BIOORGANIC CHEMISTRY 19, 418–432 (1991)

Steroidal Acetylenes: Mechanism-Based Inactivators of Lanosterol 14α-Demethylase

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Received March 6, 1991

The design rationale, synthesis, and characterization of several acetylenic substrate analogs of sterol 14α-demethylase are described. Using a tritium release assay we have been able to attain the sensitivity required to characterize (3β)-32-(ethynyl)lanost-8-en-3-ol (**1**) as a time-dependent irreversible inhibitor of sterol 14α-demethylase. Additional studies suggest that this is a general characteristic of the steroidal acetylenes reported herein. The structural requirements for potent inhibition are in qualitative agreement with the known substrate preferences of the enzyme. © 1991 Academic Press, Inc.

INTRODUCTION

Lanosterol 14α-demethylase ($P450_{14DM}$) is the rate-limiting enzyme in the conversion of lanosterol to cholesterol in mammals and to ergosterol in fungi. Both the mammalian and the fungal lanosterol 14α-demethylases are cytochrome P450 containing enzymes which oxidatively remove the C-32 methyl group of lanosterol as formic acid (*1–4*). The reaction utilizes three equivalents each of oxygen and NADPH and proceeds through three independent steps (pathway illustrated in Fig. 1.). Although the mechanism of the final oxidation step to yield formic acid and 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol is unclear, the intermediate alcohol and aldehyde of the preceding steps have been identified (*5, 6*).

The azole antifungals are an important class of clinically useful agents which appear to prevent fungal growth by blocking $P450_{14DM}$ and hence ergosterol biosynthesis (*7*). The azoles are so named because they contain an imidazole or triazole moiety as the active pharmacophore. The binding of an azole nitrogen of these inhibitors to the iron-heme center of the enzyme has been demonstrated and correlated with the inhibition of substrate turnover (*8, 9*). The high affinity of P450 iron for the lone pair electrons of nitrogen is a general property of this class of enzymes. Hence, it is not surprising that inhibitors which exploit this property to achieve tight binding often lack specificity. For example, ketoconazole, which is a potent inhibitor of the fungal $P450_{14DM}$, also inhibits the mammalian enzyme

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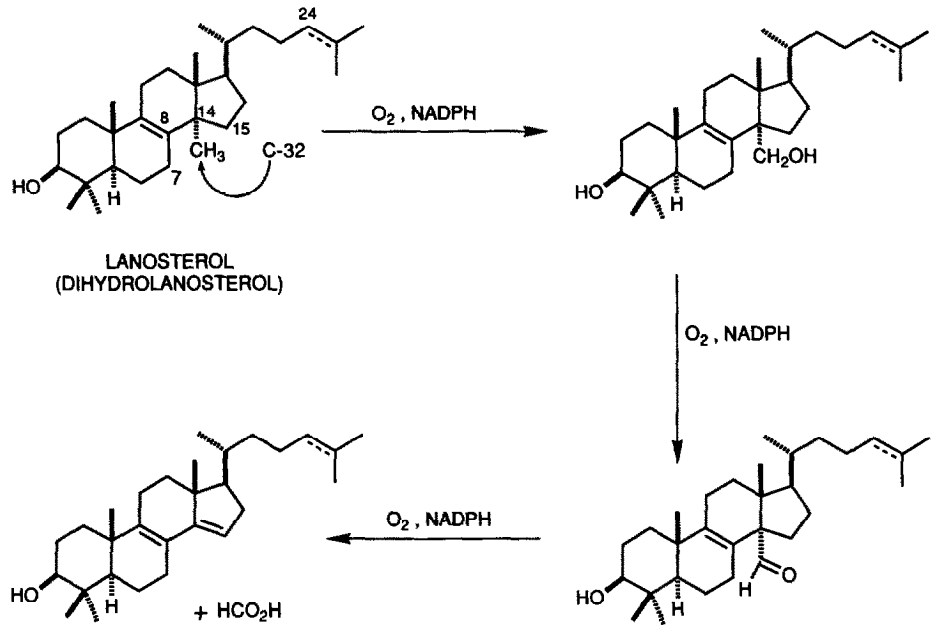
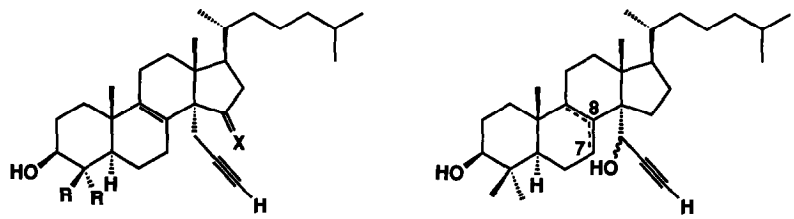


FIGURE 1

as well as many of the P450-dependent enzymes involved in steroid hormone biosynthesis (*10*).

Because of the proven therapeutic utility of the azole antifungals and the potential utility of selective inhibitors of the mammalian enzyme in the treatment of hypercholesterolemia (*11*), our goal was to prepare potent and selective inhibitors of 14α-demethylase. For the initial studies we chose to examine substrate analogs containing an acetylene as a latently reactive functional group. The terminal acetylene is a particularly attractive functional group because compounds containing this moiety are among the more efficient and predictable mechanism-based inactivators of P450 isozymes. Activation of the acetylene by P450 can lead to the formation of a covalent adduct with the active-site heme, rendering it catalytically inactive (*12*).The terminal acetylene functionality has been successfully used in the design of selective inhibitors of several P450 enzymes including the steroidal enzyme aromatase which catalyzes transformations very similar to that of $P450_{14DM}$ (*13–15*). Thus lanosterol derivatives containing an appropriately positioned acetylene group could potentially serve as mechanism-based inhibitors. This substrate analog-based approach would be expected to exhibit selectivity for 14α-demethylase and not inhibit other P450 enzymes.

