International Journal of Pharmaceutics, 71 (1991) 45–53

ADONIS 037851739100177E

IJP 02380

Prodrugs of peptides. 10. Protection of di- and tripeptides against aminopeptidase by formation of bioreversible 4-imidazolidinone derivatives

Gitte Juel Rasmussen and Hans Bundgaard

The Royal Danish School of Pharmacy, Department of Pharmaceutical Chemistry, Copenhagen (Denmark)

(Received 3 December 1990)
(Accepted 31 December 1990)

Key words: Prodrug; Peptide; 4-Imidazolidinone; Aminopeptidase; Hydrolysis

Summary

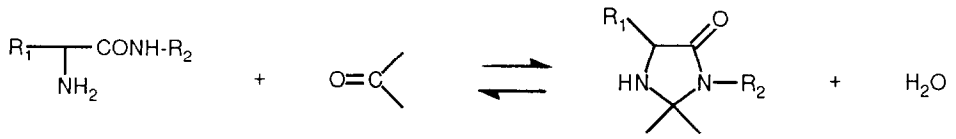
The kinetics of hydrolysis of a series of 4-imidazolidinones derived from acetone and various di- and tripeptides was studied in aqueous solution and in the presence of enzymes in order to assess their suitability as prodrug forms for the peptides. Whereas the parent di- and tripeptides were readily hydrolyzed by a purified aminopeptidase as well as in human plasma solutions and rabbit intestinal homogenates, the imidazolidinyl peptides were totally resistant to enzymatic cleavage in these media. On the other hand, these derivatives are readily bioreversible, being converted to the parent peptide by spontaneous hydrolysis. The rate of hydrolysis is greatly dependent on the structure of the peptide. For the eleven 4-imidazolidinones studied the half-lives of hydrolysis at pH 7.4 and 37°C ranged from 18 min to 545 h. The major structural factor influencing the stability was shown to be the steric properties within the α-carbon atom substituents in the amino acid residue next to the N-terminal amino acid. It is concluded that 4-imidazolidinone formation can be a useful prodrug approach to protect the N-terminal amino acid residue of peptides against cleavage by aminopeptidases and related exopeptidases.

Introduction

A major obstacle to the application of peptides as clinically useful drugs is their poor biomembrane penetration, rapid enzymatic degradation and short biological half-lives (Humphrey and Ringrose, 1986; Lee and Yamamoto, 1990). A possible approach to solve or diminish these delivery problems of peptide drugs is derivatization of the peptides to produce prodrugs or transport forms which are more lipophilic than the parent peptides and capable of protecting these against degradation by enzymes present at the mucosal absorption barrier or in the blood (Bundgaard, 1986, 1991; Bundgaard and Møss, 1989, 1990; Bundgaard and Rasmussen, 1991).

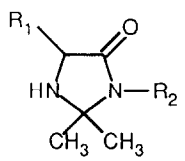
As a part of studies going on in this laboratory to identify various types of bioreversible derivatives for the functional groups or chemical entities occurring in amino acids and peptides, we have previously reported that 4-imidazolidinones may be a potentially useful prodrug type for the α-aminoamide moiety which is found in several

Correspondence: H. Bundgaard, The Royal Danish School of Pharmacy, Department of Pharmaceutical Chemistry, 2 Universitetsparken, DK-2100 Copenhagen, Denmark.



Scheme 1.

peptides (Klixbüll and Bundgaard, 1984, 1985). Such derivatives are readily formed by condensing compounds containing an α-aminoamide moiety such as peptides with a free N-terminal amino group with aldehydes or ketones (Scheme 1) (for references, see Klixbüll and Bundgaard (1984). As



