 and R_f (E) 0.70. Anal. $C_{55}H_{51}N_5O_6$: C, H. IR: 3426, 3283, 1748, 1684 and 1652 cm^{-1}. ^1H NMR: δ 7.40–7.05 [31 H, m, Ph-H and H(H)-2], 6.98 and 5.82 (1 H, two d, *J* 5.1 and 7.6 Hz respectively, CONH), 6.74 and 6.59 [1 H, two d, *J* 1 Hz, H(H)-4], 4.64 and 4.58 [1 H, one dd with *J* 5.1 and 7.6 Hz and one q with *J* 7.6 Hz, H(H)-7], 4.49 and 4.25 [1 H, two unresolved m, H(P)-4], 4.48 and 4.08

[1 H, two t, *J* 7.6 Hz, H(P)-2], 4.08 and 4.05 [1 H, two d, *J* 7.6 Hz, H(G)-5], 4.02 and 3.98 [1 H, two d, *J* 14.3 Hz, H(P)-5a], 3.75 and 3.50 (3 H, two s, OMe), 3.46 and 3.38 [1 H, two dd, *J* 4.1 and 14.3 Hz, H(P)-5b], 2.76 and 2.64 [2 H, two d, *J* 7.6 Hz, H(H)-6], 2.42–1.98 [6 H, m, H(G)-3, H(G)-4 and H(P)-3].

N-*Trityl-L-pyroglutamyl-D-histidyl*(N^{im}-*trityl*)-trans-*4-hydroxy-L-proline methyl ester* (**17b**). To an ice-cold solution of acid **15b** (1.53 g, 2 mmol) in anhydrous DMF (2.5 ml) was added HOBt (0.4 g, 3 mmol), followed by DCC (0.43 g, 2.1 mmol). The resulting mixture was stirred at 0°C for 15 min and at room temperature for 45 min. HCl·H-Hyp-OMe (0.38 g, 2.1 mmol) was then added, the mixture was cooled to 0°C, and the pH adjusted to ca. 8 by the dropwise addition of NMM (ca. 0.23 ml). The resulting mixture was allowed to attain room temperature, its pH was readjusted to 8 by the dropwise addition of further NMM and stirring was continued for 1 h at room temperature after which reaction was found to be complete (TLC). The resulting reaction mixture was then diluted with 5% aq. citric acid and extracted twice with EtOAc. The combined organic phases were washed sequentially with 5% aq. citric acid, water, 5% aq. $NaHCO_3$ and brine, dried and evaporated to leave crude product which was purified by FCC, also with the system G as the eluant, to give 1.37 g (78%) of pure **17b** and 0.26 g of **17a**.

Tripeptide derivative **17b** had m.p. 147–149°C, $[\alpha]_D^{25}$ −46.0° (*c* 0.5, MeOH), R_f (A) 0.37, R_f (B) 0.77 and R_f (E) 0.75. Anal. $C_{55}H_{51}N_5O_6$: C, H. IR: 3429, 3244, 1754, 1698 and 1653 cm^{-1}. ^1H NMR: δ 7.48–6.96 [31 H, m, Ph-H and H(H)-2], 6.32 [1 H, s, H(H)-4], 5.87 (1 H, d, *J* 5.2 Hz, CONH), 4.44 [1 H, unresolved m, H(H)-7], 4.36 [1 H, t, *J* 9 Hz, H(P)-2], 4.20 [1 H, unresolved m, H(P)-4], 3.98 [1 H, d, *J* 11.3 Hz, H(P)-5a], 3.96 [1 H, d, *J* 6.2 Hz, H(G)-5], 3.78 (3 H, s, OMe), 3.58 [1 H, dd, *J* 2.7 and 11.3 Hz, H(P)-5b], 3.11 [1 H, dd, *J* 5.0 and 13.5 Hz, H(H)-6a], 2.87 [1 H, dd, *J* 2.3 and 13.5 Hz, H(H)-6b], 2.82 [1 H, dt, *J* 7.9 and 10.1 Hz, H(G)-3a], 2.46–1.92 [4 H, H(G)-4 and H(P)-3], 1.55 [1 H, dd, *J* 9.0 and 10.1 Hz, H(G)-3b].

