SYNTHESIS OF TWO NEW HYDROXIMINOSTEROIDS FROM CHOLESTEROL AND THEIR BIOLOGICAL EVALUATION

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Abstract. A simple and efficient synthesis of two new hydroximino steroidal derivatives (11, 13) from cholesterol as starting material was described. The cytotoxicity of these compounds, their intermediates and two other known hydroximino steroids were evaluated on human cancer cell lines including hepatocellular carcinoma (Hep-G2), cervical cancer (HeLa) and glioblastoma (T98G). Compound 13 presented a potent antiproliferative effect to T98G cancer cell at 2.9 \( \mu \)M of IC\(_{50}\). Our results demonstrated that the compounds with oxime group at C-6 having better cytotoxic activity against tested cell lines. Presence of 4,5-double bond or 4,5-epoxide can increase cytotoxicity for the 3,6-dihydroximino derivatives. In addition, the antimicrobial assay showed that two new oximes 11 and 13 were active against \( E. \) coli and \( S. \) aureus strain with MIC values of 50 \( \mu \)g/mL. The active compounds may provide us a clue for a lead structure of anticancer and antimicrobial agents.

Keywords: cholesterol, hydroximinosteroiud, HeLa, HepG2, T98G.

Classification numbers: 1.1.2, 1.2.1.

1. INTRODUCTION

In recent years, marine organisms have been regarded as the sources for natural steroids with unusual and interesting functionalities [1]. A variety of natural steroids have been isolated from various marine organisms, particularly from sponges and starfish [2]. Among them, hydroximino steroids isolated from marine sponges display a wide range of biological activities including antiviral, cytotoxicity and aromatase inhibitory activity [3-5]. Especially, compound (6E)-hydroximinocholest-4-en-3-one (1) which was isolated from \( Cinachyrella alloclada \)
exhibited a strong cytotoxicity against P-388 (murine leukemia), A-549 (human lung carcinoma), HT-29 (human colorectal adenocarcinoma) and MEL-28 (human myeloma) cell lines with IC$_{50}$ value ranging between 1.25-2.5 µg/mL [3,4]. The synthesis of several steroidal oximes containing 1-2 hydroximino groups and different side chains has been reported recently. Structure – bioactivity relationship studies indicated that the position of hydroximino groups and side chain type play a critical role in their cytotoxicities [5-8]. Cytotoxicity assays against P388, A549, HT29, MEL28 and T98G cell lines showed that cholestane-type side chain at C-17 exhibited stronger cytotoxicity compared to sitostane-, stigmastane- or gorgostane-type side chain [6, 7]. Presence of oxime group at C-3 or/p) and C-6 or presence of oxygen on A-ring (C-3,4,5) could remarkably increase the cytotoxic activity (compounds 2-4) [8,9].

![Figure 1: Natural hydroximinosteroid (1) and bioactive synthetic hydroximinosteroids (2-4).](image)

In this study, we report a short and efficient synthetic pathway for the preparation of two new steroidal oximes 11 and 13 from cholesterol as starting material with hydroximino groups at C-3 or C-3/C-6 and with epoxide ring at C-4,5. The antiproliferative activity against three human cancer cell lines (Hep-G2, HeLa, T98G) as well as the antimicrobial activity against 8 bacterial and fungal strains of these steroidal oximes and all intermediates were evaluated. In order to establish structure/activity relationship, the cytotoxicity of compounds 11 and 13 was compared with compounds 3 and 4 from the same bioassay. The aim of this study was to obtain additional information about structure-activity relationships for this type of compound.

2. MATERIALS AND METHODS

**General:** All reagents and solvents were purchased from Sigma-Aldrich, Acros and Merck Co and used without pre-purification. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV 500 spectrometer (Germany) at working frequencies 500 and 125 MHz, respectively. Chemical shifts (δ) are reported in ppm and coupling constants (J) are given in Hertz (Hz). MS spectra and HPLC were recorded on a LC-MS Agilent 1100 (USA). All reactions were monitored by thin layer chromatography (TLC) using silica gel 60 coated plates F254 (Merck). Visualization was performed under UV lamp at 254 and 365 nm or by spraying with 10 % H$_2$SO$_4$ solution.
Antimicrobial activity: Antimicrobial activity assay was performed on a 96-well microplate to determine the minimum inhibitory concentration (MIC) against 8 fungal and bacterial strains, i.e. Gram-negative bacteria (Escherichia coli M42, Pseudomonas aeruginosa ATCC 25923), Gram-positive bacteria (Bacillus subtilis ATCC27212, Staphylococcus aureus ATCC12222). The fresh microorganisms were diluted with the growth medium broth to a final inoculum size of about 10^3 colony-forming units (CFU) per mL. The samples were dissolved in 5 % DMSO at various concentrations and then were loaded into 96-well microplates with test microorganisms. Doxycycline, gentamicin, and nystatin were used as positive references for bacteria, and fungi, respectively. A blank control was treated in the same way using 5 % DMSO instead of the test samples [10].

