Electronic Supporting Information for

A Chemical "Hub" for Absolute Quantification of a Targeted Protein: Orthogonal Integration of Elemental and Molecular Mass Spectrometry

Xiaowen Yan,[a] Zhaoxin Li,[a] Yong Liang,[a] Liming Yang,[a] Bo Zhang,[a] Qiuquan Wang*[ab]

[a] Department of Chemistry and the MOE Key Laboratory of Spectrochemical Analysis & Instrumentation, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China
[b] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

Materials. (N-Boc-6-azido-L-norleucine)(dicyclohexylammonium) salt, 4-[4-(1-Hydroxyethyl) -2-methoxy-5-nitrophenoxy] butyric acid, *N*-Fmoc-1,4-butanediamine hydrobromide, N.N'-Disuccinimidyl Carbonate (DSC), 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), 6-Heptynoic acid, O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), N,N-Diisopropylethylamine (DIPEA), sodium ascorbate, tris-(benzyltriazolylmethyl)amine (TBTA), copper(II) sulfate ($CuSO_4$) tris(2-carboxyethyl) phosphine (TCEP), 4-methylpiperidine, (+)-Biotin-PEG4-NHS, ribonuclease A (P61823, from bovine pancreas), cytochrome C (P00004, from equine heart), lysozyme (P00698, from chicken egg white), bovine serum albumin (P02769, fraction V), carbonic anhydrase (P00921, from bovine erythrocytes), α-chymotrypsin (P00766, from bovine pancreas, type II, ≥ 85%), insulin (P01317, from bovine pancreas), ovalbumin (P01012, from chicken egg), streptavidin-coated magnetic beads (1 μm, Binding capacity for FITC-Biotin: >600 pmol mg^{-1}), tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 4-morpholinepropanesulfonic acid (MOPS), and ammonium acetate (NH_4Ac) were purchased from Sigma-Aldrich (St. Louis, MO). 1,4,7,10-Tetraazacyclododecane-1,4,7-tris acetic acid-10-(azidopropyl-ethylacetamide) (azido-mono-amide-DOTA) was purchased from Macrocyclics (Dallas, TX). ^{153}Eu-enriched Eu_2O_3 (99.8%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). High-purity Eu_2O_3 was obtained from Changchun Institute of Applied Chemistry (Chinese Academy of Sciences, purity greater than 99.999%). Concentrated HNO_3 and HPLC grade acetonitrile (ACN) were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was prepared in a Milli-Q system (Millipore Filter Co., Bedford, MA) and used throughout this study. All chemicals and reagents were at least of analytical or higher grade and used without further purification.

Instrumentation. Chromatographic separations were carried out on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) using a SHISEIDO C18 column (2.0 I.D. × 150 mm in length) for the separation of synthetic chemicals, and a Zorbax 300SB C18 column (1.0 I.D. × 50 mm in length; particle size, 3.5 μm) for the separation of proteins. The gradient elution program for both columns was as follows: The 95% mobile phase A (0.05% TFA in UPW) was maintained for 5 min, then mobile phase B (0.05% TFA in ACN) was increased from 5 to 80% in 45 min with a flow rate of 0.05 mL min^{-1}. ESI-MS experiments were performed in positive mode on an Esquire-LC ESI ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The operational parameters were as follows: nebulizer, 65 psi; dry gas, 8 L min^{-1}; dry temperature, 300 °C; capillary voltage, -3500 V; endplate offset, -500 V. Inductively coupled plasma mass spectrometry (ICPMS) experiments were performed on an ELAN DRC II ICPMS (PerkinElmer, SCIEX, Canada) equipped with a concentric pneumatic nebulizer and a cyclonic spray chamber. The ICPMS operational parameters were as follows: nebulizer gas, 0.88 L min^{-1}; auxiliary gas, 1.0 L min^{-1}; plasma gas, 15 L min^{-1}; RF power, 1200 W; dwell time, 100 ms; lens voltage, 7.2 V. Parameters such as nebulizer gas flow and lens voltage were optimized daily to obtain the best sensitivity. ^{2}H- and ^{13}C-NMR spectra were collected on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as an internal standard.

Synthesis of 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy] butyric acid-*N*-Fmoc-1,4-butanediamine Derivative (Compound 3). 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy] butyric acid (150 mg) was dissolved in 5 mL of MeOH/ACN (1:1), and then 190 mg of HATU and 150 µL of DIPEA were added to activate the carboxyl group for 10 min. *N*-Fmoc-1,4-butanediamine (195 mg) was then added into the mixture at 0 °C. The mixture was stirred at room temperature for 3 h. The resulting product (SF-alkyne) was further purified using HPLC (Figure S1a) and confirmed using ESI-MS m/z: 592.1 (Figure S1b); ^{1}H NMR (DMSO-d_6) δ ppm (Figure S2): δ 8.17 (s, 1H), 7.89 (d, J = 7.5 Hz, 2H), 7.85 (t, J = 5.6 Hz, 1H), 7.68 (d, J = 7.4 Hz, 2H), 7.52 (s, 1H), 7.41 (t, J = 7.3 Hz, 1H), 7.36 (s, 1H), 7.35 (d, J = 1.2 Hz, 1H), 7.33 (d, J = 1.2 Hz, 1H), 7.28 (t, J = 5.7 Hz, 1H), 5.48 (d, J = 4.5 Hz, 1H), 5.31 – 5.20 (m, 1H), 4.29 (d, J = 7.0 Hz, 2H), 4.20 (t, J = 6.9 Hz, 1H), 4.03 (dd, J = 8.5, 4.4 Hz, 1H), 3.90 (s, 1H), 2.69 (s, 1H), 2.23 (t, J = 7.4 Hz, 2H), 1.99 – 1.90 (m, 2H), 1.38 (s, 2H), 1.36 (s, 1H), 1.28 (s, 1H), 1.26 (s, 2H), 1.25 (s, 1H), 1.23 (s, 1H); and ^{13}C NMR (DMSO- d_6) δ ppm (Figure S3): δ 171.90, 171.73, 153.87, 146.71, 143.03, 139.87, 139.35, 138.44, 137.88, 129.40, 127.76, 121.85, 120.49, 110.22, 109.55, 108.78, 68.73, 64.37, 56.52, 38.70, 38.30, 32.09, 27.59, 26.97, 26.68, 25.65, 25.43, 25.18, 20.69.

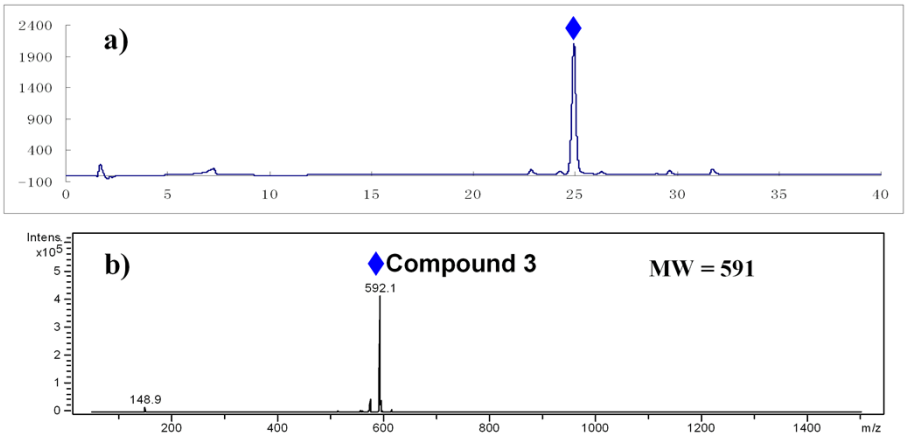

Figure S1. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of purified compound **3**.

