Prodrugs of Peptides IV: Bioreversible Derivatization of the Pyroglutamyl Group by *N*-Acylation and *N*-Aminomethylation to Effect Protection against Pyroglutamyl Aminopeptidase

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Abstract □ Various *N*-acyl derivatives and *N*-Mannich bases of the model compound L-pyroglutamyl benzylamide were synthesized to assess their suitability as prodrug forms for the *N*-terminal pyroglutamyl residue occurring in several peptides, with the aim of improving peptide delivery characteristics. Whereas pyroglutamyl benzylamide was rapidly hydrolyzed by pyroglutamyl aminopeptidase, the *N*-acyl derivatives and *N*-Mannich bases (*N*-aminomethyl derivatives) were totally resistant to cleavage by the enzyme. On the other hand, these derivatives are readily bioreversible, the conversion to the parent pyroglutamyl amide taking place either by spontaneous hydrolysis at physiological pH, as demonstrated for the *N*-Mannich bases, or by plasma enzymes, as shown for the *N*-acyl derivatives. The results suggest that by appropriate *N*-acylation or *N*-aminomethylation it may be feasible to protect pyroglutamyl-containing peptides against cleavage by pyroglutamyl aminopeptidase and to obtain a release of the parent peptide in the organism, hence improving the delivery characteristics of such peptides.

Development of peptide drugs is presently a major area in drug research and, in recent years, several biologically active peptides, including peptides consisting of only two or three amino acids, have been discovered. The application of peptides as well as proteins as clinically useful drugs is, however, seriously hampered due to substantial delivery problems. 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 due to rapid metabolism.[1-7]

A possible approach to solve these delivery problems, especially in the case of small peptides, may be derivatization of the bioactive peptides to produce prodrugs or transport forms which possess enhanced physicochemical properties in comparison with the parent compounds with regard to delivery and metabolic stability. Thus, it may be imagined that bioreversible derivatization may protect small peptides against degradation by peptidases present at the mucosal absorption barrier and render hydrophilic peptides more lipophilic and hence facilitate their absorption. To be useful, however, the derivatives should be cleaved spontaneously or enzymatically in the blood following their absorption, with release of the parent bioactive peptide.[8]

Recently, studies have been initiated in our laboratory to develop various types of bioreversible derivatives for the functional groups or chemical entities occurring in amino acids and peptides.[8-12] In the present work, various derivatives of the pyroglutamyl group have been developed. An *N*-terminal pyroglutamyl residue occurs in several peptides and proteins such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH), neurotensin, bombesin, gastrin, fibrinopeptides, and collagen.[13,14] The specific cleavage of the *N*-terminal pyroglutamyl residue from such peptides and proteins is effected by pyroglutamyl aminopeptidase (also called L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8).[13-19] The enzyme, which occurs in many different tissues such as liver and kidney,[14-16] plays a major role in the metabolism of such pyroglutamyl-containing peptides as TRH and LH-RH.[20-22] Hence, the enzyme also plays a role in the delivery problems associated with these pharmacologically interesting peptides.

Several attempts have been made to improve the resistance of pyroglutamyl-containing peptides, notably TRH, to enzymatic degradation by the development of analogues in which the pyroglutamyl group has been replaced by other ring structures.[23-26] While increased resistance to degradation by pyroglutamyl aminopeptidase has been observed in several cases for such analogues, this approach involves the design of a new peptide. In contrast, the prodrug approach to solve delivery problems implies that the parent bioactive peptide is ultimately released in the body. In this work we show that by suitable derivatization of the pyroglutamyl group it is feasible to obtain derivatives which are completely resistant to attack by pyroglutamyl aminopeptidase and, at the same time, capable of releasing the parent pyroglutamyl derivative by spontaneous or plasma-catalyzed hydrolysis.

Experimental Section

Melting points were taken on a capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were obtained with a Varian 360L instrument and ultraviolet spectra with a Shimadzu UV-190 recording spectrophotometer. High-performance liquid chromatography was done with a Kontron apparatus consisting of an LC-pump T-414, a Uvikon LC UV detector, a 20-μL loop injection valve, and a Chrompack column (100 × 3 mm) packed with Chromosphere C8 (5 μM particles). Elemental analyses (C, H, and N) were performed at the Microanalytical Laboratory, Leo Pharmaceutical Products, Ballerup, Denmark, and the results obtained are within ±0.4% of the theoretical values.

