Solution Structures of TOAC-Labeled Trichogin GA IV Peptides from Allowed ($g \approx 2$) and Half-Field Electron Spin Resonance

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Received December 8, 1998. Revised Manuscript Received June 9, 1999

Abstract: The recently isolated trichogin GA IV is a 10 amino acid, Aib-rich peptide with potent membrane-modifying properties. The peptide is too short to span lipid bilayers, so the mechanism by which trichogin GA IV interacts with biological membranes is unknown. The crystal structure has been solved, but there is much less information on the peptide's conformation in solution. This problem is addressed by examining the electron spin resonance (ESR) of single and double TOAC-labeled trichogin GA IV analogues, where TOAC is a rigid nitroxide amino acid and serves as an Aib analogue. The doubly labeled peptides, trich-1,4, -4,8 and -1,8, represent all possible trichogin GA IV analogues containing two Aib → TOAC substitutions. ESR in MeOH at 200 K of the $g \approx 2$ spectral region suggests that the N-terminus from residues one through four adopts a helical structure similar to that observed in the crystal. However, the central and C-terminal regions appear to be structurally heterogeneous. To further resolve the solution structure, we performed half-field ESR measurements in a MeOH/EtOH glass at 120 K and referenced them against similar measurements from a series of double TOAC-labeled peptides of known structure. Half-field intensities depend on electron spin dipolar coupling and scale as $1/r^6$ where r is the internitroxide distance. The combination of allowed ($g \approx 2$) and half-field ESR indicates that the trichogin GA IV C-terminal region is partially α-helical, as in the crystal structure, but is in equilibrium with unfolded conformers. It is suggested that the Gly−Gly stretch creates a hinge point between two short but stable helical regions. The combined ESR methods used here represent a new approach for determining the solution structures of partially folded peptides.

Introduction

Peptaibols are peptides of fungal origin, rich in α-aminoisobutyric acid (Aib), that possess a 1,2-amino alcohol at the C-terminus.[1] These unique peptides are of current interest because of their membrane-modifying properties and, consequently, their potential as model membrane channels and therapeutics. The longer peptaibols such as alamethicin form stable, membrane-spanning helices and are believed to assemble as barrel staves to form voltage-gated channels.[2] The recently isolated peptide trichogin GA IV brings new and unique characteristics to the peptaibol class of peptides.[3] First, it has an *n*-octanoyl group attached to the N-terminus.[4] Because of the lipophilic character of this N-terminal group, trichogin GA IV is referred to as a *lipopeptaibol*.[3] The second distinguishing feature is its short length; trichogin GA IV is composed of only 10 amino acid residues and is therefore too short to span biological lipid bilayers. Nevertheless, trichogin GA IV exhibits remarkable membrane-modifying properties similar to other peptaibols, although the mechanism by which it causes membrane leakage is unknown.

The sequence of trichogin GA IV is the following:

*n*Oct-Aib[1]-Gly-Leu-Aib[4]-Gly-Gly-Leu-Aib[8]-Gly-Ile-Lol

where *n*Oct stands for *n*-octanoyl and Lol stands for leucinol. Preliminary circular dichroism and NMR studies in methanol suggest that the peptide forms a helix as expected for sequences containing Aib residues.[3] The crystal structure was recently solved and confirmed a general helical character with residues one through four forming a distorted 3_{10}-helix ($i \leftarrow i + 3$ hydrogen bonding) and residues four through ten forming an α-helix ($i \leftarrow i + 4$ hydrogen bonding).[5] There is a kink at Gly 2 allowing for the switch from 3_{10}- to α-helix. On the basis of this crystal structure, it was proposed that a transmembrane pore could form via an alamethicin-like barrel stave mechanism but with each stave composed of a trichogin GA IV head-to-head dimer.[4,5] This proposed mechanism of pore formation remains speculative.

Spin label electron spin resonance (ESR) using the spin active TOAC residue has recently emerged as a powerful tool for determining the conformations of peptides in solution.[6−11] As shown in Figure 1, the TOAC nitroxide ring is attached rigidly

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Published on Web 07/14/1999

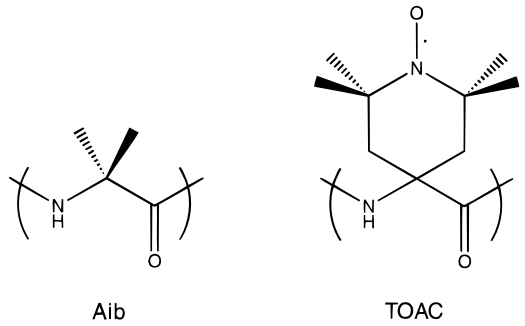

Figure 1. Chemical structures of Aib and its spin label analogue TOAC.

to the polypeptide backbone. It is a C^{α}-tetrasubstituted α-amino acid like Aib (see Figure 1) and exhibits Aib's tendency to strongly promote helical conformations.[12,13] Recent crystallographic and spectroscopic studies demonstrate that Aib → TOAC substitutions are structure-preserving.[9] When two TOACs are engineered into a peptide sequence, the intramolecular, biradical dipolar and isotropic couplings may be used to map local geometry.[7,8] Because of TOAC's rigid attachment to the peptide backbone, spectra often show strong dipolar couplings under solvent and temperature conditions in which other spin label techniques do not.[7,8,14] Past work from our lab has demonstrated the remarkable detail double TOAC labeling reveals about local peptide fold. We have been able to distinguish 3_{10}-helix from α-helix[15,16] in Ala-rich peptides[7] and to follow the solvent-induced denaturation of hexameric sequences.[8] Most recently, quantitative line shape fitting was used to analyze the structure of long, α-helical peptides in aqueous solution.[14] α-Helices in proteins usually have a pitch of approximately 3.6 residues/turn. However, comparison of the relative i, $i + 3$ and i, $i + 4$ distances in solvated Ala-rich peptides suggested backbone torsion angles of $\phi = -70°$ and $\psi = -45°$, consistent with a pitch of 3.8−3.9 residues/turn.[14] Our findings suggested that local solvation may exert substantial influence over local helix fold.

