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SYNTHESIS OF TWO NEW HYDROXIMINOSTEROIDS FROM CHOLESTEROL AND THEIR BIOLOGICAL EVALUATION

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Abstract. A simple and efficient synthesis of two new hydroximinosteroidal derivatives (**11**, **13**) from cholesterol as starting material was described. The cytotoxicity of these compounds, their intermediates and two other known hydroximinosteroids 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 μM 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 μg/mL. The active compounds may provide us a clue for a lead structure of anticancer and antimicrobial agents.

Keywords: cholesterol, hydroximinosteroid, 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, hydroximinosteroids isolated from marine sponges display a wide range of biological activities including antiviral, cytotoxicity and aromatase inhibitory activity [3-5]. Especially, compound (6*E*)-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/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].

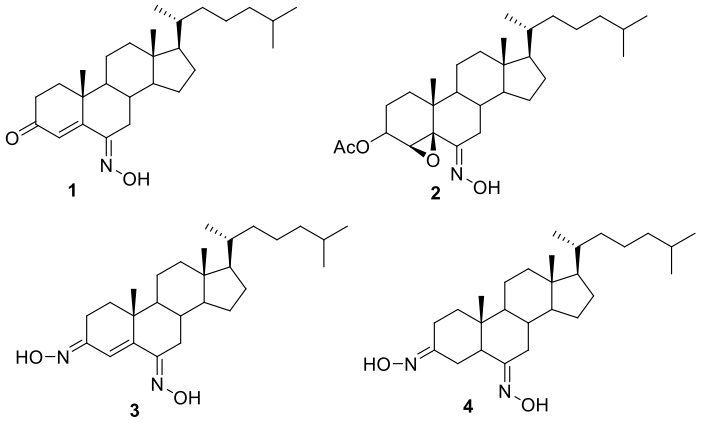


Figure 1. Natural hydroximinosteroid (**1**) and bioactive synthetic hydroximinosteroids (**2-4**).

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_2SO_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 10^5 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 cells 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 % CO_2. 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 % CO_2 at 37 ^oC. Cultured T98G cells were added in 96 well plates at the density of 5×10^3 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 CH_2Cl_2 (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 CH_2Cl_2 (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/diethylether (1:1) as the eluent. Compound **5** was obtained as pale yellow crystals (1.20 g, 80 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (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, 23), 1.53 (m, 2H, H-11, H-25), 1.40 (m, 1H, H-8), 1.38 (m, 1H, H-9), 1.36 (m, 2H, H- 23, H-22), 1.33 (m, 1H, H-16), 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 (2d, *J* = 6.5 Hz, 6H, H-26, H-27), 0.74 (s, 3H, H-18).^{13}C NMR (125 MHz, $CDCl_3$), δ_C (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 $[M+H]^+$.

(3*E*,6*E*)-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 Na_2SO_4, 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%). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H (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). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C (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-3*β*,6*α*-diol (6):1M BH_3 in THF (7 mL 7.0 mM) was added slowly to a solution of cholesterol (540 mg, 1.4 mM) in dry THF (8 mL) under argon atmosphere at 0 ^{o}C. The mixture was stirred at room temperature for 4h. Aqueous solution of NaOH (5 N, 1.5 mL) and 30 % H_2O_2 (0.75 mL) were added dropwise at 0 ^{o}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 $NaHCO_3$ solution and brine, dried over Na_2SO_4, 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 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (ppm): 3.55 (1H, m, H-3), 3.39 (1H, ddd, *J* = 4.5, 10.5, 11.0 Hz, H-6), 0.90 (3H, d, *J* = 6.5 Hz, CH_3-21), 0.86 (6H, d, *J* = 6.5 Hz, CH_3-26, CH_3-27), 0.80 (3H, s, CH_3-19), 0.65 (3H, s, CH_3-18); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C (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-21), 12.0 (C-18).

Cholestane-3,6-dione (7): Solution of **6** (100 mg, 0.25 mmol) in CH_2Cl_2 (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. ^{1}H-NMR ($CDCl_3$, 500MHz) δ_H (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)-Dihydroximinocholestane (4): Compound **7** was prepared according to the procedure of **3**. Compound **4** was obtained as off white needles (35 mg, 81 %). ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H (ppm): 3.33 (dd, 1H, *J* = 4.5, 14.0 Hz, H-7*α*), 3.27 (brd, 1H, *J* = 12.5 Hz, H-2*β*), 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). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C (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 $[M+H]^+$.

