Synthesis and Brain Targeted Chemical Delivery of [Nva^2,Pip^3]-TRH

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Thyrotropin-releasing hormone (TRH, PyroGlu-His-Pro-NH_2) was the first brain peptide isolated from hypothalamus and is primarily responsible for the neuroendocrine regulation of thyrotropin secretion hormone (TSH) from the pituitary.[1,2] Besides its function as the primary neurotrophic hormone, TRH and its analogues induce numerous behavioral effects[3,4] such as the reduction of barbiturate narcosis or haloperidol-induced catalepsy,[5,6] accelerateion of ACh turnover,[7] and improvement of memory and learning.[8] Therefore, peptides can be beneficial for treating motor neuron diseases,[9] spinal cord trauma,[10] Alzheimer's disease,[11] and West syndrome.[12]

The therapeutic use of TRH in the treatment of age-related brain dysfunction is however limited primarily by its short half-life[13] and its inability to penetrate the Blood Brain-Barrier (BBB).[14] In addition, TRH is rapidly metabolized in plasma by TRH-specific pyrogluamyl aminopeptidase and prolylendopeptidase.[15] Thus, strategies to deliver TRH to the brain must include not only protection of the peptide from the enzymes in plasma but the penetration of the BBB. The brain-enhanced delivery of TRH analogues was successfully achieved by our group[16] based on the sequential bioactivation of a brain-targeted construct with the molecular packaging method. In the case of previously reported Leu-TRH analogue,[17] the targeting construct constituted of DHT-Pro-Pro-Gln-Leu-Pro-Gly-OCh, where the c-terminal Pro residue was extended with Gly residue in order to allow the formation of the prolinamide by PAM (peptidyl glycine α-amidating monooxygenase), resulted in significant reduction in the pentobarbital induced sleeping time in mice following iv administration. In the course of improving the stability of the TRH analogue or its precursors in the brain, we have identified that the prolinamide obtained from the Pro-Gly precursor by PAM was also susceptible to degradation that involved deamination by PPCE (a.k.a. TRH deaminase).[18] Since this process was a competitive side-reaction to the "designed-in" cleavage of the spacer-Gln peptide bond, we recently replaced the Pro-Gly c-terminal with pipecolic acid (Pip) and found that the replacement resulted in better *in vivo* stability.[19] In this study, we extended our approach to [Nva^2]-TRH derivative for the further improvement of *in vivo* stability by replacement of Leu residue with Nva. Here, we report the synthesis and biological evaluation of [Nva^2, Pip^3]-TRH derivative.

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Experimental Section

Materials and Methods. All chemicals used were of reagent or peptide synthesis grade. TRH, 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. 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, U.K.). UV spectra were recorded in methanol on a Lambda 11 UV/vis Perkin Elmer (Perkin Elmer Anal. Inst., Norwalk, CT). The CDS of [Nva^2]-TRH and [Leu^2]-TRH for brain targeted delivery were similarly prepared by the following the previously reported methods;[17] 1) stepwise elongation of the peptide chain from Fmoc-Gly-O-resin with the correspondent activated ester of amino acids, 2) cleavage of peptide resin, 3) coupling reaction of the main peptide fragment with cholesterol, 4) methylation with dimethyl sulfate, and 5) reduction with sodium dithionite. Following abbreviations are used; AA, amino acid; Ch, cholesteryl; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DIC, 1,3-diisopropylcarbodiimide; DMAP, 4-(N,N-dimethylamino)pyridine; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; DHT, dihydrotrigonellyl; DMA, N,N-dimethylacetamide; DMF, N,N-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; EtOAc, ethyl acetate; IPA, isopropyl alchohol; MeOH, methanol; TFA, trifluoroacetic acid; DIEA, N,N-didisopropylethylaime, TEA, triethylamine; Nic, nicotinoyl; Su, N-hydroxysuccinyl, T^+, trigonellyl.

