Supplementary information

Detection and resolution of sub-millimolar concentration amino acids on a benchtop NMR spectrometer by nh-PHIP hyperpolarization

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1. Chemicals and materials

Structures of all tested cosubstrates are given in Scheme S1 below. All cosubstrates were obtained from standard suppliers of research chemicals, as specified below. Numbers in parenthesis denote cosubstrate (*cs*) compound numbers as used in Scheme S1 and throughout main text.

4-Acetylpyridine (20), 3-bromopyridine (15), 4-bromopyridine hydrochloride (16), 4-chloropyridine hydrochloride (14), 3-chloropyridine (13), 3,5-difluoropyridine (12), 3-Iodopyridine (17), 4-iodopyridine (18), 4-methoxypyridine (7), 4-hydroxypyridine (6), 3-fluoro-4-methylpyridine (8) and 3-chloro-5-(trifluoromethyl)pyridine (9) were acquired from TCI Europe and used without further purification.

3-Acetylpyridine (19) and 4-*t*-butylpyridine (5) were acquired from Acros Organics and used without further purification.

3-Fluoropyridine (10) and 4-fluoropyridine hydrochloride (11) were acquired from BLDpharm and used without further purification.

3-Methylpyridine (2), 4-methylpyridine (3) and 3,5-lutidine (4) were acquired from Alfa Aesar and used without further purification.

1-Methyl-1,2,3-triazol (mtz) was synthesized according to a published procedure.[1] After purification by vacuum distillation, pure *mtz* is a colourless viscous substance that slowly solidifies at +4 °C in a refrigerator.

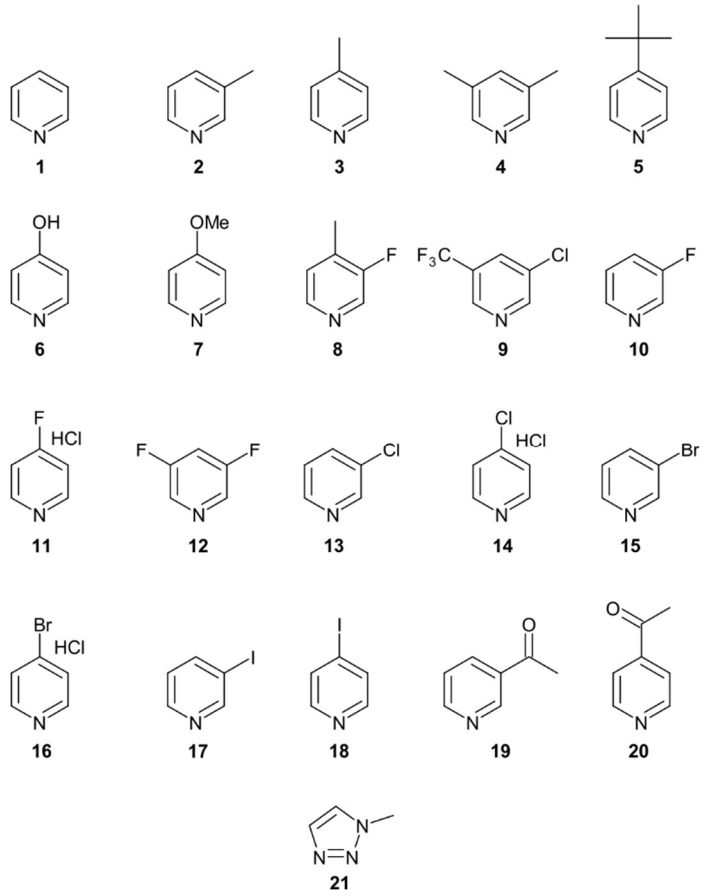
Amino acids were from a Reanal Hungary L-amino acids kit, containing 1 g bottles of 24 common α-amino acids (product code C1328).

The active catalyst precursor [Ir(Cl)(COD)(IMes)] was synthesized in house according to a published procedure.[2,3] The final product was purified by recrystallisation, which yielded a bright yellow powder.

