Chem. Pharm. Bull. **42**(5) 1028—1035 (1994) Vol. 42, No. 5

Non-stereoselective Formation of 3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic Acid during Cholic Acid Biosynthesis

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Incubation of (25*RS*)-, (25*R*)- and (25*S*)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA, 6, 6a, 6b) and (24*E*)-3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid (7) with rat liver mitochondria gave all four stereoisomers (9a, 9b, 9c, 9d) of 3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (TeHCA). The corresponding 27-nor analogs (10, 11) were also converted non-stereoselectively to a 1 : 1 mixture of the epimeric 24-hydroxy compounds (12).

Keywords bile acid; cholic acid; β-oxidation; 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid; 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid

A primary bile acid cholic acid (**5**), is biosynthesized in liver from cholesterol *via* the coenzyme A (CoA) thioester derivative (**1**) of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA). Conversion of **1** into **5** has been thought to occur by a mechanism similar to that of the β-oxidation of fatty acid (Chart 1).[1,2] The key intermediates of this pathway would be the Δ^{24}-olefin (**2**), 24-alcohol (**3**) and 24-ketone (**4**). Concerning the stereochemistry

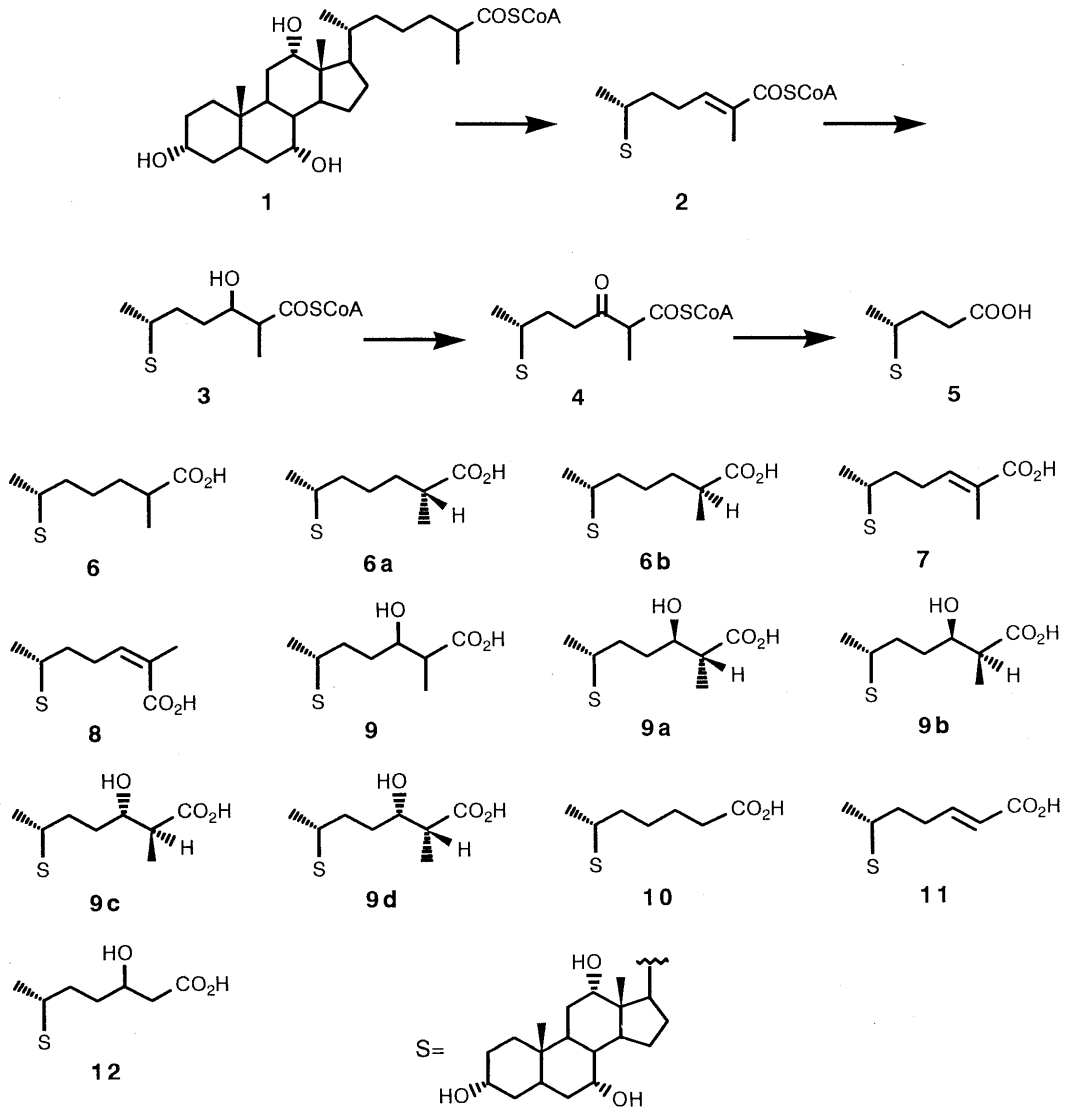

Chart 1

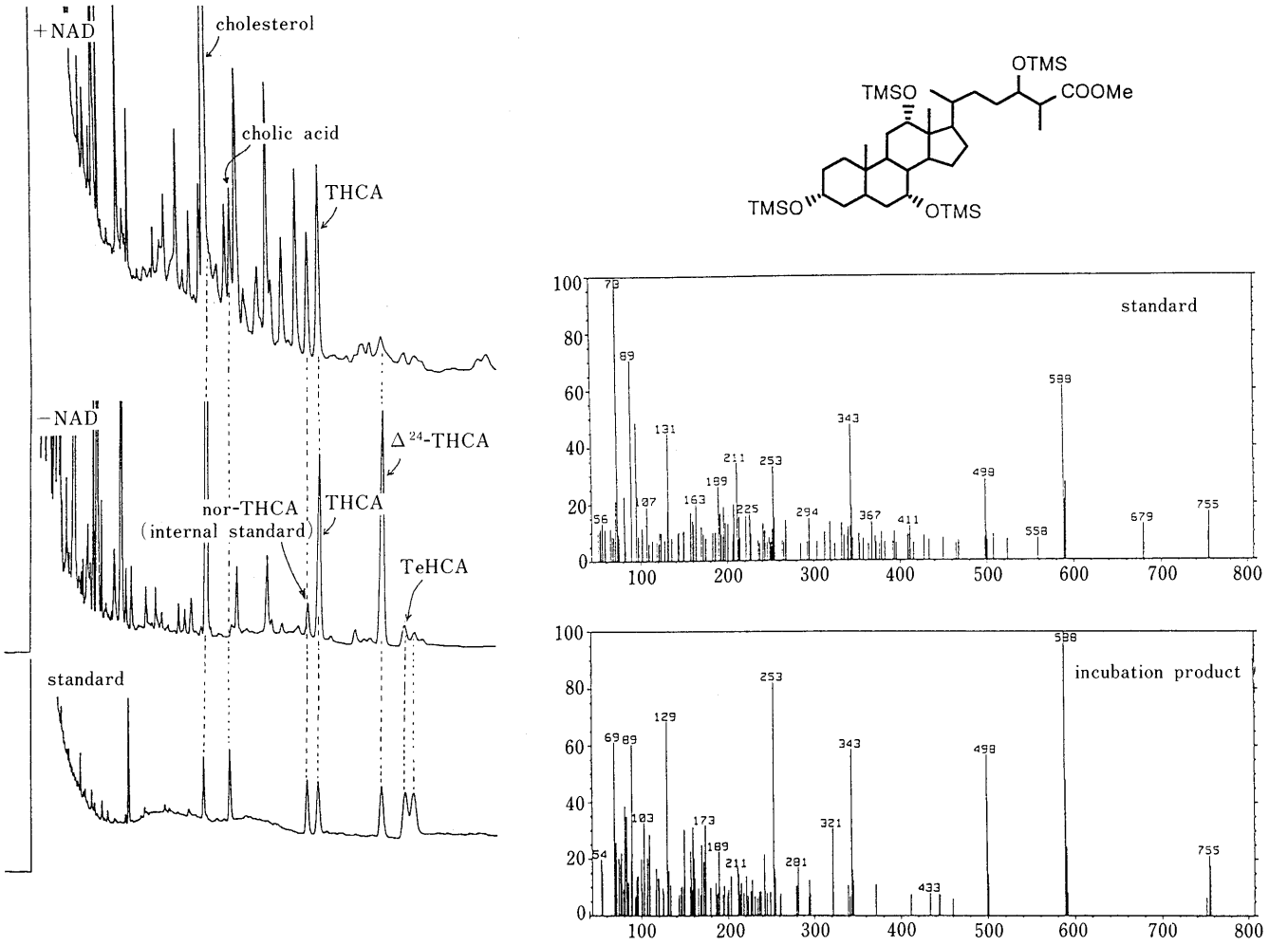

Fig. 1. GC-MS Analysis of the Mitochondrial Incubation Products from (25*RS*)-THCA

Left: GC of the products on incubation in the presence (upper) or in the absence (middle) of NAD, in comparison with a mixture of the standard samples indicated (bottom). Right: EI-MS of the GC peak corresponding to TeHCA of standard sample (upper) and of the incubation product (lower).

of these compounds, we have previously demonstrated[3] that all four diastereoisomer (**9a**—**d**) of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (TeHCA) were converted to cholic acid (**5**) on incubation with mitochondrial fraction of rat liver homogenate in the presence of CoA, nicotinamide adenine dinucleotide (NAD), adenosine triphosphate (ATP) and $MgCl_2$. However, others have found[4,5] that only the 24*R*,25*R*-isomer (**9a**) was produced from THCA on incubation with the $800 \times g$ supernatant fraction of rat liver homogenate. We report here that incubation with rat liver mitochondria produced all four stereoisomers (**9a**—**d**) from THCA (**6**)[6] and (24*E*)-3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid (24-ene-THCA, **7**).

