Synthesis and Bioevaluation of Δ^7-5-Desaturase Inhibitors, an Enzyme Late in the Biosynthesis of the Fungal Sterol Ergosterol

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Ergosterol, the predominant sterol of fungi, is postulated to have many cellular functions which include a bulk membrane role and a regulatory role. Studies with sterol auxotrophs show that, even in the presence of sterols which can fulfill the bulk membrane requirements, a small concentration of ergosterol is absolutely necessary for growth. The Δ^5-double bond appears to be required for the regulatory role of ergosterol; therefore, development of inhibitors of the enzyme that introduce this double bond, Δ^7-sterol 5-desaturase (5-desaturase), may lead to effective antifungal agents. Within is the first reported synthesis of inhibitors of fungal 5-desaturase and the development of an *in vitro* tritium efficacy radioassay. The inhibitors were of the general structure 7,22(*E*)-ergostadien-3β-ol with α-face heteroatom substituents in the vicinity of C-5. They exhibited IC_{50} values of 47−149 μM.

Introduction

Fungal infections are becoming more prevalent especially in immune-compromised individuals such as those afflicted with AIDS, undergoing chemotherapy, and receiving organ transplants. Systemic infections are not readily combated, and the physician's arsenal is limited. Current treatments suffer from several disadvantages: they are fungistatic rather than fungicidal; they interfere with mammalian steroid biochemistry; they exhibit poor drug uptake in some tissues; and they can cause severe liver or kidney damage. In addition, there are emerging reports of fungal strains resistant to current therapies.[1,2] Clearly, there is a need for the development of more effective antifungal agents.

Most compounds currently used clinically inhibit the biosynthesis of ergosterol (**1**), the principal sterol of most fungi.[3] Ergosterol is vital for fungal survival. It serves two purposes: a bulk membrane function and a sparking function.[4] Like cholesterol in mammals, ergosterol regulates the fungal cell membrane's permeability and fluidity, membrane-bound enzyme activity, and growth rate.[5−9] The sterol required for this bulk membrane function is nonspecific. Sterols, depending on their features, also spark or promote fungal growth.

In order to spark, a sterol must contain Δ^5-unsaturation which is introduced by 5-desaturase.[6] A-ring unsaturated steroids or cis AB-ring sterols are unsuitable substrates for the 5-desaturase.[10] The sparking ability of any given 5-desaturated sterol is enhanced by other structural features. These features, in order of effectiveness, are Δ^{22E}-unsaturation, Δ^7-unsaturation, and the presence of a C-24α methyl.[10] Furthermore, the sterol must contain a 3β-oxygenated substituent. An alcohol or methyl ether fulfills this requirement, whereas an ester does not.[11,12]

5-Desaturase, an enzyme found in mammals and fungi, is an unexploited target in the development of sterol biosynthesis inhibitors. This protein catalyzes one of the last steps in ergosterol biosynthesis (Δ^5-alkene formation), and thus its inhibition is the most costly in terms of energy expenditure.[13] The buildup of ergosterol intermediates may also cause the negative feedback control of sterol biosynthesis and interfere with the bulk membrane function.

The mechanism of the 5-desaturase has not been fully elucidated but has been studied using rat liver microsomes and yeast cell-free preparations. Fungal and mammalian 5-desaturases appear to achieve similar ends using different mechanisms. The fungal 5-desaturase step is accelerated by the presence of a C-24α methyl, and the enzyme tolerates either Δ^7- or Δ^8-sterols as substrates.[6,14] The mammalian enzyme accepts only Δ^7-sterols.[15] In both organisms, researchers have shown that the C-5α and C-6α protons are removed during the desaturation process and are found in the aqueous medium.[16] Mammalian desaturation is inhibited by iron chelators and enhanced by free radical inhibitors.[15] Isolation of a 5α-hydroxylated sterol from fungi and the demonstrated conversion to ergosterol implies a potential oxidation−dehydration mechanism.[17,18] Likewise, researchers have isolated a 6α-hydroxylated sterol from fungi.[19] Similar C-5- and C-6-hydroxylated cholesterols are not desaturated in mammals.[20] In fungi, lack of inhibition by carbon monoxide, involvement of an electron transport pathway, and cofactor similarities to fatty acid desaturase reaction pathway all suggest a non-heme iron-containing enzyme.[20−23]

On the basis of the differences between fungal and mammalian 5-desaturase, this paper proposes that new drugs affecting only fungal desaturase can be developed. Sterols which contain an oxygen, nitrogen, or sulfur atom near the vicinity of C-5 on the α-face of the sterol, one- or two-atom bond lengths from the steroid nucleus, should yield inhibitors structurally similar to the potential oxysterol intermediates. Being similar in structure to the natural substrate, these inhibitors should compete with the substrate and/or product for the active site of 5-desaturase and coordinate with the catalytic metal (most likely iron). The buildup of $\Delta^{7,22E}$-sterol intermediates may alter the fungal membrane stability, deplete energy reserves, exert negative feedback control

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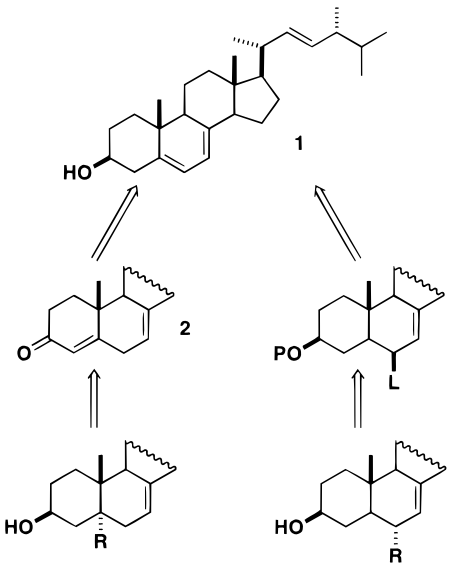
of *de novo* sterol biosynthesis, interfere with sparking, and eventually lead to cell death.

Retrosynthetically, C-5-functionalized inhibitors were envisioned to arise from the Michael addition of an appropriate oxygen, sulfur, or carbon nucleophile to enone **2**, a known compound. C-6-substituted inhibitors were thought to be accessible by displacing a C-6β leaving group (L) which was thought to be obtainable from a C-6α alcohol. This α-alcohol could be obtained from ergosterol (**1**).

Proposed Retrosynthesis

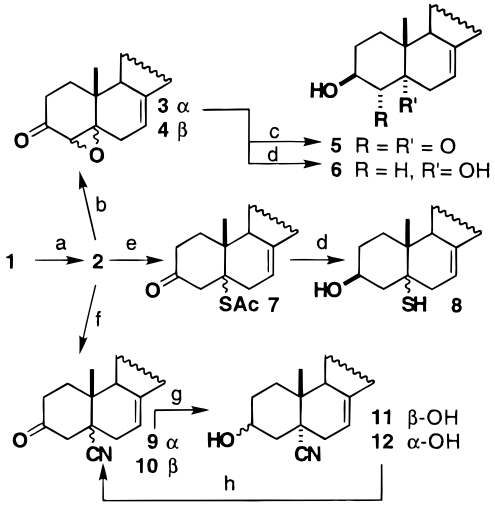


R = Nitrogen, Oxygen or Sulfur containing group

Inhibitor Synthesis

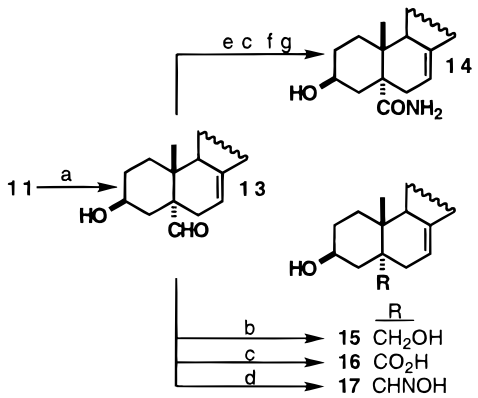
Oppenhauer oxidation of ergosterol (**1**) provided enone **2** in 53% yield[24] (Scheme 1). Alkaline epoxidation of this intermediate provided a mixture of the α- and β-epoxides **3** (31%) and **4** (17%) which were readily purified by flash chromatography. Potential inhibitor **5** was then prepared by selective reduction of the ketone in the presence of the epoxide with lithium tri-*tert*-butoxyaluminohydride (68%). Reduction of α-epoxide **3** with lithium aluminum hydride provided the known 3β,5α-diol **6** (22%).[25]

Scheme 1[a]



[a] (a) Al(O-*i*-Pr)$_3$, *N*-methyl-4-piperidone; (b) 10% NaOH, 30% H_2O_2, MeOH, 48 h, 4 °C; (c) LiAl(O-*t*-Bu)$_3$H; (d) LAH; (e) $(BzO)_2HSC(O)CH_3$; (f) Et_2AlCN; (g) $NaBH_4$; (h) PCC.

