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Synthesis and Evaluation of Some Steroidal Oximes as Cytotoxic Agents: Structure/Activity Studies (I)

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Synthesis and evaluation of some steroidal oximes as cytotoxic agents: Structure/activity studies (I)

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Introduction

A variety of steroids with unusual and interesting structures have been isolated from marine sponges recently [1–3]. Among these steroidal compounds, marine steroids with oxime groups have been reported rarely. Two steroidal oximes, (6*E*)-hydroximino-24-ethylcholest-4-en-3-one and (6*E*)-hydroximinocholest-4-en-3-one, were isolated from *Cinachyrella alloclada* and *C. apion* [4] in 1997, and another steroidal oxime, (3*E*)-hydroximinocholest-4-en-6-one, was isolated from marine sponge *Cinachyrella australiensis* of South China Sea [5] in 2005. The structures of the three compounds are shown in Fig. 1. Studies have revealed that these steroids exert interesting biological activities. For example, the compound **3** displays antiviral function to hepatitis virus (Hep G2) *in vitro* [5] and the compound **2** exerts cytotoxic activities against several types of cancer cells such as P-388 (murine leukemia), A-549 (human lung carcinoma), HT-29 (human colorectal adenocarcinoma) and MEL-28 (human myeloma) tumor cells [6].

In recent years, several 6-hydroximinosteroid analogues have been synthesized and evaluated for their cytotoxicity [7–9]. Interestingly, studies have revealed that the cytotoxicity of these compounds against cancer cells is dependent on the location of the hydroximino group on the steroidal nucleus. The parental steroids with a hydroximino group located at a different position show a remarkable difference in their cytotoxicities, suggesting the importance of a side chain location on a steroid compound in its biological functions. In this report we present more evidence that the cytotoxicity of steroidal oximes we synthesized is not only dependent on the location of a hydroximino group but also the type of a side chain at position 17 on the parental steroid. Our results may provide useful information for the design of chemotherapeutic drugs.

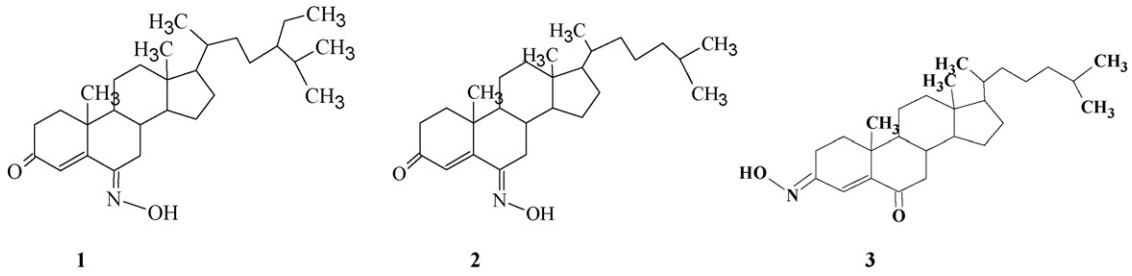


Fig. 1 – Natural steroidal oximes. (1) (6*E*)-hydroximino-24-ethylcholest-4-en-3-one; (2) (6*E*)-hydroximinocholest-4-en-3-one; (3) (3*E*)-hydroximinocholest-4-en-6-one.

Results and discussion

Chemistry

To determine the effect of the type of a side chain and the hydroximino position on the biological role of a steroidal compound, we have synthesized several analogues of steroidal oximes with the variation of the side chain at position 17 and the hydroximino group on the A ring or B ring by using cholesterol, stigmasterol and β-sitosterol.

Synthesis of analogues of (3E)-hydroximino-4-en-6-one steroids (Scheme 1)

The steroidal oxime **3** and its analogues, with a hydroxymino group on the A ring, were synthesized in two steps according to the sequence shown in Scheme 1. First, the compound **4a** was converted to the corresponding 4-en-3,6-dione (**5a**) via oxidation with pyridinium chlorochromate (PCC) in CH_2Cl_2. Next, the oxime **3** was produced in a yield of 87% by the reaction of **5a** with hydroxylamine hydrochloride in ethanol in the presence of NaOAc. At the same time, the compound **2** was obtained as a byproduct in 3% yield. The structure of **3** and **2** was confirmed by analysis of the proton and carbon NMR chemical shifts at C-2 and C-7. Resonances showing H-2 at 3.095 ppm (dd, $J = 18.0$ and 3.8 Hz) and C-3 at 155.808 ppm demonstrated a position of 3-hydroxymino in **3**, while the chemical shifts found for 7-βH and C-6 at 3.437 ppm (1H, dd, $J = 15.9$ and 4.6 Hz) and 149.173 ppm, respectively were indicative of the E-configuration of 6-hydroxymino in **2**.

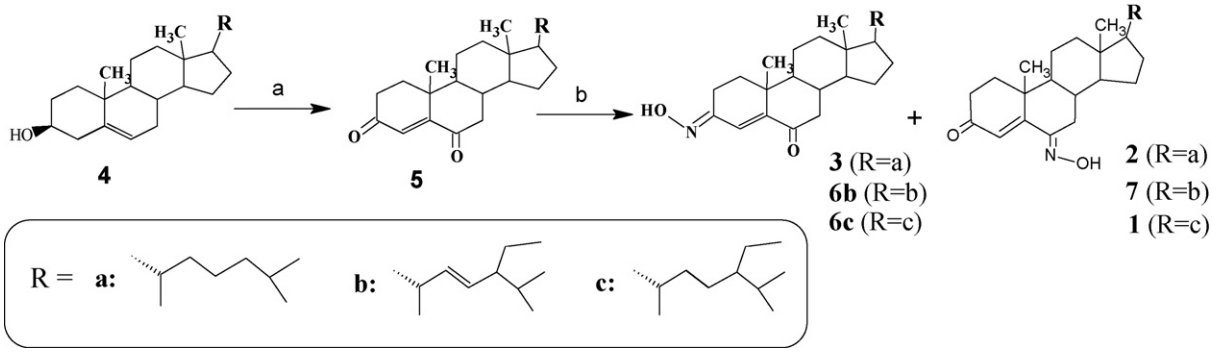
Synthesis of analogues of (6E)-hydroximino-4-en-3-one steroids (Scheme 2)

Seven steps were needed to synthesize compound **1** as reported in Ref. [4]. Here, we introduce a new synthetic method for the steroidal oxime compounds **1**, **2** and **10** with higher overall yields and fewer synthetic steps [10].

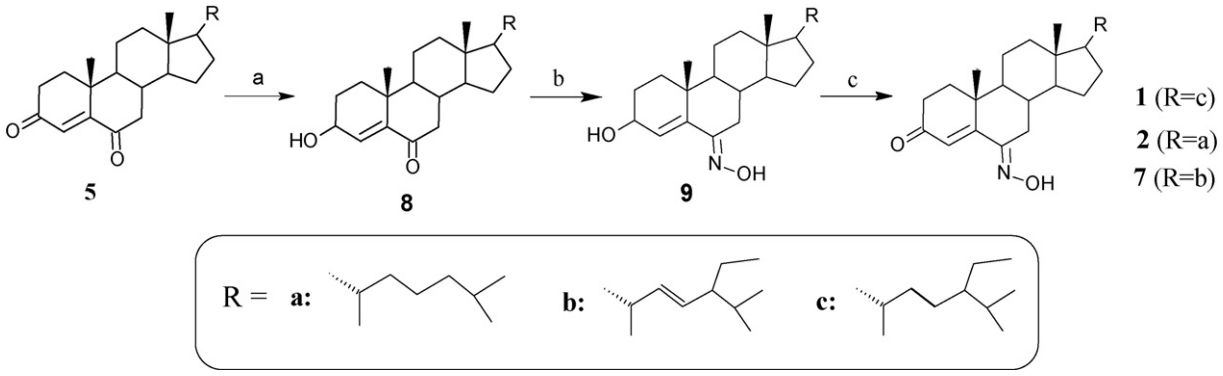
The cholest-4-en-3,6-dione (**5a**) was converted to **8a** by selective reduction using $NaBH_4$ in the presence of $CoCl_2$ according to the synthetic method we developed. The structure of **8a** was confirmed by comparing IR and ^1H NMR spectra with those of the analogous compound that was analyzed previously. The oxime **9a** was generated by the reaction of **8a** with hydroxylamine hydrochloride in ethanol in the presence of NaOAc. At the same time, Z-isomer of **9a** was yielded in the reaction with a lower yield (3%). The oxidation of **9a** with a Jones' reagent in acetone produced the compound **2**.

