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Prodrugs of peptides. 12. Bioreversible derivatization of thyrotropin-releasing hormone (TRH) by N-phthalidylation of its imidazole moiety

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Summary

N-Phthalidylation of the imidazole group in TRH (pGlu-His-Pro-NH_2) was found to be a promising approach to obtain prodrug forms of this tripeptide, with the aim of protecting it against rapid enzymatic inactivation in the systemic circulation. The N-phthalidyl derivative of TRH was shown to be resistant to cleavage by the TRH-specific pyroglutamyl aminopeptidase serum enzyme but is, on the other hand, readily bioreversible by virtue of spontaneous or plasma esterase-catalyzed opening of the phthalidyl lactone ring to yield TRH in a two-step process. The pH-rate profile for the degradation of the derivative in aqueous solution was determined and accounted for in terms of specific acid- and base-catalyzed reactions in addition to a spontaneous hydrolysis. Like previously studied N-alkoxycarbonyl derivatives the phthalidyl derivative did not protect TRH against cleavage by unspecific pyroglutamyl aminopeptidase (PAPase I) or intestinal prolyl endopeptidase.

Introduction

Thyrotropin-releasing hormone (TRH, pGlu-L-His-L-Pro-NH_2) is a potentially useful drug in the management of various neurologic and neuropsychiatric disorders but its clinical utility has been greatly hampered due to its rapid enzymatic inactivation in the blood and poor access to the brain (Metcalf, 1982; Griffiths, 1987; Metcalf and Jackson, 1989). We have recently reported that these delivery problems may be overcome by bioreversible derivatization of the peptide (Bundgaard and Møss, 1990). The derivatives developed are N-alkoxycarbonyl derivatives of TRH formed by N-acylating the imidazole group of the histidine residue with various chloroformates. These derivatives are totally resistant to cleavage by the TRH-inactivating pyroglutamyl aminopeptidase serum enzyme, but are readily bioreversible as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or by plasma esterase-catalyzed hydrolysis (Scheme 1). By appropriate selection of the alkyl moiety (R) derivatives with greatly increased lipophilicity relative to TRH and hence better blood-brain barrier penetration properties can readily be obtained (Bundgaard and Møss, 1990).

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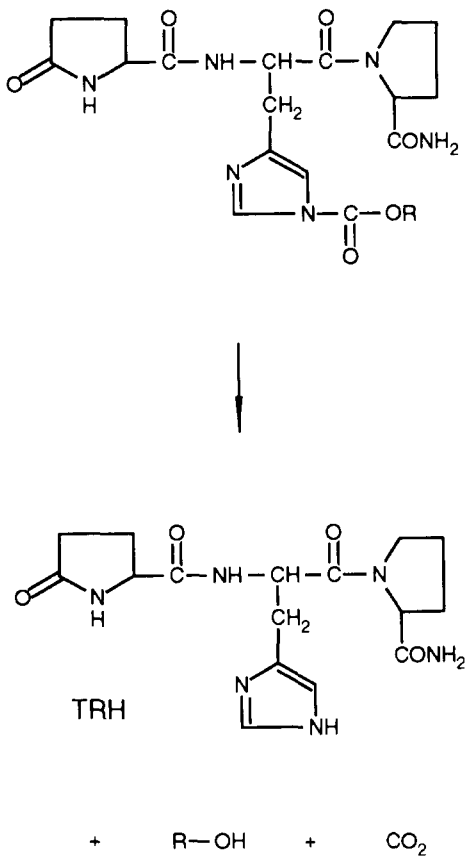


Scheme 1.

These prodrug derivatives may also be useful for achieving transdermal delivery of TRH (Møss and Bundgaard, 1990b) but are, on the other hand, not suitable for oral administration because of their facile degradation by intestinal enzymes, in particular prolyl endopeptidase (Møss et al., 1990).

A further potentially useful prodrug type for TRH may be N-α-acyloxyalkyl or N-phthalidyl derivatives formed at the imidazole moiety. The phthalidyl functionality has previously been attached to various amides (Bundgaard et al., 1988) and the NH-groups in 5-fluorouracil (Kametani et al., 1982; Kamata et al., 1985) as well as to the imidazole ring in theophylline (Tonda and Hirata, 1987) in order to obtain prodrug derivatives. The lactone ring in the phthalidyl derivatives may be readily cleaved by esterase-catalyzed hydrolysis followed by release of the parent NH-acidic compound and phthalaldehydic acid through a spontaneous decomposition of the N-α-hydroxybenzyl intermediate (Bundgaard et al., 1988).

