Synthesis and properties of some spin labeled long chain aliphatic acids

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We have synthesized and characterized a series of spin labeled aliphatic acids with variable chain lengths between the nitroxide radicals and the carboxylic group. These molecules should be of general utility in labeling a variety of substrates which can react with the free carboxylic acid group. Variable temperature esr studies have been conducted with some of these molecules to characterize molecular motion as a function of solvent viscosity.

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On synthétisé et caractérisé une série d'acides aliphatiques portant un marqueur de spin et l'on a fait varié la longueur de la chaîne entre les radicaux nitroxydes et groupement carboxylique. Les molécules devraient être d'une utilité générale pour marquer un grand nombre de substrats qui peuvent réagir avec le groupement acide carboxylique libre. On a effectué des études de rpe à température variable à l'aide de ces molécules afin de caractériser leur mouvement moléculaire en fonction de la viscosité du solvant.

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Introduction

The electron spin resonance (esr) spectra of spin labeled molecules have been used to investigate motional correlation times of a variety of biologically important compounds (1). Nitroxide radicals are generally the label of choice because extensive theoretical work has been conducted to relate experimental spectra to motional correlation times (2). The motional correlation times may depend on steric interactions between the labeled reporter group and a biological substrate. If the nitroxide group can be attached to a drug molecule or enzyme with a variable length connecting chain, one is able to monitor changes in molecular correlation times as a function of the length of the spacer group between the drug or enzyme and the nitroxide label (3). Through analysis of changes in molecular motion as a function of the spacer group's length one can obtain information about the structure of a binding site. If the spacer group is short compared to the depth of the binding site, the nitroxide label's motion will be inhibited by steric interactions with the substrate while longer spacers may allow the nitroxide to extend out of the binding site resulting in greater mobility of the labeled group. If one determines the motional correlation time as a function of the length of the spacer group one obtains a measure of the depth of the binding site.

We have synthesized a group of spin labels in which a nitroxide label is bound to a carboxylic acid functional group via a variable length aliphatic chain (Fig. 1). The carboxylic acid groups of these molecules can be used to form either ester or amide linkages with a variety of different substrates. We have used these molecules to spin label both morphine and codeine through formation of ester linkages at the 3 and 6 positions. The labeled acids should prove to be of general utility in the synthesis of a variety of other types of labeled drugs or enzymes. The labeled molecules were synthesized by three related techniques. In method A (Fig. 1) the half ester, half acid chloride of a dicarboxylic acid was allowed to react with the amino group of 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxy to form a molecule with both an amide and ester group. The ester was subsequently hydrolysed to form the desired product. In method B the acid function of the half ester of a dicarboxylic acid was condensed with the amino group of the nitroxide radical through use of *N*,*N'*-dicyclohexylcarbodiimide (DCC). Hydrolysis of the ester group again led to the formation of the desired labeled acid. In method C a dicarboxylic acid was directly condensed with the amino group of the nitroxide radical using DCC as a coupling reagent. The spin labeled acids were characterized by their ir spectra, their nmr spectra (after reduction of the nitroxide group), and by their esr spectra. A variable temperature esr study was conducted with two of the labeled acids to determine changes in motional correlation times as a function of solvent viscosity and the chain length of the spacer group.

Methods and experimental

The spin labeled acids were synthesized by three different techniques. The choice of technique for a given molecule was dictated by availability of starting materials and the overall yield of the product. The three reaction schemes are illustrated in Fig. 1. The half esters of dibasic acids with n = 2–4, 7 were commercially available, while only the diacids were available

