Studies on Orally Active Cephalosporin Esters. IV.[1] Effect of the C-3 Substituent of Cephalosporin on the Gastrointestinal Absorption in Mice

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The effect of the C-3 substituent on the oral absorbability of pivaloyloxymethyl (POM) ester of cephalosporin in mice is described. The C-3 substituent affects the physicochemical and biochemical properties of POM ester, such as lipophilicity, water solubility, chemical stability and enzymatic stability. Quantitative analyses of the relationships between these properties and the oral bioavailability have been attempted.

Lipophilicity made a parabolic contribution to the absorption. The optimum log $P_{\text{octanol/water}}$ value was estimated to be around 2.22. The chemical isomerization of the cephem double bond from Δ^3 to Δ^2 in the intestinal lumen prior to absorption contributed linearly to decrease of absorption. In the case of POM ester having a larger isomerization rate, more Δ^2 isomer was detected in feces and urine. Enzymatic hydrolysis of POM ester to the parent acid in intestinal tissue was faster for a more lipophilic ester. Hydrolytic activity, which was detected in the content of the intestinal lumen, would lower the absorption. The effect of the C-3 substituent on water solubility was not important for the absorption of cephalosporin employed in the present study.

Isomerization of the double bond, which was found to be characteristic for cephalosporin ester, presented a problem in the prodrug approach for oral use.

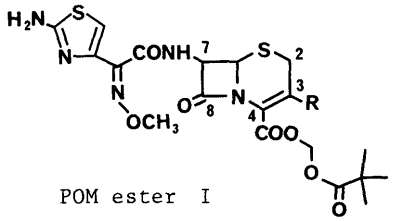
Keywords cephalosporin; prodrug; oral absorption; substituent effect; isomerization; Δ^2 cephalosporin; lipophilicity; stability; esterase

Recently ester-type prodrugs of cephalosporin have been investigated for oral use.[2-8] They are absorbed from the small intestine, hydrolyzed in the intestinal epithelial cells by esterases and transferred into the blood stream as their parent acids. In the development of cefpodoxime proxetil (CPDX-PR), a new orally active cephalosporin ester, we found that the substituent at the C-3 position plays an important role in the intestinal absorption of ester-type prodrugs of cephalosporin as well as in the antimicrobial activity of the parent acid.[4] However, the reason has remained obscure.

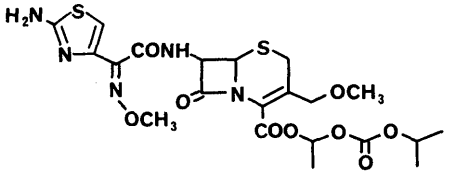
It is generally accepted that lipophilicity of a drug is an important factor in intestinal absorption.[9] If the drug is a solid with low hydrophilicity, its water solubility would also influence intestinal absorption.[6c,9] Yoshimura *et al.* reported that the oral bioavailability of cephalosporin pivaloyloxymethyl (POM) esters is linearly correlated with their water solubility.[10] Additionally, for the prodrug, it is necessary to consider its stability in the gastrointestinal lumen prior to absorption and its conversion to the parent drug after absorption.

In the preceding papers,[1] the C-3 substituent of cephalosporin POM esters Ia—k was found to influence their chemical stability in phosphate buffer solution. The stability depends on the isomerization rate from the Δ^3 cephalosporin ester I to the Δ^2 ester II. The Δ^2 ester II is readily hydrolyzed to the biologically inactive Δ^2 acid IV.

