# $^{13}C$ Spin Exchange in Amino Acids and Peptides

**M. H. Frey and S. J. Opella***

*Contribution from the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104.* *Received November 28, 1983*

**Abstract:** Rapid $^{13}C$ spin exchange occurs in natural abundance samples of the amino acid L-isoleucine·HCl and the peptide cyclic (D-Phe-Pro-Gly-D-Ala-Pro) in the presence of magic-angle sample spinning. The observation of spin exchange allows resonances from carbons bonded to each other to be identified and to have their chemical shifts correlated. It also allows the detection of carbons that are near to each other in space even when they are on separate residues. The spin exchange in $^{13}C$ resonances caused by $^{14}N$ relaxation improves resolution in the two-dimensional spectra and enables measurement of the frequency split in those resonances from carbons directly bonded to nitrogens.

The development and application of NMR experiments that detect weak homo-nuclear spin interactions offer considerable promise for spectroscopic and structural studies. These experiments identify pairs of nuclei in relatively close spatial proximity. The use of two-dimensional approaches in solution[1] and the solid state,[2,3] for $^{1}H$, $^{13}C$, $^{15}N$, and other nuclei, demonstrate the breadth of possible applications in chemistry and biochemistry. Molecules as complex as proteins are being investigated by these methods.[4,5]

The three-dimensional structures of peptides are key to understanding their chemical and biological properties. Because of the conformational flexibility of many peptides in solution, the description of their structures has proven difficult. Studies of peptides in the solid state are of interest because only one defined conformation may be present, simplifying the problem, and because direct comparisons can be made to X-ray diffraction results for those molecules that form single crystals. Peptides that cannot be crystallized in a form suitable for X-ray diffraction can be studied as polycrystalline powders. In addition, hydrophobic peptides that reside in membrane bilayers and are immobilized by their interactions with lipids can be studied by solid-state NMR.

The results presented here are for the most general case where the use of natural abundance $^{13}C$ NMR spectroscopy eliminates the need for obtaining labeled molecules and the use of powder samples means that sample handling and preparation are minimal. Two-dimensional $^{13}C$ spin-exchange spectroscopy is applied to polycrystalline powders of the amino acid isoleucine and the synthetic peptide cyclic (D-Phe-Pro-Gly-D-Ala-Pro).

Spin exchange can be caused by cross relaxation.[6] In the short correlation time limit the exchange rate depends on $1/r^6$ where $r$ is the relevant internuclear distance. In the rigid lattice time limit spin diffusion occurs as a function of $1/r^3$. Most molecules in the solid state have some sites that are completely rigid and others that undergo large-amplitude rapid motions, thus, the details of spin-exchange processes are complex. Nonetheless, spin exchange has been demonstrated to occur in liquids and solids and to occur most rapidly among nearby nuclei. Virlet and Ghesquieres[7] demonstrated that under certain conditions the dominant $^{13}C$ longitudinal relaxation mechanism is $^{13}C$–$^{13}C$ cross relaxation in unenriched organic molecules in the solid state. Maciel and co-workers[2] have shown that $^{13}C$ cross relaxation can occur in the presence of magic-angle sample spinning and that $^{14}N$ relaxation can induce spin exchange in directly bonded carbons. Ernst and co-workers[3] have used intermolecular $^{13}C$ spin exchange to describe the heterogeneity of chemical mixtures.

## Experimental Section

The experiments were carried out on a home-built double-resonance spectrometer with a 3.5 T magnet. The dilute spin-exchange experiments with magic-angle spinning were performed as described by Maciel and co-workers.[2] Phase cycling was used to remove transverse and axial interference. The results are presented as symmetrized phase-sensitive contour plots. The experiment starts with the conversion of equilibrium $^{1}H$ longitudinal magnetization into transverse $^{13}C$ magnetization by a cross-polarization sequence. The $^{13}C$ spin frequencies are "labeled" by evolving for the sequentially incremented time interval $t_1$ in order to determine the origin of $^{13}C$ magnetization observed during the $t_2$ detection period. The frequency-labeled $^{13}C$ magnetization is converted back to longitudinal magnetization with a $\pi/2$ pulse at the $^{13}C$ resonance frequency at the end of the $t_1$ interval. A fixed-spin mixing interval ($\tau_M$) allows for spin exchange among $^{13}C$ spins with different resonance frequencies. This mix period is varied only in separate experiments. Following the mix period, the $^{13}C$ magnetization is converted back to transverse magnetization with another $\pi/2$ pulse. The $^{13}C$ free induction decays affected by spin exchange are detected during time interval $t_2$. The data are processed in standard ways via two-dimensional Fourier transformation to give contour plots with the diagonal peaks representing chemical shift positions and off-diagonal cross peaks as frequencies defined by $\omega_1$ and $\omega_2$, where spin exchange occurs between sites with resonance frequencies $\omega_1$ and $\omega_2$.

