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Internuclear Distance Measurements up to 0.44 nm for Retinals in the Solid State with 1-D Rotational Resonance $^{13}$C MAS NMR Spectroscopy

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Abstract: The results presented in this paper show that accurate through-space internuclear distance measurements can be performed on doubly labeled retinals using the one-dimensional approach to the solid state magic angle spinning (MAS) rotational resonance NMR technique. The apparent splitting $\Delta \omega_1$ of the resonances at $n = 1$ rotational resonance for the labeled vinylic positions of (all-$E$)-[10,20-$^{13}$C$_2$]-retinal, (all-$E$)-[11,20-$^{13}$C$_2$]-retinal, and (all-$E$)-[12,20-$^{13}$C$_2$]-retinal can be simulated with a coherent set of parameters. From a series of simulations with different dipolar coupling constant $b_{IS}$ it appears that $b_{IS}/2\sqrt{8} = 1.15(\Delta \omega_1/2\pi) + 7$ (Hz) to a good approximation. Using this relationship as a calibration, it is demonstrated with a set of model compounds that straightforward Lorentzian fitting to measure $\Delta \omega_1$ can be used to determine internuclear distances up to 0.44 nm in doubly labeled retinals in the solid state.

Rotational resonance is a high-resolution solid state magic angle spinning (MAS) NMR technique in which the interference of the MAS with the homonuclear dipole interactions within a pair of equal spins $I$ and $S$ gives rise to dipolar recoupling.1,2 This phenomenon can be exploited to measure the internuclear distance. The rotation frequency of the sample $\omega_1$ is adjusted to match a rotational resonance condition $\omega_1 = \Delta \omega_{IS}$ where $\Delta \omega_{IS}$ is the difference between the isotropic shifts. With $n$ a small integer and a homonuclear dipole coupling

$$B_{IS} = \left( -\frac{\mu_0}{4\pi} \right) \frac{\gamma_1^2 h}{r_{IS}^3}$$

(1)

sufficiently strong compared to the average linewidths, the line shapes change at or close to a rotational resonance condition and additional fine structure or broadening can be observed. In eq 1 $\mu_0$ is the magnetic permeability in vacuum, $\gamma$ is the gyromagnetic ratio of the nuclei, and $r_{IS}$ represents the internuclear distance. To a first-order approximation, neglecting the influence of anisotropy and relaxation effects, an apparent splitting $\Delta \omega_1$ of each resonance is expected.1 At or close to $n = 1$ rotational resonance the spectrum of a doubly labeled sample can be described by

$$S(\omega) = \frac{1}{16\pi b_{IS}} \sum_{M=1}^{8} Y^{(1)}(\omega) \left( \frac{\Omega_{sl}(\omega)}{b_{IS}(2\sqrt{8})} \right)$$

(2)

with $\Omega_{sl}(\omega)$ the set of frequency offset functions for the eight spectral components and

$$Y^{(1)}(\chi) = \left[ \frac{1}{(1 - x^2)} + 1 \right]^{1/2} + \left[ \frac{1}{(1 - x^2)} - 1 \right]^{1/2}$$

(3)

the line shape function,1 which is only evaluated for $0 < |x| < 1$. The extrema of the spectrum can be found at the positions where $x = 1$ or $x = -1$; in these cases the line shape function goes to infinity. In the absence of $J$-coupling these singularities can be found at $\omega = \omega_1 \pm b_{IS}/2\sqrt{8}$ and $\omega = \omega_1 \pm b_{IS}/2\sqrt{8}$, predicting a “splitting” in the line shape $b_{IS}/2\sqrt{8}$.1

In an established approach to measure distances between a pair of $^{13}$C atoms, one of the spins is selectively inverted and the rotor-driven exchange of magnetization is followed in time by collecting a series of 1-D datasets.3 The trajectory of the dipolar splitting $\{t, S(t)\}$ may then be simulated to yield the internuclear distance.4-3 The inherently two-dimensional Zeeman polarization exchange NMR technique has already, at an early stage, been successfully applied to specifically doubly $^{13}$C-labeled retinal in the light-driven ion pump bacteriorhodopsin,4 which contains a retinoid photochemical energy converter.5 For the bacteriorhodopsin the rotational resonance technique has been used to discriminate between s-cis and s-trans conformations, providing for the first time genuine structure information to atomic resolution for an intrinsic membrane protein.6,7 The bacteriorhodopsin membrane protein is a member of an important class of biological photoreceptors. Another member of the class, rhodopsin,8 is a G-protein-coupled receptor and responsible for the photochemical energy conversion and the primary steps in visual signal transduction in vertebrates.

