Pharmaceutical Research, Vol. 8, No. 3, 1991

Research Article

Prodrugs of Peptides. 9. Bioreversible *N*-α-Hydroxyalkylation of the Peptide Bond to Effect Protection Against Carboxypeptidase or Other Proteolytic Enzymes

Hans Bundgaard[1,2] and Gitte Juel Rasmussen[1]

Received July 5, 1990; accepted August 30, 1990

Various *N*-α-hydroxyalkyl derivatives of *N*-acyl amino acids and di- and tripeptides were prepared by hydrolysis or aminolysis of *N*-acyl 5-oxazolidinones. The stability of these derivatives was studied in aqueous solution as a function of pH. The compounds were all degraded quantitatively to their parent *N*-acylated amino acid or peptide and aldehyde but with vastly different rates. At pH 7.4 and 37°C the half-lives of decomposition ranged from 4 min to 1500 hr. The structural factors influencing the stability included both steric and polar effects within the acyl and *N*-α-hydroxyalkyl moieties as well as within the amino acid attached to the *N*-α-hydroxyalkylated *N*-acyl amino acid. Whereas the *N*-benzyloxycarbonyl (Z) derivatives of the dipeptides Gly-L-Leu and Gly-L-Ala were readily hydrolyzed by carboxypeptidase A, the *N*-hydroxymethylated compounds, i.e., Z-Gly(CH_2OH)-Leu and Z-Gly(CH_2OH)-Ala, were resistant to cleavage by the enzyme as revealed by their similar rates of decomposition in the presence or absence of the enzyme at pH 7.4 and 37°C. The results suggest that *N*-α-hydroxyalkylation of a peptide bond protects not only this bond but also an adjacent peptide bond against proteolytic cleavage. Since the *N*-α-hydroxyalkyl derivatives are readily bioreversible, undergoing spontaneous hydrolysis at physiological pH, this prodrug approach promises to overcome the enzymatic barrier to absorption of various peptides.

KEY WORDS: peptides; prodrug; 5-oxazolidinones; *N*-α-hydroxyalkylation; proteolytic enzymes; peptide absorption.

INTRODUCTION

A major obstacle to the application of peptides as clinically useful drugs is their poor delivery characteristics. Most peptides are rapidly metabolized by proteolysis at most routes of administration; they are, in general, nonlipophilic compounds showing poor biomembrane penetration characteristics, and they possess short biological half-lives as a result of rapid metabolism and clearance (1–3).

A possible approach to solve these delivery problems may be derivatization of the bioactive peptides to produce prodrugs or transport forms which possess enhanced physicochemical properties in comparison to the parent compounds with regard to delivery and metabolic stability. Thus, such derivatization may, on one hand, protect small peptides against degradation by enzymes present at the mucosal barrier and, on the other hand, render hydrophilic peptides more lipophilic and hence facilitate their absorption. However, the derivatives should be capable of releasing the parent peptide spontaneously or enzymatically in the blood following their absorption (4).

In our laboratory studies have been initiated to develop various types of bioreversible derivatives for the functional groups or chemical entities occurring in amino acids and peptides (5–11). Thus, 5-oxazolidinones have been suggested as a prodrug type for the α-amido carboxy moiety in peptides (6) and various *N*-acyl, *N*-Mannich base, and *N*-α-acyloxyalkyl derivatives have been shown to be useful as prodrugs for protecting the pyroglutamyl residue against pyroglutamyl aminopeptidase (7–9). More recently, various *N*-alkoxycarbonyl derivatives of thyrotropin-releasing hormone (TRH) have been developed as a prodrug form of this tripeptide capable of protecting the peptide against enzymatic inactivation in the blood and of rendering transiently the peptide more lipophilic (10,11).

In the present work, a possible approach to develop bioreversible derivatives of the peptide bond itself has been examined. It is generally recognized that *N*-alkylation of peptide bonds usually makes them resistant to enzymatic attack (12–16). However, since *N*-methyl and similar alkyl derivatives are not bioreversible, the approach of simple *N*-alkylation implies the design of a new peptide (the analogue approach). The strategy of the present prodrug approach (Fig. 1) is to create an *N*-α-hydroxyalkyl derivative of the peptide bond since such derivatives of primary and cyclic amides are known to be spontaneously converted to the parent amide and the corresponding aldehyde in aqueous solu-

[1] Royal Danish School of Pharmacy, Department of Pharmaceutical Chemistry, 2 Universitetsparken, DK-2100 Copenhagen, Denmark.

[2] To whom correspondence should be addressed.

0724-8741/91/0300-0313$06.50/0 © 1991 Plenum Publishing Corporation

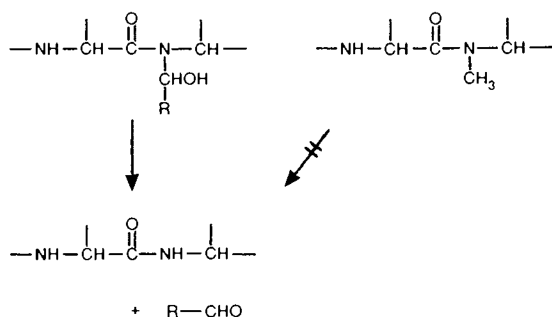


Fig. 1. Illustration of a possible prodrug approach to protect a peptide bond against enzymatic cleavage. Whereas an *N*-methyl derivative usually remains stable *in vivo*, the *N*-α-hydroxyalkyl derivative is spontaneously decomposed at physiological pH with release of the parent peptide and an aldehyde.

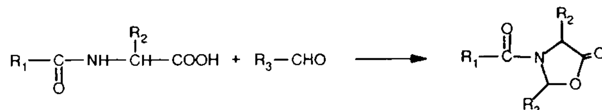
tion, with a conversion rate dependent on the nature of the alkyl group and the acidity of the amide (17–20). A major obstacle to this approach has been the difficulty in performing *N*-α-hydroxyalkylation of secondary amides such as peptide bonds (19), but this difficulty can be overcome by making 5-oxazolidinones (6). Such compounds are readily formed by condensing an α-amino acid or an *N*-acylated amino acid with an aldehyde (Scheme I). The lactone ring in *N*-acyl 5-oxazolidinones is highly reactive and is easily opened by hydrolysis or aminolysis, resulting in the intermediate formation of an *N*-α-hydroxyalkyl derivative (Scheme II) (6).

