Journal of Medicinal Chemistry

ARTICLE

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Selective Cytotoxicity of Oxysterols through Structural Modulation on Rings A and B. Synthesis, in Vitro Evaluation, and SAR

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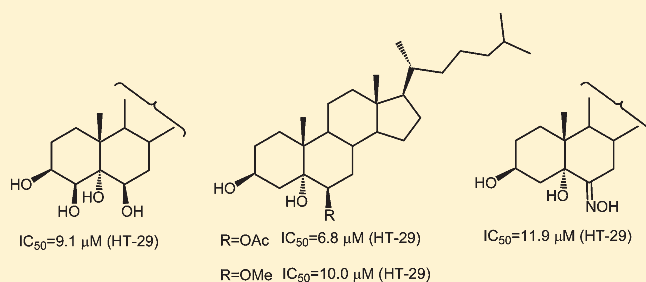
S *Supporting Information*

ABSTRACT: Chemically diverse oxysterols were prepared and evaluated for cytotoxicity, aiming to push forward potency and selectivity. They were tested against seven cancer (HT-29, HepG2, A549, PC3, LAMA-84, MCF-7, and SH-SY5Y) and two noncancerous cell lines (ARPE-19 and BJ). The influence of the oxidation pattern on rings A and B was studied. Oxygen functionalities on ring B, such as oxo, oxime, acetamide, acetate, and alkoxy, were evaluated. Most oxysterols were cytotoxic in the low micromolar range, with emphasis to the tetrols **14** and **34**, the 6β methoxy and acetoxy derivatives **21** and **45**, and the oxime **28**. In general, the oxysterols were more toxic to cancer cells and a set of compounds (**9**, **14**, **21**, **28**, **45**) with very high selectivity was identified. The cytotoxicity of 3β-acetates was lower than that of the parent alcohols, although incubation for a longer period rendered them equally cytotoxic, pointing them as potential prodrugs of oxysterols.



INTRODUCTION

Oxysterols, a group of lipids derived from cholesterol, endogenously found and formed via spontaneous and/or enzymatic oxidation processes,[1] comprise a very heterogeneous group of molecules that, according to the oxygen position in the sterol template, display different membrane biophysical properties,[2] membrane transfer abilities,[3−5] and several cellular functions.[6−10] They attract increasing interest because of the diverse biological effects observed in cell cultures and have been reported to affect the regulation of cholesterol homeostasis,[1] inflammation, cell differentiation, and proliferation.[11] Oxysterols are intermediates in the biosynthesis of bile acids[12] and steroid hormones,[13−15] participate in the Hedgehog signaling pathways,[16] and are important regulators of lipid rafts.[2,17]

Several lines of evidence point to the involvement of oxysterols in a series of pathological events, like atherosclerosis,[18] osteoporosis,[19−21] age-related macular degeneration,[11] and neurodegenerative diseases like Alzheimer, Parkinson, and multiple sclerosis.[22]

The mechanisms by which oxysterols induce cell death are still largely unknown, despite intensive research on the field.[23,24] Therefore, studies involving the cytotoxic evaluation of a library of endogenous and synthetic oxysterols and the analysis of structure−activity relationships (SARs) should give promising anticancer oxysterols and pave the way to better understand the mechanisms involved in the cytotoxic effects of oxysterols.

Our group has been interested in the study of the structural requirements of oxysterols to display cytotoxicity. In previous studies, we have correlated the cytotoxic profile of common endogenous oxysterols and synthetic polyoxygenated steroids with its oxidation pattern.[25,26] Specifically, the position and stereochemistry of the epoxide group,[25] the type of sterol side chain, and the oxygenation of ring B[26] have been correlated with selective cytotoxicity against human cancer cells. Moreover, the cytotoxicity of the most important ring B endogenous oxysterols (Figure 1) was determined for the first time under the same experimental conditions.

In the present study, we have synthesized and evaluated a library of heavily oxygenated sterols in rings A and B and their derivatives. Three oxysterols were used as scaffolds for further derivatization, specifically, 4β-hydroxycholesterol **1**, which is one of the most abundant oxysterol in plasma,[27] and 3β,5α,6β-triol **2** and 7β-hydroxycholesterol **6**, which are the most cytotoxic endogenous oxysterols.[26] The SAR analysis of the oxysterols synthesized contributes to define the sterol structural determinants for cytotoxicity and selectivity on cancer and noncancerous cells.

RESULTS AND DISCUSSION

On the basis of the oxidation state on rings A and B of the sterol nucleus and on the oxygenated groups known to be essential for cytotoxicity in natural occurring oxysterols,[26] herein other patterns and functionalities have been introduced to get a diverse and amplified synthetic library. The molecules synthesized were evaluated for cytotoxicity in several cancer and

Received: June 21, 2011
Published: July 29, 2011

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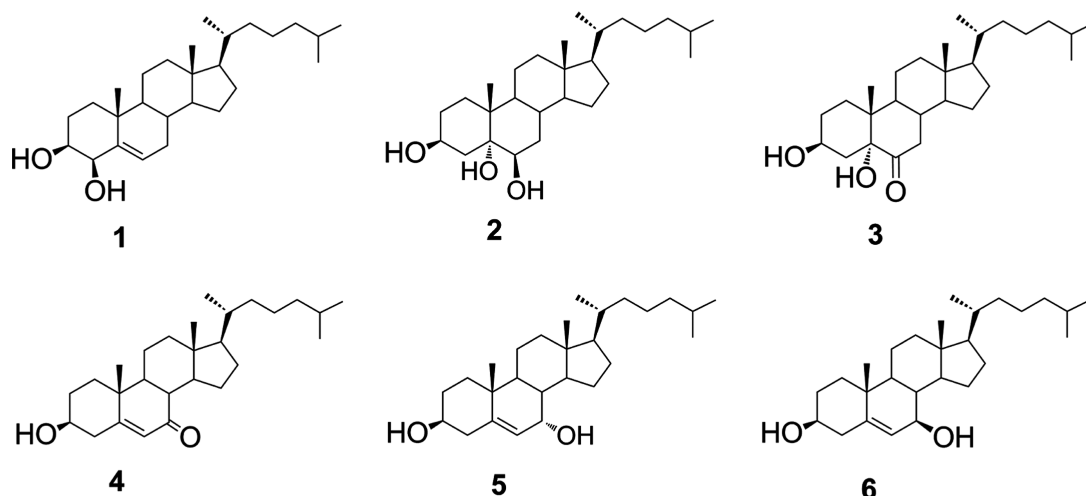
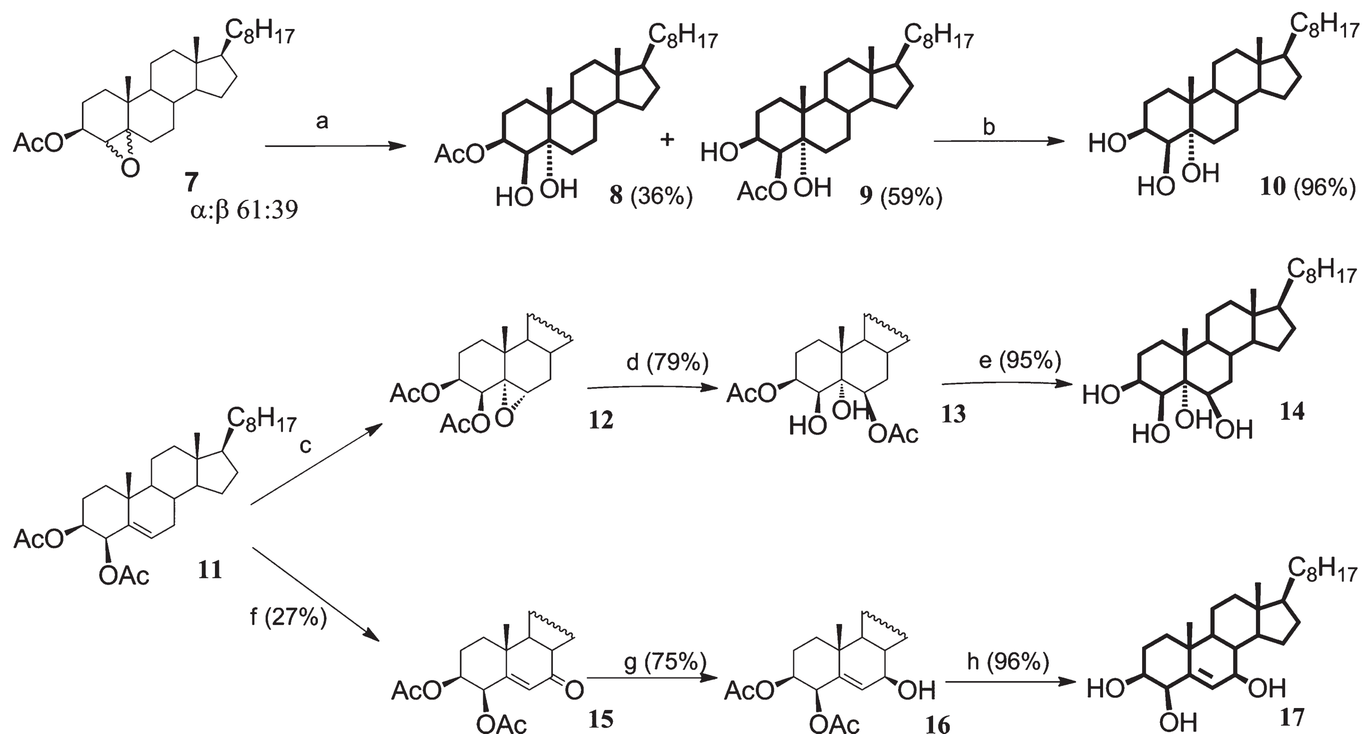


Figure 1. Endogenous ring B oxysterols.

Scheme 1. Synthesis of Ring A and Ring B Oxygenated Sterols[a]



[a] (a) $HClO_4$ (7% aq), acetone, room temp, overnight; (b) NaOH (10% aq), EtOH, CH_2Cl_2, room temp, 2 h; (c) (1) MMPP, CH_3CN, reflux, (2) crystallization (EtOH); (d) $Bi(OTf)_3$, acetone, room temp, overnight; (e) NaOH (10% aqueous solution), EtOH, CH_2Cl_2, room temp, overnight; (f) *t*BHP, CuI, CH_3CN, reflux, overnight; (g) $NaBH_4$, $CeCl_3.7H_2O$, THF, room temp, 15 min; (h) Na_2CO_3, MeOH, room temp, overnight. Structures in bold have been evaluated for cytotoxicity.

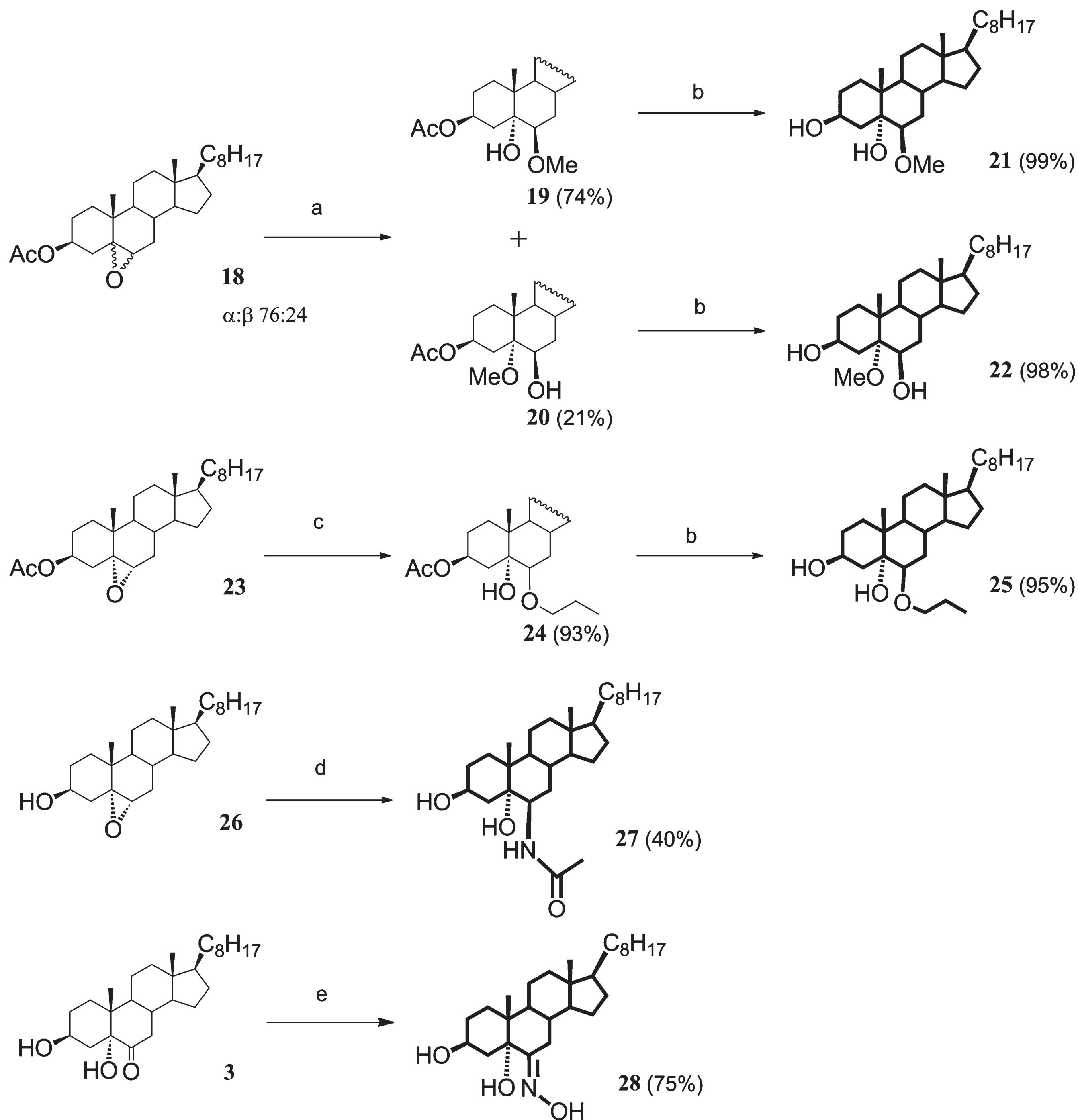
noncancerous cells. The main purpose of this study is to evaluate the structural requirements for potent and selective sterol cytotoxicity.

■ CHEMISTRY

The oxysterols reported in this study were obtained by chemical and enzymatic syntheses, and the synthetic routes are outlined in Schemes 1−4. The starting materials **1**, **3**, **4**, **7**, **11**, **18**, **23**, **26**, **29**, and **43** were prepared as described in the literature.[25,26,28,29]

Ring A acetoxycholestanes **8** and **9** were obtained by acid-catalyzed opening of the 3*β*-acetoxycholestane-4,5-epoxide mixture (**7**, 61:39 α:*β* ratio) under aqueous $HClO_4$ followed by flash chromatography, affording compounds **9** and **8** in 59% and 36% yields. These yields are consistent with the α:*β* ratio of the substrate **7**. The neighboring acetoxy group participation in the cleavage of the 4α,5α-epoxide is in agreement with the work of

Scheme 2. Synthesis of Ring B Derivatives[a]

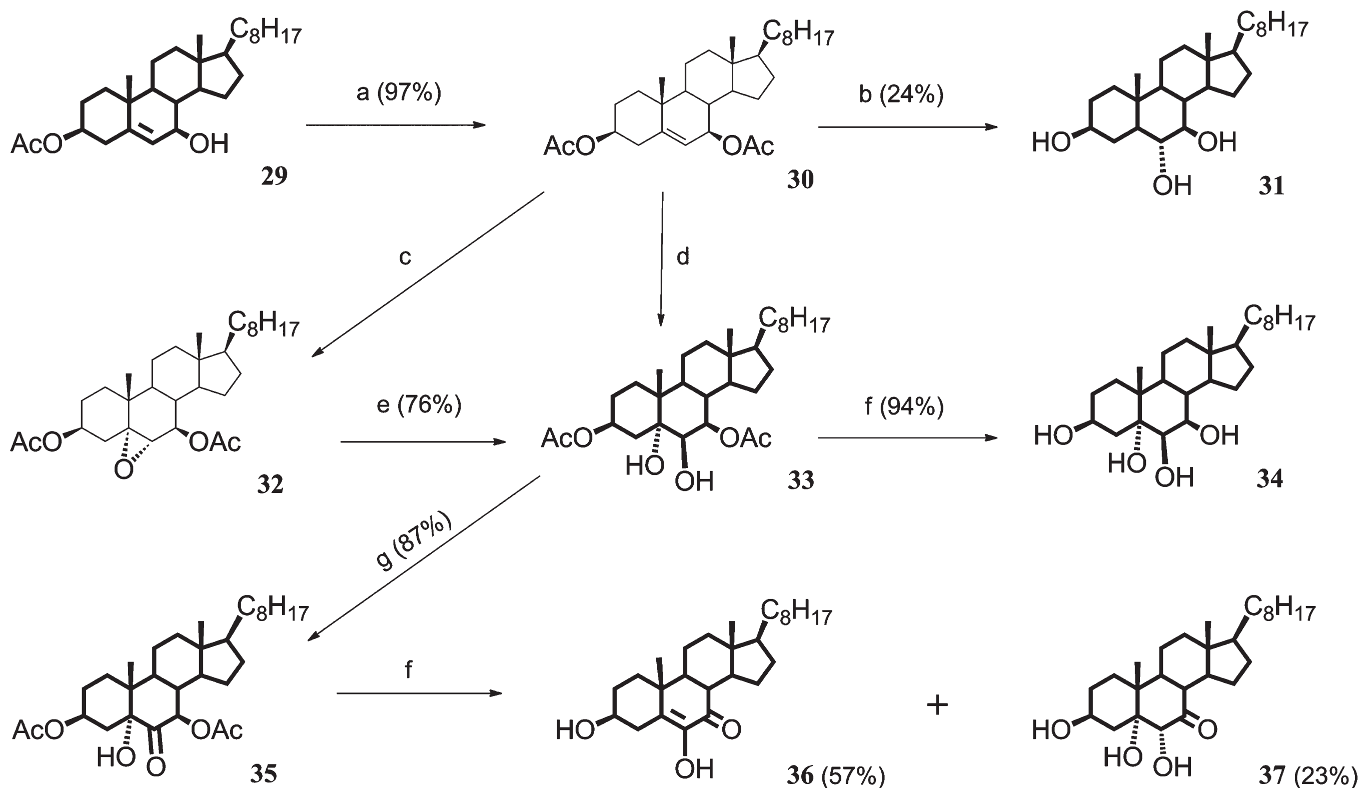


[a] (a) $Bi(OTf)_3$ (5%), MeOH, room temp, 3 h; (b) NaOH (10% aq), EtOH, CH_2Cl_2, room temp, 2 h; (c) $Bi(OTf)_3$ (5%), propanol, room temp, 12 h; (d) $Bi(OTf)_3$ (10%), CH_3CN, reflux, 3 h; (e) CH_3COONa, $NH_2OH.HCl$, pyridine, MeOH, room temp, overnight. Structures in bold have been evaluated for cytotoxicity.

