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KINETICS OF THE ALKALINE HYDROLYSIS OF SEVERAL N-BENZYLOXYCARBONYLDIPEPTIDE METHYL AND ETHYL ESTERS

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SUMMARY

The reaction rates of the alkaline hydrolysis of synthesized N-protected dipeptide methyl and ethyl esters were studied systematically. From the kinetic data the energies of activation, the pre-exponential factors and the reference values at 40 °C were calculated. The rate of hydrolysis shows to be strongly dependent on the C-terminal amino acid in the sequence Gly >> Ala/Met/Phe > Leu >> Val/Pro. Surprisingly the N-terminal amino acid also exerts an effect, but in a different sequence. N-Terminal Phe in particular shows a relative accelerating effect. Remarkable is the significantly faster ester hydrolysis of glycine containing dipeptide ethyl esters in ethanol/water compared to the corresponding methyl esters in methanol/water.

INTRODUCTION

Sequential synthesis of large peptides leads to a number of problems, such as solubility and the purification of the intermediates. In order to avoid these problems, a procedure based on the synthesis of fragments is often chosen. These fragments are then coupled to each other resulting in larger fragments. Successive coupling of the peptide fragments finally leads to the desired polypeptide[1]. The strategy of the synthesis is based on a temporary protection of the carboxylic acid function of amino acids or peptides, which is nearly always performed by esterification. In order to achieve the coupling of the desired fragments, the relevant fragments have to be activated again after saponification of the ester groups, prior to the coupling. It is obvious that for a well-considered concept of the synthesis of large peptides, the knowledge of the rates of hydrolysis of peptide esters is essential. As only little is known about these reaction rates, it is the intention of this paper to supply data on the rates of alkaline hydrolysis of N-protected dipeptide esters, which serve as model compounds of larger peptide esters.

N-Benzyloxycarbonyldipeptide methyl and ethyl esters as model compounds

Though the use of protected amino acids as model compounds would seem obvious, the protected

dipeptides were chosen for several reasons. Firstly the presence of a peptide bond makes this type of model compound more comparable with larger peptides and a possible effect of the remote amino acid on the hydrolysis of the C-terminal amino acid ester may be studied. Secondly, unlike the protected amino acids, most of the dipeptides concerned were known to be (or expected to be) amorphous or crystalline and therefore easier to purify. From the bulk of N-protecting groups the benzyloxycarbonyl function was selected, because of its general use and stability (Fig. 1). For the C-terminal protection we took methyl esters. Later on the program was extended with some ethyl esters. Only amino acids without functional side chains have been selected to avoid instability of side chain protection under the conditions used for removal of the ester functions. The amino acids taken in our research program were alanine, valine, leucine, phenylalanine, proline, glycine and later on also methionine.

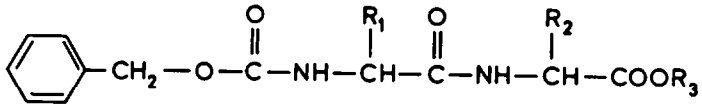

Figure 1. N-Benzyloxycarbonyldipeptide ester; R_1 and R_2 are the side chains of the respective amino acids (aa1 and aa2). R_3 is methyl or ethyl.

Synthesis of the N-benzyloxycarbonyl amino acids (as dicyclohexylammonium salts)

Reaction of N-benzyloxycarbonylchloride with the respective amino acids under Schotten-Baumann conditions yielded the corresponding N-benzyloxycarbonyl amino acids, which were converted with dicyclohexylamine into their dicyclohexylammonium salts. These readily recrystallizable compounds were used for the coupling with amino acid esters to dipeptides.

Synthesis of the amino acid esters (as hydrochloric acid salts)

According to standard procedures, the amino acids were treated with di(m)ethylsulphite in (m)ethanol, obtained by the reaction of thionyl chloride with an excess of (m)ethanol at low temperature (-15 °C). The reaction was completed by boiling the mixture for a period of 90 min. Evaporation of the volatile reagent and solvent, followed by recrystallization, mostly from (m)ethanol-diethylether mixtures, yielded the desired amino acid ester as a hydrochloride. Proline ethyl ester, however, could only be obtained as a highly viscous oil.

