formula are due to partial metalation and sulfonation of the porphyrin.

Binding of 1 to Colloidal Anion-Exchange Resins. Porphyrin 1 (1.25 mL, 2.22×10^{-3} mmol) solution was added slowly via a pipet to a latex (1.0 mL, 6 mg, $n_{R_4N^+Cl^-} = 2.53 \times 10^{-2}$ mequiv) while it was sonicated. The ratio of SO_3^- to R_4N^+ was 0.3 in the dispersion. After sonication for about 15 min the dispersion was ultrafiltered in an all glass system (Millipore) through a 0.1-μm cellulose acetate/nitrate membrane. The red-brown residue was washed with distilled water. The filtrate was diluted to 25 mL. Its visible spectrum showed 0.04% of the original porphyrin 1. However, there was about a 5-nm shift to $\lambda_{max} = 468$ nm in the spectrum relative to the $\lambda_{max} = 463$ nm spectrum of the original solution.

Binding of 1 to IRA-420C. Porphyrin 1 (4 mL of a 0.907 mM aqueous solution) and 83 mg of hydrated (40.4 mg dry) Amberlite IRA-420C were shaken for 100 h. The mixture was filtered, and from the visible absorption spectrum of the filtrate the amount of 1 bound was calculated to be 3.4 mmol.

Epoxidation of Styrene. A 25-mL single-neck round-bottomed flask was charged with pentadecane (internal standard, 60.9 mg) and latex (1.0 mL, 40.4 mg). With the flask in an ultrasonic water bath, an aqueous stock solution of 1.78×10^{-3} M porphyrin 1 (1.25 mL, 2.22×10^{-3} mmol), NaOH (0.76 mL, 2.30 mmol), and distilled water (0.90 mL) was added. (Without latex the sonication step was omitted.) The flask was equipped with a stirring bar and an adapter fitted with a septum and glass stopcock and was flushed with argon. Styrene (125 mg, 1.20 mmol) was added via a syringe. Argon-purged Clorox solution (1.95 mL, 1.50 mmol) was added via a syringe. The pH of Clorox was 11.3. The flask was protected from light by covering with a black sheet, and its contents were stirred for 1 h at ambient temperature. Alternatively the mixture was prepared in a 20-mL test tube, and the tube was sealed with a septum, wrapped with Teflon tape, and shaken with a wrist-action shaker. The reaction mixture was transferred to a 12-mL centrifuge tube with a screw cap. A few milliliters of diethyl ether was added, and the tube was shaken vigorously and centrifuged with a bench top centrifuge. The top organic phase was transferred to another flask with a disposable pipet. The extraction process was repeated 4-6 times until no styrene or product peak was seen on GLC chromatograms of the extract. The combined extract was analyzed quantitatively by GLC. Response factors of 0.99 and 0.91 relative to pentadecane were used for styrene and styrene oxide. The products were identified by ^{1}H NMR spectroscopy.

Epoxidations of substituted styrenes were performed by the same procedures. Only two components were detected from the reaction mixtures by GLC, and ^{1}H NMR spectra of the recovered product mixtures showed only the substituted styrene and its epoxide.

Spectrophotometry of Reacting Mixtures. Samples (200 μL) from test tube reaction mixtures were withdrawn with a syringe at timed intervals and diluted to 500 μL with water. The UV/visible spectrum of each sample was obtained immediately in a 1-mm path length cell. The samples were handled under air and light during preparation.

Acknowledgment. We thank the U.S. Army Research Office for support of this research and the government of Turkey for a scholarship supporting the Ph.D. study of Hayrettin Turk. The high-resolution mass spectrometer was supported in part by National Science Foundation grant BBS-8704089.

Registry No. 1, 131214-66-9; 3, 120644-24-8; $PhCH{=}CH_2$, 100-42-5; $PhC(CH_3){=}CH_2$, 98-83-9; p-$ClC_6H_4CH{=}CH_2$, 1073-67-2; p-$MeC_6H_4CH{=}CH_2$, 622-97-9; NaOCl, 7681-52-9; styrene oxide, 96-09-3; (chloromethylstyrene-divinylbenzene) copolymer-trimethylamine salt, 63453-89-4.

Assessment of the Active-Site Requirements of Lanosterol 14α-Demethylase: Evaluation of Novel Substrate Analogues as Competitive Inhibitors

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Lanosterol 14α-demethylase ($P450_{14DM}$), a cytochrome P450 enzyme, is responsible for the first stage in the biosynthesis of cholesterol (1) from lanosterol (2). Inhibitors of $P450_{14DM}$ may have therapeutic use in the treatment of familial hypercholesterolemia or as antifungal agents. The specificity of $P450_{14DM}$ has been investigated by using substrate analogues modified at the C-32 carbon. The hitherto undescribed 14α-ethyl and 14α-propyl analogues 15 and 13 of lanost-7-en-3β-ol, as well as the 14α-ethenyl and 14α-prop-2-enyl analogues 14 and 12, have been synthesized. These all proved to be good competitive inhibitors of the enzyme. A series of 32-oxiranes and 32-thiiranes was then synthesized and evaluated as inhibitors. Oxiranes 4 and 5 were excellent stereoselective competitive inhibitors of $P450_{14DM}$. The (2'*S*)-32-oxirane 4 had $K_i = 0.62$ μM, and the (2'*R*)-32-oxirane 5 showed $K_i = 2$ μM. The (2'*R*)-32-thiiranyl and (2'*S*)-32-thiiranyl compounds 10 and 11 were considerably less potent inhibitors. Comparison of the K_i values for analogues 12-15, also good competitive inhibitors of this enzyme, indicated the $P450_{14DM}$ active site to be relatively insensitive to the size and degree of unsaturation of C-14α alkyl substituents up to and including propyl.

