A Pyridinium-substituted Analog of the TRH-like Tripeptide pGlu-Glu-Pro-NH_2 and its Prodrugs as Central Nervous System Agents

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Abstract: A metabolically stable and centrally acting analog of pGlu-Glu-Pro-NH_2 ([Glu^2]TRH, a tripeptide structurally related to TRH (thyrotropin-releasing hormone)) was designed by replacing the amino-terminal pyroglutamyl residue with a pyridinium moiety. The analeptic action of the analog was used to optimize the efficacy of this novel CNS agent when administered intravenously in its CNS-permeable prodrug forms obtained via the reduction of the pyridinium moiety to the nonionic dihydropyridine and esterifying the central Glu with various alcohols. The maximum effect in antagonizing pentobarbital-induced narcosis in mice was achieved with the hexyl ester that was used subsequently for a comparative evaluation with a prodrug of the parent neuropeptide in the Porsolt swim test as a paradigm for antidepressant effect. The novel analog maintained its antidepressant potency but showed reduced analeptic action compared to [Glu^2]TRH; thus, an increase in the selectivity of CNS-action was obtained by the incorporation of the pyridinium moiety.

Key Words: [Glu^2]TRH, central nervous system permeable prodrugs, analeptic effect, antidepressant, Porsolt swim test, immobilized artificial membrane chromatography.

INTRODUCTION

The peptide pGlu-Glu-Pro-NH_2 [1], which is also known as [Glu^2]TRH (**1**) because of its structural similarity to the thyrotropin-releasing hormone (TRH), has been shown to occur in a wide range of mammalian tissues including the brain [2,3]. Levels of **1** increase, similarly to those of TRH (pGlu-His-Pro-NH_2) [4,5], in parallel with behavioral measures of an antidepressant response in various limbic brain regions during electroconvulsive seizures [6,7]. Additionally, **1** (and other TRH-like neuropeptides) has shown neuroprotective [8], locomotor [9,10] and analeptic [11-14] activities. Although these properties are also shared with TRH, the pharmacological responses after treatment with **1** are more robust and/or longer lasting compared to the mostly transient response exerted by TRH. These advantages are probably due, in part, to the increased resistance of **1** to serum enzymes responsible for the rapid degradation of TRH *in vivo* [6,7,15,16]. Moreover, and very importantly when CNS-pharmacotherapy is considered, **1** does not bind to TRH receptors [17] and, thus, does not elevate T_3 levels even at a dose 250-time larger than the effective dose of TRH [18], which indicates that this TRH analog probably exerts pharmacological effects through binding to its yet uncharacterized own receptor. After systemic administration, **1** is rapidly cleared from the circulation in rats, and the entrance of the peptide into the CNS by a yet to be identified transcellular transport mechanism is delayed (by close to two hours) resulting in a small fraction (≤0.005% dose/g wet brain tissue) of the peptide reaching certain brain regions [6].

Research in our laboratory has been focused on improving CNS-targeting of neuropeptides, with special emphasis on TRH and related compounds [19-22], by designing metabolically stable CNS-active analogs or/and improving the CNS-bioavaibility of the target compound *via* CNS-permeable prodrug (CPP) design through bioreversible chemical manipulation. In a broad sense prodrugs can be described as precursors of the parent drugs having no intrinsic activity that undergo enzymatic or/and chemical transformation to regenerate the active agent *in vivo* at the site of action (e.g. in the brain) in a preferably predictable manner. Simple prodrugs contain a covalent link between the drug and the strategically selected chemical/transport ("pro"-) moiety. However, prodrugs are frequently obtained by multiple chemical manipulations; therefore we use the prodrug term for any inactive, bioreversibly modified lead compounds independently from the number and nature of chemical manipulations and pro-moieties, respectively [23].

Peptide **1** is water-soluble and partially ionized at physiological pH on the γ-carboxyl group of the central Glu residue, which hinders its diffusion into the CNS. Therefore, we recently rendered the molecule neutral and enhanced its lipophilicity by bioreversibly masking the γ-carboxyl group through esterification with various alcohols [20] to enhance CNS penetration by passive transport. The analeptic effect, measured as reduction in pentobarbital-induced sleeping time in mice [11,12,19], was used as an experimental indicator of CPP activation and, thus, of CNS efficacy. Among the many esters we prepared, the hexyl ester pGlu-Glu(Hex)-Pro-NH_2 (**1a**) afforded the most promising CPP; it produced a tenfold decrease in ED_{50} compared to that of the unmodified TRH-like peptide **1**.

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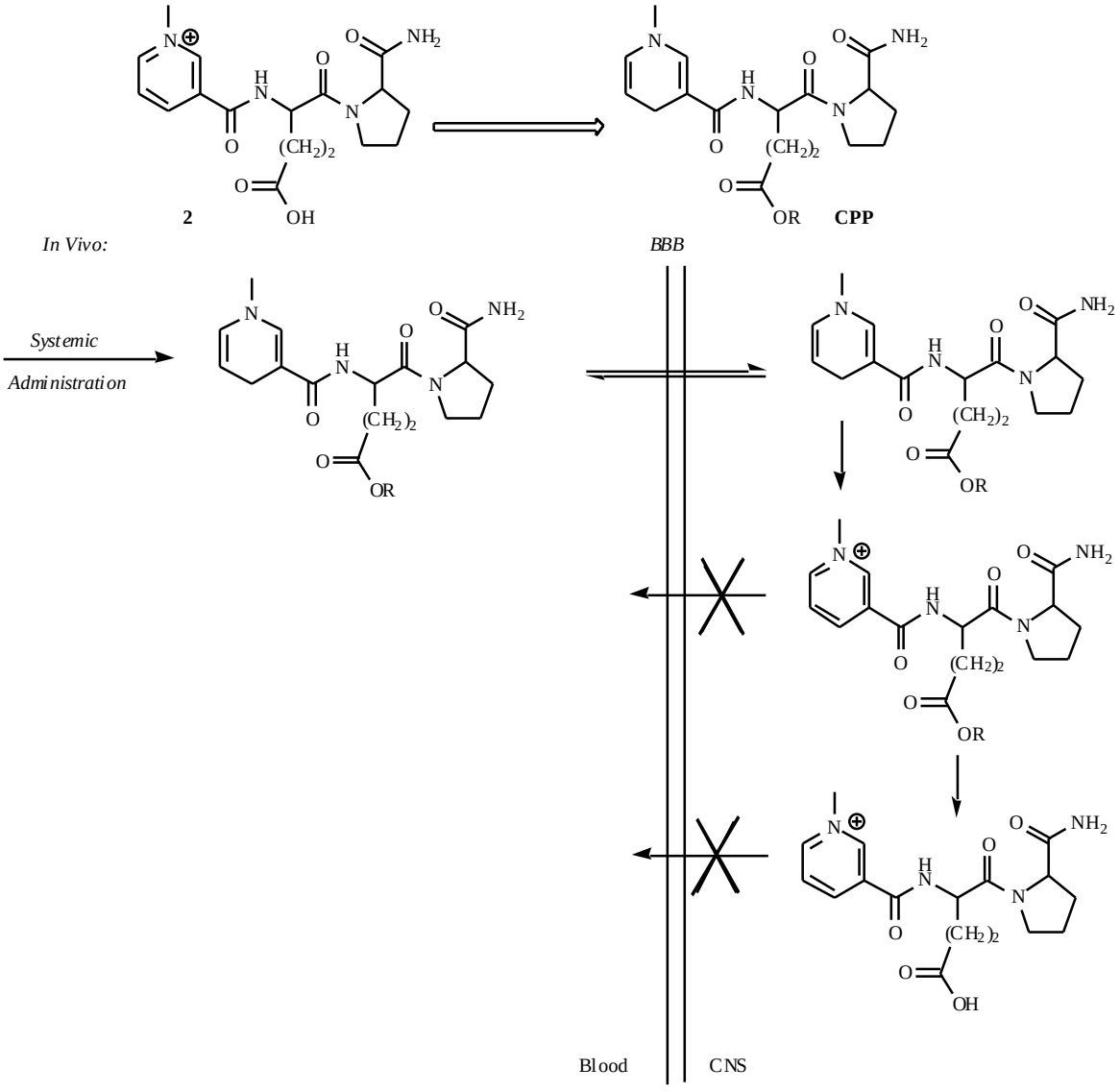
1573-4064/05 $50.00+.00