Cholest-4-ene-3*β*,6*α*-diol (8): The solution of **5** (530 mg, 1.38 mmol) in 20 mL of the solvent mixture (CH_2Cl_2:MeOH, 1:1) was stirred at room temperature. $CeCl_3.7H_2O$ (530 mg, 1.38 mmol) and $NaBH_4$ (232 mg, 6.07 mmol) were added to the solution and the mixture was stirred for 1h. The HCl aqueous solution (1N) was added dropwise to neutralize the alkaline medium. CH_2Cl_2 (15 mL) was added and the organic layer was separated and dried over Na_2SO_4. 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%). ^{1}H NMR (500 MHz, $CDCl_3$), δ_H (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). ^{13}C NMR (125 MHz, $CDCl_3$), δ_C (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), 36.1 (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-4*α*,5*α*-epoxycholestane-3-one (9) and 4*α*,5*α*-epoxycholestane-3,6-dione (10): Compound **8** (285 mg, 0.7 mmol) in CH_2Cl_2 (20 mL) was treated with *m*-CPBA (290 mg, 1.48 mmol) in CH_2Cl_2 (10 mL) by the procedure of **6** to give a mixture of 4*α*,5*α*-epoxy-cholestane-3*β*,6*α*-diol and 4*β*,5*β*-epoxy-cholestane-3*β*,6*α*-diol (250 mg, 4:1).To a stirred solution of the diol mixture (210 mg) in 10 mL of CH_2Cl_2 at 0 ^{o}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 $NaHCO_3$ (20 mL) containing $Na_2S_2O_3$ (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 $NaHCO_3$ (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-4*α*,5*α*-epoxycholestane-3-one (9): white needles. ^{1}H-NMR ($CDCl_3$, 500 MHz), δ_H (ppm): 4.17 (dd, 1H, *J* 3.0 & 10.0Hz, H-6), 3.48 (s, 1H, H-4), 1.13 (s, 3H, H-19), 0.90 (d, 3H, *J* = 6.5Hz, H-21), 0.87 (d, 3H, *J* = 6.0Hz, H-26), 0.86 (d, 3H, *J* = 6.0Hz, H-27), 0.69 (s, 3H, H-18). ^{13}C-NMR ($CDCl_3$, 125 MHz), δ_C (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 $[M+H]^+$.

4*α*,5*α*-epoxycholestane-3,6-dione (10): white needles.^{1}H-NMR ($CDCl_3$, 500 MHz), δ_H (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).^{13}C-NMR ($CDCl_3$, 125 MHz), δ_C (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 $[M+H]^+$.

4*α*,5*α*-epoxy-6-hydroxycholestane-3-oxime (11) and4,5,6-trihydroxy cholestane-3-one (12): 6-hydroxy-4*α*,5*α*-epoxycholestane-3-one (**9)** (25 mg, 0.06 mmol) was treated with hydroxylamine hydrochloride ($NH_2OH.HCl$) (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 %).

4*α*,5*α*-epoxy-6-hydroxycholestane-3-oxime (11): white needles. ^{1}H-NMR ($CDCl_3$, 500 MHz), δ_H (ppm): 4.16 (dd, 1H, *J* = 5.0, 11.5 Hz, H-6*β*), 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), δ_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 $C_{27}H_{46}NO_3$: 432.3478).

4,5,6-trihydroxy cholestane-3-one (12): white needles.^{1}H-NMR ($CDCl_3$, 500 MHz), δ_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), δ_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 $C_{27}H_{47}O_4$: 435.3474).

4*α*,5*α*-epoxycholestane-3,6-dioxime (13): 4*α*,5*α*-epoxycholestane-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), δ_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), δ_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 $C_{27}H_{45}N_2O_3$: 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_2Cl_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 dihydroxyimino with a double bondat 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_2O_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].

