C-19393 S_2 AND H_2, NEW CARBAPENEM ANTIBIOTICS

IV. INHIBITORY ACTIVITY AGAINST β-LACTAMASES

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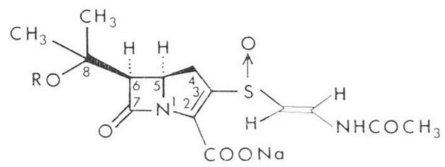
New carbapenem antibiotics, C-19393 S_2 and H_2, have been found to be potent and broad-spectrum inhibitors of β-lactamases. Among 11 types of β-lactamases tested, those from *Escherichia coli* (plasmid-bearing), *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens* and *Bacteroides fragilis* were especially sensitive. They also inhibited cephalosporinases insensitive to clavulanic acid. The inhibition by C-19393 S_2 and H_2 was of progressive type, except for the inhibition of *E. coli* enzyme (plasmid-mediated type I) by C-19393 H_2. The inhibition of *E. coli* β-lactamase by C-19393 S_2 was irreversible, while that by C-19393 H_2 was reversible.

C-19393 S_2 and H_2 are new carbapenem antibiotics with a broad antibacterial spectrum, produced by *Streptomyces griseus* subsp. *cryophilus*[1]. Chemical studies[2] established their structures to be those shown in Fig. 1. They had high affinity for penicillin-binding protein 2 of *Escherichia coli*, and inhibited peptidoglycan biosynthesis of the same organism[3]. They also acted synergistically with ampicillin and cefotiam against β-lactamase producing bacteria[1], and their strong interactions with β-lactamases were expected. The present report describes the β-lactamase inhibitory properties of these new antibiotics.

Fig. 1. Chemical structures of C-19393 S_2 and H_2.



C-19393 S_2 : R = SO_3Na
C-19393 H_2 : R = H

Materials and Methods

Antibiotics

C-19393 S_2 and H_2 were prepared from the culture broth of *Streptomyces griseus* subsp. *cryophilus* as described[2]. Clavulanic acid lithium salt was prepared in our research division. Ampicillin (Takeda Chemical Industries, Ltd.), benzylpenicillin (Meiji Seika Kaisha, Ltd.), dicloxacillin (Banyu Pharmaceutical Co., Ltd.) and cephalothin (Shionogi & Co., Ltd.) were commercial products.

β-Lactamase preparations

β-Lactamases of *Escherichia coli* TN713, *E. coli* TN649 and *Pseudomonas aeruginosa* GN3407 are plasmid-mediated, constitutive enzymes, and that of *Klebsiella pneumoniae* TN1698 is a constitutive enzyme. The β-lactamase of *Staphylococcus aureus* 1840 is an inducible one (induced by 0.5 μg/ml dicloxacillin), as are those of *Citrobacter freundii* GN1706, *P. aeruginosa* U31, *Serratia marcescens* TN81, *Proteus vulgaris* GN4413 and *Enterobacter cloacae* TN1282 (induced by 1 mg/ml benzylpenicillin).

The enzymes were prepared as follows. An overnight culture was diluted 10-fold into 250 ml of Trypticase soy broth (BBL) in a 1,000 ml flask and the flask was shaken for 3 hours on a rotary shaker at 37°C. Inducer was added, if necessary, and the organism was grown for another 2 hours. The cells were harvested from 5 liter of the culture by centrifugation, suspended in 500 ml of phosphate

buffer (0.05 M, pH 6.9), and sonically disrupted by a ultrasonic oscillator (Kaijo Denki Co., Ltd.) in an ice bath. Cell debris was discarded by centrifugation and the enzyme was purified from the supernatant fluid. The β-lactamase of *S. aureus* 1840 was purified through phospho-cellulose column chromatography as described by RICHMOND[4]; those of *P. vulgaris* GN4413 and *E. cloacae* TN1282 through CM Sephadex C-50 column chromatography and Sephadex G-100 gel filtration according to the method described by HENNESSEY *et al.*[5]; those of *E. coli* TN713, *E. coli* TN649 and *P. aeruginosa* GN3407 through DEAE cellulose column chromatography and Sephadex G-100 gel filtration as described by OGAWARA *et al.*[6] The enzymes of *K. pneumoniae* TN1698, *C. freundii* GN1706, *P. aeruginosa* U31 and *S. marcescens* TN81 were purified as was the *E. coli* TN713 enzyme, by the use of CM cellulose instead of DEAE cellulose. The *Bacteroides fragilis* V284-3 β-lactamase, which was precipitated by between 50 and 70% saturation of ammonium sulfate, was a generous gift of Dr. T. KOHATA (Gifu University).

