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Axial Isomer and ESR Spectra of the Steroid Spin Label, 3-Doxyl-5α-Cholestane

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The ESR spectra of the axial isomer of the steroid spin label 3-doxyl-5α-cholestane has been obtained by computer subtraction of the equatorial isomer from a partially purified mixture of the two isomers. The ^{14}N coupling constant is 15.03 ± 0.05 G in $CDCl_3$ at room temperature. The proton hyperfine splittings are only partially resolved. The assignment of these splittings is made by synthesizing a number of spin label analogs and comparing their hyperfine patterns and the corresponding computer simulations.

Steroid spin labels are readily diffused into membrane model systems and biological membranes. The ESR spectra provide useful information on the orientation of lipids (*1–3*), molecular motion (*4*, *5*), phase transitions (*6*), lateral diffusion rates (*7*), lipid–protein interactions (*8*, *9*), and the effects of cholesterol (*10*) and osmium tetroxide (*11*) on bilayers, and in studies of liquid crystals (*12*). The most commonly used spin label of this type is 3-doxyl-5α-cholestane.[1] There are two possible isomers formed in the synthesis (equatorial or axial) which result from the stereochemistry of attachment of the oxazolidine ring at the C_3 position of the steroid A ring, as shown in Fig. 1. Evidence has been obtained from the synthesis of closely related analogs to the two isomers (*13*) and from a single crystal ESR study (*14*), that the dominant radical in the

Ie.

Ia.

FIG. 1. The pair of isomers of 3-doxyl-5α-cholestane with the C–N bond in the equatorial (*e*) and axial (*a*) position with respect to the steroid A ring.

[1] The trivial name, *doxyl-*, refers to the 4′,4′-dimethyl-oxazolidine-*N*-oxyl derivative of the parent ketone and is used throughout this work.

synthetic product is the equatorial isomer. However, the axial isomer has not been observed. We report here the ESR spectra of the axial isomer, 3*a*-doxyl-5α-cholestane. In addition, over a period of years we have examined a number of related spin labels and deuterated analogs in order to test lineshape simulations. These data provide proof of the assignment of the coupling constants, although the assignment of the equatorial isomer is confirmatory in view of the previously published NMR and ESR results of Michon and Rassat (*13*). We include these additional ESR data in the hope that it will aid others encountering ESR lineshape problems involving steroid spin labels.

EXPERIMENTAL

Ethyl acetate, $CDCl_3$, and cyclohexanone were purchased from Mallinckrodt. Cyclohexanone-2,2,6,6-d_4 and 5α-cholestan-3-one-2,2,4,4-d_4 were custom synthesized by Merck, Sharp and Dohme of Canada, Ltd. Cyclohexanone-d_{10} is a standard product of that company. *Trans*-1-decalone, 4-*tert*-butyl-cyclohexanone, and 3,3,5,5-tetramethyl-cyclohexanone are from the Aldrich Chemical Co. 5α-Cholestan-3-one, 3-hydroxyl-5α-cholestan-6-one, and 3-keto-5α-androstan-17β-ol were purchased from Steraloids. The ketone starting materials were all used without further purification. The 2-amino-2-methyl-1-propanol (Eastman Kodak) and 2-methyl-2-butanol (Mallinckrodt) were purified by distillation (bp 153–154 and 100–101°, respectively) and rejection of the first 20% of the distillate. The *m*-chloroperoxybenzoic acid (85% pure) is an Eastman Kodak product and was not further purified.

The oxazolidine precursors of the nitroxides used in this study were synthesized by established methods (*15*, *16*). No acid catalyst was used to avoid production of amine salt contaminants during the oxazolidine ring formation and to avoid acid catalyzed proton–deuteron exchange in the deuterated ketone starting materials. This method gave high yields (>70%) in moderate lengths of time (3–4 days). Typically, 1.0 g of ketone and 1.1 mole equivalents of 2-amino-2-methyl-1-propanol were dissolved in 10 to 15 ml of spectral grade benzene. Boiling chips were added and the solution refluxed under a constant water separator (Dean Stark trap) until no additional water was produced. The reaction was then cooled and diluted with 50–75 ml of spectral grade benzene. This solution was washed four times with 50 ml of a $NaHCO_3$-saturated water solution, four times with 50 ml of a NaCl-saturated water solution, and four times with 50 ml of distilled water. The combined water washes were back-extracted two times with 50 ml portions of benzene. The washed reaction solution and the benzene back-extractions were combined and dried by stirring overnight with $MgSO_4$ or Na_2SO_4. The solution was then filtered and the benzene removed under vacuum on a rotary evaporator yielding the oxazolidine product.

