Amino Acids and Peptides. L. Development of a Novel N^{π}-Protecting Group for Histidine, N^{π}-2-Adamantyloxymethylhistidine, and Its Application to Peptide Synthesis[1-3]

Yoshio OKADA,* Jidong WANG,[4a] Takeshi YAMAMOTO,[4b] Toshio YOKOI, and Yu MU

Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Nishi-ku, Kobe 651–21, Japan.
Received August 30, 1996; accepted October 28, 1996

N^{α}-*tert*-Butyloxycarbonyl-N^{π}-2-adamantyloxymethylhistidine, Boc-His(N^{π}-2-Adom)-OH, was prepared by the reaction of Boc-His (N^{τ}-Boc)-OMe with 2-adamantyloxymethyl chloride (2-Adom-Cl), followed by saponification. The 2-Adom group was stable to trifluoroacetic acid (TFA), 1 N NaOH and 20% piperidine/DMF and easily removed by 1 M trifluoromethanesulfonic acid–thioanisole/TFA and HF. This new protecting group suppressed racemization during peptide synthesis and exhibited high solubility in organic solvents. It was applied to the synthesis of thyrotropin-releasing hormone (TRH) using both solution and solid-phase methods. The N^{π}-2-Adom group can be used for peptide synthesis in combination with the *tert*-butyloxycarbonyl group as the N^{α}-protecting group in both solution and solid-phase methods.

Key words N^{π}-2-adamantyloxymethylhistidine; new protecting group; histidine imidazole π-nitrogen; thyrotropin-releasing hormone; peptide synthesis

During peptide synthesis, the NH group in the imidazole ring of the histidine side chain should be blocked to avoid side reactions, such as acylation, formation of the DCC adduct,[5] and side-chain-induced racemization.[6] Toluene-*p*-sulfonyl (Tos),[7,8] dinitrophenyl (Dnp),[9] trityl (Trt)[10,11] and 2-adamantyloxycarbonyl (2-Adoc)[12] groups have been developed as protecting groups on N^{τ} of the imidazole function. Although these protecting groups can reduce the extent of side-chain-induced racemization due to the reduction of the basicity of the imidazole nucleus, almost complete protection against loss of chiral purity of the histidine residue can be expected in derivatives in which the side chain protecting group is on the π-nitrogen atom of the imidazole. As N^{π}-protecting groups, benzyloxymethyl (Bom)[13] and *tert*-butyloxymethyl (Bum)[14] groups have been developed. The latter protecting group (Bum) can be easily removed by TFA and is stable under alkaline conditions, such as 20% piperidine/DMF; it is used in combination with the Fmoc group as an N^{α}-protecting group in the solid-phase method. The former protecting group (Bom) is stable to TFA and alkaline conditions and cleavable by hydrogenation over a Pd catalyst or HF.[15] Therefore, His(N^{π}-Bom) can be applied for peptide synthesis in combination with a *tert*-butyloxycarbonyl (Boc) group or 9-fluorenylmethyloxycarbonyl (Fmoc) group as the N^{α}-protecting group. The latter derivative can be used in convergent solid-phase peptide synthesis, which involves the preparation of partially protected peptide fragments by stepwise synthesis, followed by purification and assembly on a solid support.[16]

The convergent solid-phase method has become the focus of increasing attention because of its usefulness in the preparation of fairly large peptides. In this method, high solubility of the protected peptide fragment is required, since the fragment must be easily purified, and after fragment condensation on the resin, it must be removed by washing with an organic solvent.[17] Under these circumstances, our studies were directed to the development of a new protecting group for π-nitrogen of the imidazole function; it shoud behave in the same way as the Bom group to acids and bases, exhibit high solubility in organic solvents and also suppress side-chain-induced racemization. Previously, we have reported that the 2-adamantyl ester employed for protection of the β-carboxy function of aspartic acid was stable to acids and sufficiently soluble in organic solvents,[18,19] while 1-adamantyl ester was susceptible to acids. The above results prompted us to design a novel N^{π}-protecting group.

This paper deals with the synthesis of N^{π}-2-adamantyloxymethylhistidine, H-His(N^{π}-2-Adom)-OH (Fig. 1), examination of its properties and its application to the synthesis of thyrotropin-releasing hormone (TRH) using solution and solid-phase methods.

First of all, 2-adamantyloxymethyl chloride (2-Adom-Cl) was prepared by the reaction of 2-adamantyloxymethyl methyl sulfide and sulfuryl dichloride as shown in Chart 1. 2-Adom-Cl was reacted with Boc-His(N^{τ}-Boc)-OMe,[20] where introduction of the 2-Adom group into π-nitrogen and removal of the Boc group from the τ-nitrogen occurred simultaneously to afford Boc-His(N^{π}-2-Adom)-OMe. The product was saponified with 1 N NaOH to give Boc-His(N^{π}-2-Adom)-OH in a good yield. H-His(N^{π}-2-Adom)-OH (Fig. 1) was derived from Boc-His(N^{π}-2-Adom)-OH by treatment with TFA. During the synthesis of H-His(N^{π}-2-Adom)-OH starting from histidine, racemization of the His residue amounted to 1.8% as determined by HPLC (see the experimental section).