L-Pyroglutamyl Benzylamide (1)—L-Pyroglutamic acid ethyl ester (15.7 g, 0.1 mol), prepared as previously described,[27] was dissolved in ethyl acetate (100 mL) and benzylamine (22 mL, 0.2 mol) was added. The mixture was stirred at 20 °C for 20 h and filtered. The filtrate was concentrated under reduced pressure to yield an oil which crystallized on trituration with ether:petroleum ether at 4 °C. Recrystallization from ethyl acetate:ethanol afforded 15.5 g (71%) of **1**, mp 133–134 °C.

Anal.—Calc. for $C_{12}H_{14}N_2O_2$.

Preparation of L-1-Acyl-5-oxoproline Benzylamides (2–6)—The *N*-acyl derivatives **2–6** were prepared by reacting L-pyroglutamyl benzylamide (**1**) with the corresponding acid anhydride or chloride.

Compounds **2–4**—A suspension of **1** (0.87 g, 4 mmol) in 3 mL of the acid anhydride (acetic, propionic, or butyric anhydride) was heated in an oil bath at 100 °C for 3 h. After cooling, water (20 mL) was added and the mixture was stirred at room temperature for 3 h. The

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precipitate was collected, washed with water, and recrystallized from ethanol:water. Yields were 65–75%.

Compounds **5** *and* **6**—A mixture of **1** (1.09 g, 5 mmol), pyridine (0.40 mL, 5 mmol), and benzoyl chloride or chloroacetyl chloride (5 mmol) in acetone (10 mL) was refluxed with stirring for 3 h. After cooling, the mixture was filtered and evaporated under reduced pressure. The residue was dissolved in ethyl acetate (30 mL) and water (30 mL). The ethyl acetate phase was separated and washed with 2 M HCl, a 5% $NaHCO_3$ solution, and water. Following drying over anhydrous Na_2SO_4, the ethyl acetate solution was evaporated under reduced pressure to yield the title compounds which were recrystallized from ethyl acetate:ether:petroleum ether. Yields were 70%.

L-1-(Methylaminomethyl)-5-oxoproline Benzylamide, HCL Salt (7)—A mixture of **1** (654 mg, 3 mmol), 37% formaldehyde solution (0.25 mL, 3 mmol), and methylammonium chloride (203 mg, 3 mmol) in water (3 mL) and ethanol (1 mL) was stirred at 80 °C for 1 h. The clear solution obtained was evaporated under reduced pressure to yield an oil which crystallized from ethanol at −20 °C. Recrystallization from ethanol gave 705 mg (80%) of **7** as the hydrochloride salt.

L-1-(Piperidinomethyl)-5-oxoproline Benzylamide, HCl Salt (8)—A mixture of **1** (1.09 g, 5 mmol), 37% formaldehyde solution (0.48 mL, 6 mmol), and piperidine (0.45 mL, 5 mmol) in water (5 mL) and ethanol (1 mL) was refluxed for 1 h. Upon cooling, the solution was extracted with ethyl acetate (20 mL). The extract was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to leave an oil which was dissolved in ether:methanol. A 2.5 M methanolic solution of HCl (2 mL, 5 mmol) was added, followed by ether. After standing at 4 °C for 3 h, the crystalline title compound was filtered off and recrystallized from methanol:ether to give 1.23 g (70%).

L-1-(Morpholinomethyl)-5-oxoproline Benzylamide, HCl Salt (9)—A mixture of **1** (1.09 g, 5 mmol), 37% formaldehyde solution (0.48 mL, 6 mmol), and morpholine (0.44 mL, 5 mmol) in water (5 mL) was refluxed for 1 h. Following the procedure described above for **8**, 1.2 g (68%) of the title compound was obtained.

Compounds **1–9** showed ^1H NMR spectra consistent with their structures. Melting points and elemental analyses are shown in Table I.

