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^{13}C hyperpolarization of a barbituric acid derivative via parahydrogen induced polarization

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ABSTRACT

Significant ^{13}C NMR signal enhancement by a factor of 5000 of a barbituric acid derivative (5-methyl-5-propenyl-barbituric acid) via parahydrogen induced polarization is presented. This hyperpolarization is achieved by hydrogenating 5-methyl-5-propargyl-barbituric acid with 98% enriched para-H_2 under elevated temperature and pressure and transferring the initially created ^{1}H hyperpolarization with an INEPT-derived pulse sequence to ^{13}C. The polarization can be selectively transferred to different carbons in the barbituric acid derivative by applying different pulse delays in the INEPT pulse sequence. These results demonstrate the potential of using hyperpolarized barbituric acid derivatives as "*active*" contrast agents in MRI and visualizing their pharmacokinetics in vivo.

1. Introduction

In comparison with other methods, ^{13}C nuclear magnetic resonance (NMR) spectroscopy and magnetic resonance imaging (MRI) are rather insensitive techniques due to the small magnetogyric ratio of the ^{13}C nucleus and its low natural abundance. Therefore, the application of ^{13}C MRI for clinical diagnosis has been limited so far due to the extremely long acquisition times that are required to obtain high signal-to-noise ratios (SNR) under physiologic conditions. However, it was recently demonstrated, that this obstacle can be overcome by in vitro hyperpolarization of a molecule with long ^{13}C spin-lattice relaxation time and subsequent injection into the animal or patient of investigation [1–7]. Hyperpolarization techniques offer the unique possibility to use "active" MRI contrast agents, which are the direct signal source and do not rely on T_1 and/or T_2 changes of surrounding water molecules like classical "passive" (Gd or Fe based) contrast agents [4].

Several possibilities are known to produce hyperpolarized molecules and enhance the sensitivity of magnetic resonance, e.g., dynamic nuclear polarization (DNP) [8–13], photochemically induced dynamic nuclear polarization (photo-CIDNP) [14–16], and parahydrogen induced polarization (PHIP) [17–26]. The latter is a versatile technique to generate hyperpolarized molecules via a chemical route and was applied in this work. PHIP requires a homogeneously catalyzed hydrogenation of an unsaturated precursor with parahydrogen ($I = 0$), which creates two magnetically non-equivalent product protons. The two inserted protons exhibit a polarization far above the Boltzmann polarization and a theoretic signal increase of up to 10^5 can be obtained. Depending on the experimental setup parahydrogenation results in two different NMR signal patterns named either adiabatic longitudinal transport after dissociation engenders nuclear alignment (ALTADENA [18], in case of parahydrogenation at low magnetic field) or parahydrogen and synthesis allow dramatically enhanced nuclear alignment (PASADENA [17], for parahydrogenation at high magnetic field). Polarization transfer from the protons to hetero-nuclei can be implemented either randomly in weak magnetic fields or selectively via special pulse sequences [27–33].

Until today, mostly components of the citrate cycle were hyperpolarized and applied for in vivo metabolic imaging [3–6]. ^{13}C hyperpolarization can also be employed, however, to study pharmaceuticals. Along these lines we present results on hyperpolarization of a barbituric acid derivative. Barbiturates are clinically applied for the treatment of epilepsy or as injection narcotics [34]. They are well suited for parahydrogen induced polarization because of the straightforward synthesis of their unsaturated precursors. Moreover, long ^{13}C spin-lattice relaxation times make these derivatives of high interest as a potential "active" contrast agent in MRI.

Barbituric acid itself is not particularly suited for application in the human body due to its high acidity which prevents it from crossing the blood–brain barrier [35]. Therefore, only barbituric

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acid derivatives are clinically relevant. On substitution of the two protons at the C5 position during the synthesis of the barbiturate, it is possible to reduce the acidity of the whole molecule and, additionally insert an unsaturated group for the later incorporation of parahydrogen into the molecule. The barbiturates, which now can pass the blood–brain barrier, bind at the $GABA_A$-receptor (GABA: gamma-aminobutyric acid) of neurocytes [35] and increase the GABA-induced chloride influx into the cell. Therefore, they can stimulate different states of central depression depending on the applied dose and can work as anticonvulsants. By a significantly high ^{13}C polarization information on the location and the mechanism of action of the barbituric acid derivatives may be obtained (LD50 of the similar and well-known barbiturate phenobarbital is 835–1003 mg/kg for a rat [36]). Moreover, selective polarization of carbon positions, which play an important role in the biotransformation of the substrate, offer the possibility to investigate the pharmacokinetic of this pharmaceutically important drug.

