methods

Chemical synthesis of (22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid and its taurine and glycine conjugates: a major bile acid in the rat

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Abstract A method for the synthesis of Δ^{22}-β-muricholic acid (Δ^{22}-β-MCA), (22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid, and its taurine and glycine conjugates (Δ^{22}-β-muricholyltaurine and Δ^{22}-β-muricholylglycine) is described. The key intermediate, 3α,6β,7β-triformyloxy-23,24-dinor-5β-cholan-22-al, was prepared from β-muricholic acid (β-MCA) via the 24-nor-22-ene and 24-nor-22,23-diol derivatives. Wittig reaction of the aldehyde with (carbomethoxymethylene) triphenylphosphorane and subsequent hydrolysis gave (unconjugated) Δ^{22}-β-MCA. Condensation reaction of the unconjugated acid with taurine or glycine methyl ester using diethylphosphorocyanide yielded the naturally occurring taurine or glycine conjugate (*N*-acylamidate) of Δ^{22}-β-MCA. These synthetic reference compounds are now available for investigation of the metabolism of β-MCA by bacterial and hepatic enzymes in the rat and should also be useful as substrates for reductive deuteration or tritiation to give the 22,23-^{2}H or ^{3}H-β-MCA.—Kakiyama, G., T. Iida, A. Yoshimoto, T. Goto, N. Mano, J. Goto, T. Nambara, L. R. Hagey, and A. F. Hofmann. **Chemical synthesis of (22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid and its taurine and glycine conjugates: a major bile acid in the rat. *J. Lipid Res.* 2004.** 45: **567–573.**

Supplementary key words unsaturated bile acid • tauro-Δ^{22}-β-muricholic acid • Δ^{22}-β-muricholyltaurine • glyco-Δ^{22}-β-muricholic acid • Δ^{22}-β-muricholylglycine

When the chemical structure of the common natural C_{24} bile acids was deduced, it was assumed that the C_5 side chain of all natural C_{24} bile acids was saturated. However, in 1973, while analyzing urinary bile acids from bile duct ligated rats, Danielsson (1) noted a bile acid with chromatographic behavior slightly different from that of β-muricholic acid (β-MCA; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) and with two fewer mass units by mass spectroscopy. He proposed that this bile acid was β-MCA with a double bond in the side chain. Similar observations were made by Kern et al. (2), who characterized the biliary bile acids of rats in whom cholestasis was induced by ethynylestradiol. In 1980, Kuriyama et al. (3) used gas chromatography-mass spectrometry to show that an unsaturated derivative of β-MCA was present as a minor constituent in the biliary bile acids of the healthy Wistar rat. In 1993, Davis and Thompson (4) used ^{1}H- and ^{13}C-NMR to establish the chemical structure of the unsaturated bile acids as Δ^{22}-β-muricholic acid [Δ^{22}-β-MCA; (22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid], and Thompson, Davis, and Morris (5) reported that its plasma concentration in female Fischer rats exceeded that of β-MCA. This observation was confirmed for Sprague-Dawley rats in 1996 by Rodrigues et al. (6). Subsequently, this group reported that the concentration of Δ^{22}-β-MCA exceeded that of β-MCA in liver homogenates (7), although no correction was made for entrapped blood, in which the bile acid concentration was likely to be much higher than in hepatocytes.

In the early studies in which Δ^{22}-β-MCA was identified in plasma or bile, it was inferred that the Δ^{22}-β-MCA had been formed by the enteric flora. This assumption was based on the finding that the compound disappeared from bile after prolonged biliary drainage (1, 8) as well as on reports from the Eyssen group that this acid was formed in vitro by bacteria (9) and in vivo in gnotobiotic rats (10). Kayahara et al. (11) confirmed and extended

Abbreviations: DEPC, diethylphosphorocyanide; EI, electron ionization; ESI, electrospray ionization; EtOAc, ethyl acetate; HR-MS, high-resolution mass spectra; IR, infrared; LR-MS, low-resolution mass spectra; β-MCA, β-muricholic acid; Δ^{22}-β-MCA, Δ^{22}-β-muricholic acid; NIM, negative ion mode; PIM, positive ion mode; UDCA, ursodeoxycholic acid; Δ^{22}-UDCA, Δ^{22}-ursodeoxycholic acid.

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Manuscript received 22 September 2003, in revised form 24 October 2003, and in re-revised form 18 November 2003.

Published, JLR Papers in Press, December 1, 2003.
DOI 10.1194/jlr.D300027-JLR200

this finding by using gnotobiotic rats to show that several bacterial species form Δ^{22}-β-MCA. This group, working independently of the Eyssen group, also established its chemical structure by ^{1}H- and ^{13}C-NMR spectroscopy.

The first evidence that Δ^{22}-β-MCA could also be formed in the liver was presented by Thompson, Davis, and Morris (5), who in 1993 showed that Δ^{22}-β-MCA could be formed by rat liver slices. Two years later, Setchell et al. (12) provided additional indirect evidence for the formation of Δ^{22}-β-MCA in hepatocytes by showing that ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid) was converted to Δ^{22}-UDCA when incubated with rat peroxisomes.

Thus, the consensus of the work to date is that Δ^{22}-β-MCA can be formed not only by the enteric flora of the distal intestine but also by hepatocyte peroxisomal enzymes, at least in the rat. In the rat, the compound is present in plasma, bile, and intestinal content in both conjugated and unconjugated forms (5), suggesting that it is absorbed from the distal intestine after formation by bacteria and then conjugated with taurine or glycine during transport through the hepatocyte; the conjugates then undergo enterohepatic circulation. However, Δ^{22}-β-MCA is sometimes absent in fecal bile acids of the rat despite being present in intestinal content (10, 11), indicating that intestinal bacteria can both desaturate and saturate the side chain of β-MCA.

