Synthesis of Thyrotropin-Releasing Hormone-Related Peptides Using N^{α}-*tert*-Butyloxycarbonyl-ω-(*N*-*tert*-butyloxycarbonylcarbamoyl)-α-amino Acids[1)]

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Application of N^{α},N^{ca}-di-*tert*-butyloxycarbonylhomoglutamine to synthesis of thyrotropin-releasing hormone (TRH) analogs was examined. The δ-lactam formation from homoglutaminylpeptides took place more easily than γ-lactam formation from glutaminylpeptides in water or dioxane containing acetic acid. [pHgu^1,Nva^2]-TRH had dose-dependent antagonistic activity against pentobarbital anesthesia in mice, but almost no binding activity to TRH receptor in rat brain.

Keywords pyrohomoglutamylpeptide; δ-lactam formation; N^{α},N^{ca}-di-*tert*-butyloxycarbonylhomoglutamine; [pHgu^1,Nva^2]-TRH; central nervous system effect; TRH receptor assay; HPLC; alumina support

Many biologically active peptides contain an N-terminal pyroglutamic acid (pGlu) residue, which is postulated to be derived from the precursor peptide by enzymatic cleavage at the N-terminus of Gln residue, followed by cyclization possibly mediated by some enzymes,[2,3)] or by a non-enzymatic reaction. The concept has been examined in a study on the formation of luteinizing hormone-releasing hormone (LH-RH), a pGlu-peptide, from the prohormone model peptide.[4)] The pGlu residue of the peptide plays an important role not only in protection against aminopeptidases but also in the structural requirement for the inherent biological activity. For studies of the structure–activity relationship of the pGlu-peptide, substitution of pyrohomoglutamic acid (pHgu: piperidonecarboxylic acid) for pGlu may be able to provide interesting analogs, exemplified by TRH analogs with pHgu at the N-terminus, which have relative selectivity for action in the central nervous system (CNS).[5)]

Recently we have briefly communicated the synthesis and chemical properties of N^{α}-*tert*-butyloxycarbonyl-ω-(*N*-*tert*-butyloxycarbonylcarbamoyl)-α-amino acids.[6,7)] In the present study, N^{α},N^{ca}-di-*tert*-butyloxycarbonylhomoglutamine [Boc-Hgn(Boc)] was employed for the synthesis of pHgu-peptides, pHgu-Nva-Pro-NH_2 (**1**) and pHgu-His-Pro-NH_2[5)] (**2**), which underwent internal cyclization. For a comparative study on γ- and δ-lactam formation, Nva^2-TRH[8)] (**3**) and TRH (**4**) were also synthesized using Boc-Gln(Boc)-OH.

Protected tripeptides (**1a**—**4a**) for TRH or the analogs (**1**—**4**) were prepared by acylation of H-Nva-Pro-NH_2[8)] or H-His-Pro-NH_2[9)] with Boc-Hgn(Boc)-OH and Boc-Gln(Boc)-OH by the mixed anhydride method[10)] (Fig. 1). These amino acid derivatives with N^{ca}-Boc have superior solubility characteristics in organic solvents such as tetrahydrofuran and dichloromethane, and do not exhibit nitrile formation during the C-terminal activation.[6)] Recently, Liberek and Kasprozykowska[11)] reported that the protection of the side chain carboxamide with N^{ca}-Z, a similar urethane-type protecting group, also prevents the dehydration reaction. Boc-Hgn(Boc)-Nva-Pro-NH_2 (**1a**), Boc-Hgn(Boc)-His-Pro-NH_2 (**2a**), Boc-Gln(Boc)-Nva-Pro-NH_2 (**3a**) and Boc-Gln(Boc)-His-Pro-NH_2 (**4a**) were obtained in 40—60% yields after purification on a Sephadex LH-20 column.

Boc-Hgn(Boc)-peptides (**1a** and **2a**) and Boc-Gln(Boc)-peptides (**3a** and **4a**) were easily converted to the corresponding trifluoroacetates of H-Hgn-peptides (**1b** and **2b**) and H-Gln-peptides (**3a** and **4b**) by treatment with trifluoroacetic acid–anisole, because of similar acid lability of N^{ca}-Boc to N^{α}-Boc.[7)]

The lactam formation from H-Hgn- and H-Gln-peptides obtained above was examined comparatively in three different concentrations of acetic acid in water or dioxane at 40 °C. The reaction was assessed by quantitative HPLC analysis of the reaction mixtures using a normal-

