Supporting Information

Enantiomeric two-armed lanthanide-binding tags for complementary effects in paramagnetic NMR spectroscopy

Michael D. Lee, Matthew L. Dennis, James D. Swarbrick* and Bim Graham*

Contents

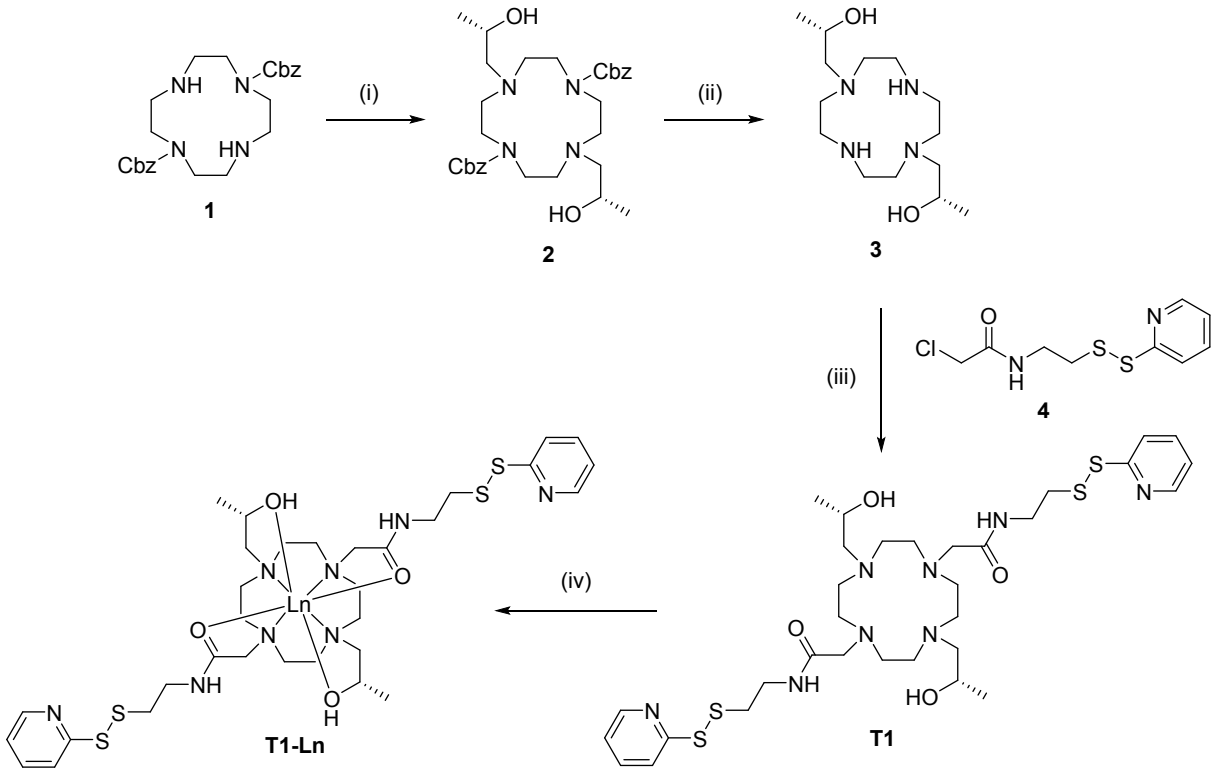
Materials and reagents..... S2
Instruments..... S2
Synthesis of **T1** and metal complexes..... S3
High-resolution mass spectrum of **T2-Yb^{3+}**..... S7
^{1}H and ^{13}C NMR spectra of novel compounds..... S8
Ubiquitin and HPPK expression, purification and tagging..... S12
Protein NMR spectroscopy and determination of Δχ and alignment tensors..... S13
Δχ-Tensor parameters for **T1**- and **T2**-tagged ubiquitin E24C/A28C and **T1**- and **T2**-tagged HPPK K76C/C80 (with metal ion coordinates and Euler angles)..... S14
Correlations between experimental and back-calculated PCSs for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/80C at pH 8.0..... S15
^{15}N-HSQC spectra of **T1**- and **T2**-tagged HPPK K76C/80C..... S16
Correlations between experimental and back-calculated RDCs for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/80C..... S17
Alignment tensor parameters for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/C80..... S18
Equation S1..... S18
Correlations between PCSs or RDCs measured with **T1** and **T2**..... S18
Orientations of the principal axes of the Δχ and alignment tensors..... S19
Structures of ubiquitin and HPPK with determined Δχ-tensor axes..... S20
^{15}N-HSQC spectra of **T1**- and **T2**-tagged ubiquitin E24C/A28C at pH 6.5..... S21
Correlations between PCSs measured at pH 8.0 and 6.5 for **T1**- and **T2**-tagged ubiquitin E24C/A28C..... S22
^{15}N-HSQC spectra of **T1**- and **T2**-tagged ubiquitin E24C/A28C at pH 8.0 and 6.5..... S23
Experimental PCSs measured for **T1**- and **T2**-tagged ubiquitin E24C/A28C and **T1**- and **T2**-tagged HPPK K76C/80C..... S24
Experimental RDCs measured for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/80C..... S29
References..... S31

Materials and reagents

Dibenzyl 1,4,7,10-tetraazacyclododecane-1,7-dicarboxylate (**1**)[1] and 2-chloro-*N*-(2-(pyridin-2-yldisulfanyl)ethyl)acetamide (**4**)[2] were prepared following literature procedures. All other starting materials, reagents and solvents were obtained from commercial suppliers and were of general reagent or analytical grade and used without further purification.

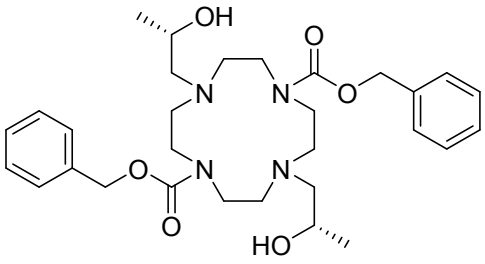
Instruments

All 400 MHz NMR spectra were recorded on a Bruker Avance III Nanobay spectrometer. NMR data was acquired using TOPSPIN/ICONNMR (Bruker), processing and plotting of the acquired data were performed using MestReNova software. Chemical shifts are quoted in units of parts per million (ppm) and were referenced internally to the residual proteo-solvent resonance; ^{1}H NMR: $CDCl_3$ (δ 7.26), D_2O (δ 4.79), MeOD (δ 3.31); ^{13}C NMR: $CDCl_3$ (δ 77.16), MeOD (δ49.00).[3] Multiplicity for NMR resonances are abbreviated as; s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet, app, apparent; br, broad. LCMS were acquired on an Agilent 1220/6120 LCMS system, using ChemStation software for instrument control and data analysis. HRMS were acquired on an Agilent 6224 TOF LCMS mass spectrometer, coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). Acquisition was performed using Agilent Mass Hunter Data Acquisition software and analyzed using Mass Hunter Qualitative Analysis software. Preparative reverse-phase HPLC was performed on an Agilent 1260 Prep HPLC using an Alltima C8 column (250 mm x 22 mm, 5 micron).



Scheme S1. Synthesis of **T1** and its lanthanide complexes. Reagents and conditions: (i) (*S*)-propylene oxide, MeOH, RT, 48 h, 88%; (ii) HCl (32%), reflux, 2 h, 91%;(iii) DIPEA, ACN, RT, 5 days, 22%; (iv) $LnCl_3$, H_2O, ACN, pH 7, 80 °C, 1 h, quant.

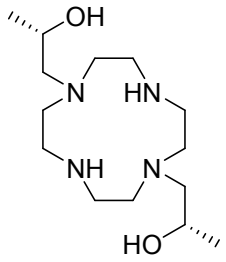
Dibenzyl 4,10-*bis*((*S*)-2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,7-dicarboxylate, 2



(*S*)-Propylene oxide (475 µL, 6.78 mmol) and **1** (500 mg, 1.13 mmol) were added to MeOH (5 mL) and stirred at room temperature for 48 h. Solvent was removed under reduced pressure and the crude oil purified by silica flash chromatography (0 to 5% MeOH in $CHCl_3$) to yield **2** as a colourless oil. Yield: 554 mg (88%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.46–7.30 (m, 10H, C_6H_5), 5.18 (d, J = 12.3 Hz, 2H,

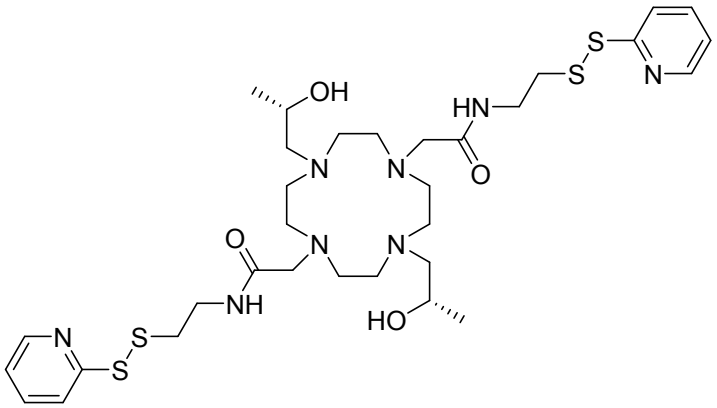
CH_2Ar), 5.11 (d, J = 12.3 Hz, 2H, CH_2Ar), 3.85 (br, 2H, CHOH), 3.41 (m, 8H), 2.92 (br, 4H), 2.40 (m, 6H), 2.05 (br 2H), 1.06 (br, 6H, CH_3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.77 (*C*=O), 136.65 (*C*), 128.53 (*C*H), 128.22 (*C*H), 128.15 (*C*H), 67.22 (*C*H_2), 63.97 (br, *C*H_2), 63.60 (*C*H), 55.31 (br, *C*H_2), 48.51 (br, *C*H2), 20.28 (*C*H_3). **LCMS**: *m/z* (ESI, 20 V) 557.4 $[M+H]^+$.

(2*S*,2'*S*)-1,1'-(1,4,7,10-tetraazacyclododecane-1,7-diyl)*bis*(propan-2-ol), 3



A solution of **2** (263 mg, 0.47 mmol) in conc. HCl (32%, 10 mL) was heated to reflux for 2 h. The reaction was cooled to room temperature and concentrated under reduced pressure to yield a beige solid. 1M NaOH (50 mL) was added and washed with $CHCl_3$ (3 x 50 mL). The combined organic layers were dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to yield **3** as a colourless oil. Yield: 127 mg (91%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 3.79 (p, J = 6.3 Hz, 2H, CH), 2.81–2.64 (m, 8H), 2.49–2.46 (m, 2H), 2.46–2.38 (m, 6H), 2.31 (d, J = 2.0 Hz, 2H), 2.29 (s, 2H), 1.05 (d, J = 6.3 Hz, 6H, CH_3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 64.59 (*C*H), 63.41(*C*H_2), 53.40 (br, *C*H_2), 45.60 (*C*H_2), 20.34 (*C*H_3). **LCMS**: *m/z* (ESI, 20 V) 289.3 $[M+H]^+$.

2,2'-(4,10-*bis*((*S*)-2-Hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl)*bis*(*N*-(2-(pyridin-2-yldisulfanyl)ethyl)acetamide), trifluoroacetate salt, T1



A solution of **3** (50 mg, 0.17 mmol), **4** (100 mg, 0.38 mmol) and DIPEA (132 µL, 0.76 mmol) in ACN (4 mL) was stirred at room temperature for 5 days. Solvent was removed under reduced pressure and the resulting residue was purified by reverse-phase HPLC (0.1% TFA and a 5–100% ACN gradient over 20 min on a C8 preparative column). Fractions containing pure product were lyophilised to afford **T1** as a brown residue. Yield: 49 mg (22%, based on a hexatrifluoroacetate salt). **^{1}H NMR** (400 MHz, MeOD) δ 8.58 (dd, *J* = 5.8, 1.0 Hz, 2H), 8.30 (td, *J* = 8.1, 1.6 Hz, 2H), 8.15 (d, *J* = 8.4 Hz, 2H), 7.70 (ddd, *J* = 7.1, 5.8, 0.9 Hz, 2H), 4.21 (m, 2H, C*H*OH), 3.72–3.54 (m, 11H), 3.40–3.20 (m, 12H), 3.14–2.90 (m, 11H), 1.21 (d, *J* = 6.2 Hz, 6H, CH_3). **^{13}C NMR** (101 MHz, MeOD) δ 171.77 (*C*=O), 155.99 (*C*), T2.53 (*C*H), 143.51 (*C*H), 124.62 (*C*H), 123.70 (*C*H), 61.15 (*C*HOH), 59.36 (*C*H_2), 55.00 (*C*H_2), 50.91 (*C*H_2), 50.22 (*C*H_2), 49.31 (*C*H_2), 46.61 (*C*H_2), 37.69 (*C*H_2), 37.40 (*C*H_2), 20.11 (*C*H_3). **HRMS** (ESI) *m/z* calcd $[M+H]^+$ $C_{32}H_{53}N_8O_4S_4$: 741.3073, found: 741.3085.