Julia and Lavaux, reporting the rearrangement of a vicinal acetate via formation of an intermediary oxonium ion.[30] The cholestane-3β,4β,5α-triol **10** resulted from the alkaline hydrolysis of the acetoxy compounds **9** and **8** (Scheme 1).

We designed compounds **14** and **17** as interesting 4β-hydroxy analogues of the natural cytotoxic oxysterols **2** and **6** (Scheme 1). The synthetic route for the synthesis of tetrol **14** started with the 3β,4β-diacetoxy derivative **11**, which was submitted to our previously described epoxidation protocol,[28] affording an epoxide mixture (**12**) enriched in the α-epoxide. Next, crystallization with ethanol afforded the pure α-epoxide **12**, which was used for the next step. Hydrolysis of the pure 5α,6α-epoxycholestane-3β,4β-diyl diacetate **12** was achieved using an equimolar amount of the strong Lewis acid, $Bi(OTf)_3$. After overnight stirring at room temperature, product **13** was obtained in 79% yield. This reaction revealed once more the neighboring group participation of a

Scheme 3. Synthesis of Ring B Oxygenated Sterols and Acetyl Derivatives[a]



[a] (a) Ac_2O, DMAP, THF, room temp, 2 h; (b) (1) BH_3−THF, THF, 0 °C (5 min), room temp (overnight), (2) NaOH (10% aqueous solution), H_2O_2 (30% aqueous solution), 0 °C (5 min), room temp (2 h); (c) *m*CPBA, CH_2Cl_2, 0 °C, 48 h; (d) MMPP, CH_3CN, reflux, 250 min; (e) $HClO_4$ (7% aqueous solution), acetone, room temp, overnight; (f) NaOH (10% aqueous solution), EtOH, CH_2Cl_2, room temp, 2 h; (g) Jones reagent (CrO_3, H_2SO_4, H_2O), acetone, 0 °C. Structures in bold have been evaluated for cytotoxicity.

vicinal acetoxyl in the acid catalyzed epoxide opening reaction. Subsequent alkaline hydrolysis afforded quantitatively the desired tetrol **14** (Scheme 1).

To access triol **17**, the diacetoxy derivative **11** was submitted to allylic oxidation to introduce a carbonyl at C-7 using a known procedure involving portionwise addition of tBHP in acetonitrile at reflux, catalyzed by CuI. Because of the low reactivity of the substrate, a 5-fold amount of the hydroperoxide usually required for this kind of reaction was necessary to access intermediary **15**, which was isolated in 27% yield. Stereoselective reduction of the C-7 carbonyl using Luche conditions[31] with increased amounts of cerium chloride and sodium borohydride afforded the 7*β*-hydroxycholest-5-ene-3*β*,4*β*-diyl diacetate **16** in 75% yield. Alkaline hydrolysis under mild conditions (Na_2CO_3/MeOH) gave the desired triol **17** quantitatively after overnight reaction (Scheme 1).

By use of epoxides as useful intermediates, several transformations were performed by exploring the catalytic properties of $Bi(OTf)_3$ in the epoxide ring-opening. The derivatives **19** (74%) and **20** (21%) were easily prepared from the 3*β*-acetoxy-5,6-epoxy epimeric mixture (**18**, 76:24, α/*β* ratio) in dry methanol at room temperature. The yields obtained reflect the diastereomeric ratio of the substrate **18**. Quantitative alkaline hydrolysis of the 3*β*-acetoxy compounds **19** and **20** gave the corresponding 3*β*-alcohol analogues **21** and **22**. The same methodology was applied to the 5α,6α-epoxy isomer **23**, in the presence of dry propanol, affording the 5α-hydroxy-6*β*-propoxycholestan-3*β*-yl acetate **24**, which upon alkaline hydrolysis yielded the 6*β*-propoxycholestane-3*β*,5α-diol **25**. The 6*β*-acetamidocholestane-3*β*,5α-diol **27** was obtained in 40% yield by trans-diaxial opening of 5α,6α-epoxycholestan-3*β*-ol **26** in acetonitrile at reflux for 3 h (Scheme 2).

The synthesis of oxime **28** was easily accomplished from ketone **3** by adapting a reported method.[32] Pure (6*E*)-hydroximino derivative **28** was isolated by flash chromatography (75%), and oxime configuration was confirmed by ^{1}H NMR analysis (Scheme 2).

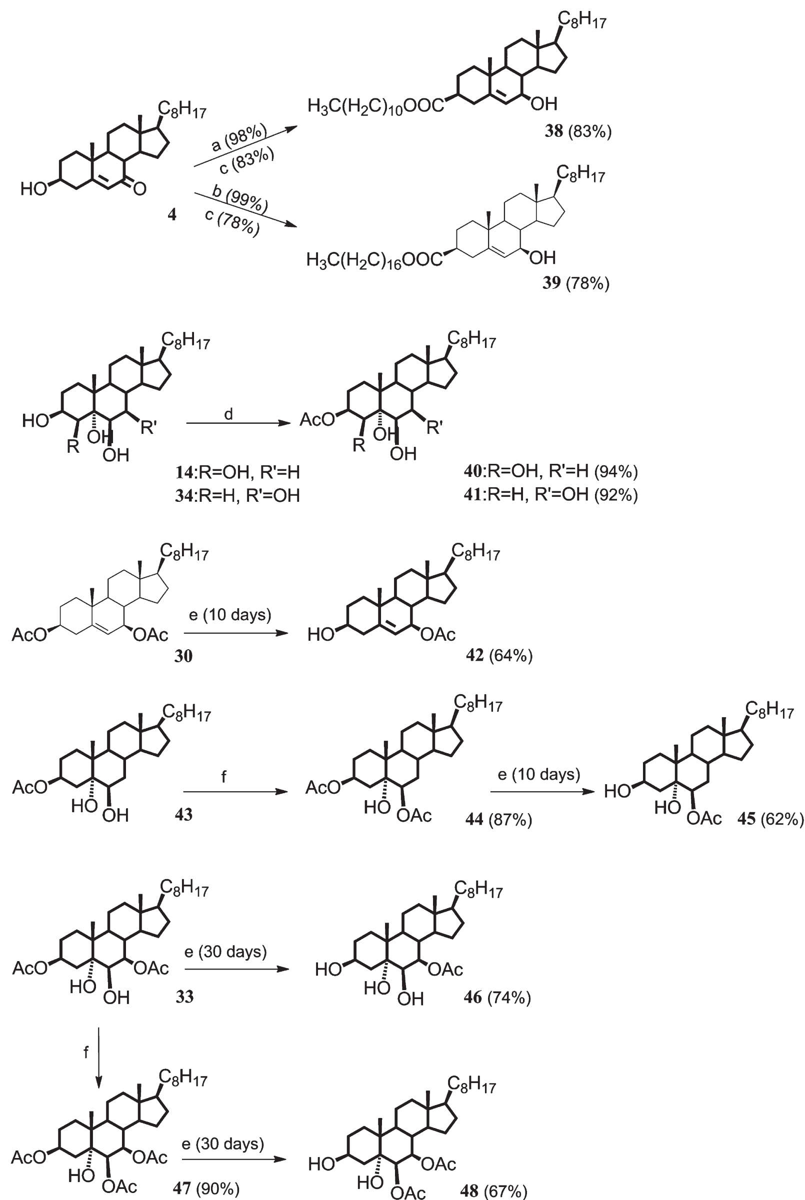
Highly polyhydroxylated sterols and their acetyl derivatives were prepared as shown in Scheme 3. The key intermediate 3*β*,7*β*-diacetoxy-5-ene **30** was obtained by acetylation of compound **29**. Hydroboration−oxidation[33] of olefin **30** led to the triol **31**, although instead of the quantitative conversion reported,[33] in our hands, a mixture of products was obtained and the desired 3*β*,6α,7*β*-triol **31** was isolated in 24% yield after careful flash chromatography.

Acidic hydrolysis (7% $HClO_4$) of the pure α-epoxydiacetoxy compound **32**, obtained from **30** by *m*-CPBA at 0 °C, gave a mixture containing the 7*β*-acetoxy **33** and the 6*β*-acetoxy counterpart in a 85:15 ratio, indicating that normal α-epoxide opening occurs followed by migration of the acetate group in acidic medium.

Nevertheless, the synthesis of diol **33** can be done directly from olefin **30**. Epoxidation of **30** with MMPP in acetonitrile at reflux, after a long reaction time (250 min), led to the formation of diol **33** in 62% yield, indicating that the epoxide formed in situ undergoes trans-diaxial epoxide opening.

The intermediate **33** yielded tetrol **34** by alkaline hydrolysis and the 6-oxo derivative **35** by Jones oxidation. Subsequent alkaline

Scheme 4. Chemoenzymatic Synthesis of Acylsterols[a]



[a] (a) Lauroyl chloride, pyridine, 0 °C, 15 min; (b) stearic anhydride, DMAP, THF, room temp, 2 h; (c) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, THF, room temp, 15 min; (d) lipase AY, vinyl acetate, toluene, 45 °C, 250 rpm, 24 h; (e) lipase AY, DIPE (aq sat.), 45 °C, 250 rpm; (f) Ac_2O, DMAP, THF, room temp, overnight. Structures in bold have been evaluated for cytotoxicity.

hydrolysis by 10% NaOH afforded, unexpectedly, two products: the 3β,6-dihydroxycholest-5-en-7-one (**36**, 57%) and the 3β,5α,6α-trihydroxycholestan-7-one (**37**, 23%). The formation of **37** should result from in situ enolization of the C-6 carbonyl and subsequent alkali-catalyzed rearrangement. The low yield (23%) is justified because, as described in literature,[34] the 7-oxotriol **37** under alkaline conditions affords the 3β,6-dihydroxycholest-5-en-7-one **36**, which seems to be the end product. Mild hydrolysis of the 6-oxodiacetoxy derivative **35** with Na_2CO_3 instead of NaOH also affords the two products **36** and **37**, after overnight stirring, with predominance of product **36**, as indicated by the ^{1}H NMR of the reaction crude.

The synthesis of acyl derivatives was performed through chemical and enzymatic reactions (Scheme 4). 3β-Lauroyl and 3β-stearoyl derivatives of 7β-hydroxycholesterol, compounds **38** and **39**, were prepared in a two-step procedure involving an acylation step (lauroyl chloride in pyridine or stearic anhydride in THF catalyzed by DMAP) followed by reduction of the C-7 carbonyl derivatives, in $CeCl_3/NaBH_4$, affording the 7β-hydroxyacyl derivatives **38** and **39** in good yields.

To further evaluate the biological relevance of the acetate substitution on ring B and keeping in mind the importance of the 3β-hydroxyl group, an enzymatic approach was pursued for the synthesis of oxysterols bearing acetyl groups at ring B and a free 3β-hydroxyl, specifically compounds **42**, **45**, **46**, and **48** (Scheme 4).

We have explored the potential of lipases in discriminating epoxide mixtures by acylation or alcoholysis of the 3β-position of the steroid template.[25,35] We have previously found that Novozym 435 is very stereoselective in the acylation of the 3β-hydroxyl of 5β,6β-epoxysterols, while the resulting 3β-acetates

were difficult to deacylate. On the other hand, lipase AY acylates preferentially the 3β-hydroxyl of 5α,6α-epoxides, even in the presence of C-7 hydroxyl groups, being also able to efficiently promote the 3β-alcoholysis reaction. Noteworthy, none of the enzymes tested are able to catalyze the acylation of hydroxyl groups at ring B.[25]

On the basis of these observations, we explored the regioselective alcoholysis reaction promoted by lipase AY, aiming the synthesis of polyacetylated oxysterols bearing a free 3β-hydroxyl group. We started the study by submitting the diacetoxy compounds **30** and **44** to alcoholysis using *n*-octanol as nucleophile, toluene as solvent, and the lipase AY as catalyst. However, the reaction rate was very low, specially for the 3β,6β-diacetoxy substrate **44**. Moving to hydrolytic conditions, using water-saturated diisopropyl ether (DIPE), we noticed an increased enzymatic activity, although high amounts of enzyme were still needed. Therefore, enzymatic hydrolysis was performed using substrates **30**, **44**, **33**, and **47** under the latter conditions and stopped when significant conversions were detected (TLC analysis). The 3β-hydroxy derivatives **42** and **45** were obtained in around 60% isolated yield after 10 days. Compounds **46** and **48** were obtained in around 70% yield after 30 days. It is worth noting that in all cases the hydrolytic reaction is very regioselective, promoting only the deacylation on the 3β-position of the sterol template.

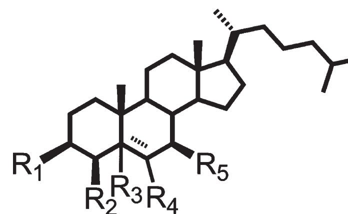
Interestingly, the reversed acylation reaction catalyzed by lipase AY proceeded in a very fast and regioselective fashion, as observed for the synthesis of the 3β-acylated polyhydroxylsterols **40** and **41** from **14** and **34**, respectively, which occurred in 1 day in the presence of lower amounts of enzyme (Scheme 4).

In Vitro Evaluation of Cytotoxicity and Structure−Activity Relationships. In the present study, 33 oxygenated sterols were evaluated in vitro for cytotoxicity in a panel of human cancer and noncancerous cells, using the Alamar Blue assay, as already described by our group.[25,26] The panel of cancer cells encompassed HT-29 (from colorectal adenocarcinoma), HepG2 (from hepatocellular carcinoma), LAMA-84 (from myeloid leukemia), A549 (from lung adenocarcinoma epithelium), PC3 (from prostate metastasis), and MCF-7 (from breast adenocarcinoma), in accordance with our previous studies. A neuroblastoma bone marrow derived cell line (SH-SY5Y) was added becaue of the role of cholesterol and a particular oxysterol, 24*S*-hydroxycholesterol, in the normal brain cellular function[36] and also because of the influence of oxysterol imbalance in neurodegenerative processes.[22] Neuroblastoma is one of the most common malignancies in childhood,[37] with generally low cure rates due to inefficient therapies as a result of the impermeable specific characteristics of the blood−brain barrier. Oxysterols, with amphiphilic properties and rapid exchange rates between membranes, are expected to cross easily the blood−brain and the brain−hematotumoral barriers and to interfere with the normal brain physiology, being potentially useful as a chemotherapeutic alternative for neuroblastoma treatment.

ARPE-19 (from retinal pigment epithelium) and BJ (a skin fibroblast cell line) were used as models of human noncancerous cells aiming at gaining new insights on the preferential cytotoxicity of the oxysterols tested against cancer cells.

A period of 48 h of drug exposure was chosen to test cytotoxicity (Tables 1−5). In order to investigate possible effects of cell metabolism on cytotoxicity, a period of 96 h was also tested (Table 4). Compounds presenting an inhibitory concentration responsible for 50% of cell death (IC_{50}) above 50 μM were considered without relevant cytotoxicity. Doxorubicin and cisplatin were used as cytotoxic drugs of reference.

