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Synthesis and Cytotoxicity Studies of Polyhydroxysterols and Their Sulfate Analogs

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ABSTRACT

The six new polyhydroxy steroids, *3β, 20(S)-dihydroxy-5α-cholest-24-ene* (19), *3β, 20(S)-dihydroxy-5α-cholestane* (20), *3β, 20(S), 24-trihydroxy-5α-cholestane* (23), *2β, 3α, 20(S)-trihydroxy-5α-cholestane* (29), *2β, 3α, 20(S)-trihydroxy-5α-cholest-24-ene* (31), *2β, 3α, 20(S), 24-tetrahydroxy-cholestane* (37) and the sulfate derivative **21** were synthesized from tigogenin. Antitumor activity against two tumor cell lines (lung cancer NCI-H187 and oral cancer KB) was evaluated. Compound **23** containing trihydroxy groups at the C-3, C-20 and C-24 positions showed strong activity against both NCI-H187 and KB cells (IC_{50} 2.11 and 5.39 μg/mL). The 3, 20-dihydroxy steroid **19** showed strong activity against NCI-H187 (IC_{50} 4.24 μg/mL) but was weakly active against KB (IC_{50} 39.12 μg/mL) whereas the analog **20** which has a saturated side chain showed moderate activity against KB (IC_{50} 20.51 μg/mL) and was inactive against the NCI-H187 cell line. Surprisingly, the sulfate derivative of **20**, compound **21,** was inactive to both tested cells. Compounds **29** and **31,** having vicinal dihydroxy groups in ring A at C-2, C-3 as well as a hydroxyl group at the C-20 position, showed similar activity against NCI-H187 (IC_{50} 9.08 and 9.59 μg/mL) but for the KB cell, only **31** showed strong activity (IC_{50} 10.14 μg/mL) whereas **29** was inactive. The analogue compound **37,** which has an extra hydroxyl group at C-24, was inactive against both tested cell lines.

Key words: steroids, synthesis, marine organisms, anticancer, biological activity

INTRODUCTION

Marine organisms have historically been a rich source of novel sterols, particularly in terms of unique side chain structures and unusual functionalities. The steroids isolated from sponges are sometimes very complex mixtures of highly functionalized compounds. Common structures of sponge steroids have been documented, including additional oxygenation of the nucleus and the side chain, the side chain modified by alkylation and degradation, and the occurrence of sulfate esters of polyoxygenated sterol (Aiello *et al*., 1999).

Up to the present, two classes of interesting new steroids—sulfated polyhydroxysterols and polyoxygenated steroids—have attracted considerable attention because most of them have shown strong biological and pharmacological activity.

Sulfated polyhydroxysterols are naturally occurring metabolites in sponges and echinoderms (D'Auria *et al*., 1993). Most of these

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compounds have shown various biological activities such as being antiviral (McKee *et al*., 1994) and inhibition of protein tyrosine kinase that result in the control of cancer and other hyperproliferative conditions (Fu and Schmitz, 1994). For instance (Figure 1), orthoesterol A (1), B (2) and C (3) exhibited *in vitro* activity against the feline leukemia virus (FeL V), mouse influenza virus (PR8) and mouse corona virus (A59) (Koehn *et al*., 1991), halistanol sulfates (4) and (5) showed anti-HIV reaction (Bifulco *et al*., 1994) and sterol sulfates **6**-**8** inhibited protein tyrosine kinase activity (Fu and Schmitz, 1994).

Polyoxygenated steroids are a large group found in marine organisms, especially in sponges. Recently our group has synthesized a series of polyoxygenated steroids **9-12** (Figure 2), a marine steroid isolated from gorgonian *Leptogorgia sarmentosa* (Garrido *et al*., 2000; Boonananwong *et al*., 2008) and new polyoxygenated aromatic steroids **13-16** (Figure 3) and also determined their activities against cancer cells (Bunyathaworn *et al*., 2010).

In this paper, the synthesis of a series of polyhydroxy sterols and a sulfate derivative from tigogenin is described and tigogenin can be obtained from fermentation of *Agave sisalana* (Blunden *et al*., 1974). In addition, the anticancer activities against the two cancer cell lines NCI-H187 and KB were determined.

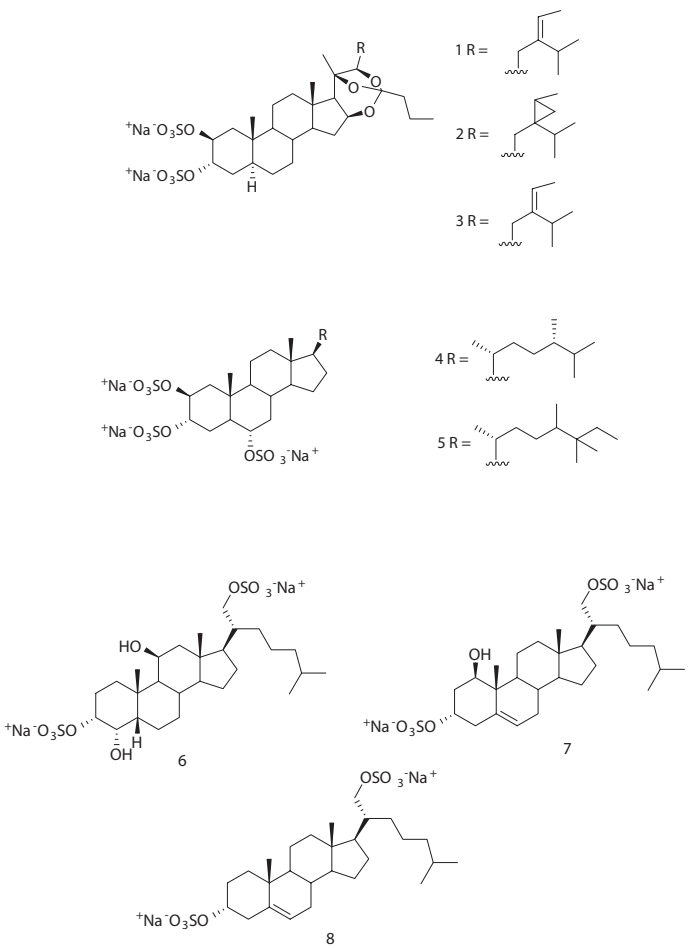


Figure 1 Steroid sulfates isolated from marine organisms.

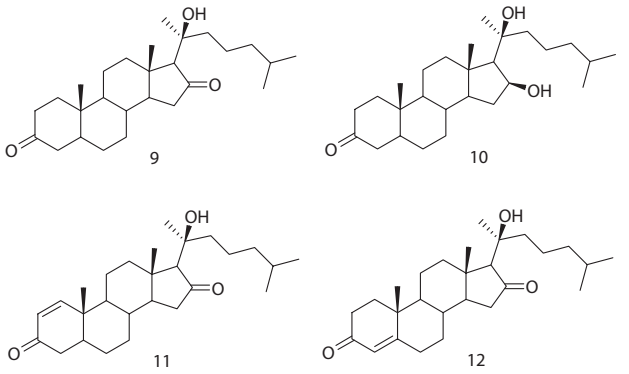


Figure 2 Steroids isolated from *Leptogorgia sarmentosa*.

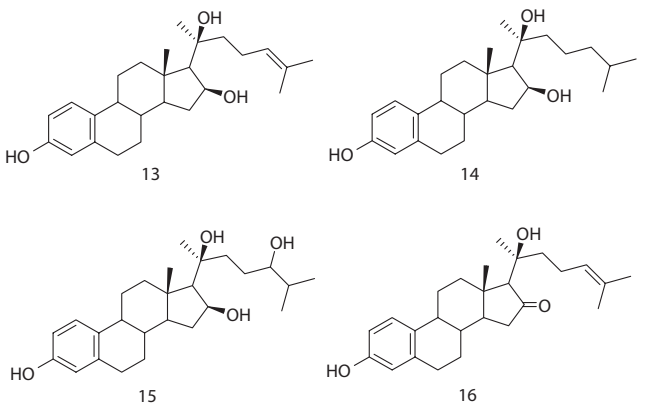


Figure 3 Synthetic polyoxygenated aromatic steroids.

MATERIALS AND METHODS

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian Gemini 300 spectrophotometer and on a 400 and 100 MHz Bruker Advance DPX-400. Chemical shifts were recorded as δ values in ppm. Spectra were acquired in $CDCl_3$ unless otherwise stated. The peak due to residual $CHCl_3$ signal (7.26 ppm for ^{1}H and 77.23 ppm for ^{13}C) was used as the internal reference. Coupling constant (*J*) values were given in Hz, and multiplicity was defined as follows: br = broad, s = singlet, d = doublet, dd = double of doublets, dt = double of triplet, t = triplet, q = quartet and m = multiplet. Infrared (IR) spectra were recorded per centimeter (cm^{-1}) on a Perkin-Elmer 2000 Fourier transform infrared spectrophotometer at the Chemistry Department, Faculty of Science, Kasetsart University. Samples were analyzed as KBr disks. Mass spectra were obtained on an Agilent Technology 1100 series LL/MSD Trap. Melting points (m.p.) were determined on the Fisher John apparatus and MEL-TEMP capillary melting point apparatus at the Chemistry Department, Faculty of Science, Kasetsart University and were reported uncorrected in degrees Celcius (°C). Analytical grade chemicals and solvents were purchased from Fluka Co. Ltd. Solvents were purified by a general method before being used.

