

Supporting Information

Real-Time Interrogation of Aspirin Reactivity, Biochemistry, and Biodistribution by Hyperpolarized Magnetic Resonance Spectroscopy

*Niki M. Zacharias⁺, Argentina Ornelas⁺, Jaehyuk Lee, Jingzhe Hu, Jennifer S. Davis, Nasir Uddin, Shivanand Pudakalakatti, David G. Menter, Jose A. Karam, Christopher G. Wood, Ernest T. Hawk, Scott Kopetz, Eduardo Vilar, Pratip K. Bhattacharya, and Steven W. Millward**

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Supporting Information

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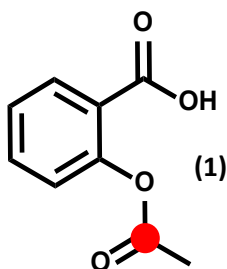
Materials and Methods

Chemicals

[1-¹³C, 99%]-acetyl chloride and [1,1'-¹³C₂, 99%]-acetic anhydride were acquired from Cambridge Isotope Laboratories. [α-¹³C]-salicylic acid was obtained from Sigma-Aldrich. All other solvents, salts, and reagents were purchased from commercial vendors and used without further purification.

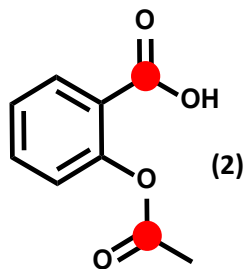
Synthesis and characterization of aspirin derivatives

Synthesis of [8-¹³C]-aspirin (**1**).



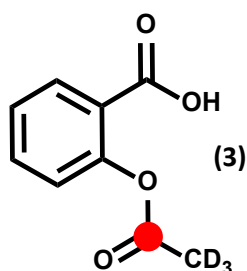
Single-labeled aspirin (**1**) was synthesized by the adaptation of previously reported methods and protocols.^{1,2} In summary, 6.2 mmol of unlabeled salicylic acid was dissolved in 20 mL of pyridine in a 50-mL round-bottom flask. The solution was capped and placed on an ice bath. To this, 2.5 equivalents of ¹³C-labeled acetyl chloride or acetic anhydride (15.5 mmol) were added slowly. The reaction was stirred for 30 minutes at 0°C. The reaction was quenched by the addition of 40 mL of ice. The reaction was worked up by adding 200 mL of ethyl acetate followed by 100 mL of 1N HCl. The solution was transferred to a separatory funnel, and the aqueous layer was removed. The organic layer was further washed with 2×100 mL of HCl/wash, followed by a brine wash (100 mL). The organic layer was dried over MgSO₄ and evaporated under reduced pressure. The white solid was redissolved in a small amount of dichloromethane and was purified by silica gel chromatography (30%-70% ethyl acetate in hexanes). The resulting product was recrystallized in a 50% solution (v/v) of ethanol in water. Purity was assessed by thin-layer chromatography and NMR analysis (>99% purity, 55% yield). [8-¹³C]-aspirin (**1**): ¹H-NMR (500 MHz, H₂O+D₂O) δ 7.67 (dt, *J* = 7.7, 1.4 Hz, 1H), 7.55 – 7.44 (m, 1H), 7.35 (td, *J* = 7.6, 1.2 Hz, 1H), 7.13 (ddd, *J* = 8.1, 1.3, 0.6 Hz, 1H), 2.32 (dd, *J* = 6.9, 1.1 Hz, 3H). ¹³C-NMR (500 MHz, H₂O + D₂O) δ 181.30, 173.44, 173.34, 147.50, 131.10, 131.07, 129.57, 126.41, 122.39, 20.75, 20.35, -2.70. ESI: Expected [M+H]⁺ = 182.00, Observed [M+H]⁺ = 182.05.

Synthesis of [7,8-¹³C]-aspirin (**2**).



Double-labeled aspirin (**2**) was synthesized by dissolving 3.4 mmol of [α -¹³C]-salicylic acid in 15 mL of pyridine in a round-bottom flask placed in an ice bath. A total of 12.58 mmol of acetyl chloride (1-¹³C, 99%) was added drop wise, and the reaction was stirred for 30 minutes at 0°C. The reaction was quenched by 30 mL of iced water slurry, followed by 200 mL of ethyl acetate. The reaction was neutralized by 100 mL of 1N HCl. The contents were transferred to a separatory funnel, and the organic layer was further washed with 2×100 mL of 1N HCl. The organic layer was then dried with 100 mL of brine solution and dried over MgSO₄. The product was concentrated under reduced pressure, redissolved in CH₂Cl₂, and purified by column chromatography with a gradient of 30%-70% ethyl acetate in hexanes. The fractions with product were pooled, and the product was obtained under reduced pressure. The resulting double-labeled aspirin was recrystallized in a 50% solution (v/v) of ethanol in water, and purity was assessed by thin-layer chromatography and NMR analysis (>99% purity). [7,8-¹³C]-aspirin (**2**): ¹H-NMR (500 MHz, D₂O+H₂O) δ 7.67 (ddt, J = 7.6, 4.2, 1.5 Hz, 1H), 7.50 (tt, J = 8.0, 1.6 Hz, 1H), 7.35 (tdd, J = 7.5, 1.2, 0.6 Hz, 1H), 7.18 – 7.09 (m, 1H), 2.32 (dd, J = 7.0, 1.2 Hz, 3H). ¹³C-NMR (500 MHz, H₂O+D₂O) δ 181.30, 173.44, 173.34, 147.50, 131.28, 131.10, 130.84, 129.57, 126.40, 122.38, 20.75, 20.35, -2.70. ESI: Expected [M+H]⁺ = 183.00, Observed [M+H]⁺ = 183.04.

Synthesis of [8-¹³C, 9-d₃]-aspirin (**3**) (single-labeled, deuterated).



¹³C single-labeled and deuterated aspirin (**3**) was synthesized by dissolving 0.909 mmol of salicylic acid in 5 mL of pyridine in a round-bottom flask placed in an ice bath. A total of 2.26 mmol of [1,1'-¹³C₂,d₆]-acetic anhydride were added drop wise, and the reaction was stirred for 30 minutes at 0°C. The reaction was quenched by adding 10 mL of iced water slurry followed by 20 mL of ethyl acetate. The reaction was neutralized by adding 10 mL of 1N HCl, and the contents were transferred to a separatory funnel. The bottom layer was removed, and the organic layer was washed with 2×15 mL 1N HCl. The organic layer was then treated with 100 mL of brine and later dried over MgSO₄. The product was concentrated under reduced pressure, redissolved in CH₂Cl₂, and purified by column chromatography with a gradient of 30%-70% ethyl acetate in hexanes. The resulting aspirin was recrystallized in a 50% solution (v/v) of ethanol in water, and purity was assessed by thin-layer chromatography and NMR analysis (>95% purity). [8-¹³C, 9-d₃]-aspirin (**3**): ¹H-NMR (500 MHz, D₂O+H₂O) δ 7.74 – 7.62 (m, 1H), 7.50 (dddd, *J* = 8.1, 7.4, 1.8, 0.8 Hz, 1H), 7.35 (ddd, *J* = 8.6, 7.1, 1.0 Hz, 1H), 7.15 – 7.10 (m, 1H). ¹³C-NMR (500 MHz, H₂O+D₂O) δ 181.25, 173.24, 164.03, 131.04, 129.56, 126.33, 122.36, 18.99, 14.89, -2.70. ESI: Expected [M+H]⁺ = 185.00, Observed [M+H]⁺ = 185.15.

Dynamic Nuclear Polarization

In an optimized hyperpolarization experiment, ^{13}C -labeled aspirin was dissolved in a 63% dimethyl sulfoxide- d_6 and water solution to a final concentration 1.5 M. Oxo63 free radical (Oxford Instruments) was added directly to the mixture to a final concentration of 15.6 mM. For transacetylation reactions, a 112 μL of this solution was mixed with 4.2 μL of 50 mM gadolinium (III) relaxation agent (Bracco Diagnostics). Samples were then loaded into the HyperSense dynamic nuclear polarizer and irradiated at 94.100 GHz for 1-1.5 hours before being dissolved in 4 mL of PBS buffer in D_2O (pH = 7.2) to a final concentration of ~ 42 mM hyperpolarized aspirin. This solution was then used directly in 300 MHz NMR and 7T MR animal experiments. Solid-state polarization values were obtained directly from the HyperSense with no correction. For blood component experiments, 89 μL of aspirin radical solution with 3.3 μL of 50 mM gadolinium relaxation agents was polarized and dissolved in 4 mL of PBS in 10% D_2O (pH = 7.2) to a final concentration of ~ 33 mM hyperpolarized aspirin.

