

Chemistry—Methods

Supporting Information

Rapidly Signal-enhanced Metabolites for Atomic Scale Monitoring of Living Cells with Magnetic Resonance

Yonghong Ding⁺, Sergey Korchak⁺, Salvatore Mamone⁺, Anil P. Jagtap⁺, Gabriele Stevanato⁺, Sonja Sternkopf⁺, Denis Moll, Henning Schroeder, Stefan Becker, André Fischer, Ellen Gerhardt, Tiago F. Outeiro, Felipe Opazo, Christian Griesinger, and Stefan Glöggler*

Supplementary Information

1. Abbreviations	3
2. Maximizing Insensitive Nuclei Enhancement Reached Via para-hydrogen Amplification (MINERVA).....	4
3. Chemistry.....	6
3.1. Development of chemical precursors	6
3.2. General materials and methods	6
3.3. Pyruvate precursors	7
3.3.1. 2-Oxopropanoic-1- ¹³ C-3,3,3-d ₃ acid (7)	8
3.3.2. 2,2-Dimethoxypropanoic-1- ¹³ C-3,3,3-d ₃ acid-d (8)	8
3.3.3. 5,5-Dimethyl-2-(methyl-d ₃)-4-(2-nitrophenyl)-1,3-dioxane-2-carboxylic- ¹³ C acid (10)...	8
3.3.4. Vinyl-d ₃ 5,5-dimethyl-2-(methyl-d ₃)-4-(2-nitrophenyl)-1,3-dioxane-2 carboxylate- ¹³ C (7)	9
3.3.5. Vinyl-d ₃ 2-oxopropanoate-1- ¹³ C-3,3,3-d ₃ (1b).....	10
3.4. Enol compounds	11
3.4.1. Ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-1- ¹³ C-d ₂ (4b).....	11
3.4.2. Ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-2- ¹³ C-d ₂ (14b)	11
3.4.3. Ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-3- ¹³ C-d ₂ (15b)	12
3.4.4. Ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-1,2- ¹³ C-d ₂ (16b)	12
3.5. Synthesis of 3-(phenyl-d ₅)prop-2-yn-1-yl-1- ¹³ C-1,1-d ₂ 2-oxopropanoate-2- ¹³ C-3,3,3-d ₃ (1b') 13	
3.6. NMR Spectra	14
3.7.1. Vinyl pyruvates	14
3.7.2. Enol-pyruvates	17
4. Biochemistry & Cells.....	22
4.1. Cell lines and media	22
4.2. Protein expression and purification	22
4.3. Protein delivery via electroporation and characterization of transduced cells.....	23
4.4. Preparation of cells for NMR experiments	23
4.5. Western blot analysis	24
4.6. Immunofluorescence	25
4.7. Imaging	25
5. Experimental Procedures	25
5.1. Estimation of the polarization level.....	25
5.2. General procedure.....	26
5.3. Para-hydrogenation of vinyl-d ₃ 2-oxopropanoate-1- ¹³ C-3,3,3-d ₃ to ethyl-1,2,2-d ₃ 2-oxopropanoate-1- ¹³ C-3,3,3-d ₃	27
5.4. Para-hydrogenation of vinyl-d ₃ 2-oxopropanoate-2- ¹³ C-3,3,3-d ₃ to ethyl-1,2,2-d ₃ 2-oxopropanoate-2- ¹³ C-3,3,3-d ₃	28

5.5.	Para-hydrogenation of vinyl-d ₃ 2-oxopropanoate-1,2- ¹³ C-3,3,3-d ₃ to ethyl-1,2,2-d ₃ 2-oxopropanoate-1,2- ¹³ C-3,3,3-d ₃	30
5.6.	Para-hydrogenation of 3-(phenyl-d ₅)prop-2-yn-1-yl-1- ¹³ C-1,1-d ₂ 2-oxopropoanoate-1- ¹³ C-3,3,3-d ₃ to (Z)-3-(phenyl-d ₅)allyl-1- ¹³ C-1,1-d ₂ 2-oxopropanoate-1- ¹³ C-3,3,3-d ₃	32
5.7.	Para-hydrogenation of ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-1- ¹³ C-d ₂ to ethyl-d ₅ 2-(acetoxo-d ₃)propanoate-1- ¹³ C-3,3-d ₂	33
5.8.	Para-hydrogenation of ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-2- ¹³ C-d ₂ to ethyl-d ₅ 2-(acetoxo-d ₃)propanoate-2- ¹³ C-3,3-d ₂	34
5.9.	Para-hydrogenation of ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-3- ¹³ C-d ₂ to ethyl-d ₅ 2-(acetoxo-d ₃)propanoate-3- ¹³ C-3,3-d ₂	35
5.10.	Para-hydrogenation of ethyl-d ₅ 2-(acetoxo-d ₃)acrylate-1,2- ¹³ C-d ₂ to ethyl-d ₅ 2-(acetoxo-d ₃)propanoate-1,2- ¹³ C-3,3-d ₂	36
6.	Hyperpolarization of 1- ¹³ C-pyruvate for cell measurements.....	38
6.1.	Residual rhodium content.....	39
6.2.	In-cell structural analysis by NMR	40
6.3.	Kinetic model and Data Analysis	41
6.4.	Data Analysis - Results.....	42
7.	Receiver gain.....	48
8.	Relaxation data.....	49
9.	References.....	50

1. Abbreviations

DIPEA	N,N-Diisopropylethylamine
EtOAc	Ethyl acetate
Et ₃ N	Triethylamine
ESI-MS	Electrospray ionization mass spectrometry
TLC	thin layer chromatography
PE	petroleum ether
MeOH	methanol
EtOH	ethanol
rt	room temperature
PBS	Phosphate-buffered saline
α -Syn	Alpha-Synuclein
NMR	Nuclear magnetic Resonance
DMEM	Dulbecco's modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
PHIP	Parahydrogen-induced polarization
WT	Wild-type
KO	Knock-out
OE	Overexpression
HEK	Human embryonic kidney
ICP-MS	Inductively coupled plasma mass spectrometry
PVDF	Polyvinylidene difluoride
SOFAST-HMQC	band-Selective Optimized-Flip-Angle Short-Transient Heteronuclear Multiple Quantum Coherence
INEPT	Insensitive nuclei enhanced by polarization transfer
MINERVA	Maximizing Insensitive Nuclei Enhancement Reached Via para-hydrogen Amplification
PHIP	Para-Hydrogen Induced Polarization
WB	Western Blot

2. Maximizing Insensitive Nuclei Enhancement Reached Via para-hydrogen Amplification (MINERVA)

In order to maximize the signal enhancement with para-hydrogen, two main aspects need to be considered: One is the use of a magnet resonance experiment that allows for an optimal transfer of polarization from protons to a desired nuclear spin to be detected and the other one is to obtain an optimal precursor molecule to achieve high polarization degrees and that supports the transfer. In the first chapter we will introduce a pulsed magnetic resonance approach that can be used to transfer spin order from para-hydrogen via a vast network of J-couplings even in inhomogeneous magnetic fields. The sequence is hence broadly applicable and achieves more than 80% transfer efficiency for at least three coupled spins (H_1 , H_2 and S_3) whereby two originate from para-hydrogen (H_1 and H_2) and one is a heteronucleus (S_3). Thereby, the spins are coupled via J-couplings J_{12} (homonuclear coupling between H_1 and H_2), J_{13} and J_{23} (heteronuclear coupling between H_1 and S_3 or H_2 and S_3 respectively) and the condition $J_{13} \neq J_{23}$ needs to be fulfilled in addition to an initial longitudinal spin order of the hydrogen state to reach the maximal transfer efficiency. In the case where $J_{13} = J_{23}$ still 50% transfer efficiency are yielded. If the magnetic field homogeneity allows for the use of selective pulses to selectively excite resonance of the molecule, they can be implemented in the sequence to achieve a full transfer of spin order, even for the case where $J_{13} = J_{23}$. Compared to previous sequences that used the longitudinal order as initial state for the transfer only maximal transfer for a strong imbalance of heteronuclear couplings could be achieved. As a result, our newly described experiment here can deliver polarization transfer for a wide range of molecules that can e.g. be used in metabolic studies as described in the main text. For the sequence we propose the acronym MINERVA (Maximizing Insensitive Nuclei Enhancement Reached Via para-hydrogen Amplification).

The sequence used to transfer the spin order from para-hydrogen to the magnetization of the heteronuclei of interest has two variants depending on the target spin being linked via J-coupling to the hydrogens directly or indirectly, see Figure S1 and Figure S2. For the latter case an additional J-coupling J_{34} exists between two heteronuclei S_3 and S_4 . The key for a broadband application of the sequence are the depicted delays τ between pulses of the sequence.

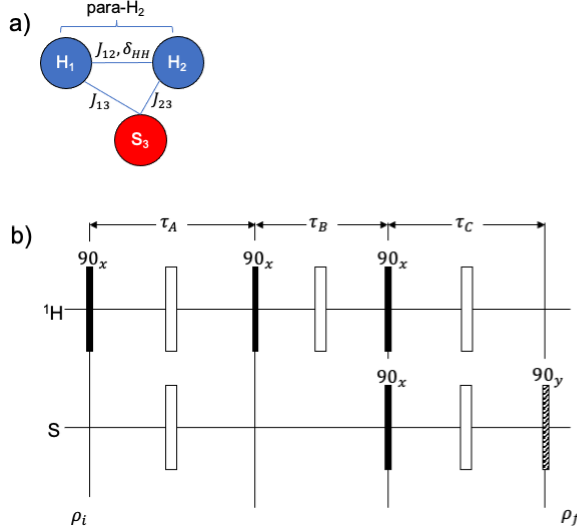


Figure S1. The panels represent a) the stylized 3-spin system after hydrogenation and b) the corresponding sequence for transfer of longitudinal spin order $\rho_i = 2I_{1z}I_{2z}$ into magnetization $\rho_f = S_{3z}$. The filled and empty rectangles are 90° and 180° pulses, respectively. The phases of the 180° pulses lay along the transverse axes of the rotating plane. The last 90° dashed pulse is removed for direct observation of heteronuclear transverse magnetization S_{3x} .

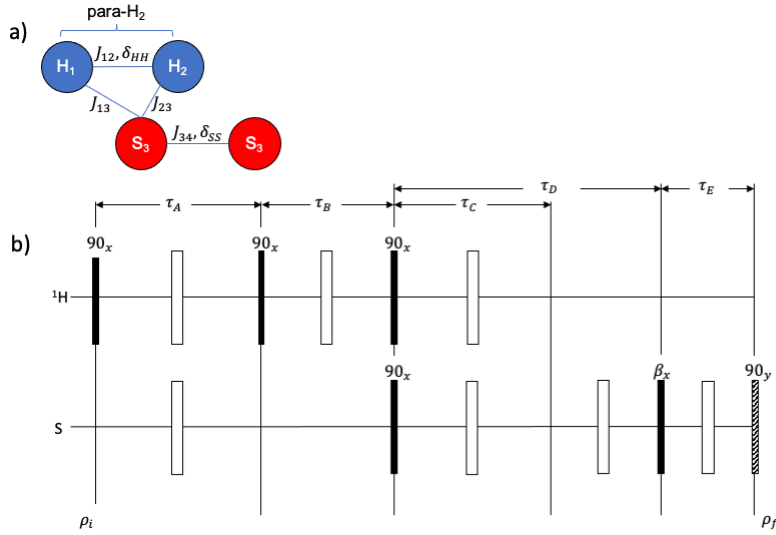


Figure S2. The panels represent a) the stylized 4-spin system after hydrogenation and b) the corresponding sequence for the transfer of longitudinal spin order $\rho_i = 2I_{1z}I_{2z}$ into magnetization $\rho_f = S_{4z}$. The filled and empty rectangles are 90° and 180° pulses, respectively. The phases of the 180° pulses lay along the transverse axes of the rotating plane. The rotation angle is chosen so that magnetization is split between S_3 and S_4 according to the cosine and sine of the flip angle β , respectively. The last 90° dashed pulse is removed for direct observation of heteronuclear transverse magnetization S_{4x} .

The sequence can be understood in the following way using the product operator formalism. The 180° pulses refocus chemical shift evolutions but leave homonuclear J-couplings active. Heteronuclear J-couplings are active only when 180° pulses are used simultaneously on the corresponding RF channels. With reference to the 3 spin system case of Figure S1a, it is possible to show that in weakly coupled approximation, the highest transfer efficiency is achieved for $\tau_B = 0.5/|J_{12}|$. By opportunely choosing the timing $\tau_A = \tau_C$ so that one of the heteronuclear couplings is refocused under J-evolution, it is possible to show that the transfer efficiency $|f_{WC}|$ remains above 80%, see Figure S3. Such efficiency is maintained for a transfer to the spin S_4 via

homonuclear INEPT. The remaining timing are set so that $\tau_D = 0.5(2n + 1)/|J_{34}| \geq \tau_C$ with n integer greater than 0 and $\tau_E = 0.5/|J_{34}|$. In this respect, it is possible to show that using a pulse with flip angle β will bring the density matrix in the state $\rho = S_{3x} \cos \beta - S_{4x} \sin \beta$ before the z-flip pulse in Figure S2b).

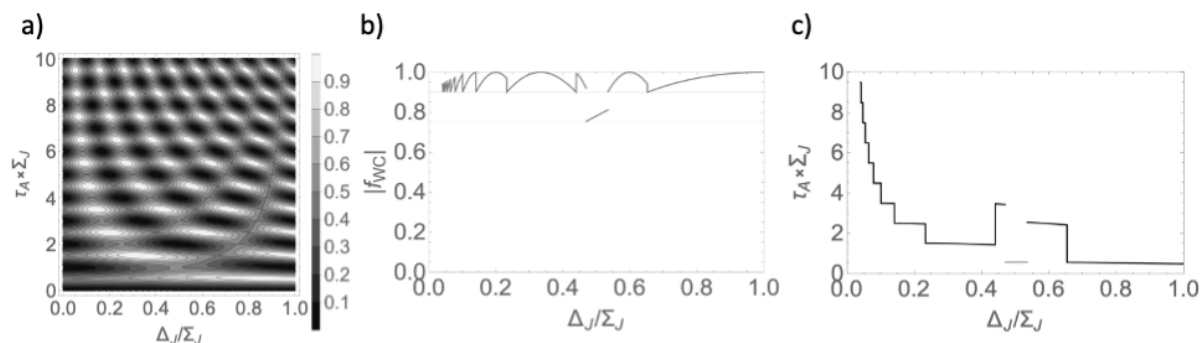


Figure S3. Efficiency for the transfer of spin order $2I_{1z}I_{2z} \rightarrow |f_{WC}|S_{3x,4x}$. Here, $\Delta_J = ||J_{13}| - |J_{23}||$ and $\Sigma_J = |J_{13}| + |J_{23}|$. In panel a), lighter areas correspond to higher efficiency. The 1D plots of panel b) and c) indicate the efficiency and corresponding time values for the delay $\tau_A = \tau_C$.

3. Chemistry

3.1. Development of chemical precursors

The optimal precursors to enhance metabolites such as pyruvate via pulsed transfer methods are thought to be ^{13}C -enriched and deuterated compounds with an unsaturated bond. Most importantly vinyl compounds provide an excellent basis but have not been available so far. This is because the vinyl precursors are obtained via transesterifications that are generally low yielding reactions and have so far been unreasonable to use if deuterated compounds want to be obtained. We have investigated several transesterification steps for reactions that lead to metabolite precursor molecules in high yields (up to 80%) and can be used in deuteration attempts. We demonstrate that ^{13}C -enriched and deuterated precursors of 1- ^{13}C -, 2- ^{13}C - and 1,2- ^{13}C -pyruvate (i.e. vinyl pyruvate) can be obtained and used to yield large spin polarizations on ^{13}C as discussed in the main text. We have furthermore investigated to enhance all ^{13}C spins in lactate via ^{13}C -enriched and deuterated enol precursors of lactate. Overall, we have synthesized precursors with the goal to enhance 1- ^{13}C -, 2- ^{13}C -, 3- ^{13}C - and 1,2- ^{13}C - lactate. Lastly, we provide the details for another 1- ^{13}C pyruvate and perdeuterated precursor based on a phenyl acetylene derivative.

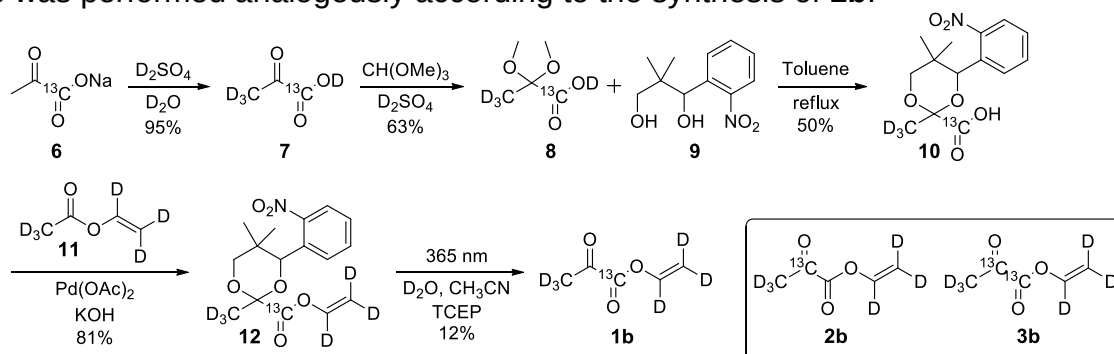
3.2. General materials and methods

If not stated otherwise all reactions were carried out in flame-dried flasks under inert nitrogen atmosphere with magnetic stirring and, if required for light sensitive compounds, brown reaction vessels were used. If not stated otherwise, reactions were

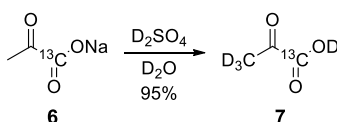
performed at 25 °C (mentioned as room temperature). Drying of the organic fractions were performed using solid Na₂SO₄ or Kinesis TELOS phase separators (15 mL). All chemicals were purchased from commercial suppliers (Sigma Aldrich, TCI Chemicals, Alfa Aesar) and were used without further purification with the exception of Pd(OAc)₂. The catalyst was freshly recrystallized from its hot filtrated benzene solution. All reactions were monitored by thin-layer chromatography using pre-coated silica gel on glass plates (Merck TLC Silica gel 60 F₂₅₄), visualized under UV light (λ =254 nm) and respective staining dips (I₂ in silica, potassium permanganate, phosphomolybdic acid or vanillin). Column chromatography was carried out by using Intershims PuriFlash XS 520 plus using prepacked silica columns (15 μ m or 30 μ m spherical silica) or flash chromatography was performed with silica gel (Sigma Aldrich 230-400 mesh, 60 Å). ¹H, ²H, ¹³C and ¹⁵N NMR spectra were recorded on Bruker Avance III HD spectrometers, at 7 T. ¹H chemical shifts (δ) were calculated relative to tetramethylsilane (TMS, δ =0.0 ppm) and the respective solvent (¹H: CDCl₃ = 7.26 ppm, (CD₃)CO = 2.20 ppm, D₂O = 4.81 ppm; ¹³C: CDCl₃ = 77.0 ppm) expressed in ppm. Spin multiplicities of the protons were reported as s (singlet), d (doublet), t (triplett), q (quartet), m (multiplet) and br (broad singlet). All coupling constants were measured in Hertz. High-resolution mass spectrometry (HR MS-ESI) spectra were taken on Thermo Scientific LTQ Orbitrap XL.

3.3. Pyruvate precursors

Synthesis Vinyl-d₃ 2-oxopropanoate-1-¹³C-3,3,3-d₃ (**1b**) was carried out as illustrated in Scheme 1. Deuteration of 2-oxopropionic-1-¹³C-acid (**6**) was performed in presence of D₂SO₄/D₂O to yield **7**, which was then treated with trimethyl orthoformate to obtain dimethyl ketal protected derivative (**8**) in quantitative yields. Installation of the photo-labile protecting group was performed by coupling of diol (**9**) with dimethyl ketal protected derivative (**8**) in toluene to yield **10** with moderate yield. Pd-catalyzed transesterification of **10** with deuterated vinyl acetate (**11**) was performed to obtain **12** with 81% yield (based on the recovery of **10**). Deprotection of **12** containing photo-labile protecting group was carried in presence of LED lamp (365 nm) to obtain desired compound (**1b**) in 12% yield. Syntheses of other differently labelled compound **2b** and **3b** was performed analogously according to the synthesis of **1b**.



Scheme S1. Synthesis of Vinyl-d₃ 2-oxopropanoate-1-¹³C-3,3,3-d₃(**1b**) and structures of differently labelled derivatives **2b** and **3b** (inset).



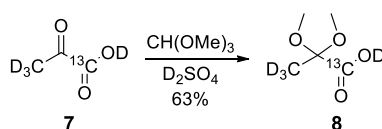
Scheme S2

3.3.1. 2-Oxopropanoic-1-¹³C-3,3,3-d₃ acid (7)

To a cold solution of **6** (0.5 g, 4.5 mmol, 1 eq.) in D₂O (15 mL) D₂SO₄ (0.109 mL, 1.98 mmol, 98% in D₂O, 0.44 eq.) was added dropwise. The resulting solution was heated at 100 °C for 2 h and cooled down to RT. The water was saturated by addition of solid NaCl and extracted with diethyl ether (15 x 10 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated in vacuo to get **7** (0.4 g, 95% yield) as colourless oil.

TLC (Silica gel, 20% EtOAc in Pet. ether), R_f (**7**) = 0.2, KMnO₄ and UV active.

²H NMR (CDCl₃, 298 K, 46.1 MHz) δ = 5.47 (s, ²H), 2.51 (s, 3 x ²H) ppm.



Scheme S3

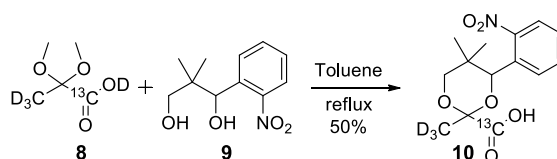
3.3.2. 2,2-Dimethoxypropanoic-1-¹³C-3,3,3-d₃ acid-d (8)

A mixture of **7** (2 g, 21.70 mmol, 1 eq.) and trimethyl orthoformate (8.31 mL, 76 mmol, 3.5 eq.) was stirred at 5-10 °C. To this cold solution, D₂SO₄ (0.108 mL, 1.98 mmol, 0.091 eq.) was added dropwise. The resulting solution was stirred at 5-10 °C for 70 min followed by the addition of brine (15 mL, D₂O) to quench the reaction. The crude mixture was extracted with CH₂Cl₂ (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated in vacuo to get **8** (1.9 g, 63% yield, found some traces of pyruvic acid) as colourless oil. The product was used for follow-up reactions without any need of purification.

