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Structure and Conformation of Nitroxyl Spin-Label Compounds in Frozen Solutions by Electron Nuclear Double Resonance Spectroscopy

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Abstract. The structure and conformation of carboxylic acid, formyl, and propenoic acid derivatives of the nitroxyl spin-label 2,2,5,5-tetramethyl-1-oxypyrroline have been determined by electron nuclear double resonance (ENDOR) spectroscopy. From ENDOR spectra of the spin-label compounds in frozen solutions, we have assigned the resonance absorption features for each class of protons. The ENDOR spectra were analyzed on the basis of their dependence on $\mathbf{H}_0$. The maximum and minimum ENDOR shifts for each proton were shown to correspond to axially symmetric principal hyperfine coupling (hfc) components, from which the dipolar contributions were estimated to calculate electron-proton separations. Conformational analysis on the basis of torsion angle search calculations constrained by the ENDOR determined electron-proton distances revealed that in all three spin-label compounds the side chains are in a planar conformation with respect to the oxypyrrolinyl ring. In the carboxylic acid and formyl derivatives the C=O group is in a *s-trans* conformation with respect to the vinyl group of the spin-label, while in the spin-labeled propenoic acid the conformation is found to be all planar *trans-s-cis*.

1. Introduction

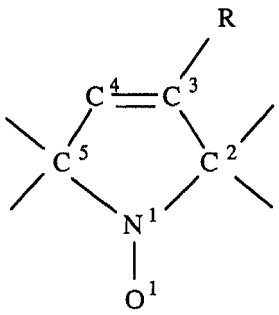
The chemically stable nitroxyl free radical species known as spin-labels are widely used in biophysical studies as spectroscopic probes of macromolecular structure and dynamics [1–3]. Since the unpaired electron of nitroxyl spin-labels is confined almost entirely to the nitroxyl nitrogen and oxygen atoms [4,5], the hyperfine interactions of nearby magnetic nuclei with the unpaired electron are largely dipole-dipole in character. We have shown in the case of nitroxyl spin-labels that the resultant, small hyperfine (hf) couplings can be accurately measured by ENDOR and that ENDOR spectroscopy of nitroxyl spin-labels provides a general method of structure determination of molecules in frozen solution [6–11]. In these studies we have dem-

onstrated that measurement of electron-nucleus distances in the 5–11 Å range can be achieved with an accuracy that is exceeded only by single-crystal X-ray diffraction methods.

In this investigation we report the structures and conformations of the five-membered oxypyrroline spin-label containing conjugated olefinic and carbonyl groups in frozen solution. We show how the conformation of these molecules can be assigned on the basis of torsion angle calculations constrained by the ENDOR determined electron-proton distances. The methods can be extended to larger and structurally more complicated molecules, including enzyme-substrate complexes.

2. Experimental Procedures

Materials. In Fig.1 are illustrated structural formulae of the spin-label compounds I–IV employed in this investigation. Compound I, 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid, was obtained by hydrolysis of the corresponding carboxamide (Aldrich Chemical Co., Inc., Milwaukee, WI 53233) according to the method of Rozantsev [12]. Compounds II–IV were



Compound	Side Chain (R)
I	$-C^{\alpha}OOH$
II	$-C^{\alpha}HO$
III	$-C^{\alpha}H=C^{\beta}H-C^{\gamma}OOH$
IV	$-C^{\alpha}D=C^{\beta}H-C^{\gamma}OOH$

Fig.1. Illustration of the chemical bonding structures and atomic numbering schemes of 2,2,5,5-tetramethyl-1-oxypyrroline-3-carboxylic acid (I), 3-formyl-2,2,5,5-tetramethyl-1-oxypyrroline (II), 3-(2,2,5,5-tetramethyl-1-oxy-pyrrolinyl)-propen-2-oic acid (III), and 3-(2,2,5,5-tetramethyl-1-oxypyrrolinyl)-[3-^{2}H]propen-2-oic acid (IV).

synthesized essentially as outlined earlier [13]. Details of an improved synthesis procedure will be published elsewhere. The deuterated solvents [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO, 99.8 % D), [^{2}H]chloroform (99.8 % D), [$^2H_8$]toluene (99.6 % D), and [$^2H_4$]methanol (99.8 % D) were obtained from Cambridge Isotope Laboratories, Inc. (Woburn, MA 01801).