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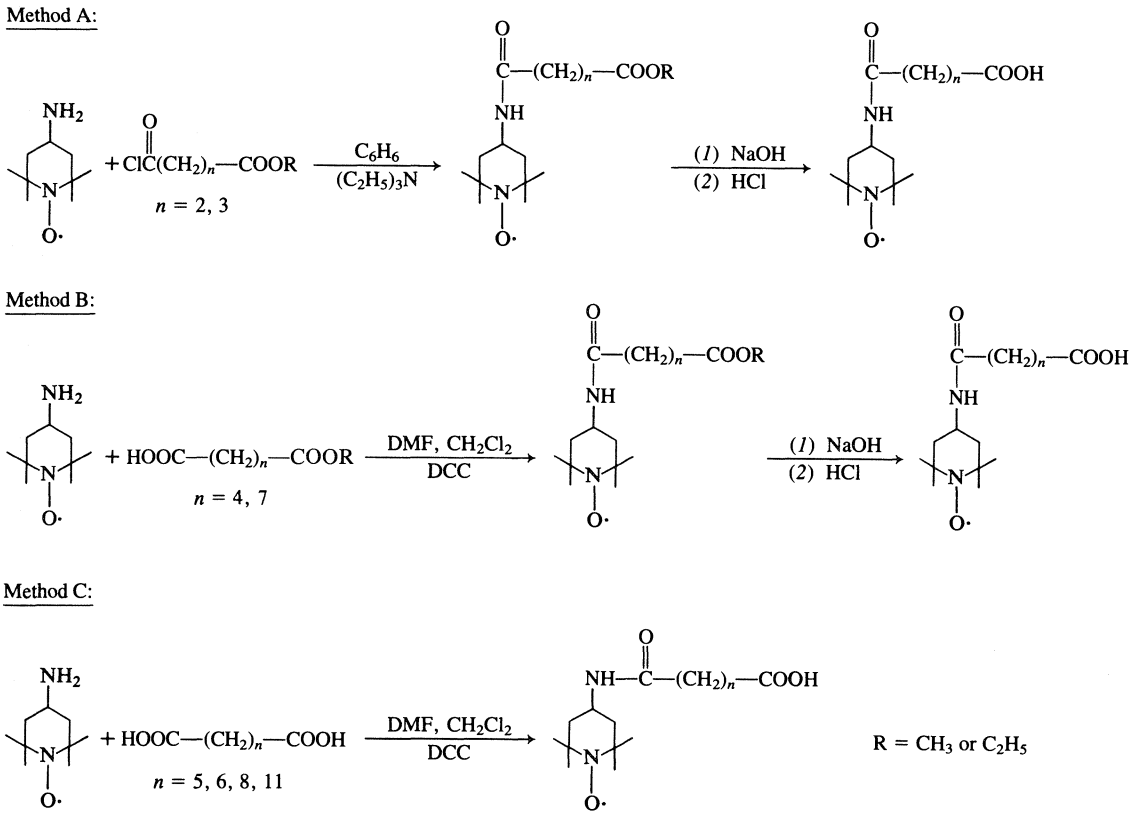

FIG. 1. Reaction schemes for synthesis of the spin labeled acids.

for the other chain lengths. The diacids can react with two molecules of the nitroxide label to yield a biradical and the yield of the desired product was lower than that from molecules in which one of the carboxylic acid groups was blocked with an ester linkage. Synthesis of the half esters of these molecules could have been carried out but the overall yield after this additional reaction would have been comparable to the yield obtained when the diacid was used directly.

All of the diacids and half acid esters were obtained from Aldrich Chemical Co. and used without further purification. The 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxy was synthesized by the technique of Rozantsev (4). Illustrations of the techniques used in the various reaction sequences are given below.

Method A: reaction of half ester, half acid chlorides (n = *2, 3*) *with the nitroxide radical*

A solution of 0.23 g (1.4 mmol) of the nitroxide radical in 1 mL of dry benzene was slowly added to a stirred solution of 0.22 g (1.4 mmol) of ethylsuccinyl chloride in 1 mL of dry benzene and 0.5 mL of triethylamine (TEA) held at 0°C. The reaction mixture was allowed to come to room temperature and stirred for an additional 12 h. Thin-layer chromatography (silica gel, CH_2Cl_2 or EtOAc) indicated completion of the reaction. Two milliliters of benzene were added to the reaction mixture and the TEA·HCl was removed by filtration. The TEA·HCl was washed with two 5-mL aliquots of ether and these washings were combined with the original filtrate. The filtrate was evaporated to dryness under vacuum to yield 0.4 g (95%) of a red oil. This oil (spin labeled ethylsuccinate) was saponified without further purification. Attempts at washing the oil with water resulted in lower yields. The spin labeled ethylsuccinate (0.33 g, 1.09 mmol) was dissolved in 5 mL of 95% ethanol and a solution containing 0.80 g (20 mmol) of NaOH in a minimum amount of 50% ethanol was added. This solution was refluxed for 18 h and then the solvent was removed by vacuum evaporation. The resulting solid was dissolved in 20 mL of 2 *N* NaOH and this solution was washed with two 5-mL aliquots of chloroform. The solution was cooled to 0°C and the pH was adjusted to 2 by addition of 1 *N* HCl. The acidified solution was extracted with 20-mL aliquots of chloroform and two 20-mL aliquots of EtOAc. The combined extracts were dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure to yield an orange solid. Recrystallization of this material from EtOAc and petroleum ether gave 0.24 g (80%) of yellow-orange spin labeled acid (mp 154–157°C). The overall yield for the two reactions to produce the desired product was 76%. In cases in which the half methyl ester of a dibasic acid was used as an initial reactant, methanol was used in place of ethanol in the saponification.

