Prodrugs of Peptides. 11. Chemical and Enzymatic Hydrolysis Kinetics of *N*-Acyloxymethyl Derivatives of a Peptide-like Bond

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Various carboxylic acid esters of the *N*-hydroxymethyl derivative of *N*-benzyloxycarbonylglycine benzylamide, used as a peptide-like model, were prepared and their decomposition kinetics studied in aqueous solution and in human plasma solutions. These *N*-acyloxymethylamide derivatives were found to undergo a facile decomposition by a pH-independent process in the pH range 4–8.5, the half-lives being 1–11hr at 37°C. The cause of this limited stability was suggested to be due to the occurrence of an elimination-addition mechanism involving a reactive *N*-acyliminium ion intermediate. In alkaline solutions (pH > 10) the derivatives showed a normal ester stability. The ester group in the *N*-acyloxymethyl derivatives was readily hydrolyzed by plasma enzymes to yield the *N*-hydroxymethyl amide, which subsequently decomposed to the parent amide. The results obtained suggest that N-acyloxymethylation of a peptide bond may be a useful prodrug approach to obtain derivatives with varying lipophilicities and a ready ability to undergo conversion to the parent peptide *in vivo*. However, the stability of the derivatives in aqueous solutions is limited.

KEY WORDS: peptides; prodrug; N-acyloxymethylation; hydrolysis kinetics; enzymatic hydrolysis.

INTRODUCTION

In a recent paper, we described N-α-hydroxyalkylation of the peptide bond as 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 (1). 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 the peptide structure and the type of aldehyde involved in the α-hydroxyalkylation (Scheme I) (1).

An interesting aspect of this prodrug approach is further derivatization of the hydroxyl group in the *N*-α-hydroxyalkyl derivatives, e.g., by esterification, to afford *N*-α-acyloxyalkyl derivatives from which the parent peptide may be released in a two-step reaction: hydrolysis of the ester grouping by unspecific esterases followed by spontaneous decomposition of the *N*-α-hydroxyalkyl derivative. In this manner not only the stability but also the lipophilicity of the prodrug derivatives can be further modified.

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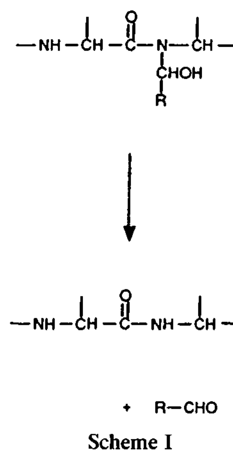


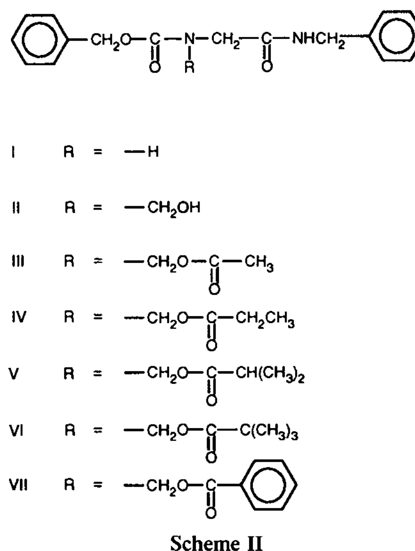
Scheme I

In the present study, we have examined this aspect, using *N*-benzyloxycarbonylglycine benzylamide (I) as a model compound. Various carboxylic acid esters of *N*-hydroxymethyl-*N*-carbobenzoxyglycine benzylamide (II) (Scheme II) have been prepared and their degradation kinetics studied in aqueous solution and in the presence of human plasma. Surprisingly, the stability of the *N*-acyloxymethyl derivatives was found to be quite poor in neutral aqueous solutions.

MATERIALS AND METHODS

Apparatus

High-performance liquid chromatography (HPLC) was done with a system consisting of a Shimadzu Model LC-6A pump, a Shimadzu SPD-6A variable-wavelength UV detector, and a Rheodyne 7125 injection valve with a 20-μl loop. The column used was a reversed-phase Resolve C-18 (100 × 8 mm; 5-μm particles) from Waters Associates. Readings of pH were carried out on a Radiometer PHM 83 Autocal instrument at the temperature of study. Microanalysis was performed at Leo Pharmaceuticals Ltd., Ballerup, Denmark.



