0090-9556/97/2501-0123–130$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 25, No. 1


# *IN VITRO* AND *IN VIVO* DISPOSITION OF 2,2-DIMETHYL-*N*-(2,4,6-TRIMETHOXYPHENYL)DODECANAMIDE (CI-976)

## Identification of a Novel Five-Carbon Cleavage Metabolite in Rats

MICHAEL W. SINZ, ANN E. BLACK, SUSAN M. BJORGE,[1] ANN HOLMES, BHARAT K. TRIVEDI, AND THOMAS F. WOOLF

*Departments of Pharmacokinetics/Drug Metabolism (M.W.S., A.E.B., T.F.W., S.M.B.) and Chemistry (A.H., B.K.T.), Parke-Davis Pharmaceutical Research, Division of Warner Lambert*

(Received July 18, 1996; accepted October 10, 1996)

**ABSTRACT:**

**The metabolism of CI-976, a potent inhibitor of liver and intestinal acyl coenzyme A:cholesterol acyltransferase, was investigated in isolated rat hepatocytes and Wistar rats after oral administration. The major metabolite observed both *in vitro* and *in vivo* was identified as the 6-carbon, chain-shortened 5,5-dimethyl-6-oxo-[(2,4,6-trimethoxyphenyl)amino]hexanoic acid (M-4). M-4 was determined to be formed from the $\omega$-carboxylic acid 11,11-dimethyl-12-oxo-12-[(2,4,6-trimethoxyphenyl)amino]dodecanoic acid (M-1) *via* the 2- and 4-carbon, chain-shortened intermediate metabolites {9,9-dimethyl-10-oxo-10-[(2,4,6-trimethoxyphenyl)amino]decanoic acid (M-2) and 7,7-dimethyl-8-oxo-8-[(2,4,6-trimethoxyphenyl)amino-]octanoic acid (M-3)}, respectively. M-1 was, in turn, determined to be derived from $\omega$-hydroxy CI-976. A minor metabolite, identified *in vitro* and *in vivo*, was a novel 5-carbon, chain-shortened derivative, 6,6-dimethyl-7-oxo-7-[(2,4,6-trimethoxyphenyl)amino]heptanoic acid (M-5). M-5 was shown not to be formed from either M-1 or the $\omega$-hydroxy derivative. Separate incubation of CI-976 ($\omega$-oxidation and $\beta$-oxidation pathways) and M-1 ($\beta$-oxidation only) indicated a potential gender difference in the $\omega$-oxidation of CI-976. Both the $\omega$-oxidation and $\beta$-oxidation pathways were enhanced by clofibrate and phenobarbital induction, and CI-976 metabolism was completely inhibited when coincubated with SKF525A pointing to cytochrome P450-mediated metabolism, presumably CYP4A. Etomoxir and L-carnitine had minor effects on the $\beta$-oxidation of M-1, indicating $\beta$-oxidation occurs predominately within peroxisomes.**

ACAT[2], (E.C. 2.3.1.1.26) is a key enzyme involved in cholesterol absorption from the gastrointestinal tract and cholesterol deposition in the body (1). The therapeutic potential of ACAT inhibitors as lipid lowering and antiatherosclerotic agents has been postulated for the treatment of hypercholesterolemia (2). The fatty acid anilide, CI-976 (fig. 1), is a potent competitive inhibitor of both liver and intestinal ACAT (3). CI-976 (50 mg/kg) has been found to lower plasma cholesterol by 60% in cholesterol-fed rats and increase levels of high density lipoprotein cholesterol by 94% (4). Preliminary studies in rat, monkey, and human liver microsomes indicate that CI-976 was metabolized predominately by $\omega$-, ($\omega$-1)-, and ($\omega$-2)-hydroxylation of the fatty acid side chain followed by subsequent oxidation to the respective carboxylic acid or ketone derivatives (5) (fig. 1). These oxidation reactions were mediated by active protein and required NADPH as a cofactor implicating CYP as an enzyme involved in the initial hydroxylation reactions and presumably some form of microsomal dehydrogenase in the subsequent oxidation steps. Interestingly, no *O*-demethylated metabolites of CI-976 were detected in the postreaction microsomal incubates.

*In vivo* pharmacokinetic studies in male rats found CI-976 to have moderate absorption and bioavailability (29%), with an intravenous elimination half-life of 8 hr (6). After intravenous or oral administration to male rats, CI-976 was extensively metabolized to a single major urinary metabolite identified as **M-4** (fig. 1), a 6-carbon, chain-shortened carboxylic acid derivative (7). The presumed metabolic route to the **M-4** metabolite is $\omega$-hydroxylation and oxidation of the terminal carbon to the carboxylic acid, followed by $\beta$-oxidation.

To understand further the metabolism of CI-976, studies to determine the disposition and metabolism in rats were performed. The metabolism of CI-976 was examined both in hepatocyte suspensions and after oral administration to both male and female rats. In these studies, the metabolic pathways leading to the formation of **M-4** were explored using metabolic intermediates as substrates, and by examining the effects of various inhibitors and inducers on the metabolism of CI-976 in hepatocyte incubations. Metabolites found in postreaction hepatocyte incubations and rat urine were characterized by HPLC, LC/MS, and GC/MS. Similar types of experiments were conducted with a new metabolite observed both *in vitro* and *in vivo*, which arises from an unusual mechanism (*i.e.* removal of 5-carbon units from the CI-976 fatty acid side chain).

### Materials and Methods

CI-976 and [$^{14}$C]CI-976 (20.72 $\mu$Ci/mg ring-labeled, 99.5% chemical and radiochemical purity); methyl-5,5-dimethyl-6-oxo-6-[(2,4,6-trimethoxyphe-

Portions of this work were presented at the 1992 North American International Society for the Study of Xenobiotics, Bar Harbour, FL (Sinz *et al.*, 1992).

[1] Present address: Bayer Research, 400 Morgan Lane, West Haven, CT.

[2] Abbreviations used are: ACAT, acyl coenzyme A:cholesterol acyltransferase; CI-976, 2,2-dimethyl-*N*-(2,4,6-trimethoxyphenyl)dodecanamide; CYP, cytochrome P450; **M-4**, 5,5-dimethyl-6-oxo-6-[(2,4,6-trimethoxyphenyl)amino]hexanoic acid; IS, internal standard; **M-1**, 11,11-dimethyl-12-oxo-12-[(2,4,6-trimethoxyphenyl)amino]dodecanoic acid; **M-5**, 6,6-dimethyl-7-oxo-7-[(2,4,6-trimethoxyphenyl)amino]heptanoic acid; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; P450, cytochrome P450; UNK, unknown; **M-2**, 9,9-dimethyl-10-oxo-10-[(2,4,6-trimethoxyphenyl)amino]decanoic acid; **M-3**, 7,7-dimethyl-8-oxo-8-[(2,4,6-trimethoxyphenyl)amino]octanoic acid.

