A C_6-Flavin Adduct Is the Major Product of Irreversible Inactivation of Cholesterol Oxidase by 2α,3α-Cyclopropano-5α-cholestan-3β-ol

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Abstract: We have synthesized 2α,3α-cyclopropano-5α-cholestan-3β-ol and tested it as a substrate and inhibitor of cholesterol oxidase. The cyclopropylsterol irreversibly inhibits cholesterol oxidase with a $k_{inact} = 0.010$ min^{-1} and $K_i = 36$ μM. Efficient inactivation requires the general base His^{447}. Two FAD−steroid adducts were isolated by reversed-phase HPLC. The UV/vis, fluorescence and mass spectra of the adducts suggest that the FAD cofactor acts as an electrophile in a cyclopropoxide ring-opening reaction to form a C_6-alkylated flavin (68%) and either an N_5 flavin adduct or a cyclic N_5-C_{4a} flavin adduct (32%). Cyclopropoxide ring-opening to form a C_6−FAD adduct represents a new approach to flavoenzyme inhibition.

Introduction

Cholesterol oxidase (EC 1.1.3.6) has emerged as a versatile enzyme with practical uses other than primary metabolism of cholesterol by the Gram-positive soil bacteria that secrete it. The enzyme is used to assay serum cholesterol levels, and recently, its larvicidal properties were discovered.[1−3] Thus, its economic impact is and will continue to be significant. In addition, cholesterol oxidase is produced by *Mycobacterium tuberculosis*, although its metabolic function in these mycobacteria has not yet been elucidated.[4,5] To gain insight into the reactivity and mechanism of this enzyme, we have synthesized cyclopropyl steroids as mechanism-based inactivators. Cyclopropanes are widely used as mechanistic probes of enzyme catalysis.[6,7] They have been employed with a variety of cofactor-dependent enzymes, ranging from P-450[8,9] and NAD[10] to PLP[11] and flavin[12] cofactors. Furthermore, in the case of cholesterol oxidase, an efficient irreversible inhibitor would allow structure determination of a cholesterol-bound enzyme by X-ray crystallography. Thus far, only a structure with dehydroepiandrosterone, a C_{17}-ketosteroid that is more soluble than cholesterol, has been obtained.[13] The structure of the cholesterol complex is of interest to be able to understand, at an atomic level, the specificity for steroids with different C_{17} substituents. We report here the synthesis of a mechanism-based, irreversible cyclopropyl steroid inhibitor of cholesterol oxidase, and the characterization of the flavin-steroid adducts formed.

Cholesterol oxidase catalyzes the oxidation and isomerization of cholesterol into cholest-4-en-3-one, via the intermediate cholest-5-en-3-one (Scheme 1).[14] It is a monomer (57 kD) with a single active site for both oxidation and isomerization, and requires one FAD per active site.[13,15,16] The oxidation of cholesterol to the intermediate, cholest-5-en-3-one, may proceed via a radical intermediate or via direct hydride transfer and is general base-catalyzed by His^{447}, Asn^{485}, and Wat^{541}.[17,18] We reasoned that a cyclopropyl steroid such as **1** could undergo anionic electrophilic or radical ring-opening in the active site. In the crystal structure, the FAD is positioned under the α-face of the sterol dehydroepiandrosterone to receive the 3α-hydrogen and could act as an electrophile to aid anionic ring-opening of the cyclopropanol (Figure 1). Thus, we undertook the synthesis of **1** and its assay with cholesterol oxidase.