Cytotoxicity assays: The cytotoxicity on hepatocellular carcinoma (Hep-G2) and cervical cancer (HeLa) cell lines was tested at Institute of Natural Products Chemistry (VAST). Cancer cell lines, i.e. Hep-G2 and HeLa, were purchased from American Type Culture Collection (ATCC, USA) and cultured in DMEM (Dulbecco’s Modified Eagle Medium) or EMEM (Eagle’s Minimum Essential Medium, Sigma-Aldrich, Singapore), 10 % heat-inactivated fetal bovine serum (FBS), at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. Cell viability was assessed through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously [11,12].

The cytotoxicity on T98G cell line (human glioblastoma cells) was tested at Korea Institute of Science and Technology (KIST) Gangneung, Institute of Natural Product. The cell viability assay was performed by image analysis using calcein AM (staining for live cells) and propidium iodide (staining for dead cells). T98G cells were cultured in DMEM media (Hyclone Co.) mixed with 10 % fetal bovine serum (Gibco Co.), 1 % penicillin/streptomycin, and 4 mM L-glutamine using an incubator with 5 % CO₂ at 37 °C. Cultured T98G cells were added in 96 well plates at the density of 5 × 10^4 cells/well and grown for 24 hours. Compounds with various concentration and control (0.5 % of dimethyl sulfoxide) were added to cells. After incubating cells for 24 hours, media was removed and cells were washed with PBS. The cells were treated with calcein AM and PI, followed by incubation for 30 min. The cells were analyzed by operetta image analysis.

Cholest-4-en-3,6-dione (5): Pyridiniumchlorochromate (PCC) (4.114 g, 19.0 mmol) was added to a solution of cholesterol (1.60 g, 3.8 mmol) in dry CH₂Cl₂ (30 mL) in one portion at room temperature. The reaction mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the residue was redissolved in CH₂Cl₂ (30 mL) and then washed with cold distilled water and brine. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure and the crude product was separated by chromatography on silica gel using dichloromethane/diethyl ether (1:1) as the eluent. Compound 5 was obtained as pale yellow crystals (1.20 g, 80 %). 1H NMR (500 MHz, CDCl₃) δH (ppm): 6.18 (H-4), 2.69 (dd, J = 4.0, 11.0 Hz, H-2), 2.53 (m, 1H, H-7), 2.47 (m, 1H, H-7), 2.16 (m, 1H, H-1), 2.11 (m, 1H, H-12), 2.05 (dd, J = 16.0, 12.5 Hz, H-2), 1.93 (m, 1H, H-1), 1.91 (m, 1H, H-20), 1.90 (m, 1H, H-16), 1.66 (m, 1H, H-11), 1.63 (m, 1H, H-1), 1.33 (m, 1H, H-12), 1.31 (m, 1H, H-12), 1.18 (s, 3H, H-19), 1.15 (m, 4H, H-15, H-23, H-14, H-17), 1.12 (m, 1H, H-24), 1.03 (m, 1H, H-22), 0.94 (d, J = 6.5 Hz, 3H, H-21), 0.88 (dd, J = 6.5 Hz, 6H, H-26, H-27), 0.74 (s, 3H, H-18). 13C NMR (125 MHz, CDCl₃), δC (ppm): 202.3 (C-3), 199.5 (C-6), 161.1 (C-5), 125.4 (C-4), 56.6 (C-14), 56.0 (C-17), 51.0 (C-9), 46.8 (C-2), 42.5 (C-13), 39.8 (C-10), 39.45 (C-24), 39.1 (C-12), 36.0 (C-22), 35.7 (C-8), 35.5 (C-1), 34.2 (C-20), 33.9 (C-7), 28.0 (C-25), 28.0 (C-16),
24.0 (C-15), 23.8 (C-23), 22.8 (C-26), 22.5 (C-27), 20.9 (C-11), 18.6 (C-21), 17.5 (C-19), 11.9 (C-18). ESI-MS (m/z): 398.62 [M+H]+.