Next, the stability and susceptibility of the 2-Adom group to various acids and bases were examined by measuring regenerated histidine and intact H-His(N^{π}-2-

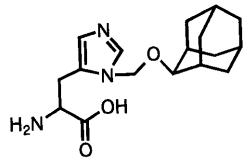

Fig. 1. Structure of H-His(N^{π}-2-Adom)-OH

* To whom correspondence should be addressed.

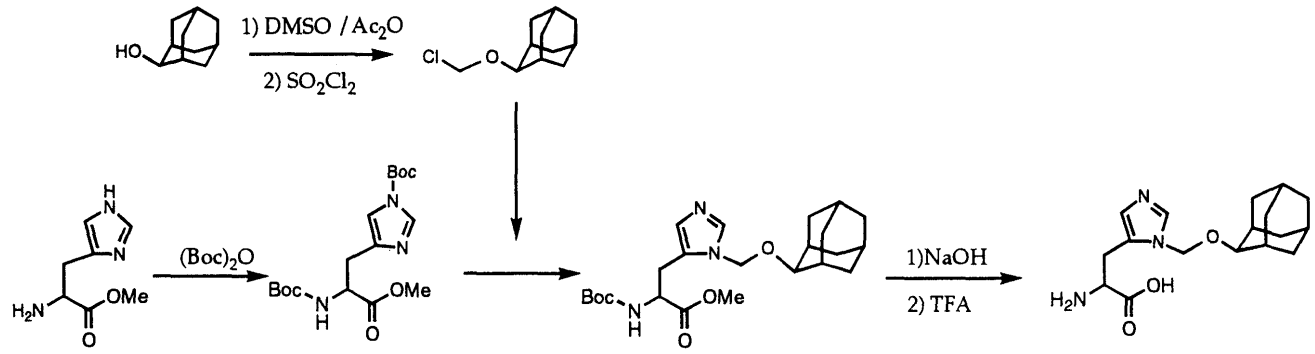

Chart 1. Synthetic Scheme for H-His(N^{π}-2-Adom)-OH

Table 1. Stability and Susceptibility of the N^{im}-2-Adom Group

Conditions	% histidine regenerated						
	15 min	30 min	45 min	60 min	12 h	24 h	48 h
TFA (800 eq)	0	0	0	0	0	0	0
25% HBr/HOAc (400 eq)	0	0	0	0	0	0	0
MSA (400 eq)	98.5	100	100	100			
TFMSA (400 eq)	100	100	100	100			
HF (400 eq, 2 eq thioanisole)	100	100					
1 N NaOH (400 eq)	0	0	0	0	0	0	0
20% piperidine/DMF (200 eq)	0	0	0	0	0	0	0

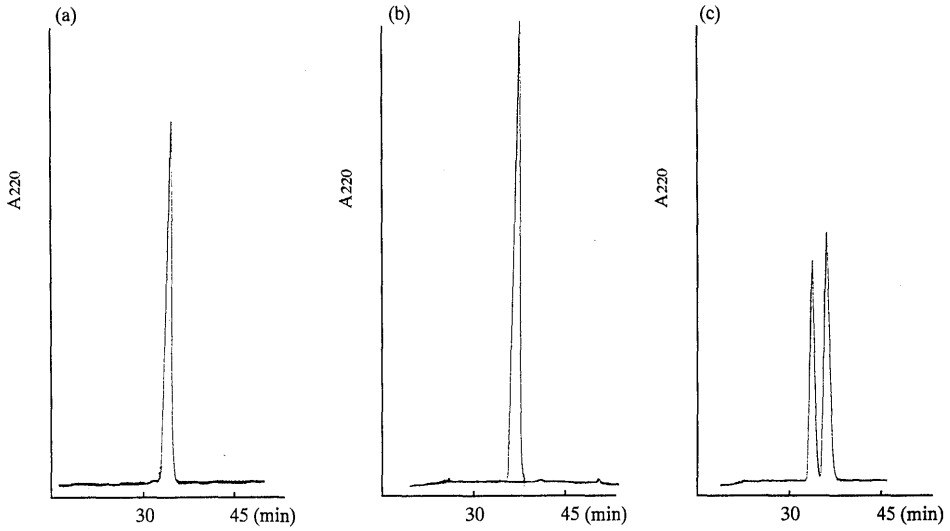

Fig. 2. HPLC Profiles of (a) Boc-L-His(N^{π}-2-Adom)-L-Gln-OBzl, (b) Boc-D-His(N^{π}-2-Adom)-L-Gln-OBzl and (c) Co-injection
The column and solvent system are described in the experimental section.

Adom)-OH (t_R, 54.2 min) on an amino acid analyzer. The results are summarized in Table 1. The 2-Adom group is stable to 7.6 N HCl/dioxane and TFA at room temperature and to hydrogenation over a Pd catalyst for up to 48 h, and rapidly cleaved by 1 M trifluoromethanesulfonic acid (TFMSA)–thioanisole/TFA or anhydrous HF. Furthermore, it is stable to alkaline conditions such as 20% piperidine/DMF, a deprotecting reagent for the N^{α}-Fmoc group, at room temperature for up to 48 h. These results indicate that His(N^{π}-2-Adom) can be used in peptide synthesis in combination with both Boc and Fmoc as N^{α}-protecting groups in solution and/or solid phase methods.