Methanol used in NMR sample preparation was HPLC grade methanol. Water used for the preparation of amino acids stock solutions was Class II purified water prepared with a Merck Millipore Elix® essential water purification system.

Methanol-d_3 that was used for deuterium locking on the 300 MHz spectrometer was acquired from Deutero GmbH.

The piperidine/piperidinium buffer solution consisted of equal concentrations of piperidine and piperidine hydrochloride. Both were acquired from Sigma Aldrich. The use of the piperidine/piperidinium buffer for amino acids nh-PHIP was adapted from Sellies et al.[4]



Scheme S1. Structures of different cosubstrates (cs) used in this work.

2. NMR instruments

80 MHz benchtop NMR experiments were conducted on a Magritek Spinsolve Carbon Ultra spectrometer that was equipped with pulsed field gradients (PFG). Sample temperature was at a fixed 26.5 ˚C.

300 MHz NMR experiments were conducted on a Bruker Avance III HD spectrometer that was equipped with a TBI probe with z-gradient and a variable temperature (VT) module with a Bruker BCU-05 VT air chiller.

3. nh-PHIP equipment

50% enriched parahydrogen (pH$_2$) was produced in flow in a previously described setup by the flow of hydrogen gas at 5 bar overpressure through a packed bed reactor filled with iron oxide-hydroxide and submerged in liquid nitrogen (77 K).[5]

The experimental setup for parahydrogen hyperpolarization consisted of a ‘‘bubbling control box’’ spectrometer accessory that allowed pH$_2$ bubbling through the NMR sample under pulse program control. The setup and its operational principles were published previously.[5] As the Magritek Spinsolve does not have a TTL output, an Arduino Uno rev.4 was used to generate a signal for the ‘‘bubbling control box.’’ Communication between the pulse sequence and the Arduino board was based on the serial port from the PC.

nh-PHIP was performed using a 600 μL total sample volume in Norell® intermediate pressure valved (IPV) 5 mm NMR tubes. The tubes were connected to a custom-built headpiece with a passage for a hydrogen capillary made of PEEK plastic to bubble pH$_2$ through the sample under pulse program control. See ESI of Reimets et al.[5] for details. The PEEK tube in the methanolic sample does not cause significant homogeneity and shimming issues, allowing for shimming by standard instrument vendor-provided shimming routines both at 300 MHz and 80 MHz.

4. Pulse sequences and their parameters

In phase 1D nh-PHIP hydride spectra (-20…-30 ppm region) were acquired with the previously published SEPP pulse sequence[4,6–8] in 64 scans (Figure 2, 80 MHz) or 128 scans (Figure 3, 300 MHz). The particular implementation of the SEPP pulse sequence element was obtained from ESI ref. 9 figure 1B. It refocuses the antiphase hydride signal responses, that would otherwise appear in a PHIP experiment, into in-phase doublets for each hydride signal. Each scan was preceded by 2 seconds of gas bubbling through the sample to refresh the dissolved parahydrogen, with bubbling performed during the relaxation delay time. Note that good signal-to-noise ratios (SNR) can be achieved with substantially fewer scans than used herein for 1D nh-PHIP spectra. The presented 1D experiments were designed to provide data with SNR, which was high enough to also observe the very weak hydride signals that were encountered during development and *cs* screening. Routine 1D analysis can be performed much faster, and quite often it can be done with only 8 scans (dictated SEPP pulse sequence phase cycle). The pulse sequence used shaped ReBurp pulses that covered the whole -20…-30 ppm hydride spectral region but that did not excite the solvent signals at 3…5 ppm (at least 800 Hz pulse bandwidth at 80 MHz and at least 3000 Hz bandwidth at 300 MHz).

