between the two stages of substitution is large enough for clear separation, it is difficult to estimate precisely the infinity absorbance for the first stage because the total change is small ($\Delta A \sim 0.060$). However, linearity over 2 half-lives suggests that the rate constant for the first stage is well estimated.

Influence of Additives of the Length of the Induction Period for the Oligomerization of Thiamin Chloride. A stock solution (0.20 M) of thiamin chloride[2] was prepared immediately before use. Solid substrate was dissolved in methanol over a 2-4-min period with the aid of a vibromixer. A stopwatch was activated when the methanol was added to solid. One-half milliliter then was added by syringe to a 5-mm NMR tube containing methanol or a methanolic solution of additive. After the sample was mixed, the total elapsed time was about 5-6 min. After a few trials to determine conditions, a set of samples was examined together; one member of the set always consisted of substrate without additive.

The tubes were inverted frequently and held to the light to facilitate detection of suspended particles; NMR tubes were advantageous in that the viewing area was maximized. The onset of precipitation (formation of a cloudy solution) was taken to be the end of the induction period. There was some day-to-day variation in the length of the induction period, especially with phenylthio compound Ib acting as inhibitor. But the order of inhibition or acceleration with respect to additive-free material was invariant.

After thiamin chloride stood for several weeks, the decomposition temperature of thiamin chloride decreased somewhat. The initial decomposition range, 165-169 °C (lit.[2] range 160-163 °C dec), could be restored by suspending finely powdered material in absolute ethanol for a few minutes prior to use. No difference could be detected in induction periods for old and new substrate.

The condition corresponding to the onset of precipitation was examined by NMR. Only small amounts (<10%) of free thiazole could be detected for the condition taken to be the end of the induction period as evidenced by the formation of precipitate. It is convenient to scan the aromatic protons at low field. The liberated thiazole signal for H-2 appears about midway between those for the thiazole and pyrimidine signals of thiamin. Even when the NMR tube contains massive amounts of precipitated pyrimidine oligomer, it still is possible to obtain good-quality signals for thiamin and free thiazole.

Curiously, one sample of 0.10 M thiamin chloride did not respond as usual, a faint cloudiness appearing only after about 2927 min. Other samples prepared from the same 0.20 M stock solution behaved normally. We have no explanation for this unexpected inhibition; an NMR spectrum obtained after 1220 min appeared to be normal.

Some samples containing inhibitor were examined by NMR to verify that little or no substitution took place at reaction times greater than that for the usual induction period. Free thiazole was detected when precipitate finally began to form, however.

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Synthesis and Characterization of a Novel Cholesterol Nitroxide Spin Label. Application to the Molecular Organization of Human High Density Lipoprotein

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Abstract: The purpose of this study was to synthesize a cholesterol nitroxide that closely mimics cholesterol in its physical and biological properties and to utilize this molecule to probe the nature of cholesterol-protein interactions in human high density lipoprotein particles. The rigidly labeled cholesterol nitroxide **15** (Δ^5-3β-hydroxy steroid) was prepared by addition of isohexylmagnesium bromide to nitrone **12**, a key intermediate readily obtained from dehydroisoandrosterone via intermediates **8** and **11**. Also formed from **12** in the Grignard reaction were hemiketal **23**, nitroxide **25**, and a tautomeric mixture of **21** and **21a**. This latter mixture was shown to serve as a precursor for **23**, and likely for **25**. Cu^{2+}-catalyzed air oxidation of **23** gave **27**. Formation of **21-21a** likely was initiated by attack of the α anion of nitrone **12** on the acetyl group of another molecule. The structure of **21-21a** was confirmed by an independent synthesis from **12** via intermediates **13**, **14**, and **32**. Nitroxide **15** was also converted into its oleate ester **16** and tritiated analogue **20** (sp act., 1.6 Ci/mMol). Nitroxide **15** both served as a substrate for cholesterol oxidase and also entered into the lecithin-cholesterol acyl transferase reaction, albeit with an efficiency less than that of cholesterol itself. The extent of hydrolysis of nitroxide oleate **16** by cholesterol esterase was about the same as that of cholesterol oleate, suggesting that toward this enzyme, **15** behaved like cholesterol. In dipalmitoylphosphatidyl choline (DPPC)-cholesterol vesicles at -196 °C, **15** showed $2A_{max}$ = 63.8 G (±0.5 G), similar to the value of 64.5 G at -196 °C for **15** in a mixed crystal with cholesterol. These values are consistent with the desired hydrophobic location of the nitroxide group in these systems. In the vesicle system at 25 °C, the ESR spectrum of **15** was similar to that of **2** (hindered rotation about the long molecular axis). At 45 °C the ESR spectral lines of **15** were much sharper than those of **2**. This is a consequence of the fact that, unlike **2**, the long molecular axis of **15** does not correspond to any of the principal axes of the nitroxide group. Thus, rotation about the long molecular axis partially averages all the x, y, and z components. Cholesterol nitroxide **15** was readily incoporated into human high density lipoproteins. Analysis of the ESR line shape showed the presence of cholesterol-lipid and cholesterol-protein contacts in HDL_3. These results are in contrast to the conclusion of other investigators, namely, that cholesterol is excluded from the immediate vicinity of a membrane protein that penetrates through cholesterol-containing phospholipid bilayers.

Cholesterol (**1**) is one of the principal lipids of mammalian membranes.[2] The nature of the molecular interactions of cholesterol with phospholipids,[2a-7] proteins,[8-11] and other molecules relevant to membrane biochemistry such as the polyene antibiotics[12-14] and certain toxins[15,16] has been investigated extensively,

(1) (a) Department of Chemistry. (b) Institute of Molecular Biology.
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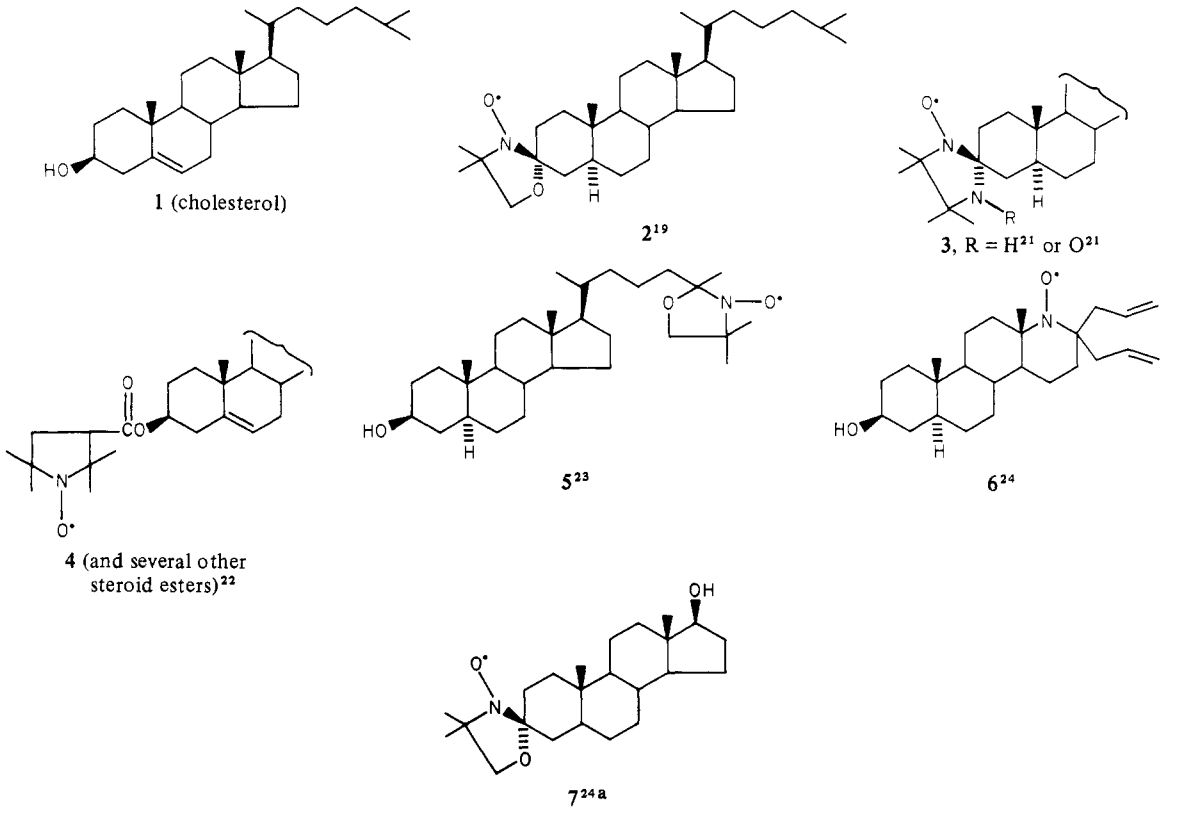
Chart I



using various biophysical techniques. It would appear that the dominant structural feature of the cholesterol molecule is the approximately planar ring system which incorporates a Δ^5-3β-hydroxy functionality. For example, a relatively minor change in this polar region of the cholesterol molecule (3β vs. 3α hydroxy groups) is accompanied by marked changes in the degree of incorporation of the sterol into lipid monolayers.[17] The overall length of the cholesterol molecule can also influence the interactions to a lesser extent, one example being the changes in ordering in bilayer systems which are observed as the side chain of cholesterol is varied.[5,7]