In this work, we have prepared a number of such *N*-α-hydroxyalkyl derivatives of peptide model compounds via 5-oxazolidinones (**1–9**) (Scheme III) and examined the influence of the structure on the kinetics of their decomposition to the parent peptide in aqueous solution. In addition, we show that such *N*-α-hydroxyalkylation of a peptide bond may be a useful prodrug approach to protect the bond or an adjacent peptide bond against specific proteolytic cleavage.

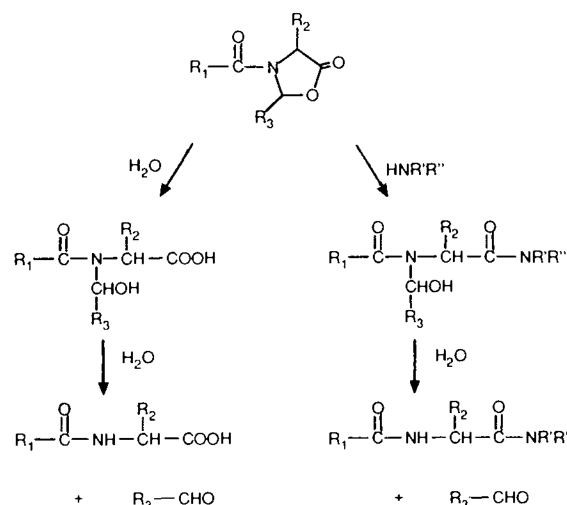
MATERIALS AND METHODS

Apparatus

High-performance liquid chromatography (HPLC) was done 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 columns were used: a Nova-Pak CN Radial Pak (Waters) C-18 column (100 × 8 mm; 4-μm particles) and a Chrompack column (100 × 3 mm) packed with Chromspher C-8 (5-μm particles). Readings of pH were carried out on a Radiometer Type PHM 26 meter. Melting points were taken on a capillary melting point apparatus and are uncorrected. Microanalysis were performed by G. Cornali, Leo Pharmaceuticals Ltd., Ballerup, Denmark.



Scheme I



Scheme II

Chemicals

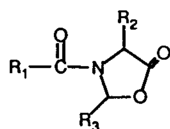
Amino acids and *N*-protected amino acids and peptides were purchased from Bachem AG, Bubendorf, Switzerland. Carboxypeptidase A (Type I; from bovine pancreas) was obtained from Sigma Chemical Company, St. Louis, MO. Chemicals and solvents used in the synthesis were from Aldrich-Chemie, F.R.G.

Preparation of 5-Oxazolidinones

The *N*-acylated 5-oxazolidinones **1–9** were prepared by reacting the *N*-benzyloxycarbonyl (**1–7**) or *N*-benzoyl (**8**) amino acid (L-configuration) with the appropriate aldehyde (paraformaldehyde, paraldehyde, chloral or benzaldehyde) as previously described (21,22). The melting points of the derivatives agreed with those reported in these references. The new *N*-acetyl-5-oxazolidinone **9** was prepared by refluxing a mixture of *N*-acetyl-L-phenylalanine (2.07 g, 0.01 mol), paraformaldehyde (400 mg), and *p*-toluenesulphonic acid (100 mg) in benzene (100 ml) for 4 hr. The mixture was cooled to 20°C and a slight, insoluble residue filtered off. The filtrate was washed with a 5% aqueous sodium bicarbonate solution and water, and then dried over anhydrous sodium sulphate. The *N*-acetyl-5-oxazolidinone **9** obtained, after removal of the benzene *in vacuo*, was crystallized from ether–petroleum ether, m.p. 77–78°C. *Anal.* Calc. for $C_{12}H_{13}NO_3$: C, 65.74; N, 5.98, N, 6.39. Found: C, 65.70; H, 5.99; N, 6.38.

Preparation of *N*-α-Hydroxyalkyl Derivatives (Table II)

N-Hydroxymethyl-*N*-carbobenzoxyglycine benzylamide (**18**) and *N*-(α-hydroxy-2,2,2,-trichloromethyl)-*N*-carbobenzoxyglycine benzylamide (**17**) were prepared by reacting the 5-oxazolidinones **1** and **3**, respectively, with an equivalent amount of benzylamine in ethanol (**18**) or benzene (**17**) as previously reported (21,22). Compound **19** was prepared in a similar way by stirring a mixture of 5-oxazolidinone **1** (331 mg, 1.5 mmol) and phenethylamine (0.19 ml, 1.5 mmol) in ethanol (5 ml) at room temperature for 20 hr. The solution was concentrated *in vacuo* and the residue obtained crystallized from chloroform–petroleum ether



	R_1	R_2	R_3
1	C_6H_5—CH_2O	H	H
2	C_6H_5—CH_2O	H	H
3	C_6H_5—CH_2O	H	CCl_3
4	C_6H_5—CH_2O	H	C_6H_5
5	C_6H_5—CH_2O	CH_3	H
6	C_6H_5—CH_2O	$CH(CH_3)_2$	H
7	C_6H_5—CH_2O	$CH_2C_6H_5$	H
8	C_6H_5	H	H
9	CH_3	$CH_2C_6H_5$	H

Scheme III

to yields 240 mg of compound **19**, m.p. 80–81°C. *Anal.* Calc. for $C_{19}H_{22}N_2O_4$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.60; H, 6.57; N, 8.22.

The *N*-α-hydroxyalkyl derivatives **20–29** were not isolated but prepared *in situ* by aminolysis of the 5-oxazolidinone **1** with the appropriate amino compound (amine, amino acid, or dipeptide) in water. Solutions of L-isoleucine, DL-norleucine, and diethylamine were prepared at a concentration of 0.1 *M* (pH 9.5), whereas solutions of L-leucine, L-leucinamide, and L-valine had a concentration of 0.2 *M* and a pH of 9.5. Solutions of glycine, glycinamide, and glycyl-glycine were prepared at a concentration of 0.5 *M*, with pH being adjusted to 9.5 for glycine and 8.5 for the two glycine derivatives. The derivatives **20–29** were obtained by adding 0.25 ml of a 0.15 *M* solution of **1** in acetonitrile to 10 ml of the amine solution described above. Following standing for 10 min at 20–25°C all oxazolidinone **1** had reacted as evidenced by HPLC analysis. These solutions were used as stock solutions for the kinetic measurements described below.

The *N*-α-hydroxyalkyl derivatives **10–16** and **30–31**, having a free carboxylic acid group, were also prepared *in situ* by hydrolysis of the appropriate *N*-acyl 5-oxazolidinone as described (6).

