Communications to the editor

FR-900318, A NOVEL PENICILLIN WITH β-LACTAMASE INHIBITORY ACTIVITY

Sir:

A new β-lactamase inhibitor which we named FR-900318[1], 6-sulfoaminopenicillanic acid, has been isolated from the cultured broth of a strain of fungi. The strain was isolated from soil collected at Hikone City, Shiga Prefecture, Japan, and was classified as *Aspergillus candidus* by taxonomic studies and direct comparison with the type strain. This paper describes the fermentation, isolation, biological properties, chemical properties and structure determination of FR-900318.

Aspergillus candidus was cultured at 28°C under agitation (300 rpm) and aeration (15 liters/minute) for 96 hours in 15 liters of a medium placed in 30-liter jar fermentor. The medium consisted of glycerol 2%, corn steep liquor 0.25%, dried yeast 0.25%, cotton seed flour 0.5% and wheat germ 0.5%. The pH of the medium was adjusted to pH 7.0 before sterilization.

Fig. 1. Isolation procedure for FR-900318.

Culture filtrate (24 liters)
↓
Activated carbon (6 liters)
→ washed with water (6 liters)
→ eluted with 60% acetone - water (6 liters)
Eluate
→ concentrated to 500 ml
DEAE-Sephadex A-25 (phosphate type; 500 ml)
→ washed with water (500 ml)
→ eluted with 0.5 M NaCl (1,500 ml)
Eluate
↓
Activated carbon (500 ml)
→ washed with water (500 ml)
→ eluted with 60% acetone - water (1,000 ml)
Eluate
→ concentrated to 10 ml
Cellulose (150 ml)
→ eluted with 85% acetonitrile - water (200 ml)
→ concentrated to dryness
Yellow solid (150 mg)
↓
Sephadex G-15 (100 ml)
↓
DEAE-Sephadex A-25 (phosphate type; 20 ml)
→ linear gradient of 0.5 M ~ 1 M NaCl
→ desalted by activated carbon
White powder (27 mg)

Table 1. Antibacterial spectrum of FR-900318.

Test organism	MIC (μg/ml)
Staphylococcus aureus 209P JC1	>1,000
Staphylococcus aureus 209P JC1 17–4*	250
Bacillus subtilis ATCC 6633	500
Escherichia coli NIHJ JC2	>1,000
Escherichia coli 114**	4
Escherichia coli 8S-1**	4
Escherichia coli J604**	
Escherichia coli 386	1,000
Proteus vulgaris IAM 1025	>1,000
Pseudomonas aeruginosa NCTC-10490	>1,000
Pseudomonas aeruginosa 3***	8
Citrobacter freundii	1,000
Serratia marcescens	>1,000

* Supersensitive mutants to β-lactam of *Staphylococcus aureus* 209P.
** Supersensitive mutants to β-lactam of *Escherichia coli*.
*** Supersensitive mutants to β-lactam of *Pseudomonas aeruginosa*.

The isolation procedure for FR-900318 is shown in Fig. 1. The amount of FR-900318 was determined by the paper disc-plate method using *Escherichia coli* 8S-1. Using this procedure, 24 liters of culture filtrate yielded 27 mg of FR-900318 in pure form as its disodium salt. Antibacterial spectrum of FR-900318 is shown in Table 1. This test was conducted by serial agar dilution method. One loopful of an overnight culture of each strain in bouillon broth (about 10^3 viable cells/ml) was streaked on Mueller-Hinton agar containing graded concentration of the drug and the minimal inhibitory concentration (MIC) was expressed terms of μg/ml after incubation at 37°C for 20 hours except fungi in which MIC was determined after 3 days of incubation.

Assay system for β-lactamase inhibitors is shown in Fig. 2. Principle of the procedure is synergy or mutual cooperation between β-lactamase inhibitors and β-lactamase-sensitive

Fig. 2. Detection procedure for β-lactamase inhibitors.

Plates containing ampicillin 10 μg, 0.1 ml solution of *S. aureus* 209P cultured at 37°C for 4 hours and dilute β-lactamase from various organisms

↓

Pulp containing test samples is placed on the agar

↓

Incubation overnight at 37°C

↓

Samples containing a diffusible β-lactamase inhibitors give rise to zone of inhibition from the protection of the penicillin present in the agar.

Table 2. β-Lactamase inhibitory activity of FR-900318.

