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Syntheses of New Arginine Derivatives as a Substrate for Trypsin or Papain[1]

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Two new enzymic substrates, tosyl-L(D or DL)-arginine-*p*-nitroanilide (L-TAPA, D-TAPA, and DL-TAPA) and acetyl-L(D or DL)-arginine-*p*-nitroanilide (L-AAPA, D-AAPA, and DL-AAPA) were synthesized by the route for benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) reported previously. The previous method for synthesis of L-BAPA was then improved by the direct benzoylation of L-arginine-*p*-nitroanilide dihydrochloride (L-APA), into which N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide was converted by the simultaneous removal of benzyloxycarbonyl and nitro groups with hydrogen fluoride. This route has the advantages of shorter reaction steps and a better over-all yield than in the case of the previous method. The method is also applicable to the synthesis of L(D or DL)-AAPA, but it is hard to prepare L(D or DL)-TAPA by the method.

L-Arginine derivatives of amide,[2-5] the methyl or ethyl ester[6-10] and α- or β-naphthylamide[11,12] have been useful as substrates for trypsin or papain. However, it has been very difficult to synthesize the derivatives of L-arginine-*p*-nitroanilide because of the racemization during the synthetic process, although these materials seem to be useful in measuring photometrically the accurate activity of enzyme.[13] Recently, we prepared benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) without any racemization.[14] Using L- and D-BAPA against papain, it was revealed that D-antipode acts as a competitive inhibitor against L-antipode. These materials were also utilized to study the reaction rate using DL-BAPA as a substrate.[15] In this paper, an improved method for the preparation of L-BAPA and the preparation of tosyl-L (D or DL)-arginine-*p*-nitroanilide hydrochloride (L-TAPA, D-TAPA, and DL-TAPA), acetyl-L (D or DL)-arginine-*p*-nitroanilide hydrochloride (L-AAPA, D-AAPA, and DL-AAPA), L-arginine-*p*-nitroanilide dihydrochloride (L-APA), and benzoyl-D-arginine-*p*-nitroanilide hydrochloride (D-BAPA)[13] will be described. These derivatives will be useful in giving some interesting information about the variation in the reaction mechanism of the enzyme according to the effect of the *N*-protecting groups and the inhibition by D-antipode.

Experimental

The ultraviolet and visible absorption spectra were measured with a Hitachi 124 spectrophotometer. The optical rotations

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were measured with a Yanagimoto Direct-reading Polarimeter, Model OR-10, and an Automatic Recording Polarimeter, Model OR-1. 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. The melting points for the samples over 220°C were determined with a Mitamura hot-stage apparatus.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine-p-nitroanilide (I). This was prepared by a method previously described.[14]

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-DL-arginine-p-nitroanilide (DL-I). This was prepared from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-DL-arginine (50.0 g, 142 mmol) and *p*-nitrophenyl isocyanate (47.0 g, 286 mmol) by the same method as I. Yield, 44 g (65.7%); mp 191°C. Found: C, 50.7; H, 4.78; N, 20.7%. Calcd for $C_{20}H_{23}N_7O_7$: C, 50.7; H, 4.90; N, 20.7%.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-D-arginine-p-nitroanilide (D-I). This was prepared from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-D-arginine[16] (3.0 g, 8.4 mmol) and *p*-nitrophenyl isocyanate (2.8 g, 17.0 mmol) by the same method as I. Yield, 3.1 g (77.5%); mp 178°C; $[\alpha]_D^{29}$ −53.0° (*c* 1.05, HMPA[17]).

N^{ω}-Nitro-L-arginine-p-nitroanilide Hydrobromide (II). This was prepared by the method previously described.[14]

N^{ω}-Nitro-DL-arginine-p-nitroanilide Hydrobromide (DL-II). This was prepared by the same method as II from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-DL-arginine-*p*-nitroanilide (DL-I) (40.0 g, 84.5 mmol), using hydrogen bromide. Yield, 32.0 g (90.1%); mp 155°C. Found: C, 34.5; H, 4.58; N, 22.9; Br, 18.8%. Calcd for $C_{12}H_{18}N_7O_5Br$: C, 34.3; H, 4.32; N, 23.3; Br, 19.0%.

