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

Jianguo Cui
Guangxi Teachers Education University

Lei Fan
Cleveland State University

Yanmin Huang
Guangxi Teachers Education University

Yi Xin
Cleveland State University

Aimin Zhou
Cleveland State University, A.ZHOU@csuohio.edu

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Introduction

The synthesis and isolation of some steroidal oximes with unusual and interesting structures have been reported recently [1–7]. These compounds exhibit valuable biological activities such as cytotoxicity and aromatase inhibitory activity. Interestingly, the analysis of the chemical structure and biofunctions has showed 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 display a remarkable difference in their cytotoxicities. In this study, we synthesized a series of hydroximinosteroid derivatives with a different functional group on the ring A or B and various side chain types at position 17, and systemically analyzed the cytotoxicity of these compounds against several types of cancer cells including 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. Our results have revealed that presence of a hydroximino at position 3 and a hydroxy at position 6, or *vice versa*, with a cholesterol-type side chain at position 17 resulted in an increase of cytotoxic activity which is consistent with our previous report [8]. Furthermore elimination of the 4,5-double bond augmented the cytotoxic activity for the steroidal oximes.

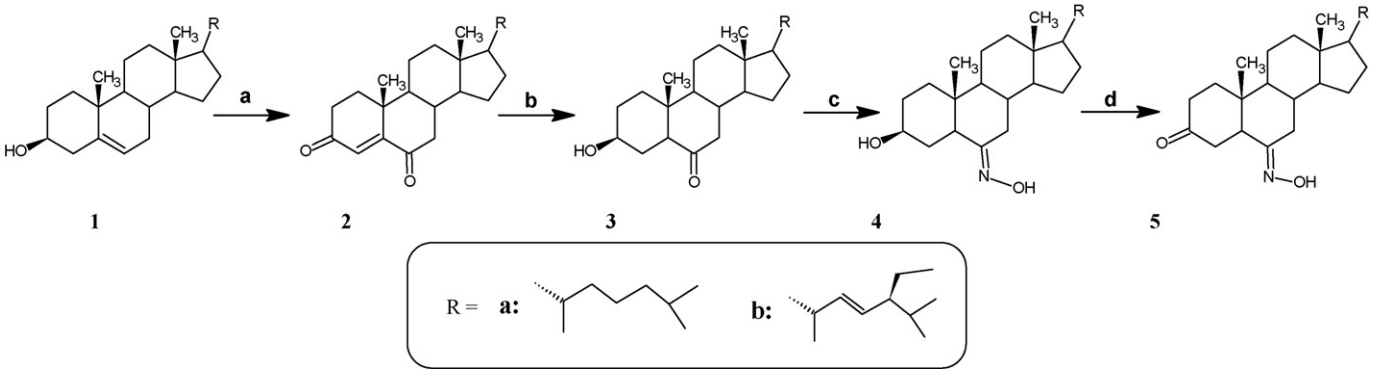
Results and discussion

Chemistry

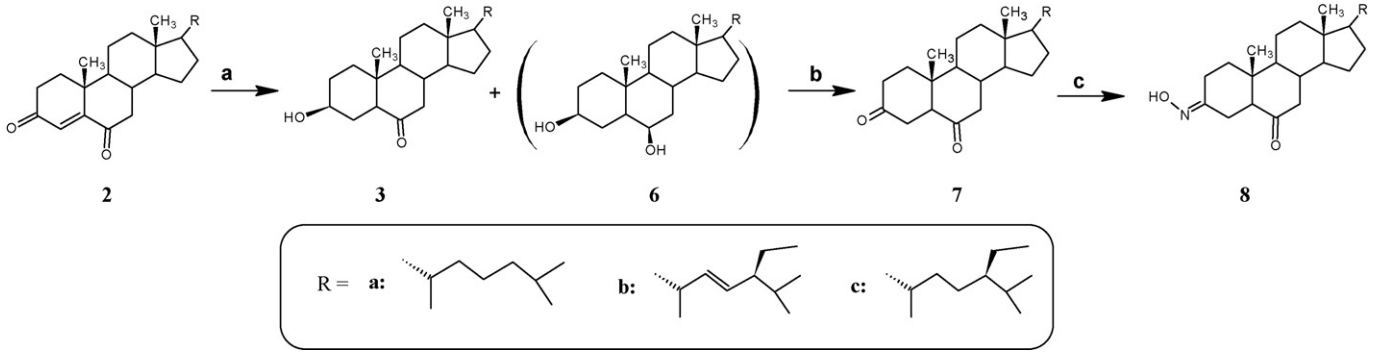
To determine the effect of the hydroximino position and the 4,5-double bond on the biological role of a steroidal compound, we synthesized several analogues of steroidal oximes with a hydroximino group on the A or B ring and the absence of the double bond at position 4 by using natural steroids.