L-Isoleucine was obtained from Sigma Chemical Co. It was recrystallized from concentrated HCl to form the hydrochloride salt. The cyclic (D-Phe-Pro-Gly-D-Ala-Pro) was synthesized by A. L. Rockwell and L. M. Geirasch in the Department of Chemistry at the University of Delaware. The actual sample used in the experiments has the Gly residue enriched in $^{15}N$ and was recrystallized from chloroform and methanol.

## Results

**A. Isoleucine.** One-dimensional chemical-shift spectra and projections from the two-dimensional spin-exchange spectra show resolution among all $^{13}C$ sites in isoleucine in Figure 1. These spectra were obtained with irradiation at the $^{1}H$ resonance frequency during data acquisition and magic-angle sample spinning so isotropic chemical-shift resonances are observed. Each resonance is a single line with the exception of the $\alpha$ carbon where the bonded $^{14}N$ of the amino group broadens and splits the $^{13}C$ resonance.[8,9] Figure 1A presents two-dimensional exchange data for a mix time of $\tau_M = 0.5$ s. The presence of off-diagonal cross peaks with this relatively short mixing interval means that the exchange process is efficient, even in the presence of the magic-angle sample spinning which is capable of averaging out static $^{13}C$–$^{13}C$ dipolar couplings. The longer mix time ($\tau_M = 1.0$ s) used in Figure 1B results in cross peaks among carbonyl and aliphatic resonances. The cross peaks as mapped out in the figure do not discriminate between inter- and intramolecular exchange. The relatively long mix interval allows the $^{13}C$ magnetization to exchange among all available carbon sites. Similar results are

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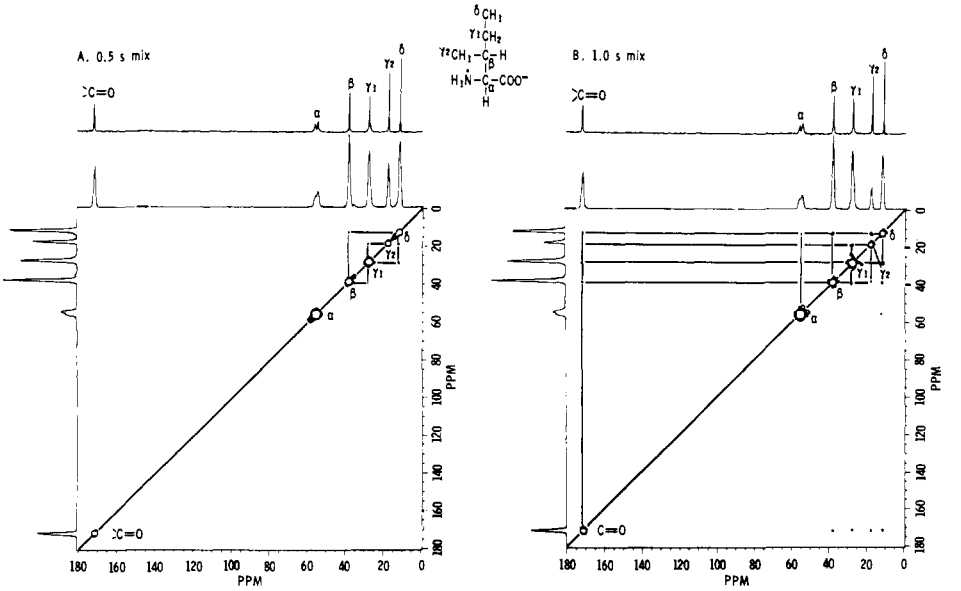


**Figure 1.** $^{13}C$ NMR spectra of polycrystalline L-isoleucine·HCl. The top one-dimensional chemical-shift spectra were obtained with proton decoupling and magic-angle sample spinning. The isoleucine resonances are labeled. The lower (and side) one-dimensional spectra are projections from the two-dimensional spin-exchange spectra. The two-dimensional contour plots were generated by using the procedure described in the Experimental Section. There were 256 $t_1$ points and 512 $t_2$ points. Both dimensions were zero filled to 2048 points. Each $t_1$ value had 64 free induction decays co-added with a recycle delay of 2 s. (A) The mix time was $\tau_M$ = 0.5 s. (B) The mix time was $\tau_M$ = 1.0 s.

