

N^α ACYL-AMINOACID AMIDES AS O-ACYLATING REAGENTS OF SERINE*

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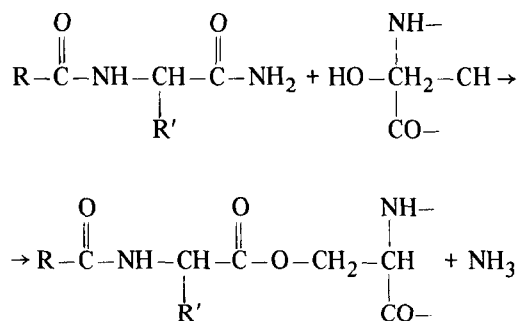
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1. Introduction

The acylating properties of amides are of great interest in connection with some fundamental problems in enzymology.

The proposed mechanism for tryptic and chymotryptic hydrolysis of simple amidic substrates [1] involves the formation of an acyl enzyme which should result from the reaction between the amide group of the specific substrate and a seryl residue of the enzyme to give an ester (the acyl-enzyme) and free ammonia. The reaction steps occurring in the mechanism of O-serine acylation by an amide, generated a controversial problem with respect to both chemical [2] and biological processes [3]. In particular, the absence of precedents in organic chemistry and the thermodynamic barrier to ester formation under mild conditions from relatively "low energy" amides have been used as arguments to exclude the possibility of acyl-enzyme formation with amide substrates [2].

In this paper we report experimental evidence that the reaction



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easily occurs if it proceeds through an acylating intermediate, an azlactone, formed from the acylaminoacid amide by an intramolecular nucleophilic displacement. This intermediate rapidly reacts in a consecutive intermolecular process with a hydroxyl group such as that of serine.

2. Materials and methods

2.1. Benzoyl aminoacid amides

Benzoyl-L-arginine amide was purchased from Cyclo Chemicals, Los Angeles, USA. The benzoylated amides of DL-alanine, DL-leucine and DL-phenylalanine have been secured by ammonolysis of the corresponding benzoyl aminoacid esters as described in the routine procedure to obtain acylated aminoacid amides [4]. The aminoacid amides were commercial products homogenous by paper chromatography.

2.2. Chromatography

Paper chromatography was performed on Whatman No. 3 paper. The solvent was 1-butanol:acetic acid:water (4:1:5 by volume). The quantitative assay of ninhydrin positive products was performed on the eluted spots after the stabilization of the blue pigment with copper nitrate [5].

For preparative purposes, column chromatography on Whatman cellulose powder was utilized using 1-butanol:acetic acid:water (4:1:5 by volume) as eluting solvent.

Thin layer chromatography was performed on silica gel (Eastman chromatogram sheet) preactivated 10 min at 100°. The solvent was light petroleum (B.P. 40–60°)

Table 1

Chromatographic characterization of the azlactones obtained from the benzoyl-aminoacyl-amides in trifluoroacetic acid. R_f are given in solvent 1: light petroleum (B.P. 40–60°)-methylene chloride (8:2 by vol); solvent 2: light petroleum (B.P. 40–60°)-methylene chloride (7:3, by volume).

Azlactone derivatives of	R_f values	
	Solvent 1	Solvent 2
Benzoyl Ala	0.43	0.52
Benzoyl Leu	0.63	0.73
Benzoyl Phe	0.46	0.58

containing different amounts of methylene chloride as specified in table 1.

Substituted azlactones were detected by spraying the chromatogram with an alcoholic solution of hydroxylamine and after drying with an alcoholic solution of FeCl_3 and acid hydrochloride [6].

Aminoacids were analyzed with a Beckman amino-acid analyzer Model 120 B using microcells with 6.6 mm light path.

2.3. Reaction of benzoyl aminoacid amides with serine

In a typical experiment a benzoylated aminoacid amide (1.5×10^{-3} moles) was dissolved in anhydrous trifluoroacetic acid* (10 ml) and the solution was allowed to stand at 50° in a tube closed with a ground glass stopcock. At suitable intervals three parallel samples were at each time withdrawn from the reaction mixture. The first one (0.5 ml) was cooled to room temperature and 0.5 ml of a solution of DL-serine 0.3 M in anhydrous TFA were added. After 1 min a dilution with 6.5 ml of ethanol was performed and 30 μl of this solution were quantitatively analyzed by paper chromatography. Calibrated solutions of the same O-serine derivatives were procured by column chromatography on cellulose (fig. 2) of large-scale preparations. The second sample (10 μl) was used to analyse quantitatively the liberated ammonia by the ninhydrin colorimetric test [7]. Chromatographic analyses were performed to rule out the presence of other ninhydrin positive material. The third sample (0.5 ml) was diluted with ethyl ether and washed