PyrNvaPro-NH_2. Rink resin (1.0 g, 0.40 mmol) was used in this synthesis. After coupling each residue to the resin step by step, the peptide was cleaved off by using TFA. After evaporation of solvent, the residue was washed with ether (3 $\times$ 30 mL) and dried over Na_2SO_4. Purification with HPLC afforded the pure compound (93.7 mg, 72% yield) as a white crystal. mp 118-120 ^{o}C, ESI MS $[M+Na]^+$ m/z 347.

Nic-ProProGlnNva-OH (3). Fmoc-Nva-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 hour, 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 a white precipitate. The precipitate was dried and recrystallized from DCM/hexane to give 350 mg of product (95% yield). HPLC showed the 99% of purity. FAB-MS: $[M + K^+]$ 583. Elemental analysis for $C_{28}H_{37}N_6O_9F_3$.0.33 hexane; Cald, C 52.42, H 6.11, N 12.23, Found, C 52.56, H 6.25, N 12.33.

(L)-H-Pip-OCh (4). The compound was prepared from *L*-pipecolic acid by following the previously reported method;[19] 1) protection of N-terminal of pipecolic acid with Boc group, 2) esterification of C-terminal pipecolic acid with cholesterol under DCC/DMAP condition, and 3) deprotection of Boc group with TFA followed by neutralization with sodium bicarbonate. mp 170.5-171.5 °C EI MS $[M + Na]^+$; m/z 520, ^{1}H NMR ($CDCl_3$) 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 $C_{33}H_{55}NO_2$; Cald, C 79.62, H 11.14, N 2.81, Found, C 79.52, H 11.08, N 2.84.

Nic-ProProGlnNvaPip-OCh (5). To a solution of TFA salt of Nic-ProProGlnNva-OH (1.32 g, 2.00 mmol) and HOBt (264 mg, 2.00 mmol) in methylene chloride (15 mL) was added DCC (412 mg, 2.00 mmol) at 0 °C. After the mixture was stirred for 30 min at room temperature, H-Pip-O-Chloresterol (500 mg, 1.0 mmol) and DIEA (1.0 mL) was added. The resulting mixture was stirred for 40 hr at room temperature under nitrogen atmosphere, and then the precipitated DCU was filtered off. The filtrate was washed with methylene chloride (2 × 10 mL). The combined organic layer was washed with 5% citric acid solution (30 mL), saturated sodium bicarbonate solution (30 mL), water (30 mL) successively, and then dried over sodium sulfate. After the solvent was filtered and concentrated, the residue was purified on neutral alumna column chromatography to give a pure compound (450 mg, 44%). FAB-MS: $[M + Na^+]$ 1047. Elemental analysis for $C_{59}H_{89}N_7O_8$; Cald, C 69.18, H 8.76, N 9.57, Found, C 69.04, H 8.79, N 9.39.

Trig-ProProGlnNvaPip-OCh (6). To a solution of Nic-ProProGlnNvaPip-OCh (1.40 g, 1.37 mmol) in 15 mL of chloroform was added dimethyl sulfate (2.59 g, 20.5 mmol) dissolved in 15 mL of chloroform. The mixture was stirred at room temperature for 14 hrs. After evaporation of solvent, ether was added. The resulting yellow precipitate was filtered off and washed with ether again. Recrystallization from chloroform/ether (1 : 20) gave the pure product (1.40 g, quantitative yield) as a crystal. FAB MS $[M + H]^+$; m/z 1039, UV (MeOH) 254 nm (max).