EPR and ENDOR. ENDOR spectra were recorded with the sample at 20 K with an X-band Bruker ER200D spectrometer equipped with an Oxford Instruments ESR10 liquid helium cryostat and a Bruker digital ENDOR accessory, as previously described [14,15]. ENDOR spectra were recorded in the first-derivative absorption mode with 1.28 mW incident microwave power, 12.5 kHz frequency modulation of the radio frequency (rf) field, and 50 W rf power with ~ 8 kHz modulation depth of the rf field. The static laboratory magnetic field was not modulated for ENDOR. Spin-labeled derivatives were dissolved to a concentration of $5 \cdot 10^{-3}$ M in [$^2H_4$]methanol, or [^{2}H]chloroform : [$^2H_8$]toluene : [$^2H_6$]DMSO (25 : 25 : 50) for spectroscopic studies.

Molecular Modeling. Atomic coordinates of nonhydrogen atoms of **I** were taken from X-ray diffraction studies [16,17]. The side chains in **II**–**IV** were constructed on the basis of atomic coordinates of molecular fragments obtained through X-ray studies of compounds containing similar, conjugated groups [18,19]. Positions of hydrogen atoms were calculated for idealized geometries with C–H bond lengths of 1.08 and 1.045 Å for sp^3 and sp^2 hybridized carbons, respectively [20]. Molecular modeling was carried out with use of the programs FRODO [21,22] and SYBYL [23] as previously described [7–8]. The basic elements and philosophy underlying the use of the program package SYBYL have been described by Naruto *et al.* [24], and parameters for van der Waals contact radii were those of Iijima *et al.* [25]. With this program package, a systematic conformational analysis was carried out with SEARCH, which checks for van der Waals contacts among nonbonded atoms by scanning all possible torsional angles around the rotatable bonds and identifies, within the van der Waals allowed conformational space, those conformations that are compatible with ENDOR determined electron-nucleus distances together with their respective uncertainties. The effective position of the unpaired spin density of the nitroxyl group as a point dipole was assigned as described earlier [26].

3. Results and Discussion

3.1. ENDOR Spectra of Nitroxyl Spin-Labels in Frozen Solutions: Selection of Molecular Orientation

We have previously outlined the stratagem of employing the magnetic field dependence of the ENDOR characteristics of nitroxyl spin-labels for selec-

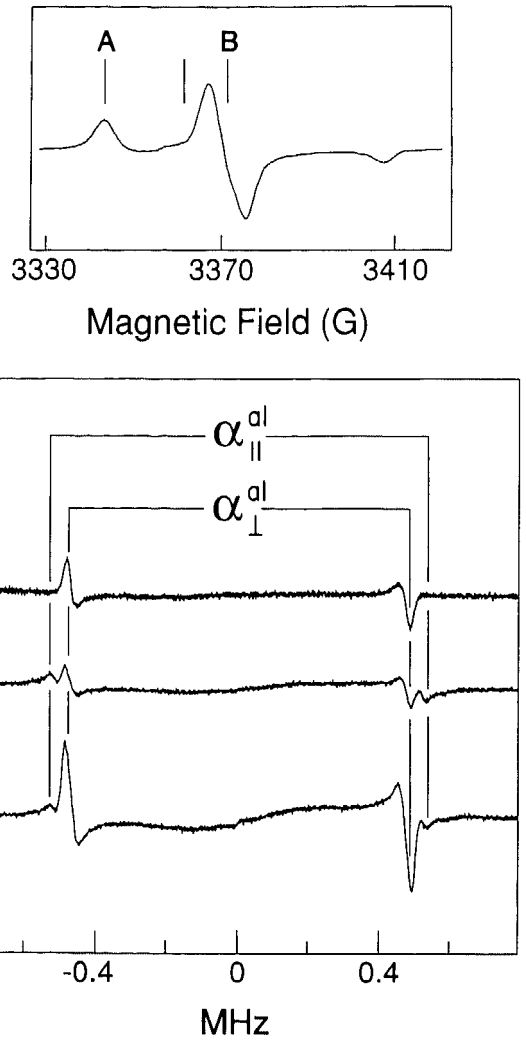


Fig.2. First-derivative EPR absorption spectrum and ENDOR spectra of **II** in fro: ($^2H_4$)methanol. The descending order of the ENDOR spectra in the lower panel correspo: to the $\mathbf{H}_0$ setting indicated by the left-to-right series of vertical lines in the top panel. Mic wave power saturation of the low-field feature of the EPR spectrum is designated as settin for ENDOR experiments, while saturation of the central feature is designated as setting The principal hfc components for the aldehydic proton of **II** are identified as $\alpha_{\parallel}^{al}$ and $\alpha_{\perp}^{a}$ the stick diagram. The abscissa indicates the ENDOR shift (measured ENDOR freque minus the free nuclear frequency).

