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AKRON, OHIO

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

p-Nitrobenzyloxycarbonyl Derivatives of Amino Acids

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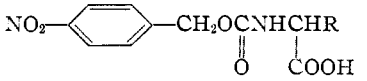
The preparation of crystalline *p*-nitrobenzyloxycarbonyl derivatives of fifteen amino acids is described.

The introduction of the use of *p*-nitrobenzyl chloroformate in the preparation of derivatives of amino acids and in peptide synthesis was reported in an earlier paper.[2] The preparation of crystalline *p*-nitrobenzyloxycarbonyl derivatives (I) of the nineteen commonly occurring amino acids has now



been completed. These derivatives include the monosubstituted derivatives of glycine, L-proline, hydroxy-L-proline, L-leucine, L- and D,L-isoleucine, previously reported,[2] D,L-methionine, D,L-valine, L-glutamic acid, D,L-aspartic acid, D,L-tryptophan, D,L-serine, D,L-threonine, D,L-phenylalanine, D,L-alanine, 3,5-diiodo-L-tyrosine and D,L-histidine and the disubstituted derivatives of L-lysine, L-cystine, D,L-tyrosine and L-arginine.

The preparation of dicarbobenzoxy-L-arginine has not yet been reported in the literature. Fruton[3] reported that he was unable to prepare dicarbobenzoxy-L-arginine by the methods which yield other disubstituted products of arginine, such as dibenzoyl-L-arginine[4] or dibenzenesulfonyl-L-arginine.[5] However, when L-arginine was treated with either one or two moles of *p*-nitrobenzyl chloroformate in the usual manner di-*p*-nitrobenzyloxycarbonyl-L-arginine was the only crystalline product isolated. Since the disubstituted derivative does not give a color in the ninhydrin reaction,[6] one of the *p*-nitrobenzyloxycarbonyl groups must be attached to the α-amino nitrogen of arginine. The other group is presumably attached to the guanido group of arginine.

Bergmann and Zervas[7] reported only a very modest yield in the preparation of carbobenzoxy-L-histidine. A yield of almost 60% was obtained in the preparation of *p*-nitrobenzyloxycarbonyl-D,L-histidine. Since this derivative gives a negative ninhydrin color test and a positive Folin color test[8] it is considered to be substituted on the α-amino group of histidine.

When the derivatives of D,L-serine, D,L-threonine and D,L-tyrosine were prepared in a strongly alkaline solution in the usual manner the yields ranged from 30–40% and a large amount of di-*p*-nitrobenzyl carbonate was found in the reaction mixture. In the cases of serine and threonine this result is believed to be due to hydrolysis of the N-*p*-nitrobenzyloxycarbonyl derivatives in the strong alkali during the course of the reaction since these derivatives were quantitatively hydrolyzed with the liberation of *p*-nitrobenzyl alcohol in less than a minute when dissolved in 4 *N* alkali at room temperature. The low yield in case of the disubstituted tyrosine derivative may have been due to the hydrolysis of the O-substituted group in the strongly alkaline solution. It was found that in the case of these three derivatives the yield could be increased several fold by performing the coupling reaction in a mixture which was buffered at *p*H 9–10.

The derivative of D,L-tryptophan was the only compound in the entire series exhibiting any appreciable visible color (a bright orange-yellow). It possessed absorption maxima at 222 and 273 mμ with molecular extinction coefficients of 32,400 and 14,100, respectively, in 95% ethanol. The derivatives of the other amino acids showed an absorption maximum at about 268 mμ with molecular extinction coefficients of 9,500 for the monosubstituted derivatives and of 19,200 for the disubstituted derivatives.

Experimental[9]

N-*p*-Nitrobenzyloxycarbonyl Derivatives.—Essentially two procedures were used in the preparation of the derivatives whose properties are described in Table I. In procedure A the reaction mixture was strongly alkaline, while in procedure B the reaction mixture was buffered to about *p*H 9–10. The derivatives of several of the amino acids, not described in Table I, were prepared by modified procedures.

Procedure A.—The amino acid was dissolved in 1.25 equivalents of 4 *N* sodium hydroxide and the resulting solution was placed in a reaction vessel so designed as to allow vigorous shaking on a mechanical shaker with simultaneous cooling in an ice-bath. The reaction vessel was constructed

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(9) All melting points were taken on the hot-stage. The analyses were performed by the Microchemical Laboratory, Department of Chemistry, University of California, Berkeley. The water analysis was by the method of Karl Fischer as modified by E. Almy, W. Griffin and C. Wilcox, *Anal. Chem.*, **12**, 392 (1940).