When we began this work the minimal structural requirements for the substrates of the fungal and mammalian enzyme had not been well defined. The literature suggested that lanosterol was the preferred substrate for both the mammalian (rat liver) and the fungal (*Saccharomyces cerevisiae*) enzyme, but that dihydrolanosterol was also turned over (*1*, *16*). Recently this has been verified using reconstitu-



1, R = Me, X = 2H
2, R = Me, X = O
3, R = H, X = O

4a,b Δ8
5a,b Δ7

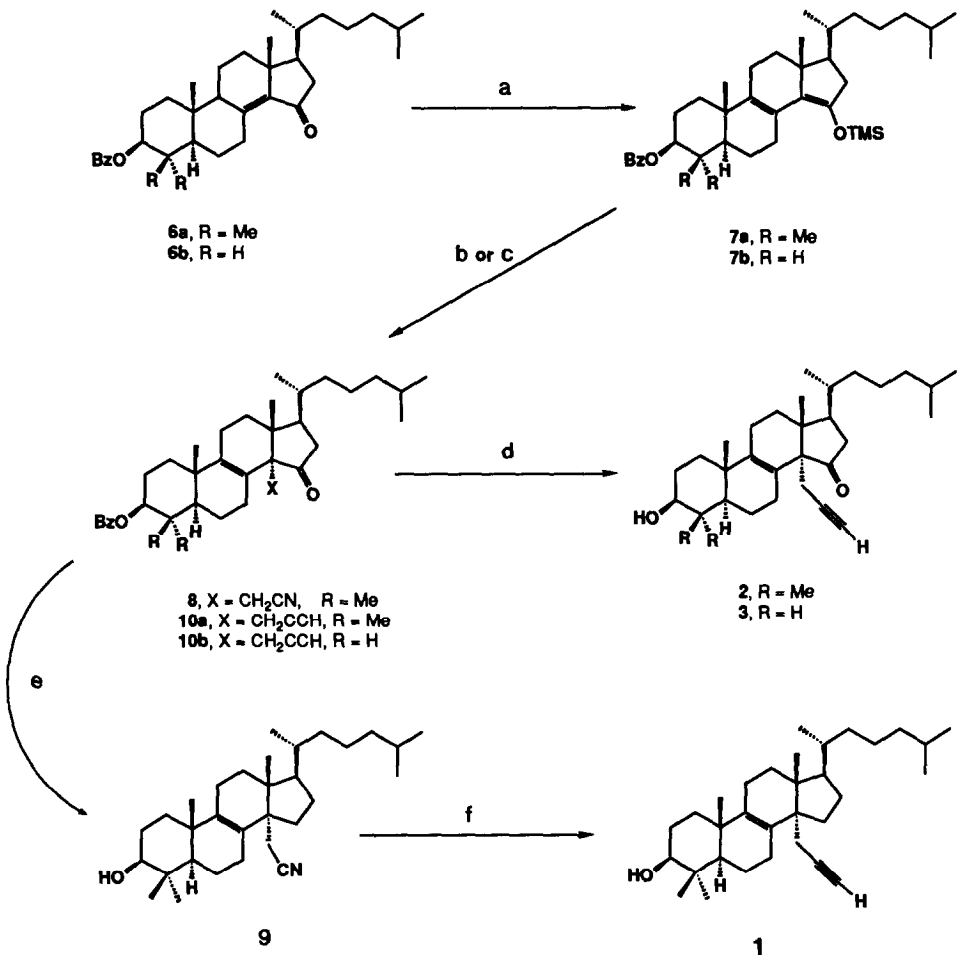
FIGURE 2

ted systems for the purified rat and yeast enzymes (*17, 18*). Furthermore, experiments undertaken to elucidate sterol biosynthetic pathways had demonstrated that 14α-methyl sterols which possessed the Δ7 nuclear double bond (*3*) or lacked the C-4 methyl groups (*19*) were converted to 14α-demethylated products, suggesting that they might also function as substrates for $P450_{14DM}$. We chose to prepare (3β)-32-(ethynyl)lanost-8-en-3-ol (**1**) and (3β,32*R,S*)-32-(ethynyl)lanost-8-en-3,32-diol (**4a** and **4b**), as potential mechanism-based inhibitors of $P450_{14DM}$, which mimic the initial substrate and the first intermediate, respectively. Compounds, **2, 3, 5a,** and **5b,** are examples of substrate-based inhibitors which were prepared to delineate and optimize the sterol structure for this class of mechanism-based inhibitors (Fig. 2). In all cases we chose to prepare analogs to 24,25-dihydrolanosterol as these compounds are more readily accessible.

This paper details the synthesis and characterization of several acetylenic inhibitors of rat liver microsomal $P450_{14DM}$. Using a tritium release assay (*20*) we have been able to attain the sensitivity required to characterize (3β)-32-(ethynyl)lanost-8-en-3-ol (**1**) as a time-dependent irreversible inhibitor of sterol 14α-demethylase. Additional studies indicate this is a general characteristic of the steroidal acetylenes reported herein. While this work was in progress, three steroidal acetylenes including compounds **5a** and **5b** were identified by Robinson as inhibitors of $P450_{14DM}$ (*21, 22*).

RESULTS AND DISCUSSION

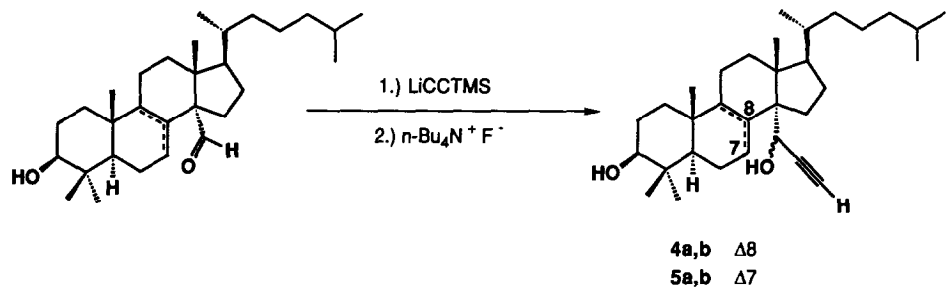
Chemistry. The development of an efficient procedure for the preparation of Δ8-14α-sterols proved to be the major synthetic problem. Of the routes which had been developed for the synthesis of 14α-functionalized sterols none was applicable to the regioselective preparation of the Δ8-isomers (*23*). Our solution was to prepare the thermodynamically more stable trimethylsilydienol ethers, **7a** and **7b,** from the enones, **6a** and **6b** (Scheme 1) using the equilibrating conditions developed by House (*24*). Using these conditions the desired 8,14-diene was formed in preference to the 7,14-diene (85 : 15 ratio). We have found that the fluoride-initiated



