C-2801X, A NEW CEPHAMYCIN-TYPE ANTIBIOTIC

II. ISOLATION AND CHARACTERIZATION

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Two new species of *Streptomyces, S. heteromorphus* and *S. panayensis,* were found to produce a new antibiotic named C-2801X together with cephamycins A and B. The antibiotics were separated from each other by column chromatography on Amberlite XAD-2 and isolated in pure form as mono-sodium salts. C-2801X mono-sodium salt has a molecular formula $C_{25}H_{23}N_3O_{12}SNa$, and exhibits antibacterial activity against Gram-positive and Gram-negative bacteria including those insensitive to cephamycins A and B. From its physicochemical and biological properties, C-2801X was considered to be a new cephamycin-type antibiotic.

In the separate paper,[1] HASEGAWA *et al.* reported on the microbiological properties of two new species of *Streptomyces*, *S. heteromorphus* and *S. panayensis*, and their fermentation for production of antibiotics.

Further investigations were made on isolation and characterizations of the antibiotics, and it was shown that both *Streptomyces* produce a new cephamycin-type antibiotic which was named C-2801X together with cephamycins A and B.[2,3] C-2801X was obtained as a mono-sodium salt by purification using column chromatography after separation from cephamycins A and B.

The present paper deals with the isolation, physicochemical and biological characterization of C-2801X.

