J. Enzyme Inhibition, 1994, Vol. 8(1), pp. 25-37
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ANTIBACTERIAL EFFECTS OF ESTERS OF GUANIDINO- AND AMIDINO-ACIDS TRYPSIN INHIBITORS

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(Received 12 August, 1993)

Inhibitory effects of various esters of *trans*-4-guanidinomethylcyclohexanecarboxylic acid and the 4-tert-butylphenyl esters of amidinopiperidine-4-alkanoic, *trans*-4-amidinocyclohexanealkanoic, *trans*-4-guanidinoethylcyclohexanecarboxylic and *trans*-4-guanidinocyclohexanealkanoic acids, all trypsin inhibitors, on the growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* were examined. 4-tert-Butylphenyl esters strongly inhibited the growth of *E. coli* and the order of the inhibitory effects correlated with that for the inhibitory effects on proteinase In, which appears immediately before initiation of DNA synthesis in *E. coli* and closely correlates with the onset of DNA synthesis. No correlation was observed between the inhibitory effects and K_i values for trypsin. The 4-tert-butylphenyl esters also strongly inhibited *B. subtilis*, *S. aureus* and *S. epidermidis*, and the order, of the inhibitory effects on these bacteria roughly coincided with that on *E. coli*. The order of the inhibitory effects of each ester, on these bacteria was *S. epidermidis* > *S. aureus* > *B. subtilis* > *E. coli*. Among the esters examined, the biphenyl ester of *trans*-4-guanidinoethylcyclohexanecarboxylic acid was the most inhibitory on these four bacteria and proteinase In. Hydrolysis of tert-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine 4-methylcoumarin-7-amide, which is a substrate for proteinase In, in crude extracts of *E. coli*, *B. subtilis* and *S. epidermidis* was examined. The order of this activity in these bacteria was *E. coli* > *B. subtilis* > *S. epidermidis*.

KEY WORDS: Trypsin inhibitor, trypsin-like proteinase, Esherichia coli, antibacterial agent, bacteria

INTRODUCTION

Many workers have reported the effects of various protease inhibitors on various cell functions and on the growth of *Escherichia coli* (*E. coli*).[1-5] However, no convincing demonstration on the exact proteinase participating or on the precise role played by the proteinase in these events has been reported.

Recently Kato *et al.* reported[6] that various aromatic esters of *trans*-4-guanidinomethyl-cyclohexanecarboxylic acid (GMCHA), which are competitive trypsin inhibitors, strongly inhibited the growth of *E. coli* K-12 IAM1264, and by using synchronized *E. coli* cells K-12 IAM1264 by phosphate starvation,[7] showed that their inhibitory effects might be due to the suppression of DNA replication by causing inhibition of a trypsin-like proteinase appearing immediately before initiation of DNA synthesis. Kato *et al.* purified the proteinase from *E. coli* and named its proteinase In.[8] These results seem to be the first example suggesting the inhibition mechanism

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of protease inhibitors on the growth of *E. coli* and the role of proteinase in the onset of DNA synthesis. Irisawa *et al.* reported[9] that 4-tert-butylphenyl ester of GMCHA (GMCHA-OPh^{t}Bu) inhibited the growth of and DNA synthesis in various *E. coli* strains, such as K-12 W3350, AB1157, JM103, W3110, C600r$^-$m$^-$ and C600r$^+$m$^+$, as well as IAM1264, and suggested that the suppression effect was caused by inhibiting proteinase In by partially purifying the proteinase from AB1157. These results suggest that proteinase In must be widely distributed in various *E. coli* strains.

Apart from these results, Kozaki *et al.* reported the effects of various esters of GMCHA amidino-piperidine-4-alkanoic acids and *trans*-4-amidinocyclohexanealkanoic acid, known trypsin inhibitors, on the growth of HeLa cells synchronized by thymidine block: (1) GMCHA-OPh^{t}Bu, 4-tert-butylphenyl esters of amidinopipridine-4-acetic and -4-propionic acids (APAA-OPh^{t}Bu and APPA-OPh^{t}Bu) caused 3h retardation of the onset of DNA synthesis by inhibiting a trypsin-like proteinase, tryptase 17:17, appearing at the pre-DNA synthetic mid phase (G1 mid phase),[10,11] (2) the ester of amidinopiperidine-4-carboxylic acid (APCA-OPh^{t}Bu) suppressed DNA synthetic phase (S phase) by inhibiting a trypsin-like proteinase appearing immediately before the onset of DNA replication, proteinase In,[12] (3) the esters of amidinopiperidine-4-butyric acid (APBA-OPh^{t}Bu) suppressed the entry of the cells from the G1 phase into S phase[11] and, (4) the esters of trans-4-amidinocyclohexane-carboxylic and propionic acids (ACHCA-OPh^{t}Bu and ACHPA-OPh^{t}Bu) suppressed the entry of the cells from post-DNA synthetic phase (G2 phase) into mitosis (M phase) by inhibiting a trypsin-like proteinase appearing at the G2 late phase, late G2 proteinase.[13] These results indicate that trypsin-like proteinases participate in the cell cycle progression of HeLa cells, although their precise roles are unclear.

The results obtained with HeLa cells suggest that it would be interesting to examine the effects of the various trypsin inhibitors described above on the growth of *E .coli* and some bacteria, and to compare their effects with those on HeLa cells.

