Effects of Synthetic Trypsin Inhibitors on the Growth of *Escherichia coli*

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The inhibitory effects of various aromatic esters of *trans*-4-guanidinomethylcyclohexanecarboxylic acid (GMCHA), potent trypsin inhibitors, on the growth of *Escherichia coli* were examined and the effects were compared with those of well known synthetic trypsin inhibitors. Various GMCHA esters strongly inhibited the growth of *E. coli* and their effects were markedly affected by the species and position of substitution on the phenyl nucleus of the GMCHA phenyl esters. No correlation was observed between the effects on the growth of *E. coli* and K_i 's for trypsin. Inhibitory effects of benzamidine, phenylmethane sulfonylfluoride and diisopropyl fluorophosphate were less than 10% at 200 μ M. 4-*tert*-Butylphenyl ester of GMCHA (GMCHA-OPh'Bu), a representative of various GMCHA esters, dose-dependently inhibited the growth of *E. coli* and the growth inhibition was preceded by a dose- and time-dependent inhibition of DNA synthesis. Tosyl-L-lysine chloromethyl ketone (TLCK) and pentamidine isethionate, potent trypsin inhibitors, also dose-dependently inhibited the growth of *E. coli* and the DNA synthesis. However, their effects were transient and disappeared after a short while. These results indicate that the effects of TLCK and pentamidine isethionate differ from those of GMCHA esters. GMCHA-OPh'Bu and pentamidine isethionate also inhibited RNA and protein synthesis.

Keywords trypsin inhibitor; *Escherichia coli*; cell growth

Introduction

One of the most promising approaches to the regulation mechanism of a cell cycle is to find a suitable and specific inhibitor which blocks a specific event, such as DNA replication and cell division, without any effect on other events in the cell cycle process. Participation of intracellular proteinase in various cellular functions in *Escherichia coli* has been reported by many workers: rapid degradation of abnormal proteins or accelerated breakdown of normal cell proteins by starvation,^{1–3} processing of secretory and membrane proteins,^{4,5} utilization of exogenous peptides,⁶ the degradation of colicins,^{7,8} viral morphogenesis,^{2,7} and the selective inactivation of certain regulatory proteins.^{2,9} Many workers have reported the effects of various protease inhibitors on various cell functions and on the growth of *E. coli*.^{10–14}

Muramatu and Fujii reported that ω -amino and ω -guanidino acid esters strongly inhibited trypsin-like proteinases,^{15,16} and Mori *et al.*¹⁷ reported that *trans*-4-guanidinomethylcyclohexanecarboxylic acid 4-*tert*-butylphenyl ester (GMCHA-OPh'Bu) competitively inhibited trypsin and suppressed the growth of HeLa cells. Kozaki *et al.*¹⁸ reported that GMCHA-OPh'Bu had no effect on any events in the first S, G2 and M phases, such as the incorporation of [methyl-³H]thymidine into DNA, the increase in the cell concentration and the appearance of trypsin-like proteinase activity. However, GMCHA-OPh'Bu retarded the onset of the second S phase and the various events in the second S, G2 and M phases for 3 h. These results stimulated us to investigate the inhibitory effects of various esters of *trans*-4-guanidinomethylcyclohexanecarboxylic acid (GMCHA) on the growth of *E. coli*.

This paper reports that various aromatic esters of GMCHA, competitive inhibitors of trypsin, strongly inhibit the growth of *E. coli*, and their effects may be due to the inhibition of DNA synthesis, suggesting that their effects differ from those by well known trypsin inhibitors, such as tosyl-L-lysine chloromethyl ketone (TLCK) and pentamidine isethionate.