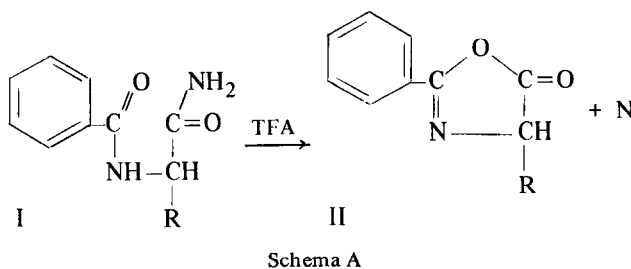
several times with cold water till a neutral pH value was reached. The ether layer, dried over Na_2SO_4 , was evaporated to dryness and the residue analyzed by thin layer chromatography for a qualitative analysis of azlactones. The azlactones utilized as reference products were synthesized by the carbodiimide method [8]. This analytical test was omitted with the arginine derivative.

When benzoylated aminoacid amides and free serine were heated together in TFA solution at 50° similar yields of O-serine derivatives were obtained as determined by quantitative chromatography at different reaction times.

The non-benzoylated amides were perfectly stable when submitted to the same experimental conditions.

3. Results and discussion

A benzoylated aminoacid amide (I) treated with TFA is specifically cleaved at its amide bond to yield free ammonia (fig. 1, curve a) and an azlactone derivative (II) (table 1) as in schema A.



This reaction, which can be deduced from the experimental evidence reported here (table 1, fig. 1) is in agreement with the one recently described for the N-acylated peptides in acidic media [9]. When free serine (III) is present, the azlactone rapidly reacts to form a benzoyl-aminoacyl-O-serine (IV) (fig. 1, curve b) as in schema B.

It is reasonable to suppose that the α -aminogroup of serine is protected by salt formation with the strong acidic solvent. These O-serine derivatives, purified by chromatography on cellulose (fig. 2) were ninhydrin positive and soluble in aqueous acidic media. The aminoacid analysis after acid hydrolysis (HCl 6N, 15 hr) gave serine in a stoichiometric ratio with the aminoacid of the parent amide as expected from their as-

* Abbreviation used, TFA = trifluoroacetic acid.

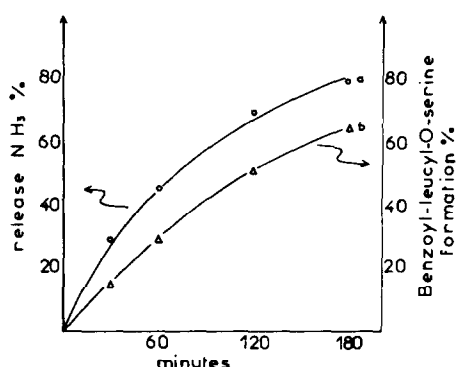
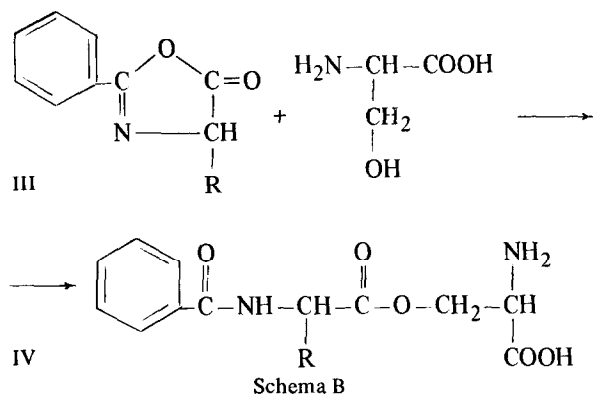


Fig. 1. Reaction of N^{α} -benzoyl leucine amide (0.15 M) and free serine in TFA, 50° as a function of time. (\circ - \circ) release of ammonia; (Δ - Δ) formation of N^{α} -benzoyl leucyl O-serine.

