ISOLATION AND STRUCTURE OF A β-LACTAMASE INHIBITOR FROM *STREPTOMYCES*

Sir:

It was found in our laboratories that *Streptomyces fulvoviridis* MC696-SY2 produced two β-lactamase inhibitors[1], designated as MC696-SY2-A and B. They strongly inhibit β-lactamases, but are extremely unstable. This communication describes the isolation and structural study of MC696-SY2-A, one of the inhibitors, which allowed us to assign structure **I**.

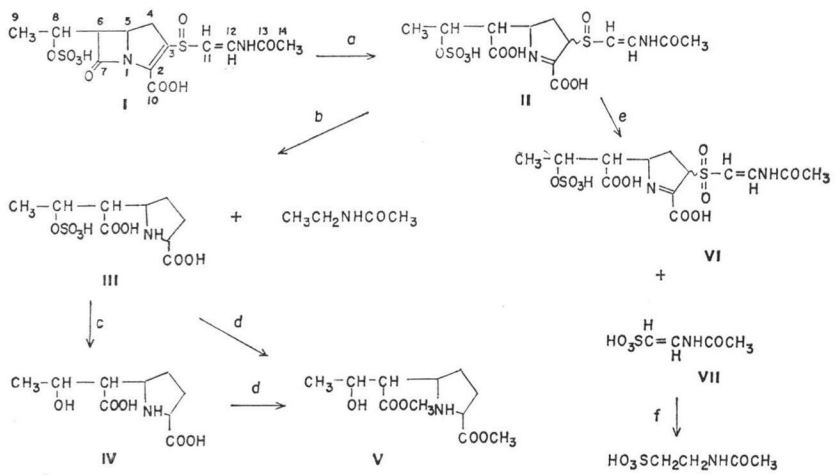
A broth filtrate was treated with Amberlite IRA-401 (SO_4^{--} form) and MC696-SY2-A (**I**) was eluted with 2 M ammonium chloride followed by adsorption on active carbon and elution with 50% aqueous acetone. The ammonium salt was converted to a rather stable barium salt with Amberlite IR-120B (Ba^{++} form), affording brownish powder. The purification of the crude barium salt of **I** was achieved by the combination or repetition of column chromatography on ECTEOLA-cellulose, Diaion HP-20 (a macroreticular resin), Sephadex LH-20, Sephadex G-25, Florisil and silica gel. The sodium salt of **I** was obtained from the purified barium salt by treatment with Dowex 50W-X4 (Na^+ form). It gave an ID_{50} to β-lactamase of *Escherichia coli* K-12 W3630R_{75}^+ of 300 pg.[1] The physical and chemical properties of the most purified barium and sodium salts of **I** are shown in Table 1. Although a tentative molecular formula was obtained for the salts, the content of oxygen remained ambiguously because of the difficulty of complete purification and unsuccessful preparation of the sample for a mass spectrum. However, the presence of O-sulfate or C-sulfonic acid was suggested from the IR spectrum and high-voltage paper electrophoresis.

The barium salt of **I** was easily hydrolyzed to a non-β-lactam compound (**II**) even at room temperature. A partial structure CH_3–CH–CH–CH–CH_2–, an isolated *trans*-olefin and an acetyl group were shown by the ^{1}H-NMR spectrum of the barium salt of **I**. Each peak of the ^{1}H-NMR spectrum of **II** was observed as a doublet, suggesting that **II** is a mixture of diastereomers and a new additional proton appeared at $\delta 4.8 \sim 5.3$ (Table 2). Analysis of ^{13}C-NMR spectra of **I** and **II** revealed that one of the olefinic carbons was transformed to sp^3 carbon (Table 3). This evidence strongly support that the conjugated double bond C_2–C_3 in the five-membered ring of **I** was converted into a N-C double bond as shown in Scheme 1. Hydrogenolysis of **II** with RANEY-Ni afforded compound **III** and N-acetylethyl amine which was identified by GC-MS (*m/e* 87). Compound **III** showed a positive ninhydrin test, suggesting the presence of a proline moiety and was treated with 1 N hydrogen chloride in metha-

Table 1. Properties of MC696-SY2-A

	Barium salt	Sodium salt
Appearance	colorless powder	colorless powder
mp	gradually decomposed over 154°C	gradually decomposed over 148°C
$[\alpha]_D^{27}$	−109° (*c* 0.56, H_2O)	−110° (*c* 0.25, H_2O)
Formula	$C_{13}H_{14}N_2O_9S_2 \cdot Ba \cdot H_2O$	$C_{13}H_{14}N_2O_9S_2 \cdot Na_2 \cdot 3H_2O$
Analysis (%)	calcd. found	calcd. found
C	27.79 27.73	30.83 31.00
H	2.87 3.33	3.98 3.98
N	4.99 4.82	5.53 5.26
S	— —	12.66 12.08
Ba	24.45 23.80	— —
UV in H_2O (nm)	240 (ε 15,200), 280 (ε 12,000)	240 (ε 15,400), 280 (ε 11,100)
IR in KBr (cm^{-1})	3500, 3000, 1770 (β-lactam), 1695, 1625, 1590, 1520, 1405, 1385, 1265, 1230 (S=O), 1070, 1040, 1025 (S=O), 980, 940, 900, 790 (S–O)	3400, 2950, 1765 (β-lactam), 1690, 1620, 1510, 1390, 1260, 1220 (S=O), 1060, 1035, 1020 (S=O), 970, 930, 900, 780 (S–O)
TLC	Silica gel (butanol - methanol - water, 4: 1: 2): Rf 0.46 Cellulose (isopropyl alcohol - water, 7: 3): Rf 0.74	
High-voltage paper electrophoresis	Rm (*p*-toluenesulfonic acid): 0.66 (3,500 V, 15 minutes, formic acid - acetic acid - water, 1: 3: 36)	

