Notes

[Chem. Pharm. Bull. 28(7)2212—2216(1980)]

Efficient Tryptic Hydrolysis of Aryl Esters with a Cationic Center in the Leaving Group. Further Characterization of "Inverse Substrates"[1]

Michiharu Nakano, Kazutaka Tanizawa, Masayuki Nozawa, and Yuichi Kanaoka

Faculty of Pharmaceutical Sciences, Hokkaido University[2]

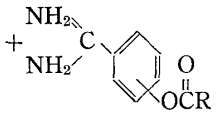
(Received December 20, 1979)

The kinetic properties of esters derived from guanidinophenol and aminomethylphenol were investigated with trypsin. These compounds, in which the site-specific groups (positive charge) are of the inverse type compared with normal substrates, were demonstrated to be specific substrates, like amidinophenyl esters. The behavior of these "inverse substrates" with trypsin and pseudotrypsin was also compared. A dramatic decrease in the efficiency of hydrolysis of the "inverse substrates" by pseudotrypsin as compared to that by trypsin was observed, which was comparable in extent to that observed for specific normal-type substrates. All these observations confirm the view that specific interaction between the positive charge at the leaving moiety of "inverse substrates" and the anionic site of the trypsin active center is an essential feature of the catalysis, just as in the case of normal-type substrates.

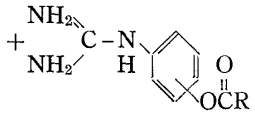
Keywords——trypsin; pseudotrypsin; synthetic substrate; enzyme kinetics; guanidinophenol ester; amidinophenol ester; acetoxybenzylamine

We have found that several acyl derivatives of *p*-amidinophenol (I-*para*), which are related to inverted-type ester substrates having the specificity function in the leaving group, are also hydrolyzable by trypsin. In these compounds the site-specific cationic center is included in the leaving group instead of in the acyl moiety. On the basis of kinetic analysis, they were demonstrated to fit well the requirements for trypsin catalysis through a pathway identical with that of normal-type substrates, and they were named "inverse substrates."[3]

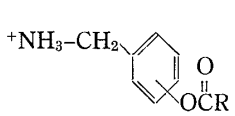
In this report, the behavior of certain esters derived from guanidinophenol(II) and aminomethylphenol (III) toward trypsin was investigated. For the further characterization of "inverse subustrates," the kinetic behavior of amidinophenyl ester with pseudotrypsin, which lacks the specificity site, was also analyzed.



Ia: R=CH_3
Ib: R=$C(CH_3)_3$



IIa: R=CH_3
IIb: R=$C(CH_3)_3$



IIIa: R=CH_3

1) Inverse Substrates. X. Part IX; T. Fujioka, K. Tanizawa, and Y. Kanaoka, *Chem. Pharm. Bull.*, **28**, 1899 (1980).
2) Location: *Kita-12, Nishi-6, Kita-ku, Sapporo, 060, Japan.*
3) *a*) K. Tanizawa, Y. Kasaba, and Y. Kanaoka, *J. Am. Chem. Soc.*, **99**, 4485 (1977); *b*) K. Tanizawa and Y. Kanaoka, *Experientia*, **35**, 16 (1979); *c*) K. Tanizawa, Y. Kasaba, and Y. Kanaoka, *J. Biochem.* (Tokyo), **87**, 417 (1980); *d*) M. Nozawa, K. Tanizawa, and Y. Kanaoka, *Biochim. Biophys. Acta*, **611**, 314 (1980); *e*) T. Fujioka, K. Tanizawa, and Y. Kanaoka, *Biochim. Biophys. Acta*, **612**, 205 (1980); *f*) H. Nakayama, K. Tanizawa, and Y. Kanaoka, *J. Am. Chem. Soc.*, **102**, 3214 (1980).

Experimental

Materials——Trypsin (twice-crystallized, lot TRL) was purchased from Worthington Biochemical Corp. 1-Chloro-3-tosylamide-7-amino-2-heptanone (TLCK) was obtained from Nakarai Chem. Corp. and *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB) was synthesized by the reported procedure.[4]

Synthesis of *m*- and *p*-Acetoxyphenylguanidine *p*-Toluenesulfonate (IIa-*meta* and IIa-*para*)——*m*- and *p*-Guanidinophenol *p*-toluenesulfonate were prepared from *m*- and *p*-aminophenol by the reported procedure.[5] Guanidinophenol *p*-toluenesulfonate (323 mg, 1 mmol) was suspended in 204 mg (2 mmol) of acetic anhydride and heated at 60—70° until a clear solution was obtained. After removal of the solvent *in vacuo*, the residue was washed with ether and recrystallized.

