Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Parahydrogen induced hyperpolarization provides a tool for NMR metabolomics at nanomolar concentrations

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Chemicals

Complex precursor [IrCl(COD)(IMes)] (IMes = 1,3-bis(2,4,6-trimethylphenyl)imidazole-2-ylidene; COD = cyclooctadiene) and co-substrate 1-methyl-1,2,3-triazole (*mtz*) were synthesized according to published methods.[1,2] All other chemicals were purchased from:

Sigma-Aldrich (methanol-d_4, pyridine, 3-methylpyridine, 4-ethylpyridine, nicotinamide, pyrazine, 2-methylpyrazine, 2-ethylpyrazine, 2,6-dimethylpyrazine, methylnicotinate, 3-hydroxypyridine, 3-methoxypyridine, imidazole, adenosine, guanosine, tryptophan),

TCI Europe N.V. (N-methylnicotinamide, N-ethylnicotinamide),

VWR International B.V. (3,5-dimethylpyridine),

Toronto Research Chemicals Inc. (1-methyladenosine),

Acros Organics BVBA (4-methylpyridine),

Selleckchem (6-methyladenosine)

and used as supplied. Parahydrogen (p-H_2) was produced with an in-house designed 2 L vessel embedded in a liquid nitrogen bath. Normal hydrogen (purity 5.0) was cooled down to 77 K in the presence of 100 mL of 4-8 MESH charcoal (Sigma-Aldrich). The resulting 51% p-H_2 was transported to an aluminum cylinder (Nitrous Oxides Systems, Holley Performance Products, Bowling Green, KY, USA),[3] with an adjustable output-pressure valve.

Sample preparation

Urine extract

The pH of urine was adjusted to 7.0 with 1 M NaOH and samples were centrifuged (3750 RCF). A Supelco Supelclean LC-8 SPE cartridge (3 mL, 500 mg) was activated with methanol (3 mL) and conditioned with water (3 mL). Urine (5 mL) was passed through with a steady flow rate of 1 mL/min and the cartridge was washed with water (3 mL), followed by drying for 60 min with a 1 L/min flow of N_2. The analytes were extracted by eluting with methanol (2 mL). The extract was dried using a speedvac and subsequently dissolved in 500 μL methanol-d_4 and centrifuged before usage of 400 μL for analysis. The SPE step resulted therefore in up to 10-fold concentration increase of the selected urine metabolites.

NMR Samples

The precursor complex [IrCl(COD)(IMes)] and *mtz* were used as synthesized. Stock solutions in methanol-d_4 were prepared gravimetrically for the precursor complex (10 mM) and for the co-substrate *mtz* (100 mM). For each NMR sample an activation mixture was prepared by adding into a quick pressure valve (QPV) NMR tube a weighed mixture of *mtz* (90 μL, 100 mM), precursor complex (50 μL, 10 mM) and methanol-d_4 (60 μL), for a final ratio of *mtz*:precursor complex=18:1. The mixture was pressurized under 4 bar of H_2, shaken and allowed to hydrogenate at room temperature for 2 h to generate a solution of active $[Ir(H)_2(IMes)(mtz)_3]^+$ catalyst. The NMR sample was obtained by adding (gravimetrically) 400 μL of urine extract in methanol-d_4 to the activated catalyst solution. Note that this results in a 66% dilution of the urine extract in the final NMR sample.

A similar protocol was followed for the other NMR samples.

NMR experiments

All NMR spectra were acquired at 298 K on an Agilent Unity INOVA spectrometer, operating at 500 MHz proton resonance frequency, equipped with a cryo-cooled HCN probe. The setup to bubble p-H_2 in the sample inside the NMR spectrometer has been previously described.[4] The 1D PHIP hydrides spectrum was acquired with the pulse sequence displayed in figure S1 below.

