The Metabolic Stability of Acyl-CoA: Cholesterol *O*-Acyltransferase (ACAT) Inhibitors, *N*-(4-Benzoyloxy-3,5-dimethoxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-708-424) and Its Derivatives in Rat Liver and Intestinal Epithelium

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The metabolic stability of the acyl-CoA: cholesterol *O*-acyltransferase (ACAT) inhibitor *N*-(4-benzoyloxy-3,5-dimethoxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-708-424) and its *n*-alkoxy derivatives containing an alkyl chain of 3 or 7 to 10 carbons, which exhibited different hypocholesterolemic activities, was investigated *in vivo* and *in vitro* in rats. After the oral administration of YIC-708-424 to rats at a dose of 5 mg/kg/d for 7 d, the parent compound was not detected in the blood. On the other hand, when the *n*-alkoxy derivatives were administered to rats, an increase in the alkyl chain length produced a progressive increase in the blood concentration of the parent compound. Both in the blood of rats administered YIC-708-424 and in the reaction mixture after the incubation of YIC-708-424 with rat hepatic 9000×*g* supernatants, an inactive major metabolite, *N*-(4-benzoyloxy-3,5-dimethoxycinnamoyl)-*N'*-(4-carboxyl-2-methylphenyl)piperazine, was observed. The ratio of the maximum velocity to the apparent Michaelis–Menten constant ($V_{\max}/K_m$) for the degradation of the *n*-propyloxy derivative in rat hepatic and intestinal microsomes was almost equivalent to that of YIC-708-424. On the other hand, an increase in the alkyl chain length of *n*-alkoxy derivatives produced a progressive decrease in $V_{\max}/K_m$ for the degradation of these compounds. Additionally, the *in vivo* hypocholesterolemic activities of YIC-708-424 and its *n*-alkoxy derivatives were positively correlated with the blood concentration of the parent compound and were negatively correlated with their $V_{\max}/K_m$. These results suggest that the metabolic stability of ACAT inhibitors in the liver and intestinal epithelium, which are the major target organs of these compounds, has a strong influence on their pharmacological activities *in vivo*.

Key words YIC-708-424; metabolic stability; hypocholesterolemic activity; acyl-CoA: cholesterol *O*-acyltransferase (ACAT) inhibitor; apparent kinetic parameter; Sprague-Dawley rat

N-(4-Benzoyloxy-3,5-dimethoxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-708-424; Fig. 1) and its derivatives which have an *n*-alkoxy residue containing an alkyl chain of 3 or 7 to 10 carbons at the 4b position in the B-ring (Table 1)¹⁾ are structurally novel acyl-CoA: cholesterol *O*-acyltransferase (ACAT) inhibitors synthesized from Yakuchinone B,²⁾ which is a component of the seeds of *Alpinia oxyphylla* MIQUEL (Zingiberaceae), as a lead compound. Although YIC-708-424 potently inhibits rat hepatic ACAT *in vitro*, it does not exhibit hypocholesterolemic activity when administered to rats fed a high cholesterol diet at a dose of 5 mg/kg/d for 7 d (Table 1).¹⁾ On the other hand, the *n*-alkoxy derivatives also exhibit potent ACAT inhibitory activity. However, the hypocholesterolemic activity of these compounds is remarkably different (Table 1).¹⁾ In addition, it is recognized that there is a definite positive correlation between the alkyl chain length of *n*-alkoxy derivatives and hypocholesterolemic activity *in vivo*, but not ACAT inhibitory activity *in vitro* (Table 1).¹⁾ Based on these results, it is assumed that it is not their specific activities with respect to ACAT inhibition, but rather other factors that may be involved in the difference in hypocholesterolemic activity among YIC-708-424 and its *n*-alkoxy derivatives. However, the exact mechanism is not clear.

Drugs that are orally administered undergo a first pass effect in the liver and intestinal epithelium before they reach the circulation. Because the liver and intestinal epithelium are also major targets of ACAT inhibitors,^{3–6)} it is speculated

Fig. 1. Chemical Structure of YIC-708-424

that the metabolic stability of ACAT inhibitors in these organs has a particularly strong influence on their pharmacological activities.

However, no reports have examined the relationships between the metabolic stability of ACAT inhibitors in the liver and intestinal epithelium and their hypocholesterolemic activities.

