On the selectivity of acylation of unprotected diamino acids

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Received October 18, 1967

p-Nitrophenyl acetate and other esters except formates, will acylate the unprotected diamino acids homolysine, lysine, and ornithine, exclusively at the ω-amino group at pH 11. At lower alkaline pH's, the reaction is preferential but not selective. The reaction with diaminobutyric acid and diaminopropionic acid is not selective at any alkaline pH. ε-*N*-Acetyllysine can be prepared directly by the action of excess phenyl acetate on lysine at pH 11.

Canadian Journal of Chemistry, 46, 1047 (1968)

We have recently described the synthesis of several ω-*N*-acetyl-α,ω-diamino acid derivatives (1). These were prepared by the action of a *p*-nitrophenyl ester on the copper salt of the amino acid. The copper was used as is done routinely in order to prevent acylation of the α-amino group (2). There exists in the literature, however, a claim to the effect that in making such compounds, selective acylation can be effected successfully even in the absence of copper (3). ω-*N*-Formyllysine and ω-*N*-formylornithine were prepared in this manner (3); moreover, there are other examples of syntheses where ε-*N*-substituted lysine derivatives were prepared without first having protected the α-amino group. These include the synthesis of carbobenzoxy- (4) and trifluoroacetyllysine (5), biocytin (6), peptides of lysine (7) and ornithine (8), and benzylidenelysine (9).

In some cases, evidence for the identity and purity of the products is presented, but only the purified products have been scrutinized. In other cases, e.g. ref. 3, no evidence at all is given. In the case of the carbobenzoxy derivative (4), the α isomer is much more soluble than the ε isomer (9), consequently the two are readily separable. In general, the question of whether the actual acylation reaction was selective remains open. In this paper are described results of a study carried out with the objective of establishing definitely whether or not the acylation of diamino acids is selective for the ω-position, and if so, under what conditions. Because its acylation presents a distinct problem, diaminopropionic acid is discussed separately.

Synthetic standards of the possible isomers in each case were prepared by unequivocal methods as described below and characterized on an amino acid analyzer. The chromatographic data appear in Table I. Acylations were carried out in aqueous solution firstly under the conditions of Okawa and Hase (3) and subsequently all at a constant pH. Reaction mixtures were placed on the amino acid analyzer column and analyzed by comparison with the standards.

The results of the acylation of diamino acids by excess *p*-nitrophenyl ester in the absence of

TABLE I

Chromatographic data from the amino acid analyzer*

Compound	Time (min)	Constant†
α-*N*-Formylornithine	146.0	7.8
α-*N*-Formyllysine	171.8	4.2
α-*N*-Acetyldiaminopropionic acid	70.5	§
α-*N*-Acetyldiaminobutyric acid	112.0	18.0
α-*N*-Acetylornithine	140.6	6.4
α-*N*-Acetyllysine	171.0	4.6
α-*N*-Benzoyllysine	126.8‡	5.7
α-*N*-Propionyllysine	82.0‡	—
α-*N*-Carbobenzoxydiaminopropionic acid	213.0	16.3
δ-*N*-Formylornithine	65.6	20.4
ε-*N*-Formyllysine	99.8	15.8
β-*N*-Acetyldiaminopropionic acid	46.0	18.9
γ-*N*-Acetyldiaminobutyric acid	57.0	18.1
δ-*N*-Acetylornithine	56.0	17.6
ε-*N*-Acetyllysine	77.4	12.1
ε-*N*-Benzoyllysine	70.5‡	22.5
ε-*N*-Chloroacetyllysine	38.0‡	6.9
ε-*N*-Propionyllysine	31.0‡	27.3
β-*N*-Carbobenzoxydiaminopropionic acid	187.0	19.5
ζ-*N*-Acetylhomolysine‖	34.5‡	22.1
Aspartic acid	44.6	20.2
Glycine	89.0	20.6

*Beckman model 120B, 50 cm spherical resin column, eluted with 0.1 *N* sodium citrate, pH 3.28, followed by pH 4.25 buffer at time 85.
†Constant = peak height × width/μmole.
‡pH 4.25 buffer at time 0.
§Constant assumed to be the same as for the β isomer.
‖See ref. 29 for details of synthesis.

base, i.e., acylation of the amino acid monohydrochloride neutralized with one equivalent of sodium hydroxide (3), appear in Table II. It is seen that in all cases, when formyl and acetyl *p*-nitrophenyl ester were used, both the α and ω derivatives were formed. The products appeared in a ratio of roughly 5 to 1 in favor of the ω derivative, but higher in the case of the acetylation of lysine. The reaction is therefore definitely not selective under these conditions.

