[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, GREECE]

On Carboxyl Activation for Peptide Synthesis[1,2]

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Although typical optically active N-acyl, *i.e.*, N-acetyl or N-benzoyl, derivatives of α-amino acids are rapidly racemized by acetic anhydride at room temperature, the initially levorotatory rotation of the corresponding carbobenzoxy derivatives of L-amino acids, after passing through the zero point, continues to increase slowly reaching high positive values. Lyophilization of such solutions within a short time after dissolution, *i.e.*, 1 to 8 hours, affords mixtures of symmetrical and unsymmetrical anhydrides which are used for coupling with amino acid esters. The peptide derivatives thus formed are chemically and optically pure.

Introduction

The very easy racemization of N-acyl derivatives, *e.g.*, N-acetyl or N-benzoyl derivatives, of α-amino acids by acetic anhydride, even in catalytic amounts, is well known.[3,4] Bergmann and Zervas,[3,4] who were among the first to work on this problem, made the suggestion that the anhydride converts the acylamino acid directly to a saturated azlactone (oxazolone) which can then be racemized by a tautomeric change through an enol structure. There is also another possibility, *i.e.*, that the acetic anhydride forms, primarily, mixed anhydrides with acylamino acids which can then rearrange to form azlactones and acetic acid.[5] Similarly, the transformation of N-acylamino acids to the corresponding chlorides is usually accompanied by racemization, since these chlorides rearrange very easily to form oxazolone hydrochlorides.[6]

Contrary to the behavior of typical N-acyl, *i.e.*, N-acetyl or N-benzoyl derivatives of α-amino acids, the corresponding N-carbobenzoxy derivatives are not racemized, within hours or even a few days, at room temperature by acetic anhydride either in catalytic amounts or in excess; on the other hand, no racemization is observed during the preparation of their chlorides. This is already stated in the first publication of Bergmann and Zervas[7] on carbobenzoxyamino acids, in which they describe the action of acetic anhydride on carbobenzoxy-L-aspartic and carbobenzoxy-L-glutamic acid, as well as the preparation of the chlorides of quite a few carbobenzoxyamino acids. In all these cases the products obtained were optically active. This retention of optical activity has been one of the reasons for the wide application of the carbobenzoxy method for peptide synthesis.

The retention of optical activity of carbobenzoxyamino acids during acetic anhydride treatment may be explained by assuming that in this case the above cyclization is not expected, or at least, can not easily take place.[7,8] Up to this time, though, the study of acetic anhydride treatment has been limited to carbobenzoxyamino acids which, on account of their structure, may not form oxazolones, but rather other types of cyclic compounds, *i.e.*, either internal anhydrides, as in the case of carbobenzoxy-L-glutamic acid and carbobenzoxy-L-aspartic acid,[7] or lactams, as in the case of some carbobenzoxy derivatives of L-arginine.[9] It is the purpose of this investigation to extend the study of the action of acetic anhydride to other carbobenzoxyamino acids.

In Table I are recorded the change of the optical rotation of acetic anhydride solutions of various carbobenzoxy-L-amino acids and of N-tosyl-L-valine at various time intervals. In all cases the initial levorotatory rotation did not stay constant (with the exception of carbobenzoxy-L-proline), but after reaching the zero point it continued to increase reaching high positive values. Of course, the rotation of carbobenzoxy-D-amino acids in acetic anhydride solution proceeds in the opposite direction (Table I). After weeks though a slow decrease of optical rotation was observed. This is evidence that parallel to the slow reaction which is responsible for the considerable increase of the rotation, another reaction (probably a racemization) proceeds with an even slower rate. It is interesting that during the initial steps of the reaction (1 to 24 hours) it is easy to isolate in very good yield the particular carbobenzoxyamino acid used, after decomposition of the acetic anhydride with water. However, the amount of the carbobenzoxyamino acid recovered rapidly decreases, when the reaction time increases.