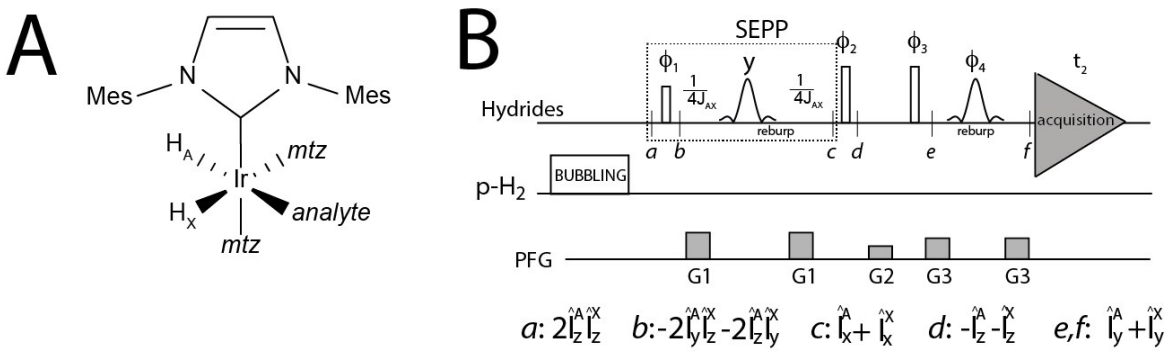

Figure S1. (**A)** Schematic representation of the asymmetric chemosensor complex formed for a dilute analyte in the presence of a large excess of 1-methyl-1,2,3-triazole (*mtz*) as co-substrate. **(B)** *S*elective *E*xcitation of *P*olarization using *P*ASADENA (SEPP)[5,6] pulse scheme to acquire a 1D PHIP NMR spectrum of the hydrides, centered at -22.235 ppm. Bubbling *p*-H_2 in the sample (typically ca. 1.0 s) occurs under spectrometer control, at the beginning of each transient. Rectangular pulses indicate low-power (RF field ca. 2.5 kHz) 45- and 90-degree pulses while shapes represent selective reburp pulses with a bandwidth of 6000 Hz. J_{AX} denotes the average inter-hydrides coupling constant (7.5 Hz). Phase cycling: ϕ_1: x, -x; ϕ_2: 2(y), 2(-y); ϕ_3: 4(x), 4(y), 4(-x), 4(-y); ϕ_4: 4(y), 4(-x), 4(-y), 4(x); $\phi_{receiver}$: x,-x,-x,x, y,-y,-y,y, -x,x,x,-x, -y,y,y,-y. Relevant terms of the density operator at different time points are indicated.

The 2D PHIP-ZQ spectrum was acquired with the pulse sequence sketched in figure S2.

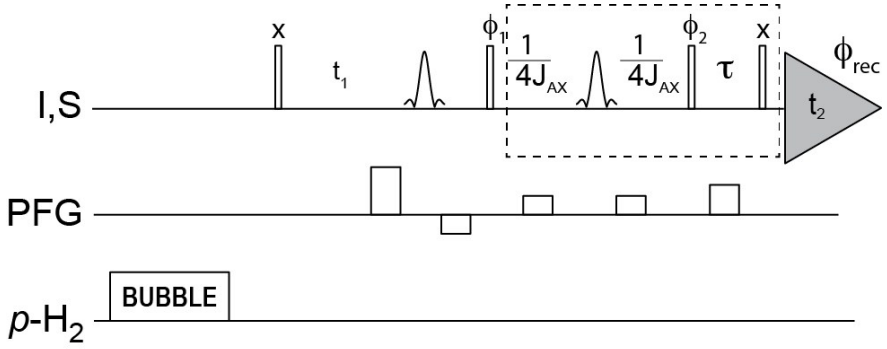

Figure S2 Pulse scheme used for the acquisition of the 2D PHIP-ZQ spectrum of the hydrides. Shaped pulses are *reburp* for refocusing, with a bandwidth of 6000 Hz. Rectangular pulses indicate low-power (RF field ca. 5 kHz) 90-degree pulses. The transmitter offset is set to -21.894 ppm. Bubbling *p*-H_2 in the sample (typically ca. 1.5 s) occurs under spectrometer control, at the beginning of each transient. Phase cycling is implemented as follows: ϕ_1 = x,x,-x,-x; ϕ_2 = x,-x; ϕ_{rec} = x,-x,-x,x.