In this study, the contribution of isomerization to the decrease of bioavailability was examined by determining the Δ^2-isomer excreted in urine and feces. Further, the effects of the C-3 substituent on partition coefficient, water solubility and stability in the gastrointestine were examined, and correlation analysis between these factors and oral bioavailability in mice is attempted. Based on the



POM ester I



cefpodoxime proxetil
(CS-807)

	-R		-R
Ia	$-CH_2OCH_3$	Ig	$-CH_2STz$
Ib	$-CH_2OCH_2CH_3$	Ih	$-CH_2STh$
Ic	$-CH_2O\overset{O}{\overset{\|}{C}}CH_3$	Ii	$-CH_2Tet$
Id	$-CH_2OIox$	Ij	$-CH_3$
Ie	$-CH_2SCH_3$	Ik	$-H$
If	$-CH_2SCH_2CN$		

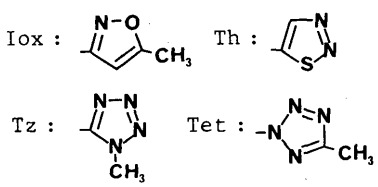


Chart 1

Δ^3 cephalosporin Δ^2 cephalosporin

I : X=POM II : X=POM

III: X=H IV : X=H

Chart 2

results, the effect of the C-3 substituent on the oral absorption of cephalosporin ester is discussed.

Experimental

Materials Cephalosporin POM esters employed in this study are listed in Chart 1. They are the same as those used in the preceding papers.[1] The structures of the Δ^3 and Δ^2 cephalosporin isomers are shown in Chart 2.

Oral Administration of POM Esters Suspension: POM ester (5 mg as the parent acid) was suspended in water with 5% tragacanth (1 ml).

Solution: POM ester was dissolved in polyethyleneglycol (PEG-400) (0.5 ml) and diluted with water (0.5 ml).

Administration: The suspension or solution prepared as mentioned above was administered orally to slc ddY mice (male, $n=5$, 50 mg/kg as the parent cephalosporin). Mice had free access to water but were fasted overnight before administration.

Determination of Parent Acid and Its Δ^2-Isomer Recovered in Urine and Feces Urine excreted from 5 mice in 24 h was collected in one bottle. Feces were collected in another bottle. The average amount of recovered cephalosporin was determined as follows.

Parent Acid (Δ^3 Acid III): Recovery was determined by a bioassay method using *Bacillus subtilis* ATCC 6633 as a test strain.

Δ^2-Isomer IV: The ratio of Δ^3 and Δ^2 isomers was determined using high performance liquid chromatography (HPLC). Recovery of the Δ^2 isomer was calculated from this ratio and the Δ^3 acid recovery.

Partition Coefficient Cephalosporin ester was dissolved in *n*-octanol (*ca.* 500 μg/ml). This solution (1 ml) and phosphate buffer (1 ml, 1/20 M, Na_2HPO_4/KH_2PO_4, pH 6.86) were shaken at 25 °C for 30 s.[11] The mixture was then centrifuged to separate the phases. The concentration of the ester in each phase was determined by HPLC. The organic phase was diluted 100 times with MeOH before analysis. The partition coefficient (*P*) was obtained by dividing the concentration in the organic phase by that in the aqueous phase.

Water Solubility Cephalosporin ester (5 mg) was suspended in phosphate buffer (1/10 M, Na_2HPO_4/NaH_2PO_4, pH 6.0, 2 ml) and gently stirred at 20 °C for 1 h. The mixture was filtered (pore size 0.2 μm, Gelman Sciences Japan (No. E134)). The concentration of the ester in the filtrate was determined using HPLC. Water solubility was expressed in μg/ml.

Preparation of Tissue Homogenate of Mouse Small Intestine Fresh small intestine of mice (1.58 g/mouse) was washed with saline (20 ml) and homogenized with 9 volumes of phosphate buffer (1/10 M, Na_2HPO_4/NaH_2PO_4, pH 6.98). The homogenate was centrifuged at 600 × *g* for 10 min. The supernatant was diluted 10 times with phosphate buffer and used in the hydrolysis experiments.

Preparation of the Washings of Mouse Small Intestinal Lumen Mouse small intestinal lumen was washed through with saline (6 ml/mouse). The washings were centrifuged at 600 × *g* for 10 min. The supernatant was used in the hydrolysis experiments.

