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Research Article

Prodrugs of Peptides. 6. Bioreversible Derivatives of Thyrotropin-Releasing Hormone (TRH) with Increased Lipophilicity and Resistance to Cleavage by the TRH-Specific Serum Enzyme

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Bioreversible derivatization of TRH (pGlu–His–Pro–NH_2) to protect the tripeptide against rapid enzymatic inactivation in the systemic circulation and to improve the lipophilicity of this highly hydrophilic peptide was performed by N-acylation of the imidazole group of the histidine residue with various chloroformates. Whereas TRH was rapidly hydrolyzed at its pGlu–His bond in human plasma by a TRH-specific pyroglutamyl aminopeptidase serum enzyme, the *N*-alkoxycarbonyl derivatives were resistant to cleavage by the enzyme. On the other hand, these derivatives are readily bioreversible as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or by plasma esterase-catalyzed hydrolysis. In addition to protecting the parent TRH against rapid inactivation in the circulation and hence potentially prolonging the duration of action of TRH *in vivo*, the *N*-alkoxycarbonyl prodrug derivatives were much more lipophilic than TRH as assessed by octanol–buffer partitioning. This property may enhance prodrug penetration of the blood–brain barrier and various other biomembranes compared to the parent peptide.

KEY WORDS: thyrotropin-releasing hormone (TRH); prodrugs; enzymatic hydrolysis; lipophilicity; peptide modification.

INTRODUCTION

Thyrotropin-releasing hormone (TRH) (pGlu–L-His–L-Pro–NH_2) is a potentially effective drug in the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma, and schizophrenia (for reviews, see Refs. 1–8). Its neuropharmacological properties, however, are hampered by its rapid metabolism and clearance as well as by its poor access to the CNS (1,5,7,9). Following parenteral administration in man TRH shows a plasma half-life of only 6–8 min (10–13), which is due mainly to rapid enzymatic degradation of the peptide in the blood, in particular by a TRH-specific pyroglutamyl aminopeptidase serum enzyme (14–18). This enzyme is responsible for the rapid metabolism of TRH in human plasma, resulting in the formation of pyroglutamic acid and His–Pro–NH_2, which is subsequently enzymatically hydrolyzed to its constituent amino acids (18, and references cited therein). The low lipophilicity of TRH (19) may be a primary reason for the limited ability of the peptide to penetrate the blood–brain barrier (19,20).

A possible approach to solving these delivery problems is derivatization of the peptide to produce prodrugs or transport forms (21) that are markedly more lipophilic than the parent peptide and resistant toward the TRH-degrading serum enzyme but remain cleavable by chemical or nonspecific enzyme-catalyzed hydrolysis to release the parent TRH *in vivo*.

In this paper, we present *N*-alkoxycarbonyl derivatives at the imidazole group of the histidine residue as suitable prodrugs of TRH (Scheme I). Their physicochemical properties and chemical and enzymatic stability were investigated. A part of this work has been published in a preliminary form (22).

MATERIALS AND METHODS

Apparatus

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Kontron 420 HPLC pump, a Kontron 432 LC detector operated at 215 nm, and a Rheodyne 7125 injection valve with a 20-μl loop. The column was a reversed-phase Nova-Pak CN HP Radial Pak column (100 × 8 mm) equipped with a Resolve CN Guard Pak precolumn (both from Waters Associates). Readings of pH were carried out on a Radiometer PHM83 Autocal instrument at the temperature of study. ^{1}H-NMR spectra were obtained with a Varian 360L instrument and ultraviolet spectra with a Shimadzu UV-190 recording spectrophotometer.

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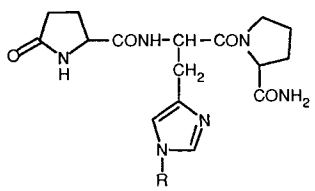


TRH	R =	H
I	R =	$-C(=O)-OCH_3$
II	R =	$-C(=O)-OC_2H_5$
III	R =	$-C(=O)-OCH(CH_3)_2$
IV	R =	$-C(=O)-OC_4H_9$
V	R =	$-C(=O)-OCH_2CH(CH_3)_2$
VI	R =	$-C(=O)-OC_6H_{13}$
VII	R =	$-C(=O)-OC_8H_{17}$
VIII	R =	$-C(=O)-OCH_2CH(C_2H_5)C_4H_9$
IX	R =	$-C(=O)-O-C_6H_5$
X	R =	$-C(=O)-OCH_2-C_6H_5$

Scheme I

Elemental analyses were performed at the Microanalytical Laboratory, University of Copenhagen.

Synthesis of TRH Derivatives I–X

The compounds were prepared by reacting TRH (purchased from Sigma Chemicals Co., St. Louis, Mo., or Carlbiotech A/S, Copenhagen) with the appropriate chloroformate in *N,N*-dimethylformamide or acetonitrile in the presence of triethylamine. Typical procedures used are as follows.