Synthesis of analogues of (7E)-hydroximino-5-en-3-ol steroids (Scheme 3)

We designed and synthesized a series of analogues of (7*E*)-hydroximino-5-en-3-ol steroids. These compounds have a hydroxyimino group at C-7 on the B ring. The following steps were used to synthesize these compounds. First, the 3β-hydroxy group of **4a** was protected by forming the acetic ester (**11a**), which was then converted to a 5-ene-7-one **12a** by oxidation with CrO_3 in pyridine and dichloromethane for 25 h at ambient temperature. The yield of the product was about 71%. The hydrolysis of **12a** with alcoholic K_2CO_3 obtained the compound **13a** in a yield of 73%. Final oximination of **12a** and **13a**



Scheme 1 – Reagents: (a) PCC; CH_2Cl_2 (4a: 84%); (b) NH2OH·HCl, AcONa (3: 87%, 2: 3%).



Scheme 2 – Reagents: (a) $NaBH_4/CH_3OH$, $CoCl_2{\cdot}6H_2O$ (8a: 88%); (b) $NaAc{\cdot}3H_2O$, 95% C_2H_5OH, $H_2OH{\cdot}HCl$ (9a: 75%); (c) Jones' reagent, acetone (2: 61%).

generated analogue **14a** and **15a**. The downfield chemical shift of H-6 at 6.568 ppm (5.706 ppm for **13a**) for **15a** confirmed the Z configuration of the oxime group because of the influence of hydroxy in the hydroxyimino group.

Synthesis of analogues of (3E)-hydroximino-4-ene steroids (Scheme 4)

The compounds **18a–c** lacking of a substituted group on B ring were synthesized from the compound **4a** which was oxidized to 5-ene-3-one (**16a**) with Jones' reagent in acetone and subsequent treatment with oxalic acid gave 4-ene-3-one (**17a**) in 83% yield. Oximination of **17a** with hydroxylamine hydrochloride produced the hydroximinosteroid analogues (**18a**) in 73% yield.

Synthesis of analogues of (3E,6E)-dihydroximino-4-ene steroids (Scheme 5)

We synthesized steroid analogues with two hydroxyimino groups on the steroidal rings. Two hydroxyimino groups were introduced by oximination of **5a** and **b** in the presence of superfluous hydroxylamine hydrochloride to generate the compound **19a** and **b**.

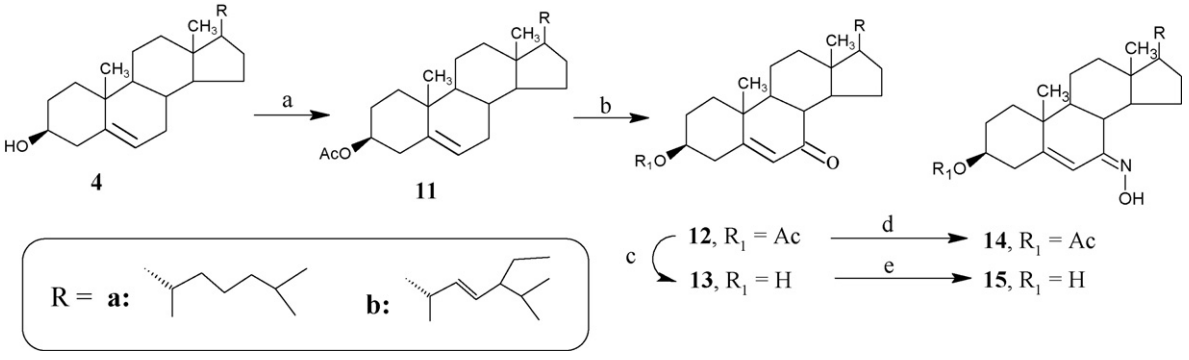
3E-Hydroximinocholest-4-en-6-ol

The compound **20** as shown in Scheme 6 was produced by the reduction of the compound **3**. In the presence of $CeCl_3{\cdot}7H_2O$ as an additive in the reaction, the compound **20** with 6β-OH was obtained as a major product.

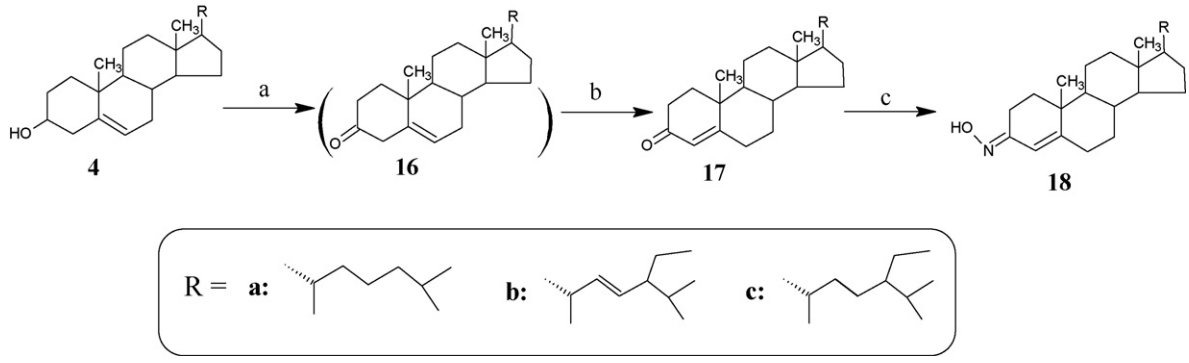
Biological evaluation

To evaluate the effect of the location of the hydroximino group(s) and the type of a side chain at position 17 on the biological functions of steroidal analogues, we determined the cytotoxicity of these compounds to a variety of cancer cell types such as Sk-Hep-1 (human liver carcinoma cell line), H-292 (human lung carcinoma cell line), PC-3 (human prostate carcinoma cell line) and Hey-1B (human ovarian carcinoma cell line) cells. Interestingly, we found that the biological activity of a steroidal oxime was significantly dependent on the location of the hydroximino group(s) and the type of a side chain at position 17 on the parental steroid. The results, expressed as IC_{50} values in μg, are summarized in Table 1.

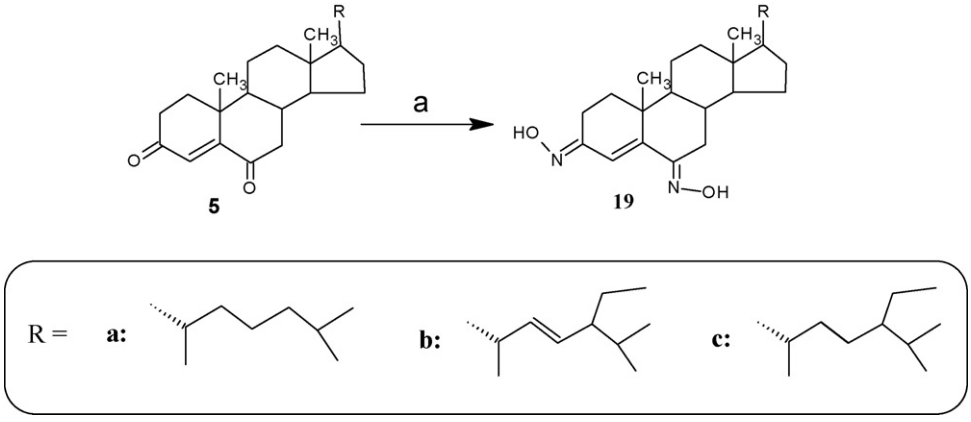
Apparently the structure of a side chain at position 17 on the steroidal oxime plays an important role in its cytotoxicity against cancer cells. An increased antineoplastic activity among these analogueues was observed along with the order of the side chain attached at position 17: cholesterol-type side chain (**2**, **15a**, **19a**) >stigmasterol-type side chain (**7**, **15b**, **19b**) >sitosterol-type side chain (**1**, **19c**). The presence of a cholesterol-type side chain appears to be necessary for the biological activity. The analogues **2**, **7**, **1**, with an oxime group at C-6, showed a remarkable increase in their cytotoxic activity in comparison with the analogues **3**, **6b**, **6c**, which have an oxime group at C-3. The compound **18a–c** without any substitute group on ring B, were found no obvious cytotoxicity against these cancer cells.



Scheme 3 – Reagents: (a) Ac_2O/Py (11a: 97%); (b) CrO_3/Py, CH_2Cl_2 (12a: 71%); (c) K_2CO_3, CH_3OH, reflux (13a: 73%); (d) $NH_2OH{\cdot}HCl$, AcONa, EtOH (14a: 95%); (e) $NH_2OH{\cdot}HCl$, NaOH, EtOH (15a: 99%).