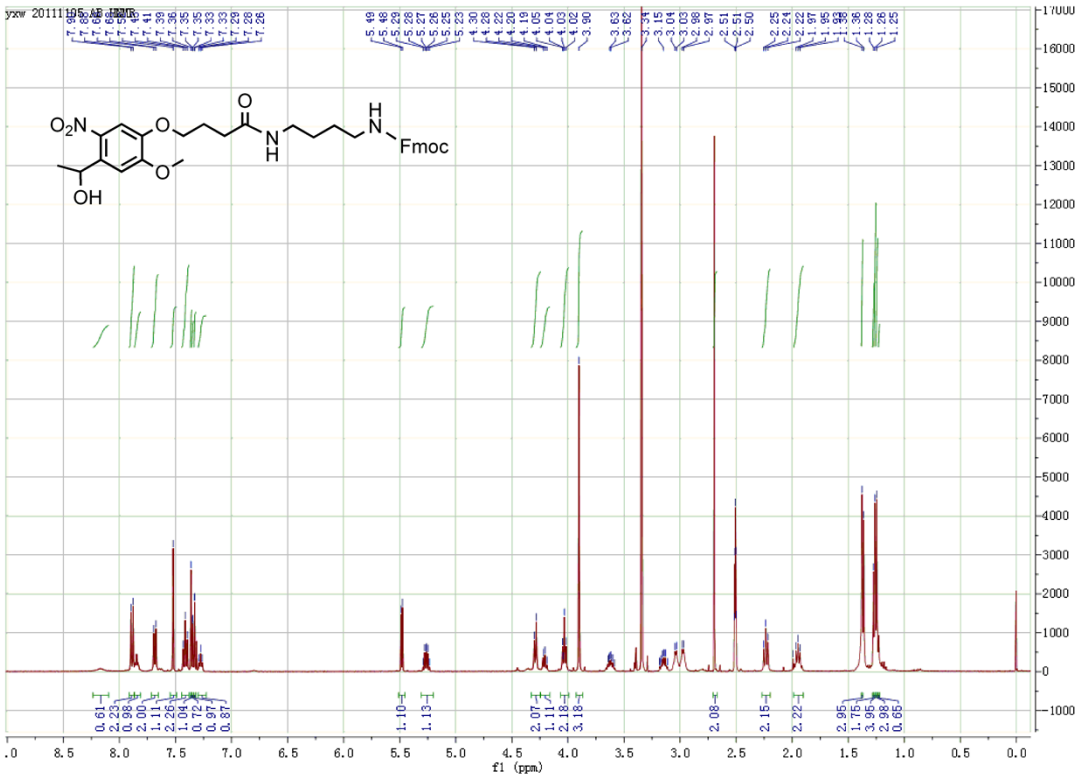

Figure S2. ^{1}H-NMR spectrum of compound **3** in DMSO-D_6.

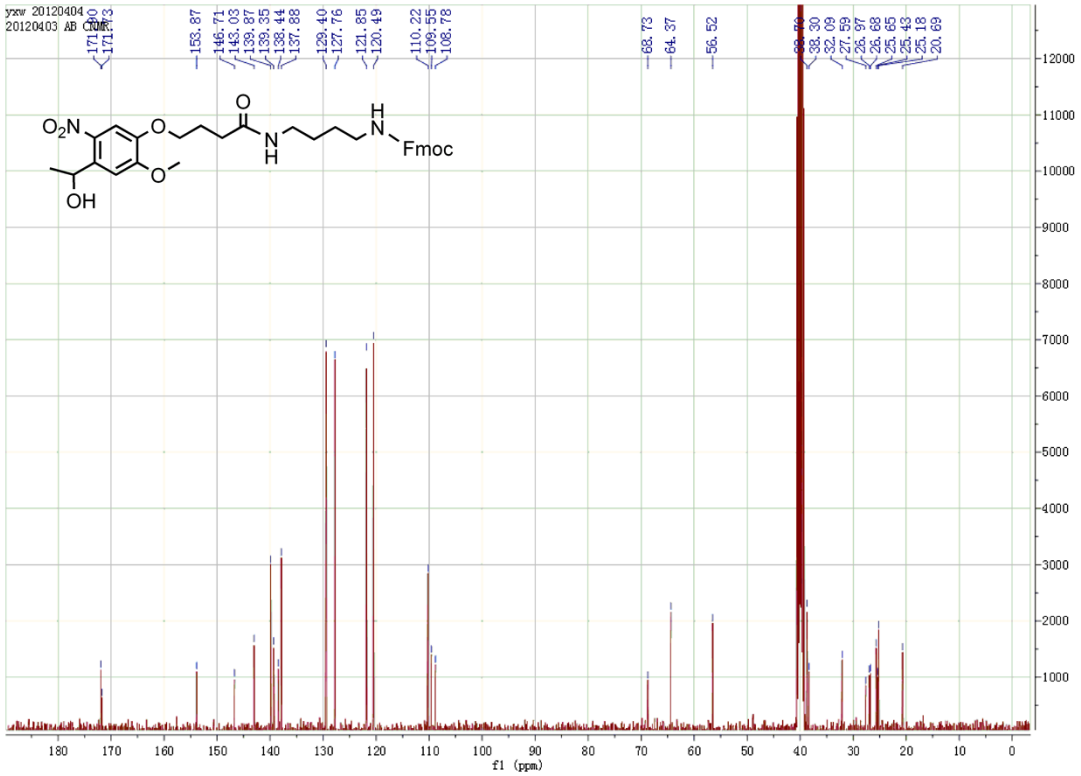

Figure S3. ^{13}C-NMR spectrum of compound **3** in DMSO-D_6.

Synthesis of photocleavable linker Fmoc-Nitrobenzyl-NHS (Compound 5). Compound **3** (96 mg) was mixed with DSC (328 mg) in 4 mL of ACN and 0.8 mL DMSO (1:1), and then 400 µL of DIPEA added. The mixture was stirred at room temperature for 2.5 h. The resulting product was further purified using HPLC (Figure S4a), and then lyophilized, and confirmed using ESI-MS m/z: 732.9 (Figure S4b); ^{1}H NMR (CD_3CN) δ ppm (Figure S5): δ 7.77 (d, J = 7.5 Hz, 2H), 7.60 (d, J = 7.4 Hz, 2H), 7.53 (s, 1H), 7.36 (d, J = 7.4 Hz, 2H), 7.30 (d, J = 7.3 Hz, 2H), 7.10 (s, 1H), 6.56 (s, 1H), 6.33 (d, J = 6.4 Hz, 1H), 5.70 (s, 1H), 4.27 (d, J = 6.8 Hz, 2H), 4.17 (d, J = 6.4 Hz, 1H), 4.02 (s, 2H), 3.93 (s, 3H), 3.04 (s, 2H), 2.27 (s, 2H), 2.00 (s, 2H), 1.93 – 1.89 (m, 1H), 1.70 (d, J = 6.4 Hz, 3H), 1.41 (s, 4H); and ^{13}C NMR (CD_3CN) δ ppm (Figure S6): δ 169.67, 154.27, 150.85, 147.87, 144.27, 141.15, 139.98, 130.12, 127.66, 127.09, 125.15, 119.97, 108.87, 108.17, 76.06, 68.55, 65.82, 56.23, 47.20, 40.14, 38.63, 26.90, 26.39, 25.27, 24.83, 20.61.

