4,5-Cyclopropanocholestan-3β-ol Substrates for Cholesterol Oxidase and Their ^{1}H NMR Assignments

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We have assayed 4,5-cyclopropanocholestan-3-ols and 4,5-cyclopropanocholestan-3-ones and tested them as substrates and inhibitors of cholesterol oxidase. The 4,5-cyclopropanocholestan-3β-ols (α and β) are substrates of cholesterol oxidase that are converted to their respective ketones 1000-fold more slowly than cholesterol. The induced ring-current effects of a cyclopropane ring are clearly illustrated in the ^{1}H NMR spectra of these sterols. These shielding effects are dramatic because of the rigidity of the steroid backbone. Assignments of the ^{1}H resonances of the A, B, and cyclopropyl rings of the sterols have been made using DQF-COSY and NOESY experiments. We have assigned the upfield multiplet at approximately 0.5 ppm to $H_{6\alpha}$ in both isomers. $H_{6\alpha}$ is shielded by the cyclopropyl σ bond. $H_{6\beta}$ is deshielded by the cyclopropane ring and appears at approximately 2.0 ppm in both isomers.

We report here the assay of 4,5-cyclopropyl steroids as substrates and inhibitors of cholesterol oxidase. These cyclopropyl steroids were designed to test the mechanism of cholesterol oxidase. Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation and isomerization of cholesterol into cholest-4-en-3-one (Scheme 1). This enzyme is part of the bacterial metabolic pathway for utilizing cholesterol as its carbon source and is secreted by Gram-positive soil bacteria, including *Brevibacterium sterolicum* (ATCC 81387) and *Streptomyces* sp. strain SA-COO. The enzyme is used to assay serum cholesterol levels, and recently, its larvicidal properties were discovered.[1,2]

Cholesterol oxidase is a monomer (57 kD) with a single active site for both oxidation and isomerization and requires one FAD per active site.[3−5] The oxidation may proceed via a radical intermediate or via direct hydride transfer. We were interested in probing how much conformational flexibility was available to the substrate in the active site and envisioned that ring-opening reactions would serve as good indicators of flexibility. These indicators could then be used in our ongoing investigation of the steroid and membrane binding properties of cholesterol oxidase. We reasoned that if there was a radical intermediate in the oxidation, a cyclopropyl substrate such as **1** might undergo ring opening. The ring-opened radical could potentially inactivate the enzyme. Alternatively, if the cyclopropyl steroids **1a** or **1b** were converted to the ketones **2a** or **2b**, the ketones might undergo nucleophilic ring opening in the active site.[6] The active site base for isomerization, glutamate-361,[7,8] is positioned over the β-face of the steroid, and inspection of the X-ray structure indicates that it is close enough to form a covalent bond. Thus, we undertook the synthesis of steroids **1**−**2** and their assay with cholesterol oxidase. In addition, spectral characterization of steroids **1a** and **1b** revealed a large perturbation of ^{1}H chemical shifts at C_6 due to the cyclopropyl ring. The results of our enzyme assays of **1a**, **1b**, **2a**, and **2b** and the spectral assignments of **1a** and **1b** are presented.

Discussion

We synthesized steroids **1a** and **1b** using a modification of the procedures of Dauben and co-workers.[9] Use of sonication in the cyclopropanation reactions resulted in an increase of 20% in product yield compared to previously used methods. Cholest-4-en-3-one was reduced with $LiAlH_4$ to yield cholest-4-en-3-ol. The 3α- and 3β-epimers were separated by silica gel chromatography. The cholest-4-en-3-ols were cyclopropanated in separate reactions using CH_2I_2 and zinc with sonication to activate the metal to yield **1b** and the epimer of **1a**. The 3-hydroxy moiety was epimerized via Collins oxidation to form the 3-ketocyclopropyl steroid **2a**, followed by a reduction with $LiAlH_4$ to provide **1a**. Ketone **2b** was prepared in an analogous manner (Scheme 2).

We envisioned that, upon incubation with cholesterol oxidase, the cyclopropyl steroids **1a**,**b** would behave as substrates and/or as irreversible inhibitors. We performed, therefore, two types of assays with the recombinant form of the *Streptomyces* cholesterol oxidase.[10] In the first assay, the rate of production of H_2O_2 was followed using horseradish peroxidase, and thus the rate of oxidation of the 3-hydroxy moiety was measured. Alcohol **1a** was oxidized at 0.1% of the rate of cholesterol oxidation; **1b** was oxidized at 0.2% of the rate for cholesterol with 50 μM substrate. The limited solubility of the steroids precluded measurement of k_{cat} and K_m. For every equivalent of alcohol consumed, 1 equiv of H_2O_2

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(1) Purcell, J. P.; Greenplate, J. T.; Jennings, M. G.; Ryerse, J. S.; Pershing, J. C.; Sims, S. R.; Prinsen, M. J.; Corbin, D. R.; Tran, M.; Sammons, R. D.; Stonard, R. J. *Biochem. Biophys. Res. Commun.* **1993**, *196*, 1406−13.

