Studies on Orally Active Cephalosporin Esters. II.[1] Chemical Stability of Pivaloyloxymethyl Esters in Phosphate Buffer Solution

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The degradation kinetics of pivaloyloxymethyl (POM) esters of cephalosporins in phosphate buffer solution (pH 6—8) were investigated. The degradation of the starting Δ^3 cephalosporin ester proceeded mainly *via* isomerization to the Δ^2 ester and subsequent hydrolysis to the Δ^2 acid. Hydrolysis to the Δ^3 acid (the parent acid) was very slow. Analysis of the rate constants indicated that the isomerization rate k_{12} was approximately equal to the apparent degradation rate of the Δ^3 ester k_{deg}, and slower than the hydrolysis rate of the Δ^2 ester k_{24}. The isomerization process to the Δ^2 ester was found to be the rate-determining step in the degradation of cephalosporin esters. The substituent at the C-3 position of the cephalosporins affected the degradation kinetics. The degradation was accelerated by increase of pH, buffer concentration and added protein.

Keywords cephalosporin; isomerization; Δ^2 cephalosporin; stability; kinetics; prodrug; cefpodoxime proxetil; CS-807

Cephalosporin antibiotics for parenteral use are not suitable for oral administration because of their poor absorption from the gastrointestinal tract. One reason for such poor absorption may be inferred from the pH-partition theory. That is, these cephalosporins in the intestinal tract exist predominantly as an ionic form of low lipophilicity due to the relatively low pK_a values of the carboxyl group at the C-4 position, which is unfavorable for passive absorption through a lipoidal membrane.[2] Esterification of the carboxyl group, which increases the lipophilicity, has been tried in many laboratories.[3] Only a few successful compounds, however, have been reported.[4] Degradation in the gastrointestinal tract prior to absorption may be a reason for the failure of this prodrug approach to cephalosporins.

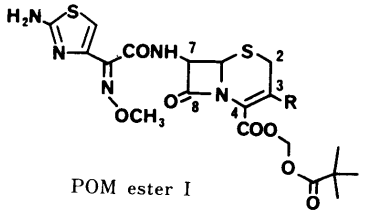
In our studies on a new, orally active cephalosporin, cefpodoxime proxetil (CPDX-PR, CS-807), the substituent at the C-3 position was found to play an important role in the intestinal absorption of the prodrug as well as in the antimicrobial activity of the parent acid.[1,5]

In this study the kinetic behavior of pivaloyloxymethyl (POM) esters of cephalosporin derivatives having various substituents at the C-3 position was investigated in phosphate buffer solution. The effects of pH, buffer concentration and addition of protein are discussed. After this manuscript had been completed, Saab *et al.* reported on the degradation kinetics of the methyl ester of cefazolin in a communication.[6]

Results and Discussion

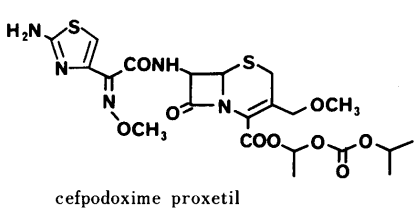
Cephalosporin esters used in this study are listed in Chart 1 along with cefpodoxime proxetil (CPDX-PR, CS-807). CPDX-PR has a methoxymethyl group as the 3-substituent and consists of a pair of diastereoisomers arising from the asymmetric center in the ester moiety, the 1-(isopropyloxycarbonyloxy) ethyl group. The ester moiety of the cephalosporins in this study was chosen as a POM group having no asymmetric center in order to simplify the experimental system. POM esterification is generally regarded as a useful method to give oral-absorbability to β-lactam antibiotics.[7] The acylamido moiety at the C-7 position was chosen as a 2-(2-aminothiazol-4-yl)-(*Z*)-2-methoxyiminoacetamido function, which is widely employed in the third generation cephalosporins used for injection.

