

Supporting Information for

Parahydrogen Induced Polarization of 1-¹³C- phospholactate-*d*₂ for Biomedical Imaging with >30,000,000-fold NMR Signal Enhancement in Water

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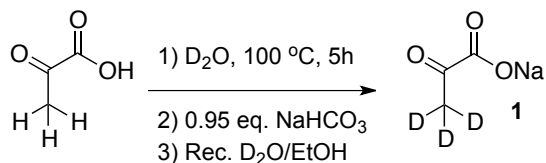
EXPERIMENTAL SECTION

1. General

All solvents were purchased from common vendors and were used as received. All reactions were performed under N₂ in oven-dried or flame-dried glassware. In particular, HPLC grade acetone (270725) and deuterium oxide (99.8%, 756822) were purchased from Sigma-Aldrich-Isotec, Miamisburg, OH. Bis(norbornadiene)rhodium(I) tetrafluoroborate ([Rh(I)(NBD)₂]BF₄, 45-0230) was purchased from Strem Chemicals Inc., Newburyport, MA. Ultra-high purity nitrogen (PGNUHP-234) was purchased from A-L Gases, Nashville, TN. Purity of isotopically labeled compounds was determined by ¹H, ¹³C and HRMS spectroscopy. High-resolution mass spectra were recorded using a Synapt hybrid quadrupole/oa-TOF Mass Spectrometer (Waters Corp., Milford, MA) equipped with a dual chemical ionization/electrospray (ESCI) source. The TOF analyzer was calibrated in negative *V* mode over a mass range of 50-1000 *m/z* using a solution of sodium formate following the manufacturer's recommended procedure. Samples were directly infused into the ion source at a flow rate of 10 μL/min; a gain correction was applied during data acquisition using a solution of ammonium citrate (citrate [M-H]⁻ exact mass 191.0197) in the lock channel. High-resolution NMR spectra were recorded using Bruker 400 MHz NMR spectrometer unless otherwise noted.

2. Synthesis and Characterization

2.1. Sodium pyruvate- d_3



Scheme S1. Synthetic scheme for preparation of sodium pyruvate- d_3 .

Pyruvic acid (2.00 g, 22.7 mmol) was dissolved in D_2O (450 mL) in a three-neck round-bottom flask equipped with reverse condenser, thermocouple (attached to heating mantle with controller) and a thermometer. Temperature controller was set to 100 °C. It was boiled gently with temperature reading ~99 °C for 5 hours. Reaction mixture was cooled to the room temperature (RT), and sodium bicarbonate (1.81 g, 21.9 mmol, 0.95 eq.) was added. The solution was evaporated at the reduced pressure, and the product was recrystallized from 15 mL of D_2O with 180 mL of absolute ethanol furnishing sodium pyruvate- d_3 (**1**, ¹ 1.39 g, 12.3 mmol, 54% yield).

HRMS calcd. for $C_3D_3O_3^-$ (M⁻): 90.0276; found 90.0276 (0 ppm);

Ratio $C_3D_3O_3^-$ to $C_3D_2HO_3^-$ (1 : 0.28);

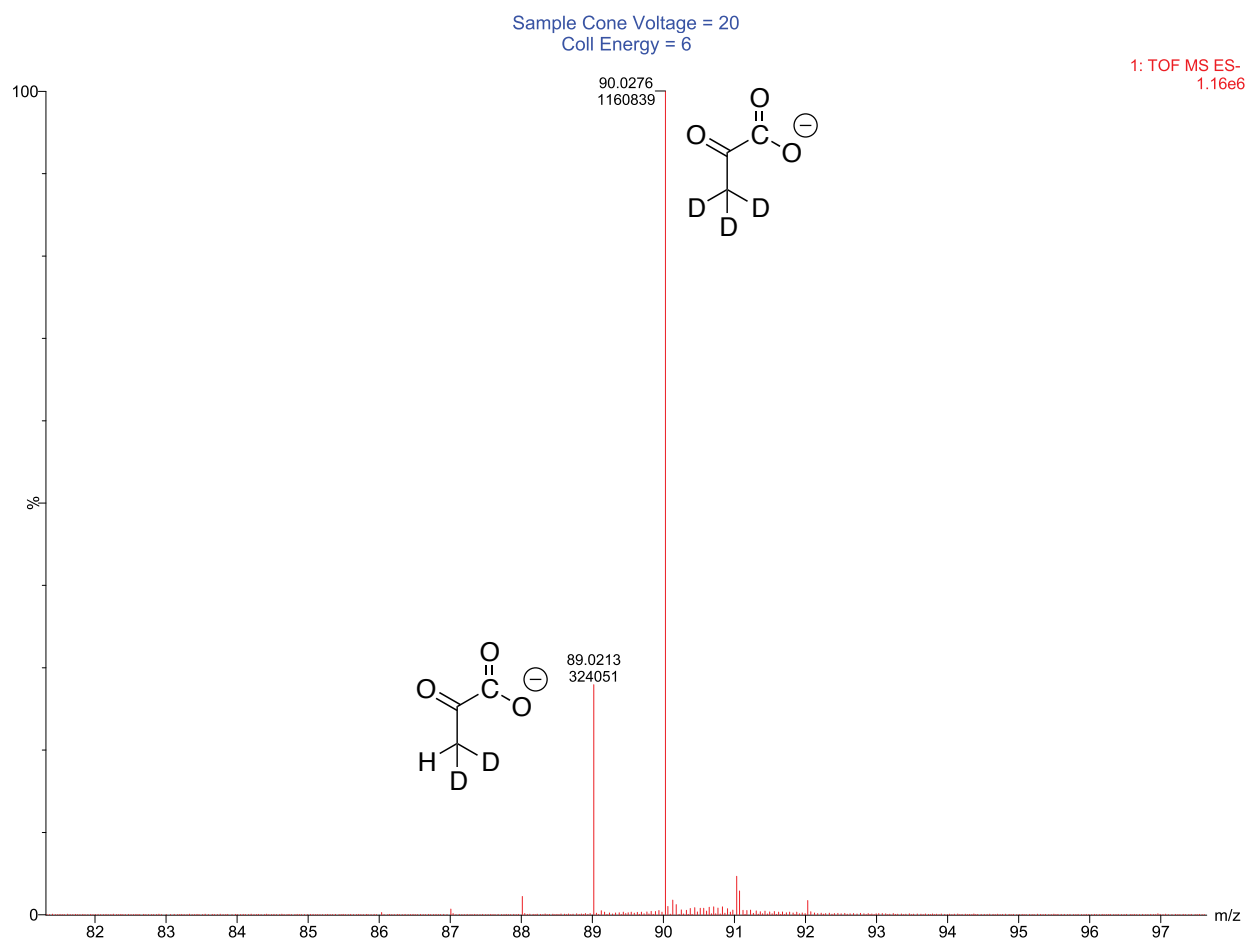
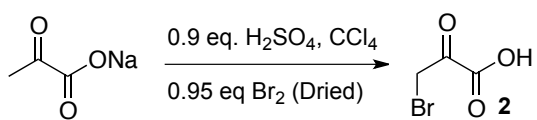


Figure S1. HR-MS negative ion spectrum of sodium pyruvate- d_3 , where the top value over each peak represents the exact mass; the bottom value represents the peak integral.

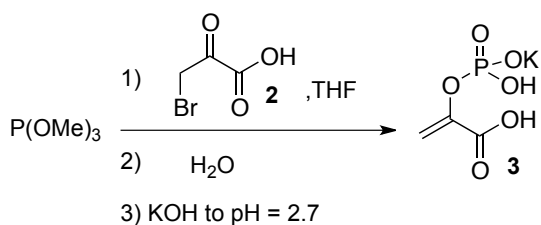
2.2. Phosphoenolpyruvate (PEP)



Scheme S2. Synthetic scheme for preparation of bromopyruvic acid.

Sodium pyruvate (1.5 g, 13.6 mmol) was suspended in the anhydrous carbon tetrachloride, CCl_4 (30 mL). Concentrated sulfuric acid (0.9 eq., 12.2 mmol, 0.67 mL) was added drop-wise. Prior to the administration, bromine was dried with concentrated sulfuric acid. Thus, Br_2 (2 mL) and concentrated sulfuric acid (4 mL) was mixed in a vial equipped with magnetic stirrer. After 10 min of vigorous stirring, stirring was stopped. Layers were separated and the top layer (sulfuric acid) was removed. Second portion of sulfuric acid (4 mL) was added, and the previous two steps were repeated.

Resulting anhydrous bromine (0.95 eq., 12.9 mmol, 0.67 mL) was added drop-wise to the pyruvic acid solution. After 24 hours, the colorless solution was evaporated under reduced pressure, and the residue was resuspended in CCl_4 (20 mL). It was evaporated, and the residue was extracted with anhydrous THF (3 × 10 mL). Combined extracts were concentrated, and crude bromopyruvic acid **2** was used without further purification in the next step.



