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Prodrugs of peptides. 5. Protection of the pyroglutamyl residue against pyroglutamyl aminopeptidase by bioreversible derivatization with glyoxylic acid derivatives

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Summary

Various *N*-hydroxyalkyl and *N*-acyloxyalkyl derivatives of L-pyroglutamyl benzylamide, used as a model pyroglutamyl peptide, were synthesised by reacting it with glyoxylic acid followed by esterification of the carboxy- or hydroxyl group. Whereas the pyroglutamyl benzylamide was rapidly hydrolyzed by pyroglutamyl aminopeptidase, the glyoxylic acid adducts were totally resistant to cleavage by the enzyme. On the other hand, these derivatives are readily bioreversible, being converted to the parent pyroglutamyl benzylamide by spontaneous or plasma-catalyzed hydrolysis. The stability of the derivatives in aqueous solution as a function of pH was studied. The results suggest that by *N*-α-hydroxyalkylation or *N*-α-acyloxyalkylation using glyoxylic acid or esters or amides thereof it may be feasible to protect pyroglutamyl-containing peptides against cleavage by pyroglutamyl aminopeptidase and hence improve the delivery characteristics of such peptides.

Introduction

The application of peptides as clinically useful drugs is associated with substantial delivery problems. Most peptides are rapidly metabolized by proteolysis at most routes of administration, they are in general non-lipophilic compounds with poor biomembrane penetration characteristics and they possess short biological half-lives due to rapid metabolism (Wiedhaup, 1981; Davis, 1986; Humphrey and Ringrose, 1986; Lee, 1986; Bundgaard, 1986). A possible approach to solve these delivery problems, especially in case of small peptides, may be derivatization of the bioactive peptides to produce prodrugs or transport forms which possess enhanced physicochemical properties in comparison to the parent compounds as regards transport and metabolic stability. Thus, such derivatization may protect small peptides against degradation by peptidases present at the mucosal barrier and render hydrophilic peptides more lipophilic and hence facilitate their absorption. To be useful, however, the prodrug derivatives should be cleaved spontaneously or enzymatically in the blood following their absorption with quantitative release of the parent bioactive peptide (Bundgaard, 1986).

As a part of studies in progress in this laboratory to develop various types of bioreversible derivatives for the functional groups or chemical entities occurring in amino acids and peptides

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(Klixbüll and Bundgaard, 1984; Bundgaard, 1985, 1986; Larsen and Bundgaard, 1986; Buur and Bundgaard, 1988), we have recently shown that *N*-acylation or *N*-aminomethylation of the pyroglutamyl group of the model peptide L-pyroglutamyl benzylamide (**I**) makes it feasible to obtain derivatives (Fig. 1) which are completely resistant to attack by pyroglutamyl aminopeptidase and at the same time capable of releasing the parent pyroglutamyl derivative by spontaneous or plasma-catalyzed hydrolysis (Bundgaard and Møss, 1989). An *N*-terminal pyroglutamyl residue occurs in several peptides and proteins such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH) and gastrin (Doolittle and Armentrout, 1968; Orlowski and Meister, 1971) and the specific cleavage of this residue is effected by pyroglutamyl aminopeptidase (also called L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8) (Doolittle and Armentrout, 1968; Orlowski and Meister, 1971; Abraham and Podell, 1981; Fujiwara et al., 1979; Browne and O'Cuinn, 1983; Griffiths and McDermott, 1983) or, in the case of TRH, by a TRH-specific pyroglutamyl aminopeptidase as well (Bauer, 1988; Wilk, 1986; Wilk et al., 1988).

