Solid-Phase Synthesis and Spectroscopic Studies of TRH Analogues Incorporating cis- and trans-4-Hydroxy-L-Proline

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An efficient solid-phase synthesis of the TRH analogue Glp-His(N°-Trt)-Hyp-OMe is described. N°-Fmoc protected amino acids and DCC/HOBT activation were employed. The bulky and mild-acid-sensitive 2-chlorotrityl resin, utilised as the solid support, completely suppressed dioxopiperazine formation. The tripeptide is a key intermediate in the synthesis of TRH analogues incorporating cis- and trans-4-hydroxy-L-proline. The tripeptide was converted, with inversion of configuration at C-4 of the Hyp residue, to Glp-His(N°-Trt)-cHyp lactone in the presence of triphenylphosphine-dieethyl azodicarboxylate (TPP-DEAD). One-pot MeOH-TPP-DEAD transesterification of the lactone, followed by N°-de-tritylation, provided Glp-His-cHyp-OMe. This ester gave the corresponding amide and acid on amonolysis and saponification, respectively. A high-field 1H NMR investigation of Glp-His-cHyp-OMe and its diastereomeric Glp-His-Hyp-OMe, obtained by N°-de-tritylation of the key tripeptide, showed that the configuration at C-4 of the prolyl residues is critical for the determination of the preferred three-dimensional structure of the molecules.

The hypothalamic tripeptide thyrotropin-releasing hormone (TRH, 1) stimulates the release of the hormones thyrotropin (TSH)1–3 and prolactin4 and exerts a number of central nervous system (CNS) effects in mammals.5 In order to elicit possible structure–function relationships in TRH, extensive structural and conformational studies have been carried out. The latter involve both mathematical and physical methods and, in particular, NMR investigations,6 aimed at determining the ‘active’ conformations of the molecule, while the former were in the form of the synthesis and biological evaluation7 of a significant number of TRH analogues. Following this line of thought, all TRH residues were replaced by a variety of other amino acids or modified amino acids in attempts to determine which and what type of residue are required to restore, enhance, or even, more recently, dissociate the hormonal-releasing activity from the CNS effects.6

The present paper describes an efficient solid-phase synthesis of a TRH analogue incorporating Hyp (2), in place of Pro, and its facile conversion into TRH analogues incorporating cHyp (3). Amino acids are abbreviated as follows: histidine (His), 4-hydroxyproline (Hyp), cis-4-hydroxyproline (cHyp), proline (Pro) and pyroglutamic acid (Glp). Evidence is provided of the importance of configuration at C-4 of the Pro residue in determining the three-dimensional structure, in solution, of the molecules under consideration. We have recently shown that cHyp and derivatives

\[
R^1 \quad R^2 \quad R^3 \quad R^4
\]

\[
\begin{align*}
2 & : \text{OH} & \text{OH} & \text{H} & \text{H} \\
3 & : \text{OH} & \text{H} & \text{OH} & \text{H} \\
4 & : \text{OMe} & \text{H} & \text{OH} & \text{H} \\
5 & : \text{NH}_2 & \text{H} & \text{OH} & \text{H} \\
6 & : \text{OH} & \text{OH} & \text{H} & \text{Trt} \\
9 & : \text{OH} & \text{OH} & \text{H} & \text{Fmoc}
\end{align*}
\]

\[
R^1 \quad R^2 \quad R^3 \quad R^4
\]

\[
\begin{align*}
7 & : \text{R} = \text{Trt} \\
14 & : \text{R} = \text{Glp-His(N°-Trt)} \\
16 & : \text{R} = \text{Ac}
\end{align*}
\]

\[
R^1 \quad R^2 \quad R^3 \quad R^4
\]

\[
\begin{align*}
1 & : \text{NH}_2 & \text{H} & \text{H} & \text{H} \\
12 & : \text{O}^\text{=O} & \text{OH} & \text{H} & \text{Trt} \\
13 & : \text{OH} & \text{H} & \text{OH} & \text{Trt} \\
15 & : \text{OMe} & \text{H} & \text{OH} & \text{Trt} \\
17 & : \text{OMe} & \text{H} & \text{OH} & \text{H} \\
18 & : \text{NH}_2 & \text{H} & \text{OH} & \text{H} \\
19 & : \text{OH} & \text{H} & \text{OH} & \text{H} \\
20 & : \text{OH} & \text{OH} & \text{H} & \text{H} \\
21 & : \text{NH}_2 & \text{H} & \text{OH} & \text{Trt}
\end{align*}
\]

Scheme 1. Structures of compounds encountered in the synthesis of 4-hydroxyproline derivatives and TRH analogues.

