Synthesis of New Arginine Derivatives as Substrates of Trypsin

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Eight new chromogenic arginine substrates: N^{α}-benzyloxy-carbonyl-L-arginine 3,5-dinitroanilide hydrochloride (L-ZADA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitroanilide hydrochloride (L-ZANA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-chloroanilide hydrochloride (L-ZANCA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-bromoanilide hydrochloride (L-ZANBA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-fluoroanilide hydrochloride (L-ZANFA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-iodoanilide hydrochloride (L-ZANIA·HCl), N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-(methylsulfonyl)anilide hydrochloride (L-ZANMA·HCl), and N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-(trifluoromethyl)anilide hydrochloride (L-ZANTA·HCl) were synthesized by coupling 5-substituted 3-nitroanilines with N^{α}-benzyloxycarbonyl-L-arginine hydrochloride. These substrates were found to be susceptible to trypsin-catalyzed hydrolysis. These series of substrates are suitable for studying electronic effects on trypsin activity.

Several workers[1-12] have studied the dependence of the electronic effects of substituents in the substrate molecules on the reactivity of chymotrypsin, but there has been very few of such systematic studies on the activity of trypsin. Studies on the relationship of the electronic effects of substituents in the substrates and enzyme activity have been very useful in elucidating the mechanism of action of enzyme catalysis. Both chymotrypsin and trypsin exhibit some remarkable similarities. For example, trypsin and chymotrypsin are proteolytic enzymes which are protein in nature, containing no co-enzyme or prosthetic group and therefore the catalytic activity of these enzymes must be associated directly with the constituents of the protein.[13] Their precursors trypsinogen and chymotrypsinogen are both important constituents of pancreatic juice. These two strikingly similar enzymes are serine proteases with unique serine residue which reacts with organophosphorus compounds to yield inactive derivatives.[14] Besides possessing identical molecular weights both enzymes possess only one active site per molecule which is defined by the reactive serine residue and the common sequence Gly–Asp–Ser–Gly.[15,16] Chymotrypsin and trypsin have mechanistic similarities as evident from the various indications in the literature that trypsin-catalyzed hydrolyses, like those of chymotrypsin, proceed through an acyl-enzyme intermediate.[16] Hence it would be expected that the electronic factors which influence the reactivity of chymotrypsin could similarly influence the reactivity of trypsin.

In order to facilitate the study of these electronic effects, chromogenic substrates would be most desirable since such substrates offer the advantage of producing their own chromogen on enzymic hydrolysis which can easily be quantitatively estimated spectrophotometrically. Most of the substrates previously reported for the studies of the electronic effects on chymotrypsin activity are not chromogenic. In this paper, synthesis of new series of chromogenic substrates suitable for studying the electronic effects of substituents in the substrate molecules on the reactivity of trypsin is reported. The series include N^{α}-benzyloxycarbonyl-L-arginine 5-substituted 3-nitroanilide derivatives. The substrates are unique because of the presence of the nitro group at the 3-position (since the nitro group confers chromogenicity on the substrate) and the presence of various substituents at the 5-position which could be conveniently modified to yield a series of substrates that are suitable for studying the electronic effects on the trypsin activity. In this study the substituents at the 5-position include nitro, methylsulfonyl, trifluoromethyl, fluoro, chloro, bromo, and iodo groups.

Abbreviations: L-ZADA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3,5-dinitroanilide hydrochloride; L-ZANA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitroanilide hydrochloride; L-ZANCA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-chloroanilide hydrochloride; L-ZANBA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-bromoanilide hydrochloride; L-ZANFA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-fluoroanilide hydrochloride; L-ZANIA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-iodoanilide hydrochloride; L-ZANMA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-(methylsulfonyl)anilide hydrochloride; L-ZANTA·HCl, N^{α}-benzyloxycarbonyl-L-arginine 3-nitro-5-(trifluoromethyl)anilide hydrochloride, and DMF, *N,N*-dimethylformamide.

Experimental

All the reagents used throughout this investigation are of analytical grades. The ultraviolet and visible absorption spectra were measured with a Zeiss PMQ3 Spectrophotometer attached to Haake F3 thermostated water bath. The optical rotations were measured with a Perkin Elmer recording polarimeter, Model 241MC. Elemental analyses were carried out by Yanagimoto CHN Corder Model MT-2 at the Analytical Laboratory of Hokkaido University, Sapporo, Japan. Melting points were determined by Reichert melting point apparatus. The elemental analysis and melting points for all known compounds were found to agree with the theoretical and literature values.