This paper reports that various esters of GMCHA and 4-tert-butylphenyl esters of amidinopiperidine-4-alkanoic, *trans*-4-amidinocyclohexanealkanoic, *trans*-4-guanidinoethylcyclohexanecarboxylic and *trans*-4-guanidinocyclohexanealkanoic acids strongly inhibit the growth of *E. coli*, *Bacillus subtilis* (*B. subtilis*), *Staphylococcus* aureus (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*), suggesting that their effects are due to the inhibition of proteinase In or similar proteinase.

MATERIALS AND METHODS

Materials

4-tert-Butylphenyl esters of *trans*-4-guanidinomethylcyclohexanecarboxylic acid (GMCHA-OPh^{t}Bu), *trans*-4-guanidinoethylcyclohexanecarboxylic acid (GECHA-OPh^{t}Bu), *trans*-4-guaniclinocyclohexaneacetic acid (GCHAA-OPh^{t}Bu), *trans*-4-guanidinocyclohexanepropionic acid (GCHPA-OPh^{t}Bu), amidinopiperidine-4-carboxylic acid (APCA-OPh^{t}Bu), amidinopiperidine-4-acetic acid (APAA-OPh^{t}Bu), amidinopiperidine-4-propionic acid (APPA-OPh^{t}Bu) and amidinopiperidin-4-butylic acid

(APBA-OPh^{t}Bu) and 4-biphenyl (GMCHA-OPhPh), 4-benzyloxycarbonylphenyl, 2-benzyloxycarbonylphenyl, 4-bromophenyl, 4-ehylphenyl, 4-methylphenyl and phenyl esters of GMCHA were obtained as hydrochlorides from Nippon Chemiphar Co., Ltd., Tokyo. 4-*tert*-Bulylphenyl esters of *trans*-4-amidinocyclohexanecarboxylic acid (ACHCA-OPh^{t}Bu) and *trans*-4-amidino-cyclohexanepropionic acid (ACHPA-OPh^{t}Bu) and the 4-biphenyl ester of GECHA (CECHA-OPhPh) were obtained as hydrochlorides from Teikoku Kagaku Sangyo Co., Ltd, Osaka. N$^\alpha$-Benzoyl-D,L-arginine *p*-nitroanilide hydrochloride and 4-methylcoumarin-7-amide of *tert*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine (Boc-Val-Pro-Arg-NH-Mec) were purchased from the Protein Research Foundation, Osaka. Trypsin (Type III) was from Sigma Chemical Co., St. Louis. Nutrient broth, nutrient agar, brain heart infusion (BHI) broth and BHI agar were from Nissui Pharmaceutical Co., Tokyo.

Bacillus subtilis 558, *Staphylococcus aureus* 209P and *Staphylococcus epidermidis* ATCC 12228 were kindly provided from Prof. K. Fukui, Department of Oral Microbiology and Immunology, School of Dentistry, Tokushima University. *E. coli* strain K-12 IAM 1264 ($F^+\lambda^+$) was from Dr. Takahashi, Godo Shusei Co., Tokyo.

Methods

Inhibition of Trypsin Activity Inhibitory effects of various esters on tryptic activity were examined with N$^\alpha$-benzoyl-D,L-arginine *p*-nitroanilide as a substrate, and K_i values were calculated from the Lineweaver-Burk plots as described previously.[14]

Preparation of Proteinase In from E. coli Proteinase In was purified as described previously.[8]

Inhibition of Proteinase In Activity Volumes of 0.9 ml of 0.05 M borate buffer, pH 9.0 containing various amounts of inhibitors and proteinase In were mixed with 0.6 ml of Boc-Val-Pro-Arg-NH-Mec, and the mixture was incubated at 37°C. After 60 min, 1 ml of 30% acetic acid was added, and the amount of 7-amino-4-methylcoumarin released was determined as described by Kanaoka *et al.*[15] The final concentration of Boc-Val-Pro-Arg-NH-Mec was 20 μM.

Growth of E. coli Volumes of 5 ml of nutrient broth medium at pH 7.0 containing various concentrations of an inhibitor were mixed with 0.1 ml of *E. coli* suspension containing about 2×10^5 cells/ml, and incubated for 7 h at 37°C with shaking. After incubation, a mixture of 0.1 ml of each *E.coli* culture diluted to $10^4 - 10^5$ – fold and 15 ml of nutrient agar were spread on 90 mm plates, and incubated overnight at 37°C. The resulting colonies were counted. Values were given as an average of five plates. Similar experiments were performed with BHI medium. IC_{50} was defined as an inhibitor concentration for 50% inhibition of colony formation.

Growth of Other Bacteria Volumes of 5 ml of nutrient broth medium at pH 7.0 containing various concentrations of an inhibitor were mixed with 0.1 ml of bacterium suspension containing about 2×10^6 cells/ml, and incubated for 8 h at 37°C with shaking. After incubation, a mixture of 0.2 ml of each bacterium culture diluted to $10^4 - 10^5$ – fold and 15 ml of nutrient agar was spread on 90 mm plates, and incubated for

36 h at 37°C. The resulting colonies were counted. Values were given as an average of five plates. Similar experiments were performed with BHI medium. IC_{50} was defined as an inhibitor concentration for 50% inhibition of colony formation.