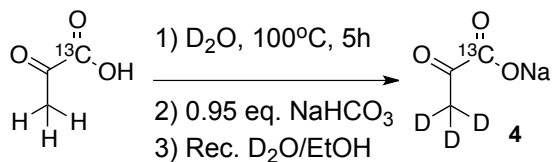
Scheme S3. Synthetic scheme for preparation of potassium phosphoenolpyruvate (PEP).

Trimethyl phosphite, P(OMe)_3 , (1.2 eq., 16.3 mmol, 1.9 mL) was placed in a three-necked round-bottom flask equipped with a reverse condenser. Solution of crude bromopyruvic acid **2** in THF (20 mL total volume) was added using glass syringe during the next 2 hours, under mild warming above 40 °C.

The above solution was left overnight at RT and evaporated under reduced pressure for 4 hours. The viscous residue was dissolved in H₂O (20 mL) and left stirring at RT overnight. After that pH was adjusted to 2.7 with aqueous solution of KOH. It was filtered and evaporated *in vacuo* (for 1 hour). The resulting viscous oil was resuspended in water (~50 mL), filtered, and the filtrate was evaporated under the reduced pressure.

The resulting viscous oil was redissolved in water (8 mL) and ethanol (60 mL), and it was added in several portions. Crystals were collected, washed with ethanol and dried *in vacuo* for 1 hour, furnishing potassium phosphoenolpyruvate^{2,3} **3** (1.45 g, 7.0 mmol, 52% over two steps).

2.3. Potassium 1-¹³C-phosphoenolpyruvate-*d*₂ (1-¹³C-PEP-*d*₂)



Scheme S4. Synthetic scheme for preparation of sodium 1-¹³C-pyruvate-*d*₃.

1-¹³C-pyruvic acid (2.00 g, 22.5 mmol) was dissolved in D₂O (500 mL) in a three-necked round-bottom flask equipped with reverse condenser, thermocouple (attached to a heating mantle with temperature controller) and a thermometer. Temperature controller was set to 100.2 °C. The solution was boiled gently with temperature reading of ~ 100 °C for 5 hours, and the solution was left at the RT for 12 h. Sodium bicarbonate (1.79 g, 21.3 mmol, 0.95 eq.) was added. The solution was evaporated at reduced pressure, and the product was recrystallized from 5 mL of D₂O with 80 mL of absolute ethanol, dried *in vacuo* for 3 hours, furnishing sodium 1-¹³C-pyruvate-*d*₃ **4** (1.93 g, 16.9 mmol, 75%).

HRMS calcd. for $C_2^{13}CD_3O_3^-$ (M⁻): 91.0310; found 91.0307 (3.8 ppm);

Ratio $C_2^{13}CD_3O_3^-$ to $C_2^{13}CD_2HO_3^-$ (1 : 0.25);

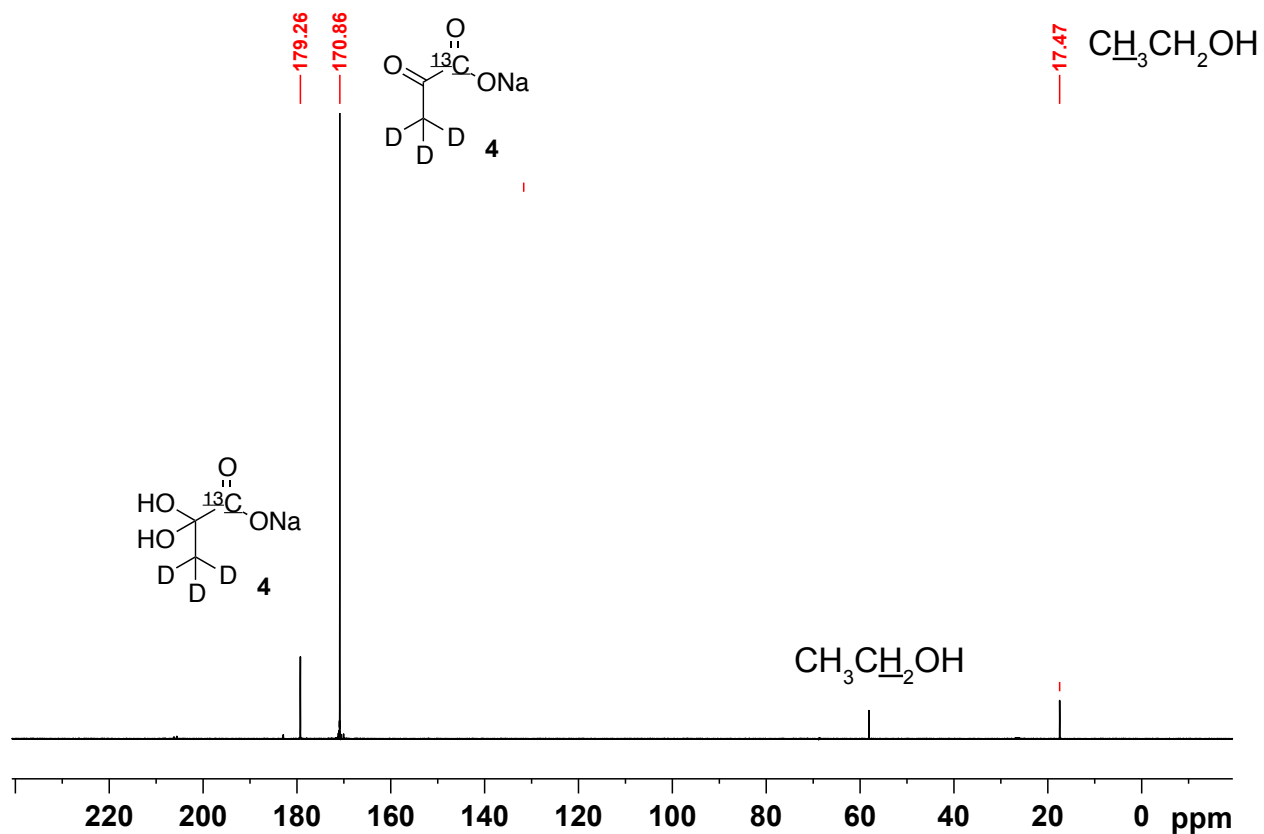


Figure S2. $^{13}C\{^1H\}$ NMR spectrum of sodium 1- ^{13}C -pyruvate- d_3 **4** in D_2O with one drop of ethanol for NMR spectrum calibration. Peak assignments of C1-carbon of two pyruvate forms was reported earlier.⁴

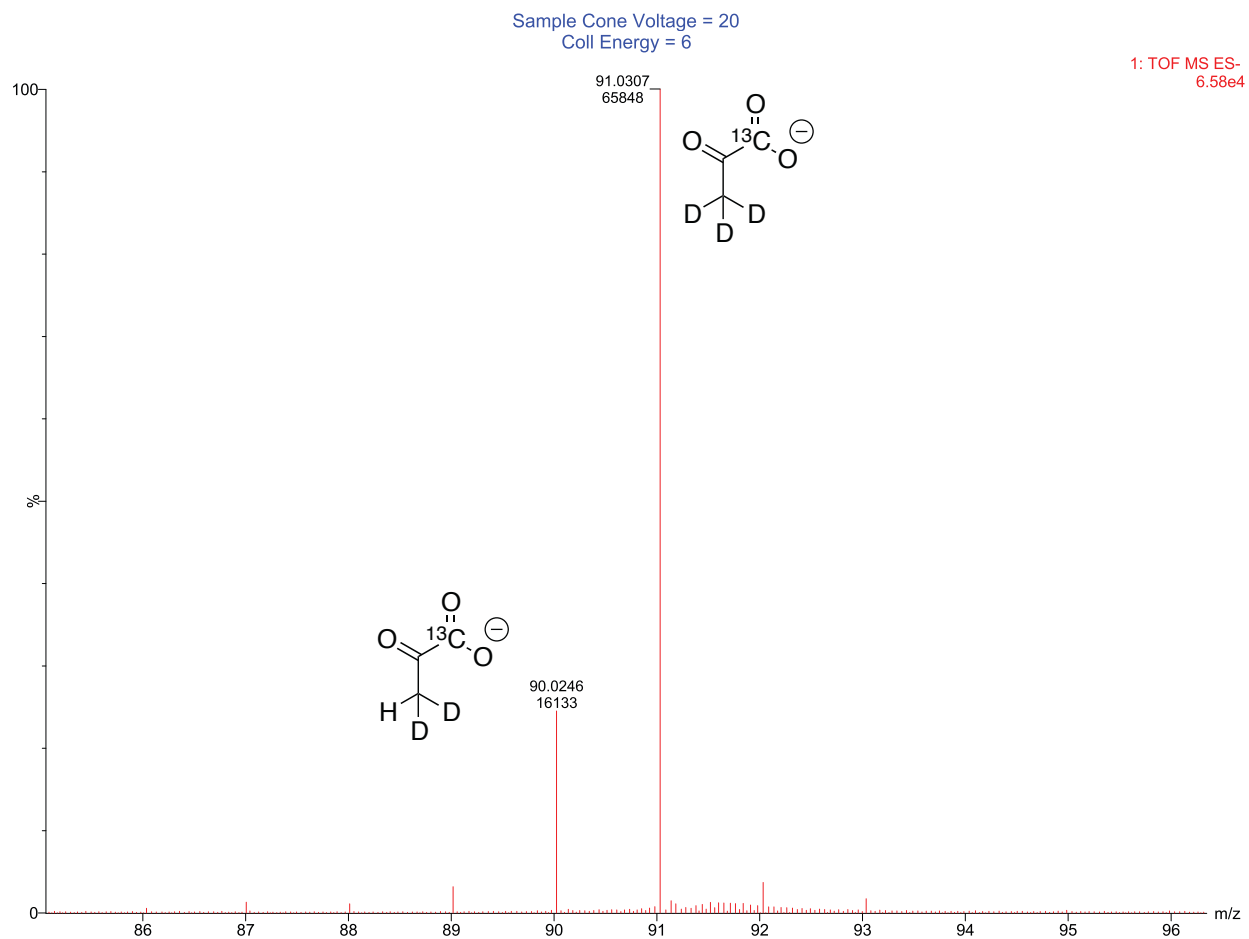
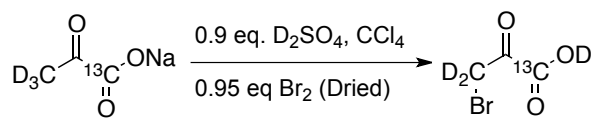