N^{ω}-Nitro-D-arginine-p-nitroanilide Hydrobromide (D-II). This was prepared by the same method as (II) from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-D-arginine-*p*-nitroanilide (D-I) (6.6 g, 13.9 mmol), using hydrogen bromide. Yield, 4.2 g (71.7%); mp 175°C; $[\alpha]_D^{25}$ −43.1° (*c* 1.0, DMF[18]). Found: C, 34.9; H, 4.41; N, 23.0; Br, 18.7%. Calcd for $C_{12}H_{18}N_7O_5Br$: C, 34.3; H, 4.32; N, 23.3; Br, 19.0%.

L-Arginine-p-nitroanilide Dihydrochloride (L-APA. III). N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (I, 15.0 g, 31.7 mmol) was treated with hydrogen fluoride at 0°C for 3 hr to remove the nitro and the benzyloxycarbonyl groups; the procedure was the same as that used for N^{α}-tosyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (V). After the evaporation of hydrogen fluoride, the residue was dissolved in 400 ml of water, then, the solution was extracted with ether to remove the anisole. To the aqueous solution, treated with charcoal, 12M hydrochloric acid (5.2 ml, 62.4 mmol) was added; the solution was then evaporated to dryness in order to convert the hydrofluoride into the hydrochloride. In order to replace the hydrofluoride completely with hydrochloride, the residue was dissolved again in 1M hydrochloric acid (60 ml, 60 mmol) and the solution was evaporated to dryness. The crystalline hydrochloride was collected with ethanol and then dried. 9.7 g (83.3%); mp 242°C. It was recrystallized from water and ethanol. Yield, 9.4 g (80.8%); mp 245°C; $[\alpha]_D^{23}$ +81.7° (*c* 1, water). Found: C, 39.5; H, 5.72; N, 22.7; Cl, 19.1%. Calcd for $C_{12}H_{20}N_6O_3Cl_2$: C, 39.2; H, 5.49; N, 22.9; Cl, 19.3%.

N^{α}-Tosyl-N^{ω}-nitro-L-arginine-p-nitroanilide (V). N^{ω}-Nitro-L-arginine-*p*-nitroanilide hydrobromide (II, 5.0 g, 11.9 mmol) was dissolved in a mixture of tetrahydrofuran and water (9: 1, 50 ml) and then cooled to −3°C. After the addition of triethylamine (3.8 ml, 27.4 mmol) to the solution, tosyl chloride (2.95 g, 15.5 mmol) in 9 ml of tetrahydrofuran was dropped with stirring over 5 min. After reaction for 2 hr, it was kept for another 3 hr at room temperature. Then the solution was evaporated to dryness at room temperature. The residue was crystallized by washing it with ether, water, 1M hydrochloric acid, water, and 5% sodium bicarbonate successively. The crystals were collected by filtration, washed with water and ether, and then dried. Yield, 5.8 g (98.8%); mp 155°C. It was recrystallized from tetrahydrofuran and water. Yield, 5.3 g (90.3%); mp 161°C; $[\alpha]_D^{20}$ −3.6° (*c* 1.0, HMPA). Found: C, 46.2; H, 4.67; N, 19.6%. Calcd for $C_{19}H_{23}N_7O_7S$: C, 46.2; H, 4.70; N, 19.9%.

N^{α}-Tosyl-N^{ω}-nitro-DL-agrinine-p-nitroanilide (DL-V). This was prepared by a method similar to that used for V from N^{ω}-nitro-DL-arginine-*p*-nitroanilide hydrobromide (DL-II) (5.0 g, 11.9 mmol) and tosyl chloride (2.94 g, 15.4 mmol). Yield, 5.2 g (88.6%); mp 214°C. Found: C, 46.1; H, 4.82; N, 19.6%. Calcd for $C_{19}H_{23}N_7O_7S$: C, 46.2; H, 4.70; N, 19.9%.

N^{α}-Tosyl-N^{ω}-nitro-D-arginine-p-nitroanilide (D-V). This was prepared from N^{ω}-nitro-D-arginine-*p*-nitroanilide hydrobromide (D-II) (1.0 g, 2.38 mmol) and tosyl chloride (0.59 g, 3.10 mmol) by the same method as was used for V. Yield, 1.0 g (85.2%); mp 161°C; $[\alpha]_D^{20}$ +3.6° (*c* 1.0, HMPA).