(2) Greenplate, J. T.; Duck, N. B.; Pershing, J. C.; Purcell, J. P. *Entomol. Exp. Appl.* **1995**, *74*, 253−258.

(3) Uwajima, T.; Yagi, H.; Terada, O. *Agric. Biol. Chem.* **1974**, *38*, 1149−1156.

(4) Vrielink, A.; Lloyd, L. F.; Blow, D. M. *J. Mol. Biol.* **1991**, *219*, 533−554.

(5) Li, J.; Vrielink, A.; Brick, P.; Blow, D. M. *Biochemistry* **1993**, *32*, 11507−11515.

(6) Liu, H.-w.; Walsh, C. T. In *The Chemistry of the Cyclopropyl Group*; Rappoport, Z., Ed.; Wiley-Interscience: New York, 1987; Vol. Part 2, p 969.

(7) Kass, I. J.; Sampson, N. S. *Biochem. Biophys. Res. Commun.* **1995**, *206*, 688−693.

(8) Sampson, N. S.; Kass, I. J. *J. Am. Chem. Soc.* **1997**, *119*, 855−862.

(9) Dauben, W. G.; Laug, P.; Berezin, G. H. *J. Org. Chem.* **1966**, *31*, 3869−3871.

(10) Nomura, N.; Choi, K.-P.; Murooka, Y. *J. Ferm. Bioeng.* **1995**, *79*, 410−416.

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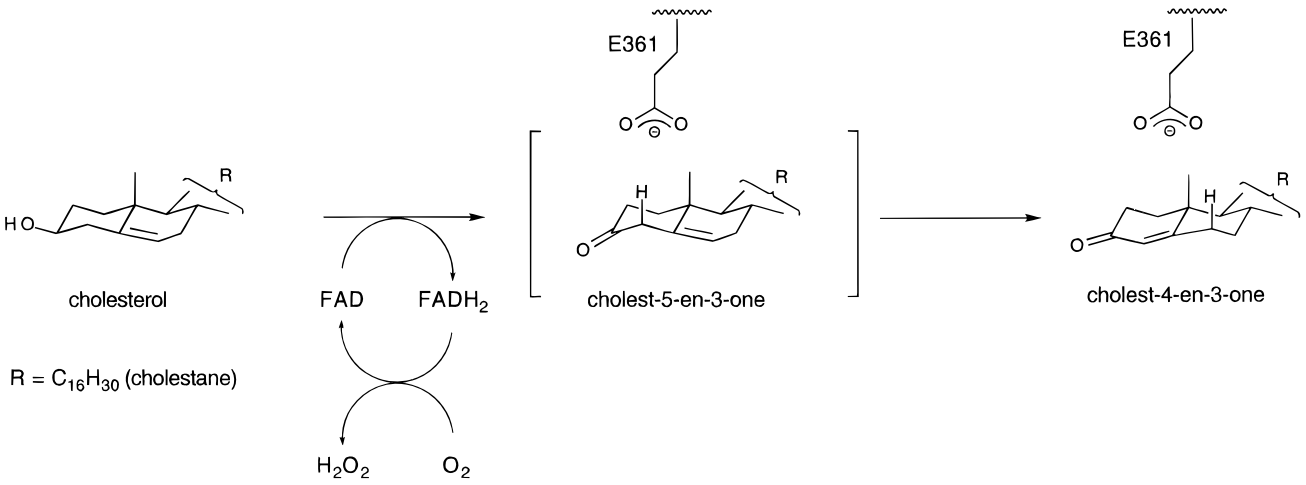
Scheme 1



Scheme 2

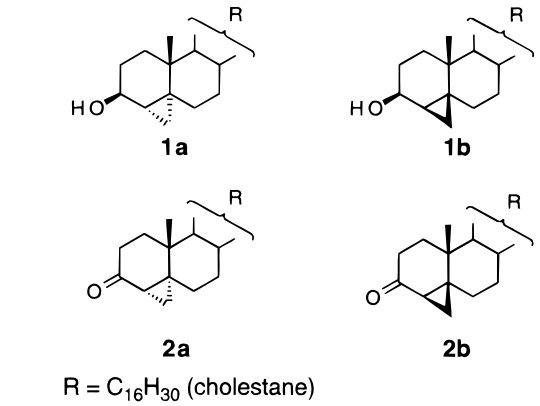


Table 1. ^{1}H Chemical Shifts (Error 0.01 ppm)

proton	1a	1b
$H_{1\alpha}$	0.86	0.99
$H_{1\beta}$	1.27	1.29
$H_{2\alpha}$	1.84	1.62
$H_{2\beta}$	1.40	1.20
$H_{3\alpha}$	4.05	4.33
$H_{4\alpha}$	na[a]	1.14
$H_{4\beta}$	0.82	na
$H_{6\alpha}$	0.48	0.54
$H_{6\beta}$	1.99	2.00
$H_{7\alpha}$	1.01	0.89
$H_{7\beta}$	1.54	1.54
H_{19}	1.15	0.94
H_{28}	0.29	0.045
H_{29}	0.092	0.67

