Supporting information

Single-armed phenylsulfonated pyridine derivative of DOTA is a rigid and stable paramagnetic tag in protein analysis

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General information

$^1$H-NMR and $^{13}$C-NMR spectra of organic ligands were recorded on a Bruker Avance (400 MHz) spectrometer in the stated solvents unless otherwise mentioned. Chemical shifts were reported in ppm on the $\delta$ scale from an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Coupling constants, J, are reported in Hz. Mass spectroscopy was performed in the large facility at State Key Laboratory of Elemento-organic Chemistry, Nankai University, Tianjin. All solvents were commercially available grade. All reactions were carried out under argon atmosphere unless otherwise mentioned.

Organic Synthesis
Scheme 1. Synthesis of T1 and T2. Reagents and conditions: (i) CH$_3$COOH, H$_2$O$_2$(30%), 80°C, 24h, 95%. (ii) H$_2$SO$_4$ (98%), fuming nitric acid, reflux, 3h, 82%. (iii) a) (CF$_3$CO)$_2$O, CH$_2$Cl$_2$, reflux, 12h; b) K$_2$CO$_3$, H$_2$O, RT, 5h; KOH, pH>11; R=CH$_3$, 83%; R=H, 62%. (iv) PhSO$_2$Na, t-Bu$_4$NBr, CH$_3$CN, Ar, reflux, 36h, 82%. (v) PBr$_3$, CHCl$_3$, 50°C, 2h, 96%. (vi) 1,4,7,10-tetraazacyclododecane, CHCl$_3$, Ar, 24h, 29%. (vii) CH$_2$Cl$_2$, Et$_3$N, 4h, 85%. (viii) (R)-ethyl 2-(tosyloxy)propanoate, K$_2$CO$_3$, CH$_3$CN, Ar, 55-60°C, 48h, 86%. (ix) NaOH, H$_2$O, EtOH, RT, 8h, 89%. (x) Ln(NO$_3$_)$_3$ 6H$_2$O, H$_2$O, pH 6-7, high-pressure reactor, 120°C, 4h, 60%.
Synthesis of DO3MA-6MePy (T1)

2,6-dimethylpyridine 1-oxide (2). 30% H$_2$O$_2$ (50 mL, 490.0 mmol) was added to a solution of 2,6-dimethylpyridine (30 mL, 258.0 mmol) in 80 mL CH$_3$COOH, and the mixture was stirred at 80 °C for 24 h. The solution was concentrated to one third of the previous volume under reduced pressure. The residue was poured into 150 mL of ice water, and the pH was adjusted to 10 with K$_2$CO$_3$. The mixture was extracted with dichloromethane and the extracts were concentrated under reduced pressure to give a pale yellow oil (30.2 g, 246.5 mmol, 95.2 %). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 8.33 (1H, d, J=7.1 Hz), 8.15 (1H, d, J=3.0 Hz), 7.98 (1H, dd, J=7.1 Hz, J=3.0 Hz), 2.58 (6H, s).

2,6-dimethyl-4-nitropyridine 1-oxide (4). A mixture of 30.2 g (246.5 mmol) 1 in 40 mL H$_2$SO$_4$ and 30 mL fuming HNO$_3$ was stirred at 110 °C for 3 h. Then the solution was mixed with ice and H$_2$O, and extracted with CH$_2$Cl$_2$. The combined extracts were washed with 250 mL saturated K$_2$CO$_3$ solution, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined extracts were dried over Na$_2$SO$_4$, filtered, and concentrated to give 34.0 g (202.4 mmol) title compound (yield 82.4 %). $^1$H-NMR (400 MHz, CDCl$_3$), $\delta$ ppm: 8.04 (2H, s), 2.59 (6H, s). $^{13}$C-NMR (100 MHz, CDCl$_3$) $\delta$ ppm: 150.32, 117.92, 18.56.

(6-methyl-4-nitropyridin-2-yl)methanol (6). Compound 2 (8.4 g, 50.0 mmol) was dissolved in 80 mL dichloromethane, and 18.0 mL trifluoroacetic anhydride (125.0 mmol, 2.5 equiv) mixed with 20 mL dichloromethane was added dropwise to the above solution. Then the reaction mixture was heated to reflux for 12 h. The resulting solution was concentrated under reduced pressure, and 80 mL H$_2$O was added to the residue. The pH was adjusted with potassium carbonate to 8, and the mixture was stirred for 5 h at ambient temperature. The pH of the above mixture was increased to 11 with KOH, and the resulting aqueous solution was extracted with dichloromethane. The combined organic phases were washed with brine, dried over Na$_2$SO$_4$, filtered and concentrated to afford a yellow solid, yield 82.6 % (6.94 g, 41.3 mmol). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 7.87 (1H, s), 7.81 (1H, s), 4.90 (2H, s), 3.43 (1H, s), 2.74 (3H, s).