Origin of β-lactamase	Rate of dilution for β-lactamase	β-Lactamase inhibitory activity (μg/ml)	
		FR-900318	CP45899*
Citrobacter freundii	1/1,000	31	16
Escherichia coli 386	1/1,000	31	125
Serratia marcescens	1/4,000	31	16
Commercial penicillinase (Difco)	1/2,000	125	16

* Structure of CP45899

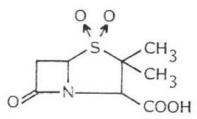


Fig. 3. Preparation of β-lactamase.

Inoculation of microorganisms in Antibiotics medium 3 (Difco)

↓ cultured at 30°C for 4~5 hours

Induction (trace of ampicillin)

↓ cultured at 30°C for 18 hours

Harvest of cells

↓ washed with 0.1 M Tris-HCl buffer (pH 7.5)

Sonic (10 minutes)

↓

Centrifuge (10,000 rpm, 15 minutes)

↓

Supernatant

↓

Milipore filter

Table 3. ^{1}H NMR data of FR-900318.

1.56 (3H, s) }	{ 2-α-CH_3
1.67 (3H, s) }	{ 2-β-CH_3
4.23 (1H, s)	H-3
5.08 (1H, d, J=4 Hz)	H-5
5.58 (1H, d, J=4 Hz)	H-6

δ In ppm downfield from internal TMS.

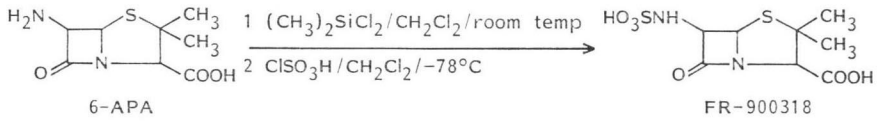
Table 4. ^{13}C NMR data of FR-900318.

26.8 }	{ α-CH_3
29.9 }	{ β-CH_3
61.3	C-6
64.0	C-2
67.8	C-5
73.4	C-3
174.7	C=O
176.1	COOH

δ In ppm downfield from internal TMS.

antibiotics against β-lactamases. The effect of β-lactamase inhibitors is shown by giving rise to zone of inhibition from protection of the penicillin present in the agar. This method is superior to the spectrophotometric assays[2,3] in regard to the sensitivity of β-lactamase inhibitory activity. The β-lactamase inhibitory activity of FR-900318, which is expressed terms of μg/ml, is shown in Table 2 and preparation of a typical β-lactamase is shown in Fig. 3.

Intravenous administration of 2,000 mg/kg of FR-900318 into mice did not result in any toxic symptom for 2 weeks after injection. FR-900318 as its disodium salt is an acidic, white amorphous powder, soluble in water, slightly soluble in methanol and insoluble in ethyl acetate and chloroform. It decomposes at 209~210°C. The optical rotation is $[\alpha]_D^{25}$ +247.5° (c 0.1, water). The antibiotic shows no ultraviolet maximum. Color reaction for including β-lactam skeleton is positive to hydroxylamine plus iron alum solution and platinous chloride plus potassium chloride solution. *Anal.* Calcd. for $C_8H_{10}N_2O_6S_2Na_2 \cdot 3H_2O$: C 24.36, H 4.06, N 7.10, S 16.20, Na 11.66. Found. C 24.65, H 4.47, N 7.17, S 16.37, Na 11.45. The molecular formula was deduced for FR-900318 on the basis of elementary analysis values, ^{1}H NMR and ^{13}C NMR spectroscopic data. The IR absorption spectrum (KBr) shows absorption bands at 1760 cm^{-1} (β-lactam); 1210, 1050, 640 and 620 cm^{-1}



6-APA FR-900318

(sulfoamide, respectively). The assignment of FR-900318 with the ^1H NMR and ^{13}C NMR in D_2O is shown in Tables 3 and 4, respectively. On TLC (Merck silica gel plates 60 F_{254}) FR-900318 has Rf 0.25 (BuOH - AcOH - H_2O, 4: 1: 2).

The structure of the compound was confirmed by derivation from 6-amino penicillanic acid (APA) as shown in following reaction.

Namely, *N,N*-dimethylaniline was added to 6-APA suspended in CH_2Cl_2 under stirring at room temperature for 30 minutes and chlorosulfonic acid added thereto at −78°C and stirring for five hours. The reaction mixture was poured into ice-water containing alkali and adjusted to pH 8. The aqueous solution was purified by DEAE-Sephadex A-25 (phosphate type). The eluate containing 0.5 ~ 0.6 M sodium chloride was collected and then treated with activated carbon to desalt.

The physico-chemical data (TLC, IR, NMR and optical rotation) were identical with those of a natural product.

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