Synthesis and mutagenicity of a ring-A-aromatized bile acid, 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid

Tetsuto Namba, Takashi Hirota, and Shohei Hayakawa

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama, 700, Japan

Abstract It has been presumed that ring-A-aromatized bile acids are produced from biliary bile acids by intestinal flora and the acids thus formed participate in the large bowel carcinogenesis. One of these acids is probably 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid, judged from the literatures. Consequently, this acid was synthesized from previously prepared 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-ol. The phenolic ether was successively oxidized with pyridinium chlorochromate and wet silver oxide to give 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oic acid in high yield, which, after successive treatments with methanol containing a catalytic amount of *p*-toluenesulfonic acid, a combination of aluminum chloride and ethanethiol, and alkali, gave the desired compound in satisfactory yield. The compound was not mutagenic in *Salmonella* tester strains TA 98 and TA 100, but it increased the mutagenicity of 2-aminoanthracene when both were applied to plates together. When compared with cholic, deoxycholic, and lithocholic acids, the investigated compound exhibited about two to threefold increase of mutagenicity in the latter assay.—**Namba, T., T. Hirota, and S. Hayakawa.** Synthesis and mutagenicity of a ring-A-aromatized bile acid, 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid. *J. Lipid Res.* 1988. **29:** 809–814.

Supplementary key words bile acid • colon cancer • *O*-demethylation • Ames test • comutagenicity • 2-aminoanthracene

Goddard and Hill (1) and Drasar and Hill (2) have found ring-A-aromatization of 3-oxo-4-cholen-24-oic acid (1, 2), 4-androstene-3,17-dione (3), and cholesterol (4) by human gut bacteria. On the basis of these results, Hill (5) has proposed an interesting hypothesis that saturated bile acids such as cholic acid may be transformed by intestinal microflora into the corresponding unsaturated various compounds bearing aromatic rings, which may play a role in the carcinogenesis of large bowel. That there is some relationship between bile acids and colon cancer has been supported by both epidemiological and experimental studies indicating that bile acids play an important role in etiology of colon cancer (6, 7). It has also been considered that bile acids act as promotors rather than direct carcinogens (8–11). In connection with the correlation between bile acids and colon carcinogenesis, the potent mutagenic *N*-nitroso derivatives of glycine and taurine conjugates of bile acids have been synthesized (12). Gupta et al. (13) and Gunatilaka, Hirai, and Kingston (14) have reported the occurrence of a strong mutagenic agent, fecapentaene, in human feces, although the latter is not a bile acid analog. In spite of the recent development of instrumental techniques in bile acid analysis (15, 16), the occurrence of ring-A-aromatized bile acid derivatives in any biological material has not been reported. Our continued interest in defining the intermediates in the microbial transformation of bile acids has prompted us to investigate the microbially ring-A-aromatized bile acid analogs that have been reported (1–5).

In this report we describe the synthesis and the mutagenicity of a new ring-A-aromatized bile acid, 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (compound IVb), which corresponds to one of the hypothetical intermediates in the microbial metabolism of bile acids by Hill (5).

RESULTS AND DISCUSSION

Synthesis of 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (compound IVb)

A synthetic route to this compound is shown in **Fig. 1.** The anisole-alcohol (Ia) was prepared as described previously (17). It should be noted that, in the preparation of this compound, the dimethoxylated ether (Ib) with mp 65–66°C is also formed as a by-product. The alcohol (Ia) was oxidized with PCC (18) to the corresponding aldehyde (II), which was further oxidized by silver oxide suspended in aqueous KOH to give the corresponding carboxylic acid

Abbreviations: CA, cholic acid; DC, deoxycholic acid; LC, lithocholic acid; PCC, pyridinium chlorochromate; 2-AA, 2-aminoanthracene; DMSO, dimethyl sulfoxide; IR, infrared; UV, ultraviolet; PMR, proton nuclear magnetic resonance; MS, mass spectrometry; TLC, thin-layer chromatography.

