Lipophilic Peptides: Synthesis of Lauroyl Thyrotropin-Releasing Hormone and Its Biological Activity

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Thyrotropin-releasing hormone (TRH) was derivatized by chemical attachment of lauric acid to the N-terminal pyroglutamyl group. The product was confirmed to be more lipophilic than TRH by high-performance liquid chromatography and measurement of partition coefficients. The central nervous system activity and endocrine activity of the lauroyl derivative were only slightly reduced, to 81 and 64% of the parent TRH, respectively. Lipophilic derivatization may be generally applicable to transmembrane delivery of peptides.

KEY WORDS: peptide delivery; lipophilic peptide; thyrotropin-releasing hormone (TRH); lipophilicity; lauroyl derivative.

INTRODUCTION

Peptides such as enkephalin, calcitonin, and insulin are hydrophilic molecules with poor penetration across biomembrane barriers. However, cyclosporin A is a highly lipophilic peptide and exhibits good penetration characteristics across the intestinal barrier (1,2). Therefore, lipophilic modification of peptide drugs by chemical derivatization may enhance their permeability across the lipid barrier membrane, such as small intestinal, rectal, and transdermal barriers.

The previously described mono- and dipalmitoyl derivatives of bovine insulin (MW 5733) were shown to be more lipophilic than the parent insulin but biologically less active. The absorption of small di- and tripeptides involves a carrier-mediated mechanism in the small intestine (4). The absorption of thyrotropin-releasing hormone (TRH; MW 362), a tripeptide, follows apparent saturation kinetics after oral administration to rats (5), with an absolute bioavailability of less than 5% (6).

TRH analogues produce central nervous and endocrine effects (7–9). In order to change the physicochemical property of TRH while maintaining its biological activities, a lipophilic lauroyl group was introduced into the N-terminal pyroglutamyl group in the peptide, resulting in a stable and biologically active derivative.

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MATERIALS AND METHODS

Materials

TRH was purchased from Peptide Institute, Inc. (Osaka, Japan), and *p*-methoxybenzyloxycarbonyl azide [Z(OMe)-N_3], *N*-hydroxysuccinimide (HOSu), *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB), and *N,N'*-dicyclohexylcarbodiimide (DCC) were purchased from Watanabe Chemical Industry (Hiroshima, Japan). Lauric acid was purchased from Sigma Chemical Company (St. Louis, Mo.), and acetic anhydride (Ac_2O), dicyclohexylamine (DCHA), triethylamine (TEA), dimethylformamide (DMF), and isoamyl nitrite (IAN) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tetrahydrofuran (THF), trifluoroacetic acid (TFA), and all other chemicals used were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and were of reagent grade. Iodine-125 was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo). A rat TSH kit was kindly supplied by the National Institute of Diabetes and Digestive and Kidney Diseases.

General Procedures

Melting points were determined on a micro melting point apparatus (Yanagimoto) and are uncorrected. The amino acid composition of acid hydrolysate was determined with a Hitachi L-8500 amino acid analyzer. Acid hydrolysis was performed in evacuated sealed glass tubes with 6 *N* HCl at 110°C for 24 hr. Mass spectra (MS) were obtained by the electron impact method, on a JEOL JMS-01SG-2 mass spectrometer. Elemental C,H,N analyses were run on a CHN corder MT-3 (Yanagimoto).

Synthetic Method for Lauroyl-TRH (Lau-TRH)

Lau-Glu-OH

Lau-OSu (14.85 g), synthesized by the method of Lapidot *et al.* (10), in dioxane (50 ml) was added to a solution of glutamic acid (14.69 g) in 1 *N* NaOH (200 ml), and the reaction mixture was stirred overnight at room temperature. After filtration, the filtrate was evaporated under reduced pressure. The residue was dissolved in 1 *N* HCl (200 ml) and adjusted to pH 3. The crystallized material was collected by filtration, washed with distilled water, and recrystallized from THF/MeOH/hexane (84.1% total yield).

Lau-Glu Anhydride

Lau-Glu-OH (11.5 g) was dissolved in Ac_2O (7.1 ml) at 40°C. The solution was stirred with additional Ac_2O (1.8 ml) at 40°C for 3 hr and then kept at room temperature. The afforded solid material was recrystallized from ether–hexane (1:1) (37.7% total yield).