In this work, we have prepared an N-phthalidyl derivative of TRH(I) and assessed its suitability as a prodrug form for the peptide. To this end, the chemical and enzymatic stability as well as the lipophilicity of the derivative were investigated.

Materials and Methods

Apparatus

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Kontron 420 HPLC pump, a Kontron 432 HPLC detector, and a Rheodyne 7125 injection valve with a 20 μl loop. A reversed-phase Supelcosil LC-8-DB column (33 × 4.6 mm) (3 μm particles) was generally used in conjunction with a Supelguard column LC-8-DB (20 × 4.6 mm), both from Supelco Inc. (U.S.A.). Readings of pH were carried out on a Radiometer PHM83 Autocal instrument at the temperature of study. Elemental analysis was performed at the Microanalytical Laboratory, University of Copenhagen.

Chemicals

TRH was obtained from Carlbiotech A/S, Copenhagen. Phthalide and phthalaldehydic acid were purchased from Fluka AG (Switzerland) whereas 3-bromophthalide was from Riedel-de Häen AG (Germany). Buffer substances and solvents used were of reagent grade.

Synthesis of N-phthalidyl TRH

3-Bromophthalide (320 mg, 1.5 mmol) was added to a mixture of TRH (543 mg, 1.5 mmol) and triethylamine (0.20 ml, 1.5 mmol) in acetonitrile (15 ml). The mixture was stirred at 60 °C for 7 h at which time HPLC analysis showed the consumption of TRH and the formation of a single major peak. The mixture was evaporated under reduced pressure and the residue taken up in acetonitrile–water and desalted on a preparative HPLC column. The fractions containing N-

phthalidyl TRH were lyophilized to give 410 mg of the compound as a hydrate, m.p. 148–150 °C.

Anal.: Calculated for $C_{24}H_{26}N_6O_6 \cdot 2.5\ H_2O$: C, 53.43; H, 5.79; N, 15.58. Found: C, 53.64; H, 5.64; N, 15.22.

Stability studies in aqueous solutions

All rate studies were performed in aqueous buffer solutions at 37.0 ± 0.2 °C. The buffers used were hydrochloric acid, acetate, phosphate and borate buffers. The total buffer concentration was generally 0.02 M and a constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The rates of degradation were followed by using an isocratic reversed-phase HPLC procedure capable of separating compound I from TRH and other degradation products. A mobile phase system of 5% (v/v) of acetonitrile in 0.1% (v/v) phosphoric acid, with triethylamine added at a concentration of 10^{-3} M, was used. The flow rate was 1.0 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 100 μl of a stock solution of compound I in acetonitrile to 10 ml of pre-heated buffer solution, the final concentration being 10^{-4} M. The solutions were kept in a water bath at 37 °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.

For the determination of TRH the column used was a reversed-phase Nova-Pak CN HP Radial Pak column (100 × 8 mm) equipped with a Resolve CN Guard Pak column (both from Waters Ass., U.S.A.). The mobile phase was the same as that mentioned above.

Degradation studies in plasma and other media

The stability of compound I was determined in human plasma and in 20% rabbit gut homogenate containing 0.05 M phosphate buffer (pH 7.40). The latter homogenate was prepared as previously described (Møss et al., 1990). Sodium edetate and dithiothreitol were added to the homogenate to activate and stabilize prolyl endopeptidase (cf., Møss et al., 1990). Compound I was incubated at 37 °C in the plasma and gut homogenate at an initial concentration of 10^{-3} and 10^{-4} M, respectively. At appropriate intervals samples of 250 μl were withdrawn and added to 250 μl of a 2% (w/v) solution of zinc sulphate in methanol–water (1 : 1 v/v) in order to deproteinize the samples. After immediate mixing and centrifugation at 13000 rpm for 3 min, 20 μl of the clear supernatant was analyzed by HPLC for remaining derivative and TRH as described above.