Assay of β-lactamase inhibition and determination of I_{50} values

β-Lactamase activity was determined by the modification of microiodometric method of NOVICK.[7] Ampicillin was used as substrate for penicillinase assay and cephalothin for cephalosporinase assay. A solution of an inhibitor (0.05 ml) was added to 0.002 units of a β-lactamase in 0.9 ml of phosphate buffer (0.05 M, pH 6.9), and the mixture was incubated at 30°C for 10 minutes unless otherwise stated. A substrate in 0.05 ml of phosphate buffer was then added to the preincubated mixture to give a final concentration of 0.1 mM, and the incubation was continued for another 10 minutes. The reaction was stopped by the addition of 0.5 ml of 0.15 M sodium tungstate in acetate buffer (2.0 M, pH 4.0). Each sample had a control in which substrate and inhibitor were incubated, enzyme being added after adding the tungstate. Then 1.5 ml of an iodine-starch solution (0.1 mM iodine, 1.6 mM potassium iodide, 0.4% starch and 0.04 M potassium phosphate buffer pH 6.0) was added. After 15 minutes or more at room temperature, absorbance was read at 620 nm in a Hitachi spectrophotometer. The enzyme activity was calculated from the differences in absorbance between control and experiment; 1 absorbance unit was equivalent to 0.0300 μmole of ampicillin and 0.0475 μmole of cephalothin hydrolyzed, respectively. One unit (U) of penicillinase or cephalosporinase is defined as the amount of enzyme that hydrolyzes 1 μmole of ampicillin or cephalothin, respectively, per minute at 30°C.

Percentage inhibition of enzyme activity was calculated against a control reaction where inhibitor was replaced by buffer. The concentration of inhibitor giving 50% inhibition (I_{50}) was calculated from a plot of the percentage inhibition against the inhibitor concentration.

Gel filtration of β-lactamase incubated with inhibitor

The *E. coli* TN713 β-lactamase (0.2 U) was incubated at 30°C with 1 μg of C-19393 S_2, 50 μg of C-19393 H_2 or 100 μg of clavulanic acid in 1 ml of phosphate buffer (0.05 M, pH 6.9). After 10 minutes incubation, 0.5 ml of the mixture was applied to a column (1.3×28 cm) of Sephadex G-25 at 4°C. The column was eluted with the same phosphate buffer at 10 ml per hour and 1 ml fractions were collected. Enzyme activity was determined microiodometrically. Fractions eluted after the peak of enzyme activity were assayed for the inhibitor by using inhibition of *E. coli* TN713 β-lactamase.

Results

β-Lactamase Inhibitory Activity

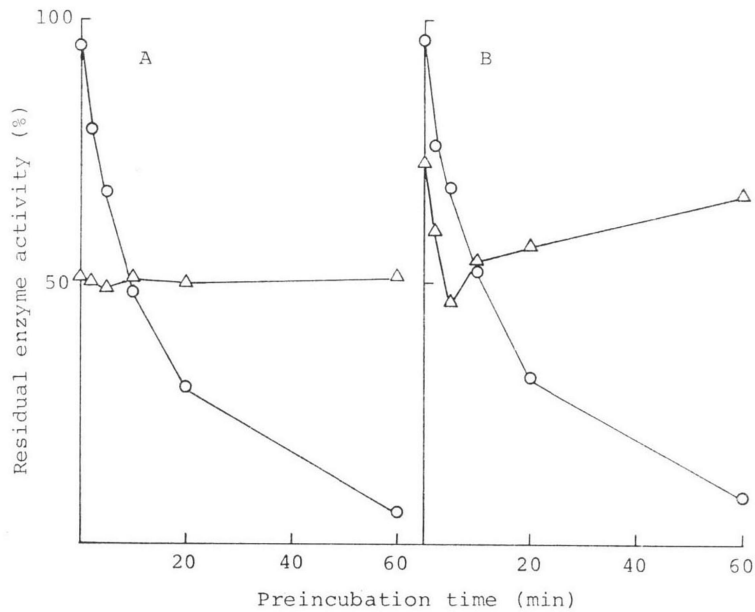
The inhibitory activities, I_{50}s, of C-19393 S_2, C-19393 H_2 and clavulanic acid against various β-lactamases were determined under the conditions described in Materials and Methods. As shown in Table 1, both C-19393 S_2 and H_2 inhibited a wide range of β-lactamases at very low concentrations. Enzymes inhibited by C-19393 S_2 and H_2 included cephalosporinases which were insensitive to clavulanic acid. Enzymes that were especially well inhibited by C-19393 S_2 and H_2 were plasmid-mediated type I and type II penicillinases[8], *K. pneumoniae* TN1698 penicillinase and cephalosporinases from *P. vulgaris* GN4413, *S. marcescens* TN81 and *B. fragilis* V284-3. The relative inhibitory activities of C-19393 S_2 and H_2 were variable, depending on the enzyme tested. Against *S. marcescens* TN81

Table 1. β-Lactamase inhibitory activities of C-19393 S_2, C-19393 H_2 and clavulanic acid.