No studies have been performed on the metabolism of Δ^{22}-β-MCA because of the absence of synthetic material. Kihira and Hoshita (13) reported the synthesis of the Δ^{22} derivatives of the common natural bile acids (cholic, deoxycholic, and chenodeoxycholic acids and UDCA) but did not extend their work to β-MCA, presumably because of the difficulty of the synthesis. Our laboratory has previously developed an improved synthesis of β-MCA and its congeners (α- and ω-muricholic acid) (14). In this paper, we report the chemical synthesis of Δ^{22}-β-MCA **[1a]** and its taurine **[1b]** and glycine **[1c]** conjugates (Δ^{22}-β-muricholyltaurine and Δ^{22}-β-muricholylglycine). Chemical structures are shown in **Fig. 1**.

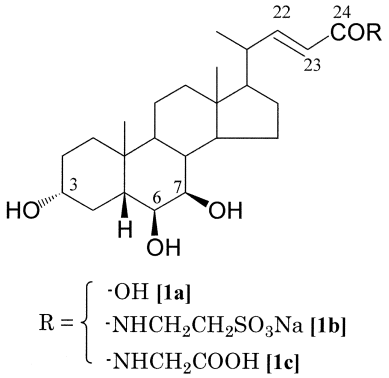

Fig. 1. Structures of Δ^{22}-β-muricholic acid (Δ^{22}-β-MCA) and its taurine and glycine conjugates **[1a–1c]**.

MATERIALS AND METHODS

Melting points (mp) were determined on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were obtained on a Bio-Rad FTS-7 FT-IR spectrometer (Philadelphia, PA) as KBr discs. ^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JNM-EX 270 FT NMR instrument (Tokyo, Japan) at 270 and 68.80 MHz, respectively, with $CDCl_3$ containing 10% CD_3OD or CD_3OD as the solvent; chemical shifts were expressed as δ ppm relative to tetramethylsilane. Low-resolution mass spectra (LR-MS) were recorded on a JEOL JMS-303 mass spectrometer with electron ionization (EI) at 70 eV under the positive ion mode (PIM). LR-MS was also obtained on a JEOL JMS-LCmate equipped with electrospray ionization (ESI) under the negative ion mode (NIM). High-resolution mass spectra (HR-MS) were recorded on a JEOL JMS-LCmate double-focusing magnetic mass spectrometer equipped with an ESI probe under the PIM or the NIM. HR-MS were also obtained on a JEOL JMS-700 mass spectrometer with an EI probe under the PIM. Sep-Pak Vac tC_{18} cartridges (adsorbent weight, 5 g) were purchased from Waters Associates (Milford, MA). Thin-layer chromatography was performed on precoated silica gel (0.25 mm layer thickness; E. Merck) using hexane-ethyl acetate (EtOAc)-acetic acid mixtures (80:20:1∼20:80:1, v/v/v) or EtOAc-methanol-acetic acid mixtures (7:2:1, v/v/v) as the developing solvents.

3α,6β,7β-Triformyloxy-5β-cholan-24-oic acid [4]

A solution of β-MCA **[3]** (1.0 g, 2.4 mmol), prepared from chenodeoxycholic acid **[2]** (see Results and Discussion), in 99% formic acid (7 ml) containing 60% perchloric acid (100 mg) was stirred at 50°C for 1.5 h. Acetic anhydride (3 ml) was added slowly with ice-bath cooling, and the mixture was poured into water. The reaction product was extracted with CH_2Cl_2, and the combined extract was washed with water, dried with anhydrous calcium sulfate (Drierite), and evaporated to dryness. The residue was recrystallized from benzene-hexane as colorless crystals: yield, 1.21 g (100%); mp, 90–92°C. IR (KBr), $\nu_{max}cm^{-1}$: 1,713 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.72 (3H, s, 18-CH_3), 0.94 (3H, d, J = 6.2 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 4.85 (1H, brm, 3β-H), 5.06 (1H, dd, J = 11.1 and 3.8 Hz, 7α-H), 5.14 (1H, m, 6α-H), 7.93, 8.02, and 8.10 (each 1H, s, 3α-, 6β-, and 7β-OCHO). LR-MS (EI-PIM), m/z: 492 (M^+, 4%), 446 (M-HCOOH, 5%), 418 (9%), 400 (M-2HCOOH, 80%), 372 (39%), 354 (M-3HCOOH, 90%), 339 (27%), 299 (100%), 272 (22%), 253 [M-3HCOOH-side chain (S.C.), 66%], 213 (22%), 211 (M-3HCOOH-S.C.-ring D, 18%), 159 (31%). HR-MS (EI-PIM), calculated for $C_{27}H_{40}O_8$ $[M]^+$: 492.2723; found, m/z: 492.2692.

3α,6β,7β-Triformyloxy-24-nor-5β-chol-22-ene [5]

