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Introduction

Nuclear magnetic resonance (NMR) spectroscopy and magnetic resonance imaging (MRI) are powerful techniques, which have widely been used for studying molecular structures associated to diseases and to visualize illnesses even in vivo.1-4 Both techniques are greatly hampered, due to their low sensitivity. This limitation can be overcome by using hyperpolarization methods, which increase signals of molecules by more than four orders of magnitude.5-13 Several hyperpolarization techniques have evolved to gain new insights e.g. in the fields of structural biology, material science, chemical analysis, biochemistry and biomedical science. With a view on the latter, hyperpolarization allows for creating new contrast agents to study and diagnose diseases in vivo.14 The technique mainly used for producing hyperpolarized contrast agents is dissolution dynamic nuclear polarization (d-DNP).5 It enables the hyperpolarization of metabolically active compounds that can be followed during *in vivo* studies.^{8,9,11,14-16} Other methods with biomedical relevance are spin exchange optical pumping (SEOP)17-20 of noble gases and para-hydrogen induced

Hyperpolarization of ¹⁵N-pyridinium and ¹⁵Naniline derivatives by using parahydrogen: new opportunities to store nuclear spin polarization in aqueous media⁺

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Hyperpolarization techniques hold the promise to improve the sensitivity of magnetic resonance imaging (MRI) contrast agents by over 10 000-fold. Among these techniques, para-hydrogen induced polarization (PHIP) allows for generating contrast agents within seconds. Typical hyperpolarized contrast agents are traceable for 2–3 minutes only, thus prolonging tracking-times holds great importance for the development of new ways to diagnose and monitor diseases. Here, we report on the design of perdeuterated ¹⁵N-containing molecules with longitudinal relaxation times (T_1) of several minutes. T_1 is a measure for how long hyperpolarization can be stored. In particular, we introduce two new hyperpolarizable families of compounds that we signal enhanced with para-hydrogen: *tert*-amine aniline derivatives and a quaternary pyridinium compound with ¹⁵N- T_1 of about 8 minutes. Especially the latter compound has great potential for applicability since we achieved ¹⁵N-polarization up to 8% and the pyridinium motif is contained in a variety of drug molecules and is also used in drug delivery systems.

polarization (PHIP).^{21–32} PHIP methods transfer nuclear spin order from para-hydrogen (para-H₂) enriched hydrogen over to target molecules for their hyperpolarization. Hydrogenative PHIP adds para-H₂ to unsaturated precursors over suitable hydrogenation catalysts, to create large spin-order in the target compounds, which can be converted into observable magnetization afterwards. Due to the design of suitable precursor molecules, this technique can now be utilized to hyperpolarize metabolically active compounds and to analyze their chemical conversion *in vivo*.^{28,31,32}

Within the past ten years, a non-hydrogenative para-H₂based hyperpolarization methods has evolved: signal amplification by reversible exchange (SABRE).³²⁻³⁷ For this method, para-H₂ and a substrate of interest coordinate to a temporarily stable transition metal complex. In this complex, the para-H₂ spin order is converted into observable magnetization at the molecule of interest. Dissociation of the complex leads to free hyperpolarized substrates that have not been altered as in the classical PHIP approach.³⁸ However, this method has not been shown to be applicable for *in vivo* applications yet since, SABRE experiments typically need to be performed in organic solvents. However, the field rapidly progressing and work is on the way to make this technique more biologically applicable in the future.^{32,39,40}

What all techniques have in common is the desire to store hyperpolarization in contrast agents for long periods of time. To this end, hyperpolarization is typically stored on hetero-nuclei such as in ¹³C and ¹⁵N, which possess longitudinal relaxation

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times (T_1) ranging from seconds to minutes. The T_1 of ¹³Cpyruvate, the metabolite most commonly hyperpolarized, for example is in the range of 40-60 s.41 For in vivo applications, this results in a time window of 2-3 minutes, during which pyruvate can be monitored.^{11,14,41} To increase tracing times, ¹⁵N nuclei are more favorable than ¹³C nuclei since T_1 can be one order of magnitude longer and $T_1 > 1200$ s (20 minutes) in water have been reported in quaternary nitrogen compounds.³⁰ Due to its longer T₁ values, ¹⁵N-derived chemical probes have been explored: with respect to PHIP N-ethyl trimethyl ammonium (NETMA) and an allyl choline derivative have been polarized in biocompatible solvents.30,42,43 Dissolution DNP has demonstrated first in vivo experiments utilizing ¹⁵N polarized choline and several other applications in vitro such as pH-sensing, Ca²⁺ monitoring and enzyme activity.44-46 Degrees of ¹⁵N-polarization have long been rather low until the advancements in crosspolarization (CP) d-DNP have overcome this challenge.¹³