Lanosterol 14α-demethylase ($P450_{14DM}$), a cytochrome P450 enzyme, is responsible for the first stage in the biosynthesis of cholesterol (1) from lanosterol (2) (Scheme I).[1] The 14α-demethylation involves three sequential steps, each requiring 1 mol of molecular oxygen and 1 mol of NADPH, with subsequent formation of 32-hydroxy and 32-oxo intermediates. The intermediate for the third step is still not established. There has been much recent interest in $P450_{14DM}$ since inhibitors of this enzyme may have therapeutic use in the treatment of familial hypercho-

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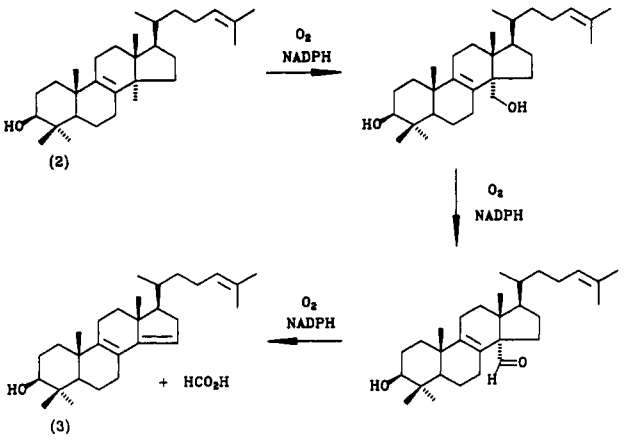
(1) Trzaskos, J. M.; Bowen, W. D.; Shafiee, A.; Fischer, R. T.; Gaylor, J. L. *J. Biol. Chem.* **1984**, *259*, 13402-13412.

0022-3263/91/1956-1260$02.50/0

Scheme I



lesterolemia[2] or as antifungal agents.[3,4]

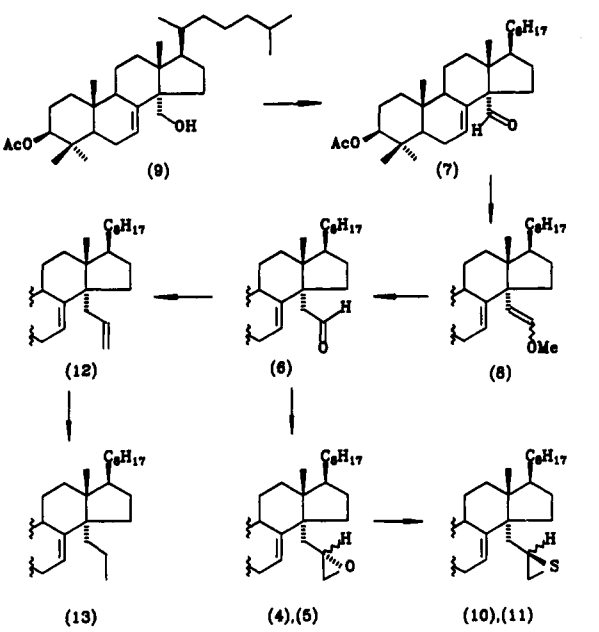
A series of steroidal inhibitors of rat liver $P450_{14DM}$ has recently been reported.[5,36] The main approach toward the development of fungal $P450_{14DM}$ inhibitors, on the other hand, has previously been the design of nonsteroidal type II inhibitors,[6] which interact with the enzyme as the sixth ligand to the heme iron as well as noncovalently with the substrate binding site.

Thus ketoconazole, miconazole, and fluconazole are all effective, nonsteroidal antimycotics. However, these compounds suffer the disadvantage of being general P450 inhibitors, their efficacy depending on a greater affinity for fungal $P450_{14DM}$ versus mammalian $P450_{14DM}$. A more exact approach has been the development of specific steroidal type II inhibitors, such as the 14α-(aminomethyl)lanosterols, which are inhibitors of ergosterol biosynthesis.[7]

Studies on steroidal type II inhibitors of human placental aromatase, a cytochrome P450 monooxygenase that acts at the C-19 position of its steroid substrate, have shown that 10β-oxiranyl- and 10β-thiiranylestr-4-ene-3,17-diones[8] and the homologated 19-oxiranyl- and 19-thiiranylandrost-4-ene-3,17-diones[9] are potent competitive inhibitors of this enzyme. Spectroscopic studies with the purified enzyme demonstrated coordination of the oxiranyl or thiiranyl heteroatom with the enzyme heme iron.[10]

Consideration of the $P450_{14DM}$ enzyme system suggested to us that the 14α-oxiranyl- and 14α-thiiranyl-4,4-dimethyl-5α-cholest-7-en-3β-ols and corresponding 32-oxiranyl- and 32-thiiranyllanost-7-en-3β-ols should be excellent targets for type II inhibitors of $P450_{14DM}$. We planned to synthesize the C-14 diastereomers of 14α-oxiranyl-4,4-dimethyl-5α-cholest-7-en-3β-ol from the known[11] 32-oxolanosterol and to use the method of Chan and Finkenbine[12] (triphenylphosphine sulfide–trifluoroacetic acid) to convert the oxiranes to the corresponding thiiranes. However, the introduction of sulfur by this method could lead to skeletal rearrangement. Thus we chose to synthesize and evaluate the homologated epoxides **4** and **5**, which were expected to lead to the corresponding thiiranes **10** and **11** without rearrangement. Further, it was hoped that these homologated compounds might be good probes to examine the tolerance of the $P450_{14DM}$ active site toward steric bulk at C-14.

Scheme II



Synthesis

Our synthetic targets were C-32 functionalized lanost-7-en-3β-ols. Oxiranes **4** and **5** were potentially available from 32-formyllanost-7-en-3β-ol (**6**) (Scheme II). The key intermediate **6** was readily prepared from the known[11] aldehyde **7**. The latter compound was conveniently obtained by catalytic oxidation of 3β-acetoxylanost-7-en-32-ol (**9**) with the recently described[13] tetra-*n*-propylammonium perruthenate (TPAP)/*N*-methylmorpholine *N*-oxide system. This offered the advantages of high yield (ca. 93%) and convenience over previously described procedure, such as the use of Fetizon's reagent.[5] Reaction of **7** with the ylide of (methoxymethyl)triphenylphosphonium chloride and cleavage of the resulting methyl enol ether **8** with perchloric acid gave **6**. Treatment of **6** with trimethylsulfonium ylide gave a mixture of the (2′*S*)- and (2′*R*)-32-oxiranyl diastereoisomers **4** and **5** in a 6:1 ratio. Protection of the 3β-hydroxyl group during reaction with the ylide was found to be unnecessary. Compounds **4** and **5** were readily separated by preparative HPLC. Use of trimethylsulfoxonium ylide also gave **4** and **5** in a 6:1 ratio. These two reagents are known to be sensitive to the steric environment of the carbonyl group, and the above results are consistent with a relatively unhindered aldehyde group in **6**. X-ray crystallographic analysis established that compound **4** is the 2′*S* diastereoisomer.

