Synthesis of Cytotoxic 6*E*-Hydroximino-4-ene Steroids: Structure/Activity Studies

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In an effort to determine the pharmaceutical utility and the structural requirements for activity against various tumor cell lines, several 6*E*-hydroximino-4-ene steroids with different side chains and degrees of unsaturation on ring A were synthesized in our laboratory. Evaluation of the synthesized compounds for cytotoxicity against P-388, A-549, HT-29, and MEL-28 tumor cells revealed that some important structural features are required for activity. The presence of a cholesterol-type side chain, which appears to play a major role in determining the biological activity, the existence of a ketone functionality at C-3, and an elevated degree of oxidation on ring A all result in higher bioactivity than other structural motifs.

Introduction

Natural products derived from marine organisms have become an important source of biologically active compounds. Especially abundant are those systems with a steroidal skeleton, which are usually isolated in large quantities from marine sponges.[1] Despite the enormous number of steroids found to have unusual and intriguing structures,[2] marine steroids with an oxime group have not been reported in the literature to date, with compounds **1** and **2** representing the first examples known (see Figure 1). These 6*E*-hydroximino-3-oxo-4-ene steroids were discovered during the chemical study of two morphospecies of the sponges *Cinachyrella alloclada* and *Cynachirella apion*, and their structures were deduced by extensive use of 1D- and 2D-NMR studies.[3]

Our interest in this type of compound was sparked by the selective cytotoxic activity shown by **1** (see Figure 1) (IC_{50} of 1.25 μg/mL against P-388, A-549, HT-29, and 2.5 μg/mL toward MEL-28 tumor cells) and the lack of activity of **2** (IC_{50} >10 μg/mL).[3] Furthermore, this group of steroids was reported to show a high affinity for human placental aromatase and to function as a competitive inhibitor of this enzyme.[4]

To explore the structure/activity relationships of this type of compound as cytotoxic agents, the naturally occurring steroids **1** and **2** were prepared along with some derivatives with different structural features (side chains and differing degrees of unsaturation on ring A).

Results and Discussion

Chemistry. The initial studies for the synthesis of this oxime-steroid system were based on the methodology developed by Holland.[4] Using as a model the active natural oxime **1**, we first investigated the variation of the side chain and observed the effect of these changes on the cytotoxicity. In this respect we prepared several systems, namely cholesterol-like, sitosterol-like, and gorgosterol-like side chains. As depicted in Scheme 1, the synthesis of these compounds began from the readily available key precursors 3β-acetoxycholest-4-en-6-one (**6a**), (24*R*)-3β-acetoxy-24-ethylcholest-4-en-6-one (**6b**), and 3β-acetoxygorgost-4-en-3-one (**6c**), obtained from cholesterol, β-sitosterol, and gorgosterol,[5] respectively. This synthetic route was undertaken using the following standard steps: acetylation followed by epoxidation of the starting materials and subsequent oxidation of compounds **4a**−**c**[6] to give the corresponding 3-acetoxy-5-hydroxy-6-one steroids **5a**−**c**. The elimination of the hydroxyl group at C-5 with thionyl chloride in pyridine afforded compounds **6a**−**c**, which, after removal of the acetate with alcoholic KOH and upon treatment with hydroxylamine hydrochloride followed by allylic oxidation with aqueous CrO_3, provided the 6*E*-hydroximino-4-en-3-oxo analogues.[7,8]

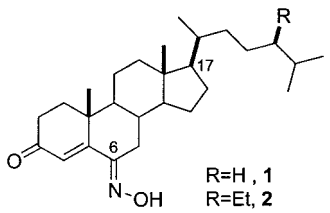

Figure 1. Natural 6*E*-Hydroximino-3-oxo-4-ene steroids from the sponges *C. apion* and *C. alloclada*.

Due to the lack of activity found in compounds **2** and **8** (see IC_{50} values in Table 1), we decided to proceed by investigating the influence of the absence of a side chain (Scheme 2). Wolff−Kishner reduction of (+)-dehydroisoandrosterone (**9**) led to the formation of 5-androsten-3β-ol (**10**), which was subsequently converted into oxime **11** by the application of the methodology already discussed for Scheme 1.

Compound **11** still showed a mild cytotoxicity (IC_{50} = 5 μg/mL against all the tumor cells tested), and so we decided to prepare compounds with different degrees of oxidation at C-17 with the aim of inducing a higher or selective cytotoxic effect. In this way, analogue **14a** was prepared by protection of the keto group at C-17 of **9** as the ketal to give compound **12**,[9] which was subjected to the synthetic strategy outlined in Scheme 1. Thus, after epoxidation and oxidation (steps a−c in

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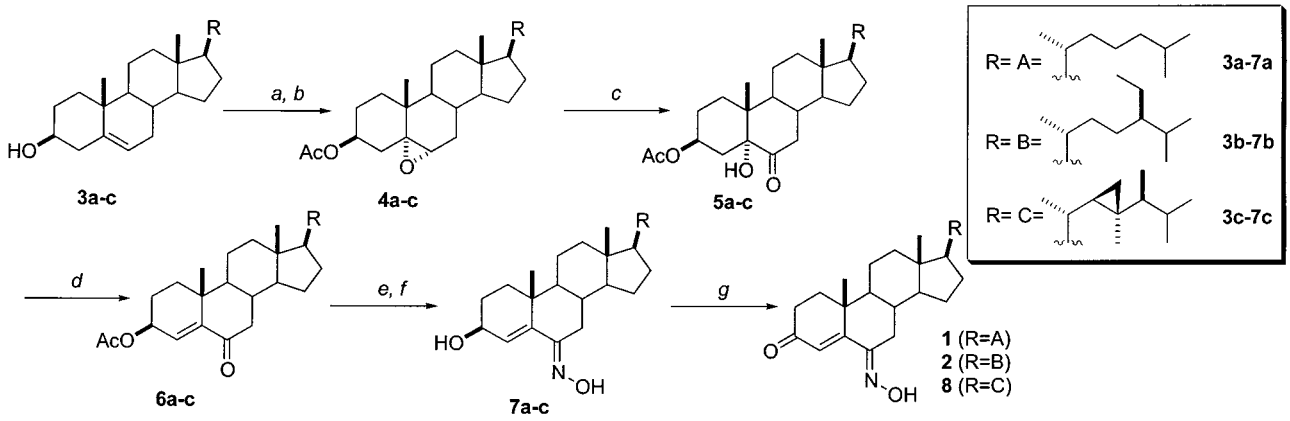
Scheme 1[a]



[a] Reagents and conditions: (a) Ac_2O, Py (99%); (b) MCPBA, CH_2Cl_2 (97%); (c) CrO_3, H_2O (90%); (d) $SOCl_2$, Py (92%); (e) KOH, MeOH (82%); (f) $NH_2OH.HCl$, EtOH, H_2O (97%); (g) CrO_3, H_2O (70%).