Kinetic Studies—The degradation of the derivatives **2–9** was studied in aqueous buffer solutions at 37 ± 2 °C. Hydrochloric acid and acetate, phosphate, borate, and carbonate buffers were used; the total buffer concentration was generally 0.02 M and a constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of KCl. The rates of hydrolysis of the derivatives were followed by using a reversed-phase HPLC procedure. Mobile phase systems of 30–45% (v/v) methanol in 0.02 M acetate buffer of pH 5.0 were used for **2–6**, while solvent systems of 35–55% (v/v) methanol in 0.01 M phosphate buffer of pH 7.0, with triethylamine added in a concentration of 10^{-3} M, were used for **7–9**. The proportion of methanol in these systems was adjusted for each compound to give a retention time of 2–5 min. With these eluants, the products of hydrolysis appeared in the solvent front. For the determination of **1** formed upon hydrolysis, a mobile phase system consisting of methanol:0.02 M acetate buffer pH 5.0 (1:5 v/v) was used. The flow rate was 0.5–1.0 mL/min and the column effluent was monitored at 215 or 254 nm. The compounds were quantified by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

The reactions were initiated by adding 100 μL of a stock solution in screw-capped test tubes, the final concentration of the compounds being ~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.

Degradation Studies in Plasma—The derivatives **2–9** were incubated at 37 °C in human plasma diluted to 80% with 0.05 M phosphate buffer of pH 7.40. The initial concentration of the derivatives was 2×10^{-4} M. At appropriate intervals, samples of 250 μL of the plasma reaction solution were withdrawn and added to 500 μL of a 2% solution of $ZnSO_4$ in methanol:water (1:1 v/v) in order to deproteinize the plasma. After mixing and centrifugation for 3 min at 13,000 rpm, 20 μL of the clear supernatant was analyzed by HPLC as described above.

Degradation Studies in the Presence of Pyroglutamyl Aminopeptidase—A preparation of pyroglutamyl aminopeptidase (EC 3.4.11.8) from calf liver was obtained from Boehringer, Mannheim, G.F.R. The enzyme assay used was that described by Martini et al.[28] The incubation mixture consisted of 5 mL of 0.1 M phosphate buffer of pH 7.40 containing 1 mM Na_2EDTA and 0.5 mM dithiothreitol, plus 500 μL of an aqueous solution of the enzyme at a concentration of 0.116 U/mL. This incubation mixture was kept at 4 °C for 3 h before assay. The degradation reactions of **1–9** were initiated by adding 50 μL of a stock solution of the compounds in acetonitrile or water to the incubation mixture (5.5 mL), the final concentration of the compounds being 10^{-4} M. The solutions were kept in a water bath at 37 °C and, at appropriate intervals, samples were taken and immediately chromatographed as described above for the degradation studies in buffer solutions. The enzyme activity was tested in separate experiments using L-pyroglutamyl-L-alanine (from Sigma Chemical) as a substrate. For the determination of this compound by HPLC, an RP-18 column and a solvent system of 1% phosphoric acid in water were used.

Results and Discussion

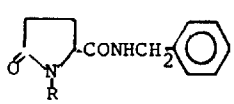
L-Pyroglutamyl benzylamide (**1**), prepared by aminolysis of the ethyl ester of L-pyroglutamic acid, was used as a model for the pyroglutamyl residue in pyroglutamyl-containing peptides. It was found to be a good substrate for pyroglutamyl aminopeptidase. Thus, at pH 7.4 and 37 °C and using the enzyme reaction conditions described in the *Experimental Section*, **1** was hydrolyzed according to first-order kinetics with a rate constant of 0.071 min^{-1}, corresponding to a half-life of 10 min. In the absence of the enzyme, the compound was completely stable. Under the same enzyme reaction conditions, pyroglutamyl-L-alanine, which has been reported to be the best substrate among various pyroglutamyl dipeptides,[29,30] was found to be hydrolyzed with a half-life of 8 min.

The derivatives of **1** assessed as potential pyroglutamyl aminopeptidase-resistant prodrugs include the *N*-acyl derivatives **2–6** and the *N*-Mannich bases **7–9** listed in Table I. The compounds were synthesized by the routes shown in Schemes I and II. Various *N*-Mannich bases of 2-pyrrolidone have previously been described,[31] and a similar procedure was used for the preparation of **7–9**.