Scheme 2[a]



[a] (a) (i) DIBAL, (ii) H_2SO_4; (b) LAH; (c) $NaClO_2$, NaH_2PO_4, *t*-BuOH, H_2O, 2-methyl-2-butene; (d) H_2NOH, pyr, EtOH, Δ; (e) AcCl, pyr; (f) (i) PCl_5, (ii) NH_3(l); (g) 10% NaOH/MeOH.

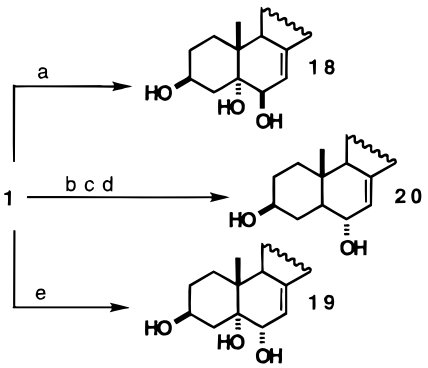
Treatment of enone **2** with thiolacetic acid and benzoyl peroxide generated C-5 thioacetate **7** (4%). Lithium aluminum hydride reduction gave thiol **8** (46%). The stereochemistry of compounds **7** and **8** has not been unequivocally determined (no observed NOE between the SAc and C-19 methyl); however, one could argue for α-stereochemistry because sulfur addition to the β-face would be blocked by the angular methyl group. Furthermore, the fact that thiol **8** inhibits 5-desaturase would imply a trans AB-ring.

The addition of diethylaluminum cyanide to enone **2** provided a mixture of α- and β-cyano ketones **9** (82%) and **10** (13%). Stereochemical assignments of the nitriles were based on analogous cholesterol compounds.[26] Sodium borohydride reduction of ketone **9** gave the optimal yield of the desired equatorial alcohol **11** (37%). Pyridinium chlorochromate oxidation of the undesired epimer 3α-alcohol **12** quantitatively regenerated α-cyano ketone **9**.

Diisobutylaluminum hydride reduction of 3β-alcohol 5α-nitrile **11** followed by acid hydrolysis provided target aldehyde **13** (86%), which was an important synthetic intermediate for further functional group transformations (Scheme 2).[27] The aldehyde was converted to amide **14** (47% overall) by acetylation, oxidation, acid chloride formation, condensation with ammonia, and alcohol deprotection.[27] Reduction of the aldehyde with lithium aluminum hydride provided diol **15** (58%), while sodium chlorite oxidation generated acid **16** (96%).[27−29] Finally, the aldehyde was also converted to hydroxylamine **17** (85%).

Inhibitors with C-5 and C-6 Functionality. Inhibitors with functionality at C-5 and C-6 were also synthesized (Scheme 3). *m*-Chloroperoxybenzoic acid oxidation of ergosterol yielded triol **18** (3%) or **19** (27%) depending on the conditions employed in addition to side chain epoxidation.[30] Under homogeneous conditions, acid-catalyzed opening of the 5α,6-epoxide intermediate generated the 5α-alcohol allylic stabilized carbocation. The *m*-chlorobenzoate anion would then add to the α-face due to the adverse steric hindrance of the C-19β methyl group. Under biphasic conditions, the epoxide initially forms but is rapidly hydrolyzed by water to provide the trans diaxial product **18**. The stereochemical assignments were confirmed by NOE studies.

Scheme 3[a]



[a] (a) MCPBA/CH_2Cl_2; (b) Ac_2O, pyr, DMAP; (c) (i) BH_3·Me_2S, (ii) 10% NaOH(aq), 30% H_2O_2; (d) 5% NaOH/MeOH; (e) MCPBA, H_2O/CH_2Cl_2, Na_2CO_3.

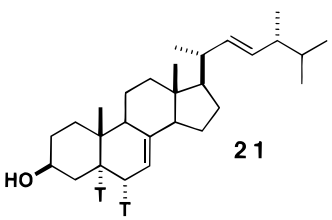
Inhibitors at C-6. Acetylation of ergosterol (**1**) followed by hydroboration−oxidation and alkaline deprotection gave the 6α-alcohol **20** (29%) (Scheme 3). The stereochemistry of the C-6α alcohol is based on the following observations. Oxidation of alcohol **20** provided the known 6-ketone compound[31−33] thereby implying hydroboration of the Δ^5-bond. Hydroboration is known to occur on the least hindered face. In this case the β-face is blocked by the C-19 methyl group. Furthermore, literature provides an analogous reaction in which 5,7-cholestadien-3β-ol is hydroborated to provide the C-6α alcohol.[34] Lastly, there is an observable NOE between the C-6 proton and the C-19 methyl group of alcohol **20**.

Unfortunately, further elaboration at C-6 failed. Reduction of the obtainable 6-ketone compound (not shown) principally formed the 6β-alcohol. Under a variety of displacement conditions, this alcohol eliminated to form a complex mixture of inseparable dienes. Other failed elaboration attempts included the conversion of the 6-ketone to an epoxide or an aldehyde equivalent (methoxymethylene) as well as hydroboration or epoxidation of the acid labile exomethylene.

Bioevaluation

As mentioned earlier, compounds with heteroatom substituents on the α-face of the sterol in the vicinity of C-5 were designed as potential inhibitors of fungal 5-desaturase. The ability of these compounds to inhibit 5-desaturase was assessed utilizing a tritium radioassay. Based on previously published whole cell and cell-free mechanistic studies, it was known that 5-desaturase removes the 5α- and 6α-protons from the natural substrate 7,22(*E*)-ergostadien-3β-ol and that the removed protons can be located in the aqueous media.[13,16,18,21] Therefore, it was thought that 5-desaturase would remove tritium from [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol (**21**) prepared as per literature with a specific activity of 44 100 cpm/mg.[20]

Sterol 21



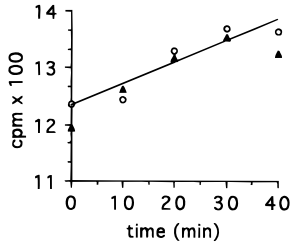


Figure 1. Incubation of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol with microsomes. Over time, incubation resulted in an increase in the radioactivity detected in the aqueous component after extraction of the organic components: (▲) run 1 and (○) run 2.

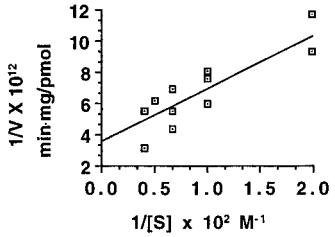


Figure 2. Plot of inverse velocity versus inverse substrate concentration for 5-desaturase.

A microsomal preparation isolated from *Saccharomyces cerevisae* was incubated with ferric chloride, NADPH, a solubilizing detergent, and substrate **21**. At various time intervals the enzymatic reaction was quenched by alkali addition, the organic components were exhaustively extracted, and the aqueous residue's radioactivity was measured. At greater time intervals there was an increase in the detected radioactivity, implying the enzyme preparation contained active 5-desaturase (Figure 1).

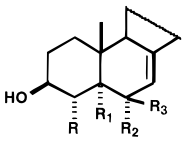
Two additional control experiments were performed to ensure that the activity was not an artifact. First, labeled sterol **21** was incubated with inactivated microsomes. Since the protein was destroyed by boiling, the radiolabel did not accumulate in the aqueous layer. Second, varying amounts of protein were incubated with identical quantities of tritiated substrate **21**. Increases in the protein concentration resulted in a corresponding increase in the radioactivity of the aqueous component.