In this present work we apply double TOAC labeling to map the solution structure of trichogin GA IV. To understand how this peptide interacts with membranes, it is imperative to determine its fold in a fluid environment. As discussed above, X-ray diffraction studies demonstrate that trichogin GA IV is helical when crystallized.[5] However, published magnetic resonance and optical spectroscopic studies in solution are qualitative and do not indicate whether details of the crystal structure are preserved in solution. And although trichogin GA IV has three Aib residues, it also has four helix breaking glycines so it may also adopt nonhelical conformations.

Table 1. Trichogin GA IV TOAC-Labeled Analogues

nOct-Aib -Gly-Leu-**TOAC**-Gly-Gly-Leu-Aib -Gly-Ile-Lol	Trich-4
nOct-**TOAC**-Gly-Leu-**TOAC**-Gly-Gly-Leu-Aib-Gly-Ile-Lol	Trich-1,4
nOct-Aib-Gly-Leu-**TOAC**-Gly-Gly-Leu-**TOAC**-Gly-Ile-Lol	Trich-4,8
nOct-**TOAC**-Gly-Leu-Aib-Gly-Gly-Leu-**TOAC**-Gly-Ile-Lol	Trich-1,8

Table 2. Reference Peptides of Known Structure

Boc-**TOAC**-**TOAC**-Ala-Ala-Ala-Ala-O*t*Bu	Hex-1,2
Boc-**TOAC**-Ala-**TOAC**-Ala-Ala-Ala-O*t*Bu	Hex-1,3
Boc-**TOAC**-Ala-Ala-**TOAC**-Ala-Ala-O*t*Bu	Hex-1,4
Boc-**TOAC**-Ala-Ala-Ala-**TOAC**-Ala-O*t*Bu	Hex-1,5
Boc-**TOAC**-Ala-Aib-**TOAC**-Ala-Ala-O*t*Bu	Hex-1,4-Aib-3
*p*BrBz-**TOAC**-Ala-Ala-**TOAC**-Ala-O*t*Bu	*p*BrBz-Pent-1,4
Ac−Ala_3-**TOAC**-Lys-Ala_2-**TOAC**-Ala-Lys-Ala_4-Lys-Ala-NH_2	3KT-4,8

Trichogin GA IV contains Aib residues at positions 1, 4, and 8. As shown in Table 1, four peptides with Aib → TOAC substitutions are examined. The trich-4 peptide contains a single TOAC substitution, which allows for determination of ESR spectra in the absence of biradical coupling, while the trich-1,4, -4,8, and -1,8 contain all possible double Aib → TOAC substitutions. A preliminary account of these TOAC-labeled peptides outlined their chemical synthesis and demonstrated that they retain the membrane-modifying properties of the parent trichogin GA IV.[17] In addition, a crystal structure of the trich-4,8 showed that it adopts a mixed 3_{10}/α fold similar to that of the parent peptide.[17,18]

There are two goals of this present study. First, we use TOAC double labeling to determine the conformations of trichogin GA IV in methanol solution. Second, as part of this structural determination, we extend double label ESR by using half-field transitions as a means independent of line shape fitting to determine an average distance between TOAC residues. The half-field absorption arises from a forbidden "$\Delta m = 2$" transition that becomes partially allowed in the presence of biradical dipolar coupling.[19−22] The integral of this signal scales as $1/r^6$ where r is the separation between spins.[19,23] Past work has demonstrated the utility of half-field measurements in the determination of internitroxide distances.[19,23] We develop a half-field transition calibration curve, a half-field molecular ruler, from a series of short Ala-rich sequences of known geometry, which are shown in Table 2. By combining allowed ESR with these half-field measurements, we show that the N-terminal region of trichogin GA IV retains its helical structure in solution. However, the central and C-terminal regions are only partially helical and appear to coexist as a superposition of helical and unfolded conformers. This study reveals trichogin GA IV conformations not previously seen by either crystallography or nuclear magnetic resonance spectroscopy. In addition, this work

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lays the foundation for a new mode of ESR spectral analysis aimed at determining the detailed conformations of peptides in solution.

Experimental Section

Synthesis and purification of the peptides used in this study have previously been reported.[17] Samples were prepared in a glassing solvent mixture consisting of 9:1 (v/v) methanol−ethanol. Samples for variable temperature measurements (40 μL) were withdrawn and sealed into 100 μL glass capillaries. Samples for half-field measurements were prepared using 300 μL of this mixed-solvent solution placed in 4 mm quartz sample tubes and quickly frozen in liquid nitrogen to form a glass. These samples were immediately placed in the cooled spectrometer cavity for spectral acquisition. Concentrations were determined by comparison of the integrated intensities of the allowed, room temperature spectra against a series of TEMPOL standards prepared in 5 mM aqueous MOPS buffer (pH 7.1; 0.1, 1, and 2 mM TEMPOL) and in 9:1 MeOH−EtOH (0.5, 1.0, and 5.0 mM TEMPOL).

Unless otherwise indicated, all spectra were recorded in continuous-wave mode on a Bruker ESP-380 X-band spectrometer equipped with a TE_{102} cavity and a ER4111 variable temperature unit. The temperatures at which this unit regulates were checked against a Cernox ceramic thermocouple purchased from and calibrated by Lakeshore Cryonics. Allowed $g \approx 2$ spectra near 200 K were recorded with a center field of 3357 G, a modulation frequency of 100 kHz, a modulation amplitude typically of 1.0 G, and a power of 2.7 mW. Care was taken to ensure that spectra were not power-saturated.

Integrated intensities for both allowed and half-field spectral regions were determined via double integration of the recorded derivative-mode spectra. For the half-field signals, a third-order polynomial baseline correction was determined using 35 G of baseline at both edges of the absorption mode spectrum and subtracting this polynomial from the entire spectrum prior to the second integration.

It has been demonstrated by the Eaton laboratory that the intensity of a half-field spectrum, when normalized against the intensity of the full field spectrum at the same temperature to give a relative intensity, directly reports on distance according to the relationship

$$\text{Relative Intensity} = \frac{A(9.1)^2}{r^6 \nu^2} \qquad (1)$$

where r is the internitroxide distance in Å, ν is the spectrometer frequency in GHz, and A is a coefficient that depends on the type of interacting radicals. For two nitroxides $A = 19.5 \pm 0.5$ (see refs 19, 23), and this value has been supported by theory.[24] We tested this approach with one of our doubly TOAC-labeled peptides, *p*BrBzHex-1,4.[8] By using a distance of 5.84 Å between the electron spins (see below) we determined $A = 8 \pm 2$, which is in reasonable agreement with the Eaton's value.