Kinetics of Hydrolysis—The kinetics of hydrolysis of the *N*-acyl derivatives **2–6** and the *N*-Mannich bases **7–9** was studied in aqueous buffer solutions at 37 °C and μ = 0.5 over a wide range of pH. At constant pH and temperature, the disappearance of the derivatives displayed strict first-order kinetics over several half-lives. An example is shown in Figure 1.

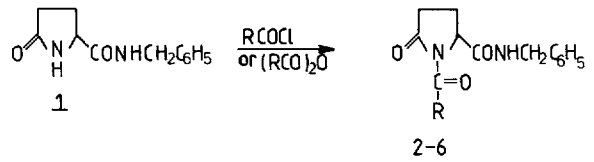
The influence of pH on the overall degradation rate of the *N*-benzoyl derivative **6** is shown in Figure 2 where the logarithm of the observed pseudo-first-order rate constant (k) is plotted against pH. The observed pH–rate relationship indicates that the overall hydrolysis can be described in

Table I—Physical Properties of Various Derivatives of L-Pyroglutamyl Benzylamide

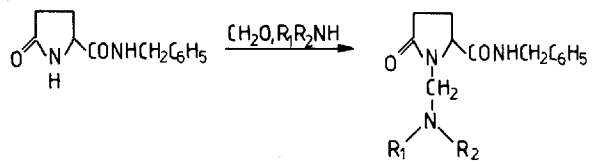


Compound	R	mp, °C	Formula[a]
2	$COCH_3$	161–162	$C_{14}H_{16}N_2O_3$
3	$COCH_2CH_3$	185–186	$C_{15}H_{18}N_2O_3$
4	$COCH_2CH_2CH_3$	138–139	$C_{16}H_{20}N_2O_3$
5	$COCH_2Cl$	193–194	$C_{14}H_{15}N_2O_3Cl$
6	COC_6H_5	165–167	$C_{19}H_{18}N_2O_3$
7	CH_2NHCH_3, HCl	165–167	$C_{14}H_{16}N_3O_2$, HCl
8	CH_2—N , HCl	154–155	$C_{18}H_{25}N_3O_2$, HCl
9	CH_2—N O, HCl	156–158	$C_{17}H_{23}N_3O_3$, HCl

[a] Analyses for C, H, and N were within ±0.4% of calculated values for formulas shown.



Scheme I



Scheme II

terms of a water-catalyzed or spontaneous reaction and specific acid- and base-catalyzed reactions according to the following rate expression:

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

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:[32]

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

The following values of the specific rate constants for **6** were obtained from the pH–rate profile and eq 1: $k_H = 7.4 \times 10^{-3}$ M^{-1} min^{-1}; $k_{OH} = 890$ M^{-1} min^{-1}; and $k_o = 1.3 \times 10^{-5}$ min^{-1}.

The other *N*-acyl derivatives were only studied in the pH range 7–11. At these pH values, the hydrolyses were entirely due to a hydroxide ion-catalyzed reaction. The k_{OH} values obtained for these compounds, as well as their half-lives of hydrolysis at physiological pH, are listed in Table II.

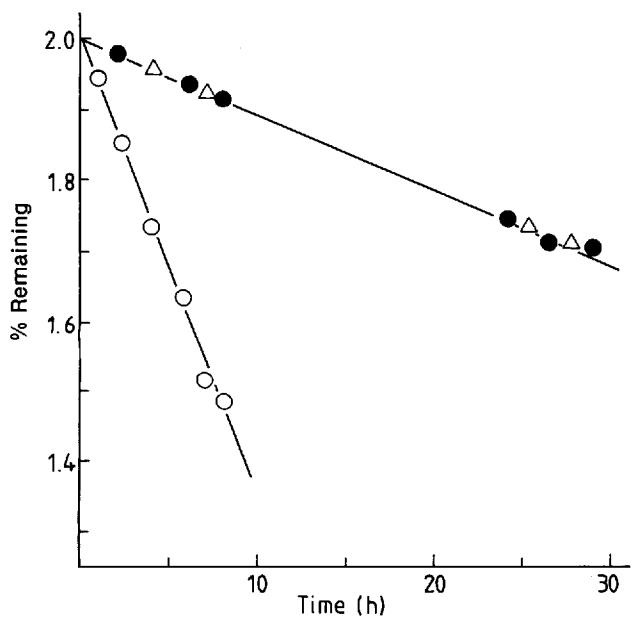


Figure 1—*Plots showing the apparent first-order kinetics of degradation of* **3** *in a 0.02 M phosphate buffer solution of pH 7.40 (●), in buffer solution containing pyroglutamyl aminopeptidase (△), and in 80% human plasma pH 7.4 solution (○) at 37 °C.*