N-(Hydroxymethyl)benzylcarbamate

This compound was prepared by stirring a mixture of benzylcarbamate (3.0 g, 0.02 mol), potassium carbonate (100 mg), 37% formaldehyde solution (3 ml), ethanol (2 ml), and water (10 ml) for 2 hr at 60°C. The resultant clear solution was cooled to 4°C and the precipitate formed upon standing overnight was filtered off, washed with water, and recrystallized from ethanol–water to give 2.1 g of the title compound, m.p. 83–84°C. *Anal.* Calc. for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.63; H, 6.10; N, 7.76.

Kinetic Measurements

The decomposition of the *N*-acyl 5-oxazolidinones **3**, **8**, and **9** and the various *N*-α-hydroxyalkyl derivatives **10–31** was studied in aqueous buffer solutions at 37.0 ± 0.2°C. Hydrochloric acid, acetate, phosphate, and borate were used as buffers; the total buffer concentration was generally 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 rates of decomposition were determined by using reversed-phase HPLC procedures capable of separating the compounds from their products of degradation. Mobile phase systems of 0.1% phosphoric acid containing acetonitrile (10–50, v/v) and methanol (0–30, v/v) were used, the acetonitrile and methanol concentration being adjusted for each compound to give an appropriate retention time (3–8 min). 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 30–100 μl of a stock solution of the derivatives in acetonitrile or 100 μl of the aqueous reaction solutions described above to 10.0 ml of buffer solution, preequilibrated at 37°C, in screw-capped test tubes, the final concentration of the derivatives in the buffer solution being 5×10^{-5}–10^{-4} *M*. The solutions were kept in a water bath at 37 ± 0.2°C, and at appropriate times samples were taken and immediately chromatographed. In some cases, the reactions were stopped by diluting the samples with an acetate buffer solution of pH 4 and then analyzing this mixture by HPLC. Pseudo-first-order rate constants for the degradation were determined from the slopes of linear plots of the logarithm of residual derivative against time.

Degradation studies in human plasma solutions were performed as earlier described (6).

In the case of the *N*-hydroxymethyl derivative of *N*-acetyl-L-phenylalanine (**31**), it was not possible to find an HPLC procedure allowing adequate separation of the compound from its product of decomposition, *N*-acetyl-L-phenylalanine. The decomposition of compound **31** was instead monitored by measuring the production of formaldehyde using a spectrophotometric method as described for other *N*-hydroxymethyl derivatives (23). The initial concentration of the *N*-acetyl-5-oxazolidinone **9** in the buffer solu-

tions was 8×10^{-4} *M*. At various intervals 1-ml samples were withdrawn and diluted to 10 ml with water. A 500-μl sample of the dilution was then immediately analyzed for formaldehyde (23).

Degradation Studies in the Presence of Carboxypeptidase A

The stability of Z-Gly-L-Leu, Z-Gly-L-Ala and the derivatives **23** and **24** in the presence of carboxypeptidase A was examined at 37°C in a 0.05 *M* phosphate buffer solution of pH 7.40 containing the enzyme at a concentration of 50 U/ml. The initial concentration of the derivatives was 5×10^{-4} *M*. The reaction solutions were kept at 37°C, and at various intervals samples of 200 μl were withdrawn and added to 1000 μl of methanol in order to stop the reaction and deproteinize the samples. After mixing and centrifugation for 3 min at 13,000 rpm, 20 μl of the clear supernatant was analyzed by HPLC as described above.

RESULTS AND DISCUSSION

Hydrolysis of *N*-Acyl 5-Oxazolidinones

The kinetics of hydrolysis of the *N*-benzyloxycarbonyl (Z) 5-oxazolidinones **1**, **2**, and **4–7** has previously been determined in aqueous buffer solutions at 37°C and $\mu = 0.5$ (6). In the present study the hydrolysis kinetics of compound **3** and two *N*-acylated 5-oxazolidinones, **8** and **9**, was examined under similar conditions.

The influence of pH on the hydrolytic opening of the lactone ring of these oxazolidinones is shown in Fig. 2, where the logarithms of the buffer-independent pseudo-first-order rate constant (*k*) are plotted against pH. The observed pH–rate relationships indicate that the 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:

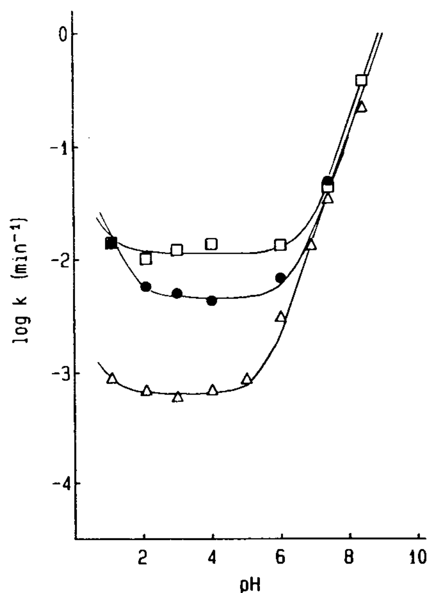


Fig. 2. The pH–rate profiles for the hydrolysis of the *N*-acyl 5-oxazolidinones **3** (●), **8** (□), and **9** (△) in aqueous solution ($\mu = 0.5$) at 37°C.

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

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 according to the following equation (24):

$$\log a_{OH} = \text{pH} - 13.62 \quad (2)$$

Values of the specific rate constants k_0, k_H, and k_{OH} obtained from the pH-rate profiles and Eq. (1) are listed in Table I along with half-lives for the hydrolysis of the derivatives at pH 4 and 7.4. For comparison, rate data for the previously studied oxazolidinones have been included in Table 1.

The rate data show that the various *N*-acylated 5-oxazolidinones are reactive compounds being easily hydrolyzed in aqueous solution. Maximal stability occurs at pH 3–5. The stability is influenced by the steric and electronic properties of the substituents R_1, R_2, and R_3 (6). By comparing the reactivity of the compounds **1** and **8**, it can be seen that the replacement of a benzyloxycarbonyl group (**1**) at the nitrogen with a benzoyl group (**8**) does not markedly change the chemical reactivity.