The oxidation of the oxazolidine to the nitroxide (*16*) was carried out by dissolving the oxazolidine from the preceding step in 100 ml of anhydrous diethyl ether and adding 1.5 mole equivalents of *m*-chloroperoxybenzoic acid in 75 ml ether dropwise to the stirred oxazolidine solution. The reaction is cooled in an ice bath during the peracid addition. The oxazolidine solution becomes yellow immediately upon addition of the peracid but was allowed to return to room temperature and stir for an additional 40 to 72 hr. (This appears to increase the nitroxide yield.) After completion of the oxidation period the ether solution was washed four times each with saturated $NaHCO_3$, saturated NaCl, and distilled water. The washes were back-extracted with ether and

the combined oxidation reaction solution and ether back-extractions were dried by stirring overnight with $MgSO_4$ or Na_2SO_4. The solution was filtered and the ether removed under vacuum on a rotary evaporator. Further purification and characterization of the nitroxide products was done as described below.

Doxylcyclohexane (**2**), doxylcyclohexane-d_{10} (**3**), doxylcyclohexane-2,2,6,6-d_4 (**4**), doxyl-4-*tert*-butyl-cyclohexane (**5**), and doxyl-3,3,5,5-tetramethylcyclohexane (**6**) were recrystallized from hexane giving orange to red-orange needles. The uncorrected melting points and chemical analyses are as follows: **2**, mp 59°, calculated for $C_{10}H_{18}NO_2$, theoretical C 65.18, H 9.84, N 7.60, analysis C 64.21, H 9.86, N 7.40; **3**, mp 59°, calculated for $C_{10}D_{10}H_3NO_2$, theoretical C 61.80, N 7.21, analysis C 61.95, N 7.26; **4**, mp 59°, calculated for $C_{10}D_4H_{14}NO_2$, theoretical C 63.79, N 7.44, analysis C 63.48, N 7.27; **5**, mp 115°, calculated for $C_{14}H_{26}NO_2$, theoretical C 69.95, H 10.90, N 5.83, analysis C 70.01, H 11.01, N 5.56; **6**, mp 74°, calculated for $C_{14}H_{26}NO_2$, theoretical C 69.96 H 10.90, N 5.83, analysis C 70.01, H 11.00, N 5.60.

The stereoisomers 1*e*-doxyl-*trans*-decalin (**7**) and 1*a*-doxyl-*trans*-decalin (**8**) were separated on a silica gel column eluted with 2.5% diethyl ether-hexane (v/v) and crystallized from that solvent giving orange plates. The uncorrected melting points and chemical analyses are as follows: **7**, mp 73°, calculated for $C_{14}H_{24}NO_2$, theoretical C 70.54, H 10.15, N 5.88, analysis C 70.82, H 10.32, N 5.94; and **8**, mp 75°, calculated for $C_{14}H_{24}NO_2$, theoretical C 70.54, H 10.15, N 5.88, analysis C 70.89, H 10.32, N 5.77.

The spin labels 6*e*-doxyl-5α-cholestan-3β-ol (**9**) and 6*a*-doxyl-5α-cholestan-3β-ol (**10**) were synthesized in low yield (<10%). Partial separation of these two was accomplished by successive recrystallizations of **9** from ethanol with **10** becoming enriched in the mother liquor. Computer subtraction of the ESR solution spectrum of **9** from the spectrum of the mother liquor containing **9** and **10** affords the solution spectrum of **10**. The product **9**, calculated for $C_{31}H_{54}NO_3$ (MW 488.78), was characterized by mass spectrometry (M^+ 489) and **10** was not characterized further.

The steroid 3*e*-doxyl-5α-androstan-17β-ol (**11**) was recrystallized from methanol giving large, yellow, rhombohedral crystals with an uncorrected melting point of 171°. Chemical analysis calculated for $C_{23}H_{36}NO_3$ gave C 73.16, H 10.21, N 3.39 (theoretical C 73.36, H 10.17, N 3.72). The deuterated steroid, 3*e*-doxyl-5α-cholestane-2,2,4,4-d_4 (**12**), was recrystallized from absolute ethanol giving small, pale yellow plates, mp 158°. Chemical analysis calculated for $C_{31}D_4H_{50}NO_2$ gave C 78.36 and N 2.51 (theoretical C 78.09, N 2.94).