Recently, we demonstrated that homogeneous hydrogenation of unsaturated barbituric acid derivatives with 50% enriched parahydrogen resulted in a substantial increase of the ^1H NMR signals of the reaction products [37]. However, ^{13}C-signal enhancement by randomly triggered polarization transfer to ^{13}C in the weak magnetic field was not observed. Hence, the aim of this study was to obtain considerable ^{13}C NMR signal enhancement of barbiturates. This was achieved by applying a closed-cycle cryostat setup for parahydrogen enrichment up to 98% in combination with effective INEPT-derived pulse sequences for polarization transfer to ^{13}C.

2. Materials and methods

2.1. Materials

Barbituric acid derivatives were synthesized from urea and unsaturated malonic acid derivatives ([37] and references therein). The synthesis of 5-methyl-5-propargylbarbituric acid is depicted in Scheme 1. For parahydrogenation reactions the commercially available rhodium catalyst system [Rh(COD)(dppb)]BF_4 was used. All chemicals were purchased from Sigma–Aldrich or Merck and used without further purification.

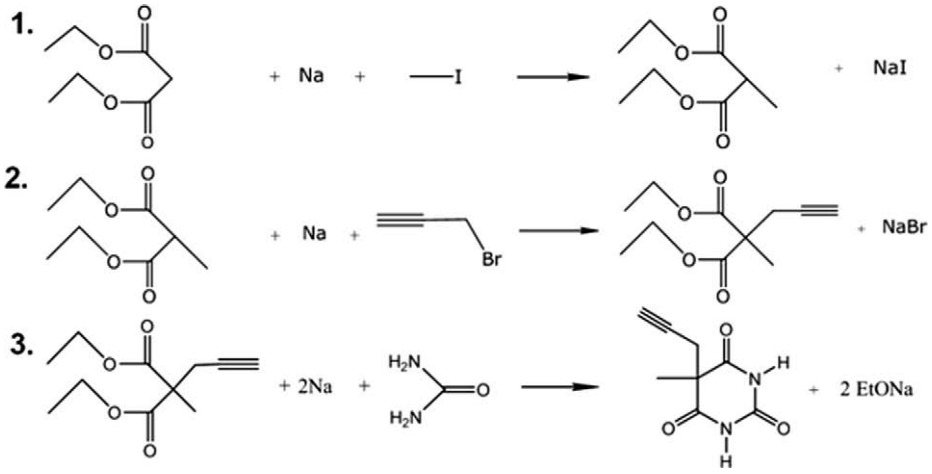
2.2. Parahydrogen enrichment and storage

Normal hydrogen with a purity of 5.0 was used as received from a commercial source (Westfalen AG, Münster, Germany). Enriched parahydrogen (98%) was generated by cooling thermal hydrogen down to 30 K with a closed-cycle cryostat setup (Advanced Research Systems, Macungie, PA, USA) in the presence of active charcoal as a catalyst for the symmetry forbidden conversion from orthohydrogen to parahydrogen. The para-H_2 can be stored for several days in transportable aluminum cylinders at 3.5 bar.

2.3. PHIP NMR experiments

NMR tubes (10 mm) were filled with 0.007 mmol (5 mg) catalyst, 0.28 mmol (50 mg) of the unsaturated barbiturate precursor and 3 ml acetone-d_6 under argon atmosphere and sealed with a septum cap. Parahydrogenation was carried out at elevated temperature and pressure in order to increase the conversion of the reaction. Therefore, the reaction tube was gently heated to 60 °C in a water bath and then pressurized with 3.5 bar of para-enriched H_2. Subsequently, the tube was shaken closely above the bore of the magnet to start the hydrogenation and immediately inserted into the spectrometer. Shaking the tube in the stray field of the magnet should create polarization in the PASADENA spin state which is necessary for polarization transfer using the PH-INEPT+ pulse sequence. All experiments were performed on a Bruker AVANCE DRX 300 MHz spectrometer. The high proton polarization was transferred to ^{13}C using the PH-INEPT+ sequence [29] with different delays $t_1/2$ ranging from 5 to 20 ms (Fig. 1). The difference between the PH-INEPT+ sequence and a standard INEPT+ sequence is the length and phase of the first pulse which is adjusted to excite a PASADENA spin system [29].

