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Chemical Shift Correlations from Hyperpolarized NMR by Off-Resonance Decoupling

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Nuclear magnetic resonance, through observation of chemical shift, allows the separate identification of each atom in a molecule. Thus, NMR spectra impart an often unrivaled wealth of information on molecular structure. A particular advantage of NMR spectroscopy is the ability to record multidimensional spectra, which provide correlations between atoms. When compared to other techniques, such as optical spectroscopy, the acquisition of NMR spectra is however an insensitive process, requiring samples of high concentration and long acquisition times. Recently, it has been demonstrated that dynamic nuclear polarization, a hyperpolarization technique, can increase the NMR signal by several orders of magnitude. Here, we present a robust method that allows recording two-dimensional chemical shift correlations from such hyperpolarized molecules. The method makes use of an apparent scaling of the scalar coupling observed on one type of atom, when an off-resonance decoupling field is applied to another type of atom. Thus, two-dimensional chemical shift correlations can be read directly from a small number of scans acquired using a hyperpolarized sample. Due to the ease of implementing this technique on commercial hyperpolarization and NMR equipment, it appears ideally suited for routine application, for example, to obtain carbon−proton chemical shift correlations in organic molecules.

NMR is a powerful analytical technique to elucidate the molecular structure and has become an indispensable tool to analyze products of organic syntheses,[1–3] to identify natural products,[4–6] and to determine biochemical processes.[7,8] However, these NMR studies are often constrained by the amount of sample available. An important limitation in sensitivity persists despite significant advances made in recent years, including the availability of high magnetic fields, cold-probe technology, and the use of microcoils.[9]

Low sensitivity in NMR derives in part from the weak interaction of nuclear spins with their environment, giving rise to a low equilibrium polarization of the spin system even in a strong magnetic field ($<10^{-4}$ at room temperature). By hyperpolarization, a spin system is prepared in a nonequilibrium, polarized state prior to the NMR experiment. Thus, the sensitivity can potentially be increased by the same order of magnitude (i.e., $>10^4$). Many hyperpolarization techniques are very selective in the types of substances that they are compatible with. Optical pumping is applicable only for polarization of noble gases.[10,11] Polarized noble gases can be targeted to specific molecular sites; for example, xenon biosensors[12] have been developed to detect polarized xenon in magnetic resonance imaging.[13] However, this technique cannot give chemical information about arbitrary molecules. Likewise, para-hydrogen-induced polarization[14] is limited to generate polarization on protons that are being attached at the sites of unsaturated bonds by chemical reaction. In contrast, dynamic nuclear polarization (DNP)[15,16] is a recently developed technology that allows polarizing nuclear spins in almost any small molecule, with subsequent NMR measurement in the liquid state.[15] Thus, it appears ideally suited for routine application in small-molecule NMR.

In most modern NMR studies, Fourier transform NMR[17] enables the acquisition of multidimensional correlation spectra, which yield important information on the structure of a molecule. A multidimensional NMR data set is acquired by repeating a one-dimensional experiment with different indirect evolution times, allowing reconstruction of the indirect dimension. Such an experiment relies on a delay between each scan, during which the spin system returns to the equilibrium state, given by the thermal distribution over the Zeeman levels in the magnetic field.

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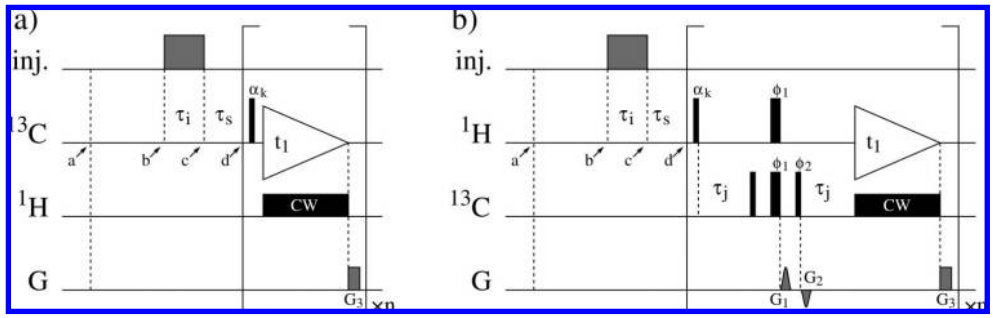