In the present study, the metabolic stability of these compounds was investigated *in vivo* and *in vitro* in rats to clarify the reason for the difference in the *in vivo* hypocholesterolemic activity among YIC-708-424 and its *n*-alkoxy derivatives.

MATERIALS AND METHODS

Materials YIC-708-424, *N*-(3,5-dimethoxy-4-*n*-propyloxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-C3-424), *N*-(3,5-dimethoxy-4-*n*-heptyloxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-C7-424), *N*-(3,5-dimethoxy-

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Table 1. Biological Profiles and Blood Concentrations of YIC-708-424 and *n*-Alkoxy Derivatives

Compounds	R	ACAT inhibition ^{a)} (nm)	Cholesterol lowering ^{b)} (% reduction)	Blood concentration ^{c)} (μM)
YIC-708-424		62	2	<0.03
YIC-C3-424	<i>n</i> -C ₃ H ₇ -O-	46	-2	<0.03
YIC-C7-424	<i>n</i> -C ₇ H ₁₅ -O-	15	25	<0.03
YIC-C8-424	<i>n</i> -C ₈ H ₁₇ -O-	15	38*	0.34
YIC-C9-424	<i>n</i> -C ₉ H ₁₉ -O-	19	49*	0.60
YIC-C10-424	<i>n</i> -C ₁₀ H ₂₁ -O-	22	65**	0.96

a) *In vitro* ACAT inhibition was measured using rat hepatic microsomes. These data were cited from reference 1. b) Each compound was administered to rats fed a high cholesterol diet (HCD) at a dose of 5 mg/kg/d for 7 d (*n*=8). Significantly different from drug-untreated control using unpaired, two-tailed Student's *t*-test, **p*<0.01, ***p*<0.001. These data were cited from reference 1. c) Each compound was administered to rats fed a HCD at a dose of 5 mg/kg/d for 7 d (*n*=8).

4-*n*-octyloxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-C8-424), *N*-(3,5-dimethoxy-4-*n*-nonyloxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-C9-424), and *N*-(4-*n*-decyloxy-3,5-dimethoxycinnamoyl)-*N'*-(2,4-dimethylphenyl)piperazine (YIC-C10-424) were synthesized at Yakult Central Institute for Microbiological Research.¹⁾ Cholesterol was obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan). Cholic acid and 6-propyl-2-thiouracil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

Analytical Procedures. HPLC HPLC analysis was performed using an HPLC system with a 2690 separation module (Waters, Milford, Massachusetts, U.S.A.); detector, 996 photodiode array detector (Waters, Milford, Massachusetts, U.S.A.); column, J's Sphere ODS-M80 (250×4.6 mm I.D.: Y.M.C., Kyoto, Japan); mobile phase, acetonitrile/methanol/H₂O/trifluoroacetic acid (54:15:31:0.085 v/v) for YIC-708-424 and YIC-C3-424, acetonitrile/methanol/H₂O/trifluoroacetic acid (66:19:15:0.085 v/v) for YIC-C7-424, YIC-C8-424, YIC-C9-424, and YIC-C10-424; flow rate, 1.0 ml/min; wavelength of detection, 235 nm, and column temperature, 40 °C.

Liquid Chromatography-Mass Spectrometry (LC-MS) The LC-MS analysis was performed using a JEOL SX-102A instrument; column, J's Sphere ODS-M80 (250×4.6 mm I.D.: Y.M.C., Kyoto, Japan); mobile phase, acetonitrile/methanol/H₂O/trifluoroacetic acid (54:15:31:0.085 v/v); flow rate, 1.0 ml/min; wavelength of detection, 235 nm. The MS conditions were as follows: accelerating voltage, 8 kV; ion source temperature, 25 °C; ionization method, FRIT-FAB; matrix, glycerin; mass range for measurement, < *m/z* 1500.

IR Spectrometry IR spectra were recorded on a Shimadzu IR435 spectrophotometer as KBr disks.

NMR Spectrometry ¹H- and ¹³C-NMR spectra were measured with a JEOL JNM-A400 (400 MHz) spectrometer. The operating conditions were as follows: internal standard, tetramethylsilane; solvent, dimethyl-*d*₆ sulfoxide (DMSO-*d*₆); temperature, 30 °C.

In Vivo Studies. Animals Male Sprague-Dawley rats weighing 120–150 g were purchased from Clea Japan Inc. (Tokyo, Japan) and maintained under the following environ-

mental conditions: room temperature of 25 °C, 55% humidity, and illumination for 12 h from 8 a.m. to 8 p.m. Rats were housed individually in a metal cage and provided with water *ad libitum*.