One of the more fruitful approaches to the study of cholesterol interactions in biological membranes has been the electron spin resonance (ESR) spin-labeling method.[18] The first of the spin-labeled cholesterol analogues, 3-doxylcholestane (**2**),[19] has provided valuable information[18,20] though the molecule differs significantly from cholesterol in the A,B rings. Subsequently, several other spin-labeled steroids, **3**–**7** (Chart I), which possess some of the structural features of cholesterol have been synthesized and used in spin-label studies. Unfortunately, none of these molecules incorporates the important Δ^5-3β-hydroxy grouping characteristic of cholesterol. Moreover, for applications in which a measure of the motion experienced by the steroid nucleus itself is important, use of spin labels such as **4** and **5** is inappropriate. This is because the nitroxide grouping in these molecules is attached to the steroid skeleton via one or more single bonds; hence the spin label can undergo rotational motion more or less independent of the motion of the skeleton.

In view of the limitations of existing spin-labeled cholesterol analogues, we have designed, synthesized, and characterized a spin-labeled cholesterol analogue, nitroxide **15**, the structure of which preserves the important Δ^5-3β-hydroxy group of cholesterol. The nitroxide, rigidly attached to the steroid skeleton is distant from this distinctive polar region. This introduces a modification only in the hydrophobic region near the other end of the steroid but also preserves the hydrocarbon side chain characteristic of cholesterol. In order to maintain the relatively low polarity and low chemical reactivity characteristic of the C-17 region of cholesterol, we chose to utilize the pyrrolidine nitroxide moiety for the spin label, as opposed to the more polar and more chemically reactive doxyl nitroxide grouping.[25]

The relationship of this analogue and cholesterol itself is evaluated by comparing them as substrates for cholesterol esterase and cholesterol oxidase. The new cholesterol spin label is also characterized by ESR spectroscopy in bilayer model systems.

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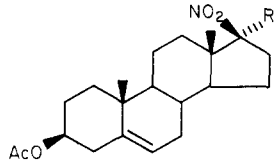
Experiments with human high density lipoprotein (HDL_3) were performed to investigate the lipid–lipid and lipid-protein interactions in the intact lipoprotein complex, and results are related to current models of HDL_3 organization.

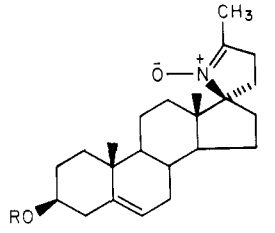
Synthesis of Cholesterol Nitroxide 15

The synthetic route to **15** parallels our recently developed routes to proxyl[25] and azethoxyl[26] nitroxide spin labels. The requisite precursor pyrrolidine nitrone **12** was synthesized as follows. Dehydroisoandrosterone (Aldrich Co.) was converted into nitro ester **8** by the method of Patchett et al.[27] Reaction of **8** with methyl-α-trimethylsilyl vinyl ketone[28] in the presence of Triton B gave directly nitro ketone **11** in 41% yield without isolation of the corresponding α-trimethylsilyl ketone.[29] Comparable yields of **11** could also be obtained from **8** by use of Triton B and a large excess of methyl vinyl ketone itself.



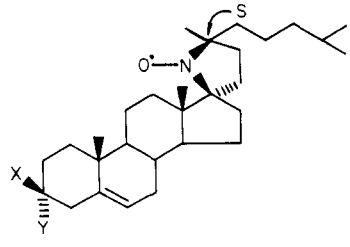
8, R = H
9, R = $CH_2CH_2CO_2CH_3$
10, R = $CH_2CH_2CO_2H$
11, R = $CH_2CH_2COCH_3$

The nitro group in **11** was assigned the β configuration by analogy to the established mode of Michael addition of methyl acrylate to **8**.[27] This assignment was confirmed by synthesis of crude **11** from the methyl acrylate adduct **9**[27] as follows. Hydrolysis of **9** followed by reacetylation gave acid **10**. The acid chloride of **10** was allowed to react with dimethylcadmium, affording nitro ketone **11** in modest overall yield. Reduction of **11** with zinc and ammonium chloride gave the key intermediate nitrone acetate **12**, mp 245–250 °C dec, in 82% yield.



12, R = Ac
13, R = H
14, R = THP

The synthesis of the desired nitroxide spin-labeled cholesterol analogue **15** was completed by the addition of the Grignard reagent isohexylmagnesium bromide to nitrone **12**. Spontaneous air



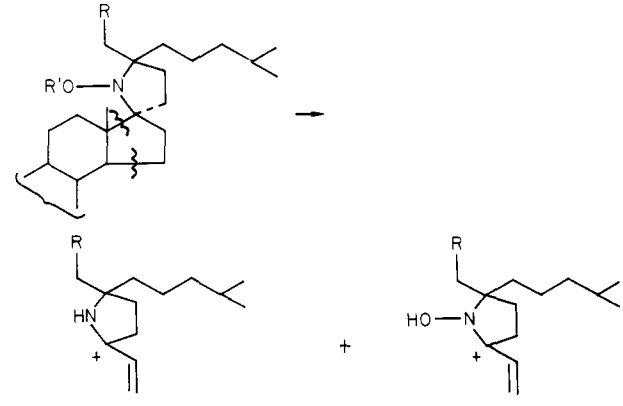
15, X = OH; Y = H
16, X = n-$C_{17}H_{33}CO_2$; Y = H
17, X = AcO; Y = H
18, X = THPO; Y = H
19, X, Y = O
20, X = OH; Y = ^3H

oxidation of the intermediate *N*-hydroxy compound afforded pure nitroxide **15**, mp 185–186 °C, in 14% yield after chromatography over silica gel. Nitroxide **15** exhibited the expected isotropic three-line ESR spectrum with a_n = 14.3 G (CH_2Cl_2, ca. one unpaired spin per molecule). Although a C, H, N elemental analysis showed crystalline **15** to be a monohydrate, the high-resolution mass spectrum exhibited a molecular ion at *m/e* 442.368, corresponding to that expected for $C_{29}H_{48}NO_2$ (calcd *m/e* 442.368).

In order for the overall molecular shape of **15** to be similar to that of the side chain extended cholesterol molecule, the newly introduced chiral center on the pyrrolidine ring must possess the *S* absolute configuration. A consideration of molecular models of nitrone **12** strongly suggests that the addition of the Grignard reagent to the carbon–nitrogen double bond should proceed in the desired sense, namely, from the less hindered right-hand side of **12** as drawn, generating the *S* configuration. It is significant that while several other pure products were isolated from the Grignard reaction mixture and characterized (vide infra), none of the *R* stereoisomer of **15** was detected among the products.

Upon closer examination, the products which accompanied **15** in the Grignard reaction proved to be quite interesting (Chart II). Three new steroids were obtained in pure form: **21**, mp 153–154 °C (23%); **23**, mp 168–170 °C (33%); and **25**, mp 131–132 °C (3%). Steroid **23** was a diamagnetic substance of formula $C_{31}H_{51}NO_3$ (*m/e* 485.390), corresponding to the composition of **15** plus C_2H_2O plus a hydrogen atom. Unlike the *N*-hydroxy intermediate corresponding to **15** (vide supra), **23** did not undergo spontaneous air oxidation to a nitroxide. However, **23** smoothly led to the corresponding nitroxide **27**, $C_{31}H_{50}NO_3$ (*m/e* 484.381), in 81% yield upon cupric acetate catalyzed air oxidation in ethanol containing ammonium hydroxide.[25,26] Whereas the infrared spectrum of neither **15** nor **23** showed carbonyl absorption, a carbonyl group was clearly indicated for nitroxide **27** (1730 cm^{-1}). The NMR spectrum of **23** showed a three-proton singlet at 1.42 ppm (hemiketal methyl) and no acetyl methyl group at about 2 ppm. That the carbonyl group of **27** was not that of an ester group was demonstrated by the observation that heating of **27** in aqueous methanol in the presence of potassium carbonate led to quantitative recovery of starting **27**.

These data collectively indicated that **23** possessed a hemiketal structure. This assignment was supported by a comparison of the mass spectral fragmentation patterns of **15** and **23**. Both molecules showed peaks corresponding to loss of the pyrrolidine ring (with or without the nitroxyl oxygen) and two of the D-ring carbon atoms. The two major fragments, **29** and **31**, derived from **23** differed from those, **28** and **30**, derived from **15** by 42 mass units, confirming that the extra C_2H_2O unit of **23** was attached to the pyrrolidine ring.