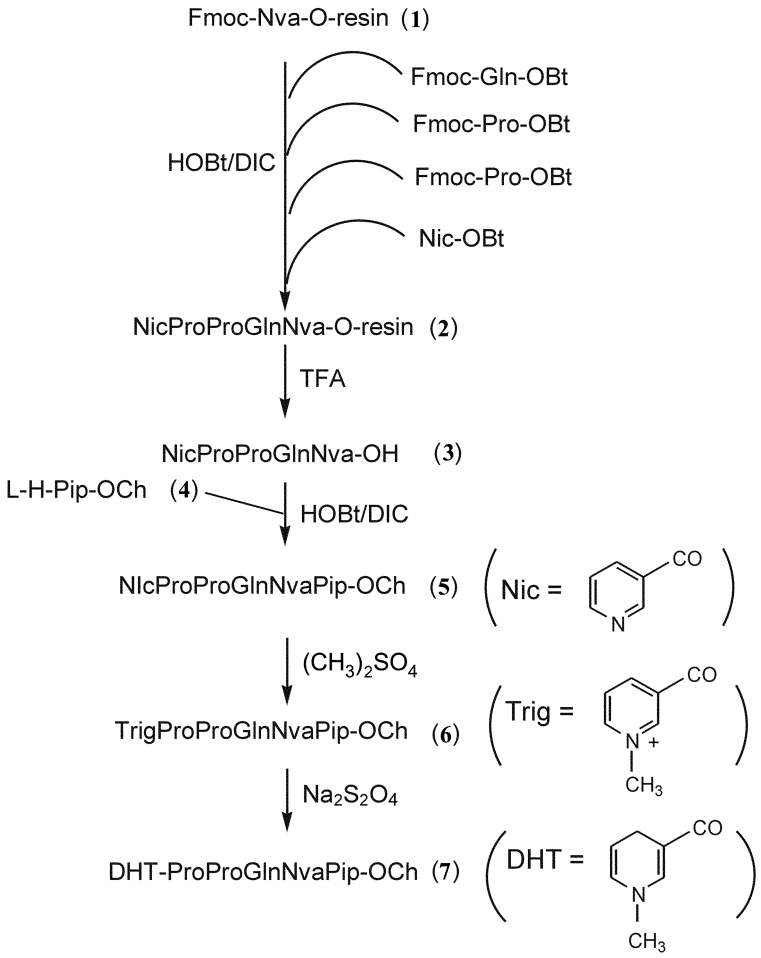
1,4-Dihydrotrigonellyl-ProProGlnNvaPip-OCh (7). To an ice-cold solution of Trig-ProProGlnNvaPip-OCh (400 mg, 0.320 mmol) in 60 mL of deariated water, sodium bicarbonate (580 mg, 7.00 mmol), sodium dithionate (2.10 g, 12.0 mmol) and 10 mL of ethyl acetate were added under nitrogen. The mixture was stirred at 0 °C for 5 min and another 100 mL of cooled ethyl acetate was added. The mixture was stirred for another 30 min at 0 °C, then for 50 min at room temperature. Ethyl acetate was separated and aqueous layer was extracted with 120 mL of ethyl acetate. The combined ethyl acetate extract was washed with 900 mL of 5% sodium bicarbonate solution, dried over sodium sulfate and distilled on a rotary evaporator to give the product (152 mg, 42% yield) as a yellowish solid. Deairated petroleum ether was added immediately to prevent the oxidation. R_f = 0.30 ($CHCl_3$/MeOH/Et_3N), UV (λ_{max}); 348 nm.

Complexation of [Nva^2,Pip^3]-TRH-CDS with HPBCD (2-hydroxypropyl-β-cyclodextrin). Saturated [Nva^2,Pip^3]-TRH-CDS in HPBCD/water solution was prepared by addition of HPBCD/water (30%, w/w) into the excess amount of [Nva^2,Pip^3]-TRH-CDS followed by ultrasonication at 10 °C for one hour. To minimize the decomposition of [Nva^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 absorbance peak height at 355 nm with UV spectrometer.

Pharmacology: Effect of [Nva^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 μL/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 tail. Ten minutes 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. Group of 6-7 animals were used for testing each compound. The students' t-test was used for the statistical analyses.