Incubation of (25*RS*)-THCA (6) (25*RS*)-THCA (**6**) was incubated with mitochondrial fraction of rat liver homogenate under various conditions, and the products were processed as described in Experimental. Figure 1 illustrates GC-MS profiles of the incubation products analyzed as their methyl ester trimethylsilyl (TMS) ether derivatives. It can be seen that in the presence of NAD, ATP and CoA, (25*RS*)-THCA (**6**) was transformed into cholic acid (**5**) in accord with the previous result,[7] whereas TeHCA (**9**) was scarcely detected. However, removal of NAD from the incubation medium induced the accumulation of **9** which was conclusively identified by the coincidence of its MS with that of a standard sample. These results are in agreement with expectation, because NAD should be a requisite cofactor of the alcohol dehydrogenase catalyzing the conversion of the 24-alcohol (**3**) to the 24-ketone (**4**). As shown in Fig. 1, GC is able to resolve the four isomers of TeHCA into two (not four) peaks. Complete separation of the four isomers of TeHCA was attained by HPLC of their *p*-bromophenacyl ester derivatives. The results of HPLC analysis of the incubation products (TeHCA fraction) are shown in Fig. 2. In accord with the GC-MS analysis (Fig. 1), TeHCAs were clearly identified among the products of incubation carried out in the absence of NAD. It should be noted that not just the single isomer, *i.e.* 24*R*,25*R*-TeHCA (**9a**),[4,5] but all four stereoisomers (**9a**—**d**) were produced from (25*RS*)-THCA (**6**). Figure 2 also indicates that incubation in the complete system (in the presence of all the cofactors) gave a much lower level of TeHCA. Under this condition, most THCA was transformed into the final product cholic acid (**5**), as evidenced by GC-MS analysis (Fig. 1), with only

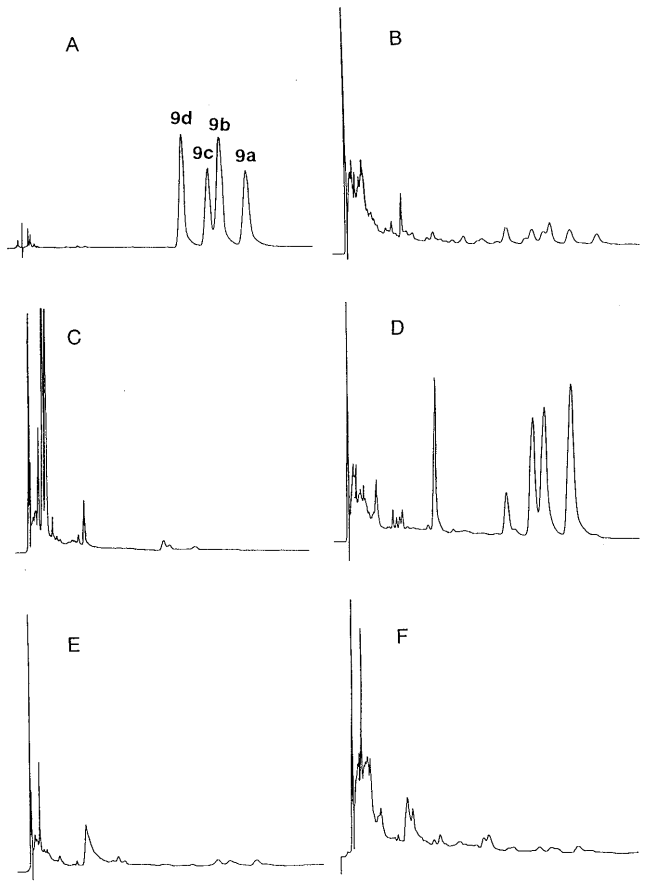

Fig. 2. HPLC Profiles of TeHCA (*p*-Promophenacyl Ester) of the Mitchondrial Incubation Products from (25*RS*)-THCA

