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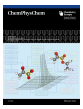

ParaHydrogen Polarized Ethyl-[1-^{13}C]pyruvate in Water, a Key Substrate for Fostering the PHIP-SAH Approach to Metabolic Imaging

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An efficient synthesis of vinyl-[1-^{13}C]pyruvate has been reported, from which ^{13}C hyperpolarized (HP) ethyl-[1-^{13}C]pyruvate has been obtained by means of ParaHydrogen Induced Polarization (PHIP). Due to the intrinsic lability of pyruvate, which leads quickly to degradation of the reaction mixture even under mild reaction conditions, the vinyl-ester has been synthesized through the intermediacy of a more stable ketal derivative. ^{13}C and ^{1}H hyperpolarizations of ethyl-[1-^{13}C]pyruvate, hydrogenated using ParaHydrogen, have been compared to those observed on the more widely used allyl-derivative. It has been demonstrated that the spin order transfer from ParaHydrogen protons to ^{13}C, is more efficient on the ethyl than on the allyl-esterdue to the larger J-couplings involved. The main requirements needed for the biological application of this HP product have been met, i.e. an aqueous solution of the product at high concentration (40 mM) with a good ^{13}C polarization level (4.8%) has been obtained. The in vitro metabolic transformation of the HP ethyl-[1-^{13}C]pyruvate, catalyzed by an esterase, has been observed. This substrate appears to be a good candidate for in vivo metabolic investigations using PHIP hyperpolarized probes.

1. Introduction

The access to hyperpolarization techniques has brought an outstanding contribution in the perspectives of providing innovative tools in the field of medical diagnoses as it allows, for the first time, the investigation of metabolic processes in vivo, in real time.[1–3] Dissolution-Dynamic Nuclear Polarization (d-DNP)[3,4] is the gold standard among the hyperpolarization methods and several in-human studies are currently ongoing,[6–10] that aim to translate this powerful diagnostic tool to the clinics. In spite of the important achievements, the distribution of d-DNP in clinical settings appears hampered by the high cost and the technical complexity of the technique as well as by the intrinsically slow polarization time. On this basis much attention is still devoted to the alternative approach to hyperpolarize molecules based on ParaHydrogen Induced Polarization (PHIP)[11–13] because this technique is simpler, faster, and cheaper than d-DNP. Moreover, the Side-Arm Hydrogenation (SAH) strategy (PHIP-SAH)[14] has extended the PHIP substrate scope to a number of biologically relevant molecules for which an unsaturated ParaHydrogen acceptor was not available.[15] Currently, among the applications of the PHIP-SAH approach, the access to HP pyruvate is obviously the one on which the attention is more focussed and metabolic studies in vivo and in-cells have been reported.[16–18] This strategy can, in principle, be applied to any molecule containing a carboxylic group and is based on the esterification of the target substrate with an unsaturated alcohol. The unsaturated ester is, then, hydrogenated using hydrogen enriched in the para-isomer, the spin order of ParaHydrogen is transferred to ^{13}C and the ester is hydrolysed to obtain the ^{13}C hyperpolarized product.

The use of propargylic esters is convenient, thanks to the simple synthetic route and because triple bonds can be hydrogenated easily. Aqueous solutions of HP [1-^{13}C]pyruvate thus obtained have already been used for in vivo and in-cells studies.[16–18] Vinyl esters have also been synthesized and hydrogenated using ParaHydrogen,[19–22] nevertheless the conditions in which they were obtained (organic solvents, low concentration) are not suitable for biological applications. Furthermore, the synthesis of vinyl-[1-^{13}C]pyruvate shows more challenges than other metabolites, due to the intrinsic instability of this molecule.

In this work we report an efficient synthetic procedure for vinyl-[1-^{13}C]pyruvate from which hyperpolarized ethyl-[1-^{13}C]

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pyruvate can be obtained by means of the PHIP procedure. Ethyl-pyruvate is able to cross the BBB (blood-brain barrier) with greater efficiency than pyruvate and this substrate, hyperpolarized by means of the d-DNP procedure, was used as a metabolic probe in the brain, to investigate the occurrence of neurological pathologies in which metabolism is dysregulated.[23,24] The application of HP substrates to biological studies requires that an aqueous solution of the HP product is obtained, endowed with sufficiently high concentration and good hyperpolarization level. All these issues will be tackled, in this work, and the differences between ^{13}C hyperpolarization in allyl- and ethyl- esters will be discussed.