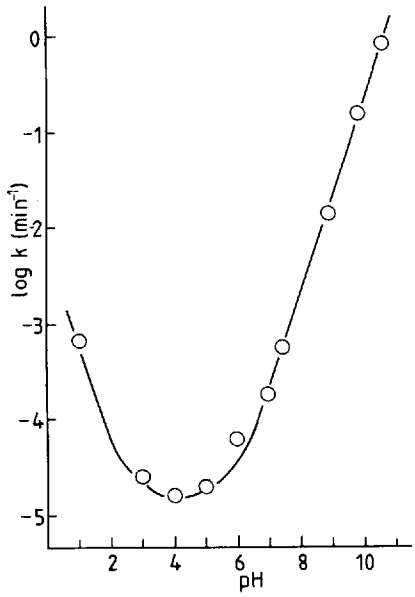


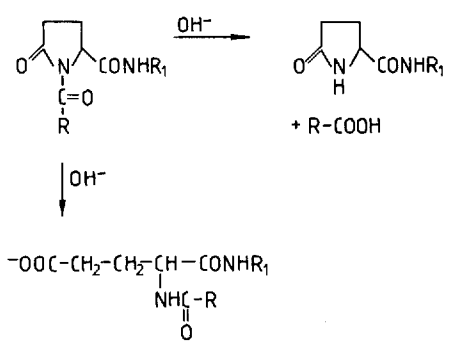
Figure 2—*The pH–rate profile for the overall degradation of* **6** *in aqueous solution (μ = 0.5) at 37 °C.*

Table II—Rate Data for the Hydrolysis of the *N*-Acyl Derivatives 2–6 at 37 °C

Compound	k_{OH}, M^{-1} min^{-1}	$t_{1/2}$, h (% Compound **1** Formed)[a]		
		Buffer pH 7.4	80% Human Plasma	Enzyme Solution[b]
2	1040	18.0 (40%)	14.0 (75%)	19.7
3	660	28.9 (72%)	4.5 (100%)	28.1
4	470	39.3 (55%)	7.8 (55%)	37.2
5	9.5×10^4	14 min (80%)	8 min (100%)	15 min
6	890	21.5 (65%)	4.3 (70%)	20.1

[a] The percentage amounts of **1** formed upon hydrolysis of the derivatives in buffer, plasma, and enzyme solutions are given in parentheses. [b] These data are half-lives for the hydrolysis in buffer solution (pH 7.4) containing pyroglutamyl aminopeptidase.

The hydrolysis of the *N*-acyl derivatives **2–6** was shown to follow two pathways, giving either the parent amide (**1**) or the corresponding *N*-acyl glutamic acid α-benzylamide which is stable at the reaction conditions used (Scheme III). The amounts of **1** formed were determined by HPLC and the data obtained at pH 7.4 are listed in Table II. Similar amounts were observed at higher pH values in accordance with the fact that the two reactions depicted in Scheme III both involve attack of hydroxide ion at one of the two carbonyl



Scheme III

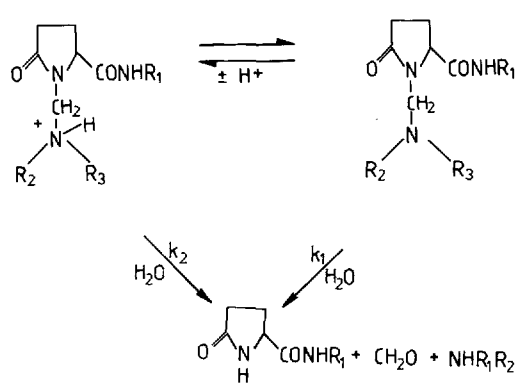
groups present. Simultaneous ring opening and hydrolysis of an *N*-acyl moiety has previously been observed for *N*-acyl phthalimides[33] and *N*-acyl succinimides.[34]