The enzyme's specific activity (K_m) was determined by correlating the amount of ^3H_2O formed in a given time (velocity) at a given substrate concentration. The information obtained was graphed on a Lineweaver−Burk plot (Figure 2). Solving for K_m gave an activity of 0.94 ± 0.39 mM and a V_{max} of 6.58 ± 2.66 pmol/min·mg.

After a K_m value was obtained, inhibition studies were undertaken. In these experiments the ability of a synthesized inhibitor to compete with the tritiated substrate **21** for the active site of 5-desaturase was evaluated. In a given experiment, all factors were held constant except for the amounts of added inhibitor. By plotting the inverse velocity versus inhibitor concentration, one can obtain the concentration required to inhibit the enzyme activity by 50% (IC_{50}).

The IC_{50} values given in Table 1 are the average of at least two experiments, and the deviation from the average value was never greater than 5%. Examination of Table 1 suggests several possible trends. One must keep in mind, however, that the error associated with the measured K_m value (±41%) may affect the validity of these trends. In addition, IC_{50} values for all of the

Table 1. IC_{50} Values of Tested Compounds



compd	R	R_1	R_2	R_3	IC_{50} (μM)
5	O		H	H	66
6	H	OH	H	H	148
8	H	SH	H	H	80
11	H	CN	H	H	131
13	H	CHO	H	H	116
14	H	$CONH_2$	H	H	114
15	H	CH_2OH	H	H	112
16	H	CO_2H	H	H	111
17	H	CHNOH	H	H	136
18	H	OH	H	OH	96
19	H	OH	OH	H	149
20	H	H	OH	H	47

evaluated compounds were within a fairly narrow range (47−149 μM), making assessment of the trends problematic.

Inhibitors with functionality at C-5, in general, appear to be less potent than those with functionality at either C-4 or C-6. The thiol **8** upsets this general trend. Several factors may be at work. A thiol, a soft base, may be able to bind more tightly than an alcohol, a hard base, to the postulated catalytic iron. A second factor may be that the longer C−S bond places the heteroatom at a more ideal location in relationship to the 5-desaturase catalytic center than a C−O bond. However, insertion of an additional carbon between C-5 and the heteroatom(s) makes sterols **11** and **13**−**17** less "potent" inhibitors than thiol **8**. Finally, sulfur, unlike oxygen, has d-orbitals available for back-bonding.

Alcohols **18** and **19** are also unusual. The cis diol **19**, with two potential chelating groups, was of "similar" potency to the 5α-alcohol **6**. However, the trans diol epimer **18** was surprisingly more effective. The increased inhibition caused by the pseudoaxial alcohol was not expected. Studies of the 5-desaturase mechanism indicate that the enzyme operated on the α-face of the sterol. β-Substituents would be thought to decrease the potency of a given inhibitor. The fact that a C-6β alcohol in addition to a C-5α alcohol lowers the IC_{50} value suggests that the enzyme may contain either a hydrophilic pocket or hydrogen bond acceptor/donor near the β-face of the sterol around C-6.

The 4α,5-epoxide **5** and C-6α alcohol **20** appear to be the most effective compounds evaluated. Why these compounds are better inhibitors of 5-desaturase is unclear. As explained earlier, one mechanism of 5-desaturation proposes hydroxylation followed by dehydration. The preponderance of evidence also pointed to hydroxylation at C-5. Although it is known that the C-5 alcohol **6** can be dehydrated to provide ergosterol,[18] analogs **5** and **20** were not expected to be substrates. Perhaps being conformationally similar to the C-5 alcohol **6**, inhibitors **5** and **20** are able to fit into the active site more readily. These compounds may also coordinate more tightly to the catalytic iron, or they may inhibit the dehydration step.

Since all the tested compounds showed inhibition, a final control experiment was performed. The procedure employed was identical with that of a competition study except that cholestanol served as the inhibitor. This sterol lacks unsaturation and the C-24 methyl substituent and should not compete for the active site of 5-desaturase. In this case, the accumulation of the radiolabel in the aqueous medium was equivalent to those cases in which no inhibitor was added. This result implied that cholestanol was not an inhibitor of 5-desaturase.

Conclusion

An *in vitro* radioassay was developed to screen for substrate-based inhibitors of Δ^7-5-desaturase, a fungal sterol biosynthetic enzyme. The tested ergosterol derivatives contained α-face heteroatoms in the vicinity of C-5 and exhibited weak potency. Additional studies employing amine and fluorinated substituents are warranted. Furthermore, the evidence that a C-6β heteroatom increases potency should also be explored. In addition, the assay may also be useful in screening for other nonsteroidal inhibitors of 5-desaturase.

Experimental Section

^{1}H NMR spectra were obtained on a Varian Unity-500 (500 MHz) NMR spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. Infrared spectra were recorded on a Perkin Elmer 298 spectrometer in $CHCl_3$ unless otherwise noted. Direct insertion probe chemical ionization (CI) using isobutane as a carrier gas and electron-impact (EI) mass spectral data were recorded using a Hewlett Packard HP 5087 GC−MS system. Melting points were determined using a Thomas Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Spang Microanalytical Laboratories, Eagle Harbor, MI, or Quantitative Technologies, Inc., Whitehouse, NJ. High-resolution mass spectra (HRMS) were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois using a VG 70-VSE instrument. Silica gel (EM Science silica gel 60, 230−400 mesh) was used for all flash chromatography.

4α,5-Epoxy-7,22(*E*)-ergostadien-3-one (3) and 4β,5-Epoxy-7,22(*E*)-ergostadien-3-one (4). 4,7,22(*E*)-Ergostatrien-3-one (**2**) (0.2 g, 0.5 mmol) in 35 mL of MeOH was cooled to 0 °C, and 30% H_2O_2 (4.7 mL, 41.3 mmol) and 10% NaOH(aq) (1.6 mL) were added. The reaction mixture was stirred at 4 °C for 48 h and then poured into ice water and extracted with 5 × 10 mL of Et_2O. The organic extracts were dried with anhydrous $MgSO_4$ and filtered, and the solvent was evaporated *in vacuo*. The residue was purified by flash chromatography (30:1 hexane:EtOAc) giving in order of elution **4** as a white solid (0.036 g, 17%) and **3** as a white solid (0.066 g, 31%).

3: mp 148−150 °C; R_f 0.60 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.23−5.13 (m, 3H, C-7, C-22, C-23), 3.06 (s, 1H, C-4), 2.88 (d, 1H, C-6, J = 18.5 Hz), 1.08 (s, 3H, C-19), 1.01 (d, 3H, C-21, J = 6.5 Hz), 0.90 (d, 3H, C-28, J = 7.0 Hz), 0.81 (t, 6H, C-26, C-27, J = 7.0 Hz), 0.58 (s, 3H, C-18); IR 1675 cm^{-1}; MS (EI) *m/z* 410 (M, 6), 283 (M − C_9H_{17}, 42). Anal. ($C_{28}H_{42}O_2$) C, H.

4: mp 147−150 °C; R_f 0.67 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.25−5.14 (m, 3H, C-7, C-22, C-23), 3.08 (s, 1H, C-4), 2.81 (d, 1H, C-6, J = 16 Hz), 1.15 (s, 3H, C-19), 1.02 (d, 3H, C-21, J = 6.5 Hz), 0.92 (d, 3H, C-28, J = 7.0 Hz), 0.83 (t, 6H, C-26, C-27, J = 7.0 Hz), 0.58 (s, 3H, C-18); IR 1703 cm^{-1}; MS (EI) *m/z* 410 (M, 14), 283 (M − C_9H_{17}, 100). Anal. ($C_{28}H_{42}O_2$) H; C: calcd, 81.90; found, 81.35.

