Mechanistic studies of lanosterol 14α-methyl demethylase: substrate requirements for the component reactions catalyzed by a single cytochrome P-450 isozyme

Robert T. Fischer, Simon H. Stam, Paul R. Johnson, Soo S. Ko, Ronald L. Magolda, James L. Gaylor,* and James M. Trzaskos[1]

Medical Products Department and Central Research and Development Department, E. I. du Pont de Nemours and Co., Du Pont Experimental Station, Wilmington, DE 19880-0400, and Johnson and Johnson, Co.,* New Brunswick, NJ

Abstract Lanosterol 14α-methyl demethylation is a cytochrome P-450-dependent process that proceeds through the oxidative sequence of alcohol, aldehyde followed by decarbonylation with formic acid release. Microsomal metabolism studies shown here indicate that only lanostenols and 32-oxy-lanostenols with unsaturation at either the Δ^7 or Δ^8 position in the sterol can be demethylated. The 14α-methyl group of either lanostan-3β-ol or Δ^6 lanostenol is not oxidized to the anticipated C-32 alcohol or aldehyde by the enzyme, nor are the corresponding 32-oxy-lanostanols demethylated when incubated with microsomal preparations. Despite the lack of metabolism, the saturated and Δ^6 sterol analogues are effective competitive inhibitors of demethylase activity. Utilizing preferred substrates, comparison of the component reactions of the demethylation sequence shows that both the oxidative function and lyase function are sensitive to common inhibitors and that both activities require NADPH. These findings strongly support the premise that a P-450 isozyme does catalyze each phase of the lanosterol 14α-methyl demethylation sequence. Collectively these results demonstrate the double-bond requirement for both components of the demethylation sequence and suggest that the olefinic electrons at Δ^7 or Δ^8 but not Δ^6 may participate directly during demethylation. This participation may involve stabilizing a transition state intermediate or directing activated oxygen insertion as part of the P-450 monoxygenase mechanism. —**Fischer, R. T., S. H. Stam, P. R. Johnson, S. S. Ko, R. L. Magolda, J. L. Gaylor, and J. M. Trzaskos.** Mechanistic studies of lanosterol 14α-methyl demethylase: substrate requirements for the component reactions catalyzed by a single cytochrome P-450 isozyme. *J. Lipid Res.* 1989. **30:** 1621–1632.

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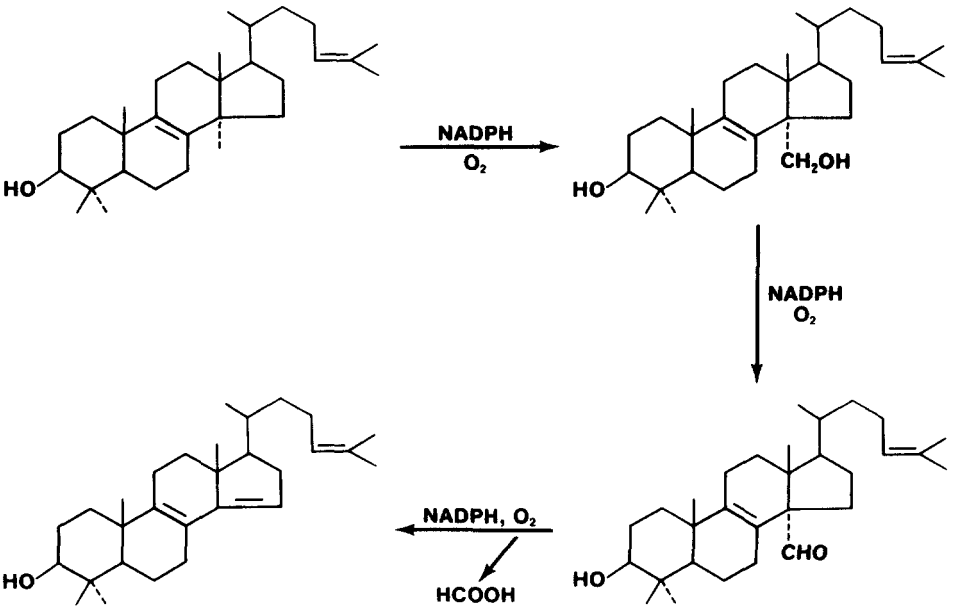
Lanosterol 14α-methyl demethylation is the first enzyme-catalyzed reaction sequence in the cholesterol biosynthetic pathway that utilizes a sterol as substrate (1). The reaction involves sequential oxidations of the initial sterol substrate, lanosterol or 24,25-dihydrolanosterol, to the corresponding 14α-hydroxymethyl, 14α-carboxaldehyde, and finally to the conjugated $\Delta^{8,14}$-diene product (**Scheme I**). Evidence that the sequential oxidations are catalyzed by a single cytochrome P-450 species is derived from purification of the enzyme to homogeneity (2–4), as well as isolation of lanosterol 14α-methyl demethylase-deficient mutants from yeast (5) and Chinese hamster ovary cells (6). Previous literature reports, however, have suggested that distinct oxidases may be associated with the individual component reactions collectively termed lanosterol demethylase (7). Similarly, we have demonstrated that various perturbations to the 14α-methyl demethylase activity in hepatic microsomes can result in selective accumulation of oxysterol intermediates over demethylated end-product even though the reaction sequence is tightly coupled. These results suggest that unique kinetic and/or mechanistic properties of the enzyme may be associated with the initial oxidations that are not associated with the final oxidative lyase activity of the enzyme.

In the present report, the biochemical properties of the lanosterol 14α-methyl demethylase are further defined. Kinetic analyses and determination of substrate requirements for the initial oxidative steps and the final lyase activity of the enzyme are reported. The results demonstrate an absolute requirement for a double-bond in the sterol nucleus in order that demethylation might occur. Kinetic analyses also

Abbreviations: HPLC, high pressure liquid chromatography; SIM, selective ion monitoring; TLC, thin-layer chromatography; GLC-MS, gas–liquid chromatography–mass spectrometry; MPLC, medium pressure liquid chromatography.

[1]To whom correspondence should be addressed.



Scheme I. Lanosterol 14α-methyl demethylase-catalyzed reaction sequence in cholesterol biosynthesis.

demonstrate that the demethylation component reactions, oxidase and lyase, are catalyzed by a single cytochrome P-450 isozyme.

