Metabolism-Based Brain-Targeting System for a Thyrotropin-Releasing Hormone Analogue†

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Gln-Leu-Pro-Gly, a progenitor sequence for the thyrotropin-releasing hormone (TRH) analogue [Leu^2]TRH (pGlu-Leu-Pro-NH_2), was covalently and bioreversibly modified on its N- and C-termini (by a 1,4-dihydrotrigonellyl and a cholesteryl group, respectively) to create lipoidal brain-targeting systems for the TRH analogue. The mechanism of targeting and the recovery of the parent peptide at the target site involve several enzymatic steps, including the oxidation of the 1,4-dihydropyridine moiety. Due to the lipid insolublity of the peptide pyridinium conjugate obtained after this reaction, one of the rudimentary steps of brain targeting (i.e., trapping in the central nervous system) can be accomplished. Our design also included spacer amino acid(s) inserted between the N-terminal residue of the progenitor sequence and the dihydrotrigonellyl group to facilitate the posttargeting removal of the attached modification. The release of the TRH analogue in the brain is orchestrated by a sequential metabolism utilizing esterase/lipase, peptidyl glycine α-amidating monooxygenase (PAM), peptidase cleavage, and glutaminyl cyclase. In addition to in vitro experiments to prove the designed mechanism of action, the efficacy of brain targeting for [Leu^2]TRH administered in the form of chemical-targeting systems containing the embedded progenitor sequence was monitored by the antagonistic effect of the peptide on the barbiturate-induced anesthesia (measure of the activational effect on cholinergic neurons) in mice, and considerable improvement was achieved over the efficacy of the parent peptide upon using this paradigm.

Introduction

The majority of hydrophilic peptides have poor access to the central nervous system (CNS); they do not cross the blood−brain barrier (BBB) due to their poor lipid solubility and due to the absence of specific transport or delivery systems present in the membrane.[1] Administration of only large doses of peptides produces neuropharmacological effects. Their metabolic instability also implies that highly active neuropeptide-degrading enzymes,[2] such as the capillary-bound aminopeptidases, endopeptidases, angiotensin-converting enzyme (ACE), etc., represent an additional "enzymatic BBB". Therefore, the use of a targeting approach is highly justified, when centrally active peptides are considered as potential neuropharmaceuticals.

Three strategies have been used to achieve drug targeting: (1) development of site-specific, pharmacologically active agents; (2) preparation of pharmacologically inactive agents that are activated preferentially at the site of action; and (3) use of biological carrier systems that selectively direct drugs to the specific site of the body.[3] When targeting peptides into the CNS, the first approach focuses only on (mostly elusive) receptor specificity and avoids implementing measures to overcome the BBB and, therefore, is of limited usefulness. Carrier-based strategies (3) also sustain several limitations (e.g., inadequate transport capacity, high cost, etc.) in brain delivery of small peptides.[1] A prodrug or related approach (2) may be considered primarily. Recently, an opioid peptide (a Leu-enkephalin analogue) has been successfully transported into the CNS[4] by using a specific prodrug (chemical-targeting system) approach based on retrometabolic design.[5] In this method, a redox-targeting moiety (1,4-dihydrotrigonellyl, **T**) is conjugated to the N-terminus of the peptide via a spacer amino acid and a lipophilic ester is anchored to the C-terminus. The nonpeptidic, lipophilic groups furnish lipid solubility to the molecule and also protect the embedded peptide from peptidases; thus, penetration through the BBB to the CNS is facilitated. Sequential metabolism, starting with the enzymatic oxidation of the dihydropyridine in the brain, affords retention ("lock-in") of the peptide conjugate and, then, the release of the pharmacologically active neuropeptide analogue at the site of action. The principles of this method can be adapted to other neuropeptides with free amino- and carboxyl-termini.[6]

TRH, pGlu-His-Pro-NH_2, is the first hypothalamic releasing factor characterized, establishing the fundamental proof for the existence of a neuroendocrine regulation of pituitary functions by hypothalamic neuronal structures.[7,8] A variety of behavioral effects are induced by peripheral and central application of TRH, such as increased locomotor activity, "wet dog" shakes, and forepaw tremor.[9,10] The most interesting and best-known CNS effect of this neuropeptide is the analeptic

† Abbreviations: DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; HOBt, 1-hydroxybenzotriazole; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; MeOH, methanol; TFA, trifluoroacetic acid; PG, propylene glycol; DIEA, *N*,*N*-diisopropylethylamine; TEA, triethylamine; DMSO, dimethyl sulfoxide; Nic, nicotinoyl; **T**$^+$, trigonellyl or *N*-methylnicotinoyl; **T**, 1,4-dihydrotrigonellyl.

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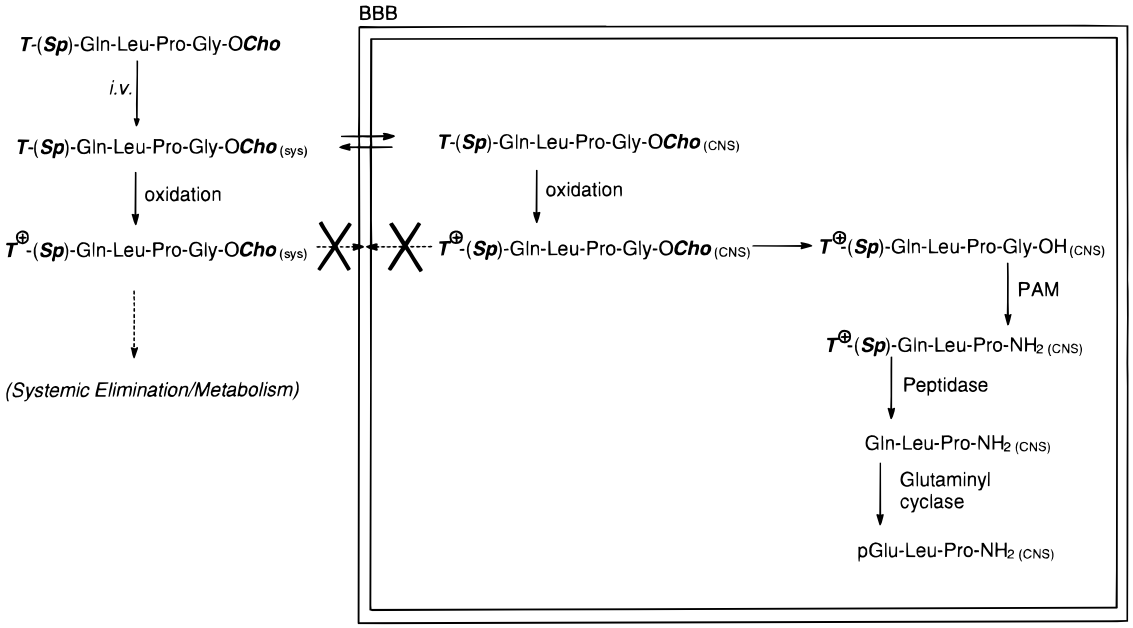
Scheme 1. Retrometabolic Design for CNS Targeting of [Leu^2]TRH[a]



[a] Where **T** is a 1,4-dihydrotrigonellyl-type targeting group, $\mathbf{T}^+$ is trigonellyl, **Sp** represents spacer amino acid residue(s), and **Cho** is cholesteryl.

action manifested by the reduction of barbiturate narcosis or haloperidol-induced catalepsy.[11,12] TRH and its analogues can also increase extracellular acetylcholine (ACh) levels, accelerate ACh turnover, improve memory and learning, and reverse the reduction in high-affinity choline uptake induced by lesions of the medial septal cholinergic neurons.[13−15] Therefore, these peptides have been indicated to be beneficial for treating motor neuron diseases,[16] spinal cord trauma,[17] and Alzheimer's disease.[18,19] Various analogues of TRH have been synthesized to separate the endocrine effect (i.e., TSH-releasing activity) from the CNS effects.[20−23] Several compounds have been described having enhanced CNS activity, but decreased or absent TSH-releasing action, which is a desired feature concerning potential treatment of CNS disorders (analogues where [His^2] is replaced by an aliphatic amino acid residue, such as by leucine, isoleucine, or norvaline,[23] are characteristic representatives of agents meeting this requirement). However, TRH and its analogues have poor access to the CNS.[24]

