Supporting Information

Biomolecular Interactions Studied by Low-Field NMR Using SABRE Hyperpolarization

Pierce Pham and Christian Hilty*

[*] Chemistry Department, Texas A&M University 3255 TAMU, College Station, TX (USA). E-mail: chilty@tamu.edu

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Synthesis and characterization of 5-fluoropyridine-3-carboximidamide hydrochloride

Characterization methods

NMR free induction decays (FIDs) were acquired by a Bruker 400 MHz NMR spectrometer (^{1}H at 400 MHz, ^{13}C{^{1}H} at 100 MHz, and ^{19}F at 376 MHz) equipped with a broad-band probe. Spectra were processed with Bruker Topspin software, version 4.1.1.

High-resolution mass spectra were measured by the Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station, TX 77840. Electrospray ionization mass spectrometry (ESI-MS) experiments were performed using a Thermo Scientific Q Exactive Focus.

Synthesis

The compound 5-fluoropyridine-3-carboximidamide hydrochloride was synthesized from a literature procedure,[1] and the synthesis procedure and any modifications were summarized in the following. First, 5-fluoropyridine-3-carbonitrile (0.5 g, 4.1 mmol, Ambeed, Arlington Heights, IL) was dissolved in 5 mL methanol. Sodium methoxide (27 mg, 0.5 mmol, Alfa Aesar, Ward Hill, MA) was added to this solution, and the mixture was stirred at room temperature overnight in a sealed flask. Ammonium chloride (0.44 g, 8.2 mmol, Alfa Aesar) was added to this mixture that was then stirred at room temperature for 2 more days in the same sealed flask. The solvent was evaporated under reduced pressure, and 10 mL of ethanol was added. The mixture was stirred at room temperature for 15 minutes, and the undissolved solid was filtered off with a 0.45 μm PES membrane. The filtrate was left at 4 ºC overnight for crystallization. The product as a white crystalline solid was filtered and washed with 5 mL cold ethanol and dried under vacuum overnight. The yield was 0.414 g or 58%.

^{1}H NMR δ (ppm) (D_2O): 8.72-8.69 (multiplet, 2H) and 8.02 (doublet of doublet of doublet, 1H, $^{3}J_{HF}$ = 8.6 Hz and $^{5}J_{HH}$ = 2.6 and 1.8 Hz)

^{13}C NMR δ (ppm) (D_2O): 163.6, 158.9 (doublet, $^{1}J_{CF}$ = 258 Hz), 143.8 (doublet, $^{4}J_{CF}$ = 4.7 Hz), 142.5 (doublet, $^{2}J_{CF}$ = 24.6 Hz), 126.4 (doublet, $^{3}J_{CF}$ = 4.4 Hz), 123.9 (doublet, $^{2}J_{CF}$ = 21.4 Hz).

^{19}F NMR δ (ppm) (D_2O): 123.87 (doublet, 2H, $^{3}J_{HF}$ = 8.6 Hz)

Positive ESI-HRMS M/Z: found 140.0616, calculated 140.0619 for $[C_6H_7N_3F]^+$ as $[M\text{-}Cl]^+$

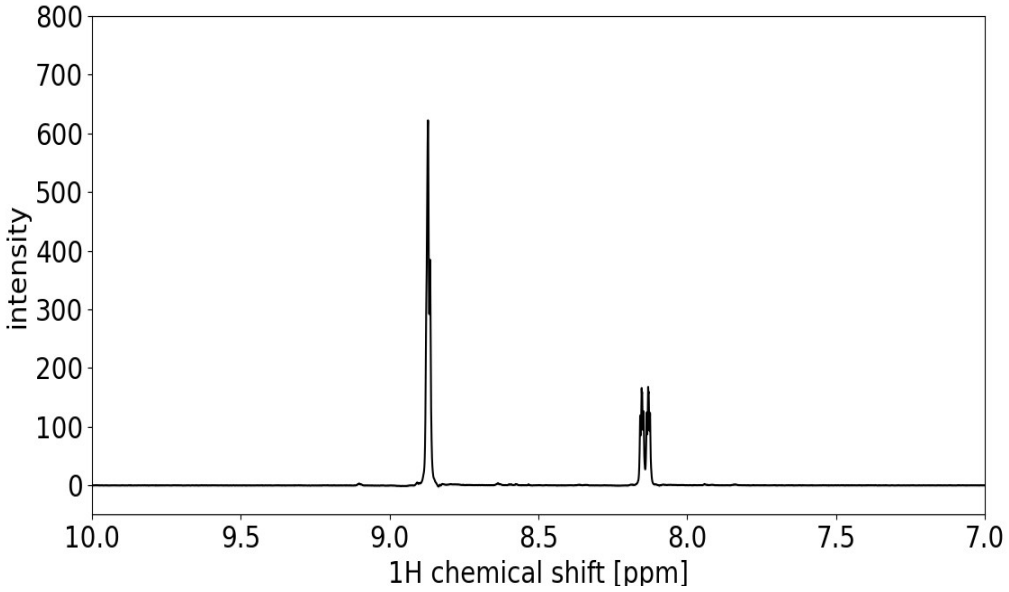

Figure S1: ^{1}H NMR spectrum of 5-fluoropyridine-3-carboximidamide hydrochloride.

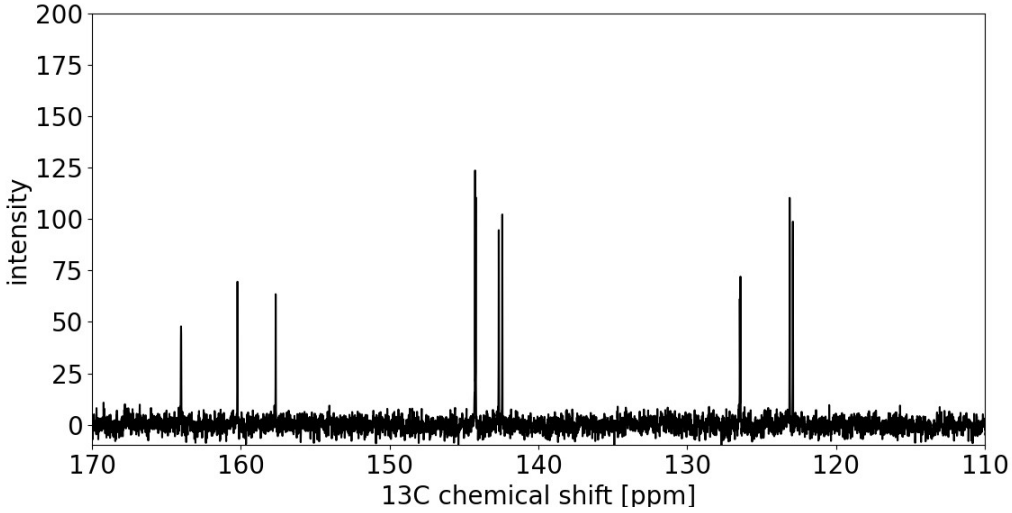

Figure S2: ^{13}C NMR spectrum of 5-fluoropyridine-3-carboximidamide hydrochloride.

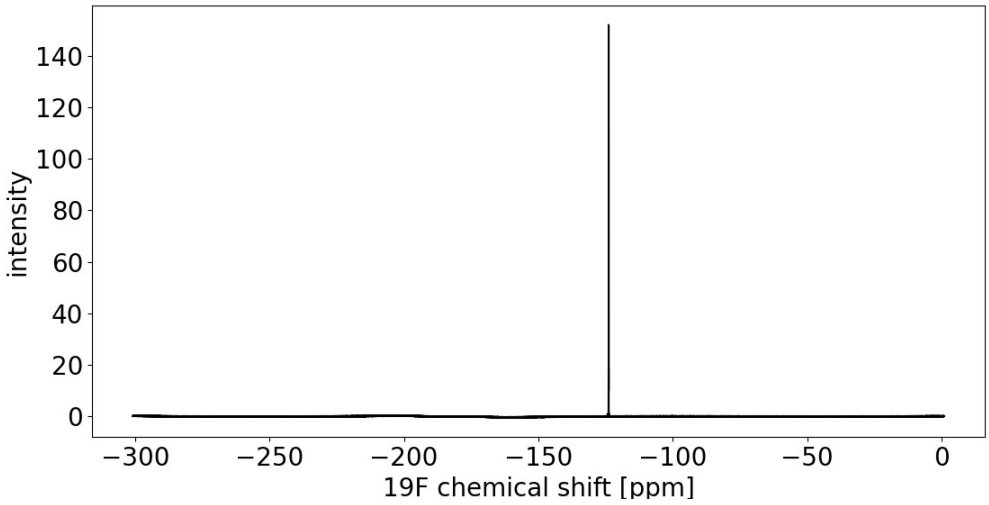

Figure S3: ^{19}F NMR spectrum of 5-fluoropyridine-3-carboximidamide hydrochloride.

SABRE hyperpolarization of 5-fluoropyridine-3-carboximidamide hydrochloride

Configuration of the low-field NMR spectrometer

The configuration of the low-filed NMR spectrometer was described in the previous publication.[2] The main components are summarized in the following. The homogeneous B_0 = 0.85 mT was created by an electromagnet as a tetra-coil that was powered with a DC supply (XDL35-5TP, Xantrax, Elkhart, IN). A shim coil that created a linear gradient in the y direction (along the axis of the NMR sample) was powered with another DC supply (DP832A, Rigol, Portland, OR). A Helmholtz pair that had 15 turns per coil, 45-mm radius, and 45-mm separation was used as an excitation coil, creating a B_1 field in the x direction. This excitation coil was driven by a data acquisition board (SCB-68, National Instruments, Austin, TX). A solenoid coil that included 252 turns on a hollow polyoxymethylene cylinder with a 6-mm radius and 20-mm length was used as the detection coil, being aligned in the y direction. This detection coil was connected with a 75-nF capacitor set to form a resonant circuit with a Q factor of ~13. This circuit generated the input to a preamplifier (SR-560, Stanford Research Systems, Sunnyvale, CA). After being filtered with a band-pass filter (10 kHz – 100 kHz) and amplified 5000 times, its output was sent to the acquisition board.

