Supporting Information

A terbium(III) complex-based time-resolved luminescence probe for selenocysteine as an inhibitor of selenoproteins

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1. Experimental Section

1.1. Reagents and Materials

Common reagents used in the experiments were all of analytical grade and purchased from commercial suppliers unless otherwise stated. Diethylenetriaminepentaacetic acid dianhydride (DTPAA) was purchased from Acros Organics. The stock solution of TbL4 (4 mM) was obtained by dissolving the compound in DMSO and filtered using a 0.22 μM filter (organic system). All aqueous solutions and buffers were obtained using Milli-Q water, and filtered through a 0.22 μM filter (Millipore) before use. Human ovarian cancer cell line A2780, breast adenocarcinoma cell line MDA-MB-231, human normal ovarian epithelial cell line IOSE80, and human normal liver cell line L02 were obtained from American Type Culture Collection (ATCC).

1.2. Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer. Electrospray ionization mass spectra (ESI-MS) were acquired on an LCQ Fleet electrospray mass spectrometer. Time-resolved luminescence spectra were recorded on a PerkinElmer FL6500 fluorescence spectrometer. MTT and protein assays were quantified by using a PerkinElmer Fusion Reader.

1.3. Synthetic procedures

*Synthesis of tert-butyl (4-aminophenyl)carbamate (**1**).* *p*-phenylenediamine (5.5 mmol, 0.595 g) was added to dichloromethane (DCM, 20 mL) in a round bottom flask under nitrogen atmosphere and cooled to 0 ℃. A solution of di-tert-butyl dicarbonate (2.1 mmol, 0.460 g) in DCM (10 mL) was slowly added to the reaction flask and stirred for 30 min. Then the reaction was allowed to warm to room temperature and stirred for an additional 4 h. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (DCM : petroleum ether = 1 : 4) to obtain a brownish product (0.385 g, yield: 88%). ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.13 (d, J = 8 Hz, 2H, benzene), 6.63 (d, J = 8.8 Hz, 2H, benzene), 1.50 (s, 9H, methyl). ^{13}C NMR ($CDCl_3$, 100 MHz, δ, ppm): 28.42, 80.02, 115.63, 120.94, 129.73, 142.40, 153.39. ESI-MS found (calcd) for $C_{11}H_{16}N_2NaO_2$ (m/z): 231.1102 (231.1109) $[M+Na]^+$.

*Synthesis of tert-butyl (4-((2,4-dinitrophenyl)sulfonamido)phenyl)carbamate (**2**).* Compound **1** (2.00 mmol, 0.416 g) and 2,4-dinitrobenzenesulfonyl chloride (2.00 mmol, 0.532 g) were dissolved in 20 mL of DCM. Pyridine (350 mL, 4.36 mol) was slowly added to the reaction mixture at 0 ℃ and stirred under nitrogen atmosphere. After 30 min, the reaction mixture was allowed to warm to room temperature and stirred for an additional 4 h. The solvent was evaporated under reduced pressure and the resulting product was purified by column chromatography (petroleum ether : ethyl acetate = 2 : 1) to obtain an orange powder (0.350 g, yield: 40%). ^{1}H NMR (DMSO-d_6, 400 MHz, δ, ppm): 10.75 (s, 1H, SO_2NH), 9.38 (s, 1H, CNHCOO), 8.88 (s, 1H, benzene), 8.58 (d, J = 8.8 Hz, 1H , benzene), 8.13 (d, J = 8.4 Hz, 1H, benzene), 7.36 (d, J = 8.8 Hz, 2H, benzene), 7.01 (d, J = 8.8 Hz, 2H, benzene), 1.44 (s, 9H, methyl). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, ppm): 28.53, 79.62, 119.25, 120.64, 123.79, 127.53, 129.88, 132.17, 136.74, 138.03, 148.31, 150.41, 153.16. ESI-MS found (calcd) for $C_{17}H_{18}N_4NaO_8S$ (m/z): 461.0731 (461.0743) $[M + Na]^+$.