Results and Discussion

Synthesis. Since the stepwise elongation of the peptide chain from Fmoc-Pip-O-resin with activated ester of amino acid resulted in diketopiperazine formation,[20] [Nva^2,Pip^3]-TRH-CDS was prepared by pentapeptide-single amino acid



Scheme 1. Synthesis of DHT-ProProGlnNvaPip-OCh.

(5 + 1) segment-coupling approach. As shown in Scheme 1, two individual segments, NicProProGlnNva-OH and H-Pip-OCh, were prepared by the combined solid phase peptide synthesis (SPPS) and solution phase synthesis. The main fragment of the peptide chain, NicProProGlnNva-OH, was prepared by SPPS using an automated Fmoc chemistry protocol. The DIC mediated coupling was accelerated with HOBt. After completion of stepwise elongation, the crude peptide of NicProProGlnNva-OH (**3**) was cleaved off from the resin with TFA and recrystallized from DCM/hexane mixture.

The two fragments, NicProProGlnNva-OH (**3**) and H-Pip-O-Cholesterol (**4**), were assembled with DCC/HOBt system in DCM to give the crude product of **5**, which was purified by column chromatography on neutral alumina with 5% MeOH/DCM eluent. Methylation of the pyridine ring (**5**) with dimethyl sulfate gave **6** in good yield. Finally, reduction of the trigonelloyl group with sodium dithionite gave the desired 1,4-dihydro product (**7**).

Pharmacology. The antagonism of barbiturate-induced sleeping time in mice was used to assess the activation effect on mice cholinergic neurons of CNS delivered [Nva^2,Pip^3]-TRH-CDS, [Nva^2]-TRH-CDS, and [Leu^2]-TRH-CDS. In mice, ten minutes after iv administration of vehicle (30% hydroxypropyl-β-cyclodextrin, 1.5 mL/kg) or drug solution (10 μmole/kg) in the tail vein, 60 mg/kg of pentobarbital was ip 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 [Nva^2]-TRH was 94.2 ± 2.5

Table 1. A comparison of the pentobarbital induced sleeping time in mice after the administration of vehicle and compounds. Ten min after iv injection of the compound (10 μmole/kg)[a], pentobarbital, 60 mg/kg, was ip injected in the animal. The sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex was regained[b,c]

Compounds	Sleeping time (min)
Vehicle	100.5 ± 6.3
Pyr-Nva-Pro-NH_2	78.2 ± 4.7*
DHT-Pro-Pro-Gln-Nva-Pip-O-cholesterol	41.4 ± 2.6*
DHT-Pro-Pro-Gln-Nva-Pro-Gly-O-cholesterol	49.8 ± 7.7*
DHT-Pro-Pro-Gln-Leu-Pro-Gly-O-cholesterol	50.8 ± 2.5*

[a]TRH 10 mmol = 3.7 mg. [b]A 30% hydroxypropyl-β-cyclodextrin solution was used as vehicle. [c]Six to seven Swiss Webster mice (30 ± 3 g) were used in each group. Table entries are mean ± SE. *$p < 0.05$ when compared to vehicle control using student t-test.

min and 79.2 ± 4.8 min respectively. In comparison with these referenced compounds, [Nva^2,Pip^3]-TRH-CDS showed a significant decrease in sleeping time (41.4 ± 2.6 min), which also imply better activity than the corresponding [Leu^2]-TRH-CDS and [Nva^2]-TRH-CDS (50.8 ± 2.5 min and 49.8 ± 7.7 min respectively). These results indicated that the replacement of Leu residue with Nva didn't change activity, but the replacement of Pro-Gly residue with Pip residue significantly increases the activity. In conclusion, the present study demonstrated the successful delivery of [Nva^2,Pip^3]-TRH-CDS to the brain, and an effective release of the active GlnNvaPip in the brain after the oxidative conversion of the dihydrotrigonellyl (DHT) to the trogonellyl (T^+) moiety, followed by removal of the cholesteryl function and cleavage of T^+-Pro-Pro by prolyl endipeptidase .

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