Scheme 4 – Reagents: (a) Jones' reagent, acetone (16a: 83%); (b) oxalic acid, EtOH (17a: 89%); (c) $NH_2OH{\cdot}HCl$, AcONa, EtOH (18a: 73%).

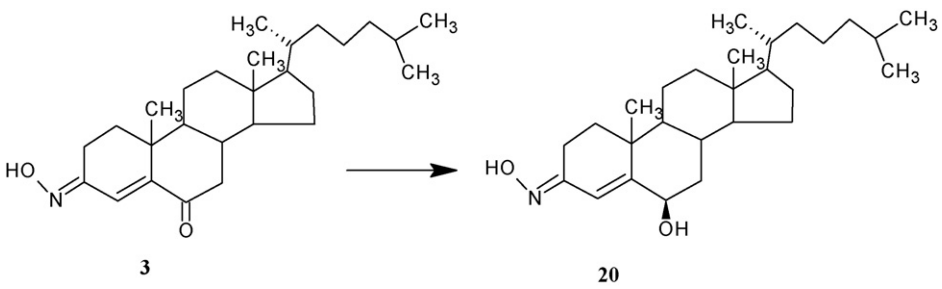


Scheme 5 – Reagents: (a) $NH_2OH{\cdot}HCl$, AcONa, EtOH (19a: 96%).

The conversion of a hydroxyl group to a keto group at C3 resulted in a dramatic loss of cytotoxic activity, suggesting the importance of the hydroxyl group in the biological function of a steroidal oxime (comparing the IC_{50} values of **9a/2**, **9b/7**, **9c/1** and **20/3** in Table 1). This result is different from the conclusion obtained by Rodriguez and co-workers [6]. The reason for that is under investigation.

Compounds **2** and **7**, with a hydroximino group at C-6, showed a slight increase in their cytotoxic activity when compared to compound **15a** and **15b** with the same group at C-7 (except of Sk-Hep-1 and Hey-1B cell lines for **2**/**15a**). However, after the 3-hydroxy on **15a** or **15b** was acetylated, the cytotoxic activity of the compounds was markedly decreased (the IC_{50} values of **15a**/**14a**, **15b**/**14b** in Table 1).

Compound **19a**, with a cholesterol-type side chain and two oxime groups at C-3 and C-6, showed a slight increase in its cytotoxicity against Sk-Hep-1 and Hey-1B cells in comparison of compound **2** with the same side chain at position 17, a keto at C-3 and a oxime group at C-6. However, **19b** with a stigmasterol-type side chain and a similar steroidal nucleus was less active than analogues **7** of compound **2**. Furthermore, conversion of the oxime group at C-6 (**19a**, **19b**) to a keto (**3**, **6b**) caused a loss of activity indicating that an oxime on ring B (analogues **2**, **7**, **15a**, **15b**, **19a** and **19b**) or a hydroxy group



Scheme 6 – Reagents: (a) $NaBH_4/CH_3OH$, $CeCl_3{\cdot}7H_2O$ (20: 82%).

Table 1 – In vitro antitumor activities (IC_{50} in μg/mL) of the synthetic hydroximinosteroid analogues.

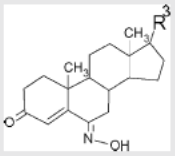
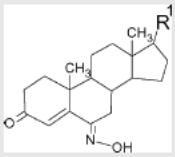
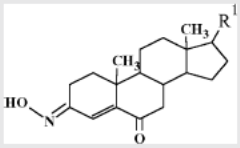
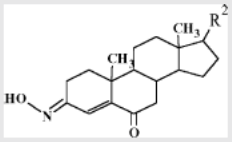
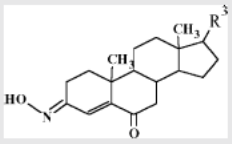
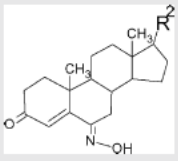
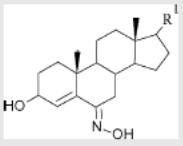
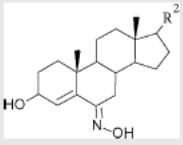
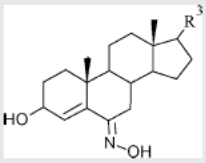
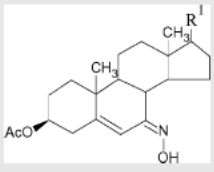
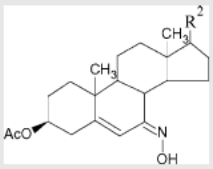
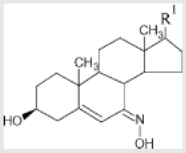
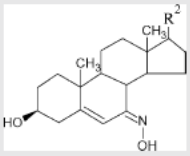
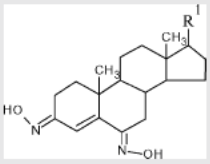
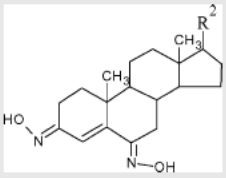
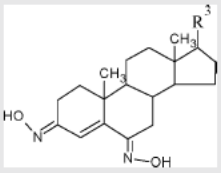
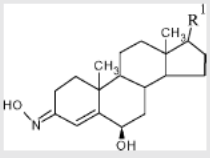
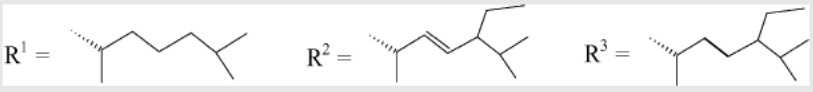
Compound	Structure[a]	Sk-Hep-1	H-292	PC-3	Hey-1B
1		>100	>100	>100	>100
2		33	32.6	35	54
3		>100	>100	>100	>100
6b		>100	>100	>100	>100
6c		>100	>100	>100	>100
7		43	59.5	44	49
9a		20.1	26.2	32.5	26.3
9b		37	37	40.5	45
9c		45	62.5	41.5	53
14a		>100	>100	>100	>100

Table 1 – (*Continued*)

Compound	Structure[a]	Sk-Hep-1	H-292	PC-3	Hey-1B
14b		>100	>100	>100	>100
15a		25	46	76	38
15b		76.8	70	>90	78
19a		24	33	36	37
19b		57	76	66	51
19c		>100	>100	>100	>100
20		34.5	53	52	45

a 

on ring B (**20**) plays a key role in enhancing the cytotoxicity of this type of compounds.

Conclusions

We have prepared a series of hydroximinosteroid derivatives with different substituted groups and the position of a hydroximino on the ring A and B, and different side chains. The cytotoxicity of the synthesized compounds against sk-Hep-1 (human liver carcinoma cell line), H-292 (human lung carcinoma cell line), PC-3 (human prostate carcinoma cell line) and Hey-1B (human ovarian carcinoma cell line) cells was investigated. The results have demonstrated that the presence of a cholesterol-type side chain is very important in determining the biological activity of these compounds. We have found that presence of a hydroximino on the B ring and a hydroxy on the A ring or B ring resulted in an increase of cytotoxic activity for the compounds against tumor cells. Our findings provide new evidence showing the relationship between the chemical structure and biological function. The information obtained from the studies may be useful for the design of novel chemotherapeutic drugs for cancer.

Experimental

Chemistry

The sterol and $NaBH_4$ were purchased from the Merck Co. All chemicals and solvents were analytical grade and solvents were purified by general methods before being used. Melting points were determined on an X_4 apparatus and were uncorrected. Infrared spectra were measured with a Nicolet FT-360 Spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AV-500 spectrometer at working frequencies 500 and 125 MHz, respectively. Chemical shifts are expressed in ppm (δ) values and coupling constants (J) in Hz. The cell proliferation assay was performed by a MTS method using 96-well plates in Beckman coulter LD400 AD/LD analysis spectrometer.

Compounds **1**, **2** and **7** were prepared according to Ref. [10].