TABLE I

p-NITROBENZYLOXYCARBONYL DERIVATIVES OF AMINO ACIDS

N-*p*-Nitrobenzyloxycarbonyl derivative of	Proc. for prep.	M.p., °C.	Yield, %	Solvent for recryst.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Neut. equiv. Calcd.	Neut. equiv. Found
D,L-Alanine	A	132.5–134	86	Chloroform	$C_{11}H_{12}O_6N_2$	49.25	49.62[a]	4.51	4.38	268.2	268
D,L-Aspartic acid	A	150–151	53	Amyl acetate–*n*-butyl ether	$C_{12}H_{12}O_8N_2$	46.16	46.39[a]	3.88	4.05	156.1	154
L-Glutamic acid[b]	A	159–161	58	Water	$C_{13}H_{14}O_8N_2$	47.85	48.11[a]	4.41	4.42	163.1	163
D,L-Histidine	A[c]	206.5–208.5	60	Water	$C_{14}H_{14}O_6N_4$[d]	50.30	50.44[e]	4.22	4.37	334.3	330
D,L-Methionine	A	117–119	83	Amyl acetate–hexane[f]	$C_{13}H_{16}O_6N_2S$	47.55	47.50[g]	4.91	4.87	328.3	329
D,L-Phenylalanine	A	134.5–136.5	82	Diisobutyl ketone	$C_{17}H_{16}O_6N_2$	59.30	59.25[a]	4.68	4.66	344.3	346
D,L-Serine	B	140.5–142.5	85	Amyl acetate	$C_{11}H_{12}O_7N_2$	46.48	46.41[a]	4.26	4.11	284.2	284
D,L-Threonine	B	141.5–143	79	Amyl acetate–hexane	$C_{12}H_4O_7N_2$	48.32	48.24[a]	4.73	4.67	298.3	299
D,L-Tryptophan	A	151–152.5	95	Acetic acid–water	$C_{19}H_{17}O_6N_3$	59.53	59.25[a]	4.47	4.41	383.4	376
D,L-Valine	A	125.5–127.5	89	*n*-Butyl ether	$C_{13}H_{16}O_6N_2$	52.70	52.82[g]	5.44	5.31	296.3	294

[a] Dried for analysis *in vacuo* over phosphorus pentoxide at 75°. [b] $[\alpha]^{25}D$ −8.2° (*c* 2, 95% ethanol). [c] Isolated directly from the alkaline reaction mixture, after removal of the crystalline by-product, by acidification to *p*H 4–4.5. [d] Calcd.: N, 16.76. Found: N, 16.54. [e] Dried for analysis *in vacuo* over phosphorus pentoxide at 100°. [f] The temperature during recrystallization was not allowed to rise above 85° because of the formation of an insoluble product at 100°. [g] Dried for analysis *in vacuo* over phosphorus pentoxide at 56°.

from a large size standard taper outer joint which was sealed at one end and was equipped with a flat stopper. The portion of the vessel below the joint was fitted by means of a rubber stopper into a wide-mouth bottle containing the ice–water mixture. The solution of the sodium salt of the amino acid was treated with 1.25 equivalents of 2 *M* *p*-nitrobenzyl chloroformate[2] in purified dioxane[10] and with 1.25 equivalents of 4 *N* sodium hydroxide in five approximately equal portions. The reaction mixture was shaken vigorously with cooling for about five minutes between each addition of reagents, except in the case of aspartic and glutamic acids where 15 minutes was allowed. At the end of the shaking period crystalline by-product, chiefly di-*p*-nitrobenzyl carbonate, was removed by centrifugation and washed with water, and the washings were added to the solution of the product. The alkaline reaction mixture was acidified with concentrated hydrochloric acid and the product was extracted with ethyl acetate. After the organic phase had been washed with *N* hydrochloric acid and water the derivative was extracted into *N* sodium bicarbonate. The alkaline solution was washed with ethyl acetate, cooled, and acidified with concentrated hydrochloric acid. The derivative was induced to crystallize and was collected by filtration.