Synthetic procedures

General procedure for preparation of compounds 22 and 28c

To a stirring suspension of Mg (687 mg, 27 mmol) and I_2 catalyst in dry THF (20 mL) was slowly added 4-bromobutene (2.5 mL, 13.9 mmol) under N_2 at room temperature. After stirring for 1 h, a solution of **18** or **27** (2.8 mmol) in dry THF (15 mL) was added dropwise at room temperature. After 20 min, the reaction was quenched by addition of saturated aqueous ammonium chloride and the solution was extracted with dichloromethane. The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate and then with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography.

3β, 20(*S*)-Dihydroxy-5α-24a-homo-chol-24-ene (22)

The crude product was purified by flash column chromatography eluting with 1:9 ethyl acetate:hexane to give **22** (639.8 mg, 61%) as a white needle after recrystallization (dichloromethane); m.p. 119–122 °C; **FTIR** (KBr) ν_{max} 3314, 2929, 1447, 1040, 895 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.80 (1H, ddt, *J* = 17.1, 10.2, 6.6 Hz, H-24), 4.99 (1H, dd, *J* = 17.1, 2.0 Hz, H-25), 4.91 (1H, dd, *J* = 10.2, 2.0 Hz, H-25), 3.56 (1H, m, H-3), 2.04 (4H, m, H-23 and H-12), 1.81-0.82 (5C<u>H</u>, 9C<u>H</u>$_2$), 1.25 (3H, s, H-21), 0.81 (3H, s, H-18), 0.78 (3H, s, H-19); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 139.1 (CH, C-24), 114.2 (CH_2, C-25), 75.1 (C, C-20), 71.3 (CH, C-3), 58.2 (CH, C-17), 56.7 (CH, C-14), 54.3 (CH, C-9), 44.9 (CH, C-5), 43.0 (C, C-13), 42.6 (CH_2, C-22), 40.1 (CH_2, C-12), 38.2 (CH_2), 37.0 (CH_2, C-1), 35.4 (C, C-10), 34.9 (CH, C-8), 31.9 (CH_2), 31.5 (CH_2), 28.7 (CH_2, C-23), 28.6 (CH_2), 26.2 (CH_3, C-21), 23.7 (CH_2), 22.4 (CH_2), 21.5 (CH_2), 13.8 (CH_3, C-19), 12.3 (CH_3, C-18); **HRMS** *m/z* $C_{25}H_{42}O_2Na$ $[M+Na]^+$, calculated 397.3083, found 397.3083.

2α, 3α-Epoxy-20(*S*)-hydroxy-5α-24a-homo-chol-24-ene (28c)

The crude product was purified by flash column chromatography eluting with 1:19 ethyl acetate:hexane to give **28c** (249 mg, 50%) as a white needle after recrystallization (hexane); m.p. 254–256 °C; **FTIR** (KBr) ν_{max} 3448, 2918, 1671, 1657, 1601, 1435, 1380, 1107 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.78 (1H, ddt, *J* = 17.0, 10.3, 6.6 Hz, H-24), 4.98 (1H, dd, *J* = 17.0, 1.8 Hz, H-25), 4.90 (1H, dd, *J* = 10.3, 1.8 Hz, H-25), 3.11 (1H, m, H-3), 3.07 (1H, m, H-2), 2.03 (3H, m, H-23 and H-12), 1.86 (2H, m, H-1), 1.73-0.88 (4C<u>H</u>, 7C<u>H</u>$_2$, H-12), 1.24 (3H, s, H-21), 0.77 (3H, s, H-18), 0.72 (3H, s, H-19), 0.58 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 139.0 (CH, C-24), 114.2 (CH_2, C-25), 75.0 (C, C-20), 58.1 (CH, C-17), 56.4 (CH, C-14), 53.6 (CH, C-9), 52.4 (CH, C-3), 51.0 (CH, C-2), 42.7 (C, C-13), 42.6 (CH_2, C-22), 40.2 (CH_2, C-12), 38.2 (CH_2, C-1), 36.2 (CH, C-5), 35.0 (CH, C-8), 33.6 (C, C-10), 31.5 (CH_2), 29.0 (CH_2), 28.6 (CH_2), 28.4 (CH_2), 26.2 (CH_3, C-21), 23.6 (CH_2), 22.3 (CH_2), 20.7 (CH_2), 13.6 (CH_3, C-19), 12.9 (CH_3, C-18); **HRMS** *m/z* $C_{25}H_{40}O_2Na$ $[M+Na]^+$, calculated 395.2926, found 395.2925.

General procedure for preparation of compounds 23 and 33

<u>Step I</u>

A mixture of **22** or **28c** (1.94 mmol) and $NaHCO_3$ (796 mg) in CH_2Cl_2 (25 mL) and methanol (5 mL) was cooled to -78 °C and ozone was bubbled through the mixture with stirring. When the reaction mixture turned blue, ozone addition was stopped. Nitrogen gas was passed through the solution until the blue color was discharged. The reaction was quenched by addition of triphenylphosphine (3.8 mmol) at -78 °C. The reaction mixture was slowly warmed to room temperature and further stirred for 1 h before concentration *in vacuo*. The residue was purified by flash column chromatography to give the aldehyde intermediate.

Step II

To a stirring suspension of Mg (1.1 g, 45 mmol) and I_2 catalyst in dry THF (20 mL) while being stirred, was slowly added 2-bromopropane (9.0 mL, 91.9 mmol) under N_2 gas atmosphere at room temperature. After stirring for 2 h, the mixture was cooled to -78 °C. A solution of the aldehyde intermediate (1.24 mmol) in dry THF (8 mL) was added slowly and kept at this temperature. After 20 min, the reaction mixture was allowed to slowly warm to room temperature and left at this temperature for 3 h. The reaction was quenched by the addition of saturated aqueous ammonium chloride and the mixture was extracted with ethyl acetate. The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate and with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography.

3β, 20(*S*), 24-Trihydroxy-5α-cholestane (23)

The crude product was purified by flash column chromatography eluting with 1:4 ethyl acetate:hexane to give **23** (231 mg, 75% (brsm)) as a white solid and the starting material was recovered (187.2 mg, 61% conversion); **FTIR** (KBr) ν_{max} 3399, 2920, 1036 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.51 (1H, m, H-3), 3.24 (1H, m, H-24), 1.98 (1H, m, H-12), 1.81-0.76 (6C<u>H</u>, 10C<u>H</u>$_2$, H-12), 1.20 (3H, s, H-21), 0.84 (6H, m, H-26 and H-27), 0.76 (3H, s, H-18), 0.74 (3H, s, H-19). **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 77.1 (CH, C-24), 75.0 (C, C-20), 71.3 (CH, C-3), 58.9 (CH, C-17), 56.6 (CH, C-14), 54.3 (CH, C-9), 44.8 (CH, C-5), 43.0 (C, C-13), 40.4 (CH_2, C-12), 39.8 (CH_2), 38.1 (CH_2), 37.0 (CH_2, C-1), 35.4 (C, C-10), 34.8 (CH, C-8), 33.6 (CH, C-25), 31.9 (CH_2), 31.5 (CH_2), 28.7 (CH_2), 28.2 (CH_2), 26.0 (CH_3, C-21) 23.7 (CH_2), 22.5 (CH_2), 21.1 (CH_2), 18.9, 17.1 (CH_3, C-26 and C-27), 13.8 (CH_3, C-18), 12.3 (CH_3, C-19); **HRMS** *m/z* $C_{27}H_{48}O_3Na$ $[M+Na]^+$, calculated 443.3501, found 443.3501.

2α, 3α-Epoxy-20(*S*), 24-dihydroxy-5α-cholestane (33)

The crude product was purified by flash column chromatography eluting with 1:13 ethyl acetate:hexane to give **33** (83.6 mg, 48% (brsm)) as a white solid and the starting material was recovered (41 mg, 74% conversion); **FTIR** (KBr) ν_{max} 3422, 2929, 1459, 1380, 1006 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.27 (1H, m, H-24), 3.11 (1H, m, H-3), 3.08 (1H, m, H-2), 2.09-0.72 (6C<u>H</u>, 10C<u>H</u>$_2$), 1.22 (3H, s, H-21), 0.87 (6H, m, H-26 and H-27), 0.78 (3H, s, H-18), 0.71 (3H, s, H-19), 0.57 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 76.7 (CH, C-24), 75.1 (C, C-20), 58.8, 58.4 (CH, C-17), 56.4 (CH, C-14), 53.5 (CH, C-9), 52.4 (CH, C-3), 51.0 (CH, C-2), 42.7 (C, C-13), 40.2 (CH_2, C-12), 39.8 (CH_2, C-22), 39.4 (CH_2), 38.2 (CH_2, C-1), 36.2 (CH, C-5), 35.0 (CH, C-8), 33.6 (CH, C-25), 33.5 (C, C-10), 31.4 (CH_2), 28.9 (CH_2), 28.3 (CH_2), 26.0 (CH_3, C-21), 23.6 (CH_2), 22.3 (CH_2), 20.7 (CH_2), 18.9, 18.8, 17.4, 17.1 (CH_3, C-26 and C-27), 13.6 (CH_3, C-18), 12.9 (CH_3, C-19); **HRMS** *m/z* $C_{27}H_{46}O_3Na$ $[M+Na]^+$, calculated 441.3345, found 441.3339.

