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Synthesis, aggregation behavior and cholesterol solubilization studies of 16-*epi*-pythocholic acid (3α,12α,16β-trihydroxy-5β-cholan-24-oic acid)

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ABSTRACT

Synthesis, aggregation behavior and *in vitro* cholesterol solubilization studies of 16-*epi*-pythocholic acid (3α,12α,16β-trihydroxy-5β-cholan-24-oic acid, EPCA) are reported. The synthesis of this unnatural epimer of *pythocholic acid* (3α,12α,16α-trihydroxy-5β-cholan-24-oic acid, PCA) involves a series of simple and selective chemical transformations with an overall yield of 21% starting from readily available cholic acid (CA). The critical micellar concentration (CMC) of 16-*epi*-pythocholate in aqueous media was determined using pyrene as a fluorescent probe. *In vitro* cholesterol solubilization ability was evaluated using anhydrous cholesterol and results were compared with those of other natural di- and trihydroxy bile acids. These studies showed that 16-*epi*-pythocholic acid (16β-hydroxy-deoxycholic acid) behaves similar to cholic acid (CA) and avicholic acid (3α,7α,16α-trihydroxy-5β-cholan-24-oic acid, ACA) in its aggregation behavior and cholesterol dissolution properties.

1. Introduction

There has been considerable interest towards the synthesis of natural and unnatural analogues of bile acids and their derivatives in recent years [1,2]. Recent advances in the field of molecular biology and genetics have greatly accelerated the knowledge related to the role of bile acids and its derivatives in a number of physiologically important processes [3]. The studies on new roles of bile acids as potential ligands for certain nuclear hormone receptors [4,5], and as pheromones [6,7] have emerged as an attractive area in steroid research. The importance of 15- and 16-hydroxy derivatives of chenodeoxycholic acid (CDCA) and 16-hydroxy derivatives deoxycholic acid (DCA) has recently been discussed in the literature [8,9]. In the majority of vertebrates, the hydroxylation of primary bile acids like chenodeoxycholic acid and certain secondary bile acids (deoxycholic acid and lithocholic acid) occurs at an unusual site on the steroid nucleus [10,11]. It has been suggested that such unusual trihydroxy bile acids are formed due to the involvement of hepatic enzymes (in CDCA) or intestinal microbial flora (in secondary bile acids) which mediates dehydroxylation at C-7 followed by absorption of the 7-deoxy bile acids and subsequent hepatic hydroxylation at additional sites.

The presence of 15α- and 15β-hydroxy derivatives have been found to be the major bile acids in certain marsupials [12], whereas, the 16-hydroxy derivatives are major bile acids in snake and avian bile. Kakiyama et al. [13] reported the presence of 15α-hydroxy derivative of lithocholic acid as a major biliary bile acid in the wombat (*Vombatus ursinus*), a common Australian marsupial. Studies on microbial transformation of lithocholic acid (LCA) into 3α,15β-dihydroxy-5β-cholan-24-oic acid by a fungus *Cunninghamella blakesleeana* ST-22 was reported in 1985 [14]. In 2002 Iida et al. published the synthesis of 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid and also its 15β-epimer (3α,7α,15β-trihydroxy-5β-cholan-24-oic acid) as a byproduct upon the synthesis of avicholic acid (3α,7α,16α-trihydroxy-5β-cholan-24-oic acid). It is interesting to note that four years later the same group identified and structurally characterized 3α,7α,15α-trihydroxy-5β-cholan-24-oic acid as a major biliary bile acid in swans, tree ducks and geese as its taurine conjugates [11]. There are several examples where unusual trihydroxy bile acids like 3α,7α,14α- or 3α,7α,16α-trihydroxy-5β-cholan-24-oic acid have been isolated and structurally characterized [15,16]. Chemical synthesis of such unusual bile acids is of considerable interest because the availability of synthetic samples provides useful reference towards a definitive structural determination of the 15- and 16-hydroxy bile acids. Unambiguous proof of many naturally isolated bile acids and their structural characterization requires chemical synthesis and demonstration of identity of the isolated compounds with synthetic ones. The chemical synthesis of these uncommon bile acids also provides a unique opportunity to study the chemical, biologi-

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cal and physicochemical properties of rare and unusual natural bile acids.

The first chemical synthesis, aggregation behavior and cholesterol solubilization studies of pythocholic acid (3α,12α,16α-trihydroxy-5β-cholan-24-oic acid, PCA) a major component in python's bile and 16α-hydroxycholic acid (3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid) a minor component in avian bile was reported from our laboratory [17]. Pythocholic acid (PCA) displayed unusual aggregation behavior and high cholesterol solubilization ability. A biomimetic remote functionalization strategy utilized towards the first chemical synthesis of PCA resulted in 5.5% overall yield [17]. Therefore, we decided to carry out the synthesis of PCA *via* an alternative synthetic route. Final characterization of the product revealed that it is the 16β-epimer of pythocholic acid. Herein we report the synthesis, aggregation properties and preliminary *in vitro* cholesterol dissolution studies of this unnatural epimer of PCA.