Materials and Methods

Materials 4-Chloro-2-isopropyl-5-methylphenyl, 4-biphenyl, 4-benzoyloxycarbonyl-ethylphenyl, 4-benzoyloxycarbonylphenyl, 3-benzoyloxycarbonylphenyl, 2,4,6-trichlorophenyl, 4-*tert*-butylphenyl, 4-phenyloxycarbonylphenyl, 2-isopropyl-5-methylphenyl, 2-biphenyl, 2-benzoyloxycarbonylphenyl, 2,4-dichlorophenyl, 4-iodophenyl, 4-bromophenyl, 2-cyanophenyl, 2-phenyloxycarbonylphenyl, 4-ethylphenyl, 4-methylphenyl and phenyl esters of GMCHA were from Nippon Chemphar Co., Ltd., Tokyo, as hydrochlorides. TLCK was from Nacalai Tesque Inc., Kyoto, and pentamidine isethionate was from Sigma Chemical Co., St. Louis. [methyl-³H]Thymidine (85 Ci/mmol), [5-³H]uridine (29 Ci/mmol) and L-[4,5-³H]leucine (160 Ci/mmol) were from Amersham International plc, Buckinghamshire. *N*²-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride was from the Protein Research Foundation, Osaka. Nutrient broth, nutrient agar, brain heart infusion (BHI) broth and BHI agar were from Nissui Pharmaceutical Co., Tokyo. Glucose minimum medium was prepared according to Davis and Mingioli.¹⁹

E. coli strains IFO 3301 and *K* strains were from Prof. Fukui, Department of Oral Microbiology and Immunology, School of Dentistry, Tokushima University. *E. coli* strains IAM 1132, K-12 IAM 1264 and K-12 W 3110 were from Dr. Takahashi, Godo Shusei Co., Tokyo.

Inhibition of Trypsin Activity Inhibitory effects of various esters of GMCHA on tryptic activity were examined with *N*²-benzoyl-DL-arginine *p*-nitroanilide as a substrate, and K_i 's were calculated from the Lineweaver-Burk plots as described previously.¹⁷

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 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 10^3 to 10^5 -fold and 8 ml of nutrient agar were spread on 90 mm agar plates, and incubated overnight at 37°C. The resulting colonies were counted. Values were given as an average of five plates with standard errors. Similar experiments were performed with glucose minimum medium or BHI medium. In the experiments with glucose minimum medium the incubation period was 16 h at 37°C. IC_{50} was defined as an inhibitor concentration for 50% inhibition of colony formation.

A volume of 40 ml of *E. coli* K-12 IAM 1264 suspension attained stationary state was added to 360 ml of glucose minimum medium and the mixture was incubated with stirring at 37°C with or without inhibitor. At one-hour intervals 3 ml samples of the growing cell suspension were withdrawn, and the turbidity was determined at 600 nm.

Uptake of [³H]Thymidine into DNA The uptake of [³H]thymidine into DNA was determined as described by Joseleau-Petit *et al.*²⁰ A volume of 0.4 ml of the growing *E. coli* suspension was withdrawn and incubated with 20 μ l of [³H]thymidine (100 μ Ci/ml) for 5 min at 37°C. The reaction was stopped by adding 0.4 ml of 10% trichloroacetic acid. After 30 min, precipitates were collected on a 0.45 μ m Millipore filter and rinsed with

5% trichloroacetic acid. Filters were dried and counted in Scintisol EX-H (Dojin Laboratories, Kumamoto).

Uptake of [5-³H]Uridine into RNA A volume of 0.4 ml of the growing *E. coli* suspension was withdrawn and incubated with a mixture of 20 μ l of [5-³H]uridine (100 μ Ci/ml) and 20 μ l of cold uridine (100 μ g/ml) for 5 min at 37°C. The reaction was stopped by adding 0.4 ml of 10% trichloroacetic acid. After 30 min, precipitates were collected on a 0.45 μ m Millipore filter and treated as described.