SCHEME 1. Reagents: (a) TMSCl, Et_3N, DMF, 140°C,18 h; (b) $BzMe_3N^+F'^-$, 4A sieves, propargyl bromide,THF; (c) $BzMe_3N^+F^-$, 4A sieves, iodoacetonitrile, THF; (d) 1 N LiOH, THF, MeOH, reflux 1–4 h; (e) (1) NH_2NH_2–HCl, NH_2NH_2, triethylene glycol, 130°C (2) KOH, 210°C; (f) (1) Dibal-H, CH_2Cl_2, 0°C, 1 h; 2 N HCl (2) $Ph_3P{=}CHCl$, THF, −78°C to 10°C; cool −78°C add LDA.

alkylation (*25*) of the dienol ethers proceeds in good to moderate yield with a variety of highly reactive electrophiles. For example, the reaction of **7a** with tritiated methyl iodide was used to prepare the radiolabeled 24,25-dihydrolanosterol used in the enzymatic assay (*20*).

Reaction of **7a** and **7b** with propargyl bromide using the fluoride-mediated alkyation provided **10a** (75%) and **10b** (46%). Small amounts (5% or less) of the Δ7-isomers arising from alkylation of the minor dienol ether isomers were detected in these alkylations. Recrystallization or a combination of chromatography and recrystallization was sufficient to remove most of the Δ7-isomers and yield compounds of high isomeric purity. Saponification of the benzoates **10a** and **10b**



SCHEME 2

produced **2** and **3** which contained no detectable Δ7 isomeric impurities (2% or less).

In an attempt to obtain **1** directly from **10a,** several deoxygenation strategies were investigated. The most direct approach, a Wolff–Kishner reduction, yielded a mixture of products including the 14α-allyl and propyl over-reduction products. A more circuitous route involving first reduction to a mixture of 15α-and β-alcohols, conversion of the major isomer (15α-isomer) to the thionophenyl carbonate, and finally deoxygenation by the procedure of Barton using tributyltin hydride was likewise unsuccessful. In this case the major products were those derived from the addition of the tin reagent to the acetylene.

These difficulties could be overcome by utilizing the nitrile functionality as a precursor for the acetylene. The reaction of iodoacetonitrile with **7a** affords **8** which is obtained directly by recrystallization in 70% yield (no detectable Δ7 isomer). The Wolff–Kishner reduction of **8** provided the deoxygenated nitrile **9** in low, but reproducible yield. Along with the desired product we obtained two side products in roughly equivalent quantity. These were the 8,14-diene formed by the elimination of the acetonitrile fragment and a product which appears to be a cyclic hydrazone resulting from an intramolecular cyclization of the initially formed hydrazone onto the nitrile. The remaining steps of DIBAL-H reduction of the nitrile, hydrolysis of the resulting imine to the aldehyde, Wittig reaction to yield the vinyl chloride, and *in situ* dehydrohalogenation of this product proceeded smoothly to afford **1** (56% from **9**).

The propargylic alcohols **4a,b** and **5a,b** were prepared from the respective 3β-hydroxy-lanost-8 or 7-en-32-al (Scheme 2).[2] Reaction of the aldehydes with an excess of the lithium anion derived from trimethylsilyl acetylene afforded a mixture of diastereomeric alcohols. In each case the diastereomers were first separated by chromatography and then deprotected with tetrabutylammonium fluoride. This provided **5a, 5b,** and **4a** as >95% pure isomers containing no more than 2% of any one isomeric impurity. The minor Δ8-isomer, **4b,** was obtained as a 9 : 1 mixture of **4b** and **4a.**

[2] The synthesis of the 32-oxolanostenols has been reported in the literature (*5*). We have developed alternative routes which will be detailed in separate manuscripts.

TABLE 1

Cofactor Requirements for 14α-Demethylase Inactivation by (3β)-32-(Ethynyl)lanost-8-en-3-ol

Condition	% Activity remaining[a]
Control	100
$-P450_{14DM}$	0
+ 100 nM inhibitor	47
− NADPH, + 100 nM inhibitor	114
− reductase, + 100 nM inhibitor	89
$-O_2$[b], + 100 nM inhibitor	84

Note. The final concentration of (3β)-32-(ethynyl)lanost-8-en-3-ol in the initial velocity assay mixture was 10 nM. A control initial velocity assay containing 10 nM (3β)-32-(ethynyl)lanost-8-en-3-ol still retained 91% activity. The control without inhibitor did not lose any activity during the incubation.

[a] Samples were preincubated for 10 min at 30°C with the buffered assay components described in the Experimental section (no substrate). Additions or omissions were as indicated in the table. Following incubation, an aliquot was removed and diluted 10-fold and the remaining initial velocity activity was determined under conditions of the standard assay.

[b] To eliminate oxygen, the sample was kept under a low stream of argon during the preincubation.

Enzymology. The concept of steroidal, mechanism-based inhibitors of 14α-demethylase was specifically addressed using (3β)-32-(ethynyl)lanost-8-en-3-ol, **1**, as a prototype. Preincubation mixtures containing **1** in addition to the normal assay components exhibited a time-dependent loss of activity (data not shown). To differentiate between a mechanism-based inhibitor and formation of a high affinity enzyme–inhibitor adduct, the cofactor requirements for inhibition were examined. Normal catalytic turnover has been shown to require cytochrome P450, NADPH, NADPH–cytochrome *c* reductase, and oxygen (*26*). As shown in Table 1, maximal time-dependent inactivation by **1** requires all the cofactors required for catalysis, indicative of a mechanism-based inhibitor.

