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Proteolytic Enzymes. II.[1] Studies on the Requirement for the Active Center of Trypsin with Amidinobenzoates as Substrates[2]

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Isomers of benzamidine series containing a hydrolyzable ester group have been synthesized: Ethyl **1a** and *p*-nitrophenyl *p*-amidinobenzoates **1b**, and ethyl **2a** and *p*-nitrophenyl *m*-amidinobenzoates **2b**. The *p*-isomers **1a**, **1b** were found to be fairly good substrates while the *m*-isomers **2a**, **2b** were poor substrates in spite of their good binding for trypsin as measured by kinetic treatment. Characteristic behaviors of these isomers to trypsin were interpreted in terms of the structural requirements of the active center of the enzyme by taking advantage of their reigid conformations.

Many papers have been published on the active center of trypsin. The role of His[40] and Ser[177] at the "catalytic site" in the enzymatic process have been largely clarified by chemical modification and kinetic methods.[4-8] Like other enzymes, however, little has been known of "specificity site" in the active site of trypsin,[9] since only limited approaches have been available for studies of this problem, among which the studies of binding of competitive inhibitors to the enzyme may be cited.[12]

In our related work,[13] the requirements for "specificity site" of the enzyme was investigated using aromatic amidines as competitive inhibitors. In the course of the studies, it was found that benzamidine derivatives containing a hydrolyzable ester bond, *i.e.*; ethyl *p*-amidinobenzoate **1a** and *p*-nitrophenyl *p*-amidinobenzoate hydrochloride (NPAB; **1b**), can be employed as useful quasi-substrates[14] in the study of the enzyme.[1]

In an effort to elucidate more detailed aspects of the active center of trypsin, alteration of relative spatial arrangement between an amidine group and an ester group in the substrate may be useful. The present paper is mainly concerned with the synthesis and measurement of the kinetic parameters of the *meta* derivative **2b** of NPAB. Although the preparation of the ethyl ester **1a** and the methyl ester **2c** has been described by Shaw, *et al.*,[15] this *p*-nitro-

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2) Presented before the Annual Meeting of the Japanese Biochemical Society, Nov. 1967, Sakai and Oct. 1968, Tokyo. For Abstracts of papers: *a*) K. Tanizawa, S. Ishii and Y. Kanaoka, *Seikagaku*, **39**, 150 (1967); *b*) *Idem, ibid.*, **40**, 275 (1968).
3) Location: *Kita-12, Nishi-6, Sapporo.*
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5) V. Tomášek, E.S. Severin and F. Šorm, *Biochem. Biophys. Res. Comm.*, **20**, 545 (1965).
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8) M.L. Bender and E.T. Kaiser, *J. Am. Chem. Soc.*, **84**, 2556 (1962).
9) Recently chemical modification techniques have provided evidences that carboxyl groups, particularly that of Asp[171], are responsible for the specificity of trypsin (*cf.* ref. 10, 11).
10) G. Feinstein, P. Bodlaender and E. Shaw, *Biochemistry.*, **8**, 4949 (1969).
11) A. Eyl and T. Inagami, *Biochem. Biophy. Res. Comm.*, **38**, 149 (1970).
12) M. Mares-Guia and E. Shaw, *J. Biol. Chem.*, **240**, 1579 (1965).
13) K. Tanizawa, S. Ishii and Y. Kanaoka, *Seikagaku*, **38**, 530 (1966); K. Tanizawa, S. Ishii, K. Hamaguchi and Y. Kanaoka, in preparation.
14) S.J. Singer, "Advances in Protein Chemistry," Vol. 22, ed. by C.B. Anfinsen, Jr., M.L. Anson, J.T. Edsall and F.M. Richard, Academic Press. New York, 1962, p. 1.
15) M. Mares-Guia, E. Shaw and W. Cohen, *J. Biol. Chem.*, **242**, 5777 (1967).