4α,5-Epoxy-7,22(*E*)-ergostadien-3β-ol (5). To 4α,5-epoxy-7,22(*E*)-ergostadien-3-one (**3**) (0.044 g, 0.11 mmol) in 25 mL of anhydrous THF was added 1 M lithium tri-*tert*-butoxyaluminohydride in THF (0.09 mL, 0.32 mmol). The reaction mixture was stirred under Ar for 11 h, at which point 4 mL of EtOAc and 3 drops of saturated Na_2SO_3(aq) were added. The resultant solution was filtered, dried with anhydrous $MgSO_4$, and filtered, and the solvent was removed *in vacuo*. The residue was purified by flash chromatography (9:1 toluene:EtOAc) to yield **5** as a white solid (0.030 g, 68%): mp 148−150 °C; R_f 0.30 (4:1 toluene:EtOAc); ^{1}H NMR δ 5.17−5.09 (m,

3H, C-7, C-22, C-23), 3.88 (m, 1H, C-3), 2.90 (d, 1H, C-4, J = 1.0 Hz), 2.68−2.65 (dd, 1H, C-6), 1.03 (s, 3H, C-19), 0.95 (d, 3H, C-21, J = 6.9 Hz), 0.85 (d, 3H, C-28, J = 6.9 Hz), 0.76 (t, 6H, C-26, C-27, J = 7.1 Hz), 0.59 (s, 3H, C-18); MS (EI) m/z 413 (M, 11), 91 (M − 322, 100). Anal. ($C_{28}H_{44}O_2$) C, H.

7,22(*E*)-Ergostadiene-3*β*,5α-diol (6). To 4α,5-epoxy-7,22-(*E*)-ergostadien-3-one (**3**) (0.066 g, 0.16 mmol) in 25 mL of anhydrous Et_2O was added $LiAlH_4$ (0.019 g, 0.50 mmol). The reaction mixture was refluxed for 3 h under Ar and then cooled to 0 °C. EtOAc (3 mL) and 3 drops of saturated Na_2SO_4(aq) were added. The resultant solution was dried with anhydrous $MgSO_4$ and filtered, and the solvent was evaporated *in vacuo*. The residue was purified by flash chromatography (3:1−0:1 hexane:EtOAc) to yield **6** as a white solid (0.015 g, 22%): mp 237−238 °C (lit.[25] mp 227−234 °C); R_f 0.55 (1:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (dd, 2H, C-22, C-23), 5.08−5.05 (m, 1H, C-7), 4.04 (m, 1H, C-3), 2.23 (d, 1H, J = 16.8 Hz), 1.03 (d, 3H, C-21, J = 6.6 Hz), 0.92 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 5.1 Hz), 0.83 (t, 6H, C-26, C-27, J = 7.3 Hz), 0.57 (s, 3H, C-18); IR 3590, 3423 cm^{-1}; MS (CI) m/z 415 (M + 1, 3), 397 (M + 1 − H_2O, 20), 379 (M + 1 − 2 × H_2O, 2), 125 (C_9H_{17}, 100). Anal. ($C_{28}H_{46}O_2$) C, H.

5-(*S*-Acetylthio)-7,22(*E*)-ergostadien-3-one (7). After 4,7,22(*E*)-ergostatrien-3-one (**2**) (0.503 g, 1.27 mmol) was stirred in 25 mL of thiolacetic acid with benzoyl peroxide (0.077 g, 0.3 mmol) for 5 days, the solvent was evaporated *in vacuo*. The resultant residue was dissolved in Et_2O and washed to neutrality with 5% NaOH(aq). The organic layer was evaporated *in vacuo*. The residue was then recrystallized from MeOH to give **7** as white needles (0.025 g, 4%): mp 120−122 °C; R_f 0.50 (1:1 hexane:EtOAc); ^{1}H NMR δ 5.25−5.14 (m, 3H, C-7, C-22, C-23), 2.26 (s, 3H, SAc), 1.08 (s, 3H, C-19), 1.02 (d, 3H, C-21, J = 6.6 Hz), 0.92 (d, 3H, C-28, J = 6.9 Hz), 0.83 (t, 6H, C-26, J = 7.1 Hz), 0.59 (s, 3H, C-18); IR 1700, 1678 cm^{-1}; MS (CI) m/z 471 (M + 1, 8), 395 (M + 1 − SAc, 100).

5-Mercapto-7,22(*E*)-ergostadien-3*β*-ol (8). A flask charged with 5-(*S*-acetylthio)-7,22(*E*)-ergostadien-3-one (**7**) (0.265 g, 0.56 mmol), 25 mL of anhydrous Et_2O, and $LiAlH_4$ (0.064 g, 1.7 mmol) was refluxed for 5 h and then allowed to cool to room temperature. EtOAc (5 mL) was added followed by 3 drops of 10% Na_2SO_3(aq). The precipitate was removed by vacuum filtration and the liquid evaporated *in vacuo*. The residue was purified by flash chromatography (12:1 toluene:EtOAc) to afford **8** as a white solid (0.111 g, 46%). Recrystallization from CH_3CN gave fine white needles: mp 139−140 °C; R_f 0.28 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 2H, C-22, C-23), 5.06 (s, 1H, C-7), 4.17 (m, 1H, C-3), 1.01 (d, 3H, C-21, J = 6.6 Hz), 0.99 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 6.9 Hz), 0.84 (d, 3H, C-26, J = 7.3 Hz), 0.82 (d, 3H, C-27, J = 7.3 Hz), 0.56 (s, 3H, C-18); IR 3440, 1057 cm^{-1}; MS (CI) m/z 431 (M + 1, 6), 413 (M + 1 − H_2O, 31), 397 (M + 1 − H_2S, 63), 379 (M + 1 − H_2O − H_2S, 13), 288 (M + 1 − H_2O − C_9H_{17}, 3), 273 (M + 1 − H_2S − C_9H_{17}, 18), 255 (M + 1 − H_2O − H_2S − C_9H_{17}, 9), 125 (C_9H_{17}, 100). Anal. ($C_{28}H_{46}OS$) C, H, S.

5α-Cyano-7,22(*E*)-ergostadien-3-one (9) and 5*β*-Cyano-7,22(*E*)-ergostadien-3-one (10). To a solution of 4,7,22(*E*)-ergostatrien-3-one (**2**) (1.5 g, 3.8 mmol) in 300 mL of anhydrous THF was added 1.0 M Et_2AlCN (11.4 mL, 11.4 mmol) in toluene. Upon addition of the cyanide reagent the solution turned dark red. After stiring for 3.5 h the mixture was washed with 2 × 40 mL of ice cold 10% NaOH(aq). (Caution! When the reaction is performed on a large scale, add the aqueous components dropwise to avoid a violent exotherm.) The aqueous layer was exhaustively extracted with CH_2Cl_2, the combined organic layers were dried with anhydrous $MgSO_4$ and filtered, and the solvent was evaporated *in vacuo*. The residue was purified by flash chromatography (8:1−0:1 hexane:EtOAc) to provide **9**, which was recrystallized from EtOH giving white plates (1.3 g, 82%), and **10**, as a white solid (0.2 g, 13%).

9: mp 280−282 °C; R_f 0.30 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 3H, C-7, C-22, C-23), 2.58 (bs, 2H, C-4), 1.13 (s, 3H, C-19), 1.03 (d, 3H, C-21, J = 6.5 Hz), 0.92 (d, 3H, C-28, J = 7 Hz), 0.84 (d, 3H, C-26, J = 6.8 Hz), 0.83 (d, 3H, C-27, J = 6.8 Hz), 0.59 (s, 3H, C-18); IR 2221, 1717 cm^{-1}; MS (EI) m/z 422 (M, 5), 109 (C_8H_{13}, 100). Anal. ($C_{29}H_{43}NO$) C, H, N.

10: mp 120−123 °C; R_f 0.15 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.78 (s, 1H, C-7), 5.18−5.07 (m, 2H, C-22, C-23), 2.94 (s, 1H, C-4 axial), 2.35 (d, 1H, C-4), 1.19 (s, 3H, C-19), 0.95 (d, 3H, C-21, J = 6.5 Hz), 0.84 (d, 3H, C-28, J = 6.5 Hz), 0.76 (t, 6H, C-26, 27 J = 7.5 Hz), 0.68 (s, 3H, C-18); IR 2240, 1670 cm^{-1}; MS (CI) m/z 422 (M + 1, 100), 396 (M + 1 − CN, 8), 296 (M + 1 − C_9H_{17}, 5), 125 (C_9H_{17}, 27). Anal. ($C_{29}H_{43}NO$) H, N; C: calcd, 82.60; found, 82.01.