Preparation of POM Ester Solution for Hydrolysis Experiment POM ester was dissolved in 70% EtOH–water using an ultrasonic bath to give a concentration of 3.7 mM (*ca.* 2 mg/ml).

Hydrolysis Experiment The homogenate or washings prepared as mentioned above (0.2 ml) was added to phosphate buffer (1.7 ml, 1/10 M, Na_2HPO_4/NaH_2PO_4, pH 6.98) and preincubated at 37 °C for 3 min. POM ester solution (0.1 ml) was added to the mixture and the whole was incubated at 37 °C for 30 min. The initial concentration of POM ester was about 100 μg/ml. Samples were taken out at suitable intervals, added to an equal amount of CH_3CN and centrifuged at 10000 rpm for 5 min. Concentrations of the remaining POM ester and the acid formed were determined by HPLC. Enzymatic hydrolysis rates of POM esters were calculated in μmol/min/mouse from the amount of acids produced in the initial 5 min.

HPLC Method HPLC conditions in the present study were the same as those reported previously.[1b]

Results and Discussion

Oral Absorption Recovery of the microbiologically active Δ^3 acid (parent acid) in urine or feces after oral dosage of POM ester in mice was determined by bioassay. Recovery after oral or parenteral dosage of parent Δ^3 acid was determined as well. Oral bioavailability of POM ester or parent Δ^3 acid was calculated according to the following equation;

$$\text{oral bioavailability (BA\%)} = \frac{\text{urinary recovery after } p.o. \text{ dosage}}{\text{urinary recovery after s.c. dosage of parent acid}} \times 100$$

The results are listed in Table I. By POM esterification, oral bioavailabilities of parent cephalosporins with various 3-substituents were enhanced to various extents. These results showed that the 3-substituent of the cephalosporin esters markedly influenced the intestinal absorption. POM esters with an alkoxymethyl or methyl group, Ia, Ib and Ij, showed good bioavailability.

Effect of Water Solubility Water solubilities of cephalosporin POM esters are listed in Table II. Water solubility was not related to oral bioavailability of POM esters in suspension, as shown in Fig. 1. The result does not agree with that of Yoshimura *et al.*[10]

In order to confirm the effect of water solubility on intestinal absorption, POM ester was administered in solution (PEG–water). Compared with the bioavailability after dosage in suspension, slight enhancement was observed. However, the relationship between bioavailabilities of POM esters and the various 3-substituents changed little (Fig. 1).

These results show that the alteration of oral bioavailability arising from the variation of the 3-substituent can not be due to changes in water solubility. Other physicochemical or biochemical properties of cephalosporin after dissolution in the intestinal tract must be responsible.

Effect of Lipophilicity Parent cephalosporins were little absorbed from the intestinal tract because of their low

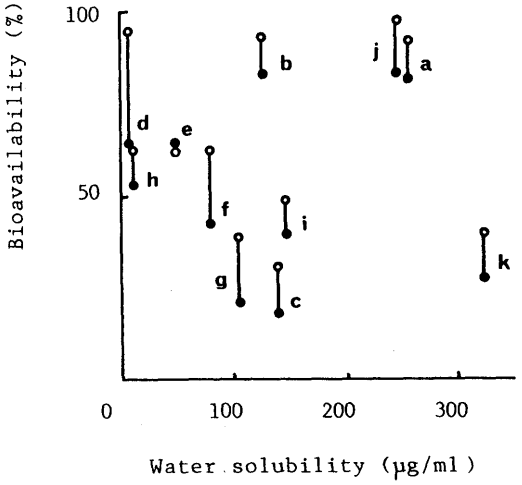


Fig. 1. Effect of Water Solubility on the Oral Absorption of Cephalosporin POM Esters Ia—k in Mice

Dosage form: ●, suspension in water with 5% tragacanth; ○, solution in PEG 400–water (1 : 1).