Method B: reaction of half ester, half acids (n = *4, 7*) *with the nitroxide radical*

A dicyclohexyl carbodiimide (DCC) solution containing 5.19 g (25 mmol) in 10 mL of CH_2Cl_2 was added to a stirred solution of 3.56 g (22.5 mmol) of mono-methyladipate in 5 mL of CH_2Cl_2 at 0°C. A white solid formed in about 15 min. A solution of 3.8 g (22 mmol) of the nitroxide radical in 10 mL of CH_2Cl_2 was slowly added to this mixture while the temperature was maintained at 0°C. The temperature was allowed to rise to room temperature

and the mixture was stirred for 24 h. The precipitate was filtered, washed with two 10-mL aliquots of CH_2Cl_2, and the filtrate was vacuum evaporated to yield a thick red oil. This oil was dissolved in chloroform and quickly washed with 15 mL of water, 15 mL of 5% $NaHCO_3$, two additional 15-mL aliquots of water, and dried over anhydrous $MgSO_4$. Thin-layer chromatographic examination (silica gel, $CHCl_3$, EtOAc, and CH_3OH) showed that more than one component was present in this solution. The solution was concentrated under reduced pressure and the resulting red oil was separated on a silica gel column using either EtOAc or 10% CH_3OH in CH_2Cl_2 as eluant to obtain 6.25 g (90%) of the spin labeled adipic acid methyl ester as a thick red oil. The ester was saponified using the general technique described under method A.

Method C: reaction of diacids (n = 5, 6, 8, 11) with the nitroxide radical

A solution of DCC (5.64 g, 27 mmol, in a minimum volume of dry DMF) was added to a stirred solution of 6.18 g (38 mmol) of pimelic acid in 10 mL of dry DMF at 0°C. A white crystalline precipitate formed in about 45 min. At this point 4.5 g (26 mmol) of the nitroxide radical in 5 mL CH_2Cl_2 was slowly added at 0°C. The reaction mixture was stirred at room temperature for 48 h and the white precipitate was removed by filtration. The precipitate was washed with three 10-mL aliquots of $CHCl_3$ and the combined filtrate was concentrated under vacuum. The resulting thick red oil was dissolved in 90 mL of $CHCl_3$ and insoluble white crystals were removed by filtration. The solution was extracted with three 60-mL aliquots of 10% NaOH and the NaOH extracts were combined, cooled to 0°C, and the pH was adjusted to 2 by titration with concentrated HCl. The acidified solution was extracted with four 60-mL aliquots of chloroform and two 60-mL aliquots of EtOAc. The extracts were washed separately with three 30-mL aliquots of water, dried over anhydrous $MgSO_4$, combined, and the solvent was removed by vacuum evaporation to yield 3.75 g (46%) of an orange-red solid (mp 105–121°C). Thin-layer chromatographic examination (silica gel, $CHCl_3$, EtOAc, CH_3OH, and 6% CH_3OH in CH_2Cl_2) showed this solid to contain small amounts of impurity. The mixture was separated on a silica gel column with either 6% or 10% CH_3OH in CH_2Cl_2 which yielded 2.05 g (25%) of the product. After evaporation of the eluant from the column one always obtained a thick red oil. Addition of about 5 mL of EtOAc to this oil yielded a crystalline product, mp 114–117°C. Recrystallization from EtOAc and petroleum ether yields pure spin labeled pimelic acid, mp 118–121°C.

