A NOVEL NATURALLY OCCURRING CARBAPENEM ANTIBIOTIC, AB-110-D, PRODUCED BY *KITASATOSPORIA PAPULOSA* NOVO SP.

Sir:

So far many carbapenem antibiotics have been discovered, such as thienamycins[1], olivanic acids[2,3], epithienamycins[4], PS series[5,6], OA-6129 series[7], C-19393 series[8,9], asparenomycins[10], carpetimycins[11], pluracidomycins[12], SF-2103 series[13] and SQ 27,860[14]. Most carbapenem antibiotics were produced by *Streptomyces*. Only SQ 27,860 was produced by *Erwinia* and *Serratia*. No other microorganism in any other genus has been reported to produce carbapenem antibiotics. Our screening program for novel carbapenem antibiotics from so-called 'rare actinomycetes' led to the discovery of a new strain named *Kitasatosporia papulosa* AB-110[15], which produces a novel carbapenem antibiotic named AB-110-D together with epithienamycins A, B, E and F[4]. The structure of AB-110-D was elucidated as the *Z* isomer of epithienamycin E in the side chain. No carbapenem antibiotic having a *Z* side chain has been isolated from the culture broth of any microorganism. This paper describes the fermentation, isolation, structure elucidation and characteristics of AB-110-D.

Carbapenem antibiotics were produced by submerged cultivation of strain AB-110 in a 500-liter fermentor containing 300 liters of SS medium supplemented with antifoam. SS medium contained starch 20 g, glucose 5 g, Soytone 12 g, yeast extract 5 g, KH_2PO_4 1 g, $CoSO_4 \cdot 7H_2O$ 0.006 g, $ZnSO_4 \cdot 7H_2O$ 0.003 g, $MnSO_4 \cdot 4 \sim 5H_2O$ 0.003 g, $FeSO_4 \cdot 7H_2O$ 0.003 g per liter adjusted to pH 7.0. The fermentation was carried out at 28°C for 60 hours with an agitation speed of 310 rpm and an air flow rate of 75 liters/minute.

Carbapenem antibiotics were detected by antibacterial activity and β-lactamase inhibitory activity during fermentation and isolation. The antibacterial activity was assayed by the paper-disc agar diffusion method using *Comamonas terrigena* AJ 2083 as the test organism. The β-lactamase inhibitory activity was also assayed by the paper-disc agar diffusion method using *C. terrigena* AJ 2083, penicillinase from *Bacillus cereus* (Sigma Chemical Co., penicillinase type I) and benzylpenicillin (Meiji Seika Kaisha, Ltd.).

The carbapenem antibiotics in the culture filtrate (220 liters) were absorbed on a column of anion exchange resin, Diaion SA 20A (30 liters, Mitsubishi Chemical Industries Limited), and eluted with 20 mM phosphate buffer (pH 7.0) containing 50% MeOH, 1 M NaCl and 0.001% EDTA. The active eluate was desalted on a column of activated charcoal (15 liters, Wako Pure Chemical Industries Co.), and the desalted fraction was applied to a column of anion exchange resin, QAE-Sephadex A-25 (7 liters, Pharmacia Co.). After washing the column with 20 mM phosphate buffer (pH 7.0) containing 0.001% EDTA, elution was performed with a linear gradient of 0~1 M NaCl in the same buffer. Five active fractions, A, B, C, D and E (in the order of elution), were eluted from the column. Each fraction was desalted on a column of high porous resin, Diaion SP-207 (Mitsubishi Chemical Industries Limited), with 50% MeOH and lyophilized to give crude powders respectively.

The crude powders of fractions A, B, C and E were further purified. AB-110-A, B, C and E were isolated from the corresponding fractions and identified as epithienamycins A, B, F and E[16] respectively.