Figure 1. NMR experiments for measurement of 2D chemical shift correlations of hyperpolarized sample. Injection ("inj.") into the NMR takes place during $\tau_i = 350$ ms, and the NMR experiment is started after a stabilization time $\tau_s = 300$ ms at time point d. Narrow and wide black bars denote $\pi/2$ and π hard pulses, unless otherwise indicated ($\gamma_H B_{1,H} = 25$ kHz; $\gamma_C B_{1,C} = 20$ kHz; 400 MHz proton frequency). Flip angles on excitation pulses are given by $\alpha_k = \arcsin(1/\sqrt{n+1-k})$, with $k = 1 \ldots n$. Pulse phases are *x*, unless indicated. A pulsed field gradient $G_3 =$ 50 G/cm, 2.5 ms duration is applied along the *z* axis. In (a), the offset on the ^{13}C channel is 120 ppm. Three spectra with off-resonance decoupling ($\gamma_H B_{1,H} = 1.67$ kHz) at offset 11, 7, and 3 ppm are acquired. The acquisition time $t_{1,max} = 324$ ms. In (b), coherence selection is achieved by the pulsed field gradients G_1 (50 G/cm, 1000 μs applied along *x*, *y*, and *z* axes) and G_2 (−37.4 G/cm, 1000 μs applied along *x*, *y*, and *z* axes). The delay $\tau_j = 1/(2J_{CH}) = 3.45$ ms. Pulse phases are $\varphi_1 = y$; $\varphi_2 = -x$. The offset on the ^{1}H channel is 6 ppm. Four spectra with off-resonance decoupling ($\gamma_C B_{1,C} = 6.0$ kHz) at offset 210, 150, 90, and 30 ppm are acquired. The acquisition time $t_{1,max} = 426$ ms.

Each scan of the experiment thus starts with the same amount of "fresh" longitudinal magnetization. When working with hyperpolarized sample, however, the initial spin state is polarized far beyond equilibrium. The ensuing large magnetization is the basis for the increased signal, but at the same time, the magnetization does not return to the hyperpolarized initial state after an NMR scan has been completed. Rather, the spin system will relax to the thermal equilibrium, which has much lower magnetization. Thus, a two-dimensional NMR spectrum of a hyperpolarized sample cannot be acquired by the means described above.

One elegant way of obtaining a two-dimensional NMR spectrum in a single scan has recently been developed by Frydman.[18] Thereby, using the methods of magnetic resonance imaging, the sample is divided by the NMR experiment into many small subvolumes. The coherences corresponding to the multiple scans that are necessary for reconstruction of an indirect spectral dimension are encoded in, and read out from these separate volumes.

Here, we propose an alternative scheme to obtain two-dimensional chemical shift correlations from hyperpolarized samples, which is particularly robust and easy to implement. By a differential scaling of scalar coupling under low-power radio frequency (rf) irradiation, a second chemical shift dimension is directly encoded into the line shape of the acquired NMR signals. In conventional biomolecular NMR, such an approach has previously been proposed for use with multidimensional correlation experiments of isotopically enriched proteins.[19] We demonstrate here that off-resonance decoupling provides a quite general and simple way of obtaining two-dimensional chemical shift correlations with hyperpolarized samples of small molecules.

