C-19393 S_2 AND H_2, NEW CARBAPENEM ANTIBIOTICS

III. MODE OF ACTION

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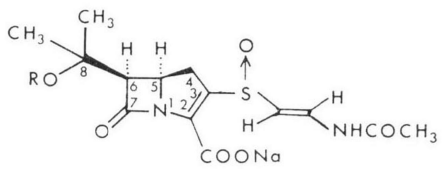
Biochemical activities of new carbapenem antibiotics, C-19393 H_2(H_2) and C-19393 S_2(S_2), were examined in comparison with those of mecillinam using *Escherichia coli*. H_2 showed remarkably high affinity for penicillin-binding protein (PBP) 2, and high affinity for PBPs 1 and 3. S_2 showed high affinity for PBP 2, moderate affinity for PBP 1 and low affinity for PBP 3. They induced ovoid cells at lower concentrations and cell lysis at higher concentrations. The inhibitory potency of H_2 for peptidoglycan synthesis was similar to that of mecillinam at lower concentrations up to 0.1 μg/ml. At concentrations higher than 0.1 μg/ml, the inhibition rate by H_2 gradually increased up to 100%, whereas that by mecillinam remained at 60% level. The MICs of H_2, S_2 and mecillinam corresponded to the lowest concentrations giving 60% of inhibition of peptidoglycan synthesis at which concentrations the function of PBP 2 seemed to be prevented completely. These findings indicate that the primary targets of H_2 and S_2 are PBP 2 involved in cell shape determination in *E. coli*.

Since the discovery of thienamycin by KAHAN *et al.*[1], several new β-lactam antibiotics having a carbapenem nucleus have been reported to be produced by various strains of the genus *Streptomyces*[2~6]. However, the mode of action of the carbapenem antibiotics has been studied only with thienamycin[7].

C-19393 S_2 and H_2 are new carbapenem antibiotics produced by *Streptomyces griseus* subsp. *cryophilus*[8]. Their structures have been determined to be those shown in Fig. 1[9]. They show potent antibacterial[8] and β-lactamase inhibitory activities[10].

Fig. 1. Structures of C-19393 S_2 and H_2.



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

Here we describe some biochemical activities of C-19393 S_2 and H_2 against *Escherichia coli*, *i.e.* their effect on bacterial morphology, their inhibition of peptidoglycan synthesis, and their affinity for penicillin-binding proteins (PBPs). We also discuss the interrelations among these activities and compare the mode of action of these carbapenem antibiotics with that of mecillinam[11].

Materials and Methods

Bacterial strains

The following *E. coli* K-12 strains were used. *E. coli* Y10[12] was the source of envelopes containing PBPs. *E. coli* LD-2 is a lysine and *meso*-diaminopimelic acid (DAP) auxotroph derived from *E. coli* Y10 in our laboratory; this mutant incorporates DAP specifically into the peptidoglycan of cell walls[13].

Observation of morphological changes

The overnight culture of *E. coli* LD-2 at 37°C was diluted 1/10 with DYA medium[13] containing

12% of sucrose. Then, 0.2 ml of an antibiotic solution was added to 0.8 ml of the above inoculated medium placed in a test tube. After cultivation at 37°C for 2 hours with shaking, the morphological changes of the cells induced by the antibiotic were observed by phase-contrast microscopy.

Assay of minimum inhibitory concentrations (MICs), peptidoglycan synthesis and PBPs

MICs, inhibitory activities for peptidoglycan synthesis, and affinities for the PBPs in *E. coli* were determined as described previously[13].

Antibiotics, labeled compounds and other chemicals

C-19393 S_2 and H_2 were obtained as described in the preceding paper[9]. Mecillinam was kindly supplied by Dr. F. LUND of Leo Pharmaceutical Co., Copenhagen, Denmark. [G-^{3}H]DAP (300 μCi/μmol) and [^{14}C]benzylpenicillin (53 μCi/μmol) were purchased from the Radiochemical Centre, Amersham, England. All other chemicals used in this study were of reagent grade.