It remained to convert the oxiranes **4** and **5** to the corresponding thiiranes **10** and **11**. The method of Chan and Finkenbine[12] proceeds with stereochemical inversion at the

(2) Blackenhorn, D. H.; Nessim, S. A.; Johnson, R. L.; Sanmarco, M. E.; Azen, S. P.; Cashin-Hemphill, L. *J Am. Med. Assoc.* **1987**, *257*, 3233.
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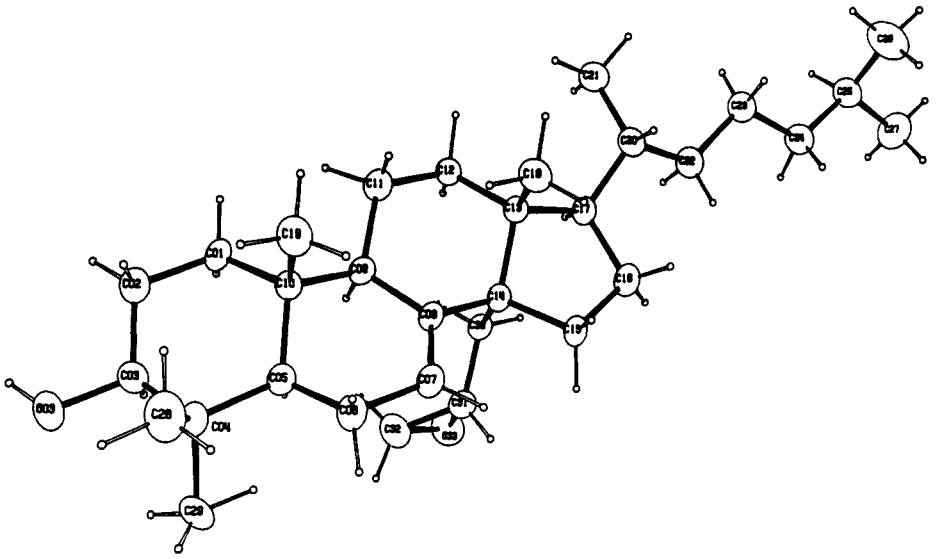


Figure 1. ORTEP drawing of (2′*S*)-32-oxiranyllanost-7-en-3β-ol (4).

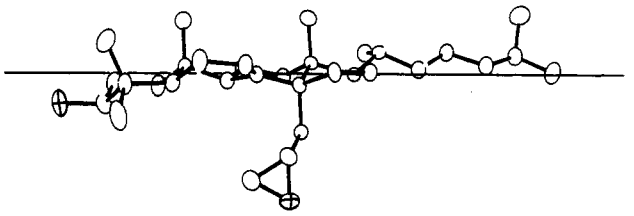


Figure 2.

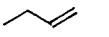
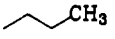
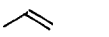
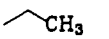
epoxide carbon. This method was used to convert (2′*S*)-32-oxirane **4** to (2′*R*)-32-thiirane **10** and (2′*R*)-32-oxirane **5** to (2′*S*)-32-thiirane **11**, both reactions proceeding stereospecifically in good yield. The stereochemistry of the thiiranes **10** and **11** follows from the established stereochemistry of the oxirane precursors and the known stereochemical course (inversion) of the thiirane-forming reaction. Each of the thiiranes was smoothly desulfurized to the independently synthesized 14α-(prop-2-enyl)lanost-7-en-3β-ol (**12**) by treatment with triphenylphosphine in refluxing toluene. The integrity of the steroid carbon skeleton was thus assured.

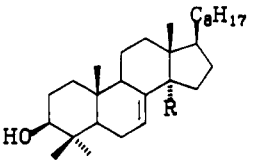
Full evaluation of the compounds described above necessitated assessment of the tolerance of the active site of $P450_{14DM}$ for the hitherto undescribed 14α-ethyl and 14α-propyl analogues of lanost-7-en-3β-ol. Consequently 14α-propyllanost-7-en-3β-ol (**13**) was prepared by selective catalytic hydrogenation of olefin **12**, and 14α-ethyllanost-7-en-3β-ol (**15**) was similarly prepared from 14α-ethenyllanost-7-en-3β-ol[5] (**14**). 14α-(Prop-2-enyl)lanost-7-en-3β-ol (**12**) was prepared by the reaction of **6** with the ylide of methyltriphenylphosphonium bromide.

X-ray Crystal Structure

Oxirane **4** is depicted with its correct absolute configuration in Figure 1 (all figures are ORTEP drawings[14]) and it will be seen that the configuration at C(31), the oxirane C atom, is *S* (chiral angle[15] is −79 (2)°). Figure 2, a projection perpendicular to the least-squares plane of rings B, C, and D, shows that the steroid moiety is fairly flat. The presence of the C(07)–C(08) double bond, in addition to flattening the B ring, has also reduced the average torsion angle to 50.3° in the C ring. The D-ring conformation is close to a half chair with Altona, Geise, and Romers parameters[16] of ϕ_m = 48.7° and 3.6°. The crystal packing is shown in Figure 3 (supplementary material) and shows that the extension outward of the oxirane ring and its movement under the main molecule are probably a consequence of the O(03)···O(33) hydrogen bond since the oxirane substituent appears to be quite flexible. The O(03)···O(33) and H(03)···O(33) distances are 2.986 (6) and 2.17 (6) Å, respectively, and the angle O(03)–H(03)···O(33) is 153 (6)°. The molecular dimensions are available in the supplementary material.

