Structural Confirmation of Ampicillin Polymers Formed in Aqueous Solution

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INTRODUCTION

The stability of ampicillin sodium in solution is dependent not only on the type of vehicle, pH, and temperature, but also on the concentration of the drug solution (1,2). A concentrated (i.e., 10–25%, w/v) aqueous solution of ampicillin sodium forms polymers during storage for a few days at room temperature (3), and these polymers are considered to be one of the causes of allergic reaction (4–6). Ampicillin polymer formation is initiated by a reaction of a side-chain amino group of one ampicillin molecule with the cleaved β-lactam moiety of a second molecule (2). Bundgaard and Larsen (7) showed the presence of homogeneous ampicillin dimer, tetramer, and hexamer using anion-exchange chromatography and high-performance liquid chromatography (8). Recently, Girona *et al.* (9) tried to determine the molecular mass of ampicillin even polymers by using a gel filtration chromatographic technique.

In this report, circular dichroism (CD) spectrometry has been used to monitor the ampicillin polymerization process and to characterize polymers in an aqueous solution of ampicillin sodium. The chemical structures of the individual polymeric substance isolated were determined with the use of both fast atom bombardment (FAB) mass and proton nuclear magnetic resonance (^{1}H-NMR) spectrometry.

MATERIALS AND METHODS

Chemicals

Ampicillin sodium was purchased from Sigma Chemical Co. (St. Louis, Mo.) and DEAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden). Diaion HP-20 was procured from Mitsubishi Chemical Industrial Co. Ltd. (Tokyo). All of the other chemicals used were of reagent grade.

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Preparation and Isolation of Ampicillin Polymers

The ampicillin polymers were formed in a 50% (w/v) aqueous solution of ampicillin sodium (initial pH 8.5) which was kept in the dark at 24°C for 24 hr. Separation was performed by anion-exchange chromatography on a column of DEAE-Sephadex A-25 using a linear sodium chloride gradient at a constant pH of 7.4 (7). Fractionation was carried out at a constant flow rate of 45 ml/hr by a peristaltic pump (LKB, Bromma, Sweden) and the absorbance of the effluent was monitored continuously at 260 nm on a UV-210 double-beam spectrophotometer (Shimadzu, Kyoto, Japan) connected to a R-11 recorder (Rikadenki, Tokyo). After passage through the spectrophotometer cuvette, the effluent was collected in 10-ml fractions using a FC-220 fraction collector (Gilson Medical Electronics Inc., Middleton, Wis.). The content of the eluates containing ampicillin polymers was characterized by CD spectrophotometry at room temperature.

Each eluate fraction containing an ampicillin polymer was lyophilized. The residue was then dissolved in a small volume of distilled water and, after adjustment to pH 3.0 with 2 *M* HCl, was desalted by a Diaion HP-20 column (30-cm length × 0.9-cm I.D.). The column was first eluted with 200 ml of water, then with 50 ml of methanol. The last 30-ml fraction was collected, and the solvent was removed by evaporation.

CD Spectrophotometry

The CD spectra of fractions obtained by ion-exchange chromatography were run on a Jasco J-500S automatic recording spectropolarimeter equipped with a Jasco DP-501N data processor (Japan Spectroscopic Co. Ltd.) at room temperature. The instrument was calibrated using 0.05% androsterone (Tokyo Chemical Industrial Co. Ltd., Tokyo) in dioxan. The CD spectra were recorded between 200 and 300 nm with a 0.1- or 1-cm path-length cell, at a time constant of 8 sec and a scan speed of 10 nm/min.

Mass Spectrometry

FAB mass spectra were measured with a JEOL JMS-DX 300 mass spectrometer (JEOL Ltd., Tokyo) equipped with a FAB ion source and a JMA-3100 data processing system. The mass range m/z 1–2000 was scanned for 10 sec at an ion source accelerating potential of 3.0 kV and averaged intensities of 10 scans were recorded. The sample was mixed with a glycerol–thioglycerol (1:1) matrix on a stainless-steel target, which was bombarded with a beam of Xenon neutral atoms accelerated to 1 keV.

