Communication

# Measurement of the principal values of the chemical-shift tensors of overlapping protonated and unprotonated carbons with the 2D-SUPER technique and dipolar dephasing (DD-SUPER)

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ABSTRACT

A modified 2D-SUPER technique is demonstrated to allow independent measurement of the principal values of the chemical-shift tensors of overlapping protonated and unprotonated carbons. The insertion of a dipolar-dephasing period into the sequence causes loss of signal from protonated carbons. The spectrum obtained with this modification allows one to determine the principal values of the unprotonated carbons with high precision. Subsequent fitting of the usual 2D-SUPER spectrum, with the chemical-shift parameters of the unprotonated carbons fixed, gives the parameters of the overlapped resonances of the protonated carbons. As an example, we report the determination of the $^{13}C$ chemical-shift parameters of the carbons of form II of piroxicam. The experimental results are compared with those obtained from calculations using the DFT/GIAO method. Potential applications of this method are discussed.

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## 1. Introduction

Accurate measurement of the principal values of $^{13}C$ and $^{15}N$ chemical-shift tensors, $\delta_{11}$, $\delta_{22}$, and $\delta_{33}$, is a challenging, but important, task for gaining insight into the electronic structure and geometry of pharmaceutically significant organic solids [1–7]. In principle, the principal values of single dilute nuclear spins (e.g., $^{13}C$ or $^{15}N$) can be measured directly from the line shape of a powder pattern obtained with high-power proton decoupling. Analysis of the relative intensities of sidebands in the spectrum of a powder sample obtained under high-power proton decoupling and magic-angle spinning also provides these parameters of molecules containing many carbons. However, in cases where the molecule contains a sufficiently large number of carbons, obtaining these parameters from a one-dimensional spectrum, either of the static powder or with magic-angle spinning, can be daunting because of overlap of signals.

Various two-dimensional MAS techniques – chemical-shift recoupling [8,9], variable-angle spinning [10], magic-angle turning [11,12], and 2D phase-adjusted spinning sidebands (PASS) [13] – have been designed to overcome the problem of chemical-shift-pattern overlap. In most cases, the isotropic chemical shift is displayed along one dimension and a representation of the separated chemical-shift dispersion is along the second dimension, providing the needed resolution. These experiments generally include high-power decoupling to suppress heteronuclear dipolar coupling to protons. Each technique has advantages and disadvantages. For example, the relationship between the magic-angle-turning method and the spinning-sideband-separation method has recently been discussed [14]. One method, recoupling of chemical shift anisotropy (ROCSA) [8], is particularly useful when the sample is spun at high spinning rates and has wide application to the determination of chemical-shift parameters of bimolecular solids [15].

Among the chemical-shift-recoupling methods, separation of undistorted powder patterns by effortless recoupling (SUPER) [9] is a convenient experiment for the two-dimensional separation of isotropic and anisotropic components of the chemical shift at moderate spinning rates (2.5–5 kHz). Because of its robustness and the reduced demands on the probe, the SUPER experiment is particularly applicable to the study of organic solids [16,17].

In general, in developing these experimental techniques for separation of the isotropic and anisotropic components of the chemi-

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cal shift, it is assumed that all carbon (or nitrogen) resonances are well-resolved in the isotropic dimension. In many cases, this assumption is valid. However, for a molecule containing a large number of carbons, it is inevitable that the problem of overlapping isotropic resonances must be addressed. Overlapped resonances lead to an inability to separate powder patterns for some carbons (as well as for nitrogens). In many cases, this lack of resolution results in the reporting of some average set of chemical-shift properties for the overlapped resonances. Attempts to extract site-specific parameters by fitting such overlapped line shapes with sums of subspectra introduce significant errors due to the increased number of fitting parameters. Chemical-shift parameters extracted in this manner tend to have large uncertainties due to the ambiguities introduced because the fitting may not be unique.