Decomposition of *N*-α-Hydroxyalkyl Derivatives

The primary objective of the present study was to determine the stability of the *N*-α-hydroxyalkyl derivatives. Since the first hydrolytic step in the overall degradation of the *N*-acyl 5-oxazolidinones proceeds much faster in neutral and alkaline solutions (except for compound **4**) (6) than the second step, the *N*-α-hydroxyalkyl derivatives were easily provided by incubating the 5-oxazolidinones in such solutions. HPLC analysis of aqueous solutions of the oxazolidinones at pH 7–10 showed that the disappearance of the peak from the oxazolidinone was accompanied by the formation of a new peak which subsequently disappeared more slowly. During the latter reaction a new peak in the chromatograms formed. It was identified as the parent *N*-acyl amino acid, e.g., Z-Gly from compound **1** and *N*-benzoyl glycine from compound **8**, by comparison of the retention time with that of an authentic sample.

The high chemical reactivity of the *N*-acyl 5-oxazolidinones is also reflected in their ease of undergoing aminolysis (21,22,25–27). By reacting the oxazolidinones **1** and **3** with one equivalent of benzylamine or phenethylamine in ethanol or benzene, the *N*-α-hydroxyalkyl derivatives of the corresponding *N*-acyl amino acid amides (**17–19**, Table II) were readily formed and isolated. It was found that this aminolysis also could be performed in aqueous solution. By using a high concentration of an amine, the aminolysis predominates over hydrolysis of the oxazolidinone ring as evidenced by HPLC analysis. Using the conditions described under Materials and Methods, the *N*-α-hydroxyalkyl derivatives **20–29** (Table II) were readily obtained in this way with a minimal concurrent formation of hydrolysis products.

The kinetics of decomposition of the various *N*-α-hydroxyalkylated peptides were examined in aqueous buffer solutions at 37°C as a function of pH. At constant pH and temperature the decomposition followed strict first-order kinetics over several half-lives.

Table I. Rate Data for the Hydrolysis of Various N-Acyl 5-Oxazolidinones in Aqueous Solution at 37°C and $\mu = 0.5$[a]

Compound	k_H (M^{-1} min^{-1})	k_0 (min^{-1})	k_{OH} (M^{-1} min^{-1})	$t_{1/2}$ (min)	
				pH 4.0	pH 7.4
1	0.037	0.051	5.0×10^4	14	8.2
2	1.5	0.27	nd	2.7	2.7
3	0.14	4.6×10^{-3}	8.0×10^4	150	14
4	2.0	0.15	3.9×10^4	4.6	4.1
5	0.014	9.0×10^{-3}	3.7×10^4	77	18
6	5.0×10^{-3}	1.2×10^{-3}	8.9×10^3	580	78
7	0.032	1.5×10^{-3}	3.9×10^4	460	30
8	0.05	0.011	6.7×10^4	63	16
9	2.5×10^{-3}	6.5×10^{-4}	6.5×10^4	1065	20

[a] The data for compounds 1, 2, and 4–7 are from a previous study (6).

As noted above the N-acyl oxazolidinones and hence the N-α-hydroxyalkyl derivatives are quantitatively converted to aldehyde and the parent N-acyl amino acid derivative in aqueous solution. This was specifically shown for the derivatives **17**, **18**, and **21**. An example of a product analysis is shown in Fig. 3. It can readily be seen that the disappearance of the N-hydroxymethyl derivative **18** is accompanied by the stoichiometric formation of N-benzyloxycarbonylglycine benzylamide (**32**) (Scheme IV). The latter compound was prepared as described by Ben-Ishai (21). Such quantitative conversion of compound **18** as well as of compound **17** to compound **32** was observed at all pH values studied (pH 1–11). Similarly, compound **21** was shown to be converted to Z-Gly-Gly as evidenced by HPLC analysis, using an authentic sample of Z-Gly-Gly for comparison.

Table II. Rate Data for the Decomposition of Various N-α-Hydroxylalkyl Derivatives of Amino Acids and Peptides in Aqueous Solution ($\mu = 0.5$) at 37°C

$$R_1{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}\underset{\displaystyle \underset{R_3}{\overset{|}{CHOH}}}{\underset{|}{N}}{-}\overset{\displaystyle R_2}{\overset{|}{CH}}{-}\overset{\displaystyle O}{\overset{\|}{C}}{-}R_4$$

Compound	R_1	R_2	R_3	R_4	k_{OH}[a] (M^{-1} min^{-1})	$t_{1/2}$ (hr)[b]
10	$C_6H_5CH_2O$	H	H	OH	32	205
11	$C_6H_5CH_2O$	CH_3	H	OH	96	199
12	$C_6H_5CH_2O$	$CH(CH_3)_2$	H	OH	114	169
13	$C_6H_5CH_2O$	$CH_2C_6H_5$	H	OH	49	391
14	$C_6H_5CH_2O$	H	CH_3	OH	5.3×10^3	3.6
15	$C_6H_5CH_2O$	H	C_6H_5	OH	2.6×10^5	0.08
16	$C_6H_5CH_2O$	H	CCl_3	OH	1.9×10^3	5.7
17	$C_6H_5CH_2O$	H	CCl_3	$NHCH_2C_6H_5$	5.7×10^4	0.18
18	$C_6H_5CH_2O$	H	H	$NHCH_2C_6H_5$	2.7×10^3	10.8
19	$C_6H_5CH_2O$	H	H	$NHCH_2CH_2C_6H_5$	8.6×10^2	22.3
20	$C_6H_5CH_2O$	H	H	$N(C_2H_5)_2$	1.1×10^3	17.5
21	$C_6H_5CH_2O$	H	H	Gly	1.3×10^3	15.3
22	$C_6H_5CH_2O$	H	H	Gly-NH_2	1.7×10^3	11.2
23	$C_6H_5CH_2O$	H	H	Ala	1.4×10^2	137
24	$C_6H_5CH_2O$	H	H	Leu	20	980
25	$C_6H_5CH_2O$	H	H	Leu-NH_2	12	1560
26	$C_6H_5CH_2O$	H	H	iso-Leu	13	1535
27	$C_6H_5CH_2O$	H	H	nor-Leu	19	1035
28	$C_6H_5CH_2O$	H	H	Val	21	910
29	$C_6H_5CH_2O$	H	H	Gly-Gly	1.4×10^3	14.1
30	C_6H_5	H	H	OH	2.9×10^3	6.6
31	CH_3	$CH_2C_6H_5$	H	OH	57	337

[a] Apparent hydroxide ion catalytic rate constant.

[b] Half-lives of decomposition at pH 7.4. Some values are determined at pH 7.4, whereas others are calculated from the k_{OH} values.

