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Synthesis and Structure–Activity Study of Protease Inhibitors. I. (Guanidinophenyl)propionate and Guanidinocinnamate Derivatives

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Guanidino-ester derivatives were synthesized and evaluated for inhibitory activities against trypsin, plasmin, kallikrein, thrombin and Cl esterase, as well as on *in vitro* complement-mediated hemolysis. Among the compounds synthesized, phenyl α-ethyl-*p*-guanidinocinnamate (IVg) and phenyl α-propyl-*p*-guanidinocinnamate (IVh) exhibited potent and selective Cl esterase inhibition (IC_{50}: 7×10^{-6} and 6×10^{-6} M, respectively) and 6-methyl-3-pyridyl α-ethyl-*p*-guanidinocinnamate (IVi) markedly suppressed complement-mediated hemolysis (86% inhibition at 1×10^{-3} M).

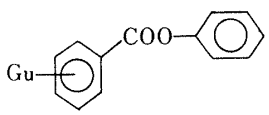
Keywords——protease inhibition; trypsin; plasmin; thrombin; Cl esterase; complement-mediated hemolysis; guanidinobenzoate; (guanidinophenyl)propionate; guanidinocinnamate; structure activity relationship

Trypsin, plasmin, kallikrein, thrombin and Cl esterase, which belong to the class of serine-proteases, play important roles in the homeostasis of the living body. Anomalous activation of these enzymes, however, is known to be involved in the pathogenesis of various diseases such as pancreatitis (trypsin), hemorrhagic diseases (plasmin), thrombus formation (thrombin) and autoimmune diseases (Cl esterase).

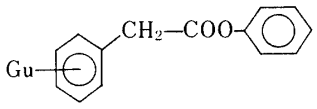
Many studies[3)] have been done by various groups of investigators in attempts to develop serine-protease inhibitors for the treatment of these diseases. In these studies, many guanidino-containing compounds, especially guanidino-ester derivatives, have been found to have potent inhibitory activities. More precisely, phenyl *p*-guanidinobenzoate[3c)] and *p*-nitrophenyl *p*-guanidinobenzoate[3e)] are well-known as potent serine-protease inhibitors and trypsin titrants. Moreover, Tamura *et al.*[3b)] reported that *N*,*N*-dimethylamino *p*-(*p'*-guanidinobenzoyloxy)benzylcarbonyloxy glycolate also has strong inhibitory activity against trypsin and Muramatsu *et al.*[3g)] reported that *trans*-4-(guanidinomethyl)cyclohexanecarboxylic acid esters inhibit the esterolytic activity of chymotrypsin.

We are also interested in the effect on serine-proteases of various guanidino-ester derivatives, especially aryl esters, and we have synthesized a series of guanidinobenzoates (I), (guanidinophenyl)acetates (II), (guanidinophenyl)propionates (III),[2)] guanidinocinnamates (IV) and (guanidinophenyl)butyrate (V) in order to examine their imhibitory activities against serine-proteases, as well as on *in vitro* complement-mediated hemolysis.

Phenyl α-ethyl-*p*-guanidinocinnamate (IVg), and phenyl α-propyl-*p*-guanidinocinnamate (IVh) were found to have potent and selective inhibitory effects on Cl esterase, and 6-methyl-3-pyridyl α-ethyl-*p*-guanidinocinnamate (IVi) markedly suppressed the complement-mediated

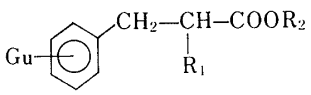


Ia, b

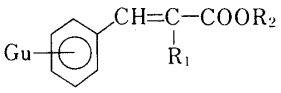


IIa, b

a : *m*-Gu , b : *p*-Gu



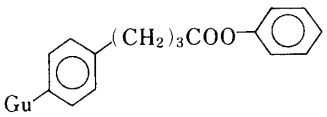
IIIa－j



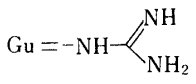
IVa－k

a : *m*-Gu, $R_1=H$, $R_2=C_6H_5$
b : *m*-Gu, $R_1=CH_3$, $R_2=C_6H_5$
c : *m*-Gu, $R_1=C_2H_5$, $R_2=C_6H_5$
d : *m*-Gu, $R_1=C_3H_7$, $R_2=C_6H_5$
e : *p*-Gu, $R_1=H$, $R_2=C_6H_5$
f : *p*-Gu, $R_1=CH_3$, $R_2=C_6H_5$

g : *p*-Gu, $R_1=C_2H_5$, $R_2=C_6H_5$
h : *p*-Gu, $R_1=C_3H_7$, $R_2=C_6H_5$
i : *p*-Gu, $R_1=C_2H_5$, $R_2=$6-MP
j : *p*-Gu, $R_1=C_2H_5$, $R_2=C_2H_5$
k : *p*-Gu, $R_1=C_2H_5$, $R_2=H$



V



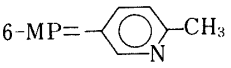
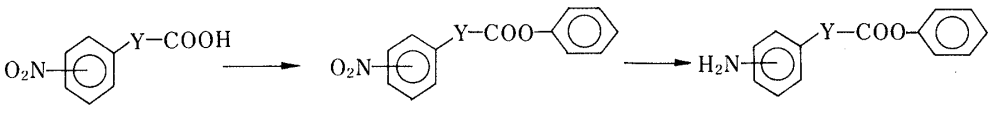