	R_1	R_2
I	$-CH_2C_6H_5$	$-CH(iC_4H_9)COOH$
II	$-CH_2C_6H_5$	$-CH(CH_3)COOH$
III	$-CH_2C_6H_5$	$-CH_2COOH$
IV	$-CH_2C_6H_5$	$-CH(CH_2C_6H_5)COOH$
V	$-CH_3$	$-CH(CH_2C_6H_5)COOH$
VI	$-H$	$-CH(CH_2C_6H_5)COOH$
VII	$-H$	$-CH(CH_2C_6H_5\text{-}4\text{-}OH)COOH$
VIII	$-CH_2C_6H_5\text{-}4\text{-}OH$	$-CH(iC_4H_9)COOH$
IX	$-H$	$-CH(CH_2C_6H_5)CONHCH(CH_2C_6H_5)COOH$
X	$-CH_3$	$-CH(CH_2C_6H_5)CONHCH_2COOH$
XI	$-CH_2C_6H_5\text{-}4\text{-}OH$	$-CH_2CONHCH_2COOH$

has been shown for some 4-imidazolidinones derived from various dipeptides and acetone the compounds are readily hydrolyzed in aqueous solution to the parent dipeptide and acetone, the rates being dependent on pH and the structure of the dipeptide (Klixbüll and Bundgaard, 1984). For the further evaluation of 4-imidazolidinones as a prodrug type for peptides it is, however, important to examine whether this prodrug approach may afford protection of the parent peptides to enzymatic degradation. To this end, we have studied the kinetics of hydrolysis of a great number of di- and tripeptides and their 4-imidazolidinones derivatives formed with acetone (**I–XI**) in human plasma, rabbit intestinal homogenates and in buffer solutions containing leucine aminopeptidase. The kinetics of hydrolysis of the 4-imidazolidinones has also been studied in aqueous solution over a wide range of pH in order to provide more information on the structural effects influencing the chemical reactivity of the prodrug derivatives.

Materials and Methods

Apparatus

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Shimadzu pump model LC-6A, a Shimadzu SPD-6A variable-wavelength UV detector, and a Rheodyne 7125 injection valve with a 20 μl loop. Two different reversed-phase columns were used: a Nova-Pak CN HP Radial column (100 × 8 mm; 4 μm particles) with a Resolve CN Guard Pak precolumn (both from Waters) and a Supelcosil LC-8-DB column (33 × 4.6 mm; 4 μm particles) with a Supelguard precolumn (both from Supelco Inc.). Readings of pH were carried out on a Radiometer PHM 83 Autocal instrument.

Chemicals

The di- and tripeptides studied (all of L-configuration) were purchased from Bachem AG, Budendorf, Switzerland or Sigma, St Louis, U.S.A. The aminopeptidase preparation used was leucine aminopeptidase (cytosol, type III-CP from porcine kidney) obtained from Sigma. Buffer substances and all other chemicals and solvents used were of reagent grade.

Preparation of 4-imidazolidinones

The 4-imidazolidinones of Phe-Leu, Ala-Phe, Phe-Ala and Phe-Gly and acetone were prepared as described by Hardy and Samworth (1977). Their melting points agreed with those reported by these authors. The remaining derivatives studied were prepared by refluxing mixtures of the corresponding di- or tripeptide (5 mg ml^{-1}) in methanol-acetone (1 : 1 v/v) for 24 h (method C in the above mentioned reference). Any undissolved peptide was filtered off and the solutions evaporated under reduced pressure. HPLC analysis of the solid residues obtained showed a content of unreacted di- or tripeptide less than 10%. No other peaks than those of the 4-imidazolidinones and the parent peptide were seen in the chromatograms.

Kinetic measurements

Hydrolysis in buffer solution. The decomposition of the 4-imidazolidinones was studied in aqueous buffer solution at 37 ± 0.2°C. The buffers used were hydrochloric acid, acetate, phosphate, borate and carbonate solutions. The buffer concentration generally used was 0.02 M. A constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The progress of hydrolysis of the derivatives was monitored by using HPLC procedures capable of separating the derivatives from their parent peptides. Mobile phase systems of 0.1% phosphoric acid containing acetonitrile (0–50 v/v) and/or methanol (0–25 v/v) were used, the concentration of acetonitrile and methanol being adjusted for each compound to give a retention time of 2.5–7 min. In all cases the 4-imidazolidinone was eluted later than the parent peptide. The flow rate was 1–2 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 50–100 μl of a stock solution of the derivatives in methanol to 10 ml of buffer solution, preequilibrated at 37°C, in screw-

capped test tubes, the initial concentration 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.