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*All amino acids in this work are of the l-configuration.
such as the ester 4 and the amide 5, suitable for use in peptide synthesis, can be simply prepared from the readily available Trt-Hyp-OH (6) through the key intermediate lactone 7' (see Scheme 1 for structures, Trt = trityl). The latter was obtained from 6 by means of an intramolecular Mitsunobu reaction which resulted in the desired configurational inversion at C-4 of Hyp. Initial attempts to incorporate cHyp directly into the TRH peptide chain by coupling either the ester 4 or amide 5 to Glp-His-OH or Z-Glp-His-OH, employing the commonly used azide 8 or dicyclohexylcarbodiimide (DCC) activation 9 methods were unsatisfactory owing to the complex product mixtures obtained. On the other hand, dioxopiperazine formation was so efficient that no trace of coupling product could be found when we attempted to couple Glp-OH with H-His (N<sup>α</sup>-Trt)-cHyp-<sub>NH2</sub>. This last compound was readily obtained by coupling Fmoc-His(N<sup>α</sup>-Trt)-OH and H-chyp-NH<sub>2</sub> followed by N<sup>α</sup>-deprotection (Fmoc = 9-fluorenylmethoxycarbonyl). Similar results were obtained when the diastereomeric amide H-Hyp-NH<sub>2</sub> was used as the starting material for either of the two described methodologies.

Prompted by these results we decided to employ the recently developed, mild-acid-sensitive, and bulky 2-chlorotryptyl resin 8<sup>10</sup> for a stepwise assembly of the peptide skeleton using N<sup>α</sup>-Fmoc protection. The resin was utilized in expectation of minimal dioxopiperazine formation and concomitant peptide losses. Although SPPS is quite popular in the synthesis of other biologically important peptides, it is only rarely used for TRH analogues. 11 Since we were aware of the tendency of the trityl group to migrate from the carboxyl to an adjacent stereochemically accessible hydroxy group 12 we preferred to anchor Hyp rather than cHyp to the resin. Thus, reaction of the polymeric chloride 8 with Fmoc-Hyp-OH (9), in the presence of diisopropylethylamine (DIEA), resulted in the clean formation of resin 10 (Scheme 2) with a substitution of 0.62 mmol Fmoc-Hyp-OH per gram of resin as determined spectrophotometrically. Fmoc-Hyp-OH was obtained, in 92 % yield, via pertrimethylsilylation with Me<sub>3</sub>SiCl/DIEA, followed by electrophilic Si–N bond fission with Fmoc-Cl. 13

N<sup>α</sup>-deprotection of resin 10 with 20 % piperidine in DMF followed by reaction with Fmoc-His(N<sup>α</sup>-Trt)-OH, preactivated with DCC/HOBt (HOBt = 1-hydroxybenzotriazole) led unexceptionally to the derivative 11. Application of another cycle of N<sup>α</sup>-deprotection followed by coupling with H-Glp-OH, this too preactivated with DCC/HOBt, afforded the resin-bound tripeptide 12. Careful examination, by TLC comparison with an authentic sample, of both the deprotection and coupling media for the latter cycle excluded the presence of cyclo[His(N<sup>α</sup>-Trt)-Hyp]. The use of the bulky 2-chlorotryptyl resin as solid support did indeed completely suppress dioxopiperazine formation.

Finally, treatment of 12 with acetic acid/2,2,2-trifluoroethanol/dichloromethane (AcOH/TFE/DCM) 1:2:7 for 20 min at room temperature provided the tripeptide 13 in 92 % yield. Physical data for this tripeptide and the compounds referred to below are provided in Table 1. Tripeptide 13 had been projected as a pivotal intermediate for the synthesis of TRH analogues incorporating cHyp and Hyp in place of Pro. The former possibility had been realised in conjunction with the application of the methodology already used by us to invert the configuration of C-4 of Hyp. 7 Treatment of the tripeptide 13 with TPP–DEAD in THF/ DMF (2:1) for 1 h at room temperature provided the expected lactone 14. The low yield (ca. 20 %) encountered on attempting to purify this lactone by flash chromatography (FC) with CHCl<sub>3</sub>/MeOH (9:1) is mainly attributed to its facile conversion into tripeptide 15 (TLC comparison with an authentic sample) on the silica gel column used. This accords with the earlier reported 14 Amberlyst 15 catalysed transesterification of the lactone 16 (Scheme 1) with ethanol.

This result prompted us to generate this lactone in situ and subsequently exploit the excess of the TPP–DEAD complex present in the reaction mixture, to catalyse transesterification with MeOH, 7 in a one-pot experiment. In practice, treatment of 13 with TPP–DEAD as above, followed by excess MeOH for 12 h at room temperature as for lactone 14, did indeed provide a 60 % yield of the tripeptide ester 15. N<sup>α</sup>-Detritylation of the latter with 20 % trifluoroacetic acid (TFA) in DCM/TFE (6:1) for 1 h at
Table 1. Yields and physical data of TRH analogues.a

