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The Synthesis of Benzoyl-L-arginine-*p*-nitroanilide*[1]

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Benzoyl-arginine-*p*-nitroanilide (BAPA) is a synthetic substrate for trypsin, papain, and other enzymes, but it has not yet been prepared in the active L-form, although the preparations of DL-form and D-form by the trypsin digestion of the DL-form have been reported. The optically active L-BAPA was prepared by the following route; N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide was prepared from N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine and *p*-nitrophenyl isocyanate, and the subsequent decarbobenzyloxylation and the benzoylation of this compound gave N^{α}-benzoyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide. The removal of the nitro group was followed by Sakakibara's method using hydrogen fluoride. The L-BAPA shows the same melting point and the same reversal optical rotation as those of D-BAPA. When N^{α}-benzoyl derivatives of L-arginine were used as starting materials in the preparation of BAPA, DL-BAPA was always obtained with complete racemization; when N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine as the starting material reacted on *p*-nitrophenyl isocyanate, the product had no racemization, but the desired L-BAPA was not obtained because one of the carbobenzyloxy groups on the guanido group was extraordinally stable to reagents and could not be removed completely.

Benzoyl-arginine-*p*-nitroanilide (BAPA) is a interesting material for use as a synthetic chromogenic substrate of trypsin and papain; DL-BAPA has been used as a substrate because the L-form has not yet been prepared. The synthesis of the L-form is important in order to see whether enzymic activity to the L-form is inhibited by the D-form or not. BAPA has thus far been synthesized from benzoyl-arginine and *p*-nitroaniline using the polyphosphoric ester[1] or phosphorous pentoxide in diethylphosphite[2] as a condensation reagent, but only the racemic compound was obtained in these methods. The optically-active D-form was prepared by the tryptic digestion of the DL-form.[2] The optically-active L-BAPA was prepared after we had examined the several synthetic routes shown in Scheme 1.

The obtained L-BAPA shows the same melting point, the same reversal optical rotation, and the same solubility to water and ethanol as D-BAPA.[2] The carboxyl group of arginine tends to form a six-membered lactam by cyclization with the δ-imino group, and the reactivity of the amino group of *p*-nitroaniline is less than that of amino acid because of its low basicity.

In this paper, *p*-nitrophenyl isocyanate was used to form an amide bond with the carboxyl group of arginine.[3] Generally, it is hard to prepare *p*-nitroanilide of amino acids by other methods, such as the use of dicyclohexyl carbodiimide[4] or Woodward's reagent.[5] When a carbobenzyloxy derivative is used on the coupling step of the carboxyl and isocyanate group, as is shown in Scheme 1-A, no racemization occurs, but when a benzoyl derivative, like N^{α}-benzoyl-N^{ω}-nitro-L-arginine (Scheme 1-B) or N^{α}-benzoyl-N^{δ}-carbobenzyloxy-L-ornithine (Scheme 1-C),[6] is used, the reaction always proceeds with racemization. When N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine is used as the starting material and is coupled with *p*-nitrophenyl isocyanate, the reaction proceeds, producing a high yield without any racemization (Scheme 1-D), but one of the carbobenzyloxy group on the guanido group is not removed by the usual method without a breakdown of the amide bond because of the extraordinally stability.[7]

*1 This work was presented at the 7th Symposium on Peptide Chemistry, Chemical Society of Japan, Tokyo, Nov. 21, 1969.

1) E. A. Bashkir, Ya. I. Lapuk and V. M. Stepanov, *Metody Poluchenіya Khim. Reactivov i Preparanov*, **13**, 76 (1965).

2) B. F. Erlanger, N. Kokowsky and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).

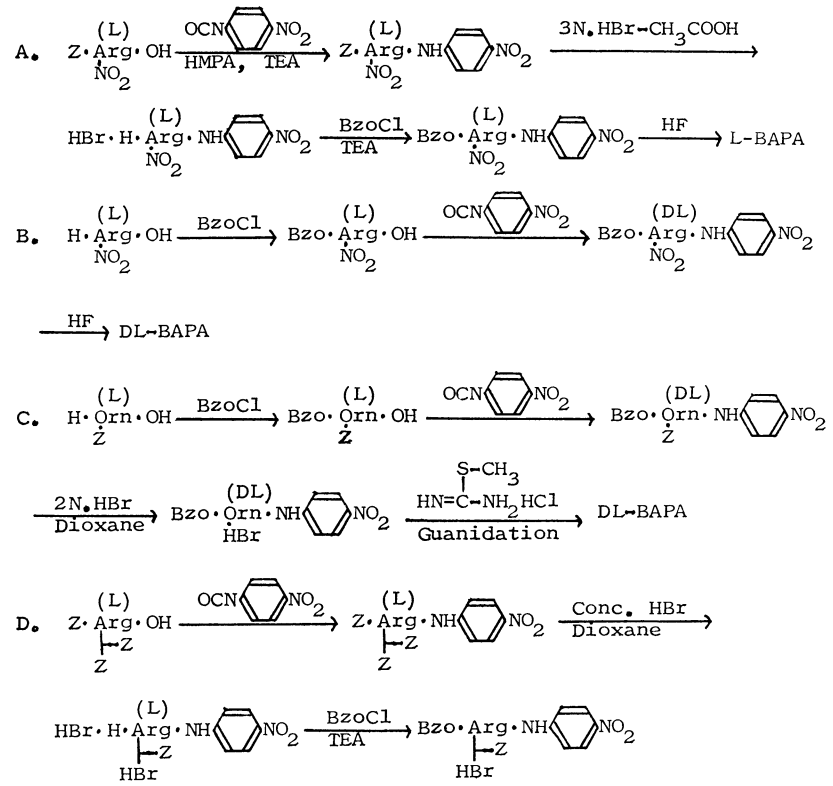
3) W. Diekmann and F. Breest, *Ber.*, **309**, 3052 (1906); S. Goldschmidt and M. Wick, *Z. Naturforsch.*, **B**, **5** 170 (1950); S. Goldschmidt and M. Wick, *Ann., Chem.*, **575**, 217 (1952).