General procedure for preparation of compounds 28a and 28b

To a stirring suspension of Mg (91 mg, 3.6 mmol) and I_2 catalyst in dry THF (4 mL) was slowly added 5-bromo-2-methyl-2-pentene or 1-bromo-4-methylpentane (3.8 mmol) under N_2 at room temperature. After stirring for 15 min at room temperature, the mixture was warmed to 45 °C for 1 h before cooling to room temperature and the solution of **27** (0.48 mmol) in dry THF (5 mL) was added dropwise and stirred for 2 h. The reaction was quenched by the addition of saturated aqueous ammonium chloride and then extracted with diethyl ether. The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate and then with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography.

2α, 3α-Epoxy-20(*S*)-hydroxy-5α-cholestane (28a)

The crude product was purified by flash column chromatography eluting with 1:49 ethyl acetate:hexane to give **28a** (97.2 mg, 50%) as a white solid; m.p. 121–123 °C; **FTIR** (KBr) ν_{max} 2942, 1468, 1382 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.12 (1H, m, H-3), 3.07 (1H, m, H-2), 2.02 (1H, m, H-12), 1.88 (1H, dd, *J* = 15.1, 5.9 Hz, H-1), 1.84 (1H, m, H of CH_2), 1.73-0.87 (5C<u>H</u>, 8C<u>H</u>$_2$, H-12, H-1, H of CH_2), 1.23 (3H, s, H-21), 0.84 (6H, d, *J* = 6.7 Hz, H-26 and H-27), 0.80 (3H, s, H-18), 0.72 (3H, s, H-19), 0.58 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 75.2 (C, C-20), 57.7 (CH, C-17), 56.4 (CH, C-14), 53.6 (CH, C-9), 52.4 (CH, C-3), 51.0 (CH, C-2), 44.2 (CH_2, C-22), 42.4 (C, C-13), 40.2 (CH_2, C-12), 39.6 (CH_2, C-24), 38.3 (CH_2, C-1), 36.2 (CH, C-5), 35.0 (CH, C-8), 33.6 (C, C-10), 31.5 (CH_2), 29.0 (CH_2), 28.4 (CH_2), 27.9 (CH, C-25), 26.4 (CH_3, C-21), 23.7 (CH_2), 22.7, 22.5 (CH_3, C-26 and C-27), 22.3 (CH_2), 22.0 (CH_2), 20.7 (CH_2), 13.6 (CH_3, C-18), 12.9 (CH_3, C-19); **CIMS**: 403 $[M+H]^+$ (4), 385 (76), 367 (100), 257 (13).

2α, 3α-Epoxy-20(*S*)-hydroxy-5α-cholest-24-ene (28b)

The crude product was purified by flash column chromatography eluting with 1:49 ethyl acetate:hexane to give **28b** (74.8 mg, 39%) as a colorless gum; **FTIR** (neat) ν_{max} 3441, 2923, 1672, 1653, 1603, 1435, 1382, 1027 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.06 (1H, m, H-24), 3.12 (1H, m, H-3), 3.08 (1H, m, H-2), 2.03 (1H, dt, *J* = 12.5, 3.4 Hz, H-12), 1.95 (2H, dt, *J* = 8.0, 7.8 Hz, H-23), 1.88 (1H, dd, *J* = 15.1, 5.9 Hz, H-1), 1.85 (1H, m, H of CH_2), 1.76-0.82 (4C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-1, H of CH_2), 1.65, 1.58 (6H, 2s, H-26 and H-27), 1.25 (3H, s, H-21), 0.80 (3H, s, H-18), 0.72 (3H, s, H-19), 0.59 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 131.5 (C, C-25), 124.5 (CH, C-24), 75.1 (C, C-20), 58.0 (CH, C-17), 56.4 (CH, C-9), 53.6 (CH, C-14), 52.4 (CH, C-3), 51.0 (CH, C-2), 43.6 (CH_2, C-22), 42.7 (C, C-13), 40.3 (CH_2, C-12), 38.2 (CH_2, C-1), 36.2 (CH, C-5), 35.0 (CH, C-8), 33.6 (C, C-10), 31.5 (CH_2), 29.0 (CH_2), 28.4 (CH_2), 26.1 (CH_3, C-21), 25.7 (CH_3, C-26 or C-27), 23.7 (CH_2), 22.9 (CH_2), 22.3 (CH_2), 20.7 (CH_2), 17.6 (CH_3, C-26 or C-27), 13.6 (CH_3, C-18), 12.9 (CH_3, C-19); **CIMS**: 401 $[M+H]^+$ (15), 383 (100), 365 (43), 283 (6), 257 (14).

General procedure for preparation of compounds 29, 30, 31, 32, 35 and 36

A solution of **28a**, **28b** or **34** (0.15 mmol) in THF (2 mL) was treated with 1M H_2SO_4 (0.8 mL) and stirred for 24 h at room temperature. After neutralization with saturated sodium hydrogen carbonate solution, the mixture was evaporated to one fifth of the initial volume, diluted with water and extracted with ethyl acetate. The combined organic layers were washed with water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography.

2β, 3α, 20(*S*)-Trihydroxy-5α-cholestane (29) and 2α, 3α, 20(*S*)-trihydroxy-5α-cholestane (30)

The crude product was purified by flash column chromatography eluting with 1:4 ethyl acetate:hexane to give **29** (36.9 mg, 51.6%) as a white solid and **30** (7 mg, 10%) as a white solid.

Compound **29**; m.p. 186–188 °C; **FTIR** (KBr) ν_{max} 3388, 2930, 1466, 1381, 1036 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.82 (1H, m, H-2), 3.78 (1H, m, H-3), 1.98 (1H, m, H-12), 1.82 (1H, ddd, *J* = 13.8, 13.5, 3.2 Hz, H-4), 1.71-0.84 (5C<u>H</u>, 9C<u>H</u>$_2$, H-12, H-4), 1.19 (3H, s, H-21), 0.92 (3H, s, H-19), 0.80 (6H, d, *J* = 6.6 Hz, H-26 and H-27), 0.77 (3H, s, H-18), 0.63 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 75.3 (C, C-20), 71.8 (CH, C-2), 70.6 (CH, C-3), 57.8 (CH, C-17), 56.6 (CH, C-14), 55.1 (CH, C-9), 44.2 (CH_2, C-22), 42.9 (C, C-13), 40.5 (CH_2, C-1), 40.4 (CH_2, C-12), 39.6 (CH_2, C-24), 38.9 (CH, C-5), 35.7 (C, C-10), 34.2 (CH, C-8), 31.8 (CH_2), 31.7 (CH_2),

28.2 (CH_2), 27.9 (CH, C-25), 26.3 (CH_3, C-21), 23.6 (CH_2), 22.7, 22.5 (CH_3, C-26 and C-27), 22.3 (CH_2), 22.0 (CH_2), 20.7 (CH_2), 14.6 (CH_3, C-19), 13.8 (CH_3, C-18); **CIMS**: 403 $[M+H-H_2O]^+$ (75), 385 (100), 367 (76).

Compound **30**; **FTIR** (KBr) ν_{max} 3394, 2937, 1450, 1362, 1258, 1107, 1040 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.82 (1H, m, H-3), 3.53 (1H, m, H-2), 1.98 (2H, m, H-12, H-4), 1.75-0.84 (5C<u>H</u>, 9C<u>H</u>$_2$, H-12, H-4), 1.19 (3H, s, H-21), 0.95 (3H, s, H-19), 0.80 (6H, d, J = 6.4 Hz, H-26 and H-27), 0.77 (3H, s, H-18), 0.63 (1H, m, H-9).

2β, 3α, 20(*S*)-Trihydroxy-5α-cholest-24-ene (31) and 2α, 3α, 20(*S*)-trihydroxy-5α-cholest-24-ene (32)

The crude product was purified by flash column chromatography eluting with 1:4 ethyl acetate:hexane to give **31** (32.9 mg, 53.1%) as a white solid and **32** (3.6 mg, 6%) as a white solid and the mixture of these two steroids (2 mg, 3.2%).