Hyperpolarized Aspirin 7T NMR Experiments

NMR spectroscopy after hyperpolarization of aspirin was performed on a Bruker Spectrospin UltraShield 300 MHz/54 mm NMR magnet system with a 10-mm probe running TopSpin 3.5. Ten-millimeter NMR tubes were equipped with tubing spanning the bottom of the tube to the outside of the magnet for injection. Before the hyperpolarization experiment, 10-mm NMR tubes were loaded with KOH hydrolysis, lysine, glycine, phosphate, or blood component (plasma, red blood cells, bovine serum albumin [BSA]) solutions. Using variable temperature control, the probe was heated to 37°C , and autotuning, gradient shimming (TopShim), and autolocking were performed before the hyperpolarization experiment. For all experiments, 500 μL of hyperpolarized solution was injected into the NMR tube. An array of single transients (6 seconds, 12° flip angle, gain 1) using one-dimensional proton-decoupled (Waltz16) ^{13}C -spectroscopy was performed for 3 minutes to monitor a reaction or determine the longitudinal relaxation time (T_1) of the hyperpolarized compound. The array experiment was initiated before injection of hyperpolarized material. After hyperpolarized PBS control experiments were completed, thermal polarization of aspirin in the NMR experiment was determined using the same parameters as in the hyperpolarization experiment (gain and flip angle) with 3200-7200 transients. Owing to the 12° flip angle, a 10- to 5-second relaxation delay between transients was used for this overnight experiment. Integration for the hyperpolarized aspirin resonance from the first hyperpolarized FID was directly compared with the integration from the thermal polarization experiment. All spectra were referenced to the $\text{DMSO-}d_6$ resonance at 39 ppm which was present in all spectra. We did not correct for the time it took for the hyperpolarized material to be delivered from the HyperSense to the NMR. Spectra were analyzed using MestreNova (Mestrelab Research, version 10.0.1).

Transacetylation reactions

The buffer used in NMR and imaging experiments was prepared from a BupH phosphate-buffered saline (PBS) packet dissolved to 10X in D_2O . At the time of the experiment, the buffer was further diluted to 1X using D_2O . The composition of 1X phosphate buffer (hereinafter referred to as PBS) is 0.1M sodium phosphate, 0.15 M NaCl, pH = 7.2. Reaction conditions are shown below:

KOH hydrolysis: 1.5 mL of 1 M KOH solution dissolved in PBS in D_2O .

Lysine reaction: 1.5 mL of 250 mM N α -acetyl lysine and 500 mM KOH in PBS in D_2O .

Glycine reaction: 1.5 mL of a 300 mM glycine and 500 mM KOH in PBS buffer in D_2O .

PBS control: 1.5 mL of PBS in D₂O.

KOH hydrolysis: 1.5 mL of 1 M KOH solution dissolved in PBS in D₂O.

Blood Component Binding Experiments

BSA: A 50 mg/mL solution was prepared by dissolving 250 mg BSA (Sigma-Aldrich A3294, heat shock fraction, protease free, pH = 7) in PBS pH = 7.2 (10% D₂O). A total of 2.5 mL of the solution was used in each hyperpolarization experiment.

Plasma and red blood cells: Adult wild-type C57B/6 mice (both male and female) were euthanized using Institutional Animal Care and Use Committee (IACUC) approved methods, and their blood was collected via cardiac puncture. To prevent clotting, blood was transferred to tubes containing 109 mM buffered sodium citrate (BD Vacutainer, product no. 363083). Blood was pooled from 4-6 mice for a volume of 4 - 5 mL per experiment. Pooled blood was centrifuged at 1,000g for 10 minutes. Plasma was transferred to a clean tube, and the remaining red cells were rinsed with 3 mL of PBS, then subjected to repeat centrifugation as described above. Plasma and red cells were transported on ice for analysis. For each hyperpolarized experiment, 2 mL of either red blood cells (RBC) or plasma was added to a 10-mm NMR tube along with 210 μ L of PBS in 100% D₂O and 300 μ L of PBS in 10% D₂O to obtain a final concentration of 10% D₂O in the sample.

Thermal Polarized Single-Labeled Aspirin (1) NMR Experiments

BSA (Figure 2C): BSA was dissolved to 50 mg/mL in PBS (10% D₂O). [8-¹³C]-aspirin (**1**) and [8-¹²C]-aspirin were dissolved in d₆-DMSO to a final concentration of 1.5 M and added immediately before the start of the ¹³C-NMR acquisition at 37°C (1,024 scans). Reactions were set up as follows:

Reaction 1: To 1 mL of PBS (10% D₂O) was added 21 μ L of d₆-DMSO and 7 μ L of 1.5 M [8-¹³C]-aspirin (**1**).

Reaction 2: To 1 mL of 50 mg/mL BSA in PBS (10% D₂O) was added 21 μ L of d₆-DMSO and 7 μ L of 1.5 M [8-¹³C]-aspirin (**1**).

Reaction 3: To 1 mL of 50 mg/mL BSA (10% D₂O) was added 21 μ L of 1.5 M [8-¹²C]-aspirin followed by 7 μ L of 1.5 M [8-¹³C]-aspirin (**1**).

Hyperpolarized Aspirin 7T Magnetic Resonance Animal Experiments

Mice

Nude mice (male, >12 weeks old) were purchased from Harlan Laboratories. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Animal Imaging and Spectroscopy

Imaging and spectroscopy were carried out in a BioSpec 7T USR 70/30 MRI (Bruker BioSpin MRI, Billerica MRI, Billerica, MA) with a dual-tuned $^1\text{H}/^{13}\text{C}$ volume coil (Doty Scientific, 35 mm ID, homogenous rf region ~ 52 mm along z-axis) and equipped with a single channel for carbon excitation/reception. Proton anatomic images were taken using a multi-slice T2-weighted RARE sequence. Unless stated otherwise, a small 8 M [^{13}C]-urea phantom doped with gadolinium-DTPA was placed next to each animal for chemical shift referencing and to determine the basic frequency for C-13 (~ 75.52 MHz) on the instrument. A series of slice-selective ^{13}C spectra (field of view 40×40 mm, slice thickness 15-20 mm) were collected immediately (**Figure 4c**) or 5 seconds (**Figure 4d**) after injection of hyperpolarized ^{13}C aspirin. A total of 90 transients were acquired with a delay of 2 seconds between each transient (total time 3 minutes). The center frequency was determined in each experiment using 8 M Each transient used a 15° - 20° flip angle excitation pulse (Gaussian pulse) and 2,048 data points. Data were processed both in MATLAB (MathWorks, Inc.) or MestreNova. The dynamic spectra were manually phased, and line-broadening was applied (10-15 Hz). ^{13}C imaging was performed by converting a ^1H Bruker interlaced echo-planar imaging sequence to ^{13}C . Dynamic sequential ^{13}C coronal images were acquired immediately or 5 seconds after the start of hyperpolarized ^{13}C aspirin injection via a tail-vein catheter. Identical single-shot echo-planar imaging readouts were executed with following parameters: field of view = 40×40 mm², image size = 20×20 , slice thickness = 32 mm, TR = 1 second, echo time = 5.8 millisecond, flip angle = 30° . These parameters allowed for an image to be taken every 1 second. All *in vivo* images were reconstructed from raw data using a Bruker 7T magnetic resonance imaging scanner. ^{13}C images were processed for resampling to 192×256 pixels, which was matched with the ^1H anatomic image. Two ^1H and ^{13}C images were aligned and cropped to remove the Nyquist ghosts of the echo-planar imaging sequence outside the body. This image processing program was implemented with Python 2.7 and image processing modules.