Based on previous work, activation of the acetylene functionality by P450 would be expected to yield a covalent adduct and thus irreversible inhibition (*12*). The irreversibility of inactivation by **1** was examined by subjecting partially inactivated enzyme to dialysis. The data in Table 2 indicate that activity is not regained under these conditions. These findings further support that (3β)-32-(ethynyl)lanost-8-en-3-ol functions as a mechanism-based inhibitor of $P450_{14DM}$.

Steroidal acetylene inhibition is comprised of both initial velocity and time-dependent components as illustrated in Table 3. These results have been previously presented in preliminary form (*27*). K_i values, which were determined under initial velocity conditions from linear Dixon analysis, are a measure of the inherent ability of these compounds to compete with substrate for binding to free enzyme. Upon

TABLE 2

Irreversibility of 14α-Demethylase Inactivation by (3β)-32-(Ethynyl)lanost-8-en-3-ol

Dialysis time (hours)	% Activity[a]		
	Control	125 nM	500 nM
0	100	27	8
4	100	6	3
8	100	3	1

Note. Lanosterol 14α-demethylase was incubated with and without inhibitor as indicated for 10 min at 30°C. At this time the tubes were put on ice and assayed for residual activity. Aliquots were put into a microdialyzer and dialyzed 1600-fold against 100 mM Tris–Cl, 20% glycerol buffer, pH 7.5, 4°C. Fresh dialysis buffer was added after 2 and 4 h. The control did not lose activity during the dialysis.

[a] Activity was determined under initial velocity conditions of the standard assay described in the Experiment section.

preincubation with 14α-demethylase, all compounds displayed saturable, time-dependent inactivation with no inactivation lag period. The K_I values (*28*) reflect the concentration of inactivator which gives half maximal rate of inactivation. K_I and k_{inact} were obtained by extrapolation to inhibitor saturation from a Kitz and Wilson plot of $t_{1/2}$ vs 1/[I] as described by Jung and Metcalf (*28–30*). The inactivation parameter k_{inact} suggests that a variety of 32-alkynyl-substituted sterols may irreversibly inhibit the enzyme. Product analysis was not performed on any of these inactivations. Although direct partition ratios were not determined, the ratio of k_{inact}/K_I suggests that compounds **2** and **4a** are the most efficient inactivators.

The potency of the inhibition of $P450_{14DM}$ by these compounds appears to be primarily a reflection of the inherent affinity of these substrate analogs for the enzyme; i.e., K_i values vary by two orders of magnitude, whereas k_{inact} changes by only a factor of two. As would be expected for inhibitors which are substrate analogs, there is a good correlation between the structural requirements for inhibitors and substrates (Table 3). For example, **4a** which contains a Δ8 olefin is a better inhibitor than **5a** which has a Δ7 olefin, and **2** which possesses the geminal dimethyl substitution at C-4 is more potent than **3**. Similarly, the presence of the 32-hydroxy group greatly enhances potency (**1** vs **4a**). The enhanced inhibitory potency of the hydroxylated compounds is in contrast to the results seen with analogous acetylenic substrate analogs for aromatase. However, aromatase binds the initial substrate, androstendione, more tightly than the subsequent 18-hydroxy-androstendione. In accordance with this substrate preference 10-[(1*S*)-1-hydroxy-2-propynyl]estr-4-en-3,17-dione is a poorer inhibitor of aromatase than 10-(2-propynyl)estr-4-en-3,17-dione (*13–15*).

TABLE 3

Inactivation Parameters for Steroidal Acetylenes

Compound	K_i[a,b] (μM)	K_I[c] (μM)	K_{inact}[d] (min^{-1})	k_{inact}/K_I ($\mu M^{-1}\ min^{-1}$)
1	1.2	0.37	0.15	0.41
2	0.019	0.086	0.093	1.1
3	3.2	6.0	0.17	0.029
4a	0.044	0.055	0.080	1.5
4b	0.44[e]			
5a	1.2	2.4	0.087	0.036
5b	6.3			

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_2}{\underset{k_3}{\rightarrow}} EI' \overset{k_4}{\rightarrow} EI''_{inact}$$
$$\downarrow$$
$$E + P$$

[a] K_i represents the dissociation constant for the reversible EI complex so long as k_1 and k_{-1} are much greater than k_2.

[b] K_i values were determined at 30°C. Due to rapid rates, the inactivations were done at 20°C.

[c] K_I is the concentration of inactivator that gives half the maximal rate of inactivation determined from a replot of $t_{1/2}$ versus 1/[I] (*28–30*) and is a reflection of *EI'*.

[d] k_{inact} is the maximum rate of inactivation at saturating inhibitor determined from the replot of $t_{1/2}$ vs 1/[I] where $k_{inact} = 0.693/t_{1/2\,(max)}$.

[e] K_i for **4b** determined on compound containing approximately 10% of **4a**.

Qualitatively the effect of adding an oxygen substitutent at C-15 (**1** → **2**) and C-32 (**1** → **4a**) is similar; both enhance potency at least 10-fold. We have prepared additional 32-alkynyl sterols which follow this same trend of enhanced binding when either a ketone or an α-hydroxy substituent is present at C-15.[3] The potent inhibition of $P450_{14DM}$ by 14α-alkyl-15-oxygenated sterols has been previously noted (*31*).

The absolute configuration of the more potent diastereoisomer of the Δ8-propargylic alcohols (**4a**) has been determined by X-ray crystallographic analysis to be 32-S.[4] This is the same stereochemistry as has been reported for the analogous allylic alcohol (substitution of vinyl for propargyl in **4a**) which was reported to be a time-dependent irreversible inhibitor of sterol 14α-demethylase (*32*). These workers have suggested a mechanism for inhibition in which the allylic alcohol is oxidized to the vinyl ketone. Subsequent 1,4-addition of an enzymatic nucleophile would afford the inactivated enzyme. A similar mechanism has been proposed by Covey for the analogous acetylenic inhibitors of aromatase (*14*). An alternative mechanism, namely activation of the acetylene function by reaction with the oxoiron(V) species, has been proposed by Metcalf for aromatase and is equally applicable to sterol 14α-demethylase (*13*). Further work is needed to distinguish between these two mechanisms.