5α-Cyano-7,22(*E*)-ergostadien-3*β*-ol (11) and 5α-Cyano-7,22(*E*)-ergostadien-3α-ol (12). To 5α-cyano-7,22(*E*)-ergostadien-3-one (**9**) (2.7 g, 6.4 mmol) were added 25 mL of CH_2Cl_2, 175 mL of 95% EtOH, and $NaBH_4$ (0.7 g, 19.2 mmol). The solution was stirred for 12 h. The solvent was evaporated *in vacuo* and the residue purified by flash chromatography (8:1−2:1 hexane:EtOAc) to yield in order of elution **11**, as a white solid which was recrystallized from Et_2O/hexane to form white needles (1.0 g, 37%), and **12**, as a white solid (1.7 g, 63%).

11: mp 170−174 °C; R_f 0.48 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 3H, C-7, C-22, C-23), 4.06 (m, 1H, C-3), 1.02 (d, 3H, C-21, J = 7 Hz), 0.94 (d, 3H, C-28, J = 8.5 Hz), 0.92 (s, 3H, C-19), 0.84 (d, 3H, C-26, J = 7 Hz), 0.82 (d, 3H, C-27, J = 7 Hz), 0.55 (s, 3H, C-18); IR 3443, 2223 cm^{-1}; MS (CI) m/z 424 (M + 1, 100), 397 (M + 1 − HCN, 20), 378 (M + 1 − HCN − H_2O, 18), 271 (M + 1 − HCN − C_9H_{17}, 20), 125 (C_9H_{17}, 80). Anal. ($C_{29}H_{45}NO$) C, H, N.

12: mp 174−176 °C; R_f 0.40 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 3H, C-7, C-22, C-23), 4.14 (m, 1H, C-3), 1.03 (d, 3H, C-21, J = 6.6 Hz), 0.92 (d, 3H, C-28, J = 7.1 Hz), 0.89 (s, 3H, C-19), 0.83 (t, 6H, C-26, C-27, J = 7.8 Hz), 0.56 (s, 3H, C-18); IR 3462, 2211 cm^{-1}; MS (CI) m/z 424 (M + 1, 100), 406 (M + 1 − H_2O, 91), 380 (M + 1 − H_2O − HCN, 5), 125 (C_9H_{17}, 40). Anal. ($C_{29}H_{45}NO$) H, N; C: calcd, 82.21; found, 81.37.

5α-Formyl-7,22(*E*)-ergostadien-3*β*-ol (13). 5α-Cyano-7,22(*E*)-ergostadien-3*β*-ol (**11**) (0.7 g, 3.1 mmol) was dissolved in 100 mL of anhydrous toluene. The solution was cooled to −10 °C, and 1.5 M DIBAL in toluene (12.3 mL, 18.4 mmol) was added. The reaction mixture was stirred for 40 min under argon at −10 °C and then refluxed with 20 mL of 1.0 M H_2SO_4(aq) for 40 min. The organic layer was washed with 40 mL of H_2O and 40 mL of saturated NaCl(aq). The aqueous layer was back-extracted with 2 × 30 mL of CH_2Cl_2. All organic layers were evaporated *in vacuo*. The residue was purified by flash chromatography (6:1−2:1 hexane:EtOAc) to yield **13** as a white solid (0.6 g, 86%): mp 149−152 °C; R_f 0.29 (3:1 hexane:EtOAc); ^{1}H NMR δ 9.59 (s, 1H, CHO), 5.24−5.13 (m, 3H, C-7, C-22, C-23), 3.78 (m, 1H, C-3), 1.02 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 6.8 Hz), 0.84 (d, 3H, C-26, J = 6.8 Hz), 0.82 (d, 3H, C-27, J = 6.8 Hz), 0.56 (s, 3H, C-18); IR 3419, 1708 cm^{-1}; MS (CI) m/z 427 (M + 1, 43), 409 (M + 1 − H_2O, 100), 381 (M + 1 − CHO, 6), 125 (C_9H_{17}, 77); HRMS m/z 425, 3417 (M + 1), calcd for $C_{29}H_{45}O_2$ 425.3419. Anal. ($C_{29}H_{45}O_2$) C: calcd, 79.44; found, 81.41. H: calcd, 10.87; found, 10.24.

3*β*-Hydroxy-7,22(*E*)-ergostadiene-5α-formamide (14). 5α-Formyl-7,22(*E*)-ergostadien-3*β*-ol (**13**) (0.9 g, 2.1 mmol), 100 mL of CH_2Cl_2, pyridine (0.19 mL, 2.3 mmol), and acetyl chloride (0.16 mL, 2.3 mmol) were combined and stirred overnight. The solution was washed with 3 × 10 mL of 5% HCl(aq), the organic layer was rotary evaporated, and the residue was purified by flash chromatography (20:1−8:1 hexane:EtOAc) to give 3*β*-acetoxy-7,22(*E*)-ergostadiene-5α-formaldehyde as a white solid (0.9 g, 90%): mp 149−152 °C; R_f 0.66 (3:1 hexane:EtOAc); ^{1}H NMR δ 9.57 (s, 1H, CHO), 5.23−5.13 (m, 3H, C-7, C-22, C-23), 4.84 (m, 1H, C-3), 2.00 (s, 3H, OAc), 1.03 (s, 3H, C-19), 1.01 (d, 3H, C-21, J = 6.4 Hz), 0.91 (d, 3H, C-28, J = 7.1 Hz), 0.83 (t, 6H, C-26, C-27, J = 7.0 Hz), 0.56 (s, 3H, C-18); IR 1720 cm^{-1}; MS (CI) m/z 469 (M + 1, 15), 409 (M + 1 − OAc, 100), 125 (C_9H_{17}, 18). Anal. ($C_{31}H_{48}O_3$) C: calcd, 79.44; found, 81.41. H: calcd, 10.32; found, 10.85.

To 3*β*-acetoxy-7,22(*E*)-ergostadiene-5α-formaldehyde (0.300 g, 0.64 mmol) in 80 mL of *t*-BuOH, 20 mL of H_2O, and 20 mL of 2-methyl-2-butene were added NaH_2PO_4 (0.691 g, 5.76 mmol) and $NaClO_2$ (0.695 g, 7.68 mmol). After stirring for 24 h, the solvents were evaporated *in vacuo*. The residue was dissolved in 100 mL of a 1:1 mixture of CH_2Cl_2 and H_2O. The aqueous layer was back-extracted with 3 × 20 mL of CH_2Cl_2.

All organic layers were combined, washed with 20 mL of 5% HCl(aq) and 20 mL of H_2O, and then rotary evaporated. The residue was purified by column chromatography (15:1−6:1 hexane:EtOAc) to yield 3β-acetoxy-7,22(*E*)-ergostadiene-5α-methanoic acid as a white solid (0.270 g, 87%): mp 198−200 °C; R_f 0.27 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.23−5.16 (m, 3H, C-7, C-22, C-23), 4.84 (m, 1H, C-3), 1.01 (d, 3H, C-21, J = 8.8 Hz), 1.01 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 6.8 Hz), 0.82 (t, 6H, C-27, C-26, J = 7.0 Hz), 0.55 (s, 3H, C-18); IR 1720 cm^{-1}; MS (CI) m/z 485 (M + 1, 4), 468 (M + 1 − OH, 2), 425 (M + 1 − OAc, 38), 384 (M + 1 − OAc − CO_2H, 18), 301 (M + 1 − OAc − C_9H_{17}, 73), 125 (C_9H_{17}, 100). Anal. ($C_{31}H_{48}O_4$) C, H.