*24-Ethylcholest-4-en-3,6-dione (**5a**)*

Pyridinium chlorochromate (PCC) (2.564 g, 2.0 mmol) was added to a solution of sitosterol (**4c**) (0.852 g, 0.50 mmol) in dried CH_2Cl_2 (40 mL) in one portion at room temperature. The reaction was completed in 26 h. To the mixture was then added 30 mL of CH_2Cl_2, and the suspension was poured over a silica gel column and eluted with CH_2Cl_2. The resulting solution was washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the resulting crude product was purified by chromatography on silica gel using petroleum ether (60–90 °C)/EtOAc (5:1) as eluent to give 0.75 g (86%) of **5a** as pale yellow crystals, θ_{mp} 172–174 °C. IR(KBr) ν: 2959, 1683, 1601, 1581, 1461, 1377, 1246, 1124, 948, 871 cm^{-1}; ^1H NMR($CDCl_3$): 0.724(s, 3H, 18-CH_3), 0.816(d, 3H, J = 7.0, 26- or 27-CH_3), 0.841(d, 3H, J = 7.0, 26- or 27-CH_3), 0.848(t, 3H, J = 8.0, 29-CH_3), 0.935(d, 3H, J = 6.5, 21-CH_3), 1.167(s, 3H, 19-CH_3), 2.13–2.17(m, 1H, 2-CαH), 2.44–2.58(m, 2H, 7-C$_\beta$H and 2-C$_\beta$H), 2.682(dd, 1H, J = 4.5, 15.5, 7-CαH), 6.170(s, 1H, 4-CH).

*Cholest-4-en-3,6-dione (**5b**)*

PCC (2.564 g, 2.4 mmol) was added to a solution of cholesterol (0.924 g, 2.2 mmol) in dried CH_2Cl_2 (40 mL) in one portion at room temperature. The reaction was completed in 28 h. The workup similar to **5a** provided 0.795 g (83.5%) of **5b** as pale yellow crystals, θ_{mp} 90–91 °C; IR(KBr) ν: 2953, 2865, 1693, 1600, 1486, 1249, 1221, 1117, 942 cm^{-1}. ^1H NMR($CDCl_3$): 0.746(s, 3H, 18-CH_3), 0.886(d, 3H, J = 6.4, 26 or 27-CH_3), 0.899(d, 3H, J = 6.4, 26 or 27-CH_3), 0.952(d, 3H, J = 6.5, 21-CH_3), 1.172(s, 3H, 19-CH_3), 2.546(dd, 1H, J = 5.2, 14.6, 2-C$_\beta$H), 2.706(dd, 1H, J = 4.0, 16.0, 7-CαH), 6.196(s, 1H, 4-CH).

*Stigmast-4,22-dien-3,6-dione (**5c**)*

5c was prepared similarly according to the procedure of **5a**. PCC (1.30 g, 6.0 mmol) was added to a solution of stigmasterol (0.50 g, 1.2 mmol) in dried CH_2Cl_2 (10 mL) in one portion at room temperature. The reaction was completed in 27 h. The workup similar to **5a** gives 0.42 g (83%) of **5c** as pale yellow crystals, θ_{mp} 134–135°C; IR(KBr) ν: 2959, 1714, 1686, 1609, 969, 864 cm^{-1}; ^1H NMR($CDCl_3$): 0.743(s, 3H, 18-CH_3), 0.805(t, 3H, J = 7.0, 29-CH_3), 0.798(d, 3H, J = 6.5, 26- or 27-CH_3), 0.849(d, 3H, J = 6.5, 26- or 27-CH_3), 1.036(d, 3H, J = 7.0, 21-CH_3), 1.169(s, 3H, 19-CH_3), 5.040(dd, 1H, J = 9.0, 15.2, 22-CH), 5.150(dd, 1H, J = 8.5, 15.2, 23-CH), 6.171(s, 1H, 4-CH).

*(3E)-Hydroximinocholest-4-en-6-one (**3**)*

5a (84 mg, 0.20 mmol) was dissolved in 10 mL 95% CH_3CH_2OH. After the mixture was heated to 60 °C $CH_3COONa{\cdot}3H_2O$(33 mg, 0.24 mmol) and $NH_2OH{\cdot}HCl$ (22.0 mg, 0.32 mmol) were added to the solution. The mixture was stirred for 1 h at 60 °C. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. Proper water was added into the reaction mixture, and the product was extracted with ethyl acetate (3× 20 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to chromatography to give 76 mg of **3** (87.4%) as pale yellow crystals, θ_{mp} 136–138 °C; IR(KBr) ν (cm^{-1}): 3264, 2941, 2868, 1683, 1658, 1585, 1462, 1384, 1245, 1176, 1025, 984; ^1H NMR($CDCl_3$) δ: 0.735(s, 3H, 18-CH_3), 0.889(d, 3H, J = 6.5 Hz, 26 or 27-CH_3), 0.892(d, 3H, J = 6.5, 26 or 27-CH_3), 0.948(d, 3H, J = 6.5, 21-CH_3), 1.064(s, 3H, 19-CH_3), 2.039–1.976(m, 1H, 7-CαH), 2.272(ddd, 1H, J = 18.0, 13.5, 5.4, 2-C$_\beta$H), 2.661(dd, 1H, J = 16.4, 3.7, 7-C$_\beta$H), 3.095(dd, 1H, J = 18.0, 3.8, 2-CαH), 6.776(s, 1H, 4-CH), 8.667(brs, 1H, N–OH); ^{13}C NMR($CDCl_3$) δ: 200.9(6-C), 155.8(3-C), 149.2(5-C), 126.3(4-C), 56.7(14-C), 56.05(17-C), 50.0(9-C), 46.0(7-C), 42.5(13-C), 39.5(24-C), 39.4(12-C), 38.9(10-C), 36.1(22-C), 35.7(20-C), 33.5(1-C), 33.4(8-C), 28.1(16-C), 28.0(25-C), 24.0(23-C), 23.8(15-C), 22.8(26-C), 22.5(27-C), 21.3(11-C), 18.9(19-C), 18.7(2-C), 18.6(21-C), 11.9(18-C).

In the reaction, the compound **2** was obtained as a byproduct in 3.4% yield.

*(3E)-Hydroximino-24-ethylcholest-4,22-dien-6-one (**6b**)*

The preparing method is similar to **3**, yield 70.6%; θ_{mp} 215–216 °C; IR(KBr) ν (cm^{-1}): 3289, 3040, 2954, 2864, 1675, 1581, 1454, 1238, 972; ^1H NMR($CDCl_3$) δ: 0.759(s, 3H, 18-CH_3), 0.824(d, 3H, J = 7.1, 26-CH_3 or 27-CH_3), 0.840(d, 3H, J = 7.7, 26-CH_3 or 27-CH_3), 0.833(t, 3H, J = 7.5, 29-CH_3), 0.874(d, 3H, J = 6.3, 21-CH_3), 1.069(s, 3H, 19-CH_3), 2.271(ddd, 1H, J = 18.5, 14.0, 5.5, 2-C$_\beta$H), 2.657(dd, 1H, J = 16.5, 3.5, 7-C$_\beta$H), 3.097(dd, 1H, J = 18.3, 3.5, 2-CαH), 5.063(dd, 1H, J = 15.1, 8.6, 22-CH), 5.179(dd, 1H, J = 15.2, 8.6, 23-CH), 6.775(s, 1H, 4-CH), 8.386(brs, N–OH). ^{13}C NMR($CDCl_3$) δ: 200.9(6-C), 155.8(3-C), 149.2(5-C), 137.9(22-C), 129.7(23-C), 126.3(4-C), 56.8(14-C), 55.9(17-C), 51.3(9-C), 50.1(24-C), 46.0(13-C), 42.4(10-C), 40.4(20-C), 39.3(7-C), 38.9(12-C), 33.6(8-C), 33.4(25-C), 31.9(1-C), 28.7(16-C), 25.4(2-C), 24.1(28-C), 21.3(15-C), 21.2(11-C), 21.1(21-C), 19.0(26-C), 19.0(27-C), 18.7(19-C), 12.2(18-C), 12.1(29-C).

