Supporting Information for:

Measurement of Motional Parameters of Invisible, Excited Protein States by NMR Spectroscopy

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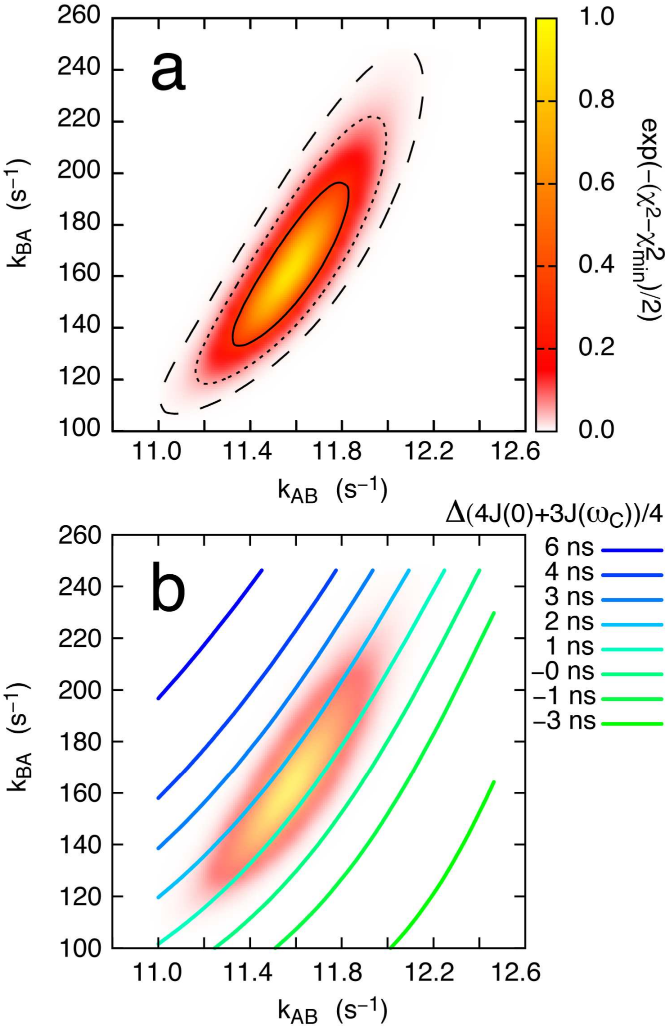

Fig. S1. Probability surface as a function of k_{AB}, k_{BA} generated from fits of the Leu18 $C^{\delta 2}$ relaxation dispersion profiles. For each set of k_{AB} and k_{BA} (that were varied systematically) dispersion data were analyzed to extract values of $(4J^{A}(0)+3J^{A}(\omega_C))/4$, $\Delta\sigma$, τ_{methyl}, $\Delta\varpi$, $\Delta\big(4J(0)+3J(\omega_C)\big)/4=(4J^{A}(0)+3J^{A}(\omega_C))/4-(4J^{B}(0)+3J^{B}(\omega_C))/4$, as well as a contribution from fast exchange in the ground state only, $R_{\mathrm{ex,fast}}$ (see Tables S1, S2), and the χ^2 from the fit obtained. (***a***) The probability density, $\exp(-(\chi^2-\chi^2_{\min})/2)$, is shown in color where $\chi^2_{\min}$ is the minimum of χ^2 (best fit). The contours represent the 68.3% confidence level (continuous line), the 95.4% level (dotted line) and the 99.7% level (dashed line). (***b***) The $\Delta\big(4J(0)+3J(\omega_C)\big)/4$ iso-lines obtained in the least-squares analysis for each combination of k_{AB} and k_{BA}. Accurate $\Delta\big(4J(0)+3J(\omega_C)\big)/4$ values (and hence $(4J^{B}(0)+3J^{B}(\omega_C))/4$ values) are obtained despite the correlation between k_{AB} and k_{BA}.