Diets Normal diet (ND) was F2 supplemented with cholic acid (0.5%, w/w), sucrose (10%, w/w), coconut oil (10%, w/w) and 6-propyl-2-thiouracil (0.005%, w/w). High cholesterol diet (HCD) was ND supplemented with cholesterol (1.5%, w/w).

Purification of a Putative Major Metabolite (M1) of YIC-708-424 from Rat Feces Fifteen rats were given a HCD supplemented with YIC-708-424 (0.1%, w/w) for 14 d. A total of 400 g of freeze-dried rat feces was crushed using a mill, added to ethyl acetate (4 l), and refluxed at 100 °C for 6 h. The filtrate was evaporated and purified by silica gel column chromatography (CHCl₃-MeOH, 2:1, v/v elution). After evaporation, the resulting residue was added to diisopropylether, and the insoluble fraction was separated. From this fraction, the fraction insoluble in toluene was obtained and washed with toluene to give 120 mg of M1 as a light yellow crystal.

Comparison of the Blood Concentrations of YIC-708-424 and Its *n*-Alkoxy Derivatives Rats were given a ND supplemented with the test compound (0.005%, w/w) for 4 d. For the three following days, the rats were given a HCD supplemented with the test compound (0.005%, w/w). For this investigation the dosage of each compound calculated from the body weight and the food consumption during the study period was 5 mg/kg/d. The blood (0.5 ml) was mixed with 2 ml acetonitrile and sonicated for 5 min. After vortexing for 15 min, the mixture was centrifuged (900×*g*, 10 min) and the supernatant was collected. The supernatant was then evaporated to dryness. The resulting residue was dissolved in acetonitrile/water (9:1, v/v) and the amounts of YIC-708-424 and its derivatives were measured by HPLC.

Measuring the Blood Concentration of YIC-708-424 and M1 YIC-708-424 dissolved in olive oil was orally administered in a single dose of 100 mg/kg to rats with a cannula surgically inserted into the femoral artery. At the designated time, blood was collected from the cannula. The blood was treated according to the above-mentioned method and then the concentrations of YIC-708-424 and M1 were mea-

sured by HPLC.

Analysis of the Putative Major Metabolite of YIC-708-424 and YIC-C3-424 Rats were given a HCD supplemented with YIC-708-424 (0.1%, w/w) or YIC-C3-424 (0.1%, w/w) for 7 d. For this investigation the dosage of each compound calculated from the body weight and the food consumption during the study period was 70 mg/kg/d. The rats were bled *via* the abdominal aorta while under pentobarbital anesthesia. The blood was treated by the above-mentioned method and then putative major metabolites of YIC-708-424 and YIC-C3-424 were analyzed by LC-MS.

In Vitro Studies. Measurement of ACAT Activity Microsome fractions were prepared from the livers of rats given a diet (F2, Funabashi Farmer Co., Ltd., Chiba, Japan) according to the method of Field and Salome.⁷⁾ ACAT activity was measured using [$1\text{-}^{14}\text{C}$]oleoyl-CoA as a substrate according to the method of Helgerud *et al.*⁸⁾

Metabolism of YIC-708-424 and Its *n*-Alkoxy Derivatives in Microsomes or 9000 $\times$ g Supernatants Microsomes and 9000 $\times$ g supernatants were prepared from the liver and the small intestine of rats given F2 according to the method described by Koudriakova *et al.*⁹⁾ The reaction mixture containing 100 mM phosphate buffer (pH 7.4), 1.2 mM NADPH, 6 μM YIC-708-424 and 1.6 mg protein/ml microsomes or 12.2 mg protein/ml 9000 $\times$ g supernatants in a volume of 0.5 ml was incubated at 37 $^{\circ}\text{C}$ for 2, 5, 10, 20, 30, and 50 min. The reaction was initiated by adding 5 μl of an ethanol solution of test compound and terminated by adding 2.0 ml acetonitrile, mixing, and cooling on ice. The mixture was sonicated for 5 min using an ultrasonic apparatus and was shaken at 200 strokes/min for 10 min. After centrifugation at 900 $\times$ g for 10 min, the supernatant was evaporated to dryness at 55 $^{\circ}\text{C}$. The residue was reconstituted in 0.5 ml acetonitrile-methanol-water (54:15:31, v/v/v), and an aliquot of 0.1 ml was analyzed by HPLC. For determination of the apparent metabolic kinetic parameters, five concentrations of the test compounds were assayed. The apparent initial velocity was determined while there was linearity between the reaction time and the residual amount of the compound. The apparent Michaelis-Menten constant (K_m) and the maximum velocity ($V_{\max}$) were obtained from a Lineweaver-Burke plot drawn from the initial concentration and the apparent initial velocity.