To a solution of the triformate **[4]** (1.0 g, 2.0 mmol) in dry benzene (18 ml), lead tetraacetate (1.44 g, 3.24 mmol), cuprous acetate (0.1 g, 0.55 mmol), and dry pyridine (0.1 ml) were added successively, and the mixture was refluxed for 12 h. Most of benzene was evaporated under reduced pressure, and the residual solution was poured onto a column of silica gel (30 g). Elution with benzene-EtOAc (9:1, v/v) afforded the title compound **[5]**, which recrystallized from ethanol-water as colorless crystals: yield, 450 mg (49%); mp, 71–73°C. IR (KBr), $\nu_{max}cm^{-1}$: 1,728 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.74 (3H, s, 18-CH_3), 1.04 (3H, d, J = 6.5 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 4.77–4.87 (3H, brm, 3β- and 23-H), 5.06 (1H, dd, J = 11.1 and 4.0 Hz, 7α-H), 5.13 (1H, m, 6α-H), 5.65 (1H, m, 22-H), 7.92, 8.02, and 8.10 (each 1H, s, 3α-, 6β-, and 7β-OCHO). LR-MS (EI-PIM), m/z: 446 (M^+, 2%), 431 (M-CH_3, 9%), 418 (18%), 400 (M-HCOOH, 4%), 389 (16%), 372 (16%), 354 (M-2HCOOH, 37%), 343 (33%), 326 (31%), 308 (M-3HCOOH, 14%), 299 (69%), 271 (21%), 253 (M-3HCOOH-S.C., 100%), 211 (M-3HCOOH-S.C-ring D, 11%), 197 (8%), 159 (21%). HR-MS (EI-PIM), calculated for $C_{26}H_{38}O_6$ $[M]^+$: 446.2669; found, m/z: 446.2665.

3α,6β,7β-Triformyloxy-24-nor-5β-cholan-22ξ,23-diol [6]

To the 24-nor-22-ene **[5]** (1.38 g, 3.0 mmol) dissolved in *tert*-butyl alcohol-tetrahydrofuran-water (34.5 ml; 7:2:1, v/v/v) was added *N*-methylmorpholine *N*-oxide (2 ml) and osmium tetroxide (30 mg, 0.12 mmol), and the mixture was allowed to stand at room temperature for 12 h. The reaction product was extracted with $CHCl_3$, and the combined extract was washed with water, dried with Drierite, and evaporated to a dark brown oily residue. Chromatography of the oil on a column of silica gel (50 g) and elution with benzene-EtOAc (6:4–1:9, v/v) afforded the desired 24-nor-22ξ,23-diol **[6]** as viscous oil, which resisted crystallization attempts: yield, 950 mg (66%). IR (KBr) $\nu_{max}cm^{-1}$: 1,713 (C=O), 3,427 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.72 (3H, s, 18-CH_3), 0.94 (3H, d, J = 6.2 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 3.65–3.75 (3H, brm, 22ξ- and 23-H), 4.81 (1H, brm, 3β-H), 5.09–5.13 (2H, m, 6α- and 7α-H), 7.93, 8.02, and 8.10 (each 1H, s, 3α-, 6β-, and 7β-OCHO). LR-MS (EI-PIM), *m/z*: 462 (M-H_2O, 2%), 444 (M-2H_2O, 5%), 434 (M-HCOOH, 8%), 416 (M-H_2O-HCOOH, 15%), 403 (48%), 388 (M-2HCOOH, 22%), 357 (69%), 342 (M-3HCOOH, 10%), 329 (39%), 311 (100%), 299 (32%), 281 (34%), 253 (M-3HCOOH-S.C., 29%), 211 (M-3HCOOH-S.C.-ring D, 18%), 159 (26%), 107 (25%). HR-MS (ESI-PIM), calculated for $C_{26}H_{40}O_8Na$ [M+Na]$^+$: 503.2621; found, *m/z*: 503.2632.

3α,6β,7β-Triformyloxy-23,24-dinor-5β-cholan-22-al [7]

To a magnetically stirred solution of sodium periodate ($NaIO_4$) (1.5 g, 7.0 mmol) dissolved in methanol (14 ml) and water (7 ml), a solution of the 24-nor-22ξ,23-diol **[6]** (900 mg, 1.9 mmol) in methanol (15 ml) was added, and the mixture was stirred at room temperature for 12 h. The reaction product was extracted with $CHCl_3$, and the combined extract was washed with water, dried with Drierite, and evaporated to dryness. Although the residual oil **[7]** was found to be homogeneous according to TLC, it resisted crystallization attempts: yield, 800 mg (95%). IR (KBr), $\nu_{max}cm^{-1}$: 1,726 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.77 (3H, s, 18-CH_3), 0.96 (3H, d, J = 6.8 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 4.81 (1H, brm, 3β-H), 5.07 (1H, dd, J = 11.3 and 3.5 Hz, 7α-H), 5.14 (1H, m, 6α-H), 7.92, 8.02, and 8.10 (each 1H, s, 3α-, 6β-, and 7β-OCHO), 9.57 (1H, d, J = 3.0 Hz, 22-CHO). LR-MS (EI-PIM), *m/z*: 448 (M$^+$, 3%), 416 (6%), 402 (M-HCOOH, 7%), 374 (M-HCOOH-CHO, 17%), 356 (M-2HCOOH, 93%), 328 (M-2HCOOH-CHO, 70%), 310 (M-3HCOOH, 42%), 282 (M-3HCOOH-CHO, 20%), 272 (37%), 253 (M-3HCOOH-S.C., 45%), 211 (M-3HCOOH-S.C.-ring D, 4%), 159 (41%), 111 (100%). HR-MS (EI-PIM), calculated for $C_{25}H_{36}O_7$ [M]$^+$: 448.2461; found, *m/z*: 448.2459.