Table 1. In Vitro Antitumor Activities (IC_{50} in μg/mL) of **1** and Its Analogues

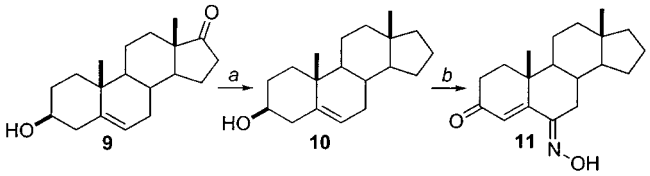
compd	P-388	A-549	HT-29	MEL-28
1	1.25	1.25	1.25	2.5
2	10	10	10	10
7a	10	10	10	10
7c	10	10	10	10
8	5	5	5	5
11	5	5	5	5
14a	5	5	5	5
14b	5	5	5	5
20a	1.25	1.25	1.25	1.25
20b	1.25	1.25	1.25	1.25
21a	0.25	0.125	0.25	0.125
21b	0.25	0.125	0.25	0.125
22	0.5	0.125	0.25	0.125
24	5	5	5	5

Scheme 1), deprotection of the ketal occurred. The presence of two keto groups, at C-6 and C-17, meant that the synthesis to obtain the oxime at position 6 was regioselective because an oxime at C-17 was not detected. The synthesis of the 17β-ol analogue **14b** was also undertaken,[4] thus completing the series of different compounds lacking a side chain in the steroidal skeleton (Scheme 3).

When the cytotoxic tests of **14a** and **14b** were performed, we found that there was no increase in the activity (see IC_{50} values in Table 1) in comparison to compound **1**. For this reason, the next step in this structure/activity relationship study involved a different approach consisting of the preparation of a number of 6*E*-hydroximino-4-ene steroids with different degrees of oxidation in ring A. At this point, and with the IC_{50} values of the previously synthesized analogues in mind, we decided to keep the cholesterol-type side chain in order to maintain a good level of cytotoxicity.

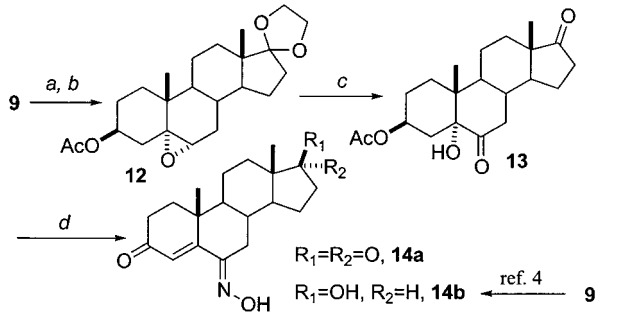
Cholesterol (**3a**) was thus converted into **16** by tosylation followed by epoxidation, oxidation, and dehydrotosylation.[10] Subsequent osmium-catalyzed dihydroxylation of the Δ^2 double bond gave, after chromatographic purification, diols 2α,3α,5α- and 2β,3β,5α-trihydroxycholestan-6-ones **17** and **18**, respectively (Scheme 4). Protection of the hydroxyl groups at C-2 and C-3 as acetates allowed us to follow the same sequence of reactions to obtain the Δ^4 double bond and the oxime group at C-6. Deprotection of the acetates with KOH/EtOH, followed by an allylic oxidation with aqueous

Scheme 2[a]



[a] Reagents and conditions: (a) DEG, $NH_2NH_2{\cdot}H_2O$, K_2CO_3 (66%); (b) Scheme 1 (30%).

Scheme 3[a]



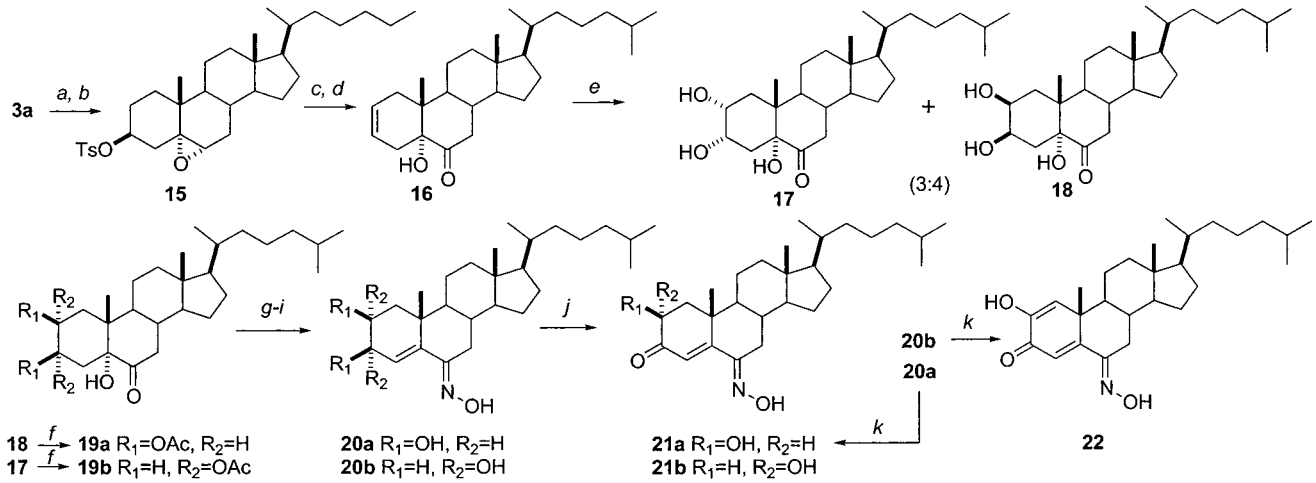
[a] Reagents and conditions: (a) $HOCH_2CH_2OH$, adipic acid, Ph Me (60%); (b) steps a−b as in Scheme 1 (96%); (c) CrO_3, H_2O (90%); (d) steps d−g as in Scheme 1 (51%).

