Hyperpolarization of Amino Acid Derivatives in Water for Biological Applications

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Supporting Information

Contents

S1 Materials and Methods

Chemicals

N,N'-Dimethylformamide (DMF), diethyl ether methylene chloride (DCM), sodium bicarbonate ($NaHCO_3$) and concentrated HCl (10 M) were purchased from Thermo Fisher Scientific. Absolute methanol under inert gas, pentane, trifluoroacetic acid (TFA), Dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), Boc-Gly-OH (**1**), Boc-Ala-OH (**2**), Ac-Ala-OH (**4**), 2-hydroxyethyl acrylate (**5**), bis(norbornadiene) rhodium(I) tetrafluoroborate (**6**) and 1,4-Bis[(phenyl-3-propanesulfonate) phosphine] butane disodium salt (**7**) were purchased from Sigma-Aldrich. Boc-Gln-Xan-OH (**3**) was purchased from Bachem and $CDCl_3$ and D_2O (99.8%) from Cambridge isotopes. All chemicals were used as received without further purification.

NMR Spectroscopy

NMR spectra were recorded on either a Bruker AV600, a Bruker DRX500 or Bruker AV500 with cryoprobe. All experiments were performed at room temperature and the chemical shift data are reported in ppm for ^{1}H and ^{13}C spectra relative to tetramethylsilane or 4,4-dimethyl-4-silapentane-1-sulfonic acid.

Mass Spectrometry

Mass Spectrometry was performed on a LCT Premier XE of Micromass MS Technologies by electrospray ionization. Masses are given in m/z and were compared to the exact calculated mass.

S2 Synthetic procedures

Synthesis of 1a

In 15 mL methylene chloride, 1.0 mmol (175.2 mg) Boc-Gly-OH and 0.25 mmol (30 mg) DMAP were dissolved and 1.0 mmol (116.1 mg, 114.8 µL) 2-hydroxyethyl acrylate added. The solutions was cooled to 0°C and 1.1 mmol (227.0 mg) dicyclohexylcarbodiimide in 5 mL methylene chloride added. It was stirred at 0°C for 1 hour, was subsequently allowed to warm up to room temperature and stirred for an additional 4 hours. Afterwards, the solution was concentrated to dryness, the residue taken up in 15 mL methylene chloride, filtered and washed two times with 5 mL HCl in water (0.5 M) and two times with a saturated aqueous $NaHCO_3$ solution. The organic phase was collected, residual solvent removed and the product further purified by column chromatography with 95% DCM and 5% MeOH. The product was received as colorless oil. ^{1}H NMR (600 MHz, [D]$CDCl_3$, 25°C): δ=6.32 (d, ^3J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^3J(H,H)=17.4 Hz, ^3J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^3J(H,H)=10.7 Hz, 1H; CH), 4.37 (m, 4H; CH_2,CH_2), 3.93 (2d, ^2J(H,H)=9.0 Hz, 2H; CH_2), 1.43 ppm (s, 9H, 3CH_3); ^{13}C NMR (151 MHz, [D]$CDCl_3$, 25°C): δ=170.45, 165.74, 155.58, 134.47,127.72, 79.95, 62.80, 61.87, 42.19, 28.16 ppm; MS: m/z (%):calculated: 296.1110, found: 296.1108 $[M+Na]^+$.

Synthesis of 2a

Synthesis of **2a** was achieved according to the same procedure as described for **1a** by using **2** as a reactant instead of **1**. The product was received as colorless oil. ^{1}H NMR (600 MHz, [D]$CDCl_3$, 25 °C): δ=6.32 (d, ^3J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^3J(H,H)=17.4 Hz, ^3J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^3J(H,H)=10.7 Hz, 1H; CH), 4.37 (m+q, 4H+1H; CH_2,CH_2,CH), 1.43 (s, 9H, 3CH_3), 1.37 ppm (d, ^3J(H,H)=7.4 Hz, 3H, CH_3); ^{13}C NMR (151 MHz, [D]$CDCl_3$, 25°C): δ=173.08, 165.70, 154.96, 131.40,127.75, 79.77, 62.70, 61.89, 49.04, 28.17, 18.43 ppm; MS: m/z (%):calculated: 310.1266, found: 310.1275 $[M+Na]^+$.

Synthesis of 3a

0.5 mmol (mg) **3** and 0.25 mmol (30 mg) DMAP were dissolved in 0.5 mL DMF, followed by addition of 0.5 mmol (58.1 mg, 57.4 µL) 2-hydroxyethyl acrylate and 15 mL DCM. The solutions was cooled to 0°C and 1.1 mmol (227.0 mg) dicyclohexylcarbodiimide in 5 mL methylene chloride added. It was stirred at 0°C for 1 hour, was subsequently allowed to warm up to room temperature and stirred for an additional 5 hours. Afterwards, the solution was concentrated to dryness, the residue taken up in 15 mL methylene chloride, filtered and washed two times with 5 mL HCl in water (0.5 M) and two times with a saturated aqueous $NaHCO_3$ solution. The organic phase was collected, residual solvent removed, washed with pentane and the product further purified by column chromatography with 95% DCM and 5% MeOH. The product was received as

a colorless solid. ^{1}H NMR (500 MHz, [D]$CDCl_3$, 25°C): δ= 7.47 (m, 2H, CH), 7.30 (m, 2H, CH), 7.11 (m, 4H, CH), 6.52 (m, 1H, CH), 6.34 (d, ^{3}J(H,H)=17.4 Hz, 1H; CH), 6.03 (dd, ^{3}J(H,H)=17.4 Hz, ^{3}J(H,H)=10.7 Hz, 1H; CH), 5.78 (d, ^{3}J(H,H)=10.7 Hz, 1H; CH), 4.35 (m+t, 4H+1H; CH_2,CH_2,CH), 2.29 (m, 2H, CH_2), 2.21 (m, 1H, CH), 2.04 (m, 1H, CH), 1.41 ppm (s, 9H, $3CH_3$); ^{13}C NMR (126 MHz, [D]$CDCl_3$, 25°C): δ=171.99, 170.68, 165.73, 155.40, 151.03, 131.59, 129.51, 129.15, 127.58, 123.48, 120.95, 116.52, 80.04, 63.05, 61.87, 52.91, 43.84, 32.34, 29.60, 28.26 ppm; MS: m/z (%):calculated: 547.2056, found: 547.2036 $[M+Na]^+$.