*Synthesis of N-(4-aminophenyl)-2,4-dinitrobenzenesulfonamide (**3**).* Compound **2** (1.00 mmol, 0.438 g) was dissolved in 20 ml of methanol solution in a reaction flask. 10 mL of concentrated hydrochloric acid was added dropwise and stirred at 0 ℃ under nitrogen atmosphere. After 15 min, the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction solution was washed with saturated

Na_2CO_3 and the pH was adjusted to neutral. The resulting mixture was extracted three times with DCM, dried over magnesium sulfate, and filtered. The solvent was removed under reduced pressure to yield a yellow product (0.305 g, yield: 90%). ^{1}H NMR (DMSO-d_6, 400 MHz, δ, ppm): 10.25 (s, 1H, SO_2NH), 8.86 (d, J = 2.4 Hz, 1H, benzene), 8.57 (dd, J = 8.7, 2.3 Hz, 1H, benzene) , 8.06 (d, J = 8.8 Hz, 1H, benzene), 6.75 (d, J = 8.8 Hz, 2H, benzene), 6.43 (d, J = 8.8 Hz, 2H, benzene), 5.15 (s, 2H, amine). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, ppm): 114.56, 120.48, 123.40, 126.27, 127.30, 132.28, 137.19, 148.01, 148.30, 150.24. ESI-MS found (calcd) for $C_{12}H_{11}N_4O_6S$ (m/z): 339.0381 (339.0399) $[M + H]^+$.

*Synthesis of 2,2'-((((carboxymethyl)azanediyl)bis(ethane-2,1-diyl))bis((2-((4-((2,4-dinitrophenyl)sulfonamido)phenyl)amino)-2-oxoethyl)azanediyl))diacetic acid (**L4-3H**).* Compound **3** (2.00 mmol, 0.676 g) in DMF (20 mL) was added dropwise to a mixture of DTPAA (0.994 mmol, 0.355 g) and triethylamine (4 mL) in DMF (10 mL) and stirred under nitrogen atmosphere at 0 ºC. After 2 h, the reaction mixture was heated to 40 ºC and stirred for an additional 48 h. The reaction was quenched with water (50 mL) and the resulting solution was concentrated to 2 mL. The product was precipitated by adding acetone (50 mL) to the above-mentioned residue. The precipitate was filtered, washed with anhydrous chloroform and diethyl ether, and dried under vacuum to afford the product as a pale yellow powder (0.719 g, yield: 70%). ^{1}H NMR (DMSO-d_6, 400 MHz, δ, ppm): 8.81 (s, 2H, benzene), 8.54 (d, J = 7.9 Hz, 2H, benzene) , 8.15 (d, J = 9.8 Hz, 2H, benzene), 7.53 (s, 4H, benzene), 7.02 (d, J = 8.9 Hz, 4H, benzene), 3.38 (s, 18H, methylene, overlapped with H_2O peak). ESI-MS found (calcd) for $C_{38}H_{38}KN_{11}NaO_{20}S_2$ and $C_{38}H_{37}KN_{11}Na_2O_{20}S_2$ (m/z) : 1094.17 (1094.13) $[M + K + Na - H]^+$, 1116.17 (1116.11) $[M + K + 2Na - 2H]^+$.

*Synthesis of Tb(L4)(H_2O) (**TbL4**).* To a solution of **L4-3H** (0.0996 mmol, 0.103 g) dissolved in 10 mL of water (pH 6), 4 mL of aqueous solution of terbium nitrate hexahydrate (0.099 mmol, 0.045 g) was slowly added. Then the reaction mixture was heated to 45 °C, stirred for 12 h, and the solvent was evaporated. The residue was dissolved in distilled water (2 mL). 50 ml of acetone was added to the solution to yield the precipitate. The precipitate was isolated through centrifugation and dried in vacuum

to afford the product (dark brown solid, 53.8 mg, yield 45%). ESI-MS found (calcd) for $C_{38}H_{36}N_{11}NaO_{20}S_2Tb$ (m/z): 1212.25 (1212.07) $[M + Na - H_2O]^+$.