CrO_3, afforded the desired 6*E*-hydroximino-2-hydroxy-4-en-3-oxo epimers **21a** and **21b** in good yields. Surprisingly, the use of MnO_2 as the oxidizing agent with **20b** afforded the 6*E*-hydroximino-2-hydroxy-3-oxo-1,4-diene **22**. However, the diasteroisomer **20a** under the same conditions yielded the expected oxidation product at the allylic position (**21a**), but compound **22** was not detected at all. The proton coupling constants of compounds **17**−**21** allowed the unequivocal assignment of the configurations at C-2 and C-3. At present, compounds **21a**, **21b**, and **22** have proven to be the most potent cytotoxic 6*E*-hydroxymino-4-ene steroids found in this work.

A final analogue, compound **24**, was synthesized (see Scheme 5) from **16** in order to study the effect on the cytotoxicity of a 2,4-diene conjugated with the oxime at C-6. Thus, elimination of the hydroxyl group at C-5 was performed with LiBr in DMF under reflux, and the resulting ketone, **23**, was converted into **24** by the standard procedure.

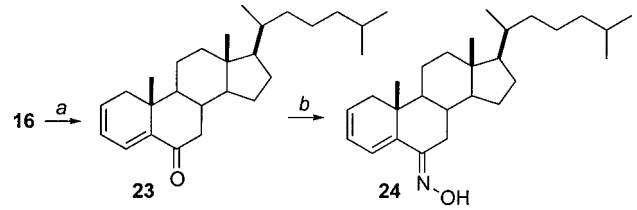
Biological Activities. All these novel 6*E*-hydroximino-4-ene steroids were studied in vitro on P-388,

Scheme 4[a]



[a] Reagents and conditions: (a) TsCl, Py (98%); (b) MCPBA, CH_2Cl_2 (97%); (c) CrO_3, H_2O (90%); (d) LiBr, DMF, reflux (93%); (e) OsO_4, NMO, THF, *t*-BuOH, H_2O, (65%); (f) Ac_2O, Py (70%); (g) $SOCl_2$, Py (90%); (h) KOH, MeOH (90%); (i) $NH_2OH{\cdot}HCl$, EtOH, H_2O (95%); (j) CrO_3, H_2O (70%); (k) MnO_2, $CHCl_3$ (50%).

Scheme 5[a]



[a] Reagents and conditions: (a) LiBr, DMF, reflux (90%); (b) $NH_2OH{\cdot}HCl$, EtOH, H_2O (94%).

A-549, HT-29, and MEL-28 tumor cells. The results, expressed as IC_{50} values in μg/mL, are reported in Table 1. Compounds **8**, **11**, **14a**, **14b**, and **24** presented moderate activity (5 μg/mL) in comparison to that of the "parent" natural compound **1** (IC_{50} 1.25 μg/mL for all the cell lines, **2** showed a very mild activity). However, the cytotoxicity data for analogues **21a**, **21b**, and **22** showed an increase in the cytotoxic activity, and these compounds were 10 times more potent in comparison to **1**, while compounds **20a** and **20b** offered similar IC_{50} values to that of **1**.

The importance of the presence of a cholesterol-like side chain (compounds **1**, **20a**, **20b**, **21a**, **21b**, and **22**) is illustrated by the lack of significant cytotoxicity in compounds **2** and **8** (sitosterol-like and gorgosterol-like side chains, respectively) and in compounds with no side chain at C-17 (**11**, **14a**, and **14b** presented IC_{50} values of 5 μg/mL).

Additional relevant evidence can be deduced by considering the overall skeleton in the derivatives with a ketone at C-3, which apparently plays a key role in increasing the activity given that the change from a keto to a hydroxyl group at this position results in loss of the activity by a factor of ~10 (see the IC_{50} values of pairs **1**/**7a**, **20a**/**21a**, and **20b**/**21b**). Besides, compound **22**, which has a higher level of oxidation on ring A, shows a very promising level of selective cytotoxicity because the IC_{50} value for the cell lines A-549 and MEL-28 for compound **22** was 0.125 μg/mL, while the values for the remaining cell lines HT-29 and P-388 are higher by 100% (0.25 μg/mL) and 400% (0.5 μg/mL), respectively.

Conclusions

In summary, we have described the chemical synthesis of a new series of 6*E*-hydroximino-4-ene steroids and have found some important features needed for cytotoxicity. As outlined in Figure 2, these first structure/cytotoxicity investigations have demonstrated that one important feature is the presence of a cholesterol-type side chain, which appears to play a major role in

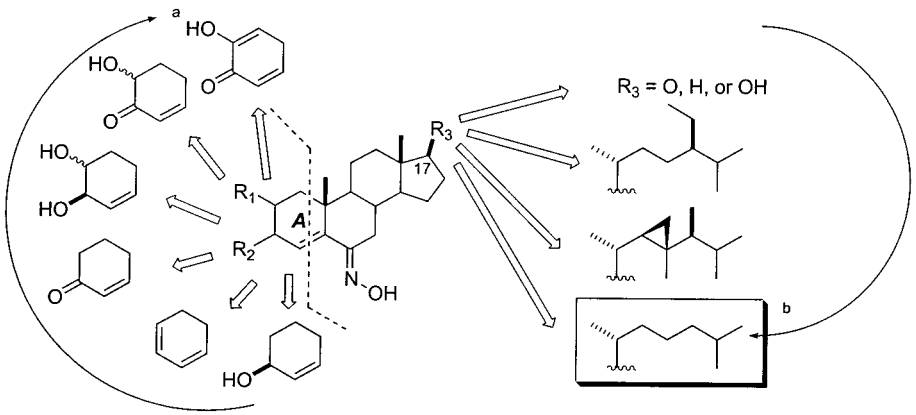


Figure 2. Pattern of nonselective cytotoxicity of 6*E*-hydroximino-4-ene steroids. a: Higher oxidation degree implies higher cytoxicity. b: Required side chain for cytoxicity.

determining the biological activity. The existence of a ketone functionality at C-3 and an elevated degree of oxidation on ring A result in higher bioactivity than other structural motifs.

Compound **22** was selected for NCI's 60 human tumor cell line panel. The results showed IC_{50} values in a range of 10^{-5} to 10^{-6} μM but did not produce any pattern of differential cytotoxicity of sufficient interest to warrant further study based upon the NCI screen.