Results and Discussion

Synthesis of 1. We synthesized steroid **1** using a modification of the procedures of Templeton et al.[19] for the cyclopropanation

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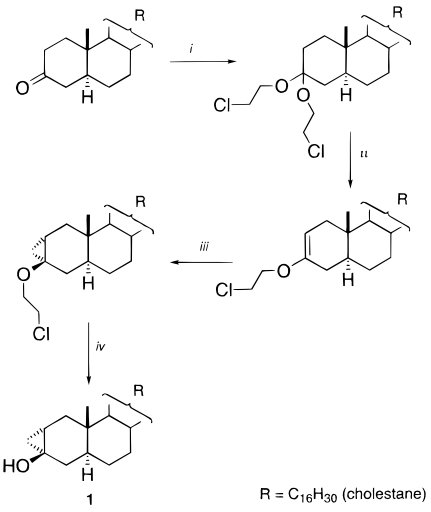
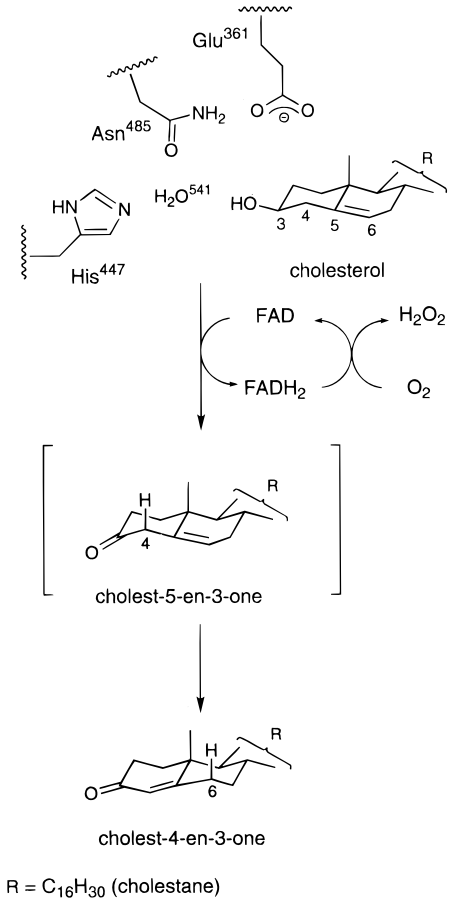


1

Figure 1. Synthetic scheme for preparation of **1**. (i) 2-chloroethanol, *p*-toluenesulfonic acid, benzene, molecular sieves. (ii) reduced pressure, reflux. (iii) Zn, CH_2I_2, sonication. (iv) *n*-BuLi, Et_2O.

Scheme 1



of androstanones (Figure 1). 5α-Cholestan-3-one was converted to the 3,3-(2-chloroethoxy) ketal under acidic conditions. Pyrolysis of the ketal yielded 3-(2-chloroethoxy)-5α-cholest-2-ene. Cyclopropanation of the enol ether under Simmons−Smith conditions and treatment of the chloroethoxy ether with *n*-butyllithium provided 2α,3α-cyclopropano-5α-cholestan-3β-ol in 13% overall yield.

Inhibition of Wild-Type by 1. We did not observe catalytic redox cycling of the flavin when **1** was incubated with recombinant *Streptomyces* cholesterol oxidase.[20] That is, **1** is not a substrate. However, incubation of cholesterol oxidase with **1** resulted in a pseudo-first-order loss of enzyme activity as determined by oxidation of cholesterol. This inactivation was concentration-dependent, and from a plot of k_{obs} vs [I] were obtained K_i and k_{inact} values of 36 μM and 0.010 min^{-1}, respectively (Table 1). No enzyme activity was recovered upon extensive dialysis, indicating that the inhibition is irreversible. Incubation of cholesterol oxidase with **1** in the presence of the substrate dehydroepiandrosterone reduced the rate of inactivation, confirming that inactivation is active-site directed. Wild-type cholesterol oxidase will catalyze the isomerization of cholest-5-en-3-one to cholest-4-en-3-one when cholest-5-en-3-one is used directly as a substrate. Cholesterol oxidase inactivated by **1** no longer catalyzes isomerization of cholest-5-en-3-one. This suggests that inhibition by **1** is active-site-directed and a covalent complex with enzyme or FAD is formed that sterically blocks the active site.