TRH analogues modified only at the His^2 residue[23] typify peptides, where no free N-terminal amino and C-terminal carboxy groups are available for apparent covalent modification. These peptides, therefore, convey problems to the design of a targeting system due to the presence of the amino-terminal pGlu residue and of the prolinamide at the carboxy-terminus. The solutions to these problems have been derived from considering the biosynthesis of TRH, which involves posttranslational cleavage of a large precursor polyprotein[25] synthesized on ribosomes. This precursor, consisting of 123 amino acid residues, contains multiple sequences of Gln-His-Pro-Gly.[26] The TRH progenitor sequence is flanked by dibasic residues that are typical sites of processing by hormone secretase enzymes in polyproteins.[27,28] Peptidyl glycine α-amidating monooxygenase (PAM) converts the C-terminal glycyl residue to an amide.[29,30] The final peptide is designed to develop from a precursor, Gln-His-Pro-NH_2. During the posttranslational processing of precursors of many secretory proteins in the endoplasmic reticulum, Golgi, or secretory vesicle, "unmasking" of an N-terminal glutamine is actually a signal causing its enzymatic cyclization to pyroglutamyl by glutaminyl cyclase.[31]

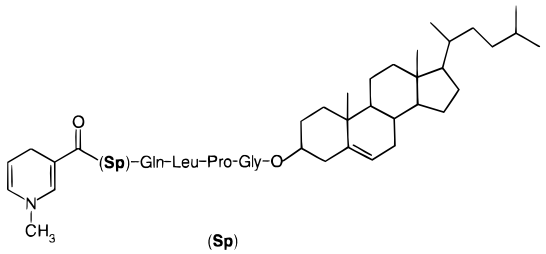
The aim of the present study is to report the synthesis and evaluation of chemical CNS-targeting systems for a centrally active TRH analogue, [Leu^2]TRH. Our design is based on the hypothesis that CNS delivery of a progenitor sequence embedded in an appropriate chemical−enzymatic-targeting system will eventually lead to the formation of the target peptide via specific enzymes inherently present at the site of action. We focused our effort to evaluate the approach by examining the occurrence and relative rates of sequential metabolic processes presumed to be involved in the formation of the specific TRH analogue in the brain. We also assessed the improvement, compared to the unmodified peptide and partially modified compounds, based on a CNS-specific effect of the target molecule.[11,23]

Results

Design. We have postulated that TRH analogues modified at the second amino acid residue can be obtained from a Gln-Xaa-Pro-Gly progenitor sequence (where Xaa is an unspecified amino acid residue). Because pGlu-Leu-Pro-NH_2 was found to exert a 2.5-fold increase in CNS activity[23] and also showed decreased TSH-releasing properties compared to TRH, we have synthesized and evaluated chemical-targeting systems based on a retrometabolic design for this CNS-active TRH analogue.[32]

In the approach selected for brain targeting (Scheme 1), a 1,4-dihydrotrigonellyl targeting group (**T**) is attached to the N-terminal Gln via spacer amino acid(s) (**Sp**). The 1,4-dihydrotrigonellyl undergoes an enzymatically mediated oxidation ($\mathbf{T} \rightarrow \mathbf{T}^+$) resulting in a hydrophilic, membrane-impermeable trigonellyl ($\mathbf{T}^+$)

Chart 1. CDSs of [Leu^2]TRH



	(Sp)
13a	Ala
13b	Ala-Ala
13c	Ala-Pro
13d	Pro
13e	Pro-Pro
13f	Pro-Ala

salt whose efflux from the brain is greatly reduced; thus, it is retained in the CNS ("lock-in"). The lipophilic cholesteryl (**Cho**) moiety on the C-terminus is removed by lipases/esterases further enhancing the lock-in.[33] Then, the peptide conjugate with the liberated Gly on the C-terminus produces the corresponding proline amide[29,30] followed by a cleavage that removes the **T**$^+$-**Sp** moiety releasing Gln-Leu-Pro-NH_2, the immediate precursor for pGlu-Leu-Pro-NH_2. The role of **Sp** in this design is to facilitate or control this cleavage via appropriate enzymes, dipeptidyl dipeptidases[34] (EC 3.4.14.2 and/or EC 3.4.14.5)[34] or proline oligopeptidase (POP, formerly known as postproline cleavage enzyme, EC 3.4.21.26),[35,36] that have specific cleaving sites (primarily after Pro) in a peptide sequence. (Theoretically, POP may also cleave after Pro^3 of the embedded progenitor sequence in **T**$^+$-(**Sp**)-Gln-Leu-*Pro*-Gly-OH. However, since N-terminal elongation of the sequence results in decreased specific rate constants,[37] this reaction is not expected to be competitive with the amidation catalyzed by PAM.) Although dipeptidyl peptidase and POP cleave primarily after Pro in the proper position,[25−27] the Pro residue may be substituted with Ala.[34,35] However, this replacement is accompanied by a decreased affinity of the substrate to the respective enzyme. To show that the expected production of Gln-Leu-Pro-NH_2 and, after formation of [Leu^2]TRH from this immediate precursor, the pharmacological effect in the CNS are associated with the given enzymatic reaction, chemical-targeting systems containing Ala as single- and double-residue spacers were also synthesized as controls for pharmacological evaluation.

Synthesis. The brain-targeting systems (**13a**−**f**, Chart 1) were obtained via stepwise elongation of the peptide chain from the direction of the C-terminus utilizing Boc chemistry in solution phase. The synthetic procedure consisted of a series of coupling/deprotection steps. Briefly, the first step involved the preparation of the cholesteryl ester of the C-terminal Gly (**1**). The intermediate peptide esters (**2**−**11**) were synthesized by DCC/HOBt activation. In the final coupling nicotinic acid was used. After each step, column chromatographic purification was applied. The Nic-terminated peptide cholesteryl esters (**11a**−**f**) were alkylated on the pyridine ring with Me_2SO_4, and the obtained pyridinium salts (**12a**−**f**) were reduced with sodium dithionite[38] to the corresponding peptide brain-targeting systems (**13a**−**f**) for [Leu^2]TRH. Other peptide conjugates important in this study were obtained by routine solid-phase peptide synthesis (SPPS) protocols.

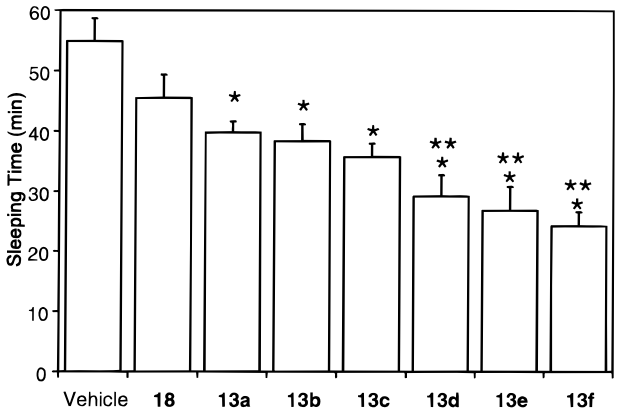


Figure 1. Comparison of barbiturate-induced sleeping times in mice after the administration of the vehicle control, [Leu^2]-TRH (**18**, in vehicle), and **13a**−**f**. Ten min after iv injection of the compound (15 μmol/kg of body weight), pentobarbital (60 mg/kg, ip) was injected. Sleeping was recorded as the time elapsed from the onset of the loss of the righting reflex until the reflex was regained. The statistical significance of differences between groups was determined using analysis of variance (ANOVA) followed by post hoc Fisher's PLSD test for multiple comparisons: *$p < 0.05$ vs vehicle control, **$p < 0.05$ vs [Leu^2]TRH.

Analeptic Activity. We used pharmacological responses[11,23] to assess the enhanced brain-targeting of this CNS-active peptide. The antagonism on the barbiturate-induced anesthesia should indicate the extent of the activation of cholinergic neurons[15] and, thus, the successful central delivery. The results shown in Figure 1 indicate that, compared to the vehicle control (54.9 ± 3.8 min), [Leu^2]TRH (**18**) only decreased the sleeping time (45.5 ± 3.8 min, a 17 ± 7% change) slightly, while a significant ($p < 0.05$) decrease was observed after the administration of the chemical-targeting systems (**13a**−**f**). Compared to **18**, peptide conjugates with the 1,4-dihydrotrigonellyl (**T**) attached to a Pro residue showed the most significant enhancement, with $p < 0.05$, of analeptic effect in both the dipeptidyl dipeptidase- and the POP-based releases of the TRH analogue. Compound **13d** with Pro, **13e** with Pro-Pro, and **13f** with Pro-Ala as the spacer portion of the chemical-targeting systems have resulted in a decrease of sleeping time to 29.2 ± 3.5 min (47 ± 6% change compared to the vehicle control), 26.8 ± 4.0 min (55 ± 7%), and 24.3 ± 2.3 min (56 ± 4%), respectively.