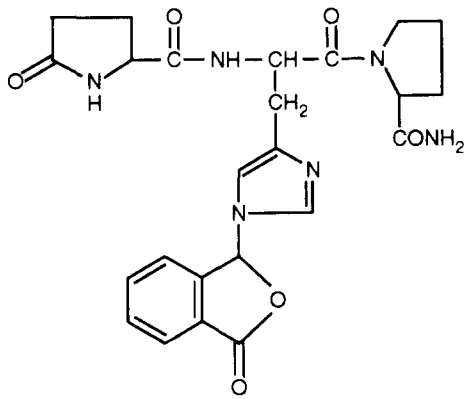
The stability of N-phthalidyl TRH in the presence of pyroglutamyl aminopeptidase (PAPase I) (a calf liver preparation obtained from Boehringer, Mannheim, Germany) was examined at 37 °C under conditions previously described (Bundgaard and Møss, 1989).

Determination of partition coefficient

The partition coefficient of N-phthalidyl TRH between octanol and 0.05 M phosphate buffer solution (pH 7.40) was determined using a previously described procedure (Bundgaard and Møss, 1990).

Results and Discussion

The N-phthalidyl derivative of TRH (I) was prepared by alkylation of its imidazole moiety with 3-bromophthalide. A similar alkylation of imidazole as well as of tertiary aliphatic amines has previously been reported (Bodor et al., 1980; Sloan and Koch, 1983). Since TRH exists in two tautomeric forms, the N(1)-H-tautomer and the N(3)-H-tautomer, alkylation of the imidazole group may occur either at N(1) or at N(3). HPLC analysis of the product formed upon reaction of TRH with 3-bromophthalide revealed only one peak. Since the tautomeric equilibrium of TRH in its free base form is shifted toward the N(3)-form (Giralt et al., 1986), the position of alkylation may most likely be assigned to the N-3 atom as shown in the structural formula of compound



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I. This assignment is also supported by steric reasons as the N(3) ring nitrogen is less hindered (cf. Emmett et al. (1979)).

Stability of N-phthalidyl TRH in aqueous solution

The kinetics of degradation of N-phthalidyl TRH was studied in aqueous solution at 37 °C over the pH-range 0.7–9.8. At constant pH and temperature the disappearance of the derivative displayed strict first-order kinetics over several half-lives. Some typical first-order plots are shown in Fig. 1.

The influence of pH on the rate of degradation is shown in Fig. 2 where the logarithm of the

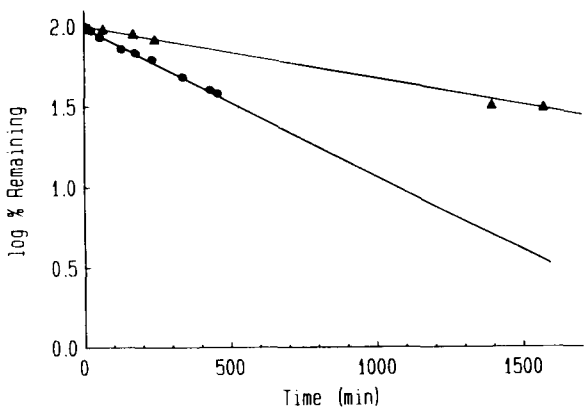


Fig. 1. Plots showing first-order kinetics of degradation of the phthalidyl TRH derivative I at 37 °C in a 0.02 M phosphate buffer solution of pH 7.40 (▲) and in human plasma (●).