The aim of this investigation is to explore the possibility of measuring through-space internuclear distances up to 0.44 nm with high accuracy from the analysis of the rotational resonance line shape, obtained through a single one-dimensional experiment on a di-labeled retinal. The one-dimensional approach to the rotational resonance distance measurements is illustrated and calibrated on a set of doubly labeled compounds, (all-$E$)-[10,20-$^{13}$C$_2$]-retinal, (all-$E$)-[11,20-$^{13}$C$_2$]-retinal, and (all-$E$)-[12,20-$^{13}$C$_2$]-retinal.
Paragraph 1:

The intramolecular structure is the X-ray structural data. Because of the antiparallel arrangement of retinal molecules, the ionone ring is not drawn completely, since structural positions labeled for this study. (b) Antiparallel arrangement of (all-E)-retinal, showing the positions labeled for this study. In Figure 1b, a representation of the relative orientation of two retinal molecules in the crystalline solid state of (all-E)-retinal is shown. In this figure the ionone ring is not drawn completely, since structural disorder prevented the determination of all atom positions with X-ray structure determination. Because of the antiparallel spatial ordering, the shortest intermolecular internuclear distance between the 10 and 20' positions in two different molecules is 0.367 nm (indicated with a dotted line). However, for a sample containing dilute highly enriched pairs, the intramolecular coupling will dominate, while the strongest dipolar interaction in a uniform [10,20-13C2]-labeled sample is associated with the intermolecular coupling.

In order to measure distances between the labeled positions in these model compounds, we carefully analyzed resonance line shapes using simulations and fit procedures. To achieve this, high-resolution CP/MAS NMR spectra were recorded for pure [12,20-13C2]-, pure [11,20-13C2]-, and [10,20-13C2]retinal diluted to 15% in natural abundance (all-E)-retinal and 85% natural abundance (all-E)-retinal (Fluka Switzerland), in n-pentane with subsequent recrystallization at -20 °C. All manipulations with isomerically pure retinals were performed in dim red light (λ > 700 nm) or in the dark. The retinals were stored in an argon atmosphere at -80 °C. Crystalline samples were packed into zirconium oxide rotors with KEL-F caps.

Materials and Methods

Synthesis of (all-E)-[10,20-13C2]retinal, (all-E)-[11,20-13C2]retinal, and (all-E)-[12,20-13C2]retinal was performed with procedures originally developed by Groesbeek et al. starting from commercially available [1-14C]acetone, [2-14C]acetone, [3-14C]acetic acid, and [3-13C]methyl iodide (Cambridge Isotope Laboratories, MA). After silica gel column chromatography purification of the retinal isomer mixture, the (all-E)-retinals were crystallized from n-pentane at -20 °C. The purity of the labeled retinals was confirmed with 300 MHz 1H NMR (CDCl3), 75.4 MHz 13C no-decoupled NMR (CDCl3), and mass spectrometry, and the isotopic incorporation is >99% at both positions. Subsequently five samples were prepared: pure (all-E)-[10,20-13C2]-retinal, pure (all-E)-[11,20-13C2]retinal, pure (all-E)-[12,20-13C2]retinal, (all-E)-[10,20-13C2]retinal diluted to 15% in natural abundance (all-E)-retinal, and (all-E)-[11,20-13C2]retinal diluted to 15% in natural abundance (all-E)-retinal. The dilute samples were prepared from a solution of 15% labeled (all-E)-retinal and 85% natural abundance (all-E)-retinal (Fluka Switzerland), in n-pentane with subsequent recrystallization at -20 °C. All manipulations with isomerically pure retinals were performed in dim red light (λ > 700 nm) or in the dark. The retinals were stored in an argon atmosphere at -80 °C. Crystalline samples were packed into zirconium oxide rotors with KEL-F caps.