Synthesis of 4a

Synthesis of **4a** was achieved according to the same procedure as described for **3a** by using **4** as a reactant instead of **3**. The product was received as colorless oil. . ^{1}H NMR (500 MHz, [D_2]D_2O, 25°C): δ=6.32 (d, ^{3}J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^{3}J(H,H)=17.4 Hz, ^{3}J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^{3}J(H,H)=10.7 Hz, 1H; CH), 4.32 (m, 4H; CH_2,CH_2), 4.27 (q, ^{3}J(H,H)=7.4 Hz, 1H, CH), 1.87 (s, 3H, CH_3), 1.26 ppm (d, ^{3}J(H,H)=7.4 Hz, 3H, CH_3); ^{13}C NMR (126 MHz, [D_2]D_2O, 25°C): δ=174.38, 173.38, 167.99, 132.80, 127.07, 63.27, 62.46, 48.66, 21.28, 15.64 ppm; MS: m/z (%):calculated: 252.0848, found: 252.0852 $[M+Na]^+$.

Amine-deprotection of 1a and 2a

The Boc-group of **1a** and **2a** was removed by dissolving them in excess of a 1:1 by volume mixture of DCM and TFA and subsequent stirring for 30 minutes at room temperature. The residues were taken up in deionized water, filtered and the solvent removed under vacuum to yield product **1b** and **2b** as colorless oils. All deprotected derivatives were found to degrade rapidly and were stored under inert gas at 4°C. **1b**: ^{1}H NMR (500 MHz, [D_2]D_2O, 25°C): δ=6.32 (d, ^{3}J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^{3}J(H,H)=17.4 Hz, ^{3}J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^{3}J(H,H)=10.7 Hz, 1H; CH), 4.42 (m, 4H; CH_2,CH_2), 3.82 ppm (s, 2H; CH_2); ^{13}C NMR (126 MHz, [D_2]D_2O, 25°C): δ=168.05, 167.80, 132.67, 127.00, 64.10, 62.34, 39.85 ppm; MS: m/z (%):calculated: 174.0766, found: 174.0762 $[M+H]^+$. **2b**: ^{1}H NMR (500 MHz, [D_2]D_2O, 25°C): δ=6.32 (d, ^{3}J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^{3}J(H,H)=17.4 Hz, ^{3}J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^{3}J(H,H)=10.7 Hz, 1H; CH), 4.40 (m, 4H; CH_2,CH_2), 4.09 (q, ^{3}J(H,H)=7.4, 1H, CH); 1.43 ppm (d, ^{3}J(H,H)=7.4, 3H; CH_3); ^{13}C NMR (126 MHz, [D_2]D_2O, 25°C): δ=170.36, 168.02, 132.70, 127.00, 64.21, 62.32, 48.56, 14.87 ppm; MS: m/z (%):calculated: 188.0923, found: 188.0915 $[M+H]^+$.

Amine-deprotection of 3a

3a was dissolved in an excess of a 1:1: by volume mixture of DCM and TFA. After 5 minutes of stirring at room temperature the solvents were removed under vacuum.

Diethyl ether was added (2mL) twice and decanted. The remaining residue was washed three times with pentane and yielded 3b as colorless wax. ^{1}H NMR (500 MHz, $[D_2]D_2O$, 25°C): δ=6.32 (d, ^{3}J(H,H)=17.4 Hz, 1H; CH), 6.07 (dd, ^{3}J(H,H)=17.4 Hz, ^{3}J(H,H)=10.7 Hz, 1H; CH), 5.87 (d, ^{3}J(H,H)=10.7 Hz, 1H; CH), 4.40 (m, 4H; CH_2,CH_2), 4.08 (t, ^{3}J(H,H)=7.0, 1H, CH), 2.36 (m, 2H, CH_2), 2.09 ppm (m, 2H, CH_2); ^{13}C NMR (126 MHz, $[D_2]D_2O$, 25°C): δ=176.57, 169.40, 167.96, 132.74, 126.97, 64.32, 62.24, 51.99, 30.08, 25.15; MS: m/z (%):calculated: 245.1102, found: 245.1105 $[M+H]^+$.

Synthesis of the water soluble PHIP catalyst

1,4-bis-[(phenyl-3-propane sulfonate) phosphine]butane (norbornadiene) rhodium(I)tetrafluoroborate (**8**), which has previously been used as water soluble PHIP catalyst has been synthesized in a different procedure as reported:[S1] Under argon atmosphere, in two different schlenk flasks, 10 µmol (7.5 mg) of **6** and 10 µmol (11.3 mg) of **7** were dissolved in 5 mL absolute methanol. After stirring for 30 minutes, **7** was slowly added over 1 minute to **6** and stirring continued for 1 hour followed by concentration to dryness under vacuum.

S3 NMR spectra

NMR spectra are shown of the reactants (**1b**-**3b**, **4a**), which have been used for the hyperpolarization experiments.