4) J. C. Sheehan, G. P. Hess and M. Goodman, *J. Amer. Chem. Soc.*, **77**, 1067 (1955); J. C. Sheehan, M. Goodman and G. P. Hess, *ibid.*, **78**, 1367 (1956).

5) R. B. Woodward, R. A. Olofson and H. Mayer, *ibid.*, **83**, 1010 (1961).

6) J. Noguchi and N. Nishi, The 5th Symposium on Peptide Chemistry, (1967), p. 36.

7) N. Nishi and J. Noguchi, The 6th Symposium on Peptide Chemistry, (1968), p. 1.



Scheme 1

HMPA: Hexamethyl Phosphoramide; TEA: Triethylamine; Z: Carbobenzyloxy; Bzo: Benzoyl.

Experimental

The melting points for the sample over 220°C were determined with a Mitamura Hot-stage Apparatus. The ultraviolet and visible absorption spectra were measured with a Hitachi 124 Spectrophotometer, while the infrared absorption spectra were measured with a Hitachi Grating Infrared Spectrophotometer, EPI-G2. The optical rotations were measured with a Yanagimoto Direct Reading Polarimeter, Model OR-10, and an Automatic Recording Polarimeter, Model OR-1. Toyo Densitorol #2 was used for the measurement of the concentration after development by ninhydrin. The elemental analysis was carried out by means of a Yanagimoto C.H.N. Corder, Model MT-2, and a Coleman Nitrogen Analyser, Model 29.

N^{ω}-Nitro-L-arginine. N^{ω}-Nitro-L-arginine was prepared according to Hayakawa *et al.*[8]

N^{α}-Carbobenzyloxy-N^{ω}-nitro-L-arginine. This material was prepared by the carbobenzyloxylation of N^{ω}-nitro-L-arginine essentially as has been described by Hofmann *et al.*[9]

***p*-Nitrophenyl Isocyanate.**[10] Hydrogen chloride was introduced into *p*-nitroaniline (20 g, 145 mmol) in dry dioxane (200 ml) until saturation at room temperature, and then phosgene was bubbled into the reaction mixture for 1 hr at 50°C until a clear solution was obtained. After expelling the excess phosgene at room temperature by bubbling with nitrogen for 30 min, the solution was evaporated to dryness below 40°C and the residue was refluxed with dry carbon tetrachloride (120 ml) for 2.5 hr. The insoluble part was removed by filtration at room temperature, the filtrate was concentrated to dryness, and the crystalline *p*-nitrophenyl isocyanate was recrystallized from carbon tetrachloride. Yield, 21.8 g (92.4%). mp 56°C.

Found: C, 51.1; H, 2.23; N, 17.3%. Calcd for $C_7H_4N_2O_3$: C, 51.2; H, 2.44; N, 17.1%.

N^{α}-Carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide. Into the solution of N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine (20.0 g, 56.7 mmol) in dry hexamethylphosphoramide (HMPA) (120 ml), triethylamine (7.92 ml, 56.7 mmol) and *p*-nitrophenyl isocyanate (18.7 g, 114 mmol) were stirred successively at 20°C. The mixture was stirred for 20 hr at room temperature, diluted with 1000 ml of 2% sodium bicarbonate to separate the product, and then cooled. The resulting precipitate was filtered and washed successively with 2% sodium bicarbonate, water, 0.5 N hy-

8) T. Hayakawa, Y. Fujiwara and J. Noguchi, This Bulletin, **40**, 1205 (1967).

9) M. Bergmann, L. Zervas and H. Rinke, *Z. Physiol. Chem.*, **224**, 40 (1934); K. Hofmann, W. D. Peckham and A. Rheiner, *J. Amer. Chem. Soc.*, **78**, 238 (1956).

10) Vittenet, *Bull. Soc. Chim.*, **(3)21**, 586 (1899); V. Hoogstraten, *Rec. Trav. Chim. Pays-Bas*, **51**, 418 (1932); Swartz, *Amer. Chem. J.*, **19**, 318 (1897); Shriner and Cox, *J. Amer. Chem. Soc.*, **53**, 1603 (1931).

drochloric acid and water. The product was extracted twice with 300-ml and 100-ml portions of hot dioxane, and the extract was concentrated to dryness. The residue was washed several times with ether and recrystallized from *n*-butanol (300 ml). The crystal was washed with ether and dried. Yield, 20.4 g (76.2%); mp 178°C, $[\alpha]_D^{29}$ +52.8° (*c* 1.05, HMPA), −1.4° (*c* 1.02, glacial acetic acid).

Found: C, 50.7; H, 4.75; N, 20.4%. Calcd for $C_{20}H_{23}N_7O_7$: C, 50.8; H, 4.87; N, 20.7%.