All operations of parahydrogen bubbling, liquid injection system, NMR spectrometer, data acquisition, and data processing were implemented by our pre-programmed software using Python language (version 3.10, Python Software Foundation, Wilmington, DE).

Pulse length determination

The pulse lengths of π/2 and π pulses were determined using a typical nutation experiment. Two SABRE-hyperpolarized samples that consisted of 1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride or ligand, and 10 mM DMSO in methanol were activated by pressurized with 120 psi parahydrogen (~95% enrichment) for 5 minutes. After that, the sample was hyperpolarized by bubbling with a continuous flow parahydrogen (0.2 slpm) at room temperature and in the B_0 field of the spectrometer (0.85 mT) for the entire experiment. Various pulse lengths from 0.05 to 2.5 ms with 0.05 ms increments and a frequency of 34 kHz were used to excite ^{19}F nuclei of the ligand, and a transient delay between scans was set at 5 s to reproduce polarization. Because the acquired FIDs included the duration of excitation pulses, the first 50-ms portions were trimmed off from the FIDs. A 50-ms portion of each FID was Fourier-transformed without applying window functions and plotted in Figure S4. The lengths of π/2 and π pulses were found at 0.665 and 0.13 ms respectively.

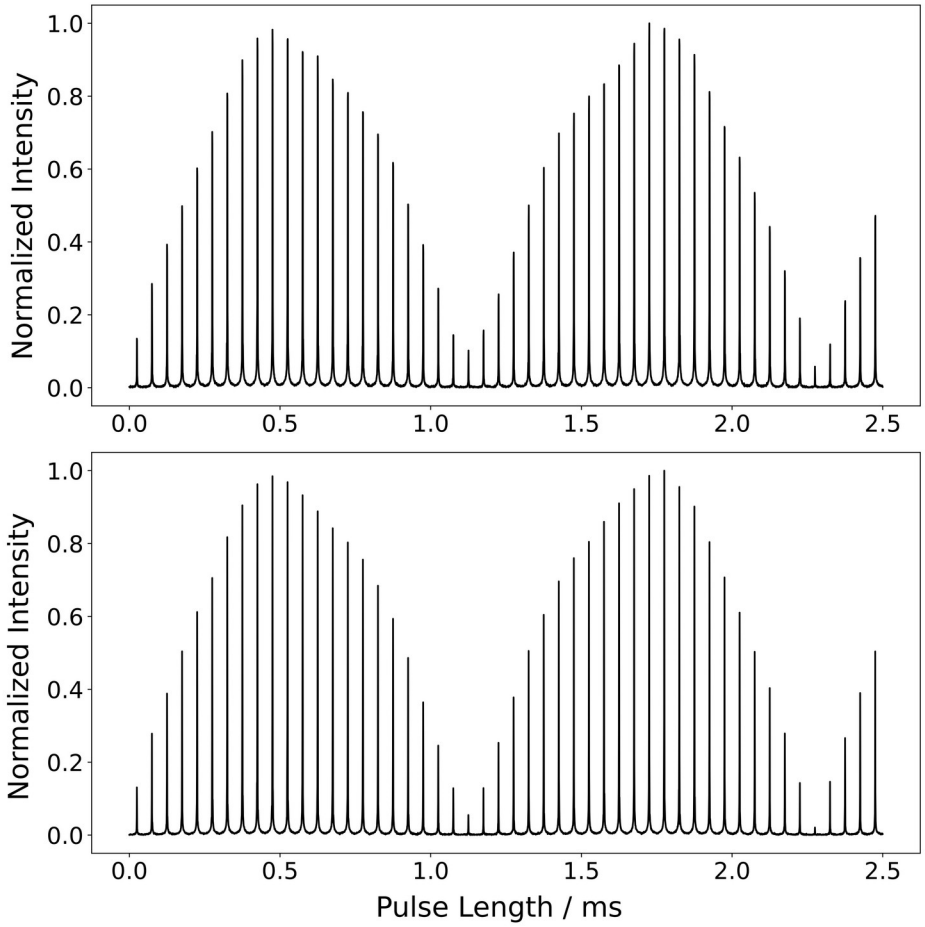

Figure S4: Pulse length calibration of the low-field NMR spectrometer. Serial ^{19}F spectra of two SABRE-enhanced samples (1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide

hydrochloride, and 10 mM DMSO in methanol) that were measured with increasing pulse lengths from 0.05 ms to 2.5 ms. The length of π/2 pulses was found at 0.665 ms.

Optimization of SABRE hyperpolarization of 5-fluoropyridine-3-carboximidamide hydrochloride

The dependencies of SABRE efficiency on temperatures, magnetic fields, and duration of bubbling were determined in the following description. Two SABRE-hyperpolarized samples that consisted of 1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride or ligand, and 10 mM DMSO in methanol were activated by pressurized with 120 psi parahydrogen for 5 minutes. After that, the sample was placed in a chamber whose temperature was adjusted by a constantly heated air-flow generator (FTS Systems Kinetic Air Jet Temperature Controller TC-84). The actual temperature of this chamber was measured with a thermocouple detector. This chamber was placed in a lab-made solenoid coil that was powered with a DC supply (6553A, Hewlett Packard, Palo Alto, CA) to create a desired magnetic field in a millitesla range. The samples were bubbled with a continuous flow parahydrogen (0.2 SLM) to create a ^{19}F polarization. After the bubbling stopped, the samples were manually transferred to the NMR spectrometer for measurements within 4 s. The data acquisition and processing were described in the previous pulse length determination section. Duplicate measurements were conducted for each sample, and the average of these measurements was presented in the following figures.

The magnetic-field dependence was measured from 1 to 10 mT with 1 mT increments when the temperature was kept at 25 ^{o}C. The results are plotted in Figure S5 and indicated the difference of magnetic fields at ~5 mT showed a minor effect on SABRE efficiency. Three temperatures of 25, 35, and 45 ^{o}C were tested for hyperpolarization when the magnetic field was set at 5 mT. The results, shown in Figure S6, indicated that temperatures above 25 ^{o}C worsened the hyperpolarization of this ligand. The duration of bubbling time from 5 to 30 s with 5 s increments was also evaluated. The results were plotted in Figure S7. Therefore, the chosen conditions for hyperpolarizing this ligand included a temperature of 25 ^{o}C, a magnetic field of 5 mT, and a bubbling duration of 25 s.

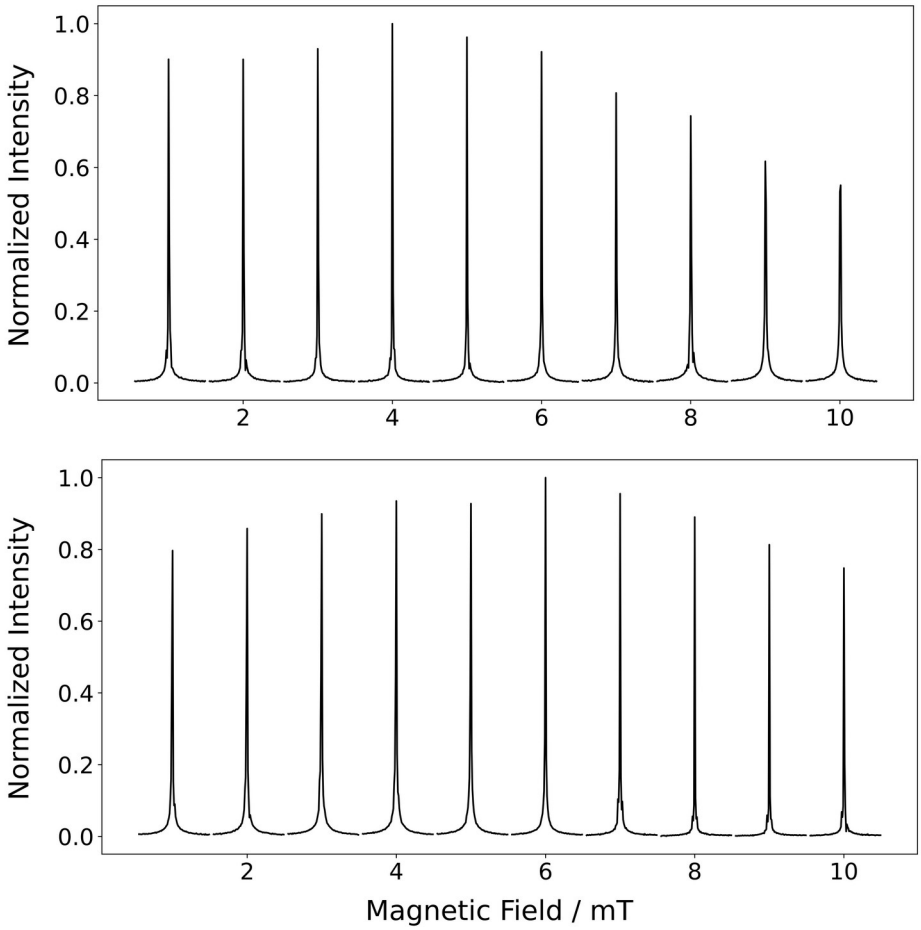

Figure S5: Dependence of SABRE efficiency on magnetic fields from 1 to 10 mT. Series of ^{19}F spectra of two SABRE-enhanced samples (1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 10 mM DMSO in methanol) that were measured after a π/2 pulse.