[3] Bossard, Gallagher, Tomaszek, Metcalf, and Adams, manuscript in preparation.

[4] Structure determined by Dr. Drake Eggleston, unpublished results.

We have demonstrated that 32-(ethynyl)lanost-8-en-3-ol (**1**) is a mechanism-based inhibitor of sterol 14α-demethylase. The time-dependent inhibition demonstrated with **2–5** suggests that these compounds are also able to irreversibly inactivate $P450_{14DM}$. The structural requirements for potent inhibition are in qualitative agreement with the known substrate preferences of the enzyme. The potent activity of the 15-oxosterols is a finding that was not predicted a priori. The SAR required for potent inhibition of the purified rat enzyme, including substituents at C-32 and C-15 and the effects of side-chain variation, will be the subject of future publications.[3]

EXPERIMENTAL

Synthesis

Melting points were determined with a Thomas–Hoover melting point apparatus and are uncorrected. Infrared spectra were recorded in KBr on a Perkin–Elmer Model 783 spectrophotometer unless otherwise noted. Proton NMR were recorded in $CDCl_3$ on either a JOEL-GX-270MHz or a Bruker-AM-250MHz instrument. Chemical shifts are reported in parts per million on the δ scale relative to TMS as the internal standard. Resonances are given for all protons downfield of the methylene envelope and all methyl groups, except for C-21 unless otherwise noted. The C-26 and -27 methyl groups, which are diastereotopic and often appear as a double doublet or a broad doublet, are designated as broad doublets. The data are given as (multiplicity, assignment, coupling constant). THF was distilled from Na/benzophenone. All other solvents were Aldrich Gold Label or the best available and were used without further purification. Unless otherwise noted all reactions were run under argon at 20°C. Standard workup refers to an aqueous/organic workup followed by washing with brine, drying over $MgSO_4$ and concentration *in vacuo* (water aspirator pressure). Chromatography refers to flash chromatography on E. Merck silica gel 60 (230–400) mesh.

(3β,5α)-4,4-Dimethyl-15-(trimethylsilyloxy)cholest-8,14-dien-3-ol benzoate (**7a**). A mixture of 3β-(benzoyloxy)lanost-8(14)-en-15-one (37.6 g, 0.071 mol), anhydrous DMF (300 ml), triethylamine (58 ml, 0.42 mol) and trimethylsilyl chloride (26.6 ml, 0.21 mol) was heated under an atmosphere of argon in an oil bath held at 140°C for 18 h. The volatiles and DMF were evaporated *in vacuo,* and the residue was chromatographed with a hexane in ethyl acetate gradient to provide **7a** (38.8 g, 91%). The product contained 13% of the Δ7,14-diene isomer [(3β,5α)-4,4-dimethyl-15-(trimethylsilyloxy)cholest-7,14-dien-3-ol benzoate] by both GLPC and HPLC analysis: mp 172–177°C; ir 1715, 1650 cm^{-1}; NMR (250 MHz) δ 8.1–7.4 (m, Ar), 5.95 (bs, H-7 of minor isomer), 4.75 (dd, 3α-H), 1.09, 1.06, 0.99, 0.82 (s, 4 quaternary methyls), 0.89 (d, C-26 and -27 methyl, J = 6.0 Hz), 0.21 and 0.19 (s, trimethylsilyl resonance of major and minor isomer, respectively). *Anal.* Calcd for $C_{39}H_{60}O_3Si$: C, 77.43; H, 10.00. Found: C, 77.60; H, 10.34.

(3β,5α)-15-(Trimethylsilyloxy)cholest-8,14-dien-3-ol benzoate (**7b**). Using the above procedure (3β,5α)-3-(benzoyloxy)cholest-8(14)-en-15-one (3.42 g) was con-

verted to **7b** (3.67 g, 93%). GLPC analysis indicated 15% of the Δ7,14-diene: mp 170–172°C; ir 1720, 1650 cm^{-1}; NMR (250 MHz) δ 8.1–7.4 (m, Ar), 5.86 (bs, H-7 of minor isomer), 4.97 (m, 3α-H), 1.04 (s, C-19 methyl), 0.93 (d, C-21 methyl, J = 6.0 Hz), 0.88 (d, C-26 and -27 methyl, J = 6.6 Hz), 0.83 (s, C-18 methyl), 0.20 and 0.17 (s, trimethylsilyl resonance of major and minor isomer, respectively). *Anal.* Calcd for $C_{37}H_{56}O_3Si$: C, 77.03; H, 9.78. Found: C, 76.94; H, 9.81.

(3β)-3-Benzoyloxy-32-cyanolanost-8-en-15-one (**8**). Benzyl trimethylammonium fluoride (5.05 g, 29.9 mmol) and 4A molecular sieves (32 g) in THF (50 ml) were vigorously stirred for 8 h at 20°C. To the stirring precooled (−10°C) mixture containing the activated fluoride catalysis was added **7a** (12.0 g, 19.9 mmol) and iodoacetonitrile (38.6 mmol) in THF (60 ml). After 15 min, the cooling bath was removed and stirring was continued an additional 20 min. The reaction was diluted with 2–3 vol of hexanes, filtered through celite, and concentrated *in vacuo*. The residue was dissolved in a minimum amount of CH_2Cl_2 and diluted first with acetone and then methanol to induce crystallization which was allowed to proceed overnight at 0°C. The solid was collected, washed with ethanol, and dried at 70°C and 1 mm Hg to yield **8** (8.08 g, 70%): mp 193–194°C; ir 2240 (nitrile), 1740 (ketone), 1710 (ester) cm^{-1}; NMR (270 MHz) δ 8.1–7.4 (m, 5H, Ar), 4.76 (dd, 3α-H, J = 4.7 and 11.1 Hz), 2.63 (ab quartet, 2H, C-32 CH_2), 1.08, 1.05, 0.99, 0.81 (s, 3H each, methyl groups at C-4, -18 and -19), 0.87 (bd, 3H each, C-26 and -27 methyl, J = 6.4 Hz). *Anal.* Calcd for $C_{38}H_{53}NO_3$: C, 79.82; H, 9.34; N, 2.45. Found: C, 79.71; H, 9.22; N, 2.49.