[a] Not applicable.

was produced. The product(s) were isolated from an incubation mixture of cholesterol oxidase and **1a** or **1b** and characterized. We determined that **1a** was converted to **2a** and **1b** is converted to **2b** by mass spectrometry and TLC comparison with authentic material. These products are consistent with the alcohol:H_2O_2 stoichiometry observed. Thus, the products were ring-intact cyclopropanocholestanones.

In a second assay, we incubated the cyclopropanosteroids with cholesterol oxidase and catalase and periodically removed aliquots. Catalase was included because it is known that extended incubation of cholesterol oxidase with H_2O_2 leads to inactivation of the enzyme.[11] The aliquots were assayed for remaining enzymatic activity by the addition of cholesterol. Incubation of cholesterol oxidase with **1a**, **1b**, **2a**, or **2b** did not result in irreversible inactivation of cholesterol oxidase.

Our assay results are consistent with either mechanism. The oxidation may proceed via a hydride transfer without formation of radical intermediate. Alternatively, the radical intermediate may not partition into a ring-opening pathway and simply undergoes a second 1 e^- transfer step. Even if ring opening does occur, the conformational constraints of the active site might prevent geometric rearrangement and favor rapid reversion to the cyclopropyl ring, resulting in no apparent partitioning into the ring-opening pathway. The question of whether oxidation proceeds via hydride transfer or radical intermediate remains open. Furthermore, we do not see nucleophilic ring opening in the presence of **2a** or **2b**. This lack of reaction could be because of improper alignnment of glutamate-361 with respect to the cyclopropyl ring or insufficient electrophilicity of the ketone.

Upon characterization of the alcohol **1b**, we discovered that the spectral assignments of the protons in the A and B steroid rings in the literature were incomplete.[9,12] Protons attached to cyclopropyl rings characteristically appear at high field. In the ^{1}H NMR spectrum of **1b**, there are three high-field resonances, but only two correspond to cyclopropyl protons. Joska and Fajkos assigned the third resonance to H_6, but did not designate whether it was $H_{6\alpha}$ or $H_{6\beta}$, and they did not report J coupling to support their assignment.[12] We have used DQF-COSY and NOESY experiments to assign the cyclopropyl and A- and B-ring proton resonances of the steroids. The peak assignments and J couplings are summarized in Tables 1 and 2. The DQF-COSY experiment was used to identify the cyclopropyl peaks at 0.045, 0.667, and 1.14 ppm. Their assignment was based on following the proton couplings in **1b** from $H_{3\alpha}$, which is distinctive with its downfield resonance at 4.33 ppm, to H_4, and from H_4 to H_{28} and H_{29} (Figure 1). The third upfield resonance at 0.54 ppm, however, was not coupled to any of the other cyclopropyl hydrogens. This peak was coupled to a downfield resonance at 2.00 ppm, with a large ^2J of 13.6 Hz, indicating that they are geminal protons. We used a NOESY experiment to assign these resonances. There is an NOE cross-peak between H_{19} and the resonance at 2.00 ppm and another between $H_{4\alpha}$ and the resonance at 0.54 ppm (Figure 2). We assigned

(11) Loubathugel, C.; Tritsch, D.; Biellmann, J. F. *C. R. Acad. Sci., Ser. III* **1994**, *317*, 299−303.

(12) Joska, J.; Fajkos *Collect. Czech. Chem. Commun.* **1980**, *45*, 1850−1859.

Table 2. ^{1}H, ^{1}H Coupling Constants (Error 0.2 Hz) Determined from 1D ^{1}H Spectra and DQF-COSY Experiment

J (Hz)		**1a**	**1b**
^2J	1α, 1β	−14.2	−14.5
	2α, 2β	−14.7[a]	−15.0
	6α, 6β	−13.5	−13.6
	7α, 7β	nm[b]	−12.3
	28, 29	−4.9	−4.6
^3J	1α, 2α	4.9	14.5
	1α, 2β	nm	3.4
	1β, 2α	3.7	3.8
	1β, 2β	nm	nm
	2α, 3α	7.3	7.2
	2β, 3α	7.4	no[c]
	3α, 4α	na[d]	7.2
	3α, 4β	no	na
	4α, 28	na	9.3
	4α, 29	na	4.7
	4β, 28	4.9	na
	4β, 29	9.8	na

[a] Not accurate due to non-first-order splitting. [b] Cross-peak observed, but coupling constant not measurable. [c] No cross-peak observed. [d] Not applicable.

