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First Chemical Synthesis, Aggregation Behavior and Cholesterol Solubilization Properties of Pythocholic Acid and 16α-Hydroxycholic Acid

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The first chemical synthesis of pythocholic acid, a major component of python's bile, and 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid (16α-hydroxycholic acid), a minor component in Shoebill stork's bile, was achieved starting from readily available cholic acid in overall yields of 5% and 5.5%, respectively, through a common intermediate. A biomimetic template-directed remote functionalization strategy was utilized to selectively functionalize C-16 of the steroid skeleton. This synthesis involves a series of regio and chemoselective transformations. Pythocholic acid showed unusually low critical micellar concentration (CMC) with high cholesterol solubilization ability.

Introduction

Bile acid science with a history of more than a century and continuing importance in biology and medicine,[1] has gained considerable attention in supramolecular and materials chemistry in recent years. Bile is produced by all vertebrates, and bile salts are one of the most important physiological constituents. Structurally modified bile acids have pharmacological potential to act as carriers of liver-specific drugs, absorption enhancers and as cholesterol lowering agents.[1] Bile salts are natural biosurfactants which act as solubilizer and emulsifier for cholesterol, lipids and proteins in the intestine. They form mixed micelles with cholesterol/lipids/fats in the intestine to enable fat digestion and absorption through the intestinal wall.[1,2]

Bile acids in vertebrates differ mainly in three respects: (i) the side-chain structure, (ii) the distribution of the number, position and stereochemistry of the hydroxyl groups on the steroid nucleus and (iii) the fusion of A/B rings (cis or trans).[3] In contrast to most of the small molecules found in vertebrates, bile acid structures are strikingly diverse.[1,3]

Recent literature reports show that bile acids act as potential ligands for nuclear hormone receptors like farnesoid X receptor (FXR). There is a growing body of evidence that bile salts are involved in the control of high-density lipoprotein (HDL) and low density lipoprotein (LDL) metabolism.[4a–4c] Bile acids secreted by male sea lamphrey act as sex pheromones.[4b]

The most abundant bile salts in humans are cholate, chenodeoxycholate and deoxycholate, and they are normally conjugated with glycine (75%) and taurine (25%). The 15-and 16-hydroxy derivatives of chenodeoxycholic acid and deoxycholic acid are of sustained interest in synthetic, biological, physiological and metabolic studies because they are major bile acids in some vertebrates.[3] An unusual 16α-hydroxy bile acid was reported and structurally identified many decades ago from the family of snakes *Biodae*.[5] It was named *Pythocholic acid* **1** (Figure 1) to signify that this class was a major component in python's bile. Certain primitive snakes (pythons and boas) including *Cylindrophis* all have pythocholic acid (3α,12α,16α-trihydroxy-5β-cholan-24-oic acid) in their bile, with the amount varying from 30% to 90% of the total bile acid. The biosynthesis of pythocholic acid in pythons was reported in 1960 by Bergström et al.[6] Using extensive labelling studies this group concluded that deoxycholic acid undergoes 16α-hydroxylation by the action of a 16α-hydroxylase enzyme. Over the last five decades there has been no work on a chemical synthesis of pythocholic acid **1**. An attempt to obtain pythocholic acid using ferro-ascorbate system resulted in the formation of 15-hydroxy bile acid.[7] Thus the synthesis of pythocholic acid and the study of its cholanological properties remained unexplored. Hagey et al. recently reported the existence of 3α,7α,12α,16α-tetrahydroxy-5β-cholan-24-oic acid (16α-hydroxycholic acid, **2**) which is a minor component (1.6%) in Shoebill stork's bile.[8] No chemical synthesis has been reported in the literature for the tetrahydroxy bile acid **2** as well.

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Supporting information for this article is available on the WWW under http://www.eurjoc.org or from the author.

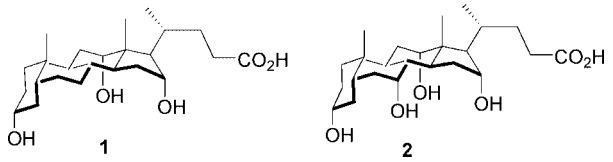


Figure 1. Pythocholic acid (**1**) and 16α-hydroxycholic acid (**2**).

Our interest in the synthesis of uncommon bile acids,[9] led us to explore a simple synthesis of both **1** and **2**. We now report the first chemical synthesis of both these bile acids via a common intermediate involving a series of regio and chemoselective transformations. Some cholanological (the term "cholanology" refers to the science of bile acids[1b]) properties of these two bile acids are also reported, and compared with other more common bile acids.

Results and Discussion

We adopted a template-directed biomimetic remote functionalization strategy to control the chemical selectivity.[10] The methyl ester of cholic acid **4** was selectively acetylated at the 3-and 12-positions using acetic anhydride and anhydrous potassium acetate in refluxing toluene.[11] Esterification of **5** with 3-iodobenzoyl chloride resulted in **6**.[12] It was crystallized using ethyl acetate in petroleum ether (3:1 v/v) and characterized by spectroscopy and single-crystal X-ray technique (Figure 2, a).