(3β)-3-Hydroxy-32-(ethynyl)lanost-8-en-15-one (**2**). Benzyl trimethylammonium fluoride (640 mg, 3.8 mmol) and 4A molecular (3.6 g) sieves in THF (8 ml) were vigorously stirred for 6 h at 20°C. To the stirring precooled (−10°C) mixture containing the activated fluoride catalysis was added a solution of **7a** (1.5 g, 2.5 mmol) and propargyl bromide (1.0 ml of an 80% solution in benzene, 9 mmol) in THF (8 ml). After 15 min, the cooling bath was removed and stirring was continued an additional 20 min. The reaction was diluted with 2–3 vol of hexanes, filtered through celite, and concentrated *in vacuo*. Crystallization from ether/methanol afforded **10a** (1.07 g, 75%) which was used without further purification. A mixture of **10a** (1.07 g), THF (60 mL), methanol (40 ml), and 1 N lithium hydroxide solution (20 ml) was refluxed for 3.5 h and then allowed to cool. The resulting solid was collected, washed with water, dissolved in ether, washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield 850 mg of an oil. The crude product was chromatographed eluting with hexanes: EtOAc to afford **2** (737 mg, 84%) from which an analytical sample obtained by recrystallization from methanol: mp 104–105°C ir (KBr) 3310 (alkyne C–H), 2110 (alkyne CC), 1745 cm^{-1}; NMR (250 MHz) δ 3.25 (m, 3α-H), 1.01, 0.99, 0.81, 0.76 (s, 3H each), 0.95 (d, C-21 methyl, J = 6.5 Hz), 0.86 (d, C-26 and -27 methyl, J = 6.6 Hz); mass spectrum *m/e* 467 (M + H, 12), 449 (M + H − H_2O, 100), 427 (M − C_3H_3, 32). *Anal.* Calcd for $C_{32}H_{50}O_2$: C, 82.35; H, 10.80. Found: C, 82.49; H, 11.15.

(3β,14α)-3-Benzoyloxy-14-(2-propynyl)-5α-cholest-8-en-15-one (**10b**). Using the preceding procedure **7b** (1.67 g, 2.89 mmol) was reacted to obtain the crude product **10b.** Chromatography of the isolated crude product with a hexane in ethyl acetate gradient provided a homogeneous product (710 mg, 46%). A crystallization from

ether/methanol gave 600 mg of **10b;** mp 152–153°C; ir 3300 (alkyne C–H), 2110 (alkyne CC), 1735 (ester), 1710 (ketone) cm^{-1}; NMR (270 MHz) δ 8.1–7.4 (m, 5H, Ar), 5.0 (m, 3α-H), 2.04 (t, -CCH, J = 2.6 Hz), 1.02 (s, C-19 methyl), 0.97 (d, C-20 methyl, J = 6.3 Hz), 0.87 (bd, C-26 and -27 methyl, J = 6.1 Hz), 0.79 (s, C-18 methyl).

(3β,5α,14α)-3-Hydroxy-14-(2-propynyl)-5-cholest-8-en-15-one (**3**). A mixture of **10b** (532 mg), THF (27 ml), methanol (15 ml), and 1 N lithium hydroxide solution (5 ml) was refluxed for 1.5 h and worked up by dilution with brine, extraction with ether, drying over $MgSO_4$, and concentration *in vacuo*. Two crystallizations of the crude product from hexanes gave **3** (387 mg, 90%): mp 91–94°C; ir 3320 and 3280 (alkyne C–H), 1740 and 1725 (ketone) cm^{-1}; NMR (250 MHz) δ 3.62 (m, 3α-H), 0.95 (s, C-19 methyl), 0.86 (bd, C-26 and -27 methyl, J = 6.5 Hz), 0.78 (s, C-18 methyl); mass spectrum *m/e* 439 (M + H, 17), 421 (M + H − H_2O, 100), 399 (M − C_3H_3, 10). *Anal.* Calcd for $C_{30}H_{46}O_2$: C, 82.14; H, 10.57. Found: C, 82.00; H, 10.97.

(3β)-32-Cyanolanost-8-en-3-ol (**9**). A mixture of **8** (104 mg, 0.22 mmol), hydrazine hydrochloride (960 mg, 14.1 mmol), anhydrous hydrazine (4.5 ml), and triethylene glycol (4.5 ml) was heated (bath 130°C) and stirred under an argon atmosphere until all the solids dissolved (1–3 h), at which point solid KOH (1.08 g) was added cautiously. A shortpath distillation condenser was attached and the reaction temperature was raised initially to 160–170°C to remove the hydrazine and then to 210°C for 2 h. Upon cooling the reaction was diluted with ether, washed with water and brine, dried over $MgSO_4$, and concentrated. Chromatography eluting sequentially with hexanes: EtOAc afforded three major products as follows: (3β, 5α)-4,4-dimethylcholest-8,14-dien-3-ol[17 mg, 19%; mp 139–140°C; mass spectrum *m/e* 413 (M + H, 39), 395 (M + H − H_2O, 100); analysis good for $C_{29}H_{48}O$], **9** [14 mg, 17%; mp 133–135°C; ir (film) 2240 (nitrile) cm^{-1}; NMR (270 MHz) δ 3.27 (dd, 3α-H, J = 4.7 and 11.2 Hz), 1.01, 1.00, 0.82, 0.72 (s, C-4, -18, and -19 methyl) 0.87 (bd, C-26 and -27 methyl, J = 6.5 Hz); mass spectrum *m/e* 454 (M + H, 16), 436 (M + H − H_2O, 50), 413 (M − CH_2CN, 100); *Anal.* Calcd. for $C_{31}H_{51}NO$: C, 82.06; H, 11.33; N, 3.09. Found: C, 82.10; H, 10.99; N, 3.01] and 18 mg of a hydrazine containing side-product [mp 180°C with decomp.; FT-ir ($CDCl_3$ on NaCl) 1673, 1634 cm^{-1}; NMR (270 mHz) δ 6.80 (dd, 1H, J = 3.8 and 2.7 Hz), 3.52 (bt, 1H, J = 7.8 Hz), 3.22 (dd, 3α-H, J = 11.0 and 4.7 Hz), 0.99, 0.97, 0.80, 0.74 (s, C-4, -18 and -19 methyls), 0.87 (d, C-20 methyl, J = 6.4 Hz), 0.86 (bd, C-26 and -27 methyl, J = 4.4 Hz); mass spectrum *m/e* 469 (M + H, 100), 451 (M + H − H_2O, 82); *Anal.* Calcd for $C_{31}H_{52}N_2O$: C, 79.43; H, 11.18; N, 5.97. Found: C, 80.17; H, 11.15; N, 5.62].