All reference spectra of the thermally polarized products were measured using the same NMR parameters as for the PHIP spectra. Signal enhancements were calculated by comparing the NMR line integrals of the recorded PHIP spectrum with a reference spectrum measured in the same sample after the hydrogenation reaction has stopped (which can take up to 20 min) and the polarization relaxed to thermal equilibrium. Therefore, the conversion of the parahydrogenation reaction at the acquisition time point of a PHIP spectrum and the reference spectrum is different, which has to be taken into account for the calculation of the ^1H-signal enhancement. The conversion at the acquisition time of the hyperpolarized PHIP spectrum was estimated to be 10% by comparison with the total conversion after 20 min (30%, calculated from the integrals of the NMR peaks of the thermally polarized product). For the carbon spectra, the signal enhancements were calculated simply by comparing the amplitudes of the NMR lines of the hyperpolarized and thermally polarized compounds. Such a thermal spectrum was recorded with 256 scans immediately after the ^{13}C polarization



Scheme 1. Three step synthesis of 5-methyl-5-propargyl-barbituric acid.

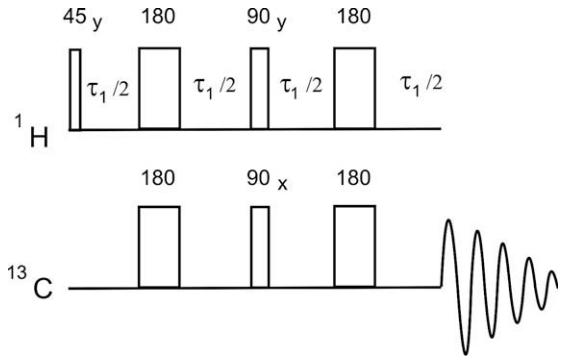


Fig. 1. NMR pulse sequence PH-INEPT+ for polarization transfer from ^{1}H to ^{13}C.

was destroyed by the read out pulse of the PH-INEPT+ sequence. This served as a reference for all hyperpolarized ^{13}C spectra.

2.4. T_1 measurements

T_1 relaxation times were measured by standard inversion recovery experiments [38]. NMR tubes (5 mm) were filled with 30 mg of 5-methyl-5-propenyl-barbituric acid and 1 ml of D_2O. For the proton measurements, inversion times τ ranging from 0.2 to 20 s and a repetition time of 40 s were applied. The carbon T_1 relaxation times were measured with τ times of 0.5–80 s and a repetition time of 320 s. The corresponding T_1-times were calculated by the Bruker Topspin 2.1 T_1-calculation software.

3. Results and discussion

The parahydrogenation of 5-methyl-5-propargyl-barbituric acid leads to the hyperpolarized product 5-methyl-5-propenyl-barbituric acid, as shown in Scheme 2 with the hyperpolarized protons depicted in red.

The ^{1}H and ^{13}C T_1 relaxation times of the hydrogenation product 5-methyl-5-propenyl-barbituric acid are presented in Table 1. The T_1-times were measured in D_2O because of the much higher solubility of the barbituric acid derivative in D_2O than in acetone resulting in a higher NMR signal and in order to confirm long-lived hyperpolarization in an aqueous environment to allow for in vivo experiments. However, so far we did not find a suitable catalyst for the parahydrogenation of 5-methyl-5-propargylbarbituric acid in water. The spin-lattice relaxation times of the protons are in the range between 0.7 and 3.8 s, whereas the carbons exhibit T_1-times of about 15 and 27 s for the quaternary carbon and the carbonyl group, respectively, which should be sufficient to perform in vivo experiments.

