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t-Amyloxycarbonyl as a New Protecting Group in Peptide Synthesis. III. An Unexpected Side-reaction during the Synthesis of *t*-Amyloxycarbonylamino Acids

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Formation of unexpected by-products was observed when *t*-amyloxycarbonyl-(AOC) amino acid esters were prepared according to the previously published method from some specific amino acid esters using AOC-chloride as the reagent. Further, it was observed that the weaker the basicity of the amino acid ester used, the more by-product was formed under the same conditions. Analyses of the infrared and NMR spectra of the by-products revealed their structures to be ureid-type compounds of the respective amino acid esters. Use of excess AOC-chloride together with triethylamine was found to be the main cause of the ureid-formation, and the side-reaction could be almost entirely suppressed by the use of *N, N*-diethylglycine ethyl ester in place of triethylamine.

In the previous papers,[1-3] it was shown that the *t*-amyloxycarbonyl (AOC) protective group has similar functions to the *t*-butyloxycarbonyl group, and that the synthesis of the AOC-amino acids is much easier than that of the *t*-butyloxycarbonyl-amino acids. A number of AOC-amino acids have been prepared from their respective amino acid esters by the original method[1] using AOC-chloride as the reagent and triethylamine as the required base. However, formation of unexpected by-products was observed in a few specific cases. For instance, when an oily reaction product from L-phenylalanine benzyl ester was treated with ether, some crystals appeared gradually in an yield of 32%, and ether-soluble material, which mainly consisted of AOC-L-phenylalanine benzyl ester, was obtained in less than 70% yield (Table 1).

The former compound was sparingly soluble in the usual organic solvents and showed no color reaction with ninhydrin after treatment with hydrogen chloride or bromide under the normal conditions. On the other hand, only about 6% yield of a by-product was obtained when L-phenylalanine methyl ester was subjected to the same reaction, and the yield of the desired product was about 88% (Table 1). Infrared spectra of these by-products showed absorption bands at 3370, 1720 and 1630 cm^{-1}, which correspond to N–H, ester-carbonyl C=O and amide-carbonyl C=O stretching bands, respectively. Figure 1a shows an NMR spectrum of a by-product which was obtained from L-phenylalanine methyl ester, and Fig. 1b is that of another by-product obtained from the benzyl ester. The former indicates clearly the presence of aromatic hydrogens (7—7.4 ppm, multiplet), amide hydrogen (5.47 ppm, doublet), α-carbon hydrogen (4.80 ppm, sextet), methyl hydrogens (3.60 ppm, singlet) and methylene hydrogens (3.0 ppm, doublet) in the molecule; the

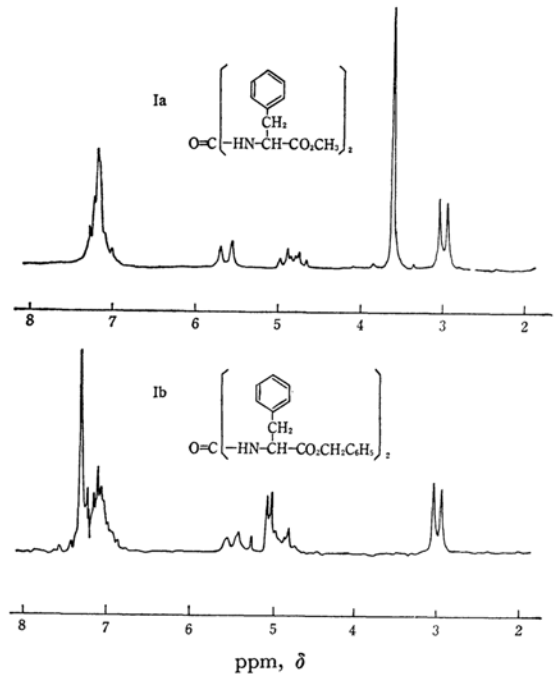

Fig. 1. NMR spectra of ureids in $CDCl_3$ at 60 Mc.

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1) S. Sakakibara, M. Shin, M. Fujino, Y. Shimonishi, S. Inouye and N. Inukai, This Bulletin, **38**, 1522 (1965).
2) S. Sakakibara and M. Fujino, *ibid.*, **39**, 947 (1966).
3) S. Sakakibara and N. Inukai, *ibid.*, **39**, 1567 (1966).

