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t-Amyloxycarbonyl as a New Protecting Group in Peptide Synthesis. IV. Synthesis and Use of *t*-Amyl Azidoformate

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t-Amyl carbazate (II) was synthesized in a satisfactory yield by the direct hydrazinolysis of *t*-amyl chloroformate (I). Reagent I was prepared by an improved method, using a stock solution of phosgene in toluene. Reagent II was converted to *t*-amyl azidoformate (III), as in the case of the *t*-butyl derivative, and reagent III was used for introducing the *t*-amyloxycarbonyl (AOC) group into free amino acids, amino acid esters, and peptides. Various new AOC-amino acids were synthesized by the present method. Chloroform was found to inhibit the reaction of reagent III with amino acid esters. A practical and safe method for obtaining large quantities of anhydrous hydrazine is described in the present paper.

Although *t*-amyl chloroformate is a useful reagent for introducing the *t*-amyloxycarbonyl (AOC) group into amino acids, the reaction has to be carried out under anhydrous conditions because of the water-sensitivity of the reagent, as has been shown previously.[1] Therefore, the AOC-introducing reaction is generally performed in chloroform, using esters of the amino acids in the presence of tertiary amine; the resulting AOC-amino acid esters are used for peptide synthesis after having been converted to the free acids by the saponification or hydrogenolysis of the ester group. This procedure, however, is not suitable for the preparation of the monoesters of AOC-glutamic acid and aspartic acid, and is not practical for the synthesis of the AOC-derivatives of glutamine, asparagine, serine, threonine, and N^G-nitroarginine. For the preparation of those complicated AOC-amino acids, it seems that *t*-amyl azidoformate will be a convenient reagent; the present paper will describe a practical method of synthesizing reagent III from the chloroformate (I).

$$t\text{-Amyl-O-CO-Cl} + NH_2\text{-}NH_2 \rightarrow \underset{[I]}{}$$

$$\underset{[II]}{t\text{-Amyl-O-CO-NH-NH}_2}$$

$$II \xrightarrow[\text{in AcOH}]{NaNO_2} t\text{-Amyl-O-CO-N}_3 \qquad [III]$$

Recently, Ovchinnikov *et al.*[2] described a direct method for synthesizing *t*-butyl carbazate from the chloroformate; in this method freshly-prepared *t*-butyl chloroformate is coupled with anhydrous hydrazine in dry ether at −60°C. In the present study, the reaction conditions of Ovchinnikov *et al.* were followed exactly in the reaction of *t*-amyl chloroformate (I) with anhydrous hydrazine, but it was observed that anhydrous hydrazine separated out as a solid at below 0°C, and the yield of *t*-amyl carbazate (II) was very low. After several tests, it was found that the reaction temperature should be kept at 0—2°C in order to keep the hydrazine in the liquid state during the reaction, and that vigorous stirring was indispensable. These improved conditions afforded an over-all yield of about 65% of the desired product from *t*-amyl alcohol; this yield compares favorably with the yield of 33% reported for *t*-butyl carbazate.[2]

The preparation of *t*-amyl chloroformate (I) was also improved during these studies. Thus, phosgene was stored as a solution in toluene, and the necessary amount of phosgene for each reaction was determined simply as the volume of the stock solution required. Otherwise the reaction was carried out as has been described in the first paper.[1] Thus, after the concentration of the reaction mixture, *t*-amyl chloroformate (I) was obtained as a toluene solution; this solvent was found to be favorable for storage of the reagent.*1 The anhydrous hydrazine which was used for the reaction was obtained without danger of explosion following a procedure of Kusama;[3] a modification of the apparatus of Deen and Stark, using a bigger receiver, was used to collect large quantities of hydrazine by azeotropic distillation with toluene.

t-Amyl azidoformate (III) was obtained from the *t*-amyl carbazate (II) as in the case of *t*-butyl azidoformate synthesis; the yield was as good as

1) S. Sakakibara, M. Shin, M. Fujino, Y. Shimonishi, S. Inouye and N. Inukai, This Bulletin, **38**, 1522 (1965).
2) Y. A. Ovchinnikov, A. A. Kiryushkin and A. I. Miroshinikov, *Experientia*, **21**, 418 (1965).