Incorporation of L-[4,5-³H]Leucine into Protein A volume of 0.4 ml of the growing *E. coli* suspension was incubated with a mixture of 20 μ l of L-[4,5-³H]leucine (100 μ Ci/ml) and 20 μ l of cold L-leucine (100 μ g/ml) for 5 min at 37°C. The labeling was terminated by the addition of an equal volume of 10% trichloroacetic acid and heated in a boiling water bath for 15 min. The resulting precipitates were collected using a Millipore filter, and rinsed twice with 5% trichloroacetic acid and once with water. The filters were dried and counted in Scintisol EX-H.

Results

Effects of GMCHA Esters on the Growth of *E. coli* The effects of 4-biphenyl, 4-*tert*-butylphenyl and 2-benzoyloxycarbonylphenyl esters of GMCHA on the growth of *E. coli* K-12 IAM 1264 in nutrient broth medium were examined by counting viable cells (Fig. 1). Inhibitor concentrations for a 50% inhibition (IC_{50}) of 4-biphenyl, 4-*tert*-butylphenyl and 2-benzoyloxycarbonylphenyl esters of GMCHA were 26, 45 and 87 μ M, respectively, and their concentrations for 100% inhibition (IC_{100}) were 40, 90 and 140 μ M, respectively. At inhibitor concentrations for 100% inhibition no increases in turbidity were observed after 24 h incubation.

Table I shows IC_{50} 's and IC_{100} 's of various GMCHA esters on the growth of *E. coli*. IC_{50} 's and IC_{100} 's were determined as shown in Fig. 1. Among the esters tested, 4-chloro-2-isopropyl-5-methylphenyl and 4-biphenyl esters were strongly inhibitory, their IC_{50} 's being 21 and 26 μ M, respectively, and their IC_{100} 's being 30 and 40 μ M, respectively. GMCHA itself, phenol and substituted phenols were not inhibitory even at 200 μ M. The parent compound, phenyl ester of GMCHA, was weakly inhibitory (about 22% inhibition at 200 μ M). K_i 's of various esters of GMCHA for trypsin are also shown in Table I. K_i values of these esters were 29 to 325 μ M, although no relationship between the inhibitory effects on trypsin and those on growth of *E. coli* was obtained. Inhibitory effects of protease inhibitors,

such as benzamidine, phenylmethane sulfonylfluoride and diisopropyl fluorophosphate, were less than 10% at 200 μ M.

The relationship between the effect of GMCHA-OPh'Bu on the growth of *E. coli* and on the cell concentration was examined in nutrient broth medium. Various concentrations of GMCHA-OPh'Bu were added to the medium containing 4×10^3 , 4×10^5 and 4×10^6 cells/ml, and the mixtures were incubated for 7, 4.5 and 3.5 h, respectively, at 37°C with shaking. Without GMCHA-OPh'Bu, cell numbers increased to approximately 2×10^8 cells/ml after each incubation time. Figure 2 represents the results. IC_{50} 's for each cell concentration of 4×10^3 , 4×10^5 and 4×10^6 cells/ml were 45, 51 and 60 μ M, respectively. Although the inhibitory effects of GMCHA-OPh'Bu decreased with increases in the

TABLE I. Inhibitory Effects of Various GMCHA Esters on Growth of *E. coli* and K_i 's for Trypsin

Ester of GMCHA	Inhibitory effects		
	IC_{50} (μ M)	IC_{100} (μ M)	K_i (μ M)
4-Chloro-2-isopropyl-5-methylphenyl	21	30	35
4-Biphenyl	26	40	54
4-Benzoyloxycarbonylphenyl	36	50	29
4-Benzoyloxycarbonylphenyl	42	60	45
3-Benzoyloxycarbonylphenyl	43	60	45
2,4,6-Trichlorophenyl	44	60	273
4- <i>tert</i> -Butylphenyl	45	90	38
4-Phenylloxycarbonylphenyl	64	100	103
2-Isopropyl-5-methylphenyl	68	120	325
2-Biphenyl	74	130	187
2-Benzoyloxycarbonylphenyl	87	140	56
2,4-Dichlorophenyl	92	140	46
4-Iodophenyl	105	>200	51
4-Bromophenyl	135	>200	113
2-Cyanophenyl	142	>200	85
2-Phenylloxycarbonylphenyl	153	>200	56
4-Ethylphenyl	167	>200	48
4-Methylphenyl	>200	>200	78
Phenyl	>200	>200	110