(3E,6E)-dihydroximinocholest-4-ene (3): Compound 5 (81 mg, 0.2 mmol) and hydroxylamine hydrochloride (141 mg, 2.0 mmol) were dissolved in dry pyridine (5 mL). The mixture was stirred at room temperature for 24 h and the solvent was removed under reduced pressure. The residue was dissolved in water and extracted with ethyl acetate (3 x 10 mL). The organic layers were combined, dried over anhydrous Na2SO4, evaporated and the crude product was purified by chromatography on silica gel using dichloromethane/methanol (20:1) as the eluent to give 3 as a white solid (75 mg, 85%). 1H-NMR (CDCl3, 500 MHz) δH (ppm): 6.53 (s, 1H, H-4), 3.33 (dd, 1H, J = 4.5, 15.5 Hz, H-7), 3.08 (brd, 1H, J = 15.0 Hz, H-2), 1.00 (s, 3H, H-19), 0.91 (d, 3H, J = 6.5 Hz, H-21), 0.87 (d, 3H, J = 2.0 Hz, H-26), 0.86 (d, 3H, J = 2.5 Hz, H-27), 0.69 (s, 3H, H-18). 13C-NMR (CDCl3, 125 MHz) δC (ppm): 157.2 (C-6), 156.8 (C-3), 147.8 (C-5), 119.2 (C-4), 56.7 (C-14), 56.1 (C-17), 51.3 (C-9), 42.6 (C-13), 39.5 (C-7, C-24), 38.3 (C-12), 36.1 (C-22), 35.7 (C-20), 33.6 (C-1), 33.0 (C-10), 29.7 (C-8), 28.2 (C-16), 28.0 (C-25), 24.1 (C-23), 23.8 (C-15), 22.8 (C-26), 22.6 (C-27), 21.3 (C-11), 18.7 (C-21), 18.6 (C-2), 17.6 (C-19), 11.9 (C-18). ESI-MS (m/z): 429.2 [M+H]+.

Cholestane-3β,6α-diol (6): 1M BH3 in THF (7 mL, 7.0 m) was added slowly to a solution of cholesterol (540 mg, 1.4 mM) in dry THF (8 mL) under argon atmosphere at 0°C. The mixture was stirred at room temperature for 4 h. Aqueous solution of NaOH (5 N, 1.5 mL) and 30% H2O2 (0.75 mL) were added dropwise at 0°C. The mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure. The residue was extracted with EtOAc (3×15 mL). The combined organic layers were washed successively with 1N HCl, saturated NaHCO3 solution and brine, dried over Na2SO4, filtered and evaporated in vacuo. The crude product was purified by chromatography on silica gel (DCM/MeOH 100:1) to yield 6 as white needles (402 mg, 71%). 1H NMR (500 MHz, CDCl3) δH (ppm): 3.55 (1H, m, H-3), 3.39 (1H, dd, J = 4.5, 10.5, 11.0 Hz, H-6), 0.90 (3H, d, J = 6.5 Hz, CH3-21), 0.86 (6H, d, J = 6.5 Hz, CH3-26, CH3-27), 0.80 (3H, s, CH3-19), 0.65 (3H, s, CH3-18); 13C NMR (125 MHz, CDCl3) δC (ppm): 71.1 (C-3), 69.4 (C-6), 56.2 (C-14), 56.18 (C-5), 53.8 (C-17), 51.6 (C-9), 42.6 (C-13), 41.5 (C-4), 39.8 (C-12), 39.5 (C-24), 37.3 (C-1), 36.3 (C-10), 36.1 (C-22), 35.7 (C-20), 34.3 (C-8), 32.1 (C-7), 30.8 (C-2), 28.1 (C-16), 28.0 (C-25), 24.2 (C-15), 23.8 (C-23), 22.8 (C-26), 22.5 (C-27), 21.1 (C-11), 18.6 (C-19), 13.4 (C-24), 12.0 (C-18).

Cholestane-3,6-dione (7): Solution of 6 (100 mg, 0.25 mmol) in CH2Cl2 (10 mL) was treated with PCC (280 mg, 1.25 mmol) in a similar procedure of 5 to give a crude product which was subjected to chromatography on silica gel (hexane/ethyl acetate, 19:1) to afford 7 (82 mg, 82%) as light yellow crystals. 1H-NMR (CDCl3, 500 MHz) δH (ppm): 2.59 (brs, 1H, H-4), 2.40 (m, 1H, H-7), 2.37 - 2.34 (m, 1H, H-7), 2.30 - 2.32 (m, 1H, H-2), 2.05 - 2.10 (m, 1H, H-2), 2.00 (t, 1H, J = 12.0 Hz, H-4), 0.96 (s, 3H, H-19), 0.92 (d, 3H, J = 6.5 Hz, H-21), 0.87 (d, 3H, J = 6.5 Hz, H-26), 0.86 (d, 3H, J = 6.5 Hz, H-27), 0.69 (s, 3H, H-18). ESI-MS (m/z): 401.2 [M+H]+.