Table I. K_i Values for Sterols 4–6 and 10–15

R	K_i	R	K_i
	(4) 0.62uM (5) 2uM		(12) 11uM
	(6) 3uM		(13) 16uM
			(14) 6uM
	(10) >50uM (11) 32uM		(15) 10uM



K_m for 24,25-DHL is 45uM

Enzyme Studies and Conclusion

The inhibitory potencies of the compounds described in this study were evaluated by a UV-HPLC assay similar to that described by Trzaskos et al.[1] Rat liver microsomal $P450_{14DM}$ preparations were used, in conjunction with an NADPH regenerating system, and with 24,25-dihydrolanosterol (24,25-DHL, K_m = 45 μM) as substrate. Values for K_i were determined by using Lineweaver–Burk analysis.[17] The results are shown in Table I. Oxiranes **4** and

(14) Johnson, C. K. *ORTEP, Technical Report, OR*-3794, Oak Ridge National Laboratory, TE, 1965.
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5 were both excellent competitive inhibitors of $P450_{14DM}$. Furthermore, stereoselective inhibition was observed. The (2′S)-32-oxirane 4 had K_i = 0.62 μM and was three times more potent than the diastereomeric (2′R)-32-oxirane 5 K_i = 2 μM, reflecting the importance of the C-32 substituent in binding to the active site of $P450_{14DM}$. It is noteworthy that strong competitive inhibition of $P450_{14DM}$ is observed when the oxirane is separated from the C-14 position by a methylene group.

We were unable to induce the high spin state of a partially purified sample of $P450_{14DM}$ with 24,25-DHL. A similar observation has been made for *S. cerevisiae* $P450_{14DM}$.[18,19] Thus, we were unable to use type I/type II spectral changes to directly examine interactions between the inhibitor epoxide oxygen atoms and the enzyme's heme iron. Studies on $P450_{CAM}$,[20] $P450_{SCC}$,[21] and other P450 isozymes[22] have established variously a water molecule or an active-site residue (such as serine, threonine or tyrosine) as the sixth ligand of the resting, low spin state, heme iron. For $P450_{CAM}$ as well as $P450_{LM2}$, a phenobarbital-induced P450 isozyme, this interaction results in a Soret band at 416–420 nm. Coordination of the epoxysteroid to the substrate-free low spin form of $P450_{14DM}$ would be expected to result in a Soret band in the 416–420-nm region. Hence, the adduct, if formed, would exhibit the same Soret band as native six-coordinate $P450_{14DM}$ and interaction of the epoxide oxygen atom with the heme would not be clearly established. Indeed, Coon and White[22] noted that the UV–vis spectrum of $P450_{LM2}$ remained essentially unchanged in the presence of oxygen-coordinating ligands.

Interestingly, upon addition of 32-hydroxy-24,25-DHL to ferric low spin *S. cerevisiae* $P450_{14DM}$, Aoyama et al.[19] observed a 1-nm red shift, from 417 to 418 nm, of the Soret peak. They suggested that the slight red shift reflected the exchange of the native sixth ligand for the 32-hydroxyl group of the sterol. Furthermore a very small spectral change was observed for the same $P450_{14DM}$ system upon addition of 32-oxo-24,25-DHL, the change being different to that observed for 32-hydroxy-24,25-DHL.[23] The latter observations led Aoyama et al. to conclude that the interaction of the 32-aldehyde with the heme iron was responsible for the spectral changes. However, given the observations[20,22] that the oxygen donor complexes have spectra that very closely resemble those of the resting enzyme, it appears questionable to conclude that interactions are indeed occurring between the 32-oxo and 32-hydroxyl oxygen atoms and the $P450_{14DM}$ heme. However, in our work, the stereoselective nature of the binding observed for 4 and 5 indicates some involvement of the oxirane group in binding, in spite of the absence of spectroscopic shifts.

The (2′R)-32-thiiranyl and (2′S)-32-thiiranyl compounds 10 and 11 proved to be considerably less potent inhibitors of $P450_{14DM}$ than the corresponding oxiranes. We found this result somewhat surprising, because a sulfur atom was expected to be a superior P450 binding ligand when compared to an oxygen atom. Indeed, this observation has been made by Childers et al. for oxiranyl and thiiranyl inhibitors of aromatase.[10] However, although the (2′S)-thiirane isomer 11, showing K_i = 32 μM, is more potent than its 2′R isomer 10 [no significant inhibitory activity even at a concentration of 50 μM], each thiirane is a less powerful inhibitor than the corresponding oxirane.

Table II. Partial Purification of $P450_{14DM}$

	[protein], mg/mL	$P450_{14DM}$ activity, pmol/mg/min
microsomes	1305	20
agarose-octylamine fraction	200	105
DEAE Sephacel fraction	10	300

The stereospecific inhibition observed for the C-32 oxiranes 4 and 5 of this work indicates a somewhat restricted binding region around C-32 of the substrate, and it is likely that the oxirane groups of 4 and 5 are tightly packed within this binding region. Replacement of the oxygen atom by sulfur, which has a larger van der Waals radius than oxygen, may exceed the tolerance of the active site for steric bulk at C-32, resulting in the poor binding affinity of the thiiranes. The longer C–S bond compared with the C–O bond may also be a factor.

It is of interest to compare the inhibitory potencies of the thiiranes and oxiranes with some simple C-14α analogues of lanosterol, such as 14α-ethyl and 14α-propyl. No information has been previously available on the sensitivity of $P450_{14DM}$ toward such analogues of lanosterol. Hence we synthesized the 14α-ethyl-, 14α-propyl-, 14α-ethenyl-, and 14α-propenyllanosterols 12–15. Comparison of the K_i values for analogues 12–15, which are all good competitive inhibitors of this enzyme, and for the homologated aldehyde 6 indicates the $P450_{14DM}$ active site to be relatively insensitive to the size and degree of unsaturation of C-14α alkyl substituents up to and including propyl. Frye and Robinson have previously reported[24] a series of C-14 derivatized lanosterols, including the 14α-propynyl, the 14α-ethynyl, the 14α-difluoromethyl, and the 14α-difluoroethyl, all of which are good inhibitors of $P450_{14DM}$. Interestingly, although the enzyme inhibits a slightly greater affinity for the acetylenic analogues, homologation of either the ethynyl to propynyl or difluoromethyl to difluoroethyl does not decrease their affinity for the enzyme. The contrast between the inhibitory potencies of the above lanosterol analogues and the thiiranes 10 and 11 is striking. We can offer no good explanation, at present. In the present work it should be noted that the observations for $P450_{14DM}$ are in contrast to the observation[25] that 10β-ethylestr-4-ene-3,17-dione has a 330-fold greater binding affinity than the 10β-propyl analogue for the P450 enzyme aromatase.