METHODS

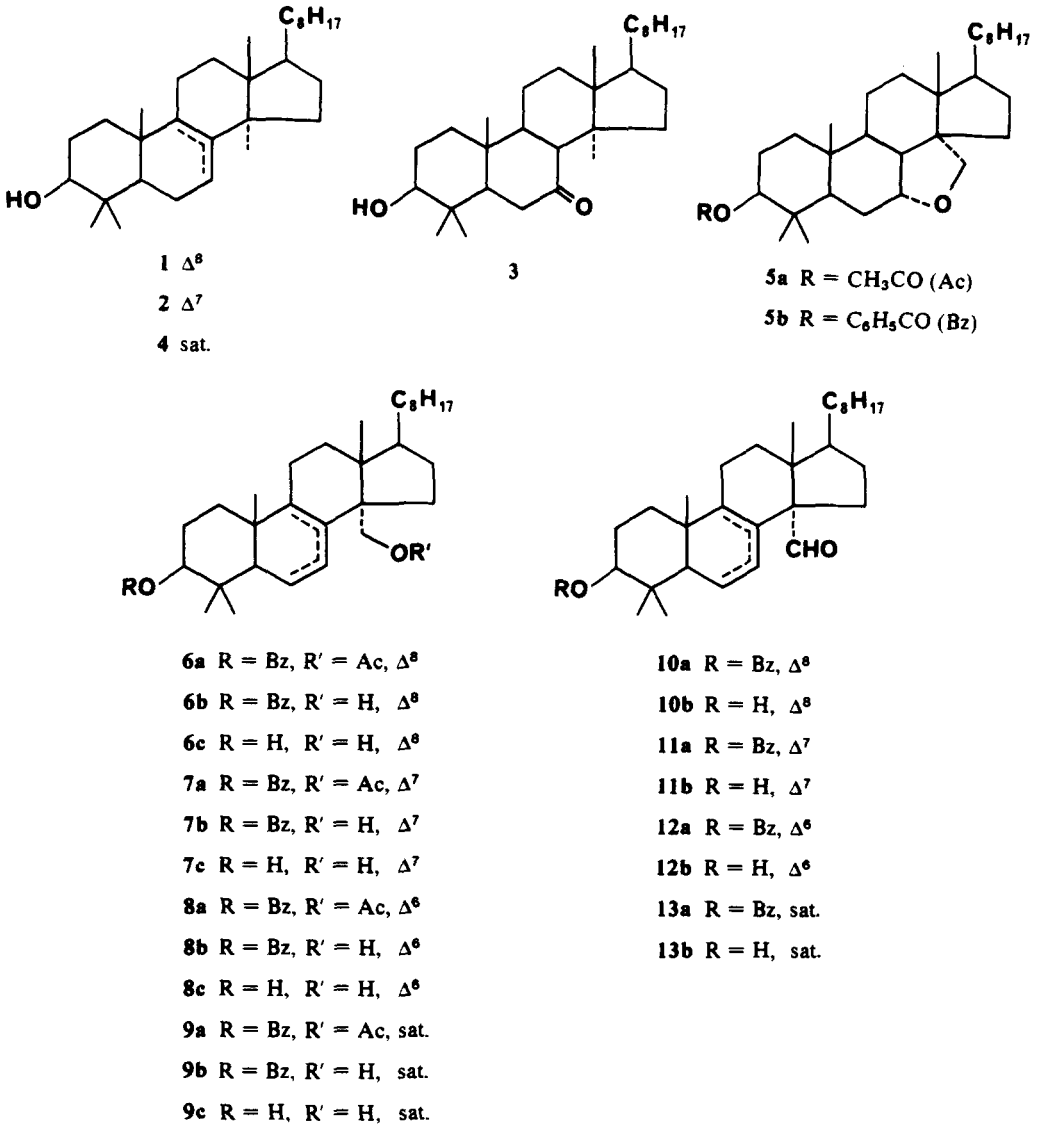
Lanosterol 14α-methyl demethylase assay

Demethylase assays were performed as described previously (8). Kinetics studies were performed with [24,25-$^3H_2$]-24,25-dihydrolanosterol as substrate with various sterols as indicated as inhibitors. Sterols were suspended in a total of 5 mg Triton WR-1339 in reaction tubes prior to the addition of assay buffer (0.1 M potassium phosphate, 0.1 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) and enzyme source. Reactions were initiated after a 5-min preincubation at 37°C by the addition of cofactors (2.0 mM NADPH, 0.3 mM NADH, 10 mM isocitrate, 4 mM $MgCl_2$, 0.5 units isocitrate dehydrogenase). Total assay volume was 0.5 ml. Incubations were terminated by the addition of 0.5 ml of 15% KOH in 95% methanol followed by saponification, extraction, and HPLC analysis of reaction products (9).

Metabolic conversions of non-radiolabeled sterols were determined by GLC–MS analysis employing selective ion monitoring (SIM). Ions used during SIM mode analyses were as follows: 412(M^+) and 397(M^+–CH_3) for Δ^6,Δ^7, and Δ^8 demethylated diene sterols (10); 486(M^+) and 471(M^+–CH_3) for Me_3Si-derivatized demethylated saturated sterols; 590(M^+), 487 (M^+–CH_2OTMS) and 397 (M^+–CH_2OTMS–HOTMS) for Me_3Si-derivatized lanostane-3β,32-diol, and 3β-hydroxylanostan-32-aldehyde following reductive treatment (see below); and 485(M^+–CH_2OTMS) and 395(M^+–CH_2OTMS–HOTMS) for Me_3Si-derivated lanostene-3β-32-diols and 3β-hydroxylanosten-32-aldehydes following reductive treatment. GLC-MS was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a 5970 mass selective detector. A capillary column of DB17-30W J&W Scientific, Inc.) was used for sterol separations with helium carrier gas. The following temperature program was used: starting temperature, 235°C; ramp rate, 5°C/min; final temperature, 265°C.

Me_3Si-derivatization of sterols

The C-32 oxysterol demethylation intermediates were unstable under the gas chromatographic conditions we used; therefore, derivatization was required prior to GLC-MS analysis. First, 3β-hydroxy-32-aldehydic sterols were reduced to the 32-hydroxy analogue by $NaBH_4$ treatment. The sterol sample obtained by extracting enzyme reaction mixtures was dissolved in 500 μl of methanol at room temperature and 5.0 mg of $NaBH_4$ was added and allowed to react for 3 h. The reaction was then quenched with 2 ml of water and sterols were extracted with 10 ml of petroleum ether. The petroleum ether extract was evaporated under N_2 gas and the reduced sterols were derivatized to their respective trimethylsilyl ethers. Sterols were dissolved in 200 μl dry pyridine and 200 μl of Tri-Sil TBT reagent (Pierce Chemicals), capped under N_2 gas, and reacted overnight at 80°C. The following day, the Me_3Si mixture was dissolved in 10 ml petroleum ether and backwashed with 2 ml of 10% sodium bicarbonate solution. The organic phase was removed and evaporated to dryness under N_2 gas. Derivatized sterols were dissolved



Scheme II. Sterol structures of synthetic intermediates and sterol substrates referred to in the text.

in 250 μl of toluene and a 4.0- μl aliquot was analyzed by GLC–MS as described above. Derivatization of 3β,32-diol sterols was accomplished by Tri-Sil TBT treatment as above on incubation extracts without the $NaBH_4$ reduction.

Enzyme source

Hepatic microsomes were used throughout these investigations as enzyme source. Microsomes from cholestyramine-fed rats were isolated as previously described (8, 9).

Protein determination

Protein was determined by the Bio-Rad dye binding assay according to the manufacturer's directions using bovine serum albumin as a standard.

MATERIALS

Synthesis of sterol substrates

Lanostan-3β-ol (**Scheme II, 4**)[2]. To 3 ml of dry diethylene glycol was added sodium (0.3 g, 13 mmol) under N_2 and the mixture was stirred at 40°C until dissolved. Then hydrazine (1.5 ml, 47 mmol) was added and the solution was heated at 100°C for 0.5 h. After cooling to 40°C, a solution of 3β-hydroxylanostan-7-one (**3**) (11) (200 mg, 0.45 mmol) was added in 2 ml of toluene and the solution was refluxed for 24 h in a 180°C oil bath. The excess hydrazine was distilled off at 200°C and the remaining solution was refluxed at 240°C for 24 h. After cooling to room tempera-

[2]Bold face numerals refer to structures given in Scheme II.

ture it was diluted with water and neutralized with 1 N HCl. The mixture was extracted with ethyl acetate (3 × 100 ml) and the combined extracts were washed with brine, dried over $MgSO_4$, and evaporated to give a solid residue. It was purified by HPLC to afford pure lanostan-3β-ol (**4**) (100 mg, 51% yield): mp 177–178°C; $[\alpha]^{25}D$ + 24.8 ± 3.3° (c 0.60, $CHCl_3$); NMR ($CDCl_3$) d 3.22 (1H, m), 2.0–0.9 (32H, m), 0.99 (3H, s), 0.92 (3H, s), 0.90–0.87 (9H, m), 0.82 (3H, d, J = 7Hz), 0.79 (3H, s); IR (KBr) 3270, 2950, 2930, 2870, 1380, 1365, 1040 cm^{-1}; MS (EI), *m/z* 430 (10), 415 (10), 290 (35), 275 (85), 206 (100); HRMS Calcd. for $C_{30}H_{54}O$: 430.4175. Found: 430.4162.

3β-Hydroxylanostan-7α,32-oxide benzoate (**5b**). To a solution of 3β-acetoxylanostan-7α,32-oxide (**5a**) (12,13) (2.85 g, 5.86 mmol) in 100 ml of toluene was added 100 ml of 2 M KOH in 95% ethanol and the mixture was stirred at room temperature for 16 h. Water and toluene were added until two layers separated and the aqueous layer was extracted with toluene (3 × 150 ml). The combined extracts were washed with brine, dried ($MgSO_4$), and evaporated to give a white solid of 3β-hydroxylanostan-7α,32-oxide (2.60 g).

The crude material was dissolved in 150 ml of pyridine at 50°C and benzoyl chloride (2 ml, 17.2 mmol) and N,N-dimethylaminopyridine (71.5 mg, 0.59 mmol) were added to the solution. It was stirred at 50°C for 18 h and 250 ml of ether was added after cooling to room temperature. Then the solution was washed with 1 N HCl, 10% $CuSO_4$, water, and brine, dried ($MgSO_4$), and evaporated to give a solid residue. Column chromatography on silica gel with elution by toluene–EtOAc 98:2 afforded pure benzoyl ester **5b** (2.40 g, 75% yield): mp 225–227°C (acetone); $[\alpha]^{25}D$ + 41 ± 0.8° (c 1.01, $CHCl_3$); NMR ($CDCl_3$) δ 8.05 (2H, d, J = 7.2 Hz), 7.64–7.39 (3H, m), 4.75 (1H, dd, J = 11.5, 4.6 Hz), 4.22 (1H, m), 4.02 (1H, d, J = 7.5 Hz), 3.38 (1H, d, J = 7.5 Hz), 2.1–0.8 (26H, m), 1.03 (3H, s), 0.92–0.87 (18H, m); IR ($CHCl_3$) 2955, 2870, 1710, 1278, 1116, 1025, 962 cm^{-1}; MS (EI), *m/z* 517 (38), 403 (64), 395 (100); HRMS Calcd for $C_{36}H_{53}O_2$ ($M-CH_2OH$): 517.4045. Found 517.3994.

32-Acetoxylanost-8-en-3β-ol benzoate (6a), 32-acetoxylanost-7-en-3β-ol benzoate (**7a**), and *32-acetoxylanost-6-en-3β-ol benzoate* (**8a**). A solution of the oxido-benzoate **5b** (1.90 g, 3.47 mmol) and pyridine hydrochloride (3.8 g, 32.9 mmol) in 380 ml of acetic anhydride was refluxed for 18 h under nitrogen and cooled to room temperature. It was poured into 400 ml of ice water and stirred for 2 h. Then the mixture was extracted with ether (4 × 150 ml) and the combined extracts were washed with cold 5% HCl (300 ml), saturated $NaHCO_3$ solution (8 × 200 ml), water (2 × 100 ml), and brine (100 ml). The ether solution was dried ($MgSO_4$) and evaporated to give a solid residue. The crude product was subjected to preparative HPLC (toluene followed by toluene–EtOAc 99.5 : 0.5 to provide 391 mg of the Δ^8 isomer **6a** (19% yield), 555 mg of the Δ^7 isomer **7a** (27% yield), and 329 mg of Δ^6 isomer **8a** (16% yield).