In principle, one should be able to use eq 1 for accurate distance determination in most cases. For our studies reported below, however, we used a slightly different approach. Our samples were often studied at submillimolar concentrations where the allowed $g \approx 2$ spectra were often significantly broadened by dipolar coupling and the half-field transitions were weak. Thus, it was convenient to obtain allowed and half-field spectra, using conditions that optimized signal reproducibility for each, with the allowed spectra used only to determine peptide concentration. Half-field spectra were recorded with a center field of 1677 G, a modulation frequency of 100 kHz, a modulation amplitude of 5.0 G, a microwave power of approximately 84 mW, and a sweep width of 200 G. Integrated intensities of the half-field transitions were normalized by dividing by the number of scans acquired, the peptide concentration, and the square root of the microwave power. The gain and modulation amplitude were held constant for all half-field spectra. Solution concentrations were determined at room temperature using methods reported previously.[25] Although this method is less general than that proposed by the Eaton laboratory and requires the availability of standards of known distance such as those employed here (see below), it is very accurate for studying samples at low concentration. Nevertheless, when standards of known distance are not available, eq 1 should provide an excellent means for distance determination in TOAC-labeled peptides.

Fits to the 200 K spectra were done according to the procedures detailed by Hustedt et al.[26] Computer models of the peptides were built and queried with MOLMOL,[27] using a library modified to contain the TOAC and Aib residues. Calculations necessary to transform the coordinates appropriately for inclusion and inspection of the models so built were aided by use of MOLMOL, MidasPlus, Perl 5, and Octave 2.0.5 on a Silicon Graphics, Inc. Indy R5000 workstation.

Results

Allowed ESR Spectra. Previous work has focused on the structure of trichogin GA IV in MeOH.[3] Methanol is an appropriate solvent for several reasons. First, it is the most helix-promoting of the various neat alcohols.[8] Second, among the alcohols, it is the closest to water in physical properties but offers much greater fluid temperature range and solubility than water for these uncharged peptides. Finally, past NMR and CD studies were performed in MeOH, and by holding close to this solvent, we can compare our present results to those obtained elsewhere. For this study we use 9:1 (v/v) MeOH−EtOH. Our past work has demonstrated that EtOH shares helix-promoting properties nearly identical to those of MeOH,[8] but this mixture allows for the formation of proper low-temperature glasses which are useful for measuring half-field transitions (see below). Room temperature spectra of the trichogin GA IV analogues in neat MeOH have already been reported, and it was demonstrated that rapid tumbling of these analogues averages the distance-dependent dipolar interaction.[17] Thus, we focus here on low-temperature spectra. Conventional continuous wave spectra were obtained in fluid solution at 200 K where rotational diffusion is too slow to average spectral rigid lattice features.[7,14] Derivative and absorption spectra for the four trichogin GA IV analogues are shown in Figure 2. The trich-4 spectrum serves as a reference monoradical spectrum devoid of dipolar coupling.

The dipolar interaction scales as $1/r^3$. At distances sufficiently long to give a weak interaction, dipolar coupling results mainly in broadening of the rigid lattice features. At shorter distances, the dipolar interaction dominates the ESR spectrum and the width between the spectrum's outer edges scales as $1/r^3$. Representative spectra have been published elsewhere[7,8,14] (see also Figure 5). The trich-1,4 spectrum is clearly broader than the trich-4 spectrum and gives a pattern that has been observed for other well-structured i, $i + 3$ doubly labeled helical peptides.[8,14] It is difficult to assign secondary structure without companion i, $i + 4$ labeling in the same region of the peptide;[7,28] however, the results can be compared to those obtained from model 3_{10}- and α-helical peptides. Ala-rich, double TOAC, hexameric peptides form well-structured 3_{10}-helices, and the i, $i + 3$ spin-labeled analogues at 200 K show much stronger dipolar coupling than that observed for the trich-1,4.[8] However, longer sixteen- and twenty-residue sequences form α-helices, and spectra from their i, $i + 3$ double TOAC analogues are quite similar to that obtained from the trich-1,4.[14]

We attempted to fit the trich-1,4 spectrum with a simulation that incorporates dipolar coupling.[26] The best simulation obtained is shown in Figure 2. Fine features in the simulation are clearly absent in the experimental derivative spectrum. It is

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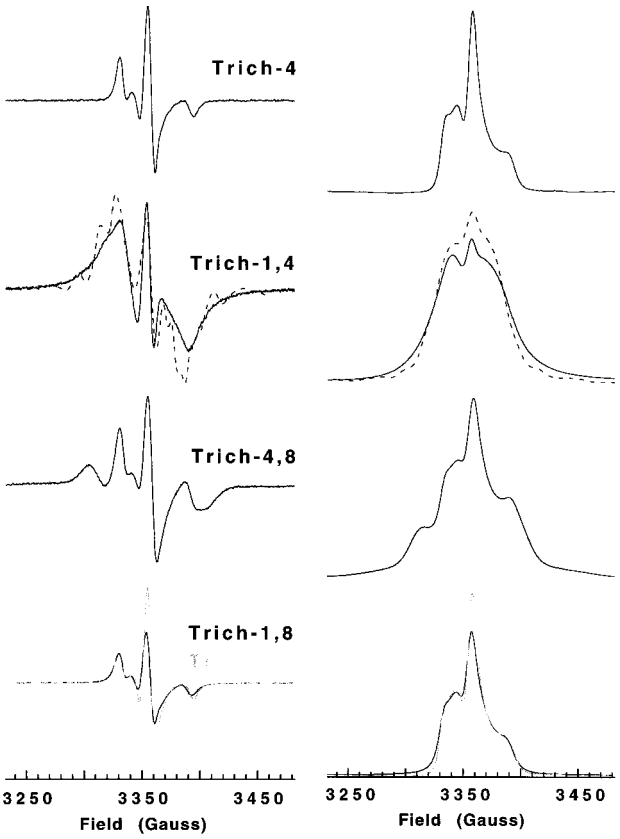

Figure 2. Full field spectra (250 G scan width) of the trichogin peptides in 9:1 (v/v) MeOH−EtOH at 200 K displayed in derivative mode (left) and absorption mode (right). The dashed line overlaying the trich-1,4 spectra is the least-squares result from a simulation incorporating dipolar coupling. The distance determined from this fit is 8.6 Å.