Lau-pGlu-OH

Lau-Glu anhydride (4.08 g) was dissolved in the mixture of dried ether (14 ml) and dried THF (7.1 ml). The solution of DCHA (2.6 ml) in dried ether (5.4 ml) was dropped into the

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solution of Lau-Glu anhydride and stirred overnight at room temperature. After filtration, the solution was evaporated under reduced pressure to give solidified residue. Ethyl acetate (50 ml) and 5% citric acid aqueous solution (50 ml) were added to the residue and stirred for 1 hr. The collected organic phase was washed with 5% citric acid aqueous solution followed by saturated NaCl solution. The organic phase was dried on anhydrous sodium sulfate and evaporated. The residue was recrystallized from hexane (24.4% total yield).

Z(OMe)-His-OMe

H-His-OMe · 2 HCl (20 g) was suspended in $CHCl_3$ (200 ml), and TEA (36.8 ml) was added. Z(OMe)-N_3 (20.54 g) was added to the mixture under ice cooling and stirred overnight. The mixture was washed with 5% sodium bicarbonate aqueous solution followed by saturated NaCl solution. The organic phase was dried and evaporated.

Z(OMe)-His-$NHNH_2$

Z(OMe)-His-OMe (27.54 g) was dissolved in isopropyl alcohol (50 ml), hydrazine (24.8 ml) was added, and the solution was kept overnight at room temperature. The ensuring solid was collected by filtration.

Z(OMe)-His-Pro-NH_2

Z(OMe)-His-$NHNH_2$ (1.87 g) in DMF solution was cooled to −20°C, then 3.5 *N* HCl-DMF (3.84 ml) and IAN (0.89 ml) were successively added, and stirred until the hydrazine test (11) was negative. After cooling to −30°C, the solution was neutralized by TEA (1.87 ml), dropped to the DMF solution of H-Pro-NH_2 (0.53 g), and stirred with additional TEA (0.93 ml) for 14 hr below 4°C. The filtrate from the reaction mixture was evaporated. The residue was dissolved in ethyl acetate and washed with 5% sodium bicarbonate (NaCl saturated) solution. The organic phase was dried over anhydrous sodium sulfate, filtrated, and evaporated. The residue was recrystallized from ether (83.5% total yield).

H-His-Pro-NH_2

Z(OMe)-His-Pro-NH_2 (1.27 g) was treated with the mixture of anisole (0.7 ml) and TFA (2.8 ml) in an ice bath for 1 hr to remove the Z(OMe) group according to usual method. The solution was evaporated and triturated with ether. The precipitate was dissolved in DMF and was neutralized with TEA (0.43 ml).

Lau-pGlu-ONB

Lau-pGlu-OH (1.05 g) and HONB (0.66 g) were dissolved in THF, DCC (0.77 g) was added, and the mixture was stirred overnight. After filtration, the solution was evaporated under reduced pressure. The residue was dissolved in DMF.

Lau-pGlu-His-Pro-NH_2(Lau-TRH)

The Lau-pGlu-ONB DMF solution was added to the H-His-Pro-NH_2 DMF solution and stirred overnight. The solution was filtrated, evaporated, and then dissolved in $CHCl_3$. This solution was washed with 5% sodium bicarbonate and saturated NaCl solution successively. The organic phase was dried and evaporated. The residue was recrystallized with ether (48.8% total yield).

Chromatographic Purification of Lau-TRH

The final product, Lau-TRH, in 0.1% TFA solution was applied to a YMC-Gel ODS-AQ 120A(S-50) column in a fast protein, peptide, and polynucleotide liquid chromatography (FPLC) system (Pharmacia Co.). The column was eluted with a linear gradient of acetonitrile (30–80%, 120 min) in 0.1% TFA at a flow rate of 3.0 ml/min. The effluent corresponding to the main peak at 80 min of retention time (monitored by UV absorption measurement at 230 nm) was collected. The solvent was evaporated, and the residue was lyophilized to give a white powder, m.p. 92–93°C. Amino acid ratios in 6 *N* HCl hydrolysate: Glu 1.06, His 1.00, Pro 1.00. MS: M^+, 544 (MW 544.678). *Anal.* Calcd. for $C_{28}H_{44}N_6O_5$, 5/2H_2O, CF_3COOH: C, 51.01; H, 6.99; N, 11.72. Found: C, 51.20; H, 7.16; N, 11.94.