Procedure B.—The amino acid was dissolved in one equivalent of 4 *N* sodium hydroxide and one equivalent of *N* sodium bicarbonate was added. Enough dioxane was added so as to make the resulting solution about 30% in dioxane. The solution was treated with 1.1 equivalents of 2 *M* *p*-nitrobenzyl chloroformate in dioxane and 1.1 equivalents of 4 *N* sodium hydroxide in five approximately equal portions. The mixture was shaken and cooled for about 20 minutes between each addition of reagents. The derivatives were then isolated as described in procedure A.

O,N-Di-*p*-nitrobenzyloxycarbonyl-D,L-tyrosine.—D,L-Tyrosine (1.81 g., 0.01 mole) was dissolved in 5 ml. of 4 *N* sodium hydroxide and 40 ml. of *N* sodium bicarbonate. A dioxane solution of *p*-nitrobenzyl chloroformate (6.47 g., 0.03 mole in total volume of 20 ml.) and 10 ml. of 4 *N* sodium hydroxide were added in five approximately equal portions. The mixture was shaken for 10–20 minutes between each addition of reagents. After the insoluble by-product had been removed, the reaction mixture was acidified and the derivative was extracted into ethyl acetate. After the organic phase had been washed with *N* hydrochloric acid and water, a 1/2 volume of hexane was added to the ethyl acetate and the derivative was extracted into *N* sodium bicarbonate. When the aqueous phase was cooled and acidified, the derivative separated as a crystalline solid, yield 3.98 g. (74%), m.p. 150–161°. After the derivative had been recrystallized from ethyl acetate–hexane, it melted at 154–162° and gave a negative Folin test for the phenolic hydroxyl group by the *p*H 8 method of Herriott.[11] A sample was dried at 56° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_{25}H_{21}O_{11}N_3$: C, 55.66; H, 3.92; neut. equiv., 539.4. Found: C, 55.55; H, 4.22; neut. equiv., 543.

Di-*p*-nitrobenzyloxycarbonyl-L-arginine.—L-Arginine free base (3.48 g., 0.02 mole) was dissolved in 6.25 ml. of 4 *N* sodium hydroxide and 4 ml. of dioxane was added. To this mixture were added in the usual manner 9.7 g. (0.045 mole) of *p*-nitrobenzyl chloroformate in dioxane (30 ml. total volume) and 12.5 ml. of 4 *N* sodium hydroxide. After the reaction was complete, the mixture was centrifuged to remove the insoluble by-product which was washed twice with water and the washings were added to the solution of the derivative. The alkaline aqueous mixture was washed twice with ethyl acetate–ether (1:1) and once with ether. The aqueous phase was then cooled and made strongly acid. The product, which first separated as an oil, soon solidified into an amorphous solid. It was collected and dried over phosphorus pentoxide *in vacuo*. The yield was 6.81 g. (64%), m.p. 123–125°. It was hygroscopic and gained about 4% in weight when allowed to stand in air. The product was precipitated several times from hot dioxane by the addition of water without any change in melting point. Then the product separated from a hot dioxane–water mixture as well-defined needle-like crystals. The solution of these crystals in dioxane was considerably hastened by addition of a small amount of water and addition of more water caused the derivative to crystallize. The melting point of the purified product was 180.5–181.5°, $[\alpha]^{25}D$ +6.3° (*c* 1, 0.6 *N* hydrochloric acid–dioxane (1:4)). A sample was dried at 75° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_{22}H_{24}O_{10}N_6$: C, 49.62; H, 4.54; N, 15.79; neut. equiv., 532.5. Found: C, 49.84; H, 4.79; N, 15.98; neut. equiv., 511.

N,N'-Di-*p*-nitrobenzyloxycarbonyl-L-cystine.—This derivative was prepared in a manner similar to procedure A starting with 6.05 g. (0.025 mole) of L-cystine and 13.48 g. (0.0625 mole) of *p*-nitrobenzyl chloroformate. The sodium salt of the product separated during the reaction. After completion of the reaction several volumes of water were added to dissolve the sodium salt and the by-product (di-*p*-nitrobenzyl carbonate) was removed by filtration. The alkaline aqueous solution was washed several times with ethyl acetate, cooled and acidified. The product separated as a mixture of crystals and oil. About half of the water was decanted off and the *p*H was adjusted to about 9. Sodium chloride was dissolved in the warm solution to aid in salting out the sodium salt of the

(10) According to E. Eigenberger, see A. Weisberger and E. Proskauer, "Organic Solvents," Oxford University Press, New York, N. Y., 1935, p. 139.