Our replacement of the central basic histidine in TRH with substituted pyridinium has also produced a metabolically stable, non-endocrine, centrally acting TRH analog [21,22]. Its CPP was obtained upon reducing the pyridinium residue to a neutral dihydropyridine. The analog, when administered intravenously (i.v.) in its dihydropyridine CPP form and formed in the brain after enzymatic oxidation analogously to that of NAD(P)H to $NAD(P)^+$ [24], not only showed TRH-equivalent potency in antagonizing pentobarbital-induced narcosis in mice but also a profoundly longer-lasting effect was measured compared to TRH due to the greatly enhanced metabolic stability.

After evaluating this promising, exclusively CNS-active TRH analog, we became interested in probing whether CPP-amenable pyridinium analog retaining or, perhaps, selectively enhancing certain TRH-related CNS-actions could also be obtained from **1** as a lead compound. Since we had already confirmed [19] that ester prodrugs significantly improved CNS targeting of **1** shown in an analeptic model, we decided to keep the Glu-Pro-NH_2 fragment and replace this time the N-terminus (pGlu) with a pyridinium–specifically with the residue derived from N-methylnicotinic acid (Trg, abbreviated from the trivial name trigonelline, the betaine of N-methylnicotinic acid). Consequently, we perceived Trg-Glu-Pro-NH_2 (**2**) as a prototype for [Glu^2]TRH-derived analogs to be explored as a potential CNS-agent**.** The presence of pyridinium and γ-carboxylic acid of Glu in the molecule allowed for bioreversible "double lipophilization" to obtain CPPs—Dht-Glu(R)-Pro-NH_2 (**3a-g**, where Dht is the 1,4-dihydrotrigonellyl residue) expected to significantly enhance brain targeting of the analog **2** by passive transport. Moreover (as shown in Scheme **1**), once the CPP diffused through the BBB, the Dht to Trg enzymatic oxidation produces ionic compounds (**2** and/or **2a-g** depending on the rate of the ester hydrolysis)



Scheme (1). CPP approach for Trg-Glu-Pro-NH_2 (**2**).

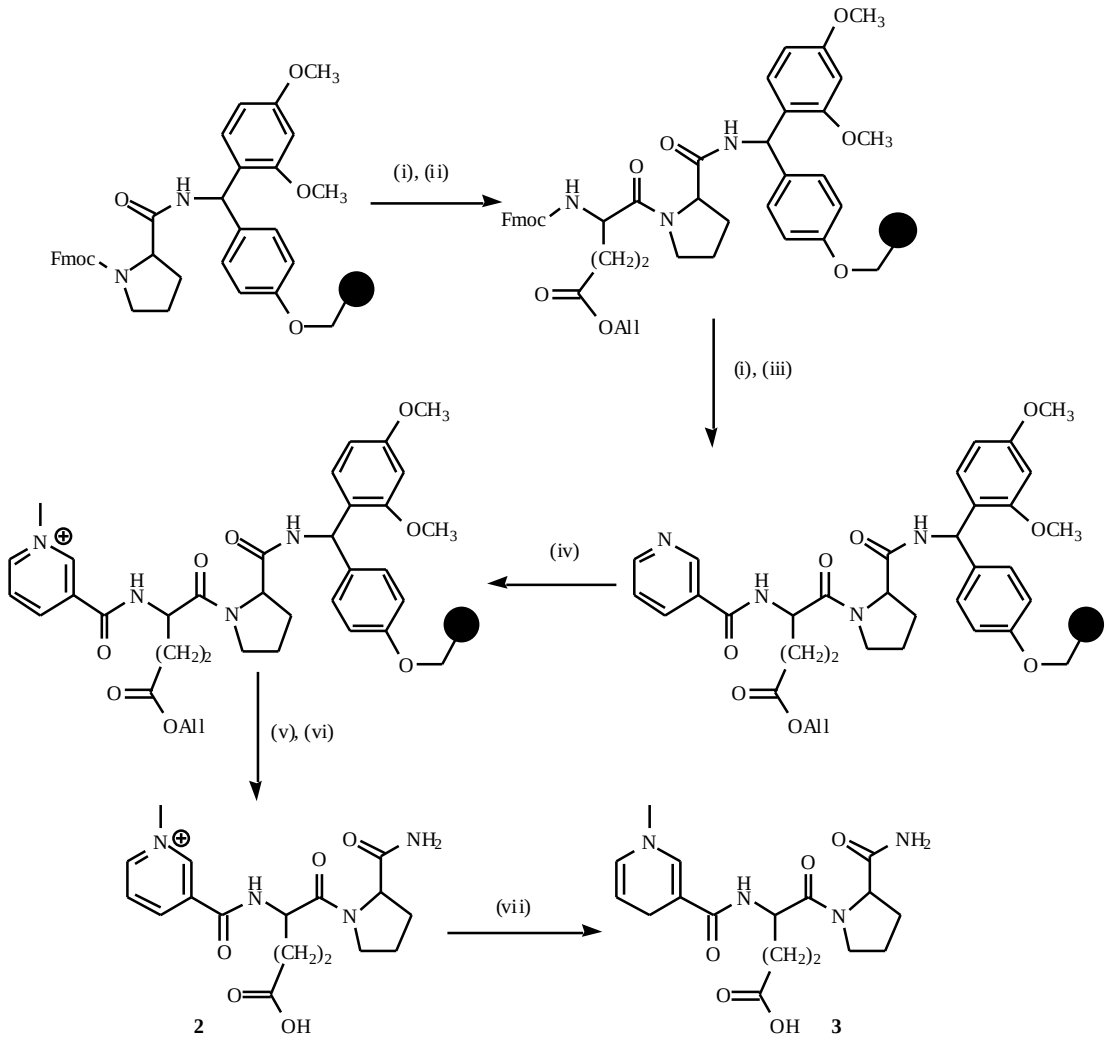
whose efflux from the brain is, therefore, hindered (unless an efficient efflux mechanism for the compounds formed *in situ* is present in the CNS).

Here we report the synthesis and initial neuropharmacological evaluations of the novel type of [Glu^2]TRH analog Trg-Glu-Pro-NH_2 (**2**), when it is administered in CPP forms (**3a-g**) obtained upon bioreversible chemical manipulations of **2** to provide non-ionic and lipophilic properties which are prerequisites for efficient BBB transport. The pharmacological consequences of replacing pGlu with Trg in the "tripeptidic" structure related to TRH were measured upon i.v. administration of **2** in its CPP forms (**3a-g**) by two experimental paradigms: the well-known analeptic potency model [11,12,19] and the Porsolt Swim Test (PST) in rodents for antidepressant potency [25,26]—in comparison with **1a** and TRH as reference agents. A systematic variation of the ester moieties allowed us, again, to find/confirm the optimum for the promoiety to modify the Glu residue and yield efficacious CPPs for an analog of **1**.