As shown in the previous study (Bundgaard and Møss, 1989), L-pyroglutamyl benzylamide (**I**) is a good substrate for pyroglutamyl aminopeptidase and therefore, it is a useful model compound

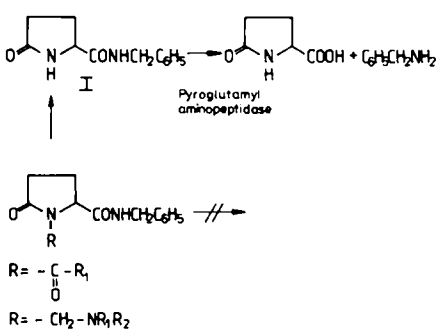
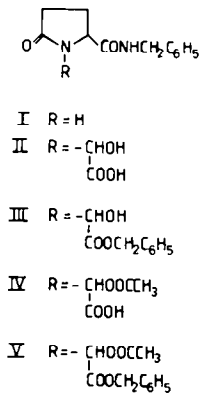


Fig. 1. L-Pyroglutamyl benzylamide (**I**) is readily hydrolyzed by pyroglutamyl aminopeptidase to L-pyroglutamic acid and benzylamine. In contrast, the *N*-acyl or *N*-Mannich base derivatives of **I** are totally resistant to undergo cleavage by the enzyme but capable of being converted to **I** by spontaneous (*N*-Mannich bases) or plasma-catalyzed (*N*-acyl derivatives) hydrolysis (Bundgaard and Møss, 1989).



for prodrug studies aiming at protecting the pyroglutamyl residue of peptides against enzymatic cleavage. In the present work, *N*-α-hydroxyalkyl derivatives of compound I derived from glyoxylic acid and esters thereof (**II**–**V**) have been explored as further potentially useful prodrug types for the *N*-terminal pyroglutamyl group in peptides.

Materials and Methods

Apparatus

Nuclear magnetic resonance spectra were obtained with a Varian 360 L instrument. Melting points were taken on a capillary melting point apparatus and are uncorrected. Readings of pH were carried out on a Radiometer Type PHM 26 meter at the temperature of study. High-performance liquid chromatography (HPLC) was done with a Kontron apparatus consisting of an LC-pump T-414, a Uvikon LC UV detector, a 20-μl injection valve and a Chrompack column (100 × 3 mm) packed with Chromspher C 18 or C 8 (5 μm particles). Elemental analysis was performed at the Microanalytical Laboratory, Leo Pharmaceutical Products, Ballerup, Denmark.

Preparation of the derivatives I–VI

L-Pyroglutamyl benzylamide (I) was prepared by aminolysis of L-pyroglutamic acid ethyl ester as previously described (Bundgaard and Møss, 1989).

The glycolic acid derivative II was obtained by refluxing a solution of compound **I** (5.45 g, 25

mmol) and glyoxylic acid monohydrate (2.54 g, 27.5 mmol) in 25 ml of acetonitrile for 8 h. Upon standing at 4° C overnight compound **II** separated from the reaction solution as a crude diastereoisomeric mixture. HPLC analysis (RP8 column eluted with 20% v/v methanol in 0.02 M acetate buffer pH 4.0) indicated a mixture of diastereoisomers in the proportion 8 : 1, the retention time being 3.5 min for the major component and 3.0 min for the minor component. By recrystallizing twice from ethanol a 60% overall yield of an essentially pure preparation of the less polar racemate (**II**) was obtained, m.p. 150–151° C.

Anal.: calc. for $C_{14}H_{16}N_2O_5$: C, 57.53; H, 5.52; N, 9.58; found: C, 57.70; H, 5.62; N, 9.40.

*Benzyl glycolate derivative **III***. A mixture of compound **II** (1.17 g, 4 mmol), triethylamine (0.56 ml, 4 mmol) and benzyl bromide (0.48 ml, 4 mmol) in 4 ml of *N,N*-dimethylformamide was stirred at room temperature overnight and then poured into water (50 ml). The crystalline precipitate formed upon cooling to 4° C was filtered off, washed with water, dried and recrystallized from aqueous ethanol to provide 0.80 g of compound **III**, m.p. 129–130° C.

Anal.: calc. for $C_{21}H_{22}N_2O_5$: C, 65.96; H, 5.80; N, 7.33; found: C, 66.11; H, 5.86; N, 7.28.