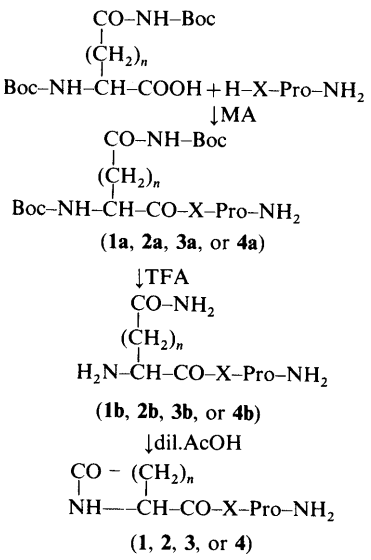

Fig. 1. Synthesis of TRH-Related Peptides

TABLE I. Conversion of H-Hgn- and H-Gln-Peptides into pHgu- and pGlu-Peptides Mediated by Acetic Acid

Substrate	Reaction time (h) for 1/2 conversion[a)]					
	1 N AcOH in		4 N AcOH in		8 N AcOH in	
	H_2O	Dioxane	H_2O	Dioxane	H_2O	Dioxane
1b	6.0	0.5	4.0	0.6	2.7	0.8
2b	4.8	0.6	2.9	0.8	2.0	0.8
3b	13.5	0.8	7.0	0.9	4.8	1.1
4b	7.4	0.8	4.5	1.2	2.8	1.5

a) Decrease of substrates was analyzed by HPLC.

phase alumina column, eluting with a descending gradient of acetonitrile concentration in triethylammonium phosphate buffer according to the method reported by Tanaka *et al.*[12] Thin layer chromatography (TLC) was also used for qualitative examination. As can be seen in Table I, the formation of pHgu–peptides (**1** and **2**) from the corresponding H–Hgn–peptides (**1b** and **2b**) was easier than that of pGlu–peptides (**3** and **4**) from (**3b** and **4b**) in all solvent systems examined. The reaction times for both γ- and δ-lactam formation in acetic acid in water were diminished significantly by the increased concentration of acetic acid. It was of interest to note that the increase of acetic acid concentration in dioxane gave rise to slower cyclization. Thus, γ- and δ-lactam formations with a weak acid catalyst seem to be facilitated in non-polar solvent systems. Similar solvent effects on pyroglutamylpeptide formation as a side reaction are well known in solid-phase peptide synthesis.[13] The results revealed that pHgu–peptides could be prepared from H–Hgn–peptides more easily than pGlu–peptides from H–Gln–peptides under mild acidic conditions, especially in non-polar solvent system.

Taking into account the results of the above analytical work and the practical solubility of peptides, preparations of [pHgu^1, Nva^2]–TRH (**1**) and the other related peptides were carried out in 4 N acetic acid in dioxane at 40 °C for 8—12 h. The products were obtained almost quantitatively after gel-filtration on a Sephadex LH-20 column (Table III, in the experimental section).

Recently Nva^1–TRH (**3**) was reported to be the first analog giving total dissociation of CNS and pituitary activities,[8] and it has no cardiovascular activity.[14] As expected from the structural similarity of [pHgu^1, Nva^2]–TRH (**1**) to **2** and **3**, a dose-dependent antagonistic effect of **1** on pentobarbital anesthesia was observed in the dose range from 0.156 to 10.0 mg/kg body weight in mice (Table II). On the other hand, in a TRH receptor assay system using rat brain synaptosomal fraction, pHgu^1–TRH (**2**) showed a slightly reduced displacing activity of ^{3}H–TRH ($IC_{50} = 4.9 \times 10^{-8}$ M) compared to unlabeled TRH (1.9×10^{-8} M), whereas the displacing activity of **1** was reduced to a level of 1/1000 of that of TRH ($IC_{50} = 1.8 \times 10^{-5}$ M). The result suggested the importance of the His residue for binding to the TRH receptor. The pHgu moiety of **1** may play a role in the selectivity for CNS effects in addition to the increased metabolic stability. The details of the biological activity of the synthetic peptides, such as the effect on release of catecholamines, will be published separately.[15]

Experimental

Melting points are uncorrected. Optical rotation was measured with a Nippon Bunko DIP-370 polarimeter. HPLC was performed on a system composed of two model 510 pumps, a model U6K injector, a model 680 gradient controller, a model 481 LC spectrophotometer and a model 730 data module (Waters). Acid hydrolysis of samples was conducted with twice-distilled 6 N HCl at 110 °C for 24 h in evacuated sealed tubes, and amino acid analysis was performed on a Beckman model 7300 amino acid analyzer system. The fast atom bombardment mass spectrum (FAB-MS) was measured with a JEOL JMS-DX-300 mass spectrometer connected with a JEOL JMA-DA5000 mass data system. Evaporation of solvents was carried out *in vacuo* below 40 °C in a rotary evaporator. The solvent systems used for HPTLC (Merck) were *n*-BuOH–AcOH–H_2O (4 : 1 : 5, the upper phase, Rf^1), *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24, Rf^2) and *n*-BuOH–AcOEt–H_2O (1 : 1 : 1 : 1, Rf^3).