Effects of Dynamic Frequency Shifts and Residual Dipolar Couplings: The fidelity of the described methodology is predicated on the fact that deviations between spin-state selective dispersion profiles reflect exclusively differences in dynamics between ground and excited states. However, variations in effective $\Delta\varpi$ values in a spin-state selective manner would also lead to differences in dispersion profiles that could erroneously be interpreted in terms of differential dynamics. Consider for example, dynamic frequency shifts (DFS) that are given by the sine Fourier transforms of time-dependent angular fluctuations of magnetic interactions [1] and that cause changes in the effective splittings between the ^{13}C multiplet components of a methyl group [2,3]. In this case each pair of neighboring lines is separated by a different $J_{\mathrm{CH,eff}}$ ($\neq J_{CH}$) that reflects the fact that each of the four transitions $\mathbf{C}_{+}^{\alpha\alpha\alpha}$, $\mathbf{C}_{+}^{\alpha\alpha\beta}$, $\mathbf{C}_{+}^{\alpha\beta\beta}$, and $\mathbf{C}_{+}^{\beta\beta\beta}$ have different DFS values. Differences in $J_{\mathrm{CH,eff}}$ between multiplet components in the ground and excited states result in spin-state selective $\Delta\varpi$ values and hence can potentially decrease the accuracy of the extracted dynamic parameters pertaining to the excited state. It can be shown that

$$\begin{aligned} J_{\mathrm{CH,\,eff}}^{\alpha\alpha\alpha,\alpha\alpha\beta} - J_{\mathrm{CH}} &= DFS(\mathbf{C}_+^{\alpha\alpha\alpha}) - DFS(\mathbf{C}_+^{\alpha\alpha\beta}) \approx \\ &-\frac{6}{5}P_2^0(\cos\theta)d_{\mathrm{CH}}(P_2^0(\cos\theta)d_{\mathrm{CH}} - c_C)\frac{S_{axis}^2\omega_{\mathrm{C}}\tau_C^2}{1+\omega_{\mathrm{C}}^2\tau_C^2} \\ &+\frac{6}{5}P_2^0(\cos\theta)d_{\mathrm{CH}}d_{\mathrm{HH}}\frac{S_{axis}^2\omega_{\mathrm{H}}\tau_C^2}{1+\omega_{\mathrm{H}}^2\tau_C^2} \end{aligned} \tag{S1}$$

$$\begin{aligned} J_{\mathrm{CH,\,eff}}^{\alpha\beta\beta,\beta\beta\beta} - J_{\mathrm{CH}} &= DFS(\mathbf{C}_+^{\alpha\beta\beta}) - DFS(\mathbf{C}_+^{\beta\beta\beta}) \approx \\ &\frac{6}{5}P_2^0(\cos\theta)d_{\mathrm{CH}}(P_2^0(\cos\theta)d_{\mathrm{CH}} + c_C)\frac{S_{axis}^2\omega_C\tau_C^2}{1+\omega_C^2\tau_C^2} \\ &-\frac{6}{5}P_2^0(\cos\theta)d_{\mathrm{CH}}d_{\mathrm{HH}}\frac{S_{axis}^2\omega_{\mathrm{H}}\tau_C^2}{1+\omega_{\mathrm{H}}^2\tau_C^2} \end{aligned} \tag{S2}$$

Assuming τ_c= 6 ns (the correlation time of the folded state of the Fyn SH3 domain considered in the text), S^2_{axis}= 0.8, β= 111°, ω_{C}= 2π 150 MHz, and ω_{H} = 2π 600 MHz a DFS = ($J_{\mathrm{CH,\,eff}}^{\alpha\beta\beta,\beta\beta\beta} - J_{\mathrm{CH,\,eff}}^{\alpha\alpha\alpha,\alpha\alpha\beta}$)/2 value of 0.56 Hz is calculated, which can be considered as an upper limit since a large S^2_{axis} value has been used. This upper limit agrees well with the largest of the experimental DFS values obtained for methyl groups of the G48M Fyn SH3 domain, where values between ~0.2 and ~0.55 Hz are measured at 11.74 T. Thus, assuming that S^2_{axis} > 0.1 in the excited state, the largest change to $\Delta\omega/(2\pi)$ that is introduced by DFS is ~0.5 Hz.