Inhibitory effects of various GMCHA esters on growth of *E. coli* K-12 IAM 1264 were examined in nutrient broth medium and IC_{50} 's and IC_{100} 's were determined as described in Fig. 1. Inhibitory effects of various esters of GMCHA on tryptic activity were examined with *N*^α-benzoyl-DL-arginine *p*-nitroanilide as a substrate, and K_i 's were calculated from the Lineweaver-Burk plots.

Fig. 1. Inhibitory Effects of 4-Biphenyl, 4-*tert*-Butylphenyl and 2-Benzoyloxycarbonylphenyl Esters of GMCHA on the 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* K-12 IAM 1264 suspension containing 2×10^5 cells/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 with standard errors. Vertical bars represent standard errors. —●—, 4-biphenyl ester; —○—, GMCHA-OPh'Bu; —▲—, 2-benzoyloxycarbonylphenyl ester.

Fig. 2. Relationship between Inhibitory Effects of GMCHA-OPh'Bu on Growth of *E. coli* and on Cell Concentration

Volumes of 5 ml of nutrient broth medium containing various concentrations of GMCHA-OPh'Bu with 4×10^3 , 4×10^5 and 4×10^6 cells/ml of *E. coli* K-12 IAM 1264 were incubated at 37°C for 7, 4.5 and 3.5 h, respectively, with shaking. Viable cells were counted by colony counting on agar plates. Each point represents an average of five plates with standard errors. Vertical bars represent standard errors. —●—, 4×10^3 cells/ml; —○—, 4×10^5 cells/ml; —▲—, 4×10^6 cells/ml.

TABLE II. Effects of GMCHA-OPh'Bu on Growth of Various Strains of *E. coli*

Strains of <i>E. coli</i>	IC ₅₀ (μM)		
	BHI medium	Nutrient broth medium	Glucose minimum medium
Kλs	14	14	
IFO 3301	44	44	
IAM 1132	41		
K-12 IAM 1264	44	45	41
K-12 W 3110	49		

E. coli strains Kλs, IFO 3301, IAM 1132, K-12 IAM 1264 and K-12 W 3110 were grown in each BHI, nutrient broth and glucose minimum media and the effects of GMCHA-OPh'Bu on the growth of these strains were examined as described in Fig. 1.

Fig. 3. Inhibitory Effects of GMCHA-OPh'Bu on Growth of *E. coli* and on the Uptake of [³H]Thymidine into DNA

A volume of 40 ml of *E. coli* K-12 IAM 1264 suspension attained stationary state was added to 360 ml of glucose minimum medium and incubated with stirring at 37°C with and without GMCHA-OPh'Bu. a) The inhibitory effect on growth of *E. coli* was determined by turbidity measurement at 600 nm. b) The inhibitory effect on the uptake of [³H]thymidine into DNA was determined by counting the radioactivity in cold trichloroacetic acid precipitable material after incubating the cells at 37°C for 5 min with 4.76 μCi/ml of [³H]thymidine. —○—, without GMCHA-OPh'Bu; —●—, with 27 μM; —△—, with 41 μM; —▲—, with 54 μM; —□—, with 81 μM of GMCHA-OPh'Bu.

cell concentrations, the extent of the decreases were small.