Table 1. Catalytic and Inactivation Rate Constants for Wild-Type and Mutant Cholesterol Oxidases

substrate/inhibitor enzyme	cholesterol k_{cat} (sec^{-1})[a]	**1** k_{inact} (min^{-1})[b]
wild-type	44 ± 2	0.010 ± 0.001
E361Q	1.4 ± 0.1	0.008 ± 0.001
H447Q	0.32 ± 0.01	0.0009 ± 0.0003[c]
H447N	0.010 ± 0.003	n.d.[d]

[a] Measured by H_2O_2 formation with HRP coupling. [b] Measured under pseudo-first-order conditions, [1] ≫ [E]. [c] Determined at [**1**] = 50 μM. [d] Not detected.

Buffer-Catalyzed Ring-Opening of 1. k_b was measured by incubating **1** in assay buffer, adding aliquots to enzyme as a function of time, and measuring k_{obs} to determine the concentration of **1** that remained in the incubation. k_b was 0.001 min^{-1}.

Inhibition of Mutants by 1. We further probed the mechanism of inactivation using mutant enzymes in which active-site residues Glu^{361} and His^{447} were substituted with amides. Glu^{361} is the base for isomerization of cholest-5-en-3-one to cholest-4-en-3-one.[14] Mutant E361Q will catalyze oxidation, but not isomerization.[14] This mutant is inactivated by **1** at the same rate as wild-type (Table 1). Thus, the mechanism of inactivation by **1** does not require the Glu^{361} base. When the general base, His^{447} is mutated, the k_{cat}'s for oxidation by mutants H447N and H447Q are 120-fold and 4400-fold less than wild-type. Primary and solvent deuterium isotope effects suggested that 3-hydroxyl deprotonation is the rate-determining step in the mutant-catalyzed reactions.[17,21] Isomerization is not affected by histidine to glutamine mutation. Interestingly, H447Q was inactivated by **1** 10 times more slowly than wild type, and no inactivation of H447N was observed (Table 1). Presumably, the rate of inactivation of H447N is sufficiently slow that buffer-catalyzed ring-opening of **1** competes effectively with ring-opening in the active site and inhibition. We conclude that efficient inactivation by **1** requires the presence of the active-site general base, His^{447}, to deprotonate the 3-hydroxyl of **1** and form a cyclopropoxide. The parallels between reduced rates of cholesterol oxidation by His^{447} mutants and rates of inactivation by **1** provide a third piece of evidence that inhibition is active-site directed.

Analysis of Inhibition Products. Mutation of His^{447} to Asn results in a 1.9 Å shift of Wat^{541} that positions the substrate relative to the FAD.[21] We reasoned that failure to inactivate H447N with **1** was because the FAD was not aligned with the

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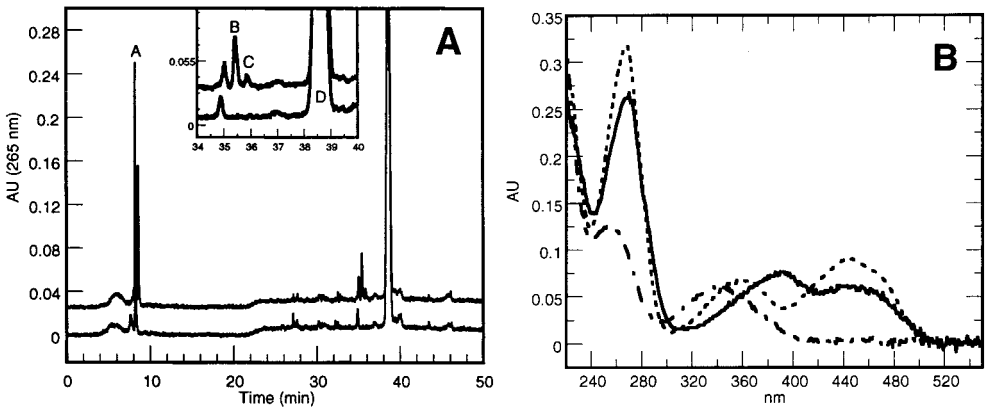