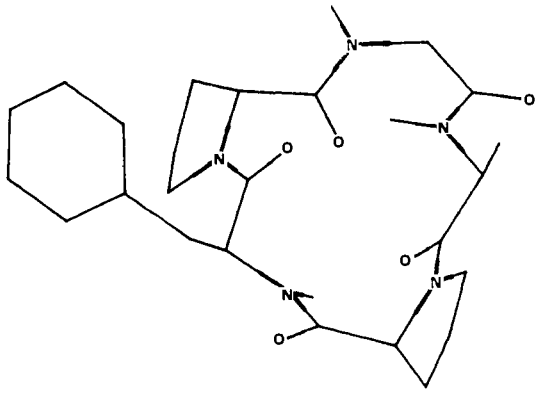

**Figure 2.** Structure of cyclic (D-Phe-Pro-Gly-D-Ala-Pro) based on the coordinates of ref 10.

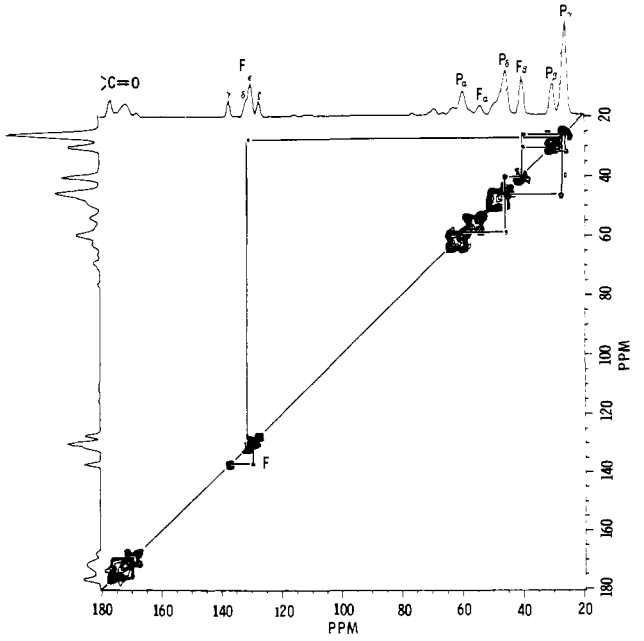


**Figure 3.** $^{13}C$ NMR spectra of *cyclo*(D-Phe-Pro-Gly-D-Ala-Pro) as described for Figure 1 with a mix time of $\tau_M$ = 4.0 s.

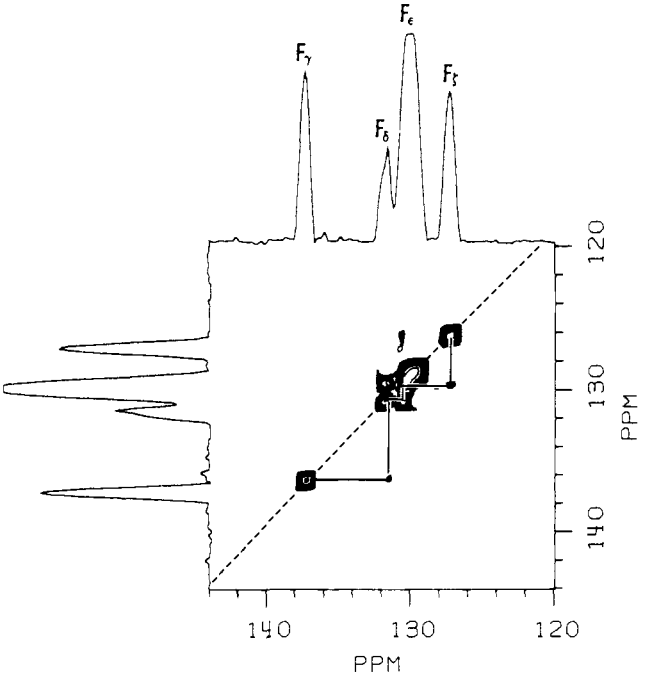


**Figure 4.** Expansion of Figure 3 showing the aromatic resonance region. The phenylalanine ring carbon resonances are labeled.

obtained for other crystalline amino acids. This lack of discrimination restricts possible interpretation of the data for small molecules in a closely packed lattice.