N-Hexyloxycarbonyl TRH (VI). To a mixture of TRH (1 mmol, 362 mg) in 20 ml of acetonitrile was added 1.2 mmol (0.17 ml) of triethylamine followed by 1.2 mmol of *n*-hexyl chloroformate. The mixture was stirred at room temperature for 5 hr and evaporated under reduced pressure. The residue obtained was taken up in water (10 ml) and ethyl acetate (20 ml). The organic phase was separated and the aqueous phase reextracted with 20 ml of ethyl acetate. The combined extracts were washed with a 2% sodium bicarbonate solution followed by water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue obtained was recrystallized from ethanol–ether–petroleum ether and finally dried *in vacuo* over phosphorous pentoxide to give the title compound in a yield of 67%. mp 71–73°C.

Anal.: Calc. for $C_{23}H_{34}N_6O_6$, $1H_2O$: C, 54.32; H, 7.14; N, 16.52. Found: C, 54.20; H, 7.25; N, 16.40.

N-Octyloxycarbonyl TRH (VII). To a solution of TRH (3 mmol, 1.09 g) in 10 ml of *N,N*-dimethylformamide was added 3.6 mmol (0.51 ml) of triethylamine followed by 3.6 mmol (0.72 ml) of *n*-octyl chloroformate. The mixture was stirred at room temperature for 2.5 hr, poured into 60 ml of water, and then extracted with ethyl acetate (2 × 75 ml). The combined ethyl acetate extracts were washed with water (2 × 50 ml), dried over anhydrous sodium sulfate, and evaporated *in vacuo*. The residue obtained was recrystallized from ethyl acetate–ether–petroleum ether to give the title compound in a yield of 70%. mp 80–82°C.

Anal.: Calc. for $C_{25}H_{38}N_6O_6$, $1H_2O$: C, 55.96; H, 7.51; N, 15.66. Found: C, 56.07; H, 7.69; N, 15.44.

The other compounds studied *(I–V, VII–X)* were prepared by the method described for compound VI. They showed all ^{1}H-NMR and UV spectra (λ_{max} at 230 nm) consistent with their structures. HPLC analysis as described below revealed a single major peak (>95%); the content of unreacted TRH was less than 1%.

Stability Studies

The kinetics of degradation of the TRH derivatives were determined in aqueous buffer solutions at constant temperature. The buffers were hydrochloric acid, acetate, phosphate, *N*-ethylmorpholine, borate, and carbonate buffers. The total concentration of the buffers was generally 0.02 *M* and a constant ionic strength (μ) of 0.5 was maintained for each buffer solution by adding a calculated amount of potassium chloride. The rates of degradation of the derivatives were followed by using an isocratic reversed-phase HPLC procedure capable of separating the derivatives from TRH. The Nova-Pak CN HP Radial Pak column was eluted with mobile phase systems consisting of 10–50% (v/v) acetonitrile in 0.1% (v/v) phosphoric acid, with triethylamine added at a concentration of 10^{-3} *M*. The concentration of acetonitrile was adjusted for each of the compounds I–X to give an appropriate retention time (3–8 min). With these eluents the products of hydrolysis appeared in the solvent front. For the determination of TRH formed upon hydrolysis of the various derivatives, a mobile phase system consisting of acetonitrile–0.1% phosphoric acid (1:20, v/v) containing 10^{-3} *M* triethylamine was used. The flow rate was 1.5 ml min^{-1} and the column effluent was monitored at 215 nm. Quantitation of the compounds was done by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

The reactions were initiated by adding 25 μl of a stock solution of the compounds in acetonitrile or water to 10 ml of preheated buffer solution in screw-capped test tubes, the final concentration of the compounds being about 2×10^{-4} *M*. The solutions were kept in a water bath at a constant temperature (±0.2°C), and at appropriate intervals samples were taken and chromatographed immediately. Pseudo-first-order rate constants for the degradation were determined from the slopes of linear plots of the logarithm of residual derivative against time.

Degradation Studies in Human Plasma

The derivatives I–X were incubated at 37°C in human plasma. The initial concentration of the derivatives was in the range of 10^{-4}–10^{-3} *M*. At appropriate intervals, samples of 250 μl of the plasma reaction solutions were withdrawn

and added to 500 μl of a 2% solution of zinc sulfate in methanol:water (1:1, v/v) in order to deproteinize the plasma. After mixing and centrifugation for 3 min at 13,000 rpm, 20 μl of the clear supernatant was analyzed for remaining derivative and TRH formed by HPLC as described above.

Degradation Studies in the Presence of Pyroglutamyl Aminopeptidase

The stability of TRH and the compounds I–X in the presence of pyroglutamyl aminopeptidase (a calf liver preparation obtained from Boehringer, Mannheim, F.R.G.) was examined at 37°C using an incubation mixture of 5 ml of 0.1 *M* phosphate buffer, pH 7.40, containing 1 m*M* disodium edetate (Na_2EDTA) and 0.5 m*M* dithiothreitol, 500 μl of an aqueous solution of the enzyme at a concentration of 0.116 U/ml, and 50 μl of a stock solution (10^{-2} *M*) of the compounds in water or acetonitrile as previously described for other compounds (23). At appropriate intervals samples were taken and immediately chromatographed as described above for the degradation studies in buffer solutions.