The coherence flow is described by means of product operator formalism as:

$$2\hat{I}_z^A\hat{S}_z^X \xrightarrow{\left(\frac{\pi}{2}\right)_x} 2\hat{I}_y^A\hat{S}_y^X \xrightarrow{t_1,PFG} \frac{1}{2}\left\{\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_y^A\hat{S}_y^X\right)c\Delta t_1+\left(2\hat{I}_x^A\hat{S}_y^X-2\hat{I}_y^A\hat{S}_x^X\right)s\Delta t_1\right\}$$

$$\xrightarrow{\left(\frac{\pi}{2}\right)_{\phi_1}} \frac{1}{2}\left\{\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_z^A\hat{S}_z^X\right)c\Delta t_1+\left(2\hat{I}_x^A\hat{S}_z^X-2\hat{I}_z^A\hat{S}_x^X\right)s\Delta t_1\right\}$$

$$\xrightarrow{\frac{1}{2J_{AX}}} \frac{1}{2}\left\{\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_z^A\hat{S}_z^X\right)c\Delta t_1+\left(\hat{I}_y^A-\hat{S}_y^X\right)s\Delta t_1\right\}$$

$$\xrightarrow{\left(\frac{\pi}{2}\right)_{\phi_2}} \frac{1}{2}\left\{\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_y^A\hat{S}_y^X\right)c\Delta t_1+\left(\hat{I}_z^A-\hat{S}_z^X\right)s\Delta t_1\right\}$$

$$\xrightarrow{\tau,PFG} \frac{1}{2}\left\{\left[\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_y^A\hat{S}_y^X\right)c\Delta\tau-\left(2\hat{I}_x^A\hat{S}_y^X-2\hat{I}_y^A\hat{S}_x^X\right)s\Delta\tau\right]c\Delta t_1+\left(\hat{I}_z^A-\hat{S}_z^X\right)s\Delta t_1\right\}$$

$$\xrightarrow{\left(\frac{\pi}{2}\right)_x} \frac{1}{2}\left[\left(2\hat{I}_x^A\hat{S}_x^X+2\hat{I}_z^A\hat{S}_z^X\right)c\Delta\tau-\left(2\hat{I}_x^A\hat{S}_z^X-2\hat{I}_z^A\hat{S}_x^X\right)s\Delta\tau\right]c\Delta t_1-\frac{1}{2}\left(\hat{I}_y^A-\hat{S}_y^X\right)s\Delta t_1$$

where I^A and S^X denote the spin angular momentum of the two hydrides, $c\Delta t_1$ and $s\Delta t_1$ signify respectively $\cos(\Omega_A-\Omega_X)t_1$ and $\sin(\Omega_A-\Omega_X)t_1$ and, analogously, $c\Delta\tau$ and $s\Delta\tau$ indicate $\cos(\Omega_A-\Omega_X)\tau$ and $\sin(\Omega_A-\Omega_X)\tau$. Phase cycles ϕ_1 and ϕ_2 suppress residual antiphase coherences (2nd term between parentheses in the final expression) that might otherwise contribute to the detected signals. Note that the signals of the two hydrides have opposite sign. Zero-quantum coherences modulate in t_1 as a sine function of the frequency difference of the two hydrides' signals. A real-FT is performed to obtain a 2D spectrum that is anti-symmetric in the indirect dimension.
The 2D PHIP-ZQ spectrum of the SPE of urine was recorded with spectral widths of 3000 Hz and 10000 Hz in the indirect (t_1) and acquisition (t_2) dimension, respectively. The 2D dataset consists of (512 real)x(5000 complex) points. Two transients were collected for each increment, for a total acquisition time of ca. 50 minutes.

A standard addition series of five 2D PHIP-ZQ experiments was acquired to determine the concentration of nicotinamide in the SPE of urine. The five 2D spectra were recorded with reduced spectral widths in both dimensions: 900 Hz in t_1 and 6000 Hz in t_2. Each 2D dataset, consisting of (96 real)x(3000 complex) points, was acquired with 2 transients per increment, for an acquisition time of 9 minutes.

The 2D PHIP-ZQ spectrum of series of homologous compounds (Figure 3 in the main text) was acquired with spectral widths of 2000 Hz and 4000 Hz in the indirect (t_1) and acquisition (t_2) dimension, respectively. The 2D dataset consists of (128 real)x(1200 complex) points. For each increment 2 transients were collected.

All 2D data sets were processed with NMRPipe[7] using 90° shifted squared sine-bell apodization in both dimensions, prior to zero filling to 4096 (t_1) × 16384 (t_2) points, and Fourier transformation. The software iNMR (http://www.inmr.net) was used for the analysis of the 2D spectra.

PHIP sensitivity enhancement

In order to estimate the PHIP sensitivity enhancement on the hydrides signals, a thermal spectrum and a PHIP-NMR spectrum of a 100 μM solution of two model compounds, nicotinamide and 2-methylpyrazine, were acquired using the pulse sequences displayed in figure S3.

