INHIBITION OF CEPHALOSPORIN β-LACTAMASE BY M4854-I AND M4854-II

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(Received for publication October 30, 1979)

By screening for inhibitors of the β-lactamase produced by *Citrobacter freundii* GN346, two water-soluble, acidic, sulfur-containing substances exhibiting strong inhibitory activity against the enzyme were obtained from a culture filtrate of *Chaetomella raphigera* M4854.

The present paper deals with the isolation and physicochemical properties and biological characterizations of M4854-I and -II potassium salts.

The β-lactamase preparations consisted of ultrasonic extracts of bacterial cells and were prepared as follows: Five-hundred-milliliter flasks containing 100 ml of heart infusion broth were inoculated with 10 ml of an 18-hour broth culture of the organisms listed in Table 1 and shaken at 37°C. After 2-hour incubation benzylpenicillin (0.5 mg/ml) was added as an inducer to all cultures except *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. The culture was incubated for a further 3 hours. The bacterial cells were collected by centrifugation, washed twice with saline and resuspended in 0.1 M phosphate buffer, pH 7.0, to a concentration of 40~60 mg dry wt/ml. The suspensions were then treated in a UR-200 P super sonic vibrator for 4 minutes in 5 ml portions and centrifuged at 10,000 *g* for 30 minutes to remove insolubles. The clear supernatant solutions were kept at −20°C until required. The enzyme of *S. aureus* 0003 was prepared from the supernatant fluid of a methicillin-induced (1 μg/ml) *S. aureus* 0003 culture.

The activity of the inhibitors, M-4854, was determined by the iodometric method[1]. The β-lactamase preparation was diluted in 0.1 M phosphate buffer (pH 7.0) until it gave about 80% hydrolysis of a 0.5 mg/ml solution of cephaloridine in 30 minutes at 30°C. Suitable dilutions of the M4854-I or -II preparation were mixed with the β-lactamase solution and incubated at 30°C for 10 minutes because of the progressive nature of the inhibition. A control with buffer in place of M4854-I or -II preparation was also incubated. The substrate in pH 7.0 buffer was then added to both inhibitor and control reaction mixtures to give a final concentration of 0.5 mg/ml, and incubation at 30°C was continued for a further 30 minutes. The residual substrate in each mixture was then estimated by the iodometric method, and the results were used to calculate the percentage of inhibition.

The I_{50} for M4854-I or -II is defined as the concentration giving 50% inhibition and was obtained from a plot of percentage of inhibition against the inhibitor concentration.

The inhibitors were produced by submerged culture of the *C. raphigera* M4854 in a medium containing 2% glucose, 1% peptone, 1% C.S.L, 0.2% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$. The whole broth culture (shake-cultured at 26°C for 3~5 days) was adjusted to pH 5.0 with hydrochloric acid and filtered. The filtrate was extracted with *n*-butanol. The extract was concentrated *in vacuo* to remove the *n*-butanol, and a brownish oily syrup of the M4854 mixture was obtained. In order to separate the mixture, the active oily syrup was chromatographed on a column of aluminum oxide, which was developed with ethanol followed by 80% ethanol. Two active fractions were separated; the faster one was called M4854-I and the other M4854-II. Each inhibitor was purified using silica gel column chromatography with chloroform - methanol (5: 2) as the developer. Both M4854-I and -II were obtained as pure white powders.

In order to obtain M4854-I potassium salt, 2-ethylhexanoic acid potassium salt solubilized in ethylacetate was added to the ethylacetate - methanol (4: 1) solution containing M4854-I. A white precipitate of M4854-I potassium salt was formed. The precipitate was collected by filtration and washed with ethylacetate and then dried under reduced pressure. M4854-II potassium salt was obtained by the same procedure described above.

M4854-I potassium salt has a mp. 240°C (decomp.) and $[\alpha]_D^{26}$ +3.8 (*c* 0.64, H_2O). It is soluble in water and DMSO, moderately soluble in lower alcohols, but insoluble in esters and nonpolar solvents. The ultraviolet absorption spectrum in water exhibits a maximum at 267 nm ($E_{1cm}^{1\%}$ 4.14) with an inflection at 273 nm ($E_{1cm}^{1\%}$

3.03), as shown in Fig. 1. The IR and NMR spectra are shown in Figs. 2 and 3, respectively. Elementary analysis data is as follows: C 50.18%, H 7.19%, S 4.20%, N 0%, K 10.24%. It gives a positive reaction with vanillin-sulphuric acid and ammonium vanadate-sulphuric acid tests, but a negative reaction with FEHLING, MOLISCH, $FeCl_3$, ninhydrin and permanganate tests.