*(3E)-Hydroximino-24-ethylcholest-4-en-6-one (**6c**)*

The preparing method is similar to **3**, yield 79.6% θ_{mp} 190–192 °C; IR(KBr) ν (cm^{-1}): 3252, 3035, 2933, 2868, 1658, 1585, 1462, 1376, 1249, 984; ^1H NMR($CDCl_3$) δ: 0.741(s, 3H, 18-CH_3), 0.843(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.865(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.874(t, 3H, J = 7.8, 29-CH_3), 0.959(d, 3H, J = 6.4, 21-CH_3), 1.067(s, 3H, 19-CH_3), 2.271(ddd, 1H, J = 19.5, 14, 5.5, 2-C$_\beta$H), 2.663(dd, 1H, J = 16.0, 3.5, 7-C$_\beta$H), 3.097(dd, 1H, J = 18.3, 4.0, 2-CαH), 6.777(s, 1H, 4-CH), 8.507(brs, N–OH). ^{13}C NMR($CDCl_3$) δ: 200.9(6-C), 155.9(3-C),

149.2(5-C), 126.3(4-C), 56.7(14-C), 56.0(17-C), 50.1(9-C), 46.0(24-C), 45.9(13-C), 42.5(10-C), 39.4(7-C), 38.9(12-C), 36.1(20-C), 33.9(8-C), 33.6(22-C), 33.4(1-C), 29.2(25-C), 28.1(16-C), 26.2(2-C), 24.0(23-C), 23.1(15-C), 21.3(28-C), 19.8(11-C), 19.1(19-C), 19.0(21-C), 18.7(26-C), 18.7(27-C), 12.0(18-C), 11.9(29-C).

*3-Hydroxycholest-5-en-7-one acetate (**12a**)*

4.400 g CrO_3 was dissolved in a mixture of 8.8 mL pyridine and 60 mL CH_2Cl_2. After stirring for 10 min, a sulution of **13** (0.640 g) in 20 mL CH_2Cl_2 was added slowly. The mixture was stirred at room temperature for 25 h. The reaction mixture was filtered and filtrate was neutralized with 5% HCl, washed (NaCl, $NaHCO_3$, and water), dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to chromatography (petroleum ether (60–90 °C)/ether 4:1) to give 480 mg of **12a** (70.9%) as a white solid, θ_{mp} 156–158 °C. IR(KBr) ν (cm^{-1}): 2969, 1731, 1668, 1467, 1374, 1190, 965, 624; ^1H NMR($CDCl_3$) δ: 0.701(s, 3H, 18-CH_3), 0.877(d, 3H, J = 2.4, 26-CH_3 or 27-CH_3), 0.890(d, 3H, J = 2.4, 26-CH_3 or 27-CH_3), 0.941(d, 3H, J = 6.5, 21-CH_3), 1.231(s, 3H, 19-CH_3), 2.077(s, 3H, CH_3CO), 4.769–4.705(m, 1H, 3-CαH), 5.724(d, 1H, J = 1.6, 6-CH).

*3-Hydroxy-24-ethylcholest-5,22-dien-7-one acetate (**12b**)*

Yield 72%, θ_{mp} 170–172 °C; IR(KBr) ν (cm^{-1}): 2962, 2872, 1728, 1675, 1458, 1377, 1254, 1037; ^1H NMR($CDCl_3$) δ: 0.713(s, 3H, 18-CH_3), 0.819(d, 3H, J = 6.4, 26-CH_3 or 27-CH_3), 0.825(t, 3H, J = 7.0, 29-CH_3), 0.863(d, 3H, J = 6.4, 26-CH_3 or 27-CH_3), 1.044(d, 3H, J = 6.5, 21-CH_3), 1.228(s, 3H, 19-CH_3), 2.078(s, 3H, CH_3CO), 4.735(m, 1H, 3-CH), 5.035(dd, 1H, J = 15.1, 8.7, 22-CH), 5.185(dd, 1H, J = 15.1, 8.7, 23-CH), 5.714(s, 1H, 6-CH).

*3-Hydroxycholest-5-en-7-one (**13a**)*

K_2CO_3 solution (13%) of 15 mL was added to a solution of **12a** (0.500 g) in CH_3OH (30 mL) at room temperature. The reaction mixture was heated under reflux for 4 h. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. CH_2Cl_2 of 60 mL was added to dissolve a solid and the resulting solution was washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the resulting crude product was purified by chromatography on silica gel using petroleum ether (60–90 °C)/EtOAc (2:1) as eluent to give 0.330 g (73%) of **13a** as white solid, θ_{mp} 171–172 °C. IR(KBr) ν (cm^{-1}): 3434, 2929, 2866, 1670, 1464, 1263, 1060, 949, 799; ^1H NMR($CDCl_3$) δ: 0.701(s, 3H, 18-CH_3), 0.875(d, 3H, J = 2.4, 26-CH_3 or 27-CH_3), 0.888(d, 3H, J = 2.4, 26-CH_3 or 27-CH_3), 0.939(d, 3H, J = 6.6, 21-CH_3), 1.216(s, 3H, 19-CH_3), 3.714–3.669(m, 1H, 3-CαH), 5.707(d, 1H, J = 1.1, 6-CH).

*24-Ethylcholest-5,22-dien-3-hydroxy-7-one (**13b**)*

Yield 95%, θ_{mp} 153–155 °C; IR(KBr) ν (cm^{-1}): 2962, 2872, 1728, 1675, 1458, 1377, 1254, 1037; ^1H NMR($CDCl_3$) δ: 0.722(s, 3H, 18-CH_3), 0.819(d, 3H, J = 6.4, 26-CH_3 or 27-CH_3), 0.826(t, 3H, J = 7.0, 29-CH_3), 0.871(d, 3H, J = 6.4, 26-CH_3 or 27-CH_3), 1.051(d, 3H, J = 6.7, 21-CH_3), 1.224(s, 3H, 19-CH_3), 3.731–3.677(m, 1H, 3-CαH), 5.043(dd, 1H, J = 15.0, 8.6, 22-CH), 5.191(dd, 1H, J = 15.0, 8.6, 23-CH), 5.714(d, 1H, J = 1.5, 6-CH).

*(7Z)-Hydroximinocholest-5-en-3-ol acetate (**14a**)*

Compound **12a** (75 mg) was dissolved in 10 mL 95% CH_3CH_2OH. After the mixture was stirred for 5 min, $CH_3COONa{\cdot}3H_2O$ (23 mg) and $NH_2OH{\cdot}HCl$ (18 mg) were added to the solution. The mixture was stirred for 3.5 h at room temperature. After removal of the majority of solvent, proper water was added into the reaction mixture, and the product was extracted with ethyl acetate (3× 15 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to column chromatography (silica gel, ethyl acetate/petroleum ether (60–90 °C) 1:6) to afford 73 mg of **14a** (95%) as white solid, θ_{mp} 136–137 °C; IR(KBr) ν (cm^{-1}): 3469, 2954, 2872, 1719, 1670, 1466, 1389, 1262, 1037, 956; ^1H NMR($CDCl_3$) δ: 0.717(s, 3H, 18-CH_3), 0.877(d, 6H, J = 1.6, 26-CH_3 or 27-CH_3), 0.890(d, 6H, J = 1.6, 26-CH_3 or 27-CH_3), 0.951(d, 3H, J = 6.5, 21-CH_3), 1.154(s, 3H, 19-CH_3), 2.064(s, 3H, CH_3CO), 4.769–4.676(m, 1H, 3-CαH), 6.591(s, 1H, 6-CH), 6.970(s, 1H, =N–OH). ^{13}C NMR($CDCl_3$) δ: 170.3(–C=O), 163.8(7-C), 152.0(5-C), 113.8(6-C), 73.0(3-C), 54.7(17-C), 50.2(14-C), 49.6(13-C), 42.9(9-C), 39.5(8-C), 38.7(10-C), 38.3(24-C), 38.1(12-C), 38.0(4-C), 37.8(1-C), 36.2(22-C), 35.7(20-C), 28.3(16-C), 28.0(25-C), 27.5(2-C), 27.2(15-C), 23.9(23-C), 22.8(26-C), 22.6(27-C), 20.8(11-C), 18.9(21-C), 17.9(19-C), 12.2(18-C).

*(7Z)-Hydroximino-24-ethylcholest-5, 22-dien-3-ol acetate (**14b**)*

Yield 93%, θ_{mp} 140–142 °C; IR(KBr) ν (cm^{-1}): 3407, 2958, 2872, 1732, 1662, 1467, 1368, 1246, 1037, 972; ^1H NMR($CDCl_3$) δ: 0.737(s, 3H, 18-CH_3), 0.820(d, 3H, J = 7.0, 26-CH_3 or 27-CH_3), 0.827(t, 3H, J = 5.5, 29-CH_3), 0.835(d, 3H, J = 7.0, 26-CH_3 or 27-CH_3), 0.871(d, 3H, J = 5.5, 21-CH_3), 1.159(s, 3H, 19-CH_3), 2.067(s, 3H, CH_3COO–), 4.749–4.700(m, 1H, 3-CαH), 5.043(dd, 1H, J = 15.0, 8.5, 22-CH), 5.201(dd, 1H, J = 15.0, 9.0, 23-CH), 6.589(s, 1H, 6-CH), 6.881(s, 1H, =N–OH). ^{13}C NMR($CDCl_3$) δ: 170.3(–C=O), 163.8(7-C), 152.1(5-C), 138.2(22-C), 129.5(23-C), 113.7(6-C), 73.0(3-C), 54.7(17-C), 51.2(14-C), 50.1(24-C), 49.7(13-C), 43.0(20-C), 42.8(9-C), 40.3(8-C), 40.2(10-C), 38.6(12-C), 38.1(4-C), 37.8(1-C), 32.0(25-C), 29.0(16-C), 27.5(2-C), 27.3(28-C), 25.4(15-C), 21.5(11-C), 21.3(CH_3–C=O), 21.1(21-C), 20.8(26-C), 19.0(27-C), 17.9(19-C), 12.4(29-C), 12.3(18-C).