Table 1. Elemental Analyses and Optical Rotations of New Chromogenic Arginine Substrates

Substrate	Mp θ_m/°C	$[\alpha]_D^{25}$	Fomula	Analyses					
				Calcd (%)			Found (%)		
				C	H	N	C	H	N
L-ZADA·HCl	222—224	−19.0°	$C_{20}H_{24}N_7O_7Cl$	47.09	4.75	19.23	47.10	4.77	19.04
L-ZANA·HCl	187—188	−19.3°	$C_{20}H_{25}N_6O_5Cl$	51.64	5.42	18.08	51.67	5.38	18.17
L-ZANCA·HCl	214—215	−15.8°	$C_{20}H_{24}N_6O_5Cl_2$	48.08	4.85	16.83	48.12	4.88	16.91
L-ZANBA·HCl	220—221	−14.6°	$C_{20}H_{24}N_6O_5ClBr$	44.15	4.45	15.45	44.09	4.38	15.35
L-ZANFA·HCl	215—216	−18.9°	$C_{20}H_{24}N_6O_5ClF$	49.72	5.01	17.40	49.88	5.09	17.66
L-ZANIA·HCl	203—204	− 9.6°	$C_{20}H_{24}N_6O_5ClI$	40.63	4.10	14.22	40.92	4.17	13.94
L-ZANMA·HCl	162—163	−20.3°	$C_{21}H_{27}N_6O_7ClS$	46.43	5.01	15.47	46.22	4.94	15.19
L-ZANTA·HCl	195—196	−14.7°	$C_{21}H_{24}N_6O_5ClF_3$	47.31	4.54	15.77	47.49	4.68	15.83

Buffer. 0.05 M(1 M=1 mol dm^{-3}) tris(hydroxymethyl)-aminomethane hydrochloride buffer solutions containing 0.04 M calcium chloride at pH values ranging from 6.5 to 9.5 were prepared.

Enzyme. Twice crystallized bovine pancreatic trypsin with a specific activity of 182 units mg^{-1} protein was purchased from Wako Pure Chemicals Industries, Japan. The trypsin activity was determined using L-TAME·HCl as a substrate according to the Worthington Procedure.[17]

Preparation of 5-Substituted 3-Nitroanilides. 3,5-Dinitroaniline, 3-nitro-5-chloroaniline 3-nitro-5-bromoaniline, 3-nitro-5-iodoaniline, 3-nitro-5-fluoroaniline, 3-nitro-5-(methylsulfonyl)aniline, and 3-nitro-5-(trifluoromethyl)-aniline were prepared according to the procedure reported by Dosumu.[18]

Synthesis of the Chromogenic Arginine Substrates, N^{α}-Benzyloxycarbonyl-L-arginine 5-Substituted 3-Nitroanilide Hydrochloride. The 3-nitro-5X-aniline (X=H, CF_3, NO_2, SO_2CH_3, F, Cl, Br, and I) was coupled to N^{α}-benzyloxycarbonyl-L-arginine hydrochloride by the method reported by Somorin et al.[19] Solution A containing the 3-nitro-5X-aniline (0.03 mol) and triethylamine (4.2 ml, 0.03 mol) in diethyl phosphonate (15 ml) was added to Solution B which contained phosphorus pentaoxide (8.55 g, 0.06 mol) in diethyl phosphonate (30 ml). Solution of N^{α}-benzyloxy-carbonyl-L-arginine (9.24 g, 0.03 mol) containing 85% phosphoric acid (2.1 ml, 0.03 mol) in diethyl phosphonate (30 ml) was added to the mixture of solutions A and B described above. The reaction mixture was heated on a water bath with stirring for 2 h in a well ventilated fume-cupboard. Diethyl phosphonate was removed in vacuo and the residual oil dissolved in 300 ml of 1M HCl after heating at 85 °C for about 5 minutes. On refrigerating, a semi-solid oil precipitated. The oil was separated by decanting off the acid solution, washed with cold water and dried. The oily product crystallized on triturating with ethyl acetate and was recrystallized from dilute hydrochloric acid. The mp, elemental analysis, and optical rotation of each chromogenic arginine substrate is recorded in Table 1.

Enzymatic Activity Determination. The initial rates of trypsin catalyzed hydrolyses of the anilide substrates were determined by continuous measurement of free 5-substituted 3-nitroaniline while the reaction proceeded in a themostated cuvette inserted in a Zeiss PMQ3 Model Spectrophotometer attached to a Haake F3 thermostated water bath. The concentration of the liberated substituted aniline was determined at 400 nm at which wavelength all the substrates have no contribution to the absorbance.

Results and Discussion

The synthesis of arginine substrates has always been a difficult task. The low basicity of the amino group in the 5-substituted 3-nitroaniline makes it difficult to couple with the carboxyl of arginine which has been activated by the azide, dicyclohexyl-carbodiimide or active ester method.[20] Therefore the activation of the amino group of the aniline derivatives seems to be essential for facilitating the coupling with the carboxyl group of arginine. The series of new arginine substrates reported in this paper were readily prepared without racemization through the condensation of 5-substituted 3-nitroanilines and N^{α}-benzyloxycarbonyl-L-arginine hydrochloride using the coupling procedure reported by Somorin et al.[19] The relative initial rates of trypsin catalyzed hydrolysis of the various substrates were determined and tabulated in Tables 2 and 3.