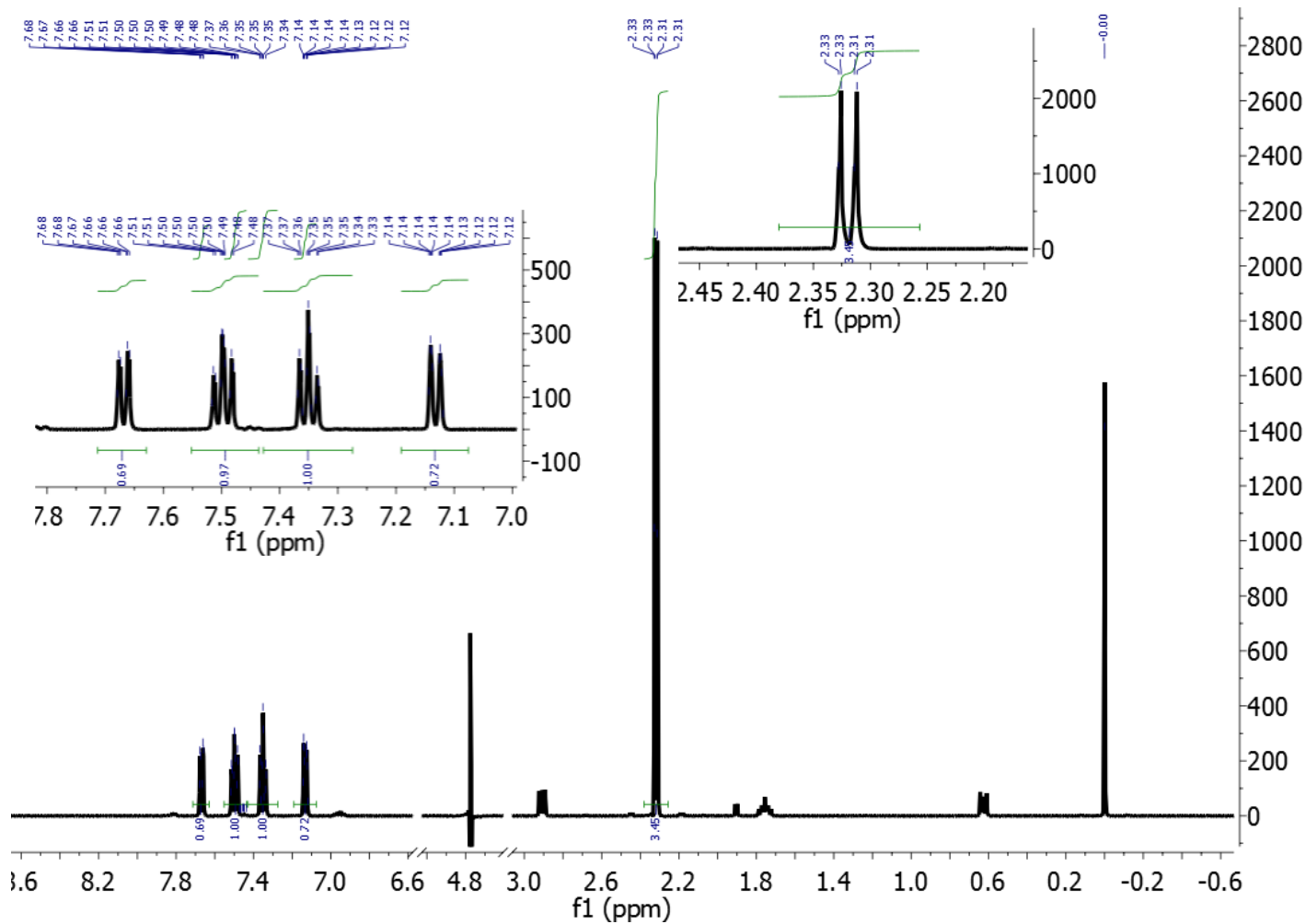


Figure S1: $^1\text{H-NMR}$ spectrum of (1). Resonances from the aromatic and acetyl protons are shown in the insets.

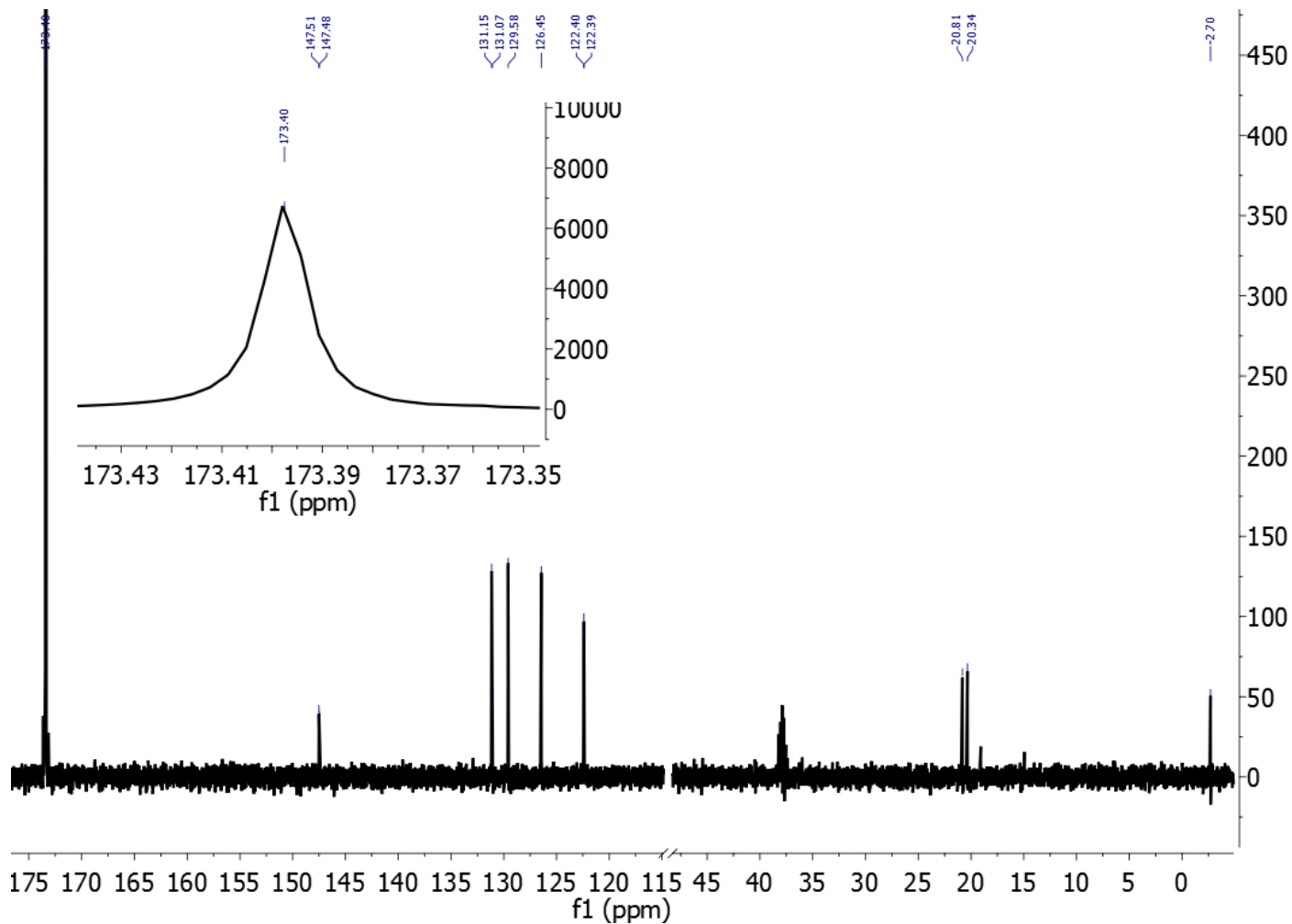


Figure S2: ^{13}C -NMR spectrum of (1). The resonance for the ^{13}C -labeled carbonyl carbon is shown in the inset.