TABLE II

Acylation of diaminoacids in absence of base*

Amino acid	Nitrophenyl ester	Derivative formed (relative %) α	ω
Lysine	formate	18	82
	acetate	6	94
Ornithine	formate	17	83
	acetate	13	87
Diaminobutyric acid	acetate	14	86
Diaminopropionic acid	acetate	10	90

*Method of Okawa and Hase (3).

Preparative runs were then worked up as described by Okawa and Hase, and the ω-*N*-formylornithine and ω-*N*-formyllysine were passed on the analyzer. Both compounds were in fact free of α isomer. The conclusion is that the α isomer is more soluble and is lost during the crystallization.

The effect of pH on the selectivity was then studied with lysine, using one equivalent of acylating agent. The results appear in Table III. It was found that, in the pH range of 10.6 to 11.7, only the ω-substituted derivative was formed. Moreover, the amount of starting material remaining was negligible indicating that the reaction was complete. At pH's near neutrality, where more α derivative was formed than at higher pH's, the reaction was far from complete and both the α and ω derivatives were formed. At pH 11, therefore, the acylation of lysine by *p*-nitrophenyl acetate is selective for the ω-position.

Having shown that the acylation of lysine is selective at pH 11, we proceeded to verify if the same was obtained for the other diamino acids. The results in Table IV show that the acylation of homolysine and ornithine is also selective, but the selectivity is lost for the diamino acids of shorter chain length. The results also indicate

TABLE III

Effect of pH on the selectivity of acylation of lysine by *p*-nitrophenyl acetate

pH	Derivative formed (relative %) α	ω	Residual amino acid %
6.2	11	89	72
7.2	13	87	43
7.5	14	86	—
8.0	8	92	—
10.6	0	100	4
11.7	0	100	—

that a one-fold excess of acylating agent is sufficient to force the reaction to completion. But they do not give any information as to whether the reaction proceeds a step further, to the formation of the disubstituted derivative. Calculations indicated that this was not the case. This was confirmed by another experiment. A sample of ε-*N*-acetyllysine was stirred at pH 11.0 in the presence of a four-fold excess of phenyl acetate for 12 h. There was absolutely no change in the ninhydrin color yield of the solution, indicating that ε-*N*-acetyllysine is completely resistant to acylation under these conditions.

TABLE IV

Acylation of diaminoacids by *p*-nitrophenyl acetate at pH 11

Amino acid	Moles of ester	Derivative formed (relative %) α	ω	Residual amino acid %
Homolysine	1	0	100	2.5
Lysine	1	0	100	4
	2	0	100	0
Ornithine	1	0	100	12
Diaminobutyric acid	2	1.2	98.8	0.2
Diaminopropionic acid	1	12	88	38

So far, we have dealt with the acylation of diamino acids by *p*-nitrophenyl acetate. We have also studied the nature of the products formed by other esters as well as by other acylating agents. The results, in Table V, show that at pH 11, the acylation of lysine was selective only if the acylating agent was an ester. Using *p*-nitrophenyl acetate, chloroacetate, or propionate, no α derivative was formed at all, and practically no unreacted amino acid remained in the reaction

mixture when a one-fold excess of reagent was employed. Using phenyl acetate or propionate, the same results were obtained, even though a larger excess of reagent was required to force the reaction to completion. A different result was obtained when acetic anhydride or benzoyl chloride was used as acylating agent. In both cases, both the α and ω derivatives were formed. In the presence of excess reagent, both were completely converted to the disubstituted derivative, as was expected from other work (10, 11). The selectivity therefore is restricted to acylating agents which are esters.

TABLE V

Acylation of lysine at pH 11

Reagent	Moles of reagent	Derivative formed (relative %) α	ω	Residual amino acid %
p-Nitrophenyl acetate	1	0	100	4
	2	0	100	0
p-Nitrophenyl chloroacetate	5	0	100	<1
p-Nitrophenyl propionate	2	0	100	0
Phenyl acetate	1	0	100	48
	5	0	100	2
Phenyl propionate	5	0	100	18
Acetic anhydride	1	38	62	—
	5	0	0	0
Benzoyl chloride	1	18.8	81.2	41.8
	5	0	0	0

The results obtained with esters of formic acid, however, proved to be different from those obtained with other ester reagents. As seen in Table VI, in the formylation of lysine at pH 11 by either *p*-nitrophenyl formate or ethyl formate, there was always some α derivative formed. In the presence of excess reagent which was required to increase the yield of product, the relative percent of α isomer also increased. The apparent low reactivity[1] of formic acid esters explains the low yields (28%) which we obtained in the synthesis of δ-*N*-formyl-L-ornithine by our method as well as by the method of Okawa and Hase (3).