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phenyl ester **2b** was especially needed for the systematic comparison of kinetic behaviors of these substrates.

$$\mathrm{Cl^- \; (H_2N)(H_2N^+{=})C{-}C_6H_4{-}CO_2R}$$ **1** (para)

$$\mathrm{Cl^- \; (H_2N)(H_2N^+{=})C{-}C_6H_4{-}CO_2R}$$ **2** (meta)

a: $R=C_2H_5$ **b**: $R=C_6H_4NO_2$ (p) **c**: $R=CH_3$

Experimental[16]

Materials——Trypsin (twice crystallized, lyophilized) was a Worthington preparation, Lot TRL-6261. *m*- and *p*-toluamidine hydrochloride were prepared from the corresponding nitriles through a common route *via* the iminoester hydrochloride, followed by ammonolysis.[13] The synthesis of ethyl *p*-amidinobenzoate hydrochloride **1a** and *p*-nitrophenyl *p*-amidinobenzoate hydrochloride **1b** were reported previously.[1] The preparation of ethyl *m*-amidinobenzoate hydrochloride **2b** was carried out by the same method as the *p*-derivatives (Chart 1).

m-$NC{-}C_6H_4{-}CO_2H$ $\xrightarrow[\text{EtOH}]{\text{HCl}}$ *m*-$(EtO)(H_2N^+{=})C{-}C_6H_4{-}CO_2Et \; Cl^-$ $\xrightarrow{NH_3}$ *m*-$(H_2N)(H_2N^+{=})C{-}C_6H_4{-}CO_2Et \; Cl^-$ (**2a**) $\xrightarrow{\text{NaOH}}$ *m*-$(H_2N)(H_2N^+{=})C{-}C_6H_4{-}CO_2^-$ $\xrightarrow{SOCl_2}$ *m*-$(H_2N)(H_2N^+{=})C{-}C_6H_4{-}COCl \; Cl^-$ $\xrightarrow[\text{pyridine}]{p\text{-nitrophenol}}$ *m*-$(H_2N)(H_2N^+{=})C{-}C_6H_4{-}CO_2{-}C_6H_4{-}NO_2 \; Cl^-$

Chart 1

m-Cyanobenzoic acid was dissolved in a large excess of EtOH and the solution was saturated with dry HCl with cooling in an ice bath. After allowed to stand over-night at room temp. the solvent was evaporated *in vacuo*. The residual *m*-ethoxycarbonylbenzimidate hydrochloride was washed with ether and dried in a dessicator over alkali. The hydrochloride was then treated with 1.36 equivalents of 3.5% w/w NH_3 in EtOH at room temp. for 12 hr. Evaporation of the EtOH *in vacuo* and recrystallization gave **2a** in pure state. **2a** was then saponified with 1N NaOH at room temp. and the carboxylic acid was converted to acid chloride with thionyl chloride as usual but a long reaction time was needed compared with the *p*-derivative. The acid chloride was coupled with an equimolar amount of *p*-nitrophenol in DMF solution in the presence of pyridine.

Ethyl *m*-Amidinobenzoate Hydrochloride **2a**: Recrystallization from EtOH–ether gave colorless prisms, mp 199—201°. IR $_{max}$ cm^{-1}: 1730 (COOEt), 1670 (C=N amidine). *Anal.* Calcd. for $C_{10}H_{11}O_2N_2\cdot HCl$: C, 52.52; H, 5.47; N, 12.35. Found: C, 52.47; H, 5.47; N, 12.33.

p-Nitrophenyl *m*-Amidinobenzoate Hydrochloride **2b**: Recrystallization from acetonitrile gave pale yellow crystalline powder, mp 211—213°. IR $_{max}$ cm^{-1}: 1750 (C=O *p*-nitrophenyl ester), 1665 (C=N amidine). *Anal.* Calcd. for $C_{14}H_{11}O_4N_3\cdot HCl$: C, 52.35; H, 3.74; N, 13.07. Found: C, 52.35; H, 3.54; N, 12.93.

Measurement of Kinetic Parameters——Rate of trypsin–catalyzed or spontaneous hydrolysis of **1a** and **2a** was measured with a Radiometer pH-stat titrator Model TTTlc at 25° in 200 ml of 0.1M KCl containing 0.02M $CaCl_2$ maintained at pH 8.2 by addition of 0.02N NaOH in atmosphere of nitrogen. Substrate concentration was 1.53×10^{-5}—2.62×10^{-3}M and trypsin concentration was 1.90×10^{-6}M. Observed initial velocities were plotted according to Lineweaver and Burk[17] so that K_m and k_{cat} defined in equation (1) can be determined as listed in Table I.