TABLE II. Effect of pHgu–Nva–Pro–NH_2 (**1**) on Pentobarbital Sleeping Time in Mice

Dose of **1** mg/kg	$N^{a)}$	Percentage$^{b)}$ of sleeping time
0.156	15	81.4$^{c)}$
0.625	16	74.5$^{c)}$
2.50	15	63.9$^{d)}$
10.0	13	52.3$^{d)}$

a) Number of animals used. *b*) Mean sleeping time of control groups was taken as 100%. Statistical significance of differences from the control: *c*) $p<0.10$, *d*) $p<0.01$.

Boc–Hgn(Boc)–Nva–Pro–NH_2 (1a) IBCF (0.158 ml, 1.20 mmol) was added to a solution of Boc–Hgn(Boc)–OH[7] (469 mg, 1.30 mmol) and NMM (0.133 ml, 1.30 mmol) in THF (6 ml) at −15 °C. After 1 min, the mixture was added to a solution of H–Nva–Pro–NH_2, prepared from Boc–Nva–Pro–NH_2[8] (313 mg, 1.0 mmol) by treatment with anhydrous TFA, in DMF (2 ml) containing NMM (0.102 ml, 1.04 mmol) at −10 °C. The mixture was stirred for 1 h in an ice bath and the solvent was removed *in vacuo*. The residue was taken up in AcOEt (30 ml) and washed successively with 1 N citric acid, saturated sodium bicarbonate and saturated sodium chloride solution. The organic phase was dried over Na_2SO_4 and the product (**1a**) was solidified with petroleum ether, and reprecipitated from AcOEt (3 ml)–ether (10 ml)–petroleum ether (10 ml). Yield 360 mg (64.7%), mp 103.0—106.0 °C; Rf^1 0.65, Rf^2 0.80, Rf^3 0.82; $[\alpha]_D^{23}$ −69.8° ($c=1$, MeOH). *Anal.* Calcd for $C_{26}H_{45}N_5O_8$: C, 56.20; H, 8.16; N, 12.60. Found: C, 55.72; H, 8.04; N, 12.42. FAB-MS *m/z*: 556 (M+1), 456 (M−99), 356 (M−199). Amino acid ratios in an acid hydrolysate: Pro 0.93 (1), Hgu 1.11 (1), Nva 0.96 (1), NH_3 2.05 (2).

Boc–Hgn(Boc)–His–Pro–NH_2 (2a) A mixed anhydride, prepared from Boc–Hgn(Boc)–OH (520 mg, 144 mmol), NMM (0.147 ml, 1.44 mmol) and IBCF (0.190 ml, 1.44 mmol) in THF (10 ml), was allowed to react with H–His–Pro–NH_2 in H_2O (0.5 ml)–DMF (2 ml), which was prepared from Z–His–Pro–NH_2[9] (535 mg, 1.2 mmol) by catalytic hydrogenation over Pd in MeOH containing 1 N HCl (2.4 ml). The mixture was stirred for 1 h under cooling and evaporated. The residue was distributed between five portions of *n*-BuOH (30 ml each) and five portions of H_2O (20 ml each). The combined organic phase was evaporated and the residue was solidified with ether. The crude product was chromatographed on a column (2.5 × 90 cm) of Sephadex LH-20 with MeOH–1% AcOH (9 : 1). The fractions (6 g each) containing the desired product (fr. No. 42—46) were combined, and evaporated, and **2a** was solidified with MeOH (3 ml) –AcOEt (10 ml)–ether (20 ml). Yield 455 mg (56.7%); mp 121.0—123.0 °C (dec.); Rf^1 0.32, Rf^2 0.64, Rf^3 0.64; $[\alpha]_D^{23}$ −39.2° ($c=1$, MeOH). *Anal.* Calcd for $C_{27}H_{43}N_7O_8 \cdot AcOH$: C, 53.28; H, 7.25; N, 15.00. Found: C, 53.61; H, 7.60; N, 14.84. FAB-MS *m/z*: 594 (M+1), 494 (M−99). Amino acid ratios in an acid hydrolysate: Pro 0.96 (1), Hgu 1.07 (1), His 0.97 (1) NH_3 2.70 (2).