Results and Discussion

Figure 2 shows the 100.6 MHz CP/MAS NMR spectra of (all-E)-[10,20-13C2]retinal (a, b), (all-E)-[11,20-13C2]retinal (c, d), and (all-E)-[12,20-13C2]retinal (e). The short intermolecular distance r_{10,20} = 0.367 nm is indicated with a dotted line. The ionone ring is not completely resolved.

### Table 1

<table>
<thead>
<tr>
<th>Label</th>
<th>δ (kHz)</th>
<th>η</th>
<th>T₂' (ms)</th>
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<tr>
<td>10</td>
<td>134.1(0.1)</td>
<td>-11.3(0.3)</td>
<td>0.7(0.1)</td>
</tr>
<tr>
<td>11</td>
<td>127.9(0.1)</td>
<td>-8.0(0.3)</td>
<td>1.0(0.1)</td>
</tr>
<tr>
<td>12</td>
<td>125.6(0.1)</td>
<td>-1.6(0.3)</td>
<td>1.0(0.1)</td>
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</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Δω/2π (Hz)</th>
<th>Γ (Hz)</th>
<th>b_92ν/σ (Hz)</th>
<th>r_90 (NMR) (nm)</th>
<th>r_90(X-ray) (nm)</th>
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</thead>
<tbody>
<tr>
<td>10,20 (15%)</td>
<td>12</td>
<td>41</td>
<td>20.8</td>
<td>0.505</td>
<td>0.440</td>
</tr>
<tr>
<td>10,20 (100%)</td>
<td>35</td>
<td>45</td>
<td>47.3</td>
<td>0.383</td>
<td>0.367</td>
</tr>
<tr>
<td>11,20</td>
<td>78</td>
<td>48</td>
<td>96.7</td>
<td>0.302</td>
<td>0.296</td>
</tr>
<tr>
<td>12,20</td>
<td>141</td>
<td>55</td>
<td>169.2</td>
<td>0.250</td>
<td>0.253</td>
</tr>
</tbody>
</table>

For comparison the X-ray distances are also listed.

Internuclear Distance Measurements for Retinals


Figure 2. Comparison of $^1$H CP/MAS data at $n = 1$ rotational resonance (solid lines) with data at $\omega / 2\pi = 10.0$ kHz (dotted lines): well off rotational resonance for pure (all-$E$)-[10,20-$^{13}$C$_2$]retinal, 99% labeled at both positions (a), (all-$E$)-[10,20-$^{13}$C$_2$]retinal diluted to 15% in natural abundance (all-$E$)-retinal (b), pure (all-$E$)-[11,20-$^{13}$C$_2$]retinal 99% dilabeled (c), (all-$E$)-[11,20-$^{13}$C$_2$]retinal diluted to 15% in natural abundance (all-$E$)-retinal (d), and (all-$E$)-[12,20-$^{13}$C$_2$]retinal (e). For all five samples spectra were accumulated with $\omega / 2\pi = 10.0$ kHz (dotted lines) and at the respective $n = 1$ rotational resonance conditions (solid lines). For the [10,20-$^{13}$C$_2$] samples $\omega / 2\pi = 11.794(2)$ kHz, for the [11,20-$^{13}$C$_2$] samples $\omega / 2\pi = 12.198(2)$ kHz, and for the [12,20-$^{13}$C$_2$] sample $\omega / 2\pi = 12.180(2)$ kHz at the $n = 1$ rotational resonance condition. The data in Figure 2 are scaled in order to keep the integrated intensities of the signals approximately equal at the different spinning speeds. The effect of the rotor-driven dipolar recoupling on the $n = 1$ line shape is now evident. The resonances of the $n = 1$ spectra are broadened compared to the 10.0 kHz spectra, and additional fine structure is observed for the [11,20-$^{13}$C$_2$] and [12,20-$^{13}$C$_2$] compounds. The $n = 1$ spectra of the [10,20-$^{13}$C$_2$] sample show a broadening of the signals, but no additional fine structure. The dipolar recoupling effects are more pronounced for (all-$E$)-[11,20-$^{13}$C$_2$]- and (all-$E$)-[12,20-$^{13}$C$_2$]retinal since the $r_{11,20} = 0.296$ nm and the $r_{2,20} = 0.253$ nm are considerably smaller than $r_{10,20} = 0.440$ nm.