Degradation Kinetics and Mechanism of POM Esters in Phosphate Buffer Solution The time courses of the POM ester degradation in phosphate buffer (1/20 M, pH 6.86) at 37 °C were followed by a high performance liquid chromatography (HPLC) method. Esters and hydrolyzed acids were measured under two different conditions. First, the degradation of the POM ester Ia was examined. HPLC patterns of the degraded POM ester after 4 h are shown in Fig. 1. Along with peak I of the starting POM ester (Ia), peaks of degraded products were observed. By comparing the retention times of the peaks with those of authentic samples, it was confirmed that the small peak II cor-



POM ester I

No.	-R	No.	-R
Ia	$-CH_2OCH_3$	Ig	$-CH_2STz$
Ib	$-CH_2OCH_2CH_3$	Ih	$-CH_2STh$
Ic	$-CH_2OCCH_3$ (O)	Ii	$-CH_2Tet$
Id	$-CH_2OIox$	Ij	$-CH_3$
Ie	$-CH_2SCH_3$	Ik	-H
If	$-CH_2SCH_2CN$		



cefpodoxime proxetil
(CS-807)

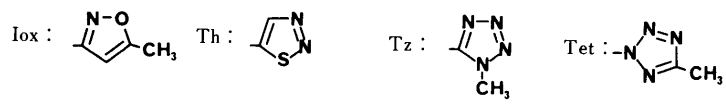


Chart 1

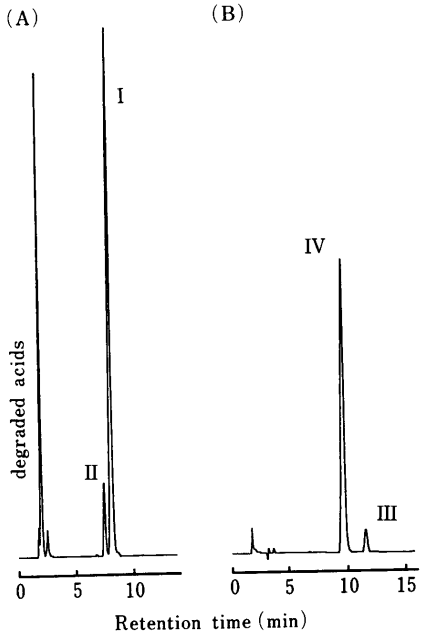


Fig. 1. HPLC Patterns of the Degraded POM Ester Ia in Phosphate Buffer Solution

1/20 M phosphate buffer, pH 6.86, after 4 h at 37 °C. HPLC conditions: column, YMC ODS A-312 (6 × 150 mm); mobile phase, (A) for esters, 60% CH_3CN–0.2% CH_3COONH_4 and (B) for acids, 10% CH_3CN–0.2% CH_3COONH_4; flow rate, 1.0 ml/min. Peak assignments: peak I, Δ^3 ester (Ia); peak II, Δ^2 ester (IIa); peak III, Δ^3 acid (IIIa); peak IV; Δ^2 acid (IVa).

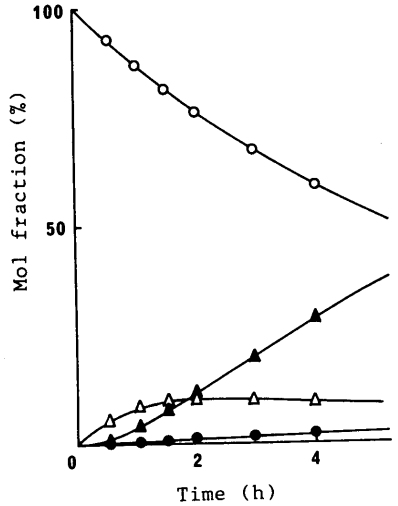


Fig. 2. Time Courses of Δ^3 Ester Ia (○), Δ^2 Ester IIa (△), Δ^3 Acid IIIa (●) and Δ^2 Acid IVa (▲) during the Degradation of POM Ester Ia

Conditions: in 1/20 M phosphate buffer solution of pH 6.86 at 37 °C. The lines were simulated based on the scheme in Chart 2 and the rate constants in Table I.

responded to the Δ^2 ester isomer (IIa), the major peak IV to the Δ^2 acid isomer (IVa) and the other peak III to the parent acid (the Δ^3 acid) (IIIa). Figure 2 shows time courses for these degraded products. Accompanying the degradation of the starting Δ^3 ester (Ia), the Δ^2 ester (IIa) increased gradually to attain a maximum at around 1.5 h and later decreased, and the Δ^2 acid (IVa) increased gradually after a short lag time. The Δ^3 acid (IIIa) increased gradually but its amount was much less than that of the Δ^2 acid (IVa). These results show that the Δ^2 ester (IIa) is an intermediate in formation of the Δ^2 acid (IVa). The other POM esters Ib—k also gave degradation patterns similar to that observed in the degradation of Ia.

Saikawa *et al.* treated the degradation of cefteram pivoxil (Ii) as a pseudo-first order process.[8] In our experiment, the degradation of POM esters Ia—k could be regarded as pseudo-first order. Their apparent degradation rate constants k_{deg} were calculated from the slope of the logarithmic plot. The degradation rate constant of Ii ($0.63\,h^{-1}$) under our conditions was similar to that ($0.6\,h^{-1}$) estimated from Saikawa *et al.*'s data.