Hydrolysis in plasma, gut homogenate and enzyme solution. The decomposition of the 4-imidazolidinones and their parent peptides was studied at 37°C in 80% human plasma solutions, 10% rabbit intestinal homogenate and in 0.05 M phosphate buffer solutions containing leucine aminopeptidase at a concentration of 20 U ml^{-1}. The intestinal homogenate was prepared as described previously by Møss et al. (1990). The initial concentration of the compounds was $1-2 \times 10^{-4}$ M. The reaction mixtures were kept in a water bath at 37°C and 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) to deproteinize the samples and stop the reactions. After immediate mixing and centrifugation for 3 min at 13000 rpm 20 µl of clear supernatant was analyzed by HPLC for remaining 4-imidazolidinone or peptide as described above. Pseudo-first-order rate constants for the degradation was determined as described above.

Results and Discussion

Kinetics and mechanism of hydrolysis

The kinetics of hydrolysis of 4-imidazolidinones derived from acetone and various di- and tripeptides was studied in aqueous solution at 37°C over the pH range 1–10. Under the experimental conditions used all derivatives were hydrolyzed with the quantitative formation of the parent peptide as revealed by HPLC analysis, the degradation displaying strict first-order kinetics. As seen from a typical plot in Fig. 1 no lag time occurred in the formation of the parent peptide.

The buffers used to maintain a constant pH had no measurable influence on the rates of hydrolysis of the 4-imidazolidinones in concentrations up to 0.1 M and accordingly, the hydrolysis is not subject to significant general acid-base catalysis.

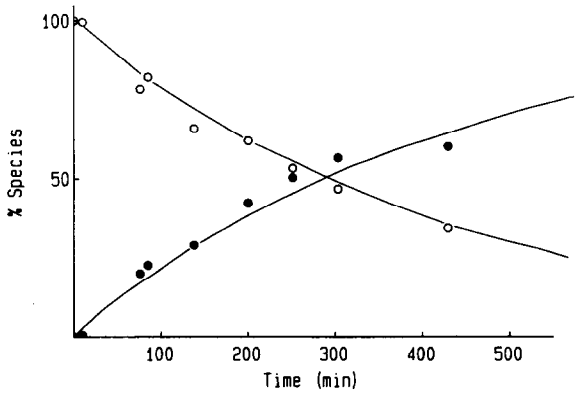


Fig. 1. Time courses for Phe-Leu (●) and the 4-imidazolidinone **I** (○) during the degradation of compound **I** in 0.02 M phosphate buffer solution of pH 7.40 (at 37°C).

The influence of pH on the hydrolysis rate is shown for some compounds in Fig. 2 where the logarithms of the observed pseudo-first-order rate constants (k_{obs}) are plotted against pH. As can be seen the pH-rate profiles have a sigmoid shape with maximum and constant rates at pH above about 4. Except for the 4-imidazolidinones derived

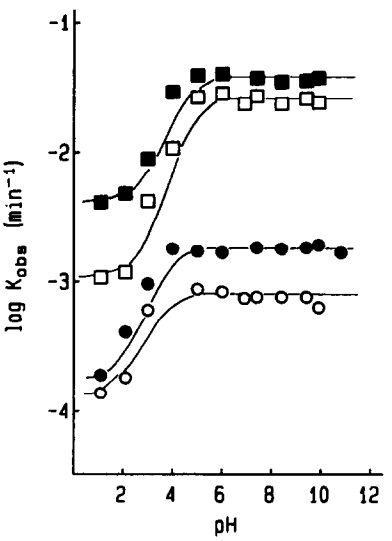


Fig. 2. The pH-rate profiles for the hydrolysis of the 4-imidazolidinones **II** (○), **IV** (●), **IX** (■) and **X** (□) in aqueous solution at 37°C.