(6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methanol (8). 80 mL CH$_3$CN was mixed with 3 (3.20 g, 19.05
mmol), sodium benzenesulfinate (7.50 g, 45.71 mmol) and tetrabutyl ammonium bromide (0.31 g, 0.96 mmol), and the mixture was heated to reflux under argon atmosphere for 36 h. Then the reaction mixture was filtered, and concentrated. The title compound was purified through a silicon column (EA:PE=1:1) to give a white powder (4.11 g, 15.63 mmol) and the yield was about 82.0 %. ¹H-NMR (400 MHz, CDCl₃) δ ppm: 7.98 (2H, d, J=7.3 Hz), 7.67 (1H, t, J=7.4 Hz), 7.60-7.56 (4H, m), 4.80 (2H, d, J=4.3 Hz), 2.65 (3H, t, J=4.9 Hz).

2-(bromomethyl)-6-methyl-4-(phenylsulfonyl)pyridine (10). PBr₃ (2.0 mL, 21.05 mmol) in 20 mL CHCl₃ was added dropwise to the solution of 4 (3.11 g, 11.83 mmol) in 100 mL CHCl₃. Then the reaction mixture was stirred at 50 °C for 2 h, and the resulting solution was poured into water and potassium carbonate was added to adjust the pH to 8. The mixture was extracted with dichloromethane. The combined organic phases were washed with brine, dried over Na₂SO₄, filtered, concentrated, and purified by chromatography column (EA:PE=1:2) to afford a white solid, yield 96.1 % (3.70 g, 11.35 mmol). ¹H-NMR (400 MHz, CDCl₃) δ ppm: 7.98 (2H, d, J=8.2 Hz), 7.73 (1H, s), 7.68 (1H, t, J=7.7 Hz), 7.60 (2H, t, J=7.5 Hz), 7.54 (1H, s), 4.55 (2H, s), 2.65 (3H, s).

1-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane (12). 5 (3.70 g, 11.35 mmol) in 20 mL CHCl₃ was added dropwise over 30 min to a solution of 1,4,7,10-tetraazacyclododecane (2.93 g, 17.03 mmol) in 40 mL CHCl₃. The mixture was stirred at ambient temperature under argon atmosphere for 24 h. The mixture was purified by chromatography column to give the title product as a white solid (1.39 g, 3.33 mmol, 29.4 %). ¹H-NMR (400 MHz, CDCl₃) δ ppm: 7.98 (2H, d, J=7.3 Hz), 7.82 (1H, s), 7.62 (1H, t, J=7.0 Hz), 7.58-7.52 (2H, t, J=7.3 Hz), 7.43 (1H, s), 3.82 (2H, s), 2.86 (4H, brs), 2.69 (8H, s), 2.62 (4H, brs); 2.58 (3H, s), 1.25 (3H, brs).

(R)-ethyl-2-hydroxypropanoate (13). A solution of (R)-ethyl 2-hydroxypropanoate (12.5 g, 106 mmol) and p-toluenesulfonyl chloride (22.3 g, 117 mmol) in dichloromethane (80 ml) was stirred at 0-10 °C for 10 min. Triethylamine (18.6 ml, 134 mmol) was then added dropwise with vigorous stirring and cooling. The
reaction mixture was then stirred for 4 h at room temperature, and filtered. The filtrate was washed with water, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. The residue was purified by chromatography column (petroleum ether: ethyl acetate=50:1, and then 10:1). The title product 7 (24.5 g, 90.07 mmol, 85.1 %) was a pale yellow oil. $^1$H-NMR spectrum (400 MHz, CDCl$_3$), $\delta$ ppm: 7.82 (2H, d, J=8.2 Hz), 7.35 (2H, d, J=8.0 Hz), 4.94 (1H, t, J=6.9 Hz), 4.13 (2H, t, J=7.1 Hz), 2.45 (3H, s), 1.52 (3H, d, J=6.9 Hz), 1.20 (3H, t, J=8.3 Hz).