TABLE I. Bioavailability after Oral Dosage of Cephalosporin Esters and Acids in Mice[a]

No.	3-Substituent	U.R. and F.R. (%)[b]			Bioavailability (%)[c]	
		Parent acid		POM ester	Parent acid	POM ester
		p.o.	s.c.	*p.o.*	*p.o.*	*p.o.*
Ia	$-CH_2OCH_3$	5 (55)	93 (2)	76 (4)	5	82
Ib	$-CH_2OCH_2CH_3$	2 (30)	84 (4)	70 (6)	3	83
Ic	$-CH_2OAc$	1 (13)	45 (2)	8 (3)	2	18
Id	$-CH_2OIox$	1 (2)	65 (1)	42 (1)	2	65
Ie	$-CH_2SCH_3$	4 (54)	84 (10)	55 (13)	5	65
If	$-CH_2SCH_2CN$	3 (19)	65 (1)	28 (2)	5	43
Ig	$-CH_2STz$	5 (33)	67 (2)	14 (3)	7	21
Ih	$-CH_2STh$	2 (12)	54 (3)	29 (3)	4	54
Ii	$-CH_2Tet$	2 (5)	98 (0)	39 (2)	2	40
Ij	$-CH_3$	4 (67)	75 (7)	62 (5)	5	83
Ik	$-H$	3 (11)	95 (1)	27 (1)	3	28

a) POM esters (dose; 50 mg/kg as the parent acid) were administered to mice ($n=5$, male, slc ddY strain) and recovery was determined by using a bioassay method. *b*) U.R and F.R. indicate urinary recovery and fecal recovery as the parent acid, respectively. F.R. are given in parentheses. *c*) Bioavailability was calculated from the following equation. $\text{bioavailability (\%)} = \frac{\text{U.R. after } p.o. \text{ dosage}}{\text{U.R. after s.c. dosage of parent acid}} \times 100.$

TABLE II. Physicochemical Properties of Cephalosporin POM Esters

No.	3-Substituent	Water solubility[a] (μg/ml)	Lipophilicity[b] log P	Chemical stability[c] Chem (h^{-1})
Ia	$-CH_2OCH_3$	256	1.93	0.134
Ib	$-CH_2OCH_2CH_3$	127	2.28	0.166
Ic	$-CH_2OAc$	139	1.90	0.276
Id	$-CH_2OIox$	7	2.55	0.301
Ie	$-CH_2SCH_3$	48	2.67	0.144
If	$-CH_2SCH_2CN$	78	1.66	0.233
Ig	$-CH_2STz$	105	1.55	0.333
Ih	$-CH_2STh$	10	2.46	0.478
Ii	$-CH_2Tet$	146	1.82	0.743
Ij	$-CH_3$	246	2.00	0.052
Ik	$-H$	325	1.52	0.144

a) Water solubility was measured in phosphate buffer (pH 6.0, 1/10 M) at 20 °C under mild stirring. *b*) Partition coefficients (P) were determined between *n*-octanol and phosphate buffer (pH 6.86, 1/20 M). *c*) Degradation rate constants (Chem) in phosphate buffer solution (pH 6.86, 1/20 M, 37 °C) were taken from reference 1*b*).

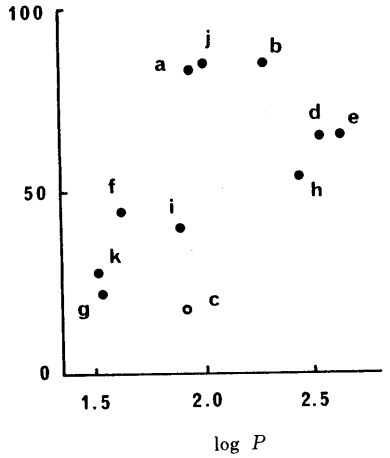


Fig. 2. Effect of Lipophilicity on the Oral Absorption of Cephalosporin POM Esters Ia—k

$BA/100 = -4.85 + 5.06 \log P - 1.14 \log P^2$. $n=10$ $s=0.130$ $r=0.866$.