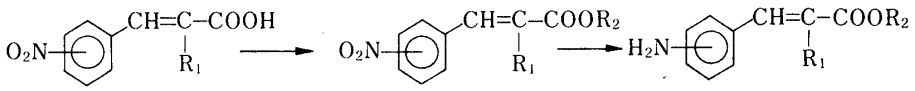
Chart 1



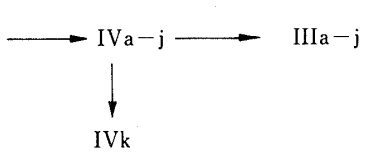
VIa－e　　VIIa－e　　VIIIa－e

Y＝－, $-CH_2-$, $-(CH_2)_3-$

Ia, b, IIa, b, V



IXa－h　　Xa－j　　XIa－j



IVa－j　　IIIa－j

IVk

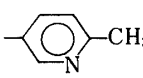
$R_1=H$, CH_3, C_2H_5, C_3H_7, $R_2=C_6H_5$,  , CH_3, C_2H_5

Chart 2

hemolysis. This paper describes the synthesis of these compounds and their serine-protease inhibitory activities. The structure–activity relationships are also discussed.

Synthesis

The principal synthetic routes for the preparation of these compounds are outlined in Chart 2 and described in detail in the experimental section.

Nitro-carboxylic acids (VI) were converted to the corresponding acid chlorides by treatment with phosphorous pentachloride (PCl_5), and the resulting acid chlorides were then subjected to a reaction with phenol to give nitro-phenyl-esters (VII). The nitro-phenyl-esters were reduced with hydrogen and palladium-on-carbon (Pd–C) catalyst to give amino-esters (VIII), which were then transformed to guanidino-esters (I, II and V) by treatment with cyanamide.

In the case of nitrocinnamic acids (IX),[4] alkyl-ester formation was performed by reaction with EtOH–HCl (Xj: $R_1=C_2H_5$, $R_2=C_2H_5$), and aryl-esters (Xa—i: $R_2=C_6H_5$, 6-methyl-3-pyridyl) were prepared by reaction of the corresponding acid chlorides with either phenol or 6-methyl-3-pyridinol. Reduction of the nitro group in X was performed by using zinc powder in acetic acid to form amino-esters (XI), which were subsequently transformed to the corresponding guanidinocinnamate derivatives (IVa—j) by treatment with cyanamide. Guanidino-carboxylic acid (IVk) was prepared from the corresponding ester, IVg, by hydrolysis with aq. NaOH.

(Guanidinophenyl)propionate derivatives (IIIa—f) were prepared by reduction of the olefinic bond of IVa—j.

Protease Inhibitory Activities

The inhibitory activities against trypsin, plasmin, kallikrein and thrombin were determined by using N^{α}-tosyl-arginine methyl ester (TAME) as the substrate according to the method of Muramatsu *et al.*,[6] and the inhibitory activities against Cl esterase were determined by using acetyl-tyrosine ethyl ester (ATEE) as the substrate according to the method of Tamura *et al.*[7] The results are shown in Tables I—III, in which the inhibitory activities are expressed in IC_{50}, namely, the molar concentration at which a compound inhibits 50% of the activity of the enzyme to hydrolyze the substrate. The potency of the compounds with IC_{50} less than 1×10^{-3} M is expressed as the percent inhibition at the concentration of 1×10^{-3} M.

The inhibitory activities against complement-mediated hemolysis were determined by using sensitized sheep erythrocytes and guinea pig serum as a complement source according to the method of Baker *et al.*[8]

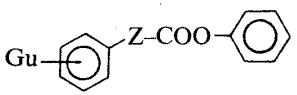
Results

Effect of Unbranched Carbon Chain Introduced between the Ester Linkage and Benzene Ring on the Inhibitory Activity (Table I)

Among nine compound tested, phenyl *p*-guanidinobenzoate (Ib)[3d] was the most potent inhibitor of protease activities and exhibited potent inhibition of trypsin activity (IC_{50}: 3×10^{-8} M). Although no systematic structure–activity relationship was revealed as a result of the introduction of unbranched carbon chain between the ester linkage and benzene ring, it was of interest to note that a high degree of Cl esterase inhibition was retained by almost all of these compounds and that the inhibitory effectiveness of cinnamates (IVa, e) was somewhat more potent as compared with that of the corresponding phenylpropionates (IIIa, e).

On the basis of these findings, we next synthesized a series of α-alkyl substituted (guanidinophenyl)propionates (IIIb—d, f—j) and guanidinocinnamates (IVb—d, f—k).

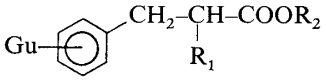
TABLE I. Enzyme Inhibition by Guanidino-esters