2. Experimental

2.1. General methods

All reactions were carried out in oven-dried glassware. TLC was checked on pre-coated glass plates (0.25 mm silica gel with fluorescent UV254) purchased from Aldrich. After elution the plates were developed using the Liebermann-Burchard reagent. Commercial grade solvents were distilled prior to use in column chromatography. Silica gel (100–200 mesh) columns were run under gravity. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on a JEOL Lambda 300 MHz spectrometer in deuterated solvents as indicated with TMS or residual solvent signals as the internal standards. Infrared spectra were recorded on a JASCO-70 FT-IR spectrophotometer, by either making a film of the compounds on a NaCl plate from a chloroform solution or using KBr pellets. ESI-QTOF MS was recorded on a Micromass Q-TOF mass spectrometer. Optical rotations were recorded on a DIP-370 digital polarimeter at 589 nm. Elemental analyses were done on a Carlo Erba Strumentazione CHNS Analyzer-model 1106 and Flash EA 1112. Double distilled water was used in fluorescence and absorption spectral measurements. Fluorescence experiments were performed using a Perkin-Elmer LS-50 B luminescence spectrometer, while absorption spectra were recorded on a Shimadzu UV200 spectrometer.

2.1.1. Methyl 3α,12α-dihydroxy-5β-chol-8(14)-en-24-oate (**2**)

A solution containing cholic acid **1** (3.0 g, 7.34 mmol) and anhydrous $ZnCl_2$ (3.0 g, 22.06 mmol) in acetone (30 mL) was refluxed at 80 °C for 2 h and the solvent was slowly distilled off until TLC showed conversion of the starting material. The solution was then cooled to room temperature and aqueous acetic acid (30 mL of 0.5%) was added. The precipitate was collected by filtration and dried in vacuum. The solid obtained was dissolved in cold methanol (15 mL) containing anhydrous HCl (generated *in situ* by the dropwise addition of AcCl into methanol). After stirring for 12 h at room temperature (~28 °C), the volatiles were removed under reduced pressure. The crude product obtained after aqueous work up was column purified on silica gel using 50–70% ethyl acetate in *n*-hexane to afford **2** in 78% yield as white solid. Mp 84–86 °C. IR, $\bar{\nu}_{max}$: 3420, 2925, 2804, 1740, 1630 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.90 (br s, 1H, 12β-H), 3.66 (s, 4H, 3β-H and $-CO_2C\underline{H}_3$), 2.2–1.2 (br m, steroidal $-C\underline{H}$ and $-C\underline{H}_2$), 1.00 (d, J = 6.0 Hz, 3H, 21-$C\underline{H}_3$), 0.865 (s, 3H, 19-$C\underline{H}_3$), 0.81 (s, 3H, 18-$C\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.74 (C-24), 137.88 (C-8), 127.40 (C-14), 72.16 (C-12), 71.85 (C-3), 51.49 ($CO_2\underline{C}H_3$), 47.19 (C-17), 42.08 (C-5), 36.02 (C-4), 35.63 (C-1), 34.44, 33.75, 31.17, 30.94 (C-23), 30.76 (C-22), 30.51, 27.24, 26.60, 26.37, 26.25, 24.56, 23.77 (C-19), 19.24 (C-21), 17.62 (C-18). $[\alpha]_D^{23}$ +53 (*c* 2.0, EtOH). HRMS: calculated for $C_{25}H_{40}O_4$ + Na: 427.2824; found: 427.2824. Analysis: calculated for $C_{25}H_{40}O_4 \cdot 1/2H_2O$: C 72.6, H 9.99; found: C 72.5, H 9.95.

2.1.2. Methyl 3α,12α-dihydroxy-5β-chol-14-en-24-oate (**3**)

A solution of compound **2** (1.0 g, 2.5 mmol) in chloroform (20 mL) was cooled to −78 °C with dry ice/acetone. A dry stream of hydrogen chloride gas was passed through the solution for 4 h followed by a stream of nitrogen to remove excess hydrogen chloride from the reaction vessel. Then, 0.5 M aqueous sodium hydrogen carbonate (10 mL) was added at low temperature (0 °C) and the mixture was allowed to attain room temperature. The organic layer was separated, washed with water, dried with $MgSO_4$ and evaporation of the volatiles under reduced pressure resulted in yellow solid. Column chromatography on silica gel using 50–75% ethyl acetate in petroleum ether furnished 0.68 g (68%) of **3** as white solid. Mp 92–95 °C. IR, $\bar{\nu}_{max}$: 3420, 2925, 2804, 1735 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 5.29 (d, J = 1.6 Hz, 1H, 15-H), 3.80 (s, 1H, 12β-H), 3.67 (s, 3H, $-CO_2C\underline{H}_3$), 3.65–3.60 (m, 1H, 3β-H), 2.5–1.1 (m, steroidal $-C\underline{H}$ and $-C\underline{H}_2$), 0.95 (d, J = 6.8 Hz, 3H, 21-$C\underline{H}_3$), 0.93 (s, 3H, 19-$C\underline{H}_3$), 0.92 (s, 3H, 18-$C\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.70 (C-24), 151.40 (C-14), 120.10 (C-15), 73.22 (C-3), 71.67 (C-12), 51.68 (C-13), 51.47 ($-CO_2\underline{C}H_3$), 46.91 (C-17), 41.90 (C-5), 36.24 (C-4), 34.87 (C-1), 34.69, 34.18, 33.49, 31.94, 31.06 (C-23), 30.86 (C-2), 30.51 (C-22), 29.20, 26.80, 23.93, 22.83 (C-19), 17.73 (C-21), 16.68 (C-18). $[\alpha]_D^{23}$ +54 (*c* 2.0, EtOH). HRMS: calculated for $C_{25}H_{40}O_4$ + Na: 427.2824; found: 427.2824. Analysis: calculated for $C_{25}H_{40}O_4$: C 74.21, H 9.96; found: C 74.22, H 10.12.