Effects of GMCHA-OPh'Bu on the growth of various strains of *E. coli*, such as Kλs, IFO 3301, IAM 1132, K-12 W 3110, were examined in BHI, nutrient broth or glucose minimum medium. GMCHA-OPh'Bu was strongly inhibitory on the growth of various strains of *E. coli* and its IC₅₀'s were as shown in Table II. The effect was most marked on strain Kλs, the IC₅₀ being 14 μM in both the BHI and in the nutrient broth media. IC₅₀'s for other strains were in the range of 40 to 50 μM. These results show that there is no significant difference between the different culture media.

Effects of GMCHA-OPh'Bu on Growth, and DNA, RNA and Protein Syntheses The effects of GMCHA-OPh'Bu, which was selected as a representative of GMCHA esters, on the growth of *E. coli* K-12 IAM 1264 and the uptake of [³H]thymidine into DNA were examined. The growth of *E. coli* was determined by an increase in turbidity at 600 nm. GMCHA-OPh'Bu dose-dependently suppressed the growth, and at a concentration of 81 μM the cell growth was completely suppressed (Fig. 3a).

Figure 3b shows the effect of GMCHA-OPh'Bu on the uptake of [³H]thymidine into DNA. Without GMCHA-

Fig. 4. Inhibitory Effects of GMCHA-OPh'Bu on RNA and Protein Syntheses

Experimental conditions were as described in Fig. 3. a) The inhibitory effects of GMCHA-OPh'Bu was determined by counting the radioactivity in cold trichloroacetic acid-precipitable materials after incubation at 37°C for 5 min with 4.56 μCi/ml of [⁵⁻³H]uridine. b) The inhibitory effects of GMCHA-OPh'Bu was determined by counting the radioactivity in hot trichloroacetic acid-precipitable material after incubation at 37°C for 5 min with 4.56 μCi/ml of L-[4,5-³H]leucine. —○—, without inhibitor; —●—, with 27 μM; —△—, with 41 μM; —▲—, with 54 μM of GMCHA-OPh'Bu.

OPh'Bu the incorporation increased time-dependently and attained a maximum after 3 h and decreased after 4 h. These results indicate that a decrease in the incorporation of [³H]thymidine into DNA precedes a decrease in the growth. GMCHA-OPh'Bu dose-dependently suppressed the uptake of [³H]thymidine into DNA. The addition of the above 54 μM of the inhibitor immediately reduced the uptake. The results shown in Figs. 3a and 3b suggest that the suppression of the cell growth by GMCHA-OPh'Bu is due to the inhibition of DNA replication, because the suppression of DNA replication by the inhibitor precedes that of cell growth.

Figures 4a and 4b show the effects of GMCHA-OPh'Bu on RNA and protein syntheses. Without inhibitor RNA, synthesis rapidly increased after 1 h and reached a maximum after 5 h, followed by a rapid fall (Fig. 4a). These results are similar to the patterns observed in the growth of *E. coli* (see Fig. 3a). GMCHA-OPh'Bu dose-dependently suppressed the RNA synthesis, and the effect was similar to that on cell growth (see Fig. 3a).

Similar effects were observed on protein synthesis (Fig. 4b). Without inhibitor protein, synthesis rapidly increased and reached a maximum after 4 to 5 h, followed by a rapid decrease. These results were closely related to RNA synthesis and reflected the cell growth. GMCHA-OPh'Bu dose-dependently suppressed the protein synthesis, and the results were similar to those on cell growth and RNA synthesis.

Effects of TLCK and Pentamidine Isethionate on Growth, and DNA, RNA and Protein Syntheses TLCK and pentamidine isethionate, potent trypsin inhibitors, strongly inhibit the growth of *E. coli* and macromolecular synthesis in *E. coli*.^{10,11} So, the effects of these compounds on the growth of *E. coli* K-12 IAM 1264 were examined. As shown in Fig. 5a, the effect of TLCK was dose-dependent, but its effect was temporary and reparable: the addition of 2 mM TLCK completely suppressed the growth of *E. coli* during 2 h. Thereafter, the growth gradually recovered and showed an exponential growth curve similar to the control.