Physical data of **6a**: R_f 0.63 (ETOAc–toluene 1:9); mp 109.5–110°C (95% ethanol); $[\alpha]^{25}D$ + 61 ± 2° (c 1.00, $CHCl_3$); NMR ($CDCl_3$) δ 8.06 (2H, d, J = 7.2 Hz), 7.60–7.40 (3H, m), 4.75 (1H, dd, J = 11.4, 4.2 Hz), 4.08 (1H, d, J = 10.5 Hz), 3.97 (1H, d, J = 10.5 Hz), 2.2–0.85 (26H, m), 2.06 (3H, s), 1.08 (3H, s), 0.97 (3H, s), 0.90 (3H, d, J = 6.3 Hz), 0.88 (6H, d, J = 6.6 Hz), 0.73 (3H, s); IR ($CHCl_3$) 2950, 2860, 1710, 1600, 1465, 1450, 1275, 1115, 1025, 980, 970 cm^{-1}; MS (EI), *m/z* 530 (2), 517 (22), 395 (100); HRMS Calcd for $C_{37}H_{54}O_2$($M-CH_3CO_2H$): 530.4142. Found 530.4116.

Physical data of **7a**: R_f 0.63 (EtOAc–toluene 1:9); mp 154–155°C (95% ethanol); $[\alpha]^{25}D$ + 50 ± 2° (c 1.03, $CHCl_3$); NMR ($CDCl_3$) δ 8.06 (2H, d, J = 7.2 Hz), 7.60–7.40 (3H, m), 5.25 (1H, d, J = 4.8 Hz), 4.79 (1H, dd, J = 10.8, 4.2 Hz), 4.59 (1H, d, J = 10.8 Hz), 3.73 (1H, d, J = 10.8 Hz), 2.15–0.85 (25H, m), 1.99 (3H, s), 1.13 (3H, s), 0.95 (3H, s), 0.94 (3H, s), 0.90 (3H, d, J = 6.0 Hz), 0.88 (6H, d, J = 7.2 Hz), 0.73 (3H, s); IR ($CHCl_3$) 2950, 2860, 1710, 1600, 1465, 1450, 1380, 1365, 1275, 1115, 1025, 965 cm^{-1}; MS (EI), *m/z* 517 (25), 395 (100); HRMS Calcd for $C_{36}H_{50}O_2$ ($M-CH_2CO_2CH_3$): 517.4045. Found 517.3999.

Physical data of **8a**: R_f 0.66 (EtOAc–toluene 1:9); mp 141–141.5°C (90% ethanol); $[\alpha]^{25}D$ – 36.6 ± 2° (c 1.01, $CHCl_3$); NMR ($CDCl_3$) δ 8.06 (2H, d, J = 7.2 Hz), 7.60–7.40 (3H, m), 5.61 (1H, d, J = 10.2 Hz), 5.52 (1H, d, J = 10.2 Hz), 4.77 (1H, dd, J = 11.4, 4.8 Hz), 4.56 (1H, d, J = 11.5 Hz), 3.96 (1H, d, J = 11.5 Hz), 2.42–0.85 (24H, m, 1.99 (3H, s), 1.03 (3H, s), 0.97 (3H, s), 0.91–0.87 (15H, m); IR ($CHCl_3$) 2950, 2860, 1720, 1600, 1470, 1450, 1385, 1370, 1315, 1275, 1115, 1025, 970 cm^{-1}; MS (EI), *m/z* 517 (9), 453 (32), 408 (30), 395 (100); HRMS Calcd for $C_{36}H_{53}O_2$ ($M-CH_2CO_2CH_3$): 517.4045. Found 517.4042.

32-Acetoxylanostan-3β-ol benzoate (**9a**). To solution of the Δ^6 acetoxybenzoate **8a** (320 mg, 0.54 mmol) in 70 ml of ethanol was added 100 mg of 10% Pd on carbon and the mixture was stirred under hydrogen (200 atm) at 80°C for 24 h. After cooling, the catalyst was filtered off through Celite and rinsed with methylene chloride. Evaporation of the solvents gave 315 mg of residue. Purification by preparative HPLC with elution by toluene provided pure saturated isomer **9a** (245 mg, 76% yield): mp 143–144°C (EtOAc–methanol 1:3); $[\alpha]^{25}D$ + 36.0 ± 2.0° (c 1.04, $CHCl_3$); NMR ($CDCl_3$) δ 8.05 (2H, d, J = 8.1 Hz), 7.56 (1H, t, J = 7.2 Hz), 7.45 (2H, t, J = 7.5 Hz), 4.73 (1H, dd, J = 11.7, 4.8 Hz), 4.20 (2H, s), 2.07 (3H, s), 2.10–0.80 (28H, m), 1.04 (3H, s), 0.99 (3H, s), 0.93 (3H, s), 0.88–0.86 (12H, m); IR ($CHCl_3$) 2950, 2870, 1712, 1602, 1586, 1468, 1451, 1372, 1320, 1315, 1118, 1025 cm^{-1}; MS (EI), *m/z* 532 (4), 519 (14), 397 (100), 386 (14), 310 (7), 297 (16), 135 (35); HRMS Calcd for $C_{37}H_{56}O_2$ ($M-CH_3CO_2H$): 532.4280. Found 532.4309.

Lanost-8-ene-3β,32-diol 3-benzoate (**6b**). To a solution of the acetoxy-benzoate **6a** (330 mg, 0.56 mmol) in 100 ml of ethanol at 5°C was added a solution of potassium hydroxide

(87%, 8.3 g) in 30 ml of 70% aqueous ethanol and the mixture was stirred at 10°C for 2 h. Then 40 ml of ice water was added and it was extracted with methylene chloride (3 × 100 ml). The combined extracts were washed with water, dried ($MgSO_4$), and evaporated to give a solid residue. MPLC on silica gel with elution by EtOAc–toluene 2:98 afforded pure hydroxy benzoate **6b** (209 mg, 68% yield): R_f 0.50 (EtOAc–toluene 1:9); mp 167–168.5°C (95% ethanol); $[\alpha]^{25}D$ +79 ± 2° (c 1.02, $CHCl_3$); NMR ($CDCl_3$) δ 8.06 (2H, d, J = 7.2 Hz), 7.60–7.40 (3H, m), 4.75 (1H, dd, J = 11.7, 4.2 Hz), 3.66 (1H, dd, J = 10.8, 9.3 Hz), 3.25 (1H, dd, J = 10.8, 3.8 Hz), 2.15–0.85 (26H, m), 1.13 (3H, s), 1.08 (3H, s), 0.98 (3H, s), 0.90 (3H, d, J = 6.5 Hz), 0.88 (6H, d, J = 6.6 Hz), 0.72 (3H, s); IR ($CHCl_3$) 3480, 2950, 2860, 1710, 1460, 1445, 1305, 1275, 1115 cm^{-1}; MS (EI), *m/z* 530 (8), 518 (42), 395 (68), 105 (100); HRMS Calcd for $C_{37}H_{54}O_2$ (M–H_2O): 530.4124. Found 530.4162.

Lanost-7-ene-3β,32-diol 3-benzoate (**7b**). By the method described above the acetoxy-benzoate **7a** was converted into the hydroxy benzoate **7b** in 73% yield: R_f 0.50 (EtOAc–toluene 1:9); mp 209.5–211°C (acetone); $[\alpha]^{25}D$ +51.1 ± 2° (c 1.01, $CHCl_3$); NMR ($CDCl_3$) δ 8.06 (2H, d, J = 7.5Hz), 7.60–7.40 (3H, m), 5.39 (1H, m), 4.75 (1H, dd, J = 11.3, 4.7 Hz), 3.66 (1H, d, J = 10.2 Hz), 3.26 (1H, t, J = 10.2 Hz), 2.15–0.85 (26H, m), 0.96 (3H, s), 0.89 (3H, d, J = 6.5 Hz), 0.87 (6H, d, J = 6.8 Hz), 0.74 (3H, s); IR ($CHCl_3$) 3500, 2940, 2860, 1705, 1600, 1465, 1315, 1275, 1115, 1020, 970 cm^{-1}; MS (EI), *m/z* 518 (22), 395 (37), 381 (19), 105 (100); HRMS Calcd for $C_{36}H_{54}O_2$ (M–CH_2O): 518.4124. Found 518.4161.

