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Prodrugs of peptides. 17. Bioreversible derivatization of the C-terminal prolineamide residue in peptides to afford protection against prolyl endopeptidase

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Summary

Various aliphatic *N*-acyl derivatives and an *N*-phthalidyl derivative of the model compound *N*-benzyloxycarbonyl-glycyl-L-prolineamide (Z-Gly-ProNH$_2$) were synthesized to assess their suitability as prodrug forms for the C-terminal prolineamide residue occurring in several peptides (e.g. TRH) with the aim of protecting the peptide against prolyl endopeptidase in the gut prior to absorption. Whereas Z-Gly-ProNH$_2$ was rapidly hydrolyzed in a rabbit gut homogenate, used as a source of prolyl endopeptidase, the *N*-acyl derivatives were found to afford protection by a factor of 1.5–6. The stability of the *N*-acyl derivatives in the gut homogenate decreased with increasing chain length within the acyl group. The *N*-phthalidyl derivative, on the other hand, degraded even faster than the parent compound. The derivatives were all converted quantitatively into the parent peptide in human plasma solutions via hydrolysis catalyzed by non-specific plasma esterases. The results suggest that by appropriate *N*-acylation it may be feasible to improve the stability of a C-terminal prolineamide moiety toward prolyl endopeptidase. The combination of increased stability in the intestine and higher lipophilicity of the *N*-acyl prodrugs might render it possible to improve the delivery characteristics of peptides containing a C-terminal prolineamide moiety.

Introduction

The potential clinical utility of thyrotropin-releasing hormone (TRH) in the management of various neurologic and neuropsychiatric disorders including depression, brain injury, acute spinal trauma, schizophrenia and Alzheimer's disease is hampered by its rapid metabolism, limited per-oral absorption and poor access to the brain (Griffiths, 1985, 1986, 1987; Horita et al., 1986; Loosen, 1988; Metcalf and Jackson, 1989). In recent papers, we have described the development of some bioreversible derivatives (prodrugs) of TRH which are completely resistant towards degradation by the TRH-specific pyroglutamyl aminopeptidase (PAPase II) in human blood and hence capable of protecting the parent TRH against rapid inactivation in vivo (Bundgaard and Møss, 1990; Møss and Bundgaard, 1991). The

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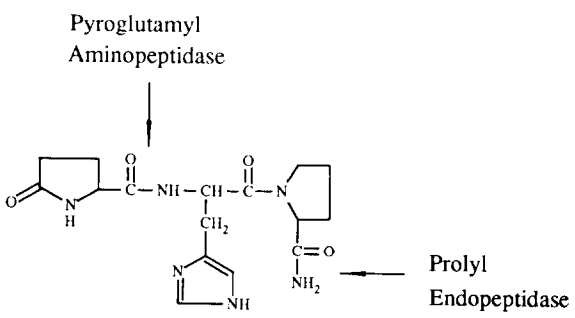
derivatives developed are *N*-alkoxycarbonyl and *N*-phthalidyl derivatives formed at the imidazole residue of TRH. These derivatives are readily bioreversible as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or plasma esterase-catalyzed hydrolysis. In addition, the prodrugs possess greatly increased lipophilicity relative to TRH which may render them more capable of penetrating the blood brain barrier or various other biomembranes such as the skin (Møss and Bundgaard, 1990) than the parent peptide.

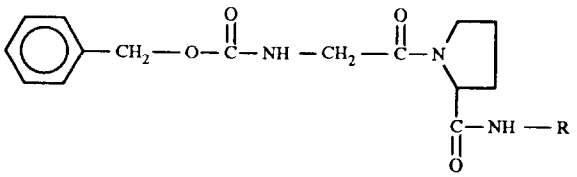
Despite having a high lipophilicity as expressed in terms of octanol-water partition coefficients these prodrug derivatives did not improve the penetration of TRH across various intestinal segments of the rat or albino rabbit as demonstrated with the modified Ussing chamber technique (Møss et al., 1990). A major reason for this was suggested to be facile degradation of the derivatives by prolyl endopeptidase in the gut tissues (Scheme 1) (Møss et al., 1990). This enzyme is a serine protease which cleaves the C-terminal prolineamide residue both in the prodrugs and in TRH to yield the respective acids. Therefore, prodrugs suitable for improving the oral absorption of TRH (which is only 1–2% in man (Yokohama et al., 1984; Duntas et al., 1988)) should not only possess a certain lipophilicity but also be resistant toward the prolyl endopeptidase enzyme present in the gut.