Determination of Lipophilicity

Retention Time of High-Performance Liquid Chromatography (HPLC)

The final products and TRH were analyzed by reversed-phase HPLC (Hitachi) on a column (4.6 × 150 mm) of YMC-AM302(ODS) (Fig. 1). The column was eluted with a linear gradient of acetonitrile (0–100%, 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min. The eluate was monitored with a UV detector at a wavelength of 230 nm.

Partition Coefficients

The partition coefficients of TRH and Lau-TRH were determined in both *n*-octanol–buffer and $CHCl_3$–buffer systems. TRH or Lau-TRH (1.0 μmol/ml each) in pH 6.5 phosphate-buffered saline and equal volumes of organic solvent (*n*-octanol or $CHCl_3$) were mixed and then shaken vigor-

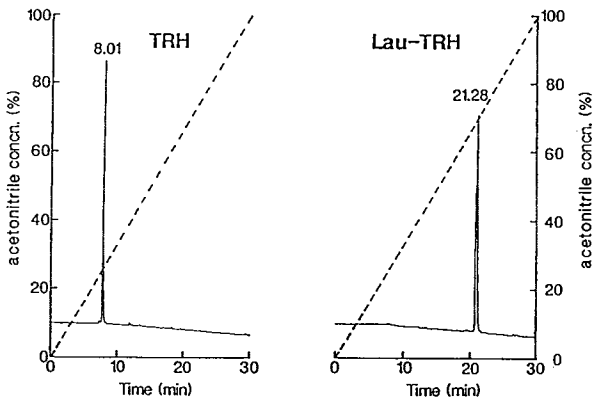


Fig. 1. Reversed-phase HPLC of TRH and lauroyl-TRH with 0.1% TFA on a column (4.6 × 150 mm) of YMC-AM302 (ODS). Detection at 230 nm (——); acetonitrile gradient (----).

ously (100 strokes/min) for 2 hr at 23°C. The contents of TRH or Lau-TRH in aqueous phase were determined at 230 nm at UV spectrum by HPLC, and partition coefficients (P) were calculated by the following equation:

$$P = \frac{C_i - C_w}{C_w}$$

where C_i and C_w represent the solute concentrations in the aqueous buffer phase and after distribution, respectively. Determinations were carried out in triplicate, and the P values obtained were reproducible to within ±5%.

Biological Evaluation

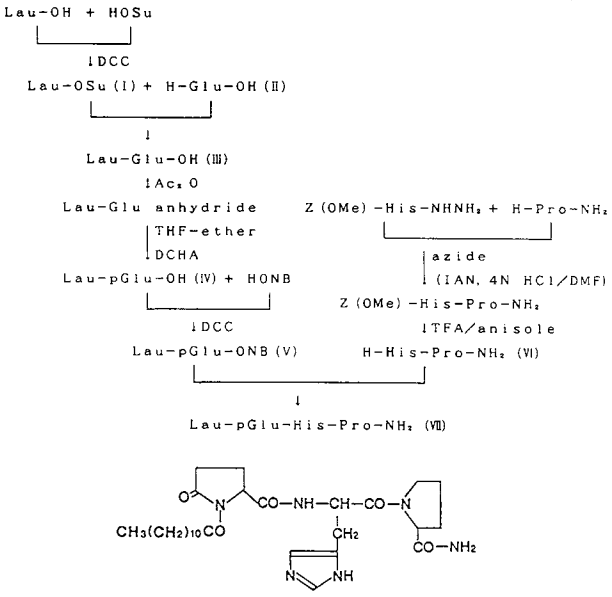
Pentobarbital Sleeping Time in Mice

Groups of five male Slc:ICR mice weighing 23–24 g were used. Animals were given intravenously various doses of a test peptide or saline as a control, at 10 min after intraperitoneal administration of 51.84 mg/kg of pentobarbital Na (Somnopentyl, Pitman-Moore), and the time from the administration of the test peptide to regaining the righting reflex was taken as the sleeping time. Percentage shortening of the sleeping time by the test peptide was calculated using the formula: percentage shortening of the sleeping time = (1 − mean sleeping time in drug-treated group/mean sleeping time in saline-treated group) × 100.

