C-19393 S_2 AND H_2, NEW CARBAPENEM ANTIBIOTICS

I. TAXONOMY OF THE PRODUCING STRAIN, FERMENTATION AND ANTIBACTERIAL PROPERTIES

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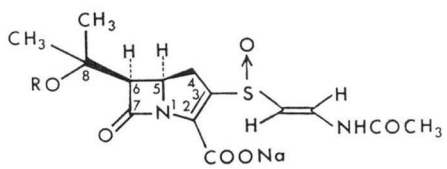
C-19393 S_2 and H_2 are new carbapenem antibiotics produced by a streptomycete. The producing strain was taxonomically studied and named *Streptomyces griseus* subsp. *cryophilus*. Cobaltous compounds were necessary for production of the antibiotics. C-19393 S_2 and H_2 showed a broad spectrum of antibacterial activities with C-19393 H_2 being 8~120 times more active than C-19393 S_2. They also exhibited β-lactamase-inhibiting activities and acted synergistically with ampicillin and cefotiam against clinical isolates resistant to β-lactam antibiotics.

We have developed a sensitive and selective screening system for finding β-lactam antibiotics in which a mutant of *Pseudomonas aeruginosa* PsC^{ss} is employed as a test organism and penicillinase and cephalosporinase as discriminating probes[1]. We have added, as a test organism, a mutant of *Escherichia coli* which lacks chromosomal β-lactamase and the penicillin-binding protein 1B and is consequently hypersensitive to β-lactam antibiotics[2]. We have screened for β-lactam antibiotics with the above agents and encountered a streptomycete, No. C-19393, that produces new β-lactam antibiotics. The present report describes the taxonomic characterization of the producing strain as well as the fermentation and the antibacterial activities of these new antibiotics. The synergistic effect of C-19393 S_2 and H_2 with common β-lactam antibiotics is also described. Chemical studies which will be reported separately established their carbapenem structures shown in Fig. 1[3]. Studies elucidating their modes of action[4] and mechanisms of β-lactamase inhibition[5] will be reported in forthcoming issues.

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



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

Materials and Methods

Microorganisms

Strain No. C-19393 was isolated from a soil sample collected in Sweden and maintained on T-agar slants. Organisms used to test for antimicrobial activities are the stock cultures maintained at our laboratories. Type and authentic cultures of *Streptomyces* were obtained from the Institute for Fermentation, Osaka.

Media

Media used for taxonomic studies were prepared as recommended by the International Streptomyces

Project (ISP)[6]. T agar, which was used additionally for characterization and for the maintenance of strain No. C-19393, was prepared as follows: Twenty grams each of oatmeal and tomato paste, and 2 g of Bovril (edible beef extract, Bovril Ltd., Burton-on-Trent) were boiled for 10 minutes in 1 liter of tap water and the mixture was filtered through gauze. The filtrate was adjusted to pH 7.0, made to 1 liter with tap water and supplemented with 20 g of Bacto agar (Difco Labs., Detroit). Agar slants were prepared after autoclaving at 120°C for 15 minutes.

Seed medium used for fermentation contained (g/liter): glucose 20, soluble starch 30, soybean flour 10, corn-steep liquor 10, Polypepton (Daigo Nutritive Chem., Osaka) 5, NaCl 3, and $CaCO_3$ (precipitated) 5. Fermentation medium contained (g/liter): glucose 30, soluble starch 30, soybean meal 15, cotton-seed meal 15, K_2HPO_4 0.6, KH_2PO_4 0.25, $CoCl_2$ 0.002, and Actcol (antifoam, Takeda Chem. Ind., Osaka) 0.5. The pH of the seed and fermentation media was adjusted to pH 7.0 with 2 N NaOH before sterilization.