It is interesting to note that compound **6** is a *hydrophobic* bile acid derivative. Supramolecular assemblies of bile acids are generally directed by hydrophobic interactions *and* hydrogen bonding.[13] A detailed analysis of the packing of the molecules in the crystal revealed the existence of C–iodine···Ar interactions (Figure 2, b). This type of C–halogen···Ar interactions is well documented in the literature.[14] A variety of proteins and organic compounds have been reported with these types of interactions. In a majority of cases the halogen is either fluorine or chlorine, but there are a few examples with iodine also.[14] C–halogen···Ar interactions are favorable when the distance between halogen and the interacting carbon/centroid of a π cloud is less than or equal to the sum of van der Waals radii (ca. 3.68 Å for C–I). A distance of 3.65 Å was observed between the iodine and the centroid of aromatic ring with an angle (∠C–I··· centroid of the aromatic ring) of 139° (Figure 2, b). An interesting zig-zag arrangement of 3-iodoaryl group can be clearly observed when the steroidal skeleton is removed (Figure 2, c) (Scheme 1).

Photochemical reaction of template **6** in dichloromethane using iodobenzene dichloride (3.0 equiv.) in the presence of *t*BuOH (0.3 M) selectively chlorinated C-17. Chlorination was not very facile (50% yield) presumably because of the deactivating effect of the 12-acetoxy group.[10b] Dehydrochlorination in refluxing pyridine followed by hydroboration–oxidation (BH_3·Me_2S in THF) of the resulting olefin **8** furnished the pentahydroxy steroid **9**. Our next task was to selectively oxidize the side chain primary hydroxyl group in the presence of four secondary hydroxyl groups. A biphasic reaction using TEMPO-mediated *N*-chlorosuccinimide oxidation with $BnEt_3N^+Cl^-$ as a phase-transfer catalyst was unsuccessful.[15] Eventually, TEMPO-mediated oxidation using CuCl in DMF under an oxygen atmo-

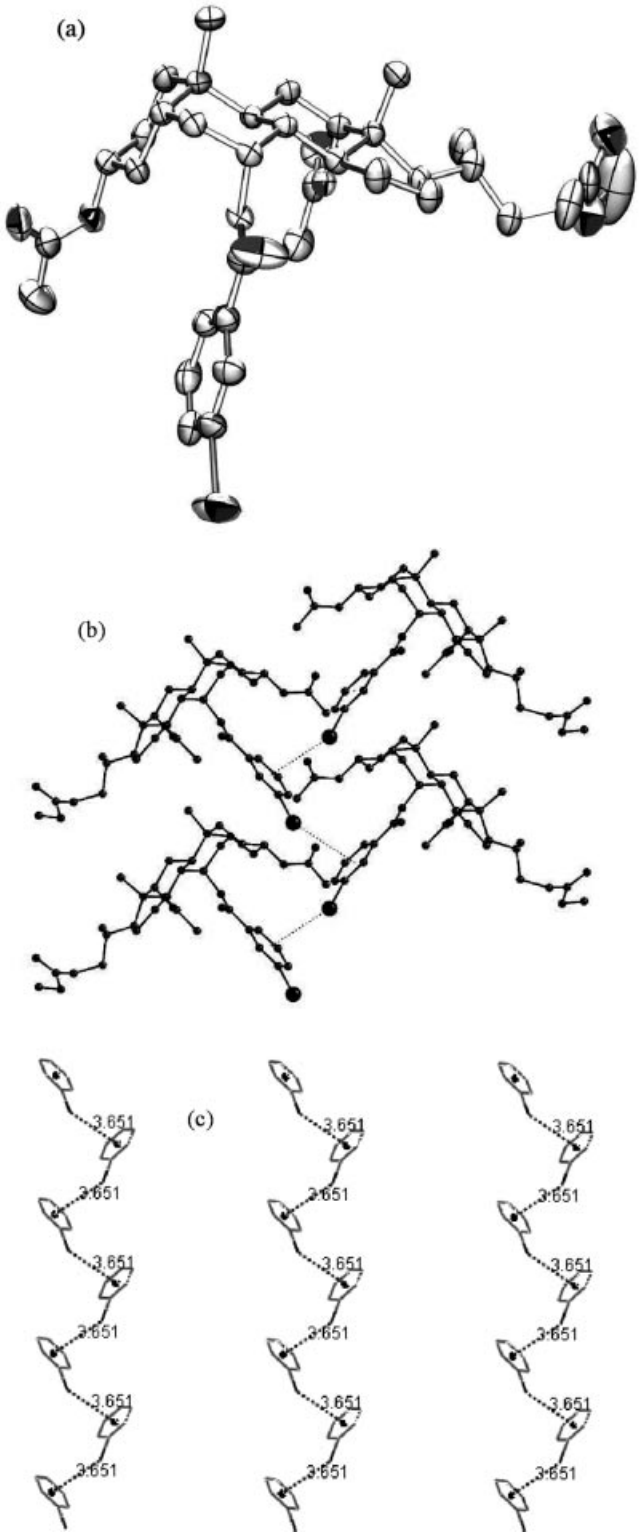
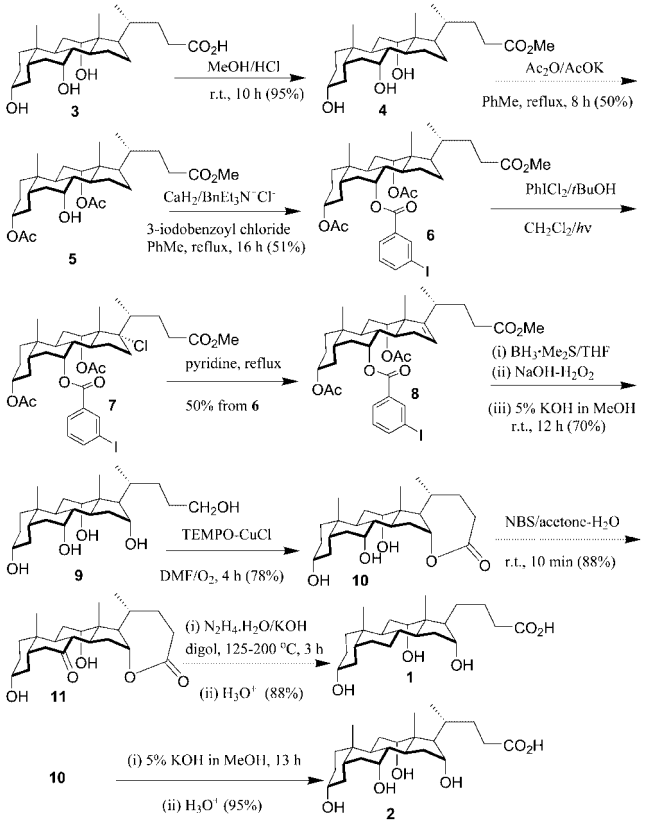