2.1.3. Methyl 3α,12α-diacetoxy-5β-chol-14-en-24-oate (**4**)

To a stirred solution of **3** (160 mg, 0.39 mmol) in pyridine (3 mL), acetic anhydride (1 mL, 10.58 mmol) and 4-dimethylaminopyridine (20 mg, 0.16 mmol) were added and the reaction mixture was stirred at 25 °C for 16 h. It was then neutralized with 1 M HCl (10 mL), extracted with ethyl acetate (3 × 5 mL) and washed with aqueous sodium bicarbonate solution. After drying the organic layer over anhydrous. Na_2SO_4, the volatiles were removed under reduced pressure. Column chromatographic purification of crude product using 15–30% ethyl acetate in *n*-hexane furnished 170 mg (90%) of **4** as a foamy solid. Mp 75–77 °C. IR, $\bar{\nu}_{max}$: 2935, 1735, 1241, 1025 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 5.28 (s, 1H, 15-H), 5.00 (br t, J = 3.0 Hz, 1H, 12β-H), 4.75–4.65 (m, 1H, 3β-H), 3.67 (s, 3H, $-CO_2CH_3$), 2.10 (s, 3H, $C\underline{H}_3COO$-12), 2.00 (s, 3H, $C\underline{H}_3COO$-3), 2.0–1.1 (m, steroidal $-C\underline{H}$ and $-C\underline{H}_2$), 0.96 (s, 3H, 19-$C\underline{H}_3$), 0.91 (s, 3H, 18-$C\underline{H}_3$), 0.81 (d, J = 6.6 Hz, 3H, 21-$C\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.43 (C-24), 170.45 ($CH_3\underline{C}OO$-12), 170.09 ($CH_3\underline{C}OO$-3), 151.63 (C-14), 119.63 (C-15), 75.90 (C-12), 73.94 (C-3), 51.40 ($CO_2\underline{C}H_3$), 49.66 (C-13), 47.65 (C-17), 41.50 (C-5), 34.74, 34.59, 34.35, 33.92, 32.91, 32.42, 31.86, 30.87, 30.74, 29.94, 26.55, 26.48, 26.38, 23.55, 22.57, 21.30 (C-19), 17.76 (C-21), 16.76 (C-18). HRMS: calculated for $C_{29}H_{44}O_6$ + Na: 511.3036: found: 511.3036. Analysis: calculated for $C_{29}H_{44}O_6$: C 71.27, H 9.07; found: C 71.20, H 9.08.

2.1.4. Methyl 3α,12α-diacetoxy-16-keto-5β-chol-14(15)-en-24-oate (**5**)

A mixture of olefin **4** (162 mg, 0.33 mmol), *N*-hydroxysuccinimide (150 mg, 1.30 mmol) and $Na_2Cr_2O_7 \cdot 2H_2O$ (250 mg, 0.83 mmol) was stirred in acetone (8 mL) at 40 °C for 48 h. A saturated solution of solution of Na_2SO_3 (12 mL) was added and the contents were extracted with ethyl acetate (2 × 10 mL). The organic layer was washed with saturated solution of NaCl (15 mL) and dried over anhydrous Na_2SO_4. The crude product obtained after the removal of volatiles was purified by column chromatography using silica gel in 20–30% ethyl acetate in petroleum ether to yield 120 mg (72%) of **5**. Mp 120–121 °C. IR $\bar{\nu}_{max}$: 2933, 1737,

1241, 1025 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 5.82 (d, J = 1.5 Hz, 15-H), 5.08 (t, J = 2.7 Hz, 12β-H), 4.75–4.68 (m, 1H, 3β-H), 2.10 (s, 3H, C$\underline{H}_3$COO-12), 2.00 (s, 3H, C$\underline{H}_3$COO-3), 2.0–1.1 (m, steroidal –C$\underline{H}$ and –C$\underline{H}_2$), 1.28 (S, 3H, 19-C$\underline{H}_3$), 1.06 (d, J = 6.6 Hz, 3H, 21-C$\underline{H}_3$), 1.00 (s, 3H, 18-C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 207.89 (C-16), 184.98 (C-14), 173.99 (C-24), 170.48 ($CH_3\underline{C}OO$-12), 170.04 ($CH_3\underline{C}OO$-3), 126.07 (C-15), 75.49 (C-12), 73.58 (C-3), 55.89 (C-17), 51.45 (–$CO_2\underline{C}H_3$), 49.89 (C-13), 41.25, 36.04, 34.59, 34.33, 32.06, 31.96, 30.87, 28.65, 26.55, 26.10, 25.43, 23.46, 22.58 (C-19), 21.35 ($\underline{C}H_3$COO-12), 21.14 ($\underline{C}H_3$COO-3), 20.48 (C-21), 19.13 (C-18). $[\alpha]_D^{23}$ +115 (c 2.0, EtOH). HRMS: calculated for $C_{29}H_{42}O_7$ + Na: 525.2828; found: 525.2828. Analysis: calculated for $C_{29}H_{42}O_7$: C 69.29, H 8.42; found: C 69.27, H 8.46.