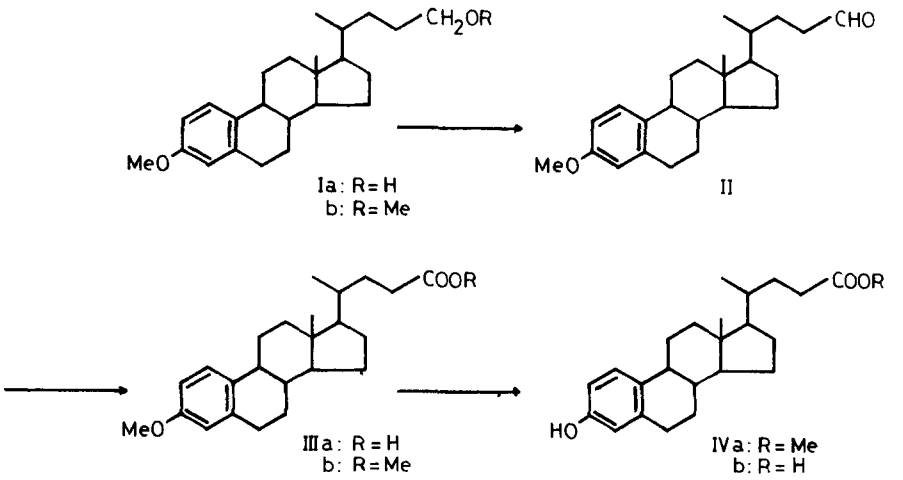

Fig. 1. Synthesis of 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid. Ia, 3-Methoxy-19-nor-1,3,5(10)-cholatrien-24-ol; Ib, 3,24-dimethoxy-19-norchola-1,3,5(10)-triene; II, 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-al; IIIa, 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oic acid; IIIb, methyl 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oate; IVa, methyl 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oate; IVb, 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid.

(IIIa) in satisfactory yield. The acid (IIIa) was converted into the corresponding methyl ester (IIIb) according to the method of Dayal et al. (19). Much work on the ether bond cleavage has recently appeared (20). We used compound Ib as a model substrate in preliminary investigations of mild methods for *O*-demethylation: *1)* a combination of aluminum chloride and ethanethiol (21); *2)* iodotrimethylsilane (22, 23); and *3)* a combination of boron trifluoride etherate and acetic anhydride (24). We chose the first method as the selective phenolic *O*-demethylation of compound IIIb. This method gave methyl 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oate (IVa) in good yield without the cleavage of an ester bond. Alkaline hydrolysis of the ester (IVa) afforded the desired ring-A-aromatized bile acid (IVb). The overall yield of this compound from compound Ia was approximately 40%. From the above chemical investigations there was no doubt as to the structure of compound IVb.

The MS data of all the newly synthesized compounds are given in **Fig. 2** and **Table 1.** The fragments were assigned according to results reported in the literature (25, 26). Sih, Wang, and Tai (25) have reported the synthesis and the MS data of methyl 3-hydroxy-19-nor-1,3,5(10)-pregnatriene-20-carboxylate, the parent acid of which corresponds to the 23,24-dinor analog of our compound IVb. The spectrum of compound IVa closely resembled that of the ester (25) in fragmentation patterns. The fragmentation patterns of these compounds were quite similar to each other.

Mutagenicity of compound IVb

Table 2 and **Table 3** indicate that, in the presence or absence of S9-mix, compound IVb revealed no mutagenicity for both strains TA 98 and TA 100. In a relatively high concentration, this compound seems to be toxic against a strain TA 100 in the presence of S9-mix, judged

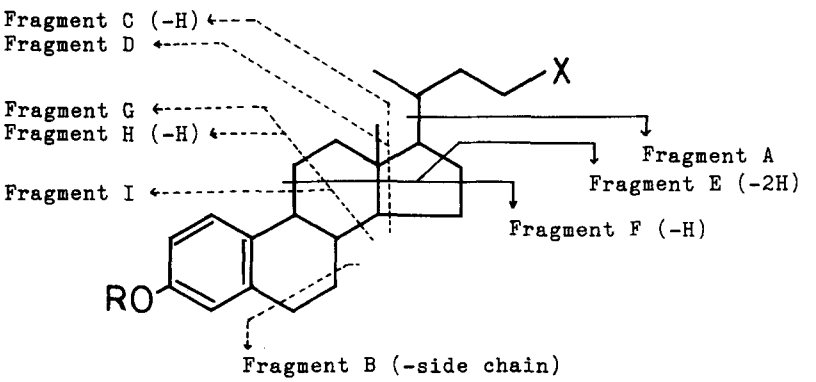

Fig. 2. Mass spectral fragmentation pattern of compounds II, IIIa, IIIb, IVa, and IVb; R = H or Me; X = CHO, COOH, or COOMe.