Figure 2. (a) ORTEP diagram of single-crystal X-ray structure; (b) GS view of C–iodine···Ar interactions (c) Packing pattern of 3-iodoaryl moiety of compound **6** using mercury 1.4.1.



Scheme 1. Synthesis of **1** and **2** from cholic acid.

sphere[16] led to the selective oxidation of the primary alcohol and resulted in the formation of ε-lactone **10**.

It is known in the literature that 16α-hydroxy bile acids undergo facile latconization.[5] This transformation was observed in several instances – the high resolution mass spectra of both bile acids showed *m*/*z* peaks of lactones with significant intensities.

The selective oxidation of the 7α-**OH** group of lactone **10** without affecting the other two secondary hydroxyl groups was readily achieved using *N*-bromosuccinimide in 25:1 (v/v) acetone/water.[17] 7-ketolactone **11** was successfully reduced using the Huang–Minlon modification of the Wolf–Kishner reduction[18] to furnish pythocholic acid **1** in an overall yield of 5% from cholic acid. Along with pythocholic acid, pythocholic lactone **12** was also isolated as a minor product and characterized by spectral techniques. On the other hand, the treatment of lactone **10** with 5% KOH in MeOH and acidic work up furnished tetrahydroxy bile acid **2** in overall yield of 5.5% from cholic acid. In order to assign the C-16 signal in ^{13}C NMR we carried out 2D-NMR studies on compound **2** using [D_6]DMSO/$CDCl_3$ (1:9, v/v) (see electronic supporting information). Interestingly, in many steroidal and other molecules with a significant amount of hydrocarbon units one often finds fewer signals than the number of carbons due to "accidental degeneracy of ^{13}C signals" which is common for steroids and triterpenes. Heteronuclear single quantum correlation (HSQC) experiments suggest that the signal from C-16 is more deshielded (δ = 76 ppm) compared to the other C–O carbon atoms.

Since the methyl ester peracetate of compound **2** has been reported in the literature,[8] synthetic **2** was converted into the same derivative **13**. Although the observed chemical shifts of the angular methyl groups of the synthetic sample are consistent with the predicted values, they differ from the data reported in the literature for the biological sample.[8] We can now confirm that our data are correct (see electronic supporting information) since compound **13** prepared from the biological sample was present only as a minor product with other impurities in that sample (Figure 3).[19]

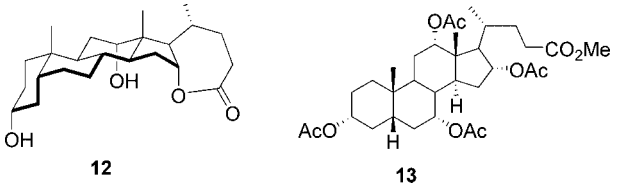


Figure 3. Pythocholic lactone and methyl 3α,7α,12α,16α-tetracetoxy-5β-cholan-24-oate.

The aggregation properties of bile salts have been studied extensively by various methods and this property of pythocholate and 16α-hydroxycholate in aqueous medium were studied using pyrene as a 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 micellar aggregates.[20] Using this technique we have measured the CMC values of cholate, deoxycholate, chenodeoxycholate (dihydroxy bile salts) and pythocholate. The CMC of pythocholate was 3 mM where as those of deoxycholate, chenodeoxycholate, cholate and 16α-hydroxycholate were 5, 5, 13.5 and 14 mM, respectively, under identical experimental conditions (Figure 4). It is interesting to note that among the trihydroxy bile acids the aggregation behavior of pythocholate is similar to deoxycholate and differs considerably from other trihydroxy bile salts (cholate and avicholate). It thus appears that the 16-hydroxy group does not significantly affect the aggregation/cholesterol solubilization *in the absence* of the 7-OH group.