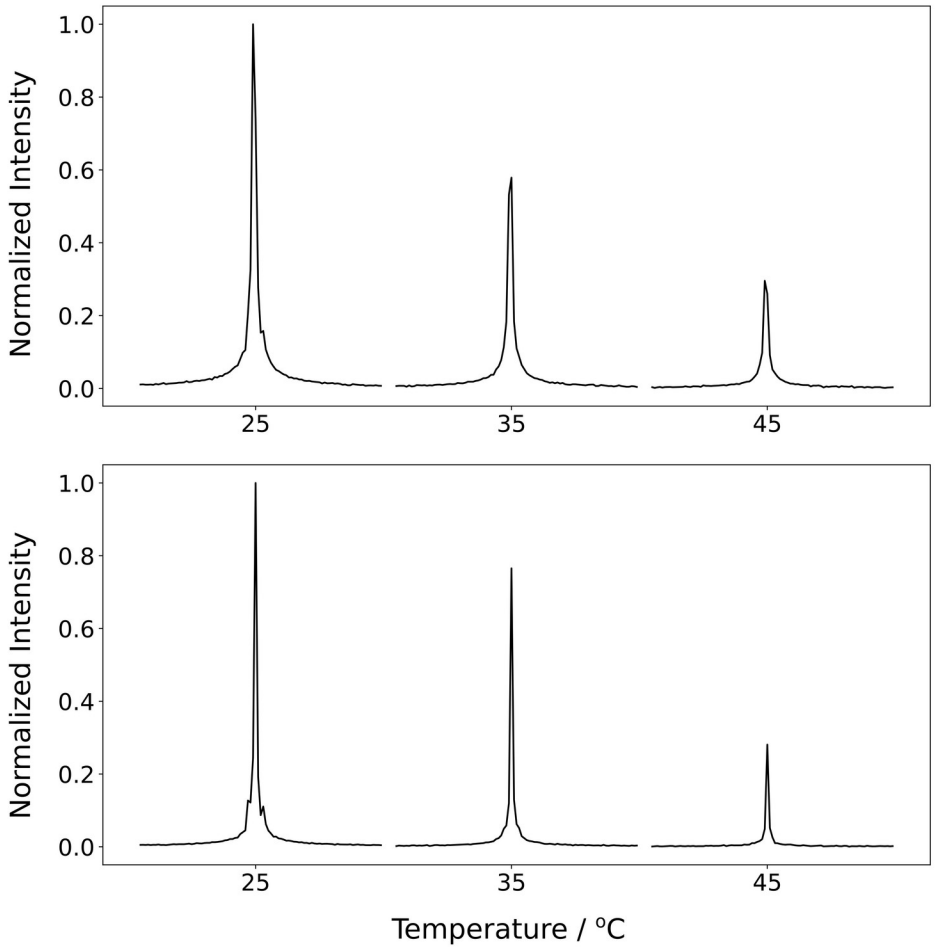

Figure S6: Dependence of SABRE efficiency on temperatures at 25, 35, and 45 °C. Series of ^{19}F spectra of two SABRE-enhanced samples (1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 10 mM DMSO in methanol) that were measured after a π/2 pulse.

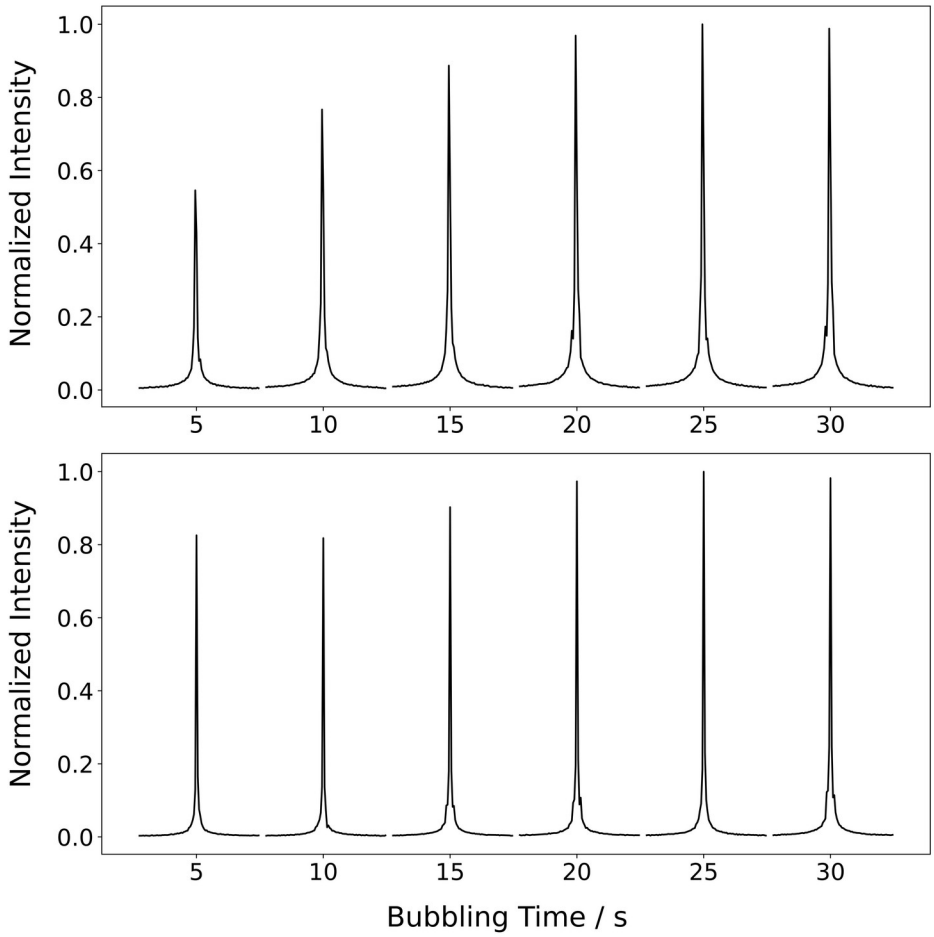

Figure S7: Dependence of SABRE efficiency on a bubbling duration from 5 to 30 seconds. Series of ^{19}F spectra of two SABRE-enhanced samples (1 mM precatalyst, 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 10 mM DMSO in methanol) that were measured after a π/2 pulse.

Activation of precatalysts and deactivation of catalysts

The determination of precatalyst activation and catalyst deactivation was interpreted from the hyperpolarization of 5-fluoropyridine-3-carboximidamide hydrochloride with respect to time. Two samples consisting of 0.05 mM precatalyst, 0.5 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 0.5 mM DMSO in methanol were pressurized and bubbled with a constant flow of parahydrogen (0.2 SLM). The hyperpolarization occurs at B_0 of the NMR spectrometer. FIDs were acquired with a 15 s transient delay, and data was processed as previously mentioned. The activation results were shown in Figure S8. The maximal enhancement was observed under 100 s which indicated the rapid activation of the precatalyst. The efficiency of the capping agents was verified using two samples consisting of 0.05 mM precatalyst, 0.5 mM 5-fluoropyridine-3-carboximidamide hydrochloride, 0.5 mM DMSO, 5 mM pyridine, and 5 mM 2,2'-bipyridine in methanol. These concentrations resembled the CPMG experiments in the main text. All parameters for hyperpolarization

and data acquisition were exact to the activation process. Hyperpolarization was not observed for these samples, which indicated the capping agents of pyridine and 2,2'-bipyridine and their concentrations were sufficient for deactivating the catalyst.

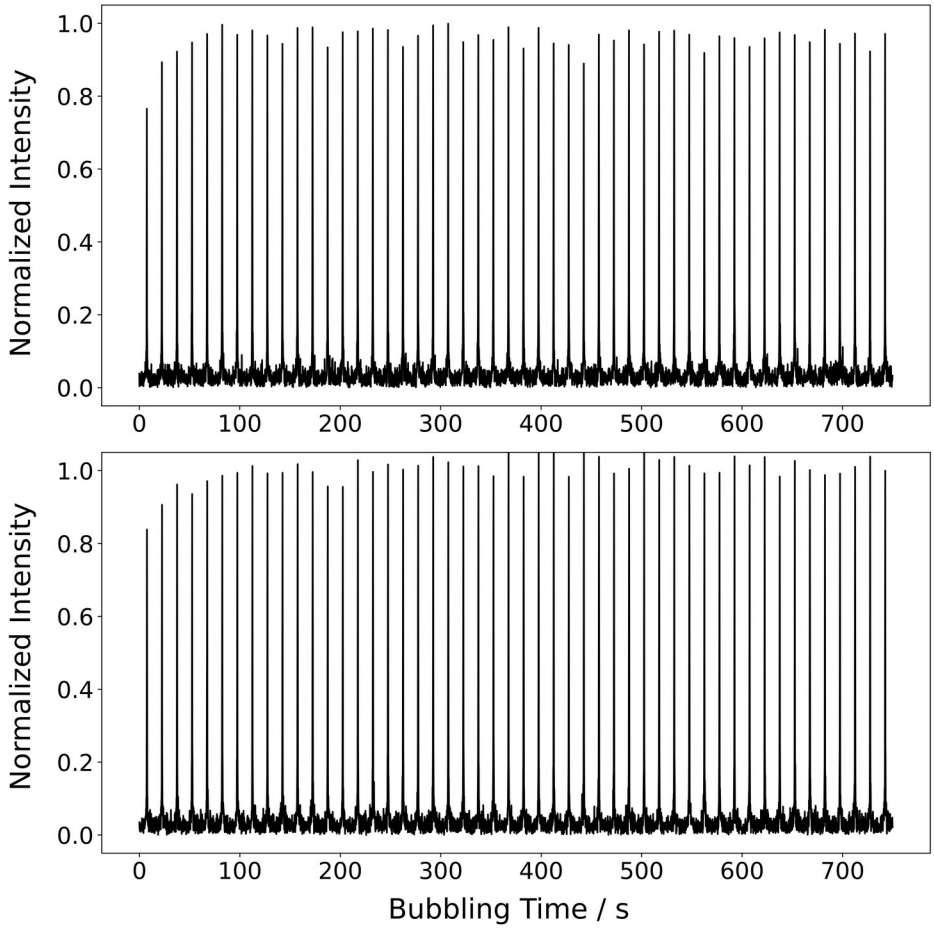

Figure S8: Activation process of two SABRE-enhanced samples (0.05 mM precatalyst, 0.5 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 0.5 mM DMSO in methanol). Series of ^{19}F spectra of two SABRE-enhanced samples that were measured after a π/2 pulse.