tion of molecular orientation [6–11]. These principles are applied for t analysis of each of the ENDOR spectra presented in this investigation. T upper panel of Fig.2 illustrates the first-derivative EPR absorption spectru of **II**. The EPR spectra of nitroxyl spin-labels is dominated by anisotro

hyperfine interactions of the ^{14}N nucleus of the nitroxyl group, and their spectra in frozen solutions are a composite of three sets of spectral components that correspond to the $m_I = +1$, 0, and -1 states of the ^{14}N nucleus. The intense central feature of the EPR spectrum arises predominantly from molecules with $m_I = 0$ while the low- and high-field features, which are well separated from the central region, are due to the A_z hf component of $m_I = +1$ and -1, respectively. For this reason microwave power saturation of the central feature of the EPR spectrum for ENDOR selects essentially all orientations of the spin-label with respect to the laboratory magnetic field while saturation of the low- or high-field regions selects molecules for which the five-membered oxypyrrolinyl ring is perpendicular to the static laboratory magnetic field.

The lower panel of Fig.2 illustrates ENDOR spectra of **II** at three different $\mathbf{H}_0$ settings. With $\mathbf{H}_0$ set to the low-field turning point of the EPR spectrum (setting A), the topmost ENDOR spectrum exhibits one set of resonances belonging to the perpendicular hfc component ($A_\perp$) of the aldehydic proton. When $\mathbf{H}_0$ is set to the central region of the EPR spectrum (setting B), both the parallel and perpendicular principal hfc components $A_\parallel$ and $A_\perp$ are observed, as shown in the ENDOR spectrum at the bottom of the lower panel. The middle ENDOR spectrum was obtained with $\mathbf{H}_0$ set to the low-field edge of the central absorption feature of the EPR spectrum. This setting corresponds predominantly to the H_x, H_y region of $m_I = +1$. The ENDOR spectrum at this magnetic field setting exhibits a larger peak-to-peak amplitude for the outermost set of ENDOR resonances but with an identical splitting to that in the bottom spectrum. By changing the magnetic field from setting B to the low-field edge of the central feature, as shown in Fig.2, the amplitude of the perpendicular resonance absorption feature is diminished with a corresponding increase for the parallel absorption feature. The splitting of the innermost ENDOR line pair assigned to the perpendicular hfc component is not altered by a change in $\mathbf{H}_0$ setting. Since the perpendicular hfc is observed for $\mathbf{H}_0$ settings A and B, the aldehydic proton of **II** must lie almost in or exactly coincident with the plane of the oxypyrrolinyl ring.

3.2. Assignment of Proton ENDOR Features and Estimation of Electron-Proton Distances

Figure 3 illustrates proton ENDOR spectra of compounds **III** and **IV** with $\mathbf{H}_0$ at setting B. The use of an aprotic solvent provides an opportunity to detect the ENDOR features of the carboxylic acid proton, as shown in the top spectrum of **III**. The carboxylic proton normally exchanges with solvent deuterons, as shown through the middle and bottom spectra of compounds **III** and **IV** in [$^2H_4$]methanol, respectively, and the ENDOR features of the carboxylic acid proton are absent. The ENDOR splittings for H^α and H^β in