Taking into account the mechanism of degradation, further kinetic analyses were carried out. In the hydrolysis experiment of each compound Ia—k, more than 97% of the degraded POM ester was recovered as Δ^2 ester (II) and Δ^3 and Δ^2 acids (III, IV), and degradation to other products was negligible. In addition, it is known that the Δ^3 and Δ^2 esters are interconverted by basic catalysis but the acid isomers are not.[9] On this basis, a kinetic model for the degradation of cephalosporin ester is postulated in Chart 2.

The first order rate constants k_{12}, k_{21}, k_{13} and k_{24} were

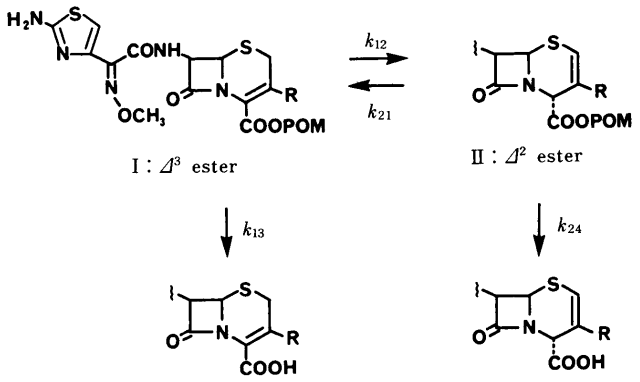


Chart 2

TABLE I. Kinetic Rate Constants for the Degradation of POM Esters

No.	3-Substituent	k_{deg}	k_{12}	k_{21}	k_{13}	k_{24}
Ia	CH_2OCH_3	1.34×10^{-1}	1.30×10^{-1}	5.68×10^{-2}	6.37×10^{-3}	0.81
Ib	$CH_2OCH_2CH_3$	1.66×10^{-1}	1.54×10^{-1}	1.37×10^{-3}	3.56×10^{-3}	2.11
Ic	CH_2OAc	2.76×10^{-1}	3.42×10^{-1}	3.41×10^{-2}	9.41×10^{-3}	1.28
Id	CH_2OIox	3.01×10^{-1}	2.85×10^{-1}	9.99×10^{-5}	1.04×10^{-2}	1.68
Ie	CH_2SCH_3	1.44×10^{-1}	1.35×10^{-1}	3.92×10^{-3}	7.03×10^{-3}	1.32
If	CH_2SCH_2CN	2.33×10^{-1}	2.17×10^{-1}	4.02×10^{-4}	1.01×10^{-2}	4.54
Ig	CH_2STz	3.33×10^{-1}	3.26×10^{-1}	4.04×10^{-6}	6.44×10^{-3}	2.34
Ih	CH_2STh	4.78×10^{-1}	4.53×10^{-1}	1.00×10^{-4}	1.21×10^{-2}	3.05
Ii	CH_2Tet	7.43×10^{-1}	6.00×10^{-1}	6.38×10^{-4}	3.52×10^{-2}	16.6
Ij	CH_3	0.52×10^{-1}	0.59×10^{-1}	1.36×10^{-1}	2.92×10^{-3}	0.85
Ik	H	1.44×10^{-1}	1.61×10^{-1}	1.82	3.54×10^{-2}	4.12

Each rate constant is given in h^{-1}. Experiments were carried out using 100 μg/ml solutions in 1/20 M phosphate buffer of pH 6.86 at 37 °C.

calculated for each compound with the aid of NONLIN 84,[10] where the data were fitted to the following differential equations. Degradation of the acids (III, IV) through β-lactam ring opening was neglected in the analysis. Results are listed in Table I.

$$d[I]/dt = -(k_{12}+k_{13})[I]+k_{21}[II]$$

$$d[II]/dt = -(k_{21}+k_{24})[II]+k_{12}[I]$$

$$d[III]/dt = k_{13}[I]$$

$$d[IV]/dt = k_{24}[II]$$

For all POM esters examined in this study, the time courses for the esters and acids (Ia—IVa) simulated using these rate constants showed good agreement with observed values (Fig. 2). This supported the validity of the kinetic model proposed in Chart 2. Comparison of the rate constants for each compound gave the following general relations.

$$k_{13} < k_{12} \ll k_{24} \text{ and } k_{24} > k_{21} \quad (1)$$

$$k_{deg} \fallingdotseq k_{12} \quad (2)$$

Relations (1) show that the main degradation product is the Δ^2 acid formed *via* isomerization to the Δ^2 ester and its subsequent hydrolysis, while little Δ^3 acid is formed *via* direct hydrolysis of the starting POM ester. The isomerization process from the starting Δ^3 ester to the Δ^2 ester was found to be the rate-determining step in the chemical degradation of cephalosporin esters in a phosphate buffer system.