N-*Trityl-L-pyroglutamyl-L- and -D-histidyl*(N^{im}-*trityl*)-cis-*4-hydroxy-L-proline methyl ester* (**20a**) *and* (**20b**). To an ice-cold solution of tripeptide **17a** (0.58 g, 0.66 mmol) in MeOH (5 ml) was added dropwise 4 M NaOH (0.5 ml). The resulting mixture was stirred at 0°C for 15 min and then left to attain room temperature and stirred for 2.5 h, after which saponification was complete (TLC). MeOH was removed under reduced pressure and the residue was diluted with water. The resulting solution was cooled to 0°C and adjusted to pH 6 by the dropwise addition of gl. AcOH. The precipitated product was extracted twice with EtOAc and the combined organic phases were washed several times with brine, dried and then evaporated to dryness to afford 0.55 g (96%) tripeptide acid **18a** as a foam. The acid obtained thus was dissolved in anhydrous THF (3 ml) cooled to 0°C, and treated sequentially with TPP (0.33 g, 1.27 mmol) and DEAD (0.20 ml, 1.27 mmol). The resulting solution was kept at that temperature for 10 min and thereafter for 20 min at room temperature. An additional portion of 0.6 mmol of each of TPP and DEAD was added and the mixture was stirred at room temperature for a further 30 min. Excess MeOH (1.5 ml) was then introduced into the solution which was then kept at room temperature for 12 h. The solvent was then evaporated off and the oily residue was subjected to FCC, using system A as the eluant, to give 0.34 g (59%) of tripeptide ester **20a**.

Tripeptide derivative **20a** had m.p. 143–146°C, $[\alpha]_D^{25}$ −95.4° (*c* 0.5, MeOH), R_f (A) 0.26, R_f (B) 0.75 and R_f (E) 0.71. Anal. $C_{55}H_{51}N_5O_6$: C, H. IR: 3442, 2260, 1742, 1684 and 1641 cm^{-1}. ^1H NMR: δ 7.40–7.02 [31 H, m, Ph-H and H(H)-2], 6.84 and 5.60 (1 H, two d, *J* 5.2 and 7.8 Hz, respectively, CONH), 6.68 and 6.60 [1 H, two d, *J* 1 Hz, H(H)-4], 4.60–4.38 [3 H, m, H(H)-7, H(P)-4 and H(P)-2], 4.13 and 4.11 [1 H, two d, *J* 7.8 Hz, H(G)-5], 3.86 and 3.72 [1 H, two dd, *J* 4.2 and 11.4 Hz, H(P)-5a], 3.76 and 3.48 (3 H, two s, OMe), 3.70 [1 H, dd, *J* 4.2 and 11.4 Hz, H(P)-5b], 2.76 [2 H, d, *J* 5.2 Hz, H(H)-6], 2.45–1.92 [6 H, m, H(G)-3, H(G)-4 and H(P)-3].

