Late-stage Deuteration of ^{13}C-enriched Substrates for T_1 Prolongation in Hyperpolarized ^{13}C MRI

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Abstract

A robust and selective late-stage deuteration methodology was applied to ^{13}C-enriched amino and alpha hydroxy acids to increase spin-lattice relaxation constant T_1 for hyperpolarized ^{13}C magnetic resonance imaging. For the five substrates with ^{13}C-labeling on the C1-position ([1-^{13}C]alanine, [1-^{13}C]serine, [1-^{13}C]lactate, [1-^{13}C]glycine, and [1-^{13}C]valine), significant increase of their T_1 was observed at 3T with deuterium labeling (+26%, 22%, +16%, +25% and +29%, respectively). Remarkably, in the case of [2-^{13}C]alanine, [2-^{13}C]serine and [2-^{13}C]lactate, deuterium labeling led to a greater than four fold increase in T_1. [1-^{13}C,2-^{2}H]alanine, produced using this method, was applied to *in vitro* enzyme assays with alanine aminotransferase, demonstrating a kinetic isotope effect.

Magnetic resonance imaging employing hyperpolarized substrates (HP MRI) has recently emerged as a powerful tool for studying metabolism in cells, animal models and patients.[1–9] Polarization of substrates can be realized through a variety of mechanisms including dissolution dynamic nuclear polarization (DNP),[10] parahydrogen induced polarization (PHIP),[11,12] or signal amplification by reversible exchange (SABRE).[13] While these are versatile methods that allow for real time imaging of metabolism, the short lifetime of the hyperpolarized signal, which decays exponentially based upon the spin lattice relaxation time T_1, remains one of the key limiting factors in the implementation of this technology. The most widely used HP ^{13}C probe is [1-^{13}C]pyruvate, a key metabolic intermediate, which has a T_1 of 67 s at 3T. However, other ^{13}C nuclei, especially those with directly attached protons, are not feasible HP ^{13}C probes due to very short T_1's (less than 5s).[14] One approach to increase T_1 is the substitution of ^{1}H with ^{2}H (or D), a quadrupolar nucleus with a gyromagnetic ratio γ about 6.5-fold smaller than the one for ^{1}H.[15–29] This use of deuterated substrates has proved particularly fruitful in the case of SABRE[13] and PHIP[11,12] methods. This approach is effective when dipolar ^{13}C-^{1}H coupling contributes substantially to T_1

relaxation. Fortunately, in the case of pyruvate, the incorporation of deuterium is straightforward owing to lack of stereocenters.[30] However, the synthesis of multiply labelled molecules containing stereocenters including both ^{13}C and ^{2}H is generally both expensive and time consuming, and most isotopically enriched molecules require multi-step syntheses. Therefore, a robust method for incorporation of deuterium in the final step of synthesis would be generally valuable in the field of HP MRI.

For the synthesis of deuterated molecules, late-stage isotopic exchange has several advantages over a synthetic pathway from enriched building blocks. Numerous methods based on homogeneous or heterogeneous catalysts for H/D exchange have already been described, but the development of a deuteration methodology with mild reaction conditions, high selectivity and deuterium incorporation is still a challenge.[31,32] In order to develop [$^{13}C,^{2}H$]labelled probes for HP MRI, we considered the regioselective deuteration, at the α-position of aliphatic alcohols and sugars, developed by Sajiki *et al.*, as a straightforward way to the deuterium labelling of ^{13}C-substrates with attached O or N.[33,34] In this manuscript, we report the application of this methodology to a variety of ^{13}C-enriched compounds, enabling high incorporation yields with retention of configuration, and demonstrate a significant increase in T_1 of the resulting deuterated substrates. One of the probes, [1-^{13}C, 2-^{2}H]alanine, was studied in an *in vitro* enzymatic assay with alanine aminotransferase (ALT), revealing a deuterium kinetic isotope effect.

Initially, we evaluated the performance of the labelling methodology with a variety of labelled substrates including α-amino and hydroxy acids. We performed the one-step deuterium labelling reaction on position C2 of several commercial ^{13}C-labeled substrates (Scheme 1, Table 1). Reactions were incubated in D_2O in the presence of RuC 5% (40 wt%), under H_2, overnight, at 80°C (Table S1, ESI†). Efficient deuterium incorporation on position C2 (95–97 %) was observed for aliphatic amino acids [1-^{13}C,2-^{2}H] and [2-^{13}C,2-^{2}H]alanine (**1** and **6**), [1-^{13}C,2-$^{2}H_2$]glycine **4** and [1-^{13}C,2-^{2}H]valine **5**, with enantiomeric excesses greater than 99%. Isotopic enrichments on position C2 of [1-^{13}C,2-^{2}H] and [2-^{13}C, 2-^{2}H]sodium lactate (**3** and **8**) were 97% and 98%, respectively, with lower enantiomeric excesses (86 and 94%). Moderate chemical yield on [1-^{13}C,2-^{2}H] valine **5**, 53%, may be due to its lower solubility in D_2O. Enantiomeric excess was 98% for both [1-^{13}C,2-^{2}H] and [2-^{13}C,2-^{2}H]serine (**2** and **7**) whereas chemical yields were 78% and 77%, respectively. Their lower isotopic enrichments on position C2 (52 and 90%) may be due to the additional deuterium labelling on their position C3. In a few cases, side reactions were encountered which led to decomposition of the desired product (ESI†). Taken together with prior reports, [27,35] our data indicate that this is a versatile method for deuterium incorporation in biologically relevant molecules.