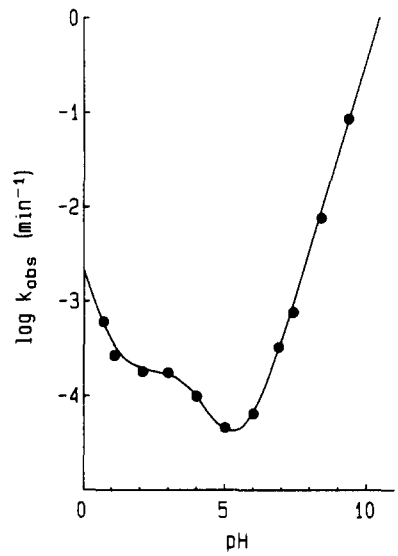


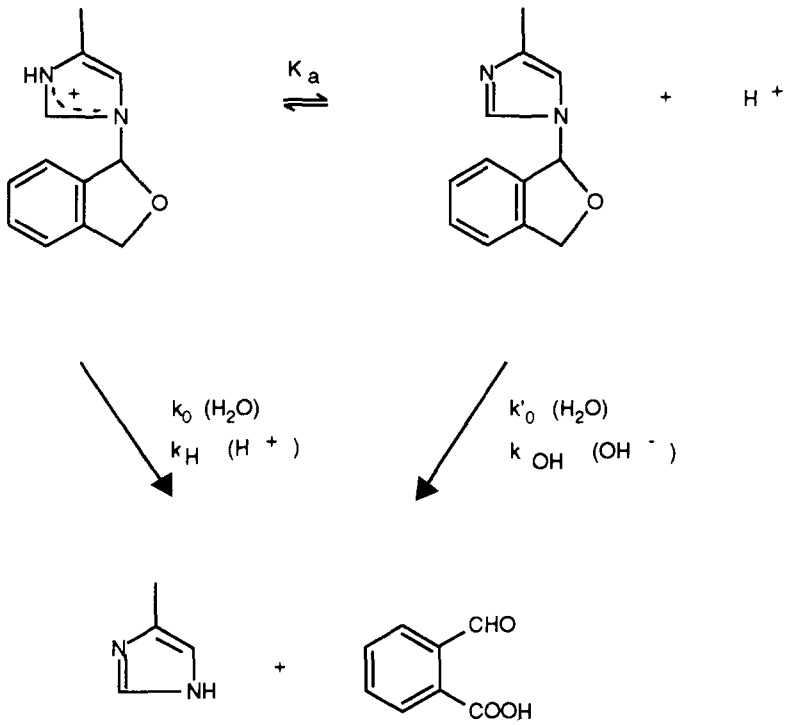
Fig. 2. The pH rate profile for the hydrolysis of N-phthalidyl TRH (I) in aqueous solution ($\mu = 0.5$) at 37 °C.

observed pseudo-first-order rate constants (k_{obs}) is plotted against pH. No significant buffer catalysis was observed at the low concentration (0.02 M) used. The shape of the pH-rate profile in Fig. 2 indicates that the degradation of N-phthalidyl TRH can be accounted for in terms of specific acid-catalyzed and water-catalyzed reactions of the protonated form and a spontaneous or water-catalyzed and a specific base-catalyzed reaction of the unprotonated form (Scheme 2). Mathematically,

$$k_{obs} = (k_H a_H + k_0)\frac{a_H}{a_H + K_a} + (k_0' + k_{OH} a_{OH})\frac{K_a}{a_H + K_a} \quad (1)$$

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 compound I, $a_H/(a_H + K_a)$ and $K_a/(a_H + K_a)$ are the fractions of the derivative in protonated and unprotonated form, respectively, and k_H, k_0, k_0' and k_{OH} are rate constants referring to the reactions shown in Scheme 2.

The following values of the specific rate and



Scheme 2.

ionization constants for I (37 °C; $\mu = 0.5$) were obtained from the pH-rate profile and Eqn 1: $k_H = 2.0 \times 10^{-3}$ M^{-1} min^{-1}; $k_0 = 1.8 \times 10^{-4}$ min^{-1}; $k'_0 = 3.0 \times 10^{-5}$ min^{-1}; $k_{OH} = 1.5 \times 10^3$ M^{-1} min^{-1}; $pK_a = 3.9$.

A kinetically equivalent reaction to the k_0 reaction is a specific acid-catalyzed degradation of the unprotonated form. The present data do not make it possible to distinguish between these reactions. The k_0-reaction is preferred, however, due to the high electron-attracting power of a protonated imidazole group, making a water reaction on the phthalidyl carbonyl group facile.

The pK_a value of the imidazole group in TRH is 6.25 at 25 °C (Grant et al., 1972). The considerable decrease of the pK_a value achieved by attachment of the phthalidyl moiety can be ascribed to the strong electron-attracting properties of this N-α-acyloxyalkyl group. A similar lowering of basicity has been seen upon N-acyloxymethylation of the imidazole group in cimetidine (Buur and Bundgaard, 1991).

