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Medicinal Chemical Studies on Synthetic Protease Inhibitors, *trans*-4-Guanidinomethylcyclohexanecarboxylic Acid Aryl Esters

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trans-4-Guanidinomethylcyclohexanecarboxylic acid (*trans*-GMCHA) aryl esters were synthesized and tested for inhibitory effects on serine proteases, trypsin, chymotrypsin, plasmin, plasma kallikrein, pancreatic kallikrein, urokinase and thrombin. In general, these compounds showed strong inhibitory effects on chymotrypsin, pancreatic kallikrein and urokinase, but the effects varied greatly depending on the substituent in the benzene nucleus.

Some of the *trans*-GMCHA aryl esters strongly inhibited compound 48/80-induced histamine release from mast cells; the *p-tert*-butylphenyl ester was especially active.

Keywords——serine protease inhibitor; *trans*-4-guanidinomethylcyclohexanecarboxylic acid aryl ester; mast cell; histamine release; anti-allergic agent; structure–activity relationship

Synthetic protease inhibitors have been used clinically in the treatment of gastric ulcers and pancreatitis,[1,2] while others obtained from microorganisms are being tested as anticancer drugs.[3] One of the authors, Muramatu,[4-8] found that esters of *trans*-4-aminomethylcyclohexanecarboxylic acid (*trans*-AMCHA) and ε-guanidinocaproic acid (ε-GCA) are stronger inhibitors of plasmin than the corresponding acids. Moreover, he showed that the phenyl and *p*-carboxyethylphenyl esters of *trans*-AMCHA are strong inhibitors of serine proteases such as plasmin and plasma kallikrein.[8] Okano *et al.* synthesized numerous amides and esters of *trans*-AMCHA and their N-substituted derivatives and showed that of these *trans*-AMCHA derivatives only the aryl esters had a strong antiplasmin effect.[9]

trans-AMCHA *p*-carboxyethylphenyl ester, which is a potent inhibitor of serine proteases,[8] was recently put on the market as an antiulcer drug (common name: cetraxate hydrochloride), though it is difficult to explain the mechanism of its antiulcer effect simply in terms of its antiplasmin action. Many other receptors which participate in the regulatory systems of living things might also interact with serine protease inhibitors to various extents. Thus, the pharmacological screening of newly designed protease inhibitors may result in the discovery of new physiologically active compounds.

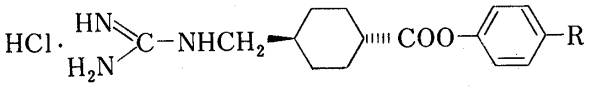
We prepared *trans*-4-guanidinomethylcyclohexanecarboxylic acid (*trans*-GMCHA) aryl esters and tested their inhibitory activities on serine proteases. *In vivo* pharmacological screening of the *trans*-GMCHA aryl esters showed that some of the esters (especially *p-tert*-butylphenyl ester) had striking preventive effects against anaphylactic shock in guinea pigs. (Details of the experiments will be reported elsewhere.) To clarify the mechanism of the preventive effects of these esters on anaphylactic shock, we examined their direct inhibitory effects on histamine release from mast cells induced by compound 48/80.

Results and Discussion

Synthesis of *trans*-GMCHA Aryl Esters

trans-GMCHA aryl esters were synthesized by the usual method from *trans*-GMCHA hydrochloride (**30**) and phenols with the aid of *N*, *N'*-dicyclohexylcarbodiimide (DCC) (Method A). Some *trans*-GMCHA aryl esters were obtained by the reaction of **30** with diarylsulfites (Method B).

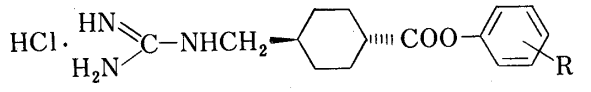
TABLE I. *trans*-GMCHA *p*-Substituted Phenyl Esters