**Send reprint requests to:** Dr. Michael W. Sinz, Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48105.

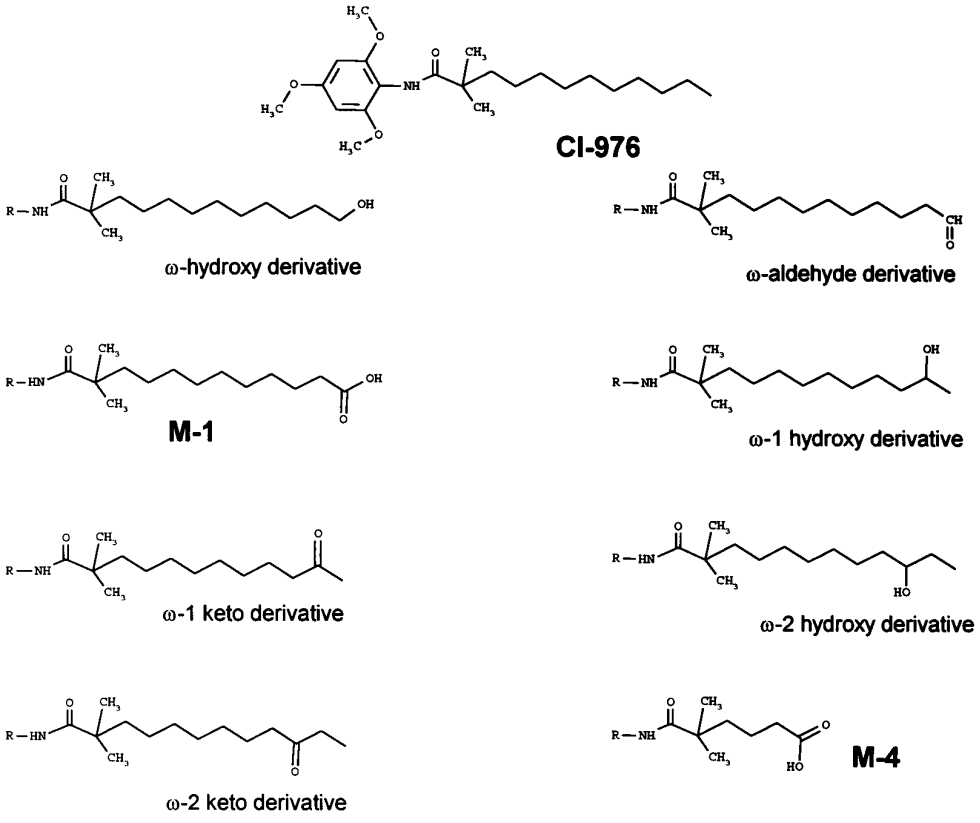


FIG. 1. *Chemical structures of CI-976 and its proposed metabolites (R = 2,4,6-trimethoxyphenyl).*

nyl)amino]hexanoate (IS); 12-hydroxy-2,2-dimethyl-*N*-(2,4,6-trimethoxyphenyl)dodecanamide; **M-1**; **M-4** (7); and **M-5** were obtained from Parke-Davis Pharmaceutical Research (Ann Arbor, MI). **M-1** was synthesized in a similar manner as previously described for **M-4** (7), starting with 1,9-dichlorononane that was converted to 1-chloro-9-iodononane by reaction with sodium iodide in refluxing acetone. **M-5** was synthesized in an analogous fashion starting with 1-bromo-4-chlorobutane.

Clofibrate, phenobarbital, L-carnitine, HEPES, Williams' Media E, collagenase Type IV, BSA, EDTA, and CMC were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, water, ethyl acetate, cyclohexane, dichloromethane, methanol, ammonium acetate, and trifluoroacetic acid (all HPLC grade) were obtained from EM Science (Gibbstown, NJ). Phosphoric acid (HPLC grade) was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Tween 20 was obtained from Aldrich Chemical Co. (Milwaukee, WI). SKF525A and Etomoxir were gifts from SmithKline Beecham Pharmaceuticals (Philadelphia, PA) and Byk Gulden, Lomberg Chemische Fabirk GmbH, respectively. Ready Safe was obtained from Beckman Instruments (Fullerton, CA). All other chemicals and materials were of the highest grade commercially available.

***In Vivo* Animal Experiments.** Four Wistar rats per gender (179–276 g) received single 50 mg/kg therapeutic oral suspension doses of [$^{14}$C]CI-976 (33–51 $\mu$Ci) in CMC/ethanol/Tween 20 (90.4:9.4:0.2), with a final concentration of CI-976 at 14.2 mg/ml (4). Animals were housed in individual stainless-steel metabolism cages for collection of urine and feces. Animals were allowed food and water *ad libitum*. Urine was collected predose and over the intervals 0–8, 8–24, 24–48, 48–72, 72–96, and 96–120 hr postdose. Urine was stored at −20°C before analysis.

**Animal Induction.** Male Wistar rats (3 animals/group) were injected intraperitoneally once daily for 3 days with either saline (phenobarbital control), sodium phenobarbital (80 mg/kg), corn oil (clofibrate control), or clofibrate (300 mg/kg). Hepatocytes were isolated from all animals 24 hr after the last injection.

**Analysis of Radioactivity in Urine and Feces.** Urine (0.1–0.2 ml aliquots) was analyzed directly for total radioactivity after addition of 16 ml of Ready Safe. Fecal samples were homogenized in 10% (w/v) water, and duplicate 1.0 ml aliquots were transferred to combustion cones, air-dried, and combusted in a Packard Tri-Carb sample oxidizer. The resulting $^{14}CO_2$ was trapped with Carbo-Sorb II and counted in Permafluor V. Each sample was counted in a Packard Tri-Carb liquid scintillation spectrometer with external standardization for quench correction.

Reversed-phase HPLC coupled to on-line radioactivity detection was used to profile urine from male and female rats. Fifty-microliter aliquots of urine collected through 72 hr were directly injected onto a 3-$\mu$m Percosphere C-18CR column (0.46 × 33 mm; Perkin-Elmer, Norwalk, CT). Separation was achieved by isocratic liquid chromatography using a mobile phase of 20% acetonitrile/80% water containing 1% acetic acid (pH 2.8) at a flow rate of 1.5 ml/min.

**Characterization of the M-5 Metabolite.** Two-milliliter aliquots of pooled female rat urine (0–8 hr) were passed through $C_{18}$ BondElut cartridges (3-ml capacity) preconditioned with one column of methanol followed by water. The retained analytes were washed successively with one column volume of 0.1 N HC1, 0.1 N HC1/methanol (1:1), twice with 0.1 N NaOH, and water. Subsequent elution with methanol resulted in a recovery of ~90% of the total radioactivity applied to the column. After concentration under nitrogen, samples were dissolved in methanol for LC/MS analysis or derivatized with diazomethane for GC/MS analysis. For GC/MS analysis, residues were treated with a solution of freshly prepared diazomethane in ether. After methylation, extracts were concentrated under nitrogen and reconstituted in ethyl acetate for injection onto the GC/MS system.

***In Vitro* Animal Studies—Hepatocyte Isolation.** Hepatocytes were obtained from male (308–401 g) and female (249–284 g) Wistar rats. Animals were fed a standard rat chow diet and were allowed food and water *ad libitum* before isolation of hepatocytes. Hepatocytes were isolated by the two-step collagenase perfusion method of Seglen (8). Digested liver was placed in a dish containing 50 ml of Williams' Medium E (25mM HEPES) and incubated for 15 min at 37°C. Cell suspension was centrifuged 3 times at 50*g* for 5 min to sediment parenchymal cells. The final supernatant was removed, and the cells resuspended in 40 ml of media from which the viability and cell number were determined by Trypan Blue exclusion (viability of all preparations were 92% ± 2 (mean ± SD)). Cells were diluted in media and incubated in 125 ml Erlenmeyer flasks containing 10 ml of 1 × $10^6$ cells/ml. Substrates were

dissolved in dimethylsulfoxide and added in a total volume not exceeding 1% (v/v) of the incubate volume. Incubate concentrations of substrates were as follows: CI-976, 50 $\mu$M; **M-1**, 24 $\mu$M; **M-4**, **M-5**, and the $\omega$-hydroxy derivative, 20 $\mu$M. Incubate concentrations of inhibitors and inducer were as follows: SKF525A, 50 $\mu$M; etomoxir, 34 $\mu$M; and L-carnitine, 1.0 mM. All incubations were performed in a shaking water bath at 37°C, 50 rpm, under an atmosphere of 95% oxygen/5% carbon dioxide. One-milliliter aliquots were removed from duplicate flasks, quenched with an equal volume of 20 mM sodium phosphate buffer (pH 1.5), and stored at −20°C before analysis.