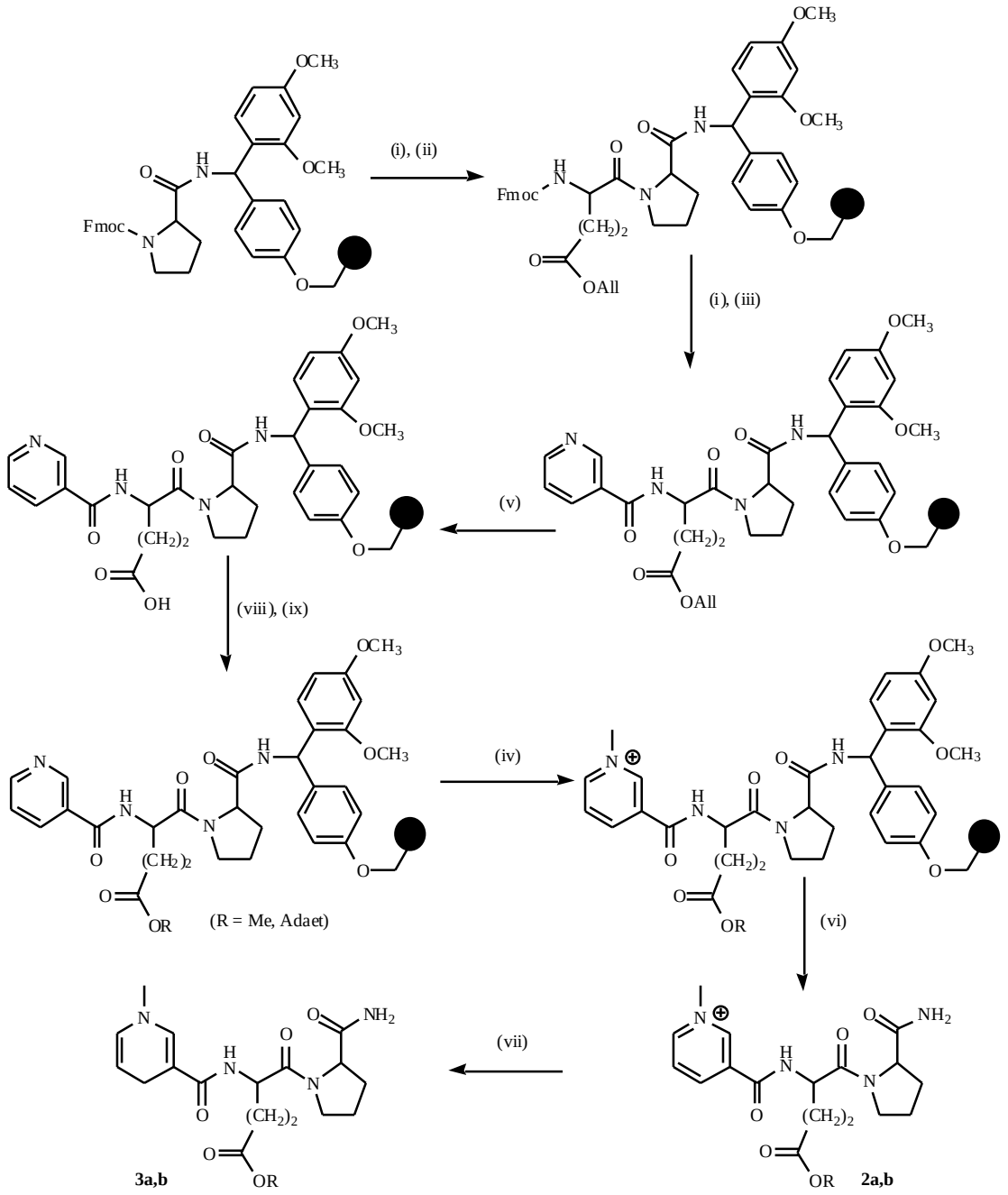
RESULTS

Synthesis

The [Glu^2]TRH analog Trg-Glu-Pro-NH_2 (**2**) as well as its numerous ester derivatives Trg-Glu(R)-ProNH_2 (**2a-g**, where R=Me/methyl, Et/ethyl, nBu/butyl, tBu/tertButyl, Hex/hexyl, Adaet/1-adamantaneethyl, and Bn/benzyl) as synthetic precursors of the corresponding CPPs were prepared by solid-phase chemistry, as shown in Scheme **2**–**4**. Assembly of **2** utilized standard 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide synthesis (Scheme **2**). Esterification on the side-chain of the central Glu was also carried out by solid-phase synthesis. Depending on the ester group, either pre-loaded Fmoc-Pro-Rink Amide-MBHA resin (**2a–c**,



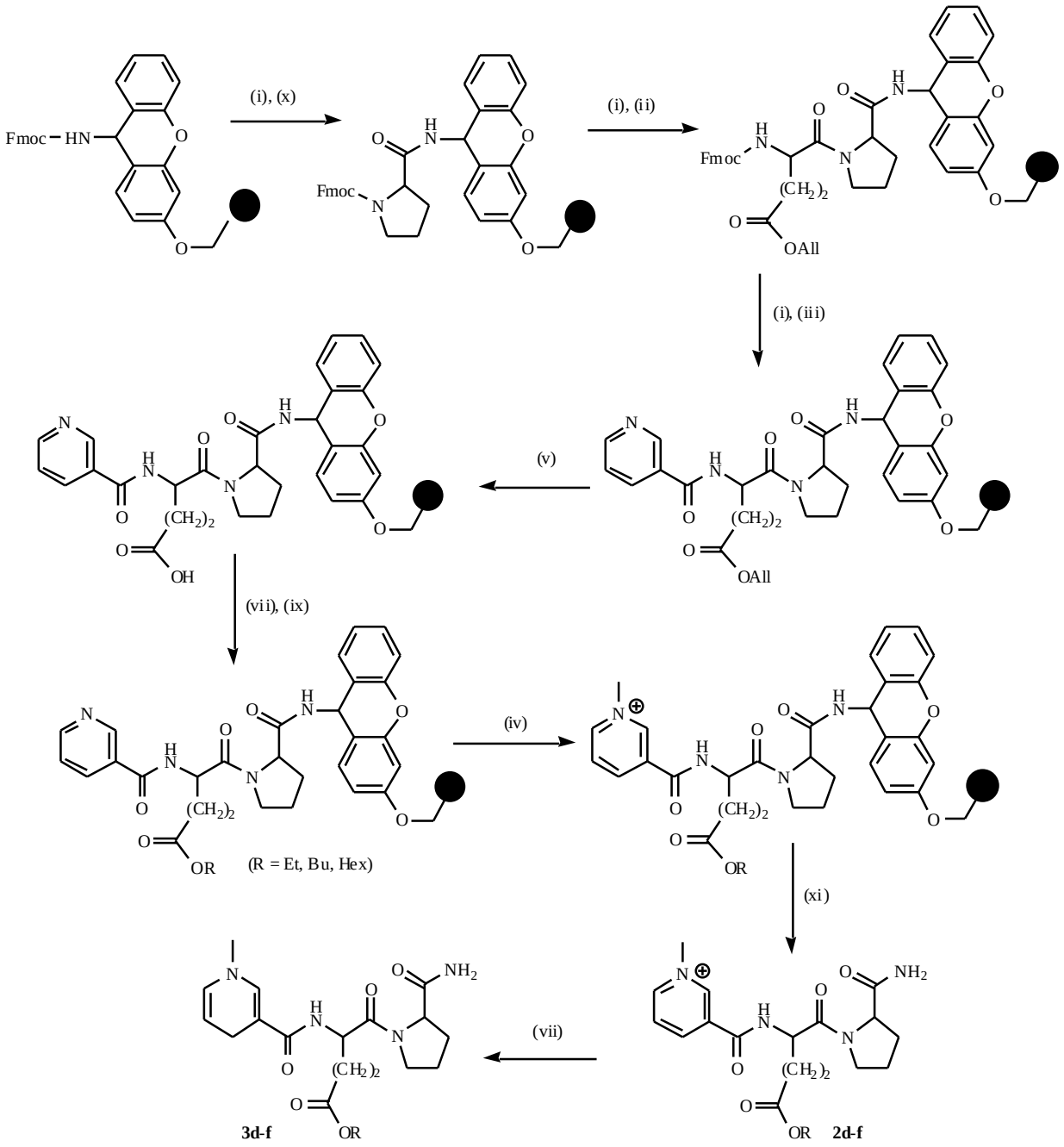
Scheme (2). Synthesis of Trg-Glu-Pro-NH_2 (**2**) and its CPP (**3**): (i) 20% (v/v) piperidine in DMF, 10 min; (ii) PyBOP:HOBt:Fmoc-Glu(All):DIPEA (4:4:4:8 eq); (iii) PyBOP:HOBt:nicotinic acid:DIPEA (4:4:4:8 eq); (iv) MeI (20 eq) in DMF, 2h; (v) $Pd(PPh_3)_4$ (0.1 eq), $PhSiH_3$ (10 eq) in DCM, 3 x 15 min; (vi) TFA:H_2O:triisopropyl silane (95 : 2.5 : 2.5, v/v), 1 h;); (vii) $Na_2S_2O_4$ (10 eq.) in H_2O:MeOH (1:1, v/v), pH 7.