Determination of Lipophilicity Parameters

The partition coefficients of the derivatives were determined in an octanol–buffer system. The aqueous phase was a 0.02 *M* phosphate buffer solution, pH 7.40. The buffer solution and octanol were mutually saturated at 20–22°C before use. The compounds were dissolved in the aqueous buffer phase at a concentration of 10^{-4} *M* and the octanol–water mixtures were shaken for about an hour to reach a distribution equilibrium. The volumes of each phase were chosen so that the solute concentration in the aqueous phase, before and after distribution, could readily be measured using the aforementioned HPLC procedures. The partition coefficients (*P*) were calculated from Eq. (1):

$$P = \left(\frac{C_i - C_w}{C_w}\right)\left(\frac{V_w}{V_o}\right) \quad (1)$$

where C_i and C_w represent the solute concentrations in the aqueous buffer phase before and after distribution, respectively, and V_w represents the volume of the aqueous and V_o the volume of the octanol phase. For each compound, determinations were carried out in triplicate, and the *P* values thereby obtained were reproducible to within ±5%.

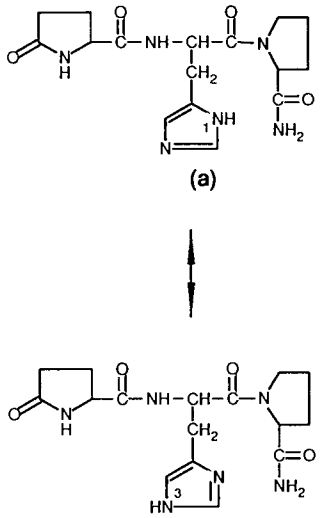
The lipophilicity of the derivatives was also evaluated by means of reversed-phase HPLC. In this method the capacity factor (*k'*) of a solute is taken as a measure for the relative lipophilicity:

$$k' = (t_R - t_0)/t_0 \quad (2)$$

where t_R is the retention time of the solute and t_0 is the elution time of the solvent. In this measurement, a mobile phase system of methanol–0.1% phosphoric acid (1:20, v/v) containing 10^{-3} *M* triethylamine was used.

RESULTS AND DISCUSSION

The various *N*-alkyloxycarbonyl derivatives (I–X) of TRH were prepared by reacting TRH with a slight excess of the appropriate chloroformates. Since TRH exists in two tautomeric forms, the *N*(1)-H-tautomer (a) and the *N*(3)-H-tautomer (b) (Scheme II), acylation of the imidazole group may occur either at N(1) or at N(3). The reaction of histidine derivatives with chloroformates is thought to yield N(3)-substituted derivatives. Thus, acylation of various α-amino and carboxy protected histidine derivatives with isobutyl chloroformate resulted in the exclusive formation of N(3)-substituted isobutyloxycarbonyl derivatives, whereas reaction of the same derivatives with adamantyloxycarbonyl fluoride resulted in an isomeric mixture consisting of N(1)- and N(3)-substituted derivatives at the ratio 1:2 (24,25). HPLC analysis of the products and the reaction mixtures of TRH revealed only one major peak, accounting for more than 95%. Further support for the assignment of the position of substitution to the N-3 atom is provided by the fact that the tautomeric equilibrium of TRH in its free-base form is shifted toward the N(3)-H form (26).



Scheme II

To assess the potential utility of the *N*-alkoxycarbonyl derivatives of TRH as prodrug forms, the stability and degradation pathways of the derivatives were investigated in aqueous buffer solutions, in human plasma, and in the presence of pyroglutamyl aminopeptidase derived from calf liver.

Hydrolysis in Aqueous Solution

The kinetics of degradation of the derivatives I–X were studied in 0.02 *M* phosphate buffer solution of pH 7.40 at 37°C. In order to examine the influence of pH and temperature on the stability, compound VII was studied over a wide range of pH and at different temperatures.

At constant pH and temperature, the disappearance of the derivatives displayed strict first-order kinetics over several half-lives. Examples are shown in Fig. 1. The half-lives of hydrolysis are listed in Table I and range from 9 to 37 hr at pH 7.40 and 37°C. As evidenced by HPLC the derivatives hydrolyzed quantitatively to the parent TRH, which is stable under the conditions used. An example of a time course of formation of TRH upon hydrolysis is shown in Fig. 2.