The objective of the present study was to evaluate various means of bioreversible derivatization



TRH

Scheme 1.



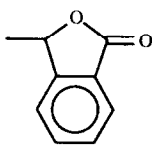
I R = H

II R = $C(=O)CH_3$

III R = $C(=O)CH_2CH_3$

IV R = $C(=O)CH(CH_3)_2$

V R = $C(=O)CH_2CH_2CH_2CH_2CH_3$

VI R = 

of the C-terminal prolineamide moiety with the aim of achieving protection of the amide bond against prolyl endopeptidase. Previously exploited bioreversible derivatives for an amide moiety include *N*-Mannich bases and *N*-hydroxymethyl derivatives (Bundgaard, 1985), glyoxylic acid adducts (Bundgaard and Buur, 1987) as well as *N*-phthalidyl derivatives (Bundgaard et al., 1988) and *N*-acyl derivatives (Kahns and Bundgaard, 1991a,b). In this work, we have used *N*-benzyloxycarbonyl-glycyl-L-prolineamide (Z-Gly-ProNH_2) (**I**) as a model compound because of its high susceptibility to prolyl endopeptidase. Various *N*-acyl derivatives (**II–V**) and an *N*-phthalidyl derivative (**VI**) have been prepared and their chemical stability as well as enzymatic stability in gut homogenates, used as a source of prolyl endopeptidase, and in human plasma determined.

Materials and Methods

Chemicals

Amino acids and *N*-protected amino acids and peptides were purchased from Bachem AG, Bubendorf, Switzerland. Chemicals and solvents used in the synthesis and the kinetic studies were from Aldrich-Chemie, Germany.

Synthesis of compounds I–VI

N-Benzyloxycarbonyl-glycyl-L-prolineamide (Z-Gly-ProNH$_2$) (**I**) was prepared by stirring a mixture of Z-Gly-*N*-hydroxysuccinimide ester (20 mmol, 6.14 g), L-prolineamide (20 mmol, 2.28 g) and triethylamine (20 mmol, 2.8 ml) in 100 ml of acetonitrile at room temperature for 2 h. The precipitate formed was filtered off and recrystallized from ethyl acetate-petroleum ether to give Z-Gly-ProNH$_2$ in a yield of 75%. M.p. 147–149°C (reported m.p. 150–151°C (Smith and Bergmann, 1944)).

The *N*-acyl derivatives (**II–V**) were prepared by reacting Z-Gly-ProNH$_2$ (1 mmol, 305 mg) with the appropriate acid anhydride (4 mmol) in a solution of acetonitrile (2 ml) containing 200 μl of sulphuric acid for 30 min at room temperature. Water (25 ml) was added and the mixture was extracted with ether. The organic phase was separated, dried over anhydrous sodium sulphate and evaporated in vacuo. The compounds were crystallized from ether-ethanol-petroleum ether, m.p. (**II**) 105–106°C. Anal.: Calc for $C_{17}H_{21}N_3O_5$: C, 58.78; H, 6.09; N, 12.10. Found: C, 58.65; H, 6.13; N, 11.99. The compounds **III-V** were contaminated with 10–20% of unreacted parent amide as revealed by HPLC analysis.

The *N*-phthalidyl derivative **VI** was prepared using the general procedure described by Wheeler et al. (1957). A mixture of equimolar amounts of Z-Gly-ProNH$_2$ and phthalaldehydic acid was kept at 135°C for 5 h. The solid obtained upon cooling to 20°C was recrystallized from ethanol-water, m.p. 138–140°C. Anal.: Calc. for $C_{23}H_{23}N_3O_6$, H_2O: C, 60.65; H, 5.53; N, 9.23. Found: C, 60.97; H, 5.54; N, 9.48.

HPLC assays

Z-Gly-ProNH$_2$ (**I**) and its derivatives were determined by isocratic reversed-phase HPLC procedures performed with a Merck Hitachi apparatus comprising a pump model L-6200, a Rheodyne 7125 injection valve with a 20 μl loop, a variable UV-detector L-4000 operated at 215 nm and an autosampler model 655A-40. For the analysis a ChromSep column (100–4.6 mm) packed with Microspher C_{18} (3-μm particles) and supplied with a ChromSep guard column (both from Chrompack) was eluted at ambient temperature with a mobile phase consisting of 0.1% v/v phosphoric acid containing acetonitrile (20–40% v/v). The flow rate was 1.0 ml min^{-1}. Quantitation of the compounds was performed by measuring the peak heights or peak areas in relation to those of standards chromatographed under the same conditions.