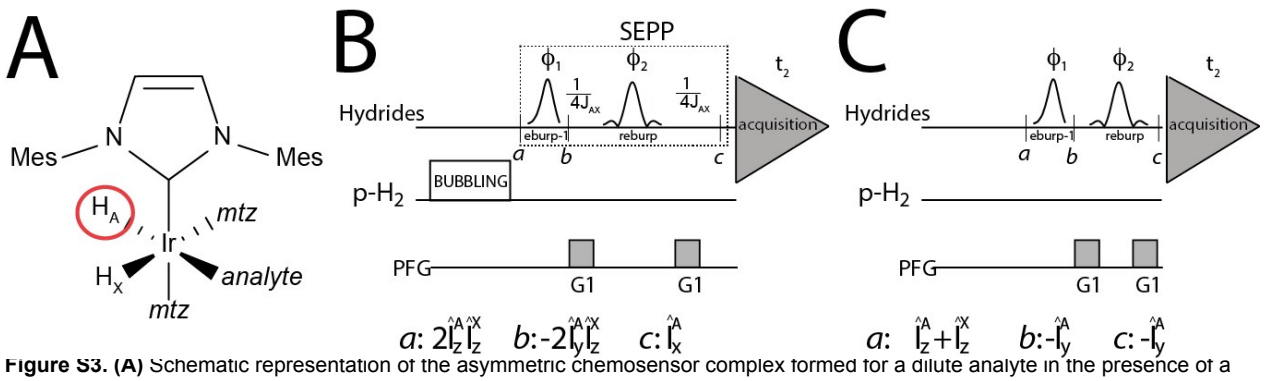

Figure S3. (A) Schematic representation of the asymmetric chemosensor complex formed for a dilute analyte in the presence of a large excess of 1-methyl-1,2,3-triazole (*mtz*) as co-substrate. The observed hydride is indicated by a red circle. **(B)** *S*elective *E*xcitation of *P*olarization using *P*ASADENA (SEPP)[5,6] pulse scheme to acquire a 1D PHIP NMR spectrum of the hydrides, centered at -22.73 ppm. Bubbling *p*-H_2 in the sample occurs for 1.5 s. The selective *eburp-1* pulse covers a bandwidth of 500 Hz, for a duration of 9 ms while the *reburp* pulse covers a bandwidth of 4000 Hz for a duration of 1.22 ms. J_{AX} denotes the average inter-hydrides coupling constant (7.5 Hz). Phase cycling: ϕ_1: x,-x,-x,x, y,-y,-y,y; ϕ_2: y,-y,-y,y, x,-x,-x,x; $\phi_{receiver}$: x,-x,-x,x, y,-y,-y,y. **(C)** Pulse scheme to acquire a thermal NMR spectrum of the hydrides. Identical pulses and pulse field gradients were used for this reference experiment. For recovery of thermal equilibrium a delay of 7.5 s at the beginning of each transient was used. Relevant terms of the density operator at different time points are indicated.

For the acquisition of the thermal spectrum 128 transients were collected, for a total duration of ca. 16 minutes. The PHIP spectrum was collected with a single scan. Both spectra are displayed in Figure S4, below.