TABLE 1. FORMATION OF BY-PRODUCTS DURING THE SYNTHESIS OF AOC-AMINO ACID ESTERS FROM VARIOUS AMINO ACID ESTERS WITH AOC-CHLORIDE AND TRIETHYLAMINE AS THE REAGENTS

Amino acid ester hydrochloride or tosylate[a]	AOC-chloride eq.	Triethyl-amine eq.	Yield of AOC-amino acid ester[b] %	Yield of by-product %	pK_a'[c]	pK_a''[d]
Ileu-OBzl·TosOH	1	1.4	100	0	7.70	7.30
Val-OBzl·TosOH	1	1.4	100	0	7.55	7.15
Try-OBzl·TosOH	1.3	1.8	93.4 (Cryst.)	Trace	—	6.95
Phe-OMe·HCl	1	1.5	88.0	6.5	7.15	6.80
Phe-OBzl·TosOH	1	1.5	70.4	31.7	6.95	6.75
Glu(OMe)ONBzl·HBr	1.4	1.5	64.2	24.4	6.95	6.60
Glu(OBzl)OBzl·TosOH	1.3	1.8	50.9	48.4	—	6.70
Asp(OMe)ONBzl·HBr	1	1.4	74.5	13.5	6.30	6.15
Asp(OMe)ONBzl·HBr	1.8	2.1	34.2	64.4	6.30	6.15

a) One eq. of triethylamine was added previously to get free base.
b) Crude yield before purification.
c) Determined in water as described in the experimental part.
d) Determined in a mixture of water and methanol as described in the experimental part.

TABLE 2. MELTING POINTS AND ANALYTICAL DATA OF UREID-COMPOUNDS DERIVED FROM AMINO ACID ESTERS

Structure of ureid	Recrystallization from	Mp, °C	Molecular formula		Anal. % C	H	N
OC[Phe-OBZL]$_2$	EtOH	154.5—155	$C_{33}H_{32}O_5N_2$	Calcd	73.86	6.01	5.22
				Found	73.74	5.93	5.25
OC[Phe-OMe]$_2$	MeOH-H_2O	159.5—160.5	$C_{21}H_{24}O_5N_2$	Calcd	65.61	6.29	7.29
				Found	65.34	6.09	7.07
OC[Glu(OBZL)-OBZL]$_2$	EtOH	112—112.5	$C_{39}H_{40}O_9N_2$	Calcd	68.81	5.92	4.12
				Found	68.62	5.92	4.00
OC[Glu(OMe)ONB]$_2$	$CHCl_3$-EtOH	175—176.5	$C_{27}H_{30}O_{13}N_4$	Calcd	52.43	4.89	9.06
				Found	52.11	4.90	9.07
OC[Asp(OMe)-ONB]$_2$	DMF-$CHCl_3$	200.5—202	$C_{25}H_{26}O_{13}N_4$	Calcd	50.85	4.44	9.49
				Found	50.89	4.41	9.30
OC[Lys(Z)-OMe]$_2$	AcOEt	132.5—133.5	$C_{31}H_{42}O_9N_4$	Calcd	60.57	6.89	9.12
				Found	60.56	6.88	9.16

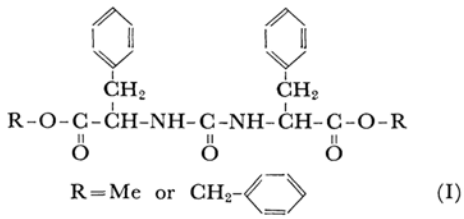
Z: Carbobenzoxy NB: *p*-Nitrobenzyl ester

TABLE 3. INTRODUCTION OF THE AOC-GROUP INTO L-ASPARTIC ACID α-*p*-NITROBENZYL β-METHYL ESTERS WITH AOC-CHLORIDE AND VARIOUS BASES[a]

Base	pK_a	AOC-chloride used eq.	Crude yield of desired product (oil) %	Yield of ureid %
Triethylamine	10.65[b]	1	74.5	13.5
Triethylamine	10.65[b]	1.67	34.2	64.4
DEG	7.90[c]	1.33	97.4 (86.0)[f]	2.5
DEG	7.90[c]	1.67	101.0 (87.1)[f]	5.0
Pyridine	5.17[d]	1	48.0	Trace
N,*N*-Dimethylaniline	5.06[e]	1	25.2	Trace

a) The starting material, α-*p*-nitrobenzyl β-methyl L-aspartate hydrobromide, was neutralized with an eq. amount of triethylamine before each reaction. Each reaction mixture was kept alkaline by respective base in slight excess.
b) J. Hanson, *Svensk Kem. Tidskrift*, **67**, 256 (1955).
c) pK_a value was determined in water as described in the experimental part.
d) H. C. Bown and X. R. Mihm, *J. Am. Chem. Soc.*, **78**, 1723 (1956).
e) N. F. Hall and M. R. Sprinkle, *ibid.*, **54**, 3469 (1932).
f) Yield of purified material by crystallization.
DEG: *N*,*N*-Diethylglycine ethyl ester.

latter spectrum also indicates the additional presence of a benzyl ester group (6.9—7, 5 ppm, multiplet due to the aromatic protons and 5.0 ppm, quartet due to the methylene protons) instead of a methyl ester group. These results all strongly support the presence of ester groups and amide bonds in the respective molecules. Finally, elemental analyses of these by-products indicated the structure to be that of ureids (I) derived from respective amino acid esters.