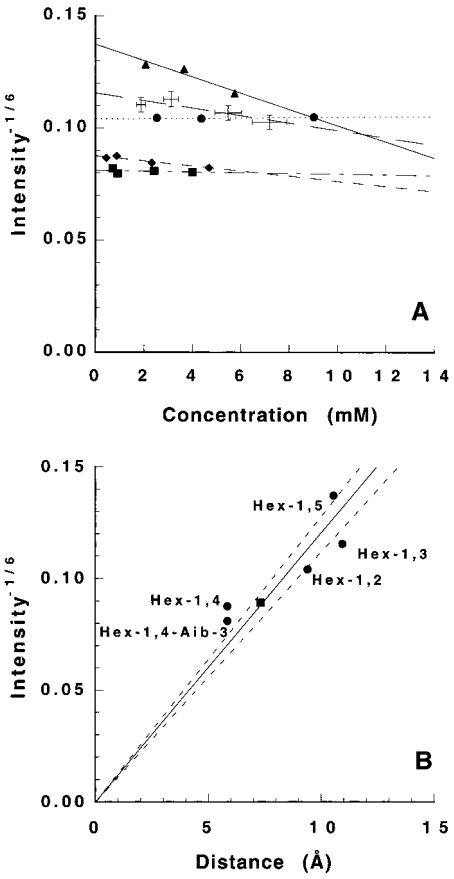

Figure 3. Distance calibration curves determined at 120 K from the five hexameric peptides in Table 2. (A) Intensity$^{-1/6}$ vs peptide concentration for Hex-1,2 (●), Hex-1,3 (error bars), Hex-1,4 (◆), Hex-1,4-Aib-3 (■), and Hex-1,5 (▲) showing the expected linear dependence. (B) Intensity$^{-1/6}$ values from A extrapolated to zero concentration and plotted against internitroxide distance. The solid line represents the resulting calibration curve, and the dashed lines represent the calculated error. Intensity$^{-1/6}$ for *p*BrBz-Pent-1,4 (■) was determined at a single concentration below 2 mM.

possible that some of these details are smoothed out by local conformation heterogeneity or small local motions. However, the absorption spectrum shows that the simulation captures the main spectral features and overall width but that the experimental spectrum is somewhat less sharp at the edges. The distance obtained from the simulation is 8.6 Å, which is consistent with the structure of an α-helix[14] (note that -1,4 spacing in a 3_{10}-helix gives a distance of approximately 5.8 Å; see Figure 3B and ref 8). Thus, by comparison with spectra obtained from α-helical peptides and from spectral fitting, it appears that the N-terminal region of the trichogin GA IV forms a stable helix with, most likely, $i \leftarrow i + 4$ hydrogen bonding.

The trich-4,8 spectrum shows prominent outer wings consistent with strong dipolar coupling.[14] However, the central region of the spectrum suggests that there is substantial contribution from a peptide species devoid of dipolar coupling. Attempts at fitting the entire spectrum failed, and the relative contributions of the dipolar coupled and uncoupled spectra exhibit a temperature dependence (see below). Thus, it appears that the central and C-terminal regions of the peptide are structurally heterogeneous with spectral contributions coming from both helical and unfolded peptide conformations. It is also possible, however, that the uncoupled contribution arises from a monoradical contaminant. Given the high purity of the peptide samples, the most probable monoradical species would be a peptide where one nitroxide has been reduced to the hydroxylamine. To rule out this type of contamination, the trich-4,8 peptide was treated with base to ensure complete oxidation of the nitroxide moiety.[10] Despite this treatment, the relative contributions of coupled and uncoupled spectra showed no variation at a fixed temperature. In addition, the peptide was mixed with a suspension of egg phosphatidyl choline (ePC) vesicles. This type of lipid environment typically stabilizes peptaibols in the helical conformation. Indeed, in ePC the trich-4,8 peptide showed a full-field dipolar spectrum at 300 K with only a negligible amount of monoradical-like signal (data shown in ref 17). Thus, we conclude that conformational heterogeneity rather than a contaminant is responsible for the trich-4,8 line shape. This is discussed in detail below.

The trich-1,8 spectrum exhibits very little dipolar coupling. In Figure 2 the trich-1,8 spectra are superimposed on the trich-4 spectra to show that it is just a little broader than that obtained from a monoradical species. Simulations were attempted, but each fitting attempt converged to a different distance. Thus, we conclude that the broadening is too small to allow for determination of a well-defined distance.

The ESR spectra are complex but suggest that the N-terminal region is probably well-structured, although no single conformation gives a simulation that provides an ideal fit to the experimental data. The central and C-terminal regions are structurally heterogeneous with a contribution from an unfolded conformation. Given that the trichogin GA IV peptide exists among multiple conformations, it would greatly refine our knowledge of its structure if we had a method for determining an *average* distance between labels. For this, we turn to measurement of half-field intensities.

Development of a "Half-field Molecular Ruler" Using 3_{10}-Helical Peptides. ESR typically detects allowed single-spin transitions characterized by $\Delta m = 1$, where m is the unpaired electron spin state. However, in the presence of strong dipolar coupling, there is a mixing among the electron two-spin states resulting in a weak transition that appears at approximately half the field of the allowed transition. This transition is often referred to as "$\Delta m = 2$", although it actually arises from absorption of a single quantum.[22] The strength of this interaction is proportional to $1/r^6$ and becomes forbidden in the high field limit (or more specifically when the Zeeman interaction is much greater than the dipolar interaction). The Eaton laboratory has performed extensive studies of this signal, and they have demonstrated that, in the limit of low concentration, the integral of the half-field absorption is directly proportional to $1/r^6$ with negligible influence from relative nitroxide orientations.[19,23]

There are three steps in setting up a half-field calibration curve. First, one must obtain a series of nitroxide biradicals where the internitroxide distance for each is known. For this we use the first five peptides in Table 2. Next, the strength of the half-field transition must be determined for each known peptide biradical as a function of peptide concentration. Extrapolation to zero concentration (infinite dilution) gives the half-field transition strength in the absence of intermolecular dipolar coupling.[19] Finally, these zero concentration integrals are plotted against known distance, thereby establishing a standard curve, that is, a half-field molecular ruler.