Scheme 1.



a. In H_2O at 37°C for 72 hours.
b. Hydrogenolysis with RANEY-Ni in 50% MeOH at 3.5 kg/cm² for 4 hours.
c. Hydrolysis with 0.5 N HCl at 97°C for 4 hours.
d. Reflux in 1 N HCl-MeOH for 20 hours.
e. In CH_3CO_3H at room temperature for 2 hours.
f. Hydrogenation with PtO_2 in 90% MeOH at 3.2 kg/cm².

Table 2. Chemical shifts and coupling constants in ^{1}H-NMR spectra

Proton	δ ppm (*J* Hz)				
	I-Ba	II-Ba	III	IV	VII
9-CH_3	1.98 d (6)	1.94 d (6) 1.97 d (6)	1.92 d (6)	1.74 d (6)	
8-CH	5.38 m (6,8)	4.8~5.3	~5.2	4.47 m (6,10)	
6-CH	4.44 dd (6,8)	3.54	3.66 dd (7,8)	2.97 dd (9,10)	
5-CH	4.96 m (6,9,10)	4.8~5.3	4.52 m (7,8,10)	~4.3	
4-CH_2	3.53 dd (10,18) 3.98 dd (9,18)	2.7~3.2	2.4~2.8	~2.2 ~2.7	
11-CH	8.07 d (14)	7.90 d (14) 8.00 d (14)			7.96 d(14)
12-CH	6.87 d (14)	6.50 d (14) 6.58 d (14)			6.64 d (14)
14-CH_3	2.60 s	2.62 s 2.64 s			2.60 s
3-CH or 3-CH_2		4.8~5.3	~2.8	~2.6	
2-CH			4.94 dd (7,8)	~4.6	

Spectra were measured in D_2O using TMS as the external reference.

nol to afford the hydrochloride of a dimethyl ester (**V**). The mass spectrum of **V** is compatible with the dimethyl ester (*m/e* 246.1323, $M^+ + 1$, calcd. for $C_{11}H_{20}NO_5$: *m/e* 246.1340) of 5-(1-carboxy-2-hydroxypropyl)-proline (**IV**), showing that the substituent at C_8 of **I** is O-sulfate and excluding the possibility of C-sulfonic acid.

The oxidation state of sulfur of the side chain at C_8 was decided as follows: Oxidation of **II** with peracetic acid afforded a sulfone derivative **VI** along with 2-acetamidoethenesulfonic acid (**VII**) which was introduced into N-acetyltaurine by catalytic hydrogenation. The UV spectra of **II** and **VI** showed almost the same characteristic absorption at λ_{max} 250 nm. Treatment of **II** with acetyl chloride and stannous chloride[2] afforded a reduced compound which showed a characteristic absorption for a conjugated sulfide compound, showing a λ_{max} at 230 and 300 nm. On the other hand, one of the isolated *trans*-olefin protons is observed at *ca.* δ 8.0 for **II** and **VI** and at δ 7.57 for the sulfide compound, suggesting that such a shift to a lower field is due to the presence of an electron-attracting group. All the evidence mentioned above combined with the more stable form of the unsaturated sulfoxide

Table 3. Chemical shifts in ^{13}C-NMR spectra

Carbon	Chemical shifts, δ (ppm)			
	I-Ba	II-Ba	III	VII
10	177.7	178.2*	173.2* s	
13	173.5	173.6*		173.7 s
7	166.1	169.4	172.6* s	
3	141.1*	72.3** 70.7**	28.1** t	
2	139.1*	168.8 168.0	60.9***d	
12	135.0	136.0 135.8		131.1 d
11	112.1	110.9 108.0		113.9 d
8	73.7	77.1** 76.8**	76.3 d	
5	59.0	70.7** 70.0**	60.6***d	
6	54.6	59.3 59.1	53.1 d	
4	29.6	25.4 24.7	27.9** t	
14	23.0	23.1		23.1 q
9	19.1	19.5 18.6	19.6 q	

δ: ppm from TMS (internal dioxane, δ=67.4) in D_2O.

*, **, ***: Assignments within any vertical column may be reversed.

of cephalosporin chemistry[3] clearly suggest that the sulfoxide side chain is a more likely structure.

On this bases, the β-lactamase inhibitor named MC696-SY2-A is considered to have structure **I**. Recently, Beecham researchers reported independently that the β-lactam inhibitors MM 4550 and MM 13902 were co-produced by *Streptomyces olivaceus*[4]. MM 4550 was assigned the same structure as **I** in the Patent literature.[5]

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