TABLE 1. Mass spectral data of compounds II-IV

Fragment Ion M^+	m/z	II 354	IIIa 370	IIIb 384	m/z	IVa 370	IVb 356
A	269	2	2	6	255	4	2
B	242	10	9	18	228	12	15
C	227	35	26	37	213	52	55
D	213	5	3	5	199	5	6
E	199	15	11	11	185	6	7
F	186	13	12	11	172	9	12
G	174	26	25	25	160	30	38
H	173	21	22	19	159	19	12
I	160	15	15	13	146	10	13

Relative intensities of molecular ion peaks (M^+) in all the compounds are 100%. See the fragmentation pattern indicated in Fig. 2 for the structure of fragment ion A-I.

from the lack of a lawn around the revertant colonies. As shown in Table 2, common bile acids such as CA, DC, and LC, like compound IVb, exhibited no mutagenic activity for a strain TA 98. The present results agree well with those of other studies (27, 28) that bile acids are not mutagenic in the Ames test.

Comutagenicity of compound IVb

As shown in **Fig. 3,** the strong comutagenicity of compound IVb toward 2-aminoanthracene (2-AA) was demonstrated, and compared with that of CA, DC, and LC. Silverman and Andrews (27) have reported that LC, glycine- and taurine-conjugated lithocholic acid, and 3-oxo-5β-cholan-24-oic acid act as comutagenic agents in the Ames test using 2-AA and *Salmonella typhimurium* TA 1538 as an indicator strain. Their results agree well with those of the present study, except for DC. The disagreement in the comutagenicity of DC is probably ascribed to a difference in the evaluation methods of this activity, since they used the original Ames test (29), while we adopted a modified test (30–32). There are several studies dealing with the comutagenicity of bile acids in the Ames test with the use of the following compounds: benzo[a]pyrene (33); 9-aminophenanthrene, 2-acetylaminoanthracene, and *N,N*-dimethyl-*p*-phenylenediamine (34); 1,2-dimethylhydrazine (35, 36); 2-aminofluorene, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (37). The comutagenicity test of compound IVb toward these compounds will be the subject of future studies.

EXPERIMENTAL

General

All melting points were determined on a Yanagimoto micro-melting point apparatus, and are uncorrected.

Optical rotations were measured in chloroform solution on a JASCO DIP-4 digital polarimeter at room temperature.

Infrared (IR) spectra were obtained with a JASCO IRA-102 spectrometer as KBr discs and the frequencies were expressed in cm^{-1}.

Ultraviolet (UV) spectra were recorded in ethanol solution on a Hitachi ESP-2 spectrophotometer. The results are expressed as λ max in nanometer (nm) with molar absorptivity (ϵ) in parentheses.

Proton nuclear magnetic resonance (PMR) spectra were measured with a Hitachi R-22 FTS instrument (90 MHz) in deuteriochloroform. The chemical shifts (δ) in ppm were measured relative to the tetramethylsilane internal standard.

Mass spectra were obtained by using a direct inlet probe with a Shimadzu LKB-9000 instrument at 70 eV.

Thin-layer chromatography (TLC) was performed on glass plates coated with silica gel (Wako gel B5-FM, 0.25 mm thickness; Wako Pure Industries, Co., Osaka) using solvent systems such as benzene–ethyl acetate 4:1, cyclohexane–ethyl acetate–acetic acid 16:8:1, isooctane–ethyl acetate–acetic acid 10:10:2, and benzene–dioxane–acetic acid 70:20:2. Spots were visualized by a PAN-UV lamp (continuous wave length from 250 nm to 400 nm; Tokyo Kogaku, Co., Tokyo) and spraying with 50% sulfuric acid, followed by heating at 150–160°C. Column chromatography was carried out on silica gel (Wako gel C-200; Wako Pure Industries, Co., Osaka) unless otherwise stated.

The phrase, "the usual workup," described in the Experimental section refers to extraction with ethyl acetate, washing with water to neutrality, drying over Na_2SO_4, and solvent evaporated in vacuo.