NMR Spectroscopy

^{1}H-NMR spectra were taken on a JEOL GX-400 interfaced with a DEC RSX-11M computer, using 5-mm spinning tubes at 35.0°C. Samples were dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6) and the spectra were measured immediately after preparation of the solution. The chemical shifts

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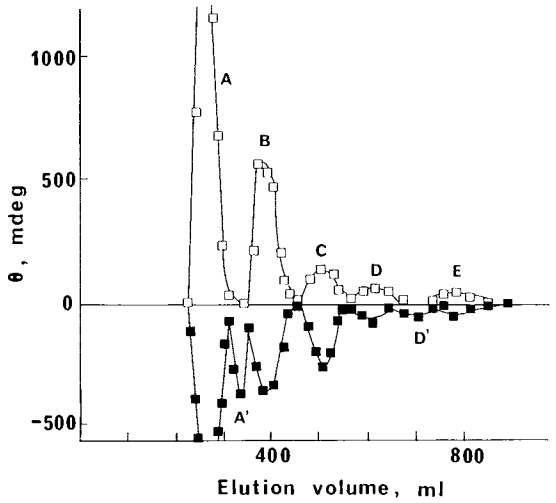

Fig. 1. Elution pattern by anion-exchange chlomatography. The points show the induced ellipticity (θ) of the fractions at both a positive peak (□) and a negative peak (■). Relative peak areas are as follows: A (ampicillin monomer), 54.9%; B (dimer), 24.2%; C (trimer), 7.1%; D (tetramer), 4.9%; E (pentamer), 2.5%; A′ (unknown), 5.9%; and D′ (unknown), 1.2%.

were assigned values based on the internal standard tetramethylsilane (TMS).

RESULTS AND DISCUSSION

Under the experimental conditions, ampicillin dimers readily formed within 1 hr, followed by the formation of polymers with higher molecular weights. Figure 1 shows the elution pattern of 0.5-ml portions of a 50% ampicillin sodium solution kept standing for 60 min, obtained from a DEAE-Sephadex A-25 column using the measurement of CD spectra. This elution profile was divided into five fractions (A to E) having both a positive and a negative peak and two fractions (A′ and D′) with a negative peak. The CD spectra for some of the fractions are shown in Fig. 2. The CD spectrum of fraction A coincided with that of intact ampicillin, where a well-defined biphasic curve could be observed: a positive peak at 232 nm arising from the Cotton effect of an intact

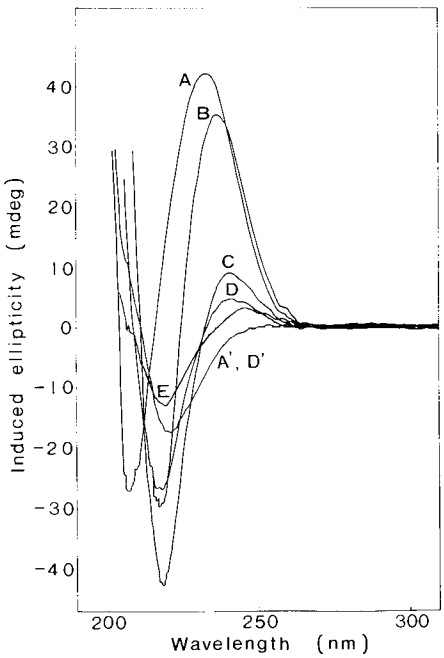

Fig. 2. The induced CD spectra of the A–E and A′ (D′) fractions. Path length, 0.1 cm; time constant, 4 sec; and scan speed, 100 nm/min.

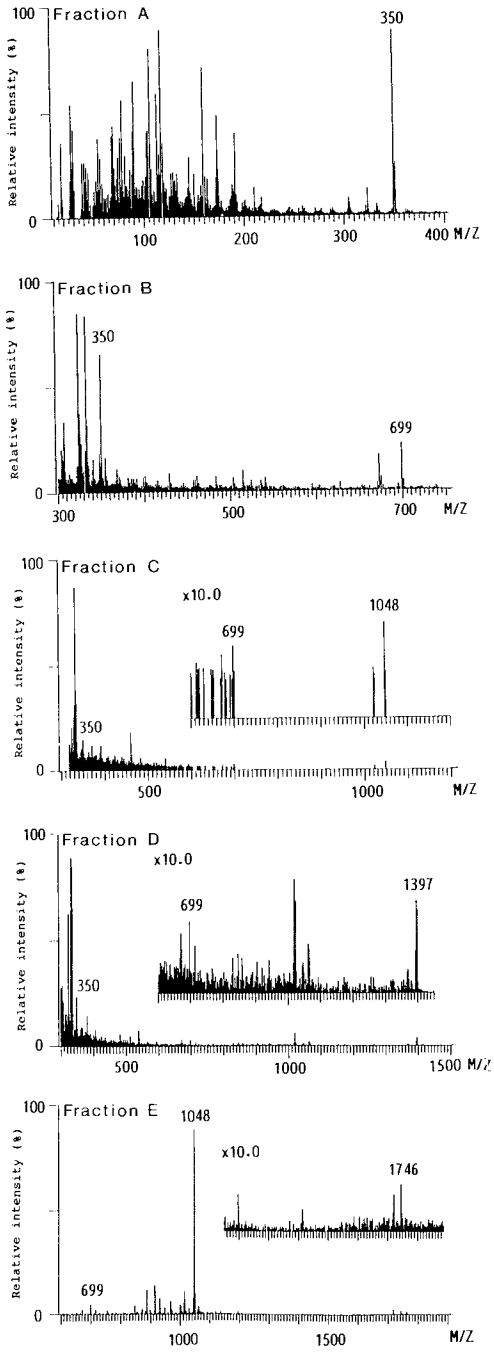

Fig. 3. FAB mass spectra of the polymeric substances isolated from the fraction peaks of A to E, having both a positive and a negative band in the CD spectra.