28, R = H (*m/e* 194.191)
29, R = CH_3CO (*m/e* 236.201)
30, R = H (*m/e* 210.186)
31, R = CH_3CO (*m/e* 252.196)

The source of **23** was likely (the salt of) **21**, a substance also isolated from the Grignard reaction. High-resolution mass spectrometry of **21** established the formula to be $C_{25}H_{37}NO_3$. The infrared spectrum ($CHCl_3$) exhibited, in addition to OH absorption, a rather weak peak at 1720 cm^{-1} together with a strong peak at 1580 cm^{-1} and a shoulder at 1610 cm^{-1}. In a KBr pellet

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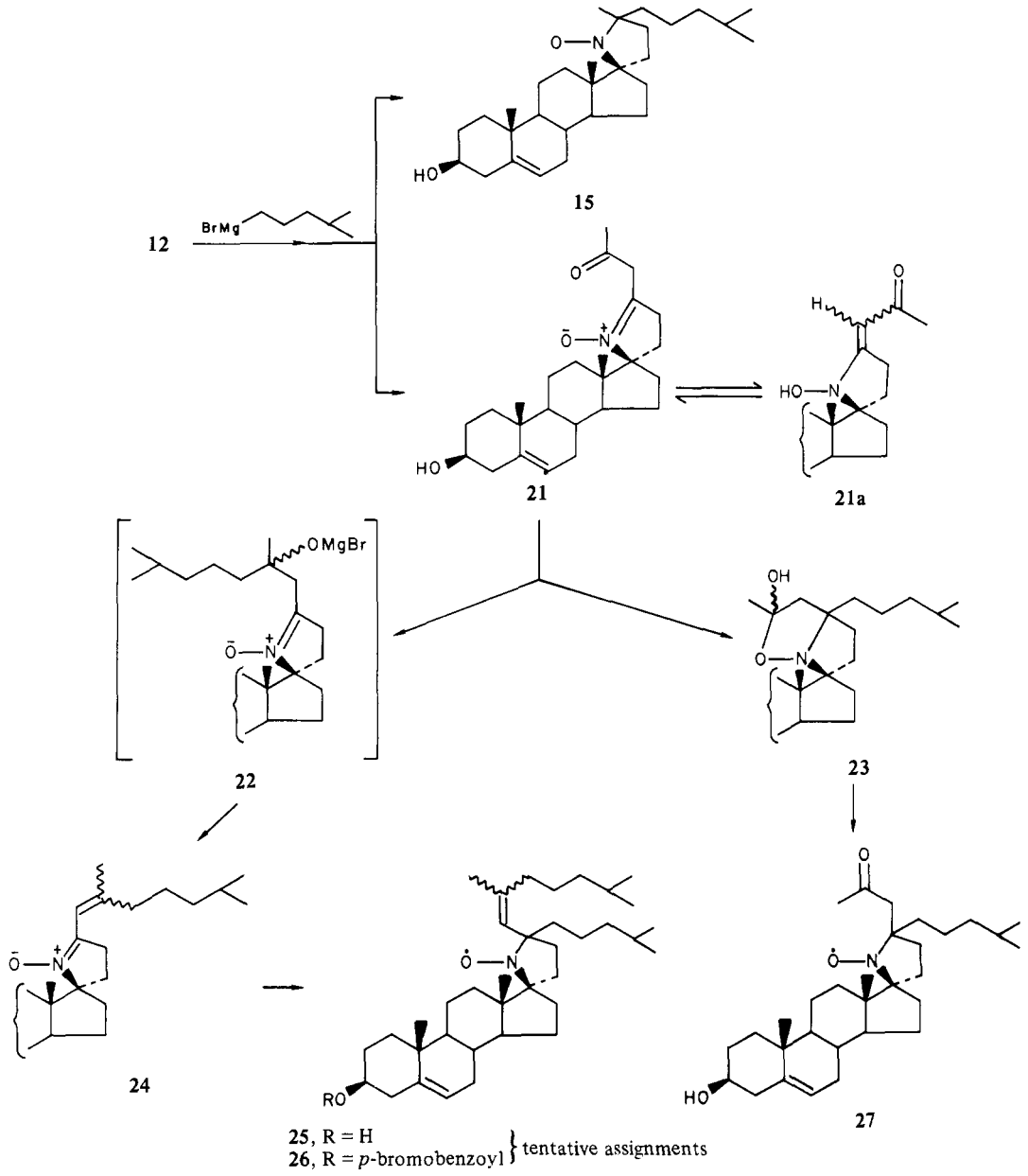
Chart II



or a Nujol mull, the 1720-cm^{-1} peak was present only as a weak shoulder. Intense absorptions were present at about 1620 and 1580 cm^{-1}. These latter peaks are consistent with the presence of an enaminone linkage[30] in **21a**. The NMR spectrum ($CDCl_3$) of **21** also supported this conclusion. An acetyl peak was present at 1.99 ppm together with a somewhat less intense acetyl singlet at 2.25 ppm. In the vinyl region, in addition to the approximate doublet due to the C-6 proton, a singlet was observed at 4.71 ppm (vinyl proton of the enaminone grouping) which integrated for 0.7–0.8 proton. This absorption slowly disappeared upon prolonged exposure of the $CDCl_3$ solution to D_2O. We conclude that **21** is in dynamic equilibrium with its tautomer, **21a**, which predominates. That **21** does, in fact, serve as a precursor for **23** was demonstrated by treatment of **21** with isohexylmagnesium bromide, a reaction which produced **23** in 77% yield, based on starting material consumed. It is interesting to note that with **21**, Grignard addition takes place more readily to the nitrone rather than to the ketone group.

A plausible explanation for the formation of **21** is as follows. Isohexylmagnesium bromide could act as a base, removing a proton from the methyl group of nitrone **12**. The resulting anion could then attack the C-3 acetyl group of another molecule, producing **21**, after cleavage of its own acetyl group. For confirmation of this proposed scheme, nitrone **12** was hydrolyzed to alcohol **13**, mp 236–242 °C, and then converted into the tetrahydropyranyl ether **14**, mp 220–224 °C. Generation of the anion of **14** with lithium diisopropylamide followed by reaction with excess acetic anhydride gave **32**, mp 138–139 °C, in 60% yield. The infrared spectrum ($CHCl_3$) of **32** showed strong absorption at 1810 cm^{-1} (*N*-acetoxy) in addition to the enaminone bands at 1650 and 1550 cm^{-1}.[30] The NMR spectrum showed singlets at 2.06 and 2.24 ppm, assigned to the *N*-acetoxy and methyl ketone groups, respectively. Hydrolysis of the THP and acetal groups of **32** with hydrochloric acid in methanol followed by methanolic potassium hydroxide gave **21**, identical with the sample obtained from the Grignard reaction above. Alternatively, reaction of **14** (no acetoxy group at C-3) with isohexylmagnesium bromide gave **18** in modest yield, unaccompanied by side products corresponding to **21**, **23**, and **25**. Acid-catalyzed hydrolysis of **18** gave **15** in 83% yield.

As stated above, a third steroid, nitroxide **25** (3% yield), was isolated from the original Grignard reaction on nitrone **12**. Mass spectrometry together with an elemental analysis of the *p*-

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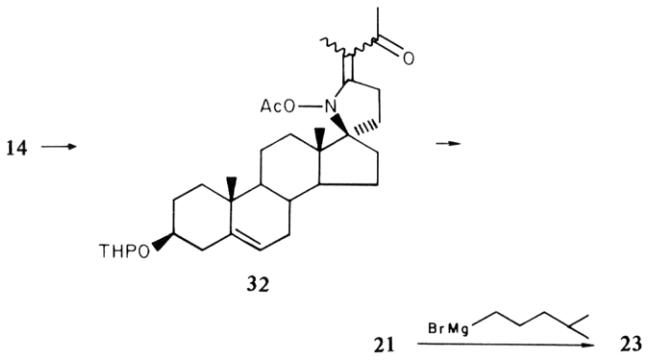

bromobenzoate ester **26**, mp 127–129 °C, indicated that **25** was a $C_{37}H_{62}NO_2$ compound. The expected three-line ESR spectrum (a_n = 14.30 G ($CHCl_3$), ca. one spin per molecule) was observed for **25**. We tentatively assign the structure to be **25**. This substance likely was formed by addition of isohexylmagnesium bromide to the ketone group of **21** (as the salt), producing **22**, which then could undergo a spontaneous β-elimination reaction to give nitrone **24**. Addition of Grignard reagent to **24** followed by an oxidation of the intermediate *N*-hydroxy compound during workup would give **25**. Probably the low yield in which **25** is formed essentially precluded its isolation from the small-scale synthesis of **23** from **21** discussed above.