Table 1. 4β-Hydroxycholesterol, 1, Related Oxysterols[a]



compd	R_1	R_2	R_3	R_4	R_5	HT-29	ARPE-19	$SI_{(ARPE-19/HT-29)}$
1	OH	OH	5-ene	H	H	>50		
8	OAc	OH	α-OH	H	H	18.6 ± 1.9	32.5 ± 4.1	1.75
9	OH	OAc	α-OH	H	H	15.2 ± 1.0	37.4 ± 5.3	2.46
10	OH	OH	α-OH	H	H	34.0 ± 7.9	-	-
14	OH	OH	α-OH	β-OH	H	9.1 ± 1.0	20.4 ± 1.4	2.24
17	OH	OH	5-ene	H	OH	15.4 ± 0.7	25.6 ± 3.9	1.66
40	OAc	OH	α-OH	β-OH	H	17.7 ± 0.8	28.8 ± 0.3	1.63

[a] IC_{50} in μM: IC_{50}, the concentration that inhibits 50% of cellular growth. Data are presented as the mean of at least three separated experiments for each cell line after 48 h of exposure. HT-29 cancer cells are from colon, and ARPE-19 normal cells are from eye. SI: selectivity index = $IC_{50(ARPE-19)}/IC_{50(HT-29)}$.

In view of our previous results,[25,26] we decided to explore strategies for recognition of a selective cytotoxic profile at rings A and B by undertaking the synthesis and evaluation of a new set of oxysterol derivatives with a wide variety of functionalities, namely, hydroxy, acetoxy, oxo, oxime, and acetamide.

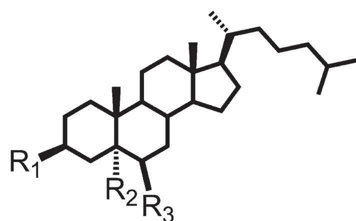
The cytotoxicity of the sterols synthesized against cancer (HT-29) and noncancer (ARPE-19) cell lines are detailed in Tables 1−3, whereas the curves of dose-dependent effects of the most representative molecules are displayed in Figure 2. The cytotoxicity of reference oxysterols **1**−**6**, previously disclosed by us,[26] was also included for comparison purposes. The cytotoxic activity of the most active oxysterols identified in Tables 1−3 was then studied against a panel of cancer (HT-29, HepG2, A549, PC3, LAMA-84, MCF-7, and SH-SY5Y) and noncancerous cells (ARPE-19 and BJ). Results are shown in Table 5.

*(a) 4β-Hydroxycholesterol, **1**, Related Oxysterols.* To gain insight on how modifications on ring A can affect cytotoxicity, HT-29 and ARPE-19 cells were incubated with four different vicinal diols, specifically cholestane-2α,3α, -2β,3β, -2α,3β-, and 3β,4α-vicinal diols previously prepared in our group.[38] Not surprisingly, no activity was observed within 48 h (data not shown). We had already reported that 4β-hydroxycholesterol, **1**, is not cytotoxic,[25] which is expected, because it is one of the most abundant oxysterols in human plasma,[27] due to its slow elimination, namely, through 7α-hydroxylation.[39] Because of its high plasma concentration, oxysterol **1** may undergo nonenzymatic oxidation similarly to cholesterol and, therefore, induce specific cellular responses through its oxidation products. Therefore, this oxysterol was used as a scaffold to design a series of related compounds.

Compared to the above-mentioned ring A vicinal diols, the presence of an additional 5α-hydroxyl group (triol **10**) led to a significant increase in activity (30 μM).

Tetrol **14**, with an additional 6β-OH compared to triol **10**, is significantly more cytotoxic against HT-29 cells, being less toxic

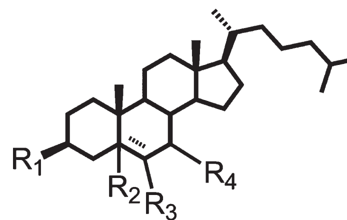
Table 2. $3\beta,5\alpha,6\beta$-Trihydroxycholestanol, 2, Related Oxysterols[a]



compd	R_1	R_2	R_3	HT-29	ARPE-19	$SI_{(ARPE\text{-}19/HT\text{-}29)}$
2	OH	OH	β-OH	12.9 ± 1.1	19.1 ± 0.9	1.48
3	OH	OH	=O	17.0 ± 0.3	33.9 ± 2.3	1.99
21	OH	OH	β-OMe	10.0 ± 1.0	25.8 ± 1.2	2.58
22	OH	OMe	β-OH	36.1 ± 5.0		
25	OH	OH	β-OPr	25.9 ± 3.5		
27	OH	OH	NHCOMe	14.5 ± 0.3	19.0 ± 3.1	1.31
28	OH	OH	=NOH	11.9 ± 0.7	23.7 ± 1.6	1.99
43	OAc	OH	β-OH	19.4 ± 2.4	>60	>3.09
44	OAc	OH	β-OAc	43.1 ± 8.4	64.1 ± 2.5	1.49
45	OH	OH	β-OAc	6.8 ± 1.4	17.9 ± 1.1	2.58

[a] IC_{50} in μM: IC_{50}, the concentration that inhibits 50% of cellular growth. Data are presented as the mean of at least three separated experiments for each cell line after 48 h of exposure. HT-29 cancer cells are from colon, and ARPE-19 normal cells are from eye. SI: selectivity index = $IC_{50(ARPE\text{-}19)}/IC_{50(HT\text{-}29)}$.

Table 3. 7β-Hydroxycholesterol, 6, Related Oxysterols[a]



compd	R_1	R_2	R_3	R_4	HT-29	ARPE-19	$SI_{(ARPE\text{-}19/HT\text{-}29)}$
4	OH	5-ene	H	=O	25.7 ± 0.2	32.3 ± 2.4	1.26
5	OH	5-ene	H	α-OH	15.5 ± 1.5		
6	OH	5-ene	H	β-OH	6.9 ± 0.9	21.6 ± 1.9	3.13
29	AcO	5-ene	H	β-OH	29.4 ± 2.2	46.5 ± 4.5	1.58
31	OH	α-H	α-OH	β-OH	14.9 ± 1.2	19.1 ± 2.6	1.28
33	OAc	α-OH	β-OH	β-OAc	20.1 ± 1.2	29.0 ± 1.2	1.44
34	OH	α-OH	β-OH	β-OH	13.9 ± 2.4	21.4 ± 0.2	1.54
35	OAc	α-OH	=O	β-OAc	27.2 ± 0.8		
36	OH	5-ene	OH	=O	36.7 ± 1.4		
37	OH	α-OH	α-OH	=O	15.2 ± 2.5	25.7 ± 4.3	1.69
38	lauroyl	5-ene	H	β-OH	>50		
41	OAc	α-OH	β-OH	β-OH	18.8 ± 4.3	30.5 ± 0.5	1.62
42	OH	5-ene	H	β-OAc	26.8 ± 6.1		
46	OH	α-OH	β-OH	β-OAc	17.0 ± 2.6	15.8 ± 0.7	0.93
47	OAc	α-OH	β-OAc	β-OAc	27.0 ± 6.7		
48	OH	α-OH	β-OAc	β-OAc	13.1 ± 1.0	11.2 ± 0.4	0.85

[a] IC_{50} in μM: IC_{50}, the concentration that inhibits 50% of cellular growth. Data are presented as the mean of at least three separated experiments for each cell line after 48 h of exposure. HT-29 cancer cells are from colon, and ARPE-19 normal cells are from eye. SI: selectivity index = $IC_{50(ARPE\text{-}19)}/IC_{50(HT\text{-}29)}$.

to noncancerous ARPE-19 cells than to HT-29 cells, and thus, the presence of a 6β-OH is beneficial for selective cytotoxicity, as we have previously noticed.[26] Tetrol **14**, being an oxidation product of the abundant oxysterol **1**, has never been detected in plasma, possiblybecause of the lack of sample reference. Moreover, the introduction of a 7β-OH into diol **1** confers cytotoxicity to the resulting compound (triol **17**) with preferential activity against cancer cells (Table 1).

*(b) $3\beta,5\alpha,6\beta$-Trihydroxycholestanol, **2**, Related Oxysterols.* The $3\beta,5\alpha,6\beta$-trihydroxylation pattern, present in the natural oxysterol **2**, has been identified as affording high cytotoxicity.[26,29] On the other hand, another endogenous oxysterol, the 6-oxo derivative **3**, is much less potent. To dissect the importance of the 5α- and 6β-hydroxyl moieties, several compounds were prepared and evaluated in HT-29 and ARPE-19 cell lines (Table 2).

The substitution of the 6β-hydroxy by a methoxy group did not interfere significantly with the cytotoxic activity, on both cancer and noncancerous cells, as observed for 6β-OMe **21**, despite a better selectivity for cancer cells was achieved. Therefore, concerning the 6β-position, modification of the hydrogen bond character from donor to acceptor and introduction of a more bulky substituent do not seem to affect the activity. However, when a bulky group is intoduced in the α-face (5α-position), a significantly less active compound, 5α-OMe **22**, was obtained. Such result is consistent with the presence of bulky substituents only on the β-face of the sterol template, particularly the C-18 and C-19 methyl groups, which reflects evolutionary optimization[40] while the presence of bulky substituents in the α-face may not be favorable for biological activity purposes. In fact, lanosterol a precursor of cholesterol, with two methyl groups on the α-face, cannot promote the formation of a liquid-ordered phase on membranes[40] and is a poor raft former.[41] Contrarily, a smooth α-face and β-methyl groups, mostly the C-18, are essential features for the remarkable ordering effect properties of cholesterol in membranes.[42] Regarding the result obtained with compound **21**, the incorporation of different bulky groups in the β-face should be explored for SAR analysis.

Replacement of the 6β-methoxy by the larger propoxy group, compound **25**, caused an almost 3-fold decrease in activity on HT-29 cells.

Introduction of a 6β-acetamide group, compound **27**, which acts as acceptor or donor for hydrogen bonds, led to a similar cytotoxic profile when compared to triol **2**.

An oxime derivative at the C-6 position was prepared, **28**, since oximes represent a very interesting functionality containing both hydrogen-bond donor and acceptor atoms and are known to possess stronger hydrogen-bonding capabilities than alcohols, phenols, and carboxylic acids.[43] Despite the fact that the oxime functionality is not very common in nature, reports on the isolation[44] and cytotoxicity evaluation of hydroximino steroids[45] stimulated the synthesis and biological studies of steroidal oxime derivatives in recent years.[46−48] The (6*E*)-oxime derivative **28** showed improved cytotoxic activity when compared to the 6-oxo analogue **3**, and a similar cytotoxic profile when compared to the 6β-counterparts, **2**, **21**, and **27** (Table 2).

*(c) 7β-Hydroxycholesterol, **6**, Related Oxysterols.* Recently, the cytotoxicity of a set of endogenous oxysterols was systematically studied in our laboratory along with other endogenous and

Table 4. Cytotoxicity of Acyl Oxysterols in HT-29 and ARPE-19 Cell Lines after 48 and 96 h of Exposure (IC_{50}[a] in μM)

position of the β-acyl group	compd	HT-29 (48 h)	HT-29 (96 h)	ARPE-19 (48 h)	ARPE-19 (96 h)
C-3	**8**	18.6 ± 1.9	17.7 ± 0.8	32.5 ± 4.1	21.8 ± 2.5
C-3	**29**	29.4 ± 2.2	12.4 ± 1.3	46.5 ± 4.5	23.6 ± 2.6
C-3	**38**	>50	>50		
C-3	**40**	17.7 ± 0.8	14.2 ± 0.6	28.8 ± 0.3	
C-3	**41**	18.8 ± 4.3	14.7 ± 1.3	30.5 ± 0.5	23.8 ± 0.7
C-3	**43**	19.4 ± 2.4	12.4 ± 0.2	>60	53.0 ± 2.5
C-3, C-7	**33**	20.1 ± 1.2	16.6 ± 1.9	29.0 ± 1.2	19.5 ± 0.6
C-3, C-6	**44**	43.1 ± 8.4	25.3 ± 6.0	64.1 ± 2.5	35.4 ± 0.6
C-3, C-6, C-7	**47**	27.0 ± 6.7	26.7 ± 6.8		
C-4	**9**	15.2 ± 1.0	18.7 ± 1.0	37.4 ± 5.3	22.3 ± 2.9
C-7	**42**	26.8 ± 6.1	24.6 ± 1.6		
C-6	**45**	6.8 ± 1.4	6.6 ± 4.8	17.9 ± 1.1	
C-7	**46**	17.0 ± 2.6	15.5 ± 1.5	15.8 ± 0.7	
C-6, C-7	**48**	13.1 ± 1.0	14.9 ± 2.6	11.2 ± 0.4	

[a] IC_{50} in μM: IC_{50}, the concentration that inhibits 50% of cellular growth. Data are presented as the mean of at least three separated experiments for each cell line after 48 h of exposure. HT-29 cancer cells are from colon, and ARPE-19 normal cells are from eye.

Table 5. Cytotoxicity[a] of a Set of Oxysterols (IC_{50} in μM), in Nine Human Cell Lines[b] after 48 h of Exposure

compd	HT-29	LAMA-84	MCF-7	SH-SY5Y	HepG2	A549	PC3	ARPE-19	BJ
2[c]	12.9 ± 1.1	5.0 ± 0.1	13.6 ± 0.8	17.2 ± 0.8	10.9 ± 1.5	17.9 ± 0.2	17.4 ± 1.3	19.1 ± 0.9	18.4 ± 0.7
6[c]	6.9 ± 0.9	4.3 ± 0.2	8.7 ± 1.7	16.4 ± 0.3	14.6 ± 2.1	13.6 ± 0.7	22.0 ± 1.7	21.6 ± 1.9	20.1 ± 0.8
10	34.0 ± 4.3				34.4 ± 3.1	31.8 ± 5.7	35.1 ± 6.2		
14	9.1 ± 1.0	5.1 ± 0.7	14.5 ± 1.0	14.5 ± 0.8	12.7 ± 1.7		17.1 ± 1.8	20.4 ± 1.4	17.8 ± 0.8
21	10.0 ± 1.0	4.3 ± 0.2			13.3 ± 0.9		17.4 ± 1.9	25.8 ± 1.2	17.4 ± 1.0
27	14.5 ± 0.3				11.6 ± 2.0	14.5 ± 0.7	19.2 ± 0.9	19.0 ± 3.1	
28	11.9 ± 0.7			16.8 ± 1.9	13.2 ± 0.4	15.0 ± 0.7	15.0 ± 0.3	23.7 ± 1.6	17.1 ± 1.4
34	13.9 ± 2.4	5.1 ± 1.0	13.1 ± 1.6	20.2 ± 1.0	13.8 ± 0.2	17.3 ± 0.8	17.4 ± 1.7	21.4 ± 0.2	19.3 ± 1.5
45	6.8 ± 0.7	3.9 ± 1.3	11.0 ± 0.2	12.2 ± 1.1	11.9 ± 1.2	13.3 ± 1.2	13.2 ± 1.2	17.9 ± 1.1	13.4 ± 1.2
48	13.1 ± 1.0	6.4 ± 0.3	13.2 ± 1.9	13.8 ± 0.3	11.7 ± 1.4	13.9 ± 0.5	10.3 ± 1.0	11.2 ± 0.4	10.2 ± 1.4
CIS[b]	13.8 ± 1.3	8.1 ± 2.9	27.0 ± 1.8	6.0 ± 1.0	9.8 ± 2.1	12.0 ± 0.9	15.9 ± 1.4	26.7 ± 0.5	15.7 ± 1.8
DOXO[b]	1.23 ± 0.4	0.74 ± 0.2	0.85 ± 0.1	0.50 ± 0.1	1.54 ± 0.1	0.95 ± 0.1	1.63 ± 0.6	0.76 ± 0.1	2.33 ± 0.2

[a] - see footnote of Table 1 [b] - **Cancer cells:** HT-29 from colon, HepG2 from liver, A549 from lung and PC3 from prostate metastasis. **Normal cells**: ARPE-19 from eye and BJ from skin. [c] - Endogenous oxysterols were previously studied[26] and are included for comparison.

synthetic oxysterols.[26] Motivated by the cytotoxicity of oxysterols **2** (Table 2) and **6**, we designed and synthesized novel oxysterols bearing both oxidation patterns (Table 3). The cytotoxicity of the endogenous 3,7-dioxygenated cholestenes **4**, **5**, and **6**, previously studied by us,[26] are included in Table 3 for comparison.

Compound **31**, bearing a 6α-hydroxyl adjacent to a 7β-hydroxyl, exhibited lower cytotoxicity than oxysterol **6**, reinforcing the importance of the 5,6-double bond in this series of compounds.

The 3β,5α,6β,7β-tetrol **34**, combining the oxidative patterns of oxysterols **2** and **6**, was not found to be more active than those compounds.

By comparison of tetrols **14** (Table 1) and **34** (Table 3) with triol **2** (Table 2), the cytotoxic profiles are similar, although **14** is more toxic and selective, revealing that the presence of an additional β-hydroxyl group at position C-4 rather than C-7 results in a stronger cytotoxic effect. Moreover, less proliferative effects on cancer cells are observed at low concentrations for tetrol **14** when compared to **34**, as evidenced by the plots of the dose-dependent effect (Figure 2).

Compounds **36** and **37**, bearing a 7-oxo functionality, display very different results according to the vicinal functionalities. While the introduction of a planar hydroxyl group into the double bond of compound **4** detracts its activity, as observed for compound **36**, the replacement of the double bond by two α-hydroxyl groups (compound **37**) increases substantially its activity as shown in Table 3.