*Synthesis of the analogues of (6E)-hydroximino-3-ol (**4**) and (6E)-hydroximino-3-one (**5**) steroids (Scheme 1)*

The steroidal oximes **4** and **5**, with a hydroxy group or carbonyl group on the A ring and a hydroxymino group on the B ring, were synthesized in 3 steps according to the sequence shown in Scheme 1. First, the compound **1a** was converted to the corresponding 4-en-3,6-dione (**2a**) via oxidation with pyridinium chlorochromate (PCC) in CH_2Cl_2. Next, cholest-4-en-3,6-dione (**2a**) was converted to **3a** by selective reduction using $NaBH_4$ in the presence of $NiCl_2$ in 72% yield according to the synthetic method we developed [9]. In the reaction, cholest-3β,6β-diol (**6a**) was obtained as a byproduct in 11% yield. The structure of **3a** was confirmed by comparing IR and ^1H NMR spectra with those analogous compounds analyzed previously. The configuration of C_3–αH was justified by its coupling constant of 10.5 Hz. (6*E*)-hydroximinocholest-3-ol (**4a)** was generated by the reaction of **3a** with hydroxylamine hydrochloride in ethanol in the presence of NaOAc in 96% yield. The structure of **4a** was confirmed by analysis of IR, the proton and carbon NMR chemical shifts at C-3 and C-7.



Scheme 1. Reagents: (a) PCC, CH_2Cl_2 (**2a**: 84%); (b) $NaBH_4/CH_3OH$, $NiCl_2{\cdot}6H_2O$ (**3a**: 72%); (c) $NH_2OH.HCl$, AcONa (**4a**: 96%); (d) PCC, CH_2Cl_2 (**5a**: 71%).



Scheme 2. Reagents: (a) $NaBH_4/CH_3OH$, $NiCl_2{\cdot}6H_2O$; (b) Jones' reagent, acetone (**7a**: 88%); (c) $NaAc{\cdot}3H_2O$, 95% C_2H_5OH, $NH_2OH{\cdot}HCl$ (**8a**: 90%).

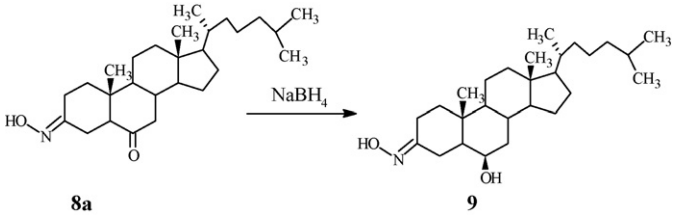
In the IR spectra, the absorptions of 1711 cm^{-1} for the original carbonyl group of **3a**, was absent and replaced by a new absorption at 1650 cm^{-1} (C = N) for **4a**. In the ^1H NMR spectrum the resonances showing of H-7 at 3.339 ppm (dd, J = 14.0, 4.0 Hz) and C-6 at 160.1 ppm demonstrated a position of 6*E*-hydroximino in **4a** [10]**.** Finally, oxidation of the 3-hydroxy of **4a** to produce **5a** was achieved under different conditions. Although the oxidation with the Jones' agent or CrO_3 in pyridine was rapid, a better yield was obtained and work up was simpler than that when PCC in dichloromethane was used. The IR absorption of **5a** at 1693 cm^{-1} showed that a 3-hydroxy in **4a** had been converted to a 3-carbonyl in **5a**.

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

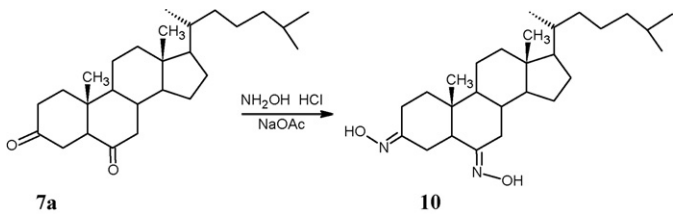
To determine the effect of the location of a hydroximino group on steroidal nucleus on the cytotoxic activity, we synthesized a new 3-hydroximine analogue **8** with an opposite arrangement of the two functional groups compared to the compound **5**. In the preparation, the compound **2** was selectively reduced to generate the crude product **3** and **6**, which were then directly oxidized to produce 3,6-diketone (compound **7**). The compound **8** was obtained by the treatment of **7** with hydroxylamine hydrochloride (1:1.1) in ethanol in the presence of NaOAc. The structure of compound **8** was confirmed by its IR and NMR spectra. In the IR spectra, the absorption of 1711 cm^{-1} for an original carbonyl group of compound **7a** was disappeared and a new absorption at 1653 cm^{-1} for compound **8a** indicating the presence of C=NOH group. ^1H NMR spectrum of **8a** showed a ddd-peak at 3.303 ppm corresponding to C_2–βH, confirming that 3-carbonyl in **7a** had been converted to 3*E*-hydroximino in **8a** (if there is a 6*E*-hydroximino in **8a,** a dd-peak would been appeared on C_7–H).

Synthesis of the analogues of (3E)-hydroximino-6-ol steroids (Scheme 3)

To evaluate the effect of different groups on the cytotoxic activity, we synthesized a new compound **9** with a hydroxy group at position 6. The compound **9** was obtained by a reduction of the carbonyl group at position 6 in **8a** using $NaBH_4$ as a reductive agent in 51% yield. The structure of compound **9** was determined by a comparison of its IR and NMR spectral data with **8a**. The chemical shift of H-6 at 3.874 ppm and C-6 at 71.2 ppm for **9**, and the lack of the chemical shift of C-6 at 210.3 ppm for **8a** confirmed that 6-carbonyl of **8a** had been converted to 6-hydroxy. The coupling constant of 2.0 Hz and a broad doublet for C_6–H revealed the presence of an equatorial hydrogen atom.