Synthesis of *m*- and *p*-Trimethylacetoxyphenylguanidine *p*-Toluenesulfonate (IIb-*meta* and IIb-*para*)——Guanidinophenol (323 mg, 1 mmol) was suspended in a mixture of pivalic acid (15 ml) and pivaloyl chloride (1 g). The mixture was heated at 60—65° with stirring until a clear solution was obtained. The precipitate obtained by the addition of ether was collected and recrystallized.

Synthesis of *m*- and *p*-Acetoxybenzylamine *p*-Toluenesulfonate (IIIa-*meta* and IIIb-*para*)——N-Carbobenzoxy-α-aminomethylphenol (*meta* and *para*) were prepared from the corresponding α-aminomethylphenol and carbobenzoxy chloride. *para*: mp 90.5—91° (dec.) recrystallized from benzene, 83% yield. *Anal.* Calcd for $C_{15}H_{15}N_1O_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 69.99; H, 5.77; N, 5.45. *meta*: mp 97.5—98.5° (dec.) crystallized from benzene, 80% yield. *Anal.* Calcd for $C_{15}H_{15}N_1O_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 69.96; H, 5.82; N, 5.34. Acetyl chloride (0.4 g, 5 mmol) was added to a solution of N-carbobenzoxy-α-aminomethylphenol (1.3 g, 5 mmol) and triethylamine (0.5 g, 5 mmol) in 12 ml of tetrahydrofuran at 0°. After standing for 45 min at room temperature, precipitated triethylamine hydrochloride was filtered off. The filtrate was concentrated, and the resulting residue was recrystallized. *para*: mp 97—98.5° (dec.) recrystallized from benzene, 89% yield. *Anal.* Calcd for $C_{17}H_{17}N_1O_4$: C, 68.21; H, 5.73; N, 4.68. Found: C, 68.33; H, 5.65; N, 4.65. *meta*: mp 73.5—75° (dec.) recrystallized from benzene, 76% yield. *Anal.* Calcd for $C_{17}H_{17}N_1O_4$: C, 68.21; H, 5.73; N, 4.68. Found: C, 68.49; H, 5.73; N, 4.79. N-Carbobenzoxy-α-aminomethylphenol (260 mg, 1 mmol) and *p*-toluenesulfonic acid hydrate (220 mg, 1.1 mmol) were dissolved in 20 ml of alcohol, and subjected to catalytic hydrogenation in the presence of 10% Pd/carbon (150 mg). After removal of the solvent, the residue was recrystallized.

Synthesis of *p*-Acetoxycresol (*p*-AcOCr)——*p*-Cresol (11 g, 0.1 mmol) was heated with acetic anhydride (31 g, 0.3 mmol) at 100° for 12 hr. After removal of the acetic anhydride, the residue was distilled *in vacuo*. bp 94—100°/15—20 mHg (lit. bp 208—209°/760 mHg.[6]).

Determination of Kinetic Parameters for "Inverse Substrates"——Analysis of K_s and k_2 values was carried out by the thionine displacement method.[7] Optical density changes at 620 nm were monitored with a Union Giken RM-401 stopped flow spectrophotometer. In these experiments, the concentrations were: enzyme, 4.5×10^{-6} M; substrate, 1.25×10^{-3}—2.25×10^{-4} M; thionine; 2.92×10^{-5} M. The values of $k_3(k_{cat})$ and K_m

Table I. Physical Constants and Elemental Analysis of "Inverse Substrates"

Substrate[a]	Formula	mp (°C)	Recryst. solv	Elemental analysis Calcd (Found) C	H	N	S
IIa-*para*	$C_{16}H_{19}N_3O_5S_1$	154—155	Acetonitrile	52.69 (52.30	5.24 5.32	11.40 11.62	8.78 9.01)
IIa-*meta*	$C_{16}H_{19}N_3O_5S_1$	158—159	Ethanol–ether	52.69 (52.51	5.24 5.26	11.40 11.56	8.78 8.67)
IIb-*para*	$C_{19}H_{25}N_3O_5S_1$	178—179	Ethanol–ether	56.00 (55.92	6.18 6.10	10.31 10.14	7.87 7.84)
IIb-*meta*	$C_{19}H_{25}N_3O_5S_1$	168—169	Acetonitrile	56.00 (55.73	6.18 6.21	10.31 10.31	7.87 7.84)
IIIa-*para*	$C_{16}H_{19}N_1O_5S_1$	215—216	Ethanol	56.96 (56.97	5.68 5.87	4.15 3.98	9.50 9.47)
IIIa-*meta*	$C_{16}H_{19}N_1O_5S_1$	163—164	Ethanol–ether	56.96 (56.87	5.68 5.69	4.15 3.98	9.50 9.23)

a) *p*-Toluenesulfonate.