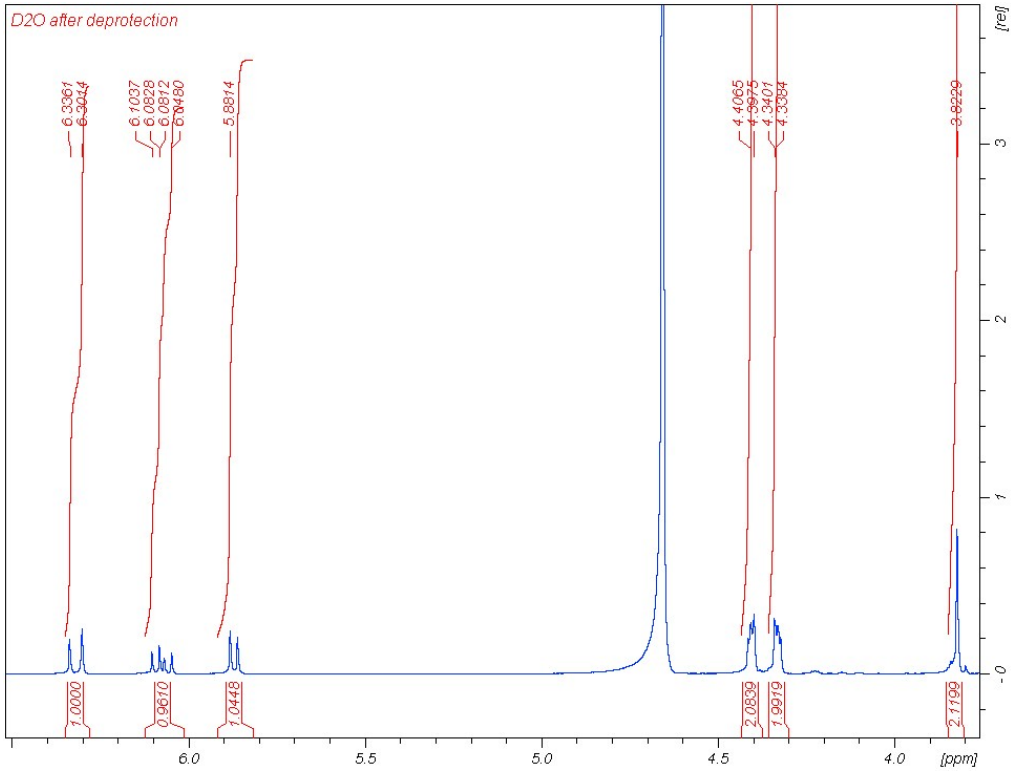


Fig. S1. ^{1}H NMR spectrum of **1b** at B_0 = 11.7 T (500 MHz ^{1}H frequency) acquired with 8 scans.

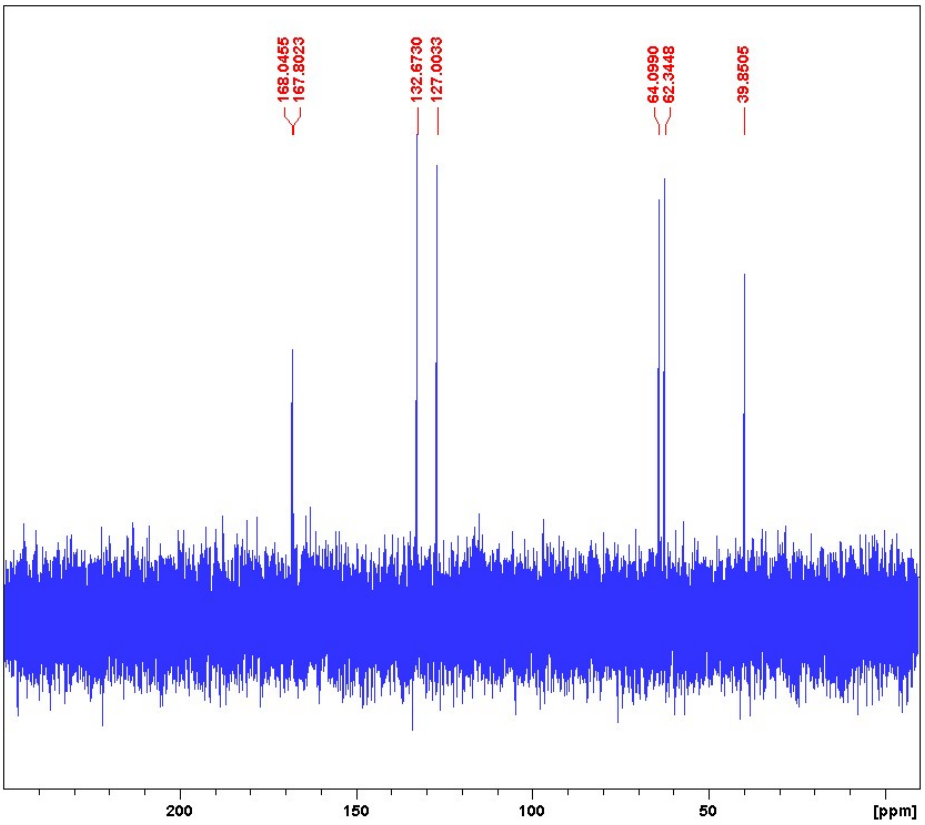


Fig. S2. ^{13}C NMR spectrum of **1b** at B_0 = 11.7 T (126 MHz ^{13}C frequency) acquired with 64 scans.

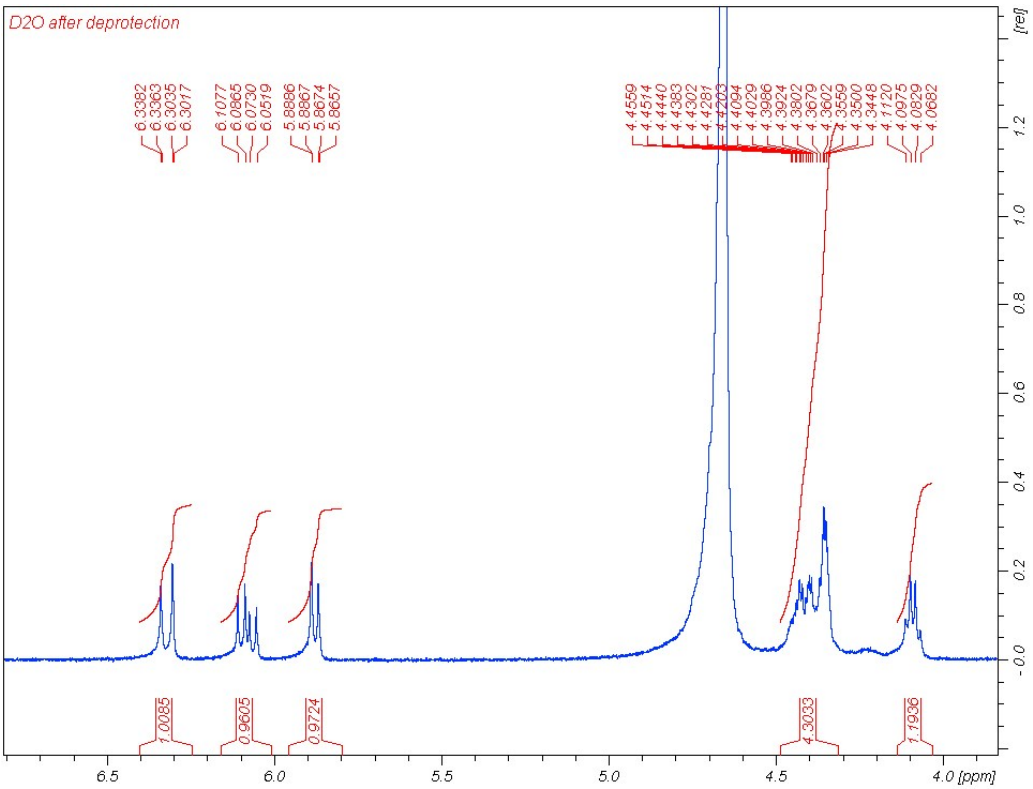


Fig. S3. ^{1}H NMR spectrum of **2b** at B_0 = 11.7 T (500 MHz ^{1}H frequency) acquired in a single scan.