Methyl (22*E*)-3α,6β,7β-triformyloxy-5β-chol-22-en-24-oate [8]

To a stirred solution of the 23,24-dinor-22-aldehyde **[7]** (370 mg, 0.6 mmol) in dry benzene (28 ml), methyl (triphenylphosphoranylidene)acetate (500 mg, 1.5 mmol) was added, and the mixture was refluxed for 12 h under a stream of N_2. After cooling at room temperature, most of the solvent was evaporated under reduced pressure, and the reaction product was poured onto a column of silica gel (20 g). Elution with benzene-EtOAc (95:5, v/v) gave the title compound **[8]** as viscous oil, which resisted crystallization attempts: yield, 350 mg (84%). IR (neat), $\nu_{max}cm^{-1}$: 1,186 (C-O), 1,651 (C=C), 1,715, 1,732 (C=O). ^{1}H-NMR ($CDCl_3$), δ: 0.75 (3H, s, 18-CH_3), 1.09 (3H, d, J = 7.0 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 3.72 (3H, s, $COOCH_3$), 4.80 (1H, brm, 3β-H), 5.06 (1H, dd, J = 10.8 and 3.8 Hz, 7α-H), 5.14 (1H, m, 6α-H), 5.74 (1H, d, J = 15.7 Hz, 23-H), 6.82 (1H, dd, J = 15.7 and 8.9 Hz, 22-H), 7.91, 8.02, and 8.10 (each 1H, s, 3α-, 6β-, and 7β-OCHO). LR-MS (EI-PIM), *m/z*: 504 (M$^+$, 2%), 473 (M-CH_3O, 2%), 458 (M-HCOOH, 1%), 412 (M-2HCOOH, 11%), 366 (M-3HCOOH, 7%), 343 (34%), 299 (M-2HCOOH-S.C., 30%), 271 (11%), 253 (M-3HCOOH-S.C., 50%), 211 (M-3HCOOH-S.C.-ring D, 6%), 159 (12%), 114 (100%). HR-MS (EI-PIM), calculated for $C_{28}H_{40}O_8$ [M]$^+$: 504.2723; found, *m/z*: 504.2722.

(22*E*)-3α,6β,7β-Trihydroxy-5β-chol-22-en-24-oic acid [1a] and its methyl ester

A solution of the triformyloxy-22-ene ester **[8]** (500 mg, 0.99 mmol) in 5% methanolic KOH (5 ml) was refluxed for 6 h. After evaporation of most of the solvent, the residue was dissolved in water and then acidified with 10% H_2SO_4 with ice-bath cooling. The precipitated solid was filtered off, washed with water, and recrystallized from aqueous methanol to give the desired Δ^{22}-β-MCA as colorless prisms: yield, 370 mg (92%); mp, 205–209°C. IR (KBr), $\nu_{max}cm^{-1}$: 1,707 (C=O), 3,342 (OH). ^{1}H-NMR (CD_3OD), δ: 0.73 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 1.11 (3H, d, J = 6.5 Hz, 21-CH_3), 3.49–3.61 (3H, brm, 3β-, 6α, and 7α-H), 5.72 (1H, d, J = 15.4 Hz, 23-H), 6.86 (2H, dd, J = 15.4 and 6.8 Hz, 22-H). ^{13}C-NMR ($CDCl_3$+10% CD_3OD), δ: 12.1 (C-18), 19.1 (C-21), 20.6 (C-11), 25.2 (C-19), 26.9 (C-15), 28.2 (C-16), 29.4 (C-2), 33.6 (C-10), 35.0 (C-1), 35.3 (C-4), 38.2 (C-8), 39.4 (C-2), 39.5 (C-9), 39.7 (C-12), 43.7 (C-13), 47.2 (C-5), 54.1 (C-17), 55.3 (C-14), 70.4 (C-3), 73.0 (C-7), 75.4 (C-6), 118.7 (C-23), 155.6 (C-22), 169.2 (C-24). LR-MS (ESI-NIM), *m/z*: 405 ([M-H]$^-$, 100%), 111 (42.3%). HR-MS (ESI-NIM), calculated for $C_{24}H_{37}O_5$ [M-H]$^-$: 405.2641; found, *m/z*: 405.2682.

A solution of the free acid **[1a]** (100 mg, 0.25 mmol) and *p*-toluenesulfonic acid (30 mg) in methanol (5 ml) was left overnight at room temperature. After evaporation of most of the solvent, the reaction product was extracted with EtOAc. The combined extract was washed with saturated brine, dried with Drierite, and evaporated to dryness. The resulting C-24 methyl ester, although homogeneous according to TLC, resisted crystallization attempts: yield, 93 mg (86%). IR (neat), $\nu_{max}cm^{-1}$: 1,651 (C=C), 1,715 (C=O), 3,364 (OH). ^{1}H-NMR ($CDCl_3$), δ: 0.73 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 1.10 (3H, d, J = 6.5 Hz, 21-CH_3), 3.44 (1H, dd, J = 10.3 and 3.8 Hz, 7α-H), 3.51 (1H, brm, 3β-H), 3.57 (1H, m, 6α-H), 3.72 (3H, s, $COOCH_3$), 5.75 (1H, d, J = 16.2 Hz, 23-H), 6.90 (1H, dd, J = 16.2 and 8.1 Hz, 22-H). ^{13}C-NMR ($CDCl_3$), δ: 12.4 (C-18), 19.4 (C-21), 20.7 (C-11), 25.4 (C-19), 27.1 (C-15), 28.4 (C-16), 29.9 (C-2), 33.8 (C-10), 35.4 (C-1), 35.4 (C-4), 38.5 (C-8), 39.6 (C-2), 39.6 (C-9), 39.8 (C-12), 44.0 (C-13), 47.2 (C-5), 51.4 ($COOCH_3$), 54.1 (C-17), 55.3 (C-14), 71.0 (C-3), 73.4 (C-7), 75.5 (C-6), 118.6 (C-23), 154.9 (C-22), 167.5 (C-24). LR-MS (EI-PIM), *m/z*: 420 (M$^+$, 2%), 402 (M-H_2O, 100%), 384 (M-2H_2O, 52%), 369 (M-2H_2O-CH_3, 14%), 366 (M-3H_2O, 4%), 347 (10%), 305 (22%), 289 (M-H_2O-S.C., 22%), 271 (M-2H_2O-S.C., 80%), 253 (M-3H_2O-S.C., 53%), 229 (11%), 211 (M-3H_2O-S.C.-ring D, 9%), 175 (12%), 147 (20%), 114 (52%). HR-MS (EI-PIM), calculated for $C_{25}H_{40}O_5$ [M]$^+$: 420.2876; found, *m/z*: 420.2893.