EXPERIMENTAL SECTION

Sample Preparation. (1) Sample for determination of ^{1}H-{^{13}C} spectra: 0.5 μL of 3.6 M vanillin (MP Biomedicals, Solon, OH) solution in 72% dimethyl sulfoxide-d_6 (DMSO-d_6; Cambridge Isotope Laboratories, Andover, MA) and 28% D_2O (Cambridge Isotope Laboratories, Andover, MA) containing 15 mM 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol; Sigma-Aldrich, St. Louis, MO) free radical. (2) Sample for ^{13}C-{^{1}H} spectra: 0.5 μL of 3.6 M vanillin solution in 72% DMSO-d_6 and 28% D_2O containing 15 mM of tris[8-carboxyl-2,2,6,6-tetramethyl-benzo(1,2-*d*:4,5-*d*′)bis(1,3)dithiole-4-yl]methyl sodium salt ("Finland"; Oxford Instruments, Tubney Woods, UK) free radical. After dissolution into the NMR spectrometer, samples were analyzed by high-pressure liquid chromatography (HPLC). A C18 column (ODS, 3 μm, 250 × 4.0 mm) from SGE (Austin, TX) was used with a mobile phase of 60% acetonitrile/40% H_2O + 0.1% trifluoroacetic acid. Vanillin concentrations were determined by comparing peak integral values for vanillin to standard samples of vanillin in mobile phase.

DNP Polarization. DNP polarization took place at a temperature of 1.4 K in a Hypersense DNP polarizer (Oxford Instruments). Samples for ^{1}H observation were polarized for 30 min at a microwave frequency of 94.270 GHz and a power of 100 mW. Samples for ^{13}C observation were polarized for 3 h at 93.976 GHz and 60 mW. Samples were dissolved in acetonitrile and transferred to the NMR spectrometer using a sample injector described elsewhere (S. Bowen and C. Hilty, *Angew. Chem., Int. Ed.* **2008**, DOI: 10.1002/anie.200801492). The total time of sample injection, from the point of dissolution to the point of NMR measurement, is <2.5 s. After sample injection, NMR experiments are automatically started.

NMR Spectroscopy. The purpose of the NMR experiment is to determine the chemical shifts of a pair of heteronuclei that are coupled via scalar coupling. The information obtained is the same as from a heteronuclear correlation spectroscopy experiment.[20,21] In contrast to the aforementioned technique, however, the chemical shift of the coupled nucleus is determined from the same transient that shows a spectrum of the directly observed nucleus. When continuous-wave (CW) decoupling is applied near the resonance frequency of the coupled nucleus, the scaling in the observed scalar coupling constant, J_{obs}, allows direct inference of the chemical shift of the coupled nucleus. Specifically, the NMR experiment (Figure 1) acquires a small number of scans, typically $n = 3-5$. The first scan is a reference scan without CW radiation,

(18) Frydman, L.; Scherf, T.; Lupulescu, A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15858–15862. Frydman, L.; Blazina, D. *Nat. Phys.* **2007**, *3*, 415–419.
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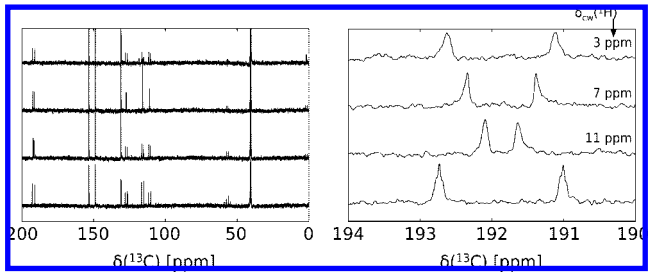

Figure 2. Series of ^{13}C NMR spectra recorded from one single DNP polarized sample, which permits the reconstruction of a two-dimensional chemical shift correlation. (a) Full spectrum. (b) Expanded spectrum around 192 ppm. The frequency for off-resonance CW irradiation is indicated near each trace. The lowest trace is the reference spectrum without irradiation. Chemical shifts were calibrated against a standard of tetramethylsilane (TMS), using the substitution method.[22]