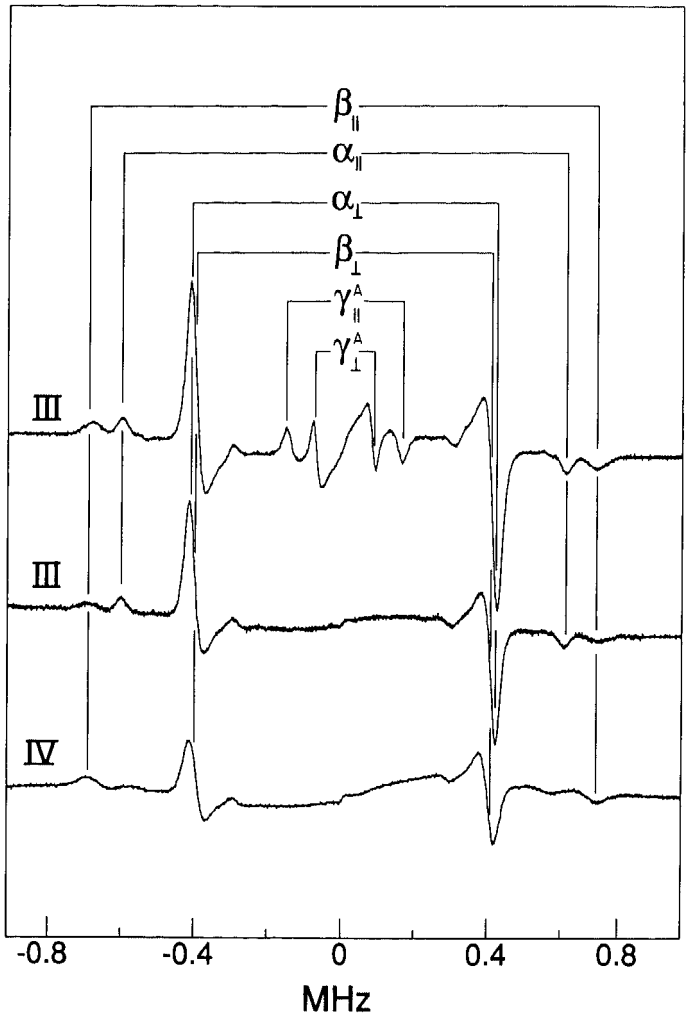


Fig.3. Proton ENDOR spectra of **III** and **IV** in frozen solutions of [^{2}H]chloroform:[$^2H_8$] luene:[$^2H_6$]DMSO (top spectrum) and [$^2H_4$]methanol (middle and bottom spectra). For ea ENDOR spectrum $\mathbf{H}_0$ was set at position B of the EPR spectrum. The ENDOR splittings tl correspond to both principal hfc components for the α, β and γ^A protons in the propen acid side chain are identified in the stick diagram.

III, however, remain identical in both of these solvent systems. The reso ance features of both protons are assigned through use of deuterated ana ogues. Because $\mathbf{H}_0$ is set to the central feature of the EPR spectrum, tl spectra in Fig.3 identify both the perpendicular and parallel hfc componen for each proton. On the other hand, ENDOR spectra under setting A ider ify only the perpendicular hfc components. In all cases, the assignment perpendicular and parallel hfc components has been made on the basis

Table 1. Summary of hfc components and estimated electron-proton distances in compounds **I–IV**.

Proton[a,b]	Compound	$A_\parallel$	$A_\perp$	A_{iso} (MHz)	$A_\parallel^D$	$A_\perp^D$	r^c (Å)
α^A	I	0.659	0.437	−0.072	0.731	−0.365	6.01±0.04
α^{al}	II	1.064	0.958	−0.284	1.348	−0.674	4.91±0.02
α	III	1.214	0.809	−0.135	1.349	−0.674	4.91±0.02
β	III, IV	1.394	0.794	−0.065	1.459	−0.729	4.76±0.02
γ^A	III, IV	0.313	0.156	0.000	0.313	−0.156	7.98±0.07

[a] See atomic numbering schemes in Fig.1 for atom designations. The superscripts "A" and "al" are used to designate the carboxylic acid and aldehydic protons, respectively.
[b] ENDOR line pairs for each proton except for the carboxylic acid proton α^A of I are seen in Figs.2 and 3.
[c] Uncertainty in frequency of 0.08–0.12 MHz due to the line width of each absorption is included in the calculation of electron-proton distances.

both setting A and setting B spectra although only spectra for setting B are presented here for purposes of brevity.

In Table 1 we have summarized the observed values of the parallel and perpendicular hfc components of each class of protons in compounds **I–IV**. The observation of only two pairs of resonance features for each proton requires that the observed ENDOR splittings correspond to axially symmetric, principal hfc components. The principal hfc components, as obtained from ENDOR, are expressed in terms of dipolar A^D and isotropic A_{iso} components. The isotropic hfc is estimated directly from the ENDOR determined principal hfc components according to the relationship $A_{iso} = (A_\parallel + 2A_\perp)/3$. In Table 1 we have also listed the values of isotropic and dipolar hfc components for each proton together with corresponding electron-proton separations r calculated on the basis of the dipolar equation.