2. Results and Discussion

2.1. Synthesis of Vinyl-[1-^{13}C]pyruvate

Vinyl esters cannot be obtained through direct esterification of the alcohol with the acid (the so-called Fischer esterification), due to the unfavorable keto-enol equilibrium between the vinyl alcohol and the more stable acetaldehyde, and they can be obtained through trans-vinylation reactions between the carboxylic acid and vinyl esters (usually, vinyl-acetate).[25,26]

Unfortunately, pyruvic acid solutions are rather unstable, and it is well-known that this α-ketoacid quickly forms dimers and oligomers, especially at high temperature and alkaline pH.[27] Due to this reason, the trans-vinylation reaction of pyruvic acid with vinyl acetate,[19,28] led, in our hands, to the quick degradation of the substrates, that made impossible the isolation of the desired product from the reaction mixture. Although the reaction yields might be improved by the use of a larger amount of the starting material, this way could not be conveniently pursued for the synthesis of the isotopically enriched vinyl-[1-^{13}C]pyruvate, on a laboratory scale.

Therefore, we decided to overcome the intrinsic instability of pyruvate by synthesizing a more stable ketal derivative (Scheme 1, **II**).[29]

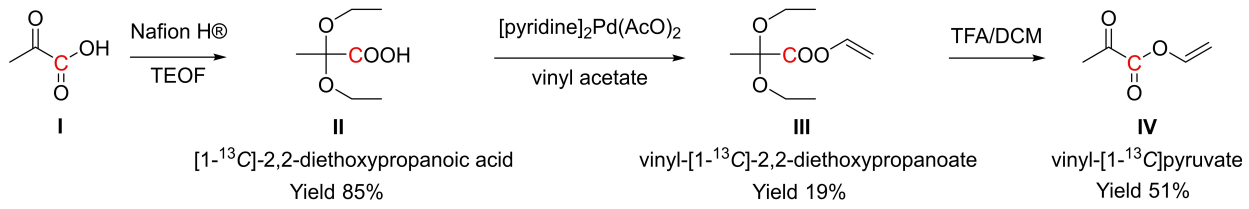
The ketalization of [1-^{13}C]pyruvic acid (**I**) was obtained by reaction with triethyl orthoformate (TEOF)[30] catalysed by Nafion H®. In the original work,[31] sulfuric acid was used to catalyse the reaction, but, in this case, a not negligible amount of ethyl-sulphate was formed, which is quite toxic and difficult to remove from the desired product. Sulphonic resins were also reported to be efficient acidic catalyst. Nafion H®, a sulfonated tetrafluoroethylene based fluoropolymer-copolymer, with excellent thermal and mechanical stability, was proven to be the most efficient, in our case, for the ketalization of pyruvic acid to [1-^{13}C]-2,2-diethoxypropanoic acid (**II**). It must be noticed that the resin can be easily removed, together with the by-products, by means of filtration and regenerated, after every use, in order to make the process more cost effective.

Compound (**II**) was then reacted with vinyl acetate using palladium diacetate as catalyst to obtain the vinyl ester (vinyl-[1-^{13}C]-2,2 diethoxypropanoate, **III**). To prevent the degradation of the catalyst to metallic Pd, the complex was stabilized by means of complexation with pyridine. As transvinylation is a reversible reaction with an equilibrium constant near to one, this feature appears as the limiting step in terms of obtainable yields. Finally, vinyl-[1-^{13}C]-2,2diethoxypropanoate (**III**) was deprotected using an excess of trifluoroacetic acid (TFA)[32] in dichloromethane (DCM). This route to obtain the deketalization was chosen to reduce as much as possible the presence of ethanol in the solution, which may yield subsequent transesterification with pyruvate. In the applied experimental design, volatile ethyl trifluoroacetate was formed, which was easily removed together with the solvent by evaporation in vacuum, to yield vinyl-[1-^{13}C]pyruvate (**IV**). The overall yield of the process, in terms of transformation of ^{13}C-labelled pyruvate into ^{13}C-labelled vinyl pyruvate, was 8.3%. To the best of our knowledge, this is the first time that vinyl [1-^{13}C]pyruvate is obtained in amounts and form suitable for a detailed study of its hyperpolarization properties, as described below.

