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Brain-Targeted Chemical Delivery of [Leu^2, Pip^3]-TRH: Synthesis and Biological Evaluation†

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Abstract—A chemical targeting system for [Leu^2, Pip^3]-TRH (Gln,Leu,Pip) was synthesized in order to allow its specific delivery to the central nervous system (CNS). Sequential metabolism of the obtained 'packaged' chemical delivery system, (CDS), DHT-Pro-Pro-Gln-Leu-Pip-OCh, should yield a 'locked-in' precursor following the oxidative conversion of the dihydrotrigonellyl (DHT) to the trigonellyl (T^+) moiety, followed by removal of the cholesteryl function and cleavage of the T^+-Pro-Pro by prolyl endopeptidase. The antagonism of barbiturate-induced sleeping time was used to assess the activity of the CDS. The sleeping time after administration of vehicle and [Leu^2]-TRH was 100.5 ± 6.3 min, and 78.2 ± 4.7 min, respectively. The [Leu^2, Pip^3]-TRH-CDS showed a significant decrease in sleeping time (58.2 ± 3.4 min) compared to the vehicle or [Leu^2]-TRH. These results indicate successful brain delivery of the precursor construct, and an effective release of the active GlnLeuPip in the brain.

Introduction

Thyrotropin-releasing hormone (TRH, PyroGlu-His-Pro-NH_2) was the first brain peptide isolated from the hypothalamus, and its function in neuroendocrine regulation of pituitary secretion of thyroid-stimulating hormone (TSH) is well established.[1-4] Besides its function as the primary neurotrophic hormone of TSH, extra hypothalamic distribution of TRH and its receptor indicates that TRH plays other roles in nervous system physiology. Support for the neurotransmitter/neuromodulatory role for TRH in the CNS comes from several lines of investigations. Direct CNS administration of TRH exerts profound pharmacological effects. TRH has been shown to increase locomotor activity and to induce 'wet dog' shakes and forepaw tremors.[5,6] TRH is an effective analeptic agent, reducing pentobarbital induced narcosis by 50% or more following peripheral administration of high doses[7,8] or central injection of lower doses in rats, rabbits, and monkeys.[9,10] A cholinergic mechanism appears to explain the ability of TRH to reduce catalepsy in experimental animals. TRH exhibits its highest potency in antagonizing pentobarbital-induced catalepsy when microinjected into the medical septum and the diagonal band of Broca,[11] two areas that are rich in cholinergic cell bodies. Additionally, the capacity of TRH to antagonize narcosis appears to depend on cholinergic neurons.[7–10] Finally, TRH has been shown to stimulate activity in brain cholinergic neurons in barbiturate-treated rats.[11–13] Collectively, these results indicate that TRH may normally serve to activate brain cholinergic neurons and hence may be a useful therapeutic agent for treating the cognitive decline associated with aging and Alzheimer's disease.

The therapeutic use of TRH in the treatment of age-related brain dysfunction is limited primarily by its short half-life[14] and its inability to penetrate the blood brain-barrier (BBB).[15] TRH is rapidly metabolized in plasma by TRH-specific pyroglutamyl aminopeptidase and prolylendopeptidase.[16] Thus, strategies to deliver TRH to the brain must include protection of the peptide in plasma as well as a way to enhance its penetration through the BBB.

The brain-enhanced delivery of a TRH analogue by molecular packaging[17] was successfully achieved by our group[18] based on the sequential bioactivation of a brain-targeted construct, containing the active peptide. As a result of the brain-targeted, retained release of the active Leu-TRH analogue, significant reduction in the pentobarbital induced sleeping time in mice was

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demonstrated, following iv administration. In the case reported,[18] the targeting construct constituted of DHT-Pro-Pro-Gln-Leu-Pro-Gly-OCh, The C-terminal extension with Gly before esterification with cholesterol was introduced in order to allow formation of the prolinamide by PAM (peptidyl glycine α-amidating monooxygenase). It was found, however, that the amide precursor was susceptible to deamination by PPCE (a.k.a. TRH deaminase),[19] a side reaction process competitive to the 'designed-in' cleavage of the spacer-Gln-peptide bond. Thus, we have investigated replacement of the Pro-Gly C-terminal with pipecolic acid (Pip), thereby allowing formation of a carboxylic analogue of [Leu^2]-TRH, that is not prone to enzymatic deactivation but it is an active TRH-analogue in the free carboxylate form. Here, we report the synthesis and biological evaluation of the packaged CDS of [Leu^2, Pip^3]-TRH analogue.