The conditions and parameters for parahydrogen hyperpolarized 2D nh-PHIP ZQ spectra (Figures 1b and 5) were derived from prior work by Sellies *et al.*,[4] which employed zero-quantum (ZQ) spectroscopy[8] for the resolution of the nh-PHIP hydride spectral regions. Implementation of the pulse sequence has been derived from figure 2 of said publication.[4] The 2D nh-PHIP ZQ sequence utilized the same shaped pulses as the SEPP sequence described above. On the 80 MHz spectrometer, the f_1 dimension spectral width was chosen individually for each sample from previously recorded 1D SEPP spectra, ensuring that all hydride resonances would equally fold into a 150…180 Hz bandwidth (for example, if the furthest hydride signals were 455 Hz apart and the closest hydride signals were 390 Hz apart, the spectrum was measured with a 170 Hz bandwidth in f1 to allow all signals to fold into the same 170 Hz window). Both spectral dimensions were zero-filled twofold, and a 90-degree shifted sine square window function was applied before Fourier transformation in spectral processing, followed by phase correction and baseline correction. The specific numbers of scans and increments are displayed in each 2D spectrum or its caption. Technical details for nh-PHIP ZQ at 300 MHz can be found in the caption of **Figure S3**.

5. nh-PHIP sample preparation

10 mM stock solutions in water were prepared for all amino acids used in the study. A 200 mM stock solution of the piperidine/piperidinium buffer was prepared in methanol by dissolving equimolar amounts of both components. 100 mM stock solutions in methanol were prepared for all tested cosubstrates (*cs*). A 10 mM stock solution in methanol was prepared from [Ir(Cl)(COD)(IMes)], the active nh-PHIP catalyst precursor. All stock solutions were prepared gravimetrically by weighing the

desired amounts of compounds into designated vials and dissolving them in a precisely measured amount of solvent (usually water or methanol).

nh-PHIP samples consisted of 1 mM of the nh-PHIP catalyst, an 18-fold excess of a cosubstrate (18 mM in the sample), 40 mM of the piperidinium buffer and 1.05 eq (relative to catalyst) of amino acids combined (0,35 eq of each amino acid for samples in Figures 2-5; 0.13 eq of each amino acid for the sample in Figure 1b). Amino acid stock solutions were in water, all other stock solutions were in methanol. This resulted in a ~10% vol water content in the final nh-PHIP sample.

The piperidine/piperidinium buffer is necessary to maintain a constant pH 11 sample environment during the nh-PHIP experiment. High pH is required to promote coordination of amino acids to the nh-PHIP catalyst. While this can also be achieved by pH adjustment with a strong base (e.g., KOH), buffering has the benefit of assuring consistent nh-PHIP performance due to uniform (pH dependent) analyte coordination efficiency from sample to sample. The effect of pH on nh-PHIP signal enhancement has been discussed in further detail by Sellies et al.[4]

For the preparation of the samples for Figures 2, 3 and 5, the following components were pipetted into an IPV tube:

- 60 μL of the catalyst precursor stock solution;
- 108 μL of the *cs* stock solution;
- 120 μL of the buffer stock solution;
- 21 μL of Leu stock solution;
- 21 μL of Pro stock solution;
- 21 uL of Sar stock solution;
- 250 μL of methanol to make up the 600 μL sample. Note that for experiments on the 300 MHz instrument, 100 μL of methanol was replaced by 100 μL of methanol-d_3 for deuterium locking. Since the Magritek Spinsolve BT instrument uses external locking, no deuterated solvent was required for BT experiments.

For the preparation of the sample in Figure 1b and Figure S1i, the following components were pipetted into an IPV tube:

- 60 μL of the catalyst precursor stock solution;
- 108 μL of the *cs* (cosubstrate **20**, 4-acetylpyridine) stock solution;
- 120 μL of the buffer stock solution;
- 7,9 μL of Leu stock solution;
- 7,9 μL of Pro stock solution;
- 7,9 uL of Sar stock solution;
- 7,9 uL of Val stock solution;
- 7,9 uL of Gly stock solution;
- 7,9 uL of Trp stock solution;
- 7,9 uL of Lys stock solution;
- 7,9 uL of Ala stock solution;
- 250 μL of methanol to make up the 600 μL sample.

