Supporting Information

Characterization of invisible reaction intermediate in decarboxylation reaction of phenylglyoxylic acid by hyperpolarized ^{13}C NMR spectroscopy

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Supporting Information

1. Dynamic nuclear polarization

20 μL of 2.2 M phenylglyoxylic acid (PhGA; Sigma Aldrich, St. Louis, MO, USA), 1 mM gadolinium(III) diethyltriaminepentaacetic acid (Gd(DTPA); Sigma Aldrich, St. Louis, MO, USA), and 15 mM tris(8-carboxyl-2,2,6,6-tetra [2-(1-hydroxymethyl)]-benzo(1,2-d:4,5-d0)bis(1,3)dithiole-4-yl) methyl sodium salt ("OX63"; Oxford Instruments, Abingdon, UK) were mixed in a glass-forming solvent (50% DMSO-d_6 and 50% D_2O (vol/vol)). 20 μL aliquots of the PhGA were polarized on ^{13}C spins in a HyperSense DNP polarizer (Oxford Instruments). During the polarization time of 1.5 hours, microwaves with 60 mW power and a frequency of 93.965 GHz were irradiated to the sample at a temperature of 1.4 K. The hyperpolarized sample was subsequently dissolved in 4 mL of a preheated buffer solution (100 mM sodium phosphate, pH 7.0) and injected into a 5 mm NMR tube pre-installed in a 400 MHz NMR magnet (Bruker Biospin, Billerica, MA). The fast injection into the NMR tube was driven by nitrogen gas pressures of 262 psi (forward pressure) and 150 psi (back pressure).[1] For the decarboxylation reaction, 25 μL of 30 % H_2O_2 (Sigma Aldrich, St. Louis, MO, USA) was preloaded in the NMR tube, and subsequently mixed with ~ 425 μL of front portion of the sample stream, yielding a total sample volume of ~ 450 μL. For relaxation measurements, 25 μL of the buffer solution was pre-loaded instead of the H_2O_2. The final concentration of the hyperpolarized PhGA was 25 mM, after a dilution of 88-fold during sample injection. For 1,2-^{13}C-pyruvate hyperpolarization, 15 mM OX63 radical was directly dissolved in a neat liquid of 1,2-^{13}C-pyruvate (Cambridge Isotope Laboratories, Andover, MA). A 1 μL aliquot of the 1,2-^{13}C-pyruvate was polarized on ^{13}C spins under the same polarization conditions as described above. The sample was dissolved in 4 mL of preheated buffer solution (100 mM sodium phosphate, pH 6.0).

2. NMR spectroscopy and data analysis

Time-resolved ^{13}C spectra were acquired on a 400 MHz NMR spectrometer equipped with a broadband probe containing three pulsed field gradients (Bruker Biospin), at a temperature of 303 K. The hyperpolarized sample was transferred from the polarizer to the sample injector during a transfer time (t_t). The sample was subsequently injected from the injection loop of the sample injector into a 5 mm NMR tube, which was preinstalled in the NMR spectrometer. NMR experiments were triggered after an injection time (t_i) of 450 ms and a stabilization time (t_s) of 500 ms. The pulse sequence for blind saturation is shown in Fig. S1. To suppress the product signal (175.7 ppm) generated during the mixing and stabilization times, three EBURP-2 shaped pulse (π/2 flip angle, 20 ms duration), each followed by a randomized pulsed field gradient $G_{x,y,z}$, were applied. This scheme enables to increase the selectivity for the detection of the saturation effect by removing the hyperpolarized product signals that formed during the dead time of the sample injection. It can also simplify analyzing the effect of the saturation of a spin in the intermediate species on the product signals, by removing contributions from uninverted intermediate species. For each scan, a series of EBURP-2 shaped pulses (π/2 flip angle, 60 ms duration for 100 Hz excitation bandwidth, or 10 ms duration for 600 Hz excitation bandwidth) followed by a randomized pulsed field gradient $G_{x,y,z}$ was applied at the resonance frequency of the expected reaction intermediate. A total of 32 transients were acquired for a total duration of 33 s. The delay time between transients was 1 s. The small flip angle α of the excitation pulse was 15.2°, and the pulse strength was 25 kHz. In each transient, 8,192 data points were acquired for an acquisition time of 170 ms. During the acquisition, WALTZ-16 ^{1}H decoupling was applied with a field strength γB_1 = 2.3 kHz. The raw data were zero filled to 16,384 complex data points, and an exponential window function with 3 Hz line broadening was applied before Fourier transform using the TOPSPIN 3.1 program (Bruker Biospin). Chemical shifts of ^{13}C were calibrated against ^{1}H spectrum of sodium trimethylsilylpropanesulfonate (DSS) in 10%/90% D_2O/H_2O according to the IUPAC recommendations.[2] Peak integration, curve fitting, and calculation of apparent reaction rates was performed using the MATLAB program (The MathWorks).