Figure S3. HR-MS negative ion spectrum of sodium 1-¹³C-pyruvate-*d*₃ **4**, where the top value over each peak represents the exact mass, and the bottom value is the peak integral value.

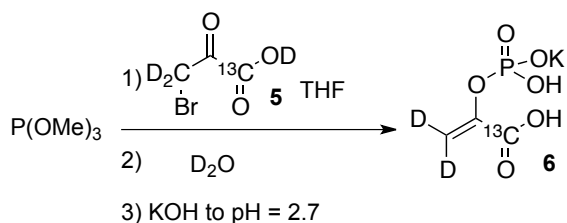


Scheme S5. Synthetic scheme for preparation of 1-¹³C-bromopyruvic-*d*₃ acid.

Sodium 1-¹³C-pyruvate-*d*₃ **4** (1.9 g, 16.6 mmol) was suspended in the anhydrous carbon tetrachloride, CCl₄ (30 mL). Concentrated sulfuric acid-*d*₂ (0.9 eq., 14.9 mmol, 0.81 mL) was added drop-wise.

Prior to the administration, bromine was dried with concentrated sulfuric acid-*d*₂. Thus, Br₂ (2 mL) and concentrated sulfuric acid-*d*₂ (4 mL) was mixed in a vial equipped with a magnetic stirrer. After 10 min of vigorous stirring, stirring was stopped. Layers were separated and the top layer (sulfuric acid-*d*₂) was removed. Second portion of sulfuric acid-*d*₂ (4 mL) was added, and the previous two steps were repeated.

Resulting anhydrous bromine (0.90 eq., 14.9 mmol, 0.77 mL) was added drop-wise to the pyruvic acid solution. After 48 hours, colorless solution was evaporated under the reduced pressure, and the residue was resuspended in CCl₄ (20 mL). It was evaporated, and the residue was extracted with anhydrous THF (3 × 15 mL). Combined extracts were concentrated and crude bromopyruvic acid **5** was used without further purification in the next step.



Scheme S6. Synthetic scheme for a large-scale preparation of mono-potassium 1-¹³C-phosphoenolpyruvate-*d*₂ (1-¹³C-PEP-*d*₂).

Trimethyl phosphite, P(OMe)₃ (1.2 eq., 16.3 mmol, 2.35 mL), was placed in a three-necked round-bottom flask equipped with a reverse condenser. Solution of crude bromopyruvic acid **5** in

THF (20 mL total volume) was added using glass syringe during the next 2 hours, under mild warming above 40 °C. It was left overnight at RT and evaporated under the reduced pressure for 4 hours. The viscous residue was dissolved in D₂O (20 mL) and left stirring at RT overnight. After that pH was adjusted to 2.7 with aqueous solution of KOH (stock solution was made from 1.5 g of KOH). It was filtered and evaporated *in vacuo* (for 1 hour). The resulting viscous oil was resuspended in water (~40 mL), filtered, and the filtrate was evaporated under the reduced pressure.

Resulting viscous oil was redissolved in water (8 mL), and ethanol (100 mL) was added in several portions. After 40 min crystals were collected, washed with ethanol and dried *in vacuo* for 1 hour, furnishing potassium 1-¹³C-phosphoenolpyruvate-*d*₂ **6** (1-¹³C-PEP-*d*₂, 1.34 g, 6.4 mmol, 43% *over two steps*). The purity of the product was determined.

HRMS calcd. for C₂¹³CH₂D₂O₆P⁻ (M⁻): 169.9910; found 169.9907 (0.59 ppm);

Ratio C₂¹³CH₂D₂O₆P⁻ to C₂¹³CH₃DO₆P⁻ to C₂¹³CH₄O₆P⁻ (1 : 0.10 : 0.05);

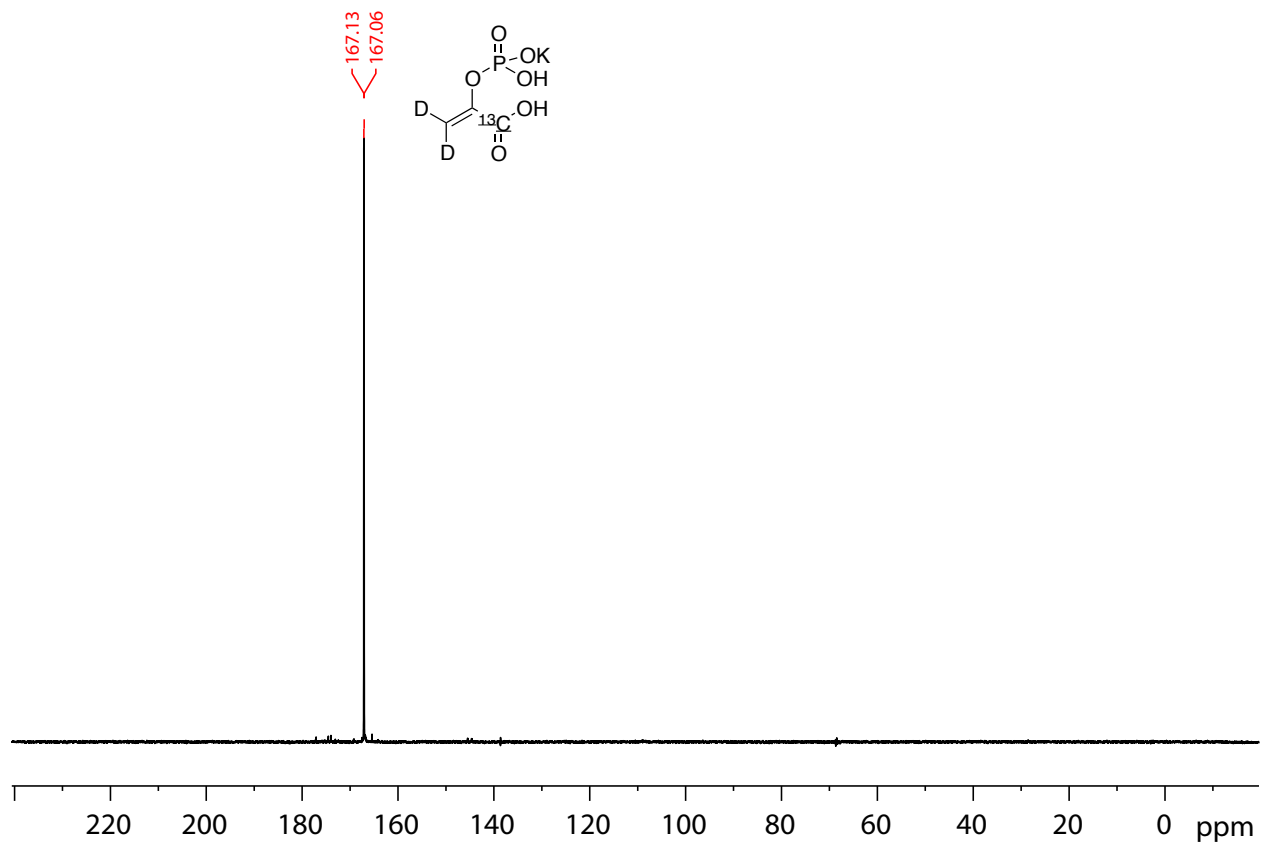


Figure S4. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of potassium 1- ^{13}C -PEP- d_2 **6** in D_2O .