Next, the efficiency of the N^{π}-2-Adom group in the prevention of side-chain-induced racemization was examined. Boc-D-His(N^{π}-2-Adom)-OH was synthesized by the procedure employed for the synthesis of the corresponding L-derivative (Chart 1). Boc-D-His(N^{π}-2-Adom)-OH was coupled with H-L-Gln-OBzl by use of the BOP reagent to afford Boc-D-His(N^{π}-2-Adom)-L-Gln-OBzl, which was completely separated from Boc-L-His(N^{π}-2-Adom)-L-Gln-OBzl on HPLC as shown in Fig. 2. Therefore, this sequence was employed for the model study on racemization. Boc-L-His(N^{π}-2-Adom)-OH was coupled with H-L-Gln-OBzl by DCC, DCC–HOBt, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP),[21] 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)[22] or di-

phenylphosphoryl azide (DPPA),[23] and then the crude product was analyzed by HPLC. The results (summarized in Table 2) show that the formation of D-L pepide was particularly low in all the coupling methods so far examined.

Finally, Boc-His(N^{π}-2-Adom)-OH was employed for synthesis of TRH using both solution and solid-phase methods. As shown in Chart 2, Boc-His(N^{π}-2-Adom)-OH was coupled with H-Pro-NH_2 using BOP to give Boc-His(N^{π}-2-Adom)-Pro-NH_2. After removal of Boc with TFA, the resultant amine was coupled with Boc-Pyr-OH (pyroglutamic acid) to give Boc-Pyr-His(N^{π}-2-Adom)-Pro-NH_2. The protected tripeptide was purified by silica gel column chromatography and the purified peptide was treated with 1 M TFMSA–thioanisole/TFA as usual to give TRH, which exhibited a single peak on analytical HPLC which coincided with that of authentic TRH purchased from Peptide Institute (Osaka, Japan), as shown in Fig. 3. For the solid-phase method, the desired sequence was constructed on 4-methylbenzhydrylamine (MBHA) resin as shown in Chart 3. The protected peptide resin thus obtained was treated with HF and the crude product was purified by preparative HPLC. The purified TRH exhibited a single peak on analytical HPLC at the same retention time as that of authentic TRH, as shown in Fig. 3.

The newly developed His(N^{π}-2-Adom) should be useful for peptide synthesis. Furthermore, His(N^{π}-2-Adom)-containing peptides exhibited high solubility in organic solvents. Therefore, it is expected that His(N^{π}-2-Adom) can be used successfully in combination with Fmoc as the N^{α}-protecting group for convergent solid-phase peptide synthesis.

Table 2. Racemization during the Coupling of Boc-His(N^{π}-2-Adom)-OH and H-Gln-OBzl

Coupling method	D-L/(D-L + L-L) %
DCC	2.15
DCC/HOBt	0.62
BOP	0.44
HBTU	0.40
DPPA	0.46

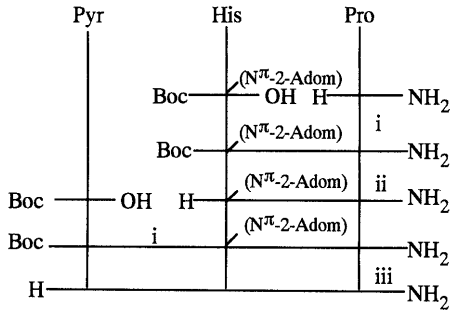

Chart 2. Synthetic Scheme for TRH in Solution Method

i, Bop (1.2 eq), HOBt (1.2 eq) and *N*-methylmorpholine (NMM, 1.2 eq). ii, TFA. iii, 1 M TFMSA–thioanisole/TFA.

Experimental

Melting points were determined on a Yanagimoto micro-melting point

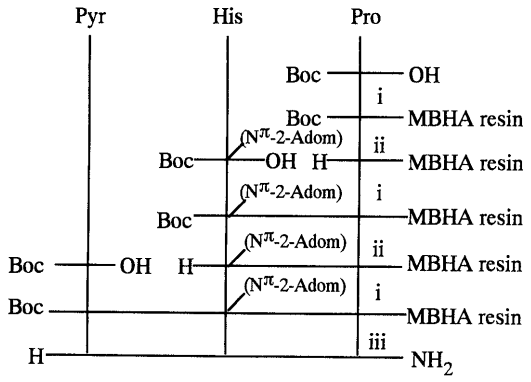

Chart 3. Synthetic Scheme for TRH in Solid-Phase Method

i, Boc-amino acid (3.0 eq), Bop (3.6 eq), NMM (5.4 eq). ii, a) 50% TFA/CH_2Cl_2, b) Et_3N/DMF. iii, HF (+2 eq thioanisole).