Cholesterol solubilization is another significant cholanological property of bile salts,[21] and this property of pythocholate and tetrahydroxy bile salts was also evaluated in a preliminary experiment using only bile salts. For this study, anhydrous cholesterol was stirred with bile salt solution in carbonate–hydrogen carbonate buffer (pH 10.0) for 24 h at 37 °C. After filtration, the solubilized cholesterol was quantified using a commercially available enzymatic assay. The maximum aqueous solubility of cholesterol was found to be much higher with pythocholate (1.8 mM, bile salt:cholesterol ≈ 28:1) than cholate (0.8 mM, bile salt: cholesterol ca. 63:1) and 16α-hydroxycholate (0.7 mM, bile salt:cholesterol ca. 70:1). This solubilization ability of pythocholate and the 16α-hydroxycholate are consistent with their CMC values.

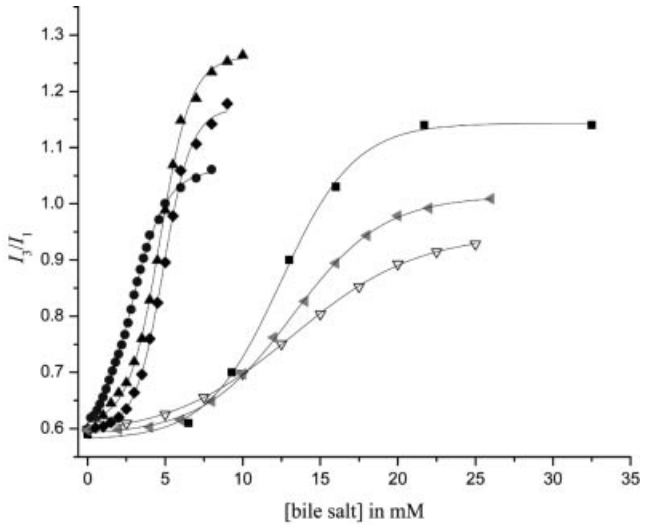


Figure 4. The ratio of vibronic bands (I_3/I_1) of pyrene fluorescence as a function of bile salt concentration. Concentration of pyrene was ca.1 μM in tris buffer at pH 9. Pythocholate (black circles), deoxycholate (black triangles), chenodeoxycholate (black diamonds), cholate (open triangles), 16α-hydroxycholate (grey triangles), avicholate (black squares).

Conclusions

We have developed a synthetic route for chemical synthesis of pythocholic acid and 16α-hydroxycholic acid. To the best of our knowledge this is the first chemical synthesis of both the bile acids. Unusual aggregation behavior and high cholesterol solubilization ability of pythocholic acid suggest that the number, position and distribution of hydroxyl groups have remarkable effect on the physicochemical properties of bile acids. It will be interesting to physicochemically characterize other rare bile acids in order to understand the structure–property landscape.

Experimental Section

General Methods: All reactions were carried out in oven-dried glassware. TLC was checked on pre-coated plates (0.25 mm silica gel with fluorescent UV_{254}) obtained from Aldrich. After elution the plates were developed using Liberman–Buchard reagent. Commercial grade solvents were distilled prior to use in column chromatography. Silica gel (100–200 mesh) columns were run under gravity. Cholic acid (Fluka, >99%), 3-iodobenzoic acid (Fluka, >98%), TEMPO (Aldrich, 98%), $BnEt_3N^+Cl^-$ (Aldrich, 99%), *N*-bromosuccinimide (Aldrich, 99%), $BH_3{\cdot}Me_2S$ (Fluka, ca. 95% in dimethyl sulfide), CuCl (S. D. fine, 96%) and CaH_2 (Aldrich, 95%) were obtained from commercial sources. Iodobenzene dichloride was prepared from iodobenzene (Lancaster, 98%) using a known procedure.[22] ^1H and ^{13}C NMR was recorded on a JEOL 300 MHz spectrometer. Infrared spectra were recorded on a JASCO spectrophotometer, either by 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 micro Mass Spectrometer. CCDC-617607 (for **6**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Methyl 3α,7α,12α-Trihydroxy-5β-cholan-24-oate (4): A water-free solution of HCl in methanol was generated by adding CH_3COCl (1 mL, 14.5 mmol) to methanol (50 mL) at 0 °C. Cholic acid (10.0 g, 24.5 mmol) was added and the mixture was stirred at room temperature for 10 h. After removal of all volatile components the residue was dissolved in EtOAc (100 mL). It was washed with satd. aq. $NaHCO_3$ (2 × 30 mL) and water (2 × 25 mL). The organic layer was separated, dried with anhyd. Na_2SO_4, filtered and volatile substances were removed under reduced pressure. The crude product was recrystallized from hot methanol (25 mL) to afford 9.9 g (95%) of compound **4**; m.p. 153–155 °C (ref.[23] m.p. 155–156 °C). ^1H NMR (300 MHz, $CDCl_3$): δ = 3.97 (br. s, 1 H, 12β-H), 3.85 (br. d, J = 2.4 Hz, 7β-H), 3.66 (s, 3 H, CO_2Me), 3.49–3.39 (br. m, 1 H, 3β-H), 2.43–1.08 (m, steroidal CH_2), 0.98 (d, J = 6 Hz, 3 H, 21-H), 0.89 (s, 3 H, 19-H), 0.68 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.8, 73.0, 71.9, 68.5, 51.5, 47.0, 46.5, 41.7, 41.5, 39.6, 39.5, 35.2, 34.7, 31.1, 30.9, 30.4, 28.2, 26.4, 23.2, 22.5, 17.3, 12.5 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3396, 1737 cm^{-1}.