In the case of *p*-nitrophenyl *m*- and *p*-amidinobenzoate hydrochloride, **2b** and **1b**, the rate assay was carried out also by photometrical determination of liberated *p*-nitrophenol at 405 or 320 mμ using an ex-

16) All melting points are uncorrected. UV and IR spectra were determined using a Hitachi EPS-3T spectrophotometer and a JASCO IR-S infrared spectrophotometer respectively.

17) L. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

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perimentally determined value 17600 as ε_{405} mμ at pH 8.2 or 8200 as ε_{320} mμ at pH 5.4 and 4.7 (reported pK_a value of *p*-nitrophenol, 7.16[18]). Three ml of buffer solution (0.05M Tris, pH 8.2 or 0.1M acetate, pH 5.4, 4.7) and 50 μl of a substrate solution (in DMF) were pipetted in a 1 cm quartz cuvette placed in a thermostated cuvette holder at 25°. The enzymatic reaction was then initiated by introduction of an aliquot (10—50 μl) of enzyme solution in 0.001N HCl. [*S*]: 1.8×10^{-5}—7.9×10^{-4}M, [*E*]: 9.35×10^{-6}—3.94×10^{-5}M. In the pH-stat titration method the rate was measured in 100 ml of 0.1M KCl, 0.02M $CaCl_2$ containing 0.025—2.0% of DMF, [*S*]: 2.5×10^{-6}—2.0×10^{-4}M, [*E*]: 3.72×10^{-7}M.

The reaction rate was calculated assuming that two equivalent of anion are released from 1 mole of substrate hydrolyzed at pH 8.2, and one equivalent at pH 4.7 and 5.4, because in the pH range above 7.16 *p*-nitrophenol ionically dissociates. The rate of hydrolysis of **1b** and **2b** was found independent of substrate concentration at every pH in either of methods indicating that they correspond to V_{max}. Revised values were shown in Table I for the *p*-derivatives instead of the previous ones.[1] Estimation of enzyme concentration was based on optical factor 0.65 at 280 mμ.

Determination of pK_a Values of *m*- and *p*-Toluamidine——About 2 mg of free amidine were used in the measurement by a Radiometer automatic titrator Model TTTlc, with 0.1N HCl as a titrant.

Determination of K_i Values of *m*- and *p*-Toluamidine——N-Benzoyl-DL-arginine *p*-nitroanilide (DL-BANA) was used as a substrate.[19] K_i values were obtained from Dixon plots following steady-state kinetic treatment. Assay was carried out at 25° in 0.05M tris buffer containing 0.02M $CaCl_2$ and 1% DMSO. Concentrations of trypsin and DL-BANA were 2.3×10^{-7}M and 4.8—1.6×10^{-4}M respectively.

Result

The pathway of the tryptic hydrolysis of substrates is considered at present to be as shown in Chart 2. The Michaelis complex, *ES*, proceeds to form an acyl-enzyme intermediate, *E*-A, with k_2 as the rate constant of acylation. In the next deacylation step, the acyl–enzyme decomposes with the rate constant k_3.[20]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow[\searrow B]{k_2} E-\mathrm{A} \xrightarrow[H_2O \swarrow]{k_3} E+\mathrm{A}$$

Chart 2

The Michaelis constant obtained experimentally in the usual way is an "apparent" one as represented in equation (2). When the rate constant k_2 is much larger than k_3, K_m (app) becomes much less than the true Michaelis constant K_m (equation (1)). The rate constant of the over–all reaction in the steady–state, k_{cat}, is written as a function of k_2 and k_3 as given in equation (3).

$$K_m = \frac{k_{-1}+k_2}{k_1} = K_s + \frac{k_2}{k_1} \tag{1}$$

$$K_m(\mathrm{app}) = \frac{k_{-1}+k_2}{k_1} \times \frac{k_3}{k_2+k_3} \tag{2}$$

$$\frac{1}{k_{cat}} = \frac{1}{k_2} + \frac{1}{k_3} \tag{3}$$

When B in Chart 2 is *p*-nitrophenol with such a substrate as **1b**, the time course of its formation is readily traceable by an optical method. Assuming $[S]\gg[E]$, the theoretical expression describing the presteady- and steady–state of formation of *p*-nitrophenol in time *t* is as follows:[21]

$$[B] = \frac{k_{cat}[E][S]t}{[S]+K_m(\mathrm{app})} + [E]\left(\frac{k_{cat}/k_3}{1+K_m(\mathrm{app})/[S]}\right)^2(1-e^{-bt}) \tag{4}$$