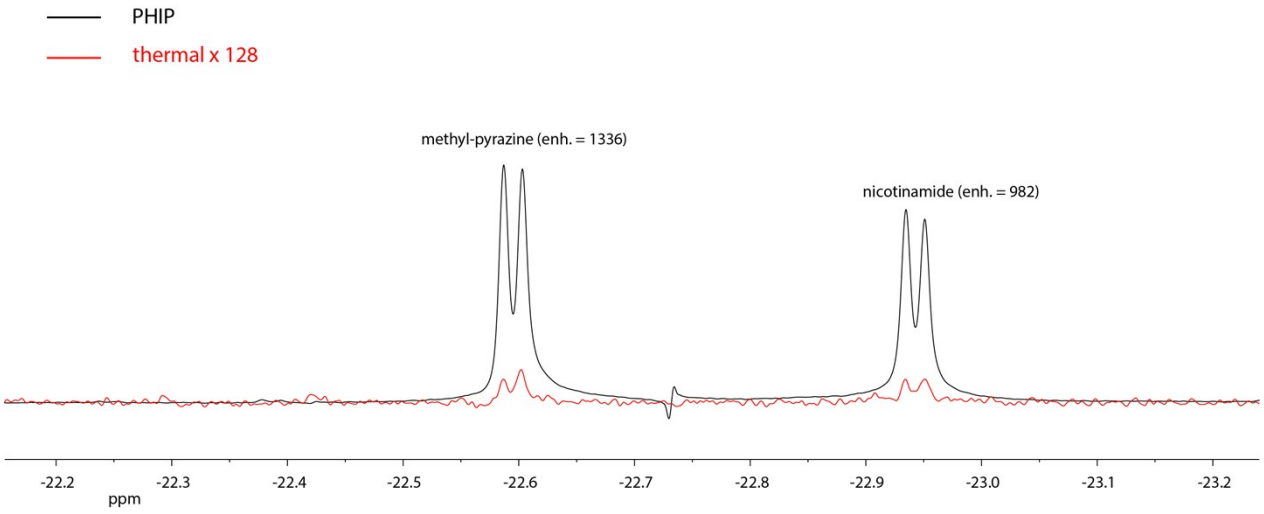

Figure S4 Comparison between a single scan PHIP spectrum (black line) and a thermal spectrum (red line, 128 transients) of the "A" hydrides signals (see Figure S3A) of nicotinamide and 2-methylpyrazine. The spectra were acquired at 35 °C in the presence of 0.2 mM of iridium catalyst, 3 mM of *mtz* and 0.1 mM of 2-methylpyrazine and nicotinamide. The PHIP spectrum was measured under 4 bar of 51%-enriched p-H_2, while thermally equilibrated hydrogen was used for the acquisition of the thermal spectrum. Note that the thermal spectrum in the comparison appears amplified by a factor 128 due to the different number of transients. This has been accounted for in the calculation of the signal enhancement (see text).

As indicated in Figure S4, a ca. 1000-fold signal enhancement was estimated by comparison of the two spectra using the following expression:

$$\text{enhancement} = 128 \times \frac{\text{Integral(PHIP)}}{\text{Integral(thermal)}}$$

This signal enhancement reported for the hydrides should be taken as a ballpark figure; as a matter of fact, it depends on a number of molecular and experimental factors, including temperature, binding constants, kinetic constants, catalyst concentration, co-substrate concentration and p-H_2 enrichment. Note, furthermore, that such sensitivity increase was determined for pyridine- and pyrazine-like compounds, which present optimal thermodynamic and kinetic properties for the PHIP experiment, while lower enhancements (down to 200-fold) have been found for compounds with multiple binding modes, or because of unfavorable steric or electronic factors.