N^{ω}-Nitro-L-arginine-*p*-nitroanilide Hydrobromide. N^{α}-Carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide (20.0 g, 42.3 mmol) was treated with 3 N hydrogen bromide in glacial acetic acid (200 ml) for 7 hr at 20—23°C while being stirred. The product was precipitated by the addition of dry ether (1000 ml); after the mixture had stood for 1 hr at 0°C, the precipitate was collected by decantation and washed with dry ether. This precipitate was easily crystallized with water, cooled, filtered, washed with a small amount of cold water, and dried. From the concentration of the mother liquor of acetic acid and ether containing HBr, an additional product was obtained. The combined product was refluxed with dioxane (40 ml) for a few minutes to remove the impurity; then it was cooled, filtered and dried. Yield, 15.8 g (88.8%); mp 174—175°C.

Found: Br, 18.7%. Calcd for $C_{12}H_{18}N_7O_5Br$: Br, 19.0%.

N^{α}-Benzoyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide. N^{ω}-Nitro-L-arginine-*p*-nitroanilide hydrobromide (19.5 g, 46.4 mmol) was dissolved in a mixture of tetrahydrofuran and water (9 : 1, 195 ml) and then cooled to −5—−7°C. After the addition of triethylamine (13.5 ml, 97.5 mmol) to the solution, benzoyl chloride (6.05 ml, 51.1 mmol) was stirred in, drop by drop, over a five-minute period. After standing for 1 hr, it was kept for 1 hr at 0°C and for another 1 hr at room temperature; then the solution was evaporated to dryness at room temperature. The residue was washed with water, dried, and crystallized by refluxing with ethanol. It was recrystallized from dimethyl formamide (DMF) and water. Yield, 15.4 g (75.2%); mp 246°C; $[\alpha]_D^{26}$ +60.7° (*c* 1.12, HMPA).

Found: C, 51.5; H, 4.41; N, 21.9%. Calcd for $C_{19}H_{21}N_7O_6$: C, 51.5; H, 4.73; N, 22.1%.

N^{α}-Benzoyl-L-arginine-*p*-nitroanilide Hydrochloride. N^{α}-Benzoyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (13 g, 29.4 mmol) was placed in a HF-reaction cylinder containing anisole (13 ml, 120 mmol). Forty milliliters of hydrogen fluoride dried with CoF_3 were collected under cooling with dry ice in the cylinder;[11] the mixture was then allowed to react at 0°C for 40 min while being stirred. The excess HF was removed under reduced pressure, and the residue was kept *in vacuo* for a further 2 hr. The residue was washed with ether, and then hydrofluoride was converted into the hydrochloride by successive treatment with 3 N HCl. It was crystallized with ethanol (100 ml) and ether (300 ml), and then left for 1 hr at 0°C. The crystal was collected by filtration and dried. The yield was 11.0 g. This crude product was extracted four times with hot ethanol (200 ml, 100 ml, 50 ml and 50 ml), and the combined extract was concentrated to dryness and recrystallized from absolute ethanol. Yield, 5.6 g (44.1%); mp 225°C; $[\alpha]_D^{25}$ +14.2° (*c* 1.0, water).

Found: C, 52.4; H, 5.23; N, 19.2; Cl, 8.1%. Calcd for $C_{19}H_{23}N_6O_4Cl$: C, 52.5; H, 5.29; N, 19.3; Cl, 8.2%.

N^{α}-Benzoyl-N^{ω}-nitro-L-arginine. A solution of N^{ω}-nitro-L-arginine (20 g, 91.5 mmol) in 1 N sodium hydroxide (91.5 ml, 91.5 mmol) was cooled to −3—−5°C. 1 N sodium hydroxide (109 ml, 109 mmol) and benzoyl chloride (12.8 ml, 109 mmol) were stirred in alternatively over a 30-min period. After stirring for an additional 90 min, the reaction mixture was extracted three times with ether in order to remove the excess benzoyl chloride. The solution was acidified to pH 1—2 with concentrated hydrochloric acid, and the precipitate was crystallized by trituration. The crystal was collected by filtration, and then washed with diluted hydrochloric acid, water, and ether. It was recrystallized from water. Yield, 27.8 g (94.5%); mp 158—159°C; $[\alpha]_D^{24}$ +1.4° (*c* 1.0, methanol).

Found C, 48.5; H, 5.47; N, 21.3%. Calcd for $C_{13}H_{17}N_5O_5$: C, 48.3; H, 5.26; N, 21.6%.

N^{α}-Benzoyl-N^{ω}-nitro-DL-arginine-*p*-nitroanilide. Into a solution of N^{α}-benzoyl-N^{ω}-nitro-L-arginine (3.2 g, 10 mmol) in 30 ml of dry dioxane, *p*-nitrophenyl isocyanate (1.64 g, 10 mmol) was stirred at 20°C. After the dissolution of *p*-nitrophenyl isocyante in the solution, the mixture was kept for a further 2 hr at 50°C. The crystal which separated out from the solution was filtered and refluxed with methanol (15 ml). It was recrystallized from nitrobenzene and washed with methanol and ether. Yield, 2.5 g (56.4%); mp 236°C; $[\alpha]_D^{25} \fallingdotseq 0$ (*c* 0.5, glacial acetic acid).

Found: C, 51.6; H, 4.78; N, 22.0%. Calcd for $C_{19}H_{21}N_7O_6$: C, 51.5; H, 4.73; N, 22.1%.

N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide Hydrochloride. N^{α}-Benzoyl-N^{ω}-nitro-DL-arginine-*p*-nitroanilide (2.0 g, 4.5 mmol) was treated with hydrogen fluoride in order to remove the nitro group by the same procedure as was used for N^{α}-benzoyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide. The product was recrystallized from 20% acetic acid. Yield, 1.5 g (76.6%); mp 262°C; $[\alpha]_D^{25}=0$ (*c* 0.4, glacial acetic acid).