No.	R	mp (°C)	Yield (%)	Method	Formula	*Anal.*	IR (KBr) $\nu_{C=O}$ cm^{-1}
1	–H	159.5—161.5	67	A	$C_{15}H_{21}N_3O_2 \cdot HCl$	C, H, N	1755
2	$-CH_2CH_2COOEt$	90—91	87	A	$C_{20}H_{29}N_3O_4 \cdot HCl$	C, H, N	1740, 1725
3	$-OCH_3$	203—205	42	A	$C_{16}H_{23}N_3O_3 \cdot HCl$	C, H, N	1745
4	$-COOCH_2$–Ph	134—138	66	A	$C_{23}H_{27}N_3O_4 \cdot HCl$	C,[a] H, N	1750, 1710
5	–COOEt	181—184	59	A	$C_{18}H_{25}N_3O_4 \cdot HCl$	C, H, N	1755, 1715
6	–COO–Ph	166—170	54	A	$C_{22}H_{25}N_3O_4 \cdot HCl$	C, H, N	1745, 1715
7	–Br	165—166	52	A	$C_{15}H_{20}BrN_3O_2 \cdot HCl$	C, H, N	1735
8	$-NO_2$	155—155.5	25	A	$C_{15}H_{20}N_4O_4 \cdot HCl$	C, H, N	1760
9	$-SO_2NH_2$	194—196	65	A	$C_{15}H_{22}N_4O_4S \cdot HCl$	C, H, N	1760
10	$-C(CH_3)_3$	208—210	70	A	$C_{19}H_{29}N_3O_2 \cdot HCl$	C, H, N	1750
11	$-CH_3$	151—153	70	B	$C_{16}H_{23}N_3O_2 \cdot HCl$	C, H, N	1742
12	–Cl	163—165	60	B	$C_{15}H_{20}N_3O_2 \cdot HCl$	C, H, N	1747

a) Calcd: 61.95; Found: 61.47.

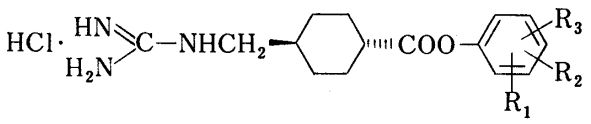
TABLE II. *trans*-GMCHA *o*- or *m*-Substituted Phenyl Esters



No.	R	mp (°C)	Yield (%)	Method	Formula	*Anal.*	IR (KBr) $\nu_{C=O}$ cm^{-1}
13	2-COOEt	110—111	73	A	$C_{18}H_{25}N_3O_4 \cdot HCl$	C, H, N[a]	1740
14	2-OCH_3	141—145	84	A	$C_{16}H_{23}N_3O_3 \cdot HCl$	C, H, N	1760
15	2-OEt	144—148	61	A	$C_{17}H_{25}N_3O_3 \cdot HCl$	C,[b] H, N	1750
16	2-$COCH_3$	159—166	44	A	$C_{17}H_{23}N_3O_3 \cdot HCl$	C, H, N	1750
17	2-CHO	135—138	39	A	$C_{16}H_{21}N_3O_4 \cdot HCl$	C,[c] H, N	1740
18	2-COO–Ph	157—162	68	A	$C_{22}H_{25}N_3O_4 \cdot HCl$	C, H, N	1750, 1740
19	2-CN	102—105	70	A	$C_{16}H_{20}N_4O_2 \cdot HCl$	C, H, N	1760
20	2-$COOCH_2$–Ph	82—84	89	A	$C_{23}H_{27}N_4O_2 \cdot HCl$	C,[d] H, N	1730
21	2-Cl	157—158	70	B	$C_{15}H_{20}ClN_3O_2 \cdot HCl$	C, H, N	1723
22	2-OCH_2–Ph	120—123	80	A	$C_{22}H_{27}N_3O_3 \cdot HCl$	C, H, N	1745
23	3-CF_3	88—91	60	A	$C_{16}H_{20}F_3N_3O_2 \cdot HCl$	C, H, N	1742
24	3-$COOCH_2$–Ph	75—80	82	A	$C_{23}H_{27}N_3O_4 \cdot HCl$	C,[e] H, N	1755, 1725

a) Calcd: N, 10.95; Found: 10.54. *b*) Calcd: C, 57.38; Found: 56.94.
c) Calcd: C, 56.55; Found: 55.98. *d*) Calcd: C, 61.95; Found: 61.38.
e) Calcd: C, 61.95; Found: 61.37.