Assay methods

Antibiotics C-19393 S_2 and H_2 were assayed by determining (1) antibacterial activity against mutants of *Escherichia coli* lacking chromosomal β-lactamase and penicillin-binding protein 1B[2] and (2) β-lactamase-inhibiting activity using *Klebsiella pneumoniae* as described by BROWN *et al.*[7] The minimum inhibitory concentrations were assayed by the conventional agar-dilution method using the medium described previously[8]. The synergistic action of C-19393 S_2 and H_2 with ampicillin and cefotiam was examined by the two-fold agar-dilution method using Trypticase soy agar (BBL, Baltimore).

Chemicals

Ampicillin is a product of our company. Cephaloridine is a product of Eli Lilly & Co. Cefotiam was prepared in our research division. Other chemicals are commercial products.

Results

Taxonomy of Strain No. C-19393

The taxonomic characterization was carried out according to the method recommended by the ISP[6]. Unless otherwise specified, the cultivation temperature was 28°C.

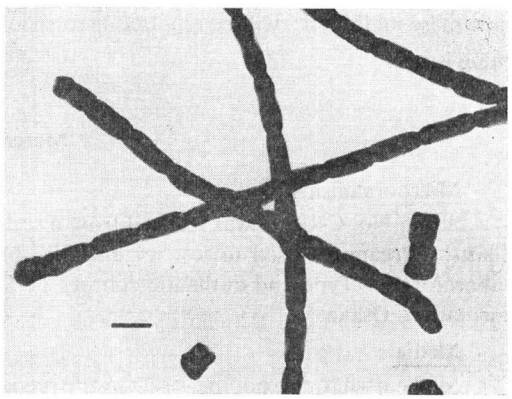
Morphological characterization

The strain produced aerial mycelium with tufts of straight to slightly wavy spore chains; it therefore belongs to the Section *Rectus-Flexibilis* (RF). The mature spore chains were generally long with more than 30 spores per chain. The spores were cylindrical ($0.35 \sim 0.55 \times 0.7 \sim 1.4$ μm) and their surfaces were smooth (Fig. 2).

Cultural characteristics

The cultural characteristics observed after a 2-weeks cultivation are shown in Table 1. The strain gave the most characteristic appearance on T agar; the color of the aerial mycelium was light grayish yellow and thus it belongs to the Yellow color-series. Neither melanin nor other soluble pigments were formed on any agar media tested.

Fig. 2. Electron microphotograph of spores of strain No. C-19393. Bar indicates 1 μm.



Physiological characteristics

The physiological characteristics are shown in Table 2. The strain contained cell walls of type I (LL-diaminopimelic acid). It is unusual

Table 1. Cultural characteristics of strain No. C-19393.

Medium	Growth	Aerial mycelium*
Sucrose-nitrate agar	moderate	moderate, white
Glucose-asparagine agar	moderate	poor, white
Glycerol-asparagine agar (ISP No. 5)	moderate	moderate, white
Inorganic salts-starch agar (ISP No. 4)	moderate	poor, white
Nutrient agar	poor	none
Tyrosine agar	moderate	moderate, pastel yellow (2 db)
Yeast-malt extract agar (ISP No. 2)	moderate	poor, parchment (1½ db)
Oatmeal agar (ISP No. 3)	moderate	poor, white-parchment (1½ db)
T agar	abundant	abundant, parchment (1½ db)

* Color determination with Color Harmony Manual.[9] Reverse color was colorless and soluble pigments were not produced on any media tested.

in growing over a lower temperature range than many actinomycetes; it grew at 4°C but not above 36°C. Sporulation was most abundant at 21~24°C. A distinctive feature in its utilization of carbon compounds was its inability to metabolize mannitol.