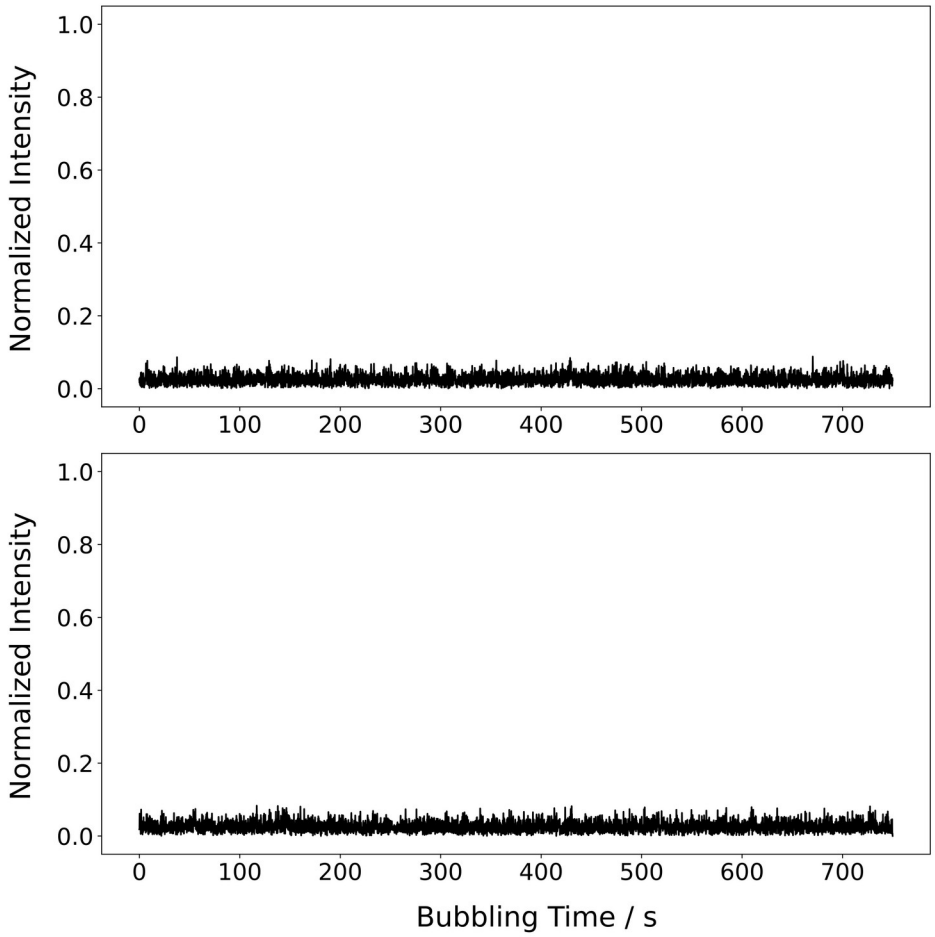

Figure S9: Deactivation process of two SABRE-enhanced samples (0.05 mM precatalyst, 0.5 mM 5-fluoropyridine-3-carboximidamide hydrochloride, 0.5 mM DMSO, 5 mM pyridine, and 5 mM 2,2'-bipyridine in methanol). Series of ^{19}F spectra of two SABRE-enhanced samples that were measured after a π/2 pulse.

For CPMG experiments, the precatalyst activation is observed by ^{19}F polarization with respect to the time of hydrogen inclusion, and the results are shown in Figure S8. The polarization grows and then stagnates under 100 seconds, so a duration of 5 minutes is used to ensure the complete activation of the precatalyst. Chelating agents such as 2,2'-bipyridine and 1,10-phenanthroline show their ability to deactivate SABRE catalysts,[3] and the quick deactivation is observed for 2,2'-bipyridine and is appropriate for investigating protein-ligand interaction.[4,5] Using 2,2'-bipyridine alone, the deactivation of iridium catalysts still needs a fraction of bound ligand, which causes a reduction of free-ligand concentrations and partly effect the determination of ligand affinity. Therefore, a mixture of pyridine and 2,2'-bipyridine with 10-fold excess to the ligand is employed to free the ligand from the deactivated catalysts. The completion of deactivation is shown in Figure S9 in which the hyperpolarization of ^{19}F is absent when pyridine and 2,2'-bipyridine with similar concentrations to the CPMG experiments are added.

Carr-Purcell-Meiboom-Gill experiments

Sample preparation, instrumental parameters, and data processing

A 0.5 mL sample for SABRE hyperpolarization consisted of 1 mM chloro(1,5-cyclooctadiene)[4,5-dimethyl-1,3-bis(2,4,6-trimethylphenyl)imidazol-2-ylidene]iridium(I) or precatalyst (Strem, Newburyport, MA), 10 mM 5-fluoropyridine-3-carboximidamide hydrochloride (synthesis and characterization details shown previously), and 10 mM dimethyl sulfoxide (DMSO) (Alfa Aesar, Ward Hill, MA) in deuterated methanol-d_4 (Cambridge Isotope Laboratories, Andover, MA). This sample was pressurized at 120 psi of hydrogen gas that was enriched to ~95% parahydrogen using a cryogenic system (Advanced Research Systems, Macungie, PA) at 29 K. This pressure was maintained during all experiments. The precatalyst was fully activated in 5 minutes at 25 ^{o}C after adding hydrogen gas. Subsequently, 5-fluoropyridine-3-carboximidamide was hyperpolarized by bubbling with 0.2 SLM of parahydrogen gas for 25 seconds at 25 ^{o}C in an external magnetic field of 5 mT that was generated by a lab-made solenoid coil.

After hyperpolarization, the sample was diluted with 0.5 mL of 100 mM pyridine (Sigma-Alrich, St. Louis, MO) and 100 mM 2,2'-bipyridine (Sigmal-Alrich) in methanol (Fisher Scientific, Waltham, MA) in 0.3 s using an automatic syringe pump (Nexus 6000, Chemyx, Stafford, TX). After dilution, this sample was pushed by the pressurized hydrogen gas into a 0.5 mL sample loop of an injection system and initiated the injection procedure.[4] From the sample loop, the hyperpolarized sample was pushed for a 1 s duration by pressurized water with a flow rate of 170 mL/min set at the pump (1000D syringe pump, Teledyne ISCO, Lincoln, NE) into a 10-mm NMR tube that was pre-loaded with 1 mL of non-hyperpolarized samples and placed in advance in the low-field NMR spectrometer. The flow path was switched using a multi-port valve (DL8UWTI, VICI/Valco, Houston, TX).[6] The R_2 relaxation measurements were initiated in a lab-made low-field (0.85 mT, f_{19F} = 34 kHz) NMR spectrometer[2,7,8] with a 0.5 s delay after the completion of injection. These non-hyperpolarized samples were a buffer (50 mM phosphate, pH=7.6), 41 μM trypsin in this buffer, and a mixture of 41 μM trypsin and 0.5 mM benzamidine hydrochloride in the same buffer for the experiments of free ligand, non-competition, and competition respectively. Protein concentrations were determined by a UV method with ε = 35.1 cm^{-1} mM^{-1} at 280-nm wavelength.[4] After mixing the hyperpolarized and non-hyperpolarized samples, the final samples had a volume of 3.5 mL. The concentrations of ligands and protein were calculated from dilution factors of 15.3 ± 0.7 and 3.53 ± 0.09 folds for the hyperpolarized and non-hyperpolarized samples respectively, which were obtained by injecting a dye using the same parameters. The final concentrations of ligand, protein, and competitor were 655 ± 31 μM, 11.7 ± 0.5 μM, and 142 ± 4 μM respectively.

Compared to previous methods for injecting samples into a 9.4 T magnet,[4,5] the low-field NMR experiment requires only one liquid pump to inject the hyperpolarized ligand into a protein solution that is preloaded in an NMR tube. This injection method resulted in thorough mixing of protein and ligand and was found to be reproducible within <5% and <3% in the concentrations of ligand and protein, respectively. Adding the mixture of dilutant and capping agent to the SABRE samples before

being delivered to a sample loop allows for a longer contact time for catalyst deactivation. Also, the exclusion of capping agents from protein samples would help retain protein integrity. The capping agents are prepared in methanol due to the low 2,2'-bipyridine solubility in water. If water-soluble capping agents and water were used, the percentage of methanol from SABRE samples could further be reduced.

For determining the detection limit, SABRE samples with 5-fold lower concentrations consisted of 0.2 mM precatalyst, 2 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 2 mM DMSO in methanol-d4. The non-hyperpolarized samples consisted of 20 μM trypsin without and with 0.15 mM benzamidine hydrochloride for the non-competition and competition experiments respectively. The final concentrations of ligand, protein, and competitor were 131 ± 6 μM, 5.7 ± 0.3 μM, and 42.5 ± 3.8 μM respectively. SABRE samples with 10-fold lower concentrations consisted of 0.1 mM precatalyst, 1 mM 5-fluoropyridine-3-carboximidamide hydrochloride, and 1 mM DMSO in methanol-d_4. The non-hyperpolarized samples consisted of 20 μM trypsin without and with 0.1 mM benzamidine hydrochloride for the non-competition and competition experiments respectively. The final concentrations of ligand, protein, and competitor were 65.5 ± 3.1 μM, 5.7 ± 0.3 μM, and 28.6 ± 2.5 μM respectively.

The SABRE hyperpolarization of 5-fluoropyridine-3-carboximidamide is optimized with the appropriate temperature, magnetic field, and polarization duration. Among the tested temperature range, the highest enhancement was obtained at 25 ^{o}C when compared with elevated temperatures in Figure S6, which is likely caused by the weak binding of this ligand. The hyperpolarization magnetic fields are screened from 1 to 10 mT, shown in Figure S5. Because the enhancement difference is insignificant in the 4 - 6 mT range, the magnetic field of 5 mT is chosen. Various durations of bubbling time are shown in Figure S7 in which the maximal enhancement is reached after 25 seconds.