M4854-II potassium salt has a mp. 193°C (decomp.) and $[\alpha]_D^{26}$ +2.6 (*c* 0.65, H_2O). The UV spectrum in water exhibits a maximum at 266 nm ($E_{1cm}^{1\%}$ 3.31) with an inflection at 273 nm ($E_{1cm}^{1\%}$ 2.38). The IR and NMR spectra are shown in Figs. 2 and 3, respectively. Elementary analysis data is as follows: C 44.35%, H 6.20%, S 5.92%, N 0%, K 11.28%. The solubility and color reaction were similar to M4854-I potassium salt. On thin-layer chromatography on silica gel F_{254} (E. Merck AG, Darmstadt, Germany) on glass plates (5 cm × 20 cm) with chloroform - methanol (8: 5), the Rf values of M4854-I and -II potassium salts were 0.50 and 0.31, respectively; with *n*-butanol - acetic acid - water (3: 1: 1) the Rf were 0.66 and 0.56. The Rf values were 0.25 and 0.08 when using a solvent system consisting of ethylacetate - isopropylalcohol - water (10: 4: 1).

Neither inhibitors had antibacterial or antifungal activities at a 2 mg/ml concentration, and no toxicity in mice has been observed with 100 mg/kg of M4854-I or -II potassium salt administered intravenously.

The enzyme-inhibitory activity of M4854-I or -II against a range of β-lactamases was determined as I_{50}. The results given in Table 1 show that chromosomally-mediated β-lactamases from Gram-negative bacteria except *K. pneumoniae* GN69 are inhibited by very low concentrations of M4854-I or -II. These enzymes were typical cephalosporinases. The β-lactamases mediated by R factors such as *E. coli* ML1410/R_{ms212} and ML1410/R_{ms213} are moderately inhibited, while neither inhibitor has an inhibitory effect at 100 μg/ml concentration on the activity of the *S. aureus* 0003 enzyme.

The kinetics of the effect of M4854-I (0.5 μg/ml) and -II (0.4 μg/ml) on the hydrolysis of cephaloridine by the β-lactamase from *C. freundii* GN346 were studied. The LINEWEAVER-BURK plot is shown in Fig. 4. Inhibition by M4854-I or -II was not competitive.

C. freundii GN346, *P. morganii* GN926 and *E. coli* ML1410/R_{ms212} (0.01 ml of a stationary growth phase culture) were inoculated into 10 ml of nutrient broth containing various concentrations of cephaloridine or ampicillin with or without M4854-I or -II and incubated with shaking at 37°C. Bacterial growth was followed by measuring the optical density at 660 nm with a Shimazu Bausch and Lomb spectronic 20.

As shown in Fig. 5, the growth of GN346 and GN926 were not inhibited by 100 μg/ml cephaloridine or 10 μg/ml of inhibitor. However the growth of those strains was inhibited by the combined use of both agents. This effect was

Fig. 1. Ultraviolet spectra of potassium salt of M4854-I and -II in water.

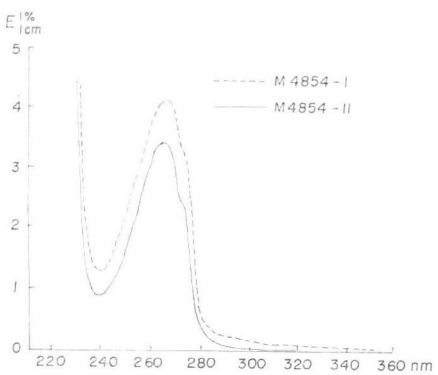


Fig. 2. Infrared spectra of potassium salts of M 4854-I and -II in KBr disk.