**Hepatocyte Sample Analysis and Analytical HPLC.** Samples obtained from incubates were assayed for unchanged CI-976, **M-1**, **M-4**, and **M-5** by a dual liquid–liquid extraction procedure followed by reversed-phase HPLC. Concentrations were determined by an IS method using peak area comparisons to standard curves for each analyte. Standard samples were extracted and analyzed in duplicate, and were in the concentration range of 0.25 to 10 $\mu$g/ml for **M-1**, **M-4**, and **M-5**, and 0.25 to 50 $\mu$g/ml for CI-976.

Hepatocyte sample aliquots (1.0 ml) were thawed on ice and 50 $\mu$l of IS (100 $\mu$g/ml) added. Five milliliters of cyclohexane/ethyl acetate (9:1) was added and the samples placed on a mechanical shaker (150 strokes/min) for 20 min. Samples were centrifuged at 1,500*g* for 10 min at room temperature and the organic supernatant transferred to clean tubes. The aqueous layer was then reextracted with 5.0 ml of dichloromethane in a similar fashion as described. Organic fractions were combined and dried under nitrogen at 40°C. Samples were initially reconstituted in 150 $\mu$l of acetonitrile followed by the addition of 150 $\mu$l of sodium phosphate buffer (20 mM, pH 2.2). Standard curve samples were treated in an identical fashion as the incubation samples. The analytical HPLC assay system was comprised of a Waters $\mu$Bondapak $C_{18}$ reversed-phase column (0.46 × 300 mm) with UV detection at 240 nm. The mobile phase consisted of acetronitrile and sodium phosphate buffer (20 mM, pH 2.2) at a flow rate of 1.5 ml/min. The initial mobile phase was 30% acetonitrile/buffer for 10 min, after which time the mobile phase was stepped to 50% acetonitrile/buffer for 15 min, and then to a final 72% acetonitrile/buffer for 15 min.

**Isolation of Microsomes from Rat Hepatocytes and Determination of Protein Concentration and Total Spectral CYP.** Forty milliliters ($1 \times 10^6$ cells/ml) of freshly isolated hepatocyte suspension was centrifuged at 1,500 rpm. The supernatant was removed, 1.0 ml of isotonic KCl buffer (pH 7.4) added, and the tube placed at −20°C for 20 min. After one freeze/thaw cycle, the suspension was sonicated for 20 sec and homogenized using a glass mortar and pestel. The homogenate was centrifuged at 10,000*g* for 20 min at 4°C. The resulting supernatant was centrifuged at 100,000*g* for 60 min at 4°C. The supernatant was discarded and the microsomal pellet resuspended in 5.0 ml of 25 mM potassium phosphate buffer (pH 7.4). Protein concentration was determined by the Bradford method (9). Standard curves were generated using BSA as the protein source. CYP concentration was determined by the method of Omura and Sato (10).

**MS.** LC/MS and GC/MS analyses of the **M-5** metabolite from female rat urine were performed on a VG Trio-2 mass spectrometer coupled to either a 600 MS multisolvent delivery system. (Waters, Milford, MA), or a Hewlett-Packard 5890 gas chromatograph (Wilmington, DE) equipped with a capillary splitless injector, respectively. For LC/MS analysis, separation was achieved with a 3 $\mu$m Percosphere C-18CR column as described previously. The mobile phase consisted of acetonitrile/0.1 M ammonium acetate (10:90; pH 1.8, adjusted with 1% trifluoroacetic acid) at a flow rate of 1.0 ml/min. The mass spectrometer was operated in the plasmaspray mode with a source temperature of 250°C, a capillary temperature 240°C, and a discharge current of 300 $\mu$A. Full scans from 600–150 Da were taken repetitively at a scan rate of 1 sec/decade.

For GC/MS analysis, a fused silica capillary column (30 m × 0.25 mm i.d., 1 $\mu$m film thickness), coated with DB-1 bonded stationary phase (J & W Scientific, Rancho Cordova, CA) was used and operated with helium (head pressure: 10 psi) as the carrier gas. Samples were injected in the splitless mode (injector temperature: 250°C) and cold-trapped on the column at 40°C for 1 min. The column temperature was raised to 180°C using a linear program of 50°C/min, followed by a second linear program to 280°C at 10°C/min. The column temperature was then held at 280°C for 50 min. The mass spectrometer was operated in the electron impact positive mode, with a filament emission current and electron energy of 1,000 $\mu$A and 70 eV, respectively. Full scans (600–150 Da) were taken repetitively at a scan rate of 1 sec/decade.

Characterization of metabolites from extracted hepatocyte preparations was achieved on a Hewlett Packard 5970A GC/mass selective detector (Wilmington, DE) with a fused silica capillary column (30 m × 0.25 mm i.d., 1-$\mu$m film thickness), coated with DB-17 bonded stationary phase (J & W Scientific). Extracted hepatocyte samples were treated with diazomethane before injection onto the GC/MS. Instrument operating conditions and sample preparations were similar to those described previously for the **M-5** metabolite.

**Analysis of Data.** Urinary excretion was reported as percentage of radioactive dose administered. The rate of disappearance of CI-976 and **M-1** from hepatocyte preparations was determined from time *vs.* concentration plots for each individual rat hepatocyte experiment performed with duplicate incubation flasks. Statistical analysis was performed by comparing the means between groups. The statistical method used was a two-tailed, unpaired *t* test.

## Results

***In Vivo* Rat Studies.** Mean cumulative urinary and fecal recoveries of radioactivity in male animals after a single 50 mg/kg oral suspension dose of [$^{14}$C]CI-976 was 44.1% (range: 41.7–47.1) and 50.0% (range: 41.0–62.6) of dose, respectively. In female animals, mean cumulative urinary recovery was 71.0% (range: 66.6–78.3), whereas cumulative fecal recovery averaged 22.8% (range: 16.0–29.1) of the dose. Excretion of radioactivity was nearly complete 48 hr postdose, with mean total recovery through 120 hr of 94.1% in males and 93.8% in females.

