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Through-Bond $^{13}C-^{13}C$ Correlation at the Natural Abundance Level: Refining Dynamic Regions in the Crystal Structure of Vitamin-D_3 with Solid-State NMR

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Two-dimensional ^{13}C correlation spectroscopy at the natural abundance isotope level has enabled the application of nuclear magnetic resonance (NMR) to industrial and academic problems that would be impractical if labeled materials were required. The challenge of natural abundance ^{13}C spectroscopy is the loss in signal intensity due to the 1% isotope concentration of the spins. This loss is particularly acute for correlation spectroscopy, which relies on pairs of nuclei to be spin active, decreasing the sensitivity of the technique by 4 orders of magnitude as compared to correlation experiments on uniformly labeled materials. Yet, liquid-state ^{13}C correlation spectroscopy continues to play an important role in the assignment and determination of structure in unenriched compounds through robust methods such as the INADEQUATE experiment,[1] which works despite this unfavorable scaling. In solids, however, there are far fewer examples of natural abundance ^{13}C correlation experiments, particularly on molecules with more than a dozen carbon sites.[2] Here, we show that ^{13}C natural abundance correlation in solids can be extended to moderately sized molecules, using the uniform-sign cross-peak double-quantum-filtered correlation spectroscopy (UC2QF COSY) to assign the 54 peaks of the solid-state NMR spectrum of microcrystalline vitamin-D_3. In this case, comparison between the assigned peaks and ab initio calculations of the chemical shifts based on the crystal coordinates permits a refinement of the average structure in dynamic regions reported as disordered in the crystal structure.[3]

Recently, we introduced the scalar-coupling-driven UC2QF COSY as an effective through-bond correlation spectroscopy for disordered solids.[4] This experiment is robust even under fast (35 kHz) magic-angle-spinning (MAS) and in the presence of dynamics. The latter is particularly relevant in the characterization of molecules such as HC_{60}^+, where anisotropic molecular motion renders $^{13}C-^{13}C$ dipolar-driven correlation ineffective. The scalar-coupling-driven UC2QF COSY, however, was shown to provide a reliable experimental characterization of the direct bond between the sp^2 cationic site and the protonated sp^3 site in a 10% ^{13}C enriched sample. At natural abundance isotope concentrations, correlation spectroscopy is more challenging. Yet the UC2QF COSY continues to perform well, in part due to the relatively low overall power delivered to the ^{13}C channel, which allows for good stability during the longer signal averaging periods necessary to increase the signal-to-noise ratio. Excellent correlation spectroscopy can typically be performed on smaller molecules (~10 unique sites) in less than 24 h on a 500 MHz spectrometer with 80 mg of sample. For larger molecules such as vitamin-D_3 (Figure 1), significantly longer signal averaging is required. With 2 weeks of signal averaging on a 500 MHz instrument, however, a full correlation spectrum and complete assignment of the 54 peaks from the cross-peak connectivities are obtained.

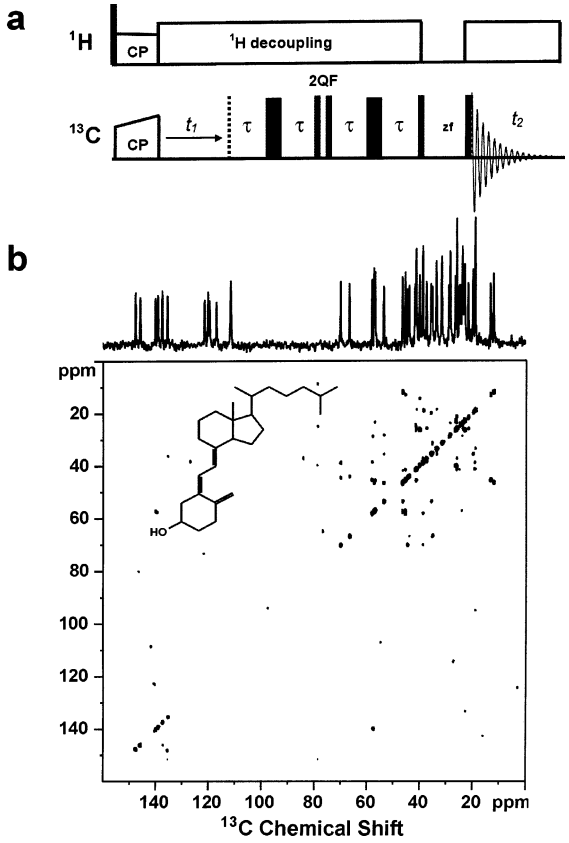

Figure 1. (a) The pulse sequence for the UC2QF COSY experiment (ref 4) appends two $\tau-\pi-\tau$ refocusing elements about the double-quantum filter (2QF) to produce a correlation spectrum with in-phase cross-peaks. Thin vertical lines indicate $\pi/2$ pulses, and thick vertical lines indicate π pulses. (b) The UC2QF COSY of microcrystalline vitamin-D_3 (Solvay Duphar) allows for the complete assignment of the 54 peaks in the solid-state spectrum. Data were acquired using 80 mg of sample and the pulse sequence from (a) on an 11.7 T Bruker DSX spectrometer (^{1}H frequency 500 MHz) equipped with a double resonance 4 mm MAS probe spinning at a MAS rate of 12 kHz. ^{13}C nutation rates of 62.5 kHz and ^{1}H decoupling at 100 kHz were used. 1024 t_2 points and 256 t_1 points were acquired with a spectral width of 20 kHz in both dimensions and a recycle delay of 2 s. τ was set to 5 ms, and, on the basis of results in glycine, we expect a transfer efficiency of ~45%.