RESULTS

Blood Concentrations of YIC-708-424 and Its Derivatives after Oral Administration The blood concentrations of YIC-708-424 or its *n*-alkoxy derivatives after the administration of each compound to rats as a dietary admixture at a dose of 5 mg/kg/d for 7 d are shown in Table 1. The blood concentration of YIC-708-424, YIC-C3-424, or YIC-C7-424 was the lower limit of detection ($<0.03 \mu\text{M}$). On the other hand, in rats administered YIC-C8-424, YIC-C9-424, or YIC-C10-424, increasing the alkyl chain length produced a progressive increase in their blood concentrations.

As shown in Fig. 2A, the parent compound and a putative major metabolite (M1) were detected in the blood of rats administered YIC-708-424 for 7 d as a dietary admixture at a dose of 70 mg/kg/d. In the LC-MS analysis, the parent compound and M1 showed an $(\text{M}+\text{H})^+$ at m/z 487 and m/z 517,

Fig. 2. HPLC Chromatogram (A) and Blood Concentrations (B) of YIC-708-424 and Its Putative Major Metabolite in Rat Blood after Oral Administration of YIC-708-424

(A) Rats were administered YIC-708-424 as a dietary admixture at a dose of 70 mg/kg/d for 7 d. (B) Rats were orally administered YIC-708-424 at dose of 100 mg/kg at once. Data are the mean $\pm$ S.D. ($n=5$).

respectively.

The blood concentrations of the parent compound and M1 after oral administration of YIC-708-424 at a dose of 100 mg/kg to rats are shown in Fig. 2B. YIC-708-424 was detected in the blood beginning 1 h after administration. The blood concentration of YIC-708-424 then sequentially increased and reached a maximum at 6 h after administration. At 11 h after administration, YIC-708-424 had disappeared from the blood.

On the other hand, M1 was detected in the blood beginning 30 min after the administration of YIC-708-424, and its concentration reached a maximum at 6 h after administration. M1 was still detected in the blood 24 h after administration, although the blood concentrations of M1 had markedly decreased.

The maximum concentration and area under the curve (AUC) of M1 were 3.7 and 6.4 times higher than those of YIC-708-424, respectively.

Isolation and Identification of the Major Metabolite In preliminary studies, M1 was detected in the bile and feces of rats administered YIC-708-424. Therefore, feces was collected from rats fed the HCD containing YIC-708-424 at a concentration of 0.1% for 14 d, and the isolation and purification of M1 were attempted.

As a result, 120 mg of M1 (purity 94%) was isolated from

A

B

Fig. 3. ^1H -NMR Spectra ($\text{DMSO}-d_6$, 400 MHz) of YIC-708-424 (A) and Purified M1 (B)

400 g of freeze-dried feces. In the LC-MS analysis, the retention time of M1 purified from feces corresponded to those of the M1 detected in the blood. Additionally, the M1 purified from feces showed an $(\text{M}+\text{H})^+$ at m/z 517, which was the same mass for the M1 detected in the blood.

The ^1H -NMR spectra of YIC-708-424 and purified M1 are presented in Fig. 3. For the purified M1, a carboxyl residue signal at 12.55 ppm was observed instead of that of a methyl residue. The ^1H chemical shift values for M1 were consistent with those of YIC-708-424, except for the signals of the aromatic protons for the A-ring (δ_{H} 6.99, 6.93, 6.91). Additionally, the ^1H detected single quantum coherence (HSQC) spectrum revealed three protons at δ_{H} 7.77, 7.75, and 7.07 coupled to three carbons at δ_{C} 132.1, 128.2, and 118.6, respectively (Fig. 4). The heteronuclear multiple bond connectivity (HMBC) spectrum revealed (i) a methyl proton (δ_{H} 2.34) coupled to two carbons at δ_{C} 154.8 and 132.1, (ii) two protons at δ_{H} 7.77 and 7.75 coupled to a carboxyl carbon at δ_{C} 167.2, and (iii) a proton at δ_{H} 7.07 coupled to a carbon at δ_{C} 124.8 (Fig. 4). Further, a nuclear overhauser effect (NOE)

was observed between a methyl proton (δ_{H} 2.34) and piperazine proton (δ_{H} 2.95) and an aromatic proton in the A-ring (δ_{H} 7.77).