Partial separation of the stereoisomers 3*e*-doxyl-5α-cholestane (**1**) and 3*a*-doxyl-5α-cholestane (**13**) was accomplished by column chromatography. The product of the oxazolidine oxidation was recrystallized three times from absolute ethanol yielding **1**. The mother liquor was then applied to a 15 × 320 mm silica gel column and eluted for 28 days with hexanes (boiling range 30–60°) at a flow rate of approximately 2 liters/day. Fractions were collected and checked by ESR. **13** comes off the column first as a mixture with **1** and is followed by pure **1**. Computer subtraction of the ESR solution spectrum of **1** from the fractions containing **1** and **13** affords the spectrum of **13**. Computer integration of the spectra allows the estimation that <1% of the spin label product is **13**. The pale yellow crystals of **1** from ethanol (mp 160°) calculated for $C_{31}H_{54}NO_2$ gave the following analytical results: theoretical C 78.75, H 11.51, N 2.96, analysis C 78.25, H 11.57, N 2.75.

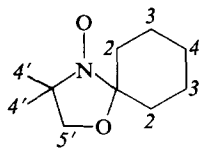
All ESR spectra were recorded at 9.5 GHz on a Varian E-line spectrometer. The samples were prepared by dissolving the nitroxide in $CDCl_3$ and diluting to 5×10^{-5} *M* with $CDCl_3$. The samples were placed in 3 mm quartz ESR tubes and deoxygenated by bubbling nitrogen through the sample. The spectra were taken with scan ranges of 40 G (includes all three ^{14}N lines) and 10 G (includes only the center ^{14}N line). Typical spectrometer settings were: modulation amplitude, 2.0×10^{-2} G; filter time constant, 1.0 sec; microwave power, 5 mW; and scan time, 15 min. All spectra were digitalized by a Varian 620/L-100 dedicated computer for later replotting. Before and after each day's run a sample of 5×10^{-4} *M* di-*tert*-butyl-nitroxide in pH 7.0 phosphate buffer was recorded to calibrate the spectrometer scan range. This nitroxide has a ^{14}N coupling constant of 17.16 ± 0.01 G (*17*).

Spectral simulations were performed with a Varian 620/L-100 computer. The simulation program (*18*) uses a first-order Hamiltonian (secular terms only) to generate first a stick spectrum and then a simulation of actual lineshapes. Gaussian or Lorentzian lineshapes, linewidths, and coupling constants were specified as input parameters and best fits to experimental spectra were determined by visual inspection.

RESULTS AND DISCUSSION

Computer Simulation of the ESR Solution Spectrum of 3e-Doxyl-5α-Cholestane

To characterize the axial isomer, which has poorly resolved proton hyperfine splittings, it is convenient to start with a discussion of the well-resolved equatorial isomer. The ESR solution spectrum of 3*e*-doxyl-5α-cholestane (**1** in Fig. 2) results from the interaction of one ^{14}N nuclear spin and several protons with the unpaired electron of the N–O group (*14*, *19*). The protons nearest the unpaired electron in the cholestane nitroxide spin label are those of the oxazolidine ring and the adjacent steroid A ring. Reference to these protons and all others in this paper is simplified by the diagram below.



Assuming the oxazolidine ring to be essentially planar (*20*) and the steroid A ring to be in a chair conformation, the cholestane spin label has a pair each of equivalent axial and equatorial protons at the *2* position and a pair of equivalent axial protons and a single equatorial proton at the *3* position in the A ring. This molecule has no protons at the *4* position. In addition, there are six equivalent 4′-methyl protons and a pair of equivalent protons at the *5′* position in the oxazolidine ring. The ESR solution spectrum of 3*e*-doxyl-5α-cholestane can be simulated by two pairs of protons with coupling constants of 0.65 and 0.72 G and a single proton with a splitting of 1.06 G. The ^{14}N coupling constant is 14.79 G. There are additional small, unresolved proton coupling constants which serve only to increase the apparent linewidth.

Assignment of All the Large Proton Coupling Constants to the 2 and 3 Positions

The simplest structural analog of the cholestane nitroxide spin label is doxylcyclohexane (**2** in Fig. 2). This molecule has six *4′* methyl protons, two *5′* protons, two pairs

each of equatorial and axial protons at the *2* and *3* positions, and one each equatorial and axial proton at the *4* position. The cyclohexane ring is assumed to be in the chair conformation with the N–O group occupying an equatorial position (*21*, *22*). Computer