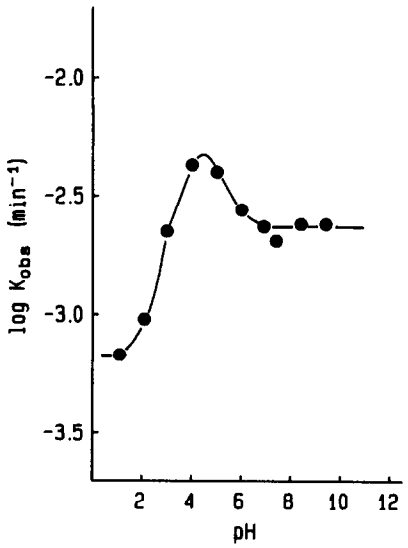


Fig. 3. The pH-rate profile for the hydrolysis of the 4-imidazolidinone **VIII** in aqueous solution at 37°C.

from Phe-Leu and Tyr-Leu all the imidazolidinones studied showed such sigmoidal pH-rate profiles. The Phe-Leu and Tyr-Leu imidazolidinone derivatives exhibited instead mixed sigmoidal and bell-shaped profiles as shown in Fig. 3.

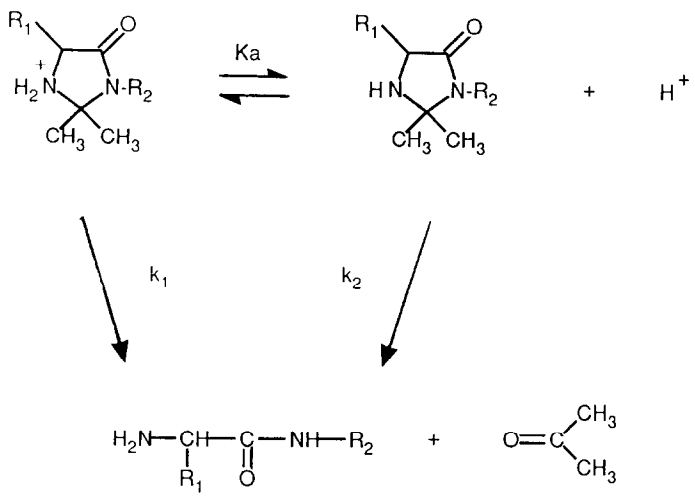
As described previously (Klixbüll and Bundgaard, 1984) the sigmoidal pH-rate profiles can be accounted for in terms of Scheme 2 where k_1 and k_2 are apparent first-order rate constants for the spontaneous degradation of the protonated and unprotonated species, respectively, of the 4-imidazolidinone. Mathemathically,

$$k_{obs} = \frac{k_1 K_a}{a_H + K_a} + \frac{k_2 a_H}{a_H + K_a} \quad (1)$$

where a_H is the hydrogen ion activity and K_a is the ionization constant of the protonated imidazolidinone.

This degradation behaviour is similar to that of various *N*-Mannich bases of amides (α-aminoalkylamides) (Bundgaard and Johansen, 1980a,b). In fact, 4-imidazolidinones can be considered as cyclic *N*-Mannich bases in which the amide and amino functions are placed in the same molecule. As previously discussed (Klixbüll and Bundgaard, 1984) the bell-shaped pH-rate profiles for the Phe-Leu and Tyr-Leu imidazolidinones may be explained by the involvement of a kinetically significant Schiff base intermediate in the reaction pathway and a change of the rate-determining step in the overall hydrolysis with pH.

The values of k_1 and k_2 as well as pK_a derived from the pH-rate profiles are listed in Table 1. The pK_a values are seen to be considerably lower than those for the amino group in the parent peptides (pK_a around 8) which is a general char-



Scheme 2.