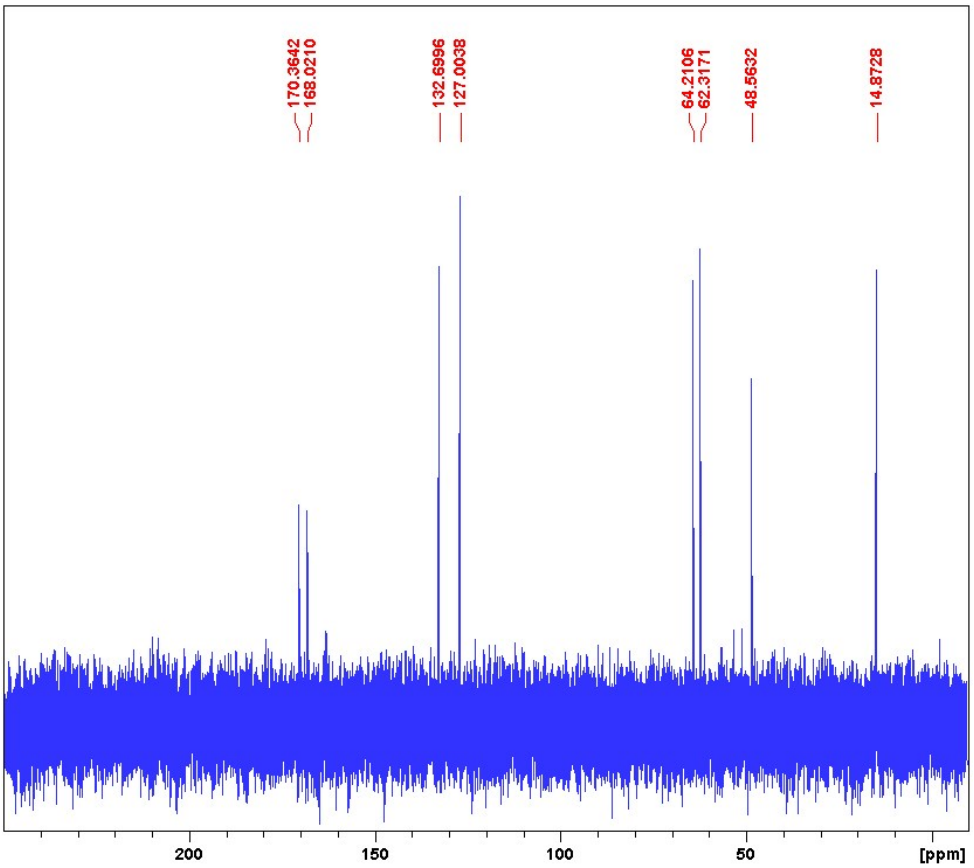


Fig. S4. ^{13}C NMR spectrum of **2b** at B_0 = 11.7 T (126 MHz ^{13}C frequency) acquired with 128 scans

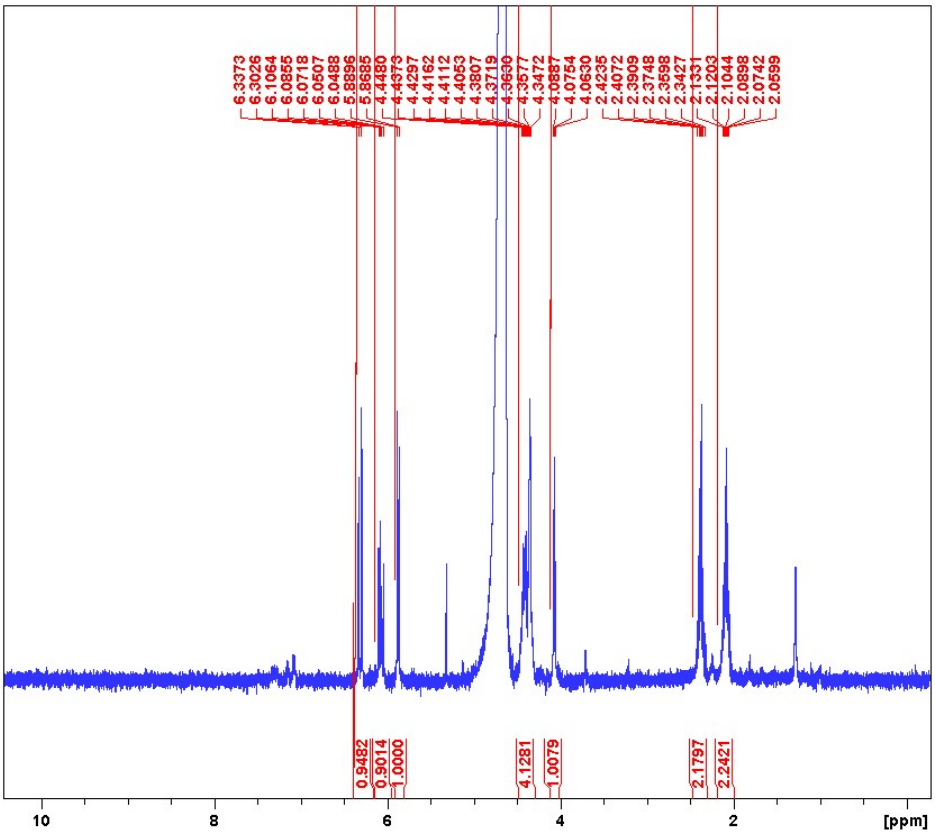


Fig. S5. ^{1}H NMR spectrum of **3b** at B_0 = 11.7 T (500 MHz ^{1}H frequency) acquired with 8 scans