Source and type of enzyme	I_{50} (μg/ml)		
	C-19393 S_2	C-19393 H_2	Clavulanic acid
S. aureus 1840 (PCase*)	0.21	0.043	0.040
E. coli TN 713 (PCase I**)	0.00027	0.0030	0.016
E. coli TN 649 (PCase II**)	0.0025	0.0014	0.30
P. aeruginosa GN 3407 (PCase IV**)	0.019	0.024	0.017
K. pneumoniae TN 1698 (PCase)	0.0052	0.0052	0.012
E. cloacae TN 1282 (CSase***)	0.043	0.034	>5
P. vulgaris GN 4413 (CSase)	0.00037	0.00060	0.045
P. aeruginosa U 31 (CSase)	2.1	0.33	>5
S. marcescens TN 81 (CSase)	0.055	0.00050	>5
C. freundii GN 1706 (CSase)	0.016	0.015	>5
B. fragilis V 284-3 (CSase)	0.00049	0.000038	0.0045

* Penicillinase.
** Classification of MITSUHASHI[8]; PCase I, II and IV are identical to TEM-1, OXA-1 and PSE-1 of MATTHEW,[9] respectively.
*** Cephalosporinase.

Fig. 2. Progressive and non-progressive inhibition of β-lactamases by C-19393 S_2 and H_2.
(A) *E. coli* TN713 β-lactamase (0.002 U/ml) was incubated with 0.3 ng/ml of C-19393 S_2 (○) or 3 ng/ml of C-19393 H_2 (△), and (B) *P. vulgaris* GN4413 β-lactamase (0.002 U/ml) was incubated with 0.4 ng/ml of C-19393 S_2 (○) or 0.6 ng/ml of C-19393 H_2 (△) for the time indicated, prior to the addition of substrate.



β-lactamase, C-19393 H_2 was 100 times more active than C-19393 S_2, whereas against *E. coli* TN713 β-lactamase, C-19393 H_2 was 10 times less active than C-19393 S_2.

Progressive and Non-progressive Inhibition of β-Lactamase

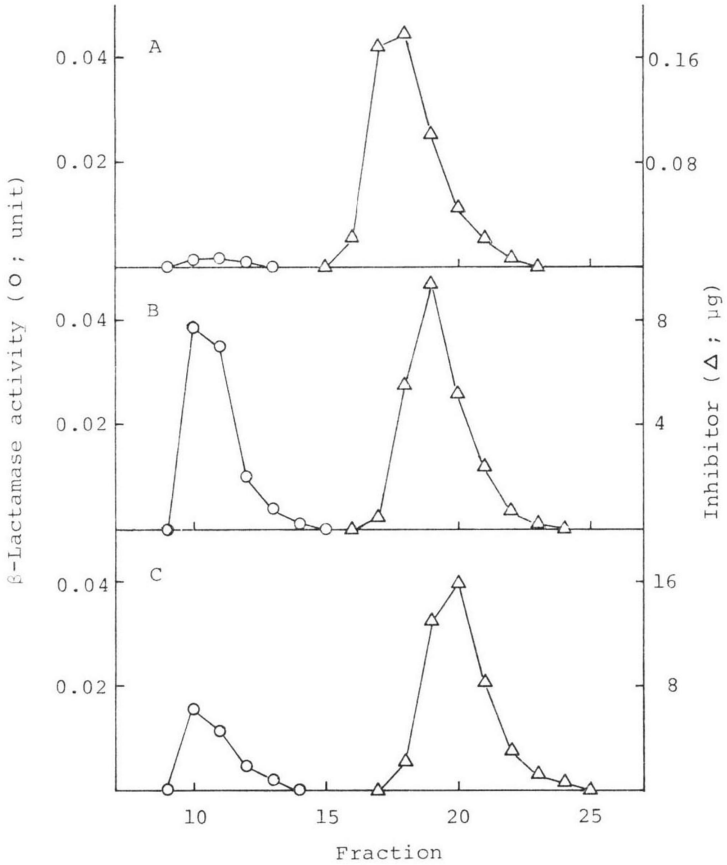
The enzyme activity remaining after various times of preincubation of β-lactamase with inhibitor

was measured. The concentration of inhibitors corresponding to the I_{50} values (see Table 1) were chosen for these experiments. As shown in Fig. 2, C-19393 S_2 inhibited both *E. coli* TN713 and *P. vulgaris* GN4413 β-lactamases in a progressive fashion. On the other hand, inhibitory activity of C-19393 H_2 against *E. coli* TN713 β-lactamase did not change during preincubation periods of up to 1 hour. *P. vulgaris* GN4413 β-lactamase was inhibited by C-19393 H_2 progressively at first, then the enzyme activity was gradually recovered. In this experiment, the molar ratio of the *P. vulgaris* enzyme and C-19393 H_2 was approximately 1: 8. Under the condition of this enzyme/inhibitor concentration ratio, C-19393 H_2 lost its antibacterial activity within 10 minutes of incubation. β-Lactamases from *S. aureus* 1840, *E. cloacae* TN1282 and *S. marcescens* TN81 were inhibited progressively by both C-19393 S_2 and H_2 (data not shown).