<table>
<thead>
<tr>
<th>No.</th>
<th>Yield (%)</th>
<th>M.p. °C</th>
<th>[α]b</th>
<th>TLC: Rf</th>
<th>IRν (ν/cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(A)</td>
<td>(B)</td>
</tr>
<tr>
<td>13</td>
<td>92</td>
<td>203 (decomp.)</td>
<td>−11.3 (c 1, MeOH)</td>
<td>0.35</td>
<td>0.20</td>
</tr>
<tr>
<td>14</td>
<td>20</td>
<td>150–152</td>
<td>−23.3 (c 0.8, MeOH)</td>
<td>–</td>
<td>0.53</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>173–175</td>
<td>−24.1 (c 0.9, MeOH)</td>
<td>–</td>
<td>0.51</td>
</tr>
<tr>
<td>17c</td>
<td>92</td>
<td>148–151</td>
<td>−53.4 (c 1, H2O)</td>
<td>–</td>
<td>0.16</td>
</tr>
<tr>
<td>18</td>
<td>94</td>
<td>193</td>
<td>−48.4 (c 0.5, H2O)</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>19</td>
<td>93</td>
<td>221 (decomp.)</td>
<td>−13.8 (c 0.5, H2O)</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>20c</td>
<td>90</td>
<td>225 (decomp.)</td>
<td>−50.8 (c 1, H2O)</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>21</td>
<td>95</td>
<td>209 (decomp.)</td>
<td>+2.1 (c 1, MeOH)</td>
<td>–</td>
<td>0.46</td>
</tr>
</tbody>
</table>

*a Amino acid analysis for the deprotected TRH analogues were as follows: 17, Glu 1.00, His 0.97, cHyp 0.98; 18, Glu 1.00, His 0.98, cHyp 1.04; 19, Glu 1.00, His 0.99, cHyp 1.05; 20, Glu 1.00, His 0.92, Hyp 0.94. bThe IR spectra of compounds 17–20 were similar to those for the corresponding trifluoroacetate derivatives apart from the fact that the absorptions at 730 and 630 cm⁻¹, due to the trityl group, were missing. cAs the corresponding trifluoroacetate salt.

room temperature provided the TRH analogue 17 incorporating cHyp. Furthermore, ammonolysis of 17 in MeOH for 12 h at room temperature afforded the tripeptide amide 18 in 92% yield, while saponification of 17 for 1.5 h at room temperature gave a 93% yield of the tripeptide acid 19. The tripeptide amide 18 could also be obtained in 86% yield in 48 h at 0°C. Overall, ammonolysis of the lactone 14, as for 17, followed by N-de-tritylation, and neutralisation of the

Fig. 1. 400 MHz ¹H NMR spectrum of Glp-His-cHyp-OH (19).
TFA salt thus obtained with ammoniacal MeOH. The TRH analogue 20, incorporating Hyp for Pro, was straightforwardly obtained in 99% yield from tripeptide 13 by N\textsuperscript{m}-detritiylation as described for 17. By way of comparison, a synthesis of the related analogue Glp-His-Hyp-NH\textsubscript{2}, based on a DCC-mediated coupling of Glp-His-OH and H-Hyp-NH\textsubscript{2}, has been reported earlier.\textsuperscript{15}

