Note

Chemical Synthesis of Uncommon Natural Bile Acids: The 9*α*-Hydroxy Derivatives of Chenodeoxycholic and Lithocholic Acids

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The chemical synthesis of the 9*α*-hydroxy derivatives of chenodeoxycholic and lithocholic acids is reported. For initiating the synthesis of the 9*α*-hydroxy derivative of chenodeoxycholic acid, cholic acid was used; for the synthesis of the 9*α*-hydroxy derivative of lithocholic acid, deoxycholic acid was used. The principal reactions involved were (1) decarbonylation of conjugated 12-oxo-$\Delta^{9(11)}$-derivatives using *in situ* generated monochloroalane (AlH_2Cl) prepared from $LiAlH_4$ and $AlCl_3$, (2) epoxidation of the deoxygenated $\Delta^{9(11)}$-enes using *m*-chloroperbenzoic acid catalyzed by 4,4′-thiobis-(6-*tert*-butyl-3-methylphenol), (3) subsequent Markovnikov 9*α*-hydroxylation of the $\Delta^{9(11)}$-enes with AlH_2Cl, and (4) selective oxidation of the primary hydroxyl group at C-24 in the resulting 3*α*,9*α*,24-triol and 3*α*,7*α*,9*α*,24-tetrol to the corresponding C-24 carboxylic acids using sodium chlorite ($NaClO_2$) in the presence of a catalytic amount of 2,2,6,6-tetramethylpiperidine 1-oxyl free radical (TEMPO) and sodium hypochlorite (NaOCl). The ^{1}H- and ^{13}C-NMR spectra are reported. The 3*α*,7*α*,9*α*-trihydroxy-5*β*-cholan-24-oic acid has been reported to be present in the bile of the Asian bear, and its 7-deoxy derivative is likely to be a bacterial metabolite. These bile acids are now available as authentic reference standards, permitting their identification in vertebrate bile acids.

Key words 9*α*-hydroxy-bile acid; C-9 5*β*-steroid hydroxylation; decarbonylation; monochloroalane; Markovnikov-hydroxylation; 2,2,6,6-tetramethylpiperidine 1-oxyl free radical

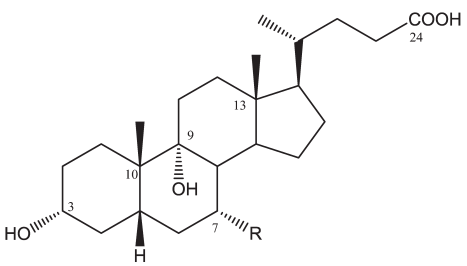
The great variety of natural C_{24} and C_{27} bile acids, as well as C_{27} bile alcohols, (together called bile salts) occurring in vertebrates can be explained by the evolution of differing biochemical pathways that serve to convert cholesterol into these multifunctional amphipathic compounds. Bile salt composition shows significant variation between orders but not between families, genera, or species, suggesting a biochemical trait providing clues to evolutionary relationships that complement anatomical and genetic analyses.[1–3] Naturally occurring bile salts differ markedly in their chemical structures, particularly the number, position and stereochemistry of hydroxyl groups on the steroid nucleus and on the branched C_5 or C_8 side-chain. Since some of the uncommon bile acids are present in the biliary and/or urinary bile salts of specific vertebrates or patients with hepatobiliary diseases, the bile acid profile can be used as a biomarker for clinical purposes, as well as for suggesting phylogenetic relationships.[4]

Nuclear hydroxylation of bile acids in the liver of vertebrates is the result of CYP-mediated hydroxylation. If one accepts the concept of a default steroid nucleus with hydroxyl groups at C-3 and C-7, then further modifications can be considered as additions to the default structure. The dominant sites of additional hydroxylation are at C-6 (*α* or *β*), C-12 (*α*), or C-16 (*α*). Other sites of additional nuclear hydroxylation in bile acids are at C-1 (1*α*-/1*β*-), C-2 (2*β*-), C-4 (4*β*-), C-5 (5*β*-), and C-15 (15*α*-).[1–3]

Recently, a new natural bile acid having a hydroxylation site at C-9 (9*α*-) in the 5*β*-steroid nucleus (*cis* A/B-ring juncture) was reported by Bi *et al.*,[5] to be present in the bile of the Asian black bear (*Ursus thibetanus*). The semitrivial name, selocholic acid, was proposed for this novel bile acid. Its structure was considered to be 3*α*,7*α*,9*α*-trihydroxy-5*β*-cholan-24-oyl taurine by MS and one and two dimensional (1D)- and (2D)-NMR spectroscopy. Nonetheless, validation of the proposed structure awaits the synthesis of an authentic reference standard.

Microbial or enzymatic biotransformation are important tools for structural modification of organic compounds, especially natural products with complex structures like steroids.[6–8] This synthetic method can be used to prepare chemical structures that are difficult to obtain by ordinary chemical methods. The observed biotransformations may suggest metabolic pathways in mammals, due to similarity between mammalian and microbial systems. However, the procedure is of limited use, because of the substrate specificity.

As part of our ongoing program of chemical synthesis of novel, uncommon natural or potentially natural bile acid metabolites for use as authentic reference standards, we now describe the chemical synthesis of 9*α*-hydroxy-chenodeoxycholic



	R
1a	H
1b	OH

Fig. 1. Chemical Structures of Synthetic 9*α*-Hydroxylated Bile Acids (**1a**, **b**)

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acid [9α-OH-CDCA; 3α,7α,11α-trihydroxy-5β-cholan-24-oic acid (**1b**)] starting from cholic acid (**2b**; CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), and 9α-hydroxy-lithocholic acid [9α-OH-LCA; 3α,9α-dihydroxy-5β-cholan-24-oic acid (**1a**)] starting from deoxycholic acid (**2a**; DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid) (Fig. 1).