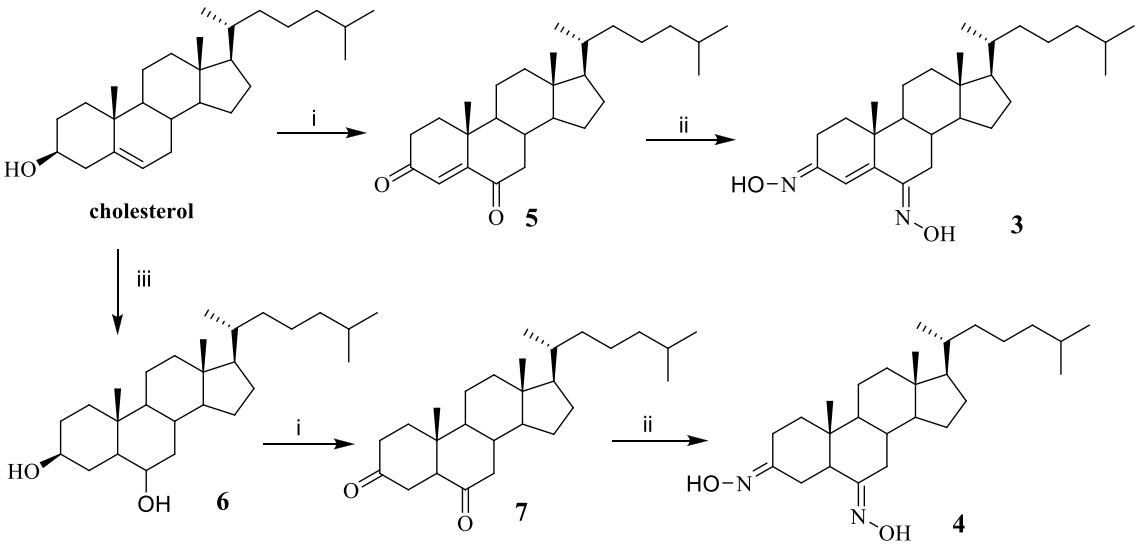


Figure 2. Reagents and conditions: (i): PCC/CH_2Cl_2, rt, 48 h; (ii): $NH_2OH.HCl$/Pyridine, rt, 24 h; (iii): $BH_3.THF/H_2O_2$, NaOH, rt, 1 h.