Preparation of Crude Extract The preparation of crude extract from *E. coli* K-12 IAM1264 was carried out as described previously.[8]

B. subtilis 558 was grown in glucose minimum medium[16] at 37°C until absorbance at 600 nm reached 0.7 (about 5×10^8 cells/ml) when, the cells were harvested by centrifugation at 7,000 × g for 30 min at 4°C. Form 1 liter of the culture medium about 1 g of cells was obtained. The cells (about 50 g) were suspended in 500 ml of 0.1 M borate buffer, pH 8.0, containing 0.15 M NaCl, and were frozen at −20°C. About 50 g of *B. subtilis* (500 ml) as prepared above were allowed to thaw and the suspension was sonicated 10 times at 100 W for 30 s in an ice-bath. The homogenate was centrifuged at 13000 × g for 30 min. The precipitate was washed thrice with 180 ml of the above buffer by sonication and centrifuging as described above. The supernatant fluid was collected and designed as the crude extract.

S. epidermidis ATCC12228 was grown in nutrient broth medium at 37°C until absorbance at 600 nm reached 0.4 (about 4×10^8 cells/ml) when the cells were harvested by centrifugation at 7,000 × g for 30 min at 4°C. From 1 liter of the culture medium about 0.5 g of cells was obtained. The cells (about 30 g) were suspended in 300 ml of 0.1 M borate buffer, pH 8.0, containing 0.15 M NaCl, and were frozen at -20°C. About 30 g of *S. epidermidis* (300 ml) as prepared above were allowed to thaw and the suspension was sonicated 10 times at 100 W for 30 s in an ice-bath. The homogenate was centrifuged at 13000 × g for 30 min. The precipitate was washed 11 times with 100 ml of the above buffer by sonication and centrifuging as deseribed above. The supernatant fluid was collected, concentrated by addition of solid ammonium sulfate to 90% saturations and dialyzed against above buffer. The dialyzate was designated as the crude extract (50 ml).

Assay of Proteinase Activity The hydrolytic activity of the proteinase on Boc-Val-Pro-Arg-NH-Mec was determined in 0.1 M borate buffer pH 8.0, containing 1 mM $CaCl_2$ and 0.08 M NaCl. Mixtures of 0.5 ml of the above buffer containing various amount of crude extract and 1 ml substrate were incubated at 37°C. After 60 min, 1 ml of 30 acetic aid was added, and the amount of 7-amino-4-methylcoumarin released was dtermined as described above. The final concentration of substrate was 66.6 μM.

RESULTS

Effects of 4-tert-Butylphenyl Esters of Various Guanidino- and Amidino-Acids on Growth of E.coli

The effects of GECHA-OPh^{t}Bu, GCHAA-OPh^{t}Bu and APBA-OPh^{t}Bu on the growth of *E. coli* K-12 IAM1264 in nutrients broth medium were examined by counting viable cells. The results are shown in Figure 1. Inhibitor concentrations for 50% inhibition (IC_{50})

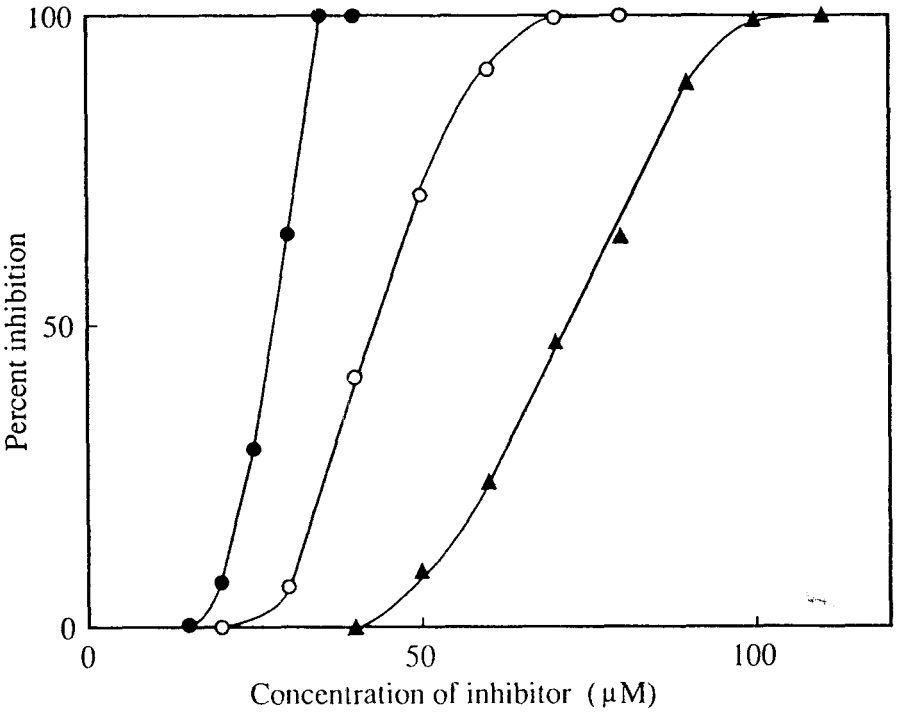

FIGURE 1 Inhibitory Effects of GECHA-OPh^{t}Bu, GCHAA-OPh^{t}Bu and APBA-OPh^{t}Bu on the Growth of *E. coli*. Volumes (5 ml) of nutrient broth medium at pH 7.0 containing various concentrations of an inhibitor were mixed wih 0.1 ml of *E. coli* K-12 IAM1264 suspension containing 2×10^5 cell/ml, and incubated at 37°C for 7 h with shaking. Viable cells were counted by colony counting on agar plates. Each point represents an average of five plates. –●–, GECHA-OPh^{t}Bu; –○–, GCHAA-OPh^{t}Bu; –▲–, APBA-OPh^{t}Bu.

of GECHA-OPh^{t}Bu, GCHAA-OPh^{t}Bu and APBA-OPh^{t}Bu were 28,43, and 71 μM, respectively, and their concentrations for 100% inhibition (IC_{100}) were 45, 90 and 130 μM, respectively. At an inhibitor concentration for 100% inhibition no increase in turbidity were observed after 24 h incubation.