$H_{6\alpha}$ to the upfield resonance at 0.54 ppm and $H_{6\beta}$ to the downfield resonance at 2.00 ppm on the basis of these NOE cross-peaks and geometric constraints. The protons resonances for **1a** were assigned in an analogous fashion, and the relevant portions of their DQF-COSY and NOESY spectra are shown in Figures 1 and 2.

The conformations of the A- and B-rings of **1a** and **1b** were determined using distance constraints from the NOESY experiment and approximate dihedral angles calculated from the J couplings in the DQF-COSY and 1D-^{1}H spectra (Scheme 3). The A-ring of **1a** is a pseudo-chair that is trans-fused to the B-ring. The A-ring of **1b** is a pseudo-chair that is cis-fused to the B-ring. These conformations corresponded to the lowest energy conformations calculated in the absence of NMR constraints. The number of A/B/cyclopropyl-ring minimum energy conformers is limited to two in each case because of the rigid framework of the fused cyclopropyl steroid. It is this same rigidity that makes the shielding effect of the cyclopropyl ring on the H_6's so pronounced.

In sterol **1b**, $H_{6\alpha}$ is positioned over the cyclopropane ring (the $C_4-C_5-C_6-H_{6\alpha}$ dihedral is −25.6°) and is shifted upfield. $H_{6\beta}$ is positioned outside the ring (the $C_4-C_5-C_6-H_{6\beta}$ dihedral is −141.5°) and is shifted downfield from the position of a typical cyclohexane methylene at 1.4 ppm. The same is true for sterol **1a** (the $C_4-C_5-C_6-H_{6\alpha}$ dihedral is +27.1° and the $C_4-C_5-C_6-H_{6\beta}$ dihedral is −88.7°). These shifts may be due to an induced ring current in the cyclopropyl ring.[13] Using the shielding contours generated by Poulter and co-workers[13] and cyclopropane−H_6 distances from our minimizations, we calculated the expected shift difference due to the cyclopropane for $H_{6\alpha}$ and $H_{6\beta}$. The calculated chemical shifts agree with those observed. Similar long range upfield and downfield shifts have been reported for other cyclopropyl steroids.[14−16]

In summary, it is remarkable that **1b** is a substrate for cholesterol oxidase. After inspection of the cholesterol oxidase X-ray crystal structure,[4,5] one might expect that **1b** would not fit in the enzyme active site because of the conformation imposed by the cis-fused A/B ring system. In fact, Slotte reported that 5β-cholestan-3β-ol is not a substrate for cholesterol oxidase.[17] However, the H_2O_2 assay used in his work was not continuous, and a very slow rate such as that for 5β-cholestan-3β-ol or **1b** would not be observed. Without more detailed kinetic assays, we cannot say exactly why **1a** and **1b** are oxidized so

