Chemical Delivery System To Transport a Pyroglutamyl Peptide Amide to the Central Nervous System

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We have recently reported[1] the delivery of an enkephalin analog to the central nervous system (CNS) following intravenous injection by a "chemical delivery system" [2] approach that involves the fundamental steps of (1) transport through the blood-brain barrier (BBB) made possible by a lipophilic steroid moiety (L) and a lipoidal 1,4-dihydrotrigonellyl targetor (T) attached to the peptide via a spacer (S), (2) retention in the brain or "lock-in" via the enzymatic oxidation of T to a membrane-impermeable pyridinium moiety (T^+), and (3) release of the biologically active peptide (P). However, the method reported can only be applied to neuropeptides with free NH_2 and COOH termini. Here we report an important extension of the method to peptides with N-terminal pyroglutamyl (Pyr) and C-terminal carboxamide functions.

In our lead compound Pyr-His-Pro-NH_2 known as thyrotropin-releasing hormone (TRH) and originally isolated from the hypothalamus,[3] no free hydroxy or amino groups are present. Besides its function as the primary neurotrophic hormone for thyroid-stimulating hormone (TSH) secretion,[3,4] the extrahypothalamic distribution of TRH and its receptors indicates that TRH plays other roles in nervous system physiology,[5] and large doses show beneficial effects in memory impairment[6] and in amyotrophic lateral sclerosis.[7] However, TRH has a short *in vivo* half-life on the order of 5 min[8] and does not effectively penetrate the BBB[9] due to its low distribution coefficient (K_D < 0.005 between 1-octanol and water) and its susceptibility to peptidase degradation.[10] There have been numerous attempts to modify the structure to obtain metabolically stable analogs, as well as to improve potency and/or CNS selectivity,[11] but without improvement in brain delivery and duration of action.

TRH is derived from posttranslational processing of a precursor polyprotein.[12] The TRH progenitor sequences (Gln-His-Pro-Gly) are flanked by dibasic residues[13] that are typical sites of processing by carboxypeptidase B-like enzymes.[14] The C-terminal glycine functions as an amide donor for proline,[15] due to an enzymatic activity designated peptidyl glycine α-amidating monooxygenase (PAM) which requires Cu^{2+}, ascorbic acid, and molecular oxygen.[16] Glutamine (Gln) is the precursor of the N-terminal pyroglutamyl,[17] and the cyclization is catalyzed by a specific enzyme, glutaminyl cyclase.[18]

The peptide delivery system (**2a**) we designed and synthesized incorporates a Gln-Leu-Pro-Gly progenitor sequence of a TRH analog Pyr-Leu-Pro-NH_2 (**1**),[19] the free COOH of the glycine residue is esterified with cholesterol, and the 1,4-dihydrotrigonellyl moiety is attached to the progenitor via an additional alanine (Ala) residue as a spacer (S). The mechanism of CNS delivery for **1** by sequential bioactivation of **2a** is shown in Scheme 1. The delivery of a pharmacologically significant amount of **1** has been indicated by the profound decrease in the barbiturate-induced sleeping time, the measure of the activational effect on cholinergic neurons, in mice.[20] At an equimolar (30 μmol/kg) dose, the intravenous administration of **1** showed only a marginal decrease (29.2 ± 2.8 min, compared to the 32.4 ± 3.4 min for the control group), while **2a** resulted in ca. 30% reduction of sleeping time (to 21.5 ± 3.7 min).

To support that the bioactivation of the CNS-delivered peptide precursor **4a** to **5a** occurs in the sequence implied by the design, **4a** was synthesized and incubated *in vitro* (20% w/w rat brain homogenate, pH 7.4, 37 °C, 30 μM substrate concentration).[1] Electrospray ionization mass spectrometric analysis has revealed that about 70% of the peptide precursor has been degraded within a half-hour, and processed mainly (by ca. 80%) to **5a**, which is relatively stable *in vitro*, by PAM. About 20% of the degradation may be due to endopeptidase cleavage (possibly by enkephalinase, EC 3.4.24.11, which attacks internal Phe, Leu, Tyr, and Trp residues[21]) yielding a T^+-Ala-Gln-Leu fragment.[22] Thus, processing to **1** is only dependent on the release of **6a** by peptidase