Nuclear magnetic resonance spectra

In order to obtain high resolution nmr spectra of the molecules for analytical purposes, it is necessary to reduce the nitroxide radical to a hydroxyl amine. The reduction was carried out in an nmr sample tube in which 30 to 50 mg of a given spin label was dissolved in 1 mL of DMSO-d_6. A solution (15 μL) of 85% hydrazine was added as the reducing agent. The reduction led to formation of gas bubbles (N_2) and loss of color from the solution. The reaction was complete in 3–5 h and the nmr spectra were taken. All of the nmr spectra showed the following general characteristics: a sharp singlet with a shoulder (1.12 to 1.18 δ) from the 12 methyl protons on the piperidine ring, a broad doublet (7.8 to 8.0 δ) from the amide proton, and a group of overlapped lines (1.2 to 2.2 δ) whose area corresponded to the protons in the methylene chain. Addition of 0.25 mL of $CDCl_3$ to the solution revealed an additional peak from the single carboxylic acid proton (8.1–8.15 δ).

Properties of specific labels

Compound ***1***, $n = 2$, prepared by method A; yield 76%; mp 154–157°C; ir (KBr): 3475, 3000, 2960, 1740, 1645, 1555 cm^{-1}; nmr δ: 1.1 (12 H), 7.9 (1 H); *Anal.* calcd. for $C_{13}H_{23}N_2O_4 \cdot 1/2H_2O$: C 55.74, H 8.60, N 9.98; found: C 56.29, H 8.54, N 9.89.

Compound ***2***, $n = 3$, prepared by method A; yield 73%; mp 117–120°C; ir (KBr); 3460, 3000, 1710, 1625, 1575 cm^{-1}; nmr δ: 1.1 (12 H), 7.8 (1 H); *Anal.* calcd. for $C_{14}H_{25}N_2O_4 \cdot 1/2H_2O$: C 57.32, H 8.92, N 9.54; found: C 57.64, H 8.83, N 9.36.

Compound ***3***, $n = 4$, prepared by method B; yield 55%; mp 99–101°C; ir (KBr): 3340, 2970, 2880, 1695, 1610, 1560 cm^{-1}; nmr δ: 1.1 (12 H), 7.7 (1 H); *Anal.* calcd. for $C_{15}H_{27}N_2O_4$: C 60.18, H 9.09, N 9.36; found: C 60.19, H 9.15, N 9.20.

Compound ***4***, $n = 5$, prepared by method C; yield 25%; mp 118–121°C; ir (KBr): 3395, 2960, 2890, 1730, 1640, 1550 cm^{-1}; nmr δ: 1.15 (12 H), 7.4 (1 H); *Anal.* calcd. for $C_{16}H_{29}N_2O_4$: C 61.32, H 9.33, N 8.94; found: C 60.83, H 9.45, N 8.78.

Compound ***5***, $n = 6$, prepared by method C; yield 28%; mp 103–105°C; ir (KBr): 3360, 2950, 2880, 1710, 1620, 1565 cm^{-1}; nmr δ: 1.0 (12 H), 7.7 (1 H); *Anal.* calcd. for $C_{17}H_{31}N_2O_4 \cdot 1/2H_2O$: C 60.69, H 9.58, N 8.32; found: C 60.61, H 9.55, N 8.31.

Compound ***6***, $n = 7$, prepared by method B; yield 42%; mp 98–99°C; ir (KBr): 3380, 3340, 2940, 2860, 1720, 1630, 1560 cm^{-1}; nmr δ: 1.15 (12 H), 7.65 (1 H); *Anal.* calcd. for $C_{18}H_{33}N_2O_4$: C 63.31, H 9.74, N 8.20; found: C 63.41, H 9.88, N 8.16.

Compound ***7***, $n = 8$, prepared by method C, catalytic amounts of 4-dimethylpyridine were used in this reaction; yield 25%; mp 99–101°C; ir (KBr): 3350, 2935, 2860, 1700, 1610, 1565 cm^{-1}; nmr δ: 1.1 (12 H), 7.7 (1 H); *Anal.* calcd. for $C_{19}H_{35}N_2O_4$: C 64.19, H 9.92, N 7.88; found: C 63.74, H 10.14, N 7.67.