In this report, we present a modified SUPER pulse sequence that includes a dipolar-dephasing delay [18]. Appropriate manipulation of spectrometer parameters allows one to obtain the powder patterns of unprotonated carbons with this sequence. With precise chemical-shift parameters of the unprotonated carbon determined in this manner, the parameters of protonated carbons whose resonances overlap the unprotonated carbons can be obtained from the usual SUPER experiment through spectral fitting, using the fixed values of the chemical-shift parameters of the unprotonated carbon to model its contribution to the line shape. Piroxicam is a non-steroidal anti-inflammatory drug. A measurement of form II of the polymorphic molecule, piroxicam, demonstrates this technique for dealing with overlapped resonances of protonated and unprotonated carbons.

## 2. Experimental

### 2.1. Sample preparation

Piroxicam occurs in at least four polymorphic structures [19]. A sample of piroxicam form I was purchased from Sigma (lot number 066K1779). Piroxicam form II was obtained from form I by recrystallization in absolute ethanol [19]. Powder X-ray diffraction (PXRD) data (not shown here) of the recrystallized product were essentially identical to previously reported data of piroxicam form II [20].

### 2.2. NMR experiments

All solid-state NMR experiments were performed with a Bruker Avance II WB400 NMR spectrometer, operating at a proton frequency of 400.13 MHz, equipped with a 4-mm CP/MAS triple resonance (XYH) probe. Standard $^{13}$C CP/MAS experiments were performed at a spinning rate of 10.000 ± 0.002 kHz. For cross-polarization, a 2.4 μs proton 90° pulse was used and the cross-polarization was carried out for 3 ms. During acquisition, protons were decoupled using the TTPM decoupling scheme [21].

The dipolar-dephasing SUPER sequence (DD-SUPER) is shown in Fig. 1. All DD-SUPER experiments were carried out at a MAS rate of 5.000 ± 0.002 Hz, which dictates the lengths of $2\pi$ pulses after cross-polarization in the X-channel for chemical-shift recoupling. A proton-decoupling field having an amplitude of approximately 117 kHz was applied during the recoupling step. For each $t_1$ increment in the anisotropic dimension, 2048 complex points were collected along the acquisition (isotropic) dimension, with 512 scans co-added for each point. There was a total of 32 complex points in the $F_1$ dimension. The recoupling step was followed by a $\gamma$-integral delay of 1 ms to eliminate spinning sidebands in the $F_1$ dimension. A TOSS sequence [22,23] was used to remove spinning sidebands along the $F_2$ dimension, as in a regular SUPER sequence. A dipolar-dephasing period (without proton decoupling) was placed prior to the last $\pi$ pulse in the TOSS sequence to suppress signals of protonated carbons [24]. The scaling factor for the DD-SUPER sequence was 0.155. It is conceivable that the use of a shorter TOSS sequence, such as a single rotor period with well separated five $\pi$ pulses, [25] would be useful particularly for carbons with a short $T_2$.

For Fourier analysis of the data, a cosine-square filter was applied in both the $t_1$ and $t_2$ dimensions. Post-transformation shearing of the spectra along the $F_1$ dimension gave chemical-shift patterns nearly centered along the $F_1$ dimension. All $^{13}$C chemical shifts were referenced externally by the high-frequency resonance of adamantane in the spectrum, whose isotropic chemical shift relative to tetramethylsilane (TMS) is 38.55 ppm. The extracted powder patterns were analyzed with the line shape analysis package of Bruker TopSpin 2.0. The chemical shift referencing of the extracted CS pattern was based on its corresponding isotropic shift obtained in the $F_2$ dimension.

### 2.3. Prediction of NMR parameters

Density functional theory (DFT) calculations of the NMR parameters were carried out with Gaussian 03 [26]. The NMR parameters were predicted using the gauge-including atomic orbitals (GIAO) method at the B3PW95 level of theory, with the 6-311++G(2d, p) basis set. All predicted chemical shifts are referred to the $^{13}$C chemical shift of tetramethylsilane calculated at the same level of theory in the same basis.