2.2. ParaHydrogen Hyperpolarization

Parahydrogenation of vinyl-[1-^{13}C]pyruvate has been carried out, then, to obtain ^{13}C hyperpolarized ethyl-pyruvate. Thanks to the larger J-couplings among the added ParaHydrogen protons and the ^{13}C carboxylate spin, we might expect a more efficient spin order transfer from protons to ^{13}C with respect to parahydrogenated allyl-[1-^{13}C]pyruvate. The relationship between the efficiency of spin order transfer (SOT) with the strength of proton-carbon J-couplings has already been discussed, for a simple three spins system.[33] Therefore, we deemed useful to compare the PHIP response of vinyl-[1-^{13}C] pyruvate with the one provided by propargyl-[1-^{13}C]pyruvate.

The hyperpolarization experiments were carried out by means of hydrogenation, using para-enriched hydrogen in chloroform solution using the commercial rhodium catalyst [1,4-Bis(diphenylphosphino)butane](1,5-cyclooctadiene) rhodium(I) tetrafluoroborate ([Rh(COD)dppb][BF_4], CAS: 79255-71-3).



Scheme 1. Synthetic pathway for the preparation of vinyl-[1-^{13}C]pyruvate. The ^{13}C label is indicated in red.

The hydrogenation reactions were carried out in an NMR sample tube equipped with a valve (Norell®), pressurized with ParaHydrogen. To initiate the hydrogenation reaction, the NMR tube was heated in a hot water bath at 85 °C for about 7 s, then shaken vigorously for 3 s.

In order to transform the proton spin order derived from the addition of ParaHydrogen into net ^{13}C magnetization, magnetic field cycling (MFC)[34,16] was applied. To do that, the NMR sample tube containing the parahydrogenation product was placed in the μ-metal shield, immediately after the release of the ParaHydrogen pressure at the end of the reaction. The μ-metal shield is equipped with a solenoid coil fed with controlled current which enables a precise control of the magnetic field variations that occur during the MFC[35] procedure (see S.I. for more details). The field, initially set at 1.5 μT, was suddenly dropped to 150 nT in less than 1 ms, then increased exponentially to 10 μT in 4 s; the obtained ^{13}C polarization was 3.8 ± 0.3 % (Table 1). When the ^{13}C hyperpolarization experiment was carried out on the propargyl ester, using an exponential remagnetization that starts from 50 nT, about 6.2 ± 0.3 % ^{13}C hyperpolarization was obtained on allyl-[1-^{13}C]pyruvate. The higher ^{13}C polarization measured on the allyl-ester, in respect to the one on the ethyl-ester, might appear in contrast with the fact that the J-couplings, between the added ParaHydrogen protons and the ^{13}C carboxylate atom, are larger for the second spin system (Figure 1) and the spin order transfer should be more efficient.

Table 1. ^{1}H and ^{13}C polarization level of ethyl- and allyl-[1-^{13}C]pyruvate at 9.4T. The experimental condition can be found in the Experimental Section.

	^{1}H polarization (ALTADENA)	^{13}C polarization
Ethyl-[1-^{13}C]pyruvate	2.4 ± 0.4 %	3.8 ± 0.3 %
Allyl-[1-^{13}C]pyruvate	6.0 ± 0.1 %	6.2 ± 0.3 %

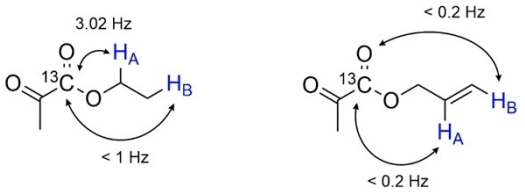


Figure 1. ^{1}H-^{13}C J-couplings of ethyl-[1-^{13}C]pyruvate and allyl-[1-^{13}C]pyruvate measured in the reaction solution after the parahydrogenation of the precursors.

We surmise that the observed heteronuclear hyperpolarization level depends on the ^{1}H spin order obtained on the product, upon the addition of ParaHydrogen to the substrate. ^{1}H hyperpolarization was assessed by carrying out ALTADENA experiments.[36,13] In these experiments, hyperpolarized ^{1}H-NMR spectra were acquired 18 s after the completion of the parahydrogenation reaction, carried out at the geomagnetic field. The typical ALTADENA pattern of hyperpolarized signals was observed (Figure 2) for the methylene and methyl groups of ethyl-pyruvate resulting in a proton polarization level of 2.4 ± 0.4 %.

Conversely, when the ALTADENA experiment was carried out using propargyl-[1-^{13}C]pyruvate, the ^{1}H hyperpolarized signals were outstandingly intense and the spectrum was characterized by damped signals. The proton polarization was measured to be 6.0 ± 0.1 %.