TLC (Silica gel, 15% MeOH in CH₂Cl₂), R_f (**8**) = 0.4, KMnO₄ active

¹H NMR (CDCl₃, 298K, 300.13 MHz) δ = 3.28 (s, 6 x ¹H) ppm

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 1.50 (s, 3 x ²H) ppm



Scheme S4

3.3.3. 5,5-Dimethyl-2-(methyl-d₃)-4-(2-nitrophenyl)-1,3-dioxane-2-carboxylic-¹³C acid (10)

A solution of **8** (0.94 g, 6.83 mmol, 1 eq.) and **9** (2 g, 8.88 mmol, 1.3 eq.) in anhydrous toluene (25 mL) was warmed to reflux for 2h. The solution was cooled down to RT and followed by concentration in vacuo to obtain the crude product. The residue was taken up in water (20 mL) and extracted with CH₂Cl₂ (3 x 15 mL). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated in vacuo to get crude material. Purification was performed by flash column chromatography (silica) using a

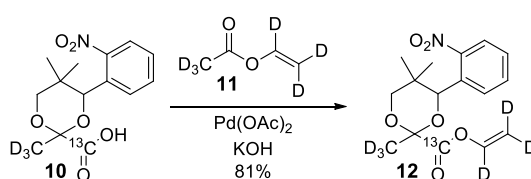
gradient solvent system (MeOH: CH₂Cl₂; 0:100 to 5:95) to yield **10** (1 g, 49% yield) as an off-white solid.

TLC (Silica gel, 15% MeOH in CH₂Cl₂), R_f (**10**) = 0.4, UV and KMnO₄ active

¹H NMR (CDCl₃, 298K, 300.13 MHz) δ = 7.78 (t, 2 x ¹H), 7.62 (t, 1 x ¹H), 7.45 (t, 1 x ¹H), 5.67 (s, 1 x ¹H), 3.76 (dd, 2 x ¹H, ²J_{1H-1H}: 11.67 Hz), 0.91 (s, 3 x ¹H), 0.57 (s, 3 x ¹H) ppm

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 1.7 (s, 3 x ²H) ppm

¹³C NMR (CDCl₃, 298 K, 75.5 MHz) = 174.41 (s, 1 x ¹³C), 149.08 (s, 1 x ¹³C), 131.32 (s, 1 x ¹³C), 130.31 (s, 1 x ¹³C), 128.74 (s, 1 x ¹³C), 124.18 (s, 1 x ¹³C), 99.69 (d, 1 x ¹³C, ¹J_{13C-13C}: 66.02 Hz), 77.27 (s, 1 x ¹³C), 75.17 (d, 1 x ¹³C, ³J_{13C-13C}: 0.78 Hz), 34.62 (d, 1 x ¹³C, ⁴J_{13C-13C}: 0.72 Hz), 25.39 (d, 1 x ¹³C, ²J_{13C-13C}: 2.64 Hz), 20.84 (s, 1 x ¹³C), 18.69 (s, 1 x ¹³C) ppm



Scheme S5

3.3.4. Vinyl-d₃ 5,5-dimethyl-2-(methyl-d₃)-4-(2-nitrophenyl)-1,3-dioxane-2-carboxylate-¹³C (7)

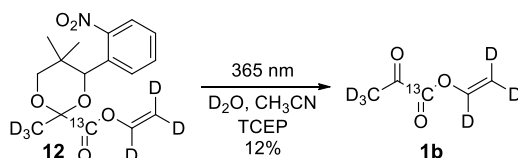
A mixture of **10** (1 g, 3.34 mmol, 1 eq.), Pd(OAc)₂ (0.008 g, 0.0334 mmol, 0.01 eq.), KOH (0.018 g, 0.334 mmol, 0.1 eq.) and vinyl acetate-d₆ (**11**, 5 mL) was stirred for 24 h at RT. The vinyl acetate-d₆ (**11**) was recovered by using short path distillation and was re-used for the later reactions of the same kind. The distillation residue was taken up in dichloromethane and washed with D₂O (15 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated in vacuo. The crude material was purified by flash column chromatography (silica) using a gradient solvent system (EtOAc: petroleum ether; 0:100 to 5:95) to yield **12** as colourless liquid. Starting material (**10**) was recovered (0.43 g) by washing column with 10% MeOH in CH₂Cl₂. The transesterification reaction was repeated with recovered starting material (**10**) and recovered vinyl acetate (**11**) to obtain **12** (0.89 g, 81 % as combined yield) as colourless oil.

TLC (Silica gel, 10% EtOAc in pet ether), R_f (**12**) = 0.4, KMnO₄ and UV active

¹H NMR (CDCl₃, 298K, 300.13 MHz) δ = 7.77 (d, 2 x ¹H), 7.63 (t, 1 x ¹H), 7.45 (t, 1 x ¹H), 5.59 (s, 1 x ¹H), 3.67 (dd, 2 x ¹H, ²J_{1H-1H}: 12 Hz), 0.91 (s, 3 x ¹H), 0.55 (s, 3 x ¹H) ppm

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 5.09 (s, 1 x ²H), 4.76 (s, 1 x ²H), 1.66 (s, 3 x ²H) ppm

¹³C NMR (CDCl₃, 298 K, 75.5 MHz) = 167.86 (s, 1 x ¹³C), 149.19 (s, 1 x ¹³C), 140.94 (d, 1 x ¹³C, ²J_{13C-13C}: 2.8 Hz), 132.27 (s, 1 x ¹³C), 131.27 (s, 1 x ¹³C), 130.32 (s, 1 x ¹³C), 128.72 (s, 1 x ¹³C), 124.06 (s, 1 x ¹³C), 99.79 (d, 1 x ¹³C, ³J_{13C-13C}: 28.64 Hz), 99.36 (d, 1 x ¹³C, ¹J_{13C-13C}: 37.76 Hz), 76.23 (s, 1 x ¹³C), 75.12 (d, 1 x ¹³C, ³J_{13C-13C}: 0.68 Hz), 34.51 (d, 1 x ¹³C, ⁴J_{13C-13C}: 0.66 Hz), 25 (d, 1 x ¹³C, ²J_{13C-13C}: 2.42 Hz), 20.83 (s, 1 x ¹³C), 18.75 (s, 1 x ¹³C) ppm



Scheme S6

3.3.5. Vinyl-d₃ 2-oxopropanoate-1-¹³C-3,3,3-d₃ (**1b**)

To a clear solution of **12** (0.40 g, 1.56 mmol, 1 eq.) in CH₃CN (8 mL), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 0.16 g, 0.55 mmol, 1.1 mL; 0.5 M solution in H₂O), 0.35 eq.]. The solution was irradiated with UV light (365 nm, LED bulb) for 4 h. The reaction mixture was diluted with CH₂Cl₂ (25 mL) and washed with D₂O (30 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated cautiously in vacuo at 30 °C. The crude material was purified by distillation utilizing a Kugelrohr distillation under reduced pressure at 55 °C at 30 mbar to get obtain a fraction containing **1b**. Isolated distillation fraction of **1b** contained residual acetonitrile and therefore to remove residual acetonitrile; it was diluted with CH₂Cl₂ (10 mL) and washed with D₂O (4 X 10 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated cautiously in vacuo to obtain **1b** (0.018 g, 12 %) as a colorless oil (has traces of CH₂Cl₂).

TLC (Silica gel, 10% EtOAc in pet ether), R_f (**12**) = 0.4, KMnO₄ and UV active

²H NMR [(CD₃)₂CO, 298 K, 46.1 MHz] = 7.30 (s, 1 x ²H), 5.10 (s, 1 x ²H), 4.84 (s, 1 x ²H), 2.44 (s, 3 x ²H) ppm

¹³C NMR [(CD₃)₂CO, 298 K, 75.5 MHz] = 190.48 (d, 1 x ¹³C, ¹J_{13C-13C}: 67.74 Hz), 158.21 (s, 1 x ¹³C), 141.55 (d, 1 x ¹³C, ²J_{13C-13C}: 2.72 Hz), 100.01 (d, 1 x ¹³C, ³J_{13C-13C}: 3.84 Hz), 30.09 (s, 1 x ¹³C) ppm

For Vinyl-d₃ 2-oxopropanoate-2-¹³C-3,3,3-d₃ (**2b**)

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 7.30 (s, 1 x ²H), 5.10 (s, 1 x ²H), 4.82 (s, 1 x ²H), 2.44 (d, 3 x ²H, ¹J_{2H-13C}: 1.05 Hz) ppm

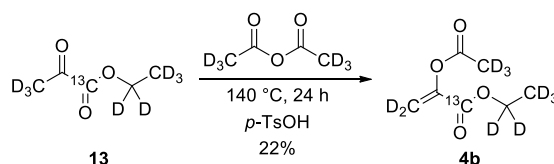
¹³C NMR (CDCl₃, 298 K, 75.5 MHz) = 190.92 (s, 1 x ¹³C), 157.74 (d, 1 x ¹³C, ¹J_{13C-13C}: 69.07 Hz), 141.56 (d, 1 x ¹³C, ³J_{13C-13C}: 1.48 Hz), 100.03 (s, 1 x ¹³C), 30.09 (s, 1 x ¹³C) ppm

For Vinyl-d₃ 2-oxopropanoate-1,2-¹³C-3,3,3-d₃ (**3b**)

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 7.30 (s, 1 x ²H), 5.10 (s, 1 x ²H), 4.83 (s, 1 x ²H), 2.45 (d, 3 x ²H, ¹J_{2H-13C}: 1.01 Hz) ppm

¹³C NMR (CDCl₃, 298 K, 75.5 MHz) = 191.39 (d, 1 x ¹³C, ¹J_{13C-13C}: 69.01 Hz), 158.65 (d, 1 x ¹³C, ¹J_{13C-13C}: 69.14 Hz), 141.58 (dd, 1 x ¹³C, ²J_{13C-13C}: 2.72 and ³J_{13C-13C}: 1.59 Hz), 100.06 (s, 1 x ¹³C, ³J_{13C-13C}: 3.78 Hz), 30.09 (s, 1 x ¹³C) ppm

3.4. Enol compounds



Scheme S7

3.4.1. Ethyl-d₅ 2-(acetoxymethyl)-2,2-d₂-¹³C-d₂ (**4b**)

To a solution of **13** (0.189 g, 1.51 mmol) in anhydrous p-TSA (0.002 g, 0.01 mmol) was added acetic anhydride-d₆ (0.285 mL, 3 mmol) dropwise under N₂. The resulted solution was heated to 140 °C for 24 h. The reaction mixture was cooled down to room temperature and quenched by adding using D₂O. The aqueous layer was extracted with CH₂Cl₂ (3 x 10 mL). The combined organic layer was dried over Na₂SO₄, filtered and concentrated in vacuo to get crude product which was purified by flash column chromatography (silica) using a gradient elution (EtOAc: pet ether; 0:100 to 5:95) to yield **4b** (0.050 g, 22 % yield) as colourless oily compound.

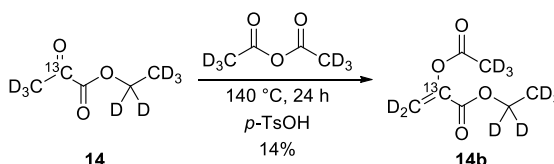
TLC (Silica gel, 20% EtOAc in Pet. ether), R_f (**4b**) = 0.6, KMnO₄ active.

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 6.09 (s, 1 x ²H), 5.52 (s, ²H), 4.26 (s, 2 x ²H), 2.23 (s, 3 x ²H), 1.3 (s, 3 x ²H) ppm

¹³C NMR (CDCl₃, 298 K, 75.5 MHz) = 170.89 (s, 1 x ¹³C), 161.63 (s, 1 x ¹³C), 145.47 (s, 1 x ¹³C, ¹J_{13C-13C}: 86.66 Hz), 113.4 (s, 1 x ¹³C, ²J_{13C-13C}: 6.7 Hz), 61.19 (s, 1 x ¹³C, ²J_{13C-13C}: 2.36 Hz), 19.9 (s, 1 x ¹³C), 13.18 (s, 1 x ¹³C, ³J_{13C-13C}: 2.13 Hz) ppm

LCMS: calculated for C₆¹³CD₁₀O₄: 169.1240, found 170.1315 (M+ H) and 192.1134 (M+Na).

Syntheses of differently labelled ¹³C-ethyl pyruvates-d₈ (**14b**, **15b** and **16b**) were prepared analogously with the synthesis of **4b**.



Scheme S8

3.4.2. Ethyl-d₅ 2-(acetoxymethyl)-2,2-d₂-¹³C-d₂ (**14b**)

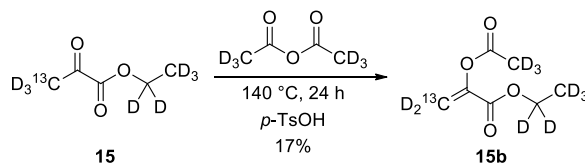
To a solution of **14** (0.13 g, 1.03 mmol) in anhydrous p-TSA (0.001 g, 0.007 mmol) was added acetic anhydride-d₆ (0.196 mL, 2.07 mmol) dropwise under N₂. The resulted solution was heated to 140 °C for 24 h. The reaction mixture was cooled down to room temperature and quenched by adding using D₂O. The aqueous layer was extracted with CH₂Cl₂ (3 x 10 mL). The combined organic layer was dried over Na₂SO₄, filtered and concentrated in vacuo to get crude product which was purified by flash column chromatography (silica) using a gradient elution (EtOAc: pet ether; 0:100 to 5:95) to yield **14b** (0.025 g, 14 % yield) as colourless oily compound (found traces of ethyl pyruvate, **14**).

TLC (Silica gel, 20% EtOAc in Pet. ether), R_f (**14b**) = 0.6, KMnO₄ active.

²H NMR (CDCl₃, 298 K, 46.1 MHz) = 5.96 (s, 1 x ²H), 5.39 (s, ²H), 4.13 (s, 2 x ²H), 2.1 (s, 3 x ²H), 1.16 (s, 3 x ²H) ppm

^{13}C NMR (CDCl_3 , 298 K, 75.5 MHz) = 169 (s, $1 \times ^{13}\text{C}$, $^2\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 4 Hz), 162.09 (s, $1 \times ^{13}\text{C}$, $^1\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 87.17 Hz), 144.78 (s, $1 \times ^{13}\text{C}$), 113.75 (s, $1 \times ^{13}\text{C}$, $^1\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 84.25 Hz), 61.05 (s, $1 \times ^{13}\text{C}$, $^2\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 1.54 Hz), 19.77 (s, $1 \times ^{13}\text{C}$, $^3\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 1.9 Hz), 13.03 (s, $1 \times ^{13}\text{C}$) ppm; found traces of ethyl pyruvate (**14**).

LCMS: calculated for $\text{C}_6^{13}\text{CD}_{10}\text{O}_4$: 169.1240, found 170.1318 ($\text{M}^+ \text{H}$) and 192.1130 ($\text{M}^+ \text{Na}$).



Scheme S9

3.4.3. Ethyl- d_5 2-(acetoxymethyl)-2,2-d $_3$ acrylate-3- ^{13}C - d_2 (**15b**)

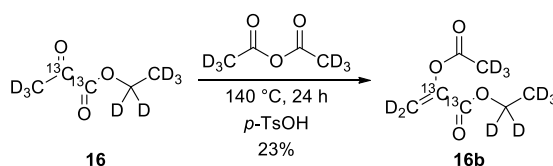
To a solution of **15** (0.12 g, 0.958 mmol) in anhydrous p-TSA (0.001 g, 0.0067 mmol) was added acetic anhydride- d_6 (0.226 mL, 2.39 mmol) dropwise under N_2 . The resulted solution was heated to 140 °C for 24 h. The reaction mixture was cooled down to room temperature and quenched by adding using D_2O . The aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layer was dried over Na_2SO_4 , filtered and concentrated in vacuo to get crude product which was purified by flash column chromatography (silica) using a gradient elution (EtOAc: pet ether; 0:100 to 5:95) to yield **15b** (0.027 g, 17 % yield) as colourless oily compound (found traces of ethyl pyruvate-**15**).

TLC (Silica gel, 20% EtOAc in Pet. ether), R_f (**15b**) = 0.6, KMnO_4 active.

^2H NMR (CDCl_3 , 298 K, 46.1 MHz) = 6.32-5.20 (m, $2 \times ^2\text{H}$), 4.21 (s, ^2H), 2.19 (s, $3 \times ^2\text{H}$), 1.25 (s, $3 \times ^2\text{H}$) ppm

^{13}C NMR (CDCl_3 , 298 K, 75.5 MHz) = 169.16 (s, $1 \times ^{13}\text{C}$, $^3\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 2.14 Hz), 161.60 (s, $1 \times ^{13}\text{C}$, $^2\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 6.40 Hz), 144.35 (t, $1 \times ^{13}\text{C}$, $^1\text{J}_{^{13}\text{C}-^{13}\text{C}}$: 42.15 Hz), 113.35 (s, $1 \times ^{13}\text{C}$), 61.18 (s, $1 \times ^{13}\text{C}$), 20.38 (s, $1 \times ^{13}\text{C}$), 13.17 (s, $1 \times ^{13}\text{C}$) ppm; found traces of ethyl pyruvate (**15**).

LCMS: calculated for $\text{C}_6^{13}\text{CD}_{10}\text{O}_4$: 169.1240, found 192.1133 ($\text{M}^+ \text{Na}$).



Scheme S10

3.4.4. Ethyl- d_5 2-(acetoxymethyl)-2,2-d $_3$ acrylate-1,2- ^{13}C - d_2 (**16b**)

To a solution of **16** (0.165 g, 1.3 mmol) in anhydrous p-TSA (0.0015 g, 0.0091 mmol) was added acetic anhydride- d_6 (0.309 mL, 3.26 mmol) dropwise under N_2 . The resulted solution was heated to 140 °C for 24 h. The reaction mixture was cooled down to room temperature and quenched by adding using D_2O . The aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layer was dried over Na_2SO_4 , filtered and concentrated in vacuo to get crude product which was purified by flash column chromatography (silica) using a gradient elution (EtOAc: pet ether; 0:100 to 5:95) to yield **16b** (0.03 g, 23 % yield) as colourless oily compound (found traces of ethyl pyruvate-**16**).

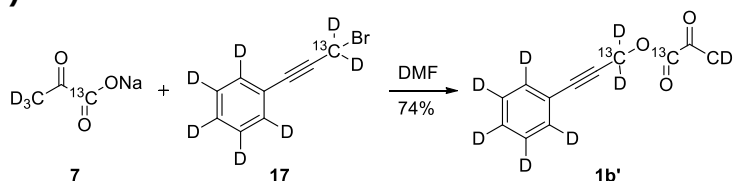
TLC (Silica gel, 20% EtOAc in Pet. ether), R_f (**16b**) = 0.6, KMnO_4 active.

^2H NMR (CDCl_3 , 298 K, 46.1 MHz) = 6.04 (s, 1 x ^2H), 5.49 (s, 1 x ^2H), 4.2 (s, 2 x ^2H), 2.18 (s, 3 x ^2H), 1.25 (s, 3 x ^2H) ppm

^{13}C NMR (CDCl_3 , 298 K, 75.5 MHz) = 171.38 (d, 1 x ^{13}C , $^2\text{J}_{13\text{C}-13\text{C}}$: 70.92 and $^3\text{J}_{13\text{C}-13\text{C}}$: 2.61 Hz), 162.23 (s, 1 x ^{13}C , $^1\text{J}_{13\text{C}-13\text{C}}$: 86.47 Hz), 145.47 (s, 1 x ^{13}C , $^1\text{J}_{13\text{C}-13\text{C}}$: 86.66 Hz), 113.93 (d, 1 x ^{13}C , $^1\text{J}_{13\text{C}-13\text{C}}$: 83.75 and $^2\text{J}_{13\text{C}-13\text{C}}$: 6.41 Hz), 61.18 (m, 1 x ^{13}C), 19.91 (s, 1 x ^{13}C , $^3\text{J}_{13\text{C}-13\text{C}}$: 2.03 Hz), 13.18 (s, 1 x ^{13}C , $^3\text{J}_{13\text{C}-13\text{C}}$: 2.21 Hz) ppm; found traces of ethyl pyruvate-**16**.

LCMS: calculated for $\text{C}_6^{13}\text{CD}_{10}\text{O}_4$: 170.1274, found 193.1170 ($\text{M}+\text{Na}$).

3.5. Synthesis of 3-(phenyl- d_5)prop-2-yn-1-yl-1- ^{13}C -1,1- d_2 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 (**1b'**)



Scheme S11

To a solution of **7** (0.299 g, 2.63 mmol) in DMF (10 mL), was added **17**^[1] (0.333 g, 1.64 mmol). The resulting suspension was heated at 50 °C for 7 h. After completion reaction, it was cooled to 0 °C and quenched by adding D_2O to it (25 mL). To this solution, EtOAc (25 mL) was added. The organic layer was separated and the aqueous layer was again extracted with EtOAc (15 mL). The combined organic layer was washed with water, brine, dried over Na_2SO_4 and was concentrated under reduced pressure (water bath temperature 30 °C). The crude product was purified by flash column chromatography (silica) using a gradient elution (CH_2Cl_2) to give **1b'** (0.260 g, 74% yield) as a colourless liquid.

TLC (Silica gel, 10% EtOAc in pet. ether), R_f (**17**) = 0.9, R_f (**1b'**) = 0.3, UV active.

^2H NMR (CDCl_3 , 298 K, 46.1 MHz) δ = 7.56-7.32 (5 x ^2H), 5.27 (d, 2 x ^2H , $^3\text{J}_{2\text{H}, \text{C}}$ = 23.53 Hz), 2.46 (s, 3 x ^2H) ppm.

^{13}C NMR (CDCl_3 , 298K, 75.5 MHz) = 191.01 (s, 1 x ^{13}C , $^1\text{J}_{\text{C}, \text{C}}$ = 66.74 Hz), 160.33 (d, 1 x ^{13}C , $^2\text{J}_{\text{C}, \text{C}}$ = 2.57 Hz), 131.67 (d, 1 x ^{13}C , $^1\text{J}_{\text{C}, \text{D}}$ = 0.77 Hz), 128.66 (s, 1 x ^{13}C), 127.97 (s, 2 x ^{13}C), 121.62 (d, 1 x ^{13}C , $^3\text{J}_{\text{C}, \text{C}}$ = 1.62 Hz), 87.62 (d, 1 x ^{13}C , $^2\text{J}_{\text{C}, \text{C}}$ = 14.08 Hz), 80.92 (dd, 1 x ^{13}C , $^1\text{J}_{\text{C}, \text{C}}$ = 81.40 and $^3\text{J}_{\text{C}, \text{C}}$ = 2.46 Hz), 54.19 (d, 1 x ^{13}C , $^3\text{J}_{\text{C}, \text{D}}$ = 2.58 Hz), 26.48 (m, 1 x ^{13}C) ppm.