Simulations were performed to determine how DFS affects the extracted dynamics parameters. Relaxation dispersion curves for the four lines ($\mathbf{C}_+^{\alpha\alpha\alpha}$, $\mathbf{C}_+^{\alpha\alpha\beta}$, $\mathbf{C}_+^{\alpha\beta\beta}$, and $\mathbf{C}_+^{\beta\beta\beta}$) were generated using the parameters, k_{ex}=300 s^{-1}, p$_{\mathrm{b}}$=5%, $(4J(0)+3J(\omega_{\mathrm{C}}))/4$ = 4 ns, τ_{methyl}= 50 ps, $\Delta\sigma$= 25 ppm, with $\Delta\varpi$ and $\Delta(4J(0)+3J(\omega_{\mathrm{C}}))/4$ varied as shown in Figure S2. DFS is included in the simulations by subtracting 0.5 Hz×18.79T/B_0 from $\Delta\nu(\mathbf{C}_+^{\alpha\alpha\alpha})$ and $\Delta\nu(\mathbf{C}_+^{\beta\beta\beta})$. The generated data were subsequently analyzed under the assumption that DFS= 0 Hz following the standard procedure described in Materials and Methods for the experimental data and the systematic errors in the fitted $(4J(0)+3J(\omega_C))/4$ values plotted in Figure S2. Further simulations establish that the systematic error scales linearly with DFS so that for average values of approximately 0.25 Hz (that takes into account differences between ground and excited states) and for $\Delta\varpi > 0.3$ ppm, errors on the order of 0.25 ns or less are predicted for $(4J(0)+3J(\omega_C))/4$, that are well within the experimental random errors (see below).

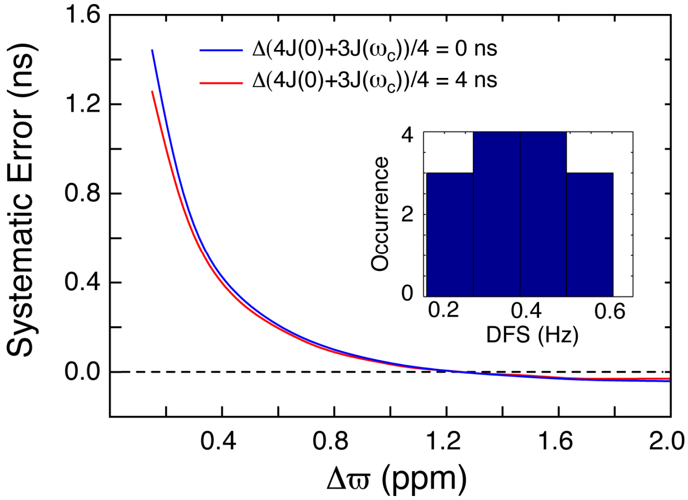

Fig. S2: Predicted upper bound of the systematic error that is introduced by a change of DFS between ground and excited states. The difference between DFS values of the ground and excited states was assumed to be 0.5 Hz that represents a maximum value. The systematic error is calculated as $\Delta(J(0)+3/4J(\omega_C))_{Fit}-\Delta(J(0)+3/4J(\omega_C))_{Input}$ where $\Delta(J(0)+3/4J(\omega_C))_{Fit}$ is the value obtained from the least-squares analysis of the computed data and $\Delta(J(0)+3/4J(\omega_C))_{Input}$ is the value of $\Delta(J(0)+3/4J(\omega_C))$ used to generate the data. The insert shows the experimental DFS = $(J_{CH,\,eff}^{\alpha\beta\beta,\beta\beta\beta} - J_{CH,\,eff}^{\alpha\alpha\alpha,\alpha\alpha\beta})/2$ obtained for the G48M Fyn SH3 domain at 11.4 T (500 MHz proton frequency), 23°C.