Finally, the ^1H - and ^{13}C -NMR signals of the M1 were assigned as shown in Table 2 and its structure was identified to be *N*-(4-benzyloxy-3,5-dimethoxycinnamoyl)-*N'*-(4-carboxyl-2-methylphenyl)piperazine in which the methyl residue at the 4a position in the A-ring of YIC-708-424 was oxidized to the carboxyl residue (Fig. 4).

On the other hand, the putative major metabolite was identified in blood after oral administration of YIC-C3-424 to rats as a dietary admixture at a dose of 70 mg/kg/d for seven days. YIC-C3-424 and its putative major metabolite showed an $(\text{M}+\text{H})^+$ at m/z 439 and m/z 469, respectively.

ACAT Inhibitory Activity of M1 The ACAT inhibitory activity of the purified M1 was compared with that of YIC-708-424. As shown in Fig. 5, YIC-708-424 strongly inhibited rat hepatic ACAT, while purified M1 did not at a high concentration (1 μM).

Metabolism of YIC-708-424 to M1 in Microsomes and

Fig. 4. Long-Range Couplings Observed in the HMBC Spectrum of Purified M1

Table 2. ^{13}C and ^1H Chemical Shifts of YIC-708-424 and Purified M1 in $\text{DMSO}-d_6^a$

Position ^{b)}	YIC-708-424		Purified M1	
	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	164.6	—	164.7	—
2	117.3	7.24 (1H, d, $J=15.6$)	117.1	7.25 (1H, d, $J=15.6$)
3	142.0	7.48 (1H, d, $J=15.2$)	142.1	7.49 (1H, d, $J=15.2$)
4	73.9	4.94 (2H, s)	73.9	4.94 (2H, s)
5	17.3	2.26 (3H, s)	17.9	2.34 (3H, s)
6	20.3	2.21 (3H, s)	167.2	12.55 (1H, br s)
7, 8	56.1	3.83 (3H, s)	56.1	3.83 (3H, s)
1' or 4'	42.2 or 45.6	3.75 (1H, br s) or 3.87 (1H, br s)	42.0 or 45.4	3.73 (1H, br s) or 3.83 (1H, br s)
2' or 3'	51.6 or 52.2	2.81 (2H, br s)	50.8 or 51.5	2.95 (2H, br s)
1a	148.4	—	154.8	—
2a	131.7	—	131.2	—
3a	131.4	6.99 (1H, d, $J=2.1$)	132.1	7.77 (1H, d, $J=2.1$)
4a	132.0	—	124.8	—
5a	126.9	6.93 (1H, dd, $J=2.1, 7.8$)	128.2	7.75 (1H, dd, $J=2.1, 8.1$)
6a	119.0	6.91 (1H, d, $J=8.0$)	118.6	7.07 (1H, d, $J=8.0$)
1b	130.8	—	130.8	—
2b, 6b	105.7	7.07 (2H, s)	105.6	7.07 (2H, s)
3b, 5b	153.2	—	153.2	—
4b	137.7	—	137.7	—
1c	137.6	—	137.6	—
2c, 6c	127.9	7.45 (1H, d, $J=8.4$)	127.9	7.45 (1H, d, $J=8.2$)
3c, 5c	128.0	7.36 (1H, t, $J=7.6$)	128.0	7.36 (1H, t, $J=8.0$)
4c	127.7	7.31 (1H, d, $J=7.2$)	127.7	7.31 (1H, d, $J=7.2$)

a) The chemical shifts are reported in δ values (ppm) relative to tetramethylsilane. b) Numbering as assigned in Fig. 1.

Fig. 5. Effects of YIC-708-424 (●) and Purified M1 (○) on Rat Hepatic ACAT

Microsome fractions were prepared from the livers of rats according to the method of Field and Salome.⁷⁾ ACAT activity was measured using $[1-^{14}\text{C}]$ oleoyl-CoA as a substrate according to the method of Helgerud *et al.*⁸⁾ Data are the mean $\pm$ S.D. of triplicate determinations.