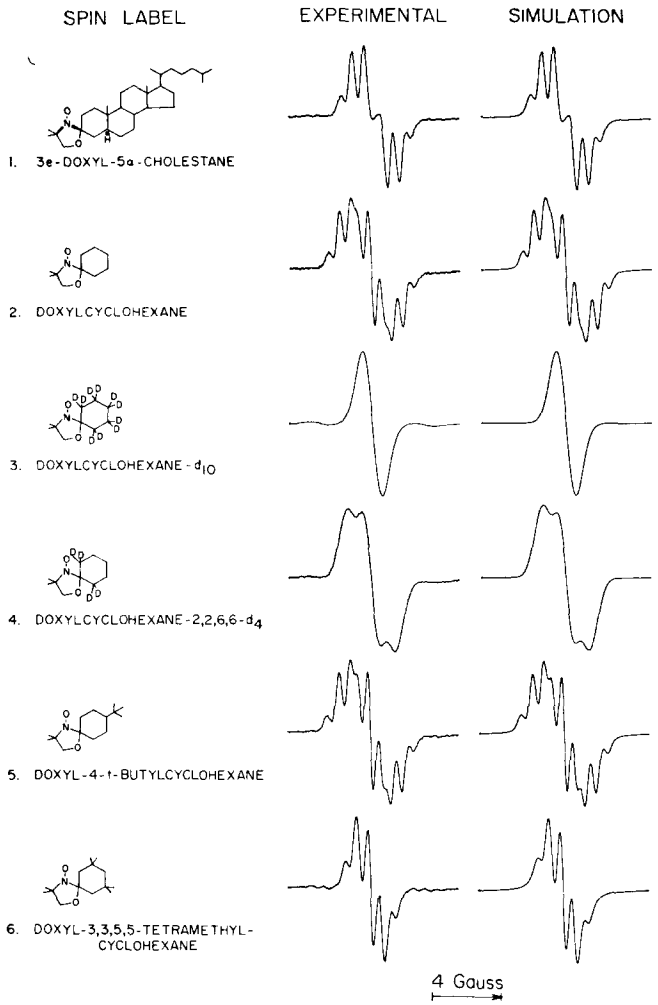


FIG. 2. The room temperature ESR solution spectra of 3*e*-doxyl-5α-cholestane and five cyclohexane related spin labels in deoxygenated $CDCl_3$. Only the center line patterns and their computer simulations are given. The complete spectra consist of three of these patterns, separated by A_N.

simulation of the experimental ESR solution spectrum in Fig. 2 indicates that the hyperfine pattern results from three pairs of protons with coupling constants of 0.63, 0.70, and 1.06 G. There are again additional small, unresolved proton coupling constants which serve only to increase the apparent linewidth. The large splittings of doxylcyclohexane are nearly identical to those of 3*e*-doxyl-5α-cholestane and certainly support the use of this simple molecular analog.

The assignment of a position for the proton pairs causing the three major splittings of doxylcyclohexane was begun by determining whether any of the splittings are associated with the oxazolidine ring. Doxylcyclohexane-d_{10} (**3** in Fig. 2) was synthesized and its solution spectrum shows the absence of any large proton coupling constants. Computer simulation of this experimental spectrum was accomplished by replacing the three proton pairs of doxylcyclohexane with deuteron pairs having coupling constants of 0.10, 0.11, and 0.16 G. These values were obtained by multiplying the proton coupling constants of doxylcyclohexane (0.63, 0.70, and 1.06 G) by the conversion factor $\gamma_D/\gamma_H = 0.1531$, where γ_D and γ_H are the magnetogyric rations of deuterium and hydrogen, respectively (*23*). There is good agreement between the experimental and simulated line shape of **3**, as seen in Fig. 2. The three major proton coupling constants clearly belong to protons on the cyclohexane ring.

Determining which protons of the cyclohexane ring are responsible for the major splittings of doxylcyclohexane is accomplished by first deuterating the axial and equatorial pairs of protons at the *2* position. The ESR solution spectrum of this molecule, doxylcyclohexane-2,2,6,6-d_4 (**4** in Fig. 2), broadened noticeably but retained the largest coupling constant of the undeuterated analog, **2**. The experimental spectrum was simulated by using two pairs of deuterons with coupling constants of 0.09 and 0.10 G and a pair of protons with a coupling constant of 1.01 G. The deuteron coupling constants were first estimated by multiplying the doxylcyclohexane splittings (0.63 and 0.70 G) by the γ_D/γ_H factor and then varied slightly to obtain the best fit. The two smaller coupling constants of doxylcyclohexane, 0.63 and 0.70 G, can thus be attributed to the equatorial and axial proton pairs at the *2* position while the large coupling constant of 1.06 G must be associated with the *3* or *4* positions.