The R_2 relaxation rates were measured with a Carr-Purcell-Meiboom-Gill (CPMG) pulse train that included 100 π pulses and a delay between pulses of 2 x τ_{CPMG} = 60 ms. The τ_{CPMG} should be chosen sufficiently short to refocus the magnetization under residual field inhomogeneity and diffusion and convective motions in the sample. Signals were acquired during the entire experiment time (> 6 s) with a sampling rate of 800 kHz. The acquisitions between the π pulses were extracted and trimmed to obtain central fractions of 48 ms, which were Fourier transformed without applying any window functions. The ^{19}F signal integrations of these spectra were plotted and fitted with single exponential functions to obtain R_2 relaxation rates.

Data of CPMG experiments with high ligand concentrations

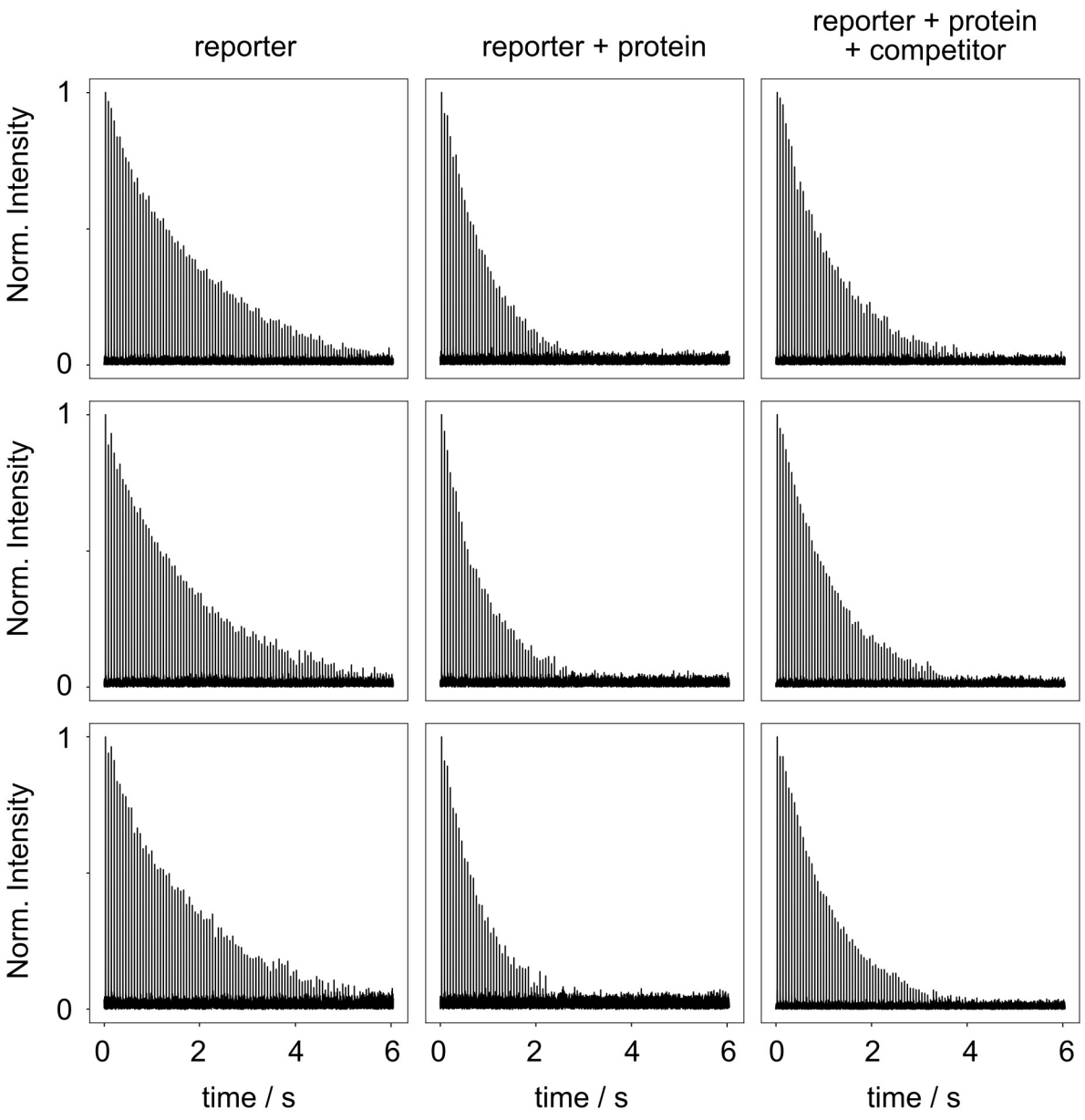

Figure S10: Series of NMR spectra of 100 CPMG echoes for the experiments of free ligand (655 ± 31 μM reporter), non-competition (ligand and 11.7 ± 0.5 μM of protein), and competition (ligand, protein, and 142 ± 4 μM of competitor). Three samples were measured for each condition.

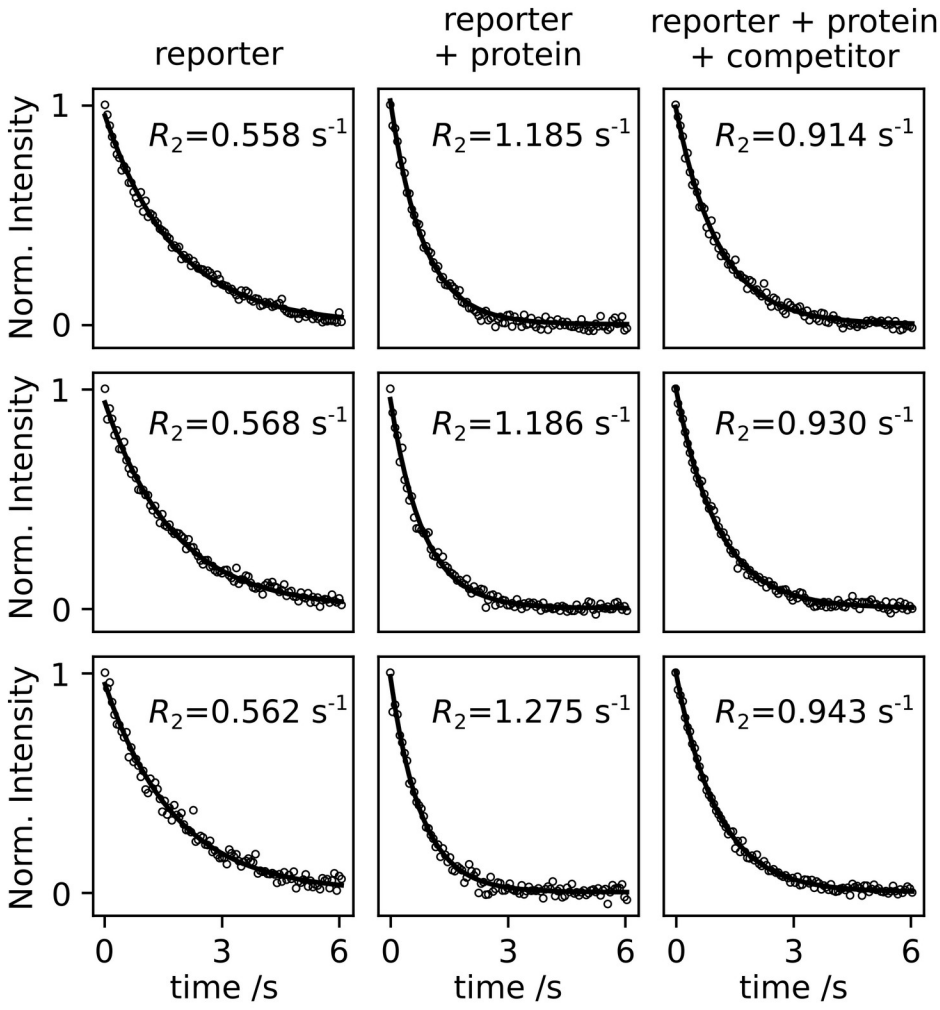

Figure S11: Fitting results of R_2 relaxation rates for the experiments of free ligand, non-competition, and competition. Signals at 34 kHz were integrated from the spectra shown in Figure S11.