TABLE 1

Rate data for the hydrolysis of various 4-imidazolidinones in aqueous solution at 37°C

4-Imidazolidinone (parent peptide)	k_1 (min^{-1})	k_2 (min^{-1})	pK_a [b]
I [a] (Phe-Leu)	9.5×10^{-4}	2.9×10^{-3}	
II (Phe-Ala)	1.4×10^{-4}	7.8×10^{-4}	3.0
III (Phe-Gly)	n.d.	2.1×10^{-5}	
IV (Phe-Phe)	1.9×10^{-4}	1.8×10^{-3}	3.1
V (Ala-Phe)	2.4×10^{-4}	1.9×10^{-3}	3.3
VI (Gly-Phe)	1.1×10^{-3}	5.5×10^{-3}	3.7
VII (Gly-Tyr)	8.8×10^{-4}	4.3×10^{-3}	3.6
VIII [a] (Tyr-Leu)	6.7×10^{-4}	2.4×10^{-3}	
IX (Gly-Phe-Phe)	4.1×10^{-3}	3.7×10^{-2}	3.7
X (Ala-Phe-Gly)	1.1×10^{-3}	2.5×10^{-2}	4.0
XI (Tyr-Gly-Gly)	1.9×10^{-4}	8.8×10^{-4}	3.9

[a] These 4-imidazolidinones showed bell-shaped pH-rate profiles. The k_2 values listed were derived from the k_{obs} values at pH 6–9.

[b] The pK_a values listed were determined kinetically.

acteristic in α-amidoalkylation of amines (Bundgaard and Johansen, 1980a,b) including 4-imidazolidinone formation of α-aminoamide compounds (Klixbüll and Bundgaard, 1984).

Structure-reactivity relationships

The data in Table 1 show that the 4-imidazolidinones are hydrolyzed with vastly different rates at physiological pH where the k_2 reaction predominates whereas the stability of the derivatives in acidic solutions (i.e., the k_1 reaction) varies only 10-fold. It has previously been established for acyclic *N*-Mannich bases that the k_2 reaction and accordingly the stability in neutral and basic solutions increase with increasing steric effects within the amine moiety, decreasing amine and hence *N*-Mannich base basicity and increasing acidity of the amide functionality (Bundgaard and Johansen, 1980a,b). Although the same structural factors may be of importance for the k_2 reaction of 4-imidazolidinones the predominant factor appears to be the steric properties within the C-terminal or the second amino acid residue (i.e., R_2). Thus, the great difference in reactivity of the 4-imidazolidinones derived from Phe-Leu, Phe-Ala, Phe-Gly and Phe-Phe (**I**–**IV**) where R_1 is the same can be accounted for in terms of differences in the steric properties of the α-carbon atom substituents in R_2 as shown in Fig. 4. The higher reactivity of the 4-imidazolidinones **IX** and **X** derived from the tripeptides Gly-Phe-Phe and Ala-Phe-Gly relative to that of compound **XI** derived from Tyr-Gly-Gly is also consistent with this structure-reactivity relationship. Besides by influencing the stability through steric effects the R_2 substituents can affect the stability via polar effects as is the case for acyclic *N*-Mannich bases (Bundgaard and Johansen, 1980a,b). Thus, the greater reactivity of the tripeptide imidazolidinones **IX** and **X** in relation to the dipeptide derivatives **VI** and **V**, respectively, may largely be ascribed to the greater polar effects of their R_2 substituents.

The α-carbon atom substituents in the N-terminal amino acid (R_1) have only a minor influence on the stability (cf. the k_2 values for the compounds **IV**, **V** and **VI** which all have Phe as the C-terminal amino acid). The differences in their reactivity can be ascribed to the different basicity of the amino group in the parent dipeptides and hence the different basicity of the 4-imidazolidinones.