Ala-rich hexamers with two or more Aib residues form stable 3_{10}-helices.[12] The first six TOAC-labeled peptides in Table 2 also form stable 3_{10}-helices as determined by X-ray crystallography on the pentamer[9] and several ESR investigations on both the pentamer and hexamers.[8] Spectra of the pentamer and hexamer sequences in Table 2 in MeOH have already been reported.[8,9] Half-field measurements are best performed at low temperature to ensure immobilization;[19] however, we have found that, upon cooling to 120 K, MeOH does not form a sufficiently good glass. Glasses are desired, rather than a crystalline matrix, to guard against solute extrusion which can locally concentrate solute molecules, thereby generating noncontrollable, spuriously high half-field signals. We found that the 10% (by volume) of EtOH added to the MeOH solvent system had little effect on the spectra of the peptides while allowing for the formation of good glass at low temperature.

The concentration dependence of the half-field signal intensity, as normalized against peptide concentration, was recorded at 120 K for the series of five TOAC containing hexamers. Figure 3A shows the inverse sixth root of the half-field signal intensity plotted against concentration for each of the hexamers. Almost all of the data points in Figure 3A represent an average of multiple measurements. To illustrate the degree of experimental uncertainty, error bars are shown for data from Hex-1,3. The relative concentration error and intensity$^{-1/6}$ error are small and less than 10% and 5%, respectively. Linear extrapolation of each curve to infinite dilution gives a value which should be directly proportional to the interspin distance within each molecule. Indeed, the relative ranking of the intercepts is consistent with that expected for 3_{10}-helical conformations. Using $D_{i,j}$ to represent internitroxide distance, Figure 3A shows that $D_{1,4} < D_{1,2} \approx D_{1,3} < D_{1,5}$, which is consistent with 3_{10}-helix and markedly different from that expected for α-helix ($D_{1,4} \approx D_{1,5} < D_{1,2} \approx D_{1,3}$).[7]

To calculate distances for the biradical hexamers, we built computer models of double TOAC-labeled peptides assuming a 3_{10}-helical conformation with uniform backbone torsion (ϕ,ψ) angles of $-57°$ and $-30°$, respectively.[15] The interspin distance was taken as the average of the internitroxide N···N and O···O distances for the TOAC residues as modeled in the twist-boat conformation found in the crystal structure of the pentamer *p*BrBzPent-1,4.[9] The half-field intensity as a function of model length is shown in Figure 3B. The data are fit with a constant slope and zero intercept corresponding to the intensity$^{-1/6} \propto$ distance relationship expected. The dashed lines in the plot represent the slope standard deviation, which incorporates the scatter in this plot as well as propagated error associated with concentration and signal integral measurements. The data in Figure 3B suggest that distances less than 10 Å may be reliably determined with error less than 1.0 Å. Longer distances may also be determined but, as expected, the error will increase proportionally.

An additional experiment was performed to test the applicability of this approach for a related peptide. Inspection of the concentration dependence curves in Figure 3A reveals that, for sufficiently dilute samples (≈2 mM or less) or for sufficiently short distances (<10 Å), the systematic error introduced by concentration-dependent intermolecular contributions is less than the random error inherent in the measurement and can, to a first approximation, be disregarded. For limited sample quantities or for samples of limited solubility in which multiple dilutions might be impractical to perform, measurements at a single, sufficiently low concentration can provide a reasonable measure of intermolecular distance. In Figure 3B, one such single-concentration measurement is shown (symbol ■) for the pentamer *p*BrBzPent-1,4 and plotted against its crystallographically determined distance. The distance of 7.4 Å determined from the half-field measurement for this peptide agrees fortuitously well with the value of 7.3 Å determined from the crystal structure.[9]

Application of Half-field Measurements to the Trichogin Peptides. Concentration-dependent half-field measurements were performed on the three trichogin GA IV analogues and extrapolated to infinite dilution. The data are shown in Figure 4 and distances reported below. Also reported below are hypothetical crystallographic distances determined from the trichogin GA IV crystal structure modeled with TOAC residues replacing the Aib's.

	half-field (Å)	crystallographic (Å)
Trich-1,4	7.9 ± 0.53	5.42
Trich-4,8	7.4 ± 0.47	7.28
Trich-1,8	12.0 ± 0.76	11.4

Along the abscissa corresponding to the half-field measurement for each peptide in Figure 4 are also plotted the crystallographic distances[5] and distances expected for ideal 3_{10}- ($\phi = -57°$, $\psi = -30°$) and α-helix ($\phi = -63°$, $\psi = -42°$) conformations.[15,29]

The trich-1,4 half-field distance of 7.9 Å agrees well with 8.6 Å obtained from fitting the allowed ESR spectrum at 200 K. However, these ESR-determined distances are substantially longer than that expected from our crystal structure-based model. Figure 4 shows that, nevertheless, these distances are quite close to that expected for an α-helix or, to a lesser extent, a 3_{10}-helix. As noted in the Introduction, the trichogin GA IV crystal structure shows a kink at Gly 2, and computer models indicate that preservation of this backbone kink in trich-1,4 would bring the two TOACs in close proximity. Since the ESR data agree well with distances expected from conventional helical structures, we conclude that this kink is not preserved in solution

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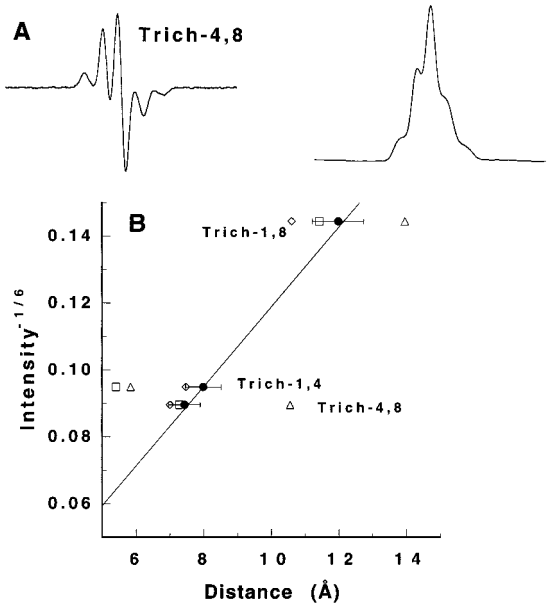

Figure 4. Half-field measurements at 120 K for the series of doubly labeled trichogin GA IV analogues. (A) Example half-field spectra from trich-4,8 at 4.3 mM (1679 G center field, 200 G scan width) showing derivative (left) and absorption (right) modes. (B) Intensity$^{-1/6}$ for the doubly labeled trichogin GA IV peptides plotted on the calibration curve from Figure 3B (●) with error bars determined from the uncertainty in the calibration curve. At the intensity$^{-1/6}$ value for each peptide are plotted the expected distances from the trichogin GA IV crystal structure (□), from ideal uniform α-helix (◇), and from ideal uniform 3_{10}-helix (△).