For the preparation of samples in Figure S1a-S1h, the following components were pipetted into an IPV tube:

- 60 μL of the catalyst precursor stock solution;
- 108 μL of the *cs* (cosubstrate **20**, 4-acetylpyridine) stock solution;

- 120 μL of the buffer stock solution;
- 62 μL of amino acid stock solution;
- 250 μL of methanol to make up the 600 μL sample.

After pipetting all solutions into an IPV tube, the tube was connected to the nh-PHIP hydrogen bubbling setup, pressurised under 4 bar overpressure of hydrogen gas, and bubbled for 3 minutes at room temperature to saturate the solution with hydrogen. The tube was then placed into a 50 °C water bath and heated for 7 minutes to complete the synthesis of the active nh-PHIP complex of each analyte (amino acid) as shown in Figure 1a. The tube was wiped dry with a lint-free paper tissue, placed into the NMR spinner and entered into the magnet for nh-PHIP analysis.

6. Supplementary Figures

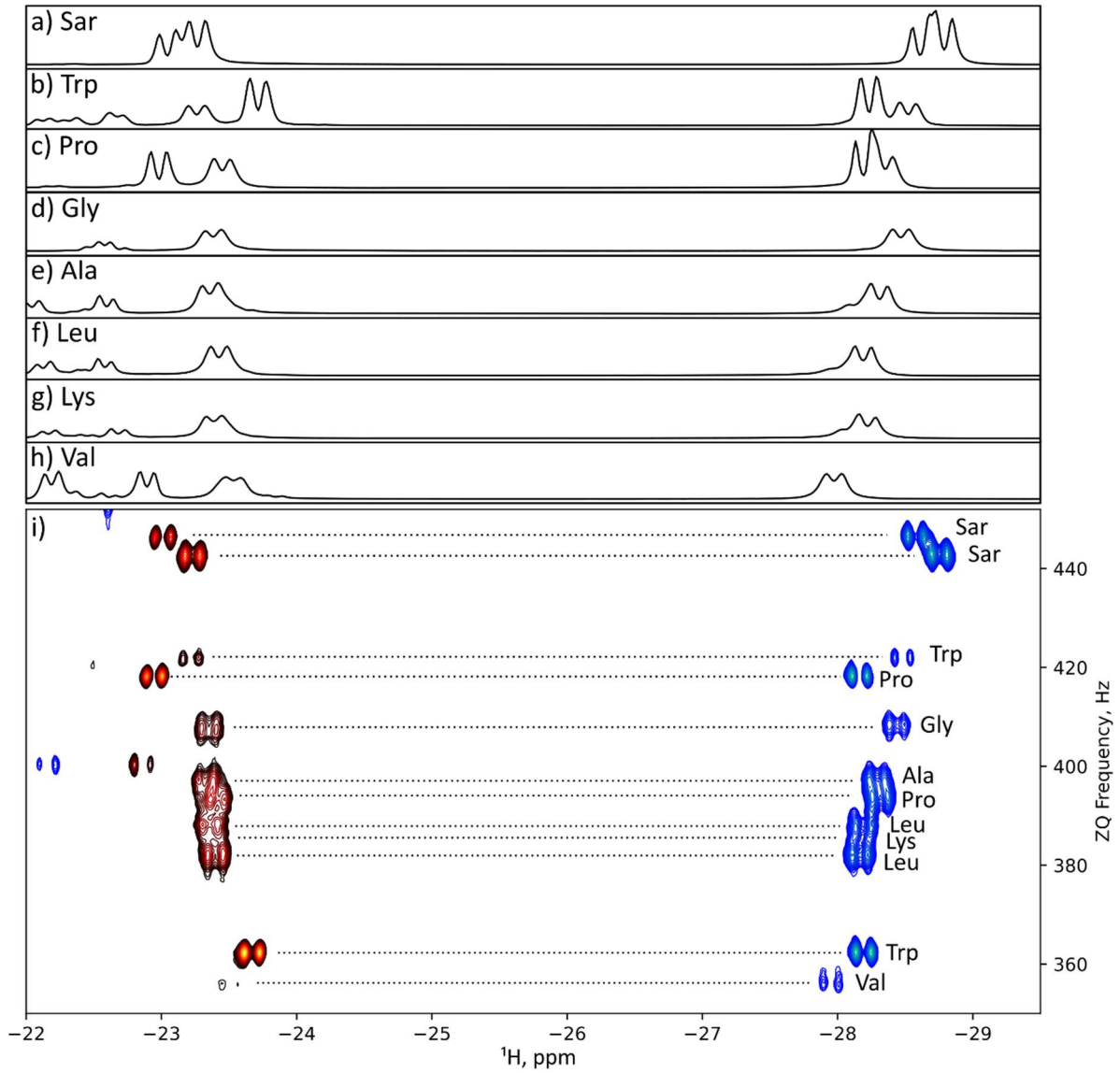


Figure S1. Panels a-h display 1D SEPP hydride spectra of individual amino acids. Note that for Gly and Val, only one of the possible analyte-catalyst complex diastereomers dominates. The less abundant diastereomer signal is low and broad for Ala and Lys and was not resolved in this experiment. Full 2D nh-PHIP ZQ spectrum of the sample in Figure 1b is displayed in panel i. The displayed 2D spectrum was acquired at 80 MHz in 16 dummy scans, 2 scans per increment, and 256 increments in a 114 Hz bandwidth in the indirect dimension, amounting to an approx. 