Table 1 shows IC_{50} and IC_{100} values for the 4-tert-butylphenyl esters of various guanidino- and amidino-acids on the growth of *E. coli*. These values were determined as shown in Figure 1. Among the esters examined, GECHA-OPh^{t}Bu was the most effective, and APCA-OPh^{t}Bu was the least active. Guanidino- and amidino-acids themselves and 4-tert-butylphenol were not inhibitory even at 200 μM. As shown in Table 1, the inhibitory effects of these esters on the growth of *E. coli* are effected by the chemical structure of the acid portion, although the effect of acid portion was not marked (see below).

TABLE 1
Inhibitory Effects of Various Synthetic Trypsin Inhibitors on the Growth of *E. coli*, the Proteinase In from *E. coli* and Trypsin

Inhibitors	Inhibitory effects (μM)			
	Growth		Proteinase In	Trypsin
	IC_{50}	IC_{100}	IC_{50}	K_i
GECHA-OPh^{t}Bu	28	45	16	1023
GCHAA-OPh^{t}Bu	43	90	37	2.7
GMCHA-OPh^{t}Bu	45	90	38	38
GCHPA-OPh^{t}Bu	58	100	40	440
APBA-OPh^{t}Bu	71	130	46	220
ACHPA-OPh^{t}Bu	75	190	46	0.7
APPA-OPh^{t}Bu	128	>200	63	0.5
ACHCA-OPh^{t}Bu	140	>200	78	55
APAA-OPh^{t}Bu	189	>200	92	6.9
APCA-OPh^{t}Bu	>200	>200	130	5.4

Inhibitory effects of various synthetic trypsin inhibitors on growth of E. coli K-12 IAM1264 were examined in nutrient broth medium and IC_{50} and IC_{100} values were determined as shown in Figure 1. Inhibitory effects of various trypsin inhibitors on tryptic activity were examined with N^{α}-benzoyl-D,L-arginine *p*-nitroanilide as a substrate, and K_i values were calculated from the Lineweaver-Burk plots. Inhibitory effects of various trypsin inhibitors on proteinase In from *E. coli* were examined with Boc-Val-Pro-Arg-NH-Mec as a substrate.

The inhibitory effects of guanidino-acid esters were stronger than those of amidino-acid esters. The IC_{50}'s of these esters on proteinase In activity and their K_i's for trysin are also shown in Table 1. The IC_{50} of GECHA-OPh^{t}Bu, the most effective inhibitor, was 16 μM and that of APCA-OPh^{t}Bu, the least effective, was 130 μM. Although their inhibitory effects on proteinase In were affected by the chemical structure of the acid portion, the effect of the acid portion was moderate: the effect of APCA-OPh^{t}Bu was 1/8 of GECHA-OPh^{t}Bu. The order for the IC_{50}'s of the esters on proteinase In was closely correlated with that on the growth of *E. coli*. These results strongly suggest that the inhibitory effects of these esters on the growth *E. coli* is caused by inhibiting proteinase In. On the other hand, the K_i's of these esters for trypsin were 0.5 – 1023 μM, and the order was APPA-OPh^{t}Bu > ACHPA-OPh^{t}Bu > GCHAA-OPh^{t}Bu > APCA-OPh^{t}Bu > APAA-OPh^{t}Bu > GMCHA-OPh^{t}Bu > ACHCA-OPh^{t}Bu > APBA-OPh^{t}Bu > GCHPA-OPh^{t}Bu > GECHA-OPh^{t}Bu. Unlike the IC_{50}'s for the growth of *E. coli* and proteinase In, K_i for trysin was greatly affected by the chemical structure of the acid portion, the K_i of APPA-OPh^{t}Bu being about 1/2000 of that of GECHA-OPh^{t}Bu. No correlation between the order of the K_i value for trypsin and the IC_{50} on the growth of *E. coli* was observed.

Effects of 4-tert-Butylphenyl Esters of Various Guanidino- and Amidino-Acids on Growth of B. subtilis, S. aureaus and S. epidermidis

The effects of 4-tert-butylphenyl esters of various guandino- and amidino-acids on *B. subtilis*, *S. aureaus* and *S. epidermidis* were examined in nutrient broth medium. IC_{50}'s were determined as shown in Figure 1. At inhibitor concentration for 100% inhibition no increase in turbidity were observed after 24 h incubation. Table 2 shows the results. The growth of *B. subtilis* was strongly inhibited by the esters tested. GECHA-OPh^{t}Bu was the most effective, the IC_{50} and IC_{100} values being 10 and 25 μM, respectively. APCA-OPh^{t}Bu had the least effect. Guanidino- and amidino-acids themselves and 4-tert-butylphenol were not inhibitory at 200 μM. The inhibitory effect of these esters on the growth of *B. subtilis* was considerably affected by the chemical structure of acid portion: the ratio of the IC_{50} of GECHA-OPh^{t}Bu to that of APCA-OPh^{t}Bu was 1:20, the effect of the acid portion being greater than on *E. coli*. The order of the inhibitory effects of the esters on the growth of *B. subtilis* completely coincided with that on *E. coli*, suggesting that these suppression effects were caused by inhibiting proteinase In-like proteinase occurring in *B. subtilis* (see DISCUSSION)