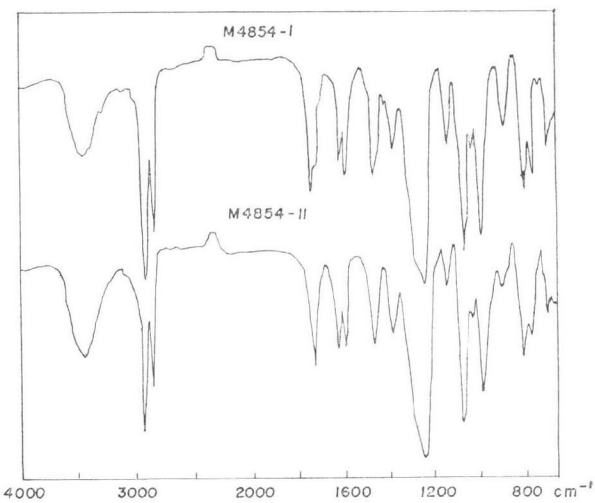


Table 1. β-Lactamase inhibitory activity of M4854-I and -II.

Source of β-lactamase	Enzyme activity[a)]	Substrate	I_{50} (μg/ml)	
			M4854-I	M4854-II
Citrobacter freundii GN346	18.33	CER[b)]	0.52	0.31
Enterobacter cloacae GN336	2.61	CER	2.6	2.4
Enterobacter aerogenes GN2132	1.48	CER	2.5	3.6
Proteus morganii GN926	1.55	CER	6.3	5.0
Proteus rettgeri GN624	3.26	CER	6.3	2.2
Proteus vulgaris GN76	0.65	CER	2.3	2.2
Pseudomonas aeruginosa GN918	0.70	CER	3.0	2.5
Escherichia coli GN206	0.21	CER	—	2.9
Escherichia coli ML1410/R_{ms212}	0.60	PCG[c)]	49	28
Escherichia coli ML1410/R_{ms213}	0.03	PCG	26	4.0
Klebsiella pneumoniae GN69	0.46	PCG	46	30
Staphylococcus aureus 0003	1.46	PCG	>100	>100

a) Units/mg dry weight of bacteria. One unit of the enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of the substrate in 1 minute at 30°C.

b) and c), CER (cephaloridine) and PCG (benzylpenicillin) were used as the substrates, respectively.

—, Not determined.

The enzyme activity used for I_{50} determination was about 0.033 units.

Fig. 3. 100 MHz NMR spectra of M4854-I and -II in d_6-DMSO with TMS as an internal standard.

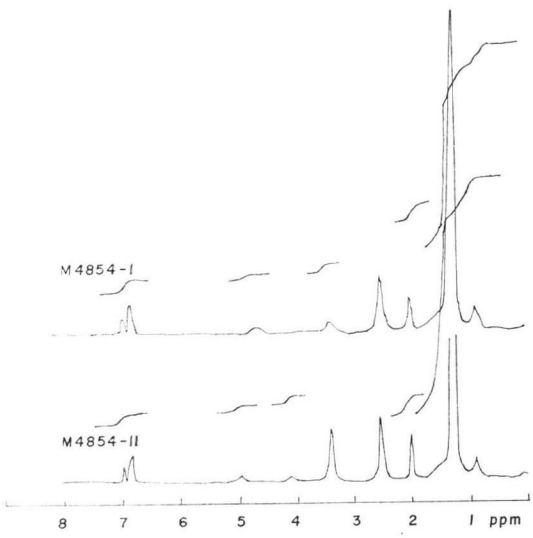
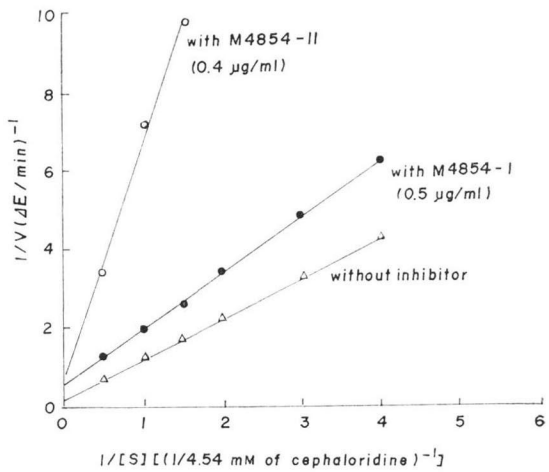


Fig. 4. Kinetics of β-lactamase inhibition by M4854-I and -II.

