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Further study on synthesis and evaluation of 3,16,20-polyoxygenated steroids of marine origin and their analogs as potent cytotoxic agents

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ABSTRACT

A series of new polyoxygenated steroid derivatives with various steroid skeleton moieties were synthesized. Antitumor activity of the compounds against three tumor cell lines (Breast cancer MCF7, lung cancer NCI and oral cancer KB) were evaluated. Compounds with aromatic A ring of this series exhibited the most potent cytotoxicities in all tested cells. The absence of OH at C-16 or lack of cholesterol like side chain at C-20 in the steroid skeleton apparently result in decreased cytotoxicity. The compound became inactive when the side chain contains double bond at C-24–C-25. When hydroxyl group at C-3 was protected no cytotoxicities against MCF7 and NCI and considerable low cytotoxicity against KB cell lines were observed.

1. Introduction

Steroids isolated from various marine organisms (marine steroids) manifest diverse biological activities [1–3]. Some of them are extremely toxic against tumor cells [4–7] and show anti-inflammatory [8] and other effects [9,10]. It is therefore not surprising that marine steroids arouse considerable interest in not only chemists, but also among pharmacologists and physicians.

A large number of polyoxygenated steroids have been found in algae, and virtually in all marine invertebrate phyla, such as Porifera, Coelenterata, Bryozoa, Molusca, Echinodermata, Arthropoda and Tunicata as well as in fish [1]. A series of polyoxygenated steroids with uncommonly present oxidation both at C-16 and C-20 were isolated from the anthozoan *Antipathes subpinnata* [11,12] and the gorgonian *Leptogorgia sarmentosa* [13–16] and two of these polyoxygenated substances were reported to exhibit cytotoxicity against P388 or mouse lymphoid neoplasma human lung cancinoma (A549), human colon carcinoma (HTG) and human metanoma (MEL 28) with ED_{50} value of 1 mg/mL in all tested cells [16]. Recently [17] we described the synthesis of four naturally occurring 3,16,20-trioxygenated cholestanes, (20*S*)-20-hydroxycholestane-3,16-dione (**1**), (16*S*,20*S*)-16,20-dihydroxy cholestan-3-one (**2**), (20*S*)-20-hydroxycholest-1-ene-3,16-dione (**3**) and (20*S*)-20-hydroxy cholest-4-ene-3,16-dione (**4**) (Fig. 1) and their antitumor activity against three cell lines [human breast cancer cell lines (MCF7), human lung cancer cell lines (NCI) and human epidermoid carcinoma cell lines (KB)]. In our study it was found that compound **1** containing keto functional group at both C-3 and C-16 lack of activity to all tested cell lines and compound **2** which contains keto functional group at C-3 and a hydroxyl group at C-16 showed activity against only NCI, whereas compounds **3** and **4** containing α,β-unsaturated ketone at ring A showed significant cytotoxicity for NCI and moderated activity for MCF7 and KB cell lines. These results provided useful information for future design. Therefore, in order to determine the role of the type and degree of unsaturation in A ring and also alkyl side chain of polyoxygenated steroids in the anticancer activity we decided to proceed in our investigation by synthesizing various types of 3,16,20-trioxygenated steroids in which, ring A was modified as quinone or aromatic ring, different functionality at C-16 and different degrees of oxidation at the cholesterol side chain were also prepared and evaluated the anticancer activity of these compounds and gain insight into structure–activity relationships (SARs).

2. Experimental

2.1. General

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 400 and 100 MHz Brucker 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 $CHCl_3$ (7.26 ppm for ^{1}H

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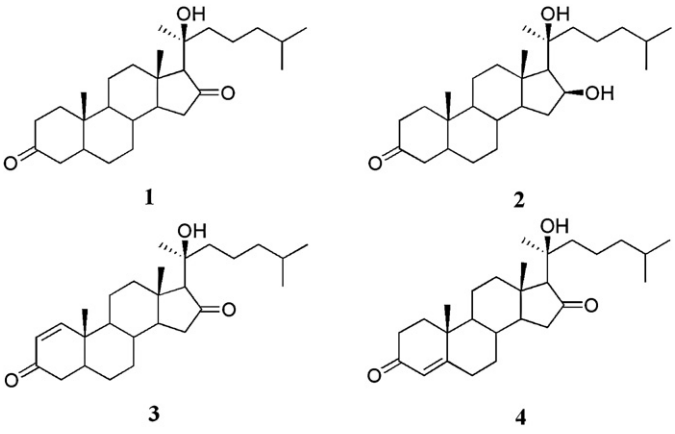


Fig. 1. Synthetic natural marine steroids.

and 77.23 ppm for ^{13}C) was used as the internal reference. Coupling constants (*J*) are given in Hz, and multiplicity is defined as follows: br = broad, s = singlet, d = doublet, dd = double of doublets, dt = double of triplet, t = triplet, q = quartet, m = multiplet. Infrared (IR) spectra were recorded in 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 a Agilent Technology 1100 series LL/MSD Trap. Melting points (mp) were determined on a Fisher John apparatus and MEI-TEMP capillary melting point apparatus at the Chemistry Department, Kasetsart University and are reported uncorrected in °C. All chemicals and solvents were purchased from the Fluka Co. Ltd. as analytical grade and solvents were purified by general method before being used. m-IBX was prepared as described by Santagostino and co-workers [18].

2.2. General procedure for preparation of compounds **10**, **29** and **35**

The mixture containing of Mg turning (338 mg), I_2 (catalytic amount) and dry THF (30 mL) was added 5-bromo-2-methylpentane or 5-bromo-2-methyl-2-pentene (9.39 mmol) at room temperature under N_2. The mixture was stirred at room temperature for 3 h and then the solution of **9**, **28** or **34** (1.87 mmol) in dry THF (20 mL) was added drowised at 0 °C. The reaction mixture was stirred at 0 °C for 20 min and then quenched with ice-water followed by neutralized with diluted HCl. The reaction mixture was then extracted with methylene chloride. The combined organic layers were washed with water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography.

2.2.1. Synthesis of 3,16S,20S-trihydroxy-24-cholestene-$\Delta^{1,3,5(10)}$-estratriene (**10**)

The crude product was purified by flash column chromatography eluting with 3:7 ethyl acetate:hexane to provide **10** as a white solid (164.5 mg, 60%), mp 220–222 °C. FTIR (KBr), ν_{max} 3427 (OH) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.00 (d, *J* = 8.32 Hz, 1H, H-1), 6.53 (dd, *J* = 8.32, 2.56 Hz, 1H, H-2), 6.47 (d, *J* = 2.56 Hz, 1H, H-4), 5.05 (m, 1H, H-24), 4.54 (m, 1H, H-16), 2.73 (m, 2H, H-6), 2.66 (m, 1H, H-15), 2.16 (m, 2H, H-9, H-12), 1.93 (m, 1H, H-11), 1.91 (m, 2H, H-23), 1.77 (m, 1H, H-22), 1.74 (m, 1H, H-7), 1.66 (s, 3H, H-26), 1.57 (m, 1H, H-22), 1.55 (s, 3H, H-27), 1.43 (m, 1H, H-8), 1.32 (m, 1H, H-17), 1.31 (m, 3H, H-7, H-11, H-15), 1.27 (m, 1H, H-12), 1.25 (s, 3H, H-21), 1.07 (s, 3H, H-18), 0.97 (m, 1H, H-14). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.1 (C-3), 137.7 (C-5), 131.7 (Me-25), 131.3 (C-10), 126.0 (C-1), 124.4 (C-24), 112.5 (C-2), 115.0 (C-4), C-20 overlap with $CDCl_3$, 73.4 (C-16), 59.8 (C-17), 53.3 (C-14), 43.6 (C-13), 43.5 (C-9), 43.1 (C-22), 40.4 (C-12), 37.6 (C-8), 36.5 (C-15), 29.4 (C-6), 27.4 (C-7), 26.3 (C-11), 26.0 (C-27), 25.4 (C-21), 23.2 (C-23), 17.4 (C-26), 14.6 (C-18). MS (APCI), *m*/*z* (relative intensity): 399 (4), 381 (98), 363 (100). HRMS *m*/*z*: $C_{26}H_{38}O_3Na$ $[M+Na]^+$, cald 421.2719, found 421.2721.

2.2.2. 3-Benzyloxy, 16R,20S-dihydroxy-24-cholestene-$\Delta^{1,3,5(10)}$-estratriene (**29**)

The crude product was purified by flash column chromatography eluting with 3:7 ethyl acetate:hexane to provide **29** (204 mg, 38.9%) as a colorless wax. FTIR (neat) ν_{max} 3411 (OH) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$ and CD_3OD) δ 7.31 (m, 5H, CH-Ar), 7.09 (d, *J* = 8.6 Hz, 1H, CH-1), 6.70 (dd, *J* = 8.6, 2.8 Hz, 1H, CH-2), 6.64 (d, *J* = 2.8 Hz, 1H, CH-4), 5.08 (m, 1H, H-24), 4.96 (s, 2H, CH_2OBn), 4.49 (m, 1H, CH-16), 2.79 (m, 2H, CH_2-6), 2.25 (m, 1H, H-9), 1.90 (m, 1H, H-11), 1.86 (m, 1H, H-12), 1.75 (m, 1H, H-15), 1.69 (m, 2H, H-7), 1.64 (s, 3H, H-26), 1.60 (m, 1H, H-11), 1.59 (m, 2H, H-14), 1.58 (s, 3H, H-27), 1.49 (m, 1H, H-15, H-8), 1.27 (s, 3H, H-21), 1.24 (m, 1H, H-12), 0.72 (s, 3H, H-18). ^{13}C NMR (100 MHz, $CDCl_3$ and CD_3OD) δ 156.7 (C-3), 138.0 (C-Ar), 137.3 (C-5), 133.0 (C-25), 131.8 (C-10), 128.5 (Ar), 127.8 (Ar), 127.4 (Ar), 126.1 (C-1), 124.4 (C-24), 114.8 (C-4), 112.3 (C-2), 76.3 (C-20), 73.4 (C-16), 69.9 (C-OBn), 69.5 (C-17), 52.8 (C-14), 45.2 (C-13), 43.9 (C-22), 43.6 (C-9), 40.0 (C-12), 37.7 (C-8), 34.6 (C-15), 29.7 (C-6), 27.2 (C-7), 26.2 (C-11), 25.7 (C-21), 25.6 (C-27), 22.1 (C-23), 17.7 (C-26), 15.2 (C-18). MS (APCI), *m*/*z* (relative intensity): 488 (28), 471 (100), 453 (73).

2.2.3. 3β,20S-Dihydroxycholestane (**35**)

The crude product was filtered through a short column (eluting with 3:7 ethyl acetate:hexane) to provide the product **35** as a white solid (67.2 mg, 60%) mp 141–143 °C [lit-141–143 °C] [19]. FTIR (KBr) υ_{max} 3366 (OH) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz). δ 3.15 (m, 1H, H-3), 1.25–1.72 (m, 20H, CH_2) 1.40–1.50 (m, 6H, CH) 1.23 (s, 3H, H-21), 1.18 (m, 2H, H-23), 1.11 (m, 2H, H-15), 1.07 (s, 3H, H-18), 0.98 (m, 1H, H-14), 0.81 (s, 3H, H-19), 0.82 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.60 (m, 1H, H-9), ^{13}C NMR ($CDCl_3$) δ 75.8 (C-20), 72.4 (C-3), 59.1 (C-17), 54.9 (C-14), 54.1 (C-9), 45.9 (C-5), 43.3 (C-13), 43.2 (C-22), 42.1 (C-4), 40.4 (C-12), 39.7 (C-24), 36.6 (C-1), 36.9 (C-2), 35.7 (C-10), 35.0 (C-8), 31.8 (C-7), 28.8 (C-6), 28.1 (C-25), 26.4 (C-21), 25.0 (C-16), 24.6 (C-15), 23.1 (C-27); 22.9 (C-26), 21.2 (C-23), 20.9 (C-11), 18.0 (C-18), 11.6 (C-19). MS (APCI), *m*/*z* (relative intensity): 404 (M^+,3), 389 (7), 386 (27), 319 (100), 301 (75), 276 (48), 258 (38). HRMS *m*/*z*: $C_{27}H_{48}O_2Na$ $[M+Na]^+$, cald 427.3552, found 427.3561.

2.3. General procedure for preparation of compound **18**, **31**

To a stirred solution of **17** or **29** (0.15 mmol)) in CH_2Cl_2 (50 mL) was added NaOAc (7.27 mmol) and PCC (8.0 mmol) at room temperature. After stirring for 1 or 3 h, the reaction mixture was filtered through celite pad. The filtrate was evaporated and purified by flash column chromatography.