Kinetic measurements

Studies in aqueous solutions The decomposition of the compounds was studied in aqueous buffer solutions at 37 ± 0.2°C. The buffers used were phosphate and borate buffers; a total ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The reactions were initiated by adding 100 μl of a stock solution of the derivatives in acetonitrile to 10 ml of preheated buffer solution, 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.

Studies in human plasma and rabbit gut homogenate The compounds were incubated at 37°C in human plasma diluted to 80% with 0.05 M phosphate buffer of pH 7.4 or in a 20% rabbit gut homogenate (diluted with a 0.05 M phosphate buffer solution of pH 7.4 containing 0.15 M sodium chloride). The gut homogenate, derived from a mixture of the jejunum, ileum and colon, was prepared as previously described (Møss et al., 1990). Sodium edetate (EDTA) (5 mM) and

dithiothreitol (2 mM) were added to the homogenates to activate and stabilize prolyl endopeptidase (Knitsatschek and Bauer, 1979; Walter et al., 1980). The initial concentration of the derivatives was about 10^{-4} M. The mixtures were kept in a water-bath at 37°C and at appropriate intervals samples of 250 μl 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 samples. After immediate mixing and centrifugation at 13000 rpm for 3 min, 20 μl of the clear supernatant was analyzed by HPLC as described above.

Results and Discussion

N-Benzyloxycarbonyl-glycyl-L-prolineamide (Z-Gly-ProNH$_2$) (**I**) was used as a model for peptides such as TRH containing a C-terminal prolineamide residue. Rabbit gut homogenate has been reported to have a high prolyl endopeptidase activity (Orlowski et al., 1979; Møss et al., 1990) and was therefore used as a source of the enzyme in this study. As seen from Fig. 1 the degradation of Z-Gly-ProNH$_2$ was accompanied by the quantitative formation of the deamidated product (Z-Gly-Pro), indicating the presence of prolyl endopeptidase in the homogenate. Compound **I** appeared to be a good substrate for prolyl endopeptidase. In a 20% rabbit gut homogenate (pH 7.4 and 37°C) it hydrolyzed according to first-order kinetics with a half-life of 6 min. In aqueous buffer solution of pH 7.4 and in 80% human plasma the compound was completely stable.

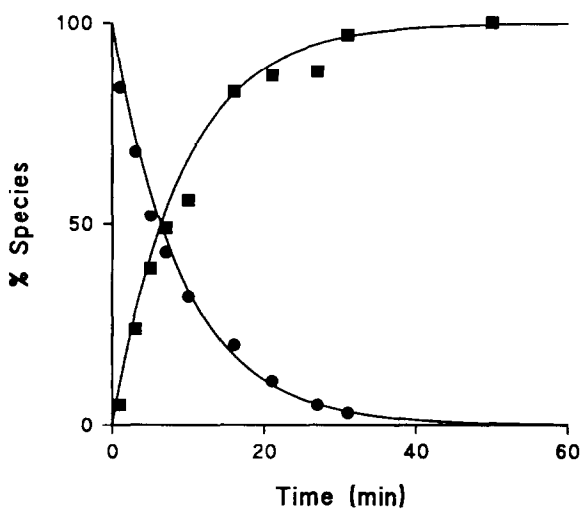


Fig. 1. Plots showing the time courses of degradation of Z-Gly-ProNH$_2$ (●) and formation of Z-Gly-Pro (■) in 20% rabbit gut homogenate (pH 7.4) at 37°C.