HPLC-radioactivity profiles of 0–72 hr rat urine indicated the presence of five radioactive components (UNK A, UNK B, UNK C, **M-4**, and **M-5**) (table 1). The 0–72 hr urine from male rats represented 43.0% of the dose, with 99% of urinary radioactivity being accounted for by the five components. In female rat urine, 69.2% of the dose was recovered in the 0–72 hr collection period, with the same five components accounting for 99% of the urinary radioactivity. In both profiles, the major radioactive metabolite was the 6-carbon, chain-shortened carboxylic acid metabolite (**M-4**) based on chromatographic and spectroscopic comparisons to synthetic material. No unchanged CI-976 was observed in male or female urine. Concentrations of the polar unknowns (UNK A, UNK B, and UNK C) were too low to allow identification. LC/MS of the **M-5** component isolated from female rat urine had a protonated molecular ion $[M + H]^+$ at *m/z* 354, 14 mass units greater than the 6-carbon, chain-shortened carboxylic acid metabolite (**M-4**) (fig. 2*A*). Figure 2*B* illustrates the LC/MS spectrum of synthetic **M-5** that also exhibits a protonated molecular ion $[M + H]^+$ at *m/z* 354. A separate **M-5** urine extract was treated with diazomethane before electron impact GC/MS analysis and displayed a molecular ion at *m/z* 367, corresponding to the methylated **M-5** (fig. 3). Diagnostic fragment ions at *m/z* 253 and 183, characteristic of the trimethoxyaniline dimethylacetamide portion of CI-976 were observed, suggesting that this portion of the molecule remained intact.

***In Vitro* Metabolism of CI-976.** In hepatocyte preparations, the

TABLE 1

*Radioactive components in male and female rat urine after a single 50 mg/kg oral suspension dose of [$^{14}$C]CI-976*

| Gender | % of CI-976 Dose | | | | |
|---|---|---|---|---|---|
| | Radioactive Components | | | | |
| | UNK A | UNK B | UNK C | **M-4** | **M-5** |
| Male | 2.4 ± 2.5 | 4.6 ± 1.3 | 1.6 ± 1.2 | 33.0 ± 2.1 | 1.0 ± 0.6 |
| Female | ND | 2.6 ± 0.5 | 1.8 ± 0.9 | 58.0 ± 4.3 | 6.3 ± 0.9 |

Values are means ± SD. ND, not detected.

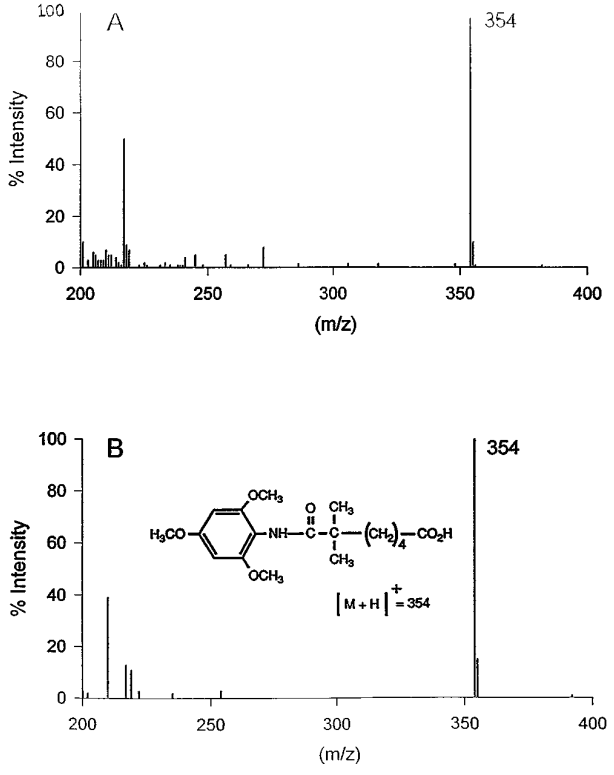


FIG. 2. *LC/MS comparison of **M-5** metabolite (A) and synthetic reference material (B).*

Analysis was conducted on a underivatized extract of female rat urine from animals given a single 50 mg/kg oral dose of [$^{14}$C]CI-976. See Materials and Methods *for conditions.*

major and minor metabolites derived from CI-976 and detected by reversed-phase HPLC were **M-4** and **M-5**, respectively (fig. 4*A*). The identity of both metabolites were characterized by HPLC cochromatography and GC/MS, and compared with synthetic reference

TABLE 2

*Rate of CI-976 (50 μM) and **M-1** (25 μM) disappearance in freshly isolated hepatocytes from male and female rats*

| Gender/Treatment | Rate of CI-976 Disappearance[a] | Rate of **M-1** Disappearance[a] |
|---|---|---|
| Male ($N$ = 7) | 0.40 ± 0.10[b] | 0.63 ± 0.20[c] |
| Male PB control ($N$ = 3) | 0.56 ± 0.07[b] | 0.69 ± 0.15[b] |
| Male PB induced ($N$ = 3) | 0.91 ± 0.19[c] | 1.33 ± 0.11[b] |
| Male CF control ($N$ = 3) | 0.36 ± 0.17[c] | 0.43 ± 0.12[d] |
| Male CF induced ($N$ = 3) | 1.49 ± 0.13[d] | 2.48 (2.30, 2.66)[e,d] |
| Female ($N$ = 3) | 0.14 ± 0.05[b] | 0.73 ± 0.28 |

Values are means ± SD. PB, phenobarbital; CF, clofibrate.

[a] Nmol/min/$10^6$ cells.

[b] $p < 0.01$ between male and female (CI-976) and PB control and induced (**M-1**).

[c] $p < 0.05$ between PB control and induced (CI-976) and the rate of CI-976 and **M-1** disappearance.

[d] $p < 0.001$ between CF control and induced (both CI-976 and **M-1**).

[e] Mean of two values.

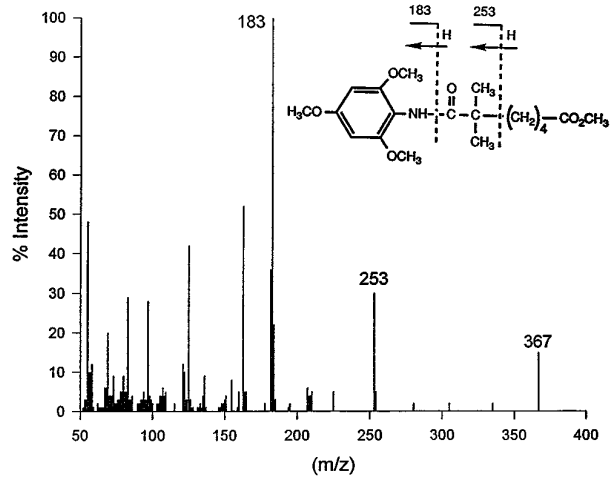


FIG. 3. *Electron impact GC/MS spectrum of the **M-5** metabolite from female rat urine after derivatization with diazomethane.*

Analysis was conducted on a diazomethane-treated extract of female rat urine from animals given a single 50 mg/kg oral dose of [$^{14}$C]CI-976. See Materials and Methods *for conditions.*

compounds. Total spectral CYP was determined in microsomes obtained from freshly prepared male rat hepatocytes, as well as hepatocytes isolated from phenobarbital- and clofibrate-induced male rats (0.73 ± 0.14, 1.84 ± 0.25, 0.90 ± 0.30 nmol P450/mg protein, respectively) to confirm CYP induction. The rate of CI-976 disappearance was significantly faster in male rat hepatocyte preparations (0.40 nmol/min/million cells), compared with the corresponding female preparations (0.14 nmol/min/million cells) (table 2). Hepatocyte incubations of CI-976 from both phenobarbital- and clofibrate-induced male rats resulted in significant increases in the rate of CI-976 disappearance. In addition, male rat hepatocytes incubated with CI-976 in the presence of the CYP inhibitor, SKF525A, exhibited complete inhibition of CI-976 metabolism (fig. 5*A*).