Under identical reaction conditions the epimeric ester **20b** was obtained from the tripeptide ester **17b** in 62% yield. Tripeptide derivative **20b** had m.p. 135–137°C, $[\alpha]_D^{25}$ −57.0° (*c* 0.5, MeOH), R_f (A) 0.22, R_f (B) 0.73 and R_f (E) 0.72. Anal. $C_{55}H_{51}N_5O_6$: C, H. IR: 3440, 3246, 1754, 1698 and 1648 cm^{-1}. ^1H NMR of the major conformer: δ 7.36–7.00 [31 H, m, Ph-H and H(H)-2], 6.58 (minor conformer) and 6.52 [1 H, two d, *J* 1 Hz, H(H)-4], 5.94 and 5.14 (minor conformer) (1 H, two d, *J* 6.6 and 8.5 Hz, respectively, CONH), 4.38 [1 H, apparent q, *J* 5.2 Hz, H(H)-7], 4.25 [2 H, one unresolved m and one dd with *J* 1.8 and 9.7, H(P)-4 and H(P)-2, respectively], 3.88 [1 H, d, *J* 8.6 Hz, H(G)-5], 3.78 and 3.75 (3 H, two s, OMe), 3.62 [1 H, dd, *J* 2.8 and 12.1 Hz, H(P)-5a], 3.20 [1 H, dd, *J* 3.5 and 12.1 Hz, H(P)-5b], 2.82 [1 H, dd, *J* 6.9 and 13.8 Hz, H(H)-6a], 2.70 [1 H, dd, *J* 5.2 and 13.8 Hz, H(H)-6b], 2.26–1.60 [6 H, m, H(G)-3, H(G)-4 and H(P)-3].

General procedure for the TFA-mediated detritylation of protected TRH **16a** *and analogues* **16b**, **17a**, **17b**, **20a** *and* **20b**. The protected tripeptide (0.25 mmol) was dissolved in an ice-cold 20% solution of TFA in 10 ml DCM–TFE (6:1) and then allowed to attain room temperature. After 1 h at room temperature, the solution was evaporated at reduced pressure and the residue was treated with anhydrous DE to provide the corresponding detritylated tripeptide methyl ester trifluoroacetate as a white powder which was, without any further purification, subjected to ammonolysis as follows.

General procedure for ammonolysis. Preparation of L-pyroglutamyl-L- and -D-histidyl-L-prolinamide (**21a**) *and* (**21b**), *L-pyroglutamyl-L- and -D-histidyl*-trans-*4-hydroxy-L-proli-*

namide (**22a**) *and* (**22b**) *and* L-*pyroglutamyl-*L- *and* -D-*histidyl-*cis-*4-hydroxy-*L-*prolinamide* (**23a**) *and* (**23b**).

The tripeptide methyl ester trifluoroacetate, obtained as described above, was dissolved in anhydrous MeOH and the resulting solution was saturated with anhydrous NH_3 and kept at room temperature for 4–5 days until reaction was complete (TLC, solvent system D). The reaction mixture was evaporated to dryness and the residue was dissolved in MeOH and reevaporated. This procedure was repeated once more and then the resulting residue was subjected to RP-FCC as described below to provide pure products as judged (Fig. 2) by RP-HPLC analysis of freshly prepared solutions of tripeptides in the eluant. This analysis was routinely performed under the following experimental conditions. The analytical column used was a Supelcosil LC-18, 5 μm particle size, 250 × 4.6 mm i.d., purchased from Supelco, equipped with an RP-18 guard column, 4 × 4.6 mm i.d., purchased from Brownlee Labs. The eluted peaks were recorded at 211 nm. Elution was carried out at 0.7 ml min^{-1} with 85% aq. MeCN. The eluant used in these HPLC analyses was degassed by vacuum filtration through a 0.2 μm membrane filter followed by agitation in an ultrasonic bath. The capacity factor $k' = (t_R - t_0)/t_0$ was used to define the relative position (retention time) of the eluted compound (t_R) to the first eluted peak (t_0).

Tripeptide **21a** (TRH). Yield 73 mg (81%), m.p. 165°C (decomp.), $[\alpha]_D^{25}$ −24.4° (*c* 0.5, DMF), R_f (B) 0.09, R_f (D) 0.29 and R_f (F) 0.49, k' 1.50. Amino acid analysis (AAA): Glu, 1.00; His, 0.96; Pro, 0.98. IR: 3426, 3303, 1682 and 1638 cm^{-1}. FAB-MS (glycerol) [*m/z* (% rel. int.)]: 363 (100, *M*H), 249 (23.5, *M*H − $ProNH_2$), 221 (32.3, *M*H − $ProNH_2$ − CO).