TABLE 2
Inhibitory Effects of Various synthetic Trypsin inhibitors on the Growth of *B. subtilis*, *S. aureus* and *S. epidermidis*

Inhibitors	Inhibitory effects (μM)					
	B. subtilis		*S. aureus*		*S. epidermidis*	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
GECHA-OPh^{t}Bu	10	25	1.6	7.5	1.2	5
GCHAA-OPh^{t}Bu	11	25	2.1	10	1.8	7.5
GMCHA-OPh^{t}Bu	26	50	3.4	15	2.9	10
GCHPA-OPh^{t}Bu	33	70	3.8	15	3.2	15
APBA-OPh^{t}Bu	36	80	8	25	7.5	25
ACHPA-OPh^{t}Bu	37	80	14	50	9.5	40
APPA-OPh^{t}Bu	79	150	26	70	24	70
ACHCA-OPh^{t}Bu	96	180	83	150	48	130
APAA-OPh^{t}Bu	147	>200	61	130	42	120
APCA-OPh^{t}Bu	200	>200	92	200	81	190

Inhibitory effects of various synthetic trypsin inhibitors on growth of *E. coli* K-12 IAM1264, *B. subtilis* 558, *S. aureus* 209P and *S. epidermidis* ATCC12228 were examined in nutrient broth medium and IC_{50} and IC_{100} were determined as shown in Figure 1.

The esters of guanidino- and amidino-acids markedly inhibited the growth of *S. aureus* and *S. epidermidis*, to a greater extent than for other bacteria tested. The effect of GECHA-OPh^{t}Bu on both strains was the greatest, the IC_{50} and IC_{100} on *S. aureus* being 1.6 and 7.5 μM, respectively, and the IC_{50} and IC_{100} on *S. epidermidis* 1.2 and 5 μM respectively. IC_{50}'s for APCA-OPh^{t}Bu on *S. aureus* and *S. epidermidis* were 92 and 81 μM, and the ratio of the IC_{50} of GECHA-OPh^{t}Bu on *S. aureus* to that of APCA-OPh^{t}Bu was 1:60 and for *S. epidermidis* the ratio was 1:70 respectively. These results indicate that among the bacteria examined, the inhibitory effects of these esters on the growth of *S. aureus* and *S. epidermidis* are most strongly affected by the chemical structure of the acid portion. The inhibitory effects of each inhibitory on both strains were closely similar. The orders of inhibitory effect on both strains coincided with those on *B. subtilis* and *E. coli* except for ACHCA-OPh^{t}Bu and APAA-OPh^{t}Bu. these results suggest that the inhibitory effects of these inhibitors on the growth of *S. aureus* and *S. epidermidis* are also caused by inhibiting proteinase In or proteinase In-like proteinase occurring in the strains.

Thus, the results shown in Table 1 and 2 indicate that 4-tert-butylphenyl esters of amidino-acids which showed various effects on cell cycle progression in HeLa cells also suppress various bacterial cell growth, although their effects are less effective than those of 4-tert-butylphenyl esters of guanidino-acids.

Inhibitory Effects of Various Esters of GMCHA on the Growth of E. coli, B. subtilis, S. aureus and S. epidermidis

In Tables 1 and 2 it was shown that the inhibitory effects of various guanidino- and amidino-acid esters on bacterial growth were affected by the chemical structure of the acid portion. Therefore, in the following, the effects of the phenolic group of GMCHA esters were examined. The results are shown in Table 3. Among the esters tested the 4-biphenyl ester was most inhibitory and the order of the inhibitory effect of the ester on bacterial cell growth was *S. epidermidis* > *S. aureus* > *B. subtilis* > *E. coli*. A similar relationship was obtained for each GMCHA ester. The inhibitory effects of the 4-methylpenyl ester on *E. coli* and *B. subtilis* were 32% and 36% at 200 μM, respectively, and those of the phenyl ester on *E. coli* and *B. subtilis* were 22% and 27% at 200 μM, respectively. From the results shown in Table 3 it is seen that the inhibitory effect of GMCHA esters on the growth of all bacteria tested was greatly affected by the nature of the substitutent on the phenlyl nucleus. The order for the inhibitory effects of GMCHA esters on *E. coli* and *B. subtilis* was the same and that for *S. aureus* and *S. epidermidis* was also the same, however, the order for the first pairs differed from that for the second pairs only in that the 4-tert-butylphenyl ester was more effective than the 4-benzyloxycarbonylphenyl ester.

Table 3 shows the IC_{50} values for the esters on proteinase In. 4-Biphenyl and 4-benzyloxycarbonylphenyl esters were strongly inhibitory, and 4-ethylphenyl, 4-methylphenyl and phenyl esters were weakly inhibitory. The inhibitory effects of 4-ethylphenyl, 4-methylphenyl and phenyl esters were 40%, 21% and 23% at 200 μM, respectively. Because the order of these inhibitory effects on the proteinase is closely correlated with those on the growth of *E. coli* and *B. subtilis*, the inhibitory effects of

TABLE 3
Inhibitory Effects of Various GMCHA Esters on the Growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* and on Proteinase In from *E. coli*.