Results and Discussion

Figure 2 shows the synthetic route of 9α-OH-CDCA (**1b**) from CA (**2b**) and 9α-OH-LCA (**1a**) from DCA (**2a**), respectively. A one step procedure for the introduction of an α-hydroxyl group at C-9 on the 5β-steroid nucleus is known.[9,10] Thus, the most feasible approach for preparing **1a** and **b** on a substantial scale appeared to be the 9α-hydroxylation of 9α,11α-epoxy intermediates (**8a**, **b**) derived from $\Delta^{9(11)}$-enes (**7a**, **b**), obtainable from **2a** and **b** in several steps.

Regioselective acetylation of methyl deoxycholate (**3a**) and methyl cholate (**3b**) under mild conditions gave the corresponding 3α-acetate (**4a**) and 3α,7α-diacetate (**4b**), respectively, which in turn were oxidized with pyridinium chlorochromate (PCC) to yield the 3α-acetoxy-12-oxo and 3α,7α-diacetoxy-12-oxo esters (**5a**, **b**) nearly quantitatively.[11] When **5a** and **b** were subjected to the selective dehydration with SeO_2 in acetic acid,[12–14] the corresponding conjugated enones, $\Delta^{9(11)}$-3α-acetoxy-12-oxo and $\Delta^{9(11)}$-3α,7α-diacetoxy-12-oxo esters (**6a**, **b**), were obtained in high yields (78–80%). The formation of the conjugated 9(11)-ene-12-oxo structure was confirmed by the ^{1}H-NMR signals arising from the 19-H_3 at δ 1.19 in **6a** and at 1.20 in **6b** and by the ^{13}C-NMR signals from the C-11 (δ 123.8) and C-9 (δ 164.1) in **6a** and the C-11 (δ 125.1) and C-9 (δ 159.8) in **6b**.

Decarbonylation at C-12 of **6a** and **b** in tetrahydrofuran (THF) for 4 h at reflux temperature with monochloroalane (AlH_2Cl), generated *in situ* from $LiAlH_4$ and $AlCl_3$,[13,15–17] gave the $\Delta^{9(11)}$-3α,24-diol (**7a**) and $\Delta^{9(11)}$-3α,7α,24-triol (**7b**) in moderate isolated yields of 52–64%, respectively, accompanied by simultaneous hydrolysis of the methyl ester at C-24 and the acetoxy groups at C-3 and C-7. The 11-H olefinic ^{1}H signal occurring at δ 5.31 in **7a** and at 5.50 in **7b** and the ^{13}C signals appearing at δ 119.6 (C-11) and 140.1 (C-9) in **7a** and at δ 121.9 (C-11) and 137.1 (C-9) in **7b** suggest that the decarbonylation at C-12 occurred successfully.

Subsequent epoxidation of the $\Delta^{9(11)}$-3α,24-diol (**7a**) and $\Delta^{9(11)}$-3α,7α,24-triol (**7b**) with *m*-chloroperbenzoic acid (*m*-CPBA) in the presence of a catalytic amount of 4,4′-thiobis-(6-*tert*-butyl-3-methylphenol)[18] afforded the corresponding 9α,11α-epoxy derivatives (**8a**, **b**) stereoselectively in reasonable isolated yields (88 and 64%), presumably by the attack of *m*-CPBA on the less sterically hindered α-face of the substrates; the β-face is more sterically crowded by the axially-oriented 18- and 19-methyl groups. The relatively low-yield (64%) of **8b**, compared to that (88%) of **8a**, is probably ascribed to the steric hindrance of an axially-oriented 7α-hydroxyl group. The stereochemical configuration of the 9α,11α-epoxy ring in **8a** was determined by measuring the nuclear Overhauser enhanced differential spectroscopy (NOEDS), in which the irradiation of the 19-H_3 signal (δ 1.15) in **8a** resulted in the formation of the correlating peaks with the 18-H_3 (δ 0.68), 5β-H (δ 1.51), 12β-H (δ 1.68), 8β-H (δ 1.93) and 11β-H (δ 3.21), thus indicating the α-configuration. Similar correlations were also observed in the NOEDS of **8b**: the 19-H_3 (δ 1.18) were correlated with the 18-H_3 (δ 0.68), 5β-H (δ 1.56), 12β-H (δ 1.69), 8β-H (δ 1.99) and 11β-H (δ 3.08).

The most promising method for preparing 9α-hydroxylated bile acids is seemed to be the application of the so-called "reductive hydroxylation" of the 9α,11α-epoxides (**8a**, **b**) with lithium-ethylamine[9,10] or $LiAlH_4$[19–21] known to a favor α-hydroxylated product to obey the Marnovnikov rule. Attempted reductive cleavage of **8a** and **b** with lithium-ethylamine or with $LiAlH_4$ alone was unsuccessful; both the reactions did not proceed at all. However, when **8a** and **b** were subjected to AlH_2Cl, generated *in situ* from $LiAlH_4$ and $AlCl_3$,[22] to afford the expected 3α,9α,24-triol (**9a**) and 3α,7α,9α,24-tetrol (**9b**) with good selectivity in fairly low isolated yields of 18 and 36%, respectively, after preparative high-performance liquid chromatography with a refractive index detector (HPLC-RI) chromatographic purification of the reaction products; the remaining compound recovered was the unreacted one (69% for **8a** and 51% for **8b**). AlH_2Cl was therefore found to be effective for the regio- and stereoselec-