Methyl 3α,12α-Diacetoxy-7α-hydroxy-5β-cholan-24-oate (5): Acetic anhydride (3.75 mL, 39.7 mmol) and anhyd. potassium acetate (5.0 g, 50.9 mmol) were added to a stirred solution of compound **4** (5.0 g, 11.8 mmol) in dry toluene (40 mL). The mixture was refluxed for 8 h and quenched with water (20 mL). After washing the organic layer with water (20 mL) and drying over anhyd. Na_2SO_4, the volatile components were removed under reduced pressure. The crude product was purified on a silica column (3 × 20 cm) using 3–5% ethyl acetate in chloroform to afford 3.0 g (50%) of compound **5**, which was recrystallized using 10% ethyl acetate in petroleum ether; m.p. 142–144 °C (ref.[24] m.p. 143–145 °C). ^1H NMR (300 MHz, $CDCl_3$): δ = 5.09 (br. s, 1 H, 12β-H) ppm. 4.56 (br. m, 1 H, 3β-H), 3.88 (br. s, 1 H, 7β-H), 3.66 (s, 3 H, CO_2Me), 2.11 (s, 3 H, 12-OAc), 2.06 (s, 3 H, 3-OAc), 1.10–2.33 (m, steroidal H), 0.89 (s, 3 H, 19-H), 0.81 (d, J = 6.6 Hz, 3 H, 21-H), 0.74 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.5, 170.6, 170.7, 75.5, 74.2, 68.1, 51.5, 47.5, 45.1, 43.5, 41.1, 39.2, 35.3, 34.7, 34.6, 34.4, 30.9, 30.8, 27.7, 27.3, 25.5, 22.6, 21.5, 21.4, 17.5, 12.3 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3126, 1734 cm^{-1}. $[\alpha]_D^{24}$ = +88 (c = 2, EtOH). HRMS: m/z calcd. for $C_{29}H_{46}O_7 + K^+$: 545.2881; found: 545.2888. $C_{29}H_{46}O_7$ (506.32): calcd. C 68.73, H 9.15; found C 68.85, H 9.16.

Methyl 3α,12α-Diacetoxy-7α-(3′-iodobenzoyl)oxy-5β-cholan-24-oate (6): 3-Iodobenzoyl chloride was prepared by adding oxalyl chloride (1.6 mL, 18.3 mmol) and dry DMF (50 μL) to a stirred solution of 3-iodobenzoic acid (2.0 g, 8.0 mmol) in dichloromethane (15 mL). After stirring at room temperature for 30 min the volatile components were removed under reduced pressure, the crude 3-iodobenzoyl chloride was dissolved in toluene (8 mL), and added to a stirred solution of compound **5** in toluene (8 mL) containing CaH_2 (1.2 g, 28.5 mmol) and $BnEt_3N^+Cl^-$ (200 mg, 0.9 mmol). The reaction mixture was refluxed for 16 h and the volatile components were removed under reduced pressure. The residue was dissolved in chloroform (15 mL), filtered through celite, washed with satd. aq. $NaHCO_3$ (2 × 10 mL), water (10 mL) and dried with anhyd. Na_2SO_4. After removing the volatile components under reduced pressure the crude product was purified on a silica column using 15–20% ethyl acetate in petroleum ether to afford 1.5 g (51%) of compound **6** as a white solid, which was recrystallized using 20% ethyl acetate in petroleum ether; m.p. 150–152 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 8.38 (t, J = 1.5 Hz, 1.8 Hz, 1 H, 2′-H), 8.05 (d, J = 7.8 Hz, 1 H, 6′-H), 7.92 (d, J = 8.1, Hz, 1 H, 4′-H), 7.24 (d, J = 7.8 Hz, 1 H, 5′-H), 5.2 (br. s, 1 H), 5.14 (br. s, 1 H, 12 β-H), 4.50 (br. m, 1 H, 3β-H), 3.63 (s, 3 H, CO_2Me), 2.19 (s, 3 H, 12-OAc), 2.03, (s, 3 H, 3-OAc), 0.97 (s, 3 H, 19-H), 0.82 (d, J = 6.6 Hz, 3 H, 21-H), 0.75 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz,

$CDCl_3$): δ = 174.5, 170.7, 170.2, 163.7, 141.6, 138.6, 132.7, 130.2, 128.9, 93.8, 75.2, 73.5, 71.9, 51.5, 47.3, 44.9, 43.1, 40.5, 38.2, 34.9, 34.5, 34.3, 31.3, 30.8, 30.7, 28.6, 27.1, 26.7, 25.2, 22.9, 22.4, 21.5, 21.5, 17.5, 12.1 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 1734 cm^{-1}. $[\alpha]_D^{24}$ = +90 (*c* = 0.5, EtOH). HRMS: *m/z* calcd. for $C_{36}H_{49}IO_8 + Na^+$: 759.2370; found: 759.2350. $C_{36}H_{49}IO_8$ (736.24): calcd. C 58.70, H 6.70; found C 58.98, H 6.77.