Scheme (3). Synthesis of **2a**,**b** and their CPPs (**3a**,**b**): See Scheme (**2**) for (i)–(vii); (viii) CDI (25 eq) in DMF, 30 min; (ix) ROH, DIPCDI (10 eq), DMAP (1 eq) in DCM, 2 x 30 min.

Scheme **3**) or acid-sensitive Sieber resin (**2d–g**, Scheme **4**) was used in order to optimize the yield, while Glu was protected on its side-chain with allyl (All) group except when R= tBu or Bn (**2g** or **2c**, respectively) was prepared. In the latter two cases, Fmoc-Glu(tBu) or Fmoc-Glu(Bn) was applied and the syntheses were carried out analogously to Scheme **3** and Scheme **4**, respectively.

In short, the pre-loaded Fmoc-Pro-Rink Amide-MBHA or Sieber resin (loaded with Fmoc-Pro in a customary manner) was deprotected with 20% (v/v) piperidine in N,N-dimethylformamide (DMF) followed by coupling to the orthogonally side chain-protected central Glu using phosphonium salt [(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, PyBOP] in the presence of a base (N,N-diisopropylethylamine, DIPEA). The peptide chain was terminated with nicotinic acid before unmasking the central side chain for the esterification. (We preferred to use nicotinic acid followed by N-methylation rather then directly introduce Trg with trigonelline hydrochloride (1-methylpyridinium-3-



Scheme (4). Synthesis of of **2d–f** and their CPPs (**3d–f**): See Scheme (**2**) for (i)–(vii); (x) PyBOP:HOBt:Fmoc-Pro:DIPEA (4:4:4:8 eq); (xi) TFA:DCM (2 : 98, v/v), 3 x 3 min.

carboxylate hydrochloride) in order to improve the efficacy of the coupling reaction). The All protecting group was then removed *via* Pd-catalyzed transfer to a nucleophilic scavenger (phenyltrihydrosilane, $PhSiH_3$) under neutral condition [27]. Esterification was done on the 1,1'-carbonyldiimidazole (CDI)-activated carboxyl group with the corresponding alcohol in the presence of 1,3-diisopropylcarbodiimide (DIPCD) and 4-(dimethylamino)pyridine (DMAP). Once this reaction was complete, the nicotinoyl residue was converted to Trg with methyl iodide (MeI) in DMF.

The esters of the Trg analog (**2a-g**) or the analog (**2**) itself were cleaved from the resin and purified by reversed-phase preparative high-performance liquid chromatography (HPLC) [22]. Identification was based on nuclear magnetic resonance (NMR) and electrospray ionization (ESI) mass spectrometry (MS). The purity of the synthetic precursors of the CPPs was verified by analytical HPLC and combustion analyses.

For the preparation of CPPs, the ionic compounds (**2** or **2a-g**) were reduced on the N-terminal Trg moiety to the

neutral 1,4-dihydrotrigonelline (Dht) with a widely reported and straightforward method using sodium dithionite [23] or polymer-supported borohydride [22]. The progress of the reduction was monitored by HLPC, UV spectroscopy (254 nm for Trg and 355 nm for 1,4-Dht) and atmospheric-pressure chemical ionization (APCI) MS.

Membrane Affinity

A measurement for the increase in the ability of the neutral CPPs compared to their cationic synthetic precursors to interact with biological membranes was done by immobilized artificial membrane chromatography (IAMC) [28] rather than by measuring or *in silico* predicting logP [22]. ESI and APCI mass spectrometry [31-33] were employed for the detection of the Trg compounds (**2**, **2a**–**g**) and their corresponding CPPs (**3**, **3a**–**g**), respectively (Table (**1**)). Dht moiety, indeed, improved the affinity of the prodrugs to membrane lipids compared to the ionic synthetic precursors by approximately three- to five-fold; however, the simple Dht-prodrug (**3**; R=H) still showed poor affinity to IAM. Esterification further increased membrane affinity, as clearly shown in Table (**1**), due to the increased lipopholicity and decreased hydrogen-bonding capacity of the resultant compounds. In agreement with our earlier finding [20], the Hex ester (**3f**) yielded more than 200-fold increase in k'_{IAM} compared to that of **3** having unmasked γ-carboxyl group of Glu (k'_{IAM} of 106.8 and 0.38, respectively). On the other hand, Adaet ester **3b** showed extremely strong attraction to IAM (i.e., too "liphophilic") under the chromatographic conditions we applied for the other CPPs.

In Vitro Stability Studies

Confirmation of prodrug activation by enzymatic oxidation and ester hydrolysis was done *in vitro* in mouse brain homogenate (20% w/v) and in freshly collected whole blood. One of the key steps in this process is the oxidation of Dht to Trg by specific enzymes after the neutral CPP has crossed the BBB to hinder efflux from the brain [23]. Once this oxidation occurred, the Trg-containing compound cannot re-cross the BBB due to its ionic character. At the same time, a rapid clearance of the oxidized species (**2**, **2a**-**g**) is expected from the periphery [23].

While the oxidation occurred rapidly ($t_{1/2}$ around 6 min in brain versus around 16 min in heparinized mouse blood) a slower hydrolysis of the ester groups was determined. This latter may imply a sustained formation of **2** from its oxidized esters (**2a**-**g**); that may be beneficial when long-lasting neuropharmacological effects are desired. Specifically, the half-lives were between 20-30 min for the esters of the primary alcohols, while **2c** (R= Bn) and **2g** (R= tBu) were quite stable ($t_{1/2}$ >70 min), thus, they are expected to be much less efficacious prodrugs than the other CPPs in our experimental models. We observed similarly long *in vitro* brain-stability for the same esters of **1** [20], which may imply that arylalkyl- and tertiary alcohol-derived esters are not "preferred" by esterases. Furthermore, because **1** is metabolically stable compared to TRH, it was expected that its Trg analog (**2**) presented in this study also retained this property. Indeed, we found that less than 10% degradation of **2** occurred in biological media over a period of 120 min.