N^{α}-Tosyl-L-arginine-p-nitroanilide Hydrochloride (L-TAPA, VIII). N^{α}-Tosyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (V, 5 g, 10.1 mmol) was mixed with anisole (5 ml, 46.2 mmol) in a HF-reaction cylinder. Forty milliliters of hydrogen fluoride dried with CoF_3 were collected in the cylinder under cooling with liquid nitrogen; the mixture was then allowed to react at 0°C for 40 min with stirring. The excess HF was removed under reduced pressure, and the residue was kept *in vacuo* for another 2 hr. The residue was crystallized by trituration with ether. The crystallized hydrofluoride was suspended in 200 ml of 3M hydrochloric acid and then stirred for one hour at room temperature to convert it into the hydrochloride. After cooling, the crystalline hydrochloride was filtered, washed with 3M hydrochloric acid and a small amount of ice cold water, and then dried. In order to complete the replacement of hydrochloride, a 75 ml portion of 3M hydrochloric acid was added to the hydrofluoride salt in glacial acetic acid and the solution was evaporated *in vacuo* to dryness at room temperature. The residue was again dissolved in a mixed solvent of acetic and hydrochloric acid, and the solvent was removed, repeatedly; then it was recrystallized from ethanol. Yield, 3.7 g (75.3%); mp 245°C; $[\alpha]_D^{23}$ −32.0° (*c* 1.0, DMF). Found: C, 47.2; H, 5.13; N, 17.0; Cl, 7.10%. Calcd for $C_{19}H_{25}N_6O_5ClS$: C, 47.1; H, 5.20; N, 17.3; Cl, 7.31%.

N^{α}-Tosyl-L-arginine-p-nitroanilide p-Toluenesulfonate (L-TAPA·CH_3·C_6H_4·SO_3H, X). L-Arginine-*p*-nitroanilide dihydrochloride (III, 1.4 g, 3.81 mmol) was dissolved in water (56 ml), and then the mixture was cooled to 0°C. After adding an ether solution (56 ml) of tosyl chloride (0.94 g, 4.95 mmol), an aqueous solution (5 ml) of sodium hydroxide (0.50 g, 12.5 mmol) was dropped in over a thirty-minute period with vigorous stirring. The reaction was continued for one hour at 0°C and for 2 hr at room temperature. Carbon dioxide was bubbled into it for a few minutes to precipitate crystals. This material was dissolved in a mixture of 1M hydrochloric acid and glacial acetic acid, and then the solution was evaporated to dryness. The product was collected with water and recrystallized from water. Yield, 0.30 g (12.7%); mp 206°C. Found: C, 50.0; H, 5.16; N, 13.4%. Calcd for $C_{26}H_{32}N_6O_8S_2$: C, 50.3; H, 5.20; N, 13.5%.

N^{α}-Tosyl-DL-arginine-p-nitroanilide Hydrochloride (DL-TAPA, DL-VIII). This was prepared by the same method as

16) H. Yajima and K. Kubo, *J. Amer. Chem. Soc.*, **87**, 2039 (1965).
17) HMPA: hexamethylphosphoramide.
18) DMF: dimethylformamide.

was used for VIII from N^{α}-tosyl-N^{ω}-nitro-DL-arginine-*p*-nitroanilide (DL-V) (4.0 g, 8.11 mmol), using hydrogen fluoride. Yield, 3.1 g (78.9%); mp 224°C. Found: C, 46.7; H, 5.11; N, 17.3; Cl, 7.20%. Calcd for $C_{19}H_{25}N_6O_5ClS$: C, 47.1; H, 5.20; N, 17.3; Cl, 7.31%.

N^{α}-*Tosyl*-D-*arginine*-p-*nitroanilide Hydrochloride (*D-*TAPA.* D-*VIII).* This was prepared by the same method as was used for VIII from N^{α}-tosyl-N^{ω}-nitro-D-arginine-*p*-nitroanilide (D-V) (0.9 g, 1.83 mmol), using hydrogen fluoride. Yield, 0.7 g (79.1%); mp 245°C; $[\alpha]_D^{23}$ +32.3° (*c* 1.0, DMF). Found: C, 47.2; H, 47.2; H, 5.18; N, 17.1; Cl, 7.10%. Calcd for $C_{19}H_{25}N_6O_5ClS$: C, 47.1; H, 5.20; N, 17.3; Cl, 7.31%.

