Structure of C-2801X, a New Cephamycin-type Antibiotic

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The C-3 side chain moiety of C-2801X, a new cephamycin-type antibiotic, was almost quantitatively obtained by Dowex 50-catalyzed hydrolysis, and its structure was established as 3,4-dihydroxy-α-methoxycinnamic acid (I) by spectroscopic analyses and synthesis. The full structure of C-2801X was also assumed to be XIII by NMR analysis.

In the course of screening for new antibiotics, two new species of *Streptomyces*, *S. heteromorphus* and *S. panayensis*, were found to produce a new cephamycin-type antibiotic C-2801X[1] together with cephamycins A and B.[2,3]

As described in detail in the paper[1] on characterization, C-2801X (Na salt: $C_{25}H_{28}N_3O_{12}SNa$) was regarded as a 7-(5-amino-5-carboxyvaleramido)-7-methoxycephem (cephamycin-type) antibiotic from the following facts: (1) C-2801X was inactivated by cephalosporinase; (2) it exhibits an IR absorption at 1760 cm^{-1} due to β-lactam, as shown in Fig. 1; (3) a signal corresponding to C-7 methoxyl group was observed in the NMR spectrum; and (4) glycine and α-aminoadipic acid in the hydrolyzate of C-2801X were determined to be 1:2 in molar ratio, as reported in cephamycin-type antibiotics.[3,4] The IR (Fig. 1) and UV [$\lambda_{max}^{H_2O}$ 234 nm (ε 12800), 318 (16900), $\lambda_{sh}^{H_2O}$ 295(15700)] spectra and paper chromatography indicated that C-2801X is a new antibiotic differing from known cephem antibiotics.

This paper describes first the elucidation of structure of the C-3 side chain moiety, and then presents evidence for the full structure for C-2801X.

Structure of C-3 Side Chain Moiety

C-2801X was almost quantitatively hydrolyzed at the C-3 side chain by treatment with Dowex 50 W×8 in methanolic solution. The hydrolysis products having amino groups were separated by adsorption on Dowex 50, and the methanolic solution containing a neutral or acidic substance was concentrated *in vacuo* to give I. The UV absorption (Table 2) was similar to that of C-2801X. Therefore, I was assumed to be the C-3 side chain moiety. The IR spectrum (Fig. 2) shows the presence of hydroxyl (3450, 1190 cm^{-1}), α,β-unsaturated carboxylic acid (1660, 1630) and aromatic ring (1605, 1510, 905). A phenolic hydroxyl group is probably present, as judged by the IR absorption and a positive color reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent.[5] The NMR spectrum in CD_3OD shows the signals of 1,2,4-*tri*-substituted benzene (δ, ppm: 7.35, 1H, d, J=1.5 Hz; 7.03, 1H, dd, J=1.5, 8 Hz; 6.75, 1H, d, J=8 Hz), a single vinylic proton (6.90, 1H, s), and methoxyl protons (3.70, 3H, s). These spectroscopic analyses suggest that I is a cinnamic acid derivative having two hydroxyl (at least one hydroxyl group is phenolic) and one methoxyl group.

Mass spectral fragmentations of I are shown in Table 1 and compared with those of *p*-hydroxy-α-methoxycinnamic acid (II) obtained from cephamycin B by hydrolysis in a similar manner. Peaks corresponding to M^+, M−15, M−32, M−43, M−60, M−87, M−88, and M−89 were observed in both mass spectra of I and II at a difference of *m/e* 16. As the peaks at *m/e* 107, 106, and 105 of II are due to fragments IIIa, IVa, and Va, the corresponding peaks at *m/e* 123, 122, and 121 of I were assigned to IIIb, IVb, and Vb, respectively. This fact indicates that I has two phenolic hydroxyl groups and that the methoxyl group is located at the α-position. A characteristic peak at M−33, observed