30 min experiment time. The concentration of each individual analyte was 130 μM, adding up to a combined amino acid concentration of 1.05 mM in the NMR sample. Although not all signals are completely resolved in the displayed 2D spectrum, we argue that this is a powerful demonstration of the possibilities of nh-PHIP on BT NMR. Higher SNR and better resolution can be obtained in the future by increasing increments in 2D acquisition. We have shown that solvent evaporation (with relatively volatile solvents like methanol or chloroform) is not an issue, even if more than 1000 scans are accumulated in a 2D experiment.[5]

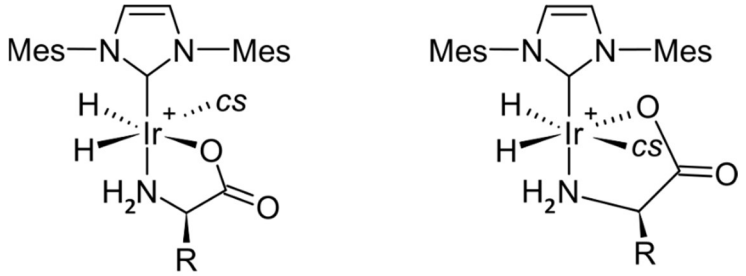


Figure S2. When a chiral chleating ligand (amino acid) coordinates to an octahedral Iridium complex, two NMR distinguishable diastereomeric complexes form as shown above.[4] *Because diastereomers have distinct physical-chemical properties, their hydride chemical shifts and binding kinetics will be different (as seen from different hydride signal nh-PHIP intensities in the in Figure S1). Therefore, each amino acid will have two HA signals (see Figure S3), but both are not necessarily always observed.*