Formation of metal complexes

A solution of **T1** (10 mg, 0.01 mmol) and $YCl_3(H_2O)_6$ (6 mg, 0.02 mmol) in a H_2O:ACN (1:1 v/v, 2 mL) mixture was adjusted to pH 7 with DIPEA and heated to 70 °C. After 15 min, the pH of the reaction was checked and readjusted to pH 7 with DIPEA. After 1 h of heating, LCMS analysis indicated no uncomplexed **T1** remained and the reaction was cooled to room temperature. The complex was then purified by reverse-phase HPLC (0.1% TFA, and a 5–100% ACN gradient over 20 min on a C8 preparative column). Fractions containing pure product were lyophilised to afford **T1-Y^{3+}** as a beige solid. Yield: 10 mg (quant., based on a pentatrifluoroacetate salt). **^{1}H NMR** (400 MHz, D_2O) δ 8.47 (d, *J* = 4.9 Hz, 2H), 8.02 (t, *J* = 7.5 Hz, 2H), 7.96 (d, *J* = 8.1 Hz, 2H), 7.45 (m, 2H), 4.43 (br, 2H), 4.12–3.86 (m, 4H), 3.61–3.25 (m, 10H), 3.08 (m, 4H), 2.77 (m, 2H), 2.65–2.43 (m, 7H), 2.25 (m, 5H), 1.15 (d, *J* = 5.4 Hz, 6H). **HRMS** (ESI) *m/z* cald $[M+H]^+$ $C_{32}H_{50}N_8O_4S_4Y$: 827.1896 , found 827.1910.

The Tm^{3+} and Yb^{3+} complexes of **T1** were formed in an analagous manner to the Y^{3+} complexes. **T2** and its lanthanide complexes were synthesised following the same methods used to synthesise **T1**, with the replacement of (*S*)-propylene oxide and with its commerically available enantiomer. **HRMS** and **^{1}H NMR** of the Yb^{3+} complexes of **T2** are shown in **Figures S1** and **S6**, respectively.

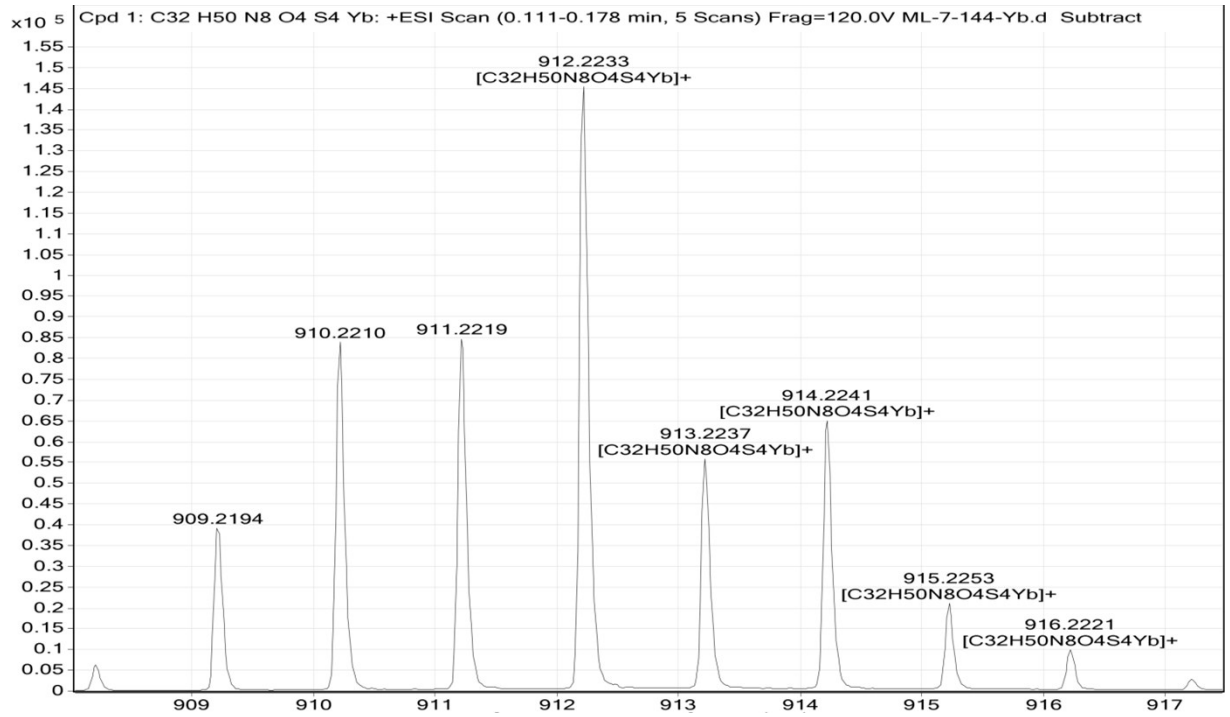
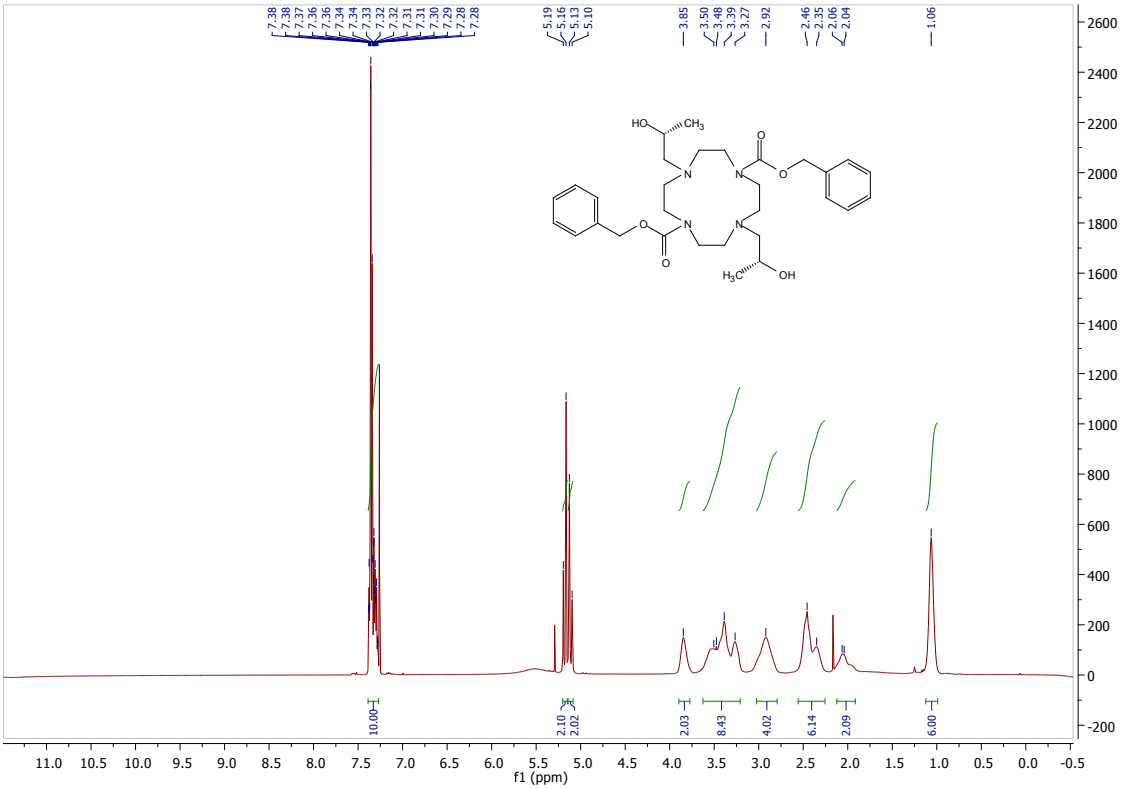


Figure S1. High-resolution mass spectrum of **T2-Yb^{3+}**.

Table S1. Predicted masses of **T0-** and **T2-Yb^{3+}** complexes.		
Complex	**Chemical formula**	**Predicted masses (relative abundance)[a]**
T2-Yb^{3+}	$[C_{32}H_{50}N_8O_4S_4Yb]^+$	912.2221 (100.0%), 910.2197 (68.6%), 911.2215 (50.7%), 909.2196 (44.9%), 914.2259 (40.1%), 913.2255 (34.6%), 911.2230 (23.7%), 914.2179 (18.1%), 912.2248 (17.5%), 910.2230 (15.5%).
[a]Only masses of the 10 highest abundance predicted species are listed.		

Figure S3. 1D ^{1}H NMR and ^{13}C NMR spectra of **2**.



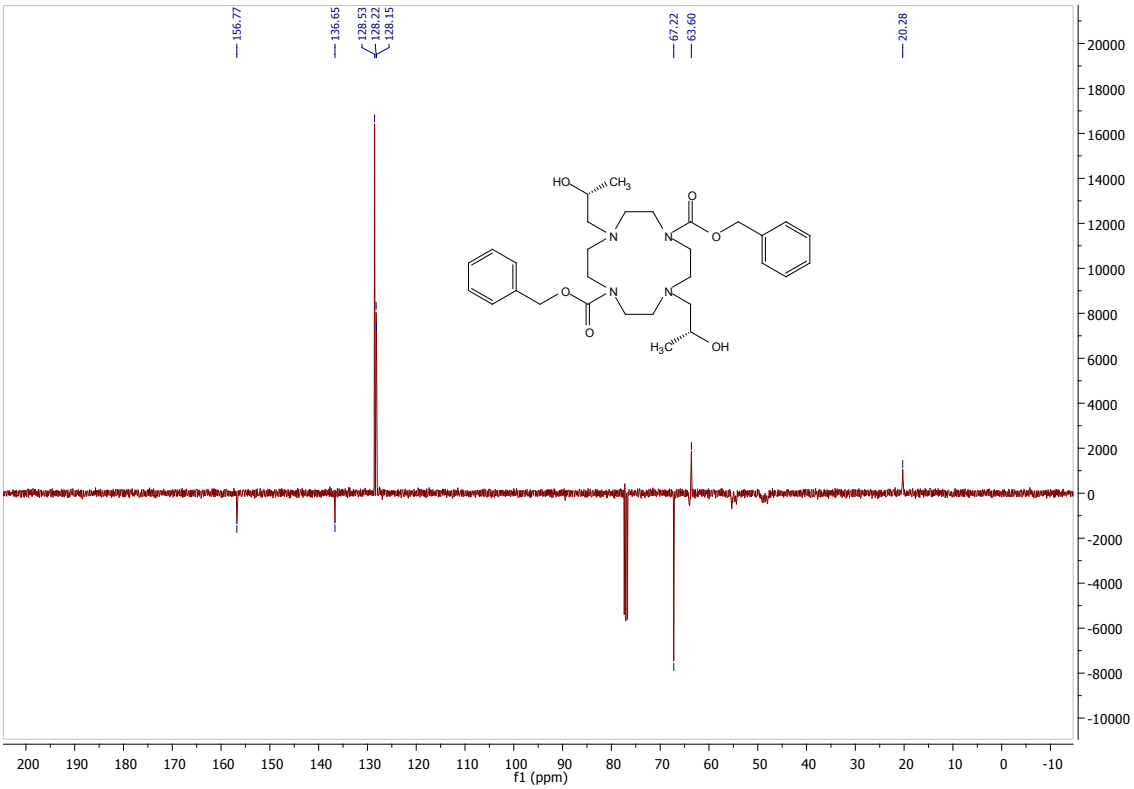
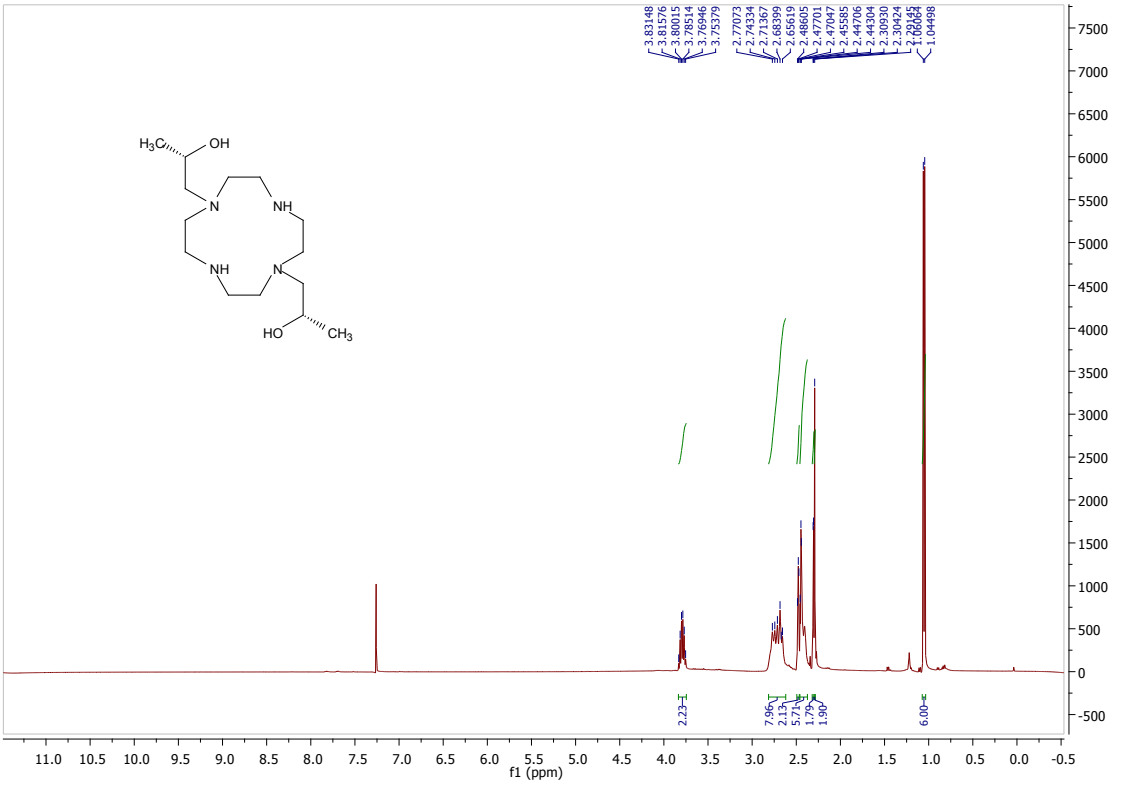