*2.1.5. Methyl 3α,12α-diacetoxy-16-keto-5β-cholan-24-oate (**7**)*

To a solution containing olefin **5** (180 mg, 0.35 mmol) in ethyl acetate (3 mL), 10% Pd–C (12 mg) was added and the vessel was evacuated and flushed with hydrogen. The reaction mixture was stirred under H_2 atmosphere for 1 h. The reaction mixture was filtered using celite over a sintered funnel. The crude product obtained after the removal of volatiles was purified by column chromatography in 20–30% ethyl acetate in petroleum ether to yield 162 mg (90%) of **7** as a white solid. IR $\bar{\nu}_{max}$: 2933, 1737, 1241, 1025 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 5.07 (br s, 1H, 12β-H), 4.77–4.64 (br m, 1H, 3β-H), 3.66 (s, 3H, –CO_2CH_3), 2.10 (s, 3H, C$\underline{H}_3$COO-12), 2.00 (s, 3H, C$\underline{H}_3$COO-3), 2.0–1.1 (m, steroidal –C$\underline{H}$ and –C$\underline{H}_2$), 0.90 (s, 3H, 19-C$\underline{H}_3$), 0.80 (d, J = 6.3 Hz, 3H, 21-C$\underline{H}_3$), 0.72 (s, 3H, 18-C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 220.20 (C-16), 173.79 (C-24), 170.62 ($CH_3\underline{C}OO$-12), 170.07 ($CH_3\underline{C}OO$-3), 78.38 (C-12), 73.84 (C-3), 61.67 (C-17), 51.59 ($CO_2\underline{C}H_3$), 45.88 (C-13), 44.23, 41.81 (C-5), 39.97, 34.18, 33.67, 33.19, 32.34, 32.04, 31.98, 28.18, 26.80, 26.60, 26.35, 25.91, 25.81, 23.0, 21.43 (C-19), 21.40 ($\underline{C}H_3$COO-12), 21.07 ($\underline{C}H_3$COO-3), 19.03 (C-21), 19.00 (C-18). $[\alpha]_D^{23}$ +112 (c 2.0, EtOH). HRMS: calculated for $C_{29}H_{44}O_7$ + Na: 527.2984: found: 527.2982.

*2.1.6. Methyl 3α,12α,16β-triacetoxy-5β-cholan-24-oate (**8**)*

To a stirred solution of **7** (62 mg, 0.12 mmol) in MeOH/THF 1:2 (v/v) (2 mL) at 0 °C was added $CeCl_3 \cdot 7H_2O$ (45 mg, 0.12 mmol) followed by $NaBH_4$ (7 mg, 0.185 mmol) and stirred at rt for 2 h. After aqueous work up the crude product was dried under vacuum and dissolved in pyridine (500 μL). Anhydrous acetic anhydride (500 μL) was added and the reaction mixture was stirred at room temperature. After stirring for 12 h, the reaction mixture was neutralized with 1 M HCl and extracted using CH_2Cl_2 (2 × 4 mL). The crude product was purified by column chromatography over silica using 15–20% ethyl acetate in *n*-hexane to yield 49 mg (72%) of **8** as gummy solid. IR $\bar{\nu}_{max}$: 2940, 1735 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 5.08 (q, J = 7.5 Hz, 1H, 16α-H), 4.77 (br s, 1H, 12β-H), 4.75–4.66 (m, 1H, 3β-H), 3.66 (s, 3H, –CO_2CH_3), 2.12 (s, 3H, C$\underline{H}_3$COO-16), 2.05 (s, 6H, C$\underline{H}_3$COO-12 and C$\underline{H}_3$COO-3), 2.0–1.1 (m, steroidal –C$\underline{H}$ and –C$\underline{H}_2$), 1.04 (s, 3H, 19-C$\underline{H}_3$), 0.90 (t, 6H, 21-C$\underline{H}_3$ and 18-C$\underline{H}_3$). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 174.38 (C-24), 171.21 ($CH_3\underline{C}OO$-16), 170.98 ($CH_3\underline{C}OO$-12), 170.91 ($CH_3\underline{C}OO$-3), 78.45 (C-12), 76.61 (C-16), 74.60 (C-3), 54.48 (C-17), 51.96 ($CO_2\underline{C}H_3$), 49.28 (C-15), 44.68 (C-14), 42.27 (C-13), 34.72 (C-1), 34.61, 33.59, 32.96, 32.87, 32.64, 29.21, 27.54, 27.42, 27.35, 27.31, 26.83, 23.41, 22.09, 21.88 (C-19), 20.50 (C-21), 20.05 (C-18). $[\alpha]_D^{23}$ +132 (c 0.5, EtOH). HRMS: calculated for $C_{31}H_{48}O_8$ + Na: 571.3247: found: 571.3247.