Compound Ic is omitted from the regression.

lipophilicities due to the dissociation of the carboxyl function at the C-4 position. Esterification of the carboxyl group is well known as a method for improving the lipophilicity.[2-9]

The partition coefficient (P) between *n*-octanol and water was measured to evaluate the lipophilicity of each POM ester. The log P values are listed in Table II. Lipophilicity of cephalosporins increased to the range of 1.52—2.67. This improvement of lipophilicity should increase bioavailability.

A rough parabolic relation between log P and oral bioavailability was observed, as shown in Fig. 2. However, the absorption of cephalosporin esters cannot be inferred only from the lipophilicity. A contribution of some additional factors is implied.

Effect of Chemical Stability Under neutral or alkaline conditions, cephalosporin ester is rapidly hydrolyzed to Δ^2 cephalosporin acid *via* isomerization from the Δ^3 to the Δ^2 ester.[1b] Recently Saab *et al.* implied that the isomerization would account for the failure of the prodrug approach to orally active cephalosprin.[12] To examine the contribution of this isomerization to the decrease of bioavailability, excretion of the Δ^2-isomer was determined by HPLC.

After oral dosage of cephalosporin esters, a significant amount of Δ^2 acid was excreted in feces (Table III). This should be caused by the chemical degradation of administered esters prior to absorption. The amount of Δ^2 acid excretion was greater in the case of a cephalosporin ester having a larger isomerization rate.

Since Δ^2 ester is rapidly hydrolyzed chemically,[1b] the amount of Δ^2-isomer absorbed and consequently excreted in urine is small. The Δ^2/Δ^3 acid ratio in urine, however, is intrinsically related to the isomerization rate, as listed in Table III.

These results show that the isomerization of cephalosporin esters occurs prior to absorption in the intestinal lumen, and consequently decreases the oral bioavailability.

Effect of Enzymatic Hydrolysis in Small Intestine Bioconversion of CPDX-PR to a microbiologically active parent acid (Δ^3 acid) has been reported to occur in epi-

thelial cells of small intestine through the action of non-specific esterases.[13] The effect of 3-substituents on enzymatic hydrolysis of POM esters was examined using 0.1% homogenate of mouse small intestinal tissue. The results are listed in Table IV. Comparison of hydrolytic activities shows that the more lipophilic ester is hydrolyzed faster (Fig. 3a). The hydrolytic activities of 3030—7580 μmol/min/mouse were high enough for administered POM esters to be completely hydrolyzed within a few minutes. Therefore, cephalosporin esters, when absorbed in intestinal cells, are rapidly hydrolyzed to regenerate the parental Δ^3 acids.

A low level of hydrolytic activity was also detected in the content of the small intestinal lumen. Its level was about one hundredth of that in the intestinal tissue (Table IV). Recently, esterase activity from rat intestinal washings which hydrolyzed cefuroxime axetil was reported by Campbell *et al.*[14] Excretion of Δ^3 acid in feces after oral dosage of POM esters would be caused by similar esterase activity. In this hydrolysis, too, a more lipophilic ester is hydrolyzed faster, as shown in Fig. 3b. This shows that a lipophilic 3-substituent may favor the degradation of POM esters by enzymatic hydrolysis in the intestinal lumen prior to absorption.

Correlation Analysis The quantitative structure–oral absorption relationship was analyzed. Good correlations were found among oral bioavailability (BA), lipophilicity (log *P*) and chemical stability (Chem); figures in parentheses indicate 95% confidence intervals, *n* the number of samples, *s* the standard deviation and *r* the correlation coefficient.

$$BA(\%) = -4.93 + 5.29 \log P - 1.19 \log P^2 - 0.53\,\mathrm{Chem}$$
$$(\pm 1.26)(\pm 1.24) \quad (\pm 0.30) \quad (\pm 0.18)$$
$$n = 10,\ s = 0.046,\ r = 0.987 \quad (\log P)_{opt} = 2.22$$