Found: C, 52.4; H, 5.16; N, 19.2; Cl, 8.1%. Calcd for $C_{19}H_{23}N_6O_4Cl$: C, 52.5; H, 5.29; N, 19.3; Cl, 8.2%.

N^{δ}-Carbobenzyloxy-L-ornithine. N^{δ}-Carbobenzyloxy-L-ornithine was prepared according to the method of Synge.[12]

N^{α}-Benzoyl-N^{δ}-carbobenzyloxy-L-ornithine. Into a cold solution (−1—−2°C) of N^{δ}-carbobenzyloxy-L-ornithine (20.0 g, 75 mmol) in 1 N sodium hydroxide (75 ml, 75 mmol), 4 N sodium hydroxide (22.5 ml, 90 mmol) and benzoyl chloride (10.6 ml, 90 mmol) were stirred alternately over a 20-min period. After the addition of ether (5 ml), the reaction proceeded for a further 3 hr at the same temperature. Then the reaction mixture was extracted three times with ether in order to remove the excess benzoyl chloride. The

11) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, This Bulletin, **40**, 2164 (1967); S. Sakakibara, Y. Kishida, R. Nishizawa and Y. Shimonishi, *ibid.*, **41**, 438 (1968); S. Sakakibara, N. Nakamizo, Y. Kishida and S. Yoshimura, *ibid.*, **41**, 1477 (1968).

12) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

solution was acidified to pH 1—2 with concentrated hydrochloric acid, and the precipitate was crystallized by trituration. The crystals were collected by filtration and washed with diluted hydrochloric acid, water and ether. They were then recrystallized from ethyl acetate. Yield, 26.7 g (96.2%); mp 145°C; $[\alpha]_D^{20}$ −1.4° (*c* 1.0 and 3.0, methanol); $[\alpha]_{508}^{20}$ −1.7° (*c* 3.0, methanol).

Found: C, 64.7; H, 5.60; N, 7.40%. Calcd for $C_{20}H_{22}N_2O_5$: C, 64.8; H, 5.95; N, 7.57%.

N^{α}-Benzoyl-N^{δ}-carbobenzyloxy-DL-ornithine-*p*-nitroanilide. Into a solution of N^{α}-benzoyl-N^{ω}-carbobenzyloxy-L-ornithine (24.0 g, 65 mmol) in dry dioxane (240 m*l*), *p*-nitrophenyl isocyanate (18.0 g, 110 mmol) was stirred at 20°C. After 2 days at 20—23°C, the product which precipitated from the solution was filtered and dried. It was then recrystallized from ethanol. Yield, 25.8 g (81.5%); mp 174°C; $[\alpha]_D^{20}$ ±0° (*c* 1.3, dioxane).

Found: C, 63.3; H, 5.02; N, 11.5%. Calcd for $C_{26}H_{26}N_4O_6$: C, 63.7; H, 5.31; N, 11.4%.

Dinitrodiphenyl Urea.[6,10] The above-mentioned part which was insoluble in hot ethanol was confirmed to be dinitrodiphenyl urea by the elemental analysis after recrystallization from dimethyl formamide. Yield, 4.8 g; mp 340°C (lit,[10] 360°C).

Found: C, 51.7; H, 3.14; N, 18.3%. Calcd for $C_{13}H_{10}N_4O_5$: C, 51.7; H, 3.31; N, 18.5%.

N^{α}-Benzoyl-DL-ornithine-*p*-nitroanilide Hydrobromide. N^{α}-Benzoyl-N^{δ}-carbobenzyloxy-DL-ornithine-*p*-nitroanilide (10.0 g, 20.4 mmol) was treated with 2 N hydrogen bromide in dioxane (300 m*l*) for 30 min at 20°C, and the N^{δ}-carbobenzyloxy group was removed. The product was then recrystallized from methanol-ethylacetate. Yield, 6.4 g (71.5%); mp 207°C.

Found: C, 49.0; H, 4.55; N, 12.8; Br, 18.5%. Calcd for $C_{18}H_{21}N_4O_4Br$: C, 49.2; H, 4.78; N, 12.8; Br, 18.2%.

The Guanidation of Benzoyl-ornithine-*p*-nitroanilide Hydrobromide. To a methanol solution (500 m*l*) of benzoyl-ornithine-*p*-nitroanilide hydrobromide (8.4 g, 19.1 mmol), 1.08 N sodium methylate in methanol (17.64 m*l*, 19.1 mmol) was added, and the sodium bromide was centrifuged. *S*-Methyl isothiourea hydrochloride (3.9 g, 28.8 mmol) in methanol (10 m*l*) was then added to the solution, and the solution was refluxed for 5 hr. After treatment with charcoal, the reaction mixture was concentrated to dryness and washed with water. The product was recrystallized from 20% acetic acid, washed with hot ethyl acetate (50 m*l*), and recrystallized again with 10% aqueous DMF. Yield, 4.8 g (57.6%); mp 225°C; $[\alpha]_D^{25}$=0° (*c* 1.0, DMF). The Sakaguchi reaction was positive, and the ninhydrin reaction was negative. R_f=0.84 (*n*-butanol: glacial acetic acid : water=4 : 1 : 5). *Cf.* Standard DL-BAPA's R_f is 0.84.

Found: Cl, 8.0%. Calcd for $C_{19}H_{23}N_6O_4Cl$: Cl, 8.2%.