It might be surprising that the hyperpolarization observed on protons is lower than that on ^{13}C. This occurs because the delay between the end of the hydrogenation and the acquisition of the ^{1}H hyperpolarized spectrum, in the ALTADENA experiment, is much longer than that between the hydrogenation end and the application of the MFC. Therefore, the spin order loss, due to relaxation, is much more prominent in the first than in the second case. Anyway, the ALTADENA experiments clearly show that the proton spin order is significantly smaller on the ethyl rather than on the allyl derivative (2.4 % on the ethyl vs 6.0 % on the allyl ester) (Table 1). Conversely, the difference between ^{13}C polarization of the two esters, is much less relevant (3.8 % on the ethyl vs. 6.2 % on the allyl) (Table 1). From these results one may draw

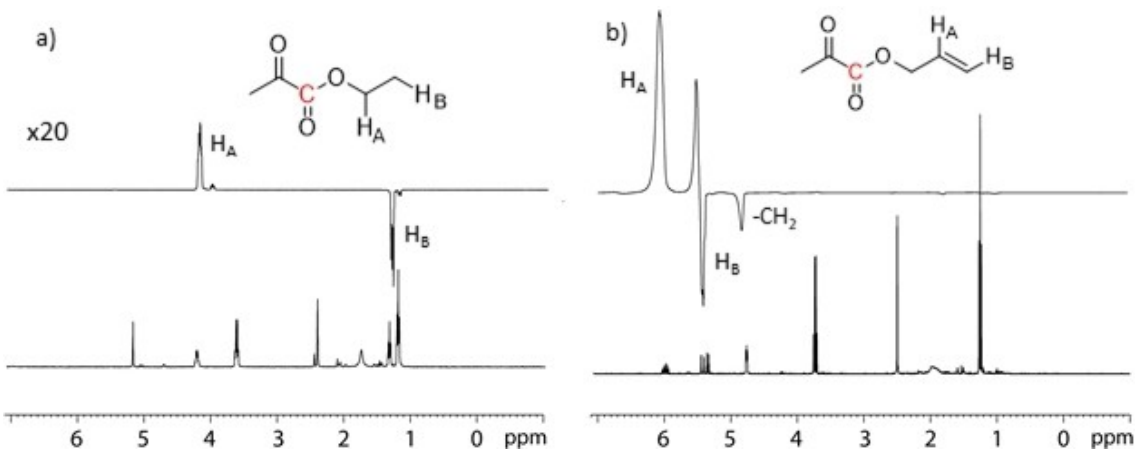


Figure 2. ^{1}H-NMR spectra of parahydrogenated ethyl-[1-^{13}C]pyruvate and propargyl-[1-^{13}C]pyruvate obtained in ALTADENA experiments. The proton polarization on the ethyl derivative (a) is considerably lower than on the parahydrogenated propargyl derivative (b).

the conclusion that the spin order transfer from the Para-Hydrogen protons to ^{13}C net magnetization is more efficient on the ethyl rather than on the allyl ester, thanks to the larger J-couplings, as expected from the theory. Due to this reason, we can also hypothesize that the application of pulsed Spin Order Transfer (SOT) methods,[33,37,38] at high field, may lead to efficient hyperpolarization of the ^{13}C signal.

The smaller ^{1}H hyperpolarization observed on the para-hydrogenated ethyl ester may be due to the lower hydrogenation efficiency of a double bond with respect to a triple bond, in homogeneously catalyzed reactions, as was already noticed in previous studies.[15] Metal complexes of the type [Rh(diene)L_n], (L_n can be a mono-dentate (n = 2) or a bi-dentate (n = 1) ligand) catalyse the hydrogenation of alkynes to alkenes much faster than the subsequent hydrogenation of alkenes to alkanes.[39] Even if the slower hydrogenation of the vinyl-derivative cannot be appreciated in our experiments, as complete hydrogenation is achieved in few seconds, a slower reaction implies the formation of more stable intermediates, on which the singlet state of ParaHydrogen is mixed with the triplet states.[40] Therefore, the population of the singlet state is depleted and the hyperpolarization level on the products is decreased.

Furthermore, the lower ^{1}H hyperpolarization on the ethyl ester may also be due to the different proton relaxation rates in the ethylene and in the allyl groups (Table 2). The relaxation rate of ParaHydrogen protons, measured at high magnetic field in the NMR spectrometer, is about two times faster in the ethyl than in the allyl ester. Nevertheless, it must be noticed that, at geomagnetic field, where the hydrogenation takes place, the relaxation processes might significantly differ from those observed at high field.