$(2S,2'S,2''S)$-triethyl-2,2',2''-(10-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)-methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoate (15). The mixture of 6 (1.02 g, 2.45 mmol), K$_2$CO$_3$ (1.69 g, 12.23 mmol), 7 (3.25 g, 11.95 mmol) and 40 mL CH$_3$CN was stirred at 55-60 °C under argon atmosphere for 48 h. The mixture was then filtered and concentrated. The residue was purified by chromatography column (EA:PE=1:1) to give a red brown solid (1.50 g, 2.09 mmol) and the yield was about 85 %. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ ppm: 8.02 (3H, brs), 7.60 (4H, brs), 4.16 ( 6H, brs), 3.88-3.37 (5H, m), 2.96 (6H, brs), 2.64 (13H, brs), 1.27 (13H, brs).

$(2S,2'S,2''S)$-2,2',2''-(10-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoic acid (17, DO3MA-6MePy, T1). 8 (1.50 g, 2.09 mmol) and NaOH (0.50 g, 12.5 mmol) was mixed with 10 mL H$_2$O and 10 mL EtOH. The solution was stirred at room temperature for 8 h, and then the pH of the solution was adjusted to 3 using hydrogen ion exchange resin. The mixture was filtered and then concentrated to give a white powder in a yield of 88 % (1.17 g, 1.85 mmol). $^1$H-NMR (400 MHz, H$_2$O+10% D$_2$O) $\delta$ ppm: 7.94 (2H, d, J=8.4 Hz), 7.89 (1H, s), 7.78 (1H, s), 7.69 (1H, d, J=7.3 Hz), 7.60-7.56 (2H, t, J=7.6 Hz), 4.21-3.47 (5H, m), 3.46-2.76 (12H, m), 2.75-2.44 (7H, m), 1.47(3H, s), 1.27 (3H, s), 1.19-1.12 (3H, d, J=7.4 Hz). $^{13}$C-NMR (100 MHz, D$_2$O) $\delta$ ppm: 161.1, 155.7, 150.9, 137.4, 135.2, 130.0, 128.1, 120.9, 119.7, 61.5, 60.8, 55.7, 55.3, 48.8, 46.8, 45.8, 45.1, 43.0, 22.8, 21.4, 10.1, 9.2, 7.8. MS-ESI (-): 632.3.
Formation of lanthanide complex.

The free tag of DO3MA-6MePy (17) (180 mg, 0.284 mmol) and Ln(NO$_3$)$_3$·6H$_2$O (0.427 mmol) was dissolved in 10 mL H$_2$O, and the pH was adjusted to 6-7 with NaOH solution. The resulting mixture was heated at 120 °C in a high-pressure reactor for 4 h. After cooling to room temperature, the pH of the solution was adjusted to 8 with NaOH solution, and centrifuged. The aqueous solution was concentrated under reduced pressure, and 20 mL dry EtOH was added. The above mixture was filtered to remove the inorganic salt, and the filtrate was concentrated to give a white fluffy solid (0.170 mmol, ~60 %).

Fig. S1 1D NMR and Mass spectra of the compounds in synthesis of T1.
Synthesis of DO3M-Py (T2)

2-methylpyridine 1-oxide (1). 30 % H$_2$O$_2$ (32 mL, 313 mmol) was added to a solution of 2-methylpyridine (15 mL, 153 mmol) in 60 mL CH$_3$COOH, and the mixture was stirred at 80 °C for 24 h. The solution was then concentrated to one third of the previous volume under reduced pressure, and the mixture was poured into 100 mL ice water, treated with K$_2$CO$_3$ to pH ~10. The mixture was then extracted with dichloromethane and the extracts were concentrated under reduced pressure to give a pale yellow oil (14.5 g, 133 mmol, 86.8 %). $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.18 (d, 1H), 7.12-7.08 (t, 2H), 7.04-7.00 (m, 1H), 2.30 (s, 3H).

2-methyl-4-nitropyridine 1-oxide (3). A solution of 14.5 g (133 mmol) of 1 in 50 mL H$_2$SO$_4$ and 60 mL fuming HNO$_3$ was stirred at 110 °C for 3 h. The mixture was added into 200 mL ice water, and extracted with CH$_2$Cl$_2$. The combined extracts were washed with 150 mL saturated K$_2$CO$_3$ solution, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined extracts were dried over Na$_2$SO$_4$, filtered, and concentrated to give 17.6 g (114 mmol) title compound in 85.9 % yield. $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.34 (d, J=6.8 Hz, 1H), 8.12(s, 1H), 8.03(s 1H), 2.60 (s, 3H).