Figure 2. (A) HPLC traces of methanol soluble fraction of (bottom) wild-type cholesterol oxidase, and (top) **1**-inactivated cholesterol oxidase (75% inhibited). Peak A is FAD, peak B is FAD−steroid adduct **2**, Peak C is FAD−steroid adduct **3/4**, and D is Triton X-100. The other small peaks are present in buffer only controls. (B) UV/vis spectrum of FAD (- - -), **2** (—), and **3/4** (—•—). The spectrum of FAD was obtained in the same solvent as that of the adducts.

inhibitor. The FAD cofactor is an excellent electrophile that, in the wild-type enzyme, is appropriately positioned to form a covalent bond with C_{28} of **1**. This is based on modeling **1** onto the dehydroepiandrosterone-bound structure of cholesterol oxidase.[13] Moreover, anionic cyclopropyl ring-openings are subject to electrophilic catalysis.[22] Therefore, we denatured the inactivated protein, and separated the protein and the cofactor fractions by addition of methanol. The MALDI mass spectrum of the cofactor fraction revealed that two new species with $MH^+ = 1184.5$ and 1186.4 were formed. These molecular weights correspond to the formation of oxidized and reduced covalent complexes between **1** and FAD, respectively. Upon reversed-phase HPLC analysis, two new FAD species were observed in the cofactor fraction (Figure 2A). The species increased retention times suggested that the species are FAD−steroid adducts in agreement with the mass spectrum. No protein adducts were observed by HPLC. The UV/visible spectrum of the major new FAD species **2** has maxima at 390 and 446 nm, in addition to the adenine absorbance at 265 nm (Figure 2B). This spectrum indicates that the isoalloxazine ring is oxidized. However, the maxima are red-shifted compared to FAD isolated from the wild-type enzyme (Figure 2B). This spectrum is consistent with alkyl substitution at C_6 of the isoalloxazine ring (Figure 3, **2**), as is the fluorescence emission maximum at 530 nm.[23−25] This species corresponds to the $MH^+ = 1184.4$ peak in the mass spectrum (1184.2 expected). The minor new FAD species **3/4** has an absorbance maximum at 340 nm (Figure 2B), and a fluorescence emission maximum at 480 nm. This is consistent with an N_5-reduced flavin adduct (Figure 3, **3**) or a cyclic N_5-C_{4a}-adduct (Figure 3, **4**).[23,26] This species corresponds to the $MH^+ = 1186.4$ peak in the mass spectrum (expected 1186.2) The fluorescence emission maximum of a C_{4a}-adduct is typically around 530 nm.[23] A similar cyclic adduct was the major product isolated when methanol oxidase was inactivated with cyclopropanol.[27] In that case, no C_6-adduct was observed. Based on integration of the HPLC chromatogram at 265 nm, 68% of the inhibited enzyme is C_6-adduct, **2**, and 32% is the N_5-C_{4a}-adduct, **3/4**.