Experimental Section

Biological Materials. Inhibition of Cell Growth by Counting Cells. This form of assay employs 24-well multidishes of 16 mm diameter.[11] The tumor cell lines employed are as follows: P-388 (ATCC CCL 46), suspension culture of a lymphoid neoplasm from a DBA/2 mouse; A-549 (ATCC CCL 185), monolayer culture of a human lung carcinoma; HT-29 (ATCC HTB-38), monolayer culture of a human colon carcinoma; and MEL-28 (ATCC HTB-72). Cells were maintained in their logarithmic phase of growth in Eagle's minimum essential medium, with Earle's balanced salts, with nonessential amino acids, with 2.0 mM L-glutamine, without sodium bicarbonate (EMEM/neaa), supplemented with 5% fetal calf serum (FCS), 10^{-2} M sodium bicarbonate and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate. For each experiment, the cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. P-388 cells were seeded into wells of 16 mm diameter at 1×10^4 cells per well in 1 mL aliquots of EMEM 5% FCS containing different concentrations of the sample to be tested. A separate set of cultures (without the drug) was seeded as a growth control to ensure that cells remained in the exponential phase of growth. All determinations were carried out in duplicate. After 3 days of incubation at 37 °C and 5% CO_2 in an atmosphere with 98% humidity, an approximate value of IC_{50} was determined by comparing the growth in wells with drug to the growth in the control wells. A-549, HT-29, and MEL-28 cells were seeded into wells of 16 mm diameter at 1×10^4 cells per well in 1 mL aliquots of EMEM 5%FCS containing different concentrations of the sample to be tested. A separate set of cultures (without drug) was seeded as a growth control to ensure that cells remained in the exponential phase of growth. All determinations were carried out in duplicate. After 3 days of incubation at 37 °C and 5% CO_2 in an atmosphere of 98% humidity, the cells were stained with 0.1% crystal violet. An approximate IC_{50} value was determined by comparing the growth in wells with drug to the growth in the control well.

To quantify the activity after completion of the incubation time, the cells were trypsinized and counted in a Coulter Counter ZM. All counts (net cells per well) represent the average of duplicate wells. The percentage growth (% *G*) is relative to cultures without drug. The results of these assays were used to generate dose−response curves from which more precise IC_{50} values were determined (sample concentration that produces 50% cell growth inhibition).

Chemistry. Nuclear magnetic resonance spectra (proton and carbon) were recorded on a Bruker AC 200F or AMX 500 spectrometer, using $CDCl_3$ as the solvent and internal standard. Multiplicities of ^{13}C signals were obtained by DEPT. Microanalyses were performed on a Carlo Erba Instruments CHNS-O 1108, and the results were within ±0.4% (for C, H, N) of the theoretical values. Medium-pressure chromatographic separations were carried out on silica gel 60 (230−400 mesh).

3*β*-Acetoxy-5α,6α-epoxycholestan-6-one (4a). Cholesterol (0.50 g, 1.29 mmol) and acetic anhydride/pyridine (1:1, 10 mL) were stirred at room temperature for 24 h. After removal of the solvents, the residue was dissolved in 20 mL of ethyl acetate and the solution was washed with $NaHCO_3$ (20 mL) and 5% HCl (20 mL) and dried over anhydrous Na_2SO_4. The resulting organic phase was evaporated under vacuum to give 3*β*-acetoxy-5-cholestene (0.52 g, 94%). This compound (0.52 g, 1.19 mmol) was dissolved in 20 mL of $CHCl_3$ at 0 °C. A solution of *m*-chloroperbenzoic acid (0.58 g, 3.36 mmol) in 20 mL of $CHCl_3$ was added dropwise to the reaction mixture, and the solution was stirred for 20 h. Na_2SO_3 (5%, 100 mL) was added to the mixture with cooling (ice/water bath), and the mixture was kept at this temperature for 6 h. The final aqueous phase was extracted twice with $CHCl_3$ (20 mL) and, after removal of the solvent, a mixture of 3*β*-acetoxy-5α,6α-epoxycholestane (**4a**) and 3*β*-acetoxy-5*β*,6*β*-epoxycholestane was obtained in a 2:1 ratio. **4a**: ^1H NMR (200 MHz, $CDCl_3$) δ_H: 4.95 (H-3, 1H, m); (2.90, H-6, 1H, d, J = 4.4 Hz); 2.03 (OAc, 3H, s); 1.08 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 5.4 Hz); 0.86 (H-26, H-27, 6H, d, J = 5.9 Hz); 0.64 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 170.4 (OAc, s); 71.5 (C-3, d); 59.2 (C-6, d). EIMS (70 eV, *m*/*z* %): 444 (M, 2); 384 (M − CH_3COOH, 42); 366 (M − CH_3COOH − H_2O, 44).

3*β*-Acetoxy-5α-hydroxycholestan-6-one (5a). A solution of chromium trioxide (0.40 g, 4.0 mmol) in 1.2 mL of water was added dropwise to a solution of a mixture 2:1 of 3*β*-acetoxy-5α,6α-epoxycholestane (**4a**) and 3*β*-acetoxy-5*β*,6*β*-epoxycholestane (0.50 g, 1.12 mmol) in 20 mL of methyl ethyl ketone at 0 °C. This addition was repeated at room temperature, and the solution was stirred for 20 min. The reaction mixture was poured into water (90 mL), and the resulting precipitate was filtered off under reduced pressure, washed with water, and subjected to chromatography (hexane/ethyl acetate, 7:3) to give **5a** (0.47 g, 90%): ^1H NMR (200 MHz, $CDCl_3$) δ_H: 5.04 (H-3, 1H, m); 2.75 (H-4*β*, 1H, t, J = 12.2 Hz); 2.02 (OAc, 3H, s); 1.05 (H-19, 3H, s); 0.94 (H-21, 3H, d, J = 5.9 Hz); 0.86 (H-26, H-27, 6H, d, J = 6.4 Hz); 0.64 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 210.0 (C-6, s); 171.1 (OAc, s); 81.3 (C-5, s); 71.7 (C-3, d). EIMS (70 eV, *m*/*z* %): 460 (M, 2); 400 (M − CH_3COOH, 34); 382 (M − CH_3COOH − H_2O, 3).

3*β*-Acetoxycholest-4-en-6-one (6a). Thionyl chloride (0.36 mL, 4.9 mmol) was added dropwise to a solution of 3*β*-acetoxy-5α-hydroxycholestan-5-one (**5a**, 0.46 g, 1.0 mmol) in 15 mL of dry pyridine at 0 °C. The mixture was stirred for 45 min and then poured into water (80 mL). The resulting precipitate was extracted with ethyl acetate (2 × 30 mL), and the combined extracts were washed (10% HCl followed by brine), dried, and evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 8:2) to give 3*β*-acetoxycholest-4-en-6-one (**6a**, 0.41 g, 92%). ^1H NMR (200 MHz, $CDCl_3$) δ_H: 6.07 (H-4, 1H, d, J = 2 Hz); 5.34 (H-3, 1H, m); 2.56 (H-7*β*, 1H, dd, J = 0.2 and 11.7 Hz); 2.07 (OAc, 3H, s); 1.02 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 6.4 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.8 Hz); 0.70 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 203.0 (C-6, s); 171.8 (OAc, s); 148.0 (C-5, s); 128.6 (C-4, d). EIMS (70 eV, *m*/*z* %): 442 (M, 36); 400 (M − CH_3COH, 100).