Fig. 5. Inhibitory Effects of TLCK on Growth of *E. coli* and on the Uptake of $[^3\text{H}]$ Thymidine into DNA, and the Effects of Reduced Glutathione

Experimental conditions and determination of inhibitory effects of TLCK on the growth of *E. coli* and on the uptake of $[^3\text{H}]$ thymidine into DNA were as described in Fig. 3. Reduced glutathione was concomitantly added with TLCK. a) Inhibitory effect on the growth of *E. coli*. b) Inhibitory effect on the uptake of $[^3\text{H}]$ thymidine into DNA. —○—, without TLCK; —●—, with 1 mM; —▲—, with 2 mM of TLCK; —△—, with 2 mM TLCK and 2 mM reduced glutathione.

Figure 5B shows the effect of TLCK on the uptake of $[^3\text{H}]$ thymidine into DNA. As compared to the control, 1 mM TLCK tentatively reduced the uptake of $[^3\text{H}]$ thymidine into DNA. However, the uptake recovered and reached a maximum after 4 h. The addition of 2 mM TLCK caused a rapid decrease in the uptake with recovery after 2 h, and the profile of the latter uptake was similar to that of the control. A maximum uptake was seen after 5 h. Inhibition of RNA synthesis by TLCK is recovered by reduced glutathione.¹¹⁾ As shown in Figs. 5a and 5b, the addition of the equimolar concentration of reduced glutathione completely abolished the effect of TLCK on the growth of *E. coli* and DNA synthesis. On the other hand, reduced glutathione (2 mM) had no effect on the inhibitory effects of GMCHA-OPh'Bu on either growth of *E. coli* or DNA synthesis (data not shown).

These results indicate that the effects of TLCK on the cell growth and the DNA synthesis markedly differ from those of GMCHA-OPh'Bu.

The effects of pentamidine isethionate on the growth of *E. coli* and the uptake of $[^3\text{H}]$ thymidine are shown in Figs. 6a and 6b, respectively. Although pentamidine isethionate dose-dependently suppressed the growth of *E. coli*, its effect seemed to be reparable: transient and strong inhibition of the growth of *E. coli* was observed at 50 μM pentamidine isethionate, but recovery was observed, and *E. coli* cells continued to grow exponentially. The effect of 100 μM was similar to that at 54 μM GMCHA-OPh'Bu, while the effect of pentamidine isethionate seemed to be abolished after 8 h. The addition of 200 μM pentamidine isethionate completely suppressed the growth of *E. coli* for at least 8 h, but after 24 h turbidity at 600 nm reached a maximum. On the other hand, GMCHA-OPh'Bu (90 μM) completely inhibited the cell growth for 24 h.

The effect of pentamidine isethionate on the uptake of $[^3\text{H}]$ thymidine was also dose-dependent and transient. At 50 μM it suppressed the uptake for 2 h, thereafter, the uptake recovered and reached a maximum after 5 h. Although the suppression of DNA replication caused by 100 μM pentamidine isethionate continued for 5 h, the effect

Fig. 6. Inhibitory Effects of Pentamidine Isethionate on the Growth of *E. coli* and on the Uptake of $[^3\text{H}]$ Thymidine into DNA

Experimental conditions, determination of inhibitory effects of pentamidine isethionate on growth of *E. coli* and the uptake of $[^3\text{H}]$ thymidine into DNA were as described in Fig. 3. a) Inhibitory effect on the growth of *E. coli*. b) Inhibitory effect on the uptake of $[^3\text{H}]$ thymidine into DNA. —○—, without pentamidine isethionate; —●—, with 50 μM ; —△—, with 100 μM ; —▲—, with 200 μM of pentamidine isethionate.