The possibility that the two equivalent protons with the 1.06 G splitting are the axial and equatorial protons at the *4* position is examined by synthesizing doxyl-4-*tert*-butylcyclohexane (**5** in Fig. 2). The experimental solution spectrum of this molecule is nearly identical to that of doxylcyclohexane and can be simulated by three pairs of protons with coupling constants of 0.64, 0.69, and 1.06 G. The fact that there is still a *pair* of protons with a splitting of 1.06 G means that the large 1.06 G splitting in doxylcyclohexane must be at the *3* position. Confirmation that the largest coupling constant (1.06 G) arises from the *3* position is achieved with doxyl-3,3,5,5-tetramethyl-cyclohexane (**6** in Fig. 2) in which the four protons at the *3* position are replaced with methyl groups. The ESR solution spectrum of this molecule is simplified by the loss of the 1.06 G splitting and can be simulated by two pairs of protons with splittings of 0.65 and 0.73 G. The assignment of the largest coupling constant to the *3* position is now certain.

The three major proton coupling constants of doxylcyclohexane are clearly identified as resulting from the protons at the *2* and *3* positions of the cyclohexane ring. The splittings we report here for molecules **2**, **4**, and **5** are in agreement with those given by Michon and Rassat (*22*), who used the NMR Knight shift technique to estimate ESR coupling constants of these three molecules. The simulation of 3*e*-doxyl-5α-cholestane has two pairs of protons with coupling constants of 0.65 and 0.72 G. On the basis of the doxylcyclohexane data these coupling constants are assigned to the *2* position. The single steroid proton splitting of 1.06 G can be unambiguously assigned to the equatorial proton at the *3* position. This is possible because the steroid spin label simulation requires only a single proton with a large coupling constant and there are two axial pro-

tons but only a single equatorial proton at the *3* position. The relatively large value of 1.06 G is evidently due to spin delocalization (*24*).

Assignment of the Equatorial and Axial Coupling Constants at the 2 Position

A simple nitroxide analog to the steroid spin label which has only one equatorial proton at the *2* position is 1*e*-doxyl-*trans*-decalin (**7** in Fig. 3). The synthesis of this molecule is complicated by the fact that two doxyl isomers are synthesized from the starting ketone, *trans*-1-decalone. These two isomers, like the two cholestane nitroxide isomers (Fig. 1), differ only in the stereochemistry of attachment of the oxazolidine

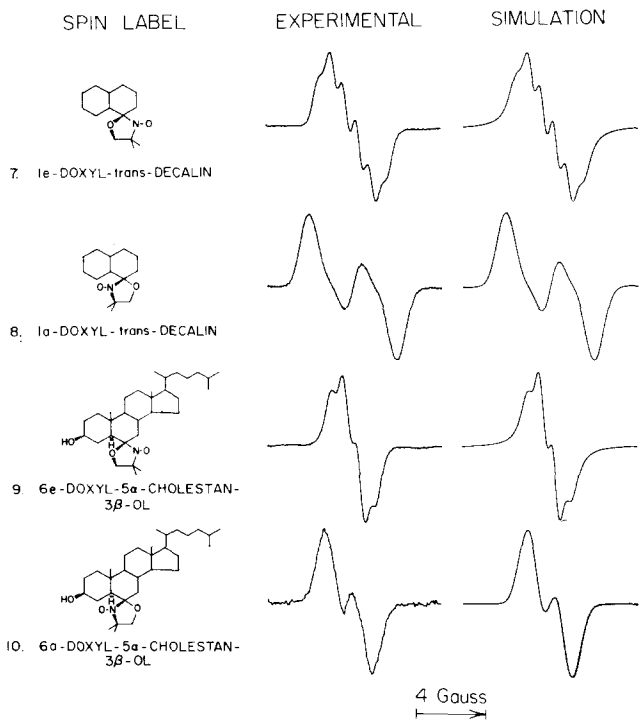


FIG. 3. The center line patterns of the ESR solution spectra of the axial and equatorial isomers of 1-doxyl-*trans*-decalin and 6-doxyl-5α-cholestan-3β-ol. These ESR solution spectra were taken at room temperature in deoxygenated $CDCl_3$.

ring to the decalin ring system at C_1. In 1*e*-doxyl-*trans*-decalin (**7**) the C_1–N bond is equatorial to the decalin ring system and is similar to 3*e*-doxyl-5α-cholestane (**1**). The other isomer, 1*a*-doxyl-*trans*-decalin (**8** in Fig. 3), has the C_1–N bond in an axial configuration. Each isomer has one equatorial and two axial protons at the *2* position and one equatorial proton at the *3* position. It was possible in this case to completely separate and purify both isomers. The ESR solution spectra of the two isomers are quite different and each experimental spectrum is assigned to a specific isomer by comparing computer simulations with experimental spectra.