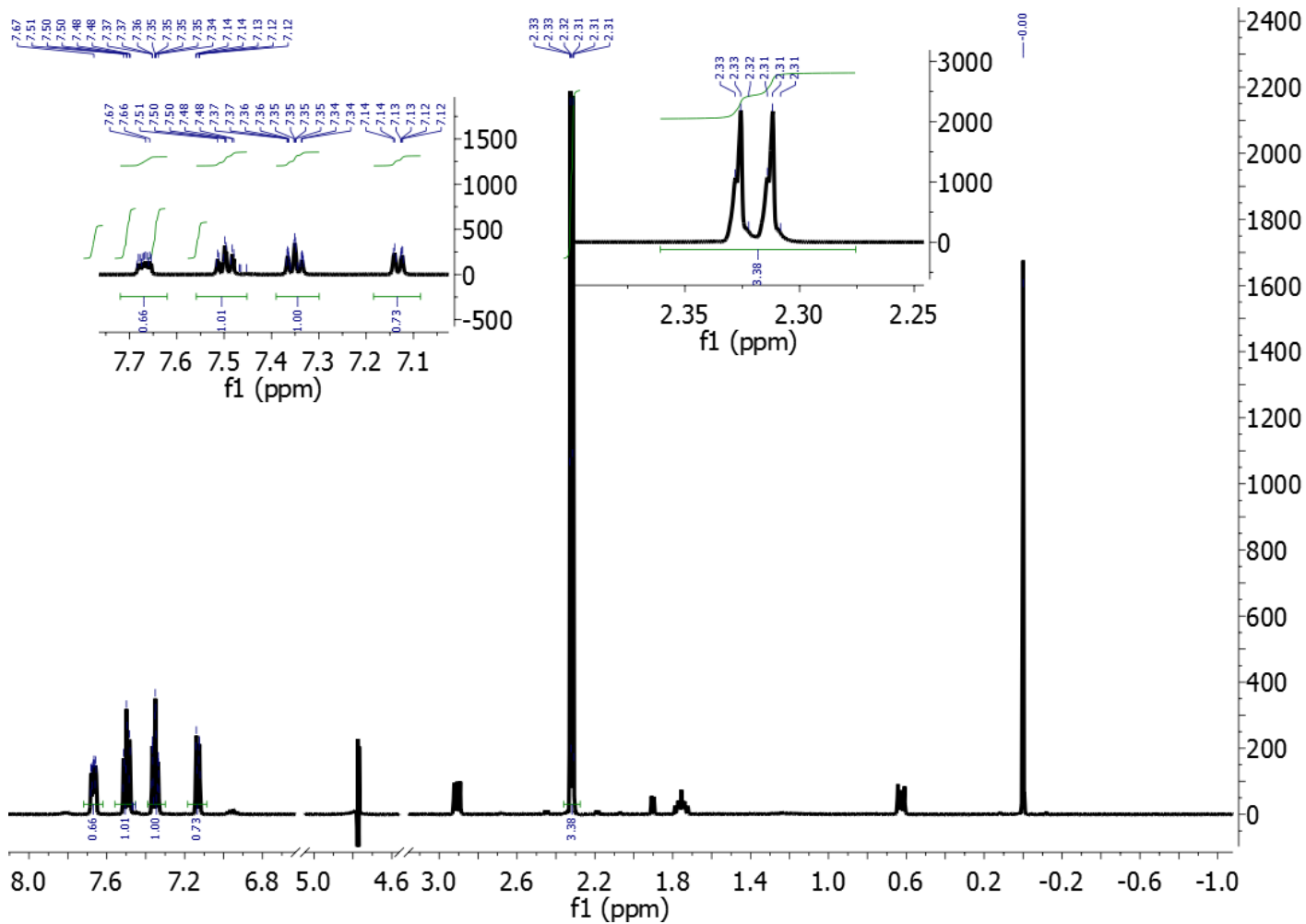


Figure S3: ^1H NMR spectrum (2). Resonances from the aromatic and acetyl protons are shown in the insets.

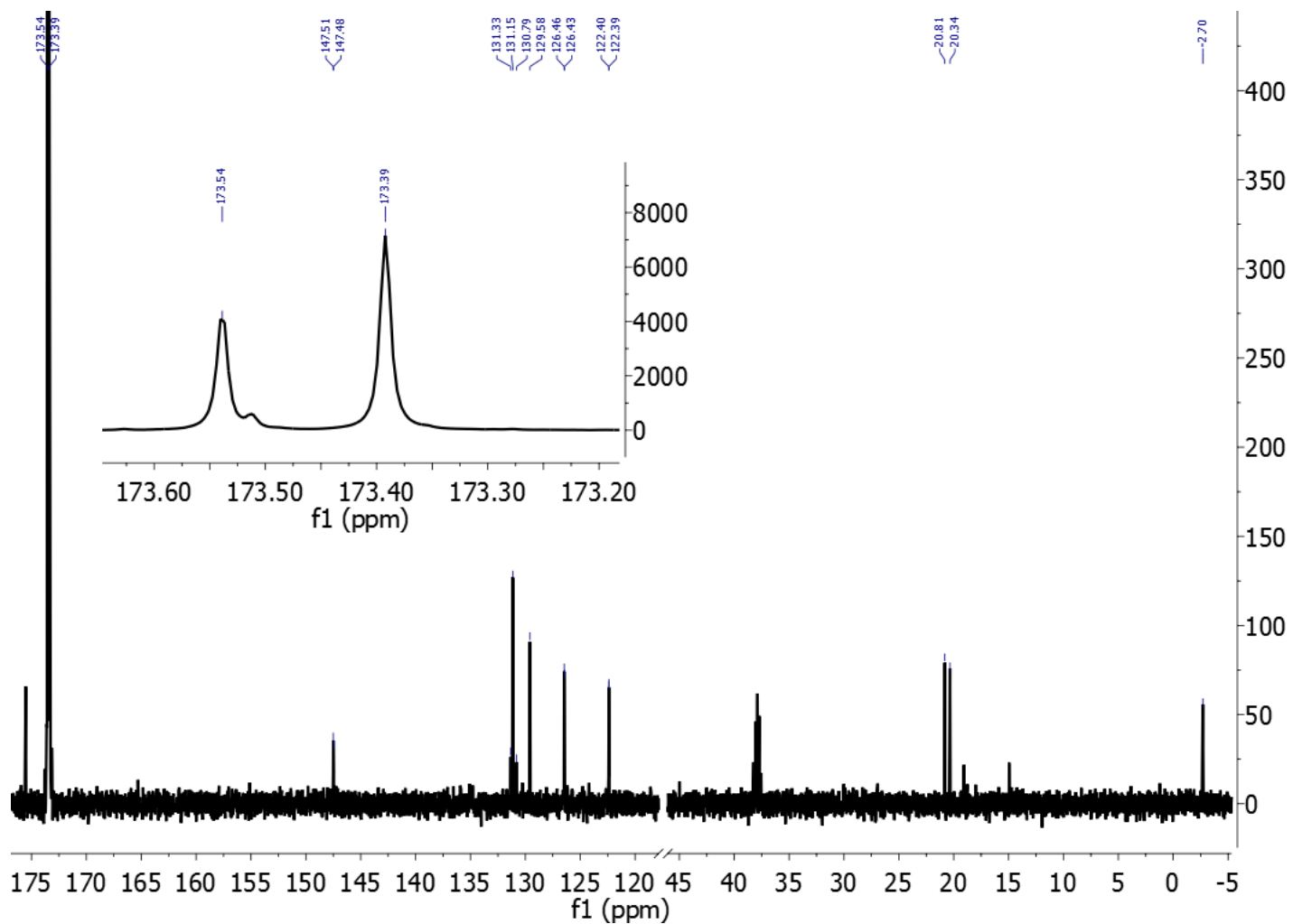


Figure S4: ^{13}C -NMR spectrum of (2). Resonances of the ^{13}C -labeled carbonyl and carboxylate carbons are shown in the inset.

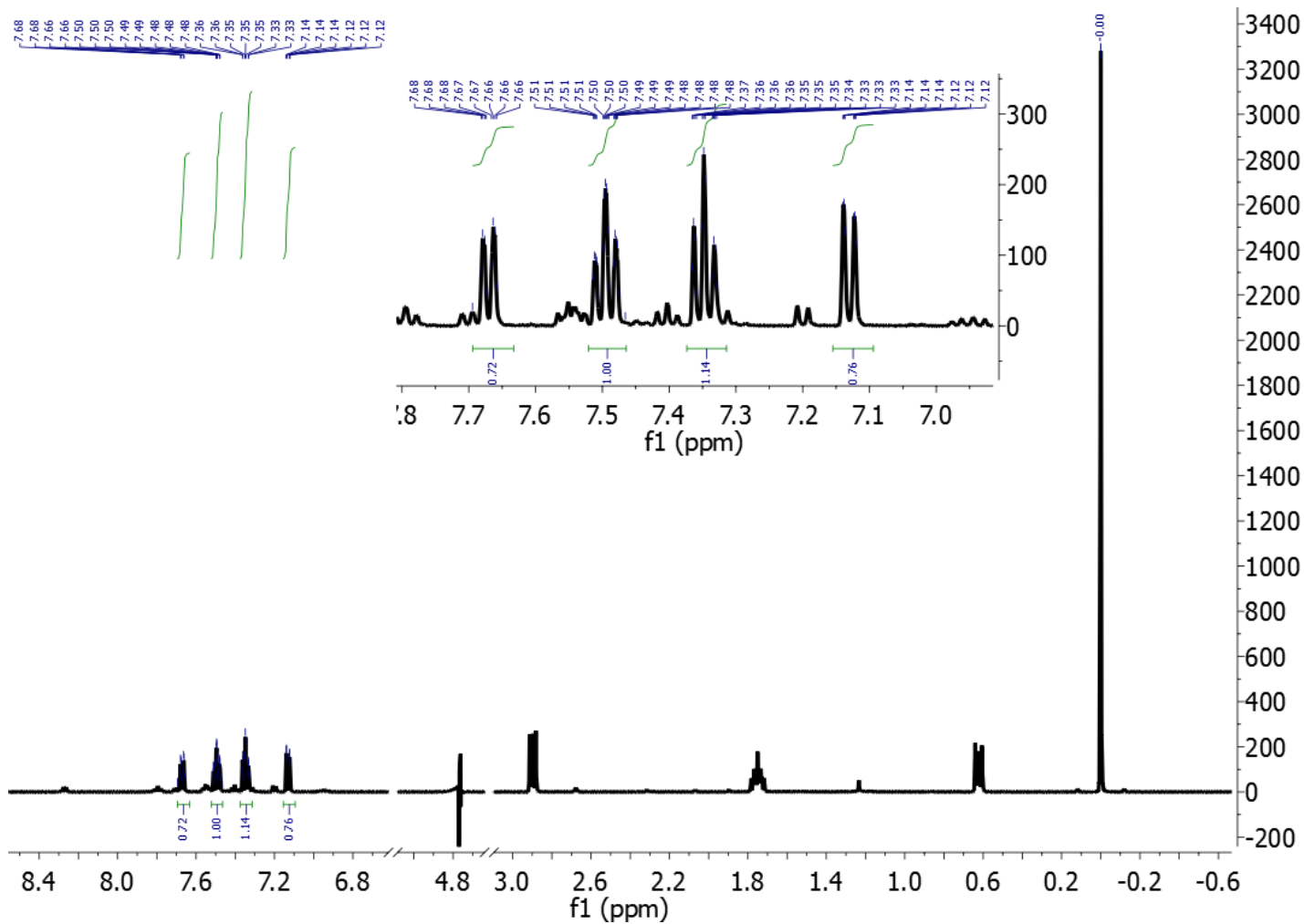


Figure S5: ¹H-NMR spectrum of (3). Resonances from the aromatic protons are shown in the inset.