Compound **31**; m.p. 163–165 °C; **FTIR** (KBr) ν_{max} 3392, 2925, 1453, 1375, 1266, 1037 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.02 (1H, m, H-24), 3.82 (1H, m, H-2), 3.79 (1H, m, H-3), 1.99 (1H, dt, J = 12.0, 3.2 Hz, H-12), 1.91 (2H, dt, J = 8.0, 8.0 Hz, H-23), 1.82 (1H, ddd, J = 13.8, 13.8, 3.2 Hz, H-4), 1.72-0.77 (4C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-4), 1.61, 1.54 (6H, 2s, H-26 and H-27), 1.21 (3H, s, H-21), 0.92 (3H, s, H-19), 0.76 (3H, s, H-18), 0.63 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 131.5 (C, C-25), 124.5 (CH, C-24), 75.2 (C, C-20), 71.8 (CH, C-2), 70.6 (CH, C-3), 58.0 (CH, C-17), 56.6 (CH, C-14), 55.1 (CH, C-9), 43.5 (CH_2, C-22), 43.0 (C, C-13), 40.5 (CH_2, C-1), 40.4 (CH_2, C-12), 38.9 (CH, C-5), 35.7 (C, C-10), 34.2 (CH, C-8), 31.8 (CH_2), 31.7 (CH_2), 28.2 (CH_2), 26.1 (CH_3, C-21), 25.7 (CH_3, C-26 or C-27), 23.6 (CH_2), 22.9 (CH_2, C-23), 22.4 (CH_2), 20.7 (CH_2), 17.6 (CH_3, C-26 or C-27), 14.6 (CH_3, C-19), 13.8 (CH_3, C-18); **HRMS** m/z $C_{27}H_{46}O_3Na$ $[M+Na]^+$, calculated 441.3345, found 441.3336.

24-Benzyloxy-2β, 3α, 20(*S*)-trihydroxy-5α-cholestane (35) and 24-benzyloxy-2α, 3α, 20(*S*)-trihydroxy-5α-cholestane (36)

The crude product was purified by flash column chromatography eluting with 3:17 ethyl acetate:hexane to give **35** (45.3 mg, 44%) as a white solid and **36** (3 mg, 3%) as a white solid.

Compound **35**; m.p. 146–150 °C; **FTIR** (KBr) ν_{max} 3391, 2927, 1699, 1454, 1369, 1273, 1038, 742, 697 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 7.27-7.25 (5H, m, H_{AR}), 4.44, 4.43 (2H, 2s, $H_{benzylic}$), 3.81 (1H, m, H-2), 3.78 (1H, m, H-3), 3.04 (1H, m, H-24), 1.98 (1H, m, H-12), 1.83 (2H, m, H-4, H-25), 1.74-0.78 (5C<u>H</u>, 8C<u>H</u>$_2$, H-12, H-4), 1.17 (3H, s, H-21), 0.91 (3H, s, H-19), 0.86, 0.85 (6H, 2d, J = 7.0, 7.0 Hz, H-26 and H-27), 0.75 (3H, s, H-18), 0.63 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 138.9 (C_{AR}), 128.3 (CH_{AR}), 127.8 (CH_{AR}), 127.4 (CH_{AR}), 84.7 (CH, C-24), 74.9 (C, C-20), 71.8 (CH_2, C_{benzyl}), 71.7 (CH, C-2), 70.6 (CH, C-3), 58.3 (CH, C-17), 56.8 (CH, C-14), 55.1 (CH, C-9), 43.0 (C, C-13), 40.5 (CH_2, C-1) , 40.4 (CH_2, C-12), 39.2 (CH_2, C-22), 38.9 (CH, C-5), 35.7 (C, C-10), 34.2 (CH, C-8), 31.8 (CH_2), 31.7 (CH_2), 30.4 (CH, C-25), 28.2 (CH_2), 26.1 (CH_3, C-21), 24.3 (CH_2), 23.6 (CH_2), 22.4 (CH_2), 20.7 (CH_2), 18.4, 18.0 (CH_3, C-26, C-27), 14.6 (CH_3, C-19), 13.8 (CH_3, C-18); **CIMS**: 401 $[M+H-BnOH-H_2O]^+$ (100), 383 (69), 365 (28).

Compound **36**; m.p. 156–160 °C; **FTIR** (KBr) ν_{max} 3417, 2938, 1699, 1455, 1370, 1261, 1067, 1044, 733, 696 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 7.27-7.25 (5H, m, H_{AR}), 4.44, 4.43 (2H, 2s, $H_{benzylic}$), 3.82 (1H, m, H-3), 3.53 (1H, m, H-2), 3.04 (1H, m, H-24), 1.98 (2H, m, H-12, H-4), 1.83 (1H, m, H-25), 1.75-0.77 (5C<u>H</u>, 8C<u>H</u>$_2$, H-12, H-4), 1.17 (3H, s, H-21), 0.95 (3H, s, H-19), 0.86, 0.85 (6H, 2d, J = 7.1, 7.0 Hz, H-26 and H-27), 0.75 (3H, s, H-18), 0.64 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 138.9 (C_{AR}), 128.3 (CH_{AR}), 127.8 (CH_{AR}), 127.4 (CH_{AR}), 84.7 (CH, C-24), 76.1 (CH), 74.9 (C, C-20), 71.8 (CH_2,

$C_{benzylic}$), 70.3 (CH), 58.2 (CH, C-17), 56.8 (CH, C-14), 55.1 (CH, C-9), 43.9 (CH, C-5), 42.9 (C, C-13), 40.3 (CH_2, C-12), 39.2 (CH_2, C-22), 35.8 (C, C-10), 34.8 (CH, C-8), 32.1 (CH_2, C-1), 31.6 (CH_2), 30.4 (CH, C-25), 26.2 (CH_3, C-21), 25.1 (CH_2), 24.5 (CH_2), 24.4 (CH_2), 23.7 (CH_2), 22.4 (CH_2), 20.0 (CH_2), 18.3, 18.1 (CH_3, C-26 and C-27), 14.1 (CH_3, C-19), 13.7 (CH_3, C-18); **CIMS**: 401 $[M+H-BnOH-H_2O]^+$ (100), 383 (68), 365 (47).

General procedure for preparation of compounds 37 and 38

To a solution of **35** or **36** (0.01 mmol) in methanol (1 mL) was added 5% Pd/C (4 mg). The black suspension was stirred under H_2 at room temperature for 2 h. The reaction mixture was filtered through celite and eluted with methanol. The filtrate was concentrated *in vacuo*.

2β, 3α, 20(*S*), 24-Tetrahydroxycholestane (37)

The filtrate was concentrated under reduced pressure to give **37** (16.9 mg, 87%) as a white solid; **FTIR** (KBr) ν_{max} 3398, 2929, 1654, 1459, 1377, 1037 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.74 (1H, m, H-2), 3.69 (1H, m, H-3), 3.19 (1H, m, H-24), 1.97 (1H, m, H-12), 1.77 (1H, m, H-4), 1.68-0.91 (5C<u>H</u>, 8C<u>H</u>$_2$, H-12, H-4), 1.18, 1.17 (3H, 2s, H-21), 0.90 (3H, s, H-19), 0.83 (6H, m, H-26 and H-27), 0.75, 0.74 (3H, 2s, H-18), 0.62 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 76.8, 76.7 (CH, C-24), 75.0, 74.8 (C, C-20), 71.0 (CH, C-2), 69.9 (CH, C-3), 58.9, 58.1 (CH, C-17), 56.4 (CH, C-14), 54.9 (CH, C-9), 42.8, 42.7 (C, C-13), 40.3, 40.2 (CH_2, C-12), 39.8 (CH_2, C-1), 39.5 (CH_2), 39.1 (CH_2), 38.7 (CH, C-5), 35.4 (C, C-10), 34.1 (CH, C-8), 33.4, 33.2 (CH, C-25), 31.6 (CH_2), 31.2 (CH_2), 28.0, 27.7 (CH_2), 25.4, 25.2 (CH_3, C-21), 23.4 (CH_2), 22.2, 22.1 (CH_2), 20.5 (CH_2), 18.6, 18.5, 17.3, 16.9 (CH_3, C-26 and C-27), 14.0 (CH_3, C-19), 13.5, 13.4 (CH_3, C-18); **CIMS**: 419 $[M+H-H_2O]^+$ (10), 401 (100), 383 (72), 365 (18).

2α, 3α, 20(*S*), 24-Tetrahydroxycholestane (38)

The filtrate was concentrated under reduced pressure to give **38** (4.2 mg, 90%) as a white solid; **FTIR** (KBr) ν_{max} 3394, 3363, 2932, 1699, 1540, 1456, 1385, 1263, 1042 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.82 (1H, m, H-3), 3.53 (1H, m, H-2), 3.24 (1H, m, H-24), 2.04-0.97 (5C<u>H</u>, 10C<u>H</u>$_2$), 1.20 (3H, s, H-21), 0.95 (3H, s, H-19), 0.85 (6H, m, H-26 and H-27), 0.77 (3H, s, H-18), 0.64 (1H, m, H-9).