*2.1.7. 3α,12α,16β-Trihydroxy-5β-cholan-24-oic acid (**9**)*

A solution of **8** (45 mg, 0.08 mmol) in 5% KOH–MeOH (2.5 mL) was stirred at 65 °C. After stirring for 16 h the volatiles were removed under reduced pressure. The residue was dissolved in ice-cold water (5 mL) and neutralized with 1 M HCl solution. The precipitate was filtered and dried to yield in 31 mg (92%) of **9** as a white solid. Mp: 192–193 °C. IR $\bar{\nu}_{max}$: 3410, 2939, 1705 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 4.15 (m, 1H, 16α-H), 3.66–3.55 (m, 2H, 12β-H + 3β-H), 1.07 (d, J = 6.5 Hz, 3H, 21-CH_3), 0.96 (s, 3H, 19-CH_3), 0.90 (s, 3H, 18-CH_3). ^1H NMR (acetone-d_6, 400 MHz) δ: 4.06 (q, J = 7.5 Hz, 1H, 16α-H), 3.57 (br s, 1H, 12β-H), 3.55–3.45 (m, 1H, 3β-H), 2.0–1.1 (m, steroidal –C$\underline{H}$ and –C$\underline{H}_2$), 1.04 (d, J = 7.2 Hz, 3H, 21-C$\underline{H}_3$), 1.00 (s, 3H, 19-C$\underline{H}_3$), 0.91 (s, 3H, 18-C$\underline{H}_3$). ^{13}C NMR (acetone-d_6, 100 MHz) δ: 174.71 (C-24), 76.47 (C-16), 73.34 (C-12), 71.47 (C-3), 61.17 (C-17), 50.42 (C-14), 46.32 (C-13), 43.09 (C-5), 38.13 (C-8), 37.17 (C-4), 35.92 (C-1), 35.59 (C-10), 34.72 (C-15), 34.35 (C-23), 33.45 (C-22), 27.98, 26.97, 26.53, 26.05, 25.72, 25.00, 23.53, 22.73 (C-19), 20.92, 20.62 (C-21), 19.53 (C-18). HRMS: calculated for $C_{24}H_{40}O_5$ + Na: 431.2773: found: 431.2772. Analysis: calculated for $C_{24}H_{40}O_5$: C 70.55, H 9.87; found: C 70.56, H 10.0.

2.2. Determination of critical micellar concentration (CMC)

Pyrene (Fluka, 99%, recrystallized from hot EtOH) was added to aqueous TRIS buffer (0.1 M, pH 9). The mixture was sonicated for ½ h and filtered through a 0.4 μm filter. Samples were made by diluting the stock solutions of bile acids with the saturated pyrene solution. The concentration of pyrene was ~0.5 μM. Samples were excited at 336 nm and the I3/I1 ratios were calculated by taking the ratio of maximum peak intensity at 384 nm to that at 373 nm. All spectra were recorded on a Perkin-Elmer LS 50B luminescence spectrometer.

2.3. In vitro cholesterol solubilization studies

A commercial 'cholesterol reagent set' based on enzymatic method (using cholesterol esterase, cholesterol oxidase and peroxidase) was used for the determination of the amount of cholesterol solubilized by bile salts [17]. Solid anhydrous cholesterol was added to bile salt solutions prepared by dissolving bile acids in carbonate buffer of pH 10. The mixtures were stirred at 37 °C for one day. These mixtures were filtered using 0.2 μm membrane filter. The filtrate (10 μL) was added to 1 mL reagent, and then it was incubated at 37 °C for 10 min. Absorbance was checked at 510 nm. The absorbance of the standard cholesterol solution (200 mg %, 5.2 mM) at 510 nm was 0.364. Conc. of cholesterol (in mg %) = [absorbance of the sample/absorbance of the standard] × 200 conc. of cholesterol (in mM) = [conc. of cholesterol (in mg %) × 1000]/[386.7 × 100].

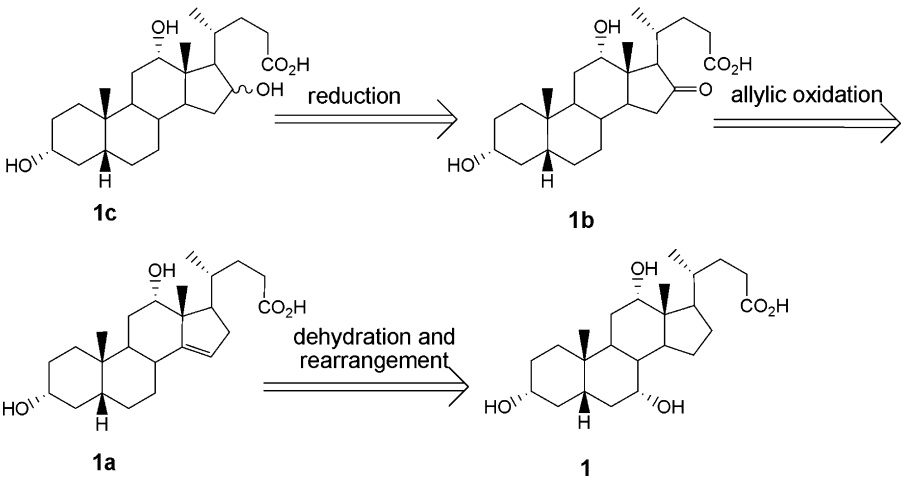
3. Results and discussions

3.1. Retrosynthetic analysis

We envisioned intermediate **1a** (Scheme 1) as a suitable precursor towards the synthesis of 16-hydroxy bile acid. Selective chemical transformation of **1a** into 16-keto derivative utilizing allylic oxidation followed by stereoselective reduction will result in the formation of 16α/β-hydroxy bile acid. During the course of our investigation, we noticed that the intermediate could easily be prepared starting from cholic acid **1**. These intermediates have been reported in the literature during the synthesis of cephalostatin [18]. Careful analysis of the structure and synthetic procedure indicated that there are two modified bile acid units where one of the intermediates used was olefin **1a**.