while the subsequent scans apply CW at different frequencies within the chemical shift range of the indirectly observed nucleus. To distribute the signal available from the hyperpolarized sample evenly across the n scans, a variable flip angle α_k is used for excitation. Since the spin polarization should not reach equilibrium conditions, there is no delay between the successive scans. Figure 1 presents two alternative schemes to obtain the chemical shift correlations. In (a), the acquired dimension is ^{13}C, and the indirect dimension is ^{1}H (^{13}C-{^{1}H} experiment). In this case, the free induction decay is obtained directly after the excitation pulse. In Figure 1b, the acquired dimension is ^{1}H (^{1}H-{^{13}C} experiment). In this experiment, the abundance of the NMR active isotope of the indirectly observed nucleus, ^{13}C, is only 1.1%. If a sequence analogous to Figure 1a were used, most of the acquired signal would not carry the desired information on the ^{13}C chemical shift. Therefore, an isotope-selective pulse sequence is used, which includes coherence selection by pulsed field gradients. By this pulse sequence, only the coherence transferred from ^{1}H to ^{13}C and then back to ^{1}H contributes to the signal.

As in any other heteronuclear experiment, this pulse sequence requires the adjustment of an additional delay, τ_j, which is a function of the J_{CH} scalar coupling constant. Since the final signal intensity depends on $\sin^2(\pi/2 \cdot J/J_0)$, where $J_0 = 1/(2\tau_j)$ is the presumed and J the actual coupling constant, this delay is however quite forgiving to small maladjustment. For example, a variation of 20% in the coupling constant translates into a sensitivity loss of only 10%.

RESULTS AND DISCUSSION

For purposes of comparison, two-dimensional chemical shift correlations of the molecule vanillin, at 0.9 mM concentration and without isotopic enrichment, were recorded. In the first case (Figure 1a), ^{13}C spectra were acquired while CW radiation was applied at different frequency values within the ^{1}H frequency range (^{13}C-{^{1}H} experiment). The obtained one-dimensional traces are shown in Figure 2, the lowest one representing the spectrum without CW. In the upper traces, the offsets of CW irradiation on the ^{1}H channel are indicated on the right. From the expanded view of the resonance around 191.9 ppm (Figure 2b), the effect of the ^{1}H CW irradiation can be observed directly. The distance between the two observed peaks in the lowest trace corresponds to the actual $^{1}H-^{13}C$ coupling constant $J_0 = 173.6$ Hz. In the traces

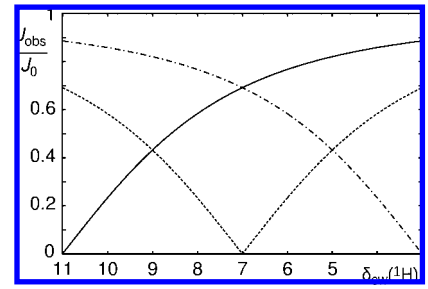

Figure 3. Scaling of the observed J-coupling constant as a function of resonance offset of the CW irradiation. The scaling factor is plotted against ^{1}H chemical shift for CW frequencies corresponding to 11 (—), 7 (— —), and 3 ppm (— · —).

with CW irradiation, this coupling constant is scaled depending on the difference between the CW frequency and the ^{1}H chemical shift attached to the observed carbon.

The observed coupling constant, J_{obs}, is given by

$$\frac{J_{obs}}{J_0} = \frac{|\delta - \delta_{CW}|}{\sqrt{(\delta - \delta_{CW})^2 + (10^6 B_{1,CW}/B_0)^2}} \quad (1)$$

where $B_{1,CW}$ is the amplitude of the decoupling field ($B_{1,CW} = 1/4\gamma\tau_{90}$) with τ_{90} the 90° pulse length corresponding to the applied power), δ is the chemical shift of the spin, and δ_{CW} the chemical shift at which CW irradiation takes place.[23] Figure 3 shows the dependence of the scaling factor on chemical shift of the indirectly observed ^{1}H nucleus, plotted using the parameters δ_{CW} and $B_{1,CW}$ from the ^{13}C-{^{1}H} experiments. The offset frequencies are chosen so that at any point in the desired frequency range, the scaling factors in at least two scans are strongly dependent on the chemical shift.