6*E*-Hydroximinocholest-4-en-3*β*-ol (7a). 3*β*-acetoxycholest-4-en-6-one (**6a**, 0.40 g, 0.90 mmol) was dissolved in a 10% methanolic potassium hydroxide solution (20 mL) under argon. The mixture was stirred at room temperature for 1 h, and then the solvent was evaporated under reduced pressure to a volume of 10 mL. This solution was poured into ice/water (50 g), and the product was extracted with ethyl acetate (2 × 20 mL). The combined extracts were washed with saturated brine, dried, and evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 7:3) to give 3*β*-hydroxycholest-4-en-6-one (0.32 g, 82%).

A solution of 3*β*-hydroxycholest-4-en-6-one (0.29 g, 0.65 mmol) in ethanol (15 mL) was treated with a solution of hydroxylamine hydrochloride (0.34 g, 4.9 mmol) in 50% aqueous ethanol (4 mL) and trihydratated sodium acetate (0.40 g, 2.9 mmol) in 50% aqueous ethanol (11 mL). The resulting mixture was stirred at room temperature for 24 h, and the solvent was removed under reduced pressure. The residue was diluted with water (20 mL) and extracted with ethyl acetate (20 mL). The extract was dried and evaporated, and the residue was subjected to chromatography (hexane/ethyl acetate, 7:3) to give 6*E*-hydroximinocholest-4-en-3*β*-ol (**7a**, 0.29 g, 97%). ^1H NMR (200 MHz, $CDCl_3$) δ_H: 5.78 (H-4, 1H, s); 4.18 (H-3, 1H, m); 3.32 (H-7*β*, 1H, dd, J = 4.4 Hz and 10.3 Hz); 0.98 (H-19, 3H, s); 0.92 (H-21, 3H, s); 0.87 (H-26, H-27, 6H, d,

J = 6.8 Hz); 0.68 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 158.0 (C-6, s); 146.6 (C-5, s); 122.5 (C-4, d); 67.5 (C-3, d). EIMS (70 eV, *m*/*z* %): 415 (M, 4); 398 (M − OH, 27).

6*E*-Hydroximinocholest-4-en-3-one (1). A solution of 6*E*-hydroximinocholest-4-en-3β-ol (**7a**, 0.28 g, 0.67 mmol) in 5 mL of pyridine was added dropwise to the chromium trioxide/pyridine complex prepared by the addition of CrO_3 (0.49 g, 4.9 mmol) to 5 mL of pyridine at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then diluted with 10 mL of ethyl acetate. The resulting precipitate was filtered off, and the filtrate was washed (10% HCl, 10% $NaHCO_3$, brine), dried with anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 7:3) to give 6*E*-hydroximinocholest-4-en-3-one (0.19 g, 70%). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 9.71 (OH, 1H, br s); 6.54 (H-4, 1H, s); 3.43 (H-7β, 1H, dd, J = 3.7 and 16.0 Hz); 2.54 (H-2, 2H, m); 2.04 (H-1, 2H, m); 1.19 (H-19, 3H, s); 0.91 (H-21, 3H, d, J = 6.4 Hz); 0.86 (H-26, H-27, 6H, d, J = 6.8 Hz); 0.70 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 201.6 (C-3, s); 162.8 (C-5, s); 155.3 (C-6, s); 122.4 (C-4, d); 56.5 (C-14, s); 55.9 (C-17, d); 51.0 (C-9, d); 42.4 (C-13, s); 39.4 (C-24, t); 39.3 (C-12, t); 38.6 (C-10, s); 36.0 (C-22, t); 35.6 (C-20, d); 34.7 (C-1, t); 33.5 (C-2, t); 32.5 (C-8, d); 29.5 (C-7, t); 28.1 (C-16, t); 28.0 (C-25, q); 24.0 (C-15, t); 23.7 (C-23, t); 22.8 (C-26, q); 22.5 (C-27, q); 20.8 (C-11, t); 18.6 (C-21, q); 16.5 (C-19, q); 11.9 (C-18, q). EIMS (70 eV, *m*/*z* %): 413 (M, 21); 396 (M − OH, 42); 370 (M − NOH − H_2O, 100).

Compounds **2** and **8** were obtained in a similar way, and for this reason, only experimental details for the synthesis of compound **1** are reported.

6*E*-Hydroximino-24-ethylcholest-4-en-3-one (2). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 10.43 (OH, 1H, br s); 6.43 (H-4, 1H, s); 3.42 (H-7β, 1H, dd, J = 3.7 and 16.0 Hz); 2.53 (H-2, 2H, m); 2.05 (H-1, 2H, m); 1.13 (H-19, 3H, s); 0.93 (H-21, 3H, d, J = 6.3 Hz); 0.86 (H-29, 3H, t; d, J = 6.6 Hz); 0.84/0.81 (H-26, H-27, 6H, d, J = 6.6 Hz); 0.70 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 201.0 (C-3, s); 162.3 (C-5, s); 156.0 (C-6, s); 122.6 (C-4, d); 56.6 (C-14, s); 55.9 (C-17, d); 51.2 (C-9, d); 46.1 (C-24, t); 42.5 (C-13, s); 39.3 (C-12, t); 38.7 (C-10, s); 36.2 (C-20, d); 34.8 (C-1, t); 33.8 (C-22, t); 33.6 (C-2, t); 32.7 (C-8, d); 29.6 (C-7, t); 29.0 (C-25, q); 28.1 (C-16, t); 26.4 (C-23, t); 24.0 (C-15, t); 23.1 (C-28, t); 20.8 (C-11, t); 19.0 (C-26, q); 19.6 (C-27, q); 18.7 (C-21, q); 16.6 (C-19, q); 12.3 (C-29, q); 12.0 (C-18, q). EIMS (70 eV, *m*/*z* %): 441 (M, 17); 425 (M − OH, 23); 399 (M − NOH − H_2O, 78).