A: standard samples of **9a**, **9b**, **9c** and **9d**, B: incubation in the presence of ATP, $MgCl_2$, CoA and NAD (complete system), C: omitting the substrate from the complete system, D: omitting NAD, E: omitting CoA and NAD, F: omitting CoA. Omitting ATP gave a similar profile to E and F.

a small accumulation of TeHCA (**6**). The requirement of CoA and ATP for TeHCA production is also suggested from Fig. 2. Since the observed non-stereoselective formation of TeHCA is in conflict with the results of others (*vide supra*),[4,5] we then examined the reaction under their experimental conditions, especially fraction of rat liver homogenate. As shown in Fig. 3, the yields of TeHCAs with $700 \times g$ supernatant were lower in comparison with mitochondrial incubation, and the peak due to (24*R*,25*R*)-TeHCA (**9a**) was more prominent, although the other three isomers (**9b—d**) were also produced in significant amounts. Further, incubation products of the $10000 \times g$ supernatant fraction showed HPLC profiles similar to those of $700 \times g$ supernatant fraction (Fig. 3). Therefore we are inclined to conclude that the four stereoisomers are always produced, irrespective of the enzyme preparation used, although the relative yields are variable.

Incubation of (25*R*)-THCA (6a) and (25*S*)-THCA (6b) The above finding of non-stereoselective formation of the four stereoisomers (**9a—d**) of TeHCA was obtained by the use of a *ca.* 1 : 1 mixture of C-25 stereoisomers of THCA (**6a**, **6b**) as the substrate. It might be that two out of the four isomers of TeHCA could be formed from (25*R*)-THCA (**6a**) and the other two from (25*S*)-THCA (**6b**). In order to examine this possibility, we prepared

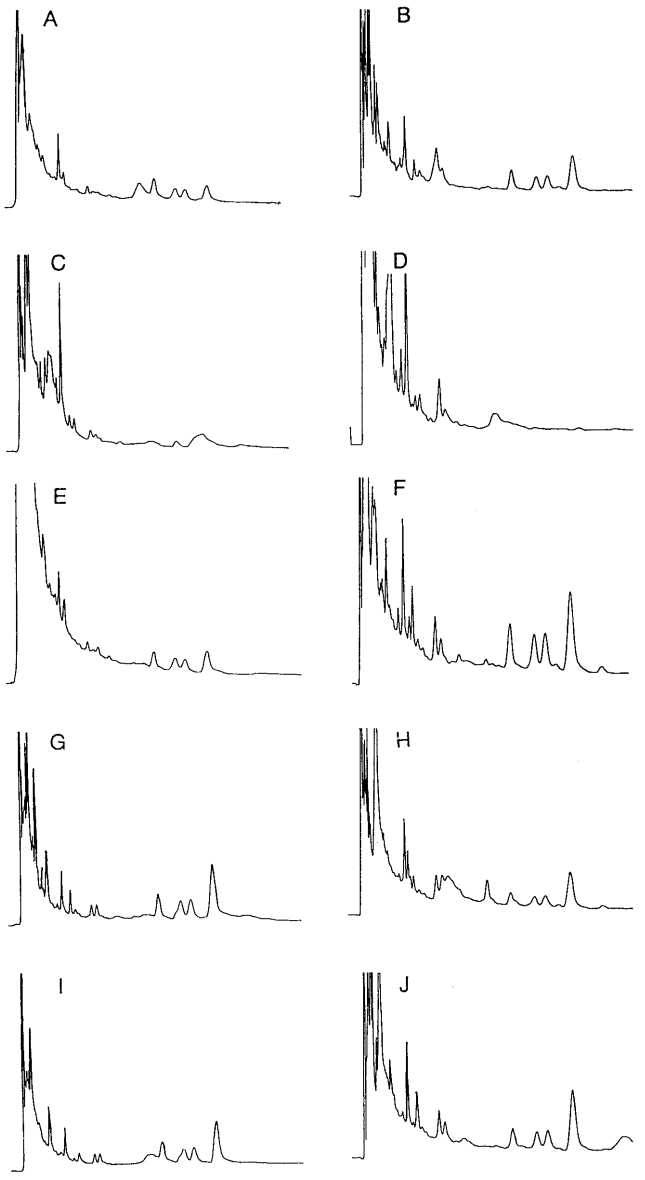

Fig. 3. HPLC Profiles of TeHCA (*p*-Bromophenacyl Ester) Produced on Incubation of $700 \times g$ (Left) or $10000 \times g$ (Right) Supernatant Fraction with (25*RS*)-THCA

A and B: incubation in the presence of ATP, $MgCl_2$, CoA and NAD. C and D: omitting the substrate. E and F: omitting NAD. G and H: omitting CoA. I and J: omitting NAD and CoA.

(25*R*)- and (25*S*)-THCA (Chart 2) and incubated them under the same conditions as described for (25*RS*)-THCA. For this purpose, the chemically synthesized (25*RS*)-THCA (**6**)[6] was derivatized to the *p*-bromophenacyl ester, which was then subjected to HPLC separation[8] utilizing a linear combination of two columns of STR PREP-ODS (25 cm × 20 mm i.d.). The resolved esters, the less mobile (25*R*)- and the more mobile (25*S*)-epimers, were separately hydrolyzed to give (25*R*)-THCA (**6a**) mp 187—189 °C (lit.[6,9] 183—184 °C) and (25*S*)-THCA (**6b**) mp 204—206 °C (lit.[6,9] 199—201 °C). Attempted preparative resolution of the (25*RS*)-THCA in the form of the free acid (**6**) (ODS column, eluted with methanol–trifluoroacetic acid–triethylamine–water, pH 2.9[10]) failed due to broadening of the peaks. Under this condition, (25*S*)-