HRMS: calculated for $\text{C}_{10}^{13}\text{C}_2\text{D}_{10}\text{O}_3\text{Na}^+$ [$\text{M}+\text{Na}$]⁺ 237.13, found 237.12

3.6. NMR Spectra

3.6.1. Vinyl pyruvates

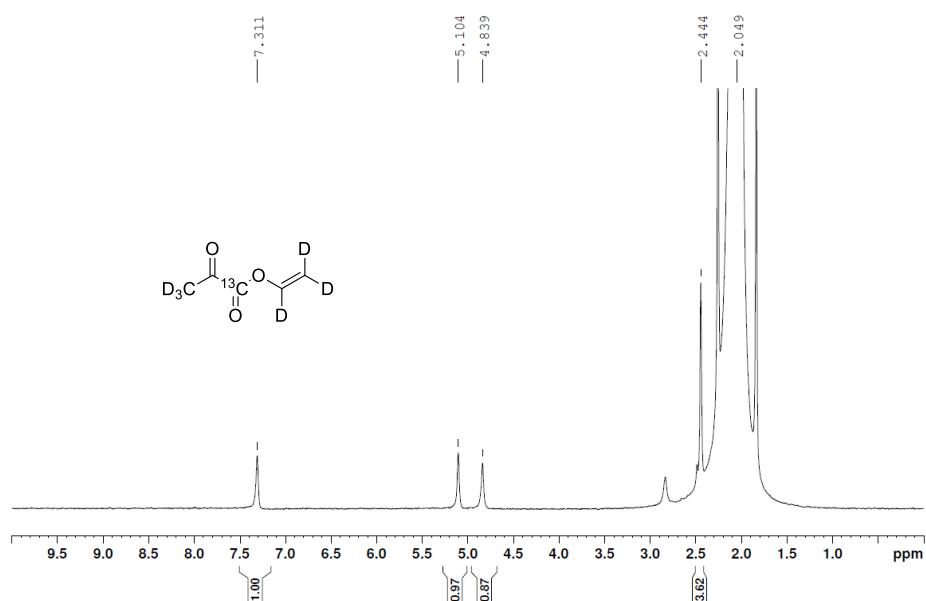


Figure S4 ^2H -NMR of Vinyl- d_3 2-oxopropanoate-1- ^{13}C -3,3,3- d_3 (**1b**) in Acetone.

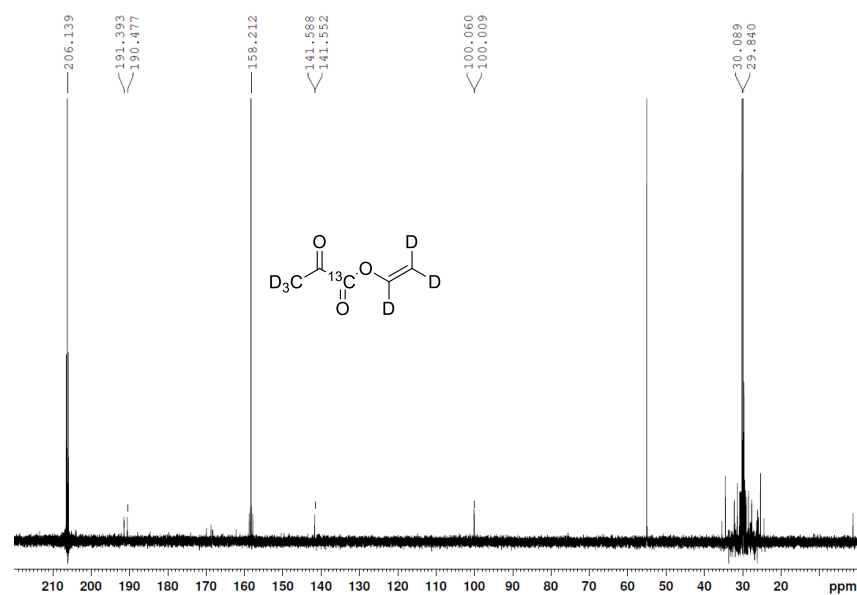


Figure S5 ^{13}C -NMR of Vinyl- d_3 2-oxopropanoate-1- ^{13}C -3,3,3- d_3 (**1b**) in Acetone.

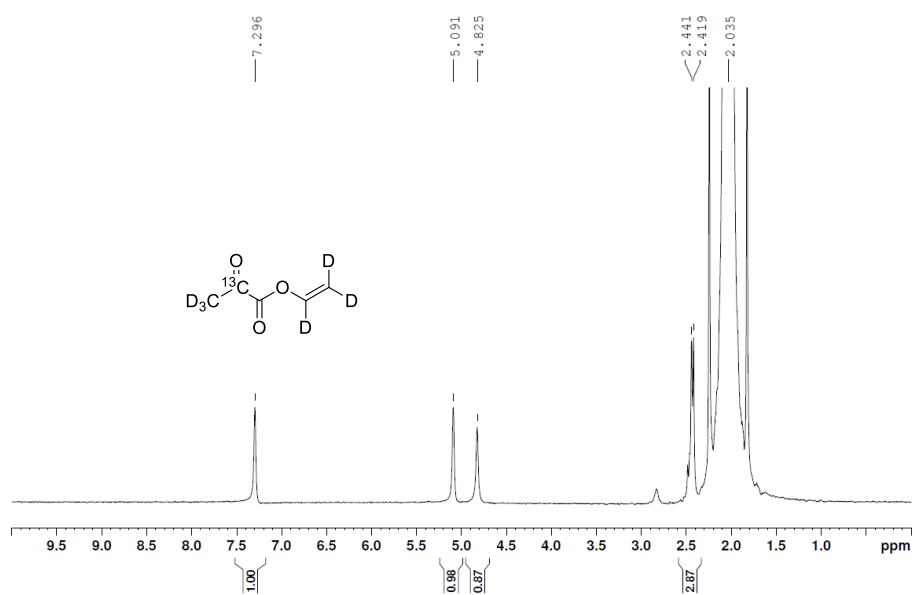


Figure S6 2H -NMR of Vinyl- d_3 2-oxopropanoate- $2-^{13}C$ -3,3,3- d_3 (**2b**) in Acetone.

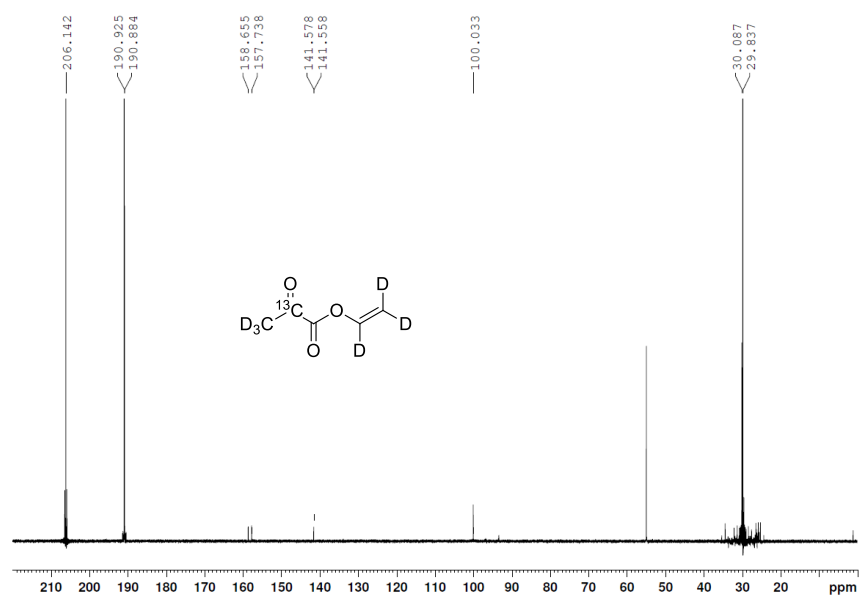


Figure S7 ^{13}C -NMR of Vinyl- d_3 2-oxopropanoate- $2-^{13}C$ -3,3,3- d_3 (**2b**) in Acetone.

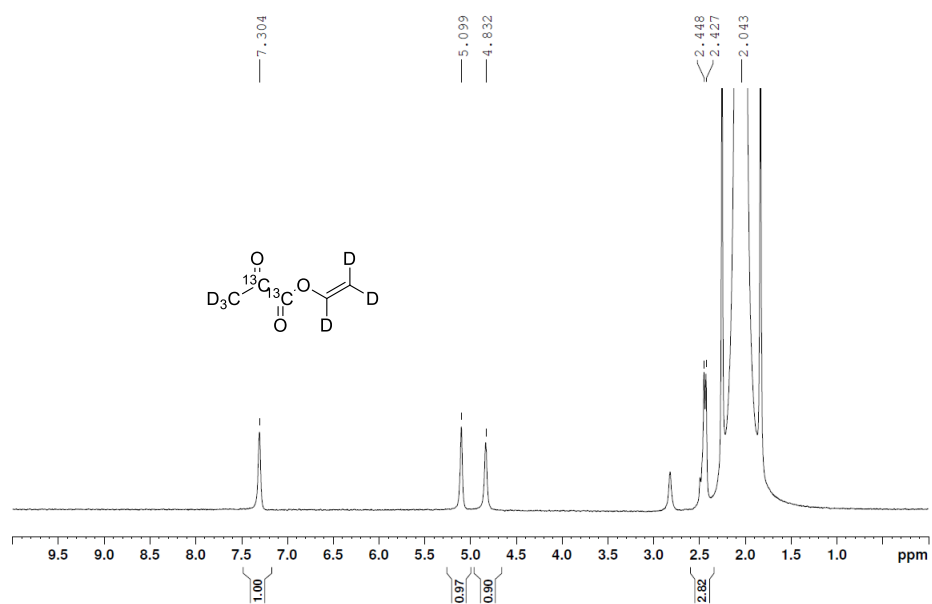


Figure S8 2H -NMR of Vinyl- d_3 2-oxopropanoate-1, 2- ^{13}C -3,3,3- d_3 (**3b**) in Acetone.

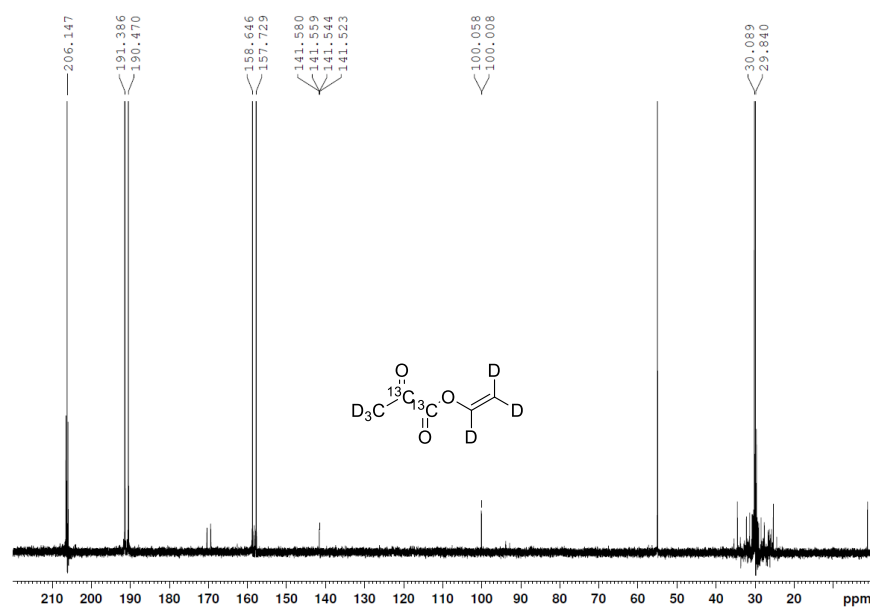


Figure S9 ^{13}C -NMR of Vinyl- d_3 2-oxopropanoate-1, 2- ^{13}C -3,3,3- d_3 (**3b**) in Acetone.

3.6.2. Enol-pyruvates

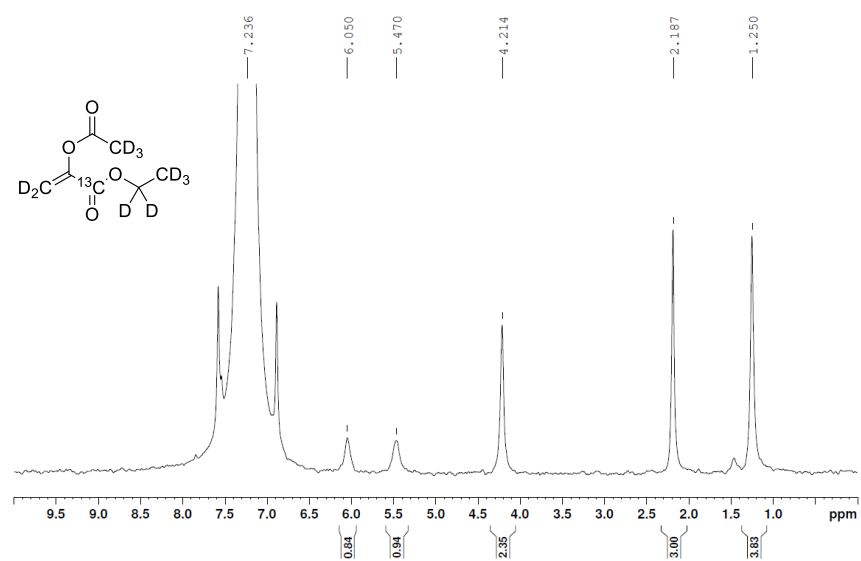


Figure S10 ²H-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-1-¹³C-d₂ (**4b**) in CDCl₃.

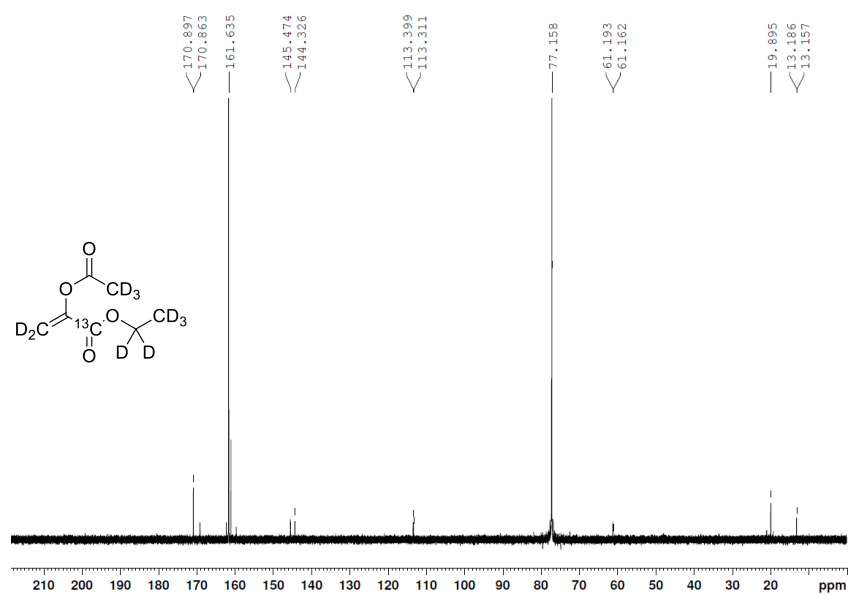


Figure S11 ¹³C-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-1-¹³C-d₂ (**4b**) in CDCl₃.

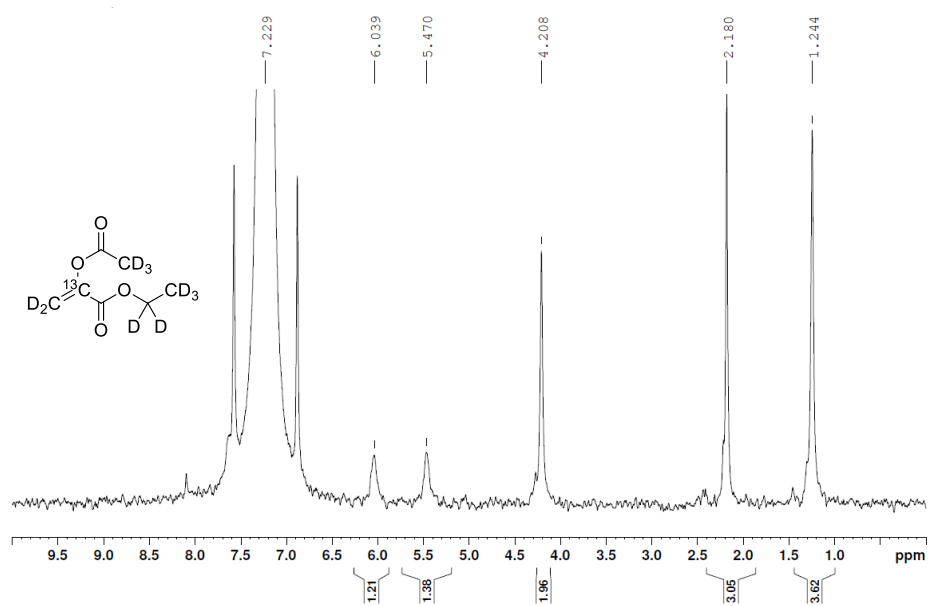


Figure S12 ¹H-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-2-¹³C-d₂ (**14b**) in CDCl₃.

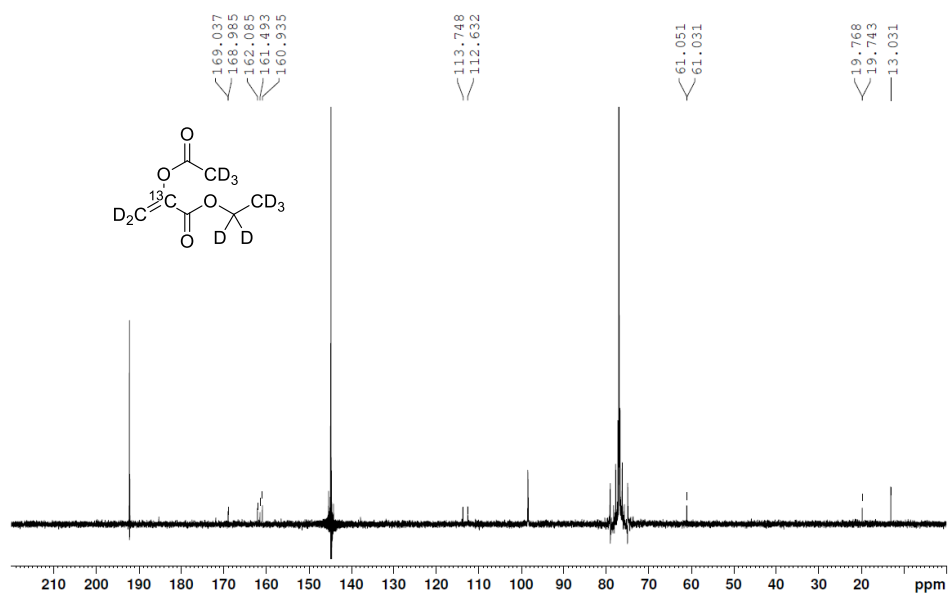


Figure S13 ¹³C-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-2-¹³C-d₂ (**14b**) in CDCl₃.

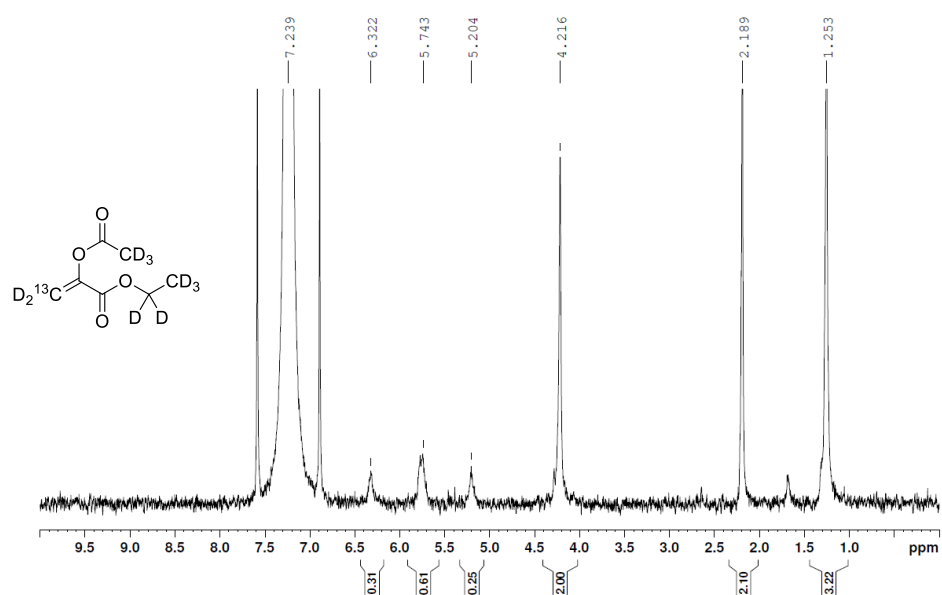


Figure S14 ¹H-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ (**15b**) in CDCl₃.

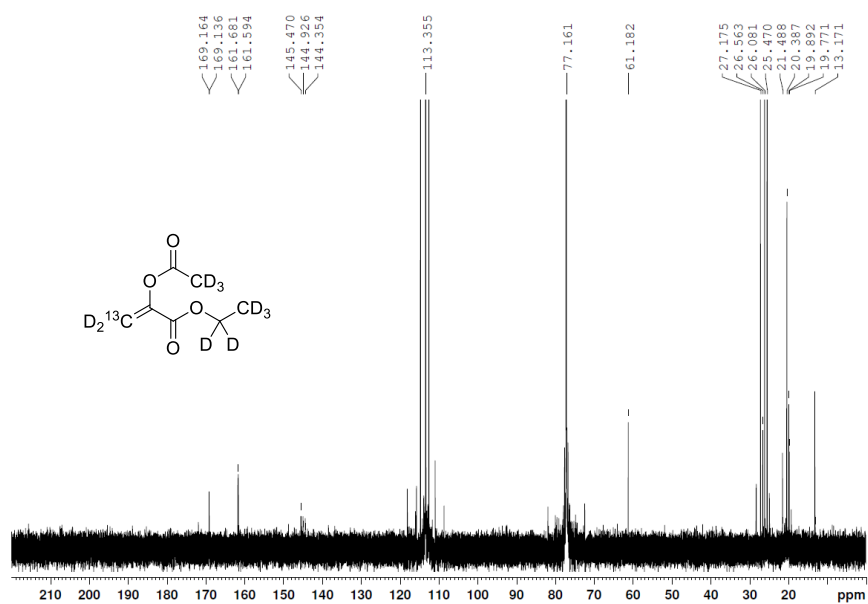


Figure S15 ¹³C-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ (**15b**) in CDCl₃.