We have also studied the analeptic effect of the partially derivatized [Leu^2]TRH analogues to demonstrate, again, the need for a bulky, highly lipophilic moiety (**Cho**) together with the 1,4-dihydrotrigonellyl (**T**) unit for successful brain-targeting of the parent peptide, as shown in Figure 2. Other lipophilic compounds such as Boc-Gln-Leu-Pro-Gly-OCho (**4**, one of the synthetic intermediates containing the full progenitor sequence), as well as **T**$^+$-Pro-Pro-Gln-Leu-Pro-Gly-OCho (**12e**) and **T**-Pro-Pro-Gln-Leu-Pro-Gly(-OH) (**15**), did not show significant decrease in sleeping time under the same experimental conditions that we used for testing the analeptic activity of **13a**−**f**.

In Vitro Stability and Metabolism. To prove that the release of the parent peptide in the brain after the systemic administration of **13a**−**f** was according to the designed sequential metabolism (Scheme 1), a series of in vitro experiments were performed by using the

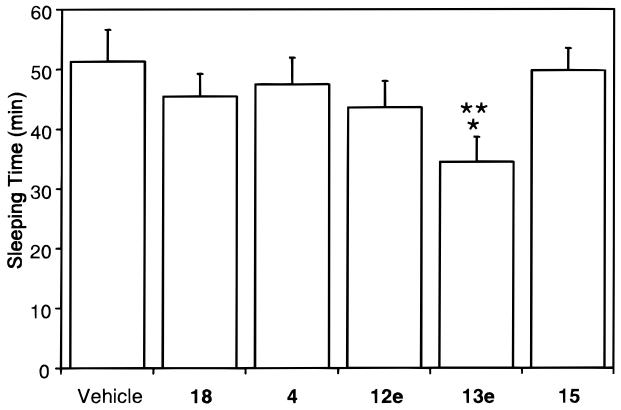


Figure 2. Comparison of barbiturate-induced sleeping times in mice after the administration of the vehicle control, **18**, **4**, **12e**, **13e**, and **15** under the same experimental conditions as given in Figure 1.

appropriate synthetic peptide conjugates as substrates. Because of the limited weight of organs and tissue that can be obtained from mice, brain, liver, and blood were obtained from rats for these studies. The analeptic effect of **13a** and **13b** was similar in rats[32] and in mice (present study); therefore, we considered the mechanism of action in the two rodent species similar in principle.

By analogy of brain targeting of a [Leu^5]enkephalin analogue,[4] our studies focused on the sequential metabolic conversion of the intermediate **T**$^+$-(**Sp**)-Gln-Leu-Pro-Gly-OH obtained after the oxidation of **T** and the subsequent rapid removal of the C-terminal ester function. One of the critical steps in the sequential metabolism is the prerequisite for a rapid conversion of the (Pro-)Gly carboxy-terminus to prolinamide in the brain. In vitro studies on the synthetic **T**$^+$-Ala-Gln-Leu-Pro-Gly-OH (**14a**, *m*/*z* 605) as a model compound were used to confirm this hypothesis. Electrospray ionization (ESI) mass spectrometric (MS) analyses have revealed that the conversion of this peptide conjugate to **T**$^+$-Ala-Gln-Leu-Pro-NH_2 (**16a**, *m*/*z* 547) is only significant in brain tissue.[39] In blood and liver, the major peptide-metabolizing tissues in the rest of the body, degradation occurs primarily by removal of the C-terminal Gly that yields **T**$^+$-Ala-Gln-Leu-Pro-OH (*m*/*z* 548). The latter could only provide further inactive metabolites. The metabolism of **14a** also is slower in the liver than in the brain, and the compound is relatively stable in plasma (less than 10% of degradation in 2 h). Thus, the systemic formation of the parent peptide (**18**) is practically prevented. Consequently, this CDS also has mechanistic aspects that are characteristic to enzymatic activation preferentially at the target site, because PAM processing of the intermediate (formed postdelivery by the loss of cholesteryl from the C-terminal portion of the peptide conjugate) is significant only in the brain.

The subsequent release of Gln-Leu-Pro-NH_2 (**17**), the immediate precursor of the TRH analogue, involves a "designed-in" peptidase cleavage. The half-lives of **T**$^+$-(**Sp**)-Gln-Leu-Pro-Gly-OH (**14**) intermediates in brain homogenate at pH 7.40 and 37 °C were in the 20−40 min range. Therefore, the **T**$^+$-(**Sp**)-Gln-Leu-Pro-NH_2 (**16**) intermediates, which have half-lives ranging from about 1 to 4 h, should be considered the "depot" forms in the process of sequential metabolism in the brain. ESI-MS studies (including tandem mass spectrometric

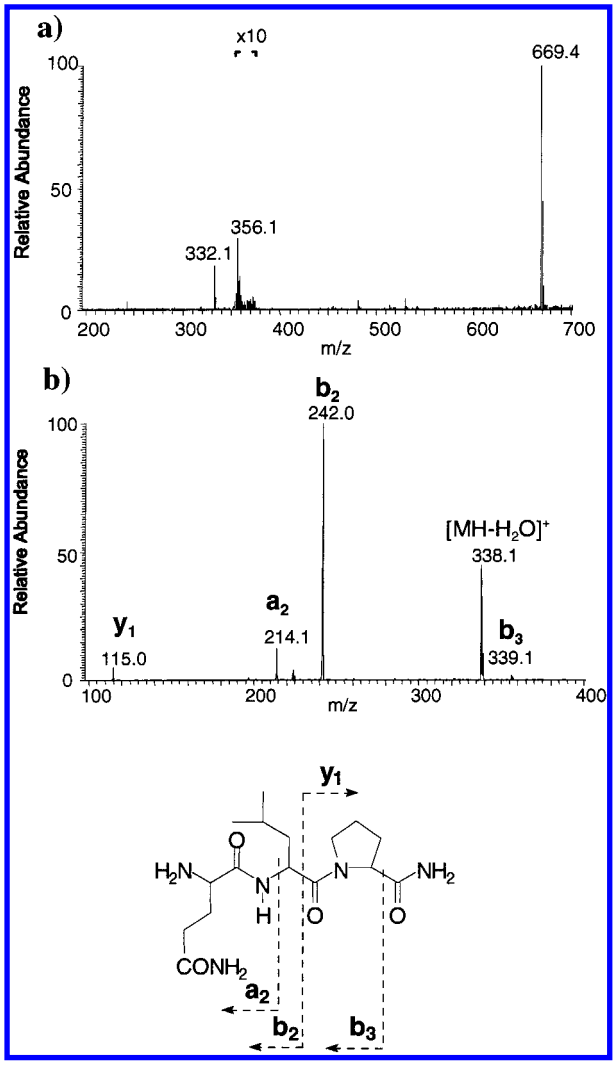


Figure 3. (a) ESI mass spectrum demonstrating the formation of the metabolites **T**$^+$-Pro-Pro-OH (T$^+$-PP, *m*/*z* 332) and Gln-Leu-Pro-NH_2 (**17**, *m*/*z* 356; 10 times magnification in the mass range indicated) from **T**$^+$-Pro-Pro-Gln-Leu-Pro-NH_2 (**16e**, *m*/*z* 669) in rat brain homogenate (incubation: 37 °C, 30 min). (b) MS/MS (product ions) of *m*/*z* 356 showing characteristic fragment ions of Gln-Leu-Pro-NH_2 (according to the nomenclature introduced by Roepstorff and Fohlman[45]), which are identical to those of the synthetic peptide **17**.