Reversible and Irreversible Inhibition of β-Lactamase

In order to elucidate how the natures of inhibition by C-19393 S_2 (progressive) and C-19393 H_2 (non-progressive) of the *E. coli* TN713 β-lactamase differ, we analyzed the reversibility of the inhibition by those two antibiotics. The reversibility of the inhibition by clavulanic acid which is a pro-

Fig. 3. Gel filtration of *E. coli* TN713 β-lactamase incubated with inhibitors.
The enzyme (0.2 U/ml) was incubated with 1 μg of C-19393 S_2 (A), 50 μg of C-19393 H_2 (B) or 100 μg of clavulanic acid (C) per ml at 30°C for 10 minutes. A 0.5 ml portion of the mixture was applied to a column of Sephadex G-25 (1.3×28 cm) and eluted with phosphate buffer (0.05 M, pH 6.9). Fractions (1 ml) were assayed for enzyme and inhibitor activities as described in Materials and Methods.



gressive inhibitor of the β-lactamase[10] was also examined. The enzyme was incubated with a large excess of C-19393 S_2, C-19393 H_2 or clavulanic acid at 30°C for 10 minutes and the mixture was applied to gel-filtration as described in Materials and Methods. As shown in Fig. 3, when the enzyme was reacted with C-19393 S_2, only 4% of the activity was recovered after the gel-filtration, whereas with C-19393 H_2 all the activity was recovered. Recovery of enzyme activity incubated with clavulanic acid was 39%. All the inhibitory activities of both C-19393 S_2 and H_2 were recovered after the gel filtration. Recovery of clavulanic acid was slightly low.

Discussion

Both C-19393 S_2 and H_2, at very low concentration, inhibited all of the 11 types of the β-lactamases tested. They also inhibited cephalosporinases which were insensitive to clavulanic acid[10]. Inhibitory activities of C-19393 S_2 and H_2 against most β-lactamases were far stronger than that of clavulanic acid. C-19393 S_2 is a methylated compound of antibiotic MM 4550[11] and C-19393 H_2 is a desulfo derivative of C-19393 S_2. I_{50} values of C-19393 S_2 and H_2 against several β-lactamases were almost the same as or slightly lower than that of MM 4550 reported by HOOD *et al*[12], although exact comparison is difficult because of differences in the assay method and the source of enzyme. The synergistic activities of C-19393 S_2 and H_2 with ampicillin and cefotiam against β-lactamase producing bacteria[1] were apparently due to the β-lactamase inhibitory activities of the drugs.

The inhibitory activity of C-19393 S_2 against *E. coli* TN713 β-lactamase was 10 times as great as that of C-19393 H_2, whereas C-19393 H_2 was 100 times more active in its ability to inhibit *S. marcescens* TN81 β-lactamase than C-19393 S_2. This difference in the relative inhibitory activities between C-19393 S_2 and H_2 against the two enzymes can be used for differential determination of the drugs in culture broth of the producing organism, which produces the two antibiotics simultaneously[1].

C-19393 S_2 inhibited all β-lactamases tested in progressive fashion, and C-19393 H_2 was also a progressive inhibitor of β-lactamases from *S. aureus* 1840, *E. cloacae* TN1282, *S. marcescens* TN81 and *P. vulgaris* GN4413. However, the inhibition of *E. coli* TN713 β-lactamase by C-19393 H_2 was not progressive. Gel filtration clearly revealed that C-19393 S_2 inhibited *E. coli* TN713 β-lactamase irreversibly but that the inhibition by C-19393 H_2 was reversible. Clavulanic acid also exerted an irreversible inhibition, though its extent was partial. This indicates that the binding of clavulanic acid is less tight than that of C-19393 S_2.

C-19393 H_2 inhibited *P. vulgaris* GN4413 β-lactamase progressively for a few minutes, but the enzyme activity was recovered gradually by prolonged incubation. This phenomenon is also observed with clavulanic acid[13] and PS-5[14]. The regeneration of the enzyme activity and the data suggesting a slow hydrolysis of C-19393 H_2 (data not shown) indicate that this compound is also a substrate for *P. vulgaris* β-lactamase, as are clavulanic acid and PS-5.

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