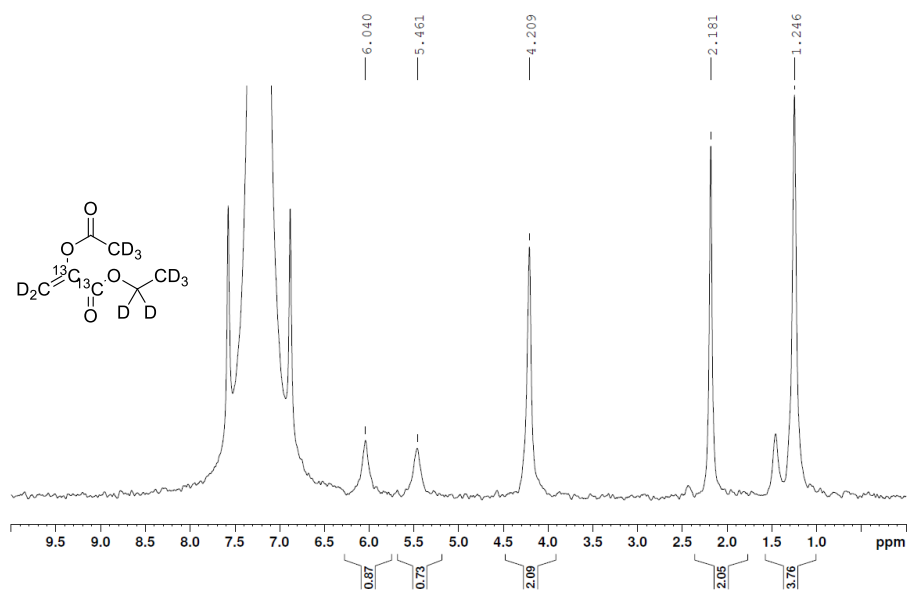


Figure S16 ¹H-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ (**16b**) in CDCl₃.

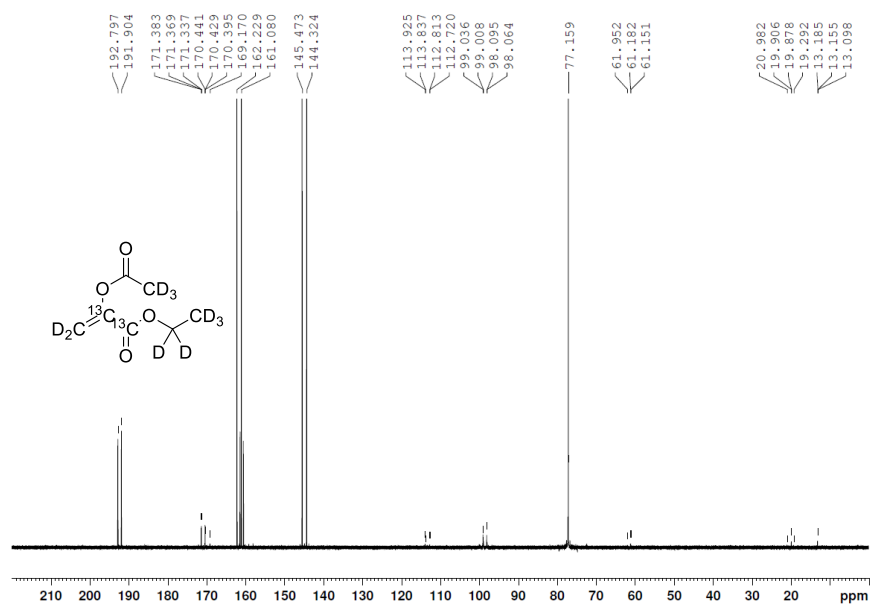


Figure S17 ¹³C-NMR of Ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ (**16b**) in CDCl₃.

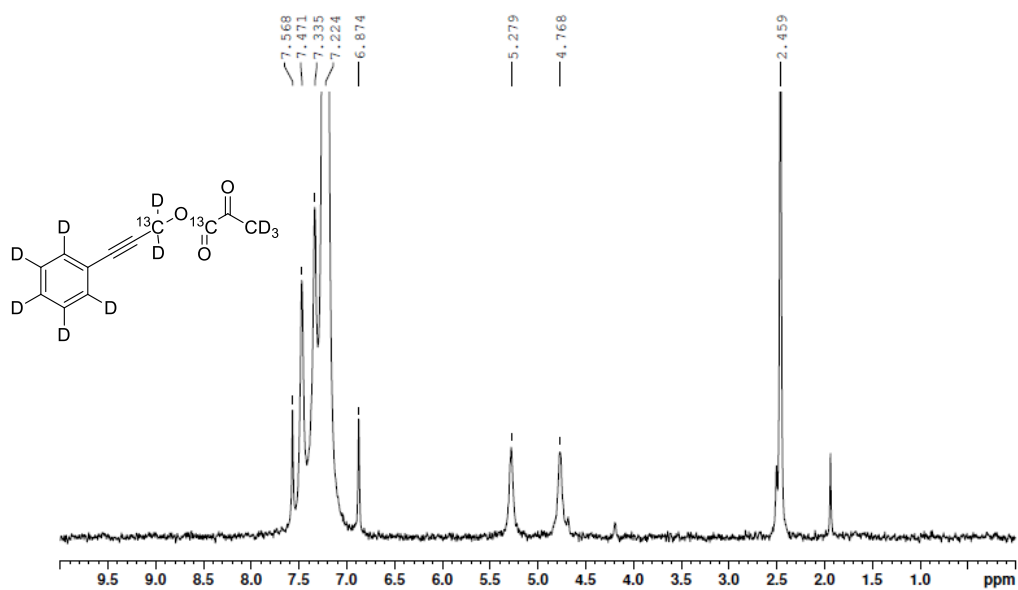


Figure S18 ^2H -NMR of 3-(phenyl- d_5)prop-2-yn-1-yl- ^{13}C -1,1- d_2 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 (**1b'**) in CDCl_3 .

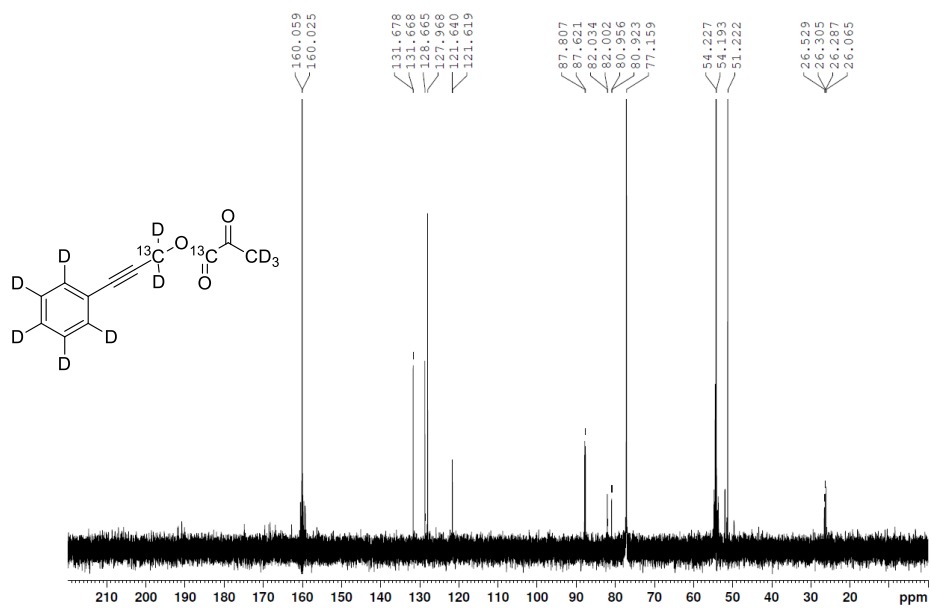


Figure S19 ^{13}C -NMR of 3-(phenyl- d_5)prop-2-yn-1-yl- ^{13}C -1,1- d_2 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 (**1b'**) in CDCl_3 .

4. Biochemistry & Cell Culture

4.1. Cell lines and media

For the culture of Hela Kyoto cells, the Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 4.5 g/L D-Glucose, 2 mM glutamine, 1 mM Sodium Pyruvate, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL Penicillin and Streptomycin (all the components were purchased from Thermo-Fisher scientific). If not mentioned otherwise, the term DMEM medium used in the following text includes all the aforementioned supplements. The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere to around 70-80% confluency before NMR experiments.

Human embryonic kidney 293 cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Invitrogen), supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin–streptomycin, and (1x) Minimal Essential Medium-Non essential Amino acids (MEM NEAA; Gibco) at 37 °C, and 5% CO₂ humidified atmosphere.

CRISPR/Cas9-Mediated SNCA knock down

HEK293T cells were transiently transfected with the “all in one” vector PX459, expressing the guide RNA (gRNA) targeting sequence 5'-TGCTGCTGAGAAAACCAAAC-3' in exon 2 of the α -synuclein (α -Syn) SNCA gene using Metafectene according to the manufacturer's instructions. (pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene plasmid #62988; <http://www.addgene.org/62988/>; RRID: Addgene 62988). After 48 h, cells were selected using 2 μ g/mL puromycin (P11-0-19, PAA Laboratories) for 48 h. Cells were plated on 10-cm Petri dishes at a density of 1 cell/cm² and grown until they formed distinct colonies. Part of each colony was used to extract DNA, followed by sequencing of exon 2 of the SNCA gene. Successfully edited colonies were further grown for subsequent experiments, e.g. Western blotting experiments using the α -Syn specific Antibody (BD Bioscience, Clone 42/ α -Syn).

Lentiviral mediated overexpression of α -Syn

For overexpression human α -Syn, HEK293T cells were infected using a lentivirus encoding human α -Syn wildtype. The lentivirus was based on lentiviral transfectovector FUGW (Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D, Science. 2002 Feb 1. 295(5556):868-72) where the GFP cassette was exchanged by the full-length SNCA cDNA sequence, driven under the Ubiquitin promoter, for 24 h with a MOI of 100. Successful overexpression of human α -Syn was evaluated by Western blotting experiments using the α -Syn specific Antibody (BD Bioscience, Clone 42/ α -Syn) in comparison to naïve Hek293T cells.

4.2. Protein expression and purification

Human α -Syn was expressed recombinantly in minimal medium in E. coli strain BL21(DE3). To produce uniformly ¹⁵N-labelled wild-type (WT) α -Syn, minimal medium supplemented with ¹⁵NH₄Cl (Cambridge Isotope Laboratories) was employed. Expression and purification of the protein were performed as previously described^[2]. Briefly, the cells from 1 L expression culture were lysed by freeze-thaw cycles and

sonication, boiled for 15 minutes and centrifuged at 48.000 x g for 45 minutes. From the supernatant DNA was precipitated by adding streptomycin sulfate (10 mg/ml). After another centrifugation step α -Syn was precipitated from the supernatant by adding ammonium sulfate to 0.36 g/ml. The pellet was resuspended in 25 mM Tris/HCl, pH 7.7 and further purified by anion exchange chromatography on a POROS HQ column (PerSeptive Biosystems) followed by gelfiltration on a SD 75 16/60 column (GE Healthcare). The produced U-¹⁵N α -Syn was dialyzed against the electroporation buffer (100 mM sodium phosphate, pH 7.0, 15 mM HEPES, pH 7.0, 5 mM KCl, 15 mM MgCl₂) its concentration adjusted to 7 mg/ml, sterile filtered and stored in aliquots at -80°C.

4.3. Protein delivery via electroporation and characterization of transduced cells

The U-15N α -Syn protein was delivered into Hela cells (HeLa 'Kyoto' cells kindly provided by the Live-cell Imaging Facility of MPI-BPC) via electroporation using Neon transfection system (Thermo-Fisher scientific), according to the protocols provided by the manufacturer and published previously (10). For the structural and metabolomic studies combined, around 100-200 million cells are needed as starting material. Before electroporation, Hela cells were detached and harvested from the cell culture flasks using trypsin/EDTA (0.05%/0.02%), centrifuged under 130 g for 3 min at room temperature, washed once with PBS and counted on a haemocytometer.

Subsequently, the cells were pelleted again by centrifugation and the supernatant was removed. A solution mixture of 100 μ L Buffer R of the transfection kit (Thermo-Fisher scientific) and 400 μ L 7 mg/mL U-15N α -Syn was used to resuspend the cells. The mixture was then subjected to two pulses electroporation under 1300 V with 15 ms pulse duration in the 100 μ L transfection tips. After electroporation, the cells were immediately transferred to a 12 mm culture plate with 15 mL pre-warmed cell culture medium without antibiotics. The cells were kept in incubator at 310 K in a humidified 5% CO₂ atmosphere for at least 5 hours for recovery of the cell membrane. Upon use, the cells were collected and washed for at least three times by PBS buffer, in order to remove the extracellular recombinant proteins in the medium.

4.4. Preparation of cells for NMR experiments

For structural analysis by 2D NMR, around 40-60 million cells were resuspended in 200 μ L DMEM medium and transferred to a 5 mm NMR tube, while for the metabolomic experiments, around 20-30 million cells were used as one replicate. To probe the impact of rotenone (Sigma-aldrich) on the pyruvate-to-lactate conversion in the cells, the cells were grown to around 70% confluency, transferred to fresh cell culture medium containing 2 μ M rotenone for 24 hours. The same cell lines were grown at the same condition without rotenone as control experiments. Cell viability was probed before and after the PHIP experiment the rotenone treatment using trypan blue staining (cell viability provided in **Table S**). At least three replicates were performed for each cell line and for each condition.

Table S1 Cell viability before and after PHIP experiments (top); cell viability before and after treatment of 2 μ M rotenone for 24 hour (middle); cell viability before and after 2D SOFAST-HMQC NMR measurement of 2-3 hours (bottom)

	Before PHIP experiment	After PHIP experiment
Hela cells	>99%	95-98%
HEK variants	>98%	~95%
	Before rotenone treatment	After rotenone treatment
HEK variants	>98%	92-95%
	Before 2D NMR analysis	After 2D NMR analysis
Hela cells	>99%	~95%

4.5. Western blot analysis

The WB was performed according to the previously published protocol^[3]. Briefly, the HeLa cells containing exogenously delivered α -Syn (details of electroporation method mentioned in 5.3) were lysed in RIPA buffer (150mM NaCl, 50 mM Tris/HCl pH 7.4, 0.25% (w/v) sodium deoxycholate, 1% NP40/IGEPAL, (Sigma-Aldrich)) supplemented with a protease inhibitor cocktail (Roche). The lysate was centrifuged at 50,000 g at 4 °C for 30 min. In addition, the supernatant of the HeLa sample was collected by centrifugation at 130 g for 4 min at different time points post in-cell NMR measurement and analysed by WB. Subsequently, the samples were separated by a 10-well 4-12% bis-tris PAGE gel (Invitrogen), transferred to PVDF membrane and blocked in 5% bovine serum albumin (Sigma) in PBS buffer. After blocking for 1-2 hour, the primary mouse antibody against α -Syn (Santa Cruz; catalog: sc-69977; final concentration: 0.1 μ g/mL) was incubated with the membrane at 4°C for overnight. Rabbit anti-mouse HRP-conjugated secondary antibody (Agilent Dako; catalog: P026002-2) was used to treat the membrane at a final concentration of 0.1 μ g/mL at 21°C for 2 h. The membranes were then developed by Pierce ECL blotting reagents (Thermo Scientific). The chemiluminescence signals were recorded by Amersham Imager 600 and processed by Image J, as shown in Figure S20.

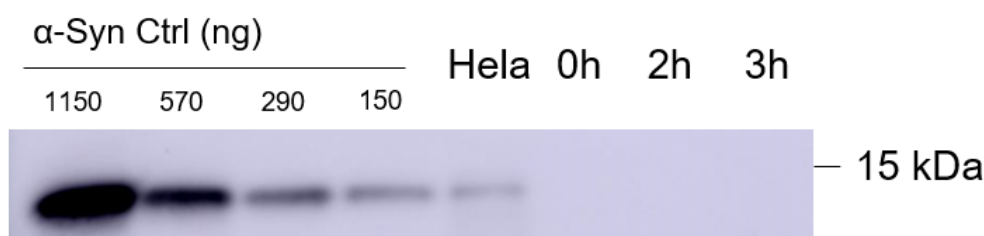


Figure S20 WB of the exogenously delivered α -Syn in 25 million HeLa cells via electroporation. With the present protocol ~5 μ M α -Syn was introduced into the cells (1 pg/cell). Purified recombinant α -Syn was employed for calibration, respectively. To check leakage of the α -Syn in the cells during NMR measurement, the supernatants

(15 μL from $\sim 350 \mu\text{L}$) were collected from the NMR tube and probed 0 h, 2 h and 3 h post NMR measurement. No obvious leakage from cells into the solvent was detected.

4.6. Immunofluorescence

Staining of the HEK 293T α -Syn KO and OE cells were performed similarly as previously described [4]. Briefly, around 0.1 million cells were seeded on glass coverslips (18 mm) previously coated poly-D-lysine and incubated overnight at 37°C and 5%CO₂. Cells were shortly washed with PBS, and fixed in PBS with 4% paraformaldehyde for 30 minutes at room temperature. Remaining reactive aldehyde groups were quenched with PBS supplemented with 0.1 M glycine for 30 minutes at room temperature. Cells were permeabilized and block using 0.3% Triton X-100 and 2% BSA in PBS for 30 minutes at room temperature. Fixed and permeabilized cells were further incubated with a mouse monoclonal anti- α -Syn antibody (BD Biosciences; catalog: 610787; final concentration: 0.25 $\mu\text{g/mL}$) for 3 h in PBS containing 0.15% TritonX-100 and 1 % BSA. After five thorough washing steps using PBS, cells were further incubated for 1.5 h with a Cy3-labeled secondary donkey anti-mouse antibody (Jackson ImmunoResearch; catalog: 715-165-150; final concentration: 1.5 $\mu\text{g/mL}$). The samples were again subjected to five thorough washing steps with PBS. Before mounting the coverslips or before imaging, cells were stained with Hoechst 33342 (Lot # TB2522291; Thermo Scientific; Germany) for 5 minutes (2 μM in PBS). After one thorough washing with PBS, coverslips were finally mounted in Mowiol mounting media (6 g glycerol, 6 ml deionized water, 12 ml 0.2 M Tris buffer pH 8.5, 2.4 g Mowiol 4-88 from Merck).

4.7 Imaging

For laser scanning confocal microscopy, an inverse 4-channel Expert Line easy3D STED setup (Abberior Instruments GmbH, Göttingen, Germany) was used. The setup was based on an Olympus IX83 microscope body equipped with a plan apochromat 100 $\times$ 1.4 NA oil-immersion objective (Olympus). Fluorescence excitation lines 561 (for Cy3) nm 405 nm (for Hoechst). Fluorescence signal was detected using APD detectors in predefined channels. The operation of the setup and the recording of images were performed with the Inspector software (Abberior Instruments GmbH). All images were acquired and processed using the same settings for direct comparisons.

5. Experimental Procedures

5.1. Estimation of the polarization level

The thermal polarisation P_{th} level for a spin-1/2 system at the magnetic field B_0 and temperature T is given by:

$$P_{th} = \tanh\left(\frac{h\omega_0}{2k_B T}\right) \quad (1)$$

$$\omega_0 = -\gamma B_0 \quad (2)$$

With γ the gyromagnetic ratio, h and k_B the Planck and Boltzmann constant respectively.

In a hyperpolarization experiment the signal of interest is integrated in the appropriate region (e.g. for the ¹³C NMR signal of pyruvate at around 170 ppm) returning I_{hyp} . The thermal experiment is acquired on the same sample by a sequence of pulse acquire experiments with an interdelay d_1 of $5T_1$ (for ¹³C pyruvate $d_1=300$ s). Integration in the

same spectral region yields I_{th} . The polarisation in the PHIP experiment P_{hyp} is calculated as follows:

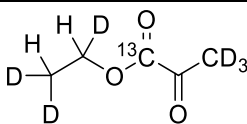
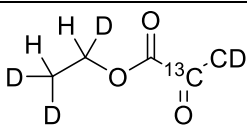
$$P_{hyp} = \frac{I_{hyp}}{I_{th}} \times \frac{rg_{th}}{rg_{hyp}} \times \frac{ns_{th}}{ns_{hyp}} \times \frac{\sin(\theta_{th})}{\sin(\theta_{hyp})} \times P_{th} \quad (3)$$

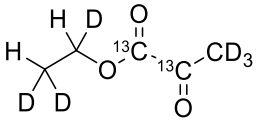
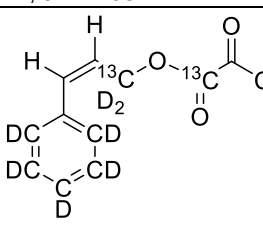
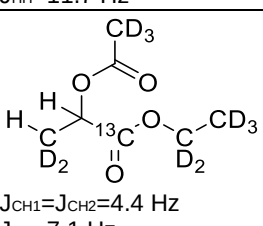
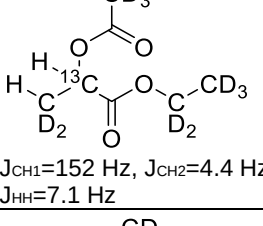
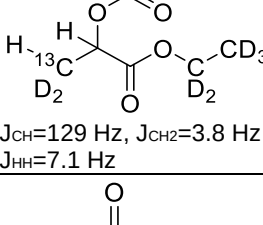
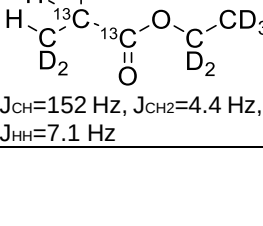
,where rg is the receiver gain (see section 7 for linearity proof) of the spectrometer, $\sin(\theta_{th})=1$ and $ns_{hyp}=1$ as the thermal experiments were acquired with a 90° degree flip angle and a single scan is used in hyperpolarisation experiments.