2.3.1. Synthesis of 3-tert-butyl-dimethylsiloxy-(16S,20S)-16,20-acetonide-24-one-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**18**)

The crude product was purified by flash column chromatography eluting with 1:19 ethyl acetate:hexane to provide **18** as a white solid (100 mg, 60%) mp 54–55 °C. FTIR (KBr) ν_{max} 1708 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 6.91 (d, *J* = 8.44 Hz, 1H, H-1), 6.41 (dd, *J* = 8.44, 2.64 Hz, 1H, H-2), 6.36 (d, *J* = 2.64 Hz, 1H, H-4), 4.36 (m, 1H, H-16), 2.26 (m, 2H, H-6), 2.43 (d, *J* = 6.90 Hz, 1H, H-25), 2.42 (m, 2H, H-23), 2.10 (m, 1H, H-22), 2.09 (m, 1H, H-15), 2.03 (m, 1H, H-11), 2.00 (m, 1H, H-9), 1.92 (m, 1H, H-12), 1.68 (m, 1H, H-7), 1.46 (m, 1H, H-22), 1.42 (m, 1H, H-11), 1.32 (m, 1H, H-8), 1.25 (m, 1H, H-15), 1.22 (s, 3H, Me), 1.19 (m, 1H, H-12), 1.15 (s, 3H, Me), 1.10 (s, 3H, H-21), 0.93 (s, 3H, H-18), 0.92 (d, *J* = 6.9 Hz, 6H, H-26, H-27), 0.90 (m, 1H, H-14), 0.80 (m, 1H, H-17), 0.79 (s, 6H, $\underline{Me_3}$CSi), 0.0 (s, 3H, $\underline{Me_2}$Si).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 214.8 (C-24), 153.3 (C-3), 137.8 (C-5), 133.1 (C-10), 126.0 (C-1), 117.1 (C-4), 120.0 (C-2), 97.15 (C), 74.3 (C-20), 68.7 (C-16), 56.0 (C-17), 53.8 (C-14), 44.0 (C-9), 42.7 (C-13), 41.9 (C-25), 40.3 (C-12), 37.9 (C-8), 36.8 (C-22), 34.7 (C-23), 33.5 (C-15), 31.7 (Me), 29.6 (C-6), 27.6 (C-7), 26.1 (C-11), 25.8 (C-21), 25.7 (MeCSi), 25.7 (MeCSi), 25.7 (MeCSi), 23.9 (Me), 18.5 (C-27), 18.4 (C-26), 18.2 (CSi), 14.9 (C-18), −0.40 (MeSi), −0.40 (MeSi). MS (APCI), *m*/*z* (relative intensity): 569 (100), 493 (87).

2.3.2. 3-Benzyloxy, 20S-hydroxyl-24-cholestene-16-one-$\Delta^{1,3,5(10)}$-estratriene (**31**)

The crude product was purified by flash column chromatography (3:17 ethyl acetate:hexane) to yield **31** (39.8 mg, 80%) as a white solid. 85–86 °C FTIR (neat) υ_{max} 3439, 1727 cm^{-1}, ^{1}H NMR (400 MHz, $CDCl_3$ and CD_3OD) δ 7.30 (m, 5H, CH-Ar), 7.08 (d, *J* = 8.4 Hz, 1H, H-1), 6.70 (dd, *J* = 8.4, 2.7 Hz, 1H, H-2), 6.65 (d, *J* = 2.7 Hz, 1H, H-4), 5.10 (m, 1H, H-24), 5.01 (s, 2H, CH_2OBn), 2.80 (m, 2H, H-6), 1.65 (s, 3H, H-26), 1.59 (s, 3H, H-27), 1.31 (s, 3H, H-21), 1.00 (s, 3H, H-18). ^{13}C NMR (100 MHz, $CDCl_3$ and CD_3OD) δ 220.7 (C-16), 156.7 (C-3), 138.2 (C-Ar), 137.2 (C-5), 133.3 (C-25), 132.0 (C-10), 128.6 (CH-Ar), 127.7 (CH-Ar), 127.3 (CH-Ar), 126.2 (C-1), 124.8 (C-24), 114.7 (C-4), 112.4 (C-2), 73.6 (C-20), 220.7 (C-16), 70.1 (CH_2OBn), 71.9 (C-17), 52.6 (C-14), 45.2 (C-13), 43.4 (C-22), 43.5 (C-9), 40.1 (C-12), 37.8 (C-8), 39.1 (C-15), 30.0 (C-6), 27.3 (C-7), 26.5 (C-11), 25.8 (C-21), 24.6 (C-27), 22.2 (C-23), 17.6 (C-26), 15.0 (C-18).

2.4. General procedure for preparation of compounds **11**, **13**, **21**, **27**, **30**, **32** and **34**

To a stirred suspension of 5% Pd/C (0.0064 mmol) in ethanol (3 mL) was added a solution of **10**, **12**, **16**, **26**, **29**, **31** or **33** (0.126 mmol) in ethyl acetate (3 mL). The reaction mixture was treated with hydrogen ballon and stirred for 16 h. The reaction mixture was filtered through celite pad and rinsed with ethyl acetate. The filtrate was concentrated by using rotary evaporator. The crude product was purified by flash column chromatography.

2.4.1. Synthesis of 3,16S,20S-trihydroxycholestene-$\Delta^{1,3,5(10)}$-estratriene (**11**)

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **11** (11 mg, 100%) as a white solid, mp 245–246 °C. FTIR (KBr) υ_{max} 3389 (OH) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 7.02 (d, *J* = 8.4 Hz, 1H, H-1), 6.54 (dd, *J* = 8.4, 2.7 Hz, 1H, H-2), 6.47 (d, *J* = 2.6 Hz, 1H, H-4), 4.52 (m, 1H, H-16), 2.71 (m, 2H, H-6), 2.23 (m, 1H, H-12), 2.15 (m, 1H, H-11), 2.07 (m, 1H, H-9), 1,74 (m, 2H, H-7), 1.49 (m, 1H, H-11), 1.48 (m, 1H, H-8), 1.43 (m, 1H, H-25), 1.40 (m, 2H, H-22), 1.29 (m, 2H, H-24), 1.27 (m, 1H, H-12), 1.23 (m, 1H, H-17), 1.22 (s, 3H, H-21), 1.18 (m, 2H, H-23), 1.11 (m, 2H, H-15), 1.07 (s, 3H, H-18), 0.98 (m, 1H, H-14), 0.80 (d, *J* = 6.6 Hz, 6H, H-26, H-27). ^{13}C NMR ($CDCl_3$) δ 154.0 (C-3), 137.5 (C-5), 131.4 (C-10), 125.8 (C-1), 114.8 (C-4), 112.3 (C-2), 76.7 (C-20), 73.1 (C-16), 59.4 (C-17), 53.1 (C-14), 44.0 (C-13), 43.5 (C-9), 42.9 (C-22), 40.2 (C-12), 39.4 (C-15), 37.5 (C-8), 36.3 (C-24), 29.3 (C-6), 27.6 (C-25), 27.3 (C-7), 26.1 (C-11), 25.7 (C-21), 22.2 (Me × 2), 22.1 (C-23), 14.3 (C-18). MS (APCI), *m*/*z* (relative intensity): 401 (7), 383 (90), 365 (100). HRMS *m*/*z*: $C_{26}H_{40}O_3Na$ [M+Na]$^+$, cald 423.2875, found 423.2862.

2.4.2. 3-Hydroxy-(16S,20S)-16,20-acetonide-cholestan-$\Delta^{1,3,5(10)}$-estratriene (**13**)

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **13** (8 mg. 99%) as a colorless syrup. FTIR (KBr) υ_{max} 3380 (OH) cm^{-1} ^{1}H NMR ($CDCl_3$) δ 7.06 (m, 1.6H, H-1), 6.55 (dd, *J* = 8.4, 2.76 Hz, 1.6H, H-2), 6.49 (d, *J* = 2.7 Hz, 1.6H, H-4), 4.45 (m, 1H, H-16), 2.73 (m, 2H, H-6), 2.19 (m, 1H, H-15) 2.14 (m, 1H, H-9), 2.12 (m, 1H, H-11), 2.09 (m, 1H, H-12), 1.82 (m, 2H, H-7), 1.46 (m, 2H, H-8, H-11), 1.43 (m, 1H, H-25), 1.42 (m, 1H, H-15) 1.40 (m, 2H, H-22), 1.29 (m, 2H, H-24), 1.27 (s, 9H, Me, Me, H-21), 1.18 (m, 2H, H-23), 1.12 (s, 3H, Me-18), 1.02 (m, 1H, H-14), 0.85 (m, 1H, H-17). 0.80 (d, *J* = 6.6 Hz, 6H, H-26, H-27), ^{13}C NMR ($CDCl_3$) δ 153.4 (C-3), 138.1 (C-5), 132.6 (C-10), 127.6 (C-1), 115.2 (C-4), 112.3 (C-2), 78.3 (C-20), 74.1 (C-16), 60.3 (C-17), 53.5 (C-14), 44.4 (C-13), 43.7 (C-9), 43.3 (C-22), 40.5 (C-12), 39.6 (C-15), 37.6 (C-8), 37.1 (C-24), 29.5 (C-6), 27.9 (C-25), 27.5 (C-7), 26.9 (C-11), 26.4 (C-21), 22.7 (Me), 22.6 (Me), 22.4 (C-23), 15.1 (C-18). HRMS *m*/*z*: $C_{29}H_{44}O_3Na$ [M+Na]$^+$, cald 463.3188, found 463.3191.

2.4.3. 3-tert-Butyl-dimethylsiloxy-(16S,20S)-16,20-acetonide-cholestane-$\Delta^{1,3,5(10)}$-estratriene (**21**)

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **21** (70 mg. 99%) as a colorless syrup. FTIR (KBr), υ_{max} 1497, 1246 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.02 (d, *J* = 8.45 Hz, 1H, H-1), 6.52 (dd, *J* = 8.45, 2.66 Hz, 1H, H-2), 6.47 (d, *J* = 2.66 Hz, 1H, H-4), 4.47 (m, 1H, H-16), 2.78 (m, 2H, H-6), 2.20 (m, 1H, H-9), 1.95 (m, 1H, H-11),1.90 (m, 1H, H-15), 1.85 (m, 1H, H-12), 1.82 (m, 2H, H-7), 1.81 (m, 1H, H-22), 1.61 (m, 1H, H-11), 1.48 (m, 1H, H-15), 1.45 (m, 1H, H-8), 1.38 (m, 1H, H-22), 1.31 (m, 2H, H-21), 1.28 (m, 2H, H-24), 1.24 (m, 1H, H-12), 1.19 (m, 2H, H-23), 1.13 (s, 1H, H-18), 1.10 (m, 1H, H-14), 1.00 (s, 9H, Me_3CSi), 0.86 (m, 1H, H-17), 0.85 (d, *J* = 6.6 Hz, 6H, H-26, H-27), 0.82 (s, 6H, Me_2Si). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.4 (C-3), 137.3 (C-5), 131.9 (C-10), 126.2 (C-1), 119.9 (C-4), 117.1 (C-2), 97.2 (C), 79.0 (C-20), 74.9 (C-16), 60.6 (C-17), 53.8 (C-14), 44.2 (C-9), 43.8 (C-13), 43.0 (C-22), 40.0 (C-12), 37.8 (C-8), 37.2 (C-24), 33.7 (C-15), 31.8 (Me), 29.9 (C-6), 28.1 (C-25), 27.4 (C-7), 27.1 (C-11), 26.5 (C-21), 25.8 (MeCSi), 24.2 (Me), 22.7 (C-27), 22.6 (C-26), 22.5 (C-23), 17.7 (CSi), 15.3 (C-18), −0.40 (MeSi), −0.4 (MeSi). MS (APCI), *m*/*z* (relative intensity): 554 (100), 494 (30).

2.4.4. 3,16α-Dihydroxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**27**)

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **27** (19.24 mg, 99%) as a white solid, mp 94–96 °C. FTIR (KBr), υ_{max} 3436 (OH), 1699 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.16 (d, *J* = 8.53 Hz, 1H, H-1), 6.66 (dd, *J* = 8.53, 2.74 Hz, 1H, H-2), 6.48 (d, *J* = 2.74 Hz, 1H, H-4), 4.87 (m, 1H, H-16), 2.85 (m, 2H, H-6), 2.83 (m, 1H, H-9), 2.63 (m, 1H, H-17), 2.20 (m, 1H, H-15), 2.16 (s, 3H, H-21), 2.10 (m, 1H, H-11), 2.08 (m, 1H, H-12), 1.77 (m, 2H, H-7), 1.62 (s, 3H, Me), 1.55 (s, 3H, Me), 1.48 (m, 1H, H-11), 1.47 (m, 1H, H-8), 1.43 (m, 1H, H-15), 1.29 (m, 1H, H-12), 1.23 (s, 3H, Me), 1.15 (s, 3H, Me), 1.08 (s, 3H, H-18), 1.02 (m, 1H, H-14), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.6 (C-20), 156.9 (C-3), 137.8 (C-10), 132,2 (C-5), 126.2 (C-1), 115.3 (C-4), 112.8 (C-2), 71.8 (C-16), 55.4 (C-14), 46.3 (C-13), 44.5 (C-9), 39.2 (C-8), 37.1 (C-17), 34.6 (C-12), 32.1 (C-15), 28.2 (C-6), 27.5 (C-11), 26.2 (C-21), 26.1 (C-7), 15.8 (C-18). HRMS *m*/*z*: $C_{20}H_{26}O_3Na$ [M+Na]$^+$, cald 337.18780, found 337.18786.