**B. Cyclic (D-Phe-Pro-Gly-D-Ala-Pro).** The pentapeptide cyclic (D-Phe-Pro-Gly-D-Ala-Pro) has the structure shown in Figure 2 in crystals.[10] This peptide has both $\beta$ and $\gamma$ turns, and the phenylalanine and proline side-chain rings are in relatively close spatial proximity. We have previously examined various aspects of this molecule by solid-state NMR.[11-13]

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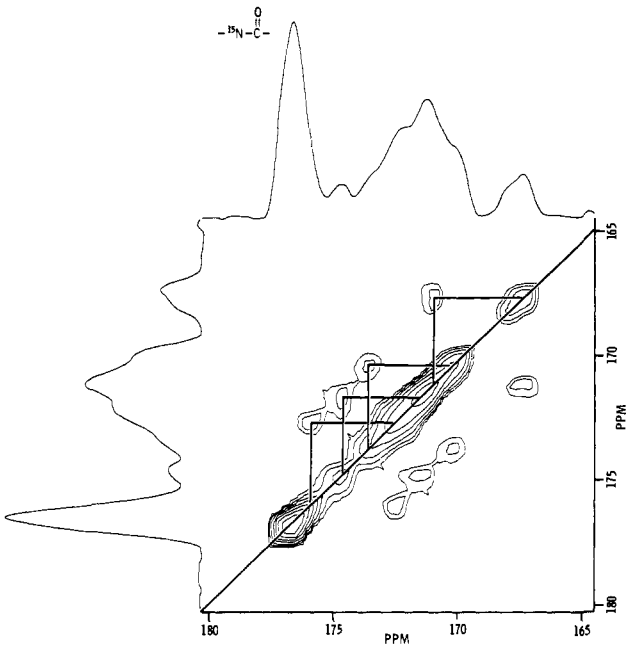


**Figure 5.** Expansion of Figure 3 showing the carbonyl resonance region. The resonance from the Pro-2 carbonyl carbon bonded to the $^{15}N$ of Gly is marked.

The results for the cyclic pentapeptide indicate more selectivity in the spin exchange of a larger, more complex molecule. Both spectroscopic and structural information are available from the two-dimensional spin-exchange spectra. The complete spectra in Figure 3 are too crowded to appreciate the resolution obtained in two dimensions; therefore, expansions of the aromatic (Figure 4) and carbonyl (Figure 5) regions are presented.

The two-dimensional spin-exchange spectrum in Figure 3 clearly shows the presence of cross peaks between phenylalanine $C_\delta$ and proline $C_\gamma$ resonances and phenylalanine $C_\beta$ and proline $C_\beta$, $C_\gamma$, and $C_\delta$ resonances. These cross peaks indicate that spin exchange occurs through-space. The through-space interaction observed by spin exchange between the phenylalanine ring and proline ring resonances shows the close spatial proximity of side chains from two different residues. The juxtaposition of molecules in the crystal lattice[10] makes it impossible to unambiguously assign this interaction to inter- or intramolecular spin exchange. Nonetheless, spin exchange between Phe and Pro residues demonstrates the possibility for obtaining spatial information by this method. In general, the success in identifying conformational features of individual molecules will depend on their relative isolation from their neighbors in the crystal lattice. Large intermolecular distances would reduce the influence of competing inter- and intramolecular exchange, although in some cases the intermolecular interactions are of interest.[3] The spectrum in Figure 3 also indicates spin exchange between various sites on the proline rings.

The aromatic region is shown in an expanded form in Figure 4. All of the cross peaks observed here are due to spin exchange between directly bonded carbons in the phenylalanine ring. Since the resonance at 137 ppm is from a carbon without a bonded proton on the basis of the results of a Selpen experiment,[14] it can be assigned to the phenylalanine $\gamma$ carbon. The entire aromatic region can be assigned on the basis of the data in Figure 4 by tracing the $^{13}C$ through-space connectivities from the $C_\gamma$ resonance to the resonances from $C_\delta$, $C_\epsilon$, and $c_\zeta$ sites. This particular phenylalanine is static on the NMR time scales[12,13] and potentially has two $C_\delta$ and two $C_\epsilon$ resonances; however, the lack of chemical-shift differences in the one-dimensional spectrum means that the extra cross peaks do not occur because they can be present only if the carbons have different $\omega_1$ and $\omega_2$ frequencies.