Analeptic Effects

The best-documented effect of TRH is its analeptic action manifested by the reduction of barbiturate narcosis or haloperidol-induced catalepsy and mediated primarily by a cholinergic mechanism [29,30]. Similarly, [Glu^2]TRH (**1**) also exerts a robust analeptic effect, although its potency as an analeptic agent is less than that of TRH [31]. We explored the antagonism on the barbiturate-induced anesthesia to survey the potency of the new analog (**2**) as a CNS-agent administered upon i.v. administration to the animals in its CPP forms.

As shown in Fig. (**1**), the most efficacious prodrugs in this experimental paradigm were **3f** (Hex), **3e** (nBu) and **3b** (Adaet)—the primary alcohol-derived esters that have also shown high k'_{IAM} values and, thus, strong membrane affinity. Among them, **3f** produced the largest decrease in sleeping time, analogously to that of **1a** obtained in our earlier study [20]. This observation indicated that, while bringing the lipophilicity of the CPP into the range preferred for optimum BBB transport by diffusion (logP ~ 2) [32] was advantageous, there was no benefit from an extreme "lipophilization" of the molecule by making the Adaet (**3b**) ester of the γ-carboxylic group of Glu. Additionally, despite

Table 1. IAMC Capacity Factors (k'_{IAM}) for Trg-Glu(R)-ProNH$_2$ (2, 2a-g) and Dht-Glu(R)-ProNH$_2$ (3, 3a-g).

Compound	k'_{IAM}	Compound	k'_{IAM}
2 (R= H)	0.1	**3** (R= H)	0.4
2a (R= Me)	0.7	**3a** (R= Me)	3.0
2b (R= Adaet)	>199	**3b** (R= Adaet)	n/d
2c (R= Bz)	8.8	**3c** (R= Bz)	15.0
2d (R= Et)	1.1	**3d** (R= Et)	5.3
2e (R= But)	4.8	**3e** (R= But)	10.2
2f (R= Hex)	28.6	**3f** (R= Hex)	107
2g (R= tBu)	4.6	**3g** (R= tBu)	14.0

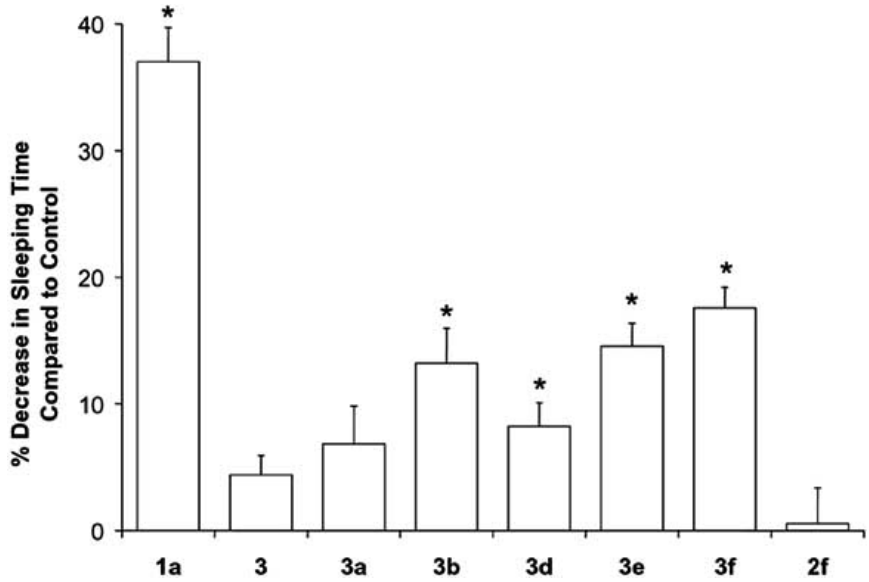


Fig. (1). Analeptic effects measured in mice at equimolar doses of 10 μmol/kg body weight after the i.v. injection of **1a**, **3** and the CPPs indicated. *Statistically significant difference from control (ANOVA followed by Dunnett's test, $p < 0.05$, N=6–10).

the enhanced membrane affinity of **3c** (R= Bn) and **3g** (R= tBu) as shown in Table (**1**), there was no reduction in the pentobarbital-induced sleeping time compared to that of control, when these CPPs were administered (data not shown). Based on the long (>70 min) half-lives for ester hydrolysis in the brain *in vitro*, esterases apparently did not remove Bn or tBu fast enough from **2c** and **2g**, respectively, to establish pharmacologically significant concentration of the target analog **2**. This observation implicated that the presence of free γ-carboxylic group of Glu is essential for the manifestation of analeptic action of the molecule. On the other hand, it was also apparent that **2** did not reach the potency of **1** in this neuropharmacological model.

In a proof-of-concept experiment regarding our CPP design, we compared the potency of the "best-performing" Hex ester CPP (**3f**) with its synthetic precursor counterpart (**2f,** Fig. (**1**)). Although Hex significantly enhanced membrane affinity compared to that of **2** (see Table (**1**)), **2f** was ineffective when administered i.v. due to its inability to cross the BBB because of its ionic nature (Trg).

Porsolt Swim Test

Because the analeptic action in mice was used to select the most efficacious CPP for CNS-targeting of **2**, Dht-Glu(Hex)-Pro-NH_2 (**3f**) as an "optimized" molecular architecture to deliver the analog through the BBB was chosen for screening **2** in the PST—also in mice. At equimolar (3 μmol/kg) doses, we compared **3f** to TRH and **1a** (Table (**2**)). In order to allow for easier comparisons, data were expressed as percentage decrease in immobility time. Immobility time of the control group (saline injection) was counted as 100%. To validate our experimental conditions, we also administered amitriptiline, a tricyclic antidepressant drug [33] and found 59 ± 2 % decrease in the immobility time compared to that of saline.

Interestingly, the hexyl ester we found to be an "optimum lipophilizer" on the γ-carboxyl group of Glu for making CPPs of both **1** [20] and its [Trg^1]- analog (**2**) reported here (i.e., **1a** and **3f**, respectively) produced an identical and statistically significant decrease in the immobility time (44 ± 2% and 43 ± 3%, respectively) compared to that of control.

Table 2. Comparison of Antidepressant and Analeptic Effects of Selected Compounds in Mice After Systemic (i.v.) Administration.

Compound	PST: % Decrease in Immobility[a]	Analeptic Effect: % Decrease in Sleeping Time[a]
TRH	36* ± 2	50* ± 2
1a	44* ± 2	37* ± 3
2f	2 ± 1	1 ± 3
3f	43* ± 3	18* ± 2

[a]Compared to saline control as 100%; *Statistically significant difference from control (ANOVA followed by Dunnett's test, $p < 0.05$, N=6–16).

At the same time, the analeptic effect of the latter was significantly less than that of **1** (Fig. (**1**)). As usual, we also injected (i.v.) the Trg counterpart of the CPP (**2f**) at an equimolar dose as a second control, and did not record any antidepressant-like response in the PST.