N^{α},N^{ω},$N^{\omega'}$-Tricarbobenzyloxy-L-arginine. This material was prepared according to the method of Zervas *et al.*[13]

N^{α},N^{ω},$N^{\omega'}$-Tricarbobenzyloxy-L-arginine-*p*-nitroanilide. Into a dry dioxane solution (68 m*l*) of N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine (11.3 g, 19.6 mmol), *p*-nitrophenyl isocyanate (4.4 g, 26.8 mmol) was stirred at 20°C. After the mixture had then stood for 20 hr at 20°C, a small excess of water corresponding to the excess isocyanate was added. 150 m*l* of ether were added to the reaction mixture, it was stirred for 1 hr, and the insoluble part was filtered off. The clear filtrate was concentrated to dryness *in vacuo* and the residue was washed with 0.1 N hydrochloric acid and water. The crude product was dissolved in methanol and reprecipitated with water. The precipitate was crystallized with cold water, and the crystals were filtered out, washed with water, and dried. Yield, 13.4 g (97.8%); mp 64°C; $[\alpha]_D^{25}$ +4.1° (*c* 1.0, chloroform). Found: C, 61.8; H, 5.31; N, 12.0%. Calcd for $C_{36}H_{36}N_6O_9$: C, 62.1; H, 5.18; N, 12.1%.

Decarbobenzyloxylation of N^{α},N^{ω},$N^{\omega'}$-Tricarbobenzyloxy-L-arginine-*p*-nitroanilide by Hydrogen Bromide and its Benzoylation. A solution of N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide (5 g, 8.7 mmol) in dioxane was saturated with dry HBr and then heated at 50°C for 6 hr. The progress of the reaction was followed by paper chromatography every hour. A spot of the reaction mixture was applied to paper in performing ascending chromatography in a *n*-butanol : glacial acetic acid : water (4 : 1 : 5), and the quantity of the color developed with ninhydrin was measured by means of Densitorol at 563 mμ. The ratio of each spot to the total color is plotted against the time in Fig. 4. After six hr, the reaction mixture was concentrated, treated with dry ether (150 m*l*), washed with water saturated with NaCl, and dried. The product was dissolved in ethanol and precipitated with ether. The precipitate was crystallized from ether. The crystals were centrifuged, washed with ether, and dried. Yield, 2.7 g (63.7%); mp 75—78°C (hygroscopic). Found: Br, 27.4%. Calcd for $C_{20}H_{26}N_6O_5Br_2$ ($N^{\omega'}$-carbobenzyloxy-L-arginine-*p*-nitroanilide dihydrobromide): Br, 27.1%.

The hydrobromide (1 g, 1.7 mmol) was dissolved in 10 m*l* of tetrahydrofuran-water (9 : 1), and the mixture was cooled at −8°C. Triethylamine (0.71 m*l*, 5.1 mmol) and benzoyl chloride (0.20 m*l*, 1.7 mmol) were then stirred in. The solution was allowed to stand for 30 min at −8°C, and then for another hour at room temperature. The reaction mixture was concentrated to dryness, washed with 1 N hydrochloric acid and water, and then dried. The product was recrystallized twice from 20% acetic acid. Yield, 330 mg; mp 157°C. Found: C, 52.2; H, 4.92; N, 13.4; Br, 13.1%. Calcd for $C_{27}H_{29}N_6O_6Br$ (N^{α}-benzoyl-$N^{\omega'}$-carbobenzyloxy-L-arginine-*p*-nitroanilide hydrobromide): C, 52.8; H, 4.73; N, 13.7; Br, 13.0%.

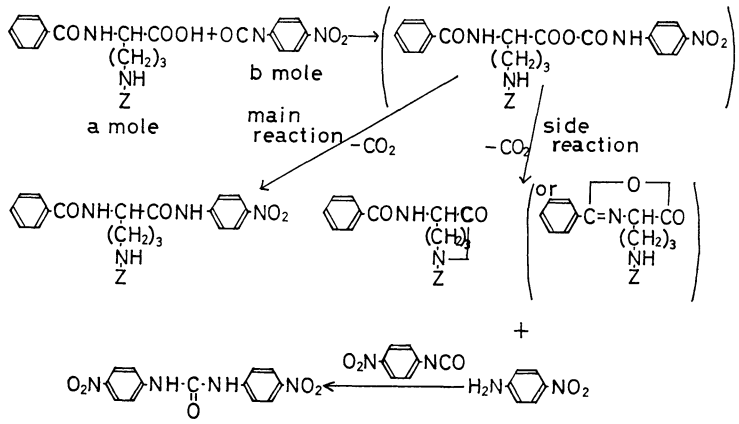
After the hydrolysis of a sample (6.14 mg) with 4 m*l* of 6 N HCl at 110°C for 15 hr, the quantity of arginine was determined by studying ninhydrin color on a paper chromatogram, while that of *p*-nitroaniline was measured by means of the absorption at 380 mμ; Found: Arg. 1.67 mg (27.2%); Calcd for $C_{27}H_{29}N_6O_6Br$: Arg. 1.74 mg (28.4%), Found: *p*-nitroaniline, 1.33 mg (21.7%); Calcd: 1.38 mg (22.5%).

Results and Discussion

North and Young[14] have reported that in acyl

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

14) M. B. North and G. T. Young, *Chem. Ind.* (London), **1955**, 1597.



Scheme 2. The main and side reaction during the coupling reaction of N^{α}-benzoyl-N^{ω}-carbobenzyloxy-L-ornithine with *p*-nitrophenyl isocyanate.

amino acids of the urethane type, such as carbobenzyloxy amino acid, no racemization occurs on the coupling of the carboxyl group with an other amino group because the carbobenzyloxy derivative can not form the oxazolone as an intermediate. Recently optically-active acetyl-L-phenylalanine-*p*-nitroanilide[15] and acetyl-D-phenylalanine-*p*-nitroanilide[16] were prepared by Ramenskii and Botovinik *et al.* They used carbobenzyloxy-L- or D-phenylalnine as the starting material, and coupled it with *p*-nitroaniline before the decarbobenzyloxylation and the acetylation. This is consistent with our method. Optically-active L-BAPA could not be prepared by the benzoyl derivatives, but it was successfully prepared by carbobenzyloxylation at the first step and by the displacement of the carbobenzyloxy group with the benzoyl group at the last step.