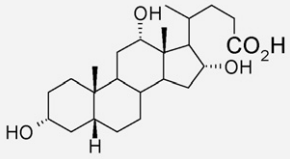
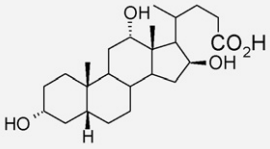
3.2. Synthesis

Selective dehydration of cholic acid **1** was performed using anhydrous $ZnCl_2$ in refluxing acetone to furnish the olefin and the crude product was stirred in methanol containing anhydrous HCl to yield the methyl ester **2** [18]. Compound **2** was subjected to isomerization using dry HCl in chloroform at −78 °C to yield the desired



Scheme 1. Retrosynthetic approach.

Table 1
Comparison of ^{1}H NMR (in $CDCl_3$, 300 MHz) chemical shift values (in ppm) of pythocholic acid [Ref. [17]] and its 16β-epimer **9**.

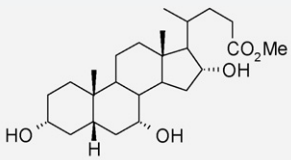
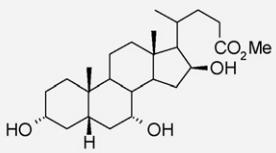
			$\Delta\delta$
18-H	0.70 (s)	0.90 (s)	0.20
19-H	0.89 (s)	0.96 (s)	0.07
21-H	1.00 (d)	1.07 (d)	0.07
3β-H	3.71–3.61 (br m)	3.66–3.55 (merged with 12β-H)	–
12β-H	3.88 (br s)	3.66–3.55 (merged with 3β-H)	–
16β/α-H	4.0 (t, 6.6 Hz)	4.15 (br m)	0.15

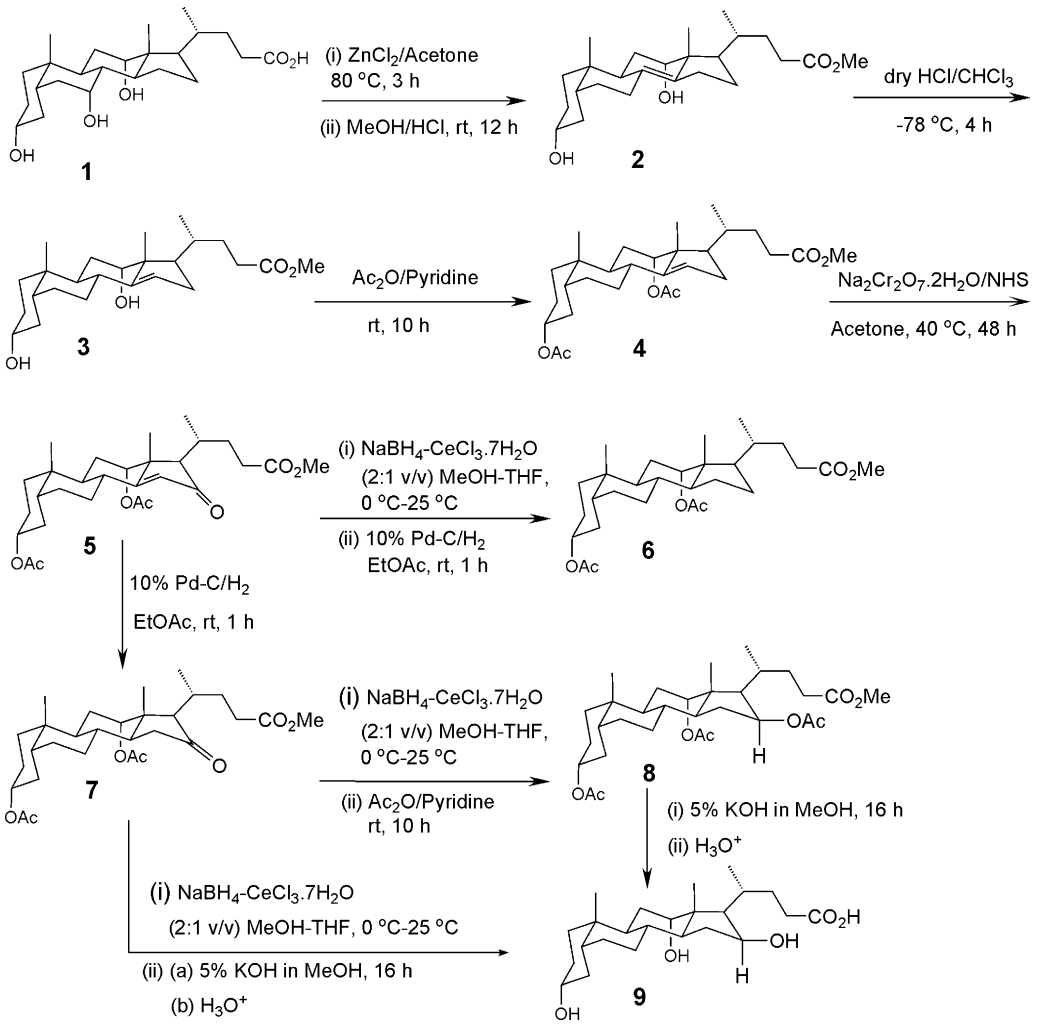
precursor **3** [18]. Our next aim was to carry out selective functionalization at C-16. In order to achieve that, the olefin **3** was converted into its diacetyl derivative **4** under pyridine-acetic anhydride condition. The selective oxidation of **4** was obtained using a mixture sodium dichromate dihydrate and *N*-hydroxysuccinimide (NHS) in acetone at 40 °C for 48 h to obtain the enone **5** [19]. Compound **5** upon reduction using $NaBH_4$–$CeCl_3{\cdot}7H_2O$ in methanol followed by hydrogenation resulted in dehydroxylation and furnished the undesired diacetyl derivative of deoxycholic acid **6**. The structure of **6** was confirmed by comparing the spectral data of authentic diacetyl derivative of methyl deoxycholate prepared from commercial deoxycholic acid.