2D PHIP-ZQ spectrum of the SPE of urine

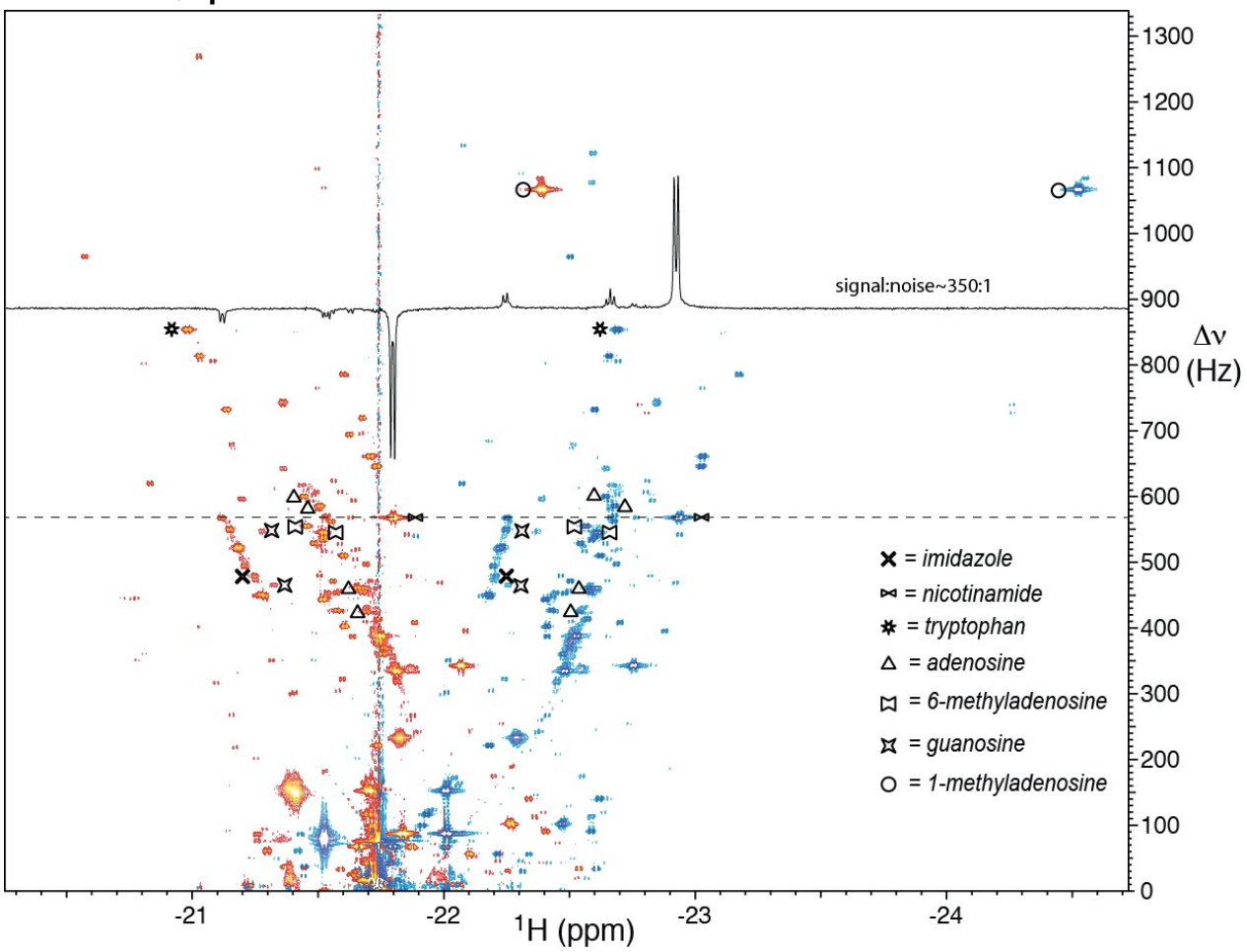

Figure S5. 2D PHIP-ZQ hydrides' spectrum recorded on a methanolic solid phase extract of urine. Signals marked have been assigned by spiking. The 1D trace displays the hydrides' signals of nicotinamide.

Substrate	Hydride A (ppm)	Hydride X (ppm)
Adenosine (I)	-21.444	-22.644
Adenosine (II)	-21.509	-22.681
Adenosine (III)	-21.603	-22.624
Adenosine (IV)	-21.657	-22.578
N1-methyladenosine	-22.390	-24.524
N6-methyladenosine (I)	-21.458	-22.566
N6-methyladenosine (II)	-21.525	-22.613
Guanosine (I)	-21.330	-22.252
Guanosine (II)	-21.288	-22.369
Tryptophan	-20.983	-22.690
Imidazole	-21.251	-22.209
Nicotinamide	-21.800	-22.935

Table S1. ^{1}H chemical shifts of identified hydrides in the 2D PHIP-ZQ acquired for the SPE of urine.

