Effect of the side-chain structure on the specificity of β-oxidation in bile acid biosynthesis in rat liver homogenates

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Abstract 3α, 7α, 12α-Trihydroxy-5β-cholestan-26-oic acid (C_{27}-5β-cholestanoic acid) derivatives with different carbon-number side chains were incubated with rat liver 800 *g* supernatant to study the effect of the side-chain length on the β-oxidation system in bile acid biosynthesis. The intermediate α, β-unsaturated and β-hydroxylated bile acids, and the corresponding degradation products, were quantitatively determined by gas chromatography. The longer side-chained derivatives (C_{28}- and C_{29}-5β-cholestanoic acids) were converted into corresponding side-chain degradation products, and the α,β-unsaturated and β-hydroxylated intermediates were also produced. On the other hand, the shorter side-chained derivative (C_{26}-5β-cholestanoic acid) only gave α,β-unsaturated intermediate. The total formation of intermediates and degradation products from corresponding substrates was in the order of C_{27}- > C_{28}- > C_{29}- > C_{26}-5β-cholestanoic acids. In the case of clofibrate-treated rat liver 800 *g* supernatant, the formation of intermediates and final degradation products from C_{28}- and C_{29}-5β-cholestanoic acids increased significantly. These longer side-chained analogues seemed to be subjected to β-oxidation system(s) induced with clofibrate treatment. The effect of a terminal methyl group in the side chain of 5β-cholestanoic acid on the β-oxidation system was also investigated using 3α, 7α, 12α-trihydroxy-27-*nor*-5β-cholestanoic acid derivatives as enzymatic substrates. These derivatives gave corresponding side chain degradation products, but the formation of intermediates was not detected. The formation of side chain cleavage products from 27-*nor*-5β-cholestanoic acid derivatives increased to 10 to 25-fold that of the controls by treatment with clofibrate. The results suggested that the β-oxidation system for 27-*nor*-5β-cholestanoic acid derivatives was different from that for C_{27}-5β-cholestanoic acid, despite their bile acid steroidal structure.—**Kurosawa, T., M. Sato, T. Watanabe, T. Suga, and M. Tohma.** Effect of the side-chain structure on the specificity of β-oxidation in bile acid biosynthesis in rat liver homogenates. *J. Lipid Res.* 1997. **38:** 2589–2602.

Supplementary key words bile acid biosynthesis • 5β-cholestanoic acid • side-chain degradation intermediate • β-oxidation • substrate specificity • 27-*nor*-5β-cholestanoic acid

Primary bile acids (CA and CDCA) are synthesized from cholesterol via C_{27}-bile acids (3α, 7α, 12α-trihydroxy- and 3α, 7α-dihydroxy-5β-cholestan-26-oic acids, 27-THCA and DHCA) (1–4) by the side chain degradation (β-oxidation). This side-chain degradation of bile acids has been thought to proceed by a mechanism similar to that of β-oxidation of fatty acids in peroxisome (5, 6). The β-oxidation system contains several enzymes such as oxidase, bifunctional hydratase/dehy-

Abbreviations: 26-THCA, 3α, 7α, 12α-trihydroxy-24-methyl-26,27-*dinor*-5β-cholestan-25-oic acid; 27-THCA, 3α, 7α, 12α-trihydroxy-5β-cholestan-26-oic acid; 28-THCA, 3α, 7α, 12α-trihydroxy-26-methyl 26a-homo-27-*nor*-5β-cholestan-26a-oic acid; 29-THCA, 3α, 7α, 12α-trihydroxy-26a-methyl-26a,26b-*dihomo*-27-nor-5β-cholestan-26b-oic acid; nor-26-THCA, 3α, 7α, 12α-trihydroxy-27-*nor*-5β-cholestan-26-oic acid; nor-27-THCA, 3α, 7α, 12α-trihydroxy-26a-*homo*-27-*nor*-5β-cholestan-26a-oic acid; nor-28-THCA, 3α, 7α, 12α-trihydroxy-26a,26b-*dihomo*-27-*nor*-5β-cholestan-26b-oic acid; 26-Δ-THCA, (23*E*)-3α, 7α, 12α-trihydroxy-24-methyl-26,27-*dinor*-5β-cholest-23-en-25-oic acid; 27-Δ-THCA, (24*E*)-3α, 7α, 12α-trihydroxy-5β-cholest-24-en-26-oic acid; 28-Δ-THCA, (25*E*)-3α, 7α, 12α-trihydroxy-26-methyl-26a-*homo*-27-*nor*-5β-cholest-25-en-26a-oic acid; 29-Δ-THCA, (26*E*)-3α, 7α, 12α-trihydroxy-26a-methyl-26a,26b-*homo*-27-*nor*-5β-cholest-26(26a)-en-26b-oic acid; nor-26-Δ-THCA, (23*E*)-3α, 7α, 12α-trihydroxy-26,27-*dinor*-5β-cholest-23-en-25-oic acid; nor-27-Δ-THCA, 3α, 7α, 12α-trihydroxy-26a-*homo*-27-*nor*-5β-cholest-25-en-26a-oic acid; nor-28-Δ-THCA, 3α, 7α, 12α-trihydroxy-26a,26b-*dihomo*-27-*nor*-5β-cholest-26(26a)-en-26b-oic acid; 26-VA, 3α, 7α, 12α, 23-tetrahydroxy-24-methyl-26,27-*dinor*-5β-cholestan-25-oic acid; 27-VA, 3α, 7α, 12α,24-tetrahydroxy-5β-cholestan-26-oic acid; 28-VA, 3α, 7α, 12α,25-tetrahydroxy-26-methyl-26a-*homo*-27-*nor*-5β-cholestan-26a-oic acid; 29-VA, 3α, 7α, 12α, 26-tetrahydroxy-26a-methyl-26a,26b-*dihomo*-27-*nor*-5β-cholestan-26b-oic acid; nor-26-VA, 3α, 7α, 12α, 24-trihydroxy-27-*nor*-5β-cholestan-26-oic acid; nor-27-VA, 3α, 7α, 12α, 25-tetrahydroxy-26a-*homo*-27-*nor*-5β-cholestan-26a-oic acid; nor-28-VA, 3α, 7α, 12α, 26-tetrahydroxy-26a,26b-*dihomo*-27-*nor*-5β-cholestan-26b-oic acid; nor-CA, 3α, 7α, 12α-trihydroxy-24-*nor*-5β-cholan-23-oic acid; CA, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid; homo-CA, 3α, 7α, 12α-trihydroxy-24a-*homo*-5β-cholan-24a-oic acid.

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drogenase, and thiolase. The initial enzyme (acyl-CoA oxidase) in the β-oxidation system of bile acids has been shown to be distinct from that of fatty acids (7, 8), and its substrate specificity has ben investigated (9). Recent studies have shown the specificity of peroxisomal trihydroxy-5β-cholestanoyl-CoA oxidase for 25*S*-configuration at the C25-position (10, 11). Other enzymes (bifunctional hydratase/dehydrogenase and thiolase) in the β-oxidation system of bile acids have also been reported, and their subcellular localization and approximate molecular weights have been determined (12–14). Although the specificity of enzymes responsible for β-oxidation of fatty acids with different chain lengths has been investigated (15, 16), the substrate specificities of the enzymes for the side-chain length of bile acids have not been well studied. The aim of the present study was to investigate the substrate specificity for the side-chain length of bile acids and the effect of a terminal methyl group on the β-oxidation system in rat liver homogenate by quantitative determination of β-oxidation products using our previously reported method (17).

EXPERIMENTAL

Chemicals

ATP, NAD, and coenzyme A (CoA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other materials and solvents were of analytical grade, and deionized water was used throughout the study.

Bile acids

Bile acids used in this study were all synthesized in our laboratory except CA and the abbreviations of the bile acids are listed in the footnote. Melting points were measured on a Mitamura melting point apparatus (Mitamura Riken Co., Tokyo, Japan). The optical rotations were measured by a DIP-360 digital polarimeter (JASCO, Tokyo, Japan) in MeOH. Infrared (IR) spectra were recorded using FT/IR-300 spectrometer (JASCO). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were recorded at 400 MHz with a JEOL JNM-EX 400 spectrometer (JEOL, Tokyo, Japan) and chemical shifts are given at the δ-value, with tetramethylsilane as the internal standard. Mass spectra were measured using a JMS-AM 150 spectrometer (JEOL) as methyl ester-dimethylethyl-silylether derivatives (see analytical procedure).