***In Vitro* Metabolism of M-1.** When **M-1** (ω-carboxylic acid) was used as substrate in these same hepatocyte preparations, **M-4** was identified as the major metabolite formed along with two new metabolites (fig. 4*B*). No detectable amounts of **M-5** were observed when **M-1** was used as substrate in either male or female rat hepatocyte suspensions. An extracted male rat hepatocyte sample was treated with diazomethane to derivatize acidic functionalities before electron impact GC/MS. The two new metabolites observed in these chromatograms, characterized by GC/MS, were the C-10 and C-8 carboxylic acid intermediates (**M-2** and **M-3**, respectively) along the β-oxidation pathway of **M-1** (C-12) to **M-4** (C-6). The methylated **M-2** and **M-3** metabolites displayed molecular ions of *m/z* 409 and 381, respectively (fig. 6). Fragment ions at *m/z* 253 and 183, characteristic of the trimethoxyaniline dimethylacetamide portion of CI-976, were present with both metabolites.

The rate of **M-1** disappearance was similar between male and female hepatocyte preparations (table 2). Hepatocyte incubations of **M-1** from both phenobarbital- and clofibrate-induced male rats resulted in significant increases in the rate of **M-1** metabolism (*i.e.* increased rate of β-oxidation) (table 2). Male rat hepatocytes incubated with **M-1** in the presence of the mitochondrial β-oxidation inhibitor, etomoxir, exhibited a 25% decrease in the rate of **M-1** metabolism (fig. 5*B*). Also, male rat hepatocytes incubated with **M-1** in the presence of the mitochondrial β-oxidation enhancer, L-carnitine, resulted in a minor 5% increase in the rate of **M-1** metabolism (fig. 5*B*).

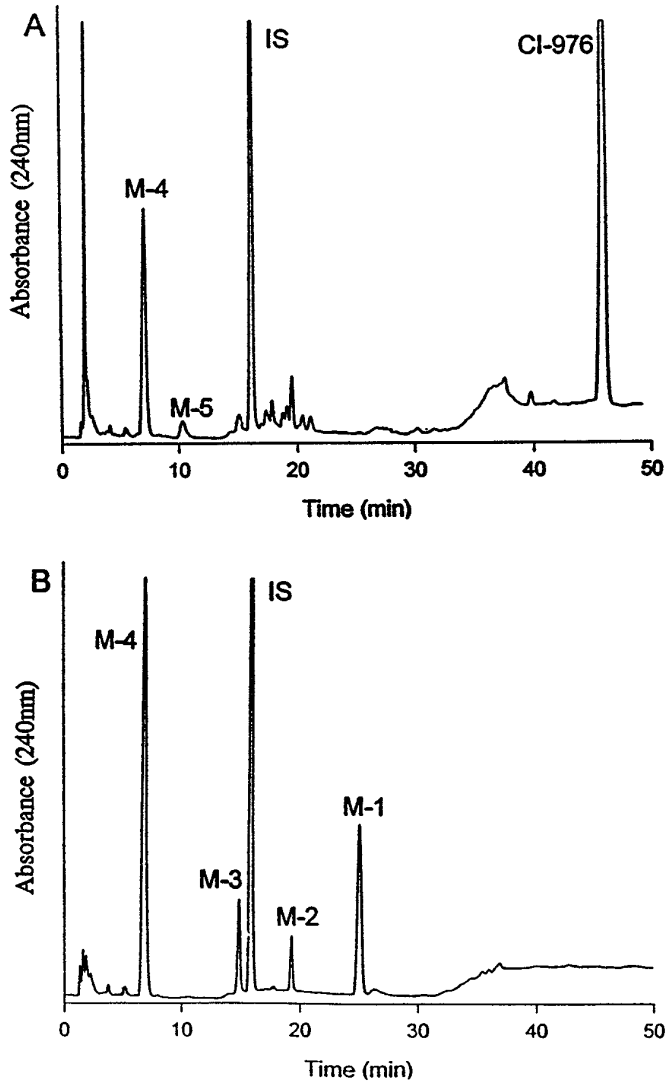


FIG. 4. *HPLC/UV chromatograms of a 60-min extracted male rat hepatocyte sample incubated with CI-976 (A) and a 30-min extracted male rat hepatocyte sample incubated with* ***M-1*** *(B).*

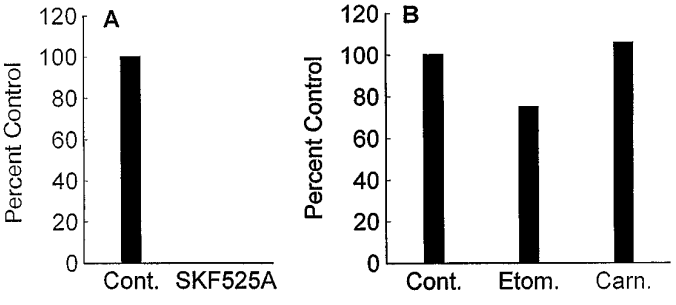


FIG. 5. *Effect of SKF525A on the metabolism of CI-976 in isolated rat hepatocytes (A) and the effects of etomoxir (Etom.) and* L*-carnitine (Carn.) on the metabolism of* ***M-1*** *in isolated rat hepatocytes (B).*

Cont., control. All data are the mean of duplicate incubations.

***In Vitro*** **Metabolism of M-4, M-5, and the ω-Hydroxy Derivative of CI-976.** The ω-hydroxy derivative of CI-976 was incubated in female rat hepatocytes to both confirm its role as an intermediate in the metabolism of CI-976 and determine if the novel **M-5** could arise from this hydroxy intermediate. Incubations using the ω-hydroxy

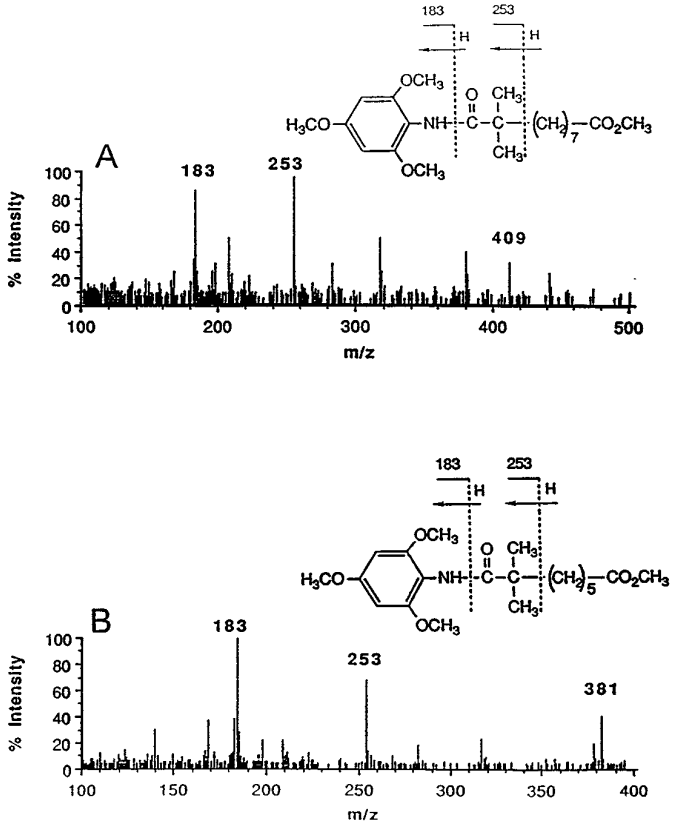


FIG. 6. *Electron-impact GC/MS spectrum of the* ***M-2*** *and* ***M-3*** *β-oxidation intermediates extracted from male rat hepatocytes.*

Analysis was conducted on a diazomethane-treated extract of male rat hepatocytes incubated with 50 μM CI-976 for 60 min. See Materials and Methods *for conditions.*

derivative resulted in formation of **M-1** and **M-4** only; no **M-5** was observed (data not shown). **M-4** and **M-5** were also used as substrates in separate incubations of female rat hepatocytes and were not further biotransformed.