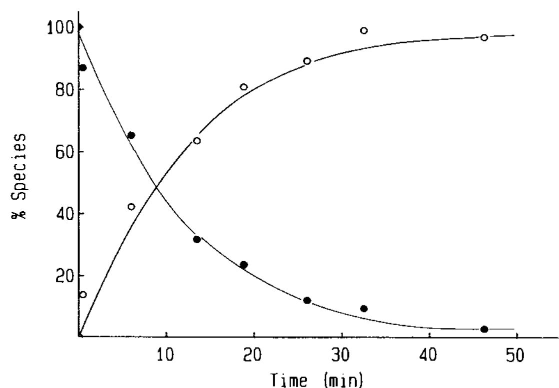


Fig. 3. Time courses for the N-hydroxymethyl derivative **18** (●) and N-benzyloxycarbonylglycine benzylamide (**32**) (○) during decomposition of compound **18** in a 0.02 M borate buffer solution of pH 9.4 (37°C).

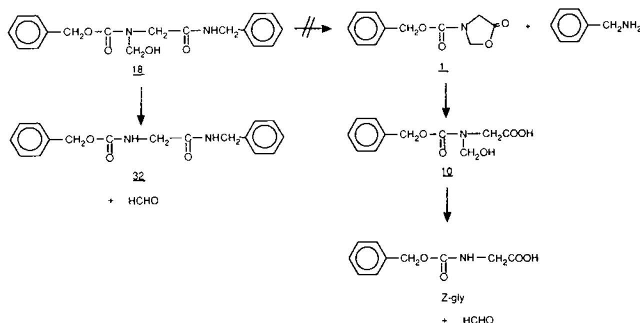
As illustrated in Scheme IV a possible additional degradation route of compound **18** and similar N-α-hydroxyalkyl amino acid amides might be an intramolecular attack of the hydroxyl group on the neighboring amide moiety to give the original N-acyl 5-oxazolidinone and benzylamine. However, such a reaction can be totally excluded since the HPLC analysis of the reaction solutions of **18** revealed not even traces of either compound **1**, compound **10**, Z-Gly, or benzylamine. In addition, the observed quantitative formation of compound **32** excludes the occurrence of this hypothetic reaction.

The influence of pH on the decomposition rate of some N-α-hydroxyalkyl derivatives is shown in Figs. 4 and 5, where the logarithms of the observed pseudo-first-order rate constants (k_{obs}) are plotted against pH. The shape of the pH–rate profiles indicates the occurrence of specific acid- and base-catalyzed reactions as well as a spontaneous or water-catalyzed decomposition:

$$k_{obs} = k_H a_H + k_0 + k_{OH} a_{OH} \tag{3}$$

where a_H, a_{OH}, and the parameters k are as defined above in connection with Eq. (1).

The derivatives **10–16** and **30–31** contain an ionizable carboxylic acid group, and as shown for **21** and **30** (Fig. 4), this results in a curvature of the pH–rate profiles at a pH around the pK_a value for the carboxy group. The rate expression for these compounds can be formulated as



Scheme IV

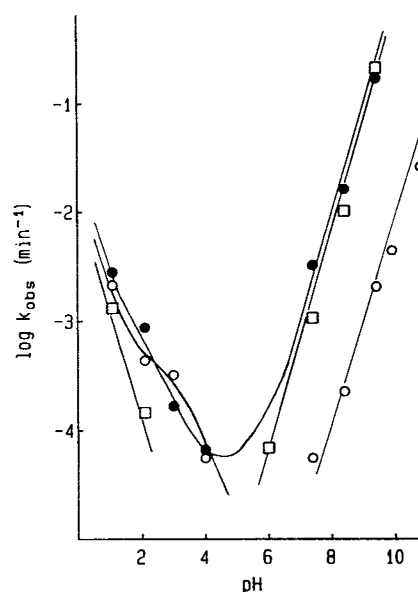


Fig. 4. The pH–rate profiles for the decomposition of the N-hydroxymethyl derivatives **30** (●), **18** (□), and **21** (○) in aqueous solution (μ = 0.5) at 37°C.

$$k_{obs} = k_H a_H \frac{a_H}{a_H + K_a} + (k'_H a_H + k_0 + k_{OH} a_{OH}) \frac{K_a}{a_H + K_a} \tag{4}$$

where $a_H/(a_H + K_a)$ and $K_a/(a_H + K_a)$ are the fractions of the free acid and dissociated forms, respectively, k_H is the specific acid-catalytic rate constant for the free acid form, and k'_H, k_0, and k_{OH} are the catalytic rate constants for the anionic form. The values of the various rate constants derived from the pH–rate profiles are listed in Table III. Most derivatives were studied only at pH 7–10 and for these compounds only a k_{OH} value was thus obtained. These values

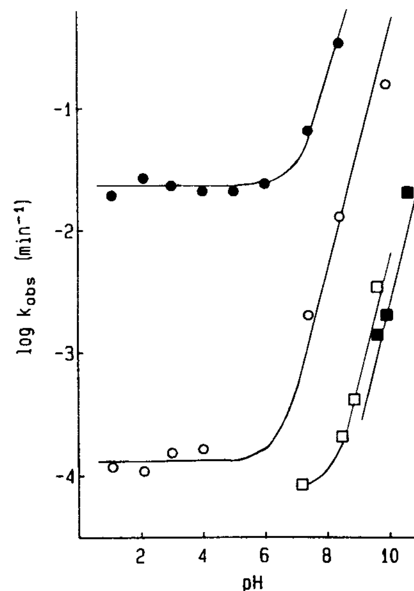


Fig. 5. The pH–rate profiles for the decomposition of the N-α-hydroxyalkyl derivatives **16** (○), **17** (●), **23** (□), and **24** (■) in aqueous solution (μ = 0.5) at 37°C.

Table III. Rate Data for the Decomposition of Various N-α-Hydroxyalkyl Derivatives in Aqueous Solution at 37°C and $\mu = 0.5$

Compound	k_H (M^{-1} min^{-1})	k'_H (M^{-1} min^{-1})	K_0 (min^{-1})	k_{OH} (M^{-1} min^{-1})	pK_a
10	1.9×10^{-2}	0.7	2.2×10^{-5}	32	3.2
13	1.6×10^{-2}	0.4		49	3.3
15	65	5.9×10^{2}		2.6×10^{5}	3.3
16			1.3×10^{-4}	1.9×10^{3}	
17			2.3×10^{-2}	5.7×10^{4}	
18	1.4×10^{-2}			2.7×10^{3}	
30	3.0×10^{-2}	0.2	0.5×10^{-5}	2.9×10^{3}	2.6

are given in Table II along with half-lives of decomposition at pH 7.4 and 37°C. These half-lives were either observed or calculated on the basis of the k_{OH} values obtained at higher pH values.