In conclusion, we have reported a series of potent competitive inhibitors of $P450_{14DM}$. This series includes the first described oxiranyl and thiiranyl inhibitors, compounds that are of considerable interest as active-site probes for this enzyme. Apparently the active site can readily accommodate C-14α substituted analogues of lanosterol of sizes up to 14α-propyl and 32-oxiranyl. Surprisingly the C-32 thiiranes are poorly accommodated. It should also be noted that compounds 4–15 have not yet been tested as substrates for $P450_{14DM}$.

Experimental Section

General. Melting points are uncorrected. HPLC separations were performed on Altex analytical or semipreparative octyl

(17) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.
(18) Yoshida, Y.; Aoyama, Y. *J. Biol. Chem.* **1984**, *259*, 1655–1660.
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(25) Marcotte, P. A.; Robinson, C. H. *Steroids* **1982**, *39*, 325–343.

columns or on a Whtman Magnum 10 partisil column. Flash chromatography was performed on silica gel (Baker) as described by Still.[26]

Isolation of Rat Liver Microsomes. Rat liver microsomes were isolated as described by Trzaskos et al.[1] from male Sprague Dawley rats sacrificed by asphyxiation with carbon dioxide. The microsomes could be stored at −80 °C for several months without significant loss of $P450_{14DM}$ activity.

Purification of $P450_{14DM}$. Microsomes were prepared as above except that the rats were maintained ona diet containing 5% cholestyramine and 3% corn oil.[1] $P450_{14DM}$ was purified to electrophoretic homogeneity according to Trzaskos et al.[27] Purification up to and including the DEAE sephacel chromatography step yielded $P450_{14DM}$ sufficiently active for our needs (see Table II). SDS-PAGE employing 8% acrylamide separation gel showed a single band of M_r 51 000, in agreement with the results of the du Pont group.

Assay of $P450_{14DM}$ Activity. $P450_{14DM}$ was assayed by a modified literature procedure.[1] The incubation flasks contained in a final volume of 0.5 mL of 0.1 M potassium phosphate buffer containing 1 mM glutathione, 0.1 mM EDTA, pH 7.4, and the following substances: 0.5 mg of microsomal protein, 0.2 mM NAD, 0.1 mM NADH, 2.0 mM NADPH, 0.3 mM NADP, 10 mM isocitrate, 0.125 unit of isocitrate dehydrogenase, 0.4 mM magnesium chloride, 1 mM NaCN, and 50 μM AY9944. Incubations were started by the addition of steroid substrate (24,25-DHL) suspended with the aid of the detergent Triton WR-1339 in the ratio of 40:1 (w/w) detergent to steroid. For enzyme assays a fixed quantity of detergent was used, determined by a 40:1 ratio to the highest substrate concentration employed. This was necessary to overcome a noncompetitive inhibition effect of the detergent on $P450_{14DM}$, observed by us and others.[28] Incubations were continued at 37 °C for 45 min, and reactions were terminated by the addition of 0.5 mL of 15% potassium hydroxide (w/w) in 95% MeOH. The vortexed mixtures were boiled for 30 min and the nonsaponifiable material was extracted 3 times with a total of 6 mL of petroleum ether. Petroleum extracts were evaporated under a stream of N_2 at 40 °C, the residue being dissolved in 150 μL of absolute ethanol.

The presence of the 4-methylsteroid oxidase and Δ^{14}-reductase inhibitors, NaCN[29] and AY9944,[30] respectively, caused accumulation of the immediate product of 14α-demethylation, the 8,14-diene **3**. $P450_{14DM}$ activity was determined by the extent of formation of **3**. Hence, 50 μL of the ethanol-soluble extraction residue was analyzed by reverse-phase HPLC on an octyl column (4.6 mm × 25 cm). Chromatography was performed at 25 °C and 0.7 mL/min on an octyl column, using a mobile phase of MeOH. $P450_{14DM}$ activity was calculated from the peak area for **3**. The system was quantified with authentic **3**. Formation of product **3** followed linear first-order kinetics for the 60-min time period examined. A single time point of 45 min was used for data collection. For all assays the substrate concentration varied from 5 to 200 μM, and inhibitor concentrations were chosen so as to achieve detectable and quantifiable decreases in enzyme activity. Values for K_m, K_i, and V_{max} were determined by using a double reciprocal analysis. Typically data are accurate to within 10% of the reported values.

3β-Acetoxy-32-oxolanost-7-ene (7).[11] To a stirred solution of 3β-acetoxylanost-7-en-32-ol (**9**)[31] (65 mg, 0.13 mmol) and 4-methylmorpholine *N*-oxide (24 mg, 0.2 mmol) in CH_2Cl_2 (3 mL) containing 4A molecular sieves was added tetrapropylammonium perruthenate (2.5 mg, 0.007 mmol). The reaction was stirred at room temperature for 1 h, after which time TLC indicated that the reaction was complete. The solution was diluted with CH_2Cl_2 (25 mL) and washed with sodium sulfite solution (10 mL), brine (10 mL), and finally saturated copper(II) sulfate solution (10 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was evaporated in vacuo to give a white solid residue. Column chromatography on silica gel (EtOAc/hexanes 2:98) gave pure **7** (60 mg, 93%): mp 143–144 °C (from MeOH) (lit.[11] mp 144–145 °C; IR ($CHCl_3$) 1725 (C=O, acetate) and 1705 cm^{-1} (C=O, aldehyde); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.11 (3 H, S, 3-CH_3COO), 4.48 (1 H, dd, J = 11 and 3 Hz, C3-H), 5.42 (1 H, m, C7-H), 9.58 (1 H, s, C32-H); mass spectrum m/z 484 (M^+).