Figure S4. 1D ^{1}H NMR and ^{13}C NMR spectra of **3**.



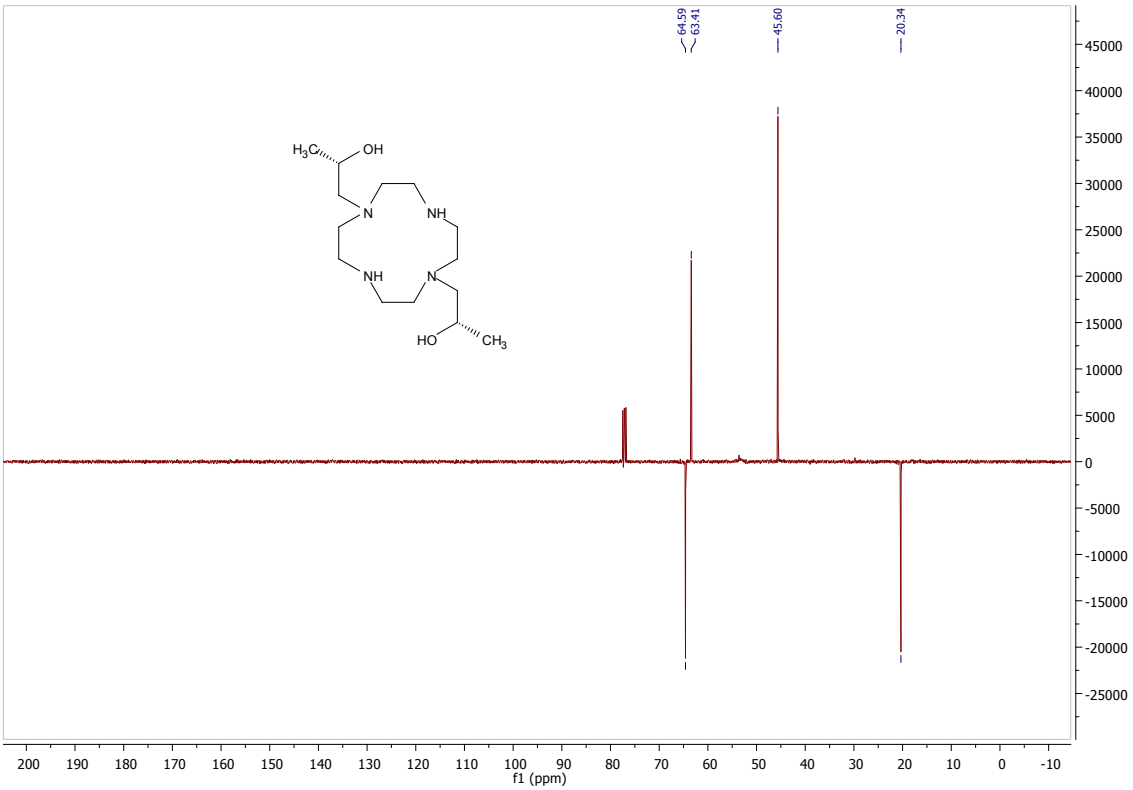
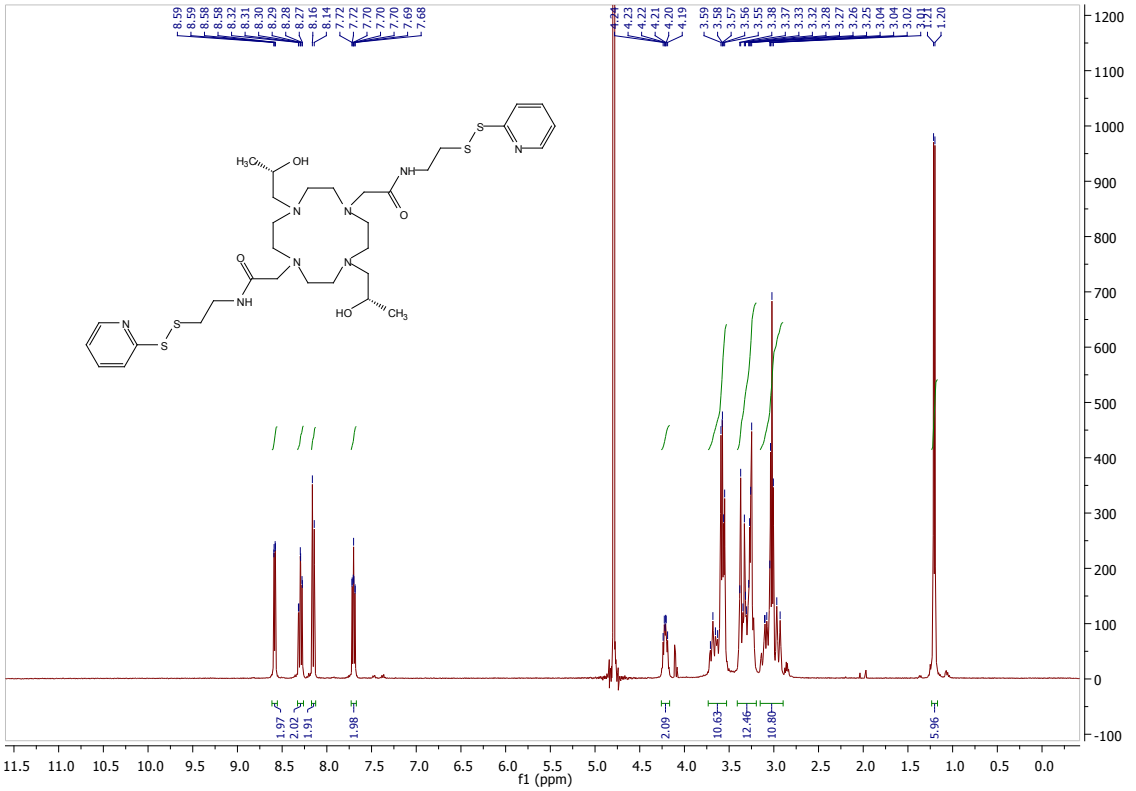


Figure S5. 1D ^{1}H NMR and ^{13}C NMR spectra of **T1**.