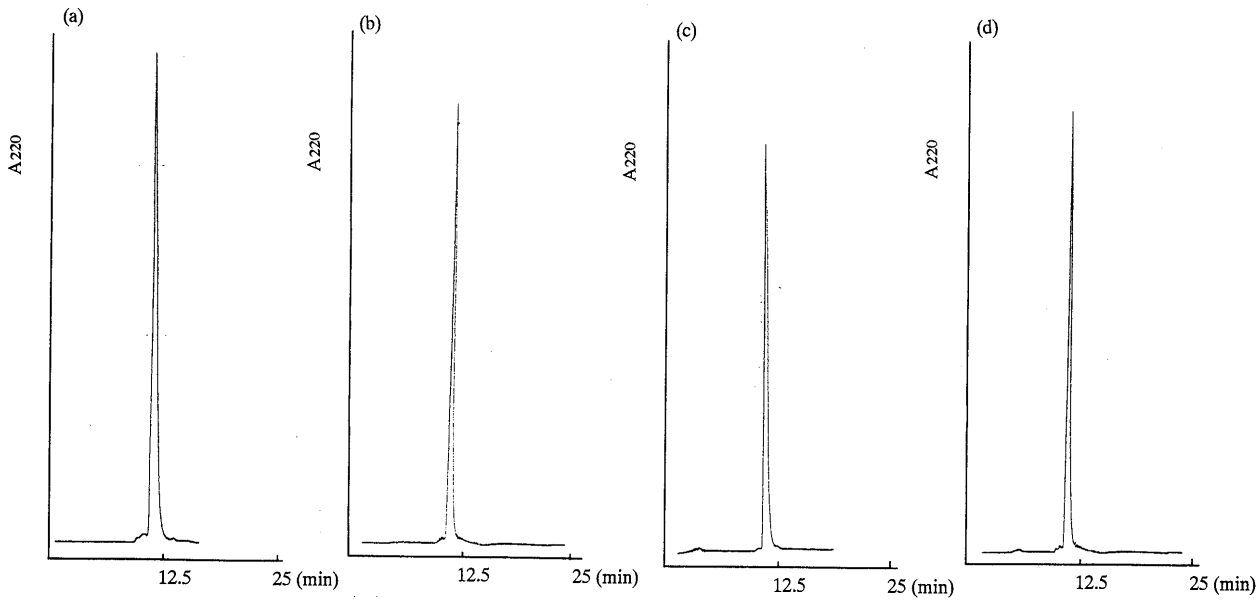

Fig. 3. HPLC Profiles of Synthetic and Authentic TRH

(a), synthetic in solution method; (b), synthetic in solid-phase method; (c), authentic and (d), co-injection. The column and solvent system are described in the experimental section.

apparatus and are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). ^{1}H-NMR (400, 500 MHz) spectra were recorded on a Bruker AM400 or an ARX500 spectrometer. Chemical shift values are expressed as ppm downfield from tetramethylsilane used as an internal standard (δ-value). The J values are given in Hz. Mass spectra were determined on a Hitachi M-2000 mass spectrometer. Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) of peptides or samples for study of the stability and susceptibility of His(N^{π}-2-Adom) to acids and bases were determined with an amino acid analyzer, K-202 SN (Kyowa Seimitsu Co.). On TLC (Kieselgel G, Merck), Rf^1, Rf^2 and Rf^3 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase) and Et_2O and hexane (1 : 2), respectively. On HPLC, solvent A and solvent B are 0.05% aqueous TFA and 0.05% TFA in MeCN, respectively.

2-Adamantyloxymethyl Methyl Sulfide[24] A mixture of DMSO (80 ml), Ac_2O (20 ml) and AcOH (10 ml) was stirred at room temperature for 6 h. To the above solution, 2-adamantanol (5.00 g, 33 mmol) and Ac_2O (40 ml) were added and the mixture was stirred at room temperature for an additional 53 h. After addition of 3 N NaOH (250 ml) to the above mixture, an oily material was extracted with hexane. The extract was washed with 1 N NaOH and water and evaporated down. The residue in 3.0 N NaOH (100 ml) was stirred overnight and extracted with hexane. The extract was washed with water, dried over Na_2SO_4 and evaporated down. The residue was applied to a silica gel column (3 × 25 cm), equilibrated and eluted with hexane and ether (8 : 1). The eluate containing the desired sulfide was collected and the solvent was removed to give 2-adamantyloxymethyl methyl sulfide as a colorless oil, yield 4.5 g (65%), Rf^3 0.54. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (2H, m, adamantyl), 1.59 (2H, m, adamantyl), 1.77—1.98 (10H, m, adamantyl), 2.18 (3H, s, SCH_3), 3.78 (1H, m, adamantyl), 4.68 (2H, s, OCH_2S). *Anal.* Calcd for $C_{12}H_{20}OS$: C, 67.9; H, 9.49. Found: C, 67.9; H, 9.32.

2-Adamantyloxymethyl Chloride[25] A solution of SO_2Cl_2 (1.0 ml, 13 mmol) in CH_2Cl_2 (10 ml) was added dropwise to a solution of 2-adamantyloxymethyl methyl sulfide (1.80 g, 8.48 mmol) in CH_2Cl_2 (42 ml) over 10 min. The reaction mixture was stirred at room temperature for 20 min. The solvent was removed under reduced pressure below 15 °C to give 2-Adom-Cl (1.70 g, quantitative), which was used without further purification. ^{1}H-NMR ($CDCl_3$) δ: 1.45—2.03 (14H, m, adamantyl), 3.93 (1H, s, CH); 5.58 (2H, s, OCH_2Cl).