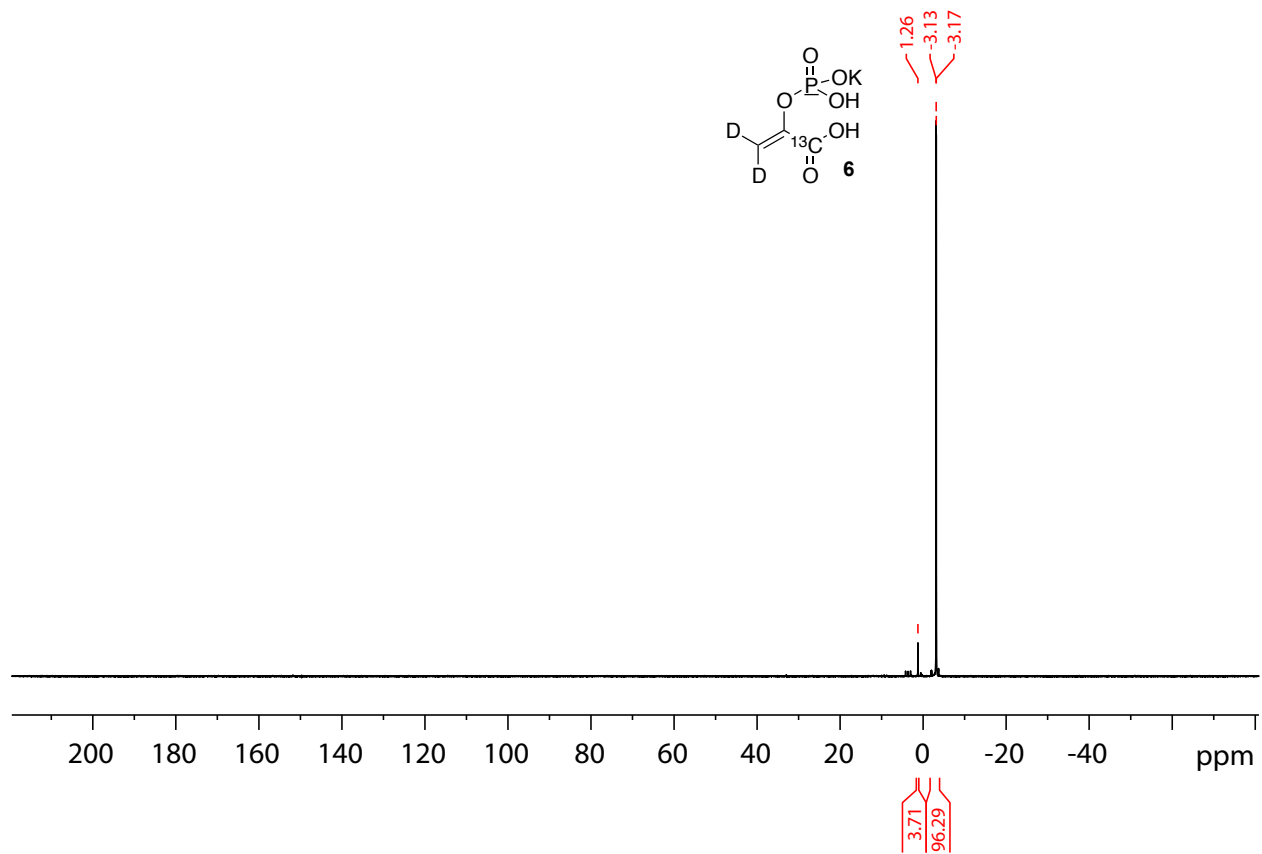


Figure S5. ³¹P{¹H} NMR spectrum of potassium 1-¹³C-PEP-*d*₂ **6** in D₂O.

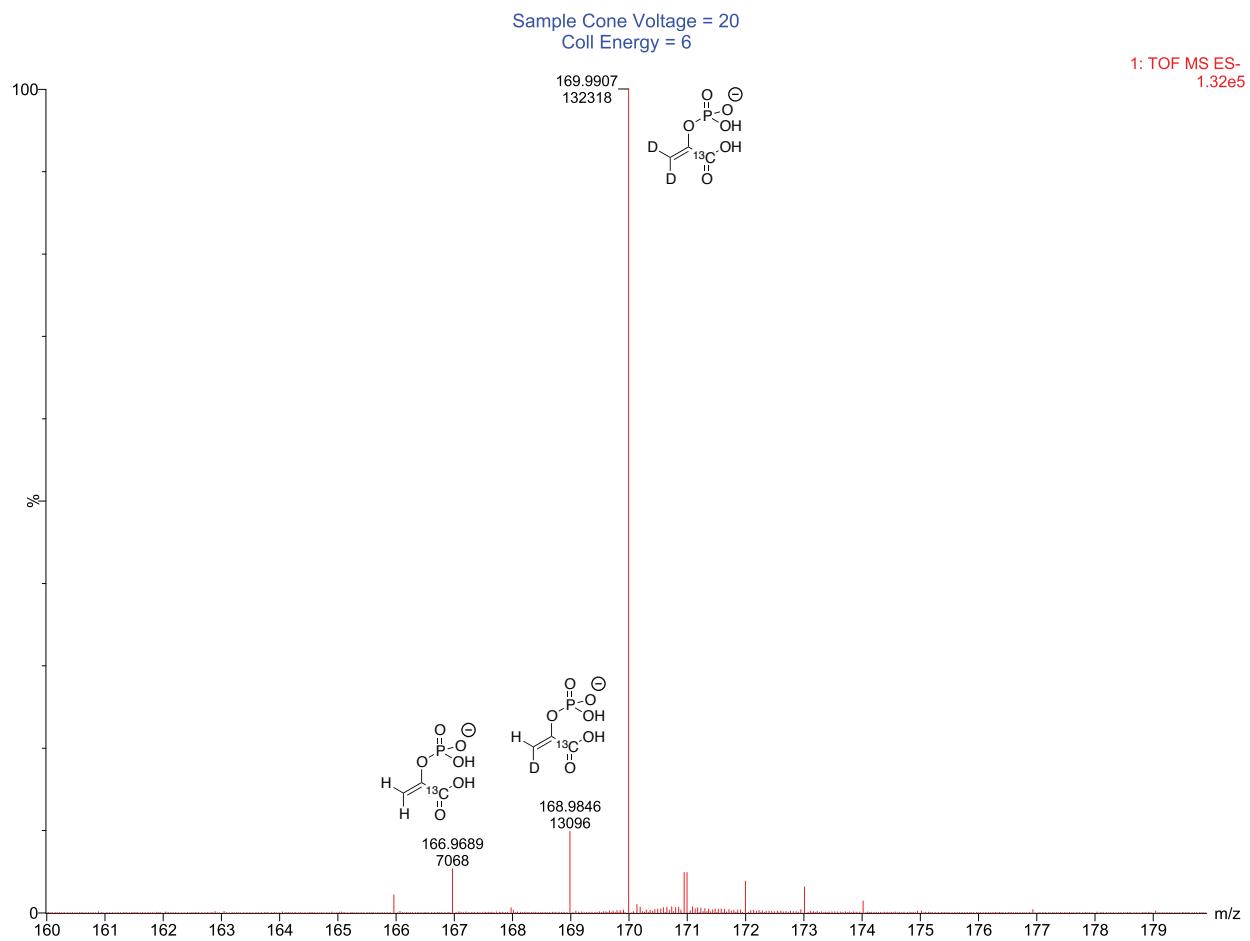


Figure S6. HR-MS negative ion spectrum 1-¹³C-PEP-*d*₂ **6**, where the top value over each peak represents the exact mass, and the bottom value is the peak integral value.