Data of CPMG experiments with lower ligand concentrations

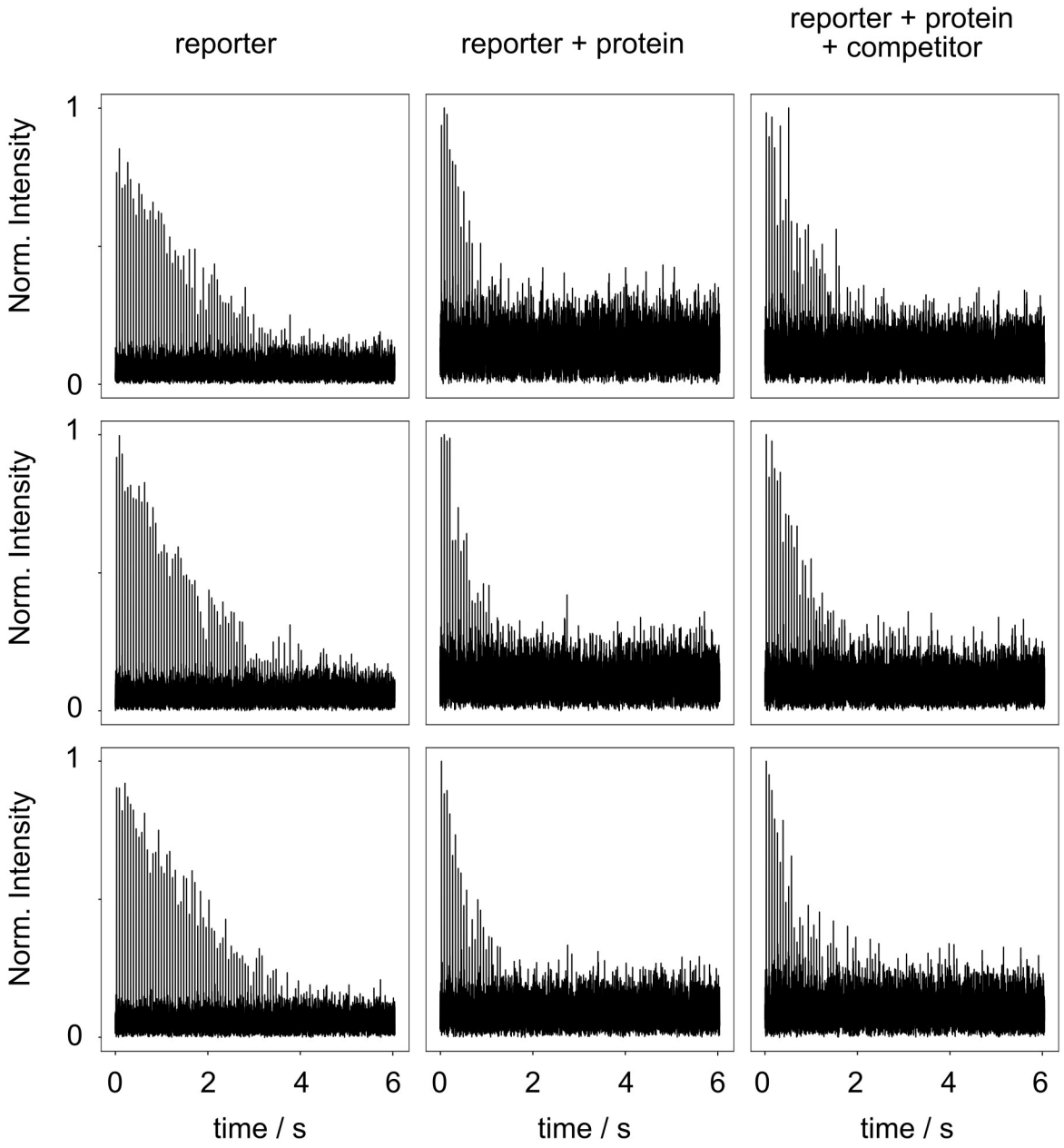

Figure S12: Series of NMR spectra of 100 CPMG echoes for the experiments of free ligand (131± 6 µM reporter), non-competition (reporter and 5.7 ± 0.3 µM protein), and competition (reporter, protein, and 42.5 ± 3.8 µM competitor). Three samples were measured per condition.

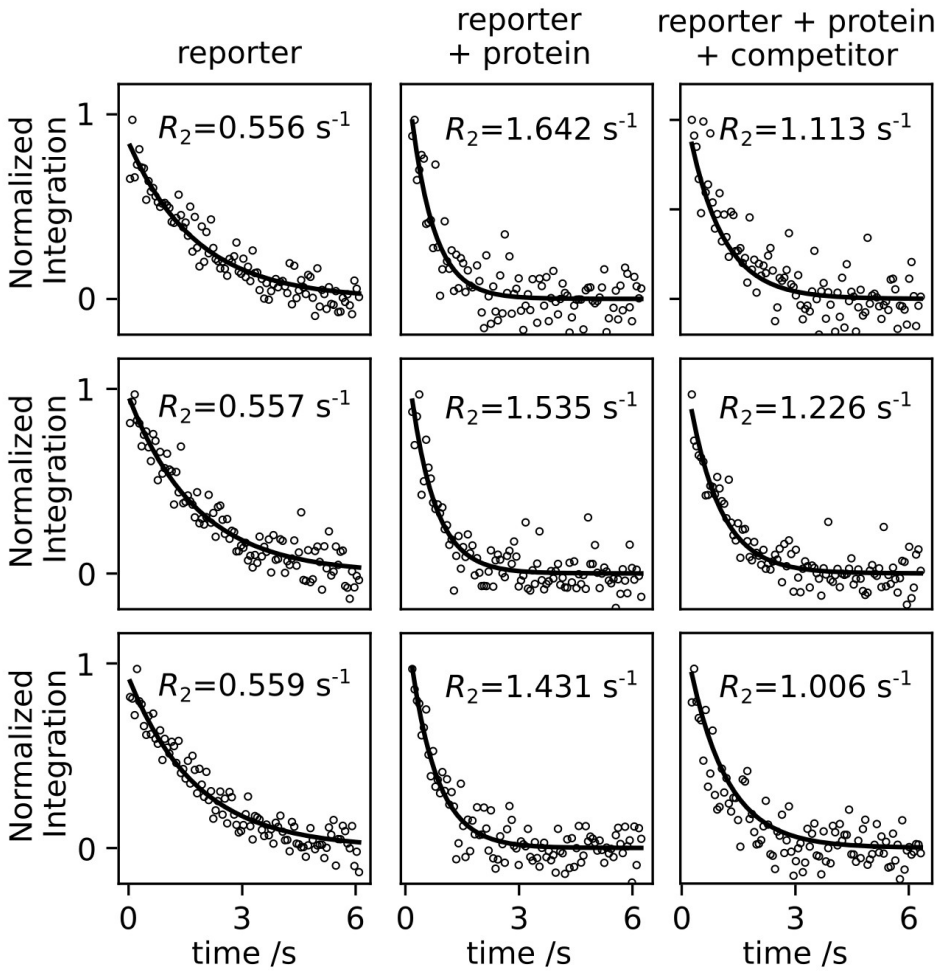

Figure S13: Fitting results of R_2 relaxation rates for the experiments of free ligand, non-competition, and competition. Signals at 34 kHz were integrated from the spectra shown in Figure S12.

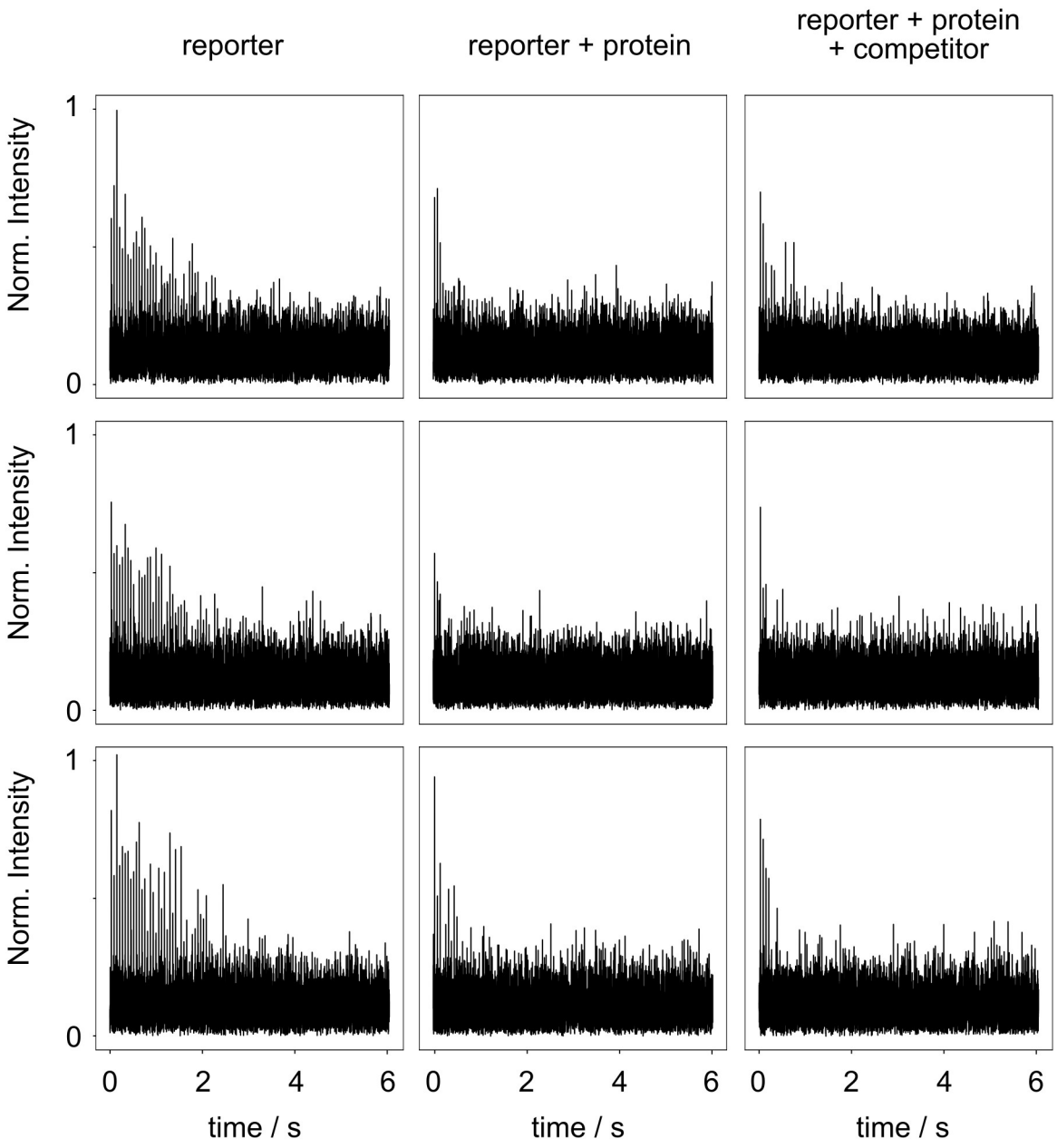

Figure S14: Series of NMR spectra of 100 CPMG echoes for the experiments of free ligand (65.5 ± 3.1 μM reporter), non-competition (reporter and 5.7 ± 0.3 μM protein), and competition (reporter, protein, and 28.6 ± 2.5 μM competitor). Three samples were measured per condition.