Compd. No.	Gu[a]	Z	Trypsin	Plasmin	Kallikrein	Thrombin	Cl esterase	Inhibition of complement-mediated hemolysis (%) 1×10^{-3} M[b] Inhibition	1×10^{-3} M[b] Lysis[c]	1×10^{-4} M Inhibition
Ia	*m*	—	28[d]	21	43	31	48	<10	0	<10
Ib	*p*	—	(3×10^{-8})[e]	(5×10^{-7})	(1×10^{-3})	(6×10^{-6})	(2×10^{-5})	80	0	14
IIa	*m*	$-CH_2-$	27	<10	49	<10	(2×10^{-4})	25	0	<10
IIb	*p*	$-CH_2-$	(5×10^{-5})	49	11	34	(5×10^{-4})	<10	0	<10
IIIa	*m*	$-(CH_2)_2-$	<10	<10	<10	<10	(4×10^{-4})	<10	0	17
IIIe	*p*	$-(CH_2)_2-$	26	<10	<10	<10	<10	11	0	17
IVa	*m*	$-CH=CH-$	39	38	28	19	(7×10^{-4})	<10	0	<10
IVe	*p*	$-CH=CH-$	35	<10	(9×10^{-4})	33	44	<10	0	<10
V	*p*	$-(CH_2)_3-$	25	(7×10^{-4})	(3×10^{-4})	23	(8×10^{-4})	<10	100	73

a) Gu=$-NH-C(=NH)-NH_2$. *b*) Molar concentration of the compound.
c) % hemolysis by the compound itself at a given concentration.
d) % inhibition at a concentration of the compound of 1×10^{-3} M. *e*) IC_{50}.

TABLE II. Enzyme Inhibition by (Guanidinophenyl)propionates



Compd. No.	Gu[a]	R_1	R_2	Trypsin	Plasmin	Kallikrein	Thrombin	Cl esterase	Inhibition of complement-mediated hemolysis (%) 1×10^{-3} M[b] Inhibition	1×10^{-3} M[b] Lysis[c]	1×10^{-4} M Inhibition
IIIa	*m*	H	C_6H_5	<10[d]	<10	<10	<10	(4×10^{-4})[e]	<10	0	17
IIIb	*m*	CH_3	C_6H_5	<10	36	49	<10	(2×10^{-4})	<10	0	14
IIIc	*m*	C_2H_5	C_6H_5	40	<10	22	<10	(6×10^{-4})	<10	0	19
IIId	*m*	C_3H_7	C_6H_5	(5×10^{-4})	20	<10	<10	(4×10^{-4})	<10	100	22
IIIe	*p*	H	C_6H_5	26	<10	<10	<10	10	11	0	17
IIIf	*p*	CH_3	C_6H_5	34	<10	21	<10	(1×10^{-4})	<10	0	16
IIIg	*p*	C_2H_5	C_6H_5	39	<10	23	<10	(2×10^{-4})	64	0	24
IIIh	*p*	C_3H_7	C_6H_5	(5×10^{-4})	27	<10	<10	(2×10^{-4})	0	100	49
IIIi	*p*	C_2H_5	6-MP[f]	(4×10^{-4})	39	(2×10^{-4})	<10	(5×10^{-4})	86	0	45
IIIj	*p*	C_2H_5	C_2H_5	<10	<10	<10	<10	26	<10	0	<10

a) Gu=–NH–C(=NH)–NH_2. *b*) Molar concentration of the compound.

c) % hemolysis by the compound itself at a given concentration.

d) % inhibition at a concentration of the compound of 1×10^{-3} M. *e*) IC_{50}.

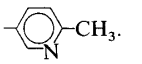
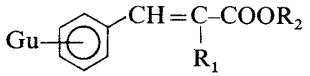
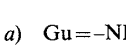
f) 6-MP= 

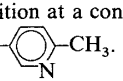
TABLE III. Enzyme Inhibition by Guanidinocinnamates



Compd. No.	Gu[a]	R_1	R_2	Trypsin	Plasmin	Kallikrein	Thrombin	Cl esterase	Inhibition of complement-mediated hemolysis (%) 1×10^{-3} M[b] Inhibition	Lysis[c]	1×10^{-4} M Inhibition
IVa	*m*	H	C_6H_5	39[d]	38	28	19	(7×10^{-4})[e]	<10	0	<10
IVb	*m*	CH_3	C_6H_5	22	24	37	43	(7×10^{-4})	<10	0	<10
IVc	*m*	C_2H_5	C_6H_5	23	27	44	49	(9×10^{-5})	<10	100	<10
IVd	*m*	C_3H_7	C_6H_5	27	49	46	(2×10^{-4})	(4×10^{-5})	<10	100	<10
IVe	*p*	H	C_6H_5	35	<10	(9×10^{-4})	33	44	<10	0	<10
IVf	*p*	CH_3	C_6H_5	(6×10^{-4})	29	(1×10^{-3})	(8×10^{-5})	(8×10^{-5})	84	63	<10
IVg	*p*	C_2H_5	C_6H_5	(3×10^{-4})	(7×10^{-4})	43	(3×10^{-4})	(7×10^{-6})	<10	100	<10
IVh	*p*	C_3H_7	C_6H_5	(7×10^{-5})	(3×10^{-5})	37	(2×10^{-4})	(6×10^{-6})	10	100	12
IVi	*p*	C_2H_5	6-MP[f]	(1×10^{-4})	(4×10^{-4})	16	(5×10^{-4})	(3×10^{-5})	86	0	34
IVj	*p*	C_2H_5	C_2H_5	<10	<10	<10	<10	27	42	0	<10
IVk	*p*	C_2H_5	H	40	<10	<10	<10	27	20	10	<10

a) Gu=  *b*) Molar concentration of the compound.

c) % hemolysis by the compound itself at a given concentration.
d) % inhibition at a concentration of the compound of 1×10^{-3} M. *e*) IC_{50}.

f) 6-MP= 

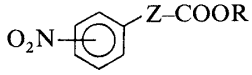
(Guanidinophenyl)propionates (Table II)

It was again noticed that eight of ten compounds tested exhibited moderate inhibition of Cl esterase activity. In a series of *m*-guanidino derivatives, introduction of an α-propyl group was found to provide a molecule with moderate trypsin inhibitory activity. In a series of *p*-guanidino derivatives, it was found that the presence of an α-alkyl substituent was necessary for the molecules to act as inhibitors of Cl esterase activity. Interestingly, the alkyl ester, *i.e.* ethyl α-ethyl-(*p*-guanidinophenyl)propionate (IIIj), was inactive as a Cl esterase inhibitor, in contrast to aryl esters. Among the compounds tested, two *i.e.* phenyl α-ethyl-(*p*-guanidinophenyl)propionate (IIIg) and 6-methyl-3-pyridyl α-ethyl-(*p*-guanidinophenyl)-propionate (IIIi), showed moderate suppression of the *in vitro* complement-mediated hemolysis.