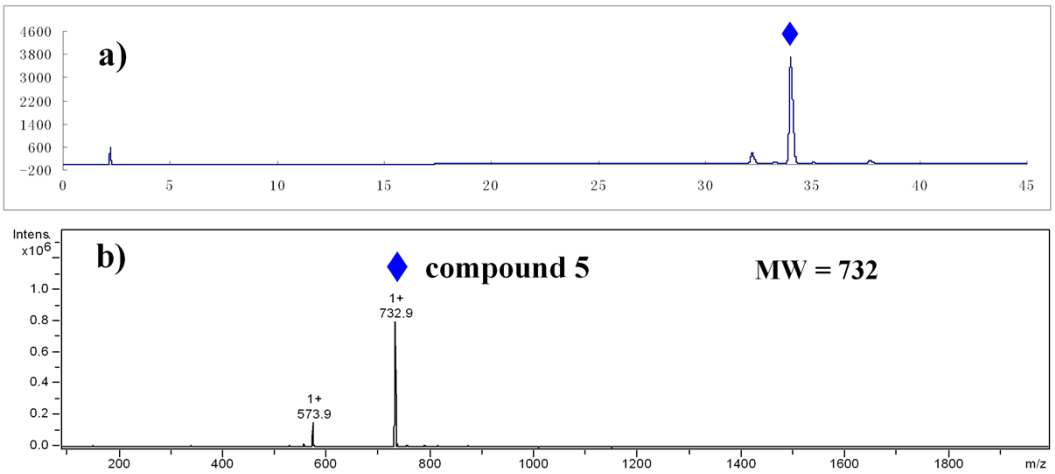

Figure S4. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of purified compound **5**.

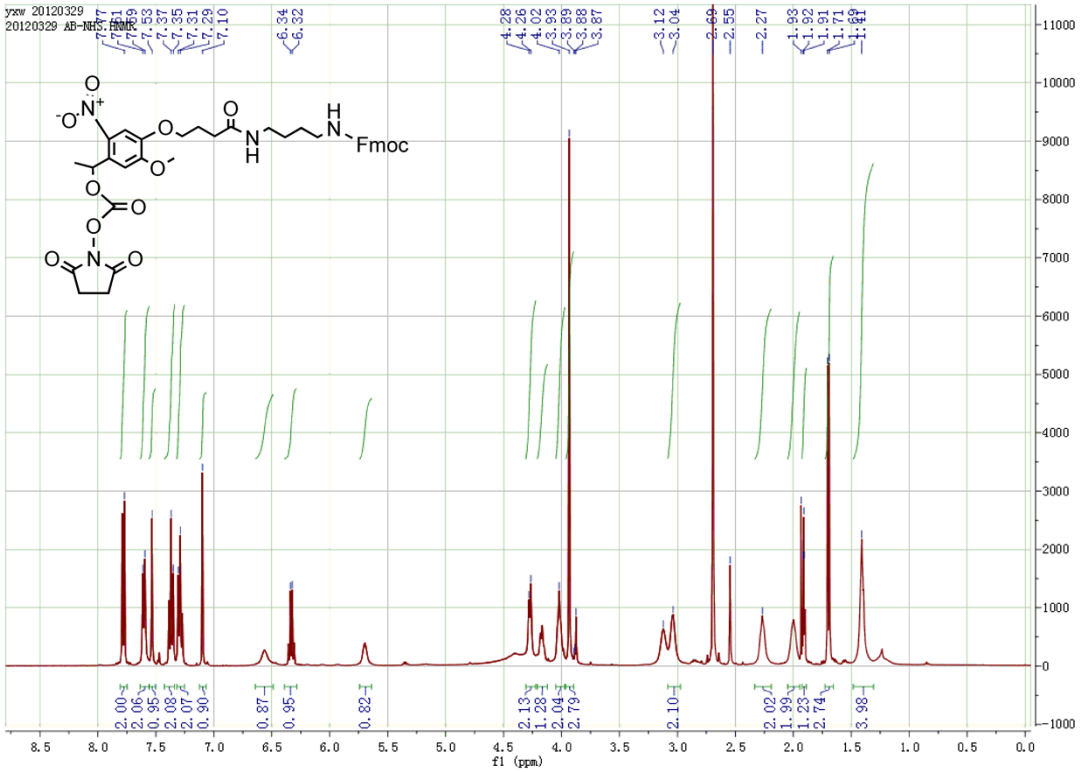

Figure S5. ^{1}H-NMR spectrum of compound **5** in CD_3CN.

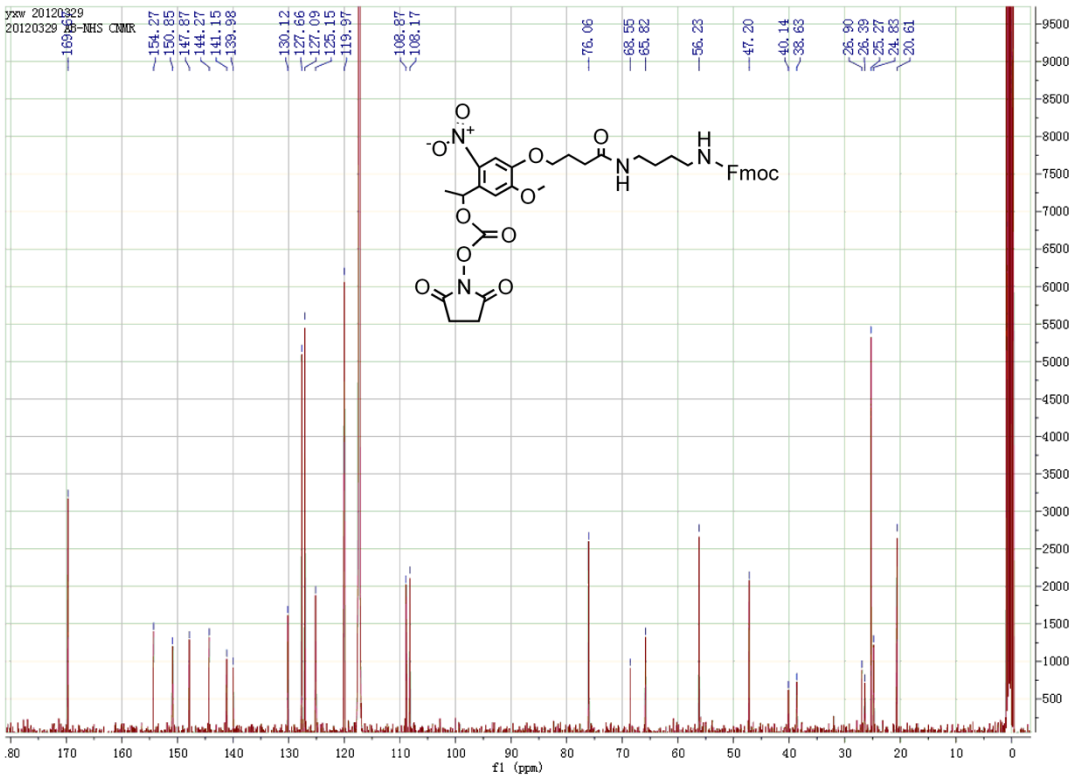

Figure S6. ^{13}C-NMR spectrum of compound **5** in CD_3CN.