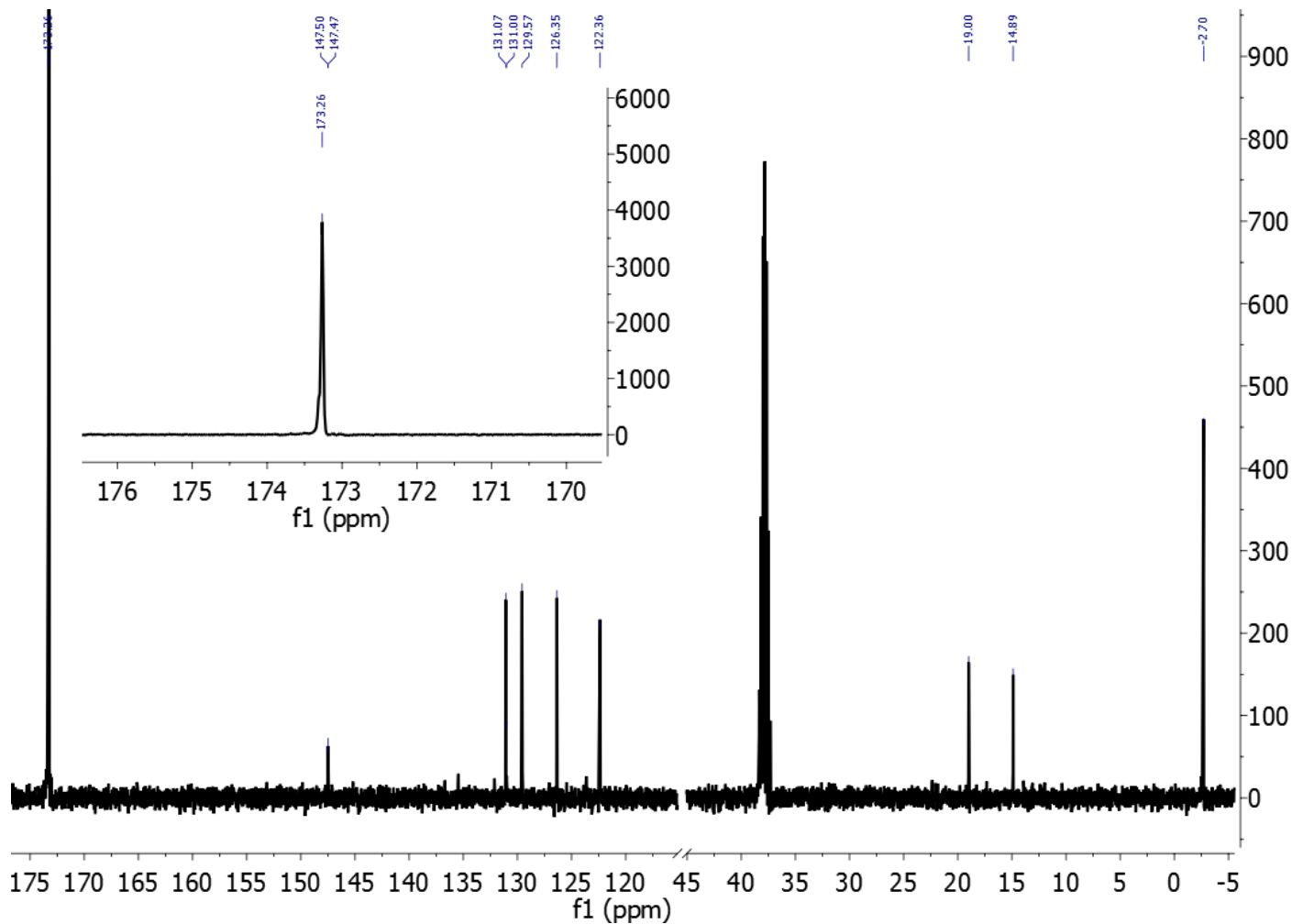


Figure S6: ^{13}C -NMR spectrum of (3). The resonance for the ^{13}C -labeled carbonyl carbon is shown in the inset.

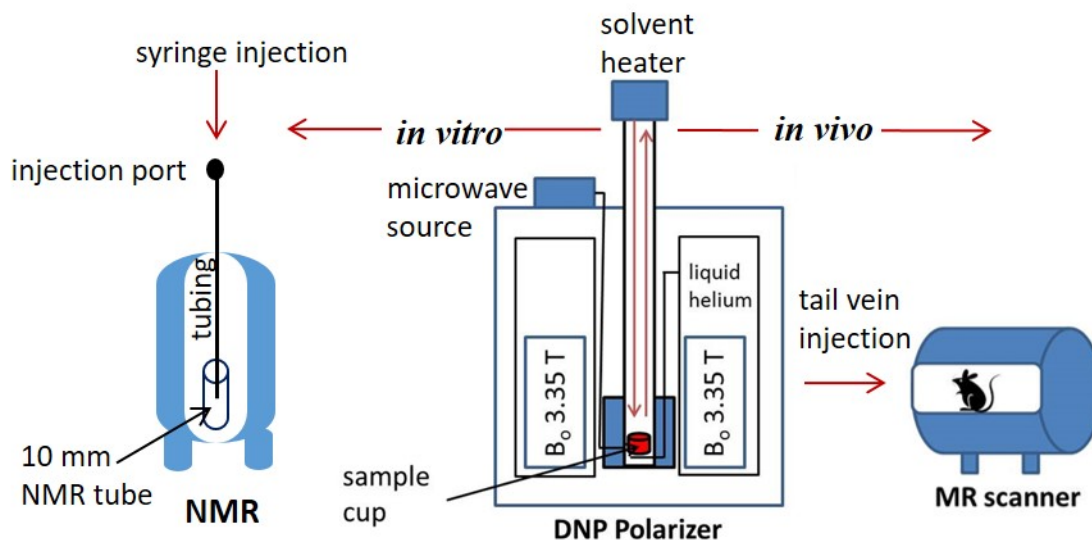
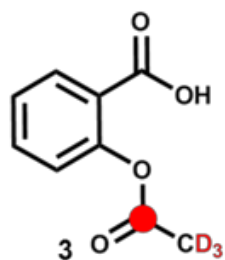


Figure S7: Schematic of the experimental setup including the DNP polarizer, NMR and MR instruments. ^{13}C -labeled aspirin is dissolved in a DMSO- d_6 water solution (1.5 M) along with Oxo63 (15.6 mM) and 1.8 mM Prohance were added to a DNP sample cup and inserted into polarizer. The sample is then irradiated using microwave irradiation while in a high magnetic field ($B_0 \sim 3.35$ T) and in a liquid helium bath. After solid state polarization buildup is complete, heated solvent is utilized to quickly dissolve frozen sample and to remove it from the polarizer. After dissolution, hyperpolarized aspirin solution is directly injected into an NMR tube for *in vitro* experiments or through tail vein catheter into a mouse for *in vivo* experiments.



[8-¹³C, 9d₃]-aspirin
(single label, deuterated)

Solid State Polarization Value

3050

Longitudinal Relaxation Constant (T_1)

8-¹³C 29 s

Figure S8: Characterization of compound (3). Solid-state dynamic nuclear polarization values for 168 μmol of deuterated, ¹³C-labeled aspirin derivative (3) ($n = 1$) along with its T_1 longitudinal relaxation constant at 7T field strength ($n = 1$).