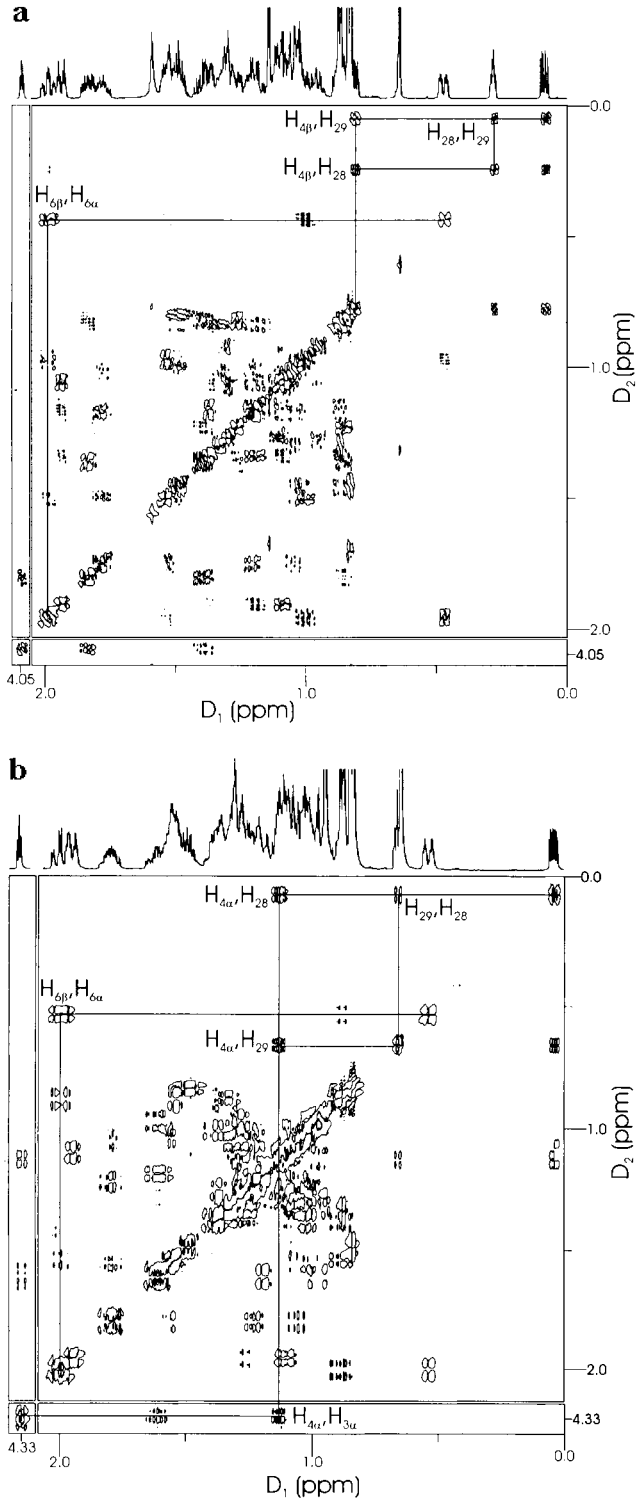


Figure 1. ^{1}H NMR DQF-COSY spectra of (a) **1a** (600 MHz) and (b) **1b** (500 MHz) at 25 °C. Cross-peaks between H_3, H_4, H_6, H_{28}, and H_{29} are labeled with their assignments.

(13) Poulter, C. D.; Boikess, R. S.; Brauman, J. I.; Winstein, S. *J. Am. Chem. Soc.* **1972**, *94*, 2291−2296.
(14) Bhacca, N. S.; Wolff, M. E.; Ho, W. *Tetrahedron Lett.* **1968**, *52*, 5427−30.
(15) Templeton, J. F.; Paslat, V. G.; Wie, C. W. *Can. J. Chem.* **1978**, *56*, 2058−64.
(16) Marat, K.; Templeton, J. F.; Ling, Y.; Lin, W.; Gupta, R. K. *Magn. Reson. Chem.* **1995**, *33*, 529−40.
(17) Slotte, J. P. *J. Steroid Biochem. Mol. Biol.* **1992**, *42*, 521−526.

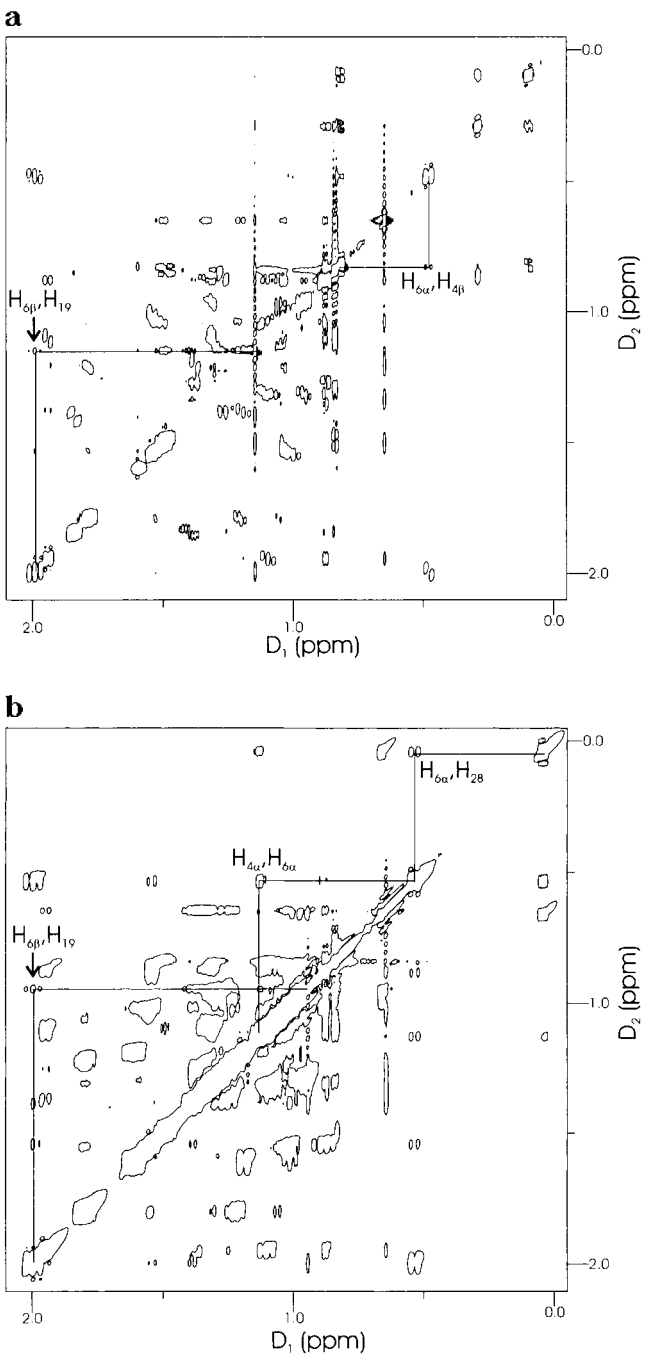


Figure 2. ^{1}H NMR NOESY spectra of (a) **1a** (600 MHz) and (b) **1b** (500 MHz) with 1100 ms mixing time at 25 °C. Cross-peaks between H_4, H_6, H_{19}, H_{28}, and H_{29} are labeled with their assignments.