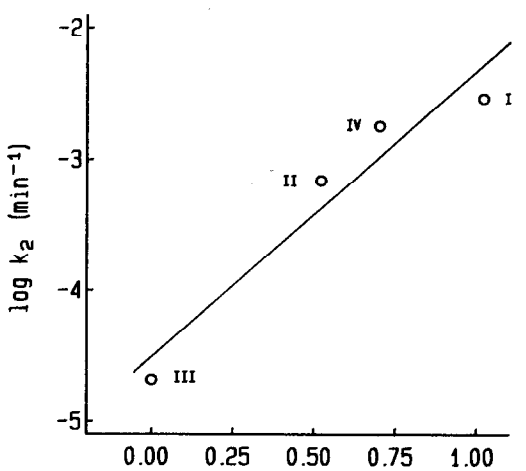


Fig. 4. Plot of log k_2 against the steric substituent parameter ν for the 4-imidazolidinones **I**–**IV**. The ν values were taken from Charton (1977) and refer to the α-carbon atom alkyl substituents in R_2 (i.e., isobutyl, methyl, hydrogen and benzyl). The correlation equation for the line is: $\log k_2 = 2.18\nu - 4.51$ ($r = 0.965$; $n = 4$).

Hydrolysis of peptides and 4-imidazolidinones in the presence of enzymes

The kinetics of hydrolysis of the 4-imidazolidinones and their parent di- or tripeptides was determined in 80% human plasma solutions, 10% rabbit intestinal homogenates as well as in 0.05 M phosphate buffer solutions containing leucine aminopeptidase at a concentration of 20 U ml^{-1}. Under the experimental conditions used the hydrolysis of the compounds proceeded according to first-order kinetics. Examples of typical first-order plots are shown in Fig. 5.

The half-lives of hydrolysis observed at 37°C are shown in Tables 2 and 3. As seen from the data, the rates of degradation of the 4-imidazolidinones are not increased in these media but are either the same or even slower than the rates of hydrolysis in buffer solutions of the same pH and temperature (Table 2). The non-catalytic effect of plasma has also been observed for acyclic *N*-Mannich bases (Johansen et al., 1983; Bundgaard and Møss, 1989). The significantly longer half-lives of hydrolysis in plasma solutions relative to buffer solutions as observed for some of the 4-imidazolidinones (**I, IV, VIII** and **XI**) may be ascribed to binding of the compounds to plasma proteins.

In contrast to the 4-imidazolidinones, the parent di- and tripeptides are readily hydrolyzed enzymatically (Table 3). The dipeptides Phe-Leu and Tyr-Leu are especially good substrates for the purified leucine aminopeptidase used. No correlation appears to exist between the reactivity of the peptides toward leucine aminopeptidase and their hydrolytic lability in plasma or intestinal homo-

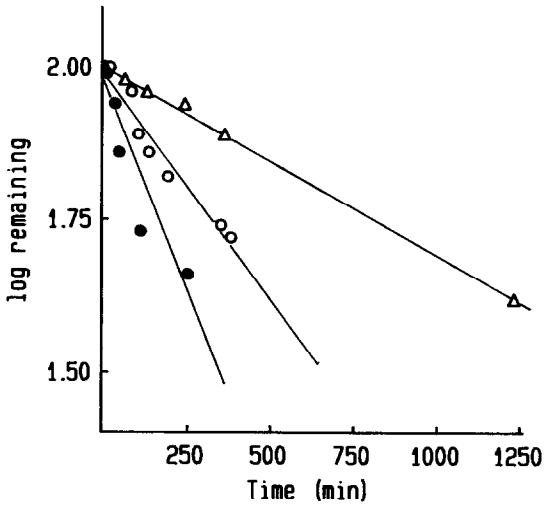


Fig. 5. First-order plots for the hydrolysis of compound **V** in 80% human plasma (○), compound **VI** in 10% rabbit intestinal homogenate (●) and compound **XI** (△) in phosphate buffer (pH 7.40) containing leucine aminopeptidase (20 U ml^{-1}) at 37°C.