The influence of pH on the rate of degradation of the

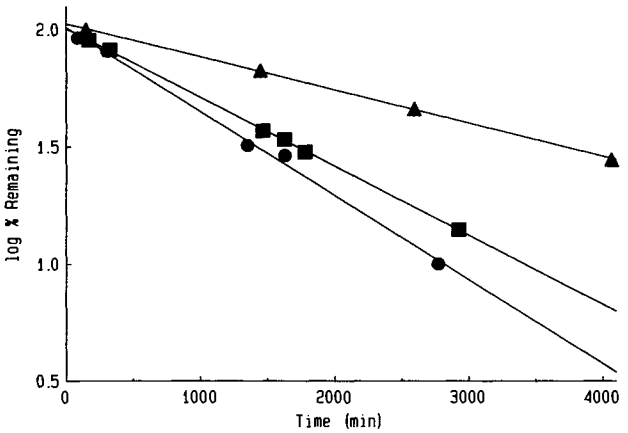


Fig. 1. Plots showing first-order kinetics of degradation of the TRH derivatives II (●), III (▲), and VII (■) in 0.02 *M* phosphate buffer solution, pH 7.40 (at 37°C).

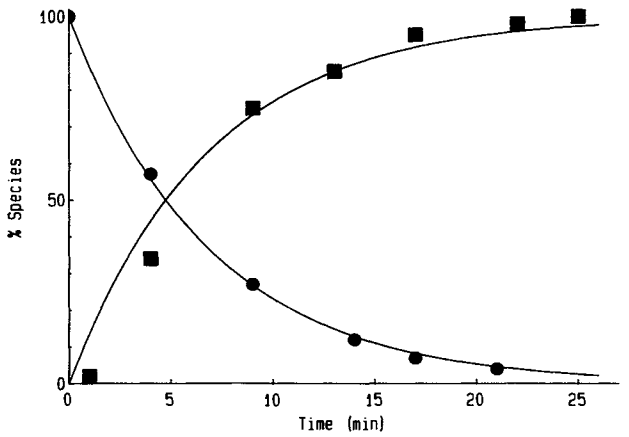


Fig. 2. Time courses for *N*-octyloxycarbonyl TRH (VII) (●) and TRH (■) during hydrolysis of compound VII in 0.02 *M* borate buffer, pH 9.80 (at 37°C).

N-octyloxycarbonyl derivative VII is shown in Fig. 3 where the logarithm of the observed pseudo-first-order rate constants (k_{obs}) is plotted against pH. No significant buffer catalysis was observed at a low concentration (0.02 *M*). As can be seen the rate of hydrolysis increases sharply above and below pH 5–6, whereas it approaches a plateau below pH 2. The shape of the pH–rate profile is similar to that of *N*-acetylimidazole (27), as well as those of *N*-ethoxycarbonyl imidazole (28) and similar imidazole carbamates formed with hydrocortisone and testosteron (29). The hydrolysis of *N*-acetylimidazole has been demonstrated to involve a water-catalyzed or spontaneous hydrolysis of acetylimidazolium cation and a spontaneous as well as a hydroxide ion-catalyzed reaction of unprotonated acetylimidazole (27,30). Accordingly, the reactions depicted in Scheme III are suggested to account for the hydrolysis of the alkoxycarbonyl derivative VII and the following rate expression may be formulated:

Table I. Rate Data for the Hydrolysis of TRH and Its Various *N*-Alkoxycarbonyl Derivatives

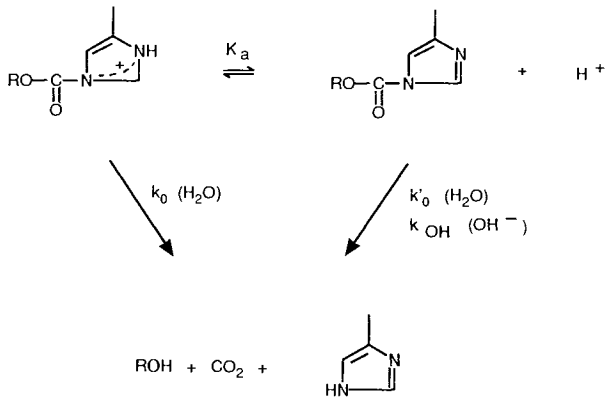
Compound	Half-lives at 37°C		
	pH 7.40 buffer	Human plasma	Pyroglutamyl aminopeptidase I[a]
TRH	—	9.4 min[b]	4 min
I	9.3 hr	2.8 hr	12 min
II	14.2 hr	3.8 hr	23 min
III	35.8 hr	6.6 hr	29 min
IV	19.0 hr	4.3 hr	11 min
V	19.0 hr	4.3 hr	11 min
VI	17.9 hr	1.2 hr	8 min
VII	17.5 hr	0.4 hr	24 min
VIII	20.4 hr	1.6 hr	24 min
IX	36.8 hr	6.4 hr	22 min
X	9.0 hr	1.6 hr	7 min

[a] These data are half-lives for the degradation in buffer solution (pH 7.40) containing calf liver pyroglutamyl aminopeptidase (0.01 U/ml).