We should now like to report some work related to the products formed during the first few hours of the reaction. The acetic anhydride solution of carbobenzoxyglycine, 1 to 8 hours after dissolution, was mixed with benzene and lyophilized; the sym-

(1) A summary of this paper was presented at the 3rd European Peptide Symposium, Basle, Switzerland, Sept., 1960; G. C. Stelakatos, *Chim. (Switz.)*, **14**, 370 (1960).

(2) This investigation was partly supported by the Royal Hellenic Research Foundation; it started in Bethesda, Md., the time the author was working at the National Cancer Institute with Professor L. Zervas, Dr. M. Winitz and the late Dr. J. P. Greenstein to whom I am indebted.

(3) M. Bergmann and L. Zervas, *Biochem. Z.*, **203**, 280 (1928).

(4) M. Bergmann and L. Zervas, *Z. physiol. Chem. Hoppe-Seyler's*, **175**, 154 (1928).

(5) A. Neuberger, *Adv. in Protein Chem.*, **4**, 297 (1948).

(6) H. T. Clarke, J. R. Johnson and R. Robinson, editors, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, pp. 731, 746.

(7) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(8) G. W. Kenner, in "Symposium on Peptide Chemistry," Special Publication No. 2, The Chemical Society, London, 1955, p. 106.

(9) L. Zervas, T. T. Otani, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959).

TABLE I[a]

	αD, deg. (c 10, in acetic anhydride, temp. 17°, l 2 dm.)								
Δt	Z-L-ala	Z-L-met	Z-L-val	Z-D-val	Z-L-phe	Z-D-phe	Z-L-pro	Ts-L-val	Z-Gly-L-pro[b]
0.3 hr.	− 3.2	− 5.5					− 9.9	−0.5	
0.5 hr.			− 0.3	+ 0.3					−1.6
8 hr.	− 2.6	− 4.7	+ 0.05	− 0.15	− 2.2	+ 2.2	−10.10	−1.7	−1.7
24 hr.	− 0.15	− 1.9	+ 1.7	− 1.8	+ 4.2	− 4.3	−10.0	−0.9	−1.7
3 days	+ 9.6	+ 6.9	+ 6.4	− 6.4	+22.9	−23.1	−10.05	+1.4	
5 days	+13.8	+13.2	+ 9.8	− 9.8	+35.3	−35.3	−10.1	+3.4	−1.6
9 days					+45.2	−45.3			
10 days	+16.4	+18.6	+16.3	−16.4			−10.1	+7.6	
12 days					+48.3	−48.3			
13 days			+19.1	−19.2					
15 days					+49.5	−49.5			
43 days	+11.7								
4 mo.			+10.7		+22.0		−10.5		

[a] Z = carbobenzoxy, Ts = tosyl, ala = alanine, met = methionine, val = valine, phe = phenylalanine, pro = proline, Gly-pro = glycylproline. [b] c 1, in acetic anhydride.

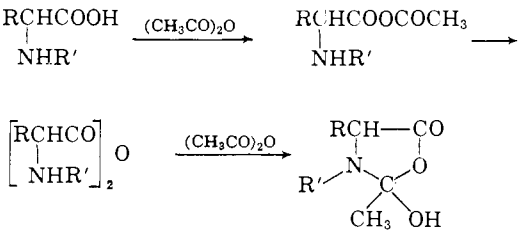
TABLE II

Carbobenzoxy-L-dipeptide esters, carbobenzoxy-	Yield, %	M.p., °C.	[α]D	t, °C.	c, chloroform
Alanylglycine benzyl ester	73,[a] 40[b]	111–112[a,b,d]	−15.7°,[a] −16.2°[b]	30	5
Valylglycine ethyl ester	82,[a] 15[b]	166–167[a,b,e]	−10.2,[a] −12.8[b]	17	2
Methionylmethionine methyl ester	20[b]	102–104[b,f,g]	−27.3[b,g]	30	1[j]
Prolylglutamic acid dibenzyl ester[h]	77,[a] 60[b,c]	89–90[a,b]	−49.2,[a] −49.3[b]	25	2
Phenylalanylglycine ethyl ester	36[b]	112–114[b,i]	−16.3[b,i]	19	2[k]