(MS/MS) confirmation of the structure of the key compounds) on the in vitro metabolism of **T**$^+$-Pro-Pro-Gln-Leu-Pro-NH_2 (**16e**), where POP is the apparent processing enzyme, provided unequivocal evidence to the specific peptidase cleavage leading to the release of **17** (Figure 3). The occurrence of this enzymatic reaction was verified by incubating the peptide conjugate both in rat brain homogenate and in pH 7.4 HEPES buffer containing 25 μg/mL recombinant porcine brain POP (JM-105 strain). On the basis of the mass spectrometric analysis and the kinetic profiles obtained for the metabolism of **16e** in brain homogenate and for the POP-catalyzed degradation,[40] respectively, the "designed-in" cleavage of the substrate and the concomitant formation of both hydrolysis products (**T**$^+$-Pro-Pro-OH and Gln-Leu-Pro-NH_2) were found to occur without significant side reactions such as the C-terminal deamidation of **16e** by POP. We observed that, after its formation, deamidation of Gln-Leu-Pro-NH_2 by POP to produce Gln-Leu-Pro-OH did occur, but at a much slower rate

than the intended primary cleavage. These observations were consistent with prior studies about the substrate specificity of POP.[37] Conversion of N-terminal Gln to pGlu by glutaminyl cyclase in the brain, the final step in the sequential metabolism shown in Scheme 1, has been well-documented for peptides containing an N-terminal Gln.[31,41,42] We also observed by using an HPLC/UV assay that **17** (Gln-Leu-Pro-NH_2) was processed very rapidly ($t_{1/2}$ of 0.8 min at 37 °C in 20% brain homogenate) and with high degree of conversion (>70%, based on determining the respective areas-under-the-curves in the kinetic profiles) to **18** ([Leu^2]TRH) in vitro. However, the protonated pGlu-Leu-Pro-NH_2 ([**18** + H]$^+$, m/z 339) was not detected in the ESI mass spectrum of the sample obtained after the incubation of **16e** in the brain homogenate (Figure 3), because ionization of this compound via ESI was hampered by the lack of basic nitrogen necessary for the detection of the compound with an adequate sensitivity by ESI-MS. Evidence for the sequential formation of **18** from **16e** was obtained by an alternative mass spectrometric assay that utilized atmospheric pressure chemical ionization (APCI) to generate protonated molecules and showed high sensitivity in detecting the analyte (**18**).[43] For consecutive first-order reactions that describe the process shown below,

$$\underset{\textbf{(16e)}}{\text{T}^+\text{-Pro-Pro-Gln-Leu-Pro-NH}_2} \xrightarrow{k_1} \underset{\textbf{(17)}}{\text{Gln-Leu-Pro-NH}_2} \xrightarrow{k_2} \underset{\textbf{(18)}}{\text{pGlu-Leu-Pro-NH}_2} \xrightarrow{k_3}$$

the expected concentrations of **17** and **18** in the brain homogenate versus time of incubation without considering side reactions are as follows:

$$c_{(17)} = c_0 \cdot k_1/(k_2 - k_1) \cdot [\exp(-k_1 t) - \exp(-k_2 t)]$$

$$c_{(18)} = c_0 \cdot [k_1 \cdot k_2/(k_2 - k_1)/(k_3 - k_1) \cdot \exp(-k_1 t) + k_1 \cdot k_2/(k_3 - k_2)/(k_1 - k_2) \cdot \exp(-k_2 t) + k_1 \cdot k_2/(k_2 - k_3)/(k_1 - k_3) \cdot \exp(-k_3 t)]$$

where c_0 is the initial concentration of **16e** (30 μM) and k_1 (0.012 min^{-1}), k_2 (0.9 min^{-1}), and k_3 (0.01 min^{-1}) are the respective first-order rate constants determined separately for the reaction steps indicated. The measured and predicted kinetic profiles for the sequential metabolism of **16e** leading to the formation of pGlu-Leu-Pro-NH_2 (**18**) are summarized in Figure 4. In agreement with the ESI mass spectrum shown in Figure 3a, the concentration of the intermediate Gln-Leu-Pro-NH_2 (**17**) should, indeed, remain low during the incubation based on the calculated $c_{(17)}$ values. The concentration of **18** determined in the brain homogenate by LC−APCI−MS/MS at the given times of incubation and comparison to the calculated $c_{(18)}$ values indicated about 50% overall conversion of **16e** to the pharmacologically active TRH analogue.

Discussion

Our studies on analeptic activities of the compounds synthesized for evaluation have demonstrated that the chemical-targeting systems (**13a**−**f**) produced significant pharmacological response. Comparison with related prodrug forms of the TRH analogue selected has, again, confirmed the rationale of a brain-targeting design for peptides; a lipophilic prodrug without a targeting moiety can efflux from the CNS, and a 1,4-dihydrotrigonellyl-conjugated peptide is not sufficiently lipophilic and/or not camouflaged against neuropeptide-degrading enzymes without the bulky lipid-soluble moiety to enable brain delivery by the passive transport through the BBB. The major goal of the present paper has been to show that a progenitor sequence is a suitable basis for the rational design of brain-targeting systems for peptides that are not amenable to straightforward chemical modifications. In vitro experiments indicated that our model peptide pGlu-Leu-Pro-NH_2 (**18**) was, indeed, formed and released in the brain according to a predictable metabolic pathway (Scheme 1) from its progenitor sequence "inlayed" in an appropriate chemical-targeting system. The bioactivation ultimately leading to the regeneration of [Leu^2]TRH in the brain was also affected predictably (i.e., Pro is preferred in the **Sp**)[44] by the specific substrate properties in the metabolic step utilized to remove the modifying moiety conjugated to the target peptide sequence.

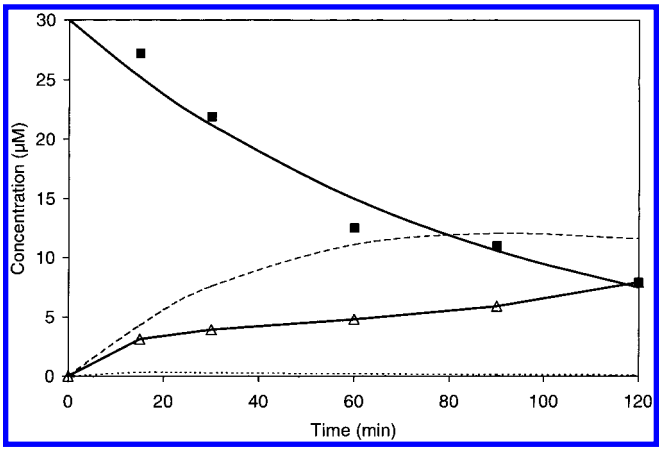


Figure 4. Kinetics of the conversion of **T**$^+$-Pro-Pro-Gln-Leu-Pro-NH_2 (**16e**) to [Leu^2]TRH (**18**) in rat brain homogenate at 37 °C. The concentrations of **16e** and **18** measured in brain homogenate by LC−APCI−MS/MS are shown by the closed squares (■) and the open triangles (Δ), respectively. The predicted concentration−time profile of **17** and **18** for an ideal consecutive process (without side reactions) is given by the dashed and dotted curves, respectively.

In conclusion, CNS delivery of peptides based on covalent conjugation to specific carrier or targeting functions may utilize appropriate progenitors, when coupling of the mature peptide to such functions appears to be cumbersome. Peptides with C-terminal amide and N-terminal pGlu may rely on a Gly-extended sequence and Gln as natural precursors, respectively, within the framework of a rational design approach, as demonstrated here through the example of chemical-targeting systems for pGlu-Leu-Pro-NH_2, a centrally active TRH analogue.