Tripeptide **21b**. Yield 70 mg (77%), m.p. 172°C (decomp.), $[\alpha]_D^{25}$ −71.2° (*c* 0.2, DMF), R_f (B) 0.09, R_f (D) 0.25 and R_f (F) 0.51, k' 1.53. AAA: Glu, 1.00; His, 0.98; Pro, 1.02. IR: 3424, 3262, 1680 and 1638 cm^{-1}. FAB-MS (glycerol) [*m/z* (% rel. int.)]: 363 (100, *M*H), 249 (14.0, *M*H − $ProNH_2$), 221 (17.6, *M*H − $ProNH_2$ − CO).

Tripeptide **22a**. Yield 80 mg (85%), m.p. 240°C (decomp.), $[\alpha]_D^{25}$ −22.4° (*c* 0.2, DMF), R_f (B) 0.08, R_f (D) 0.22 and R_f (F) 0.41, k' 1.47. AAA: Glu, 1.00; His, 0.98; Hyp, 1.05. IR: 3454, 3210, 1680 and 1642 cm^{-1}. FAB-MS [*m/z* (% rel. int.)]: 379 (100, *M*H), 249 (16.4, *M*H − $HypNH_2$), 221 (23.3, *M*H − $HypNH_2$ − CO).

Tripeptide **22b**. Yield 78 mg (83%), m.p. 160°C (decomp.), $[\alpha]_D^{25}$ +8.9° (*c* 0.5, DMF), R_f (B) 0.08, R_f (D) 0.20 and R_f (F) 0.43, k' 1.80. AAA: Glu, 1.00; His, 0.94; Hyp, 0.97. IR: 3420, 3250, 1680 and 1637 cm^{-1}. FAB-MS [*m/z* (% rel. int.)]: 379 (100, *M*H), 249 (30.2, *M*H − $HypNH_2$), 221 (37.8, *M*H − $HypNH_2$ − CO).

Tripeptide **23a**. Yield 76 mg (80%), m.p. 245°C (decomp.), $[\alpha]_D^{25}$ −21.1° (*c* 0.1, DMF), R_f (B) 0.07, R_f (D) 0.18 and R_f (F) 0.38, k' 1.47. AAA: Glu, 1.00; His, 0.98; cHyp, 1.03. IR: 3422, 3282, 1680 and 1643 cm^{-1}. FAB-MS [*m/z* (% rel. int.)]: 379 (100, *M*H), 249 (21.8, *M*H − $cHypNH_2$), 221 (31.6, *M*H − $cHypNH_2$ − CO).

Tripeptide **23b**. Yield 73 mg (77%), m.p. 141°C (decomp.), $[\alpha]_D^{25}$ −8.9° (*c* 0.5, DMF), R_f (B) 0.07, R_f (D) 0.16 and R_f (F) 0.47, k' 1.43. AAA: Glu, 1.00; His, 0.94; cHyp, 0.96. IR: 3444, 3277, 1676, 1658 and 1638 cm^{-1}. FAB-MS [*m/z* (% rel. int.)]: 379 (100, *M*H), 249 (30.6, *M*H − $cHypNH_2$), 221 (35.0, *M*H − $cHypNH_2$ − CO).

General procedure for purification of TRH and analogues **21–23** *by RP-FCC.* RP-FCC was performed using reversed-phase silica gel (RP-SG) obtained as described in Ref. 15. The column (23 mm i.d.) was packed with a slurry made up of RP-silica gel and MeOH–distilled water (1:9) by applying a gentle pressure on the top of the column. The packed column obtained thus was 35 cm in length. The crude product from the ammonolysis was dissolved in the minimum volume of 20% aq. MeOH. The column was first eluted with two column volumes of distilled water. Elution was then initially performed with 5, 10 and 15% aq. MeOH (two column volumes each time) and finally with 20% aq. MeOH. Fractions of 1 ml were collected and checked first by the Kaiser test and then on TLC plates by charring. The ninhydrin-positive fractions (containing ammonium trifluoroacetate) were rejected whereas those giving solely positive charring test were pooled and freeze-dried to give pure products as white powders.