TABLE 2. Mutagenicity[a] of bile acids and compound IVb in the Ames test

Compound	Amount of Bile Acid per Plate	His$^+$ Revertants per Plate[b] – S9-mix	+ S9-mix
	μg		
None[c]		17	25
LC	50	26	27
LC	100	21	32
LC	200	24	28
DC	50	25	41
DC	100	21	42
DC	200	23	58
CA	50	38	39
CA	100	24	24
CA	200	22	36
IVb	25	27	57
IVb	50	25	50
IVb	100	27	46
IVb	200	25	41
IVb	300	15[d]	30

[a] A strain TA 98 was used.
[b] Average of two independent experiments with two plates.
[c] Solvent control (DMSO, 100 μl).
[d] Probably due to the killing of organisms.

TABLE 3. Mutagenicity[a] of compound IVb in the Ames test

Compound	Amount of Bile Acid per Plate	His⁺ Revertants per Plate[b]	
		– S9-mix	+ S9-mix
	μg		
None[c]		148	117
IVb	25	173	134
IVb	50	164	124
IVb	100	109	90[d]
IVb	200	106	104[d]
IVb	300	107[d]	100[d]

[a] A strain TA 100 was used.
[b] Average of two independent experiments with two plates.
[c] Solvent control (DMSO, 100 μl).
[d] Probably due to the killing of organisms.

Materials

3-Methoxy-19-nor-1,3,5(10)-cholatrien-24-ol (Ia) was synthesized as described by Hayakawa, Kanematsu, and Fujiwara (17). Bile acids (CA, DC, and LC) employed for mutagenicity tests chromatographed as a single spot by TLC using the solvent systems described above. All standard chemical reagents for synthesis were used without purification. All solvents were distilled before use. Other chemical reagents for a mutagenicity test were analytical grade.

Mutagenicity of bile acids and compound IVb

Salmonella typhimurium TA 98 and TA 100 for the Ames test (29) and the S9 fraction prepared from the liver of rats treated with polychlorinated biphenyls were kindly supplied from Prof. H. Hayatsu of our Faculty. The mutagenic activity of bile acids and compound IVb was examined by the preincubation method (30) reported by Yahagi et al. (31) who modified the original Ames test (29). The mixture containing bacteria, a test compound, and S9-mix (or only sodium phosphate buffer, pH 7.4) was incubated at 37°C for 20 min. The S9-mix (500 μl per plate) contained S9 fraction (50 μl), 0.25 M sodium phosphate buffer (200 μl), 0.075 M KCl (225 μl), 0.16 M $MgCl_2$ (25 μl), NADPH (2.0 μmol), NADH (2.0 μmol), glucose 6-phosphate (2.5 μmol), and ATP (2.5 μmol). The background lawn was routinely checked by microscopic examination to confirm that the test compound had no lethal effect. As positive controls, 2-nitrofluorene was used in the case of absence of S9-mix, and 2-aminoanthracene (2-AA) was used in the case of presence of S9-mix.

Comutagenicity of bile acids and compound IVb toward 2-AA

Comutagenic effects of bile acids and compound IVb on the mutagenicity of 2-AA were assayed with a method similar to the mutagenicity test described above. According to the method of Negishi and Hayatsu (32), a mixture containing bacteria, S9-mix, each test compound, and a solution of 2-AA (0.5 μg per plate) in DMSO was preincubated at 37°C for 20 min. The total amount of DMSO used as solvent for both a test compound and 2-AA was always 100 μl per plate.