## Discussion

Mean cumulative urinary and fecal recoveries of radioactivity in male and female animals after a single 50 mg/kg oral suspension dose of [$^{14}$C]CI-976 indicated that, in males, nearly equal amounts of radioactivity were excreted by means of the urine and feces (44% and 50%, respectively); whereas in females, a greater amount of the radioactivity was excreted in the urine (71%), compared with feces (23%). The major metabolite observed in male and female rat urine was the **M-4** derivative and to a lesser extent **M-5**. A gender difference was observed in the urinary profiles with **M-4** and **M-5**. Both metabolites were excreted in greater amounts in female rat urine (58% *vs.* 33% and 6.3% and 1.0%, **M-4** and **M-5**, respectively), compared with male urine. Several unknown metabolites eluting near the solvent front were observed in the HPLC-radioactivity profile (UNK A, UNK B, and UNK C). These unknowns were not present in sufficient quantities, nor were they well resolved chromatographically to allow identification, but may constitute conjugates of various metabolites. In general, the *in vivo* data illustrated that significantly more of the CI-976 dose was excreted in female rat urine, where the major difference in excretion occurs with **M-4** and to some extent **M-5**. Interpretation of these results are unclear, but may be due to metabolic, extrahepatic, elimination, or pharmacokinetic differences in the

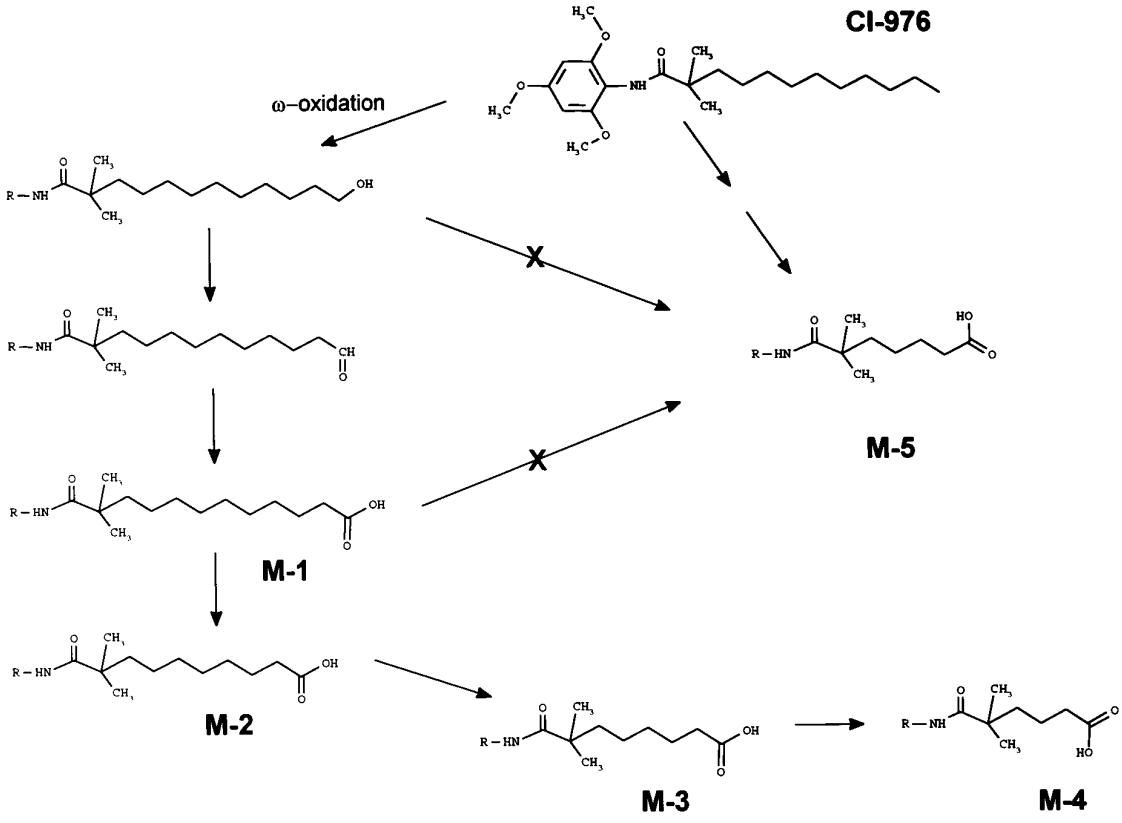


FIG. 7. *Proposed metabolic scheme of CI-976 rat metabolism (R = 2,4,6-trimethoxyphenyl).*

biotransformation/disposition of CI-976.

The CI-976 metabolic profiles observed from extracted male and female rat hepatocyte incubations were similar to those observed in the *in vivo* experiments (*i.e.* **M-4** was observed as the major metabolite) (fig. 4*A*). An additional metabolite, **M-5**, eluting after **M-4** on the HPLC-UV chromatogram, was also observed as a minor metabolite from the hepatocyte suspensions.

By analogy to fatty acid metabolism, the major metabolite, **M-4**, is presumably formed *via* initial $\omega$-carbon oxidation to the carboxylic acid derivative that then undergoes three rounds of $\beta$-oxidation. This was confirmed by synthesizing the $\omega$-carboxylic acid (**M-1**), incubating it with rat hepatocytes, and examining the products formed. When **M-1** was used as substrate, **M-4** was the major metabolite formed; however, no **M-5** was detected (fig. 4*B*). These results suggest that **M-5** is not formed from **M-1**, but is produced by an alternate metabolic route. When the $\omega$-hydroxy derivative of CI-976 was incubated with female rat hepatocytes, the metabolites observed included **M-1** and **M-4**. No **M-5** was detected in incubations of the $\omega$-alcohol, indicating that the **M-5** metabolite does not arise from the initial $\omega$-oxidation of CI-976, but that **M-1** is derived from the $\omega$-alcohol. **M-4** and **M-5** were also incubated with female rat hepatocyte preparations and were found not to be further metabolized, thus indicating that these were metabolic endproducts.

In addition to **M-4**, two new metabolites were observed in the incubations of **M-1** with chromatographic properties between those of **M-1** and **M-4** (fig. 4*B*). These new metabolites were further characterized by GC/MS as the $\beta$-oxidation intermediates **M-2** and **M-3** that are derived from one and two rounds of $\beta$-oxidation, respectively, and occur metabolically between **M-1** and **M-4**.