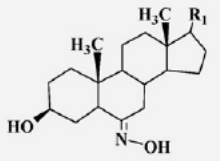
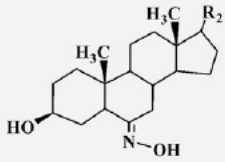
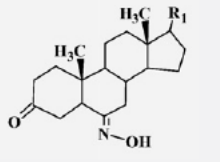
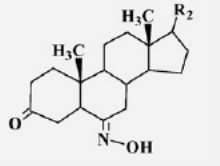
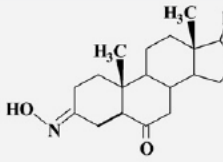
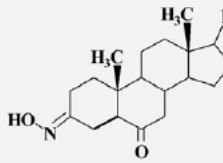
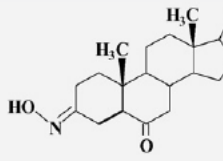
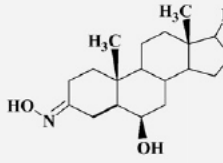
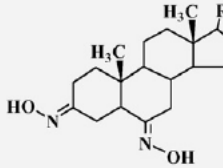


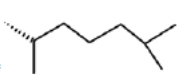
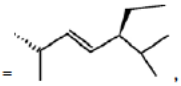
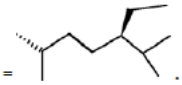
Scheme 3.



Scheme 4.

Table 1
In vitro antitumor activities (IC_{50} in nmol) of the synthetic hydroximinosteroids analogues.

Compound	Structure[a]	Sk-Hep-1	H-292	PC-3	Hey-1B
4a		39.5	41.9	49.8	47.9
4b		>250	>250	>250	>250
5a		156	>250	>250	>250
5b		>250	>250	>250	>250
8a		33.7	34.2	103	45.7
8b		125	168	154	112
8c		97	>250	196	118
9		35.4	65.8	60.1	56.3
10		48.8	78.9	62.7	61. 5

[a] R_1 =  , R_2 =  , R_3 =  .

Synthesis of the analogues of (3E,6E)-dihydroximinosteroids (Scheme 4)

To further evaluate the role of the hydroximino location in the cytotoxic activity of hydroximinosteroids, we synthesized the compound **10** with two hydroximino groups at positions 3 and 6 on the steroidal ring. The hydroximino groups were introduced by oximination of **7a** in the presence of superfluous hydroxylamine hydrochloride. In the ^{13}C NMR spectra a singlet at 157.2 ppm (C-3) and a singlet at 157.4 ppm (C-6) in **10** indicated that 3,6-dicarbonyl groups in **7a** had been converted to 3,6-dihydroximino groups. The downfield chemical shift of C_2–H at 3.088 ppm and C_7–H at 3.180 ppm in the ^1H NMR spectrum of **10** showed the *E*-configuration of two oxime groups due to the deshielding influence of the OH in the hydroximino group.

Biological evaluation

To determine 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 against to a variety of cancer cell types including 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. In this study, we demonstrated 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 as we previously reported [8]. The results expressed as IC_{50} values in nanomoles are summarized in Table 1.

Apparently, increased antineoplastic activity among these analogues was observed along with the order of the side chain at position 17: cholesterol-type side chain (**4a**, **8a**) > stigmasterol-type side chain (**4b**, **8b**) > sitosterol-type side chain (**8c**). The presence of a cholesterol-type side chain appears to be necessary for achieving a higher cytotoxicity. Interestingly, our results revealed that position of hydroximino on ring A or B have remarkably distinct cytotoxicities against cancer cells. Presence of an oxime group at position 3 on the ring A and a carbonyl group at position 6 on the ring B (**8a**) exerted significantly higher cytotoxicity than **5a** with an opposite arrangement of the two functional groups.

The analogue **4a** with a hydroxyl group at C-3 and an oxime group at C-6 showed a remarkable increase in their cytotoxic activity in comparison with **5a** with a keto group at C-3, suggesting the importance of the hydroxyl group in the biological function of a steroidal oxime. Nevertheless, compound **4a** and the analogue **9** with an opposite arrangement of the two functional groups did not show a distinct difference in its cytotoxic activity. Furthermore, when the 3-hydroxy in **4a**, or the 6-hydroxy in **9**, is substituted by an oxime (compound **10**), there was no obvious change in the cytotoxic activity (the IC_{50} values of **4a**/**10**, **9**/**10** as shown in Table 1).

Interestingly, our results indicated that the double bond between position 4 and 5 on A ring confers a negative effect to the cytotoxicity against cancer cells for 3-hydroximino-substituted compounds. Presence of the 4,5-double bond in the analogues **8a**–**c** with 3-hydroximino-6-carbonyl resulted in a remarkable decrease of the cytotoxicity of these compounds (comparing relevant compounds we had reported previously in [8]). Similarly, compounds **9** and **10** with 3-hydroximino showed a slight increase in their cytotoxicity against these cancer cells in comparison of the analogues with the similar structure and a 4,5-double bond [8].