N^{α}-*Acetyl*-N^{ω}-*nitro*-L-*arginine*-p-*nitroanilide (VI).* N^{α}-Nitro-L-arginine-*p*-nitroanilide hydrobromide (II, 10 g, 23.8 mmol) was dissolved in a mixture of tetrahydrofuran and water (9: 1, 100 ml), and then the mixture was cooled to −3°C. After the addition of triethylamine (7.7 ml, 55.6 mmol) to the solution, acetic anhydride (3.2 ml, 31.7 mmol) was dropped into it with stirring in 5 min. After 4 hr, the pH was adjusted to 4 by the addition of 1M hydrochloric acid, and then the solution was evaporated to dryness at room temperature. The residue was crystallized by treatment with 1M hydrochloric acid, filtered, washed with water, and then dried. Yield, 7.8 g (86.0%); mp 185°C. It was then recrystallized from DMF and water. Yield, 7.5 g (82.7%); mp 193°C; $[\alpha]_D^{29}$ +61.7° (*c* 1.0, HMPA). Found: C, 44.5; H, 4.70; N, 25.4%. Calcd for $C_{14}H_{19}N_7O_6$: C, 44.1; H, 5.02; N, 25.7%.

N^{α}-*Acetyl*-N^{ω}-*nitro*-DL-*arginine*-p-*nitroanilide (DL-VI).* This was prepared by the same method as was used for VI from N^{ω}-nitro-DL-arginine-*p*-nitroanilide hydrobromide (DL-II) (5.0 g, 11.9 mmol) and acetic anhydride (1.8 ml, 17.8 mmol). Yield, 3.5 g (77.2%); mp 215°C. Found: C, 44.2; H, 4.93; N, 25.4%. Calcd for $C_{14}H_{19}N_7O_6$: C, 44.1; H, 5.02; N, 25.7%.

N^{α}-*Acetyl*-N^{ω}-*nitro*-D-*arginine*-p-*nitroanilide (D-VI).* This was prepared by the same method as was used for VI from N^{ω}-nitro-D-arginine-*p*-nitroanilide hydrobromide (D-II) (1.0 g, 2.38 mmol) and acetic anhydride (0.36 ml, 3.56 mmol). Yield, 0.6 g (66.2%); mp 193°C, $[\alpha]_D^{29}$ −61.9° (*c* 1.0, HMPA).

N^{α}-*Acetyl*-L-*arginine*-p-*nitroanilide Hydrochloride (*L-*AAPA. IX).* (Method A). N^{α}-Acetyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (VI, 5.0 g, 13.1 mmol) was treated with hydrogen fluoride to remove the nitro group by the same procedure as was used for N^{α}-tosyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (V). After treatment with ether, the dried residue of hydrofluoride was dissolved in a mixture of 1M hydrochloric acid (70 ml) and glacial acetic acid (30 ml): then the solution was evaporated to dryness *in vacuo* at room temperature to convert it into its hydrochloride. The residue was crystallized with acetone, filtered, washed with acetone, and then dried. Yield, 4.6 g (85.5%); mp 135—136°C. This crude material was then dissolved in a small amount of 1M hydrochloric acid, and acetone was added to the mixture until the solution was faintly clouded. N^{α}-Acetyl-L-arginine-*p*-nitroanilide hydrochloride was crystallized out on standing at 0°C with trituration by repeating the gradual addition of acetone and cooling. The crystals were filtered out, washed with acetone, and then dried. Yield, 3.9 g (72.8%); mp 139°C; $[\alpha]_D^{20}$ −19.0° (*c* 1.0, water). Found: C, 41.0; H, 6.00; N, 20.4; Cl, 9.00%. Calcd for $C_{14}H_{21}O_4N_6Cl \cdot 2H_2O$: C, 41.1; H, 6.16; N, 20.6; Cl, 8.67%.