Boc–Gln(Boc)–Nva–Pro–NH_2 (3a) The mixed anhydride, prepared from Boc–Gln(Boc)–OH[6] (267 mg, 0.77 mmol), NMM (0.078 ml, 0.77 mmol) and IBCF (0.102 ml, 0.77 mmol) in THF (5 ml), was allowed to react with H–Nva–Pro–NH_2 (0.70 mmol) in DMF (23 ml) for 1 h in an ice bath, and the product (**3a**) was isolated in the same manner as described for **1a**. Yield 220 mg (58.0%), mp 166.0—168.0 °C; Rf^1 0.64, Rf^2 0.80, Rf^3 0.81; $[\alpha]_D^{23}$ − 56.5° ($c=1.0$, MeOH). *Anal.* Calcd for $C_{25}H_{43}N_5O_8$: C, 55.44; H, 8.00; N, 12.93. Found: C, 55.65; H, 7.95; N, 13.39. FAB-MS *m/z*: 542 (M+1), 442 (M−99). Amino acid ratios in an acid hydrolysate: Glu 1.02 (1), Pro 0.95 (1), Nva 1.04 (1), NH_3 2.26 (2).

Boc–Gln(Boc)–His–Pro–NH_2 (4a) A mixed anhydride, prepared from Boc–Gln(Boc)–OH (762 mg, 2.2 mmol), NMM (0.224 ml, 2.2 mmol) and IBCF (0.277 ml, 2.1 mmol) in THF (10 ml), was allowed to react with H–His–Pro–NH_2 (2 mmol) in H_2O (1 ml)–DMF (3 ml) for 1 h in an ice bath, and the product (**4a**) was isolated in the same manner as described for **2a**. Yield 593 mg (46.4%), mp 132.0—135.0 °C (dec.); Rf^1 0.32, Rf^2 0.64, Rf^3 0.62; $[\alpha]_D^{23}$ −33.9° ($c=1.0$, MeOH) *Anal.* Calcd for $C_{26}H_{41}N_7O_8 \cdot AcOH$: C, 52.57; H, 7.09; N, 15.33. Found: C, 52.63; H, 7.23; N, 15.75. FAB-MS *m/z*: 580 (M+1), 480 (M−99). Amino acid ratios in an acid hydrolysate: Glu 1.14 (1), Pro 0.93 (1), His 0.93 (1), NH_3 2.19 (2).

Analysis of Lactam Formation The reaction times for the formation of pHgu– and pGlu–peptides from H–Hgn– and H–Gln–peptides were determined as follows: 1 mg of **1a**, **2a**, **3a** and **4a** were dissolved in TFA

TABLE III. Characterization of Synthetic TRH-Related Peptides

	Yield (%)	$[\alpha]_D^{23}$ $c=1$ (MeOH)	HPTLC Rf^1	HPTLC Rf^2	HPTLC Rf^3	HPLC[a] t_{R_1}	HPLC[a] t_{R_2}	FAB-MS (M+1)	Amino acid ratios in acid hydrolysate: Hgu	Glu	Nva	His	Pro	NH_3
1	84	−78.3°	0.13	0.55	0.31	8.00	23.38	339	1.04	—	1.00	—	0.96	1.40
2	86	−49.0°	0.00	0.30	0.12	30.35	9.91	377	1.06	—	—	0.97	0.97	1.56
3	75	−86.0°	0.13	0.53	0.30	8.53	18.91	325	—	1.00	1.04	—	0.95	1.25
4	81	−55.5°	0.00	0.27	0.12	30.95	6.38	363	—	1.01	—	1.01	0.99	1.06

a) HPLC conditions: flow rate, 1 ml; detection, 210 nm; t_{R_1}, retention time (min) in Spherisorb A5Y (4.6 × 250 nm) HPLC with the decreasing mode of gradient elution with acetonitrile from 87.5% to 35.0% in 50 mM TEAP buffer (pH 2.1) during 40 min. t_{R_2}, retention time in Nova pak C_{18} (4.6 × 150 mm) HPLC with linear gradient elution with acetonitrile from 1% to 10% in 20 mM sodium phosphate buffer (pH 3.0) during 20 min, followed by the final solution.