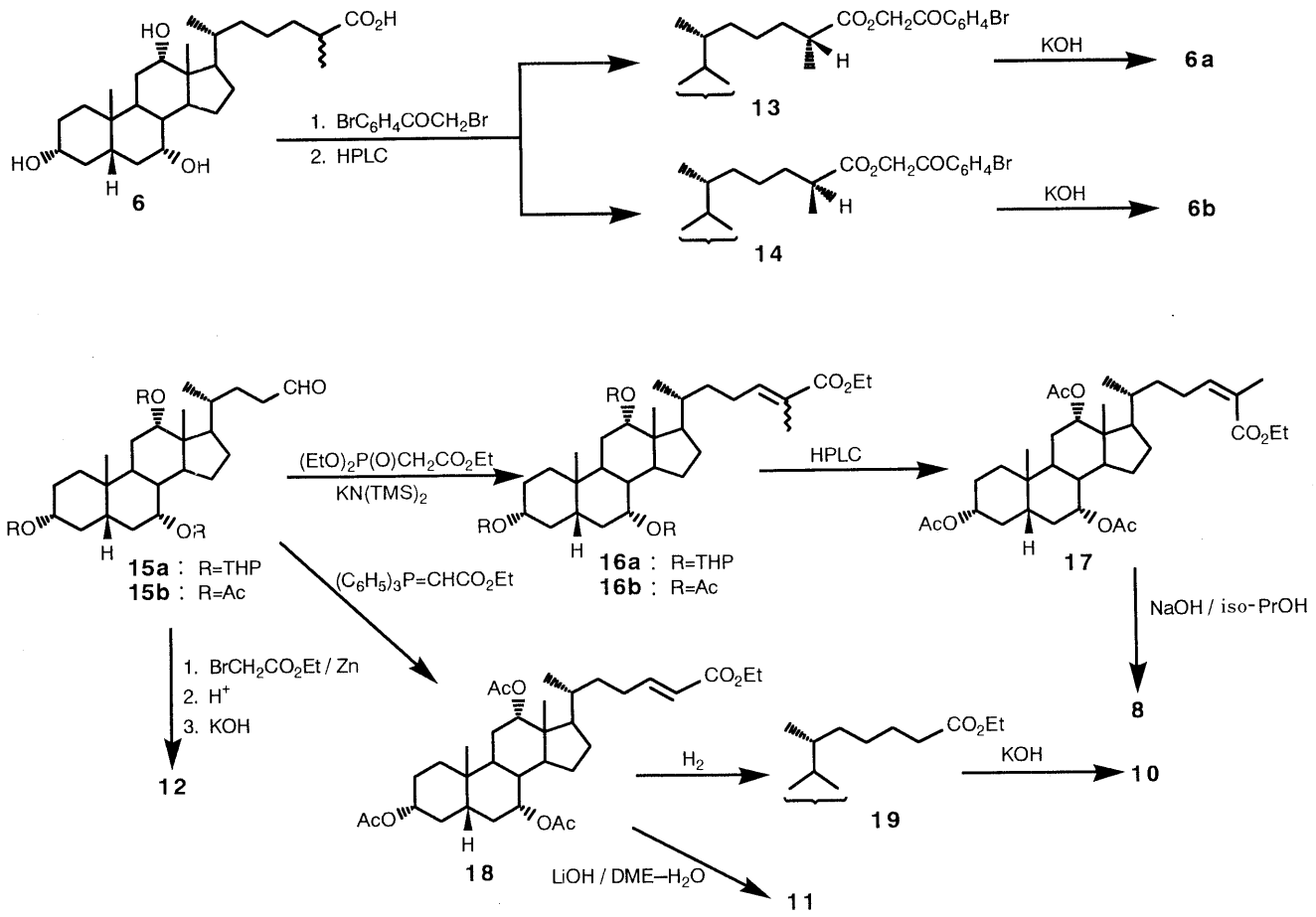

Chart 2

THCA was found to move faster than the (25*R*)-epimer, and this behavior is opposite to that described in the literature.[10] Incubation products from (25*R*)-THCA (**6a**) and (25*S*)-THCA (**6b**) showed almost identical HPLC profiles to that from (25*RS*)-THCA (**6**), and four (not two) peaks of TeHCA were observed (Fig. 4). These results suggest that (25*R*)- and (25*S*)-THCA are both transformed into the four stereoisomers of TeHCA through a common intermediate, most probably the (24*E*)-olefin (**2** or **7**).[10] HPLC analysis (Fig. 4 right) of the less polar fraction of the incubation products revealed the peak of (24*E*)-olefin (**7**), accompanied by a small peak of (24*Z*)-olefin (**8**). At the longer retention time a pair of small peaks appeared, and these were found to be due to the substrate THCAs remaining in the incubation medium. It should be noted that the level of the remaining (25*S*)-THCA (**6b**) seems to be always lower than that of (25*R*)-THCA (**6a**). This was further confirmed by incubation for shorter times (data not shown). These results suggest that the 25*S*-isomer (**6b**) is consumed faster than the 25*R*-isomer (**6a**), and are reminiscent of the recent report[11] that the activity of acyl-CoA synthetase for (25*S*)-THCA is 1.4-times higher than that for (25*R*)-THCA.

Incubation of 24-Ene-THCA (7, 8) According to Chart 1, the direct precursor of the 24-alcohol (**3**) should be the 24-olefin (**2**). When (24*E*)-24-ene-THCA (**7**) was incubated in the complete system, cholic acid was conclusively identified as a product by GC-MS and HPLC (data not shown). Under the same conditions, the (24*Z*)-isomer (**8**), of which the chemical preparation is outlined in Chart 2, was not significantly metabolized. Incubation of the (24*E*)-olefin (**7**) in the absence of NAD accumulated TeHCA as shown in Fig. 5. Formation of TeHCA from the (24*Z*)-olefin (**8**) was also observed albeit with much lower effectiveness, and this TeHCA could arise from the (24*E*)-olefin (**7**) contaminating (less than 5%, Experimental) the substrate. The HPLC profile of Fig. 5B is remarkably similar to that of Fig. 2D, the relative peak heights of the four TeHCAs being almost identical. This strongly suggests that the (24*E*)-olefin (**2** or **7**) is an obligatory intermediate in the transformation of THCA (**6**) into TeHCA (**9**); the non-stereoselectivity should arise at the stage of hydration of the (24*E*)-olefin.

Incubation of 27-Nor Compounds (10, 11, 12) The apparent non-stereoselectivity in β-oxidation of THCA (**6**) described above is in marked contrast to the strict stereoselectivity found in β-oxidation of fatty acid, where the 3*S*- but not the 3*R*-hydroxyacyl-CoA is established as the intermediate.[12] This discrepancy could be due to the presence of the "extra" methyl group (C-27) in the steroidal substrate THCA (**6**). Then 27-nor-THCA (**10**), (24*E*)-27-nor-olefin (**11**) and (24*RS*)-27-nor-TeHCA (**12**) were chemically prepared (Chart 2, Experimental), and incubated in the same manner as described above. In the presence of NAD, these 27-nor compounds were effectively converted to cholic acid; the yields of cholic acid from **10**,