Structural Effects on Reaction Rate

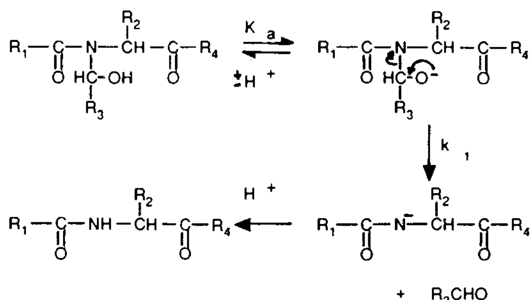
In considering the concept of using N-α-hydroxyalkyl derivatives of peptides as prodrugs, the most important parameter is the stability of the derivatives at physiological pH and temperature. Inspection of the half-lives at pH 7.4 and 37°C (Table II) shows that the derivatives studied possess vastly different stabilities and that all the substituents (R_1–R_4) influence the stability.

It has been established that the reaction mechanism for the decomposition of N-α-hydroxyalkyl derivatives of amides, imides, and similar NH-acidic compounds in neutral and alkaline solutions involves a stepwise pathway with an N-α-hydroxyalkyl anion as an intermediate undergoing rate-determining N–C bond cleavage as illustrated in Scheme V (17,18,28). In Scheme V K_a is the ionization constant for the hydroxyl group and k_1 is a first-order rate constant for cleavage of the ionized N-α-hydroxyalkyl compound. According to this mechanism the rate law for the reactions occurring in neutral and alkaline solutions can be written as

$$k_{obs} = k_1 \frac{K_a}{a_H + K_a} \quad (5)$$

At pH values up to 10–11, $a_H \gg K_a$, which means that Eq. (5) can be reduced to

$$k_{obs} = k_1 K_a / a_H \quad (6)$$



Scheme V

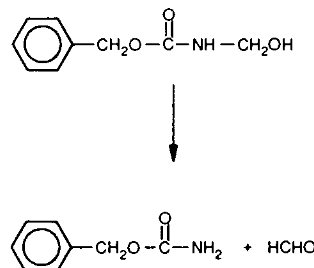
or

$$k_{obs} = \frac{k_1 K_a}{K_w} a_{OH} \quad (7)$$

where K_w is the autoprotolysis constant of water. Equation (7) predicts that the rate of decomposition is directly proportional to a_{OH} at pH $\ll$ pK_a as actually observed for the N-α-hydroxyalkyl derivatives of the present study.

It has previously been shown that the rate of decomposition of various N-hydroxymethylated primary amides increases with increasing acidity of the parent amide (17,18), which can be explained by increased k_1 values through increased leaving ability of the amide anion. Such an effect may partly explain the striking difference in the stability of the N-benzyloxycarbonyl and N-benzoyl derivatives **10** and **30** since benzamide is more acidic than benzylcarbamate. To test this explanation the N-hydroxymethyl derivative of benzylcarbamate was prepared and its decomposition (Scheme VI) studied in aqueous solution (pH 9–11) at 37°C. A k_{OH} value of 17 M^{-1} min^{-1} was found. The corresponding k_{OH} value for N-(hydroxymethyl)benzamide is 120 M^{-1} min^{-1} (29). Thus, the latter is sevenfold more reactive than the corresponding carbamate derivative. This may be compared with the 31-fold greater reactivity of compound **30** relative to compound **10**. The intermediary reactivity of the N-acetyl derivative **31** can also be rationalized in terms of parent amide acidity since acetamide is less acidic than benzamide (30).

Comparison of the rate data for the compounds **10** and **18–29** reveals a dramatic influence of R_4 on the stability. By substituting the carboxy group in **10** with a benzylamide group (**18**), the reactivity increases by a factor of 19. A similar trend is seen for the derivatives **16** and **17**. This increased reactivity of derivatives with a C-terminal amide moiety instead of a free carboxylic acid can probably be ascribed to



Scheme VI

the stronger electron-attracting properties of an amido group relative to an ionized carboxy group, which in turn increases the leavability of the amide anion and hence the rate constant k_1 (cf. Scheme V).

However, whereas compound **18** with a benzylamide group possesses almost the same reactivity as compounds with a glycine group or related moieties (**21**, **22**, and **29**), the stability of compounds formed with other amino acids (**23**–**28**) is much greater. Thus, the leucine derivatives **24**–**27** are more stable than the free acid **10**. Apparently, steric effects within the R_4 moiety greatly influence the reactivity. As seen from Fig. 6 the different reactivities of compounds **21**, **23**, **24**, and **26**–**28** can be adequately correlated with the steric effects of the α-substituents in the *C*-terminal amino acids. The regression equation between log $t_{1/2}$ and Charton's steric parameter ν (31) is

$$\log t_{1/2} = 1.98\,\nu + 1.28 \qquad (r = 0.950;\ n = 6) \qquad (8)$$

No explanation can presently be offered for this structural influence on the decomposition rate. It is of interest to note that the apparent steric influence is not reflected in substituents on the nitrogen since the *N*,*N*-diethylamide derivative **20** shows a reactivity similar to the monosubstituted amides **18** and **19**.

The structure of the R_3 substituent and hence the aldehyde component is also seen to have a pronounced influence on the stability of *N*-α-hydroxyalkylated peptides in aqueous solution. Similar differences in reactivity have previously been observed for such derivatives of benzamide and thiobenzamide (19) and may be due largely to steric effects within the α-substituents, although polar effects may also play a role.

Finally, it is of interest to note the much greater reactivity of the *N*-(hydroxymethyl) derivative **30** compared with *N*-(hydroxymethyl)benzamide. Whereas the latter is degraded with a half-life of 160 hr at pH 7.4 and 37°C, compound **30** shows a half-life of 6.6 hr. This large difference in stability appears to be due—in an as yet unknown manner—to the fact that whereas *N*-(hydroxymethyl)benzamide is derived from a primary amide, compound **30** is from a secondary amide. Thus, *N*-(hydroxymethyl)-*N*-methylbenzamide has been found to degrade with a half-life of only 4.2 hr at pH 7.4 and 37°C (unpublished observation).

Whereas plasma enzymes catalyze the hydrolysis of the *N*-acyl 5-oxazolidinones considerably (6), the rate of decomposition of the *N*-α-hydroxyalkyl derivatives is only slightly affected by the presence of human plasma, which is similar to the behavior of other *N*-α-hydroxyalkyl compounds (9, 19, 32). Thus, compound **18** showed a half-life of decomposition of 4.8 hr in 80% human plasma, compared with 10.8 hr in pure pH 7.4 buffer solution.