3β-Acetoxy-5α-pregnan-20-one (18)

To a solution of **17** (47 mg, 0.13 mmol) in ethyl acetate (2 mL) and methanol (6 mL) was added 5% Pd/C (6.5 mg, 2.62×10^{-3} mmol) and the suspension mixture was stirred at room temperature under H_2 for 16 h. Then, the reaction was filtered through celite and eluted with dichloromethane; the filtrate was concentrated *in vacuo*. The residue was purified by flash column chromatography (1:19 ethyl acetate: hexane) to give **18** (43 mg, 91%) as a white solid; 149–150 °C; **FTIR** (KBr) ν_{max} 2925, 1723, 1705, 1362, 1261, 1037 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 4.62 (1H, m, H-3), 2.45 (1H, t, J = 9.0 Hz, H-17), 2.08 (1H, m, H-16), 2.04 (3H, s, H-20), 1.95 (3H, s, C<u>H</u>$_3$COO), 1.91 (1H, m, H-12), 1.75 (1H, m, H of CH_2), 1.70-0.78 (3C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-16, H of CH_2), 0.75 (3H, s, H-19), 0.63 (1H, m, H-9), 0.53 (3H, s, H-18); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 209.6 (CO, C-20), 170.7 (COO), 73.6 (CH, C-3), 63.8 (CH, C-17), 56.6 (CH, C-14), 54.1 (CH, C-9), 44.6 (CH, C-5), 44.2 (C, C-13), 39.0 (CH_2, C-12), 36.7 (CH_2, C-1), 35.5 (C, C-10), 35.4 (CH, C-8), 33.9 (CH_2), 31.9 (CH_2), 31.5 (CH_3, C-21), 28.4 (CH_2), 27.4 (CH_2), 24.7 (CH_2), 22.8 (CH_2, C-16), 21.4 (<u>C</u>H$_3$COO), 21.2 (CH_2), 13.4 (CH_3, C-18), 12.2 (CH_3, C-19); **CIMS**: 301 $[M+H-AcOH]^+$ (81), 283 (100).

3β, 20(*S*)-Dihydroxy-5α-cholest-24-ene (19)

To a stirring suspension of Mg (101 mg, 3.9 mmol) and I_2 catalyst in dry THF (6 mL) while being stirred, was slowly added 5-bromo-2-methyl-2-pentene (0.43 mL, 4.16 mmol) under N_2 at room temperature. After stirring for 1 h, a solution of **18** (150 mg, 0.42 mmol) in dry THF (5 mL) was added slowly. After 5 h, the reaction mixture was quenched by the addition of saturated aqueous ammonium chloride and extracted with ethyl acetate (4 × 50 mL). The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate and then with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (1:13 ethyl acetate:hexane) to give **19** (60 mg, 36%) as a white solid; **FTIR** (KBr) ν_{max} 3431, 2929, 1449, 1373, 1037 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.02 (1H, m, H-24), 3.52 (1H, m, H-3), 1.98 (1H, m, H-12), 1.91 (2H, m, H-23), 1.77-0.75 (4C<u>H</u>, 9C<u>H</u>$_2$, H-12), 1.61 (3H, s, H-26), 1.54 (3H, s, H-27), 1.20 (3H, s, H-21), 0.77 (3H, s, H-18), 0.73 (3H, s, H-19), 0.55 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 131.5 (C, C-25), 124.5 (CH, C-24), 75.2 (C, C-20), 71.3 (CH, C-3), 58.1 (CH, C-17), 56.6 (CH, C-14), 54.3 (CH, C-9), 44.9 (CH, C-5), 43.5 (CH_2, C-22), 42.9 (C, C-13), 40.4 (CH_2, C-12), 38.2 (CH_2), 37.0 (CH_2), 35.4 (C, C-10), 34.9 (CH, C-8), 31.9 (CH_2), 31.5 (CH_2), 28.7 (CH_2), 26.2 (CH_3, C-21), 25.7, 17.6 (CH_3, C-26 and C-27), 23.7 (CH_2), 22.9 (CH_2), 22.4 (CH_2), 21.1 (CH_2), 13.8 (CH_3, C-19), 12.3 (CH_3, C-18). **CIMS**: 385 $[M+H-H_2O]^+$ (100), 367 (57).

3β, 20(*S*)-Dihydroxy-5α-cholestane (20)

To a solution of **19** (20 mg, 0.05 mmol) in methanol (2 mL) was added 5% Pd/C (10 mg, 6.8×10^{-3} mmol). The black suspension was stirred under H_2 at room temperature for 2 h. The reaction mixture was filtered through celite and eluted with dichloromethane, the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatrography (1:19 ethyl acetate:hexane) to give **20** (13 mg, 64%) as a white solid; m.p. 115–117 °C; **FTIR** (KBr) ν_{max} 3306, 2929, 1462, 1382, 1256, 1096, 836, 775 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.52 (1H, m, H-3), 1.98 (1H, dt, *J* = 12.1, 3.3 Hz, H-12), 1.73 (1H, m, H of CH_2), 1.68-0.84 (5C<u>H</u>, 10C<u>H</u>$_2$, H-12, H of CH_2), 1.19 (3H, s, H-21), 0.80 (6H, d, *J* = 6.6 Hz, H-26 and H-27), 0.77 (3H, s, H-18), 0.74 (3H, s, H-19), 0.55 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 75.2 (C, C-20), 71.4 (CH, C-3), 57.8 (CH, C-17), 56.6 (CH, C-14), 54.3 (CH, C-9), 44.9 (CH, C-5), 44.2 (CH_2, C-22), 42.9 (C, C-13), 40.4 (CH_2, C-12), 39.6 (CH_2, C-24), 38.2 (CH_2), 37.0 (CH_2, C-1), 35.5 (C, C-10), 34.9 (CH, C-8), 31.9 (CH_2), 31.5 (CH_2), 28.7 (CH_2), 27.9 (CH, C-25), 26.4 (CH_3, C-21), 23.7 (CH_2), 22.7, 22.6 (CH_3, C-26 and C-27), 22.4 (CH_2), 22.0 (CH_2), 21.1 (CH_2), 13.8 (CH_3, C-18), 12.3 (CH_3, C-19); **CIMS**: 387 $[M+H-H_2O]^+$ (29), 369 (100).

Sodium 3β, 20-dihydroxy-cholestane 3-sulfate (21)

Triethylamine-sulfur trioxide complex (13.5 mg, 0.064mmol) was added to a solution of 3β, 20-dihydroxy-cholestane (20) (13 mg, 0.032 mmol) in DMF (1 mL). The reaction mixture was stirred at room temperature for 24 h, and then quenched with water (1 mL). After evaporation of solvent to dryness the residue was eluted through Amberlite CG-120 (Na form) with methanol. The residue was purified by flash column chromatrography (5:95 methanol: dichloromethane) to give **21** (10 mg, 61%) as a white solid; **FTIR** (KBr) ν_{max}, 2947, 1467, 1383, 1244, 1218, 1060, 972 cm^{-1}; **^{1}H NMR** (CD_3OD, 400 MHz) δ 4.26 (1H, m, H-3), 2.03 (1H, m, H-12), 1.83-0.90 (6C<u>H</u>, 11C<u>H</u>$_2$, H-12), 1.21 (3H, s, H-21), 0.88 (6H, d, *J* = 6.6 Hz, H-26 and H-27), 0.83 (6H, s, H-18 and H-19), 0.69 (1H, m, H-9). HRMS *m/z* $C_{27}H_{46}O_3Na$ $[M+Na]^+$, calculated 483.3144, found 483.3144.

3β-Hydroxy-5α-pregnan-20-one (24)

1M KOH (30 mL) was added slowly to a solution of **18** (2.70 g, 7.5 mmol) in methanol (25 mL) and dichloromethane (25 mL) at room temperature and stirred for 6 h. Methanol and dichloromethane were removed *in vacuo*, then the residue was diluted with dichloromethane and washed with water. The aqueous layer was extracted with dichloromethane (3 × 100 mL). The combined organic layers were washed with saturated aqueous ammonium chloride, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatrography (3:17 ethyl acetate:hexane) to give **24** (2.1 g, 90%) as a white needle after recrystallization (dichloromethane:hexane); m.p. 195–197 °C; **FTIR** (KBr) ν_{max} 3441, 2930, 1700, 1353, 1039 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.56 (1H, m, H-3), 2.48 (1H, t, *J* = 9.0 Hz, H-17), 2.12 (1H, m, H-16), 2.07 (3H, s, H-21), 1.97 (1H, m, H-12)), 1.81-0.82 (3C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-16), 0.77 (3H, s, H-19), 0.65 (1H, m, H-9), 0.57 (3H, s, H-18); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 209.6 (CO, C-20), 71.2 (CH, C-3), 63.8 (CH, C-17), 56.7 (CH, C-14), 54.2 (CH), 44.8 (CH), 44.2 (C, C-13), 39.0 (CH_2, C-12), 38.1 (CH_2), 37.0 (CH_2, C-1), 35.48 (C, C-10), 35.47 (CH), 32.0 (CH_2), 31.5 (CH_3, C-21), 31.4 (CH_2), 28.6 (CH_2), 24.4 (CH_2), 22.8 (CH_2, C-16), 21.2 (CH_2), 13.4 (CH_3, C-18), 12.2 (CH_3, C-19); **CIMS**: 317 $[M-H]^+$ (11), 301 $[M+H-H_2O]^+$ (100), 283 (22), 257 (7).