*(7Z)-Hydroximinocholest-5-en-3-ol (**15a**)*

NaOH solution (0.25 mol/L) of 1.3 mL was added to a solution of **13a** (83 mg) in 95% CH_3CH_2OH (15 mL) at room temperature. After the mixture was stirred for 10 min, $NH_2OH{\cdot}HCl$ (40 mg) were added to the solution, and the mixture was heated at 78 °C for 11 h. After removal of the majority of solvent, proper water was added into the reaction mixture, and the product was extracted with ethyl acetate (3× 15 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was recrystallized and 84 mg of **15a** (99%) was obtained as a white crystal, θ_{mp} 235–236 °C; IR(KBr) ν (cm^{-1}): 3378, 2937, 2864, 1711, 1466, 1384, 1262, 1172, 1021, 959, 796; ^1H NMR($CDCl_3$) δ: 0.723(s, 3H, 18-CH_3), 0.882(d, 3H, J = 1.6, 26-CH_3 or 27-CH_3), 0.895(d, 3H, J = 1.6, 26-CH_3 or 27-CH_3), 0.955(d, 3H, J = 6.5, 21-CH_3), 1.149(s, 3H, 19-CH_3), 3.708–3.628(m, 1H, 3-CαH), 6.568(s, 1H, 6-CH), 7.119(s, 1H, 7

=N–OH); ^{13}C NMR($CDCl_3$) δ: 158.2(7-C), 153.5(5-C), 112.8(6-C), 71.2(3-C), 54.8(17-C), 50.3(14-C), 49.8(13-C), 42.9(4-C), 42.2(9-C), 39.5(8-C), 38.6(10-C), 38.4(24-C), 38.0(12-C), 36.7(1-C), 36.2(22-C), 35.6(20-C), 31.3(2-C), 28.3(16-C), 28.0(25-C), 27.2(15-C), 23.8(23-C), 22.8(26-C), 22.6(27-C), 20.8(11-C), 19.0(21-C), 18.0(19-C), 12.2(18-C).

*(7Z)-Hydroximino-24-ethylcholest-5, 22-dien-3-ol (**15b**)*

Yield 99%, θ_{mp} 232–233 °C; IR(KBr) ν (cm^{-1}): 3378, 2937, 2864, 1711, 1466, 1384, 1262, 1172, 1021, 959, 796; ^{1}H NMR($CDCl_3$) δ: 0.739(s, 3H, 18-CH_3), 0.821(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.828(t, 3H, J = 7.5, 29-CH_3), 0.870(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 1.060(d, 3H, J = 6.6, 21-CH_3), 1.149(s, 3H, 19-CH_3), 3.702-3.635(m, 1H, 3-CαH), 5.046(dd, 1H, J = 15.0, 8.5, 22-CH), 5.200(dd, 1H, J = 15.0, 9.0, 23-CH), 6.566(s, 1H, 6-CH), 7.093(s, 1H, =N–OH); ^{13}C NMR($CDCl_3$) δ: 158.1(7-C), 153.4(5-C), 138.2(22-C), 129.4(23-C), 112.8(6-C), 71.2(3-C), 54.7(17-C), 51.2(14-C), 50.4(24-C), 49.8(13-C), 42.8(20-C), 42.2(4-C), 40.1(9-C), 38.5(10-C), 38.4(12-C), 38.0(1-C), 36.7(8-C), 31.9(25-C), 31.4(2-C), 28.8(16-C), 27.3(28-C), 25.4(15-C), 21.5(11-C), 21.0(21-C), 20.8(26-C), 19.0(27-C), 18.0(19-C), 12.4(29-C), 12.2(18-C).

*Cholest-4-en-3-one (**17a**)*

The Jones' reagent of 1 mL (0.267 mol/L) was gradually added into the solution of **4a** (386 mg, 1 mmol) in 50 mL of acetone in 10 min. The reaction mixture was stirred at 0 °C for 15 min and then neutralized with 10% K_2CO_3 solution. The majority of solvent was evaporated under reduced pressure and then the product was extracted with ethyl acetate (3× 20 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The crude product was recrystallized in CH_3OH to obtain **16a** as a white crystal. The white crystal was dissolved in 5 mL 95% CH_3CH_2OH, and subsequent treatment with oxalic acid gave cholest-4-en-3-one **17a** as pale yellow crystals in 89% yield. θ_{mp} 84–85 °C; IR(KBr) ν (cm^{-1}): 3019, 2945, 2864, 1670, 1609, 1462, 1376, 1333, 1266, 1225, 1192, 1026, 951, 922, 865; ^{1}H NMR($CDCl_3$) δ: 0.737(s, 3H, 18-CH_3), 0.886(d, 3H, J = 2.5, 26-CH_3 or 27-CH_3), 0.899(d, 3H, J = 2.5, 26-CH_3 or 27-CH_3), 0.938(d, 3H, J = 6.5, 21-CH_3), 1.207(s, 3H, 19-CH_3), 2.428(dd, 1H, J = 14.5, 5.3, 2-CβH), 2.461(dd, 1H, J = 15.0, 5.3, 2-CαH), 5.747(s, 1H, 4-CH).

*24-Ethylcholest-4,22-dien-3-one (**17b**)*

Yield 85%, θ_{mp} 121–122 °C; IR(KBr) ν (cm^{-1}): 2969, 2937, 2871, 1679, 1619, 1462, 1446, 1435, 1384, 1270, 1229, 994, 961, 868; ^{1}H NMR($CDCl_3$) δ: 0.748(s, 3H, 18-CH_3), 0.816(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.824(t, 3H, J = 7.3, 29-CH_3), 0.866(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 1.037(d, 3H, J = 6.6, 21-CH_3), 1.203(s, 3H, 19-CH_3), 2.282(ddd, 1H, J = 14.5, 4.0, 2.5, 6-CαH), 2.356(dt, 1H, J = 16.5, 4.0, 6-CβH), 2.426(dd, 1H, J = 14.5, 5.2, 2-CβH), 2.459(dd, 1H, J = 15.0, 5.2, 2-CαH), 5.040(dd, 1H J = 15.1, 8.7, 22-CH), 5.165(dd, 1H, J = 15.1, 8.7, 23-CH), 5.743 (s, 1H, 4-CH).

*24-Ethylcholest-4-en-3-one (**17c**)*

Yield 85%, θ_{mp}161–163 °C; IR(KBr) ν (cm^{-1}): 2957, 2039, 2867, 2852, 1681, 1620, 1466, 1438, 1384, 1367, 1271, 1120, 1030, 867; ^{1}H NMR($CDCl_3$) δ: 0.719(s, 3H, 18-CH_3), 0.836(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.867(t, 3H, J = 7.5, 29-CH_3), 0.859(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.930(d, 3H, J = 7.5, 21-CH_3), 1.120(s, 3H, 19-CH_3), 6.486(s, 1H, 4-CH).