In order to uncover the possible importance of the configuration at C-4 of the 4-OH-prolyl residue on the definition of their preferred conformations, NMR investigations of the epimeric tripeptides 19 and 20 were carried out. Details of chemical-shift data assigned on the basis of 2D COSY spectra and NOE experiments are given in Table 2. The \textsuperscript{1}H NMR spectrum of 19 and 2D COSY spectrum of 20 may be found in Fig. 1 and Fig. 2, respectively. The results showed that, while the part of the spectrum associated with the pyroglutamyl moiety in the two molecules is almost unchanged, important differences in splitting patterns and/or chemical shifts of the ring protons of the prolyl and the side chain of the histidyl residues are observed, reflecting significant changes in the preferred conformations of 19 and 20. Considerable diagnostic value attached to the resonances assigned to the methylene histidyl protons H(H)-6. (The parenthesised letters are used here, as elsewhere, to indicate the residue, (G) glutamine, (H) histidine and (P) hydroxyproline or cis-hydroxyproline, to which the nucleus belongs.) These protons resulted in a single 2-proton doublet at 3.286 ppm for the tripeptide 19, while two doublets of doublets at 3.222 and 3.116 ppm, one for each proton, were observed for 20. These results were taken to indicate free rotation of the histidyl side chain in the former and restriction of rotation in the latter compound. A deeper insight into the preferred conformations of the two molecules was obtained with the aid of NOE experiments. The most important feature of these studies was the proximity of protons H(P)-5a and H(H)-6 for 19 and of protons H(P)-5a and H(H)-7 for 20 (Table 2). Fig. 3 and Fig. 4 give proposed conformations for the two molecules, based on Orbit Molecular Building System models (Cochranes of Oxford Ltd., 1972), which accommodate the above NMR data. A noticeable feature of the proposed model, and one which differs from that proposed for TRH itself,\textsuperscript{16} is that both the carboxy and hydroxy groups of the 4-OH-prolyl residues are involved in H-bonding, although to different groups in the Glp-His residue in each case. It is worth noting that the model proposed for 20 involves a cis His-Hyp peptide
Table 2. High-field NMR data for TRH analogues 19 and 20,\textsuperscript{a} in D\textsubscript{2}O.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Proton</th>
<th>δ-value (coupling)</th>
<th>Carbon</th>
<th>δ-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tripeptide acid 19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glp H(G)-2</td>
<td>4.393</td>
<td>(dd, J 9.1 and 4.7 Hz)</td>
<td>C(G)-2</td>
<td>59.434</td>
</tr>
<tr>
<td>Glp H(G)-3a</td>
<td>2.554</td>
<td>(m, J 9.1, 12.1 and 8.8 Hz)</td>
<td>C(G)-3</td>
<td>28.016</td>
</tr>
<tr>
<td>Glp H(G)-3b</td>
<td>2.018</td>
<td>(m, J 4.7, 12.1 and 7.9 Hz)</td>
<td>C(G)-4</td>
<td>32.027</td>
</tr>
<tr>
<td>Glp H(G)-4ab</td>
<td>2.432</td>
<td>(apparent t, J 8.8 and 7.9 Hz)</td>
<td>C(G)-5</td>
<td>173.067</td>
</tr>
<tr>
<td>His H(H)-2</td>
<td>8.470</td>
<td>(s)</td>
<td>C(H)-2</td>
<td>137.350</td>
</tr>
<tr>
<td>His H(H)-4</td>
<td>7.369</td>
<td>(s)</td>
<td>C(H)-4</td>
<td>121.074</td>
</tr>
<tr>
<td>His H(H)-6ab</td>
<td>3.286</td>
<td>(d, J 9.3 Hz)</td>
<td>C(H)-5</td>
<td>131.092</td>
</tr>
<tr>
<td>His H(H)-7</td>
<td>4.929</td>
<td>(t, J 6.6 Hz)</td>
<td>C(H)-6</td>
<td>29.230</td>
</tr>
<tr>
<td>cHyp H(P)-2</td>
<td>4.448</td>
<td>(dd, J 9.5 and 4.2 Hz)</td>
<td>C(P)-2</td>
<td>63.558</td>
</tr>
<tr>
<td>cHyp H(P)-3a</td>
<td>2.527</td>
<td>(dd, J 9.5 and 14.0 Hz)</td>
<td>C(P)-3</td>
<td>39.502</td>
</tr>
<tr>
<td>cHyp H(P)-3b</td>
<td>2.079</td>
<td>(dt, J 4.2, 14.0 and 3.8 Hz)</td>
<td>C(P)-4</td>
<td>72.579</td>
</tr>
<tr>
<td>cHyp H(P)-4</td>
<td>4.507</td>
<td>(apparent quintet, J 5.6, 3.8, 4.9 and 3.2 Hz)</td>
<td>C(P)-5</td>
<td>57.734</td>
</tr>
<tr>
<td>cHyp H(P)-5a</td>
<td>3.962</td>
<td>(dd, J 4.9 and 11.4 Hz)</td>
<td>C(P)-6</td>
<td>185.146</td>
</tr>
<tr>
<td>cHyp H(P)-5b</td>
<td>3.384</td>
<td>(dd, J 3.2 and 11.4 Hz)</td>
<td>C(P)-7</td>
<td>181.940</td>
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<tr>
<td>(Tripeptide acid 20)</td>
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<tr>
<td>Glp H(G)-2</td>
<td>4.278</td>
<td>(dd, J 9.3 and 4.9 Hz)</td>
<td>C(G)-2</td>
<td>59.621</td>
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<tr>
<td>Glp H(G)-3a</td>
<td>2.450</td>
<td>(m, J 9.3, 13.2 and 8.5 Hz)</td>
<td>C(G)-3</td>
<td>28.158</td>
</tr>
<tr>
<td>Glp H(G)-3b</td>
<td>1.902</td>
<td>(m, J 4.9, 13.2 and 7.9 Hz)</td>
<td>C(G)-4</td>
<td>32.104</td>
</tr>
<tr>
<td>Glp H(G)-4ab</td>
<td>2.322</td>
<td>(apparent t, J 8.5 and 7.9 Hz)</td>
<td>C(G)-5</td>
<td>172.830</td>
</tr>
<tr>
<td>His H(H)-2</td>
<td>8.582</td>
<td>(d, J 1.0 Hz)</td>
<td>C(H)-2</td>
<td>136.653</td>
</tr>
<tr>
<td>His H(H)-4</td>
<td>7.300</td>
<td>(s)</td>
<td>C(H)-4</td>
<td>120.699</td>
</tr>
<tr>
<td>His H(H)-6a</td>
<td>3.222</td>
<td>(dd, J 6.1 and 15.4 Hz)</td>
<td>C(H)-5</td>
<td>130.804</td>
</tr>
<tr>
<td>His H(H)-6b</td>
<td>3.116</td>
<td>(dd, J 7.9 and 15.4 Hz)</td>
<td>C(H)-6</td>
<td>28.912</td>
</tr>
<tr>
<td>His H(H)-7</td>
<td>4.999</td>
<td>(apparent t, J 6.1 and 7.9 Hz)</td>
<td>C(H)-7</td>
<td>53.307</td>
</tr>
<tr>
<td>Hyp H(P)-2</td>
<td>4.479</td>
<td>(apparent t, J 8.3 and 9.0 Hz)</td>
<td>C(P)-2</td>
<td>62.226</td>
</tr>
<tr>
<td>Hyp H(P)-3a</td>
<td>2.340</td>
<td>(overlapped m)</td>
<td>C(P)-3</td>
<td>39.778</td>
</tr>
<tr>
<td>Hyp H(P)-3b</td>
<td>2.094</td>
<td>(m, J 13.9, 9.0 and 4.4 Hz)</td>
<td>C(P)-4</td>
<td>72.755</td>
</tr>
<tr>
<td>Hyp H(P)-4</td>
<td>4.536</td>
<td>(unresolved m)</td>
<td>C(P)-5</td>
<td>58.094</td>
</tr>
<tr>
<td>Hyp H(P)-5a</td>
<td>3.760</td>
<td>(d, J 11.4 Hz)</td>
<td>C(P)-6</td>
<td>185.203</td>
</tr>
<tr>
<td>Hyp H(P)-5b</td>
<td>3.669</td>
<td>(dd, J 11.4 and 4.0 Hz)</td>
<td>C(P)-7</td>
<td>179.650</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The unambiguous assignments of the proton spectra are based on 2D COSY spectra and NOE experiments. The NOE data suggest that H(P)-3a, H(P)-4 and H(P)-5a are on the same face of the Hyp ring for 19, while, in contrast, H(P)-3b, H(P)-4 and H(P)-5b are on the same side of the ring for 20. Chemical shifts and coupling constants were obtained by spectral analysis. Carbon spectra were assigned by means of heteronuclear shift-correlation 2D-experiments. The only uncertainty is in the assignment of C(G)-6 and C(H)-8 which may be reversed. \textsuperscript{b}G, H and P are used to designate protons and carbons of Glp, His and cHyp/Hyp residues, respectively. \textsuperscript{c}As the trifluoroacetate salt. The CF\textsubscript{3}-resonance coalesces with that for C(H)-4 whereas the COO\textsuperscript{\textsuperscript{-}} resonance is found at 172.130 ppm.