[b] Half-life of hydrolysis at a TRH concentration less than 5×10^{-6} *M*.

$$k = k_0 \frac{a_H}{a_H + K_a} + k'_0 \frac{K_a}{a_H + K_a} + k_{OH} a_{OH} \frac{K_a}{a_H + K_a} \quad (3)$$

where a_H and a_{OH} refer to the hydrogen ion and hydroxide ion activity, respectively, K_a is the apparent ionization constant of the protonated imidazole group in VII, $a_H/(a_H + K_a)$ and $K_a/(a_H + K_a)$ are the fractions of the derivative in protonated and unprotonated form, and k_0, k'_0, and k_{OH} are rate constants referring to the reactions shown in Scheme III.



Scheme III

The following values of the specific rate and ionization constants for VII (37°C; $\mu = 0.5$) were obtained from the pH–rate profile and Eq. (3):

$$k_0 = 2 \times 10^{-2}\ \text{min}^{-1}$$
$$k'_0 = 6 \times 10^{-5}\ \text{min}^{-1}$$
$$k_{OH} = 1.2 \times 10^{3}\ M^{-1}\ \text{min}^{-1}$$
$$pK_a = 2.7$$

The pK_a value of both *N*-acetylimidazole and *N*-ethoxycarbonyl imidazole is 3.6 (27,28). The lower pK_a value for VII in relation to these imidazole derivatives parallels the lower pK_a value of TRH (6.25) (31) compared with that of imidazole (7.05) (31).

The effect of temperature (37–60°C) on the rate of hydrolysis of compound VII was studied in 0.02 *M* phosphate buffer of pH 5.8, at which pH the compound showed maxi-

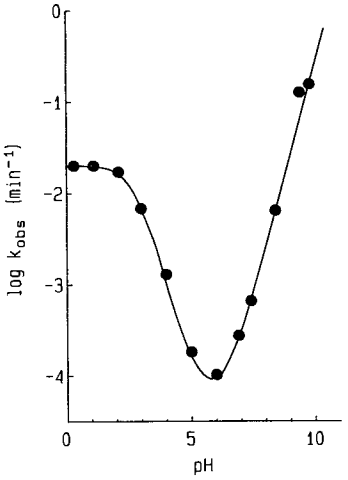


Fig. 3. The pH–rate profile for the hydrolysis of *N*-octyloxycarbonyl TRH (VII) in aqueous solution (μ = 0.5) at 37°C.

mal stability. From the Arrhenius-type plot obtained (Fig. 4) an activation energy (E_a) of 67.4 kJ mol^{-1} and a frequency factor (A) of 2.45 × 10^7 min^{-1} were obtained. From these values and the Arrhenius equation,

$$\log K = \log A - E_a/2.303\,RT \quad (4)$$

where R is the gas constant and T is the absolute temperature in K, the time for 10% degradation of *N*-octyloxycarbonyl TRH in aqueous solution at pH 5.8 and 20°C can be estimated to be 76 hr. At 5°C and pH 5.8 the estimated $t_{10\%}$ is 14 days.

Stability Toward Pyroglutamyl Aminopeptidase I

The hydrolysis of TRH at the pGlu–His bond is catalyzed by at least two different pyroglutamyl aminopeptidases, PAPase I and II (14,15,32). PAPase I is a cysteine protease that occurs in many different tissues such as liver, kidney, and brain but not in the blood. It cleaves almost all pGlu–peptide bonds including that in TRH (32–34). In contrast, pyroglutamyl aminopeptidase II (EC 3.4.19-), which is found predominantly in brain (35–37), and the TRH-specific serum enzyme [also termed thyroliberinase (38)] exhibit a high degree of substrate specificity (14,15,32,36,39). These pyroglutamyl aminopeptidases exhibit similar physical and chemical characteristics and are probably identical or derived from the same gene (15). The enzyme(s) catalyzes the hydrolysis of TRH but does not hydrolyze other pGlu-containing peptides (15).

The stability of the alkoxycarbonyl derivatives of TRH was determined at pH 7.40 in the presence of pyroglutamyl aminopeptidase I derived from calf liver to evaluate whether the modification made at the imidazole group of TRH effected the enzymatic lability of its pGlu–His bond. Under the reaction conditions used, both TRH and the alkoxycarbonyl derivatives were fairly rapidly degraded by the enzyme; the rates of degradation followed first-order kinetics (Fig. 5). The half-lives of degradation observed are listed in Table I. All the derivatives proved more stable than unmodified TRH, the most stable compound being the isopropoxycarbonyl derivative III. The relatively low degree of protection against PAPase I achieved by modification of the imidazole group of TRH is not unexpected since the specificity of this enzyme encompasses almost all pyroglutamyl-containing peptides as noted above.

Stability in Human Plasma

The degradation of TRH in human plasma or blood is due entirely to hydrolytic cleavage of its pGlu–His bond by a TRH-specific serum enzyme (PAPase II). We found this reaction to follow classical Michaelis–Menten kinetics, the K_m and V_{max} values being 1.9 × 10^{-5} M and 1.4 × 10^{-6} M min^{-1}, respectively (18). At high TRH concentrations ($>K_m$) the rate of hydrolysis of the peptide followed zero-order kinetics with a rate constant of 1.4 μmol min^{-1}, whereas at a low substrate concentration ($<K_m$), the enzymatic reaction is first-order with a rate constant of 0.074 min^{-1}, which corresponds to a half-life of 9.4 min (18).