Ester of GMCHA	Inhibitory effects (μM)								
	E. coli[a]		*B. subtilis*		*S. aureus*		*S. epidermidis*		*Proteinase In*[b]
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}
4-Biphenyl	26	40	4	10	0.6	2	0.4	1.5	17
4-Benzyloxycarbonylphenyl	42	60	22	50	4	40	3.6	30	22
4-*tert*-Butylphenyl	45	90	26	50	3.4	15	2.9	10	38
2-Benzyloxycarbonylphenyl	87	140	55	100	8	30	8	30	80
4-Bromophenyl	135	>200	63	120	8	30	8	30	115
4-Ethylphenyl	167	>200	129	>200	15	50	14	50	>200
4-Methylphenyl	>200	>200	>200	>200	47	120	48	120	>200
Phenyl	>200	>200	>200	>200	151	>200	128	>200	>200

Inhibitory effects of various synthetic trypsin inhibitors on growth of *E. coli* K-12 IAM1264, *B. subtilis* 558, *S. aureus* 209P and *S. epidermdis* ATCC12228 were examined in nutrient broth medium and IC_{50} and IC_{100}, values determined as described in Figure 1.[a] Reference.[b] Reference.[8]

these esters on the growth of B. subtilis seem to be caused by inhibiting proteinase In or proteinase In-like proteinase occurring in *B. subtilis*. The inhibitory effects of these esters on the growth of *S. aureus* and *S. epidermidis* may be due to a similar cause.

The relationships between the inhibitory effects of GMCHA-OPhPh on the growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* and on bacterial cell concentration were examined in nutrient broth medium (Table 4). Although the inhibitory effects of GMCHA-OPhPh decreased with increase in the concentration of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis*, the extent of the reduction were small.

TABLE 4
Relationship between Inhibitory Effects of GMCHA-OPhPh on Growth of Bacteria and on Bacterial Concentration.

	Inhibitory effects (μ M)							
	E. coli		*B. subtilis*		*S. aureus*		*S. epidermidis*	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
4×10^4 cells/ml	28	50	4	10	0.6	2	0.4	1.5
4×10^5 cells/ml	31	60	4.8	12.5	0.9	3	0.6	2
4×10^6 cells/ml	33	60	6.6	15	1.4	6	0.9	4

Inhibitory effects of various synthetic trypsin inhibitors on growth of *E. coli* K-12 IAM1264, *B. subtilis* 558, *S. aureus* 209P and *S. epidermidis* ATCC12228 were examined in nutrient broth medium and IC_{50} and IC_{100} values were determined as shown in Figure 1. Various concentrations of GMCHA-OPhPh were added to the medium containing about 4×10^4, 4×10^5 and 4×10^6 cells/ml. The mixture were incubated for 5.5, 4.5 and 3.5 h, respectively for *E. coli* and 8, 5.5 and 3.5 h, for *B. subtilis*, *S. aureus*, and *S. epidermidis* respectively at 37°C with shaking. Without GMCHA-OPhPh, cell numbers increased to approximately 2×10^8 cell/ml after each incubation.

Effects of GMCHA-OPhPh on the growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* were examined in BHI medium. IC_{50}'s for *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* were 26, 4.8, 1.8 and 1.1 μM, respectively, and their IC_{100}'s were 40, 10, 7 and 4 μM, respectively. These results show that there is no significant difference between the different culture media (c.f. Table 3).

Inhibitory effects of GECHA-OPhPh on growth of E. coli, B. subtilis, S. aureus and S. epidermidis

From the results shown in Table 3, it was anticipated that GECHA-OPhPh would have a stronger inhibitory effect on the growth of bacteria than GECHA-OPh^{t}Bu. GECHA-OPhPh was prepared and its effects on the growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis* were examined. The results are shown in Table 5. GECHA-OPhPh strongly inhibited the growth of *E. coli*, *B. subtilis*, *S. aureus* and *S. epidermidis*, and was the most effective among the esters examined in this paper. It also strongly inhibited proteinase In and its inhibitory effect was the greatest among the esters examined, although its K_i for trypsin was 1351 μM, and the inhibitory effect was the poorest (Table 5).

Trypsin-like Proteinase in E. coli, B. subtilis and S. epidermidis

Crude extracts of *E. coli* K-12 IAM1264, *B. subtilis* and *S. epidermidis* were prepared and trypsin-like proteinase activity was determined with Boc-Val-Pro-Arg-NH-Mec which is a substrate for proteinase In. *E. coli* and *B. subtilis* contain a trypsin-like proteinase, protease II, which hydrolyzes N$^{\alpha}$-benzoyl-D,L-arginine *p*-nitroanilide.[17,18]

TABLE 5
Inhibitory effect of the 4-biphenyl ester of GECHA

A. Inhibitory Effects on Growth of *E. coli*, Proteinase In and Trypsin

Inhibitory effects (μM)			
E. coli		Proteinase In	Trypsin
IC_{50}	IC_{100}	IC_{50}	$K-i$
13	25	11	1351

B. Inhibitory Effects on Growth of *B. subtilis*, *S. aureus* and *S. epidermidis*

Inhibitory effects (μM)					
B. subtilis		*S. aureus*		*S. epidermidis*	
IC_{50}	IC_{100}	IC_{50}	IC_{100}	IC_{50}	IC_{100}
3	8	0.4	1.5	0.4	1.5

Inhibitory effects of GECHA-OPhPh on growth of *E. coli* K-12 IAM1264, *B. subtilis* 558, *S.aureus* 209p and *S. epidermidis* ATCC12228 and proteinase In, and trypsin were determined as described in Table 1 and 2.