In assessing *N*-acyl derivatives of pyroglutamyl peptides as potential prodrugs, it is important to consider the possibility of enzymatic hydrolysis. Therefore, the stability of the *N*-acyl derivatives **2–6** was also studied in 80% human plasma (pH 7.4) at 37 °C. Under these conditions, strict first-order kinetics was observed, as exemplified for **3** in Figure 1. As it appears from the rate data obtained (Table II), plasma enzymes catalyze the rate of hydrolysis considerably. That the effect of plasma was a true enzymatic catalysis was supported by the finding that when the plasma was preheated to 80 °C for 2 h, no effect on the rate of degradation was observed. As revealed by HPLC analysis for **1** of completed reaction solutions, the enzymatic catalysis was predominantly a catalysis of the *N*-acylamide hydrolysis. As can be seen from Table II, the parent compound **1** was formed in larger amounts in the plasma solutions than in the buffer solutions. In the case of the *N*-propionyl derivative (**3**) and the *N*-chloroacetyl derivative (**5**), the plasma-catalyzed *N*-acylamide hydrolysis is so dominant that the parent compound **1** is obtained in quantitative amounts. For **6**, on the other hand, plasma appears to catalyze both routes of degradation. It should be added that **1** is completely stable in human plasma, indicating the absence in plasma of significant pyroglutamyl aminopeptidase activity. There are only a few examples of plasma-catalyzed hydrolysis of *N*-acylated amides,[10,35,36] but in view of the present results, *N*-acylation of amides (including cyclic amides such as 2-pyrrolidone) should receive more attention as a prodrug approach.

The *N*-Mannich bases **7–9** were prepared as prodrug derivatives in view of our previous studies[37] showing facile hydrolysis of such derivatives of amides, imides, and various other NH-acidic compounds. Compounds **7–9** were found to be hydrolyzed according to Scheme IV, with quantitative formation of **1** irrespective of the pH of solution. The pH–rate profiles for the compounds are shown in Figure 3. The sigmoidal shapes of these profiles are similar to those previously obtained for *N*-Mannich bases of, for example benzamide,[37] and can be accounted for by assuming spontaneous decomposition of the free base species (B) and their conjugate acids (BH^+):

$$k = \frac{k_1 K_a}{a_H + K_a} + \frac{k_2 a_H}{a_H + K_a} \qquad (3)$$

where K_a is the apparent ionization constant of the protonated *N*-Mannich bases, and k_1 and k_2 are the apparent first-order rate constants for the spontaneous degradation of B and BH^+, respectively (Scheme IV). Values of pK_a and the



Scheme IV

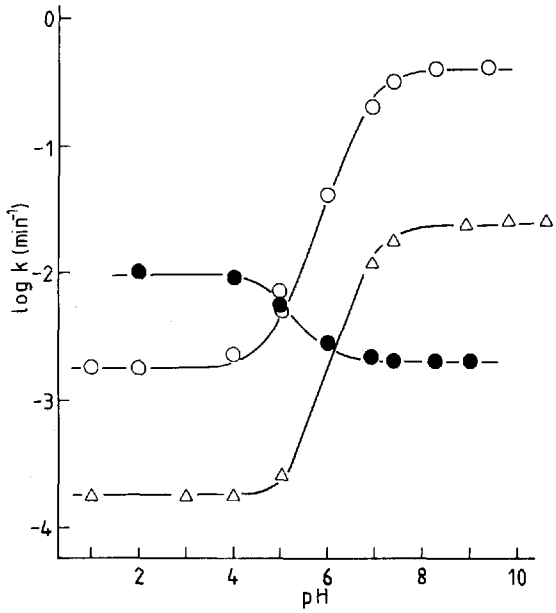


Figure 3—*The pH–rate profiles for the decomposition of the N-Mannich base derivatives* **7** (○), **8** (△), *and* **9** (●) *in aqueous solution* (μ = 0.5) *at 37 °C.*

rate constants k_1 and k_2, as well as half-lives of hydrolysis at physiological pH, are listed in Table III.