[a] Yield, m.p., [α]D for compound prepared by the carbodiimide method. [b] Yield, m.p., [α]D for compound prepared by the acetic anhydride method. As a rule, about half of the starting material (carbobenzoxy-L-proline is an exception) is recovered; consequently, the yield in dipeptide ester is almost double. [c] The higher yield (over 50%) indicates that the residue at the lyophilization step contains appreciable amounts of mixed anhydride. [d] Reported m.p. 111°; B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.*, **73**, 3508 (1951). [e] Reported m.p. 166°; W. Grassmann and E. Wünsch, *Chem. Ber.*, 91, 449 (1958). [f] Recrystallized from acetone–ether. [g] Reported m.p. 104–105°, $[\alpha]^{19}D$ −28.0° ± 2° (c 1 in methanol); M. Brenner and R. W. Pfister, *Helv. Chim. Acta*, **34**, 2085 (1951). [h] *Anal.* Calcd. for $C_{32}H_{34}O_7N_2$: C, 68.80; H, 6.13; N, 5.01 Found: C, 69.00; H, 6.24; N, 5.11. [i] Reported m.p. 109–110°, $[\alpha]^{25}D$ −16.0° (c 2 in ethanol); G. W. Anderson and R. W. Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952). [j] In methanol. [k] In ethanol.

metrical anhydride of carbobenzoxyglycine[10] (m.p. 118–119°) was isolated in good yield after recrystallization of the crude product (m.p. 94–97°) from dry ether. By the action of aqueous ammonia on this crude product more than 50% of carbobenzoxyglycinamide was obtained; this is proof that the crude product was contaminated with some mixed anhydride. In the case of other carbobenzoxyamino acids, the isolation of the anhydride formed was not attempted, but the residue from the lyophilization was used for coupling with other amino acid esters. In all cases tried, chemically pure and optically homogeneous carbobenzoxydipeptide esters were isolated (Table II). The yields can be considered good, since half of the amino acid used can be recovered; they become lower, though, when the lyophilization of the acetic anhydride solution is performed after more than 24 hours.

The above experiments show that by the dissolution of carbobenzoxyamino acids in acetic anhydride, mixed or the most stable symmetrical anhydrides, or both of them, are at first formed which are not racemized under the conditions used. These anhydrides are subsequently transformed under the action of acetic anhydride very slowly to products possessing high values of optical activity. We have not yet elucidated the structure of those compounds; however, the high values of rotation indicate that the products are of cyclic structure, for instance an O-acetylated product of the formula:



R′ = carbobenzoxy or tosyl group

The case of carbobenzoxy-L-proline is quite interesting. Since acetyl-L-proline resists racemization,[11,12] it was expected that carbobenzoxy-L-proline will behave differently in acetic anhydride solution. Actually, the rotation stays practically constant after reaching a certain value (Table I). In other words, the reaction seems to stop at the formation of mixed or symmetrical anhydride.

The procedure already described is, in principle, an application of the mixed anhydride method for peptide synthesis[10,13] and constitutes, in our experience, a rather simple way for the lenghthening of the peptide chain step by step from the

(10) Th. Wieland, W. Kern and R. Sehring, *Ann.*, **569**, 117 (1950).

(11) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **99**, 143 (1932).

(12) The initial angular rotation, αD, of an acetic acid solution of acetyl-L-proline (c 5) containing 1 to 1.5 equivalents of acetic anhydride was −5.7°; after 3 days it still was −5.2° (unpublished data). Other acetyl-L-amino acids under the same conditions are fully racemized within 4 to 6 hours; ref. 3.