6*E*-Hydroximinogorgost-4-en-3-one (8). ^{1}H NMR (200 Hz, $CDCl_3$) δ_H: 6.36 (H-4, 1H, s); 3.43 (H-7β, 1H, dd, J = 3.9 and 11.2 Hz); 2.48 (H-2, 2H, m); 2.07 (H-2, 2H); 1.14 (H-19, 3H, s); 0.94 (H-21, 3H, d, J = 6.3 Hz); 0.88 (H-26, H-27, 6H, d, J = 6.8 Hz); 0.77 (H-30, 3H, d, J = 6.3 Hz); 0.71 (H-18, 3H, s); 0.46 (H-28, 1H, m); 0.21 (H-22, 1H, m); −0.12 (H-28, 1H, m). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 200.6 (C-3, s); 161.9 (C-6, s); 156.4 (C-5, s); 122.8 (C-4, d); 56.5; 55.8; 51.3; 42.5; 39.2; 39.0; 38.8; 36.1; 34.8; 33.7; 33.6; 32.8; 32.0; 31.4; 30.5; 29.6; 29.3; 28.0; 24.0; 20.8; 20.5 (C-26, q); 18.8 (C-21, q); 17.6 (C-29, q); 16.6 (C-19, q); 15.4; 11.9 (C-18, q). EIMS (70 eV, *m*/*z* %): 453 (M, 3); 410 (M − NOH − H_2O, 13).

Androst-5-en-3β-ol (10). Hydrazine monohydrate (0.40 mL, 8.25 mmol) was added dropwise to a solution of dehydroisoandrosterone (**9**, 0.50 g, 1.73 mmol) and K_2CO_3 (0.20 g, 1.45 mmol) diluted in 15 mL of diethylene glycol. The resulting mixture was heated to 125 °C and stirred for 5 h with a Liebig condenser fitted to the reaction vessel. The excess hydrazine monohydrate was distilled off, and the temperature was then raised to 200 °C. The solution was stirred for 15 h at this temperature. The reaction mixture was cooled to room temperature and poured into water (100 mL). The resulting precipitate was extracted with diethyl ether (3 × 40 mL), and the combined extracts were washed with water, dried, and evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 7:3) to give androst-5-en-3β-ol (**10**, 0.32 g, 66%). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 5.37 (H-6, 1H, d, J = 6.0 Hz); 3.50 (H-3, 1H, m); 1.02 (H-19, 3H, s); 0.72 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 140.7 (C-5, s); 120.8 (C-6, d); 71.9 (C-3, d). EIMS (70 eV, *m*/*z* %): 274 (M, 21); 259 (M − CH_3, 16).

6*E*-Hydroximinoandrost-4-en-3-one (11). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.50 (H-4, 1H, s); 3.45 (H-7β, 1H, dd, J = 0.8 and 11.8 Hz); 2.48 (H-2, 2H, m); 2.10 (H-1, 2H, m); 1.15 (H-19, 3H, s); 0.75 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 201.3 (C-3, s); 162.2 (C-6, s); 155.5 (C-5, s); 122.5 (C-4, d); 54.7; 51.4; 40.7; 40.0; 38.8; 38.1; 34.8; 33.6; 33.0; 30.0; 25.3; 20.9; 20.4; 17.3 (C-19, q); 16.5 (C-18, q). EIMS (70 eV, *m*/*z* %): 301 (M^+, 13); 284 (M − OH, 19); 270 (M − NOH, 25); 258 (M − NOH − H_2O, 78).

3β-Acetoxy-17,17-ethylenedioxoandrost-5-ene (12). A mixture of 3β-hydroxyandrost-5-en-17-one (**9**, 0.87 g, 3.0 mmol), adipic acid (0.23 g, 1.6 mmol), and ethylene glycol (9 mL, 161 mmol) was dissolved in 40 mL of benzene. A Liebig condenser and a Dean Stark apparatus were fitted to the reaction vessel, and the reaction temperature was raised at 100 °C. After 28 h, the mixture was washed with saturated $NaHCO_3$ and water and dried over Na_2SO_4. The mixture was filtered and the solvent removed under reduced pressure to give a white solid, which was purified by flash chromatography (hexane:EtOAc, 8:2) to afford 3β-hydroxy-17,17-ethylenedioxo-5-androstene (0.60 g, 60%). This compound was further elaborated in a way to that for **3a**−**c** (i.e., acetylation and epoxidation).

3α-Acetoxy-5α-hydroxyandrosten-6,17-dione (13). Spectral data similar to those reported in the literature.[12]

6*E*-Hydroximinoandrost-4-en-3,17-dione (14a). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.53 (H-4, 1H, s); 3.57 (H-7β, 1H, dd, J = 4.4 and 11.2 Hz); 2.50 (H-2, 2H, m); 2.09 (H-1, 2H, m); 1.17 (H-19, 3H, s); 0.92 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 220.0 (C-17, s); 201.1 (C-3, s); 161.7 (C-6, s); 154.7 (C-5, s); 122.8 (C-4, d); 51.5; 51.1; 47.6; 38.7; 35.7; 34.7; 33.5; 32.3; 31.0; 28.4; 21.7; 20.2; 16.6 (C-19, q); 13.7 (C-18, q). EIMS (70 eV, *m*/*z* %): 315 (M, 16); 298 (M − OH, 30); 272 (M − NOH − H_2O, 83).

17β-Hydroxy-6*E*-hydroximinoandrost-4-en-3-one (14b). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.38 (H-4, 1H, s); 3.68 (H-17, 1H, m); 3.43 (H-7β, 1H, dd, J = 4.4 and 11.2 Hz); 2.48 (H-2, 2H, m); 2.13 (H-1, 2H, m); 1.16 (H-19, 3H, s); 0.80 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 201.1 (C-3, s); 162.1 (C-6, s); 155.4 (C-5, s); 122.7 (C-4, d); 81.5 (C-17, d); 51.3; 51.2; 42.9; 38.7; 36.1; 34.8; 33.6; 32.7; 30.4; 29.1; 23.2; 20.5; 16.6 (C-19, q); 11.0 (C-18, q). EIMS (70 eV, *m*/*z* %): 317 (M, 15); 300 (M − OH, 18).