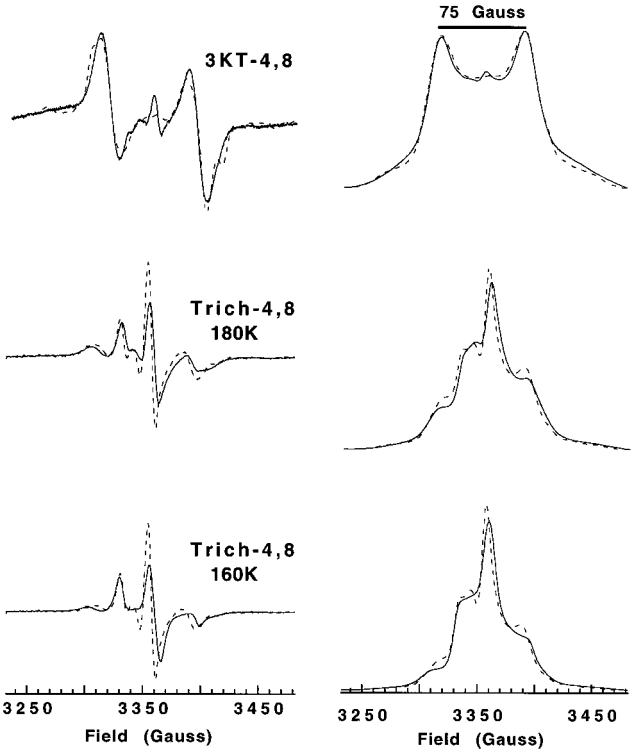

Figure 5. ESR spectra (250 G scan width) of the 3KT-4,8 at 200 K and trich-4,8 at two temperatures below 200 K. The dashed line overlaying the 3KT-4,8 spectrum is from spectral fitting with dipolar coupling. The dashed lines overlaying the trich-4,8 spectra are least-squares fits to a superposition of two spectra where one corresponds to an uncoupled biradical and the other to the simulation of the 3KT-4,8 spectrum.

but instead the N-terminal region of the peptide relaxes and forms a well-structured helical turn.

That reliable distances were obtained for the trich-4,8 and -1,8 peptides highlights the power of the half-field approach. As discussed above, allowed ESR spectra for these peptides (Figure 2) were either too complicated or failed to yield sufficient line broadening to be amenable to standard line shape analysis. In addition, Figures 3 and 4 show that half-field distance determinations are useful out to 12 Å, which is a distance regime that challenges other techniques.

The distance determined for the trich-4,8 matches well to that expected from the crystal structure. As shown in Figure 4, the trich-4,8 distance suggests that α-helix is the dominant folded structure in the C-terminal region of the peptide. There may be structural contributions from 3_{10}-helix or unfolded conformations, but in the presence of α-helix, these may not contribute significantly to the half-field intensity given the $1/r^6$ dependence. Allowed ESR spectra (see above) suggest that this region of the peptide is conformationally heterogeneous, but clearly, α-helix is a significant conformer. This is discussed further below.

Finally, the trich-1,8 distance of 12.0 Å is only a little longer than the 11.4 Å expected from the crystal structure. The inherent error of 0.8 Å suggests that it is not worthwhile trying to interpret any differences in these two values. Again, there may be unfolded conformers contributing to the overall structure, but the approximate agreement between the half-field and crystallographic values suggests that there is a significant population of peptide that is fully helical in solution.

Conformational Heterogeneity of the Central and C-Terminal Regions. Half-field measurements on the trich-4,8 peptide suggest an α-helical structure for this region of the peptide. However, the allowed ESR spectrum at 200 K (Figure 2) is complicated and clearly indicates a mixture of conformations. In addition, the half-field distance of 12.0 Å determined for the trich-1,8 peptide should give an average dipolar broadening of the allowed ESR spectrum of approximately 10 G if the peptide were structured as a uniform α-helix. However, such broadening is not observed, and instead, the trich-1,8 spectrum is only slightly broader than a monoradical spectrum. Thus, the final task in the structural assessment of the trichogin GA IV peptide is to reconcile these results with the distance measurements of the previous section.

The prominent outer wings observed in the trich-4,8 spectrum arise from significant dipolar coupling. We recently reported ESR measurements and spectral fitting on a series of doubly TOAC-labeled, sixteen-residue Ala-rich peptides, and the results of these measurements provide a reference point for understanding the trich-4,8 spectrum. The Ala-rich peptides are called 3KT and are based on the original 3K design of Marqusee et al.[30] The sequence of one member of the TOAC-labeled series relevant to the study here, the 3KT-4,8, is given in Table 2. Circular dichroism and distance hierarchies from double TOAC experiments establish clearly that these peptides fold as well-structured α-helices.[14] The ESR spectrum of the 3KT-4,8 and a simulation incorporating dipolar coupling is given in Figure 5. The spectrum is dominated by the dipolar interaction, as indicated by the large 75 G splitting, and the distance determined from the fit is 6.6 Å.

The outermost features of the trich-4,8 spectrum match well to the 75 G splitting in the 3KT-4,8 spectrum. This suggests that the distance for the folded conformer contributing to the trich-4,8 spectrum adopts approximately the same geometry as

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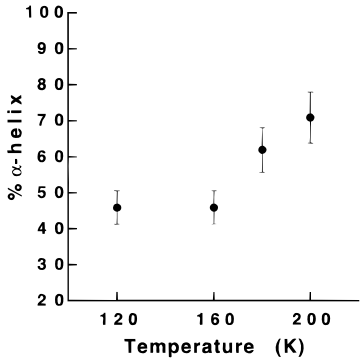

Figure 6. Percent of α-helix as determined from the trich-4,8 fits in Figure 5. An interesting feature is the decrease in the fraction of folded, helical peptide at low temperature.

the 3KT-4,8, which is α-helix. As discussed in sections above, the central region of the spectrum appears to be that from an uncoupled biradical, which most likely arises from a population of unfolded conformers. Thus, the most conservative way to interpret the trich-4,8 spectrum is to model it as a superposition of only two spectra where one corresponds to that of an ideal 4,8 doubly labeled α-helix and the other to that of an uncoupled biradical.