Tauro-(22*E*)-3α,6β,7β-trihydroxy-5γ-chol-22-en-24-oic acid sodium salt [(22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oyl taurine] [1b]

To a magnetically stirred solution of the nonamidated Δ^{22}-β-MCA **[1a]** (100 mg, 0.25 mmol) in dry *N*,*N*-dimethylformamide (DMF) (9 ml) were successively added powdered taurine (80 mg, 0.64 mmol), diethylphosphorocyanide (DEPC) (75 μl), and anhydrous Et_3N (150 μl), and the resulting suspension was stirred at room temperature for 60 min. The reaction mixture was adjusted to pH 12–14 with 1 M NaOH and then to pH 8–9 with 10% HCl. The resulting solution was diluted with water (90 ml), passed through a preconditioned Sep-Pak Vac tC_{18} cartridge,

and eluted successively with water (20 ml), 20% methanol (20 ml), and 50% methanol (25 ml). The last fraction, which contains the desired component, was evaporated under reduced pressure, and the residue was recrystallized from methanol-ether to give the taurine-conjugated Δ^{22}-β-MCA sodium salt **[1b]** as colorless crystals: yield, 82 mg (62%); mp, 283–285°C. IR (KBr), $\nu_{max}cm^{-1}$: 1,624 (C=C), 1,664 (C=O), 3,385 (OH). ^{1}H-NMR (CD_3OD), δ: 0.75 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 1.11 (3H, d, J = 7.0 Hz, 21-CH_3), 3.03 (2H, t, J = 7.0 Hz, CH_2S), 3.48 (1H, dd, J = 10.0 and 3.8 Hz, 7α-H), 3.55 (1H, brm, 3β-H), 3.62 (1H, m, 6α-H), 5.83 (1H, d, J = 15.1 Hz, 23-H), 6.70 (2H, dd, J = 15.1 and 8.9 Hz, 22-H). ^{13}C-NMR (CD_3OD) δ: 12.9 (C-18), 20.2 (C-21), 21.9 (C-11), 26.1 (C-19), 28.1 (C-15), 29.5 (C-16), 30.6 (C-2), 34.9 (C-10), 36.4 (CH_2N), 36.5 (C-1), 39.4 (C-8), 40.7 (C-20), 41.1 (C-9 and C-12), 44.9 (C-13), 49.1 (C-5), 51.5 (CH_2S), 55.8 (C-17), 56.9 (C-14), 71.7 (C-3), 74.2 (C-7), 77.1 (C-6), 122.2 (C-23), 151.8 (C-22), 168.9 (C-24). LR-MS (ESI-NIM), *m/z*: 512 ($[M-H]^-$). HR-MS (ESI-NIM), calculated for $C_{26}H_{41}NO_7S$ $[M-H]^-$: 512.2682; found, *m/z*: 512.2665.

Glyco-(22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oic acid [(22*E*)-3α,6β,7β-trihydroxy-5β-chol-22-en-24-oyl glycine] [1c]

To a magnetically stirred solution of Δ^{22}-β-MCA **[1a]** (120 mg, 0.29 mmol) in dry DMF (4 ml), glycine methyl ester hydrochloride (100 mg, 0.65 mmol), DEPC (120 μl), and Et_3N (0.5 ml) were added successively, and the resulting suspension was stirred at room temperature for 1 h. The reaction product was extracted with EtOAc, and the combined extract was washed with water, dried with Drierite, and evaporated to dryness. The residue was then refluxed in 5% methanolic KOH (10 ml) for 30 min. Most of the solvent was evaporated under reduced pressure, and the hydrolysis product dissolved in water (5 ml) was acidified by 5% H_2SO_4 with ice-bath cooling. After stirring for 10 min at room temperature, the precipitated solid was filtered, washed with water, and dried to give the glycine-conjugated Δ^{22}-β-MCA **[1c]**, which was recrystallized from 1,4-dioxane-ether as colorless crystals: yield 120 mg (89%); mp, 234–235°C. IR (KBr), $\nu_{max}cm^{-1}$: 1,624 (C=C), 1,666, 1,732 (C=O), 3,375 (OH). ^{1}H-NMR (as the methyl ester in $CDCl_3$), δ: 0.73 (3H, s, 18-CH_3), 1.10 (3H, d, J = 7.0 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 3.76 (2H, d, J = 4.1 Hz, CH_2N), 3.78 (1H, s, $COOCH_3$), 5.77 (1H, d, J = 15.4 Hz, 23-H), 6.06 (1H, t, J = 8.9 Hz, -NH-), 6.76 (2H, dd, J = 15.4 and 6.22 Hz, 22-H). ^{13}C-NMR (as the methyl ester in $CDCl_3$), δ: 12.3 (C-18), 19.5 (C-21), 20.7 (C-11), 25.4 (C-19), 27.1 (C-15), 28.5 (C-16), 29.3 (C-2), 33.8 (C-10), 35.4 (C-1), 35.6 (C-4), 39.4 (C-2), 39.6 (C-9), 39.8 (C-12), 41.3 (-CH_2COOCH_3), 43.9 (C-13), 47.2 (C-5), 52.4 (-CH_2COOCH_3), 54.2 (C-17), 55.3 (C-14), 71.0 (C-3), 73.5 (C-7), 75.5 (C-6), 120.5 (C-23), 151.2(C-22), 166.2 (C-24), 170.6 (-CH_2COOCH_3). LR-MS (ESI-NIM), *m/z*: 462 ($[M-H]^-$). HR-MS (ESI-NIM), calculated for $C_{26}H_{40}O_6N$ $[M-H]^-$: 462.2855; found: *m/z*, 462.2827.