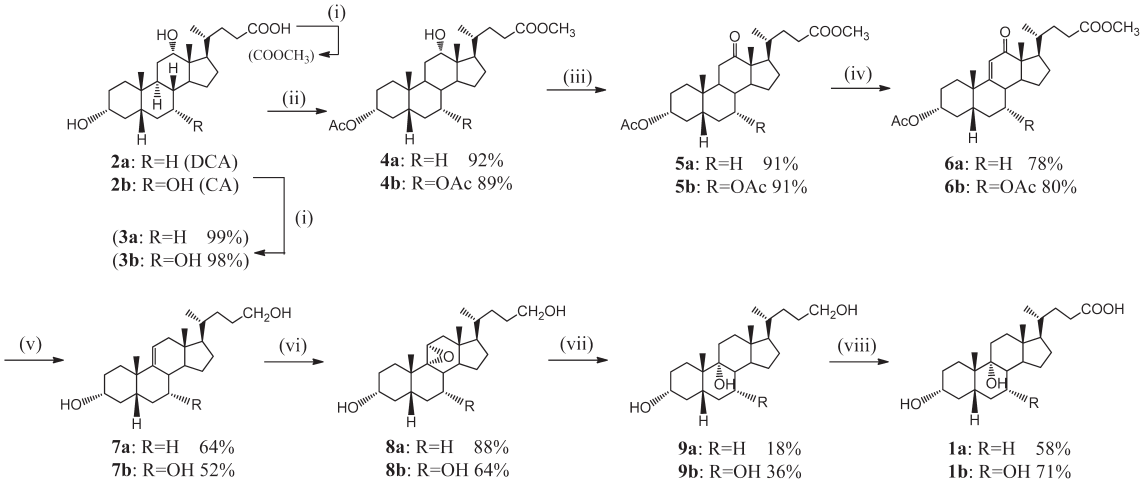

Fig. 2. Synthetic Route to 9α-Hydroxylated Bile Acids (**1a**, **b**) from DCA (**2a**) and CA (**2b**)

Reagents and conditions: (i) *p*-Toluenesulfonic acid/MeOH, at r.t. for 12 h. (ii) Ac_2O/pyridine/benzene, at r.t. for 12 h. (iii) PCC/CH_2Cl_2, at r.t. for 12 h. (iv) SeO_2/AcOH, at reflux for 18 h. (v) $AlCl_3$/$LiAlH_4$/THF, ar reflux for 4 h. (vi) *m*-Chloroperbenzoic acid/4,4′-thiobis(6-*tert*-butyl-3-methylphenol)/chloroform, at r.t. for 1 h (at reflux for 20 min). (vii) $AlCl_3$/$LiAlH_4$/THF, at reflux for 48 h (at reflux for 12 h). (viii) $NaClO_2$/TEMPO/NaClO/CH_3CN/THF, at 35°C for 3 h (at 35°C for overnight).

tive hydroxylation of the sterically crowded epoxides. An appreciable difference in the isolated yields of **9a** (18%) and **9b** (36%) suggests that the substrate **8b** is more unstable and reactive than **8a**.

The complete ^{1}H- and ^{13}C-NMR signal assignments for compounds **9a** and **b**, which were confirmed by comparison of bile acid analogs reported previously,[23–25] were compiled in Table 1. The ^{1}H-NMR spectra of the compounds exhibited the ^{1}H-signals arising from the 3β-H (br m) at δ 3.57 (**9a**) and 3.47 (**9b**) and the 24-H_2 (br m) at δ 3.50 (**9a**) and 3.49 (**9b**), both of which showed essentially identical ^{1}H signal patterns. The stereochemical configuration of the 9α-hydroxyl group in **9b** were determined by measuring the nuclear Overhauser effect spectroscopy (NOESY), in which the distinct correlation peaks were detected between the 19-H_3 (δ 0.96)/18-H_3 (δ 0.68), 19-H_3/5β-H (δ 1.58), 19-H_3/8β-H (δ 1.62), and 19-H_3/12β-H (δ 1.74). Essentially identical NOESY was also observed for **9a**: 19-H_3 (δ 0.96)/18-H_3 (δ 0.69), 19-H_3/5β-H (δ 1.42), 19-H_3/8β-H (δ 1.62) and 19-H_3/12β-H (δ 1.72). Meanwhile, the ^{13}C-NMR signals were appeared at δ 71.6 (**9a**) and 71.8 (**9b**) for the methine C-3, at δ 77.0 (**9a**) and 79.5 (**9b**) for the quaternary C-9, and at δ 62.2 (**9a**, **b**) for the methylene C-24 in the distortionless enhancement by polarization transfer (DEPT) spectra. These observations provide strong evidence for the presence of an axially-oriented 9α-hydroxyl group in the *cis* 5β-steroid nucleus (see below).