Rewriting equation 1 yields

$$|\delta_{CW,i} - \delta_i| = 10^6 \frac{B_{1,CW}}{B_0} \tan\left(\arcsin\left(\frac{J_{obs}}{J_0}\right)\right) \quad (2)$$

$\delta_{CW,i}$ is the chemical shift at which CW irradiation takes place in a given trace i, and δ_i is the chemical shift value of the spin to be determined from trace i. From measurement of J_{obs} in a one-dimensional trace, the difference between the ^{1}H chemical shift and the respective offset of CW irradiation can thus be determined. Comparing these differences from all the acquired traces allows unambiguous determination of the ^{1}H chemical shift values.

Since the accuracy of each obtained chemical shift difference depends on the slope of the respective curve shown in Figure 3, it is possible to use a weighted average that depends on this slope, for determining the final chemical shift value,

$$\delta = \frac{\sum_{i=1}^{n} \alpha_i \delta_i}{\sum_{i=1}^{n} \alpha_i} \quad (3)$$

(22) Harris, R. K.; Becker, E. D.; De Menezes, S. M. C.; Goodfellow, R.; Granger, P. *Pure Appl. Chem.* **2001**, *73*, 1795–1818.
(23) Cavanagh, J.; Fairbrother, W. J.; Palmer, A. G.; Skelton, N. J. *Protein NMR Spectroscopy: Principles And Practice*; Academic Press, Inc.: San Diego, 1996; pp 172−173, 141.

with the weighting factor

$$\alpha_i = \left(\frac{1}{\sqrt{(\delta_i - \delta_{CW,i})^2 + (10^6 B_{1,CW}/B_0)^2}} \right)^3 \quad (4)$$

Using this procedure has the further advantage that an estimate for the error in the indirectly detected chemical shift can be obtained,

$$\Delta\delta = \frac{\sum_{i=1}^{n} \alpha_i |\delta_i - \delta|}{\sum_{i=1}^{n} \alpha_i} \quad (5)$$

The observed coupling constants and the derived chemical shifts for the ^{13}C-{^{1}H} data set in Figure 2, are presented in Table 1. The assignments of these chemical shifts to the atoms in the vanillin molecule are shown in Figure 4.[24,25] It can be seen that the indirectly detected chemical shifts could be determined with an error of 0.1 ppm for all resonances, using a total of four scans on a single hyperpolarized sample.

The inverse experiment, where a proton spectrum is acquired (^{1}H-{^{13}C} experiment; Figure 5 and Table 2) works by the same principle. However, since 98.9% of the observable ^{1}H nuclei are attached to a NMR inactive ^{12}C nucleus, a heteronuclear ^{13}C filter element was included in this experiment. This filter element suppresses the large central peak that would otherwise be present in the spectra. Due to the larger ^{13}C chemical shift dispersion (0−200 ppm), it was necessary to acquire five instead of four traces. In Table 2, it can be seen that chemical shifts could be determined with an uncertainty of 3 ppm.

The concentration of vanillin was determined by HPLC to be 0.9 mM in both samples. Since the compound was not ^{13}C enriched, the concentration of the observable ^{13}C nuclei was 10 μM. For calculation of the obtained polarization level, it is necessary to compare the S/N ratio to a conventionally acquired spectrum. Due to the low concentration, it was however not practical to use the samples that were previously polarized for this purpose, as an excessively long measurement time would be needed. Therefore, we acquired a one-dimensional ^{13}C spectrum, as well as a one-dimensional ^{13}C-filtered ^{1}H spectrum using reference samples of vanillin at concentrations of 69 and 45 mM, respectively. The S/N ratio of the reference ^{13}C spectrum was 13−32, depending on the resonance line, after an acquisition time of 12 h under Ernst angle conditions.[23] This compares to a S/N ratio of 8−20 in the first trace of the data set obtained from hyperpolarized sample shown in Figure 2.