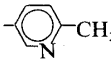
Guanidinocinnamates (Table III)

Overall, the potency of Cl esterase inhibitory activities of (guanidinophenyl)propionates

TABLE IV. Physical Data for Nitro-esters



Compd. No.	NO_2	Z	R	mp (°C)	Method	IR ν_{max}^{KBr} cm^{-1}	Formula	Analysis (%) Calcd (Found)		
								C	H	N
VIIa	*m*	—	C_6H_5	95—96[a]	A	1725, 1608	$C_{13}H_9NO_4$	64.20	3.73	5.76
								(63.82	3.75	5.72)
VIIb	*p*	—	C_6H_5	127—129[b]	A	1732, 1600	$C_{13}H_9NO_4$	64.20	3.73	5.76
								(63.89	3.76	5.73)
VIIc	*m*	$-CH_2-$	C_6H_5	48—50	A	1760	$C_{14}H_{11}NO_4$	65.37	4.31	5.44
								(65.27	4.21	5.46)
VIId	*p*	$-CH_2-$	C_6H_5	76—78[c]	A	1740, 1604	$C_{14}H_{11}NO_4$	65.37	4.31	5.44
								(64.95	4.32	5.39)
Xa	*m*	$-CH=CH-$	C_6H_5	117—119	D	1734, 1644, 1622	$C_{15}H_{11}NO_4$	66.91	4.12	5.20
								(66.95	4.12	5.20)
Xb	*m*	$-CH=C(CH_3)-$	C_6H_5	65—66	D	1722, 1642, 1620	$C_{16}H_{13}NO_4$	67.84	4.63	4.94
								(67.78	4.61	4.95)
Xc	*m*	$-CH=C(C_2H_5)-$	C_6H_5	Syrup	D	1720, 1627	[e]			
Xd	*m*	$-CH=C(C_3H_7)-$	C_6H_5	60—61	D	1724, 1620	$C_{18}H_{17}NO_4$	69.44	5.50	4.50
								(69.32	5.46	4.45)
Xe	*p*	$-CH=CH-$	C_6H_5	140—142	D	1720, 1640, 1602	$C_{15}H_{11}NO_4$	66.91	4.12	5.20
								(66.81	4.10	5.17)
Xf	*p*	$-CH=C(CH_3)-$	C_6H_5	79—80	D	1716, 1624	$C_{16}H_{13}NO_4$	67.84	4.63	4.94
								(67.53	4.60	4.88)
Xg	*p*	$-CH=C(C_2H_5)-$	C_6H_5	80—81	D	1722, 1624	$C_{17}H_{15}NO_4$	68.68	5.09	4.71
								(68.55	5.08	4.71)
Xh	*p*	$-CH=C(C_3H_7)-$	C_6H_5	64	D	1720	$C_{18}H_{17}NO_4$	69.44	5.50	4.50
								(69.17	5.46	4.46)
Xi	*p*	$-CH=C(C_2H_5)-$	6-MP[d]	124—125	D	1720, 1625	$C_{17}H_{16}N_2O_4$	65.38	5.16	8.97
								(65.29	5.16	8.91)
Xj	*p*	$-CH=C(C_2H_5)-$	C_2H_5	54—56	H	1705, 1630	$C_{13}H_{15}NO_4$	62.64	6.07	5.62
								(62.57	6.02	5.58)
VIIe	*p*	$-(CH_2)_3-$	C_6H_5	Syrup	A	1750	[f]			

a) Lit.,[9] 95—96 °C. *b*) Lit.,[9] 126—127 °C. *c*) Lit.,[10] 79—82 °C. *d*) 6-MP=  .

e) The structure of Xc was confirmed as its amino derivative (obtained in the next step).

f) The structure of VIIe was confirmed by the following spectral data: ^{13}C-NMR (in d_6-DMSO) δ: 25.6, 32.9, 34.1 ($-\underline{C}H_2-\underline{C}H_2-\underline{C}H_2-$).