4) T. Chase, Jr. and E. Shaw, *Biochem. Biophys, Res. Comm.*, **29**, 508 (1967).
5) H.C. Beyerman and J.S. Bontekoe, *Rec. Trav. Chim.*, **72**, 643 (1953).
6) J. Thiele and E. Winter, *Ann.*, **311**, 353 (1900).
7) K.G. Brandt, A. Himoe, and G.P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967).

were determined potentiometrically using a Radiometer TTT-2 pH-stat under steady-state conditions following the reported procedure.[3a] In these experiments the enzyme concentration was 6.0×10^{-6} M, and the substrate concentration was 9.0×10^{-5}—3.6×10^{-4} M. Statistical analysis of the data was performed according to the method of Wilkinson.[8]

Preparation of Pseudotrypsin——Pseudotrypsin was prepared by limited autolysis of β-trypsin and subsequent treatment with TLCK following the reported procedure.[9] Active forms (α- and β-trypsin) were eliminated during the procedure, since the preparation was inactive toward benzoyl-DL-arginine *p*-nitroanilide (BANA) and did not show a "rapid burst" in the hydrolysis of NPGB. The purity of the sample was confirmed by SDS gel-electrophoresis after treatment with β-mercaptoethanol.[10] The characteristic electrophoretic pattern due to the split between Lys-176 and Asp-177 was detected.

Results and Discussion

The kinetics of tryptic hydrolysis of synthetic ester substrates can be described by a three-step mechanism involving an acyl-enzyme intermediate (ES') in addition to the classical Michaelis complex (ES), as given in Eq. (1):

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{\underset{P_1}{+}}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where P_1 and P_2 are the reaction products (alcohol and acid, respectively). As discussed previously[3a] the paramaters K_s (dissociation constant of ES) and k_2 (acylation rate constant) can both be used for the evaluation of substrate specificity. The former provides information on the strength of binding of the substrate, which is a characteristic of the enzyme action, while the latter directly reflects the accessibility of the carbonyl function of the substrate to the catalytic residue(s) of the enzyme in the ES complex. As shown in Table II, the esters of *p*-guanidinophenol and *p*-hydroxybenzylamine were susceptible to efficient tryptic hydrolysis. All the compounds in series II and III were found to have strong affinity for trypsin, with K_s values in the range of 10^{-4}—10^{-5} M. These K_s values are comparable with those for lysine and arginine derivatives. The acylation rate constant, k_2, for IIa-*para* was not markedly different from those of normal-type substrates; NPGB, *p*-nitrophenyl α-amino-*p'*-toluate (NPMT) and *p*-nitrophenyl *p'*-amidinobenzoate (NPAB) (see Table III).

In contrast, the *meta* derivatives showed much lower acylation rates (*ca.* 1/3000—1/300 of that of the *para*-isomer) although there was almost no difference in the binding process. These different responses of the enzyme to *meta* and *para* isomers are probably due to the strict stereochemical requirements of the active site, *i. e.*, inaccessibility of the unfavorably positioned carbonyl carbon of the *meta* isomers to the enzyme catalytic residue(s), even though the binding itself is tight and specific. These properties are very similar to those of amidinophenol esters.[3a] It is clear that tryptic hydrolysis arises from the site-specific positive charge, since *p*-acetoxycresol (*p*-AcOCr) was shown to be completely insensitive to tryptic hydrolysis. Less efficient acylation of IIa-*para* than IIb-*para* was observed. The same response was seen in the series of amidinophenol esters, in which involvement of the steric bulkiness of the substituent was suggested.[3a]

Deacylation rate constants for Ia, IIa and IIIa in the *para*-series were almost identical and were very close to that of *p*-nitrophenyl acetate (AcONP), the tryptic hydrolysis of which is known to proceed by way of acetyltrypsin. The values for various acetate esters will not depend on the nature of the leaving group if the condition $k_2 \gg k_3$ is satisfied, and presumably reflect the deacylation rate constants of the common intermediate, acetyltrypsin. Almost identical deacylation rate constants were also observed within the Ib-*para* and IIb-*para* series.

8) G.N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
9) R.L. Smith and E. Shaw, *J. Biol. Chem.*, **244**, 4704 (1969).
10) K. Wober and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).