TABLE 2

Half-lives of hydrolysis of various 4-imidazolidinones in 80% human plasma, 10% rabbit intestinal homogenate and 0.05 M phosphate buffer solution (pH 7.4) with or without aminopeptidase (37°C)

4-Imidazolidinone (parent peptide)	$t_{1/2}$ (h)			
	80% human plasma	10% rabbit intestinal homogenate	Aminopeptidase (20 U ml^{-1})	pH 7.4 buffer
I (Phe-Leu)	15.5		4.7	4.8
II (Phe-Ala)	16.3	13.9	14.4	15.2
III (Phe-Gly)				545
IV (Phe-Phe)	18.9			6.4
V (Ala-Phe)	7.0	6.4	5.9	6.9
VI (Gly-Phe)	2.0	2.0	1.9	2.0
VII (Gly-Tyr)	2.8			2.8
VIII (Tyr-Leu)	12.0			4.9
IX (Gly-Phe-Phe)	0.3	0.3	0.3	0.3
X (Ala-Phe-Gly)	0.3	0.4	0.4	0.4
XI (Tyr-Gly-Gly)	18.6	8.4	13.2	11.2

TABLE 3

Half-lives of hydrolysis of various di- and tripeptides in 80% human plasma, 10% rabbit intestinal homogenate and in buffer solution (pH 7.4) containing aminopeptidase at 37°C

Peptide	$t_{1/2}$ (min)		
	80% human plasma	10% rabbit intestinal homogenate	Aminopeptidase (20 U ml^{-1})
Phe-Leu	115		0.1 [a]
Phe-Ala	42	4.6	1.7
Phe-Gly	245		
Phe-Phe	260		
Ala-Phe	18	2.0	1.9
Gly-Phe	210	2.8	480
Gly-Tyr	120		
Tyr-Leu	190		0.1 [a]
Gly-Phe-Phe	3.9	1.6	55
Ala-Phe-Gly	2.7	1.1	0.9
Tyr-Gly-Gly	14	40	54

[a] Calculated from a half-life of 2.1 min observed at an aminopeptidase concentration of 1.0 U ml^{-1}.

genate. This is, on the other hand, also not to be expected since these tissues are known to contain various other aminopeptidases or dipeptidases capable of hydrolyzing di- and tripeptides (Delange and Smith, 1971; McDonald and Schwabe, 1977; Stratford and Lee, 1986). That the plasma-catalyzed degradation of the tripeptides Gly-Phe-Phe and Ala-Phe-Gly primarily involves the attack of an exopeptidase at their N-terminal bond was evidenced by the formation of large amounts of Phe-Phe and Phe-Gly, respectively, as revealed by HPLC analysis of the reaction solutions.

The observed resistance of the 4-imidazolidinones to enzymatic hydrolysis is in accordance with the general substrate specificities of aminopeptidases and other exopeptidases cleaving peptides at their N-terminals. These specificities include a free N-terminal amino group, preferably a primary amino group, and an unmodified peptide bond (Delange and Smith, 1971). In the 4-imidazolidinone structure the primary amino group of the parent peptide has been transformed into a secondary amino group and most importantly, the vulnerable N-terminal peptide bond has been alkylated.

In conclusion, the results described show that 4-imidazolidinone derivatization may be a useful prodrug approach to protect the N-terminal amino acid residue of di- and tripeptides against enzymatic cleavage by aminopeptidases and plasma or intestinal tissue enzymes. This derivatization is readily bioreversible, the parent peptide being formed by spontaneous hydrolysis at physiological pH and temperature. Studies are in progress to apply this approach to pharmacologically interesting peptides such as oxytocin and enkephalines and to examine the effect of the carbonyl component used in the imidazolidinone formation on the rate of hydrolysis. Obviously, besides a possible influence on the reactivity, the type of this component can greatly influence the lipophilicity of the prodrug derivatives by its substituents.