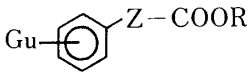
TABLE V. Physical Data for Amino-esters H_2N-(C$_6$H$_4$)-Z-COOR

Compd. No.	NH_2	Z	R	Salt	mp (°C)	Method	IR ν_{max}^{KBr} cm^{-1}	Formula	Analysis (%) Calcd (Found)		
									C	H	N
VIIIa	*m*	—	C_6H_5	HCl	177—179[a]	B	1722, 1650	$C_{13}H_{11}NO_2 \cdot HCl$	62.53	4.84	5.61
									(62.07	4.87	5.56)
VIIIb	*p*	—	C_6H_5	HCl	266—[b]	B	1724, 1612	$C_{13}H_{11}NO_2 \cdot HCl$	62.53	4.84	5.61
									(62.45	4.85	5.61)
VIIIc	*m*	$-CH_2-$	C_6H_5	MSA[c]	152—154	B	1762, 1610	$C_{14}H_{13}NO_2 \cdot CH_4O_3S$	55.72	5.30	4.33
									(55.25	5.31	4.32)
VIIId	*p*	$-CH_2-$	C_6H_5	HCl	180—182	B	1750, 1652, 1620	[e]			
XIa	*m*	$-CH=CH-$	C_6H_5	HCl	208—209	E	1718, 1640	$C_{15}H_{13}NO_2 \cdot HCl$	65.34	5.12	5.08
									(64.95	5.02	5.10)
XIb	*m*	$-CH=C(CH_3)-$	C_6H_5	MSA	137—139	E	1722, 1624	$C_{16}H_{15}NO_2 \cdot CH_4O_3S$	58.44	5.48	4.01
									(57.94	5.50	3.94)
XIc	*m*	$-CH=C(C_2H_5)-$	C_6H_5	HCl	188—190	E	1726, 1630	$C_{17}H_{17}NO_2 \cdot HCl$	67.21	5.97	4.61
									(66.92	5.85	4.59)
XId	*m*	$-CH=C(C_3H_7)-$	C_6H_5	HCl	161—163	E	1724, 1626	[f]			
XIe	*p*	$-CH=CH-$	C_6H_5	MSA	186—188	E	1724, 1640	$C_{15}H_{13}NO_2 \cdot CH_4O_3S$	57.12	5.39	4.16
									(56.79	5.10	4.07)
XIf	*p*	$-CH=C(CH_3)-$	C_6H_5	MSA	204—206	E	1734, 1640	$C_{16}H_{15}NO_2 \cdot CH_4O_3S$	58.44	5.48	4.01
									(58.10	5.57	4.01)
XIg	*p*	$-CH=C(C_2H_5)-$	C_6H_5	MSA	184—186	E	1730, 1630	$C_{17}H_{17}NO_2 \cdot CH_4O_3S$	59.49	5.82	3.85
									(59.29	5.92	3.83)
XIh	*p*	$-CH=C(C_3H_7)-$	C_6H_5	HCl	187—188	E	1728, 1630	$C_{18}H_{19}NO_2 \cdot HCl$	68.03	6.34	4.41
									(67.73	6.39	4.43)
XIi	*p*	$-CH=C(C_2H_5)-$	6-MP[d]	2HCl	100—103	E	1727, 1610	[g]			
XIj	*p*	$-CH=C(C_2H_5)-$	C_2H_5	MSA	183—184	E	1698, 1616, 1602	$C_{13}H_{17}NO_2 \cdot CH_4O_3S$	53.32	6.71	4.44
									(53.23	6.78	4.46)
VIIIe	*p*	$-(CH_2)_3-$	C_6H_5	HCl	201—203	B	1742	[h]			

a) Lit.,[11] oil (free base). *b*) Lit.,[11] 172 °C (free base). *c*) MSA = CH_3SO_3H. *d*) 6-MP = 6-methylpyridyl. *e*, *f*) These structures were confirmed by the following spectral data:

VIIId, ^{1}H-NMR (d_6-DMSO) δ: 4.02 (2H, S, $-C\underline{H}_2-$), XI, ^{13}C-NMR (d_6-DMSO) δ: 13.9, 21.9, 29.0 ($\underline{C}H_3-\underline{C}H_2-\underline{C}H_2-$). *g*, *h*) These structures were confirmed as their guanidino derivatives (obtained in the next step).

TABLE VI. Physical Data for Guanidino-esters



Compd. No.	Gu[a]	Z	R	Salt	mp (°C)	Method	IR ν_{max}^{KBr} cm^{-1}	^{13}C-NMR δ (d_6-DMSO)	Formula	Analysis (%) Calcd (Found)		
										C	H	N
Ia	*m*	—	C_6H_5	MSA[b]	152—155[d]	C	1740, 1673, 1622		$C_{14}H_{13}N_3O_2 \cdot CH_4O_3S$	51.27 (50.90	4.88 4.91	11.96 11.75)
Ib	*p*	—	C_6H_5	MSA	136—138[e]	C	1730, 1680, 1657		$C_{14}H_{13}N_3O_2 \cdot CH_4O_3S$	51.27 (50.86	4.88 4.89	11.96 11.74)
IIa	*m*	$-CH_2-$	C_6H_5	MSA	Syrup	C	1740, 1664	34.3	[g]			
IIb	*p*	$-CH_2-$	C_6H_5	MSA	148—149	C	1755, 1670, 1628		$C_{15}H_{15}N_3O_2 \cdot CH_4O_3S$	52.59 (52.33	5.24 5.24	11.50 11.47)
IIIa	*m*	$-CH_2-CH_2-$	C_6H_5	MSA	126—127	J	1752, 1666, 1624	29.8, 34.6	$C_{16}H_{17}N_3O_2 \cdot CH_4O_3S$	53.81 (53.77	5.58 5.57	11.07 10.94)
IIIb	*m*	$-CH_2-\underset{CH_3}{CH}-$	C_6H_5	MSA	142—143	J	1742, 1670, 1626	16.5, 38.5, 40.3	$C_{17}H_{19}N_3O_2 \cdot CH_4O_3S$	54.95 (54.46	5.89 5.94	10.68 10.58)
IIIc	*m*	$-CH_2-\underset{C_2H_5}{CH}-$	C_6H_5	H_2CO_3	93—95	J	1744, 1686, 1622	11.4, 24.8, 37.3, 48.2	$C_{18}H_{21}N_3O_2 \cdot CH_2O_3$	61.11 (60.93	6.21 6.24	11.25 11.21)
IIId	*m*	$-CH_2-\underset{C_3H_7}{CH}-$	C_6H_5	H_2CO_3	80—83	J	1750, 1670, 1626	13.8, 20.0, 33.9, 37.5, 46.5	$C_{19}H_{23}N_3O_2 \cdot CH_2O_3$	62.00 (62.16	6.50 6.57	10.85 11.10)
IIIe	*p*	$-CH_2-CH_2-$	C_6H_5	MSA	134—135	J	1740, 1670, 1626	29.4, 34.7	$C_{16}H_{17}N_3O_2 \cdot CH_4O_3S$	53.81 (53.34	5.58 5.58	11.07 10.92)
IIIf	*p*	$-CH_2-\underset{CH_3}{CH}-$	C_6H_5	MSA	123—124	J	1752, 1670	16.5, 40.4	[h]			
IIIg	*p*	$-CH_2-\underset{C_2H_5}{CH}-$	C_6H_5	MSA	140—142	J	1755, 1672, 1628	11.4, 24.7, 48.0	$C_{18}H_{21}N_3O_2 \cdot CH_4O_3S$	56.00 (55.58	6.18 6.24	10.31 10.22)
IIIh	*p*	$-CH_2-\underset{C_3H_7}{CH}-$	C_6H_5	MSA	107—109	J	1752, 1672, 1630	13.8, 19.9, 33.8, 37.0, 46.4	$C_{19}H_{23}N_3O_2 \cdot CH_4O_3S$	56.99 (56.67	6.46 6.51	9.97 9.92)