32-(Methoxyethylene)lanost-7-en-3β-ol (8). To a solution of (methoxymethyl)triphenylphosphonium chloride (0.91 g, 2.7 mmol) in dry THF (5 mL) under argon was added 2.5 M *n*-BuLi (0.95 mL, 2.4 mmol), dropwise with stirring. After being stirred at room temperature for 30 min, a solution of aldehyde **7** (129 mg, 0.274 mmol) in dry THF (1 mL) was added dropwise. The reaction was stirred at room temperature for 15 min, after which time TLC indicated complete reaction. The reaction mixture was evaporated in vacuo, the residue dissolved in CH_2Cl_2 and washed with brine. The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel (EtOAc–hexanes, 1:19) to give two pure products, the 32-methoxyethylene 3β-ol **8** (41 mg, 33%) [mp 149 °C (from MeOH); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 80 MHz) δ 3.28 (1 H, m, C3-H), 3.51 (3 H, s, C33-OCH_3), 4.55 (1 H, d J = 7 Hz, C32-H), 5.39 (1 H, m, C7-H), 5.65 (1 H, d, J = 7 Hz, C33-H); mass spectrum m/z 470 (M^+)] and the corresponding 3β-acetate **8a** (61 mg, 47%) [mp 137–139 °C from ($MeOH/CHCl_2$); IR ($CHCl_3$) 1725 cm^{-1} (C=O); ^1H NMR ($CDCl_3$, 80 MHz) δ 2.06 (3 H, s, C3-CH_3COO), 3.46 (3 H, s, C32-OCH_3), 4.50 (1 H, d, J = 6 Hz, C32-H), 5.32 (1 H, m, C7-H), 5.60 (1 H, d, J = 6 Hz, C33-H); mass spectrum m/z 512 (M^+)].

The 3β-acetate **8a** (61 mg, 0.13 mmol) was dissolved in 20% KOH in dry MeOH (20 mL) and stirred at room temperature under argon for 16 h, after which time TLC indicated complete reaction. The solvent was removed in vacuo and the residue was dissolved in EtOAc and washed with saturated NH_4Cl solution. The organic layers were dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel (EtOAc–Hexanes, 1:9) to give **8** (55 mg, 98%).

32-Formyllanost-7-en-3β-ol (6).[5] Compound **8** (97 mg, 0.21 mmol) was dissolved in dry ether (10 mL) under argon. The solution was cooled to 0 °C and 70% aqueous perchloric acid (150 μL) was added dropwise with stirring. The reaction was stirred for a further 15 min at 0 °C under argon, after which time TLC indicated complete reaction. EtOAc (25 mL) was added and the solution washed with 10% aqueous $NaHCO_3$ solution to remove the perchloric acid. The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. The residue was chromatographed on silica gel (EtOAc–hexanes, 1:4) to give **6** (57 mg, 60%): mp 141–142 °C (from MeOH); IR ($CHCl_3$) 3600 (OH) and 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.22 (1 H, dd, J = 14 and 4 Hz, C3-H), 5.35 (1 H, m, C7-H), 9.53 (1 H, t, J = 2 Hz, C-32 formyl); mass spectrum m/z 456 (M^+); HRMS required 456.3967 ($C_{31}H_{52}O_2$), found 456.3974.

32(*R* and *S*)-Oxiranyllanost-7-en-3β-ols (4 and 5). In a 10-mL round-bottom flask, under argon, was placed a 50% oil dispersed sodium hydride (48 mg, 1 mmol). This dispersion was washed twice with dry THF (3 mL), the solvent being removed each time under argon. Dry DMSO (1 mL) was added and the mixture heated to 70–75 °C under argon with stirring for 30 min. The reaction was cooled to 25 °C, dry THF (1 mL) was added, and the mixture was cooled to 0 °C. A solution of trimethylsulfonium iodide (230 mg, 1.12 mmol) in dry DMSO (1 mL) was added dropwise with stirring. After complete addition, stirring was continued, and after 5 min at 0 °C a solution of aldehyde **6** (0.51 mg, 0.11 mmol) in dry THF (1 mL) was added dropwise. The resulting mixture was stirred at 0 °C for 30 min followed by 16 h at room temperature under argon. The solution was concentrated in vacuo, and the residue was taken up in CH_2Cl_2, washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The residue was purified by chromatography on silica gel (EtOAc–hexanes, 1:4) followed by HPLC (silica, EtOAc–hexanes 1:3) to give pure (2′*S*)-32-oxiranyllanost-7-en-3β-ol (**4**) (31 mg, 59%) [mp 177 °C; IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.42 (1 H, m, epoxide-H), 2.65 (1 H, m, epoxide-H), 2.81 (1 H, m, epoxide-H), 3.23 (1 H, m, C3-H), 5.36 (1 H, m, C7-H); mass spectrum m/z 470 (M^+); HRMS required 470.4124 for ($C_{32}H_{54}O_2$),

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found 470.4130. Anal. Calcd for $C_{32}H_{54}O_2$: C, 81.64; H, 11.56. Found: C, 81.79; H, 11.62.] and (2'*R*)-32-oxiranyllanost-7-en-3β-ol (**5**) (5 mg, 10%) [mp 152 °C (from MeOH); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.42 (1 H, m, epoxide-H), 2.78 (1 H, m, epoxide-H), 2.81 (1 H, m, epoxide-H), 3.28 (1 H, m, C3-H), 5.27 (1 H, m, C7-H); mass spectrum *m/z* 470 (M^+); HRMS required 470.4124 ($C_{32}H_{54}O_2$), found 470.4130. Anal. Calcd for $C_{32}H_{54}O_2$: C, 81.64; H, 11.56. Found: C, 81.76; H, 11.44.].

(2'*R*)-32-Thiiranyllanost-7-en-3β-ol (10). To a solution of oxirane **4** (10 mg, 0.02 mmol) in dry benzene (2 mL) under argon were added triphenylphosphine sulfide (15 mg, 0.05 mmol) and trifluoroacetic acid (3 μL, 0.02 mmol). The reaction was stirred under argon for 30 min, when TLC indicated that the reaction was complete. The mixture was diluted with EtOAc and washed with aqueous 5% sodium bicarbonate solution to remove trifluoroacetic acid. The organic phase was dried ($MgSO_4$) and evaporated in vacuo. The residue was chromatographed on silica gel (EtOAc–hexanes, 1:9) to give **10** (8 mg, 78%): mp 167–168 °C (from EtOAc); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.51 (1 H, d, *J* = 8 Hz, thiirane-H), 2.72 (1 H, m, thiirane-H), 3.27 (1 H, dd, *J* = 11 and 3 Hz, C3-H), 5.27 (1 H, m, C7-H); mass spectrum *m/z* 486 (M^+); HRMS calculated 486.3895 ($C_{32}H_{54}O_2$), found 486.3902. Anal. Calcd for $C_{32}H_{54}OS$: C, 78.97; H, 11.18. Found: C, 79.28; H, 11.41.