5.2. General procedure

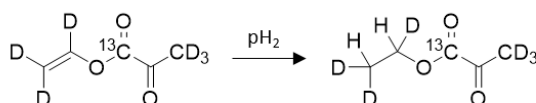
Several metabolite precursors were para-hydrogenated to generate maximal possible polarization on the metabolite after cleaving auxiliary groups by following procedure. Almost 100% enriched pH_2 was produced using a He-cooled (20 K) para-hydrogen generator provided by a Cryocooler system (Sumimoto HC-4A helium compressor, Sumimoto Cold Head CH-204 with pH_2 reaction chamber by ColdEdge Technologies, temperature controller Lake Shore Cryotronics, Inc.) and delivered by a home-built valve and tubing system. Desired amount of hydrogenation catalyst ([1,4-bis(diphenylphosphino) butane] (1,5-cyclooctadiene)rhodium(I) tetrafluoroborate, Sigma-Aldrich) was dissolved in acetone- d_6 or ethanol. Samples were degassed by bubbling N_2 gas for 1 minute and hydrogenated at 8 bar for 5-20 s in a 5 mm nmr tube by bubbling pH_2 using a home-built setup inside a probehead of a 7 T Bruker spectrometer (Avance III) at elevated temperature. The 1H spin order of pH_2 -stemming protons was transferred to the desired ^{13}C moiety by various MINERVA pulse sequences, of which the general principle is described in Section 2. The cleavage is done by adding aqueous solution of base (NaOH or Na_2CO_3). In case of acetone, solvent was removed from the water mixture by evaporation in vacuum for 10 s while the sample was resting inside the probehead under heating of the temperature regulator of the spectrometer. Hyperpolarization levels were calculated by comparing HP signal to thermally polarized signals according Section 5.1.

Table S2 Polarization levels achieved in different metabolic molecules before and after cleavage using appropriate pulse sequences to transfer polarization from protons to carbons. Para-hydrogenation was done in acetone- d_6 if not otherwise stated.

Hydrogenation product and relevant J-couplings	Sample volume and concentrations	1H spin order, measured by PHIP-echo	^{13}C polarization before cleavage	^{13}C polarization after cleavage and evaporation
 $J_{CH}=3.0\text{ Hz}$, $J_{CH_2}<0.1\text{ Hz}$, $J_{HH}=7.05\text{ Hz}$	0.1 ml 13 mM 4 mM Cat	60.5±9.3%	MINERVA-Ref: 59.7±2.5%	27±1%
 $J_{CH1}=J_{CH2}=0.33\text{ Hz}$ $J_{HH}=7.05\text{ Hz}$	0.1 ml 10 mM 4 mM Cat	47.3±4.2%	MINERVA-Ref: 10.7±0.7%	3.9±0.4%
	0.4 ml 1 mM 2 mM Cat	62.7±7.0%	MINERVA-Ref: 31% MINERVA-Sel: 51.2±5.0%	N/A

 $J_{CH1}=3.1$ Hz, $J_{CH2}<0.1$ Hz, $J_{CC}=68.6$ Hz, $J_{HH}=7.05$ Hz	0.1 ml 10mM 4mM Cat	48.9±4.2%	MINERVA-Split, split between two carbons: -23.5±1.6% +24.4±0.4% $T_1=48s$ and $57s$	Split between two carbons -7.4±1.0% and +6.5±0.4%
 $J_{CH1}=11.7$ Hz, $J_{CH2}\sim 0.1$, $J_{CC}=2.7$ Hz, $J_{HH}=11.7$ Hz	0.1 ml 10 mM 4 mM Cat	62.2±2.8%	MINERVA: 17.4±0.6%	8.6±0.7%
 $J_{CH1}=J_{CH2}=4.4$ Hz $J_{HH}=7.1$ Hz	0.1 ml in Ethanol- d_6 10 mM 2 mM Cat	~40%	MINERVA-Ref: 30.3±2.2%	without evaporation 10.3±2.0%
	0.4 ml in Acetone- d_6 1 mM 2 mM Cat	~43%	MINERVA-Sel2: 50.9±0.9%	N/A
 $J_{CH1}=152$ Hz, $J_{CH2}=4.4$ Hz $J_{HH}=7.1$ Hz	0.1 ml 10mM, 2mM Cat	43.6±1.6%	MINERVA-Ref: 53.5±4.2%	N/A
 $J_{CH}=129$ Hz, $J_{CH2}=3.8$ Hz $J_{HH}=7.1$ Hz	0.1 ml 10 mM 4 mM Cat	56.1±6.0%	MINERVA-Ref: 49.4±0.8%	N/A
 $J_{CH}=152$ Hz, $J_{CH2}=4.4$ Hz, $J_{CC}=64.5$ Hz, $J_{HH}=7.1$ Hz	0.1 ml 10 mM 4 mM Cat	22.5±1.7%	CO: -24.7% MINERVA-Split, split between two carbons: -11.1±0.7% +12.5±0.5% CH: 24.2%	N/A

5.3. Para-hydrogenation of vinyl- d_3 2-oxopropanoate-1- ^{13}C -3,3,3- d_3 to ethyl-1,2,2- d_3 2-oxopropanoate-1- ^{13}C -3,3,3- d_3



Scheme S12

13 mM of precursor and 4 mM of catalyst were dissolved in acetone- d_6 . 0.1 ml of the sample were para-hydrogenated within 5 s bubbling time at 330 K according to the general procedure. The polarization transfer to the pyruvate carbon was done using the MINERVA-Ref sequence (Figure S21) with a constant gradient of 5 mG/cm and ^2H -decoupling. After hydrogenation and polarization transfer, the side-arm was cleaved off by adding 0.1 ml of 50 mM Na_2CO_3 in H_2O .

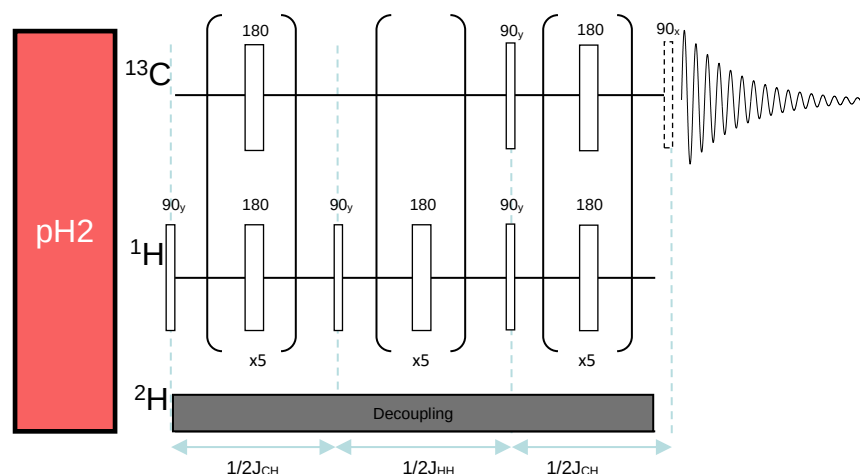


Figure S21 MINERVA-Ref pulse sequence used to generate and transfer polarization in the three-spin systems. 90° at carbon in the end of the sequence can be used to generate carbon magnetization.

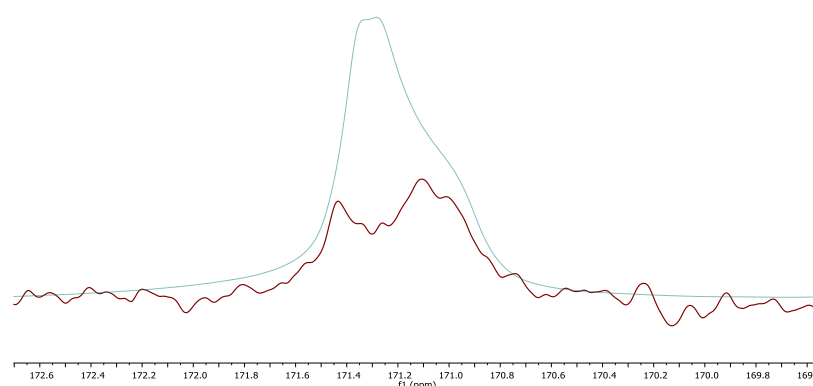
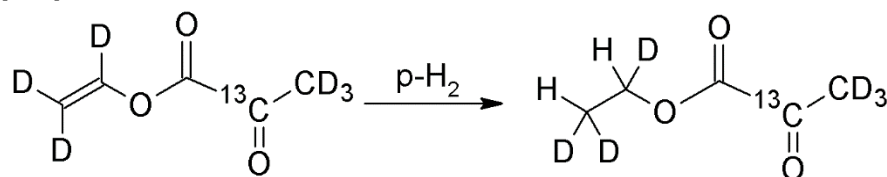


Figure S22 ^{13}C spectrum of 0.1 ml sample after hydrogenation of 13 mM of vinyl- d_3 -1- ^{13}C -pyruvate- d_3 with 4 mM of catalyst in acetone- d_6 , cleavage and acetone evaporation (green) and its thermal spectrum with 200 accumulations and 100-fold enlarged (red). Polarization of pyruvate 1- ^{13}C is 28%. Shimming was not conducted after the described steps hence the lineshapes are distorted.

5.4. Para-hydrogenation of vinyl- d_3 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 to ethyl-1,2,2- d_3 2-oxopropanoate-2- ^{13}C -3,3,3- d_3



Scheme S13

The experiments were done for two concentrations of precursor: 8.5 mM and 1.0 mM in acetone- d_6 . 0.1 ml of the 8.5 mM sample was para-hydrogenated within 20 s bubbling time at 330 K according general procedure. The polarization transfer to the pyruvate carbon was done using the MINERVA-Ref sequence (Figure S21) with 2H -decoupling. Because the J_{CH} =0.33 Hz) the delay in the MINERVA-Ref sequence was twice shorter and theoretically could transfer only half of the proton spin order. Additional losses came from long evolution times ~ 1.5 s under conditions of broad lines of the 0.1 ml sample, leading to only $\sim 10\%$ carbon polarization. After hydrogenation and polarization transfer the side-arm was cleaved off by adding 0.1 ml of 50 mM Na_2CO_3 in H_2O . In another experiments a 0.4 ml sample volume was used to achieve a good field homogeneity. Additionally, a small concentration of precursor (1 mM) was used to omit radiation damping problems. Under these settings high carbon polarization of 31% for the MINERVA-Ref sequence was obtained, proving importance of a good field homogeneity for long evolution times. The limit of 50% polarization transfer for spin systems with equal CH J-couplings can be surpassed by using selective pulses on one of the proton in a modified pulse sequence. In Figure S23 the MINERVA-Sel is shown that allowed to reach 51% carbon polarization in ethyl-1,2,2- d_3 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 .

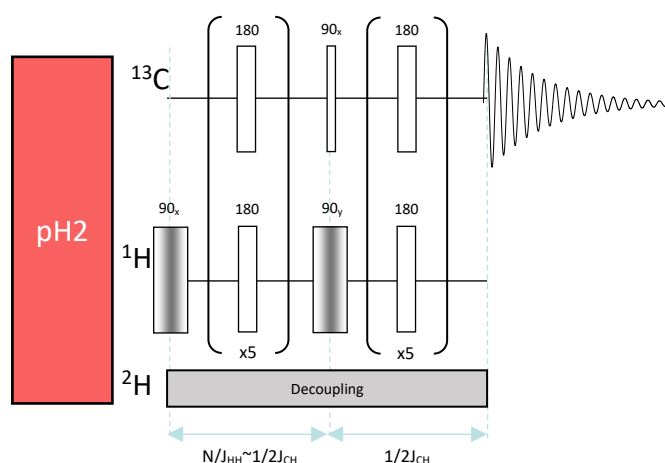


Figure S23 MINERVA-Sel pulse sequence used to generate and transfer polarization in the three-spin systems with equivalent J_{CH} couplings that are much smaller than J_{HH} . Selective pulses operate only on one and the same of protons. The first period has to be exactly equal to N/J_{HH} to refocus the proton evolution.

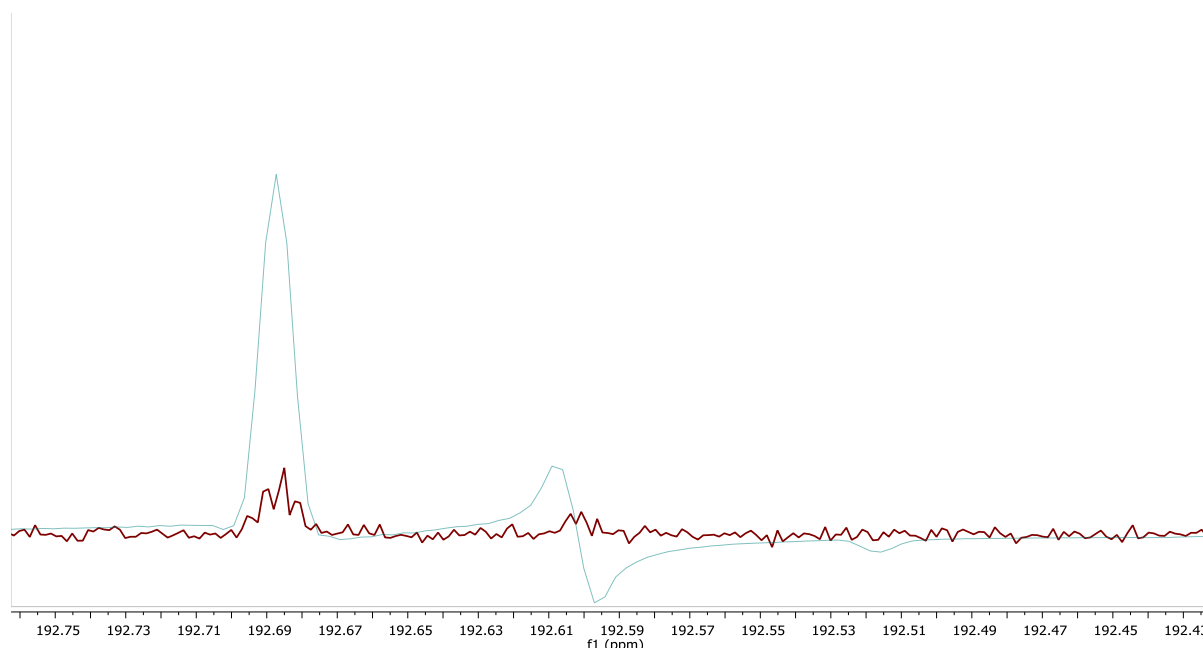
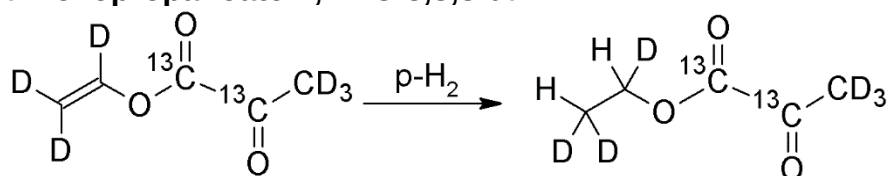


Figure S24 ^{13}C $\{^2\text{H}\}$ spectrum of 0.4 ml sample after hydrogenation of 1 mM of vinyl- d_3 2-oxopropanoate- $1\text{-}^{13}\text{C}$ -3,3,3- d_3 with 2 mM of catalyst in acetone- d_6 and polarization transfer using MINERVA-Sel pulse sequence (green) and its thermal spectrum with 150 accumulations and 100-fold enlarged (red). 192.7 ppm is the product with CD_3 -group, 192.6 ppm – with CD_2H , and 192.5 ppm – with CDH_2 .

5.5. Para-hydrogenation of vinyl- d_3 2-oxopropanoate- $1,2\text{-}^{13}\text{C}$ -3,3,3- d_3 to ethyl-1,2,2- d_3 2-oxopropanoate- $1,2\text{-}^{13}\text{C}$ -3,3,3- d_3



Scheme S14

10 mM of precursor and 4 mM of catalyst were dissolved in acetone- d_6 . 0.1 ml of the sample were para-hydrogenated within 20 s bubbling time at 330 K according to the general procedure.

The polarization transfer to the pyruvate carbon was done using the MINERVA-Split sequence (Figure S25) with a constant gradient of 1 mG/cm and ^2H -decoupling. By varying pulses at the middle of the last evolution period one can distribute polarization between two coupled carbons. Because of the large CC J-coupling caution has to be taken in order to refocus its influence during the first and third periods.

After hydrogenation and polarization transfer the side-arm was cleaved off by adding 0.1 ml of 50 mM Na_2CO_3 in H_2O .

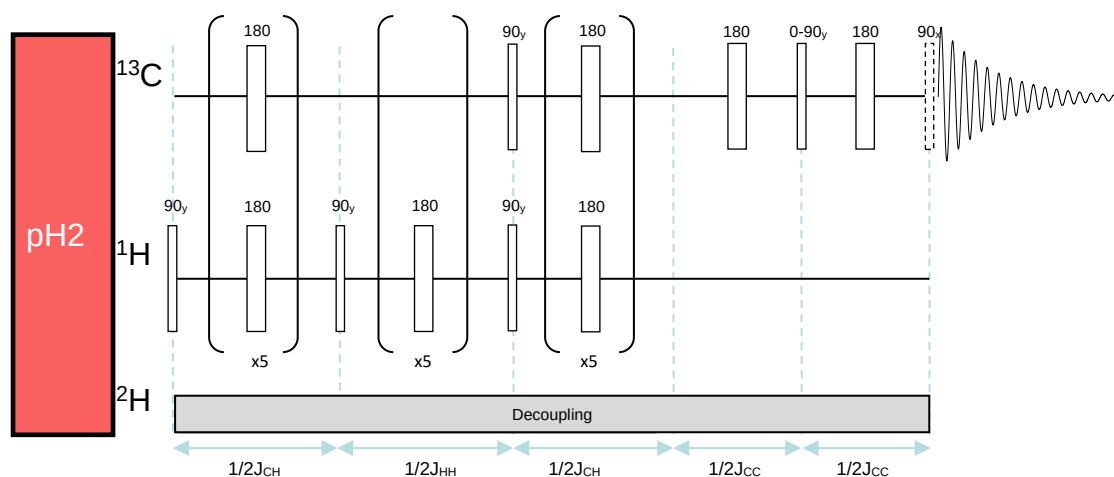


Figure S25 MINERVA-Split pulse sequence used to generate and transfer polarization in the four-spin systems and to distribute the spin order between two carbons depending on flip angle in the middle of the last evolution period. Note, the sign of the two carbon polarizations are always opposite.

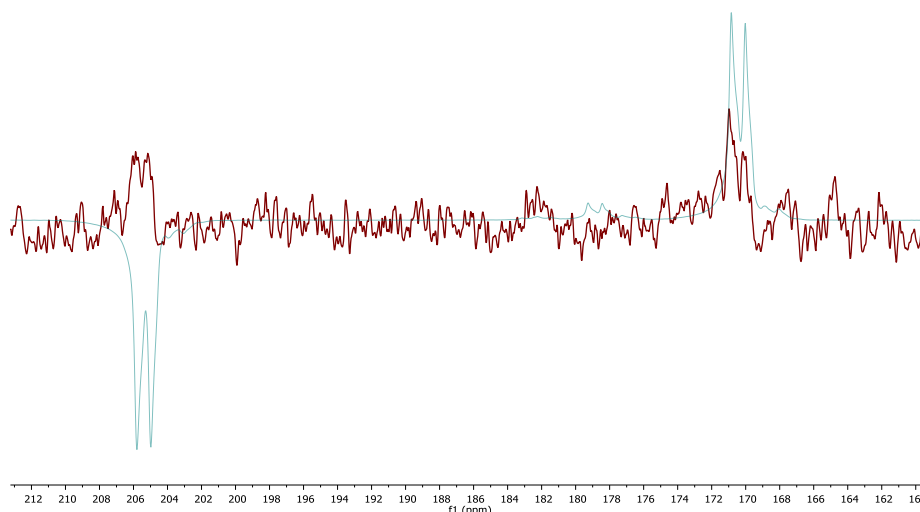
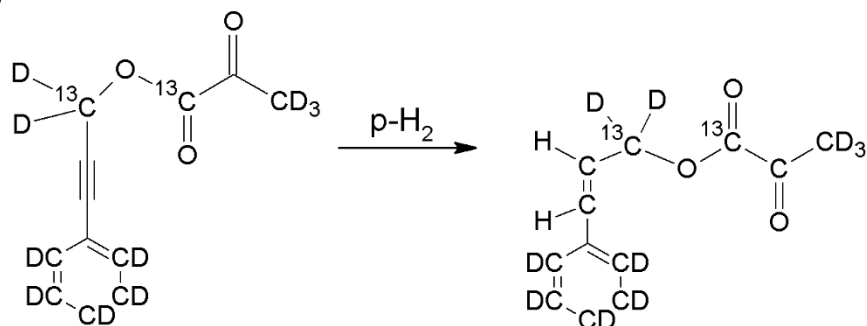


Figure S26. ^{13}C { ^2H } spectrum of 0.1 ml sample after hydrogenation of 10 mM of vinyl- d_3 2-oxopropanoate-1,2- ^{13}C -3,3,3- d_3 with 4 mM of catalyst in acetone- d_6 , cleavage and acetone evaporation (green) and its thermal spectrum with 200 accumulations and 20-fold enlarged (red). Polarizations of pyruvate carbons are -7.7% for 2- ^{13}C and 6.3% for 1- ^{13}C .

5.6. Para-hydrogenation of 3-(phenyl-d₅)prop-2-yn-1-yl-1-¹³C-1,1-d₂ 2-oxopropoate-1-¹³C-3,3,3-d₃ to (Z)-3-(phenyl-d₅)allyl-1-¹³C-1,1-d₂ 2-oxopropoate-1-¹³C-3,3,3-d₃



Scheme S15

10 mM or 50 mM precursor and 4mM of catalyst were dissolved in acetone-d₆. 0.1 ml of the sample were para-hydrogenated within 15 s bubbling time at 330 K according to the general procedure.

The polarization transfer to the labeled carbon of pyruvate was accomplished using the MINERVA sequence (Figure S27) with a constant gradient of 5 mG/cm and ²H-decoupling. After hydrogenation, the side-arm was cleaved off by adding 0.1 ml of 50 mM Na₂CO₃ in H₂O.