Fig. 7. Inhibitory Effects of Pentamidine Isethionate on RNA and Protein Syntheses

Experimental conditions were as described in Fig. 4. a) The inhibitory effect on the uptake of $[5\text{-}^3\text{H}]$ uridine into RNA. b) The inhibitory effect on the uptake of L-[4,5- ^3H]leucine into protein. —○—, without inhibitor; —●—, with 50 μM ; —△—, with 100 μM of pentamidine isethionate.

disappeared after 6 h. A higher concentration of pentamidine isethionate (200 μM) induced abrupt and marked reduction of DNA replication and the effect continued for at least 6 h. These results seem to closely correlate with the effect of pentamidine isethionate on the growth of *E. coli*, moreover, these results indicate that the effects of pentamidine isethionate on the growth of *E. coli* and DNA synthesis differ from those of GMCHA-OPh'Bu. The effects of pentamidine isethionate on RNA and protein syntheses were also dose-dependent (Figs. 7a and 7b) and similar to that on the cell growth (Fig. 6a).

Discussion

In this study, the effects of various esters of GMCHA on the growth of *E. coli* were examined by viable cell counting. Various esters suppressed the growth of *E. coli* but the effects were varied. The results shown in Table I indicate that the inhibitory effects of GMCHA esters are markedly affected by the species and position of substitution on the phenyl nucleus of GMCHA phenyl esters, and the effects

seem to be correlated with the hydrophobic or steric factor of the substituent, although details were not examined in this paper.

Prouty and Goldberg¹⁰⁾ reported that various protease inhibitors including potent trypsin inhibitors such as TLCK and aromatic diamidines, inhibited protein breakdown in starving *E. coli* cells at doses which did not inhibit the growth in glucose minimum medium, and higher concentrations of TLCK markedly reduced the growth. Rossman *et al.*¹¹⁾ examined the inhibitory effect of chloromethyl ketone protease inhibitors on β -galactosidase induction, growth, RNA synthesis and protein synthesis in *E. coli*, and stated that at least some biological effects of the chloromethyl ketones were due to activities other than the inhibition of a proteinase, and their effects were recovered by reduced glutathione. So, the effects of GMCHA-OPh'Bu on the growth, and DNA, RNA and protein syntheses were examined as a representative of various GMCHA esters, and the results were compared with those of TLCK and pentamidine isethionate. TLCK abruptly and strongly suppressed the growth and the DNA synthesis in *E. coli*. However, its effects were transient, and in a short time the growth and DNA replication recovered to the control level, and the effects were abolished by the addition of reduced glutathione. Pentamidine isethionate strongly inhibited the growth, but recovery occurred in a short time. Although DNA synthetic activity was markedly reduced when pentamidine isethionate was added, after an hour signs of recovery were observed and synthetic activity began to return to normal. On the other hand, GMCHA-OPh'Bu strongly and time-dependently inhibited the growth and the DNA replication in *E. coli*, and its inhibitory effects were not affected by reduced glutathione. These results indicate that the inhibitory effects of GMCHA-OPh'Bu differ from those of TLCK and pentamidine isethionate. The inhibition mechanisms of TLCK and pentamidine isethionate are complicated and unclear at this time. The inhibition of DNA synthesis by GMCHA-OPh'Bu preceded that of the growth of *E. coli*. These results strongly suggest that

the inhibitory effect of GMCHA-OPh'Bu on the growth is caused by the suppression of DNA replication, and the latter effect is due to inhibition of a specific trypsin-like proteinase(s) involved in DNA replication. To confirm the suggestion we examined the inhibitory effects of GMCHA-OPh'Bu with synchronized *E. coli* cells, and found a trypsin-like proteinase which participates in the onset of DNA synthesis. The inhibitory effects of TLCK and pentamidine isethionate on the trypsin-like proteinase are weakly or not seen [M. Kato, T. Irisawa, Y. Morimoto and M. Muramatsu unpublished].

GMCHA-OPh'Bu and pentamidine isethionate strongly inhibited RNA and protein syntheses in *E. coli*. Their inhibition mechanisms are unclear at this time.

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