(2'*S*)-32-Thiiranyllanost-7-en-3β-ol (11). Compound **11** was prepared from **5** exactly as described above for the preparation of **10** from **4**. The residue was chromatographed on silica gel (EtOAc–hexanes, 1:9) to yield pure **11** (8 mg, 85%): mp 149–150 °C (from EtOAc); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.37 (1 H, d, *J* = 5.3 Hz, thiirane-H), 2.69 (1 H, m, thiirane-H), 3.21 (1 H, dd, *J* = 11 and 3 Hz, C3-H), 5.31 (1 H, m, C7-H); mass spectrum *m/z* 486 (M^+); HRMS calculated 486.3895 ($C_{32}H_{54}OS$), found 486.3895. Anal. Calcd for $C_{32}H_{54}OS$: C, 78.97; H, 11.18. Found: C, 79.02; H, 11.04.

4,4-Dimethyl-14α-(prop-2-enyl)-5α-cholest-7-en-3β-ol (12). To a solution of methyltriphenylphosphonium bromide (157 mg, 0.44 mmol) in dry THF (5 mL) under argon was added 2.5 M *n*-BuLi (168 μL, 0.42 mmol) dropwise with stirring. The solution was stirred for a further 1 h at room temperature, and a solution of aldehyde **6** (50 mg, 0.09 mmol) in dry THF (200 μL) was then added. TLC indicated complete reaction after 1 h. The solution was evaporated in vacuo and the residue was chromatographed on silica gel (EtOAc–hexanes 1:4) to give **12** (31 mg, 63%): mp 142–143 °C (from MeOH); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.35 (1 H, m, C32-H), 3.23 (1 H, m, C3-H), 4.92 (2 H, m, olefinic H), 5.11 (1 H, M, C7-H), 5.57 (1 H, m, olefinic H); mass spectrum *m/z* 454 (M^+); HRMS calculated 454.4175 ($C_{32}H_{54}O$), found 454.4175. Anal. Calcd for $C_{32}H_{54}O$: C, 84.51; H, 11.96. Found: C, 84.53; H, 12.11.

Desulfurization of 10 and 11. To a solution of thiirane **10** (12 mg, 0.02 mmol) in dry toluene (3 mL) was added triphenylphosphine (26 mg, 0.1 mmol). The system was refluxed for 12 h, when TLC indicated complete reaction. The solvent was removed in vacuo and the residue was chromatographed on silica gel (EtOAc–hexanes, 1:9) to give **12** (10 mg, 89%): mp 142 °C (from MeOH); IR ($CHCl_3$) 3600 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 300 MHz) δ 2.35 (1 H, m, C32-H), 3.23 (1 H, m, C3-H), 4.92 (2 H, m, olefinic H), 5.11 (1 H, m, C7-H), 5.57 (1 H, m, olefinic H); mass spectrum *m/z* 454 (M^+) identical with the authentic material.

Desulfurisation of **11** (3 mg, 0.006 mmol) exactly as described above for thiirane **10** also gave **12**.

4,4-Dimethyl-14α-ethyl-5α-cholest-7-en-3β-ol (15). A solution of olefin **14** (6 mg, 0.014 mmol) in EtOAc (1 mL) containing 10% Pd/C (1 mg) was stirred for 18 h at room temperature under an atmosphere of hydrogen. The suspension was then filtered through Celite and the filtrate evaporated in vacuo to give a white solid, which was purified on silica gel (EtOAc–hexanes, 15:85) to give pure **15** (5 mg, 79%): mp 135–137 °C (from CH_2Cl_2/MeOH); IR ($CHCl_3$) 3200 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.60 (3 H, t, *J* = 10 Hz, C32-CH_3), 3.22 (1 H, m, C3-H), 5.09 (1 H, m, C7-H); mass spectrum *m/z* 442 (M^+). Anal. Calcd for $C_{31}H_{54}O$: C, 84.09; H, 12.29. Found C, 83.99; H, 12.31.

4,4-Dimethyl-14α-propyl-5α-cholest-7-en-3β-ol (13). Compound **13** was prepared from olefin **12** (7 mg, 0.015 mmol) by hydrogenation in EtOAc using 10% Pd/C, exactly as described for the preparation of **15** from **14** (6 mg, 86%): mp 110–111 °C (from CH_2Cl_2/MeOH); IR ($CHCl_3$) 3200 cm^{-1} (OH); ^1H NMR ($CDCl_3$, 400 MHz) δ 3.26 (1 H, dd, *J* = 11 and 8 Hz, C3-H), 5.10 (1 H, m, C7-H); mass spectrum *m/z* 456 (M^+); HRMS calculated 456.4331 ($C_{32}H_{56}O$), found 456.4338. Anal. Calcd for $C_{32}H_{56}O$: C, 84.14; H, 12.36. Found: C, 83.87: H, 11.92.

X-ray Crystal Structure. The crystals of isomer **4** are orthorhombic plates elongated along *a*. A crystal, used for both cell dimensions and data collection, was 0.375 × 0.20 × 0.03 mm and was mounted approximately along the *a* axis. All X-ray measurements were performed with an ENRAF-NONIUS CAD4 diffractometer and graphite-monochromated Cu Kα radiation (λ = 1.5418 Å). The space group was $P2_12_12_1$ and cell dimensions are *a* = 10.390 (1), *b* = 11.075 (1), and *c* = 24.595 (2) Å and were obtained by least-squares refinement using 16 reflections with Bragg angles between 20 and 26° measured at ±θ. With the formula $C_{33}H_{54}O_2$, M_r = 482.76, the cell volume of 2830.13 $Å^3$ corresponds to an X-ray density of 1.133 g cm^{-1} for *Z* = 4. The data collection was carried out with $\theta/2\theta$ scans, a scan angle of $(1 + 0.035 \tan\theta)°$, a counter aperture of 1.5 mm and a scan speed designed to produce 3% statistical accuracy based on a fast prescan and subject to a maximum time of 60 s. The intensities of three standard reflections (4 1 6, 3 4 1, and −1 0 12) were measured every 2 h. After 1300 reflections had been measured, the intensities of the standards had fallen linearly to 80% of their original values and the crystal was replaced with another slightly smaller in size. Again, after 2625 measurements, another decrease of nearly 80% led to a third replacement with a crystal 0.25 × 0.25 × 0.05 mm, which sufficed to complete the data collection. The maximum Bragg angle was 74°, maximum $\sin\theta/\lambda$ was 0.6232 $Å^{-1}$ and the maxima of *h,k,l* were 12, 13, and 30, respectively. Data reduction by local programs yielded 3240 unique reflections with 1394 having $I \leq \sigma(I)$. Standard deviations of intensities were statistical with a Peterson–Levy factor[32] included. Weights were assigned by Bayesian methods from the measured intensities and standard deviations.