3.3. Conformational Analysis Using ENDOR Constrained Molecular Modeling

To determine the conformations of compounds **I–III**, we have carried out a computational analysis on the basis of torsion angle calculations for all bonds. The torsion angle search calculations were carried out within van der Waals hard-sphere limits constrained by the ENDOR determined electron-proton separations in Table 1. In the conformational analysis of **III**, the C^α–C^β portion was treated as singly bonded. Fig.4 illustrates the angle maps for torsion angle calculations around the C(3)–C^α and C^α–O bonds of **I** and around the C^α–C^β and C^γ–O bonds of **III**. As seen in Fig.4, the distance constraint to the acid proton of **I** resulted in a value of 180° ± 15° for the [C(3)–C^α–O–H] torsion angle. This conformation thus corresponds to the classical Z conformer of a –COOH group. The electron-proton distance

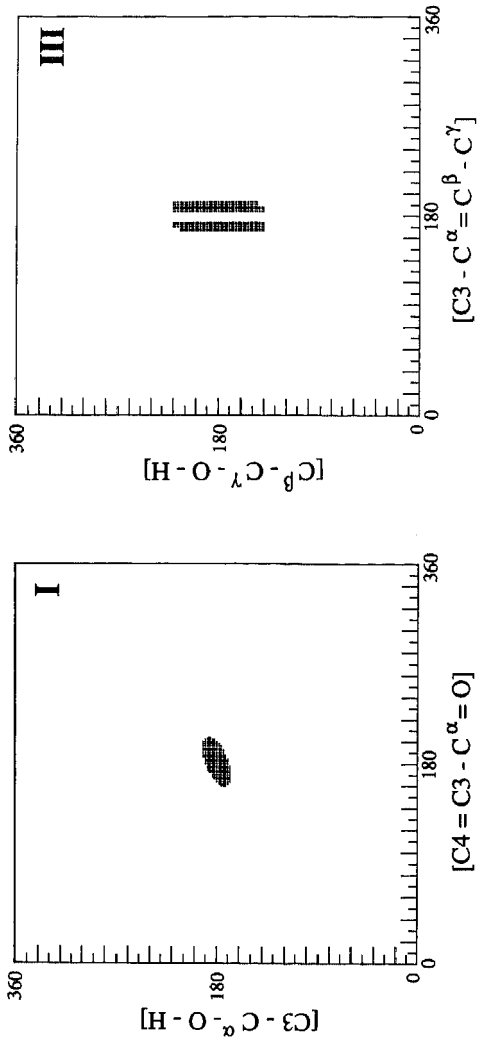


Fig.4. The torsion angle maps of compounds I and III. The axes represent 0°–360° rotation around the bonds as labelled in the angle maps (see Fig.1). The dots represent conformational space compatible with both van der Waals nonbonded constraints and the electron-proton distance constraints determined by ENDOR.

requires that the C=O group is in an *s-trans* orientation with respect to the olefinic group in the five-membered ring. The *Z* conformation is energetically favored over the *E* conformation of carboxylic acids [27]. The distance constraint to the aldehydic proton of **II** resulted in a value of $0° \pm 20°$ for the [C(4)–C(3)–C^{α}–H] dihedral angle and a value of $180° \pm 20°$ for the [C(4)–C(3)–C^{α}–O] dihedral angle, indicating that the aldehydic C=O group is also in an *s-trans* conformation.

The ENDOR determined distance constraints to H^{α}, H^{β}, and H^{γ} in compound **III**, as seen in Fig.4, resulted in an essentially planar structure of the C^{α}=C^{β} olefinic group with the oxypyrrolinyl ring. The torsion angle calculations show that the plane of the H^{α}–C^{α}–C^{β}–H^{β} group of atoms assumes a small, mirror symmetric tilt with respect to the plane of the oxypyrrolinyl ring. In fact, the [H^{α}–C^{α}–C^{β}–H^{β}] torsion angle is $170° \pm 6°$ and, therefore, the olefinic group is tilted only approximately $\pm 10°$ with respect to the five-membered ring. The essential coplanar conformation of the two olefinic groups, as measured by the [C(4)–C(3)–C^{α}–C^{β}] torsion angle is not less precisely defined than in high resolution X-ray studies of compounds with similarly conjugated olefinic groups [19]. Inspection of a space filling model of **III** by molecular graphics suggests that the slight tilt of the C^{α}=C^{β} olefinic group is due to steric interactions of H^{α} with the H(4) atom of the oxypyrrolinyl ring.