The ^{1}H NMR spectrum of the hyperpolarized product 5-methyl-5-propenyl-barbituric acid acquired using a 45° pulse is presented in Fig. 2. It was acquired 35 s after the parahydrogenation reaction was initiated by shaking the NMR tube containing the reaction mixture charged with 3.5 bar of 98% enriched parahydrogen in the stray field of the NMR magnet. The reference spectrum (which is also depicted in Fig. 2) was obtained from the very same reaction using the identical sample tube 20 min after the start of the parahydrogenation reaction. This delay is necessary to insure both the termination of the reaction inside the pressurized tube and a complete decay of the hyperpolarization.

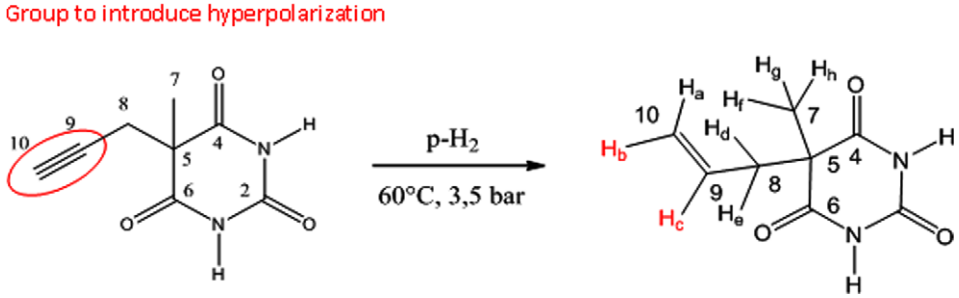
Table 1
T_1 relaxation times of the parahydrogenation product 5-methyl-5-propenyl-barbituric acid dissolved in D_2O.

T_1-times of the protons (s)				T_1-times of the carbons (s)					
H_a/H_b	H_c	H_d/H_e	$H_f/H_g/H_h$	C_4	C_5	C_7	C_8	C_9	C_{10}
1.8	3.8	0.9	0.8	27.3	15.5	1.0	0.7	2.1	1.0

The ^{1}H signal enhancements of H_b and H_c at the resulting double bond are 640 and 1025, respectively, and therewith much larger than the ^{1}H signal enhancement of 40 which we had achieved in our first attempt to hyperpolarize barbiturates [37]. This improvement in signal enhancement was achieved by optimizing the reaction conditions (elevated temperature and pressure) of the parahydrogenation reaction in combination with the application of 98% enriched para-H_2. Further improvement of the reaction conditions as were realized, e.g., in a recently built PASADENA polarizer [39,40] could maximize the conversion of the parahydrogenation reaction and would result in even higher NMR signal enhancements.

A closer look at the ^{1}H PHIP signals of the hyperpolarized spectrum in Fig. 2 suggests, however, that the spectrum is not the result of pure PASADENA conditions: Instead, the peak pattern suggests that the eventually recorded signals stem from a mixture, i.e., from a superposition of PASADENA and ALTADENA conditions. This indicates that the stray field of the magnet is not high enough to insure PASADENA conditions but give rise to ALTADENA polarization in the beginning of the parahydrogenation. However, if the reaction started at ALTADENA conditions and then continued inside the NMR magnet (PASADENA condition) for 20 s (time necessary to insert the NMR tube in the coil) most of the ALTADENA polarization should be decayed due to the short T_1 relaxation times of the protons. The proceeding of the reaction inside the magnet should result in the build-up of PASADENA polarization prior to the NMR experiment. The superposition of PASADENA and ALTADENA spin states can be concluded from the antiphase signal of C_{10} (PASADENA peak pattern) and from the spread of the hyperpolarization over the whole molecule (see emission peak at 2.6 ppm), since the latter takes place only under ALTADENA conditions.

In view of the considerable linewidths of the resonances in the hyperpolarized spectrum we expect that a certain amount of the



Scheme 2. Parahydrogenation of 5-methyl-5-propargyl-barbituric acid at optimized reaction conditions. The hyperpolarized protons are highlighted in red.