If the spin pair involves a methyl and a vinyl carbons, $\Delta\sigma_{IS}$ $\approx |\delta_1| + |\delta_3|$, the sum of the absolute values of the chemical shift anisotropies. To first order a splitting of the resonance at the $n = 1$ rotational resonance condition is expected for the stronger dipolar couplings. Since the dipolar interaction is symmetric in the two spins, a similar rotational resonance line shape for both label signals, for $n = 1$, is in principle expected in the case of small shift anisotropy.

In Figure 2c, d the C-11 resonance is apparently a doublet, but the C-20 line shape is more complicated. It is immediately obvious that for the [11,20-$^{13}$C$_2$] compound only the response from the vinyl C-11 resonance is in line with the generally accepted theory, which predicts a neat splitting of both lines. For the [12,20-$^{13}$C$_2$] compound both labeled sides of the $n = 1$ rotational resonance give rise to a doublet type contribution to the spectrum, although the C-20 response appears distorted.

The $n = 1$ experimental data can be compared with theoretical predictions in detail to address the applicability of the theoretical approaches and to calibrate a method for measuring distances. It should be stressed here that the parameters used for these simulations are not fitted to the rotational resonance data, but are from independent sources. All parameters used for the simulations are from X-ray and separate MAS experiments and are listed in Tables 1 and 2. The isotropic chemical shifts $\sigma_i$ were measured by fitting the MAS centerbands for the $\omega / 2\pi = 10.0$ kHz datasets with Lorentzians. The anisotropy parameters $\delta$ and $\eta$ were obtained by a Herzfeld–Berger analysis $\delta$ of datasets collected with $\omega / 2\pi = 2.149$ kHz, $\omega / 2\pi = 1.564$ kHz, and $\omega / 2\pi = 1.937$ kHz for the [10,20-$^{13}$C$_2$], [11,20-$^{13}$C$_2$], and [12,20-$^{13}$C$_2$] compounds, respectively (data not shown). Pseudotransverse relaxation times $T_2^*$ used for the simulations were estimated from the Lorentzian line widths $\Gamma$ at $\omega / 2\pi = 10.0$ kHz according to $T_2^* = (\pi \Gamma)^{-1}$. For all the compounds used in our study, the $J$ couplings are very small and can be neglected. In the rotational resonance simulations, the dipolar coupling $b_{IS}$ is calculated from the internuclear distance using eq 1. A right-handed principal axis frame for the dipolar recoupling tensor was taken as the frame of reference with the positive $x$ axis along the vector connecting the vinyl C-20 and the $y$ axis perpendicular to the $x$ axis in the conjugated plane (Figure 3). The orientations of the principal axis frames of the chemical shift tensors with respect to the molecular frame are not known exactly. For a vinylic carbon, the $\sigma_{11}$ axis, corresponding to the most upfield principal component, is almost perpendicular to the plane of conjugation. The $\sigma_{33}$ axis, for the most downfield component, is approximately in the conjugated plane perpendicular to the double bond, and finally the $\sigma_{22}$ axis is along this bond (see, e.g., Veeman $\delta$). To obtain the reduced tensor elements for the simulations, $\sigma_{xx}$, $\sigma_{yy}$, and $\sigma_{zz}$, with $|\sigma_{zz}| \geq |\sigma_{xx}| \geq |\sigma_{yy}|$, the isotropic chemical shift $\sigma_i$ was subtracted and the principal axis components were rearranged.

The resulting orientations of the principal axes for C-12, C-11, and C-10 are also depicted in Figure 3. The effect of the methyl anisotropy on the line shape is negligible since $\Delta\sigma_{IS}$ is much larger than the methyl anisotropy $\delta$. An approximate orientation of the principal axis frame for the 20-methyl was inferred from the various compilations of chemical shift anisotropy data presented in the literature, indicating that the $\sigma_{11}$ axis should be roughly along the chemical bond, while the $\sigma_{33}$ axis is most likely perpendicular to the bond in the plane of the molecule. This choice parallels earlier rotational resonance investigations.$^4$ After subtraction of $\sigma_i$ and rearrangement of the principal components, the principal axis frame orientation depicted in Figure 3 results.