Nor-CA was synthesized by the reported procedure (18). CA was purchased from Sigma Chemical Co. and used after recrystallization. *Homo*-CA was prepared from CA by the combination of following procedure: formylation (19), reduction (20), mesylation with methanesulfonyl chloride, substitution reaction with KCN in the presence of 18-crown-6 and subsequent hydrolysis. *Nor*-26-THCA and *nor*-27-THCA were synthesized by the Wittig reaction of the aldehydes prepared from *nor*-CA and CA, respectively (21). *Nor*-28-THCA was also prepared from *nor*-27-THCA by the same procedure as above. 27-THCA, 27-Δ-THCA, and 27-VA were synthesized as previously reported (22, 23). The C_{26}-bile acids (26-THCA, 26-Δ-THCA, 26-VA), C_{28}-bile acids (28-THCA, 28-Δ-THCA, 28-VA), and C_{29}-bile acids (29-THCA, 29-Δ-THCA and 29-VA) were synthesized from the above synthesized *nor*-CA, *homo*-CA, and *nor*-26-THCA, respectively, by the previously reported method (17). *Nor*-26-Δ-THCA, *nor*-27-Δ-THCA, *nor*-28-Δ-THCA, *nor*-26-VA, *nor*-27-VA, and *nor*-28-VA were also synthesized by the same procedure. The internal standard (3α, 12α-dihydroxy-24-methyl-26,27-*dinor*-5β-cholestan-25-oic acid : *nor*-DHCA) for the quantitative analysis was synthesized from 3α, 12α-dihydroxy-24-*nor*-5β-cholan-23-oic acid (18) by the same procedure as the synthesis of 26-THCA. The physical and spectral data of the above synthesized bile acids were summarized in Table 1 (^{1}H-NMR, IR $[\alpha]_D$ and mp) and Table 2 (mass spectra as methyl ester-dimethylethylsilyl ether derivatives). The elemental analyses of all the above synthesized compounds were in good accordance with the calculated values within 0.3% (data not shown).

Preparation of rat liver 800 *g* supernatant

Male Wistar rats (200–230 g) were divided into control and clofibrate-treated groups. The control group (n = 4) was given a commercial standard powdered diet (Type MF, Oriental Yeast Co., Tokyo, Japan) for 7 days, and the clofibrate-treated group (n = 4) was given a commercial standard powdered diet containing 0.25% clofibrate (Wako Chemical Co., Tokyo, Japan). The clofibrate-powdered diet was prepared by spraying a clofibrate solution in acetone (2.5 g in 10 ml) on powdered diet (1 kg) with mixing and drying for 1 day at room temperature. The control diet was also treated in the same manner as above without clofibrate. After 7 days of treatment, the animals were killed by decapitation. The liver was minced and homogenized in 9 volumes (w/v) of 0.25 M sucrose in a Potter-Elvehjem homogenizer (1,000 rpm, two strokes) while cooling on ice. Cellular debris and nuclei were removed by centrifugation at 800 *g* for 10 min at 4°C, and the supernatant was used in the incubation experiments. The protein concentration was determined by the method of Lowry et al. (24) using bovine serum albumin as a standard protein.

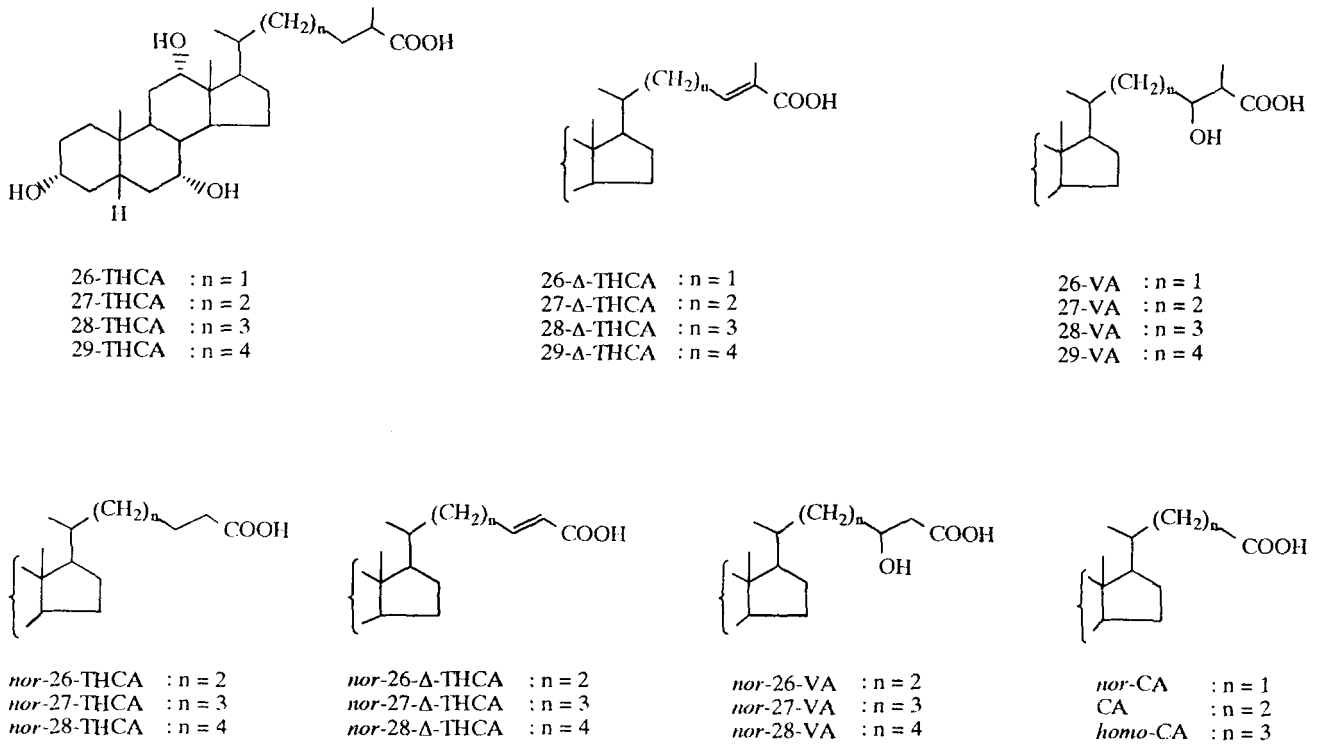

Fig. 1. Substrates and expected intermediates in β-oxidation. The abbreviations of the compounds used are listed in the footnote. The number in the abbreviation represents the carbon numbers of the compound, and the prefix *nor*-indicates bile acids without an α-methyl group in the side chain except *nor*-CA.