Boc-His(N^{π}-2-Adom)-OMe A solution of Boc-His(N^{τ}-Boc)-OMe[20,26] (4.1 g, 11.0 mmol) and Adom-Cl (3.4 g, 17 mmol) in CH_2Cl_2 (20 ml) was stirred at room temperature for 8 h. Saturated $NaHCO_3$ solution (30 ml) was added and the mixture was stirred for 30 min. The organic layer was washed with water, dried over Na_2SO_4 and evaporated *in vacuo*. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (3.5 × 15 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$, followed by 1.5% MeOH in $CHCl_3$. The effluent containing the desired product was collected and the solvent was removed *in vacuo*. Petroleum ether was added to the residue to afford crystals, yield 3.1 g (64.5%), mp 114—115 °C, $[\alpha]_D^{25}$ −8.2° (c = 0.5, MeOH), Rf^1 0.64. MS (SIMS) m/z: 467 (M^+). ^{1}H-NMR δ: 1.42 (9H, s, *tert*-butyl); 1.48—2.02 (14H, m, adaman tyl), 3.12—3.23 (2H, m, CH_2CH), 3.51 (1H, m, CH-O), 3.73 (3H, s, OCH_3), 4.59—4.60 (1H, m, $CHCH_2$), 5.29—5.33 (3H, m, CONH + CH_2OAda), 6.83 (1H, s, 5^{im}-H), 7.50 (1H, s, 2^{im}-H). *Anal.* Calcd for $C_{23}H_{35}N_3O_5$: C, 63.7; H, 8.14; N, 9.69. Found: C, 63.5; H, 8.27; N, 9.60.

Boc-His(N^{π}-2-Adom)-OH A solution of Boc-His(N^{π}-2-Adom)-OMe (3.0 g, 7.0 mmol) in MeOH (10 ml) containing 1 N NaOH (7.0 ml) was stirred at room temperature for 30 min. After removal of the solvents, the residue was dissolved in water (100 ml). The pH of the solution was adjusted with 1 N HCl to 7.0—7.5 (pH 7.2 is preferable) to give a precipitate, which was collected by filtration and dried over KOH pellets *in vacuo*, yield 1.8 g (62.9%), mp 174—175 °C, $[\alpha]_D^{25}$ +5.6° (c = 1.0, MeOH), Rf^2 0.50. ^{1}H-NMR ($CDCl_3$) δ: 1.45—1.99 (23H, m, *tert*-butyl + adamantyl), 3.26—3.34 (2H, m, CH_2CH), 3.52 (1H, s, CH-O), 4.37—4.38 (1H, m, $CHCH_2$), 5.39—5.48 (2H, m, CH_2OAda), 5.75—5.76 (1H, m, CONH), 6.98 (1H, s, 5^{im}-H), 8.06 (1H, s, 2^{im}-H), 10.91 (1H, br s, COOH). *Anal* Calcd for $C_{22}H_{33}N_3O_5 \cdot 1/5H_2O$: C, 62.5; H, 7.96; N, 9.93. Found: C, 62.5; H, 7.93; N, 9.90. After removal of the Boc group, the optical purity of H-His(N^{π}-2-Adom)-OH was examined by HPLC analysis and 1.8% of H-D-His(N^{π}-2-Adom)-OH was detected. Retention time: L, 44.176 min; D, 31.310 min. Conditions: column, Crownpak CR(+) (Dicel Chemical Industries, Ltd.); eluent, aqueous $HClO_4$ (pH 1.5) (85%, v/v) and MeOH (15%, v/v); flow rate, 1 ml/min.

Examination of Stability and Susceptibility of H-His(N^{π}-2-Adom)-OH to Acids and Bases 1) A solution of Boc-His(N^{π}-2-Adom)-OH (4.1 mg, 10 μmol) in TFA (1 ml) was stored at room temperature for 1 h. Dry ether was added to the solution to give H-His(N^{π}-2-Adom)-OH · 2TFA, which was collected by filtration, dried over KOH pellets and used for subsequent examination.

2) In the Case of Acidic Solution: H-His(N^{π}-2-Adom)-OH · 2TFA (5.5 mg, 10 μmol) was dissolved in acid (Table 1) at room temperature. Then 0.01 ml of each solution was diluted with water or 0.02—0.5 M Na_2CO_3 (0.09 ml) to adjust the pH to about 2. This solution (0.01—0.02 ml) was injected into the amino acid analyzer and the amount of regenerated His residue and intact H-His(N^{π}-2-Adom)-OH (52.4 min) were measured as a function of time.

3) In the Case of Basic Solution: H-His(N^{π}-2-Adom)-OH · 2TFA (5.5 mg, 10 μmol) was dissolved in a base (Table 1) at room temperature. Then 0.01 ml of each solution was diluted with 0.1—1 N HCl (0.09 ml) to adjust the pH to about 2. This solution (0.01—0.02 ml) was used for amino acid analysis.