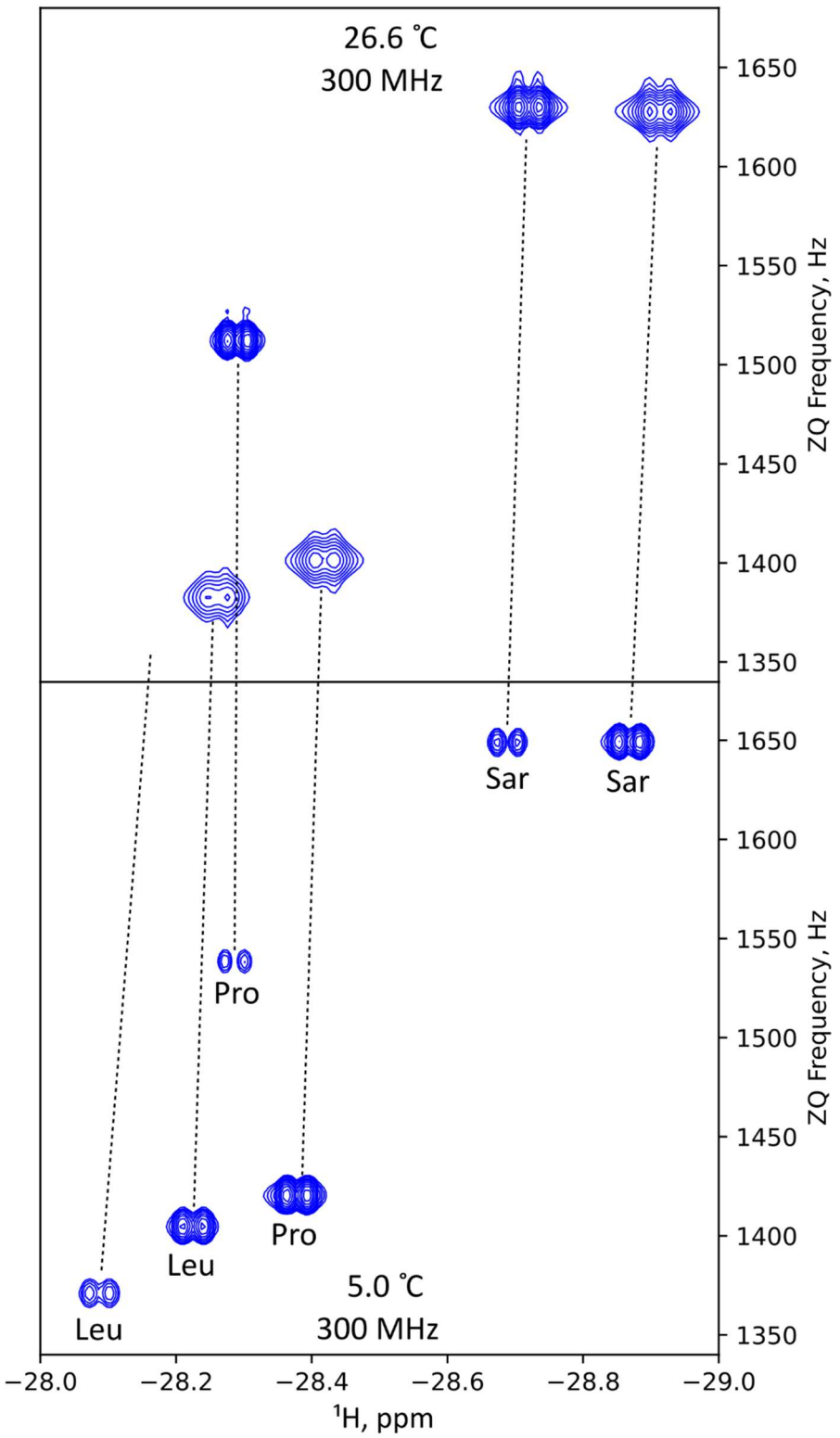


Figure S3. Comparison of 2D nh-PHIP ZQ spectra from the same samples as in main text Figure 3, showing the influence of temperature on the nh-PHIP hydride signal intensities and line widths at 5 ˚C and 26.5 ˚C. Spectra acquired with 96 increments in a 420 Hz f_1 spectral width and 2 scans per increment.

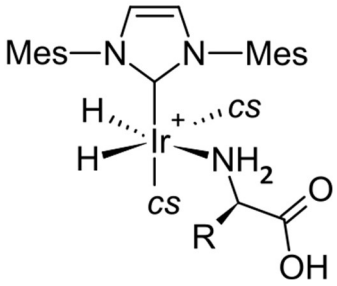


Figure S4. Amino acids in main text Figure 2 and ESI Figures S1 and S6 display doublet pairs with both hydride counterparts resonating in between -22…-23.5 ppm. The nature of these signals has been discussed by Sellies et al. in ESI Ref. 4. In short, these signals arise due to complexes where the amino acid is coordinated to the iridium catalyst with its amino moiety in one of the equatorial positions of the complex. These are undesirable byproduct complexes that form kinetically upon mixing the sample components. Heating the samples to 50 °C before nh-PHIP will convert most of these kinetic products into their thermodynamically favoured counterparts shown in Figure S2. The conversion is, however, usually not complete and a reproducible distribtuion of kinetic (Figure S4) and thermodynamic (Figure S2) complexes forms. 2D nh-PHIP ZQ analysis of amino acids is usually done by observing the -28…-29 ppm signals of the termodynamic complexes as they are more intense and provide better spectral dispersion.