For enhancement of the versatility of spin-label studies with nitroxide **15** in biological systems, a tritiated derivative of **15** was desirable. The nitroxide grouping, in general, is unstable toward the usual reagents[31,32] employed for the oxidation of cholesterol to the Δ^5-3-one derivative, for example, chromium trioxide or Me_2SO–oxalyl chloride.[33] However, oxidation of **15** with the Corey–Kim dimethyl sulfide–*N*-chlorosuccinimide reagent[34] afforded ketone **19** in near quantitative yield. Reduction of **19** with sodium boro[^{3}H]hydride in isopropyl alcohol followed by chromatographic separation of the C-3 epimers and crystallization gave radiolabeled **20** (sp act., 1.6 Ci/mM) in 45% yield.

Enzymatic Studies with Nitroxide 15

Our first indication that nitroxide **15** was quite similar to cholesterol in terms of physical behavior was the observation that it was possible to incorporate **15** into crystals of cholesterol (vide infra). From the biological viewpoint it was relevant to see whether or not **15** would serve as a substrate for enzyme systems which operate on cholesterol or cholesterol esters. The qualitative behavior of nitroxide oleate **16** toward cholesterol esterase (EC 3.1.1.13) and nitroxide **15** toward cholesterol oxidase (EC 1.1.3.6) was therefore examined. Preliminary studies[35] also showed that nitroxide **15** (and its tritiated analogue **20**) enters into the lecithin-cholesterol acyl transferase (LCAT) (EC 2.3.1.43)[36] reaction. LCAT catalyzes the transfer in plasma of the acyl chain of the 2-position of lecithin to the hydroxy group of cholesterol and as such plays a central role in lipid metabolism.

Cholesterol esterase[37] catalyzes both the esterification of cholesterol, typically with fatty acids such as oleic acid, and the hydrolysis of cholesterol esters. For the esterification reaction preferred substrates possess a 3β-hydroxy-Δ^5 or 5α structure and alteration of the side chain tends to reduce reactivity. While less information is available on substrate specificity in the hydrolysis reaction, it has been reported[38] that with pancreatic cholesterol esterase the extent of hydrolysis at 37 °C after 2 h of oleate esters of cholesterol, sitosterol, stigmasterol, and ergosterol was respectively 30.7, 28.3, 23.7, and 9.4%.

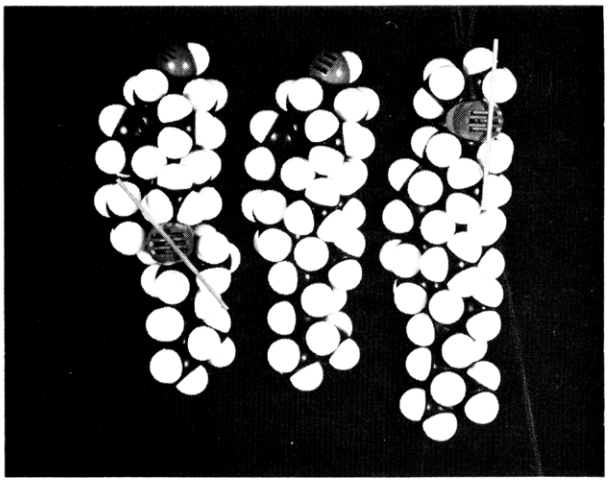

Figure 1. Models illustrating the similarity in gross structure between nitroxide **15** (left) and cholesterol (middle). 3-Doxylcholestane (**2**) (right) is shown for purposes of comparison. The markers indicate approximately the direction of the nitroxide *y* axis.

In our experiments with cholesterol esterase and nitroxide oleate **16**, after 60 min at 37 °C, the ratio of **16** to **15** was 55:45, whereas under the same conditions the ratio of cholesterol oleate to cholesterol was 60:40. Thus, the extent of hydrolysis vs. time of **16** was about the same as that of cholesterol oleate, suggesting that, toward cholesterol esterase, **15** behaves like cholesterol.

Cholesterol oxidase[39] is an enzyme which utilizes O_2 in the oxidation of cholesterol to 4-cholesten-3-one. One equivalent of H_2O_2 is liberated, which, in the presence of horseradish peroxidase, 4-aminoantipyrine, and phenol, generates a dye with A_{max} = 500 nm. The amount of cholesterol present in a sample is linearly related to the observed optical density at 500 nm.[40] This reaction, in combination with cholesterol esterase, constitutes the basis of a widely used clinical method for the determination of serum cholesterol. While cholesterol oxidase is not specific for cholesterol, the enzyme clearly prefers steroid substrates with a 3β-hydroxy group and side chains similar to that of cholesterol.[39]

Our experiments with cholesterol oxidase and nitroxide **15** under the cholesterol assay conditions[40] indicated that **15** did serve as a substrate for the enzyme, although the reaction was difficult to quantitate. The oxidations were complicated by the formation of a cloudy suspension or fine precipitate as the reactions progressed, especially in the runs with the larger amounts of substrate. The formation of chromogen proceeded at a rate 5–10-fold slower than in the case of cholesterol and in no instance was the final optical density at 500 nm more than about one-third that observed for equivalent amounts of cholesterol. Also, under the standard assay conditions, as amounts of **15** were increased, the final optical density approached a limiting value of 0.2 unit. When the amount of enzyme was increased by a factor of 110, the final maximum absorbance significantly increased, though again a precipitate formed. However, in none of the experiments with **15** did the enzyme become largely inactivated, since cholesterol itself was rapidly and quantiatively oxidized when added to the suspensions.

ESR Spectral Characterization of Nitroxide 15

The close similarity in gross structure between cholesterol and nitroxide **15** can be seen in Figure 1. For purposes of ESR spectral interpretation discussed below, it is important to note that in 3-doxycholestane (**2**) the long molecular axis lies approximately

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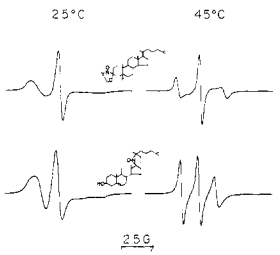
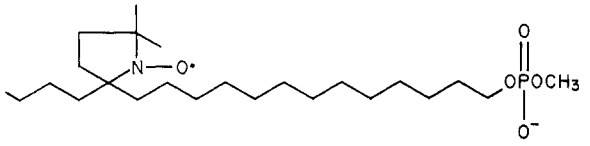


Figure 2. X-band ESR spectra of 3-doxylcholestane (**2**) and nitroxide **15** in a dipalmitoylphosphatidyl choline–cholesterol–nitroxide (100/28/1) vesicle model membrane system at 25 and 45 °C.

parallel to the nitroxide *y* axis. In nitroxide **15** the long molecular axis lies approximately in the nitroxide *y*,*z* plane and at an angle of about 40° to the nitroxide *y* axis.[41]

The ESR specttral behavior of **15** was compared with that of 3-doxylcholestane (**2**) at −196, 25, and 45 °C in a phospholipid vesicle model membrane system made from dipalmitoylphosphatidyl choline (DPPC), cholesterol, and nitroxide in a molar ratio of 100:28:1. All reported splitting values are ±0.5 G. Spectra of all mononitroxides are similar at −196 °C (except for the measured splittings). For **15**, $2A_{max}$ (at −196 °C, $2A_{max} = 2A_{ZZ}$) was estimated from the spectrum to be 63.8 G, whereas for **2**, $2A_{max}$ was 68.5 G. This value for **15** compares well with those obtained in a different way. Mixed crystals of **15** and cholesterol were readily prepared by recrystallization of a mixture of **15** and cholesterol (ratio, 1:500) from ethanol. The ESR spectrum of the powdered mixed crystal at 25 °C gave $2A_{max}$ = 62.0 G, whereas the value of $2A_{max}$ at −196 °C was 64.5 G.