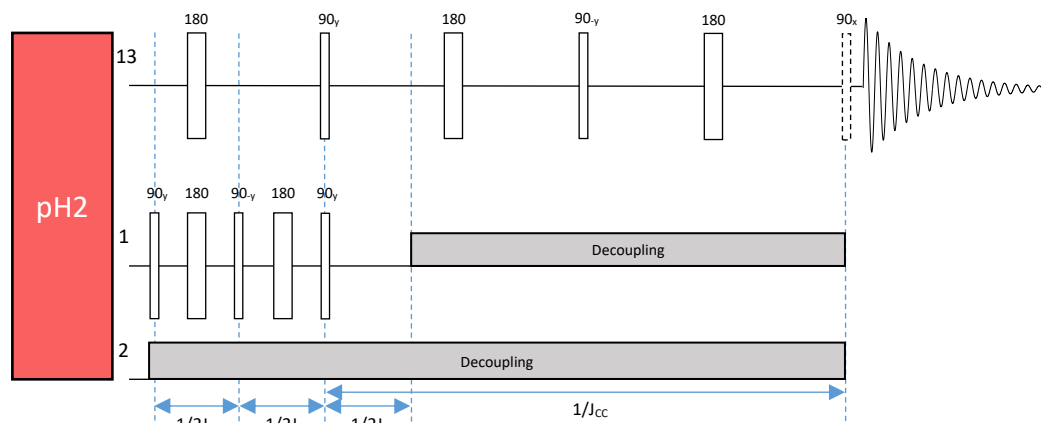


Figure S27 MINERVA pulse sequence used to generate and transfer polarization to pyruvate moiety in cis-1-¹³C-cinnamyl-d₇-1-¹³C-pyruvate-d₃.

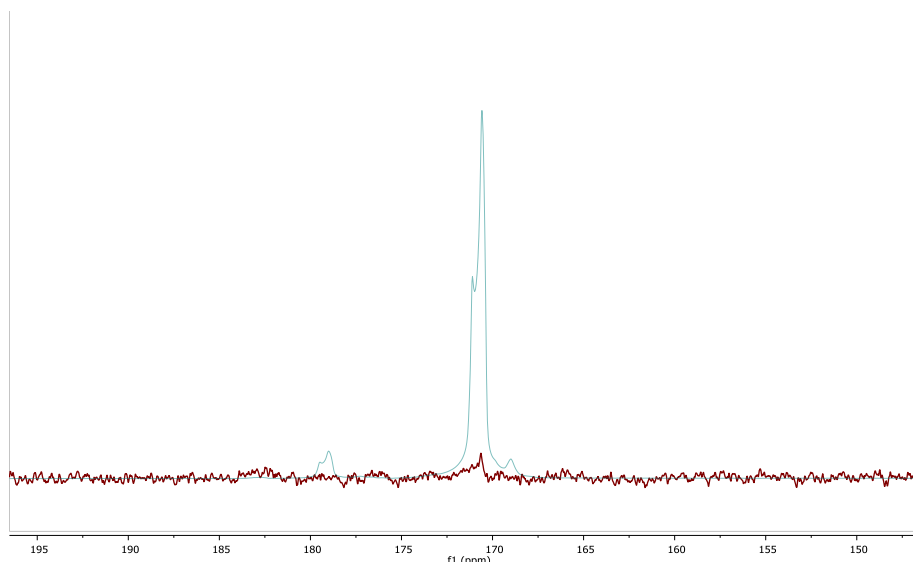
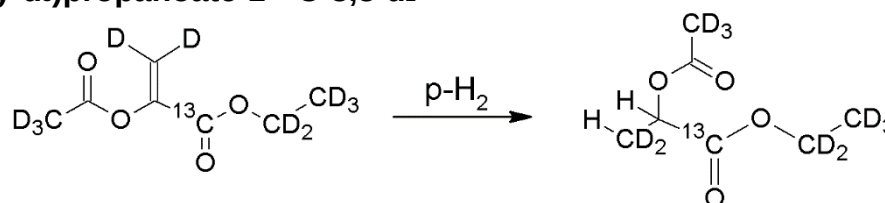


Figure S28 ^{13}C spectrum of 0.1 ml sample after hydrogenation of 10 mM of 3-(phenyl- d_5)prop-2-yn-1-yl-1- ^{13}C -1,1- d_2 2-oxopropanoate-1- ^{13}C -3,3,3- d_3 with 4 mM of catalyst in acetone- d_6 , cleavage and acetone evaporation (green) and its thermal spectrum with 200 accumulations and 80 times enlarged (red). Polarizations of pyruvate 1- ^{13}C is 9.4%.

5.7. Para-hydrogenation of ethyl- d_5 2-(acetoxyl- d_3)acrylate-1- ^{13}C - d_2 to ethyl- d_5 2-(acetoxyl- d_3)propanoate-1- ^{13}C -3,3- d_2



Scheme S16

The experiments were performed for two concentrations of precursor: 10 mM and 1.0 mM. 0.1 ml of the 10 mM sample were para-hydrogenated within 5 s bubbling time at 330 K according to the general procedure. The polarization transfer to the pyruvate carbon was done using the MINERVA-Ref sequence (Fig. S5) with ^2H -decoupling. Because the J-coupling between both protons and carbon are the same ($J_{\text{CH}}=4.4$ Hz) the first and the third delays in the MINERVA-Ref sequence were twice shorter and theoretically could transfer only half of proton spin order thus leading to only 30% carbon polarization.

After hydrogenation and polarization transfer the two auxiliary groups were cleaved off in two steps. In the first 10 s period 0.1 ml of 1 M NaOH in ethanol- d_6 was added to cleave off the acetyl group. In the second 10 s period 0.3 ml of H_2O was added to cleave remaining ethyl group. Hyperpolarized signal of lactic acid was measured without evaporation of ethanol.

In another experiments a 0.4 ml sample volume was used to achieve good field homogeneity. Additionally, small concentration of precursor was used to omit radiation damping problems. As in case of ethyl-1,2,2- d_3 2-oxopropanoate-2- ^{13}C -3,3,3- d_3 the limit of 50% transfer for spin systems with equal CH J-couplings can be surpassed by using selective pulses on one of the protons in a modified MINERVA-Sel pulse sequence. In Figure S29 MINERVA-Sel2 is shown that allowed to reach 51% carbon polarization in ethyl- d_5 2-(acetoxyl- d_3)propanoate-1- ^{13}C -3,3- d_2 measured in acetone- d_6 .

In contrast to MINERVA-Sel, an additional period is used to refocus proton-proton interaction when CH and HH j-couplings are similar in value.

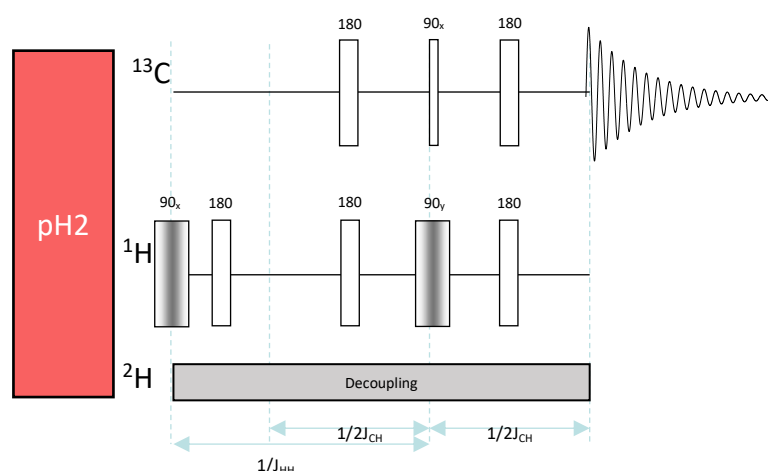


Figure S29 MINERVA-Sel2 pulse sequence used to generate and transfer polarization in the three-spin systems with equivalent J_{CH} couplings. It is more suitable for molecule where J_{CH} are comparable to J_{HH} . Selective pulses operate only on one of the protons.

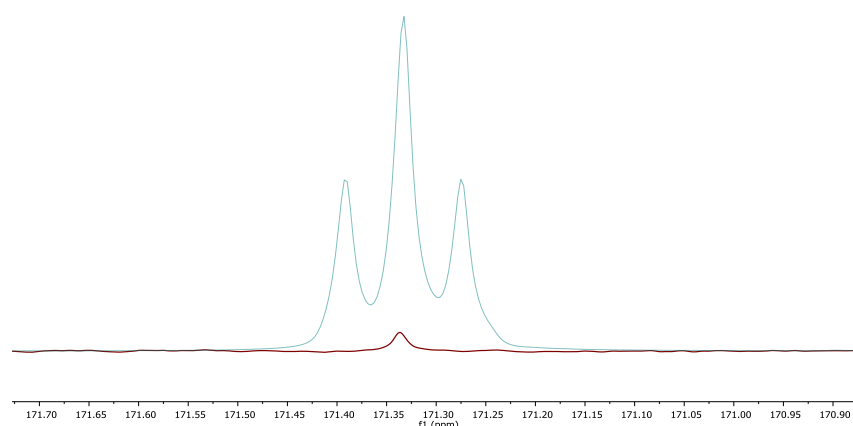
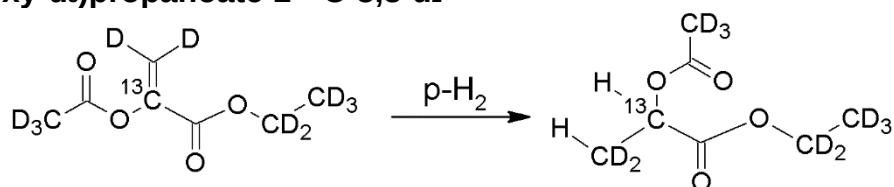


Figure S30 ^{13}C $\{^2\text{H}\}$ spectrum of 0.4 ml sample after hydrogenation of 1 mM of ethyl- d_5 2-(acetoxy- d_3)acrylate- $1\text{-}^{13}\text{C}\text{-d}_2$ with 2 mM of catalyst in acetone- d_6 (green) and its thermal spectrum ($\{^1\text{H}, ^2\text{H}\}$) with 200 accumulations and 20-fold enlarged (red). Polarizations of ethyl- d_5 2-(acetoxy- d_3)propanoate- $1\text{-}^{13}\text{C}\text{-}3,3\text{-d}_2$ is 50.4%.

5.8. Para-hydrogenation of ethyl- d_5 2-(acetoxy- d_3)acrylate- $2\text{-}^{13}\text{C}\text{-d}_2$ to ethyl- d_5 2-(acetoxy- d_3)propanoate- $2\text{-}^{13}\text{C}\text{-}3,3\text{-d}_2$



Scheme S17

10 mM of precursor and 4 mM of catalyst were dissolved in acetone- d_6 . 0.1 ml of the sample were para-hydrogenated within 5 s bubbling time at 330 K according to the general procedure. Relatively high concentration of catalyst was used to speed up the reaction to compete with proton relaxation (relaxation of $I_{1z}I_{2z}$ is 17s).

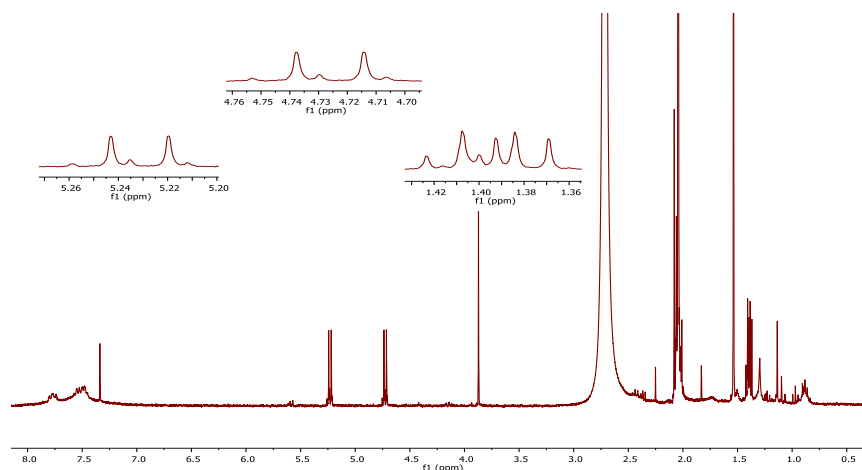


Figure S31 ^1H $\{^2\text{H}\}$ spectrum of 0.4 ml sample after hydrogenation of 10 mM of ethyl- d_5 2-(acetoxyl- d_3)acrylate-2- ^{13}C - d_2 with 4 mM of catalyst in acetone- d_6 . Enlarged are peaks of the product ethyl- d_5 acetyl- d_3 -lactate-2- ^{13}C - d_2 . The side bands show that 80% of the precursor is fully deuterium labeled and 20% contains one proton in acrylate moiety. ^{13}C labeling is 100%.

The polarization transfer to the labeled carbon was achieved with the MINERVA-Ref sequence with constant gradient of 1 mG/cm and ^2H -decoupling.

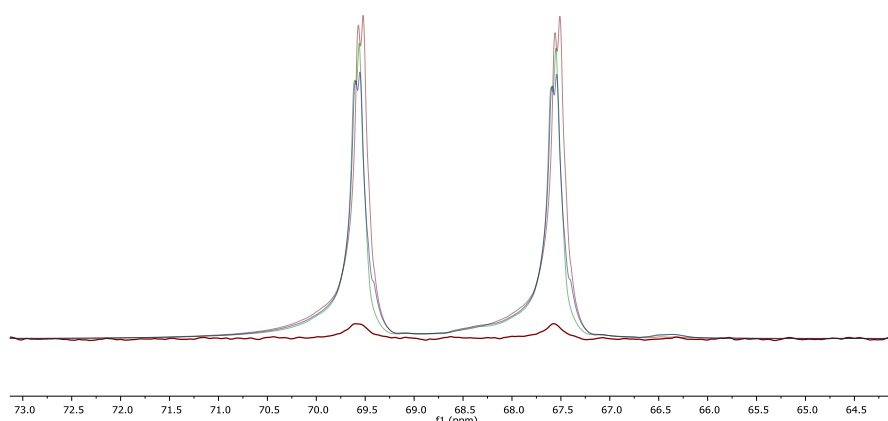
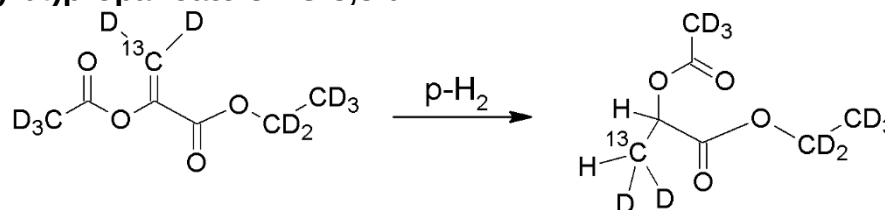


Figure S32 ^{13}C $\{^2\text{H}\}$ spectra of 0.1 ml sample after hydrogenation of 10 mM of ethyl- d_5 2-(acetoxyl- d_3)acrylate-2- ^{13}C - d_2 with 4 mM of catalyst in acetone- d_6 (magenta, blue and green) and its thermal spectrum with 300 accumulations and 20 times enlarged (red). Polarization of ethyl- d_5 2-(acetoxyl- d_3)propanoate-2- ^{13}C -3,3- d_2 is $53.5 \pm 4.2\%$.

5.9. Para-hydrogenation of ethyl- d_5 2-(acetoxyl- d_3)acrylate-3- ^{13}C - d_2 to ethyl- d_5 2-(acetoxyl- d_3)propanoate-3- ^{13}C -3,3- d_2



Scheme S18

10 mM of precursor ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ and 4 mM of catalyst were dissolved in acetone-d₆. 0.1 ml of the sample were para-hydrogenated within 5 s bubbling time at 330 K according to the general procedure. Relatively high concentration of catalyst was used to speed up the reaction to compete with proton relaxation (relaxation of I_{1z}I_{2z} is 16s). The polarization transfer to the labeled carbon was achieved using the MINERVA-Ref sequence with constant gradient of 1 mG/cm and ²H-decoupling.

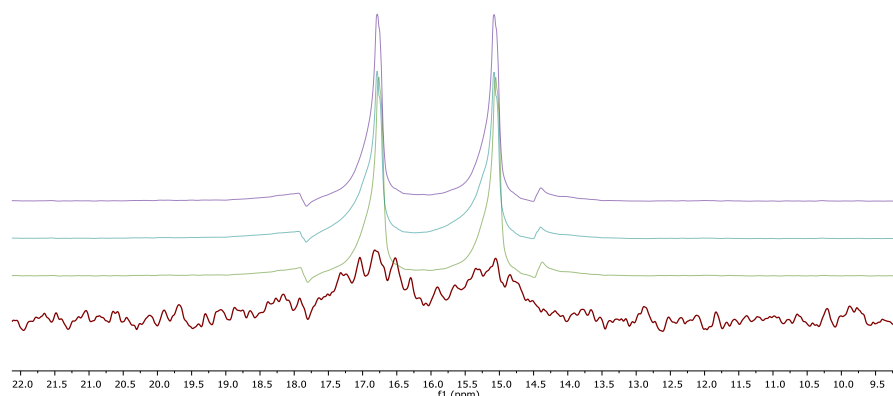
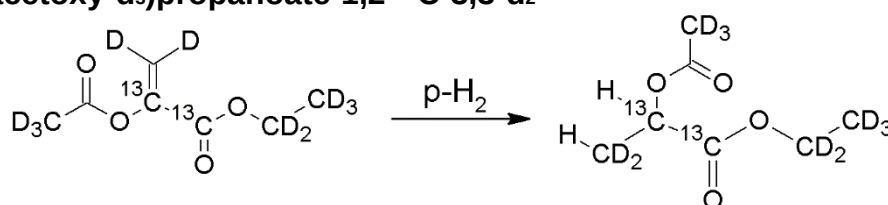


Figure S 33 ¹³C {²H} spectra of 0.1 ml sample after hydrogenation of 10 mM of ethyl-d₅ 2-(acetox-d₃)acrylate-3-¹³C-d₂ with 4 mM of catalyst in acetone-d₆ (magenta, blue and green) and its thermal ¹³C spectrum with 400 accumulations and 203 times enlarged (red). Polarizations of ethyl-d₅ 2-(acetox-d₃)propanoate-3-¹³C-3,3-d₂ is 49.4±0.8%.

5.10. Para-hydrogenation of ethyl-d₅ 2-(acetox-d₃)acrylate-1,2-¹³C-d₂ to ethyl-d₅ 2-(acetox-d₃)propanoate-1,2-¹³C-3,3-d₂



Scheme S19

10 mM of precursor and 4 mM of catalyst were dissolved in acetone-d₆. 0.1 ml of the sample were para-hydrogenated within 5 s bubbling time at 330 K according general procedure. Relatively high concentration of catalyst was used to speed up the reaction to compete with proton relation (relaxation of CH carbon is 19.5 s).

The polarization transfer to the lactate carbons was done using the MINERVA-Split sequence (Figure S25) with constant gradient of 1 mG/cm and ²H-decoupling. By varying pulse at the middle of the last evolution period one can distribute polarization between two coupled carbons.

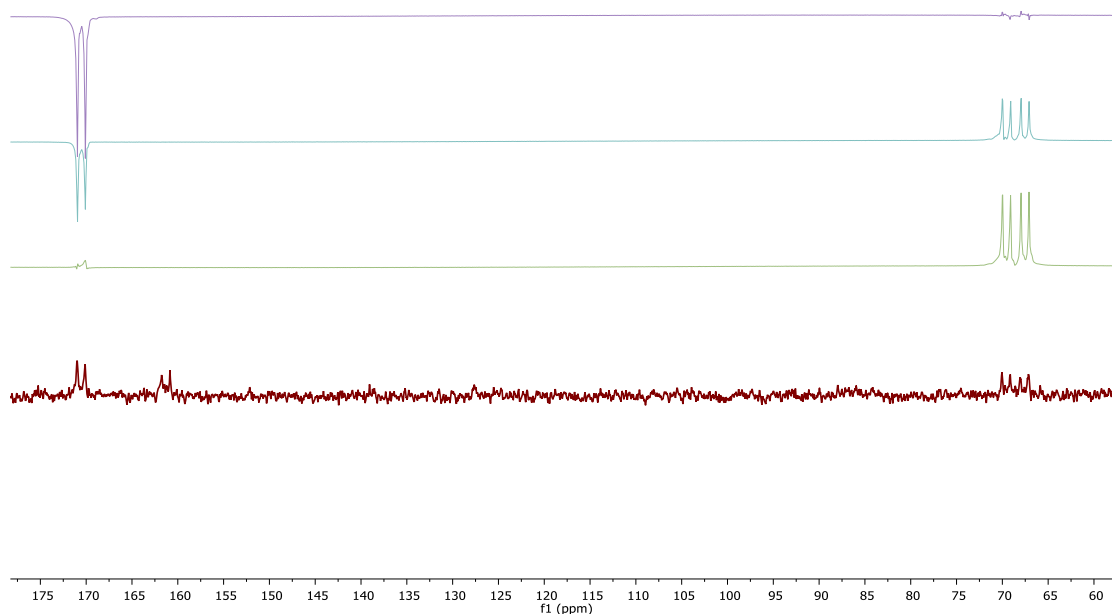


Figure S 34 ^{13}C { ^2H } spectra of 0.1 ml sample after hydrogenation of 10 mM of ethyl- d_5 2-(acetoxy- d_3)acrylate-1,2- ^{13}C - d_2 with 4 mM of catalyst in acetone- d_6 with different pulse degrees at the middle of the last evolution period (magenta, blue and green) and its thermal ^{13}C { ^2H } spectrum with 150 accumulations and 200 times enlarged (red).

6. Hyperpolarization of 1-¹³C-pyruvate for cell measurements

1-¹³C Pyruvate precursor was used for the hyperpolarization experiments and we generated 99% p_{H2} using a He-cooled p_{H2}-generator (generator T=20 K), equipped with a cryocooler system (Sumimoto HC-4A helium compressor, Sumimoto Cold Head CH-204 with p_{H2} reaction chamber by ColdEdge Technologies), temperature controller (Lake Shore Cryotronics, Inc.) and home built valve and tubing system. 10 mM of hydrogenation catalyst ([1,4-bis(diphenylphosphino) butane] (1,5-cyclooctadiene)rhodium(I) tetrafluoroborate) was dissolved in acetone-d₆. For each sample, 1 µl (~1.2mg) of precursor was added to 0.1 ml of the catalyst solution to obtain a concentration of 55 mM. Samples were placed in a 5mm NMR-tube, degassed by bubbling N₂ gas for 1 minute and hydrogenated at 8 bar for 20 s by bubbling using a home-built setup inside a probehead of 7 T Bruker spectrometer (Avance III) heated to 310 K. By using the pulse sequence described above in section SI2, the ¹H polarization is transferred to the 1-¹³C moiety of the pyruvate precursor.