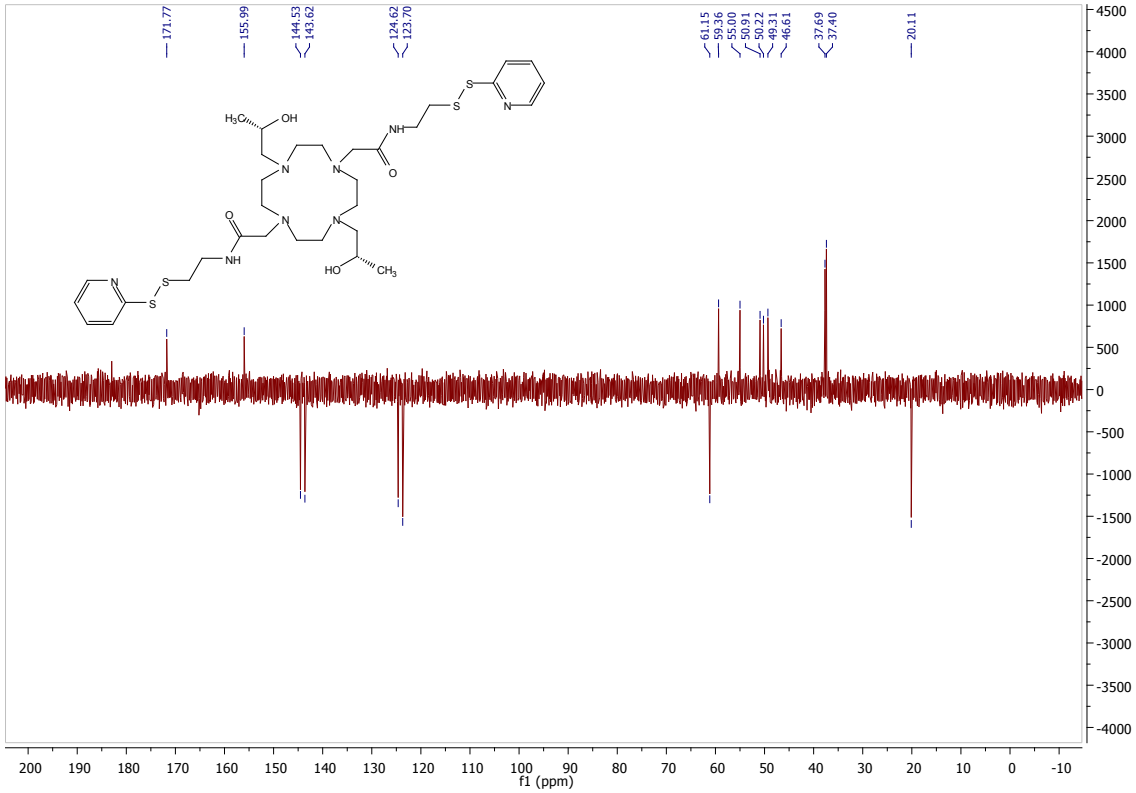
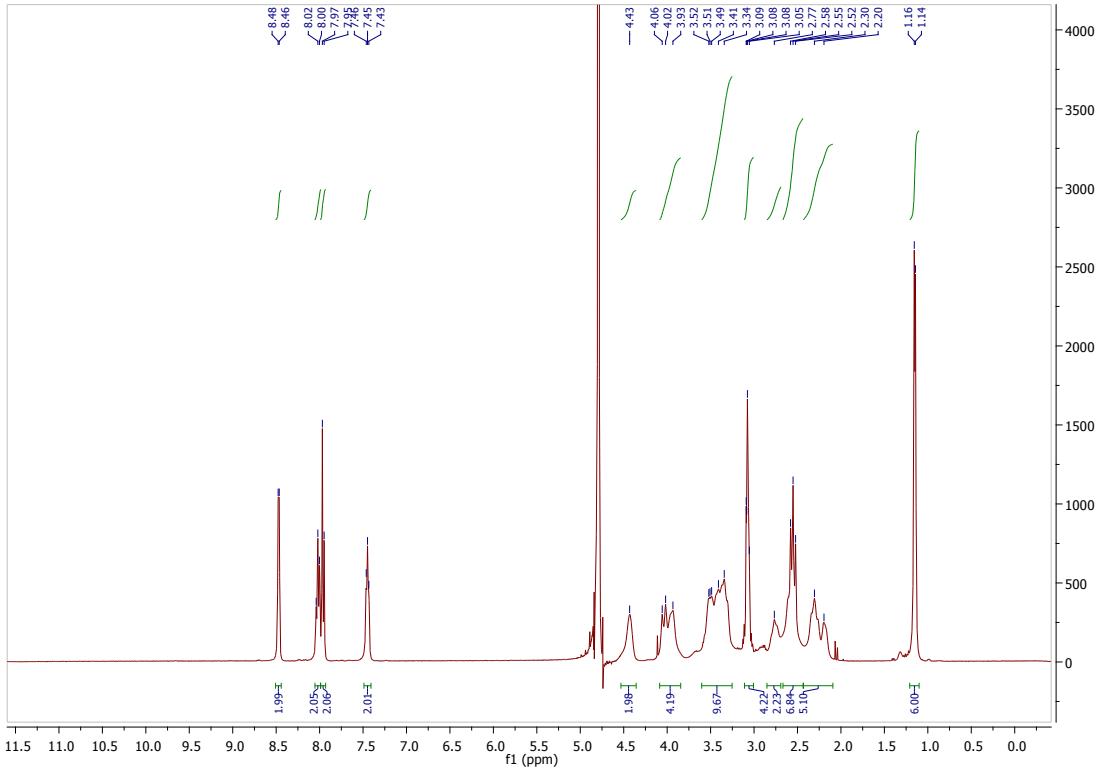
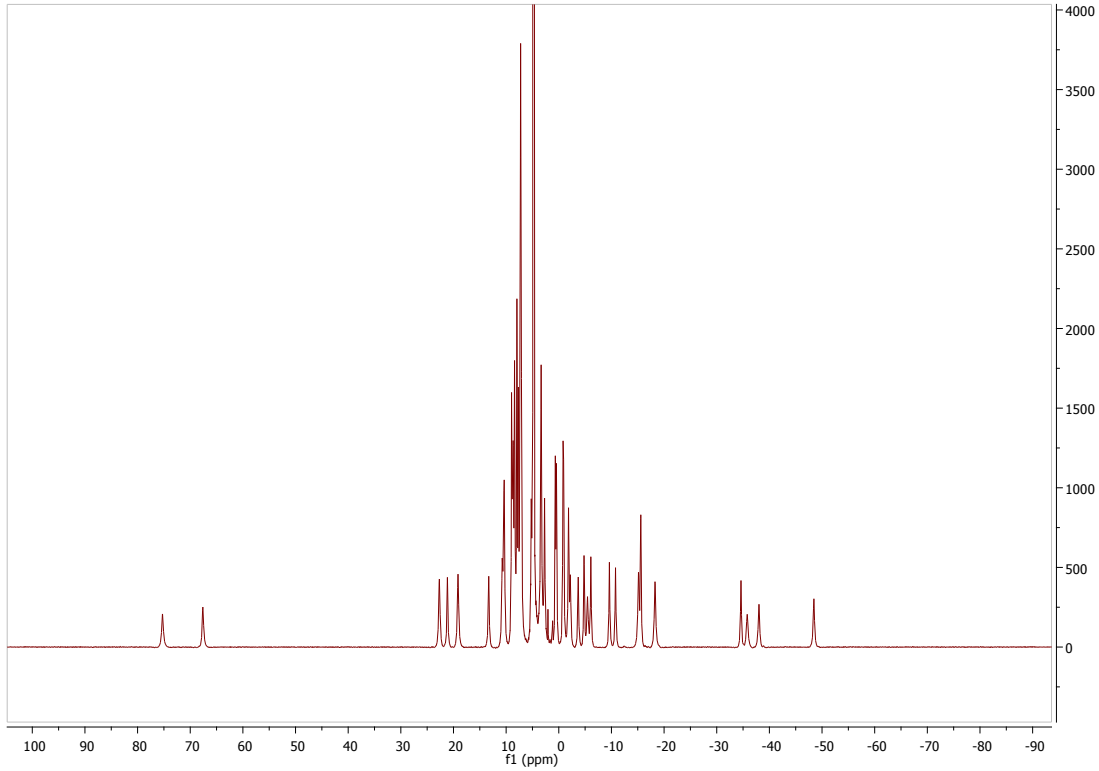


Figure S6. 1D ^{1}H NMR spectra of Y^{3+} (upper) and Yb^{3+} (lower) complexes of **T1**.





Ubiquitin E24C/A28C expression, purification and tagging

Uniformly ^{15}N-labelled ubiquitin E24C/A28C prepared as described.[4] The protein was first reduced by stirring with 20 equivalents of DTT for 1 h at room temperature. The protein was then passed through a PD 10 column equilibrated with degassed buffer (50 mM HEPES, pH 8.0). Five equivalents of **T1** or T**2** loaded with Tm^{3+}, Yb^{3+} or Y^{3+} were diluted in 300 µL of buffer and added to the protein, and the solutions stirred over ice for 3 h. Excess tag was removed by passage through a PD10 column equilibrated with either 50 mM HEPES, pH 8.0, or 50 mM MES, pH 6.5 and the eluate concentrated in an Amicon ultrafiltration centrifugal tube with a molecular weight cutoff of 3 kDa, to a final protein concentration of approximately 100 µM. Prior to NMR measurements, each sample was made to 10% D_2O (v/v).

HPPK K76C/C80 expression, purification and tagging

The HPPK 76C/C80 mutant gene cloned into a pET28a vector was purchased from Geneart. Uniformly ^{15}N-labelled HPPK 76C/C80 was expressed and purified following established protocols for the wild-type protein.[5]

Purified, uniformly ^{15}N-labelled HPPK K76C/C80 was passed through a PD10 column equilibrated with degassed buffer (50 mM HEPES, pH 8.0) to remove DTT present in the storage buffer. The eluate was then made to 10 mM magnesium chloride and 100 µM α,β-methyleneadenosine 5'-triphosphate. Three equivalents of **T1** or **T2** loaded with Tm^{3+}, Yb^{3+} or Y^{3+} were diluted in 300 µL of buffer and added to the protein, and the solutions stirred over ice for 15 min. Excess tag was removed by passage through a PD10 column (50 mM HEPES, pH 8.0). The eluate was again made to 10 mM magnesium chloride and 100 µM α,β-methyleneadenosine 5'-triphosphate, before concentrating in an Amicon ultrafiltration centrifugal tube with a molecular weight cutoff of 3 kDa, to a final protein concentration of approximately 100 µM. Prior to NMR measurements, the samples were made to 1 mM α,β-methyleneadenosine 5'-triphosphate and 400 µM of 8-mercaptoguanine (a small molecule HPPK inhibitor) and the sample adjusted to 10% D_2O (v/v).

Protein NMR spectroscopy and Δχ-tensor and alignment tensor determination

Spectra of differently tagged ubiquitin E24C/A28C and HPPK K76C/C80 samples were recorded at 25 °C and 22 °C, respectively, on a Bruker Avance 600 MHz NMR spectrometer equipped with a cryogenic probe and Z axis gradient. $^{1}H^{N}$ PCSs and $^{1}D_{HN}$ couplings were measured by recording ^{15}N-fast-HSQC and IPAP-[^{15}N, ^{1}H]-HSQC spectra. Data were processed with NMRPipe[6] and analyzed with SPARKY.[7]

Calculation of Δχ-tensors was carried out within the program Numbat.[8] The Δχ-tensors were fitted to the first conformer of the NMR structure of ubiquitin (PDB 2MJB[9]) or the X-ray crystal structure of HPPK (PDB 3QBC[5]). Unambiguous PCS assignments were used to calculate an initial estimate of the Δχ-tensor, which was used to predict PCSs of other nuclei to assist the assignment of further PCSs and refine the Δχ-tensors in an iterative manner. The measured PCSs are listed in **Tables S5** and **S6**.

Backbone amide $^{1}D_{HN}$ RDCs were fitted to the first conformer of the NMR structure of ubiquitin (PDB 2MJB[9]) or the X-ray crystal structure of HPPK (PDB 3QBC[6]) using single value decomposition *via* the "-bestFit" flag in PALES.[10] The measured RDCs are listen in **Tables S7** and **S8**.

Table S2. Δχ-Tensor parameters for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/C80[a,b]

Protein	Tag	pH	Ln^{3+}	#PCS	$\Delta\chi_{ax}$	$\Delta\chi_{rh}$	*Q*	*x*	*y*	*z*	*α*	*β*	*γ*
Ubi	**T1**	8.0	Tm^{3+}	32	49.9 (0.4)	8.8 (0.5)	0.02	4.984	2.764	-13.298	58	147	27
			Yb^{3+}	52	-9.1 (0.2)	-4.2 (0.1)	0.05	4.948	2.764	-13.298	135	79	48
	T2	8.0	Tm^{3+}	41	35.3 (0.5)	4.0 (0.4)	0.03	4.357	1.918	-12.052	24	152	1
			Yb^{3+}	43	5.5 (0.1)	2.1 (0.2)	0.07	4.357	1.918	-12.052	18	164	159
Ubi	**T1**	6.5	Tm^{3+}	40	30.0 (0.2)	9.4 (0.2)	0.01	4.874	2.612	-12.944	56	153	123
			Yb^{3+}	40	6.3 (0.1)	2.7 (0.2)	0.05	4.874	2.612	-12.944	59	146	11
	T2	6.5	Tm^{3+}	35	26.5 (0.2)	4.8 (0.4)	0.02	5.125	1.433	-12.604	19	147	1
			Yb^{3+}	43	5.4 (0.1)	1.0 (0.1)	0.07	5.125	1.433	-12.604	18	153	178
HPPK	**T1**	8.0	Tm^{3+}	67	41.0 (1.0)	6.8 (0.7)	0.08	-16.282	13.879	4.888	122	134	35
			Yb^{3+}	117	7.1	4.6	0.07	-16.282	13.879	4.888	114	120	66
	T2	8.0	Tm^{3+}	94	58.0 (0.9)	9.9 (1.4)	0.05	-14.385	14.943	5.265	125	82	133
			Yb^{3+}	106	9.5	5.4	0.03	-14.385	14.943	5.265	129	65	138

[a] The axial and rhombic components of the Δχ-tensors are reported in units of 10^{-32} m^3, and the Euler angles in degrees, using the *zyz* convention and unique tensor representation.[8] Standard deviations (in brackets) were determined from random removal of 10% of the PCSs and recalculating the Δχ-tensors 1,000 times, in some cases the *z* and *y* axes of the tensors were of similar magnitude and swapped in different fits, thus standard deviations were not determined. Quality factors (*Q*) were calculated as the root-mean-square deviation between the experimental and back-calculated PCSs divided by the root-mean-square of the experimental PCSs.

[b] Metal coordinates (*x*, *y*, *z*) are reported relative to the NMR structure of ubiquitin (PDB ID 2MJB[9]) and the crystal structure of HPPK (PDB ID 3QBC[5]), respectively.