Effect of pH, Buffer Concentration and Added Protein on the Degradation Rate In the intestinal tract it is known that the pH is raised to about 8 by secretion of pancreatic juice, that the ionic strength is raised by secretion of digestive juice, and that proteins or peptides exist abundantly as constituents of the mucosal layer or digestive fluid. Thus, the effects of pH, buffer concentration and added protein were examined to simulate intestinal conditions.

As shown in Figs. 3 and 4, both the pH and buffer concentration of the media affected the degradation rate of POM esters. The degradation proceeded faster at high pH and high buffer concentration. Similar effects of pH and buffer concentration were reported for cefteram pivoxil (Ii).[8] Our kinetic analysis clarified that the increase of pH increased the rate constants of all processes (Table II), whereas only the isomerization rate constants were increased by the increase of buffer concentration and the rates of hydrolysis were not (Table III). The results showed that the isomerization process is a base-catalyzed reaction and phosphate anion served as a catalyst in that process.

An increase of degradation rate was also observed on adding a standard protein, bovine serum albumin (BSA), to

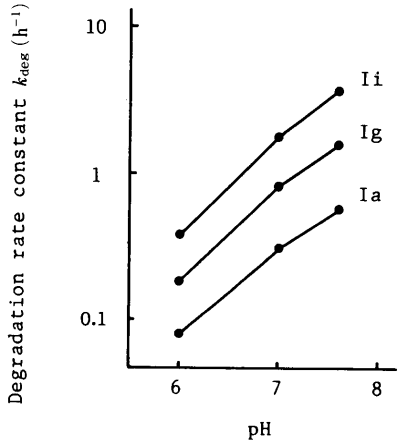


Fig. 3. Effect of pH on Degradation of POM Esters

Conditions: in 1/10 M phosphate buffer solutions at 37 °C. k_{deg}: degradation rate constants (h^{-1}) of Δ^3 esters.

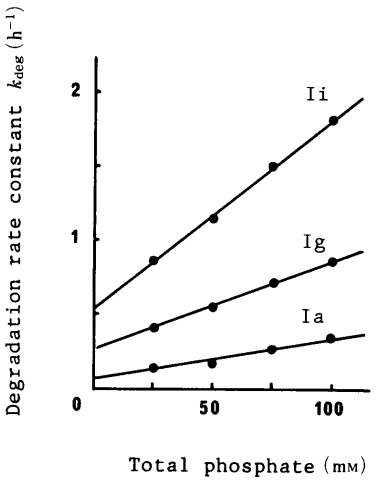


Fig. 4. Effect of Buffer Concentration on the Degradation of POM Esters

Conditions: in phosphate buffer solutions of pH 7.0 at 37 °C. k_{deg}: degradation rate constants (h^{-1}) of Δ^3 esters.

Table II. Effect of pH on the Degradation Kinetics of POM Ester Ia

pH	k_{12}	k_{21}	k_{13}	k_{24}
6.0	0.063	0.0531	0.0030	0.364
7.0	0.321	0.1028	0.0207	1.870
7.6	0.538	0.1583	0.0528	5.750

Each rate constant is given in h^{-1}. Experiments were carried out using 100 μg/ml solutions in 0.1 M phosphate buffer at 37 °C.

Table III. Effect of Buffer Concentration on the Degradation Kinetics of POM Ester Ia

Buffer conc. (mM)	k_{12}	k_{21}	k_{13}	k_{24}
25	0.112	0.0745	0.0164	1.482
50	0.190	0.0889	0.0165	1.634
75	0.264	0.0924	0.0176	1.735
100	0.321	0.1028	0.0207	1.870

Each rate constant is given in h^{-1}. Experiments were carried out using 100 μg/ml solutions in phosphate buffer of pH 7.0 at 37 °C.