*(3E)-Hydroximinocholest-4-en (**18a**)*

Compound **17a** (60 mg, 0.156 mmol) was dissolved in 10 mL 95% CH_3CH_2OH. After the mixture was heated to 60 °C, $CH_3COONa{\cdot}3H_2O$ (25 mg, 0.18 mmol) and $NH_2OH{\cdot}HCl$ (15 mg, 0.21 mmol) were added into the solution. The mixture was stirred for 1 h at 60 °C. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. Proper water was added into the reaction mixture, and the product was extracted with ethyl acetate (3× 20 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to chromatography to produce 46 mg of **18a** (73%) as pale yellow crystals, θ_{mp} 158–159 °C; IR(KBr) ν (cm^{-1}): 3276, 3066, 2933, 2864, 1629, 1466, 1376, 1291, 1237, 1200, 1134, 997, 967, 930, 857; ^{1}H NMR($CDCl_3$) δ: 0.722(s, 3H, 18-CH_3), 0.884(d, 3H, J = 2.0, 26-CH_3 or 27-CH_3), 0.897(d, 3H, J = 2.0, 26-CH_3 or 27-CH_3), 0.934(d, 3H, J = 6.7, 21-CH_3), 1.084(s, 3H, 19-CH_3), 2.137(ddd, 1H, J = 17.0, 14.0, 5.2, 6-CβH), 2.232(ddd, 1H, J = 14.0, 4.0, 2.0, 6-CαH), 2.324(ddd, 1H, J = 14.0, 5.0, 2.0, 2-CβH), 3.061(ddd, 1H, J = 18.5, 5.0, 2.5, 2-CαH), 5.777(s, 1H, 4-CH); ^{13}C NMR($CDCl_3$) δ: 156.9(3-C), 155.7(5-C), 117.2(4-C), 56.2(14-C), 56.1(17-C), 53.8(9-C), 42.4(13-C), 39.9(10-C), 39.6(12-C), 38.0(24-C), 36.2(22-C), 35.9(8-C), 35.8(20-C), 34.7(7-C), 32.5(1-C), 32.2(6-C), 28.2(2-C), 28.0(25-C), 24.3(16-C), 23.9(15-C), 22.9(23-C), 22.6(26-C), 21.4(27-C), 18.9(11-C), 18.7(19-C), 17.8(21-C), 12.0(18-C).

In the reaction, the 3Z-isomer of **18a** was obtained in 24% yield, θ_{mp} 97–98 °C; IR(KBr) ν (cm^{-1}): 3276, 3060, 2933, 2855, 1634, 1462, 1376, 963, 841; ^{1}H NMR($CDCl_3$) δ: 0.722(s, 3H, 18-CH_3), 0.883(d, 3H, J = 2.0, 26-CH_3 or 27-CH_3), 0.896(d, 3H, J = 2.0, 26-CH_3 or 27-CH_3), 0.931(d, 3H, J = 6.5, 21-CH_3), 1.125(s, 3H, 19-CH_3), 2.030(dd, 1H, J = 13.0, 3.5, 6-CβH), 2.288–2.246(m, 1H, 6-CαH), 2.395–2.314(m, 1H, 2-CH), 6.484(s, 1H, 4-CH).

*(3E)-Hydroximino-24-ethylcholest-4,22-dien (**18b**)*

Yield 70%, θ_{mp} 168–169 °C; IR(KBr) ν (cm^{-1}): 3285, 3046, 2951, 2883, 1629, 1466, 1437, 1372, 1295, 1237, 1218, 1126, 995, 930, 873; ^{1}H NMR($CDCl_3$) δ: 0.740(s, 3H, 18-CH_3), 0.821(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.829(t, 3H, J = 8.0, 29-CH_3), 0.871(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 1.039(d, 3H, J = 6.6, 21-CH_3), 1.085(s, 3H, 19-CH_3), 2.136(ddd, 1H, J = 17.0, 14.0, 5.0, 6-CβH), 2.230(ddd, 1H, J = 14.0, 4.0, 2.5, 6-CαH), 2.323(ddd, 1H, J = 14.0, 4.5, 2.0, 2-CβH), 3.065(ddd, 1H, J = 17.0, 4.5, 3.0, 2-CαH), 5.040(dd, 1H J = 15.2, 9.0, 22-CH), 5.170(dd, 1H, J = 15.2, 8.5, 23-CH), 5.778(s, 1H, 4-CH); ^{13}C NMR($CDCl_3$) δ: 157.2(3-C), 155.9(5-C), 138.2(22-C), 129.4(23-C), 117.1(4-C), 56.2(14-C), 56.0(17-C), 53.8(9-C), 51.3(24-C), 42.3(13-C), 40.5(10-C), 39.8(20-C), 38.0(12-C), 35.9(8-C), 34.7(7-C), 32.6(1-C), 32.3(25-C), 31.9(6-C), 28.9(2-C), 25.4(28-C), 24.3(16-C), 21.4(15-C), 21.2(11-C), 21.1(21-C), 19.0(27-C), 18.7(19-C), 17.8(26-C), 12.3(18-C), 12.2(29-C).

The 3Z-isomer of **18b** was obtained in 29% yield, θ_{mp} 166–168 °C; IR(KBr) ν (cm^{-1}): 3281, 3055, 2933, 2862, 1630, 1462, 1377, 995, 868; ^{1}HNMR($CDCl_3$) δ: 0.737(s, 3H, 18-CH_3), 0.818(d, 3H, J = 6.0, 26-CH_3 or 27-CH_3), 0.826(t, 3H, J = 7.5, 29-CH_3), 0.868(d, 3H, J = 6.0, 26-CH_3 or 27-CH_3), 1.034(d, 3H, J = 6.6, 21-CH_3), 1.123(s, 3H, 19-CH_3), 2.305-2.335(m, 1H, 2-CαH),

4.146(N–OH), 5.036(dd, 1H, J = 15.1, 8.7, 22-CH), 5.165(dd, 1H, J = 15.1, 8.7, 23-CH), 6.482(s, 1H, 4-CH).

*(3E)-Hydroximino-24-ethylcholest-4-en (**18c**)*

Yield 70%, θ_{mp} 174–175 °C; IR(KBr) ν (cm^{-1}): 3195, 2958, 2929, 2864, 1660, 1629, 1454, 1397, 1295, 1237, 1200, 1130, 1022, 967, 930, 868; ^{1}H NMR($CDCl_3$) δ: 0.720(s, 3H, 18-CH_3), 0.836(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.859(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.868(t, 3H, J = 7.5, 29-CH_3), 0.938(d, 3H, J = 6.5, 21-CH_3), 1.081(s, 3H, 19-CH_3), 2.136(ddd, 1H, J = 17.0, 14.0, 5.0, 6-C_βH), 2.250–2.200(m, 1H, 6-CαH), 2.357–2.289(m, 1H, 2-C_βH), 3.063(ddd, 1H, J = 17.3, 4.5, 3.0, 2-CαH), 5.776(s, 1H, 4-CH); ^{13}C NMR($CDCl_3$) δ: 157.3(3-C), 155.9(5-C), 117.1(4-C), 56.1(14-C), 56.0(17-C), 53.8(9-C), 45.9(24-C), 42.4(13-C), 39.9(10-C), 38.0(12-C), 36.2(20-C), 35.9(8-C), 34.7(22-C), 34.0(7-C), 32.6(1-C), 32.3(6-C), 29.2(25-C), 28.2(2-C), 26.2(23-C), 24.3(16-C), 23.1(15-C), 21.4(28-C), 19.8(11-C), 19.1(27-C), 18.8(19-C), 18.7(26-C), 17.8(21-C), 12.0(29-C), 12.0(18-C).

The 3*Z*-isomer of **18c** was obtained in 28% yield, θ_{mp} 162–163 °C; IR(KBr) ν (cm^{-1}): 3456, 2966, 2934, 2867, 1629, 1456, 1400, 1291, 1012, 973, 935, 864; ^{1}H NMR($CDCl_3$) δ: 0.721(s, 3H, 18-CH_3), 0.837(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.859(d, 3H, J = 6.8, 26-CH_3 or 27-CH_3), 0.869(t, 3H, J = 7.5, 29-CH_3), 0.936(d, 3H, J = 6.5, 21-CH_3), 1.124(s, 3H, 19-CH_3), 2.284-2.243(m, 1H, 6-CH), 2.383–2.328(m, 2H, 2-CH), 6.482(s, 1H, 4-CH).