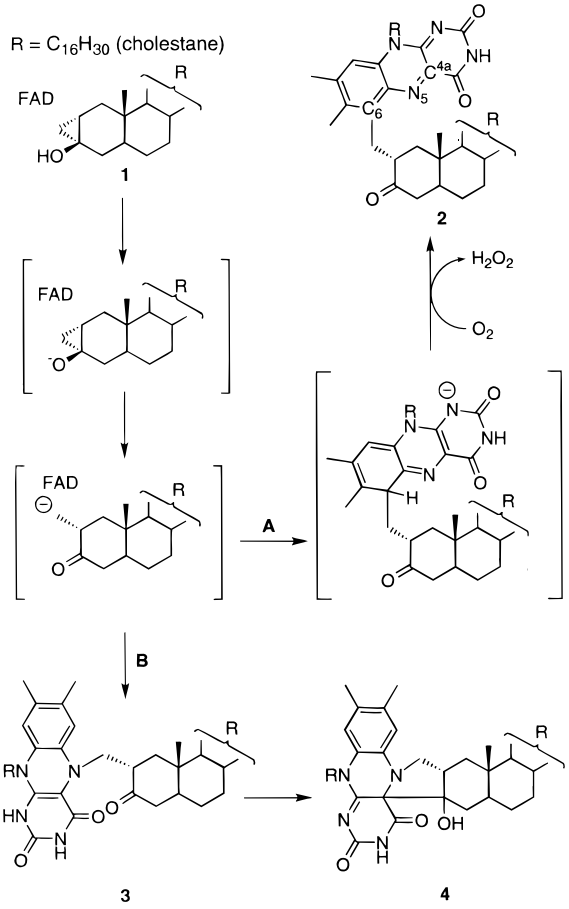

Figure 3. Proposed mechanism for inactivation of cholesterol oxidase by 2α,3α-cyclopropano-5α-cholestan-3β-ol, **1** and structures of adducts **2, 3** and **4**.

Dynamic Light Scattering of Inactivated Enzyme Complex. To assess the amount of aggregation of the inactivated enzyme complex, dynamic light scattering experiments were performed. A 36 μM solution of cholesterol oxidase in 50 mM phosphate buffer was assayed. At 22 °C, the wild-type enzyme had a polydispersity of 9.8% which corresponds to an apparent molecular weight of 49 kD. This measurement is consistent with the monomeric molecular weight of cholesterol oxidase, 55 kD.

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Under the same conditions, the inactivated enzyme had a polydispersity of 52%, which corresponds to an apparent molecular weight of 2000 kD. Thus, the inactivated enzyme aggregates into a high-molecular weight complex. Addition of organic cosolvent, sodium chloride, or β-octylthioglucoside did not reduce the size of the aggregate. The aggregation state was equally insensitive to temperature. This aggregation precluded further spectral characterization of the inactivated complex.

Proposed Mechanism of Inactivation. There are two other examples of irreversible inhibitors that result in C_6−FAD adducts. The flavoenzyme D-lactate dehydrogenase from *Megasphaera elsdenii* is irreversibly inactivated by 2-hydroxy-3-butynoate, presumably via an allenic intermediate.[28] After $NaBH_4$ reduction, an adduct that bridges N_5 to C_6 was isolated. In a preliminary report, a C_6−FAD adduct was identified as a minor product of general acyl-CoA dehydrogenase inhibition by methylenecyclopropylacetyl-CoA.[29] The ring-opened adduct formed results from carbon deprotonation catalyzed by the enzyme. Covalent C_6-adducts are also naturally occurring. Both triethylamine and diethylamine dehydrogenases contain FMN that is covalently linked to a protein cysteine thiol via a thioether at C_6.[30] Thus, there is precedent for nucleophilic substitution at C_6 of enzyme-bound flavins. Moreover, in model systems, C_6 is also an electrophilic position.[31]

On the basis of our inhibition and spectral data, we propose a mechanism in which anionic ring-opening and attack of the methylene anion on C_6 of the isoalloxazine ring is followed by O_2 oxidation to generate an oxidized C_6−flavin adduct, **2**, (Figure 3). The N_5−adduct, **3**, would be formed in an analogous fashion via anionic attack at N_5. The C_{4a} carbon then becomes nucleophilic, and **3** could further react via cyclization with the steroid ketone to form the hemi-ketal **4** (Figure 3). Mewies et al. have recently suggested that a flavin iminoquinone methide is the intermediate in the formation of covalently modified enzyme-bound flavins.[32] The iminoquinone methide could also be an intermediate during inactivation by **1**.

Alternatively, reaction of the ring-opened anion with the FAD could occur in two steps. First, single e^- transfer from the methylene anion to the FAD could form the semiquinone and methylene radicals. Second, collapse of the radicals would form either adduct. Sherry and Abeles suggested that inactivation of methanol oxidase by cyclopropanol proceeds via a radical mechanism because the rate of inactivation was much greater than the rate of anionic ring-opening.[27] In the case of cholesterol oxidase, these two mechanistic possibilities cannot be distinguished kinetically because the rate of inactivation (2×10^{-4} sec^{-1}) is much slower than radical ring-opening (1.8×10^8 s^{-1}) and is comparable to the rate of anionic ring-opening (1.5×10^{-4} s^{-1}).[22] Detailed knowledge of the rates of individual steps during inactivation are required to make this distinction.