## 3. Results and discussion

The molecular structure of piroxicam is shown in Fig. 2. This polymorphic material exists in at least four distinct solid-state structures: forms I–III, and as the monohydrate. Forms I and II have similar conformations, and the free-energy difference between these two polymorphs is small [19]. The major structural difference has been suggested to be the hydrogen-bonding patterns of the two forms.

Fig. 3 shows the region from 100 ppm to 180 ppm of the solid-state CP/MAS spectra of forms I and II. The spectra are nearly identical to those previously reported [27]. The differences in these
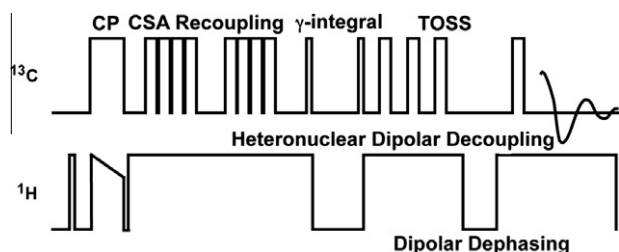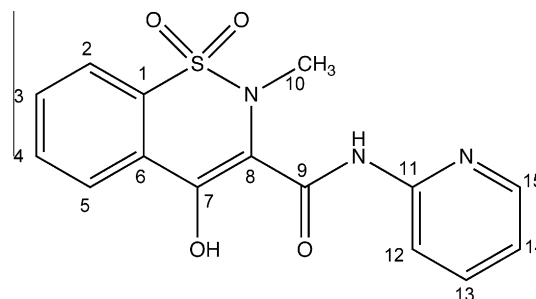


Fig. 1. Pulse sequence of the DD-SUPER experiment. The position of the insertion of the dipolar-dephasing period into the SUPER sequence [9] is indicated.



Fig. 2. The molecular structure of piroxicam, showing the numbering of the carbons.
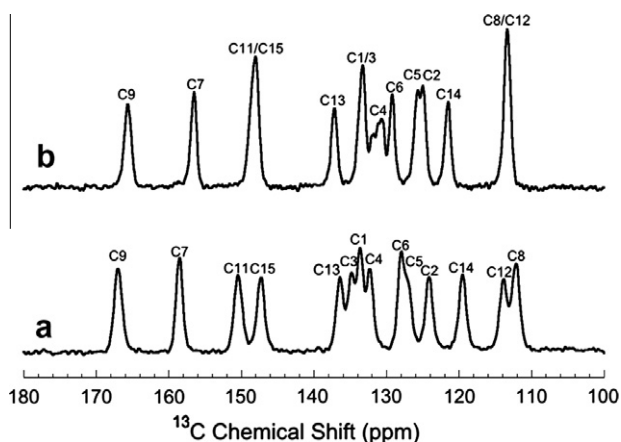
**Fig. 3.** CP/MAS spectra of two polymorphs of piroxicam: (a) form I and (b) form II.

spectra are likely the result of different hydrogen bonding interactions in the two forms.

The CP/MAS $^{13}$C chemical-shift assignments of form I, also known as β-piroxicam, have been reported previously [28]. We have verified these assignments using solution-state $^{1}$H, $^{13}$C, and heteronuclear-correlation (HSQC and HMBC, data not shown) NMR methods.

The CP/MAS spectrum of form II in Fig. 3 has significant differences from the spectrum of form I. The resonances of some carbons are shifted significantly from the positions of the equivalent carbons in form I. In particular, the spectrum contains severely overlapped resonances attributable to three pairs (C11/C15, C1/C3, and C8/C12) of carbons. Each pair contains an unprotonated carbon (C1, C8, and C11) and a protonated carbon (C3, C12, and C15). We attempted to extract the chemical-shift tensor principal values of these six carbons from a standard 2D-SUPER spectrum by fitting the overlapping patterns to a sum of subspectra. This attempted fitting required seven adjustable parameters (two sets of CS parameters and the relative-intensity ratio). The results of the fitting procedure had an arbitrariness due to strong correlated fitting that produced large uncertainties in the values of the parameters.