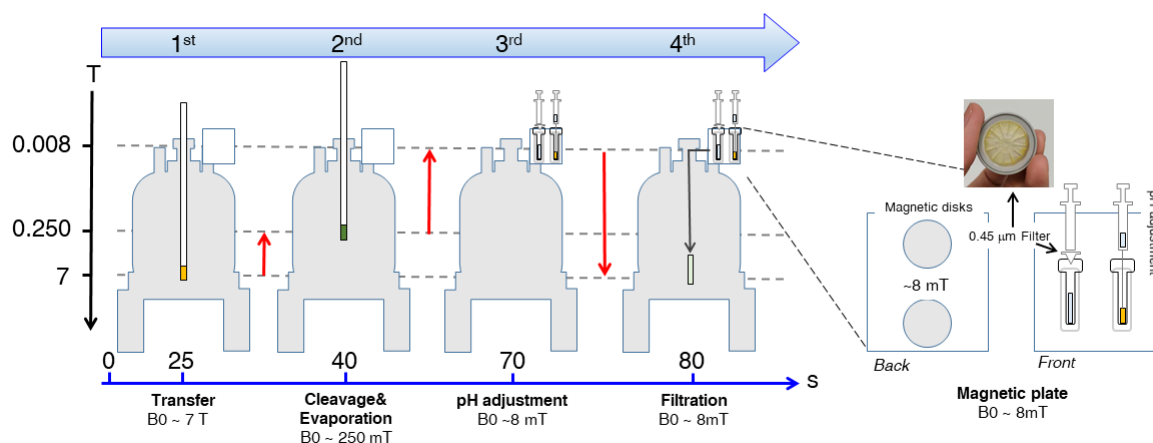


Figure S 35 1st: The initial 100 µL pyruvate precursor d₆-acetone solution is hyperpolarized at 7T by bubbling >99% polarized p_{H2} for 20 seconds. 2nd: The sample is then moved to 250 mT by manual lifting and 100 µL of 100 mM Na₂CO₃ in D₂O is injected to cleave the hyperpolarized precursor and return hyperpolarized pyruvate. Subsequently, a vacuum pump is switched on for a 10-second evaporation of acetone-d₆. 3rd: The sample is lifted out of the magnet and the remaining hyperpolarized ~ 100 µL aqueous solution is mixed with 100 µL of pH adjusting solution to return an isotonic and circumneutral pH final solution ready to be passed through a 0.45 µm filter and injected into a different 5mm NMR tube containing the cell suspension. 4th The sample is finally dropped into the 7T magnet and a pulse scheme of consecutive 22.5 degree pulses every 2 seconds is used to monitor the kinetic pyruvate to lactate conversion (see Section SI2). The top horizontal bar indicates the number of steps in the proposed protocol. The bottom horizontal bar gives the time evolution of each step in seconds. The left vertical bar returns the z-magnetic field in Tesla. The expansion indicates the magnetic plate used to perform steps number 3 and 4. The picture shows the 0.45 µm filter used to remove the Rh catalyst prior to injection into cells.

When polarization transfer was completed (1st step in Figure S35), the sample was lifted manually inside the magnet to a stray field strength of approximately 250 mT. 0.1 ml of 100 mM Na₂CO₃ in D₂O (at room temperature) was added to cleave off the sidearm, followed by 10-12 s of acetone evaporation with a vacuum pump (2nd step in Figure S35). For workup, the sample was removed out of the magnet and held in front of a magnetic plate with a homogeneous field of approximately 8 mT (3rd step in Figure S35). The sample was pulled into a syringe containing 0.1 ml of isotonic buffer to adjust the pH to around 7.4 and administered to the cell sample through a PVDF filter

(filter diameter: 13 mm; pore size: 0.45 μm , fisher scientific) to remove catalyst and other solid particles. The presence of the magnetic field plate was essential in preserving the ^{13}C polarization created in the previous steps by increasing its T_1 . After rapid mixing of the cell suspension with hyperpolarized solution, the sample was moved back into the probehead of the spectrometer to acquire ^{13}C -spectra with a flip angle of 22.5° and a repetition delay of 2 s.

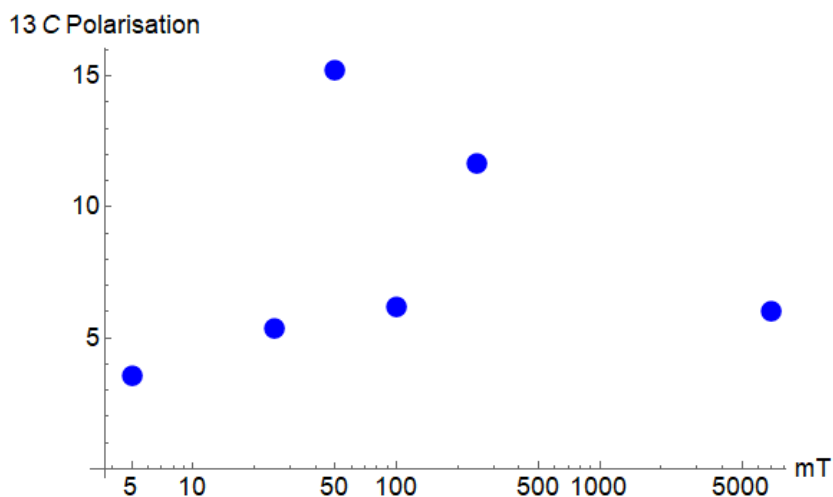


Figure S36 ^{13}C pyruvate polarization (%) after the cleavage and evaporation steps at different magnetic fields. The magnetic field has been measured with a Hall probe (**specs Xyz**). The polarization at intermediate magnetic fields between 50-250 mT is as good or greater than the one at 7T. The cleavage and evaporation step in the cell experiments have been performed at 250 mT. For the experiments here the cinnamyl pyruvate was used and these experiments were tested in D_2O . Note that all values reported in the main text are in H_2O .

Before performing cell experiments we further proved that the obtained hyperpolarized pyruvate is indeed the claimed compound by performing the pyruvate to lactate conversions with the enzyme lactate dehydrogenase (LDH). The result for pyruvate obtained from vinyl pyruvate is shown below in Figure S37.

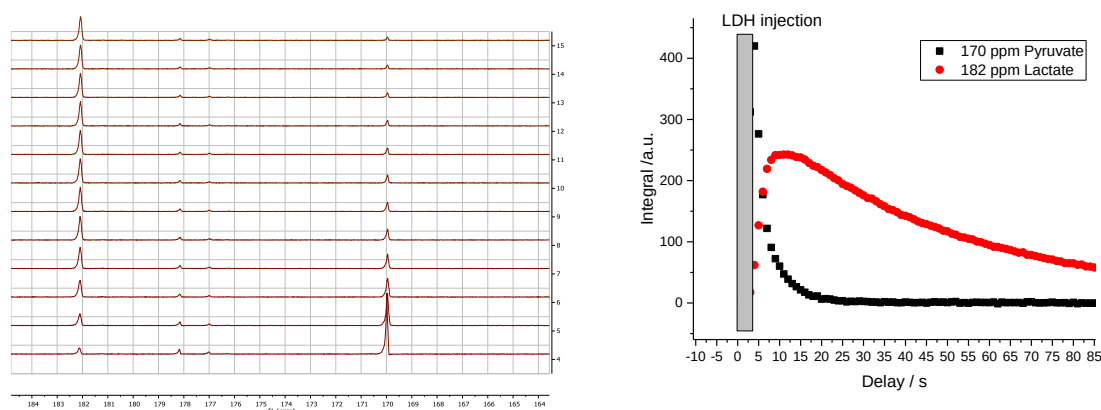


Figure S37 Enzymatic conversion of the obtained pyruvate to lactate. 3 mM of vinyl- d_3 2-oxopropanoate-1- ^{13}C -3,3,3- d_3 and 4 mM of catalyst in acetone- d_6 is hydrogenated at 330 K for 20 s according general procedure. Then 0.1 ml of 10 mM Na_2CO_3 in H_2O is added and the acetone is evaporated. Then 0.3 ml of LDH enzyme (~ 180 units) with 10 mM NADH in phosphate buffer ($\sim 100\text{mM}$) in D_2O is added. On the left, several spectra after LDH injection are showing conversion of pyruvate to lactate. Kinetics are detected with 5° pulse for 85 s and are shown on the right.

6.1. Residual rhodium content

ICP-MS analysis for residual rhodium content of the purified pyruvate in water was performed by MVZ Medizinisches Labor Bremen GmbH. When a 0.45 µm pore size filter was used, 50 +/-19 µM residual rhodium were obtained. This result was further improved by using 0.22 µm pore size filters which yielded 17 +/- 5 µM residual rhodium.

6.2. In-cell structural analysis by NMR

Followed by the PHIP experiments, in-cell NMR experiments were directly performed on the cells containing U-¹⁵N α-Syn at 800MHz (Avance III Basal) equipped with a cryogenically cooled triple resonance ¹H-{¹³C/¹⁵N} TCI probe (Bruker). Due to high sensitivity, the 2D ¹H-¹⁵N SOFAST-HMQC pulse sequence (sfhmqcf3gpqh)^[5] was chosen to measure the U-¹⁵N α-Syn in the cells. The NMR spectra were recorded with an inter-scan delay of 0.2 s and the shaped pulses Pc9_4_90.1000 and Rsnob.1000 for selective ¹H inversion and refocusing, respectively. The excitation width and offset were set to 4 and 8.3 ppm, respectively. To minimize potential cell death and protein leakage, the 2D NMR measurement time was set within 2-3 hours. Cell viability before and after the 2D NMR measurement was probed and shown in Table S1 (bottom). The potential impact of PHIP experiments on the downstream structural analysis of the α-Syn in cells was evaluated by an overlay of the 2D in-cell NMR spectra of the cells sample collected from PHIP experiment and the freshly prepared control cells sample (Figure S38) recorded under the same experimental conditions, indicating no obvious change caused by the PHIP experiment. The chemical shifts in the 2D NMR spectra were assigned according to previous publications^[6,7]. The mean weighted chemical shift differences of in-cell α-Syn was calculated as $\Delta\delta = [(\Delta\delta^1\text{H})^2 + (\Delta\delta^{15}\text{N} \cdot 0.2)^2]^{1/2}$ (in ppm) with reference to the same α-Syn in buffer (Figure 3A in the main text).

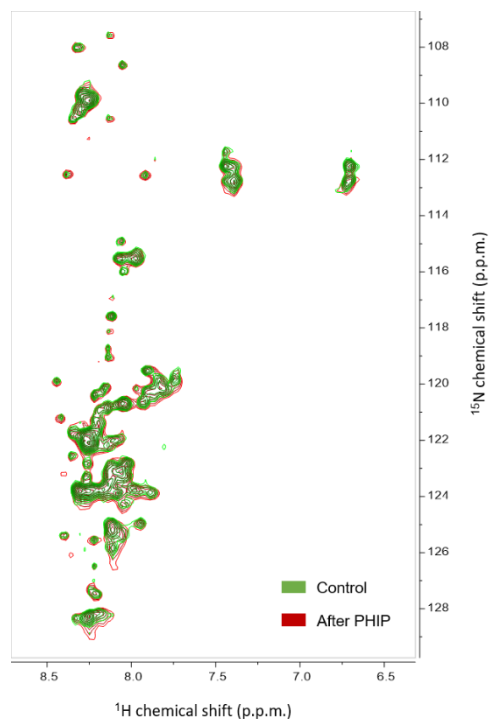


Figure S38 Overlay of ¹H-¹⁵N NMR spectra of the cells sample harvested after the PHIP experiment (red) and the freshly prepared cells sample as a control (green) recorded under the same experimental conditions.

6.3 Kinetic model and Data Analysis

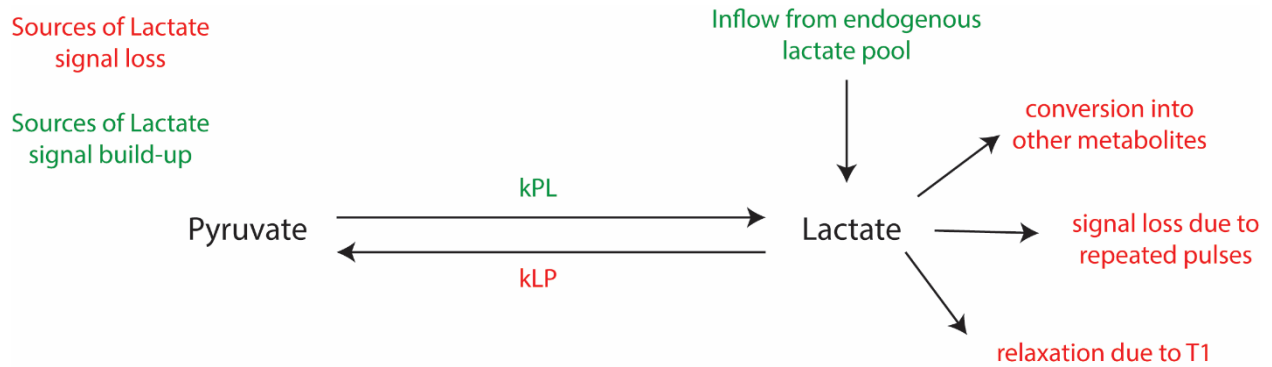


Figure S39 Scheme of two-site exchange model for enzymatic conversion from pyruvate to lactate, including sources of lactate signal build-up (green) and loss (red)

To extract rate constants for enzymatic conversion of pyruvate to lactate, a time-discretized formulation^[8] of the standard two-site exchange model was used, as illustrated in Figure S39. As opposed to the often used one-way model ($k_{LP}=0$), the two-site exchange model was shown to yield accurate results in a complete range of enzymatic activity and endogenous lactate concentration in vitro and in vivo^[9]. We therefore decided to use the formulation of the two-site exchange model derived by Khagai et al, summarizing all sources of lactate signal loss into an effective relaxation rate R_{eff} :

$$\frac{dLac(t)}{dt} = k \cdot Pyr(t) - R_{eff} \cdot Lac(t) \quad (4)$$

Together with the apparent build-up rate k , only two parameters need to be found, which leads in a finite, time-discretized formulation to an overdetermined, linear equation system with the elements:

$$\frac{\Delta Lac_i}{\Delta t} = k \cdot Pyr_i - R_{eff} \cdot Lac_i \quad (5)$$

With ΔLac_i being the difference between two consecutive lactate datapoints, Δt the time increment, and Pyr_i and Lac_i the i th datapoint of the respective metabolite. This leads to the following equation system, which can be solved for R_{eff} and k using the pseudoinverse:

$$\begin{pmatrix} (Lac_2 - Lac_1)/TR \\ \vdots \\ (Lac_i - Lac_{i-1})/TR \end{pmatrix} = \begin{pmatrix} Pyr_1 & -Lac_1 \\ \vdots & \vdots \\ Pyr_{i-1} & -Lac_{i-1} \end{pmatrix} \cdot \begin{pmatrix} R_{eff} \\ k \end{pmatrix} \quad (6)$$

Here, the time increment Δt is replaced with the constant repetition time in between pulses. To visualize the results, we used the same linear equation system to recalculate the lactate data. Only the first experimental lactate data point and the full pyruvate data need to be given, in addition to the obtained rate constants k and R_{eff} . Data for all conditions and replicates with respective k and R values is shown in Table -Table .

Before fitting with the described model using custom Matlab R2019b routines (The Mathworks, Inc.), data was preprocessed in Topspin 4.0.8 (Bruker Corporation) by applying baseline and phase correction as well as integration of pyruvate and lactate

peaks. Additionally, integrals were normalized on the highest pyruvate integral, which corresponds to the first spectrum after addition of hyperpolarized pyruvate to the cells. For better visualization, lactate integrals were multiplied by 10, unless otherwise indicated.

To compare different experiments, we calculated the flux V using the build-up rate k and normalizing on initial pyruvate concentration $[Pyr]$, sample volume (vol) and number of cells ($\#cells$):

$$V = \frac{k \cdot [Pyr] \cdot vol}{\#cells} \quad (7)$$

These were then further analyzed for statistical significance using unpaired one-tailed t-test and the box graph (Figure 2 of the main text) was prepared in Graphpad Prism 9.

6.4 Data Analysis - Results

Table S3 Replicates of HEK 293T α -Syn knock-out cells. Time-course of pyruvate and lactate integrals analyzed as described in 0 with corresponding values for k , R and Mean-Standard-Error.

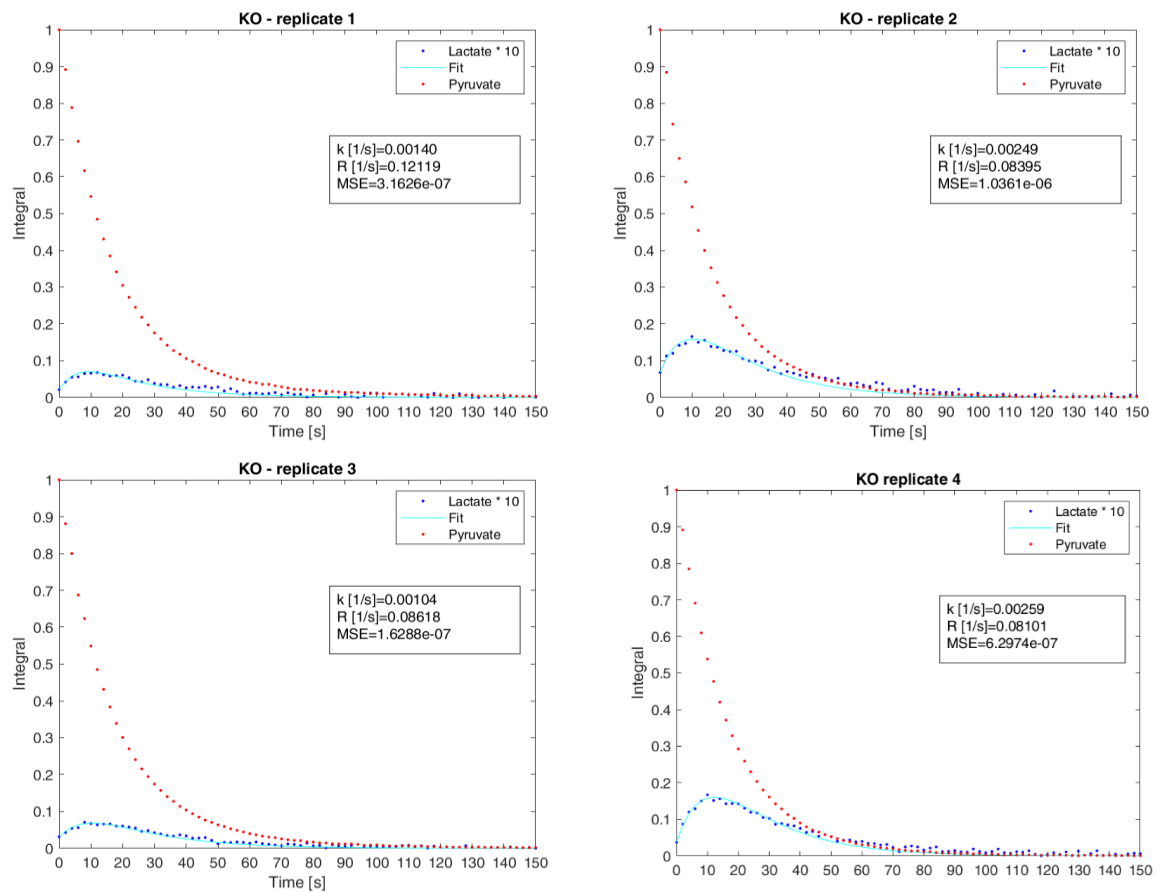


Table S4 Replicates of HEK 293T α -Syn knock-out cells treated with rotenone. Time-course of pyruvate and lactate integrals analyzed as described in 0 with corresponding values for k, R and Mean-Standard-Error.

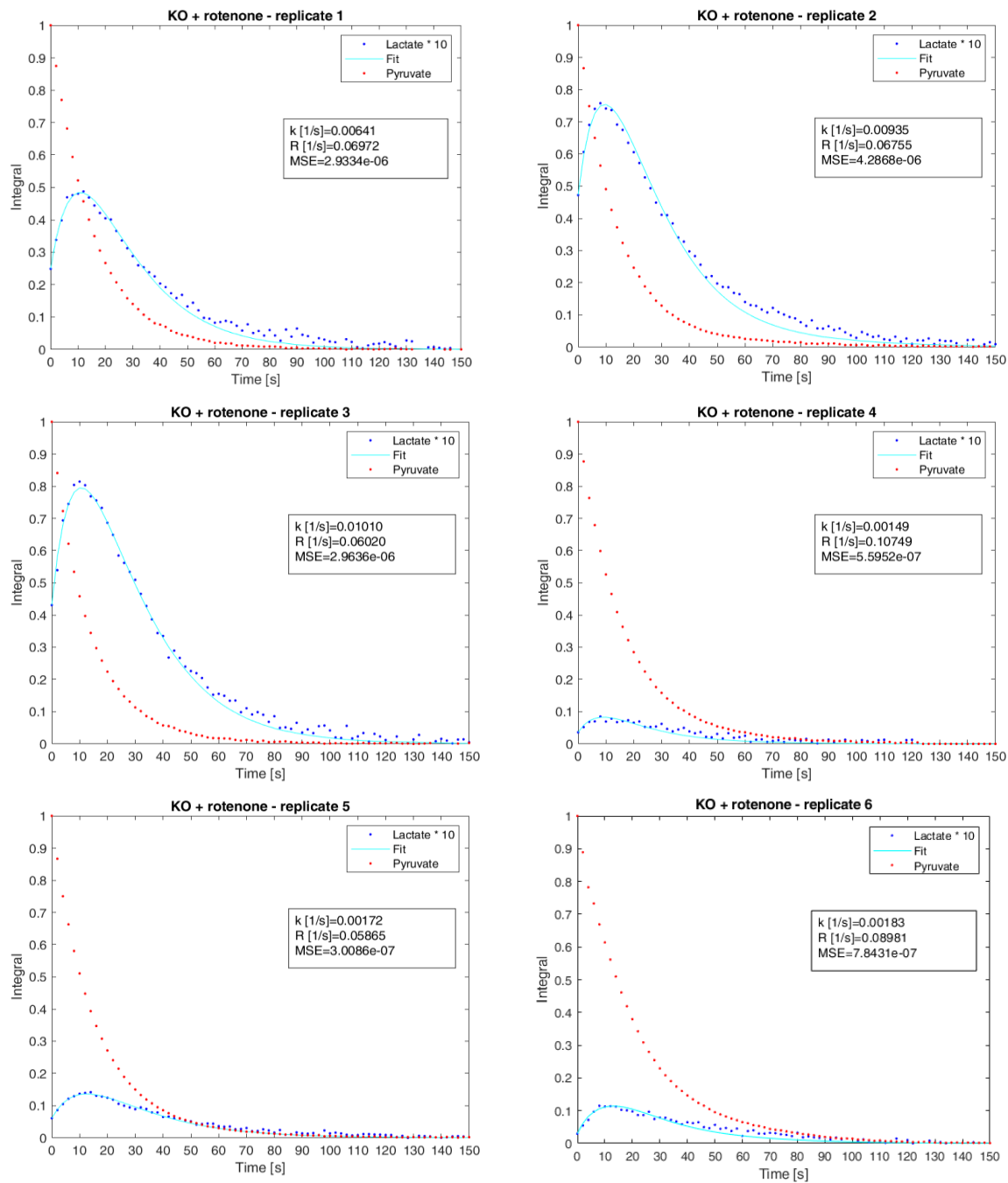


Table S5 Replicates of HEK 293T α -Syn over-expressing. Time-course of pyruvate and lactate integrals analyzed as described in 0 with corresponding values for k, R and Mean-Standard-Error.