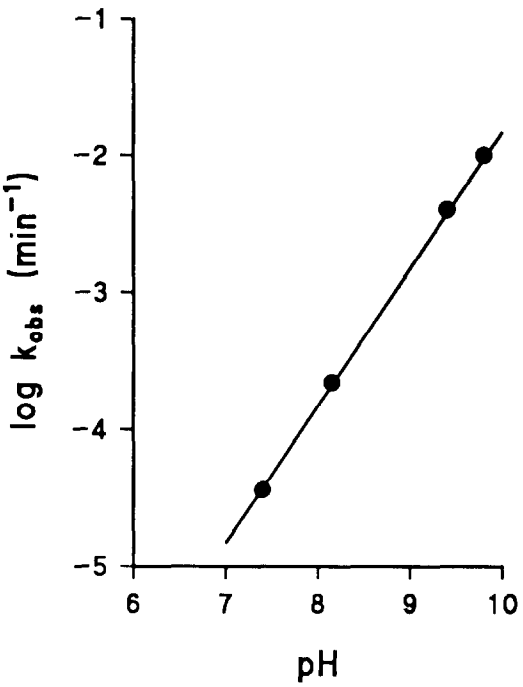


Fig. 2. The pH-rate profile for the decomposition of compound **V** in aqueous buffer solution (μ = 0.5) at 37°C.

Stability of the prodrug derivatives

The kinetics of degradation of the *N*-acyl derivatives (**II–V**) and the phthalidyl derivative (**VI**) of Z-Gly-ProNH$_2$ was studied in aqueous solution at 37°C over the pH range 7.4–10.0. At constant pH and temperature the disappearance of the derivatives displayed strict first-order kinetics over several half-lives and all reactions proceeded to completion.

The influence of pH on the rate of hydrolysis of compound **V** is shown in Fig. 2 where the logarithm of the observed pseudo-first-order constants (k_{obs}) is plotted against pH. The pH-rate

profiles obtained for the other *N*-acyl derivatives and the phthalidyl derivative **VI** had a similar shape, indicating the occurrence of a specific base-catalyzed degradation in the pH range investigated according to the following rate expression:

$$k_{obs} = k_{OH} a_{OH} \quad (1)$$

where a_{OH} refers to the hydroxide ion activity and k_{OH} is the second-order rate constant for the specific base-catalyzed decomposition. The values of k_{OH} and the half-lives of hydrolysis of the compounds at pH 7.4 are listed in Table 1. The degradation of all derivatives in neutral and alkaline aqueous solutions proceeded with the quantitative formation of compound **I** as revealed by HPLC analysis of the reaction solution. An example of a product analysis is shown in Fig. 3 for compound **III**. Thus, hydrolysis of the *N*-acylated amides, which can be regarded as unsymmetrical acyclic imides, to yield Z-Gly-Pro does not occur to any significant extent.

The half-lives observed for the hydrolysis of the compounds in 80% human plasma at 37°C are included in Table 1. As can be seen from the data the hydrolysis is markedly catalyzed by plasma enzymes as has previously been found for other *N*-acylated amides (Kahns and Bundgaard, 1991a). Also in plasma solutions the hydrolysis of the derivatives proceeded with the quantitative formation of compound **I**. The phthalidyl derivative **VI** showed a particularly high susceptibility to undergo plasma-catalyzed degradation. As described before for such derivatives of other amides (Bundgaard et al., 1988) this effect can be ascribed to enzymatic hydrolysis of the lactone ring to yield an *N*-α-hydroxyalkyl derivative which quickly decomposes to the amide and phthalaldehydic acid.

TABLE 1

Half-lives of decomposition and k_{OH} values of compounds I–VI in buffer solutions, 80% human plasma and 20% rabbit gut homogenate at 37°C

Compound	k_{OH} (M^{-1} min^{-1})	Half-lives		
		Aqueous buffer (pH 7.4)	80% human plasma (pH 7.4)	20% rabbit gut homogenate (pH 7.4)
I	–	stable	stable	6.4 min
II	98	195 h	14 h	36 min
III	80	240 h	24 h	23 min
IV	44	430 h	31 h	16 min
V	61	316 h	42 h	8.7 min
VI	912	21 h	7 min	3.6 min

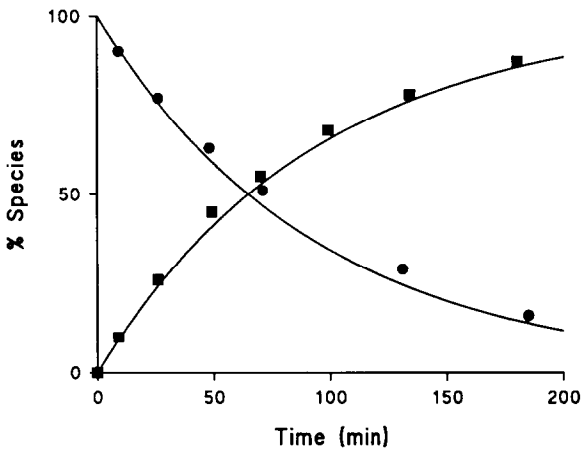


Fig. 3. Plots showing the time courses of degradation of compound **III** (●) and formation of Z-Gly-ProNH$_2$ (**I**) (■) in a 0.02 M borate buffer solution of pH 10.0 at 37°C.