(13) Th. Wieland and R. Sehring, *Ann.*, **569**, 122 (1950); G. W. Kenner and R. J. Stedman, *J. Chem. Soc.*, 2069 (1952); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951).

amino end. However, the lengthening of di- or polypeptide chains deserves special attention, since carbobenzoxypeptides are expected to racemize in acetic anhydride solution like the N-acylamino acids and N-acyl peptides, as has been shown by Bergmann and Zervas.[3] An exception would be carbobenzoxy peptides with proline as the C-terminal amino acid[14] which will behave like acetyl-L-proline; as a matter of fact, such peptides do not racemize in acetic anhydride solution at room temperature and for a rather short period of time (Table I). Therefore, lyophilization of an acetic anhydride solution of carbobenzoxyglycyl-L-proline followed by coupling with glycine ethyl ester afforded the corresponding tripeptide derivative in optically pure form and in good yield.

Experimental

The melting points are not corrected. Prior to analysis[15] the compounds were dried at room temperature over phosphorus pentoxide in high vacuum.

Synthesis of Carbobenzoxy-L-alanylglycine Benzyl Ester, Carbobenzoxy-L-valylglycine Ethyl Ester, and Carbobenzoxy-L-prolyl-L-glutamic Acid Dibenzyl Ester by the Carbodiimide Method.[16]—The following procedure is typical; the carbobenzoxydipeptide esters thus prepared are listed in Table II.

Into a solution of 3.4 g. (0.01 mole) of glycine benzyl ester tosylate[17] in 30 ml. of anhydrous chloroform and 1.4 ml. of triethylamine, 2.2 g. of carbobenzoxy-L-alanine (0.01 mole) was added, followed by the addition of 2.2 g. of N,N'-dicyclohexylcarbodiimide. After standing at room temperature for about 3 hours, a few drops of acetic acid and water were added. The precipitated N,N'-dicyclohexylurea (2.2 g., m.p. above 230°) was filtered off and the filtrate was washed successively with dilute hydrochloric acid, potassium hydrogen carbonate, and water. The chloroform solution was dried over sodium sulfate, evaporated to dryness *in vacuo*, the residue was dissolved in ethanol, and re-evaporated to dryness. Upon dissolving the residue in ethyl acetate and adding petroleum ether, 2.7 g. (73%) of carbobenzoxy-L-alanylglycine benzyl ester was obtained.

Synthesis of Carbobenzoxy-L-alanylglycine Benzyl Ester, Carbobenzoxy-L-valylglycine Ethyl Ester, Carbobenzoxy-L-methionyl-L-methionine Methyl Ester, Carbobenzoxy-L-prolyl-L-glutamic Acid Dibenzyl Ester and Carbobenzoxy-L-phenylalanylglycine Ethyl Ester by the Acetic Anhydride Method.—The following procedure is typical; the carbobenzoxydipeptide esters thus prepared are listed in Table II.

Into 10 ml. of acetic anhydride, 2.2 g. (0.01 mole) of carbobenzoxy-L-alanine was added. It was dissolved within a few minutes. The solution was kept for 1 hour at room temperature. It was mixed with dry benzene and lyophilized. Complete removal of the acetic anhydride was ensured by repeated addition (three times) of benzene and re-lyophilization. The residue was dissolved in 10 ml. of anhydrous chloroform, and it was added all at once into a precooled (0–5°) solution of 3.4 g. (0.01 mole) of glycine benzyl ester tosylate and 4.2 ml. of anhydrous triethylamine in 10 ml. of anhydrous chloroform. The mixture was kept for 10 minutes at the ice-bath temperature, and afterward for about 3 hours at room temperature. The chloroform solution was washed successively with water, dilute potassium hydrogen carbonate, dilute hydrochloric acid, water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. Complete removal of chloroform was ensured by the addition of a few ml. of alcohol and repetition of the evaporation *in vacuo*. Upon dissolving the residue in ethyl acetate and adding petroleum ether 1.4 g. (40%) of carbobenzoxy-L-alanylglycine benzyl ester was obtained.

The combined potassium hydrogen carbonate layers were acidified with 5 *N* hydrochloric acid. The carbobenzoxy-L-alanine recovered was 0.8 g., m.p. 83–84°, $[\alpha]^{24}D$ −14.6° (*c* 9 in glacial acetic acid); reported[7] m.p. 84° and $[\alpha]^{17}D$ −14.3° (*c* 9 in glacial acetic acid).