Conclusion

We have prepared a series of hydroximinosteroid derivatives with different substituted groups on the ring A or B, and various side chain types at position 17. 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. Our results have demonstrated that the elimination of the 4,5-double bond enhanced the cytotoxic activity for the 3,6-substituted steroidal oximes with a 3-hydroximino and a different 6-substituted functional group, and that the presence of a hydroxy on the A or B ring augmented the cytotoxic activity for these compounds. 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 sterols and $NaBH_4$ were purchased from the Merck Co. The compounds **2a** and **b** were prepared according to reference [11]. All chemicals and solvents were analytical grade and the 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 Hertz. LREIMS were recorded on a Thermo-DSQ instrument, while HREIMS were measured on a Thermo-MAT95XP instrument. The cell proliferation assay was performed by a MTS method using 96-well plates in Beckman coulter LD400 AD/LD analysis spectrometer.

*3β-Hydroxycholest-6-one (**3a**)*

$NaBH_4$ (0.035 g, 0.92 mmol) was added to a solution of **2a** (0.119 g, 0.3 mmol) and $NiCl_2{\cdot}6H_2O$ (0.071 g, 0.3 mmol) in CH_3OH (15 mL) in the interval of 5 min at room temperature. After 5 min, the reaction was stopped. The solution was neutralized with 1 M HCl. After evaporation of the majority of MeOH under reduced pressure, water of 15 mL was added. Then the residue was extracted with ethyl acetate (15 × 3 mL). The resulting solution was washed with cold water and saturated brine. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the resulting crude product was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (1:4) as the eluent. The **3a** was obtained as a white solid (0.087 g, 72%), θ_{mp} 161–162 °C. IR (KBr) ν: 3378, 2937, 2864, 1711, 1466, 1384, 1262, 1172, 1021, 959, 796 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.666(3H, s, 18-CH_3), 0.756(3H, s, 19-CH_3), 0.871(6H, d, J = 6.5, 26 and 27-CH_3), 0.918(3H, d, J = 6.5, 21-CH_3), 1.955(1H, t, J = 13.0, C_8–βH), 2.044(1H, brd, J = 12.5, C_7–αH), 2.207(1H, brd, J = 12.0, C_7–βH), 2.318(1H, dd, J = 13.5, 4.0, C_5–αH), 3.575(1H, tdd, J = 10.5, 5.0, 3.5, C_3–αH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 210.9(C-6), 70.7(C-3), 56.8(C-5), 56.7 (C-14), 56.2(C-17), 54.0(C-9), 49.6(C-10), 46.7(C-13), 42.9(C-12), 39.5(C-24), 38.0(C-8), 36.7(C-1), 36.1(C-20), 35.7(C-22), 31.6(C-7), 30.7(C-2), 30.1(C-4), 28.1(C-16), 28.0(C-25), 24.0(C-15), 23.8(C-23), 22.8(C-26), 22.6(C-27), 21.6(C-11), 18.7(C-21), 13.2(C-19), 12.0(C-18).

*3-Hydroxy-24-ethylcholest-22-en-6-one (**3b**)*

Compound **3b** was prepared similarly according to the procedure of **3a**. Yield 73%, θ_{mp} 172–173 °C; IR(KBr) ν: 3411, 2958, 2864, 1707, 1458, 1380, 1229, 1066, 963 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.702(3H, s, 18-CH_3), 0.779(3H, s, 19-CH_3), 0.812(3H, d, J = 6.0, 26 or 27-CH_3), 0.821(3H, t, J = 7.5, 29-CH_3), 0.862(3H, d, J = 6.0, 26

or 27-CH_3), 1.041(3H, d, J = 6.5, 21-CH_3), 2.230(1H, dd, J = 12.5, 2.0, C_7–βH), 2.239(1H, dd, J = 13.5, 4.5, C_5–αH), 3.622–3.560(1H, m, C_3–αH), 5.040(1H, dd, J = 15.6, 8.5, C_{22}–H), 5.158(1H, dd, J = 15.6, 8.6, C_{23}–H).