2.4.5. 3-Hydroxy, 16R,20S-trihydroxy-cholestane-$\Delta^{1,3,5(10)}$-estratriene (**30**)

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **30** (50 mg, 99%) as a white solid, mp 94–96 °C. FTIR (KBr), υ_{max} 3347 (OH) cm^{-1}. ^{1}H NMR ($CDCl_3$ and CD_3OD) δ 7.00 (d, *J* = 8.48, Hz, 1H, H-1), 6.53 (dd, *J* = 8.36, 2.64 Hz, 1H, H-2), 6.47 (d, *J* = 2.52 Hz, 1H, H-4), 4.42 (m, 1H, H-16), 2.72 (m, 2H, H-6), 2.16 (m, 1H, H-9), 2.14 (m, 1H, H-11), 1.94 (m, 1H, H-12), 1.74 (m, 1H, H-7), 1.62 (m, 2H, H-23), 1.61 (m, 1H, H-17), 1.60 (m, 2H, H-14), 1.58 (m, 3H, H-22), 1.47 (m, 1H, H-25), 1.43 (m, 2H, H-24), 1.38 (m, 1H, H-12), 1.34 (m, 1H, H-8), 1.29 (m, 1H, H-7), 1.22 (s, 3H, H-21), 1.20 (m, 2H, H-15), 0.81 (d, *J* = 6.64 Hz, 6H, H-26, H-27), 0.71 (s, 3H, H-18). ^{13}C NMR ($CDCl_3$ and CD_3OD) δ 154.9 (C-3), 137.6 (C-5), 131.3 (C-10), 125.8 (C-1), 114.9 (C-4), 112.4 (C-2),

75.5 (C-20), 72.8 (C-16), 67.6 (C-17), 52.6 (C-14), 43.5 (C-13), 43.4 (C-9), 43.3 (C-22), 39.7 (C-12), 39.3 (C-15), 37.6 (C-8), 34.3 (C-24), 29.3 (C-6), 27.8 (C-25), 27.3 (C-7), 25.8 (C-11), 24.7 (C-21), 22.3 (C-26), 22.2 (C-27), 20.6 (C-23), 14.8 (C-18). HRMS *m*/*z*: $C_{26}H_{40}O_3Na$ $[M+Na]^+$, cald 423.2875, found 423.2874.

*2.4.6. 3-Hydroxy, 20S-hydroxyl-cholestane-16-one-$\Delta^{1,3,5(10)}$-estratriene (**32**)*

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **32** (47.0 mg, 99%) as a white solid, mp 94–96 °C. FTIR (KBr), υ_{max} 3347 (OH), 1727 cm^{-1}. ^1H NMR ($CDCl_3$ and CD_3OD) δ 7.06 (d, *J* = 8.34, Hz, 1H, H-1), 6.52 (dd, *J* = 8.34, 2.57 Hz, 1H, H-2), 6.48 (d, *J* = 2.57 Hz, 1H, H-4), 2.81 (m, 2H, H-6), 2.21 (dd, *J* = 18.4, 13.4, 1H, H-15), 2.18 (m, 1H, H-9), 2.12 (m, 1H, H-11), 2.12 (d, *J* = 6.8 Hz, 1H, H-17), 1.81 (dd, *J* = 13.4, 8.8 Hz 1H, H-15), 1.63 (m, 2H, H-14), 1.46 (m, 1H, H-25), 1.46 (m, 1H, H-11), 1.43 (m, 2H, H-22), 1.39 (m, 2H, H-12), 1.36 (m, 1H, H-8), 1.31 (m, 1H, H-7), 1.31 (s, 3H, H-21), 1.29 (m, 2H, H-23), 1.09 (m, 1H, H-24), 0.82 (d, *J* = 6.60 Hz, 6H, H-26, H-27), 0.90 (s, 3H, H-18). ^{13}C NMR ($CDCl_3$ and CD_3OD) δ 220.5 (C-16), 154.9 (C-3), 137.6 (C-5), 131.3 (C-10), 125.8 (C-1), 114.9 (C-4), 112.4 (C-2), 75.5 (C-20), 72.8 (C-16), 67.6 (C-17), 52.6 (C-14), 43.5 (C-13), 43.4 (C-9), 43.3 (C-22), 39.7 (C-12), 39.3 (C-15), 37.6 (C-8), 34.3 (C-24), 29.3 (C-6), 27.8 (C-25), 27.3 (C-7), 25.8 (C-11), 24.7 (C-21), 22.3 (C-26), 22.2 (C-27), 20.6 (C-23), 14.8 (C-18) MS (APCI), *m*/*z* (relative intensity): HRMS *m*/*z*: $C_{26}H_{38}O_3Na$ $[M+Na]^+$, cald 421.2719, found 421.2721.

*2.4.7. 3β-Acetoxyallopregnan-20-one (**34**)*

The crude product was purified by flash column chromatography (3:7 ethyl acetate:hexane) to afford **34** (150 mg, 99%) as a white solid, mp 146–147 °C. FTIR (KBr), ν_{max} 1738, 1691 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 4.59–4.73 (m, 1H, C3), 2.29–2.40 (m, 17H, CH), 2.26 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 1.15–1.86 (m, 17H, CH and CH_2) 0.91–1.06 (m, 2H, CH), 0.7–0.77 (m, 1H, CH_2), 0.81 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 209.7 (C=O), 170.7 (C=O), 155.5 (C-3), 148.4 (C-10), 73.6 (CH), 70.8 (CH), 56.3 (C-13), 54.7 (CH), 46.3 (CH), 44.9 (CH), 36.6 (CH), 35.7 (CH_2), 34.7 (CH_2), 29.3 (CH_2), 27.5 (CH_2), 27.1 (CH_3), 26.2 (CH_2), 21.0 (CH_3), 15.8 (CH_3). MS (APCI), *m*/*z* (relative intensity): 360 (M^+,39), 315 (49), 43 (100).

*2.5. General procedure for preparation of compounds **15** and **22***

To a stirred solution of compounds **14** or **21** (0.083 mmol) in dioxane (4 mL) was added 70% aqueous acetic acid (4 mL) and the mixture was heated at 50 °C for 24 h. The reaction mixture was neutralized with saturated aqueous sodium hydrogen carbonate and extracted with ethyl acetate. The combined organic layer was washed with water and the solvent was removed under reduced pressure. The crude residue was purified by flash column chromatography.

*2.5.1. 3,16S,20S,24-Tetrahydroxy-24-cholestan-$\Delta^{1,3,5(10)}$-estratriene (**15**)*

The crude residue was purified by flash column chromatography eluting with 1:1 ethyl acetate:hexane to provide **15** (19.4 mg, 56%) as a white solid, mp 224–226 °C. FTIR (KBr) υ_{max} 3366 (OH) cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.02 (d, *J* = 8.44 Hz, 1H, H-2), 6.54 (dd, *J* = 8.4, 2.6 Hz, 1H, H-2), 6.48 (d, *J* = 2.64, Hz, 1H, H-4), 4.55 (m, 1H, H-16), 3.24 (m, 1H, H-24), 2.76 (m, 2H, H-6), 2.22 (m, 1H, H-15), 2.16 (m, 1H, H-12), 2.11 (m, 1H, H-11), 2.08 (m, 1H, H-9), 1.52 (m, 1H, H-22), 1.75 (m, 1H, H-7), 1.60 (m, 1H, H-25), 1.58 (m, 1H, H-22), 1.52 (m, 1H, H-11), 1.45 (m, 1H, H-8), 1.40 (m, 1H, H-15), 1.30 (m, 1H, H-12), 1.29 (m, 2H, H-7, H-23), 1.27 (m, 1H, H-17), 1.25 (s, 3H, H-21), 1.20 (m, 1H, H-23), 1.07 (s, 1H, H-18), 0.95 (m, 1H, H-14), 0.85 (m, 6H, H-26, H-27). ^{13}C NMR ($CDCl_3$) δ: 154.0 (C-3), 137.8 (C-5), 131.7 (C-10), 126.1 (C-1), 115.1 (C-4), 112.6 (C-2), 77.2 (C-24), 76.5 (C-20), 73.2 (C-16), 61.1 (C-17), 53.2 (C-14), 43.6 (C-9), 43.2 (C-13), 40.4 (C-12), 39.87 (C-22), 37.6 (C-8), 36.5 (C-15), 33.6 (C-25), 29.5 (C-6), 27.5 (C-7), 26.3 (C-11), 25.5 (C-21), 23.3 (C-23), 18.7 (C-27), 17.7 (C-26), 14.6 (C-18). MS (APCI), *m*/*z* (relative intensity): 417 (4), 381 (100). HRMS *m*/*z*: $C_{26}H_{40}O_4Na$ $[M+Na]^+$, cald 439.2824, found 439.2804.

*2.5.2. 3-tert-Butyl-dimethylsiloxy-16S,20S-trihydroxycholestene-$\Delta^{1,3,5(10)}$-estratriene (**22**)*

The crude residue was purified by flash column chromatography eluting with 1:1 ethyl acetate:hexane to provide **22** (19.4 mg, 60%) as a colorless syrup. FTIR (neat) ν_{max} 3411 (OH) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.02 (d, *J* = 8.42 Hz, 1H, H-1), 6.52 (dd, *J* = 8.42, 2.59 Hz, 1H, H-2), 6.49 (d, *J* = 2.59 Hz, 1H, H-4), 3.73 (m, 1H, H-16), 2.80 (m, 2H, H-6), 2.15 (m, 1H, H-9), 1.96 (m, 1H, H-12), 1.92 (m, 1H, H-11), 1.74 (m, 1H, H-15), 1.68 (m, 2H, H-7), 1.62 (m, 1H, H-17), 1.61 (m, 2H, H-14, H-23), 1.60 (m, 1H, H-11, H-22), 1.47 (m, 1H, H-8), 1.45 (m, 2H, H-15,H-24), 1.40 (m, 2H, H-11, H-21), 1.24 (m, 1H, H-12), 1.00 (s, 9H, Me_3CSi), 0.80 (d, *J* = 6.65 Hz, 6H, H-26, H-27), 0.79 (s, 6H, Me_2Si), 0.73 (s, 3H, H-18), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.1 (C-3), 137.7 (C-5), 131.6 (C-10), 125.9 (C-1), 119.7 (C-4), 117.3 (C-2), 97.2 (C), 75.3 (C-20), 72.5 (C-16), 67.5 (C-17), 52.5 (C-14), 44.1 (C-9), 43.5 (C-22), 43.1 (C-13), 39.8 (C-12), 39.2 (C-15), 37.7 (C-8), 34.2 (C-24), 31.9 (Me), 29.5 (C-6), 27.6 (C-25), 27.4 (C-7), 27.2 (MeCSi), 25.8 (C-11), 25.5 (MeCSi), 25.5 (MeCSi), 24.9 (C-21), 22.2 (C-26), 22.1 (C-27),20.5 (C-23), 17.8 (CSi), 14.9 (C-18), −0.40 (MeSi), −0.4 (MeSi). HRMS *m*/*z*: $C_{32}H_{54}O_3SiNa$ $[M+Na]^+$, cald 537.3740, found 537.3715.

*2.6. (20S)-20-Hydrooxycholest-1-ene-3,16-dione (**3**), (20S)-20-hydroxy cholest-4-ene-3,16-dione (**4**) and (20S)-20-hydroxy cholest-1,4-diene-3,16-dione (**5**)*

A mixture of meta-iodoxybenzoic acid (76 mg, 0.027 mmol) and diphenyl diselenide (8.0 mg, 0.02 mmol) in toluene (5 mL) was refluxed until the yellow color of diphenyl diselenide disappeared. The solution of (20S)-20-hydroxycholestane-3,16-dione (**1**) (95 mg, 0.23 mmol) in toluene (3 mL) was introduced to this mixture. The mixture was further heated under reflux for 3 h. The reaction mixture was cooled to room temperature and partitioned with methylene chloride and water. The organic layer was separated and dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (2:8 ethyl acetate:hexane) to yield (*20S*)-20-hydrooxycholest-1-ene-3,16-dione (**3**) (17.8 mg, 18.9%), (*20S*)-20-hydroxy cholest-4-ene-3,16-dione (**4**) (12.1 mg, 12.9%), and (*20S*)-20-hydroxy cholest-1,4-diene-3,16-dione (**5**) (29.2 mg, 31.2%) as a pale yellow wax.

Compound **3**. FTIR (KBr), υ_{max} 3510, 1729, 1679 cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.05 (1H, d, *J* = 10.2 Hz, H-1), 5.80 (1H, d, *J* = 10.2 Hz, H-2), 2.18 (m, 2H, H-4), 2.20 (m, 1H, H-15), 2.14 (s, 1H, H-17), 2.08 (m, 1H, H-12), 1.87 (m, 1H, H-5), 1.85 (m, 1H, H-11), 1.83 (dd, *J* = 18.5, 14.1 Hz, 1H, H-15), 1.82 (m, 1H, H-8), 1.59 (m, 2H, H-7), 1.55 (m, 2H, H-22), 1.48 (m, 1H, H-25), 1.47 (m, 2H, H-11, H-12), 1.45 (m, 2H, H-6), 1.40 (m, 1H, H-14), 1.30 (m, 2H, H-23), 1.19 (s, 3H, H-21), 1.10 (m, 1H, H-9), 1.08 (m, 2H, H-24), 1.03 (m, 1H, H-8), 0.98 (s, 3H, H-19), 0.89 (s, 3H, H-18), 0.81 (d, *J* = 6.6 Hz, 3H, H-27), 0.80 (d, *J* = 6.6 Hz, 3H, H-26). ^{13}C NMR ($CDCl_3$) δ 220.8 (C-16), 199.9 (C-3); 157.3 (C-1), 127.7 (C-2), 73.9 (C-20), 71.4 (C-17), 50.8 (C-14), 49.7 (C-9), 44.2 (C-5), 43.1 (C-13), 42.4 (C-22), 40.8 (C-4), 39.5 (C-24), 39.3 (C-12), 39.2 (C-15), 39.0 (C-10), 34.81 (C-8), 31.3 (C-7), 28.0 (C-25), 27.3 (C-6), 25.4 (C-21), 22.7 (C-27), 22.6 (C-26), 20.6 (C-23), 21.0 (C-11), 14.8 (C-18), 13.40 (C-19). MS (APCI), *m*/*z* (relative intensity): 415 [$(M+H)^+$, 65], 397 [$(M+H)^+ - H_2O$, 100], 329 (6), 313 ($M^+ - C_6H_{13}$, 5), 287 (33).

