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Prodrugs of peptides obtained by derivatization of the *C*-terminal peptide bond in order to effect protection against degradation by carboxypeptidases

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Abstract

Two *N*-Z-protected dipeptides were *N*-hydroxymethylated at the *C*-terminal peptide bond. The stability of the derivatives was investigated in aqueous buffer solutions as a function of pH. The compounds degraded quantitatively to their parent *N*-Z-protected dipeptide. The stability was also examined in buffer solutions containing different concentrations of carboxypeptidase A. It was shown that the half-lives of degradation in buffer with and without carboxypeptidase A were of the same order. These results demonstrate that the enzymatic reactivity of CPA is influenced if the *C*-terminal peptide bond is modified as it is here by *N*-hydroxymethylation. It is suggested that *N*-hydroxymethylation of the *C*-terminal peptide bond may become a useful approach to improve absorption characteristics by reducing the enzymatic degradation of peptides.

Keywords: Peptide; Prodrug; Bioreversible derivatization; 5-Oxazolidinones; *N*-hydroxyalkylation; Carboxypeptidase A; Stability

1. Introduction

Recently peptides have attracted considerable attention as a new class of drugs due to increasing synthetic accessibility. The application of the compounds as clinically useful drugs is, however, hampered due to extensive proteolysis at most routes of administration. Peptides are also, in general, very polar compounds resulting in poor biomembrane penetration characteristics. Administration of peptides is therefore, in general, limited to the parenteral route (Humphrey and Ringrose, 1986; Banga and Chien, 1988; Lee et al., 1991).

The development of prodrugs is a possible way to reduce the problem. Thus, it has been shown that bioreversible derivatization, i.e. prodrug formation, can protect smaller peptides against degradation by peptide hydrolases. The approach

Abbreviations: Z, benzyloxycarbonyl; CPA, carboxypeptidase A.

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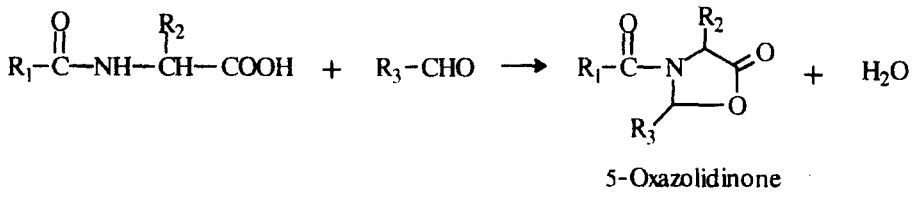


Scheme 1.

can also render peptides more lipophilic and hence facilitate their absorption (Bundgaard, 1992; Kahns et al., 1993a; Oliyai and Stella, 1993; Delie et al., 1994; Møss, 1995).

Previously, it has been shown that 5-oxazolidinone derivatization is a bioreversible prodrug approach. Such compounds are readily formed by condensing α-amino acids or *N*-acylated amino acids with an aldehyde (Buur and Bundgaard, 1988; Bundgaard and Rasmussen, 1991a) (Scheme 1). The heterocyclic ring is, however, highly reactive and is easily opened to form an *N*-hydroxyalkyl derivative as an intermediate. Such derivatives are difficult to synthesize of secondary amides such as peptide bonds (Bundgaard and Johansen, 1984). This difficulty can be overcome by making 5-oxazolidinones. The *N*-hydroxyalkyl derivative is further degraded quantitatively to the parent compounds, i.e. the parent peptide and the aldehyde used for the synthesis (Buur and Bundgaard, 1988; Bundgaard and Rasmussen, 1991a) (Scheme 2).