3β-Tosyloxy-5α-pregnan-20-one (25)

To a solution of **24** (1.5 g, 4.7 mmol) in dry dichloromethane (20 mL) and dry pyridine (8 mL) under N_2 gas atmosphere was added a solution of *p*-tolulenesulfonyl chloride (3.42 g, 14.2 mmol) in dry pyridine (8 mL) and dry dichloromethane (10 mL). The resulting mixture was stirred at room temperature for 24 h. The reaction was quenched by addition of water and extracted with dichloromethane (3 × 150 mL). The combined organic layers were washed with 10% HCl, saturated aqueous sodium hydrogen carbonate and then with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (1:9 ethyl acetate:hexane) to give **25** (1.8 g, 81%) as a white needle after recrystallization (dichloromethane:hexane); m.p. 126–127 °C; **FTIR** (KBr) ν_{max} 2942, 1706, 1654, 1352, 1172, 1098, 935, 670, 556 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 7.71 (2H, d, *J* = 8.2 Hz, H-2' and H-6'), 7.25 (2H, d, *J* = 8.2 Hz, H-3' and H-5'), 4.35 (1H, m, H-3), 2.43 (1H, t, *J* = 8.6 Hz, H-17), 2.37 (3H, s, C<u>H</u>$_3$-Ph), 2.12 (1H, m, H-16)), 2.07 (3H, s, H-21), 1.90 (1H, m, H-12) 1.74-0.74 (3C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-16), 0.70 (3H, s, H-19), 0.57 (1H, m, H-9), 0.51 (3H, s, H-18); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 209.5 (CO, C-20), 144.3 (C, C-4'), 134.7 (C, C-1'), 129.7 (CH, C-3' and C-5'), 127.5 (CH, C-2' and C-6'), 82.3 (CH, C-3), 63.7 (CH, C-17), 56.5 (CH), 53.9 (CH), 44.7 (CH), 44.1 (C, C-13), 38.9 (CH_2), 36.7 (CH_2), 35.3 (CH), 35.2 (C, C-10), 34.7 (CH_2), 31.7 (CH_2), 31.4 (CH_3, C-21), 28.3 (CH_2), 28.2 (CH_2), 24.3 (CH_2), 22.7 (CH_2), 21.6 (CH_3, C-7'), 21.1 (CH_2), 13.4 (CH_3, C-18), 12.0 (CH_3, C-19); **HRMS** *m/z* $C_{28}H_{40}O_4SNa$ $[M+Na]^+$, calculated 495.2545, found 495.2548.

2-Pregnen-20-one (26)

Lithium bromide (3.2 g, 34 mmol) and lithium carbonate (3.0 g, 34 mmol) were added to a solution of **25** (1.8 g, 3.8 mmol) in dimethylformamide (60 mL). The resulting mixture was refluxed for 6 h. After the reaction mixture was allowed to cool to room temperature, the suspension was filtered and the filtrate was poured into 10% HCl, extracted with dichloromethane (3 × 150 mL). The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate and with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (1:99 ethyl

acetate:hexane) to give **26** (982 mg, 86%) as a white needle after recrystallization (ethanol); m.p. 124–125 °C; **FTIR** (KBr) ν_{max} 2913, 1704, 1444, 1352, 1152 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 5.57 (2H, m, H-2, H-3), 2.50 (1H, t, *J* = 9.0 Hz, H-17), 2.13 (1H, m, H-16), 2.09 (3H, s, H-21), 1.98 (1H, m, H-12), 1.87 (2H, m, H-1), 1.75-0.82 (3C<u>H</u>, 5C<u>H</u>$_2$, H-12, H-16), 0.74 (1H, m, H-14), 0.73 (3H, s, H-19), 0.59 (3H, s, H-18); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 209.7 (CO, C-20), 125.9 (CH), 125.7 (CH), 63.9 (CH, C-17), 56.7 (CH, C-14), 53.9 (CH), 44.2 (C, C-13), 41.4 (CH), 39.8 (CH_2, C-1), 39.1 (CH_2, C-12), 35.6 (CH), 34.6 (C, C-10), 31.8 (CH_2, C-16), 31.5 (CH_3, C-21), 30.2 (CH_2), 28.6 (CH_2), 24.4 (CH_2), 22.8 (CH_2), 20.9 (CH_2), 13.3 (CH_3, C-18), 11.7 (CH_3, C-19); **HRMS** *m/z* $C_{21}H_{32}ONa$ $[M+Na]^+$, calculated 301.2531, found 301.2531.

2α, 3α-Epoxy-5α-pregnan-20-one (27)

To a solution of **26** (1.71 g, 5.65 mmol) in dichloromethane (20 mL) was added water (10 mL) and sodium carbonate (3.02 g, 28.5 mmol). The reaction mixture was stirred vigorously and *m*-chloroperbenzoic acid (1.33 g, 7.7 mmol) was added slowly. The reaction mixture was stirred at room temperature for 4–6 h. The aqueous layer was separated and extracted with dichloromethane (3 × 200 mL). The combined organic layers were washed with 5% sodium sulfite solution, saturated aqueous sodium hydrogen carbonate and with water, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by flash column chromatography (1:19 ethyl acetate:hexane) to give **27** (744 mg, 54% brsm) as a white needle after recrystallization (hexane); m.p. 152–153 °C and the starting material was recovered (426 mg, 75% conversion); **FTIR** (KBr) ν_{max} 2940, 1699, 1653, 1450, 1357, 1185 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 3.11 (1H, m, H-3), 3.08 (1H, m, H-2), 2.48 (1H, t, *J* = 9.0 Hz, H-17), 2.10 (1H, m, H-16), 2.07 (3H, s, H-21), 1.97 (1H, m, H-12), 1.87 (2H, m, H-1), 1.67-1.00 (2C<u>H</u>, 6C<u>H</u>$_2$, H-12, H-16), 0.72 (3H, s, H-19), 0.65 (1H, m, H-9), 0.56 (3H, s, H-18); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 209.5 (CO, C-20), 63.7 (CH, C-17), 56.5 (CH), 53.6 (CH), 52.3 (CH, C-3), 50.9 (CH, C-2), 44.0 (C, C-13), 38.9 (CH_2), 38.2 (CH_2), 36.2 (CH), 35.6 (CH), 33.6 (C, C-10), 31.6 (CH_2), 31.5 (CH_3, C-21), 29.0 (CH_2), 28.2 (CH_2), 24.3 (CH_2), 22.7 (CH_2, C-16), 20.8 (CH_2), 13.3 (CH_3, C-18), 12.9 (CH_3, C-19); **HRMS** *m/z* $C_{21}H_{32}O_2Na$ $[M+Na]^+$, calculated 339.2300, found 339.2300.

24-Benzyloxy-2α, 3α-epoxy-20(*S*)-hydroxy-5α-cholestane (34)

NaH (155.7 mg, 3.8 mmol) was washed with dry THF (4 mL). The suspension was stirred vigorously for 10 min before removing the solvent.

To the suspension of NaH in dry THF was slowly added the solution of **33** (165.3 mg, 0.38 mmol) in dry THF and the mixture was stirred under refluxing for 2 h. After the reaction mixture was cooled down to room temperature, benzyl bromide (0.28 mL, 1.9 mmol) was added and stirred for an additional 30 min at room temperature and then heated for 4 h under reflux. The reaction was quenched by pouring into water and extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with water, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (1:32 ethyl acetate:hexane) to give **34** (124.2 mg, 64%) as a colorless gum; **FTIR** (neat) ν_{max} 2930, 1455, 1382, 1069, 803, 735, 697 cm^{-1}; **^{1}H NMR** ($CDCl_3$, 400 MHz) δ 7.27-7.25 (5H, m, H_{AR}), 4.44, 4.43 (2H, 2s, $H_{benzylic}$), 3.08 (1H, m, H-3), 3.03 (2H, m, H-2 and H-24), 1.97 (1H, m, H-12), 1.82 (3H, m, H-1, H-25, H of CH_2), 1.68-0.78 (5C<u>H</u>, 7C<u>H</u>$_2$, H-12, H-1, H of CH_2,), 1.17 (3H, s, H-21), 0.87, 0.84 (6H, 2d, *J* = 6.8, 6.8 Hz, H-26 and H-27), 0.74 (3H, s, H-18), 0.68 (3H, s, H-19), 0.54 (1H, m, H-9); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ 139.1 (C_{AR}), 128.2 (CH_{AR}), 127.8 (CH_{AR}), 127.4 (CH_{AR}), 84.8 (CH, C-24), 75.1 (C, C-20), 71.8 (CH_2, $C_{benzylic}$), 58.2 (CH, C-17), 56.4 (CH, C-14), 53.6 (CH, C-9), 52.4 (CH, C-3), 51.0 (CH,

C-2), 42.7 (C, C-13), 40.3 (CH_2, C-12), 39.4 (CH_2, C-22), 38.2 (CH_2, C-1), 36.2 (CH, C-5), 35.0 (CH, C-8), 33.6 (C, C-10), 31.5 (CH_2), 30.6 (CH, C-25), 29.0 (CH_2), 28.4 (CH_2), 26.1 (CH_3, C-21), 24.6 (CH_2), 23.6 (CH_2), 22.3 (CH_2), 20.7 (CH_2), 18.6, 18.1 (CH_3, C-26 and C-27), 13.6 (CH_3, C-18), 12.9 (CH_3, C-19). **CIMS**: 401 $[M+H-BnOH]^+$ (5), 383 (100), 365 (34).