Symmetrical Carbobenzoxyglycine Anhydride.—Carbobenzoxyglycine (4.2 g., 0.02 mole) was dissolved in 20 ml. of acetic anhydride after shaking at room temperature for 1 hour, and lyophilized after addition of benzene, as described above. The solid residue (approximately 4.5 g.) melted at about 94–97°, and consisted mostly from the symmetrical carbobenzoxyglycine anhydride mixed, probably with the mixed anhydride of carbobenzoxyglycine and acetic acid. By dissolving the above crude product in absolute ether and *in vacuo* concentrating the filtrate to a small volume, the symmetrical anhydride of carbobenzoxyglycine was obtained in good yield, m.p. 118–119°, reported[10] m.p. 118°.

Anal. Calcd. for $C_{20}H_{20}O_7N_2$: N, 6.99. Found: N, 7.16.

Upon adding the above crude product into aqueous ammonia precooled at 0°, the water-insoluble carbobenzoxyglycinamide precipitated out. The yield was 2.3 g., m.p. 135–136°, reported[18] m.p. 138–139° for the recrystallized product.

Glycyl-L-prolylglycine Ethyl Ester Hydrochloride.—A suspension of 3.1 g. (0.01 mole) of carbobenzoxyglycyl-L-proline[19] in 15 ml. of acetic anhydride and 15 ml. of dioxane was shaken at room temperature until the substance was dissolved (about 4 hours). The solution was mixed with dry benzene and lyophilized as described above. After complete removal of the acetic anhydride, the residue was dissolved in 20 ml. of anhydrous chloroform, and it was coupled with glycine ethyl ester hydrochloride (1.4 g., 0.01 mole) in the presence of 4.2 ml. of triethylamine. The mixture was worked up the way described for the preparation of the carbobenzoxydipeptide esters. The amount of carbobenzoxyglycyl-L-proline recovered was 1.5 g. (m.p. 156°, $[\alpha]^{18}D$ −91.5°, *c* 2 in glacial acetic acid; reported[19] m.p. 156°, $[\alpha]^{18}D$ −89.2°, *c* 2 in glacial acetic acid). The amount of the oily carbobenzoxytripeptide ester obtained was 2 g., or 50% assuming that the sirup was pure carbobenzoxytripeptide ester; taking into account the amount of carbobenzoxyglycyl-L-proline recovered, the yield reaches 93%.

The oily carbobenzoxyglycyl-L-prolyl glycine ethyl ester (2 g.) was dissolved in 40 ml. of ethanol and 1 ml. of concd. hydrochloric acid, and the solution was hydrogenized in the presence of palladium black catalyst. As soon as the hydrogenolysis had been completed, the mixture was carefully heated, filtered, and the catalyst was washed with hot alcohol. The combined filtrates were concentrated to dryness *in vacuo*. The crystalline residue was mixed with ether, and it was filtered off affording 1.2 g. of glycyl-L-prolylglycine ethyl ester hydrochloride, m.p. 210–212° dec., and $[\alpha]^{17}D$ −105.9° (*c* 1.52 in water). After recrystallization from ethanol the m.p. was 216–217° dec., $[\alpha]^{20}D$ −106.5°; reported[20] m.p. 214° dec., $[\alpha]^{25}D$ −104.0° (*c* 1.52 in water).

(14) Carbobenzoxypeptides with glycine as the C-terminal amino acid are also not exposed to racemization for obvious reasons.

(15) Microanalyses were carried out by Mr. H. Mantzos in the Analytical Laboratory of the Royal Hellenic Research Foundation.

(16) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

(17) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

(18) J. Fruton, R. Johnston and M. Fried, *J. Biol. Chem.*, **190**, 39 (1951).

(19) M. Bergmann, L. Zervas, H. Schleich and F. Leinert, *Z. physiol. Chem. Hoppe-Seyler's*, **212**, 72 (1932). The specific rotation of the substance was found to be $[\alpha]^{18}D$ −89.2° (*c* 2 in glacial acetic acid).

(20) H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, 3642 (1956).