*The α-acetoxyglycolic acid derivative **IV*** was obtained by acetylation of compound **II**. A mixture of compound **II** (1.17 g, 4 mmol), acetic anhydride (1 ml) and pyridine (2 ml) was stirred at room temperature overnight. The solution was concentrated in vacuo, poured into 0.5 M hydrochloric acid (30 ml) and extracted with ethyl acetate (50 ml). The ethyl acetate solution was washed with water (2 × 30 ml), dried over anhydrous sodium sulphate and evaporated under reduced pressure to leave a residue which crystallized from ethanol–ether–petroleum ether to give 0.8 g of compound **IV**, m.p. 113–114° C.

Anal.: calc. for $C_{16}H_{18}N_2O_6$: C, 57.49; H, 5.43; N, 8.38; found: C, 57.29; H, 5.52; N, 8.27.

*The α-acetoxy glycolate ester **V*** was prepared by acetylation of compound **III** by a procedure similar to that used in the preparation of compound **IV**. The oil obtained after evaporation of the ethyl acetate extract crystallized from ethyl acetate–ethanol–petroleum ether, m.p. 105–106° C.

Anal.: calc. for $C_{23}H_{24}N_2O_6$: C, 65.08; H, 5.70; N, 6.60; found: C, 65.16; H, 5.69; N, 6.53. The ^{1}H-NMR spectra of compounds **I–V** were in agreement with their structures.

*N-(Hydroxymethyl)-2-pyrrolidinone (**VI**)* was prepared by reacting pyrrolidone with paraformaldehyde as described by Böhme et al. (1961), m.p. 83–84° C (from benzene), rep. m.p. 83–84° C.

Kinetic measurements

The degradation of the derivatives **II–V** was studied in aqueous buffer solutions at 37 ± 0.2° C. Hydrochloric acid, acetate, phosphate, borate and carbonate buffers were used; 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 hydrolysis of the derivatives were followed by using a reversed-phase HPLC procedure. Mobile phase systems of 20–25% v/v methanol in 0.02 M acetate buffer pH 4.0 were used for compounds **II** and **IV** whereas for compounds **III** and **V**, mixtures of methanol, acetonitrile (10%) and 0.02 M acetate buffer pH 4.0 were used, the concentration of methanol being adjusted for each compound to give an appropriate retention time (2–5 min). The solvent systems used allowed separation of the compounds from their products of degradation. The column effluent was monitored at 215 or 254 nm and the flow rate was 1 ml/min. For the determination of compound **I** formed upon hydrolysis a mobile phase system consisting of methanol–0.02 M acetate buffer pH 4.0 (1 : 5 v/v) was used. The flow rate was 0.8 ml/min 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 the compounds in acetonitrile to 10 ml of preheated buffer solution in screw-capped test tubes, the final concentration of the compounds being about 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 stability studies in plasma, the derivatives II–V were incubated at 37 °C in human plasma diluted to 80% with 0.05 M phosphate buffer of pH 7.40. The initial concentration of the derivatives was 2×10^{-4} M. At appropriate intervals samples of 250 μl of the plasma reaction solution were withdrawn and added to 500 μl of a 2% solution of zinc sulphate 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 by HPLC as described above.

Degradation studies in the presence of pyroglutamyl aminopeptidase

The stability of the compounds **I–V** 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 of pH 7.4 containing 1 mM disodium edetate and 0.5 mM dithiothreitol, 500 μl of an aqueous solution of the enzyme at a concentration of 0.116 U per ml and 50 μl of a stock solution (10^{-2} M) of the compounds in acetonitrile as previously described for compound **I** (Bundgaard and Møss, 1989). At appropriate intervals samples were taken and immediately chromatographed as described above for the degradation studies in buffer solutions.