The modified dipolar-dephasing version of the SUPER technique (called DD-SUPER; see Fig. 1) was employed to aid in the measurement of the CS parameters of form II. The only difference from the usual SUPER sequence is the insertion of a delay of 40 μs without proton decoupling prior to the last π pulse of the TOSS sequence. During this period, signals of carbons strongly coupled to protons by the dipole–dipole interaction decay rapidly and are lost from the signal at the end of the TOSS sequence. Signals attributable to unprotonated carbons survive and are represented in the spectrum obtained with this sequence. A delay of 40 μs was empirically chosen. The $^{13}$C–$^{1}$H dipolar coupling constant of carbon directly bonded to a proton is approximately 20–30 kHz, [29] implying that the dipolar oscillation period is roughly 40 μs, a time frame for $^{13}$C nuclei to precess and decay in the local proton dipolar field. Our experimental results show that a 20-μs delay was insufficient to suppress all protonated carbon signals, and a 50-μs delay led to a significant signal reduction of the unprotonated carbons.

The DD-SUPER spectrum of form II is shown in Fig. 4. Only signals of unprotonated carbons are present, except for a resonance at 40.4 ppm due to the carbon of the N-methyl group (C10). In general, internal rotation of a methyl group reduces the dipole–dipole coupling to such an extent that dipolar dephasing is much less effective than for other types of protonated carbons. An artifact appearing at 40 ppm and −10 kHz may arise from imperfect sideband suppression along the $F_1$ dimension when the sample spin-
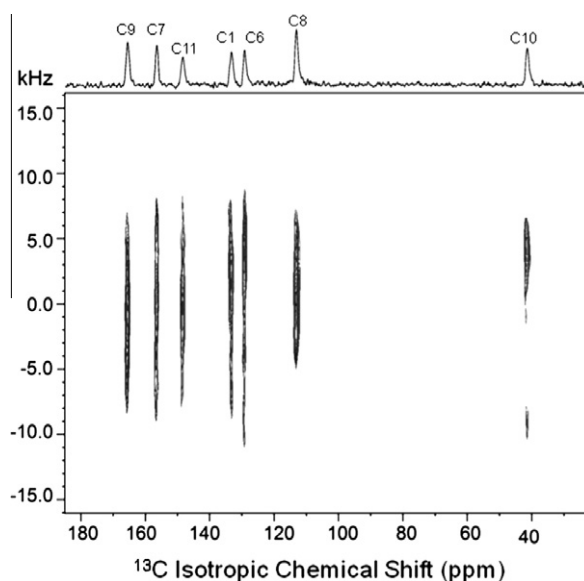


**Fig. 4.** DD-SUPER spectrum of form II of piroxicam. Only the resonances of unprotonated carbons (and the protonated carbon C10, see text) appear in the spectrum. The resonances of other protonated carbons have been suppressed by dipolar dephasing.

**Table 1**
Principal values of the chemical-shift tensors of selected carbons of form II of piroxicam.[a]

| Carbon | Method | $\delta_{11}$ (ppm) | $\delta_{22}$ (ppm) | $\delta_{33}$ (ppm) | $\delta_{iso}$ (ppm) |
|---|---|---|---|---|---|
| C6 | SUPER | 216 | 163 | 8 | 129 |
|  | DD-SUPER | 218 | 164 | 6 | 129 |
| C7 | SUPER | 247 | 161 | 62 | 157 |
|  | DD-SUPER | 247 | 161 | 61 | 157 |
| C9 | SUPER | 244 | 164 | 90 | 166 |
|  | DD-SUPER | 243 | 164 | 90 | 166 |
| C1 | DD-SUPER | 212 | 152 | 36 | 133 |
| C8 | DD-SUPER | 176 | 103 | 60 | 113 |
| C11 | DD-SUPER | 241 | 138 | 67 | 148 |
| C3 | SUPER[b] (fixed) | 245 | 124 | 30 | 133 |
| C12 | SUPER[b] (fixed) | 201 | 129 | 10 | 113 |
| C15 | SUPER[b] (fixed) | 261 | 146 | 38 | 148 |