Stability in gut homogenate

The stability of the derivatives **II–VI** was determined in 20% rabbit intestinal homogenate and compared with that of the parent peptide **I**. Under the experimental conditions used the disappearance of the compounds followed first-order kinetics as illustrated in Fig. 4. The observed half-lives for the degradation are listed in Table 1. The data show that the *N*-acyl derivatives are more resistant to degradation than Z-Gly-ProNH$_2$ although to varying extents. The degree of stabilization is seen to decrease with increasing alkyl group. The *N*-phthalidyl derivative **VI**, however, was found to be even more unstable than the parent Z-Gly-ProNH$_2$. These findings parallel those observed with various *N*-3^{im}-alkoxycarbonyl and *N*-3^{im}-phthalidyl derivatives of TRH where it was found that the more hydrophobic or lipophilic derivatives possessed the greatest susceptibility to

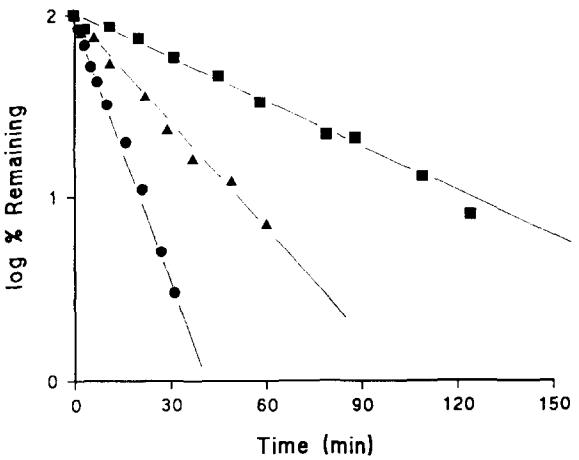


Fig. 4. First-order kinetic plots for the hydrolysis at 37°C of compounds **I** (●), **II** (■) and **IV** (▲) in 20% rabbit gut homogenate (pH 7.4) at 37°C.

undergo cleavage by prolyl endopeptidase (Møss et al., 1990; Møss and Bundgaard, 1991). The specificity of this enzyme is rather broad in terms of groups attached to the proline amide core but hydrophobic substituents in the moiety generally increase the substrate reactivity (Walter et al., 1980; Wilk; 1983).

Whereas the disappearance of the *N*-acyl derivatives in the gut homogenate proceeded according to strict first-order kinetics the mechanism of degradation remains somewhat obscure. Monitoring the degradation course by HPLC showed that the disappearance of the derivatives was accompanied by the formation of an unknown peak which then disappeared much more slowly. The ultimate degradation product was in all cases found to be Z-Gly-Pro. The retention time of the intermediate was in each case longer than that of the individual *N*-acyl derivative and different for each compound.

In conclusion, the results obtained suggest that *N*-acylation of a C-terminal prolineamide group in peptides may be a possible approach to obtain derivatives which on the one hand are more stable than the parent peptide toward prolyl endopeptidase present in the intestinal tissue and, on the other, are bioreversible, being capable of releasing the parent peptide by plasma enzyme-catalyzed hydrolysis. The most promising derivatives appear to be those with the smallest and less hydrophobic acyl substituents. Studies are in progress to prepare such derivatives as well as other non-hydrophobic *N*-acyl derivatives at the prolineamide moiety of TRH.

Acknowledgements

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References

Bundgaard, H., Design of prodrugs: Bioreversible derivatives for various functional groups and chemical entities. In Bundgaard, H. (Ed.), *Design of Prodrugs*, Elsevier, Amsterdam, 1985, pp. 1–92.

Bundgaard, H. and Buur, A., Prodrugs as drug delivery systems. 65. Hydrolysis of α-hydroxy- and α-acyloxy-*N*-benzoylglycine derivatives and implications for the design of prodrugs of NH-acidic compounds. *Int. J. Pharm.*, 37 (1987) 185–194.