Similarly to **I**, the distance constraint to H^{γ} in **III** places the carboxylic acid group into a classical *Z* conformation, although, as seen in Fig.4, the single distance constraint of 7.98 ± 0.07 Å for the electron–H^{γ} separation results in a considerably larger region of ENDOR compatible conformational space. This circumstance arises because rotation around the C^{β}–C^{γ} bond essentially keeps the carboxylic acid proton nearly equidistant from the nitroxyl group. A similar observation was reported for the oxypyrrolinyl spin-labeled form of *L*-phenylalanine [11]. It is of interest to note that for both **I** and **III** the carboxylic acid group is assigned on the basis of ENDOR constraints to the *Z* conformation and that the *E* conformation requiring an

Table 2. Values of dihedral angles of the ENDOR constrained conformations of **I–III.**

Compound	Atoms defining dihedral angle	τ (degrees)
I	C(4)=C(3)–C^{α}=O	$180° \pm 20°$
	C(3)–C^{α}–O–H	$180° \pm 15°$
II	C(4)=C(3)–C^{α}=O	$180° \pm 20°$
	C(4)=C(3)–C^{α}–H	$0° \pm 20°$
III	C(4)=C(3)–C^{α}=C^{β}	$180° \pm 10°$
	C^{α}=C^{β}–C^{γ}=O	$0° \pm 120°$
	C^{β}–C^{γ}–O–H	$180° \pm 40°$

[C(3)–C^{α}–O–H], *viz.*, [C^{β}–C^{γ}–O–H] torsion angle of ~ 0° is ruled out. In contrast to **I**, the C=O bond in **III** is placed into an *s-cis* conformation with respect to the C^{α}=C^{β} bond, according to the ENDOR constraints. The overall conformation of **III**, therefore, is *trans-s-cis*.

A summary of the dihedral angles characterizing compounds **I**–**III** and determined on the basis of ENDOR constrained torsion angle calculations is given in Table 2.

3.4. Comparison of ENDOR Determined Structures of Spin-Labeled Derivatives with Similar Compounds

The spin-label compounds investigated here exhibit conjugated olefinic bonds. An important consequence of the delocalization of the conjugated π-electrons is the planar equilibrium configuration of the molecule, which can exist in both *cis* or *trans* conformations. The results presented here indicate that the side chains of **I**–**III** are planar with respect to the oxypyrrolinyl ring. The ultraviolet absorption spectrum of **II** showed a single band with a maximum extinction coefficient of ~ 17830 $M^{-1}cm^{-1}$ at 227 nm. This is identical to the corresponding values found for the planar *s-trans* conformation of crotonaldehyde [28].

With application of ENDOR spectroscopy, we have shown that it is feasible to identify multiple conformers of molecules in solution [6,8]. In this investigation, however, the ENDOR resonance features observed for each compound studied corresponded to only a single conformation. The ENDOR determined structure of **I** is identical to the corresponding structure derived from X-ray diffraction studies [17]. Wagner and coworkers have estimated that the potential energy of *s-trans* acrolein is about 2.5 kcal/mol lower than the *s-cis* conformer, and only about 2 % of the molecules in solution at room temperature are in the *s-cis* conformation [29]. On this basis we anticipate that the *s-trans* conformation of **II** found in this study is similarly energetically favored over the *s-cis* conformation. The conformation of **III** was found to be planar *trans-s-cis*. A similar structure for 5-phenyl-2,4-pentadienoic acid has been reported on the basis of X-ray diffraction studies [30]. In the latter compound the conjugated olefinic groups are found in a planar *trans* structure, while the C=O group is in the *s-cis* conformation with respect to the olefinic bond. It is, thus, evident that ENDOR spectroscopy in conjunction with conformational analysis constrained by ENDOR electron-nucleus distance measurements provides a general method of accurate structure determination applicable to molecules in frozen glassy solutions or polycrystalline systems.

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