Communication to the editor

TWO β-LACTAMASE INHIBITORS PRODUCED BY A STREPTOMYCES

Sir:

By the screening for inhibitors to the β-lactamase produced by *Escherichia coli* K-12 W3630 R_{75}^{+}[1], two water-soluble, acidic, labile substances exhibiting the strong inhibitory activity against the enzyme were obtained from the cultured broth of a streptomyces, designated MC696-SY2. The strain was isolated from a soil sample collected in Okazaki-city, Japan, and classified as *Streptomyces fulvoviridis* MC696-SY2. The inhibitors isolated are not completely pure at present, but have an extremely strong inhibitory activity against β-lactamase. Therefore, in the present paper, the production, purification and biological properties are described.

The activity of the inhibitors, MC696-SY2, was determined by the iodometric titration method and by the agar plate method.

(1) Iodometric titration method: A solution of 0.1 ml of 0.1 M phosphate buffer (pH 7.0) containing the inhibitor, 0.25 ml of penicillinase in the same buffer (8,000 units/ml) prepared from penicillinase (300 units/mg; Nutritional Biochemical Corp., Ohio, U.S.A.) or from β-lactamase$_{75}$ produced by *E. coli* K-12 W3630 R_{75}^{+}[1], and 0.4 ml of the buffer was placed in a test tube, and shaken gently at 37°C for 5 minutes. To the mixture, 0.25 ml of potassium benzylpenicillin (8,000 units/ml) in the same buffer was added, shaken continously for 35 minutes and then heated at 100°C for exactly one minute in a boiling water bath to stop the reaction. A control without an inhibitor, a blank without penicillinase and another blank without the inhibitor or penicillinase were prepared and treated the same as the sample solution. To all test tubes containing the control and the blanks, 5 ml of 0.01 N iodine was added. After 15 minutes, the excess iodine was titrated with 0.01 N sodium thiosulfate, using 1% starch solution as an indicator. The value T_C was calculated from the titre of the blank (without penicillinase and the inhibitor) minus the control (without the inhibitor) and the value Ts was calculated from the titre of another blank (without penicillinase) minus the sample containing penicillin, penicillinase and the inhibitor. The percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - \frac{Ts}{Tc} \times 100$$

In this procedure, 1.0~0.5 mcg of purest inhibitors, MC696-SY2-A and -B showed 50% inhibition.

(2) Agar plate method: Penicillinase (500,000 units/ml) purchased from Tokyo Kasei Co. was used. *Staphylococcus aureus* FDA 209P on an agar slant was suspended in 10 ml saline solution and used as the test organism. The agar medium for the penicillin assay was utilized. Ten ml of melted agar medium, at 48°C, containing 500 units of penicillinase, 100 units of potassium benzylpenicillin and the suspension of the test organism (1%) was plated. Within 15 minutes to 2 hours after the preparation of the plates, a disc containing a suitable amount of an inhibitor was placed on the agar. After incubation overnight at 37°C, a clear inhibition zone appeared. The relation shown by the formula, $d = \alpha \log C + \beta$ (α, β = constants) was found between the diameter of the inhibition zone (d) and the concentration of the inhibitor (C) in the range of 3~150 units/ml. A sample which was a mixture of two inhibitors, MC696-SY2-A and -B, was designated as the stardard: 1,000 units/mg. The potency of the purest MC696-SY2-A obtained was 1127 units/mg.

The strain producing the inhibitors was inoculated to a medium consisting of 2% glycerol, 1.5% soybean meal, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4 and 0.02% silicone oil (pH 6.8), and shake-cultured at 28°C for 3~5 days, producing 250~300 units/ml. The cultured broth was filtered at 0~5°C, and the filtrate adjusted to pH 5~6. To the filtrate, 2% (w/v) of active carbon was added. After stirring in an ice bath for

20~30 minutes, the carbon was collected by centrifugation at <5°C, washed with water and treated with ten volumes (v/w of carbon added) of 30 % *n*-propanol in water at pH 7 and 40~45°C. The eluate was concentrated to 1/10 volume at <40°C. The yield of actitivy from the filtrate was about 50 %. The concentrate was diluted with three volumes of 0.01 M phosphate buffer (pH 7.0) and poured on a column of DEAE-cellulose (OH^- or Cl^- form, produced by Brown Co., Ltd.). The column was washed with the buffer, and eluted by increasing the concentration of NaCl from 0 to 0.2 M NaCl in the buffer. Two main active fractions were separated; the faster one was named MC 696-SY2-A and the other MC696-SY2-B. In general, a minor active fraction appeared before MC696-SY2-A. In only one case among several preparations tested, another minor fraction appeared between MC696-SY2-A and -B. Each active substance was adsorbed on active carbon, eluted with 30 % *n*-propanol in water from the carbon cake which was thoroughly washed with water, concentrated at <40°C, suspended in methanol, precipitated by addition of ether and then dried under reduced pressure to a powder. If necessary, active substances obtained by the procedure described above were further purified by column chromatography on Sephadex G-25 or G-10 (fine; manufactured by Pharmacia Fine Chemicals), using distilled water as developer, and the active fractions were lyophilized. All the above operations were done in a cold room at 3°C. Gross yield of final powders was 5~10 %, and the potency of the purest MC 696-SY2-A and -B was 1127 and 867 units/mg, respectively.