IIIi	*p*	$-CH_2-CH(C_2H_5)-$	6-MP[c]	MSA	97—98	J, I	1760, 1676, 1632, 1610	11.4, 23.2, 24.5	$C_{18}H_{22}N_4O_2 \cdot CH_4O_3S \cdot H_2O$	51.81 (51.94	6.41 6.38	12.72 12.81)
IIIj	*p*	$-CH_2-CH(C_2H_5)-$	C_2H_5	MSA	132—133	J	1726, 1668, 1626	11.3, 14.0, 24.6, 48.0, 59.6	$C_{14}H_{21}N_3O_2 \cdot CH_4O_3S$	50.12 (49.88	7.01 7.12	11.69 11.73)
IVa	*m*	$-CH=CH-$	C_6H_5	MSA	179—180	F	1720, 1670, 1630		$C_{16}H_{15}N_3O_2 \cdot CH_4O_3S$	54.10 (53.80	5.07 5.07	11.13 11.05)
IVb	*m*	$-CH=C(CH_3)-$	C_6H_5	MSA	143—144	F	1722, 1680, 1620	14.0	$C_{17}H_{17}N_3O_2 \cdot CH_4O_3S$	55.23 (54.85	5.41 5.39	10.74 10.48)
IVc	*m*	$-CH=C(C_2H_5)-$	C_6H_5	MSA	143—144	F	1708, 1688, 1616	13.5, 20.6	$C_{18}H_{19}N_3O_2 \cdot CH_4O_3S \cdot H_2O$	53.89 (53.92	5.95 5.50	9.92 9.82)
IVd	*m*	$-CH=C(C_3H_7)-$	C_6H_5	MSA	133—134	F	1720, 1704, 1680, 1616	13.9, 21.9, 29.1	$C_{19}H_{21}N_3O_2 \cdot CH_4O_3S$	57.26 (57.01	6.01 6.05	10.02 9.77)
IVe	*p*	$-CH=CH-$	C_6H_5	MSA	190—192[f]	F	1738, 1720, 1666, 1630		$C_{16}H_{15}N_3O_2 \cdot CH_4O_3S$	54.10 (53.86	5.07 5.01	11.13 11.05)
IVf	*p*	$-CH=C(CH_3)-$	C_6H_5	MSA	199—200	F	1708, 1670, 1616	14.0	$C_{17}H_{17}N_3O_2 \cdot CH_4O_3S$	55.23 (54.73	5.41 5.40	10.74 10.88)
IVg	*p*	$-CH=C(C_2H_5)-$	C_6H_5	MSA	166—168	F	1704, 1682, 1620, 1600	13.5, 20.6	$C_{18}H_{19}N_3O_2 \cdot CH_4O_3S$	56.28 (56.19	5.72 5.75	10.36 10.26)
IVh	*p*	$-CH=C(C_3H_7)-$	C_6H_5	MSA	163—164	F	1716, 1686, 1660, 1628	13.9, 21.9, 29.1	$C_{19}H_{21}N_3O_2 \cdot CH_4O_3S$	57.26 (57.23	6.01 6.04	10.02 9.97)
IVi	*p*	$-CH=C(C_2H_5)-$	6-MP	MSA	198—199	F, I	1734, 1692, 1620	13.4, 20.5, 23.3	$C_{18}H_{18}N_4O_2 \cdot CH_4O_3S$	54.27 (53.90	5.75 5.80	13.33 13.09)
IVj	*p*	$-CH=C(C_2H_5)-$	C_2H_5	MSA	167—168	F	1686, 1618, 1604	13.4, 14.1, 20.3, 60.4	$C_{14}H_{19}N_3O_2 \cdot CH_4O_3S$	50.40 (50.27	6.49 6.61	11.76 11.62)
IVk	*p*	$-CH=C(C_2H_5)-$	H	MSA	196—198	G	1670, 1617, 1605	13.5, 20.2	$C_{12}H_{15}N_3O_2 \cdot CH_4O_3S$	47.40 (47.60	5.81 5.87	12.76 12.51)
V	*p*	$-(CH_2)_3-$	C_6H_5	MSA	82—84	C	1755, 1663, 1622	25.9, 32.7, 33.6	[i]			