Boc-His(N^{π}-2-Adom)-Gln-OBzl To an ice-cooled solution of Boc-His(N^{π}-2-Adom)-OH (500 mg, 1.2 mmol), H-Gln-OBzl (320 mg, 1.2 mmol) in DMF (5 ml) containing Et_3N (0.34 ml, 2.4 mmol), DPPA (387 mg, 1.44 mmol) was added. The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford an amorphous powder, yield 443 mg (58.0%), $[\alpha]_D^{25}$ −11.6° (c = 1.0, MeOH), Rf^2 0.71. HPLC [column, Cosmosil Pack 5C 18-AR (4.6 × 250 mm); eluent, A : B from 63 : 37 to 50 : 50 for 50 min and to 63 : 37 for 5 min; flow rate, 1 ml/min] t_R 32.97 min. *Anal.* Calcd for $C_{34}H_{47}N_5O_7 \cdot 2.5H_2O$: C, 59.8; H, 7.61; N, 10.3. Found: C, 59.5; H, 7.41; N, 9.97.

Boc-D-His(N^{τ}-Boc)-OMe The title compound was prepared by the procedure described above for the synthesis of the corresponding L-compound. Yield 85.1%, mp 87—91 °C, $[\alpha]_D^{25}$ −3.9° (c = 1.0, MeOH), Rf^2 0.76. *Anal.* Calcd for $C_{17}H_{27}N_3O_5$: C, 55.3; H, 7.32; N, 11.4. Found: C, 55.6; H, 7.37; N, 11.4.

Boc-D-His(N^{π}-2-Adom)-OMe The title compound was prepared by the procedure described above for the synthesis of the corresponding L-compound, yield 56%, mp 111—114 °C, $[\alpha]_D^{25}$ +4.4° (c = 1.0, MeOH). Rf^1 0.59. *Anal.* Calcd for $C_{23}H_{35}N_3O_5$: C, 63.7; H, 8.08; N, 9.70. Found: C, 63.6; H, 8.11; N, 9.58.

Boc-D-His(N^{π}-2-Adom)-OH The title compound was prepared by the procedure described above for the synthesis of the corresponding L-compound. Yield 90.5%, mp 180—183 °C, $[\alpha]_D^{25}$ −7.7° (c = 1.0, MeOH), Rf^1 0.50. *Anal.* Calcd for $C_{22}H_{33}N_3O_5$: C, 61.7; H, 7.94; N, 9.81. Found: C, 61.8; H, 7.86; N, 9.81.

Boc-D-His(N^{π}-2-Adom)-Gln-OBzl The title compound was prepared by the procedure described above for the synthesis of its stereoisomer, L-L, yield (50%), amorphous powder, $[\alpha]_D^{25}$ −4.4° (c = 1.0, MeOH), Rf^2 0.71. HPLC [Conditions were the same as those in the case of L-L compound] t_R 34.476 min. *Anal.* Calcd for $C_{33}H_{47}N_5O_7 \cdot 1/4H_2O$: C, 63.6; H, 7.40; N, 10.9. Found: C, 63.6; H, 7.66; N, 10.8.

Racemization Analysis during the Coupling of Boc-His(N^{π}-2-Adom)-OH An ice-cooled solution of Boc-His(N^{π}-2-Adom)-OH (50 mg, 0.12 mmol), H-Gln-OBzl · HCl (39 mg, 0.14 mmol) and Et_3N (0.02 ml, 0.143 mmol) in DMF (3 ml) was prepared. To this were added 1) DCC (29.5 mg, 0.143 mmol), 2) DCC (29.5 mg, 0.143 mmol) and HOBt (21.9 mg, 0.143 mmol), 3) Bop (63.23 mg, 0.143 mmol) and Et_3N (0.02 ml, 0.143 mmol) 4) HBTU (54.2 mg, 0.143 mmol) and Et_3N (0.02 ml, 0.143 mmol) or 5) DPPA (30 mg, 0.143 mmol) and Et_3N (0.02 ml, 0.143 mmol). The reaction mixtures were stirred at 4 °C overnight. After removal of the solvent, each residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated *in vacuo*. The amorphous residue was dissolved in MeCN and analyzed by HPLC [column, Cosmosil Pack (4.6 × 250 mm); eluent, A : B, from 63 : 37 to 50 : 50 for 50 min and to 63:37 for 5 min; flow rate, 1 ml/min] to determine the percentage of D-L peptide [= peak area of D-L × 100/(peak area of D-L + peak area of L-L)]. The results are summarized in Table 2 using corrected values.

Boc-His(N^{π}-2-Adom)-Pro-NH_2 BOP (0.63 g, 1.43 mmol) and HOBt (0.22 g, 1.43 mmol) were added to an ice-cooled solution of Boc-His(N^{π}-2-Adom)-OH (0.5 g, 1.19 mmol), H-Pro-NH_2 (0.18 g, 1.43 mmol) in DMF (5 ml) containing *N*-methylmorpholine (0.23 ml,

2.15 mmol). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated *in vacuo*. The residue in $CHCl_3$ was applied to a silica gel column (2 × 45 cm) equilibrated with $CHCl_3$ and eluted with $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase, 500 ml). The effluent containing the desired product was collected and the solvents were removed *in vacuo*. Petroleum ether was added to the residue to afford crystals, which were collected by filtration, yield 1.2 g (79.4%), mp 135—140 °C, $[\alpha]_D^{25}$ −23.4° (c=1.0, MeOH), Rf^2 0.65. Amino acid analysis: His, 0.95; Pro, 1.00 (average recovery 85%). *Anal.* Calcd for $C_{27}H_{41}N_5O_5 \cdot 1/4H_2O$: C, 62.3; H, 7.89; N, 13.5. Found: C, 62.2; H, 7.96; N, 13.2.