DISCUSSION

TRH and its endogenous analogs (e.g., **1**) are intriguing neuropeptides due to their multi-faceted action in the CNS. In order to dissociate endocrine activity (if present) from CNS-actions and to improve metabolic stability, discovery of analogs/mimetics or related small molecules that not only retain TRH-related neuropharmacological responses but are also suitable for adequate CNS-targeting due to their more favorable physicochemical properties is of great interest [34]. If these properties (e.g., enhanced lipophilicity, lack of charge, etc.) are not inherently present in the molecular architecture of the novel entities, bioreversible chemical manipulations may then be applied, which essentially endow the target compound with properties preferential for BBB transport. Therefore, prodrug approaches have become an integral part of neuropeptide-based drug design and discovery [23,35-38].

The present paper focuses on a novel agent derived from the non-endocrine analog structurally related to TRH ([Glu^2]TRH; **1**). The design of Trg-Glu-Pro-NH_2 (**2**) was based on our previous experience involving innovative neuropeptide-based CPP approaches [19,21,22,39,40] utilizing a pyridinium moiety that is either covalently attached to the target neuropeptide to create CPP upon reducing the pyridinium to dihydropyridine, or is strategically incorporated into the sequence of the neuropeptide to produce a CPP-amenable analog. Additionally, properly selected and placed bioreversible lipophilizers further adjust the physicochemical properties of the molecular entity to enhance BBB transport via diffusion.

In the sequence of **2**, the N-terminal pGlu of **1** was replaced with Trg moiety that allowed for an instant CPP creation upon reducing Trg to the neutral and more lipophilic Dht (**3**). The presence of central Glu, however, called for further masking to avoid the detrimental effect of the occurrence of ionization on its γ-carboxyl group at psychological pH. Esterifcation of this group assured "neutrality" and, at the same time, adequate lipophilicity–prerequisites for efficient BBB transport.

Analog **2** as well as several ester derivatives (**2a**-**g**) as synthetic precursors for the corresponding CPPs were prepared by solid phase chemistry (Scheme **2**). From the Trg-containing intermediates, the CPPs can be obtained by straightforward reduction to Dht. Analogously to the oxidation of NAD(P)H to $NAD(P)^+$ after passing the BBB [23], Dht apparently oxidizes back to Trg, which restricts the elimination from the brain due to the ionic nature of the compound formed. Therefore, Trg-containing entities maintain pharmacologically significant concentrations at the site of action for a prolonged period of time. *In vitro* assessment of this crucial step in mouse brain homogenate and whole blood indicated that the longer $t_{1/2}$ of CPPs in blood compared to that of in brain, the more beneficial the CNS-sequestration of the oxidized species is after systemic administration. Enzymes remove the ester group during or after BBB transport completing the CPP to **2** conversion; however, we found that hydrolysis of **2c** (R=Bn) and **2g** (R=tBu) to **2** occurred at least two- to three-fold slower (half-lives >70 min) than that of other esters derived from primary alcohols.

An assessment of the membrane affinity of the compounds was done by IAMC (Table (**1**)). The measured k'_{IAM} chromatographic capacity factors clearly and consistently showed an enhanced attraction of the CPPs to the immobilized lipid when compared to the corresponding cationic synthetic precursors (which also represented the expected *in vivo* metabolites of the CPPs). IAMC measures the partitioning into monolayers of cell membrane phospholipids immobilized by covalent binding on silica particles and mimics membrane interactions better than partitioning in the isotropic n-octanol/water system. The k'_{IAM} chromatographic capacity factor obtained for a compound is directly related to its partition coefficient between the aqueous phase and the chemically bonded membrane phase and, ultimately, to the K_m value representing its fluid membrane partition coefficient. This technique has been applied for the assessment of penetration across the BBB for structurally diverse drugs including peptide analogs [41-45].

The CNS-targeting of **2** by CPPs was tested by two neuropharmacological (analeptic and PST) models. The maximum effect for antagonizing pentobarbital-induced narcosis in mice was achieved with **3f** [Dht-Glu(Hex)-Pro-NH_2]. Predictably, CPPs having long *in vitro* half-lives for ester hydrolysis were ineffective in this experimental model (Fig. **1**).

CPP **3f** was further used for a comparative evaluation of the CNS-targeted analog (**2**) with TRH and **1a** (Hex ester of **1**) in the PST in mice. This novel analog afforded reduction in the immobility time identical to that of **1** (around 43–44% decrease compared to that of control), when both were administered systemically (i.v.) in their Hex ester CPP forms (**3f** and **1a**, respectively) at equimolar doses. Additionally, both CPPs outperformed TRH in the test employed. PST is an established behavioral paradigm to screen compounds for antidepressant activity and potency [24,25]. For the evaluation, rodents (rats or mice) are put singly in an inescapable cylinder of water for a given period of time, and the time (duration) that they swim until remain floated (immobile) is measured. Immobility is considered to reflect a "depressive mood." In the control group, animals are injected with saline only. Reduction in the duration of immobility time by the test compound compared to that of saline-injected animals is considered an antidepressant-like effect.

Table (**2**) summarizes our initial neuropharmacological evaluation involving **2**-a prototype for pyridinium-containing, CPP-amenable and metabolically stable [Glu^2]TRH-analogs as potential CNS agents. Data indicating analeptic and antidepressant potencies of **2** were presented and compared to those of TRH and **1**. When **2** and **1** were administered as properly optimized CPPs (**3f** and **1a**, respectively), they

performed identically in the PST, but **3f** produced at the same time only half of the analeptic effect of **1a**. Therefore, Trg-Glu-Pro-NH_2 (**2**) may represent a centrally active TRH analog with improved selectivity of its CNS-action and a lead for a novel type of antidepressants.

EXPERIMENTAL

Instruments, Materials and Methods

All chemicals used were reagent grade or peptide synthesis grade. Solvents were obtained from Fisher Scientific (Atlanta, GA). Fmoc-Pro-Rink-MBHA and Sieber resins (loading: 0.5 mmol/g) as well as amino acids, TRH, [Glu^2]TRH (**1**) were purchased from Bachem BioSciences (Torrance, CA). Polymer-supported (Amberlyst A-26) supported borohydride (loading: 2mmol/g BH_4), nicotinic acid and methyl iodide were purchased from Aldrich Chem. Co. (Milwaukee, WI). Hexyl ester of [Glu^2]TRH (**1a**) was prepared as reported before [21]. UV spectra were recorded in methanol on a Cary 3E UV-Visible Spectrophotometer (Varian, Walnut Creek, CA). The peptides were purified on a preparative RP-HPLC system consisting of a Thermo-Separation/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 220 nm. The semi-preparative reversed-phase column (Waters RCM DeltaPack C18, 250 mm x 100 mm i.d.) was obtained from Waters (Hesperia, CA). 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 3.0 ml/min flow rate. Analytical HPLC was performed using a Hewlett Packard series 1050 chromatography system with a Thermo Hypersil-Keystone Betabasic-8 (100mm x 4.6 mm) 5-μm column, with UV detection at 220nm. Depending on the chemical nature of the analyte, ESI (**2**, **2a**–**g**) or APCI ionization (**3**, **3a**–**g**) methods were employed for the compounds reported here. Mass spectra were obtained by a quadrupole ion trap instrument (LCQ, ThermoFinnigan, San Jose, CA, USA) equipped with Xcalibur v1.3 software. Full-scan mass spectra were acquired from *m/z* 200 to 800 using the automatic gain control mode of ion trapping. ^{1}H-NMR-spectra were recorded on a Varian XL-300 instrument. The samples were dissolved in D_2O.