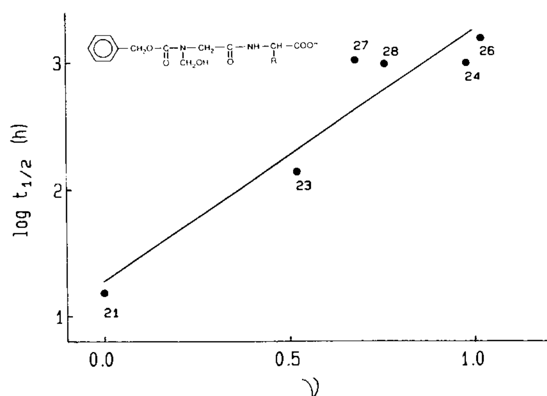


Fig. 6. Plot of log $t_{1/2}$ against the steric substituent parameter ν for the decomposition of various *N*-hydroxymethylated Z-protected peptides in aqueous solution at pH 7.40 and 37°C. The parameter ν refers to R in the formula shown.

Stability Toward Carboxypeptidase A

Carboxypeptidase A (CPA) is a pancreatic proteolytic enzyme whose primary function is that of a *C*-terminal exopeptidase. The enzyme catalyzes the hydrolysis of almost any peptide having a terminal free carboxyl group and a *C*-terminal residue of the L configuration (33,34). The rate of hydrolysis is usually enhanced if the terminal residue is aromatic or branched aliphatic (33,34).

To investigate whether *N*-α-hydroxyalkylation of the second peptide bond in a dipeptide would make the terminal bond stable toward CPA, the rates of decomposition of the derivatives **23** and **24** were determined in the presence of CPA at a concentration of 50 U/ml. Under these conditions (pH 7.4 and 37°C) the parent *N*-acylated dipeptides Z-Gly-L-Leu and Z-Gly-L-Ala were found to degrade to yield Z-Gly with first-order half-lives of 6 min and 8.7 hr, respectively. The greater reactivity of the leucine derivative is in accordance with previous studies of these dipeptides (35). Under identical reaction conditions the alanine compound **23** was found to degrade with a half-life of 141 hr, which is similar to that observed in enzyme-free solution (137 hr). Compound **24** showed an extent of degradation of 5% after incubation for 66 hr in the enzyme solution, which corresponds to a half-life about 924 hr. This is also similar to that in pure buffer solution (980 hr).

These results demonstrate that *N*-hydroxymethylation of the *N*-terminal peptide bond in the *N*-acylated dipeptides Z-Gly-L-Leu and Z-Gly-L-Ala completely protects the *C*-terminal peptide bond against CPA (Fig. 7). Thus, the presence and integrity of the second peptide bond appear to be important for the enzymatic reactivity. This is in harmony with the findings that the rate of the CPA-catalyzed hydrolysis of *N*-acyl dipeptides is greatly decreased if *N*-methylglycine (sarcosine) or β-alanine is the second amino acid (36,37). Direct protection of the susceptible *C*-terminal peptide bond of an *N*-acylated dipeptide (or a higher homologue) by *N*-α-hydroxyalkylation of this bond should also be possible by transforming an *N*-acyl dipeptides into the corresponding 5-oxazolidinone through condensation with an aldehyde and subsequent hydrolysis, but this remains to be studied.

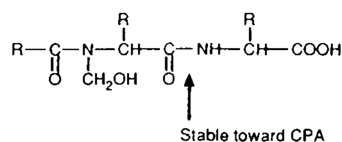


Fig. 7. Stabilization of a *C*-terminal peptide bond toward carboxypeptidase A by *N*-hydroxymethylation of the second peptide bond.

CONCLUSIONS

The prodrug approach described protects peptides against specific proteolytic cleavage as illustrated with carboxypeptidase A. Further, *N*-α-hydroxyalkylation of a peptide bond via 5-oxazolidinone formation may also protect peptides against hydrolysis by other proteolytic enzymes, such as αchymotrypsin and trypsin, since these enzymes usually also require the free NH-moiety of the peptide bond (38). In contrast to *N*-methylation or similar *N*-alkylation, *N*-α-hydroxyalkylation is a bioreversible approach, the *N*-α-hydroxyalkyl derivatives being converted quantitatively to the parent peptides via a spontaneous, i.e., nonenzymatic, reaction. The stability of the derivatives is high in weakly acidic solutions and can be controlled by selection of appropriate aldehydes for the initial 5-oxazolidinone formation. Thus, by using acetaldehyde or chloral instead of formaldehyde, a higher rate of prodrug conversion is achieved.

An interesting aspect of this approach to protect the peptide bond against rapid proteolytic cleavage is further derivatization of the hydroxyl group in the *N*-α-hydroxyalkyl derivatives, e.g., by esterification to afford *N*-α-acyloxyalkyl derivatives which are cleavable by unspecific esterases. In this manner not only the stability but also the lipophilicity can be further modified. Studies are in progress to examine this aspect.

ACKNOWLEDGMENTS

This work has been supported by PharmaBiotec Research Centre and the Lundbeck Foundation.