In summary, cyclopropoxide ring-opening to form a C_6−FAD adduct represents a new approach to flavoenzyme inhibition. Future experiments will focus on finding conditions under which the inactivated enzyme is monomeric to facilitate structure elucidation.

Experimental Section

Materials. Cholest-4-en-3-one, cholest-5-en-3-one, 4-aminoantipyrine, *p*-hydroxyphenylacetic acid, and Triton X-100 were from Aldrich Fine Chemical Co., (Milwaukee, WI). Cholesterol and horseradish peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). The plasmid for heterologous expression of *Streptomyces* cholesterol oxidase, pCO202 has been described previously,[33] and is a derivative of pCO117, a generous gift from Y. Murooka.[20] All other chemicals and solvents, of reagent or HPLC grade, were supplied by Fisher Scientific (Pittsburgh, PA). Water for assays and chromatography was distilled, followed by passage through a Barnstead NANOpure filtration system to give a resistivity better than 18 MΩ.

General Methods. DME was dried over and distilled from sodium/benzophenone ketyl under N_2 prior to use. Benzene was dried over and freshly distilled from calcium hydride under N_2 prior to use. A Shimadzu UV2101 PC spectrophotometer was used for assays. Fluorescence measurements were taken on an Spex Fluorolog 3-11 spectrofluorometer. Dynamic light scattering measurements were obtained on a Protein Solutions DynaPro instrument. A Rainin HPLC with a PDA-1 photodiode array detector and a C_4 Vydac column was used to obtain chromatograms and spectra of inhibition reactions. The ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini-300 referenced to $CDCl_3$ as the internal standard. ^1H NMR data are reported in the following manner: chemical shift in ppm (multiplicity, integrated intensity, coupling constant in Hz). Only resolved steroid resonances are given. The buffers used were A: 50 mM sodium phosphate, pH 7.0 + 0.025% Triton X-100 (w/v); B: buffer A + 0.020% BSA (w/v); C: 95:5, 5 mM ammonium acetate, pH 6.5: CH_3CN; D: 85:15, CH_3CN:propan-2-ol. E: 95:5, H_2O:CH_3CN.

3,3-Bis(2-chloroethoxy)-5α-cholestane. In a 100 mL two-neck flask fitted with a Dean−Stark apparatus, dry benzene (20 mL) was added to cholestan-3-one (1.00 g, 2.60 mmol), *p*-toluenesulfonic acid (100 mg, 0.64 mmol) and crushed molecular sieves. 2-Chloroethanol (10 mL, 150 mmol) was added and the reaction heated at reflux for 26 h. The reaction was cooled and poured into excess cold 5% $NaHCO_3$ (100 mL). The resulting solution was filtered, extracted with Et_2O (3 ×), washed (3 ×), dried (Na_2SO_4), filtered and concentrated in vacuo. 3,3-Bis(2-chloroethoxy)-5α-cholestane (32% yield) was recovered as a yellowish solid after flash chromatography (95:5 hexanes:EtOAc). ^1H NMR (300 MHz) δ 3.74 (t, 2, $J = 5.9$), 3.64 (t, 4, $J = 3.9$), 3.58 (t, 2, $J = 5.9$); LRMS (EI) *m*/*z*, 529 (MH^+ 7%).

3-(2-Chloroethoxy)-5α-cholest-2-ene. The reaction was carried out according to the procedure of Templeton et al.[19] Eighty-five percent of the enol was isolated after column purification (95:5, hexanes:EtOAc) as a mixture of 3-(2-chloroethoxy)-5α-cholest-2-ene and 3-(2-chloroethoxy)-5α-cholest-3-ene (11:2). ^1H NMR (3-(2-Chloroethoxy)-5α-cholest-2-ene) δ 4.47 (d, 1, $J = 5.7$), 3.88 (m, 2), 3.67 (t, 2, $J = 6.0$). ^{13}C NMR δ 152.675, 93.218, 66.260, 56.419, 56.309, 53.798, 42.489, 42.068, 41.723, 40.012, 39.515, 38.187, 36.184, 35.801, 35.570, 34.708, 32.170, 31.696, 28.506, 28.229, 28.001, 24.234, 23.866, 22.811, 22.561, 21.211, 18.692, 11.981, 11.628. LRMS (FAB, NBA) *m*/z 449 (MH^+, 100%).