To 3β-acetoxy-7,22(*E*)-ergostadiene-5α-methanoic acid (0.059 g, 0.12 mmol) in 7 mL of anhydrous CH_2Cl_2 was added PCl_5 (0.025 g, 0.12 mmol). The solution was stirred overnight under argon. Anhydrous ammonia (10 mL) was condensed into the reaction mixture. The solution was then allowed to warm to room temperature and the solvent removed by rotary evaporation. The residue was purified by flash chromatography (7:1−2:1 hexane:EtOAc) to yield 3β-acetoxy-7,22(*E*)-ergostadiene-5α-formamide as a white solid (0.47 g, 80%): mp 235−237 °C; R_f 0.12 (3:1 hexane:EtOAc); ^{1}H NMR δ 6.02 (bs, 1H, NH), 5.53 (bs, 1H, NH), 5.38 (m, 1H, C-3), 5.27−5.10 (m, 3H, C-7, C-22, C-23), 1.97 (s, 3H, OAc), 1.02 (s, 3H, C-19), 0.98 (d, 3H, C-21, J = 6.6 Hz), 0.88 (d, 3H, C-28, J = 6.8 Hz), 0.80 (d, 3H, C-26, J = 6.8 Hz), 0.78 (d, 3H, C-27, J = 6.8 Hz), 0.53 (s, 3H, C-18); IR 3498, 3399, 1712, 1658 cm^{-1}; MS (CI) m/z 485 (M + 1, 1), 426 (M + 1 − OAc, 4), 383 (M + 1 − OAc − $CONH_2$, 6). Anal. ($C_{31}H_{49}NO_3$) C, H, N.

3β-Acetoxy-7,22(*E*)-ergostadiene-5α-formamide (0.047 g, 0.10 mmol) in 25 mL of MeOH and 1.5 mL of 10% K_2CO_3(aq) was stirred overnight and the solvent evaporated *in vacuo*. The resultant residue was purified by flash chromatography (3:1 hexane:EtOAc to 9:1 EtOAc:MeOH) to afford **14** as a white solid (0.039 g, 91%). Recrystallization from CH_3CN gave fine white needles: mp 244−248 °C; R_f 0.63 (EtOAc); ^{1}H NMR δ 5.97 (bs, 1H, NH), 5.23−5.13 (m, 4H, C-7, C-22, C-23, NH), 4.33 (m, 1H, C-3), 1.04 (s, 3H, C-19), 1.01 (d, 3H, C-21, J = 6.4 Hz), 0.91 (d, 3H, C-28, J = 6.9 Hz), 0.83 (t, 6H, C-27, C-26, J = 7.5 Hz), 0.56 (s, 3H, C-18); Ir 3498, 3403, 3340, 1660 cm^{-1}; MS (CI) m/z 443 (M + 1, 69), 425 (M + 1 − H_2O, 100), 406 (M + 1 − 2 × H_2O, 30), 125 (C_9H_{17}, 30); HRMS m/z 442.3666 (M + 1), calcd for $C_{29}H_{45}NO_2$ 442.3685. Anal. ($C_{29}H_{47}NO_3$) H, N; C: calcd, 78.86; found, 78.12.

5α-(Hydroxymethyl)-7,22(*E*)-ergostadien-3β-ol (15). To 5α-formyl-7,22(*E*)-ergostadien-3β-ol (**13**) (0.019 g, 0.04 mmol) in 25 mL of anhydrous Et_2O was added $LiAlH_4$ (0.13 mL, 1.0 M in Et_2O, 0.13 mmol). The mixture was refluxed for 4 h under Ar and cooled to room temperature and the solvent removed *in vacuo*. The residue was purified by flash chromatography (3:1−0:1 hexane:EtOAc) to yield **15** as a white solid (0.011 g, 58%): mp 227−231 °C; R_f 0.10 (1:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 2H, C-22, C-23), 5.11 (m, 1H, C-7), 3.90 (m, 1H, C-3), 3.58 (dd, 2H, CH_2OH, J = 11 Hz), 1.02 (d, 3H, C-21, J = 6.0 Hz), 1.02 (s, 3H, C-19), 0.92 (d, 3H, C-28, J = 6.5 Hz), 0.84 (d, 3H, C-26, J = 8.0 Hz), 0.82 (d, 3H, C-27, J = 8.0 Hz), 0.56 (s, 3H, C-18); IR 3592, 3452 cm^{-1}; MS (CI) m/z 429 (M + 1, 32), 411 (M + 1 − H_2O, 38), 393 (M + 1 − 2 × H_2O, 3), 303 (M + 1 − C_9H_{17}, 2), 287 (M + 1 − H_2O − C_9H_{17}, 36), 125 (C_9H_{17}, 100). Anal. ($C_{29}H_{48}O_2$) C, H.

3β-Hydroxy-7,22(*E*)-ergostadiene-5α-methanoic Acid (16). 5α-Formyl-7,22(*E*)-ergostadien-3β-ol (**13**) (0.080 g, 0.19 mmol) in 20 mL of *t*-BuOH, 5 mL of H_2O, and 10 mL of 2-methyl-2-butene was stirred for 26 h with sodium phosphate monobasic (0.202 g, 1.69 mmol) and sodium chlorite (0.203 g, 2.25 mmol). The solvent was evaporated *in vacuo*, and the residue was dissolved in 30 mL of CH_2Cl_2 and 30 mL of H_2O. The aqueous layer was extracted with 3 × 10 mL of CH_2Cl_2. The combined organic layers were washed with 15 mL of 5% HCl(aq) and 15 mL of H_2O. The solvent was removed *in vacuo* and the residue purified by flash chromatography (8:1−0:1 hexane:EtOAc) to provide **16** as a white solid (0.080 g, 96%). The steroid was further purified by recrystallization from CH_3CN to provide fine white needles: mp 244−246 °C; R_f 0.61 (1:1 hexane:EtOAc); ^{1}H NMR δ 5.23−5.15 (m, 3H, C-7, C-22, C-23), 3.74 (m, 1H, C-3), 1.01 (d, 3H, C-21, J = 6.9 Hz), 1.01 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 6.6 Hz), 0.82 (t, 6H, C-27, C-26, J = 7.4 Hz), 0.55 (s, 3H, C-18); IR 3111, 1721 cm^{-1}; MS (CI) m/z 443 (M + 1, 3), 425 (M + 1 − H_2O, 7), 381 (M + 1 − H_2O − CO_2H, 2), 301 (M + 1 − H_2O − C_9H_{17}, 23), 125 (C_9H_{17}, 41); HRMS m/z 441.3355 (M + 1), calcd for $C_{29}H_{45}O_3$ 441.3368. Anal. ($C_{29}H_{45}O_3$) H, N; C: calcd, 78.68; found, 78.12.

5α-Formyl-7,22(*E*)-ergostadien-3β-ol Oxime (17). To 5α-formyl-7,22(*E*)-ergostadien-3β-ol (**13**) (0.066 g, 0.19 mmol) in 15 mL of EtOH were added pyridine (4.95 mmol, 0.4 mL) and hydroxylamine hydrochloride (0.098 g, 1.4 mmol). The solution was refluxed for 25 h and then evaporated *in vacuo*. The residue was dissolved in 30 mL of H_2O and extracted with 3 × 10 mL of EtOAc. The EtOAc extracts were rotary evaporated, and the residue was purified by flash chromatography (8:1−0:1 hexane:EtOAc) to provide **17** as a white solid (0.058 g, 85%): mp 218−221 °C; R_f 0.11 (3:1 hexane:EtOAc); ^{1}H NMR δ 7.56 (s, 1H, C*H*NOH), 5.21−5.17 (m, 2H, C-22, C-23), 5.13 (d, 1H, C-7, J = 3.5 Hz), 4.09 (m, 1H, C-3), 1.02 (s, 3H, C-21), 1.02 (d, 3H, C-21, J = 4.4 Hz), 0.92 (d, 3H, C-28, J = 6.8 Hz), 0.84 (d, 3H, C-26, J = 6.7 Hz), 0.82 (d, 3H, C-27, J = 6.7 Hz), 0.57 (s, 3H, C-18); IR 3338, 3010, 1519 cm^{-1}; MS (CI) m/z 442 (M + 1, 69), 424 (M + 1 − H_2O, 100), 397 (M + 1 − HCNOH, 13), 379 (M + 1 − H_2O − HCNOH, 4), 125 (C_9H_{17}, 10). Anal. ($C_{29}H_{47}NO_2$) C, H, N.