General Procedure for Solid Phase Synthesis of 2 and its Esters (2a-g)

Solid phase peptide synthesis utilizing standard Fmoc-chemistry with PyBOP/HOBt/DIPEA activation (where HOBt represents 1-Hydroxybenzotriazole Hydrate) was used manually in polypropylene syringes fitted with polyethylene porous disks. Fmoc-Pro-Rink Amide- MBHA (0.48 mmol/g) resin for **2** and **2a-c** (Trg-Glu(R)-Pro-NH_2; R = H, Me, Adaet and Bn, respectively) and Fmoc-Sieber resin (0.52 mmol/g) for **2d-g** (R = Et, nBu, tBu and Hex, respectively) were used. Solvents and soluble reagents were removed by suction. Washing after each deprotection and coupling was done with DMF (5 x 0.5 min) and dichloromethane (DCM, 5 x 0.5 min) using 10 ml solvent/g resin. Removal of the Fmoc protecting group was done with 20 % (v/v) piperidine in DMF. For the coupling step (including loading the Sieber resin), the corresponding amino acid or nicotinic acid (4 eq), PyBOP (4 eq) and HOBt (4 eq) dissolved in DMF (1-3 ml/g resin) were sequentially added to the resin, followed by DIPEA (8 eq). The mixture was allowed to react with intermittent manual stirring for 2 h. The solvent was then removed by suction, the resin was thoroughly washed and the reaction was monitored with ninhydrin test. If it was necessary, the coupling procedure was repeated again. The orthogonally protected N-α-Fmoc-N-γ-allyl-glutamic acid [Fmoc-Glu(All)] was used to introduce the central residue except for **2g** [Fmoc-Glu(tBu)] and for **2c** [Fmoc-Glu(Bn)]. Nicotinic acid was used for terminating the peptide-like chain. After this final coupling step, the All group was removed with $Pd(PPh_3)_4$ (0.1 eq) and $PhSiH_3$ (10 eq) in anhydrous DCM under nitrogen atmosphere (3 x 15 min). Esterification of the carboxylic group was done in two step; first the carboxylic group was activated with CDI (25 eq) in DMF for 30 min, after which the solvent was removed by suction and the resin was washed. In the second step, the corresponding alcohol (10 eq), DIPCDI (10 eq) and DMAP (1 eq) dissolved in DCM (1-3 ml/g resin) were sequentially added to the resin. The mixture was allowed to react with intermittent manual stirring for 2 X 30 min. N-methylation on the nicotinoyl residue to produce the desired Trg moiety was also done on solid phase using a large excess (>20-fold) of MeI in DMF (1-3 ml/g resin) for 2 h. The target compounds, **2** and its esters were (**2a-g**) removed from the solid support with trifluroacetic acid (TFA) / water/ triisopropyl silane 95 : 2.5 : 2.5 (v/v, 10 ml/g resin) for 1 h when Rink-amide resin was used and with TFA / DCM 2 : 98 (v/v, 10 ml/g resin) for 3 x 3 min when Sieber resin was used. The crude peptides were subjected to RP-HPLC purification.

Trg-Glu-Pro-NH_2 Trifluoroacetate (2)

^{1}H-NMR: δ (ppm) 9.23 (s, 1H, Trg-2); 8.95 (d, J= 6.00 Hz, 1H, Trg-6); 8.86 (d, J= 8.7 Hz, 1H, Trg-4); 8.2 (dd, J= 8.4 and 6.1 Hz, 1H, Trg-5); 4.97-4.92 (m, 1H, H_α Pro); 4.46 (s, 3H, Trg-N^+CH_3); 4.43-4.41 (m, 1H, H_α Glu); 3.96-3.78 (m, 2H, $H_{2\delta}$ Pro); 2.617 (t, J= 7.6 Hz, 2H, $CH_{2\gamma}$ Glu); 2.37-2.17 (m, 2H, $CH_{2\gamma}$ Pro); 2.15-1.90 (m, 4H, $CH_{2\beta}$ Pro and Glu). MS (ESI) m/z (C^+) 363; Anal Calc for $C_{19}H_{23}N_4O_7F_3$ x 2 H_2O: C, 44.53, H, 5.31; N, 10.93. Found C, 44.67; H, 5.11; N, 10.98.

Trg-Glu(Me)-Pro-NH_2 Trifluoroacetate (2a)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 3.71 (s, 3H, CH_3 of Me); MS (ESI) m/z C^+ 377; Anal Calc for $C_{20}H_{25}N_4O_7F_3$ x 0.25 TFA x 1.5 H_2O: C, 45.10, H, 5.22; N, 10.26. Found C, 45.01; H, 5.07; N, 10.39.

Trg-Glu(Adaet)-Pro-NH_2 Trifluoroacetate (2b)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** plus 4.18 (t, J= 6.8 Hz, 2H, $CH_{2\alpha}$ of et of Adaet); 1.88- 1.82 (m, 3H, CH of Ada of Adaet); 1.69-1.56 (m, 6H, CH_2 of Ada of Adaet); 1.54-1.42 (m, 6H, CH_2 of Ada of Adaet); 1.42 (t, J= 7.1 Hz, 2H, $CH_{2\beta}$ of et of Adaet); MS (ESI) m/z C^+ 525; Anal Calc for $C_{31}H_{41}N_4O_7F_3$ x 1.25 H_2O: C, 54.98; H, 6.47; N, 8.27. Found C, 54.77; H, 6.20; N, 8.06.

Trg-Glu(Bn)-Pro-NH_2 Trifluoroacetate (2c)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 7.41-7.37 (m, 5H, aromatic ring of Bn); 5.19 (d, J= 4.5 Hz, 2H, CH_2 of Bn); MS (ESI) m/z C^+ 453; Anal Calc for $C_{26}H_{29}N_4O_7F_3$ x 0. 5 TFA x 0.5 H_2O: C, 51.27; H, 4.86; N, 8.86. Found C, 51.34; H, 4.88; N, 8.81.

Trg-Glu(Et)-Pro-NH_2 Trifluoroacetate (2d)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 4.17 (q, J= 6.9 Hz, 2 H, CH_2 of Et); 1.24 (t, J= 6.7 Hz, CH3 of Et); MS (ESI) m/z C^+ 391; Anal Calc for $C_{21}H_{27}N_4O_7F_3$ x 0.25 TFA x 1.5 H_2O: C, 47.46; H, 5.69; N, 10.35. Found C, 47.44; H, 5.41; N, 10.35.

Trg-Glu(nBu)-Pro-NH_2 Trifluoroacetate (2e)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 4.15 (t, J= 6.9 Hz, 2H, $CH_{2\alpha}$ of nBu); 1.62 (qn, J= 6.8 Hz, 2H, $CH_{2\beta}$ of nBu); 1.36 (qn, J= 7.1 Hz, 2 H, $CH_{2\gamma}$ of nBu); 0.88 (t, J= 7.2 Hz, 3H, CH_3 of nBu); MS (ESI) m/z C^+ 419; Anal Calc for $C_{23}H_{31}N_4O_7F_3$ x 0.25 TFA x 1.25 H_2O: C, 48.75; H, 5.79; N, 9.68. Found C, 48.37; H, 5.83; N, 9.60.

Trg-Glu(Hex)-Pro-NH_2 Trifluoroacetate (2f)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 4.14 (t, J= 6.6 Hz, 2H, $CH_{2\alpha}$ of Hex); 1.63 (qn, 2H, J= 6.6 Hz, 2H, $CH_{2\beta}$ of Hex); 1.35-1.25 (m, 6H, $CH_{2\gamma\text{-}\varepsilon}$ of Hex); 0.83 (t, J= 7.5 Hz, 2H, $CH_{2\zeta}$ of Hex); MS (ESI) m/z C^+ 447; Anal Calc for $C_{25}H_{35}N_4O_7F_3$ x 0.25 TFA x 1 H_2O: C, 51.82; H, 6.36; N, 9.48. Found C, 51.91; H, 6.22; N, 9.49.