Molecular alignment due to non-zero magnetic susceptibility anisotropy can also lead to spin-state selective differences in $\Delta\varpi$. For example, under conditions of magnetic alignment the $\mathbf{C}_{+}^{\alpha\alpha\alpha}$, $\mathbf{C}_{+}^{\alpha\alpha\beta}$, $\mathbf{C}_{+}^{\alpha\beta\beta}$, and $\mathbf{C}_{+}^{\beta\beta\beta}$ lines become centered at the frequencies $\{\omega - 3\pi(J_{CH} + D_{CH}), \omega - \pi(J_{CH} + D_{CH}), \omega + \pi(J_{CH} + D_{CH}), \omega + 3\pi(J_{CH} + D_{CH})\}$, where D_{CH} is the one-bond ^{1}H-^{13}C dipolar coupling. It is clear that for $\Delta D_{CH} = D_{CH}^{A} - D_{CH}^{B} \neq 0$ the values of $\Delta\varpi_{\square\square\square}$. □$\Delta\varpi \pm 3\Delta D_{CH}$ for the outer pair of lines, $\Delta\varpi \pm \Delta D_{CH}$ for the inner pair) are spin-state selective, leading to differences in dispersion profiles for each multiplet component, and potentially to errors in dynamics parameters. Note, however, that ΔD_{CH} contributes in opposite ways to each of the outer/inner lines so that by replacing the two outer (inner) lines by their sum the small effects due to alignment average out to first order. The dynamical parameters of the excited state extracted from fits of the four individual and the two 'averaged' dispersions can be compared to establish that errors due to potential alignment are small. In the case of the Fyn SH3 domain very similar motional parameters are fitted from the 'averaged' and set of 4 dispersion profiles. In order to examine the magnitude of errors when $\Delta D_{CH} \neq 0$ we have simulated dispersion profiles (at fields corresponding to ^{1}H frequencies of 600 and 800 MHz) for each of the four multiplet components assuming k_{ex}=300 s^{-1}, p_B=5%, $(4J(0)+3J(\omega_C))/4$ = 4 ns, τ_{methyl}= 50 ps, $\Delta\sigma$= 25 ppm, $\Delta\varpi$ = 1 ppm, $\Delta(4J(0)+3J(\omega_C))/4$=2,0,-2 ns and D_{CH} = 0.66 Hz. Values of $D_{CH} \approx 0.7$ Hz are on the order of the largest ^{13}C$^{\alpha}$-^{1}H$^{\alpha}$ RDC values measured in ubiquitin aligned by the magnetic field[4] (750 MHz ^{1}H frequency). In the case of methyl groups these couplings are expected to be much smaller due to averaging of and about the methyl axis [5]. Nevertheless, even for this exaggerated D_{CH} value simultaneous fits of the eight calculated dispersions (four each at 600 and 800 MHz) produced $\Delta(4J(0)+3J(\omega_C))/4$ that were only very slightly in error, by ≈ 0.1 ns ($\frac{d\Delta(J(0)+\frac{3}{4}J(\omega_C))}{dD_{CH}}$ =0.16 ns/Hz).

Validity of an Exchange Model which Includes Differential Dynamics Between States: Separate fits of dispersion data based on models that either include or do not include differential dynamics between exchange states have been performed and *p*-values calculated. Figure S3 plots *p*-values *vs.* $\Delta\left(4J(0)+3J(\omega_C)\right)/4$ obtained in a comparison of fits of Fyn SH3 domain dispersions, 23°C (no GdnHCl). Note that the improvement in fit by including separate relaxation terms for ground and excited states is valid at a confidence level of 1-*p*, so that low values of *p* indicate that the model with differential dynamics is to be chosen (at a 1-*p* certainty level).

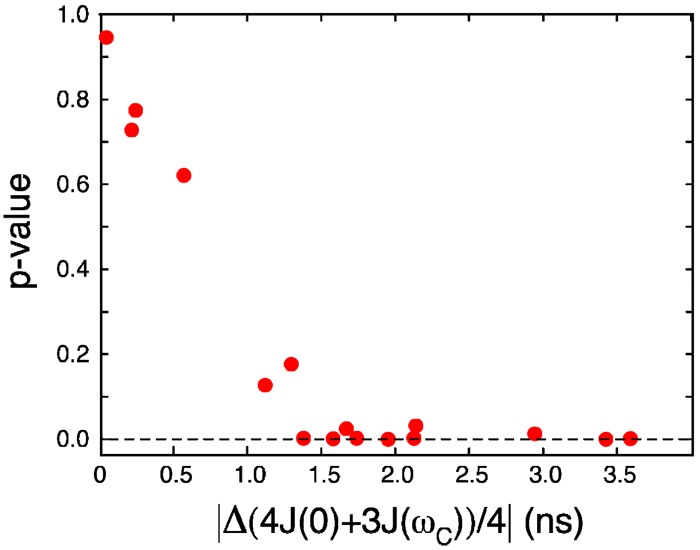

Fig. S3. The statistical significance of a two-site exchange model that includes different dynamics for each of the chemically exchanging sites.

Table S1a: Parameters obtained for the ground state of the G48M Fyn SH3 domain, 23°C.