(4-nitropyridin-2-yl)methanol (5). 25 mL trifluoroacetic anhydride (171.4 mmol) in 30 mL dichloromethane was added dropwise to the solution of 3 (13.2 g, 85.7 mmol) in 150 mL dichloromethane. The reaction mixture was then heated to reflux for 12 h. The resulting mixture was concentrated under reduced pressure, and 120 mL H$_2$O were added to the residue, potassium carbonate was added to make the pH of the system to 8 and stirred for 5 h at ambient temperature. Treated with KOH to improve pH to 11 and the resulting aqueous solution was extracted with dichloromethane. The combined organic phases were washed with brine, dried over Na$_2$SO$_4$, filtered and concentrated to afford a yellow solid, yield 62.6 % (8.26 g, 62.6 mmol). $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.89 (1H, d, J=5.2 Hz), 8.11 (1H, s), 7.97 (1H, d, J=5.3 Hz), 4.96 (2H, s), 3.32 (1H, s).
(4-(phenylsulfonyl)pyridin-2-yl)methanol (7). A mixture of 5 (3.08 g, 20.0 mmol), sodium benzenesulfinate (7.87 g, 48.0 mmol), tetrabutyl ammonium bromide (0.31 g, 0.96 mmol) and 100 mL CH$_3$CN was heated to reflux under argon atmosphere for 36 h. The resulting mixture was filtered, and purified by chromatography column (EA:PE=1:1) to give a white powder (4.18 g, 16.79 mmol) with a yield of 84.0 %. $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.77 (1H, d, J=5.1 Hz), 7.99 (2H, d, J=7.4 Hz), 7.81 (1H, s), 7.70 (1H, d, J=5.6 Hz), 7.66 (1H, d, J=7.3 Hz), 7.59 (2H, t, J=7.6 Hz), 4.86 (2H, d, J=4.9 Hz), 3.34 (1H, t, J=5.2 Hz).

2-(bromomethyl)-4-(phenylsulfonyl)pyridine (9). PBr$_3$ (2.3 mL, 24.00 mmol) in 20 mL CHCl$_3$ was added dropwise to the solution of 7 (3.00 g, 12.00 mmol) in 80 mL CHCl$_3$. The reaction solution was stirred at 50 °C for 2 h. Then the mixture was poured into water, and potassium carbonate was added to adjust the pH to 10. The above solution was extracted with CH$_2$Cl$_2$. The combined organic phases were washed with brine, dried over Na$_2$SO$_4$, filtered, concentrated and purified by chromatography column (EA:PE=1:2) to afford a white solid, yield 95.3 % (3.57 g, 11.44 mmol). $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.79 (1H, d, J=5.1 Hz), 8.00 (2H, d, J=7.8 Hz), 7.94 (1H, s), 7.70 (1H, d, J=5.1 Hz), 7.67 (1H, d, J=6.8 Hz), 7.60 (2H, t, J=7.6 Hz), 4.60 (2H, s).

1-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane (11). Compound 9 (3.60 g, 11.54 mmol) in 20 mL CHCl$_3$ was added dropwise to the solution of 1,4,7,10-tetraazacyclododecane (3.09 g, 18.00 mmol) in 40 mL CHCl$_3$. Then the solution was stirred for 24 h under argon atmosphere at room temperature. The title compound was purified by chromatography column as a white solid (1.65 g, 4.10 mmol, 35.5 %). $^1$H-NMR (400 MHz, CDCl$_3$) δ ppm: 8.53 (1H, d, J=5.5 Hz), 7.80 (2H, d, J=8.1 Hz), 7.69 (1H, s), 7.65 (1H, d, J=5.5 Hz), 7.59 (1H, t, J=7.4 Hz, 7.2 Hz), 7.48 (2H, t, J=7.6 Hz, 7.9 Hz), 3.60 (2H, s), 2.56 (4H, s), 2.40 (8H, s), 2.35 (4H, s). MS-ESI-(+): 404.3.

(2S,2'S,2''S)-triethyl-2,2',2''-(10-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)m-ethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoate (14). The mixture of 60 mL CH$_3$CN, 11 (1.50 g, 3.72
mmol), K₂CO₃ (5.10 g, 37.00 mmol) and 13 (4.10 g, 14.89 mmol) was stirred at 55-60 °C under argon atmosphere for 48 h. The mixture was then filtered, and purified by chromatography column to give a red brown solid (2.34 g, 3.33 mmol) in a yield of 89.3 %. MS-ESI-(+): 690.4, 704.4, 712.4, 726.5.