Biological assays

KB (Human epidermoid carcinoma of cavity, ATCC CCL-17), and NCI-H 187 (Human small cell lung carcinoma, ATCC CRL-5804) were determined by resazurin microplate assay (REMA) which was a modified method of the use of a fluorescent dye for mammalian cell cytotoxicity study according to Brien *et al*. (2000) (Brien *et al*., 2000). Ellipticine and doxorubicin were used as positive control substances. DMSO and sterile distilled water were used as negative controls. Briefly, cells at a logarithmic growth phase were harvested and diluted to10^5 cells/mL in fresh medium and gently mixed. Test compounds were diluted in culture medium in a ratio of 1:2 giving eight concentrations. Five μL of test sample and 45 μL of cells were put into 384-well microtiter plates in total volume of 50 μL/well. Plates were incubated at 37 °C, 5% CO_2, for 72 h for KB and 5 d for NCI-H187. After the incubation periods, 12.5 μL of resazurin solution was added to each well and the plates were incubated at 37 °C for 4 h. The plates were then processed for optical density absorbance analysis using a Victor 3 Microplate reader at dual wavelengths of 530 and 590 nm.

RESULTS AND DISCUSSION

Chemistry

The preparation of 3, 20-dihydroxy steroids **19-20** and 3, 20, 24-trihydroxy steroid **23** from pregnenolone **17** is shown in Figure 4. The keto ester **17** was prepared from tigogenin by the method developed by Mićović *et al*. (1990) and modified by Kim *et al*. (1999). Hydrogenation of pregnenolone **17** in 5% Pd/C gave ketone **18** with an excellent yield. Grignard reaction of **18** with 5-bromo-2-methyl-2-pentene, Mg in THF gave 3, 20-dihydroxy steroid **19** with 36% yield whereas a Grignard reaction with 4-bromopentene provided steroid **22** with 61% yield. Hydrogenation of **19** using 5% Pd/C in methanol gave the corresponding saturated side chain steroid **20** with 64% yield. Sulfation of **20** was performed using triethylamine-sulfur trioxide complex in *N,N*-dimethyl-formamide as the sulfating agent to give the monosodium salt derivative **21** with 61% yield. Ozonolysis of **22** followed by the Grignard reaction of the resulting aldehyde intermediate with 2-bromopropane and Mg yielded trihydroxy compound **23** with 75% yield as a diastereomeric mixture.

Syntheses of 2β, 3α, 20-trihydroxy steroids **29**, **31** and 2β, 3α, 20, 24-tetrahydroxy steroid **37** were started from compound **18** (Figures 5, 6 and 7). Hydrolysis of acetate in **18** by KOH/MeOH at room temperature gave the corresponding alcohol **24** with good yield. The reaction of **24** with *p*-toluenesulfonyl chloride followed by an elimination reaction by refluxing with lithium bromide, lithium carbonate in *N,N*-dimethylformamide at reflux afforded exclusively the desired Δ-2-alkene **26** with good yield. Treatment of **26** with *m*-chloroperbenzoic acid gave rise to the α-epoxy derivative **27** with 54% yield. This reaction occurred with high regioselectivity, such that the corresponding Δ-3 alkene was not detected. The Grignard reaction of keto epoxide **27** with various alkyl bromides provided the corresponding alcohols **28a**, **28b** and **28c** with moderate yields (Figure 5).

Opening of epoxides **28a** and **28b** to diols was accomplished by treatment with 1M H_2SO_4 in THF at room temperature to produce stereoisomeric mixture of 2, 3-vicinal diols **29** and **30** (5:1 ratio), and of **31** and **32** (9:1 ratio), respectively (Figure 6).

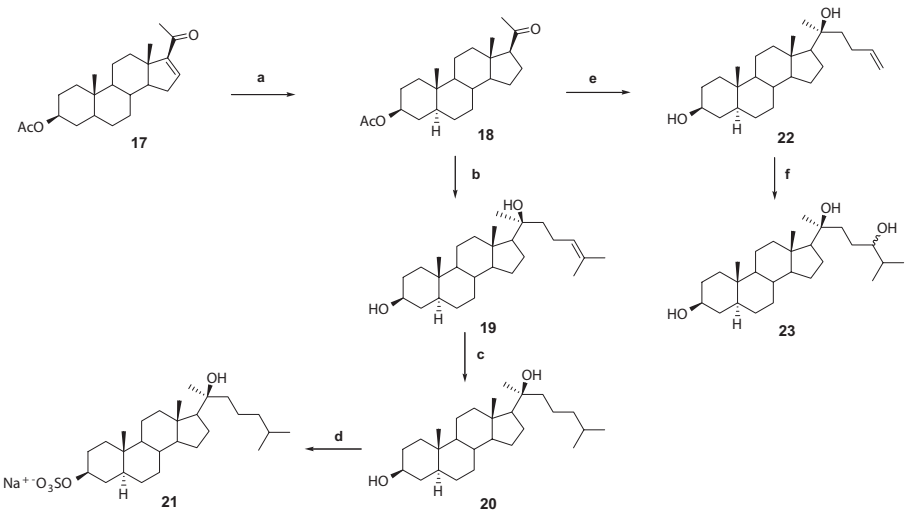


Figure 4 Synthesis of 3, 20-dihydroxy sterols **19**, **18**, **23** and a monosulfate **21**
Reagents and conditions: a) H_2, 5%Pd/C, EtOAc, EtOH, rt, 16 h. 99%; b) 5-bromo-2-methyl-2-pentene, Mg, I_2, THF, rt, 5h, 36%; c) H_2, 5% Pd/C, methanol, rt, 2h, 64%; d) $SO_3.NEt_3$, DMF, rt, 24h, 61%; e) 4-bromobutene, Mg, I_2, THF, rt, 20 min, 61%; f) (i) O_3, CH_2Cl_2, PPh_3, -78 °C, 3h, 68%; (ii) 2-bromopropane, Mg, I_2, THF, -78 °C, 1.5h, 75%.

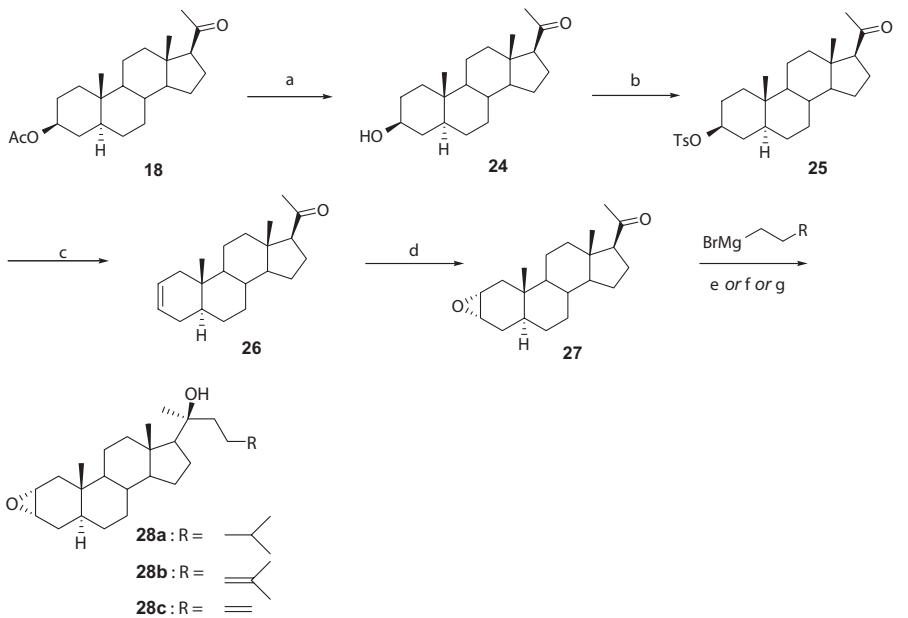


Figure 5 Synthesis of steroid epoxides **28**.
Reagents and conditions: a) 1M KOH, MeOH, CH_2Cl_2 (1:1), rt, 6h, 90%; b) *p*-toluenesulfonyl chloride, CH_2Cl_2, pyr, rt, 24h, 81%; c) LiBr, Li_2CO_3, DMF, reflux 6h, 86%; d) *m*CPBA, Na_2CO_3, CH_2Cl_2, H_2O, rt, 6h, 54% (brsm), 75% conversion; e) 1-bromo-4-methylpentane, Mg, I_2, THF, rt, 2h, 50%; f) 5-bromo-2-methyl-2-pentene, Mg, I_2, THF, rt, 2h, 39%; g) 4-bromobutene, Mg, I_2, THF, rt, 20 min, 50%.