Recently successful use of sodium chlorite ($NaClO_2$) catalyzed by 2,2,6,6-tetramethylpiperidine 1-oxyl free radical (TEMPO) and sodium hypochlorite (NaClO; bleach) for oxidation of alcohols[26–30] suggested that a selective oxidation of a primary hydroxyl group to its carboxyl group without oxidizing a secondary hydroxyl group might be feasible. As expected, mild reaction of **9a** and **b** with TEMPO in the presence of the co-reagents resulted in successful selective oxidation of the primary 24-hydroxyl group to afford the desired 3α,9α-dihydroxy and 3α,7α,9α-trihydroxy acids (**1a**; 58%, **1b**; 71%), respectively. The presence of a hydroxyl group at the C-9 position and its stereochemical configuration as α in **1a** and **b** was determined by a combined use of several 1- and 2D-NMR techniques (Table 1). The ^{13}C signals appearing at δ 71.6, 77.0, and 176.9 in **1a** were tentatively assigned to the C-3, C-9, and C-24 bearing hydroxyl groups, respectively: δ 71.8, 69.7, 79.5, and 175.2 in **1b** were due to the C-3, C-7, C-9, and C-24, respectively. The presence of a tertiary hydroxyl group at C-9 was confirmed by the appearance of the ^{1}H/^{13}C correlated peak arising from the 19-H_3/C-9 in the ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) and DEPT spectra of **1a** and **b**. In addition, the correlation peaks observed for the 18-H_3/C-12 (δ 34.9 in **1a** and 35.0 in **1b**) in the HMBC, the C-12/12-H_2 [δ 1.56 (α) and 1.73 (β) in **1a** and δ 1.59 (α) and 1.77 (β) in **1b**] in the ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and then the 12-H_2/C-9 in the HMBC provided further confirmatory evidence for the presence of the 9-hydroxyl group. In the NOESY of **1a**, the distinct correlation peaks were observed between the 19-H_3 (δ 0.96)/18-H_3 (δ 0.70), 19-H_3/5β-H (δ 1.42), 19-H_3/8β-H (δ 1.63) and 19-H_3/12β-H (δ 1.73), strongly indicating that the configuration of the hydroxyl group at C-9 is α. As shown

Table 1. Complete ^{1}H- and ^{13}C-NMR Spectral Data for Synthetic 9α-Hydroxylated Compounds (**9a**, **b**, **1a**, **b**)[a]

No	9a				9b				1a				1b			
	Type	Carbon	Proton		Type	Carbon	Proton		Type	Carbon	Proton		Type	Carbon	Proton	
			α	β			α	β			α	β			α	β
1	CH_2	34.31	2.09	1.02	CH_2	35.07	2.03	1.09	CH_2	34.34	2.09	1.03	CH_2	35.04	2.05	1.09
2	CH_2	33.14	1.99	1.63	CH_2	32.92	2.01	1.58	CH_2	33.14	1.99	1.63	CH_2	32.92	2.02	1.62
3	CH	71.55	—	3.57	CH	71.75	—	3.47	CH	71.55	—	3.57	CH	71.76	—	3.48
4	CH_2	38.54	2.26	1.41	CH_2	41.85	2.23	1.77	CH_2	38.55	2.26	1.41	CH_2	41.88	2.23	1.72
5	CH	43.50	—	1.42	CH	42.05	—	1.58	CH	43.49	—	1.42	CH	42.05	—	1.58
6	CH_2	28.13[b]	1.37[c]	1.90[c]	CH_2	34.21	2.06	1.73	CH_2	28.16[b]	1.36[c]	1.89[c]	CH_2	34.20	2.06	1.73
7	CH_2	21.27	1.56	1.19	CH	69.73	—	3.91	CH_2	21.28	1.56	1.19	CH	69.72	—	3.91
8	CH	39.75	—	1.62	CH	40.73	—	1.62	CH	39.76	—	1.63	CH	40.70	—	1.64
9	C	77.03	—	—	C	79.49	—	—	C	77.00	—	—	C	79.47	—	—
10	C	38.01	—	—	C	38.56	—	—	C	38.01	—	—	C	38.54	—	—
11	CH_2	28.06[b]	1.56[c]	1.63[c]	CH_2	27.80[b]	1.60[c]	1.71[c]	CH_2	27.95[b]	1.56[c]	1.62[c]	CH_2	27.77[b]	1.60[c]	1.70[c]
12	CH_2	34.97	1.56	1.72	CH_2	35.07	1.59	1.74	CH_2	34.94	1.56	1.73	CH_2	35.04	1.59	1.77
13	C	42.41	—	—	C	42.39	—	—	C	42.46	—	—	C	42.42	—	—
14	CH	47.99	1.61	—	CH	44.72	2.00	—	CH	47.99	1.62	—	CH	44.72	2.00	—
15	CH_2	23.84	1.56	1.06	CH_2	23.09	1.68	1.10	CH_2	23.85	1.56	1.08	CH_2	23.03	1.69	1.09
16	CH_2	28.49[b]	1.94[c]	1.33[c]	CH_2	28.03[b]	1.91[c]	1.30[c]	CH_2	28.50[b]	1.93[c]	1.32[c]	CH_2	27.91[b]	1.95[c]	1.35[c]
17	CH	56.10	1.20	—	CH	56.10	1.23	—	CH	55.90	1.20	—	CH	55.95	1.23	—
18	CH_3	10.28	0.69		CH_3	10.20	0.68		CH_3	10.33	0.70		CH_3	10.18	0.70	
19	CH_3	27.15	0.96		CH_3	27.75	0.96		CH_3	27.16	0.96		CH_3	27.17	0.96	
20	CH	35.68	1.43		CH	35.74	1.43		CH	35.41	1.45		CH	35.53	1.43	
21	CH_3	17.81	0.95		CH_3	17.83	0.97		CH_3	17.43	0.95		CH_3	17.42	0.94	
22	CH_2	31.84	1.04/1.45		CH_2	31.85	1.07/1.48		CH_2	30.99	1.28/1.79		CH_2	31.24	1.35/1.78	
23	CH_2	28.92	1.33/1.38		CH_2	28.92	1.37/1.43		CH_2	30.69	2.16/2.33		CH_2	31.24	2.17/2.34	
24	CH_2	62.24	3.50		C	62.23	3.49		C	176.87	—		C	175.23	—	

a) Measured in CD_3OD at 500.2 MHz in ^{1}H-NMR and at 125.8 MHz in ^{13}C-NMR. Chemical shifts were expressed as δ ppm relative to TMS. *b*, *c*) Assignments along a vertical column bearing the same superscript may be interchanged.