(d) Effect of Acyl Derivatization. Cholesterol cell toxicity is known to be affected by C-3 esterification.[49] In vivo, esterification is catalyzed by acyl coenzyme A, cholesterol acyl transferase (ACAT) intracellularly or by lecithin cholesterol acyltransferase (LCAT) in plasma. Cholesteryl esters and oxysteryl esters of long-chain fatty acids, particularly oleyl esters, have been found in lipoproteins and oxidized lipoproteins, respectively. Cholesteryl esters represent the way by which cholesterol can be accumulated in cells, included in lipid droplets. On the other hand, 3β-acylation of oxysterols has been shown to impair their cytotoxicity,[50] and esterification may provide in vivo a protective mechanism against oxysterol toxicity. Therefore, we decided to study the effect of acyl derivatization on rings A and B on cytotoxicity at two incubation periods, 48 and 96 h.

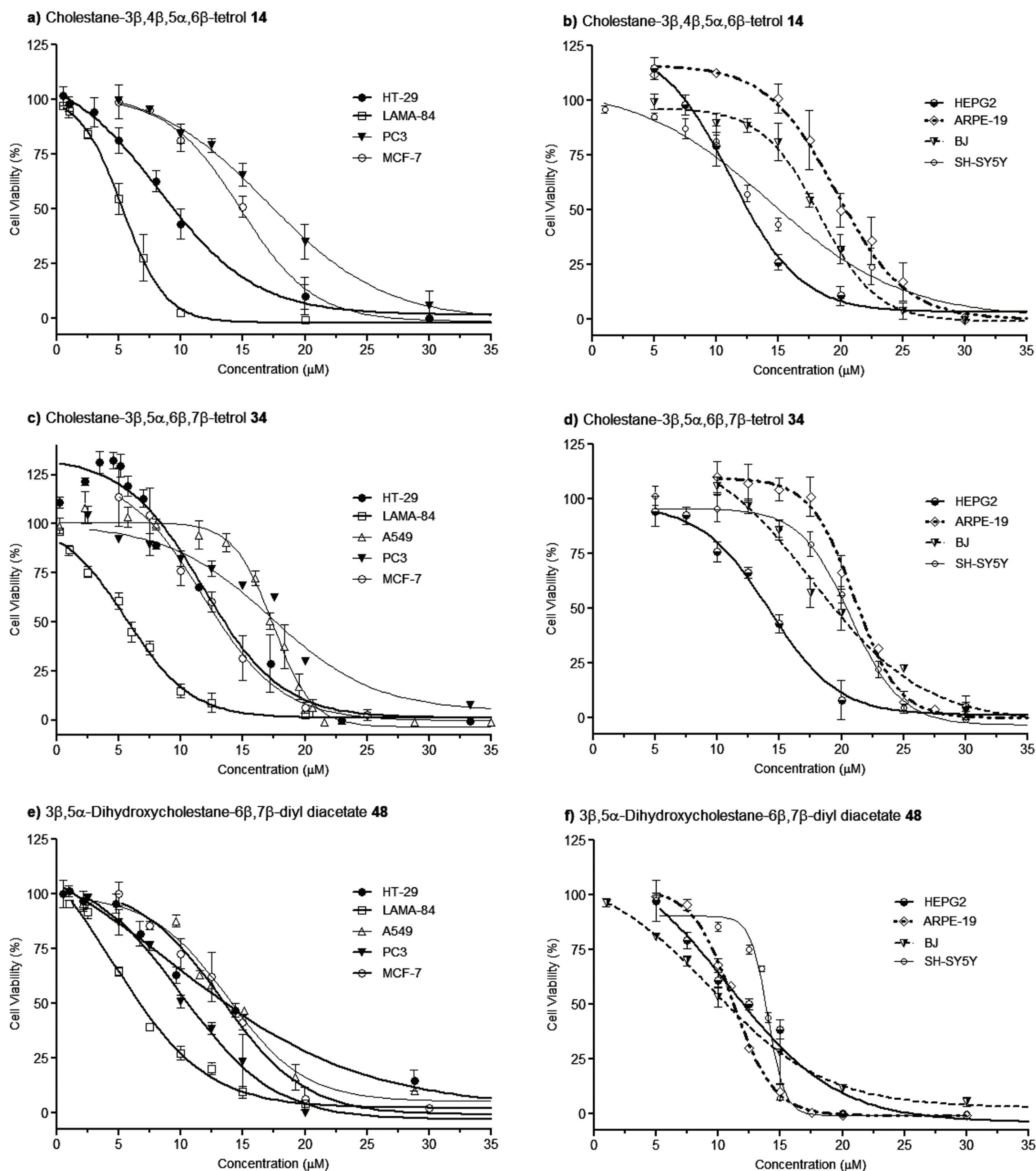


Figure 2. Dose-dependent effects of oxysterol (**14**, **34**, and **48**) on cell viability of different cell lines (cancer, HT-29, LAMA-84, A549, PC3, MCF-7, HEPG2, SH-SY5Y; noncancer, ARPE-19, BJ). Data shown are mean ± standard error of at least three independent experiments. Cancer cells and normal cells are represented by continuous and discontinuous curves, respectively.

The cytotoxicity of the diverse acyl derivatives against HT-29 and ARPE-19 cells, after 48 h of incubation, is shown in Tables 1−3. To assess the influence of cell metabolism on the activity of these derivatives, the cytotoxicity was also evaluated after 96 h (Table 4).

Three nonacylated oxysterols, **2**, **6** and **34**, were also incubated at 48 and 96 h with HT-29 cells, and no significant changes were noticed (data not shown).

3*β*-Monoacetyl derivatives of diverse oxysterols were synthesized. The 3*β*-acetyl derivative **8** is more cytotoxic than the parent triol **10**, while **40**, also bearing a 3*β*-acetyl group, is less toxic to both cell lines than the nonacylated tetrol **14** (Table 1).

The oxysterol **2** was converted in the acyl derivatives **43**, **44**, and **45**. The 3*β*-acetyl derivatives **43** and **44** afforded lower

cytotoxicities in both cell lines than the one observed for the parent triol (Table 2).

Two long chain 3β-acyl derivatives, **38** and **39**, of the cytotoxic oxysterol **6** were synthesized. Because of solubility problems, the cytotoxic evaluation of the stearoyl derivative **39** has not been determined. The lauroyl derivative **38** was deprived of toxicity, indicating that 3β-acylation with long fatty acids impairs cytotoxicity (Table 3).

The same trend was found for the 3β-acetyl derivatives **29** and **41**, when compared to the parent compounds, diol **8** and tetrol **34** (Table 3).

The cytotoxicity of these 3β-acetyl derivatives over time was then evaluated. From the results shown in Table 4, it can be observed that generally cytotoxicity is lower at 48 h but increases with the incubation time, approaching the cytotoxic activity of the parent alcohol at 96 h (3β-acetates **29**, **33**, **40**, **41**, **43**, and **44**). The exceptions are the 3β-acetoxy,4β-hydroxy derivative **8**, the 3β-lauroyl **38**, and the 3β,6β,7β-triacetylated **47**, since the cytotoxic activities remained stable for the whole time of the experiment.

In previous studies[25,26] we have shown that cytotoxic activity is strongly hampered in vitro when the 3β-hydroxyl group is masked by an acetyl function. Herein we show that cytotoxic potency can be recovered after a longer incubation period. These results suggest the involvement of a hydrolase in the removal of the 3β-acetyl groups and a steric hindrance in the access of compounds **8**, **38**, and **47** to the active site of the hydrolase. Noteworthy, the 3β-acetyl derivatives show preference for cancer cells and the selectivity is maintained over time or even improved (compound **29**). These results emphasize the potential of 3β-acetylated oxysterols as prodrugs.

Then we moved our attention to the influence of the acetoxy group on position C-4 and ring B.

The 4β- and the 6β-acetyl derivatives **9** and **45** are more toxic to HT-29 cells than the parent alcohols **10** and **2**.

On the contrary, the 7β-acetyl derivatives **42** and **46** are less toxic to HT-29 cells than the parent alcohols **6** and **34**. The 6β,7β-diacetyl derivative **48** exerts a very similar toxicity in HT-29 cells, compared to the parent tetrol **34**. However, compounds **46** and **48** are equally toxic to HT-29 and ARPE-19 cells, in contrast with **34**, which is selective to cancer cells (Table 3).

When incubating these compounds, acylated at C-4, C-6, or/and C-7 (compounds **9**, **42**, **45**, **46**, and **48**) for a longer period, no significant changes in cytotoxicity against HT-29 cells were noticed (Table 4), indicating that the overall molecule does not undergo structural changes, specifically hydrolysis reactions, which is in agreement with the preference of hydrolases for the C-3 position.

(e) Cytotoxicity of Oxysterols against a Panel of Cancer and Noncancerous Cell Lines. The best oxysterols identified in Tables 1−3 were then tested in a panel of cancer and noncancerous cell lines (Table 5). In general, the oxysterols tested were more toxic to cancer cells, specifically HT-29 and LAMA-84, being SH-SY5Y, HepG2, A549 and PC3 cells less sensitive than the other cancer cell lines. The noncancer cell lines ARPE-19 and BJ presented higher resistance to oxysterol cytotoxicity, in agreement with our previous study.[26] The MCF-7 cell line was quite sensitive to oxysterol **6** but less sensitive to the other oxysterols.

It is interesting to point out that the 6β,7β-diacetoxy derivative **48** was equally cytotoxic to cancer and noncancer cells, being the most toxic oxysterol against PC3, ARPE-19, and BJ cells. In fact, the 6β,7β-diacetoxy derivatization seems to revert the preferential toxicity of oxysterols toward cancer cells, as can be seen in Figure 2, with the shift to the left of the dose response curves (discontinuous lines) for normal cells displayed by compound **48**, when compared to the tetrols **14** and **34**.

Despite the important role of cholesterol in the central nervous system, the neuroblastoma cells (SH-SY5Y) showed resistance to the majority of the oxysterols, in contrast with other cell lines from tissues where cholesterol also plays key roles, such as HT-29 and HepG2. Interestingly, ring B acetyloxysterol derivatives **45** and **48**, exerted increased cytotoxicity, with **45** being the most active compound in this cell line. Since the central nervous system is the most cholesterol-rich organ in the body,[51] the observed resistance may be due to the condensed properties of nervous cell membranes, highly enriched in cholesterol. In parallel, prostate cancer cells are also known to possess cholesterol-rich membranes,[52] and that may explain the oxysterol resistance observed.

Noteworthy, LAMA-84 cells, derived from myeloid leukemia are quite sensitive to the oxysterols studied herein, specially to compounds **14**, **21**, **34**, and **45**, with cytotoxicities ranging from 3.9 to 5.1 μM (Table 5), which is in agreement with our previous results.[25,26]

■ CONCLUSION

By modification of the oxidation state of oxysterols, new SARs were set up throughout this work, contributing to identification of novel compounds with good cytotoxic activities and better selectivity for cancer cells. Indeed, the oxysterols studied showed a broad antiproliferative activity in the low micromolar range with increased activities on LAMA-84, HT-29, HepG2, and MCF-7 cells. We found that oxidative changes in ring A alone do not affect cytotoxicity. Of particular relevance is the enhancement of toxicity observed when the C-5 position is hydroxylated, as in compound **10**. A stronger cytotoxicity was achieved by further acetylation at either 3β- or 4β-position (compounds **8** and **9**).

Different chemical modifications involving ring B led to even higher toxicities toward cancer cells.

Tetrols **14** and **34** have shown a good cytotoxic profile, as well as the C-6 derivatives, β-methoxy **21**, β-acetoxy **45**, β-acetamido **27**, and oxime **28**.

Although less toxic than the parent alcohols, the 3β-acetyl derivatives are generally able to recover the toxicity after a longer incubation period. This effect, probably due to enzymatic hydrolysis of the ester group, points to 3β-acetates as potential prodrugs of oxysterols.

Throughout this work, we have identified oxysterol derivatives with a high selectivity index, calculated by the ratio of cytotoxicities against ARPE-19 and HT-29 cells. Specifically, compounds **9**, **14**, **21**, **28**, and **45** were 2.0- to 2.5-fold more toxic to cancer than to noncancer cells.

In summary, we have presented a detailed study that clarifies the impact of structural modifications of synthetic oxysterols on their selective cytotoxicity. The SAR analysis of the compounds studied herein is comprehensively presented in Figure 3. This study will further assist the design of more potent and selective cytotoxic oxysterols and will allow us to correlate the structural features with the biological chemistry of oxysterols.

■ EXPERIMENTAL SECTION

Chemistry. General Methods. All reagents and solvents were purchased from Sigma-Aldrich Co., with the exception of lipase AY,

4β-OAc favours selective cytotoxicity and OH decreases it

6β-OAc gives the best combination of selectivity and toxicity for this position

3β-OH is crucial for cytotoxicity and acylation delays it

7β-OH substitution provides the best selective cytotoxicity and OAc decreases it.

5α-OH contributes to cytotoxicity, while a bulky group decreases it

R, R_1, R_2, R_3, R_4

Figure 3. Graphical depiction of the general SAR for cell cytotoxicity based on the IC_{50} results on cancer cells.

which was obtained from Amano. Enzymatic reactions and compound aqueous stability studies were performed in a New Brunswick Scientific C24 incubator shaker. All commercial available chemicals were used without purification. Reactions were controlled by TLC using silica gel 60 F_{254} aluminum sheets. Reaction yields correspond to the compound isolation by flash column chromatography, performed in a Büchi automated system using a borosilicate 3.3 column and silica gel 60 (230−400 mesh ASTM). The following starting materials were prepared as described in literature: **1**, **7**, **11**, **18**, **23**, **26**,[28] **29**,[25] **43**,[29] and **3** and **4**.[26] Melting points were determined on a Buchi melting point B-540 instrument and are uncorrected. IR spectroscopy was performed on a Jasco FT/IR 420 spectrophotometer in the range of 600−4000 cm^{-1}, and compound measurements were realized as films on sodium chloride plates. ^1H, ^{13}C, and DEPT NMR spectra were recorded in a Bruker Avance instrument at 300, 400, and 500 MHz. Sample solutions were prepared in $CDCl_3$ or DMSO-d_6. Chemical shifts (δ) are given in ppm and coupling constants (J) in hertz. Deuterated solvents were used as internal reference (7.26 and 77.0 for $CDCl_3$, 2.5 and 39.4 for DMSO-d_6). Mass spectra were recorded on a Thermo Finnigan mass spectrometer, model LCQ Advantage MAX, and were obtained using the ESI method. HR mass spectra were recorded on a FTICR-MS Bruker Apex Ultra 7 T equipment. HPLC analysis, carried out on a C18 reversed-phase column (150 mm × 2.1 mm, particle size 5 μm, pore size 175 Å) at 25 °C coupled with a DAD detector from 200 to 600 nm, was used to determine the purity of final compounds. Purity was confirmed to be ≥95% for all compounds.

Synthetic Procedures. 4β,5α-Dihydroxycholestan-3β-yl Acetate (8) and 3β,5α-dihydroxycholestan-4β-yl Acetate (9). To a solution of 4,5-epoxycholestan-3β-yl acetate (**7**; α/β ratio 61:39, 200 mg, 0.500 mmol) in acetone (15 mL), $HClO_4$ (7%, aqueous solution, 161 μL, 0.112 mmol) was added. Reaction mixture was stirred for 6 h at room temperature, stopped with the addition of Et_3N and then silica gel, and evaporated under vacuum. FCC (petroleum ether, ethyl acetate 4:1 to 1:1) afforded, accordingly to the elution run, compounds **8** and **9**.

*4β,5α-Dihydroxycholestan-3β-yl Acetate (**8**).* Yield 83.3 mg, 36%. Mp 198−199.5 °C (EtOH); lit.,[30] 188−193 °C. IR (film) 3484, 2930, 2867, 1716, 1454, 1373, 1279, 1129, 1036, 955, 838, 740 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 0.65 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.17 (3H, s, 19-CH_3), 2.08 (3H, s, 3β-CH_3COO), 3.65 (1H, dd, J = 3.6, 1.0 Hz, 4α-H), 5.30 (1H, ddd, J = 12.0, 5.5, 3.6 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 15.5, 18.6, 20.4 (CH_2), 21.4, 22.1 (CH_2), 22.5, 22.8, 23.8 (CH_2), 24.1 (CH_2), 25.6 (CH_2), 27.9, 28.2 (CH_2), 30.6 (CH_2), 31.4 (CH_2), 34.5, 35.8, 36.1 (CH_2), 38.4 (C), 39.4 (CH_2), 39.8 (CH_2), 42.7 (C), 46.6, 56.1, 56.1, 72.8, 75.6 (C5), 76.4, 170.3 (CH_3COO). MS m/z (%): 463.8 (24) $[M + H]^+$, 456.5 (12), 450.1 (20), 442.3 (100), 435.2 (25), 427.6 (24), 418.8 (88), 413.8 (12), 405.0 (15), 390.8 (13), 367.4 (60), 241.2 (11).