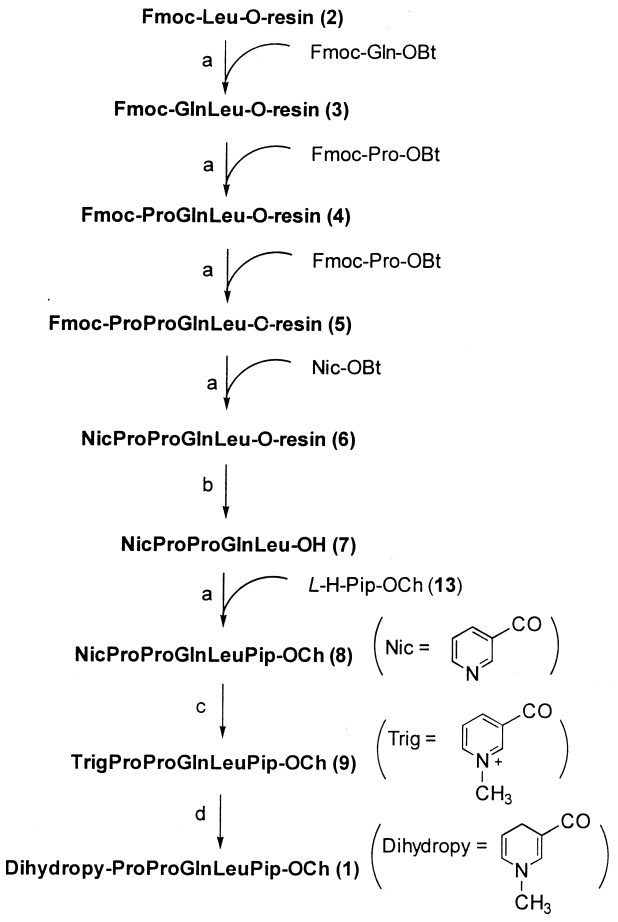
Results and Discussion

Synthesis

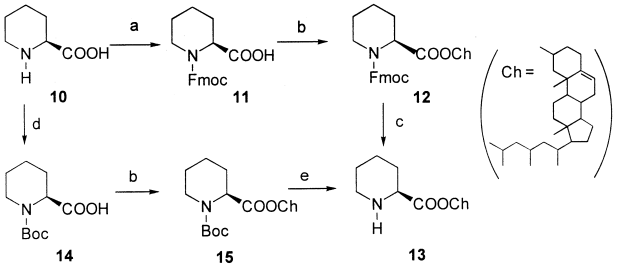
The CDS of [Leu^2, Pip^3]-TRH for brain targeted delivery was prepared by pentapeptide–single amino acid (5 + 1) segment-coupling approach because we found that stepwise elongation of the peptide chain from Fmoc-Pip-O-resin with activated ester of amino acid resulted in diketopiperazine formation.[20] Thus, the two individual segments, NicProProGlnLeu-OBt and H-Pip-OCh, were prepared by the combining solid phase peptide synthesis (SPPS) with solution phase synthesis (Scheme 1). The main fragment of the peptide chain, NicProProGlnLeu-OBt, was prepared from Fmoc-Leu-O-resin by SPPS using an automated Fmoc chemistry protocol followed by the DIC mediated coupling which was accelerated with HOBt. After completion of stepwise elongation, the crude peptide NicProProGlnLeu-OH (**7**) was cleaved off from the resin with TFA, and identified by EI–MS. Since the purity of the crude peptide was about 99% by HPLC, it was used for next reaction without further purification.

The lipophilic ester of the C-terminal amino acid, H-Pip-O-cholesterol, was prepared from L-pipecolic acid (Scheme 2). The Fmoc group was initially selected as the protecting group of the N-terminal of the pipecolic acid before esterification of the C-terminal with cholesterol. Thus, after the reaction of **10** with Fmoc-OSu in basic conditions, the esterification was carried out with cholesterol:DCC:DMAP to afford **12** in 25% yield from the starting material. The usual Fmoc group deprotection condition of piperidine/DCM or piperidine/DMF resulted in the cleavage of the cholesterol group as well as the Fmoc group. Selective deprotection of Fmoc group from **12** was achieved with a 1% piperidine/DCM using longer reaction time (2 days). The yield in this case was 50%.