3. Kinetic modeling

For estimating the temporal dependence of the benzoate signal in the decarboxylation of hyperpolarized phenylglyoxylic acid (PhGA) at a range of kinetic rates, a two-site, irreversible kinetic model was utilized (Eq. 1). This model includes two species A and B, whose NMR signals are denoted as S_A and S_B, respectively. A kinetic rate constant k indicates a conversion rate of A to B, and a signal pathway described by a decay rate r_A or r_B for each species accounts for the signal loss through spin-lattice relaxation (T_1). Another pathway for signal depletion is caused by detection pulses applied throughout the D-DNP NMR measurements. A hard radiofrequency pulse with a fixed small flip angle α converts sin(α) of the total longitudinal magnetization to the observable transverse magnetization, while cos(α) of the longitudinal magnetization is preserved for the following scan. This pulse effect is characterized by an exponential decay rate λ defined as $\lambda = \frac{-\ln(\cos(\alpha))}{\Delta t}$ where α is a constant small flip angle and Δt the time delay between scans.[3] Differential equations for S_A and S_B are expressed in Eq. 2 and 3. Since the goal of this kinetic modeling was to provide an approximate range for the product signal intensities as a reference for the blind saturation experiments. Thus, the simplest, one-step model was chosen for the analysis despite the complexity of the mechanism of the PhGA decarboxylation reaction. To obtain the heat map of temporal signal intensities of C1-benzoate shown in Figure 4 in the main text, the apparent conversion rates of PhGA to benzoate ($k_{\text{PhGA}\rightarrow\text{Benzoate}}$) were first determined from the decaying C2-PhGA signals by solving the differential equation Eq. 2 with the knowledge of $r_{A\ (\text{A=C2-PhGA})}$. Then, the product signal intensity (S_B, B = C1-benzoate) was calculated for a range of $k_{\text{PhGA}\rightarrow\text{Benzoate}}$ including the experimentally determined $k_{\text{PhGA}\rightarrow\text{Benzoate}}$ values, using Eq. 3. Signal depletion due to the detection pulses was taken into account when solving the differential equations. The T_1 relaxation rates and the experimentally determined $k_{\text{PhGA}\rightarrow\text{Benzoate}}$ values are summarized in Table S1.

$$\begin{array}{ccc} S_A & \xrightarrow{k} & S_B \\ \downarrow\downarrow & & \downarrow\downarrow \\ r_A\ \lambda & & r_B\ \lambda \end{array} \qquad \text{(Eq. 1)}$$

$$\frac{dS_A(t)}{dt} = -(k + r_A + \lambda) \cdot S_A(t) \qquad \text{(Eq. 2)}$$

$$\frac{dS_B(t)}{dt} = k \cdot S_A(t) - (r_B + \lambda) \cdot S_B(t) \quad \text{(Eq. 3)}$$