*1 A solution of the reagent prepared by this improved procedure was kept in a deep freezer for more than one month with a loss of less than 10% of the effective compound.

3) K. Kusama, *J. Biochem.*, **44**, 375 (1957).

TABLE 1. YIELDS AND PROPERTIES OF *N*-*t*-AMYLOXYCARBONYLAMINO ACIDS SYNTHESIZED BY THE GENERAL PROCEDURE WITH *t*-AMYL AZIDOFORMATE

Compound	Recryst. solvent	Yield %	Mp °C	$[\alpha]_D$ (*c* in ethanol)	Temp. °C
AOC-glycine	Ethyl acetate	72.5	84—85.5		
	Pet. ether		84—85.5[a]		
			82.5—84[b]		
AOC-L-leucine[c]	Ethyl acetate	75.6	67—67.5	−17.0 (2.0)	27
	Pet. ether		66—68[a]	−17.3 (2.0)	20
			61—63[b]	−18.6 (1.9)	21
AOC-L-Proline	Ethyl acetate	83.0	99—99.5	−47.6 (1.0)	20
	Pet. ether		98—99[a]	−47.6 (2.0)	20
			94—94.5[b]	−47.2 (1.7)	21
AOC-L-tryptophane	Ethyl acetate	74.0	126—127	+7.1 (1.2)	21
	Pet. ether		124—125[a]	+7.2 (2.0)	21
			121—123[b]	+7.1 (1.2)	21
AOC-L-alanine DCHA	Ether	85.1	126—129	+3.7 (4.4)	24
	Pet. ether		126.5—128.5[a]	+4.3 (2.0)	20
			124—126[b]	+4.7 (2.4)	22
AOC-L-isoleucine DCHA	Ether	56.8	111—112	+2.4 (1.6)	24
	Pet. ether		110—112[a]	+4.1 (2.0)	20
			101.5—102[b]	+3.9 (1.6)	22
AOC-L-methionine DCHA	Ethyl acetate	83.0	112—112.5	+17.0 (3.1)	22
	Pet. ether		112—112.5[a]	+17.2 (3.0)	21
			105—107[b]	+17.4 (1.3)	21
AOC-L-phenylalanine DCHA	Ethanol	94.8	208.5—210.5	+40.3 (0.67)	24
	Ethyl acetate		209—211[a]	+40.6 (0.67)	20
			198—199[b]	+27.9 (0.79)	21
AOC-L-serine DCHA[d]	Ethyl acetate	87.5	98—100	+13.5 (2.1)	21
	Pet. ether				
AOC-*O*-Benzyl-L-serine DCHA[e]	Ether	85.2	121—122	+26.6 (2.1)	21
	Pet. ether				
AOC-L-threonine DCHA[f]	Ethyl acetate	79.3	110—112	+12.0 (2.8)	21
	Pet. ether				
AOC-L-valine DCHA	Ether	80.0	123—124	+0.9 (4.1)	22
	Pet. ether		122—123[a]	+2.1 (2.0)	20
			118—121.5[b]	+2.0 (1.7)	21

a) Improved value obtained by the *t*-amyl chloroformate method.[1]
b) Value from Ref. 1.
c) Obtained as crystals of the monohydrate and optical rotation was calculated for the hydrate.
d) Found: C, 62.39; H, 9.99; N, 6.59%. Calcd for $C_{21}H_{40}O_5N_2$: C, 62.97; H, 10.07; N, 6.99%.
e) Found: C, 68.97; H, 9.33; N, 5.60%. Calcd for $C_{28}H_{46}O_5N_2$: C, 68.54; H, 9.45; N, 5.71%.
f) Found: C, 63.72; H, 10.06; N, 6.62%. Calcd for $C_{22}H_{42}O_5N_2$: C, 63.73; H, 10.21; N, 6.76%.
DCHA: Dicyclohexylamine salt.

that reported for the *t*-butyl derivative.[4] In more than twenty runs, there was no explosion during the distillation of the *t*-amyl azidoformate.