As an alternative synthetic route, we chose Boc as the protecting group of the nitrogen in pipecolic acid as the cholesteryl moiety could be stable in TFA. Therefore, after the Boc group was attached to pipecolic acid,



Scheme 1. (a) HOBt, DIC, DMA; (b) TFA; (c) CH_3I, $CHCl_3$, 99%; (d) $Na_2S_2O_4$, $NaHCO_3$, water, ethyl acetate.



Scheme 2. (a) Fmoc-Osu, 9% Na_2CO_3, DMF, 83%; (b) cholesterol, DCC, DMAP, CH_2Cl_2, 83%; (c) 1% piperidine, CH_2Cl_2, 50%; (d) $(Boc)_2CO$, K_2CO_3, DMF, 80%; (e) TFA:CH_2Cl_2 (1:1), 90%.

esterification was successfully achieved in the same way as in the synthesis of **15**. Deprotection of Boc followed by neutralization with sodium bicarbonate afforded **13** in 90% yield. The two fragments, **7** and **13**, were assembled using DCC/HOBt in DCM to give the crude **8**, which was purified by column chromatography on neutral alumina with 5% MeOH/DCM eluent. Methylation of the pyridine ring in **8** with methyl iodide produced **9** while methylation with dimethyl sulfate resulted in partial methylation. Finally, reduction of the trigonellyl group with sodium dithionite gave the desired 1,4-dihydro product (**1**).

The CDS of [Leu^2]-TRH for brain targeted delivery was prepared following the previously reported methods,[18] (1) stepwise elongation of the peptide chain from Fmoc-Gly-O-resin with the corresponding activated ester of amino acids, (2) cleavage of peptide resin with TFA, (3) coupling the NicProProGlnLeuProGlyOH with cholesterol, (4) methylation with dimethyl sulfate, and (5) reduction with sodium dithionite.

Pharmacology

The antagonism of barbiturate-induced sleeping time in mice was used to assess the activation effect on mice CNS cholinergic neurons by [Leu^2, Pip^3]-TRH-CDS. In mice, 10 min after iv administration of the vehicle (30% hydroxypropyl-β-cyclodextrin, 1.5 ml/kg) or drug solution (10 μmol/kg) in the tail vein, 60 mg/kg of pentobarbital was i.p. injected. The sleeping time was recorded as the time elapsed from the onset of the righting reflex until the reflex was regained. As shown in Table 1, the sleeping time after administration of vehicle and [Leu^2]-TRH was 100.5 ± 6.3 min and 78.2 ± 4.7 min, respectively. The [Leu^2, Pip^3]-TRH-CDS showed a significant decrease in sleeping time (58.2 ± 3.4 min) compared to the vehicle or [Leu^2]-TRH. The sleeping time after administration of the previously used [Leu^2]-TRH-CDS was 62.0 ± 3.9 min. These results indicate that the successful delivery of [Leu^2, Pip^3]-TRH-CDS to the brain, followed by its 'locked-in' and sequential bioactivation yielded the active TyrLeuPip in the brain effectively. The substitution of Pro-Gly with Pip resulted increased in vivo stability.

In conclusion, the molecular packaging/sequential metabolism method successfully delivered [Leu^2, Pip^3]-TRH-CDS to the brain, and an effective release of the active TyrLeuPip in the brain was achieved.

Experimental

Instrument and materials

All chemicals used were of reagent or peptide synthesis grade. Fmoc-amino acids and other chemicals were purchased from Chem-Index Inc., USA. Solvents were purchased from Fisher Scientific Inc. Thin layer chromatography was performed on either silica gel coated (Merck Kiesel 60 F254, 0.2 mm thickness) plates or neutral alumina coated (Merck Kiesel 60 F254, 0.2 mm thickness) plates and developed with DCM:MeOH. The melting points were determined on a Fisher–Johns apparatus and are uncorrected. Elemental analyses were supplied by Atlantic Micro labs. Inc. (Norcross, GA). Mass spectra were recorded on a Kratos MS80RFA instrument (Kratos Analyticals, Manchester, UK). UV spectra were recorded in methanol on a Lambda 11 UV–vis Perkin–Elmer (Perkin–Elmer Anal. Inst., Norwalk, CT). Following abbreviations are used: CDS, chemical delivery system; Ch, cholesteryl; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DIC, 1,3-diisopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; DMA, *N,N*-dimethylactamide; DMF, *N,N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; EtOAc, ethyl acetate; HPBCD (2-hydroxypropyl-β-cyclodextrin); IPA, isopropyl alchohol; MeOH, methanol; TFA, trifluoroacetic acid; DIEA, *N,N*-didisopropylethylaime, TEA, triethylamine; Nic, nicotinoyl; Su, *N*-hydroxysuccinyl, DHT, dihydrotrigonellyl; T^+, trigonellyl.