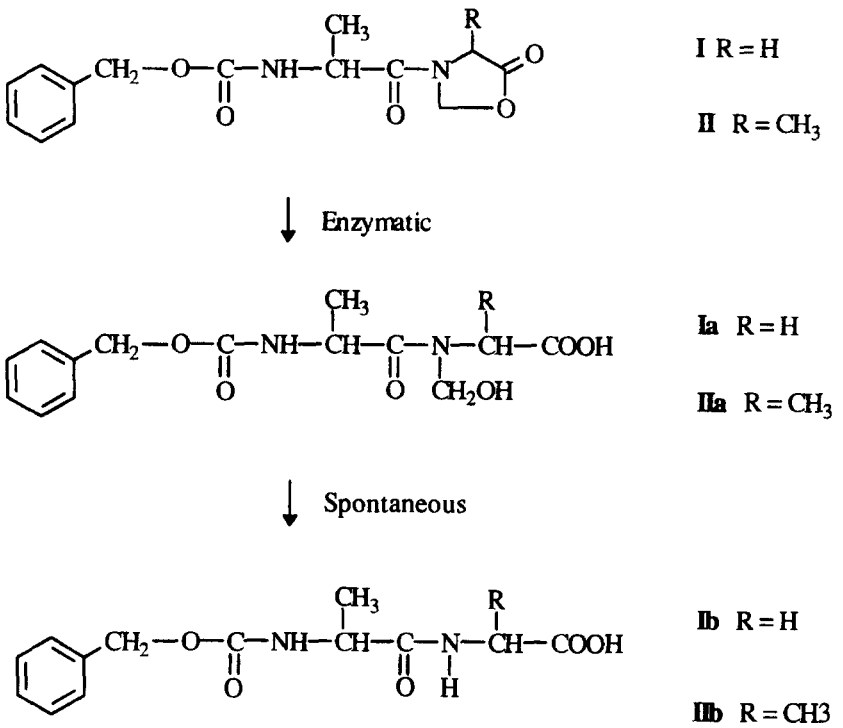
N-hydroxyalkylation of the peptide bond can be used to protect the peptide against degradation by the *C*-terminal exopeptidase carboxypeptidase A (CPA) (Rasmussen and Bundgaard, 1991). CPA is a pancreatic peptide hydrolase which primary function is as a *C*-terminal exopeptidase. It catalyses the hydrolysis of any peptide having a free terminal carboxyl group and a *C*-terminal residue of L-configuration. The rate of hydrolysis is enhanced if the terminal residue is branched or aromatic (Smith, 1951; Hartsuck and Lipscomb, 1971). CPA degrades a number of bioactive peptides such as the enkephalins that are degraded very rapidly at the *C*-terminal peptide bond by this enzyme (Rasmussen and Bundgaard, 1991). *N*-Hydroxyalkylation can also protect peptides against degradation by other peptide hydrolases such as α-chymotrypsin (Kahns et al., 1993b). Previously two *N*-Z-protected dipeptides Z-Gly-L-Leu and Z-Gly-L-Ala were *N*-hydroxymethylated at the *N*-terminal peptide-like bond through 5-oxazolidinone formation. It was shown that the derivatization completely protected the *C*-terminal peptide bond against degradation by CPA (Bundgaard and Rasmussen, 1991a).

The purpose of this study is to examine if it is possible to achieve direct protection of the susceptible *C*-terminal peptide bond by *N*-hydroxymethylation using *N*-Z-protected dipeptides as model peptides. The model peptide is transformed into the corresponding 5-oxazolidinone derivative through condensation with formaldehyde. The *N*-hydroxymethylated peptide is formed by subsequent hydrolysis. Two different 5-oxazolidinones have been made of Z-L-Ala-Gly (**I**) and Z-L-Ala-L-Ala (**II**) and formaldehyde (Scheme 2 and Fig. 1). An interesting aspect of *N*-hydroxyalkylation of peptide bonds to protect against rapid proteolytic cleavage by CPA is the possibility for further derivatization of the hydroxyl group by esterification to achieve derivatives with higher lipophilicity (Bundgaard and Rasmussen, 1991b).

2. Materials and methods

2.1. Chemicals

Z-Ala-Gly-OH (**Ib**) and Z-Ala-Ala-OH (**IIb**), both having L-configuration, were purchased from



Scheme 2.

Bachem AG (Bubendorf, Switzerland). Carboxypeptidase A (type I; from bovine pancreas) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Solvents for NMR were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Buffer substances and all other chemicals were of reagent grade.