(11) R. M. Herriott, *J. Gen. Physiol.*, **19**, 283 (1935).

derivative. The solution was allowed to cool to room temperature and then cooled at 4° overnight. The sodium salt of the derivative was collected by filtration and the mother liquor was saved for isolation of the mono-*p*-nitrobenzyloxycarbonyl derivative (see below). The sodium salt was washed successively with water, ethanol and ether, and dried *in vacuo* over phosphorus pentoxide, 10.3 g. (64%). When the product was dissolved in water and acidified, an oil separated which was extracted into ethyl acetate. The organic phase was dried over anhydrous magnesium sulfate and the solvent was removed *in vacuo*. The residue crystallized after it had been allowed to stand overnight, m.p. 95–110°. After five recrystallizations from ethyl acetate–hexane, the derivative melted at 99–115°, $[\alpha]^{24}D$ −129.8° (*c* 1, 95% ethanol). A sample was allowed to dry in air for analysis.

Anal. Calcd. for $C_{22}H_{22}O_{12}N_4S_2$: C, 44.14; H, 3.71; S, 10.71; neut. equiv., 299.3. Found: C, 44.04; H, 3.72; S, 10.49; neut. equiv., 305.

When the mother liquor from the sodium salt of the derivative above was cooled and acidified a crystalline product (2.02 g., m.p. 187–190°) was obtained. It was recrystallized from dioxane–water; m.p. 203.5–204.5°, $[\alpha]^{24}D$ −125.2° (*c* 1, *N* sodium hydroxide). The derivative which gave a positive ninhydrin test, analyzed as mono-*p*-nitrobenzyloxycarbonyl-L-cystine. A sample was dried at 100° *in vacuo* over phosphorus pentoxide for analysis.

Anal. Calcd. for $C_{14}H_{17}O_8N_3S_2$: C, 40.09; H, 4.09; S, 15.29. Found: C, 40.37; H, 4.26; S, 15.19.

Nα,Nε-Di-*p*-nitrobenzyloxycarbonyl-L-lysine Monohydrate.—L-Lysine monohydrochloride (4.57 g., 0.025 mole) was dissolved in 15.6 ml. of 4 *N* sodium hydroxide. To this solution in the usual manner were added 13.5 g. (0.0625 mole) of *p*-nitrobenzyl chloroformate in dioxane (32 ml. total volume) and 15.6 ml. of 4 *N* sodium hydroxide. The sodium salt of the product separated as an oil. The crystalline by-product, di-*p*-nitrobenzyl carbonate, was removed by centrifugation. The reaction mixture was then acidified with concentrated hydrochloric acid and the oily product was extracted into ethyl acetate. The organic phase was washed twice with *N* hydrochloric acid and once with water. Since the derivative was not readily extracted from ethyl acetate with sodium bicarbonate, an approximately equal volume of ether was added to the ethyl acetate solution and the derivative then extracted with 0.5 *N* sodium bicarbonate. The aqueous phase was washed twice with ethyl acetate–ether (1:1), then cooled and acidified. The product separated as an oil which failed to crystallize unless seeded. After the oil had been seeded with crystals obtained in a manner described below, it gradually crystallized. After seeding crystallization could be greatly hastened by vigorously stirring the oil in water since the derivative crystallizes as a monohydrate. The yield was 10.18 g. (78%), melting range 55–69°. Drying this derivative *in vacuo* over phosphorus pentoxide reduced it to an anhydrous oil.

Seed crystals were obtained by partial purification of a portion of the crude product through its benzylamine salt. A dioxane solution of the derivative was treated with a dioxane solution of benzylamine. Ether was then added to opalescence and crystallization was readily induced. The product was collected and washed with dioxane–ether, m.p. 80–100°. After the benzylamine salt had been recrystallized several times from dioxane–ether it melted at 102–108°. It was converted to the free derivative by dissolving the benzylamine salt in water containing an equivalent amount of sodium hydroxide, followed by acidification of the cooled mixture with concentrated hydrochloric acid. Crystallization was induced by cooling and scratching the wall of the flask.

For analysis the derivative was recrystallized from acetic acid–water. One gram was recrystallized from about 250 ml. of acetic acid and about 750 ml. of water. The dilute solution was necessary to prevent oiling. Recovery was about 75% and a second crop was obtained by adding more water to the mother liquor, increasing the recovery to 90–95%. The purified monohydrate slowly melted over a range from 56–72°, $[\alpha]^{23}D$ −5.4 (*c* 1, pyridine). The specific rotation is based on the amount of anhydrous material present as calculated from its water content. For analysis a sample was allowed to dry in air.