Table I. Physicochemical Data of Fractions A to E for Ampicillin Polymers

Compound (fraction)	FAB mass $[M + H]^+$ m/z	mp (°C)	TLC (R_f)[a]	Elemental anal. C:H:N (%)	CD spectra $\lambda_{max}([\theta])^b$ (nm)
Ampicillin	350	219.0	0.44	55.01:5.44:12.03 $C_{16}H_{19}N_3O_4S$	232 (42.5), 205 (−35.9)
Monomer (A)	350	219.0	0.46	47.53:6.30:10.41 $C_{16}H_{19}N_3O_4S \cdot 3.0H_2O$	232 (41.8), 205 (−30.8)
Dimer (B)	699	221.4	0.48	50.73:5.98:10.91 $(C_{16}H_{19}N_3O_4S)_2 \cdot 3.2H_2O$	236 (16.3), 215 (−13.7)
Trimer (C)	1048	251.2	0.55	51.02:5.94:11.02 $(C_{16}H_{19}N_3O_4S)_3 \cdot 4.5H_2O$	240 (6.7), 218 (−36.1)
Tetramer (D)	1397	268.0	0.62	51.09:6.01:11.02 $(C_{16}H_{19}N_3O_4S)_4 \cdot 6.0H_2O$	242 (3.3), 218 (−25.7)
Pentamer (E)	1746	277.2	0.65	50.70:5.89:11.12 $(C_{16}H_{19}N_3O_4S)_5 \cdot 8.2H_2O$	245 (1.3), 220 (−15.2)

[a] Butanol:acetic acid:water (3:1:1, v/v).
[b] deg cm^2 $dmol^{-1}$.

β-lactam ring and a negative peak at 205 nm reflecting a D-(−) form of ampicillin which has a stereochemical environment of a dyssymmetrically perturbed carbonyl group in the side chain of ampicillin (10).

The substances in the fractions having both positive and negative bands were isolated and desalted, and then the molecular mass was determined by FAB mass spectrometry. Figure 3 shows the FAB mass spectra in the fractions from A to E, respectively. The spectra gave a base peak at m/z 350, which is assignable to $[M + H]^+$ of $C_{16}H_{19}O_4N_3S$ (calc. 349.412). This molecular formula corresponds to ampicillin. The other $[M + H]^+$ peaks at m/z 699, 1048, 1397, and 1746 were found to correspond to the dimer, trimer, tetramer, and pentamer of ampicillin, respectively. Fragment peaks of intact ampicillin, dimer, and trimer are observed in the respective FAB mass spectra.

The analytical data of the isolated solids from fractions A to E are listed in Table I together with CD characteristics. The C:H:N ratios obtained from elemental analysis of the polymers are coincident with that of ampicillin, indicating that the compounds are essentially homogeneous and have been isolated without the occurence of any structural changes. The polymerization of ampicillin increases the melting points and the TLC R_f values and causes both positive and negative bands of the CD spectrum to shift to a slightly higher wave length (red-shifted). The molar ellipticity ([θ]) of the positive Cotton effect decreases with an increasing degree of polymerization, which is due to cleavage of the β-lactam ring. Insufficient quantities of fractions A′ and D′ with a negative peak (Fig. 1) were obtained to determine their chemical structures. CD spectra of these fractions show a negative band around 215 nm, suggesting substances with an opened β-lactam ring in the terminal unit.

^{1}H-NMR spectra were analyzed to clarify the chemical structure of the polymers. Figure 4 shows a 400-MHz ^{1}H-NMR spectrum of ampicillin dimer in DMSO-d_6 and the expected structure. The assignments were determined by comparison of ^{1}H-NMR spectra of ampicillin and the penicilloates (11–13). However, as the assignment of each H5 and H6 proton cannot be reliably made on the basis of ^{1}H chemical shift data alone, these resonances were thus assigned using a two-dimensional NMR (^{1}H-^{13}C COSY) spectrum. All other chemical shifts are summarized in Table II. The signals of the H5 and H6 protons on the β-lactam ring appear as doublets at 5.34 and 5.46 ppm, respectively, and the singlet signals at 1.40, 1.55, and 4.10 ppm correlate with those of the 2α-, 2β-CH_3, and H3 protons on the thiazolidine ring of the parent molecule [I] (Fig. 4). The signals completely agreed with those of ampicillin (Table II). The signal corresponding to the H10 proton on the side chain, which was noted in