2.2. Apparatus

High-performance liquid chromatography (HPLC) was carried out using two different instruments: a Merck Hitachi L-6000 pump connected to a Merck Hitachi L-4000 UV-detector and a Shimadzu LC-6A pump connected to a Shimadzu SPD-6A UV-detector. Both instruments were equipped with a Rheodyne 7125 injection valve with a 20 μl loop. Two different RP-C_{18} columns were used: a Chrompack (100 × 4.6 mm) packed with Chromsphere C-18 (3-μm particles) and a Spherisorb-ODS-2 (250 × 4.6 mm) packed with 5 μm particles. The ^{1}H-NMR spectra were recorded on a Bruker AC 200 instrument (**I**) or on a AMX-400 WB Bruker instrument equipped with a BZH 400/89 Bruker cryomagnet (**II**). FAB(+)-MS was recorded on a JEOL JMS-AX-505W instrument. Melting points were taken in unsealed capillary test tubes and are uncorrected.

2.3. Preparation of 5-oxazolidinones (Ben-Ishai, 1957)

The 5-oxazolidinones of Z-Ala-Gly (*I*) and Z-Ala-Ala (*II*) were prepared by refluxing 2.5 mmol of the *N*-benzyloxycarbonyl-protected dipeptide with 1.7 mmol paraformaldehyde and 0.2 mmol toluene-4-sulphonic acid added as a catalyst in 50 ml toluene for 2 h. The reaction mixture was then extracted with 2 × 35 ml 0.05 M phosphate buffer pH 7.45 and 2 × 35 ml water. The organic phase was evaporated to dryness and the rema-

nence recrystallized from ether/petroleum ether. The melting points were determined to be 138–141°C for compound **I** and 127–131°C for compound **II**. The structures were confirmed by FAB(+)-MS and ^{1}H-NMR. MS: Compound **I**: MW 291. Found MW 292. Compound **II**: MW 305. Found MW 306.

2.3.1. ^{1}H-NMR data

2.3.1.1. Compound I (chloroform). δ 7.34 (5H, m, aromatic H); δ 6.51 (1H, Broad d, $-CO-\underline{NH}-CH(CH_3)-$); δ 5.06 (2H, s, $Ar-\underline{CH_2}-O-CO-$); δ 5.55 and δ 5.18 (2X1 H, d, $-N-\underline{CH_2}-O-CO-$ in the oxazolidinone ring); δ 4.44 and δ 4.12 (2X1 H, d, $-N-\underline{CH_2}-CO-O-$ in the oxazolidinone ring); δ 4.32 (1H, q, $-N-\underline{CH}(CH_3)-CO-$); δ 1.38 (3H, d, $-N-CH(\underline{CH_3})-CO-$).

2.3.1.2. Compound II (DMSO). δ 7,63 (1H, Broad m, $-CO-\underline{NH}-CH(CH_3)-$); δ 7.35 (5H, m, aromatic H); δ 5.04 (2H, s, $Ar-\underline{CH_2}-O-CO-$); δ 5.62 and δ 5.04 (2X1 H, d, $-N-\underline{CH_2}-O-CO-$ in the oxazolidinone ring); δ 4.21 (1 H, Broad m, $-N-\underline{CH}(CH_3)-CO-O-$ in the oxazolidinone ring); δ 4.33 (1H, q, $-N-\underline{CH}(CH_3)-CO-$); δ 1.21 and δ 1.41 (3H, d, $-N-CH(\underline{CH_3})-CO-$ on the oxazolidinone ring or 3H, d, $-N-CH(\underline{CH_3})-CO-$ outside the oxazolidinone ring, interchangeable signals).

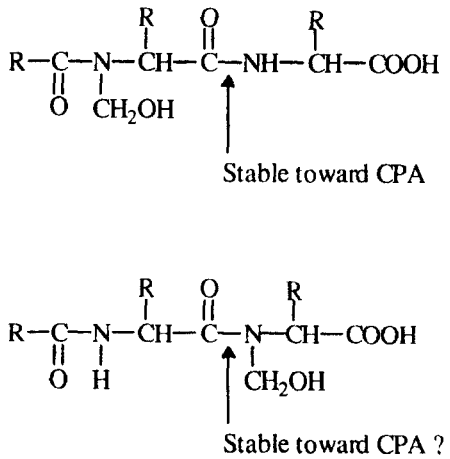


Fig. 1. Stabilization of the *C*-terminal peptide bond toward carboxypeptidase A (CPA) degradation can be achieved by *N*-hydroxymethylation of the second *C*-terminal peptide bond. Protection should be possible by direct *N*-hydroxymethylation of the *C*-terminal peptide bond.