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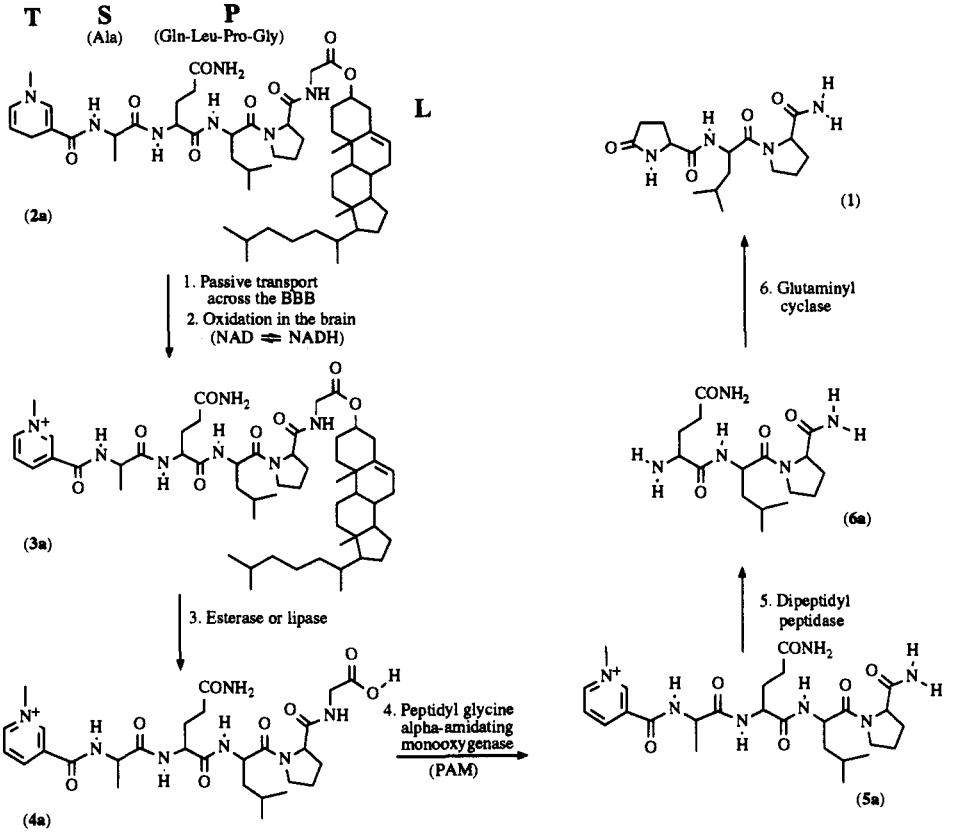
Scheme 1



cleavage of **5a**, which proceeds similarly to that of the corresponding enkephalin analog.[1]

For the facile release of the final precursor (**6a**) after the formation of the amide terminus, we considered the modification of the spacer (S). Prolyl endopeptidase (EC 3.4.21.26) plays a role in neuropeptide metabolism[23] and cleaves Pro–Xaa bonds (Xaa is an amino acid residue) with the exception of the Pro–Pro bond. The alanine (Ala) can substitute Pro.[24] However, N-blocked peptides with the general formula Y-Pro-Xaa, or Y-Ala-Xaa, are not easily attacked by this endopeptidase, where Y represents an N-terminal protecting group (Y here is T^+). Therefore, the brain-targeted and "locked-in" peptide precursors with a single Ala spacer are not readily metabolized by this enzyme. However, sensitivity to prolyl endopeptidase can be restored by placing Ala-Ala between the targetor (T) and the Gln residue of the progenitor sequence. The corresponding chemical delivery system (**2b**, Ala-Ala spacer), whose bioactivation proceeds similarly to that of **2a** (Scheme 1), has resulted in a more than 50% decrease (to 15.3 ± 1.8 min) of the barbiturate-induced sleeping time in mice, compared to the control group. It is, indeed, an improvement over **2a**, where a single Ala separates the N-terminal residue (Gln) of the peptide precursor from the targetor (T) function.

In conclusion, chemical delivery systems for peptides with no suitable functional groups for covalent bonding can be assembled by using an appropriate progenitor that undergoes predictable bioactivation. The reaction may also be controlled by allowing specific enzymes to interact with the adjustable portion of the molecule: the spacer amino acid residues.

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Supplementary Material Available: Synthetic procedure and analytical data (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(22) In liver homogenate and in plasma, **4a** is processed mainly to C-terminal proline, not to prolinamide.

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