TABLE 1. Physicochemical data of synthesized compounds

Bile Acid	mp[a] °C	^{1}H-NMR(pyridine-d_5) δ 18-H	19-H	21-H (J)	α-Methyl (J)[b]	3β-H	7β-H	12β-H	β-OH[c]	Olefinic-H(J)	IR(cm^{-1}) (Nujol)	$[a]_D$(°)[d] (MeOH)
nor-CA	190–192	0.85	1.00	1.45 (6.4)		3.72	4.07	4.24			3530, 3400, 1700	
homo-CA	201–203	0.82	1.02	1.19 (6.8)		3.73	4.10	4.26			3520, 3350, 1710	+30.3
nor-DHCA	194–195	0.76	0.96	1.23 (6.4)	1.34 (6.8)	3.85		4.21			3350, 1700	+57.0
26-THCA	123–125	0.75	0.94	1.14 (6.4)	1.25 (6.8)	3.57	3.95	4.11			3400, 3250, 1680	+25.7
26-Δ-THCA	162–164	0.83	1.02	1.23 (6.4)	2.04	3.74	4.10	4.23		7.29 (7.3)	3500, 3250, 1700, 1640	+31.6
26-VA	127–133[e]	0.79	0.95	1.20, 1.24 (6.6)	1.46, 1.58 (7.1)	3.83	4.04	4.14	3.82		3400, 1705	
28-THCA	175–176	0.77	0.96	1.13 (6.4)	1.28 (6.8)	3.62	4.01	4.16			3360, 3230, 1690	+28.1
28-Δ-THCA	197–198	0.83	1.02	1.21 (d, 6.4)	2.07	3.79	4.11	4.27		7.21 (7.3)	3350, 3250, 1680, 1640	+29.6
28-VA	96–100[e]	0.79	0.99	1.20, 1.21 (6.4)	1.42, 1.54 (7.3)	3.75	4.06	4.21	3.85		3350, 1700	
29-THCA	110–111	0.84	1.02	1.21 (d, 6.4)	1.33 (6.8)	3.75	4.11	4.27			3450, 3300, 1700	+30.6
29-Δ-THCA	217–218	0.85	1.02	1.21 (d, 6.4)	2.08	3.75	4.11	4.27		7.18 (7.8)	3350, 1675, 1635	+32.3
29-VA	77–84[e]	0.80	1.00	1.17, 1.18 (6.4)	1.44, 1.56 (7.3)	3.76	4.08	4.23	3.86		3350, 1700	
nor-26-THCA	198–200	0.82	1.02	1.20 (6.8)		3.75	4.11	4.26			3500, 3250, 1690	+31.8
nor-26-Δ-THCA	214–215	0.75	1.01	1.18 (6.4)		3.75	4.10	4.23		6.20 (15.6), 7.24	3450, 3200, 1675, 1645	+31.3
nor-26-VA	199–201	0.82	1.02	1.26 (6.8)		3.75	4.10	4.26	4.12		3400, 1700	+32.1
nor-27-THCA	201–203	0.82	1.02	1.19 (6.6)		3.73	4.10	4.26			3520, 3350, 1710	+30.3
nor-27-Δ-THCA	193–195	0.82	1.01	1.17 (6.3)		3.72	4.09	4.24		6.17 (16.6), 7.24	3450, 3300, 1690, 1640	+36.3
nor-27-VA	95–107[e]	0.79	0.99	1.20, 1.21 (6.6)		3.68	4.06	4.21	4.18		3350, 1700	
nor-28-THCA	175–171	0.84	1.02	1.21 (6.4)		3.73	4.11	4.27			3250, 1700	+33.2
nor-28-Δ-THCA	143–146	0.81	1.01	1.19 (6.4)		3.76	4.08	4.18		6.19 (16.3), 7.23	3330, 1690, 1635	+34.0
nor-28-VA	67–74[e]	0.73	0.91	1.06 (6.8)		3.70	3.90	4.06	4.16		3350, 1700	

[a] Uncorrected.
[b] Signals of terminal methyl protons in side chain.
[c] Signals of protons attached to the same carbon atom with a β-hydroxyl group of carboxylate.
[d] c = 0.8–1.1 and measured at 22°C.
[e] Amorphous powder.

TABLE 2. Selected mass spectral data of the methyl ester-dimethylethylsilyl ether derivatives of standards for the determination of the incubation products in rat liver 800 *g* supernatant

Bile Acid	*m/Z*[a] (relative intensity, %)	Expected M^+
26-THCA	679 (100), 575 (10.8), 500 (9.5), 471 (4.2), 397 (65), 365 (20), 253 (65.2)	708
26-Δ-THCA	677 (100), 573 (8.3), 498 (2.1), 395 (19.8), 253 (20)	706
26-VA	781 (76), 677 (7.3), 602 (5.2), 498 (7.2), 395 (22), 355 (18.3), 307 (19.1), 253 (40), 157 (100)	810
27-THCA	693 (76), 589 (14.5), 514 (13.1), 411 (100), 379 (28), 253 (81)	722
27-Δ-THCA	691 (100), 587 (19), 512 (12.5), 409 (52.7), 253 (43.3)	720
27-VA	795 (10.5), 691 (15.7), 616 (17.5), 512 (46.7), 409 (48.3), 357 (52.5), 321 (50), 253 (100)	824
28-THCA	707 (100), 603 (19.6), 528 (16.2), 425 (79.8), 253 (57.6)	736
28-Δ-THCA	705 (83.3), 601 (22.9), 526 (18.8), 423 (94), 253 (100)	734
28-VA	809 (6.3), 705 (6.4), 630 (11.4), 526 (48.3), 495 (10), 439 (15), 423 (22.9), 357 (41.6), 253 (100)	838
nor-26-THCA	679 (53.6), 575 (10.9), 500 (13.4), 397 (100), 365 (34.1), 253 (62.2)	708
nor-26-Δ-THCA	677 (100), 573 (19.5), 498 (9.7), 395 (64.8), 363 (19.5), 253 (62.2)	706
nor-26-VA	781 (10.3), 677 (13.4), 602 (12.9), 498 (48.8), 467 (13.4), 357 (52.4), 395 (52.3), 355 (53), 321 (32.9), 253 (100)	810
nor-27-THCA	693 (30), 589 (9.8), 514 (12.2), 483 (7.9), 411 (100), 379 (48.3), 357 (22), 253 (63.4)	722
nor-27-Δ-THCA	691 (63), 587 (32.1), 512 (12.2), 481 (9.1), 409 (95.1), 393 (12.2), 377 (21.3), 357 (25.6), 253 (100)	720
nor-27-VA	795 (7.3), 691 (7.9), 617 (9.2), 512 (52.4), 481 (17) 439, (13.4), 409 (28), 357 (42.7), 253 (100)	824
nor-28-THCA	707 (17), 603 (9.1), 528 (11), 497 (7.3), 425 (100), 409 (7.3), 393 (37.8), 357 (19.5), 253 (62.2)	736
nor-28-Δ-THCA	705 (43.2), 601 (17), 526 (7.3), 495 (12.2), 423 (100), 407 (11), 391 (23.1), 357 (21), 253 (92.7)	734
nor-28-VA	809 (6.1), 705 (5.5), 631 (12.1), 526 (62.2), 495 (17.2), 453 (21.9), 423 (22), 367 (18.3), 357 (34.1), 253 (100)	838

[a] These values correspond to the elimination of Et• (29), dimethylethylsilanol (DMESOH, 104), dimethylethylsiloxy group (DMESO•, 103) and/or side chain cleavage.

Incubation conditions

A solution (total volume 0.25 ml) containing substrate (20 μg), 800 *g* supernatant (150 μl 2.4–4.4 mg of protein), NAD (2.0 mM), ATP (4.0 mM), EDTA (7.5 mM), $MgCl_2$ (20 mM), and phosphate buffer (pH 7.6, 40 mM) was incubated at 37°C for 60 min. All incubations were carried out in duplicate using disposable borosilicate glass culture tubes (12 × 75 mm), and blank tests were performed under the same conditions without substrates.

Preparation of analytical samples

After incubation for 60 min internal standard (2 μg) and EtOH (3 ml) were added to an ice-cooled reaction solution successively, and the mixture was allowed to stand for 30 min at 0°C. Then the solution was centrifuged at 1,000 *g* for 20 min. The separated supernatant was evaporated by a centrifugal evaporator (Tokyo Rika Co., Tokyo, Japan). NaOH (2 M, 1.5 ml) in MeOH–H_2O 1:1 (v/v) was added to the residue and heated at 80°C for 16 hr. The solution was concentrated to about one-third volume with the centrifugal evaporator and acidified to pH 5 with 6 M HCl while cooling. The solution was then extracted with ethyl acetate (5 ml), and the extract was washed with H_2O (1 ml × 2). After evaporation of the solvent, the residue was dissolved in 90% EtOH (0.4 ml) and applied to a piperidinohydroxypropyl Sephadex LH-20 column (40 mm × 6 mm i.d.) (25). The column was washed with 90% EtOH (2 ml) and eluted with 0.1 M acetic acid in 90% EtOH (6.0 ml). The eluate was evaporated and the residue was treated with diazomethane–ether (0.5 ml) at room temperature for 5 min and then evaporated. The residue was then treated with dimethylethylsilylimidazole (Tokyo Kasei Organic Chemicals Co., Tokyo, Japan) (30 μl, 60°C for 45 min). The reaction mixture was diluted with 0.5 ml n-hexane–ethyl acetate 3:1 (v/v) and applied to a short silica gel column (40 nn × 6 mm i.d.), eluted with 5 ml n-hexane–ethyl acetate 3:1 (v/v). The eluate was evaporated and dissolved in n-hexane (50 μl) and analyzed by capillary gas chromatography (GC) as previously reported (17). The calibration curves were constructed by plotting the peak area ratios of authentic samples for an internal standard. Relative recoveries were carried out under the above same conditions by the addition of 2.5 μg of each expected product into heat-treated rat liver 800 *g* supernatant.