The variable Chem represents the first-order rate constant of chemical degradation in 1/20 M phosphate buffer (pH 6.86, at 37 °C). The rate constant of isomerization from the Δ^3 to the Δ^2 ester is also available as a parameter of Chem to give a similar correlation, because the degradation depends mainly on isomerization of the cephem double bond.[1b] Ester Ic was omitted from the regression, because its degradation pattern is different from others; the acetoxy group in the 3-substituent is known to be hydrolyzed in the systemic circulation.[15]

This correlation shows that the decrease of chemical stability of POM esters linearly contributes to the lowering

Table III. Excretion of Δ^2-Isomer after Dosage of Cephalosporin POM Esters[a]

No.	3-Substituent	Recovery in feces Δ^2 acid (%)	Recovery in urine Δ^3/Δ^2 acid ratio	Isomerization rate[b] k_{12} (h^{-1})
Ij	$-CH_3$	4	59	0.06
Ia	$-CH_2OCH_3$	7	48	0.13
Ig	$-CH_2STz$	12	40	0.45
Ii	$-CH_2Tet$	15	33	0.60

a) Recovery of Δ^2 isomer was determined by HPLC. *b*) The rate constants of isomerization from Δ^3 to Δ^2 cephalosporin ester k_{12} were taken from reference 1*b*).

Table IV. Hydrolytic Activities against Cephalosporin POM Esters in Small Intestinal Tissue and Intestinal Content of Mouse

No.	3-Substituent	Hydrolytic activity (μmol/min/mouse)	
		Intestinal tissue[a]	Intestinal content[b]
Ia	$-CH_2OCH_3$	6020	59
Ib	$-CH_2OCH_2CH_3$	6760	58
Ic	$-CH_2OAc$	6940	50
Id	$-CH_2OIox$	7410	77
Ie	$-CH_2SCH_3$	7330	119
If	$-CH_2SCH_2CN$	4290	46
Ig	$-CH_2STz$	4880	37
Ih	$-CH_2STh$	7580	70
Ii	$-CH_2Tet$	3030	30
Ij	$-CH_3$	6710	47
Ik	$-H$	3510	21

a) Hydrolysis rate in 0.1% homogenate of intestinal tissue in phosphate buffer (pH 6.98, 1/10 M, 37 °C) was converted into the activity. The initial concentration of cephalosporin ester was 100 μg/ml. *b*) Hydrolysis rate with 10% intestinal tract content in phosphate buffer was converted into the activity.

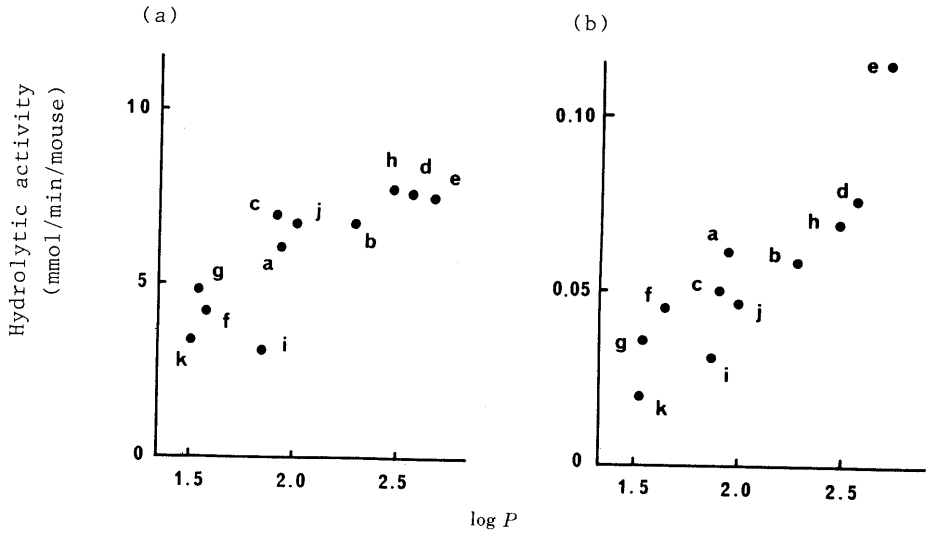


Fig. 3. Effect of Lipophilicity on the Enzymatic Hydrolysis of Cephalosporin POM Esters Ia—k in Mouse Small Intestine