In a single molecule of vitamin-D_3 there are 27 carbon sites, but vitamin-D_3 crystallizes in a unit cell with two inequivalent molecular conformations (α and β),[3,5] so there are two peaks for each carbon in this microcrystalline sample.[12] To determine which set of assignments for the two overlapping carbon spectra corresponds

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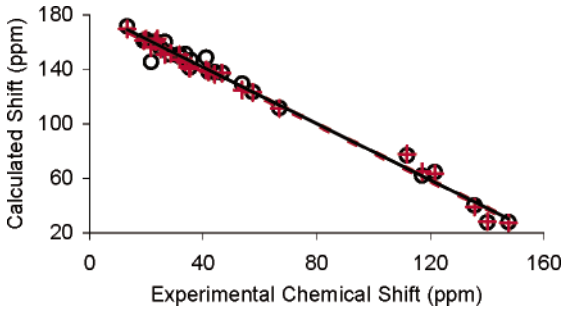

Figure 2. Calculated chemical shifts based on the crystal (○) and refined (+) coordinates versus experimental chemical shift for the β conformation of vitamin-D_3. Although no attempt is made to include the effect of vibrational motion on the chemical shifts, the sum of squared residuals for the refined side-chain structure is 1/10 that for the crystal geometry. The particularly ill-defined nature of the aliphatic side chain for the α conformation requires structural refinement before the chemical shifts can be calculated, while the fully refined solid-state structure again agrees well with the assigned chemical shifts.

to the α and β conformations, we compare the calculated chemical shifts from ab initio theory (B3LYP functional, 6-311+G(2d) basis, Gaussian 98[6]) based on the reported crystal coordinates[3] to the assigned shifts. A clear match is evident, although within each conformer there are several points that are in poor agreement, as shown in Figure 2 for the β conformation. These discrepancies correspond to the regions of disorder reported for the aliphatic side chain, and the averaged X-ray coordinates clearly underestimate certain bond lengths in this crystal structure. Interestingly, the disorder noted in the crystal structure is not reflected in the NMR spectral line widths or in additional peaks. NMR line widths are typically a good measure of conformational heterogeneity,[7] implying that the crystal disorder results purely from dynamic motion of the side chain.

To better define the side-chain conformation, we make use of CPMD,[8] a solid-state density functional theory pseudopotential structural package. This ab initio program implements periodic boundary conditions and implicitly includes condensed phase intermolecular (crystal packing) forces. For our calculations, a Troullier−Martins norm-conserving pseudopotential[9] with an energy cutoff of 70 Ry and the BLYP gradient corrected functional[10] are used. Regions of the molecule showing no conformational disorder are fixed according to the crystal coordinates, giving a framework in which the side-chain conformational space can be explored. The calculated chemical shifts for the refined molecular geometry show improved consistency with the assigned carbon spectrum (Figure 2), decreasing the sum of the squared residuals for the side chain by a factor of 10. Most significantly, C−C bond lengths that were short in the disordered regions of the X-ray structure are corrected in the minimized structure, while the torsion angles along the aliphatic side chain are not significantly altered (<5°). The good agreement between the refined and experimental chemical shifts implies that there is a single ground-state conformation undergoing significant vibrational motion rather than multiple conformations undergoing fast exchange.

Together, X-ray crystallography, ab initio structural minimization, and solid-state NMR provide complementary techniques for defining solid-state structures. Crystallography gives excellent overall structural information for well-ordered regions, but underestimates bond lengths due to molecular motion and does not necessarily differentiate dynamics and disorder. NMR can distinguish fast molecular motion from disorder, and the average chemical shift is not particularly sensitive to small-amplitude conformational averaging and can be used to ensure consistency in ab initio structural refinements. To assign larger molecules, two-dimensional correlation is imperative, and the ability to accomplish this at natural abundance levels for molecules between 10 and 100 carbons in size promises chemically and biologically significant applications. Many steroid and pharmacological agents are found in this size range, and outstanding questions often include the assignment of spectra for different crystal isomorphs and hydrates as well as the definition of disordered regions in crystal structures.[11] While week-long acquisition times for these experiments may seem time-consuming, in comparison to the synthetic efforts that would be necessary to selectively or uniformly label molecules such as the vitamin-D_3 sample, they are far less daunting. As probe sensitivity and magnetic fields continue to increase, we expect that the time for full correlation of moderately sized molecules will become even more favorable.

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Supporting Information Available: Expanded 2D spectrum, chemical shift assignments, and refined crystal coordinates (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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