Synthesis of the N-benzyloxycarbonyldipeptide (m)ethyl esters

The amino acid ester hydrochloric acid salt and the N-benzyloxycarbonyl amino acid dicyclohexylammonium salt were brought together in dichloromethane, whereupon the suspension obtained was stirred. After removal of the precipitated dicyclohexylammoniumchloride, N,N'-

dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were added to accomplish the coupling. The N-benzyloxycarbonyldipeptide ester was obtained after the usual filtration, washing and recrystallization procedures. Before use in the kinetic experiments the structure of the N-benzyloxycarbonyldipeptide esters was checked by proton NMR and MS. The purity was checked by TLC and melting point references. If necessary, e.g. by lack of references, the compounds were recrystallized until a constant melting point was reached. The proline containing N-benzyloxycarbonyldipeptide esters could only be obtained as viscous oils (Table I and II).

Table I Melting points and kinetic data of the N-benzyloxycarbonyldipeptide methyl esters synthesized (Z = benzyloxycarbonyl; k_0 = Arrhenius pre-exponential factor).

	Found(°C)	Lit.(ref.)	Activation energy (kJ/mole)	ln k_0 (k_0:l/mol min)
Z-Ala-Ala-OMe	105-106	107[2]	62.2	26.46
Z-Ala-Gly-OMe	96-97	96-97[3]	62.3	27.58
Z-Ala-Leu-OMe	oil	163-165[4]	58.4	24.22
Z-Ala-Met-OMe	77-78	81[3]	62.3	26.44
Z-Ala-Phe-OMe	102-103	103-104[5]	60.5	25.66
Z-Ala-Pro-OMe	oil	--	65.6	24.97
Z-Ala-Val-OMe	81-82	83[3]	62.1	24.19
Z-Gly-Ala-OMe	92-94	62-63[6]	67.2	29.20
Z-Gly-Gly-OMe	65-66	67-68[7]	64.1	28.51
Z-Gly-Leu-OMe	oil	62-65[8]	51.0	21.68
Z-Gly-Phe-OMe	oil	oil[9]	62.9	27.15
Z-Gly-Val-OMe	oil	oil[10]	36.1	13.63
Z-Leu-Ala-OMe	96-97	95-96[10]	53.9	23.56
Z-Leu-Gly-OMe	100-102	92-93[6]	59.5	26.49
Z-Leu-Leu-OMe	95-96	97-98[6]	68.3	27.63
Z-Leu-Phe-OMe	85-89	86-90[11]	67.1	28.00
Z-Leu-Pro-OMe	oil	--	53.3	20.91
Z-Leu-Val-OMe	oil	oil[12]	61.3	23.72
Z-Met-Gly-OMe	n.d.	70-73[13]	53.7	23.81
Z-Met-Phe-OMe	109-110	--	56.5	24.30
Z-Met-Val-OMe	n.d.	103.5[14]	99.2	37.77
Z-Phe-Ala-OMe	130-131	131-133[5]	59.5	25.78
Z-Phe-Gly-OMe	119-120	119-120[8]	83.1	31.69
Z-Phe-Leu-OMe	107-108	111-113[11]	78.4	31.79
Z-Phe-Met-OMe	121-122	--	71.5	30.27
Z-Phe-Phe-OMe	148-150	148-150[15]	56.3	24.39
Z-Phe-Pro-OMe	oil	oil[16]	19.3	8.61
Z-Pro-Ala-OMe	61-62	79-80[17]	59.6	25.51
Z-Pro-Gly-OMe	oil	oil[18]	60.8	26.64
Z-Pro-Leu-OMe	79-80	77-78[19]	65.8	26.74
Z-Pro-Met-OMe	n.d.	--	67.3	28.32
Z-Pro-Pro-OMe	oil	76-78[6]	71.3	26.83
Z-Pro-Val-OMe	oil	oil[20]	57.8	22.16
Z-Val-Ala-OMe	162-163	162-3[19]	58.5	24.99
Z-Val-Gly-OMe	159-161	159-160[21]	71.8	30.52
Z-Val-Leu-OMe	102-104	101-102[5]	79.5	31.48
Z-Val-Met-OMe	131-133	--	58.7	24.96
Z-Val-Phe-OMe	141-143	139-140[22]	77.5	33.12
Z-Val-Pro-OMe	oil	oil[23]	58.4	22.13
Z-Val-Val-OMe	105-109	107-109[24]	66.8	25.33