4. Data normalization

Time-resolved ^{13}C NMR spectra from the blind saturation experiments were normalized in order to correct for different polarization levels of the hyperpolarized PY or PhGA in each experiment. The C2 peak of the reactants was utilized for calibrating signal intensities. The signal loss of the same nuclei in the reaction products (acetate and benzoate in PY and PhGA experiments, respectively) were observed in the experiments. This signal loss was caused by the selective saturations of the spins in the reaction intermediate. Scaling factors for data normalization were obtained by fitting the reactant signal integrals to an exponential decay based on the kinetic model (Eq.2). In this approach, the estimated peak integral at t = 0, rather than the first data point at t = 725 ms after the mid-point of sample mixing, was scaled to unit value. It was assumed that no reaction had occurred at t = 0. This normalization method may include bias in the estimation of the scaling factors because (*i*) a potential reversibility of the reaction step between the reactant and the intermediate was not considered and (*ii*) signal attenuation on the C2 reactant (*i.e.* C2-PY and C2-PhGA) caused by the saturation of the C2-intermediate in the on-resonance experiments was not taken into account. To evaluate the effect of data normalization using different reactant, the ratio of the scaling factors from C1 and C2 peak based normalizations ($S.F._{C1}/S.F._{C2}$) was calculated. The values of $S.F._{C1}/S.F._{C2}$ were found to be 0.87 and 0.93 from the data with pyruvate reactions in on-resonance and off-resonance experiments, respectively. For the case of PhGA reactions, the value of $S.F._{C1}/S.F._{C2}$ from the on-resonance experiment (f_{sat} = 103 ppm) was 0.87, and the mean value of $S.F._{C1}/S.F._{C2}$ from the off-resonance experiments (f_{sat} = 99, 100, 101, 102, 104, 105 ppm) was 0.88 ± 0.04. Based on these results, the variation in the normalized product signal intensities associated with different normalization methods (C2-PY vs. C1-PY normalization) was less than 13 %.

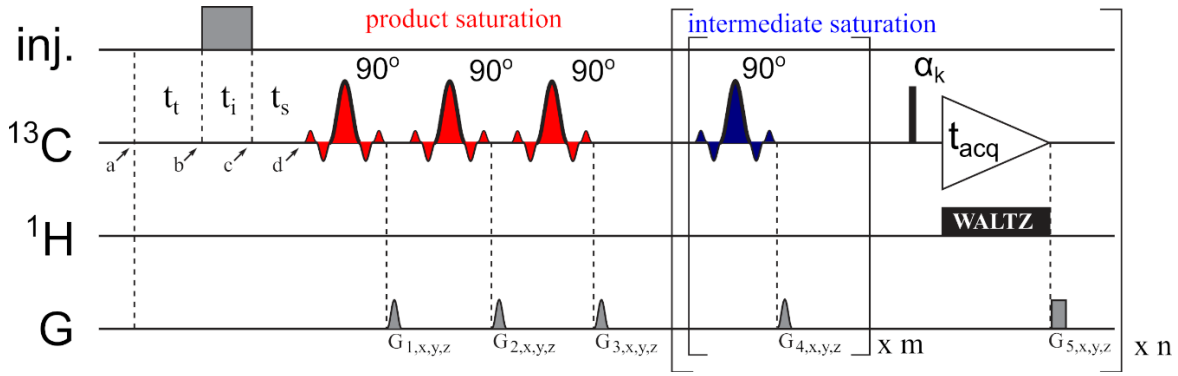

Fig. S1. NMR pulse sequence for blind selective saturation. The hyperpolarized sample is delivered from the DNP polarizer to the sample injector during a transfer time (t_t) (time points between *a* and *b*). The sample is subsequently injected from the injection loop of the sample injector into a 5 mm NMR tube, during an injection time (t_i).[1] The NMR experiment is triggered at time point d after a sample stabilization time (t_s). The pulse sequence starts with three EBURP-2 shaped pulses ($\pi/2$ flip angle) to selectively suppress the signal of hyperpolarized product that forms before the saturation of signals in the intermediate species. For each scan, a series of EBURP-2 shaped pulses with $\pi/2$ flip angle are applied at the resonance frequency of the expected reaction intermediate. The number of pulses is m = 12 with an excitation bandwidth of 100 Hz, or m = 72 with an excitation bandwidth of 600 Hz. The pulses are followed by a randomized pulsed field gradient $G_{x,y,z}$. A total of 32 transients (n = 32) are acquired for a duration of 33 s.