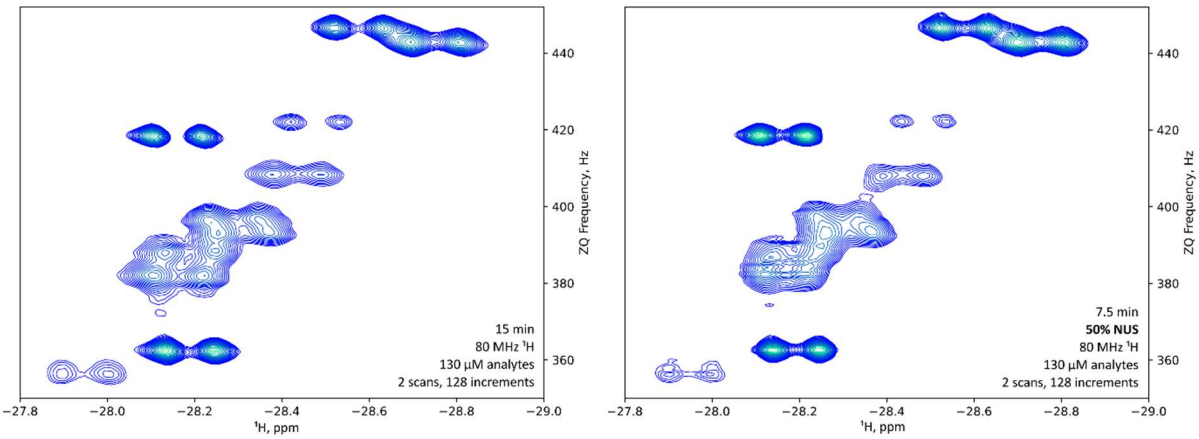


Figure S5. Comparison of nh-PHIP spectra with (right panel) and without (left panel) 50% NUS applied. The sample composition is identical to Figure 1b and Figure S1i. The f_1 dimension was sampled in 114 Hz bandwidth in both cases, the same as in Figure 1b and S1i. Acquiring half the increments compared to Figure 1b resulted in poorer resolution of the closely placed Ala, Leu, Lys and Pro signals in the middle of the plotted spectral region. NUS degrades spectral quality further, but importantly, signals of the better resolved hydrides remain resolved. Achieving such spectra of sub—millimolar analytes in less than 10 minutes at 80 MHz demonstrates the potential of nh-PHIP on BT. Alternatively, NUS schemes could potentially be used to increase the resolution for Ala, Leu, Lys and Pro in Figure 1b and Figure S1i by recording twice the increments in the same experimental time (with 50% NUS). Unless linewidth in the f_1 dimension is limited by relaxation or cs exchange in the particular sample, it may lead to better resolution in the same experimental time. Presented NUS data was reconstructed using the low-rank method implemented in the MddNMR package.[10]