18) J. Hine, "Physical Organic Chemistry" 2nd. ed., McGraw–Hill, New York, 1962, p. 64.
19) B.F. Erlanger, N. Kokowsky and W. Cohen, *Arch. Biochem. Biophys.*, **95**, 271 (1961).
20) T.C. Bruice and S.J. Benkovic, "Bioorganic Mechanism," W.A. Benjamin, Inc., New York, 1966, p. 220.
21) M.L. Bender, M.L. Begué–Cantón, R.L. Blakely, L.J. Brubacher, J. Feder, C.R. Gunter, F.J. Kézdy, J.V. Killhefer, Jr., T.H. Marshall, C.G. Miller, R.W. Roeske and J.K. Stoops, *J. Am. Chem. Soc.*, **88**, 5890 (1966).

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where $b=k_3+k_2/(1+(K_s/[S])$.[18] The first term is the steady-state rate, the second term is the displacement from the origin of the extrapolated steady-state plot, and the exponential part of the second term describes the approach to the steady-state. When k_2 is so larger compared with k_3 that the predominance of the second term over the first one exists, an initial "burst" formation of B, the extent of which approximately corresponds to $[E]$, may be clearly observable distinguished from the steady-state increase of B. This is the case of **1b** shown in Fig. 1. The first order rate constant of this presteady-state reaction at a given substrate concentration $[S]$ is represented by b. The subsequent steady-state formation of B must represent the deacylation step which determines over-all reaction rate afterward in this substrate. It was observed that the over-all reaction rate is independent of the substrate concentration within the range employed in this experiment, indicating a very small K_m (app) of this system. The deacylation rate constant k_3 of **1b** was then estimated directly from its observed over-all reaction rate. This value may be regarded as k_3 for **1a** too, because the same acyl-enzyme intermediate is expected for the both substrates. k_2 of **1a** is calculated by equation 3 using this assumed k_3 and observed k_{cat}. The kinetic parameters of **1a** and **1b**, thus obtained, are listed in Table I, which includes the published ones of **1a** and **2c**[15] as reference.

Table I. Kinetic Parameters of Synthetic Trypsin Substrates at 25°

Substrate	K_m(app) M	k_{cat} (over all) min^{-1}	k_2, min^{-1} (calcd. and assumed)	k_3, min^{-1} (calcd. and assumed)	k (spont. hyd.) min^{-1}	pH	Method
1a	1.4×10^{-4}	0.398	0.473	2.53	not obsd.	8.2	a[a]
1b	$5\times10^{-7}\gg$	2.57	$600\ll$	2.57	1.3×10^{-2}	8.2	b[b]
	$5\times10^{-7}\gg$	2.53	$600\ll$	2.53	0.7×10^{-2}	8.2	a[a]
	$5\times10^{-7}\gg$	0.166	$600\ll$	0.166	not obsd.	5.4	b[c]
	$5\times10^{-7}\gg$	0.139	$600\ll$	0.139	not obsd.	5.4	a[a]
	$5\times10^{-7}\gg$	0.0509	$600\ll$	0.0509	not obsd.	4.7	b[c]
2a	——	not obsd.	——	——	not obsd.	8.2	a[a]
2b	——	not obsd.	1.0	not obsd.	7.4×10^{-3}	8.2	b[b]
	——	not obsd.	not obsd.	not obsd.	not obsd.	5.4	b[c]
	——	not obsd.	not obsd.	not obsd.	not obsd.	4.7	b[c]
1a[d]	6.43×10^{-4}	0.426	——	——	——	8.0	a[e]
2c[d]	——	not obsd.	——	——	——	8.0	a[e]

a: pH-stat titration **b**: optical assay
a) 0.1M KCl; *b*) 0.05M Tris, 0.02M $CaCl_2$; *c*) 0.1M acetate; *d*) lit. 15; *e*) 0.05M $CaCl_2$