(3E,6E)-Dihydroximincholestane (4): Compound 7 was prepared according to the procedure of 3. Compound 4 was obtained as off white needles (35 mg, 81%). 1H-NMR (CDCl3, 500 MHz) δH (ppm): 3.33 (dd, 1H, J = 4.5, 14.0 Hz, H-7α), 3.27 (brd, 1H, J = 12.5 Hz, H-2β), 0.91 (d, 3H, J = 6.5 Hz, H-21), 0.87 (d, 3H, J = 6.0 Hz, H-26), 0.86 (d, 3H, J = 6.0 Hz, H-27), 0.85 (s, 3H, H-19), 0.67 (s, 3H, H-18). 13C-NMR (125 MHz, CDCl3) δC (ppm): 160.5 (C-6), 159.6 (C-3), 56.6 (C-14), 56.2 (C-17), 54.0 (C-9), 50.9 (C-5), 42.9 (C-10), 39.7 (C-13), 39.5 (C-24, C-12), 36.6 (C-22), 36.1 (C-20), 35.7 (C-8), 35.6 (C-16), 29.5 (C-25), 28.3 (C-7), 28.1 (C-2), 28.0 (C-1), 24.1 (C-19), 23.8 (C-15), 22.8 (C-23), 22.5 (C-4), 21.4 (C-27), 19.9 (C-26), 18.6 (C-11), 12.1 (C-21), 11.8 (C-18). ESI-MS (m/z): 431.2 [M+H]+.
Hydroximinosteroids from cholesterol and their biological activity

Cholest-4-ene-3β,6α-diol (8): The solution of 5 (530 mg, 1.38 mmol) in 20 mL of the solvent mixture (CH2Cl2:MeOH, 1:1) was stirred at room temperature. CeCl3·7H2O (530 mg, 1.38 mmol) and NaBH4 (232 mg, 6.07 mmol) were added to the solution and the mixture was stirred for 1 h. The HCl aqueous solution (1N) was added dropwise to neutralize the alkaline medium. CH2Cl2 (15 mL) was added and the organic layer was separated and dried over Na2SO4. The solvent was removed in vacuo to yield a white solid which was purified by flash column chromatography on silica gel (n-hexane/EtOAc, 5:1) to give 8 as white needles (580 mg, 89%).

1H NMR (500 MHz, CDCl3), δH (ppm): 5.65 (H-4), 4.22 (m, H-3), 4.19 (m, H-6), 1.04 (s, 3H, H-19), 0.90 (d, J = 6.0 Hz, 3H, H-21), 0.865 (d, J = 7.0 Hz, 3H, H-26), 0.860 (d, J = 6.5 Hz, 3H, H-27), 0.68 (s, 3H, H-18). 13C NMR (125 MHz, CDCl3), δc (ppm): 149.2 (C-5), 119.8 (C-4), 68.6 (C-3), 67.9 (C-6), 56.2 (C-14), 55.8 (C-17), 54.2 (C-9), 42.5 (C-13), 42.1 (C-2), 39.7 (C-10), 39.5 (C-24), 37.9 (C-12), 36.0 (C-22), 36.0 (C-8), 35.8 (C-1), 34.4 (C-20), 29.0 (C-7), 28.1 (C-25), 28.0 (C-16), 24.2 (C-15), 23.8 (C-23), 22.8 (C-26), 22.5 (C-27), 21.0 (C-11), 19.7 (C-21), 18.7 (C-19), 12.0 (C-18).

6-hydroxy-4α,5α-epoxycholestane-3-one (9) and 4α,5α-epoxycholestane-3,6-dione (10): Compound 8 (285 mg, 0.7 mmol) in CH2Cl2 (20 mL) was treated with m-CPBA (290 mg, 1.48 mmol) in CH2Cl2 (10 mL) by the procedure of 6 to give a mixture of 4α,5α-epoxycholestane-3β,6α-diol and 4β,5β-epoxycholestane-3β,6α-diol (250 mg, 4:1). To a stirred solution of the diol mixture (210 mg) in 10 mL of CH2Cl2 at 0°C was added Dess-Martin periodinane (420 mg, 0.96 mmol). After 1 h, the homogeneous solution was diluted with diethylether (10 mL) and poured into saturated aqueous NaHCO3 (20 mL) containing Na2SO3 (4 g). The mixture was stirred for 15 min. The organic layers were separated and extracted with ether 10 mL. The organic phase was washed with saturated NaHCO3 (30 mL) and water (30 mL), dried, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, n-hexane/EtOAc, 9:1) to give 9 (36 mg, 12.3%) and 10 (60 mg, 14.5%).