Values for $2A_{max}$ of about 64 G are consistent with a hydrophobic location of the proxyl nitroxide group. Thus, in the vesicles, **15** is oriented with the nitroxide group immersed among the hydrocarbon chains of the phospholipid molecules. By way of comparison, a value of 71.5 G for $2A_{max}$ was observed for proxyl nitroxide **33** associated with bovine serum albumin at −196 °C.[42]



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In this instance the proxyl nitroxide is assumed to be exposed to a more polar environment; hence the larger $2A_{max}$ value.[43] Under similar conditions 5-doxylstearic acid has a $2A_{max}$ value of 67.0 G.[44]

The ESR spectra recorded for the DPPC–cholesterol–nitroxide vesicle system at 25 and 45 °C are shown in Figure 2. These temperatures are, respectively, well below and well above the T_c of 41.4 °C for DPPC bilayers, and this transition, although broadened, persists in the presence of the 20 mol % cholesterol used here.[45] The spectra of **15** and **2** recorded at 25 °C were qualitatively quite similar, indicating that under these conditions, motion about the long molecular axis is quite restricted.[46]

At 45 °C the spectra of **15** and **2** in the vesicle system differed significantly from each other. The spectrum for doxylcholestane (**2**) corresponded well to that expected for a randomly oriented

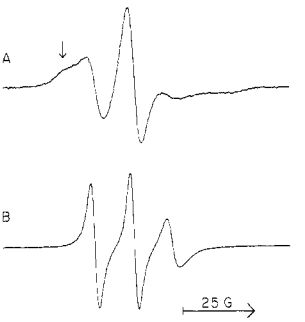

Figure 3. (A) ESR spectrum of cholesterol nitroxide **15** incorporated into human serum high density lipoprotein (HDL_3, 30 mg of protein/mL of buffer). The composite line shape is clearly evident when compared to (B): nitroxide **15** in vesicles of the polar lipids and free cholesterol extracted from human HDL_3 (phospholipid/cholesterol, mole ratio 4/1) after separation from the apolar core lipids and resuspension in buffer. Final phospholipid/label ratio of 120/1; both spectra were recorded at 37 °C.

collection of nitroxides undergoing rapid rotational motion about the *y* axis.[47] The spectral lines from **15** were markedly sharper than those for **2**, beginning to approach the isotropic limit. This is a consequence of the fact that the long molecular axis in **15** does not correspond to any of the principal axes of the nitroxide group but rather lies between the *y* and *z* axes (Figure 1). Thus, rapid rotation about the long molecular axis of **15** partially averages *all* the *x*, *y*, and *z* components. An approximately analogous relationship is present with cis and trans azethoxyl nitroxides.[48]

Evidence for Cholesterol–Protein Interactions in Human High Density Lipoprotein, HDL_3, Using Cholesterol Nitroxide 15

The human high density lipoprotein complex, HDL_3, is composed of proteins (55% by weight) and lipids (phospholipids, cholesterol, cholesterol esters, and triglycerides) organized into discrete spherical particles of 80–120-Å diameter. The organization of the molecular components is generally considered to consist of an inner core of cholesterol esters and triglycerides, surrounded by a monolayer of phospholipid with free cholesterol intercalated into this monolayer.[49] Except for the high radius of curvature, the organization of this monolayer resembles that of a phospholipid bilayer containing cholesterol. The HDL_3 proteins are associated with this outer monolayer and are responsible for maintenance of the uniform spherical morphology. Molecular models suggest that segments of the two main peptides may penetrate into the outer monolayer of the particle.[50]

Lipid spin labels in contact with protein penetrating into bilayers can be detected by the restriction of motion of the spin label.[51] In the case of steroid analogues, changes in motion are detected (at 9.5 GHz) as restriction of motion around the long axis of the steroid, except for the theoretical case where the *z* axis of the nitroxide approximately coincides with the molecular axis. With the cholesterol nitroxide **15**, the *z* axis is 50° off the steroid axis, and hindered molecular rotation is readily detected.

The ESR spectrum of cholesterol nitroxide **15** in lipid vesicles of the polar lipids (phospholipid and cholesterol) extracted from human HDL_3 is shown in Figure 3B, where the line shape is similar to the bilayer line shape of Figure 2, lower right, and is the expected line shape for rapid rotation about the long molecular axis. In the intact HDL_3 particle, the experimental spectrum (Figure 3A) exhibits a composite character, with the broad splitting (arrow) resulting from restriction of motion about the

(41) Following the usual convention, the *x* axis of a nitroxide group lies along the N–O bond; the *z* axis lies along the 2p orbital on nitrogen; the *y* axis passes through the nitrogen atom and is perpendicular to the *xz* plane.
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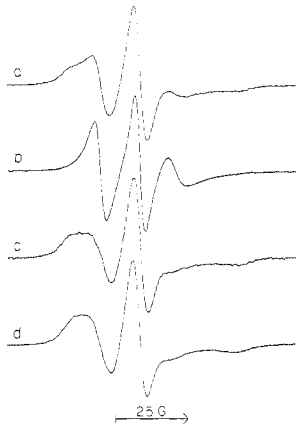

Figure 4. Spectral analysis of the composite line shape obtained with cholesterol nitroxide **15** incorporated into human HDL_3 at 37 °C (a) (same as Figure 3A). An experimental line shape (b) obtained at 20 °C with cholesterol nitroxide **15** in vesicles made from lipid extracted from human HDL_3 was identified as closely resembling an underlying component in composite a; the resulting difference spectrum (c) is characteristic of cholesterol nitroxide **15**, exhibiting reduced axial rotation. This can be seen by comparison of the difference spectrum c with line shape d, nitroxide **15** in vesicles of extracted lipids lowered to 7 °C to greatly reduce axial rotation.

long steroid axis, such that the z-axis component is not fully averaged. The splitting of this broad component is approximated by the spectrum of cholesterol nitroxide **15** in lipid bilayers below the transition temperature (Figure 2, lower left). Spectral analysis of the composite spectrum for HDL_3 is shown in Figure 4, where the two separated spectral components (b and c) are shown below the experimental spectrum (a). At the bottom, d is an experimental reference spectrum obtained from the extracted lipids resuspended as vesicles but with the temperature lowered sufficiently to match the restriction of axial motion seen in the broader component obtained from the HDL_3 spectrum.

The spectral analysis of these data is consistent with the interpretation that lateral diffusion of the cholesterol analogue in the outer monolayer of polar lipids brings it into contact with HDL_3 protein(s), and this steroid–protein contact restricts axial motion on the ESR time scale. Similar qualitative results have been reported for androstan nitroxide **7** in membranes containing cytochrome oxidase.[51] Quantitatively, the steroid molecule equilibrates between regions of lipid–lipid interactions (bilayer or monolayer) and lipid–protein interactions with a ratio of approximately 1:2.6. This is in agreement with our preliminary data obtained with phospholipid spin labels in this system. The large proportion of the broad component (72 ± 4%) suggests substantial penetration of hydrophobic segments of the HDL_3 protein(s) into the polar lipid monolayer. In another system (the calcium pump) where the hydrophobic protein penetrates through a cholesterol-containing phospholipid bilayer, it has been concluded that cholesterol is excluded from contact with the protein surface.[52] In contrast, the results we have obtained with HDL_3 show that a labeled molecule that retains most of the true cholesterol features exhibits substantial steroid–protein interactions.

Conclusions

The newly synthesized Δ^5-3β-hydroxy steroid nitroxide **15** resembles cholesterol in overall molecular shape and polarity. Mixed crystals of **15** and cholesterol may be prepared. The orientation of **15** in bilayers is the same as that of cholesterol. The analogue functions in the LCAT reaction and serves as a substrate for cholesterol oxidase, although the efficiency is lower than that of cholesterol. Cholesterol esterase hydrolysis of nitroxide oleate **16** proceeds similarly to that of cholesterol oleate. Thus, among the several nitroxide spin-labeled steroids presently available, **15** is probably the most similar to cholesterol in terms of chemical and biological properties. Cholesterol nitroxide **15** was incorporated into human high density lipoproteins (HDL_3). Analysis of the ESR line shape showed the presence of cholesterol–lipid and cholesterol–protein contacts, consistent with current models for lipoprotein organization. The evidence for cholesterol–protein contact is in contrast to the conclusion by other investigators that cholesterol is excluded from the immediate vicinity of protein that penetrates through cholesterol-containing phospholipid bilayers.

Experimental Section

Infrared spectra were recorded on a Beckman IR-10 spectrometer. NMR spectra were obtained in $CDCl_3$ on a Varian Associates XL-100 spectrometer using Me_4Si as an internal standard. X-band ESR spectra were recorded on a Varian E-line spectrometer with a dedicated Varian 620/L computer, Varian field/frequency lock, and temperature controller. ESR spectra were integrated and analyzed by spectral titration by published methods.[53] Visible spectra were determined on a Cary-15 spectrometer. High-pressure liquid chromatography was performed in methanol (flow rate, 1.2 mL/min) with a Waters Associates instrument equipped with a μ-Bondapak C_{18} (3.9 × 100 mm) reverse-phase column. Peaks were monitored at either 230 or 254 nm. Preparative TLC was performed with Analtech Inc. 1000 μ 20 × 20 cm silica gel GF plates. Elemental analyses and mass spectra were determined at the University of Oregon by Dr. R. Wielesek.