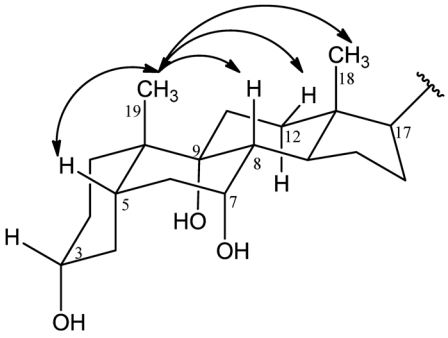

Fig. 3. NOESY Correlations Observed for 9α-OH-CDCA (**1b**)

in Fig. 3, essentially identical NOESY correlations were also observed for **1b**: 19-H_3 (δ 0.96)/18-H_3 (δ 0.70), 19-H_3/5β-H (δ 1.58), 19-H_3/8β-H (δ 1.64) and 19-H_3/12β-H (δ 1.77). To conclude, the 3α,9α,24-triol (**9a**) and 3α,7α,9α,24-tetrol (**9b**) were successfully converted to the desired 9α-OH-LCA (**1a**) and 9α-OH-CDCA (**1b**), respectively.

As note **1b** has been reported to be present in the biliary bile acids of the Asian black bear. **1a** has not been reported to occur naturally, but 7-dehydroxylation is a dominant bacterial biotransformation in the anaerobic colon,[31] leading to the prediction that **1a** should be present in the fecal bile acids of *Ursus thibetanus*. Hydroxylation of steroids at C-9 by microbial enzymes[32] or by simulated microsomal oxidation[33] has also been reported.

Thus, the availability of 9α-OH-LCA (**1a**) and 9α-OH-CDCA (**1b**) should facilitate the identification of these uncommon bile acids in body fluids of vertebrates and also highlights an unusual site of steroid hydroxylation.

Experimental

Materials DCA (**2a**) and CA (**2b**) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of analytical reagent grade and available from commercial sources. All compounds were dried by azeotropic distillation before use in reactions.

Instruments All melting points (mp) were determined on a micro hot stage apparatus and are uncorrected. Specific rotations were measured on a ATAGO AP-300 and ATAGO DP-63 auto-recording polarimeter. IR spectra, on a JASCO FT-IR-4100 spectrophotometer (samples were absorbed on a powdered KBr surface and measured with the diffusion reflection method).

^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL ECA 500 FT instrument operated at 500 and 125 MHz, respectively, with $CDCl_3$ or CD_3OD containing 0.1% Me_4Si as the solvent; chemical shifts were expressed in δ (ppm) relative to Me_4Si (0 ppm). The ^{13}C DEPT spectra were measured to determine the exact ^{13}C signal multiplicity and to differentiate among CH_3, CH_2, CH, and C based on their proton environments. In order to further confirm the ^{1}H and ^{13}C signal assignments for some of compounds, NOEDS, and 2D, ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{1}H NOESY, HMQC (^{1}H/^{13}C coupling) and HMBC (long-range ^{1}H–^{13}C coupling) spectra were also performed.

High-resolution liquid chromatography-mass spectrometry by electrospray ionization (HR-LC/ESI-MS) source was carried out using a JEOL AccuTOF JMS-T100LC liquid chromatography-mass spectrometer (JEOL, Tokyo, Japan) coupled to an Agilent 1200 series binary pump (Agilent Technologies Inc., Santa Clara, CA, U.S.A.) operated in the negative ion mode or positive ion mode.

The preparative HPLC-RI apparatus consisted of a Hitachi L-7100 pump (Tokyo, Japan), a Shodex RI-102 detector, and a Capcell Pak AQ RP-C_{18} column (250 mm×10 mm i.d.; particle size 5 μm; Shiseido).

Normal-phase TLC was performed on pre-coated Kieselgel 60F_{254} plates (E. Merck, Darmstad, Germany) using a mixture of EtOAc–hexane (8 : 2, v/v), EtOAc–hexane–acetic acid (9 : 1 : 0.1, v/v/v), EtOAc–methanol (95 : 5, v/v) or EtOAc–methanol–acetic acid (95 : 5 : 0.1, v/v/v) as the developing solvent.

Methyl 3α-Acetoxy-12-oxo-5β-chol-9(11)-en-24-oate (6a) A mixture of the 3α-acetoxy-12-oxo **5a** (1.0 g; 2.2 mmol) and selenium dioxide (SeO_2) (500 mg, 4.6 mmol) dissolved in acetic acid (40 mL) was refluxed for 18 h. After cooling at room temperature, the insoluble matter was filtered off, and the mother liquor was extracted with EtOAc. The combined extract was washed with 5% $NaHCO_3$ solution and water, dried with Drierite, and evaporated to dryness. Chromatography of the crude residue over a column of silica gel (50 g) eluting with EtOAc–hexane (3 : 7, v/v) resulted in a single component, which was identified as the conjugated $\Delta^{9(11)}$-12-ketone **6a** which was recrystallized from methanol as colorless needles: mp 149–152°C; yield, 835 mg (78%). $[\alpha]_D^{23}$+140.0 (*c* 0.10, MeOH). FT-IR 1740, 1726 (C=O), 1645 (C=C) cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.90 (s, 3H, 18-H_3), 1.00 (d, 3H, *J*=6.2 Hz, 21-H_3), 1.19 (s, 3H, 19-H_3), 1.99 (s, 3H, 3-OCO$\underline{CH_3}$), 3.66 (s, 3H, 24-COO$\underline{CH_3}$), 4.71 (brm, 1H, 3β-H), 5.70 (s, 1H, 11-H). ^{13}C-NMR (125.8 MHz, $CDCl_3$) δ: 10.8 (C-18), 19.6 (C-21), 21.4 (C-19), 24.3, 26.3, 26.6, 27.5, 27.8, 29.8, 30.7, 31.6, 34.1, 35.1, 35.4, 37.9, 40.1, 41.9, 47.4, 51.5 (C-25), 53.2, 53.6, 73.9 (C-3), 123.8 (C-11), 164.1 (C-9), 170.7 (3-O$\underline{CO}CH_3$), 174.8 (C-24), 205.2 (C-12). HR-ESI-MS, Calcd for $C_{27}H_{40}O_5Na$ $[M+Na]^+$, 467.2773. Found, *m/z* 467.2746.