2.4. Conversion of 5-oxazolidinones to the corresponding N-hydroxymethyl derivative

The *N*-hydroxymethyl derivatives of Z-Ala-Gly-OH (**Ia**) and Z-Ala-Ala-OH (**IIa**) were not isolated but prepared in situ by hydrolysis of the 5-oxazolidinones at 37°C ± 0.2°C in a borate buffer (pH 9.5). The buffer concentration was 0.02 M and the ionic strength (μ) was 0.5. Then, 1 ml of 10^{-2} M stock solution of the 5-oxazolidinone in acetonitrile was added to 1 ml buffer solution (pH 9.5), preequilibrated at 37°C. The mixture was allowed to react until all the 5-oxazolidinone had disappeared from the reaction mixture as evidenced by HPLC analysis. The mixture was immediately used as stock solution for the kinetic measurements described below.

2.5. Kinetic measurements

2.5.1. Hydrolysis in buffer solutions

The decomposition of compound **I**, **Ia**, **II** and **IIa** was studied in aqueous buffer solutions at 37°C ± 0.2°C. Buffers containing hydrochloric acid, acetate, phosphate, borate or carbonate buffers were used. The total buffer concentration was 0.02 M. A constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The reactions were initiated by adding 100 μl of a stock solution of the derivative **I** or **II** in acetonitrile (10^{-2} M) or 200 μl of the buffer solution described in Section 2.4 (**Ia** or **IIa**) to 10.0 ml of the buffers described above, preequilibrated at 37°C, giving a final concentration of 10^{-4} M for compound **I** and **II**, and approximately 10^{-4} M for compound **Ia** and **IIa**. The rates of decomposition were determined by using HPLC procedures capable of separating the derivatives from their parent peptides. The eluents used were 15–40% (v/v) acetonitrile in 0.08% phosphate buffer (v/v) pH 5.0. Triethylamine in a concentration of 10^{-3} M was added to prevent tailing. The acetonitrile content was adjusted for each compound to give appro-

priate retention times (3–9 min for the compounds in question). Compound **I**, **Ia**, **II** and **IIa** were all eluted later than the parent peptide. The flow rate was 1.2 ml/min and the column effluent was monitored at 215 nm. Quantitation of the compounds were carried out by measuring the peak heights or by automatic integration of the peaks. Pseudo-first-order rate constants for the degradation were determined from the slopes of linear plots of the logarithm of the peak heights or integrals against time.

2.5.2. Hydrolysis in 80% human plasma and in buffer solutions containing CPA

The degradation of compound **Ia**, **IIa**, **Ib** and **IIb** were studied at 37°C ± 0.2°C in 0.05 M phosphate buffer solution (pH 7.4) containing 25 and 50 U/ml carboxypeptidase A. The reactions were initiated by adding 100 μl of a stock solution (10^{-2} M) of compound **Ib** or **IIb** in acetonitrile or 200 μl of the buffer solution described in section 2.4 (**Ia** or **IIa**) to the reaction solution described above, giving a final concentration of 10^{-4} M. The reaction mixtures were kept in a waterbath at 37°C ± 0.2°C and at appropriate intervals samples of 250 μl were withdrawn and added to 250 μl of 5% (v/v) solution of perchloric acid in water in order to deproteinize the samples and stop the reactions. After immediate mixing and centrifugation for 3 min at 13000 revs./min, 20 μl of the clear supernatant was analyzed by HPLC using the same HPLC methods as previously described. The degradation of Z-Ala-Gly (**Ib**) was determined by initial rate by measuring the formation of Z-Ala. Pseudo-first-order rate constants for the degradation was determined as described above.

The hydrolysis of compound **II** and **IIa** was also studied in 80% human plasma containing 0.05 M phosphate buffer pH 7.4. The initial concentration was 10^{-4} M. The samples were treated as described above for the enzyme experiments.

3. Results and discussion

3.1. Hydrolysis in buffer solution

The kinetics of hydrolysis of compound **I**, **Ia**, **II** and **IIa** were studied in aqueous buffer solutions over a wide pH range (pH 1–11) at 37°C and μ = 0.5. At constant temperature and pH the disappearance of the compounds displayed strict first-order kinetics over several half-lives.