*(6E)-Hydroximinocholest-3β-ol (**4a**)*

$CH_3COONa{\cdot}3H_2O$ (0.027 g, 0.20 mmol) and $NH_2OH{\cdot}HCl$ (0.021 g, 0.30 mmol) were added to the solution of **3a** (0.080 g, 0.20 mmol) in 15 mL 95% ethanol. After the solution was heated to 55 °C, the mixture was stirred at the temperature for 3 h. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. Distilled 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, and evaporated under reduced pressure. The residue was subjected to chromatography using petroleum ether/ethyl acetate (2:1) as the eluent to give 0.078 g of **4a** (96%) as a white solid, θ_{mp} 202–204 °C. IR(KBr) ν: 3379, 2941, 2864, 1650, 1466, 1385, 1249, 1061, 988 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.676(3H, s, 18-CH_3), 0.778(3H, s, 19-CH_3), 0.886(3H, d, J = 4.3, 26-CH_3 or 27-CH_3), 0.888(3H, d, J = 4.3, 26-CH_3 or 27-CH_3), 0.927(3H, d, J = 5.3, 21-CH_3), 3.339(1H, dd, J = 14.0, 4.0, C_7–αH), 3.624–3.578(1H, m, C_3–αH), 8.374(1H, s, N–OH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 160.1(C-6), 71.2(C-3), 56.7(C-14), 56.2(C-17), 49.6(C-9), 46.3(C-13), 42.9(C-10), 42.3(C-5), 39.5(C-12), 39.2(C-24), 38.9(C-22), 38.8(C-4), 36.1(C-1), 35.8(C-2), 35.7(C-20), 31.6(C-8), 30.9(C-7), 28.1(C-16), 28.0(C-25), 24.1(C-15), 23.8(C-23), 22.8(C-26), 22.5(C-27), 21.3(C-11), 18.7(C-21), 12.6(C-19), 12.1(C-18).

*(6E)-Hydroximino-24-ethylcholest-22-en-3β-ol (**4b**)*

Compound **4b** was prepared similarly according to the procedure of **4a**. Yield 98%, θ_{mp} 196–198 °C. IR(KBr) ν: 3403, 2954, 2872, 1646, 1462, 1380, 1057, 976 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.698(3H, s, 18-CH_3), 0.783(3H, s, 19-CH_3), 0.812(6H, d, J = 6.0, 26-CH_3 or 27-CH_3), 0.829(3H, t, J = 7.5, 29-CH_3), 0.862(6H, d, J = 6.0, 26-CH_3 or 27-CH_3), 1.037(3H, d, J = 6.5, 21-CH_3), 3.338(1H, dd, J = 14.0, 4.5, C_7–βH), 3.524–3.625(1H, m, C_3–αH), 5.036(1H, dd, J = 15.0, 8.5, C_{22}–H), 5.161(1H, dd, J = 15.0, 8.5, C_{23}–H), 7.737(1H, brs, N–OH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 160.1(C-6), 138.1(C-23), 129.5(C-22), 71.2(C-3), 56.8(C-14), 56.0(C-17), 51.2(C-9), 46.2(C-24), 42.8(C-13), 42.3(C-5), 39.6(C-12), 38.9(C-10), 38.8(C-4), 36.3(C-20), 35.8(C-1), 33.6(C-2), 31.6(C-8), 30.9(C-7), 29.6(C-25), 28.8(C-16), 24.2(C-15), 21.5(C-28), 21.2(C-11), 21.0(C-27), 19.0(C-26), 18.7(C-21), 16.8(C-19), 12.6(C-29), 12.3(C-18).

*(6E)-Hydroximinocholest-3-one (**5a**)*

Pyridinium chlorochromate (PCC) (0.034 g) was added to a solution of **4a** (0.043 g, 0.1 mmol) in dried CH_2Cl_2 (10 mL) in one portion at room temperature. The reaction was completed in 4 h. The suspension was poured over a silica gel column and eluted with EtOAc. 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 (4: 1) as eluent to give 0.030 g (71%) of **5a** as white solid, θ_{mp} 169–170 °C. IR (KBr) ν: 2953, 2865, 1693, 1600, 1486, 1249, 1221, 1117, 942 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.708(3H, s, 18-CH_3), 0.885(6H, d, J = 6.5 Hz, 26-CH_3 and 27-CH_3), 0.938(3H, d, J = 6.0, 21-CH_3), 0.976(3H, s, 19-CH_3), 2.618(2H, m, C_4–αH and C_5–αH), 3.510(1H, m, C_7–αH); LREIMS(70 eV, m/z): 415(M^+), 397(M^+–H_2O); HREIMS: m/z 415.3447 $[M]^+$ (calcd for $C_{27}H_{45}O_2N$, 415.3445).

*(6E)-Hydroximino-24-ethylcholest-22-en-3-one (**5b**)*

CrO_3 (0.200 g) was dissolved in a mixture of 6 mL pyridine and 20 mL CH_2Cl_2. After stirring for 10 min, the solution of **4b** (0.050 g) in 5 mL CH_2Cl_2 was added slowly. The mixture was stirred at room temperature for 3.5 h. The reaction mixture was filtered with CH_2Cl_2 and filtrate was washed with saturated brines, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to chromatography (petroleum ether (60–90 °C)/EtOAc 3:1) to give 0.038 g of **5b** (76%) as a white solid, θ_{mp} 185–187 °C. IR(KBr) ν: 3391, 2983, 2868, 1703, 1612, 1458, 1397, 1270, 1143, 1025, 976 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.726(3H, s, 18-CH_3), 0.824(3H, d, J = 6.5 Hz, 26-CH_3 or 27-CH_3), 0.831(3H, t, J = 7.0, 29-CH_3), 0.874(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.982(3H, s, 19-CH_3), 1.044(3H, d, J = 7.0, 21-CH_3), 2.125–2.018(2H, m, C_2–H), 2.449–2.331(2H, m, C_4–H), 2.595(1H, dd, J = 15.0, 13.5, C_5–αH), 3.373(1H, dd, J = 13.8, 4.5, C_7–αH), 5.053(1H, dd, J = 15.2, 8.6, C_{22}–H), 5.175(1H, dd, J = 15.2, 8.6, C_{23}–H), 7.529(1H, brs, N–OH); LREIMS(70 eV, m/z): 441(M^+), 423(M^+–H_2O), 398(M^+-C_3H_7); HREIMS: m/z 441.3602 $[M]^+$ (calcd for $C_{29}H_{47}O_2N$, 441.3601).