However, the protease does not hydrolyze the fluorogenic substrate.[8] The proteinase activities on Boc-Val-Pro-Arg-NH-Mec occuring in *E. coli*, *B. subtilis* and *S.epidermidis* were 3.1, 1.4 and 0.1 pmol/min/10^9 cells, respectively, showing that the order of the activity is *E. coli* > *B. subtilis* > *S. epidermidis*. These results may explain the fact that the order of the inhibitory effects of the trypsin inhibitors described in this paper on the growth of bacteria is *S. epidermidis* > *B. subtilis* > *E. coli*. However, a more convincing demonstration is required.

DISCUSSION

In this paper we reported the inhibitory effects of various esters of guanidino- and amidino- acids on the growth of some bacteria. *E. coli* was selected as a representative of gram negative bacilli, *B. subtilis* for gram positive bacilli and *S. aureus* and *S. epidermidis* for gram positive cocci. Previously, we reported the inhibitory effects of various GMCHA esters on the growth of *E. coli*[6,9] and suggested that their effects were due to the suppression of DNA synthesis caused by inhibition of proteinase In which was closely related with the onset of DNA replication.[7,8] Recent advances in the study of DNA synthesis in prokaryotic cells has led to the identification and characterization of elements required for chromosome replication,[19] although the mechanism of control of such replication is still unclear. Therefore, the above findings will provide a clue to resolving the initiation mechanism of DNA synthesis in bacteria and an approach to developing new antibacterial agents.

4-tert-Butylphenyl esters of GMCHA and various amidino-acids showed very interesting effects on the cell cycle progression of HeLa cells synchronized by double-thymidine block.[12,13] GMCHA-OPh^{t}Bu strongly inhihited the cell growth of various *E. coli* cells including K-12 IAM1264, and it was suggested that its effect was due to suppression of DNA synthesis caused by inhibiting proteinase In.[7,9] These results and the results shown in Table 1 suggested that the inhibitory effects of all of the 4-tert-butylphenyl esters were due to suppression of DNA synthesis caused by inhibiting proteinase In. On the other hand, in HeLa cells only APCA-OPh^{t}Bu inhibited the onset of DNA replication caused by inhibiting proteinase In, and it also strongly inhibited proteinase In obtained from HeLa cells (IC_{50} = 0.6 μM and IC_{100} = 5 μM).[12] However, the effects of APCA-OPh^{t}Bu on bacteria growth and proteinase In from *E. coli* were weakly inhibitory (Tables 1 and 2). These discrepancies could be due to the difference between prokaryotes and eukaryotes. Moreover, in the cell cycle process of *E. coli* only one trypsin-like proteinase which hydrolyzes Boc-Val-Pro-Arg-NH-Mec occurred[7–9] whereas, in HeLa cells, at least three trypsin-like proteinase which attacks the fluorogenic substrate were observed.[10,12,13] These facts also would represent differences between prokryotes and eukaryotes including enzyme property, membrane transportation etc.

The inhibitory effects of various esters on trypsin and proteinase In provides an interesting insight into their enzymic targets. The order of the effects of amidino-piperidine-4-alkanoic acid esters on trypsin was APPA-OPh^{t}Bu > APCA-OPh^{t}Bu > APAA-OPh^{t}Bu > APBA-OPh^{t}Bu, and that on proteinase In was APBA-OPh^{t}Bu > APPA-OPh^{t}Bu > APAA-OPh^{t}Bu > APCA-OPh^{t}Bu. These results indicate that the

inhibitory effects of amidinopiperidine-4-alkanoi acid esters on proteinase In correlate with the length of the alkanoic acid portion but that this is not so for, too long an alkanoic portion, such as APBA-OPh^{t}Bu, markedly reduced the inhibitory effects. The ratio of the IC_{50} of APBA-OPh^{t}Bu on proteinase In to that of APCA-OPh^{t}Bu on proteinase In was 1:3, however, the ratio of the K_i of APPA-OPh^{t}Bu for trypsin to that of APBA-OPh^{t}Bu for trypsin was 1:440. A similar relationship is seen with the guanidino-acid esters. The order of the effect of guanidino-acid esters on trypsin was GCHAA-OPh^{t}Bu > GMCHA-OPh^{t}Bu > GCHPA-OPh^{t}Bu > GECHA-OPh^{t}Bu, and that on proteinase In was GCHA-OPh^{t}Bu > GCHAA-OPh^{t}Bu > GMCHA-OPh^{t}Bu > GCHPA-OPh^{t}Bu. The ratio of the IC_{50} of GCHA-OPh^{t}Bu on poteinase In to the IC_{50} of GCHPA-OPh^{t}Bu was 1:2.5, however, the ratio of the K_i of GCHAA-OPh^{t}Bu on trypsin to the K_i of GECHA-OPh^{t}Bu was 1:380. These results indicate that in the esters examined, the inhibitory effect of the esters on proteinase In is not so much affected by the length of the guandino- or amidino-acid portion, whereas for trypsin a larger effect is observed.