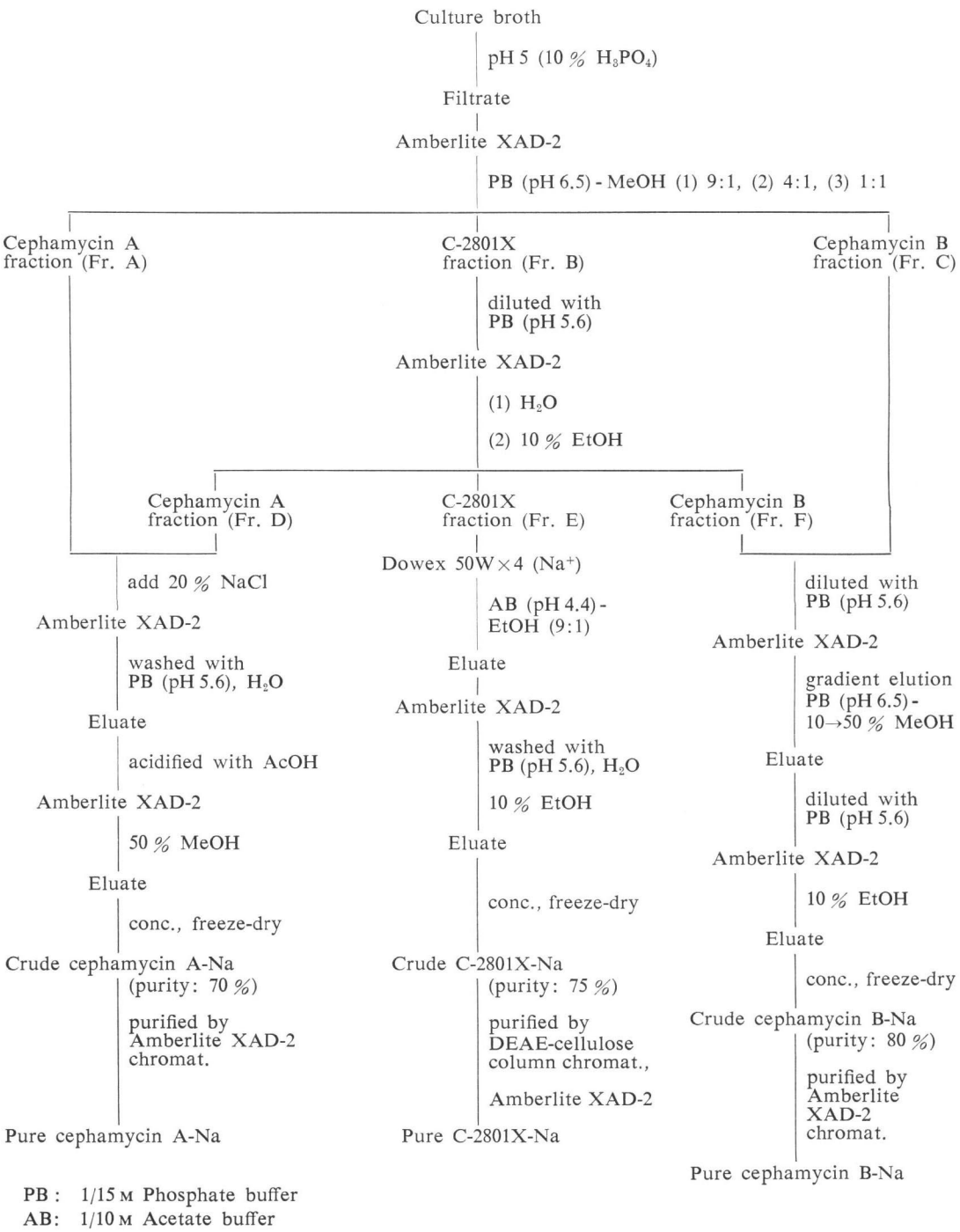
Isolation

According to the isolation method outlined in Chart 1, crude component antibiotics, *i.e.* C-2801X (75 % pure), cephamycin A (70 % pure) and cephamycin B (80 % pure), were obtained in the form of mono-sodium salts. The culture broth was acidified at harvest to obtain maximal stability of the antibiotics. The broth was filtered and the filtrate containing C-2801X, cephamycins A and B was passed through a column of Amberlite XAD-2. When a stepwise elution was performed using successive 9:1, 4:1 and 1:1 mixtures of 1/15 M phosphate buffer (pH 6.5) and methanol, cephamycin A, C-2801X and cephamycin B were eluted in the order mentioned. The fraction containing C-2801X was still contaminated with a small amount of cephamycins A and B; it was again passed through Amberlite XAD-2 after dilution with acidic phosphate buffer (pH 5.6). Complete separation of C-2801X from cephamycins A and B was achieved by elution first with water and then with 10 % aqueous ethanol. Purification by Dowex 50 column chromatography, followed by desalting using an Amberlite XAD-2 column afforded a crude powder (75 % pure) of C-2801X monosodium salt.

Final purification of C-2801X was effected by either diethylaminoethyl (DEAE)-cellulose

chromatography using 1/10 M acetate buffer (pH 4.4) containing 1/10 M sodium chloride as a developer or by cellulose column chromatography using the upper phase of *n*-butanol - acetic acid - water (4:1:5) as a developer. The fractions containing C-2801X obtained by these chromato-

Chart 1. Isolation of C-2801X, cephamycins A and B



PB : 1/15 M Phosphate buffer
AB: 1/10 M Acetate buffer

graphy steps were subjected to Amberlite XAD-2 chromatography for desalting purpose to give a colorless powder of pure C-2801X mono-sodium salt.

In order to obtain pure cephamycin A or B mono-sodium salt, Amberlite XAD-2 chromatography was applied effectively using 3 % aqueous methanol as developer for the former and 7 % aqueous ethanol for the latter.

Physicochemical Properties

C-2801X mono-sodium salt is a colorless powder with a molecular formula $C_{25}H_{28}N_3O_{12}SNa$ estimated from elemental analysis and the amount of α-aminoadipic acid by amino acid analysis. Table 1 shows the comparison of physicochemical properties of C-2801X mono-sodium salt with those of cephamycins A and B isolated together. C-2801X is unstable in a strongly acidic (pH<2)

Table 1. Physicochemical properties of mono-sodium salt of C-2801X, cephamycins A and B.

