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New Synthetic Methods for Benzyloxycarbonyl-L-arginine-*p*-nitroanilide, Benzoyl-L-arginine-*p*-nitroanilide, and Acetyl-L-arginine-*p*-nitroanilide*

Oyin SOMORIN,** Norio NISHI, and Junzo NOGUCHI

Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-ku, Sapporo 060

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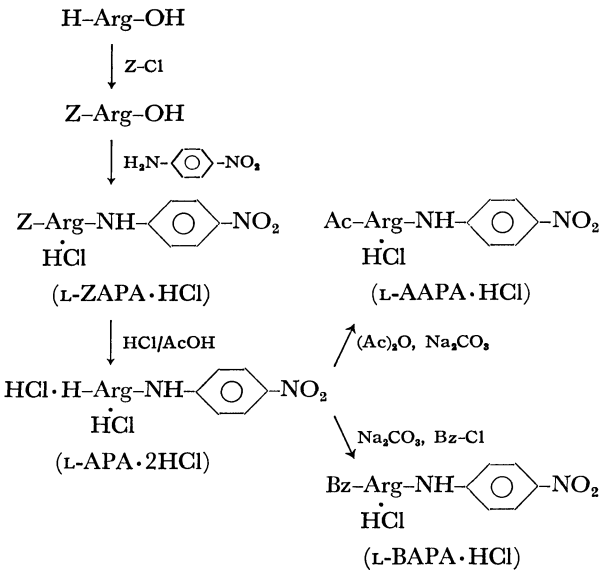
Synopsis. The optically active substrates for two peptidases, benzoyl-L-arginine-*p*-nitroanilide hydrochloride (L-BAPA·HCl) and acetyl-L-arginine-*p*-nitroanilide(L-AAPA·HCl), were readily prepared through the synthesis of benzyloxycarbonyl-L-arginine-*p*-nitroanilide hydrochloride(L-ZAPA·HCl). Benzyloxycarbonyl-L-arginine and *p*-nitroaniline were directly coupled without any racemization. The product, L-ZAPA·HCl, was quantitatively debenzyloxycarbonylated with hydrogen chloride in glacial acetic acid at 50 °C to L-arginine-*p*-nitroanilide dihydrochloride(L-APA·2HCl) which gave, on benzoylation or acetylation, L-BAPA·HCl or L-AAPA·HCl respectively.

N^{α}-Acylated arginine-*p*-nitroanilides are well known to be suitable substrates for trypsin and papain.[1,2] The low basicity of the amino group of *p*-nitroaniline makes it difficult for it to couple with the carboxyl group of arginine activated by the DCCI-, azide-, or active ester-method. The activation of the amino group of *p*-nitroaniline seems to be necessary to facilitate coupling with the carboxyl group of arginine. Hence, in our previous papers on the syntheses of L-BAPA·HCl[3] and other acylated L-arginine-*p*-nitroanilides,[4] the amino group of *p*-nitroaniline was activated by converting it to the isocyanate and the guanidino group of arginine was masked with the nitro group. The synthetic routes reported previously,[3,4] however, are complicated because of the several reaction steps involved.

Convenient syntheses of L-ZAPA·HCl, L-BAPA·HCl, and L-AAPA·HCl have now been achieved, as is shown in Scheme 1. L-ZAPA·HCl was prepared by the direct coupling of N^{α}-benzyloxycarbonyl-L-arginine and *p*-nitroaniline using a modification of the procedure of Erlanger *et al.*[5] for the synthesis of DL-BAPA·HCl. As it had been expected, there was no racemization in the synthesis of L-ZAPA·HCl, unlike as in the case of the benzoyl analog. In the modified coupling procedure, only one equivalent mole of the tertiary amine was used, since two equivalent moles as in the procedure of Erlanger *et al.*[5] caused a decrease in the yield of L-ZAPA·HCl. The order of the addition of reagents affected the yield of L-ZAPA·HCl. The optimum yield was obtained by the following procedure: a mixture of triethylamine and *p*-nitroaniline in diethyl phosphite was added to a solution of phosphorus pentoxide in diethyl phosphite. A solution of benzyloxycarbonyl-L-arginine containing phosphoric acid in diethyl phosphite was then added to the above mixture. The addition of reagents in this order is consistent with the mechanism of reaction proposed by Schramm and Wissmann.[6] L-ZAPA·HCl was successfully debenzyloxycarbonylated to L-APA·2HCl with hydrogen chloride in acetic acid at 50 °C. The yield of L-APA·2HCl was quantitative, and its melting point and its specific optical rotation were identical with those previously reported.[4] L-AAPA·HCl and L-BAPA·HCl have the same physical constants (melting points and specific optical rotations) as those reported by Nishi *et al.*[4]



Scheme 1. Improved and simplified synthetic routes for benzoyl-L-arginine-*p*-nitroanilide hydrochloride (L-BAPA·HCl) and acetyl-L-arginine-*p*-nitroanilide hydrochloride (L-AAPA·HCl). Z: Benzyloxycarbonyl, Ac: acetyl, Bz: benzoyl.

From the result of the enzymatic hydrolysis of L-ZAPA·HCl by trypsin, the new substrate, L-ZAPA·HCl seems to be more excellent than L-BAPA·HCl and tosyl-L-arginine-*p*-nitroanilide hydrochloride (L-TAPA·HCl) because it can be prepared easily and because of its higher solubility in water and higher susceptibility to tryptic hydrolysis, as is shown in Fig. 1. The details of L-ZAPA·HCl as a substrate for peptidases are under investigation.