(Method B). L-Arginine-*p*-nitroanilide dihydrochloride (III, 0.3 g, 0.82 mmol) was dissolved in water (12 ml), the solution was cooled to 0°C after ether (12 ml) had then been added. Acetic anhydride (0.09 ml) and then an aqueous solution (1.4 ml) of sodium carbonate (0.14 g, 1.27 mmol) were added into it with vigorous stirring. The reaction mixture was kept for 1 hr at 0°C and then for another hour at room temperature. The solution was evaporated to dryness after being acidified to pH 3 with concentrated hydrochloric acid. The residue was dissolved in a mixture of 1M hydrochloric acid and glacial acetic acid (1:1 v/v, 20 ml) and the solution was evaporated to dryness. After this procedure had been repeated once more, the residue was dissolved in ethanol to remove the sodium chloride and the solution was evaporated. The crude product was recrystallized from 1M hydrochloric acid and acetone by the same procedure as that of Method A. Yield, 0.25 g (74.8%); mp 139°C; $[\alpha]_D^{20}$ −19.0° (*c* 1.0, water).

N^{α}-*Acetyl*-DL-*arginine*-p-*nitroanilide Hydrochloride (*DL-*AAPA,* DL-*IX).* This was prepared by the same method as was used for IX from N^{α}-acetyl-N^{ω}-nitro-DL-arginine-*p*-nitroanilide (DL-VI) (3.0 g, 7.87 mmol), using hydrogen fluoride. Yield, 2.8 g (91.1%); mp 230°C. Found: C, 42.9; H, 5.60; N, 21.2; Cl, 9.00%. Calcd for $C_{14}H_{21}O_4N_6Cl \cdot H_2O$: C, 43.0; H, 5.93; N, 21.5; Cl, 9.07%.

N^{α}-*Acetyl*-D-*arginine*-p-*nitroanilide Hydrochloride (*D-*AAPA,* D-*IX).* This was prepared by the same method as was used for IX from N^{α}-acetyl-N^{ω}-nitro-D-arginine-*p*-nitroanilide (D-VI) (0.5 g, 1.31 mmol), using hydrogen fluoride. Yield, 0.3 g (56.0%); mp 139°C; $[\alpha]_D^{20}$ +19.0° (*c* 1.0, water). This product was lyophilized and stored in a dark place. Found: C, 41.3; H, 5.93; N, 20.1; Cl, 9.0%. Calcd for $C_{14}H_{21}O_4N_6Cl \cdot 2H_2O$: C, 41.1; H, 6.16; N, 20.6; Cl, 8.67%.

N^{α}-*Benzoyl*-L-*arginine*-p-*nitroanilide Hydrochloride (*L-*BAPA,* VII).* (Method A). L-Arginine-*p*-nitroanilide dihydrochloride (III, 5.0 g, 13.6 mmol) was dissolved in water (200 ml) and then cooled to 0°C. After the addition of benzoyl chloride (1.75 ml, 15.2 mmol) and ether (200 ml) to the solution, an aqueous solution (23 ml) of sodium carbonate (2.25 g, 21.2 mmol) was dropped into it with vigorous stirring in 15 min. The reaction mixture was kept for 1 hr at 0°C and for 3 hr at 20°C. The precipitate was then collected by filtration and washed with a small amount of water. To a hot solution of this material in 50% acetic acid (100 ml), 1M hydrochloric acid (50 ml) was added and nitrogen was bubbled in for a few minutes. After it had then been cooled to room temperature, the solution was evaporated to dryness and the residue was treated with ethanol and evaporated repeatedly to remove the acetic acid completely. It was then recrystallized from ethanol. Yield, 4.6 g (77.7%); mp 225°C; $[\alpha]_D^{25}$ +14.2° (*c* 1.0, water). Found: C, 52.2; H, 5.35; N, 19.2; Cl, 8.00%. Calcd for $C_{19}H_{23}N_6O_4Cl$: C, 52.2; H, 5.33; N, 19.3; Cl, 8.15%.