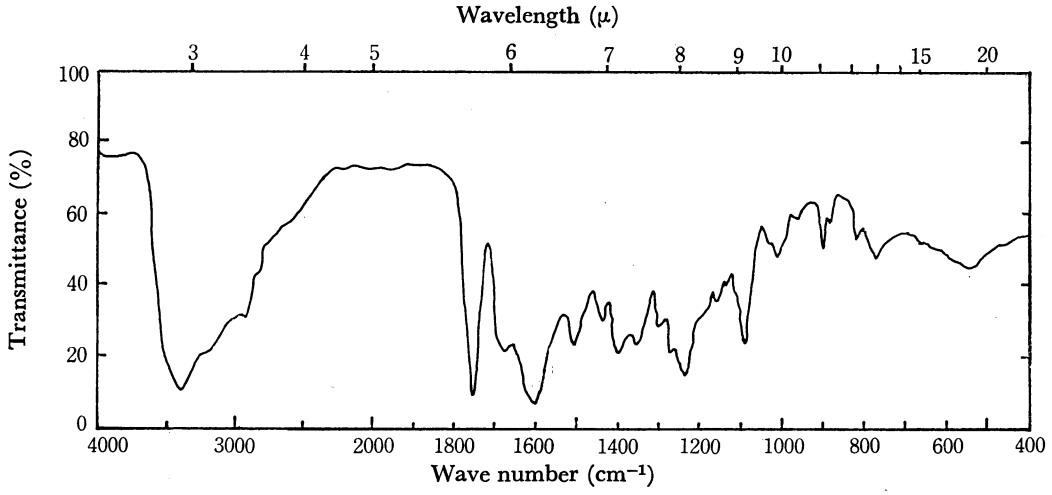


Fig. 1. IR spectrum of C-2801X mono-Na salt (KBr).

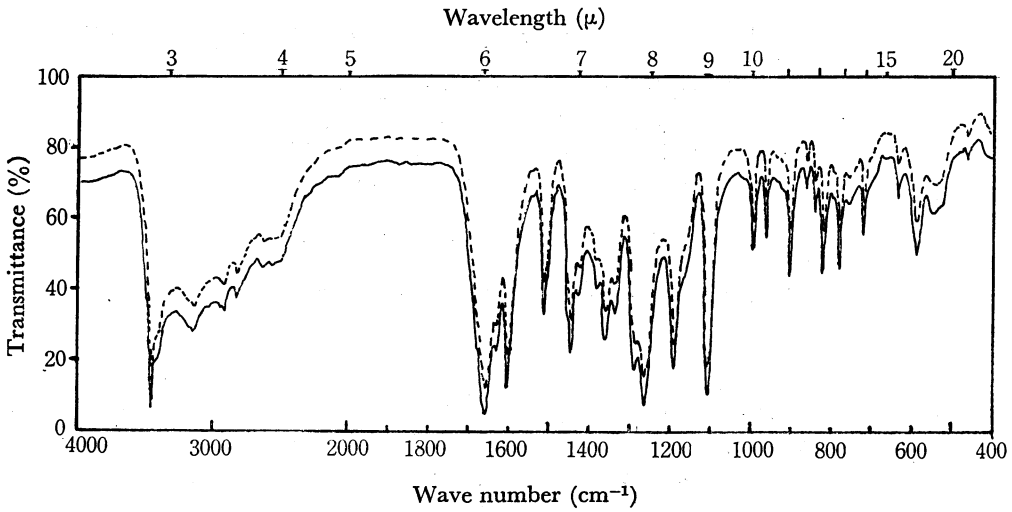


Fig. 2. IR spectra of natural and synthetic 3,4-dihydroxy-α-methoxycinnamic acid (I) (KBr).
—: Natural, ---: synthetic.

TABLE 1. MASS SPECTRAL FRAGMENTATIONS OF I AND *p*-HYDROXY-α-METHOXYCINNAMIC ACID (II)

Fragments	I	II
M^+	210 *m/e*	194 *m/e*
M−15 (CH_3)	195	179
M−32 (OCH_3+H)	178	162
M−33 (OCH_3+2H)	177	—
M−43 (CH_3+CO)	167	151
M−60 (CO_2H+CH_3)	150	134
M−87	123	107
M−88	122	106
M−89	121	105

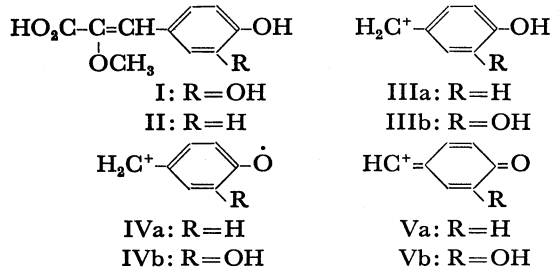
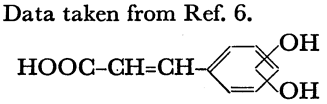


TABLE 2. COMPARISON OF UV ABSORPTION MAXIMA OF I WITH THOSE OF DIHYDROXYCINNAMIC ACIDS[a] (VI)

Dihydroxycinnamic acid	λ_{max}^{MeOH} nm	$\lambda_{max}^{MeOH-NaOH}$ nm
3, 4-	230, 290, 320	240, 305, 345
2, 4-	225, 280	
2, 5-	248, 258	
3, 5-	225, 279	
2, 6-	227, 290	
2, 3-	225, 279	
I	218, 294, 318	250sh, 300, 335

a) Data taken from Ref. 6.