Table IV. Effect of Protein on the Degradation Kinetics of POM Ester Ia

BSA conc. (%)	k_{12}	k_{21}	k_{13}	k_{24}
0	0.321	0.1028	0.0207	1.870
0.1	0.450	0.1231	0.0128	1.930
0.4	0.764	0.1770	0.0164	1.911
1.0	1.308	0.3250	0.0152	2.069

Each rate constant is given in h^{-1}. Experiments were carried out using 100 μg/ml solutions in 0.1 M phosphate buffer at 37 °C. The protein used was bovine serum albumin (fraction V).

the phosphate buffer (Fig. 5). In this case, too, only the rate constants of the isomerization process were accelerated (Table IV). Interaction with the protein surface seems to accelerate the isomerization reaction. Catalysis by the basic amino acid residues, conformational change to a form suitable for isomerization or increase of ionic strength are possible reasons.

These results indicate that the chemical degradation of cephalosporin esters would proceed more rapidly at higher pH and ionic strength and by the addition of protein to the phosphate buffer solution. These factors accelerated the isomerization process. This tendency is higher for esters having larger isomerization rate since the slopes of the rate plots were larger for those esters.

Effect of the 3-Substituent on the Degradation Rate In a series of POM esters, the degradation rates (k_{deg}) varied with the 3-substituents. The ester Ij with a methyl group as the 3-substituent was relatively stable. The esters Id, Ig, Ih and Ii with heterocyclic oxymethyl, thiomethyl or methyl groups were labile, as well as the esters Ic and If with acetoxymethyl or cyanomethylthiomethyl groups. On the other hand, the ester Ia with a methoxymethyl group, the 3-substituent of CPDX-PR, had moderate stability as well as the esters Ib, Ie and Ik with ethoxymethyl, methylthiomethyl or hydryl groups. The electron-withdrawing character of the 3-substituent seems to affect the degradation rate, *i.e.*, the isomerization rate.

The effect of the 3-substituent on each rate constant, especially on the isomerization rate constants (k_{12}, k_{21}), will be discussed quantitatively in the next paper.

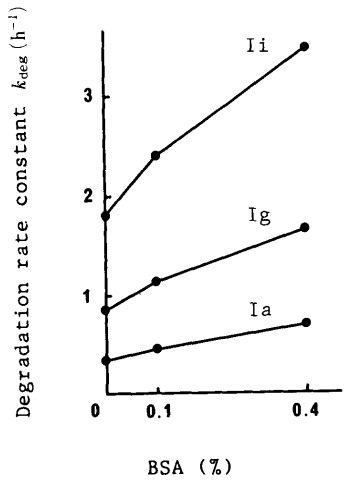


Fig. 5. Effect of Protein on the Degradation of POM Esters

Conditions: in 1/10 M phosphate buffer solutions of pH 7.0 at 37 °C. k_{deg}: degradation rate constants (h^{-1}) of Δ^3 esters. Protein: bovine serum albumin (fraction V).

Experimental

General Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a JEOL GX-270 spectrometer using tetramethylsilane as an internal standard. Infrared (IR) spectra were recorded on a JASCO IRA-120 spectrometer. Ultraviolet (UV) spectra were taken on a Shimadzu UV-3100 spectrometer. HPLC was performed using a Waters chromatography system (6000A pump, 440 absorbance detector (254 nm)) and a Shimadzu C-R3A Chromatopac.

Preparation of Cephem POM Esters POM esters Ia—k were prepared by a method similar to that reported.[1,3f,4b] ^{1}H-NMR and IR data are listed in Table V.

Preparation of the Δ^2 Isomer of the Parent Acid The Δ^2 isomers were prepared by the following method, which is a modification of Morin *et al.*'s method.[9a] POM ester (1 mmol) was dissolved in 20 ml of pyridine–water (1 : 1) and 1.2 mmol of $NaHCO_3$ was added. The mixture was stirred at room temperature for 20 h. After addition of 1.2 mmol of HCl (1 N), the mixture was concentrated *in vacuo* and chromatographed on a silica gel column (50 g) using EtOAc–EtOH–Water (5 : 2 : 1) as an eluent. After removal of the solvent *in vacuo*, the residue was dissolved in 5 ml of water with 1 mmol of $NaHCO_3$. The solution was absorbed on a column of CHP-20P (100 ml) and eluted successively with water, 5% CH_3CN and 10% CH_3CN in water. The eluate was concentrated *in vacuo* to 50 ml and then lyophilized to give the sodium salt of Δ^2 acid IV as a colorless powder. ^{1}H-NMR and IR spectra are listed in Table VI.