Synthesis of N-Boc-6-azido-L-norleucine-*N*-Fmoc-1,4-butanediamine Derivative (Compound 7). (N-Boc-6-azido-L-norleucine)(dicyclohexylammonium) salt (180 mg) was dissolved in 5 mL of ACN, and then 152 mg of HATU and 0.8 mL of DIPEA were added to activate the carboxyl group for 10 min. *N*-Fmoc-1,4-butanediamine (156 mg) was then added into the mixture at 0 °C. The mixture was stirred at room temperature for 3 h. The resulting product was further purified using silica chromatography (Ethyl Acetate: Petroleum Either = 7:3) (Figure S7a) and confirmed using ESI-MS m/z: 565.2 (Figure S7b); ^{1}H NMR (DMSO-d_6) δ ppm (Figure S8): δ 7.89 (d, J = 7.5 Hz, 2H), 7.79 (t, J = 5.5 Hz, 1H), 7.69 (d, J = 7.4 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.37 – 7.30 (m, 2H), 7.28 (t, J = 5.5 Hz, 1H), 6.76 (d, J = 8.2 Hz, 1H), 4.29 (d, J = 6.9 Hz, 2H), 4.21 (t, J = 6.8 Hz, 1H), 3.85 (dd, J = 13.7, 8.4 Hz, 1H), 3.29 (t, J = 6.8 Hz, 2H), 2.98 (d, J = 5.4 Hz, 2H), 1.54 – 1.44 (m, 4H), 1.37 (s, 13H); and ^{13}C NMR (DMSO- d_6) δ ppm (Figure S9): δ 172.27, 156.55, 155.73, 144.40, 141.20, 128.05, 127.51, 125.60, 120.57, 78.40, 65.64, 54.53, 51.02, 47.25, 38.62, 32.12, 28.64, 28.37, 27.21, 26.83, 23.19.

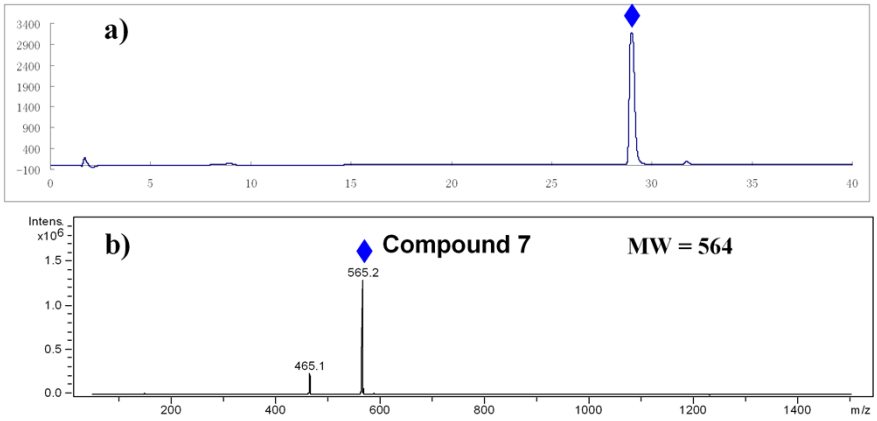

Figure S7. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of purified compound **7**.

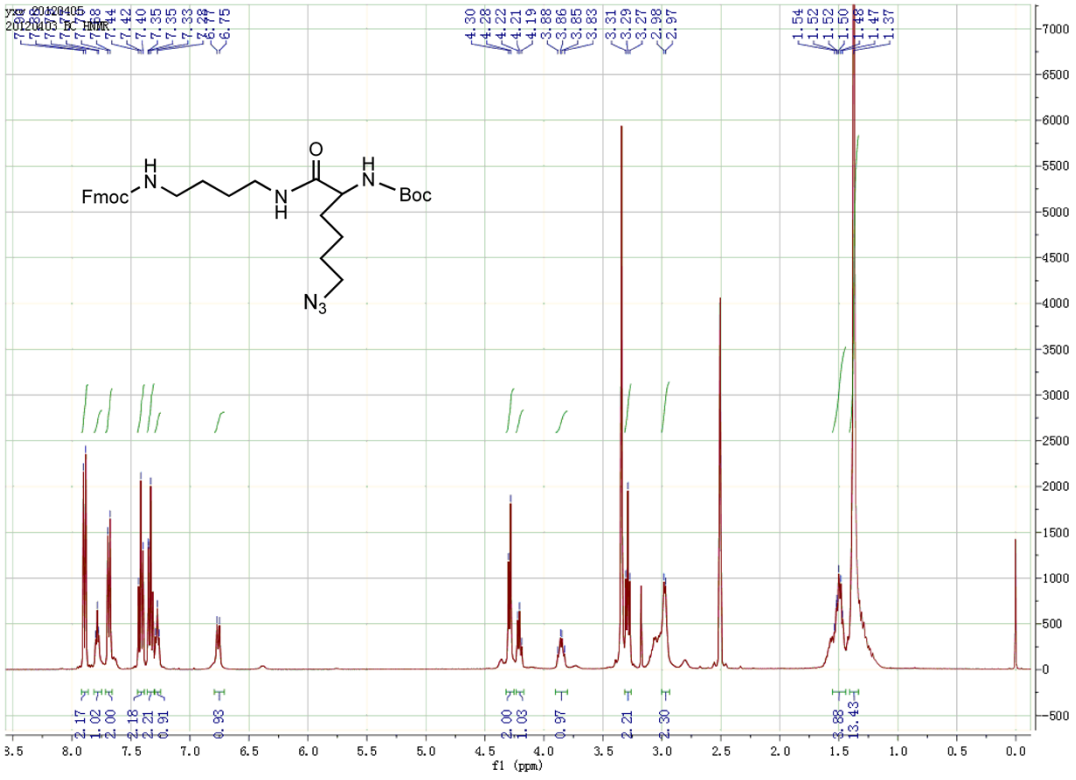

Figure S8. ^{1}H-NMR spectrum of compound **7** in DMSO-d_6.