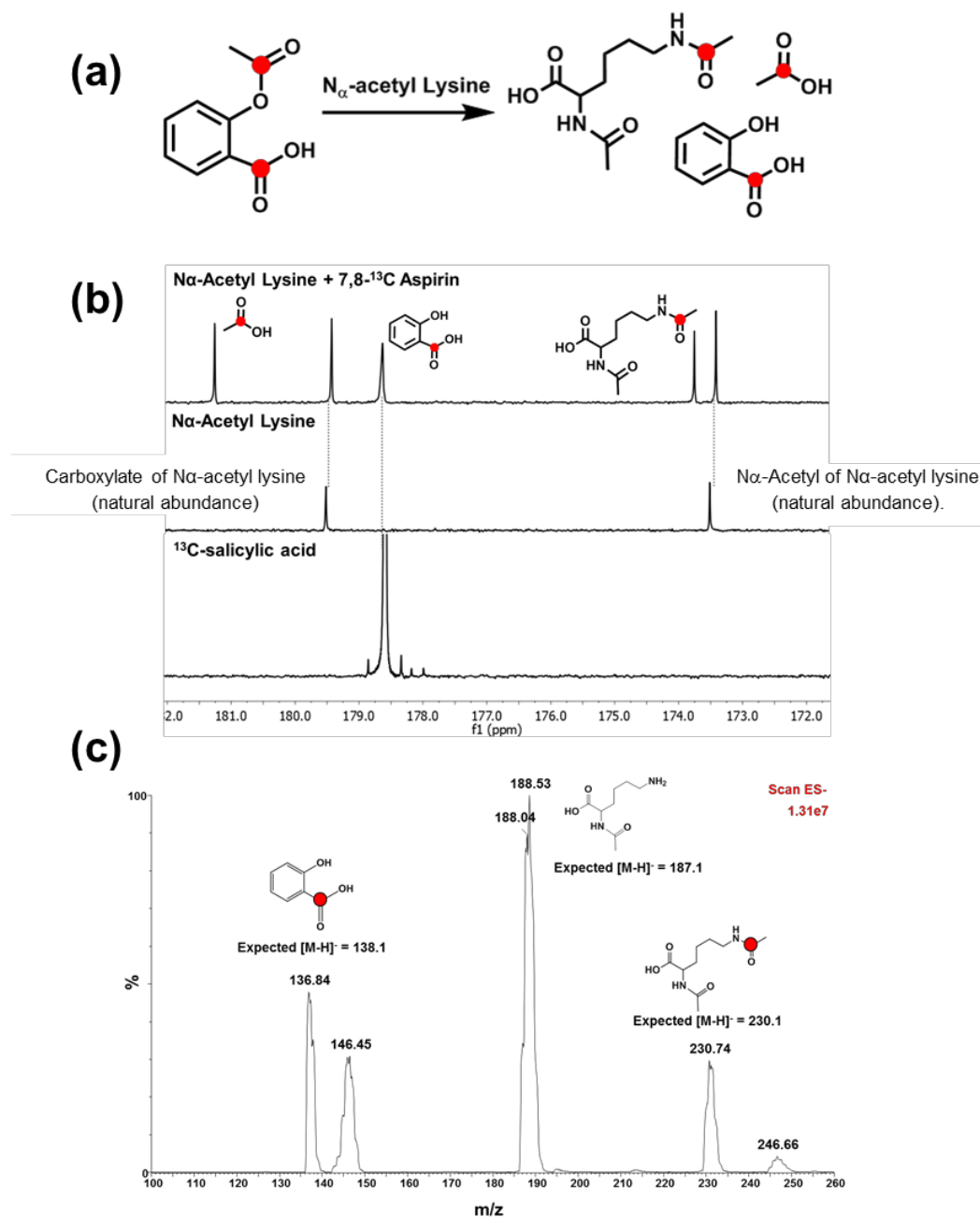


Figure S9: Monitoring the transacetylation of $N\alpha$ -acetyl lysine by [7,8- ^{13}C]-aspirin using ^{13}C -NMR spectroscopy and mass spectrometry. a) Transfer of ^{13}C -labeled acetate group from double-labeled aspirin derivative (2**) to the ϵ -amino group of $N\alpha$ -acetyl lysine. b) A model reaction consisting of 10 mM (**2**) and 160 mM $N\alpha$ -acetyl lysine in 0.5 M KOH (D_2O , pH = 12) was monitored in a 500 MHz NMR immediately after addition of (**2**). One hundred twenty-eight-scan spectrum was acquired, and the new peaks that appeared in the reaction were determined to be ^{13}C acetate, δ : 181.3 ppm; ^{13}C salicylic acid, δ : 178.7 ppm; and ϵ - ^{13}C acetyl-labeled $N\alpha$ -diacetyl lysine, δ : 173.8 ppm. The natural abundance carboxylate (δ : 179.4 ppm) and $N\alpha$ -acetyl carbons (δ : 173.4 ppm) of $N\alpha$ -acetyl lysine were also observed. All spectra were referenced to DSS. c) After the reaction was completed by ^{13}C -NMR, it was analyzed by electrospray ionization mass spectrometry (ESI, negative ion mode) to confirm the presence of ^{13}C -labeled salicylic acid and N,N -diacetyl lysine.**

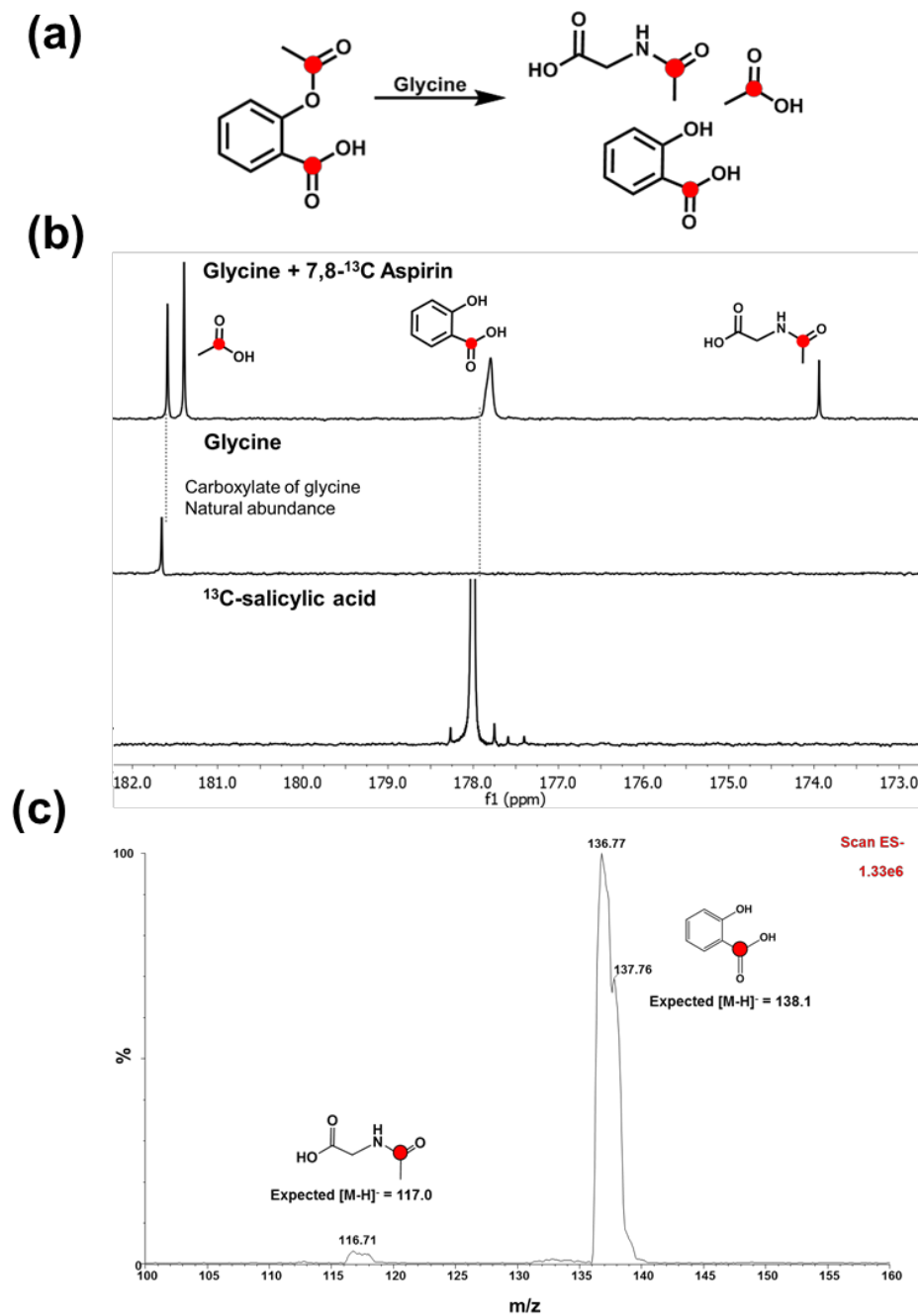


Figure S10: Monitoring the transacetylation of glycine by [7,8- ^{13}C]-aspirin using ^{13}C -NMR spectroscopy and mass spectrometry. a) Transfer of ^{13}C -labeled acetate group from double-labeled aspirin derivative (2**) to the α -amino group of glycine. b) A model reaction consisting of 10 mM (**2**) and 160 mM glycine in 0.5 M KOH (D_2O , pH = 12) was monitored in a 500 MHz NMR immediately after addition of (**2**). One hundred twenty-eight-scan spectrum was acquired, and the new peaks that appeared in the reaction were determined to be ^{13}C acetate, δ : 181.3 ppm; ^{13}C salicylic acid, δ : 177.8 ppm; and ^{13}C -labeled $\text{N}\alpha$ -acetyl glycine peak, δ : 173.9 ppm. The natural abundance carboxylate carbon of glycine (δ : 181.6 ppm) was also observed. All spectra were referenced to DSS. c) After the reaction was completed by ^{13}C -NMR, it was analyzed by ESI-MS (negative ion mode) to confirm the presence of ^{13}C -labeled salicylic acid and $\text{N}\alpha$ -acetyl glycine.**