Boc-Pyr-His(N^{π}-2-Adom)-Pro-NH_2 BOP (0.39 g, 0.87 mmol) and HOBt (0.13 g, 0.87 mmol) were added to an ice-cooled solution of H-His(N^{π}-2-Adom)-Pro-$NH_2 \cdot$TFA [prepared from Boc-His(N^{π}-2-Adom)-Pro-NH_2 (1.36 g, 0.66 mmol) and TFA (1.0 ml) as usual], Boc-Pyr-OH (0.18 g, 0.79 mmol) in DMF (5 ml) containing *N*-methylmorpholine (0.13 ml, 1.19 mmol). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated *in vacuo*. Petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt and petroleum ether, yield 0.34 g (78.9%), mp 168—171 °C, $[\alpha]_D^{25}$ −39.2° (c=0.5, MeOH), Rf^2 0.50. Amino acid analysis: Glu, 1.07; His, 0.91; Pro, 1.00 (average recovery 70.1%). *Anal.* Calcd for $C_{32}H_{46}N_6O_7 \cdot 3/2H_2O$: C, 58.8; H, 7.55; N, 12.9. Found: C, 58.9; H, 7.27; N, 12.6.

H-Pyr-His-Pro-NH_2(TRH) Boc-Pyr-His(N^{π}-2-Adom)-Pro-NH_2 (45 mg, 0.07 mmol) was dissolved in 1 M TFMSA–thioanisole/TFA (7 ml, 7.02 mmol) containing *m*-cresole (0.75 ml, 7.02 mmol). The reaction mixture was kept at room temperature for 1 h. Dry ether was then added to afford a precipitate, which was collected by centrifugation. The residue in water (5 ml) was treated with Amberlite IRA-93ZU (acetate form) for 1 h. After removal of the resin, the water layer was lyophilized to give a fluffy powder, yield 21.2 mg (80.3%), $[\alpha]_D^{25}$ −63.3° (c=0.5, H_2O) [authentic TRH purchased from Peptide Institute, Osaka, Japan: $[\alpha]_D^{25}$ −61.2° (c=0.5, H_2O)]. HPLC [column, Cosmosil packed 5C 18-AR (4.6 × 250 mm); eluent, isocratic A : B = 90 : 10; flow rate, 0.5 ml/min]. Synthetic TRH exhibited a single peak on HPLC at the same retention time as authentic TRH (Fig. 3).

Boc-Pyr-His(N^{π}-2-Adom)-Pro-MBHA Resin HCl·BMHA resin (625 mg, 0.5 mmol) was neutralized with 10% *N*-methylmorpholine in DMF (5 ml, 1 min × 1, 10 min × 1), then washed with DMF (5 ml × 2). The resin was suspended in DMF (2.5 ml), then Boc-Pro-OH (0.43 g, 2.0 mmol), HOBt (0.27 g, 2.0 mmol), BOP (0.90 g, 2.0 mmol) and *N*-methylmorpholine (0.44 ml, 4.0 mmol) were added. The mixture was shaken overnight, and the resin was filtered off and washed with DMF (5 ml, 1 min × 10). The Boc-Pro-MBHA resin thus obtained was treated with 50% TFA/CH_2Cl_2 (5 ml, 1 min × 1, 30 min × 1) and washed with CH_2Cl_2 (5 ml, 1 min × 10). After neutralization and washing, Boc-His(N^{π}-2-Adom)-OH (0.61 g, 1.5 mmol), BOP (0.80 g, 1.8 mmol), HOBt (0.24 g, 1.8 mmol) and *N*-methylmorpholine (0.36 ml, 3.3 mmol) in DMF (2.5 ml) were added to a suspension of H-Pro-MBHA resin in DMF (5 ml). The reaction mixture was shaken overnight, then the resin was collected by filtration and washed with CH_2Cl_2 (5 ml, 1 min × 10). Boc-His(N^{π}-2-Adom)-Pro-MBHA resin thus obtained was treated with 50% TFA in CH_2Cl_2, and then neutralized as described above. Boc-Pyr-OH (0.42 g, 2.0 mmol), Bop (0.97 g, 2.2 mmol), HOBt (0.30 g, 2.2 mmol) and *N*-methylmorpholine (0.46 ml, 4.2 mmol) were added to a suspension of H-His(N^{π}-2-Adom)-Pro-MBHA resin in DMF (5 ml). The mixture was shaken for 6 h, then the resin was collected by filtration, washed with CH_2Cl_2 and dried over KOH pellets *in vacuo*. The weight of the resin was 910 mg (70%). Amino acid analysis: Glu, 1.10; His, 0.90; Pro, 1.09.