(0.2 ml)–anisole (0.02 ml) and kept at room temperature for 45 min. The excess of TFA was removed *in vacuo* and the residue was washed with anhydrous ether and dried up *in vacuo* over KOH for 5 h. The resulting TFA salts of H–Hgn–Nva–Pro–NH_2 (**1b**: Rf^1 0.18, Rf^2 0.48, Rf^3 0.44), H–Hgn–His–Pro–NH_2 (**2b**: Rf^1 0.04, Rf^2 0.24, Rf^3 0.11), H–Gln–Nva–Pro–NH_2 (**3b**: Rf^1 0.18, Rf^2 0.48, Rf^3 0.45) and H–Gln–His–Pro–NH_2 (**4b**: Rf^1 0.04, Rf^2 0.24, Rf^3 0.12) were dissolved in various concentrations of AcOH in H_2O or dioxane (0.500 ml, Table II), and the mixtures were kept in stoppered containers in a bath at 40 °C. Aliquots of the solution were taken at various time intervals, and immediately subjected to quantitative analysis by HPLC (2.0 μl was injected), or kept at −20 °C until the analysis, which was carried out on a column of Spherisorb A5Y (4.6 × 300 mm) using a solvent system of 50 mM TEAP buffer (pH 2.1)–acetonitrile.[12] Linear descending gradient (20 min) elution was adopted from 90% to 50% acetonitrile for **1b** and **3b**, and 56% to 28% for **2b** and **4b**. The peak areas of the substrates detected at 210 nm at various time intervals were plotted. The time required for the amount of the starting materials to decrease to half the initial value is shown in Table I.

Preparation of [pHgu^1, Nva^2]–TRH (1), pHgu^1–TRH (2), Nva^2–TRH (3) and TRH (4) For preparative work, 100—300 mg of protected peptide (**1a**, **2a**, **3a** or **4a**) was treated with TFA (5—10 ml) and anisole (0.2—0.5 ml) in the same manner as described above for analytical work. Cyclization of the resulting H–Hgn–peptides (**1b** or **2b**) into **1** or **2** and H–Gln–peptides (**2b** or **4b**) into **3** or **4** was performed in 4 N AcOH in dioxane (20—30 ml) at 40 °C for 8—12 h until the starting material was no longer detectable on TLC. The reaction mixture was diluted with the same volume of dioxane and lyophilized.

The product was passed through a column of Sephadex LH-20 (1.2 × 90 cm) using *n*-BuOH–MeOH–AcOH–H_2O (6:6:18:70) as the eluent. The fractions containing the desired product were combined, and evaporated, and the residue was lyophilized from 10% AcOH. Yield and characterization data are given in Table III.

New analogs of TRH, [pHgu^1, Nva^2]–TRH (**1**), were confirmed by elemental analysis. *Anal.* Calcd for $C_{16}H_{26}N_4O_4$: C, 56.79; H, 7.74; N, 16.56. Found: C, 56.79; H, 7.72; N, 16.53. mp 202—205 °C (dec.).

Effect of 1 on Pentobarbital Sleeping Time Groups of 7—11 male ICR mice (26—33 g) were given intraperitoneal injections of **1** (10, 2.5, 0.625 or 0.156 mg/kg body weight of each animal) in saline (0.26—0.33 ml) or saline alone (control group), 8 min after intraperitoneal administration of 55 mg/kg body weight of pentobarbital Na (Nembutal, Abbot Lab.) at room temperature (21—26 °C). The sleeping time was taken as the period from the time of injection of the peptide or saline alone to the regaining of righting reflex. The percentage was calculated by taking the mean sleeping time × 100 of peptide-treated mice divided by the mean sleeping time of control group (47—64 min), assayed on the same day (Table II).

TRH Receptor Assay Rat brain synaptosomal fraction was prepared by the method of De Robertis *et al.*,[16] and used as the TRH receptor preparation. The assay was carried out by Ogawa *et al.*'s method,[17] measuring the displacement of ^{3}H–TRH (specific activity 55.6 Ci/m mol, New England Nuclear) by unlabeled TRH or analogs **1** and **2** (10^{-10}—10^{-4} M), and IC_{50}, the concentration causing 50% inhibition of ^{3}H–TRH binding, was determined (the results are described in the text).

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. Abbreviations used are as follows: DMF, dimethylformamide; Hgn, homoglutamine residue; pHgu, pyrohomoglutamic acid residue; Hgu, homoglutamic acid residues; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin layer chromatography; IBCF, isobutylcholoroformate; NMM, *N*-methylmorpholine; Nva, norvaline residue; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran.
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