SABRE has made great progress in polarizing ¹⁵N spins in the past years.^{34,35,47} Demonstrations of over 40% polarization in ¹⁵N pyridine and more than 30% for imidazole have been accomplished in methanol.^{48,49} Prospective applications may include pH-sensing⁵⁰ or probing of hypoxia.^{47,49} The later may in particular become feasible *via* storage of polarization in a ¹⁵N-nitro group of metronidazole which has a T_1 of about 10 minutes in methanol.⁵¹

Currently the main challenge is to discover molecules that are biological relevant, have long T_1 and can be hyperpolarized to a large degree. Here, we are tackling this challenge and introduce classes of compounds that meet these requirements. Our particular focus is thereby on pyridinium, a compound already relevant in drug applications.^{52–56}

Experimental

The synthesis of the labelled compounds was conducted as follows: to yield **1**, we prepared ¹⁵N-pyridine- d_5 starting from protonated ¹⁵N-pyridine, oxidation with *meta*-chloroperoxybenzoic acid (*m*-CPBA) followed by H–D exchange reaction under microwave condition in D₂O (Scheme 1A). Further reduction with PCl₃ in CH₂Cl₂ yielded ¹⁵N-pyridine- d_5 .⁵⁷ Finally, quaternization of **5** was accomplished by the treatment with allyl bromide- d_5 (**6**) in EtOAc to yield **1** as colourless solid.⁵⁸ In order to synthesize the aniline derivatives **2** and **3**, we first synthesized ¹⁵N-aniline- d_5 (**7**) in a two-step procedure from benzene- d_5 .⁵⁹ Mono-allylation of **7** with allyl bromide- d_5 (**6**) in the presence of K₂CO₃ and further treatment with CD₃I in presence of DIPEA, yielded **2** (Scheme 1B). Stirring of **2** in neat CD₃I leads to the quaternary aniline derivative **3**. Further experimental details can be found in the ESI.[†]

Result and discussion

We have synthesized and investigated a library of ¹⁵N-enriched compounds and report on two novelties: firstly, we have discovered an aniline derivative containing a tertiary amine with a long T_1 of about 10 minutes in methanol- d_4 (MeOD). This is of particular interest since it demonstrates that uncharged



Scheme 1 Syntheses of ¹⁵N-pyridinium derivative (A) and -aniline based (B) derivatives; MW: microwave.

nitrogen species, in addition to quaternary compounds, have potential to store polarization for long periods and opens up new possibilities to design contrast agents with lipophilic moieties. Secondly, we are introducing a new class of compounds that can be hyperpolarized and possesses a T_1 of about 8 minutes in water: quaternary pyridinium derivatives. Quaternary pyridinium is a core structure found in many molecules which has been used for investigations of neurodegenerative diseases⁶⁰ as well as in drug design and drug-delivery approaches.^{52–56} We furthermore present the hyperpolarization of the library of compounds *via* PHIP and a pulsed transfer method to enhance the ¹⁵N signals. Generating contrast agents in aqueous media becomes possible by utilizing rhodium nanocatalysts (NAC@Rh) that promote the hydrogenation reaction with para-H₂ in water.³⁰

Table 1 presents the investigated compounds and at the top the general scheme of how the investigated compounds are hyperpolarized with para-H₂. The precursor compounds prior to hydrogenation are a pyridinium derivative (**1**), a *tert*-amine derivative of aniline (**2**) and a quaternary nitrogen derivative of aniline (**3**). We have perdeuterated all of the precursors to prolong ¹⁵N- T_1 by weakening dipolar couplings, as compared to the protonated counterparts. As an unsaturated moiety to which para-H₂ will be added during the hydrogenation step, we have chosen deuterated allyl groups. The rationale behind this choice is twofold: first, the added protons from para-H₂ after the hydrogenation will be one extra bond away as compared to the vinyl derivatives, thus reducing dipolar interactions that potentially shorten T_1 . Second, the scalar coupling network in