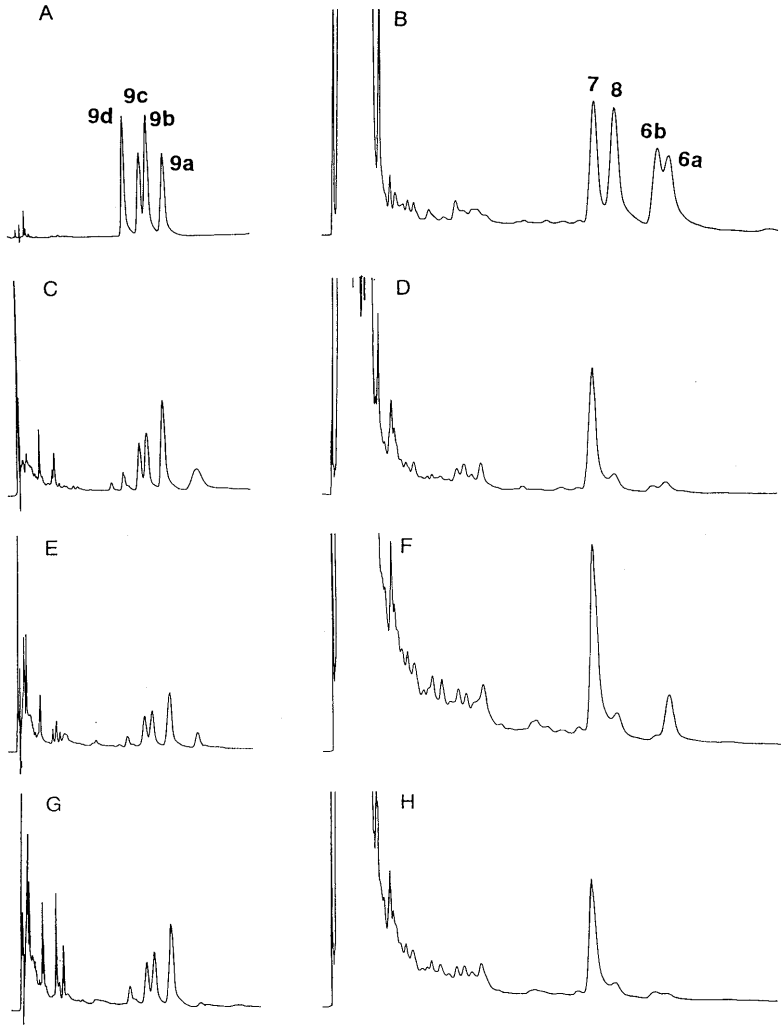

Fig. 4. HPLC Profiles of TeHCA (*p*-Bromophenacyl Ester) (Left) and the Less Polar Fraction (Right) of the Mitochondrial Incubation (in the Absence of NAD) Products from (25*R*)-THCA or (25*S*)-THCA

A: standard samples of **9a**, **9b**, **9c** and **9d**. B: standard samples of **6a**, **6b**, **7** and **8**. C and D: incubation products from (25*RS*)-THCA. E and F: from (25*R*)-THCA, G and H: from (25*S*)-THCA.

11, **12** and **9d** estimated by the reported method[3] were 9, 19, 17 and 11%, respectively. In the absence of NAD, 27-nor-THCA (**10**) and 27-nor-24-olefin (**11**) yielded a mixture (*ca.* 1:1) of the C-24 stereoisomers of 27-nor-TeHCA (**12**) as shown in Fig. 6. Thus non-stereoselectivity was again observed with the 27-nor series, and the above hypothesis is invalid.

Conclusion

The present data together with the previous results[3] indicate that all four stereoisomers of TeHCA (**9a**—**d**) are produced from THCA (**6**) and (24*E*)-24-ene-THCA (**7**) on incubation with rat liver mitochondria, and transformed into cholic acid (**5**). Thus all of them, in the form of CoA thioester derivatives, are possible intermediates of cholic acid biosynthesis. The genuine intermediate *in vivo* or under physiological conditions remains to be identified.

Experimental

Incubations Male rats of the Wistar strain weighing *ca.* 180 g were used. Rat liver homogenate 50% (w/v) was prepared in 0.25 M sucrose solution with a loose-fitting glass homogenizer. The homogenate was centrifuged at 700 × *g* for 15 min at 4 °C. The supernatant was used as such for incubation (Fig. 3) or recentrifuged at 10000 × *g* for 20 min. The new supernatant layer was used as such for incubation (Fig. 3) or discarded. The residual mitochondrial fraction was suspended in 0.1 M Tris–HCl buffer, pH 8.5 (1 ml of mitochondrial suspension corresponds to 0.6 g of rat liver). Incubations were initiated by addition to the enzyme solution (4 ml) of substrate (100 μg) solution in methanol (50 μl) and a Tris–HCl buffer solution (1.35 ml, pH 8.5) containing ATP (25 mg), $MgCl_2$ (8 mg), CoA (2 mg) and NAD (5 mg). Incubations were conducted for 1 h at 37 °C.

GC-MS Analysis Incubations were terminated by addition of 2 ml of 5% NaOH. An internal standard, 27-nor-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (20 μg), was added and the mixture was heated at reflux for 5 h under nitrogen. The cooled mixture was washed with ether (30 ml × 2) and acidified with 2 N HCl. The aqueous layer was extracted with ethyl acetate (30 ml × 3), and the extract was washed with brine, dried over $MgSO_4$ and concentrated. The residue was treated with an excess of diazomethane solution in ether. The reagent and solvent were removed by flushing with nitrogen and the residue was transferred into a micro test tube. Trimethylsilylimidazole (30 μl) was added and the sealed vessel was warmed at *ca.* 50 °C for 1 min. The mixture was diluted with hexane (*ca.* 100 μl) and washed with water. An aliquot was analyzed with a JEOL DX303 GC-MS (Fig. 1).

HPLC Analysis Incubations were terminated by addition of ethanol (2 ml) and 10% NaOH (1 ml). The mixture was acidified with 3 N HCl (2 ml), diluted with water (30 ml) and extracted with ethyl acetate

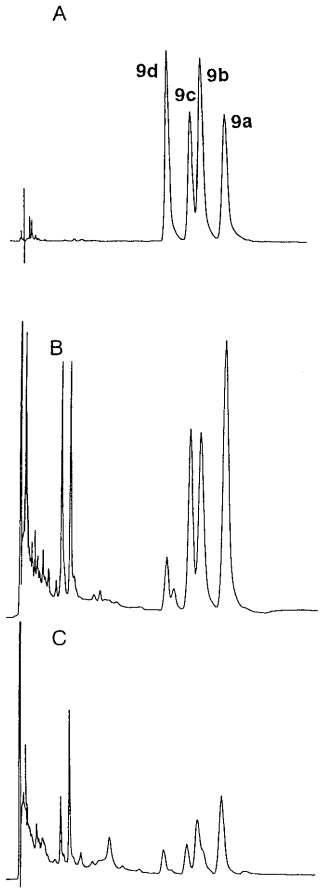

Fig. 5. HPLC Profiles of TeHCA (*p*-Bromophenacyl Ester) Produced from 24-Ene-THCA on Mitochondrial Incubation (in the Absence of NAD)

A: standard samples of **9a**, **9b**, **9c** and **9d**. B: incubation products from (24*E*)-24-ene-THCA. C: from (24*Z*)-24-ene-THCA.