Ozonolysis of **28c** followed by the Grignard reaction of the resulting aldehyde intermediate with 2-bromopropane and Mg yielded dihydroxy compound **33** with 48% yield as a diastereomeric mixture. Selective benzylation of the diol **33** with benzyl bromide in THF at refluxing temperature provided benzyl ether **34** with moderate yield. Hydrolysis of **34** with 1M H_2SO_4 in THF at room temperature afforded isomeric 2, 3-diols **35** and **36** in a 15:1 ratio. Debenzylation of **35** and **36** was accomplished by hydrogenation using 5% Pd/C in methanol to provide tetrahydroxy compounds **37** and **38** with good yield (Figure 7).

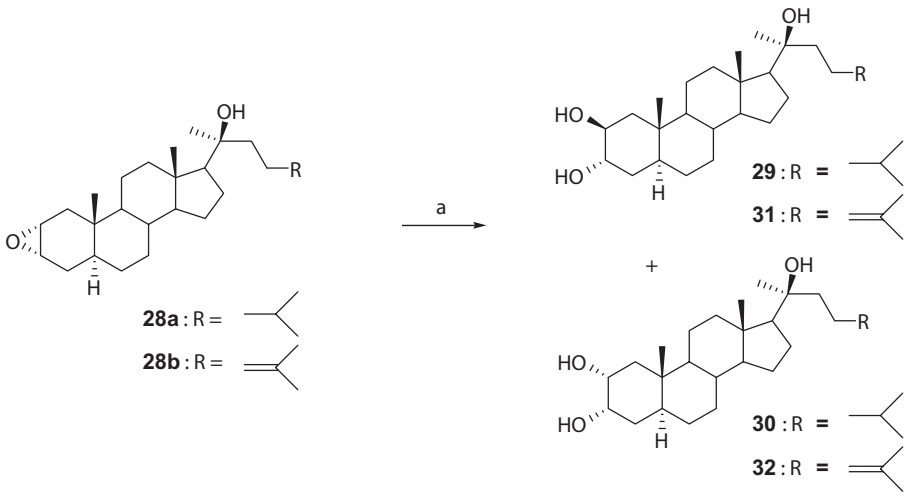


Figure 6 Synthesis of 2, 3, 20-trihydroxy steroids **29**, **30**, **31** and **32** *Reagents and conditions*: a) 1M H_2SO_4, THF, rt, 24h, (75.6%, **29**:**30** = 5:1) and (62.3%, **31**:**32** = 9:1).

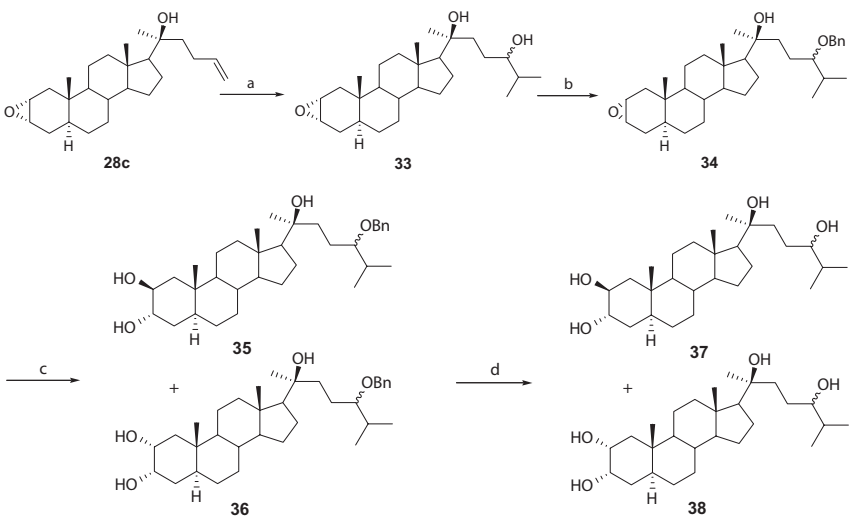


Figure 7 Synthesis of 2, 3, 20, 24-tetrahydroxy steroids **37** and **38** *Reagents and conditions*: a) (i) O_3, $NaHCO_3$, CH_2Cl_2, -78 °C; (ii) 2-bromopropane, Mg, I_2, THF, -78 °C-rt, 3h, 48%; b) BnBr, NaH, THF, reflux, 5h, 64%; c) 1M H_2SO_4, THF, rt, 24h, 57% (**35**:**36** = 15:1); d) H_2, 5% Pd/C, MeOH, rt, 2h, 87% for **37** and 90% for **38**.

Biological activity

The synthesized polyoxygenated steroids (**19, 20, 23, 29, 31, 37**) and monosulphate derivative **21** were treated *in vitro* against NCI-H187 and KB tumor cell lines (Table 1). Compound **23** containing trihydroxy at C-3, C-20 and C-24 showed strong activity against both NCI-H187 and KB (IC_{50} 2.11 and 5.39 μg/mL) whereas the analogue dihydroxy compounds **19** and **20** showed moderate activities against KB. However, **19** was strongly active against NCI-H187, but **20** was inactive. The trihydroxy steroids which contain hydroxyl groups at C-2, C-3 and C-20, compound **29** and **31** showed strong activity against NCI-H187 (IC_{50} 9.08, 9.59 μg/mL), but against KB, **31** showed strong activity and **29** was inactive. The only difference between the two compounds is that **31** contains an unsaturated side chain. The tetrahydroxy analogue **37** did not show activities against the tested cells. Likewise compound **21,** the sulphate salt of **20,** showed no activity against all tested cells.

CONCLUSION

The synthesis of six polyoxygenated steroids and one sulfate derivative were accomplished. Compound **23** containing a trihydroxy group at C-3, C-20 and C-24 showed strong activity against both NCI-H187 and KB cells. 3β, 20- Dihydroxy steroid **19** showed strong activity against NCI-H187 but was weakly active against KB whereas the analog **20** which has a saturated side chain showed moderate activity against KB and was inactive against NCI-H187 cell line. The sulfate derivative of **20**, compound

Table 1 Cytotoxicity of the synthetic steroids against human carcinoma cell lines NCI-H187 and KB.

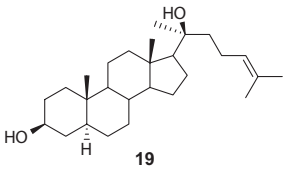
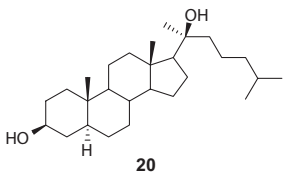
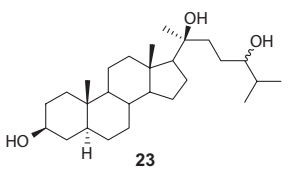
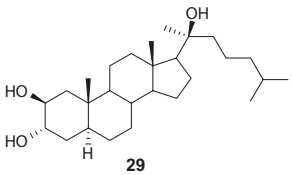
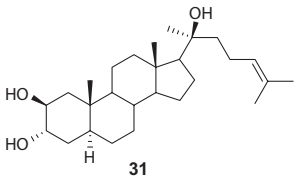
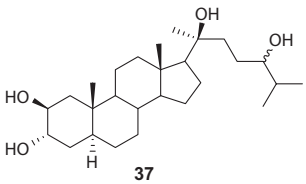
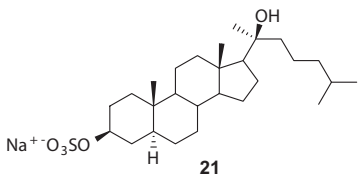
Entry	Compound	IC_{50} (μg/mL)[a]	
		NCI-H187	KB
1	 **19**	4.24	39.12
2	 **20**	Inactive[b]	20.51
3	 **23**	2.11	5.39

Table 1 Cytotoxicity of the synthetic steroids against human carcinoma cell lines NCI-H187 and KB.

Entry	Compound	IC_{50} (μg/mL)[a]	
		NCI-H187	KB
4	 **29**	9.08	Inactive[b]
5	 **31**	9.59	10.14
6	 **37**	Inactive[b]	Inactive[b]
7	 **21**	Inactive[b]	Inactive[b]
8	Ellipticine[c]	0.57	0.393
9	Doxorubicine[c]	0.43	0.16

NCI-H187, human small cell lung carcinoma;
KB, human epidermoid carcinoma of cavity.
[a] Data are typical values from six replicate experiments.
[b] Inactive = inhibition < 50%.
[c] Positive control compound.

21, showed no cytotoxic potency for both NCI-H187 and KB cells. Compounds **29** and **31,** containing vicinal dihydroxy groups in ring A and another OH at C-20, showed similar activity against NCI-H187 but only **31** displayed strong cytotoxic potency for KB whereas **29** was inactive. The analogue compound **37**, which has an extra hydroxyl group at C-24, was inactive against both tested cell lines.

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