The solution spectrum of one isomer is simulated by a single proton with a 0.63 G coupling constant, a pair of protons with a coupling constant of 0.69 G, and a single proton with a 1.45 G coupling constant. These coupling constants are in general agreement with those of 3*e*-doxyl-5α-cholestane (0.65, 0.72, and 1.06 G), although the 1.45 G coupling constant is comparatively large. We therefore associate these coupling constants with 1*e*-doxyl-*trans*-decalin (**7**), assigning the 0.63 G (1H) coupling constant to the 2*e* position, the 0.69 G (2H) coupling constant to the 2*a* position, and the 1.45 G (1H) coupling constant to the 3*e* position. Simulation of the experimental spectrum of the other isomer, now assigned as **8**, requires a single proton coupling constant of 1.20 G, a pair of protons with a 0.50 G coupling constant, and a single proton coupling constant of 2.65 G. Clearly, these coupling constants are very different from those of 3*e*-doxyl-5α-cholestane and cannot logically be assigned to 1*e*-doxyl-*trans*-decalin.

Experimental evidence supporting these assignments is obtained from the solvent dependence of the ^{14}N coupling constant (A_N) in protic and aprotic solvents. A_N of di-*tert*-butyl-nitoxide in solvents of comparable dielectric constant (ε) is dependent on the ability of the solvent molecules to form hydrogen bonds with the nitroxide (*24*). We examined the ^{14}N coupling constants of 1*e*-doxyl-*trans*-decalin (**7**) and 1*a*-doxyl-*trans*-decalin (**8**) in 2-methyl-2-butanol ($\varepsilon_{25°} = 5.8$) and ethyl acetate ($\varepsilon_{25°} = 6.0$) (*25*). It is predicted that the change in A_N, $\Delta A_N = A_N$ (2-methyl-2-butanol) $- A_N$ (ethyl acetate), will be greater for the spin label with the N–O group more accessible for hydrogen bonding. Thus, ΔA_N of the equatorial isomer **7** is expected to be greater than ΔA_N of the axial isomer **8**. The experimental results are summarized in Table 1 along with those

TABLE 1

SOLVENT DEPENDENCE OF ^{14}N COUPLING CONSTANTS

Spin label	A_N[a]		ΔA_N[b]
	2-Methyl-2-butanol	Ethyl acetate	
7. 1*e*-doxyl-*trans*-decalin	14.67	14.25	0.42
8. 1*a*-doxyl-*trans*-decalin	14.48	14.21	0.27
1. 3*e*-doxyl-5α-cholestane	14.76	14.37	0.39

[a] All ^{14}N coupling constants (A_N) are ±0.05 G.
[b] $\Delta A_N = A_N$ (2-methyl-2-butanol) $- A_N$ (ethyl acetate).

of 3*e*-doxyl-5α-cholestane (**1**). (It was not possible to obtain accurate data on 3*a*-doxyl-5α-cholestane (**13**).) Table 1 shows that ΔA_N of the equatorial isomer 1*e*-doxyl-*trans*-decalin (**7**) is larger than ΔA_N of the axial isomer 1*a*-doxyl-*trans*-decalin (**8**), as expected. Additionally, ΔA_N of **7** is nearly the same as ΔA_N of the equatorial isomer 3*e*-doxyl-5α-cholestane (**1**). These facts support assigning proton coupling constants of 0.63 G (1H), 0.69 G (2H), and 1.45 G (1H) to 1*e*-doxyl-*trans*-decalin (**7**) and coupling constants of 1.20 G (1H), 0.50 G (2H), and 2.65 G (1H) to 1*a*-doxyl-*trans*-decalin (**8**).

These assignments are further supported by an analysis of the solution spectra of 6*e*-doxyl-5α-cholestan-3β-ol (**9** in Fig. 3) and 6*a*-doxyl-5α-cholestan-3β-ol (**10** in Fig.

3). These two isomers differ only in the stereochemistry of attachment of the oxazolidine ring to the steroid ring system. Both isomers have one equatorial proton and two axial protons at the *2* position and neither has an equatorial proton at the *3* position. The ESR solution spectrum of one isomer (**9**) was obtained by redissolving the recrystallized reaction product. The spectrum of the second isomer (**10**) was obtained by computer subtraction of the spectrum of **9** from the spectrum of the mother liquor containing both isomers. The two ESR spectra exhibit different proton hyperfine patterns (Fig. 3.) One spectrum (**9**) can be simulated using a single proton with a 0.67 G coupling constant and a pair of protons with a coupling constant of 0.72 G. The other spectrum, **10** in Fig. 3, is derived from a single proton coupling constant of 1.40 G and a proton pair with a 0.50 G coupling constant. A comparison of these coupling constants with those of 1*e*-doxyl-*trans*-decalin (0.63 and 0.69 G) leads to the assignment of the 0.67 and 0.72 G coupling constants to the *2e* and *2a* positions of 6*e*-doxyl-5α-cholestan-3β-ol, respectively. The 1.40 and 0.50 G splittings are assigned to 6*a*-doxyl-5α-cholestan-3β-ol. These proton coupling constants for the *2* position are consistent with those arrived at for 1*a*-doxyl-*trans*-decalin.