2D PHIP-ZQ spectrum of series of homologous iridium ligands

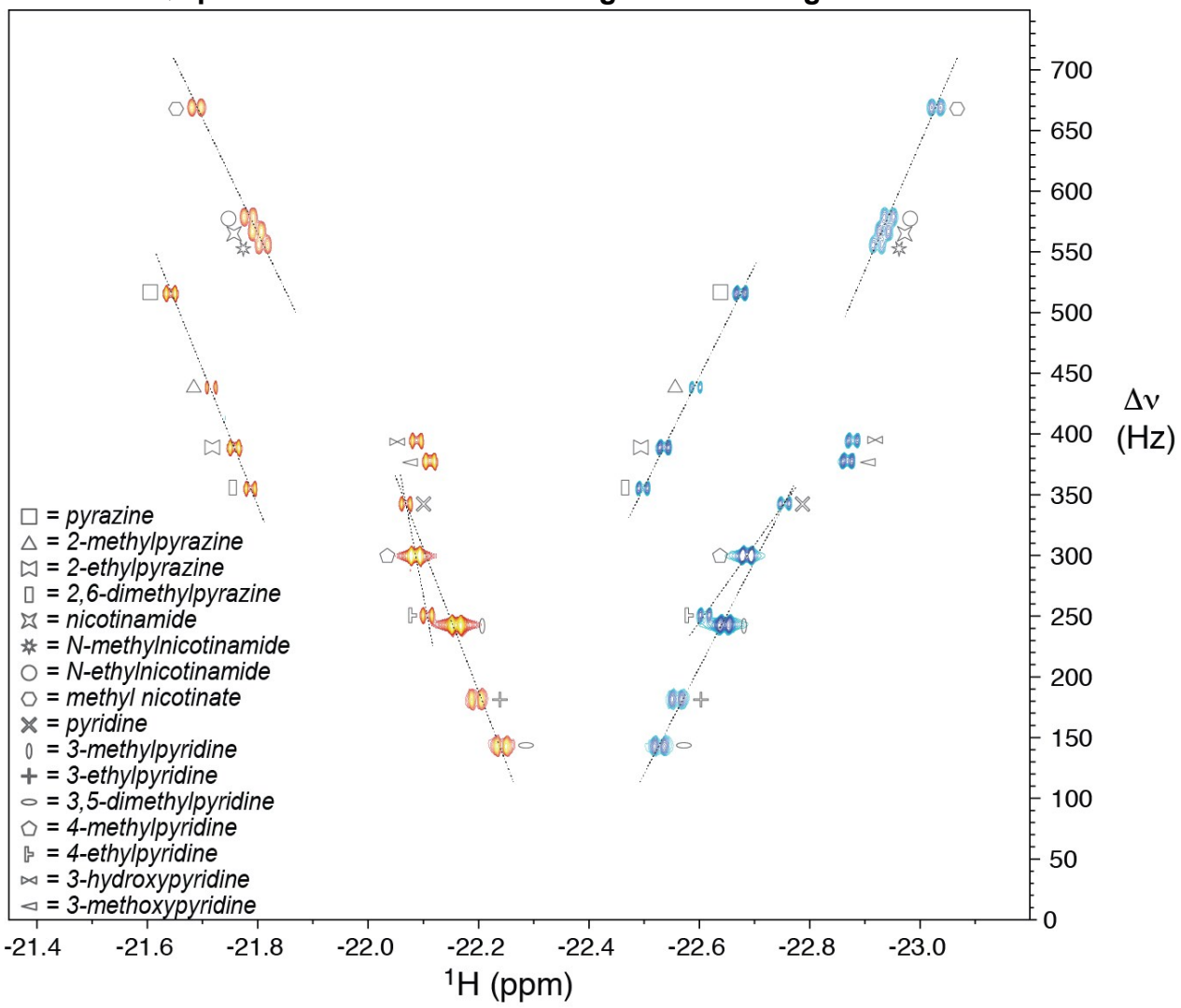

Figure S6. 2D PHIP-ZQ hydrides' spectrum recorded on a mixture of iridium ligands in methanol-d_4, in the presence of 0.8 mM of iridium catalyst, 15 mM of *mtz* and 4 bar of 51%-enriched p-H_2. The signals of structurally homologous compounds are connected with dotted lines. Signal assignment is indicated.

The structure of these compounds is sketched in Figure S7.

