Supporting Information for

Determination of Ligand Binding Modes using Relaxation Dispersion of Hyperpolarized ^{13}C Spins

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1. Experimental

For hyperpolarization, a 10 µL solution of 300 mM 4-(trifluoromethyl)benzene-1-carboximidamide hydrochloride hydrate (TFBC·HCl; Maybridge, U.K.) was prepared with 15 mM OX063 radical and 1 mM Gd-DTPA in ethylene glycol/H_2O (v/v = 3:2) solvent. This solvent forms glassy matrix when frozen. The sample was hyperpolarized on ^{13}C in a HyperSense system (Oxford Instruments, Abingdon, U.K.) by irradiating 60 mW 93.974 GHz microwaves in a 3.35 T magnetic field for 2 hours, at a temperature of 1.4 K. The hyperpolarized sample was rapidly dissolved in 5 mL preheated Tris buffer (50 mM Tris, 10 mM $CaCl_2$, pH 8) and injected into an NMR tube. For the measurement of R_2 in the presence of protein, a volume of 15 µL of 1.38 mM trypsin (AMERSCO, Solon, OH) solutions in 50 mM Tris buffer at pH 8 were preloaded into the NMR tube before injection. For experiments in Figure 3, the amount of TFBC ligand loaded in sample cup and trypsin preloaded in NMR tube was reduced to 4 µL and 6 µL, respectively.

^{13}C NMR spectra were acquired on a 400 MHz NMR spectrometer (Bruker Biospin, Billerica, MA) at a termperature of 303 K. For determination of the R_2 rates, single-scan CPMG data was acquired with the pulse sequence p1 – [$\tau_{cp}/2$ – p2 – $\tau_{cp}/2$] × n, where p1 is a selective 23040 µs π/2 with shape Ebrup2, and p2 is a hard π pulse. The pulsing delay τ_{cp} can be varied by changing the number of data points acquired between two p2 pulses (Figure S2). The values of τ_{cp} were 693.9, 3366.7, 6656.3, 13235.5 µs. In experiments detecting C3 and C4, a WALTZ16 decoupling sequence was applied on ^1H during the selective pulse p1. In the experiments selecting two peaks, a combination of two EBURP2 pulses with frequency offset of 1740 Hz was applied to selectively excite peak 1 and 2, and peak 3 and 4 were pre-suppressed to eliminate the undesired signals.

2. Calculation of DNP Signal Enhancement

The ^{13}C signal enhancement from DNP hyperpolarized samples of 4-(trifluoromethyl)benzene-1-carboximidamide (TFBC) was determined as the ratio of the hyperpolarized to the back calculated, non-hyperpolarized NMR signals. For this purpose, the concentration ratio of TFBC and ethylene glycol, which which was used as a glassing matrix in the DNP experiment, was determined from a non-hyperpolarized ^1H spectrum (Figure S1a). The ^{13}C NMR signal of TFBC, which was not observable without hyperpolarization, was then back calculated using this ratio and the signals of ethylene glycol (Figure S1b). By comparing the area of the hyperpolarized ^{13}C signal (Figure S1c) and the back calculated non-hyperpolarized signal of TFBC, the signal enhancement of C1-5 was determined to be factors of 3110±200, 4940±410, 6250±410, 6230±450, and 5920±420.

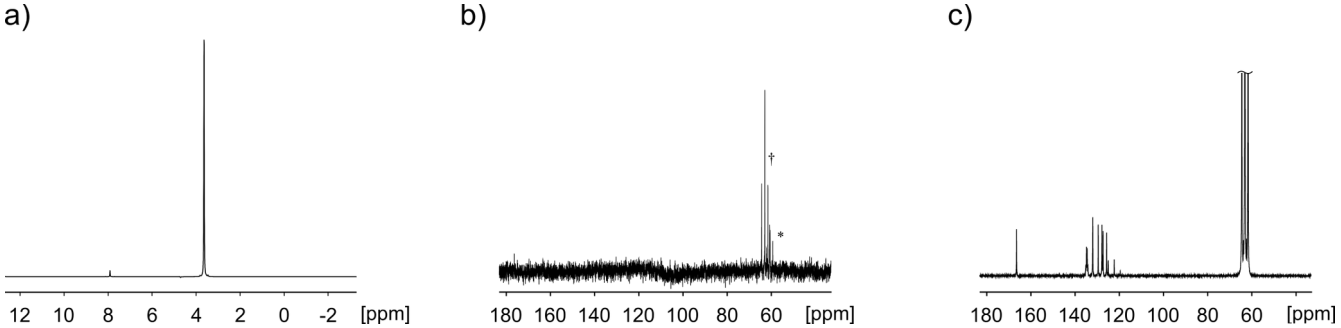

Figure S1. *a) 1H NMR spectrum of a DNP hyperpolarized sample of TFBC, measured after decay of hyperpolarization. Signals of TFBC and ethylene glycol appear near 8 ppm and 4 ppm, respectively. The spectrum was acquired in 8 scans, using watergate W5 pulse sequence water suppression. b) and c) 13C NMR thermal and hyperpolarized spectrum of the same sample as in (a), showing a triplet signal for ethylene glycol (†) near 60 ppm. (*: tris(hydroxymethyl)aminomethane)*