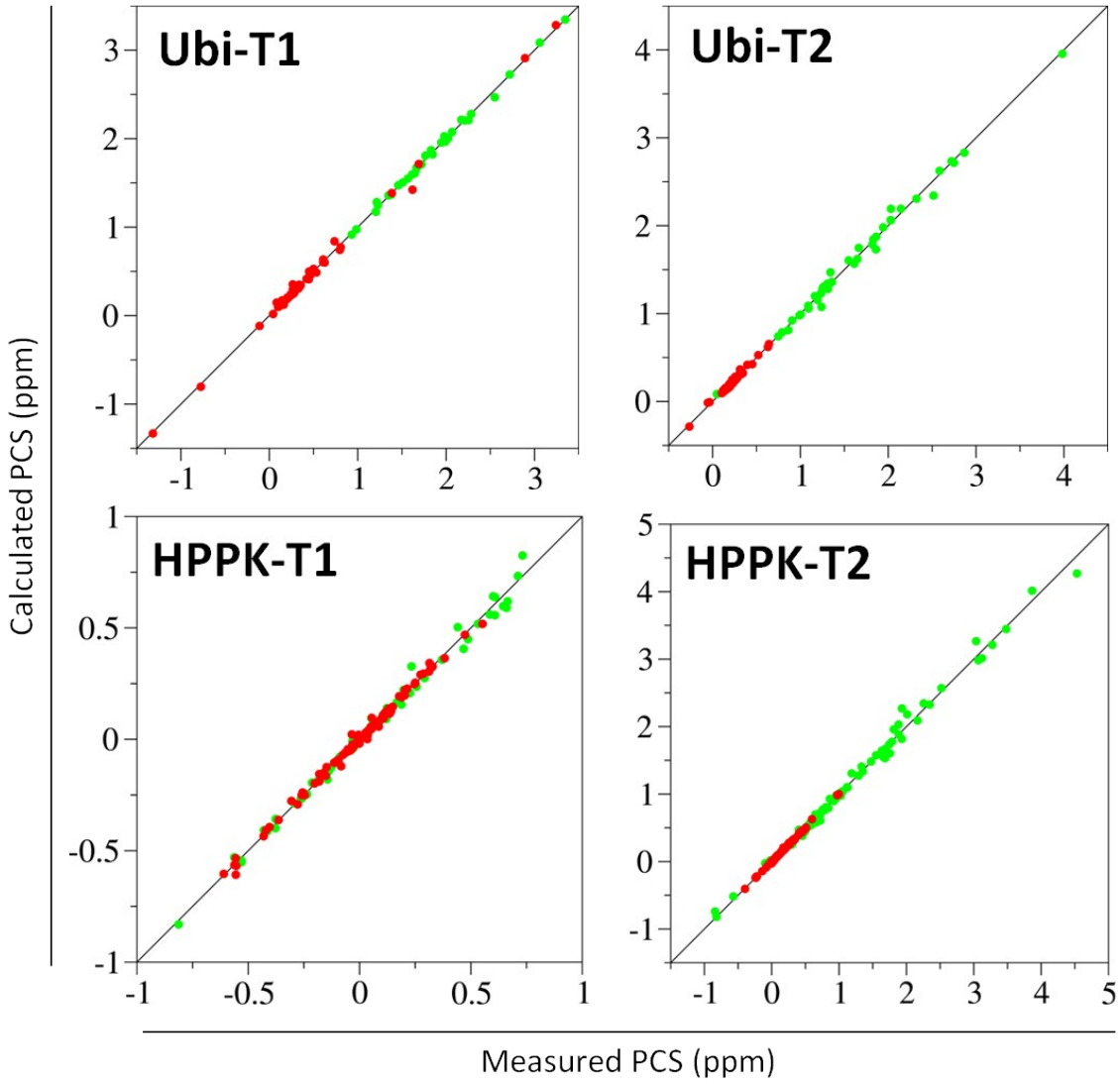


Figure S9. Correlations between experimental and back-calculated PCSs for **T1**- and **T2**-tagged ubiquitin E24C/A28C and HPPK K76C/C80 at pH 8.0, loaded with either Tm^{3+} (green) or Yb^{3+} (red). Solid lines represent perfect correlation.

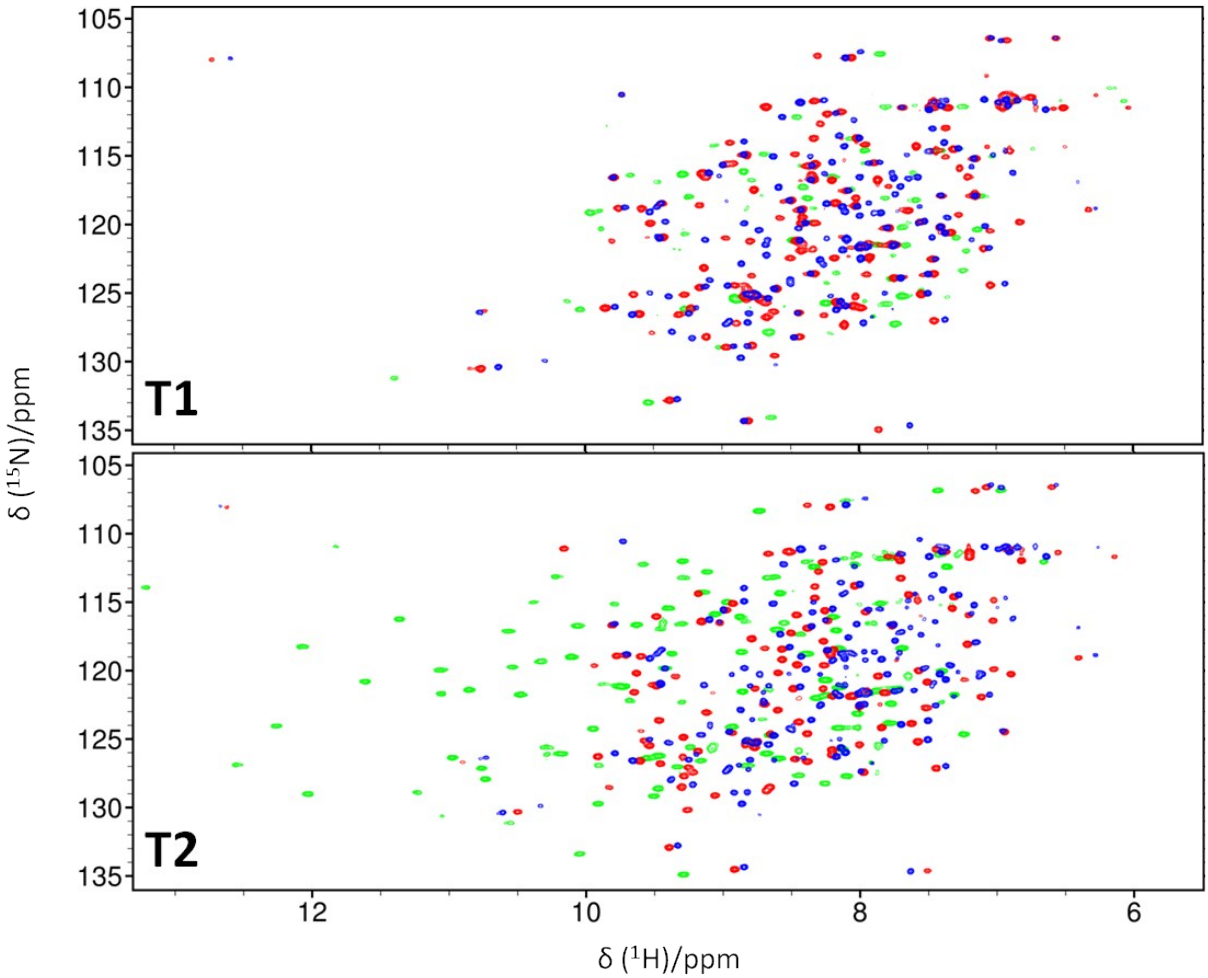


Figure S10. Overlays of ^{15}N-HSQC spectra of **T1**- (top) and **T2**- (bottom) tagged HPPK K76C/C80, loaded with either Y^{3+} (blue), Tm^{3+} (green) or Yb^{3+} (red). The spectra were recorded at 22 °C and pH 8.0 at a ^{1}H NMR frequency of 600 MHz, in the presence of 10 mM $MgCl_2$, 1 mM α,β-methyleneadenosine 5'-trisphosphate and 400 μM of a small-molecule inhibitor, 8-mercaptoguanine.

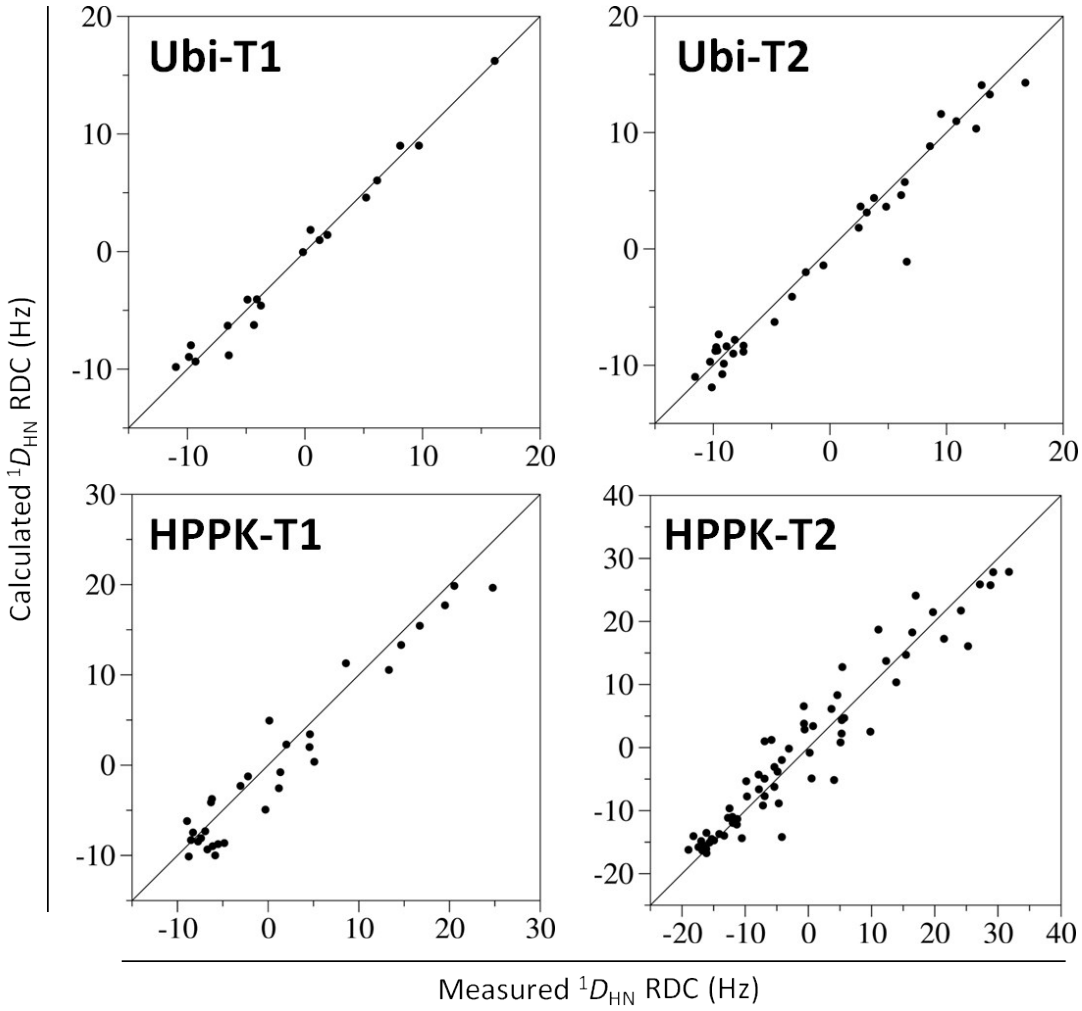


Figure S11. Correlations between experimental and calculated $^1D_{HN}$ RDCs recorded at a ^{1}H NMR frequency of 600 MHz and pH 8.0 for **T1-Tm^{3+}**- and **T2-Tm^{3+}**-tagged ubiquitin E24C/A28C and HPPK K76C/C80 loaded with Tm^{3+}. Solid lines represent perfect correlation.

Table S3. Alignment tensor parameters for **T1-Tm^{3+}**- and **T2-Tm^{3+}**-tagged ubiquitin E24C/A28C and HPPK K76C/C80[a]

Protein	Tag	# RDC	A_{ax}	A_{rh}	Q	α	β	γ	$\Delta\chi_{ax}$[b]	$\Delta\chi_{rh}$[b]
Ubi	**T1**	19	11.3	1.9	0.07	155	147	119	44.1	7.4
	T2	33	7.6	2.3	0.17	131	153	165	29.6	9.0
HPPK	**T1**	31	9.6	0.5	0.20	137	139	57	36.9	1.9
	T2	66	13.3	1.9	0.22	31	79	53	51.2	7.3

[a]The axial and rhombic components of the alignment tensor are reported in units of 10^{-4} and the Euler angles in degrees, using the *zyz* convention, values were determined by fitting the measured RDCs to the NMR structure of ubiquitin (PDB ID 2MJB[9]) or the X-ray crystal structure of HPPK (PDB ID 3QBC[5]) respectively, using the "–bestFit" flag within PALES.[10] Quality factors (Q) were calculated as the root-mean-square deviation between the experimental and back-calculated RDCs divided by the root-mean-square of the experimental RDCs.
[b]$\Delta\chi$-Tensor parameters in units of 10^{-32} m^3 determined from the A_{ax} and A_{rh} using **Equation S1**.