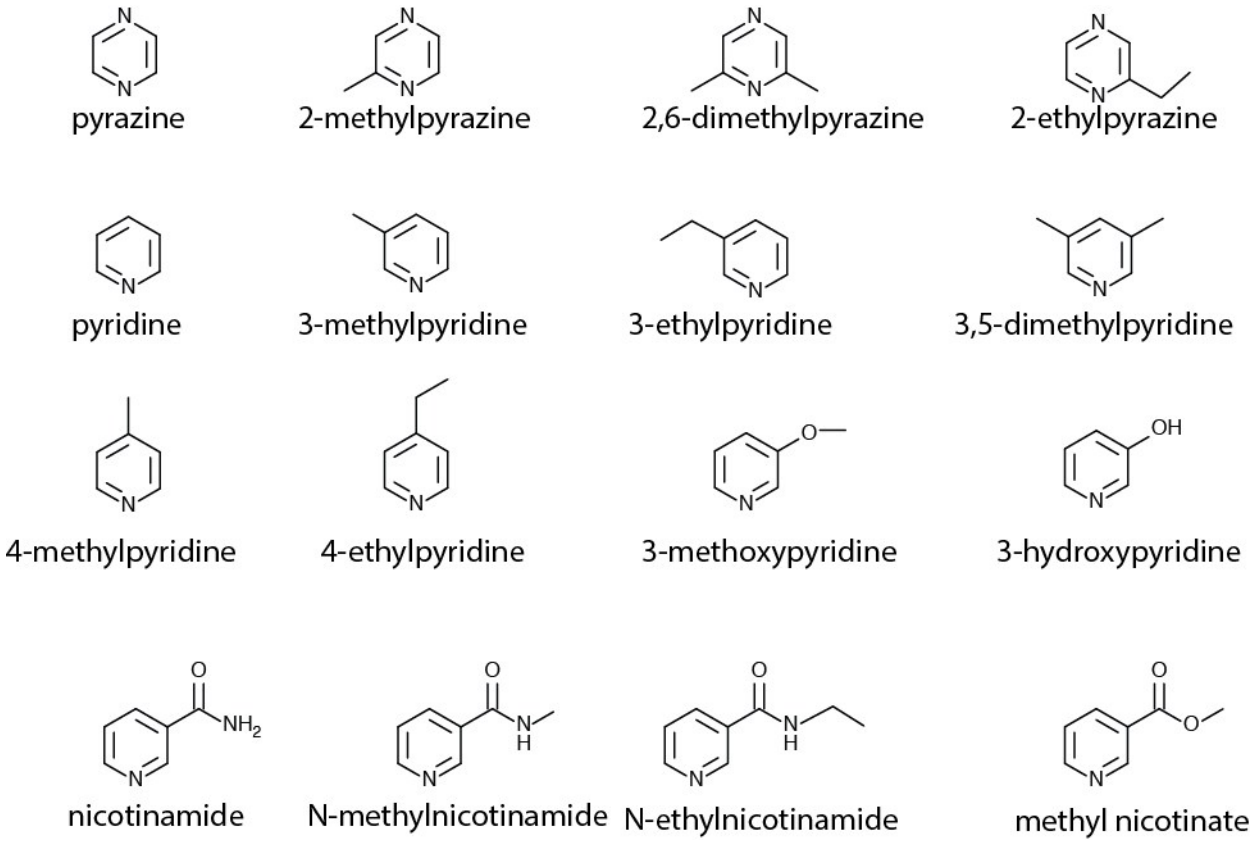

Figure S7. Structures of the iridium ligands used for the spectrum displayed in Figure S6.

Quantification by standard addition

Gravimetric standard addition was performed based on the theoretical description by Hauswaldt *et al*.[8], and was previously described in the Supporting Information of reference [4].[4] The concentration of nicotinamide in the SPE of urine was estimated from the abscissa intercepts of the standard addition curve.

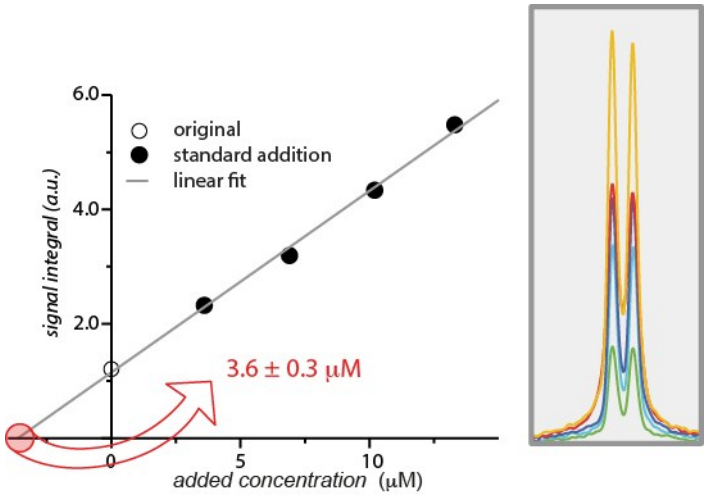

Figure S8. Standard addition to determine the concentration of nicotinamide in the SPE of urine. The plot at the left displays the integral of the signal at -22.935 ppm (high-field hydride) of nicotinamide in a series of 2D PHIP-ZQ spectra as a function of added concentration. The signals at the right hand side correspond to 1D traces of the 2D PHIP-ZQ series displaying the integrated hydride signals.

In order to assess the reproducibility of this method, we have determined the standard deviation of the integrals for a set of ten well-resolved 2D signals over five NMR samples from the same urine batch. Standard deviations in the range 4% - 12% were found for the ten signals, in good agreement with the precision of the concentration estimate for nicotinamide.

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