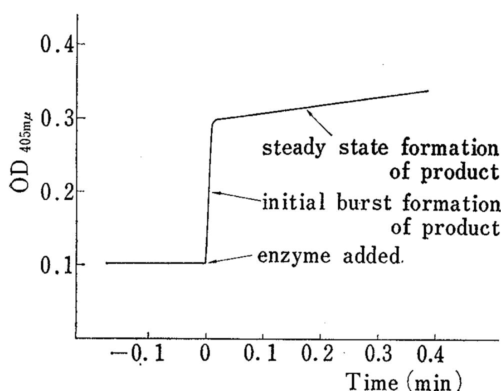

Fig. 1. Enzymatic Hydrolysis of *p*-Nitrophenyl *p*-Amidinobenzoate Hydrochloride **1b**

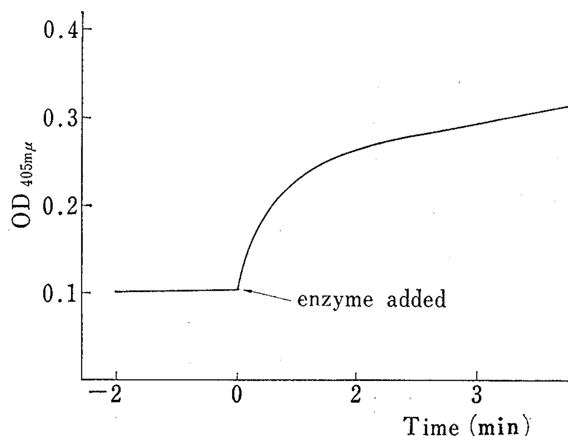

Fig. 2. Enzymatic Hydrolysis of *p*-Nitrophenyl *m*-Amidinobenzoate Hydrochloride **2b**

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Table I also demonstrates the data obtained with the *m*-derivative. No appreciable enzymatic hydrolysis of **2a** was observed in the pH range from 4.7 to 8.2 in contrast to that of the *p*-derivatives, in good agreement with the reported results for **2c**.[15] By sharp contrast, the rate constant of the liberation of *p*-nitrophenol was observable in the case of *p*-nitrophenyl *m*-amidinobenzoate hydrochloride, **2b** (Fig. 2). As shown in Fig. 2 the pattern of the time course of *p*-nitrophenol formation from **2b** is essentially the same as that of **1b**. In this presteady-state reaction, **b**, is found to be independent of substrate concentration, again indicating small K_m. Whereas the presteady-state reaction demonstrating the acylation step is rather slow in this case, it is followed by the negligibly slow reaction of deacylation ($k_2 \gg k_3$). In addition, the following condition holds: $k_{cat} \simeq 0$, $k_{cat} = k_3$ (see equation (3)), $K_m/[S] \simeq 0$ ($[S] \gg K_m$). Thus the first term in equation (4) becomes negligible and an initial slow "burst" formation of B nearly corresponds to $[E]$. The value of $[E]$, determined by extrapolating plots of very small steady-state rate to time zero is about 60% of that estimated on an absorbancy basis. This value is comparable to that from the *p*-derivative.[1] Calculation of the acylation rate constant was possible in this case by equation (5).

$$\tau = \ln 2/k \qquad (5)$$

Thus observed value, 1.0 min^{-1} is unusually small for such an active ester, especially in comparison with that of the *p*-derivative **1b**.

Discussion

The marked contrast in kinetic behaviors of the *m*- and *p*-derivatives as above cannot be ascribed to the difference in their chemical properties involving amidinium and ester moieties, since both of the two isomers have almost the same pK_a values and rate constants of spontaneous hydrolysis (Table I). Their different behavior in tryptic hydrolysis might be due to a difference in their binding to the enzyme. This is unlikely, however, because *p*- and *m*-toluamidine, simple models of **1b** and **2b**, have nearly the same binding constants (Table II). It may be reasonable to assume that the conformational requirements of the enzyme active site are reflected in the kinetic behavior of the isomeric substrates. Therefore it may be concluded that the conformation of the active center, that is the spatial arrangement of anionic and hydrophobic parts and a hydroxyl group of Ser[177], fits more favourably for the *p*-derivative than for the *m*-derivative.