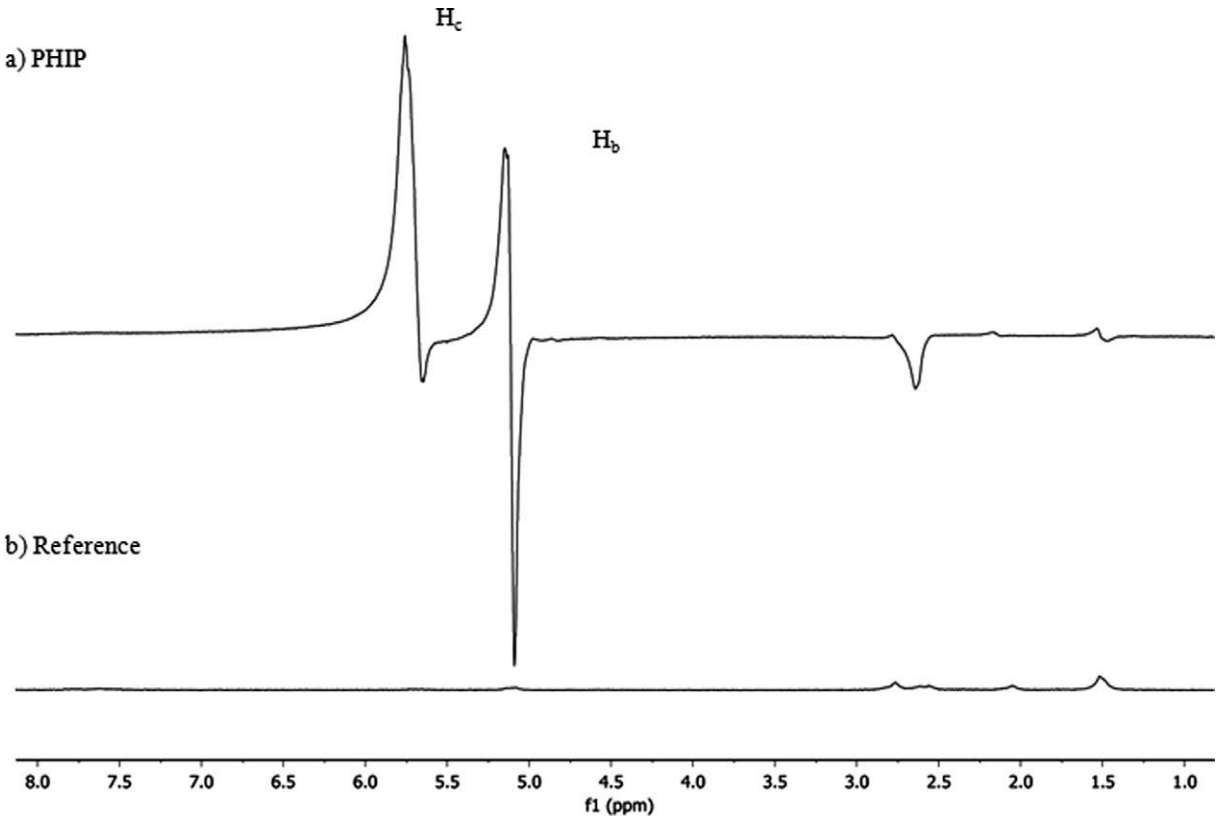


Fig. 2. ^{1}H NMR spectra acquired using only one pulse (a) upon parahydrogenation of 5-methyl-5-propargylbarbituric acid at 60 °C with 3.5 bar of 98% enriched para-H_2, and (b) the reference spectrum of the same reaction mixture acquired 20 min afterward under the same conditions. The signal enhancement of the PHIP spectrum amounts to 640 for H_b and 1025 for H_c.

achieved hyperpolarization is not observed due to cancelation of the antiphase signals of the multiplets due to their closely spaced peaks. Thus, better shimming should result in even higher ^{1}H NMR signal enhancements. Since our aim was to polarize ^{13}C positions, however, we did not optimize recording the ^{1}H NMR spectra.

The signal enhancements of the ^{13}C PHIP NMR spectra after polarization transfer from the initially hyperpolarized protons to the carbons via the PH-INEPT+ sequence are presented in Fig. 3.

All spectra depicted in Fig. 3 were recorded under the same reaction conditions, namely upon one scan but with different $t_1/2$ delays in the PH-INEPT+ sequence. As noted before, the reference spectrum was recorded with 256 scans immediately after the hyperpolarized single scan spectrum and was scaled in order to show the same noise level as the hyperpolarized spectra. The strong signal at 30 ppm in the reference spectrum belongs to the solvent acetone-d_6. For a better comparison of the signal enhancements of the various carbons obtained with different $t_1/2$ times in the PH-INEPT+ sequence Table 2 provides a summary of these data.