Analytical apparatus and conditions

GC was performed using a Shimadzu GC 14A gas chromatograph equipped with a flame-ionization detector (Shimadzu Co., Kyoto, Japan). An Ulbon HR-1 (25 m × 0.25 mm i.d., Shinwa Co., Kyoto, Japan) fused silica capillary column bonded with methyl silicone was used as the chromatographic column. The carrier gas was helium at a linear velocity of 45 cm/s. The column oven program was 230°C (3 min hold) followed by temperature ramps at 10°C/min to 270°C and then

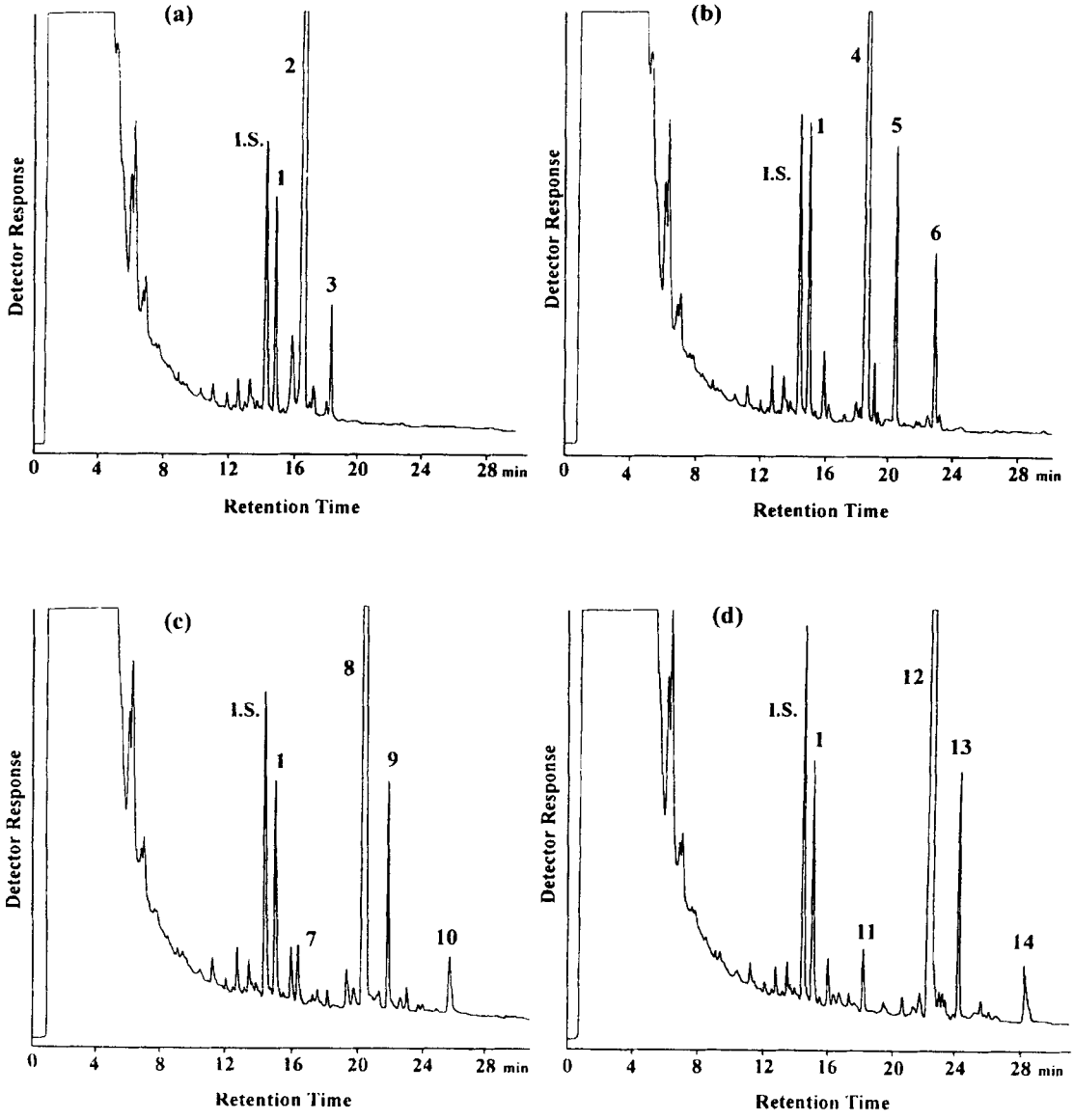

Fig. 2. Gas chromatograms of the methyl ester-dimethylethylsilyl ether derivatives of the products from 26-THCA (a), 27-THCA (b), 28-THCA (c), and 29-THCA (d) by rat liver 800 *g* supernatant. I.S., internal standard (3α,12α-dihydroxy-24-methyl-26,27-*dinor*-5β-cholestan-25-oic acid); 1, CA; 2, 26-THCA(substrate); 3, 26-Δ-THCA; 4, 27-THCA (substrate); 5, 27-Δ-THCA; 6, 27-VA; 7, *homo*-CA; 8, 28-THCA (substrate); 9, 28-Δ-THCA; 10, 28-VA; 11, *nor*-26-THCA; 12, 29-THCA (substrate); 13, 29-Δ-THCA; 14, 29-VA. Unnumbered peaks are due to the impurities from rat liver 800 *g* supernatant and derivatizing reagent.

2°C/min to 310°C. The injection and detector temperature were set at 300°C and 310°C, respectively. Gas chromatography–mass spectrometry (GC–MS) was performed under the same conditions as above using a JMS-AM 150 spectrometer (JEOL, Tokyo, Japan) to confirm the structures of products. The ionization energy and ion source temperature were 70 eV and 280°C, respectively.

RESULTS

Bile acids and their quantitative analysis

Figure 1 shows the synthetic bile acids used in this study. The compounds 26-THCA, 28-THCA, and 29-THCA are the derivatives of 27-THCA, known as a starting compound for β-oxidation in bile acid biosynthesis (26). The compounds *nor*-26-THCA, *nor*-27-THCA,

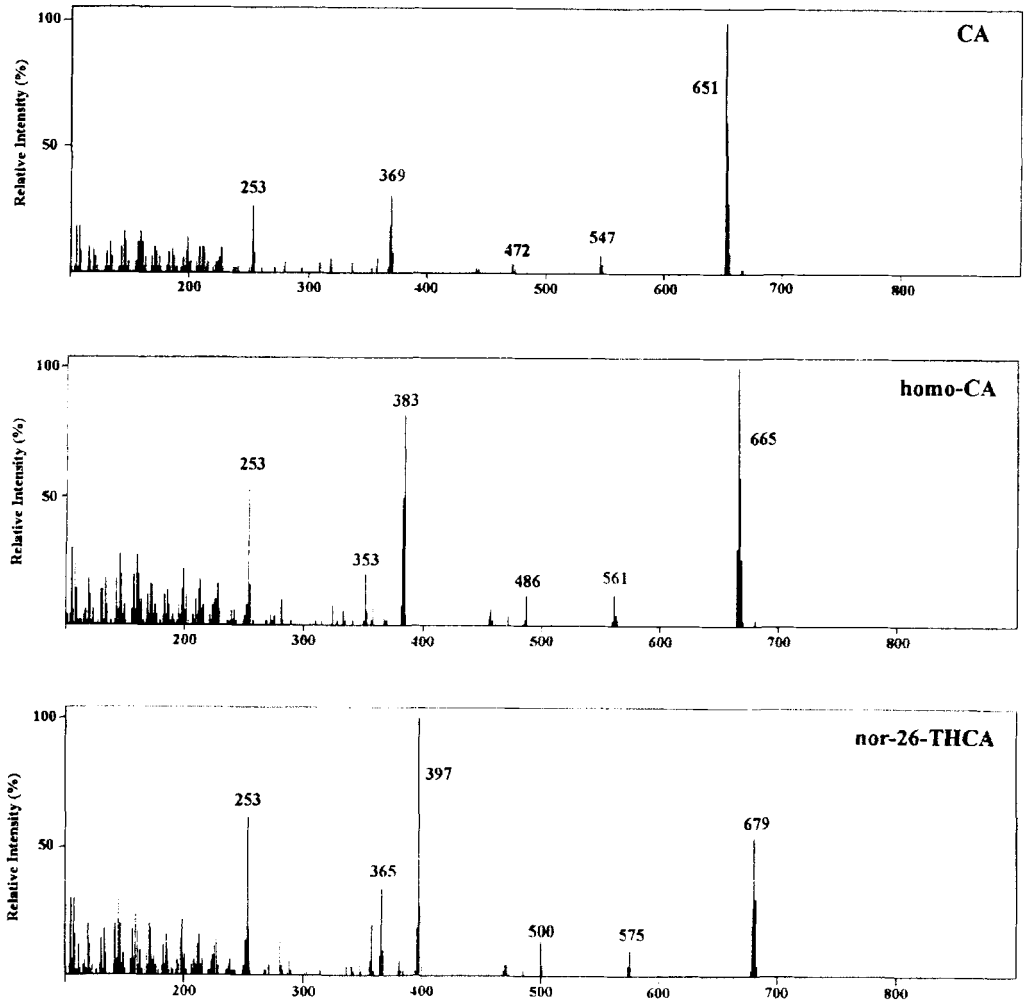

Fig. 3. Mass spectra of methyl ester-dimethylethyl silylether derivatives of the side-chain degraded products of β-oxidation by rat liver 800 *g* supernatant. (a) CA, (b) *homo*-CA, (c) *nor*-26-THCA.