Scheme II

Preparation of Derivatives I–VII

N-Benzyloxycarbonylglycine benzylamide (I) and its *N*-hydroxymethyl derivative (II) were prepared as previously described (2). The esters III–VII were prepared by reacting the alcohol II with the appropriate acid anhydride or acid chloride (in the case of compound VII) in pyridine. The general procedure used was as follows. A mixture of compound II (2 mmol), acid anhydride (2.5 ml), and pyridine (5 ml) was stirred at room temperature for 20hr and then concentrated *in vacuo*. The residue was taken up in ethyl acetate (30 ml) and water (30 ml). The organic phase was washed with 1 *M* hydrochloric acid, 5% aqueous sodium bicarbonate, and water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue obtained was recrystallized from ether–petroleum ether. The yields were about 70%. Melting points and elemental analysis of the esters are listed in Table I. The ^1H nuclear magnetic resonance (NMR) spectra of the compounds were consistent with their structures.

Kinetic Measurements

The decomposition of the esters was studied in aqueous buffer solutions at 37 ± 0.2°C. The buffers used were hydrochloric acid, acetate, phosphate, borate, and carbonate solutions. 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 a reversed-phase HPLC procedure capable of separating the esters from their products of degradation. Mobile-phase systems of 0.1% phosphoric acid containing acetonitrile (45–60, v/v) and methanol (0–10, v/v) were used, the concentration of acetonitrile and methanol being adjusted for each compound to give a retention time of 3–8 min. The flow rate was 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.

Table I. Physical Data of Various *N*-Acyloxymethyl Derivatives, III–VII

Compound	mp (°C)	Formula		Analysis (%) Calc.	Found
III	88–89	$C_{20}H_{22}N_2O_5$	C	64.85	64.65
			H	5.99	5.95
			N	7.56	7.62
IV	72–73	$C_{21}H_{24}N_2O_5$	C	65.61	65.40
			H	6.29	6.33
			N	7.29	7.40
V	78–79	$C_{22}H_{26}N_2O_5$	C	66.32	66.22
			H	6.58	6.54
			N	7.03	7.21
VI	83–84	$C_{23}H_{28}N_2O_5$	C	66.97	67.13
			H	6.84	6.76
			N	6.79	6.91
VII	126–127	$C_{25}H_{24}N_2O_2$	C	69.43	69.65
			H	5.59	5.72
			N	6.48	6.60

The reactions were initiated by adding 50 μl of a stock solution of the derivatives in acetonitrile to 10.0 ml of buffer solution, preequilibrated at 37°C, in screw-capped test tubes, the initial concentration being about 5×10^{-5} *M*. The solutions were kept in a water bath at 37°C, and at appropriate times samples were taken and immediately chromatographed. 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 (3).

RESULTS AND DISCUSSION

Kinetics of Decomposition of Compounds III–VII

The kinetics of decomposition of the various *N*-acyloxymethyl derivatives III–VII was studied in aqueous solution at 37°C over a wide pH range. At constant temperature and pH, the disappearance of the esters displayed strict first-order kinetics over several half-lives. The rates of decomposition at a fixed pH value were found to be independent of buffer concentration at the concentration used (0.02 *M*).

The influence of pH on the rate of hydrolysis of compounds III, V, and VII is shown in Fig. 1, where the logarithms of the observed pseudo-first-order rate constants (k_{obs}) are plotted against pH. The pH–rate profiles for the other derivatives (IV and VI) have a similar shape, indicating the occurrence of apparent specific acid and base catalysis as well as a spontaneous or water-catalyzed reaction according to the following expression:

$$k_{obs} = k_0 + k_H a_H + k_{OH} a_{OH} \quad (1)$$

where a_H and a_{OH} refer to the hydrogen ion and hydroxide ion activity, respectively. The latter was calculated from the measured pH (1). Values of the second-order rate constants for the specific acid (k_H)- and specific base (k_{OH})-catalyzed decomposition were determined from the straight-line portions of the pH–rate profiles at low and high pH values, respectively, whereas the value of the apparent first-order rate constant for spontaneous decomposition (k_o) was obtained from the large plateau regions of the pH–rate profiles.