6-hydroxy-4α,5α-epoxycholestane-3-one (9): white needles. 1H-NMR (CDCl3, 500 MHz), δH (ppm): 4.17 (dd, 1H, J 3.0 & 10.0 Hz, H-6), 3.48 (s, 1H, H-4), 1.13 (s, 3H, H-19), 0.90 (d, 3H, J = 6.5 Hz, H-21), 0.87 (d, 3H, J = 6.0 Hz, H-26), 0.86 (d, 3H, J = 6.0 Hz, H-27), 0.69 (s, 3H, H-18). 13C-NMR (CDCl3, 125 MHz), δc (ppm): 206.0 (C=O, C-3), 72.4 (C-5), 64.7 (C-6), 56.7 (C-4), 56.1 (C-14), 55.6 (C-17), 46.3 (C-9), 42.8 (C-13), 39.5 (C-2), 39.4 (C-24), 38.2 (C-12), 37.5 (C-10), 36.1 (C-22), 35.7 (C-8), 34.1 (C-20), 32.6 (C-1), 28.0 (C-25, C-7), 28.01 (C-16), 26.6 (C-15), 24.2 (C-15), 23.8 (C-23), 22.8 (C-27), 22.6 (C-26), 21.6 (C-11), 19.2 (C-19), 18.6 (C-21), 12.0 (C-18). ESI-MS (m/z): 417.2 [M+H]+.

4α,5α-epoxycholestane-3,6-dione (10): white needles. 1H-NMR (CDCl3, 500 MHz), δH (ppm): 3.13 (s, 1H, H-4), 1.15 (s, 3H, H-19), 0.92 (d, 3H, J = 6.5 Hz, H-21), 0.87 (d, 3H, J = 6.5 Hz, H-26), 0.86 (d, 3H, J = 6.5 Hz, H-27), 0.72 (s, 3H, H-18). 13C-NMR (CDCl3, 125 MHz), δc (ppm): 203.2 (C=O, C-6), 202.8 (C=O, C-3), 71.4 (C-5), 60.1 (C-4), 56.4 (C-17), 56.0 (C-14), 46.15 (C-2), 46.13 (C-9), 42.9 (C-13), 39.5 (C-24), 39.11 (C-12), 39.07 (C-10), 36.1 (C-22), 35.7 (C-8), 35.6 (C-20), 32.5 (C-7), 28.0 (C-25), 27.9 (C-16), 27.6 (C-1), 24.0 (C-15), 23.8 (C-23), 22.8 (C-27), 22.6 (C-26), 21.7 (C-11), 18.7 (C-19), 18.6 (C-21), 12.0 (C-18). ESI-MS (m/z): 415.2 [M+H]+.

4α,5α-epoxy-6-hydroxycholestane-3-oxime (11) and 4α,5α-epoxycholestane-3-one (12): 6-hydroxy-4α,5α-epoxycholestane-3-one (9) (25 mg, 0.06 mmol) was treated with hydroxylamine hydrochloride (NH2OH.HCl) (60 mg, 0.7 mmol) in pyridine (3 mL) by the procedure of 3 to give a residue which was subjected to column chromatography on silica gel (n-hexane/EtOAc, 3:1) to give 11 (5 mg, 19.3%) and 12 (3.5 mg, 13.4%).
**4α,5α-epoxy-6-hydroxycholestan-3-one (11):** white needles. $^1$H-NMR (CDCl$_3$, 500 MHz), $\delta_H$ (ppm): 4.16 (dd, 1H, $J = 5.0$, 11.5 Hz, H-6/$\beta$), 3.81 (1H, s, H-4), 2.59 (dd, 1H, $J = 4.0$, 19.5 Hz, H-8), 1.06 (s, 3H, H-19), 0.90 (d, 3H, $J = 6.0$ Hz, H-21), 0.86 (2d, 6H, $J = 1.5$, 6.0 Hz, H-26, 27), 0.68 (s, 3H, H-18). $^{13}$C-NMR (CDCl$_3$, 125 MHz), $\delta_C$ (ppm): 154.9 (C-3), 89.7 (C-5), 69.5 (C-4), 64.8 (C-6), 56.2 (C-14), 55.6 (C-17), 54.2 (C-9), 45.7 (C-2), 42.8 (C-13), 39.5 (C-10), 39.4 (C-24), 38.0 (C-12), 37.4 (C-22), 36.1 (C-1), 35.8 (C-8), 34.1 (C-7), 28.1 (C-25), 28.0 (C-16), 25.7 (C-20), 24.2 (C-15), 23.8 (C-23), 22.8 (C-26), 22.6 (C-27), 21.5 (C-11), 18.6 (C-21), 17.9 (C-19), 12.0 (C-18). (+)-HR-ESI-MS: 432.3474 [M+H]$^+$ (calculated for C$_{27}$H$_{45}$NO$_5$: 432.3478).