In a separate experiment, the pH rate profile for the degradation of N-phthalidyl imidazole (prepared as described by Sloan and Koch (1983)) was determined. The stability of this compound turned out to be almost identical to that of N-phthalidyl TRH at pH 1–9.8, thus showing a lack of influence of the rest of the structure of TRH upon the reactivity of the phthalidyl group. At pH 7.4 and 37 °C the half-life of degradation of N-phthalidyl TRH is 15.2 h whereas that of N-phthalidyl imidazole was found to be 20.0 h.

Comparing the stability of N-phthalidyl TRH with that of N-alkoxycarbonyl TRH derivatives previously studied (Bundgaard and Møss, 1990) shows that the stability is almost the same in neutral and alkaline solutions, whereas the phthalidyl derivative is considerably more stable at acidic pH values. Both types of derivatives

show maximal stability at pH 5–6. At the pH of maximal stability and 37 °C the phthalidyl derivative shows a half-life of 270 h whereas that for N-octyloxycarbonyl TRH is 126 h (Bundgaard and Møss, 1990).

The degradation of N-phthalidyl TRH in aqueous solution proceeded with the quantitative formation of TRH and phthalaldehydic acid as revealed by HPLC analysis of the reaction solutions. An example of a product analysis is shown in Fig. 3. As can be seen the rate of formation of both TRH and phthalaldehydic acid followed strict first-order kinetics with no occurrence of any lag period. In agreement with earlier findings of N-phthalidyl derivatives of amides (Bundgaard et al., 1988) the mechanism of degradation most likely involves hydrolytic opening of the lactone ring as the rate-determining step to give an N-hydroxybenzyl derivative which spontaneously decomposes to TRH and phthalaldehydic acid (Scheme 3).

Stability in human plasma and other media

At initial concentrations up to 10^{-3} M N-phthalidyl TRH was found to degrade according to first-order kinetics in human plasma solutions at 37 °C (Fig. 1). The half-life observed (5.4 h) was significantly shorter than that in a pH 7.4 buffer solution (Table 1), indicating the occurrence of plasma catalysis. The reaction occurring in human plasma was found to be due exclusively to hydrolysis of the lactone ring with the quantitative formation of TRH. The time-course of TRH formation observed upon degradation of N-phthalidyl TRH at an initial concentration of 10^{-3} M is shown in Fig. 4. The solid curve for TRH in Fig. 4 was drawn on the basis of the assumption that N-phthalidyl TRH degraded exclusively to TRH, using the procedure of kinetic analysis previously described (Bundgaard and Møss, 1990). The good agreement observed between the experimental data and the kinetic model for a quantitative conversion to TRH and

TABLE 1

Half-lives ($t_{1/2}$) for the degradation of TRH and N-phthalidyl TRH in various media at 37 °C

Compound	$t_{1/2}$			
	Buffer pH 7.4	Human plasma	20% rabbit gut homogenate	PAPase I solution [a]
TRH	stable	9.4 min [b]	91 min	4 min
N-Phthalidyl TRH (I)	15.2 h	5.4 h	86 min	5 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 $^{-1}$).

[b] Half-life of hydrolysis at a TRH concentration less than 5×10^{-6} M (from Bundgaard and Møss (1990)).

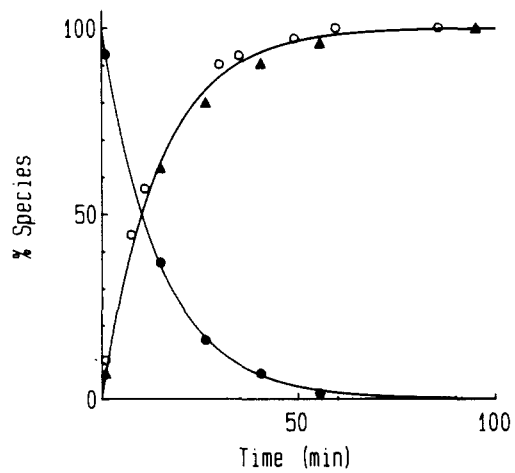


Fig. 3. Time courses for N-phthalidyl TRH (●), TRH (○) and phthalaldehydic acid (▲) during hydrolysis of compound I in 0.02 M borate buffer, pH 9.4 (at 37 °C).