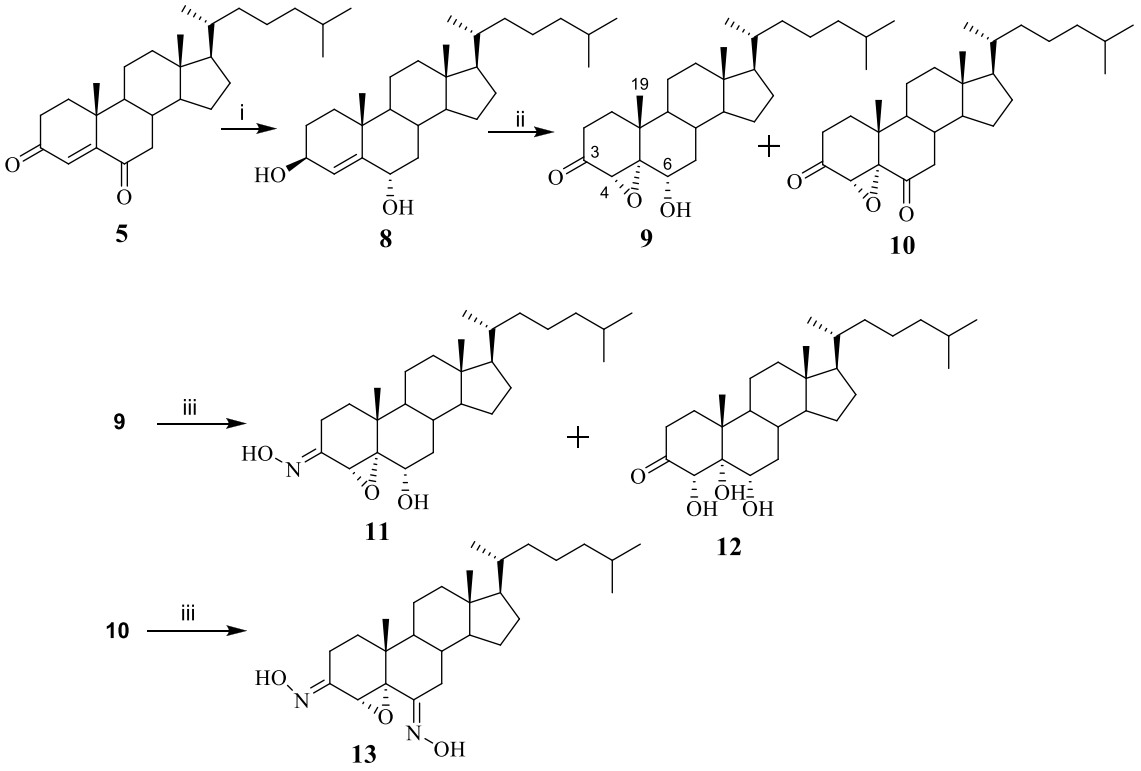


Figure 3. Reagents and conditions: (i): $CeCl_3.7H_2O/NaBH_4$, CH_2Cl_2&MeOH, rt, 1 h; (ii): 1. *m*-CPBA/CH_2Cl_2, 0 ^oC then rt, 4 h; 2. Dess-Martin/CH_2Cl_2, 0 ^oC, 1 h; (iii): $NH_2OH.HCl$/Pyridine, rt, 24 h.

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 $CeCl_3.7H_2O$ gave the cholest-4-en-3,6-diol (**8**) in 89 % yield.

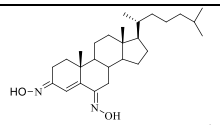
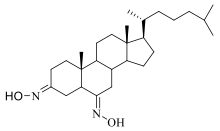
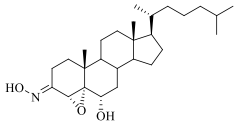
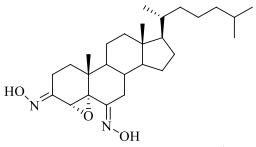
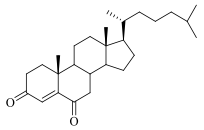
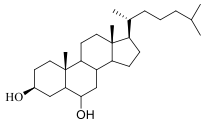
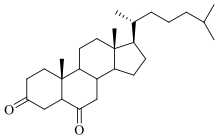
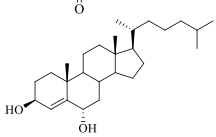
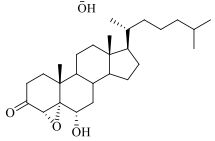
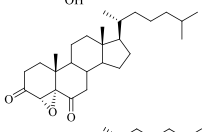
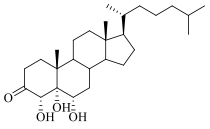
The signals of C=O groups at δ_C 202.3 and 199.5 ppm in the ^{13}C NMR spectrum of **5** were replaced by two characteristic signals of CH-OH groups at δ_C 68.6 (C-3) and 67.9 (C-6) in the ^{13}C NMR spectrum of **8**. The α-configuration of H-3 and β-configuration of H-6 of compound **8** was comfirmed 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 ^{o}C for 1 h gave the mixture of 6-hydroxy-4α,5α-epoxycholestane-3-one (**9**) and 4α,5α-epoxycholestane-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 (δ_C 206.0) and compound **10** contained two carbonyl group at C-3 (δ_C 203.2) and C-6 (δ_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 α-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 δ_H 3.48 to δ_H 3.81 ppm. Likewise, the carbonyl group's resonance signal (C-3) of compound **9** at δ_C 206.0 ppm disappeared and replaced by a quaternary carbon at δ_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 specctrum of product **13**, the presence of two quaternary carbon signals at δ_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 (δ_C 205.6) and three C-OH groups (δ_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}.

Apparently, among 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 on3,6-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-dihydroxyimino **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.

Compounds	Structure	IC_{50} (μM)		
		HeLa	Hep-G2	T98G
3		**68.6**	**42.4**	**70.3**
4		>100	>100	**69.8**
11		>100	>100	>100
13		>100	>100	**2.9**
5		>100	>100	>100
6		>100	>100	>100
7		>100	>100	>100
8		>100	>100	>100
9		**72.4**	**41.8**	>100
10		**74.6**	> 100	>100
12		>100	>100	**18.5**
Paclitaxel[a]		**0.031**	**0.040**	**0.023**

[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**.