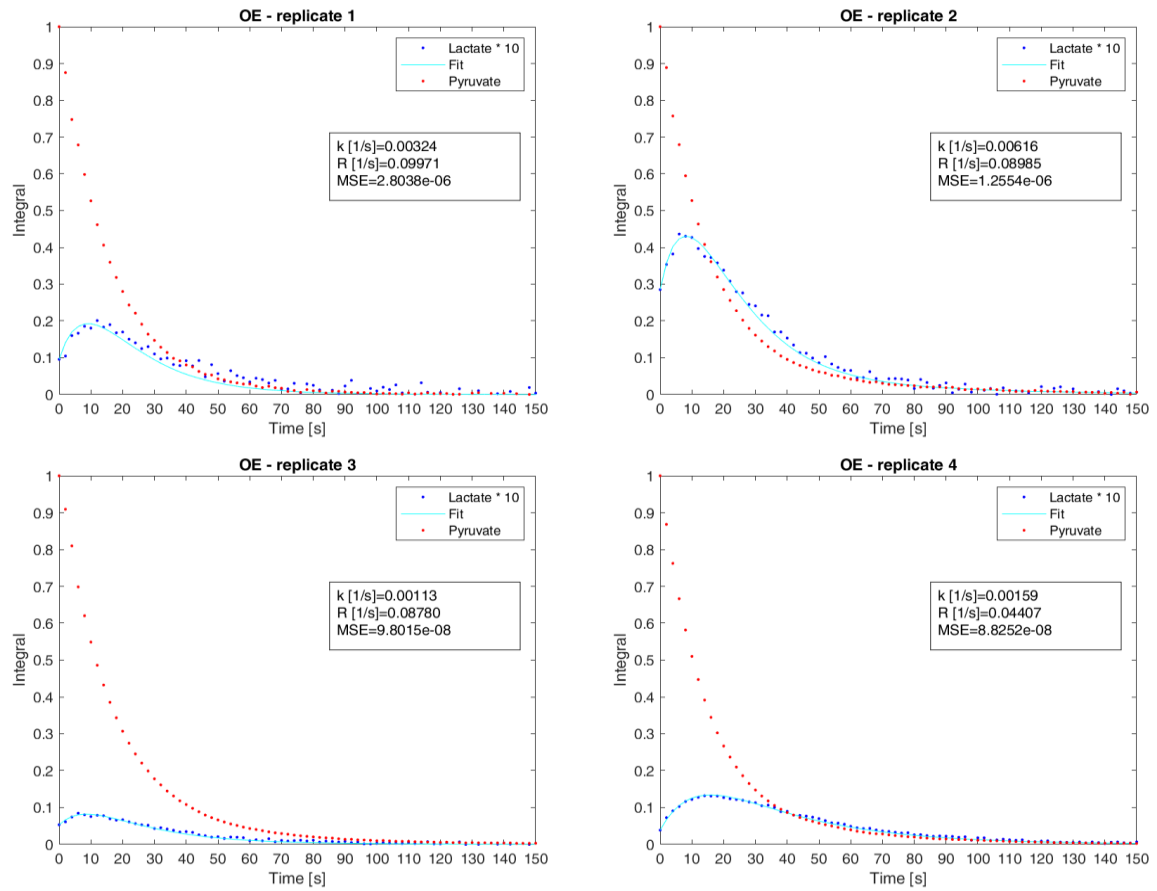


Table S6 Replicates of HEK 293T α -Syn over-expressing cells treated with rotenone. Time-course of pyruvate and lactate integrals analyzed as described in 0 with corresponding values for k, R and Mean-Standard-Error.

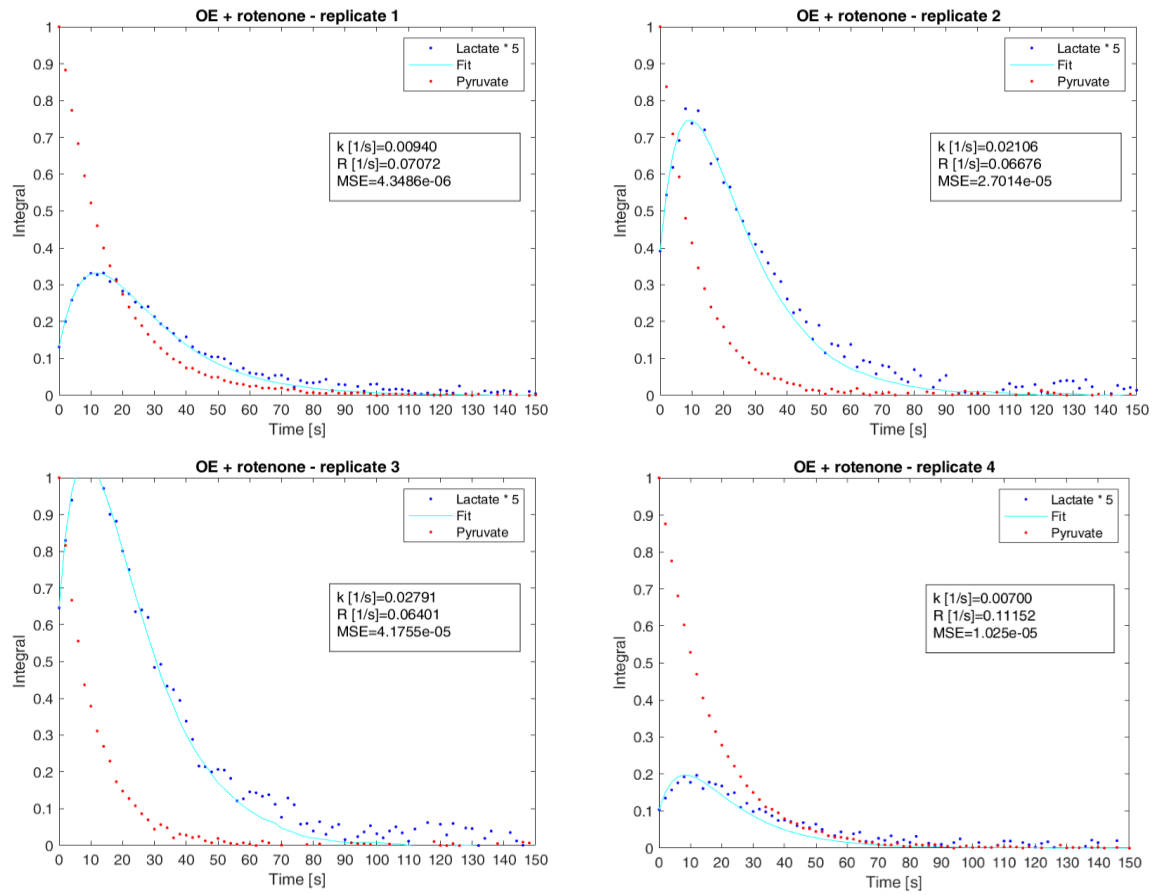
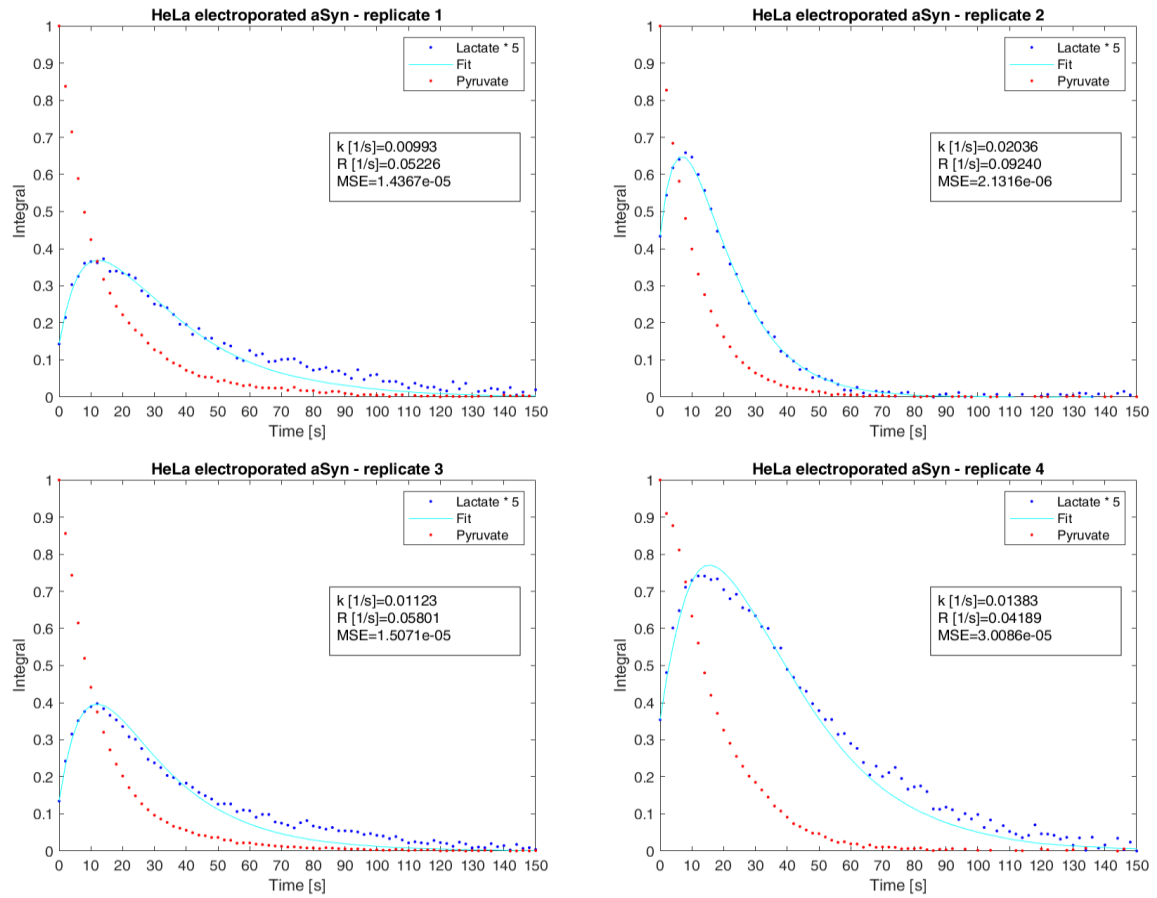


Table S7 Flux V calculated as described in 0 for all conditions of HEK293T cells.

Cell line	Replicate number	Flux V [fmol/(cell*min)]
KO	1	3.09
	2	5.75
	3	2.62
	4	5.27
OE	1	11.06
	2	16.40
	3	4.08
	4	8.25
KO + Rotenone	1	9.06
	2	10.21
	3	13.04
	4	4.81
	5	4.36
	6	7.68
OE+Rotenone	1	25.00
	2	15.94
	3	21.28
	4	16.03

Table S8 Replicates of HeLa cells electroporated with α -Syn. Time-course of pyruvate and lactate integrals analyzed as described in 0 with corresponding values for k , R and Mean-Standard-Error.



7. Receiver gain

In case that a different receiver gain setting had to be used to calculate the polarization in 5.1 it is crucial to provide proof the receiver gain is linear. The proof is delivered in figure S40.

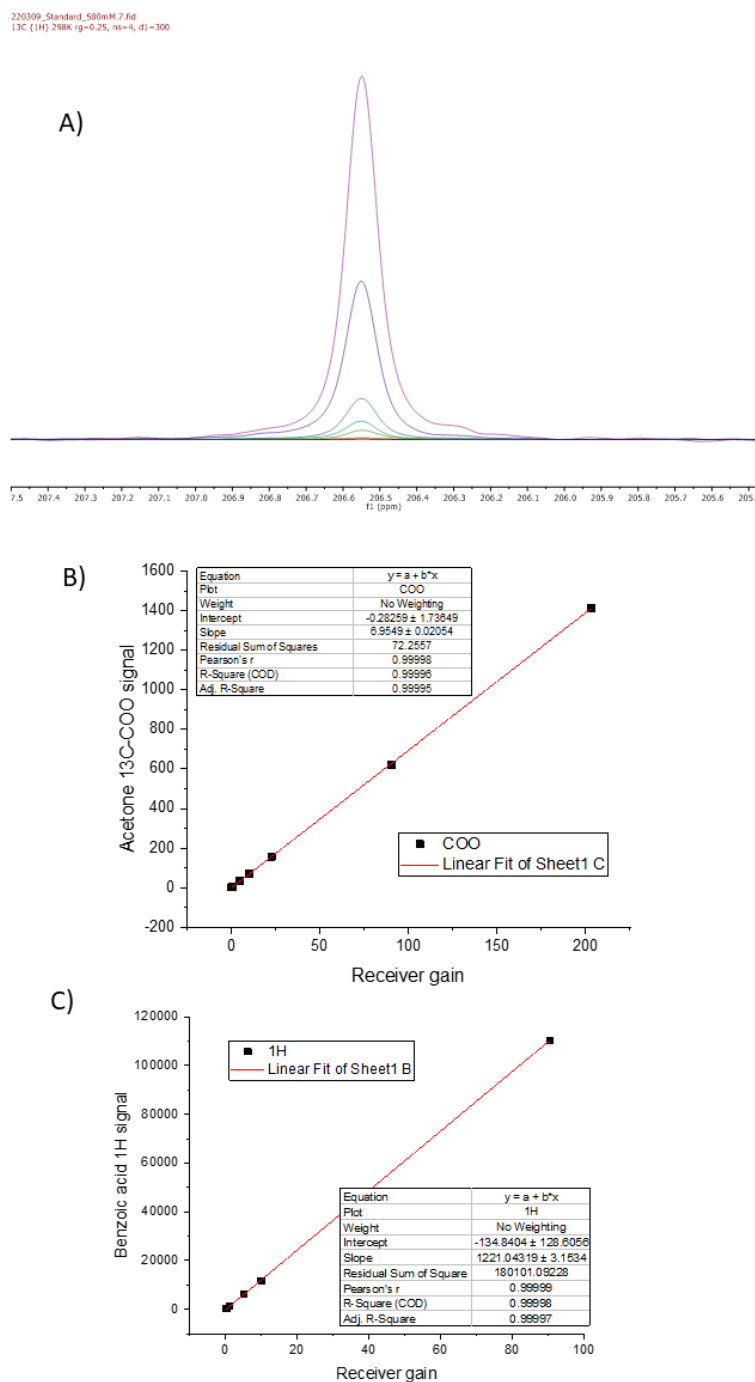
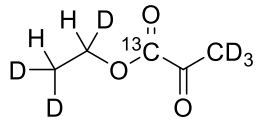
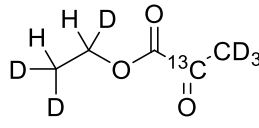
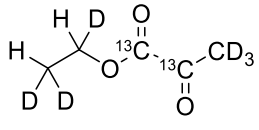
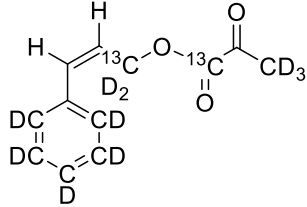
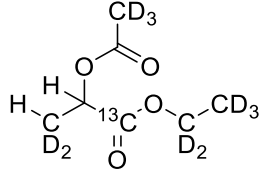
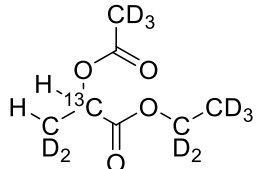
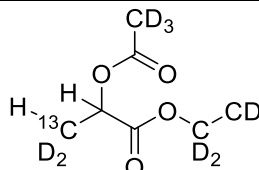
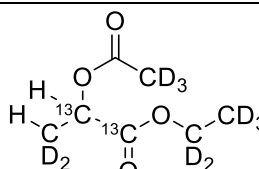


Figure S40. Proof of linearity of the receiver gain for 1H and ^{13}C signals. A sample of 500 mM benzoic acid in acetone where investigated with four scans each. A) spectra of benzoic acid at different receiver gains. B) Carbon signal of benzoic acid as a function of receiver gain and C) proton signal of benzoic acid as a function of the receiver gain. Both channels show a perfect match to a linear function.

8. Relaxation data

Table S9. ^1H and ^{13}C T_1 data for the precursors

Precursor and its enrichment	Hydrogenation product	^1H T_1	^{13}C T_1
vinyl- d_3 -pyruvate-1- ^{13}C - d_3		$T_1(\text{CDH})=140.3\pm0.3\text{ s}$ $T_1(\text{CD}_2\text{H})=100.4\pm0.2\text{ s}$	1- ^{13}C $T_1=67.8\pm0.4\text{ s}$
vinyl- d_3 -pyruvate-2- ^{13}C - d_3		$T_1(\text{CDH})=140.6\pm0.3\text{ s}$ $T_1(\text{CD}_2\text{H})=107.1\pm0.2\text{ s}$	2- ^{13}C $T_1=51.2\pm0.2\text{ s}$
vinyl- d_3 -pyruvate-1,2- ^{13}C - d_3			1- ^{13}C $T_1=66.0\pm0.1\text{ s}$ 2- ^{13}C $T_1=54.0\pm0.1\text{ s}$
3-phenylprop-2-yn-1-yl 2-oxopropanoate, phenylpropargyl pyruvate		$T_1(\text{CH})=19.3\pm0.1\text{ s}$ $T_1(\text{CH})=15.7\pm0.1\text{ s}$	1- ^{13}C -Pyr $T_1=73.3\pm0.3\text{ s}$ 13C-CD2 $T_1=63.2\pm0.2\text{ s}$
Ethyl- d_5 2-(acetoxyl- d_3)acrylate-1- ^{13}C - d_2		In Acetone- d_6 $T_1(\text{CH})=93.8\pm0.6\text{ s}$ $T_1(\text{CD}_2\text{H})=45.9\pm0.3\text{ s}$	In $\text{C}_2\text{H}_5\text{OH}$ 1- ^{13}C $T_1=56.7\pm0.2\text{ s}$
Ethyl- d_5 2-(acetoxyl- d_3)acrylate-2- ^{13}C - d_2		$T_1(\text{CH})=17.7\pm0.1\text{ s}$ $T_1(\text{CD}_2\text{H})=56.2\pm0.2\text{ s}$	2- ^{13}C $T_1=20.1\pm0.2\text{ s}$
Ethyl- d_5 2-(acetoxyl- d_3)acrylate-3- ^{13}C - d_2		$T_1(\text{CH})=85.7\pm2\text{ s}$ $T_1(\text{CD}_2\text{H})=12.6\pm0.2\text{ s}$	3- ^{13}C $T_1=17.0\pm0.1\text{ s}$
Ethyl- d_5 2-(acetoxyl- d_3)acrylate-1,2- ^{13}C - d_2		$T_1(\text{CH})=16.5\pm0.1\text{ s}$ $T_1(\text{CD}_2\text{H})=52.0\pm0.4\text{ s}$	2- ^{13}C $T_1=20.9\pm0.2\text{ s}$ 1- ^{13}C $T_1=80.5\pm0.1\text{ s}$

9. References

- [1] S. Korchak, A. Jagtap, S. Glöggler, *Chem. Sci.* **2021**, *12*, 314.
- [2] W. Hoyer, T. Antony, D. Cherny, G. Heim, T. M. Jovin, V. Subramaniam, *J. Mol. Bio.* **2002**, *322*, 383.
- [3] D. Clift, W. A. McEwan, L. I. Labzin, V. Konieczny, B. Mogessie, L. C. James, M. Schuh, *Cell* **2017**, *171*, 1692-1706 e1618.
- [4] C. Gerdes, N. Waal, T. Offner, E. F. Fornasiero, N. Wender, H. Verbarg, I. Manzini, C. Trenkwalder, B. Mollenhauer, T. Strohaker, M. Zweckstetter, S. Becker, S. O. Rizzoli, F. B. Basmanav, F. Opazo, *Nat. Commun.* **2020**, *11*, 2729.
- [5] P. Schanda, E. Kupce, B. Brutscher, *J. Biomol. NMR* **2005**, *33*, 199-211.
- [6] F.-X. Theillet, A. Binolfi, B. Bekei, A. Martorana, H.M. Rose, M. Stuiiver, S. Verzini, D. Lorenz, M. Van Rossum, D. Goldfarb, P. Selenko, *Nature* **2016**, *530*, 45.
- [7] B. M. Burmann, J. A. Gerez, I. Matecko-Burmann, S. Campioni, P. Kumari, D. Ghosh, A. Mazur, E. E. Aspholm, D. Sulskis, M. Wawrzyniuk, T. Bock, A. Schmidt, S. G. D. Rüdiger, R. Riek, S. Hiller, *Nature* **2020**, *577*, 127.
- [8] O. Khagai, R. F. Schulte, M. A. Janich, M. I. Menzel, E. Farrell, A. M. Otto, J. H. Ardenkjaer-Larsen, S. J. Glaser, A. Haase, M. Schwaiger, F. Wiesinger, *NMR Biomed.* **2014**, *27*, 1256.
- [9] C. J. Daniels, M. A. McLean, R. F. Schulte, F. J. Robb, A. B. Gill, N. McGlashan, M. J. Graves, M. Schwaiger, D. J. Lomas, K. M. Brindle, F. A. Gallagher, *NMR Biomed.* **2016**, *29*, 387.