The *t*-amyl azidoformate thus obtained was used as a reagent for introducing the AOC-group into amino acids directly, and many AOC-amino acids were synthesized smoothly. Although the use of various bases has been reported for the preparation of BOC-amino acids with *t*-butyl azidoformate, we found triethylamine to give generally the best results with *t*-amyl azidoformate (III). Because of their poor solubility, nitroarginine and *O*-benzyltyrosine were dissolved in aqueous dioxane with the

4) L. A. Carpino, C. A. Giza and B. A. Carpino, *J. Am. Chem. Soc.*, **81**, 955 (1959).

aid of sodium hydroxide before being submitted to the reaction. The AOC-amino acids which were previously synthesized with *t*-amyl chloroformate were also synthesized by the azide procedure, and the physical constants of materials prepared by the two procedures were compared (see Table 1). Although some data published previously[1] were inferior to those obtained by the present method, the differences were not due to the difference in methods, but to a difference in the recrystallization technique, which was gradually improved during repeated preparations. The data for the improved chloroformate procedure are also presented in the table.

During the reactions of *t*-amyl azidoformate (III) with the glycine ethyl ester or the phenylalanine methyl ester, the interesting phenomenon was observed that this reaction proceeds freely in usual organic solvents such as dioxane, tetrahydrofuran, dimethylformamide, ethanol, acetonitrile, methylene chloride, and ethyl acetate, but that chloroform inhibits the reaction strongly. This may be the main reason why *t*-butyl azidoformate has not been used for the acylation of amino acid esters or peptide esters. The mechanism of the inhibition reaction has not yet been elucidated.

Experimental

t-Amyl Chloroformate (I). A solution of phosgene (1200 m*l*, 4.0 mol) in toluene was mixed with a solution of *t*-amyl alcohol (176 g, 2.00 mol) in dry ether (*ca.* 1500 m*l*), and into the mixture, a solution of pyridine (182 g, 2.30 mol) in dry ether (*ca.* 800 m*l*) was vigorously stirred, drop by drop, at about −50—−60°C. The reaction mixture was allowed to stand overnight in a deep freezer at −20°C. The pyridine hydrochloride which formed was filtered off, and the filtrate was concentrated to about 1200 m*l* under reduced pressure at below 0°C. This solution was stored in a deep freezer at −20°C, and was used in following reactions without further purification.

Anhydrous Hydrazine. Calcium oxide (2 kg, 35 mol) and toluene (2 *l*) were placed in a two-necked flask (capacity: 5 *l*) equipped with a dropping funnel and a reflux condenser, and the top of the condenser was sealed with a sodium hydroxide tube.

The flask was heated gently with a heating mantle, and then 90% hydrazine hydrate (1 kg; containing about 18 mol of hydrazine and 24 mol of water) was added slowly over a 3 hr period. During the addition, the toluene gradually began to boil. After the addition of the hydrazine, the mixture was refluxed for another 10 hr, and then the reflux condenser was replaced by a modification of the apparatus of Dean and Stark, using a bigger receiver than normal. The anhydrous hydrazine which had accumulated in the receiver was collected through a bottom cock; the amount of anhydrous hydrazine recovered was about 75% of the theoretical value.*2