Compound ***8***, $n = 11$, prepared by method C, catalytic amounts of 4-dimethylpyridine were used in this reaction; yield 26%; mp 114–116°C; ir (KBr): 3380, 3320, 2935, 2860, 1720, 1630, 1560 cm^{-1}; nmr δ: 1.2 (12 H), 7.5 (1 H); *Anal.* calcd. for $C_{22}H_{41}N_2O_4$: C 66.46, H 10.36, N 7.05; found: C 66.17, H 10.67, N 6.95.

Electron spin resonance spectra

The esr spectra of this group of radicals in solution at room temperature were essentially identical ($A(N) = 16.2$ G; $g = 2.005$ in 50:50 methanol–ethanol). Variable temperature studies of two of the radicals ($n = 2$ and 11) were conducted to obtain information about the rotational motion of the labels as a function of the viscosity of the solvent. Figure 2 shows spectra of these two radicals at a series of temperatures while Fig. 3 shows plots of the ratio of intensities of the high field to center line and the low field to center line as a function of temperature. The signal intensities are inversely proportional to the square of the linewidth and measurement of relative intensities gives a good measure of line broadening produced by incomplete averaging of anisotropic interactions. If the molecules tumble isotropically so that the components of the dipolar and g anisotropy are averaged equally, one expects to observe the greatest broadening in the high field ($m = -1$) line, the second greatest broadening in the low field ($m = 1$) line, and the least broadening in the center line ($m = 0$) (5). The experimental results show the greatest broadening in the high field line, the second greatest broadening in the center line, and the least broadening in the low field line (Fig. 3).