*3β,5α-Dihydroxycholestan-4β-yl Acetate (**9**).* Yield 136.5 mg, 59%. Mp 192−193.5 °C (EtOH); lit.,[30] 190−191 °C. IR (film) 3503, 2942, 2867, 1722, 1454, 1373, 1262, 1129, 1053, 972, 937, 751 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ ppm 0.63 (3H, s, 18-CH_3), 0.84 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 1.09 (3H, s, 19-CH_3), 2.11 (3H, s, 4β-CH_3COO), 4.27 (1H, ddd, J = 10.1, 6.3, 4.0 Hz, 3α-H), 4.94 (1H, d, J = 4.0 Hz, 4α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.0, 15.3, 18.6, 20.5 (CH_2), 21.3, 22.5, 22.8, 23.8 (CH_2), 24.1 (CH_2), 25.6 (CH_2), 25.7 (CH_2), 27.9, 28.2 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 34.4, 35.8, 36.1 (CH_2), 38.5 (C), 39.4 (CH_2), 39.8 (CH_2), 42.6 (C), 46.3, 56.1, 56.1, 67.2, 75.6 (C5), 78.2, 171.6 (4β-CH_3COO). MS m/z (%): 463.6 (39) $[M + H]^+$, 457.7 (13), 453.5 (18), 441.9 (100), 433.7 (16), 418.5 (86), 403.7 (22), 400.0 (34), 385.7 (12), 101.7 (39), 65.9 (12).

Cholestane-3β,4β,5α-triol (10). To a solution of 4β,5α-dihydroxycholestan-3β-yl acetate (**8**, 40 mg, 0.086 mmol) or 3β,5α-dihydroxycholestan-4β-yl acetate (**9**, 40 mg) in a mixture of ethanol (2 mL) and dichloromethane (1 mL), NaOH (10% aqueous solution, 138 μL, 0.346 mmol) was added. The mixture was stirred at room temperature and stopped after 2 h by addition of silica gel and evaporation under vacuum. FCC (petroleum ether, ethyl acetate 1:1 to 1:2) afforded the cholestane-3β,4β,5α-triol (**10**, 35 mg, 96%). Mp 211−212 °C (EtOH/AcOEt); lit.,[30] 209−210 °C. IR (film) 3268, 2930, 2867, 1457, 1379, 1041, 956, 926, 832, 670 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/MeOD) δ ppm 0.66 (3H, s, 18-CH_3), 0.86 and 0.87 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.13 (3H, s, 19-CH_3), 1.98 (1H, m), 2.16 (1H, m), 3.43 (1H, d, J = 3.9 Hz, 4α-H), 4.07 (1H, ddd, J = 10.7, 6.4, 3.9 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$/MeOD) δ ppm 12.2, 15.6, 18.7, 20.6 (CH_2), 22.6, 22.9, 24.0 (CH_2), 24.2 (CH_2), 25.6 (CH_2), 25.7 (CH_2), 28.1, 28.4 (CH_2), 30.8 (CH_2), 31.4 (CH_2), 34.8, 35.9, 36.3 (CH_2), 38.4 (C), 39.6 (CH_2), 40.1 (CH_2), 42.8 (C), 46.7, 56.3, 56.4, 68.2, 75.8 (C5), 77.8. MS m/z (%): 419.9 (100) $[M - H]^+$, 397.5 (78), 376.7 (62), 361.2 (79), 351.3 (45), 246.7 (50), 229.1 (42), 188.8 (77), 140.9 (74).

Cholestane-3β,4β,5α,6β-tetrol (14). To a solution of 5α,6α-epoxycholestane-3β,4β-diyl diacetate (**12**, 50 mg, 0.099 mmol) in acetone (5 mL), $Bi(OTf)_3$ (65.6 mg, 0.099 mmol) was added. The reaction mixture was stirred at room temperature overnight and stopped with the addition of Et_3N until neutralization and silica gel and evaporated under vacuum and FCC (petroleum ether, ethyl acetate 10:1), affording the 4β,5α-dihydroxycholestane-3β,6β-diyl diacetate (**13**, 40.7 mg, 79%). IR (film) 3471, 2938, 2867, 1719, 1460, 1372, 1270, 1036 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ ppm 0.69 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.5 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.39 (3H, s, 19-CH_3), 2.05 and 2.06 (each 3H, 2s, 3β-CH_3COO and 6β-CH_3COO), 3.89 (1H, d, J = 3.2 Hz, 4α-H), 5.0 (1H, t, J = 2.3 Hz, 6α-H), 5.22 (1H, ddd, J = 12.3, 5.0, 3.2 Hz, 3α-H). Subsequent alkaline hydrolysis of the 4β,5α-dihydroxycholestane-3β,6β-diyl diacetate (**13**, 25 mg, 0.048 mmol) as described for the preparation of compound **10** using NaOH (10% aqueous solution, 154 μL, 0.384 mmol), afforded after overnight reaction and FCC (petroleum ether, ethyl acetate 1:3) the cholestane-3β,4β,5α,6β-tetrol (**14**, 19.9 mg, 95%). Mp 228−230 °C (EtOH); lit.,[53] 173−176 °C. IR (film) 3359, 2933, 2869, 1466, 1375, 1207, 1156, 1015, 956, 749 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$/MeOD) δ ppm 0.69 (3H, s, 18-CH_3), 0.86 and 0.87 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.91 (3H, d, J = 6.5 Hz, 21-CH_3), 1.38 (3H, s, 19-CH_3), 3.77 (1H, d, J = 3.5 Hz, 4α-H), 3.81 (1H, t, J = 2.8 Hz, 6α-H), 4.03 (1H, m, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$/MeOD) δ ppm 12.2, 15.6, 18.8, 20.5 (CH_2), 22.6, 22.9, 24.0 (CH_2), 24.3 (CH_2), 25.7 (CH_2), 28.2, 28.4 (CH_2), 30.6, 32.1 (CH_2), 34.1 (CH_2), 36.0, 36.3 (CH_2), 38.0 (C), 39.7 (CH_2), 40.1 (CH_2), 42.9 (C), 46.4, 56.2, 56.4, 68.4, 73.1 (C5), 77.6, 79.4. MS m/z (%): 435.2 (100) $[M - H]^+$, 402.9 (9), 387.2 (11), 338.3 (16). HRMS (ESI), positive mode, m/z $[M+Na]^+$ calcd for $C_{27}H_{48}O_4Na$: 459.3450, found: 459.3461.

Cholest-5-ene-3β,4β,7β-triol (17). To a mixture of cholest-5-ene-3β,4β-diyl diacetate (**11**, 300 mg, 0.616 mmol) and copper iodide (141 mg, 0.739 mmol) in CH_3CN (30 mL) at reflux temperature,

tert-butyl hydroperoxide (70% wt soln) was added portionwise (11 × 269 μL each 45 min, 30.8 mmol). After the last addition, the reaction mixture was stirred at reflux temperature overnight. Then the reaction was stopped with the addition of water. The appropriate portion was extracted with chloroform and then concentrated under vacuum. The residue was dissolved in diethyl ether and filtered under Celite to eliminate the copper iodide. The resulting organic phase was left stirring with Na_2SO_3 (10% aqueous solution) overnight, then extracted with diethyl ether, sequentially washed with $NaHCO_3$ (saturated aqueous solution) and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 8:1) afforded the 7-oxocholest-5-ene-3β,4β-diyl diacetate (**15**, 83.3 mg, 27%). Mp 231−232.5 °C (EtOH); lit.,[54] 215−225 °C. IR (film) 2950, 2867, 1751, 1730, 1673, 1466, 1375, 1254, 1216, 1170, 1051, 1018, 978 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.92 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.32 (3H, s, 19-CH_3), 2.03 and 2.09 (each 3H, 2s, 3β-CH_3COO and 4β-CH_3COO), 4.79 (1H, ddd, *J* = 12.3, 4.4, 3.5 Hz, 3α-H), 5.61 (1H, d, *J* = 3.5 Hz, 4α-H), 5.92 (1H, s, 6-H). MS *m*/*z* (%): 501.2 (94) $[M + H]^+$, 443.6 (100), 418.0 (86), 367.0 (85), 222.0 (37). Then to a solution of the 7-oxocholest-5-ene-3β,4β-diyl diacetate (**15**, 75 mg, 0.150 mmol) in THF (9 mL) and MeOH (3 mL) containing $CeCl_3 \cdot 7H_2O$ (167.7 mg, 0.450 mmol), $NaBH_4$ (56.7 mg; 1.500 mmol) was added slowly. The mixture was stirred at room temperature for 15 min, then stopped with the addition of acetone and Et_3N and concentrated under vacuum. The residue was dissolved in diethyl ether, filtered, and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (10% aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and evaporated. FCC (petroleum ether, ethyl acetate 6:1 to 4:1) afforded the 7β-hydroxycholest-5-ene-3β,4β-diyl diacetate (**16**, 56.5 mg, 75%). Mp 197−198.5 °C (EtOH). IR (film) 3442, 2936, 2861, 1747, 1721, 1457, 1370, 1267, 1224, 1123, 1038, 969, 874 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.92 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 2.00 and 2.06 (each 3H, 2s, 3β-CH_3COO and 4β-CH_3COO), 3.86 (1H, dd, *J* = 8.0, 2.2 Hz, 7α-H), 4.74 (1H, ddd, *J* = 12.5, 4.5, 3.5 Hz, 3α-H), 5.52 (1H, d, *J* = 3.5 Hz, 4α-H), 5.71 (1H, d, *J* = 2.2 Hz, 6-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 11.8, 18.7, 20.1, 20.6 (CH_2), 21.0, 21.4, 22.5 (CH_2), 22.5, 22.8, 23.8 (CH_2), 26.3 (CH_2), 28.0, 28.5 (CH_2), 35.7, 36.1 (C), 36.2 (CH_2), 36.5 (CH_2), 39.4 (CH_2), 39.5 (CH_2), 40.7 (C), 42.8, 49.0, 55.4, 55.9, 72.5, 73.1, 74.7, 133.8 (C6), 140.3 (C5), 169.9 (CH_3COO), 170.2 (CH_3COO). MS *m*/*z* (%): 503.6 (63) $[M + H]^+$, 483.3 (100), 445.2 (44), 441.4 (79), 424.5 (75), 418.7 (42), 392.9 (91), 382.0 (43), 340.5 (47), 333.8 (57), 296.8 (46), 200.3 (49), 176.0 (67). Finally, to a solution of 7β-hydroxycholest-5-ene-3β,4β-diyl diacetate (**16**, 36 mg, 0.072 mmol) in methanol (8 mL), Na_2CO_3 (30.4 mg, 0.286 mmol) was added. The mixture was stirred overnight at room temperature and stopped with addition of silica gel and evaporation under vacuum. FCC (chloroform, ethanol 15:1 to 5:1) afforded the cholest-5-ene-3β,4β,7β-triol (**17**, 28.8 mg, 96%). Mp 185.5−187.5 °C (MeOH); lit.,[55] 188−190 °C. IR (film) 3365, 2937, 2867, 1457, 1373, 1053, 972 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.63 (3H, s, 18-CH_3), 0.84 and 0.84 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.89 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.13 (3H, s, 19-CH_3), 3.26 (1H, m, 3α-H), 3.58 (1H, ddd, *J* = 8.0, 6.8, 2.5 Hz, 7α-H), 3.86 (1H, dd, *J* = 3.6, 2.7 Hz, 4α-H), 4.29 (1H, d, *J* = 6.8 Hz, 7-OH), 4.33 (1H, d, *J* = 2.7 Hz, 4-OH), 4.45 (1H, d, *J* = 5.3 Hz, 3-OH), 5.34 (1H, d, *J* = 2.5 Hz, 6-H). ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 11.6, 18.6, 20.2 (CH_2), 20.2, 22.3, 22.6, 23.2 (CH_2), 25.0 (CH_2), 26.0 (CH_2), 27.3, 28.1 (CH_2), 35.2, 35.5 (C), 35.7 (CH_2), 37.0 (CH_2), 38.9 (CH_2), 39.1 (CH_2), 39.7, 42.2 (C), 48.8, 55.0, 56.0, 71.4, 71.7, 75.9, 131.1 (C6), 143.2 (C5). MS *m*/*z* (%): 417.6 (100) $[M - H]^+$, 352.4 (20), 325.4 (36), 265.4 (51).

5α-Hydroxy-6β-methoxycholestan-3β-yl Acetate (19) and 6β-hydroxy-5α-methoxycholestan-3β-yl Acetate (20). To a solution of 5,6-epoxycholestan-3β-yl acetate (**18**; α/β ratio 76:24, 300 mg, 0.675 mmol) in dry methanol (15 mL), $Bi(OTf)_3$ (22.2 mg, 0.034 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. The reaction was stopped with addition of a drop of Et_3N and silica gel. The mixture was evaporated under vacuum, and subsequent FCC (petroleum ether, ethyl acetate 8:1) afforded, accordingly to the elution run, the 5α-hydroxy-6β-methoxycholestan-3β-yl acetate (**19**, 237 mg, 74%). Mp 142.5−144 °C (EtOH). IR (film) 3449, 2936, 2867, 1738, 1713, 1468, 1381, 1364, 1264, 1246, 1165, 1097, 1030, 957, 758 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.89 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.08 (3H, s, 19-CH_3), 2.02 (3H, s, 3β-CH_3COO), 2.18 (1H, dd, *J* = 12.9, 11.8 Hz), 2.94 (1H, t, *J* = 2.8 Hz, 6α-H), 3.26 (3H, s, 6β-OCH_3), 5.13 (1H, tt, *J* = 11.1, 5.5 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 16.2, 18.6, 21.0 (CH_2), 21.5, 22.5, 22.8, 23.9 (CH_2), 24.2 (CH_2), 26.6 (CH_2), 28.0, 28.2 (CH_2), 29.1 (CH_2), 30.5, 31.8 (CH_2), 35.8 (C), 36.1 (CH_2), 37.1 (CH_2), 38.5, 39.5 (CH_2), 39.9 (CH_2), 42.7 (C), 45.5, 55.8, 56.2, 57.8, 71.3, 75.9 (C5), 85.7, 170.9 (CH_3COO). Also produced was 6β-hydroxy-5α-methoxycholestan-3β-yl acetate (**20**, 68 mg, 21%). IR (film) 3468, 2938, 2867, 1729, 1712, 1468, 1381, 1363, 1239, 1160, 1077, 1028, 961, 737 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.89 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.19 (3H, s, 19-CH_3), 2.03 (3H, s, 3β-CH_3COO), 3.21 (3H, s, 5α-OCH_3), 3.88 (1H, t, *J* = 2.8 Hz, 6α-H), 4.87 (1H, tt, *J* = 10.6, 5.3 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 17.6, 18.6, 21.0 (CH_2), 21.4, 22.5, 22.8, 23.8 (CH_2), 24.2 (CH_2), 26.6 (CH_2), 28.0, 28.2 (CH_2), 29.8 (CH_2), 29.9, 31.9 (CH_2), 34.4 (CH_2), 35.8, 36.1 (CH_2), 39.4 (C), 39.5 (CH_2), 39.9 (CH_2), 42.7 (C), 44.6, 48.4, 55.8, 56.2, 69.9, 71.0, 78.6 (C5), 170.8 (CH_3COO).

6β-Methoxycholestane-3β,5α-diol (21). To a solution of 5α-hydroxy-6β-methoxycholestan-3β-yl acetate (**19**, 197 mg, 0.413 mmol) in a mixture of ethanol (4 mL) and dichloromethane (2 mL), NaOH (10% aqueous solution, 661 μL, 1.653 mmol) was added. The mixture was stirred at room temperature and stopped after 2 h by evaporation under vacuum. The white residue was dissolved in ethyl acetate (saturated aqueous solution) and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum, affording the 6β-methoxycholestane-3β,5α-diol (**21**, 178 mg, 99%). Mp 152−153 °C (MeOH); lit.,[56] 150.5 °C. IR (film) 3398, 2935, 2867, 1468, 1381, 1212, 1164, 1095, 1037, 1009, 956, 760 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.89 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.07 (3H, s, 19-CH_3), 2.96 (1H, t, *J* = 2.8 Hz, 6α-H), 3.28 (3H, s, 6β-OCH_3), 4.08 (1H, tt, *J* = 10.5, 5.2 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 16.3, 18.6, 21.1 (CH_2), 22.5, 22.8, 23.8 (CH_2), 24.1 (CH_2), 28.0, 28.2 (CH_2), 29.3 (CH_2), 30.5, 30.7 (CH_2), 32.1 (CH_2), 35.8, 36.1 (CH_2), 38.4 (C), 39.5 (CH_2), 39.9 (CH_2), 40.8 (CH_2), 42.7 (C), 45.9, 55.9, 56.2, 57.9, 67.8, 76.3 (C5), 85.5. MS *m*/*z* (%): 435.2 (94) $[M + H]^+$, 415.9 (11), 392.6 (22), 360.6 (100), 298.5 (31), 167.4 (17).

5α-Methoxycholestane-3β,6β-diol (22). Alkaline hydrolysis of the 6β-hydroxy-5α-methoxycholestan-3β-yl acetate (**20**, 50 mg, 0.105 mmol), as described for the preparation of compound **21**, afforded after aqueous workup the 5α-methoxycholestane-3β,6β-diol (**22**, 44.7 mg, 98%). Mp 204−205 °C (MeOH); lit.,[57] 198−199 °C. IR (film) 3373, 2933, 2867, 1455, 1381, 1339, 1268, 1165, 1123, 1077, 1036, 957, 857 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, *J* = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, *J* = 6.5 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 3.14 (3H, s, 5α-OCH_3), 3.81 (1H, tt, *J* = 10.8, 5.2 Hz, 3α-H), 3.88 (1H, t, *J* = 2.8 Hz, 6α-H).