Formation of *p*-nitroanilide by a Reaction between Carboxyl and Isocyanate Groups. N^{α}-Carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide was prepared by the reaction of N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine and *p*-nitrophenyl isocyanate in hexamethyl phosphoramide (HMPA). When dioxane, pyridine, and *N*-methyl pyrolidone but not HMPA were used as solvents, this reaction did not proceed at all, even upon the addition of triethylamine or acids as a catalyst; only HMPA gave a good result in spite of the fact that N^{α}-benzoyl-N^{ω}-nitroarginine, N^{α}-benzoyl-N^{ω}-carbobenzyloxy-ornithine, and N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine reacted with *p*-nitrophenyl isocyanate in dioxane. By another route, one *via* such benzoyl derivatives as N^{α}-benzoyl-N^{ω}-nitro-L-arginine and N^{α}-benzoyl-N^{δ}-carbobenzyloxy-L-ornithine, as is shown in Scheme 1, DL-BAPA was always prepared with racemization. In the reaction of N^{α}-benzoyl-N^{δ}-carbobenzyloxy-L-ornithine with *p*-nitrophenyl isocyanate, dinitrodiphenyl urea was obtained as its by-product in addition to the main product, N^{α}-benzoyl-N^{δ}-carbobenzyloxy-L-ornithine-*p*-nitroanilide. This dinitrodiphenyl urea seems to be formed through a side reaction, as is shown in scheme 2. Since the *p*-nitroaniline produced by the side reaction reacts with *p*-nitrophenyl isocyanate, the yield of the main product was only 43 percent when equivalent moles (a : b= 1 : 1) of isocyanate were used, but if the reaction was carried out with 1.7 equivalent moles (a : b= 1 : 1.7) of isocyanate, the amount of *p*-nitrophenyl isocyantate was sufficient and the main product was obtained in an 82 percent yield. N^{α},N^{ω},$N^{\omega'}$-

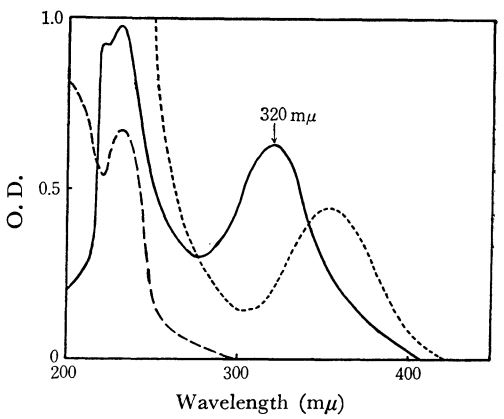


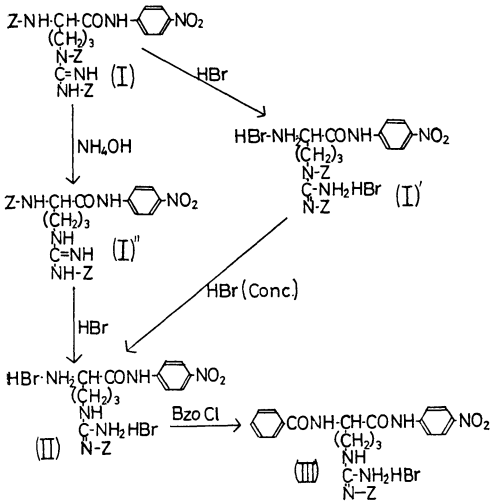
Fig. 1. The absorption spectra of N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide, N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine and *p*-nitroaniline in dioxane.
PNA: -*p*-nitroanilide, Z: carbobenzyloxy.
---- *p*-nitroaniline, ——— tri-Z-Arg
—— tri-Z-Arg·PNA

15) E. V. Ramenskii, M. M. Botovinik and R. U. Beisembaeva, *Khim. Prir. Soedin.*, **4(1)**, 23 (1968).
16) M. M. Botovinik and E. V. Ramenskii, *Ber. II*, **21 (5)**, 127 (1966).

tricarbobenzyloxy-L-arginine gave $N^{\alpha},N^{\omega},N^{\omega'}$-tri carbobenzyloxy-L-arginine-*p*-nitroanilide in a good yield of 98 percent through a coupling reaction with *p*-nitrophenyl isocyanate. The absorption spectrum in dioxane shows its maximum at 320 mμ, as is shown in Fig. 1.

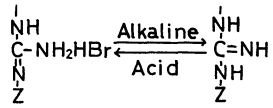
Removal of the Protected Groups in L-Arginine-*p*-nitroanilide Derativises. The removal of the carbobenzyloxy group in N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide was more difficult than that of the usual N^{α}-carbobenzyloxy group; upon standard treatment with 2 N HBr-glacial acetic acid at room temperature for 30 min, the decarbobenzyloxylation of the N^{α}-position did not proceed completely. The treatment of 3 N HBr-glacial acetic acid at 20—23°C for 7 hr was necessary in order to remove it. This is likely to be related to the difficult decarbobenzyloxylation of $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide which will be described below. After the benzoylation of nitro-L-arginine-*p*-nitroanilide, benzoyl-L-arginine-*p*-nitroanilide hydrochloride was obtained by the removal of the nitro group through the HF method of Sakakibara *et al.*[11] However, the yield of L-BAPA was about 44 percent on the removal of the nitro group.