TABLE III. *trans*-GMCHA Di- and Trisubstituted Phenyl Esters



No.	R_1	R_2	R_3	mp (°C)	Yield (%)	Method	Formula	*Anal.*	IR (KBr) $\nu_{C=O}$ cm^{-1}
25	2-OCH_3	4-CHO	-H	110—111	87	A	$C_{17}H_{23}N_3O_4 \cdot HCl$	C,[a] H, N	1760
26	2-CH_3	4-CH_3	-H	151—153	73	A	$C_{17}H_{25}N_3O_2 \cdot HCl$	C, H, N	1745
27	2-$CH(CH_3)_2$	5-CH_3	-H	158—160	39	A	$C_{19}H_{29}N_3O_2 \cdot HCl$	C, H, N	1730
28	2-$CH(CH_3)_2$	4-Cl	5-CH_3	185—187	29	A	$C_{19}H_{28}ClN_3O_2 \cdot HCl$	C, H, N	1735
29	2-Cl	4-Cl	6-Cl	151—155	46	A	$C_{15}H_{18}Cl_3N_3O_2 \cdot HCl$	C, H, N	1760

a) Calcd: C, 55.21; Found: 54.74.

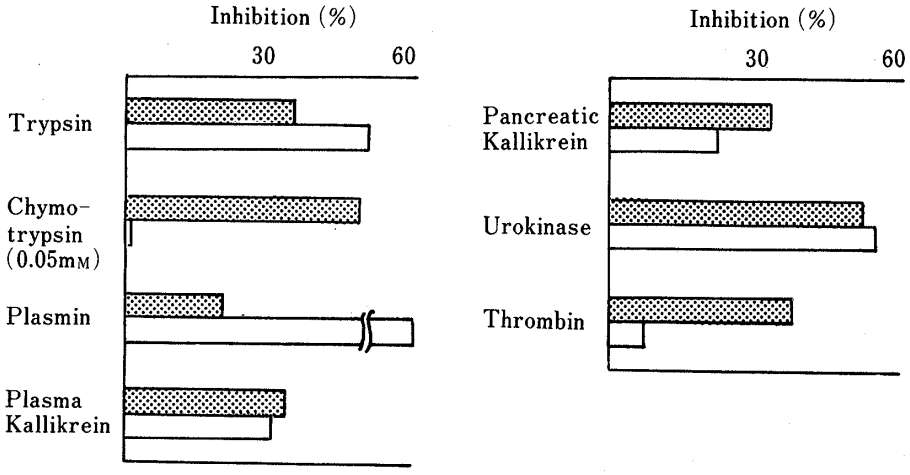
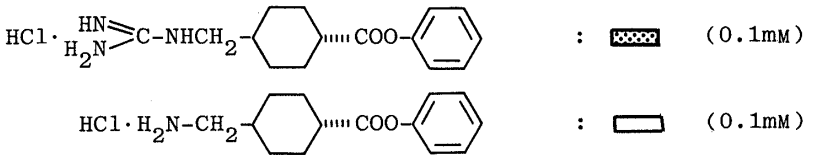


Fig. 1. Comparison of Inhibitory Patterns on Serine Proteases between *trans*-GMCHA and *trans*-AMCHA Phenyl Esters



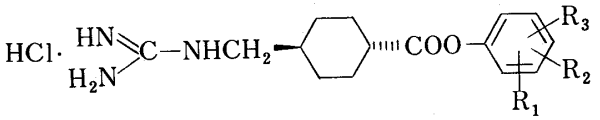
Tables I, II, and III show data on the melting points, yields, elemental analyses and infrared (IR) spectra of *trans*-GMCHA aryl esters.

Inhibitory Effects of *trans*-GMCHA Aryl Esters on Serine Proteases

First, the differences in the inhibitory effects on serine proteases between *trans*-GMCHA and *trans*-AMCHA aryl esters were studied by using the corresponding phenyl esters. Their inhibitory patterns are shown in Fig. 1.

trans-GMCHA phenyl ester (**1**) and *trans*-AMCHA phenyl ester (AMCHA-PHE) showed similar patterns of inhibition of trypsin, kallikreins and urokinase, but had different inhibitory effects on chymotrypsin, plasmin and thrombin, that is, **1** inhibited chymotrypsin strongly and plasmin weakly, while *trans*-AMCHA-PHE inhibited chymotrypsin weakly and plasmin strongly.