slowly by cholesterol oxidase. Their turnover does indicate, however, that the active site can accommodate bulky substrates and that the protein is flexible. The rigid frameworks of **1a** and **1b** illustrate the induced ring-current effects of a cyclopropane on neighboring protons. $H_{6\alpha}$ and $H_{6\beta}$ are a clear case of induced ring current effects as opposed to shielding by σ bonds, because we see both downfield and upfield shifts, depending on the position of the proton relative to the ring.

Experimental Methods

General Procedures. Cholesterol and horseradish peroxidase were from Sigma Chemical Co., St. Louis, MO. pCO117 was a generous gift from Y. Murooka.[10] All other chemicals were supplied by Aldrich Chemical Co., Milwaukee, WI, and solvents, of reagent or HPLC grade, were supplied by Fisher Scientific, Pittsburgh, PA, unless otherwise specified. Water for assays and chromatography was distilled, followed by passage through a Barnstead NANOpure filtration system to give a resistivity better than 18 MΩ.

All reactions were carried out in oven-dried glassware under a dry N_2 or Ar atmosphere. Solvents were dried and distilled from Na/benzophenone ketyl. Thin layer chromatography was performed with precoated silica plates (silica gel 60 F_{254}, Merck). Flash chromatography was performed according to the established protocol[18] with silica gel 60 (Mallinckrodt) at an average height of 20 cm. A Fisher ultrasonic bath (Model FS9H) was used as a source of ultrasound. Melting points were determined on Thomas Hoover capillary melting point apparatus (Philadelphia, PA) and are uncorrected.

NMR Spectroscopy. All spectra were acquired in $CDCl_3$ and were externally referenced to TMS in $CDCl_3$. ^{1}H, ^{1}H−^{1}H DQF-COSY,[19,20] and ^{1}H−^{1}H NOESY[21] spectra were performed on Varian INOVA 500 and INOVA 600 spectrophotometers (500 and 600 MHz) using the parameters supplied by the manufacturer. ^{13}C spectra and DEPT experiments were recorded on a Varian GEMINI 2300 spectrophotometer (75.45 MHz). ^{1}H−^{1}H DQF-COSY spectra were performed with a 90° pulse width and 3 s relaxation delay. The NOESY spectra were performed with a 1 s relaxation delay and 1100 ms mixing times. Both ^{1}H−^{1}H DQF-COSY and NOESY spectra were acquired with a homospoil−90°−homospoil sequence preceding d_1. Datasets were transformed using a shifted sine bell with digital resolutions of 0.6 Hz or better. ^{1}H NMR data are reported in the following manner: chemical shift in ppm (multiplicity, integration, coupling constant in hertz). Only resolved resonances are reported. ^{13}C NMR data are reported in the following manner: chemical shift in ppm (multiplicity).

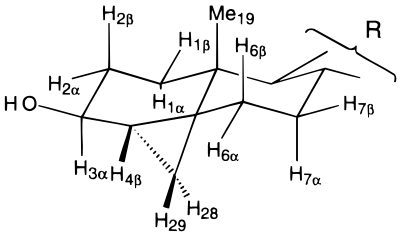
Energy Minimization of Structures. Steroids **1a** and **1b** were built and energy minimized using the Builder and Energy modules of InsightII v 95.0 by Biosym, Inc., San Diego, CA. The cvff parameter set was used for all calculations. Different starting conformers were minimized using molecular mechanics to determine their relative energies.

Cholest-4-en-3α-ol and Cholest-4-en-3*β*-ol. The reduction of cholest-4-en-3-one (1.00 g, 2.6 mmol) was performed according to the procedure of Dauben.[9] The mixture was purified by silica gel chromatography (EtOAc−hexanes 1:13 v/v) to give 95 mg (9%) of cholest-4-en-3α-ol as a colorless oil: [^{1}H NMR δ 5.25 (d, 1, J = 1.6), 4.13 (ddd, 1, J = 5.9, 6.9, 1.6)] and 873 mg (86%) of cholest-4-en-3β-ol as a white solid: mp 126.0−126.5 °C (lit.[9] mp 131−132 °C); ^{1}H NMR δ 5.43 (d, 1, J = 2.4), 4.05 (bm, 1).