It was very much harder to remove two of the three carbobenzyloxy groups of $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide in Scheme 3 completely than it was to remove the ordinary N^{α}-carbobenzyloxy group ((I)′ in Scheme 3).



Scheme 3. Decarbobenzyloxylation of $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide and its benzoylation.

This might be due to the transfer of a double bond in the guanido group $(-\overset{Z}{\underset{|}{N}}-C(=NH)-NH-Z\rightarrow -\overset{Z}{\underset{|}{N}}-C(NH_2HBr)=N-Z)$ in HBr-dioxane, which stabilises the guanido group to acid, because the carbobenzyloxylated nitrogens are accompanied by no proton. Under even more drastic conditions, one N^{ω}-carbobenzyloxy group was removed ((II) in Scheme 3). The third carbobenzyloxy group was removed in concentrated HBr-glacial acetic acid at a high temperature, the process being accompanied by the cleavage of the amide bond and by the decomposition of the guanido group. When I in Scheme 3 was treated with aqueous ammonia, $>N^{\omega}-Z$, without a proton, was split into I″ without any cleavage of the –NH–Z groups, which were stable in alkaline. By the treatment of I″ in Scheme 3 with HBr, the transfer of the double bond in the guanido group was induced, as is shown in I′ of Scheme 3, and only the N^{α}-carbobenzyloxy group was removed (II). As is shown in Scheme 4, one of the carbobenzyloxy group on the guanido group was made very stable to acid or alkaline by the transformation of guanido group, and the final product was N^{ω}-monocarbobenzyloxy-L-arginine-*p*-nitroanilide. The course of the decarbobenzyloxylation of $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide with hydrogen bromide at 50°C is shown in Fig. 2.



Scheme 4. The transfer reaction of carbobenzyloxylated guanide group with acid and alkaline.

An aliquot of the reaction mixture was spotted on paper (Toyo Roshi No. 51) every hour, used in ascending chromatography in a mixture of *n*-butanol : glacial acetic acid : water (4 : 1 : 5) and developed with ninhydrin. The ratios of the color intensities of the spots, (A) (R_f=0.65), (C) (R_f=0.51) and (B) (several spots in the closed R_f values) to the total spots were then plotted against the reaction time. The (A) spot corresponding to I′ in Scheme 3, disappeared after 4—5 hr. The (C) spot corresponded to II in Scheme 3; it increased with time and reached a constant value after 6 hr. The (B) spot was the side product of the cleavage of the amide bond and the decomposition of the guanido group. At the end of the reaction, the (C) and (B) spots were given instead of the (A) spot and the density of the (C) spot to the total density was 64 percent. From the reaction mixture, a product corresponding to the (C) spot was isolated, it was then benzoylated into compound III. The infrared absorption spectrum III showed a sharp absorption at 1740 cm^{-1} corresponding to the *N*-carbobenzyloxy group, as is shown in Fig. 3.

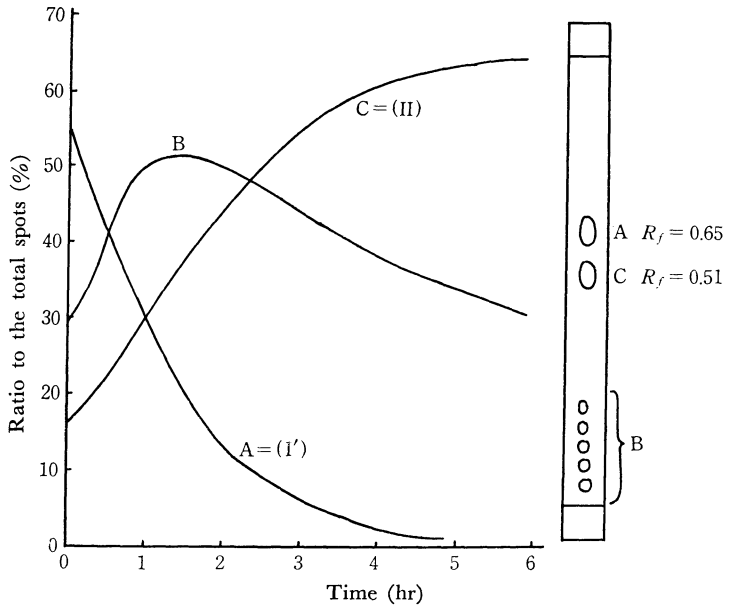


Fig. 2. The time course of decarbobenzyloxy reaction of N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide with HBr saturated dioxane at 50°C.

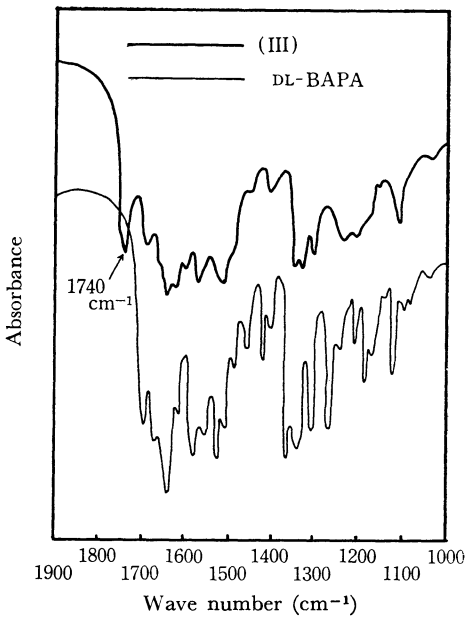


Fig. 3. The infrared absorption spectrum of III and DL-BAPA.