3β-Acetoxy-17β-nitro-17α-(3-oxobutyl)-5-androstene (11). Method A. To a stirred solution of **8**[27] (36.1 mg) (25 °C, N_2) in *tert*-butyl alcohol (0.3 mL) was added a solution of methyl-α-trimethylsilyl vinyl ketone[28] (21.3 mg) in *tert*-butyl alcohol (0.1 mL) followed by 0.04 mL of benzyltrimethylammonium hydroxide (Triton B) (35% in MeOH). After 1.5 h of stirring at 25 °C, ice and 5% aqueous HCl were added. The mixture was extracted with ethyl acetate and the extract was washed with aqueous HCl, water, and brine and dried ($MgSO_4$). Removal of solvent gave an oil (50 mg), which was purified by preparative TLC (CH_2Cl_2). Ketone **11** was obtained as an oil (18 mg, 41%), which was crystallized from EtOH, mp 148–151 °C. Recrystallization from EtOH gave the analytical sample: mp 157–158 °C; NMR δ 5.38 (1 H, H-6), 4.60 (1 H, H-3), 2.12 (3 H, s), 2.00 (3 H, s), 1.00 (3 H, s, H-19), 0.76 (3 H, s, H-18); m/e 371.247 (calcd for $C_{23}H_{33}NO_3$ = M – AcOH, 371.246). Anal. Calcd for $C_{25}H_{37}NO_5$: C, 69.58; H, 8.64; N, 3.25. Found: C, 69.44; H, 8.85; N, 3.07.

Method B. To a solution of **8** (0.560 g) in THF (7 mL) and methyl vinyl ketone (10 mL) was carefully added 0.05 mL of benzyltrimethylammonium hydroxide (35% in MeOH) (**caution**: exothermic polymerization of MVK sometimes occurred when >0.05 mL of Triton B was used) dropwise over a 10-min period at 0 °C under N_2. After 15 min of stirring at 0 °C, cold 5% aqueous HCl was added and the product was extracted into ethyl acetate. The extract was washed with HCl, water, and brine, and dried (Na_2SO_4). The above reaction was repeated twice on the same scale. The products were combined and purified by silica gel column chromatography. Elution with hexane–CH_2Cl_2 (3/1) gave crystalline starting **8** (0.98 g). Elution with CH_2Cl_2 gave 0.76 g (38%, 88% based on consumed **8**) of crystalline **11**.

Method C. A solution of methyl acrylate adduct **9** (2.48 g)[27] and K_2CO_3 (3.0 g) in 165 mL of MeOH and 25 mL of H_2O was refluxed overnight. The cooled solution was neutralized by dropwise addition of concentrated HCl. Evaporation of the resulting mixture to dryness followed by trituration with hot 10% MeOH in ethyl acetate afforded 2.4 g of white solid after removal of the solvent. The crude product was treated with 60 mL of 1/1 acetic anhydride–pyridine. After 12 h, the solution was poured into ice water and, after 30 min, extracted with ether. The extract was washed (5 °C) with water, 3% aqueous HCl, and brine and was dried (Na_2SO_4). Evaporation of solvent afforded 1.75 g (73%) of **10** as a colorless powder, which was recrystallized from benzene, giving pure **10**: mp 205–207 °C; NMR δ 0.79 (3 H, s, H-18), 1.03 (3 H, s, H-19), 2.04 (3 H, s). To a solution of 343 mg of **10** in 10 mL of CH_2Cl_2 was added at 0 °C (N_2) a solution of oxalyl chloride (freshly distilled, 148 mg) dissolved in 0.9 mL of CH_2Cl_2 followed by slow dropwise addition of a solution of 0.10 mL of pyridine in 0.9 mL of CH_2Cl_2 over a 5-min period. After 30 min at 0 °C, the solution was evaporated to dryness. Trituration of the resulting foam with dry benzene followed by evaporation gave 340 mg (95%) of the corresponding acid chloride as a foam: NMR δ 0.84 (3 H, s, H-18), 1.09 (3 H, s, H-19), 2.09 (3 H, s), 2.85 (2 H, m); IR ($CHCl_3$) 1800, 1725 cm^{-1}. The foam was used for the next reaction without further purification.

To a stirred solution (N_2) of 0.87 mmol of Me_2Cd in 4 mL of benzene was added dropwise over 5 min 264 mg of the foam in 3.25 mL of benzene. The mixture was stirred at 25 °C for 15 min and then at 70

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°C for 1 h. Ice was added and the colorless mixture was stirred for 10 min. After dilution with 4 mL of ethyl acetate, 3 mL of 2% aqueous HCl was added, and the layers were separated. The organic layer was washed with 5% aqueous HCl, 5% aqueous $NaHCO_3$, and brine, dried (Na_2SO_4), and evaporated to give 202 mg of a colorless oil. Preparative TLC eluted with $CHCl_3$ led to crude oily **11** (96 mg, 38%), the NMR spectrum of which was identical with that of **11** prepared by method A.

3′,4′-Dihydro-5′-methyl-3β-acetoxy-(17*R*)spiro[5-androstene-17,2′-(2′*H*)-pyrrole] 1′-Oxide (12). To a stirred solution (0 °C, N_2) of **11** (0.76 g) in 25 mL of THF was added a solution of NH_4Cl (95 mg) dissolved in 4.7 mL of water followed by portionwise addition of Zn dust (0.395 g) over a 1-h period. The resulting mixture was stirred at 25 °C for 5 h and then evaporated to dryness. The residue was triturated with CH_2Cl_2 and the extract was washed once with water and then dried ($MgSO_4$). Removal of solvent gave crystalline **12** (0.58 g, 82%). Recrystallization from EtOH gave the analytical specimen: mp 245–250 °C dec; NMR δ 5.38 (1 H, H-6), 4.60 (1 H, H-3), 2.06 (3 H, s), 2.02 (3 H, s), 1.02 (3 H, s), 0.98 (3 H, s); IR ($CHCl_3$) 1725, 1590 cm^{-1}; *m/e* 399.279 (calcd for $C_{25}H_{37}NO_3$, 399.277). Anal. Calcd for $C_{25}H_{37}NO_3$: C, 75.15; H, 9.33; N, 3.51. Found: C, 75.07; H, 9.12; N, 3.35.

Reaction of 12 with Isohexylmagnesium Bromide. To a 20-mL aliquot of isohexylmagnesium bromide–THF solution (25 °C, N_2), prepared by using Mg (0.40 g), isohexylbromide (2.20 g), and THF (24 mL) in a usual manner, was added a suspension of **12** (300 mg) in 60 mL of THF over a 45-min period. After a 2-h stir, the mixture was concentrated to ~30 mL, cooled, treated with saturated aqueous NH_4Cl, and extracted with $CHCl_3$. The extract was washed with water and brine, dried ($MgSO_4$), and concentrated to dryness. Chromatography over silica gel with elution by hexane–ether (2/1) gave crude **25**, which was further purified by preparative TLC (hexane–ether, 7/3). Crystallization from CH_3CN afforded pure 25 (13 mg, 3%): mp 131–132 °C; *m/e* 552; ESR ($CHCl_3$), a_n = 14.3 G. The *p*-bromobenzoate derivative **26** was prepared by using *p*-bromobenzoyl chloride and pyridine in toluene: mp 127–129 °C (CH_3CN); IR ($CHCl_3$) 1710 cm^{-1}. Anal. Calcd for $C_{44}H_{65}NO_3Br$: C, 71.81; H, 8.90; N, 1.90. Found: C, 71.40; H, 8.87; N, 1.75.

Continued elution of the silica gel column with hexane–ether (2/1) gave nitroxide **15**, which was also further purified by preparative TLC (hexane–ether, 3/7). Pure 5′-methyl-5′-(4-methylpentyl)-3β-hydroxy-(17*R*)-spiro[5-androstene-17,2′-pyrrolidin]-1′-yloxy **15** (48 mg, 14%) was obtained by recrystallization from CH_3CN: mp 185–186 °C; IR ($CHCl_3$) 3600, 3450, 1600 cm^{-1}; ESR (CH_2Cl_2), a_n = 14.3 G; *m/e* 442.368 (calcd for $C_{29}H_{48}NO_2$, 442.368). Anal. Calcd for $C_{29}H_{48}NO_2 \cdot H_2O$: C, 75.60; H, 10.94; N, 3.04. Found: C, 75.65; H, 11.11; N, 2.92.

Elution of the silica gel column with hexane–ether (1/1) gave 2′,3′,3′a,4′,5′,6′-hexahydro-2′-hydroxy-2′-methyl-3′a-(4-methylpentyl)-3β-hydroxy-(17*R*)-spiro[5-androstene-17,6′-pyrrolo[1,2-*b*]isoxazole] **23** (84 mg, 23%). Recrystallization from ether gave the analytical specimen of **23**: mp 153–154 °C; IR ($CHCl_3$) 3600, 3400 cm^{-1}; NMR δ 5.36 (1 H, H-6), 1.42 (3 H, s, 2′-CH_3), sharp resonances at 1.07, 1.02, 0.90, and 0.83; *m/e* 485.390 (calcd for $C_{31}H_{51}NO_3$, 485.387).