^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 12.2, 17.7, 18.7, 21.1 (CH_2), 22.5, 22.8, 23.9 (CH_2), 24.2 (CH_2), 28.0, 28.3 (CH_2), 30.0, 31.2 (CH_2), 32.2 (CH_2), 33.5 (CH_2), 34.4 (CH_2), 35.8, 36.2 (CH_2), 39.4 (C), 39.5 (CH_2), 40.0 (CH_2), 42.8 (C), 44.7, 48.1, 55.9, 56.3, 67.7, 70.0, 78.9 (C5). MS m/z (%): 435.8 (13) $[M + H]^+$, 418.6 (65), 403.1 (12), 384.7 (32), 367.9 (100), 334.7 (12), 301.7 (14), 256.1 (15).

6*β*-Propoxycholestane-3*β*,5α-diol (25). Alcoholysis of 5α,6α-epoxycholestan-3*β*-yl acetate (**23**, 70 mg, 0.157 mmol) by $Bi(OTf)_3$ (5.2 mg, 7.87×10^{-3} mmol) and dry propanol (6 mL) under N_2 afforded, after the mixture was stirred for 12 h at room temperature, aqueous workup, and FCC (petroleum ether, ethyl acetate 15:1), the 5α-hydroxy-6*β*-propoxycholestan-3*β*-yl acetate (**24**, 74 mg, 93%). Mp 112−113 °C (EtOH). IR (film) 3446, 2936, 2869, 1739, 1713, 1467, 1377, 1246, 1165, 1093, 1031, 958, 874, 759, 670 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (d, J = 6.5 Hz, 1H), 0.91 (3H, t, J = 7.4 Hz, CH_3), 1.12 (3H, s, 19-CH_3), 2.03 (3H, s, 3β-CH_3COO), 2.21 (1H, dd, J = 13.1, 11.6 Hz), 3.02 (1H, t, J = 2.8 Hz, 6α-H), 3.15 and 3.43 (each 1H, 2td, J = 9.0, 6.3, 6.3 Hz, OCH_2), 5.14 (1H, tt, J = 10.9, 5.4 Hz, 3α-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 11.0, 12.1, 16.3, 18.6, 21.1 (CH_2), 21.5, 22.5, 22.8, 23.4 (CH_2), 23.9 (CH_2), 24.2 (CH_2), 26.7 (CH_2), 28.0, 28.2 (CH_2), 29.8 (CH_2), 30.6, 31.9 (CH_2), 35.8, 36.2 (CH_2), 37.2 (CH_2), 38.5 (C), 39.5 (CH_2), 39.9 (CH_2), 42.7 (C), 45.7, 55.9, 56.2, 71.3, 71.6 (CH_2), 76.1 (C5), 83.6, 170.8 (CH_3COO). Successive alkaline hydrolysis of 5α-hydroxy-6*β*-propoxycholestan-3*β*-yl acetate (**24**, 55 mg, 0.109 mmol), as described for the preparation of compound **21**, afforded after aqueous workup and FCC (petroleum ether, ethyl acetate 4:1 to 2:1) the desired 6*β*-propoxycholestane-3*β*,5α-diol (**25**, 48 mg, 95%). Mp 122−124 °C (EtOH). IR (film) 3396, 2934, 2867, 1468, 1379, 1164, 1092, 1036, 1007, 955, 874, 757 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 0.91 (3H, t, J = 7.6 Hz, CH_3), 1.11 (3H, s, 19-CH_3), 1.98 (1H, m), 2.14 (1H, dd, J = 12.9, 11.6 Hz), 3.03 (1H, t, J = 2.7 Hz, 6α-H), 3.17 and 3.44 (each 1H, 2td, J = 8.9, 6.3, 6.3 Hz, OCH_2), 4.06 (1H, tt, J = 10.9, 5.3 Hz, 3α-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 11.0, 12.1, 16.5, 18.6, 21.1 (CH_2), 22.5, 22.8, 23.4 (CH_2), 23.8 (CH_2), 24.2 (CH_2), 28.0, 28.2 (CH_2), 29.9 (CH_2), 30.6, 30.9 (CH_2), 32.2 (CH_2), 35.8, 36.2 (CH_2), 38.4 (C), 39.5 (CH_2), 40.0 (CH_2), 40.9 (CH_2), 42.7 (C), 46.1, 56.0, 56.2, 67.8, 71.7 (CH_2), 76.4 (C5), 83.5. MS m/z (%): 463.7 (44) $[M + H]^+$, 441.6 (99), 428.8 (30), 420.6 (25), 385.2 (30), 323.9 (79), 265.7 (25), 240.2 (41), 102.2 (100). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{30}H_{54}O_3Na$, 485.3971; found, 485.3988.

6*β*-Acetamidocholestane-3*β*,5α-diol (27). To a solution of 5α,6α-epoxycholestan-3*β*-ol (**26**, 100 mg, 0.248 mmol) in acetonitrile (4 mL), $Bi(OTf)_3$ (16.4 mg, 0.025 mmol) was added. The reaction mixture was stirred at reflux temperature for 3 h, stopped with a drop of Et_3N and silica gel, evaporated under vacuum and careful FCC (petroleum ether, ethyl acetate 1:1 to 1:3), affording the 6*β*-acetamidocholestane-3*β*,5α-diol (**27**, 45.8 mg, 40%). Mp 266.5−268 °C (EtOH); lit.,[58] 257 −260 °C. IR (film) 3436, 2937, 2867, 1656, 1519, 1466, 1373, 1164, 1089, 1036, 1007, 966, 878, 734 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.89 (3H, d, J = 6.5 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 1.99 (3H, s, CH_3CO), 4.09 (2H, m, 3-H and 6-H), 5.68 (1H, d, J = 9.9 Hz, 6-NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.2, 18.0, 18.6, 21.0 (CH_2), 22.5, 22.8, 23.8, 23.9 (CH_2), 24.2 (CH_2), 28.0, 28.2 (CH_2), 30.2 (CH_2), 31.0, 31.8 (CH_2), 32.9 (CH_2), 35.8, 36.1 (CH_2), 38.5 (C), 39.5 (CH_2), 39.8 (CH_2), 40.8 (CH_2), 42.7 (C), 44.9, 54.0, 55.5, 56.1, 67.2, 75.8 (C5), 169.6 (CH_3CO). MS m/z (%): 460.6 (57) $[M - H]^+$, 338.7 (47), 327.6 (49), 313.8 (41), 298.4 (42), 277.7 (44), 96.6 (100) .

6*E*-Hydroximinocholestane-3β,5α-diol (28). To a solution of 3,5-dihydroxycholestan-6-one (**3**, 70 mg, 0.167 mmol) in pyridine (10 mL) and MeOH (1 mL) containing sodium acetate (54.9 mg, 0.669 mmol), hydroxylamine hydrochloride (46.5 mg, 0.669 mmol) was added. The mixture was stirred overnight at room temperature. Then the reaction mixture was evaporated under vacuum and the residue dissolved in ethyl acetate (aqueous, saturated) and sequentially washed with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 1:1 to 1:3) afforded the 6*E*-hydroximinocholestane-3β,5α-diol (**28**, 54.3 mg, 75%). Mp 237−238 °C (EtOH); lit.,[59] 227−229 °C. IR (film) 3378, 2937, 2867, 1468, 1372, 1048, 1010, 990, 940, 911, 704 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.60 (3H, s, 18-CH_3), 0.69 (3H, s, 19-CH_3), 0.84 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 2.93 (1H, dd, J = 13.3, 4.7 Hz, 4β-H), 3.76 (1H, m, 3α-H), 4.24 (1H, d, J = 5.6 Hz, 3-OH), 4.61 (1H, s, 5-OH), 10.36 (1H, s, NOH); ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 11.8, 13.9, 18.4, 20.9 (CH_2), 22.3, 22.6, 23.1 (CH_2), 23.6 (CH_2), 24.4 (CH_2), 27.3, 27.7 (CH_2), 29.6 (CH_2), 30.6 (CH_2), 34.4, 35.1, 35.5 (CH_2), 38.1 (CH_2), 38.8 (CH_2), 39.4 (CH_2), 40.4 (C), 42.4 (C), 44.1, 55.5, 55.8, 65.3 (C3), 75.5 (C5), 159.9 (C6). MS m/z (%): 432.7 (74) $[M - H]^+$, 374.0 (73), 295.4 (100), 264.9 (68), 172.3 (88). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{27}H_{47}NO_3Na$, 456.3448; found, 456.3438.

Cholest-5-ene-3*β*,7*β*-diyl Diacetate (30). To a solution of 7β-hydroxycholest-5-en-3β-yl acetate (**29**, 1000 mg, 2.249 mmol) and 4-dimethylaminopyridine (DMAP, 125.5 mg, 1.027 mmol) in THF (11 mL), acetic anhydride 98.5% (394 μL, 4.108 mmol) was added. The reaction mixture was stirred at room temperature for 2 h, stirred with MeOH for 30 min, then concentrated under vacuum. The residue was dissolved in diethyl ether. The resulting organic phase was stirred with NH_4Cl (saturated aqueous solution) for 1 h and then washed sequentially with $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtered, and evaporated. FCC (petroleum ether, ethyl acetate 20:1 to 9:1) yielded the cholest-5-ene-3β,7β-diyl diacetate (**30**, 1061 mg, 97%). IR (film) 2949, 2870, 1736, 1467, 1371, 1238, 1139, 1021, 950, 757 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.85 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.07 (3H, s, 19-CH_3), 2.01 (3H, s, CH_3COO), 2.02 (3H, s, CH_3COO), 4.59 (1H, m, 3α-H), 5.02 (1H, td, J = 8.7, 1.8, 1.8 Hz, 7α-H), 5.23 (1H, t, J = 1.8 Hz, 6-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 11.7, 18.7, 19.0, 21.0 (CH_2), 21.3, 21.6, 22.5, 22.8, 23.7 (CH_2), 25.1 (CH_2), 27.6 (CH_2), 28.0, 28.3 (CH_2), 35.6, 36.1 (CH_2), 36.4, 36.5 (C), 36.5 (CH_2), 37.5 (CH_2), 39.3 (CH_2), 39.4 (CH_2), 42.8 (C), 48.0, 55.3, 55.4, 73.2, 75.5, 122.2 (C6), 144.2 (C5), 170.3 (CH_3COO), 171.1 (CH_3COO). MS m/z (%): 485.4 (85) $[M - H]^+$, 468.2 (54), 439.8 (37), 326.2 (100), 293.3 (71).

Cholestane-3*β*,6α,7*β*-triol (31). To a solution of cholest-5-ene-3β,7β-diyl diacetate (**30**, 220 mg, 0.452 mmol) in dry THF (10 mL) cooled to 0 °C, the BH_3−THF complex (1 M in THF, 4.5 mL, 4.5 mmol) was added dropwise. The mixture was stirred overnight at room temperature. The reaction mixture was cooled to 0 °C, and NaOH (10% aqueous solution, 4.5 mL) was added followed by H_2O_2 (30% aqueous solution, 4.5 mL). The mixture was stirred vigorously for 6 h. Then the mixture was neutralized with HCl (5% aqueous solution) and evaporated under vacuum. Aqueous phase was extracted with ethyl acetate (aqueous, saturated). To the resulting organic phase, Na_2SO_3 (10% aqueous solution) was added. The mixture was stirred for 2 h, the organic phase separated and then washed with water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 1:1 to 1:4) afforded the cholestane-3β,6α,7β-triol (**31**, 45.6 mg, 24%). Mp 230−231 °C (AcOEt); lit.,[60] 230.5−231.5 °C. IR (film) 3337, 2932, 2867, 1471, 1379, 1089, 1053, 983, 949, 868, 681 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.64 (3H, s, 18-CH_3), 0.81 (3H, s, 19-CH_3), 0.82 and 0.83 (each 3H, 2d, J = 6.6 Hz,

26-CH_3 and 27-CH_3), 0.87 (3H, d, J = 6.5 Hz, 21-CH_3), 1.96 (1H, m), 2.05 (1H, m), 3.01 (1H, dd, J = 9.6, 8.6 Hz), 3.15 (1H, dd, J = 10.6, 8.6 Hz), 3.49 (1H, m, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 13.5, 18.7, 21.3 (CH_2), 22.4, 22.7, 23.8 (CH_2), 26.7 (CH_2), 27.9, 28.5 (CH_2), 30.4 (CH_2), 32.0 (CH_2), 35.5 (C), 35.6, 36.1 (CH_2), 37.2 (CH_2), 39.4 (CH_2), 39.7 (CH_2), 40.8, 43.4 (C), 47.5, 52.0, 55.2, 55.8, 70.7, 74.5, 80.3. MS m/z (%): 421.6 (100) $[M + H]^+$, 411.6 (39), 398.6 (22), 396.0 (18), 387.0 (17), 367.2 (18), 363.6 (20), 316.3 (19), 263.6 (45), 241.2 (25), 187.7 (29).

5α,6α-Epoxycholestane-3*β*,7*β*-diyl Diacetate (32). To a solution of cholest-5-ene-3β,7β-diyl diacetate (**30**, 200 mg, 0.411 mmol) in dichloromethane (20 mL), *m*CPBA 77% (425.5 mg, 2.465 mmol) was added. The mixture was left at 0 °C until complete substrate consumption. After 2 days, the reaction was stopped with the addition of Et_3N and solvent was evaporated under vacuum. The resulting residue was dissolved with ethyl acetate and washed sequentially with Na_2SO_3 (10% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 8:1, with 0.1% of Et_3N) allowed the isolation of a mixture of epoxides in 91% yield (188.0 mg, α/β ratio 93:7). A second FCC (petroleum ether, ethyl acetate 20:1 to 8:1) afforded the pure 5α,6α-epoxycholestane-3β,7β-diyl diacetate (**32**, 142.6 mg, 69%). IR (film) 2926, 2855, 1738, 1457, 1367, 1235, 1030, 803, 728, 668 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.63 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 1.13 (3H, s, 19-CH_3), 2.01 (3H, s, CH_3COO), 2.06 (3H, s, CH_3COO), 2.17 (1H, dd, J = 12.8, 11.8 Hz), 2.74 (1H, s, 6β-H), 4.75 (1H, d, J = 8.1 Hz, 7α-H), 4.91 (1H, tt, J = 11.5, 4.9 Hz, 3α-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 11.8, 15.9, 18.7, 20.5 (CH_2), 21.3, 21.3, 22.5, 22.8, 23.8 (CH_2), 24.2 (CH_2), 27.1 (CH_2), 28.0, 28.1 (CH_2), 32.0 (CH_2), 34.7 (C), 35.4 (CH_2), 35.7, 35.8, 36.1 (CH_2), 39.1 (CH_2), 39.4 (CH_2), 39.4, 42.7 (C), 55.4, 55.5, 59.9, 65.1 (C5), 71.0, 71.9, 170.1 (CH_3COO), 170.3 (CH_3COO). MS m/z (%): 502.1 (57) $[M - H]^+$, 440.4 (61), 364.7 (31), 359.1 (100), 339.1 (66), 326.4 (23), 124.3 (70), 69.1 (65).

5α,6*β*-Dihydroxycholestane-3*β*,7*β*-diyl Diacetate (33). *Method 1.* To a solution of 5α,6α-epoxycholestane-3β,7β-diyl diacetate (**32**, 120 mg, 0.239 mmol) in acetone (11.9 mL), $HClO_4$ (7% aqueous solution, 85.7 μL, 0.0598 mmol) was added. The mixture was stirred at room temperature overnight, stopped by neutralization with Et_3N, and evaporated under vacuum. The resulting white residue was dissolved with ethyl acetate, filtered and the organic phase washed with water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 1:2 to 1:4) afforded a white solid (114.5 mg, 92%). ^{1}H NMR analysis revealed a mixture (85:15) of the 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**) and 5α,7β-dihydroxycholestane-3β,6β-diyl diacetate. Crystallization from acetone afforded the pure 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**, 63.5 mg, 51%). Mp 200−201 °C (acetone). IR (film) 3463, 2951, 2867, 1738, 1712, 1466, 1375, 1265, 1141, 1077, 1028, 891, 758 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.69 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.18 (3H, s, 19-CH_3), 2.02 (3H, s, CH_3COO), 2.06 (3H, s, CH_3COO), 2.21 (1H, dd, J = 12.6, 11.1 Hz), 3.63 (1H, dd, J = 3.7, 2.5 Hz, 6α-H), 5.13 (1H, m, 3α-H), 5.14 (1H, dd, J = 10.8, 3.7 Hz, 7α-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 17.1, 18.7, 21.1 (CH_2), 21.5, 21.8, 22.5, 22.8, 23.8 (CH_2), 25.9 (CH_2), 26.6 (CH_2), 28.0, 28.6 (CH_2), 32.0 (CH_2), 35.0, 35.6, 36.1 (CH_2), 37.3 (CH_2), 37.7 (C), 39.4 (CH_2), 39.7 (CH_2), 43.5 (C), 44.1, 54.4, 55.2, 71.0, 75.7, 76.2 (C5), 76.4 (s,1C), 170.9 (CH_3COO), 171.1 (CH_3COO). MS m/z (%): 519.3 (90) $[M - H]^+$, 494.6 (43), 465.0 (50), 446.9 (58), 316.9 (100), 248.9 (44).