Table II Melting points and kinetic data of the N-benzyloxycarbonyldipeptide ethyl esters synthesized.

	Found (°C)	Lit.(ref.)	Activation energy (kJ/mole)	ln k_0 (k_0:l/mol min)
Z-Ala-Ala-OEt	114-115	114-115[25]	57.4	24.82
Z-Ala-Gly-OEt	99-100	98-99[26]	49.0	23.43
Z-Ala-Met-OEt	oil	--	52.6	22.79
Z-Gly-Gly-OEt	80-82	79-80[23]	70.3	31.63
Z-Gly-Met-OEt	oil	--	66.6	28.31
Z-Leu-Ala-OEt	89-95	66-67[27]	67.5	28.32
Z-Leu-Met-OEt	76-78	--	54.0	23.29
Z-Met-Gly-OEt	95-96	93-94[23]	58.3	26.74
Z-Met-Met-OEt	n.d.	--	69.0	29.11
Z-Phe-Ala-OEt	120-122	118-119[28]	41.2	18.93
Z-Phe-Gly-OEt	109-110	109[6]	53.8	25.14
Z-Phe-Val-OEt	102-104	103-104[29]	46.8	18.48
Z-Pro-Ala-OEt	oil	oil[25]	63.9	27.33
Z-Pro-Gly-OEt	oil	oil[30]	51.0	23.49
Z-Val-Ala-OEt	164-165	167[31]	46.4	20.07
Z-Val-Gly-OEt	163-164	162[30]	58.7	26.73
Z-Val-Met OEt	136-137	--	69.8	29.02
Z-Val-Pro-OEt	oil	oil[32]	59.7	22.58
Z-Val-Val-OEt	85-86	90-92[33]	54.6	21.19

DISCUSSION AND CONCLUSION

In aqueous alcohol solutions, basic hydrolysis and alcoholysis of esters, occur simultaneously. Preliminary experiments carried out on the saponification of ethyl esters in a water-methanol mixture reveal a variation in the order of the reaction of 1.5 to 2.0, dependent on the initial concentrations of the reactants. This phenomenon is due to the differences in the rate of attack by ethoxide ions and methoxide ions. Therefore, kinetic experiments had to be performed in different aqueous alcohol solutions: methyl esters in methanol/water and ethyl esters in ethanol/water. Within each of the experiments, k-values were obtained with an accuray of 3-10%. However, Arrhenius plots for the separate dipeptide esters yield straight lines with correlation coefficients of at least 0.99. The regression of ln k on 1/T shows on average errors of about 5% in the activation energies and in ln k_0, although in some cases the errors mount to 10%. Calculated rate constants of the alkaline hydrolysis of N-protected dipeptide methyl esters at 40 °C in 60 vol. % methanol-water are given in Table III. This temperature of reference is chosen because it is situated within each of the experimental ranges of temperatures, by which errors due to extrapolation can be avoided. According to the estimated standard deviations, most of the standard rate coefficients show an accuray of 5-8%.

It is seen from the data in the rows of Table III, that the rate constants follow in general the order Gly >> Ala / Met / Phe > Leu >> Val / Pro.

This result confirms our expectation in view of the increasing electronic effect and steric hindrance. Particularly Pro and Val show a pronounced retardation of the rate of reaction.