t-Amyl Carbazate (II). Reagent I (1200 m*l*) was vigorously stirred, drop by drop, into a suspension of anhydrous hydrazine (192 g, 6.0 mol) in dry ether (500 m*l*) at 0—2°C. After the addition of I, stirring was continued for about 1 hr at room temperature, and then water (*ca.* 500 m*l*) was added to the reaction mixture. Using a continuous extraction apparatus, the product was extracted with ether for about ten hours. The ether layer was then dried over anhydrous sodium sulfate, and the dried solution was concentrated to a small volume at 20°C under reduced pressure (20 mmHg). When the product was purified by fractional distillation, a fraction with bp 85—86°C/4—5 mmHg was collected; yield, 190 g (65.0%, the theoretical yield from *t*-amyl alcohol). Found: C, 49.23; H, 9.81; N, 18.75%. Calcd for $C_6H_{14}O_2N_2$: C, 49.30; H, 9.65; N, 19.17%.

t-Amyl Azidoformate (III). A saturated solution of sodium nitrite (22.8 g, 0.33 mol) in water was stirred, drop by drop, into a solution of II (43.8 g, 0.30 mol) in 60% aqueous acetic acid (250 m*l*) at below 0°C. After stirring had been continued for another 30 min at room temperature, the reaction mixture was covered with ether and sodium bicarbonate (*ca.* 240 g) was added slowly. The insoluble material which formed was removed by filtration, and the residue was washed thoroughly with ether. The lower layer of the filtrate was extracted twice with fresh ether, and the extracts were combined with the ether layer of the filtrate. After drying over anhydrous sodium sulfate, the combined solution was concentrated to a small volume at 100 mmHg on a water bath at room temperature, and the residual oil was purified by fractional distillation; yield, 42.4 g (90%); bp 75—78°C/55—60 mmHg. Boiling point of this material rised to 81.5—82.0°C/51—53 mmHg on refractionation. Found: C, 45.50; H, 7.30%. Calcd for $C_6H_{11}O_2N_3$: C, 45.85; H, 7.05%.

General Procedure for the Synthesis of AOC-amino Acids. A mixture of amino acid (0.01 mol), reagent III (about 0.015 mol), and triethylamine (0.025 mol) in 50% aqueous dioxane (40 m*l*) was allowed to react for about 24 hr at 35°C with stirring. The reaction mixture was then neutralized to pH 7 with N hydrochloric acid, and the dioxane was removed by distillation under reduced pressure. The residual solution was acidified to pH 2 with N hydrochloric acid, and the product was extracted with ethyl acetate. The extract was washed with water, dried over anhydrous sodium sulfate, and concentrated to a residue, which was then crystallized from a suitable solvent system. If the product did not crystallize at this stage, the residue was neutralized with a calculated amount of dicyclohexylamine in ether, and the AOC-amino acid was crystallized as the dicyclohexylamine salt.

AOC-derivatives of the following amino acids were synthesized by this general procedure (their physical constants are listed in Table 1): glycine, L-leucine, L-proline, L-tryptophan, L-alanine, L-isoleucine, L-methionine, L-phenylalanine, L-valine, L-serine, L-threonine, and *O*-benzyl-L-serine.

t-Amyloxycarbonyl-L-asparagine. A mixture of L-asparagine monohydrate (15.0 g, 0.10 mol), reagent III (31.4 g, 0.20 mol), and triethylamine (42.0 m*l*, 0.30 mol) in 50% aqueous dioxane (300 m*l*) was allowed

*2 Since the toluene which was recovered from the reaction mixture should be saturated with hydrazine, the yield of anhydrous hydrazine may be increased by using the same toluene in the next run.

to react for 24 hr at 35°C with stirring. Then the reaction mixture was neutralized to pH 7 with N hydrochloric acid and concentrated under reduced pressure. The oily material was acidified to pH 2—3 with N hydrochloric acid, and crystallized in an ice box. The crude crystals collected by filtration (23.8 g; mp 132—136°C) were recrystallized from ethanol - ethyl acetate; yield, 20.50 g (83.3%); mp 151.5—152.5°C; $[\alpha]_D^{28}$ −8.2° (*c* 3.4, ethanol). Found: C, 48.97; H, 7.25; N, 11.17%. Calcd for $C_{10}H_{18}O_5N_2$: C, 48.77; H, 7.37; N, 11.38%.