	C-2801X		Cephamycin A		Cephamycin B	
Appearance	White powder		White powder		White powder	
Elemental analysis	Found	Calcd	Found	Calcd	Found	Calcd
C	48.46	48.62	44.08	44.06	49.66	49.91
H	4.85	4.57	4.53	4.14	5.03	4.69
N	6.98	6.79	6.20	6.16	7.09	6.98
S	5.13	5.17	9.67	9.41	5.31	5.33
Na	3.66	3.72	3.21	3.37	3.73	3.82
Molecular formula	$C_{25}H_{28}N_3O_{12}SNa$		$C_{25}H_{28}N_3O_{14}S_2Na$		$C_{25}H_{28}N_3O_{11}SNa$	
Optical rotation $[\alpha]_D^{25}$ (c 0.5, H_2O)	+124.4°		+141.4°		+137.0°	
UV absorption	Fig. 4					
$\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	234 (208*a))		285 (430)		226 (300)	
	318 (274*b))				305 (520)	
$\lambda_{sh}^{H_2O}$ nm ($E_{1cm}^{1\%}$)	295 (255*c))		220 (223)			
Color reaction						
Ninhydrin	+		+		+	
$FeCl_3$-$K_3Fe(CN)_6$*d)	+		−		+	
Paper chromatography (Whatman No. 1) Rf						
n-BuOH-AcOH-H_2O (4:1:5)	0.12		0.05		0.30	
n-BuOH-AcOH-H_2O (2:1:1)	0.42		0.30		0.58	
70 % Isopropanol	0.33		0.29		0.42	
Amino acid analysis*e) (μmoles/mg)						
α-Aminoadipic acid	1.61		1.30		1.65	
Glycine	0.84		0.68		0.88	

*a) ε=12,800
*b) ε=16,900
*c) ε=15,700
*d) Sprayed with a mixture of equal volume of 1 % $FeCl_3$ and 1 % $K_3Fe(CN)_6$
*e) Hydrolized with 6 N HCl at 110°C for 16 hours

or alkaline (pH>8) solution in analogy with cephamycins A and B. It is soluble in water, methanol and ethanol, but insoluble in other organic solvents.

A characteristic absorption at 1760~1780 cm^{-1} due to β-lactam is observed in common in the IR spectra (Figs. 1, 2, 3) of the three antibiotics. UV absoption spectrum (Fig. 4) is obviously

Fig. 1. IR spectrum of C-2801X mono-sodium salt (KBr disk).

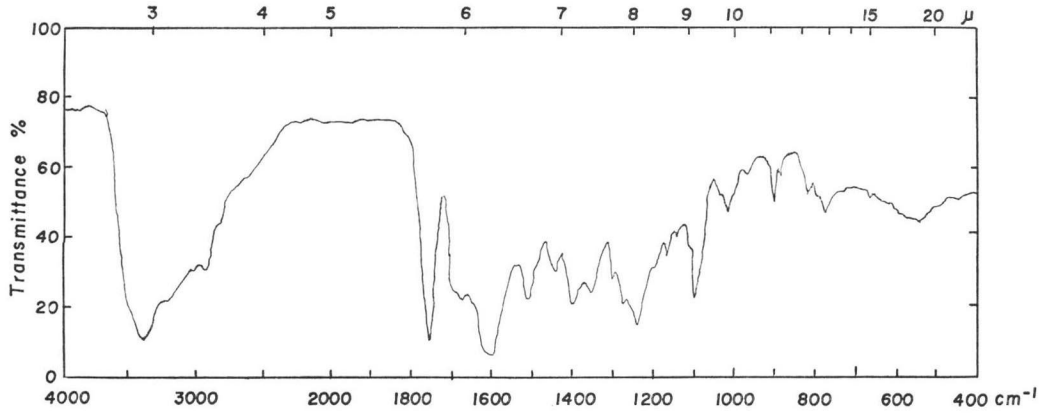


Fig. 2. IR spectrum of cephamycin A mono-sodium salt (KBr disk).