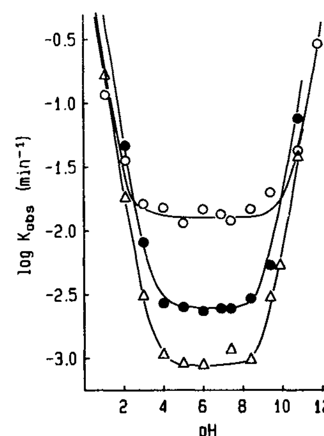
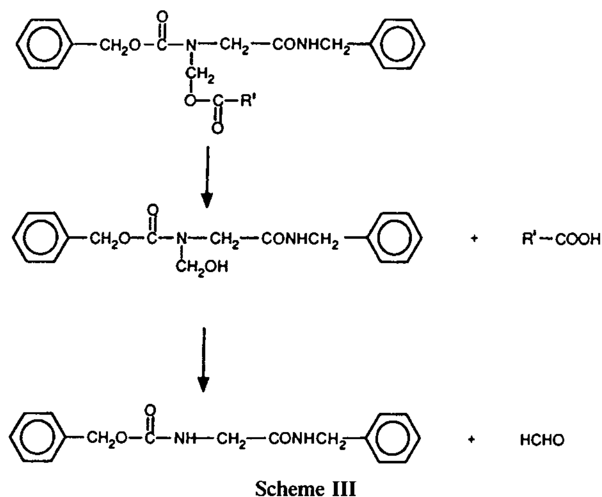


Fig. 1. The pH–rate profiles for the hydrolysis of compound III (●), compound V (△), and compound VII (○) in aqueous solution at 37°C.

The values of the derived rate constants are listed in Table II. In Fig. 1 the solid curves were constructed from these values and Eq. (1).

Mechanism of Decomposition

The decomposition of the derivatives III–VII in acidic and neutral solutions proceeded with the quantitative formation of the *N*-hydroxymethyl derivative II, which subsequently decomposed to the parent amide I and formaldehyde, as depicted in Scheme III. This was shown by HPLC analysis of the reaction solutions, using solvent systems affording separation of the esters and compounds I and II. An example of a product analysis is shown in Fig. 2 for compound III. As can be seen the *N*-hydroxymethyl derivative II is produced in quantitative amounts, the rate of formation following strict first-order kinetics with no occurrence of any lag period. Following its formation compound II decomposes quantitatively to compound I at a rate highly dependent on pH. As shown previously (1), the decomposition of compound II to compound I is subject to both specific acid and apparent specific base catalysis, the k_H and k_{OH} values being 0.014 and 2.7×10^3 M^{-1} min^{-1}, respectively. In the pH range 3–6 and at 37°C the half-life for the decomposition of compound II is greater than 200hr, whereas it is 10.8hr at pH 7.4 (1). Thus, compound II is much more stable than its esters III–VII at pH values up to about 7. At higher pH values the esters become more stable than compound II, and therefore, the formation of II from these derivatives in alkaline solution was followed by its immediate conversion to compound I.