Table 1. A comparison of the pentobarbital induced sleeping time in mice after the administration of vehicle and test compounds[a,b]

Compounds	Sleeping time (min)
Vehicle	100.5 ± 6.3
Pyr-Leu-Pro-NH_2	78.2 ± 4.7[c]
Dhtr-Pro-Pro-Gln-Leu-Pip-O-cholesterol	58.2 ± 3.4[c]
Dhtr-Pro-Pro-Gln-Leu-Pro-O-Cholesterol	62.0 ± 3.9[c]

[a]Ten min after iv injection of the compound (10 μmol/kg) (TRH 10 μmol = 3.7 mg), pentobarbital, 60 mg/kg, was ip injected in the animal.
[b]The sleeping time was recorded as the time elapsed from the onset of loss to regain of the righting reflex. Six to seven Swiss Webster mice (30 ± 3 g)were used in each group. Table entries are mean ± SE.
[c]$P \sim 0.05$ when compared to vehicle control using student *t*-test.

Synthesis of [Leu^2, Pip^3]-TRH-CDS

NicProProGlnLeu-OH (7). Fmoc-Leu-Wang resin was placed in a 200 mL reaction vessel, and the following steps were carried out: (1) deprotection of Fmoc group with piperidine/DMF (1:1, v/v, 3 min and then 7 min treatment), (2) washing, DMF (4×1 min), IPA (1×1 min), DMF (1×1 min), and DCM (4×1 min), (3) coupling with activated acid esters which were prepared from the reaction of the corresponding Fmoc-amino acids and nicotinic acid (3.0 equiv., HOBt (3.0 equiv), and DIC (3.0 equiv) in 10 mL of DMA, and (4) washing, DMF (4×1 min), IPA (1×1 min), DMF (1×1 min), and DCM (4×1 min). The coupling time was for about 2 h, and usually double coupling was applied. The completion of reaction was monitored by the Kaiser test. After coupling each residue to the resin step by step, the desired peptide was cleaved off by using TFA for 80 min. The TFA solution was concentrated and the residue was washed with ethyl ether three times to give the product as a white precipitate. HPLC showed the 99% of purity of the precipitate. Mp 128–130 °C, EI MS$[M+H]^+$ m/z 559. Elemental analysis for $C_{27}H_{38}N_60_7$. 0.55TFA; cald, C 54.32, H 6.25, N 13.52, found, C 54.68, H 6.42, N 13.15.

Nic-ProProGlnLeuPiP-OCh (8). To a solution of TFA.NicProProGlnLeu-OH (1.70 g, 2.52 mmol) in DCM (20 mL) was added HOBt (332 mg, 2.52 mmol) and DCC (332 mg, 2.52 mmol) in an ice water bath. After the solution was stirred for 5 min in an ice water bath and then for 40 min at room temperature, H-Pip-OCh (13, 630 mg, 1.26 mmol) in DCM (3 mL) was added. The mixture was basified with DIEA to pH ca. 7.5, then stirred for 3 h at room temperature. The mixture was filtered to remove DCU after completion of the reaction and washed with DCM (30 mL). After the

organic layer was washed with 5% citric acid solution, saturated sodium bicarbonate solution, and water successively, it was dried and evaporated to give the crude product which was purified by column chromatography with neutral alumina (Brockman activity I, 60–320 mesh) with 5% MeOH/DCM to give pure product (1.3 g, 99% yield). $R_f = 0.47$ (5% MeOH/DCM, on neutral alumina), mp 115–117 °C, EI MS$[M+H]^+$ m/z 1038, elemental analysis for $C_{60}H_{91}N_7O_8.H_2O$; calc, C 68.21, H 8.85, N 9.28, found, C 68.12, H 9.04, N 9.58.

Trigonellyl-ProProGlnLeuPip-OCh (9). Nic-ProProGln LeuPip-OCh (206 mg, 1.00 mmol) was dissolved in chloroform (10 mL), and methyl iodide (20 equiv, 2.8 mL) was added. The solution was stirred at room temperature for 2 days. After evaporation of the solvent, ether (30 mL) was added. The resulting yellow precipitate was filtered and washed with ether again. The product was obtained in a quantitative yield. $R_f = 0.05$ (5% MeOH/DCM, on neutral alumina), mp 166–169 °C, MALDI MS $[M+H]^+$; m/z 1055 (quarternary pyridium ion), UV (MeOH); 254 nm (λ_{max}).