3. NMR Pulse Sequence

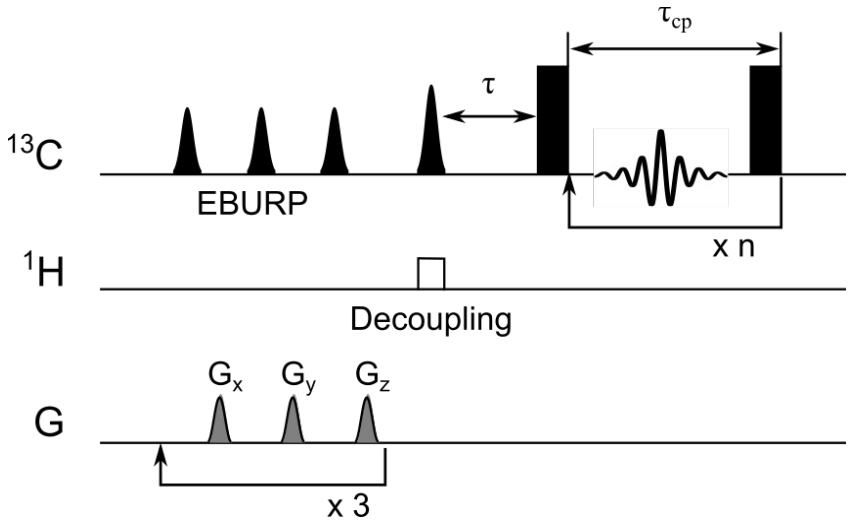

Figure S2. *CPMG Pulse sequence for R_2 relaxation rate measurements.*

A Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to measure R_2 relaxation rates for carbon spins of TFBC. The pulse sequence is shown in Figure S2. Solvent suppression was performed by selectively exciting the chemical shift region of ethylene glycol solvent peaks with a sequence of 3 × 3 EBURP2-shaped π/2 pulses (5000 μs duration), and dephasing the spins with pulsed field gradients G_x, G_y, G_z (1 ms, 35, 36.75, and 62.3 G/cm). Subsequently, a 23040 μs EBURP2-shaped $\pi/2_x$ pulse (140 Hz excitation bandwidth) as applied to selectively excite the target ligand spins. The pulse was centered at 166.38 ppm, 131.8 ppm, 128.5 ppm, 126.3 ppm, and 134.5 ppm for atoms C1 – C5, respectively. In the experiment with double-peak excitation, in addition to the solvent suppression, a similar suppression sequence with 8000 μs EBURP2-shaped π/2 pulses was applied to remove peaks from C3 and C4. A combination of two 23040 μs EBURP2-shaped π/2 pulses with frequency offset of 1740 Hz centered at 149.09 ppm was then used to simultaneously excite C1 and C2 peaks at 166.38 ppm and 131.8 ppm. In the spectrum of TFBC, both C3 and C4 appear as a doublet caused by C-H coupling (Figure S1c). For selective excitation of these signals, a WALTZ16 composite pulse decoupling sequence was looped three times on the ^{1}H channel, to cover the time of the selective excitation pulse. After excitation, all experiments applied a series of π_y hard pulses to produce an echo train, during which signal was acquired. The R_2 relaxation rates were measured at different pulsing delays τ_{cp}. The τ_{cp} was set equal to the sampling interval (dwell time) multiplied with the number of data points in each echo, plus 3 × dwell time to compensate the π pulse length and start/stop receiver for acquisition. In the experiments with single-peak excitation, τ_{cp} = 693.9, 3366.7, or 13235.5 μs was used, for corresponding number of complex data points in each echo of 24, 128 and 512, respectively. In the experiments with double-peak excitation, τ_{cp} was chosen to be 1721.9, 3366.7, 6656.3, 13235.5 μs, and 64, 128, 256, and 512 complex data points were acquired between two successive π pulses.

4. Data Processing

The acquired signal containing a series of echoes was separated according to the number of complex data points in each acquisition. A sine-shaped window function with maximum at the center was applied to each echo. Zero filling was added to the start and end of every processed echo. Each echo was Fourier transformed to yield a single spectrum. After phase correction, the imaginary signal of target carbon spin was minimized.[1] The integrals of peaks in the selected region from each spectrum were collected to fit a R_2 relaxation curve.

At short τ_{cp}, fewer data points were acquired resulting in a lower resolution (Figure S3). For example, in Figure S3a, at τ_{cp} = 488.3 μs, C2 peak sightly overlaps with ethylene glycol peaks due to resolution limit. Whereas, at τ_{cp} = 693.9 μs, the separation of C2 peak from solvent peaks indicates that τ_{cp} = 693.9 μs is the short pulsing limit. Similarly, in the experiments with double-peak excitation (Figure S3b), τ_{cp} = 1721.9 μs was determined to be the short pulsing limit, since it allowed the separation of C1 and C2 peaks. In addition, the oscillation in Figure S4b indicates that τ_{cp} = 26393.9 μs is beyond the long pulsing limit. Therefore, τ_{cp} = 13235.5 μs was applied to CPMG experiments as long pulsing limit.

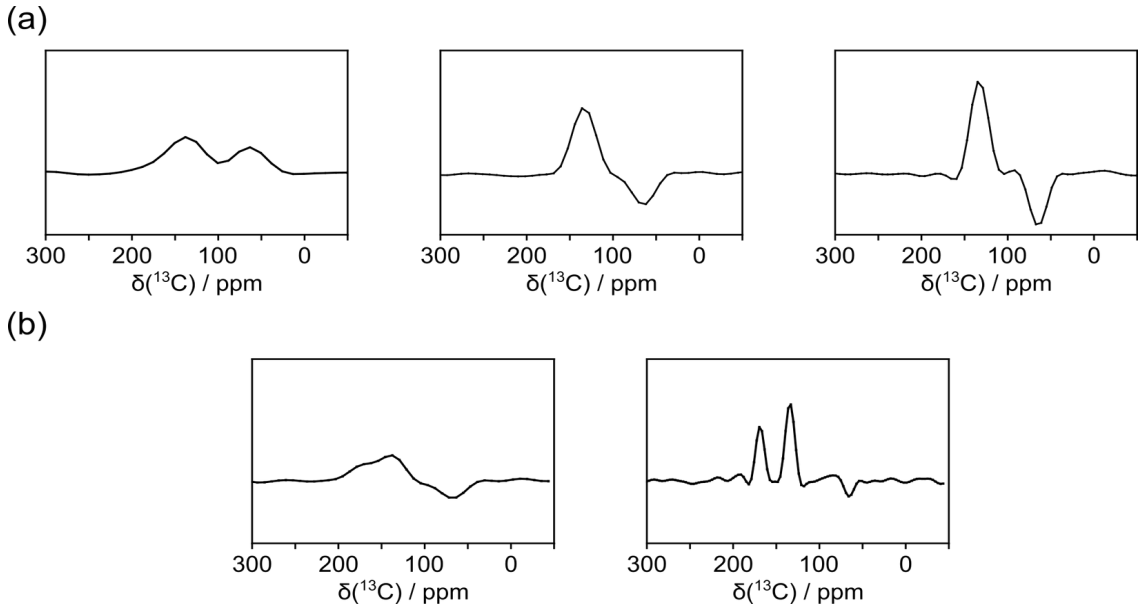

Figure S3*. a) Spectra measured in the experiments with single-peak excitation, with chemical shift selection for the C2 signal at pulsing delay τ_{cp} = 488.3, 693.9, and 899.5 μs (from left to right). Each spectrum is from Fourier Transform of the 10th echo measured by the CPMG experiments. b) Spectra measured in the experiments with double-peak excitation at pulsing delay τ_{cp} = 693.9, 1721.9 μs (left and right). Each spectrum is from Fourier Transform of the 10th echo measured by the CPMG experiments.*

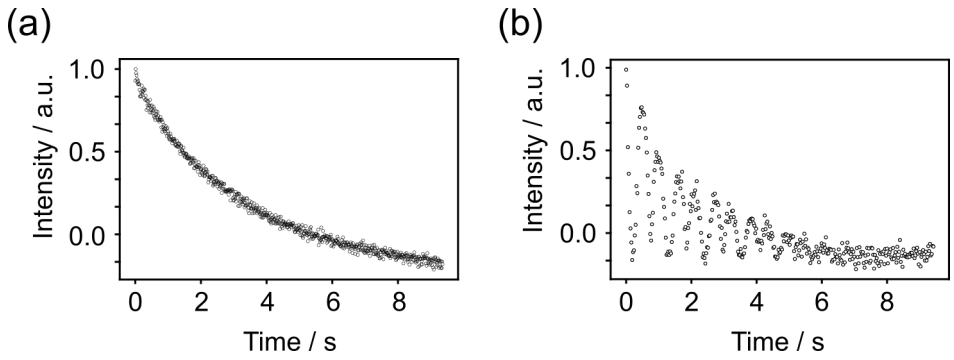

***Figure S4**. Raw time domain points measured by CPMG pulse sequence with chemical shift selection for the C2 signal at pulsing delay a) τ_{cp} = 13235.5 μs and b) τ_{cp} = 26393.9 μs.*