(3β)-32-(Ethynyl)lanost-8-en-3-ol (**1**). Into an stirring, ice-cooled solution of **9** (295 mg, 0.65 mmol) in CH_2Cl_2 (20 ml) was added diisobutylaluminum hydride (1.3 ml of a 1.5 M solution in toluene). The reaction was quenched 1 h later by the addition of methanol, reduced 50% in volume *in vacuo*, diluted with ether (50 ml) and 2 N HCl (10 ml), and stirred for 0.5 h to effect hydrolysis of the imine. Further standard workup produced the crude aldehyde (286 mg), which was used directly in the next step. To a stirring, −78°C solution of the ylide prepared from (chloromethyl)triphenylphosphonium chloride (1.04 g, 3.0 mmol) and *n*-BuLi (1.2 ml, 3.0

mmol) in THF (10 ml) was added the aldehyde in THF (3–4 ml). The reaction was allowed to warm slowly to 10°C at which point by GPLC all the starting material had been consumed and then cooled back to −78°C. After the addition of lithium diisopropyl amide (5 mmol, prepared from 0.7 ml of diisopropyl amine and 2.5 ml of *n*-BuLi in 5 ml of THF), the reaction was warmed to 0°C to effect the elimination of the vinyl chloride. Following an ether/2 N HCl aqueous workup and chromatography, **1** (166 mg, 56%) was obtained. An analytical sample was prepared by recrystallization from hexanes: mp 125–127°C, ir (KBr) 3310 (alkyne-CH), 2115 (alkyne, CC) cm^{-1}; NMR (270 MHz) δ 3.26 (dd, 3α-H, J = 4.7 and 11.3 Hz), 1.91 (t, CH_2CCH, J = 2.7 Hz), 1.00, 0.99, 0.81, 0.69 (s, C-4, -18, and -19 methyl), 0.87 (bd, C-26 and, -27 methyl, J = 6.7 Hz); mass spectrum *m/e* 453 (M + H, 10), 435 (M + H − H_2O, 75), 413 (M − CH_2CCH, 100), 395 (6). *Anal.* Calcd for $C_{32}H_{52}O$: C, 84.89; H, 11.58. Found: C, 84.42; H, 11.49.

(3β,32R,S)-32-(Ethynyl)lanost-8-en-3,32-diol (**4a** *and* **4b**). To a stirring −78°C solution of lithiotrimethylsilyl acetylene (generated by the addition of 0.80 ml of 2.5 M *n*-BuLi to 0.40 ml of trimethylsilyl acetylene in 3 ml of THF at −78°C) was added a solution 3β-hydroxylanost-8-en-32-al (*5*) (60 mg, 0.14 mmol) in THF (3 ml). The reaction was allowed to warm slowly to 0°C over a 1-h period whereupon workup yielded 78 mg of crude product as a 3–4 : 1 ratio of diastereomeric alcohols. Chromatography (hexanes: EtOAc) produced pure **4a** (40 mg), and **4b** (7 mg, approximately a 9 : 1 mixture of **4b** and **4a**) plus an intermediate fraction containing both isomers (18 mg). The major higher R_f isomer (40 mg) was dissolved in THF (4 ml) and treated with tetrabutylammonium fluoride (0.40 ml of a 1.0 M solution in THF) for 10 min. Recrystallization (ether/hexanes) of the 32 mg of product obtained following workup and chromatography (hexanes : EtOAc) afforded an analytical sample of **4a** (19 mg): mp 156–158°C; ir (film) 3310 cm^{-1}; NMR (250 MHz) δ 4.38 (bd, -CH(OH)CCH, J = 4.2 Hz), 3.23 (dd, 3α-H, J = 5.5 and 10.5 Hz), 2.40 (d, -CCH, J = 2.1 Hz), 1.00, 0.99, 0.87, 0.84, 0.81, 0.71 (s, C-4, -18, -19, -26, and −27 methyl); mass spectrum *m/e* 469 (M + H, 6), 451 (M + H − H_2O, 100), 433 (25), 413 (M + H − $HOCH_2CCH$, 81). *Anal.* Calcd for $C_{33}H_{52}O_2$: C, 82.44; H, 10.90. Found: C, 80.30; H, 11.35. Analysis consistent for 1 eq ether; NMR shows 0.5 eq ether. The minor lower R_f isomer (7 mg) was reacted in a similar manner and chromatographed to yield **4b** (6 mg). By NMR and HPLC **4b** was shown to be contaminated with 10% **4a:** ir (film) 3320 (alkyne C–H), 2110 (alkyne CC) cm^{-1}; NMR (250 MHz) δ 4.52 (bs, -CH(OH)CCH), 3.26 (dd, 3α-H, J = 4.9 and 11.2 Hz), 2.42 (d, −CCH, J = 2.2 Hz), 1.07, 1.03, 0.84, 0.75 (s, C-4, -18 and -19 methyl), 0.88 (bd, C-26 and -27 methyl).