The N-terminal amino acid, however, also shows an effect on the values of the rate constants, as is apparent from the columns of Table III and IV. Comparatively, Gly exhibits

the strongest influence as it is the substituent with the smallest contribution to the steric hindrance. A deviating behaviour is found for N-terminal phenylalanine, which exerts an accelerating effect on the hydrolysis of the ester. Further research, however, is needed to confirm our hypothesis that this unexpected behaviour is caused by spatial electronic factors as a result of a bending back of the aromatic ring of phenylalanine bringing it in the immediate neighbourhood of the reaction centre. Pilot experiments with N-protected dipeptide ethyl esters in an ethanol-water medium (Table IV) give similar results.
Another remarkable result is the significant faster hydrolysis of glycine ethyl esters in ethanol/water in comparison to the corresponding methyl esters in methanol/water. From the point of view of an easy removal of the ester moiety of a peptide fragment, it is advisable to prefer glycine and as second best alanine, phenylalanine and methionine as C-terminal amino acids. It is preferable to hydrolyse the often used glycine ethyl esters in ethanol/water instead of in the usual methanol/water mixtures.
In our opinion the low hydrolysis rate of proline esters can cause such serious problems in obtaining fragments suitable for coupling that even the advantage of the inability of racemisation via the oxazolone mechanism might be cancelled.

aa1 \ aa2	Gly	Ala	Met	Phe	Leu	Val	Pro
Gly	47.7	28.9		19.4	7.9	0.78	
Ala	37.6	12.8	12.0	11.0	5.9	1.4	0.78
Met	23.7			13.2		0.69	
Phe	47.9	18.3	16.1	15.6	5.2		3.3
Leu	37.1	17.1		9.0	3.9	1.2	1.5
Val	18.5	12.2	10.9	10.2	2.5	0.70	0.72
Pro	26.2	13.4			4.2	0.94	0.56

Table III. The reaction rate constants in $l\ mol^{-1}\ min^{-1}$ at 40 °C of the alkaline hydrolysis of N-benzyloxycarbonyldipeptide methyl esters; aa1 is referring to the N-terminal and aa2 to the C-terminal amino acid.

aa1 \ aa2	Gly	Ala	Met	Phe	Leu	Val	Pro
Gly	107.4		15.0				
Ala	98.6	15.7	13.0				
Met	73.2		13.2				
Phe	86.0	21.2				1.6	
Leu		10.7	12.5				
Val	64.2	9.3	8.9			1.2	0.69
Pro	48.5	15.8					

Table IV. The reaction rate constants in 1 mol^{-1} min^{-1} at 40 °C of the alkaline hydrolysis of N-benzyloxycarbonyldipeptide ethyl esters; aa1 is referring to the N-terminal and aa2 to the C-terminal amino acid.

EXPERIMENTAL PART

Synthesis of N-benzyloxycarbonylamino acids (as dicyclohexylammonium salts)

To a chilled solution (5 °C) of 0.1 mole of an amino acid in 25 ml of 4N NaOH, 30 ml of 4N NaOH and 0.11 moles of benzyl chloroformate were simultaneously added over a period of 30 min, keeping the temperature at ca. 5 °C. After an additional 15 min of stirring the solution was washed with diethylether. The aqueous layer was acidified to pH 2 at 0 °C. The N-benzyloxycarbonylamino acid was extracted from the mixture by three 75 ml portions of ethyl acetate, which were thoroughly rewashed with water. After drying over anhydrous magnesium sulphate, the organic phase was evaporated *in vacuo* at 40 °C. The residue was dissolved in ethyl acetate and 18.1 g (0.1 mole) of dicyclohexylamine in 30 ml of diethylether was added. After 5 min of stirring the precipitated N-benzyloxycarbonylamino acid dicyclohexylammonium salt was removed by filtration, washed with diethylether and dried *in vacuo* over P_2O_5. Methanol-diethylether mixtures proved to be a suitable recrystallization medium.

Synthesis of amino acid methyl esters (as hydrochloric acid salts)

At -10 °C 9.8 ml of thionyl chloride was added to 87 ml of methanol. After the addition of 0.11 moles of the amino acid the mixture was boiled for a period of 2 hours, evaporated *in vacuo*, and the residue was recrystallized from methanol-diethylether.