Acknowledgements

This work has been supported by the Pharma-Biotec Research Centre and the Lundbeck Foundation.

References

Bundgaard, H., Bioreversible derivatization of peptides. In S.S. Davis, L. Illum and E. Tomlinson (Eds), *Delivery Systems for Peptide Drugs*, Plenum, New York, 1986, pp. 49–68.

Bundgaard, H., Prodrugs as a means to improve the delivery of peptide drugs. *Adv. Drug Delivery Rev.*, (1991) 313–322.

Bundgaard, H. and Johansen, M., Prodrugs as drug delivery systems. IV. *N*-Mannich bases as potential novel prodrugs for amides, ureides, amines and other NH-acidic compounds. *J. Pharm. Sci.*, 69 (1980a) 44–46.

Bundgaard, H. and Johansen, M., Prodrugs as drug delivery systems. X. *N*-Mannich bases as novel prodrug candidates for amides, imides, urea derivatives, amines and other NH-acidic compounds. Kinetics and mechanisms of decomposition and structure-reactivity relationships. *Arch. Pharm. Chem. Sci. Ed.*, 8 (1980b) 29–52.

Bundgaard, H. and Møss, J., Prodrugs of peptides. IV. Bioreversible derivatization of the pyroglutamyl group by *N*-acylation and *N*-aminomethylation to effect protection against pyroglutamyl aminopeptidase. *J. Pharm. Sci.*, 76 (1989) 122–125.

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.

Bundgaard, H. and Rasmussen, G.J., Prodrugs of peptides. 9. Bioreversible *N*-α-hydroxyalkylation of the peptide bond to effect protection against carboxypeptidase or other proteolytic enzymes. *Pharm. Res.*, 8 (1991) 313–322.

Charton, M., The prediction of chemical lability through substituent effects. In E.B. Roche (Ed.), *Design of Biopharmaceutical Properties though Prodrugs and Analogs*, American Pharmaceutical Association, Washington, DC, 1977, pp. 228–280.

Delange, R.T. and Smith, E.L., Leucine aminopeptidase and other N-terminal exopeptidases. In P.D. Boyer (Ed.), *The Enzymes*, Vol. III, 3rd Edn., Academic Press, New York, 1971, pp. 81–118.

Hardy, P.M. and Samworth, D.J., Use of *N,N'*-isopropylidene dipeptides in peptide synthesis. *J. Chem. Soc. Perkin Trans. I*, (1977) 1954–1960.

Humphrey, M.J. and Ringrose, P.S., Peptides and related drugs: A review of their absorption, metabolism and excretion. *Drug Metab. Rev.*, 17 (1986) 283–310.

Johansen, M., Bundgaard, H. and Falch, E., Spectrophotometric determination of the rates of hydrolysis of aldehyde-releasing prodrugs in aqueous solution and plasma. *Int. J. Pharm.*, 13 (1983) 89–98.

Klixbüll, U. and Bundgaard, H., Prodrugs as drug delivery systems. 30. 4-Imidazolidinones as potential bioreversible derivatives for the α-aminoamide moiety in peptides. *Int. J. Pharm.*, 20 (1984) 273–284.

Klixbüll, U. and Bundgaard, H., Kinetics of reversible reactions of ampicillin with various aldehydes and ketones with formation of 4-imidazolidinones. *Int. J. Pharm.*, 23 (1985) 163–173.

Lee, V.H.L. and Yamamoto, A., Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug. Delivery Rev.*, 4 (1990) 171–207.

McDonald, J.K. and Schwabe, C., Intracellular exopeptidases. In A.J. Barrett (Ed.), *Proteinases in Mammalian Cells and Tissues*, Elsevier, Amsterdam, 1977, pp. 311–391.

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.

Stratford, R.E. and Lee, V.H.L., Aminopeptidase activity in homogenates of various absorptive mucosal in the albino rabbit: Implications in peptide delivery. *Int. J. Pharm.*, 30 (1986) 73–82.