9000 $\times$ g Supernatants from the Liver of Rats After the incubation of YIC-708-424 with microsomes or 9000 $\times$ g supernatants prepared from the liver of rats, the concentrations of the parent compound and M1 in the reaction mixture were measured.

When YIC-708-424 was incubated with microsomes, the concentration of the parent compound in the reaction mixture decreased by only 11% of the initial concentration in 10 min and it disappeared 30 min later (Fig. 6A). In this time, the generation of M1 accompanying the disappearance of YIC-708-424 was observed, but the amount was very small compared to that of YIC-708-424 consumed.

On the other hand, in the case of the incubation of YIC-708-424 with 9000 $\times$ g supernatants, the concentration of the parent compound also decreased by 23% of the initial concentration in 10 min and it remained only 1% at 50 min (Fig. 6B). The generation of M1 was observed beginning 2 min after the start of the reaction, following which its concentration sequentially increased until 20 min. The maximum concentration of M1 was 35% of the initial concentration of YIC-708-424.

When YIC-708-424 was incubated with either microsomes or 9000×*g* supernatants in the absence of NADPH, there was no change in the concentration of YIC-708-424 in the reaction mixture (Fig. 6).

Kinetic Parameters for the Metabolism of YIC-708-424

Fig. 6. Concentrations of YIC-708-424 (● or ○) and M1 (■) in the Reaction Mixture after Incubation with Microsomes (A) and 9000×*g* Supernatants (B) from the Liver of Rats

Microsomes and 9000×*g* supernatants were prepared from the liver of rats according to the method described by Koudriakova *et al.*⁹⁾ YIC-708-424 (6 μM) was incubated with microsomes (1.6 mg protein/ml) or 9000×*g* supernatants (12.2 mg protein/ml) in the presence (closed symbols) or absence (open symbols) of NADPH (1.2 mM) at 37 °C for 0–50 min. Analyses were performed using HPLC as described in the Materials and Methods. Data are the mean ± S.D. of triplicate determinations.

and Its Derivatives in Microsomes Apparent kinetic parameters for the degradation of YIC-708-424 and its *n*-alkoxy derivatives were examined using rat hepatic and intestinal microsomes.

As shown in Table 3, $V_{\max}$ and $V_{\max}/K_m$ for the degradation of YIC-708-424 in hepatic microsomes were 3.02 nmol/min/mg protein and 0.785 ml/min/mg protein, respectively. These values for the degradation of YIC-C3-424 were almost equivalent to those of YIC-708-424. $V_{\max}$ and $V_{\max}/K_m$ for the degradation of *n*-alkoxy derivatives in hepatic microsomes were negatively correlated with their alkyl chain length. $V_{\max}$ and $V_{\max}/K_m$ for the degradation of YIC-C10-424 were 50.3 and 49.1 times lower than those of YIC-708-424, respectively.

On the other hand, a correlation between the apparent K_m for the degradation of *n*-alkoxy derivatives in hepatic microsomes and alkyl chain length was not observed.

$V_{\max}$ and $V_{\max}/K_m$ but not K_m for the degradation of *n*-alkoxy derivatives in intestinal microsomes were also negatively correlated with their alkyl chain length (Table 3).

DISCUSSION

To clarify the reason for the difference in *in vivo* hypocholesterolemic activity among YIC-708-424 and its derivatives which have an *n*-alkoxy residue at the 4b position in the B-ring, the metabolic stability of these compounds was compared *in vivo* and *in vitro* in rats.

After the administration of YIC-708-424 or its *n*-alkoxy derivatives to rats at a dose of 5 mg/kg/d for seven days, the blood concentrations of each compound were compared (Table 1). YIC-708-424 was not detected in the blood. On the other hand, a definite positive correlation was observed between alkyl chain length and the blood concentrations of *n*-alkoxy derivatives. These results suggest the possibility that residues at the 4b position in the B-ring influence their bioavailability.