The selectivity of the acylation of lysine at pH 11 has permitted us to simplify the synthesis of

[1]The reviewer has suggested that the apparent low reactivity of formate esters with amino groups is probably due to the competitive hydrolysis of the reagent by base. Thus, formate esters are actually more reactive (and less specific) than acetate esters.

TABLE VI

Formylation of lysine at pH 11

Reagent	Moles of reagent	Derivative formed (relative %) α	ω	Residual amino acid %
p-Nitrophenyl formate	1	<1	>99	79.6
	2	10	90	53.5
Ethyl formate	1	<1	>99	62
	5	2.5	97.5	18.4
	10	3	97	16.8

ε-*N*-acetyl-L-lysine (1). The latter was prepared in 76% yield by stirring a mixture of lysine with an excess of phenyl acetate for 45 min while keeping the pH at 11 with a pH-stat charged with 2 *N* sodium hydroxide. After the reaction the product was desalted using Dowex 50. Precipitation at the isoelectric point without desalting led to too substantial a loss of material. This method obviates the use of copper, the complete removal of which is often tedious. Phenyl acetate is also preferable in some ways as the acylating agent.

Likely mechanisms which have been postulated for the aminolysis of esters assume a nucleophilic attack by the amine on the carbonyl group of the ester (12, 13). In the present case, for acylation to have occurred exclusively at the ω-position, the nucleophile required would have been the $NH_2(CH_2)_nNH_3^+COO^-$ ion. But this ion does not exist at any pH because the p*K* of the α-amino group is always lower than that of the ω-amino group. Moreover, at high pH's, both amino groups are unprotonated so there is no basis for discriminatory acylation that might depend on the relative concentrations of the protonated and unprotonated forms of the amino groups. However, Bruice *et al.* (14) have noted that the rate constants for the amination of esters depend on the basicity of the nucleophile. Therefore, it would be expected that aminolysis of the ester by the ω-amino group would have been favored. This is what we have observed in all cases. When an acid chloride or anhydride is the acylating agent, the attacking nucleophile is believed to be an acylium ion, $R{=}\overset{+}{C}{=}O$ (15), which because of its high reactivity, attacks any nucleophile without discrimination.

With regard to the preparation of its monosubstituted derivatives, diaminopropionic acid is

quite different from the other diamino acids. This is due to the unique nature of its copper salt. For the general case, in alkaline solution copper binds to the α-amino group permitting and resulting in reaction exclusively at the ω-amino group (2). But for diaminopropionic acid under these conditions the acylation not only is not selective but it fails completely. This is due to the fact that both the α- and β-amino groups are so close together that they are both bound by the copper (16). The β-amino group is, however, not bound at pH 4 (16). Workers have taken advantage of this to prepare the β-oxalyl (17) and β-ureido (18) derivatives. But in many instances, direct acylation in the absence of copper has been the only recourse. Kjaer (19) has observed that in strongly alkaline solution, the β-position of diaminopropionic acid is preferentially acylated. Other results corroborate this. The β-tosyl (20) and β-chloroacetyl (21) derivatives have been prepared by direct acylation, but the reactions also produced substantial amounts of disubstituted derivatives.

As can be seen in Table IV, the acetylation of diaminopropionic acid with *p*-nitrophenyl acetate at pH 11 has been shown not to be selective. Though it was certainly preferential for the β-position, a substantial amount of α isomer was formed, and much unreacted amino acid remained. Under the same conditions at pH's 7 and 8, only about 10% of the amino acid had reacted, and the relative amounts of α isomer had increased.