Methyl 3α,7α-Diacetoxy-12-oxo-5β-chol-9(11)-en-24-oate (6b) The 3,7-diacetoxy-12-oxo **5b** (1.0 g; 2.0 mmol), subjected to the dehydrogenation with SeO_2 in acetic acid and processed as described for the preparation of **6a**, gave a crude product. Chromatography of the product on a column of silica gel (50 g) and elution with EtOAc–hexane (3 : 7, v/v) afforded the title compound **6b** which was recrystallized from methanol as colorless needles: mp 159–160°C (159–161°C)[11]; yield, 797 mg (80%). $[\alpha]_D^{31}$+80.0 (*c* 0.10, MeOH). FT-IR 1735 (C=O), 1671 (C=C) cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.92 (s, 3H, 18-H_3), 1.00 (d, 3H, *J*=6.3 Hz, 21-H_3), 1.20 (s, 3H, 19-H_3), 1.99 (s, 6H, 3- and 7-OCO$\underline{CH_3}$), 3.65 (s, 3H, 24-COO$\underline{CH_3}$), 4.59 (brm, 1H, 3β-H), 5.15 (m, 1H, 7β-H), 5.82 (s, 1H, 11-H). ^{13}C-NMR (125.8 MHz, $CDCl_3$) δ: 10.8 (C-18), 19.4 (C-21), 21.4 (C-19), 21.5, 23.7, 27.5, 27.8, 30.1, 30.7, 30.9, 31.5, 35.0, 35.4, 36.2, 40.1, 40.8, 41.1, 46.8, 47.1, 51.6 (C-25), 53.3, 70.2 (C-7), 73.8 (C-3), 125.1 (C-11), 159.8 (C-9), 170.1 (7-O$\underline{CO}CH_3$), 170.7 (3-O$\underline{CO}CH_3$), 174.7 (C-24), 204.6 (C-12). HR-ESI-MS, Calcd for $C_{29}H_{43}O_7$ $[M+H]^+$, 503.3009. Found, *m/z* 503.3086.

5β-Chol-9(11)-en-3α,24-diol (7a) To an ice-cooled solution of $AlCl_3$ (4.2 g; 31.5 mmol) in dry THF (25 mL) was added $LiAlH_4$ (0.5 g; 13.2 mmol) gradually, and the resulting solution was stirred 10 min at room temperature and was subsequently refluxed during 30 min. A solution of the $\Delta^{9(11)}$-3α-acetoxy-12-ketone **6a** (1.0 g, 2.25 mmol) in THF (25 mL) was added drop-

wise to the AlH_2Cl solution, and after the addition was complete, reflux was maintained during 4 h: the reaction was monitored by TLC. The reaction mixture was cooled with an ice-bath, and water was carefully added. The reaction product was extracted with CH_2Cl_2, and the combined extract was washed with 5% H_2SO_4 and water, dried with Drierite, and evaporated to dryness. The crude residue, which consisted essentially of a single spot on TLC was chromatographed on a column of silica gel (50 g). Elution with EtOAc–hexane (1 : 1, v/v) gave the desired $\Delta^{9(11)}$-3*α*,24-diol **7a** which was recrystallized from EtOAc–hexane as colorless needles: mp 179–180°C; yield, 510 mg (64%). $[\alpha]_D^{23}$+40.0 (*c* 0.10, MeOH). FT-IR 3324 (OH), 1647 (C=C) cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*: 0.58 (s, 3H, 18-H_3), 0.92 (d, 3H, *J*=6.2 Hz, 21-H_3), 1.05 (s, 3H, 19-H_3), 3.61 (br m, 3H, 3*β*-H and 24-H_2), 5.31 (d, 1H, *J*=5.8 Hz, 11-H). ^{13}C-NMR (125.8 MHz, $CDCl_3$) *δ*: 11.7 (C-18), 18.4 (C-21), 25.4 (C-19), 27.0 (×2), 28.5, 29.5, 29.7, 31.9, 32.0, 35.5, 35.8, 36.7, 38.1, 38.6, 41.0, 42.1, 42.2, 53.4, 56.4, 63.7 (C-24), 72.4 (C-3), 119.6 (C-11), 140.1 (C-9). HR-ESI-MS, Calcd for $C_{24}H_{40}O_2Na$ $[M+Na]^+$, 383.2926. Found, *m/z* 383.2943.