Table II. Kinetic Parameters for the Trypsin–Catalyzed Hydrolysis of "Inverse Substrates"

Substrate	$K_s(K_m)$ (M)	k_2 (s^{-1})	$k_3(k_{cat.})$ (s^{-1})	k_2/K_s ($s^{-1}M^{-1}$)	Reference
IIa-*para*	$8.84\pm0.20\times10^{-4}$	$8.16+1.17\times10$	$1.25\pm0.04\times10^{-2}$	9.23×10^{4}	This work
IIa-*meta*	$8.83\pm1.80\times10^{-5}$	$2.70\pm0.21\times10^{-2}$	$(2.44\pm0.06\times10^{-3})$ a)	3.06×10^{2}	This work
IIb-*para*	$1.37\pm0.18\times10^{-3}$	$1.71\pm0.42\times10$	$3.03\pm0.07\times10^{-4}$	1.25×10^{3}	This work
IIb-*meta*	—	~0	~0	—	This work
IIIa-*para*	$5.00\pm0.24\times10^{-4}$	1.14 ± 0.03	$9.88\pm0.65\times10^{-3}$	2.28×10^{3}	This work
IIIa-*meta*	$(1.72\pm0.23\times10^{-4})$ b)	$1.93\pm0.09\times10^{-3}$	—	1.12×10^{1}	This work
Ia-*para*	$3.87\pm0.29\times10^{-5}$	$1.70\pm0.34\times10$	$9.26\pm0.97\times10^{-3}$	4.39×10^{5}	3a)
Ia-*meta*	$3.03\pm0.78\times10^{-5}$	$3.01\pm0.38\times10^{-2}$	$(4.98\pm0.50\times10^{-3})$ a)	9.93×10^{2}	3a)
Ib-*para*	$1.62\pm0.42\times10^{-4}$	1.15 ± 0.22	$2.59\pm0.43\times10^{-4}$	7.10×10^{3}	3a)
Ib-*meta*	$8.02\pm0.98\times10^{-5}$	$1.67\pm0.09\times10^{-3}$	$(2.10\pm0.60\times10^{-4})$ a)	2.08×10^{1}	3a)
p-AcOCr	—	~0	~0	—	This work

The reactions were carried out in 0.05 M Tris, 0.02 M $CaCl_2$, (pH 8.0) at 25°. $k_3(k_{cat.})$ was calculated based on the $[E]_0$ values obtained by active-site titration with NPAB[c] and NPAB.[4] The means and standard errors are given for 7 data points.
a) Overall $k_{cat.}$: k_2 is not much larger than k_3 *b*) K_m: obtained by analysis under steady-state conditions.
c) K. Tanizawa, S. Ishii, and Y. Kanaoka, *Biochem. Biophys. Res. Comm.*, **32**, 893 (1968).

In these cases the observed rates represent the rate of hydrolysis of the common intermediate, pivaloyltrypsin.

It is wothwhile to compare the kinetic behavior with that of normal-type substrates which carry a positive charge at the acyl moiety. The reported kinetic parameters are listed in Table III. Comparing their specificity indices (k_2/K_s),[11] the value for NPGB is 40—80 times that for the corresponding "inverse substrate," acetoxyphenylguanidine (IIa-*para*). This ratio would seem to be large compared with that observed in the amidino series, NPAB and Ia-*para* (14 times). However, the kinetic parameters for IIa-*para* may still be taken to qualify the compound as a specific substrate for trypsin. Compound IIIa-*para* seems to be a less efficient tryptic substrate: *i. e.*, the specificity index of IIIa-*para* is 1/500 of that of the normal-type substrate NPMT. Although the acylation rate by IIIa-*para* is low, this process is considered to be rather specific by virtue of the site-specific aminomethyl substituent, taking into account the result for *p*-acetoxycresol (*p*-AcOCr).

Table III. Kinetic Parameters for the Trypsin-Catalyzed Hydrolysis of Normal-Type Substrates

Substrate	K_s (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/K_s ($s^{-1}M^{-1}$)	pH	Reference
NPGB	3.65×10^{-5}	269	4.1×10^{-5}	7.37×10^{6}	7.8	a)
	4.0×10^{-5}	150	3.4×10^{-5}	3.8×10^{6}	8.45	12)
NPMT	5.89×10^{-5}	64.25	3.90×10^{-3}	1.09×10^{6}	7.8	a)
NPAB	5.03×10^{-6}	30.4	6.53×10^{-2}	6.04×10^{6}	8.0	3a)

a) T.J. Ryan, J.W. Fenton, II, T.-L. Chang, and R.D. Feinman, *Biochemistry*, **15**, 1337 (1966).