Name	$(4J^A(0)+3J^A(\omega_C))/4$ (ns)	τ_{Me} (ps)	Λ (s^{-1})[a]	$\Delta\sigma$ (ppm)
$3C^{\delta1}$	2.441±0.033	36.9±0.9	0.31±0.07	32.23±0.26
$3C^{\delta2}$	3.377±0.030	53.5±0.9	0.00±0.08	26.22±0.22
$7C^{\delta1}$	3.912±0.025	40.4±0.6	-0.05±0.06	30.75±0.14
$7C^{\delta2}$	4.175±0.021	33.0±0.9	-0.14±0.07	28.25±0.17
$18C^{\delta1}$	2.169±0.023	30.4±0.5	0.54±0.04	30.33±0.19
$18C^{\delta2}$	2.334±0.019	33.6±0.9	0.39±0.07	26.64±0.26
$28C^{\delta1}$	5.040±0.035	47.5±2.2	0.15±0.15	7.80±0.21
$29C^{\delta1}$	3.690±0.020	38.0±0.6	0.03±0.06	32.03±0.15
$29C^{\delta2}$	3.402±0.018	37.6±0.6	-0.05±0.05	26.00±0.16
$42C^{\delta1}$	1.485±0.042	47.7±0.9	-0.32±0.05	21.78±0.30
$42C^{\delta2}$	1.805±0.027	46.1±0.7	-0.19±0.06	19.10±0.26
$50C^{\delta1}$	4.118±0.027	23.4±0.8	0.39±0.08	16.60±0.16
$55C^{\gamma1}$	6.100±0.041	57.5±0.8	0.04±0.07	25.07±0.11
$55C^{\gamma2}$	5.736±0.035	79.7±1.7	0.20±0.12	17.77±0.19
$58C^{\gamma1}$	4.156±0.038	67.6±0.7	-0.52±0.06	24.67±0.12
$58C^{\gamma2}$	4.490±0.030	60.3±1.2	-0.32±0.09	24.52±0.17

[a] Lower bound of the contributions from fast exchange (faster than ~ 2000/s) at a static magnetic field of 14.1 T (corresponding to a ^{1}H frequency of 600 MHz). See Materials and Methods for details.

Table S1b: Chemical exchange parameters for the G48M Fyn SH3 domain, 23^{o}C.

Name	$\lvert\Delta\varpi\rvert$ (ppm)	$\Delta(4J(0)+3J(\omega_C))/4$ (ns)	k_{AB} (s^{-1})	k_{BA} (s^{-1})
$3C^{\delta1}$	0.448±0.008	-0.25±0.87	14.5±0.5	363±11
$3C^{\delta2}$	0.671±0.007	3.43±0.77	12.1±0.3	294±11
$7C^{\delta1}$	0.474±0.005	2.15±0.69	12.3±0.3	318±8
$7C^{\delta2}$	1.519±0.010	1.96±0.53	12.6±0.1	225±21
$18C^{\delta1}$	0.387±0.006	-0.23±0.63	11.5±0.4	314±8
$18C^{\delta2}$	1.679±0.009	1.37±0.46	12.9±0.1	236±19
$28C^{\delta1}$	2.687±0.033	2.95±1.34	13.2±0.3	309±77
$29C^{\delta1}$	0.821±0.005	1.77±0.56	11.8±0.1	299±8
$29C^{\delta2}$	1.106±0.005	1.58±0.47	12.1±0.1	267±9
$42C^{\delta1}$	0.248±0.016	-0.61±1.25	9.0±1.2	286±16
$42C^{\delta2}$	0.442±0.006	0.00±0.68	13.5±0.4	336±8
$50C^{\delta1}$	0.892±0.007	1.12±0.73	11.9±0.2	285±11
$55C^{\gamma1}$	0.398±0.008	3.67±1.14	11.7±0.5	325±12
$55C^{\gamma2}$	2.098±0.025	2.08±1.01	12.9±0.2	251±51
$58C^{\gamma1}$	0.336±0.009	-1.25±1.00	13.8±0.8	343±12
$58C^{\gamma2}$	1.112±0.008	1.68±0.76	12.9±0.2	260±18

Table S2a: Parameters obtained for the ground state of the G48M Fyn SH3 domain, 500 mM GdnHCl, 23°C