R=Me or CH_2- (I)

The same type of by-products were also obtained from several amino acid esters as can be seen in Table 1, and the values of their elemental analyses were all identical with their calculated values from the ureid structure (Table 2).

Since only certain specific amino acid esters gave ureid-compounds with variable yields, the ureid formation could not be attributed to the presence of phosgene which might be expected in the AOC-chloride reagent used; for example, only a trace of ureid was detected in the reaction mixture of L-tryptophan benzyl ester. In this case, all possible products were easily detectable by thin-layer chromatography using Ehrlich's reagent, and this was advantageous for checking ureid formation. Treatment of cyclohexylamine with AOC-chloride in chloroform also gave only a trace of dicyclohexylurea, which is well-known as a final reaction product from dicyclohexylcarbodiimide and is easily detectable by crystallization.

The amount of the by-product formed was likely to increase with the amount of AOC-chloride and triethylamine used (Table 1). This fact suggested that once formed AOC-amino acid ester can be converted to ureid in the presence of excess reagents. This was tested by treating once-purified AOC-L-aspartic acid α-*p*-nitrobenzyl β-methyl esters in chloroform with the following reagents; (1) AOC-chloride (1 eq.). (2) Triethylamine (1/2 eq.). (3) AOC-chloride (1 eq.) and triethylamine (1/2 eq.). (4) Carbobenzoxy chloride (1 eq.) and triethylamine (1/2 eq.). (5) Acetyl chloride (1 eq.) and triethylamine (1/2 eq.). These experiments clearly indicated that no ureid formation was observed with reagents (2), (4) and (5), only a trace of ureid was detected after treatment with (1), and a considerable amount (about 25% yield) of ureid was obtained by treatment with (3). This result can be interpreted to indicate that AOC-chloride itself is not a main cause of the ureid formation, and that the presence of triethylamine is indispensable for the ureid formation in concert with

TABLE 4. INTRODUCTION OF THE AOC-GROUP INTO DIBENZYL L-GLUTAMATE WITH AOC-CHLORIDE AND VARIOUS BASES[a]

Base	pK_a	AOC-chloride used eq.	Yield of desired product %	Yield of ureid %
Triethylamine	10.65	1.25	50.9[b] (43.0)	48.4
DEG	7.90[d]	1	80.2 (65.6)	27.9
DEG[c]	7.90[d]	1	80.0 (67.8)	0
N-Ethylmorpholine	7.90[d,e]	1	56.5	41.8

a) See Ref. a in Table 3.
b) Yield of oily product before purification. Yields of the crystallized product are in parentheses.
c) The starting material, dibenzyl L-glutamate tosylate, was also neutralized with DEG in this case, and no triethylamine was used throughout the reaction.
d) The pK_a' value was determined in water as described in the experimental part.
e) Cited: pK_a 7.70; H. K. Hall, Jr., *J. Phys. Chem.*, **60**, 63 (1956).

TABLE 5. PHYSICAL CONSTANTS AND ANALYTICAL DATA OF SOME AOC-AMINO ACID ESTERS NEWLY OBTAINED AS CRYSTALS

AOC-Derivative of	Recrystallized from	Mp, °C	$[\alpha]_D$ in ethanol (°C, concn.)	Molecular formula		Anal., % C	H	N
L-Glutamic acid dibenzyl esters	AcOEt - Pet. ether	56—58	−18.6 (24, 2.043)	$C_{25}H_{31}O_6N$	Calcd	68.00	7.08	3.17
					Found	68.21	7.05	3.15
L-Aspartic acid α-*p*-nitrobenzyl β-methyl esters	Ether - Pet. ether	48—50	−19.1 (26, 2.0)	$C_{18}H_{24}O_8N_2$	Calcd	54.54	6.10	7.07
					Found	54.67	6.02	7.08
L-Tryptophan benzyl ester	AcOEt - Pet. ether	90.5—93	− 2.8 (24, 2.3)	$C_{24}H_{28}O_4N_2$	Calcd	70.56	6.91	6.86
					Found	70.00	6.94	6.69

AOC-chloride. Elucidation of the mechanism of the new ureid-forming reaction was, however, found to be complicated, and still remains unsolved.