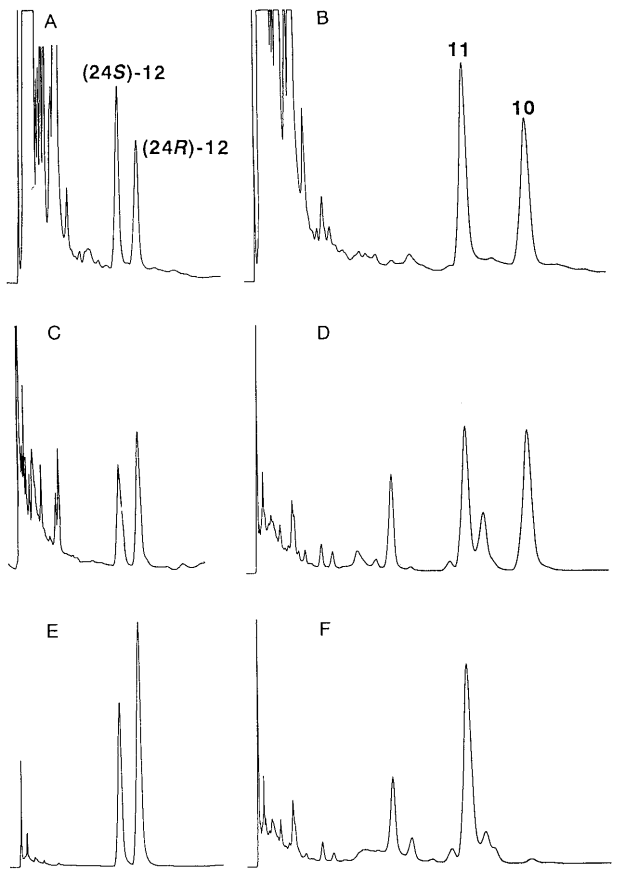

Fig. 6. HPLC Profiles of 27-Nor-TeHCA (*p*-Bromophenacyl Ester) (Left) and the Less Polar Fraction (Right) of the Mitochondrial Incubation (in the Absence of NAD) Products from 27-Nor Compounds

A: standard sample of **12** (stereochemical assignment and elution order were reported in refs. 8 and 17). B: standard samples of **10** and **11**. C and D: incubation products from 27-nor-THCA. E and F: from 27-nor-24-ene-THCA.

(30 ml × 4). The extract was shaken with brine, dried over Na_2SO_4 and concentrated. The residue was applied to a Sephadex LH-20 column (1.2 cm × 17 cm) and eluted with CH_2Cl_2–methanol (10 : 1, v/v). The initial fraction (20 ml) containing cholesterol was discarded. Subsequent fraction (40 ml) containing cholic acid, THCA, 24-ene-THCA and TeHCA were collected and concentrated. To the residue was added a mixture of *p*-bromophenacyl bromide (2 mg), acetonitrile (0.9 ml) and methanol (0.1 ml). Then *N*,*N*-diisopropylethylamine (10 μl) was added and the mixture was stirred at room temperature for 15 h. The mixture was diluted with ethyl acetate (*ca.* 10 ml), washed with water and concentrated. The residue was submitted to preparative HPLC using a Shim-pack CLC-SIL column (6.0 × 150 mm) with CH_2Cl_2–methanol (20 : 1, v/v). The fraction (containing THCA and 24-ene-THCA) moving faster than standard TeHCA and the fraction corresponding to TeHCA were each collected and concentrated. The resolved two fractions were separately analyzed by HPLC using a Shim-pack CLC-ODS column (6.0 × 150 mm) with methanol–water (4 : 1, v/v) at the flow rate of 2.2 ml/min, with a UV detector (254 nm).

Chemicals (24*E*)-24-Ene-THCA was obtained by the hydrolysis of ethyl 3α,7α,12α-triacetoxy-5β-cholest-24-en-26-oate.[13] (25*RS*)-THCA (**6**)[6] was prepared from the above ester in two steps (i, hydrogenation over 10% Pd/C, ii, hydrolysis with KOH–MeOH). TeHCA (**9**), (24*R*,25*R*)-, (24*R*,25*S*)-, (24*S*,25*R*)-, and (24*S*,25*S*)-TeHCAs were prepared as described previously.[14]

Synthesis Extractive work-up (solvent) refers to the following operations: the reaction was terminated by the addition of appropriate solvent and/or water, and the mixture was extracted with the given solvent, then the extract was washed with dilute HCl, saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to dryness. Melting points were determined on a Yazawa BY-1 hot-stage microscope and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GSX-500 spectrometer in CD_3OD or $CDCl_3$ solution unless otherwise stated, with tetramethylsilane as an internal reference. The ^{13}C chemical shifts measured in CD_3OD are expressed with reference to CD_3OD (δ 49.0). Preparative HPLC was performed with a Shimadzu LC-6A liquid chromatograph equipped with an SPD-6A UV detector [Shim-pack CLC ODS column (two 25 cm × 20 mm i.d. columns were joined), CH_3CN–H_2O (20 : 1) as a solvent, flow rate 6 ml/min, monitored at 254 nm].

(25*R*)- and (25-*S*)-THCAs (6a, 6b) A mixture of (25*RS*)-THCA[6] (**6**) (89 mg, 0.20 mmol) and *p*-bromophenacyl bromide (110 mg, 0.40 mmol) in acetonitrile–methanol (9 : 1, 10 ml) containing *N*,*N*-diisopropylethylamine (70 μl) was stirred for 48 h at room temperature. The solvent was removed under vacuum and the residue was chromatographed on a silica gel column with chloroform–methanol (10 : 1) to give the C-25 epimeric mixture of esters (**13**, **14**, 154 mg). These esters were separated by preparative HPLC[8] (acetonitrile–water (20 : 1) as an eluent, flow rate 6 ml/min) to give **14** (shorter retention time) (56 mg) and **13** (longer retention time) (36 mg). **13**: mp 160—162 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-H_3), 0.88 (3H, s, 19-H_3), 0.98 (3H, d, J = 6.8 Hz, 21-H_3), 1.23 (3H, d, J = 6.8 Hz, 27-H_3), 2.61 (1H, sextet, J = 6.8 Hz, 25-H), 3.43 (1H, m, 3β-H), 3.82 (1H, m, 7β-H), 3.98 (1H, m, 12β-H), 5.28 (2H, s, CH_2CO), 7.63 (2H, d, J = 8.8 Hz, ArH), 7.78 (2H, d, J = 8.8 Hz, ArH). **14**: mp 175—177 °C. The ^{1}H-NMR spectrum was essentially identical with that of **13**. The ^{13}C-NMR data for **13** and **14** are listed in Table I.