Elution of the silica gel column with MeOH–ether (5/95) gave 3′,4′-dihydro-5′-(2-oxopropyl)-3β-hydroxy-(17*R*)-spiro[5-androstene-17,2′(2′*H*)-pyrrole] 1′-oxide, **21** (98 mg, 33%), which was recrystallized from CH_3CN to give pure nitrone **21**, which exists largely as tautomer **21a**: mp 168–170 °C; IR ($CHCl_3$) 3600, 3400, 1720, 1610, 1580 cm^{-1}; IR (KBr) 3400, 1620, 1580 cm^{-1}; NMR δ (see text) 5.34 (1 H, H-6), 4.71 (0.7–0.8 H, enaminone vinyl), 2.25 (s), 1.99 (s); *m/e* 399.276 (calcd for $C_{25}H_{37}NO_3$, 399.277).

5′-(2-Oxopropyl)-5′-(4-methylpentyl)-3β-hydroxy-(17*R*)-spiro[5-androstene-17,2′-pyrrolidin]-1′-yloxy (27). To a solution of **23** (43 mg) dissolved in 5 mL of EtOH was added NH_4OH (0.3 mL) and $Cu(OAc)_2 \cdot H_2O$ (3 mg). This was stirred under air for 30 min. After dilution with water, the product was extracted into $CHCl_3$. The extract was washed with water and brine and dried ($MgSO_4$). Removal of the solvent followed by crystallization from CH_3CN gave 35 mg (81%). Recrystallization from CH_3CN gave pure **27**: mp 158–159 °C; IR ($CHCl_3$) 3600, 3450, 1730 cm^{-1}; ESR (CH_2Cl_2), a_n = 14.0 G; *m/e* 484.381 (calcd for $C_{31}H_{50}NO_3$, 484.379). Anal. Calcd for $C_{31}H_{50}NO_3 \cdot {}^1/_5H_2O$: C, 76.25; H, 10.40; N, 2.87. Found: C, 76.32; H, 10.77; N, 2.76.

Reaction of 21 with Isohexylmagnesium Bromide. To a solution of **21** (16 mg) dissolved in 0.8 mL of THF was added (N_2) a solution (1 mL) of isohexylmagnesium bromide (same as above) in THF. Initially some precipitate formed, which gradually disappeared with stirring for 6 h at 25 °C. After quenching with aqueous NH_4Cl, the mixture was extracted with CH_2Cl_2. The extracts were washed with water and brine and dried (Na_2SO_4). Removal of the solvent gave an oil, which was purified by preparative TLC (ether). The yield of **20**, identical with that obtained above, was 10 mg (52%, 77% based on recovered starting **21** (5 mg)).

3′,4′-Dihydro-5′-methyl-3β-hydroxy-(17*R*)-spiro[5-androstene-17,2′-(2′*H*)-pyrrole] 1′-Oxide (13). To a stirred suspension of **12** (40 mg) in 5 mL of MeOH–water (4/1) (N_2) was added 35 mg of K_2CO_3. The mixture was heated at 40–45 °C for 45 min. After concentration to half-volume, 40 mL of $CHCl_3$ was added. The $CHCl_3$ layer was washed with water and brine and dried (Na_2SO_4). Removal of solvent followed by trituration with ether gave **13** as colorless crystals (32.5 mg, 91%). Recrystallization from isopropyl alcohol–hexane gave the analytical specimen: mp 236–242 °C; NMR δ 5.31 (1 H, H-6), 2.04 (3 H, nitrone CH_3), 0.96–0.98 (6 H, H-18, 19); *m/e* 357.267 (calcd for $C_{23}H_{35}NO_2$, 357.267).

3′,4′-Dihydro-5′-methyl-3β-(tetrahydro-2*H*-pyran-2-yl)oxy-(17*R*)-spiro[5-androstene-17,2′(2′*H*)-pyrrole] 1′-Oxide (14). A catalytic amount of *p*-toluenesulfonic acid monohydrate was added to a solution of **13** (37 mg) and dihydropyran (87 mg) in 4 mL of CH_2Cl_2. After a 12-h stir at 25 °C, 20 mL of CH_2Cl was added. The solution was washed with 10% aqueous $NaHCO_3$ and water and dried ($MgSO_4$). Removal of solvent gave crystalline **14**. Recrystallization from ethyl acetate gave pure **14** (39 mg, 86%): mp 220–224 °C; NMR δ 5.34 (1 H, H-6), 4.70 (1 H, THP proton), 2.06 (3 H, nitrone methyl), 0.99–1.01 (6 H, H-18, 19); *m/e* (441.322 (calcd for $C_{28}H_{43}NO_3$, 441.324).

5′-Methyl-5′-(4-methylpentyl)-3β-(tetrahydro-2*H*-pyran-2-yl)oxy-(17*R*)-spiro[5-androstene-17,2′-pyrrolidin]-1′-yloxy (18). Isohexylmagnesium bromide in THF (2 mL, prepared same as above) was added to a solution of **14** (28 mg) in 4 mL of THF (N_2) at 25 °C. After a 3-h stir, saturated aqueous NH_4Cl was added and the product was extracted into CH_2Cl_2. The CH_2Cl_2 layer was washed with water and dried ($MgSO_4$). Removal of solvent gave a colorless oil, which was treated under air with $Cu(OAc)_2 \cdot H_2O$ (3 mg) in EtOH (4 mL) and NH_4OH (0.1 mL). After workup, the product was purified by preparative TLC (hexane–ether, 3/7). Nitroxide **18** (3 mg) was isolated in addition of unreacted **14** (10 mg). Recrystallization from hexane gave pure **18**: mp 194–196 °C; ESR (CH_2Cl_2), a_n = 14.3 G; *m/e* 526.422 (calcd for $C_{34}H_{56}NO_3$, 526.426).

Hydrolysis of 18 to 15. To a solution of **18** (11 mg) in 3 mL of ether was added 4 mL of a solution of 0.1 N HCl–MeOH (1/9). After a 24-h stir at 25 °C, 20 mL of ether was added. The ether layer was washed with aqueous $NaHCO_3$ and water and dried (Na_2SO_4). Removal of solvent gave crystalline **15** (8 mg, 86%), which was identical with that obtained in the original Grignard reaction described above.

1′-Acetoxy-5′-(2-oxypropylidene)-3β-(tetrahydro-2*H*-pyran-2-yl)-oxy-(17*R*)-spiro[5-androstene-17,2′-pyrrolidine] (32). To a stirred solution of hexane–BuLi (0.7 mM) in 1.5 mL of THF at −60 °C was added (N_2) a solution of diisopropylamine (0.14 mL) in 0.5 mL of THF. After a 10-min stir, a solution of **14** (30 mg) in 3 mL of THF was added at −60 °C. After 20 min, a solution of acetic anhydride (0.1 mL) in 0.5 mL of THF was slowly added. After 20 min, the reaction was allowed to warm to 0 °C. After 30 min, water was added and the product was extracted into CH_2Cl_2. The extract was washed with water and brine and dried ($MgSO_4$). Removal of solvent gave a yellow oil, which was purified by preparative TLC (ether), giving **20** mg (60%) of **32**. Crystallization from CH_3CN afforded pure **32**: mp 138–139 °C: IR ($CHCl_3$) 1800, 1645, 1550 cm^{-1}; *m/e* 525 (M^+); NMR δ 5.35 (1 H, H-6), 5.03 (1 H, enaminone vinyl), 4.72 (1 H, THP proton), 2.25 (3 H, CH_3CO), 2.08 (3 H, s, AcO), 1.04 (3 H, s), 0.89 (3 H, s).

Preparation of 21 from 32. A solution of **32** (7 mg) dissolved in 2 mL of MeOH–0.1 N HCl (8/1) was stirred for 25 h at 25 °C and then poured into cold 5% aqueous $NaHCO_3$. The product was extracted into CH_2Cl_2. Removal of solvent gave a brown oil to which was added 0.5 mL of MeOH followed by 0.2 mL of a KOH–MeOH solution (1/40). After a 1-h stir at 25 °C, water was added and the mixture was extracted with CH_2Cl_2. The extract was washed with water and brine and dried ($MgSO_4$). Removal of solvent gave an oil, which was purified by preparative TLC (ether) to afford 3 mg (57% from **32**) of **21**, identical with that obtained in the Grignard reaction of **16**.

5′-Methyl-5′-(4-methylpentyl)-3β-oleoyloxy-(17*R*)-spiro[5-androstene-17,2′-pyrrolidin]-1′-yloxy 16. To a solution of the nitroxide **15** (10 mg) in 1 mL of toluene were added a solution (4 equiv) of 0.5 M pyridine–toluene and a solution (3 equiv) of oleoyl chloride in toluene. After a 20-h stir at 25 °C and the usual workup, preparative TLC (hexane–ether, 3/7), gave crystalline **16** (11 mg 70%) in addition to recovered **15** (2 mg). Recrystallization from CH_3CN afforded pure **16**: mp 105–110 °C; IR ($CHCl_3$) 1720 cm^{-1}; ESR (*i*-PrOH), a_n = 14.4 G. Anal. Calcd for $C_{47}H_{80}NO_3$: C, 79.83; H, 11.40; N, 1.98. Found: C, 79.66; H, 11.13; N, 1.65.