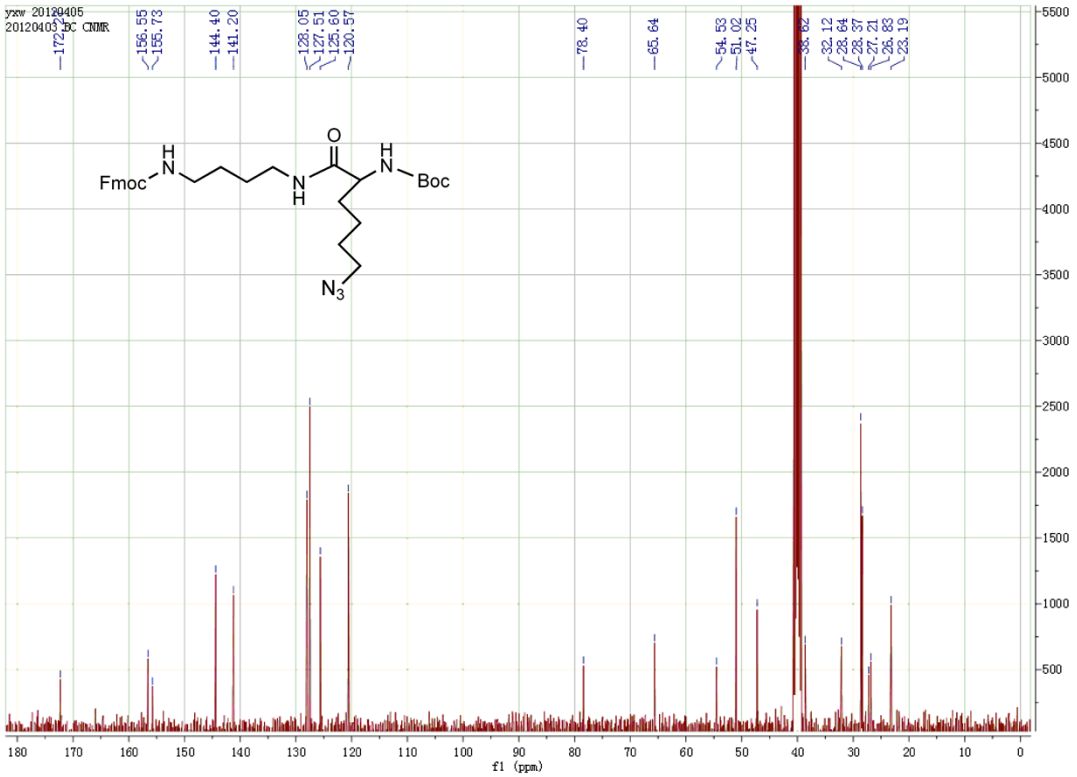

Figure S9. ^{13}C-NMR spectrum of compound **7** in DMSO-d_6.

Synthesis of amine-DOTA-Azide (Compound 10). Boc protection of Compound **7** was removed in TFA to give compound **8** with an m/z of 465.0. Then compound **8** (46.4 mg) was mixed with DOTA-NHS in 2 mL of MeOH/ACN (1/1), and then 100 µL of DIPEA were added. The mixture was stirred at room temperature overnight. 4-methylpiperidine (0.5 mL) was finally added to remove the Fmoc protection. The resulting product was further purified using HPLC (Figure S10a) and confirmed using ESI-MS m/z: 565.2 (Figure S10b); ^{1}H NMR (CD_3CN) (Figure S11) and ^{13}C NMR (CD_3CN) δ ppm (Figure S12): δ 160.46, 160.12, 118.38, 115.46, 54.16, 50.92, 48.56, 48.35, 48.14, 47.92, 47.71, 47.50, 47.29, 42.33, 30.80, 28.10, 22.88, 17.58, 16.31, 11.62, 9.42, 8.77.

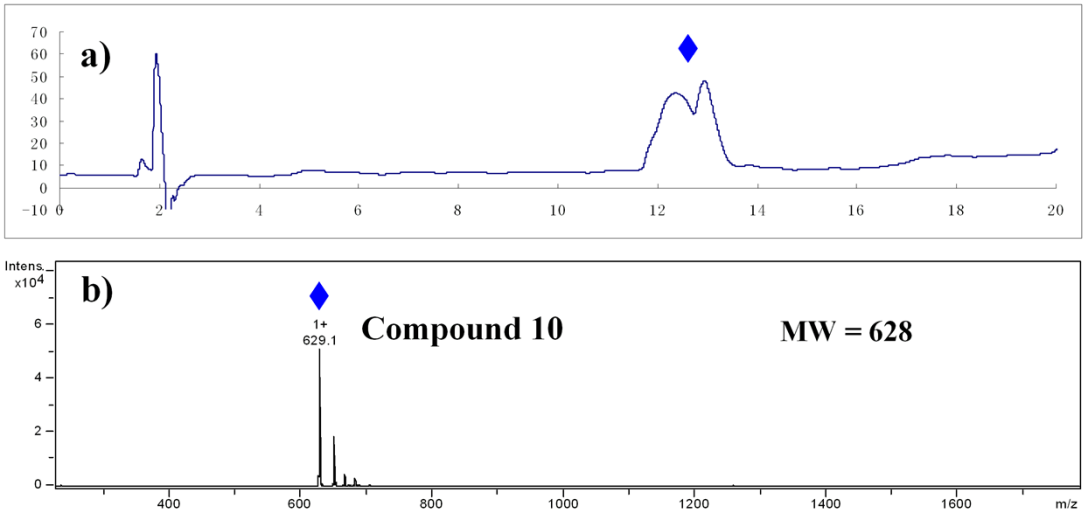

Figure S10. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of purified compound 10.

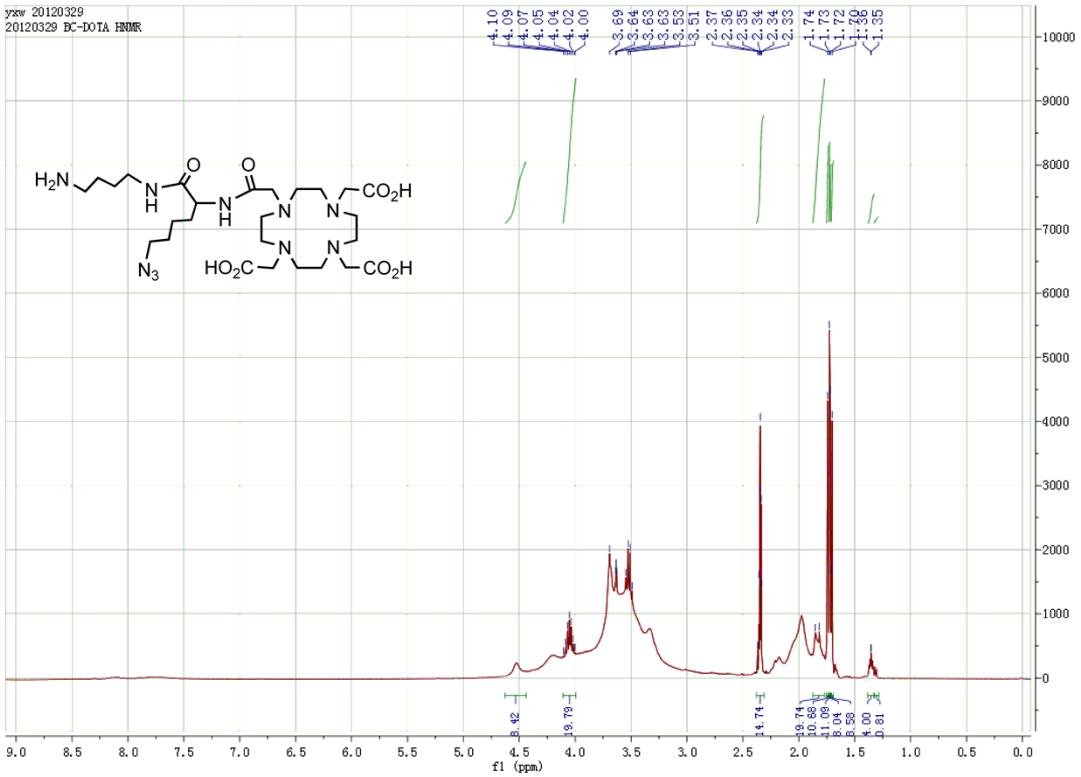

Figure S11. ^{1}H-NMR spectrum of compound **10** in CD_3CN.