Methyl 3α,12α-Diacetoxy-7α-(3′-iodobenzoyl)oxy-5β-chol-16-en-24-oate (8): Iodobenzene dichloride (1.5 g, 5.4 mmol) was added to a solution of compound **6** (1.5 g, 2.0 mmol) in dichloromethane (150 mL) containing *t*BuOH (5 mL, 52 mmol) and deoxygenated by bubbling dry N_2 for 3 min. The mixture was irradiated with two tungsten lamps (200 W each, placed 14 cm from the reaction flask) at 0 °C (ice-bath) for 2 h. A spot more polar than **6** on the TLC plate indicated the progress of the reaction. After removing the volatile components under reduced pressure the crude product was purified quickly on a silica column (3 × 16 cm) using 20–30% ethyl acetate in petroleum ether to yield 1.0 g of the 17-chlorosteroid, which was dissolved in 30 mL of dry pyridine and refluxed for 14 h. No major side products were observed during photochemical reaction and unreacted starting material was recovered. Pyridine was removed under reduced pressure and the crude product was purified on a silica column (3 × 12 cm) using 15–20% ethyl acetate in petroleum ether to afford 0.70 g (50% from **6**) of compound **8** as a white solid; m.p. 140–142 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.37 (br. s, 1 H, 2′-H), 8.04 (d, *J* = 7.8 Hz, 1 H, 6′-H), 7.91 (d, *J* = 7.5 Hz, 1 H, 4′-H), 7.22 (d, *J* = 8.1 Hz, 1 H, 5′-H), 5.32 (s, 1 H, 12β-H), 5.29 (br. d, 1 H, 16-H), 5.10 (s, 1 H, 7β-H), 4.51 (m, 1 H, 3β-H), 3.65 (s, 3 H, CO_2*Me*), 2.17 (s, 3 H, 12-OAc), 2.07 (s, 3 H, 3-OAc), 2.04–1.00 (m, steroidal CH_2), 1.01 (s, 3 H, 19-H), 0.93 (d, *J* = 6.3 Hz, 3 H, 21-H),87 (s, 3 H, 18-H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.1, 170.6, 170.5, 163.8, 156.3, 141.6, 138.6, 132.6, 130.2, 128.9, 123.3, 93.8, 73.4, 72.9, 51.5, 51.1, 43.3, 40.7, 3.8, 35.0, 34.6, 32.6, 31.9, 31.4, 30.7, 29.9, 26.7, 25.6, 22.5, 21.7, 21.5, 20.5, 17.2 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 1735, 1250 cm^{-1}. $[\alpha]_D^{24}$ = +92.0 (*c* = 2, EtOH). HRMS: *m/z* calcd. for $C_{36}H_{47}IO_8 + Na^+$: 757.2213; found: 757.2203. $C_{36}H_{47}IO_8$ (734.23): calcd. C 58.83, H 6.45; found C 58.55, H 6.61.

3α,7α,12α,16α,24-Pentahydroxy-5β-cholane (9): BH_3·Me_2S (1 mL, 10.5 mmol) was added to a stirred solution of compound **8** (0.23 g, 0.56 mmol) in dry THF (2 mL) at 0 °C under nitrogen and the reaction mixture was stirred for 20 h at room temperature. The mixture was oxidized by dropwise addition of a mixture (13 mL) of 1:1 (v/v) 4 M aq. NaOH and 30% H_2O_2 at 0 °C. After stirring at room temperature for 2 h it was neutralized by adding 1 M HCl (25 mL). The precipitate obtained was filtered, dried under vacuum and dissolved in 5% KOH in MeOH (15 mL) and refluxed for 12 h. The solid residue obtained after removing volatile components under vacuum was dissolved in water (10 mL), neutralized with 1 M HCl and filtered. The crude product was purified on a silica column (2 × 14 cm) using 10% EtOH in ethyl acetate to afford compound **9** (98 mg, 70%) as a white solid; m.p. 201–203 °C. ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 4.32 (d, *J* = 4.5 Hz, 1 H, 16-O*H*), 4.49 (d, *J* = 4.8 Hz, 1 H, 12-O*H*), 4.16 (d, *J* = 3.6 Hz, 1 H, 7-O*H*), 4.05 (d, *J* = 5.1 Hz, 1 H, 3-O*H*), 3.90 (d, *J* = 2.4 Hz, 1 H, 24-O*H*), 3.72 (br. d, *J* = 6.3 Hz, 1 H, 16β-H), 3.67 (br. s, 1 H, 12β-H), 3.57 (br. s, 1 H, 7β-H), 3.19 (m, 1 H, 3β-H), 2.04–1.00 (m, steroidal CH_2), 0.90 (d, *J* = 6.6 Hz, 3 H, 21-H), 0.78 (s, 3 H, 19-H), 0.57 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 75.4, 70.8, 70.4, 66.4, 61.5, 55.9, 47.3, 41.5, 35.9, 35.2, 34.7, 34.4, 33.9, 31.3, 30.4, 29.8, 28.2, 26.1, 22.6, 17.9, 13.9 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3423 cm^{-1}. $[\alpha]_D^{24}$ = +11 (*c* = 2, EtOH). HRMS: *m/z* calcd. for $C_{24}H_{42}O_5 + Na^+$: 433.2930; found: 433.2930. $C_{24}H_{42}O_5$·H_2O (428.31): calcd. C 67.24, H 10.35; found C 67.28, H 10.07.