Thyroid-Stimulating Hormone (TSH) Releasing Activity in Rats

Male Wistar strain rats (Std:Wistar/ST) weighing 180–220 g at 7 weeks of age were intravenously administered various doses of a test peptide via the femoral vein. Control animals received only saline. Fifteen minutes after the administration, blood was taken from the femoral aorta. Plasma was rapidly separated and stored at −20°C until analysis. Plasma TSH levels were determined by the double-antibody radioimmunoassay method of Midgley (12) using the rat TSH kit. Rat TSH was labeled with ^{125}I according to the chloramine-T method of Hunter and Greenwood (13).

Potency ratio relative to TRH was calculated by the parallel-line assay method (14).



Scheme I. Synthetic route to Lau-TRH. Lauroyl group is abbreviated Lau.

RESULTS AND DISCUSSION

Chemistry

Lau-TRH was synthesized stepwise by a solution method starting with *N*-hydroxysuccinimide esters of lauric acid (Lau-OSu; I) and glutamic acid (II) (Scheme I).

Synthesized Lau-Glu-OH (III) was converted to Lau-pGlu-OH (IV) via the anhydride and then to Lau-pGlu-ONB (V) using HONB and DCC. H-His-Pro-NH_2 (VI) was prepared by azide method from Z(OMe)-His-$NHNH_2$ and prolinamide. Lau-TRH (VII) was obtained from the reaction of V with VI by the active ester method, and purified Lau-TRH was obtained by the FPLC system in a 3.3% final yield.

Physicochemical properties of TRH and Lau-TRH were compared to ascertain the improvement of lipophilic characteristics. Partition coefficients were determined using *n*-octanol and $CHCl_3$ as organic phase. The partition coefficients of Lau-TRH were 1.910 and 2.144 on *n*-octanol and $CHCl_3$, respectively, whereas those of TRH were 0.068 and

Table I. Antagonism of Pentobarbital Sleep

Compound	Dose (μg/mouse, i.v.)[a]	N[b]	Sleeping time (min, mean ± SE)[c]	% Shortening	Potency ratio relative to TRH (95% confidence limits)
TRH	0.125	5	55.2 ± 1.7	19.5	
	0.25	5	44.5 ± 2.3	35.1	1.0
	1.0	5	34.4 ± 2.0	49.8	
Lau-TRH	0.125	5	56.8 ± 1.3	17.0	
	0.25	5	48.5 ± 1.3	29.1	0.81 (0.60–0.99)
	1.0	5	37.7 ± 1.4	45.0	

[a] Doses are expressed in terms of TRH.
[b] Number of animals used.
[c] Control (saline): 68.5 ± 3.0 min.

Table II. TSH-Releasing Activity

Compound	Dose (μg/rat, i.v.)[a]	N[b]	Plasma TSH (ng/ml, mean ± SE)[c]	Potency ratio relative to TRH (95% confidence limits)
TRH	0.25	7	469 ± 86	
	1	7	631 ± 85	1.0
	4	7	732 ± 61	
Lau-TRH	1	7	595 ± 67	
	4	7	605 ± 93	0.64 (0.20–4.13)
	16	7	945 ± 72	

[a] Doses are expressed in terms of TRH.
[b] Number of animals used.
[c] Control (saline): 87.6 ± 6 ng/ml.

0.088, respectively. In HPLC analysis, the retention time of Lau-TRH (21.28 min) was delayed compared to that of TRH (8.01 min). These data indicated that lauroyl derivatization of TRH enhanced the lipophilicity of TRH.

Pharmacology

We first injected three different doses intravenously and estimated the antagonism of pentobarbital sleep, which is the most characteristic pharmacological property of TRH on the central nervous system (CNS). Table I shows the central nervous activity of Lau-TRH in comparison with that of TRH. The sleeping time of the saline control mice was 68.5 min. The potency of CNS activity of Lau-TRH was slightly reduced, to 81% of the TRH potency.

TSH-releasing endocrine activity of the TRH derivative was also compared to that of TRH at different doses as the endocrine activity. Table II shows the hormonal activities expressed as plasma TSH levels. The saline control values were as low as 87 ng/ml. The data indicated that Lau-TRH possessed 64% of the TSH-releasing activity of the parent TRH.

In conclusion, acylation of the N-terminal pyroglutamyl residue of TRH by lauric acid reduced CNS activity by only 19% and endocrine activity by only 36%. The novel lipophilic TRH derivative, Lau-TRH, may be generally applicable to the transmembrane intestinal delivery of peptides.

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