H-Pyr-His-Pro-NH_2(TRH) Boc-Pyr-His(N^{π}-2-Adom)-Pro-MBHA resin (850 mg) obtained above was suspended in anhydrous HF (15 ml) containing thioanisole (0.11 ml, 2 eq) and the suspension was stirred at 0 °C for 1 h. After removal of HF, the residue was washed with dry ether and dried over KOH pellets *in vacuo*. The desired peptide was extracted with 3% AcOH (20 ml) and the solution was applied to an Amberlite IRA-93ZU column (0.9 × 10 cm), equilibrated and eluted with water. The eluent was lyophilized to give a fluffy powder, yield 56 mg, $[\alpha]_D^{25}$ −64.0° (c=1.0, H_2O) [authentic TRH purchased from Peptide Institute (Osaka, Japan): $[\alpha]_D^{25}$ −61.2° (c=1.0, H_2O), lit.[27] $[\alpha]_D^{25}$ −65.5° (c=1.0, H_2O). On HPLC, the synthetic TRH exhibited a single peak at the same position as that of authentic sample (Fig. 3).

Acknowledgement This work was supported in part by a grant from the Science Research Promotion Fund of The Japan Private School Promotion Foundation.

References and Notes

1) Part XLIX: Okada Y., Mu Y., *Chem. Pharm. Bull.*, **45**, 88—92 (1997).
2) Preliminary communication: Okada Y., Wang J., Yamamoto T., Mu Y., *J. Chem. Soc., Perkin Trans. 1*, **1996**, 753—754.
3) The customary L-configuration for amino acid residues is omitted; only D isomers are indicated. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1996); **6**, 362—364 (1967); **11**, 1726—1732 (1972). The following additional abbreviations are used: AcOEt, ethyl acetate; DMF, dimethylformamide; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-Su, 9-fluorenylmethyl *N*-succinimidyl carbonate; 2-Adom, 2-adamantyloxymethyl; DCC, *N,N'*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DPPA, diphenylphosphoryl azide; NMM, *N*-methylmorpholine.
4) *a*) JW is a visiting scientist from the Department of Molecular Biology, Jilin University, China; *b*) Present address: *New Drug Research Laboratory, Kanebo Ltd., 5-9, Tomobuchi-cho, 1-chome, Miyakojima-ku, Osaka 534, Japan.*
5) Rink H., Riniker B., *Helv. Chim. Acta*, **57**, 831—835 (1974).
6) Jones J. H., Ramage W. I., Witty M. J., *Int. J. Peptide Protein Res.*, **15**, 301—303 (1980).
7) Sakakibara S., Fujii T., *Bull. Chem. Soc. Jpn.*, **42**, 1466 (1969).
8) Beyerman H. C., Hirt J., Kranenberg P., Syrier J. L. M., Van Zon A., *Recl. Trav. Chim. Pays-Bas*, **93**, 256—257 (1974).
9) Shaltiel S., *Biochem. Biophys. Res. Commun.*, **29**, 178—183 (1967).
10) Zervas L., Theodoropoulos D. M., *J. Am. Chem. Soc.*, **78**, 1359—1363 (1956).
11) Mamos P., Sanika C., Barlos K., *Liebigs Ann. Chem.*, **1988**, 1083—1084.
12) Nishiyama Y., Shintomi, N., Kondo Y., Izumi T., Okada Y., *J. Chem. Soc., Perkin Trans. 1*, **1995**, 2309—2313.
13) Brown T., Jones J. H., Richards J. D., *J. Chem. Soc., Perkin Trans. 1*, **1982**, 1553—1561.
14) Colombo R., Colombo F., Jones J. H., *J. Chem. Soc., Chem. Commun.*, **1984**, 292—293.
15) Sakakibara S., Shimonishi Y., Kishida Y., Okada M., Sugihara U., *Bull. Chem. Soc. Jpn.*, **40**, 2164—2167 (1967).
16) Lloyd-William P., Albericio F., Giralt E., *Tetrahedron*, **49**, 11065—11133 (1993).
17) Nishiyama Y., Okada Y., *Tetrahedron Lett.*, **35**, 7409—7412 (1994).
18) Okada Y., Iguchi S., Kawasaki K., *J. Chem. Soc., Chem. Commun.*, **1987**, 1532—1534.
19) Okada Y., Iguchi S., *J. Chem. Soc., Perkin Trans. 1*, **1988**, 2129—2136.
20) Brown T., Jones J. H., *J. Chem. Soc., Chem. Commun.*, **1981**, 648—649.
21) Castro B., Dormoy J. R., Evin G., Selve C., *Tetrahedron Lett.*, **1975**, 1219—1222.
22) Knorr R., Trzeciak A., Bannwarth W., Gillessen, D., *Tetrahedron Lett.*, **30**, 1927—1930 (1989).
23) Shioiri T., Ninomiya K., Yamada S., *J. Am. Chem. Soc.*, **94**, 6203—6205 (1972).
24) *a*) Niwa H., Miyachi Y., *Bull. Chem. Soc. Jpn.*, **64**, 716—718 (1991); *b*) Yamada K., Kato K., Nagase H., Hirata Y., *Tetrahedron Lett.*, **1976**, 65—66.
25) Jones J. H., Thomas D. W., Thomas R. M., Wood M. E., *Syn. Commun.*, **16**, 1607—1610 (1986).
26) Handford B. O., Hylton T. A., Wang K. T., Weinstein B., *J. Org. Chem.*, **33**, 4251—4255 (1968).
27) Flouret G., *J. Med. Chem.*, **13**, 843—845 (1970).