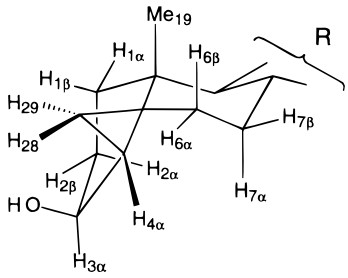
***4β,5β*-Methano-5α-cholestan-3*β*-ol (1b).** A mixture of Zn powder (2.03 g, 31.0 mmol) and dry DME (30 mL) maintained under a static atmosphere of Ar was sonicated for 20 min at 60 °C. Alcohol cholest-4-en-3β-ol (600 mg, 1.55 mmol) and a small crystal of I_2 were added to the solution, followed by dropwise addition of CH_2I_2 (936 μL, 11.6 mmol) over 5 min with continuous sonication and heating. After the DME reached reflux, sonication and heating were continued for another 4 h. The mixture was then diluted with Et_2O (40 mL), followed by slow addition of saturated aqueous NH_4Cl (30 mL). The mixture was filtered through a pad of silica. The pad was washed three times with Et_2O. The combined organic extracts were washed with 5% (w/v) aqueous $Na_2S_2O_3$, H_2O and brine, dried ($MgSO_4$), and concentrated by rotary evaporation. The yellow residue was immediately chromatographed on silica gel (EtOAc−toluene 1:9 v/v) to afford 517 mg of **1b** as a clear colorless oil (83%). Subsequent recrystallization from acetone produced white crystals: mp 87.0−88.5 °C (lit.[9] mp 94.0−95.0 °C); ^{1}H NMR see Tables 1 and 2; ^{13}C NMR δ 63.87 (d), 56.54 (d), 56.29 (d), 46.01 (d), 42.34 (s), 40.07 (t), 39.47 (t), 36.12 (t),

(18) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923−2924.
(19) Rance, M.; Sørensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479−485.
(20) Piatini, U.; Sørenson, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800−6801.
(21) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286−292.

Scheme 3



1a



1b

35.76 (d), 35.59 (d), 34.29 (t), 32.96 (s), 30.70 (t), 30.43 (s), 28.25 (t), 27.98 (d), 27.46 (d), 26.36 (t), 26.35 (t), 24.27 (t), 23.79 (t), 22.79 (q), 22.53 (q), 21.54 (t), 21.36 (q), 18.63 (q), 11.88 (q), 10.55 (t). Anal. Calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 83.93; H, 12.07.

4α,5α-Methano-5β-cholestan-3-one (2a). Treatment of cholest-4-en-3α-ol (263 mg, 0.7 mmol) in DME (6 mL) with Zn (891 mg, 13.6 mmol), a small crystal of I_2, and CH_2I_2 (406 μL, 5.04 mmol), under identical conditions as those described for **1b**, yielded 213 mg of 4α,5α-cyclopropano-5β-cholestan-3α-ol (78%) as a white solid: mp 102.5−103.0 °C (lit.[9] mp 105−106 °C). Careful addition of CrO_3 (300 mg, 3.0 mmol) to a solution of dry pyridine (485 μL, 6.0 mmol) in CH_2Cl_2 (5 mL) resulted in a deep red solution that was stirred for 15 min at rt. To this mixture was added a solution of 4α,5α-cyclopropano-5β-cholestan-3α-ol (213 mg, 0.53 mmol) in CH_2Cl_2 (5 mL), which upon addition of 4α,5α-cyclopropano-5β-cholestan-3α-ol changed from deep red to black. The formation of a black precipitate was observed. After 2 h of continuous stirring, the solution was decanted from the residue. The residue was washed with Et_2O (3×), and the combined organic extracts were washed with 5% NaOH (3×), 5% HCl, 5% $NaHCO_3$, and brine, dried ($MgSO_4$), and evaporated to dryness to afford 206 mg of **2a** (97%) as a white solid: mp 134.0−134.5 °C (lit.[9] mp 136−137 °C); ^{1}H NMR δ 2.19 (dd, 1, J = 9.75, 5.25), 2.09 (ddd, 1, J = 13.5, 3.9, 3.6), 1.97 (ddd, 1, J = 12.3, 3.0, 3.0), 1.80 (bm, 1); ^{13}C NMR δ 209.71 (s), 56.27 (d), 56.01 (d), 51.99 (d), 42.59 (s), 39.88 (t), 38.47 (t), 36.11 (t), 35.90 (d), 35.76 (d), 34.93 (d), 34.43 (s), 32.73 (t), 32.32 (t), 30.76 (t), 30.71 (t), 28.20 (t), 27.98 (d), 24.20 (t), 23.81 (t), 22.80 (q), 22.54 (q), 21.67 (t), 18.62 (q), 18.62 (t), 17.80 (q), 12.01 (q); mass spectrum (EI, 70 eV) m/z 398. Anal. Calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 84.15; H, 11.69.