[a] All chemical shifts in ppm relative to TMS, with a standard error of ±3 ppm.
[b] The chemical shifts of C3, C12, and C15 were determined from fitting the line shapes in the SUPER spectra by fixing the parameters of the unprotonated carbons C1, C8, and C11, respectively, at the values determined from fitting the DD-SUPER line shapes.

ning rate is comparable to the line width of the chemical-shift tensor pattern of the methyl group [9]. With a 40-μs delay, greater than 90% of the resonance signal of unprotonated carbons is retained in the DD-SUPER experiment.

Since C6, C7, and C9 are well-resolved in either the SUPER or DD-SUPER spectrum, we compared the fitted CS parameters of these carbons for the two different experiments. As can be seen from the data in Table 1, both experiments give essentially the same values of the chemical-shift parameters of these three carbon sites.

From the CP/MAS spectrum in Fig. 3, one sees that C1, C8, and C11 of form II overlap with C3, C12, and C15, respectively. The CS parameters of these carbons extracted from the SUPER spectrum using a two-tensor model for each set of overlapped resonances either failed to converge or resulted in an arbitrary set of values due to strong correlation in the fitting. The set of fitted CS values of the overlapped patterns depended on the initial guesses given for the fitting.
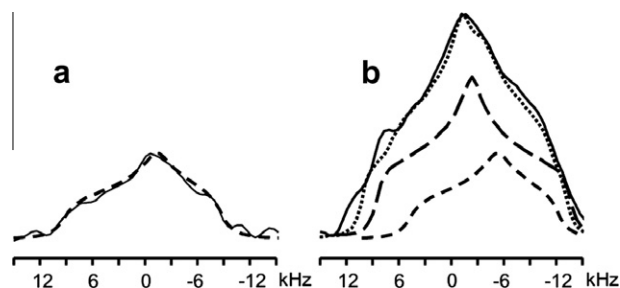
**Fig. 5.** (a) Experimental and simulated CS powder patterns of C11 derived from the DD-SUPER experiment and (b) experimental and simulated CS powder patterns of C11 and C15 derived from the SUPER spectrum. The simulated powder pattern of C11 (as determined in part (a)) is shown, as well as the simulated powder pattern determined for C15 (dashed lines). The sum of these two bands (dotted line) is compared with the SUPER spectrum to show the successful fitting.
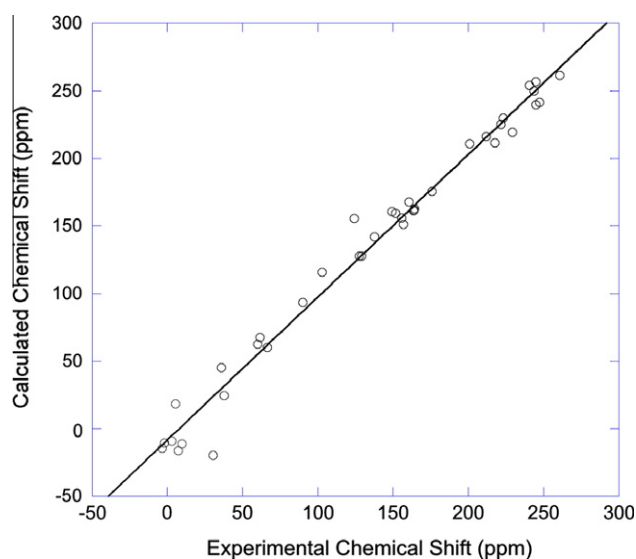


**Fig. 6.** Correlation of experimental and calculated chemical-shift parameters ($\delta_{11}$, $\delta_{22}$, and $\delta_{33}$) for form II of piroxicam.