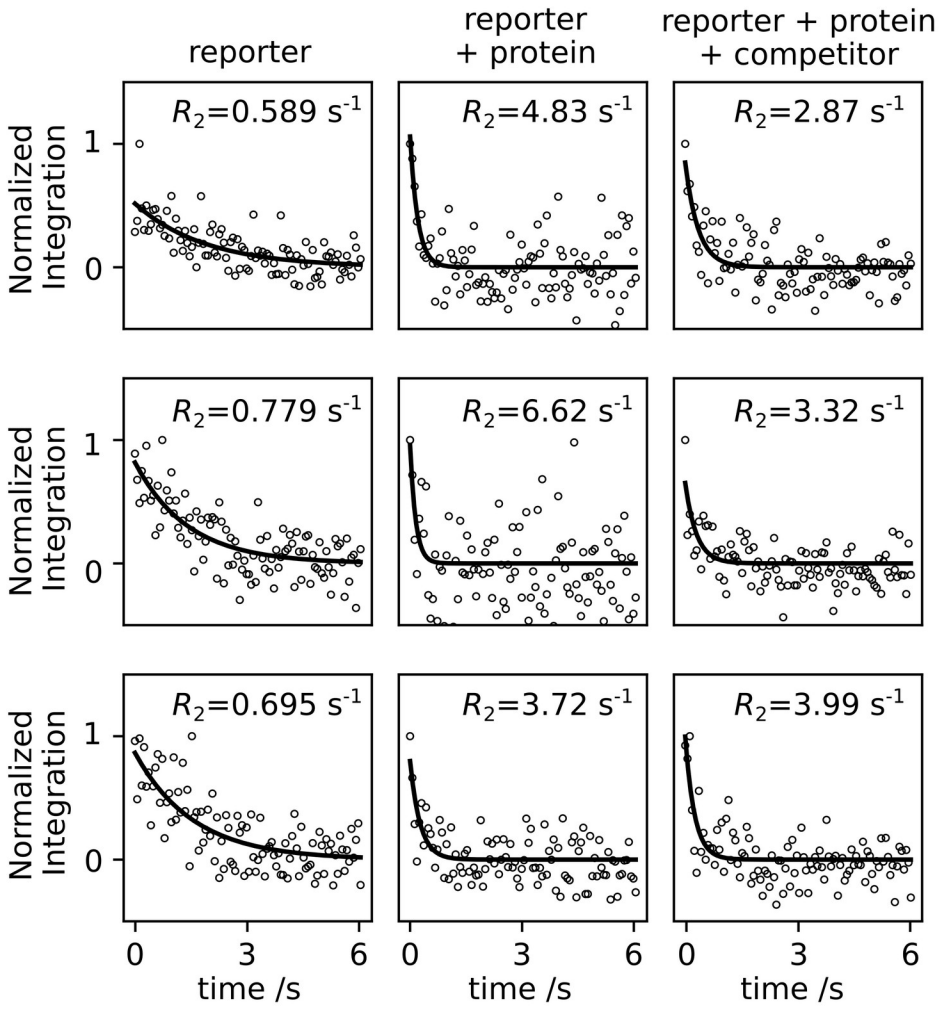

Figure S15: Fitting results of R_2 relaxation rates for the experiments of free ligand, non-competition, and competition. Signals at 34 kHz were integrated from the spectra shown in Figure S14.

Determining the R_1 and R_2 relaxation rates of trifluoroacetic acid and the signal enhancement of 5-fluoropyridine-3-carboximidamide hydrochloride

A 4 mL sample of 0.5 M aqueous trifluoroacetic acid was polarized in a permanent 1 T magnet for about 1 minute and then was manually transferred to a low-field NMR spectrometer within 2 seconds. CPMG measurements were conducted and analyzed as in the previous description. The measurement and fitting results are shown in Figure S16. The average R_2 relaxation rate of trifluoroacetic acid was 0.497 ± 0.016 s^{-1} from three measurements, and the highest signal-to-noise ratio was 19 for the CPMG experiments. The R_1 relaxation rate of trifluoroacetic acid was determined by applying a serial $\pi/6$ pulse train with 0.5 s transients, during which FIDs were acquired. The first 50 - 300 ms portions of FIDs were Fourier-transformed into frequency spectra. The signals at 34 kHz were integrated and fitted for a

single exponential decay as R_{obs}, shown in Figure S17. The R_1 relaxation rates are determined using equation 1.

$$R_1 = R_{obs} + \ln(\cos(\alpha))/\Delta t \quad (1)$$

where α is the small flip angle and Δt is the time separation between these pulses.

The R_1 relaxation rate of trifluoroacetic acid is found as 0.511 ± 0.046 s^{-1}. The ^{19}F polarization is 2.74 ppb, 3.22 ppm, and 30.3 ppm at 0.85 mT, 1 T, and 9.4 T respectively according to Boltzmann distribution. Accounting for 2-second delays prior to CPMG experiments of 0.5 M trifluoroacetic acid, the estimated ^{19}F polarization is 1.16 ppm for the highest SNR of 19. For the same CPMG experiments of 5-fluoropyridine-3-carboximidamide hydrochloride, the highest SNR is 7.8 for 65.5 ± 3.1 μM. These results yield an estimated polarization of 1.09% of this ligand or the enhancements of $3.97 \cdot 10^6$, 3380, and 359 fold in comparison to the normal polarization at 0.85 mT, 1 T and 9.4 T, respectively.

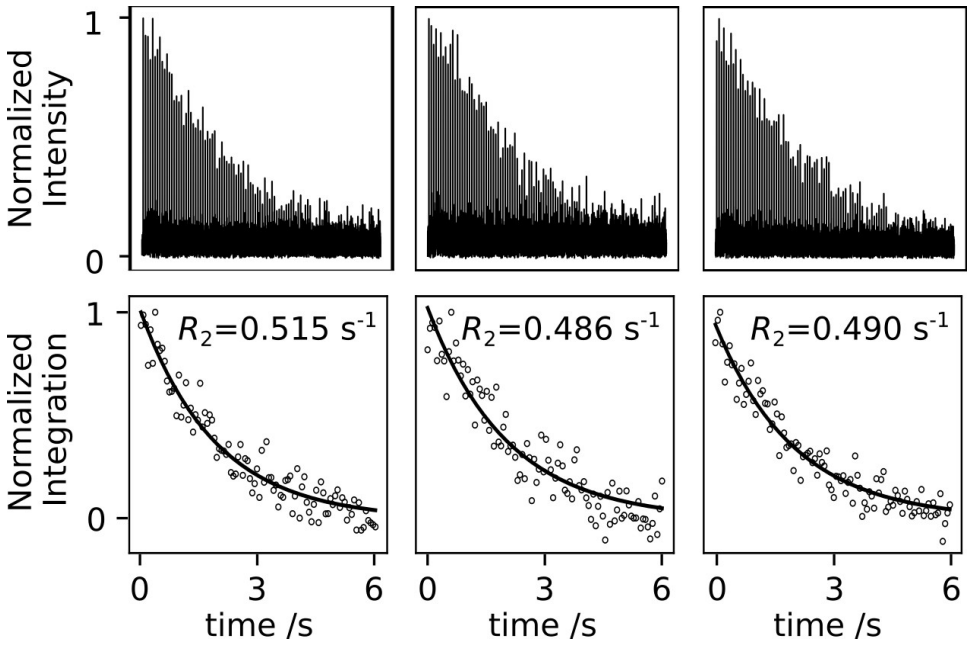

Figure S16: Series of NMR spectra of 100 CPMG echoes for three measurements of a 0.5 M trifluoroacetic acid solution, shown on the top panels, and the fitting results of R_2 relaxation rates for these CPMG experiments, shown in the bottom panels.

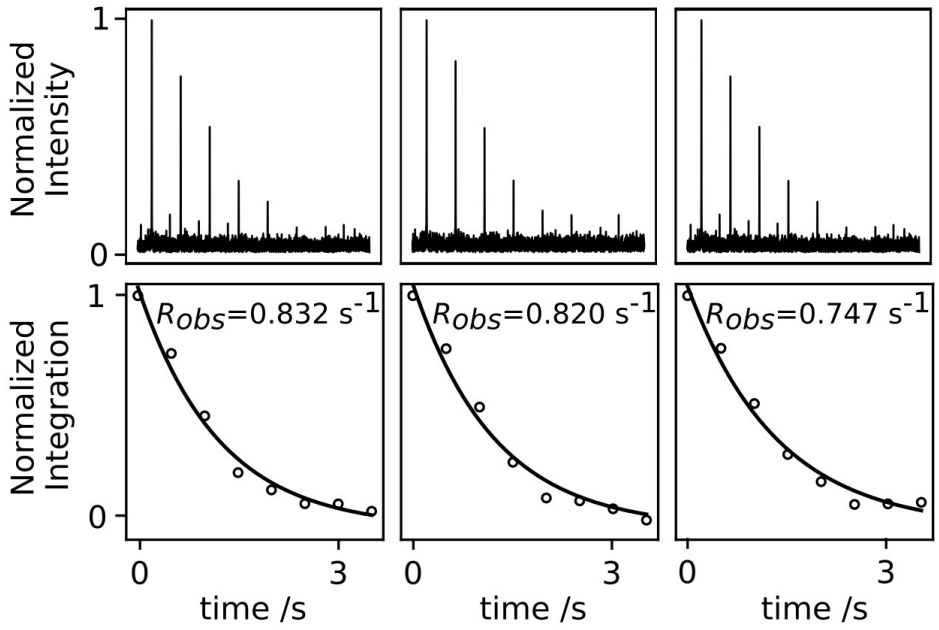

Figure S17: NMR spectra of FIDs acquired after π/6 flip angles for three measurements of a 0.5 M trifluoroacetic acid solution (the top panels) and the fitting results of a single relaxation (the bottom panels).