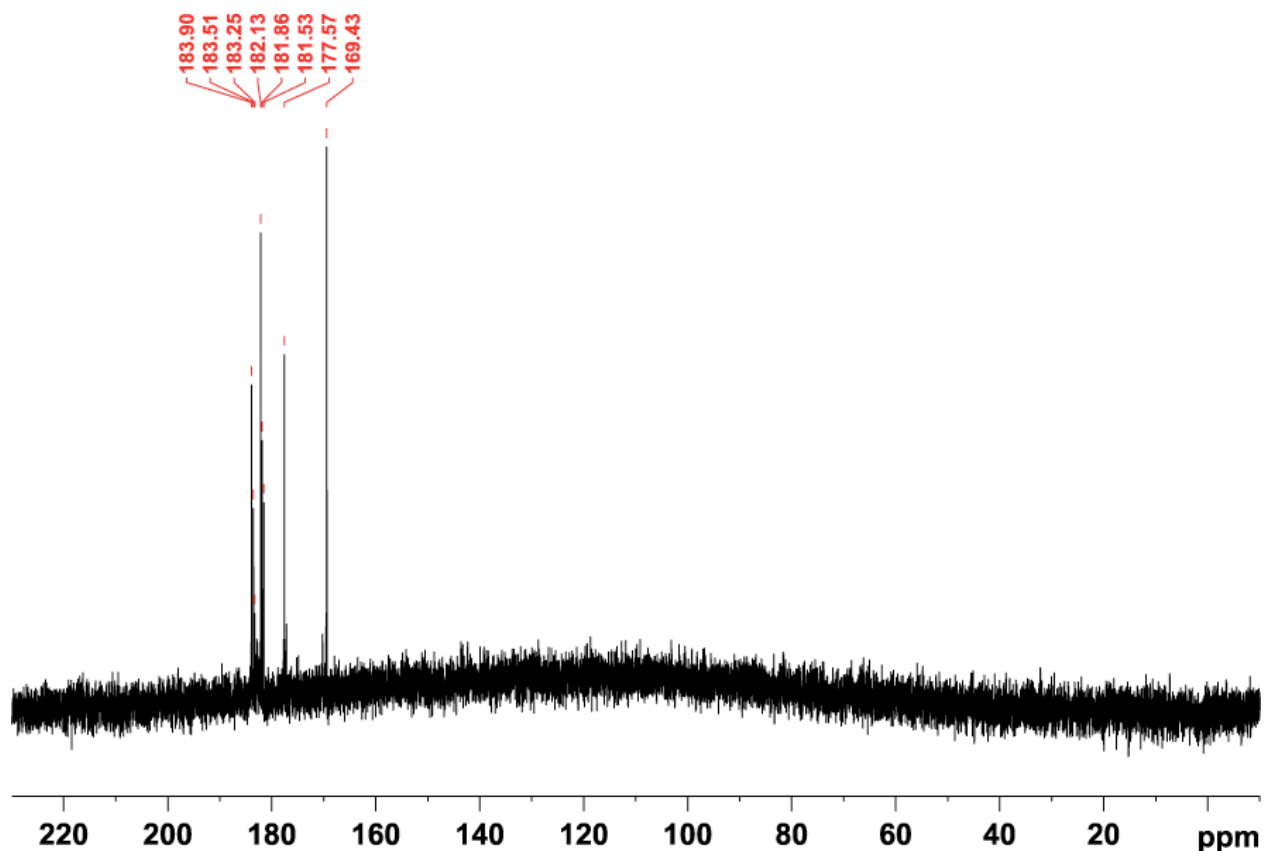
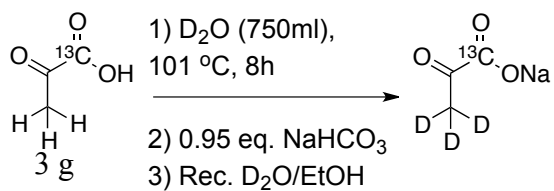


Figure S7. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum recorded using Bruker 600 MHz NMR spectrometer equipped with cryogenic RF probe. The NMR spectrum demonstrates partial polymerization (seen as additional multiple resonances of di-, tri-, and other oligomers) of sodium 1- ^{13}C -pyruvate- d_3 **4** due to exposure to NaOH.

2.4. Large-scale synthesis of potassium 1-¹³C-phosphoenolpyruvate-*d*₂ (1-¹³C-PEP-*d*₂)



Scheme S7. Synthetic scheme for optimized preparation of sodium 1-¹³C-pyruvate-*d*₃ on a 3-g production scale.

1-¹³C-Pyruvic acid (3.07 g, 34.5 mmol) was dissolved in D₂O (750 mL) in a three-necked round-bottom flask equipped with reverse condenser, thermocouple (attached to a heating mantle with controller) and a thermometer. Temperature controller was set to 101.0 °C. The solution was boiled gently with temperature reading ~100 °C for 8 hours and left at RT for 12 h. Sodium bicarbonate (2.75 g, 32.8 mmol, 0.95 eq.) was added. The solution was evaporated at reduced pressure, and the product was recrystallized from 8 mL of D₂O with 120 mL of absolute ethanol, dried under *in vacuo* for 3 hours, furnishing sodium 1-¹³C-pyruvate-*d*₃ (3.18 g, 27.9 mmol, 81%).

HRMS calcd. for C₂¹³CD₃O₃⁻ (M⁻): 91.0310; found 91.0305 (5.0 ppm);

Ratio C₂¹³CD₃O₃⁻ to C₂¹³CD₂HO₃⁻ (1 : 0.03), 97.5% isotopic purity;

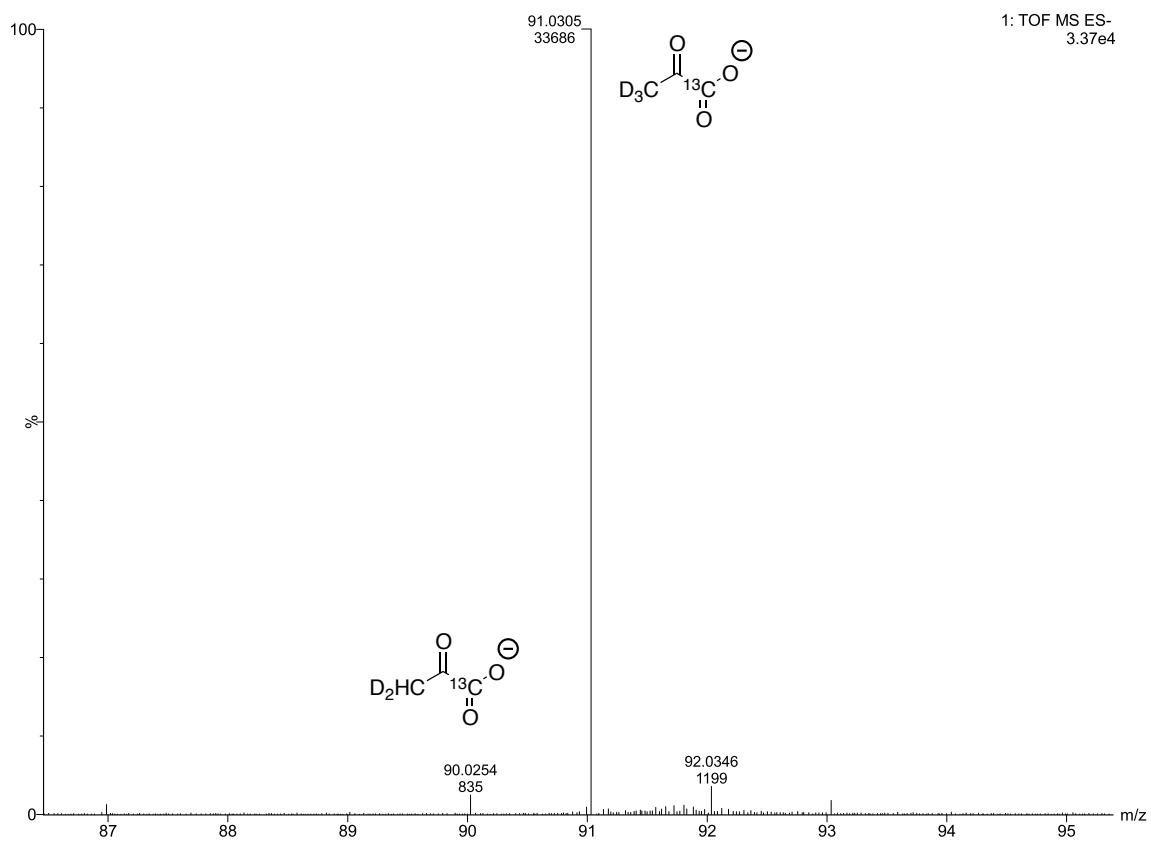
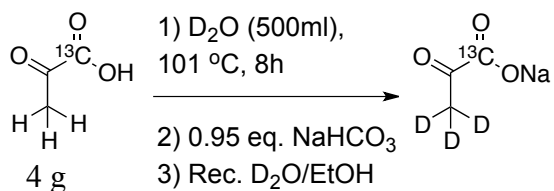


Figure S8. HR-MS negative ion spectrum of sodium 1-¹³C-pyruvate-*d*₃ **4**, where the top value over each peak represents the exact mass, and the bottom value is the peak integral value.



Scheme S8. Synthetic scheme for an optimized preparation of sodium 1-¹³C-pyruvate-*d*₃ on a 4-g production scale.

1-¹³C-Pyruvic acid (3.97 g, 44.6 mmol) was dissolved in D₂O (750 mL) in a three-necked round-bottom flask equipped with reverse condenser, thermocouple (attached to a heating mantle with temperature controller) and a thermometer. Temperature controller was set to 101.0 °C. The solution was boiled gently with temperature reading of ~100 °C for 8 hours and left at the RT for 12 h. Sodium bicarbonate (MW = 84, 3.56 g, 42.4 mmol, 0.95 eq.) was added. The solution was evaporated at reduced pressure, and the product was recrystallized from 10 mL of D₂O with 160 mL of absolute ethanol, dried under *in vacuo* for 3 hours, furnishing sodium 1-¹³C-pyruvate-*d*₃ (4.33 g, 38.0 mmol, 85%).