Table 1
Rate data for the decomposition of compound **I**, **II**, **Ia** and **IIa** in aqueous buffer solution at 37°C and μ = 0.5

Compound	k_H (M^{-1} min^{-1})	k_O (min^{-1})	k_{OH} (M^{-1} min^{-1})
I	1.0×10^{-2}	1.5×10^{-3}	2.5×10^{4}
Ia	1.0×10^{-2}	3.0×10^{-5}	2.0×10^{3}
II	1.2×10^{-2}	9.4×10^{-4}	7.5×10^{4}
IIa	3.0×10^{-2}	1.3×10^{-4}	8.0×10^{2}

The influence of pH on the rate of hydrolysis of compound **I**, **Ia**, **II** and **IIa** are shown in Figs. 2 and 3, where the logarithms of the observed pseudo-first-order rate constants (k_{obs}) are plotted against pH. The rate data obtained for the various derivatives show that the stability is maximal in the pH-range 2–5 for compound **I** and **II** and at pH around 3–5 for compound **Ia** and **IIa**. The observed pH-rate relationship indicate that the hydrolysis for compound **I**, **Ia**, **II** and **IIa** can be described in terms of a water-catalyzed or spontaneous reaction (k_0) and specific acid- (k_H) and base-catalyzed (k_{OH}) reactions according to the following rate expression:

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

where a_H and a_{OH} refer to the hydrogen ion and hydroxide ion activity, respectively. The latter was calculated from the measured pH (Bundgaard and Rasmussen, 1991a). Values of the specific rate constants k_H, k_0 and k_{OH} obtained from the pH-rate profiles and Eq. (1) are listed in Table 1. In Figs. 2 and 3, the solid curves were constructed from the rate constants for compound **I**, **Ia**, **II**, **IIa** and Eq. (1).

Derivative **Ia** and **IIa** contain an ionizable carboxylic acid group. No significant difference in the rate of degradation between the protonated and the unprotonated form of the compounds

Table 2
Half-lives ($t_{1/2}$) for the degradation of compound **Ia**, **IIa**, **Ib** and **IIb** in 0.05 M phosphate buffer pH 7.4 with or without CPA at 37°C

Compound	pH 7.4 buffer[a]	CPA[b] (25 U/ml)	CPA[b] (50 U/ml)
Z-Ala-Gly (**Ib**)	Stable[c]	80.8 h	39.6 h
Z-Ala-(N-CH_2OH)Gly (**Ia**)	8.8 h	9.1 h	9.4 h
Z-Ala-Ala (**IIb**)	Stable[c]	47 min	23 min
Z-Ala-(N-CH_2OH)Ala (**IIa**)	12.4 h	11.6 h	12.6 h

[a]0.05 M phosphate buffer pH 7.4
[b]CPA, carboxypeptidase A in 0.05 M phosphate buffer pH 7.4
[c]No degradation was seen after 24 h.

could be observed. Apparently this results in a lack of a shoulder around the pK_a value for the carboxylic acid as seen in Figs. 2 and 3. Another reason for the absence of a shoulder in the pH-rate profiles could be explained by the relative high k_0-value seen for compound **IIa** ($k_0 = 1.3 \times 10^{-4}$ min^{-1}). In comparison the k_0-value found for the degradation of *N*-hydroxymethyl-Z-glycine in buffer is 2.2×10^{-5} min^{-1} (Bundgaard and Rasmussen, 1991a) and k_0 is 3.0×10^{-5} min^{-1} for compound **Ia** as seen in Table 2.