5α-Hydroxycholest-2-en-6-one (16). A solution of cholesterol (**3a**, 1.5 g, 3.9 mmol) in 20 mL of dry pyridine was treated with *p*-toluenesulfonyl chloride, and the mixture was stirred at room temperature for 22 h. The reaction mixture was poured into 5% aqueous $NaHCO_3$ (100 mL) and was left to stand for 1 h. The solid was filtered off, washed with water, dried, and subjected to chromatography (hexane/ethyl acetate, 9:1) to give 3β-*p*-toluenesulfonyloxycholest-5-ene (2.0 g, 95%). This compound was subjected to epoxidation and oxidation, as shown in Scheme 1, to afford 3β-*p*-toluenesulfonyloxy-5α-hydroxycholestan-6-one. A solution of 3β-*p*-toluenesulfonyloxy-5α-hydroxycholestan-6-one (1.54 g, 2.68 mmol) in 40 mL of dry DMF was treated with LiBr (0.63 g, 7.25 mmol) under argon, and the solution was refluxed in the dark with stirring for 2 h. The reaction mixture was poured into 250 mL of water and extracted with ethyl acetate (2 × 40 mL). The combined extracts were washed (5% HCl and 5% $NaHCO_3$) and dried, and the solvent evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 8:1) to give 5α-hydroxycholest-2-en-6-one (**16**, 1.00 g, 83%). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 5.62 (H-2, H-3, 2H, m); 2.68 (H-7β, 1H, dd, J = 4.4 and 12.7 Hz); 0.92 (H-21, 3H, d, J = 6.3 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.71 (H-19, 3H, s); 0.65 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 211.5 (C-6, s); 125.8 (C-3, d); 122.9 (C-2, d); 78.0 (C-5, s). EIMS (70 eV, *m*/*z* %): 400 (M, 20); 382 (M − H_2O, 6).

2α,3α,5α-Trihydroxycholestan-6-one (17) and 2β,3β,5α-Trihydroxycholestan-6-one (18). *N*-Methylmorpholine *N*-oxide (10.0 g, 85.3 mmol) and tetrabutylammonium hydrogen

sulfate (5.18 g, 15.2 mmol) were dissolved in a mixture of 37 mL of THF (free of peroxides), 43 mL of *t*-BuOH, and 9 mL of water. To the resulting solution was added osmium tetroxide (0.25 g, 0.98 mmol) followed by the dropwise addition of a solution of 5α-hydroxycholest-2-en-6-one (**16**, 1.00 g, 2.49 mmol) in 20 mL of THF. The mixture was stirred for 86 h at 0 °C in the dark under an argon atmosphere. The reaction mixture was poured into 5% Na_2SO_3 (300 mL), stirred for 1 h, and extracted with CH_2Cl_2 (2 × 150 mL). The combined extracts were evaporated, and the residue was redissolved in 400 mL of ethyl acetate, washed (5 M H_2SO_4, saturated $NaHCO_3$ and saturated brine), and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue subjected to chromatography (CH_2Cl_2/dimethyl ketone, 90:10) to give 2α,3α,5α-trihydroxycholestan-6-one (**17**, 0.31 g, 29%) and 2*β*,3*β*,5α-trihydroxycholestan-6-one (**18**, 0.40 g, 36%). **17**: ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 4.19 (H-3, 1H, m); 3.96 (H-2, 1H, m); 2.71 (H-4α, 1H, t, J = 12.2 Hz); 0.92 (H-21, 3H, d, J = 6.3 Hz); 0.86 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.78 (H-19, 3H, s); 0.65 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 211.5 (C-6, s); 79.4 (C-5, s); 71.0 (C-3, d); 68 (C-2, d). EIMS (70 eV, *m*/*z* %): 434 (M, 35); 416 ($M^+ - H_2O$, 16); 398 ($M^+ - 2H_2O$, 9); 380 ($M - 3H_2O$, 3). **18**: ^{1}H NMR (200 MHz, $CDCl_3$/CD_3OD) δ_H: 3.35 (H-3, 1H, m); 3.10 (H-2, 1H, m); 2.71 (H-4α, 1H, t, J = 12.2 Hz, 12.7 Hz); 0.95 (H-19, 3H, s); 0.86 (H-21, 3H, d, J = 6.3 Hz); 0.82 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.60 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD 4:1) δ_C: 214.6 (C-6, s); 79.0 (C-5, s); 69.8 (C-3, d); 68.1 (C-2, d). EIMS (70 eV, *m*/*z* %): 434 (M, 37); 416 ($M - H_2O$, 7); 398 ($M - 2H_2O$, 7).

2*β*,3*β*-Dihydroxy-6*E*-hydroximinocholest-4-ene (20a). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 5.63 (H-4, 1H, br s); 4.21 (H-3, 1H); 4.15 (H-2, 1H, m); 3.30 (H-7*β*, 1H); 0.92 (H-19, 3H, s); 0.91 (H-21, 3H, d, J = 6.8 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.8 Hz); 0.68 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 159.2 (C-6, s); 143.2 (C-5, s); 124.0 (C-4, d); 67.7 (C-3, d); 66.4 (C-2, d). EIMS (70 eV, *m*/*z* %): 431 (M, 6); 414 ($M - OH$, 22); 413 ($M - H_2O$, 5); 400 ($M - NOH$, 5); 382 ($M - H_2O - NOH$, 6).

2α,3α-Dihydroxy-6*E*-hydroximinocholest-4-ene (20b). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 5.93 (H-4, 1H, d, J = 5.4 Hz); 4.17 (H-3, 1H, t, J = 4.4 Hz); 3.89 (H-2, 1H, dt, J = 3.9 and 12.7 Hz); 3.34 (H-7*β*, 1H, dd, J = 4.4 and 10.2 Hz); 1.02 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 6.8 Hz); 0.88 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.68 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 159.0 (C-6, s); 146.3 (C-5, s); 122.5 (C-4, d); 66.8 (C-3, d); 65.9 (C-2, d). EIMS (70 eV, *m*/*z* %): 431 (M, 10); 414 ($M - OH$, 47); 413 ($M - H_2O$, 11); 400 ($M - NOH$, 13); 396 ($M - H_2O - OH$, 30); 382 ($M - H_2O - NOH$, 11).