As can be seen in Table 1, there should be some relationship between the yield of ureid and the pK_a' value of the amino acid ester used; this seems to indicate that the weaker the basicity of the amino acid ester, the more ureid was formed under the same conditions. From the above experiments and considerations, it was supposed that the basicity of the tertiary base used for the acylating reaction should mainly affect ureid formation. This was tested, one step at a time, using AOC-chloride and L-aspartic acid α-*p*-nitrobenzyl β-methyl esters as the starting materials together with several tertiary bases possessing various pK_a values; the results are listed in Table 3. This experiment revealed that a mild base, *N*, *N*-diethylglycine ethyl ester[4] (DEG) was superior for suppressing ureid formation. Although pyridine or dimethylaniline gave no ureid, these compounds are too weak in basicity to give a satisfactory yield of the desired compound. The same results were obtained with dibenzyl L-glutamate, which produced the highest yield of ureid among the many amino acid esters so far tested with triethylamine. The superiority of DEG was also confirmed, in this case, as shown in Table 4. Although the pK_a' value of *N*-ethylmorpholine is the same as that of DEG, *N*-ethylmorpholine behaved like triethylamine and was not replaceable by DEG. Ureid formation from dibenzyl L-glutamate hydrochloride was found to depend on the conditions of neutralization of the ester hydrochloride; when triethylamine was used for liberation of the free ester from the hydrochloride, the yield of ureid was up to 28% even if DEG was used as the required base for the acylation reaction; but, when DEG was used not only for liberation of the free ester but also for the required base during the acylation reaction, no ureid formation was observed and the yield of the desired compound was satisfactory. On the other hand, there was no trouble in the AOC-reaction with α-*p*-nitrobenzyl β-methyl L-aspartate hydrobromide when DEG was used for the reaction, regardless of whether the hydrobromide was neutralized with triethylamine or with DEG. Table 5 presents data on AOC-amino acid esters which were obtained as crystals during this study.

The mild base DEG shows apparent pK_a values of 7.90 in water and 7.40 in methanolic water, which are close to those of the usual amino acid esters (Table 1); therefore, it is suggested that this mild base may be useful in general peptide synthesis for minimizing racemization during the reaction, since racemization may be due mainly to triethylamine whose basicity (pK_a 10.65) is greater than necessary.

4) R. Willstätter, *Chem. Ber.*, **35**, 584 (1902).

Experimental

AOC-chloride Reagent. This reagent was prepared by the previous method.[1] Six milliliters of the reagent was found to be able to acylate 0.01 mol of amino acid esters.

***N*, *N*-Diethylglycine Ethyl Ester.** This material was prepared following the description of Willstätter.[4] Ethyl monochloroacetate (200 g, 1.63 mol) was added dropwise into a solution of diethylamine (238 g, 3.26 mol) in ether (120 m*l*) below 10°C with stirring. After being kept overnight at room temperature, the reaction mixture was diluted with ether (300 m*l*), and the formed crystals were filtered out. The crystals were dissolved in a minimum volume of water, and the solution was extracted with ether. The ether extract was combined with the above ether filtrate, and the combined solution was washed with water (100 m*l*). Then, the ether solution was carefully treated with 20% sulfuric acid (about 250 m*l*) with cooling to take up the product into the water phase. Then, the product was re-extracted from the sulfate solution with ether (400 m*l*), after adding 20% sodium hydroxide solution (400 m*l*); the ether extract was washed with sodium chloride-saturated water, and then dried over anhydrous magnesium sulfate. Distillation out of ether left an oily product (175 g) which was purified by distillation under reduced pressure; bp 82—85°C/27 mmHg, wt 161 g (62%).

AOC-amino Acid Esters. The salt of the amino acid ester (0.02 mol) was suspended in chloroform or (50—100 m*l* and triethylamine (2.8 m*l*, 0.02 mol) DEG (3.5 m*l*), 0.02 mol) was added to the suspension with cooling. If no clear solution was obtained in this stage, addition of dimethylformamide (about 10 m*l*) was effective for obtaining complete dissolution. Into the mixture, AOC-chloride reagent (0.02 mol) and triethylamine or DEG (0.02—0.025 mol) were added alternately with stirring at −5°C unitl ninhydrin-positive material disappeared from the reaction mixture. After additional stirring for 30 min, the reaction mixture was washed with water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated to a syrup, which was dissolved in ether to separate ureid, if present as crystals. (Table 2). The ether solution was washed with 0.5 N hydrochloric acid and water, and dried over magnesium sulfate. Removal of ether left the product as an oil which was crystallized gradually from suitable solvent systems. (Tables 1, 3, 4 and 5).

Determination of Apparent pK_a Values. Each sample (0.00015 mol) was dissolved in 0.1 M sodium chloride solution (20.0 m*l*), in which N hydrochloric acid (0.20 m*l*) was added, and the mixture was titrated with N-sodium hydroxide solution using a Radiometer titrator. The pK_a' value was calculated from the titration curve by the method of Parke and Davis.[5] Since some samples were hard to dissolve in the above solution, methanol (10.0 m*l*) was added to the mixture to get a clear solution, and titration was carried out in the same manner. In this case, the observed pK_a value was designated as pK_a''. (Table 1).

5) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).