TABLE V. ^{1}H-NMR and IR Spectral Data for POM Esters Ia—Ik

No.	3-Substituent	^{1}H-NMR[a]										IR[b]		
		2-CH_2	6-CH (d)	7-CH (dd)	CONH (d)	Th-H (s)	OCH_3 (s)	$COO-CH_2-O_2C-tert$-Bu (ABq)	(s)	3-Substituent		Lactam (S)	amide (S)	ester (M)
Ia	CH_2OCH_3	3.58 (s)	5.08 (4.8)	6.02 (4.8, 9.1)	7.56 (9.1)	6.87	4.08	5.86, 5.92 (5.5)	1.23	4.31, 4.34 (2H, ABq, 13.9)	3.34 (3H, s)	1786	1752	1681
Ib	$CH_2OCH_2CH_3$	3.60 (s)	5.08 (4.8)	6.03 (4.8, 8.8)	7.64 (8.8)	6.82	4.07	5.86, 5.90 (5.5)	1.23	4.33, 4.40 (2H, ABq, 13.6)	3.48 (2H, q, 7.0) 1.20 (3H, t, 7.0)	1787	1753	1680
Ic	CH_2OAc	3.45, 3.58 (ABq, 18.3)	5.09 (5.1)	6.06 (5.1, 9.1)	7.79 (9.1)	6.81	4.06	5.86, 5.93 (5.5)	1.23	4.83, 5.12 (2H, ABq, 13.6)	2.09 (3H, s)	1788	1747	1681
Id	CH_2OIox	3.57, 3.66 (ABq, 18.5)	5.09 (4.9)	6.03 (4.9, 8.8)	7.47 (8.8)	6.89	4.07	5.86, 5.94 (5.6)	1.22	4.98, 5.29 (2H, ABq, 13.4)	2.33 (3H, s) 5.62 (1H, s)	1788	1752	1682
Ie	CH_2SCH_3	3.58, 3.71 (ABq, 18.0)	5.12 (4.8)	5.97 (4.8, 8.8)	7.47 (8.8)	6.93	4.09	5.87, 5.90 (5.5)	1.23	3.44, 3.78 (2H, ABq, 13.7)	2.06 (3H, s)	1784	1751	1681
If	CH_2SCH_2CN	3.60, 3.73 (ABq, 18.3)	5.14 (4.8)	6.01 (4.8, 8.8)	7.58 (8.8)	6.89	4.06	5.89, 5.94 (5.5)	1.24	3.35—4.12 (4H, m)		1783	1749	1677
Ig	CH_2STz	3.77, 3.81 (ABq, 19.2)	5.08 (4.8)	6.05 (4.8, 8.8)	7.65 (8.8)	6.83	4.05	5.89, 5.94 (5.5)	1.24	4.30, 4.49 (2H, ABq, 13.5)	3.93 (3H, s)	1787	1751	1680
Ih	CH_2STh	3.55, 3.64 (ABq, 18.1)	5.10 (4.9)	6.02 (4.9, 8.8)	7.74 (8.8)	6.86	4.07	5.84, 5.81 (5.6)	1.22	4.10, 4.19 (2H, ABq, 13.2)	8.52 (1H, s)	1787	1750	1680
Ii	CH_2Tet	3.27, 3.34 (ABq, 18.7)	5.08 (4.8)	6.04 (4.8, 9.2)	7.57 (9.2)	6.89	4.06	5.91, 5.97 (5.5)	1.24	5.52, 5.84 (2H, ABq, 15.4)	2.54 (3H, s)	1791	1751	1681
Ij	CH_3	3.28, 3.52 (ABq, 18.5)	5.08 (4.8)	5.99 (4.8, 9.2)	7.65 (9.2)	6.82	4.06	5.86, 5.92 (5.5)	1.23	2.15 (3H, s)		1781	1752	1680
Ik	H	3.47, 3.64 (ABX, 19.0 (AB))	5.08 (4.8)	6.09 (4.8, 8.8)	7.66 (8.8)	6.85	4.07	5.85, 5.95 (5.5)	1.23	6.65 (1H, ABX, 6.6 (AX), 2.6 (BX))		1788	1751	1680

a) Spectra were measured in $CDCl_3$ at 270 MHz with tetramethylsilane as an internal standard. Chemical shifts are given in δ (ppm). Coupling patterns and constants (Hz) are given in parentheses. *b*) Spectra were taken in a KBr pellet. Characteristic absorption bands are given in wave number (cm^{-1}).