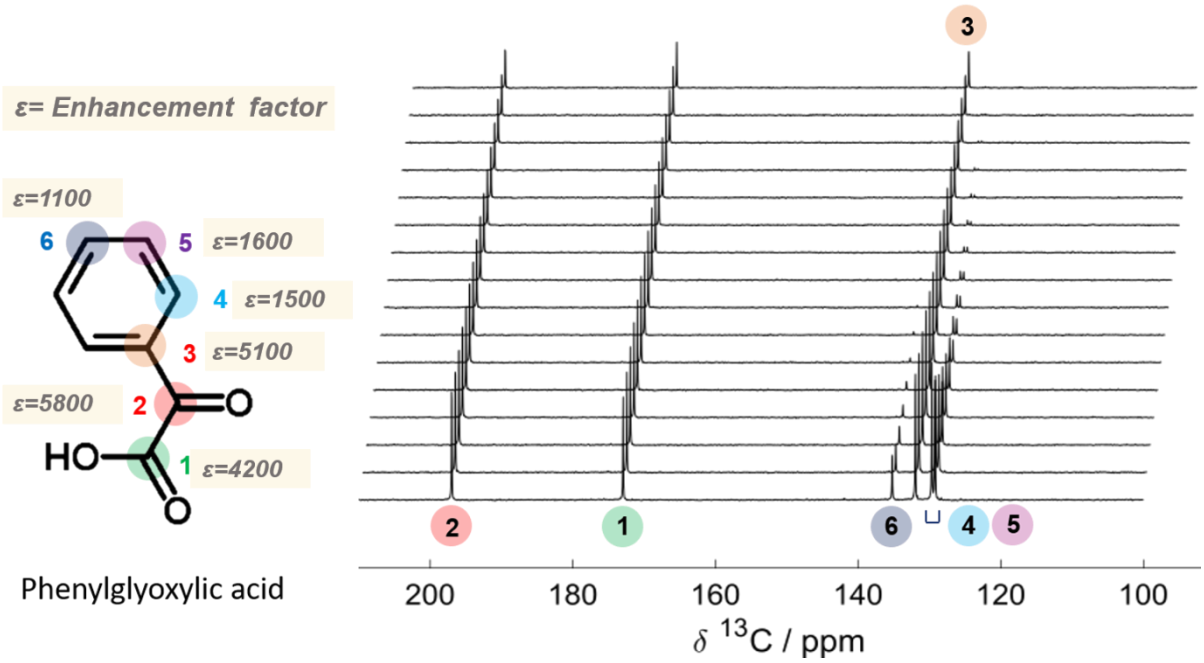

Fig. S2. Hyperpolarized ^{13}C NMR spectra of phenylglyoxylic acid. The C2 signal showed signal enhancements of > 5000-fold relative to a conventional NMR spectrum measured of a sample of 2.5 M phenylglyoxylic acid, after accounting the the concentration difference.