The stability of the *N*-alkoxycarbonyl derivatives I–X in human plasma solutions at 37°C differed greatly from that of TRH. The kinetics of degradation followed good first-order kinetics at substrate concentrations even up to 10^{-3} M (Fig.

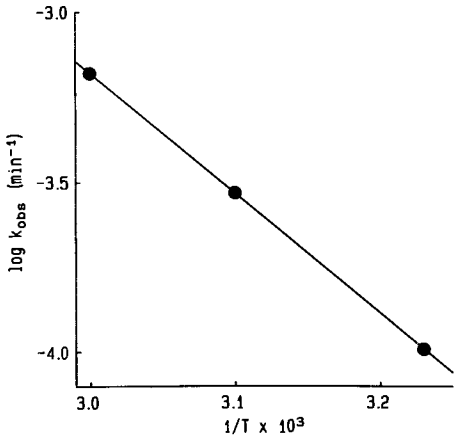


Fig. 4. Arrhenius-type plot for the rate of hydrolysis of compound VII in 0.05 M phosphate buffer, pH 5.80.

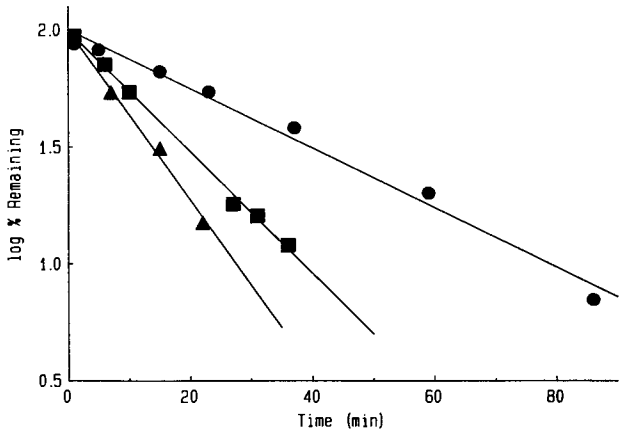


Fig. 5. Plots showing first-order kinetics of degradation of the TRH derivatives IV (■), VI (▲), and VII (●) (initial concentration: 8.8 × 10^{-5} M) in the presence of pyroglutamyl aminopeptidase I (0.01 U/ml) from calf liver (at 37°C).

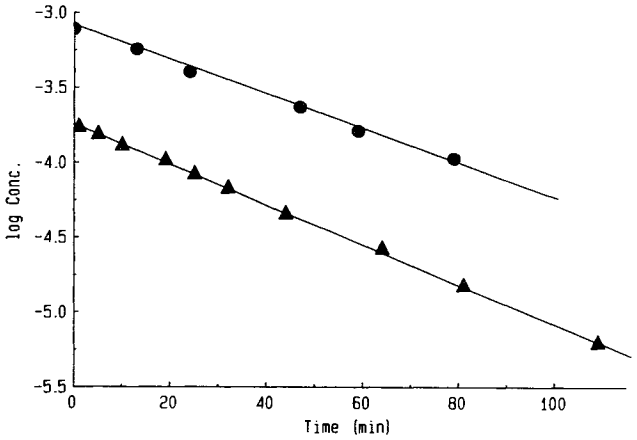


Fig. 6. Plots showing the first-order kinetics of degradation of the *N*-octyloxycarbonyl derivative of TRH (VII) in human plasma (37°C) at initial concentrations of 2×10^{-4} *M* (▲) and 8×10^{-4} *M* (●).

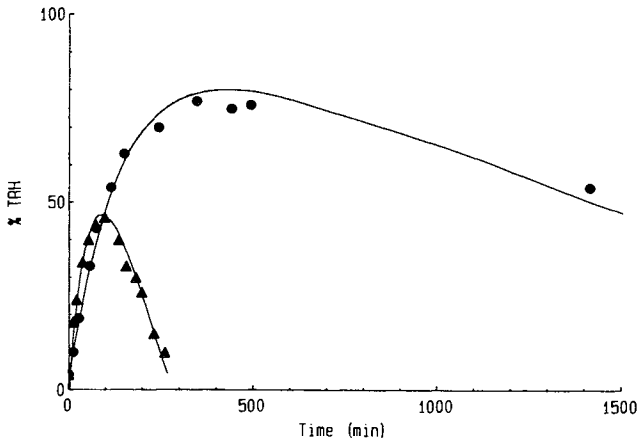


Fig. 7. Plots showing the time courses of TRH formation following incubation of *N*-hexyloxycarbonyl TRH (VI) in human plasma at 37°C at an initial concentration of the derivative of 2×10^{-4} *M* (▲) and 1×10^{-3} *M* (●). The curves are calculated from Eq. (5), while the points are experimental data.