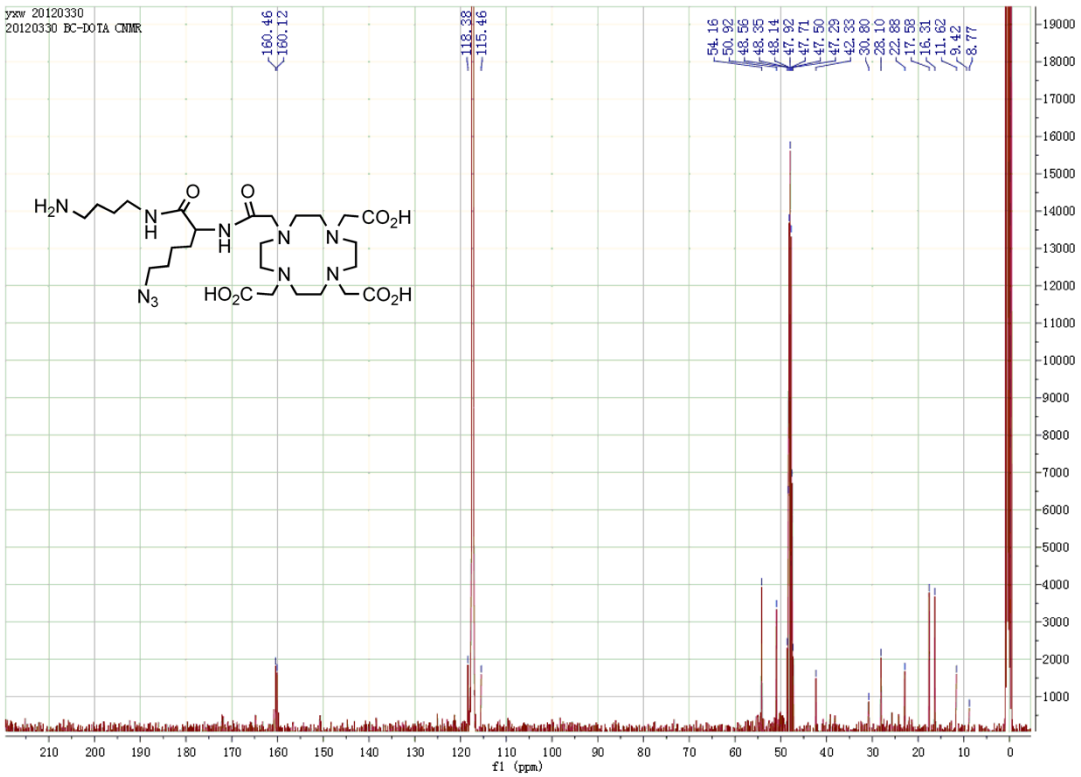

Figure S12. ^{13}C-NMR spectrum of compound **10** in CD_3CN.

Synthesis of Fmoc-Nitrobenzyl-DOTA-Azide (Compound 11). Compound **5** (29.2 mg) and compound **10** (25.0 mg) were dissolved in 4 mL of MeOH/ACN (1:1), and then DIPEA (200 µL) was added. The mixture was stirred at room temperature for 3 h. The resulting product was further purified using HPLC (Figure S13a) and confirmed using ESIMS m/z: 565.2 (Figure

S13b); ^{1}H NMR (DMSO-d_6) δ ppm (Figure S14): δ 8.73 (s, 1H), 8.09 (s, 1H), 7.88 (d, J = 7.4 Hz, 2H), 7.68 (d, J = 7.4 Hz, 2H), 7.56 (s, 1H), 7.43 (s, 1H), 7.40 (d, J = 7.1 Hz, 2H), 7.34 (d, J = 7.3 Hz, 2H), 7.30 (d, J = 8.8 Hz, 1H), 7.11 (S, 1H), 6.11 (q, J = 6.1 Hz, 1H), 4.29 (d, J = 6.8 Hz, 2H), 4.21 (d, J = 6.6 Hz, 2H), 4.08 – 4.01 (m, 3H), 3.90 (s, 3H), 3.47 (M, 12H), 3.30 (t, J = 6.6 Hz, 2H), 3.17 (s, 1H), 3.15 – 2.85 (m, 16H), 2.49 (t, J = 10.8 Hz, 4H), 2.23 (t, J = 7.3 Hz, 2H), 2.00 – 1.86 (m, 2H), 1.52 (d, J = 6.3 Hz, 6H), 1.42 – 1.28 (m, 8H); and ^{13}C NMR (DMSO- d_6) δ ppm (Figure S15): δ 171.72, 170.92, 156.56, 155.63, 154.03, 147.24, 144.40, 141.19, 139.74, 133.84, 128.05, 127.50, 125.60, 120.56, 108.93, 68.84, 67.47, 65.62, 56.63, 53.33, 51.14, 50.97, 47.24, 38.68, 32.38, 32.05, 28.35, 27.34, 26.93, 26.72, 25.18, 22.94, 22.33.

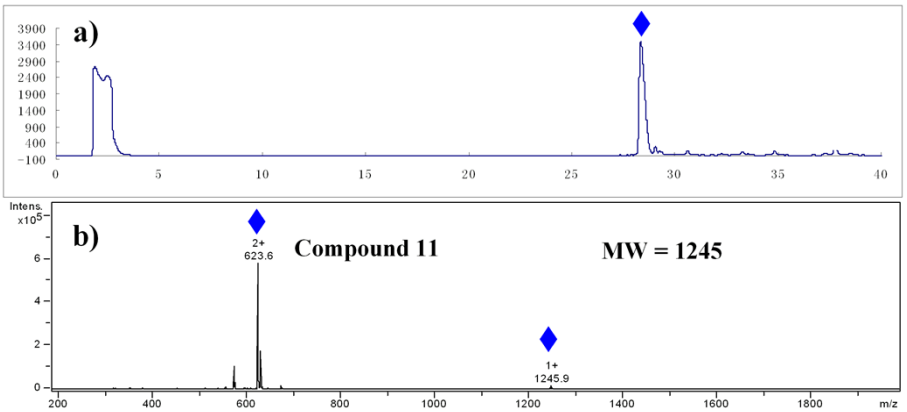

Figure S13. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of purified compound **11**.

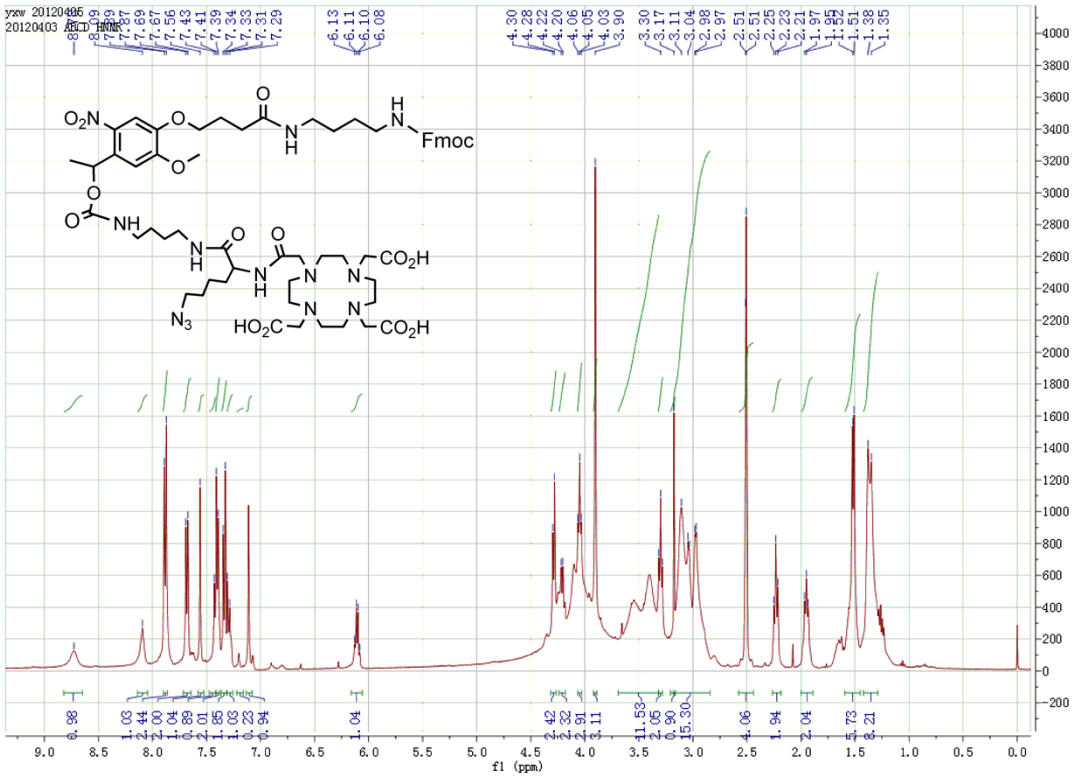

Figure S14. ^{1}H-NMR spectrum of compound **11** in DMSO-d_6.