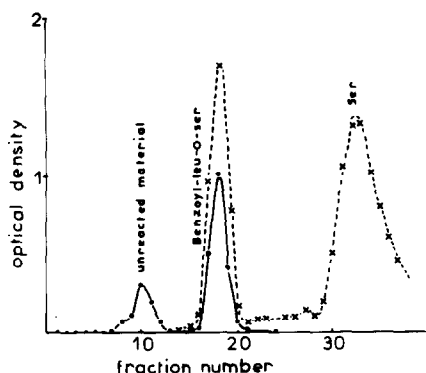


Fig. 2. Reaction products from N^{α} -benzoyl leucine amide (500 μ moles) and serine (1000 μ moles) in TFA 180 min, 50° , fractionated on cellulose column (1.8 \times 35 cm). Eluting solvent, upper layer of 1-butanol:acetic acid:water (4:1:5, by volume); fraction size 3 ml. (\bullet - \bullet) absorption at 265 $m\mu$ on 0.1 ml diluted to 3 ml by ethanol; (\times - \times) absorption at 510 $m\mu$ on 20 μ l eluted with 5 ml of methanol after paper-chromatographic separation and ninhydrin reaction [5].

Table 2

Yields and chromatographic properties (1-butanol:acetic acid: water, 4:1:5, by volume) of O-seryl derivatives obtained from benzoylated aminoacid amides (0.15 M) and free serine (0.30 M) in anhydrous TFA.

Compound	Yield		R_f values (Leu: R_f = 1)
	*	**	
Benzoyl Phe-O-Ser	58	55	1.11
Benzoyl Arg-O-Ser	53	53	0.71
Benzoyl Leu-O-Ser	63	58	1.20
Benzoyl Ala-O-Ser	65	64	0.97

* Benzoylated aminoacid amides incubated for 180 min at 50° in presence of serine.

** Benzoylated aminoacid amides incubated for 180 min at 50° and then treated with free serine at room temperature for 1 min.

signed structure (IV). The benzoyl aminoacyl-O-serine derivatives, which are perfectly stable in anhydrous TFA, are not stable under mild basic conditions, since an O \rightarrow N shift [10] rapidly occurs to give the corresponding benzoylated dipeptides, as observed in separate experiments.

The two reaction steps, A and B, were performed separately as well as at the same time (fig. 1 and table 2). Since these O-serine derivatives (IV) are immediately synthesized when the azlactone (II) is present it is reasonable to conclude that the azlactone formation is the rate-determining step of the overall reaction.

Acylated aminoacid amides are often utilized as substrates to test or to study enzymatic proteolysis. Therefore, the results of this work lead to a chemical model for obtaining a peptidyl-serine analogous to the acyl-enzyme proposed as intermediate of the proteolytic processes [1].

Furthermore, the necessity of the formation of an azlactone as intermediate in the O-acylation of serine by an amide has an interesting connection with the presence of an intermediate between the Michaelis complex and the acyl-enzyme, as deduced from a series of experiments with chromophoric substrates for trypsin and chymotrypsin [3,11]. This, together with the fact that α -chymotrypsin catalyses the hydrolysis of azlactone through acyl enzyme formation [12,13], constitutes an attractive possibility to use the chemical reaction reported here for understanding enzymatic mechanisms.

References

- [1] M.L.Bender and J.Kezdy, *Ann. Rev. Biochem.* 34 (1965) 49.
- [2] W.P.Jencks, *Ann. Rev. Biochem.* 32 (1963) 648.
- [3] H.Gutfreund, *Biochem. J.* 110 (1968) 2P.
- [4] J.P.Greenstein and M.Winitz, *Chemistry of the Amino-acids*, Vol. 2 (Wiley, New York, 1961) p. 1110.
- [5] E.Kaweran and T.Wieland, *Nature* 168 (1951) 77.
- [6] P.Nassi, C.Treves and G.Ramponi, *J. Chromatogr.* 33 (1968) 533.
- [7] S.Moore and W.H.Stein, *J. Biol. Chem.* 211 (1954) 908.
- [8] D.F.De Tar, R.Silverstein and F.F.Rogers, *J. Am. Chem. Soc.* 88 (1966) 1024.
- [9] A.Previero, M.-A.Coletti-Previero and L.G.Barry, *Biochim. Biophys. Acta* 181 (1969) 361.
- [10] E.Schröder and K.Lübke, *The Peptides*, Vol. 1 (Academic Press, New York, 1965) p. 213.
- [11] S.A.Bernhard, *The Structure and Function of Enzymes* (Benjamin, New York, 1968) p. 248.
- [12] J.de Jersey, M.T.C.Runnegar and B.Zerner, *Biochem. Biophys. Res. Commun.* 25 (1966) 383.
- [13] A.Previero and M.A.Coletti-Previero, *Compt. Rend.* 268 (1969) 1798.