5*β*-Chol-9(11)-en-3*α*,7*α*,24-triol (7b) The $\Delta^{9(11)}$-3*α*,7*α*-diacetoxy-12-ketone **6b** (1.0 g, 2.0 mmol) was subjected to the deoxygenation reaction with $LiAlH_4$ and $AlCl_3$ in THF and processed as described for the preparation of **7a** to yield an oily residue. The oil was chromatographed on a column of silica gel (50 g) and eluted with EtOAc–hexane (1 : 1, v/v). Recrystallization of the product from EtOAc–hexane gave the $\Delta^{9(11)}$-3*α*,12*α*,24-diol **7b** as colorless needles: mp 154–156°C; yield, 390 mg (52%). $[\alpha]_D^{31}$+20.0 (*c* 0.10, MeOH). FT-IR 3270 (OH), 1639 (C=C) cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) *δ*: 0.61 (s, 3H, 18-H_3), 0.99 (d, 3H, *J*=6.2 Hz, 21-H_3), 1.06 (s, 3H, 19-H_3), 3.48 (br m, 1H, 3*β*-H), 3.62 (br m, 2H, 24-H_2), 3.97 (m, 1H, 7*β*-H), 5.51 (d, 1H, *J*=5.8 Hz, 11-H). ^{13}C-NMR (125.8 MHz, $CDCl_3$) *δ*: 11.4 (C-18), 18.3 (C-21), 24.7 (C-19), 28.4, 29.4, 29.9, 31.8, 31.9, 34.3, 35.5, 35.6, 38.7, 41.1 (×2), 41.4, 41.6, 41.7, 46.5, 56.1, 63.6 (C-24), 68.8 (C-7), 72.4 (C-3), 121.9 (C-11), 137.1 (C-9). HR-ESI-MS, Calcd for $C_{24}H_{39}O_3$ $[M-H]^-$, 375.2899. Found, *m/z* 375.3173.

9*α*,11*α*-Epoxy-5*β*-cholan-3*α*,24-diol (8a) A mixture of the 9(11)-en-3*α*,24-diol **7a** (300 mg, 0.84 mmol), 4,4′-thio-bis-(6-*tert*-butyl-3-methylphenol) (10 mg, 0.03 mmol) and 75% *m*-chloroperbenzoic acid (*m*-CPBA, 400 mg, 1.74 mmol) in $CHCl_3$ (30 mL) was stirred at 35°C for 1 h; the reaction was monitored by TLC. The organic layer was washed with 5% $Na_2S_2O_3$ solution, 5% $NaHCO_3$ solution, and water, dried with Drierite, and evaporated to dryness. Chromatography of the residue using a column of silica gel (60 g) and elution with EtOAc–hexane (1 : 1, v/v) gave the 9*α*,11*α*-epoxide **9a** which was crystallized from methanol as colorless needles: mp 187–189°C; yield, 280 mg (88%). $[\alpha]_D^{23}$+40.0 (*c* 0.10, MeOH). FT-IR 3357 (OH) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) *δ*: 0.68 (s, 3H,18-H_3), 0.93 (d, 3H, *J*=6.3 Hz, 21-H_3), 1.15 (s, 3H, 19-H_3), 3.21 (d, *J*=5.2, 11*β*-H), 3.49 (br m, 3H, 3*β*-H and 24-H_2). ^{13}C-NMR (125.8 MHz, CD_3OD) *δ*: 13.1 (C-18), 17.4 (C-21), 23.9 (×2), 26.0 (C-19), 28.1, 28.3, 28.9, 31.7, 32.2, 34.8, 35.3, 35.4, 36.6, 37.1, 40.0, 40.4, 42.1, 45.1, 51.3, 56.5 (C-11), 62.2 (C-24), 67.4 (C-9), 70.8 (C-3). HR-ESI-MS, Calcd for $C_{24}H_{40}O_3Na$ $[M+Na]^+$, 399.2875. Found, *m/z* 399.2884.

9*α*,11*α*-Epoxy-5*β*-cholan-3*α*,7*α*,24-triol (8b) The 9(11)-en-3*α*,7*α*,24-triol (100 mg, 0.27 mmol) was converted to its 9*α*,11*α*-epoxy-3*α*,7*α*,24-triol (**8b**) (at reflux for 20 min) by the method described for the preparation of **8a**. After chromatographic separation on silica gel (30 g) eluting with EtOAc–hexane (75 : 25, v/v), the major reaction product was recrystallized from methanol–water as colorless needles: mp 170–173°C; yield, 69 mg (64%). $[\alpha]_D^{30}$+10.0 (*c* 0.10, MeOH). FT-IR 3244 (OH) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) *δ*: 0.68 (s, 3H, 18-H_3), 0.93 (d, 3H, *J*=6.9 Hz, 21-H_3), 1.18 (s, 3H, 19-H_3), 3.08 (d, 1H, *J*=5.7 Hz, 11*β*-H), 3.36 (br m, 1H, 3*β*-H), 3.48 (br m, 2H, 24-H_2), 3.97 (d, 1H, *J*=2.8 Hz, 7*β*-H). ^{13}C-NMR (125.8 MHz, CD_3OD) *δ*: 12.9 (C-18), 17.9 (C-21), 23.1, 26.3 (C-19), 28.1, 28.8, 31.7, 32.3, 34.3, 35.2, 35.4, 35.5, 39.4, 39.9 (×2), 40.6, 41.1, 41.2, 48.6 (C-11), 56.4, 62.2 (C-24), 65.4 (C-9), 68.5 (C-7), 71.3 (C-3). HR-ESI-MS, Calcd for $C_{24}H_{40}O_4Na$ $[M+Na]^+$, 415.2824. Found, *m/z* 415.2805.