and *nor*-28-THCA were the derivatives without a terminal methyl group. The possible α,β-unsaturated and β-hydroxylated intermediates (Δ-THCA and VA derivatives) and final degradation products (*nor*-CA, CA, and *homo*-CA) in β-oxidation were also prepared as authentic specimens for quantitative determination. Although the above substrates and β-hydroxylated compounds were mixtures of diastereoisomers, the isomers were not separated by GC and were used without further separation. The chemical structures of these compounds were established by their spectral data as shown in **Table 1**. **Table 2** also shows characteristic fragment ions of mass spectra of Me-DMES derivatives of these synthesized compounds.

Quantitative analysis of these products was carried out by GC as previously reported (17). The calibration curves for the quantitative determination of expected products showed good linearities in the range of 5–200 ng ($r = 0.999$), and the detection limit was about 0.5 ng for each compound (S/N = 5). The relative recoveries of related compounds for the internal standard were 95–104%, when 2.5 μg of each compound was added to an incubation mixture. Under these analytical conditions, only endogenous CA in rat liver 800 *g* supernatant seemed to interfere with the determination, although it was possible to quantify CA produced from substrates by correction with a blank test.

The substrates were incubated with 800 *g* supernatant of rat liver 800 *g* supernatant under the modified conditions reported by Une et al. (27), in which β-hydroxylated intermediate 27-VA could be formed in sufficient amount to analyze. The enzymatic reaction conditions were set to convert ca. 10% of substrates into the total products. In order to clarify the enzyme system related to the β-oxidation of used substrates, control rat liver and clofibrate-treated rat liver 800 *g* su-

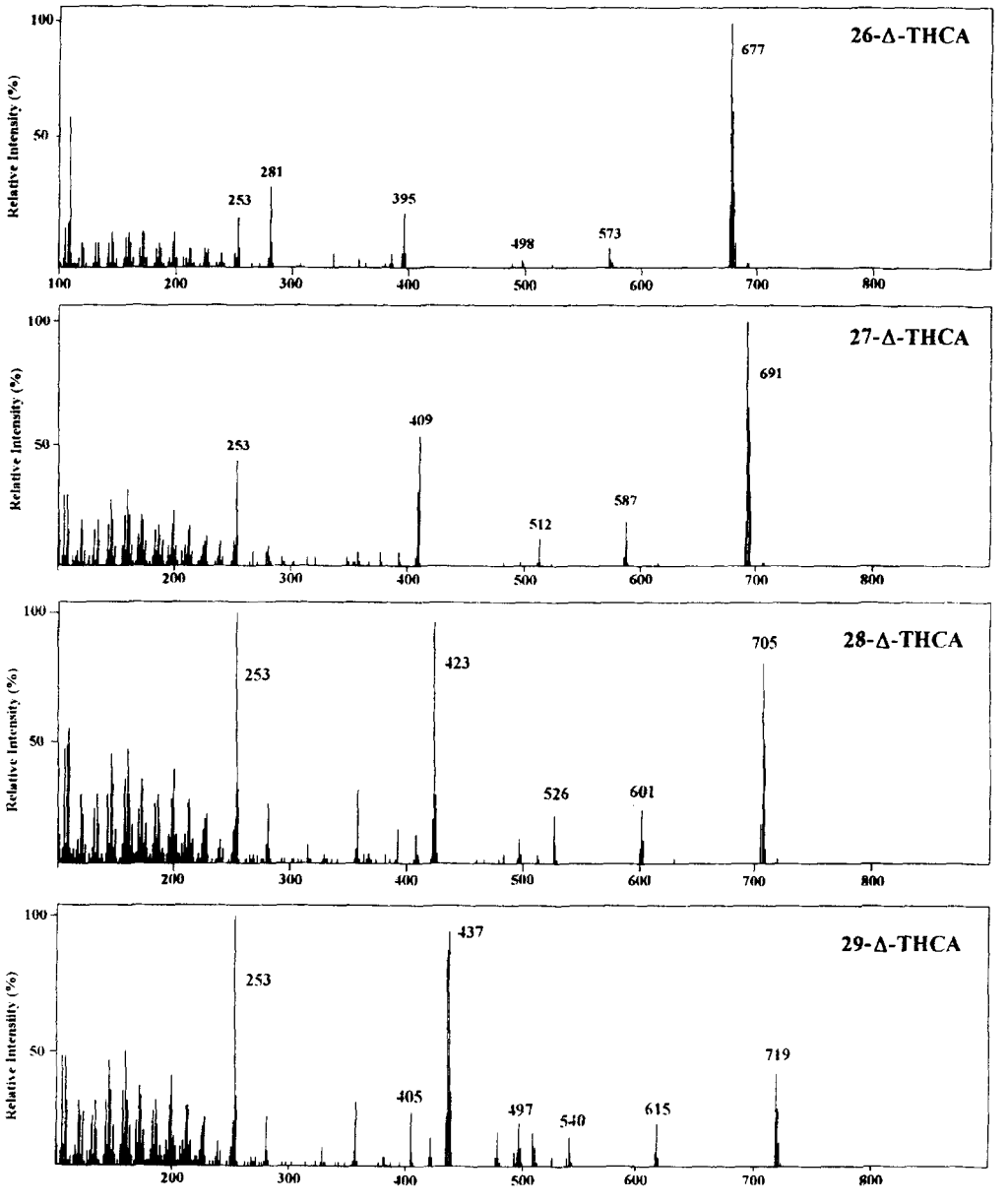

Fig. 4. Mass spectra of methyl ester-dimethylethyl silylether derivatives of the α,β-unsaturated intermediates of β-oxidation by rat liver 800 *g* supernatant. (a) 26-Δ-THCA, (b) 27-Δ-THCA, (c) 28-Δ-THCA, (d) 29-Δ-THCA.

pernatants were used as the enzymatic source. Clofibrate is known to induce peroxisomal enzymatic activity of β-oxidation for fatty acids at a much higher level than that of control (28).

Analysis of the incubation products from 5β-cholestanoic acid (27-THCA) derivatives

Figure 2 shows gas chromatograms of the methyl ester-dimethylethylsilylether (Me-DMES) derivatives of the products obtained after incubation of 27-THCA derivatives with a control rat liver 800 *g* supernatant. When 26-THCA was used as a substrate, only 26-Δ-THCA was detected as a product of β-oxidation as shown in Fig. 2a. 27-THCA gave 27-Δ-THCA and 27-VA as intermediates of β-oxidation, and the final degradation product CA was also produced (Fig. 2b). The other substrates (28-THCA and 29-THCA) also gave the intermediates (28-Δ-THCA, 29-Δ-THCA, 28-VA, and 29-VA) and final degradation products (*homo*-CA and *nor*-26-THCA) as shown in Fig. 2c and 2d, respectively. The chemical structures of these compounds were confirmed by their mass spectra. **Figure 3**, **Fig. 4**, and **Fig. 5**