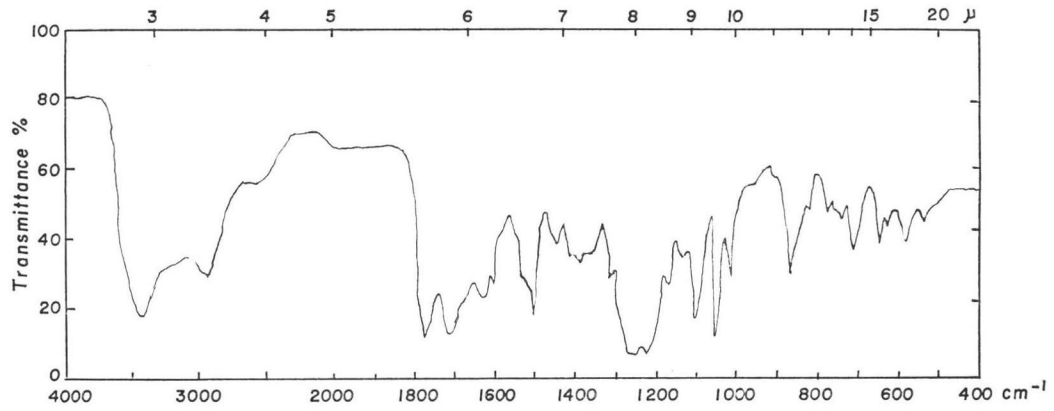
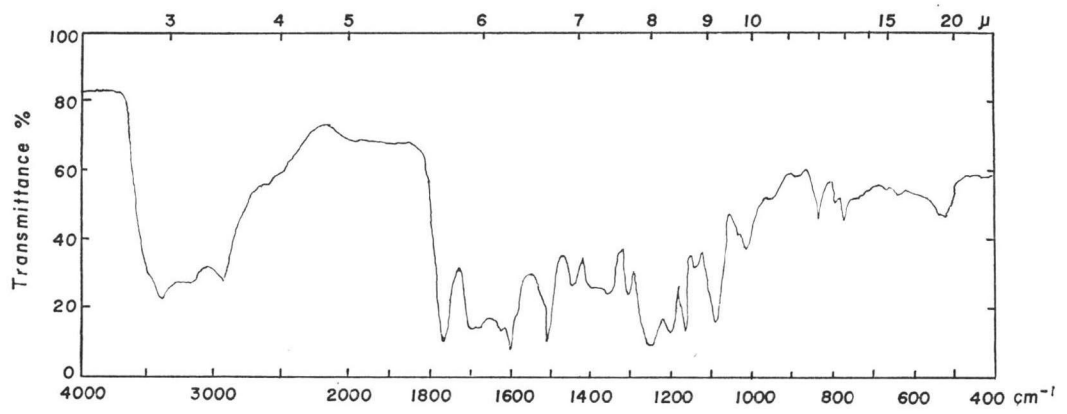


Fig. 3. IR spectrum of cephamycin B mono-sodium salt (KBr disk).



different from those of cephamycins A and B. Positive reactions with ninhydrin and $FeCl_3$-$K_3Fe(CN)_6$ reagent[4] of C-2801X indicate a structural resemblance to cephamycin B having a phenolic hydroxyl group. C-2801X has an Rf slower than cephamycin B, but faster than cephamycin A when chromatographed on Whatman No. 1 developed with *n*-butanol - acetic acid - water or 70 % isopropanol (Table 1). Therefore C-2801X was postulated to be more polar than cephamycin B, but less polar than cephamycin A.

By amino acid analysis (Table 1) of the hydrolyzate obtained by heating with 6 N hydrochloric acid, α-aminoadipic acid and glycine were determined to be 2:1 in molar ratio in all cases of the three antibiotics. This phenomenon is known to be characteristic of 7-methoxycephem antibiotics.[5]

NMR spectrum (100 MHz, in D_2O) of C-2801X mono-sodium salt also indicates the presence of 7-methoxycephem skeleton by signals (δ, ppm) at 3.34, 3.72, 2H, dd. J=18 Hz; 5.21, 1H, s; 3.60, 3H, s.

As reported in detail,[6] the chemical structure of C-2801X has recently been elucidated as shown in Fig. 5.