(3β,32R,S)-32-(Ethynyl)lanost-7-en-3,32-diol (**5a** and **5b**). Using the above procedure 3β-hydroxylanost-7-en-32-al (200 mg) was converted to a mixture of diastereomeric alcohols which were chromatographically separated. Treatment of the high R_f diastereomer with tetrabutylammonium fluoride afforded following workup and recrystallization from methanol **5a** (15 mg): mp 160–161.5°C; ir (KBr) 3310 (alkyne C–H) cm^{-1}; NMR (250 MHz) δ 5.35 (m, H-7), 4.37 (dd, CHOHCCH, J = 2 and 10 Hz), 3.26 (m, 3α-H), 2.58 (d, -CCH), 0.99, 0.89, 0.88, 0.85, 0.75 (s, C-4, -18, -19, -26, and -27 methyl); mass spectrum *m/e* 469 (M + H, 30), 451 (M + H − H_2O, 100), 433 (31), 413 (M + H − CH_2OHCCH, 61), 397 (32), 395 (35). Similarly, the

lower R_f isomer was converted to **5b** (35 mg): mp 136–136.5°C; ir (KBr) 3315 (alkyne C–H), 2110 alkyne CC) cm^{-1}; NMR (250 MHz) δ 5.48 (m, H-7), 4.52 (bs, CHOHCCH), 3.23 (m, 3α-H), 2.43 (d, -CCH, J = 2 Hz), 0.99, 0.92, 0.85, 0.72 (s, C-4, -18 and -19 methyl), 0.89 (bd, C-26 and -27 methyl); mass spectrum *m/e* 469 (M + H, 19), 451 (M + H − H_2O, 100), 433 (62), 413 (M + H − CH_2OHCCH, 75), 397 (46), 395 (57).

Enzymology

Lanosterol 14α-demethylase and NADPH–cytochrome c reductase from rat liver were purified as previously described (*20*). NADPH–cytochrome c was assayed by the method of Kubota *et al.* at 30°C (pH 7.7) (*34*). Protein concentrations were determined using Pierce bicinchoninic acid reagent (*35*) with bovine serum albumin as a standard. Cytochrome P450 content was determined by the method of Omura and Sato (*36*).

Lanosterol 14α-demethylase was assayed by the tritium release assay as described by Bossard *et al.* (*20*) using 24,25-dihydrolanosterol as the substrate. Inhibitors were dissolved in ethanol at a concentration of 7.5 mM. Prior to use the inhibitor solutions were diluted as described above for the substrate. Initial velocity assays contained 100 mM Tris–HCl, 20% glycerol, 1.0 mM NADPH, 0.2 mM DTT, 0.2 mM EDTA, 10 mM glucose 6-phosphate, and 0.4 Units/ml glucose-6-phosphate dehydrogenase, pH 7.5. For the initial velocity assays, lanosterol 14α-demethylase (6–12 μg), NADPH–cytochrome c reductase (0.35 units), and all buffered assay mix components were incubated for 10 min at 30°C. The reaction was then initiated by the addition of the above mixture to substrate or substrate plus inhibitor. The reaction time was 15 min and the concentration of substrate was 37.5 μM which is just slightly above the K_m for dihydrolanosterol. Final ethanol and Emulgen 913 concentrations were 1% and 0.2%, respectively. Reactions (0.1 ml) were quenched with 1% Emulgen 913 (0.01 ml) and 40% trichloroacetic acid containing 0.25% formic acid (0.025 ml). Following centrifugation, 0.1 ml of supernatant was applied to a SEP PAK C_{18} cartridge. Vacuum was applied and the cartridge was washed twice with 1.0 ml of water into the same collection tube. Samples were counted in Beckman Ready-Solve.

The determination of K_i values was done by Dixon analysis assuming competitive inhibition (*37*). For the determination of $t_{1/2}$ and K_{inact} values (*28–30*), lanosterol 14α-demethylase,NADPH–cytochrome c reductase (0.92 Units), and all buffered assay mix components were preincubated at 20°C. Inactivation (20°C) was initiated by addition of inhibitor in buffered solutions. At time intervals aliquots were removed for residual activity analysis and diluted 10-fold into a mixture containing 90 mM Tris–HCl, 18% glycerol, 0.9 mM NADPH, 0.18 mM DTT, 0.18 mM EDTA, 9 mM glucose 6-phosphate, 0.36 Units/ml glucose-6-phosphate dehydrogenase, 37.5 μM 24,25-dihydrolanosterol, and 0.09 Units NADPH–Cytochrome c reductase. The initial velocity reaction time was 15 min at 30°C. Final ethanol and Emulgen 913 concentrations were again 1 and 0.02%, respectively. Reactions were quenched, processed, and counted as before.

Due to the rapid rates of inactivation it was necessary to lower the inactivation

temperature to 20°C in order to obtain measurable rates of inactivation for k_{inact} determinations. The activity assays could be done at 30°C since the effect of residual inactivator in the activity assay was minimized by dilution and the protective effect of 37.5 μM substrate, dihydrolanosterol. Although oxygen is also a substrate, we did not examine the effects of varying oxygen and all experiments (except as noted in Table 1) were done at air saturation.

Materials. Emulgen 913 was kindly supplied by Kao-Atlas Co., Tokyo. 24,25-[32-^{3}H]dihydrolanosterol was synthesized at Moravek Biochemicals, Inc. Brea, California. Commercial 24,25-dihydrolanosterol was purified by high performance liquid chromatography. SEP-PAK cartridges were purchased from Waters Associates. Glucose-6-phosphate dehydrogenase and NADH type III were purchased from Sigma. All other chemicals were obtained from commercial sources and were of the highest grade available.

ACKNOWLEDGMENTS

We thank Dr. D. Eggleston for determination of the X-ray structure of **4a**, L. Killmer for mass spectral analyses, Dr. C. DeBrosse and D. Staiger for ^{1}H NMR spectra data, and E. Reich for microanalyses.

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