Experimental

The ultraviolet and visible absorption spectra were measured with a Hitachi 323 Automatic Spectrophotometer. The optical rotations were measured with a Yanagimoto Automatic

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** Japan Society for the Promotion of Science Visiting Scholar from Chemistry Department, University of Lagos, Nigeria.

Recording Polarimeter, Model OR-1. The elemental analyses were carried out by means of a Yanagimoto CHN Corder, Model MT-2; the results for the known products agreed with the theoretical values. The melting points for samples over 220 °C were determined with a Mitamura hot-stage apparatus.

*N^{α}-Benzyloxycarbonyl-*L*-arginine-*p*-nitroanilide Hydrochloride (*L*-ZAPA·HCl).* Solution A, containing *p*-nitroaniline(4.14 g, 0.03 mol) and triethylamine(4.2 ml, 0.03 mol) in diethyl phosphite(15 ml), was added to Solution B, containing phosphorus pentaoxide(8.55 g, 0.06 mol) in diethyl phosphite(30 ml). A solution of N^{α}-benzyloxycarbonyl-L-arginine(9.24 g, 0.03 mol) containing 85% phosphoric acid (2.1 ml, 0.03 mol) in diethyl phosphite(30 ml) was then added to the mixture of Solution A and B. The reaction mixture was heated on a water bath with stirring for 2 h in a well-ventilated hood. Diethyl phosphite was removed *in vacuo*, and the residual oil dissolved in 300 ml of 1 M HCl after heating at 85 °C for about 5 min. After the solution had stood in the refrigerator overnight, a semisolid oil was precipitated. This semisolid oil was separated by decanting off the acid solution, washed with cold water, and then dried. The oily product which crystallized on triturating with ethyl acetate was filtered and washed with small quantities of ethyl acetate and cold acetone. The crystalline product was recrystallized by dissolving it in hot water, and after it had been cooled to about 50 °C, an equal volume of 1M HCl was added After the solution had stood at 4 °C overnight, an almost colorless crystalline product was obtained. Yield, 7.2 g (50%); mp 181—182 °C; $[\alpha]_D^{20}-16.0°$ (*c* 1, ethanol) Found: C, 51.62; H, 5.29; N, 18.05; Cl, 7.59%. Calcd for $C_{20}H_{25}N_6O_5Cl$: C, 51.64; H, 5.42; N, 18.08; Cl, 7.64%.

L-Arginine-p-nitroanilide Dihydrochloride(L-APA·2HCl). A solution of L-ZAPA·HCl (5 g) in pure glacial acetic acid (200 ml) was saturated with dry hydrogen chloride at 50 °C for 90 min. The reaction mixture was then evaporated *in vacuo* to a small volume, and the crystalline product was precipitated with dry ether. The product was recrystallized from water/acetone. Yield, 3.55 g (90%); mp 245 °C; $[\alpha]_D^{20}+80.6°$ (*c* 1, water)

Benzoyl-L-arginine-p-nitroanilide Hydrochloride(L-BAPA·HCl) and Acetyl-L-arginine-p-nitroanilide Hydrochloride(L-AAPA·HCl). L-BAPA·HCl and L-AAPA·HCl were prepared from L-APA·2HCl by the procedures reported by Nishi and Noguchi.[4] The melting points and specific optical rotations were identical with those previously reported.[4]

The Absorption Spectra and Tryptic Digestion of L-ZAPA·HCl and Other L-Arginine-p-nitroanilides. The wavelength of maximum absorption, λ_{max}, for L-ZAPA·HCl at a concentration of 7.8×10^{-5} M was 315 nm with a molar extinction coefficient of 1.41×10^4. The enzymatic hydrolyses of L-ZAPA·HCl, L-BAPA·HCl, and L-TAPA·HCl are shown in Fig. 1; they were determined by the measurement of *p*-nitroaniline at 410 nm, at which wavelength neither the L-ZAPA·HCl nor any of the other L-arginine-*p*-nitroanilides[4] has any absorbance.

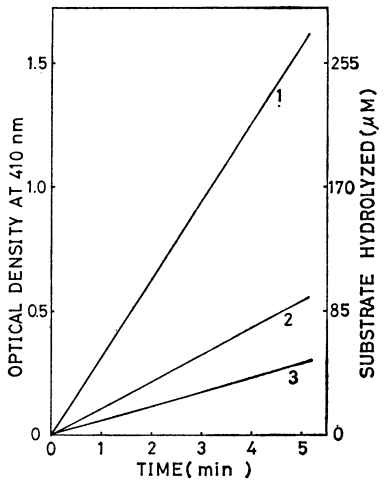

Fig. 1. Tryptic hydrolyses of 1. L-ZAPA·HCl, 2. L-BAPA·HCl, and 3. L-TAPA·HCl as a function of time. Initial concentration of *p*-nitroanilide substrate: 10^{-3}M in 0.05M tris-HCl buffer (pH 8.2) containing 0.02M $CaCl_2$ and 1% dimethylformamide. Trypsin concentration was 10 μg/ml and incubation period was 300 s at 25 °C.

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