2.3. ^{13}C hyperpolarization in aqueous medium and enzymatic reactions

For the intended biological applications, the hyperpolarized ethyl-[1-^{13}C]pyruvate has to be used in aqueous solution. Therefore, vinyl-[1-^{13}C]pyruvate (80 mM) was hydrogenated using the water-soluble rhodium complex [Rh(NBD)L_2]BF_4 (L_2 = 1,4-bis[(phenyl-3- propanesulfonate) phosphine] butane).[41] Following the reaction with ParaHydrogen, the solution underwent MFC. The ^{13}C-NMR spectra acquired immediately after the spin order transfer step, showed 12.3 ± 0.3% polarization on the ^{13}C-carboxylate signal of ethyl pyruvate, with a hydrogenation yield of 55 ± 3%. The higher polarization obtained in the aqueous solvent might be attributed to a higher efficiency of the catalyst. As clearly assessed from the ^{13}C-NMR spectrum in aqueous solution, the hydrated form of the ethyl-ester of pyruvate is about 76% of the total amount, and its polarization is 2.4 ± 0.1%. The degree of hydration of the α-ketoacids is sensitive to electronic/resonance effects and hydration is greater in the acidic form than in the carboxylate form,[42] therefore it is not surprising that a high percentage of the ethyl-ester of [1-^{13}C]pyruvate is in the hydrated form, in a large range of pH. Considering that the hydrated and non-hydrated forms of ethyl-pyruvate are exchanging one with the other,[29] the average ^{13}C polarization of ethyl-pyruvate in aqueous solution is 4.8%.

The relaxation rate of ParaHydrogen protons was also measured at high magnetic field, in the conditions of the aqueous hydrogenation, and it resulted comparable to the relaxation rate previously measured in $CDCl_3$ (Table 3).

Finally, the esterase catalyzed conversion of the HP ethyl ester to [1-^{13}C]pyruvate was tested. The build-up of the signals of [1-^{13}C]pyruvate was clearly observed in the series of ^{13}C-NMR spectra acquired immediately after the addition of the HP-ester to the enzyme containing solution (Figure 3). Conversely, no transformation was observed when the solution of the HP substrate was added to the HEPES buffer solution. However, we have to remind that, albeit that early studies have shown low toxicity of the hydrogenation catalyst,[43] the rhodium complex would ideally be removed before the injection in-vivo.

3. Conclusions

^{13}C labelled vinyl-ester of pyruvate has been obtained through a synthetic pathway in which the intrinsic instability of this metabolite has been circumvented thanks to the formation of its ketalic form, that is more stable. This substrate has been isolated and ^{13}C hyperpolarization of ethyl-[1-^{13}C]pyruvate has been obtained, by means of ParaHydrogen, in both organic and aqueous solutions. It has also been shown that the spin order transfer, from the ParaHydrogen protons to the ^{13}C carboxylate spin, is more efficient in the parahydrogenated ethyl-[1-^{13}C] pyruvate than in the allyl-[1-^{13}C]pyruvate derived from para-hydrogenation of the propargyl-ester. This is due to the larger J-couplings involved in the ethyl- rather than in the allyl-derivative.

PHIP polarized ethyl-[1-^{13}C]pyruvate obtained at high concentration (40 mM) and good polarization level (4.8%) in aqueous solution, allowed to demonstrate the conversion of HP ethyl-[1-^{13}C]pyruvate into [1-^{13}C]pyruvate by a buffered solution of esterase.

Table 2. T_1 of protons in $CDCl_3$ hydrogenation conditions, measured at 9.4T.

	$T_1(H_A)$ [s]	$T_1(H_B)$ [s]
Ethyl-[1-^{13}C]pyruvate	4.32 ± 0.02	3.61 ± 0.03
Allyl-[1-^{13}C]pyruvate	10.42 ± 0.01	7.44 ± 0.01

Table 3. T_1 of protons in aqueous hydrogenation conditions, measured at 9.4T.