TABLE IV. Inhibitions of Serine Proteases by *trans*-GMCHA Aryl Esters



No.	R_1	R_2	R_3	Inhibition (%) by 0.1 mM *trans*-GMCHA aryl esters						
				Trypsin	Chymotrypsin[a]	Plasmin	Plasma kallikrein	Pancreatic kallikrein	Urokinase	Thrombin
1	–H	–H	–H	35	48	20	33	33	52	38
2	4-CH_2CH_2COOEt	–H	–H	50	20	40	48	32	55	43
3	4-OCH_3	–H	–H	26	38	23	29	19	51	30
4	4-$COOCH_2$–Ph	–H	–H	50	99	40	34	93	76	36
5	4-COOEt	–H	–H	23	82	26	36	53	50	43
6	4-COOPh	–H	–H	64	100	63	37	45	—[b]	53
7	4-Br	–H	–H	30	95	16	20	38	61	35
8	4-NO_2	–H	–H	53	100	49	23	85	83	55
9	4-SO_2NH_2	–H	–H	—	56	7	28	25	95	38
10	4-$C(CH_3)_3$	–H	–H	51	50	33	35	60	72	32
13	2-COOEt	–H	–H	28	60	14	30	35	25	29
14	2-OCH_3	–H	–H	26	20	22	31	28	49	30
15	2-OEt	–H	–H	22	28	26	20	30	84	35
16	2-$COCH_3$	–H	–H	31	50	15	38	26	22	28
17	2-CHO	–H	–H	23	70	20	10	27	67	39
18	2-COOPh	–H	–H	38	90	10	40	45	35	34
19	2-CN	–H	–H	0	90	26	23	91	47	38
20	2-$COOCH_2$–Ph	–H	–H	80	72	27	35	56	32	36
21	2-Cl	–H	–H	30	92	13	27	52	—	40
22	2-OCH_2–Ph	–H	–H	0	54	14	7	50	86	48
23	3-CF_3	–H	–H	42	58	47	28	55	23	36
24	3-$COOCH_2$–Ph	–H	–H	32	100	18	46	75	—	40
25	2-OCH_3	4-CHO	–H	72	70	31	20	37	72	38
27	2-$CH(CH_3)_2$	5-CH_3	–H	37	—	21	12	38	30	38
28	2-$CH(CH_3)_2$	4-Cl	5-CH_3	42	43	24	3	44	—	34
29	2-Cl	4-Cl	6-Cl	0	61	17	6	63	—	65

a) 0.05 mM. *b*) Not determined.

TABLE V. Inhibitory Activity of *trans*-GMCHA Aryl Esters[a] on Histamine Release from Rat Mast Cells Induced by Compound 48/80

No.	Inhibitory activity (%)	No.	Inhibitory activity (%)
1	22	**16**	14
2	13	**17**	1
3	17	**18**	3
4	16	**19**	0
5	9	**20**	—[b]
6	8	**21**	2
7	0	**22**	0
8	9	**23**	0
9	0	**24**	1
10	80	**25**	8
11	3	**26**	0
12	5	**27**	0
13	8	**28**	39
14	13	**29**	6
15	9	DSCG[c]	7

a) The concentration of test sample: 10 μg/ml.
b) Not determined. *c*) DSCG: disodium chromoglycate.

Table IV shows the inhibitory effects of various *trans*-GMCHA aryl esters.

As mentioned above, *trans*-GMCHA aryl esters strongly inhibited trypsin, chymotrypsin, pancreatic kallikrein and urokinase, but their inhibitory activities varied, apparently depending on the kinds and positions of substituents in the phenyl moiety. Compounds **4**—**8**, **12**, **13**, **17**—**21**, **25**, and **29** strongly inhibited chymotrypsin. These compounds all have electron-withdrawing substituents on the benzene nucleus and the electronic parameters of the substituents seem to influence the inhibitory effects on the enzyme. In contrast, among the potent inhibitors for trypsin (**6**, **11**, **20**, **25**, **26**), pancreatic kallikrein (**4**, **8**, **19**, **24**, **29**) and urokinase (**9**—**11**, **15**, **17**, **22**, **25**), there was no obvious correlation between the electronic nature of the substituents and the inhibitory effects.