To perform this modeling, we used the simulation of the 3KT-4,8 for the α-helical spectral component $\mathbf{S}_\alpha$ and the monoradical trich-4 spectrum to represent the uncoupled biradical component $\mathbf{S}_\mathbf{u}$. Given these two normalized basis spectra, the normalized trich-4,8 absorption mode spectra were fit with the relation

$$\mathbf{S} = p_\alpha \mathbf{S}_\alpha + (1 - p_\alpha)\mathbf{S}_\mathbf{u} \qquad (2)$$

where the single fitting parameter p_α represents the fractional population of α-helix. The resulting least-squares fits are shown in Figure 5. The absorption mode spectra show that this fitting procedure captures the main features of the trich-4,8 spectra. In contrast, the derivative spectra show that this fitting procedure does not reproduce the central line of the uncoupled component. The central line of the simulated spectrum is sharper by approximately 2 G than the central line of the experimental trich-4,8 spectrum, which, in turn, indicates that there is still residual biradical broadening in the central region of the trich-4,8 spectrum.

Comparison of the trich-4,8 spectra at 200 (Figure 2), 180, and 160 K (the latter two in Figure 5) reveals a temperature dependence where the population of the strongly coupled component decreases as the temperature is lowered. This temperature dependence is plotted in Figure 6, and below 160 K, no further decrease in p_α was observed. An interesting feature of Figure 6 is the demonstration of a "cold denaturation" trend. This is discussed further below.

Figure 5 demonstrates that the allowed ESR spectrum of the trich-4,8 peptide fits reasonably well to a superposition of a spectrum of an α-helix and an unfolded component where the latter exhibits little detectable biradical coupling. We can now use these results to further interpret the half-field result for this peptide. Distances determined from half-field intensities represent an average where each component is weighted by $1/r^6$. For the two species identified in the trich-4,8 spectra, the average half-field-determined distance is given by

$$\frac{1}{r_{\mathrm{avg}}^6} = p_\alpha \frac{1}{r_\alpha^6} + (1 - p_\alpha)\frac{1}{r_\mathrm{u}^6} \qquad (3)$$

where r_α and r_u correspond to the 4,8 distances for α-helix and unfolded conformers, respectively. Studies with the 3KT-4,8 suggest that r_α is approximately 6.6 Å. It is impossible to know r_u, but we can estimate an upper bound on r_{avg} by setting $r_\mathrm{u} = \infty$. By using $p_\alpha = 0.46$ determined at 120 K (where the half-field measurements were obtained), we calculate $r_{\mathrm{avg}} = 7.5$ Å, which agrees well with the distance determined from the half-field measurements. Thus, there is no discrepancy between the half-field and allowed ESR measurements. The central and C-terminal regions of trichogin GA IV are conformationally heterogeneous in solution, and the half-field measurement gives a slightly longer than expected distance because of the presence of unfolded conformers.

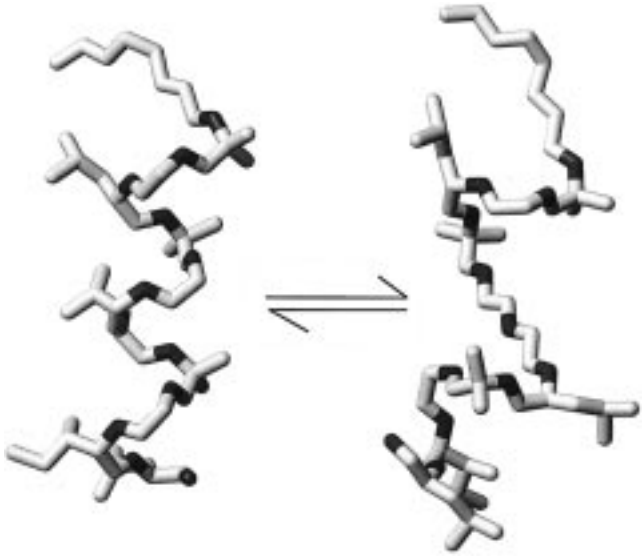

Figure 7. Schematic representation of the two trichogin conformers suggested by this study. The amide nitrogens are shown in black, and the remaining backbone and side chain atoms are shown in gray. On the left is the folded conformer in a predominatly α-helical conformation ($\phi = -63°$, $\psi = -42°$) and lacking the N-terminal kink identified in the crystal structure. On the right is the same structure, with helical turns at the N- and C-termini but with fully extended backbone torsion angles ($\phi = 180°$, $\psi = 180°$) for Gly 5 and Gly 6. Coexistence of these two conformers in nearly equal proportions is consistent with the allowed and half-field ESR measurements on the TOAC-labeled analogues.

Discussion

Allowed $g \approx 2$ ESR spectra and distances determined from half-field transitions offer complementary views of peptide structure. Allowed spectra are excellent for measuring relatively close distances (<8 Å) and for identifying conformational heterogeneity. In contrast, half-field measurements can detect longer distances, out to 12 Å in this present study, and yield a $1/r^6$ weighted view of local structure. Combining these techniques to study a partially folded peptide gives a structural perspective that would not be accessible to either technique alone. For example, the half-field distances suggest that trichogin GA IV adopts a rather uniform α-helical structure in MeOH, a result that would agree with published NMR and circular dichroism studies.[3] However, the trich-4,8 and -1,8 spectra do not support the assignment of uniform α-helix to trichogin GA IV. The trich-4,8 spectrum in particular suggests a heterogeneous structure. We reconcile these disparate results by assigning a well-ordered helical structure to the N-terminal region of the peptide (positions 1 through 4) and a mixed helical/unfolded structure to the central and C-terminal regions (positions 4 through 8), as shown schematically in Figure 7. Thus, even in

a helix-promoting solvent such as MeOH, trichogin GA IV with its three Aib residues does not fold to a well-ordered helix.