***t*-Amyloxycarbonyl-L-glutamine.** A mixture of L-glutamine (1.46 g, 0.01 mol), reagent III (3.14 g, 0.02 mol), and triethylamine (4.20 m*l*, 0.03 mol) in 50% aqueous dioxane (40 m*l*) was allowed to react for 24 hr at 35°C with stirring. The same procedure as that used for the synthesis of *t*-amyloxycarbonyl-L-asparagine was followed; the crude product obtained (2.17 g; mp 114.5—116.5°C) was recrystallized from ethyl acetate - ether; yield, 2.04 g (78.5%); mp 115.5—116.5°C; $[\alpha]_D^{20}$ −19.4° (*c* 4.8, dimethylformamide). Found: C, 51.01; H, 7.78; N, 11.09%. Calcd for $C_{11}H_{20}O_5N_2$: C, 50.75; H, 7.75; N, 10.76%.

***N*$^{\alpha}$-*t*-Amyloxycarbonyl-*N*^{G}-nitro-L-arginine.** A suspension of *N*^{G}-nitro-L-arginine (21.9 g, 0.10 mol) in a mixture of N sodium hydroxide (100 m*l*), reagent III (18.8 g, 0.12 mol), triethylamine (16.8 m*l*, 0.12 mol), and 50% aqueous dioxane (200 m*l*) was allowed to react for 48 hr at 35°C with stirring. The reaction mixture was then treated following the general procedure; the required compound was thus obtained as a syrup, which was triturated in ether for crystallization. The crude crystals (23.9 g) obtained melted at 63—70°C, solidified again at 100°C, and decomposed at 118°C. This material was recrystallized from ethyl acetate; yield, 20.5 g (61.6%); mp 139—141°C (decomp.); $[\alpha]_D^{18}$ −28.2° (*c* 2.0, pyridine). Found: C, 42.99; H, 7.12; N, 21.06%. Calcd for $C_{12}H_{23}O_6N_5$: C, 43.24; H, 6.96; N, 21.01%.

***N*-*t*-Amyloxycarbonyl-*O*-benzyl-L-tyrosine Dicyclohexylamine Salt.** A suspension of *O*-benzyl-L-tyrosine (5.43 g, 0.02 mol) in a mixture of N sodium hydroxide (20 m*l*), reagent III (6.28 g, 0.04 mol), triethylamine (5.60 m*l*, 0.04 mol), and 50% aqueous dioxane (80 m*l*) was allowed to react for 40 hr at 35°C with stirring. When the reaction mixture was treated following the general procedure, a crude product was obtained as the dicyclohexylamine salt; yield, 10.2 g; mp 107—113°C. This material was recrystallized from ethyl acetate - petroleum ether (60—80°C); yield, 9.80 g (86.4%); mp 113.5—115.5°C; $[\alpha]_D^{21}$ +43.1° (*c* 5.0, ethanol). Found: C, 71.96; H, 8.77; N, 4.77%. Calcd for $C_{34}H_{50}O_5N_2$: C, 72.05; H, 8.89; N, 4.94%.

***N*-*t*-Amyloxycarbonyl-L-aspartic Acid β-Benzyl Ester.** A suspension of the L-aspartic acid β-benzyl ester (4.46 g, 0.02 mol) in a mixture of reagent III (6.28 g, 0.04 mol), triethylamine (5.60 m*l*, 0.04 mol), and 50% aqueous dioxane (80 m*l*) was allowed to react for 24 hr at 35°C with stirring. When the reaction mixture was then treated following the general procedure, a crude product was obtained (4.9 g, mp 48—53°C) which was recrystallized from ethyl acetate - petroleum ether (60—80°C); yield, 4.22 g (62.6%); mp 58—60°C; $[\alpha]_D^{17}$ +2.7° (*c* 2.6, ethanol). Found: C, 60.76; H, 6.96; N, 4.31%. Calcd for $C_{17}H_{23}O_6N$: C, 60.52; H, 6.87; N, 4.15%.