Lanost-6-ene-3β,32-diol 3-benzoate (**8b**). By the method described above the acetoxy-benzoate **8a** was converted into the hydroxy benzoate **8b** in 62% yield: R_f 0.55 (EtOAc–toluene 1:9); mp 222–223°C (ethanol); $[\alpha]^{25}D$ −0.9 ± 2.0° (c 1.02, $CHCl_3$); NMR ($CDCl_3$) δ 8.05 (2H, d, J = 7.5 Hz), 7.60–7.40 (3H, m), 5.85 (1H, d, J = 10.2 Hz), 5.69 (1H, d, J = 10.2 Hz), 4.78 (1H, dd, J = 11.6, 4.7 Hz), 4.20 (1H, d, J = 11.5 Hz), 3.46 (1H, t, J = 11.5 Hz), 2.40 (1H, m), 2.12–0.85 (24H, m), 1.04 (3H, s), 0.98 (3H, s), 0.93 (3H, s), 0.90–0.84 (12H, m); IR ($CHCl_3$) 3690, 3540, 2960, 2870, 1710, 1600, 1580, 1470, 1450, 1315, 1280, 1120 cm^{-1}; MS (EI), *m/z* 530 (5), 517 (17), 408 (15), 403 (8), 395 (100) HRMS Calcd for $C_{37}H_{54}O_2$ (M–H_2O): 530.4124. Found 530.4093.

Lanostane-3β,32-diol 3-benzoate (**9b**) *and Lanostane-3β,32-diol* (**9c**). To a solution of the acetoxy-benzoate **9a** (205 mg, 0.35 mmol) in 20 ml of toluene and 30 ml of ethanol at 0°C was added ethanolic KOH (3.3 g in 10 ml of 90% aqueous ethanol) and the mixture was stirred at 0°C–15°C for 6 h. After addition of ice, it was extracted with toluene (50 ml) and then with methylene chloride (2 × 50 ml). The combined extracts were washed with water, dried ($MgSO_4$), and evaporated to give 179 mg of solid residue. Separation of the crude product by MPLC on silica gel with elution by 1%, 2%, 5%, and 10% EtOAc in toluene successively afforded 21 mg of the unreacted **9a** (10%), 119 mg of the hydroxy-benzoate **9b** (69% yield based on the recovered starting material), and 29 mg of the diol **9c** (21% yield).

Physical data of **9b**: mp 204.5–205°C (EtOAc); $[\alpha]^{25}D$ +41.2 ± 2.0°C (c 1.00, $CHCl_3$); NMR ($CDCl_3$) δ 8.05 (2H, d, J = 8.1 Hz), 7.56 (1H, t, J = 7.2 Hz), 7.45 (2H, t, J = 7.5 Hz), 4.74 (1H, dd, J = 11.4, 4.5 Hz), 3.91 (1H, d, J = 11.8 Hz), 3.60 (1H, d, J = 11.8 Hz), 1.95–0.80 (29H, m), 1.04 (3H, s), 1.00 (3H, s), 0.92 (3H, s), 0.88 (6H, d, J = 6.6 Hz), 0.88 (3H, d, J = 6.6 Hz), 0.85 (3H, s); IR ($CHCl_3$) 3630, 2948, 1706, 1600, 1582, 1465, 1450, 1318, 1312, 1117, 1025, 1010, 965 cm^{-1}; MS (EI), *m/z* 519 (26), 397 (100), 261 (7); HRMS Calcd for $C_{36}H_{55}O_2$ (M–CH_2OH): 519.4202. Found 519.4145.

Physical data of **9c**: mp 230–232°C (EtOAc); $[\alpha]^{25}D$ +22.4 ± 4.0°C (c 0.5, $CHCl_3$); NMR ($CDCl_3$ δ 3.89 (1H, d, J = 12 Hz), 3.58 (1H, d, J = 12 Hz), 3.21 (1H, dd, J = 11.7, 4.5 Hz), 1.90–0.80 (30H, m), 0.97 (3H, s), 0.92 (3H, s), 0.87 (6H, d, J = 6.3 Hz), 0.87 (3H, d, J = 6.3 Hz), 0.83 (3H, s), 0.80 (3H, s); IR ($CHCl_3$) 3624, 2945, 2865, 1477, 1466, 1383, 1375, 1369, 1364, 1044, 1010 cm^{-1}; MS (EI), *m/z* 428 (5), 415 (27), 397 (100), 301 (16), 135 (26); HRMS Calcd for $C_{30}H_{52}O$ (M–H_2O): 428.4018. Found 428.4019.

Lanost-8-en-3β-ol-32-al benzoate (**10a**). To a solution of the hydroxy-benzoate **6b** (200 mg, 0.37 mmol) in 100 ml of acetone at −10°C was added 2.3 ml of 1.1 M Jones reagent dropwise and the mixture was stirred at −10°C for 15 min. Then the reaction mixture was diluted with 100 ml of water and quickly extracted with toluene (3 × 50 ml). The combined extracts were washed with water (2 × 50 ml), dried ($MgSO_4$), and evaporated to give a solid residue. Purification by MPLC on silica gel with elution by toluene afforded pure aldehyde-benzoate **10a** (185 mg, 93% yield): mp 206–207°C (acetone); $[\alpha]^{25}D$ −243 ± 4°C (c 1.00, $CHCl_3$); NMR ($CDCl_3$) δ 9.47 (1H, s), 8.05 (2H, d, J = 7.5 Hz), 7.60–7.40 (3H, m), 4.73 (1H, dd, J = 11.6, 5.1 Hz), 2.40–0.85 (26H, m), 1.14 (3H, s), 1.07 (3H, s), 0.94 (3H, s), 0.90 (3H, d, J = 6.3 Hz), 0.87 (6H, d, J = 6.6 Hz), 0.77 (3H, s); IR ($CHCl_3$) 2940, 2860, 1710, 1690, 1465, 1450, 1275, 1115 cm^{-1}; MS (EI), *m/z* 517 (37), 395 (100); HRMS Calcd for $C_{36}H_{53}O_2$ (M–CHO): 517.4046. Found 517.4076.

3β-Hydroxylanost-8-en-32-al (**10b**). A solution of the aldehyde-benzoate **10a** (100 mg, 0.18 mmol) in 52 ml of 1 M KOH in 95% ethanol was stirred at 50°C for 3.5 h and cooled to room temperature. After dilution with 50 ml of water, the mixture was extracted with methylene chloride (3 × 50 ml) and the combined extracts were dried ($MgSO_4$) and evaporated to give 83 mg of solid residue. Purification by MPLC on silica gel with elution by EtOAc–toluene 1:9 afforded pure unprotected aldehyde **10b** (78 mg, 96% yield): mp 178–178.5°C (acetone) [lit.(12), 159–160°C]; $[\alpha]^{25}D$ −283.6 ± 2.0°C (c 1.03, $CHCl_3$); NMR ($CDCl_3$) δ 9.45 (1H, s), 3.23 (1H, dd, J = 11.4, 4.5 Hz), 2.30–0.70 (27H, m), 1.06 (3H, s), 0.98 (3H, s), 0.90 (3H, d, J = 7.2 Hz),

0.86 (6H, d, J = 6.9 Hz), 0.83 (3H, s), 0.76 (3H, s); IR (KBr) 3492, 2954, 2932, 2899, 2871, 2731, 1700, 1467, 1457, 1372, 1040, 1028 cm^{-1}; MS (EI), *m/z* 413 (100), 395 (53), 273 (10), 241 (11), 215 (12), 119 (22); HRMS Calcd for $C_{29}H_{49}O$ (M-CHO): 413.3783. Found 413.3761.

Lanost-7-en-3β-ol-32-al benzoate (**11a**). By the method described for **10a**, the hydroxy-benzoate **7b** was converted into the aldehyde-benzoate **11a** in 85% yield; mp 193.5–195.5°C (acetone); $[\alpha]^{25}D$ +46.5 ± 2°C (c 0.99, $CHCl_3$); NMR ($CDCl_3$) δ 9.66 (1H, s), 8.05 (2H, d, J = 7.5 Hz), 7.60–7.40 (3H, m), 5.45 (1H, m), 4.76 (1H, dd, J = 11.1, 4.2 Hz), 2.20–0.85 (25H, m), 1.14 (3H, s), 0.98 (3H, s), 0.95 (3H, s), 0.93 (3H, d, J = 6.5 Hz), 0.87 (6H, d, J = 6.6 Hz), 0.75 (3H, s); IR ($CHCl_3$) 2950, 2860, 1705, 1600, 1275, 1115, 970 cm^{-1}; MS (EI), *m/z* 518 (5), 517 (13), 395 (68), 105 (100); HRMS Calcd for $C_{36}H_{55}O_2$ (M-CHO): 517.4045. Found 517.4052.

3β-Hydroxylanost-7-en-32-al (**11b**). To a solution of the aldehyde-benzoate **11a** (40.4 mg, 0.074 mmol) in 7 ml of toluene was added 32 ml of 1 M KOH in 95% ethanol and the mixture was stirred at 50°C for 2.5 h. The reaction mixture was worked up and the product was purified as described for the compound **10b** to give pure unprotected aldehyde **11b** (26 mg, 79% yield): mp 126–128°C (acetone) [lit.(12), 119–120°C]; $[\alpha]^{20}D$ +18.9 ± 2.0°C (c 0.95, $CHCl_3$); NMR ($CDCl_3$. δ 9.62 (1H, s), 5.44 (1H, brs), 3.25 (1H, dd, J = 11.4, 4.5 Hz), 2.10–0.70 (26H, m), 0.98 (3H, s), 0.92 (3H, d, J = 7 Hz), 0.87 (3H, d, J = 6.6 Hz), 0.86 (3H, d, J = 6.6 Hz), 0.74 (3H, s); IR (KBr) 3408, 3375, 2957, 2934, 2883, 2872, 2742, 1712, 1467, 1445, 1382, 1367, 1055, 1028 cm^{-1}; MS (EI), *m/z* 442 (1), 414 (34), 413 (100), 395 (36); HRMS Calcd for $C_{30}H_{50}O_2$ (M^+): 442.3811. Found 442.3807.