Experimental Section

Instruments and Materials. All chemicals used were reagent grade or peptide synthesis grade. Solvents were obtained from Fisher Scientific (Atlanta, GA). Resins and amino acids were purchased from Bachem BioSci (Torrance, CA). Cholesterol was purchased from Eastman Kodak Co. (Rochester, NY). Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was done on silica gel-coated (Kieselgel 60 F254, 0.2-mm thickness) glass or aluminum plates (EM

Science, Gibbstown, NJ) using the following solvent systems: (a) $CHCl_3$/MeOH (98:2); (b) $CHCl_3$/MeOH (90:10); (c) $CHCl_3$/MeOH (88:12). Column chromatography was performed using Davisil grade 643 silica gel (for compounds **1**−**11**) or neutral alumina (for compound **12**). UV spectra were recorded in MeOH on Cary 210 and Cary 3E UV−visible spectrophotometers (Varian, Walnut Creek, CA). Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA) and the Department of Chemistry, University of Florida (Gainesville, FL). Organic solutions that had been previously extracted with aqueous solution were dried over anhydrous sodium sulfate. A Coupler 1000 (DuPont-VEGA, Wilmington, DE) and Synthor 2000 (Peptide International, Louisville, KY) instruments were applied for solid-phase peptide synthesis (SPPS) using standard Fmoc chemistry for obtaining **T**-(**Sp**)-Gln-Leu-Pro-NH_2 (**16**), Gln-Leu-Pro-NH_2 (**17**), and pGlu-Leu-Pro-NH_2 (**18**). Preparative RP-HPLC purification was performed on a system consisting of a ThermoSeparation/SpectraPhysics (Fremont, CA) SpectraSERIES P200 binary gradient pump, a Rheodyne (Cotati, CA) model 7125 injector valve equipped with a 5-mL loop, and a Spectra 100 UV/VIS detector set at 216 nm. The preparative column (25-mm × 100-mm cartridge with Guard-Pak insert from Waters Chrom) was packed with 15-μm Delta-Pak C_{18}. The mobile phase was mixed from 0.1% (v/v) TFA in H_2O and 0.08% (v/v) of TFA in CH_3CN at a 5.0 mL/min flow rate. Molecular weight of the synthetic compounds was confirmed by ESI mass spectrometry on a Vestec 200 ES instrument (Perspective Biosystem/Vestec, Houston, TX) using MeOH or MeOH/$CHCl_3$ as a solvent (to promote cation attachment as a mechanism of ionization for lipophilic peptide derivatives, the spraying solvent contained ca. 10^{-4} M sodium iodide). All other samples were sprayed from a methanolic solution containing 2% (v/v) acetic acid.

Synthesis. Boc-Gly-OCho (1). A solution of Boc-glycine (24.71 g, 14.11 mmol), DMAP (24.01 g, 196.53 mmol), and DCC (43.73 g, 21.16 mmol) in DCM (165 mL) was cooled to 0 °C with an ice bath. Cholesterol (28 g, 72.41 mmol) dissolved in DCM (100 mL) was added and the mixture was stirred at 0 °C for 30 min, then at room temperature for 24 h. The precipitate (DCU) was filtered off, and the filtrate was washed with citric acid (5%, 5 × 100 mL), saturated sodium bicarbonate (5 × 100 mL), and water (5 × 100 mL). The volatiles were removed in vacuo. The crude product was purified by column chromatography on silica gel (CCSG) with DCM/MeOH (99:1, v/v) eluent affording 29.6 g (yield 75%) of white solid: TLC R_f (a) 0.61; mp 115−116 °C; MS *m*/*z* 567 $[M + Na]^+$. Anal. ($C_{34}H_{57}O_4N \cdot 0.5H_2O$) C, H, N.

General Procedure for the Preparation of Peptide Cholesteryl Esters (Compounds 2−11). The Boc-protected peptide cholesteryl ester, dissolved in DCM, was deprotected with TFA (60 min at room temperature). The volatiles were removed in vacuo. The residue was dissolved in DCM and neutralized with DIEA. A solution of Boc-protected amino acid or, in the final step, nicotinic acid (1.5 equiv, 1.5:1 molar ratio to the cholesteryl ester), HOBt (1.7 equiv), and DCC (2.8 equiv), dissolved in DCM, was added and the mixture was stirred at room temperature for 24 h. The precipitate (DCU) obtained was separated and the filtrate was diluted with DCM and washed with 5% citric acid solution (4×), saturated aqueous sodium bicarbonate (4×), and water (4×). The organic layer was separated and the solvent was removed in vacuo. The residue was purified by column chromatography on silica gel (CCSG).

Boc-Pro-Gly-OCho (2): CCSG DCM/MeOH (99:1, v/v), yield 63%; TLC R_f (a) 0.3; mp 173−175 °C; MS *m*/*z* 664 $[M + Na]^+$. Anal. ($C_{39}H_{64}O_5N_2$) C, H, N.

Boc-Leu-Pro-Gly-OCho (3): CCSG DCM/MeOH (98:2, v/v), yield 75%; TLC R_f (b) 0.65; mp 59−61 °C; MS *m*/*z* 777 $[M + Na]^+$. Anal. ($C_{45}H_{75}O_6N_3 \cdot H_2O$) C, H, N.

Boc-Gln-Leu-Pro-Gly-OCho (4): CCSG DCM/MeOH (93:7, v/v), yield 76%; TLC R_f (b) 0.4; mp 105−107 °C; MS *m*/*z* 905 $[M + Na]^+$. Anal. ($C_{50}H_{83}O_8N_5 \cdot H_2O$) C, H, N.

Boc-Ala-Gln-Leu-Pro-Gly-OCho (5): CCSG DCM/MeOH (92:8, v/v), yield 87%; TLC R_f (b) 0.34; mp 110−112 °C; MS *m*/*z* 976 $[M + Na]^+$. Anal. ($C_{53}H_{88}O_9N_6 \cdot H_2O$) C, H, N.

Boc-Ala-Ala-Gln-Leu-Pro-Gly-OCho (6): CCSG DCM/MeOH (92:8, v/v), yield 87%; TLC R_f (b) 0.25; mp 183−185 °C; MS *m*/*z* 1047 $[M + Na]^+$. Anal. ($C_{56}H_{93}O_{10}N_7 \cdot H_2O$) C, H, N.

Boc-Pro-Ala-Gln-Leu-Pro-Gly-OCho (7): CCSG DCM/MeOH (88:12, v/v), yield 83%; TLC R_f (c) 0.41; mp 135−137 °C; MS *m*/*z* 1073 $[M + Na]^+$. Anal. ($C_{58}H_{95}O_{10}N_7 \cdot 2H_2O$) C, H, N.

Boc-Pro-Gln-Leu-Pro-Gly-OCho (8): CCSG DCM/MeOH (92:8, v/v), yield 75%; TLC R_f (b) 0.35; mp 128−130 °C; MS *m*/*z* 1002 $[M + Na]^+$. Anal. ($C_{58}H_{95}O_{10}N_7 \cdot H_2O$) C, H, N.

Boc-Pro-Pro-Gln-Leu-Pro-Gly-OCho (9): CCSG DCM/MeOH (90:10, v/v), yield 81%; TLC R_f (b) 0.47; mp 137−139 °C; MS *m*/*z* 1099 $[M + Na]^+$. Anal. ($C_{60}H_{97}O_{10}N_7 \cdot 0.5\ H_2O$) C, H, N.

Boc-Ala-Pro-Gln-Leu-Pro-Gly-OCho (10): CCSG DCM/MeOH (90:10, v/v), yield 63%; TLC R_f (b) 0.42; mp 135−137 °C; MS *m*/*z* 1073 $[M + Na]^+$. Anal. ($C_{58}H_{95}O_{10}N_7 \cdot H_2O$) C, H, N.

Nicotinoyl-Ala-Gln-Leu-Pro-Gly-OCho (11a): CCSG DCM/MeOH (90:10, v/v), yield 49%; TLC R_f (c) 0.34; mp 160−162 °C dec; MS *m*/*z* 981 $[M + Na]^+$. Anal. ($C_{54}H_{83}O_8N_{10} \cdot 2\ H_2O$) C, H, N.

Nicotinoyl-Ala-Ala-Gln-Leu-Pro-Gly-OCho (11b): CCSG DCM/MeOH (88:12, v/v), yield 65%; TLC R_f (c) 0.42; mp 169−171 °C dec; MS *m*/*z* 1052 $[M + Na]^+$. Anal. ($C_{57}H_{88}O_9N_8$) C, H, N.

Nicotinoyl-Pro-Ala-Gln-Leu-Pro-Gly-OCho (11c): CCSG DCM/MeOH (88:12, v/v), yield 65%; TLC R_f (c) 0.33; mp 156−158 °C; MS *m*/*z* 1078 $[M + Na]^+$. Anal. ($C_{59}H_{90}O_9N_8 \cdot H_2O$) C, H, N.

Nicotinoyl-Pro-Gln-Leu-Pro-Gly-OCho (11d): CCSG DCM/MeOH (88:12, v/v), yield 71%; TLC R_f (c) 0.36; mp 140−142 °C; MS *m*/*z* 1007 $[M + Na]^+$. Anal. ($C_{56}H_{85}O_8N_7 \cdot 1.5H_2O$) C, H, N.