(2S,2'S,2"S)-2,2',2"-(10-((6-methyl-4-(phenylsulfonyl)pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tripropanoic acid (16, DO3MA-Py, T2). The mixture of 14 (1.60 g, 2.28 mmol), NaOH (0.50 g, 12.50 mmol), 10 mL H₂O and 10 mL EtOH was stirred at ambient temperature for 8 h. Then the pH of the above solution was adjusted to 3 using hydrogen ion exchange resin. The mixture was filtered and then concentrated to give a white powder in a yield of 85.5 % (1.21 g, 1.95 mmol). ¹H-NMR (400 MHz, H₂O+10% D₂O) δ ppm: 8.97 (1H, d, J=5.4 Hz), 7.85 (1H, s), 7.83 (1H, d, J=1.4 Hz), 7.59 (3H, m), 7.48 (2H, t, J=7.9 Hz), 3.58-3.30 (3H, m), 2.76-1.66 (18H, m), 1.03-0.76 (9H, m). MS-ESI (-): 618.3.

Formation of lanthanide complex. The free tag T2 (16) (180 mg, 0.291 mmol) and Tm(NO₃)₃·6H₂O (202 mg, 0.436 mmol) was dissolved in 10 mL H₂O and the pH was adjusted to 6-7. The resulting mixture was heated at 120 °C in a high-pressure reactor for 4 h. After cooling to room temperature, the pH of the solution was adjusted to 8 with NaOH solution, and centrifuged. The aqueous solution was concentrated under reduced pressure, and 20 mL dry EtOH was added. The above mixture was filtered to remove the inorganic salt, and the filtrate was concentrated to give a white fluffy solid (141 mg, 0.180 mmol, 62 %). ESI (+): 808.4, 809.5, 824.3, 825.3.
Fig. S2 1D NMR and Mass spectra of the compounds in synthesis of T2.
m/z: 619.2676 (100.0%), 620.2709 (31.4%)
**Protein expression and purification**

All oligonucleotides used for mutagenesis were purchased from Promega. $^{15}$NH$_4$Cl was purchased from Aldrich-Sigma ISO-TECH. The plasmid for expression of the target protein was constructed using a PET3a vector for expression under control of the T7 promoter. *E. coli* BL21 (Rosetta) strain was used for protein expression. Recombinant ubiquitin and GB1 mutants were cloned into the PET3a vector and the proteins were expressed in *E. coli* with induction by isopropyl-D-1-thiogalactopyranoside (IPTG). $^{15}$N-labeled protein was prepared by growing cells in M9 medium following an established high cell-density protocol.$^1$ The protein was first purified through a DEAE column. Pure protein was obtained through G50 gel filtration. Approximately 28 mg of purified uniformly $^{15}$N-labeled GB1 and 18 mg ubiquitin was obtained from 250 mL M9 medium.

**Protein ligation: site-specifically labeling of ubiquitin and GB1 with a paramagnetic tag (Scheme S1)**

2 mL 0.3 mM $^{15}$N-ubiquitin G47C or GB1 T11C in 20 mM tris (hydroxymethyl) aminomethane (Tris) buffer was mixed with 3 equivalents of T1-Ln or T2-Ln tag (in 30 mM stock of aqueous solution) and 0.2 mM tris(2-carboxyethyl)phosphine (TCEP), and the pH was adjusted to 9-9.5. The reaction was monitored by recording $^{15}$N-HSQC spectra and MALDI-TOF mass spectra. After incubation at room temperature for about 20 h, excess of free paramagnetic complex was removed using a short PD-10 desalting column. The overall yield of ligated protein was about 85%.

**NMR measurements.**

Samples for NMR measurements generally contained 0.2 mM or 0.3 mM $^{15}$N-protein-T1(orT2)-Ln in 20 mM MES buffer at pH 6.4. All NMR spectra were recorded at 298K with Bruker Avance 600 MHz NMR spectrometer equipped with a QCI-cryoprobe. PCSs of backbone amide protons were measured as the chemical shift differences between the paramagnetic sample and free protein. RDCs of backbone amide groups were measured with IPAP pulse sequence and calculated as the coupling constant differences between the free protein and paramagnetic sample. The NMR data were processed with Topspin 2.1 and analysed with Sparky.$^2$
Supplementary Figures and Tables.