Modified trypsin produced by limited autodigestion, resulting in an interchain split between Lys-176 and Asp-177, is known as pseudotrypsin. This enzyme was characterized as being defective in the recognition of the site-specific group, whereas the catalytic function toward non-specific substrates is preserved.[9] The behavior of "inverse substrates" with this enzyme is thus of interest. The kinetic characteristics of IIa-*para* with trypsin and pseudotrypsin were compared. Pseudotrypsin was shown to be greatly quenched both in the binding and acylation processes compared with the unmodified trypsin, as shown in Table IV. The acylation is rate-determining in this case. As shown in the table, the k_2/K_s values differ by more

11) F.E. Brot and M.L. Bender, *J. Am. Chem. Soc.*, **91**, 7187 (1969).

TABLE IV. Kinetic Characteristics of "Inverse Substrate" Ia-*para* with Trypsin and Pseudotrypsin

Enzyme	K_S (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/K_S ($s^{-1}M^{-1}$)
Trypsin[a)]	$3.87\pm0.29\times10^{-5}$	$1.70\pm0.34\times10$	$9.26\pm0.97\times10^{-3}$	4.39×10^{5}
Pseudotrypsin[b)]	$1.82\pm0.37\times10^{-3}$	$2.87\pm0.33\times10^{-3}$ [c)]	—	1.57

The reaction was carried out in 0.05 M Tris, 0.02 M $CaCl_2$ (pH 8.0) at 25°.
a) See reference 3*a*).
b) Assay was carried out at $[E]_0$: 5.45×10^{-6} M, $[S]_0$: 2.08×10^{-3}—5.53×10^{-4} M.
c) Overall reaction rate.

than 2.8×10^5 times between trypsin and pseudotrypsin. Comparative kinetic properties of pseudotrypsin and trypsin were reported by Foucault *et al.* in the hydrolysis of normal-type substrates.[12] In this study, kinetic parameters in the pseudotrypsin-catalyzed hydrolysis of NPGB were: k_2, 0.14 s^{-1}; k_3, 6.4×10^{-5} s^{-1}; K_s, 5.7×10^{-3} M, k_2/K_s; 24.6. The k_2/K_s values can thus be calculated to differ by 1.5—3.0×10^5 times between trypsin and pseudotrypsin (*cf.* Table III). This difference is comparable to that for the "inverse substrate" IIa.

Our present observations show that not only amidinium, but also guanidinium and ammonium, which are related to the side chains of arginine and lysine, can provide the site-specific positive charge at the leaving portion of an "inverse substrate," and that loss of catalytic activity followed modification in a way that eliminated the charge in either the substrate or the enzyme. This study provides further detailed evidence for the concept of "inverse substrates."

Acknowledgement We are grateful to the staff of the Center of Instrumental Analysis of this University for elemental analyses. Our thanks are also due to Mr. T. Kumazaki of this Faculty for his advice on the preparation of pseudotrypsin. This work was supported in part by grants from the Yamada Science Foundation, the Foundation for the Promothion of Research on Medicinal Resources, and the Ministry of Education, Science and Culture of Japan (Grant-in-Aid for Scientific Research No. 447120).

12) G. Foucault, F. Seydoux, and J. Yon, *Eur. J. Biochem.*, **47**, 295 (1974).

[Chem. Pharm. Bull. 28(7)2216—2220(1980)]

Electron Spin Resonance Study of the Conformations of Anion Radicals derived from Some Thermochromic Ethylenes[1)]

SHUNSUKE KAZAMA, EIJI SATO, MAMORU KAMIYA and YUKIO AKAHORI

Shizuoka College of Pharmacy[2)]

(Received January 10, 1980)

Electron spin resonance (ESR) spectra of the anion radicals of 10(9*H*)-xanthene-9-ylidene-9(10*H*)-anthracenone, 10-diphenylmethylene-9(10*H*)-anthracenone, 9-diphenylmethylene-9*H*-xanthene, 9,9′-bixanthene and diphenylfulvene were measured. These compounds, except for diphenylfulvene, are thermochromic ethylenes. On the basis of calculation of the spin densities by McLachlan's method and h.f.s. analysis of the ESR spectra, the anion radicals were found to be substantially twisted about the central double bond.

1) A part of this work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August 1979.
2) Location: *2-2-1, Oshika, Shizuoka-shi 422, Japan.*