In order to overcome this problem the enone **5** was selectively reduced using Pd–C/H_2 condition to yield the 16-ketosteroid **7**. The ketosteroid **7** was then subjected for reduction under $NaBH_4$/MeOH followed by hydrolysis using 5% KOH in methanol. This procedure gave the 16-hydroxy bile acid **9** with an overall yield of 21%. On the other hand $NaBH_4$ reduction of the 16-ketosteroid **7**, followed by peracetylation in pyridine/Ac_2O furnished the triacetyl derivative **8**.

After successful synthesis we compared the NMR spectral data of compound **9** with that of pythocholic acid. Interestingly ^{1}H NMR spectral data of **9** showed a considerable difference in the chemical shift values of angular methyl protons and in the splitting pattern of 16-H compared to that of pythocholic acid [17]. Careful analysis of spectral data with pythocholic acid revealed a significant difference in the chemical shift values (0.20 ppm) of angular methyl 18-CH_3. However, the difference in the chemical shift values of other angular methyl protons (19-CH_3 and 21-CH_3) was much smaller. A similar difference was also observed in the chemical shift values of 16-H (0.15 ppm) compared to other β-protons (Tables 1 and 2). It clearly suggests that the stereochemistry at C_{16} might be different. A detailed analysis of *avicholic acid* and its 16β-

Table 2
Comparison of ^{1}H NMR (in $CDCl_3$) chemical shift values (in ppm) of avicholic acid and its 16β-epimer [Ref. [8]].

			$\Delta\delta$
18-H	0.67 (s)	0.87 (s)	0.20
19-H	0.89 (s)	0.91 (s)	0.02
21-H	0.95 (d)	1.07 (d)	0.02
3β-H	3.43 (br m)	3.46	0.03
7β-H	3.84 (br s)	3.88	0.04
16β/α-H	4.01 (t, 6.6 Hz)	4.50 (br m)	0.49

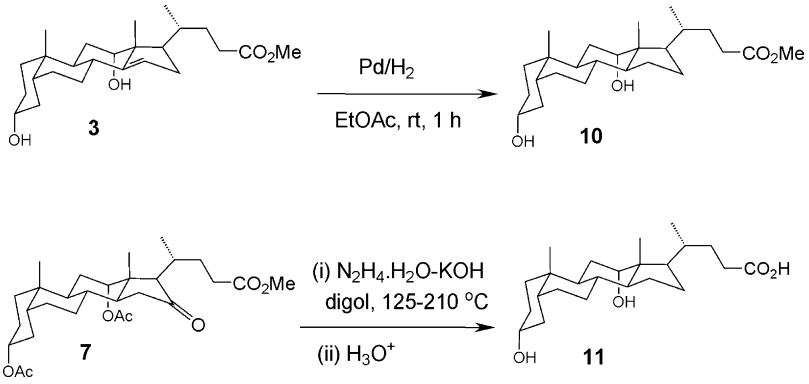


Scheme 2. Synthesis of 16-*epi*-pythocholic acid from **1**.

epimer reported in the literature also revealed similar difference in the chemical shift values [8]. On the basis of the above results conclude that compound **9** is the 16β-epimer of pythocholic acid.

Since the β-face of the steroid is sterically more hindered, the hydride attack occurs predominantly from the α-face. Spartan' 06 energy minimized structure of compound **7** also showed that the α-face of the steroidal skeleton is sterically less hindered (Fig. 1). Therefore, the reduction of 16-ketosteroid **7** results in the formation of 3α,12α,16β-trihydroxy-5β-cholan-24-oic acid **9**, an unnatural epimer of pythocholic acid.

In order to ensure that the steroidal skeleton remained unchanged during the course of our synthesis, some of the intermediates were converted into known bile acid derivatives (Scheme 2). The olefin **3** upon hydrogenation furnished the methyl ester of deoxycholic acid **10**. Wolf-Kishner reduction of ketone **8** resulted in the formation of deoxycholic acid **11** [20]. The structure of compounds **10**, **11** (Scheme 2) and **6** (Scheme 3) was confirmed by comparing the spectral data of standard derivatives obtained from deoxycholic acid.



Scheme 3. Conversion of intermediates into known bile acid derivatives.

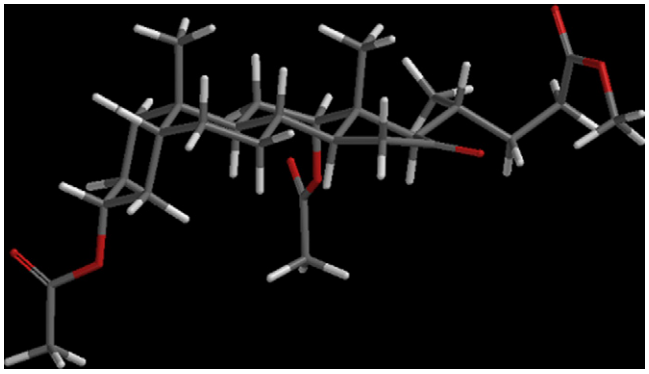


Fig. 1. Spartan' 06 energy minimized structure of **7**.