Lanost-6-en-3β-ol-32-al benzoate (**12a**). To a solution of the hydroxy-benzoate **8b** (73 mg, 0.13 mmol) in 5 ml of dry methylene chloride were added pyridinium dichromate (74 mg, 0.19 mmol) and 72 mg of powdered 4A molecular sieves and the mixture was stirred at room temperature for 2 h. After adding 40 ml of ether, the mixture was filtered through Florisil and the solvents were evaporated off. The residue was purified by MPLC on silica gel with elution by toluene to afford pure aldehyde-benzoate **12a** (43 mg, 60% yield): mp 175–177°C (acetone); $[\alpha]^{20}D$ −26.8 ± 2.0°C (c 1.01, $CHCl_3$); NMR ($CDCl_3$) δ 9.97 (1H, s), 8.05 (2H, d, J = 7.5 Hz), 7.60–7.40 (3H, m), 5.69 (1H, d, J = 10.7 Hz), 5.64 (1H, d, J = 10.7 Hz), 4.74 (1H, dd, J = 11.7, 4.8 Hz), 2.46 (1H, m), 2.23–0.85 (23H, m), 1.03 (3H, s), 0.97 (3H, s), 0.95–0.90 (12H, m), 0.87 (6H, d, J = 6.6 Hz); IR (KBr) 2950, 2870, 1718, 1710, 1600, 1580, 1465, 1450, 1275, 1115 cm^{-1}; MS (EI), *m/z* 546 (1), 518 (29), 396 (44), 381 (100); HRMS Calcd for $C_{37}H_{54}O_3$ (M^+): 546.4073. Found 546.4099.

3β-Hydroxylanost-6-en-32-al (**12b**). To a solution of the aldehyde-benzoate **12a** (3.6 mg, 0.0066 mmol) in 0.6 ml of toluene was added 1.9 ml of 1 M KOH in 95% ethanol and the mixture was stirred at room temperature for 2.5 h and at 50°C for 1.5 h. The reaction mixture was worked up as described for the compound **10b** and purified by preparative TLC with EtOAc–toluene 1:9 to afford pure unprotected aldehyde **12b** (2.3 mg, 79% yield): mp NMR ($CDCl_3$) d 9.92 (1H, s), 5.68 (1H, d, J = 10.5 Hz), 5.60 (1H, d, J = 10.5 Hz), 3.23 (1H, dd, J = 10.8, 4.8 Hz), 2.45–0.80 (25H, m), 1.00 (3H, s), 0.91 (3H, d, J = 6.6 Hz), 0.90 (3H, s), 0.86 (6H, d, J = 6.6 Hz), 0.84 (3H, s), 0.78 (3H, s); IR (KBr) 3479, 3030, 2952, 2930, 2870, 2720, 1720, 1468, 1381, 1065 cm^{-1}; MS (EI), *m/z* 442 (2), 414 (100), 399 (52), 396 (39), 381 (30); HRMS Calcd for $COH_{50}O_2$ (M^+): 442.3811. Found 442.3803.

Lanostan-3β-ol-32-al benzoate (**13a**). To a solution of the hydroxy-benzoate **9b** (99 mg, 0.18 mmol) in 20 ml of acetone at 0°C was added 1.6 ml of 1.1 M Jones reagent dropwise and the mixture was stirred at 0°C for 0.5 h. Then the mixture was diluted with 20 ml of water and quickly extracted with toluene (3 × 20 ml). The combined extracts were washed with water (2 × 15 ml), dried ($MgSO_4$), and evaporated to give a solid residue of chromatographically pure aldehyde-benzoate **13a** (97 mg, 98% yield): mp 184– 186°C (EtOAc); $[\alpha]^{25}D$ +0.6 ± 2.0°C (c 1.04, $CHCl_3$); NMR ($CDCl_3$) δ 10.24 (1H, s), 8.05 (2H, d, J = 7.5 Hz), 7.56 (1H, t, J = 7.2 Hz), 7.45 (2H, t, J = 7.5 Hz), 4.71 (1H, dd, J = 11.7, 4.5 Hz), 2.15–0.80 (28H, m), 1.03 (3H, s), 0.98 (3H, s), 0.90–0.85 (15H, m); IR ($CHCl_3$) 3020, 2946, 2866, 2750, 1705, 1600, 1582, 1466, 1450, 1318, 1312, 1116, 1069, 1024, 964 cm^{-1}; MS (EI), *m/z* 548 (13), 519 (17), 397 (100), 313 (17); HRMS Calcd for $C_{37}H_{56}O_3$ (M^+): 548.4230. Found 548.4242.

3β-Hydroxylanostan-32-al (**13b**). To a solution of the aldehyde-benzoate **13a** (80 mg, 0.15 mmol) in 10 ml of toluene was added 21 ml of 1 M KOH in 95% ethanol and the mixture was stirred at 50°C for 8 h. The reaction mixture was worked up as described for compound **10b** to give the unprotected aldehyde **13b** (63 mg, 97% yield): mp 157–159°C (hexane); $[\alpha]^{25}D$ −24.9 ± 2.0° (c 0.99, $CHCl_3$); NMR ($CDCl_3$) δ 10.25 (1H, s), 3.20 (1H, dd, J = 11.4, 4.8 Hz), 2.13–0.75 (29H, m), 0.94 (3H, s), 0.91 (3H, s), 0.88–0.84 (12H, m), 0.79 (3H, s); IR ($CHCl_3$) 3610, 2945, 2865, 2752, 1702, 1465, 1445, 1383, 1035, 1025 cm^{-1}; MS (EI), *m/z* 444 (20), 415 (29), 397 (100), 331 (24); HRMS Calcd for $C_{30}H_{52}O_2$ (M^+): 444.3947. Found 444.3967.

Other materials

24,25-Dihydrolanosterol (**1**) was prepared from commercial sources by preparative HPLC (9). [24,25-$^3H_2$]-24,25-Dihydrolanosterol was from New England Nuclear (Lot #1574-252). Miconazole was from Sigma. Ketoconazole was obtained from Janssen Pharmaceuticals (Beesee, Belgium). Lanost-7-en-3β-ol (2) was prepared as described by Woodward (14). All other reagents were of the best grade commercially available and from sources described (8).

RESULTS

Chemical synthesis of sterols

A convenient synthesis of the double-bond isomers of 32-hydroxy-dihydrolanosterol has been reported by Parish and Schroepfer (12) and a modified procedure by Sonoda et al. (13) has appeared. These procedures provide the necessary Δ^6, Δ^7, and Δ^8 isomers of the oxylanosterol in a single operation. The saturated compound, lanostan-3β-ol (**4**) was synthesized by a reduction of 3β-hydroxylanostan-7-one (**3**) (11). However, because the above procedures do not distinguish the two hydroxy groups at C-3 and C-32, further selective elaboration of the hydroxy group at C-32 was troublesome (13). Thus, we have modified the reported synthesis to obtain selectively protected products, which allow easy modifications at C-32 position.

The acetyl group of 3β-acetoxylanostan-7α,32-oxide (**5a**) (12,13) was converted to a benzoyl derivative by treatment with base followed by re-esterification with benzoyl chloride to give the corresponding benzoate **5b**. The suitably protected oxide **5b** was treated with pyridine hydrochloride in refluxing acetic anhydride to afford Δ^8, Δ^7, and Δ^6 isomers of 32-acetoxylanosten-3β-ol benzoate **6a, 7a**, and **8a** in 19%, 27%, and 16% yields, respectively. The corresponding saturated compound **9a** was prepared by a catalytic hydrogenation of the Δ^6 isomer **8a** with 10% palladium on carbon in ethanol at 80°C in 76% yield. Selective hydrolysis of the acetate groups of **6a, 7a, 8a,** and **9a** with KOH in ethanol at 10°C freed the hydroxy group at C-32 to provide 3β-benzoyl derivatives **6a, 7b, 8b**, and **9b** for further elaboration.

The double-bond isomers of lanostene-3β,32-diol **6c, 7c**, and **8c** were synthesized either by treating **6a, 7a**, and **8a** with KOH at 50°C or more directly by following the procedure for **5a** (12). Experimental details and analytical data for these three diols are found in reference 11 and are not provided in this paper. The saturated isomer **9c** could be prepared by double hydrolysis of **9a** with KOH. However, during the selective hydrolysis of **9a** to give the monoprotected **9b**, the diol **9c** was obtained as a side product conveniently providing the material for biochemical studies.

3β-Hydroxylanost-8-en-32-al (**10b**) was obtained by an oxidation of the corresponding alcohol **6b** with Jones reagent in acetone at -10°C to give lanost-8-en-3β-ol-32-al benzoate (**10a**) followed by a saponification of the benzoate with KOH in ethanol–toluene 2:1 at 50°C in 89% overall yield. Δ^7 and saturated isomers **11b** and **13b** were also prepared in the same manner from **7b** and **9b** in 67% overall yields, respectively. Jones oxidation of the Δ^6 alcohol **8b**, on the other hand, was less satisfactory and pyridinium dichromate was used instead to provide lanost-6-en-3β-ol-32-al benzoate (**12a**) in 60% yield. Hydrolysis of **12a** with KOH afforded the unprotected Δ^6 aldehyde **12b** in near quantitative yield.