Equation S1. For comparison of $A_{ax,rh}$ and $\Delta\gamma_{ax,rh}$

$$\Delta\chi_{ax,rh} = A_{ax,rh}\frac{15\mu_0 KT}{B_0^2}$$

where B_0 is the field strength (14.1 T), μ_0 is the magnetic permeability of vacuum (12.566×10^{-7} T^2 m^3 J^{-1}), k is the Boltzmann constant (1.38×10^{-23} J K^{-1}), T is temperature (in Kelvin), $\Delta\chi_{ax,rh}$ are the axial and rhombic components of the magnetic susceptibility anisotropy tensor (in m^3) respectively and $A_{ax,rh}$ are the axial and rhombic components of the alignment tensor respectively.

Table S4. Correlations between PCSs or RDCs measured with **T1** and **T2** (see **Figure 3**).

Protein	Ln^{3+}	# PCS[a]	Slope	R^2	#RDCs[a]	Slope	R^2
Ubi	Tm^{3+}	31	0.21	0.28	17	0.65	0.51
	Yb^{3+}	42	0.62	0.29			
HPPK	Tm^{3+}	66	0.22	0.32	26	-0.12	0.02
	Yb^{3+}	105	0.10	0.14			

[a] Number of residues for which the amide proton PCS or RDC was assigned for both **T1**- and **T2**-tagged protein.

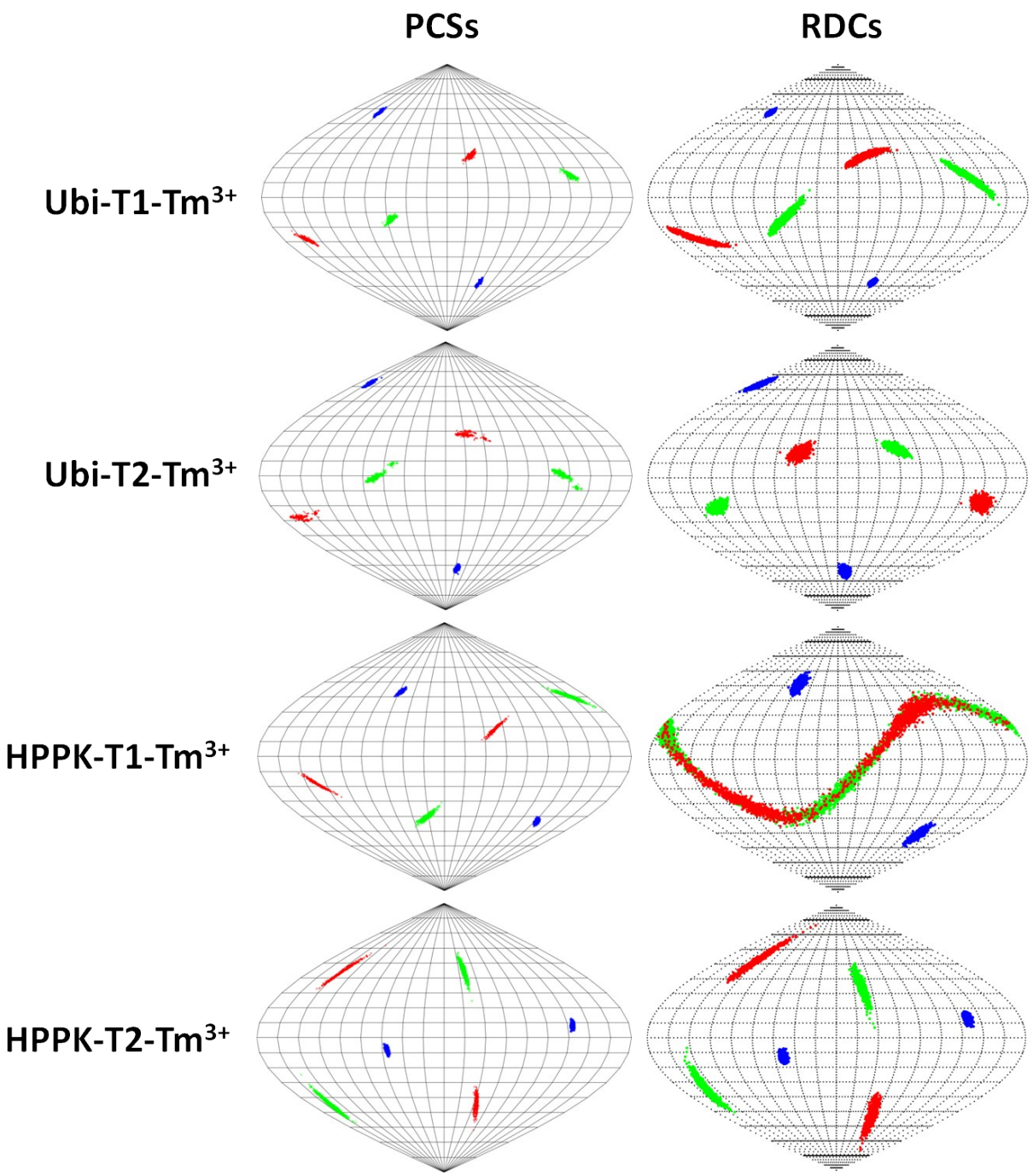


Figure S12. Orientations of the principal axes of the Δχ- (left) and alignment (right) tensors. The points show where the principal axes of the tensors penetrate the sphere with the axes colored as follows: *z* (blue), *y* (green), *x* (red). For the alignment tensors, 1000 replicates of SVD calculation using the structural noise Monte-Carlo method ('-mcStruc') within PALES[10] are shown. For the Δχ-tensors, 1000 replicates with a random 10% of the PCS data removed each time are shown.

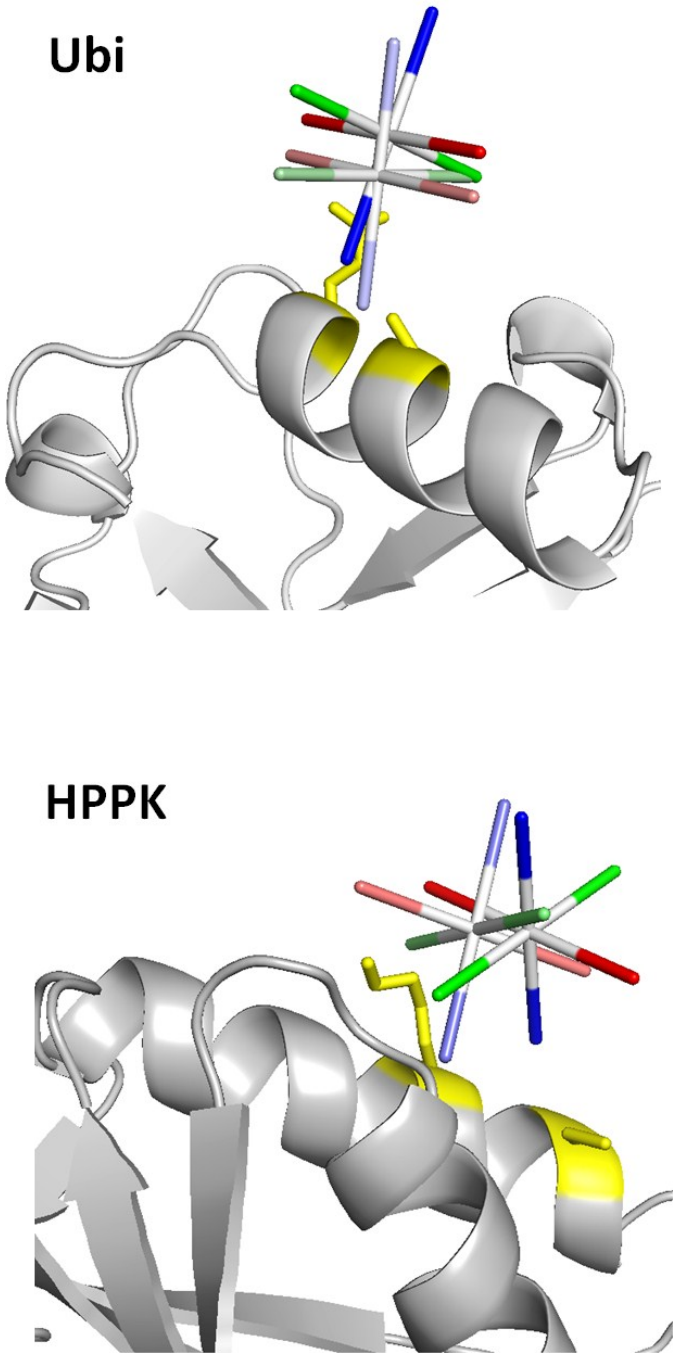


Figure S13. Structures of ubiquitin (top) and HPPK (bottom) and the Δχ-tensor axes of the Tm^{3+} complexes of **T1** and **T2** at pH 8.0. The backbone structures of ubiquitin (PDB ID 2MJB[9]) and HPPK (PDB ID 3QBC[5]) are shown in grey, the side chain residues of E24 and A28 of ubiquitin, and K76 and C80 of HPPK are shown in yellow, the Δχ-tensor axes are shown as sticks colored *z* (blue), *y* (green) and *x* (red) with the **T1** and **T2** axes shown in bold and pastel colors, respectively.

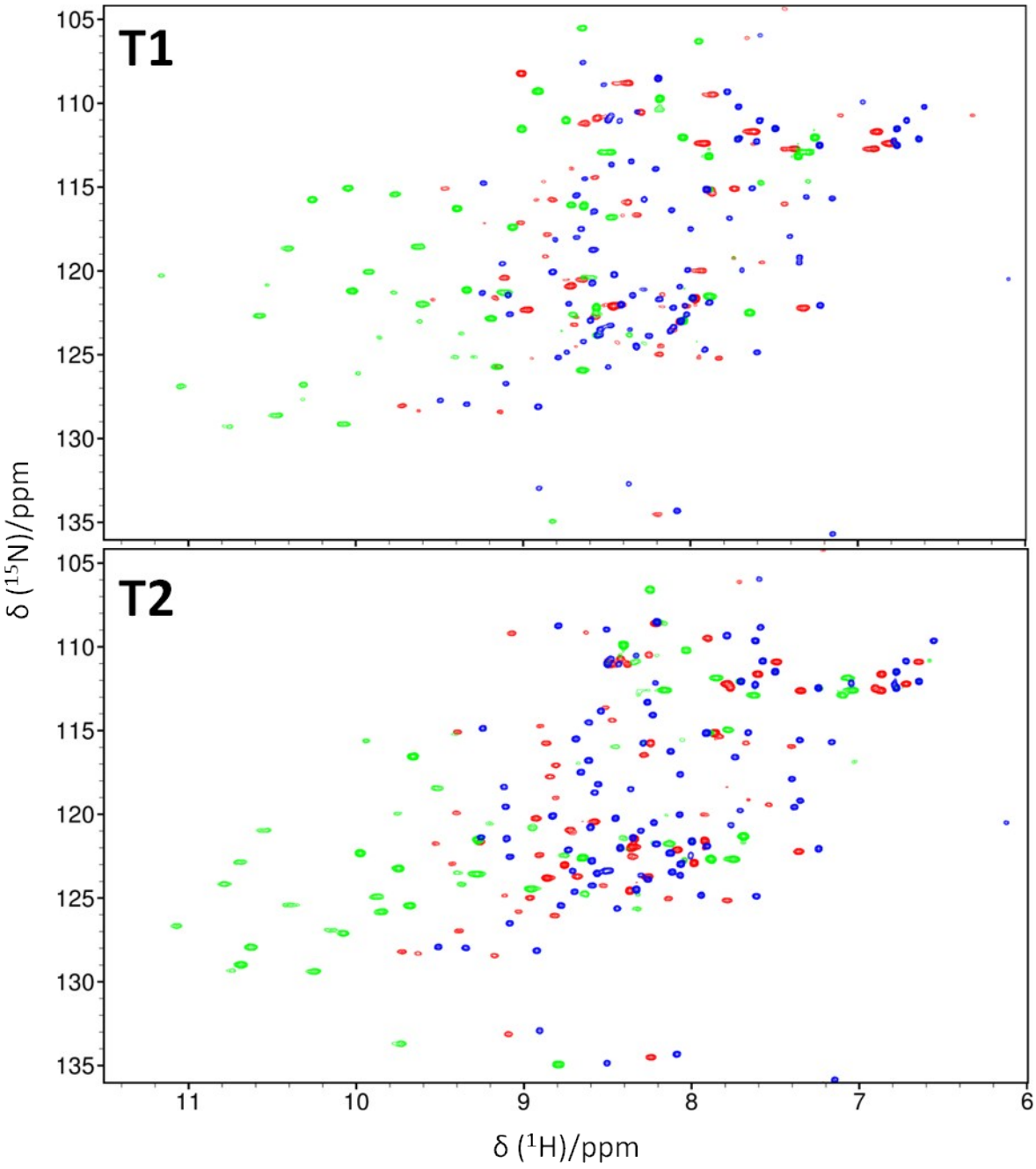


Figure S14. Overlays of ^{15}N-HSQC spectra of **T1-** (top) and **T2-** (bottom) tagged ubiquitin E24C/A28C, loaded with Y^{3+} (blue), Tm^{3+} (green) or Yb^{3+} (red). The spectra were recorded at 25 °C and pH 6.5 at a ^{1}H NMR frequency of 600 MHz.