RESULTS AND DISCUSSION

The synthetic route to Δ^{22}-β-MCA **[1a]** is shown in **Fig. 2**. The key starting compound, β-MCA **[3]**, was prepared according to the established procedures (14) reported previously by us from chenodeoxycholic acid **[2]** as follows: chenodeoxycholic acid **[2]** was converted to its 3α-cathyloxy-7-oxo ester; bromination of the resulting ketone with bromine in the presence of HBr afforded the 6α-bromo-3α-cathyloxy-7-oxo ester; reduction of the bromoketone with $Zn(BH_4)_2$ gave 6α-bromo-3α-cathyloxy-7α-hydroxy ester; treatment of the bromohydrin in acetic acid with zinc powder yielded 3α-cathyloxy-Δ^6 ester; β-face *cis*-dihydroxylation of the Δ^6 ester with osmium tetroxide-*N*-methylmorpholine *N*-oxide afforded the 3α-cathyloxy-6β,7β-dihydroxy ester, which in turn was hydrolyzed to give the corresponding free acid **[3]** (total yield of 30% from chenodeoxycholic acid **[2]**).

Two methods have been reported for the introduction of a (22*E*)-double bond in the bile acid side chain (C_4H_8COOH) of β-MCA **[3]**. The first method involves an ene reaction with (*Z*)-ethylidene-($\Delta^{17(20)}$)-steroids and methyl propiolate ($HC{\equiv}CCOOCH_3$) under the presence of ethyl aluminum dichloride ($EtAlCl_2$) or diethyl aluminum chloride as Lewis acid-catalyzed conditions (15, 16). The second method consists of condensation of (20*S*)-20-methyaldehyde pregnane derivatives with a Wittig reagent, (carbomethoxymethylene)triphenylphosphorane

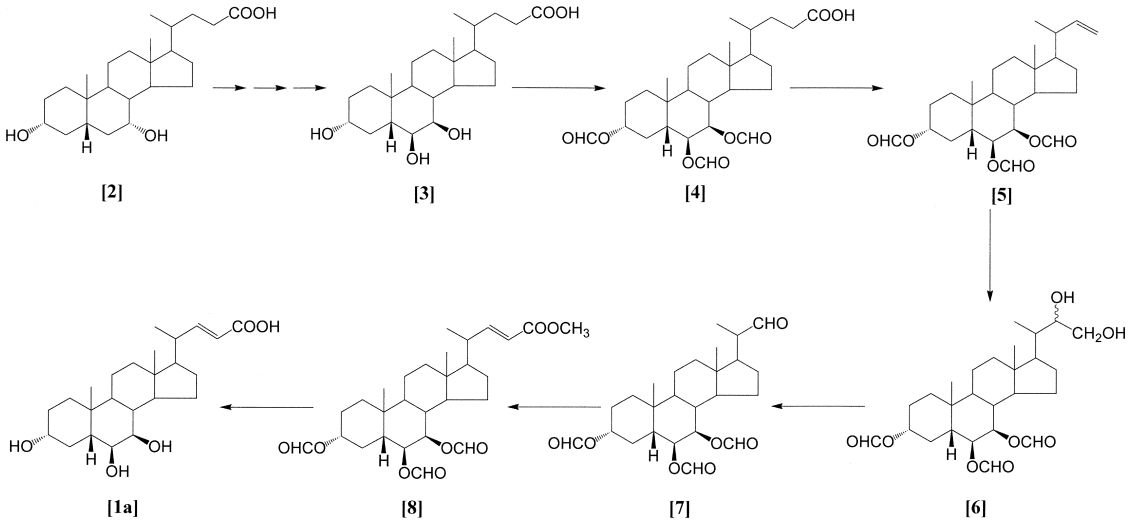

Fig. 2. Synthetic route to unconjugated Δ^{22}-β-MCA **[1a]**.

$[(C_6H_5)_3P{=}CHCOOCH_3]$ (17). Of the two methods, the latter appeared to be much more practical than the former, because of easy preparation of the key intermediate, (20*S*)-23,24-dinoraldehyde **[7]**.

Based on the above assumption, our next effort was directed to the preparation of the 24-nor-triformyloxy-22-ene intermediate **[5]** from β-MCA **[3]**. Oxidative decarboxylation of the β-MCA triformate derivative **[4]** with lead tetraacetate and cuprous acetate in refluxing pyridine (18) yielded 24-nor-triformyloxy-22-ene **[5]** in an isolated yield of 46% after chromatographic purification on a column of silica gel. The triformate **[4]** was obtained in nearly quantitative yield by treatment of **[3]** with 99% formic acid in the presence of 60% perchloric acid (19).

Vicinal dihydroxylation of the unsaturated 22-ene **[5]** in *tert*-butyl alcohol-tetrahydrofuran-water mixtures with osmium tetroxide and *N*-methylmorphorine *N*-oxide produced exclusively an epimeric mixture (22*R* and 22*S*) of vicinal 22ξ,23-diols **[6]**, without being accompanied by a simultaneous hydrolysis of the formyloxy protecting groups at the C-3, C-6, and C-7 positions, as indicated by the ^{13}C-NMR spectrum. The one-step dihydroxylation is much more simple and straightforward than the two-step procedure reported by Kihira and Hoshita (13) and Kihira and others (20, 21), in which epoxidation of the 22-ene **[5]** with hydrogen peroxide and subsequent methanolic KOH cleavage of the resulting epoxide were used. When the triformyloxy-22ξ,23-diol **[6]** (both epimers) was subjected to oxidation with $NaIO_4$ in methanol-water mixtures (13), both the 22*R*- and 22*S*-epimers were quantitatively converted into the 23,24-dinor-triformyloxy-22-aldehyde **[7]** in an excellent yield of 92%.