(Method B). N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (I, 5 g, 10.5 mmol) was treated with hydrogen fluoride as has been described in the case of L-arginine-*p*-nitroanilide dihydrochloride (L-APA, III). After the evaporation of the hydrogen fluoride, the residue was dissolved in water (150 ml) and the solution was extracted with ether to remove the anisole. The aqueous solution was then cooled to 0°C, and the pH was adjusted to 9 by the addition of sodium carbonate. Benzoyl chloride (1.25 ml, 10.8 mmol) and ether (10 ml) were vigorously stirred into it, and the reaction was continued for 2 hr. The precipitate in a syrup was crystallized by acidification to pH 1—2 with concentrated hydrochloric acid and then kept overnight in an ice box. The crystalline product was filtered, washed successively with dilute hydrochloric acid, a small amount of ice water, and ether, and then dried. It was subsequently recrystallized from ethanol. Yield, 2.5 g (53.8%); mp 224°C. The product was dissolved in hot water (50 ml) and treated with charcoal; the solution was concentrated to about 15 ml and then crystallized with

trituration. To the mixture, 1M hydrochloric acid (15 ml) was added, after which the mixture was cooled for one hour at 0°C. The crystalline N^{α}-benzoyl-L-arginine-*p*-nitroanilide hydrochloride was filtered, washed with a small amount of water, and then dried. Yield, 2.4 g (52.3%); mp 225°C; $[\alpha]_D^{25}$ +14.2° (*c* 1.0, water). Found: C, 52.7; H, 5.47; N, 19.4; Cl, 8.1%. Calcd for $C_{19}H_{23}N_6O_4Cl$: C, 52.5; H, 5.33; N, 19.3; Cl, 8.15%.

N^{α}-*Benzoyl*-N^{ω}-*nitro*-D-*arginine*-p-*nitroanilide* (D-*IV*). This was prepared by the same method as was used for IV[14] from N^{ω}-nitro-D-arginine-*p*-nitroanilide hydrobromide (D-II) (1.0 g, 2.38 mmol) and benzoyl chloride (0.31 ml, 2.62 mmol). Yield, 0.7 g (66.4%); mp 246°C; $[\alpha]_D^{26}$ −61.0° (*c* 1.12, HMPA).

N^{α}-*Benzoyl*-D-*arginine*-p-*nitroanilide Hydrochloride* (D-*BAPA*, D-*VII*). This compound was prepared by the same method as was used for VII[14] from N^{α}-benzoyl-N^{ω}-nitro-D-arginine-*p*-nitroanilide (D-IV) (0.65 g, 1.47 mmol), using hydrogen fluoride. It has no crystalline water and is sparingly soluble in water. Yield, 0.4 g (62.7%); mp 225°C; $[\alpha]_D^{25}$ −14.2° (*c* 1.0, water). Found: C, 52.7; H, 5.47; N, 19.0; Cl, 8.0%. Calcd for $C_{19}H_{23}N_6O_4Cl$: C, 52.5: H, 5.33; N, 19.3; Cl, 8.15%.

To make it easily soluble in water, it was redissolved in water and lyophilized. The crystals contain crystalline water and were stored in a brown sample bottle. Found: C, 47.9; H, 5.94; N, 17.5; Cl, 7.20%. Calcd for $C_{19}H_{23}N_6O_4Cl \cdot 2.5H_2O$: C, 47.6; H, 5.88; N, 17.5; Cl, 7.39%, mp 100—150° (broad).

The Ultraviolet and Visible Absorption Spectra of L-*TAPA*, L-*AAPA, and* L-*APA*. The ultraviolet and visible absorption spectra of L-TAPA, L-AAPA, and L-APA in an aqueous sodium chloride solution are shown in Fig. 1. The absorption spectra of L-BAPA[14] and *p*-nitroaniline[13] are also shown in Fig. 1. The values of the molar extinction coefficients, ε, at the optimum absorptions are as follows:

L-TAPA: 317 mμ, $\varepsilon = 1.10 \times 10^4$
L-AAPA: 316 mμ, $\varepsilon = 1.14 \times 10^4$
L-APA: 304 mμ, $\varepsilon = 1.14 \times 10^4$