**4,5,6-trihydroxy cholestan-3-one (12):** white needles. $^1$H-NMR (CDCl$_3$, 500 MHz), $\delta_H$ (ppm): 4.05 (m, 1H, H-6), 3.28 (d, 1H, $J = 3.5$ Hz, H-4), 2.60 (dd, 1H, $J = 3.5$ & 15.0Hz, H-7), 2.08 (d, 1H, $J = 3.0$ Hz, H-12), 2.06 (d, 1H, H-24), 1.96 (t, 1H, $J = 12.5$ Hz, H-7), 1.89 (m, 1H, H-8), 1.87 (m, 1H, H-25), 1.64 (m, 1H, H-2), 1.56 (m, H-15), 1.53 (m, H-2), 1.52 – 1.50 (m, 2H, H-11), 1.03 (s, 3H, H-19), 0.92 (d, 3H, $J = 6.5$ Hz, H-21), 0.87 (d, 3H, $J = 6.5$ Hz, H-26), 0.86 (d, 3H, $J = 6.0$ Hz, H-27), 0.70 (s, 3H, H-18). $^{13}$C-NMR (CDCl$_3$, 125 MHz), $\delta_C$ (ppm): 205.6 (C=O, C-3), 69.9 (C-5), 64.7 (C-6), 62.6 (C-4), 56.5 (C-14), 56.1 (C-17), 48.1 (C-9), 46.5 (C-7), 42.7 (C-13), 39.5 (C-12), 39.3 (C-24), 38.1 (C-10), 36.1 (C-22), 35.7 (C-20), 34.8 (C-8), 29.2 (C-1), 28.01 (C-23), 28.0 (C-25), 25.7 (C-2), 24.1 (C-15), 23.8 (C-16), 22.8 (C-26), 22.6 (C-27), 21.5 (C-11), 18.6 (C-21), 18.3 (C-19), 11.9 (C-18). (+)-HR-ESI-MS: [M+H]$^+$ 435.3633 (calculated for C$_{27}$H$_{46}$O$_4$: 435.3474).

**4α,5α-epoxycholestan-3,6-dione (13):** 4α,5α-epoxycholestan-3,6-dione 30 mg (10, 0.07 mmol) was treated with hydroxylamine hydrochloride (50 mg, 0.7 mmol) in pyridine (3 mL) by the procedure of 3 to give a crude product which was subjected to chromatography on silica gel (DCM/EtOAc, 6:1) to give 13 as white needles (7.0 mg, 22.5 %). $^1$H NMR (CDCl$_3$, 500 MHz), $\delta_H$ (ppm): 4.44 (s, 1H, H-4), 2.64 (m, 1H, H-7), 2.58 (m, 1H, H-2), 2.40 (dd, 1H, $J = 5.0$, 19.5 Hz, H-2), 2.14 (m, 1H, H-7), 2.10 (m, 1H, H-8), 2.00 (dt, 1H, $J = 3.5$, 6.0 Hz, H-12), 1.86 (m, 1H, H-9), 0.91 (d, 3H, $J = 6.5$ Hz, H-21), 0.88 (d, 3H, $J = 6.5$ Hz, H-26), 0.87 (s, 3H, H-19), 0.86 (d, 3H, $J = 6.5$ Hz, H-27), 0.69 (s, 3H, H-18). $^{13}$C-NMR (CDCl$_3$, 125 MHz), $\delta_C$ (ppm): 161.3 (C=NOH, C-6), 154.6 (C=NOH, C-3), 91.0 (C-5), 85.7 (C-4), 57.5 (C-14), 55.9 (C-17), 49.0 (C-9), 42.4 (C-13), 40.7 (C-10), 39.5 (C-12, C-24), 39.3 (C-2), 36.1 (C-22), 35.7 (C-8), 32.1 (C-20), 30.4 (C-23), 28.0 (C-7), 27.97 (C-25), 27.8 (C-16), 24.2 (C-15), 23.8 (C-23), 22.8 (C-2), 22.54 (C-27), 22.47 (C-26), 19.4 (C-11), 18.7 (C-21), 14.8 (C-19), 11.8 (C-18). (+)-HR-ESI-MS: [M+H]$^+$ 445.3427 (calculated for C$_{27}$H$_{48}$N$_2$O$_5$: 445.3430).