bond, while that for 19 involves a \textit{trans} His-cHyp peptide bond. However, both conformers around this bond are observed in the \textsuperscript{1}H NMR spectra of both compounds 19 and 20 in the ratio of \textit{1:5.7 and 2.4:1 cis/trans}, respectively.

In contrast with the \textsuperscript{1}H NMR spectra, the corresponding \textsuperscript{13}C NMR spectra showed only minute differences and were of no significant diagnostic value. The assignment of the resonances follow from the heteronuclear shift correlation of the 2D experiments. The most important feature of both spectra was the absence of resonances due to a possible δ-histidyl residue which could have been generated by racemisation during coupling.\textsuperscript{17} In particular, the resonance for C-4 of histidine was an intense sharp singlet. This means that no detectable racemisation occurred on using the derivative Fmoc-His(N\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{n}}} Trt)-OH for incorporation of His into the peptide chain and this accords with related studies by other investigators.\textsuperscript{18}

Synthesis and \textsuperscript{1}H NMR-aided studies on further TRH analogues incorporating derivatives of cHyp and Hyp as well as biological evaluation of the presently reported analogues is now in progress.
Experimental

General. Capillary melting points were taken on a Buchi SMP-20 apparatus and are uncorrected. Optical rotations were determined with a Carl Zeiss precision polarimeter.

IR spectra were recorded as Nujol mulls on a Perkin-Elmer 457 grating spectrophotometer. UV spectra were obtained on a Cary 219 (Varian) spectrophotometer in DCM/TF/A/MeOH (7:1:2). One- and two-dimensional 1H NMR spectra were obtained at 400.13 MHz on a Bruker AM-400 WB instrument using D$_2$O as the solvent and sodium 3-trimethylsilylpropan-2-ylsilane (TSP) as an internal standard at the sample temperature of 298 K. Exceptionally acetone-d$_6$ was used as the solvent with tetramethylsilane (TMS) as the standard. The 1H spectral dimensions were performed by means of the PANIC program available from the Bruker Software library. The samples for the NMR experiments were prepared as follows: 14.0 mg and 13.5 mg of the trans- and cis-isomers, respectively, were each lyophilised twice from 99.9% D$_2$O subsequently dissolved in 400 µl of D$_2$O and transferred to 5 mm NMR tubes (Wilmad). The final concentration of each tripeptide was ca. 0.09 M.