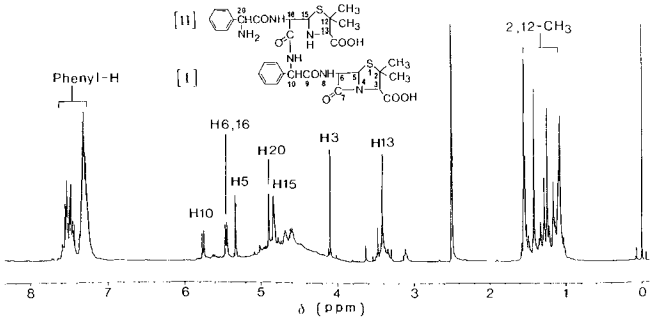

Fig. 4. The 400-MHz ^{1}H-NMR spectrum of ampicillin dimer in DMSO-d_6 and the expected chemical structure.

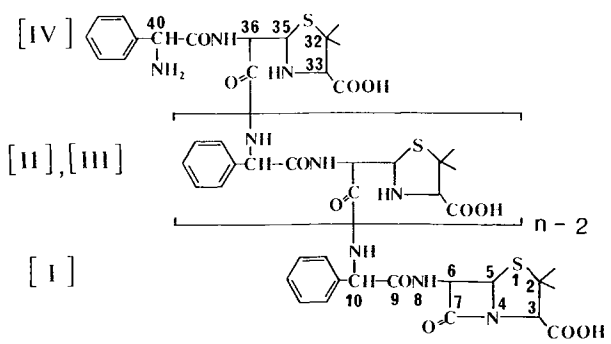

Fig. 5. Structure of ampicillin polymer ($n \geqq 2$).

Table II. ^{1}H Chemical Shifts from 400-MHz NMR Spectra of Ampicillin Polymers[a] in DMSO-d_6

Compound (fraction)		Chemical shift, δ ppm[b]						
	[I] [II] [III] [IV]	H3 (H13) (H23) (H33)	H5 (H15) (H25) (H35)	H6 (H16) (H26) (H36)	H10 (H20) (H30) (H40)	2α-CH_3 (12α-CH_3)	2β-CH_3 (12β-CH_3)	Phenyl-H
Ampicillin		4.10s	5.37d	5.45d	4.78s	1.41s	1.51s	7.43d, 7.35t, 7.32m
Monomer (A)		4.10s	5.37d	5.46d	4.78s	1.40s	1.50s	7.45d, 7.35t, 7.30m
Dimer (B)	[I]	4.10s	5.34d	5.46d	5.75d	1.41s	1.54s	7.53m, 7.47m, 7.31b
	[II]	(3.43s)	(4.84q)	(5.40d)	(4.89s)	(1.07s)	(1.24s)	
Trimer (C)	[I]	4.08s	5.28d	5.44d	5.76d	1.35s	1.54s	7.51m, 7.32m, 7.22b
	[II]	(3.45s)	(4.86q)	(5.67q)	(5.76d)			
	[III]	(3.31s)	(4.86q)	(5.67q)	(4.98s)			
Tetramer (D)	[I]	4.05s	5.30d	5.48q	5.71m	1.40s	1.53s	7.50b, 7.33b, 7.25b
	[II–III]	(3.37m)	(4.76m)	(5.64m)	(5.71m)			
	[IV]	(3.37m)	(4.76m)	(5.64m)	(4.86s)			

[a] Chemical structures are shown in Fig. 5.
[b] The ratio of signal intensities was correct for the number of protons assigned in all spectra.

order to characterize the polymerization of ampicillin, shows a doublet signal at 5.75 ppm. The corresponding signals of the second molecule [II] were widely shifted upfield, i.e., δ1.07 (s, 12α-CH_3), δ1.24 (s, 12β-CH_3), δ3.43 (s, H13), δ5.40 (d, H16), and δ4.84 (d, H15), reflecting the cleavage of the β-lactam ring (12). A chemical structure of the ampicillin polymer is shown in Fig. 5. Although results corresponding to those described for the dimer were obtained in the ^{1}H-NMR spectra of the trimer and tetramer of ampicillin (Table II), the resolution was not adequate to determine reliably the individual shifts.

The results indicated that ampicillin polymers in aqueous solution are formed through a chain process by linkage of the amino group on the side chain in one ampicillin molecule with the carbonyl group of the cleaved β-lactam ring in the second molecule and supported the previous studies (2,7,9). With regard to the chemical structures of ampicillin polymers, it became clear that the polymers formed in an aqueous solution under the conditions described are dimer, trimer, tetramer, and pentamer with an intact β-lactam ring in the terminal unit.

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