1,4-Dihydrotrigonellyl-ProProGlnLeuPip-OCh (1). To an ice cold solution of Trig-ProProGlnLeuPiP-OCh (200 mg, 0.168 mmol) in dearated water (30 mL), sodium bicarbonate (294 mg, 3.5 mmol), sodium dithionate (1.04 g, 6.0 mmol) and ethyl acetate (5 mL) were added under nitrogen. The mixture was stirred at 0 °C for 5 min and another cooled ethyl acetate (50 mL) was added. The mixture was stirred for another 30 min at 0 °C, then for 50 min at room temperature. The ethyl acetate layer was separated and the aqueous layer was further extracted with ethyl acetate (60 mL). The combined ethyl acetate extract was washed with 5% sodium bicarbonate solution (540 mL), dried over sodium sulfate and evaporated on a rotary evaporator to give the product as a yellow solid (70 mg, 38.5% yield). Dearriated petroleum ether was added immediately to prevent the oxidation. $R_f = 0.30$ ($CHCl_3$:MeOH:Et_3N = 10:1:0.1, v/v), UV (MeOH, λ_{max}); 348 nm.

(L)-Fmoc-Pip-OH (11). To a cold solution of L-pipecolic acid (0.50 g, 3.8 mmol) in 9% Na_2CO_3 (10 mL) solution was added Fmoc-Osu (1.30 g, 3.8 mmol) in DMF (2 mL) at ice water bath temperature. After the mixture was stirred for 5 min at ice water bath temperature and for 1 h at room temperature, it was diluted with water (25 mL), and then extracted with ethyl ether (10 mL) followed by ethyl acetate (2×10 mL). The aqueous layer was cooled down and acidified to pH 2 with 1N HCl. The acidic aqueous layer was extracted with ethyl acetate (4×30 mL). The organic layer was washed with saturated NaCl solution (3×30 mL) and dried over anhydrous Na_2SO_4. After evaporation of solvent, white solid was obtained (1.20 g, 83% yield). Mp 161–164 °C, FAB MS$[M+H]^+$; m/z 352.4.

(L)-Fmoc-Pip-OCh (12). The mixture of Fmoc-Pip-OH (1.20 g, 3.42 mmol), cholesterol (1.32 g, 3.42 mmol), DMAP (0.42 g, 3.42 mmol), and DCC (1.08 g, 3.42 mmol) in DCM (18 mL) was stirred for 10 min at 0 °C and for 2 h at room temperature. After additional DCC (0.50 g, 1.7 mmol) was added, the mixture was stirred for additional 2 h at room temperature. After completion of the reaction, the mixture was filtered to remove DCU and then washed with DCM. The combined organic layer was washed with 5% citric acid solution, saturated sodium bicarbonate solution, and water. After drying over anhydrous Na_2SO_4, the organic layer was concentrated to give the brownish residue, which was purified with column chromatography on silica gel with DCM/hexane (2:1, v/v) to give a white product (825 mg, 34% yield). Mp 161–164 °C, FAB MS$[M+H]^+$ m/z 720.

(L)-Boc-Pip-OH (14). The mixture of L-pipecolic acid (1.23 g, 9.50 mmol) and di-*t*-butylcarbamate (1.84 g, 9.50 mmol) in 9% Na_2CO_3 solution (11 mL) and DMF (11 mL) was stirred for 5 min at ice water bath temperature and 2 h at room temperature. The slurry mixture was diluted with water (100 mL) to give clear solution, which was extracted with ethyl ether (10 mL) followed by ethyl acetate (2×10 mL). The aqueous layer was cooled down and acidified to pH 2 with c-HCl. The acidic aqueous layer was extracted with ethyl acetate (4×50 mL). The organic layer was washed with half brine (3×30 mL) and dried over anhydrous Na_2SO_4. After evaporation of solvent, white solid was obtained (1.75 g, 80% yield). Mp 133–135×C, EI MS $[M+Na]^+$ m/z 252, ^{1}HNMR ($CDCl_3$); δ 1.20–1.50 (2H, m), 1.45 (9H, s), 1.50–1.70 (3H, m), 2.22 (1H, m), 2.90–2.97 (1H, m), 3.95 (1H, m), 4.80 (1H, d), 9.25 (1H, br, COOH).