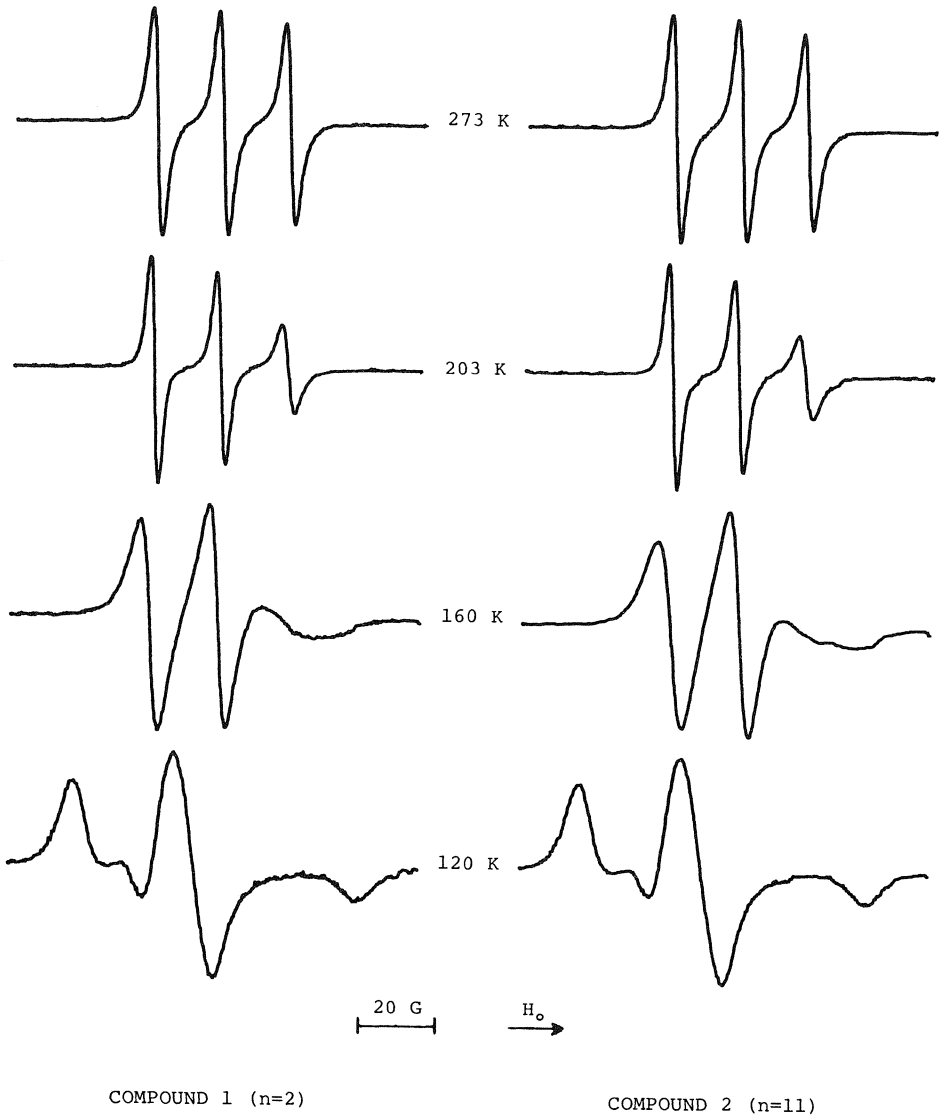

FIG. 2. The esr spectra of compound **1** (n = 2) and compound **8** (n = 11) in 50:50 methanol–ethanol as a function of temperature.

This type of behavior is observed when rotation around the x axis (defined by the N—O bond direction) is preferred over rotation around the other two axes (6) (Fig. 4). The x axis is roughly colinear with the direction (long axis) of the aliphatic connecting chain if this chain extends out into solution and is not wrapped back around the radical. This type of motion is often observed from labels with long chains which are introduced into lipid bilayers where motion around the long axis is much more facile than rotation around the other axes. The motion of the longer chain molecules (n = 11) appears to be slightly slower than that of the short chain acid (n = 2) at a given temperature, which may reflect differences in the steric bulk or configuration of the connecting chain. One cannot obtain values for motional correlation times from the simple formulas used for isotropic rotation and a complete lineshape analysis would be necessary to determine accurate correlation times.

Conclusions

A group of spin labeled long chain aliphatic acids has been synthesized and characterized by their ir and esr spectra, by elemental analysis, and by the nmr spectra of hydroxyl amines formed by reduction of the radicals. The esr results indicate that molecules preferentially rotate around the x mo-