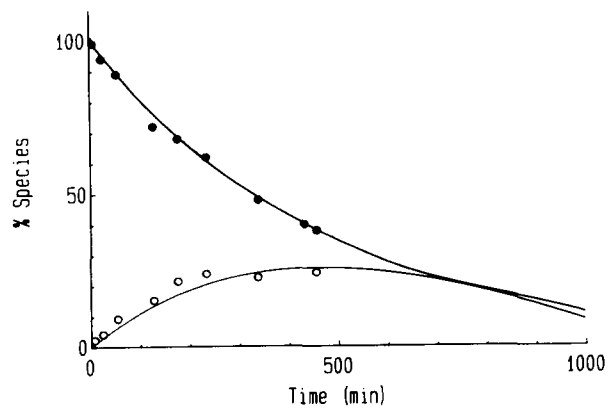
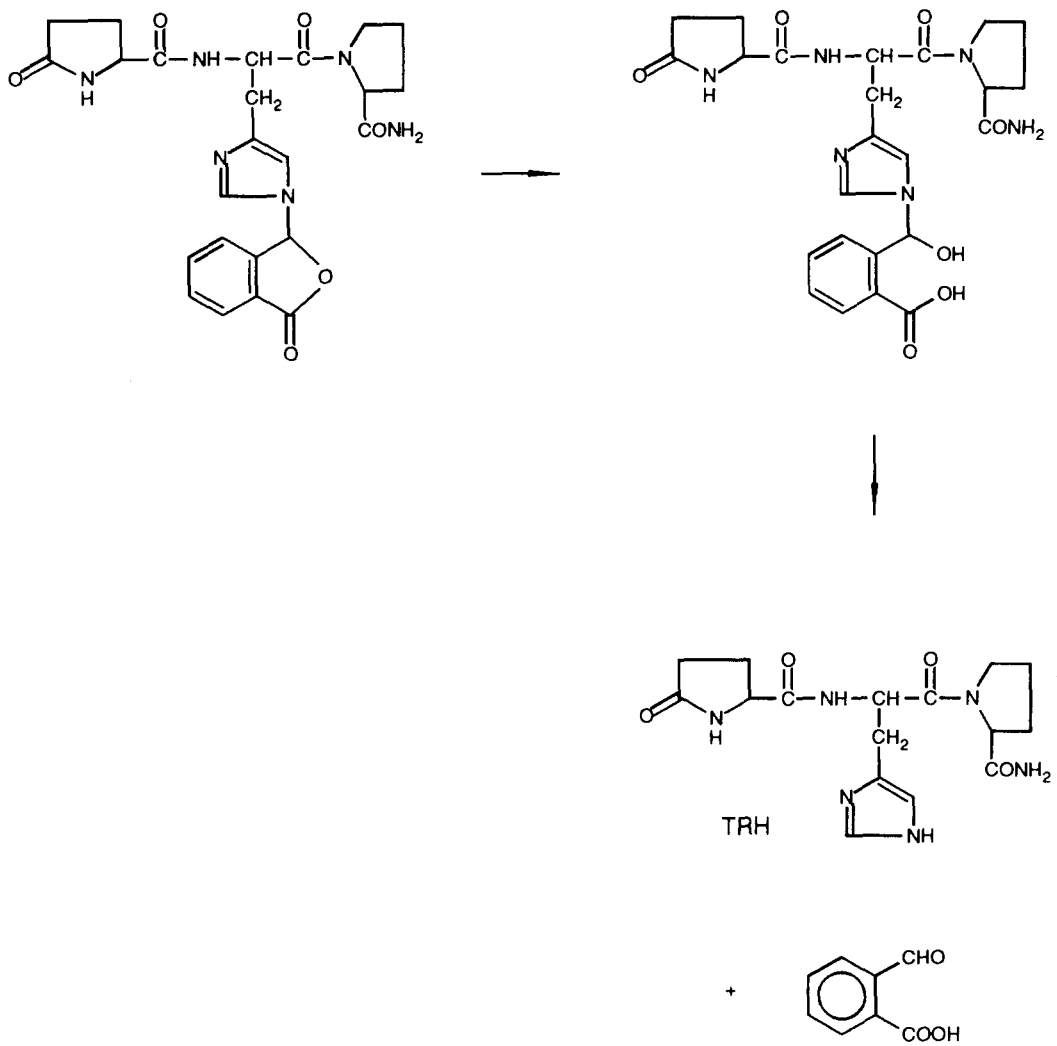


Fig. 4. Time courses for N-phthalidyl TRH (●) and TRH (○) following incubation of compound I in human plasma at 37 °C at an initial concentration of 10^{-3} M.