Identification

Analysis of the above results indicated that strain No. C-19393 is a member of the genus *Streptomyces* of the Yellow color-series with chains of RF-type smooth-surface spores and without melanoid pigment formation. It can thus be regarded as a species related to those listed in Table 17.43 by PRIDHAM and TRESNER in BERGEY's Manual, 8th ed.[10] However, strain No. C-19393 is not identical with any species in that table, nor with those in the recent literature. It can be regarded as a species close to *Streptomyces griseus* in its gross cultural characteristics (grayish yellow color and powdery appearance) and morphology (formation of tufts). Although it has these typical characters of *S. griseus*, strain No. C-19393 differed in being unable to use D-mannitol and to grow at 37°C, the optimum temperature for *S. griseus*. In addition, strain No. C-19393 grew at 4°C. Examination of 13 authentic cultures from the Institute for Fermentation, Osaka, including the type strain, ISP 5236, for growth at different temperatures showed none able to grow at 6°C (data not shown). Accordingly, strain No. C-19393 was named *Streptomyces griseus* subsp. *cryophilus*. The type strain,

Table 2. Physiological characteristics of strain No. C-19393.

Test	Characteristic
Temperature requirement	growth: 4~35°C (good sporulation at 21~24°C)
Gelatin	liquefied
Starch	hydrolyzed
Milk	peptonized but not coagulated
Melanin pigment	not produced
Cell wall type	I (containing LL-DAP)
Utilization of carbon compounds	
Glycerol	++
i-Inositol	±
D-Mannitol	−
D-Xylose	+
L-Arabinoe	++
D-Glucose	++
D-Galactose	++
D-Fructose	++
Maltose	+
Sucrose	−
Rhamnose	++
Raffinose	−
Starch	++

Symbols: ++, efficient utilization; +, utilization; ±, doubtful utilization; −, no utilization.

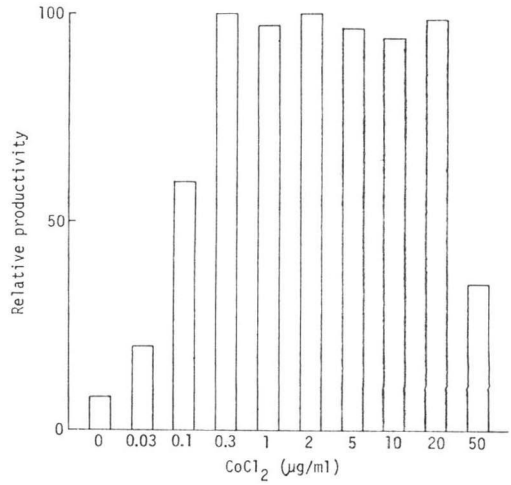
No. C-19393, has been deposited in the Institute for Fermentation, Osaka, under accession number IFO 13886.

Fermentation

Preliminary studies on the fermentation conditions revealed that the combination of glucose and starch was a good carbon source and that soybean meal mixed with cotton-seed meal was a good nitrogen source. The addition of $CoCl_2$ greatly stimulated production of the antibiotics (Fig. 3). The optimum concentration was between 0.3 and 20 μg/ml. The effect of $CoCl_2$ could be replaced by vitamin B_{12}.

Fig. 3. Effect of $CoCl_2$ on the production of antibiotics.

Cultivation was carried out at 30°C for 5 days in fermentation media supplemented with various levels of $CoCl_2$. Potency was measured as β-lactamase-inhibiting activity and is expressed relative to that in the medium which showed the maximum potency of 100.



The fermentation was optimized from preliminary experiments and was carried out as follows: Strain No. C-19393 was grown on a slant of T agar for 1 week and the spores generated were added to 500 ml of seed medium in a 2-liter Sakaguchi flask which was then shaken at 28°C for 2 days on a reciprocating shaker. The culture was transferred into 30 liters of the seed medium in a 50-liter stainless steel fermentor. After cultivation at 28°C for 2 days with aeration (30 liters/min) and agitation (280 rpm), the culture was transferred into 1,200 liters of fermentation medium in a 2,000-liter stainless steel fermentor. The fermentor was operated at 30°C for 5 days with aeration (840 liters/min) and agitation (180 rpm).

The antibiotics accumulated in the culture broth were isolated by chromatographic techniques, as described in the accompanying paper[3].