Nicotinoyl-Pro-Pro-Gln-Leu-Pro-Gly-OCho (11e): CCSG DCM/MeOH (86:14, v/v), yield 94%; TLC R_f (c) 0.37; mp 150−152 °C; MS *m*/*z* 1104 $[M + Na]^+$. Anal. ($C_{61}H_{92}O_9N_8 \cdot H_2O$) C, H, N.

Nicotinoyl-Ala-Pro-Gln-Leu-Pro-Gly-OCho (11f): CCSG DCM/MeOH (83:17, v/v), yield 68%; TLC R_f (c) 0.51; mp 146−147 °C; MS *m*/*z* 1078 $[M + Na]^+$. Anal. ($C_{59}H_{90}O_9N_8 \cdot 1.5H_2O$) C, H, N.

General Procedure for the Preparation of T^+-(Sp)-Gln-Leu-Pro-Gly-OCho 12a−f. Compounds **11a**−**f** (1 equiv) were alkylated in DCM with approximately 15 equiv of dimethyl sulfate at room temperature until the reaction was completed. Then the solvent was removed in vacuo and the solid residue obtained was washed with ether. The crude products were recrystallized from chloroform and ether. The products crystallized with 0.5−1 equiv of alkylating agent.

Trigonellyl-Ala-Gln-Leu-Pro-Gly-OCho (12a): yield 98%; mp 172−174 °C; MS *m*/*z* 973 (Q^+); $UV_{\lambda max}$ 265 nm.

Trigonellyl-Ala-Ala-Gln-Leu-Pro-Gly-OCho (12b): yield 98%; mp 173−175 °C; MS *m*/*z* 1044 (Q^+); $UV_{\lambda max}$ 265 nm.

Trigonellyl-Pro-Ala-Gln-Leu-Pro-Gly-OCho (12c): yield 98%; mp 176−178 °C; MS *m*/*z* 1070 (Q^+); $UV_{\lambda max}$ 265 nm.

Trigonellyl-Pro-Gln-Leu-Pro-Gly-OCho (12d): yield 99%; mp 194−196 °C; MS *m*/*z* 999 (Q^+); $UV_{\lambda max}$ 268 nm.

Trigonellyl-Pro-Pro-Gln-Leu-Pro-Gly-OCho (12e): yield 92%; mp 186−188 °C; MS *m*/*z* 1096 (Q^+); $UV_{\lambda max}$ 264 nm.

Trigonellyl-Ala-Pro-Gln-Leu-Pro-Gly-OCho (12f): yield 98%; mp 181−183 °C; MS *m*/*z* 1070 (Q^+); $UV_{\lambda max}$ 264 nm.

General Procedure for the Sodium Dithionite Reduction (13a−f). Under continuous nitrogen atmosphere the pyridinium salts **12** (1 equiv) were dissolved in 50% (v/v) aqueous MeOH; then the mixture of sodium bicarbonate (12 equiv) and sodium dithionite (10 equiv) was added under ice cooling. After 15 min the cooling was removed and the stirring was continued at room temperature for 40 min under nitrogen atmosphere. Then the reaction mixture was extracted several times with cold, degassed DCM. The combined organics were

washed several times with cold, degassed water, and then the solvent was removed in vacuo. The crude products were purified by column chromatography on neutral alumina with DCM/MeOH (97:3) (v/v).

1,4-Dihydrotrigonellyl-Ala-Gln-Leu-Pro-Gly-OCho (13a): yield 35%; TLC R_f (c) 0.42; mp 138−140 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{55}H_{87}O_8N_7 \cdot 2H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Ala-Ala-Gln-Leu-Pro-Gly-OCho (13b): yield 34%; TLC R_f (c) 0.46; mp 150−152 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{58}H_{92}O_9N_8 \cdot 2.5H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Pro-Ala-Gln-Leu-Pro-Gly-OCho (13c): yield 25%; TLC R_f (c) 0.43; mp 145−147 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{60}H_{94}O_9N_8 \cdot 3H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Pro-Gln-Leu-Pro-Gly-OCho (13d): yield 27%; TLC R_f (c) 0.4; mp 142−144 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{57}H_{89}O_8N_7 \cdot 2.5H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Pro-Pro-Gln-Leu-Pro-Gly-OCho (13e): yield 24%; TLC R_f (c) 0.4; mp 140−142 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{62}H_{96}O_9N_8 \cdot 3.5H_2O$) C, H, N.

1,4-Dihydrotrigonellyl-Ala-Pro-Gln-Leu-Pro-Gly-OCho (13f): yield 21%; TLC R_f (c) 0.45; mp 163−165 °C dec; $UV_{\lambda max}$ 348 nm. Anal. ($C_{60}H_{94}O_9N_8 \cdot 3H_2O$) C, H, N.

Trigonellyl-(Sp)-Gln-Leu-Pro-Gly-OH 14a−f. Componds **12a−f** (200 mg) in methanesulfonic acid (2.8 mL) containing anisole (0.3 mL) were kept at 0 °C for 10 min and at room temperature for 30 min. Then 400 mL of diethyl ether was added. The precipitated amorphous solids were separated, washed with diethyl ether, and dried in vacuo. The solids (identified by MS) were obtained with approximately 95% yield; $UV_{\lambda max}$ 264 nm.

1,4-Dihydrotrigonellyl-Pro-Pro-Gln-Leu-Pro-Gly-OH (15). Compound **14e** (100 mg, 0.14 mmol) was dissolved in degassed distilled water (25 mL) at 0 °C; then the mixture of sodium bicarbonate (236.0 mg, 2.8 mmol) and sodium dithionite (407.4 mg, 2.3 mmol) was added under argon followed by the addition of DCM (20 mL). The mixture was stirred at 0 °C for 20 min and then at room temperature for 40 min. Methylene chloride (30 mL) and 1 g of sodium chloride were added. The layers were separated and the organic layer was washed several times with deaerated water, then evaporated to dryness resulting in 40 mg (yield 40%) of fine yellowish powder: MS *m*/*z* 753 $[M + Na]^+$; $UV_{\lambda max}$ 348 nm; peptide content (spectrophotometric titration with methanolic 0.1 M $AgNO_3$) > 90%.

Trigonellyl-(Sp)-Gln-Leu-Pro-NH_2 (16): prepared by SPPS utilizing standard Fmoc protocol; after purification (RP-HPLC) the white solids obtained were identified by ESI-MS and APCI-MS.

Gln-Leu-Pro-NH_2 (17) trifluoroacetate: semisolid; MS *m*/*z* 356 $[M + H]^+$. Anal. ($C_{18}H_{30}O_6N_5F_3 \cdot 1.5H_2O$) C, H, N.

pGlu-Leu-Pro-NH_2 (18): yellowish semisolid; MS *m*/*z* 339 $[M + H]^+$. Anal. ($C_{16}H_{27}O_4N_4 \cdot H_2O$) C, H, N.

Analeptic Activity. The effect on pentobarbital-induce sleeping times was studied in Swiss Webster mice (Harlan Sprague−Dawley). All animals experiments were conducted in accordance with the guidelines set forth in the *Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals* (DHEW Publication, NIH 80-23). Ten to sixteen Swiss Webster mice (30 ± 2 g) were used in each group. Test compounds were dissolved in a vehicle consisting of PG and DMSO (2:1) (v/v). The vehicle alone (1.5 mL/kg of body weight) was administered to the animals in the control group. Equimolar doses of test compounds including [Leu^2]TRH itself (**18**; 15 μmol/kg of body weight) were injected through the tail vein. After 10 min, each animal received an intraperitoneal (ip) injection of sodium pentobarbital solution at a dose of 60 mg/kg of body weight. The sleeping time was recorded as the time elapsed from the onset of loss of the righting reflex until the reflex was regained. The significance of differences between groups was determined using analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test for multiple comparisons.