Fig. 4. UV spectra of mono-sodium salts of C-2801X, cephamycins A and B in aqueous solution.

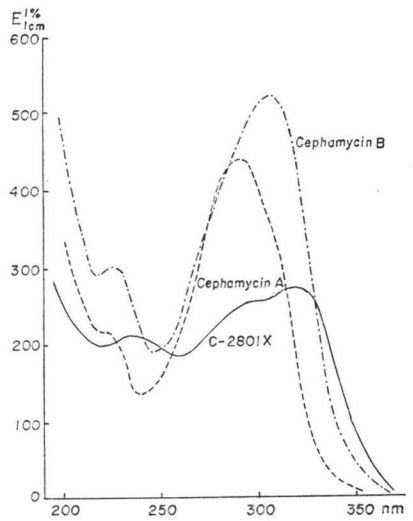
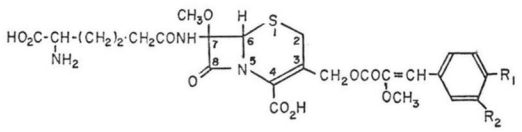


Fig. 5. Structures of C-2801X, cephamycins A and B.



C-2801X $R_1 = R_2 = OH$
Cephamycin A $R_1 = OSO_3H$ $R_2 = H$
Cephamycin B $R_1 = OH$ $R_2 = H$

Biological Properties

The antibacterial spectrum of C-2801X determined by the conventional serial agar dilution method was shown in Table 2 in comparison with those of cephamycins A and B. Among the three antibiotics, C-2801X bearing two phenolic hydroxyl groups shows the strongest activity against Gram-negative bacteria, although their activities against Gram-positive bacteria are almost indistinguishable.

No toxicity in mice has been observed up to 400 mg/kg of C-2801X mono-sodium salt when administered intravenously.

The therapeutic activity (ED_{50}) of C-2801X mono-sodium salt on the experimental infection in mice caused by *Escherichia coli* was about 30 mg/kg by subcutaneous injection, while those of cephamycins A and B are more than 100 mg/kg.

From these physicochemical and biological properties, C-2801X was considered to be a new cephamycin-type antibiotic differing from the reported 7-methoxycephem antibiotics.[2,7~9]

Experimental

Isolation: The culture broth (93 liters) was adjusted to pH 5 with 10 % phosphoric acid

Table 2. Antimicrobial activities of mono-sodium salts of C-2801X, cephamycins A and B.
Medium: Trypticase Soy Agar (BBL), pH 6.5, 37°C

Test organisms	MIC (mcg/ml)		
	C-2801X	Cephamycin A	Cephamycin B
Staphylococcus aureus 209P	50	50	50
Staphylococcus aureus 209P (chloramphenicol, streptomycin, tetracycline and erythromycin-resistant)	50	50	25
Staphylococcus aureus No. 87	50	50	50
Bacillus subtilis PCI 219	12.5	25	25
Bacillus pumilus IFO 3813	50	100	25
Escherichia coli NIHJ	50	>100	>100
Escherichia coli (clinical isolate, antibiotic-susceptible strain)	25	>100	>100
Escherichia coli (clinical isolate*)	25	>100	>100
Escherichia coli (clinical isolate**)	25	>100	>100
Proteus vulgaris IFO 3988	12.5	6.25	50
Proteus mirabilis IFO 3849	3.13	6.25	50
Proteus morganii IFO 3848	25	50	50
Klebsiella pneumoniae IFO 3512	6.25	100	25
Pseudomonas aeruginosa IFO 3080	>100	>100	>100
Pseudomonas aeruginosa NCTC 10490	>100	>100	>100
Salmonella typhimurium IFO 12529	6.25	>100	>100
Salmonella enteritidis IFO 3313	6.25	>100	>100
Aerobacter cloacae IFO 12009	25	>100	>100
Alcaligenes faecalis IFO 13111	12.5	25	50