7,22(*E*)-Ergostadiene-3β,5α,6β-triol (18). To ergosterol (0.591 g, 1.5 mmol) were added 20 mL of CH_2Cl_2, 20 mL of H_2O, Na_2CO_3 (0.125 g, 1.5 mmol), and MCPBA (0.257 g, 1.5 mmol). The mixture was stirred for 0.5 h, and then the phases were separated. The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated *in vacuo*. The residue was purified by flash chromatography (3:1−0:1) hexane:EtOAc to 19:1 EtOAc:MeOH) to yield 0.075 g of crude **18** as a white solid. Recrystallization from EtOAc provided **18** as fine white needles (0.019 g, 3%): mp 243−245 °C; R_f 0.00 (1:1 hexane:EtOAc); ^{1}H NMR δ 5.36 (m, 1H, C-7), 5.25−5.14 (m, 2H, C-22, C-23), 4.08 (m, 1H, C-3), 3.63 (m, 1H, C-6), 1.09 (s, 3H, C-19), 1.02 (d, 3H, C-21, J = 6.6 Hz), 0.91 (d, 3H, C-28, J = 6.8 Hz), 0.84 (d, 3H, C-26, J = 7.0 Hz), 0.82 (d, 3H, C-27, J = 7.0 Hz), 0.60 (s, 3H, C-18); IR (neat) 3315 cm^{-1}; MS (CI) m/z 429 (M + 1, 2), 411 (M + 1 − H_2O, 18), 393 (M + 1 − 2 × H_2O, 5), 377 (M + 1 − 3 × H_2O, 88). Anal. ($C_{28}H_{46}O_3$) C, H.

7,22(*E*)-Ergostadiene-3β-5α,6α-triol (19). To ergosterol (0.250 g, 0.63 mmol) in 25 mL of anhydrous CH_2Cl_2 was added MCPBA (0.109 g, 0.63 mmol). The reaction mixture was stirred for 1.5 h and then the solvent removed *in vacuo*. The residue was purified by flash chromatography (4:1−0:1 hexane:EtOAc to 9:1 EtOAc:MeOH). The second product to elute from the column was isolated (white solid, 0.127 g, R_f 0.52, 1:1 hexane:EtOAc). This steroid was subjected to methanolysis (40 mL of MeOH/2.5 mL of 5% KOH/MeOH for 16 h). The solvent was removed *in vacuo* and the residue purified by flash chromatography (4:1−0:1 hexane:EtOAc to 19:1 EtOAc:MeOH) to provide **19** as a white solid (0.072 g, 27%): mp 210−213 °C; R_f 0.22 (19:1 EtOAc:MeOH); ^{1}H NMR δ 5.24−5.13 (m, 2H, C-22, C-23), 5.02 (s, 1H, C-7), 4.01−3.97 (m, 2H, C-3, C-6), 1.02 (d, 3H, C-21, J = 6.7 Hz), 0.97 (s, 3H, C-19), 0.91 (d, 3H, C-28, J = 6.1 Hz), 0.84 (d, 3H, C-26, J = 7.1 Hz), 0.82 (d, 3H, C-27, J = 7.1 Hz), 0.56 (s, 3H, C-18); IR 3399 cm^{-1}; MS (CI) m/z 431 (M + 1, 1), 413 (M + 1 − H_2O, 20), 395 (M + 1 − 2 × H_2O, 38), 377 (M + 1 − 3 × H_2O, 100), 289 (M + 1 − 2 × H_2O − C_9H_{17}, 10), 271 (M + 1 − 3 × H_2O − C_9H_{17}, 14), 125 (C_9H_{17}, 73). Anal. ($C_{28}H_{46}O_3$) C, H.

7,22(*E*)-Ergostadiene-3β,6α-diol (20). Ergosterol (5.0 g, 12.5 mmol) dissolved in 200 mL of anhydrous CH_2Cl_2, pyridine (1.50 mL, 18.8 mmol), and acetyl chloride (0.85 mL, 15.0 mmol) were stirred overnight, and the solvent was removed *in vacuo*. The residue was purified by flash chromatography (10:1−3:1 hexane:EtOAc) to yield 3β-acetoxy-5,7,22(*E*)-ergostatriene as a white solid (5.4 g, 98%). Recrystallization from acetone gave fine white needles: mp 170−172 °C; R_f 0.60 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.56 (bs, 1H, C-6 or C-7), 5.38 (bs, 1H, C-6 or C-7), 5.25−5.15 (m, 2H, C-22, C-23), 4.70 (m, 1H, C-3), 2.50 (d, 1H, J = 12.0 Hz), 2.37 (t, 1H, J = 12.2 Hz), 2.17 (s, 3H, OAc), 1.03 (d, 3H, C-21, J = 6.4 Hz), 0.95 (s, 3H, C-19), 0.92 (d, 3H, C-28, J = 6.3 Hz), 0.83 (t, 6H, C-26, C-27, J = 7.1 Hz),

0.63 (s, 3H, C-18); MS (CI) *m/z* 439 (M + 1, 3), 379 (M + 1 − HOAc, 100).

Ergosterol acetate (0.040 g, 0.09 mmol) in 10 mL of anhydrous Et_2O was cooled to 0 °C under Ar. To the mixture was added 2.0 M BH_3·Me_2S in THF (0.09 mL, 0.18 mmol). The vessel was stirred for 2 h while warming to room temperature. After cooling to 0 °C 1 mL of H_2O, 1 mL of 10% NaOH(aq), and 30% H_2O_2 (0.14 mL, 4.56 mmol) were added. The heterogeneous solution was stirred for 2 h while warming to room temperature. (Caution! When the reaction is performed on a large scale, add the aqueous components dropwise to avoid a violent exotherm.) The phases were separated, and the organic layer was washed with 3 mL of 10% Na_2SO_3(aq) and 3 mL of saturated NaCl(aq) and then evaporated *in vacuo*. The residue was purified by flash chromatography (15:1−3:1 hexane:EtOAc) to provide the 3β-acetoxy-7,22(*E*)-ergostadien-6α-ol as a white solid (0.012 g, 29%): mp 177−180 °C; R_f 0.32 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.24−5.14 (m, 3H, C-7, C-22, C-23), 4.72 (m, 1H, C-3), 3.79 (d, 1H, C-6, J = 9.3 Hz), 2.28 (m, 1H), 2.04 (s, 3H, OAc), 1.02 (d, 3H, C-21, J = 6.9 Hz), 0.91 (d, 3H, C-28, J = 6.6 Hz), 0.86 (s, 3H, C-19), 0.83 (t, 6H, C-26, C-25, J = 7.1 Hz), 0.55 (s, 3H, C-18); IR 3416, 1705 cm^{-1}; MS (CI) *m/z* 457 (M + 1, 2), 440 (M + 1 − H_2O, 35), 397 (M + 1 − OAc, 10), 379 (M + 1 − H_2O − OAc, 100), 271 (M + 1 − OAc − C_9H_{17}, 4), 255 (M + 1 − OAc − H_2O − C_9H_{17}, 3), 125 (C_9H_{17}, 22). Anal. ($C_{30}H_{48}O_3$) C, H.

To 3β-acetoxy-7,22(*E*)-ergostadien-6α-ol (0.088 g, 0.19 mmol) in 50 mL of MeOH was added 1 mL of 5% NaOH(aq). The mixture was stirred for 24 h and the solvent removed by rotary evaporation. The residue was purified by flash chromatography (3:1 hexane:EtOAc to 9:1 EtOAc:MeOH) to provide the diol **20** as a white solid (0.071 g, 89%): mp 180−182 °C; R_f 0.00 (3:1 hexane:EtOAc); ^{1}H NMR δ 5.25−5.14 (m, 3H, C-7, C-22, C-23), 3.80 (d, 1H, C-6, J = 8.9 Hz), 3.58 (m, 1H, C-3), 2.25 (m, 1H), 1.02 (d, 3H, C-21, J = 6.3 Hz), 0.92 (d, 3H, C-28, J = 5.8 Hz), 0.84 (s, 3H, C-19), 0.82 (t, 6H, C-26, C-25, J = 7.8 Hz), 0.55 (s, 3H, C-18); IR 3595, 3407 cm^{-1}; MS (CI) *m/z* 415 (M + 1, 3), 397 (M + 1 − H_2O, 100), 379 (M + 1 − 2 × H_2O, 8), 271 (M + 1 − H_2O − C_9H_{17}, 8), 125 (C_9H_{17}, 22). Anal. ($C_{28}H_{46}O_2$) C, H.