It has been reported that trypsin catalyses the hydrolysis of anilides via the acyl-enzyme intermediate.[16] The overall mechanism can be represented by the equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^1 + P_1 \xrightarrow{k_3} E + P_2,$$

where k_2 and k_3 are the first-order rate constants for the acylation and deacylation steps respectively; P_1 and P_2 are products. It is known that the acylation step is the rate limiting step for amide and anilide substrates.[21,22] The same mechanism operates in the chymotrypsin-catalyzed hydrolysis of anilides and its acylation step has been studied in detail by varying the leaving group P_1. Through the determination of rate constant for the acylation step, k_2, several workers concluded that a proton transfer was involved in the acylation step.[5,6,8,11] Studies of

Table 2. Relative Rates of Trypsin Catalyzed Hydrolysis of Chromogenic Substrates

Substrate	Rate	Relative rates
	μmoles min^{-1}	ratio
L-ZANA·HCl	100	1.43
L-ZANTA·HCl	92	1.31
L-ZANMA·HCl	76	1.09
L-ZANFA·HCl	70	1.0

Table 3. Relative Rates of Trypsin Catalyzed Hydrolysis of the Halogen Derivatives of the New Arginine Chromogenic Substrates

Substrate	Rate	Relative rates
	μmoles min^{-1}	ratio
L-ZANFA·HCl	70	1.0
L-ZANCA·HCl	134	1.91
L-ZANBA·HCl	161	2.30
L-ZANIA·HCl	189	2.70

Parker and Wang[8] on the acylation step of α-chymotrypsin catalyzed hydrolysis of anilides suggest a pre-transition state protonation of the substrate followed by the usual nucleophilic attack of the protonated substrate by the serine residue.

The results in Table 1 suggest that the electron-withdrawing substituents (-F, $-CF_3$, and $-SO_2Me$) inhibit the rate of trypsin catalysed hydrolysis of the anilide substrates. It has been suggested that electron-withdrawing substituents hinder the rate of chymotrypsin catalyzed hydrolysis of anilides by inhibiting the proton transfer in the removal of the substituted aniline.[9] This is consistent with the mechanism proposed by Inward and Jencks[23] in which a conjugate acid of the catalyst (histidinium cation) donates a proton to the leaving group which is 5-substituted 3-nitroaniline.

The results in Table 2 show that the rates of enzyme-catalyzed hydrolysis of the halogen derivatives of the arginine substrates decrease in the order: iodo>bromo>chloro>fluoro. This unique sequence, which is characteristic of the halogen substituted chromogenic substrates, is the reverse of the order for the inductive effect which follows the order: fluoro>chloro>bromo>iodo. The details of the kinetics for the trypsin-catalyzed hydrolysis of these new arginine chromogenic substrates will be published elsewhere.

References

1) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).
2) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962).
3) W. F. Sager and P. C. Parks, *J. Am. Chem. Soc.*, **85**, 2678 (1963).
4) W. F. Sager and P. C. Parks, *Proc. Nat. Acad. Sci. U.S.A.*, **52**, 408 (1964).
5) T. Inagami, S. S. York, and A. Patchornik, *J. Am. Chem. Soc.*, **87**, 126 (1965).
6) H. F. Bundy and C. L. Moore, *Biochemistry*, **5**, 808 (1966).
7) J. H. Wang and L. Parker, *Proc. Natl. Acad. Sci. U.S.A.*, **58**, 2451 (1967).
8) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).
9) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem.*, **65**, 809 (1969).
10) M. Caplow, *J. Am. Chem. Soc.*, **91**, 3639 (1969).
11) A. Williams, *Biochemistry*, **9**, 3383 (1970).
12) M. Philipp, R. M. Pollack, and M. L. Bender, *Proc. Nat. Acad. Sci., U.S.A.*, **70**, 517 (1973).
13) M. L. Bender, G. R. Schonbaum, and G. A. Hamilton, *J. Polymer Sci.*, **49**, 75 (1961).
14) B. S. Hartley, *Ann. Rev. Biochem.*, **29**, 45 (1960).
15) G. H. Dixon, D. L. Kauffman, and H. Neurath, *J. Am. Chem. Soc.*, **80**, 1260 (1958).
16) M. L. Bender, and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).
17) Worthington Descriptive Manual, (1972).
18) M. Dosumu, Ph. D. Thesis submitted to Chem. Dept. University of Lagos (1975).
19) O. Somorin, N. Nishi, and J. Noguchi, *Bull. Chem. Soc. Jpn.*, **51**, 1255 (1978).
20) J. P. Greenstein, and M. Winitz, "Chemical Procedures for Synthesis of Peptides" in "Chemistry of the Amino Acids," John Wiley and Sons, Inc. (1961) Vol. 2. p. 763.
21) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **85**, 356 (1963).
22) T. Inagami, and J. M. Sturtevant, *Biochem. Biophys. Res. Commun.*, **14**, 69 (1964).
23) P. W. Inward and W. P. Jencks, *J. Biol. Chem.* **240**, 1986 (1965).