5. Spectra and fitted curves from CPMG experiments

5.1 Experiments with Single-Peak Excitation

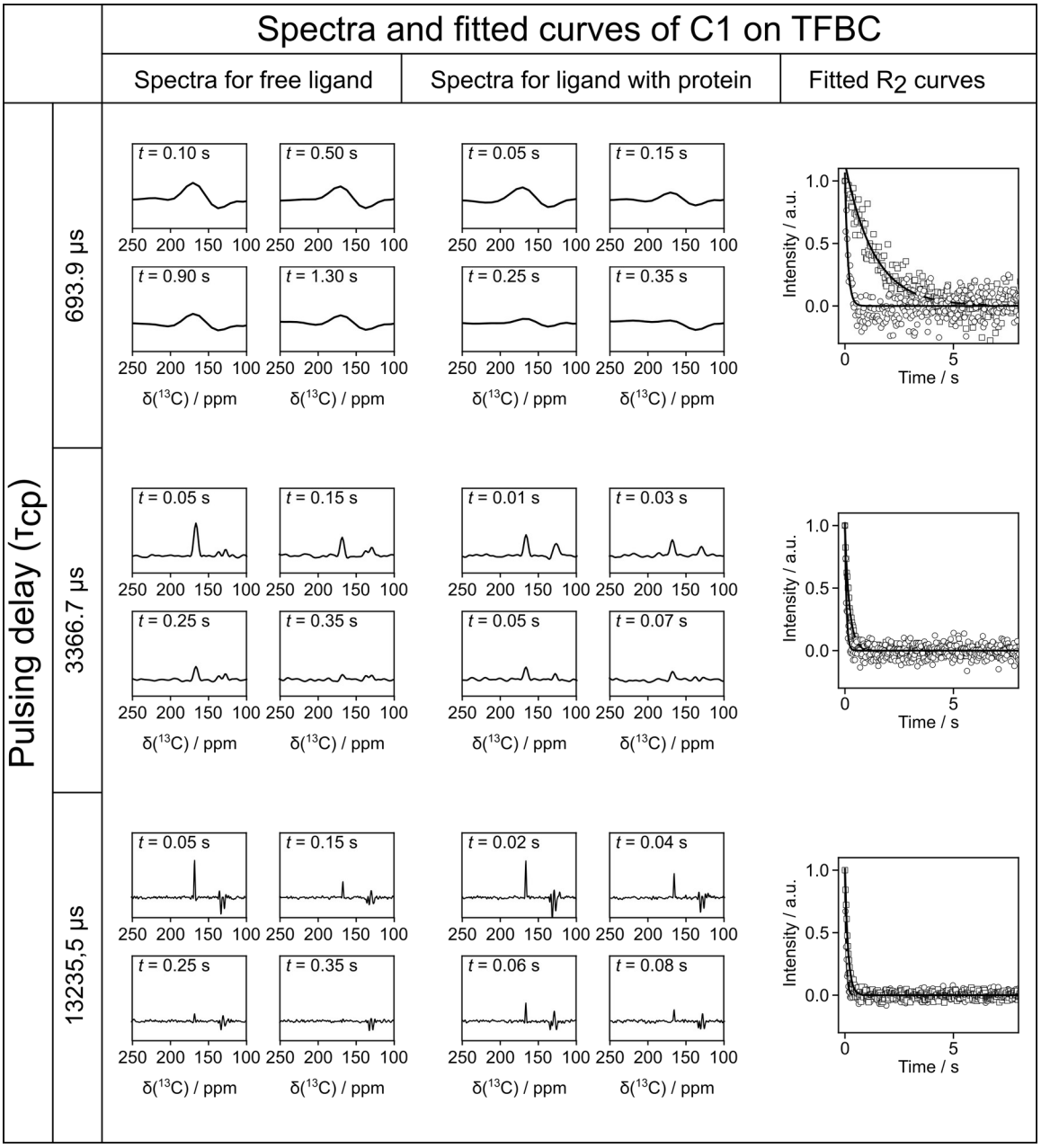

Figure S5. *Selected spectra and fitted R_2 curves for C1 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin. Data were measured with pulsing delays τ_{cp} = 693.9, 3366.7, and 13235.5 µs. Correspondingly, 2.52, 2.06, and 2.41 mM TFBC were used in the free ligand experiments, while 2.55, 2.38, 2.62 mM TFBC and 47.1, 37.8, 37.8 µM trypsin were used in the ligand with protein experiments. Fitted R_2 with standard deviation in the free ligand experiments were 0.76 ± 0.01, 4.79 ± 0.06, and 6.95 ± 0.15 s^{-1}, while those in the ligand with protein experiments were 7.69 ± 0.15, 14.02 ± 0.64, and 16.28 ± 0.59 s^{-1}, respectively.*

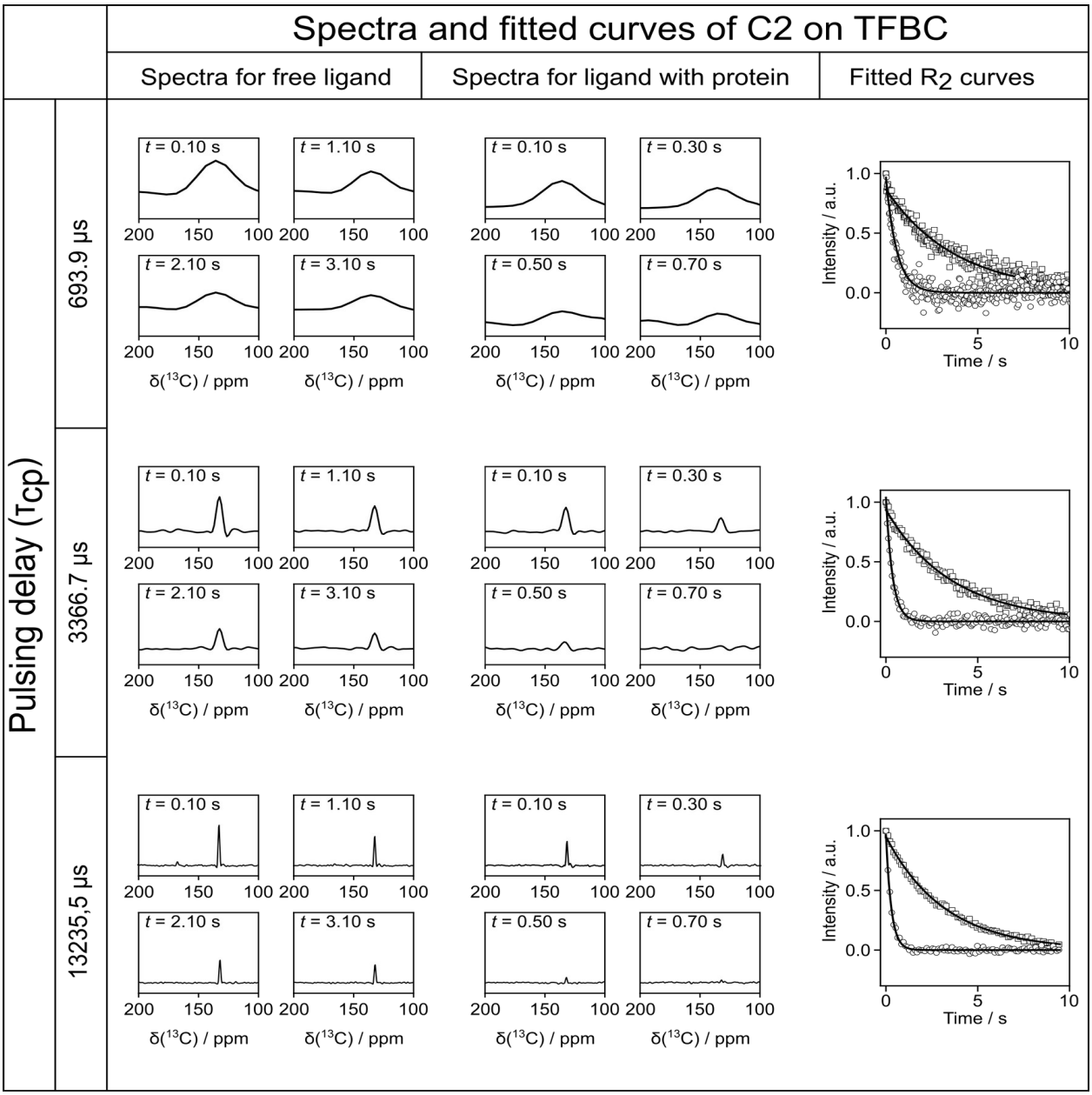

Figure S6. *Selected spectra and fitted R_2 curves for C2 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin. Data were measured with pulsing delays τ_{cp} = 693.9, 3366.7, and 13235.5 µs. Correspondingly, 2.39, 2.24, and 2.48 mM TFBC were used in the free ligand experiments, while 2.64, 2.54, 2.61 mM TFBC and 45.8, 42.1, 45.4 µM trypsin were used in the ligand with protein experiments. Fitted R_2 with standard deviation in the free ligand experiments were 0.80 ± 0.01 0.27 ± 0.00, and 6.95 ± 0.15 s^{-1}, while those in the ligand with protein experiments were 1.68 ± 0.01, 2.76 ± 0.03, and 3.43 ± 0.04 s^{-1}, respectively.*