	$T_1(H_A)$ [s]	$T_1(H_B)$ [s]
Ethyl-[1-^{13}C]pyruvate	5.32 ± 0.13	5.02 ± 0.12
Ethyl-[1-^{13}C]pyruvate hydrated	3.68 ± 0.10	4.18 ± 0.10

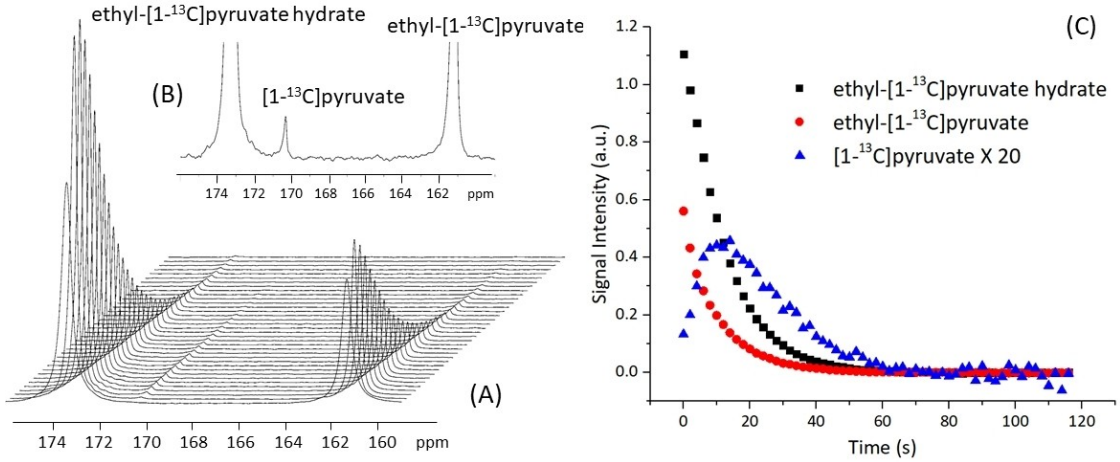


Figure 3. (A) Series of ^{13}C-NMR spectra acquired upon mixing the pyruvate esterase containing solution (400 U) with the aqueous solution of hyperpolarized ethyl-[1-^{13}C]pyruvate. Spectra were acquired using small flip angle pulse (10°) and 2 s delays between successive scans. (B) expanded ^{13}C-NMR spectrum at maximum intensity of the [1-^{13}C]pyruvate signal; (C) Plot of the time dependent changes of the signals of ethyl-[1-^{13}C]pyruvate, its hydrated form, and of the [1-^{13}C]pyruvate (obtained from the integrals of the peaks in the ^{13}C-NMR spectra reported in (A)); the [1-^{13}C]pyruvate signal intensity is shown multiplied by 20.

All these features made this ParaHydrogen hyperpolarized molecule suitable for biological applications and a promising candidate for in-vivo metabolic studies, thus widening the scope of PHIP polarized substrates.

Experimental Section

[1-^{13}C]pyruvic acid was purchased from CortecNet. All other chemicals were purchased from Merck KGaA and were used without purification. All the NMR spectra of the synthesized materials were acquired at 14.1T using a 600 MHz Bruker Avance NMR spectrometer.

Synthesis of Vinyl-[1-^{13}C]pyruvate

The synthesis pathway is described in Scheme 1.

[1-^{13}C]-2,2-diethoxypropanoic acid (II) The [1-^{13}C]pyruvic acid (CAS: 99124-30-8) (2 g; 22.45 mmol) was dissolved in triethyl orthoformate (TEOF) (CAS: 122-51-0) (10 ml; 57 mmol) and was cooled to 0–5 °C with an ice bath; 200 mg of Nafion H® resin were added and the reaction was stirred at room temperature under argon atmosphere. After 2 h and after 5 h, 120 mg of Nafion H® resin and 4 ml of TEOF were added. After one night, the mixture was filtered and 25 ml of DCM were added: the organic solution was washed with water (3×12 ml); the aqueous phase was extracted with DCM (12 ml). All the organic phases were put together, washed with brine (12 ml), dried over Na_2SO_4 and evaporated to residue (3.1 g, yield 85%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.24 (t, 6H, J = 7.08 Hz), 1.57 (d, 3H, J = 3.45 Hz), 3.55 (m, 4H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 15.24 (2C), 21.78 (1C), 58.79 (2C), 99.75 (d, 1C, J = 67.29 Hz), 171.07 (1C).