*Cholest-3,6-dione (**7a**)*

To the stirred solution of **2a** (0.100 g, 0.25 mmol) and $NiCl_2{\cdot}6H_2O$ (0.060 g, 0.25 mmol) in CH_3OH (15 mL) was added $NaBH_4$ (0.030 g, 0.79 mmol) in one time at room temperature. After 30 min, the reaction was stopped. The solution was neutralized with 1 M HCl. After evaporation of the majority of MeOH under reduced pressure, the residue was extracted with ethyl acetate (15 × 3 mL). The combined extracts were washed with cold water and saturated brines. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure. The resulting solid was dissolved in 10 mL of acetone. The Jones' reagent was gradually added into the solution of acetone at 0–5 °C until the orange of solution was not faded. The reaction mixture was continually stirred at 0 °C for 10 min and then neutralized with saturated $NaHCO_3$ 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 water and saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was subjected to chromatography (petroleum ether (60–90 °C)/EtOAc 6:1) to give 0.088 g of **7a** (88%) as a white solid, θ_{mp} 172–173 °C. IR(KBr) ν: 2962, 2864, 1720, 1711, 1462, 1440, 1384, 1258, 1237, 1127, 1094, 992, 923, 612, 514 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.704(3H, s, 18-CH_3), 0.873(3H, d, J = 2.0, 26 or 27-CH_3), 0.886(3H, d, J = 2.0, 26 or 27-CH_3), 0.933(3H, d, J = 6.5, 21-CH_3), 0.972(3H, s, 19-CH_3), 2.344–2.307(1H, m, C_2–H), 2.372–2.347(2H, m, C_7–H), 2.396(1H, dd, J = 13.0, 4.5, C_7–αH), 2.610(1H, brs, C_4–αH).

*24-Ethylcholest-22-en-3,6-dione (**7b**)*

Compound **7b** was prepared similarly according to the procedure of **7a**. Yield 82%, θ_{mp} 190–192 °C. IR(KBr) ν: 2962, 2864, 1731, 1718, 1682, 1609, 972, 864 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.736(3H, s, 18-CH_3), 0.821(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.829(3H, t, J = 7.5, 29-CH_3), 0.870(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.984(3H, s, 19-CH_3), 1.052(3H, d, J = 6.5, 21-CH_3), 2.136–2.058(2H, m, C_2–H), 2.357–2.317(1H, m, C_7–βH), 2.387–2.357(1H, m, C_4–βH), 2.405(1H, dd, J = 13.0, 4.5, C_7–αH), 2.644–2.579(2H, m, C_4–αH and C_5–αH), 5.058(1H, dd, J = 15.0, 9.0, C_{22}–H), 5.172(1H, dd, J = 15.0, 8.5, C_{23}–H).

*24-Ethylcholest-3,6-dione (**7c**)*

Compound **7c** was prepared similarly according to the procedure of **7a**. Yield 83%, θ_{mp} 163–164 °C. IR(KBr) ν: 2962, 2868, 1707, 1458, 1421, 1394, 1266, 1237, 1168, 1123, 1090 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.714 (3H, s, 18-CH_3), 0.837(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.859(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.868(3H, t, J = 7.8, 29-CH_3), 0.949(3H, d, J = 6.5, 21-CH_3), 0.978(3H,

s, 19-CH_3), 2.134–2.077(2H, m, C_2–H), 2.364–2.302(1H, m, C_7–βH), 2.452–2.347(2H, m, C_4–βH and C_7–αH), 2.640–2.575(2H, m, C_4–αH and C_5–αH).

*(3E)-Hydroximinocholest-6-one (**8a**)*

Compound **7a** (0.064 g, 0.16 mmol) was dissolved in 10 mL 95% CH_3CH_2OH. After the mixture was heated to 60 °C, $CH_3COONa{\cdot}3H_2O$ (0.022 g, 0.16 mmol) and $NH_2OH{\cdot}HCl$ (0.013 g, 0.18 mmol) were added into the solution in 10 min. The mixture was stirred for 1 hour at 60 °C. Then the reaction was terminated and the majority of solvent was evaporated under reduced pressure. Distilled 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 (petroleum ether (60–90 °C)/EtOAc 3:1) to produce 0.060 g of **8a** (90%), θ_{mp} 180–181 °C. IR(KBr) ν: 3411, 2949, 2929, 2864, 1719, 1653, 1462, 1397, 1337, 1286, 1237, 1163, 1027, 951, 759 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.696(3H, s, 18-CH_3), 0.870(3H, s, 19-CH_3), 0.882(3H, d, J = 2.0, 26-CH_3 or 27-CH_3), 0.895(3H, d, J = 2.0, 26-CH_3 or 27-CH_3), 0.937(3H, d, J = 6.5, 21-CH_3), 2.387–2.330(3H, m, C_5–H and C_7–H), 3.303(1H, ddd, J = 16.0, 4.0, 1.5, C_2–βH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 210.3(C-6), 159.4(C-3), 57.9(C-5), 56.7(C-14), 56.2(C-17), 53.7(C-13), 46.7(C-9), 43.0(C-7), 41.9(C-10), 39.5(C-12), 39.4(C-24), 38.1(C-22), 38.0(C-8), 37.0(C-20), 36.1(C-25), 35.7(C-16), 28.0(C-4), 26.9(C-2), 24.0(C-1), 23.8(C-15), 22.8(C-23), 22.6(C-26), 21.5(C-27), 19.7(C-11), 18.7(C-21), 12.4(C-19), 12.0(C-18); LREIMS(70 eV, m/z): 415(M^+), 398(M^+–OH); HREIMS: m/z 415.3446 [M] $^+$ (calcd for $C_{27}H_{45}O_2N$, 415.3445).