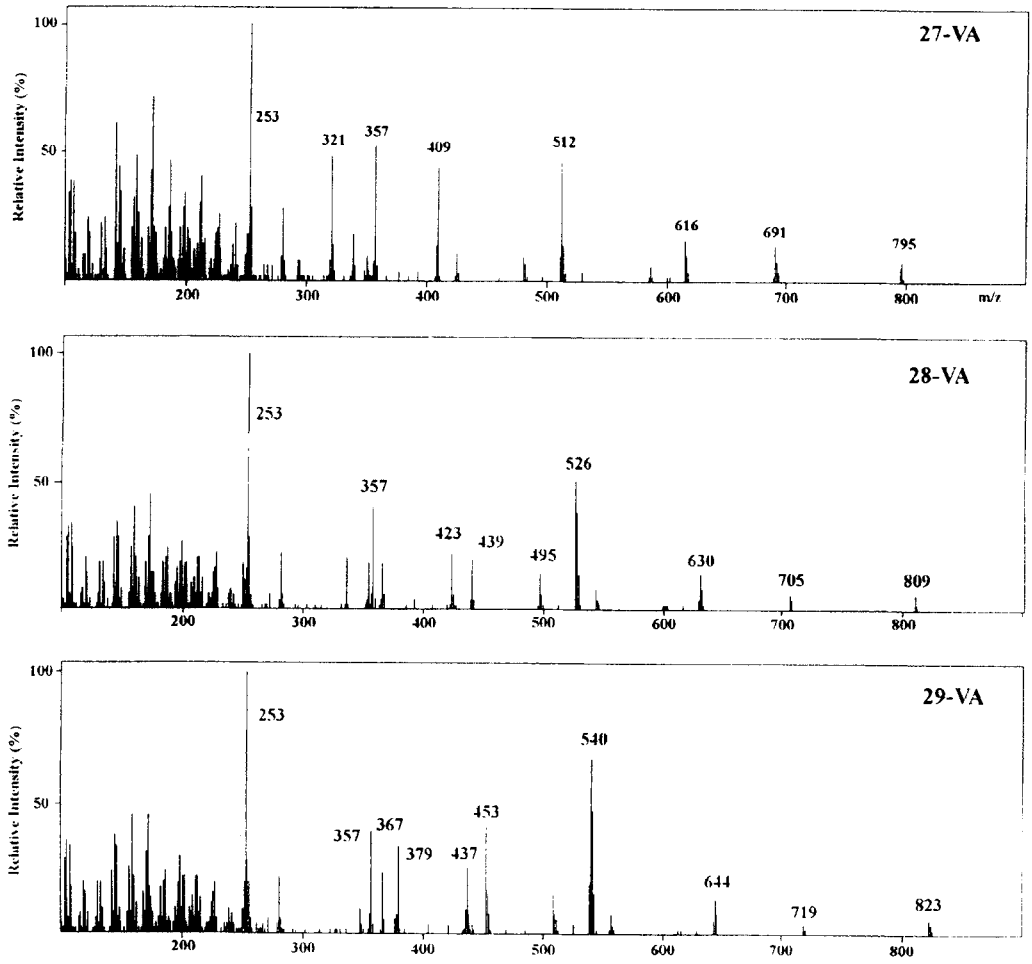

Fig. 5. Mass spectra of methyl ester-dimethylethyl silylether derivatives of the β-hydroxylated intermediates of β-oxidation by rat liver 800 *g* supernatant. (a) 27-VA, (b) 28-VA, (c) 29-VA.

show mass spectra of Me-DMES derivatives of the above final degradation products (CA, *homo*-CA, *nor*-26-THCA), α,β-unsaturated products (26-, 27-, 28- and 29-D-THCA) and β-hydroxylated intermediates (27-, 28- and 29-VA), respectively. The characteristic fragment ions were completely identical to authentic specimens as shown in Table 2.

The results of the quantitative determination of the above products are summarized in **Table 3**. The formation of α,β-unsaturated intermediates (27-Δ-, 28-Δ- and 29-Δ-THCA) were more than two times higher than that of 26-Δ-THCA. The highest rate of producing α,β-unsaturated intermediates was observed for the C_{27}-bile acid (27-THCA). The formation of β-hydroxylated intermediates were in the order of 27-VA > 28-VA > 29-VA. The ratios against those of corresponding α,β-unsaturated bile acids (27-Δ-, 28-Δ-, and 29-Δ-THCA) were 0.46 0.32, and 0.28, respectively. The degradation products (CA, *homo*-CA, and *nor*-26-THCA) were also produced from corresponding substrates (27-, 28-, 29-THCA). The formation ratios of CA, *homo*-CA, and *nor*-26-THCA against the formation of β-hydroxylated products were about 0.73, 1.24, and 0.91, respectively. The total formation of the products is in the order of 27-THCA > 28- THCA > 29-THCA > 26-THCA. These results indicated the specificities for side-chain length in β-oxidation system of bile acids.

Table 3 also shows the results from incubation with clofibrate-treated 800 *g* supernatant. 26-THCA gave 26-Δ-THCA as a product of β-oxidation, and the formation was approximately the same as that of the control. The formation of 27-Δ-THCA and 27-VA from 27-THCA was also the same as that of the control. The enzymes catalyzing these dehydrogenation and hydration reactions were not affected by clofibrate treatment. However, the formation of CA increased to about three times higher than that of the control. It seemed that the enzyme of this final step was induced

TABLE 3. Determination of products in β-oxidation of 5β-cholestanoic acid derivatives

Substrate	Inducer	Formation of α,β-Unsaturated Intermediate	Formation of β-hydroxylated Intermediate	Formation of Final Product
			pmol/min^{-1} mg^{-1}	
26-THCA	control	5.6 ± 1.7	n.d.	n.d.
	clofibrate	5.0 ± 1.7	n.d.	n.d.
		(26-Δ-THCA)	(26-VA)	(*nor*-CA)
27-THCA	control	16.7 ± 0.9	7.7 ± 1.9	5.6 ± 3.6
	clofibrate	17.1 ± 2.1	6.8 ± 1.7	17.7 ± 4.0
		(27-Δ-THCA)	(27-VA)	(CA)
28-THCA	control	12.8 ± 2.1	4.1 ± 0.6	5.1 ± 1.9
	clofibrate	28.1 ± 3.8	9.4 ± 1.5	20.8 ± 8.0
		(28-Δ-THCA)	(28-VA)	(*homo*-CA)
29-THCA	control	12.0 ± 5.3	3.4 ± 1.0	3.1 ± 1.2
	clofibrate	21.0 ± 2.9	6.4 ± 1.2	17.8 ± 2.6
		(29-Δ-THCA)	(29-VA)	(*nor*-26-THCA)

The values represent mean ± SD (n = 4); n.d., not detectable.

by clofibrate treatment. This is in good accordance with the reported fact that the final enzyme, acyl-CoA thiolase, may be the same as that of the fatty acid peroxisomal β-oxidation enzyme (14).

On the other hand, the induction of desaturation, hydration, and final cleavage steps was observed for 28-THCA and 29-THCA. The formation of intermediates (28-Δ-THCA, 29-Δ-THCA, 28-VA, and 29-VA) and final products (*homo*-CA and *nor*-26-THCA) was 2- to 4-fold higher than that of the control. In the final step of β-oxidation of the C_{29}-bile acid (29-THCA), the formation of CA was also observed by further side-chain cleavage of *nor*-26-THCA. The data indicated for the formation of *nor*-26-THCA are calculated from the total formation of *nor*-26-THCA and CA. The formation of 28-Δ- and 29-Δ-THCA was stimulated to about two times higher than that of control. Moreover, the formation of 28- and 29-VA was also about two times higher than that of the control. This suggested that the enzymes induced with clofibrate for desaturation and hydration of 28-THCA and 29-THCA are distinct from those for 27-THCA. The order of the total formation of products by clofibrate treatment was changed to 28-THCA > 29-THCA > 27-THCA > 26-THCA, which also indicated that an enzyme system different from that for 27-THCA is induced with clofibrate.

Analysis of incubation products from 27-demethylated 5β-cholestanoic acid (*nor*-26-THCA) derivatives

Figure 6 shows the gas chromatograms of the Me-DMES derivatives of products from *nor*-26-THCA, *nor*-27-THCA, and *nor*-28-THCA. The intermediate α, β-unsaturated bile acids (*nor*-26-Δ-, *nor*-27-Δ-, and *nor*-28-Δ-THCA) and β-hydroxylated bile acids (*nor*-26-, *nor*-27-, and *nor*-28-VA) were not detected, and only the degradation products (CA, *homo*-CA, and *nor*-26-THCA) were observed. The absence of the intermediates suggested that the β-oxidation system for these derivatives without a terminal methyl group might be different from that for 27-THCA. The formation of degraded bile acids was in the order of *nor*-27-THCA > *nor*-26-THCA > *nor*-28-THCA, as summarized in **Table 4**. The above order indicated that the enzyme system responsible for the side-chain cleavage of these demethylated derivatives showed specificities for the side-chain length (maybe in this system, carbon 27 is most favorable). In the case of clofibrate treatment, the formation of degradation products (CA, *homo*-CA, and *nor*-26-THCA) was significantly increased. This strongly suggests that the enzyme system for *nor*-26-, *nor*-27-, and *nor*-28-THCA is the same as that for long-chain fatty acids.