Name	$1/4(4J^A(0)+3J^A(\omega_C))$ (ns)	τ_{Me} (ps)	Λ (s^{-1})[a]	$\Delta\sigma$ (ppm)
$3C^{\delta1}$	2.559±0.027	21.8±1.1	1.01±0.09	34.69±0.28
$3C^{\delta2}$	3.623±0.039	52.1±1.5	-0.05±0.13	26.08±0.29
$7C^{\delta1}$	4.087±0.034	39.2±1.2	0.10±0.10	31.91±0.20
$7C^{\delta2}$	4.523±0.031	34.8±2.0	0.17±0.13	28.48±0.24
$18C^{\delta1}$	2.287±0.026	29.9±0.9	0.63±0.07	31.98±0.26
$18C^{\delta2}$	2.493±0.026	35.4±1.5	0.77±0.11	27.06±0.35
*28*$C^{\delta1\mathit{b}}$	*5.395±0.060*	*56.7±3.9*	*0.01±0.29*	*8.36±0.37*
$29C^{\delta1}$	3.971±0.028	38.3±1.3	0.15±0.10	32.93±0.22
$29C^{\delta2}$	3.584±0.026	34.0±1.3	0.52±0.10	26.95±0.23
$42C^{\delta1}$	1.419±0.030	50.9±1.0	-0.45±0.06	23.48±0.31
$42C^{\delta2}$	1.766±0.028	37.7±1.1	0.09±0.09	20.44±0.33
$50C^{\delta1}$	4.359±0.034	24.7±1.7	0.66±0.13	17.09±0.21
$55C^{\gamma1}$	6.458±0.061	57.6±1.3	0.16±0.13	24.80±0.16
$55C^{\gamma2}$	6.109±0.053	85.0±3.4	0.44±0.25	17.70±0.31
$58C^{\gamma1}$	4.348±0.047	47.1±1.2	0.26±0.10	25.56±0.18
$58C^{\gamma2}$	4.692±0.044	58.3±2.0	-0.14±0.16	24.65±0.30

[a] Lower bound of the contributions from fast exchange (faster than ~ 2000/s) at a static magnetic field of 14.1 T (corresponding to a ^1H frequency of 600 MHz). See Materials and Methods for details.

[b] Dispersion profiles for Ile28 $C^{\delta1}$ could not be properly quenched at the maximum ν_{CPMG} value of 1 kHz, which results in large uncertainties of the extracted parameters.

Table S2b: Chemical exchange parameters for the G48M Fyn SH3 domain, 500 mM GdnHCl, 23^{o}C

Name	$\Delta\varpi$ (ppm)	$\Delta(4J(0)+3J(\omega_C))/4$ (ns)	k_{AB} (s^{-1})	k_{BA} (s^{-1})
3$C^{\delta1}$	0.521±0.005	1.25±0.53	13.8±0.3	261±8
3$C^{\delta2}$	0.651±0.008	2.81±0.71	12.2±0.3	201±14
7$C^{\delta1}$	0.459±0.006	1.59±0.68	11.9±0.3	215±9
7$C^{\delta2}$	1.510±0.022	3.40±0.98	11.1±0.2	191±43
18$C^{\delta1}$	0.408±0.004	0.17±0.48	12.0±0.3	214±7
18$C^{\delta2}$	1.503±0.022	1.46±0.54	11.6±0.2	162±32
*28*$C^{\delta1a}$	*2.765±0.050*	*1.40±3.26*	*13.1±0.4*	*427±143*
29$C^{\delta1}$	0.813±0.006	2.17±0.65	11.9±0.2	233±14
29$C^{\delta2}$	1.122±0.009	2.59±0.65	11.4±0.2	217±21
42$C^{\delta1}$	0.248±0.011	-2.59±0.78	7.2±0.7	186±14
42$C^{\delta2}$	0.500±0.004	-1.76±0.54	14.5±0.3	260±7
50$C^{\delta1}$	0.893±0.008	1.47±0.77	11.4±0.2	203±18
55$C^{\gamma1}$	0.333±0.011	4.04±1.21	11.7±0.9	231±14
55$C^{\gamma2}$	1.990±0.032	1.84±2.23	12.6±0.3	355±86
58$C^{\gamma1}$	0.321±0.008	2.03±0.84	13.1±0.7	234±10
58$C^{\gamma2}$	1.184±0.014	0.98±1.02	12.5±0.3	220±32

[a] Dispersion profiles for Ile28 $C^{\delta1}$ could not be properly quenched at the maximum ν_{CPMG} value of 1 kHz leading to large uncertainties in extracted parameters.

References

(1) Abragam, A. *Principles of Nuclear Magnetism*; Clarendon Press: Oxford, 1961.

(2) Chenon, M.; Dunkel, R.; Grant, D.; Webelow, L. *J. Phys. Chem* 1999, *103*, 1447-1451.

(3) Tugarinov, V.; Ollerenshaw, J. E.; Kay, L. E. *Magn Reson Chem* 2006, *44 Spec No*, S122-129.

(4) Tjandra, N.; Bax, A. *J. Magn. Reson.* 1997, *124*, 512-515.

(5) Ottiger, M.; Bax, A. *J. Am. Chem. Soc.* 1999, *121*, 4690-4695.