The concentration of cephaloridine varied from 0.44 mM to 0.055 mM.

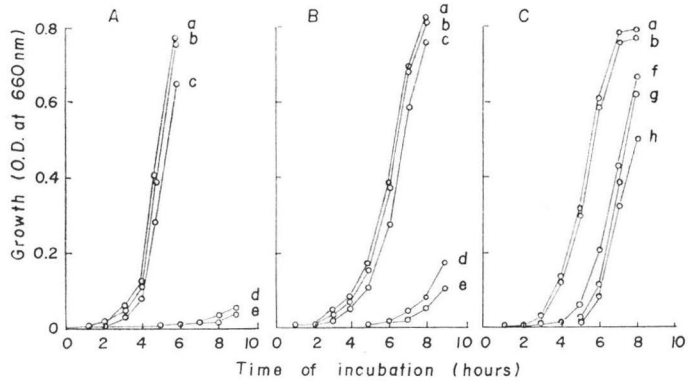
β-Lactamase was prepared from *C. freundii* GN346. The reaction was continued in 0.1 M phosphate buffer (pH 7.0) at 30°C.



not found in the *E. coli* ML1410/R_{ms212} strain when 12.5 μg/ml ampicillin, which could be inactivated by R_{ms212} penicillinase, was used.

Recently, Beecham researchers,[2,3,4] UMEZAWA *et al.*[5], MAEDA *et al.*[6] and OKAMURA *et al.*[7] have reported the isolation of the low molecular weight β-lactamase inhibitors clavulanic acid, MM4550, MM13902, MM17880, MC-696-SY-A and -B, and PS-5 from *Streptomyces* species. These substances were naturally occurring β-lactamase-inhibiting β-lactams with antibacterial activity.

Fig. 5. Combined effect of M4854-I or -II on the antibacterial activity of β-lactam antibiotics.
A, *C. freundii* GN346; B, *P. morganii* GN926; C, *E. coli* ML1410/R_{ms212}.
a, Control growth without drug; b, growth in nutrient broth containing 10 μg/ml of M4854-I; c, growth in nutrient broth containing 100 μg/ml of cephaloridine; d, growth in nutrient broth containing 10 μg/ml of M4854-I and 100 μg/ml of cephaloridine; e, growth in nutrient broth containing 10 μg/ml of M4854-II and 100 μg/ml of cephaloridine; f, growth in nutrient broth containing 12.5 μg/ml of ampicillin; g, growth in nutrient broth containing 10 μg/ml of M4854-I and 12.5 μg/ml of ampicillin; h, growth in nutrient broth containing 10 μg/ml of M4854-II and 12.5 μg/ml of ampicillin.



Accordingly, M4854-I and -II are clearly different from these metabolites.

M4854-I and -II are sulfur-containing compounds which are stable to alkali but less stable to acid. The presence of a strong absorption band at 1240 cm^{-1} in their IR spectra indicated the presence of a sulfate ester group in M4854-I and -II. From these properties and the UV and NMR spectra, the substances M4854-I and -II are found to belong to the alkylbenzene di-sulfate group and to be related to panosialin[8] which exhibits inhibition of sialidase. Their physicochemical properties are sufficient to differentiate M4854-I and -II from panosialin such as the presence of a strong absorption band at 1730 cm^{-1} in their IR spectra, specific rotation and elemental analysis.

The fact that the typical cephalosporinases are strongly inhibited by low concentrations of M4854-I or -II but penicillinases are only moderately inhibited, is interesting in view of the difference in structure between penicillinase and cephalosporinase. It is known that clavulanic acid has potent inhibitory activity against penicillinase but not against cephalosporinase.

Data in Table 1 and Fig. 5 show that where good inhibition was seen against isolated enzymes, the organisms producing those enzyme were rendered susceptible to cephaloridine in the presence of M4854-I or -II. Accordingly, in the strains of bacteria that owe their resistance to the production of cephalosporinase, it is thus possible, by adding M4854-I or -II at low concentrations, to demonstrate marked improvement in the activity of cephalosporinase-labile cephalosporins.

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