Inhibition by palmitic acid

Figure 7 shows the results of analysis of the products from 27-THCA and *nor*-26-THCA in the presence of various concentration of palmitic acid with clofibrate-treated 800 *g* supernatant. The β-oxidation of 27-THCA was not affected in the presence of palmitic acid (Fig. 7a). However, the β-oxidation of *nor*-26-THCA was strongly inhibited in the presence of palmitic acid (Fig. 7b). These results suggested that β-oxidation of the demethylated derivatives may proceed through the fatty acid β-oxidation.

DISCUSSION

Incubation of 5β-cholestanoic acid (27-THCA) derivatives

The side-chain degradation reaction (β-oxidation) for bile acid biosynthesis is localized in the liver peroxi-

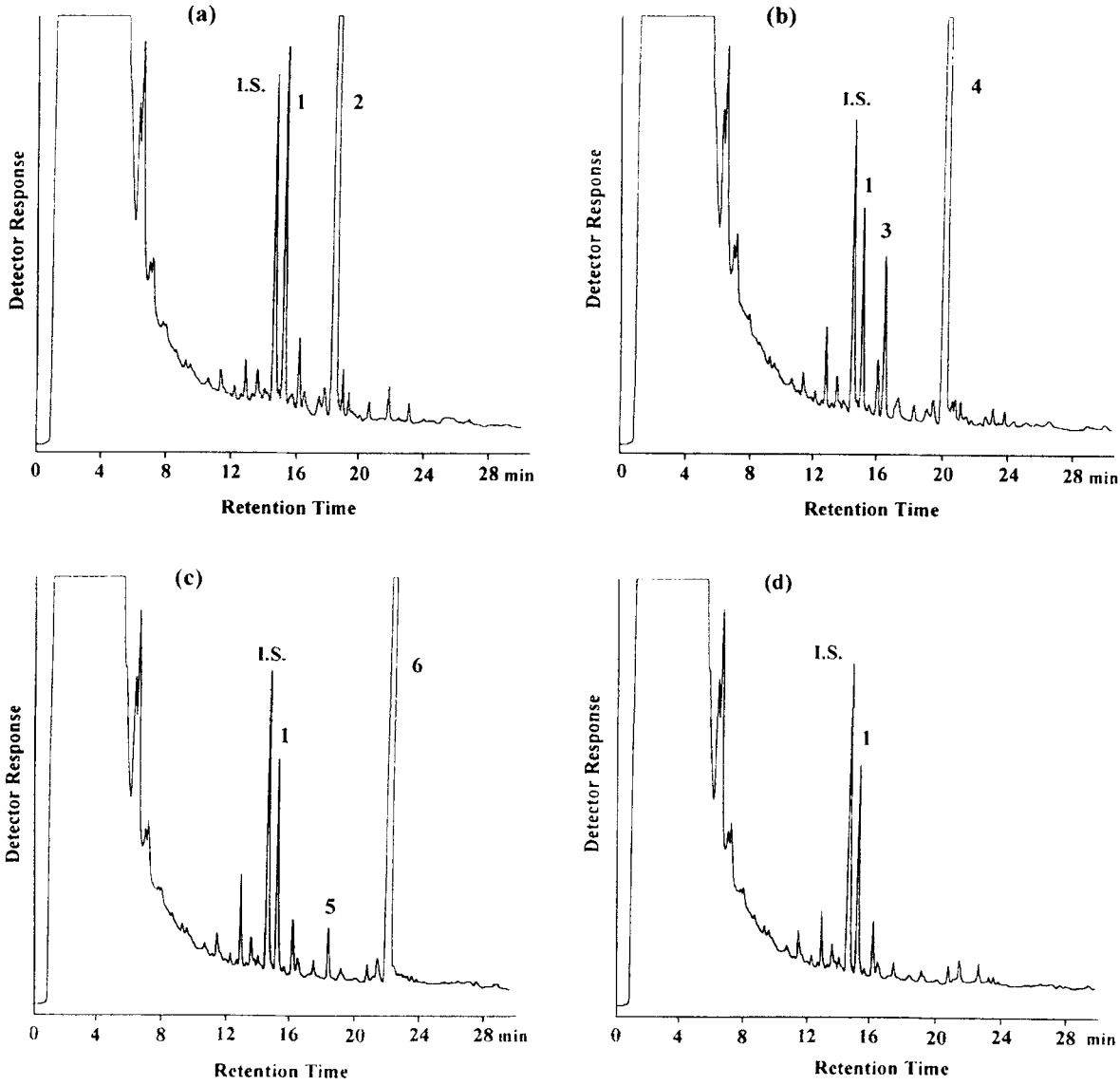

Fig. 6. Gas chromatograms of the methyl ester-dimethylethylsilyl ether derivatives of the products from *nor*-26-THCA (a), *nor*-27-THCA (b), *nor*-28-THCA (c), by rat liver 800 *g* supernatant and without substrate (d). I.S., internal standard (3α,12α-dihydroxy-24-methyl-26,27-*dinor*-5β-cholestan-25-oic acid); 1, CA; 2, *nor*-26-THCA (substrate); 3, *homo*-CA; 4, *nor*-28-THCA (substrate); 5, *nor*-26-THCA; 6, *nor*-28-THCA (substrate). Unnumbered peaks are due to the impurities from rat liver 800 *g* supernatant and derivatizing reagent.

some (12, 26, 29), and it involves several reaction steps. Analysis of the products of each reaction step in bile acid β-oxidation in the rat liver homogenate, therefore, may be useful for the study of peroxisomal enzymatic function. One of the aims of this study was to identify and quantify the products by the side-chain degradation step of bile acid in the intact rat liver. For this purpose, rat liver 800 *g* supernatant was used as an enzymatic source of β-oxidation without further fractionation to investigate the substrate specificities. Thus, the present results may represent various enzymatic reactions not only in peroxisomes but also in other organelles such as mitochondria and endoplasmic reticulum.

Although the stereoisomeric specificity of β-oxidation (acyl-CoA oxidase) is known (10), it is thought that the enzymatic epimerization (30) of the activated form in β-oxidation (CoA ester of bile acid) eliminates the effect of the above stereospecificity. Therefore, the substrates with a terminal methyl group in the side chain (26-THCA, 28-THCA, and 29-THCA) were used as a diastereomixture, except for 27-THCA (25*R*-isomer was used).

The formation of the products as shown in Table 3

TABLE 4. Determination of metabolites in β-oxidation of 27-nor-5β-cholestanoic acid derivatives

Substrate	Inducer	Formation of α,β-Unsaturated Intermediate	Formation of β-Hydroxylated Intermediate	Formation of Final Product
nor-26-THCA	control	n.d.	n.d.	8.8 ± 4.2
	clofibrate	n.d.	n.d.	93.7 ± 38.6
		(*nor*-26-Δ-THCA)	(*nor*-26-VA)	(CA)
nor-27-THCA	control	n.d.	n.d.	11.8 ± 4.7
	clofibrate	n.d.	n.d.	119.0 ± 5.1
		(*nor*-27-Δ-THCA)	(*nor*-27-VA)	(*homo*-CA)
nor-28-THCA	control	n.d.	n.d.	3.2 ± 1.8
	clofibrate	n.d.	n.d.	78.7 ± 15.5[a]
		(*nor*-28-Δ-THCA)	(*nor*-28-VA)	(*nor*-26-THCA)

The values represent mean ± SD (n = 4); n.d., not detectable.
[a]The value was calculated from the total amounts of *nor*-26-THCA and CA which were produced by the further oxidation of *nor*-26-THCA.

may not show the true activities of the enzymes, although we consider that these values reflect the specificities for different side-chain lengths in the whole enzymatic system(s). The α,β-unsaturated intermediates (26-Δ-, 27-Δ-, 28-Δ-, and 29-Δ-THCA) were detected in both the control and clofibrate-treated rat liver 800 *g* supernatant. In the control, the highest formation was observed when 27-THCA was used as a substrate. As 27-THCA is the endogenous substrate of β-oxidation in bile acid biosynthesis, it may be reasonable that the first reaction step represents the highest activity for 27-THCA. The above data indicated that acyl-CoA oxidase, the first enzyme of β-oxidation of bile acid, could distinguish the side-chain length of the substrate when the same enzyme related to the production of the above four α,β-unsaturated intermediates. On the other hand, the highest formation of 28-Δ-THCA was observed with clofibrate-treated rat liver 800 *g* supernatant. It was considered that another acyl-CoA oxidase for 28- and 29-THCA might be induced by clofibrate treatment.