For the short pulse delay of 5 ms the polarization is almost equally transferred to both carbons of the resulting double bond yielding a signal enhancement of 1930 for the CH-group. With the somewhat longer delay of 10 ms in the PH-INEPT+ sequence polarization transfer to the carbons of the double bond as well as to the neighbor carbon C_8 occurs. Under these conditions the highest signal enhancement is obtained for the CH_2-group C_{10} amounting to 1700. Compared to the high proton polarization of more than 1000 the PH-INEPT+ polarization transfer to ^{13}C does not seem to be very efficient for these delays.

However, implementing a longer delay of 15 ms most of the polarization is transferred to the vicinal CH_2-group C_8 of the product featuring the dramatic enhancement factor of 5200. Indeed, this represents the highest signal gain achieved for the barbituric acid derivative in our study and proves the effectiveness of the PH-INEPT+ sequence for polarization transfer to ^{13}C when using an appropriate timing. This result demonstrates that the investigation of the target location and the mechanism of action of this molecule by MRI should be feasible with ^{13}C enriched barbiturates.

Finally, the even longer delay of 20 ms resulted in a large signal enhancement of the C_{10} of approximately 3400 and even more interestingly polarization is also transferred to the carbonyl group of the molecule which takes part in the biotransformation of the molecule. This provides an opportunity to store the polarization on this nucleus with its long T_1 time in order to investigate the substrate's metabolic pathway subsequently.

Haake et al. pointed out that the transfer efficiency of the PH-INEPT+ sequence critically depends on the ^{1}H–^{1}H coupling strength of the initially polarized protons [29]. However, the transfer efficiencies to the various carbon positions in the molecule also depend on the J couplings between the carbons and the polarized protons. This is clearly reflected in our data, since longer delays in the PH-INEPT+ sequence allow polarization transfer through weaker couplings and hence polarization of more distant carbons (compare Table 2). More advanced polarization transfer sequences derived by optimal control theory could optimize the polarization transfer efficiency and selectivity from the initially created ^{1}H polarization to the carbon position of choice [32,33].

4. Conclusion

Recently, we demonstrated that homogeneous hydrogenation of an unsaturated barbituric acid derivative with 50% parahydrogen resulted in a signal enhancement of around 40 for the ^{1}H