Double quantum filtered COSY spectra were collected in the pure-absorption mode into 1024 complex points for 700 and 560 $t_2$-values for the trans- and cis-isomers, respectively. Quadrature detection in the $f_1$ dimension was achieved by employing the TPPI method. Sixteen transients were averaged for each $t_1$ increment. The spectral width was 4000 Hz. A recycle delay of 2 s was used. The data were processed on a Micro Vax II computer using the program FIDNMR. The 700 (or 560) $t_1$-incremented FIDs from the DQF-COSY experiments were multiplied by an exponential window prior to a complex Fourier transform along $t_2$. 2 Hz was added to the linewidths. The $f_2$ spectra were carefully phase-adjusted and the baselines corrected by a 3rd order polynomial fit. In the $t_2$-dimension the data were apodised with a squared, skewed (0.7) and phase-shifted (75°) sine-bell function. Prior to a real Fourier transform along $t_2$, the first $t_1$-increment ($f_1$ spectrum) was multiplied by 0.5 in order to suppress $t_2$ noise. No baseline correction was applied to the data along $f_1$.

The homonuclear Overhauser enhancements were measured by means of NOE difference spectroscopy. The saturation was built up line by line, each line of a particular multiplet being subjected to a 0.5 s burst of weak irradiation, and this process was repeated many times to give a total pre-irradiation period longer than nine times the estimated $T_1$. Subtraction of the unperturbed FID with off-resonance irradiation from the perturbed FID, followed by Fourier transformation and integration yielded the NOE values. The broad-band decoupled 13C NMR spectra were obtained at 100.63 MHz using standard one-pulse and spin-echo experiments. The latter technique was used in order to distinguish methyl and methine carbons from methylene and quaternary carbons.

The carbon–proton chemical shift correlations were established using the heteronuclear shift correlation pulse sequence. The recycle delay was 7 s, and the equipment was optimised for a one-bond proton–carbon coupling constant of 142 Hz. 128 FIDs were recorded in $t_1$ and 1K data points in $t_2$. The spectral widths were ±1725 Hz in $f_1$ and 19230 Hz in $f_2$. 80 transients were collected for each $t_1$ increment. The 2D FIDs were zero filled in both dimensions to 256 in $t_1$ and 2K in $t_2$, multiplied by a sine-bell function prior to Fourier transformation, and processed in the magnitude mode.

Mass spectra were recorded at 70 eV with a JEOL JMS D-100 instrument. FC was performed on Merck silica gel 60 (230–400 mesh) and TLC on Merck silica gel 60F$_{254}$ films (0.2 mm) precoated on aluminium foil. The solvent systems used were: (A) BuOH/AcOH/H$_2$O (4:1:1), (B) CH$_3$CN/H$_2$O (5:1), (C) CHCl$_3$/MeOH/HC(OH) (85:10:5) and (D) CHCl$_3$/MeOH (6:1). Spots were visualised with UV light at 254 nm and the charring reagent (NH$_4$)$_2$SO$_4$ conc. H$_2$SO$_4$/H$_2$O (20 g/4 ml/100 ml).

All solvents (Merck, p.a. grade) were dried and/or purified according to standard procedures prior to use. Fmoc-His(NCA-Trt)-OH and the 2-chlorotrityl chloride resin were purchased from BIOCHEM. Other reagents were obtained as follows: trifluoroacetic acid (99%), 2,2,2-trifluoroethanol (99%) diethyl azodicarboxylate (95%), trimethylsilyl chloride (98%), triphenylphosphine (99%) (Alrich); dicyclohexylcarbodiimide (synthesis grade) and disopropyl(ethyl)amine (p.a.) (Merck); 9-fluorenylmethoxycarbonyl chloride, 1-hydroxybenzotriazole, hydroxyproline and pyroglyutamic acid (Sigma).

Amino acid analyses were performed on a Beckmann Model 120C amino acid analyser using a three-buffer column system. cHyp was detected as the corresponding lactone in the extent of 88%. Yields, physical constants and spectral data for the THR analogues described below are given in Table 1.