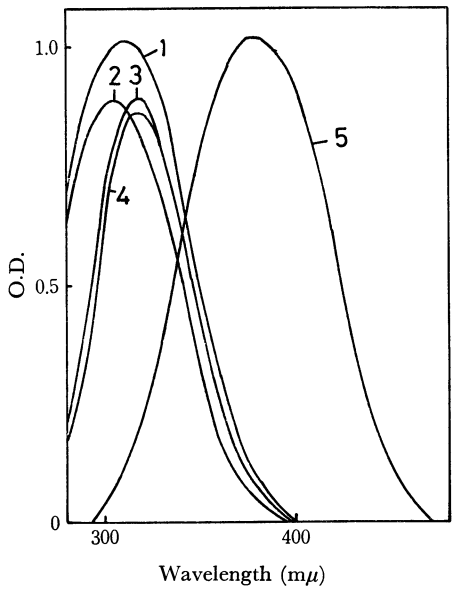
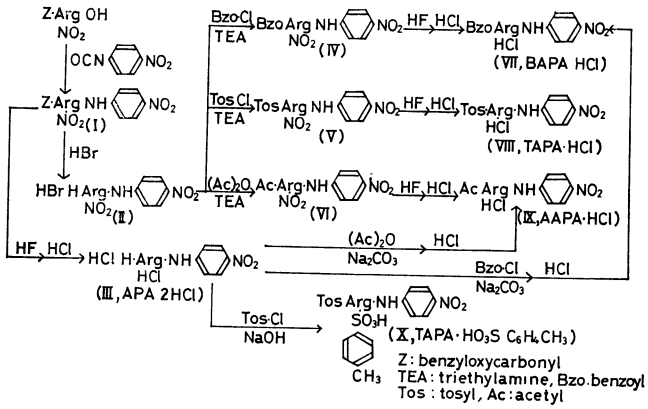


Fig. 1. Absorption spectra of L-AAPA, L-TAPA, and L-APA, all at concentration of 7.8×10^{-5}M in 0.05M NaCl solution, pH 6.2 and 22°C. 1: L-BAPA[12,1.)], 2: L-APA, 3: L-AAPA, 4: L-TAPA, 5: *p*-nitroaniline.[12)]

Results and Discussion

The synthetic routes shown in this paper are summarized in Scheme 1.



Scheme 1.

The Syntheses of L(D *or* DL)-*TAPA*, L(D *or* DL)-*AAPA, and* D-*BAPA*. The synthetic route of optically active L-BAPA described previously[14] was followed in order to avoid the racemization through the formation of oxazolone in the reaction. N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-agrinine-*p*-nitroanilide was prepared by a reaction between N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine and *p*-nitrophenyl isocyanate, because the α-carboxyl group of N^{α}-benzyloxycarbonyl-N^{ω}-nitro-arginine can easily be cyclized into its δ-lactam, also, the amino group of *p*-nitroaniline is too inactive to form an amide bond in the ordinary way. The benzyloxycarbonyl group was removed with hydrogen bromide, and then the product was benzoylated by benzoyl chloride; finally, the nitro group was removed with hydrogen fluoride[19] into L-BAPA. This route was also applicable to the synthesis of L-AAPA (IX) and L-TAPA (VIII) by acetylation or tosylation. The nitro group with hydrogen fluoride could be sufficiently removed in 40 min at 0°C, as was L-BAPA. The hydrofluoride of L-TAPA formed by denitration with hydrogen fluoride is convertible into its hydrochloride up to about 90% by stirring the suspension of the salt in 3M hydrochloric acid for 1 hr at room temperature. Then, the product was dissolved in a mixture of glacial acetic acid and 3M hydrochloric acid (1: 1 v/v) at room temperature and the solvent was evaporated *in vacuo*. Since L-AAPA is soluble in hydrochloric acid, L-AAPA hydrofluoride can be converted into its hydrochloride by the repeated concentration of the solution in a mixture of glacial acetic acid and 1M hydrochloric acid (1: 1 v/v) at room temperature. The calcium nitrate test of hydrogen fluoride showed negative in the L-TAPA hydrochloride and L-AAPA hydrochloride produced. DL-TAPA, and DL-AAPA, and D-TAPA, D-AAPA, and D-BAPA, were also prepared from DL-arginine hydrochloride and D-arginine hydro-

19) 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).

chloride respectively by the same method. These properties are summarized below:

Compound	Mp (°C)	$[\alpha]_D$ (°)	ε at optimum absorption
L-TAPA·HCl	245	−32.0 (*c* 1.0, DMF)	1.10×10^4 (317 mμ)
D-TAPA·HCl	245	+32.3 (*c* 1.0, DMF)	
DL-TAPA·HCl	224	0	
L-AAPA·HCl·$2H_2O$	139	−19.0 (*c* 1.0, water)	1.14×10^4 (316 mμ)
D-AAPA·HCl·$2H_2O$	139	+19.0 (*c* 1.0, water)	
DL-AAPA·HCl·H_2O	230	0	
L-APA·2HCl	245	+81.7 (*c* 1.0, water)	1.14×10^4 (304 mμ)
D-BAPA·HCl	225	−14.2 (*c* 1.0, water)	

The Improved Synthetic Method of L-*BAPA and* L-*AAPA.* The method of synthesizing L-BAPA through the (I)—L-APA(III)—L-BAPA(VII) route had shorter reaction steps and a better overall yield than the previous method.[14] L-Arginine-*p*-nitroanilide dihydrochloride (L-APA, III) was derived from N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine-*p*-nitroanilide (I) by the simultaneous removal of the benzyloxycarbonyl group and the nitro group with hydrogen fluoride, after which the hydrofluoride was converted into its hydrochloride by a method similar to that described above. In this case, the protecting groups were removed with hydrogen fluoride for 3 hr at 0°C, because the complete reaction had hardly proceeded at all in 40 min at 0°C. L-BAPA(VII) was obtainable from L-APA (III) by the benzoylation of the α-amino group with sodium carbonate as a base. The reaction was carried out homogeneously in emulsion by vigorous stirring in a mixture of water and ether (1: 1 v/v). By this route, L-BAPA can be prepared in one step from I without isolating L-APA. The yield of L-BAPA in the method is 52—63%. Moreover, the experimental treatment was simplified exceedingly. L-AAPA was also prepared by the same method, but the expected results were not obtained in the preparation of L-TAPA by the direct tosylation of L-APA. L-APA does not react with tosyl chloride in sodium carbonate, but forms *p*-toluenesulfonate of L-TAPA in sodium hydroxide; the yield was very low and it was very difficult to convert it into its hydrochloride. Therefore, this method seems to be unsuitable for the preparation of L-TAPA.

The Attempts at the Preparations of D-*TAPA and* D-*AAPA by Enzymic Digestion.* Erlanger *et al.* prepared D-BAPA by the enzymic digestion of DL-BAPA.[13] We have now used the method in the preparation of D-TAPA and D-AAPA from DL-TAPA and DL-AAPA, but we found it to be almost entirely unsuitable in both cases. In DL-TAPA, the reaction proceeds very slowly and, a large amount of enzymes and a long reaction time were necessary to complete the reaction; moreover, it was necessary to use a lot of solution for the reaction, because DL-TAPA was more sparingly soluble in water than DL-BAPA. In the case of DL-AAPA, the self-decomposition of DL-AAPA was assumed in a phosphate buffer and the D-AAPA produced was easily soluble in water and was very difficult to separate from the sub-products, such as acetyl-L-arginine or *p*-nitroaniline, and enzymes. Although D-BAPA was prepared from DL-BAPA by the biochemical method, the direct synthesis seems to be more easier than the enzymic preparation. The absorption spectra of TAPA, AAPA, and APA have their bases at about 400 mμ much like BAPA, and the enzymic activity can be measured by means of the amount of *p*-nitroaniline formed at 410 mμ, the same as with BAPA. When TAPA is used as a substrate, DMF or DMSO should be used to increase the solubility in water. AAPA and APA are easily soluble in water and may be used as substrate or inhibitor, although AAPA tends to self-decompose into the deacetylation or cleavage of the amide bond in a phosphate buffer. When L(D)-BAPA was stocked in a crystalline form, a part of it sometimes became sparingly soluble in water, although no changes were assumed in the chromatography or absorption spectrum. The lyophilized crystal contains crystalline water and is more easily soluble in water than the crystal without water. Therefore, it is desirable to use the lyophilize method in the preparation of L(D)-BAPA as a substrate. The details of the substrates will be published elsewhere.

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