$^{13}$ Veeman, W. S. Prog. NMR Spectrosc. 1984, 16, 193.
For the methyl principal axis frame into the dipolar reference frame.


The methyl principal axis frame into the dipolar reference frame.


In Figure 5, the C-11 position and simulations is illustrated in Figure 5. In

negligable differences in the spectra.

with different sets of Euler angles were generated, giving almost

different values for the parameters. In particular, simulations
calculating theoretical predictions for the line shapes using

the vector connecting C-10 and C-20

dipolar frame. Finally, for the pure [10,20-13 C 2 ]retinal, in which

that in the case of dilute [10,20-13 C 2 ]retinal. The deviation of

the intermolecular coupling dominates, the orientation of the
dipolar tensor and therefore the reference frame is different from

the 20-methyl, the deviation may still be larger. However, since

the anisotropy on the rotational

line shapes is only minor. This was verified by calculating theoretical predictions for the line shapes using different values for the parameters. In particular, simulations with different sets of Euler angles were generated, giving almost negligible differences in the spectra.

For instance, a comparison between experimental data for the C-11 position and simulations is illustrated in Figure 5. In

this figure, the signals of the vinylic and methyl labels are shown, with ωr/2π = 10 kHz and at n = 1 rotational resonance.

In addition, the first and second derivatives of the signals are plotted. All resonances are scaled so that the amplitudes of the C-11 resonances coincide. For the vinylic C-11 the agreement between the data and simulations is quantitative with respect to the positions of the maxima and qualitative with respect to the overall line shape.

From the comparison of the experimental results with the simulated data in Figure 5, it is evident that it is impossible to simulate the experimentally observed line shapes of both spins in detail for internuclear distances in the 0.3 nm range with the rotational resonance theory that was originally developed for the description of this phenomenon within a dilute spin pair, for short internuclear distances and correspondingly strong dipolar couplings, as for adjacent carbons in one molecule. A similar conclusion was reached independently by Peersen et al., who studied the rotational resonance effects for pairs of nuclei in a small peptide.

The n = 1 second-derivative line shape for the methyl side apparently contains an off rotational resonance type single line, superimposed on the n = 1 rotational resonance "doublet". The discrepancies between experiment and isolated spin-pair theory for the methyl response originate from additional field-dependent interactions interfering with the very weak homonuclear dipolar couplings. For instance, the linewidths for the two labels are different, and for the ωr/2π = 10 kHz data the vinylic resonance has extended wings at its bottom, which are not reproduced by the theory. In contrast, the methyl side of the spin pair response is much narrower. Interestingly, the separation between the two outer lines in the second-derivative methyl response at rotational resonance matches the simulations and the characteristic splitting of the vinyl part (Figure 5c). In this investigation we focus on the extrema and ∆ω1. A discussion of the details of the line shape is beyond the scope of the present study, and will be reported elsewhere.

Interestingly, despite the obvious differences between the experimental data and the theoretical predictions with respect to the overall line shape, the ∆ω1 matches the simulations quite well. To enhance the extrema for further analysis, we now often

use second derivatives of the data and the corresponding simulations. This is illustrated in Figure 6, which shows the second derivatives of the experimental data (solid lines) and simulations (dotted lines) for pure (all-E)-[10,20-13C2]retinal and pure (all-E)-[11,20-13C2]retinal with \( \omega_i/2\pi = 10.0 \) kHz (a), at \( n = 1 \) rotational resonance \( \tau \), \( +25 \) Hz off \( \tau \) (c), \( +100 \) Hz off \( \tau \) (d), \( -25 \) Hz off \( \tau \) (e), and \(-100 \) Hz off \( \tau \) (f).

Figure 6. Comparison of second derivatives of experimental data (solid lines) and simulations (dotted lines) for pure (all-E)-[10,20-13C2]retinal and pure (all-E)-[11,20-13C2]retinal with \( \omega_i/2\pi = 10.0 \) kHz (a), at \( n = 1 \) rotational resonance condition, \( +25 \) Hz off \( \tau \) (c), \( +100 \) Hz off \( \tau \) (d), \( -25 \) Hz off \( \tau \) (e), and \(-100 \) Hz off \( \tau \) (f).