Compound **4**. FTIR (KBr), υ_{max} 3447, 1725, 1676 cm^{-1}. ^1H NMR ($CDCl_3$) δ 5.68 (s, 1H, H-4), 2.37 (m, 1H, H-2), 2.27 (m, 1H, H-2), 2.24 (m, 1H, H-15), 2.22 (m, 2H, H-6), 2.13 (s, 1H, H-17), 2.08 (m, 1H, H-12), 1.96 (m, 1H, H-1), 1.85 (dd, *J* = 18.4, 13.4 Hz, 1H, H-15), 1.68 (m, 1H, H-11), 1.58 (m, 1H, H-7), 1.48 (m, 2H, H-22), 1.47 (m, 3H, H-8, H-12, H-25), 1.44 (m, 1H, H-11), 1.40 (m, 1H, H-14), 1.30 (m, 2H, H-23), 1.29 (m,1H, H-1), 1.19 (s, 3H, H-21), 1.15 (s, 3H, H-19), 1.09 (m, 1H, H-9), 1.08 (m, 2H, H-24, 1.02 (m, 1H, H-7), 0.90 (s, 3H, H-18), 0.80 (d, 6H, *J* = 6.6 Hz, H-26, H-27). ^{13}C NMR ($CDCl_3$) δ 220.8 (C-16), 199.7 (C-3), 169.9 (C-5), 124.1 (C-4), 73.9 (C-20), 71.2 (C-17), 50.3 (C-14), 49.7 (C-9), 43.1 (C-13), 42.4 (C-22), 40.9 (C-2), 39.4 (C-24), 39.2 (C-12), 39.1 (C-15), 38.8 (C-1), 38.5 (C-10), 34.0 (C-8), 33.8 (C-6), 32.5 (C-7), 28.0 (C-25), 25.4 (C-21), 22.7 (C-27), 22.6 (C-26), 20.9 (C-23), 21.0 (C-11), 14.8 (C-18), 13.40 (C-19). MS (APCI), *m*/*z* (relative intensity): 415 [$(M+H)^+$, 100)], 397 [$(M+H)^+ - H_2O$, 93], 345 (45), 313 ($M^+ - C_6H_{13}$, 21), 287 (23).

Compound **5**. FTIR (KBr), υ_{max} 3455 (OH), 1726 (C=O), 1662 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$) δ 6.97 (d, *J* = 10.2 Hz, 1H, H-1), 6.19 (dd, *J* = 10.12, 1.88 Hz, 1H, H-2), 6.02 (d, m, 1H, H-4), 0.93 (s, 3H, H-18), 0.80 (d, 6H, *J* = 6.6 Hz, H-26, H-27). ^{13}C NMR ($CDCl_3$): δ: 220.8 (C-16), 186.1 (C-3); 170.7 (C-5), 154.9 (CH-1), 127.8 (CH-4), 124.3 (CH-2), 73.8 (C-20), 71.4 (CH-17), 51.9 (CH-14), 49.9 (CH-9), 43.4 (C-10), 43.1 (C-13), 42.4 (CH_2-22), 39.4 (CH_2-24), 39.3 (C H_2-12), 38.9 (CH_2-15), 33.9 (C-8), 33.4 (CH_2-6), 32.0 (C-7), 28.0 (C-25); 25.3 (C-21), 22.7 (C-27), 22.6 (C-26), 22.2 (C-11), 21.1 (C-23), 18.7 (C-19), 14.6 (C-18). MS (APCI), *m*/*z* (relative intensity): 413 (62), 412 (13), 395.2 (100), 285.2 (89). HRMS *m*/*z*: $C_{27}H_{40}O_3Na$ $[M+Na]^+$, cald 435.2875, found 435.2883.

2.7. Spirosta-$\Delta^{1,4}$-diene-3-one (7)

A mixture of meta-iodoxybenzoic acid (43 g, 153.85 mmol) and diphenyl diselenide (972 mg, 3.08 mmol) in toluene (50 mL) was refluxed until the yellow color of diphenyl diselenide disappeared. The solution of tigogenin (**6**) (6.4 g, 15.38 mmol) in toluene (50 mL) was introduced to this mixture. The mixture was further heated under reflux for 7 h. The reaction mixture was cooled to room temperature and partitioned with methylene chloride and water. The organic layer was separated and dried over anhydrous sodium sulphate and concentrated under *vacuo*. The residue was purified by flash column chromatography to provide **7** as a white solid (4.37 g, 70%), mp 182–184 °C. FTIR (KBr), ν_{max} 1664 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 6.97 (d, *J* = 10.14 Hz, 1H, H-1), 6.2 (dd, *J* = 10.2 Hz, 1.99 Hz, 1H, H-2), 5.99 (m, 1H, H-4), 4.31 (m, 1H, H-16), 3.39 (m, 1H, H-26), 3.28 (m, 1H, H-26), 1.94 (m,1H, H-15), 1.27 (m, 1H, H-15), 1.18 (s, 3H, H-19), 0.89 (d, *J* = 7 Hz, 3H, H-27,), 0.78 (s, 3H, H-18), 0.71 (d, *J* = 6.36 Hz, 3H, H-21). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.1 (C-3), 168.8 (C-10), 155.6 (C-1), 127.3 (C-4), 123.7 (C-2), 109.6 (C-5), 80.3 (C-16), 66.7 (C-26), 61.9 (CH), 55.1 (CH), 52.2 (CH), 43.4 (C), 42.5 (CH), 40.3 (C-15), 39.3 (C), 34.9 (CH), 33.6 (CH_2), 32.6 (CH_2), 31.8 (CH_2), 31.2 (CH_2), 30.1 (CH), 28.7 (CH_2), 22.6 (CH_2), 18.6 (C-19), 16.9 (C-21), 16.7 (C-18), 14.3 (C-27). MS (EI), *m*/*z* (relative intensity): 410 (18), 351 (14), 289 (10), 181 (28), 139 (100).

2.8. 19-Nor-$\Delta^{1,3,5(10)}$-22α-spirostatriene-3-ol (8)

A lithium was cut into small pieces and introduced to the mixture of biphenyl (17.3 g, 112.1 mmol) in dry tetrahydrofuran (100 mL) under N_2 atmosphere at room temperature. The mixture was stirred under reflux for 0.5 h and the solution had turned blue. The mixture was treated dropwise, under N_2, with the solution of **7** (6.5 g, 16.0 mmol) and diphenyl methane (7.8 mL) in dry tetrahydrofuran. After refluxing for 2 h and cooled down, methanol was added to destroy the excess lithium. The solvent was removed and the residue was dissolved in 10% aq. HCl and extracted with methylene chloride. The organic layer was washed with water, dried over anhydrous sodium sulphate and the solvent was removed *in vacuo*. Purification of the crude product by flash column chromatography eluting with ethyl acetate:hexane (1:9) provided the desired product **8** as a white solid (2.47 g, 44%), mp 244–246 °C [lit.145–147 °C] [20]. FTIR (KBr), ν_{max} 3402 (OH), 3062 (CH-Ar), 1609 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.06 (d, *J* = 8.36 Hz, 1H, H-1), 6.54 (dd, *J* = 2.76 Hz, 8.36 Hz, 1H, CH-2), 6.48 (d, *J* = 2.76 Hz, 1H, CH-4), 4.67 (s, OH), 4.37 (m, 1H, H-16), 3.44 (m, 1H, H-26), 3.32 (t, *J* = 10.83 Hz, 1H, H-26), 2.76 (m, 2H, H-6), 2.01 (m, 1H, H-15), 1.83 (m, 1H, H-17), 1.30 (m, 1H, H-15), 0.92 (d, *J* = 6.84 Hz, 3H, H-27,), 0.75 (s, 3H, H-18), 0.72 (d, *J* = 6.32 Hz, 3H, H-21). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.7 (C-3), 138.3 (C-5), 132.8 (C-10), 126.3 (C-1), 115.2 (C-4), 112.6 (C-2), 109.3 (C-22), 80.9 (C-16), 66.9 (C-26), 62.3 (CH), 55.3 (C-14), 43.7 (C-9), 41.6 (CH), 40.8 (C-13), 39.9 (C-12), 38.3 (C-8), 31.53 (C-15), 31.40 (CH_2), 30.3 (CH), 29.5 (C-6), 28.8 (CH_2), 27.8 (C-7), 26.4 (C-11), 17.1 (C-21), 16.4 (C-18), 14.5 (C-21). MS (EI), *m*/*z* (relative intensity): 396 (13), 282 (48), 139 (100).

2.9. 3-Acetoxy-16β-acetoxymethylvaleroyloxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (9)

A mixture of 19-nor-$\Delta^{1,3,5(10)}$-spirostatriene-3-ol (**8**) (300 mg, 0.78 mmol), acetic anhydride (5.4 mL), ammonium chloride (83.14 mg, 1.56 mmol) and pyridine (0.06 mL) was heated to 125–135 °C and kept at that temperature for 12 h. After cooling down, the reaction mixture was neutralized with saturated sodium hydrogen carbonate then extracted with methylene chloride. The organic layer was washed with water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude residue was used in the next step without further purification.

The crude residue was dissolved in 1,2-dichloroethane (0.7 mL), water (0.1 mL) and acetic acid (0.25 mL). The mixture was cooled to 0 °C. A solution of chromium trioxide (105 mg, 1.05 mmol) in water (2.19 mL) and acetic acid (0.25 mL) was added (the temperature was kept below 7 °C). The mixture was allowed to warm to room temperature and stirred for another 2 h. A solution of sodium chloride (124 mg) in water (1.66 mL) and methanol (1.66 mL) was introduced and the mixture was stirred for 1 h. The reaction mixture was neutralized using sodium bicarbonate and extracted with methylene chloride and the organic phase was washed with water, dried over anhydrous sodium sulphate and filtered. The filtrate was evaporated off solvent and the crude residue was purified by flash column chromatography eluting with ethyl acetate:hexane (1:9) to provide **9** (148 mg, 38%) as a pale yellow syrup. FTIR (neat), υ_{max} 1756 (C=O), 1734 (C=O), 1712 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.19 (d, *J* = 8.48, 1H, CH-1), 6.76 (dd, *J* = 8.48, 2.56, 1H, CH-2), 6.70 (d, *J* = 2.48, 1H, CH-4), 5.49 (m, 1H, CH-16), 3.81 (d, 6.12, 2H, CH_2-26), 2.78 (m, 2H, CH_2-6), 2.46 (m, 1H, $C\underline{H}_AH_B$-15), 2.40 (d, 7.60, 1H, CH-17), 2.21 (m, 1H, CH-9), 2.20 (s, 3H, $\underline{CH_3}C{=}O$), 2.19 (m, 1H, $C\underline{H}_AH_B$-11), 2.15 (m, 1H, $C\underline{H}_AH_B$-7), 2.09 (m, 1H, $C\underline{H}_AH_B$-12), 2.01 (s, 3H, Me-21), 1.96 (s, 3H, $\underline{CH_3}C{=}O$), 1.77 (m, 3H, CH_2-22, CH-27), 1.63 (m, 1H, $C\underline{H}_AH_B$-23), 1.50 (m, 2H, CH_AH_B-7, CH-8), 1.37 (m, 1H, CH_AH_B-15, $CH_A\underline{H}_B$-23), 1.35 (m, 1H, CH_AH_B-11), 1.19 (m, 1H, $CH_A\underline{H}_B$-12), 1.05 (m, 1H, CH-14), 0.99 (s, 3H, Me-18), 0.85 (s, 3H, Me-25). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 260.0 (CO), 172.9 (CO), 171.1 (CO), 169.8 (CO), 148.45 (C-3), 137.9 (C-5), 137.6 (C-10), 126.24 (C-1), 121.5 (C-4), 118.61 (C-2), 74.3 (C-16), 68.7 (C-26), 66.7 (C-17), 53.0 (C-14), 43.9 (C-9), 42.6 (C-13), 37.97 (C-12), 37.1 (C-8), 34.8 (C-15), 32.0 ($\underline{CH_3}C{=}O$), 31.9 (C-22), 30.5 (C-24), 29.3 (C-6), 28.2 (C-23), 27.3 (C-7), 25.7 (C-11), 21.0 (C-21), 20.8 ($\underline{CH_3}C{=}O$), 16.3 (C-25), 13.4 (C-18). MS (APCI), *m*/*z* (relative intensity): 512 (52), 339 (100), 297 (34). HRMS *m*/*z* $C_{30}H_{40}O_7Na$ $[M+Na]^+$, calcd 535.2672, found 535.2673.