The higher k_0-value and at the same time a possibly higher pK_a-value for compound **IIa** compared to similar compounds will possibly result in a very little contribution from the reaction between $R-COO^-$ and H^+ to the overall reaction (pK_a for *N*-α-hydroxymethyl-Z-glycine can be estimated to be around 3 (Bundgaard and Rasmussen, 1991a)). A plateau is therefore seen for compound **IIa** around the pK_a-value for the carboxylic acid group instead of a shoulder. However, a relative high k_0-value can not explain the

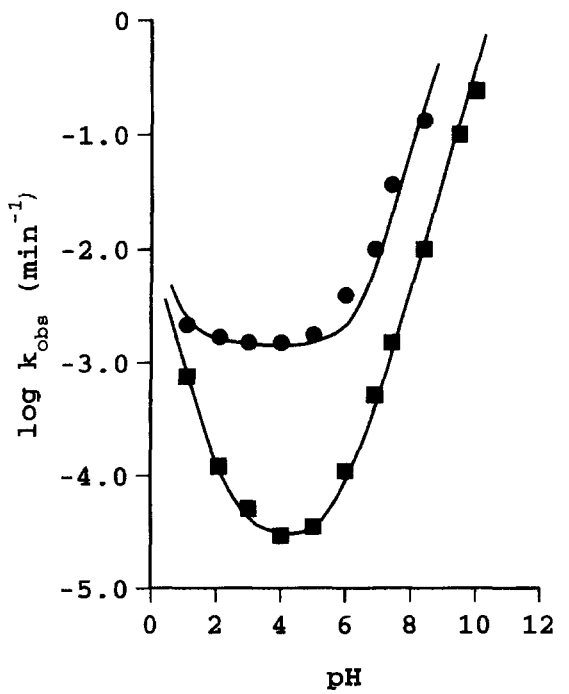


Fig. 2. The pH-rate profile for the hydrolysis of compound **I** (●) and compound **Ia** (■) in aqueous solution at 37°C.

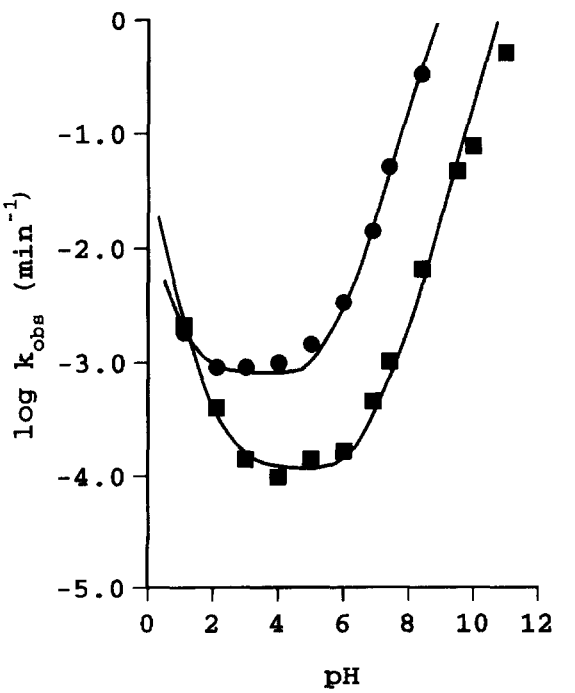


Fig. 3. The pH-rate profile for the hydrolysis of compound **II** (●) and compound **IIa** (■) in aqueous solution at 37°C.

lack of a shoulder seen for compound **Ia**. In contrary a relative higher pK_a-value of the carboxylic acid group may result in a shoulder later than usual. Therefore it will probably be hidden under the k_0 reaction that will dominate in the pH-range in question.

3.2. Mechanism of degradation

From Figs. 2 and 3 it is seen that the compound **Ia** and **IIa** are degraded slower in buffer solution than the corresponding 5-oxazolidinone which is in accordance with what previously has been shown (Buur and Bundgaard, 1988; Bundgaard and Rasmussen, 1991a). It was shown that the degradation appears to take place via a two-step reaction as shown in Scheme 2 (Buur and Bundgaard, 1988; Bundgaard and Rasmussen, 1991a). First the heterocyclic ring is opened by a plasma-catalyzed reaction followed by a spontaneous degradation of the intermediary *N*-hydroxymethyl derivative. Thus, compound **II** showed a half-life of decomposition in 80% human plasma of 1.4 min compared to 22.1 min in pure buffer solution pH 7.4. The degradation of compound **IIa** was, in contrast, only slightly influenced by the presence of plasma enzymes. This is in accordance with previous results (Johansen and Bundgaard, 1981; Bundgaard and Johansen, 1984; Bundgaard and Buur, 1987; Møss and Bundgaard, 1989).