(a) Homogenate of small intestinal tissue. (b) Content of small intestinal lumen in phosphate buffer (0.1 M, pH 6.98).

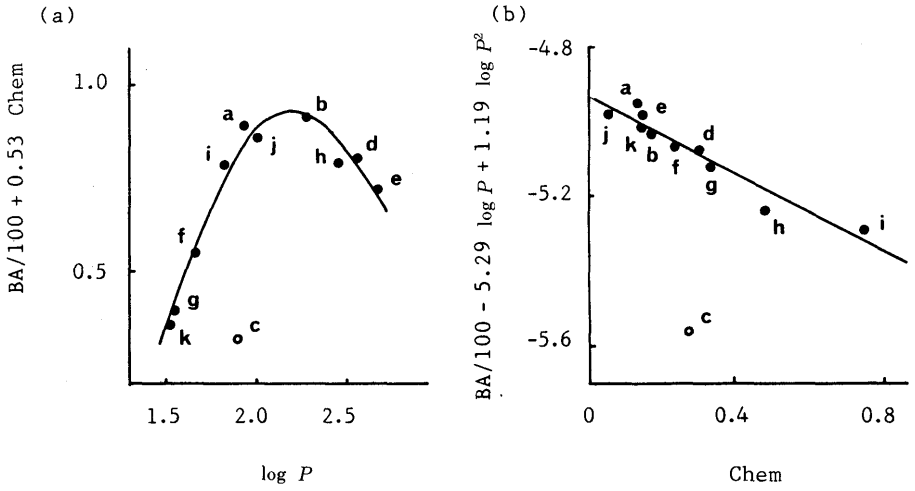


Fig. 4. Contributions of Lipophilicity (a) and Chemical Stability (b) to the Oral Absorption of Cephalosporin POM Esters Ia—k in Mouse

Chem is the degradation rate constant in phosphate buffer solution (1/20 M, pH 6.86, 37 °C).

$BA/100 = -4.93 - 0.53\,\mathrm{Chem} + 5.29\log P - 1.19\log P^2$. $n=10$ $s=0.046$ $r=0.987$ $\log P_{opt}=2.22$.

of oral bioavailability (Fig. 4b) and that lipophilicity makes a parabolic contribution to bioavailability with an optimum log P value of 2.22 (Fig. 4a). Permeability through the lipoidal membrane is known to be high in compounds possessing large log P values. Decrease of bioavailability in the log P region larger than 2.22 would be attributable to the following effects; lipophilic POM esters easily suffer enzymatic hydrolysis in the intestinal lumen and are converted into the unabsorbable parent acids as discussed above, and/or the hydrophilic mucosal layer on the intestinal membrane acts as a barrier for the absorption of highly lipophilic POM esters.

Conclusion

3-Substituents of cephalosporins have been found to determine the oral bioavailability of the ester-type prodrugs by influencing their chemical stability and lipophilicity. Isomerization of double bond, which is characteristic of cephalosporin esters, represents a serious problem in the ester-type prodrug approach for oral use. The effect of the 3-substituent on water solubility is not so important for the absorption of the cephalosporin esters employed in the present study.

The methoxymethyl group ($-CH_2OCH_3$) of cefpodoxime proxetil (CPDX-PR in Chart 1), which contributes to the stability of the ester,[1] the reactivity of the β-lactam,[4] adequate lipophilicity and moderate water solubility, is a well-balanced 3-substituent for the ester-type prodrug of cephalosporin.

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