2.10. 3-Hydroxy-(16S,20S)-16,20-acetonide-24-cholestene-$\Delta^{1,3,5(10)}$-estratriene (**12**)

To a stirred solution of **10** (250 mg, 0.63 mmol) in 2,2-dimethoxypropane (7.7 mL) was added *p*-toluenesulphonic acid (6 mg, 0.032 mmol) at room temperature. The mixture was stirred for 2 h and triethylamine was added (3 drops). The mixture was added to the ice-water and extracted with methylene chloride. The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 1:9 ethyl acetate:hexane to provide **12** as a colorless solid (192.7 mg, 70%), mp 68–70 °C. FTIR (KBr), ν_{max} 3373 (OH) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.06 (d, J = 8.40 Hz, 1H, H-1), 6.55 (dd, J = 8.40, 2.68 Hz, 1H, H-2), 6.48 (d, J = 2.68 Hz, 1H, H-4), 5.01 (m, 1H, H-24), 4.44 (m, 1H, H-16), 2.75 (m, 2H, H-6), 2.19 (m, 1H, H-15), 2.14 (m, 1H, H-9), 2.12 (m, 1H, H-11), 2.09 (m, 1H, H-12), 1.97 (m, 2H, H-23), 1.83 (m, 1H, H-22), 1.80 (m, 2H, H-7), 1.62 (s, 3H, Me), 1.55 (s, 3H, Me), 1.50 (m, 1H, H-22), 1.46 (m, 2H, H-8, H-11), 1.42 (m, 1H, H-15), 1.41 (s, 3H, H-21), 1.29 (m, 1H, H-12), 1.28 (s, 3H, H-27), 1.27 (s, 3H, H-26), 1.06 (s, 3H, H-18), 1.02 (m, 1H, H-14), 0.98 (m, 1H, H-17). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.3 (C-3), 138.2 (C-5), 132.7 (C-10), 131.4 (C25), 126.4 (C-1), 124.4 (C-24), 115.2 (C-4), 112.6 (C-2), 97.2 (C), 75.2 (C-20), 68.7 (C-16), 55.6 (C-17), 53.7 (C-14), 44.2 (C-22), 43.9 (C-9), 42.6 (C-13), 40.0 (C-12), 37.9 (C-8), 33.5 (C-15), 31.8 (C-26), 29.6 (C-6), 27.5 (C-7) 26.2 (C-11),), 25.7 (C-27), 25.6 (Me), 25.2 (C-21), 22.6 (C-23), 17.7 (Me), 14.8 (C-18). MS (APCI), *m*/*z* (relative intensity): 439 (30), 381 (100), 363 (76).

2.11. 3,24-Dihydroxy-(16S,20S)-16,20-acetonide-cholestan-$\Delta^{1,3,5(10)}$-estratriene (**14**)

To a solution of **12** (100 mg, 0.228 mmol) in dry THF (5.8 mL) was added portionwise, with stirring, BH_3.THF complex (ca. 1 M in THF, 0.22 mL) during a period of 2 h at 0 °C. Hydrogen peroxide solution (35%, 1.2 mL) and 1N sodium hydroxide (1.6 mL) was then added and continued stirring for 16 h. Water was added and the mixture was extracted with methylene chloride. The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 1:4 ethyl acetate:hexane to provide **14** as a colorless wax (67.1 mg, 64%). FTIR (KBr), υ_{max} 3411 (OH) cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.05 (d, J = 8.4 Hz, 1H, H-1), 6.55 (dd, J = 8.32, 2.40 Hz, 1H, H-2), 6.49 (d, J = 2.16 Hz, 1H, H-4), 4.43 (m, 1H, H-16), 3.26 (m, 1H, H-24), 2.73 (m, 2H, H-6), 2.18 (m, 1H, H-15), 2.15 (m, 1H, H-11), 2.08 (m, 1H, H-9), 2.04 (m, 2H, H-12), 1.64 (m, 2H, H-7), 1.60 (m, 1H, H-25), 1.52 (m, 1H, H-11), 1.43 (m, 1H, H-8), 1.41 (s, 3H, H-30), 1.34 (m, 6H, H-15, H-22, H-29), 1.17 (s, 3H, H-21), 1.05 (s, 3H, H-18), 1.02 (m, 1H, H-14), 1.0 (m, 1H, H-17), 0.87 (m, 6H, H-26, H-27). ^{13}C NMR ($CDCl_3$) δ 153.6 (C-3), 138.1 (C-5), 132.5 (C-10), 126.3 (C-1), 115.3 (C-4), 112.7 (C-2), 97.6 (C), 77.4 (C-24), 76.5 (C-20), 68.8 (C-16), 56.2 (C-17), 53.6 (C-14), 43.8 (C-9), 42.7 (C-13), 40.9 (C-12), 40.0 (C-22), 37.8 (C-8), 33.5 (C-25), 33.3 (C-15), 31.5 (Me), 29.5 (C-6), 28.3 (C-23), 27.5 (C-7), 26.2 (C-11), 25.8 (C-21), 24.4 (Me), 18.9 (Me-26), 17.0 (C-27), 15.1 (C-18). MS (APCI), *m*/*z* (relative intensity):457 (31), 456 (11), 381 (100). HRMS *m*/*z*: $C_{29}H_{44}O_4Na$ $[M+Na]^+$, cald. 479.3137, found 479.3141.

2.12. 3-tert-Butyl-dimethylsiloxy-(16S,20S)-16,20-acetonide-24-cholestene-$\Delta^{1,3,5(10)}$-estratriene (**16**)

To a stirred solution of **12** (107 mg, 0.24 mmol) in DMF (0.4 mL) and methylene chloride (5 mL) was added imidazole (50 mg, 0.73 mmol) and tert-butyldimethylsilyl chloride (73 mg, 0.49 mmol). The reaction mixture was stirred at ambient temperature for 3 h, then water was added (3 drops). The reaction mixture was extracted with methylene chloride. The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 2:98 ethyl acetate:hexane to provide **16** (106 mg, 79%) as a colorless syrup. FTIR (KBr), ν_{max} 1607, 1496, 1247 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.03 (d, J = 8.44 Hz, 1H, H-1), 6.53 (dd, J = 8.44, 2.68 Hz, 1H, H-2), 6.47 (d, J = 2.56 Hz, 1H, H-4), 5.01 (m, 1H, H-24), 4.45 (m, 1H, H-16), 2.73 (m, 2H, H-6), 2.20 (m, 1H, H-15), 2.15 (m, 1H, H-9), 2.14 (m, 2H, H-23), 1.97 (m, 1H, H-12), 1.93 (m, 1H, H-11), 1.83 (m, 2H, H-7), 1.81 (m, 1H, H-22), 1.62 (s, 3H, H-27), 1.56 (s, 3H, H-26), 1.44 (m, 1H, H-15), 1.43 (m, 1H, H-22), 1.42 (m, 1H, H-8), 1.40 (m, 4H, H-11, H-21), 1.29 (m, 1H, H-12), 1.06 (s, 9H, H-18, MeX2), 1.02 (m, 1H, H-14), 0.90 (s, 9H, Me_3CSi), 0.89 (m, 1H, H-17), 0.80 (s, 6H, Me_2Si). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.3 (C-3), 137.8 (C-5), 131.4 (C-10), 131.1 (C-25), 126.0 (C-1), 124.5 (C-24), 119.9 (C-4), 117.1 (C-2), 97.1 (C), 75.1 (C-20), 68.7 (C-16), 55.6 (C-17), 53.9 (C-14), 44.2 (C-22), 44.0 (C-9), 42.6 (C-13), 40.0 (C-12), 37.9 (C-8), 33.5 (C-15), 31.8 (Me), 29.6 (C-6), 26.2 (C-11), 27.5 (C-7), 25.7 (MeCSi), 25.7 (MeCSi), 25.7 (MeCSi), 25.7 (C-27), 25.6 (C-21), 24.2 (Me), 22.6 (C-23), 18.2 (C-26), 17.7 (CSi), 14.8 (C-18), −0.40 (MeSi), −0.4 (MeSi). MS (APCI), *m*/*z* (relative intensity): 553 (100), 495 (30).

2.13. Synthesis of 3-tert-butyl-dimethylsiloxy-(16S,20S)-16,20-acetonide-24-ol-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**17**)

To a solution of **16** (106 mg, 0.20 mmol) in dry THF (5.6 mL) was added portionwise, with stirring, BH3.THF complex (ca 1 M in THF, 0.2 mL) during a period of 2 h. Hydrogen peroxide solution (35%, 0.6 mL) and 1N sodium hydroxide (0.8 mL) was then added and stirring was continued for 16 h. Water was added and the reaction mixture was extracted with methylene chloride. The combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 1:19 ethyl acetate:hexane to provide **17** as a white solid (53 mg, 48%) mp 69–71 °C. FTIR (KBr), ν_{max} 3429 (OH) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 6.90 (d, J = 8.44 Hz, 1H, H-1), 6.42 (dd, J = 8.44, 2.44 Hz, 1H, H-2), 6.36 (d, J = 2.48, 1H, H-4), 4.33 (m, 1H, H-16), 3.13 (m, 1H, H-24), 2.26 (m, 2H, H-6), 2.06 (m, 2H, H-11, H-15), 2.05 (m, 1H, H-22), 2.00 (m, 1H, H-9), 1.93 (m, 1H, H-12), 1.67 (m, 2H, H-7), 1.49 (m, 1H, H-25), 1.42 (m, 2H, H-23), 1.37 (m, 2H, H-22), 1.30 (s, 3H, Me), 1.33 (m, 1H, H-8), 1.27 (s, 3H, Me), 1.16 (s, 3H, H-21), 1.13 (m, 1H, H-15), 1.11 (m, 1H, H-11), 1.10 (m, 1H, H-12), 1.08 (m, 1H, H-14), 0.95 (s, 3H, H-18), 0.86 (m, 1H, H-17), 0.79 (s, 9H, Me_3CSi), 0.75 (s, 6H, H-26, H-27), 0.66 (s, 6H, Me_2Si). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.3 (C-3), 137.8 (C-5), 133.1 (C-10), 125.6 (C-1), 119.9 (C-4), 117.1 (C-2), 97.5 (C), 77.6 (C-24), 74.3 (C-20), 68.8 (C-16), 56.0 (C-17), 53.7 (C-14), 44.0 (C-9), 42.7 (C-13), 44.1 (C-22), 40.1 (C-12), 37.8 (C-8), 33.9 (C-25), 33.6 (C-15), 31.6 (Me), 29.6 (C-6), 28.4 (C-23), 27.6 (C-7), 26.1 (C-11), 25.9 (C-21), 25.7 (MeCSi), 25.7 (MeCSi), 25.7 (MeCSi), 24.4 (Me), 19.0 (C-27), 15.0 (C-18), 18.2 (CSi), 17.4 (C-26), −0.40 (MeSi), −0.40 (MeSi). MS (APCI), *m*/*z* (relative intensity): 571 (100), 495 (40). HRMS: 593.4003 $C_{35}H_{58}O_4NaSi$ require 593.4002.

2.14. Synthesis of 3-hydroxy (16S,20S)-16,20-acetonide-24-ol-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**19**)

To a stirred solution of 3-*tert*-butyl-dimethylsiloxy-(16*S*,20*S*)-16,20-acetonide-24-one-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**18**) (60 mg, 0.088 mmol) in THF was added TBAF (0.44 mmol) at room temperature. After stirring for 10 h, water was added and the reaction mixture was extracted with ether. The combined organic phases were washed with water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 3:97

ethyl acetate:hexane to provide compound **19** as a colorless syrup. (18.6 mg, 60%), FTIR (KBr), ν_{max} 3373 (OH), 1708 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.05 (d, J = 8.42 Hz, 1H, H-1), 6.54 (dd, J = 8.42, 2.65 Hz, 1H, H-2), 6.48 (d, J = 2.65 Hz, 1H, H-4), 4.42 (m, 1H, H-16), 2.80 (m, 2H, H-6), 2.45 (m, J = 6.80 Hz, 1H, H-25), 2.43 (m, 2H, H-23), 2.20 (m, 1H, H-15), 2.16 (m, 1H, H-9), 2.12 (m, 1H, H-22), 2.10 (m, 1H, H-11), 2.08 (m, 1H, H-12), 1.77 (m, 2H, H-7), 1.50 (m, 1H, H-22), 1.48 (m, 1H, H-11), 1.47 (m, 1H, H-8), 1.43 (m, 1H, H-15), 1.29 (m, 1H, H-12), 1.23 (s, 3H, Me), 1.15 (s, 3H, Me), 1.18 (s, 3H, H-21), 1.08 (s, 3H, H-18), 1.02 (m, 1H, H-14), 0.95 (m, 1H, H-17), 0.94 (d, J = 6.8 Hz, 6H, H-26, H-27). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 214.2 (CH-24), 154.0 (C-3), 138.1 (C-5), 132.9 (C-10), 126.2 (C-1), 115.0 (C4), 112.7 (C-2), 97.0 (C-28), 74.5 (C-20), 68.8 (C-16), 56.1 (C-17), 53.8 (C-14), 43.9 (C-9), 42.8 (C-13), 41.5 (C-25), 40.1 (C-12), 37.9 (C-8), 36.5 (C-22), 34.6 (C-23), 33.5 (C-15), 29.6 (C-6), 28.7 (Me), 27.6 (C-7), 26.1 (C-11), 25.7 (C-21), 24.1 (Me), 18.3 (C-27), 18.2 (C-26), 18.3 (C-18). HRMS m/z: $C_{29}H_{42}O_4Na$ $[M+Na]^+$, cald 477.2981, found 477.2991.