Inhibition by *trans*-GMCHA Aryl Esters of Histamine Release from Mast Cells Induced by Compound 48/80

The inhibitory effects of *trans*-GMCHA aryl esters on histamine release from mast cells induced by compound 48/80 were examined. Mast cells are known to be histamine storage sites in mammals and histamine is known to be released from these cells by the actions of various exogenous substances[10,11] or in the antigen–antibody reaction,[12,13] causing allergy or anaphylaxis. Participation of a chymotrypsin-like enzyme in histamine release from mast cells had been suggested.[14,15]

Of the compounds tested, **1**, **10** and **28** inhibited histamine release by more than 20%, and *trans*-GMCHA *p-tert*-butylphenyl ester (**10**) inhibited it up to 80%. Under the same conditions, disodium chromoglycate (DSCG), a known inhibitor of histamine release from mast cells, caused weak inhibition (7%).

These findings suggest that the effect of **10** in preventing anaphylaxis is closely related to its inhibition of histamine release from mast cells induced by compound 48/80. The possible interaction of **10** with receptors involved in the histamine release process should also be considered, because no marked difference in enzyme inhibition profile could be found between **10** and the other *trans*-GMCHA esters tested.

Experimental

Melting points were obtained with a Büchi melting point apparatus and are given as uncorrected values. IR spectra were obtained with a Shimadzu model IR-27G.

Materials——Phenols and *trans*-4-aminomethylcyclohexanecarboxylic acid (*trans*-AMCHA) were purchased from commercial sources. Substrates for enzymic reactions such as N^{α}-tosyl-L-arginine methyl ester HCl (TAME), N^{α}-benzoyl-L-arginine methyl ester (BAEE), N^{α}-acetyl-L-tyrosine ethyl ester (ATE) and N^{α}-acetylglycyl-L-lysine methyl ester HCl (GLME) were from the Protein Research Foundation, Osaka. Compound 48/80 was purchased from Sigma Chemical Co., St. Louis. Trypsin,[7] plasmin,[4] plasma kallikrein[8] and thrombin[8] were as described previously. Chymotrypsin was purchased from Sigma Chemical Co., St. Louis, and pancreatic kallikrein for injection was from E. Bayer, Leverkusen. Urokinase was purchased from Godo Shusei Co., Tokyo; its specific activity was 9800 i.u./mg protein.

trans-4-Guanidinomethylcyclohexanecarboxylic acid (*trans*-GMCHA) and its hydrochloride were synthesized as follows: 2 N NaOH solution (36 ml) was added to a solution of methyl isothiourea disulfate (10.0 g, 0.07 mol) in water (36 ml) with cooling in ice and stirring, and then *trans*-AMCHA (10.8 g, 0.069 mol) in boiling water (54 ml) was added dropwise. The mixture was left to stand overnight at room temperature and then chilled in ice water for 1 h. The precipitated white crystals were filtered off and washed with cold water. The yield of *trans*-GMCHA (mp 300 °C) was 11.4 g (83%). *Anal.* Calcd for $C_9H_{17}N_3O_2$: C, 54.25; H, 8.60; N, 21.09. Found: C, 54.01; H, 8.45; N, 21.28.

trans-GMCHA (10.0 g, 0.05 mol) was dissolved in warm 1 N HCl (76 ml) and insoluble material was removed by filtration. The solution was concentrated *in vacuo* to *ca.* 25 ml. Colorless prisms formed when the solution was cooled in ice water. These crystals, 6.7 g (57%), mp 234—235 °C, were collected and recrystallized from H_2O to obtain pure *trnas*-GMCHA HCl (**30**) (mp 234—235.5 °C).

Synthesis of *trans*-GMCHA Aryl Ester Hydrochlorides——Most *trans*-GMCHA aryl esters were synthesized by method A, but some by method B.

Method A——*trans*-4-Guanidinomethylcyclohexanecarboxylic Acid Phenyl Ester (1): *trans*-GMCHA HCl (11.8 g, 0.05 mol) (**30**), phenol (5.6 g, 0.06 mol) and *N*,*N'*-dicyclohexylcarbodiimide (DCC) (12.4g, 0.06 mol) were dissolved in a mixture of pyridine (75 ml) and DMF (75 ml) with stirring. The reaction mixture was evaporated *in vacuo*, and the residue was suspended in cold 0.1 N HCl (200 ml) then filtered. The filtrate was concentrated to 100 ml and chilled in ice water to obtain white crystals.