In order to determine the impact of deuterium incorporation on T_1, we then prepared the labelled substrates for hyperpolarization. Solutions of 4 to 6 M substrate with 1 to 1.2 equivalents NaOH and 23 to 24 mM free radical (OX063) were prepared for

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†Electronic Supplementary Information (ESI) available: Reagents and procedures for deuteration reaction, deuterium incorporation quantification, characterization for compounds 1 to 8, experimental details for T_1 measurements in solution, *in vivo* and *in vitro* enzyme experiments. See DOI: 10.1039/x0xx00000x

hyperpolarization using DNP.[36] Following polarization, T_1 measurements were performed on a 3T preclinical MR scanner. Deuterium substitution at the C2 position yielded significant improvements of the T_1 with ^{13}C at the C1 position, ranging from 16–29% (Figure 1). The relatively modest improvement in T_1 yielded larger signal gains at later time points. For example, in the case of [1-^{13}C]alanine, deuteration yielded an increase in signal to noise ratio of 60% at 90s after the start of the experiment (Figure S68c, ESI†). Remarkably, in the case of [2-^{13}C,2-^{2}H]alanine **6**, [2-^{13}C,2,3-^{2}H]serine **7** and [2-^{13}C, 2-^{2}H]lactate **8**, deuterium labelling led to a greater than four-fold increase in T_1. Due to rapid signal decay on [2-^{13}C]alanine, [2-^{13}C]serine and [2-^{13}C]lactate, their T_1 could not be measured using a hyperpolarized method[36] and were instead assayed using an inversion recovery-sequence. Part of the reason why the T_1 gains due to deuteration are relatively limited is because of chemical shift anisotropy (CSA) which is likely the dominant relaxation mechanism at 3T.[37,38] Therefore, at 1.5T, there could be further improvements in T_1 prolongation.[39]

We then evaluated the T_1 of one of our substrates, [1-^{13}C,2-^{2}H]alanine **1**, in an *in vivo* experiment in a mouse model and compared its properties with those of [1-^{13}C]alanine. MR measurements where performed on a preclinical 3T scanner (Figure S71, ESI†). 300 μL of 80 mM solutions of hyperpolarized [1-^{13}C]alanine and [1-^{13}C,2-^{2}H]alanine **1** were injected intravenously immediately followed by dynamic acquisition of ^{13}C MRS spectra. As expected, based on the *in vitro* studies, we found an increase in the apparent *in vivo* T_1 at 3T, from 32 s, for [1-^{13}C]alanine, to 42 s, for [1-^{13}C,2-^{2}H]alanine **1**.

As a demonstration of the utility of the deuteration method, we next applied the labelled alanine probes in an *in vitro* enzyme assay using alanine transaminase (ALT). ALT is an abundant enzyme and a biomarker for liver disease, which converts alanine and α-ketoglutarate to pyruvate and glutamate, respectively (Figure 2a). Previous reports have studied this enzyme both *in vitro* and *in vivo* using hyperpolarized ^{13}C methods.[36,40,41] Therefore, we developed an assay for the detection of ^{13}C pyruvate production by incubation of polarized [1-^{13}C]alanine or [1-^{13}C,2-^{2}H]alanine **1** with α-ketoglutarate, glutamate and ALT based on prior reports.[42] As expected, ^{13}C pyruvate was rapidly formed during the time course of the hyperpolarized experiment (Figures 2b-d). Furthermore, the initial rate of pyruvate signal growth, which approximates the forward conversion rate, was about 2.42-fold lower for the [1-^{13}C,2-^{2}H]alanine **1** as compared with the [1-^{13}C]alanine ($n = 3$ each, p < 0.002, neutral pH). This agrees closely with the previously reported kinetic isotope effect of 2.3.[42] In order to confirm these findings, we fit the dynamic alanine and pyruvate MRS data to a kinetic model accounting for HP signal exchange between protonated and deuterated [1-^{13}C]alanine and [1-^{13}C]pyruvate pools as well as signal loss due to RF sampling and T_1 loss (Fig. S72, ESI†).[43] We thus obtained pseudo-first order rate constants of $(1.87 \pm 0.174) \times 10^{-3}$ s^{-1} ($n = 3$) and $(0.736 \pm 0.015) \times 10^{-3}$ s^{-1} ($n = 3$) for protonated and deuterated alanine, respectively. This difference in kinetic rates suggested a kinetic isotope effect of 2.53, in close agreement with our previous analysis and with the literature. [42]