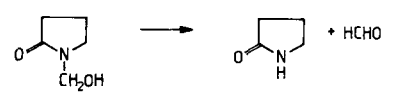
Degradation of N-(hydroxymethyl)-2-pyrrolidinone (VI)

The kinetics of degradation of compound **VI** was determined in aqueous buffer solutions of pH 9–11 at 37 °C. The progress of hydrolysis was monitored by determining formaldehyde formed during the hydrolysis by the spectrophotometric method previously described (Johansen and Bundgaard, 1979; Johansen et al., 1983).

Results and Discussion

L-Pyroglutamyl benzylamide (**I**) was used as a model for the pyroglutamyl residue in pyroglutamyl-containing peptides. As previously reported (Bundgaard and Møss, 1989), compound **I** is a good substrate for pyroglutamyl aminopeptidase. At the enzyme reaction conditions described above the compound hydrolyzed according to first-order kinetics to give L-pyroglutamic acid and benzylamide, the half-life of hydrolysis being 10 min. In the absence of the enzyme compound **I** is completely stable (Bundgaard and Møss, 1989).

The derivatives of **I** assessed as potential pyroglutamyl aminopeptidase-resistant prodrugs (**II–V**) are *N*-α-hydroxyalkyl compounds formed by reaction of **I** with glyoxylic acid followed by esterification of the carboxy or hydroxy group in compound **II**. The weakly acidic character of the NH-group in the pyrrolidone moiety of **I** (pK_a being estimated to be in the range of 15–17) suggested *N*-α-acyloxyalkylation to be a useful means of obtaining bioreversible derivatives of the pyroglutamyl group. Simple *N*-hydroxymethylation might, in contrast, not be a useful approach because of the too weak acidity of the secondary amide group in the pyrrolidone ring of pyroglutamyl peptides, resulting in a too high stability of an *N*-hydroxymethyl derivative at physiological pH and temperature (Johansen and Bundgaard, 1979; Bundgaard, 1985). Thus, *N*-hydroxymethyl derivatives of benzamide and other carboxamides (pK_a 14–15) show half-lives of hydrolysis of 100–200 h at pH 7.4 and 37 °C (Johansen and Bundgaard, 1979; Bundgaard and Buur, 1987). In agreement with this prediction, the *N*-hydroxymethyl derivative of pyrrolidone (compound **VI**) proved to be highly stable in aqueous solution. At 37 °C and at pH 9–11 the hydrolysis of compound **VI** (Scheme 1) was found to be specific base-catalyzed, the second-order rate constant k_{OH} being 7.9 M^{-1} min^{-1}. From this value a half-life of hydrolysis of 6×10^3 h at pH 7.4 and 37 °C can be estimated.



VI

Scheme 1.

In contrast to *N*-hydroxymethylation, *N*-hydroxyalkylation using glyoxylic acid or esters and amides thereof as the aldehyde component should be a more promising approach in view of a previous study (Bundgaard and Buur, 1987) in which it was shown that glyoxylate adducts of benzamide are readily hydrolyzed at pH 7.4 and 37 °C. As described below a similar facile hydrolysis occurs for the glyoxylate adducts or glycolic acid derivatives **II** and **III**.

Kinetics of hydrolysis of II–V

The kinetics of hydrolysis of the glyoxylate adducts **II** and **III** and their acetate esters (**IV** and **V**) were studied in aqueous buffer solutions at 37 °C over a wide range of pH. At constant pH and temperature the disappearance of the derivatives displayed strict first-order kinetics over several half-lives. An example is shown in Fig. 2. No significant buffer catalysis was observed at buffer concentrations up to 0.05 M.

The influence of pH on the hydrolysis rate is shown in Fig. 3, where the logarithms of the observed pseudo-first-order rate constants (k_{obs}) are plotted against pH. The observed pH-rate relationships indicate that the overall hydrolysis can be described in terms of a water-catalyzed or spontaneous reaction and specific acid- and base-catalyzed reactions according to the following rate expression:

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

where a_H and a_{OH} refer to the hydrogen ion and hydroxide ion activity, respectively. The latter was calculated from the measured pH at 37 °C as previously described (Bundgaard and Buur, 1987). Values of the second-order rate constants k_H and k_{OH} and the first-order rate constant for spontaneous hydrolysis (k_0) determined from the pH-rate profiles and on the basis of Eqn. 1 are listed in Table 1. In Fig. 3 the solid curves drawn for compounds **II**, **III** and **V** were constructed from these constants and Eqn. 1. In Table 2 the half-lives of hydrolysis of the compounds at pH 7.4 and 37 °C are given.