Finally, in Figure 7 a graph of the \( b_{\text{IS}}/2\pi \sqrt{8} \) in the compounds versus the \( \Delta\omega_1/2\pi \) is presented. To compare these data with the rotational resonance theory, a series of \( n = 1 \) rotational resonance second-derivative simulations was performed, with \( 10 \) Hz \(< b_{\text{IS}}/2\pi \sqrt{8} \approx 190 \) Hz (see the Supporting Information). It appears that \( \Delta\omega_1 \) in the simulations varies linearly with \( b_{\text{IS}}/\sqrt{8} \), according to the solid line in Figure 7, and can be described

\[
\Delta\omega_1 \approx 10.0 \text{ kHz}
\]

resonance second-derivative simulations was performed, with \( 10 \) Hz \(< b_{\text{IS}}/2\pi \sqrt{8} \approx 190 \) Hz (see the Supporting Information). It appears that \( \Delta\omega_1 \) in the simulations varies linearly with \( b_{\text{IS}}/\sqrt{8} \), according to the solid line in Figure 7, and can be described
2. For pure \([10,20-13\, C_2]\), \([11,20-13\, C_2]\), and \([12,20-13\, C_2]\) retinal, simulations. Applying this calibration, distances can be calculated as a good calibration for measuring distances from the data and simulations in our labeled retinals, since for practical purposes a simple expression, \(b_0/2\pi\sqrt{8} = 1.15(\Delta\omega/2\pi) + 7\) (Hz), can now be used instead of full numerical simulations. Applying this calibration, distances can be calculated for the set of model compounds which are listed in Table 2. For pure \([10,20-13\, C_2]\), \([11,20-13\, C_2]\), and \([12,20-13\, C_2]\) retinal, the results are in close agreement with the X-ray data, as can already be expected on the basis of the close correspondence between the data and simulations in the distance. Additional systematic errors arising from the deviation between the line in Figure 7 and the experimental points yield an overall error estimate of \(\sim 0.01 \pm 0.02\) nm for distances below 0.4 nm.

It thus appears that a determination of the location of the maxima associated with the singularities in the rotational resonance line shape provides a good route to the measurement of homonuclear dipolar interactions in the quasi-two-spin system at rotational resonance and internuclear distances. In this respect, peaks and shoulders in a spectrum are generally considered a source of valuable information, even when the associated spectral lines are heavily distorted, also, e.g., in optical spectroscopy.

In our study we have chosen to evaluate the particular problem of measuring distances between vinylic carbons and methyl carbons in some depth, from a practical perspective and aiming for an optimization with respect to accuracy. Other investigations that have been performed in the past have largely neglected the possibility for extracting distances from the analysis of rotational resonance line shapes, in favor of magnetization exchange experiments. The results presented here suggest that line shape analysis will be quite useful for pairs of carbons provided that essential features of the response can be described in terms of the two-spin approximation. Testing with model compounds may help to determine the accuracy that may be achieved for other specific cases, involving different types of carbons.

Conclusion

The results discussed in this paper show that it is possible to measure the internuclear distance between a vinylic and a methyl carbon in doubly labeled retinals accurately from the apparent splitting in a 1-D rotational resonance dataset. A calibration procedure is worked out and tested for four independent model distances. The advantage of this technique over the more widely used magnetization exchange experiments is that the distance information can be obtained in a single experiment and can already be extracted using straightforward fitting procedures. It should be possible to use this approach for collecting structural constraints in complex systems, for instance ligands bound to receptor proteins.

Acknowledgment. We thank M. H. Levitt, S. Vega, and their co-workers Y. Lee, X. Feng, and G. Goobes for making their simulation programs available to us and for extensive discussions. This work was financed in part by Biotechnology Grant PL920467 of the European Community.

Supporting Information Available: Figures showing measurements of the apparent splitting in the experimental data, the effect of dipolar coupling, and simulations of a series of \(n = 1\) rotational resonance spectra (3 pages). See any current masthead page for ordering and Internet access instructions.