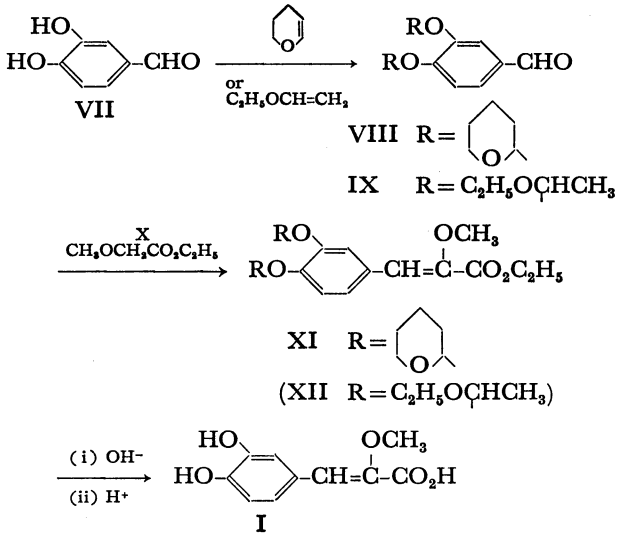
VI

only in the spectrum of I, is consistent with the structure having two hydroxyl groups.

To confirm the positions of the two hydroxyl groups on the benzene nucleus, UV absorption maxima[6] of six isomers of dihydroxycinnamic acid (VI) were compared with that of I (Table 2). The absorption maxima of I are obviously similar to that of 3,4-dihydroxycinnamic acid (caffeic acid).

From these data the chemical structure of I was assumed to be 3,4-dihydroxy-α-methoxycinnamic acid (I).

Since I is a hitherto unreported compound, 3,4-dihydroxy-α-methoxycinnamic acid was synthesized from protocatechualdehyde (VII) and ethyl methoxyacetate (X) as shown in the Scheme. As I is considerably labile in an alkaline solution, the two hydroxyl groups of VII were protectedby converting into hemiacetals which revert easily to hydroxyl by mild acidic hydrolysis. The addition reaction of dihydropyran[7] and ethyl vinyl



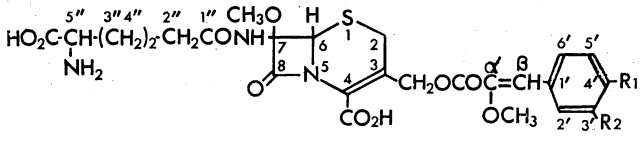
Scheme. Synthesis of 3,4-dihydroxy-α-methoxycinnamic acid.

ether[8] onto VII in the presence of phosphoric acid yielded VIII and IX, respectively. Condensation of VIII and IX with ethyl methoxyacetate (X) was carried out in toluene in the presence of metallic sodium to give XI and XII (not isolated), respectively.

Saponification of ester XI or XII and subsequent acid hydrolysis of the acetals gave 3,4-dihydroxy-α-methoxycinnamic acid (I). NMR, Mass, UV, and IR spectra (Fig. 2) of the synthetic compound were identical with those of the natural specimen (I).

Full Structure of C-2801X

In the Dowex 50-catalyzed hydrolysis described above, compounds adsorbed on the resin were eluted with 1 M sodium acetate. The investigation by paper chromatography of the eluate revealed that it contains α-aminoadipic acid and 7-methoxycephem moieties showing the same R_f values as those of hydrolysis products obtained by the same treatment of cephamycin B.