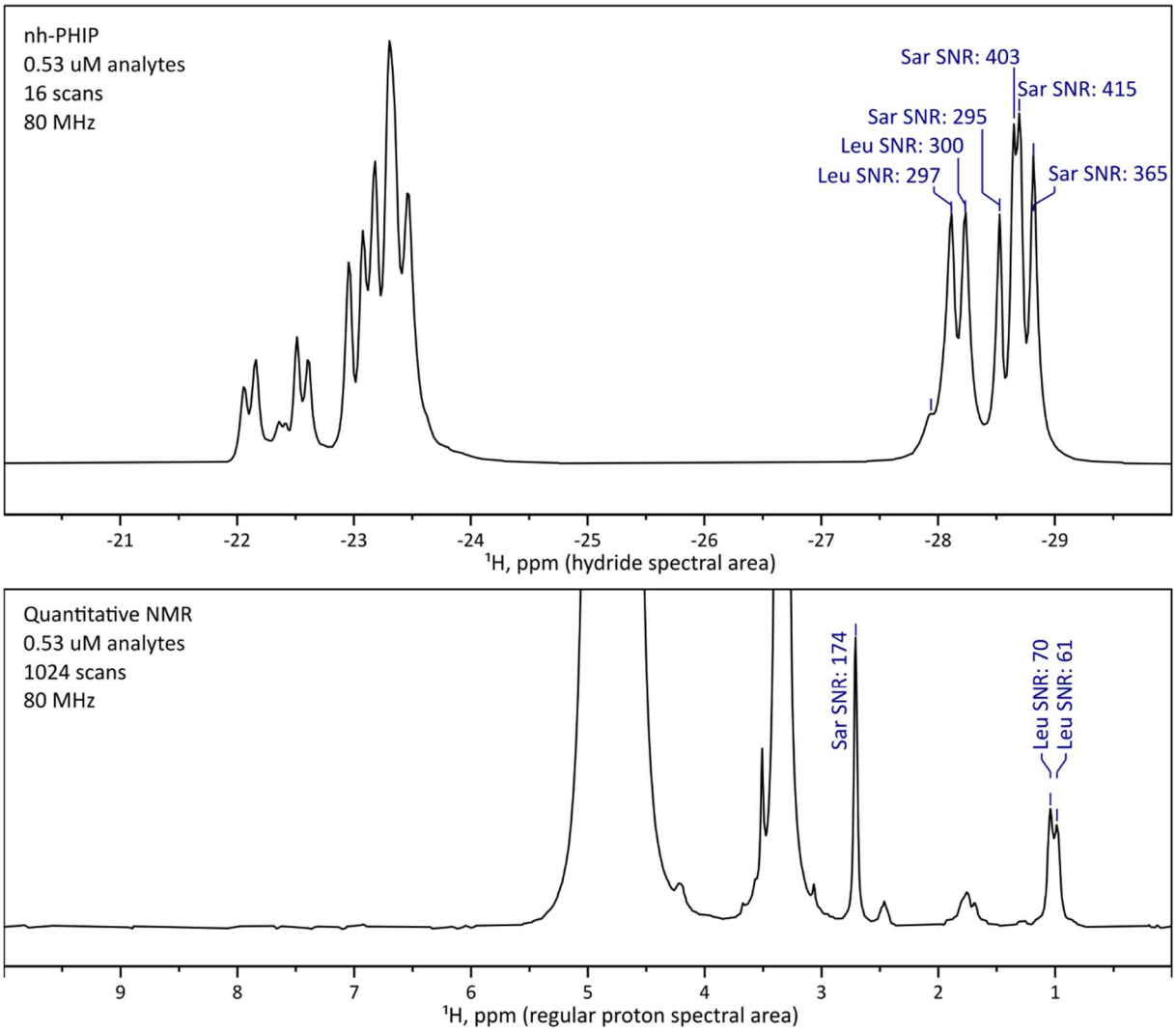


Figure S6. Comparison of sensitivity and signal enhancement between nh-PHIP signals at -20…-30 ppm region (upper panel) and thermally polarized quantitative ^1H NMR (lower panel). If we normalize SNR in regular NMR according to the 64-fold difference in scans and the number of protons (3 for each signal), we arrive at per proton SNR of 2.9 for Leu and 6.9 for Sar (see formula below). Comparing these numbers with the nh-PHIP SNR value, we get a 60-fold signal enhancement for Sar and a 103-fold enhancement for Leu. These numbers are somewhat smaller than what has been reported at high-field spectrometers.[4] However, they still offer substantial benefits for BT spectrometers.

Formula for normalizing SNR for thermally polarized NMR:

$$Normalized\ SNR = \frac{^1H\ qNMR\ SNR}{\sqrt{64}\cdot 3}$$

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