Compounds	Minimum inhibitory concentration (MIC, μg/ml)				Compounds	MIC (μg/ml)			
	E. coli	*P.aeruginosa*	*B. subtillis*	*S. aureus*		*E. coli*	*P. aeruginosa*	*B. subtillis*	*S. aureus*
3	>50	> 50	> 50	> 50	**9**	**50**	> 50	> 50	> 50
4	> 50	> 50	> 50	> 50	**10**	**25**	> 50	> 50	> 50
5	**50**	> 50	> 50	>50	**11**	**50**	> 50	> 50	**50**
6	> 50	> 50	> 50	**50**	**12**	>50	> 50	> 50	> 50
7	> 50	> 50	> 50	> 50	**13**	**50**	> 50	> 50	**50**
8	> 50	> 50	> 50	> 50	**Positive control**	**0.06-0.1** (Doxycycline)		**0.5-0.9** (Gentamicin)	

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

1. Sarma N. S., Krishna M. S. R., Rao S. R. - Sterol ring system oxidation pattern in marine sponges, Marine Drugs **3** (2005) 84-111.
2. Ivanchina N. V., Kicha A. A., Stonik V. A. – Steroid glycosides from marine organisms, Steroids **76** (2011) 425-454.
3. Rodriguez J., Nunez L., Pexinho S., Jimenez C. – Isolation and synthesis of the first natural 6-hydroximino-4-en-3-one steroids from the sponges *Cinachyrella* spp., Tet. Lett. **38** (1997) 1833-1836.
4. Xiao D. J., Peng X. D., Deng S. Z., Ma W. Z., Wu H. M. – Structure elucidation of (3*E*)-cholest-4-en-3,6-dione-3-oxime in marine sponge *Cinachyrella australiensis* from the south china sea, Chin. J. Org. Chem. **25** (2005) 1606-1609.
5. Cui J. G., Huang L., Fan L., Zhou M. A. - A facile and efficient synthesis of some (6E)-hydroximino-4-en-3-one steroids, steroidal oximes from *Cinachyrella* sp. Sponges, Steroids **73** (2008) 252-256.
6. Noé Deive, Jaime Rodríguez, and Carlos Jiménez – Synthesis of cytotoxic 6*E*-Hydroximino-4-ene steroids: Structure/Activity studies, J. Med. Chem. **44** (2001) 2612-2618.
7. Cui J. G., Fan L., Huang L., Liu L., Zhou M. A. – Synthesis and evaluation of some steroidal oximes as cytotoxic agents: Structure/activity studies (I), Steroids **74** (2009) 62-72.
8. Cui J. G., Fan L., Huang M. Y., Xin Y., Zhou M. A. - Synthesis and evaluation of some steroidal oximes as cytotoxic agents: Structure/activity studies (II), Steroids **74** (2009) 989-995.
9. Javier P., Miriam R., Vanessa P., Beatriz A., Jaime R., Nelida S., Antonio F., Carlos J. – Synthesis and evaluation of new 6-hydroximinosteroids analogs as cytotoxic agents, Bioorg. Med. Chem. **15** (2007) 4722-4740.
10. Vanden Bergher D.A., Vlietinck A. J. - Screening Methods of Antibacterial and Antiviral Agents from Higher Plants, Methods in Plant biochemistry, Academic Press, USA, V.6 1991.
11. Do Huu Nghi, Tran Thi Nhu Hang, Vu Dinh Giap, Do Thi Nhuan - Screening for antiproliferative and antimicrobial activity of total lipids of some marine invertebrates collected from Vietnam's North Central Coast. Vietnam J. Chem. **55** (2017) 124-130.
12. Mosman T. - Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. J. Immunol. Methods **65** (1983) 55-63.
13. Cui J. G., Cui W. L., Long M. Z., Jing Y. S. - Synthesis of polyhydroxysterols (III): synthesis and structural elucidation of 24-methylenecholest-4-en-3β,6α-diol, Steroids **67** (2002) 1015-1019.