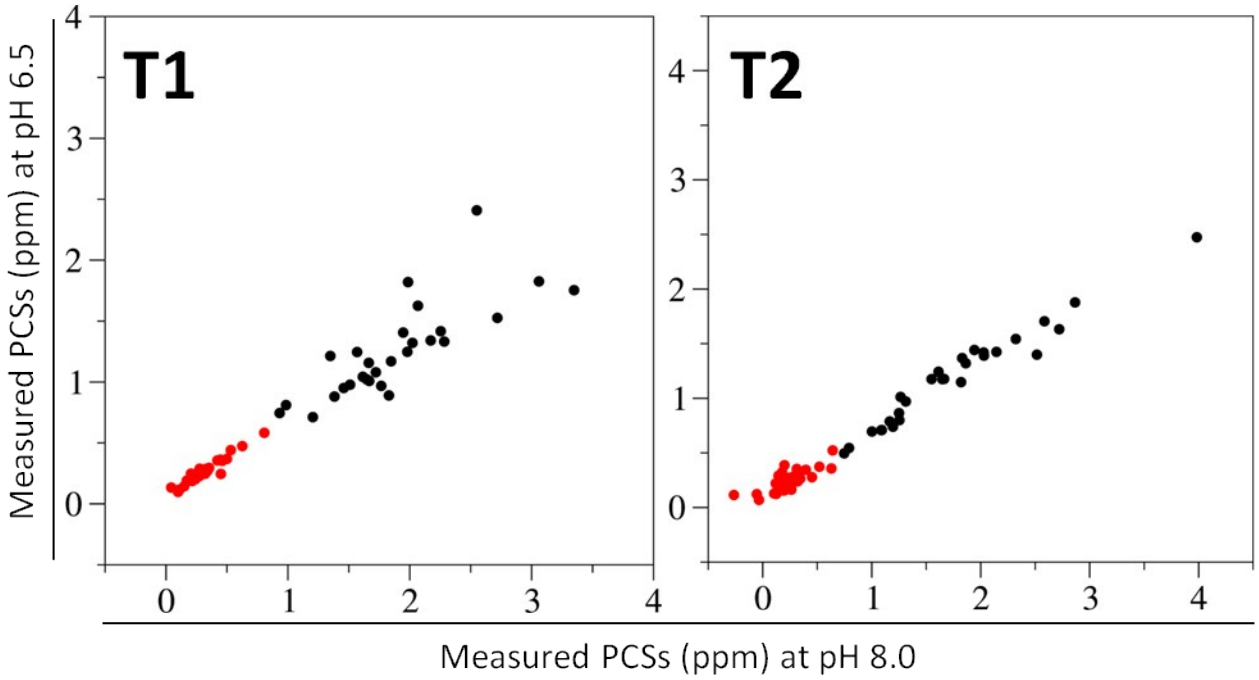


Figure S15. Correlations between the PCSs measured at pH 8.0 and pH 6.5 for **T1**/**T2**-tagged ubiquitin E24C/A28C, loaded with Tm^{3+} (black) or Yb^{3+} (red). Only PCSs which were assigned at both pH values are shown.

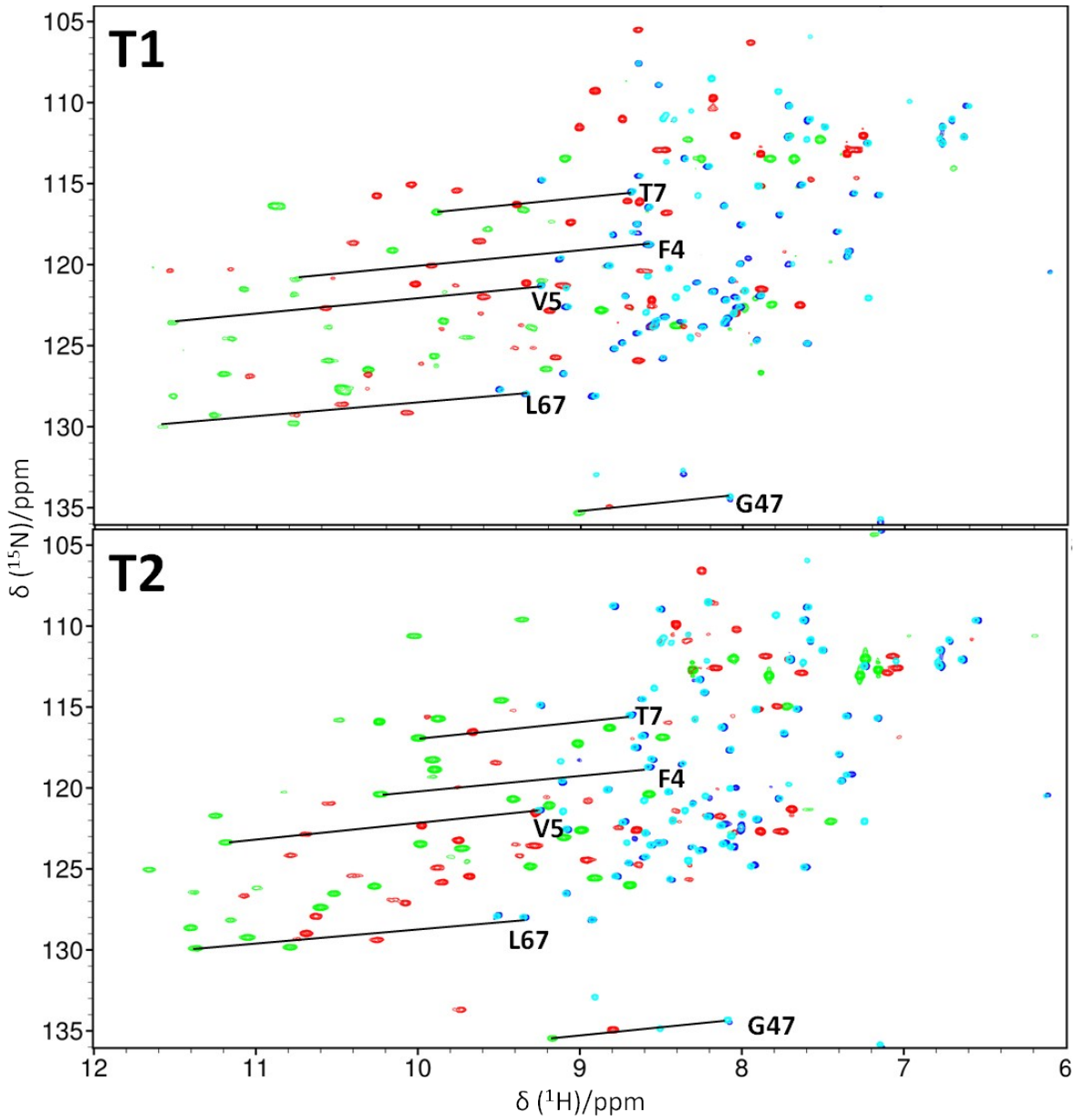


Figure S16. Overlays of ^{15}N-HSQC spectra of **T1**- (top) and **T2**- (bottom) tagged ubiquitin E24C/A28C, loaded with Y^{3+} (pH 8.0 = blue, pH 6.5 = cyan) or Tm^{3+} (pH 8.0 = green, pH 6.5 = red). The spectra were recorded at 25 °C at a ^{1}H NMR frequency of 600 MHz. Lines connecting the PCSs of selected residues at different pH are indicated.

Table S5. Experimental PCSs for tagged ubiquitin E24C/A28C.

		pH 8.0				pH 6.5			
		T1		T2		T1		T2	
Residue		Tm^{3+}	Yb^{3+}	Tm^{3+}	Yb^{3+}	Tm^{3+}	Yb^{3+}	Tm^{3+}	Yb^{3+}
2	GLN	1.67	0.28	0.79	0.11	1.01		0.55	0.13
3	ILE		0.45	1.65	0.23	1.56	0.36	1.18	0.24
4	PHE	2.17	0.34	1.66	0.23	1.34	0.29	1.18	0.27
5	VAL	2.28	0.35	1.94	0.25	1.33	0.30	1.44	0.25
6	LYS	1.85	0.25	1.86	0.25	1.17	0.23	1.32	0.18
7	THR	1.21	0.15	1.31	0.16	0.71	0.14	0.97	0.16
8	LEU					0.52	0.08	0.87	0.12
9	THR					0.37	0.08	0.65	0.11
10	GLY					0.40	0.09	0.62	0.12
11	LYS		0.10			0.42	0.10	0.64	0.12
12	THR					0.52	0.13	0.67	0.22
13	ILE	1.77	0.27	1.55	0.19	0.97	0.23	1.18	0.17
14	THR	1.83	0.32	1.27	0.14	0.89	0.25	1.01	0.25
15	LEU	2.72	0.46	1.83	0.23	1.53	0.35	1.37	0.25
16	GLU	3.35	0.63	1.61	0.18	1.75	0.47	1.24	0.23
17	VAL	3.06	0.53	1.31	0.15	1.83	0.44		0.19
18	GLU		0.61	1.36	0.12	2.86	0.53		0.22
20	SER		0.28	0.05	-0.03	1.49	0.29		0.07
21	ASP		0.61		0.15				0.19
27	LYS		2.89						
29	LYS		3.24						
30	ILE		1.69						
31	GLN		1.39						
32	ASP		1.62						
33	LYS		0.74		0.18				0.32
34	GLU	2.21	0.45	1.86	0.15		0.25		0.29
35	GLY		0.27	0.86	-0.05				0.12
36	ILE		0.08	1.34					0.17
40	GLN		-1.32		-0.26				0.11
41	GLN		-0.78		0.20				0.39
42	ARG	1.22	-0.11	2.87	0.31			1.88	0.35
43	LEU	2.55	0.16	3.98	0.64	2.41		2.47	0.52
44	ILE	2.07	0.22	2.59	0.40	1.63		1.71	0.35
45	PHE	1.57	0.19	1.82	0.35	1.25	0.21	1.15	0.27
46	ALA						0.17	0.82	0.19
47	GLY	0.93	0.11	1.09	0.19	0.75	0.12	0.71	0.16
48	LYS	0.99	0.13	1.20	0.24	0.81		0.74	0.18
49	GLN				0.26	0.77	0.10	0.69	0.16

50	LEU	1.99	0.21	2.72	0.52	1.82	0.25	1.63	0.37
51	GLU	1.99	0.30	2.52	0.63			1.40	0.36
52	ASP	1.23	0.14						
54	ARG		0.81	2.03			0.58		0.54
55	THR		0.50	1.24	0.45		0.37		0.28
56	LEU		0.80	2.75		3.16	0.67		0.51
57	SER		0.42	1.23	0.28	1.69	0.36		0.25
58	ASP	1.98	0.34	0.99	0.26	1.25	0.27		0.20
59	TYR	2.02	0.35	1.33	0.32	1.32	0.29		0.24
60	ASN	1.46	0.24	0.91	0.21	0.95	0.20		0.16
61	ILE		0.27	1.25	0.22	1.28	0.23	0.80	0.19
62	GLN	1.62	0.25	1.09	0.20	1.04	0.23	0.71	0.18
63	LYS	1.38	0.22	0.75	0.12	0.88	0.19	0.50	0.12
64	GLU	1.65	0.27	1.00	0.15	1.02	0.23	0.70	0.15
65	SER	1.72	0.27	1.17	0.18	1.08	0.24	0.79	0.17
66	THR	1.51	0.22	1.25	0.20	0.98	0.20	0.86	0.19
67	LEU	2.25	0.33	2.03	0.30	1.42	0.29	1.39	0.29
68	HIS	1.95	0.24	2.14	0.34	1.41	0.24	1.43	0.30
69	LEU	1.66	0.17	2.03	0.26	1.16	0.19	1.42	0.27
70	VAL	1.35	0.04	2.32	0.31	1.21	0.13	1.54	0.30

Table S6. Experimental PCSs for tagged HPPK K76C/C80.