Trg-Glu(tBu)-Pro-NH_2 Trifluoroacetate (2g)

^{1}H-NMR: δ (ppm): essentially identical to that of **2** and 1.45 (s, 9H, CH_3 of tBu); MS (ESI) m/z C^+ 419; Anal Calc for $C_{23}H_{31}N_4O_7F_3$ x 0.25 TFA x 0.75 H_2O: C, 49.13; H, 5.75; N, 9.75. Found C, 49.01; H, 5.67; N, 10.08.

General Procedure for the Preparation of CPPs (3 or 3a-g)

Under continuous nitrogen atmosphere and ice-cooling, **2** or **2a-g** was dissolved in 50% (v/v) aqueous methanol and then sodium dithionite (10 eq) was added together with enough sodium bicarbonate to adjust the pH to 6.5-7.0. The progress of the reaction was monitored by UV, MS and HPLC. Once the reduction was complete (usually within 4 h) the reaction mixture was extracted with cold, degassed DCM. The solvent was then removed with a stream of nitrogen and the residual CPP was subjected to analytical HPLC and thin layer chromatographic (TLC) analyses before administering it to the experimental animals.

Alternatively, reduction of the Trg moiety could also be carried out with polymer-supported borohydride; 0.25 eq of borohydride-resin was added to 1 eq of Trg-compound dissolved in degassed methanol under nitrogen stream and ice-cooling. After 6 hrs, the resin was filtered off and the solvent was blown away with a nitrogen stream. The residue was then taken up in cold, degassed DCM and extracted with ice-cold, degassed water. The organic phase was separated, dried and the solvent was blown away with a nitrogen stream. When it was necessary, preparative TLC purification (Silica Gel GF, Analtech, Inc., Newark, DE) was applied [DCM/methanol 9:1 (v/v) eluent]. Pale yellow semi-solids were obtained.

Dht-Glu-Pro-NH_2 (3)

^{1}H-NMR: characteristic resonances for 1,4-Dht (δ, ppm, $CDCl_3$): 7.28 (d, 1H, J=1.6 Hz, 1H, Dht-2), 5.95 (dd, 1H, J= 8.06 Hz and 1.6 Hz, 1H, Dht-6), 4.91–4.84 (unresolved m, 1H, Dht-5), 3.06–2.92 (brs, 2H, Dht-4), 2.94 (s, 3H, DHt-N-CH_3). MS (APCI) *m/z* 365 $[M+H]^+$; UV_{max}: 365 nm.

IAM Chromatography

A 1-cm x 3.0 mm i.d. IAM.PC.DD2 column (Regis Technologies, Morton Grove, IL) was employed. An isocratic solvent delivery (10 mM ammonium acetate adjusted to pH 5.4 with acetic acid) of 1.0 ml/min was provided by a Pharmacia-LKB (Bromma, Sweden) HPLC pump (model 2150). IAMC capacity factors (k_{IAM}') were calculated as follows: $k_{IAM}' = (t_{R(X)} - t_{R([1)} / t_{R(([1)}$, where $t_{R(X)}$ and $t_{R([1)}$ are the retention times for the compound of interest and the void volume marker [Glu^2]TRH (**1**) [21], respectively. ESI and APCI mass spectrometry were used for detection of the compounds [22, 44].

***In Vitro* Metabolic Stability Studies**

Stability studies were performed in freshly prepared mouse brain homogenate and whole blood. Approximately 1 μM of test compound was added to 0.5 ml of brain homogenate (20% w/w, pH 7.4, 100 mM phosphate buffer) or heparinized whole blood and the mixture was incubated at 37^{o}C in a temperature-controlled, shaking water bath. Aliquots (50 μl) were removed after 2, 5, 15, 30, 45, 60 and 90 min of incubation, and transferred into a 1.5 ml Eppendorf tube containing 200 μl of ice-cold acetonitrile. The samples were centrifuged for 15 min and the supernatant was removed and analyzed by HPLC to monitor the decline in the concentration of the compound added.

Animals

Swiss-Webster mice (30±2 g body weight) were used for all experiments 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) and approved by the Institutional Animal Care and Use Committee at the University of Florida Health Science Center.

Analeptic Activity

Eight mice were used in each group. Test compounds were dissolved in degassed saline. The vehicle alone (1.5 ml/kg body weight) was administered to the animals in the control group. Equimolar doses of test compounds (10 μmol/kg body weight) were injected through the tail vein. After 10 min, each animal received an i.p. injection of sodium pentobarbital solution at a dose of 60 mg/kg body weight. The sleeping time was recorded from the onset of loss of the righting reflex until the reflex was regained.

Porsolt Swim Test (PST).

We modified the original PST for mice by introducing a "pre-test session" similarly to PST used for rats to avoid variations and for maintaining consistency in the immobility time among different group of mice. In this "pre-swim test session," mice were placed to swim individually in a glass cylinder (22 x 25 cm) containing fresh water up to the height of 15 cm and a temperature of 25 ± 2 ^{o}C. Upon removal, the animals were hand-dried with a towel and placed on warming pads for 15 min prior to returning them to their cage. Twenty four hours later, mice were treated with either the test compound (3 μmol/kg, i.p.) or the vehicle (saline, 50 μl i.p., control group) 10 min before the actual test and were subsequently placed into the water-filled cylinder to swim again. For 6 min, the immobility time (defined as the duration of floating making only those movements necessary to keep the head above the water) was recorded simultaneously by two trained observers who were unaware ("blinded") which animals were injected with the test compound and the saline control, respectively. Data were expressed as percentage change in immobility time counting data for the control group as 100%. Each group consisted of 6 to 10 mice.

Statistical Analysis

Analysis of variance (ANOVA) followed by *post hoc* Dunnett's test was done to compare treatments to the control group. Differences were considered significant when $P<0.05$.

ACKNOWLEDGEMENTS

The authors thank the National Institutes of Health (MH59380) for financial support. We also thank to Mr. Jose Vera for running the NMR spectra.

ABBREVIATIONS

All	=	Allyl
Adaet	=	1-Adamantaneethyl
APCI	=	Atmospheric-pressure chemical ionization
BBB	=	Blood-brain barrier
CNS	=	Central nervous system
CPP	=	Central nervous system permeable prodrug
CDI	=	1,1-Carbonyldiimidazole
DCM	=	Dichloromethane
DIPEA	=	N,N-diisopropylethylamine
DIPCD	=	1,3-Diisopropylcarbodiimide
DMAP	=	4-(Dimethylamino)pyridine
DMF	=	N,N-dimethylformamide
ESI	=	Electrospray ionization
Fmoc	=	9-Fluorenylmethyloxycarbonyl
Glu	=	Glutaminyl
HOBt	=	1-Hydroxybenzotriazole hydrate
His	=	Histidyl
IAM(C)	=	Immobilized artificial membrane (chromatography)
i.v.	=	Intravenous
pGlu	=	Pyroglutamyl
logP	=	Logarithm of the n-octanol/water partition coefficient
Pro	=	Prolyl
PST	=	Porsolt swim test
PyBOP	=	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
T_3	=	Triiodothyronine
TRH	=	Thyrotropin-releasing hormone
Trg	=	Trigonellyl (residue of N-methylnicotinic acid betaine)

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Received: 03 September, 2004 Accepted: 05 November, 2004