The resonances of the unprotonated carbons C1, C8, and C11 are clearly resolved in the DD-SUPER experiment as shown in Fig. 4. The CS parameters of each carbon were independently determined with a single-tensor model. The results are given in Table 1. The chemical-shift tensors of carbons C3, C12, and C15 were subsequently determined from fitting the overlapped regions of the SUPER spectrum, using the CS parameters of C1, C8, and C15 as fixed parameters. With this reduced number of fitting parameters, we were consistently able to achieve a well-behaved fit to the line shapes of the overlapping patterns, giving parameters for C3, C12, and C15, regardless of the initial guesses. These results are also given in Table 1.

Fig. 5 shows the results for the overlap of the resonances of carbons C11 and C15. In Fig. 5a the simulated and experimental powder patterns for C11, as determined with the DD-SUPER experiment, are compared. In Fig. 5b, the results of the SUPER experiment, from which the parameters of C15 are determined, are shown. The agreement between experimental and simulated overlapped CS powder patterns is satisfactory. The relative-intensity ratio required for fitting is 1:2.1, instead of 1:1. This ratio most likely depends on the cross-polarization time used in the SUPER experiment; in principle it should not affect the CS parameters determined by this method. The fact that a two-tensor model must be used to fit the experimental line shape (Fig. 5b) further validates our chemical-shift assignment of overlapped isotropic shifts of C11

and C15. A similar procedure gave similar results for the overlapped resonances of C1 and C3 and the overlapped resonances of C8 and C12.

The crystallographic structural parameters of form II of piroxicam have been reported previously [30]. The principal values of the $^{13}C$ chemical-shift tensors for the carbons of form II of piroxicam were calculated from the reported structural parameters without geometry optimization (except for the positions of hydrogens) using Gaussian 03 at the B3YPW95 level, using the 6–311++G(2d, p) basis set. A plot of the experimental principal values of the chemical-shift tensors of *all* carbons except C4 versus the calculated principal values is given in Fig. 6. Because of the multiple-resonance characteristics of C4, it is difficult to obtain accurate CS parameters for this site. The plot is linear with an intercept of $-8.09 \pm 3.72$ ppm and a slope of $1.06 \pm 0.02$. For calculations with prior geometry optimization, the linear correlation between experimental and calculated chemical-shift principal values is improved to give an intercept of $1.86 \pm 2.51$ and a slope of $1.00 \pm 0.02$. The excellent agreement between experimental and calculated CS parameters further suggests that combining the SUPER and the DD-SUPER techniques to measure the CS parameters of a molecular system such as form II of piroxicam (which has severe overlap of several $^{13}C$ resonances of unprotonated and protonated carbons) can produce accurate chemical-shift results for all carbons.

## 4. Conclusions

We have demonstrated that insertion of a dipolar-dephasing delay into the SUPER sequence (DD-SUPER) is an effective way of suppressing signals of protonated carbons to allow observation of the unprotonated carbons in a complex molecule with many sites. This method is particularly useful to measure the CS powder patterns of unprotonated carbons that overlap the resonance lines of protonated carbons. The combination of fitting the DD-SUPER spectrum with subsequent fitting of a normal SUPER spectrum allows precise determination of both the protonated and unprotonated carbons. The CS principal values of all the carbons of form II of piroxicam, measured using this method, were in excellent agreement with calculated CS parameters using DFT/GIAO methods.

This method is applicable to any molecular system in which there is overlap of resonances of protonated and unprotonated nuclei. It is particularly applicable to $^{13}C$ in large biological molecules and in complex pharmaceuticals. The method may also be appropriate to resolution of other protonated and unprotonated nuclei, particularly $^{15}N$, although we have not demonstrated that. Finally, this method of insertion of a dipolar-dephasing delay may also be useful with other techniques designed to separate the anisotropic and isotropic chemical-shift parameters by the 2D approach, such as 2D ROCSA, when there is overlap of the resonances of protonated and unprotonated nuclei.

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