With respect to the monoacylation of diaminopropionic acid, Kjaer (19) had suggested that since the pK values of the amino groups are 6.8 and 9.6, at neutral pH, one should be able to expect preferential acylation of the α-amino group since it is uncharged and the β-amino group is charged at this pH. The results cited above indicate that this is not so. Moreover, the authors themselves have shown exactly the opposite, namely that carbobenzoxylation of diaminopropionic acid in a phosphate buffer at pH 7 is highly selective for the β-position (18). They have succeeded in preparing β-carbobenzoxydiaminopropionic acid in 77% yield by this procedure. We have studied the selectivity of the acylation of a dilute solution of diaminopropionic acid with carbobenzoxy chloride in excess sodium bicarbonate and at pH's 7 and 8 using the pH-stat. In all cases, both β and α isomers were formed, in a ratio of about 2 to 1. In more concentrated solutions as described (18), at pH 7, the β-carbobenzoxy derivative precipitates out of solution during the reaction and is, in fact, pure after one recrystallization. However, in all our attempts, there was always some α-isomer present in the filtered reaction mixture. On some occasions, some disubstituted derivative was also formed. We have failed to obtain yields as high as those reported (18). Our conclusion is that acylation of diaminopropionic acid is preferential but not selective at any pH.

Experimental

Materials

Except as otherwise indicated, all ω-*N*-acyl derivatives used as standards were prepared by the action of the corresponding *p*-nitrophenyl ester on the copper salt of the amino acid as has been described for the acetyl derivatives (1). The *p*-nitrophenyl esters were prepared in 70% yield from the acid chloride and sodium *p*-nitrophenylate as described for the chloroacetyl ester (22), except for *p*-nitrophenyl formate (3). ε-*N*-Formyl-L-lysine was purchased from Sigma Chemical Co., St. Louis. ε-*N*-Benzoyl-L-lysine was made as reported (23).

α-*N*-Acetyl-L-lysine (24), α-*N*-acetyl-L-ornithine (24), α-*N*-acetyl-L-diaminobutyric acid (24), α-*N*-benzoyl-L-lysine (25), and α-*N*-formyl-L-lysine (26) were obtained from the corresponding carbobenzoxy derivative followed by hydrogenation. α-*N*-Formyl-L-ornithine was prepared as for the lysine derivative (26), except that contaminating ornithine was removed with Amberlite IRC-50; yield, 59%.

Anal. Calcd. for $C_6H_{12}N_2O_3$: N, 17.5. Found: N, 17.6.

α-*N*-Carbobenzoxy-DL-diaminopropionic acid was prepared from L-cystine as described (27), and acetylated in the presence of *N* NaOH with acetic anhydride. After acidification, the solution was concentrated, the product extracted into ethyl acetate, the solvent evaporated, and the residue hydrogenated over Pd/C to give β-*N*-acetyl-DL-diaminopropionic acid from water–ethanol; yield, 60%.

Anal. Calcd. for $C_5H_{10}N_2O_3$: C, 41.1; H, 6.9; N, 19.2. Found: C, 41.2; H, 7.1; N, 19.2.

β-*N*-Carbobenzoxy-DL-diaminopropionic acid was prepared by the action of phosphorus pentachloride on the dicarbobenzoxy derivative (28).

Acylation Experiments

A solution of diamino acid (1.0 mmole) in 10 ml of water was brought to the required pH with a pH-stat charged with 2 *N* NaOH. Acylating agent was added, and the mixture was magnetically stirred 5 h while the pH was being kept constant. The reaction was usually complete after 3/4 h. When a large excess of reagent was used (Tables V and VI), the reaction was left for 12 h. The solution was then transferred and completed to 100 ml. One ml aliquots were diluted to 5 ml with 0.20 *N* sodium citrate, pH 2.2, and samples were assayed for the appropriate products with a Beckman Amino Acid Analyzer, model 120B.

ε-*N-Acetyl*-L-*lysine*

A mixture of lysine monohydrochloride (1.83 g; 0.01 mole) and phenyl acetate (6.4 ml; 0.05 mole) was stirred for 1 h at pH 11.0 (pH-stat). The solution was removed from the pH-stat and then stirred for an additional hour in the presence of 80 ml of Dowex 50 (H^+ form). The resin was filtered off, washed several times with water, and stirred in 3 *N* NH_4OH for 1/4 h. The resin was removed by filtration, the filtrate evaporated to dryness, and the residue crystallized twice from water–ethanol; yield, 1.37 g (76%).

Anal. Calcd. for $C_8H_{16}N_2O_3$: N, 14.9. Found: N, 14.9.

Acknowledgments

Financial support of this work by the Medical Research Council of Canada and the Department of Education of the Province of Quebec (a scholarship to J. L.) is gratefully acknowledged. We thank Mr. J. H. Seely of this department for the homolysine.

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