*(3E,6E)-Dihydroximinocholest-4-ene (**19a**)*

Compound **5a** (80 mg, 0.2 mmol) was dissolved in 10 mL of 95% CH_3CH_2OH. After the mixture was heated to 60 °C, $CH_3COONa{\cdot}3H_2O$ (54 mg, 0.4 mmol) and $NH_2OH{\cdot}HCl$ (42 mg, 0.6 mmol) were added. The mixture was stirred for 1 h at 60 °C. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. Proper water was added into the reaction mixture, and the product was extracted with ethyl acetate (3× 20 mL). The combined extracts were washed with saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to recrystallize in methanol to give 82 mg of **19a** (96.4%) as pale yellow crystals, θ_{mp} 141–142 °C. IR(KBr) ν (cm^{-1}): 3554, 3178, 3039, 2941, 2864, 1634, 1458, 1376, 1307, 1000, 792; ^{1}H NMR ($CDCl_3$) δ: 0.709(s, 3H, 18-CH_3), 0.885(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.889(d, 3H, J = 6.5, 26-CH_3 or 27-CH_3), 0.936(d, 3H, J = 6.4, 21-CH_3), 1.051(s, 3H, 19-CH_3), 3.103(d, 1H, J = 17.6, C_2-αH), 3.353(dd, 1H, J = 15.6, 4.3, C_7-$_\beta$H), 6.544(s, 1H, 4-CH), 6.780(s, 1H, 6-NOH), 6.948(s, 1H, 3-NOH); ^{13}C NMR($CDCl_3$) δ: 157.3(6-C), 156.8(3-C), 147.8(5-C), 119.3(4-C), 56.7(14-C), 56.1(17-C), 51.4(9-C), 42.6(13-C), 39.5(7-C), 39.4(24-C), 38.3(12-C), 36.1(22-C), 35.7 (20-C), 33.6(1-C), 33.0(10-C), 29.6(8-C), 28.1(16-C), 28.0(25-C), 24.1(23-C), 23.8(15-C), 22.8(26-C), 22.5(27-C), 21.3(11-C), 18.5(21-C), 17.6(2-C), 17.5(19-C), 11.9(18-C).

*(3E,6E)-Dihydroximino-24-ethylcholest-4,22-dien (**19b**)*

Yield 98%, θ_{mp} 140–141 °C; IR(KBr) ν (cm^{-1}): 3317, 2953, 2864, 1629, 1454, 1376, 1298, 1176, 959; ^{1}H NMR($CDCl_3$) δ: 0.731(s, 3H, 18-CH_3), 0.826(d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.876(d, 3H, J = 6.3, 26-CH_3 or 27-CH_3), 0.833(t, 3H, J = 7.0, 29-CH_3), 1.026(s, 3H, 19-CH_3), 1.047(d, 3H, J = 6.0, 21-CH_3), 2.222-2.148(m, 1H, 7-CαH), 2.436–2.407(m, 1H, 2-C_βH), 3.101(dd, 1H, J = 14.9, 2.5, C_2-αH), 3.369(ddd, 1H, J = 17.5, 15.0, 4.5, 7-C_βH), 5.057(dd, 1H, J = 15.1, 8.7, 22-CH), 5.179(dd, 1H, J = 15.1, 8.6, 23-CH), 6.528(s, 1H, 4-CH), 6.961(–OH); ^{13}C NMR($CDCl_3$) δ: 157.2(6-C), 156.6(3-C), 147.6(5-C), 138.1(22-C), 129.6(23-C), 119.3(4-C), 56.8 (14-C), 55.9(17-C), 51.4(9-C), 51.3(24-C), 42.5(10-C), 40.4(13-C), 39.4(20-C), 38.4(12-C), 33.7(25-C), 33.1(8-C), 31.9(1-C), 29.7(16-C), 28.8(2-C), 25.4(7-C), 24.2(28-C), 21.3(15-C), 21.2 (21-C), 21.1(11-C), 19.1(27-C), 18.6(19-C), 17.6(26-C), 12.3(18-C), 12.2(29-C).

*(3E,6E)-Dihydroximino-24-ethylcholest-4-en (**19c**)*

Yield 93%, θ_{mp} 207–208 °C; IR(KBr) ν (cm^{-1}): 3542, 3340, 3061, 2964, 2861, 1642, 1455, 1377, 1307, 1275, 1175, 1005, 956; ^{1}H NMR($CDCl_3$) δ: 0.721(s, 3H, 18-CH_3), 0.846(d, 3H, J = 7.0, 26-CH_3 or 27-CH_3), 0.867(d, 3H, J = 7.0, 26-CH_3 or 27-CH_3), 0.877(t, 3H, J = 8.0, 29-CH_3), 0.951(d, 3H, J = 6.0, 21-CH_3), 1.034(s, 3H, 19-CH_3), 2.227–2.152(m, 1H, 7-CαH), 2.436–2.402(m, 1H, 2-C_βH), 3.111(dd, 1H, J = 16.5, 2.0, 2-CαH), 3.382(ddd, 1H, J = 17.0, 15.0, 4.5, 7-C_βH), 6.532(s, 1H, 4-CH), 6.963(N–OH); ^{13}C NMR($CDCl_3$) δ: 157.3(C-6), 156.7(3-C), 147.7(5-C), 119.3(4-C), 56.7(14-C), 56.1(17-C), 51.4(9-C), 45.9(24-C), 42.6(10-C), 39.5(13-C), 38.4(12-C), 36.1(20-C), 34.0(22-C), 33.7(8-C), 33.1(1-C), 29.6(25-C), 29.3(16-C), 28.1(2-C), 26.2(7-C), 24.1(23-C), 23.1(15-C), 21.3(28-C), 19.8(11-C), 19.1(27-C), 18.7(19-C), 18.5(26-C), 17.6(21-C), 12.0(29-C), 11.9(18-C).

*(3E)-Hydroximinocholest-4-en-6α-ol (**20**)*

$NaBH_4$ (19 mg, 0.28 mmol) was added to a solution of **3** (140 mg, 0.339 mmol) and $CeCl_3{\cdot}7H_2O$ (126 mg, 0.339 mmol) in CH_3OH (20 mL) in the interval of 5 min at room temperature. After 30 min, the reaction was terminated. The solution was neutralized with 1 M HCl. After evaporation of the majority of the MeOH under reduced pressure, ethyl acetate (30 mL) was added to the residue. The resulting solution was washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the resulting crude product was purified by chromatography. The **20** was obtained as pale yellow crystals (115 mg, 82%), θ_{mp} 172–173 °C; IR(KBr) ν (cm^{-1}): 3374, 3043, 2933, 2905, 2872, 1629, 1462, 1376, 1315, 1287, 1154, 1123, 1074, 972, 886, 849; ^{1}H NMR($CDCl_3$) δ: 0.704(s, 3H, 18-CH_3), 0.877(d, 3H, J = 2.0, 26 or 27-CH_3), 0.890(d, 3H, J = 2.0, 26 or 27-CH_3), 0.923(d, 3H, J = 6.5, 21-CH_3), 1.044(s, 3H, 19-CH_3), 2.153–2.089(m, 1H, 2-C_βH), 3.026(ddd, 1H, J = 13.6, 6.8, 3.5, 2-CαH), 4.243(dd, 1H, J = 14.5, 3.5, 6-C_βH), 6.233(s, 1H, 4-CH); ^{13}C NMR($CDCl_3$) δ: 157.0(5-C), 156.9(3-C), 113.8(4-C), 68.9(6-C), 56.3(17-C), 55.8(14-C), 53.6(9-C), 42.5(13-C), 41.2(10-C), 39.7(24-C), 39.5(12-C), 38.5(7-C), 36.2 (22-C), 35.8(20-C), 35.2(1-C), 34.3(8-C), 28.2(2-C), 28.1(25-C), 28.0(16-C), 24.3(15-C), 23.9(23-C), 22.8(26-C), 22.6(27-C), 21.4(11-C), 18.7(19-C), 18.6(21-C), 12.0(18-C).

Antiproliferative activity

Materials and methods

Stock solutions of compounds **1**, **2** and **4**, were prepared in sterile dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 10 mg/mL and afterwards diluted with complete nutrient medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate.

Cell culture

Sk-Hep-1, H-292, PC-3 (ATCC) and Hey-1B (A gift from Dr. Yan Xu, University of Indiana) cells were cultured in a proper medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C.

Treatment of cancer cells

Cancer cells (4×10^3 cells/200 μL) were seeded into each well of a 96-well microtiter plate. After incuation for 24 h, the compounds with a series of concentrations (range 20–80 μg/mL) were added to the cells. An equal amount of DMSO was added to the cells used as negative controls. All were treated in triplicate.

Determination of cell viability

MT stetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium) (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Cat.# G5421, Promega Corporation) dye reduction assay was used. The assay is dependent on the MTS being reduced to an aqueous, soluble formazan by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Briefly, after treatment (see Section 4.2.3) for 72 h, the medium was removed and the cells were incubated with 100 μL of fresh medium plus 20 μL of MTS solution according to the instruction provided by the manufacturer for additional 4 h. The absorbance (A) at 490 nm was measured using an Beckman coulter LD400 AD/LD analysis spectrometer. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared to a control.

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