The crude powder of fraction D was further purified by column chromatography on Toyopearl HW-40 (400 ml, Tosoh Co.) with 20 mM phosphate buffer (pH 7.0) containing 0.001% EDTA. The active eluate was desalted and concd *in vacuo*. Final purification was achieved by preparative reverse phase HPLC on YMC-PAK D-ODS-5 column (20×250 mm, Yamamura Chemical Laboratories) with 5 mM phosphate buffer (pH 7.0) containing 8% MeOH. The peak fraction of AB-110-D was desalted on the same column with water followed by 50% MeOH and lyophilized to give 0.2 mg of substantially pure AB-110-D as the dipotassium salt.

HPLC retention times of those carbapenem antibiotics isolated from culture broth of *K. papulosa* AB-110 are shown in Table 1.

The UV spectrum of AB-110-D in water shows maxima at 229 nm and 305 nm, and resembles those of AB-110-B and E (epithienamycins B and E[16] respectively).

A molecular weight of 392 (the same as that

of epithienamycin E[16]) for AB-110-D was suggested by the fast atom bombardment mass spectrum (FAB-MS): *m/z* 431 $(M+K)^+$, 469 $(M+2K-H)^+$.

Table 1. Retention times of carbapenem antibiotics in HPLC.

	Retention time (minutes)
AB-110-A (epithienamycin A)	22.5
AB-110-B (epithienamycin B)	37.3
AB-110-C (epithienamycin F)	10.0
AB-110-D	25.2
AB-110-E (epithienamycin E)	19.3

HPLC was carried out on a YMC R-ODS column (4×250 mm) eluting with 2.5% MeOH in 5 mM potassium phosphate buffer (pH 7.0) containing 1 mg/liter of EDTA at flow rate of 1.0 ml/minute. The effluent was monitored with UV absorption at 300 nm.

The ^{1}H NMR spectrum of AB-110-D is closely related to that of AB-110-E (epithienamycin E[16]), as shown in Table 2, but some differences were observed in the chemical shift of the proton at C-12 and in the coupling constant of vinyl protons at C-12 and C-13. The smaller coupling constant of the vinyl protons (J=7.7 Hz) suggested the presence of the *Z* configuration for the C-12 – C-13 double bond in the antibiotic. The configuration of the β-lactam hydrogen atoms at C-5 – C-6 in the antibiotic was determined to be *cis* by virtue of the observed coupling

Fig. 1. Structure of AB-110-D.

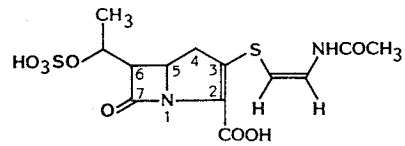

Table 2. ^{1}H NMR chemical shifts of AB-110-D and AB-110-E (epithienamycin E).

	AB-110-D	AB-110-E (epithienamycin E[16])
9-H_3	1.52 (d, J=6.1)	1.50 (d, J=6.5)
$COCH_3$	2.14 (s)	2.04 (s)
4-H_a	3.10 (dd, J=18, 9)	3.06 (dd, J=18, 9)
4-H_b	3.32 (dd, J=18, 9)	3.32 (dd, J=18, 9)
5-H	4.31 (dt, J=5.5, 9.3)	4.29~4.85 (m)
6-H	3.87 (dd, J=5.5, 9.3)	3.84 (dd, J=5, 10)
8-H	4.8 (m)	4.29~4.85 (m)
12-H	5.71 (d, J=7.7)	6.07 (d, J=13.8)
13-H	7.18 (d, J=7.7)	7.16 (d, J=13.8)

The ^{1}H NMR spectrum was recorded at 400 MHz in D_2O with a Jeol JNM-GX-400 spectrometer. Chemical shifts are given in ppm relative to external DSS, and coupling constants are given in Hz.

Table 3. Antimicrobial activity of AB-110-D.