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The cross peaks described above result from interactions between nearby carbons. Cross peaks also result from $^{14}N$ relaxation inducing interchange between the upfield (±1 states) and downfield (0 state) resonances from those carbons directly bonded to nitrogen.[2] Figure 5 is an expansion of the carbonyl resonance region of Figure 3. The one sharp single carbonyl carbon resonance line is from the Pro-2 carbonyl group which forms a peptide bond with the $^{15}N$-Gly. The splitting and broadening of all of the other carbonyl carbon resonances results in an overlapping one-dimensional chemical-shift spectrum with poor resolution. The off-diagonal peaks in Figure 5 are a consequence of the $^{14}N$ relaxation influencing the split $^{13}C$ resonances.[2] The two-dimensional spectrum is remarkable in having complete resolution of the various carbonyl resonances and unique assignment of both components of each carbonyl resonance because of the $^{14}N$-induced spin connectivity. The one non-split carbonyl resonance from the carbonyl bonded to the one $^{15}N$ in the molecule is readily seen in both the one- and two-dimensional plots. The magnitude of the splitting between the two compounds of each carbonyl resonance can be measured directly from the two-dimensional contour plot. Such measurements are impossible in the one-dimensional spectrum because of spectral overlaps.

## Discussion

Natural-abundance $^{13}C$ spin-exchange experiments provide two distinct types of information. Under experimental conditions where short-range interactions are emphasized, the connectivities between resonances from adjacent carbons and between the two parts of the resonances from carbons bonded to nitrogen can be established. This is primarily of spectroscopic importance for enhancing resolution amd making resonance assignments. In addition, the two-dimensional spectra where the off-diagonal peaks are from $^{14}N$ relaxation causing exchange of the $^{13}C$ spins can be used to accurately measure the $^{14}N$-induced $^{13}C$ resonance splittings. These splittings are useful in determining various molecular features, such as quadrupole coupling constants, bond lengths, and geometry of the C–N bond.[8,9]

The presence of cross peaks due to spin exchange between carbons that are relatively close spatially and the absence of cross peaks for carbons that are distant provide the basis for a general method of determining the structure of molecules. The results for the crystalline amino acid isoleucine suggest difficulties in fully exploiting these methods for small molecules that are closely packed in crystals, because both inter- and intramolecular exchange occur with similar rates.

The results for the cyclic pentapeptide are more promising, suggesting that this approach will be more selective on larger molecules. Short-range interactions provide resonance assignments as shown for the phenylalanine ring (Figure 4). They are also useful in quite a different way with the $^{14}N$-induced $^{13}C$ spin exchange in the carbonyl region providing extraordinary resolution enhancement and a method for measuring $^{14}N$-induced splitting (Figure 5). The through-space interaction between phenylalanine and proline resonances shows that side chains from two different residues are close to each other. Considering the importance of conformations to the properties of peptides, proteins, and other biomolecules, it is valuable to be able to obtain spatial information from powder samples. The success of the experiments in obtaining intramolecular structural information will depend in general on the relative isolation of the molecules from their neighbors in the crystal lattice. Large intermolecular distances would reduce the influence of competing intermolecular processes. However, there are cases where the relative intermolecular distances are of interest.[3]

Ideal samples for homonuclear spin-exchange studies have well-defined conformations and are relatively isolated in the solid state. Many peptides fulfill these conditions, and the experimental isolation of molecules can be arranged. Possibilities include frozen solutions of peptides in aqueous or nonaqueous solvents or even inert gases. Peptides immobilized in membrane bilayers are also appropriate samples, since the phospholipid molecules will serve to separate the peptide molecules. Protein crystals with their high

water content are also likely candidates. The use of single or multiple isotopic labels will improve selectivity and sensitivity by providing more spins in a particular location and by increasing the probabilities for spin exchange between nearby spins.

**Acknowledgment.** We thank A. L. Rockwell and L. M. Geirasch for synthesizing the sample of cyclic (D-Phe-Pro-Gly-D-Ala-Pro) and helpful discussions, D. Eggleston for help in analysis of the diffraction data, and J. Hempel for preparing Figure 2. This research is being supported by the National Institutes of Health (GM-24266 and GM-29754). S.J.O is a Senior Fellow at Smith, Kline, and French Laboratories. M.H.F. was supported by a Cell and Molecular Biology Training Grant.