* The strain is resistant to tetracycline, streptomycin, kanamycin, lividomycin, aminobenzylpenicillin and sulbenicillin.
** The strain is resistant to sulfonamide, streptomycin and sulbenicillin.

and filtered with the aid of Hyflo-Super Cel. The filtrate (80 liters) was passed through an 8-liter bed of Amberlite XAD-2 at a flow rate of 16 liters per hour. The resin bed was washed with 16 liters of 1/15 M phosphate buffer (pH 6.5) and then eluted with a 9:1 mixture (16 liters), a 4:1 mixture (16 liters) and a 1:1 mixture (16 liters) of 1/15 M phosphate buffer (pH 6.5) and methanol, successively. After 6 liters of the forerun, 14-liter fractions (Fr. A) of the eluate contained cephamycin A. Next, 20-liter fractions (Fr. B) were found to contain C-2801X together with a small amount of cephamycins A and B. Cephamycin B was found in the last 4-liter eluate (Fr. C).

Fr. B (20 liters), containing C-2801X as a main component, was diluted with 60 liters of 1/15 M phosphate buffer (pH 5.6) and then passed through a 2-liter column of Amberlite XAD-2 at a flow rate of 4 liters per hour. After being washed with 1/15 M phosphate buffer (pH 5.6) (1 liter), the column was eluted first with water (2 liters) and then with 10% aqueous ethanol (4 liters). The eluate was collected in 500 ml fractions to obtain fractions 1 to 12. When analyzed by ascending paper chromatography (Whatman No. 1) using an *n*-butanol-acetic acid-water (2:1:1) system, fractions 2 through 5 (Fr. D) were found to contain cephamycin A, fractions 6 through 10 (Fr. E) to contain C-2801X and fractions 11 and 12 (Fr. F) to contain cephamycin B.

Fr. E (2.5 liters), containing C-2801X, was passed through a column (3×55 cm) of Dowex

50W×4 (100~200 mesh, 250 ml) equilibrated with 1/10 M sodium acetate buffer (pH 4.4) at a flow rate of 250 ml per hour. The resin was washed with 10 % aqueous ethanol, and then eluted with a solvent system of 1/10 M acetate buffer (pH 4.4) - ethanol (9:1). The active fractions were collected and charged to a column (3×45 cm) of Amberlite XAD-2 (200 ml) to adsorb C-2801X. The resin was washed with 1/15 M phosphate buffer (pH 5.6) until the pH of the effluent was 5.6 and then washed with water. Elution was carried out with 10 % aqueous ethanol and the fractions (100 ml) showing antibacterial activity were concentrated under reduced pressure at a temperature below 15°C to remove ethanol. The resulting aqueous solution was freeze-dried to give a crude powder (75 % pure) of C-2801X mono-sodium salt (1.2 g).

Purification of C-2801X: (i) A 500-mg portion of the crude powder of C-2801X mono-sodium salt was dissolved in 3 ml of a pH 4.4 solution of 1/10 M sodium acetate buffer containing 1/10 M sodium chloride and applied to a column (2.2×55 cm) of DEAE-cellulose on the chloride cycle. The column was developed with the same buffer, and the active fractions were collected and adsorbed on a column (2×20 cm) of Amberlite XAD-2. The column was washed with 1/15 M posphate buffer (pH 5.6) until the pH of the effluent was 5.6, and then washed with 100 ml of water. Elution was performed with 5 % aqueous ethanol, and the fractions showing positive reactions with ninhydrin and $FeCl_3$-$K_3Fe(CN)_6$ reagent[4] were combined, concentrated under reduced pressure and freeze-dried to afford a colorless powder of pure C-2801X mono-sodium salt (275 mg). (ii) Another 500-mg portion of the crude powder of C-2801X mono-sodium salt obtained above was mixed well with 5 g of cellulose powder (Tōyō Kagaku Co.) and the mixture was placed on the top of a column (3×45 cm) of cellulose powder packed previously with the upper phase of the system *n*-butanol - acetic acid - water (4:1:5). A small amount of the solvent was added and, after stirring with caution so that the column top would not be disturbed, the column was developed with the same solvent. The eluate fractions showing positive reactions with ninhydrin and $FeCl_3$-$K_3Fe(CN)_6$ reagent[4] were collected and the butanol was removed by evaporation *in vacuo*. The resulting aqueous solution was subjected to an Amberlite XAD-2 column chromatography in a similar manner as described in (i) to give a colorless powder of pure C-2801X mono-sodium salt (200 mg).