Subsequent Wittig reaction of the triformyloxy-22-aldehyde **[7]** in benzene with a reagent, methyl (triphenylphosphoranylidene)acetate, led to the formation of the (*E*)-isomer of the 3α,6β,7β-triformyloxy-22-ene methyl ester **[8]**. The resulting reaction product, which consisted essentially of a single component, was purified by passing through a column of silica gel and eluting with a mixture of benzene-EtOAc (95:5, v/v). Alkaline hydrolysis of 3α,6β,7β-triformyloxy-22-ene methyl ester **[8]** with 5% methanolic KOH followed by acidification with 10% H_2SO_4 resulted in the simultaneous hydrolysis of the methyl ester at C-24 and the formyloxy protecting group at the C-3, C-6, and C-7 positions to give the desired (unconjugated) Δ^{22}-β-MCA **[1a]**. Esterification of **[1a]** using *p*-toluenesulfonic acid in methanol yielded the corresponding methyl ester derivative. In the Δ^{22}-β-MCA **[1a]** and its methyl ester, the stereochemical configuration of the Δ^{22}-double bond as an *E*-form was determined on the basis of a large coupling constant (*J*, 15–16 Hz) arising from a doublet signal of the 23-H in the ^{1}H-NMR (13, 22) and of characteristic olefinic carbon signal pairs appearing at ~119 and 155 ppm, respectively assigned to C-23 and C-22, in the ^{13}C-NMR (4, 11).

The unconjugated Δ^{22}-β-MCA **[1a]** was then transformed into the corresponding taurine- and glycine-amidated conjugates. Condensation of the carboxyl group at C-24 in the Δ^{22}-β-MCA **[1a]** with the amino group of taurine (or of glycine methyl ester) was successfully attained by the use of DEPC as a coupling reagent and Et_3N as a catalyst (23). The *N*-acylamidation reaction involves the formation of intermediary acyl phosphates as carboxyl-activated intermediates by the interaction of carboxylic acid with DEPC in the presence of Et_3N and subsequent coupling with amines to give the corresponding amides in one step. The condensation reaction proceeded cleanly and rapidly by simple mixing of the unconjugated Δ^{22}-β-MCA **[1a]**, taurine (or glycine methyl ester), DEPC, and Et_3N in DMF at room temperature for 60 min. After the amidation reaction, sample clean up, crystallization, and/or hydrolysis were effected as follows. Because the free sulfonic acid form of tauro-Δ^{22}-β-MCA **[1b]** is highly hygroscopic, it was converted to the sodium salt by treatment with NaOH and then purified and isolated as a crystalline product by passing through a prepacked Sep-Pac Vac tC_{18} cartridge for reversed-phase solid extraction, eluting with 50% aqueous methanol. On the other hand, the glycine methyl ester form of glyco-Δ^{22}-β-MCA **[1c]** was hydrolyzed with methanolic KOH, followed by acidification with H_2SO_4 to give the free acid form of glyco-Δ^{22}-β-MCA **[1c]** as a crystalline product.

The ^{1}H-NMR spectrum of tauro-Δ^{22}-β-MCA **[1b]** exhibited a triplet signal (*J*, 7 Hz) at 3.03 ppm arising from CH_2S protons, whereas that of glyco-Δ^{22}-β-MCA **[1c]** showed a doublet signal (*J*, 4.1 Hz) at 3.76 ppm and a triplet signal (*J*, 8.9 Hz) at 6.06 ppm, attributable to CH_2N and NH, respectively. The ^{13}C-NMR spectrum of synthetic tauro-Δ^{22}-β-MCA **[1b]** is very compatible with that reported for the natural compound that was isolated from serum of female rats treated with α-naphthylisothiocyanate (4) as well as from bile, small and large intestinal content, and feces of gnotobiotic rats monocontaminated with *E. coli*, *B. longum*, *B. vulgatus*, *C. ramosum*, *P. productus*, or *L. gasseri* (11). Particularly noteworthy NMR features of the amidated Δ^{22}-β-MCA are carbon signals occurring at 36.4 (CH_2N), 51.5 (CH_2S), and 168.9 (C-24) ppm in the tauro-Δ^{22}-β-MCA **[1b]** and at 41.3 (-$\underline{C}H_2COO$-), 166.2 (C-24), and 170.6 (-$CH_2\underline{C}OO$-) ppm in the glyco-Δ^{22}-β-MCA **[1c]**. **Figure 3** shows ESI-NIM mass spectra of unconjugated Δ^{22}-β-MCA acid and its taurine and glycine conjugates (Fig. 3A–C). Each of the spectra is very simple, giving only a $[M\text{-}H]^-$ as the principal fragment ion.