In contrast to the ester derivative **III** the free acid derivative **II** exhibits no significant specific acid-catalyzed hydrolysis. A similar finding was made for the corresponding glyoxylate derivatives of benzamide (Bundgaard and Buur, 1987). Also, since there is no break in the pH–rate profile for

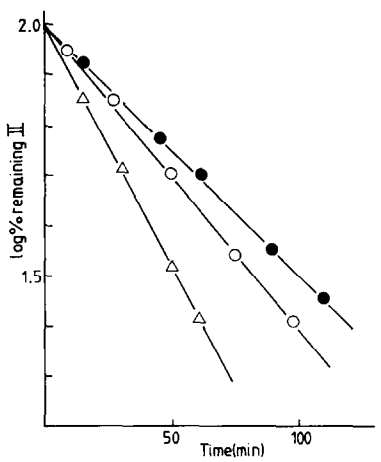


Fig. 2. Plots showing the apparent first-order kinetics of degradation of compound **II** at 37 °C in a 0.02 M phosphate buffer solution of pH 7.40 (○), in 80% human plasma (△) and in buffer solution containing pyroglutamyl aminopeptidase (●).

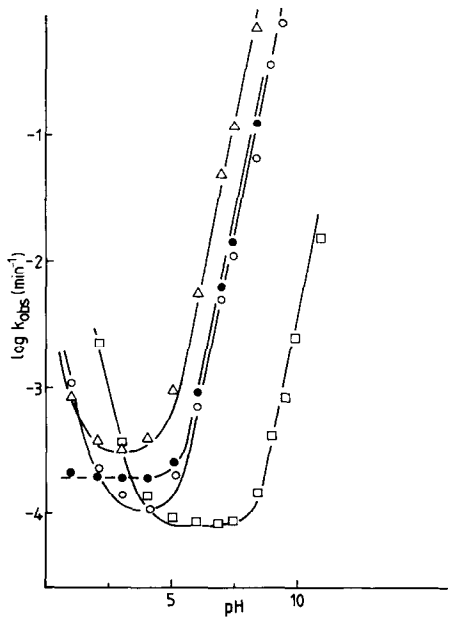


Fig. 3. The pH-rate profiles for the degradation of compound **II** (●), compound **III** (△), compound **IV** (□) and compound **V** (○) in aqueous solution at 37 °C.

TABLE 1

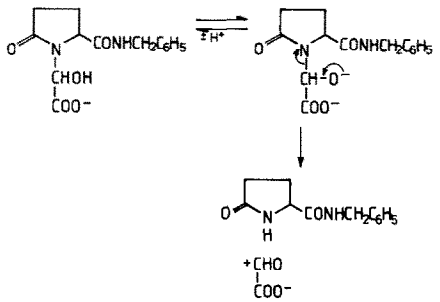
Rate data for the hydrolysis of compounds II–V in aqueous solution at 37 °C and μ = 0.5

Compound	k_H ($M^{-1}min^{-1}$)	k_0 (min^{-1})	k_{OH} ($M^{-1}min^{-1}$)
II	–	2.0×10^{-4}	3.3×10^{4}
III	8.5×10^{-3}	3.2×10^{-4}	2.1×10^{5}
IV	2.5×10^{-1}	9.1×10^{-5}	16.6
V	1.1×10^{-2}	1.2×10^{-4}	2.1×10^{4}

compound **II** around pH values corresponding to the pK_a for its carboxylic acid group (3–3.5, cf. Bundgaard and Buur (1987)), there is no measurable difference in the reactivity of the ionized and unionized species. Interestingly, the isomeric form of compound **II** formed to a minor extent during the synthesis (see Materials and Methods) was about 7-fold more labile at pH 7.4 than the diastereomeric form actually isolated.