3α,7α,12α-Trihydroxy-5β-cholane *O*-24,16α-Lactone (10): CuCl (1.6 mg, 0.016 mmol) was added to a solution containing TEMPO (2.5 mg, 0.016 mmol) and compound **9** (20 mg, 0.04 mmol) in dry DMF (1 mL) and the mixture was stirred at room temperature for 3 h under an oxygen atmosphere. The reaction mixture was diluted by adding diethyl ether (8 mL), washed with satd. aq. $CuSO_4$ (4 mL) and water (4 mL). The organic layer was dried with anhyd. Na_2SO_4 and the volatile components were removed under reduced pressure. The crude product was purified on a silica column (1 × 12 cm) with 90–100% EtOAc/petroleum ether to afford 16.4 mg (78%) of compound **10** as a solid; m.p. 277–279 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.63 (t, *J* = 7.5 Hz, 1 H, 16β-H), 3.88 (br. s, 2 H, 12β-H & 7β-H), 3.42 (br., m, 1 H, 3β-H), 2.04–1.00 (m, steroidal CH_2), 1.09 (d, 3 H, 21-H), 0.87 (s, 3 H, 19-H), 0.76 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 176.3, 84.1, 72.6, 71.7, 68.0, 55.9, 48.7, 41.3, 39.3, 38.9, 38.5, 35.9, 35.1, 34.5, 34.3, 33.9, 32.9, 31.5, 30.2, 27.3, 26.2, 22.1, 19.3, 13.3 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3430, 1713, 1704 cm^{-1}. $[\alpha]_D^{24}$ = +28 (*c* 2, EtOH). HRMS: *m/z* calcd. for $C_{24}H_{38}O_5 + Na^+$: 429.2617; found: 429.2595. calcd. for $C_{24}H_{38}O_5 + K^+$: 445.2356; found 445.2365. $C_{24}H_{38}O_5$·H_2O (424.28): calcd. C 67.88, H 9.50; found C 67.89, H 9.15.

3α,7α,12α,16α-Tetrahydroxy-5β-cholan-24-oic Acid (2): Compound **10** (40.0 mg, 0.098 mmol) was dissolved in 5% KOH in MeOH (5 mL) and stirred at room temp. for 12 h. After removing the volatile components under reduced pressure, the solid residue was dissolved in water (10 mL) and neutralized by adding 1 M HCl in an ice-water bath with stirring and extracted using ethyl acetate (2 × 10 mL). The organic layer was dried with anhyd. Na_2SO_4, volatile components were removed under reduced pressure to afford 39.6 mg (95%) of compound **2** as a white solid; m.p. 205–206 °C. ^{1}H NMR (300 MHz, [D$_6$]acetone): δ = 3.99 (t, *J* = 7.5 Hz, 1 H, 16β-H), 3.89 (br. s, 1 H, 12β-H), 3.79 (br. q, 1 H, 7β-H), 3.35–3.06 (br. m, 1 H, 3β-H), 1.02 (d, *J* = 6.6 Hz, 3 H, 21-H), 0.89 (s, 3 H, 19-H), 0.72 (s, 3 H, 18-H) ppm. ^{1}H NMR (300 MHz, $CDCl_3$ + 10% [D$_6$]DMSO): δ = 3.99 (t, *J* = 6.3 Hz, 1 H, 16β-H), 3.88 (br. s, 1 H, 12β-H), 3.81 (br. s, 1 H, 7β-H), 3.47–3.37 (br. m, 1 H, 3β-H), 0.98 (d, *J* = 6 Hz, 3 H, 21-H), 0.87 (s, 3 H, 19-H), 0.68 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, [D$_6$]acetone): δ = 206.2, 175.5, 77.1, 72.7, 72.2, 68.3, 57.1, 48.8, 42.9, 40.5, 40.2, 39.8, 36.9, 36.3, 35.5, 34.7, 31.5, 31.4, 27.3, 23.1, 17.9, 14.3 ppm. IR (KBr): $\tilde{\nu}$ = 3414, 1704, 1668 cm^{-1}. $[\alpha]_D^{24}$ = +11 (*c* = 2, EtOH). HRMS: *m/z* calcd. for $C_{24}H_{40}O_6 + Na^+$: 447.2723; found 447.2731.