Sterol nuclear double-bond requirements for lanosterol 14α-methyl demethylase component oxidase and lyase activities

The component reactions of the lanosterol 14α-methyl demethylase are divided into an oxidative sequence with conversion of the initial 14α-methyl group to the corresponding 14α-hydroxymethyl and 14α-carboxaldehyde, and a lytic step with oxidative bond cleavage of the 14α-carboxaldehyde resulting in conjugated diene formation. By using synthetic substrates for each phase of the demethylation sequence, we have defined preferred substrates for the demethylase component reactions. The data are summarized in **Table 1**. Metabolism studies conducted under initial rate conditions show that a homoallylic double-bond is absolutely required in the sterol nucleus for both components of the demethylation reaction sequence. This is in keeping with previous observations made by Sharpless et al. (15), who demonstrated that lanostanol was not converted to cholesterol by liver preparations. Additionally, the double-bond regioisomers show rather large differences in metabolic activity which results in a reactivity order of $\Delta^8 > \Delta^7 > \Delta^6$ = saturated. This ordering could be due to decreased enzyme affinity for the sterols or lack of metabolic conversion of the respective sterol by the enzyme due to mechanistic requirements of the homoallylic double-bond. Decreased demethylase affinity for the various lanostanols or the Δ^6-regioisomer, however, is not substantiated by kinetic data (**Table 2**). The results clearly show that the series of lanostanols are very fine competitive inhibitors of the demethylase with K_i values comparable to the Δ^8 and Δ^7 counterparts and that the Δ^6-lanosterol is also a very potent inhibitor. Similarly, the lanost-7-en-3β-ol displays comparable affinity to the Δ^8-isomer for the demethylase with a K_m of 338 μM versus 312 μM, respectively. In contrast, a much lower V_{max} is observed for the Δ^7-isomer (Δ^7 V_{max} = 0.087 nmol/min per mg; Δ^8 V_{max} = 0.645 nmol/min per mg) which accounts for the lower reactivity.

Kinetics of lanosterol 14α-methyl demethylase lyase activity

In order to probe the positional double-bond requirement of the demethylase enzyme further, the lyase component of the reaction sequence was evaluated with both the Δ^7- and Δ^8-32-aldehyde oxysterols. As shown in **Fig. 1**, a vast difference in reactivity of the two substrates is observed. The Δ^8-isomer is clearly the preferred substrate for the lyase reaction with essentially 100% conversion of substrate to demethylated product occurring in just 5 min under defined conditions. This compares with the Δ^7-isomer which is metabolized less than 1% under these same conditions. The disparity in reactivity observed is reflected primarily in the kinetic constant V_{max} as shown in **Fig. 2**. A 125-fold lower V_{max} is observed for the Δ^7-alde-

TABLE 1. Lanosterol 14α-methyl demethylase activity

Sterol	Products (% of Total Added Sterol) C-32-ol	C-32-al	Demethylated Sterol	Rate nmol × min^{-1} × mg^{-1}
Lanost-8-en-3β-ol (30 min)	0.00	1.80	29.10	0.643
Lanost-7-en-3β-ol	0.80	4.90	3.00	0.091
Lanostan-3β-ol	0.00	0.00	0.00	0.00
Lanost-8-ene-3β,32-diol (1 min)	n.d.	n.d.	17.07	10.660[a]
Lanost-7-ene-3β,32-diol	n.d.	n.d.	26.16	0.109[a]
Lanost-6-ene-3β,32-diol	n.d.	0.00	0.00	0.000
Lanostane-3β,32-diol	n.d.	0.00	0.00	0.000
3β-Hydroxylanost-3-en-32-al (2 min)	n.d.	n.d.	42.62	13.320
3β-Hydroxylanost-7-en-32-al (2 min)	n.d.	n.d.	0.11	0.034
3β-Hydroxylanost-6-en-32-al	n.d.	n.d.	0.54	0.005
3β-Hydroxylanostan-32-al	n.d.	n.d.	0.00	0.000

The ability of lanosterol 14α-methyl demethylase to metabolize double-bond regioisomers of 24,25-dihydrolanosterol and C-32 oxysterols of 24,25-dihydrolanosterol was assessed using 250 μM concentrations of sterols listed here. All assays were performed with 1 mg microsomal protein for 60 min except where indicated by the times in parentheses. GLC–MS selective ion monitor analysis of the extracted sterols was performed for high sensitivity detection of anticipated metabolites; n.d., not determined.

[a]Value does not include 3β-hydroxylanost-8 or 7-en-32-aldehyde derived from diol substrate.

hyde compared to the Δ^8-aldehyde (0.17 nmol/min per mg vs. 20.6 nmol/min per mg) while a K_m less than 2-fold greater has been determined (615 μM vs. 368 μM). Such great differences in reactivity may be due in part to thermodynamic stability of the conjugated diene products or transition state intermediates that result during demethylation. These metabolites would appear to be unfavorable in the case of the Δ^7-isomer. Alternatively, the data may reflect the poor ability of the Δ^7-substrate to undergo oxidative attack by the demethylase enzyme due to conformational constraints.

Inhibitor sensitivity and cofactor requirements of lanosterol 14α-methyl demethylase component oxidase and lyase activities

Since the two portions of the demethylation sequence are catalyzed by the same enzyme (2–4), one would anticipate comparable sensitivity to inhibitors and cofactor dependence. Previous reports, however, have shown that the oxidase function and lyase function have different sensitivity to CO inhibition, thus suggesting two distinct oxidases in the demethylation sequence (7). The data

TABLE 2. Kinetic constants for various 14α-methyl sterols and double-bond regioisomers for lanosterol 14α-methyl demethylase

Sterol	Affinity K_i	14α-Methyl Demethylase K_m	V_{max}
	μM	μM	nmol × min^{-1} × mg^{-1}
Lanost-8,24-dien-3β-ol[a]	100	165	3.390
Lanost-8-en-3β-ol		312	0.645
Lanost-7-en-3β-ol	1600	338	0.087
Lanostan-3β-ol	345		
Lanost-8-ene-3β,32-diol	6	56	7.000
Lanost-7-ene-3β,32-diol	45		
Lanost-6-ene-3β,32-diol	1		
Lanostane-3β,32-diol	6		
3β-Hydroxylanost-8-en-32-al	80	368	20.600
3β-Hydroxylanost-7-en-32-al	161	615	0.165
3β-Hydroxylanostan-32-al	160		

Inhibition K_i values were determined with 83–500 μM [24,25-$^3H_2$]dihydrolanosterol as substrate and sufficient concentrations (20–200 μM) of the evaluated compounds to obtain measurable inhibition of lanosterol demethylase.

[a]Previously reported by Trzaskos, J. M., R. T. Fischer, and M. F. Favata, ref. 8.

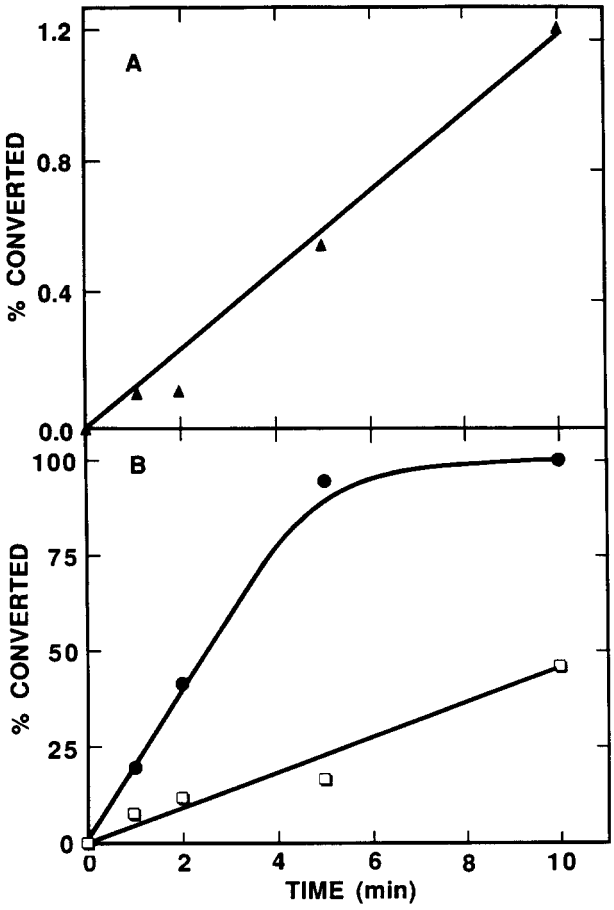


Fig. 1. Linearity of lanosterol 14α-methyl demethylase lyase activity. An assay was performed as outlined in Methods with 250 μM substrate concentrations in 5.0 mg of WR-1339. Subsequent to starting the assay with NADPH cofactor, 100-μL aliquots of the reaction mixture were removed at timed intervals and quenched in 500 μL of 15% KOH in 90% methanol. A blank determination for each substrate was performed without the addition of microsomal protein. A) Conversion of 3β-hydroxylanost-7-en-32-al with 2.0 mg of microsomal protein; B) conversion of 3β-hydroxylanost-8-en-32-al with 0.5 mg (□) and 2.0 mg (●) of microsomal protein. GLC-MS selective ion monitor analysis of the sterol extracts was performed to detect coeluting 412 and 397 amu ions, which confirmed the formation of the respective conjugated diene products.

presented in **Table 3** demonstrate that this discrepancy can be resolved when the lyase function is evaluated under conditions where first order rates are maintained. Metabolism of both 24,25-dihydrolanosterol and of 3β-hydroxylanost-8-en-32-aldehyde are inhibited to the same extent by CO and have a preference for NADPH as cofactor. Sensitivity to azole inhibitors is also maintained with the aldehyde substrate when evaluated under first order conditions.