It has been shown that the coupling constant of the equatorial proton at the *2* position is 0.63 G in **7** and 0.67 G in **9**. The coupling constant of the axial pair of protons at the *2* position is 0.69 G in **7** and 0.72 G in **9**. Therefore, the 0.65 G coupling constant in 3*e*-doxyl-5α-cholestane is assigned to the equatorial pair of protons at the *2* position. The 0.72 G coupling constant is assigned to the axial pair of protons at the *2* position in this molecule.

Supportive Observations on Steroid Analogs of 3e-Doxyl-5α-Cholestane

Another useful steroid spin label is 3*e*-doxyl-5α-androstan-17β-ol (*14*) (**11** in Fig. 4). This molecule is identical to **1** in the region of the doxyl group and the ESR solution spectra of these two are nearly identical. Simulation of the experimental spectrum of **11** requires a proton pair with a coupling constant of 0.65 G, a proton pair with a coupling constant of 0.72 G, and a single proton coupling constant of 1.06 G. The coupling constants are identical to those of 3*e*-doxyl-5α-cholestane.

Another molecule of interest is 3*e*-doxyl-5α-cholestane-2,2,4,4-d_4 (**12** in Fig. 4). This spin label has been deuterated at the *2* position. The ESR solution spectrum of this molecule is broad and can be simulated by two pairs of deuteron coupling constants of 0.10 and 0.11 G and a single proton coupling constant of 1.06 G. The 0.10 and 0.11 G coupling constants were obtained by multiplying the 0.65 and 0.72 G coupling constants of **1** by the factor γ_D/γ_H. The simulation of the solution spectrum of **12** with these coupling constants supports the assignment of the 0.65 and 0.72 G coupling constants of **1** to the *2* position and the 1.06 G coupling to the equatorial proton at the *3* position.

The ESR Solution Spectrum of 3a-Doxyl-5α-Cholestane

With the above set of ESR spectra on closely related spin labels **1–12**, it is straightforward to discuss the more troublesome axial isomer. The partial separation of 3*a*-doxyl-5α-cholestane (**13**) from **1** was accomplished by column chromatography. The ESR solution spectrum of **13** is different from that of **1** and was obtained by subtracting the spectrum of **1** from that of the column effluent containing **1** and **13**. Simulation of the solution spectrum of **13** is difficult because the proton hyperfine pattern is

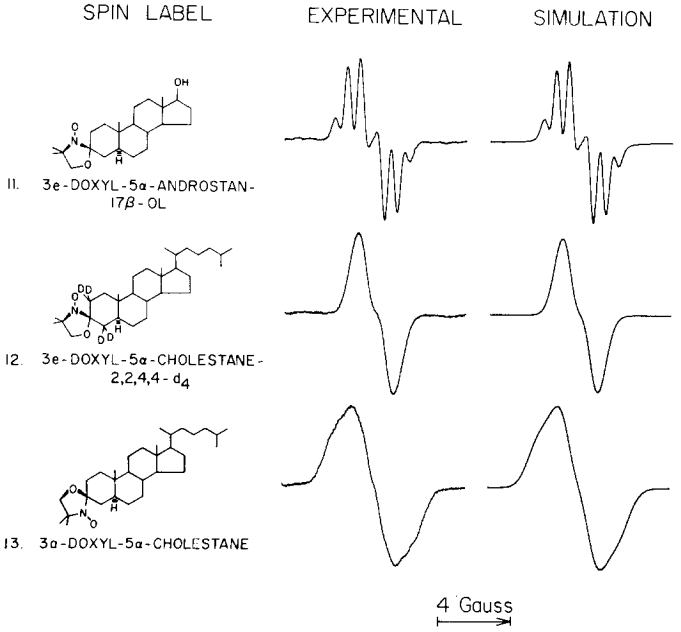
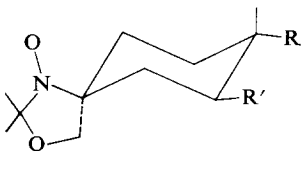


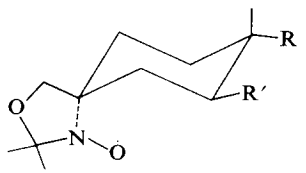
FIG. 4. The room-temperature ESR solution spectra of three steroid spin labels in deoxygenated $CDCl_3$. As in Figs. 2 and 3, only the center line pattern and its computer simulation are given.

almost unresolved (Fig. 4). Approximate assignments of 1.05 G for the equatorial proton pair at the *2* position, 0.45 G for the axial proton pair at the *2* position, and 1.40 G for the single equatorial proton at the *3* position, can, however, be made. These coupling constants are consistent with those of **8** and **10**, although the 1.40 G splitting is low. These coupling constants and assignments are summarized in Table 2. The coupling constants of all the equatorial and axial isomers are internally consistent.