There also seemed to be different specificities for α,β-unsaturated intermediates in the second reaction step, the hydration step, as the formation of the β-hydroxylated products decreased with elongation of the side chain. It is also considered that shortening of the side chain of 27-THCA extinguished the enzymatic activity for hydration. It seemed that the total formation of β-hydroxylated products and final degraded products much more clearly represented the

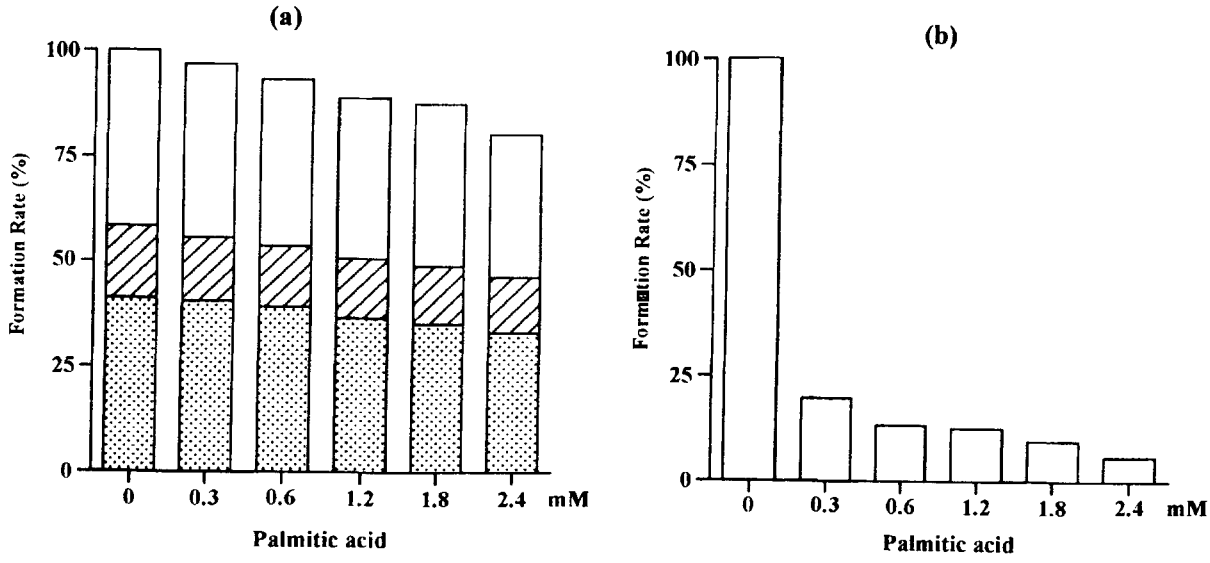

Fig. 7. Formation of intermediates and degradation products from 27-THCA (a) and *nor*-26-THCA (b) by clofibrate-treated rat liver 800 *g* supernatant under the various concentrations of palmitic acid. The formation rate of total products in the absence of palmitic acid was taken as 100%. The concentrations of both bile acids were 0.2 mM, □; formation of CA, ▨; formation of 27-VA, ■, formation of 27-Δ-THCA.

real activities of the related enzyme(s) in this hydration step. The total formation in these two steps (13.3, 9.2, and 6.5 pmol/mg protein per min for 27-VA + CA, 28-VA + *homo*-CA, and 29-VA + *nor*-26-THCA, respectively) against the corresponding formation of the α,β-unsaturated products was about 0.80, 0.72, and 0.54, respectively, which indicated the different specificities in the hydration step for side-chain length. In the case of clofibrate treatment, the highest total formation was observed for 28-THCA, which may suggest that the induced enzyme(s) are responsible for hydration step.

The final side-chain cleavage of the β-hydroxylated intermediate is catalyzed formally with two enzymes (dehydrogenase and thiolase). Evaluation of the above two enzymatic activities was impossible in the present work. However, this step may represent the activity of the final thiolytic cleavage, because the β-oxo-intermediate (3α, 7α, 12α-trihydroxy-24-oxo-5β-cholestan-26-oic acid) produced from VA derivatives (27-, 28-, and 29-VA) by dehydrogenase were not detected. It is shown that the β-oxo-intermediate from 27-THCA was easily decarboxylated to produce the 24-oxo-cholestan derivative (31). We attempted to determine the decarboxylated products, but the amounts were under the limits of quantitative determination. The β-oxo-intermediate might be smoothly subjected to thiolytic cleavage under the present conditions. From the results of both control and clofibrate treatment, it was considered that the enzymes in the last cleavage step did not show remarkable specificities for different side-chain length.

It is known that the β-oxidation system of fatty acids has substrate specificity for different carbon-chain lengths (15, 16). In order to clarify the difference from fatty acid β-oxidation, the above 27-THCA derivatives were incubated with clofibrate-treated rat liver 800 *g* supernatant. Clofibrate is known to significantly induce the peroxisomal β-oxidation system of fatty acids but to induce that of bile acids only slightly (32, 33). The induced β-oxidation system for 28-THCA and 29-THCA seemed likely to be the β-oxidation system for long, straight-chained fatty acids. However, the induction rate of the fatty acid β-oxidation system is generally much higher than that of control (28). In this respect, the induced β-oxidation system for 28-THCA and 29-THCA is somewhat different from the fatty acid β-oxidation system. In the above induced β-oxidation system, slightly induced pristanoyl-CoA oxidase (9) may concern the desaturation step. And two multifunctional enzymes (2-enoyl-CoA hydratases) catalyzing the hydration of 27-Δ-THCA were also known (34). The inducible multifunctional enzyme displays L-3-hydroxyacyl-CoA dehydrogenase activity and another displays D-3-hydroxyacyl-CoA dehydrogenase activity. The former (inducible) dose does not catalyze the dehydrogenation of its hydration product (35). It was considered that the above inducible multifunctional enzyme may also catalyze the hydration of 28- and 29-Δ-THCA and the equilibrium reaction between its substrates (28-Δ-THCA and 29-Δ-THCA) and products (28-VA and 29-VA).

Incubation of 27-demethylated 5β-cholestanoic acid (*nor*-26-THCA) derivatives

The substrates (*nor*-26-THCA, *nor*-27-THCA, and *nor*-28-THCA) used in this experiment have both a bile-acid structure and a fatty acid-like straight side-chain. As the enzymatic systems for fatty acids and for bile acids are known, it is interesting to clarify whether the above derivatives are subject to β-oxidation as bile acids or as fatty acids. These demethylated derivatives gave only side-chain degradation products and intermediates were not detected. These results indicate that the β-oxidation system for these derivatives is different from that for 27-THCA. The order of the formation rates in the control rat liver 800 *g* supernatant showed specificities for the side-chain length. The highest rate was observed in *nor*-27-THCA (C_{27}-bile acid) as in the same case of 27-THCA. The formation of degraded products of these derivatives was significantly stimulated (10- to 20-fold that of the control) by treatment with clofibrate (Table 4). Moreover, the formation of CA was strongly inhibited in the presence of palmitic acid as shown in Fig. 7. These results indicate that there are different β-oxidation systems for 5β-cholestanoic and demethylated 5β-cholestanoic acid derivatives.

In conclusion, specificities for the side-chain length in β-oxidation systems were observed. It is noteworthy that 27-THCA and *nor*-27-THCA, which have the same 27 carbon units, exhibited the highest activities for each β-oxidation system. The β-oxidation for demethylated 5β-cholestanoic acid derivatives may proceed by the fatty acid β-oxidation system, in spite of its bile acid structure.

The β-oxidation system of bile acid formation has been reported to be mainly localized in the peroxisome (12, 26, 29). However, it has been shown that the formation of 27-VA from 27-Δ-THCA occurs in the microsomal fraction fortified with 100,000 *g* supernatant fluid (36). And it is also known that mitochondrial enzymes are able to oxidize branched fatty acids (37). Under the present experimental conditions, it is uncertain whether these enzymatic reactions proceed in a special organelle, such as peroxisome, and therefore, the present results may contain the formation of intermediates in peroxisome, mitochondria, and microsomes. Further study using a purified peroxisomal fraction is now in progress.

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