2α,3α-methano-3β-(2-chloroethoxy)-5α-cholestane. Zn powder (562 mg, 8.6 mmol) and a small crystal of I_2 were added to a 10 mL oven-dried pear shaped flask fitted with a reflux condenser. The flask was flushed with Ar, DME (1.0 mL) was added, and the solution was sonicated at 65 °C for 15 min to activate the Zn/I_2 complex. A solution of 3-(2-chloroethoxy)-5a-cholest-2-ene (193 mg, 0.430 mmol) in DME (2.5 mL) was added, followed by dropwise addition of CH_2I_2 (346 μL, 4.30 mmol). After the reaction was sonicated at reflux for 20 h, an additional aliquot of CH_2I_2 was added and the reaction allowed to proceed for an additional 6 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 chromatographed on silica gel (hexanes:EtOAc, 95:5) to afford

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145 mg of 2α,3α-methano-3β-(2-chloroethoxy)-5α-cholestane as a clear colorless oil (73%). ^{1}H NMR δ 3.7 (m, 2), 3.5 (t, 2, J = 5.5), 0.43 (ddd, 1, J = 11.1, 11.1, 4.5), 0.15 (dd, 1, J = 5.4, 5.4); ^{13}C NMR δ 66.681, 60.695, 56.416, 56.275, 53.840, 43.252, 42.356, 39.982, 39.944, 39.868, 39.504, 36.161, 35.774, 34.746, 31.825, 31.139, 29.348, 28.195, 27.990, 24.234, 23.840, 22.808, 22.553, 20.972, 18.669, 18.646, 18.627, 17.144, 12.307, 11.924.

2α,3α-Cyclopropano-5α-cholestan-3β-ol (1). A N_2 flushed 10 mL oven-dried flask with syringe outlet was fitted with a reflux condenser and magnetic stir bar. 2α,3α-methano-3β-(2-chloroethoxy)-5α-cholestane (126 mg, 0.281 mmol) was added in DME (3.0 mL), stirred, and chilled to 0 °C for 30 min. The reaction was initiated by slow dropwise addition of 2.5 M *n*-butyllithium in hexanes (675 μL, 1.69 mmol) with stirring. After 50 min, the reaction was quenched with saturated NH_4Cl (3.0 mL), diluted with Et_2O, and transferred to a separatory funnel. The organic layer was washed with H_2O and brine, dried ($MgSO_4$), and concentrated in vacuo. The crude solid was chromatographed on silica gel (toluene:EtOAc, 9:1) to afford 74 mg of 2α,3α-cyclopropano-5α-cholestan-3β-ol as a white solid (65%): mp 123.5−127.0 °C. ^{1}H NMR δ 0.46 (ddd,1, J = 10.8, 10.8, 4.5), 0.17 (dd,1, J = 5.7, 5.7); ^{13}C NMR δ 56.431, 56.279, 54.819, 53.715, 42.353, 40.198, 40.148, 39.989, 39.504, 36.165, 35.801, 35.778, 35.539, 34.906, 31.818, 39.234, 28.195, 27.990, 24.242, 23.840, 22.808, 22.553, 20.9732, 20.114, 18.669, 18.149, 12.292, 11.916; HRMS (EI) Calcd: 400.370517 ($C_{28}H_{48}O$). Found: 400.369800.