C-2801-X (XIII) $R_1 = R_2 = OH$

cephamycin A $R_1 = OSO_3H$ $R_2 = H$

cephamycin B $R_1 = OH$ $R_2 = H$

Table 3. NMR assignments of monosodium salts of C-2801X, cephamycin A and B
NMR (in D_2O) δ, ppm Mono–Na salt

Proton	C-2801X	Cephamycin A	Cephamycin B
2-C**H**$_2$	3.72, 3.34 (dd, J=18 Hz)	3.74, 3.35 (dd, J=18)	3.64, 3.31 (dd, J=18)
6-C**H**	5.21 (s)	5.15 (s)	5.10 (s)
7-OC**H**$_3$	3.60 (s)[a]	3.57 (s)[a]	3.59 (s)[a]
α-OC**H**$_3$	3.70 (s)[a]	3.65 (s)[a]	3.62 (s)[a]
β-C**H**	6.90 (s)	6.92 (s)	6.83 (s)
2′-C**H**	7.35 (d, 1.5)	7.68 (d, 8)[a]	7.52 (d, 8)[a]
3′-C**H**	—	7.32 (d, 8)[a]	6.85 (d, 8)[a]
5′-C**H**	6.90 (d, 8)	7.32 (d, 8)[a]	6.85 (d, 8)[a]
6′-C**H**	7.15 (dd, 1.5, 8)	7.68 (d, 8)[a]	7.52 (d, 8)[a]
2″-C**H**$_2$	2.4—2.7 (m)	2.4—2.7 (m)	2.4—2.7 (m)
3″,4″-(C**H**$_2$)$_2$	1.7—2.2 (m)	1.6—2.2 (m)	1.7—2.3 (m)
5″-C**H**	4.0—4.2 (m)	4.0—4.2 (m)	4.0—4.3 (m)

a) Tentative assignment. The figures in parentheses are coupling constants. s=singlet, d=doublet, dd=double doublet, m=multiplet

The NMR assignments of C-2801X are shown in Table 3, for comparison with those of cephamycin A and B. The chemical shifts, multiplicity, and coupling constants of each proton of C-2801X except the aromatic protons of the C-3 side chain were in fair agreement with those of cephamycin A and cephamycin B; *i.e.* 7-OC**H**$_3$, 6-C**H**, and 2-C**H**$_2$ of methoxycephem nucleus, α-OC**H**$_3$ of the C-3 side chain, and 2″-C**H**$_2$, 3″,4″-(C**H**$_2$)$_2$, and 5″-C**H** of the C-7 aminoadipoyl moiety. The chemical shifts of the aromatic protons of the C-3 side chain were assigned as shown in Table 3, in comparison with the NMR of I. The C-3′ of C-2801X has no proton, so the 2′-benzene proton does not show any *ortho*-coupling in J=8 Hz, but has a *meta*-coupling with the 6′-benzene proton in J=1.5 Hz. As the chemical shifts of the 2′-C**H**, 5′-C**H**, and 6′-C**H** are closer to those of cephamycin B than to those of cephamycin A, the two phenolic hydroxyl groups were supposed to be free. UV absorption and phenolic color reaction[5] of C-2801X also support this structure. Consequently, C-2801X was assumed to have the chemical structure shown as XIII.

Among C-2801X, cephamycin A, and cephamycin B, C-2801X (having two phenolic hydroxyl groups) shows the strongest activity against gram-negative bacteria, although their activities against gram-positive bacteria are almost indistinguishable.[1] This phenomenon is very interesting from the viewpoint of the structure-activity relationship.

Experimental

Hydrolysis of C-2801X. In a suspension of Dowex 50W×8 (H^+) (1 ml) in methanol (3 ml), the sodium salt (3 mg) of C-2801X was dissolved and the mixture was stirred at room temperature for 3 h. After filtration, the resin was washed with methanol (10 ml). The filtrate and the washings were combined and concentrated *in vacuo* to dryness. Addition of benzene to the residue, followed by standing at 10 °C overnight, afforded colorless prisms (1.1 mg) of I, mp 215 °C (dec.).

Found: C, 57.22; H, 4.67%; (M^+) *m/e* 210. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80%; mol wt, 210.18.

To Dowex resin separated by filtration was added 1M sodium acetate solution (5 ml), and the mixture was stirred for 30 min. The mixture was poured into a glass column (0.9×10 cm) and additional 1M sodium acetate solution (3 ml) was passed over the resin. The resulting eluates were combined and concentrated to *ca.* 1 ml. Ascending paper chromatography of the concentrate was carried out using 1-butanol–acetic acid–water (4: 1: 5) as a developing solvent. By spraying ninhydrin reagent, spots corresponding to α-aminoadipic acid and 7-methoxycephem moieties were observed, showing the same R_f values as those of the hydrolysis products of cephamycin B.