As previously discussed for benzamide-glyoxylic acid adducts (Bundgaard and Buur, 1987), the decomposition of compound **II** (and **III**) in neutral and alkaline solutions most likely involves a stepwise pathway with anionic *N*-hydroxyalkyl amide as an intermediate undergoing rate-determining *N*-*C* cleavage (Scheme 2).

Esterification of the hydroxyl group in compounds **II** and **III** giving the derivatives **IV** and **V**, respectively, is seen to afford a stabilization against hydrolytic decomposition. By blocking the hydroxyl group the decomposition along the pathway shown in Scheme 2 can no longer take place, and since the amide portion in the pyrrolidone moiety of the compounds contains no free hydrogen atoms an S_N1 reaction with the formation of an *N*-acylimine intermediate is furthermore not possible. Such a reaction has recently been shown to account for the extremely rapid degradation of *N*-acyloxyalkyl derivatives of primary amides including benzamide glyoxal adducts (Bundgaard and Nielsen, 1987).



Scheme 2.

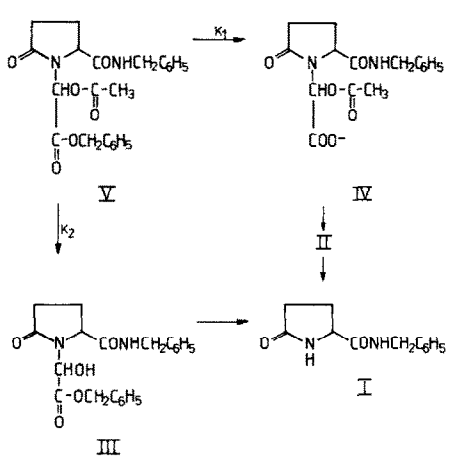
The stabilization observed by acetylation of the hydroxyl groups in compounds **II** and **III** is much more pronounced for the acid **II**, cf. the half-lives at pH 7.4 given in Table 2. The less marked stabilization seen for the acetyl ester (**V**) of compound **III** was shown to be due to a rather facile hydrolysis of the benzyl ester moiety in **V**. Thus, in neutral and alkaline solutions compound **V** hydrolyzed by two parallel pathways as depicted in Scheme 3 with the k_1-pathway predominating.

TABLE 2

Rate data for the hydrolysis of various glyoxylic acid derivatives of L-pyroglutamyl benzylamide at 37 °C

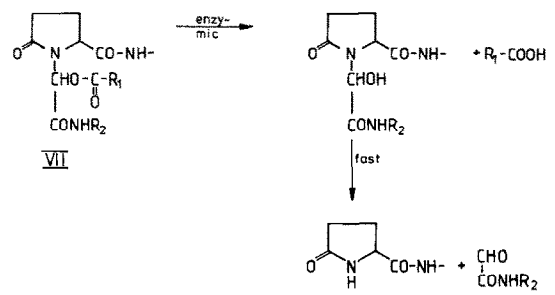
Compound	Half-lives		
	Buffer pH 7.4	80% human plasma	Enzyme solution
II	51 min	32 min	62 min
III	6 min	–	6 min
IV	133 h	121 h	143 h
V	64 min	12 min	61 min

Enzyme solution data are half-lives for the degradation in buffer solution (pH 7.4) containing pyroglutamyl aminopeptidase. Under identical conditions the half-life of hydrolysis of compound **I** is 10 min.



Scheme 3.

By HPLC analysis of the reaction solutions compound **IV** was found to be formed in an amount of 90%, indicating a k_1/k_2-ratio of 9 (at pH 6–10). Compound **V** can be regarded as a substituted *O*-acetyl glycolate ester and the greater susceptibility of the terminal ester moiety to undergo hydrolysis parallels the behaviour of various O-benzoyl glycolate esters (Nielsen and Bundgaard, 1987).



