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PROTECTION OF THE PEPTIDE BOND AGAINST α-CHYMOTRYPSIN BY THE PRODRUG APPROACH

Anne H. Kahns, Gitte J. Friis and Hans Bundgaard*
Royal Danish School of Pharmacy, Department of Pharmaceutical Chemistry,
2 Universitetsparken, DK-2100 Copenhagen, Denmark

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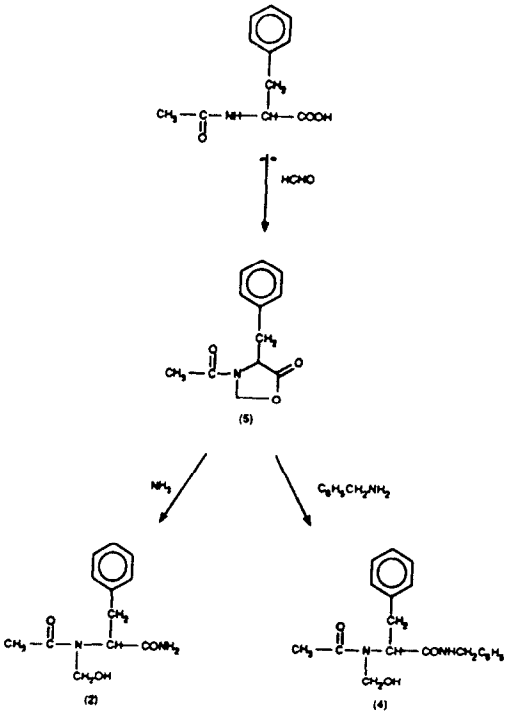
Abstract: N-Hydroxymethylation of the N-terminal peptide bond in N-acetyl-L-phenylalanine amides was found to protect the C-terminal amide bond against cleavage by α-chymotrypsin. The N-hydroxymethyl derivatives are readily bioreversible, undergoing spontaneous hydrolysis at physiological pH, and are thus promising prodrugs to overcome the enzymatic barrier to absorption of various peptides.

A major challenge in peptide and protein drug delivery is to overcome the enzymatic barrier that limits peptide and protein drugs to be delivered to their targets of action in effective amounts.[1,2] The enzymatic degradation usually begins at the site of administration and can be so extensive that it completely hinders any absorption of intact peptide or protein.

In recent years, studies in our laboratory have been performed with the aim of diminishing the problem of proteolytic degradation of peptide drugs at the mucosal barrier by the prodrug approach.[3] Of particular interest is the finding of a strategy to protect the peptide bond itself against enzymatic cleavage. While it is generally recognized that N-alkylation of peptide bonds usually makes them resistant to enzymatic attack,[4] such N-methyl and similar N-alkyl derivatives are not bioreversible. One the other hand, N-α-hydroxyalkylation of the peptide bond is a promising prodrug approach to protect this bond as well as an adjacent peptide bond in peptide drugs against cleavage by proteolytic enzymes such as carboxypeptidase A.[5] The N-α-hydroxyalkylated peptides are readily hydrolyzed nonenzymatically in aqueous solution at physiological pH to the parent peptide and the corresponding aldehyde, the rate being dependent on pH, the peptide structure and the type of aldehyde involved in the α-hydroxyalkylation.[5] In our previous work[5] we showed the applicability of this prodrug approach to stabilize peptide bonds against carboxypeptidase A. We now wish to report that the prodrug principle also promises to protect peptides against degradation by another pancreatic proteolytic enzyme, α-chymotrypsin.

N-Acetylphenylalanineamide (1) and N-acetylphenylalanine benzylamide (3) were used as α-chymotrypsin-reactive model peptides. The N-hydroxymethyl prodrug derivatives 2 and 4 were prepared by aminolysis of the N-acetyl-5-oxazolidinone (5), obtained by condensation of N-

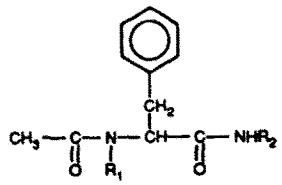


Scheme 1

acetyl-L-phenylalanine with paraformaldehyde, as previously described[5] (Scheme 1).[6]

The stability of the compounds 2 and 4 was studied in aqueous buffer solutions at 37°C and in pH 7.4 buffer solutions containing varying amounts of α-chymotrypsin (Type II; from bovine pancreas; Sigma Chemical Co., U.S.A.). The initial concentrations of the compounds were 2×10^{-4} M and the progress of reaction was followed by using reversed-phase HPLC procedures capable of separating the compounds from their products of degradation.[7] Under the experimental conditions used both compound 2 and 4 were found to degrade according to strict first-order kinetics and to convert quantitatively to the parent N-acetyl-L-phenylalanine-amide (1 or 3). The pH-rate profile observed for compound 2 is shown in Fig. 1. Its shape is similar to that of the pH-rate profiles for other N-hydroxymethylated peptides[5] with maximal stability occurring at pH around 4 At physiological pH 7.4 and 37°C the half-life of degradation is 38 min and 63 min for compounds 2 and 4, respectively (Table 1). The half-lives of degradation in 0.1 M phosphate buffer solutions pH 7.4 containing varying amounts of α-chymotrypsin are listed in Table 1 The data readily demonstrate thet whereas the stability of the model peptides 1 and 3 decreases linearly with increasing α-chymotrypsin concentration, the stability of the N-hydroxymethylated compounds is totally uneffected by the enzyme.