In Vitro Stability and Metabolism Studies. Stability studies of selected compounds have been performed in phosphate buffer, plasma, and brain and liver homogenates. Approximately 50 nmol of the compound (dissolved in water) was added to 1 mL of rat plasma or brain or liver homogenate (20%, w/w, in pH 7.4 Tris buffer), respectively, and the mixture was incubated at 37 °C in a temperature-controlled, shaking water bath. For the experiment involving recombinant porcine brain POP (JM-105 strain), the enzyme (25 μg/mL) was dissolved in pH 7.4 HEPES buffer, and the solution was preincubated at 37 °C for 5 min before the addition of the compound studied. Aliquots (100 μL) were removed after 5, 15, 30, 45, 60, and 90 min of incubation, respectively, and transferred into 1.5-mL plastic centrifuge tubes containing 200 μL of ice-cold 1 M aqueous acetic acid. After centrifugation at 12500*g* for 15 min, the supernatant was removed and analyzed by microbore HPLC. Analyses were done on a system consisting of a ThermoSeparation/SpectraPhysics (Fremont, CA) SpectraSERIES P200 binary gradient solvent delivery system, a Rheodyne (Cotati, CA) model 7125 injector valve equipped with a 5-μL sample loop, a Spectroflow 757 variable wavelength UV/VIS detector (Kratos Analytical, Manchester, U.K.) operated at 216 nm, and a Hewlett-Packard model HP 3395 computing integrator (Palo Alto, CA). A 30-cm × 1.0-mm i.d. Supelcosil LC-18 (5-μm) reversed-phase column (Supelco, Bellefonte, PA) was used at a flow rate of 50 μL/min that was maintained by using a dynamic split (before the injection valve) via a balance column (25-mm × 4.6-mm i.d.) connected in parallel with the microbore column. The mobile phase was mixed from 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile, and gradient elution was done from 5% to 95% of organic modifier that changed in a linear profile at a rate of 1%/min. HPLC calibration curves for determining concentrations were obtained by adding known amount of peptide into aliquots of brain homogenate transferred into ice-cold 1 M acetic acid solution and analyzing the supernatant after centrifugation. Concentration−time profiles were analyzed by exponential fitting, assuming a pseudo-first-order degradation. Half-lives ($t_{1/2}$) were calculated from the rate constants (k) as $0.693/k$. Supernatants obtained from samples removed from the incubation mixtures were desalted on reversed-phase cartridges (1-mL Supelclean LC-18, Supelco, Bellefonte, PA) for ESI mass spectrometry. After treating the cartridge by washing with methanol (2 mL) followed by 3% (v/v) aqueous acetic acid (2 mL), the sample was adsorbed. Poorly retained sample components were removed by washing with 3% aqueous acetic acid solution (2 mL), and the analytes were obtained by water/methanol/acetic acid (49/49/2, v/v, 400 μL) as an eluent. The sample solution was supplied for analysis at 3−5 μL/min by syringe pumps fitted with a 100- or 250-μL gastight syringe (Hamilton, Nevada) and connected to the ESI source of a mass spectrometer via fused silica tubing (100-μm i.d.). ESI mass spectra were recorded on a Vestec/PerSeptive Biosystems (Houston, TX) model 200 ES instrument controlled by a Vector/Two data system (Teknivent, St. Louis, MO) and/or on an LCQ (Finnigan MAT, San Jose, CA) quadrupole ion trap controlled by a Microsoft Windows NT-based system (Navigator 1.1, Finnigan MAT). The mass spectra were acquired in the 100−1000 mass-to-charge (*m*/*z*) range. On the LCQ, narrow mass-range scans (ZoomScan; covering an *m*/*z* range of 10 u) were also recorded for the ESI ions of interest in order to achieve the resolution of the isotope peaks. APCI mass spectra were acquired on the LCQ. LC−MS and LC−MS/MS analyses were performed by using a 5-cm × 4.0-mm i.d. C_{18} reversed-phase cartridge (SGE International, Ringwood, Australia). The mobile phase was 25% (v/v) acetonitrile and 2 % (v/v) acetic acid in deionized water, the flow rate was maintained at 1.0 mL/min by a Spectroflow 400 (Kratos Analytical) isocratic solvent delivery system, and 5 μL of the desalted sample solution was injected for analysis.

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Supporting Information Available: Mass spectrometric, LC−MS, and kinetic analyses that show the involvement of PAM, peptidase cleavage, and glutaminyl cyclase in the designed sequential metabolic process leading to the formation of [Leu^2]TRH in rat brain in vitro according to Scheme 1. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Prokai, L. Delivery of peptides into the central nervous system. *Drug Discovery Today* **1996**, *1*, 161−167. (b) Prokai, L. Peptide delivery into the central nervous system: invasive, physiological and chemical approaches. *Exp. Opin. Ther. Patents* **1997**, *7*, 233−245. (c) Prokai, L. Peptide drug delivery into the central nervous system. In *Progress in Drug Research*; Jucker, E. M., Ed.; Birkhäuser: Basel, 1998; Vol. 51, pp 95−131.

(2) (a) Brownless, J.; Williams, C. H. Peptidase, peptides, and the mammalian blood-brain barrier. *J. Neurochem*. **1993**, *60*, 793−803. (b) Bertler, A.; Falck, B.; Owman, C.; Rosengreen, E. The localisation of monoaminergic blood-brain barrier mechanism. *Pharmacol. Rev*. **1966**, *18*, 369−385.

(3) Kumar, V.; Banker, G. S. Target-oriented drug delivery systems. In *Modern Pharmaceutics*, 3rd ed.; Banker, G. S., Rhodes, C. T., Eds.; Marcel Dekker: New York, 1996; p 611.

(4) (a) Bodor, N.; Prokai, L.; Wu, W. M.; Farag, H.; Jonalogadda, S.; Kawamura, M.; Simpkins, J. A strategy for delivering peptides into the central nervous system by sequential metabolism. *Science* **1992**, *257*, 1698−1670. (b) Prokai-Tatrai, K.; Prokai, L.; Bodor, N. Brain-targeted delivery of a Leu-enkephalin analogue by retrometabolic design. *J. Med. Chem*. **1996**, *39*, 4775−4782. (c) Preliminary pharmacokinetic analysis indicated that at least 6 nmol of CDS/g of tissue reached and "locked-in" the brain to reach the 600 pmol/g concentration measured for the immediate precursor of the enkephalin analogue 15 min after injection,[4a] which was about 0.3% of the injected dose. An unusual feature of the brain-targeting CDS is that, because of the involvement of oxidation producing membrane-impermeable ("locked-in") compound(s) in the sequential metabolism, blood pharmacokinetics is not of crucial importance for adequate delivery.

(5) (a) Bodor, N. Retrometabolic approaches to drug targeting. *NIDA Res. Monogr. Ser*. **1995**, *154*, 1−26. (b) Bodor, N. Drug targeting and retrometabolic drug design approaches. *Adv. Drug Del. Rev*. **1994**, *14*, 157−166.

(6) Chen, P.; Bodor, N.; Wu, W.-M.; Prokai, L. Strategies to target kyotorphin analogues to the brain. *J. Med. Chem*. **1998**, *41*, 3773−3781.

(7) Schally, A. V.; Bowers, C. Y.; Redding, T. W.; Barrett, J. F. Thyrotropin releasing factor (TRF) from porcine hypothalamus. *Biochem. Biophys. Res. Commun*. **1966**, *25*, 165−169.

(8) Böler, J.; Enzmann, F.; Folkers, K.; Bowes, C. Y.; Schally, A. V. The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem. Biophys. Res. Commun*. **1969**, *37*, 705−710.

(9) Andrews, J. S.; Sahgal, A. The effects of thyrotropin-releasing hormone, metabolites and analogues on locomotor activity in rats. *Regul. Pept*. **1984**, *7*, 97−109.

(10) Webster, V. A. D.; Griffiths, E. C.; Slater, P. Induction of wet-dog shaking in rats by analogues and metabolites of thyrotropin-releasing hormone (TRH). *Regul. Pept*. **1984**, *5*, 43−51.

(11) Schmidt, D. Effects of thyrotropin releasing hormone (TRH) on pentobarbital-induced decrease in cholinergic neuronal activity. *Commun. Psychopharmacol*. **1977**, 469−473.

(12) Horita, A.; Carino, M.; Zabawska, J.; Lai, H. TRH analogue MK-771 reverses neurochemical and learning deficits in medial septal-lesioned rats. *Peptides* **1989**, *10*, 121−124.

(13) Atweh, S.; Simon, J.; Kuhar, M. Utilization of sodium-dependent high-affinity choline uptake in vitro as a measure of the activity of cholinergic neurons in vivo. *Life Sci*. **1975**, *17*, 1535−1544.

(14) Itoh, Y.; Ogasawara, T.; Mushiori, A.; Yamazaki, A.; Ukai, Y.; Kimura, K. Effect on NS-3, a thyrotropin-releasing hormone analogue, on in vivo acetylcholine release in rat brain: regional difference and its sites of action. *J. Pharmacol. Exp. Ther*. **1994**, *271*, 884−890.