### 3. RESULTS AND DISCUSSION

Two known steroidal oximes (3, 4) with two hydroximino groups at C-3 and C-6 were prepared from cholesterol as starting material by methods described by Cui et al. (Figure 2) [7, 8].

Oxidation of cholesterol with pyridinium chlorochromate (PCC) in CH$_2$Cl$_2$ gave compound 4-en-3,6-dione (5) with 80 % yield. Next, the dione 5 was treated with hydroxylamine hydrochloride in pyridine to afford the dihydroxymino with a double bond at C-4,5 position in 3 (85 %). The structure of 5 and 3 were confirmed by analysis of $^1$H, $^{13}$C NMR, ESI-MS spectra and by comparing with reported data [7]. Reduction of cholesterol to 3,6-diol 6 by using BH$_3$ in THF, then treated with H$_2$O$_2$/NaOH. Oxime derivative 4 was prepared from diol 6 by two synthetic steps as in the case of 3 in a yield of 82 % and 81 %, respectively [8].

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Hydroximinosteroids from cholesterol and their biological activity

**Figure 2.** Reagents and conditions: (i): PCC/CH$_2$Cl$_2$, rt, 48 h; (ii): NH$_2$OH.HCl/Pyridine, rt, 24 h; (iii): BH$_3$.THF/H$_2$O$_2$, NaOH, rt, 1 h.

Two new steroidal oximes 11 and 13, with an epoxide ring at C-3,4, were prepared in 3 steps from diketone 5 according to the sequence shown in Figure 3. First, the reduction of 5 using NaBH$_4$ in the presence of CeCl$_3$.7H$_2$O gave the cholest-4-en-3,6-diol (8) in 89 % yield.
The signals of C=O groups at $\delta_C$ 202.3 and 199.5 ppm in the $^{13}$C NMR spectrum of 5 were replaced by two characteristic signals of CH-OH groups at $\delta_C$ 68.6 (C-3) and 67.9 (C-6) in the $^{13}$C NMR spectrum of 8. The $\alpha$-configuration of H-3 and $\beta$-configuration of H-6 of compound 8 was confirmed by comparing the coupling constance, chemical shifts and multiplicities with reported data by Cui [13]. Oxidation of 8 with m-CPBA and then by Dess-Martin in dichloromethane at 0 °C for 1 h gave the mixture of 6-hydroxy-4α,5α-epoxycholestan-3-one (9) and 4α,5α-epoxycholestan-3,6-dione (10). $^{13}$C NMR spectra of 9 and 10 revealed that compound 9 was incomplete oxidation product with only one group carbonyl at C-3 ($\delta_C$ 206.0) and compound 10 contained two carbonyl group at C-3 ($\delta_C$ 203.2) and C-6 ($\delta_C$ 202.8). It was indicated that 8 was first converted to a mixture of 4α,5α-epoxide-3,6-diol and 4β,5β-epoxide-3,6-diol (TLC analysis monitoring) under treatment with m-CPBA [9], and then the CH-OH groups at C-3 and C-6 were oxidized by Dess Martin reagent into C=O group. It was surprising that only oxidative products from 4α,5α-epoxide were observed from the results reactional medium. NOESY spectra of 9 and 10 with the correlation between H-4 and H-19 confirmed $\alpha$-configuration of epoxide ring in two molecules.