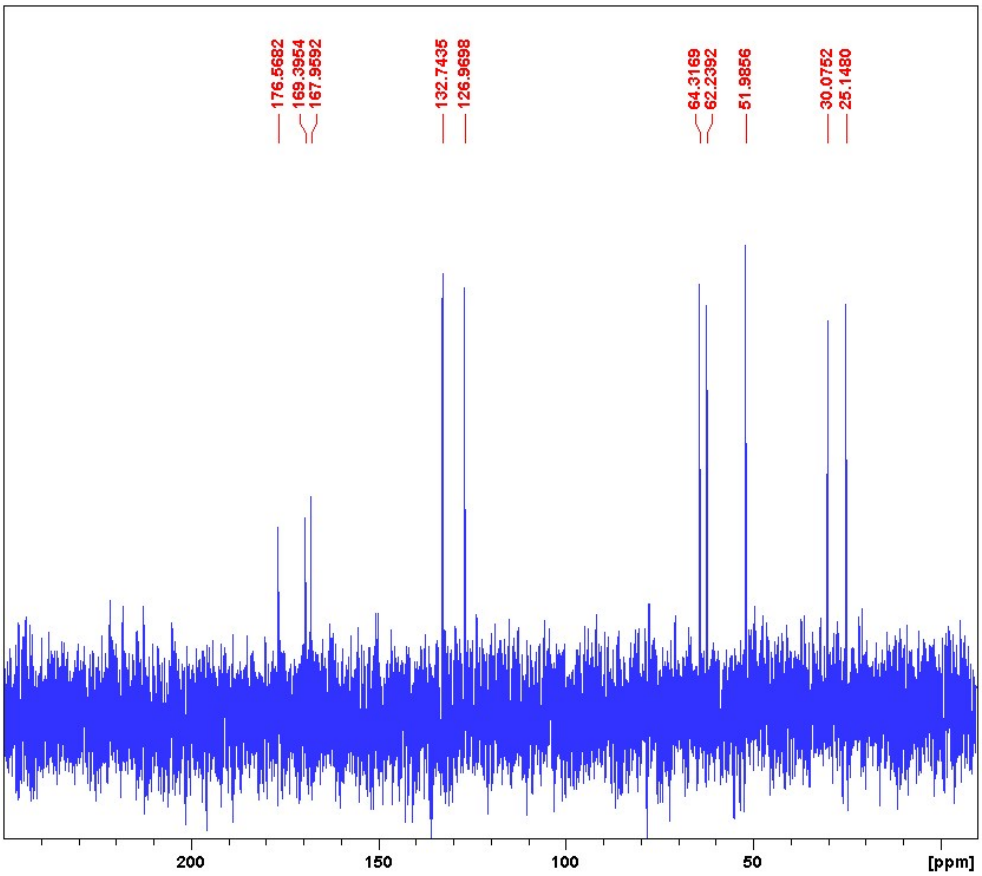


Fig. S6. ^{13}C NMR spectrum of **3b** at B_0 = 11.7 T (126 MHz ^{13}C frequency) acquired with 512 scans

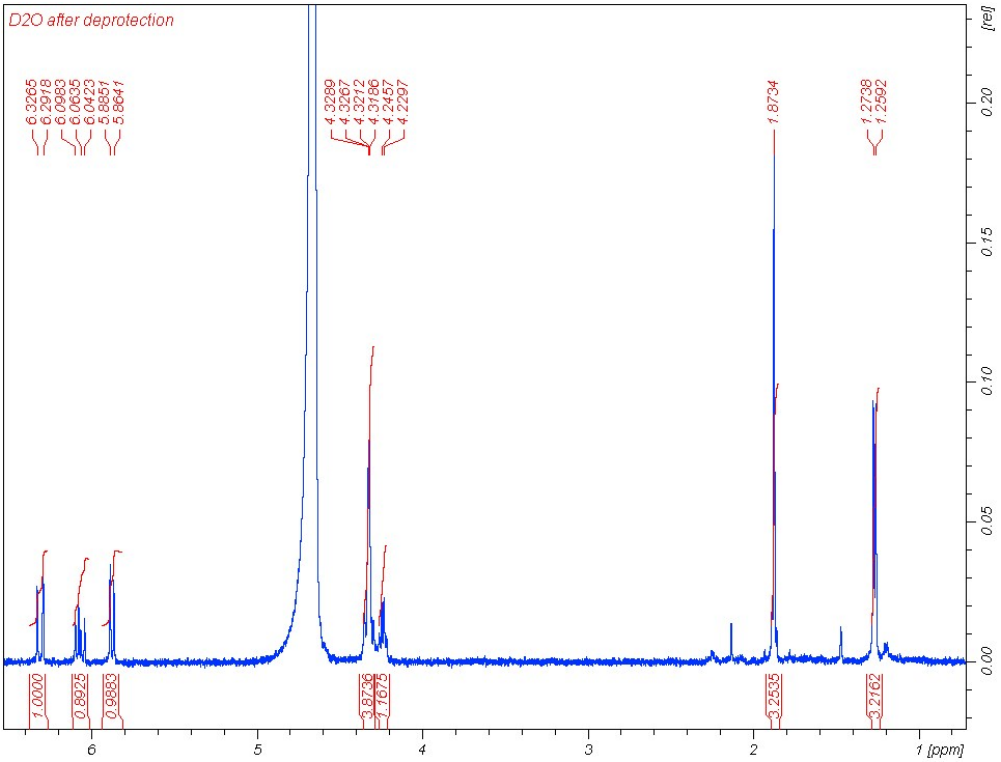


Fig. S7. ^{1}H NMR spectrum of **4a** at B_0 = 11.7 T (500 MHz ^{1}H frequency) acquired with 8 scans.