We estimate that a conventional spectrum with the same S/N ratio would require >200 days when acquired on the same NMR spectrometer. In the case of the ^{1}H experiment, the S/N ratio was 26−88 for the ^{13}C filtered ^{1}H experiment after an acquisition time of 1 h, and 11−21 for the first trace of the hyperpolarized experiment. Based on these numbers, a conventionally acquired spectrum with the same S/N ratio would thus take 4−18 days.

In both cases, a significant gain in sensitivity is achieved by hyperpolarization, since the polarization time is only on the order of hours (3 h for ^{13}C polarization and 30 min for ^{1}H polarization), while the actual NMR measurement time for the two spectra is below 3 s.

Table 1. Observed Coupling Constants and Chemical Shifts in the ^{13}C-{^{1}H} Spectrum[a]

^{13}C chemical shift/ppm	^{1}H CW offset/ppm[a], J/Hz				^{1}H chemical shift/ppm	group
	no CW	11	7	3		
191.9	173.6	44.8	94.6	150.1	9.8 ± 0.1	E
153.2	no proton is attached to these carbons					G
148.9						H
130.9						F
127.3	161.9	102.6	18.8	116.4	7.5 ± 0.1	D
115.8	162.4	108.0	0	109.7	7.0 ± 0.1	C
111.0	161.9	103.5	18.8	114.3	7.4 ± 0.1	B
56.8	145.5	123.8	84.9	30.7	4.0 ± 0.1	A

[a] The offset values in the table are nominal values. The values after calibration against the standard of TMS are 10.92, 6.92, and 2.92 ppm.

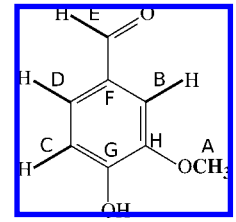

Figure 4. Vanillin, with CH groups indicated as in Tables 1 and 2.

The level of polarization can be calculated from this comparison, using

$$p_p = p_t \sqrt{n_t} \frac{1 - e^{-t_r/T_1}}{1 - e^{-t_r/T_1} \cos(\alpha_t)} \frac{\sin(\alpha_t)}{\sin(\alpha_p)} \frac{s_p}{s_t} \frac{c_t}{c_p} \quad (6)$$

with the thermal polarization

$$p_t = \frac{\hbar \gamma B_0}{2kT}^{23} \quad (7)$$

Here, the subscripts p and t refer to the polarized and the thermal samples, respectively. n is the number of scans, s the obtained signal-to-noise ratio, c the concentration, α the flip angle for excitation, t_r the recycle time in the conventional experiment, T_1 the spin−lattice relaxation time, and T the temperature of NMR measurement. Equations 6 and 7 yield polarization levels between 2 and 7% for ^{13}C and between 2 and 3% for ^{1}H, depending on the observed resonance line.

The level of polarization that is observed in the NMR depends on the polarization in the solid state, and on losses due to T_1 relaxation during dissolution and injection into the NMR spectrometer. Because of the latter process, the liquid-state polarization level is generally higher for spins with longer relaxation time. This observation stands in contrast to the situation in a conventional NMR spectrum, where it is the signals from spins with long relaxation time that are typically attenuated due to incomplete recovery during the delay between subsequent scans.

For demonstration of the present experiments, we have chosen the vanillin molecule because it contains a variety of proton- and carbon-containing chemical groups, including a methyl group, an

(24) AIST. Spectral Database for Organic Compounds, SDBS. http://riodb.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng; accessed 02−25−2008.
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Table 2. Observed Coupling Constants and Chemical Shifts in the ^{1}H-{^{13}C} Spectrum

	^{13}C CW offset/ppm[a], J/Hz						
1H chemical shift/ppm	no CW	210	150	90	30	^{13}C chemical shift/ppm	group
9.81	175.9	49.9	94.5	147.9	162.2	190 ± 3	E
7.43	162.3	129.1	56.4	79.8	143.0	129 ± 3	D
7.43	161.1	135.4	85.4	47.8	125.2	111 ± 3	B
6.99	162.3	133.9	77.6	59.4	129.9	116 ± 3	C
3.93	145.4	134.2	120.9	69.1	53.8	58 ± 3	A

[a] The offset values in the table are nominal values. The values after calibration against the standard of TMS are 211.0, 151.0, 91.0, and 31.0 ppm.