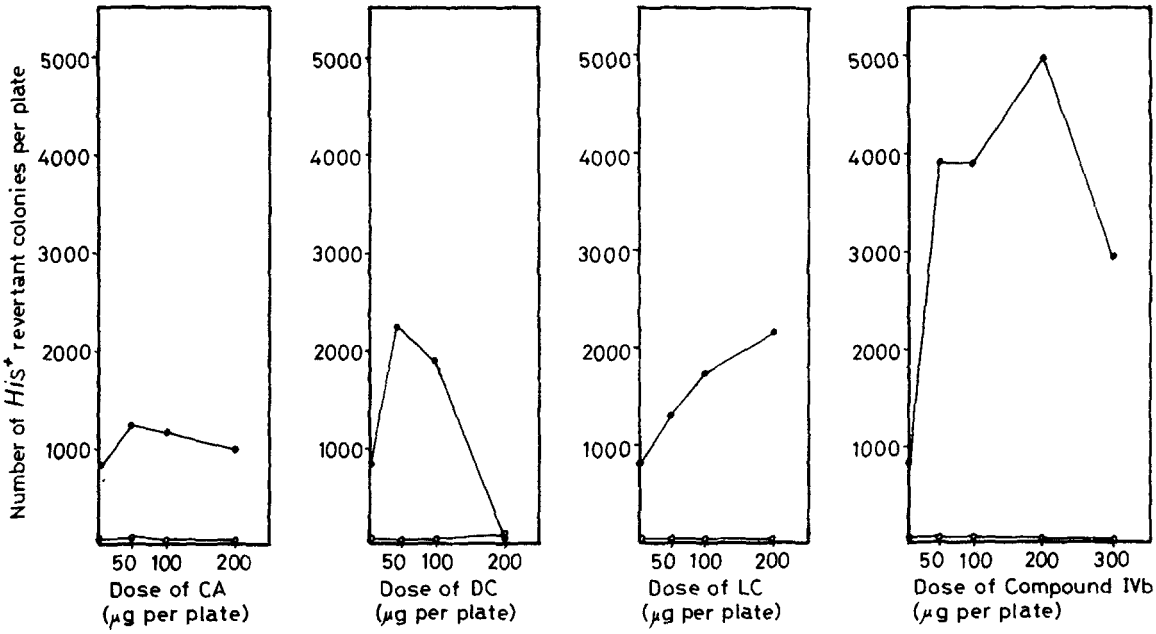

Fig. 3. Comutagenic effect of bile acids and compound IVb on the mutagenicity of 2-aminoanthracene (2-AA) with S9-mix in the Ames test (*S. typhimurium* TA 98); (●—●) with 2-AA (5 μg per plate); (○—○) without 2-AA. The number of revertant colonies observed for 2-AA in the absence of test compound was 838.

Synthesis of 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (compound IVb)

3-Methoxy-19-nor-1,3,5(10)-cholatrien-24-al (II). A solution of the anisole (Ia; 1.45 g, 4.07 mmol) in dry dichloromethane (15 ml) was added in one portion to pyridinium chlorochromate (PCC; 2.47 g, 11.5 mmol) suspended in dry dichloromethane (12 ml) under ice-cooling and with stirring. After 2.5 hr at room temperature, dry diethyl ether (50 ml) was added and the supernatant liquid was decanted from the black gum. The insoluble residue was washed with dry diethyl ether. The combined organic solution was passed through a short pad of Florisil and the solvent was evaporated to yield a crystalline residue, which crystallized from acetone to give 1.25 g (86%) of 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-al (II) as colorless prisms, mp 113.5–114°C. $[\alpha]_D$ = +159.7° (c = 1.02). UV: 278 (3660), 286 (3270). IR: 1715, 1616. PMR: 0.70 (3H, s, 18-Me), 0.97 (3H, d, J =6 Hz, 21-Me), 3.77 (3H, s, 3-OMe), 6.63 (1H, br s, 4-H), 6.68 (1H, br d, J = 10 Hz, 2-H), 7.20 (1H, br d, J = 10 Hz, 1-H), 9.79 (1H, t, J = 3 Hz, 24-CHO). Anal. calc. for $C_{24}H_{34}O_2$ (354.51): C, 81.31; H, 9.67. Found: C, 81.36; H, 9.83.

3-Methoxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (IIIa). A solution of the aldehyde (II; 1.00 g, 2.82 mmol) in a mixture of dioxane (20 ml) and ethanol (20 ml) was added to a suspension of wet silver oxide, which was freshly prepared from a solution of silver nitrate (1.04 g, 6.12 mmol) in water (4 ml) and 1 N KOH (13 ml), under ice-cooling and with stirring. After stirring for 4 hr at room temperature, the mixture was passed through a pad of Celite. Additional boiling water (200 ml) was passed through the pad and the combined effluents were evaporated in vacuo to approximately one-half of the initial volume. After extraction with diethyl ether to remove neutral materials, the aqueous layer was acidified with 1 N HCl. The usual workup gave a crystalline residue, which crystallized from acetone to give 0.8 g (94%) of 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (IIIa) as colorless prisms, mp 178.5–180.5°C. $[\alpha]_D$ = +180.0° (c = 1.00). IR: 3450 (br), 1717, 1641, 1615. UV: 278 (2790), 286 (2410). PMR: 0.70 (3H, s, 18-Me), 0.96 (3H, d, J = 5.5 Hz, 21-Me), 3.77 (3H, s, OMe), 6.65 (1H, br s, 4-H), 6.69 (1H, br d, J = 9.5 Hz, 2-H), 7.20 (1H, br d, J = 9.5 Hz, 1-H). Anal. calc. for $C_{24}H_{34}O_3$ (370.51): C, 77.80; H, 9.25. Found: C, 77.75; H, 9.35.