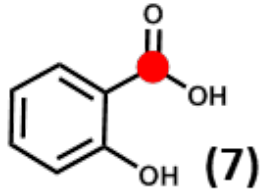
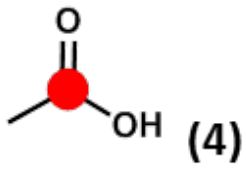
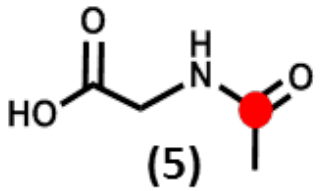
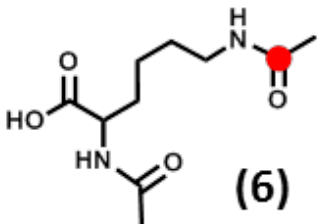
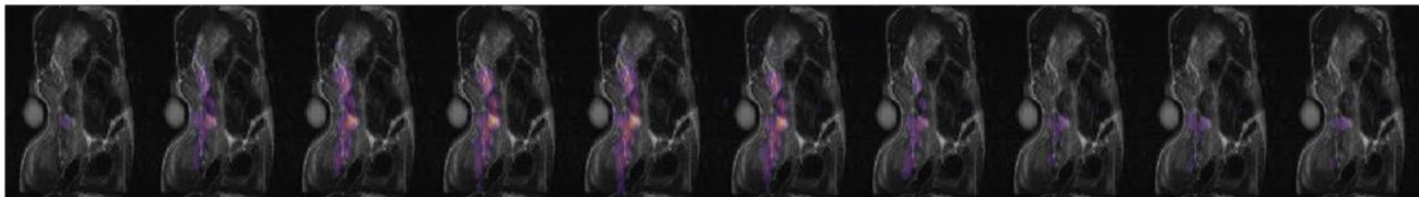
	Name	T_1
	1- ^{13}C salicylic acid	22
	1- ^{13}C acetate	77 ± 22
	^{13}C $N\alpha$ -acetyl glycine	30 ± 8
	$N\alpha$, ^{13}C $N\epsilon$ -acetyl lysine	16 ± 6

Table S1: Longitudinal relaxation (T_1) constants for aspirin hydrolysis and transacetylation products. T_1 values were obtained by exponential decay of the hyperpolarized product (12° flip angle). Errors are presented as \pm standard error of the mean for experiments where multiple replicates were carried out.

Start of acquisition approximately 20 seconds prior to injection

1 s

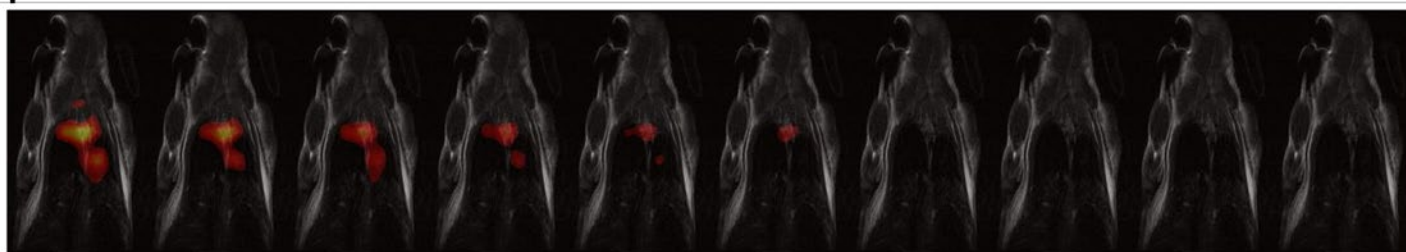


↑ Hyperpolarized 7,8-¹³C aspirin injection

Figure S11: Time course scans of imaging experiment described in Figure 4a. In this experiment, start of acquisition began several seconds before injection of hyperpolarized (**2**). The hyperpolarized signal was observed after 2 seconds and persisted for approximately 10 seconds (eight scans), with the signal observed primarily in the inferior vena cava. Scans were performed every 1 second.

Start of acquisition

1 s



Injection of Hyperpolarized 7,8-¹³C double labeled aspirin approximately 5 seconds before beginning of acquisition.

Figure S12: Time course scans of imaging experiment described in Figure 4b. In this experiment, the start of acquisition began 5 seconds after injection of hyperpolarized (2). The hyperpolarized signal was detected immediately and persisted for approximately 6 seconds (six scans), with the signal observed primarily in the heart. Scans were performed every 1 second.

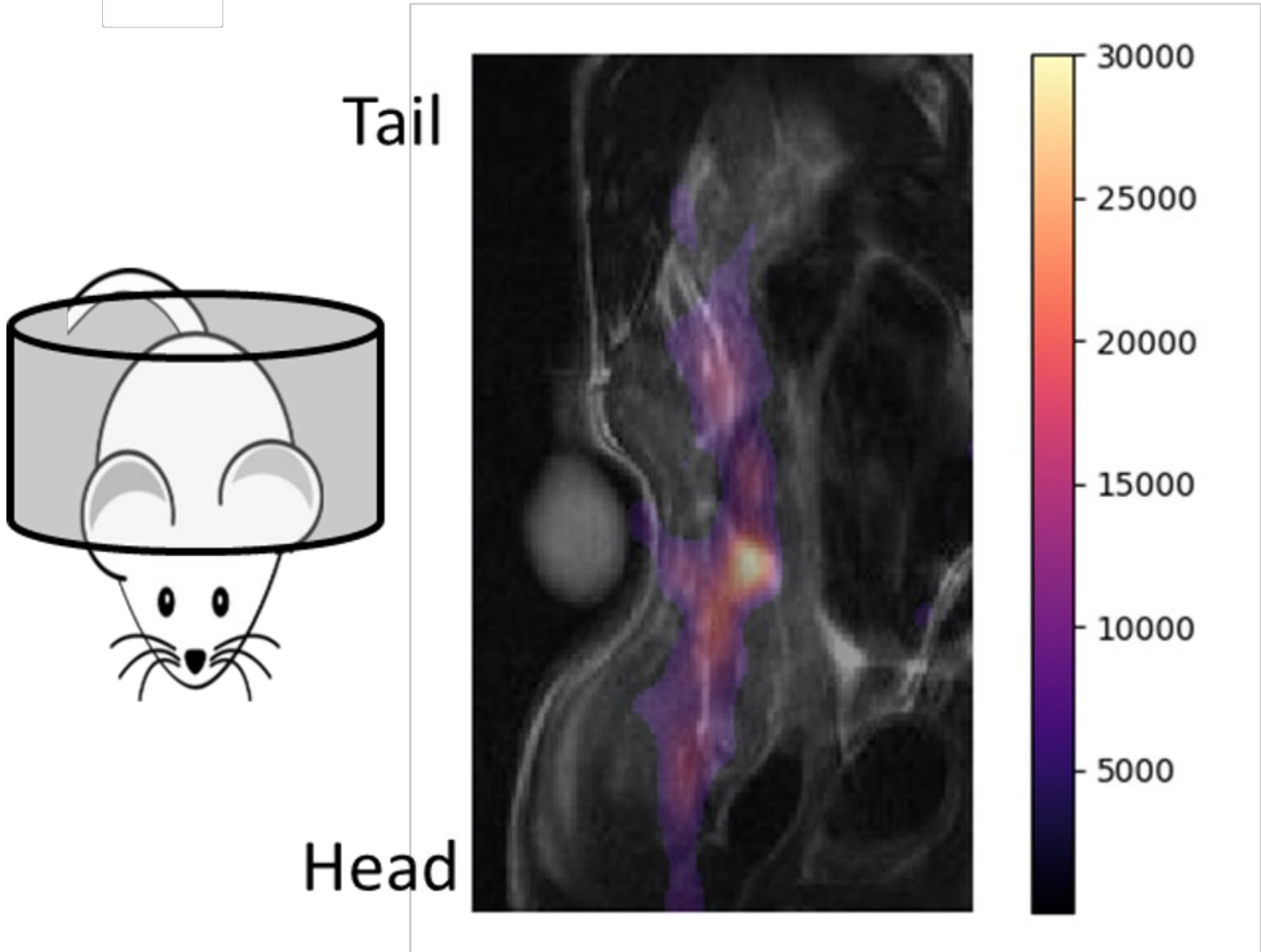


Figure S13: Enlarged Version of Figure 4a.