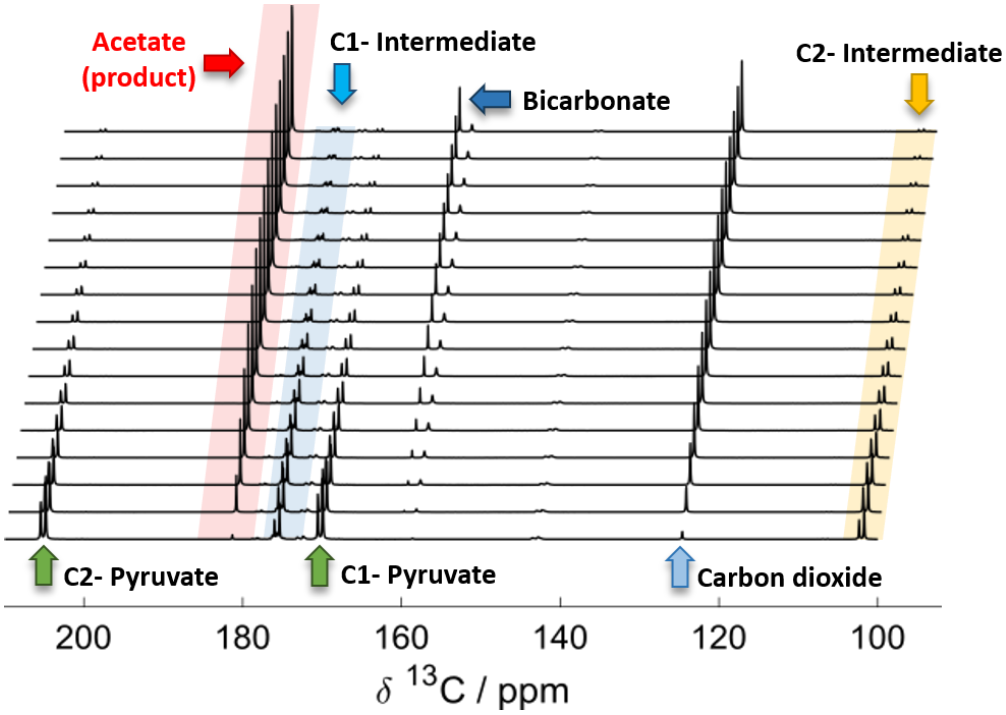

Fig. S3. Time-resolved ^{13}C NMR spectra of hyperpolarized 1,2-^{13}C-pyruvic acid mixed with hydrogen peroxide.