The phase problem was solved with MITHRIL (Gilmore, (1983)[37] in its "Hard" configuration, i.e., using negative quartets and NQEST[33] as one of the figures of merit. The best *E* map had peaks corresponding to all expected atoms and a selection was readily made. Isotropic refinement with labels assigned on a basis of peak size in the *E* map terminated at $R = 0.124$, $R_w = 0.122$ with the *U* values of O(32) and C(33) being 0.07 and 0.08, respectively. When the labels were interchanged *R* and R_w became 0.128 and 0.127, respectively, and the *U* values for O(33) and C(32) were 0.148 and 0.037, respectively, thus confirming the original choice. The absolute configuration was assigned to correspond to the known steroid conformation, which is also that determined for 24,25-dibromolanosterol.[34] The final refinement operations were anisotropic refinement of the C and H atoms, location of all H atoms in a difference map and refinement to convergence at $R = 0.059$, $R_w = 0.054$, and $R(F^2) = 0.055$. Three scale factors were used (one for each crystal) and *U* values for H atoms were assigned rather than refined. Because refined geometries were not completely satisfactory, the positions of methyl H atoms were recalculated and not refined. Since O(03) refined to within 2.99 Å of O(33) in an other molecule related by the *a* axis, it was possible to deduce a probable position for H(03) and a peak was found in a difference map. The final value of *S* was 1.622 and the maximum value of Δ/σ for C and O positions was 0.05. A table of coordinates is available in the supplementary material. Except as otherwise indicated, all programs came from XTAL 2.4.[35]

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Supplementary Material Available: Crystal packing diagram, table of coordinates and equivalent isotropic temperature factors for carbon and oxygen atoms, table of molecular dimensions, and tables of positional parameters for carbon and oxygen atoms and hydrogen atoms of 4 (6 pages). Ordering information is given on any current masthead page.

Substituent Effects in Organic Vanadate Esters in Imidazole-Buffered Aqueous Solutions

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The spontaneous formation of vanadate esters and diesters in imidazole-buffered aqueous solutions was observed by using ^{51}V NMR spectroscopy. The ^{51}V NMR chemical shifts of monoesters varied as follows: primary alcohols > vanadate monomer ≃ primary alcohols substituted with electronegative substituents > secondary alcohols > tertiary alcohols. Vanadate esters formed from primary alcohols were more stable than the vanadate esters formed from secondary or tertiary alcohols. This order of stability in solution containing high ionic strength and imidazole is contrary to the order of stability observed in low ionic strength solutions containing Hepes. The role of imidazole in vanadate ester reactions was examined by using relaxation times and 1D and 2D ^{51}V NMR spectroscopy. The effects of imidazole on the relaxation times and the concentration of vanadate oligomers suggest that imidazole may interact directly with vanadium. The presence of imidazole or salts significantly affects the reactions of vanadate with organic molecules and may be important for the observed catalytic and biological effects of vanadate derivatives.

Introduction

Spontaneously formed organic vanadate esters have recently been found to have substrate activity and consequently possible synthetic utility[1] and biological activity.[2] The spontaneously formed organic vanadates are structural analogues of the corresponding organic phosphates, and this similarity presumably explains the substrate activity with enzymes that convert organic phosphates.[3] The structural analogies do not extend to the kinetic properties of these compounds. Vanadate esters form with rate constants 10^{10} times that of the corresponding organic phosphates in aqueous solutions.[4] Solutions of vanadate and alcohols reach equilibrium within milliseconds as would organic vanadates prepared by other methods on addition to aqueous solutions. Vanadate esters have been reported to form from alcoholic solutions containing ethanol,[5] ethylene glycol,[6] lactate,[7] glyceric acid,[7] glucose,[8] citrate,[9] and glycosides.[10] The vanadate-alcohol complexes have been characterized by using ^{51}V NMR spectroscopy and UV spectroscopy, but no other structural information is available on these derivatives. In this paper we will describe the complexes between an alcohol and vanadate as organic vanadate esters in analogy with organic phosphates, although the structure of these vanadium complexes in aqueous solution at high alcohol concentrations are not known.

Table I. K_{eq1} and K_{eq2} for a Series of Alcohols[a]

compound	K_{eq1}	K_{eq2}	compound	K_{eq1}	K_{eq2}
CH_3CH_2OH	9.4	7.2	$(CH_3)_3COH$[b]	3.4[b]	1.4[b]
$CH_3(CH_2)_2OH$	6.1	2.8	$HOCH_2CH_2OH$[c]	2.3[c]	19
$(CH_3)_2CHOH$	5.0	3.5			

[a] Samples contained 10 mM vanadate, 0.35 M KCl, 0.10 M imidazole, and various concentrations of alcohol and had a final pH of 7.00 ± 0.05. [b] These equilibrium constants were determined in samples containing 0.1 mM vanadate, 0.35 M KCl, 0.10 M imidazole, and various concentrations of alcohol and had a final pH of 7.00 ± 0.05. [c] Chemical shift for the monoester could not be distinguished from the chemical shift of the vanadate monomer.

The reaction of a simple alcohol at neutral pH with vanadate is presumed to be a reaction between the monomeric vanadate species (diprotonated or monoprotonated vanadate anion) and the alcohol, ROH, although in a solution of several equilibrating vanadate species other reaction pathways are possible. The reaction generates a vanadate monoester (eq 1) and a diester (eq 2).[5-8,10]

$$ROH + H_2VO_4^- \overset{K_{eq1}}{\rightleftharpoons} ROVO_3H^- + H_2O \qquad (1)$$

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