Antimicrobial Activities

As seen in Table 3, both antibiotics exhibited antibacterial activities against a wide variety of Gram-positive and Gram-negative bacteria. C-19393 H_2 was 8~120 times stronger than C-19393 S_2. It inhibited all the tested bacteria at concentrations below 10 μg/ml and most at below 1 μg/ml. On the other hand, no activities were observed against fungi and yeasts. The activity of C-19393 H_2 against clinically isolated bacteria resistant to β-lactam antibiotics was compared with that of cephaloridine. As shown in Table 4, C-19393 H_2 was active against bacteria which were resistant to cephaloridine.

Several carbapenem antibiotics have been discovered as inhibitors of β-lactamases[11~14]. C-19393 S_2 and H_2 also strongly inhibited several β-lactamases[5] and acted synergistically with ampicillin and cefotiam against bacteria resistant to β-lactam antibiotics due to production of β-lactamases. Some of the results are shown in Table 5. The potentiating effects were dramatic in several cases. For example, the addition of 0.5 μg/ml of C-19393 H_2 increased the activity of cefotiam against *Proteus mirabilis* TN 265 more than 1,000 times. The potentiating effects of C-19393 S_2 and H_2 varied with the organisms tested; the synergistic activity against *P. vulgaris* GN 4413 was stronger with C-19393 S_2 while that

Table 3. Antimicrobial activities of C-19393 S_2 and H_2.

Test organism	Minimum inhibitory concentration, μg/ml	
	C-19393 S_2	C-19393 H_2
Staphylococcus aureus FDA 209P	6.25	0.39
Micrococcus luteus IFO 12708	25	0.39
Bacillus subtilis IFO 3513	6.25	0.78
Bacillus cereus IFO 3466	>25	6.25
Escherichia coli NIHJ JC 2	6.25	0.1
Salmonella typhimurium IFO 12529	12.5	0.1
Proteus vulgaris IFO 3988	>25	3.13
Proteus mirabilis ATCC 21100	>25	1.56
Enterobacter cloacae IFO 12937	25	0.78
Serratia marcescens IFO 12648	12.5	0.2
Klebsiella pneumoniae IFO 3318	12.5	0.1
Alcaligenes faecalis IFO 13111	>25	1.56
Pseudomonas aeruginosa IFO 3080	>25	6.25
Comamonas terrigena IFO 13299	12.5	0.39
Acinetobacter calcoaceticus IFO 12552	>25	1.56
Candida albicans IFO 0583	>25	>25
Saccharomyces cerevisiae IFO 0209	>25	>25
Aspergillus niger IFO 4066	>25	>25
Penicillium chrysogenum IFO 4626	>25	>25

Table 4. Antibacterial activities of C-19393 H_2 and cefotiam against clinical isolates resistant to β-lactam antibiotics.

Test organism	Minimum inhibitory concentration, μg/ml	
	C-19393 H_2	Cefotiam
E. coli TN 649 (producer of PCase*)	0.31	2.5
K. pneumoniae TN 1698 (high producer of PCase)	1.25	>80
S. marcescens TN 81 (high producer of CSase**)	10	>80
P. mirabilis TN 265	2.5	>80
P. morganii GN 4738 (high producer of CSase)	2.5	>80
P. rettgeri TN 344 (producer of CSase)	1.25	>80
E. cloacae TN 587 (producer of CSase)	0.63	>80
Citrobacter freundii TN 515 (high producer of CSase)	2.5	>80

* Penicillinase.
** Cephalosporinase.

against *P. rettgeri* TN 344 was stronger with C-19393 H_2.