Table II. Binding Constant and pK_a Value of Toluamidines

Inhibitor	K_i (DL-BANA)[a]	pK_a
m-Toluamidine	1.37×10^{-5}	11.4
p-Toluamidine	1.20×10^{-5}	11.2

a) pH 8.2 0.05M Tris, 0.02M $CaCl_2$

Table III. Comparison of Kinetic Parameters of Trypsin Substrates

Substrate	K_m M	k_{cat} min^{-1}	k_2 min^{-1}	k_3 min^{-1}	pH
1b	5×10^{-7}	2.57	$600 \leqslant$	2.57	8.2
2b		~ 0	1.0	~ 0	8.2
3	10^{-6}	7.8	$600 \leqslant$	7.8	6.6
4	7.7×10^{-5}	—	$k_2/k_3 = 15.2$		7.8

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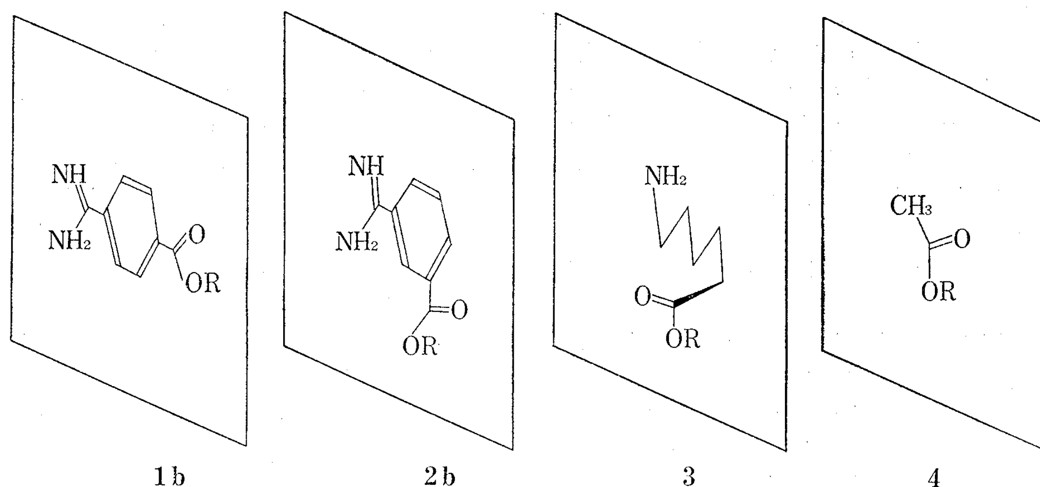

Fig. 3. Possible Shapes of Substrates Interacting with the Active Center of Trypsin

Benzamidine exhibited absorption maximum at 280 mμ with ε 1000, whereas benzylamine exhibits benzenoid absorption; λ_{max} 256 mμ and ε: 200. The fact indicates that the phenyl ring conjugates to the amidine moiety with coplaner conformation. Based on this assumption, conformation of the *p*- and *m*-derivatives and other substrates reported previously are shown in Fig. 3. Kinetic parameters of these compounds are listed in Table III. By using Dreiding model the distance between the positive charge and the carbonyl carbon of compound **2b** is observed shorter than that of **1b** by 0.8 Å. The distance between the carbonyl carbons of **2b** and **1b** is about 3 Å when they are superimposed at the amidinium and the benzene moieties. Ishii, *et al.* reported *p*-nitrophenyl ε-aminocapronate **3** as a trypsin substrate with $K_m=10^{-6}$M, $k_3=7.8$ min^{-1} at pH 6.6.[22] Since this aliphatic compound can change its conformation easily unlike **1b** and **2b**, it is unable to sketch an equivocal shape interacting with the active center. In the tentative conformation in Fig. 3 the alkylamine is on a plane according to Lawson's hypothesis.[23]

The kinetic parameters of *p*-nitrophenyl acetate **4** as a trypsin substrate are reported as follows: $K_m=7.7\times10^{-5}$M, $k_2/k_3=15.2$ at pH 7.8.[22] This nonspecific substrate lacking both positive charge and hydrophobic part is likely to interact with fairly large freedom in orientation to the active center. Although **2b** is a specific substrate, its ester group is perhaps unfavourably situated in the molecule for the interaction with the enzyme, so that it cannot easily approach the catalytic Ser^{177} locus. The results in Table III can be well explained by the above postulation. Thus the substrates in the benzamidine series can be conveniently employed to estimate the structural requirements for the active center of trypsin by taking advantage of their rigid conformations.

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