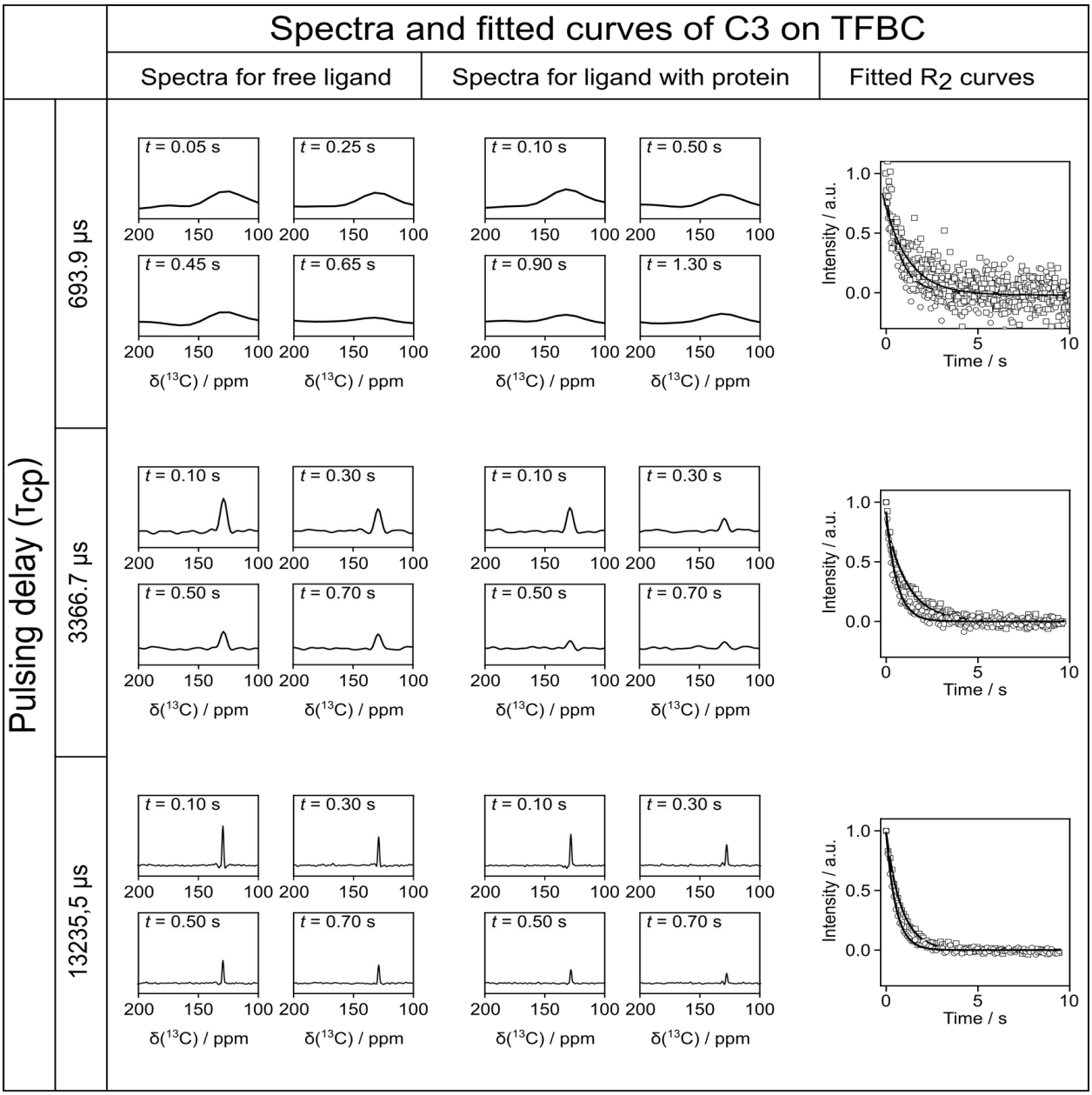

Figure S7. *Selected spectra and fitted R_2 curves for C3 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin. Data were measured with pulsing delays τ_{cp} = 693.9, 3366.7, and 13235.5 µs. Correspondingly, 2.73, 2.33, and 2.56 mM TFBC were used in the free ligand experiments, while 2.64, 2.55, 2.45 mM TFBC and 46.7, 45.8, 45.1 µM trypsin were used in the ligand with protein experiments. Fitted R_2 with standard deviation in the free ligand experiments were 0.80 ± 0.01, 0.90 ± 0.01, and 1.20 ± 0.01 s^{-1}, while those in the ligand with protein experiments were 1.37 ± 0.01, 1.65 ± 0.02, and 1.88 ± 0.02 s^{-1}, respectively.*