This finding of partially folded conformers is relevant to the general study of peptides in solution. Peptides rich in Aib, such as the peptaibols, are usually studied by X-ray crystallography. Crystallographic work has revealed a wealth of information including identification of different helical pitches, α- and 3_{10}-helix, and the determinants that favor various secondary structures (see, for example, ref 12). It is often assumed that the crystallographically identified structure will apply to solution. While this appears to be a safe assumption for large proteins and nucleic acids, such an equivalence between solution and crystal structure may not hold for peptide folds of limited thermodynamic stability. Indeed, there has been considerable interest in developing NMR and ESR methods for studying Aib-rich peptides and, only recently, have such methods revealed detailed polypeptide backbone folds.[7,8,31−33] (Note that NMR studies of Aib-rich peptides can be hampered by Aib's lack of a Cα proton.) The methods presented here represent a new approach for exploiting TOAC labeling to reveal detailed solution peptide conformations.

Similar to the longer peptaibol alamethicin, the crystal structure of trichogin GA IV reveals a mixed 3_{10}-/α-helix. Alamethicin adopts a helical structure in both the crystal and in solution, although a recent paramagnetic relaxation NMR study indicates that it exhibits limited internal flexibility.[32] Our results indicate that trichogin GA IV in solution may be substantially more flexible than alamethicin and, perhaps, significantly unfolded over the central and C-terminal regions of the peptide. Such a result is not surprising given the peptide's short sequence and consecutive Gly residues at positions five and six. Despite this apparent internal flexibility, however, trichogin GA IV remains a potent membrane-modifying peptide. Although existing models propose a helical structure for trichogin GA IV in membranes, the results presented here suggest that perhaps other conformations should be considered when developing biophysical theories of peptaibol channel formation.

It is interesting to compare our results with previous conformational studies of the trichogin GA IV peptide. Preliminary NMR work on trichogin GA IV in MeOH, based on ROESY connectivities, hydrogen exchange, and NH temperature coefficients, suggests a fairly uniform α-helical structure.[3] Circular dichroism[3,4] and infrared spectroscopy[4,17] support this assignment. The crystal structure also identifies a primarily α-helical structure but with a 3_{10}-helical N-terminus.[5] On the basis of the crystal structure, it was suggested that ion channels might form with aligned Gly residues forming the channel lumen. Our results indicate that α-helix is the primary folded component but it is in equilibrium with central and C-terminal unfolded conformers. Of course, the ESR data presented here were obtained at 200 K and below, whereas the NMR and other spectroscopic data were obtained at 298 K. Is it possible, then, that uniform α-helix is dominant at room temperature and the unfolded conformers observed here are a result of the low-temperature condition? This issue is addressed in our preliminary ESR work on this series of peptides where spectra were obtained at 298 K. We found that at 298 K the trich-4,8 peptide gave weak biradical coupling with much less coupling than has been observed for well-structured α-helical or 3_{10}-helical peptides.[17] Only at 200 K does the spectroscopic signature of a strongly coupled component become apparent. Thus, the 298 K ESR data also support the existence of unfolded conformers. It is interesting to note that, in the preliminary NMR work, there was a lack of medium-range connectivities and the authors interpreted this to indicate possible flexibility.[3]

Whereas this present study clearly documents the existence of central and C-terminal unfolded conformers, it is difficult at this stage to determine the nature of the unfolded conformers. We can consider, however, two limiting cases. In the first case, residues 5−10 are classical random coil and exist among a random ensemble of conformers. On average for this model, the distance between residues 4 and 8 is substantially greater than that for a folded α- or 3_{10}-helix. In the second case, we focus on the consecutive Gly residues at positions five and six. Gly is a strong helix breaking residue in media of both low and high dielectric constant,[34] and -Gly-Gly- may serve as a hinge point between two short but stable helical stretches. The short C-terminal helical region would be defined by α-helical hydrogen bonds 6 ← 10 and 7 ← Lol. We have constructed molecular models and, by allowing Gly 5 and Gly 6 to adopt a fully extended conformation ($\phi = 180°$, $\psi = 180°$), the internitroxide distance in trich-4,8 exceeds 12 Å. Such a distance would give only weak dipolar coupling in the allowed ESR spectrum as observed. We favor the second case, shown in Figure 7, because it preserves a largely hydrogen-bonded structure as suggested by the published infrared spectra. In either case, it is very likely that the flexibility in the central region would be reduced by Gly → Ala or similar helix-favoring substitutions at positions five or six. Such substitutions may offer a new strategy for modulating the membrane-modifying properties of trichogin GA IV.

Finally we note that Figure 6 reveals an interesting and unexpected feature of trichogin GA IV. Typically, the folded helical state is favored at low temperature. For example, in aqueous solution, structural studies on Ala-rich peptides demonstrate an increase in helix content as the temperature is reduced (see, for example, refs 30, 35). For trichogin GA IV, Figure 6 suggests a gentle trend in the opposite direction; the fraction of helix decreases as the temperature is reduced. In other words, below 200 K, trichogin GA IV exhibits cold denaturation. There is only one previous report for cold denaturation of helical peptides, and this was for a series of mainly Ala-rich helices in water/hexafluoro2-propanol.[36] Cold denaturation, which arises when the unfolded state has a higher heat capacity than the folded state, plays a key role in the thermodynamics of protein folding because it provides insight into the temperature dependence of the folding free energy.[37] Our results on trichogin GA IV in methanolic solution suggest that cold denaturation may be a general property of helical peptides. Above 200 K we do not know whether this trend continues, but as discussed above, ESR spectra at 298 K suggest a persistence of the unfolded conformers.

Conclusion

By combining allowed $g \approx 2$ ESR with half-field distance measurements, we have developed a new strategy for characterizing multiple conformations in partially folded peptides. This

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approach has been applied to trichogin GA IV and suggests a folded structure, similar to that identified by X-ray crystallography, coexisting with a partially unfolded structure with perhaps a hinge point at residues five and six. Modification of this hinge point may offer a new strategy for modulating the biological activity of this peptide.

Acknowledgment. This work was supported by a grant from the NIH (GM 46870). Acknowledgment is also made to the donors of the Petroleum Research Fund, administered by the ACS, for partial support of this research (32164-AC4). Financial support from the MURST (Ministry of University and the Scientific and Technological Research) of Italy is also gratefully acknowledged. The authors wish to convey their gratitude to Dr. Eric Hustedt for providing the computer program used for simulating the dipole coupled spectra and to Drs. Sandra and Gareth Eaton for helpful comments on the manuscript.

JA984255C