Although the occurrence of Δ^{22}-β-MCA in rat is well established (see introduction), there have been no reports of Δ^{22}-β-MCA occurring in the mouse, a species in which β-MCA is a dominant primary bile acid. β-MCA has been detected in trace amounts in the urine of a cholestatic patient (24) and was also reported to be formed from administered UDCA in another cholestatic patient, based on its appearance in urinary bile acids during UDCA ingestion (25). As yet, Δ^{22}-β-MCA has not been reported to occur in humans. Even when β-MCA was administered to human volunteers, Δ^{22}-β-MCA was not identified as a metabolite (26). When rats ingest UDCA, Δ^{22}-UDCA is a major metabolite formed in the liver and possibly also by the enteric flora (12). Yet, the formation of Δ^{22}-UDCA has not been observed in the biliary bile acids (27), urinary bile

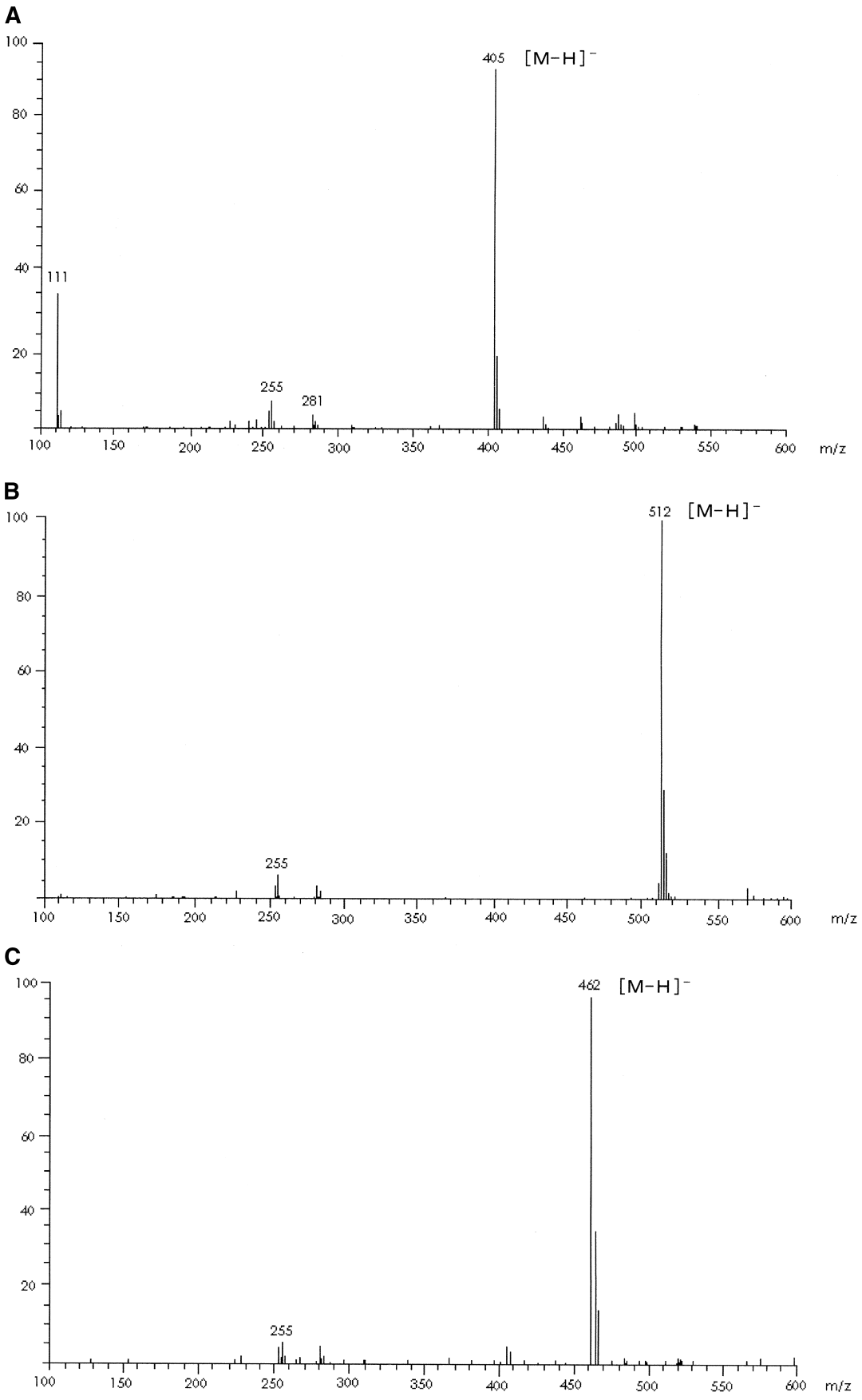

Fig. 3. Electrospray ionization-negative ion mode mass spectra of unconjugated and taurine and glycine conjugates of Δ^{22}-β-MCA [A–C].

acids (25), or liver biopsies (7) of patients with liver disease who have received UDCA as therapy.

The availability of Δ^{22}-β-MCA **[1a]** as well as its taurine **[1b]** and glycine **[1c]** conjugates should facilitate the characterization of its metabolism and physiological properties. Δ^{22}-β-MCA can also be used as a substrate for reductive deuteration or tritiation, thereby forming 22,23-^{2}H-β-MCA or 22,23-^{3}H-β-MCA. The latter should be useful for transport studies (28). The tritium atoms of 22,23-^{3}H label of cholic acid and its principal metabolite deoxycholic acid have been shown to be stable in vivo during enterohepatic cycling in human (29), confirming the view (12) that bacterial desaturation of the side chain of these two bile acids does not occur. The stability of the label of 22,23-^{3}H-β-MCA during enterohepatic cycling is uncertain, because of the ability of the enteric flora to desaturate the side chain of this bile acid (11). Indeed, the availability of 22,23-^{3}H-β-MCA should permit the metabolism of β-MCA by bacteria and by hepatic enzymes to be characterized.

Bile acids with a Δ^{22} side chain occur only rarely in the bile of other vertebrates. In a survey of the biliary bile acids of some 900 vertebrate species (30), only some caviomorph rodents were found to have Δ^{22} bile acid as their dominant biliary bile acid. For example, the mountain paca, a large South American rodent, was found to have 3α-hydroxy-7-oxo-5β-chol-22-en-24-oic acid (31) as its major biliary bile acid.

This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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