A solution of the ester **13** (55 mg, 0.085 mmol) in 5% aqueous KOH (4 ml) and methanol (8 ml) was stirred for 1 h at 0 °C, and for 12 h at room temperature. Acidification with 2 N HCl and extractive work-up (ethyl acetate) gave the residue, which was purified on a Sephadex LH-20 column with methanol to give the acid **6a** (36 mg, 94%). The other isomer **6b** (20.0 mg, 82%) was obtained in the same manner from the corresponding ester **14** (35 mg). **6a**: mp 187—189 °C (from ethyl acetate) (lit. 180—182 °C,[7] 183—184 °C[9]) ^{1}H-NMR (CD_3OD) δ: 0.71 (3H,

TABLE I. ^{13}C-NMR Chemical Shifts of Side-Chain Carbons (125 MHz, CD_3OD)

C	*p*-Bromophenacyl ester		Acid	
	13	**14**	**6a**	**6b**
20	37.11	37.07	37.07	37.06
21	18.09	18.07	18.04	18.01
22	35.43	35.43	35.44	35.50
23	24.80	24.83	24.90	24.95
24	37.01	37.01	37.00	37.02
25	40.57	40.57	40.74	40.78
26	17.55	17.75	17.55	17.75
27	177.86	177.75	180.90	180.87

s, 18-H_3), 0.92 (3H, s, 19-H_3), 1.00 (3H, d, J=6.3 Hz, 21-H_3), 1.13 (3H, d, J=6.8 Hz, 27-H_3), 2.39 (1H, sextet, J=6.8 Hz, 25-H), 3.37 (1H, m, 3β-H), 3.80 (1H, m, 7β-H), 3.96 (1H, m, 12β-H). **6b**: mp 204—206 °C (from ethyl acetate) (lit. 199—201 °C[6,9]). The ^{1}H-NMR spectrum of **6b** was essentially identical with that of **6a**. The ^{13}C-NMR data for **6a** and **6b** are listed in Table I.

(24*Z*)-24-Ene-THCA (8) Although Une *et al.*[13] claimed that (24*Z*)-24-ene-THCA (**8**) was obtained as a minor product in the Wittig reaction of 3α,7α,12α-triacetoxy-5β-cholestan-24-al (**15b**) with α-(ethoxycarbonylethylidene)triphenylphosphorane followed by saponification, only a negligible amount of the 24*Z* derivative (**16**) was formed by an analogous Wittig reaction of 3α,7α,12α-tritetrahydropyranyloxy-5β-cholestan-24-al (**15a**) in our hands. Therefore, the corresponding Horner–Emmons reaction[15] of **15a** was carried out. A solution of triethylphosphonopropionate (1.15 m, 5 mmol), 18-crown-6 (2.77 g, 10 mmol) in anhydrous tetrahydrofuran (THF) (12 ml) was cooled to −78 °C under nitrogen, and treated with 5 mmol of $KN(TMS)_2$ (0.5 M in toluene, Aldrich Company). Then a solution of the aldehyde (**15b**, 0.70 g, 1.04 mmol) in THF (3 ml) was added slowly through a syringe. The resulting mixture was stirred at −78 °C for 30 min and the temperature was allowed to rise to 20 °C. Extractive work-up (ether) gave a crude product, which was chromatographed on silica gel with hexane–ethyl acetate (10 : 1) to give the esters (**16a**, 0.76 g), ^{1}H-NMR δ: 6.76, 5.91 (*ca.* 1 : 1, 24-H of the *E*- and *Z*-isomer, respectively). A solution of the esters in CH_2Cl_2 (10 ml)–methanol (10 ml) was treated with concentrated HCl (0.1 ml) and the mixture was stirred for 1 h. Extractive work-up (CH_2Cl_2) gave the crude triol (0.37 g), a part (250 mg) of which was heated in a mixture of pyridine (3 ml), acetic anhydride (1.5 ml) and *N*,*N*-dimethyl-4-aminopyridine (20 mg) at 60 °C for 15 h. Extractive work-up (ethyl acetate) gave the crude triacetate (**16b**, 310 mg), which showed on HPLC [Shim-pack CLC-ODS (46 × 150 mm) with 95% aqueous CH_3CN (0.3 ml/min)] twin peaks (*ca.* 1 : 1) at the retention times of 13.2 and 14.4 min. A part (200 mg) of **16b** was chromatographed on silica gel with *n*-hexane–ethyl acetate (10 : 1). The faster-running 24*Z*-isomer (**17**, 50 mg) was obtained in more than 95% purity as tested by HPLC. A mixture of the (24*Z*)-ester (**17**, 21 mg), isopropanol (3.0 ml) and 4 N NaOH (1.0 ml) was heated at 80 °C for 15 h, and then acidified by addition of concentrated HCl. Extractive work-up (ethyl acetate) gave amorphous (24*Z*)-24-ene-THCA (**8**, 17 mg), ^{1}H-NMR (pyridine-d_5–$CDCl_3$) δ: 0.79 (3H, s, 18-H_3), 0.98 (3H, s, 19-H_3), 1.24 (3H, d, J=5.6 Hz, 21-H_3), 2.08 (3H, s, 26-H_3), 3.68 (1H, m, 3-H), 4.05 (1H, m, 7-H), 4.20 (1H, m, 12-H), 5.98 (1H, m, 24-H).

Ethyl 27-Nor-3α,7α,12α-triacetoxy-5β-cholest-24-en-26-oate (18) A mixture of 3α,7α,12α-triacetoxy-5β-cholan-24-al (**15b**)[13] (965 mg, 1.86 mmol) and ethoxycarbonylmethylenetriphenylphosphorane (1.30 g, 3.72 mmol) in toluene (15 ml) under nitrogen was heated for 1 h at 80 °C and then cooled to room temperature. Extractive work-up (ether) gave a crude product, which was chromatographed on a silica gel column with hexane–ethyl acetate (3 : 1) as an eluent to give the unsaturated ester (**18**) as a white solid (852 mg, 78%). mp 136—138 °C (from $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s, 18-H_3), 0.83 (3H, d, J=6.3 Hz, 21-H_3), 0.92 (3H, s, 19-H_3), 1.28 (3H, t, J=7.4 Hz, $OCH_2C\underline{H}_3$), 2.05, 2.09, 2.14 (3H each, s each, $OCOCH_3$ × 3), 4.18 (2H, q, J=7.4 Hz, $OC\underline{H}_2CH_3$), 4.58 (1H, m, 3β-H), 4.91 (1H, m, 7β-H), 5.10 (1H, m, 12β-H), 5.80 (1H, d, J=15.6 Hz, H-25), 6.94 (1H, dt, J=15.6, 6.8 Hz, H-24). *Anal.* Calcd for $C_{34}H_{52}O_8$: C, 69.36; H, 8.90. Found: C, 69.16; H, 9.20.