Table 1. Half-lives ($t_{1/2}$) for the decomposition of various N-acetyl-L-phenylalanine derivatives in the presence of α-chymotrypsin and in buffer solution at 37°C



Compound	R_1	R_2	$t_{1/2}$			
			Buffer[a] pH 7.4	Buffer[a] with α-chymotrypsin		
				0.25 mg/ml	0.50 mg/ml	1.0 mg/ml
1	H	H	stable	9.6 h	4.9 h	2.3 h
2	CH_2OH	H	38 min	33 min	35 min	32 min
3	H	$CH_2C_6H_5$	stable	15.0 h	7.5 h	3.9 h
4	CH_2OH	$CH_2C_6H_5$	63 min	65 min	64 min	63 min

[a] Phosphate buffer, 0.1 M (pH 7.4).

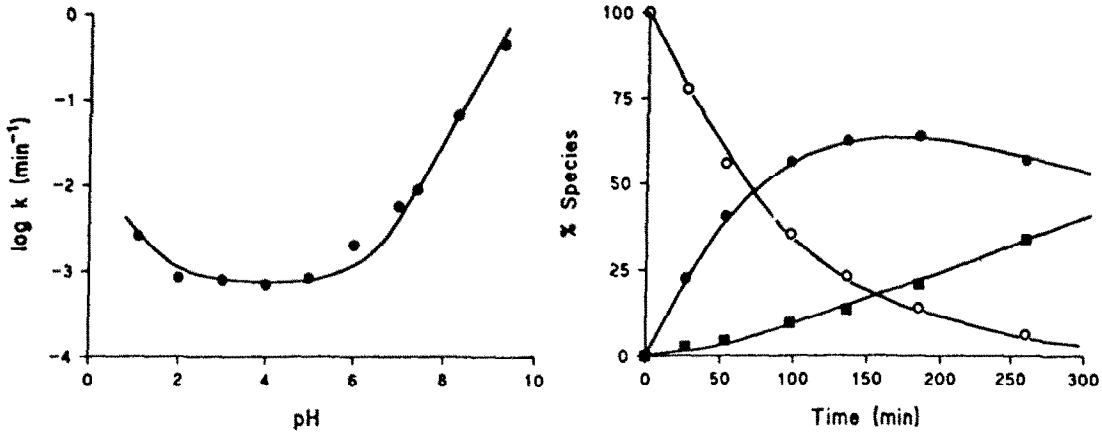


Fig. 1. The pH-rate profile for the conversion of compound 2 to 1 in aqueous solution at 37°C.

Fig. 2. Plots showing the time courses of degradation of compound 4 (○) to N-acetyl-L-phenylalanine (■) via the intermediate formation of compound 3 (●) in 0.1 M phosphate buffer solution (pH 7.4) containing 0 50 mg/ml of α-chymotrypsin (at 37°C)

Furthermore, product analysis of the reaction solutions by HPLC revealed a quantitative conversion of the compounds 1-4 to N-acetyl-L-phenylalanine, with the amides 1 and 3 being formed as intermediates in stoichiometric amounts from 2 and 4, respectively (Fig. 2).

In conclusion, N-hydroxymethylation of a peptide bond has been shown to make the adjacent peptide bond completely stable toward attack by α-chymotrypsin just as it is the case for carboxypeptidase A-sensitive substrates.[5] While direct protection of the susceptible peptide bond by N-hydroxymethylation of this bond should also be possible, it is thus of interest to note that derivatization of an adjacent peptide bond affords the same result. Studies are in progress to investigate whether N-hydroxymethylation of a peptide bond further away from the enzyme-sensitive bond may also result in its stabilization.

Acknowledgement: This work has been supported by PharmaBiotec Research Centre.

References and Notes

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6. Compound 4 was prepared by stirring a solution of the 5-oxazolidinone[5] (0.44 g, 2 mmol) and benzylamine (0.22 ml, 2 mmol) in 10 ml of benzene at room temperature for 20 h. The solution was evaporated **in vacuo** and the residue crystallized from ethanol-ether-petroleum ether to give 0.32 g of compound 4, m.p. 81-82°C. Anal.: Calc. for $C_{19}H_{21}N_2O_3$: C, 70.13; H, 6.51; N, 8.61. Found: C, 70.10; H, 6.62; N, 8.62.
   Compound 2 was prepared **in situ**[5] by adding 0.50 ml of a solution (20 mg/ml) of 5 in acetonitrile to 10 ml of a 5 M solution of ammonia (pH 9.0). Following standing for 1 min at 20-25°C all oxazolidinone 5 had reacted as evidenced by HPLC analysis and the solution was used as stock solution for the kinetic measurements of compound 2.
7. The HPLC procedures used were as previously described: Kahns, A.H.; Bundgaard, H. *Pharm. Res.* **1991**, *8*, 1533.