Recrystallization of the material from iso-PrOH–iso-Pr ether afforded **1** (10.5 g, mp 159.5—161.5 °C).

trans-4-Guanidinomethylcyclohexanecarboxylic Acid *p*-Nitrophenyl Ester Hydrochloride (**8**): *p*-Nitrophenyl (6.95 g, 0.05 mol) and DCC (6.18 g, 0.03 mol) were added to a suspension of **30** (5.0 g, 0.021 mol) in dry pyridine (100 ml), and the mixture was stirred for 40 h at room temperature. Insoluble material was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was suspended in CH_3COCH_3 (30 ml) and insoluble material was removed by filtration. The CH_3COCH_3 was then evaporated off, and AcOEt (20 ml) was added to the residue to obtain slightly yellowish needless, which were recrystallized from iso-PrOH to afford 1.9 g of **8**, mp 155—155.5 °C.

trans-4-Guanidinomethylcyclohexanecarboxylic Acid *p-tert*-Butylphenyl Ester Hydrochloride (**10**): *p-tert*-Butylphenol (7.2 g, 0.048 mol) and DCC (10.0 g, 0.04 mol) were added to a mixure of dry pyridine (61 ml) and dry DMF (61 ml) **30** (9.4 g, 0.04 mol), and the mixture was stirred for 24 h at room temperature. The solvents were removed by evaporation *in vacuo* and 0.1 N HCl (240 ml) was added to the residue. The solution was stirred for 1 h at room temperature, and then insoluble material was removed by filtration and washed with AcOEt. The filtrate and washings were combined and shaken, and the aqueous layer was separated, concentrated, *in vacuo* and stored in a cold place to obtain a crystalline mass. This was filtered and washed with ether. Recrystallization from EtOH–Et_2O afforded colorless prisms of **10** (10.2 g, mp 208—210 °C).

Method B——*trans*-4-Guanidinomethylcyclohexanecarboxylic Acid *p*-Chlorophenyl Ester Hydrochloride (**12**): **30** (2.0 g, 0.0085 mol) and bis(*p*-chlorophenyl) sulfite (8.5 g, 0.025 mol) were added to a mixture of dry pyridine (9 ml) and dry DMF (18 ml), and the solution was stirred for 1 h at room temperature. The solvents were removed *in vacuo*, and the residue was washed with Et_2O to obtain crystals, which were filtered off and washed with Et_2O and AcOEt. Recrystallization from MeOH–Et_2O afforded **12** (1.77 g, mp 163—165 °C).

The bis(*p*-chlorophenyl) sulfite used in this experiment was prepared from *p*-chlorophenol and $SOCl_2$ as follows: a mixture of dry Et_2O (100 ml), *p*-chlorophenol (25.6 g, 0.2 mol) and $SOCl_2$ (11.8 g, 0.1 mol) was cooled to 0 °C, then a solution of Et_3N (20 g) in dry Et_2O (100 ml) was added over a period of 30 min, and the resulting mixture was stirred overnight at room temperature. After removal of $Et_3N \cdot HCl$, the filtrate was evaporated to dryness under reduced pressure to give bis(*p*-chlorophenyl) sulfite (30 g).

Preparation of Mast Cells——Rat mast cells were prepared as described by Johnson and Moran[16] and Baxter and Admik.[17]

Assay of Inhibition of Various Serine Proteases——The effects of *trans*-GMCHA aryl esters on TAME hydrolysis by trypsin, plasmin, plasma kallikrein and thrombin were assayed by Hesterin's method as modified by Roberts,[18] as described previously.[19] Inhibition of BAEE hydrolytic activity of pancreatic kallikrein was assayed by Hesterin's