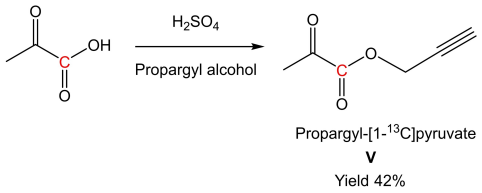
[1-^{13}C]-2,2-diethoxypropanoic acid vinyl ester (III) The [1-^{13}C]-2,2-diethoxypropanoic acid (**II**) (3.1 g; 19 mmol) was dissolved in vinyl acetate (CAS: 108-05-4) (24 ml; 0.26 mol); freshly prepared $[pyridine]_2Pd(AcO)_2$[44] (40 mg; 0.1 mmol) was added every 4 hours for a total of 280 mg; the third day a little amount of NaAcO was added, the solution was filtered and washed with NaAcO 10% (3× 20 ml) and brine (20 ml). The solution was evaporated at 25 °C and 100 mbar then the product was distilled at 50 °C and 18 mbar with a Claisen apparatus and the collection flask cooled to −80 °C. (788 mg; 85% w/w pure, yield 19%; the impurity is represented by the ethyl ester). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.24 (t, 6H, J = 6.96 Hz), 1.58 (d, 3H, J = 3.38 Hz), 3.47–3.64 (m, 4H), 4.68 (d, ^{1}H, J = 6.29 Hz), 5.02 (d, ^{1}H, J = 13.89 Hz), 7.29 (ddd, ^{1}H, J = 2.5 – 6.29–13.89 Hz). ^{13}C NMR (600 MHz, $CDCl_3$): δ = 15.34 (2C), 22.00 (1C), 58.34 (2C), 99.3 (1C), 141.29 (1C), 167.46 (1C).

Vinyl-[1-^{13}C]pyruvate (IV) The [1-^{13}C]-2,2-diethoxypropanoic acid vinyl ester (**III**) (788 mg; 3.56 mmol) was dissolved in DCM (25 ml), cooled to 0 °C with an ice bath and trifluoroacetic acid (TFA) (6.3 ml; 84 mmol) was added. The reaction was followed by means of NMR and after 2 h at room temperature the deprotection was complete. The organic solution was gently dropped into $NaHCO_3$ 5%, vigorously stirred, keeping the pH 6–7 by adding $NaHCO_3$ 5%. The organic phase was separated from the aqueous one and evaporated at 25 °C and 400 mbar. The product was distilled at 40 °C and 18 mbar with a Claisen apparatus and the collection flask cooled to −80 °C. Compound (**IV**) was recovered (376 mg 55.7% w/w pure, yield 51%. Ethyl pyruvate, 11.9% w/w, and dichloromethane, 32.4% w/w, are the only impurities, but they do not affect polarization experiments. Nevertheless, their amount is taken in account for the evaluation of the signal enhancement). The overall yield of the process, in terms of transformation of ^{13}C-labelled pyruvate into ^{13}C-labelled vinyl pyruvate was 8.3%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 2.52 (d, 3H, J = 1.42 Hz), 4.82 (dd, ^{1}H, J = 2.10–6.14 Hz), 5.17 (dd, ^{1}H, J = 2.10–13.70 Hz), 7.28 (ddd, ^{1}H, J = 2.10 – 6.14–13.70 Hz). ^{13}C NMR (600 MHz, $CDCl_3$): δ = 26.79 (d, 1C, J = 17.3 Hz), 101.41 (1C), 140.70 (1C), 157.56 (1C).

Synthesis of Propargyl-[1-^{13}C]pyruvate

The synthesys pathway is reported in Scheme 2.

Propargyl-[1-^{13}C]pyruvate (V) [1-^{13}C]pyruvic acid (2 g; 22.45 mmol) was dissolved in propargyl alcohol (CAS: 107-19-7) (6.52 ml; 113 mmol) and cooled to −10 °C with an ice/NaCl bath; H_2SO_4 conc. (1.58 ml; 29.6 mmol) was slowly added dropwise, then the reaction was stirred at room temperature under argon atmosphere for 2 days. The mixture was then cooled in ice and 8 ml of water were



Scheme 2. Synthetic pathway for the preparation of propargyl-[1-^{13}C] pyruvate. The ^{13}C label is indicated in red

added; the product was extracted with DCM (3×8 ml), washed with water (8 ml), $NaHCO_3$ 10% (3×8 ml) and brine (8 ml). The organic layer was evaporated at room temperature and the product was distilled with the Hickman apparatus at 80–110 °C 33 mbar (1.18 g, yield 42%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 2.50 (d, 3H, J = 1.48 Hz), 2.55 (t, ^{1}H, J = 2.56 Hz), 4.84 (t, 2H, J = 2.94 Hz). ^{13}C NMR (600 MHz, $CDCl_3$): δ = 26.9 (d, 1C, J = 16.9 Hz), 53.75 (d, 1C, J = 2.71), 76.24 (1C), 76.40 (1C), 159.83 (1C), 190.85 (d, 1C, J = 67.14 Hz).