Scheme 3.

its subsequent degradation demonstrates that the sole or predominant (> 90%) reaction of N-phthalidyl TRH in human plasma is hydrolysis of its lactone ring to yield TRH.

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) (Møss and Bundgaard, 1990a). This reaction follows Michaelis-Menten kinetics and at TRH concentrations higher than its K_m value (1.9×10^{-5} M) the rate of hydrolysis follows zero-order kinetics with a rate constant of 1.4 μmol min^{-1} (Møss and Bundgaard, 1990a). At low substrate concentrations ($< K_m$), the enzymatic reaction is first-order with a half-life of 9.4 min (Møss and Bundgaard, 1990a). As the degradation of the N-phthalidyl derivative proceeds according to first-order kinetics with a half-life of 5.4 h at all concentrations up to at least 10^{-3} M it can readily be seen that this derivative greatly stabilizes TRH against inactivation at pharmacologically relevant concentrations and can serve as a depot for the supply of the peptide.

The stability of N-phthalidyl TRH was also

determined and compared with that of TRH in 20% rabbit gut homogenate and in the presence of pyroglutamyl aminopeptidase PAPase I. The latter is a cysteine protease which is less specific than PAPase II and which occurs in many different tissues such as liver and brain but not in blood (for references, see Bundgaard and Møss (1990)). The results obtained are listed in Table 1. It is seen that the attachment of a phthalidyl group to the imidazole moiety of TRH does not provide any significant stabilization of the peptide towards degradation by PAPase I or by enzymes, e.g., prolyl endopeptidase, present in the gut homogenate. In this regard N-phthalidyl TRH resembles N-alkoxycarbonyl TRH derivatives (Bundgaard and Møss, 1990; Møss et al., 1990).

Lipophilicity

The logarithmic value of the partition coefficient (P) of N-phthalidyl TRH between octanol and pH 7.4 aqueous buffer was determined to be −1.25. The corresponding log P value for TRH is −2.46 (Bundgaard and Møss, 1990).

Conclusions

The results obtained show that attachment of a phthalidyl group to the imidazole moiety of TRH makes its pyroglutamyl peptide bond resistant toward cleavage by the TRH-specific pyroglutamyl aminopeptidase serum enzyme. This parallels the behaviour of N-alkoxycarbonyl derivatives (Bundgaard and Møss, 1990) but contrasts with simple methylation of the imidazole group which only affords a very minor degree of protection against plasma-catalyzed inactivation (Dvorak and Utiger, 1977; Morley et al., 1979). Apparently, the PAPase II enzyme tolerates the small methyl group but not the large phthalidyl group. Since the N-phthalidyl group is readily removed from TRH by spontaneous or enzymatic hydrolysis effected by plasma enzymes (esterases) not attacking the pyroglutamyl peptide bond, N-phthalidylation of TRH may thus be a promising approach to obtain prodrug forms of TRH with the aim of protecting the peptide against rapid inactivation in the systemic circulation. Regarding the feasibility of obtaining lipophilic prodrug forms capable of penetrating the blood-brain barrier, the lipophilicity of N-phthalidyl TRH is certainly too low. However, it should be recognized that it is readily possible to increase the lipophilicity by introducing, e.g., alkyl groups in the phthalidyl phenyl moiety. In several respects, the N-phthalidyl derivative shares the properties of N-alkoxycarbonyl derivatives of TRH (Bundgaard and Møss, 1990). Both types of derivatives protect TRH against cleavage by PAPase II but not against PAPase I or prolyl endopeptidase present in intestinal homogenates. Future studies in this laboratory will focus on the creation of bioreversible derivatives that can bring about such protection.

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