The proposed two-step degradation suggested above was studied in greater details for compound **II**. Using a HPLC system as described above it was possible to monitor the disappearance of this compound. The disappearance was accompanied by the formation of compound **IIa** which was subsequently transformed to compound **IIb**. No other peaks were seen in the chromatogram. In Fig. 4, the time-courses for the compounds upon degradation of **II** in 0.02 M borate buffer pH 8.50 (μ = 0.5) and 37°C are shown.

3.3. Hydrolysis in the presence of carboxypeptidase A (CPA)

The kinetics of the hydrolysis of compound **Ia**, **IIa**, **Ib** and **IIb** was determined in 0.05 M phosphate buffer pH 7.4 as well as in 0.05 M phosphate buffer containing CPA in a concentration of 25 U/ml and 50 U/ml. Under the experimental conditions mentioned above the hydrolysis of the compounds followed first-order kinetics.

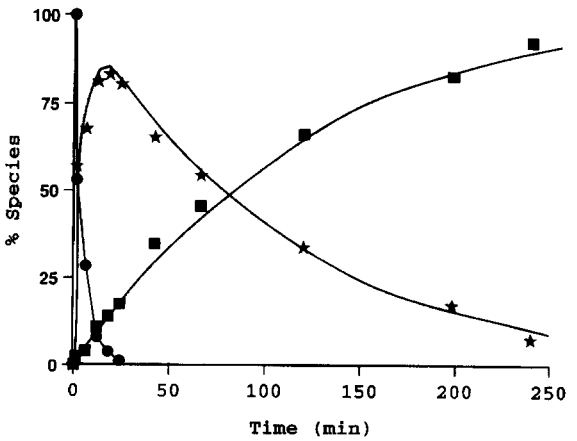


Fig. 4. Time courses for compound **II** (●), compound **IIa** (★) and compound **IIb** (■) during the degradation of compound **II** in 0.02 M borate buffer solution of pH 8.50 (μ = 0.5) at 37°C. The curve for compound **IIa** was constructed from data based on HPLC measurements and the identity: % derivative = 100 − (**II** + **IIb**)%.

It is seen in Table 2 that the parent dipeptides Z-Ala-Gly (**Ib**) and Z-Ala-Ala (**IIb**) are degraded with vastly different half-lives in the presence of two different concentrations of CPA. In agreement with the substrate specifity, compound **IIb** is degraded much faster than compound **Ib**. The greater reactivity of Z-Ala-Ala (**IIb**) compared to Z-Ala-Gly (**Ib**) is in accordance with previous studies of similar Z-protected dipeptides (Z-Gly-Gly and Z-Gly-Ala) (Stahmann et al., 1946). Under identical reaction conditions it is observed that the *N*-hydroxymethylated compounds **Ia** and **IIa** is degraded with a similar half-life to that observed in buffer solution pH 7.4, i.e. enzyme-free solution.

The results demonstrate that *N*-hydroxymethylation of the *C*-terminal peptide bond makes the derivatives stabile against degradation by CPA. Thus, it is also possible to achieve protection by direct derivatization at the *C*-terminal peptide bond. Previously, it has been shown that the presence and integrity of the second peptide bond

seen from the *C*-terminal end of the peptide is important for the enzymatic reactivity of CPA (Hanson and Smith, 1948; Snoke and Neurath, 1949; Bundgaard and Rasmussen, 1991a). The presented results demonstrate that the enzymatic reactivity of CPA against the dipeptides also is influenced if the *C*-terminal peptide bond is modified as shown here by *N*-hydroxymethylation.

4. Conclusions

It has been shown that *N*-hydroxymethylation of the *C*-terminal peptide bond via 5-oxazolidinone formation protects peptides against degradation by carboxypeptidase A (CPA). In contrast to *N*-methylation or similar *N*-alkylation of the peptide bond *N*-hydroxymethylation is a bioreversible modification. The parent peptide is formed in quantitative amounts via a spontaneous reaction, i.e. non-enzymatic. Studies are in progress to apply this approach to pharmacologically interesting peptides. Further derivatization of *N*-hydroxymethyl derivatives producing ester derivatives is another interesting aspect of this approach (Bundgaard and Rasmussen, 1991b). This approach is currently being investigated further in our laboratory.

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