3α,12α-Dihydroxy-7-oxo-5β-cholane *O*-24,16α-Lactone (11): *N*-Bromosuccinimide (25 mg, 0.140 mmol) was added to a solution of compound **10** (42 mg, 0.10 mmol) in 25:1 (v/v) acetone/water (2.8 mL) and stirred at room temperature for 10 min. After adding water (2 mL) the crude product was extracted with diethyl ether and washed with satd. aq. $NaHCO_3$ (2 × 4 mL). The organic layer was dried with anhyd. Na_2SO_4 and the crude product was purified using 45–60% ethyl acetate in petroleum ether to afford 34 mg (88%) of compound **11** as a white solid; m.p. 268–269 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.60 (t, *J* = 7.8 Hz, 1 H, 16β-H), 3.97 (br. t, 1 H, 12β-H), 3.58–3.50 (br. m, 1 H, 3β-H), 2.04–1.02 (m, steroidal CH_2), 1.17 (s, 3 H, 19-H), 1.08 (d, *J* = 6.6 Hz, 3 H, 21-H), 0.76 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 212.0, 176.0, 84.2, 77.2, 70.8, 54.7, 49.1, 48.9, 46.1, 45.1, 37.8, 37.7, 35.9, 34.6, 34.0, 33.1, 32.9, 29.9, 29.6, 29.3, 22.8, 19.6, 13.7 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3419, 1712 cm^{-1}. $[\alpha]_D^{24}$ = +32 (*c* = 0.5, EtOH). HRMS: *m/z* calcd. for $C_{24}H_{36}O_5 + Na^+$: 427.2460; found. 427.2449.

3α,12α,16α-Trihydroxy-5β-cholan-24-oic Acid (1): To a solution of compound **11** (50 mg, 0.12 mmol) in digol (1 mL), hydrazine hydrate (0.1 mL, 2.0 mmol) and KOH (100 mg, 1.8 mmol) were added. The reaction mixture was refluxed at 125 °C for 3 h and then heated to 205 °C for 1 h on a sand bath. After cooling to room temperature and adding water (2 mL) it was neutralized with 6 M HCl. The acid was extracted with ethyl acetate (3 × 3 mL) and dried with anhyd. Na_2SO_4. After removing the volatile components under reduced pressure, it was dried under vacuum to afford 45 mg (88%) of compound **1** as a white solid; m.p. 188–189 °C (ref.[2] m.p. 186–187 °C). ^{1}H NMR (300 MHz, [D$_6$]acetone): δ = 3.99 (t, J = 7.8 Hz, 1 H, 16β-H), 3.89 (br. t, 1 H, 12β-H), 3.57–3.46 (m, 1 H, 3β-H), 2.04–1.00 (m, steroidal CH_2), 1.01 (d, J = 6.3 Hz, 21-H), 0.91 (s, 3 H, 19-H), 0.723 (s, 3 H, 18-H) ppm. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.03 (t, J = 7.5 Hz, 1 H, 16β-H), 3.88 (br. s, 1 H, 12β-H), 3.71–3.61 (br. m, 1 H, 3β-H), 1.00 (d, J = 6.6 Hz, 21-H), 0.89 (s, 3 H, 19-H), 0.70 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, [D$_6$]-acetone): δ = 175.6, 76.7, 72.7, 71.6, 57.0, 48.8, 45.8, 43.1, 37.7, 37.3, 36.3, 36.1, 34.9, 34.5, 34.2, 31.5, 31.3, 31.0, 28.0, 27.2, 23.6, 18.0, 14.6 ppm. $[\alpha]_D^{24}$ = +28 (c = 0.5, EtOH). IR (KBr): $\tilde{\nu}$ = 3415, 1704 cm^{-1}. HRMS: m/z calcd. for $C_{24}H_{40}O_5$ + Na^+: 431.2773; found: 431.2757. $C_{24}H_{40}O_5$ (408.28): calcd. C 70.54, H 9.87; found C 70.65, H 10.00.

Characterization of Pythocholic Lactone (12): Wolf–Kishner reduction followed by acidic work up of compound **11** afforded a less polar product along with the desired pythocholic acid. Spectral characterization confirmed that it to be pythocholic lactone **12**; m.p. 260–261 °C. (ref.[4] m.p. 263–264 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.60 (t, J = 7.5 Hz, 1 H, 16β-H), 3.93 (t, J = 3.3 Hz, 1 H, 12β-H), 3.67–3.59 (br. m, 1 H, 3β-H), 2.04–1.16 (steroidal CH_2), 1.07 (d, J = 6.3 Hz, 3 H, 21-H), 0.91 (s, 3 H, 19-H), 0.77 (s, 3 H, 18-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 175.7, 83.6, 72.3, 71.7, 55.9, 48.8, 44.9, 41.9, 36.3, 35.9, 35.4, 35.1, 34.1, 33.6, 32.9, 32.2, 30.3, 28.3, 26.9, 25.9, 23.0, 19.6, 13.7 ppm. IR ($CHCl_3$): $\tilde{\nu}$ = 3430, 1713, 1704 cm^{-1}. $[\alpha]_D^{24}$ = +48 (c = 0.5, EtOH). HRMS: m/z calcd. for $C_{24}H_{38}O_4$ + Na^+: 413.2668; found: 413.2662.

Supporting Information (see also the footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of compounds **1**, **2**, **6**, **8**, **9**, **10**, **11**, **12**, 2D-NMR spectrum of compound **2**, crystal data of compound **6**.

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