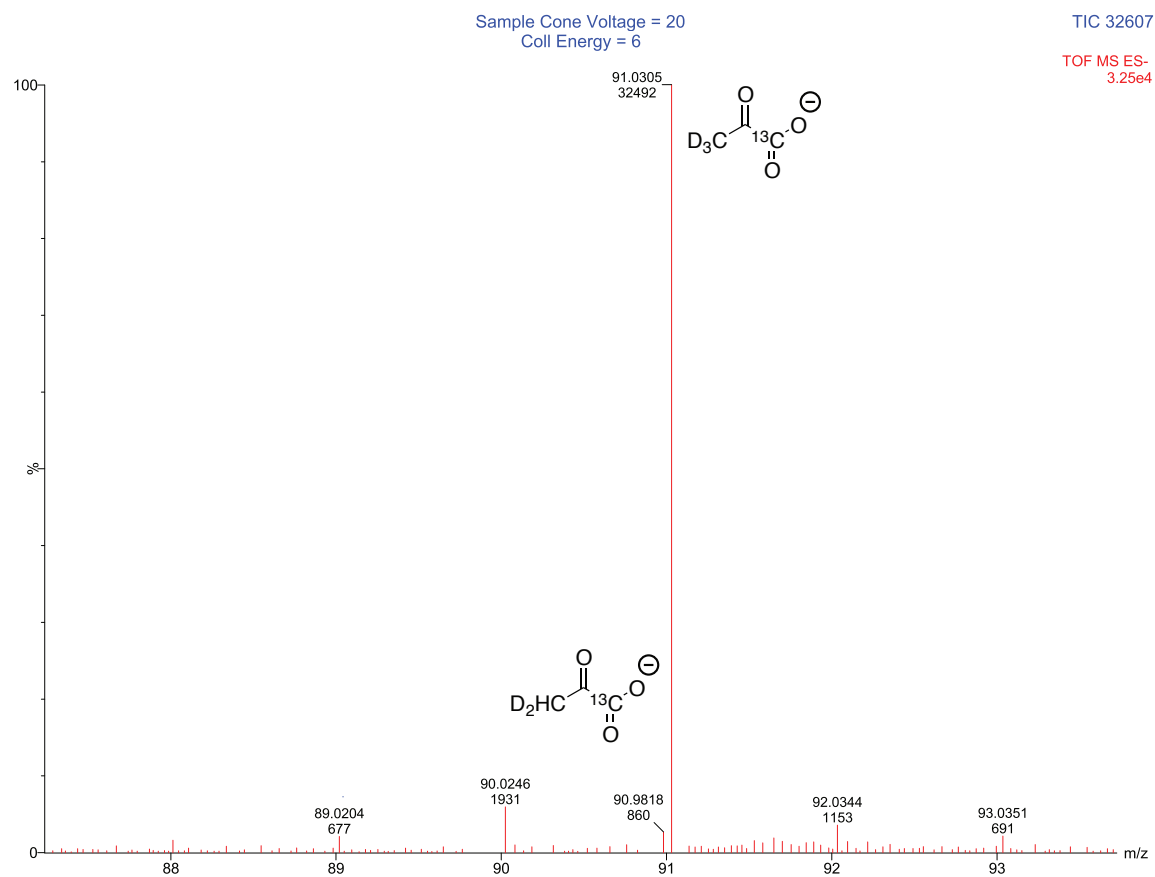
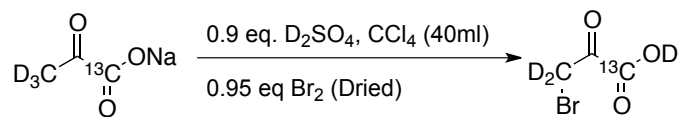


Figure S9. HR-MS negative ion spectrum of sodium 1-¹³C-pyruvate-*d*₃ **4**, where the top value over each peak represents the exact mass, and the bottom value is the peak integral value.

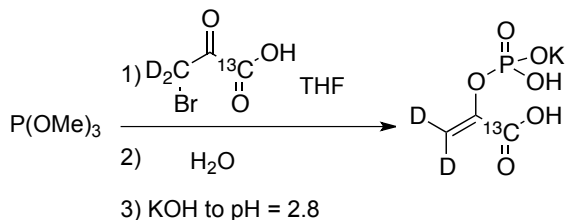


Scheme S9. Synthetic scheme for a large-scale preparation of 1-¹³C-bromopyruvic-*d*₃ acid.

Sodium 1-¹³C-pyruvate-*d*₃ (7.00 g, 61.4 mmol) was suspended in anhydrous carbon tetrachloride, CCl₄ (100 mL). Concentrated sulfuric acid-*d*₂ (0.9 eq., 55.3 mmol, 3.01 mL) was added drop-wise.

Prior to the administration, bromine was dried with concentrated sulfuric acid. Thus, Br₂ (4 mL) and concentrated sulfuric acid acid-*d*₂ (6 mL) were mixed in a vial equipped with magnetic stirrer. After 10 min of vigorous stirring, stirring was stopped. Layers were separated, and the top layer (sulfuric acid) was removed using a plastic syringe. Second portion of sulfuric acid acid-*d*₂ (6 mL) was added, and the previous two steps were repeated.

Resulting anhydrous bromine (0.95 eq., 58.3 mmol, 3.00 mL) was dissolved in CCl₄ (7 mL) and added drop-wise to the pyruvic acid solution. After 4 days, colorless solution was evaporated under reduced pressure, and the residue was resuspended in CCl₄ (40 mL). It was evaporated, and the residue was extracted with anhydrous THF (3 × 50 mL). Combined extracts were concentrated, and crude bromopyruvic acid was used without further purification in the next step.



Scheme S10. Synthetic scheme for a large-scale preparation of mono-potassium 1-¹³C-phosphoenolpyruvate-*d*₂ (1-¹³C-PEP-*d*₂).

Trimethyl phosphite, P(OMe)₃, (1.2 eq., 73.7 mmol, 8.70 mL) was placed in a three-necked round-bottom flask equipped with a reverse condenser. Solution of crude bromopyruvic acid in THF (80 mL total volume) was added with glass syringe during the next 1 hours, under mild solution warming to ~50 °C. It was left overnight at RT, and it was evaporated under reduced pressure for 4 h. The viscous residue was dissolved in D₂O (80 mL), and it was left stirring at RT overnight. After that pH was adjusted to 2.7 with aqueous solution of KOH (stock solution was made from 6 g of KOH), it was filtered and evaporated *in vacuo* (for 1 hour). The resulting viscous oil was resuspended in water (~160 mL), filtered, and the filtrate was evaporated under reduced pressure.

Resulting viscous oil was redissolved in water (22 mL), and ethanol (400 mL) was added in several portions. After, 40 min crystals were collected, washed with ethanol and dried *in vacuo* for 1 h furnished 6.82 g as a mixture of 1-¹³C-PEP-*d*₂ : 1-¹³C-PLAC-*d*₂ (1-¹³C-phospholactate-*d*₂) in 85% to 15% ratio. *Note: The formation of 1-¹³C-PLAC-*d*₂ is presumably due to the reduction of 1-¹³C-PEP-*d*₂ methylated precursor by P(OMe)₃. The reaction mechanism of this transformation is a subject further investigation.*

HRMS calcd. for $C_2^{13}CH_2D_2O_6P^-$ (M⁻): 169.9910; found 169.9906 (-2.4 ppm);

Ratio $C_2^{13}CH_2D_2O_6P^-$ to $C_2^{13}CH_3DO_6P^-$ (1 : 0.05);