The **M-5** metabolite was present in limited amounts in extracted hepatocyte samples; therefore, **M-5** was characterized from female rat urine, where it was present in greater amounts. **M-5** was identified as a 5-carbon cleavage product of CI-976. **M-5** constitutes a novel metabolite due to the fact that it is formed by the removal of an odd number of carbon units (five methylene groups) from the CI-976 fatty acid side chain in contrast to the major metabolite **M-4**, where an even 6 carbon units are removed by $\beta$-oxidation. Examples exist of compounds whose metabolites contain side chains, where both odd and even numbers of carbon units have been removed by metabolism. For example, the pentyl side chain of cannabidiol and its derivatives have been shown to undergo $\beta$-oxidation (removal of an even number of carbon units), in addition to metabolism that generates products with an odd number of carbon units removed (11–15). The mechanism for removal of an odd number of carbon units has not been well characterized; however, with cannabidiol and its analogs, the mechanism does not seem to involve $\omega$- ($\omega$-1)-oxidation, but may involve an intermediate derived from initial ($\omega$-2)-hydroxylation (13, 14). Consistent with these results, our data shows that **M-5** does not arise from either the $\omega$-hydroxy or $\omega$-carboxylic acid. Perhaps formation of **M-5** involves the ($\omega$-1)- or ($\omega$-2)-intermediates, both of which have been observed in rat liver microsomes (5). Another pathway, common with branched-chain fatty acids such as phytanic acid, involves $\alpha$-oxidation followed by elimination of the terminal carboxylic acid as $CO_2$ subsequent to $\beta$-oxidation (16, 17).

A significant gender difference was observed in the overall rate of CI-976 disappearance between male and female hepatocyte preparations (table 2). In contrast to the CI-976 data, there was no significant difference in the overall rate of disappearance of **M-1** between male and female suspensions when **M-1** was used as substrate (table 2). Taken together, these results suggest that the *in vitro* gender difference may be due to a difference in the rate of hepatocyte uptake of CI-976 or **M-1**, or a more likely scenario may involve a metabolic difference between the $\beta$-oxidation and $\omega$-oxidation pathways. Kawashima *et al.* (18) have shown with the fatty acid palmitoyl-coen-

zyme A that no difference in the peroxisomal β-oxidation of this substrate exists between male and female rats, supporting the hypothesis that the gender difference arises during formation of the ω-carboxylic acid. The steps leading to formation of **M-1** include the initial ω-hydroxylation, oxidation of the alcohol, and subsequent oxidation of the aldehyde to **M-1**. Previous *in vivo* rat studies have postulated that the ω-oxidation of CI-976 is CYP-mediated; in particular, the lauric acid hydroxylase: CYP4A (7). Coincubation of SKF525A, a mechanism-based inhibitor of CYP, with male rat hepatocyte, resulted in complete inhibition of CI-976 metabolism, pointing to CYP involvement in the biotransformation of CI-976. The gender difference observed in these *in vitro* experiments may be a result of differences between male and female rats in their level of CYP450 responsible for CI-976 ω-oxidation. The relative amount of CYP4A isoforms responsible for ω-fatty acid oxidation may impart a significant effect between male and female rat liver metabolism of CI-976. Consistent with this theory, Sundeth and Waxman (19) have reported a gender difference in the constitutive expression and inducibility of CYP4A isoforms (CYP4A1, CYP4A2, and CYP4A3) in rats. Their results indicate that CYP4A1, CYP4A2, and CYP4A3 are present in male rat liver, but only CYP4A1 and CYP4A3 are present in female liver tissue. Therefore, the absence of CYP4A2 in female liver may exert a greater influence on the rate at which CI-976 is metabolized by the ω-oxidation pathway. Phenobarbital has been shown in rats to induce predominately CYP2B, as well as CYP2C, CYP3A, and CYP4A isoforms (20). With respect to CYP4A, phenobarbital induction leads to increased (ω-1)-oxidation, to a lesser extent ω-oxidation, and has not been shown to induce β-oxidation (21, 22). Our studies with phenobarbital-induced hepatocytes resulted in less than a 2-fold increase in the metabolism of both CI-976 and **M-1**. Although direct induction of the ω-oxidation pathway cannot be concluded from these results, the induction observed by phenobarbital on the β-oxidation of **M-1** is unique, whereas phenobarbital has not been previously shown to induce β-oxidation (22, 23). Clofibrate has been shown to be a very potent inducer of CYP4A and β-oxidation (23–25). Intact male animals induced with clofibrate followed by subsequent isolation and incubation of CI-976 or **M-1** in hepatocyte suspensions resulted in a significant increase in the rates of CI-976 and **M-1** disappearance (4-fold and 6-fold increases, respectively). These data suggest that the β-oxidation and CYP inducers clofibrate and, to a lesser extent, phenobarbital have an effect on the metabolism of CI-976, in particular the β-oxidation pathway as demonstrated by the increases in **M-1** metabolism.

β-Oxidation of fatty acids can occur in either the mitochondria or peroxisomes (26). Coincubation of **M-1** with the mitochondrial β-oxidation inhibitor etomoxir resulted in a 25% reduction in **M-1** metabolism. The moderate inhibition observed with etomoxir is far less than would be expected (typically 90% inhibition) if the β-oxidation were occurring within the mitochondria (27, 28). Coincubation of **M-1** with the mitochondrial β-oxidation enhancer, L-carnitine (29–31), resulted in no significant increase in **M-1** metabolism. In addition, similar fatty acid xenobiotics, such as *N*-(α-methylbenzyl)azelaamic acid and $PCA_{16}$, have been shown to be preferentially metabolized by peroxisomal β-oxidation (32–35). These data would suggest that the β-oxidation of **M-1** occurs within the peroxisomes and not the mitochondria, albeit further studies would be necessary to confirm these results.

A proposed scheme for the metabolism of CI-976 consistent with the *in vivo* and *in vitro* results accumulated in these studies is presented in fig. 7. These results suggest that CYP4A and peroxisomal β-oxidation are involved in the biotransformation of CI-976. All of the metabolites/intermediates along the ω-/β-oxidation pathway leading to formation of the major metabolite, **M-4**, have been identified. Although the mechanism by which **M-5** is produced could not be delineated, several possible metabolic routes were eliminated. It is clear ω-oxidation is not involved in the formation of **M-5**. By following similar *in vitro* protocols as those used herein, but using (ω-1)- and (ω-2)-oxidation products of CI-976, it may be possible to elucidate further the metabolic route leading to formation of the unusual 5-carbon cleavage product.

**Acknowledgments.** We gratefully acknowledge the technical assistance of Mr. James Burleigh, Mr. Robert Bonczyk, and Ms. Jeannette Roesner.