Purification of Cephamycin A: Cephamycin A-containing fractions (Fr. A and D, 12 liters) described above were combined and, after addition of equal volume of 20 % sodium chloride solution, the mixture was passed through a 3-liter bed of Amberlite XAD-2. The resin was washed with 6 liters of 1/15 M phosphate buffer (pH 5.6) and then eluted with water. The active fractions obtained (3 liters) was acidified with 10 ml of acetic acid and was rechromatographed on 300 ml of Amberlite XAD-2. The resin was washed with 600 ml of water and then eluted with 50 % aqueous methanol. The eluate (200 ml) was concentrated *in vacuo* to remove the methanol and freeze-dried to yield a crude powder (70 % pure) of cephamycin A mono-sodium salt (10.8 g). A 5-g portion of the crude powder was dissolved in 20 ml of 3 % aqueous methanol and applied to a column (6.5×60 cm) of Amberlite XAD-2 packed previously with 3 % aqueous methanol. The column was develoved with the same solvent, and ninhydrin-positive fractions were collected, concentrated *in vacuo*, and freeze-dried to yield 2.5 g of pure cephamycin A mono-sodium salt.

Purification of Cephamycin B: Cephamycin B-containing fractions (Fr. C and F, 5 liters) described above were combined and, after addition of 15 liters of 1/15 M phosphate buffer (pH 5.6), the mixture was passed through a 1-liter bed (4.5×50 cm) of Amberlite XAD-2 at a flow rate of 2 liters per hour. Separation of the active substances was effectively achieved by gradient elution using a mixture of methanol and 1/15 M phosphate buffer (pH 6.5) changing from 10 to 50 % in the methanolic concentration. Fractions containing cephamycin B were collected, and, after dilution to four-fold volume with 1/15 M phosphate buffer (pH 5.6), the mixture was rechromatographed on a 500 ml column of Amberlite XAD-2. The resin was washed with water and eluted with 10 % aqueous ethanol. The eluate was concentrated *in vacuo* and freeze-dried to yield a crude powder (80 % pure) of cephamycin B mono-sodium salt (3.5 g). A 1-g portion

of the crude powder was dissolved in 5 ml of 7 % aqueous ethanol and applied to a column (2.1×60 cm) of Amberlite XAD-2 packed previously with 7 % aqueous ethanol. The column was developed with the same solvent, and ninhydrin- and $FeCl_3$-$K_3Fe(CN)_6$-positive fractions were collected, concentrated *in vacuo* and freeze-dried to afford 480 mg of pure cephamycin B mono-sodium salt.

Hydrolysis of the Antibiotics: A 5-mg portion of C-2801X, cephamycin A or cephamycin B was suspended in 0.3 ml of 6 N hydrochloric acid, sealed in a small glass tube, and placed in an oven at 110°C for 16 hours. The solution was transferred to a round-bottom flask with water and evaporated several times from water to remove excess hydrochloric acid. The residue was dried in the desiccator containing sodium hydroxide pellets and subjected to amino acid analysis. The results are shown in Table 1.

In Vivo Protection Test: Four week old male mice weighing 18~22 g were infected intraperitoneally with 316 times the number of *Escherichia coli* O-111 that should kill 50 % of the infected, nontreated animals (LD_{50}). Each test compound was injected intravenously immediately after infection. Five mice were used at each of the four-fold drug dilutions test. Seven days after infection, the test was considered complete, and survival records of that day were used to calculate ED_{50}, *i.e.*, the amount of compound that should protect 50 % of the infected, treated animals.

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