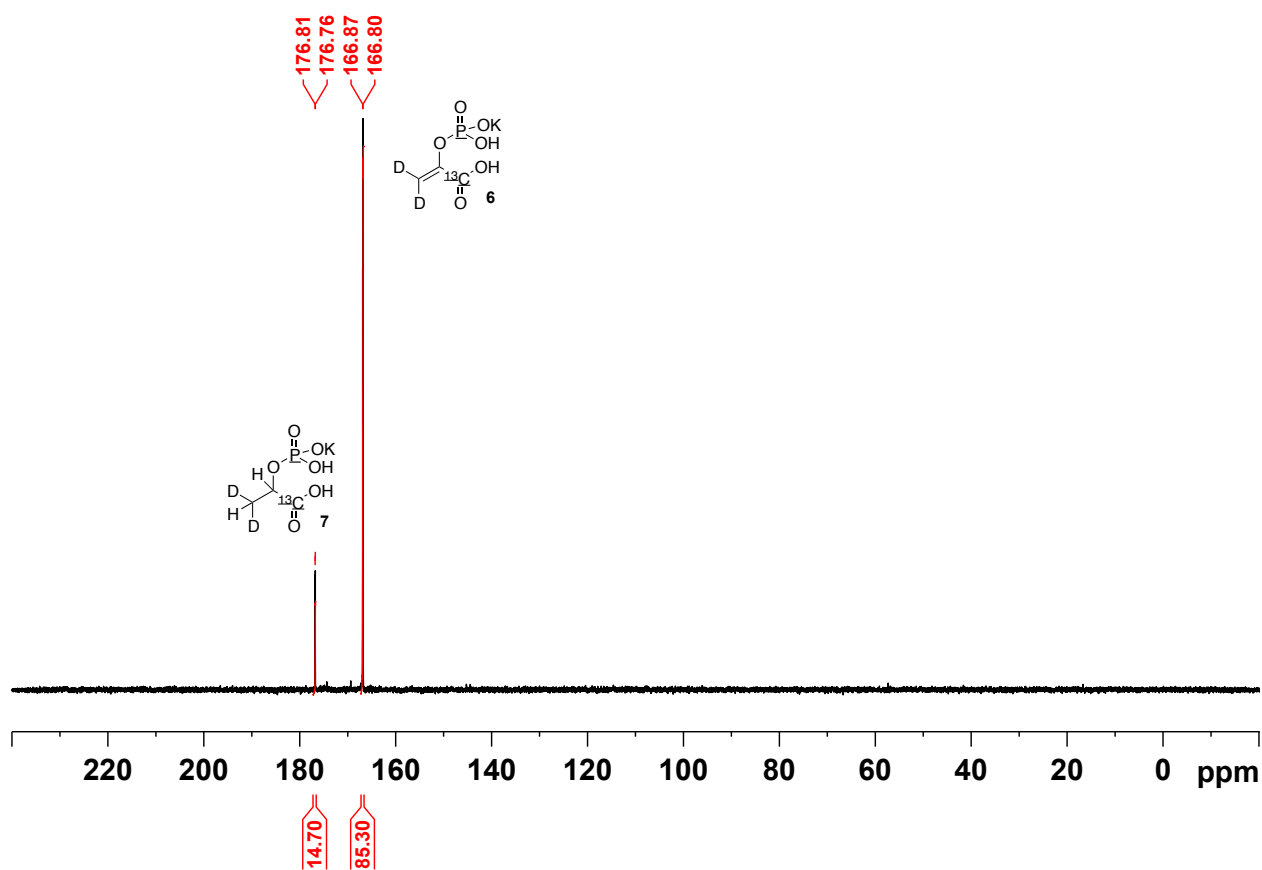


Figure S10. $^{13}C\{^1H\}$ NMR spectrum of potassium 1- ^{13}C -PEP- d_2 **6** in D_2O .

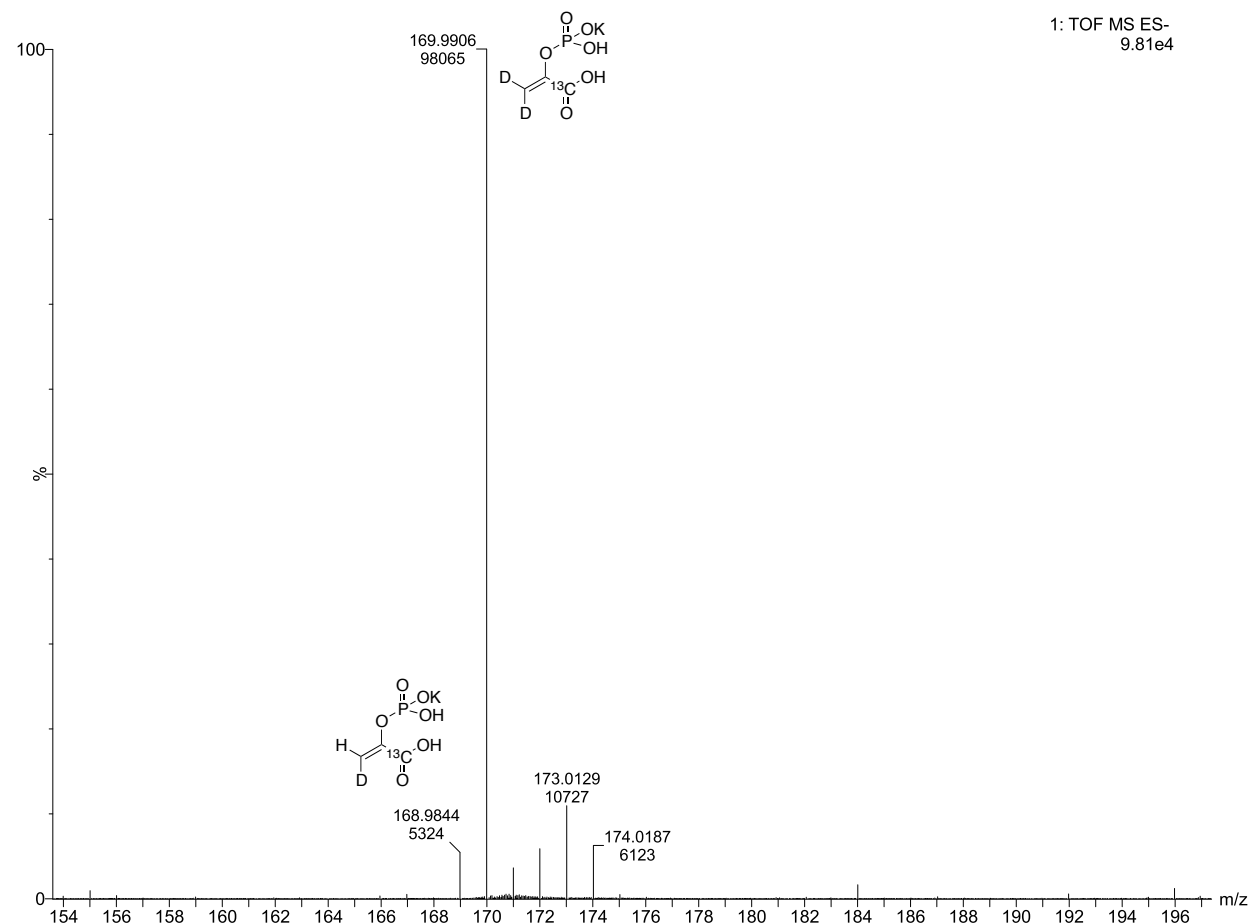


Figure S11. HR-MS negative ion spectrum 1-¹³C-PEP-*d*₂ **6** produced via the large-scale procedure; the top value over each peak represents the exact mass, and the bottom value is the peak integral value.

3. Monitoring % PHIP conversion using *ex situ* NMR spectroscopy: pH study

3.1. Preparation of buffered PEP solutions (blank PHIP precursor) at 'low' concentrations

Stock solution (500 mL) was prepared by dissolving PEP (0.0025 mol, 0.515 g) and Na₃PO₄ (0.125 mol, 4.715 g) in D₂O (300 mL) in a glass beaker equipped with magnetic stir bar. The solution was transferred to a volumetric flask and adjusted to 500 mL volume. Fractions of the

stock solution (50 mL each) were adjusted to a desired pH using small volumes of NaOD solution and a pH meter.

3.2. Preparation of buffered PEP solutions (blank PHIP precursor) at 'high' concentration

Stock solution (250 mL) was prepared by dissolving PEP (0.015 mol, 3.092 g) and Na₃PO₄ (0.015 mol, 5.700 g) in D₂O (150 mL) in a beaker equipped with magnetic stir bar. The solution was transferred to a volumetric flask and adjusted to 250 mL volume. Fractions of the stock solution (50 mL each) were adjusted to a desired pH with using small volumes of NaOD solution and a pH meter, and the volume of every fraction was brought to 100 mL with D₂O. Percentage conversion of PEP to PLAC after passing the PHIP polarizer was estimated using the integrated peak intensities of PEP and PLAC of ¹H NMR spectra of the PHIP polarizer output solutions. The example is provided below in Fig. S12.