In the blood of rats administered YIC-708-424 for seven days as a dietary admixture at a dose of 70 mg/kg/d, the parent compound and a putative major metabolite (M1) were de-

Table 3. Kinetic Parameters for Degradation of YIC-708-424 and *n*-Alkoxy Derivatives in Microsomes

Compounds	R	Liver			Intestine		
		K_m^a (μM)	$V_{\max}^b$ (nmol/min/mg protein)	$V_{\max}/K_m$ (ml/min/mg protein)	K_m^a (μM)	$V_{\max}^b$ (nmol/min/mg protein)	$V_{\max}/K_m$ (ml/min/mg protein)
YIC-708-424		3.84 ± 0.20	3.02 ± 0.15	0.785 ± 0.010	1.42 ± 0.11	0.395 ± 0.024	0.278 ± 0.012
YIC-C3-424	<i>n</i> -C ₃ H ₇ -O-	3.11 ± 0.43	2.36 ± 0.31	0.761 ± 0.032	1.05 ± 0.07	0.277 ± 0.014	0.263 ± 0.011
YIC-C7-424	<i>n</i> -C ₇ H ₁₅ -O-	3.60 ± 0.16	0.37 ± 0.02	0.103 ± 0.001	2.55 ± 0.46	0.032 ± 0.006	0.013 ± 0.001
YIC-C8-424	<i>n</i> -C ₈ H ₁₇ -O-	5.60 ± 0.50	0.30 ± 0.03	0.053 ± 0.001	0.93 ± 0.20	0.004 ± 0.001	0.005 ± 0.001
YIC-C9-424	<i>n</i> -C ₉ H ₁₉ -O-	5.59 ± 0.66	0.15 ± 0.02	0.027 ± 0.001	1.36 ± 0.25	0.004 ± 0.001	0.003 ± 0.001
YIC-C10-424	<i>n</i> -C ₁₀ H ₂₁ -O-	3.86 ± 0.55	0.06 ± 0.01	0.016 ± 0.001	1.41 ± 0.33	0.003 ± 0.001	0.002 ± 0.001

a) Michaelis-Menten constant. *b*) Maximal velocity. Kinetic parameters (± S.E.) were obtained from Lineweaver-Burke plot drawn from the initial concentration and apparent initial velocity calculated from the residual amount of each compound. Data are the mean ± S.E.M. of triplicate determinations.

tected concurrently (Fig. 2A). After a single oral administration of YIC-708-424 at a dose of 100 mg/kg to rats, the maximum blood concentration and AUC of M1 were higher than those of YIC-708-424 by 3.7 and 6.4 times, respectively. Similar to the parent compound, M1 was detected in the blood stream beginning 30 min after the administration of YIC-708-424 and its concentration reached a maximum 6 h after administration (Fig. 2B). From these results, it appears that most of the YIC-708-424 is metabolized to M1 in the intestinal epithelium and the liver before reaching the blood circulation by the first pass effect. The hepatic and intestinal clearances of YIC-708-424 still however need to be estimated.

Next, M1 was isolated and its structure was analyzed. M1 had a molecular weight of 516, which was 30 higher than that of YIC-708-424 (MW 486), and its structure was identified to be *N*-(4-benzyloxy-3,5-dimethoxycinnamoyl)-*N'*-(4-carboxyl-2-methylphenyl)piperazine in which the methyl residue at the 4a position in the A-ring of YIC-708-424 was oxidized to the carboxyl residue (Fig. 4). As shown in Fig. 5, M1 was inactive against rat hepatic ACAT. From these results, it was found that the ACAT inhibitory activity of YIC-708-424 was markedly decreased by oxidation of the methyl residue at the 4a position in the A-ring into the carboxyl residue.

As in the case of YIC-708-424, a major metabolite having a molecular weight 30 mass higher than the parent compound was also detected in the blood of rats administered YIC-C3-424 as a dietary admixture at a dose of 70 mg/kg/d for 7 d. From this result, it is speculated that the methyl residue at the 4a position in the A-ring of YIC-C3-424 also might be oxidized to the carboxyl residue in the liver and intestines of rats.

The conversion of an aromatic methyl residue to a carboxyl residue is a general metabolic pathway widely reported for many drugs such as the oral antidiabetic drug tolbutamide. In the metabolic pathway of tolbutamide, an aromatic methyl residue is oxidized to a hydroxymethyl residue by the action of cytochrome P450 (CYP) in microsomal fractions followed by conversion to a carboxyl residue *via* an aldehyde residue by the action of the alcohol dehydrogenase in cytosol fractions.¹⁰⁾ As shown in Fig. 6A, only a little M1 was generated by the incubation of YIC-708-424 with microsomes in the presence of NADPH, while the concentrations of the parent compound in the reaction mixture rapidly decreased. On the other hand, high concentrations of M1 were generated by the incubation of YIC-708-424 with the 9000 $\times$ *g* supernatants (Fig. 6B). From these results, it was concluded that both the microsomal and cytoplasmic enzymes were needed to oxidize the methyl residue at the 4a position in the A-ring to the carboxyl residue. Therefore, it appears that YIC-708-424 is metabolized to M1 through steps similar to those of tolbutamide. However, the amount of M1 generated was lower than that of YIC-708-424 consumed, suggesting the possibility that metabolites other than M1 are generated concurrently.