***N*-*t*-Amyloxycarbonyl-L-glutamic Acid γ-Benzyl Ester Dicyclohexylamine Salt.** A suspension of the L-glutamic acid γ-benzyl ester (4.75 g, 0.02 mol) in a mixture of reagent III (6.28 g, 0.04 mol), triethylamine (5.60 m*l*, 0.04 mol), and 65% aqueous dioxane (100 m*l*) was allowed to react for 10 hr at 35°C with stirring. Triethylamine (2.80 m*l*, 0.02 mol) was then added, drop by drop, to the reaction mixture, and stirring was continued for a further 15 hr. When the reaction mixture was treated following the general procedure, a crude compound was obtained (8.5 g; mp 119—122°C). This compound was recrystallized from ethyl acetate - petroleum ether (60—80°C); yield, 8.31 g (78.0%); mp 120.5—122°C; $[\alpha]_D^{14}$ +11.2° (*c* 1.7, ethanol). Found: C, 67.66; H, 9.13; N, 5.18%. Calcd for $C_{30}H_{48}O_6N_2$: C, 67.64; H, 9.08; N, 5.26%.

Reaction of *t*-Amyl Azidoformate with Glycine Ethyl Ester Hydrochloride. A solution of glycine ethyl ester hydrochloride (1.40 g, 0.01 mol), reagent III (1.88 g, 0.012 mol), and triethylamine (3.08 m*l*, 0.022 mol) in 90% aqueous dioxane (45 m*l*) was allowed to react for 24 hr at 35°C with stirring. The reaction mixture was then concentrated to a small volume under reduced pressure, and the oily material formed was extracted with ethyl acetate. The extract was washed with 0.5 N hydrochloric acid, 5% sodium bicarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was then concentrated under reduced pressure to produce the *N*-*t*-amyloxycarbonylglycine ethyl ester as a syrup. This was saponified with N sodium hydroxide (10 m*l*) in acetone (20 m*l*) for 2 hr at room temperature. The reaction mixture was adjusted to pH 7, concentrated under reduced pressure, and then acidified to pH 2—3 with N hydrochloric acid. The product was extracted with ethyl acetate, and the organic layer was washed with water and dried over anhydrous sodium sulfate. The dried solution was concentrated under reduced pressure, and the crude product was recrystallized from ethyl acetate - petroleum ether (60—80°C); yield, 1.70 g (90.0%); mp 84—85°C. The mixture of this material with the authentic compound caused no depression of the melting point.

***N*-*t*-Amyloxycarbonylglycylglycine.** A suspension of glycylglycine (1.32 g, 0.01 mol), reagent III (3.14 g, 0.02 mol), and triethylamine (2.10 m*l*, 0.015 mol) in 50% aqueous dioxane (40 m*l*) was allowed to react for 20 hr at 35°C with stirring. The mixture was kept at pH 8—9 by the intermittent addition of triethylamine (total 1.4 m*l*, 0.01 mol) during the reaction. When the reaction mixture was then treated following the general procedure, a crude product was obtained (1.56 g; mp 89—91°C) which was recrystallized from ethyl acetate - petroleum ether (60—80°C); yield, 1.34 g (52.5%); mp 93—95°C. Found*3: C, 47.00; H, 7.36; N, 11.49%. Calcd for $C_{10}H_{18}O_5N_2 \cdot {}^1/_2H_2O$: C, 47.05; H, 7.50; N, 10.98%.

*3 This material was dried over phosphorus pentoxide *in vacuo* at room temperature for 20 hr.