*(3E)-Hydroximino-24-ethylcholest-22-en-6-one (**8b**)*

Compound **8b** was prepared similarly according to the procedure of **8a**. Yield 93%, θ_{mp} 179–180 °C. IR(KBr) ν: 3223, 2953, 2868, 1707, 1666, 1468, 1425, 1397, 1286, 1237, 1172, 1074, 972, 906, 730 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.716(3H, s, 18-CH_3), 0.819(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.826(3H, t, J = 7.5, 29-CH_3), 0.867(3H, d, J = 6.5, 26-CH_3 or 27-CH_3), 0.873(3H, s, 19-CH_3), 1.045(3H, d, J = 6.5, 21-CH_3), 2.384–2.329(3H, m, C_5–H and C_7–H), 3.302(1H, ddd, J = 15.3, 3.5, 0.5, C_2–βH), 5.052(1H, dd, J = 15.0, 9.0, C_{22}–H), 5.168(1H, dd, J = 15.0, 8.5, C_{23}–H), 7.912(brs, N–OH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 210.2(C-6), 159.5(C-3), 137.9(C-22), 129.7(C-23), 57.9(C-5), 56.8(C-14), 56.0(C-17), 53.7(C-24), 51.7(C-13), 46.8(C-20), 42.9(C-9), 41.9(C-7), 40.4(C-10), 39.4(C-12), 38.0(C-8), 37.0(C-25), 31.9(C-16), 28.7(C-4), 27.2(C-2), 26.9(C-28), 25.4(C-1), 24.1(C-15), 21.5(C-11), 21.2(C-21), 21.1(C-26), 19.7(C-27), 19.0(C-19), 12.4(C-18), 12.2(C-29); LREIMS(70 eV, m/z): 441(M^+), 424(M^+–OH), 398(M^+-C_3H_7); HREIMS: m/z 441.3605 [M] $^+$ (calcd for $C_{29}H_{47}O_2N$, 441.3601).

*(3E)-Hydroximino-24-ethylcholest-6-one (**8c**)*

Compound **8c** was prepared similarly according to the procedure of **8a**. Yield 91%, θ_{mp} 168–170 °C. IR(KBr) ν: 3362, 2962, 2929, 2864, 2839, 1699, 1645, 1462, 1433, 1380, 1335, 1286, 1237, 1168, 1074, 963, 943, 906, 694 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.689(3H, s, 18-CH_3), 0.829 (3H, d, J = 6.5, 26 or 27-CH_3), 0.851 (3H, d, J = 6.5, 26 or 27-CH_3), 0.857(3H, s, 19-CH_3), 0.859(3H, t, J = 7.5, 29-CH_3), 0.937(3H, d, J = 6.5, 21-CH_3), 2.380–2.316(3H, m, C_5–H and C_7–H), 3.293(1H, ddd, J = 15.8, 3.8, 1.5, C_2–βH), 8.830(1H, brs N–OH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 210.2(C-6), 159.3(C-3), 56.7(C-5), 56.7(C-14), 56.1(C-17), 53.8(C-24), 46.8(C-13), 45.9(C-9), 43.0(C-7), 41.9(C-10), 39.5(C-12), 38.1(C-20), 38.1(C-8), 36.1(C-22), 33.9(C-25), 29.2(C-16), 28.1(C-4), 27.2(C-2), 26.2(C-23), 24.0(C-1), 23.1(C-15), 21.5(C-28), 19.8(C-11), 19.8(C-26), 19.1(C-27), 18.7(C-21), 12.5(C-19), 12.1(C-29), 12.0(C-18); LREIMS(70 eV, m/z): 443(M^+), 426(M^+–OH); HREIMS: m/z 443.3757 [M] $^+$ (calcd for $C_{29}H_{49}O_2N$, 443.3758).