TABLE VI. Elemental Analysis

No.	Formula	Calcd (%)			Found (%)		
		C	H	N	C	H	N
1	$C_{15}H_{21}N_3O_2 \cdot HCl$	57.78	7.11	13.48	57.49	7.25	13.27
2	$C_{20}H_{29}N_3O_4 \cdot HCl$	58.32	7.34	10.20	57.98	7.10	10.13
3	$C_{16}H_{23}N_3O_3 \cdot HCl$	56.22	7.08	12.29	56.19	7.01	12.35
4	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.47	6.18	9.35
5	$C_{18}H_{25}N_3O_4 \cdot HCl$	56.32	6.83	10.95	56.21	6.79	11.03
6	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	61.09	6.12	9.78
7	$C_{15}H_{20}BrN_3O_2 \cdot HCl$	46.11	5.42	10.75	45.92	5.31	11.14
8	$C_{15}H_{20}N_4O_4 \cdot HCl$	50.49	5.93	15.70	50.21	5.84	15.83
9	$C_{15}H_{22}N_4O_4S \cdot HCl$	46.09	5.93	14.33	46.29	6.05	14.62
10	$C_{19}H_{29}N_3O_2 \cdot HCl$	62.03	8.22	11.42	62.20	8.29	11.73
11	$C_{16}H_{23}N_3O_2 \cdot HCl$	58.98	7.42	12.90	59.09	7.53	12.61
12	$C_{15}H_{20}ClN_3O_2 \cdot HCl$	52.03	6.11	12.14	52.18	6.17	12.03
13	$C_{18}H_{25}N_3O_4 \cdot HCl$	56.32	6.83	10.95	55.97	6.72	10.54
14	$C_{16}H_{23}N_3O_3 \cdot HCl$	56.22	7.08	12.29	56.18	7.01	12.31
15	$C_{17}H_{25}N_3O_3 \cdot HCl$	57.38	7.37	11.81	56.94	7.01	12.11
16	$C_{17}H_{23}N_3O_3 \cdot HCl$	57.71	6.84	11.88	57.47	6.78	12.03
17	$C_{16}H_{21}N_3O_3 \cdot HCl$	56.55	6.53	12.37	55.98	6.31	12.63
18	$C_{22}H_{25}N_3O_4 \cdot HCl$	61.18	6.07	9.73	61.01	5.95	9.79
19	$C_{16}H_{20}N_4O_2 \cdot HCl$	57.06	6.29	16.63	57.17	6.32	16.97
20	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.38	6.38	9.19
21	$C_{15}H_{20}ClN_3O_2 \cdot HCl$	52.03	6.11	12.14	52.25	6.18	12.03
22	$C_{22}H_{27}N_3O_3 \cdot HCl$	63.23	6.75	10.05	63.41	6.79	9.82
23	$C_{16}H_{20}F_3N_3O_2 \cdot HCl$	50.60	5.57	11.06	50.75	5.63	11.24
24	$C_{23}H_{27}N_3O_4 \cdot HCl$	61.95	6.33	9.42	61.37	6.18	9.85
25	$C_{17}H_{23}N_3O_4 \cdot HCl$	55.21	6.54	11.36	54.74	6.66	11.22
26	$C_{17}H_{25}N_3O_2 \cdot HCl$	60.08	7.71	12.36	60.25	7.78	12.15
27	$C_{19}H_{29}N_3O_2 \cdot HCl$	62.03	8.22	11.42	62.29	8.27	11.30
28	$C_{19}H_{28}ClN_3O_2 \cdot HCl$	56.72	7.26	10.44	56.82	7.30	10.63
29	$C_{15}H_{18}N_3O_2 \cdot HCl$	43.40	4.61	10.12	43.59	4.75	9.87

method with incubation at 37 °C for 30 min in 0.1 M borate buffer, pH 8.0. A substrate concentration of 15 mM was used. Inhibition of urokinase was assayed by the chromotropic acid method[20] after incubation of 60 i.u. of urokinase with 10 mM AGLME at 37 °C for 30 min in 0.06 M phosphate buffer, pH 7.5, containing 0.09 M NaCl.

Assay of the Effects on Histamine Release from Mast Cells Induced by Compound 48/80[21]——Released histamine was determined fluorometrically as follows: 1.5 ml of mast cell suspension (3.5×10^5/ml) was incubated with 1 ml of a physiological solution containing the test sample for 7 min at 37 °C. Then 1 ml of compound 48/80 (3.5 μg/ml) was added. After incubation for 20 min at 37 °C, the suspension was cooled to 4 °C and the histamine release was determined as described by Shore *et al.*[22] None of the compounds tested interfered with the fluorometric assay.

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