Cholesterol Esterase Procedure. The chemicals were purchased from Supelco. The enzyme reagent was made up by combining sodium cholate (32 mg), Na_2HPO_4 (1978 mg), NaH_2PO_4 (168 mg), Carbowax 6000 (26 mg), and cholesterol esterase (EC 3.1.1.13) (0.83 unit) in 25 mL of water. To 2.5 mL of the enzyme reagent was added 50 μL of a solution of ester **16** (1.805 mg) in 0.60 mL of *i*-PrOH followed by incubation of 37 °C for 60 min. The reaction mixture was then thoroughly extracted

with ether. The extract was washed once with water and evaporated. The residue was dissolved in 0.1 mL of *i*-PrOH and analyzed by HPLC. Comparison with HPLC generated standard curves using pure **16** and **15** showed that the ratio of **16** to **15** was 55/45.

The cholesterol oleate hydrolysis experiments were performed as above, using 50-μL aliquots of a solution of cholesterol oleate (2.350 mg) dissolved in 0.58 mL of *i*-PrOH. After 60 min at 37 °C, the mixture was thoroughly extracted with CH_2Cl_2 (ether is not suitable owing to the presence of traces of peroxides). The extract was washed with water and evaporated. The residue was homogenized in an *i*-PrOH–water (1/1) solution (0.1 mL). Free cholesterol was then determined by using the cholesterol oxidase enzyme method described below. The ratio of cholesterol oleate to cholesterol was 60/40.

Cholesterol Oxidase Procedure. The reagent solution was made up by combining sodium cholate (64.5 mg), 4-aminoantipyrine (8.5 mg), phenol (61.5 mg), Na_2HPO_4 (355 mg), NaH_2PO_4 (335 mg), Carbowax 6000 (51 mL), cholesterol oxidase (EC 1.1.3.6) (5.85 units), and horseradish peroxidase (3350 units) in water (50 mL) as described by Allain et al.[40] A 10 × 10 × 30 mm cuvette was charged with 2.4 mL of the reagent solution (~37 °C) followed by 0.3 mL of *i*-PrOH (~37 °C) to which had been added either 0, 5, 10, 30, 50, or 60 μL of stock substrate solution (3.0 mg of substrate in 1.0 mL of *i*-PrOH). After the solution was mixed (37 °C), the absorbance at 500 nm due to the formation of the chromogen was monitored until a constant value was observed. Cholesterol required 15–25 min. Reactions involving nitroxide **15** required up to 90 min. A linear response was obtained for cholesterol: 5 μL gave A_{500} = 0.12; 10 μL, 0.24; 30 μL, 0.76; 60 μL, 1.58. With **15**, the maximum absorbance at 500 nm approached a limiting value: 5 μL gave A_{500} = 0.03; 10 μL, 0.14; 30 μL, 0.22; 50 μL, 0.25. The oxidation studies with **15** were complicated by formation of suspensions or fine precipitates. Another set of experiments were performed in which the concentrations of both cholesterol oxidase and horseradish peroxidase were increased 110-fold. Cholesterol behaved normally, with 30 μL of stock solution giving A_{500} = 0.78. Nitroxide **15** behaved as follows: 10 μL gave A_{500} = 0.12; 30 μL, 0.18; 50 μL, 0.35 (cloudy).

Synthesis of Tritium-Labeled Cholesterol Nitroxide 20. To a cooled suspension of *N*-chlorosuccinimide (7 mg) in 0.2 mL of dry toluene at −15 °C (N_2) was added 70 μL of a 1 M dimethyl sulfide solution in toluene. Then a solution of **15** (5 mg) in 1.0 mL of dry toluene was added dropwise. After a 2-h stir at −23 °C, 52 μL of 1 M triethylamine in toluene was added at −23 °C. The cooling bath was removed, and after 5 min, ether (15 mL) was added. The organic layer was washed with cold water and brine and dried ($MgSO_4$). After removal of solvent, **19** (5.5 mg) was obtained as a solid: mp 156–160 °C; IR ($CHCl_3$) 1710 cm^{-1}. This was used immediately for the following reduction. Sodium boro[^{3}H]hydride (100 mCi) (Amersham, sp act., ~5 Ci/mMol) was transferred to a centrifuge tube with the aid of some *i*-PrOH. A solution of **19** (5.5 mg) in 0.8 mL of *i*-PrOH was added. After a 1.5-h stir at 25 °C, 0.3 mL of 0.1 N NaOH and 0.1 mL of water were added, and then all was extracted with ether. The extract was washed with brine and evaporated, giving a residue which was purified by preparative TLC (hexane–ether, 3/7). Tritiated nitroxide **20** (2.5 mg) and its C-3 epimer (1.5 mg) were both isolated in pure form. After recrystallization of **20** from CH_3CN, the specific activity was 1.6 Ci/mM.

Preparation of Human High Density Lipoprotein (HDL_3) and HDL_3 Lipids. Human high density lipoprotein, HDL_3, was kindly supplied by Dr. Angelo M. Scanu[54] and stored at 4 °C in a high salt solution consisting of 98 mg of NaCl, 200 mg of NaBr, and 0.01 mL of 5% EDTA, pH 7.0, per milliliter (final density of 1.21 g/mL). Before use, HDL_3 was dialyzed against 0.15 M NaCl, 10 mM phosphate, 1 mM EDTA, pH 7.4, and adjusted to 30 mg of protein/mL by ultracentrifugation. The same buffer was used in all experimental procedures. Cholesterol nitroxide **15** was introduced into the lipoprotein particles at a molar ratio of less than one spin label per two HDL_3 via ethanolic injection of the label [1% (v/v) final ethanol concentration]. Full incorporation of nitroxide **15** required incubation for 8 h at 37 °C as monitored by ESR. Control experiments showed that the ESR spectrum of **7** in HDL_3 was not altered by the addition of 1% ethanol.

HDL_3 lipids were extracted by the method of Bligh and Dyer,[55] dried, resuspended in buffer, and centrifuged (1 h, 12000*g*) to separate the vesicles containing phospholipid and cholesterol from the floating apolar lipids. The phospholipid–cholesterol-rich fraction was reextracted, characterized by thin-layer chromatography, and stored in choloroform–methanol (4/1). Aqueous vesicles of the phospholipid–cholesterol HDL_3 lipid fraction and cholesterol nitroxide **15** were prepared by mixing 685 nmol of HDL_3 lipid phosphorus and 5.7 nmol of nitroxide **15** in chloroform–methanol (4/1) and drying the lipids under nitrogen followed by water aspiration (1 h). Buffer (40 μL) was added to the dry lipid film and the sample was vortexed. This procedure yields multilamellar vesicles with the same phospholipid to cholesterol ratio as the intact HDL_3.

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Comparative NMR Studies of Cytochrome *c* and Its Active Site Octapeptide

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Abstract: The source of the asymmetric electron spin density distribution in cytochrome *c* has been investigated with use of the active site heme octapeptide (OP) as a model system. The paramagnetic ^{1}H NMR spectra of OPL where L = CN^-, pyridine, N_3^-, and *N*-acetyl-DL-methionine are reported and compared to the spectra of analogous cytochrome *c* derivatives. The temperature and concentration dependences of the OPCN and OPpyr heme methyl resonances were used to characterize the model system. Low-temperature EPR spectra of OPCN and OPpyr are very similar to their respective cytochrome *c* analogues. The *g* values were used to calculate the dipolar contribution to the isotropic shifts. Large isotropic shift differences between the appropriate model system and cytochrome *c* derivative primarily reflect greater contact shifts in the peptide systems, in contrast to previous model system studies. Heme methyl group resonances were assigned for OPCN, OPpyr, azide–cytochrome *c*, and pyridine–cytochrome *c*, using either Gd^{3+} as a relaxation probe or saturation transfer for N_3^- and pyridine–cytochrome *c* complexes. The pattern of methyl group resonances with increasing field presumably is 5, 8, 1, 3 for the model systems as well as for the protein derivatives, compared to 8, 3, 5, 1 for native cytochrome *c*. The detailed analysis of these results demonstrates that the orientation of the axial methionine in cytochrome *c* and heme-protein contacts are important determinants of the electronic structure of the heme in native cytochrome *c*.

NMR spectroscopy can provide a uniquely sensitive probe of the electronic structure of paramagnetic heme proteins.[1-20] This ability has been elegantly exploited in studies of cytochrome *c*. One finding of such studies is that the spin density in cytochrome

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