2.15. Attempted hydrolysis of 3-tert-butyl-dimethylsiloxy-(16S,20S16S,20S)-16,20-acetonide-24-one-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**20**)

To a stirred solution of 3-*tert*-butyl-dimethylsiloxy-(16*S*,20*S*)-16,20-acetonide-24-one-chlorestane-$\Delta^{1,3,5(10)}$-estratriene (**18**) (56 mg, 0.1045 mmol) in THF (3 mL) was added 10% aqueous hydrochloric acid (3 drops) at room temperature. After being stirred for 5 min, the solvent was removed. The reaction mixture was neutralized by sodium hydrogen carbonate and extracted with methylene chloride. The combined organic layers were washed with water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 3:97 ethyl acetate:hexane to provide cyclic compound **20** as a pale yellow solid (25.8 mg, 48.4%), mp 65–67 °C. FTIR (KBr) ν_{max}1653, 1607, 1498 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.06 (d, J = 8.4 Hz, 1H, H-11), 6.53 (dd, J = 8.4 Hz, 1H, H-2), 6.47 (d, J = 2.68 Hz, 1H, H-4), 4.31 (m, 1H, H-16), 2.75 (m, 2H, H-6), 2.14 (m, 1H, H-11), 2.13 (m, 2H, H-15), 2.10 (m, 1H, H-9), 2.07 (m, 1H, H-12), 1.88 (m, 2H, H-22), 1.79 (m, 2H, H-7), 1.75 (m, 1H, H-25), 1.53 (m, 1H, H-8), 1.14 (m, 1H, H-11), 1.37 (s, 3H, H-21), 1.32 (m, 1H, H-15), 1.28 (m, 2H, H-23), 1.05 (m, 1H, H-14), 1.03 (s, 3H, H-18). 0.92 (m, 1H, H-17), 0.91 (d, J = 6.92 Hz, 3H, H-26), 0.90 (s, 9H, $\underline{Me}_3CSi$), 0.68 (d, J = 6.9 Hz, 3H, H-27), 0.60 (s, 6H, $(CH_3)_2Si$). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.3 (C-3), 137.8 (C-5), 133.2 (C-10), 126.9 (C-1), 120.0 (C-4), 117.1 (C-2), 108.9 (C-24), 82.0 (C-20), 72.1 (C-16), 58.5 (C-17), 54.0 (C-14), 44.0 (C-2), 42.4 (C-13), 40.0 (C-12), 39.8 (C-22), 37.8 (C-8), 35.8 (C-25), 33.2 (C-15), 32.0 (C-23), 29.6 (C-6), 27.7 (C-7), 26.2 (C-11), 25.7 ($Me_3\underline{C}Si$), 25.1 (C-21), 18.2 (CSi), 17.3 (C-26), 17.0 (C-27), 14.7 (C-18), −0.4 (($\underline{C}H_3)_2Si$).

2.16. 3,17-Acethyl-$\Delta^{1,3,5(10),16}$-estratetraene (**23**)

A mixture of 19-nor-$\Delta^{1,3,5(10)}$-22α-spirostatriene-3-ol (**8**) (300 mg, 0.78 mmol), acetic anhydride (5.4 mL), ammonium chloride (83.14 mg, 1.56 mmol) and pyridine (0.06 mL) was heated to 125–135 °C and kept at this temperature for 12 h. After cooling down, the reaction mixture was dissolved in 1,2-dichloroethane (0.7 mL), water (0.083 mL), acetic acid (0.25 mL) and acetone (2 mL) and cooled to 0 °C. A solution of chromium trioxide (105 mg, 1.05 mmol, 2.9 eq) in water (2.19 mL) and acetic acid (0.25 mL) was added (kept the temperature less than 7 °C) then the reaction mixture was stirred in cooled water until TLC indicated the completion of the reaction. Then a solution of sodium chloride (124 mg) in water (1.66 mL) and methanol (1.66 mL) was introduced and stirred for 1 h. The reaction mixture was neutralized using sodium bicarbonate and extracted with methylene chloride and the organic phase was washed with water, dried over anhydrous sodium sulphate, filtered and the filtrate was concentrated. The residue was dissolved in benzene (30 mL) and basic alumina (1 g) was added and stirred for 16 h. Filtered and plenty rinsed with methylene chloride. The filtrate was evaporated off solvent. The crude product was purified by flash column chromatography eluting with 1:9 ethyl acetate:hexane to give **23** as a white solid (146.6 mg, 58%), mp 154–156 °C [lit 161–162 °C] [21] FTIR (KBr), ν_{max} 3062, 1609 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.32 (d, J = 8.43 Hz, 1H, CH-1), 6.88 (dd, J = 8.4, 2.6 Hz, 1H, CH-2), 6.83 (d, J = 2.4 Hz, H, CH-4), 6.78 (dd, J = 3.3, 1.8 Hz, 1H, CH-16), 2.93 (m, 2H, CH_2-6), 2.32 (s, 3H, Me), 2.32 (s, 3H, Me), 0.95 (s, 3H, Me). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 196.7 (C=O), 169.8 (C=O), 155.4 (C-3), 148.4 (C-10), 148.4 (C-17), 144.1 7 (C-16), 137.9 (C-5), 126.1 (C-1), 121.4 (C-4), 118.5 (C-2), 55.6 (C-13), 46.4 (CH), 44.3 (CH), 36.6 (CH), 34.7 (CH_2), 31.9 (CH_2), 29.3 (CH_2), 27.5 (CH_2), 27.1 (CH_3), 26.2 (CH_2), 21.0 (CH_3), 15.8 (CH_3). MS (EI), m/z (relative intensity): 296 (100), 159 (94).

2.17. 16α,17α-Epoxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**24**)

To a stirred solution of **23** (45 mg) in methanol (5 mL) was added 2.5N potassium hydroxide (1 mL) at room temperature. The reaction mixture was cooled to 0 °C and then was treated with 30% hydrogen peroxide (1 mL). The mixture was stirred for 16 h. The solvent was removed, extracted with methylene chloride, dried over anhydrous sodium sulphate and concentrated *in vacuo*. The crude product was purified by flash column chromatography eluting with 3:7 ethyl acetate:hexane to give **24** as a white solid (37 mg, 90%), mp 224–226 °C [lit 234.6 °C] [22] FTIR (KBr), ν_{max} 3468 (OH), 3018 (CH-Ar), 1686 (C=O), 1620 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.15 (d, J = 8.46 Hz, 1H, H-1), 6.65 (dd, J = 8.36, 2.74 Hz, 1H, H-2), 6.58 (d, J = 2.66 Hz, 1H, H-4), 4.80 (s, 1H), 3.76 (s, 1H, H-16), 2.84 (m, 2H, H-6), 2.08 (s, 3H, H-21), 1.08 (s, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 204.9 (C-20), 153.4 (C-3), 137.9 (C-10), 132,4 (C-5), 126.3 (C-1), 115.2 (C-4), 112.7 (C-2), 71.1 (CH), 60.5 (CH), 44.5 (CH), 44.0 (CH), 42.1 (C-13), 36.3 (CH), 31.5 (CH_2), 29.4 (CH_2), 27.4 (CH_2), 27.2 (CH_2), 26.0 (C-21), 25.9 (CH_2), 15.3 (C-18). MS (EI), m/z (relative intensity): 312 (15), 251 (3).

2.18. 3-Benzyloxy-16α,17α-epoxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**25**)

To a mixture of 16α,17α-epoxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**24**) (20 mg, 0.064 mmol), K_2CO_3 (13 mg, 0.096 mmol) in acetone (2 mL) was added benzyl bromide (0.01 mL, 0.076 mmol) and refluxed for 6 h. The reaction mixture was quenched with saturated aqueous ammonium chloride and the solvent was removed under reduced pressure. The mixture was extracted with methylene chloride and the combined organic layers were washed with water, dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure. The crude product was purified by flash column chromatography (2:8 ethyl acetate:hexane) to provide **25** as a colorless wax (25 mg, 97%), mp 100–101 °C. FTIR (KBr), ν_{max} 1702 (C=O) cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.23 (m, 5H, ArH), 7.10 (d, J = 8.42 Hz, 1H, CH-1), 6.70 (dd, J = 8.60, 2.68 Hz, CH-2), 1H), 6.63 (d, J = 2.72 Hz, 1H, CH-4), 4.95 (s, 2H, CH_2-Ar), 3.64 (s, 1H, CH-16), 2.78 (m, 2H, CH_2-6), 1.98 (s, 3H, CH_3-21), 0.99 (s, 3H, CH_3-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.0 (C=O), 158.2 (C-3), 137.9 (C-10), 137.5 (C-Ar), 133.3 (C-5), 128.7 (CH-Ar), 128.1 (CH-Ar), 127.6 (CH-Ar), 126.3 (C-1), 115.1 (C-4), 112.4 (C-2), 70.1 (CH_2OBn), 60.7 (C-16), 56.2 (C-16), 44.7 (CH), 44.3 (CH), 42.2 (C-13), 36.5 (CH), 31.7 (CH_2), 29.8 (C-6), 27.7 (CH_2), 27.4 (CH_2), 26.2 (C-21), 26.1 (CH_2), 15.5 (C-18). HRMS m/z: $C_{27}H_{30}O_3Na$ $[M+Na]^+$, cald 425.2093, found 425.2098.

2.19. 3-Benzyloxy-16α-hydroxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**26**)

To a solution of **25** (26 mg, 0.0647 mmol) in ethanol (1 mL) and methylene chloride (0.2 mL) was added hydrazine hydrate (0.24 mL, 4.975 mmol) at room temperature. After stirring for 5 h, the solvent was evaporated and stirred vigorously for 30 min with the mixture of ethyl acetate (3 mL) 10% aqueous hydrochloric acid (3 mL). The organic layer was separated and the aqueous layer was washed with ethyl acetate. The combined organic layers were washed with brine and water, dried over anhydrous sodium sulphate. Concentration of the organic extract under reduced pressure, followed by purification by flash column chromatography using ethyl acetate:hexane (3:7) afforded the product **26** (24 mg, 92%) as a white solid, mp 224–226 °C. FTIR (KBr), ν_{max} 3436 (OH), 1699 (C=O) cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.46–7.30 (m, 5H, Ar (H)), 7.19 (d, J = 8.40 Hz, 1H, H-1), 6.65 (dd, J = 8.8, 2.8 Hz, 1H, H-2), 6.55 (d, J = 2.80 Hz, 1H, H-4), 5.05 (s, 2H, CH_2Ar), 4.90 (m, 1H, H-16), 2.87 (m, 2H, H-6), 2.63 (d, J = 6.8 Hz, H-17), 2.15 (s, 3H, H-21), 0.68 (s, 3H, H-18). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 177.0 (C=O), 156.8 (C-3), 137.9 (C-10), 137.3 (C-Ar), 133.1 (C-5), 128.7 (CH-Ar), 128.6 (CH-Ar), 127.4 (CH-Ar), 126.1 (C-1), 114.9 (C-4), 112.2 (C-2), 72.1 (C-16), 70.0 (CH_2OBn), 55.6 (CH), 46.5 (C-13), 44.2 (CH), 38.9 (CH), 37.0 (C-17), 34.9 (CH_2), 32.0 (CH_2), 29.7 (C-6), 27.7 (CH_2), 26.4 (CH_3), 26.2 (CH_2), 15.9 (CH_3). MS (APCI), m/z (relative intensity): 387 (100). HRMS m/z: $C_{27}H_{32}O_3Na$ $[M+Na]^+$, cald 427.2249, found 427.2252.

2.20. Benzyloxy-16α-acetoxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**28**)

To a stirred solution of 3-benzyloxy-16α-hydroxy-17-acetyl-$\Delta^{1,3,5(10)}$-estratriene (**26**) (404 mg, 1 mmol) in pyridine (2 mL) was added acetic anhydride (2 mL) at 0 °C. The resulting mixture was continued stirring for 3 h. Ice-water was added and the reaction mixture was diluted with methylene chloride and extracted with water. The combined extracts were dried over anhydrous sodium sulphate, filtered and concentrated under reduce pressure. The crude product was purified by flash column chromatography (2:8 ethyl acetate:hexane) to afford **28** (430.4 mg, 96.5%) as a white solid, mp 144–146 °C. FTIR (neat), ν_{max} 1736 (C=O), 1707 (C=O) cm^{-1}. ^{1}H NMR (400 MHz) δ 7.35–7.23 (m, 5H, ArH), 7.10 (d, J = 8.6 Hz, CH-1), 6.70 (dd, J = 8.6, 2.4 Hz, 1H, CH-2), 6.64 (d, J = 2.8 Hz, 1H, CH-4), 5.45 (m, 1H, CH-16), 4.95 (s, 2H, CH_2OBn), 2.78 (m, 2H, CH_2-6), 2.67 (d, J = 6.4 Hz, 1H, CH-17), 2.11 (s, 3H, Me-21), 1.94 (s, 3H, Me), 0.61 (s, 3H, Me). ^{13}C NMR (100 MHz) δ 206.4 (C=O), 170.6 (C=O), 156.8 (C-3), 137.7 (C-10), 137.2 (C-Ar), 132.2 (C-5), 128.5 (CH-Ar), 8 (CH-Ar), 3 (CH-Ar), 126.1 (C-1), 114.8 (C-4), 112.3 (C-2), 75.7 (C-16), 70.1 (C-17), 69.9 (CH_2OBn), 53.3 (CH), 44.9 (CH), 43.5 (C-13), 38.8 (CH_2), 38.0 (CH), 33.1 (CH_2), 31.4 (Me), 25.5 (CH_2), 27.5 (CH_2), 26.2 (CH_2), 21.1 (CH_3), 14.5 (CH_3). MS (APCI), m/z (relative intensity): HRMS: 469.2363 $C_{29}H_{34}O_4Na$ require 469.2355.