Note added in proof. The preparation of *N*-(9-fluorenylmethoxycarbonyl)pyroglutamic acid and derivatives, which has recently appeared, is relevant to the present work.[22]

References

1. Barlos, K., Papaioannou, D., Patrianakou, S. and Tsegenidis, T. *Liebigs Ann. Chem.* (1986) 1950.
2. Baldwin, J. E., North, M., Flinn, A. and Moloney, M. G. *Tetrahedron 45* (1989) 1453 and references cited therein.
3. Papaioannou, D., Stavropoulos, G., Sivas, M., Barlos, K., Francis, G. W., Aksnes, D. W. and Maartmann-Moe, K. *Acta Chem. Scand., Ser. B 45* (1991) 99.
4. Papaioannou, D., Stavropoulos, G., Karagiannis, K., Francis, G. W., Brekke, T. and Aksnes, D. W. *Acta Chem. Scand., Ser. B 44* (1990) 243.
5. Barlos, K., Papaioannou, D. and Theodoropoulos, D. *J. Org. Chem. 47* (1982) 1324.
6. Wünsch, E. *Methoden der organischen Chemie, Synthese von Peptiden I*, Houben-Weyl-Müller, 4. Aufl., Bd. XV/I, Thieme, Stuttgart 1974.
7. Coppola, G. M. and Schuster, H. F. *Asymmetric Synthesis — Construction of Chiral Molecules Using Amino Acids*, Wiley Interscience, New York 1987.
8. Menyhart, J. *Endogenous Regulatory Peptides — Chemistry, Biology and Medical Significance*, Akademiai Kiado, Budapest 1989.
9. Barlos, K., Papaioannou, D. and Theodoropoulos, D. *Int. J. Peptide Protein Res. 23* (1984) 300.
10. Sieber, P. and Riniker, B. *Tetrahedron Lett.* (1991) 739.
11. Stavropoulos, G., Karagiannis, K., Vynios, D., Papaioannou, D., Aksnes, D. W., Frøystein, N. A. and Francis, G. W. *Acta Chem. Scand., Ser. B 45* (1991) 1047.

12. Barlos, K., Mamos, P., Papaioannou, D., Patrianakou, S., Sanida, C. and Schäfer, W. *Liebigs Ann. Chem.* (1987) 1025.
13. Sieber, P. and Riniker, B. *Tetrahedron Lett.* (1987) 6031.
14. Castro, B., Dormoy, J.-R., Evin, G. and Selve, C. *Tetrahedron Lett.* (1975) 1219.
15. O'Neil, I. A. *Synlett* (1991) 661.
16. Chang, J.-K., Sievertsson, H., Currie, B., Folkers, K. and Bowers, C. *J. Med. Chem. 14* (1971) 484.
17. Flouret, G., Morgan, R., Gendrich, R., Wilber, J. and Seibel, M. *J. Med. Chem. 16* (1973) 1137.
18. Bankowski, K. and Drabarek, S. *Acta Pol. Pharm. 33* (1976) 81; *Chem. Abstr. 86* (1977) 55688m.
19. Brown, D. W., Nakashima, T. T. and Rabenstein, D. L. *J. Magn. Reson. 45* (1981) 302.
20. Perrin, D. D., Armarego, W. L. F. and Perrin, D. F. *Purification of Laboratory Chemicals*, 2nd ed., Pergamon, Oxford 1980.
21. Boissonnas, R. A., Guttmann, S., Jaquenoud, P. A. and Waller, J. P. *Helv. Chim. Acta 38* (1955) 1491.
22. Benoiton, N. L. and Chen, F. M. F. *Int. J. Peptide Protein Res. 43* (1994) 321.

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