Similar procedure as above using hydroxylamine hydrochloride in pyridine on compound 9 and 10 afforded corresponding hydroximino products 11 and 13. The $^1$H NMR spectrum of compound 11 showed all proton signals as in the $^1$H NMR spectrum of 9, but with a deshielding of H-4 signal from $\delta_H$ 3.48 to $\delta_H$ 3.81 ppm. Likewise, the carbonyl group’s resonance signal (C-3) of compound 9 at $\delta_C$ 206.0 ppm disappeared and replaced by a quaternary carbon at $\delta_C$ 154.9 (C-3) which assigned to the C=N-OH groups of the expected product. Moreover, the carbon C-4 and C-5 signals of compound 11 move slightly towards the lower field comparing to compound 9. Similarly, in the $^{13}$C NMR spectrum of product 13, the presence of two quaternary carbon signals at $\delta_C$ 154.6 (C-3) and 161.3 (C-6) confirmed that 2 C=O groups of 10 were converted to C=N-OH groups. Additionally, HRMS data of 11 and 13 correspond to expected molecular formulas. A side-product by the epoxide opening reduction (12) was suprisingly obtained together with 13 from the reaction of 9. $^{13}$C NMR spectrum of 12 showed the presence of one C=O group ($\delta_C$ 205.6) and three C-OH groups ($\delta_C$ 69.9 (C-5), 64.7 (C-6) and 62.6 (C-4)).

**Biological evaluation:** Cytotoxicity of prepared hydroximinosteroidal derivatives (3, 4, 11, 13) and their intermediates (5-10, 12) against three human cancer cell lines including hepatocellular carcinoma (Hep-G2), cervical cancer (HeLa) and glioblastoma (T98G) were studied. The results which were expressed as IC$_{50}$ values in µM revealed that compounds 3, 4, 9, 10, 12 and 13 exhibited a moderate activity against at least one tested cancer cell line at concentrations of up to 50 µg/mL (Table 1). In particular, hydroximinosteroidal 13 shows a potent antiproliferative effect to T98G cancer cells at 2.9 µM of IC$_{50}$.

Amongst, apparently the hydroximinosteroidal derivatives (3, 4, 11, 13), compounds with oxime group at C-6 (3, 4, 13) exhibited stronger cytotoxicity against 3 tested cancer cell lines compare to compound with only oxime group at C-3 (11). However, the absence of 4,5-double bond on 3,6-dihydroximino 4 caused loss of cytotoxic activity against Hela and Hep-G2 cell lines in comparison of 3,6-dihydroximino 3. Cui et al. previously also observed the decrease of cytotoxicity of 4 in comparison of 3 while testing against Sk-Hep-1, H-292, PC-3 and Hey-1B cancer cell lines [7, 8]. It was interesting that compound 11 with only 3-hydroximino group and 4,5-epoxide ring was found inactive, while 3,6-dihydroximino 13 with 4,5-epoxide ring was selectively cytotoxic against T98G (IC$_{50}$: 2.9 µM).
**Table 1.** Cytotoxic activity of synthetic compounds against three human cancer cell lines.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC$_{50}$ (µM)</th>
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<th>Hep-G2</th>
<th>T98G</th>
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$^a$ Used as positive control. Hela: cervical cancer cell line; Hep G2: hepatocellular carcinoma cell line; T98G: glioblastoma cell line. Paclitaxel: positive control.
Among the intermediates (6-10, 12), the results demonstrated that compounds with the presence of oxygen at C-4 or C-5 (9, 10, 12) showed a moderate cytotoxic activity against at least one of tested cell lines whereas the others were found no obvious cytotoxicity against these cancer cells. These results coincide with previously reported researches, have revealed that the hydroximino group at C-6 is important for determination of cytotoxic activity of this type of compound. The 4,5-double bond or oxygen at C-4 or C-5 may have positive effects on cytotoxicity against tested cancer cells [8, 9]. This study gave supplement information for the structure – activity relationship and need further investigation.

Preliminary antimicrobial assays indicated that six of tested compounds, i.e. 5, 6, 9, 10, 11, 13, were active against at least one microorganism strain with MIC values ranging between 12.5 - 50 µg/mL (Table 2). Two new oximes 11 and 13 were found active against E. coli and S. aureus strain.

Table 2. Antimicrobial activity of compounds 3-13.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimum inhibitory concentration (MIC, µg/ml)</th>
<th>MIC (µg/ml)</th>
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4. CONCLUSIONS

Two new hydroximino steroid derivatives of cholesterol (11, 13) with 1-2 hydroximino groups at C-3 (ring A) and C-6 (ring B), and with epoxide ring at C-4,5 were prepared by short and efficient synthetic pathway. The cytotoxicity against HepG2, Hela and T98G of synthesized compounds and their intermediates together with 2 known hydroximinosteroids (3, 4) were studied for the first time. Compound 13 presented a potent antiproliferative effect to T98G cancer cell with IC_{50} value of 2.9 µM. In addition, two new oximes 11 and 13 and compounds 5, 6, 9, 10 were shown active in antimicrobial assay against at least one tested strain with MIC values ranging between 12.5 - 50 µg/mL.

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REFERENCES