Scheme III

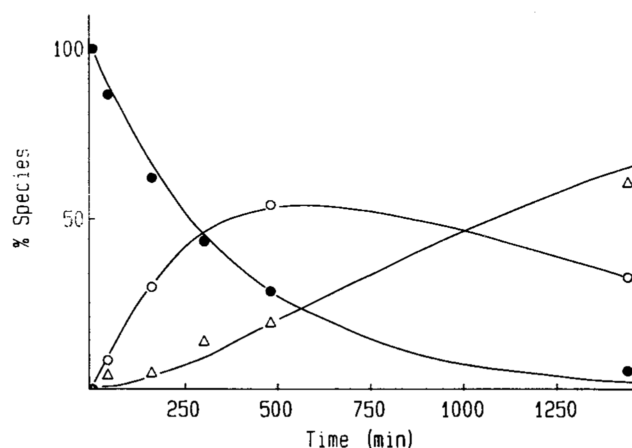
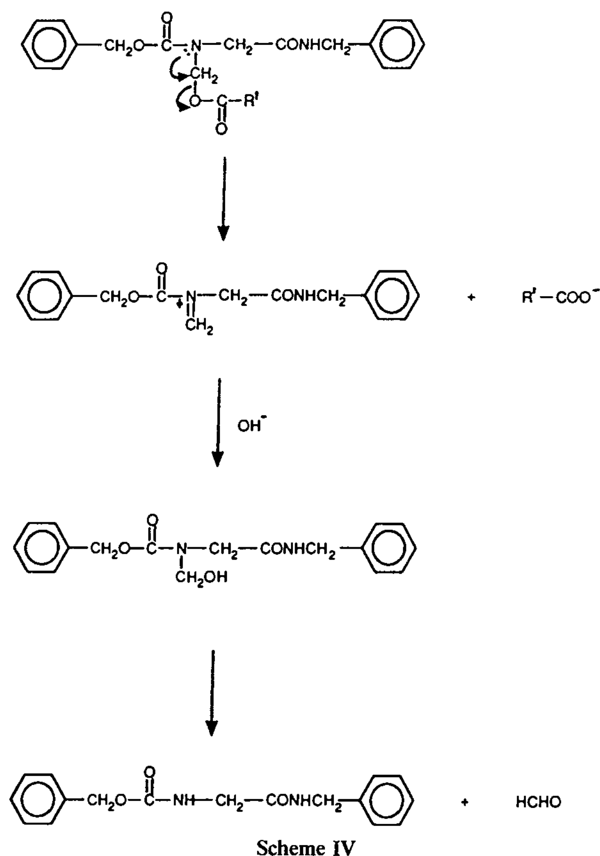


Fig. 2. Time courses for compound III (●), compound II (○), and compound I (△) during the degradation of compound III in 0.02 *M* phosphate buffer solution of pH 7.40 (at 37°C).

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

Compound	k_H (M^{-1} min^{-1})	k_o (min^{-1})	k_{OH} (M^{-1} min^{-1})
III	5.5	2.5×10^{-3}	60
IV	2.5	1.2×10^{-3}	65
V	2.2	9.0×10^{-4}	25
VI	0.40	1.6×10^{-3}	5.0
VII	1.9	1.3×10^{-2}	22

The most remarkable feature in the degradation of the esters III–VII is their high k_o value and consequently high lability in the pH range 4–8. Thus, the half-lives of the ester cleavage in this pH range at 37°C are only 1–11hr. This high reactivity is indicative of the involvement of another mechanism of hydrolysis than the nucleophilic S_N2 reaction normally occurring in ester hydrolysis. The most likely mechanism involved is suggested to be a unimolecular elimination-addition process, which can be regarded as an S_N1 reaction, with the formation of a transient *N*-acyliminium ion intermediate as depicted in Scheme IV. In this mechanism the



Scheme IV

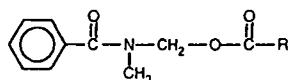
rate-determining step involves elimination of carboxylate anion to give an iminium ion, which in a subsequent fast step undergoes attack by hydroxide ions, giving the *N*-hydroxymethyl amide II. During the present investigation, Iley and co-workers (4) presented, in a poster communication, several lines of argument for the involvement of such a mechanism in the pH-independent hydrolysis of various *N*-acyloxymethyl derivatives of *N*-methylbenzamide (VIII). These compounds are tertiary acyloxymethylated amides like III–VII. We have previously (5,6) reported on the occurrence of an elimination-addition mechanism in both the neutral and the alkaline hydrolysis of *N*-acyloxymethyl derivatives formed from primary benzamides, the rate-determining step in this case being the formation of an unprotonated acylimine intermediate.