2.21. 3β-Acetoxy-5α-pregn-16-ene-20-one (**33**)

A mixture of tigogenin (**6**) (3.52 g, 8.45 mmol), acetic anhydride (17 mL), ammonium chloride (0.45 g, 8.45 mmol) and pyridine (1.47 mL) was heated to 125–135 °C and kept at this temperature for 9 h. After cooling down, the mixture was dissolved in 1,2-dichloroethane (7 mL), water (1 mL), acetic acid (1 mL) and cooled to 0 °C. A solution of chromium trioxide (1.62 g, 16.2 mmol, 2.9 equiv.) in water (2 mL) and acetic acid (0.7 mL) was added (kept the temperature less than 7 °C) then the reaction mixture was stirred in cooled water until TLC indicated the completion of the reaction. Then a solution of sodium chloride (124 mg) in water (1.66 mL) and methanol (1.66 mL) was introduced and stirred for 1 h. The reaction mixture was extracted with 1,2-dichloroethane and washed with water. Solid sodium acetate (1.38 g, 16.9 mmol) was added to the organic phase and the solvent was distilled off azeotropically to remove 1,2-dichloroethane then washed with water, dried over anhydrous sodium sulphate, filtered and the filtrate was concentrated. The crude product was purified by flash column chromatography eluting with 1:2 ethyl acetate:hexane to give **33** as a white solid (1.67, 55%), mp 166–168 °C. FTIR (KBr), ν_{max} 1738, 1665, 1592 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.70 (s, 3H, C16), 4.59–4.73 (m, 1H, C3), 2.29–2.40 (m, 10H, CH), 2.26 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 1.15–1.86 (m, 17H, CH and CH_2) 0.91–1.06 (m, 2H, CH_2), 0.7–0.77 (m, 1H, CH), 0.85 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 196.8 (C=O), 170.7 (C=O), 155.5 (C-3), 148.4 (C-10), 145.4 (C-17), 144.1 (C-16), 73.6 (CH), 56.3 (C-13), 54.7 (CH), 46.3 (CH), 44.9 (CH), 36.6 (CH), 35.7 (CH_2), 34.7 (CH_2), 29.3 (CH_2), 27.5 (CH_2), 27.1 (CH_3), 26.2 (CH_2), 21.0 (CH_3), 15.8 (CH_3). MS (APCI), m/z (relative intensity): 358 (M^+,40), 315 (36), 43 (100).

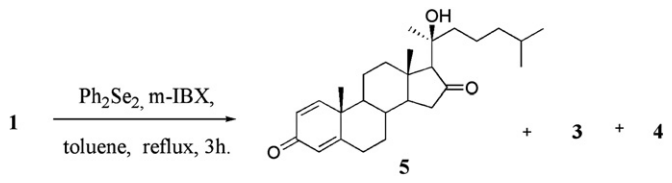
2.22. Biological assays

KB (human epidermoid carcinoma of cavity, ATCC CCL-17), MCF7 (human breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (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 according to Brien et al. [23]. Ellipticine and doxorubicin were used as positive controls. 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 8 concentrations. Five microliters 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 MCF7, and 5 days 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.

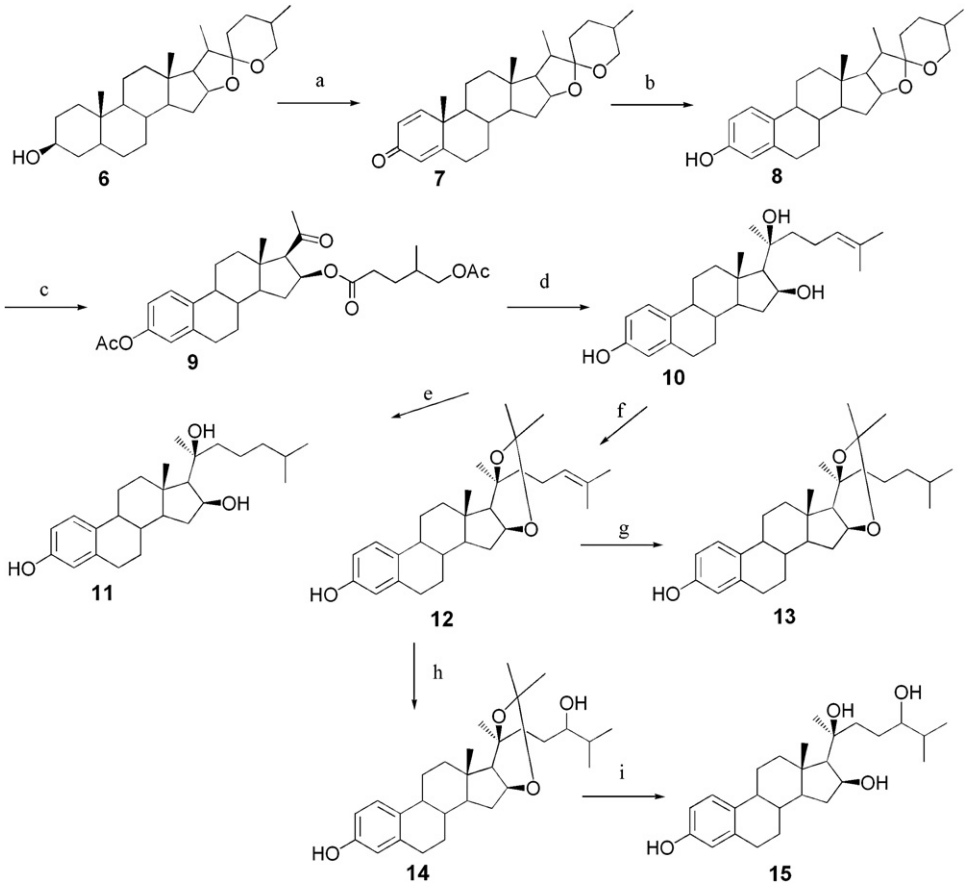
3. Results and discussion

3.1. Chemistry

The initial structure–activity relationship studies of 3,16,20-polyoxygenated steroids system were undertaken. We first investigated the different structural features of ring A and observed the effect of these altered systems. In this respect, we prepared two new steroids, **5** and **32**, with differences in the oxidation and unsaturation on ring A as depicted in Schemes 1 and 4. Oxidation of **1** using *meta*-iodoxybenzoic acid and diphenyl diselenide [24] in refluxed toluene until the reaction was completed (monitoring by TLC), smoothly afforded a mixture of (*20S*)-20-hydroxycholest-1-ene-3,16-dione (**3**), (*20S*)-20-hydrooxycholest-4-ene-3,16-dione (**4**) and (*20S*)-20-hydroxycholest-1,4-diene-3,16-dione (**5**) in 3:2:5 ratio. The ^{1}H NMR of **5** showed three olefinic quinone protons at 6.97, 6.19 and 6.02 ppm.



Scheme 1.



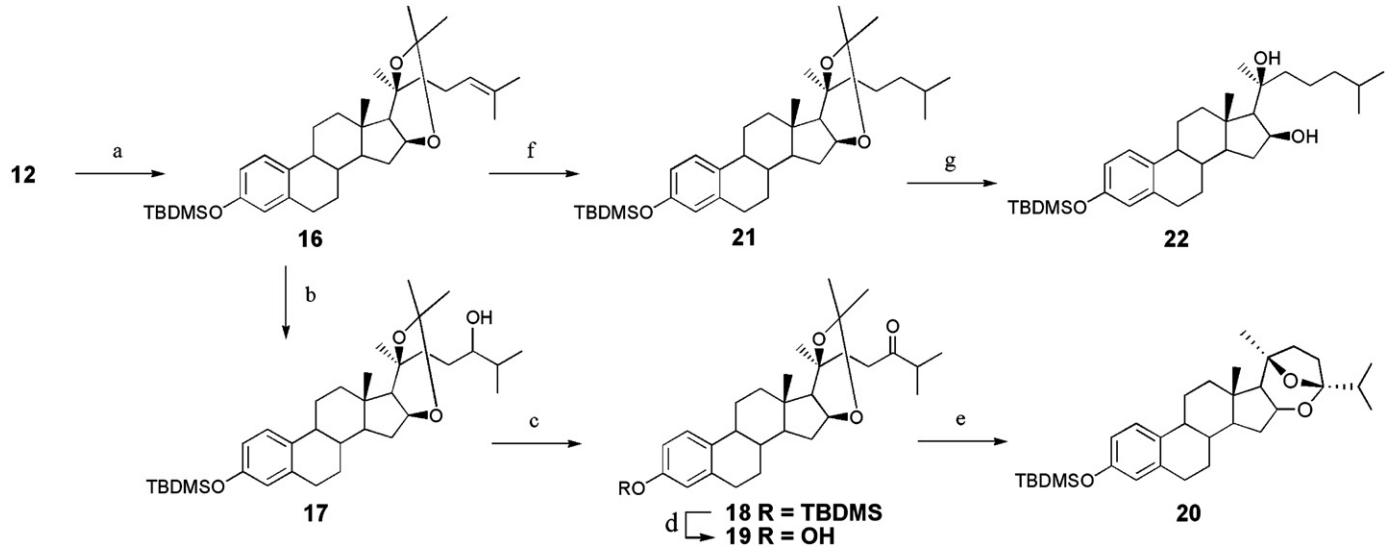
Scheme 2. Reagents and conditions: (a) m-IBX, $(PhSe)_2$, toluene, reflux, 7 h, 70%; (b) Li, Ph_2, Ph_2CH_2, THF, reflux, 2 h, 44%; (c) (i) NH_4Cl.py, 135°C, 12 h.; (ii) CrO_3, AcOH, H_2O, $(CH_2Cl)_2$, 0 °C, 2 h. (d) 5-bromo-2-methyl-2-pentene, Mg, THF, 0 °C, 20 min, 60%; (e) H_2, 5%Pd/C, EtOAc, EtOH, rt. 16 h, 100%; (f) 2,2-dimethoxypropane, TsOH, rt, 70%; (g) H_2, 5% Pd/C, EtOH, rt, 16 h, 99%; (h) BH_3, THF (1 M in THF), THF, 0 °C, 2 h, then 35% H_2O_2, 1N NoOH, 64%; (i) 70% AeOH, 50 °C, 24 h, 56%.

The aromatic steroid analogues **11** were synthesized from tigogenin (**6**) (Scheme 2) by first oxidation using m-iodoxybenzoic acid (*m*-IBX) and diphenyl diselenide [24] to give quinone **7**. Aromatization of **7** by treating with lithium, biphenyl in the presence of diphenylmethane provided compound **8** in moderate yield. Transformation of the spiro ketal moiety in **8** to the keto ester moiety in **9** was performed in a one pot preparation by the method developed by Micovic et al. [25] and modified by Fuchs. [26]. Grignard reaction of **9** with 2-bromo-5-methyl-2-pentene and Mg in THF gave 3,16,20-trihydroxy-1,3,5(10)-estratriene (**10**). Hydrogenation of unsaturated side chain was achieved by using Pd/C catalyst in ethanol and ethyl acetate at atmospheric pressure and room temperature. The reaction provided cholesterol-like side chain of **11** in quantitative yield.

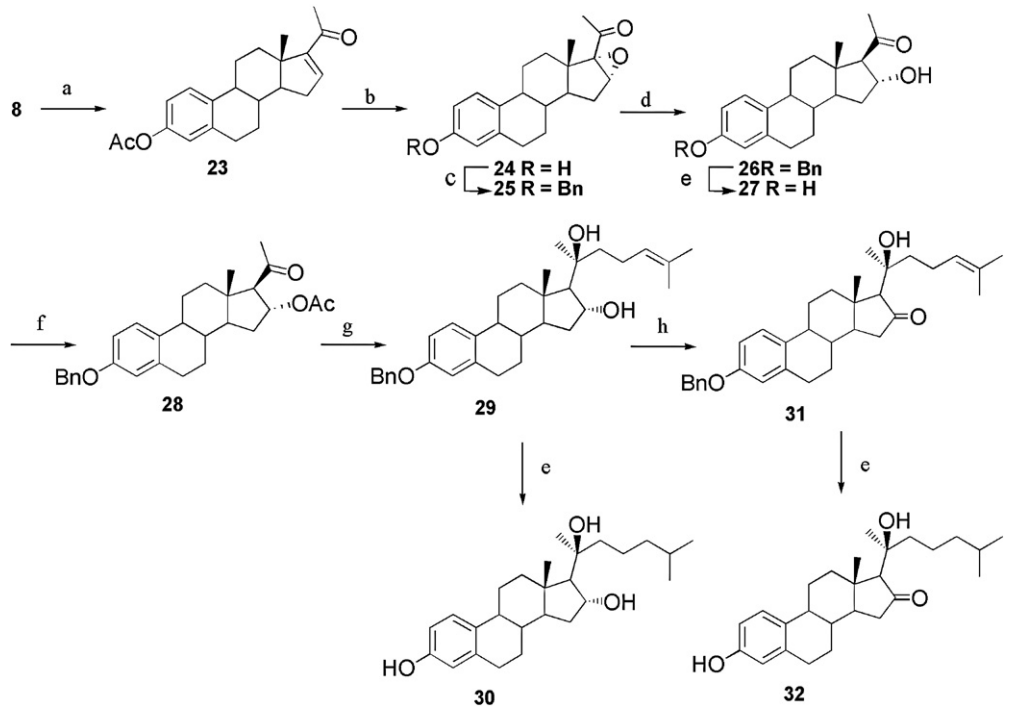
In order to study the effect on the cytotoxicity of a ketone or hydroxyl at C-24 of cholestane side chain, 1,3-diol of **10** was protected as acetonide by treatment with 2,2-dimethoxy propane and TsOH to furnish the corresponding acetonide **12** in 70% yield (Scheme 2). Hydrogenation of **12** using 5% Pd/C in EtOAc-EtOH at room temperature provided the corresponding **13** in almost quantitative yield. Hydroboration of **12** followed by oxidation using H_2O_2 provided alcohol **14**. Removal of cyclic ketal in **14** with 70% acetic acid gave the corresponding 16,20,24-triol **15**. An attempt to oxidize 2° alcohol at the side chain of **14** to the corresponding ketone with CrO_3 was unsuccessful probably due to the phenolic hydroxyl group. Therefore the phenolic OH must be protected.