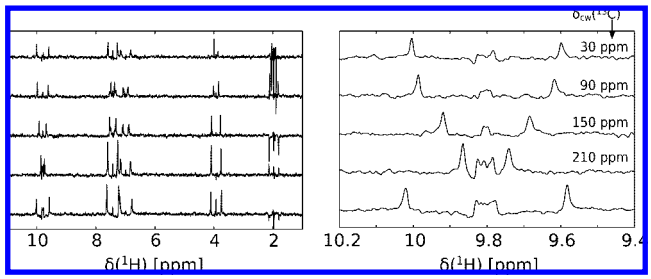

Figure 5. Series of ^{13}C filtered ^{1}H NMR spectra for the reconstruction of two-dimensional chemical shift correlation.

aromatic ring, and an aldehyde group. In our experience, most small molecules are polarizable, and the presented method should be quite generally applicable. The most limiting factor determining the type of molecule suitable for solid-to-liquid state DNP polarization is the loss to relaxation during the time required for sample dissolution and injection into the NMR spectrometer. Using our present, "home-built" sample injector, this time is ~2.5 s. Spins with a T_1 relaxation time of a few seconds are thus ideal for polarization using our DNP instrument. Chemical groups belonging to this class include aromatic rings, ketones or aldehydes, and also many aliphatics. The limitation imposed by T_1 relaxation is not as stringent as it may seem at first glance, as appreciable signal gain can still be realized for spins with a T_1 relaxation time as low as one-third of the injection time (i.e., currently $T_1 \approx 700$ ms). We have also successfully polarized molecules containing spins with relaxation times in this range, including peptides and glucose.

CONCLUSIONS

Using the presented method, the entire set of ^{13}C,^{1}H chemical shift correlations from a single hyperpolarized sample, here 0.9 mM vanillin, can be determined with high sensitivity. In the spectra shown, 10 μM ^{13}C atoms were observed with a total experiment time of 3 h, including the time required for hyperpolarization. Certainly, this method does not resolve overlapping resonances in the second dimension, as would be done in a two-dimensional data set with indirect chemical shift evolution. However, the hyperpolarization offers the ability to directly detect the ^{13}C spectrum, rather than the ^{1}H spectrum. Therefore, the ^{13}C-{^{1}H} experiment yields chemical shifts of carbon atoms without attached protons, in addition to the ^{13}C,^{1}H chemical shift correlations. At the same time, the large chemical shift range of the carbon spectrum alleviates potential signal overlap problems. To further reduce the number of lines in the spectra, it would also be possible to include a spin-state selective pulse sequence[19,26,27] to select only one component of the peak doublet. However, this was not done here because it would require the acquisition of additional one-dimensional traces and thus decrease the overall sensitivity of the experiment.

Even though we determined both the ^{1}H-{^{13}C} and the ^{13}C-{^{1}H} spectra, either one is sufficient to reconstruct the ^{1}H−^{13}C chemical shift correlations. Comparing the spectra from Figures 3 and 4, the signal-to-noise ratio of an individual trace of the ^{1}H-{^{13}C} and the ^{13}C-{^{1}H} experiment is comparable under the experimental conditions used. The latter, however, has the advantage that (1) it enables at the same time the observation of the chemical shifts of carbons without attached protons, and (2) signal overlap is reduced due to the larger chemical shift range.

A major advantage of this method over other techniques of NMR spectroscopy with hyperpolarized samples lies in its robustness and ease of implementation. For this reason, the presented technique seems to lend itself well for routine use in the identification and structure determination of compounds originating from organic synthesis, as well as for other applications to small molecules, where the obtainable sample quantity is severely limited.

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