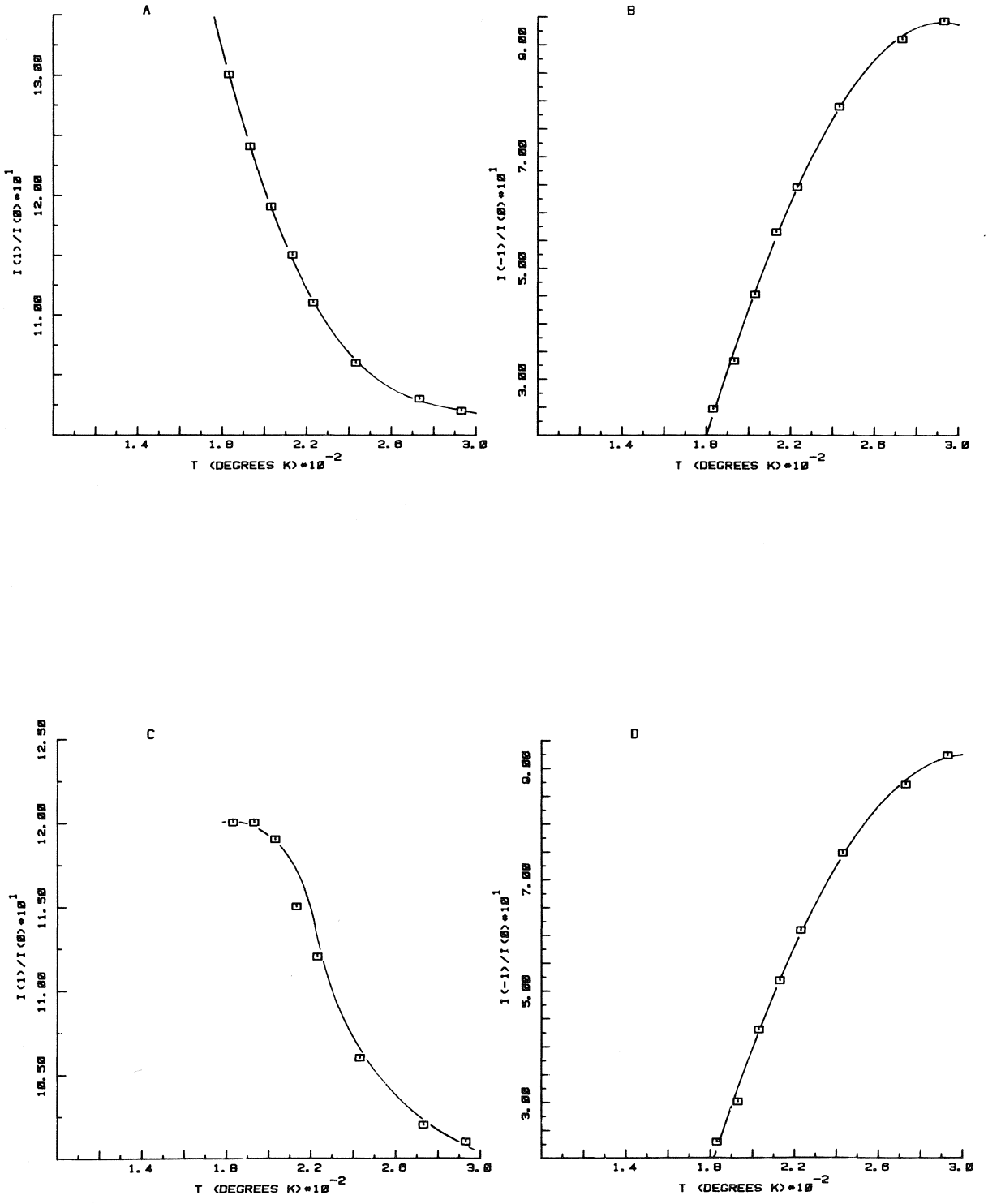

FIG. 3. Plots of the ratio of signal intensities as a function of temperature. A: Compound **1** ($n = 2$) low field line/center line. B: Compound **1** ($n = 2$) high field line/center line. C: Compound **8** ($n = 11$) low field line/center line. D: Compound **8** ($n = 11$) high field line/center line.

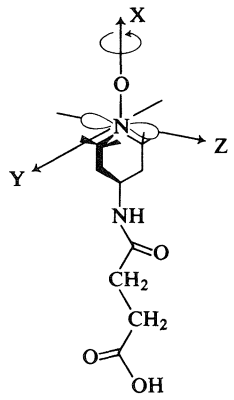

FIG. 4. Schematic representation of axis of preferred rotation for this group of spin labels.

lecular axis. These molecules should be of use as starting material for a variety of spin labeling studies. These spin labels can readily be attached to the head groups of lipids for studies of labeled membranes. The molecules can also be attached to a variety of drug molecules and some enzymes. We have used this group of molecules to produce spin labeled derivatives of both morphine and codeine. The synthesis and properties of these labeled opiates will be reported in a later paper.

Acknowledgement

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1. L. J. BERLINER (*Editor*). Spin labeling, theory and applications. Vols. 1 and 2. Academic Press, New York. 1976 and 1979; A. D. KEITH, M. SHARNOFF, and G. E. COHN. Biochim. Acta, **300**, 379 (1973); B. J. GAFFNEY and C. J. MCNAMEE. Methods Enzymol. **32**B, 161 (1974).
2. J. A. FREED. Ann. Rev. Phys. Chem. **23**, 265 (1972).
3. J. C. HSIA and L. H. PIETTE. Arch. Biochem. Biophys. **129**, 296 (1969); L. J. BERLINER and S. S. WONG. J. Biol. Chem. **249**, 1668 (1974).
4. E. G. ROZANTSEV. Free nitroxyl radicals. Plenum Press, New York. 1970.
5. J. H. FREED. J. Chem. Phys. **41**, 2077 (1964); S. A. GOLDMAN. G. V. BRUNO, and J. H. FREED. J. Phys. Chem. **76**, 1858 (1972).
6. G. B. BIRRELL, S. P. VAN, and O. H. GRIFFITH. J. Am. Chem. Soc. **95**, 2451 (1973); G. B. BIRRELL, O. H. GRIFFITH, and D. FRENCH. J. Am. Chem. Soc. **95**, 8171 (1973).