(L)-Boc-Pip-OCh (15). The mixture of Boc-Pip-OH (1.70 g, 7.4 mmol), cholesterol (2.85 g, 7.4 mmol), DMAP (0.90 g, 7.4 mmol), and DCC (1.53 g, 7.4 mmol) in DCM (30 mL) was stirred for 10 min at 0 °C and 2 h at room temperature. After additional DCC (0.50 g, 1.7 mmol) was added, the mixture was stirred for additional 2 h at room temperature. After completion of the reaction, the mixture was filtered to remove DCU and then washed with DCM. The combined organic layer was washed with 0.2 N HCl solution, saturated sodium bicarbonate solution, and water. After drying over anhydrous Na_2SO_4, the organic layer was concentrated to give a white residue, which was purified with column chromatography on silica gel with DCM to give the pure product (3.7 g, 83% yield). mp 159–160 °C, EI MS$[M+Na]^+$ m/z 621, ^{1}HNMR ($CDCl_3$); δ 5.4 (1H, m, cholesterol alkenyl-H).

(L)-H-Pip-OCh (13). (1) From (L)-Boc-Pip-OCh: The solution of (L)-Boc-Pip-OCh (3.6 g, 6.0 mmol) in TFA:DCM (60 mL, 1:1, v/v) was stirred for 50 min at room temperature. After concentration of the solution, the resulting purple colored oil was dissolved in water (20 mL). After the aqueous layer was basified with saturated sodium bicarbonate solution, it was extracted with ethyl acetate (3×40 mL). The organic layer was washed with saturated sodium bicarbonate solution, and half brine. After drying over anhydrous Na_2SO_4, the organic layer was concentrated to give the product. Recrystallization from ether/hexane gave an analytically pure compound (2.7 g, 90% yield). mp 170.5–171.5 °C, EI MS$[M+Na]^+$; m/z 520, ^{1}HNMR ($CDCl_3$); δ 1.20–1.50

(2H, m), 1.45 (9H, s), 1.50–1.70 (3H, m), 2.22 (1H, m), 2.90–2.97 (1H, m), 3.95 (1H, m), 4.80(1H, d), 9.25 (1H, br, COOH), elemental analysis for $C_{33}H_{55}NO_2$; calc, C 79.62, H 11.14, N 2.81, found, C 79.52, H 11.08, N 2.84.

(2) From (L)-Fmoc-Pip-Och: To the solution of (L)-Fmoc-Pip-OCh (120 mg, 0.170 mmol) in dry DCM (5 mL) were added 4 drops of piperidine, and the resulting solution was stirred for 2 days. After concentration of the solvent, the yellow residue was diluted with ether to give a white precipitate, which was dissolved in DCM (10 mL). The DCM layer was washed with 0.1 N HCl, saturated $NaHCO_3$ solution, and half brine. After drying over anhydrous Na_2SO_4, the organic layer was evaporated to give the product (30 mg, 50% yield). Analytical data were the same as method (1) above.

Complexation of [Leu^2,Pip^3]-TRH-CDS with HPBCD (2-hydroxypropyl-β-cyclodextrin). Saturated [Leu^2,-Pip^3]-TRH-CDS in HPBCD/water solution was prepared by addition of HPBCD/water (30%, w/w) into the excess amount of [Leu^2,Pip^3]-TRH-CDS followed by ultrasonication at 10 °C for 1h. To minimize the decomposition of [Leu^2,Pip^3]-TRH-CDS during the complexation, de-gassed water and argon atmosphere were used during the process. After equilibration, the complex solution was filtered through HV polyethylene filter (pore size = 0.45 μm). Aliquot of the filtrate was freeze-dried, and then immediately stored at −15 °C. The concentration was analyzed by comparing the characteristic absorbance peak UV maxima height at 355 nm.

Pharmacology

Effect of [Leu^2, Pip^3]-TRH-CDS on the barbiturate induced sleeping time in mice. Swiss Webster mice (body weight, 30 ± 3 g) were used. Freeze-dried CDS-HPBCD (2-hydroxypropyl-β-cyclodextrin) complexes of TRH analogues were reconstituted by adding appropriate amount of HPBCD solution to obtain desired concentration of CDS in 30% HPBCD aqueous solution. Vehicle (3.0 mL/kg) only, or compounds at a dose of 10 μmol/kg (equimolar to 3.6 mg/kg of TRH) were injected to the animals through the tail. Ten min after iv injection of the compound, each animal received an intraperitoneal injection of sodium pentobarbital solution (30 mg/mL) at a dose of 60 mg/kg. The sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex until the reflex was regained. Groups of 6–7 animals were used for testing each compound. The student *t*-test was used for the statistical analyses.

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