6) and the compounds showed a markedly higher stability than TRH when the comparison was made under conditions where the degradation of TRH also occurs according to first-order kinetics. The half-lives of degradation range from 0.4 to 6.6 hr, whereas TRH degrades with a half-life of 9.4 min (Table I). Comparison of the plasma and buffer rate data for the derivatives reveals a significant plasma-catalyzed hydrolysis, especially for compounds VI–VIII containing long alkyl chains.

The plasma-catalyzed degradation observed may be due to a catalyzed hydrolysis of the acyl bond at the imidazole moiety with liberation of TRH or it may be due to other degradation processes such as hydrolysis of the pGlu–His bond. Ideally, the compounds should be hydrolyzed in plasma at the imidazole-protecting moiety to release TRH in quantitative amounts.

Since the degradation of TRH proceeds at a rather slow rate at higher concentrations (10^{-4}–10^{-3} *M*), whereas the rate of degradation of the derivatives I–X remain independent of concentration up to at least 10^{-3} *M*, any TRH formed from the degradation of the derivatives should be readily measurable when using a high substrate concentration. Using such conditions and analyzing the reaction solutions for TRH by HPLC as described above, a quantitative (i.e., >90%) conversion of the derivatives to TRH in plasma was demonstrated.

Examples of the time courses of TRH formation following incubation of the derivatives VI and VII in human plasma at initial concentrations of 2×10^{-4} or 10^{-3} *M* are shown in Figs. 7 and 8. The formation of TRH in appreciable amounts is readily seen. The greater amounts of TRH occurring in solutions with the highest initial concentration of the derivatives are due to saturation of the inactivation pathway of TRH. If it is assumed that the derivatives are hydrolyzed exclusively to yield TRH, the time dependency of the concentrations of TRH as the percentage of the initial derivative concentration is given by the following expression:

$$\% \text{ TRH} = 100\left[(1 - e^{-k_1 t}) - \frac{k_2}{A_0} t\right] \qquad (5)$$

where k_1 is the pseudo-first-order rate constant for the disappearance of the TRH derivatives, k_2 is the zero-order rate constant for the degradation of TRH in plasma, and A_0 is the initial concentration of the TRH prodrug. Using the k_1 values experimentally determined and a k_2 value of 1.4×10^{-6} *M* min^{-1} [equal to V_{max} as previously determined for TRH (18)], the solid curves drawn in Figs. 7 and 8 were constructed on the basis of Eq. (5). The good agreement observed between the experimental and the calculated data demonstrates that the sole or predominant (>90%) reaction of the TRH derivatives in human plasma is hydrolysis at the imidazole carbamate moiety to yield TRH. Similar results were obtained for the other *N*-alkoxycarbonyl derivatives.

These experiments show that the *N*-alkoxycarbonyl derivatives are totally resistant toward degradation by the TRH-inactivating serum enzyme. This finding further underlines the high substrate specificity of the enzyme. The experiments also demonstrate that the *N*-alkoxycarbonyl de-

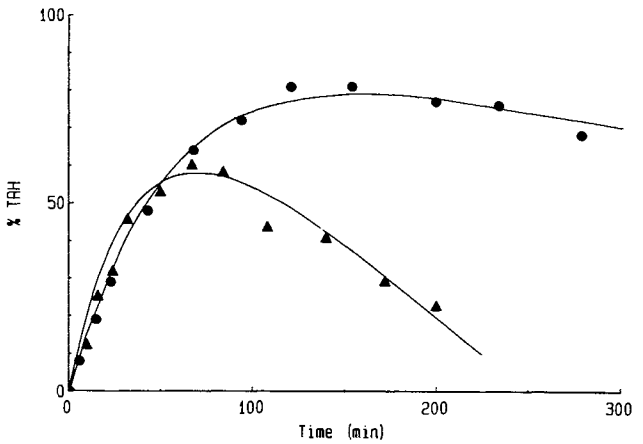


Fig. 8. Plots showing the time courses of TRH formation following incubation of *N*-octyloxycarbonyl TRH (VII) in human plasma at 37°C at an initial concentration of the derivative of 2×10^{-4} *M* (▲) and 1×10^{-3} *M* (●). The curves are calculated from Eq. (5), while the points are experimental data.

rivatives function as prodrug forms of TRH in that the parent peptide is released under conditions similar to those prevailing *in vivo*. The catalytic effect of plasma on the rates of hydrolysis of the derivatives is most likely due to nonspecific esterases.

It is of interest to note that whereas acylation of the imidazole moiety of TRH to give *N*-alkoxycarbonyl derivatives makes the pGlu–His bond resistant to cleavage by the TRH-specific pyroglutamyl aminopeptidase serum enzyme, methylation of the imidazole group at N-3 to give *N*-methyl TRH affords only a stabilization of a factor of about 2 toward plasma-catalyzed inactivation (11,40). Apparently, the enzyme tolerates a methyl group but not an alkoxycarbonyl group in the imidazole moiety. These two types of substitution are chemically quite different but also the basic properties of the imidazole group are very different.