^{13}C Hyperpolarization of Ethyl- and Allyl-[1-^{13}C]pyruvate

The hyperpolarization experiments were carried out by means of hydrogenation, using para-enriched hydrogen (Bruker BPHG generator, 85% enrichment), of the substrates (200 mM) in chloroform solution (100 µl $CDCl_3$), catalysed by the commercial rhodium complex [Rh(COD)dppb][BF_4] (13.8 mM). Ethanol (5 µl, 85 mM) was also added, as hydrogenation co-solvent, in order to increase the polarization level.

The hydrogenation reactions were carried out in a NMR sample tube equipped with a valve (Norell®). ParaHydrogen (2 bar) was added to the NMR tube by keeping it in a liquid nitrogen bath. To initiate the hydrogenation reaction, the NMR tube was heated in a hot water bath at 85 °C for 7 s, then shaken vigorously for 3 s. The tube was opened to release the ParaHydrogen pressure and placed in the µ-Metal shield for the application of the MFC. Then it was quickly placed in the 400 MHz NMR spectrometer (Bruker Avance III) and a 90° pulse was applied at the ^{13}C NMR frequency, after the addition of 250 µl of $CDCl_3$ (added to obtain an adequate volume for the acquisition of a ^{13}C-NMR spectrum). ^{13}C polarization has been referred to the thermal spectra acquired on each hyperpolarized sample (6 transients, inter-scan delay 300 s) and the ParaHydrogen enrichment was not used to normalize the results.

^{1}H Hyperpolarization of Ethyl- and Allyl-[1-^{13}C]pyruvate

The hydrogenation was carried out under the same conditions reported for ^{13}C HP. Immediately after hydrogenation completion, the NMR tube was opened to release the ParaHydrogen pressure and $CDCl_3$ was added (250 µl) in order to obtain an adequate volume for the acquisition of a ^{1}H-NMR spectrum. Then the tube was quickly placed in the 400 MHz NMR spectrometer (Bruker Avance III) and a 90° pulse was applied at the proton NMR frequency. The time lapse between the end of the hydrogenation step and the acquisition of the ^{1}H-NMR spectrum was 18 s.

^{13}C Hyperpolarization in Aqueous Medium and Enzymatic Reactions

The substrate vinyl-[1-^{13}C]pyruvate was hydrogenated using the water-soluble rhodium complex [Rh(NBD)L2]BF_4 (L2 = 1,4-bis [(phenyl-3- propanesulfonate) phosphine] butane). In these experiments, the NMR tubes were charged with the catalyst solution (300 µl, 5 mM in D_2O) and the substrate (80 mM). When para-hydrogenation was completed, the NMR tube was opened to release the ParaHydrogen pressure, placed in the µ-Metal shield, MFC was applied and then it was quickly placed in the 600 MHz NMR spectrometer (Bruker Avance III) for the acquisition of the ^{13}C NMR spectrum.

To observe the enzyme catalyzed conversion to [1-^{13}C]pyruvate, 300 µl of a buffered solution of esterase (60–400 U in 100 µl HEPES buffer, pH 6.5) was placed in the NMR spectrometer and kept at 25 °C. The aqueous solution of ^{13}C hyperpolarized ethyl pyruvate was prepared as described in the previous paragraph, collected in a syringe containing 100 µl of HEPES (430 mM) and immediately injected into the enzyme solution by means of a PTFE tube, using a set-up already described.[39]

Using this procedure, the final pH of the hyperpolarized aqueous solution was 6.5–7. At this pH the hydrate form of ethyl-[1-^{13}C] pyruvate correspond to 74–77% of the total ethyl-pyruvate in the aqueous solution, and this percentage does not change over a pH range from 1.5 to 7, as shown in the S.I.

MFC Setup

To perform the MFC, after the hydrogenation, the tube was immediately placed in a three-layers µ-Metal shield (Aspect Imaging, Shoham, Israel). The complete magnetic field cycling is obtained by controlling the current in a coaxial solenoid by means of a custom-written function (Microsoft Visual Basic) that drives an Arbitrary Waveform Generator (Keysight Technologies 33220A 20 MHz, Keysight Technologies, Santa Rosa, U.S.). The sample was then lifted out of the shield and placed in the NMR spectrometer for the acquisition of the ^{13}C-NMR spectrum.

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Conflict of Interest

The authors declare no conflict of interest.

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