3.3. Determination of critical micellar concentration (CMC)

Being facially amphiphilic, bile salts show unique aggregation behavior compared to conventional surfactants. Bile salts carry extensive hydrophobic segments, which are responsible for reduced contact with water. Bile salts undergo rapid, dynamic association–dissociation equilibrium to form self-aggregates or micelles as the total concentration of the bile salt are increased [21]. The aggregation properties of bile salts have been studied extensively by various methods and this property of 16-*epi*-pythocholate in aqueous medium was studied using pyrene as the fluorescent probe. The ratio of the two vibronic bands (I_3/I_1) in the fluorescence spectrum is indicative of the polarity experienced by the probe solubilized in the micellar aggregates [22]. Using this technique we have measured the CMC values of cholate, deoxycholate, and pythocholate. The CMC of 16-*epi*-pythocholate was 14 mM, whereas pythocholate and cholate showed CMC of 3–4 mM and 15 mM respectively under similar experimental conditions (Fig. 2). It is interesting to note that pythocholate shows very low CMC [17], whereas its epimer behaves similar to other trihydroxy bile acids. These results are consistent with the hypothesis that aggregation behavior of bile salts depends on the number, position and the stereochemistry of hydroxyl groups [23].

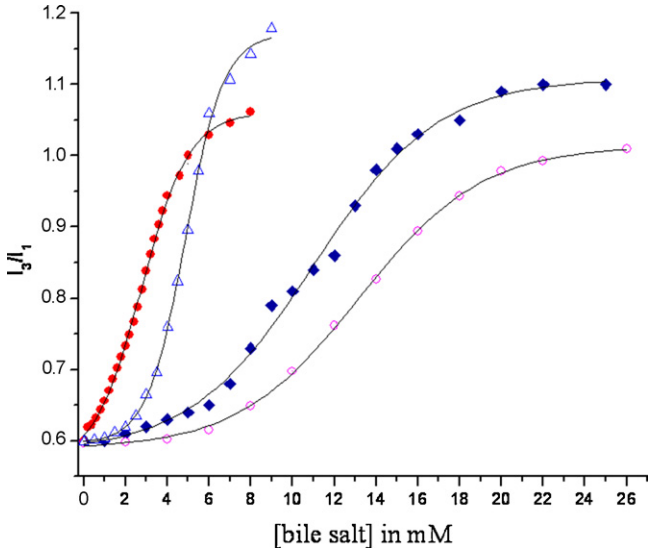


Fig. 2. The ratio of vibronic bands (I_3/I_1) of pyrene fluorescence as a function of bile salt concentration at pH 9.0 (TRIS buffer) at 25 °C; (●) PCA; (△) DCA; (♦) 16-EPCA; (○) CA.

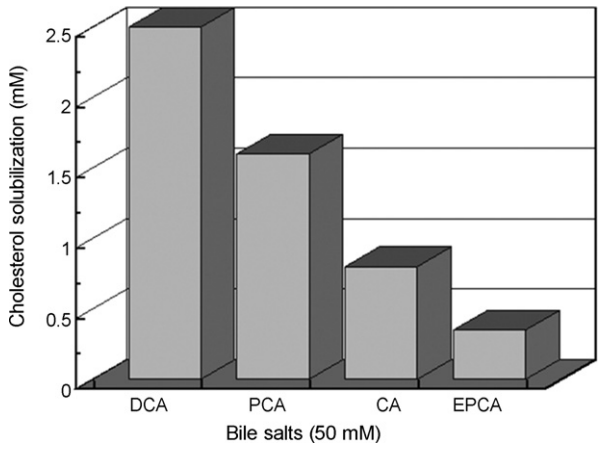


Fig. 3. Solubilization of cholesterol by bile salts (50 mM bile salt, pH 10.0 at 37 °C).

3.4. In vitro cholesterol solubilization studies

Mixed micelle formation leading to solubilization of lipolysis products (fatty acids and monoglycerides) is one of the significant properties of bile salts. Bile salt micelles increase the solubility of cholesterol (aqueous solubility is 1 nM) by more than a million fold. The solubility of cholesterol is better in dihydroxy bile salts compared to trihydroxy bile salts. *In vitro* cholesterol solubilization study of *epi*-pythocholate was evaluated using anhydrous cholesterol with bile salt solution in carbonate-bicarbonate buffer (pH 10.0) Maximum aqueous solubility of cholesterol was found to be much less with *epi*-pythocholate (0.35 mM, bile salt: cholesterol ~114:1) as compared to cholic acid (0.8 mM, bile salt: cholesterol ~63:1) (Fig. 3).

4. Conclusions

In summary, we have developed a synthetic route for the chemical synthesis of 16-*epi*-pythocholic acid, an unnatural analogue of pythocholic acid starting from readily available cholic acid. The aggregation behavior in aqueous media and preliminary cholesterol dissolution studies revealed high CMC values and low cholesterol solubilization ability of 16-*epi*-pythocholate compared to pythocholate. Synthesis and characterization of unusual bile acids will provide an easy access towards the identification and characterization of bile acids from biological sources.

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