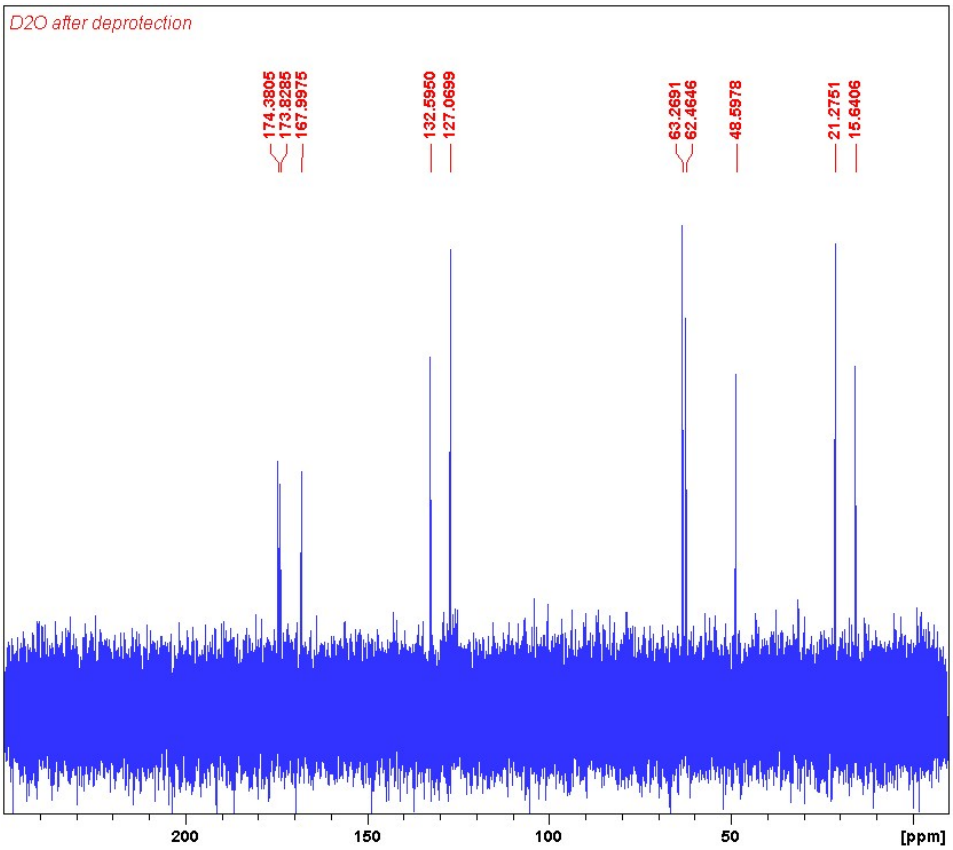
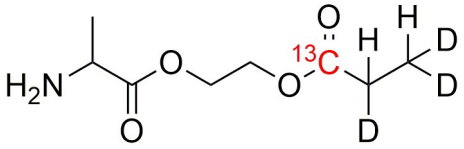


Fig. S8. ^{13}C NMR spectrum of **4a** at B_0 = 11.7 T (126 MHz ^{13}C frequency) acquired with 512 scans.

S4 *Para*-hydrogen experiments

Proton *para*-hydrogen experiments were performed on a Bruker AV600 spectrometer (B_0 = 14.1 T). *Para*-hydrogen of about 95% *para*-state enrichment was produced using a commercial polarizer located at Cedars Sinai Medical Center.[S2] Samples were prepared in 5 mm J. Young tubes from New Era under inert gas with sample (**1b**-**3b**, **4a**) concentrations of 25 mM and 2.5 mM catalyst concentration in 0.5 mL D_2O. Each sample was pressurized with 5 bars of *para*-hydrogen, shaken for 10 s in the earth's magnetic field (ALTADENA conditions) and transported to the center of the magnet within 10 s, where the spectrum was recorded in a single scan (45°-pulse). The experiments were repeated three times with different samples. After the hyperpolarization experiment, a spectrum was recorded with the formed product in thermal equilibrium, the signal enhancement and the corresponding nuclear magnetic polarization calculated. All experiments were conducted at pH = 6.5±0.5. Polarization transfer experiments were performed on a home-built polarizer[S2] with a 10 mM solution of the unprotected alanine derivative and 2.2 mM catalyst concentration. The sample was heated up to 60°C and mixed with 5 bars of *para*-hydrogen. Subsequently a polarization sequence introduced by Goldman *et al.* was applied.[S3] After a 20 s transport the NMR signal was detected in a 9.4 T 94/20 Bruker Biospec. This experiment was performed twice after optimization of the pulse sequence. In order to determine the polarization, the hyperpolarized signal was compared to its signal in thermal equilibrium averaged 200 times. *J*-couplings and

parameters applied for the sequence are as follows (refer to scheme S1 for the derivative) and a schematic drawing of the sequence can be found in figure S9:



Scheme S1. Alanine-derivative that was polarized at the indicated ^{13}C nuclei using a polarization transfer sequence.

J-couplings: $^{3}J_{H,H}$ = 7.24 Hz; $^{3}J_{13C,H}$ = -5.62 Hz; $^{2}J_{13C,H}$ = 7.57 Hz

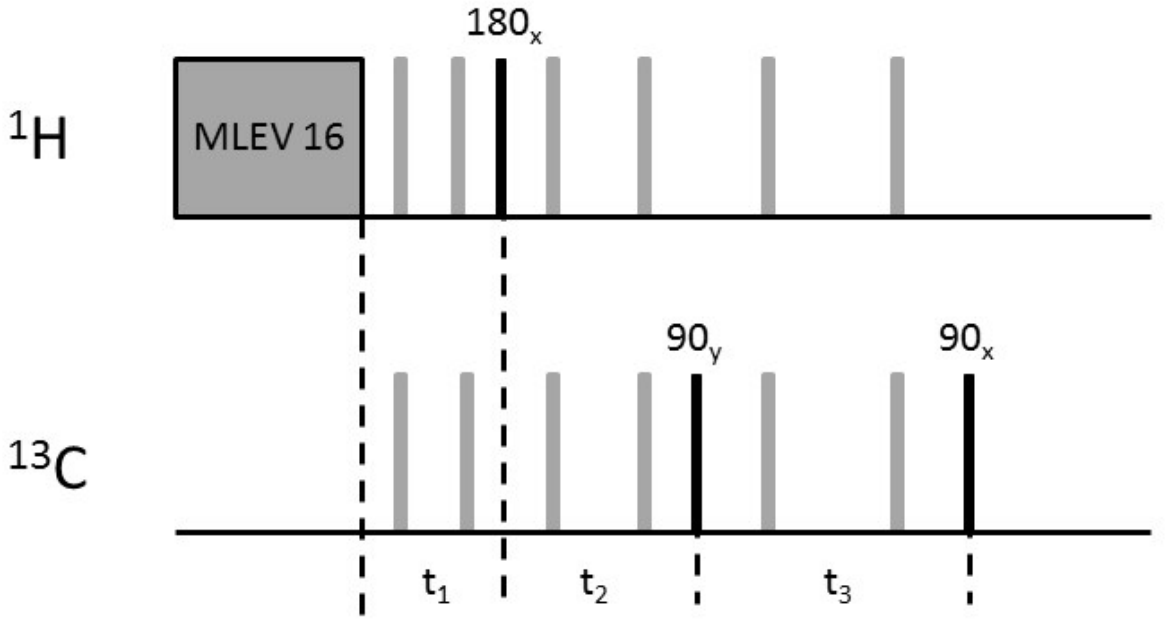


Fig. S9. Schematic of the Goldman sequence used. Grey bars indicate 180° pulses at one fourth and three forth of the evolution periods. The black bars are the pulses important for the polarization transfer.[S5]