**Registry No.** (D-Phenyl-Pro-Gly-D-Ala-Pro), 75929-66-7; L-isoleucine hydrochloride, 17694-98-3.

# High-Affinity Binding of Quinine to Iron(III) Porphyrins: Novel Formation of Alkoxide Complexes from Alcohols and Amines

**D. V. Behere and Harold M. Goff***

*Contribution from the Department of Chemistry, University of Iowa, Iowa City, Iowa 52242.*
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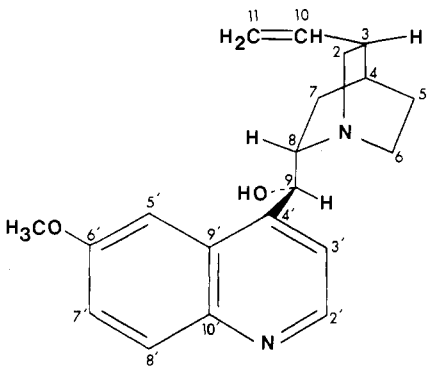
**Abstract:** The antimalarial drug quinine is shown to have a high affinity for binding to iron(III) porphyrin derivatives in nonaqueous solution. Various spectroscopic measurements are consistent with coordination through a deprotonated benzylic alcohol residue rather than through quinoline or quinuclidine nitrogen atoms. Deprotonation of the alcohol is facilitated by the basic quinuclidine moiety. Addition of simple alcohols and amines to iron(III) porphyrins in aprotic solvents likewise yields alkoxide complexes rather than mixed alcohol–amine complexes. Optical spectra for the quinine adducts resemble those of previously characterized alkoxide iron(III) porphyrin complexes. Proton NMR spectra show hyperfine-shifted porphyrin signals that are distinctive for alkoxide complexes. Hyperfine-shifted quinine resonances also provide compelling evidence for adduct formation. The stoichiometry for iron(III) coordination is 1:1, and the resulting complex is in the high-spin $S = {}^5/_2$ state on the basis of NMR hyperfine-shift patterns, magnetic measurements, and EPR spectra. Quinine binding to $\mu$-oxo dimeric iron(III) porphyrins was not detected. It is thus demonstrated that quinine interactions with heme-containing malarial pigments in the red blood cell are unlikely to involve coordination of drug nitrogen residues or ligation to a $\mu$-oxo dimeric iron(III) porphyrin.

## Introduction

The antimalarial drug quinine reportedly exhibits high-affinity binding to iron(III) porphyrin species in both aqueous and nonaqueous solution.[1,2] Aside from the possibility of only $\pi$–$\pi$ stacking interactions between quinoline and porphyrin rings,[3] three basic residues are available for coordination as is apparent in the quinine structure shown below. Suggestions of quinuclidine




nitrogen coordination to iron(III) porphyrins contained in "wet" benzene solution[2] has been challenged[4] on the basis of comparative optical spectra. A 1:1 iron(III) protoporphyrin–quinine aggregated complex is formed at neutral pH in aqueous solution and this adduct is optically active in the Soret wavelength region (negative circular dichroism band) by virtue of coordination of the chiral quinine ligand.[5] The molecular basis for quinine–iron(III) porphyrin interactions has not been unambiguously demonstrated by previous workers for either aqueous or nonaqueous solutions.

A variety of physical measurements reported here for nonaqueous solutions have served to define the quinine binding site in aprotic media. Proton and carbon-13 NMR spectroscopic methods are emphasized, and magnetic, electron paramagnetic resonance, optical spectral, and electrochemical techniques are utilized in a complementary fashion. The vast literature relating molecular and electronic iron porphyrin structures with results for the various physical methods has made possible detailed characterization of the quinine–iron(III) porphyrin interaction. A novel example of iron(III) porphyrin–alkoxide coordination induced by an adjacent amine residue is recognized here for the first time. On the basis of comparative spectroscopic measurements the previously reported mixed "alcohol–amine" iron(III) porphyrin complexes[6] are more reasonably described as alkoxide complexes. In general, alkoxide iron(III) porphyrin complexes can be generated in aprotic media through addition of an alcohol and small quantities of an aliphatic amine. The relevance of quinine alkoxide binding to cellular antimalarial drug receptors cannot be directly evaluated from the nonaqueous model study, but this newly discovered mode of interaction must now be considered among the various possibilities.

## Experimental Section

Quinine (Sigma Chemical Co.) was vacuum dried at 125 °C overnight prior to transfer under a nitrogen atmosphere to the drybox. Quinuclidine (Sigma) was recrystallized from *n*-hexane in the drybox. Quinoline

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