Bundgaard, H., Buur, A., Hansen, K.T., Larsen, J.D., Möss, J. and Olsen, L., Prodrugs as drug delivery systems. 77. Phthalidyl derivatives as prodrug forms for amides, sulfonamides, carbamates and other NH-acidic compounds. *Int. J. Pharm.*, 45 (1988) 47–57.

Bundgaard, H. and Møss, J., Prodrugs of peptides. 6. Bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by the TRH-specific serum enzyme. *Pharm. Res.*, 7 (1990) 885–892.

Duntas, L., Keck, F.S., Loos, U. and Pfeiffer, E.F., Pharmakokinetik und Pharmakodynamik von Protirelin (TRH) beim Menschen. *Dtsch. Med. Wschr.*, 113 (1988) 1354–1357.

Griffiths, E.C., Thyrotrophin releasing hormone: Endocrine and central effects. *Psychoneuroendocrinology*, 10 (1985) 225–235.

Griffiths, E.C., Thyrotrophin-releasing hormone: New applications in the clinic. *Nature*, 322 (1986) 212–213.

Griffiths, E.C., Clinical applications of thyrotrophin-releasing hormone. *Clin. Sci.*, 73 (1987) 449–457.

Horita, A., Carino, M.A. and Lai, H., Pharmacology of thyrotropin-releasing hormone. *Annu. Rev. Pharmacol. Toxicol.*, 26 (1986) 311–332.

Kahns, A.H. and Bundgaard, H., *N*-Acyl derivatives as prodrug forms for amides: Chemical stability and enzymatic hydrolysis of various *N*-acyl and *N*-alkoxycarbonyl amide derivatives. *Int. J. Pharm.*, 71 (1991a) 31–43.

Kahns, A.H. and Bundgaard, H., Prodrugs of peptides. 13. Stabilization of peptide amides against α-chymotrypsin by the prodrug approach. *Pharm. Res.*, 8 (1991b) 1533–1538.

Knitsatschek, H. and Bauer, K., Characterization of 'thyroliberin-deamidating enzyme' as a postproline cleaving enzyme. *J. Biol. Chem.*, 254 (1979) 10936–10943.

Loosen, P.T., TRH: behavioral and endocrine effects in man. *Prog. Neuro-Psychopharmacol. Biol. Psychiatr.*, 12 (1988) s87–s117.

Metcalf, G. and Jackson, I.M.D., Thyrotropin-releasing hormone. Biomedical significance. *Ann. N.Y. Acad. Sci.*, 553 (1989) 1–631.

Møss, J. and Bundgaard, H., Prodrugs of peptides. 7. Transdermal delivery of thyrotropin releasing hormone (TRH) via prodrugs. *Int. J. Pharm.*, 66 (1990) 39–45.

Møss, J. and Bundgaard, H., Prodrugs of peptides. 12. Bioreversible derivatization of thyrotropin-releasing hormone (TRH) by N-phthalidylation of its imidazole moiety. *Int. J. Pharm.*, 74 (1991) 67–75.

Møss, J., Buur, A. and Bundgaard, H., Prodrugs of peptides. 8. In vitro study of intestinal metabolism and penetration of thyrotropin-releasing hormone (TRH) and its prodrugs. *Int. J. Pharm.*, 66 (1990) 183–191.

Orlowski, M., Wilk, E., Pearce, S. and Wilk, S., Purification and properties of a prolyl endopeptidase from rabbit brain. *J. Neurochem.*, 33 (1979) 461–469.

Smith, E.L. and Bergmann, M., The peptides of intestinal mucosa. *J. Biol. Chem.*, 153 (1944) 627–651.

Walter, R., Simmons, W.H. and Yoshimoto, T., Proline specific endo- and exopeptidases. *Mol. Cell. Biochem.*, 30 (1980) 111–127.

Wheeler, D.D., Young, D.C. and Erley, D.S., Reactions of phthalaldehydic acid. *J. Org. Chem.*, 22 (1957) 547–556.

Wilk, S., Minireview: prolyl endopeptidase. *Life Sci.*, 33 (1983) 2149–2157.

Yokohama, S., Yamashita, K., Toguchi, H., Takeuchi, J. and Kitamori, N., Absorption of thyrotropin-releasing hormone after oral administration of TRH tartrate monohydrate in the rat, dog and human. *J. Pharm. Dyn.*, 7 (1984) 101–111.