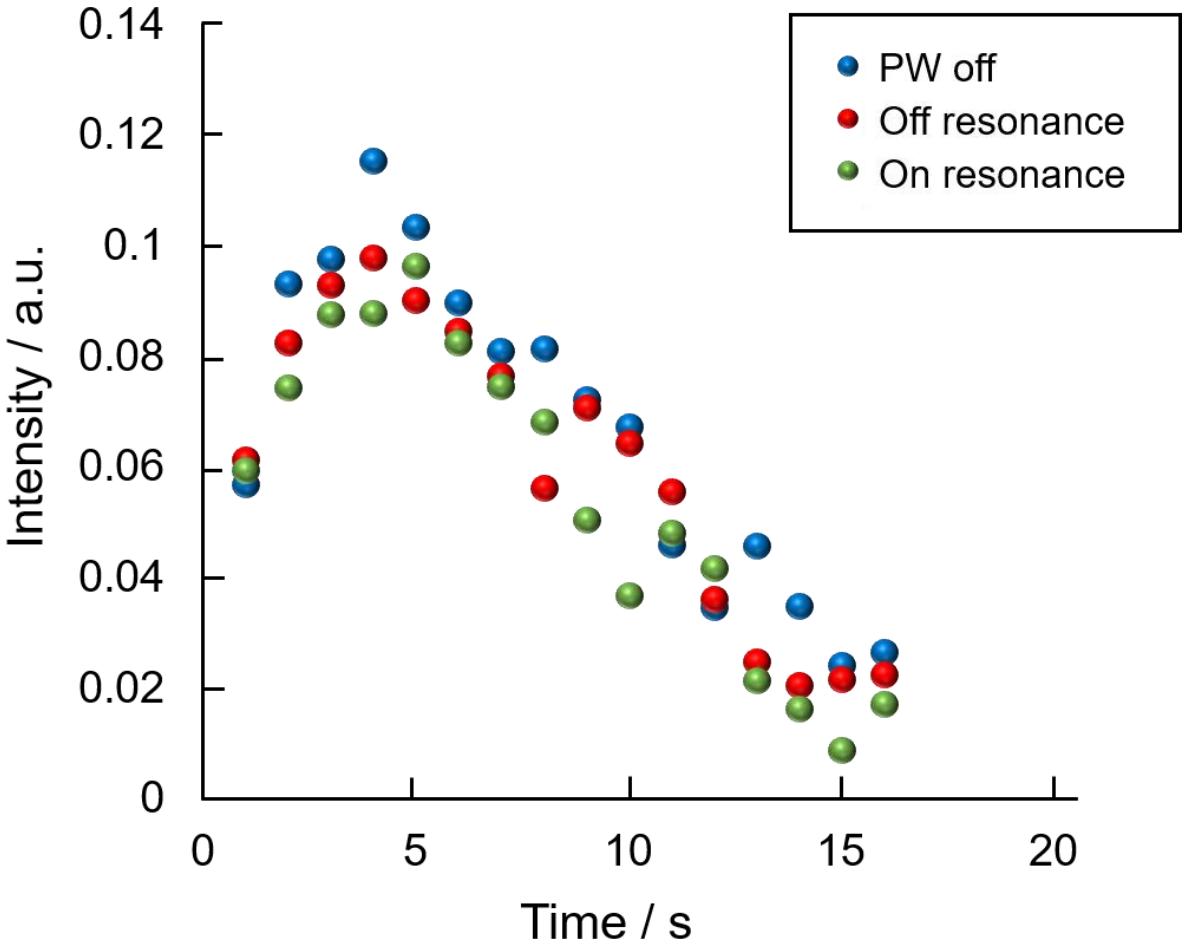

Fig. S4. Signal intensities of carbon dioxide from the blind saturation (On resonance) and control experiments (Off resonance, Pulse power-off). The center frequency of the saturation pulse (f_{sat}) was set to 102 ppm, and the bandwidth of the selective saturation pulse was 600 Hz. The frequency of the off-resonance saturation was set to -20 ppm away from the expected intermediate signal regions.