Fig. S3 MALDI-TOF mass spectra of ubiquitin G47C mutant (red) and G47C-T1/(T2)-Ln conjugate (blue). The molecular masses of the $^{15}$N-labelled protein samples are indicated. The mass experiment was performed during the ligation reaction for the reaction mixture of $^{15}$N-labelled G47C mutant and its T1/T2-Ln conjugate. A) T1-Dy; B) T1-Tm. C) T2-Tm. The theoretical molecular mass differences between the G47C mutant and its T1/T2-Ln conjugates are 654.8, 661 and 647 for T1-Dy, T1-Tm and T2-Tm, respectively.
Fig. S4 Superimposition of $^{15}$N-HSQC spectra of samples of 0.2 mM solutions of the $^{15}$N-labeled G47C mutant (black) and G47C-Ti-Ln: Dy(red), Tm (magenta) and Yb(blue), of which the first two spectra were zoomed Fig. 2. NMR spectra were recorded for protein samples in 20 mM MES buffer, pH 6.4, at 298 K and at a proton frequency of 600 MHz.
Fig. S5 Superimposition of $^{15}$N-HSQC spectra of samples of 0.2 mM solutions of the $^{15}$N-labeled G47C mutant (black) and G47C-T2-Tm (magenta). NMR spectra were recorded for protein samples in 20 mM MES buffer, pH 6.4, at 298 K and at a proton frequency of 600 MHz.
Fig. S6 Zoomed $^{15}$N-HSQC spectra of Fig. 3.
**Fig. S7** Superimposition of $^{15}$N-HSQC spectra of samples of 0.3 mM solutions of the $^{15}$N-labeled GB1 T11C (black) and T11C-T2-Tm (magenta). NMR spectra were recorded for protein samples in 20 mM MES buffer, pH 6.4, at 298 K and at a proton frequency of 600 MHz.
**Fig. S8** Correlations of the experimental PCSs (PCS\(_{\text{exp}}\)) and back-calculated PCSs (PCS\(_{\text{calc}}\)) using Numbat program. Quality factors (Q=sqrt{\Sigma*(PCS_{\text{calc}}-PCS_{\text{exp}})^2}/sqrt{\Sigma*(PCS_{\text{exp}})^2}) are shown as insert in figure. PCSs were measured for the protein samples of 0.2 mM ubiquitin G47-T1-Ln in 20 mM MES buffer at pH 6.4 and 298 K.
Fig. S9 Correlations of the experimental PCSs (PCS\text{exp}) and back-calculated PCSs (PCS\text{calc}) using Numbat program. Quality factors (Q=\sqrt{\frac{\sum (\text{PCS}_{i\text{calc}}-\text{PCS}_{i\text{exp}})^2}{\sum (\text{PCS}_{i\text{exp}})^2}}) are shown as insert in figure. PCSs were measured for the protein samples of 0.3 mM GB1 T11C-T1-Ln in 20 mM MES buffer at pH 6.4 and 298 K
Fig. S10 Correlations of the experimental PCSs (PCS\textsubscript{exp}) and back-calculated PCSs (PCS\textsubscript{calc}) using Numbat program. Quality factors (Q=\sqrt{\sum (PCS\textsubscript{calc}-PCS\textsubscript{exp})^2}/\sqrt{\sum (PCS\textsubscript{exp})^2}) are shown as insert in figure. PCSs were measured for the protein samples of 0.2 mM ubiquitin G47C-T2-Tm in 20 mM MES buffer at pH 6.4 and 298 K.
**Fig. S11** Correlations of the experimental PCSs (PCS\(_{\text{exp}}\)) and back-calculated PCSs (PCS\(_{\text{calc}}\)) using Numbat program. Quality factors (Q=\(\sqrt{\sum(\text{PCS}\_{\text{calc}}-\text{PCS}\_{\text{exp}})^2}\)/\(\sqrt{\sum(\text{PCS}\_{\text{exp}})^2}\)) are shown as insert in figure. PCSs were measured for the protein samples of 0.3 mM GB1 T11C-T2-Tm in 20 mM MES buffer at pH 6.4 and 298 K.
Fig. 12 Correlations of the experimental RDCs (RDC\_exp) and back-calculated RDCs (RDC\_calc) using Module program. Quality factors (Q=\sqrt{\sum (RDC\_calc - RDC\_exp)^2}/\sqrt{\sum (RDC\_exp)^2}) are shown as insert in figure. RDCs of backbone amide groups were measured for the protein samples of 0.2 mM ubiquitin G47C-T1-Ln in 20 mM MES buffer at pH 6.4 and 298 K.
Supplementary Reference