	MIC (μg/ml)				
	AB-110-A (epithienamycin A)	AB-110-B (epithienamycin B)	AB-110-C (epithienamycin F)	AB-110-D	AB-110-E (epithienamycin E)
Escherichia coli NIH JC-2	0.1	0.2	0.2	0.2	0.2
Proteus vulgaris N-29	0.2	0.78	0.39	0.78	0.39
P. mirabilis N-76	0.2	0.39	0.39	0.78	0.39
Alcaligenes faecalis H-30	0.2	0.2	0.39	0.39	0.2
Bacillus subtilis ATCC 6633	0.2	0.78	0.78	0.78	0.39
Micrococcus luteus AJ 2020	0.2	0.78	0.78	0.78	0.39
Staphylococcus aureus 209P	0.2	0.39	0.39	0.39	0.39

MICs (μg/ml) were determined by the serial agar dilution method using Brain Heart Infusion medium (Difco). Plates were inoculated with 1 μl of an undiluted overnight broth culture (~10^6 cfu) and incubated at 37°C aerobically for 18 hours. The lowest concentration of antibiotic which inhibited macroscopic growth of bacteria was regarded as the MIC.

Table 4. Stability of AB-110-D to β-lactamases.

	Penicillinase from *Bacillus cereus*	Cephalosporinase from *Enterobacter cloacae*
AB-110-A (epithienamycin A)	50,000	200,000
AB-110-B (epithienamycin B)	25,000	200,000
AB-110-C (epithienamycin F)	150,000	700,000
AB-110-D	200,000	700,000
AB-110-E (epithienamycin E)	250,000	500,000
Benzylpenicillin	100	200
Cephalosporin C	100,000	100

The stability of the antibiotics was determined microbiologically[20]. Agar plates contained a range of concentrations of penicillinase or cephalosporinase with *Comamonas terrigena* AJ 2083. The concentration of β-lactamase that resulted in 50% inactivation of the antibiotic was determined. Values were expressed as relative stability, taking the stability for benzylpenicillin (penicillinase) or cephalosporin C (cephalosporinase) as 100.

Table 5. β-Lactamase inhibitory activity of AB-110-D.

	I_{50} (ng/ml)	
	Penicillinase from *Bacillus cereus*	Cephalosporinase from *Enterobacter cloacae*
AB-110-A (epithienamycin A)	3.5	6.7
AB-110-B (epithienamycin B)	5.3	4.5
AB-110-C (epithienamycin F)	2.0	0.3
AB-110-D	1.5	0.2
AB-110-E (epithienamycin E)	3.5	3.1

β-Lactamase inhibitory activity was determined by the spectrophotometric method of READING[21]. The concentration of the inhibitor required to cause 50% inhibition of hydrolysis of benzylpenicillin by penicillinase or cephalosporin C by cephalosporinase were obtained when the inhibitor was preincubated with penicillinase or cephalosporinase for 10 minutes at 30°C before substrate addition.

constant ($J_{5,6}$=5.5 Hz) in the ^{1}H NMR spectrum.

From those spectral data mentioned above, the structure of AB-110-D was elucidated to be (*Z*)-3-[[2-(acetylamino)ethenyl]thio]-6-[1-(sulfooxy)ethyl]-7-oxo-1-azabicyclo[3,2,0]hept-2-ene-carboxylic acid as shown in Fig. 1.

AB-110-D exhibits strong antimicrobial activity against Gram-positive and Gram-negative bacteria as shown in Table 3. Also AB-110-D exhibits high stability to penicillinase from *B. cereus* (penicillinase type I, Sigma Chemical Co.) and cephalosporinase from *Enterobacter cloacae* (penicillinase type III, Sigma Chemical Co.), as shown in Table 4. And AB-110-D exhibits powerful β-lactamase inhibitory activity against both penicillinase and cephalosporinase as shown in Table 5.

The compound, AB-110-D, has been synthesized chemically by Beecham's[17] and Takeda's researchers[18,19]. However, there has been no report of production of this compound by any microorganism.

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