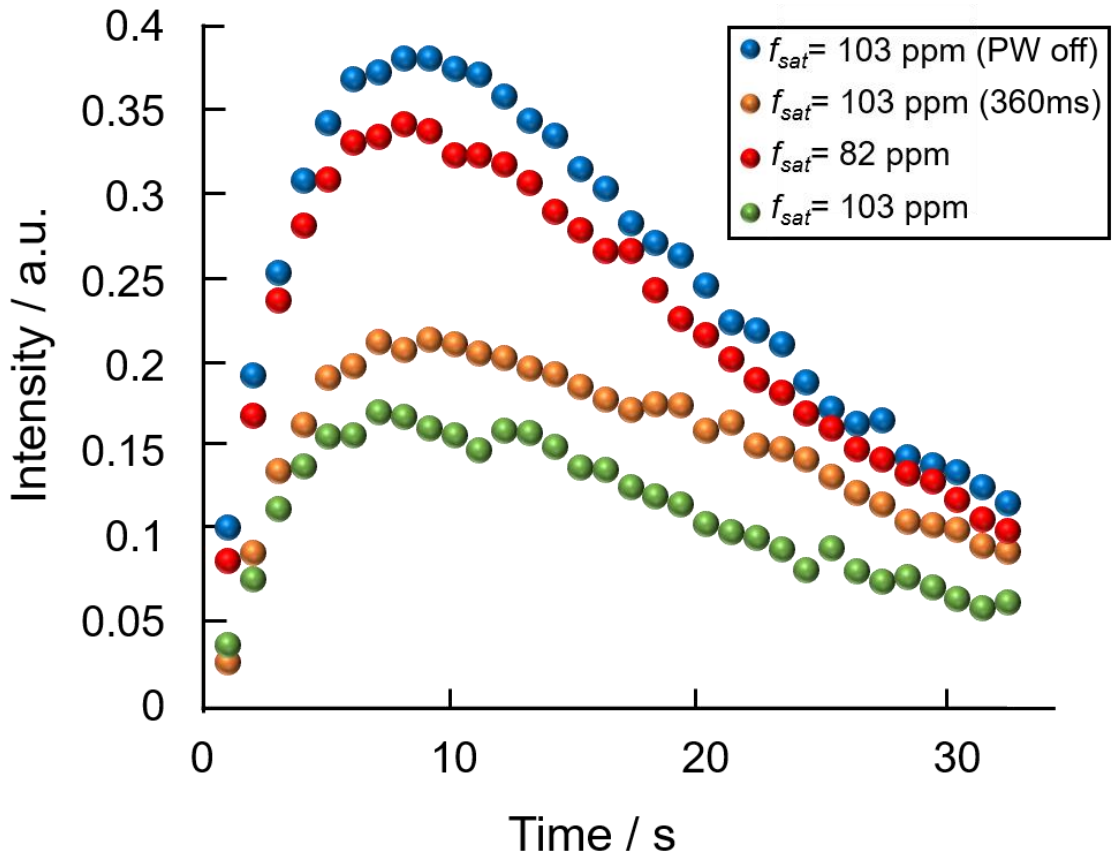

Fig. S5. Signal intensities of benzoate from the blind saturation and control experiments (f_{sat} = 82 ppm, f_{sat} = 103 ppm with power off, and f_{sat} = 103 ppm with decreasing the saturation pulse time by half (360 ms)). The center frequency of the saturation pulse (f_{sat}) was set to 103 ppm, and the bandwidth of the selective saturation pulse was 100 Hz. The center frequency of the off-resonance saturation pulse was set to -20 ppm away from the expected intermediate signal regions.

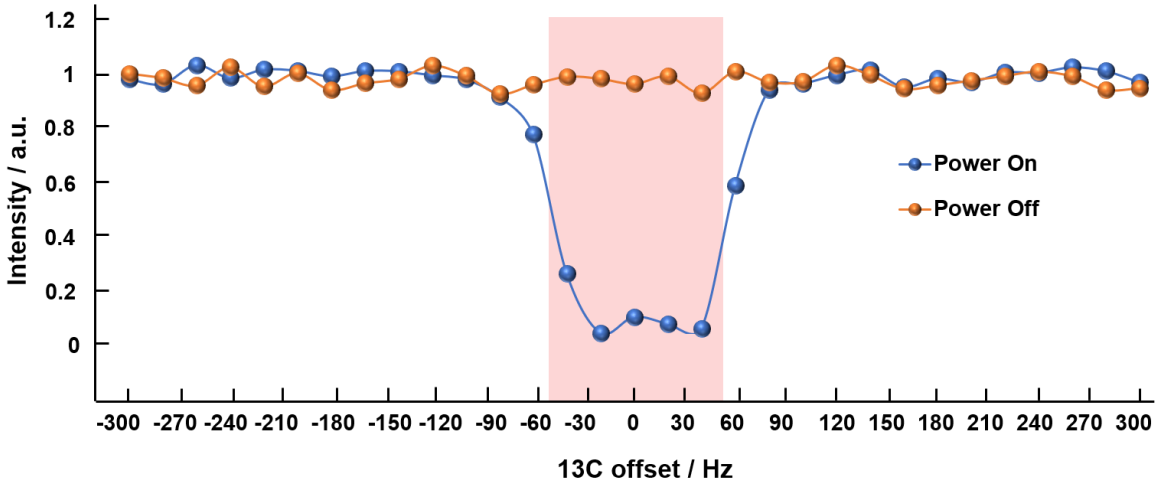

Fig. S6. Excitation bandwidth of the selective saturation pulse characterized with the 1-^{13}C signal of bicarbonate at 160.4 ppm (0 Hz in x-axis). The center frequency of the saturation pulse (saturation time: 720 ms) was varied from -300 Hz to 300 Hz in increments of 20 Hz. As a control, the same experiment was also conducted with power-off of the saturation pulses. The colored area represented a 100 Hz width.