In summary, these data indicate that the RuC labelling method represents a versatile method for high-yield deuteration of ^{13}C labelled substrates, ideal for application to hyperpolarized

^{13}C MRI. When the deuterium was incorporated adjacent to a ^{13}C-enriched carbonyl, the effect on T_1 prolongation was moderate, ranging from 16–29%. In contrast, when applied to ^{13}C nuclei with directly attached protons ([2-^{13}C,2-^{2}H]alanine **6**, [2-^{13}C,2,3-^{2}H]serine **7** and [2-^{13}C,2-^{2}H]lactate **8**), an approximately 4-fold increase in T_1 was observed. To further study the behavior of doubly-enriched substrates, we applied [1-^{13}C]alanine and [1-^{13}C, 2-^{2}H]alanine **1** to an *in vitro* enzyme assay with purified ALT enzyme, demonstrating a kinetic isotope effect, in agreement with prior reports. We anticipate that this versatile method will find application to a variety of substrates for hyperpolarized ^{13}C MRI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Notes and references

‡ C.T. carried out the experiments and wrote the manuscript with support from D.E.K. R.R.F. and D.M.W. designed and directed the project. H.V.B, D.B.V., S.M.R., R.S. and J.K. helped supervise the project and helped edit the manuscript. C.V.M., J.Y., S.W. J.E.B., S.S. and R.B. helped with characterization of obtained compounds, T_1 measurements and *in vivo* experiments. C.N. and S.M.R. provided critical feedback and helped shape the research, notably for the study of [2-^{13}C,2-^{2}H]enriched substrates.

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Scheme 1:
Regioselective catalytic deuterium labelling via 1H/2H exchange using ruthenium on carbon (RuC).
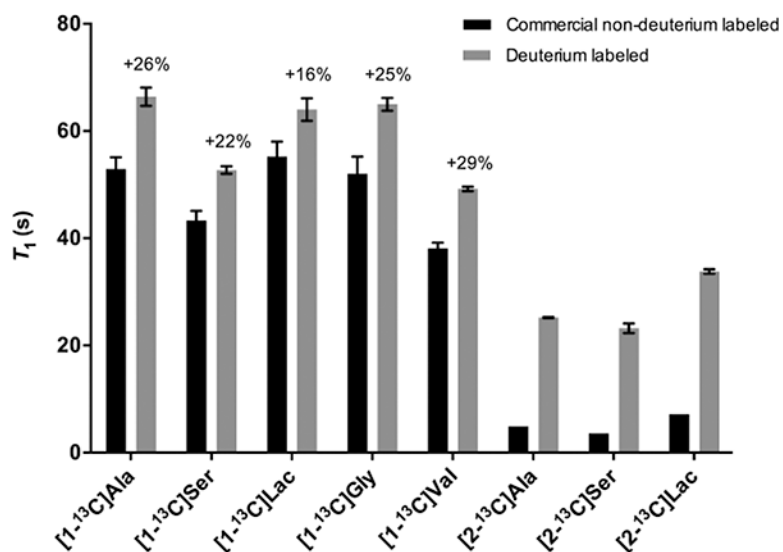
Figure 1:

T_1 relaxation times at 3T for proton and deuterium-labelled ^{13}C-substrates ($n = 3$, ± s.d., $p < 0.02$). Due to very low polarization for commercial non-deuterium labeled [2–^{13}C]alanine, [2–^{13}C]serine and [2–^{13}C]lactate, T_1 could not be evaluated using hyperpolarized methods, and inversion-recovery was used at 11.7T: $T_1 = 4.9$ s, 3.6 s and 7.2 s, respectively.
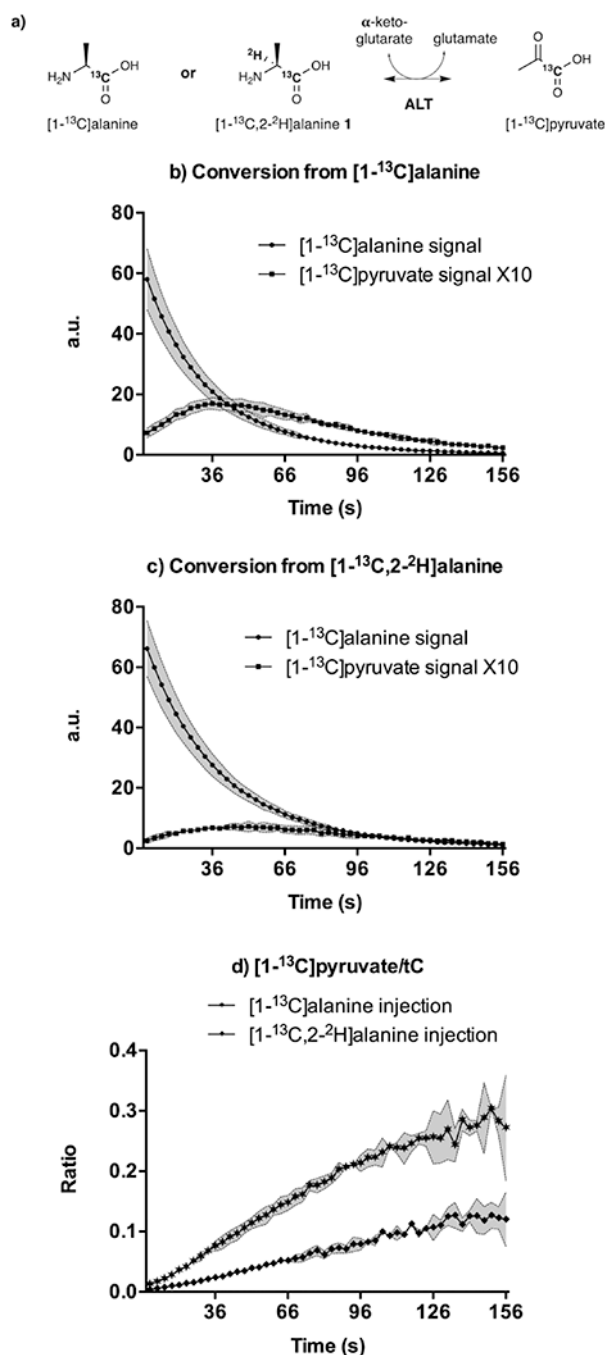
Figure 2:
Influence of deuterium labelling on [1–^{13}C]pyruvate formation after conversion from [1–^{13}C]alanine and from [1–^{13}C,2–^{2}H]alanine **1** in solution in the presence of ALT enzyme. a) Metabolic pathways of hyperpolarized [1–^{13}C]alanine and [1–^{13}C,2–^{2}H]alanine **1** via ALT. b) and c) Time courses of integrated spectra showing the evolution of HP [1–^{13}C]alanine,[1–^{13}C,2–^{2}H]alanine **1** and their metabolite [1–^{13}C]pyruvate (normalized peak integrations) (*n = 3*, ± s.d.). For clarity, pyruvate integrals were ten-fold upscaled. Shaded areas denote the experimental error bars. Spectral acquisition started 11s (b) and 10 s (c) after incubation of

the HP probe and the enzyme solution in an NMR tube. d) Measurements of [1–^{13}C]pyruvate/total ^{13}C-labeled signals (tC) ratios ($n = 3$, ± s.d.).
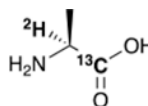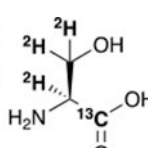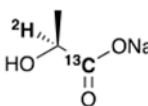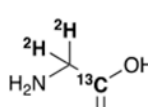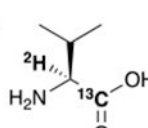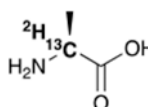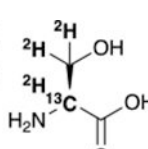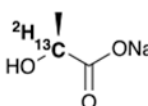
Table 1:

Structures of ^{13}C-enriched molecules after deuterium enrichment. The bracketed number indicates the isotopic enrichment determined by ^{1}H,^{13}C NMR and HRMS (analyses described in the ESI†). ee: enantiomeric excess.

Molecule	Structure	Chemical yield	ee
[13]**C on position C1 and** [2]**H on position C2**			
[1-^{13}C,2-^{2}H]alanine **1**	[97]	99 %	99 %
[1-^{13}C,2,3-[2]H$_3$]serine **2**	[26] [52]	78 %	98 %
[1-^{13}C,2-^{2}H]lactate **3**	[97]	98 %	86 %
[1-^{13}C,2-[2]H$_2$]glycine **4**	[97]	79 %	-
[1-^{13}C,2-^{2}H]valine **5**	[95]	53 %	99 %
[13]**C on position C2 and** [2]**H on position C2**			
[2-^{13}C,2-^{2}H]alanine **6**	[97]	89 %	99 %
[2-^{13}C,2,3-[2]H$_3$]serine **7**	[65] [90]	77 %	98 %
[2-^{13}C,2-^{2}H]lactate **8**	[98]	99 %	94 %