Results

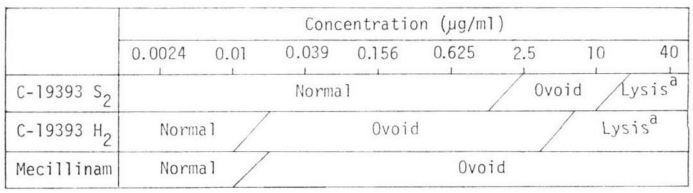
Antibacterial Activities

Antibacterial activities against *E. coli* LD-2 of the antibiotics used in the present study are shown in Table 1. C-19393 H_2 exerted a potent antibacterial activity, while C-19393 S_2 showed rather weaker activity, indicating that the sulfate group in C-19393 S_2 (Fig. 1) gives a negative effect on the antibacterial activity against *E. coli*.

Table 1. Antibacterial activities of C-19393 S_2 and H_2, and mecillinam against *E. coli* LD-2.

	MIC (μg/ml)
C-19393 S_2	6.25
C-19393 H_2	0.1
Mecillinam	0.2

Fig. 2. Morphological changes induced by C-19393 S_2 and H_2, and mecillinam.



a: Ghosts of ovoid-shaped cells and spheroplasts.

Morphological Changes

Fig. 2 shows morphological changes in *E. coli* LD-2 induced by the antibiotics tested. Mecillinam, having the high affinity specific for PBP 2, induced "ovoid cells" (osmotically stable round cells)[11].

C-19393 H_2, like mecillinam, induced ovoid cells over a wide range of lower concentrations (0.039~2.5 μg/ml). Although C-19393 S_2 also induced ovoid cells, the concentration range of the induction was very narrow (2.5~10 μg/ml). Above concentrations of 10 μg/ml of C-19393 H_2 and 40 μg/ml of C-19393 S_2, cell lysis occurred and production of ghosts and spheroplasts were observed.

Inhibitory Activity for Peptidoglycan Synthesis

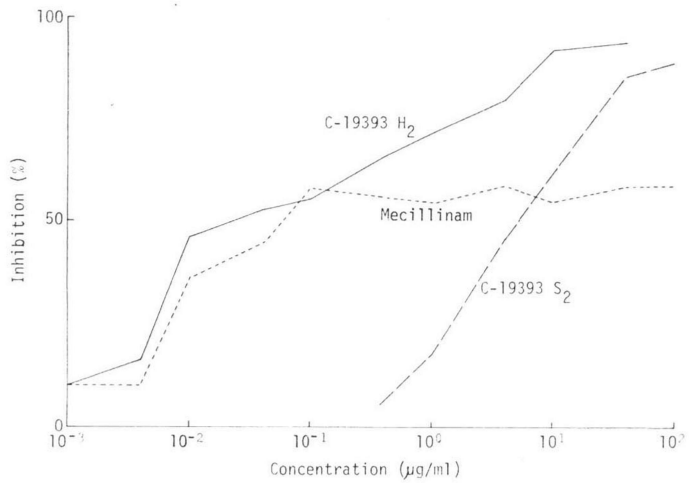
As shown in Fig. 3, C-19393 H_2 exerted a very strong inhibitory activity for peptidoglycan synthesis; the concentration required to inhibit peptidoglycan synthesis by 50% (PGI_{50}) was 0.024 μg/ml. The inhibition curve of C-19393 H_2 followed that of mecillinam up to 0.1 μg/ml, when the inhibition was about 60%. Above this concentration, the inhibition by C-19393 H_2 gradually increased to nearly 100%, but the inhibition by mecillinam, a specific blocker of the function of PBP 2 in *E. coli*[11], showed no further increase even at 100 μg/ml (the highest concentration tested) (Fig. 3).

C-19393 S_2 showed rather weaker activity (PGI_{50}: 5.0 μg/ml). However, it should be pointed out that the inhibition by C-19393 S_2 and H_2 at their MICs were both about 60% as in the case of

Fig. 3. Comparison of C-19393 S_2 and H_2, and mecillinam in their activities to inhibit peptidoglycan synthesis in *E. coli*.

Cells of *E. coli* LD-2 grown in DYA medium to exponential growth phase were harvested, washed twice with YA medium (DAP-free DYA medium), and suspended × 5/4 strength YAB medium to an extinction of 0.5 at 600 nm with a Spectronic 20 colorimeter. The incubation mixture, containing 0.4 ml of the above cell suspension, 0.05 ml of a [G-^{3}H]DAP solution (300 μCi/μmole, 30 μCi/ml), and either 0.05 ml of water (control) or an antibiotic solution, was incubated at 37°C for 2 hours without shaking.