On thin-layer chromatography on silica gel GF_{254} (E. Merck AG, Darmstadt, Germany) on glass plates (5 cm×20 cm) with *n*-propanol - 0.1 M pH 7 phospharte buffer (7:3), the Rf values of MC696-SY2-A and -B were 0.32~0.37 and 0.41~0.46, respectively, with *n*-butanol - methanol - water (4:1:2) the Rf was 0.20~0.23 and 0.23~0.25. The active materials were demonstrated by bioautography. After thin-layer chromatography on Spotfilm (5 cm×20 cm) (Tokyo Kasei Kogyo Co., Ltd., DEAE-cellulose), MC696-SY2-A and -B gave spots at Rf 0.54~0.60 and Rf 0.42~0.53, respectively with 0.2 M NaCl in 0.01 M phosphate buffer (pH 7).

The kinetics of the effect of MC696-SY2-A (2.23 units/ml) and -B (1.47 units/ml) on hydrolysis of benzylpenicillin by β-lactamase$_{75}$ from *E. coli* K-12 W3630 R^+_{75} were studied. The LINEWEAVER-BURK plot is shown in Fig. 1. Inhibition by MC696-SY2-A was competitive, but MC696-SY2-B was not completely competitive.

Both MC696-SY2-A and -B were not destroyed by penicillinase at 37°C for 3 hours, while benzylpenicillin was completely hydrolyzed under these conditions in one hour.

A mixture of MC696-SY2-A and -B (233 units/mg) was dissolved in pH 7.0 phosphate buffer (100 mcg/ml, 23.3 units/ml). A cephalosporinase-producing strain, *Escherichia freundii*, was incubated for 16 hours at 37°C, diluted 10-fold with bouillon, and incubated for 2 hours to prepare a cephalosporinase solution. When the inhibitory activity against cephalosporinase was determined by the iodometric titration method as described above, using 0.25 ml of the inhibitor solution, 0.25 ml of the diluted cephalosporinase solution and 0.5 ml of cephazoline solution (5 mg/ml), 6 units of inhibitor gave 20 % inhibition of the cephalosporinase.

Fig. 1. Kinetics of inhibition by MC-696-SY2-A and -B of β-lactamase$_{75}$-benzylpenicillin system.

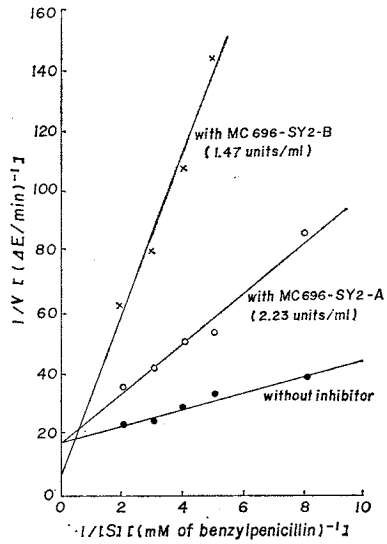

Table 1. Stability of broth filtrate at various pH's

pH	Activity remaining %	
	60°C, 30 min.	50°C, 30 min.
2.0	0	—
5.0	43	65
7.0	—	80
7.8	38	—
9.0	—	30

MC696-SY2-A and -B are labile substances. Their activity in the culture filtrate decreased to 60.5, 34.5 and 15.1 % after 3, 8 and 10 days' storage in a refrigerator (5°C). Even as a dry powder, the activity rapidly disappeared at room temperature in a desiccator. However, they were stable at −20°C. The stability of the broth filtrate (180 units/ml) and of aqueous solutions (500 mcg/ml) of a crude mixture (13.3 units/mg) and partially purified powder of MC696-SY2-A (184 units/mg) and -B (363 units/mg) was tested at various pH's as shown in Tables 1 and 2. The influence on the activity of calf serum was tested with a solution containing 10 mg/ml of crude powder (6.7 units/mg) by the disc method. The activity was reduced to 100, 88.5, 69.6 and 51.5 % in 6.25, 12.5, 25 and 50 % calf serum-bouillon solutions, respectively.

A crude powder (28.3 units/mg) was dissolved in 0.1 M phosphate buffer (pH 7) at 20 mg/ml. To portions of the solution, 0.01 M 2-mercaptoethanol, dithiothreitol or glutathione were added, and the mixtures allowed to stand in a refrigerator overnight. The activity in all solutions completely disappeared, while a solution with no reducing agent retained 57.5 % of the original activity.