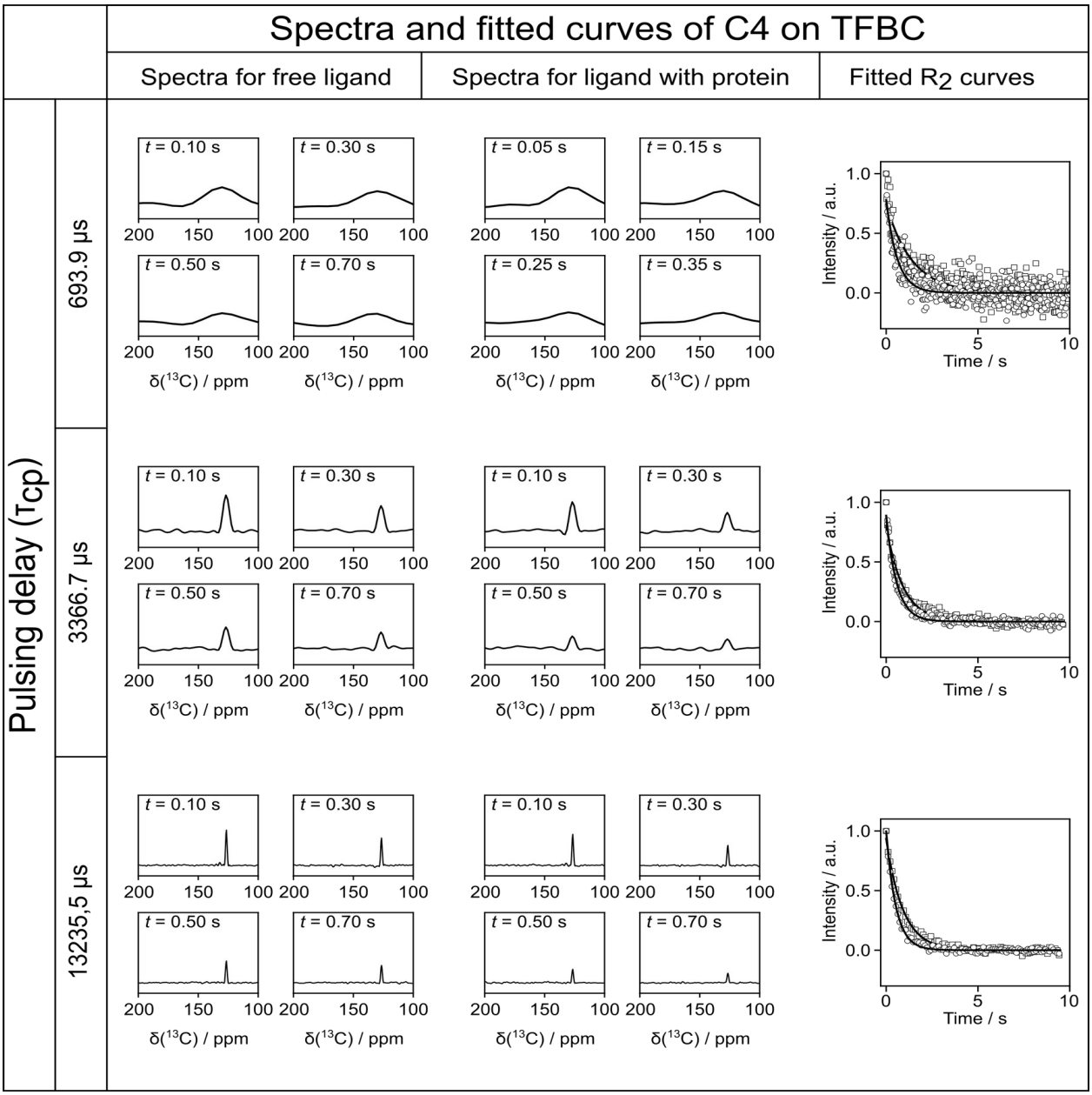

Figure S8. *Selected spectra and fitted R_2 curves for C4 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin. Data were measured with pulsing delays τ_{cp} = 693.9, 3366.7, and 13235.5 µs. Correspondingly, 2.50, 2.54, and 2.60 mM TFBC were used in the free ligand experiments, while 2.51, 2.75, 2.64 mM TFBC and 50.6, 40.3, 46.0 µM trypsin were used in the ligand with protein experiments. Fitted R_2 with standard deviation in the free ligand experiments were 0.76 ± 0.01, 1.19 ± 0.01, and 1.10 ± 0.01 s^{-1}, while those in the ligand with protein experiments were 1.74 ± 0.02, 1.79 ± 0.02, and 1.88 ± 0.02 s^{-1}, respectively.*

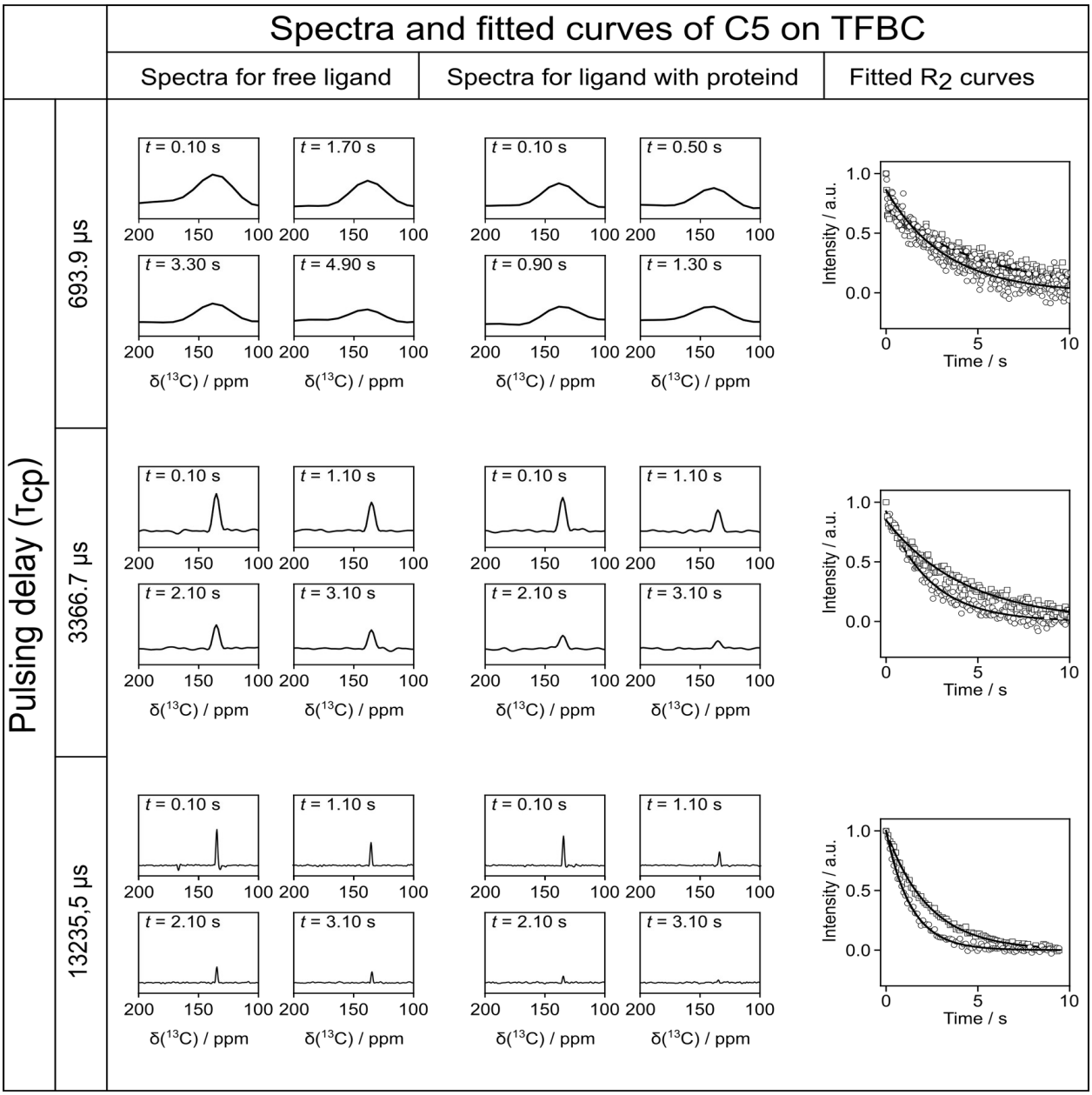

Figure S9. *Selected spectra and fitted R_2 curves for C5 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin. Data were measured with pulsing delays τ_{cp} = 693.9, 3366.7, and 13235.5 µs. Correspondingly, 2.27, 2.20 and 2.66 mM TFBC were used in the free ligand experiments, while 2.37, 2.58, 2.77 mM TFBC and 44.2, 40.0, 4.15 µM trypsin were used in the ligand with protein experiments. Fitted R_2 with standard deviation in the free ligand experiments were 0.18 ± 0.00, 0.24 ± 0.00, and 0.43 ± 0.00 s^{-1}, while those in the ligand with protein experiments were 0.30 ± 0.00, 0.42 ± 0.00, and 0.74 ± 0.01 s^{-1}, respectively.*

Table S1. *Relaxation rates for C1 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Fitted R_2 for C1 on TFBC						
Free ligand experiment			Ligand with protein experiment			
τ_{cp} / µs	$[TFBC]_0$ / mM	$R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	$R_{2,obs}$ / s^{-1}
693.9	2.52	0.76 ± 0.01	693.9	2.55	47.1	7.69 ± 0.15
3366.7	2.06	4.79 ± 0.06	3366.7	2.38	37.8	14.02 ± 0.64
13235.5	2.41	6.95 ± 0.15	13235.5	2.62	37.8	16.28 ± 0.59

Table S2. *Relaxation rates for C2 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Fitted R_2 for C2 on TFBC						
Free ligand experiment			Ligand with protein experiment			
τ_{cp} / µs	$[TFBC]_0$ / mM	$R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	$R_{2,obs}$ / s^{-1}
693.9	2.39	0.25 ± 0.00	693.9	2.64	45.8	1.68 ± 0.01
3366.7	2.24	0.27 ± 0.00	3366.7	2.54	42.1	2.76 ± 0.03
13235.5	2.48	0.31 ± 0.00	13235.5	2.61	45.4	3.43 ± 0.04