2α-Hydroxy-6*E*-hydroximinocholest-4-en-3-one (21b). A solution of 6*E*-hydroximinocholest-4-en-2α,3α-diol (**20b**, 0.050 g, 0.11 mmol) in 4 mL of pyridine was added dropwise to the chromium trioxide/pyridine complex prepared by the addition of CrO_3 (0.49 g, 4.9 mmol) to 4 mL of pyridine at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then diluted with 10 mL of ethyl acetate. The resulting precipitate was filtered off, and the filtrate was washed (10% HCl, 10% $NaHCO_3$, and brine), dried with anhydrous Na_2SO_4, evaporated under reduced pressure, and subjected to chromatography (hexane/ethyl acetate, 1:1) to give 2α-hydroxy-6-hydroximinocholest-4-en-3-one (**21b**, 0.030 g, 60%). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.24 (H-4, 1H, s); 4.35 (H-2, 1H, dd, J = 5.4 and 7.8 Hz); 3.40 (H-7*β*, 1H, dd, J = 3.9 and 11.2 Hz); 2.41 (H-1α, 1H, dd, J = 5.4 and 7.3 Hz); 1.13 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 6.3 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.71 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 199.9 (C-3, s); 162.8 (C-6, s); 156.8 (C-5, s); 119.9 (C-4, d); 69.6 (C-2, d); 56.4; 55.9; 51.8; 42.5; 40.9; 39.4; 39.1; 36.0; 35.7; 32.6; 29.7; 29.6; 28.1; 28.0; 23.9; 23.8; 22.8 (C-26, q); 22.5 (C-27, q); 20.7 (C-21, q); 18.6 (C-19, q); 17.3; 11.9 (C-18, q). EIMS (70 eV, *m*/*z* %): 411 ($M - H_2O$, 10); 384 ($M - NOH - Me$, 63).

2*β*-Hydroxy-6*E*-hydroximinocholest-4-en-3-one (21a). Treatment of 6*E*-hydroximinocholest-4-en-2*β*,3*β*-diol (**20a**) with CrO_3/pyridine or MnO_2 gave, in both cases, 2*β*-hydroxy-6-hydroximinocholest-4-en-3-one (**21a**). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.06 (H-4, 1H, s); 4.25 (H-2, 1H, dd, J = 5.9 and 6.3 Hz); 3.42 (H-7*β*, 1H, dd, J = 4.4 and 10.7 Hz); 2.47 (H-1α, 1H, dd, J = 5.9 and 8.3 Hz); 0.95 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 6.3 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.72 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 199.6 (C-3, s); 163.8 (C-6, s); 157.4 (C-5, s); 120.4 (C-4, d); 68.7 (C-2, d); 56.4; 55.9; 49.0; 42.9; 41.2; 39.4; 39.3; 39.1; 36.0; 35.7; 33.9; 30.6; 28.1; 28.0; 24.0; 23.8; 22.8 (C-26, q); 22.7 (C-27, q); 22.5 (C-21, q); 18.6 (C-19, q); 12.1 (C-18, q). EIMS (70 eV, *m*/*z* %): 411 ($M - H_2O$, 20); 384 ($M - NOH - Me$, 28).

2-Hydroxy-6*E*-hydroximinocholesta-1,4-dien-3-one (22). A mixture of 6*E*-hydroximinocholest-4-en-2α,3α-diol (**20b**, 0.10 g, 0.23 mmol) and activated MnO_2 (0.39 g, 4.55 mmol) in dry chloroform (25 mL) was shaken vigorously at room temperature for 17 h. The reaction was subjected to chromatography (hexane/ethyl acetate, 1:1) to give 2-hydroxy-6-hydroximinocholest-1,4-dien-3-one (**22**, 0.050 g, 50%). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.72 (H-1, 1H, s); 6.42 (H-4, 1H, s); 3.47 (H-7*β*, 1H, dd, J = 4.4 and 11.2 Hz); 0.95 (H-19, 3H, s); 0.92 (H-21, 3H, d, J = 6.3 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.8 Hz); 0.73 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 181.8 (C-3, s); 163.7 (C-6, s); 156.3 (C-5, s); 146.4 (C-2, s); 124.1 (C-1, d); 121.5 (C-4, d); 56.1; 56.0; 49.8; 44.5; 42.8; 39.4; 39.2; 36.0; 35.7; 33.2; 30.4; 28.1; 28.0; 24.1; 23.8; 23.3; 22.8 (C-26, q); 22.5 (C-27, q); 19.9 (C-21, q); 18.6 (C-19, q); 12.0 (C-18, q). EIMS (70 eV, *m*/*z* %): 427 (M, 65); 410 ($M - H_2O$, 48).

6*E*-Hydroximinocholesta-2,4-diene (24). A solution of 5α-hydroxycholest-2-en-6-one (**16**, 0.1 g, 0.25 mmol) in 10 mL of dry DMF was treated with LiBr (0.63 g, 0.67 mmol) under an argon atmosphere. The reaction mixture was refluxed in the dark with stirring for 2 h and then poured into 25 mL of water. The product was extracted with ethyl acetate (2 × 10 mL), and the combined extracts were washed (5% HCl and 5% $NaHCO_3$), dried, and evaporated under reduced pressure. The residue was subjected to chromatography (hexane/ethyl acetate, 8:1) to give 2,4-cholestadien-6-one (**23**, 0.086 g, 90%). A solution of **23** (0.030 g, 0.08 mmol) in ethanol (3 mL) was treated with a solution of hydroxylamine hydrochloride (0.041 g, 0.60 mmol) in 50% aqueous ethanol (1 mL) and trihydratated sodium acetate (0.048 g, 0.34 mmol) in 50% aqueous ethanol (3 mL). The resulting mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure, and the residue diluted with water (3 mL) and extracted with ethyl acetate (3 mL). The extract was dried, evaporated, and subjected to chromatography (hexane/ethyl acetate, 7:3) to give 6*E*-hydroximinocholesta-2,4-diene (**24**). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 6.32 (H-4, 1H, d, J = 4.9 Hz); 5.88 (H-3, H-2, 2H, m); 3.42 (H-7*β*, 1H, dd, J = 0.5 and 11.2 Hz); 0.94 (H-19, 3H, s); 0.90 (H-21, 3H, d, J = 6.3 Hz); 0.87 (H-26, H-27, 6H, d, J = 6.3 Hz); 0.68 (H-18, 3H, s). ^{13}C NMR (50 MHz, $CDCl_3$) δ_C: 157.7 (C-6, s); 138.6 (C-5, s); 127.2 (C-4, d); 123.3 (C-3, d); 119.5 (C-2, d); 56.5; 56.1; 52.0; 42.6; 39.6; 39.5; 37.5; 36.8; 36.1; 35.7; 33.1; 29.1; 28.1; 28.0; 24.2; 23.8; 22.8; 22.5; 21.3; 18.7 (C-19, q); 16.8; 11.9 (C-18, q). EIMS (70 eV, *m*/*z* %): 397 (M, 34); 380 ($M - OH$, 29).

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Supporting Information Available: NMR spectra and elemental analysis data for some of the compounds reported here are available free of charge via the Internet at http://pubs.acs.org.

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