The metabolic rates of YIC-708-424 and its *n*-alkoxy derivatives were investigated using rat hepatic and intestinal microsomes (Table 3). In the hepatic microsomes, $V_{\max}$ and $V_{\max}/K_m$ for the degradation of YIC-C3-424 were almost equivalent to those of YIC-708-424. On the other hand, the

longer the alkyl chain in the *n*-alkoxy derivatives was the smaller the $V_{\max}$ and $V_{\max}/K_m$. Similarly, in the intestinal microsomes, an increase in the alkyl chain length of the *n*-alkoxy derivatives produced progressive decreases in $V_{\max}$ and $V_{\max}/K_m$. These results indicate that an *n*-alkoxy residue with long alkyl chain contributes to a progressive increase in metabolic stability in the intestinal epithelium and liver. At present, the reason why the metabolism of the methyl residue at the 4a position in the A-ring is suppressed by the structural conversion at the 4b position in the B-ring is unknown. However, it was found that $V_{\max}$ rather than K_m for the degradation of these compounds in microsomes varied significantly with the structural conversions (Table 3). From this result, it seems that the residue at the 4b position in the B-ring has considerable influence on the rate of the oxidative reaction of the methyl residue and/or the rate of the dissociation of the oxidative product from the enzyme rather than the affinity of the substrate to the enzyme. In this respect, it is necessary to identify the CYP catalyzing this oxidation and to conduct more detailed kinetics studies.

The $V_{\max}$ and $V_{\max}/K_m$ for the degradation of YIC-708-424 and its *n*-alkoxy derivatives in rat hepatic and intestinal microsomes (Table 3) were negatively correlated with the blood concentration of the parent compound (Table 1). Therefore, it is thought that one of the causes of the difference in blood concentration of parent compound is the difference in the $V_{\max}$ and $V_{\max}/K_m$ in hepatic and intestinal microsomes of these compounds. However, the blood concentration of YIC-C8-424 was more than 10 times greater than that of YIC-C7-424, while the $V_{\max}/K_m$ for the degradation of these compounds in hepatic and intestinal microsomes differed by 1.9-fold and 2.6-fold, respectively. These results suggest the possibility that the residue at the 4b position in the B-ring affects not only metabolic parameters in the microsomes but also other factors such as the unbound fraction in blood and the fraction absorbed from the intestinal tract which determines their bioavailability. Therefore, the effects of the residue at the 4b position in the B-ring on these factors should be investigated.

Not only our *n*-alkoxy derivatives but also many other ACAT inhibitors that have an alkyl chain, such as CI-976¹¹⁾ and FR129169,¹²⁾ have been reported. In these compounds, it is speculated that a long-chain aliphatic hydrocarbon moiety with a structure similar to an acyl residue of an acyl-CoA, which is one of the substrates for ACAT, is introduced to increase the specificity of the compound for ACAT inhibition. In fact, the ACAT inhibitory activity of the *n*-alkoxy derivatives containing an alkyl chain length of more than 7 carbons is 3–4 times higher than that of YIC-708-424 (Table 1).¹⁾ However, a definite correlation between ACAT inhibitory activity *in vitro* and hypocholesterolemic activities *in vivo* has not been observed for YIC-708-424 and its *n*-alkoxy derivatives (Table 1).¹⁾ On the other hand, it was found that the hypocholesterolemic activities of YIC-708-424 and *n*-alkoxy derivatives were positively correlated with their blood concentrations (Table 1) and were negatively correlated with $V_{\max}$ and $V_{\max}/K_m$ in hepatic and intestinal microsomes (Table 3). Based on these results, it is thought that one of the causes of the difference in hypocholesterolemic activity among these compounds is the difference in the metabolic stability in the intestinal epithelium and liver. These results also sug-

gest that the metabolic stability of ACAT inhibitors in the liver and intestinal epithelium, which are major target organs for these compounds, has a particularly strong influence on their pharmacological activities *in vivo*.

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