Methyl 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oate (IIIb). A solution of the anisole-carboxylic acid (IIIa; 0.98 g, 2.65 mmol) in methanol (10 ml) containing *p*-toluenesulfonic acid (100 mg, 0.53 mmol) was allowed to stand overnight at room temperature. The mixture was concentrated to a quarter of its original volume. After dilution with water, the usual workup gave a crystalline residue, which crystallized from acetone to give 0.92 g (90%) of methyl 3-methoxy-19-nor-1,3,5(10)-cholatrien-24-oate (IIIb) as colorless plates, mp 108.5–111°C. $[\alpha]_D$ = +161.7° (c = 1.06). IR: 1737, 1610. UV: 278 (2520), 287 (2240). PMR: 0.70 (3H, s, 18-Me), 0.94 (3H, d, J = 6 Hz, 21-Me), 3.66 (3H, s, COOMe), 3.77 (3H, s, 3-OMe), 6.66 (1H, br s, 4-H), 6.70 (1H, br d, J = 10 Hz, 2-H), 7.21 (1H, br d, J = 10 Hz, 1-H). Anal. calc. for $C_{25}H_{36}O_3$ (384.54): C, 78.08; H, 9.44. Found: C, 77.83; H, 9.55.

Methyl 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oate (IVa). To a stirred solution of the anisole-ester (IIIb; 152 mg, 0.39 mmol) in ethanethiol (11 ml) was added anhydrous aluminum chloride (370 mg, 2.8 mmol) under ice-cooling and the reaction process was monitored by TLC. After stirring for 90 min at room temperature, the mixture was poured into ice-water and treated by the usual workup to give a crude residue, which was chromatographed on a silica gel. Elution with benzene–ethyl acetate 19:1 afforded 130 mg (91%) of a crude product, which crystallized from ethyl acetate –hexane to give methyl 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oate (IVa) as colorless prisms, mp 141.5–143.5°C. $[\alpha]_D$ = +186.0° (c = 1.06). IR: 3360, 1700, 1608. UV: 282 (2930), 287 (sh). PMR: 0.69 (3H, s, 18-Me), 0.95 (3H, d, J = 5.5 Hz, 21-Me), 3.67 (3H, s, COOMe), 4.89 (1H, br s, 3-OH, disappeared with D_2O), 6.55 (1H, br s, 4-H), 6.61 (1H, br d, J = 8 Hz, 2-H), 7.14 (1H, br d, J = 8 Hz, 1-H). Anal. calc. for $C_{24}H_{34}O_3$ (370.51): C, 77.80; H, 9.25. Found: C, 77.60; H, 9.35.

3-Hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (IVb). The ester (IVa; 224 mg, 0.6 mmol) was hydrolyzed with 5% (w/v) methanolic KOH (20 ml) for 3 hr under reflux. After the mixture was concentrated in vacuo, diluted with water, and acidified with 1 N HCl, the usual workup gave a crystalline residue. Repeated crystallization from acetone–hexane afforded 175 mg (82%) of 3-hydroxy-19-nor-1,3,5(10)-cholatrien-24-oic acid (IVb) as colorless plates, mp 208.5 –210°C. $[\alpha]_D$ = +214.3° (c = 0.88). IR: 3250, 1698. UV: 282 (2640), 287 (sh). PMR: 0.70 (3H, s, 18-Me), 0.97 (3H, d, J = 6 Hz, 21-Me), 6.56 (1H, br s, 4-H), 6.61 (1H, br d, J = 8.5 Hz, 2-H), 7.15 (1H, br d, J = 8.5 Hz, 1-H). Anal. calc. for $C_{23}H_{32}O_3$ (356.49): C, 77.49; H, 9.05. Found: C, 77.23; H, 9.13.

The treatment of this acid with diazomethane in diethyl ether under ice-cooling gave the parent methyl ester (IVa) without methoxylation at the C-3 position.

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