By varying the *R* substituent in the derivatives, prodrug forms with different rates of hydrolysis can be obtained. To give an impression of the effect of rate of hydrolysis of the prodrugs on the time course of TRH formation in human plasma at pharmacologically relevant prodrug concentrations, the curves shown in Fig. 9 were constructed. The time courses of TRH were calculated from the following expression:

$$\% \text{ TRH} = \frac{k_1 \times 100}{k_2 - k_1}(e^{-k_1 t} - e^{k'_2 t}) \qquad (6)$$

where k_1 is the first-order rate constant for the hydrolysis of the derivatives in plasma and k'_2 is the first-order rate constant for the degradation of TRH in plasma at low concentrations (i.e., 0.074 min^{-1}, corresponding to a half-life of 9.4 min). The data clearly show that the prodrug derivatives may serve as depots for the supply of TRH.

Lipophilicity of the TRH Prodrugs

The lipophilicity of the derivatives and of TRH was assessed by measuring the partition coefficients (*P*) between octanol and 0.02 *M* phosphate buffer of pH 7.40. At this pH the derivatives are present as their free-base forms, and more than 90% of TRH is also present in this form. The log *P* values obtained are shown in Table II. The results show that the derivatives are much more lipophilic than the highly hydrophilic TRH and that *N*-alkoxycarbonyl derivatives with varying lipophilicity can be obtained by selecting appropriate *R* substituents. The log *P* values agree well with values calculated from the π substituent values (41). For example, going from the hexyl derivative VI to the octyl derivative VII, log *P* increases by 1.16, which corresponds to the π value for two methylene groups (1.0). Thus, it is possible to predict the partition coefficients of other *N*-alkoxycarbonyl-TRH derivatives on the basis of the additive substituent principle.

The increased lipophilicity of the *N*-carboalkoxy derivatives relative to the parent TRH is also illustrated by the chromatographic capacity factors (k') listed in Table II. As has been observed for many different types of compounds (42–44), a linear relationship exists between log k' and log *P* for the TRH derivatives.

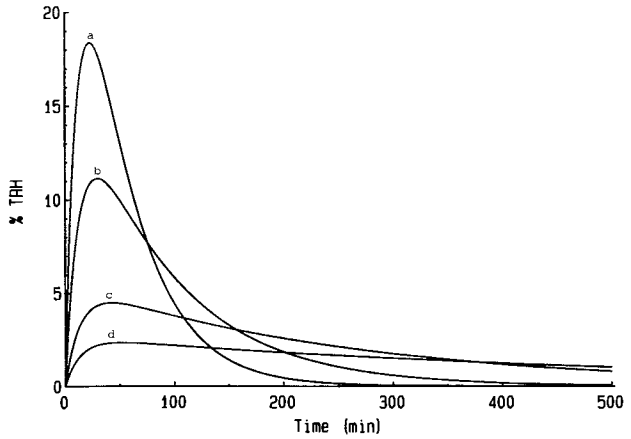


Fig. 9. Plots of TRH concentration versus time during hydrolysis of *N*-alkoxycarbonyl prodrug derivatives of TRH in human plasma at 37°C. The curves are calculated from Eq. (6) and represent cases where the half-life of hydrolysis of the prodrug derivative is 0.5 hr (a), 1 hr (b), 3 hr (c) and 6 hr (d). In all instances, the formation of TRH is assumed to be quantitative.

CONCLUSIONS

By forming *N*-alkoxycarbonyl derivatives at the imidazole moiety of TRH, the great susceptibility of the pyroglutamyl peptide bond in TRH to cleavage by the TRH-specific serum enzyme is abolished. The modification is readily bioreversible, as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or by enzymatic hydrolysis effected by plasma enzymes (esterases) not attacking the pyroglutamyl peptide bond. The increased lipophilicity of the derivatives relative to the TRH may render it feasible to deliver TRH to the brain in the form of the prodrugs. Further, the *N*-alkoxycarbonyl moieties are hydrolyzed to innocuous products (carbon dioxide and alcohols).

The poor oral absorption (0.2–2%) of TRH in man and in rats (45) is probably not due to enzymatic inactivation at the absorption site (46) but, rather, to the low lipophilicity of the peptide making a passive transport across the intestinal membrane difficult. We are currently studying the use of the more lipophilic *N*-alkoxycarbonyl-TRH derivatives to improve TRH bioavailability by the oral and other routes such as the dermal route.

Table II. Partition Coefficients (*P*) and Chromatographic Capacity Factors (k') of TRH and Its Various *N*-Alkoxycarbonyl Derivatives

Compound	log P^a	log k'
TRH	−2.46	<0.4
I	−1.88	0.71
II	−1.30	0.85
III	−0.80	0.96
IV	−0.47	1.07
V	−0.44	1.09
VI	0.71	1.29
VII	1.88	1.50
VIII	1.82	1.48
IX	0.60	1.28
X	−0.11	1.10

[a] Between octanol and 0.02 *M* phosphate buffer, pH 7.40 (21°C).

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