(15) Santori, E.; Schmidt, D. Effects of MK-771, a TRH analogue on pentobarbital-induced alteration of cholinergic parameters in discrete regions of the brain. *Regul. Pept*. **1980**, *1*, 69−74.

(16) Yarbrough, G. Minireview. Thyrotropin releasing hormone and CNS cholinergic neurons. *Life Sci*. **1983**, *33*, 111−118.

(17) Faden, A. I.; Vink, R.; McIntosh, T. K. Thyrotropin-releasing hormone and central nervous system trauma. *Ann. N. Y. Acad. Sci*. **1989**, *553*, 380−384.

(18) Sunderland, A.; Mellow, A.; Cohen, R.; Lawlor, B.; Jill, J.; Newhouse, P.; Cohen, M.; Murphy, D. Acute effects of high-dose thyrotropin releasing hormone infusions in Alzheimer's disease. *Psychopharmacology* **1989**, *98*, 403−407.

(19) Lampe, T.; Norris, J.; Risse, S.; Owens-Williams, E.; Keenan, T. Basic mechanisms, diagnosis and therapeutic strategies. In *Alzheimer's Disease;* Iqbal, K., McLachlan, D. R. C., Winbald, B., Wisniewski, H. M., Eds.; John Wiley and Sons, Ltd.: New York, 1991; p 643.

(20) Yamamoto, M.; Shimizu, M. Effects of a new TRH analogue, YM-14673 on the central nervous system. *Naunyn-Schmiedebergs Arch. Pharmacol*. **1987**, *336*, 561−565.

(21) Friedrichs, E.; Schwertner, E.; Herrling, S.; Gunzler, W.-A.; Ottig, F.; Flohe, L. In *Structure and activity of natural peptides*; Voelter, W., Weitzel, G., Eds.; Walter de Gruyter: Berlin, Germany, 1981; p 461.

(22) Nutt, R. F.; Holly, F. W.; Homnick, C.; Hirschmann, R.; Veber, D. F. Synthesis of thyrotropin-releasing hormone analogues with selective central nervous system effects. *J. Med. Chem*. **1981**, *24*, 692−698.

(23) Szirtes, T. L.; Kisfaludy, L.; Palosi, E.; Szporny, L. Synthesis of thyrotropin-releasing hormone analogues. 1. Complete dissociation of central nervous system effect from thyrotropin-releasing activity. *J. Med. Chem*. **1984**, *27*, 741−745.

(24) (a) Zlokovic, B. V.; Segal, M. B.; Begley, D. J.; Davson, H.; Rakic, L. Permeability of the blood-cerebrospinal fluid and the blood-brain barriers to thyrotropin-releasing hormone. *Brain Res*. **1985**, *358*, 191−199. (b) Zlokovic, B. V.; Lipovac, M. N.; Begley, D. J.; Davson, H.; Rakic, L. Slow penetration of thyrotropin-releasing hormone across the blood-brain barrier of an in situ perfused guinea pig brain. *J. Neurochem*. **1988**, *51*, 252−257.

(25) Jackson, I. Controversies in TRH biosynthesis and strategies towards the identification of a TRH precursor. *Ann. N. Y. Acad. Sci*. **1989**, *553*, 71−75.

(26) Richter, K.; Kawashima, E.; Egger, R.; Kreil, G. Biosynthesis of thyrotropin-releasing hormone in the skin of xenopus laevis: partial sequence of the precursor deduced from cloned cDNA. *EMBO J*. **1994**, *3*, 617−621.

(27) Docherty, K.; Steiner, D. F. Posttranslational proteolysis in polypeptide hormone biosynthesis. *Annu. Rev. Physiol*. **1982**, *44*, 625−638.

(28) Gainer, H.; Russell, J.; Loh, Y. The enzymology and intracellular organization of peptide precursor processing: the secretory vesicle hypothesis. *Neuroendocrinology* **1985**, *40*, 171−184.

(29) (a) Bradbury, A.; Finnie, M.; Smith, D. Mechanism of C-terminal amide formation of pituitary enzyme. *Nature* **1982**, *298*, 686−688. (b) Martinez, A.; Treston A. M. Where does amidation take place? *Mol. Cell. Endocrinol*. **1996**, *123*, 113−117.

(30) Husain, I.; Tate, S. S. Formation of the COOH-terminal amide group of thyrotropin-releasing-factor. *FEBS Lett*. **1983**, *152*, 277−281.

(31) (a) Fischer, W.; Spiess, J. Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutaminyl peptides. *Proc. Natl. Acad. Sci. U.S.A*. **1987**, *84*, 3628−3632. (b) Busby, Jr. W. H.; Quackenbush, G. E.; Humm, J.; Youngblood, W.; Kizer, J. S. An enzyme(s) that converts glutaminyl-peptides into pyroglutamyl peptides. Presence in pituitary, brain, adrenal medulla, and lymphocytes. *J. Biol. Chem*. **1982**, *262*, 8532−36.

(32) Prokai, L.; Ouyang, X.; Wu, W.-M.; Bodor, N. Chemical delivery system to transport a pyroglutamyl peptide amide to the central nervous system. *J. Am. Chem. Soc*. **1994**, *116*, 2643−2644.

(33) (a) Eto, Y.; Suzuki, K. Cholesterol ester metabolism in rat brain. A cholesterol ester hydrolase specifically localized in the myelin sheath. *J. Biol. Chem*. **1973**, *248*, 1986−1991. (b) Johnson, R. C.; Shah, S. N. Cholesterol ester metabolizing enzymes in human brain: properties, subcellular distribution and releative levels in various diseased conditions. *J. Neurochem*. **1978**, *31*, 895−902.

(34) Kenny, A. J.; Booth, A. G.; George, S. G.; Ingram, J.; Kershaw, D.; Wood, E. J.; Young, A. R. Dipeptidyl peptidase IV, a kidney brush-border serine peptidase. *Biochem. J*. **1976**, *157*, 169−182.

(35) Walter, R.; Simmons, W. H.; Yosimoto, T. Proline specific endo- and exopeptidase. *Mol. Cell Biochem*. **1980**, *30*, 111−127.

(36) Rawlings, N. D.; Polgar, L.; Barrett, A. J. A new family of serine-type peptidases related to prolyl oligopeptidase. *Biochem. J*. **1991**, *279*, 907−911.

(37) Polgar, L. Unusual secondary specificity of prolyl oligopeptidase and the different reactivities of its two forms toward charged substrates. *Biochemistry* **1992**, *31*, 7729−7735.

(38) Eisner, V.; Kutha, J. The chemistry of dihydropyridine. *Chem. Rev*. **1972**, *72*, 1−42.

(39) Refer to the Supporting Information (Figure S-1) for ESI mass spectra. Mass resolution sufficient to resolve isotope profiles (due to the presence of the natural ^{13}C, ^{15}N, and ^{18}O isotopes) allowed us to conclude that the product with the carboxamide-terminus

(**16a**) was in large excess compared to the one with the carboxy-terminus upon incubation of **14a** in rat brain homogenate, but it was formed in a small proportion in rat plasma and rat liver homogenate. The ratio of the amide (**16a**, monoisotopic nominal *m*/*z* 547) to the acid (*m*/*z* 548) after 30 min of incubation in brain homogenate was calculated to be 4.3:1, while the ratio in plasma and liver homogenate after 60 min of incubation was 1:6.3 and 1:6.7, respectively.

(40) See Supporting Information (Figure S-2) for details.

(41) Yoo, O.; Powell, C.; Argarwal, K. Molecular cloning and nucleotide sequence of full-length of cDNA coding for porcine gastrin. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1049−1053.

(42) Seeburg, P.; Adelman, J. Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* **1984**, *311*, 666−668.

(43) See Supporting Information (Figure S-3) for chromatogram and mass spectra.

(44) It has been noted that a significant difference in analeptic activity was obtained between the targeting system containing both Pro and Ala as spacer (**13c** and **13f**). Although this observation could not be rationalized based on our prior knowledge about the affinity of the enzymes possibly involved in the cleavage toward the peptide bonds of the respective substrates, its detailed study was beyond the scope of this paper.

(45) Roepstorff, P.; Fohlman, J. Proposal for a common nomenclature for sequence ions in mass-spectra of peptides. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

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