Table 1	¹⁵ N-T ₁ values for	^r pyridinium and p	henylammonium	compounds along with	their reduced p	products using para-H ₂ ^a
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	ו ¹⁵ ר R	$\begin{array}{c} R' & D \\ V \\ D & D \\ D & D \\ X \end{array} \xrightarrow{Para-H_2} R^{T} \\ Polarization \\ R \\ Polarization \\ R \\ D \\ D \\ D \\ D \\ X \end{array} \xrightarrow{Polarization} R^{T} \\ R^{T} \\ R^{T} \\ D \\ $	$\stackrel{n}{\rightarrow} \stackrel{15}{R^{\prime}} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{D}{\rightarrow} \stackrel{H}{\rightarrow} \stackrel{H}{\rightarrow}$	
X	Structure	¹⁵ N- <i>T</i> ₁ in s	Mag. field in T	Solvent and temp. in K
1		$\begin{array}{c} 140 \pm 20 \\ 220 \pm 30 \end{array}$	7 7	MeOD at 320 D ₂ O at 353
1a	$D \qquad D \qquad D \qquad H \qquad H \\ D \qquad H \qquad N \qquad D \qquad D \qquad D \qquad D \qquad Br$	$\begin{array}{l} 240\pm10^b\\ 360\pm50^b\\ 570\pm110^b\\ 380\pm30^b\\ 120\pm10^c\\ 330\pm20^c\\ 500\pm30^c\\ 290\pm20^c\end{array}$	7 1 0.1 0.01 7 1 0.1 0.01	MeOD at 320 MeOD at 320 MeOD at 320 MeOD at 320 D ₂ O at 353 D ₂ O at 353 D ₂ O at 353 D ₂ O at 353 D ₂ O at 353
2	$D \xrightarrow{CD_3} D \xrightarrow{D} D$	570 ± 40	9.4	MeOD at 298
2a		$\frac{150 \pm 20^c}{90 \pm 40^c}$	7 0.1	MeOD : D ₂ O at 320 MeOD : D ₂ O at 320
3	$\begin{array}{c} D & D_3 C & C D_3 \\ D & 15 H & D \\ D & 0 & D \\ D & 0 & D \\ D & 0 & 0 \\ D & 0 & 0 \\ \end{array}$	420 ± 100	9.4	D ₂ O at 298
3a	$\begin{array}{c} D_{3}C \\ CD_{3} \\ D \\ H \\ D \\ D$	Decomposed	_	_

^{*a*} A general scheme of hyperpolarization followed by polarization transfer to ¹⁵N nuclei; X = 1 (R, R' = pyridinium); X = 2 (R = phenyl, R' = -CD₃) and X = 3 (R = phenyl, R' = -(CD₃)₂); Mag.: magnetic; temp.: temperature. ^{*b*} In the presence of 2 mM [Rh(dppb)(COD)][BF₄]. ^{*c*} In the presence of 0.5 mg mL⁻¹ NAC@Rh.

the hydrogenation products **1a–3a** are thought to be an ideal spin system to apply the recently developed ESOTHERIC (efficient spin order transfer to heteronuclei *via* relayed INEPT chains) spin order transfer sequence to hyperpolarize the ¹⁵N spins.^{61,62} This is because the ³ $J_{\rm H,N}$ coupling is larger than ⁴ $J_{\rm H,N}$ (see ESI†) and the protons are weakly coupled.

Prior to performing hyperpolarization experiments, we determined ¹⁵N- T_1 for the unsaturated precursor molecules **1–3** in D₂O, MeOD or mixtures thereof to increase the molecule's solubility. The ¹⁵N- T_1 values obtained in different solvents and at various magnetic fields are summarized in Table 1. For the precursor molecules it is noteworthy to mention that ¹⁵N- T_1 of the *tert*-amine **2** has a ¹⁵N- T_1 of 570 ± 40 s in MeOD (this compound was not soluble in water) at high field and the quaternary ammonium compound **3** displays a ¹⁵N- T_1 of 420 ± 100 s in D₂O. For the unsaturated pyridinium derivative **1**, we discovered a ¹⁵N- T_1 of 220 ± 30 s at high field in D₂O.

Since we found ¹⁵N-T₁ values of several minutes for all precursor compounds, we performed hydrogenation reactions and investigated 15 N- T_1 of the hydrogenation products. This was done by hyperpolarizing the ¹⁵N nuclei and measuring the polarization decay with low flip angle pulses as described in the next paragraph and in the ESI.[†] Our first observation was that the anilinium derivative 3a decomposes upon hydrogenation. This may reflect that trimethylanilinium is typically used as a methylation agent⁶³ and not stable enough for hyperpolarization studies with para-H2. Moreover, a similar kind of degradation was reported on ¹⁵N-propargylcholine while performing PHIP.42 In addition to this, Shchepin et. al. reported lack of the successful ¹⁵N hyperpolarization on other choline derivatives using ¹⁵N-enriched PHIP precursors.⁶⁴ The ¹⁵N-T₁ of the tert-amine 2a is strongly reduced after hydrogenation to 150 \pm 20 s. Lastly, the pyridinium derivative **1a** has a T_1 of 120 \pm 10 s at high field in D_2O , but reaches 500 \pm 30 s (about 8 minutes) when the field is lowered to 0.1 T (see also Fig. S1[†]). With

respect to T_1 , the main relaxation source at high field appears to be chemical shift anisotropy (CSA). This offers possibilities to make the compound applicable for studies in clinical scanners. Given its long ¹⁵N- T_1 at low field in water and being an important structure in a variety of biomolecules or drugs, the pyridinium derivative is the most promising compound discovered among the investigated compounds here for future applications.