trans-4-Hydroxy-N-9-fluorenylmethoxycarbonyl-L-proline (9). Powdered crystalline Hyp (2.23 g, 17 mmol) was suspended in dry DCM (35 ml) and treated sequentially with Me$_3$SiCl (7.6 ml, 60 mmol) and DIEA (10.2 ml, 60 mmol). The resulting mixture was refluxed, with exclusion of moisture, for 30 min to ensure solution. Fmoc-Cl (4.66 g, 18 mmol) was then added in a single portion at room temperature and the resulting solution stirred for a further 1.5 h, prior to treatment with MeOH (3 ml) for 15 min. The solvents were removed under reduced pressure and the oily residue was taken up in 2.5%aq. NaHCO$_3$ (50 ml) and washed twice with diethyl ether. The aqueous phase was cooled to 0°C and acidified with 1 M HCl to pH 2. The mixture obtained in this way was extracted twice with ethyl acetate and the combined organic layers were washed with saturated aqueous NaCl, dried (Na$_2$SO$_4$) and evaporated to leave pure Fmoc-Hyp-OH (5.5 g, 92% yield) as a white powder. Fmoc-Hyp-OH had m.p. 187–189°C, $\left[\alpha\right]_D^{38}$ −60.1 (c 1, MeOH), $R_1$ (A) 0.62, (B) 0.42, (C) 0.21, Anal. C$_{29}$H$_{21}$NO$_5$: C, H.
UV [abs (ε)]: 267sh (15 300), 277 (18 600), 288sh (11 600), 299 (5 200), 311 (6 000). IR (Nujol): 3400-3100, 2750-2200, 1725, 1655 cm⁻¹. MS [IP 70 eV; m/z (% rel. int.]): 243 (78), 179 (85), 178 (90), 177 (83), 176 (87), 175 (71), 165 (100), 163 (52), 152 (83), 151 (80), 150 (76). ¹H NMR (400.13 MHz, acetone-δ:): 7.90-7.25 [arom C-H], 4.60 and 4.44 [J, J 7.8 Hz, H(P)-2], 4.53 and 4.50 [m, H(P)-4], 4.38-4.26 [m, O-CH₂], 4.26-4.17 [m, fluorenyl, H-9], 3.69 [dd, J 4.5 and 10.9 Hz, H(P)-5a], 3.61 [overlapped dd, J 10.9 and 2.4 Hz, H(P)-5b], 3.63-3.56 [m, H(P)-5ab], 2.42 [m, J 8.1, 3.1 and 12.6 Hz, H(P)-3a], 2.32 [m, J 8.1, 3.5 and 13.0 Hz, H(P)-3a], 2.19 [m, J 7.4, 4.8 and 12.6 Hz, H(P)-3b], 2.13 [m, J 7.6, 4.9 and 13.0 Hz, H(P)-3b].³¹C NMR (100.63 MHz, acetone-δ): 174.34 and 173.76 [COOH], 155.6 and 155.25 [O-CO-N], 145.22-142.01 [arom C], 128.57-120.79 [arom C-H], 70.25 and 69.44 [C(P)-4], 68.38 and 67.99 [O-C=H], 58.85 and 58.46 [C(P)-2], 55.94 and 55.52 [C(P)-5], 48.09 and 47.98 [fluorenyl C-9], 40.30 and 39.13 ppm [C(P)-3]. [Paired signals result from the presence of an equilibrium mixture (55:45) of isomers about the partial C-N double bond. The signal identified with the major isomer is given in italics.]

Esterification of 2-chlorotriyl resin with Fmoc-Hyp-OH. Polymeric 2-chlorotriyl chloride [5 g, 1.6 mequiv. Cl per gram of resin (2-chloro-4-poly(styryl)phenylmethyl resin)] was swollen with DCM (30 ml) and treated sequentially with Fmoc-Hyp-OH (1.6 g, 4.5 mmol) and DIEA (3.5 ml, 20.3 mmol) and the resulting mixture shaken for 1 h at room temperature. Anhydrous MeOH (3 ml) was added and the mixture shaken for a further 15 min and then filtered. The resulting resin was washed, sequentially, twice with 40 ml of each of the following: DCM, DMF, MeOH, DMF, MeOH and diethyl ether. After overnight drying under reduced pressure (10⁻² mmHg) the resin 10 (6.4 g) was obtained [substitution: 0.62 (by weight) and 0.64 (spectrophotometrically) mmol Fmoc-Hyp-OH per gram of resin].

The spectrophotometric estimation of the substitution of resin 10 was carried out as follows. Resin 10 (23 mg) was treated three times with DCM/TFA/MeOH (7:1:2), 10 ml for 10 min on each occasion, and the filtrates pooled and then diluted to 100 ml with the same solvent mixture. The concentration was then calculated on the basis of the absorbance of this solution at 311 nm; the UV absorbance of standard Fmoc–Hyp–OH solutions in this solvent mixture has previously been shown to have a linear relationship to concentration at this wavelength.

l-Pyroglutamyl-l-histidyl(N⁵-trityl)-trans-4-hydroxy-l-proline (13). Resin 10 (4.80 g) was swollen with DMF (30 ml), filtered, and twice treated (10 and 15 min, respectively) with 20% piperidine in DMF (40 ml) at room temperature in order to effect Fmoc-deprotection. The resin thus obtained was sequentially washed for 2 min each time with two portions of 40 ml of DMF, 2-ProH, DMF, 2-ProH and DMF. The resin was subsequently shaken with Fmoc-His(N⁵-Trt)-OH (4.21 g, 6.8 mmol), preactivated with HOBT (1.47 g, 10.9 mmol) and DCC (1.55 g, 7.5 mmol) in DMF (30 ml) for 10 min at 0°C and then for 20 min at room temperature. The precipitated bicyclohexylurea was removed by filtration immediately before mixing this active ester with the swollen resin. After being shaken for 3.5 h, the mixture was filtered and the resulting resin 11 washed as described above for deprotection. This coupling step and the one following were routinely checked by means of the Kaiser test to ensure complete reaction. Fmoc-deprotection of 11 and coupling with Glp-OH (1.46 g, 11.3 mmol), as described for Fmoc-His(⁵-NHTrt)-OH, was followed by a second coupling cycle (reaction time 1 h) with Glp-OH (1.16 g, 9 mmol) and washing as described above gave the resin 12 (5.50 g).