Method 2. To a solution of cholest-5-ene-3β,7β-diyl diacetate (**30**, 100 mg, 0.205 mmol) in acetonitrile (4.1 mL), MMPP (202.8 mg, 0.410 mmol) was added. The mixture was stirred at reflux temperature for 250 min and stopped by evaporation under vacuum. The resulting white residue was dissolved with ethyl acetate, filtered and the resulting organic phase washed sequentially with Na_2SO_3 (10% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 1:1 to 1:4) afforded a mixture of 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**) and 5α,7β-dihydroxycholestane-3β,6β-diyl diacetate, as revealed by ^{1}H NMR (75:25, 88.6 mg, 83%). 5α,7β-Dihydroxycholestane-3β,6β-diyl diacetate (chemical shifts depicted from mixture): ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.69 (3H, s, 18-CH_3), 0.85 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.11 (3H, s, 19-CH_3), 2.00 (3H, s, CH_3COO), 2.12 (3H, s, CH_3COO), 4.01 (1H, dd, J = 9.8, 4.2 Hz, 7α-H), 4.98 (1H, d, J = 4.2 Hz, 6α-H), 5.14 (1H, m, 3-H).

Cholestane-3*β*,5α,6*β*,7*β*-tetrol (34). Alkaline hydrolysis of the 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**, 90 mg, 0.173 mmol), as described for the preparation of compound **21**, using NaOH (10% aqueous solution, 554 μL, 1.384 mmol) afforded after FCC (chloroform, ethanol 15:1 to 10:1) the cholestane-3β,5α,6β,7β-tetrol (**34**, 70.9 mg, 94%). Mp 226−227 °C (EtOH); lit.,[61] 219−222 °C. IR (film) 3396, 2936, 2867, 1466, 1385,1135, 1083, 1041, 966, 897, 803, 757, 693 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.61 (3H, s, 18-CH_3), 0.84 and 0.84 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 3.22 (1H, t, J = 4.0, 3.4 Hz, 6α-H), 3.49 (1H, brs, OH), 3.52 (1H, m, 7α-H), 3.79 (1H, m, 3α-H), 3.84 (1H, s, 5-OH), 4.21 (1H, d, J = 5.7 Hz, OH), 4.39 (1H, d, J = 4.0 Hz, 6-OH). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm 12.0, 16.7, 18.6, 20.8 (CH_2), 22.3, 22.6, 23.3 (CH_2), 26.8 (CH_2), 27.4, 28.3 (CH_2), 31.0 (CH_2), 32.1 (CH_2), 35.3, 35.7 (CH_2), 37.2 (C), 37.9, 38.9 (CH_2), 39.8 (CH_2), 41.0 (CH_2), 43.0 (C), 43.3, 55.0, 55.4, 65.4, 71.4, 75.2 (C5), 77.5. MS m/z (%): 437.3 (100) $[M + H]^+$, 419.7 (52), 408.2 (36), 370.5 (71), 287.9 (46), 59.9 (26). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{27}H_{48}O_4Na$, 459.3450; found, 459.3473.

5α-Hydroxy-6-oxocholestane-3*β*,7*β*-diyl Diacetate (35). To a solution of 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**, 180 mg, 0.346 mmol) in acetone (15 mL) at 0 °C, Jones reagent[62] was added dropwise until the reaction medium became brown-red. After total substrate consumption (confirmed by TLC), reaction was stopped with the addition of methanol. The mixture was neutralized with Et_3N and then evaporated under vacuum. The residue was dissolved in ethyl acetate and washed sequentially with Na_2SO_3 (10% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum to yield an oily crude product. FCC (petroleum ether, ethyl acetate 5:1 to 1:1) afforded the 5α-hydroxy-6-oxocholestane-3β,7β-diyl diacetate (**35**, 156 mg, 87%). IR (film) 3389, 2952, 2872, 1735, 1716, 1460, 1374, 1243, 1106, 1077, 1053, 1030, 960, 757, 722 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.65 (3H, s, 18-CH_3), 0.78 (3H, s, 19-CH_3), 0.85 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 2.01 and 2.12 (each 3H, 2s, 3β- and 7β-CH_3COO), 5.08 (1H, m, 3α-H), 5.64 (1H, d, J = 10.4 Hz, 7α-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.0, 14.0, 18.7, 21.0, 21.3, 21.5 (CH_2), 22.5, 22.8, 23.8 (CH_2), 25.3 (CH_2), 26.3 (CH_2), 27.9, 28.3 (CH_2), 29.8 (CH_2), 32.2 (CH_2), 35.6, 36.0 (CH_2), 39.4 (CH_2), 39.5 (CH_2), 42.1, 42.3 (C), 43.1, 43.8 (C), 55.3, 55.4, 70.3, 77.7, 80.3 (C5), 170.8 and 171.2 (3β- and 7β-CH_3COO), 205.5 (C6).

3*β*,6-Dihydroxycholest-5-en-7-one (36) and 3*β*,5α,6α-Trihydroxycholestan-7-one (37). Alkaline hydrolysis of the 5α-hydroxy-6-oxocholestane-3β,7β-diyl diacetate (**35**, 95 mg, 0.183 mmol), as described for the preparation of compound **21**, using NaOH (10% aqueous solution, 1.118 mmol) afforded after FCC (petroleum ether, ethyl acetate, 4:1 to 1:3) and according to the elution run the 3β,6-dihydroxycholest-5-en-7-one (**36**, 43.5 mg, 57%). Mp 154−155 °C (EtOH); lit.,[34] 155−156 °C. IR (film) 3415, 2950, 2872, 1669, 1643,

1468, 1383, 1315, 1262, 1193, 1135, 1100, 1062, 1012 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 0.86 and 0.87 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.92 (3H, d, J = 6.5 Hz, 21-CH_3), 1.17 (3H, s, 19-CH_3), 3.23 (1H, m, 4-H), 3.67 (1H, tt, J = 11.2, 4.5 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 11.9 (C18), 17.2 (C19), 18.8 (C21), 21.1 (CH_2), 22.5 and 22.8 (C26 and C27), 23.8 (CH_2), 26.2 (CH_2), 28.0, 28.5 (CH_2), 30.8 (CH_2), 32.7 (C4, CH_2), 35.7, 36.1 (CH_2), 36.6 (C), 37.1 (CH_2), 38.7 (CH_2), 39.4 (CH_2), 43.3 (C), 43.6, 49.7, 50.4, 54.7, 70.0 (C3), 134.1 (C5), 141.8 (C6), 197.1 (C7). MS m/z (%): 415.9 (100) $[M - H]^+$, 400.8 (20), 341.6 (18), 326.0 (26), 311.9 (22), 293.4 (24). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{27}H_{44}O_3Na$: 439.3188; found 439.3177. Also produced was the 3β,5α,6α-trihydroxycholestan-7-one (**37**, 18.3 mg, 23%). Mp 188−189 °C (EtOH). IR (film) 3431, 2936, 2867, 1716, 1466, 1373, 1256, 1135, 1059, 1024, 978, 926, 780 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.66 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.91 (3H, d, J = 6.5 Hz, 21-CH_3), 1.28 (3H, s, 19-CH_3), 4.01 (1H, m, 3α-H), 4.09 (1H, s, 6-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.0, 16.0, 18.7, 21.7 (CH_2), 22.5, 22.8, 23.8 (CH_2), 24.8 (CH_2), 28.0, 28.4 (CH_2), 30.4 (CH_2), 30.9 (CH_2), 35.6, 36.1 (CH_2), 38.7 (CH_2), 38.8 (CH_2), 39.0 (C), 39.4 (CH_2), 42.7 (C), 46.8, 47.6, 48.6, 54.9, 67.1, 77.0, 81.7 (C5), 210.4 (C7). MS m/z (%): 433.9 (38) $[M - H]^+$, 417.3 (29), 354.2 (53), 325.5 (20), 321.7 (21), 294.9 (38), 265.0 (100). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{27}H_{46}O_4Na$, 457.3288; found, 457.3298.

7β-Hydroxycholest-5-en-3β-yl Laurate (38). To a solution of 7-oxocholesterol (**4**, 250 mg, 0.624 mmol) in pyridine (10 mL) at 0 °C, lauroyl chloride (297 μL, 1.250 mmol) was slowly added. Reaction mixture was left stirring for 15 min, then stopped with the addition of methanol and evaporated under vacuum. The oily residue was dissolved with a mixture of diethyl ether, petroleum ether 1:1 and sequentially washed with HCl (5% aqueous solution), $NaHCO_3$ (saturated aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. FCC (petroleum ether, ethyl acetate 30:1 to 20:1) afforded the 7-oxocholest-5-en-3β-yl laurate (356.5 mg, 98%). Mp 115−118 °C (MeOH/AcOEt). IR (film) 3018, 2928, 2867, 1740, 1672, 1632, 1468, 1381, 1161, 1014, 870, 759 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.87 (3H, m, CH_3CH_2) 0.92 (3H, d, J = 6.5 Hz, 21-CH_3), 1.21 (3H, s, 19-CH_3), 1.26 (18H, m, $CH_3(CH_2)_9$), 2.28 (2H, t, J = 7.5 Hz, CH_2CO), 4.72 (1H, tt, J = 11.2, 4.5 Hz, 3α-H), 5.70 (1H, d, J = 1.4 Hz, 6-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.0, 14.1, 17.3, 18.8, 21.2 (CH_2), 22.5, 22.7 (CH_2), 22.8, 23.8 (CH_2), 25.0 (CH_2), 26.3 (CH_2), 27.4 (CH_2), 28.0, 28.5 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (2CH_2), 31.9 (CH_2), 34.5 (CH_2), 35.7, 36.0 (CH_2), 36.2 (CH_2), 37.8 (CH_2), 38.3 (C), 38.7 (CH_2), 39.5 (CH_2), 43.1 (C), 45.4, 49.8, 49.9, 54.8, 71.9, 126.7 (C6), 163.9 (C5), 173.1 (RCH_2COO), 201.9 (C7). Then to a solution of 7-oxocholest-5-en-3β-yl laurate (250 mg, 0.429 mmol) in THF (5 mL) and MeOH (1 mL) containing $CeCl_3 \cdot 7H_2O$ (239.8 mg, 0.644 mmol), $NaBH_4$ (64.9 mg, 1.716 mmol) was slowly added. The mixture was stirred at room temperature for 15 min, then stopped with the addition of acetone and Et_3N and concentrated under vacuum. The residue was dissolved in a mixture of diethyl ether, petroleum ether 2:1, filtered, and washed sequentially with HCl (5% aqueous solution), $NaHCO_3$ (10% aqueous solution), and water, dried over anhydrous Na_2SO_4, filtered, and evaporated. FCC (petroleum ether, ethyl acetate 20:1 to 10:1) afforded the 7β-hydroxycholest-5-en-3β-yl laurate (**38**, 208.1 mg, 83%). Mp 89−92 °C (EtOH/Et_2O). IR (film) 3496, 2924, 2852, 1712, 1676, 1468, 1384, 1294, 1183, 1085, 1038, 1022, 760 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 0.87 and 0.87 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, t, J = 7.0 Hz, CH_3), 0.92 (3H, d, J = 6.6 Hz, 21-CH_3), 1.06 (3H, s, 19-CH_3), 1.26 (18H, m, $CH_3(CH_2)_9$), 2.27 (2H, t, J = 7.5 Hz, RCH_2COO), 3.85 (1H, td, J = 8.0, 2.1, 2.1 Hz, 7α-H), 4.63 (ddt, J = 10.4, 10.4, 5.9, 4.3 Hz, 3α-H), 5.31 (1H, t, J = 2.1 Hz, 6α-H). ^{13}C NMR (126 MHz, $CDCl_3$) δ ppm 11.8, 14.1, 18.8, 19.1, 21.1 (CH_2), 22.5, 22.7 (CH_2), 22.8, 23.9 (CH_2), 25.1 (CH_2), 26.4 (CH_2), 27.8 (CH_2), 28.0, 28.5 (CH_2), 29.1 (CH_2), 29.3 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 29.6 (2CH_2), 31.9 (CH_2), 34.7 (CH_2), 35.7, 36.2 (CH_2), 36.6 (C), 36.7 (CH_2), 37.7 (CH_2), 39.5 (CH_2), 39.6 (CH_2), 40.9, 43.0 (C), 48.2, 55.5, 56.0, 73.2, 73.3, 126.3 (C6), 142.5 (C5), 173.2 (RCH_2COO). MS m/z (%): 583.6 (16) $[M - H]^+$, 554.4 (100), 488.5 (23), 452.1 (16), 426.3 (22), 371.8 (14), 256.8 (16), 210.0 (16). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{39}H_{68}O_3Na$, 607.5066; found, 607.5114.

7β-Hydroxycholest-5-en-3β-yl Stearate (39). To a solution of 7-oxocholesterol (**4**, 250 mg, 0.624 mmol) in THF (20 mL) containing DMAP (35 mg, 0.286 mmol), stearic anhydride (687.5 mg, 1.248 mmol) was added. The mixture was stirred overnight at room temperature and then stirred with MeOH for 30 min, concentrated under vacuum and the residue dissolved in a mixture of diethyl ether, petroleum ether 1:1. The resulting organic phase was stirred with NH_4Cl (saturated aqueous solution) for 1 h and then washed sequentially with $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtered, and evaporated under vacuum. FCC (petroleum ether, ethyl acetate 20:1) afforded the 7-oxocholest-5-en-3β-yl stearate (412 mg, 99%). Mp 93−96 °C (EtOH/AcOEt). IR (film) 2916, 2849, 1738, 1672, 1463, 1373, 1164, 931, 861, 809, 724 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, t, J = 7.0 Hz, CH_3CH_2), 0.92 (3H, d, J = 6.5 Hz, 21-CH_3), 1.21 (3H, s, 19-CH_3), 1.25 (30H, m, $CH_3(CH_2)_{15}$), 2.28 (2H, t, J = 7.5 Hz, RCH_2COO), 4.72 (1H, tt, J = 11.0, 4.5 Hz, 3α-H), 5.71 (1H, d, J = 1.4 Hz, 6-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.0, 14.1, 17.3, 18.9, 21.2 (CH_2), 22.5, 22.7 (CH_2), 22.8, 23.8 (CH_2), 24.7 (CH_2), 25.0 (CH_2), 26.3 (CH_2), 27.4 (CH_2), 28.0, 28.5 (CH_2), 29.1 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.4 (CH_2), 29.6 (CH_2), 29.7 (4CH_2), 29.7, 31.9 (CH_2), 33.9 (CH_2), 34.5 (CH_2), 35.7, 36.0 (CH_2), 36.2 (CH_2), 37.8 (CH_2), 38.3 (C), 38.7 (CH_2), 39.5 (CH_2), 43.1 (C), 45.4, 49.8, 50.0, 54.8, 71.9 (C3), 126.7 (C6), 164.0 (C5), 173.1 (RCH_2COO), 202.0 (C7). Then stereoselective reduction of 7-oxo-cholest-5-en-3β-yl stearate (250 mg, 0.375 mmol), as described for the preparation of compound **38**, afforded the 7β-hydroxycholest-5-en-3β-yl stearate (**39**, 195.7 mg, 78%) after FCC. Mp 60−65 °C (EtOH/Et_2O). IR (film) 3364, 2917, 2850, 1736, 1468, 1377, 1175, 1089, 1047, 881, 759, 721 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.69 (3H, s, 18-CH_3), 0.86 and 0.87 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.87 (3H, m, CH_3CH_2), 0.92 (3H, d, J = 6.5 Hz, 21-CH_3), 1.06 (3H, s, 21-CH_3), 1.25 (30H, m, $CH_3(CH_2)_{15}$), 2.27 (2H, t, J = 7.5 Hz, RCH_2COO), 3.85 (1H, td, J = 7.9, 2.2, 2.2 Hz, 7α-H), 4.63 (1H, ddt, J = 10.4, 10.4, 6.3, 4.3 Hz, 3α-H), 5.31 (1H, t, J = 2.2 Hz, 6-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 11.8, 14.1, 18.8, 19.1, 21.0 (CH_2), 22.5, 22.7 (CH_2), 22.8, 23.8 (CH_2), 25.0 (CH_2), 26.4 (CH_2), 27.7 (CH_2), 28.0, 28.5 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (3CH_2), 29.7 (4CH_2), 31.9 (CH_2), 34.7 (CH_2), 35.7, 36.2 (CH_2), 36.5 (C), 36.7 (CH_2), 37.6 (CH_2), 39.5 (2CH_2), 40.8, 42.9 (C), 48.2, 55.4, 55.9, 73.1, 73.2, 126.2 (C6), 142.5 (C5), 173.3 (RCH_2COO).