To obtain the hyperpolarized products, compounds 1–3 were hydrogenated with para-H₂ under two experimental conditions: for preparation in MeOD, we used the homogeneous Rh-catalyst [Rh(dppb)(COD)][BF₄] (dppb: diphenylphosphino butane, COD: cyclooctadiene). For hyperpolarization in D₂O, we used an *N*-acetylcysteine-capped Rh-nano-catalysts (NAC@Rh).³⁰ The enrichment of H₂ in its *para*-state was 80%, as determined experimentally. At first, we have investigated the ¹H polarization and subsequently the ¹⁵N polarization following the ESOTHERIC sequence.^{61,62} The results are summarized in Table 2.

As compound **3a** did not form during hydrogenation, no hyperpolarization data is reported here for either the homogeneous or heterogeneous catalyst. Compound **2** turned out to be insoluble in D_2O ; therefore, we chose an equimolar mixture of MeOD and D_2O for dissolving the heterogeneous catalyst for PHIP experiments. We have found 1% polarization of ¹H and ¹⁵N nuclei respectively in the hydrogenated compound **2a**, whereas multiple polarized products were observed in MeOD with the homogeneous catalyst. This result demonstrates that heterogeneous catalysts provide new opportunities for polarizing nitrogen containing compounds that may not be accessible with the standard homogeneous catalyst.

With respect to the pyridinium derivative, we observed significant ¹H polarization of $11\% \pm 1.3\%$ in **1a** using the homogeneous catalyst in MeOD. We succeeded in transferring this polarization to the ¹⁵N-spin with a signal enhancement (ε) of 32 000 ($P = 7.4\% \pm 0.6\%$) compared to thermal polarization at $B_0 = 7$ T at 320 K in MeOD. For improved biocompatibility, we performed polarization experiments with the heterogeneous catalyst in water and achieved a highest polarization of 3.1% ($\varepsilon = 15$ 000-fold compared to the thermal signal at 353 K, Fig. 1) and an average 2.3% polarization. The spectrum of the hyperpolarized compound in water as well as the T_1 -experiment (inset) with small tip angle pulses at 0.1 T is depicted in Fig. 1.

 Table 2
 PHIP enhancements by using the homogenous and heterogeneous catalysts

	Compd.	¹ H <i>P</i> %	¹⁵ N <i>P</i> %
PHIP (homogeneous) MeOD at 320 K	1a	11 ± 1.3	7.4 ± 0.6
	2a 3a	a	a
PHIP (heterogeneous) D ₂ O at 353 K	1a 2a	$egin{array}{c} 2.1 \pm 1.2 \ 1.3 \pm 0.2^b \end{array}$	$2.3 \pm 1.1 \\ 0.8 \pm 0.1^b$
	3a	a	a

^{*a*} Multiple products or decomposition. ^{*b*} Measured in MeOD : $D_2O(1:1)$ at 320 K. Compd.: compound.



Fig. 1 Hyperpolarized ¹⁵N spectrum of **1a** (blue) by using PHIP in D₂O and thermally polarized ¹⁵N spectrum of the unsaturated precursor **1** (red) at 7 T. The inset shows the ¹⁵N- T_1 relaxation data measured at 0.1 T, using a hyperpolarized sample of **1a** which was collected using sample shuttling and small flip angle pulses (see ESI[†] for further details).

Conclusions

In conclusion, we have introduced and synthesized perdeuterated ¹⁵N-allyl-pyridinium (1) and -aniline derivatives (2 & 3). We succeeded in forming hyperpolarized addition products of 1 and 2 utilizing para-H₂. Most notably, a ¹⁵N-pyridinium derivative (1a) provided strong ¹⁵N-polarization of P = 7.4% in methanol and P = 2.3% in water compared to thermal polarization. Polarization in water was achieved via rhodium nanocatalysts that although heterogeneous PHIP catalysts are still in an early development stage show here the possibility to signal enhance molecules that are not polarizable with standard homogeneous metal complexes. In water at 0.1 T field, we discovered a long 15 N- T_1 of about 8 min. We also found that the tert-amine 2 features notably a slow relaxation time of 10 min for ¹⁵N-nuclei in methanol. This is despite the fact that it is not a quaternary nitrogen compound, and thus could be used as a hydrophobic ¹⁵N-labelled tracer. Overall, our presented studies introduce new possibilities for the molecular design of contrast agents and storage capabilities of hyperpolarized spin states. It is noteworthy to mention that out of all compounds studied here, the highest levels of hyperpolarization (¹H and ¹⁵N) were found in pyridinium derivatives, a molecular species present in many bio-relevant molecules. Longer relaxation times of ¹⁵N nuclei of these compounds in combination with targeting moieties will potentially in the future ensure long traceability and opportunity to deliver the hyperpolarization in organisms for biomedical imaging applications.

Conflicts of interest

There are no conflicts to declare.

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