DISCUSSION

With the use of synthetic substrates, this study has defined the substrate specificity and metabolic requirements of the component reactions of lanosterol 14α-methyl demethylase. The results demonstrate an absolute requirement for a homoallylic double-bond in the sterol nucleus in order to detect either initial oxidations at C-32 or subsequent decarbonylation products by the enzyme. These findings substantiate those of Sharpless et al. (15) who previously showed a requirement for the nuclear olefin in lanosterol in order that the C_{30}-sterol might be converted to cholesterol. The data presented here extend these observations by defining the selectivity of this double-bond requirement in the component reactions of the lanosterol 14α-methyl demethylase enzyme.

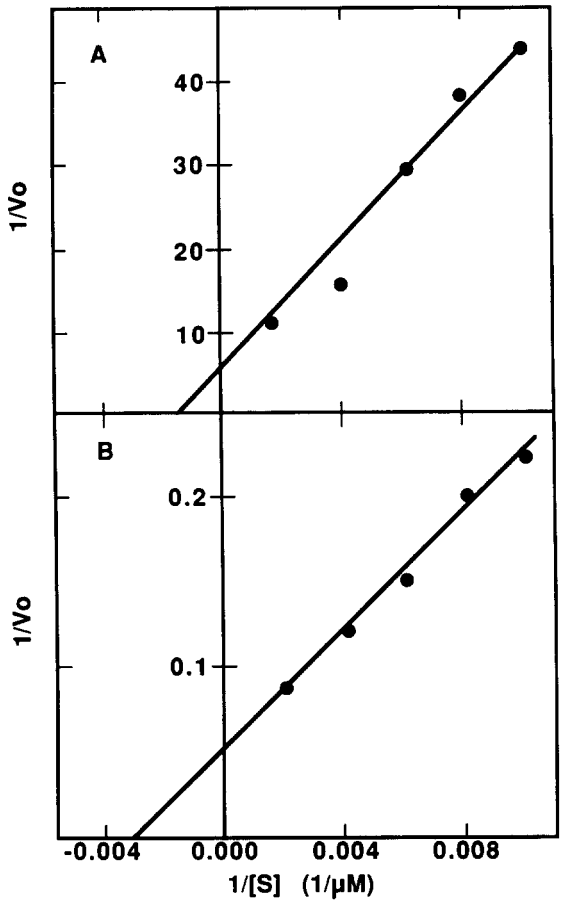


Fig. 2. Lanosterol 14α-methyl demethylase substrate kinetics of C-32 aldehyde oxysterols. Enzyme activities were evaluated with 100–500 μM concentrations of both substrates in the presence of 2.0 mg of microsomal protein and 5.0 mg of WR-1339 detergent. Lyase kinetics of 3β-hydroxylanost-7-en-32-al (A) was performed using a 45-min assay, and of 3β-hydroxylanost-8-en-32-al (B) in a 1.0-min assay. The formation of their respective conjugated diene products was measured by GLC-MS selective ion monitor analysis as described under Methods. The enzyme is significantly more suited to metabolism of the Δ^8-32-aldehyde sterol with a V_{max} of 20.6 nmol × min^{-1} × mg^{-1} as compared to 0.165 nmol × min^{-1} × mg^{-1} for the Δ^7 isomer. As with the K_i values in Table 3, the K_m values for these oxysterols indicate approximately a twofold difference in affinity for the P-450_{14DM}. The K_m for the Δ^8-32-al substrate is 368 μM whereas the K_m of Δ^7-32-al is 615 μM.

TABLE 3. Lanosterol 14α-methyl demethylase metabolism sensitivity comparing 3β-hydroxylanost-8-en-32-al and 24,25-dihydrolanosterol

Assay Conditions	Activity	Activity
	nmol × min^{-1} × mg^{-1}	*% control*
24,24-Dihydrolanosterol		
Control	1.153	100.0
+ CO/air (8:2)	0.063	5.5
− NADPH	0.000	0.0
3β-Hydroxylanost-8-en-32-al		
Control	11.280	100.0
+ CO/air (8:2)	0.560	5.0
− NADPH	0.000	0.0
− NADPH + 2.0 mM NADH	0.690	6.1
+ 20 μM Ketoconazole	0.100	0.9

A comparison of lanosterol demethylase 14α-methyl oxidation activity on 24,25-dihydrolanosterol and lyase activity of 3β-hydroxylanost-8-en-32-al under metabolic conditions that characterize the enzyme's requirements and sensitivities. The assays were performed as described in Methods with 250 μM substrate concentrations along with the additions or deletions to the enzyme reaction as shown.

In contrast to the nuclear double-bond requirement for metabolic transformation, all sterols tested either with or without a nuclear double-bond were found to be good competitive inhibitors of the 14α-methyl demethylase. Thus, it appears unlikely that the nuclear double-bond is involved in binding of the substrate to the enzyme active site. The possibility that the double-bond directs or imparts constraints necessary for catalysis, however, seems plausible by virtue of the large differences seen in the catalytic efficiency of the Δ^8, Δ^7, Δ^6, and the saturated isomers. Both Δ^8 and Δ^7 sterols undergo catalysis, yet the Δ^8 isomers are by far the preferred substrates. This is in keeping with results obtained on the conversion of Δ^8 and Δ^7 lanostenols to cholesterol by liver S-10 preparations (16). Whether these are steric or electronic constraints remains to be resolved. It seems likely that the orientation of the 14α-methyl group of lanosterol is undoubtedly affected by the position of the double-bond. This is only a slight effect, however, based upon molecular modeling calculations (R. L. Magolda, unpublished observation). Conceivably the small steric change imparted by the positional double-bond isomer could dramatically alter catalysis. In this regard, the placement of the double-bond in the B-ring has been shown to affect the catalytic rate for the $\Delta^{8,14}$-steroid 14-reductase (10). Preference for the Δ^8-isomer over the Δ^7-isomer is observed. Interestingly, the regioselectivity of this enzymic transformation is lost when the 4,4-bis normethyl diene substrates are compared (10), thus suggesting a steric rather than electronic effect. Unfortunately, regio double-bond specificity of other enzymes in the sterol biosynthetic pathway has not been investigated owing to the difficulty of substrate preparations.

Alternatively, a direct electronic effect of the double-bond may be responsible for the great difference in isomer reactivity seen in the demethylation process. Formation of a transition state intermediate arising during normal metabolism of the 14α-methyl group could be stabilized to different degrees based upon the presence or position of the unsaturation. One would envision such an electronic effect to be important in the final lytic reaction of the demethylation reaction where formic acid loss and conjugated diene formation proceeds upon abstraction of the 15α- hydrogen. The stability of the transition state and final conjugated diene product would be the driving force for decarbonylation. Electronic effects are not as easily invoked during the initial oxidations where oxygen insertion should proceed without involvement of the double-bond. One has to ask, then, why the saturated lanostanol is not metabolized at least to the aldehyde level of oxidation. Our failure to detect oxygenated lanostanol intermediates would suggest that constraints on metabolism are inherent in the structure of the saturated sterol which prevent its enzymic metabolism. Alternatively, metabolism to a product not detected by our analysis could also explain our results. We are in the process of investigating these possibilities.

The results presented here also show the relative substrate specificity of the demethylase for the various oxysterol demethylation intermediates. In either the Δ^8 or Δ^7 series, it is observed that the oxidized sterols are the preferred substrate over the initial, unoxidized lanostenol. This is reflected primarily in the kinetic constant V_{max} which shows a 2- to 30-fold increase as the oxidation state at C-32 increases. Gibbons, Mitropoulos, and Pullinger (17) also observed a similar increase in substrate reactivity with increasing oxidation state, although the apparent K_m values were larger for the oxidized sterols than for the corresponding unoxidized substrate. In our hands, the lowest K_m observed was for the C-32 alcohol. This observation explains kinetically what has been observed in situ with cultured cells. Namely, under conditions when the demethylase reaction sequence is interrupted with inhibitors (8, 18) or substrate overload (18, 19), it is the C-32 aldehyde that accumulates. The greater affinity of the C-32 alcohol over the C-32 aldehyde favors this situation despite the greater reactivity of the aldehyde. Thus, kinetic properties of the demethylase determine which oxysterol accumulates under conditions when demethylation is perturbed.