Ethyl 27-Nor-3α,7α,12α-triacetoxy-5β-cholestan-26-oate (19) A solution of **18** (406 mg, 0.69 mmol) in ethyl acetate (10 ml) was hydrogenated in the presence of 10% Pd/C (60 mg) under atmospheric pressure for 25 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated to give the saturated ester (**19**) as colorless needles (377 mg, 93%). mp 137—139 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-H_3), 0.79 (3H, d, J=6.3 Hz, 21-H_3), 0.92 (3H, s, 19-H_3), 1.25 (3H, t, J=7.4 Hz, $OCH_2C\underline{H}_3$), 2.05, 2.09, 2.13 (3H each, s each, $OCOCH_3$ × 3), 2.28 (2H, t, J=7.4 Hz, 25-H_2), 4.12 (2H, q, J=7.4 Hz, $OC\underline{H}_2CH_3$), 4.58 (1H, m, 3β-H), 4.91 (1H, m, 7β-H), 5.09 (1H, m, 12β-H). *Anal.* Calcd for $C_{34}H_{54}O_8$: C, 69.12; H. 9.21. Found: C, 69.04; H, 9.50.

27-Nor-3α,7α,12α-trihydroxy-5β-cholestan-26-oic Acid (10) A solution of the ester **19** (362 mg, 0.61 mmol) in 5% methanolic KOH (15 ml) and water (4 ml) was refluxed for 12 h. The mixture was cooled to room temperature and acidified with 2 N HCl. Extractive work-up (ethyl acetate, washed only with brine) gave a crude product, which was chromatographed on a silica gel column with chloroform–methanol (4 : 1) to give the acid (**10**) as a white solid (178 mg, 67%). mp 198—199 °C (from ethyl acetate) (lit.[16] 194—195 °C). ^{1}H-NMR (CD_3OD) δ: 0.71 (3H, s, 18-H_3), 0.91 (3H, s, 19-H_3), 1.00 (3H, d, J=6.3 Hz, 21-H_3), 2.27 (2H, t, J=7.3 Hz, 25-H_2), 3.38 (1H, m, 3β-H), 3.80 (1H, m, 7β-H), 3.96 (1H, m, 12β-H). ^{13}C-NMR (CD_3OD) δ: 13.03 (C-18), 18.03 (C-21), 23.19 (C-19), 24.23 (C-15), 26.66, 26.86 (C-23, 24), 27.81 (C-9), 28.82 (C-11), 29.55 (C-16), 31.12 (C-2), 35.39 (C-22), 35.83 (C-6), 35.88 (C-10), 36.49 (C-1), 36.79 (C-25), 37.07 (C-20), 40.40 (C-4), 40.98 (C-8), 42.90 (C-5), 43.13 (C-14), 47.41 (C-13), 48.25 (C-17), 69.05 (C-7), 72.84 (C-3), 74.04 (C-12), 178.42 (C-26).

(24*E*)-27-Nor-3α,7α12α-trihydroxy-5β-cholest-24-en-26-oic Acid (11) A solution of the ester **18** (245 mg, 0.41 mmol) in 1,2-dimethoxyethane (DME) (15 ml) and 2.0 M aqueous LiOH (13 ml) was heated at 110 °C for 14 h. The mixture was cooled to room temperature and acidified with 2 N HCl. Extractive work-up (ethyl acetate) gave a crude product, which was recrystallized from ethyl acetate to give the acid (**11**) as a white solid (80 mg, 45%). mp 201—203 °C. ^{1}H-NMR (CD_3OD) δ: 0.70 (3H, s, 18-H_3), 0.90 (3H, s, 19-H_3), 1.01 (3H, d, J=6.4 Hz, 21-H_3), 3.37 (1H, m, 3β-H), 3.79 (1H, m, 7β-H), 3.95 (1H, m, 12β-H), 5.77 (1H, d, J=15.6 Hz, H-25), 6.93 (1H, dt, J=15.6, 6.8 Hz, H-24). ^{13}C-NMR (CD_3OD) δ: 13.01 (C-18), 17.79 (C-21), 23.18 (C-19), 24.23 (C-15), 37.88 (C-9), 28.76 (C-11), 29.59 (C-16), 29.99 (C-23), 31.19 (C-2), 35.57 (C-22), 35.86 (C-6), 35.91 (C-10), 36.50 (C-1), 36.85 (C-20), 40.47 (C-4), 41.03 (C-8), 42.98 (C-5), 43.20 (C-14), 47.51 (C-14), 48.13 (C-17), 69.06 (C-7), 72.89 (C-3), 74.04 (C-12), 122.22 (C-25), 151.82 (C-24), 170.18 (C-26). *Anal.* Calcd for $C_{26}H_{42}O_5 \cdot \frac{1}{2}H_2O$: C, 70.39; H, 9.77. Found: C, 70.00; H, 9.66. Hydrolysis of **11** with KOH–MeOH was not satisfactory, affording the 24-methoxy compound [^{13}C-NMR (CD_3OD) δ: 18.1 (C-21), 31.5, 32.5 (C-22, C-23), 37.3 (C-20), 42.8 (C-25), 57.0 (OMe), 79.9 (C-24a), 80.2 (C-24b), 176.3 (C-26)] by the addition of methanol to the unsaturated acid (or ester) together with **11**.

(24*RS*)-27-Nor-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic Acid (12) The (24*RS*)-acid was prepared from 3α,7α,12α-tritetrahydropyranyloxy-5β-cholan-24-al (**15a**) in three steps according to the published method.[17] A mixture of the acid (153 mg, 0.34 mmol) and *p*-bromophenacyl bromide (191 mg, 0.69 mmol) in acetonitrile–methanol (9 : 1, 5 ml) containing *N*,*N*-diisopropylethylamine (120 μl) was stirred for 48 h at room temperature. The mixture was concentrated and the residue was chromatographed on a silica gel column with chloroform–methanol (10 : 1) to give the C-24 epimeric mixture of esters (128 mg). The esters were separable by HPLC (see Fig. 6) and it is reported that the (24*S*)-compound is more mobile than the (24*R*)-epimer.[8]

Acknowledgements We are grateful to Dr. T. Kurosawa, Higashi Nippon Gakuen University for his advice on preparing (24*Z*)-24-ene-THCA. Thanks are also due to Miss T. Utsunomiya, T. Hiraga, H. Ohkubo, E. Hasegawa, A. Kusaka and M. Nakajho for their contributions at the early stage of this work.

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