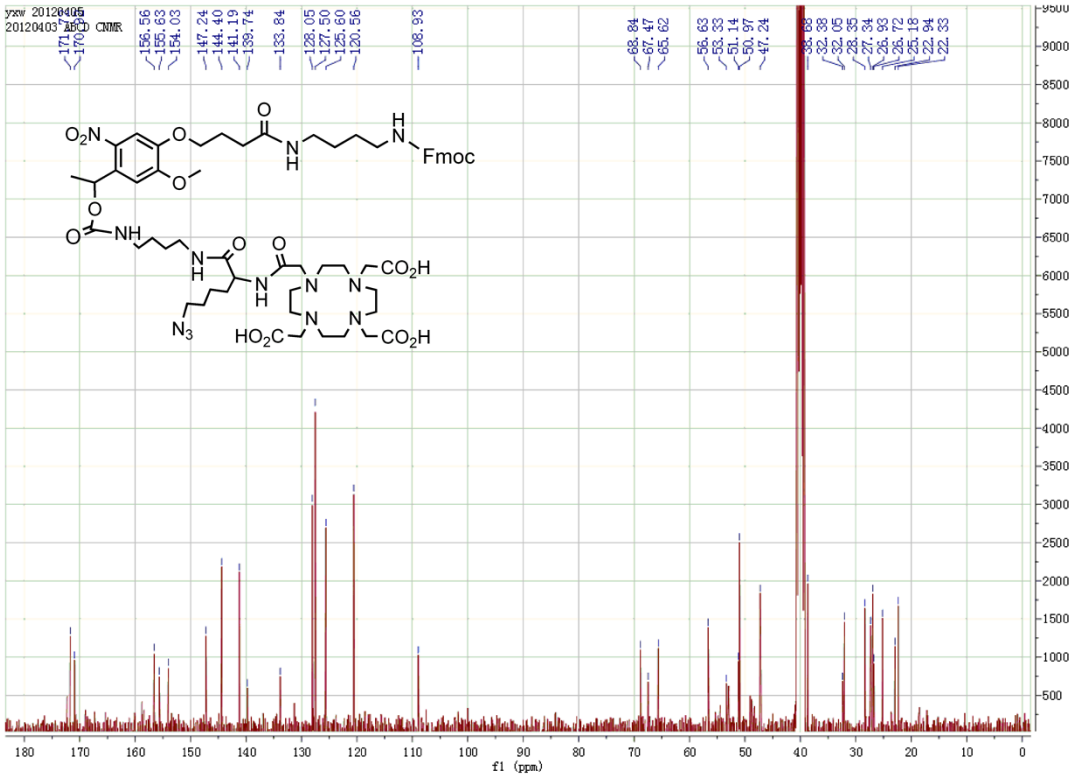

Figure S15. ^{13}C-NMR spectrum of compound **11** in DMSO-d_6.

Synthesis of Nitrobenzyl-DOTA-Azide (Compound 11-Fmoc), Biotin-Nitrobenzyl-DOTA- Azide (Compound 12), Biotin-Nitrobenzyl-DOTA-Eu-Azide (Compound 12-Eu) and Biotin-PEG-Nitrobenzyl-DOTA-Eu-SF (Compound 14). Fmoc group of Compound **11** (23.6 mg) was quantitatively removed using 4-methylpiperidine, and the product (Compound **11**-Fmoc) was analyzed using HPLC-UV (Figure S16a) and ESI-MS m/z: 1024.2 (Figure S16b). To synthesize Biotin-Nitrobenzyl-DOTA-Azide (Compound **12**), Compound **11**-Fmoc (20.5 mg) was mixed with (+)-Biotin-PEG4-NHS (58.9 mg) in 2 mL MeOH/DMSO (3:1), then 0.5 mL of DIPEA was added. The mixture was shaken at room temperature for 3 h. Compound **12** was purified with HPLC and confirmed using ESI-MS m/z: 1498 (Figure S17a). 0.2 mL of Compound **12** (18 mM) was mixed with 0.4 mL of $Eu(NO_3)_3$ solution (10 mM in 2 % HNO_3), 2 mL of 100 mM MES buffer (pH = 6.0) and 1 mL ACN. Compound **12**-Eu was confirmed using ESI-MS m/z: 1646 (Figure S17b). To synthesize Biotin-PEG-Nitrobenzyl-DOTA-Eu-SF (Compound **14**), 1.44 mL of compound **12**-Eu (1 mM) was mixed with 136 µL of compound **13** (40 mM in DMSO, synthesized as described in Figure S18), 42 µL TBTA (20 mM in t-butanol), 168 µL $CuSO_4$ (50 mM in 100 mM, pH = 7.5 Tris-HCl) and 282 µL ascorbate (150 mM in H_2O) in 1.68 mL of 100 mM Tris-HCl (pH = 9.0) with 1.2 mL t-butanol for 1h at room temperature. The pH value of the reaction was adjusted to 6.0 with TFA to prevent the hydrolysis of the sulfonyl fluoride group in compound **14**. The final product of compound **14** was purified using HPLC (Figure 2a), freeze-dried and confirmed using ESIMS m/z: 1957 (Figure 2c).

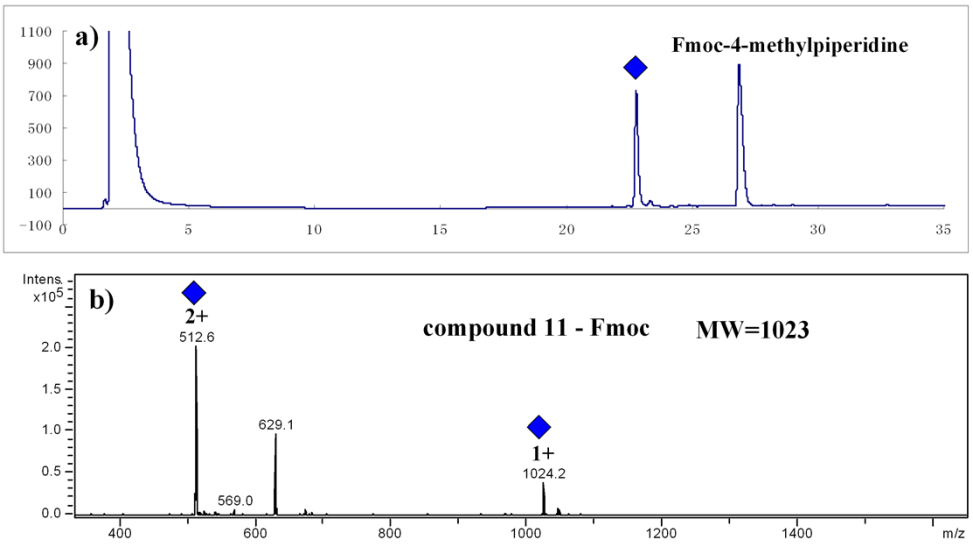

Figure S16. (a) HPLC-UV (214 nm) chromatogram and (b) ESI-MS spectrum of Fmoc-deprotected compound **11**.