Additional support for the elimination-addition mechanism is provided by the influence of the acyl moiety on the k_0 reaction. As seen from the following relationship between k_0 and the pK_a values of the corresponding acids (taken from Ref. 7),

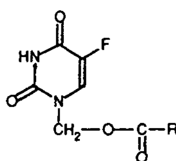
$$\log k_0 = -1.28\, pK_a + 3.42 \quad (r = 0.922;\ n = 5) \qquad (2)$$

the k_0 values increase with increasing leavability of the carboxylate anions. This is in accordance with the elimination mechanism depicted in Scheme IV. Furthermore, if a normal nucleophilic ester hydrolysis was occurring, the sterically hindered pivalate ester VI should be much more stable in neutral solutions than the other esters as it is in alkaline solutions but this is evidently not the case.

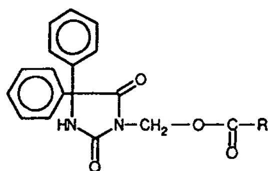
The high lability of the acyclic derivatives III–VII in the neutral pH range contrasts greatly with the stability of similar *N*-acyloxymethyl derivatives formed with cyclic secondary amides such as the 5-fluorouracil derivatives IX (3) and the phenytoin derivatives X (8) (Scheme V). Whereas the



VIII



IX



X

Scheme V

latter show about the same k_{OH} values as those for the esters III–VII, they are severalfold more stable at pH 4–8 than the acyclic analogues. The reason for this is probably not a matter of being cyclic or acyclic but can rather be ascribed to the different acidities of the parent NH-acidic compound. The electronic rearrangement depicted in Scheme IV for the acyliminium ion formation should be depressed by increasing polar effects of the substituents attached to the $-$CON⟨ moiety, i.e., by increasing acidity of the $-$CONH$-$ moiety. The pK_a values for the NH functions in both 5-fluorouracil and phenytoin (about 8–10) are much smaller than those (about 14–15) for the amide moiety in compound I. It would certainly be of interest to determine systematically the influence of amide acidity on the stability of the corresponding *N*-acyloxymethyl derivatives.

The k_{OH} values observed for the esters III–VII are in the range expected for the normal ester hydrolysis mechanism involving acyl–oxygen bond cleavage (9), the variation in the values being due largely to the varying steric properties within the acyl moieties.

Plasma-Catalyzed Hydrolysis

The decomposition of the acyloxymethyl derivatives III–VII was found to be subject to marked enzymatic catalysis by human plasma. The degradation of the compounds in 80% plasma solutions (pH 7.4) followed strict first-order kinetics, the half-lives being in the range of 9–240 min (Table III). As seen from the data in Table III the catalytic effect by plasma was most pronounced for the propionyl ester IV and least for the sterically hindered pivalate ester VI. HPLC analysis of the reaction solution showed that the product of degradation was compound II, which subsequently decomposed to compound I. Thus, plasma enzymes catalyze the first step in the reactions shown in Scheme III.

In conclusion, the results obtained show that esterification of N-hydroxymethylated acyclic secondary amides like the peptide bond does not result in a stabilization of the hydroxymethyl derivatives toward decomposition in acidic and neutral aqueous solution due to the occurrence of a facile elimination-addition mechanism. However, the ester functionality is readily hydrolyzed by plasma enzymes and the N-acyloxymethylation or, generally, N-α-acyloxyalkylation may thus be considered as a double-prodrug principle (10). By selecting various acyl groups, this approach makes it feasible to obtain prodrug derivatives for the peptide bond with varying lipophilicities. The compounds inves-

Table III. Half-Lives of Decomposition of Various *N*-Acyloxymethyl Derivatives in 80% Human Plasma (pH 7.4) and Buffer (pH 7.4) Solutions at 37°C

Compound	Half-life (min)	
	Buffer	80% plasma
III	260	36
IV	530	10
V	670	17
VI	400	240
VII	58	9

tigated in this study are essentially *N*-acyloxymethyl derivatives of a secondary urethane bond, and not of a secondary carboxamide (peptide) bond. However, the degradation mechanism of these derivatives is most likely similar [cf. the results reported with compound VIII (4)], but the overall stability may differ and depend on the substituents attached to the urethane or amide bond.

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