The reaction mechanism for the decomposition of *N*-Mannich bases in neutral and basic solution involves, as the rate-determining step, a unimolecular N—C bond cleavage with formation of an amide anion and an immonium cation.[37,38] As expected from this mechanism, the rates of hydrolysis of **7–9** in human plasma solutions were found to be quite similar to those in buffer solutions of the same pH (Table III). These results are in agreement with similar findings for other *N*-Mannich bases.[39]

The relative stabilities of the three *N*-Mannich bases **7–9** parallel the behavior of similar *N*-Mannich bases of benzamide.[37] The reactivity at physiological pH increases with increasing steric effect and basicity of the amine component and with increasing acidity of the amide component.[37] Compared with analogous *N*-Mannich bases of benzamide,[37] **7–9** are more reactive by a factor of 5–20, depending on the amine; this most probably reflects the greater acidity of the ring amide group in **1** relative to benzamide. On the basis of previous studies with benzamide *N*-Mannich bases,[37] it should be readily feasible to select *N*-Mannich bases of pyroglutamyl compounds with widely different chemical stabilities.

Stability Toward Pyroglutamyl Aminopeptidase—Whereas the parent pyroglutamyl benzylamide (**1**) was rapidly hydrolyzed to pyroglutamic acid in the presence of pyroglutamyl aminopeptidase ($t_{1/2}$ = 10 min), none of the *N*-substituted derivatives **2-9** were attacked by the enzyme under identical reaction conditions. As seen from the results given in Figure 1 and Tables II and III, the rates of hydrolysis of the derivatives in the presence of the enzyme were quite similar to those occurring in buffer solutions. This finding is most important since it shows that the enzyme does not tolerate *N*-acylation or *N*-aminomethylation of the pyrrolidone ring nitrogen. It may well be that any replacement of the hydrogen in the cyclic amide moiety would lead to derivatives totally resistant to pyroglutamyl aminopeptidase. It has previously been reported[40] that a pyroglutamyl aminopeptidase preparation from *B. amyloliquefaciens* was

Table III—Rate Data for the Decomposition of the *N*-Mannich Base Derivatives 7–9 at 37 °C

Compound	k_1, min^{-1}	k_2, min^{-1}	pK_a	$t_{1/2}$, min[a]		
				Buffer pH 7.4	80% Human Plasma	Enzyme Solution[b]
7	0.024	1.8×10^{-4}	7.2	41	43	44
8	0.42	1.7×10^{-3}	7.2	2.1	2	2
9	2.5×10^{-3}	0.010	5.1	282	300	290

[a] The hydrolysis of the *N*-Mannich bases proceeded in all solutions with the quantitative formation of **1**. [b] Buffer solution (pH 7.4) containing pyroglutamyl aminopeptidase.

completely inert toward various *N*-benzyloxycarbonyl pyroglutamyl dipeptides.

Conclusions

The results described show, apparently for the first time, that the prodrug approach may be useful for protection of a peptide or peptide model against specific enzymatic cleavage. By *N*-acylation or *N*-aminomethylation of the pyroglutamyl residue, the suspectibility of the pyroglutamyl amide bond to pyroglutamyl aminopeptidase cleavage is completely lost, as shown with the model compound pyroglutamyl benzylamide. This modification is readily bioreversible, the parent pyroglutamyl amide being formed either by spontaneous hydrolysis at physiological pH, as demonstrated for the *N*-Mannich bases, or by enzymes such as those in plasma which do not attack the pyroglutamyl amide bond. As shown above for the model substance, *N*-propionylation fulfills the requirement of the prodrug approach in that the pyroglutamyl aminopeptidase-resistant *N*-propionyl derivative is quantitatively cleaved to the parent compound in plasma. It can readily be envisaged that by appropriate *N*-acylation or *N*-aminomethylation it may be feasible both to protect pyroglutamyl-containing peptides against cleavage by pyroglutamyl aminopeptidase and to obtain an enzymatic or chemical release of the parent peptide. Furthermore, such derivatization may be useful in modifying the lipophilicity of a pyroglutamyl peptide and hence in improving its bioavailability to the requisite sites of action.

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