Anal. Calcd. for $C_{22}H_{24}O_{10}N_4 \cdot H_2O$: C, 50.57; H, 5.02; H_2O, 3.45; neut. equiv., 522.5. Found: C, 50.67; H, 4.92; H_2O, 3.5; neut. equiv., 515.

N-*p*-Nitrobenzyloxycarbonyl-3,5-diiodo-L-tyrosine Ethyl Ester.—This derivative was prepared by a method similar to that used by Fox[12] in the preparation of the N-benzoyl derivative of diiodotyrosine ethyl ester. The hydrochloride of diiodotyrosine ethyl ester (5.0 g., 0.01 mole) was allowed to react with *p*-nitrobenzyl chloroformate (2.16 g., 0.01 mole) in a chloroform–sodium carbonate mixture. After the chloroform layer had been washed successively with 2 *N* sodium carbonate, 1 *N* sulfuric acid and water, it was dried over anhydrous magnesium sulfate and the chloroform was removed *in vacuo* to give 5.6 g. (87%) of the crystalline compound. The compound was recrystallized several times from toluene to give N-*p*-nitrobenzyloxycarbonyl-diiodo-L-tyrosine ethyl ester with m.p. 151–152°, $[\alpha]^{22}D$ −40.5° (*c* 1, pyridine), which was dried at 78° *in vacuo* over phosphoric anhydride for analysis.

Anal. Calcd. for $C_{19}H_{18}O_7N_2I_2$: C, 35.64; H, 2.83; I, 39.65; sapn. equiv., 640.2. Found: C, 36.05; H, 2.76; I, 39.26; sapn. equiv., 650.

This material gave a positive test for the phenolic hydroxyl group[11] and a negative ninhydrin test for amino groups.[6]

N-*p*-Nitrobenzyloxycarbonyl-3,5-diiodo-L-tyrosine Acetate.—N-*p*-Nitrobenzyloxycarbonyldiiodo-L-tyrosine ethyl ester (3.2 g., 0.005 mole) was dissolved in 110 ml. of 90% ethanol and 7.0 ml. of 1.86 *N* ethanolic potassium hydroxide. The solution was allowed to stand at room temperature for 20 hours and then it was acidified with 1 *N* hydrochloric acid and diluted with an equal volume of water. A crop of crystals separated which weighed 3.05 g. (99%). The material was recrystallized from amyl acetate–heptane to give material with a m.p. of 167–171° but which turned brown on standing in air. When the material was recrystallized from acetic acid, it separated as white crystals which were stable to storage but which contained a molecule of acetic acid of crystallization. When observed on the hot-stage, this air-dried material melted at 100–118° and resolidified to long needles which were transformed at 157–161° to large plates which in turn melted at 172–174°. After the material had been dried at 120° *in vacuo* over sodium hydroxide, it melted at 170–174°. A sample was dried in air for analysis, $[\alpha]^{22}D$ +7.0° (*c* 1, *N* sodium hydroxide).

Anal. Calcd. for $C_{17}H_{14}O_7N_2I_2 \cdot CH_3COOH$: C, 33.95; H, 2.70; I, 37.76; neut. equiv., 224.1. Found: C, 34.05; H, 2.85; I, 38.08; neut. equiv., 225.

Methyl-*p*-nitrobenzyl Carbonate.—*p*-Nitrobenzyl chloroformate (1.0 g.) was dissolved in 15 ml. of methanol and allowed to stand at room temperature. After about 15 minutes a mass of crystals separated when the wall of the flask was scratched. The mixture was allowed to stand overnight at room temperature and then cooled in the refrigerator. The crop was collected and washed with methanol. The yield was 0.76 g. (77%), m.p. 97–103°. A second crop was obtained by concentration of the mother liquor and addition of water to the hot concentrate. It amounted to 0.13 g., m.p. 99.5–103°. The total yield was 91%. After the ester had been recrystallized from methanol, it melted at 102.5–103°. A sample was allowed to dry in air for analysis.

Anal. Calcd. for $C_9H_9O_5N$: C, 51.19; H, 4.30. Found: C, 51.35; H, 4.33.

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(12) S. W. Fox, THIS JOURNAL, **68**, 194 (1946).