Table S3. *Relaxation rates for C3 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Fitted R_2 for C3 on TFBC						
Free ligand experiment			Ligand with protein experiment			
τ_{cp} / µs	$[TFBC]_0$ / mM	$R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	$R_{2,obs}$ / s^{-1}
693.9	2.73	0.80 ± 0.01	693.9	2.64	46.7	1.37 ± 0.01
3366.7	2.33	0.90 ± 0.01	3366.7	2.55	45.8	1.65 ± 0.02
13235.5	2.56	1.20 ± 0.01	13235.5	2.45	45.1	1.88 ± 0.02

Table S4. *Relaxation rates for C4 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Fitted R_2 for C4 on TFBC						
Free ligand experiment			Ligand with protein experiment			
τ_{cp} / µs	$[TFBC]_0$ / mM	$R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	$R_{2,obs}$ / s^{-1}
693.9	2.50	0.76 ± 0.01	693.9	2.51	50.6	1.74 ± 0.02
3366.7	2.54	1.19 ± 0.01	3366.7	2.75	40.3	1.79 ± 0.02
13235.5	2.60	1.10 ± 0.01	13235.5	2.64	46.0	1.88 ± 0.02

Table S5. *Relaxation rates for C5 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Fitted R_2 for C5 on TFBC						
Free ligand experiment			Ligand with protein experiment			
τ_{cp} / μs	$[TFBC]_0$ / mM	$R_{2,f}$ / s^{-1}	τ_{cp} / μs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / μM	$R_{2,obs}$ / s^{-1}
693.9	2.27	0.18 ± 0.00	693.9	2.37	44.2	0.30 ± 0.00
3366.7	2.20	0.24 ± 0.00	3366.7	2.58	40.0	0.42 ± 0.00
13235.5	2.66	0.43 ± 0.00	13235.5	2.77	41.5	0.74 ± 0.01

Table S6. *ΔR_2 for five carbon spins of hyperpolarized TFBC at different pulsing delays τ_{cp}.ΔR_2 was from $\Delta R_2 = R_{2,obs} - X_f R_{2,f}$, assuming $X_f = 1$.*

ΔR_2 of TFBC spins					
τ_{cp} / μs	ΔR_2 for C1	ΔR_2 for C2	ΔR_2 for C3	ΔR_2 for C4	ΔR_2 for C5
693.9	6.94 ± 0.15	1.41 ± 0.01	0.57 ± 0.02	0.97 ± 0.02	0.13 ± 0.00
3366.7	9.23 ± 0.65	2.49 ± 0.03	0.77 ± 0.02	0.60 ± 0.03	0.19 ± 0.00
13235.5	9.33 ± 0.61	3.12 ± 0.04	0.68 ± 0.03	0.78 ± 0.03	0.31 ± 0.01

5.2 Experiment with Double-Peak Excitation

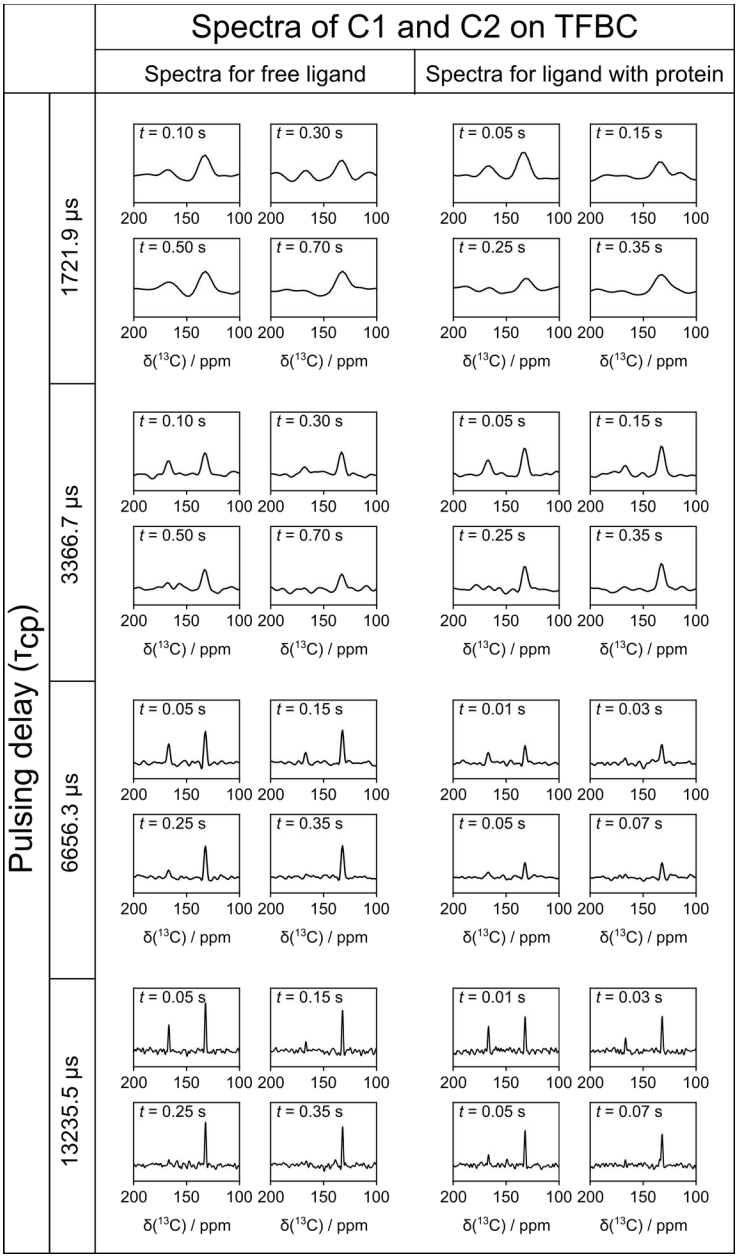

Figure S10. *Selected spectra for C1 and C2 of hyperpolarized TFBC with and without trypsin. Data were measured with pulsing delays τ_{cp} = 1721.9, 3366.7, 6656.3, 13235.5 µs. Correspondingly, 0.82, 0.72, 0.82, and 0.71 mM TFBC were used in the free ligand experiments, while 0.72, 0.75, 0.68, 0.71 mM TFBC and 16.0, 15.2, 13.4, 17.0 µM trypsin were used in the ligand with protein experiments.*