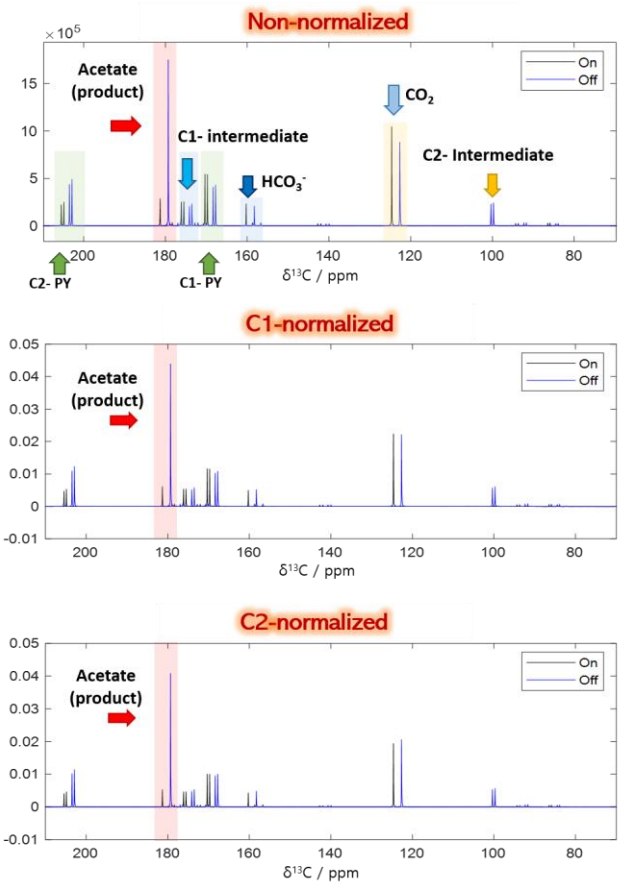

Fig. S7. Selective saturation with the pulse applied off-resonance (blue) and on-resonance (black) with respect to the 2-^{13}C signal of the intermediate species in the reaction with pyruvate. The spectra were analyzed by different normalization methods (Non-normalized, C1-PY normalized, and C2-PY normalized). To allow for the comparison of peak intensities, the "off-resonance" spectra are shifted by – 2 ppm along the x-axis.

Table S1. Apparent conversion rates of PhGA to Benzoate ($k_{PhGA \rightarrow Benzoate}$) in blind saturation experiments. The center of the saturation frequency was changed from 99 ppm to 105 ppm, in steps of 1 ppm. The effective bandwidth of the selective saturation pulse was 100 Hz. R_1 values of C1-PhGA and C2-PhGA are 0.0315 s^{-1} and 0.0395 s^{-1}, respectively.

Center Frequency	99 ppm	100 ppm	101 ppm	102 ppm	103 ppm	104 ppm	105 ppm
Apparent C2-PhGA conversion rate (s^{-1})	0.124	0.138	0.125	0.167	0.201	0.149	0.130
Apparent C1-PhGA conversion rate (s^{-1})	0.129	0.139	0.128	0.160	0.138	0.144	0.125

References

(1) Bowen, S.; Hilty, C. *Phys. Chem. Chem. Phys.* 2010, **12**, 5766–5770.

(2) Harris, R.; Becker, E.; de Menezes, S.; Goodfellow, R.; Granger, P. *Pure Appl. Chem.* 2001, **73**, 1795–1818.

(3) Zeng, H.; Lee, Y.; Hilty, C. *Anal. Chem.* 2001, **82**, 8897–8902.