TABLE VI. ^{1}H-NMR and IR Spectral Data for Δ^2 Cephalosporin Acids IVa—IVk

No.	3-Substituent	^{1}H-NMR[a] 2-CH (br s)	4-CH (br s)	6-CH (d)	7-CH (dd)	CONH (d)	Th-H (s)	OCH_3 (s)	3-Substituent			IR[b] Lactam amide (S)	(M)	carboxyl (S)
IVa	CH_2OCH_3	6.12	4.34	5.30—5.34 (m)		9.55 (7.8)	6.78	3.82	3.78, 4.31 (2H, ABq, 11.7)	3.16 (3H, s)		1758	1670	1619
IVb	$CH_2OCH_2CH_3$	6.21	4.49	5.33—5.41 (m)		9.60 (7.8)	6.78	3.83	3.87, 4.26 (2H, ABq, 11.7)	3.36 (2H, q, 6.8)	1.09 (3H, t, 6.8)	1762	1671	1619
IVc	CH_2OAc	6.28	4.38	5.30—5.37 (m)		9.56 (7.8)	6.78	3.83	4.58, 4.89 (2H, ABq, 12.0)	2.01 (3H, s)		1758	1666	1618
IVd	CH_2Olox	6.38	4.44	5.32—5.35 (m)		9.56 (7.8)	6.78	3.83	4.63, 5.12 (2H, ABq, 11.2)	5.99 (1H, s)	2.29 (3H, s)	1761	1670	1620
IVe	CH_2SCH_3	5.99	4.59	5.31—5.34 (m)		9.56 (7.8)	6.76	3.82	3.15, 3.83 (2H, ABq, 13.2)	1.89 (3H, s)		1757	1668	1616
IVf	CH_2SCH_2CN	6.09	4.56	5.33—5.38 (m)		9.44 (7.8)	6.78	3.84	3.38—4.05 (4H, m)			1758	1669	1619
IVg	CH_2STz	6.60	5.06	5.18 (3.9)	5.56 (3.9, 7.8)	9.62 (7.8)	6.75	3.83	4.11, 4.22 (2H, ABq, 13.9)	3.92 (3H, s)		1769	1673	1629
IVh	CH_2STh	6.35	4.58	5.33 (3.9)	5.39 (3.9, 7.8)	9.63 (7.8)	6.77	3.84	3.99, 4.48 (2H, ABq, 13.7)	8.90 (1H, s)		1759	1669	1619
IVi	CH_2Tet	6.24	4.31	5.26—5.36 (m)		9.58 (7.8)	6.76	3.82	5.31, 5.89 (2H, ABq, 14.2)	2.44 (3H, s)		1761	1666	1622
IVj	CH_3	6.19	4.67	5.16 (3.9)	5.53 (3.9, 7.8)	9.58 (7.8)	6.76	3.83	1.85 (3H, s)			1770	1660	1625
IVk	H	6.17 (dd) (10.3, 2.4)	4.28 (dd) (2.4, 3.9)	5.13 (3.9)	5.38 (3.9, 7.8)	9.55 (7.8)	6.75	3.83	5.95 (1H, dd, 10.3, 3.9)			1755	1657	1607

a) Spectra were measured in DMSO-d_6 at 270 MHz with tetramethylsilane as an internal standard. Chemical shifts are given in δ (ppm). Coupling patterns and constants (Hz) are given in parentheses. *b*) Spectra were taken in a KBr pellet. Characteristic absorption bands are given in wave number (cm^{-1}).

TABLE VII. Conditions of HPLC Analyses

No.	3-Substituent	[A] for ester Mobile phase[a]	Flow rate[b]	Column[c]	[B] for acids Mobile phase	Flow rate	Column
Ia	CH_2OCH_3	CH_3CN (60)	1.0	B	CH_3CN (10)	1.0	B
Ib	$CH_2OCH_2CH_3$	CH_3CN (70)	1.0	A	CH_3CN (15)	1.2	B
Ic	CH_2OAc	CH_3OH (70)	1.0	A	CH_3CN (15)	1.2	B
Id	CH_2Olox	CH_3OH (70)	1.0	A	CH_3CN (20)	1.2	B
Ie	CH_2SCH_3	CH_3CN (70)	1.0	A	CH_3CN (15)	1.2	B
If	CH_2SCH_2CN	CH_3OH (70)	1.0	A	CH_3CN (15)	1.2	B
Ig	CH_2STz	CH_3OH (70)	1.0	A	CH_3CN (15)	1.2	B
Ih	CH_2STh	CH_3OH (70)	1.0	A	CH_3CN (20)	1.2	B
Ii	CH_2Tet	CH_3OH (70)	1.0	A	CH_3CN (15)	1.2	B
Ij	CH_3	CH_3OH (70)	1.0	A	CH_3CN (15)	1.0	B
Ik	H	CH_3OH (70)	1.0	A	CH_3OH (18)	1.0	C

a) Mobile phases used were (A) CH_3CN–0.2% $AcONH_3$ and (B) CH_3OH–0.2% $AcONH_4$. Percentage (%) of CH_3CN or CH_3OH is given in parentheses. *b*) Flow rates are given in ml/min. *c*) Column A, YMC ODS AQ-312 (6 × 150 mm); B, YMC ODS A-312 (6 × 150 mm); C, NAKARAI COSMOSIL 5PE (4.5 × 150 mm).