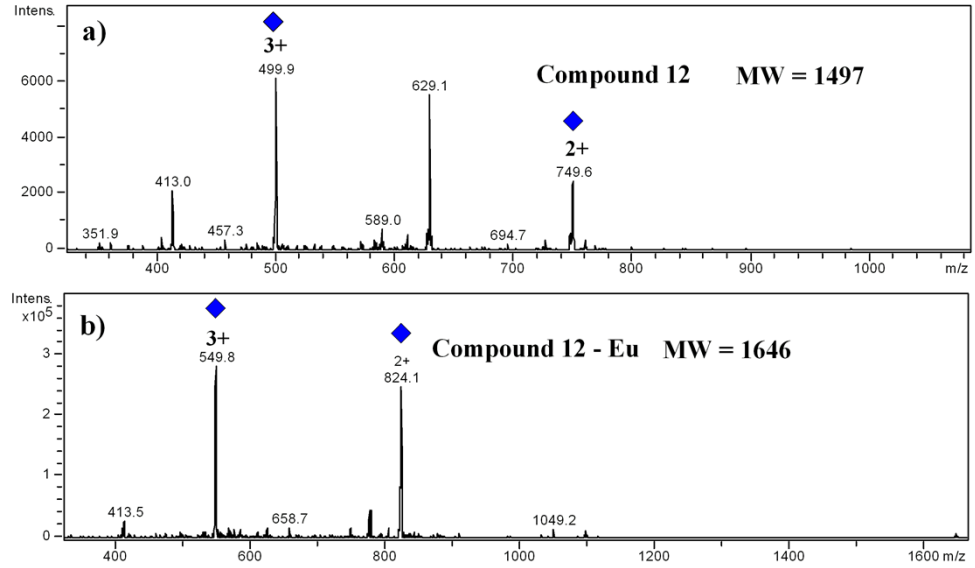

Figure S17. ESI-MS spectrum of (a) compound **11** and (b) Eu-loaded compound **11**.

Synthesis of AEBSF-6-Heptynoic acid Derivative (Compound 13). Briefly, 6-Heptynoic acid (126 µL) was dissolved in 5 mL of N, N-Dimethylformamide (DMF), and then 380 mg of HATU and 300 µL of DIPEA were added to activate the carboxyl group of 6-Heptynoic acid for 10 min. 203 mg of AEBSF was then added into the mixture at 0 °C. The mixture was stirred at room temperature for 3 h. The resulting product was purified using HPLC.

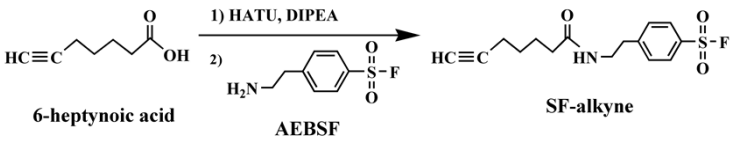

Figure S18. Synthesis of AEBSF-6-Heptynoic acid Derivative (Compound **13**).

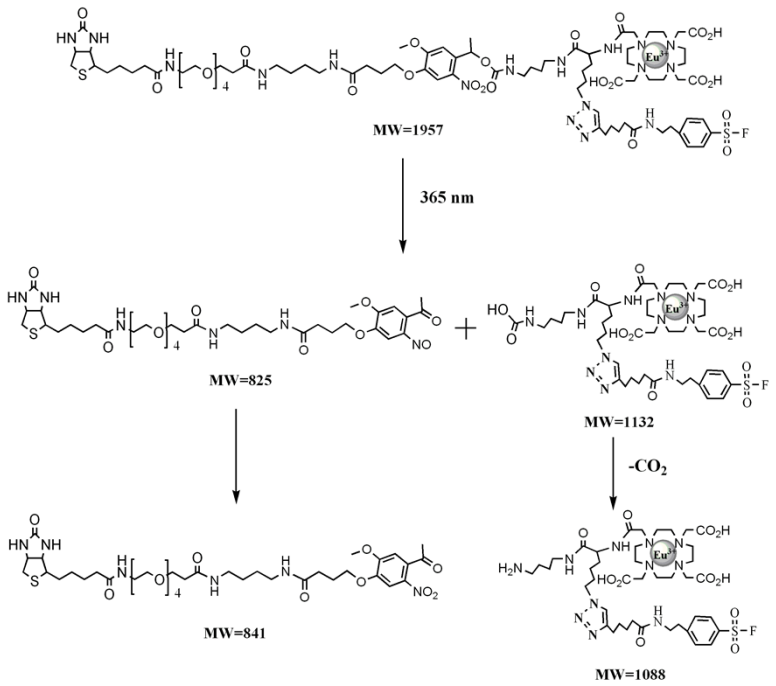

Figure S19. Photocleavage products of tetrafunctional probe **14** and their theoretical molecular weights (TM) under UV irradiation based on the photocleavage mechanism of o-nitrobenzyl ether compound (Ref. S1-S2).

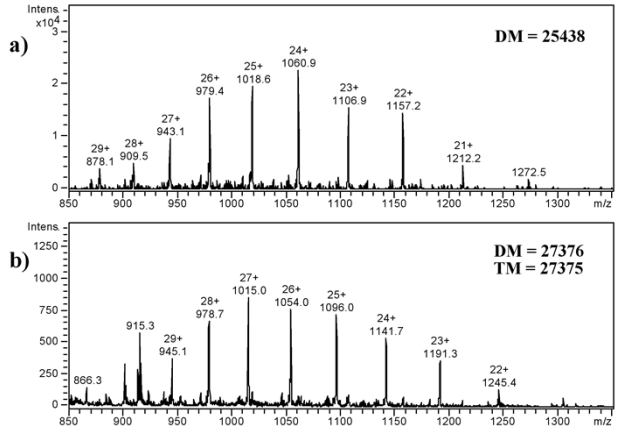

Figure S20. Mass spectra of (a) intact chymotrypsin and (b) **14**-tagged chymotrypsin. DM and TM in the mass spectra denote devolution and theoretical molecular weights.

Table S1. The model peptides/proteins used.

No.	Name[a]	Number of serine residues	Molecular weight (KDa)
1	RNase A	15	13.7
2	insulin	3	5.7
3	Cyt C	0	12.3
4	lysozyme	10	14.3
5	BSA	28	66.4
6	chymotrypsin	26	25.4
7	carbonic anhydrase	30	29.0
8	ovalbumin	38	42.7

[a] The SWISS-PROT numbers for the proteins are indicated in Materials Section.

References

[S1] J. E. Corrie, A. Barth, V. R. Munasinghe, D. R. Trentham, M. C. Hutter, *J. Am. Chem. Soc.,* 2003, **125**, 8546-8554.

[S2] Y. V. Il'ichev, M. A. Schwörer, J. Wirz, *J. Am. Chem. Soc.,* 2004, **126**, 4581-4595.