### References

1. K. E. Suckling, and E. F. Stange: Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J. Lipid Res.* **26,** 647–671 (1985).
2. D. R. Sliskovic, and A. D. White: Therapeutic potential of ACAT inhibitors as lipid lowering and anti-atherosclerotic agents. *Trends Pharmacol. Sci.* **12,** 194–199 (1991).
3. B. R. Krause, M. Anderson, C. L. Bisgaier, T. Bocan, R. Bousley, P. DeHart, A. Essenburg, K. Hamelehle, R. Homan, K. Kieft, W. McNally, R. Stanfield, and R. S. Newton: In vivo evidence that the lipid-regulating activity of the ACAT inhibitor CI-976 in rats is due to inhibition of intestinal and liver ACAT. *J. Lipid Res.* **34,** 279–294 (1993).
4. B. D. Roth, C. J. Blankley, M. L. Hoefle, A. Holmes, W. H. Roark, B. K. Trivedi, A. D. Essenburg, K. A. Kieft, B. R. Krause, and R. L. Stanfield: Inhibitors of acyl-CoA:cholesterol acyltransferase. 1. Identification and structure-activity relationships of a novel series of fatty acid anilide hypocholesterolemic agents. *J. Med. Chem.* **35,** 1609–1617 (1992).
5. T. F. Woolf, S. M. Bjorge, and T. Chang: Metabolism of the acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide, CI-976: species differences in omega oxidation pathway. 8th International Symposium on Microsomes and Drug Oxidations, Stockholm, Sweden, 1990.
6. T. F. Woolf, A. Black, Y. Y. Shum, W. McNally, H. Lee, and T. Chang: Biodisposition studies with the acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide, CI-976. *Drug Metab. Dispos.* **21,** 1112–1118 (1993).
7. T. Woolf, S. Bjorge, A. Black, A. Holmes, and T. Chang: Metabolism of the acyl-CoA:cholesterol acyltransferase inhibitor 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide in rat and monkey. *Drug Metab. Dispos.* **19,** 696–702 (1991).
8. P. O. Seglen: Preparation of isolated rat liver cells. *Methods Enzymol.* **13,** 29–83 (1976).
9. M. Bradford: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–255 (1976).
10. T. Omura, and R. Sato: The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239,** 2370–2378 (1964).
11. D. J. Harvey, and R. Mechoulam: Metabolites of cannabidiol identified in human urine. *Xenobiotica* **20,** 303–320 (1990).
12. D. J. Harvey, and J. T. A. Leuschner: Studies on the β-oxidative metabolism of $\Delta^1$- and $\Delta^6$-tetrahydrocannabinol in mouse. *Drug Metab. Dispos.* **13,** 215–219 (1985).
13. E. Samara, M. Bialer, H. Bar-On, and D. J. Harvey: Identification of metabolites of the 1″, 1″-dimethylheptyl analogue of cannabidiol in rat and dog in vivo. *Xenobiotica* **20,** 447–455 (1990).
14. D. J. Harvey: Further studies on the oxidative cleavage of the pentyl side-chain of cannabinoids: identification of new biotransformation pathways in the metabolism of 3′-hydroxy-delta-9-tetrahydrocannabinol by the mouse. *Xenobiotica* **19,** 1437–1447 (1989).
15. D. J. Harvey: Oxidative cleavage of the pentyl side-chain of cannabinoids. *Drug Metab. Dispos.* **18,** 350–355 (1990).
16. J. Vamecq, and J. P. Draye: The enzymatic and mass spectrometric identification of 2-oxophytanic acid, a product of the peroxisomal oxidation of L-2-hydroxyphytanic acid. *Biomed. Environ. Mass Spectrom.* **15,** 345–351 (1988).

17. R. J. A. Wanders, and C. W. T. Van Roermund: Studies on phytanic acid $\alpha$-oxidation in rat liver and cultured human skin fibroblasts. *Biochim. Biophys. Acta* **1167,** 345–350 (1993).
18. Y. Kawashima, N. Uy-Yu, and H. Kqauka: Sex-related difference in the inductions by perfluoro-octanoic acid of peroxisomal beta oxidation, microsomal 1-acylglycerophosphocholine acyltransferase and cytosolic long-chain acyl-CoA hydrolase in rat liver. *Biochem. J.* **261,** 595–600 (1989).
19. S. S. Sundseth, and D. J. Waxman: Sex-dependent expression and clofibrate inducibility of cytochrome P450 4A fatty acid omega-hydroxylases. *J. Biol. Chem.* **267,** 3915°–3921 (1992).
20. D. J. Waxman, and L. Azaroff: Phenobarbital induction of cytochrome P450 gene expression. *Biochem. J.* **281,** 577–592 (1992).
21. R. T. Okita, and B. S. S. Masters: Effect of phenobarbital treatment and cytochrome P-450 inhibitors on the laurate $\varpi$- and ($\varpi$-1)-hydroxylase activities in rat liver microsomes. *Drug Metab. Dispos.* **8,** 147–151 (1980).
22. G. Heinemeyer, H. Nau, A. G. Hildebrandt, and I. Roots: Oxidation and glucuronidation of valproic aid in male rats—influence of phenobarbital, 3-methylchlolanthrene, beta-naphthoflavone and clofibrate. *Biochem. Pharmacol.* **34,** 133–139 (1985).
23. I. Close, G. Shackleton, P. S. Goldfarb, G. G. Gibson, R. Sharma, and D. Howes: Influence of single and concurrent clofibrate and phenobarbital administration on cytochrome P450-dependent mixed function oxidase activities and peroxisome proliferation in male rat liver. *J. Biochem. Toxicol.* **7,** 193–198 (1992).
24. T. A. Kocarek, and D. R. Feller: Quantitative assessment of enzyme induction by peroxisome proliferators and application to determination of effects on triglyceride biosynthesis in primary cultures of rat hepatocytes. *Biochem. Pharmacol.* **38,** 4169–4176 (1989).
25. R. Sharma, B. G. Lake, and G. G. Gibson: Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid $\beta$-oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate, dose response studies. *Biochem. Pharmacol.* **37,** 1203–1206 (1988).
26. H. Osmundsen, J. Bremer, and J. I. Pedersen: Metabolic aspects of peroxisomal beta-oxidation. *Biochim. Biophys. Acta* **1085,** 141–158 (1991).
27 C. Skorin, C. Necochea, V. Johow, U. Soto, A. M. Grau, J. Bremer, and F. Leighton: Peroxisomal fatty acid oxidation and inhibitors of the mitochondrial carnitine palmitoyltransferase I in isolated rat hepatocytes. *Biochem. J.* **281,** 561–567 (1992).
28 L. Agius, E. J. Meredith, and H. S. A. Sherratt: Stereospecificity of the inhibition by etomoxir of fatty acid and cholesterol synthesis in isolated rat hepatocytes. *Biochem. Pharmacol.* **42,** 1717–1920 (1991).
29 K. F. Buechler, and J. M. Lowenstein: The involvement of carnitine intermediates in peroxisomal fatty acid oxidation: a study with 2-bromofatty acids. *Arch. Biochem. Biophys.* **281,** 233–238 (1990).
30 M. Bronfman, M. N. Morales, L. Amigo, A. Orellana, L. Nunez, L. Cardenas, and P. C. Hidalgo: Hypolipidaemic drugs are activated to acyl-CoA esters in isolated rat hepatocytes. *Biochem. J.* **284,** 289–295 (1992).
31. G. P. Mannearts, L. J. Debeer, J. Thomas, and P. J. De Schepper: Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate treated rats. *J. Biol. Chem.* **254,** 4585–4595 (1979).
32. Y. Yoshida, J. Yamado, T. Watanabe, T. Suga, and H. Takayama: Participation of the peroxisomal beta-oxidation system in the chain-shortening of PCA16, a metabolite of the cytosine arabinoside prodrug, YNK01, in rat liver. *Biochem. Pharmacol.* **39,** 1505–1512 (1990).
33. J. Yamado, S. Ogawa, S. Horie, T. Watanabe, and T. Suga: Participation of peroxisomes in the metabolism of xenobiotic acyl compounds: comparison between peroxisomal and mitochondrial beta-oxidation of omega-phenyl fatty acids in rat liver. *Biochim. Biophys. Acta* **921,** 292–301 (1987).
34. J. Yamada, S. Itoh, S. Horie, T. Watanabe, and T. Suga: Chain-shortening of a xenobiotic acyl compound by the peroxisomal beta-oxidation system in rat liver. *Biochem. Pharmacol.* **35,** 4362–4368 (1986).
35. H. Suzuki, K. Mori, J. Yamada, and T. Suga: Contribution of peroxisomal beta-oxidation system to the chain-shortening of N-($\alpha$-methylbenzyl)azelaamic acid in rat liver. *Biochem. Pharmacol.* **39,** 1975–1981 (1990).