Changes in catalytic efficiency and enzyme affinity with increasing substrate oxidations have also been observed for other cholesterol biosynthetic and metabolizing enzymes (20–26). In the case of 4-methyl sterol oxidase, increases in oxidation state from the free methyl to the carboxylic acid result in increased catalytic efficiency, as well as greater enzyme affinity (20–23). Analogue results are observed with the cytochrome P-450-dependent generation of pregnenolone from cholesterol in steroid hor-

mone biosynthesis (24). Binding of hydroxycholesterol intermediates to the cytochrome P-450 is 100 to 300 times tighter than for cholesterol itself, while catalytic efficiency is approximately equal for the various hydroxysteroid intermediates (24). In both examples, kinetic parameters prevent competition by substrate or product and consequent accumulation of hydroxysteroid intermediates.

In contrast to these examples, the cytochrome P-450 for C_{21} steroid side-chain cleavage (17α-hydroxylase/$C_{17,20}$-lyase) shows a preference for the initial, unoxidized substrate (25). In fact, it has been shown that for this hydroxylase/lyase system that initial substrates are strong inhibitors of the forward lyase reaction (26). Thus, pregnenolone or progesterone may be important in regulating synthesis of androgens in vivo due to enzyme kinetic preferences. This represents a kinetic situation analogous to the lanosterol demethylase. Substrate inhibition of the forward lyase reaction by lanosterol or dihydrolanosterol leads to accumulation of the aldehyde intermediate (18) in situ. The ability of lanosterol aldehyde to regulate expression of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, suggests that formation of the regulatory oxysterol is a designed mechanistic feature of the demethylase which serves to regulate cholesterol formation in vivo under conditions of altered carbon flow through the biosynthetic pathway.

The demonstrated sensitivity of the final lyase reaction to CO inhibition confirms with synthetic substrates the cytochrome P-450 involvement in all phases of the 14α-methyl demethylase reaction. A previous report (7) which showed an insensitivity of the final lyase to CO can now be explained by a lack of adherence to first order kinetic conditions. When the inhibition studies are performed under proper, first order reaction conditions, the noted sensitivity to CO is observed as expected from data demonstrating a single cytochrome P-450 in lanosterol demethylation (3-6).

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REFERENCES

1. Trzaskos, J. M., and J. L. Gaylor. 1985. Membrane-bound enzymes of cholesterol biosynthesis: resolution and identification of the components required for cholesterol biosynthesis from squalene. *In* The Enzymes of Biological Membranes. Vol. 2. A. N. Martanosi, editor. Plenum Press, New York. 177–204.
2. Yoshida, Y., and Y. Aoyama. 1984. Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation. I. Purification and spectral properties. *J. Biol. Chem.* **259:** 1655–1660.
3. Aoyama, Y., Y. yoshida, and R. Sato. 1984. Yeast cytochrome P-450 catalyzing lanosterol 14α-demethylation. II. Lanosterol metabolism by purified P-450 14DM and by intact microsomes. *J. Biol. Chem.* **259:** 1661–1666.
4. Trzaskos, J. M., S. Kawata, and J. L. Gaylor. 1986. Microsomal enzymes of cholesterol biosynthesis: purification of lanosterol 14α-methyl demethylase cytochrome P-450 from hepatic microsomes. *J. Biol. Chem.* **261:** 14651–14657.
5. Aoyama, Y., Y. Yoshida, T. Nishino, H. Katsuki, U. S. Maitra, V. P. Mohan, and D. B. Sprinson. 1987. Isolation and characterization of an altered cytochrome P-450 from a yeast mutant defective in lanosterol 14α-demethylation. *J. Biol. Chem.* **262:** 14260–14264.
6. Chen, H. W., D. A. Leonard, R. T. Fischer, and J. M. Trzaskos. 1988. A mammalian mutant cell lacking detectable lanosterol 14α-methyl demethylase activity. *J. Biol. Chem.* **263:** 1248–1254.
7. Gibbons, G. F., C. R. Pullinger, and K. A. Mitropoulos. 1979. Studies on the mechanism of lanosterol 14α-demethylation. A requirement for two distinct types of mixed-function oxidase systems. *Biochem. J.* **193:** 309–315.
8. Trzaskos, J. M., R. T. Fischer, and M. F. Favata. 1986. Mechanistic studies of lanosterol C-32 demethylation: conditions which promote oxysterol intermediate accumulation during the demethylation process. *J. Biol. Chem.* **261:** 16937–16942.
9. Trzaskos, J. M., W. D. Bowen, A. Shafiee, R. T. Fischer, and J. L. Gaylor. 1984. Cytochrome P-450-dependent oxidation of lanosterol in cholesterol biosynthesis: microsomal electron transport and C-32 demethylation. *J. Biol. Chem.* **259:** 13402–13412.
10. Paik, Y-K., J. M. Trzaskos, A. Shafiee, and J. L. Gaylor. 1984. Microsomal enzymes of cholesterol biosynthesis: characterization, stabilization, and partial purification of NADPH-dependent $\Delta^{8,14}$-steroid 14-reductase. *J. Biol. Chem.* **259:** 13413–13423.
11. Barton, D. H. R., J. M. McGhie, P. L. Batten. 1970. On the oxidation of hydrazones by lead tetra-acetate. *J. Chem. Soc.* **(C):** 1033–1042.
12. Parish, E. J., and G. J. Schroepfer. 1981. Sterol synthesis. A simplified method for the synthesis of 32-oxygenated derivatives of 24,25-dihydrolanosterol. *J. Lipid Res.* **22:** 859–868.
13. Sonoda, Y., Y. Tarroue, M. Yamaguchi, and Y. Sato. 1987. A simplified synthesis of 32-oxygenated lanosterol derivatives. *Chem. Pharm. Bull.* **35:** 394–397.
14. Woodward, R. B., A. A. Patchett, D. H. R. Barton, and R. B. Kelley. 1957. Synthesis of lanosterol (lanostadienol). *J. Chem. Soc.* **(Land) I:** 1131–1144.
15. Sharpless, K. B., T. E. Snyder, T. A. Spencer, K. K. Maheshwari, G. Gahn, and R. B. Clayton. 1968. Biological demethylation of 4,4-dimethyl sterols. Initial removal of the 4α-methyl group. *J. Am. Chem. Soc.* **90:** 6874–6875.
16. Akhtar, M., C. W. Freeman, D. C. Wilton, R. B. Boar, and D. B. Copsey. 1977. The pathway for the removal of the 14α-methyl group of lanosterol. The role of lanost-8-ene-3β, 32-diol in cholesterol biosynthesis. *Bioorganic Chem.* **6:** 473–481.
17. Gibbons, G. F., K. A. Mitropoulos, and C. R. Pullinger. 1976. Lanosterol 14α-demethylase. The metabolism of some potential intermediates by cell-free systems from rat liver. *Biochem. Biophys. Res. Commun.* **69:** 781–789.
18. Trzaskos, J. M., M. F. Favata, R. T. Fischer, and S. H. Stam. 1987. In situ accumulation of 3β-hydroxylanost-8-en-32-aldehyde in hepatocyte cultures. A putative regulator of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity. *J. Biol. Chem.* **262:** 12261–12268.
19. Saucier, S. E., A. A. Kandutsch, S. Phirwa, and T. A. Spencer. 1987. Accumulation of regulatory oxysterols, 32-oxylanos-

terol and 32-hydroxylanosterol in mevalonate-treated cell cultures. *J. Biol. Chem.* **262:** 14056–14062.

20. Miller, W. L., and J. L. Gaylor. 1970. Investigation of the component reactions of oxidative sterol demethylation. Oxidation of a 4α-methyl sterol to a 4α-carboxylic acid during cholesterol biosynthesis. *J. Biol. Chem.* **245:** 5369–5374.
21. Miller, W. L., and J. L. Gaylor. 1970. Investigation of the component reactions of oxidative sterol demethylation. Oxidation of a 4,4-dimethyl sterol to a 4β-methyl-4α-carboxylic acid during cholesterol biosynthesis. *J. Biol. Chem.* **245:** 5375–5381.
22. Miller, W. L., D. R. Brady, and J. L. Gaylor. 1971. Investigation of the component reactions of oxidative demethylation of sterols. Metabolism of 4α-hydroxymethyl steroids. *J. Biol. Chem.* **246:** 5147–5153.
23. Williams, M. T., J. L. Gaylor, and H. P. Morris. 1977. Investigation of the rate-determining microsomal reaction of cholesterol biosynthesis from lanosterol in Morris hepatomas and liver. *Cancer Res.* **37:** 1377–1383.
24. Lambeth, J. D., S. E. Kitchen, A. A. Farooqui, R. Tuckey, and H. Kamin. 1982. Cytochrome P-450$_{scc}$-substrate interactions. Studies of binding and catalytic activity using hydroxycholesterols. *J. Biol. Chem.* **257:** 1876–1884.
25. Nakajin, S., and P. F. Hall. 1981. Microsomal cytochrome P-450 from neonatal pig testis. Purification and properties of a C_{21} steroid side-chain cleavage system (17α-hydroxylase-$C_{17,20}$ lyase). *J. Biol. Chem.* **256:** 3871–3876.
26. Nakajin, S., P. F. Hall, and M. Onoda. 1981. Testicular microsomal cytochrome P-450 for C_{21} steroid side chain cleavage. Spectral and binding studies. *J. Biol. Chem.* **256:** 6134–6139.