5*β*-Cholan-3*α*,9*α*,24-triol (9a) To a magnetically stirred dry THF solution, at −5°C, was added slowly $AlCl_3$ (4.41 g, 33 mmol). Then, $LiAlH_4$ (500 mg, 13 mmol) was added slowly, and the mixture was stirred at room temperature for 30 min. A solution of the 3*α*,24-dihydroxy-9*α*,11*α*-epoxide **8a** (850 mg, 2.26 mmol) in dry THF (20 mL) was added dropwise to the AlH_2Cl solution, and the mixture was refluxed for 48 h. After cooling the solution at room temperature, the reaction product was extracted with CH_2Cl_2. The combined extract was washed with 5% H_2SO_4 and water, dried with Drierite, and evaporated. The residue was subjected to preparative HPLC-RI on a Capcell Pak AQ RP-C_{18} column. Elution with methanol–H_2O (85 : 15, v/v) afforded the desired compound **9a** which crystallized from methanol as colorless needles; mp 174–177°C: yield, 154 mg (18%); the remaining compound recovered was the unreacted one **8a** (584 mg, 69%). $[\alpha]_D^{23}$+50.0 (*c* 0.10, MeOH). FT-IR 3321 (OH) cm^{-1}, 1709. ^{1}H-NMR (500 MHz, CD_3OD) *δ*: 0.69 (s, 3H, 18-H_3), 0.95 (d, 3H, *J*=5.2 Hz, 21-H_3), 0.96 (s, 3H, 19-H_3), 3.50 (br m, 2H, 24-H_2), 3.57 (br m, 1H, 3*β*-H). ^{13}C-NMR: see Table 1. HR-ESI-MS, Calcd for $C_{24}H_{42}O_3Na$ $[M+Na]^+$, 401.3032. Found, *m/z* 401.3000.

5*β*-Cholan-3*α*,7*α*,9*α*,24-tetrol (9b) The 3*α*,7*α*,24-trihydroxy-9*α*,11*α*-epoxide **8b** (100 mg, 0.25 mmol), subjected to reductive cleavage with AlH_2Cl at reflux condition for 12 h and processed as described for the preparation of **9a**, afforded an oily residue. Preparative HPLC-RI of the oily residue on a Capcell Pak AQ RP-C_{18} column and elution with methanol–water (80 : 20, v/v) afforded the desired compound **9b** which crystallized from methanol–water as colorless needles; mp 175–176°C; yield, 43 mg (36%); the remaining compound recovered was the unreacted one **8b** (51 mg, 51%). $[\alpha]_D^{30}$+20.0 (*c* 0.10, MeOH). FT-IR 3293 (OH) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) *δ*: 0.68 (s, 3H, 18-H_3), 0.95 (d, 3H, *J*=6.9 Hz, 21-H_3), 0.97 (s, 3H, 19-H_3), 3.47 (br m, 1H, 3*β*-H), 3.49 (br m, 2H, 24-H_2), 3.91 (m, 1H, 7*β*-H). ^{13}C-NMR: see Table 1. HR-ESI-MS, Calcd for $C_{24}H_{42}O_4Na$ $[M+Na]^+$, 417.2981. Found, *m/z* 417.2998.

3*α*,9*α*-Dihydroxy-5*β*-cholan-24-oic Acid (1a) To a magnetically stirred solution of the 3*α*,11*α*,24-triol **9a** (20 mg, 53 *μ*mol) in dry THF (1 mL) and CH_3CN (0.25 mL) was added TEMPO (1 mg, 6 *μ*mol) and a 0.2 M sodium phosphate buffer (pH, 6.7; 40 *μ*L). Solutions of 2% NaClO (25 *μ*L) and $NaClO_2$ (7 mg dissolved in 50 *μ*L of H_2O) were added simultaneously at 35°C to the solution over 0.5 h, and the reaction mixture was further stirred at 35°C for 3 h; the reaction was monitored by TLC. The reaction was quenched by adding a cold saturated solution of $Na_2S_2O_3$. After stirring for 0.5 h at room

temperature, the reaction product was extracted with EtOAc. The combined extract was washed with a saturated brine, dried with Drierite, and evaporated to dryness. The residue was chromatographed on a column of silica gel (10 g) eluting with EtOAc–hexane–acetic acid (8 : 2 : 0.1, v/v/v). Recrystallization of the product from methanol–water gave the titled compound **1a** as colorless needles: mp 88–91°C; yield, 12 mg (58%). $[\alpha]_D^{23}$+40.0 (*c* 0.10, MeOH). FT-IR 3447 (OH), 1709 (C=O) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.70 (s, 3H, 18-H_3), 0.95 (d, 3H, J=6.3 Hz, 21-H_3), 0.96 (s, 3H, 19-H_3), 3.57 (br m, 1H, 3β-H). ^{13}C-NMR: see Table 1. HR-ESI-MS, Calcd for $C_{24}H_{39}O_4$ $[M-H]^-$, 391.2848. Found, *m/z* 391.2847.

3α,7α,9α-Trihydroxy-5β-cholan-24-oic Acid (1b) The 3α,7α,9α,24-terol **9b** (40 mg, 0.10 mmol) was subjected to the selective oxidation (at 35°C overnight) with $NaClO_2$ in the presence of TEMPO and NaClO in sodium phosphate buffer and processed as described for the preparation of **1a**; the reaction product was an oily residue. Chromatography of the oil on a preparative HPLC-RI on a Capcell Pak AQ RP-C_{18} column and elution with a mixture of methanol–water–acetic acid (8 : 2 : 0.2, v/v/v) afforded the desired compound **1b** which was crystallized from methanol–EtOAc as colorless needles; mp 139–142°C; yield, 29 mg (71%). $[\alpha]_D^{29}$+40.0 (*c* 0.10, MeOH). FT-IR 3320 (OH), 1713 (C=O) cm^{-1}. ^{1}H-NMR (500 MHz, CD_3OD) δ: 0.70 (s, 3H, 18-H_3), 0.94 (d, 3H, J=6.9 Hz, 21-H_3), 0.96 (s, 3H, 19-H_3), 3.48 (br m, 1H, 3β-H), 3.91 (m, 1H, 7β-H). ^{13}C-NMR: see Table 1. HR-ESI-MS, Calcd for $C_{24}H_{39}O_5$ $[M-H]^-$, 407.2796. Found, *m/z* 407.2822.

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