3,4-Bis(tetrahydropyranyloxy)benzaldehyde (VIII). A few drops of 80% phosphoric acid was added to a suspension of VII (2.7 g) in dihydropyran (10 ml) and the mixture was stirred at room temperature until in solution. After stirring at room temperature for an additional 3 h, the reaction mixture was poured into ice water (500 ml). The oily substance which separated out was extracted with ether, and after washing with 5% sodium hydrogencarbonate solution, the ether extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to dryness. The residue was dissolved in ethyl acetate–benzene (1: 1) (10 ml) and charged to a column containing Merck's silica gel (250 ml). The column was developed with ethyl acetate–benzene (1: 1) and the eluate was evaporated *in vacuo* to give VIII (4.3 g) as an oil (yield: (72%).

Found: C, 66.79; H, 7.28%. Calcd for $C_{17}H_{22}O_5$: C, 66.65; H, 7.24%.

3,4-Bis(α-ethoxyethyloxy)benzaldehyde (IX). A few drops of 80% phosphoric acid was added with stirring and cooling

below 5 °C to a suspension of VII (2.7 g) in ethyl vinyl ether (10 ml). The suspension was stirred below 10 °C overnight and the resulting clear reaction mixture was poured into ice water (500 ml). The mixture was extracted with ether and the extracts, after washing with 5% sodium hydrogencarbonate solution, were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The oily residue was purified by vacuum distillation, bp 134—137 °C/1 mmHg, to give IX (3.0 g) as a colorless oil (yield: 43%).

Found: C, 63.71; H, 7.86%. Calcd for $C_{15}H_{22}O_5$: C, 63.81; H, 7.85%.

Ethyl 3,4-Bis(tetrahydropyranyloxy)-α-methoxycinnamate (XI). To a mixture of metallic sodium (2.5 g), anhydrous toluene (50 ml), and VIII (3.0 g), anhydrous ethanol (0.1 ml) was added with stirring. Under cooling with ice and stirring, X (3.6 g) was added dropwise to the mixture. The condensation reaction was performed by stirring the whole mixture under ice-cooling for 2 h and then at room temperature for an additional 6 h. The reaction mixture was poured into ice water (200 ml) and the product was extracted with benzene. After washing with 0.1 M hydrochloric acid and 5% sodium hydrogencarbonate solution, the benzene extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to dryness. The residue was purified by column chromatography using Merck's silica gel (250 ml) as adsorbent and ethyl acetate–benzene (1: 9) as developer to obtain XI (1.8 g) as a colorless oil (yield: 43%).

Found: C, 64.94; H, 7.57%. Calcd for $C_{22}H_{30}O_7$: C, 65.01; H, 7.44%.

3,4-Dihydroxy-α-methoxycinnamic Acid (I). (i) Aqueous 1 M sodium hydroxide solution (50 ml) was added to a solution of XI (1.0 g) in methanol (50 ml) and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated (*ca.* 20 ml) under reduced pressure. After acidification of the concentrate with 1 M hydrochloric acid, the resulting oil was extracted with ethyl acetate, and the extracts were concentrated *in vacuo*. The residue obtained was dissolved in methanol (20 ml) and, after addition of 0.2 M hydrochloric acid (20 ml), the mixture was warmed at 60 °C for 15 min. The reaction mixture was adjusted to pH 4 with 0.1 M sodium hydroxide solution on cooling with ice-water, and concentrated to dryness under reduced pressure. The residue was extracted with ethanol and the ethanol extracts were concentrated to dryness. The residue was crystallized from water to yield I (310 mg) as colorless prisms (yield: 60%), mp 215 °C (dec.).

Found: C, 57.40; H, 4.78%. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80%.

(ii) The condensation reaction of IX with X was carried out in a similar manner to that described in the preparation of XI to obtain XII (*ca.* 3.5 g) in a crude oil. Without further purification, a mixture of a solution of XII in methanol (100 ml) and aqueous 1M sodium hydroxide solution (100 ml) was stirred overnight at room temperature. The reaction mixture was concentrated (30 ml) *in vacuo*, neutralized with hydrochloric acid, and evaporated to dryness. The residue was dissolved in acetic acid (50 ml) and the solution was warmed at 60 °C for 10 min. The reaction mixture was concentrated to dryness and the residue was extracted with ethanol. The ethanol extracts were evaporated to dryness, and the residue was crystallized from water to obtain I (1.0 g) as colorless prisms (yield 48%), mp 215 °C (dec.).

Found: C, 56.88; H, 4.74%. Calcd for $C_{10}H_{10}O_5$: C, 57.14; H, 4.80%.

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