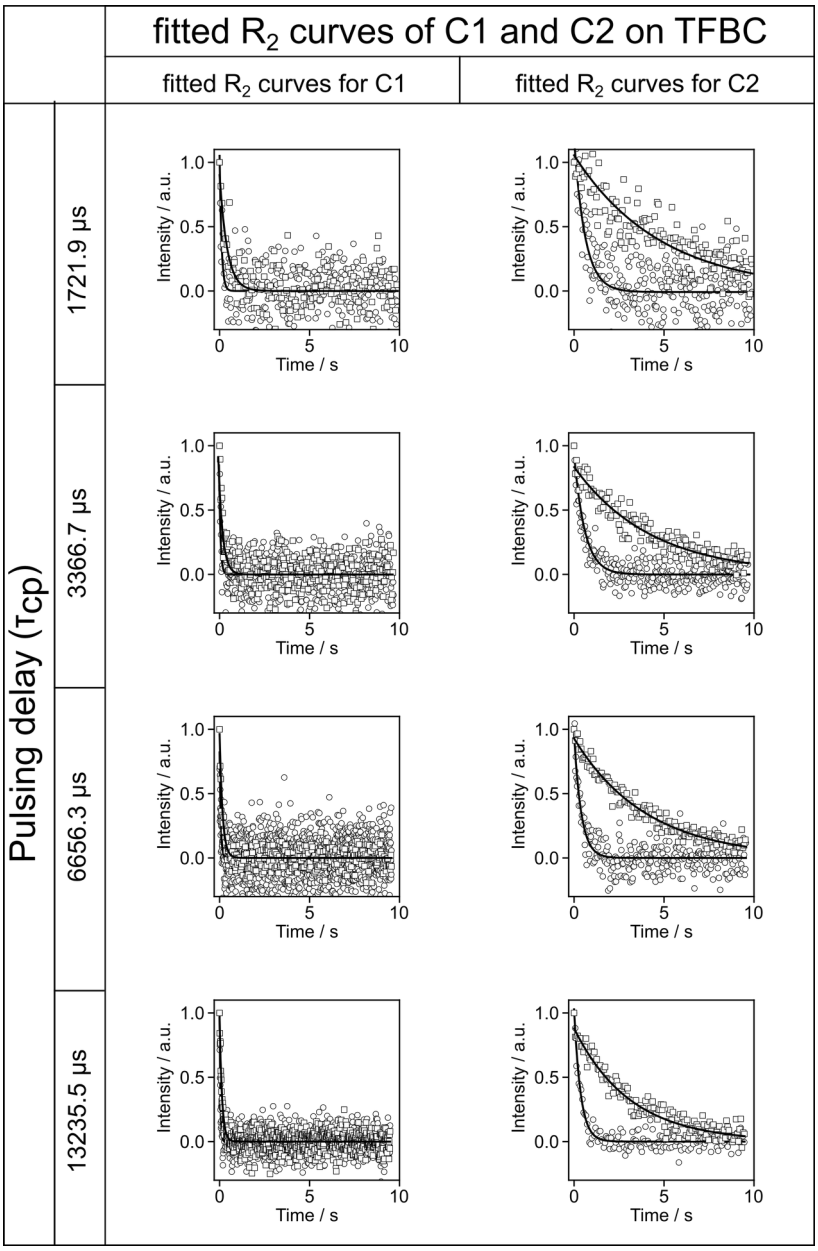

Figure S11. *Fitted R_2 curves for C1 and C2 of hyperpolarized TFBC with (open squares) and without (open circles) trypsin corresponding to Figure S10. Fitted R_2 of C1 with standard deviation in the free ligand experiments were 2.38 ± 0.07, 4.42 ± 0.28, 5.84 ± 0.34, and 7.14 ± 0.46 s^{-1}, and fitted R_2 of C1 with standard deviation in the ligand with protein experiments were 10.43 ± 0.81, 14.14 ± 1.12, 16.11 ± 2.16, and 17.95 ± 1.34 s^{-1}, respectively. Fitted R_2 of C2 with standard deviation in the free ligand experiments were 0.24 ± 0.01, 0.21 ± 0.01, 0.28 ± 0.01, and 0.36 ± 0.01 s^{-1}, while those in the ligand with protein experiments were 2.17 ± 0.05, 2.14 ± 0.04, 2.75 ± 0.10, and 2.88 ± 0.08 s^{-1}, respectively.*

Table S7. *Relaxation rates for C1 and C2 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Free ligand experiment				Ligand with protein experiment				
τ_{cp} / µs	$[TFBC]_0$ / mM	C1 $R_{2,f}$ / s^{-1}	C2 $R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	C1 $R_{2,obs}$ / s^{-1}	C2 $R_{2,obs}$ / s^{-1}
1721.9	0.82	2.38 ± 0.07	0.24 ± 0.01	1721.9	0.72	16.0	10.43 ± 0.81	2.17 ± 0.05
3366.7	0.72	4.42 ± 0.28	0.21 ± 0.01	3366.7	0.75	15.2	14.14 ± 1.12	2.14 ± 0.04
6656.3	0.82	5.84 ± 0.34	0.28 ± 0.01	6656.3	0.68	13.4	16.11 ± 2.16	2.75 ± 0.10
13235.5	0.71	7.14 ± 0.46	0.36 ± 0.01	13235.5	0.71	17.0	17.95 ± 1.34	2.88 ± 0.08

Table S8. *ΔR_2 for C1 and C2 of hyperpolarized TFBC at different pulsing delays τ_{cp}. ΔR_2 was from $\Delta R_2 = R_{2,obs} - X_f R_{2,f}$, assuming $X_f = 1$.*

ΔR_2 for C1 and C2		
τ_{cp} / µs	ΔR_2 for C1	ΔR_2 for C2
1721.9	8.05 ± 0.82	1.93 ± 0.05
3366.7	9.72 ± 1.16	1.93 ± 0.04
6656.3	10.27 ± 2.20	2.47 ± 0.10
13235.5	10.81 ± 1.44	2.52 ± 0.08

6. Supplemental R_2 data from Experiments with double-peak excitation

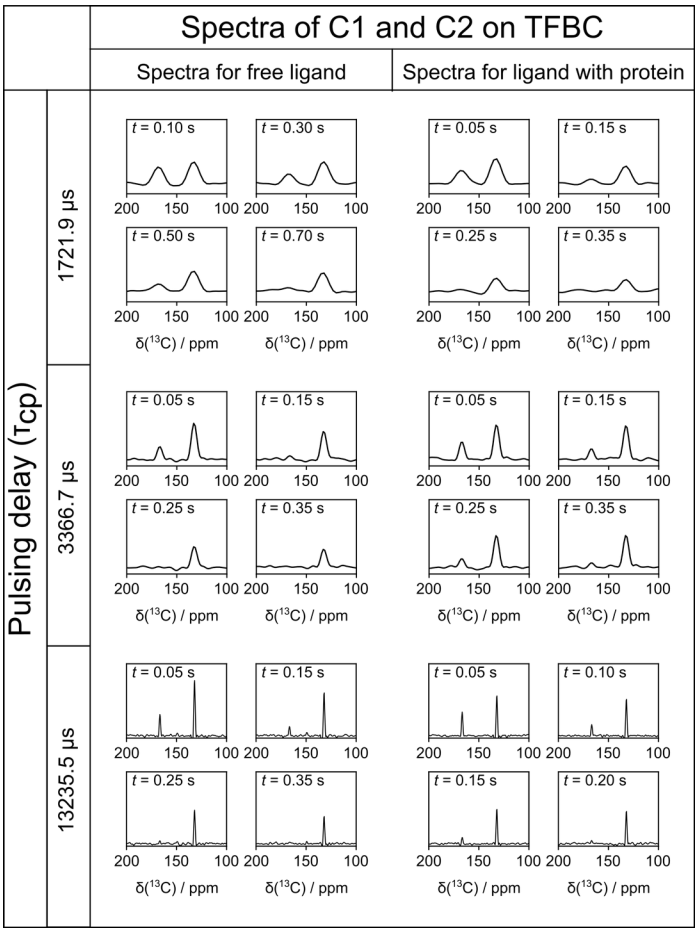

Figure S12. *Selected spectra for C1 and C2 of hyperpolarized TFBC with and without trypsin. Data were measured with pulsing delays τ_{cp} = 1721.9, 3366.7, 13235.5 µs. Correspondingly, 2.42, 2.69, and 2.54 mM TFBC were used in the free ligand experiments, while 2.53, 2.27, 2.43 mM TFBC and 50.9, 53.2, 50.9 µM trypsin were used in the ligand with protein experiments.*