Residue		T1		T2	
		Tm^{3+}	Yb^{3+}	Tm^{3+}	Yb^{3+}
2	ILE		-0.28		0.26
3	GLN	-0.81	-0.18	0.66	0.21
4	ALA		-0.25	1.68	0.40
5	TYR		-0.16	1.93	0.35
6	LEU		-0.15	3.12	0.51
7	GLY		-0.01	3.48	0.43
8	LEU		0.06	3.28	0.43
9	GLY		0.31	3.87	0.43
10	SER		0.25	2.34	0.26
11	ASN		0.32	1.78	0.15
12	ILE		0.33	1.55	0.15
13	GLY		0.25	0.86	0.09
14	ASP		0.32	1.19	0.16
15	ARG	0.73	0.21	1.33	0.18
16	GLU	0.66	0.18	1.75	0.21
17	SER	0.59	0.20		0.25
18	GLN		0.31		0.35
19	LEU		0.29		0.46
20	ASN		0.20	3.03	0.48
21	ASP		0.27	4.53	0.60
22	ALA		0.38		0.97
23	ILE		0.09		1.00
24	LYS		-0.08		
33	SER		-0.61		
34	VAL		-0.56		
35	SER		-0.31	1.88	0.50
36	ASN		-0.20	1.89	0.42
37	ILE	-0.53	-0.11	1.66	0.31
38	SER		-0.05	1.81	0.34
40	ILE	-0.03	0.03	1.35	0.21
41	TYR	0.23	0.07	1.48	0.21
42	GLU	0.26	0.07	0.96	0.13
43	THR	0.49	0.11	1.06	0.12
44	ALA	0.37	0.08	0.73	0.07
46	VAL	0.60	0.12	0.76	0.04
47	GLY	0.44	0.10	0.43	0.00
48	TYR	0.71	0.14	0.46	-0.02
49	THR	0.53	0.09	0.30	-0.02
50	GLU	0.61	0.11	0.29	-0.02

51	GLN	0.67	0.12	0.42	0.01
53	ASN	0.65	0.12	0.65	0.06
54	PHE	0.61	0.13	1.02	0.10
55	LEU		0.19	1.66	0.19
56	ASN	0.47	0.11	1.64	0.21
57	LEU	0.23	0.10	2.52	0.34
58	CYS		-0.01	2.16	0.33
59	VAL		-0.08	3.07	0.49
60	GLU		-0.18	1.93	0.45
61	ILE		-0.26	2.01	0.50
62	GLN		-0.40	1.74	
63	THR		-0.36		0.40
64	THR		-0.56		
65	LEU		-0.56		
66	THR		-0.43		
67	VAL		-0.26	-0.82	-0.01
68	LEU		-0.25		-0.23
69	GLN		-0.42		-0.22
70	LEU		-0.55		
95	ASP		0.47		
96	VAL		0.55		
97	ASP		0.15		0.45
98	ILE		-0.04		0.17
99	LEU			2.26	0.31
100	LEU		-0.10	1.69	0.24
101	TYR		-0.11	0.79	0.14
102	GLY		-0.15	0.84	0.20
103	GLU	-0.56	-0.10	0.26	0.08
104	GLU	-0.53	-0.10	0.26	0.08
105	MET	-0.43	-0.06	-0.08	-0.01
106	ILE	-0.38	-0.05	-0.10	-0.05
107	ASP	-0.42	-0.04	-0.57	-0.14
108	LEU	-0.38	-0.02	-0.84	-0.24
110	LYS		0.04		
111	LEU		0.00		-0.40
112	SER		0.04		-0.23
113	VAL		-0.03	-0.02	-0.08
115	HIS	-0.14	-0.01	0.92	0.06
117	ARG	0.19	0.04	0.40	0.00
118	MET	0.12	0.03	0.72	0.03
119	ASN	0.06	0.02	0.59	0.04
120	GLU	0.13	0.04	0.57	0.03
121	ARG	0.17	0.04	0.72	0.06

122	ALA	0.21	0.06	0.72	0.06
123	PHE	0.29	0.07	0.94	0.10
124	VAL	0.20	0.05	1.12	0.12
125	LEU	0.12	0.04	1.03	0.11
126	ILE	0.09	0.03	0.96	0.12
128	LEU	-0.03	0.01	1.29	0.18
129	ASN	-0.10	0.00	1.00	0.14
130	ASP	-0.14	-0.01	1.03	0.16
131	ILE	-0.25	-0.04	1.13	0.19
132	ALA	-0.26	-0.04	1.03	0.18
133	ALA	-0.24	-0.04	0.78	0.14
134	ASN	-0.26	-0.04	0.64	0.12
135	VAL	-0.26	-0.04	0.65	0.12
136	VAL	-0.29	-0.05	0.50	0.10
137	GLU	-0.21	-0.03	0.44	0.07
139	ARG	-0.18	-0.03	0.31	0.03
140	SER	-0.13	-0.01	0.27	0.02
141	LYS	-0.16	-0.02	0.25	0.03
142	LEU	-0.13	0.00	0.31	0.05
143	LYS	-0.13	-0.02	0.36	0.06
T2	VAL	-0.19	-0.03	0.57	0.09
145	LYS	-0.14	-0.02	0.56	0.09
146	ASP	-0.11	-0.01	0.44	0.07
147	LEU	-0.09	0.00	0.50	0.07
148	VAL	-0.04	0.00	0.54	0.06
149	PHE	0.05	0.02	0.57	0.07
150	VAL	0.05	0.02	0.42	0.05
151	ASP	0.11	0.03	0.54	0.07
152	ASP	0.12	0.04	0.46	0.05
153	SER	0.20	0.05	0.53	0.06
154	VAL	0.19	0.05	0.63	0.07
155	LYS	0.21	0.06	0.81	0.10
156	ARG	0.05	0.04	0.71	0.10
157	TYR	0.11	0.06	0.99	0.14
158	LYS	0.06	0.05	0.95	0.14

Table S7. Experimental $^{1}D_{HN}$ RDCs of tagged ubiquitin E24C/A28C measured at 600 MHz.

T1-Tm^{3+}			T2-Tm^{3+}		
Residue		$^{1}D_{HN}$ RDC (Hz)	Residue		$^{1}D_{HN}$ RDC (Hz)
2	GLN	16.1	2	GLN	6.1
4	PHE	-6.6	3	ILE	3.2
5	VAL	-11.0	4	PHE	-4.8
6	LYS	-9.9	5	VAL	-9.2
7	THR	1.2	6	LYS	-11.6
13	ILE	-9.3	7	THR	-7.4
14	THR	-9.7	13	ILE	-10.1
15	LEU	5.2	14	THR	-9.7
43	LEU	8.1	15	LEU	4.8
44	ILE	-4.9	16	GLU	2.6
45	PHE	-0.2	17	VAL	10.8
47	GLY	-6.5	20	SER	-9.1
48	LYS	-4.1	34	GLU	-8.2
60	ASN	9.7	35	GLY	-2.1
62	GLN	-3.8	44	ILE	-8.3
65	SER	0.5	45	PHE	-7.4
66	THR	1.9	48	LYS	-8.9
68	HIS	-4.3	50	LEU	-9.5
69	LEU	6.1	54	ARG	6.6
			55	THR	-9.8
			57	SER	13.0
			58	ASP	6.4
			59	TYR	-3.3
			60	ASN	13.7
			61	ILE	16.8
			62	GLN	8.6
			64	GLU	12.5
			65	SER	9.5
			66	THR	2.5
			67	LEU	-9.8
			68	HIS	-10.3
			69	LEU	-0.6
			70	VAL	3.8

Table S8. Experimental $^1D_{HN}$ RDCs of tagged HPPK K76C/C80 measured at 600 MHz.

T1-Tm^{3+}			T2-Tm^{3+}			T2-Tm^{3+}		
Residue		$^1D_{HN}$ RDC (Hz)	Residue		$^1D_{HN}$ RDC (Hz)	Residue		$^1D_{HN}$ RDC (Hz)
16	GLU	4.6	3	GLN	-12.5	115	HIS	-15.3
17	SER	13.3	4	ALA	-19.0	119	ASN	9.8
41	SER	-0.3	5	TYR	-14.1	120	GLU	31.8
42	GLU	-4.8	6	LEU	-7.2	121	ARG	-4.9
44	ALA	-5.8	7	GLY	13.9	122	ALA	-12.0
51	GLN	-6.7	10	SER	29.3	124	VAL	-10.5
53	ASN	1.2	12	ILE	-5.4	125	LEU	-4.2
104	GLU	-7.4	13	GLY	-5.8	126	ILE	-9.7
105	MET	2.0	15	ARG	-0.7	128	LEU	-16.1
119	ASN	-6.9	16	GLU	4.1	129	ASN	-12.7
120	GLU	1.4	20	ASN	-5.4	130	ASP	-4.2
122	ALA	19.5	21	ASP	-6.9	131	ILE	-12.0
129	ASN	16.7	37	ILE	4.6	132	ALA	-6.9
130	ASP	-6.2	43	THR	25.3	133	ALA	-16.8
133	ALA	-8.9	44	ALA	16.4	134	ASN	-13.3
134	ASN	5.1	46	VAL	-0.7	135	VAL	-11.3
135	VAL	-2.2	49	THR	-17.4	136	VAL	0.5
136	VAL	24.8	50	GLU	0.2	137	GLU	27.2
137	GLU	0.1	51	GLN	24.2	140	SER	-14.9
140	GLU	-7.7	56	ASN	28.8	141	LYS	11.1
141	LYS	-6.3	57	LEU	17.0	142	LEU	3.7
142	LEU	-8.5	58	CYS	5.4	143	LYS	21.5
143	LYS	-6.1	59	VAL	-7.9	T2	VAL	5.1
T2	VAL	20.5	100	LEU	-18.2	145	LYS	-7.8
146	ASP	8.6	101	TYR	-15.7	148	VAL	-11.3
148	VAL	14.7	102	GLY	-16.1	149	PHE	15.5
150	VAL	-5.5	103	GLU	5.7	150	VAL	0.7
151	ASP	4.6	104	GLU	-16.8	151	ASP	-16.2
153	SER	-8.8	105	MET	-4.7	153	SER	-16.7
154	VAL	-8.3	106	ILE	5.3	154	VAL	12.3
158	LYS	-3.1	107	ASP	-0.6	155	LYS	19.7
			108	LEU	-3.1	157	TYR	5.3
			113	VAL	-6.9	158	LYS	-9.8

References

1. L. M. De León-Rodríguez, Z. Kovacs, A. C. Esqueda-Oliva and A. D. Miranda-Olvera, *Tetrahedron Lett.*, **2006**, 47, 6937–6940.

2. B. Graham, C. T. Loh, J. D. Swarbrick, P. Ung, J. Shin, H. Yagi, X. Jia, S. Chhabra, N. Barlow, P. Guido, T. Huber and G. Otting, *Bioconjugate Chem.*, **2011**, 22, 2118–2125.

3. H. E. Gottleib, V. Kotlyar and A. Nudelman, *J. Org. Chem.* **1997**, 62, 7512–7515.

4. J. D. Swarbrick, P. Ung, X. Su, A. Maleckis, S. Chhabra, T. Huber, G. Otting and B. Graham, *Chem. Commun.*, **2011**, 47, 7368–7370.

5. S. Chhabra, O. Dolezal, B. M. Collins, J. Newman, J. S. Simpson, I. G. Macreadie, R. Fernley, T. S. Peat and J. D. Swarbrick, *PLoS One*, 2012, **7**, e29444.

6. F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer and A. Bax, *J. Biomol. NMR*, 1995, **6**, 277–293.

7. T.D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco

8. C. Schmitz, M. J. Stanton-Cook, X.-C. Su, G. Otting and T. Huber, *J. Biomol. NMR*, 2008, **41**, 179–189.

9. A. S. Maltsev, A. Grishaev, J. Roche, M. Zasloff and A. Bax, *J. Am. Chem. Soc.*, 2014, **136**, 3752–3755.

10. M. Zweckstetter, *Nat. Protoc.*, 2008, **3**, 679–690.