Discussion

A number of streptomycetes have been reported to produce carbapenem antibiotics. *S. griseus* subsp. *cryophilus* subsp. nov. can be differentiated from these as follows: *S. cattleya*, the producer of thienamycin[15], is a Violet color-series streptomycete with spiral spore chains. *S. olivaceus*, *S. flavogriseus* and *S. fulvissimus*, the producers of olivanic acids[11,16] and epithienamycins[17], are Gray color-series streptomycetes. *S. cremeus* subsp. *aurantilis*, the producer of PS-5[18], is a Red color-series streptomycete with RA spore chains.

Table 5. Potentiation of antibacterial activities of ampicillin and cefotiam by C-19393 S_2 and H_2.

Test organism, 10^8 CFU/ml	Addition, μg/ml	Minimum inhibitory concentration, μg/ml	
		Ampicillin	Cefotiam
S. aureus 1840 (producer of PCase)	None	25	1.56
	S_2* 0.1	6.25	0.78
	0.5	0.78	0.78
	H_2** 0.1	0.78	0.78
	0.5	0.1	0.2
E. coli TN 649	None	>800	0.78
	S_2 0.1	800	0.39
	0.5	50	0.2
	H_2 0.1	25	0.1
	0.5	—***	—
K. pneumoniae IFO 3512**** (producer of PCase)	None	200	0.2
	S_2 0.1	0.39	0.1
	0.5	0.39	0.1
	H_2 0.1	0.2	≤0.02
	0.5	—	—
P. mirabilis TN 265	None	>800	>800
	S_2 0.1	12.5	3.13
	0.5	6.25	1.56
	H_2 0.1	100	25
	0.5	1.56	0.78
P. vulgaris GN 5297 (producer of CSase)	None	200	100
	S_2 0.1	3.13	3.13
	0.5	6.25	3.13
	H_2 0.1	3.13	3.13
	0.5	1.56	1.56
P. vulgaris GN 4413 (high producer of CSase)	None	>800	>800
	S_2 0.1	12.5	6.25
	0.5	12.5	3.13
	H_2 0.1	400	50
	0.5	200	25
P. rettgeri TN 344	None	200	200
	S_2 0.1	200	200
	0.5	100	50
	H_2 0.1	25	12.5
	0.5	0.78	0.78
C. freundii TN 512	None	100	25
	S_2 0.1	6.25	0.39
	0.5	3.13	0.20
	H_2 0.1	1.56	0.78
	0.5	—	—

* C-19393 S_2.
** C-19393 H_2.
*** Test organism was inhibited by 0.5 μg/ml C-19393 H_2.
**** Not a clinical isolate.

Fermentative production of antibiotics C-19393 S_2 and H_2 was greatly stimulated by addition of $CoCl_2$. This compound was also added to the fermentation media for the production of other carbapenem antibiotics, such as thienamycin[15] and olivanic acids[13], presumably based on the finding of similar effects. We do not know, however, through what mechanism(s) $CoCl_2$ exerts its pronounced stimulatory effect.

C-19393 S_2 is the 8-methyl derivative of MM 4550[11] and, like MM 4550, it strongly inhibited β-lactamases. The antibacterial activity of C-19393 S_2 was rather low. In contrast, C-19393 H_2, which is the desulfonated derivative of C-19393 S_2, exhibited very strong antibacterial activity against a wide variety of bacteria, including several clinical isolates resistant to β-lactam antibiotics. The marked difference between the antibacterial activities of C-19393 H_2 and S_2 will be analyzed separately[4]. C-19393 H_2 inhibited β-lactamases and acted synergistically in combination with ampicillin and cefotiam against β-lactamase-producing clinical isolates, as did C-19393 S_2. The activities of C-19393 S_2 and H_2 as potentiators of ampicillin or cefotiam were variable, depending on the bacteria tested; C-19393 S_2 showed a stronger effect than C-19393 H_2 for some bacteria and *vice versa*. Such variations are probably due to the difference between the two antibiotics in β-lactamase-inhibiting activity or in permeability.

The *in vivo* effectiveness of C-19393 S_2 and H_2 as antibacterial agents, either alone or as potentiators of classical penicillins and cephalosporins, is now being investigated.

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