The effect of a mixture of MC696-SY2-A and -B on the inhibitory activity of penicillin against two strains of penicillin-resistant *Staphylococcus aureus* was tested. *S. aureus* #B154 (*S. aureus* 193 resistant to penicillin, streptomycin and tetracycline) and #B337 (*S. aureus* S-642 resistant to erythromycin, oleandomycin, leucomycin and penicillin) were used as test organisms. Plates were prepared with 10 ml of melted nutrient agar containing 1,300, 650, 325, 163 or 0 units of the mixture of the inhibitors and 1 % of a 16-hour test organism culture. Discs soaked in 800, 400, 100, 25 and 6.25 units/ml of standard benzylpenicillin solutions were placed on the agar plates. After incubation at 37°C for 16 hours, the diameter of inhibition zones was measured. The results, as shown in Table 3, indicated that inhibitors enhanced the activity of penicillin against penicillin-resistant *S. aureus*. The activity against the sensitive *S. aureus* FDA 209P was also enhanced.

When 5 mg of MC696-SY2-A (129 units/mg) was injected intravenously into mice weighing 20 g, no toxicity was seen in 10 days.

Table 2. Stability of aqueous solutions containing crude or purified powder (at 50°C)

pH	Activity remaining %				
	Crude powder	MC696-SY2-A		MC696-SY2-B	
	30 min.	30 min.	2 hours	30 min.	2 hours
2.3	—	0	—	0	—
3.0	25	0	—	0	—
4.0	61	8	—	15	10
5.0	84	—	—	—	—
5.2	—	65	11	62	28
6.0	85	82	66	100	69
6.8	—	100	76	100	64
7.0	99	—	—	—	—
8.0	49	100	70	100	71
9.0	20	—	—	—	—
10.5	—	61	39	84	28

Table 3. Effect of MC696-SY2 on activity of penicillin G against *S. aureus* by disc method

S. aureus No.	MC696-SY2 in agar units/ml	Diameter of inhibition zone (mm) and penicillin G (u/ml) applied to discs				
		800	400	100	25	6.25
B154	0	(10.5)	(9.25)	0	0	0
	16.3	12.25	10.25	0	0	0
	32.5	13.0	12.5	10.0	0	0
	65.0	16.5	14.5	12.5	10.5	0
	130	24.5	22.5	20.5	17.75	15.0
B337	0	13.0	11.5	9.5	0	0
	16.3	14.5	12.75	11.0	9.25	0
	32.5	17.75	15.5	12.25	10.25	0
	65.0	19.25	18.0	15.5	13.25	11.0
	130	24.5	22.5	19.5	17.5	14.5
209P sensitive strain	0	—	34.0	30.75	27.25	22.25
	16.3	—	—	33.5	30.25	25.0
	32.5	—	—	36.0	32.5	28.0
	65.0	—	—	36.0	32.5	28.0
	130	—	—	—	34.0	30.0

Table 4. Comparison of inhibitory activity against penicillinase and bacteria by MC696-SY2-A and -B with that of cephalosporin analogues

Substance	Inhibitory activity against penicillinase		M. I. C. (mcg/ml)*	
	Inhibition %/mcg (wt) by iodometric	MC696-SY2 units/mg by agar plate**	*S. aureus* FDA 209P	*E. coli* NIHJ
A16886A	8/200	17.9	125	62.5
A16886B	50/220	13.3	500	8
A16884	50/280	10.7	250	4
Cephamycin A	13.5/200	6.0	125	31.3
Cephamycin B	18/200	10.0	125	16
Cephamycin C	50/215	2.0	>500	16
MC696-SY2-A	50/2.6	161	1,000	250
MC696-SY2-B	50/0.78	867	93.5	23.4

* Bouillon dilution method ** Units/mg means the value calculated as the potency of MC696/SY2

Recently, the cephalosporin analogues A16886A, A16886B and A16884[2,3,5], and cephamycins A, B and C[4,6] have been isolated from *Streptomyces*. These materials were obtained and compared with MC696-SY2-A and -B. The results are shown in Table 4. The MC696-SY2 inhibitors were stronger inhibitors of penicillinase than the cephalosporin analogues, but much weaker in inhibiting the growth of the bacteria. MC696-SY2-A and -B could be differentiated from the cephalosporin analogues by thin-layer chromatography.

HATA and others[7] have reported a penicillinase inhibitor (KA-107) produced by *Streptomyces gedanensis*. KA-107 is differentiated from MC696-SY2 because KA-107 is a macromolecular substance, while MC696-SY2-A and -B are dialysable and have molecular weights around 400 as determined by ultracentrifuge and Sephadex G-25 chromatography.

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