Determination of the ligand dissociation constant

The ligand dissociation constants (K_D) were determined from a typical NMR titration method. The 1D ^{19}F spectra were measured for samples with various ligand concentrations, 0.5 to 1.5 mM, and fixed concentrations of trypsin (80.5, 82.3, and 85.4 μM). The spectra of the free ligand were fitted with Lorentzian models in equation (2), where I is intensity, A is amplitude, f_0 is central frequency, and $\Delta\upsilon_{1/2max}$ is the width of the half-maximal intensity.

$$I(f) = \frac{2A}{\pi} \cdot \frac{\Delta\nu_{1/2max}}{4(f-f_0)^2 + \Delta\nu_{1/2max}^2} \tag{2}$$

Both peaks of the free ligand are restricted with the same A and $\Delta\upsilon_{1/2max}$ for fitting. The fitting results, shown in Figure S18, yield $\Delta\upsilon_{1/2max}$ = 1.99 ± 0.09 Hz and the coupling constant $^3J_{HF}$ = 8.59 ± 0.05 Hz. The spectra for NMR titration with trypsin were fitted with a double-Lorentzian model that was restricted with the same A and $\Delta\upsilon_{1/2max}$ and a separation of $^3J_{HF}$. The fitting results are also plotted in Figure S18, and the $\Delta\upsilon_{1/2max}$ results were further used as inputs for equations 3 and 4 to determine K_D. All fitting was performed in the Python language using a least-squares method in the Scipy library.

$$\Delta\nu_{1/2max,obs} = (1-\rho_b)\Delta\nu_{1/2max,f} + \rho_b\Delta\nu_{1/2max,b} \tag{3}$$

where ρ_b is the protein-bound fraction of ligand and the subscripts obs, f, and b stand for observed, free, and bound.

$$\rho_b = \frac{K_D + [P]_0 + [L]_0 - \sqrt{(K_D + [P]_0 + [L]_0)^2 - 4 + [P]_0[L]_0}}{2[L]_0} \quad (4)$$

where $[P]_0$ and $[L]_0$ are the total concentrations of protein and ligand respectively. The fitting results are plotted in Figure S19, and K_D = 179 ± 12 μM.

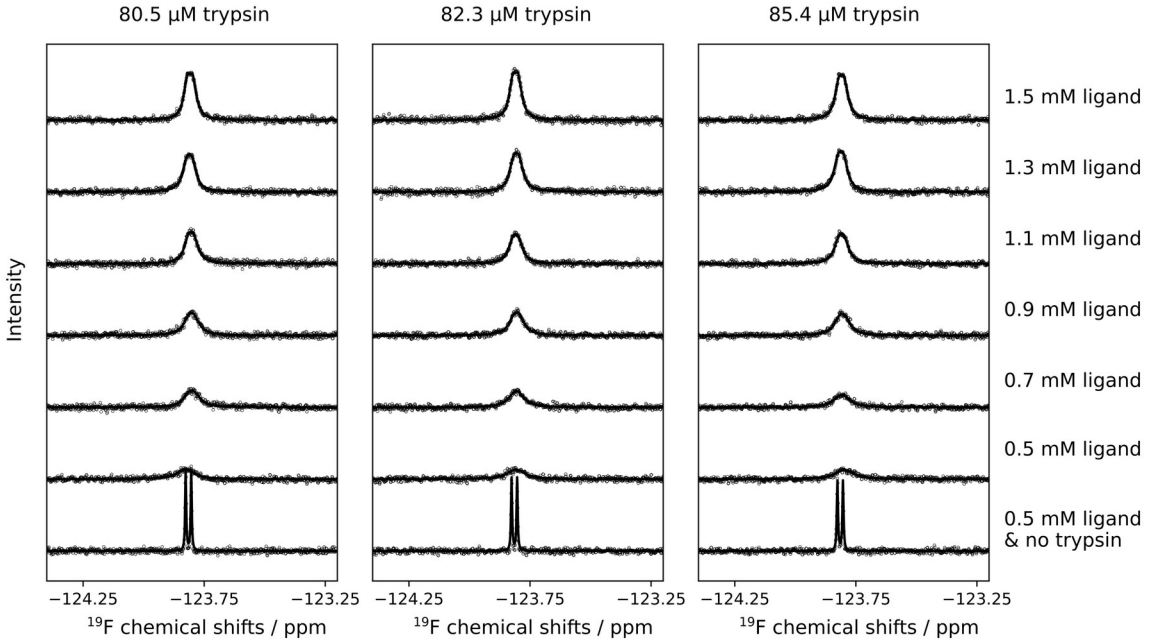

Figure S18: Lorentzian-fitting results of ligand ^{19}F peaks in three titration experiments with various ligand concentrations and fixed trypsin concentrations.

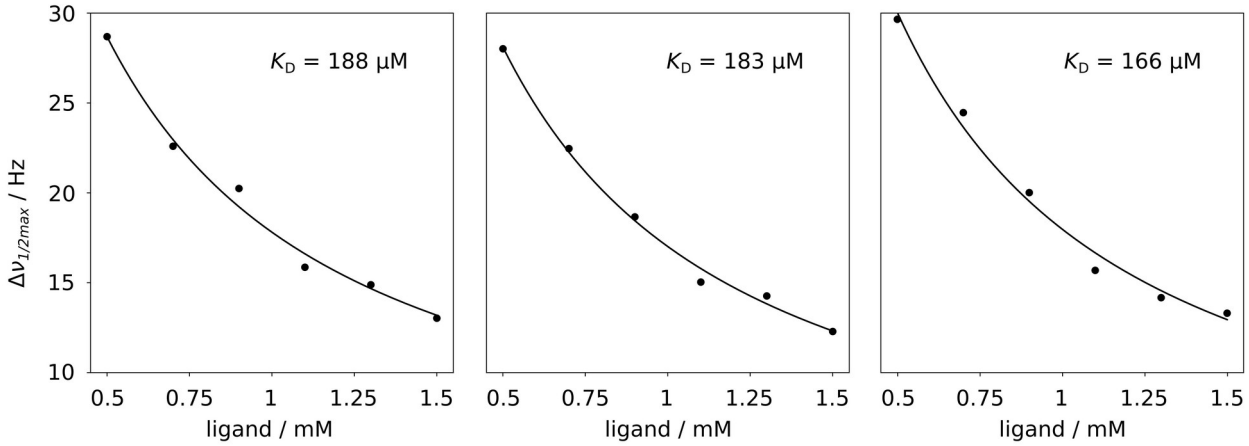

Figure S19: Fitting results for the dissociation constants (K_D) from three titration experiments. Each titration used various concentrations (0.5 – 1.5 mM) of5-fluoropyridine-3-carboximidamide hydrochloride and fixed concentrations of trypsin (80.5, 82.3, and 85.4 μM from left to right respectively).

Simulation of competitor binding

The accuracy in determining K_D of the competitor ($K_{D,c}$) depends on the experimental conditions including the concentrations of the reporter, protein, and competitor and the K_D of the reporter ($K_{D,r}$). The optimal experimental conditions should result in the relative fraction of the bound reporter ligand (f) in the competing and noncompeting experiments, shown in Equation 4, to fall in a range 0.2 - 0.8.

$$f = \frac{p_{b,r}^{(c)}}{p_{b,r}^{(nc)}} \tag{5}$$

where the superscripts c and nc stand for competition and non-competition experiments respectively and $p_{b,r}$ stands for the protein-bound fraction of reporter ligand. The details of this simulation were previously published.[5]

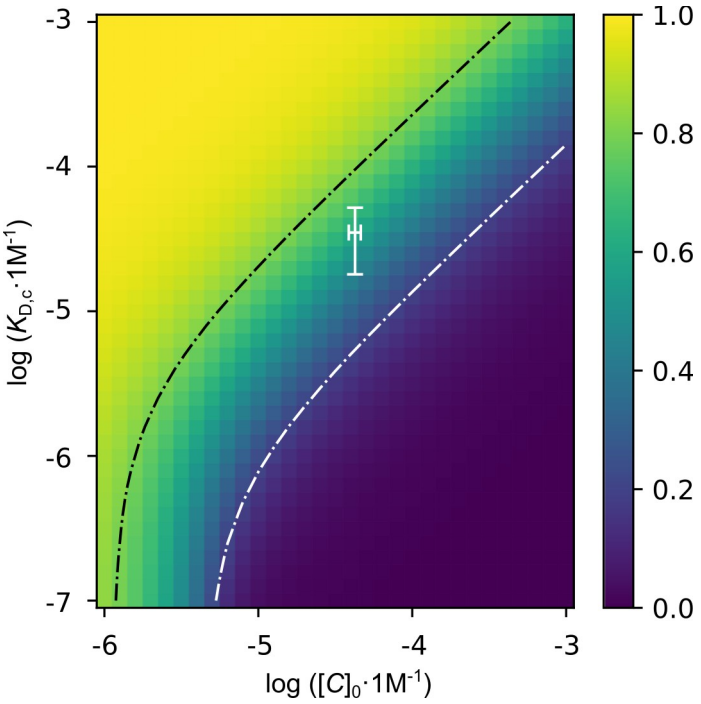

***Figure S20**:Calculated relative fraction of the bound reporter between the competition and noncompetition experiments, $f = p^{(c)}_{b,r}/p^{(nc)}_{b,r}$, plotted as a function of competitor concentration $[C]_0$ and dissociation constant $K_{D,c}$ of the competing ligand of interest. For the calculation, a total concentration $[P]_0$ = 5.7 µM of trypsin, $[R]_0$ = 131 µM of the reporter ligand, and $K_{D,r}$ = 179 µM were used. The experimental condition is indicated for benzamidine ($[C]_0$ = 42.5 ± 3.8 µM, $K_{D,c}$ = 35 ± 17 µM). The f values in the range of 0.2 – 0.8, corresponding to partial displacement, are enclosed between dashdotted curves.*

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