Compared with the IC_{50} of each inhibitor on proteinase In, the lC_{50} on the growth of *E. coli* was generally high. This discrepancy may be explained by the effect of permeability of the cell wall or cell membrane for each inhibitor.

tert-Butylphenyl esters of guanidino- and amidino-acids suppressed the growth of *B. subtilis* and their suppression effects were more effective than those on *E. coli*. The order of their inhibitory effects on the growth of *B. subtilis* was similar to that on *E. coli*, suggesting that their suppression effects were caused by inhibiting proteinase In or proteinase In-like proteinase occurring in *B. subtilis*. Recently, Irisawa *et al.* reported that CMCHA-OPh^{t}Bu inhibited the growth of and DNA, RNA and protein synthesis in *B. subtilis*, and they partially purified a trypsin-like proteinase from *B. subtilis*, though the molecular weight of this protease differed from proteinase In purified from *E. coli* K-12 IAM1264, its enzyme properties such as substrate specificity and inhibitory effects of various protease inhibitors including GMCHA esters were closely similar to proteinase In.[20] These results seem to confirm the above suggestion.

4-tert-Butylphenyl esters of various guanidino- and amidino-acids and various GMCHA esters strongly suppressed the growth of *S. aureus* and *S. epidermidis*, and the effects on both strains were greatest among the bacteria examined. The orders of the inhibitory effects on both strains (Table 2 and 3) were similar to those on *E. coli* and *B. subtilis* with some exception. As described in this paper, a trypsin-like proteinase, which hydrolyzes Boc-Val-Pro-Arg-NH-Mec, a specific substrate for proteinase In, was found in *S. epidermidis*. These results suggest that the inhibitory effects of various guanidino- and amidino-acid esters on the growth of *S. aureus* and *S. epidermidis* are caused by inhibiting proteinse In occurring in Staphylococci as well as *E. coli* and *B. subtilis*.

Two factors may be responsible for the effective inhibitory effects of various esters guanidino- and amidino-acids on the growth of *S. aureus* and *S. epidermidis*. The first is the small amount of proteinase In or proteinase In-like activity present as shown in *S. epidermidis*, and the second is the marked inhibitory effects of these esters on proteinase In occurring in *S. aureus* and *S. epidermidis*. Permeability of these esters

through cell wall or cell membrane should also be considered. Further work is required to consolidated these views.

References

1. Prouty, W.F. and Goldberg, A.L. (1972) *J. Biol. Chem.*, **247**, 3341–3352.
2. Rossman, T., Norris, C. and W. Troll, W. (1974) *J. Biol. Chem.*, **249**, 3412–3417.
3. Ito, K. (1977) *J. Bacteriol.*, **132**, 1021–1023.
4. Ito, K. (1978) *Biochem. Biophys. Res. Commun.*, **82**, 99–107.
5. Meyn, M.S., Rossman, T. and Troll, W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1152–1156.
6. Kato, M., Irisawa, T. and Muramatu, M. (1993) *Biol. Pharm. Bull.*, **16**, 120–124.
7. Kato, M., Irisawa, T., Morimoto, Y. and M. Muramatu, M. (1993) *Biol. Pharm. Bull.*, **16**, 552–557.
8. Kato, M., Irisawa, T., Ohtani, M. and Muramatu, M. (1992) *Eur. J. Biochem.*, **210**, 1007–1014.
9. Irisawa, T., Kato, K., Moroishi, J. and Muramatu, M (1993) *Biol. Pharm. Bull.*, **16**, 621–626.
10. Muramatu, M. and Kozaki, Y. (1990) *Biochim Biophys. Acta*, **1087**, 87–90.
11. Kozaki, Y., Kubo, M. and Muramatu, M. (1993) *Biol. Pharm. Bull.*, **16**, 357–361.
12. Kozaki, Y., Kubo, M., Fukuda, Y., Onishi, T. and Muramatu, M. (1993) *Biol. Pharm. Bull.*, **16**, 558–564.
13. Kozaki, Y., Ariki, T. Kubo, M, Onishi T. and Muramatu, M. (1993) *Biol. Pharm. Bull.*, **16**, 829–833.
14. Mori, S., Kozaki, Y., Kato, M., Tendo, A. Kikawa, Y., Sekine, H. and Muramatu, M. (1984) *J. Biochem.*, **95**, 1617–1623.
15. Kanaoka, Y., Takahashi, T., Nakayama, H., Takada, K., Kimura, T. and Sakakibara, S. (1977) *Chem. Pharm. Bull.*, **25**, 3126–3128.
16. Spizizen, J. (1958) *Proc. Natl. Acad. Sci. USA*, **44**, 1072–1078.
17. Pacaud, M. and Richaud, C. (1975) *J. Biol. Chem*, **250**, 7771–7779.
18. Srivastava, O.P. and Aronson, A.I. (1981) *Arch. Microbiol.*, **129**, 227–232.
19. Campbel, J.L. (1986) *Ann. Rev. Biochem.*, **55**, 733–771.
20. Irisawa, T., Kato, M. and Muramatu, M. (1993) *Biol. Pharm. Bull*, **16**, 1211-1215