Synthesis of N-benzyloxycarbonyldipeptide esters (general procedure)

Under stirring 10 mmoles of the N-benzyloxycarbonylamino acid (dicyclohexylammonium salt) were added to 10 mmoles of the amino acid ester (HCl-salt) in 40 ml of dry methylene chloride. After 10 min of stirring the dicyclohexylammonium chloride precipitated was filtered off and washed with 10 ml of cold methylene chloride. To the combined filtrates 2.7 g (20 mmoles) of 1-hydroxybenzotriazole was added. After cooling down to approximately -10 °C, a solution of 2.20 g (11 mmoles) of N,N'-dicyclohexylcarbodiimide in 15 ml of dry methylene chloride was added. The reaction mixture was stirred for two hours whilst keeping the temperature below 0 °C. After standing overnight at ambient temperature the N,N'-dicyclohexylurea precipitated was removed by filtration and washed with 10 ml of ethyl acetate, whereupon the combined filtrates were evaporated in vacuo to dryness. The residue was dissolved in 75 ml of ethyl acetate; insoluble residual N,N'-dicyclohexylurea was removed by filtration. The filtrate was extracted successively with: a saturated sodium carbonate solution (3 times), a solution containing 5% $KHSO_4$ and 10% K_2SO_4 (2 times), and distilled water (2 times). Losses were limited by a thorough rewashing procedure. After drying over anhydrous magnesium sulphate the ethyl acetate fraction was evaporated in vacuo and the residue recrystallized from a suitable solvent, mostly ethyl acetate/ligroin 60-80.

Kinetic experiments and methods

The saponification of the esters and dipeptides was performed in a 50 ml double walled glass reaction vessel. The vessel was kept at a constant temperature (± 0.05 °C) by a thermostat and had been equipped with an Anschutz thermometer, a conductivity cell and a magnetic stirring device. The cell was connected to a Philips laboratory conducting meter (PW 9501). All experiments were carried out in a dry nitrogen atmosphere. Practically in all cases, equimolar amounts of both reactants were used at the beginning of the reaction, in aqueous alcohol containing 60 per cent of alcohol by volume. The initial concentrations varied in a range of $5 * 10^{-1}$ to $5 * 10^{-3}$ M, dependent on the rate of conversion. Some of the crystalline products, however, showed a low solubility in the reaction mixture and smaller concentrations had to be used. As the rate of reaction became too low, experiments were carried out with unequal starting concentrations.

Saponification of the methyl esters and ethyl esters was carried out in aqueous methanol and aqueous ethanol, respectively. Bulk solutions of the dipeptide esters were prepared by dissolving the appropriate amount in the respective alcohol. The sodium hydroxide solution was prepared by an accurate dilution with distilled water, free from carbon dioxide, from 0.1000 M Titrisol (Merck). 25.00 ml of the respective alcohol and 20.00 ml of the aqueous solution of sodium hydroxide were transferred to the reaction vessel. After reaching a constant temperature, the reaction was started by adding 5.00 ml of the ester solution. Conductance readings registered by a recorder were made as soon as possible after the mixing of the reactants by a magnetic stirrer. The boiling point of the reaction mixture as well as the combination of solubility and acceptable time of reaction restrained the range of temperatures of the experiments. In general, for each of the dipeptide esters five temperatures were chosen in the range of 20-55 °C. The value of the rate constant was obtained by applying the method of Tobey[34] for reactions carried out with equal starting

concentrations, while an optimisation procedure[35] was used for the remaining experiments. In both cases only those experimental results were used that lie in between 20-80 per cent conversion.

The kinetic experiments furnished a set of reaction rates at distinct temperatures from which the corresponding activation energies and the Arrhenius pre-exponential factors (k_0) were calculated (Table I and II).

For the ease of the peptide chemist who is planning the synthesis of a protein or polypeptide we have calculated from the kinetic data the rate of hydrolysis at 40 °C (Table III and IV).

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