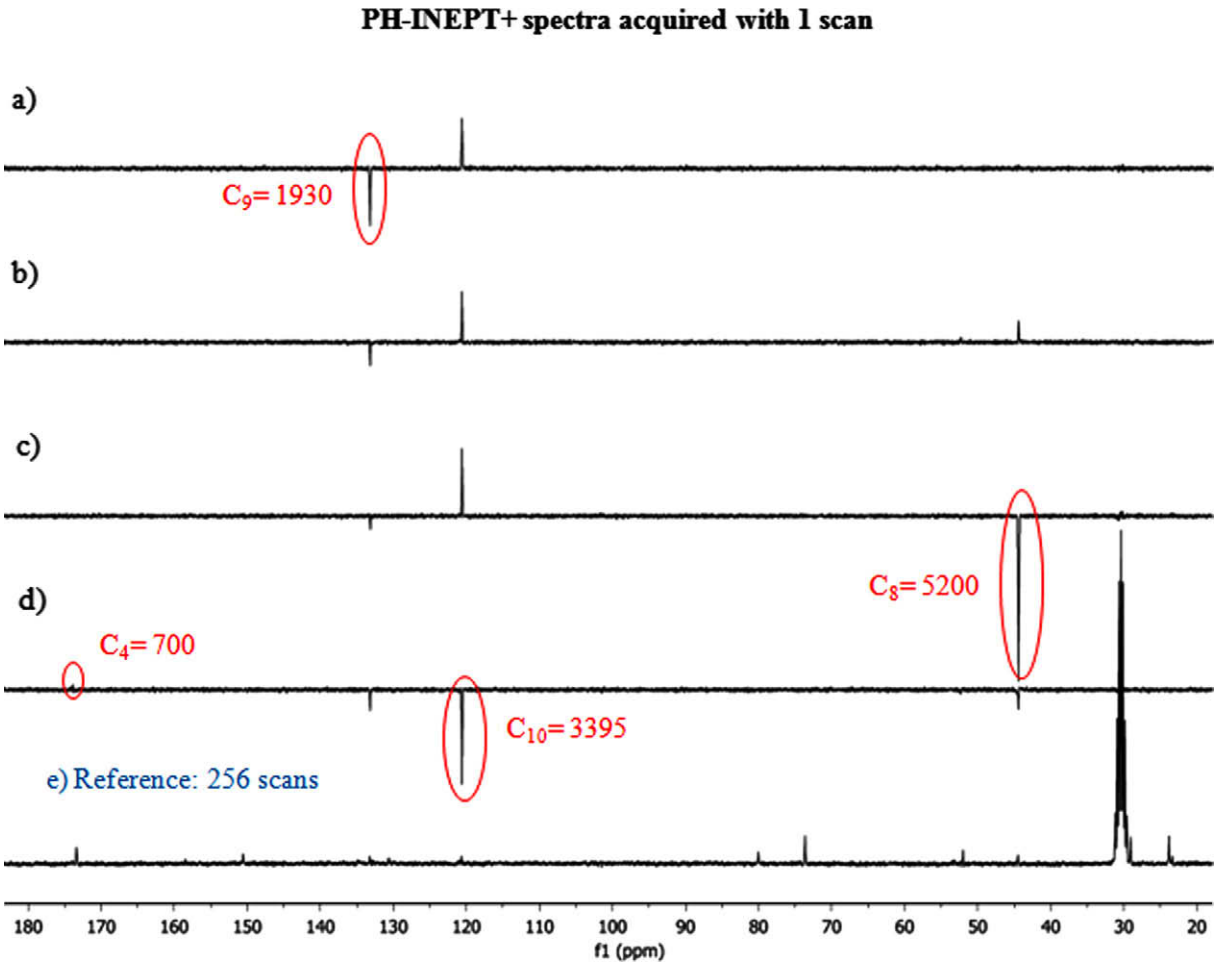


Fig. 3. ^{13}C PHIP NMR spectra of hyperpolarized 5-methyl-5-propargyl-barbituric, all recorded upon one pulse but using different delays $t_1/2$ for the PH-INEPT+ sequence (a) $t_1/2$ = 5 ms, (b) $t_1/2$ = 10 ms, (c) $t_1/2$ = 15 ms, and (d) $t_1/2$ = 20 ms. (e) Reference spectrum of the thermally polarized product acquired using 256 scans. The highlighted peaks correspond to the highest observed signal enhancements of the carbons C_4, C_8, C_9, and C_{10}.

Table 2
Comparison of the ^{13}C signal enhancements of the parahydrogenation product 5-methyl-5-propenyl-barbituric acid for different delays of the PH-INEPT+ sequence.

Delay (ms)	$C_{10}(CH_2)$ 120 ppm	$C_9(CH)$ 132 ppm	$C_8(CH_2)$ 43 ppm	$C_4(C{=}O)$ 174 ppm
5	1720	1930	–	–
10	1700	790	605	–
15	2100	450	5200	–
20	3395	765	–	700

NMR signals of the reaction product [37]. However, no signal enhancement by randomly triggered polarization transfer to ^{13}C in the weak magnetic field could be achieved. In our present study we demonstrated significantly larger signal enhancements around 1000 of ^1H in barbiturates by using 98% enriched para-H_2 in combination with optimized reaction conditions (elevated temperature and pressure). Moreover, this optimization together with the application of an INEPT-derived pulse sequence for effective polarization transfer allowed for dramatic ^{13}C NMR signal enhancements up to 5200 of the different sites of the product 5-methyl-5-propenyl-barbituric acid. The implementation of different delays in the PH-INEPT+ sequence showed that the polarization can be selectively transferred to different carbons in the barbituric acid derivative. Notably, even polarization of the carbonyl group featuring a long T_1 time was achieved, which is of high importance for storing the polarization within this molecule. Optimized polarization transfer sequences based on optimal control theory could be beneficial in order to improve the efficiency and selectivity of polarization transfer [32,33]. The presented results open up the opportunity to use barbituric acid derivatives as "*active*" contrast agents in MRI and enable investigations of the function of an important pharmaceutical substrate in vivo.

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