a) Gu=–NH–C(=NH)–NH_2. *b*) MSA=CH_3SO_3H. *c*) 6-MP=  . *d*) Lit.,[12] 214—215 °C (nitrate). *e*) Lit.,[12] 163—170 °C (nitrate). *f*) Lit.,[13] 95 °C (nitrate).

g—*i*) These structures were confirmed by the IR and NMR spectral data.

increased with increasing carbon chain length of α-substituents, *p*-guanidino derivatives were more potent than *m*-guanidino derivatives, cinnamates were more potent than the corresponding phenylpropionates, and the inhibitory potency was higher toward Cl esterase than toward other serine-proteases. Thus, IVg and IVh proved to be the most active inhibitors of Cl esterase (with IC_{50} values of 7×10^{-6} and 6×10^{-6} M, respectively), as well as of other serine-proteases among the compounds synthesized in this study. The 6-methyl-3-pyridyl ester (IVi) was nearly as potent as the phenyl ester of the same α-ethyl derivative (IVg) in terms of Cl esterase inhibition. As in the series of phenylpropionate derivatives, the ethyl ester (IVj) and free carboxylic acid (IVk) were practically inactive as serine-protease inhibitors. Remarkable suppression of *in vitro* complement-mediated hemolysis was shown by IVi and IVf with inhibitors of 86 and 84% at 1×10^{-3} M, respectively. However, IVc, IVd, IVg, and IVh showed no detectable suppression of the hemolysis, although these compounds exhibited Cl esterase inhibition as potent as that of IVi and IVf, with IC_{50} values of the order of 10^{-5}—10^{-6} M. The discrepancy is presumably attributable to strong spontaneous hemolysis by the former compounds themselves at a concentration of 1×10^{-3} M.

Experimental

Melting points were determined on a Yamato MP-21 melting point apparatus and are uncorrected. The infrared (IR) spectra were determined on a Shimadzu IR-430 and ^{13}C nuclear magnetic resonance (^{13}C-NMR) on a JEOL FX-60Q (15 MHz) spectrometer (with tetramethylsilane as an internal standard). The physical data are summarized in Tables IV—VI.

Phenyl *p*-Nitrobenzoate (VIIb: *p*-NO_2, Y=-) (Method A)——A solution of 12.1 g of triethylamine in 50 ml of ethyl acetate (AcOEt) was added to a solution of 18.6 g of *p*-nitrobenzoyl chloride and 9.4 g of phenol in 70 ml of AcOEt. The mixture was stirred for 3 h at room temperature, then washed with 10% HCl, 5% NaOH and satd. NaCl, dried over anhyd. $MgSO_4$ and concentrated to dryness *in vacuo*. Recrystallization of the residue from ethanol (EtOH) gave 16.5 g (68%) of VIIb (*p*-NO_2, Y=-) as pale yellow prisms.

Phenyl *p*-Aminobenzoate Hydrochloride (VIIIb: *p*-NH_2, Y=-) (Method B)——A solution of 15 g of VIIb (*p*-NO_2, Y=-) in 100 ml of dimethylformamide (DMF) containing 2.5 g of HCl was hydrogenated at room temperature under atmospheric pressure in the presence of 10% Pd–C catalyst (2.0 g). The solution was filtered and the filtrate was poured into 200 ml of AcOEt to give an amorphous powder. Recrystallization from EtOH–ethylether (Et_2O) gave 10.8 g (70%) of VIIIb (*p*-NH_2, Y=-) as colorless powder.

Phenyl *p*-Guanidinobenzoate Methanesulfonate (Ib) (Method C)——A mixture of 10.0 g of VIIIb (*p*-NH_2, Y=-) and 2.5 g of cyanamide in 50 ml of EtOH was stirred overnight at 50 °C. The mixture was filtered and the filtrate was poured into satd. $NaHCO_3$. Precipitates were collected and washed with water and acetone. A suspension of the precipitates in 50 ml of EtOH was treated with 4.2 g of methanesulfonic acid with stirring. Et_2O was added to the solution and precipitates were collected and recrystallized from EtOH to give 6.5 g (48%) of Ib as colorless prisms.

Phenyl α-Ethyl-*p*-nitrocinnamate (Xg: *p*-NO_2, $R_1=C_2H_5$, $R_2=C_6H_5$) (Method D)——A suspension of 11 g of α-ethyl-*p*-nitrocinnamic acid in 150 ml of AcOEt was treated with 15 g of PCl_5. After being stirred for 3 h at room temperature, the mixture was concentrated to dryness *in vacuo* and the residue was recrystallized from AcOEt–hexane to give the acid chloride. Next, a solution of 10 g of triethylamine in 100 ml of AcOEt was added to a solution of the acid chloride and 5 g of phenol in 300 ml of AcOEt. After being stirred for 3 h at room temperature, the mixture was washed with 10% HCl, 5% NaOH and satd. NaCl, dried over anhyd. $MgSO_4$ and concentrated to dryness *in vacuo*. Recrystallization of the residue from EtOH gave 13.3 g (86%) of Xg (*p*-NO_2, $R_1=C_2H_5$, $R_2=C_6H_5$) as pale yellow prisms.