Scheme 4.

Hydrolysis in plasma solutions

The rates of decomposition of compounds **II**, **IV** and **V** were determined in 80% human plasma (pH 7.4) and 37 °C. The half-lives of hydrolysis observed are shown in Table 2. The carboxylic acid **II** was slightly more labile in plasma than in buffer solution whereas compound **V** was hydrolyzed markedly faster. HPLC analysis of the reaction solutions showed that compound **IV** was formed quantitatively from compound **V**, thus showing that the plasma-catalyzed degradation of **V** is solely or predominantly a catalysis of the benzyl ester hydrolysis (the k_1-pathway in Scheme 3). The hydrolysis of the ester moiety in compound **IV** is, on the other hand, not significantly catalyzed by plasma. The high resistance towards enzymatic hydrolysis by plasma may be due to the negative charge of the compound at pH 7.4 as is the case for benzoylglycolic acid and other esters containing an ionized carboxylate group (Nielsen and Bundgaard, 1987). It should be noted that although pyroglutamyl aminopeptidase occurs in many different tissues such as liver and kidney its activity in plasma is very low (Szewczuk and Kwiatkowska, 1970; Bauer and Nowak, 1979; Friedman et al., 1985). In accordance with this, the pyroglutamyl benzylamide (**I**) was found to remain completely stable in human plasma after incubation for 24 h at 37 °C.

Stability toward pyroglutamyl aminopeptidase

As stated above the parent pyroglutamyl benzylamide (**I**) is rapidly hydrolyzed to pyroglutamic acid and benzylamine in the presence of pyroglutamyl aminopeptidase, the half-life being 10 min at the conditions specified. Under the same conditions none of the derivatives **II**–**V** were found to be attacked by the enzyme. This is evident from the finding that the half-lives of hydrolysis of the compounds in the presence of pyroglutamyl aminopeptidase were quite similar to those of hydrolysis in buffer solutions without the enzyme (Table 2 and Fig. 2). Combined with the previous finding (Bundgaard and Møss, 1989) that *N*-acylation or *N*-aminomethylation of the pyrrolidone ring nitrogen makes compound **I** resistant toward pyroglutamyl aminopeptidase, this result indicates that the enzyme does not tolerate any replacement of the hydrogen in the cyclic amide moiety.

Conclusions

The results obtained suggest that *N*-α-hydroxyalkylation of pyroglutamyl peptides by glyoxylic acid or derivatives thereof may be a useful approach to obtain prodrug derivatives which on one hand are stable against pyroglutamyl aminopeptidase and on the other hand are readily bioreversible, releasing the parent pyroglutamyl peptide either by spontaneous hydrolysis at physiological pH or by hydrolysis catalyzed by hydrolytic enzymes in the organism. The prodrug concept described can be appropriately modified in order to match the delivery and release characteristics aimed at for a pyroglutamyl peptide. Thus, as demonstrated above for compound **IV**, a high stability can be achieved by esterification of the hydroxyl group in the glyoxylic acid adduct. Derivatives obtained with a glyoxylic acid ester such as compound **III** are more readily converted to the parent compound at physiological pH than a de-

rivative formed with glyoxylic acid itself, but due to the lability of its ester bond *O*-acylation does not result in the desirable combination of high stability in vitro and high lability in vivo (i.e. in the presence of plasma enzymes). By selecting a sterically hindered ester or, more preferably, an amide in combination with *O*-acylation, derivatives with this characteristic can certainly be obtained. For example, a derivative of the structure **VII** may be envisaged to be a pyroglutamyl peptidase resistant prodrug or double prodrug (Bundgaard, 1987) possessing a high chemical stability and a ready capability of releasing the parent peptide in vivo through a sequential process of enzymatic hydrolysis followed by a spontaneous decomposition (Scheme 4). Studies are in progress to apply this prodrug approach to clinically interesting pyroglutamyl peptides such as TRH.

Acknowledgements

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