It is of interest to compare these results with the work of Michon and Rassat (*13*) on the 3-doxyl-5α-cholestane analogs where R and R′



14e



14a

signify the remainder of the cholestane molecule. The only difference from the structures of Fig. 1 is that the methylene group and oxygen of the oxazolidine ring have been permuted. Structure **14a** is the dominant free radical whereas only small quantities of **14e** are present, which is exactly the reverse situation for the equatorial and axial

TABLE 2

SUMMARY OF PROTON HYPERFINE COUPLING CONSTANTS, COMPUTER SIMULATION PARAMETERS, AND ^{14}N COUPLING CONSTANTS

Spin label	Proton coupling constants[a]			Linewidth[b]	A_N[c]
	2*e*	2*a*	3*e*		
1. 3*e*-doxyl-5α-cholestane	0.65 (2H)	0.72 (2H)	1.06 (1H)	0.20	14.84
2. doxylcyclohexane	0.63 (2H)	0.70 (2H)	1.06 (2H)	0.14	14.84
3. doxylcyclohexane-d_{10}	0.10 (2D)	0.11 (2D)	0.16 (2D)	1.00	14.94
4. doxylcyclohexane-2,2,6,6,-d_4	0.09 (2D)	0.10 (2D)	1.01 (2H)	0.95	14.80
5. doxyl-4-*t*-butylcyclohexane	0.64 (2H)	0.69 (2H)	1.06 (2H)	0.17	14.82
6. doxyl-3,3,5,5-tetramethylcyclohexane	0.65 (2H)	0.73 (2H)	—	0.34	14.96
7. 1*e*-doxyl-*trans*-decalin	0.63 (1H)	0.69 (2H)	1.45 (1H)	0.50	14.72
8. 1*a*-doxyl-*trans*-decalin	1.20 (1H)	0.50 (2H)	2.65 (1H)	0.95	14.53
9. 6*e*-doxyl-5α-cholestan-3β-ol	0.67 (1H)	0.72 (2H)	—	0.50	14.67
10. 6*a*-doxyl-5α-cholestan-3β-ol	1.40 (1H)	0.50 (2H)	—	0.90	15.13[d]
11. 3*e*-doxyl-5α-androstan-17β-ol	0.65 (2H)	0.72 (2H)	1.06 (1H)	0.15	15.09
12. 3*e*-doxyl-5α-cholestane-2,2,4,4-d_4	0.10 (2D)	0.11 (2D)	1.06 (1H)	1.10	14.81
13. 3*a*-doxyl-5α-cholestane	1.05 (2H)	0.45 (2H)	1.40 (1H)	1.10	15.03[d]

[a] The equatorial and axial proton positions are designated *e* and *a*, respectively, and all proton coupling constants are ±0.04 G.

[b] Molecules **3**, **4**, **8**, **12**, and **13** were simulated using a Gaussian lineshape with these linewidths. All others were Lorentzian lineshapes. All linewidths are ±0.01 G.

[c] All ^{14}N coupling constants (A_N) are ±0.05 G unless otherwise noted.

[d] ±0.10 G.

isomers of 3-doxyl-5α-cholestane. Spin label **14e** has a hyperfine pattern very similar to 3*e*-doxyl-5α-cholestane, whereas **14a** is a broad single-line spectrum, similar to **13** (Fig. 4) except that no inflection point is evident. Thus, although the radicals are present in very different proportions, it is clear that all of these observations and assignments are in agreement.

The main interest in these spin labels is the accurate interpretation of lineshapes when dissolved in membrane model systems and biological membranes. The lineshapes are dependent on the underlying proton hyperfine pattern so it is necessary to know the ratios of the isomers present. Using computer addition and subtraction techniques we

find that as little as 20% contamination of 3*a*-doxyl-5α-cholestane visibly distorts the solution spectrum of 3*e*-doxyl-5α-cholestane. Computer subtraction is sensitive enough to detect easily a 5% contamination of the axial isomer. We find no evidence of the axial isomer in the solution spectrum of 3-doxyl-5α-cholestane prepared from the crystalline product according to the synthesis of Keana *et al.* (*16*), and we conclude that it does not interfere with the lineshape analysis of 3*e*-doxyl-5α-cholestane in membrane spin labeling studies.

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