Phenyl α-Ethyl-*p*-aminocinnamate Hydrochloride (XIg: *p*-NH_2, $R_1=C_2H_5$, $R_2=C_6H_5$) (Method E)——A suspension of 9 g of Xg (*p*-NO_2, $R_1=C_2H_5$, $R_2=C_6H_5$) in 100 ml of acetic acid was stirred overnight with 9 g of zinc powder at room temperature, then the mixture was filtered, and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in AcOEt and the solution was washed with 5% NaOH and satd. NaCl, then dried over anhyd. $MgSO_4$, and concentrated to dryness. The residue was dissolved in Et_2O, the solution was filtered, and dry HCl was passed into the filtrate. The precipitates formed were collected to give 6.8 g (74%) of XIg (*p*-NH_2, $R_1=C_2H_5$, $R_2=C_6H_5$), which was used for the next step without further purification.

Phenyl α-Ethyl-*p*-guanidinocinnamate Methanesulfonate (IVg) (Method F)——A mixture of 6 g of XIg (*p*-NH_2, $R_1=C_2H_5$, $R_2=C_6H_5$) and 2.5 g of cyanamide in 40 ml of MeOH was stirred overnight at 50 °C. The mixture was concentrated to half the initial volume and poured into satd. $NaHCO_3$. The precipitates were collected and washed with water and acetone, then 2.5 g of methanesulfonic acid was added to a suspension of the precipitates in 50 ml of

EtOH with stirring. Et_2O was added to the mixture and the precipitates were collected. Recrystallization from EtOH gave 1.6 g (23%) of IVg as colorless prisms.

α-Ethyl-*p*-gluanidinocinnamic Acid Methanesulfonate (IVk) (Method G)——A mixture of 1.0 g of IVg in 5 ml of EtOH and 10 ml of 10% NaOH was stirred overnight at room temperature. The mixture was then neutralized with 10% HCl, and the precipitates were collected. A suspension of the precipitates in 5 ml of DMF was treated with 0.5 g of methanesulfonic acid to give a clear solution, to which Et_2O was added. The precipitates were collected and recrystallized from MeOH–Et_2O to provide 0.7 g (86%) of IVk as colorless prisms.

Ethyl α-Ethyl-*p*-nitrocinnamate (Xj: *p*-NO_2, $R_1=C_2H_5$, $R_2=C_2H_5$) (Method H)——A suspension of 10 g of α-ethyl-*p*-nitrocinnamic acid in 200 ml of EtOH was bubbled through with dry HCl until saturation. After being stirred overnight at room temperature, the mixture was concentrated to dryness *in vacuo*. The residue was dissolved in AcOEt and the solution was washed with 5% NaOH and satd. NaCl, dried over anhyd. $MgSO_4$ and concentrated to dryness *in vacuo*. Recrystallization from hexane gave 6.5 g (58%) of Xj (*p*-NO_2, $R_1=C_2H_5$, $R_2=C_2H_5$) as yellow plates.

6-Methyl-3-pyridyl α-Ethyl-*p*-guanidinocinnamate Methanesulfonate (IVi) (Method I)——A DMF (10 ml) solution of 1.4 g of 6-methyl-3-pyridyl α-ethyl-*p*-guanidinocinnamate bis(methanesulfonate) [prepared from 6-methyl-3-pyridyl α-ethyl-*p*-aminocinnamate dihydrochloride according to method F] was mixed with 10 ml of pyridine, then 100 ml of Et_2O was added. The precipitates were collected and recrystallized from EtOH–H_2O–AcOEt to give IVi as colorless prisms.

Phenyl α-Ethyl-*p*-guanidinophenylpropionate Methanesulfonate (IIIg) (Method J)——A suspension of 5.0 g of IVg in MeOH was hydrogenated at room temperature under atmospheric pressure in the presence of 10% Pd–C (1.0 g). The mixture was filtered and the filtrate was concentrated to dryness *in vacuo*. Recrystallization of the residue from EtOH–Et_2O gave 4.5 g (90%) of IIIg as colorless prisms.

Enzyme Inhibition——Bovine trypsin was purchased from Sigma Chemical Co., St. Louis, U.S.A. and dissolved in 0.1 M borate buffer containing 0.01 M $CaCl_2$, pH 8.5. Human plasmin was purchased from Green Cross Co., Osaka, Japan, and porcine kallikrein from Bayer, and they were each dissolved in 0.1 M borate buffer, pH 8.5. Bovine thrombin was purchased from Mochida Pharmaceutical Co., Ltd., Tokyo, Japan, and dissolved in 0.02 M phosphate buffer, pH 7.4. Human Cl esterase was prepared by the method of Sumi *et al.*[5] The rates of hydrolysis of TAME by trypsin, plasmin, kallikrein, and thrombin were determined as described by Muramatsu *et al.*,[6] and that of ATEE by Cl esterase as described by Tamura *et al.*,[7] at a substrate concentration of 10 mM.

Inhibition of Complement-Mediated Hemolysis——Sheep erythrocyte was purchased from Tokyo Faruma Co., Tokyo, Japan and hemolysin from Denka Seiken Co., Ltd., Tokyo, Japan. Complement-mediated hemolytic activities were determined as described by Baker *et al.*[8]

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