Cleavage of the tripeptide 13 from the resin 12 was achieved by treatment of the latter (5.50 g) with DCM/TFA/AcOH (7:2:1 v/v, 40 ml) for 20 min at room temperature. The mixture thus obtained was diluted with water (40 ml) and evaporated to half its volume under reduced pressure, and the resulting solution lyophilised to give the tripeptide 13 (1.70 g, 92%).

l-Pyroglutamyl-l-histidyl(N⁵-trityl)-cis-4-hydroxy-l-proline lactone or 5-l-Pyroglutamyl-l-histidyl(N⁵-trityl)-2-oxa-5-azabicyclo[2.2.1]heptan-3-one (14). The tripeptide 13 (0.47 g, 0.75 mmol) was dissolved in DMF/THF (1:2, 3 ml), whereupon TPP (0.39 g, 1.5 mmol) was added and then DEAD (0.24 ml, 1.5 mmol) was added dropwise with stirring to the resulting solution at 0°C. Stirring was continued for a further 15 min at 0°C and then for 20 min at room temperature. Additional quantities of TPP (0.39 g, 1.5 mmol) and DEAD (0.24 ml, 1.5 mmol) were added as above and stirring was continued at room temperature for a further 30 min. After this time, the solvents were removed under reduced pressure and the residual oil subjected to FC with solvent system D as the eluant to give the lactone 14 (90 mg).

l-Pyroglutamyl-l-histidyl(N⁵-trityl)-cis-4-hydroxy-l-proline methyl ester (15). The tripeptide 13 (0.99 g, 1.6 mmol) was treated with TPP–DEAD as in the preparation of the lactone 14. The resulting reaction mixture was further treated with an excess of MeOH (2 ml) for 12 h at room temperature. Solvents were then removed and the residue chromatographed as for the lactone 14 to give the ester 15 (0.61 g).

N⁵-Detritylation of the TRH analogues 13 and 15 with trifluoroacetic acid. The protected analogues 13 (0.12 g, 0.2 mmol) and 15 (0.38 g, 0.6 mmol) were dissolved in 20% TFA in DCM/TFE (6:1, 5 ml for 13 and 15 ml for 15) and kept at room temperature for 1 h to give complete deprotection (TLC, solvent system B). The resulting solutions
were concentrated under reduced pressure at room temperature and triturated with diethyl ether. The precipitated trifluoroacetates of the TRH analogues 20 (89 mg) and 17 (0.28 g) were collected by filtration.

1- pyroglutamyl-3- histidyl-[N<sup>ω</sup>-trityl]-cis-4-hydroxy-1-prolinamide (21). The lactone 14 (72 mg, 0.12 mmol) was dissolved in MeOH (5 ml) and the solution was saturated with anhydrous NH<sub>3</sub>. The reaction mixture was allowed to stand overnight at room temperature and then concentrated to a small volume. Fresh MeOH was added and the resulting solution reevaporated to dryness to leave an oil. FC with system D, followed by CHCl<sub>3</sub>/MeOH (1:1) gave the amide 21 (69 mg) after pooling of the fractions, concentration and trituration with diethyl ether.

1-pyroglutamyl-3-histidyl-cis-4-hydroxy-1-prolinamide (18). The ester 17 (0.10 g, 0.2 mmol) was ammonolysed, as described for the lactone 14, for 48 h. Concentration to small volume, addition of fresh MeOH and reevaporation left a residue which was redissolved in MeOH, cooled to 0°C, and triturated with diethyl ether to provide the amide 18 (71 mg). Amide 18 could also be obtained in 90% yield from 21 by detritylation, as for 13, followed by neutralisation with NH<sub>3</sub>.

1-pyroglutamyl-3-histidyl-cis-4-hydroxy-1-prolin (19). The ester 17 (0.10 g, 0.2 mmol) was dissolved in water (3.7 ml) and treated with 1 M NaOH (0.3 ml) for 30 min at 0°C and then for a further 1.5 h at room temperature, after which time reaction was complete as shown by TLC (system B). Neutralisation with 5% HCl at 0°C and concentration under reduced pressure provided a residue to which MeOH was added. After removal of NaCl by filtration, the resulting filtrate was concentrated to a small volume and triturated with diethyl ether to give the TRH analogue 19 (71 mg).

References
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