4β,5β-Methano-5α-cholestan-3-one (2b). Treatment of **1b** (100 mg, 0.25 mmol) in CH_2Cl_2 (1 mL) with CrO_3·pyridine complex (1:2) under identical conditions as those described for **2a** gave 91 mg of **2b** (91%) as a white solid: mp 82.0−84.0 °C (lit.[9] mp 89.0−89.5 °C); ^{1}H NMR δ 2.09 (m, 3), 1.96 (dd, 1, J = 12.7, 3.42), 1.81 (bm, 1), 1.62 (m, 2); ^{13}C NMR δ 210.11(s), 56.17 (d), 56.12 (d), 45.86 (d), 42.39 (s), 39.75 (t), 39.46 (t), 36.81 (s), 36.08 (t), 35.76 (d), 35.50 (d), 35.08 (s), 34.28 (s), 32.47 (t), 32.28 (t), 30.10 (t), 28.18 (t), 27.96 (d), 27.60 (t), 24.16 (t), 23.81 (t), 22.77 (q), 22.52 (q), 21.65 (t), 20.63 (q), 18.59 (q), 17.83 (t), 11.91 (q); mass spectrum (EI, 70 eV) m/z 398. Anal. Calcd for $C_{28}H_{46}O$: C, 84.36; H, 11.63. Found: C, 84.39; H, 11.67.

4α,5α-Methano-5β-cholestan-3β-ol (1a). The reduction of **2a** (148 mg, 0.371 mmol) was performed using the procedure of Dauben.[9] Recrystallization from MeOH and acetone produced 143 mg of **1a** (96%) as colorless plates: mp 126.0−127.0 °C (lit.[12] mp 131−132 °C); ^{1}H NMR see Tables 1 and 2; ^{13}C NMR δ 6.87 (d), 56.29 (d), 56.26 (d), 52.04 (d), 42.68 (s), 40.00 (t), 39.47 (t), 36.13 (t), 36.06 (d), 35.77 (d), 33.25 (s), 32.93 (t), 30.82 (s), 30.14 (t), 29.37 (t), 28.21 (t), 27.97 (d), 27.97 (t), 26.69 (d), 24.14 (t), 23.81 (t), 22.78 (q), 22.52 (q), 21.15 (t), 19.20 (q), 18.62 (q), 13.08 (t), 12.11 (q). Anal. Calcd for $C_{28}H_{48}O$: C, 83.93; H, 12.07. Found: C, 83.78; H, 12.04.

Activity Assay with 1a and 1b. Recombinant *Streptomyces* cholesterol oxidase was purified as described from *Escherichia coli*.[8] The assay conditions were 1.13 mM phenol, 0.87 mM 4-aminoantipyrine, and 10 U of horseradish peroxidase in 50 mM sodium phosphate, pH 7.0 buffer, with 0.025% (w/v) Triton X-100 at 37 °C, 2.1 nM cholesterol oxidase. Steroids were added as a propan-2-ol solution to a final concentration of 50 μM. The final assay mixture was never more than 1.6% propan-2-ol. The activities of the cyclopropyl steroids **1a**,**b** were determined by following the rate of formation of H_2O_2 and thus, indirectly, 3β-alcohol oxidation. The formation of quinone imine at 510 nm was followed as a function of time.

Analysis of Products of the Reaction Catalyzed by Cholesterol Oxidase with 1a and 1b as Substrates. The product of enzymatic turnover was isolated from an activity assay mixture that did not contain horseradish peroxidase, phenol, or 4-aminoantipyrine. The reaction product was isolated as previously described.[7] The product from the enzymatic reaction of **1a** co-spotted on TLC (1:9 EtOAc:toluene) with **2a** and had m/z 398 (EI, 70 eV). Upon treatment of the enzymatic product with $LiAlH_4$ (as described for the preparation of **1a**), the major product co-spotted with **1a**. The product from the enzymatic reaction of **1b** co-spotted on TLC (1:9 EtOAc:toluene) with **2b** and had m/z 398 (EI, 70 eV). Upon treatment of the enzymatic product with $LiAlH_4$ (as described for the preparation of **1a**), the major product co-spotted with **1b**.

Assay for Irreversible Inhibition. Steroids **1a**, **1b**, **2a**, and **2b** were incubated separately with 90 nM cholesterol oxidase in 50 mM sodium phosphate, pH 7.0 buffer, with 0.025% (w/v) Triton X-100 at 37 °C. At various time intervals, the amount of active cholesterol oxidase remaining was measured by removing an aliquot (20 μL), diluting to 1 mL with assay buffer, adding 50 μM cholesterol, and following the appearance of cholest-4-en-3-one at 240 nm (ϵ_{240} = 12 100 M^{-1} cm^{-1} [22]). The incubations were followed for 5 days.

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Supporting Information Available: Reaction scheme and ^{1}H NMR, DQF-COSY, and NOESY spectra of all compounds (11 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS. See any current masthead page for ordering information.

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(22) Smith, A. G.; Brooks, C. J. W. *Biochem. J.* **1977**, *167*, 121−129.