The mixture was cooled in an ice bath, and 0.125 ml of 25% trichloroacetic acid was added. After heating at 90°C for 10 minutes, acid-insoluble material was spun down, washed twice with 0.1 M ammonium acetate, and its radioactivity was counted in a liquid scintillation spectrometer.



mecillinam.

Affinity for PBPs

Fig. 4 shows the competition of the increasing concentrations of (A) C-19393 S_2 and (B) C-19393 H_2 for the binding of [^{14}C]benzylpenicillin to PBPs of *E. coli* Y10[12]. This strain is the parent of *E. coli* LD-2 and these strains showed almost the same sensitivity to various antibiotics. The binding affinities of C-19393 S_2 and H_2 for each PBP of *E. coli* were quantitated from the fluorograms in Fig. 4, in terms of I_{50} (μg/ml) which is the concentration required to inhibit the binding of [^{14}C]benzylpenicillin by 50%[14] (Table 2).

Table 2. Binding affinities of C-19393 S_2 and H_2 for penicillin-binding proteins in *E. coli*.

Penicillin-binding protein	I_{50}* (μg/ml)	
	C-19393 S_2	C-19393 H_2
1A	0.86	0.22
1B	>20 (*ca.* 30**)	3.6
2	5.1	0.0072
3	>20	1.3
4	8.5	0.017
5/6	n.d.***	n.d.

* Concentration required to inhibit binding of [^{14}C]benzylpenicillin to each protein by 50%.

** Determined by extraporation of the competition curve.

*** Not determined.

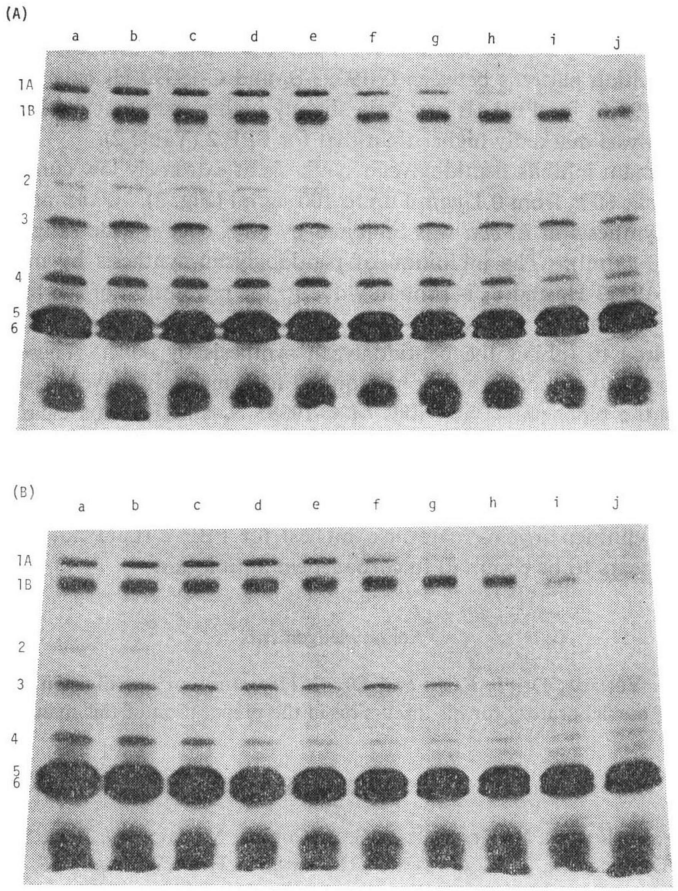
Among the possible lethal targets of *E. coli*, PBPs 1, 2 and 3, C-19393 H_2 showed remarkably high affinity for PBP 2 (I_{50}: 0.0072 μg/ml) and good affinity for PBPs 1 and 3. In contrast, C-19393 S_2 had rather lower affinity for all PBPs. It showed the highest affinity for PBP 2 (I_{50}: 5.1 μg/ml) like C-19393 H_2.

Fig. 4. Competition of (A) C-19393 S_2 and (B) C-19393 H_2 for [^{14}C]benzylpenicillin binding.