Biological Experimental. Reagents purchased from Sigma and used as received are as follows: poly(oxyethylenesorbitan) monolaurate (Tween 20), Agar (A-5054), β-nicotinamide adenine dinucleotide phosphate (reduced form, tetrasodium salt; NADPH), $FeCl_3$·$6H_2O$, and Sephadex G-50-150.

Tris(hydroxymethyl)aminomethane (Tris) was purchased from Research Organics. Bacteriological peptone was purchased from Oxoid. Yeast extract, technical, was purchased from Difco Labs. D-Glucose was purchased from Fisher Scientific. *S. Cerevisiae* [JC530-4b (*Mata leu 2-3,122 his4 ura3-52*)] was kindly supplied by Dr. Leo Parks, Department of Microbiology, North Carolina State University. Flo-Scint III scintillation cocktail was purchased from Packard Instrument Co. and used as received. All liquid scintillation counting was performed using a Packard Tri-Carb 4640 liquid scintillation counter.

Protein Isolation. The procedure was modified from Katsuki *et al.* and carried out at 4 °C unless otherwise noted.[21] *S. cerevisiae* was cultivated aerobically at 37 °C in YPD broth (20 g of bacteriological peptone, 10 g of yeast extract, and 20 g of glucose per liter of H_2O) as below.

vol of broth in flask (mL)	flask size (mL)	no. of flasks	time grown (h)	vol of broth transferred (mL)
25	250	1	40	10
100	500	1	28	10
725	2000	5	48	

Harvested cells were centrifuged at 2600*g* (10 min, Sorvall RC5C), decanted, and placed in a 250 mL bead beater chamber (Biospect Products) half-filled with 0.5 mm glass beads; 0.1 M Tris-HCl buffer, pH 7.4 (Tris), was added until the chamber was filled. The vessel was then cooled to 0 °C. When well cooled, the cells were disrupted by 6 × 1 min pulses with 1 min intervals between pulses. The solution was decanted, and the beads were washed until the rinse solution became clear.

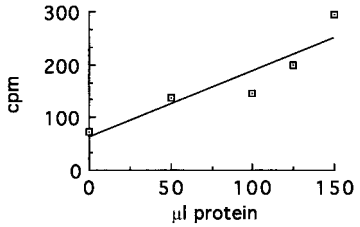


Figure 3. Incubation of varying amounts of protein solution with set amounts of sterol **21** and cofactors at 37 °C for 20 min followed by alkali quenching and then organic extraction, showing that the aqueous residue had greater radioactivity with increasing protein concentration.

After centrifugation at 2 × 4100*g* (10 min, Sorvall RC5C) the resulting supernatant was further centrifuged at 94500*g* (60 min, Beckmann L8-60M). The precipitate was suspended in a minimum of Tris and passed through a Sephadex G-50-150 column (25 g, column diameter = 6 cm) to remove low molecular weight substances (collected 7 mL fractions). Each fraction was examined by UV absorption. All fractions with an absorbance ratio ($\lambda_{280}/\lambda_{210}$) greater than 0.56 were combined (tubes 5−12).

The protein concentration was determined to be 3.54 mg/mL (Pierce BCA protein assay). All isolated protein (≈150 mL) was stored at −80 °C until needed. Live cultures were maintained on plates made of YPD broth (as above with 15 g of Agar) and stored at 4 °C.

Time Study Using [5α,6α-^{3}H]-7,22(*E*)-Ergostadien-3β-ol. A test tube was charged with 50.0 mg of Tween 20, 1.20 μmol of NADPH, 3.70 of μmol $FeCl_3$·$6H_2O$, 200 μL of Tris, 800 μL of protein solution, and 0.500 mg of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol. Sterol and Tween 20 were added as ethanol solutions. Prior to the addition of the other components, the ethanol was evaporated under a steady stream of air. $Iron^{3+}$ and NADPH were added as Tris solutions. The total volume was 1.0 mL. The tube was incubated at 37 °C, and 100 μL aliquots were removed every 10 min. Each aliquot was treated with 100 μL of 15% KOH(aq) (w/w). To each aliquot was added 1.0 mL of EtOAc. The aliquot was mixed, and then 0.7 mL of EtOAc was removed. The remaining solution was extracted with EtOAc (3 × 0.7 mL), and any remaining EtOAc was removed. The radioactive aqueous residue was counted with a scintillation counter. Over time there was an increase in the radioactivity of the aqueous medium (Figure 1).

Inactivated Microscome Study. Scintillation vials were charged with 14.0 mg of Tween 20, 1.06 μmol of $FeCl_3$·$6H_2O$, 0.23 μmol of NADPH, 60 μL of Tris, 60 μL of inactivated protein solution (previously placed in boiling water for 30 min), and 0.045 mg of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol. The sterol and Tween 20 were added as ethanol solutions. Prior to the addition of the other components, the ethanol was evaporated under a steady stream of air. $Iron^{3+}$ and NADPH were added as Tris solutions. The total volume was 120 μL. The vials were incubated at 37 °C. After 20 min 200 μL of 15% KOH/MeOH (w/w) was added to each vial, and then each vial was extracted with 4 × 1 mL of EtOAc. The aqueous residue was counted with a scintillation counter. There was no accumulation of radioactivity in the aqueous layer.

Varying Protein Concentration Study. The procedure is identical with that of the Inactivated Microsome Study except that 0.241 mg of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol and varying amounts of Tris and protein solution were used, and the total volume was 200 μL. Increases in the protein concentration resulted in a corresponding increase in the radioactivity of the aqueous residue (Figure 3).

***K*$_m$ Studies.** Scintillation vials were charged with 25.0 mg of Tween 20, 1.06 μmol of $FeCl_3$·$6H_2O$, 0.23 μmol of NADPH, 100 μL of Tris, 100 μL of protein solution, and varying amounts of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol (0.050−0.250 mg). The sterol and Tween 20 were added as ethanol solutions. Prior to the addition of the other components, the ethanol was

evaporated under a steady stream of air. $Iron^{3+}$ and NADPH were added as Tris solutions. The total volume was 200 μL. The vials were incubated at 37 °C. After 20 min the reaction was arrested with 100 μL of 15% KOH(aq) (w/w). To each aliquot was added 1.5 mL of EtOAc. The aliquot was mixed, and then 1.0 mL of EtOAc was removed. The remaining solution was extracted with EtOAc (4 × 0.75 mL), and any remaining EtOAc was removed. The aqueous residue was counted with a scintillation counter (Figure 2).

Competition Studies (IC_{50} Experiments). In a typical experiment, scintillation vials were charged with 15.0 mg of Tween 20, 0.64 μmol of $FeCl_3 \cdot 6H_2O$, 0.14 μmol of NADPH, 60 μL of Tris, 60 μL of protein solution, 0.045 mg of [5α,6α-^{3}H]-7,22(*E*)-ergostadien-3β-ol, and varying amounts of inhibitor (0−20 mol % of the substrate). The sterols and Tween 20 were added as ethanol solutions. Prior to the addition of the other components, the ethanol was evaporated under a steady stream of air. $Iron^{3+}$ and NADPH were added as Tris solutions. The total volume was 120 μL. Each vial was incubated for 20 min at 37 °C and then saponified with 60 μL of 15% KOH(aq) (w/w). To each vial was added 1.5 mL of EtOAc. The vial was mixed, and then 1.0 mL of EtOAc was removed. The remaining solution was extracted with EtOAc (4 × 0.75 mL), and any remaining EtOAc was removed. The aqueous residue was counted with a scintillation counter.

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