Preparation of the Δ^2 Isomer of the POM Ester Triethylamine (0.1 ml) was added to a solution of POM ester Ia (1.0 g) in dichloromethane (10 ml) at ice-bath temperature and the mixture was stirred for 1 h. After removal of the solvent, the mixture containing Ia and IIa was chromatographed on a silica gel column (60 g) using benzene–acetonitrile (2 : 3) as an eluent. Fractions mainly containing IIa were collected (310 mg). These combined fractions were chromatographed on an RP-8 column (100 g) using acetonitrile–water (55 : 45) as an eluent to give IIa (220 mg) as an amorphous powder. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (9H, s, $-C(CH_3)_3$), 3.29 (3H, s, $-OCH_3$), 3.87 and 4.05 (2H, ABq, J = 12.2 Hz, 3′-CH_2), 4.07 (3H, s, $=NOCH_3$), 5.09 (1H, d, J = 1.5 Hz, 4-CH), 5.36 (1H, d, J = 4.4 Hz, 6-CH), 5.50 (2H, b s, NH_2), 5.78—5.84 (3H, m, 7-CH and CH_2OCO), 6.36 (1H, d, J = 1.0 Hz, 2-CH), 6.94 (1H, s, thiazole-H), 7.37 (1H, d, J = 8.8 Hz, CONH). IR (KBr) cm^{-1}: 1775 (β-lactam), 1760 (ester), 1678 (amide).

The Δ^2 ester IIg was also prepared by the procedure described above. ^{1}H-NMR ($CDCl_3$) δ: 1.23 (9H, s, $-C(CH_3)_3$), 3.91 (3H, s, N–CH_3), 4.02 (3H, s, N–OCH_3), 4.01 and 4.34 (2H, ABq, J = 14.6 Hz, 3′-CH_2), 5.25 (1H, d, J = 1.5 Hz, 4-CH), 5.30 (1H, d, J = 3.9 Hz, 6-CH), 5.45 (2H, b s, NH_2), 5.78 (1H, dd, J = 3.9, 8.8 Hz, 7-CH), 5.84 (2H, s, OCH_2OCO), 6.61 (1H, s, 4-CH), 6.87 (1H, s, thiazole-H), 7.46 (1H, d, J = 8.8 Hz, CONH). IR (KBr) cm^{-1}: 1780 (β-lactam), 1760 (ester), 1680 (amide).

Molar absorbancy values (ε) at 254 nm of these Δ^2 esters were almost equal to those of the corresponding Δ^3 esters (Ia: 14942, IIa: 14088, Ig: 16374, IIg: 17114). So the peak areas of the Δ^3 esters were used as the standard in the determination of the Δ^2 esters.

Procedure for Kinetic Study i) General: A solution of POM ester in *N,N*-dimethylformamide (10 mg/ml) was diluted to a final concentration of 100 μg/ml with 1/20 M phosphate buffer (pH 6.86) preincubated in a water bath held at 37 °C. The solution thus obtained was incubated at 37 °C with stirring. Samples were taken at suitable intervals, diluted with an equal amount of water and stored at −20 °C. Concentrations of the POM esters (Δ^2 ester and Δ^3 ester) and the parent acids (Δ^2 acid and Δ^3 acid) were determined by a HPLC method. Conditions of HPLC analysis for each compound are listed in Table VII. ii) Effect of pH: 1/10 M phosphate buffers of pH 6.0, 7.0 and 7.6 were used. iii) Effect of buffer concentration: 25, 50, 75 and 100 mM phosphate buffers (pH 7.0) were used. iv) Effect of added protein: 0.1, 0.4 and 1.0% solutions of bovine serum albumin (Fraction V; Sigma A-4503) in 1/10 M phosphate buffer (pH 7.0) were used. HPLC analysis was done after removal of the protein by the addition of an equal amount of CH_3CN followed by centrifugation at 3000 rpm for 3 min.

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