Envelopes (12.9 mg of protein per ml) of *E. coli* Y10 were preincubated at 37°C for 10 minutes with water (a) or increasing concentrations of C-19393 S_2 and H_2, and the PBPs remaining accessible were labeled with a saturating concentration (50 μCi/ml, 53 μCi/μmol) of [^{14}C]benzylpenicillin.

PBPs were detected by exposure of the dried gel to X-ray films for 22 days at −80°C.

The final concentration of C-19393 S_2 and H_2 were (μg/ml): (b) 0.002, (c) 0.008, (d) 0.02, (e) 0.08, (f) 0.2, (g) 0.8, (h) 2, (i) 8, and (j) 20.



Discussion

In order to elucidate the mode of action of the newly discovered carbapenem antibiotics, C-19393 S_2 and H_2, some of the biochemical activities of these compounds were examined and compared with those of mecillinam.

The PBP assay developed by SPRATT and PARDEE[15] has been a powerful probe to explain the mode of action of β-lactam antibiotics[7,11,13,16~18]. Among the seven PBPs in *E. coli*, PBPs 1(1A, 1B), 2 and 3 are thought to be the possible lethal targets of β-lactam antibiotics[19,20]; PBP 4[21,22] and PBP 5/6[23] seem not to be essential for normal growth of *E. coli*. PBP 1 consisting of 1A and 1B[24], compensatory for each other[25], is involved in cell elongation, PBP 2 in cell morphogenesis, and PBP 3 in septum formation.

Here we have demonstrated that both C-19393 S_2 and H_2 show the highest affinities for PBP 2 among essential PBPs in *E. coli* (Table 2). Especially, C-19393 H_2 in an extremely broad concentration range blocked the function of PBP 2 without interference with the functions of other essential PBPs (Fig. 4). This finding indicates that C-19393 H_2 may act in a similar fashion to mecillinam.

The morphological changes induced by C-19393 S_2 and H_2 can be well understood by their affinity profiles for PBPs; the ovoid cell-inducing activities are correlated with their high affinities for PBP 2. C-19393 H_2 induced the ovoid cells over a wide range of lower concentrations as shown in Fig. 2.

Moreover, C-19393 H_2 was an extremely potent inhibitor of peptidoglycan synthesis (PGI_{50}: 0.024 μg/ml). As shown in Fig. 3, the inhibition curve of C-19393 H_2 followed that of mecillinam up to 0.1 μg/ml which was the lowest concentration giving 60% inhibition. The inhibition above 60% at concentrations higher than 0.1 μg/ml and the decrease in the slope of the curve seem to be a consequence of the blocking of PBP 1, the possible main peptidoglycan synthesizing enzyme[25,26].

In contrast, C-19393 S_2 showed an inhibition curve without a decrease in slope (Fig. 3). The difference in the inhibition patterns between C-19393 S_2 and C-19393 H_2 may be due to the fact that the I_{50} value of C-19393 S_2 for PBP 1B was only slightly higher than that for PBP 2, while that of C-19393 H_2 for PBP 1B was markedly higher than that for PBP 2 (Table 2).

Although mecillinam inhibits peptidoglycan synthesis at extremely low concentrations, the degree of inhibition remains at 60% from 0.1 μg/ml up to 100 μg/ml (Fig. 3). PARK and BURMAN[27] reported that peptidoglycan synthesis in *E. coli* was inhibited by 50% over a wide range of concentrations of mecillinam (1~1,000 μg/ml). The inhibition of peptidoglycan synthesis by mecillinam and by low concentrations of C-19393 H_2 and S_2 is probably due to the prevention of the function of PBP 2.

It is interesting that all the MICs of C-19393 S_2 and H_2, and mecillinam are close to the lowest concentrations required to inhibit the peptidoglycan synthesis by 60%. These concentrations probably correspond to the lowest concentrations required to completely prevent the function of PBP 2.

The studies on the biochemical activities of C-19393 S_2 and H_2 thus clearly indicate that their primary targets are PBP 2 involved in cell shape determination in *E. coli*.

SPRATT *et al.*[7] reported that a carbapenem antibiotic, thienamycin, showed the highest affinity for PBP 2 among PBPs in *E. coli*, and induced large osmotically stable round cells. We have found that the affinities of epithienamycins[5] are also highest for PBP 2 (data not shown). Thus, a high affinity for PBP 2 appears to be common to carbapenem antibiotics.

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