N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy or N^{α},N^{ω}-dicarbobenzyloxy-L-arginine-*p*-nitroanilide was treated with hydrogen fluoride instead of hydrogen bromide, and the reaction was followed by thin-layer chromatography or by paper electrophoresis. However, one carbobenzyloxy group on the guanido group was too stable to be removed by hydrogen fluoride as it had been by hydrogen bromide. Hayakawa *et al.*[17] succeeded in removing two carbobenzyloxy groups on the guanido group of poly-N^{ω},$N^{\omega'}$-dicarbobenzyloxy-L-arginine by the treatment of 6 N HBr-glacial acetic acid at 50°C for 90 min, and Sakakibara *et al.*[11] have reported that carbobenzyloxy groups are easily removed from N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginyl-peptide by means of treatment with hydrogen fluoride at 0°C for 30 min. In the case of N^{α},N^{ω},$N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide, the difficulty in completely removing the three carbobenzyloxy groups is due to the steric hindrance of *p*-nitroanilide.

Properties of L-BAPA. The melting point of L-BAPA, 225°C, is the same as that of D-BAPA reported by Cohen *et al.*[2] and is considerably lower than the melting point of 262°C for DL-BAPA. The specific optical rotation of the L-form is +14.2° (*c* 1.0, water, 25°), while that of the D-form is −14.2° under the same conditions.

The absorption spectrum of L-BAPA in water is the same as that of DL-BAPA; it shows its maximum absorption at 312 mμ, as is shown in Fig. 4.

The infrared absorption spectra of the DL-form and the L-form in the solid state (KBr pellet) give almost the same pattern, except for the absorptions of amide I at 1630 cm^{-1} and of the guanido group at 1660 cm^{-1} in DL-BAPA, which shift to the shorter wavelengths of 1640 cm^{-1} and 1680 cm^{-1} respectively in L-BAPA.

These results seem to indicate that DL-BAPA forms a stable racemic compound by means of the

17) T. Hayakawa, Y. Kondo, H. Yamamoto and Y. Murakami, This Bulletin, **42**, 478 (1967).

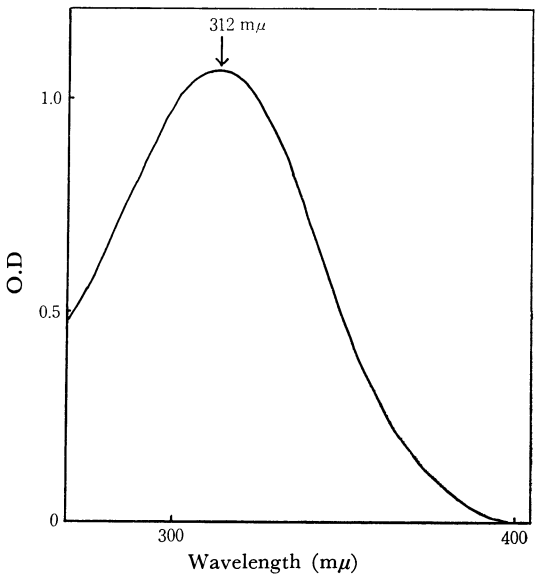


Fig. 4. The absorption spectrum of L-BAPA and DL-BAPA, 8.1×10^{-5} M in water at 22°C, pH 5.60.

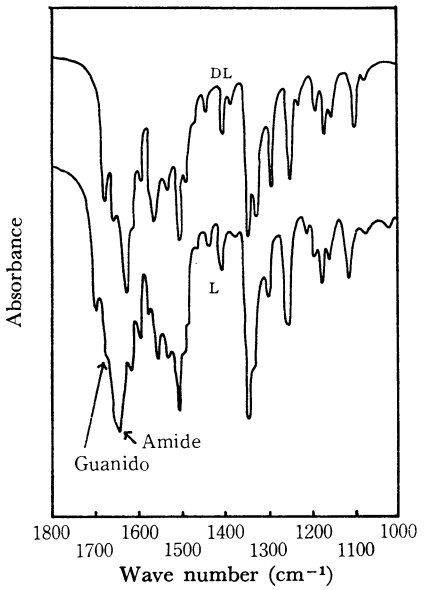


Fig. 5. The infrared absorption spectrum of L- and DL-BAPA.

guanido group; L-BAPA is more easily soluble in water and ethanol than is DL-BAPA.

Conclusions

Optically-active N^{α}-benzoyl-L-arginine-*p*-nitroanilide hydrochloride was synthesized through N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine-*p*-nitroanilide, which had been prepared from N^{α}-carbobenzyloxy-N^{ω}-nitro-L-arginine and *p*-nitrophenyl isocyanate in hexamethyl phosphoramide. The N^{α}-carbobenzyloxy group was removed with HBr-glacial acetic acid and converted to the N^{α}-benzoyl derivative, and then the nitro group was removed with hydrogen fluoride. If the benzoyl derivative is used as the starting material, racemization is unavoidable, and so the urethane type of N^{α}-protecting group should be used in order to prevent racemization upon the coupling between carboxyl and isocyanate groups. The coupling reaction proceeded smoothly only in hexamethyl phosphoramide. $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine-*p*-nitroanilide was prepared from $N^{\alpha},N^{\omega},N^{\omega'}$-tricarbobenzyloxy-L-arginine, but the complete removal of the carbobenzyloxy group into L-arginine-*p*-nitroanilide was unsuccessful. The enzymatic study of trypsin or papain to L-BAPA or DL-BAPA is still in progress.

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