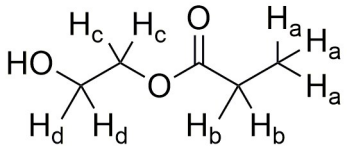
The timings are: t_1 = 28.28 ms , t_2 = 36.20 ms, t_3 = 50.34 ms

S5 Relaxation times of the investigated molecules

The longitudinal relaxation times (T_1) of the synthesized molecules were investigated after hydrogenation with an AV500 system (B_0 = 11.7 T) with cryoprobe for the protonated derivatives with an inversion recovery experiment and one scan. For carbon experiments 8 scans were used with an inversion recovery experiment with inverse gated decoupling . The T_1 for the deuterated and ^{13}C labelled derivative was recorded with an AV600 system (B_0 = 14.1 T) with 1 scan for protons and 8 scans for carbon. All experiments were performed at room temperature. HEP, Ala-HEP and Ac-Ala-HEP were investigated in D_2O in the presence of air whereas for solubility and stability reasons T_1 of the N-protected Gly-HEP and Gln-HEP derivatives were measured in methanol-d_4. The pH of

the samples was 6.5±0.5 and the catalyst concentration 10 mol% of the material, which means 2.5 mM.

HEP:



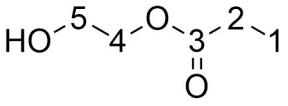


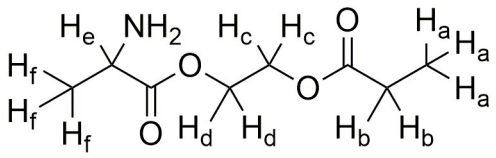
Table S1. Longitudinal ^{1}H relaxation times of HEP

^{1}H position	a	b	c	d
T_1/s	4.0	3.5	2.3	2.1

Table S2. Longitudinal ^{13}C relaxation times of HEP

^{13}C position	1	2	3	4	5
T_1/s	5.5	4.6	15.9	1.9	2.0

Ala-HEP:



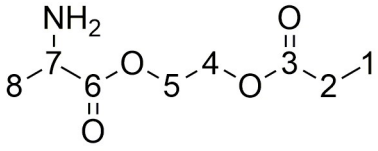


Table S3. Longitudinal ^{1}H relaxation times of Ala-HEP

^{1}H position	a	b	c	d	e	f
T_1/s	4.1	3.0	0.9	0.9	5.9	1.2

Table S4. Longitudinal ^{13}C relaxation times of Ala-HEP

^{13}C position	1	2	3	4	5	6	7	8
T_1/s	5.9	5.8	14.2*	1.0	1.1	10.2	2.4	1.3

*T1 for the deuterated derivative corresponds to 21.5s

Ac-Ala-HEP:

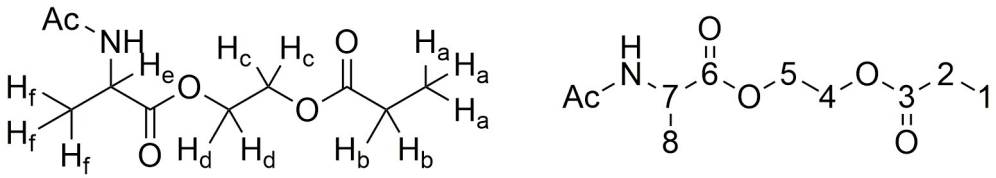


Table S5. Longitudinal ^{1}H relaxation times of Ac-Ala-HEP

^{1}H position	a	b	c	d	e	f
T_1/s	3.4	3.2	2.4*	1.0	2.4*	0.7

*chemical shift values of c and d are merged. Therefore the values reported reflect a time extracted from a monoexponential fit over the two proton signals

Table S6. Longitudinal ^{13}C relaxation times of Ac-Ala-HEP

^{13}C position	1	2	3	4	5	6	7	8
T_1/s	4.8	4.3	15.6	0.8	0.9	4.2	4.3	1.1

Boc-Gly-HEP:

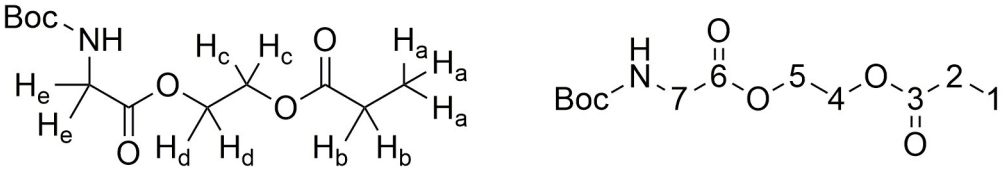


Table S7. Longitudinal ^{1}H relaxation times of Boc-Gly-HEP

^{1}H position	a	b	c	d	e
T_1/s	5.5	5.1	2.0	1.7	1.7

Table S8. Longitudinal ^{13}C relaxation times of Boc-Gly-HEP

^{13}C position	1	2	3	4	5	6	7
T_1/s	6.3	6.1	16.7	1.6	1.5	11.1	1.8

Boc-Gln(Xan)-HEP:

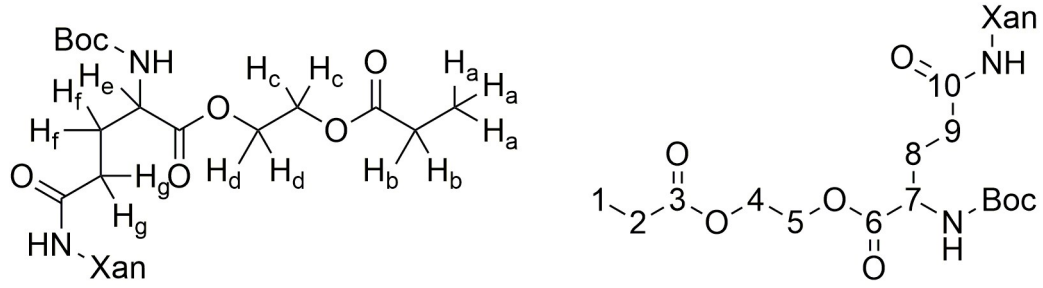


Table S9. Longitudinal ^{1}H relaxation times of Boc-Gln(Xan)-HEP

^{1}H position	a	b	c	d	e	f	g
T_1/s	3.6	2.8	1.2	1.2	2.0	4.3	4.3

Table S10. Longitudinal ^{13}C relaxation times of Boc-Gln(Xan)-HEP

^{13}C position	1	2	3	4	5	6	7	8	9	10
T_1/s	6.6	4.8	15.6	0.4	0.4	7.9	3.3	1.8	2.9	11.1

S6 Catalyst decomposition

When an NMR spectrum is recorded as part of a PHIP experiment in which the reaction mixture was heated up to 80 °C, additional hyperpolarized peaks could be observed at 0.9, 1.8, 3.1 and 6.2 ppm. Norbornadiene is part of the used metal complex that catalyzes the hydrogenation of the amino acid derivatives. The observed additional peaks correspond to norbornene indicating that at elevated temperature the norbornadiene is partly hydrogenated as the catalyst decomposes.[S4,S5] Figure S10 shows a ^{1}H NMR spectrum of the hyperpolarized Ala-HEP derivative and hyperpolarized norbornene.

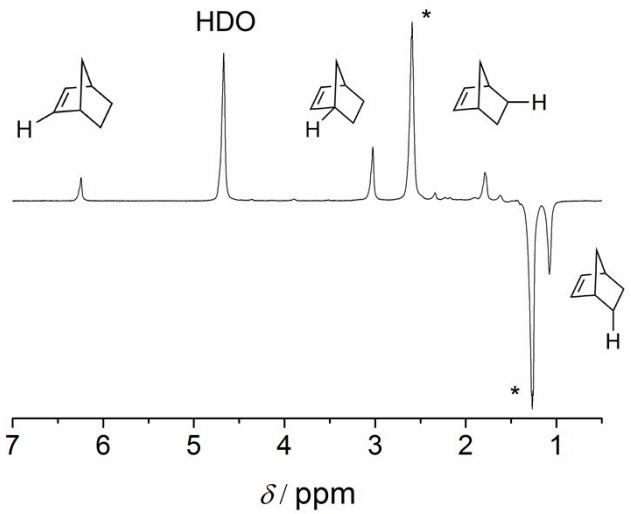


Fig. S10. ^{1}H NMR spectrum of hyperpolarized norbornene and Ala-HEP at B_0 = 14.1 T (600 MHz ^{1}H frequency) after a 45° pulse. Asterisks indicate the propionate part of the hyperpolarized amino acid derivative.

S7 Remarks on previous amino acid PHIP

PHIP studies on amino acids (non-derivatives) in water have been demonstrated as a proof of principle, but the achieved polarization showed only small improvements over thermal equilibrium conditions, which prohibited any useful *in vivo* applications.[S6] Currently, the most potent r.f. polarization transfer sequence from ^{1}H to ^{13}C was developed by Goldman and coworkers.[S3] Except for the previously polarized γ-aminobutyric acid (GABA), it is unlikely that this sequence can be used to generate high polarization levels in the carbonyl ^{13}C (typically a long-lived ^{13}C species) of the reported unprotected amino acids. This is due to the small *J*-couplings (in particular of ^{4}J of aliphatic protons to ^{13}C) leading to unfavorable conditions (e.g., timing and angle) with regards to transfer of proton polarization (see above for pulse sequence details or ref [S3]). γ-aminobutyric acid has the right structure for efficient polarization transfer from protons to the desired long-lived carbonyl ^{13}C. But the chemical reaction appears to be too slow to efficiently transfer spin order of p-H_2 and it is necessary to protect the catalyst by working in a very acidic environment to achieve any polarization at all.[S3] Working in an acidic environment, in the context of potential *in vivo* applications, means that the polarized substrate needs to be diluted in a buffer as an additional step before injection, which may be a source of polarization loss. No reported research has achieved a ^{13}C PHIP polarization of N-unprotect amino acids on the order of 1%, a prerequisite for in vivo applications.

S8 Toxicity and Metabolic considerations of hydrolysis

Previous PHIP studies utilizing esters revealed that esters are quickly taken up in cells and are hydrolyzed fast by enzymatic processes.[S7] As the synthesized amino acid derivatives are based on esters we would like to provide some considerations regarding the time of the hydrolysis, potential metabolites and their potential toxicity.

The synthesized derivatives contain two ester moieties that may potentially be hydrolyzed. It is plausible that the following chemicals could be observed due to enzymatic hydrolysis: free amino acid, ethylene glycol and propionic acid. Ethylene glycol and propionic acid originate from HEP. The amino acids used in this study can be seen as non-toxic as glycine, glutamine and alanine are metabolites in the human body. Ethylene glycol has been identified to have toxic effects on humans. Studies have revealed that a threshold for ethylene glycol to have toxic effects on humans is a concentration of about 3.2 mM in the plasma.[S8] In typical *in vivo* experiments utilizing hyperpolarized contrast agents, concentrations of 25-300 mM in 1 mL are administered.[S9] Considering that humans carry multiple liters of blood plasma, the concentration of formed ethylene glycol in a potential *in vivo* experiment is more than one order of magnitude lower than the described threshold and thus the expected toxicity should be low. Propionic acid is a metabolite that is transformed into succinyl-CoA, which is an intermediate of the citric acid cycle.[S10] Therefore, propionic can be seen as a substrate with very low toxicity and can also be seen as a potential new tracer for the citric acid cycle.

Regarding the speed of hydrolysis, we estimate it to be on a one to several minutes timescale, which would be sufficient for the proposed *in vivo* experiments. Experiments with esters of fatty acids have shown that a half-life of 58 s can be expected for simple esters derived from ethanol.[S11] With the more sterically demanding properties of the synthesized esters, the hydrolysis may take longer as has also been observed in the context of prodrug research making use of ester moieties.[S12] Although parts of the potential contrast agents will be hydrolyzed, we expect that sufficient polarization can be delivered *in vivo* in the expected time scale.

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