REFERENCES

1. K. Wiedhaup. The stability of small peptides in the gastrointestinal tract. In D. D. Breimer and P. Speiser (eds.), *Topics in Pharmaceutical Sciences,* Elsevier, Amsterdam, 1981, pp. 307–324.
2. M. J. Humphrey and P. S. Ringrose. Peptides and related drugs: A review of their absorption, metabolism and excretion. *Drug Metabl. Rev.* 17:283–310 (1986).
3. V. H. L. Lee and A. Yamamoto. Penetration and enzymatic barriers to peptide and protein absorption. *Adv. Drug Deliv. Rev.* 4:171–207 (1990).
4. H. Bundgaard. Bioreversible derivatizaiton of peptides. In S. S. Davis, L. Illum, and E. Tomlinson (eds.), *Delivery Systems for Peptide Drugs,* Plenum Press, New York, 1986, pp. 49–68.
5. U. Klixbüll and H. Bundgaard. Prodrugs as drug delivery systems. 30.4-Imidazolidinones as potential bioreversible derivatives for the α-aminoamide moiety in peptides. *Int J Pharm.* 20:273–284 (1984).
6. A. Buur and H. Bundgaard. Prodrugs of peptides. III. 5-Oxazolidinones as bioreversible derivatives for the α-amido carboxy moiety in peptides. *Int. J. Pharm.* 46:159–167 (1988).
7. H. Bundgaard and J. Møss. 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.* 78:122–126 (1989).
8. H. Bundgaard and J. Møss. Prodrug derivatives of thyrotropin-releasing hormone and other peptides. *Biochem. Soc. Trans.* 17:947–949 (1989).
9. J. Møss and H. Bundgaard. Prodrugs of peptides. 5. Protection of the pyroglutamyl residue against pyroglutamyl aminopeptides by bioreversible derivatization with glyoxylic acid derivatives. *Int. J. Pharm.* 52:255–263 (1989).
10. H. Bundgaard and J. Møss. 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.* 7:885–892 (1990)
11. J. Møss and H. Bundgaard. Prodrugs of peptides. 7. Transdermal delivery of thyrotropin-releasing hormone (TRH) via prodrugs. *Int. J. Pharm.* 66:39–45 (1990).
12. P. S. Farmer. Bridging the gap between bioactive peptides and nonpeptides: some perspectives in design. In E. J. Ariëns (ed.), *Drug Design, Vol. X,* Academic Press, London, 1980, pp 119–143.
13. B. E. B. Sandberg C.-M. Lee, M. R. Hanley, and L. L. Iversen. Synthesis and biological properties of enzyme-resistant analogues of substance P. *Eur. J. Biochem.* 114:329–337 (1981).
14. S. Thaisrivongs, D. T. Pals, D. W. Harris, W. M. Kati, and S. R. Turner. Design and synthesis of a potent and specific renin inhibitor with a prolonged duration of action *in vivo. J. Med. Chem.* 29:2088–2093 (1986).
15. D. F. Veber and R. M. Freidinger. The design of metabolically-stable peptide analogs. *Trends Neurosci.* 8:392–396 (1985).
16. J.-L. Fauchère. Elements for the rational design of peptide drugs. *Adv. Drug Res.* 15:29–69 (1986).
17. M. Johansen and H. Bundgaard. Prodrugs as drug delivery systems. VI. Kinetics and mechanism of the decomposition of *N*-hydroxymethylated amides and imides in aqueous solution and assessment of their suitability as possible prodrugs. *Arch. Pharm. Chem. Sci. Ed.* 9:40–42 (1981).
18. H. Bundgaard and M. Johansen. Prodrugs as drug delivery systems. VIII. Bioreversible derivatization of hydantoins by *N*-hydroxymethylation. *Int. J. Pharm.* 5:67–77 (1980).
19. H. Bundgaard and M. Johansen. Hydrolysis of *N*-(α-hydroxybenzyl)benzamide and other *N*-(α-hydroxyalkyl)-amide derivatives: Implications for the design of *N*-acyloxyalkyl-type prodrugs. *Int. J. Pharm.* 22:45–56 (1984).
20. H. Bundgaard. Design of prodrugs: Bioreversible derivatives for various functional groups and chemical entities. In H. Bundgaard (ed.), *Design of Prodrugs,* Elsevier, Amsterdam, 1985, pp. 1–92.
21. D. Ben-Ishai. Reaction of acylamino acids with paraformaldehyde. *J. Org. Chem.* 79:5736–5738 (1957).
22. F. Micheel and W. Meckstroth. Peptidsynthesen nach dem Oxazolidon-Verfahren. III. *Chem. Ber.* 92:1675–1679 (1959).
23. M. Johansen, H. Bundgaard, and E. Falch. Spectrophotometric determination of the rates of hydrolysis of aldehyde-releasing pro-drugs in aqueous solution and plasma. *Int. J. Pharm.* 13:89–98 (1983).
24. H. S. Harned and W. J. Hamer. The ionization constant of water in potassium chloride solution from electromotive forces of cells without liquid junctions. *J. Am. Chem. Soc.* 53:2194–2206 (1933).
25. F. Micheel and S. Thomas. Eine neue Peptid-Synthese. *Chem. Ber.* 90:2906–2909 (1957).
26. E. Dane, R. Heiss, and H. Schäfer. Peptid-Synthesen unter Verwendung von Chloral. *Angew. Chem.* 71:339 (1959).
27. M. Itoh. Peptides. I. Selective protection of α- or side-chain carboxyl groups of aspartic and glutamic acid. A facile synthesis of β-aspartyl and γ-glutamyl peptides. *Chem. Pharm. Bull.* 17:1679–1686 (1969).
28. J. Ugelstad and J. de Jonge. A kinetical investigation of the reaction between amides and formaldehyde. *Rec. Trav. Chem.* 76:919–945 (1957).
29. H. Bundgaard and A. Buur. 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.* 37:185–194 (1987).
30. H. Bundgaard and M. Johansen. Pro-drugs as drug delivery systems. X. N-Mannich bases as novel pro-drug candidates for amides, imides, urea derivatives, amines and other NH-acidic compounds. Kinetics and mechanisms of decomposition and structure-activity relationships. *Arch. Pharm. Chem. Sci. Ed.* 8:29–52 (1980).
31. M. Charton. The prediction of chemical lability through substituent effects. In E. B. Roche (ed.), *Design of Biopharmaceutical*

Properties Through Prodrugs and Analogs, American Pharmaceutical Association, Washington, D.C., 1977, pp. 228–280.
32. M. Johansen and H. Bundgaard. Decomposition of rolitetracycline and other N-Mannich bases and of *N*-hydroxymethyl derivatives in the presence of plasma. *Arch. Pharm. Chem. Sci. Ed.* **9**:40–42 (1981).
33. E. L. Smith. The specificity of certain peptidases. *Adv. Enzymol.* **12**:191–257 (1951).
34. J. A. Hartsuck and W. N. Lipscomb. Carboxypeptidase A. In P. D. Boyer (ed.), *The Enzymes, Vol. III,* Academic Press, New York, 1971. pp. 1–56.
35. M. A. Stahmann, J. S. Fruton, and M. Bergmann. The specificity of carboxypeptidase. *J. Biol. Chem.* **164**:753–760 (1946).
36. H. T. Hanson and E. L. Smith. The application of peptides containing β-alanine to the study of the specificity of various peptidases. *J. Biol. Chem.* **175**:833–848 (1948).
37. J. E. Snoke and H. Neurath. Structural requirements of specific substrates for carboxypeptidases. *J. Biol. Chem.* **181**:789–802 (1949).
38. D. M. Blow. The structure of chymotrypsin. In P. D. Boyer (ed.), *The Enzymes, Vol. III,* Academic Press, New York, 1971, pp. 185–212.