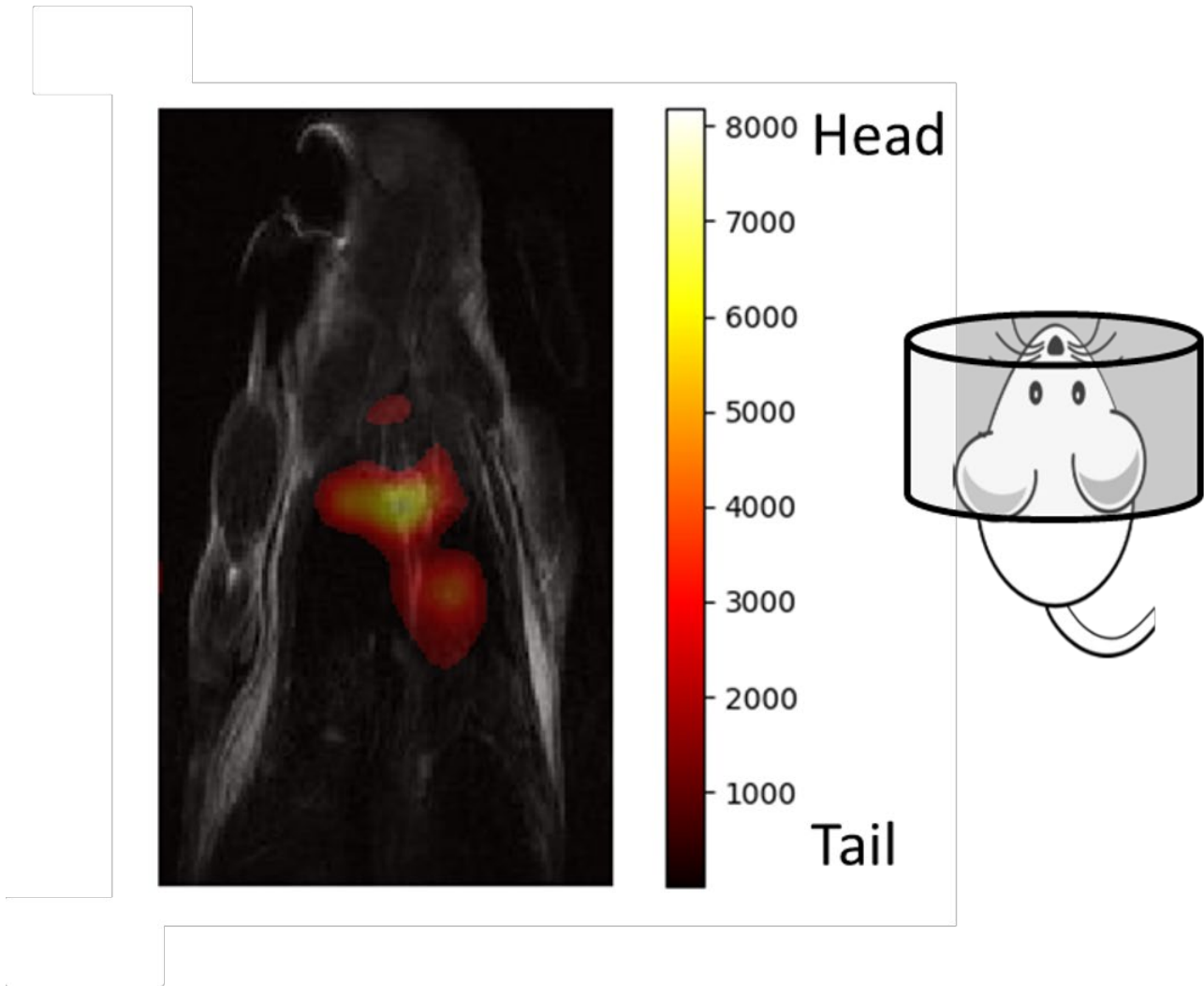


Figure S14: Enlarged Version of Figure 4b.

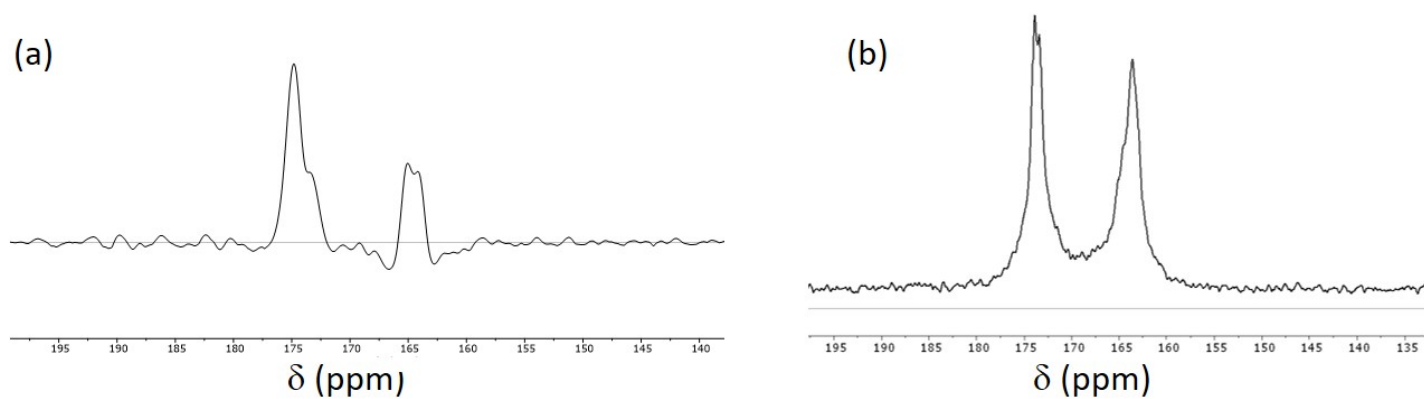


Figure S15: Summation of *in vivo* spectra. *In vivo* spectra shown in **Figure 4c (a)** and **Figure 4d (b)** were summed together to confirm that no metabolism was observed 30 seconds following injection of hyperpolarized aspirin. The hyperpolarized aspirin resonance at 175 ppm and the resonance for the ^{13}C -urea phantom at 165 ppm are easily observable.

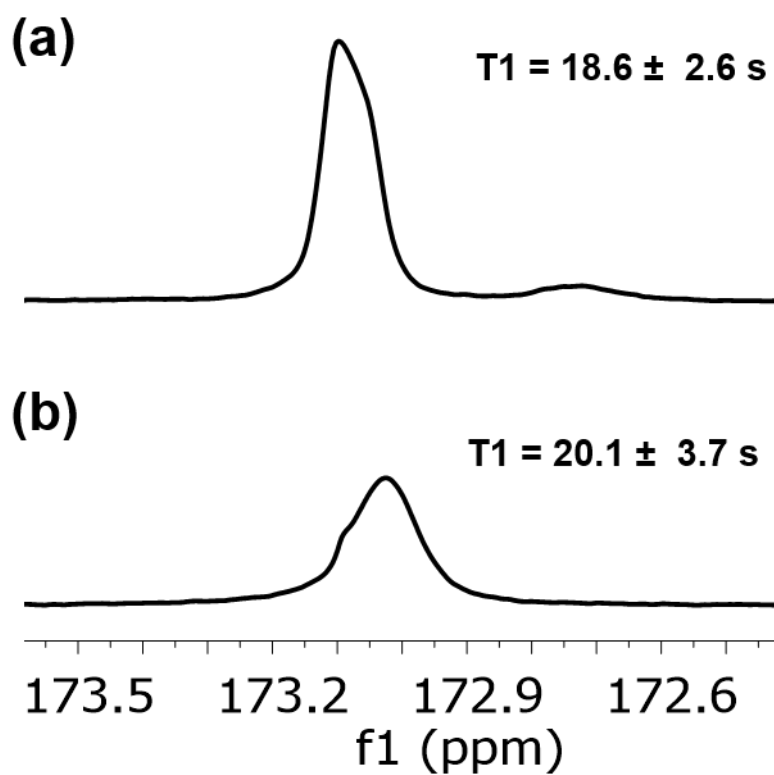


Figure S16: Interaction between hyperpolarized (1) and mouse blood components. Hyperpolarized (1) was incubated with freshly prepared mouse red blood cells (a) and mouse plasma (b) and monitored by ^{13}C -NMR. In both cases, the T_1 is significantly reduced relative to hyperpolarized (1) in PBS (~27 seconds, see **Figure 3a**).

References

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