Silyllation of **12** with tert-butyldimethylsilyl chloride, potassium carbonate gave **16** in good yield (Scheme 3). Hydroboration oxidation of **16** as described above provided alcohol **17**. Oxidation of **17** to ketone **18** was accomplished by using PCC in methylene chloride. Silyl protecting group of **18** was removed by treatment with TBAF to give the corresponding phenol **19**. Unfortunately deprotection of cyclic ketal in **18** by 10% HCl did not give the expected dihydroxy ketone but a new cyclic ketal **20** was formed instead. In order to study the effect on the cytotoxicity of phenolic hydroxyl group, the protected phenolic compound was prepared. The unsaturated side chain of **16** was hydrogenated to give **21** and then 1,3-acetal was deprotected to provide 17,20-dihydroxy steroid (**22**).

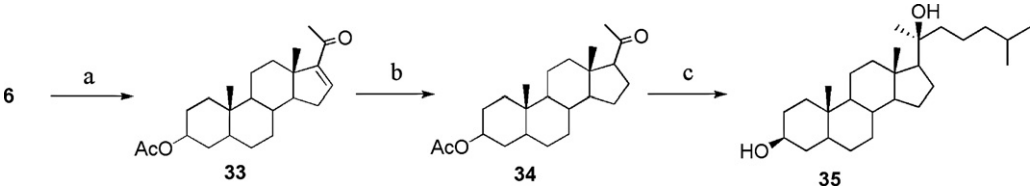
Apart from the effect of the side chain, we have also examined the effect of functionality at C16. Therefore the synthesis of the 16α-ol and 16-keto analogues were undertaken as shown in Scheme 4. Transformation of the spiro ketal moiety in **8** to acetyl side chain in **23** was performed in one pot preparation by the method developed by Micovic et al. [25], and modified by Fuchs et al. [26]. Epoxidation of α,β-unsaturated ketone **23** gave epoxide **24** then protection of hydroxyl as benzyl ether gave α-epoxide **25**. Rigeoselective ring opening of the epoxide by hydrazine in ethanol afforded α-hydroxy ketone **26** in good yield. Debenzylation of **26** by hydrogenation using Pd/C catalyst provided **27** in nearly quantitative yield. Acetylation of **26** with Ac_2O gave acetate **28** in 97% yield. Steroid ketone **28** was converted to **29** by the methodology already discussed in Scheme 2. Hydrogenation of **29** by using Pd/C catalyst gave **30**. Whereas oxidation of **29** with PCC in sodium acetate provided 16-ketosteroid **31** in good yield. Debenzylation of **31** by hydrogenation using Pd/C catalyst provided **32** in nearly quantitative yield.



Scheme 3. Reagents and conditions: (a). TBDMSCI, imidazole, DMF, CH_2CL_2, rt, 36 h, 79%; (b) BH_3. THF (1M in THF), THF, rt, 2 h, then 35% H_2O_2, 1N NaOH, 48%; (c) NaOAc, PCC, CH_2Cl_2, rt, 1 h, 60%; (d) TBAF, THF, AcOH, CH_3CN, 70%; (e) 10% HCl, CH_2Cl_2, rt, 5 min, 49%; (f) H_2, 5% Pd/C, EtOAc, EtOH, rt, 16 h, 99%; (g) 70% AcOH, dioxane, 50 °C, 24 h, 70%.



Scheme 4. Reagents and conditions: (a) (i) NH_4Cl, py, Ac_2O, 135 °C, 12 h, (ii) CrO_3, AcOH, H_2O, $(CH_2Cl_2)_2$, 0 °C, 2 h and (iii) Al_2O_3, benzene, rt, 16 h. 58% 3 steps; (b) H_2O_2, NaOH, MeOH, rt, 16 h, 90%; (c) BnBr, K_2CO_3, acetone, reflux, 6 h, 97%; (d) $NH_2NH_2{\cdot}H_2O$, EtOH, CH_2Cl_2, rt, 30 min, 92%; (e) H_2, 5% Pd/C, EtOAc, EtOH, rt, 16 h, 99%; (f) Ac_2O, py, 0 °C, 3 h, 97%; (g) 5-bromo-2-,ethyl-2-pentene, Mg, THF, 0 °C, 20 min, 39%; (h) PCC, NaOAc, CH_2Cl_2, rt, 3 h, 70%.



Scheme 5. Reagents and conditions: (a) (i) NH_4Cl, py, Ac_2O, 135 °C, 9 h; (ii) CrO_3, AcOH H_2O, $(CH_2Cl_2)_2$, rt, 1 h. (iii) Al_2O_3, benzene, rt, 16 h. 55% 3 steps; (b) H_2, 5%Pd/C, EtOAc, EtOH, rt, 16 h, 99%; (c) 5-bromo-2-methylpentane, Mg, THF, 0 °C, 5 min, 87%.

Table 1
Cytotoxicity of the synthetic steroids **5**, **10**, **11**, **13**, **15**, **19**, **22**, **27**, **30**, **32** and **35** against human carcinoma cell lines (MCF7, NCI and KB).

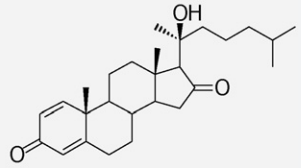
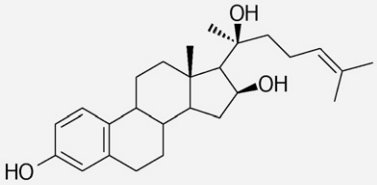
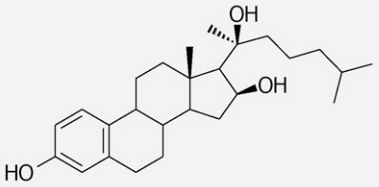
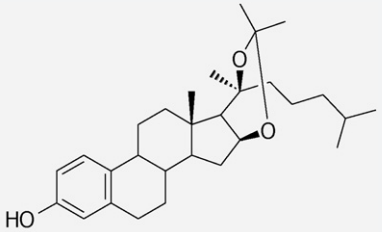
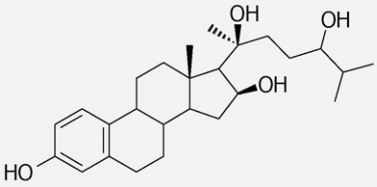
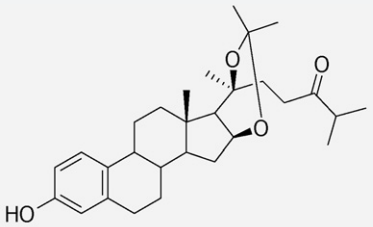
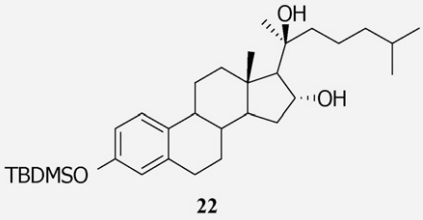
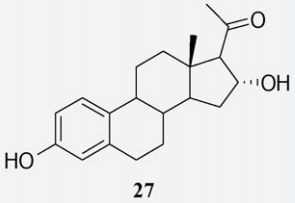
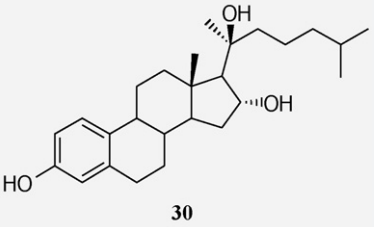
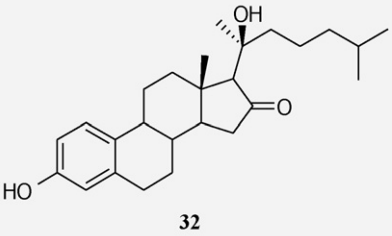
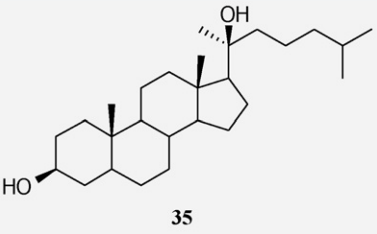
Compound	IC_{50} (μM)[a]		
	MCF7	NCI	KB
 **5**	55.53	55.75	48.40
 **10**	Inactive[b]	Inactive[b]	Inactive[b]
 **11**	Inactive[b]	Inactive[b]	11.73
 **13**	12.66	51.18	16.64
 **15**	42.43	Inactive[b]	20.41
 **19**	21.41	16.69	48.04

Table 1 (*Continued*)

Compound	IC_{50} (μM)[a]		
	MCF7	NCI	KB
 **22**	Inactive[b]	Inactive[b]	56.13
 **27**	Inactive[b]	Inactive[b]	Inactive[b]
 **30**	30.2	11.95	39.45
 **32**	12.66	17.43	26.43
 **35**	Inactive[b]	53.59	53.99
Ellipticine[c]	ND	1.79	1.72
Doxorubicine[c]	1.51	0.16	0.33

MCF7, human breast adenocarcinoma; NCI, 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] Used as reference.

Moreover the analogue without functional group at C-16 was also synthesized as shown in Scheme 5. Transformation of tigogenin (**6**) to unsaturated ketone **33** was performed by the previously described method in Scheme 4. Hydrogenation of **33** provided the corresponding ketone **34** in nearly quantitative yield. Gridnard reaction of **34** with 5-bromo-2-methylpentane and Mg in THF gave 3β,20-dihydroxylcholestane (**35**) in good yield.

3.2. Biological activity

All synthetic compounds as described above were subjected to an in vitro cytotoxic evaluation against KB (human epidermoid carcinoma), NCI (human lung cancer) and MCF-7 (human breast carcinoma) cell lines by using resazurin microplate assay (REMA) which was a modified method of fluorescent dye for the mam-

malian cell cytotoxicity according to Brien et al. [23]. Ellipticine and doxorubicin were used as positive control. The results are summarized in Table 1.

In our previous work compound **1** bearing a ketone at C-3 showed no cytotoxicity (IC_{50} > 100 μM) against all tested cells, whereas the analogues **3** and **4** bearing α,β-unsaturated ketone in ring A showed an increase in the cytotoxic activity against all tested cells [MCF7 (30.65 and 31.43 μM), NCI (6.16 and 10.51 μM) and KB (47.22 and 41.74 μM)] [17]. The quinone steroid **5** synthesized from this work which has a higher level of oxidation on ring A, showed a considerably lower cytotoxicity than **3** and **4** against MCF7 and NCI but similar activity for KB. In contrast when ring A is aromatic as steroid **32**, it exhibited the most potent cytotoxicities in all tested cells (see Table 1).

Comparison on cytotoxicity of A-ring aromatic compounds which have different functional group or stereochemistry at C-16 (**11**, **30** and **32**) showed that compound **32** with ketone group at C-16 is very active to all tested cells whereas **11** bearing β-OH group at C-16 showed no activity against NCI and MCF7 but two times more active against KB cell lines than **32**. Interestingly, 16α-OH steroid (**30**) exhibited higher cytotoxicity against MCF7 and NCI than the corresponding 16β-OH isomer (**11**) but lower for KB cell lines (see Table 1).

The absence of OH at C-16 (**35**) and cholesterol like side chain at C-20 (**27**) in the steroid skeleton apparently resulted in decreased cytotoxicity, indicating that both OH and side chain must be present to retain cytotoxicity against the tested cancer cell lines. However it was found that steroids which have both hydroxyl groups at C-16 and C-20 protected as cyclic ketal such as compounds **13** and **19** still had activities. Compound **13** showed very strong cytotoxicity against MCF7 and KB but moderately active against NCI whereas **19** showed strong activity against MCF7, NCI and moderate activity against KB.

Steroid analogues, compounds **10** and **15** which have functional group on side chain and **11** which has no functional group. Compound **10** containing double bond at C-24 on side chain showed no cytotoxicity against all tested cell lines whereas compound **15** with hydroxyl group at C-24 showed very potent cytotoxicity against KB, moderate against MCF7 and was inactive to NCI. Compound **11** showed no activity against both MCF7 and NCI but was very active against KB cell lines. These results indicated that functional group on steroids side chain have some kind of cytotoxic selectivity against the tested cell lines. The importance of the presence of a cholesterol like side chain and hydroxyl group at C-3 is illustrated by the lack of activity in compound **27** and no cytotoxicity against both MCF7 and NCI and considerable low cytotoxicity against KB cell lines of compound **22** when hydroxyl group at C-3 was protected.

In conclusion we have described the chemical synthesis of a series of polyoxygenated steroid derivatives with various steroid skeleton moieties. Based on this SAR study, compounds with A-ring aromatic of this series exhibited the most potent cytotoxicities in all tested cells. The hydroxyl group at C-16 and cholesterol like side chain at C-20 of the steroids is at least partly responsible for the observed activity. The existence of hydroxyl group at C-3 was also crucial for cytotoxic activity.

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