4β,5α,6β-Trihydroxycholestan-3β-yl Acetate (40). To a solution of cholestane-3β,4β,5α,6β-tetrol (**14**, 40 mg) in toluene (8 mL) and vinyl acetate (300 μL), lipase AY (400 mg) was added. The mixture was shaken at 200 rpm at 45 °C. After 24 h, the enzyme was filtered and the solvent evaporated. FCC (petroleum ether, ethyl acetate 1:1 to 1:3) afforded the 4β,5α,6β-trihydroxycholestan-3β-yl acetate (**40**, 41.2 mg, 94%). Mp 214−215 °C (EtOH/AcOEt). IR (film) 3384, 2936, 2867, 1710, 1460, 1373, 1262, 1210, 1158, 1036, 1012 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.6 Hz, 21-CH_3), 1.44 (3H, s, 19-CH_3), 2.09 (3H, s, 3β-CH_3COO), 3.82 (1H, t, J = 2.8 Hz, 6α-H), 3.97 (1H, d, J = 3.6 Hz, 4α-H), 5.24 (1H, ddd,

J = 12.1, 5.3, 3.6 Hz, 3α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 15.4, 18.6, 20.3 (CH_2), 21.4, 22.2 (CH_2), 22.5, 22.8, 23.8 (CH_2), 24.1 (CH_2), 28.0, 28.2 (CH_2), 30.3, 31.9 (CH_2), 34.3 (CH_2), 35.8, 36.1 (CH_2), 38.1 (C), 39.5 (CH_2), 39.8 (CH_2), 42.7 (C), 46.7, 56.0, 56.2, 72.2, 73.4 (C5), 77.5, 77.9, 170.2 (CH_3COO). MS m/z (%): 479.9 (33) $[M + H]^+$, 441.6 (53), 419.1 (19), 102.6 (100), 100.6 (38). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{29}H_{50}O_5Na$, 501.3556; found, 501.3586.

5α,6β,7β-Trihydroxycholestan-3β-yl Acetate (41). To a solution of cholestane-3β,5α,6β,7β-tetrol (**34**, 40 mg) in toluene (8 mL) and vinyl acetate (300 μL), lipase AY (400 mg) was added. The mixture was shaken at 200 rpm at 45 °C. After 24 h, the enzyme was filtered and the solvent evaporated. FCC (petroleum ether, ethyl acetate 1:2 to 1:3) afforded the 5α,6β,7β-trihydroxycholestan-3β-yl acetate (**41**, 40.3 mg, 92%). Mp 178−179 °C (EtOH); lit.,[61] 179−180 °C. IR (film) 3442, 2927, 2855, 1733, 1460, 1379, 1272, 1123, 1070, 1030, 891, 740 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.91 (3H, d, J = 6.5 Hz, 21-CH_3), 1.16 (3H, s, 19-CH_3), 2.03 (3H, s, 3β-CH_3COO), 2.22 (1H, dd, J = 13.0, 11.6 Hz), 3.46 (1H, d, J = 3.7 Hz, 6α-H), 3.83 (1H, dd, J = 9.4, 3.7 Hz, 7α-H), 5.13 (1H, m, 3α-H). ^{13}C NMR (101 MHz, DMSO-d_6) ppm 12.0, 16.6, 18.6, 20.7 (CH_2), 21.1, 22.3, 22.6, 23.2 (CH_2), 26.6 (CH_2), 26.7 (CH_2), 27.3, 28.3 (CH_2), 31.6 (CH_2), 35.2, 35.6 (CH_2), 37.0 (CH_2), 37.1 (C), 37.8, 38.9 (CH_2), 39.7 (CH_2), 43.0 (C), 43.2, 54.9, 55.3, 70.6, 71.2, 75.0 (C), 77.1, 169.8 (C). MS m/z (%): 477.9 (21) $[M - H]^+$, 407.9 (32), 378.9 (28), 339.4 (58), 337.5 (22), 325.3 (35), 311.3 (46), 165.0 (65), 149.0 (100).

3β-Hydroxycholest-5-en-7β-yl Acetate (42). To a solution of cholest-5-ene-3β,7β-diyl diacetate (**30**, 120 mg) in DIPE (aqueous saturated, 10 mL), lipase AY (2500 mg) was added. The mixture was shaken at 200 rpm at 45 °C. After 10 days, the enzyme was filtered and the solvent evaporated. FCC (petroleum ether, ethyl acetate 8:1 to 1:1) afforded the 3β-hydroxycholest-5-en-7β-yl acetate (**42**, 70 mg, 64%). Mp 132−133 °C (MeOH); lit.,[63] 83−84 °C. IR (film) 3402, 2948, 2867, 1733, 1466, 1372, 1239, 1175, 1141, 1057, 1018, 949, 757 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 0.83 and 0.84 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 1.04 (3H, s, 19-CH_3), 2.00 (3H, s, 7β-CH_3COO), 3.51 (1H, tt, J = 11.4, 4.6 Hz, 3α-H), 4.99 (1H, td, J = 8.7, 1.6 Hz, 7α-H), 5.17 (1H, t, J = 1.6 Hz, 6-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 11.7, 18.7, 19.0, 21.0 (CH_2), 21.6, 22.5, 22.8, 23.7 (CH_2), 25.1 (CH_2), 27.9, 28.3 (CH_2), 31.3 (CH_2), 35.6, 36.0 (CH_2), 36.3 (C), 36.4, 36.7 (CH_2), 39.3 (CH_2), 39.4 (CH_2), 41.5 (CH_2), 42.7 (C), 48.1, 55.3, 55.5, 71.0, 75.7, 121.2 (C6), 145.3 (C5), 171.3 (CH_3COO). MS m/z (%): 443.9 (85) $[M - H]^+$, 431.1 (63), 369.7 (37), 353.8 (64), 339.7 (100), 310.4 (99), 136.6 (63), 116.9 (45). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{29}H_{48}O_3Na$, 467.3501; found, 467.3526.

5α-Hydroxycholestane-3β,6β-diyl Diacetate (44). To a solution of 5α,6β-dihydroxycholestan-3β-yl acetate (**44**, 120 mg, 0.259 mmol) and DMAP (15.8 mg, 0.130 mmol) in THF (2 mL), acetic anhydride 98.5% (99.4 μL, 1.036 mmol) was added. The reaction mixture was stirred overnight at room temperature, then stirred with MeOH for 30 min, concentrated under vacuum, and the residue was dissolved in diethyl ether. The resulting organic phase was stirred with NH_4Cl (saturated aqueous solution) for 1 h and then washed sequentially with $NaHCO_3$ (saturated aqueous solution) and water, dried with anhydrous Na_2SO_4, filtered, and evaporated. FCC (petroleum ether, ethyl acetate 4:1) yielded the 5α-hydroxycholestane-3β,6β-diyl diacetate (**44**, 113.9 mg, 87%). Mp 168−169 °C (EtOH); lit.,[64] 165−167 °C. IR (film) 3446, 2937, 2867, 1734, 1716, 1458, 1375, 1242, 1164, 1031, 960 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.67 (3H, s, 18-CH_3), 0.85 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.14 (3H, s, 19-CH_3), 2.01 (3H, s, CH_3COO), 2.06 (3H, s, CH_3COO), 4.69 (1H, m, 6α-H), 5.14 (1H, tt, J = 10.8, 5.3 Hz, 3α-H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.2, 16.3, 18.6, 21.0 (CH_2), 21.4, 21.4, 22.5, 22.8, 23.9 (CH_2), 24.1 (CH_2), 26.6 (CH_2), 28.0, 28.2 (CH_2), 30.7, 31.4 (CH_2), 31.8 (CH_2), 35.8, 36.1 (CH_2), 36.8 (CH_2), 38.4 (C), 39.5 (CH_2), 39.8 (CH_2), 42.7 (C), 45.0, 55.7, 56.2, 70.7, 74.9 (C5), 76.2, 170.2 (CH_3COO), 170.7 (CH_3COO). MS m/z (%): 505.3 (55) $[M + H]^+$, 447.6 (79), 429.0 (100), 413.3 (84), 382.2 (73), 322.0 (90), 180.5 (91).

3β,5α-Dihydroxycholestan-6β-yl Acetate (45). To a solution of 5α-hydroxycholestane-3β,6β-diyl diacetate (**44**, 85 mg) in DIPE (aqueous, saturated, 20 mL), lipase AY (3600 mg) was added. The mixture was shaken at 200 rpm at 45 °C. After 10 days the enzyme was filtered and the solvent evaporated. FCC (petroleum ether, ethyl acetate 1:1) afforded the 3β,5α-dihydroxycholestan-6β-yl acetate (**45**, 48.3 mg, 62%). Mp 145−146 °C (EtOH); lit.,[65] 143 − 144 °C. IR (film) 3474, 2935, 2863, 1716, 1457, 1464, 1372, 1266, 1248, 1160, 1038, 928 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 0.68 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.14 (3H, s, 19-CH_3), 2.06 (3H, s, CH_3COO), 4.08 (1H, tt, J = 10.4, 5.1 Hz, 3α-H), 4.71 (1H, m, 6α-H); ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 12.2, 16.5, 18.6, 21.1 (CH_2), 21.4, 22.5, 22.8, 23.8 (CH_2), 24.1 (CH_2), 28.0, 28.2 (CH_2), 30.6 (CH_2), 30.7, 31.4 (CH_2), 32.0 (CH_2), 35.8, 36.1 (CH_2), 38.4 (C), 39.5 (CH_2), 39.9 (CH_2), 40.5 (CH_2), 42.7 (C), 45.4, 55.8, 56.2, 67.3, 75.3 (C5), 76.1, 170.3 (CH_3COO). MS m/z (%): 463.5 (51) $[M + H]^+$, 413.1 (44), 384.9 (50), 358.0 (73), 328.5 (41), 259.9 (100), 224.5 (50), 109.3 (46), 85.2 (59), 60.8 (46).

3β,5α,6β-Trihydroxycholestan-7β-yl Acetate (46). To a solution of 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**, 130 mg) in DIPE (aqueous, saturated, 30 mL), lipase AY (2730 mg) was added . The mixture was shaken at 200 rpm at 45 °C. After 30 days the enzyme was filtered and the solvent evaporated. FCC (petroleum ether, ethyl acetate 1:1 to 1:5) afforded the 3β,5α,6β-trihydroxycholestan-7β-yl acetate (**46**, 88.4 mg, 74%). Mp 218−219 °C (EtOH). IR (film) 3478, 2951, 2872, 1714, 1468, 1374, 1266, 1142, 1086, 1031, 973, 888, 757, 695 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.63 (3H, s, 18-CH_3), 0.83 and 0.84 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.88 (3H, d, J = 6.5 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 1.93 (3H, s, CH_3COO), 3.41 (1H, dd, J = 5.0, 3.5 Hz, 6α-H), 3.76 (1H, m, 3α-H), 4.13 (1H, s, 5-OH), 4.21 (1H, dd, J = 5.7 Hz, 3-OH), 4.63 (1H, dd, J = 5.0 Hz, 6-OH), 4.90 (1H, dd, J = 10.7, 3.5 Hz, 7α-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm 11.8, 16.8, 18.6, 20.8, 21.7 (CH_2), 22.3, 22.6, 23.2 (CH_2), 25.5 (CH_2), 27.3, 28.2 (CH_2), 31.0 (CH_2), 32.0 (CH_2), 34.6, 35.0, 35.6 (CH_2), 37.2 (C), 38.9 (CH_2), 39.6 (CH_2), 40.6 (CH_2), 43.1 (C), 43.7, 54.5, 54.7, 65.2, 74.3, 75.1 (C5), 75.8, 169.9 (CH_3COO). MS m/z (%): 477.8 (100) $[M - H]^+$, 468.3 (83), 384.9 (81), 326.7 (69), 316.7 (51), 265.1 (56), 263.3 (75), 237.9 (64), 236.6 (81). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{29}H_{50}O_5Na$, 501.3556; found, 501.3540.

5α-Hydroxycholestane-3β,6β,7β-triyl Triacetate (47). Acetylation of 5α,6β-dihydroxycholestane-3β,7β-diyl diacetate (**33**, 150 mg, 0.288 mmol), as described for the preparation of compound **44**, using acetic anhydride (114.6 μL, 1.152 mmol) afforded, after FCC (chloroform, ethanol 100:0 to 95:5), the 5α-hydroxycholestane-3β,6β,7β-triyl triacetate (**47**, 145.9 mg, 90%). Mp 144.5−146 °C (MeOH); lit.,[61] 100−103 °C. IR (film) 3396, 2934, 2867, 1742, 1716, 1457, 1364, 1241, 1135, 1070, 1028, 879 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.70 (3H, s, 18-CH_3), 0.86 and 0.86 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.16 (3H, s, 19-CH_3), 1.91 (3H, s, CH_3COO), 2.03 (3H, s, CH_3COO), 2.10 (3H, s, CH_3COO), 5.02 (1H, d, J = 4.0 Hz, 6α-H), 5.19 (1H, m, 3α-H), 5.20 (1H, dd, J = 10.8, 4.0 Hz, 7α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.2, 17.1, 18.7, 21.1, 21.2 (CH_2), 21.3, 21.4, 22.5, 22.8, 23.8 (CH_2), 25.7 (CH_2), 26.6 (CH_2), 28.0, 28.5 (CH_2), 31.9 (CH_2), 35.5, 35.7, 36.1 (CH_2), 36.6 (CH_2), 37.9 (C), 39.4 (CH_2), 39.7 (CH_2), 43.5 (C), 44.0, 54.4, 55.2, 70.4, 73.0, 74.7, 75.3 (C5), 170.3 (CH_3COO), 170.7 (CH_3COO), 171.0 (CH_3COO). MS m/z (%): 561.6 (23) $[M - H]^+$, 515.1 (26), 487.1 (100), 361.1 (25), 326.2 (40).

3β,5α-Dihydroxycholestane-6β,7β-diyl Diacetate (48). To a solution of 5α-hydroxycholestane-3β,6β,7β-triyl triacetate (**47**, 120 mg) in DIPE (aqueous, saturated, 30 mL), lipase AY (4000 mg) was added. The mixture was shaken at 200 rpm at 45 °C. After 30 days the enzyme was filtered and the solvent evaporated. FCC (chloroform, ethanol 99:1 to 95:5) afforded the 3β,5α-dihydroxycholestane-6β,7β-diyl diacetate (**48**, 74.4 mg, 67%). Mp 171−172 °C (EtOH). IR (film) 3365, 2951, 2867, 1743, 1722, 1457, 1374, 1262, 1070, 1041, 879, 693 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.69 (3H, s, 18-CH_3), 0.85 and 0.85 (each 3H, 2d, J = 6.6 Hz, 26-CH_3 and 27-CH_3), 0.90 (3H, d, J = 6.5 Hz, 21-CH_3), 1.14 (3H, s, 19-CH_3), 1.91 and 2.08 (each 3H, 2s, 6β-CH_3COO and 7β-CH_3COO), 4.09 (1H, tt, J = 10.3, 5.0 Hz, 3α-H), 5.00 (1H, d, J = 4.0 Hz, 6α-H), 5.16 (1H, dd, J = 10.9, 4.0 Hz, 7α-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 12.1, 17.2, 18.7, 21.1, 21.2 (CH_2), 21.4, 22.5, 22.8, 23.8 (CH_2), 25.8 (CH_2), 28.0, 28.5 (CH_2), 30.2 (CH_2), 32.1 (CH_2), 35.5, 35.6, 36.1 (CH_2), 37.9 (C), 39.4 (CH_2), 39.8 (CH_2), 40.5 (CH_2), 43.5 (C), 44.1, 54.4, 55.1, 66.7, 73.4, 74.9, 75.6 (C5), 171.1 (CH_3COO), 170.7 (CH_3COO). MS m/z (%): 521.7 (57) $[M + H]^+$, 477.1 (80), 445.4 (57), 419.1 (49), 391.1 (64), 385.6 (79), 279.2 (87), 177.2 (100). HRMS (ESI), positive mode, m/z $[M + Na]^+$ calcd for $C_{31}H_{52}O_6Na$: 543.3662, found: 543.3696.

Biology. General Methods. Methods and conditions were used as recently described by our group.[25,26] SH-SY5Y cell line was purchased from ATCC. Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12, 1:1) with L-glutamine and without sodium bicarbonate was obtained from Sigma-Aldrich Co.

Cell Lines and Culture Condition. As previously described.[26] SH-SY5Y cells were cultured in DMEM/F12 medium supplemented with 10% of heat inactivated fetal bovine serum (iFBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin, with pH adjusted to 7.4.

Cell Viability Assay. Stock solutions of the synthesized sterols were prepared in DMSO, except for compound **38**, which was dissolved in a 1:1 mixture of DMSO and THF, and stored at −20 °C. All experiments were performed in 96-well culture plates, and cells were seeded in a total volume of 100 μL of culture medium, with the following densities, according to the cell line and period of drug exposure: SH-SY5Y at 5 × 10^3 cells/well for 48 h and ARPE-19 cells seeded at 5 × 10^3 cells/well for 96 h.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. NMR spectra of the compounds synthesized and plots of dose-dependent effects for oxysterols **21**, **28**, and **45** in the different cell lines tested. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

We thank Fundação para a Ciência e Tecnologia (FCT), Portugal, through POCI for financial support. We acknowledge the Laboratório de Espectrometria de Massa do Centro de Estudos Farmacêuticos, Universidade de Coimbra, Portugal, for providing data from the LC-MSn (QITMS), and Laboratório de FTICR e Proteómica Avançada, Faculdade de Ciências, Universidade de Lisboa, Portugal, for providing data from the FTICR-MS, both integrated in the National Mass Spectrometry Network (RNEM). We thank J. Silva Ramalho, Laboratório de Biologia Celular e Molecular, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Portugal, for the ARPE-19 cell line. J.F.S.C. thanks FCT for Grant SFRH/BD/18263/2004.

■ ABBREVIATIONS USED

ACAT, acyl coenzyme A: cholesterol acyltransferase; $Bi(OTf)_3$, bismuth(III) triflate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MMPP, magnesium bis(monoperoxyphthalate) hexahydrate

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