Activity Assay with 1. Recombinant *Streptomyces* cholesterol oxidase was purified as described from *Escherichia coli*.[14] **1** was 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 activity was determined by following the rate of formation of H_2O_2, and thus indirectly, of $FADH_2$ oxidation. The standard assay conditions were the same as the UV A_{240} assay with the addition of 1.0 mM *p*-hydroxyphenylacetic acid and 10 U of horseradish peroxidase. The reaction was initiated by the addition of 36 nM cholesterol oxidase. The reaction was followed by excitation at 325 nm and monitoring the fluorescence emission at 415 nm (slits = 3.0 nm) using an HRP-coupled assay to quantitate the rate of formation of H_2O_2.

Activity Assay with Cholesterol and Cholest-5-en-3-one. Recombinant *Streptomyces* cholesterol oxidase was purified as described from *E. coli*.[14] The assay conditions were in buffer B 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 activity was determined by following the rate of formation of cholest-4-en-3-one at 240 nm (ϵ_{240} = 12 100 M^{-1} cm^{-1}).[34]

Assay for Irreversible Inhibition. Steroid **1** was incubated with 90 nM cholesterol oxidase in buffer A 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 buffer B containing 50 μM cholesterol, and following the appearance of cholest-4-ene-3-one at 240 nm (ϵ_{240} = 12 100 M^{-1} cm^{-1}).[34] The incubations were followed for 24 h. Inhibitor concentrations ranging from 5 μM to 150 μM were analyzed in the above manner.

Characterization of FAD Adduct by HPLC. Cholesterol oxidase (2 mM) was incubated with 150 μM inhibitor in buffer A. When the remaining enzyme activity was less than 50% of the initial value, the reaction was terminated by the addition of MeOH (80% total volume). The protein was allowed to precipitate overnight in the dark. The solution was then passed through a 0.45 μ filter and concentrated to the initial reaction volume (0.5 mL) under a stream of N_2. The concentrated solution was loaded onto a C_4 RP-HPLC column and eluted with 100% buffer C for 10 min, followed by a linear gradient to 100% buffer D over 40 min, and was held at 100% D for 10 min.

Determination of k_{hyd} of 1. Steroid **1** was incubated in buffer A. Aliquots were removed and cholesterol oxidase added. The k_{obs} was measured as described above and used as to determine the concentration of **1** remaining.

Isolation of Inactivated Enzyme for Dynamic Light Scattering and Mass Spectrometry. The inactivated enzyme complex was precipitated with 3 M $(NH_4)_2SO_4^{2-}$ and centrifuged at 40 000 rpm in a swinging bucket rotor. The aggregated protein was resuspended in buffer A and loaded onto a Sephadex G-75 (32 cm × 20 mm) column equilibrated in buffer A. Fractions containing protein were combined and ultrafiltered (Ammicon, YM30 membrane) into buffer A. The inactivation and purification were repeated to ensure that the enzyme was completely inactivated. For dynamic light scattering, the solution was filtered (0.2 μm), and a 15 μL sample (36 μM) was loaded into the cuvette and the signal recorded. Light scattering measurements were performed at 22 and 4 °C in buffer E, buffer E + 6% *i*-propanol, buffer E + 500 mM NaCl, and buffer E + 0.4% β-octylthioglucoside. The apparent molecular weight was determined using the manufacturer's software. For mass spectrometry, 4 vol of MeOH were added to the inactivated enzyme, and the mixture was allowed to stand at 4 °C for 1 h. The mixture was centrifuged at 15 000 g, and the supernatant removed for mass spectral analysis. The concentrated solution was loaded onto a C_4 RP-HPLC column and eluted with 100% buffer E for 10 min, followed by a linear gradient to 100% buffer D over 40 min, and was held at 100% D for 10 min. The two adducts elute as a single peak under these conditions. The mixture was analyzed by MALDI-TOF mass spectroscopy. The positive ion spectrum of the cofactor fraction was acquired in the linear mode with a 300 ns delayed extraction on a Perseptive Voyager. A saturated CH_3CN solution of the matrix sinnapinic acid was used. The standards for calibration were insulin and the matrix ions.

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Supporting Information Available: Copies of spectra of all synthesized compounds (PDF). This material is available free of charge via the Internet, http://pubs.acs.org.

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