*(3E)-Hydroximinocholest-6-ol (**9**)*

To a solution of **8a** (0.300 g, 0.72 mmol) in CH_3OH (30 mL) was added $NaBH_4$ (0.055 g, 1.45 mmol) in the interval of 8 min at room temperature. After 20 min, the reaction was stopped. The solution was neutralized with 1 M HCl. After evaporation of the majority of MeOH under reduced pressure, water of 15 mL was added. Then the residue was extracted with ethyl acetate (15 × 3 mL). The resulting solution was washed with cold water and saturated brine. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the resulting crude product was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (3:1) as the eluent. The compound **9** was obtained as a white solid (0.154 g, 51.1%), θ_{mp} 191–192 °C. IR (KBr) ν: 3387, 2937, 2864, 1662, 1466, 1380, 1045, 951 cm^{-1}; ^1H NMR($CDCl_3$, 500 MHz) δ: 0.717(3H, s, 18-CH_3), 0.873(3H, d, J = 2.0, 26 or 27-CH_3), 0.886(3H, d, J = 2.0, 26 or 27-CH_3), 0.925(3H, d, J = 6.5, 21-CH_3), 1.117(3H, s, 19-CH_3), 2.283(1H, dd, J = 12.0, 5.0, C_4–αH), 3.055(1H, dd, J = 15.0, 3.5, C_2–βH), 3.874(1H, brd, J = 2.0, C_6–αH), 8.111(1H, brs, =NOH); ^{13}C NMR($CDCl_3$, 125 MHz) δ: 161.1(C-3), 71.2(C-6), 56.3(C-14), 56.1(C-17), 54.0(C-9), 47.8(C-5), 42.7(C-13), 39.9(C-10), 39.8(C-7), 39.7(C-12), 39.5(C-24), 36.2(C-22), 36.1(C-20), 35.8(C-8), 30.2(C-16), 28.2(C-25), 28.0(C-4), 27.8(C-2), 24.5(C-1), 24.3(C-15), 23.9(C-23), 22.8(C-26), 22.6(C-27), 21.0(C-11), 18.7(C-21), 15.0(C-19), 12.1(C-18); LREIMS(70 eV, m/z): 417(M^+), 400(M^+–OH), 399(M^+–H_2O), 382(M^+–H_2O + OH)); HREIMS: m/z 417.3600 [M] $^+$ (calcd for $C_{27}H_{47}O_2N$, 417.3601).

*(3E,6E)-Dihydroximinocholestane (**10**)*

Compound **7a** (0.202 g, 0.5 mmol) was dissolved in 20 mL of 95% CH_3CH_2OH. After the mixture was heated to 60 °C, $CH_3COONa{\cdot}3H_2O$ (0.272 g, 2 mmol) and $NH_2OH{\cdot}HCl$ (0.138 g, 2 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. Distilled water was added into the reaction mixture, and the product was extracted with ethyl acetate. The combined extracts were washed with water and saturated brine, dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate (1:1) as the eluent. The compound **10** was obtained as a white solid (0.369 g, 86%), θ_{mp} 194–195 °C. IR(KBr) ν: 3293, 3174, 3109, 2949, 2868, 1658, 1466, 1392, 1290, 1209, 1160, 1104, 959, 927, 710 cm^{-1}; ^1H NMR (DMSO-d6, 500 MHz) δ: 0.633(3H, s, 18-CH_3), 0.754(3H, s, 19-CH_3), 0.851(6H, d, J = 3.0, 26- and 27-CH_3), 0.893(3H, d, J = 4.0, 21-CH_3), 3.088(1H, brd, J = 12.5, C_2–βH), 3.180(1H, brd, J = 13.4, C_7–αH), 10.122(1H, s, 6-NOH), 10.377(1H, s, 3-NOH); ^{13}C NMR(DMSO-d6, 125 MHz) δ: 157.4(C-6), 157.2(C-3), 56.4(C-14), 56.2(C-17), 53.4(C-9), 50.8(C-5), 42.8(C-10), 39.6(C-13), 39.4(C-24), 39.3(C-12), 36.5(C-22), 36.1(C-20), 35.6(C-8), 35.5(C-16), 29.4(C-25), 28.8(C-7), 28.2(C-2), 27.9(C-1), 24.2(C-19), 23.7(C-15), 23.1(C-23), 22.9(C-4), 21.4(C-27), 20.0(C-26), 19.0(C-11), 12.3(C-21), 11.8(C-18); LREIMS(70 eV, m/z): 430(M^+), 413(M^+–OH), 398(M^+–NOH_2), 396(M^+–2OH); HREIMS: m/z 430.3553 [M] $^+$ (calcd for $C_{27}H_{46}O_2N_2$, 430.3554).

Antiproliferative activity

Materials and methods

Stock solutions of the compounds 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 (4×10^4 cells/mL, 200 μL) were seeded into each well of a 96-well microtiter plate. After incubation for 24 h, the compounds with a series of concentrations (range 5–80 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 to determine the cell viability. The assay is dependent on the reduction of MTS by mitochondrial dehydrogenases of viable cells to an aqueous, blue, soluble formazan product, which can be measured spectrophotometrically. The absorbance of the formazan product at 490 nm is in linear proportion to cell numbers. 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 for additional 4 h according to the instruction provided by the manufacturer. The absorbance (A) at 490 nm was measured using a LD400 AD/LD analysis spectrometer (Beckman coulter). The IC_{50} value was calculated as the concentration of drug yielding 50% cell survival.

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