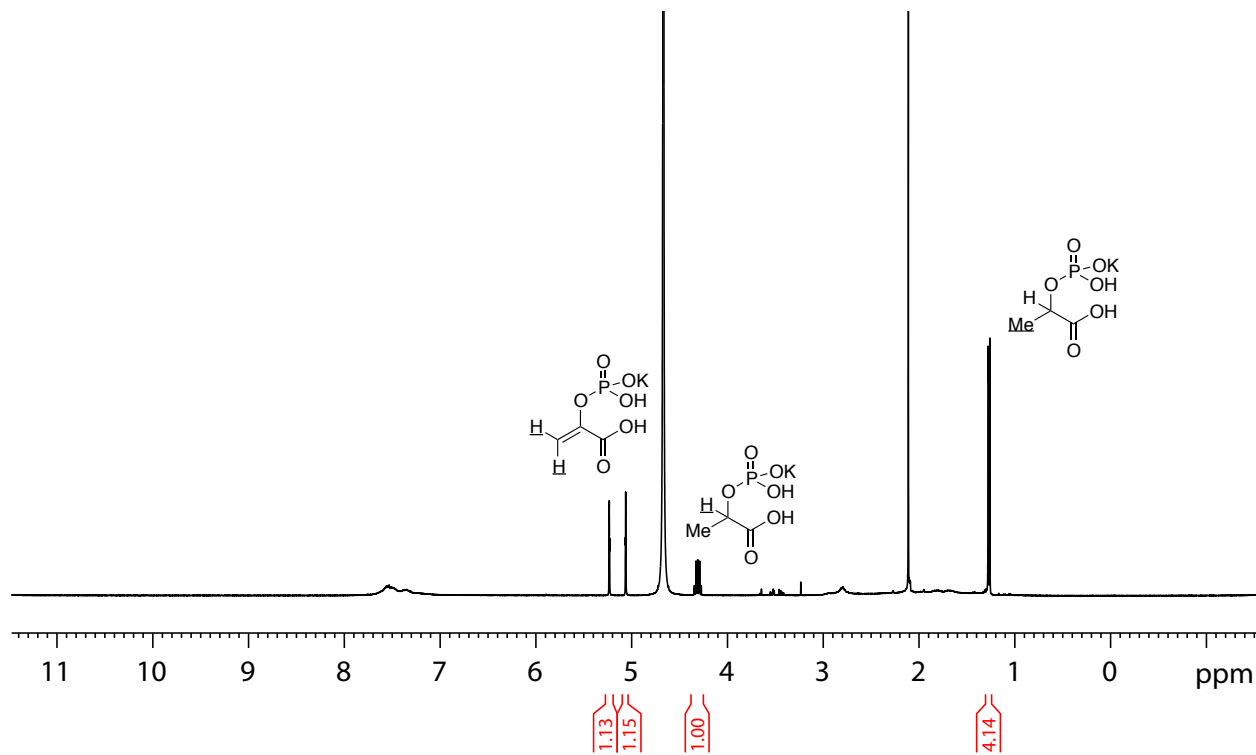


Figure S12. Representative ^1H NMR spectrum of buffered PEP solution after passing the PHIP polarizer, where molecular hydrogenation with hydrogen (H_2) gas occurs.

3.3. Preparation of buffered 'high'-concentration $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** solutions (PHIP precursor)

The solution (50 mL) was prepared by dissolving $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** (0.0015 mol, 0.314 g) and Na_3PO_4 (0.0015 mol, 0.570 g) in D_2O (25 mL) in a glass beaker equipped with magnetic stir bar. Its pH was adjusted to 10.3, and the solution was transferred to a volumetric flask, and the volume was adjusted to 50 mL with D_2O . Percentage conversion of $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** to $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$ **8** after passing the PHIP polarizer was estimated using the integrated peak intensities of $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** to $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$ **8** of ^{13}C NMR spectra of the polarizer output solutions. The example is provided below in Fig. S13.

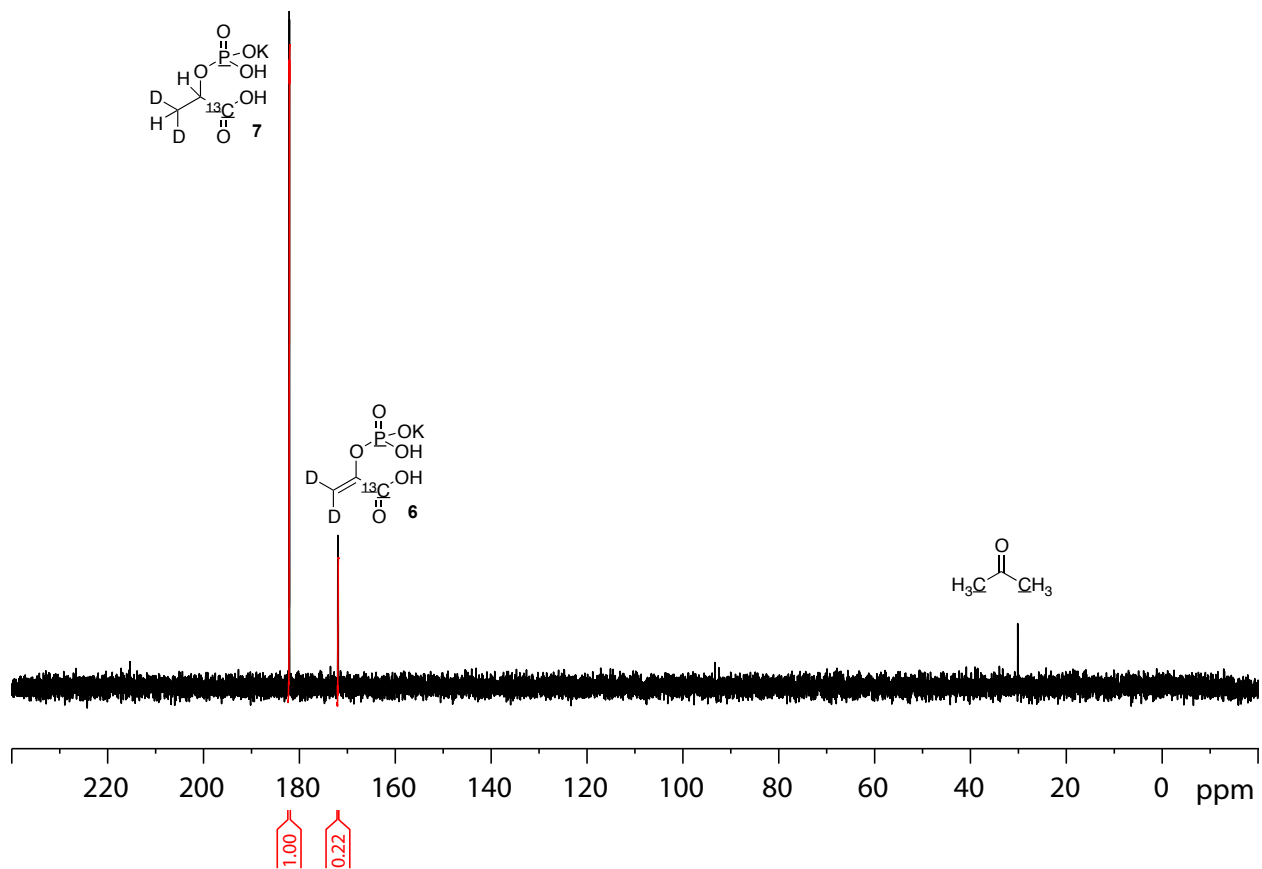


Figure S13. ^{13}C NMR spectrum of buffered $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** solution after passing the PHIP polarizer, where molecular hydrogenation with hydrogen gas occurred at $t = 68 \pm 1$ °C and 7 atm of hydrogen partial pressure. This representative spectrum corresponds to the final solution after exiting the PHIP polarizer output solution at $\text{pH} = 10.3$ and in D_2O .

Table S1. Integral values of high-resolution NMR peaks and % yield calculated for ^{13}C (carbonyl) peak of $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$, and $1\text{-}^{13}\text{C}\text{-PEP-}d_2$. NMR spectra were recorded from buffered $1\text{-}^{13}\text{C}\text{-PEP-}d_2$ **6** solution after passing the PHIP polarizer, where molecular hydrogenation with hydrogen gas occurred at ~ 7 atm of parahydrogen partial pressure for ~ 5 seconds during PHIP process and ~ 5 more seconds during *in situ* NMR spectroscopy. These results corresponds to the $1\text{-}^{13}\text{C}\text{-PEP-}d_2/1\text{-}^{13}\text{C}\text{-PLAC-}d_2$ solution in D_2O at $\text{pH} = 10.3$ and $t = 68 \pm 1$ °C after it exited the polarizer. % conversion was calculated as an average of two injections per each pH value except for the entry labeled with an asterisk (*), where the data for one sample was available.

Experiment Number	^{13}C integral of $1\text{-}^{13}\text{C}\text{-PEP-}d_2$, (carbonyl peak)	^{13}C integral of $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$, (carbonyl peak)	Conversion (%)
1	0.13	1	88*
2	0.27	1	79
3	0.22	1	82
4	0.19	1	84

4. ^{13}C T_1 measurement *in situ* of automated PHIP polarizer at 5.75 mT

The decay rate of ^{13}C hyperpolarization of $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$ **8''** was measured *in situ* of the PHIP polarizer using 30° RF excitation pulse at 5.75 mT, Fig. 4d. The ^{13}C T_1 decay of $1\text{-}^{13}\text{C}\text{-PLAC-}d_2$ was simulated using a mono-exponential fitting function after accounting for polarization loss due to RF pulses and yielding $T_1 = 51 \pm 2$ s. The 95% upper confidence limit (UCL) and lower confidence limit (LCL) of the fitted curve are provided by the green traces in Fig. 4d.

References

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