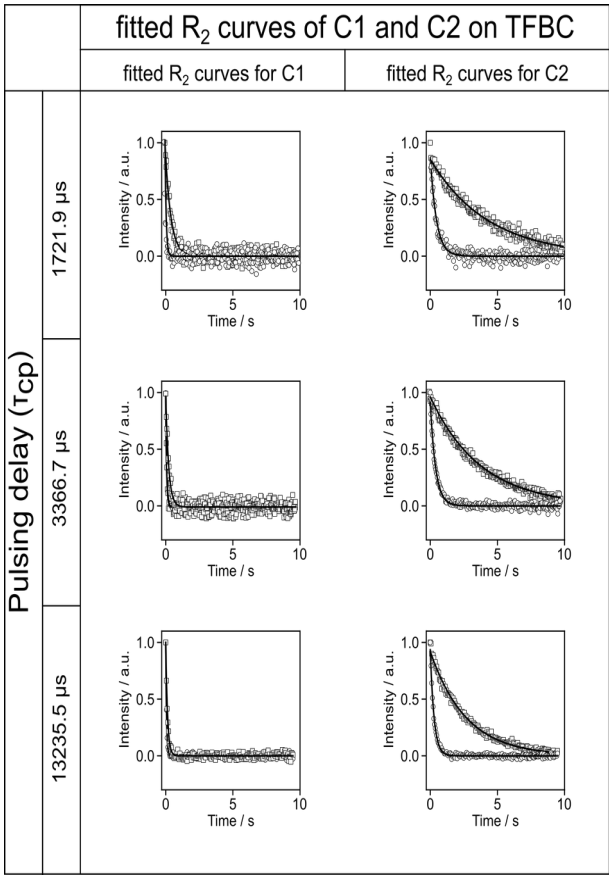

Figure S13. *Fitted R_2 of C1 with standard deviation in the free ligand experiments were 2.39 ± 0.02, 4.55 ± 0.09, and 7.64 ± 0.40 s^{-1}, while those in the ligand with protein experiments were 12.00 ± 0.35, 14.51 ± 0.39, and 17.78 ± 0.41 s^{-1}, respectively. Fitted R_2 of C2 with standard deviation in the free ligand experiments were 0.23 ± 0.00, 0.27 ± 0.00, and 0.37 ± 0.00 s^{-1}, while those in the ligand with protein experiments were 2.21 ± 0.02, 2.46 ± 0.02, and 3.60 ± 0.03 s^{-1}, respectively.*

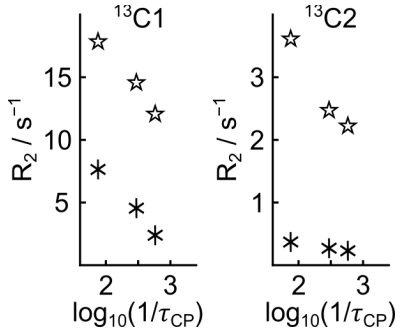

Figure S14. *R_2 relaxation rates of C1 and C2 measured in the absence (asterisks) and presence of protein (stars), at pulsing delays τ_{cp} = 1721.9, 3366.7, 13235.5 µs.*

Table S9. *Relaxation rates for C1 and C2 of hyperpolarized TFBC in the absence and presence of trypsin measured at different pulsing delays τ_{cp}. Standard errors from R_2 fitting are indicated.*

Free ligand experiment				Ligand with protein experiment				
τ_{cp} / µs	$[TFBC]_0$ / mM	C1 $R_{2,f}$ / s^{-1}	C2 $R_{2,f}$ / s^{-1}	τ_{cp} / µs	$[TFBC]_0$ / mM	$[Trypsin]_0$ / µM	C1 $R_{2,obs}$ / s^{-1}	C2 $R_{2,obs}$ / s^{-1}
1721.9	2.42	2.39 ± 0.02	0.23 ± 0.00	1721.9	2.53	50.9	12.00 ± 0.35	2.21 ± 0.02
3366.7	2.69	4.55 ± 0.09	0.27 ± 0.00	3366.7	2.27	53.2	14.51 ± 0.39	2.46 ± 0.02
13235.5	2.54	7.64 ± 0.40	0.37 ± 0.00	13235.5	2.43	50.9	17.78 ± 0.41	3.60 ± 0.03

Table S10. *ΔR_2 for C1 and C2 of hyperpolarized TFBC at different pulsing delays τ_{cp}. ΔR_2 was from $\Delta R_2 = R_{2,obs} - X_f R_{2,f}$, assuming $X_f = 1$.*

ΔR_2 for C1 and C2		
τ_{cp} / µs	ΔR_2 for C1	ΔR_2 for C2
1721.9	9.61 ± 0.35	1.98 ± 0.02
3366.7	9.96 ± 0.40	2.19 ± 0.02
13235.5	10.14 ± 0.57	3.23 ± 0.03

7. Chemical Shift Difference

Chemical shift differences in samples of TFBC with and without trypsin were measured without hyperpolarization. Figure S15 shows the chemical shift of C1 spins at varied concentration of TFBC. The chemical shift of other carbon positions on TFBC (regions were not shown in Figure S15) can be obtained in the same experiments. Figure S15 indicated that the line broadening effect made the chemical shift measurement for the bound ligand difficult. In addition, concentration difference also contributes to the chemical shift changes of carbon spins on TFBC.

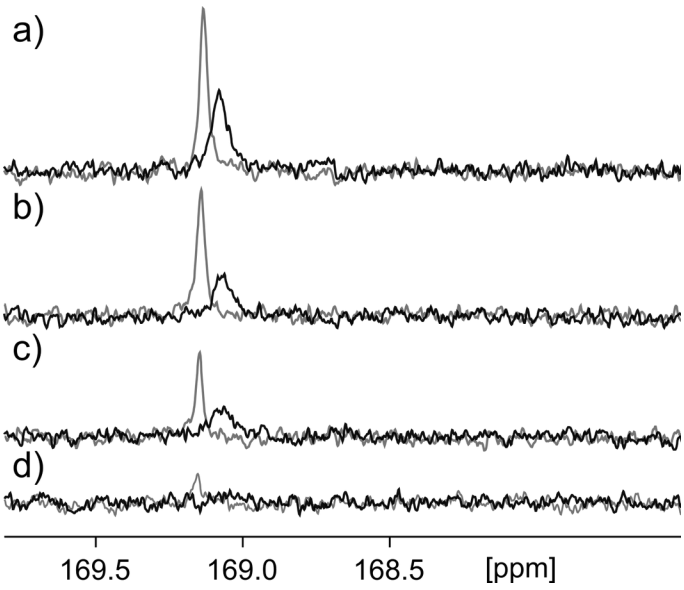

Figure S15. *C1 signals measured at 500 MHz NMR with π/6 hard pulse excitation from a) 32, b) 24, c) 16 and d) 8 mM TFBC in Tris buffer at pH = 8.0. Gray and black spectra are of samples without and with 0.5 mM trypsin, respectively. In the free ligand measurement, 1% DSS was added to the ligand sample as an internal reference, whereas in the bound ligand measurement, 2% DSS was added to the 1mM trypsin stock sample only to make the final DSS concentration at the level of 1%. The DSS in the trypsin stock sample act as an internal reference and was used to calculate the actual concentration of trypsin in the sample.*

Table S11. *^{13}C Chemical shift differences for carbon spins 1-5 on TFBC in the presence of 0.5 mM trypsin compared to free TFBC.*

C_{ligand} /mM	$\Delta\nu_{obs}$ /Hz				
	C1	C2	C3	C4	C5
32	7.52	5.82	1.99	2.10	1.38
24	8.50	6.55	2.30	2.42	1.46
16	10.29	7.91	2.83	2.97	1.64
8	—	—	3.99	—	—

8. References

1. Liu, M.; Kim, Y.; Hilty, C. Characterization of Chemical Exchange Using Relaxation Dispersion of Hyperpolarized Nuclear Spins. *Anal. Chem.* **2017**, *89* (17), 9154–9158.