1.4. Time-resolved luminescent recognition of benzenemethaneselenol (BzSeH)

The solution of BzSeH was freshly prepared each time before use by the pre-incubation of equal molar amount of dibenzyldiselenide (DBDS) (1 mM) with dithiothreitol (DTT) (1 mM) in buffer solutions at 37 ℃ for 5 min. The concentration-dependent emission spectra of TbL4 towards BzSeH were acquired by incubating TbL4 (20 μM) with increasing concentration of BzSeH (0 to 60 μM) at 37 ℃ for 0.5 h in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). The time-dependent emission spectra (λ_{ex} = 260 nm) of TbL4 in response to BzSeH were obtained with the following procedures: TbL4 (20 μM) was incubated in the presence of BzSeH (50 μM) at 37 ℃ in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). The emission spectra of TbL4 were recorded at different time intervals. The pseudo-first-order rate constants (k_{obs}) were obtained from slope of the plot of $\ln[(F_{max}-F_t)/F_{max}]$ *vs* time. F_{max} is maximum luminescence intensity during the measurement time, and F_t are the luminescence intensities at different time points. The pH-dependence of TbL4 (20 μM) emissions towards BzSeH (30 μM) were measured after the coincubation in Tris-HCl buffer of different pH at 37 ℃ for 0.5 h (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO).

The detection limit (3σ/slope) of TbL4 for BzSeH was determined by the reported method.[1] The time-resolved luminescence spectra of TbL4 (20 μM, λ_{ex} = 260 nm) in the absence of BzSeH in buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4) were collected for 20 times to determine the background noise σ. The time-resolved luminescence spectra of TbL4 upon addition of BzSeH at various concentration were measured. A linear regression curve was fitted according to the emission intensity at 546 nm in the range of 0 − 90 μM, and the slope of curve was obtained using Origin 8.5.

The selectivity of TbL4 towards different analytes was investigated under the following conditions. The emission intensity ratio (I/I_0) at 546 nm (λ_{ex} = 260 nm) was determined after mixing TbL4 (20 μM) with various biorelated molecules at 37 ℃ for

0.5 h in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). The concentrations of all biorelated molecules are as follows: [amino acids] = 1 mM or 30 μM, [thiols] = 10, 1 mM, or 30 μM, [proteins] = 20 μM, [selenocompounds] = 1 mM.

1.5. Time-resolved luminescence recognition of selenocysteine (Sec)

Sec was freshly prepared prior to each analysis. The stock solution of Sec was obtained by mixing an equal molar amount of $(Sec)_2$ (selenocystine dimethyl ester) with DTT (1 mM) and incubated at 37 ℃ for 5 min. The concentration-dependent emission spectra of TbL4 towards Sec were acquired by incubating TbL4 (20 μM) with increasing concentration of Sec (0 to 100 μM) at 25 ℃ for 2 h in PBS buffer (5‰ v/v DMSO, pH 7.4). The time-dependent emission spectra (λ_{ex} = 260 nm) of TbL4 in response to Sec were obtained by incubating TbL4 (20 μM) with Sec (100 μM) at 25 and 37 ℃ in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). The emission intensities of TbL4 at 546 nm were recorded at different time intervals. The kinetics analysis was conducted by the same method as that for recognition of BzSeH.

1.6. TrxR enzyme activity assay

The TbL4 complex and rat liver TrxR (0.15 U) were dissolved in 50 μL of reaction buffer. Then 25 μL of DTNB (disulfide 5,5′-dithio-bis(2-nitrobenzoic acid)) stock solution (20 mM) was added to 225 μL of the above-mentioned mixture. The reaction was allowed to proceed for 5 min at 37 ℃. The absorption at 405 nm was recorded on a microplate reader. The increase in TNB concentration (by measuring absorbance at 405 nm) over time followed a linear trend, and the enzyme activity was calculated as its slope (increased absorbance per thirty seconds).

1.7. Cytotoxicity assay

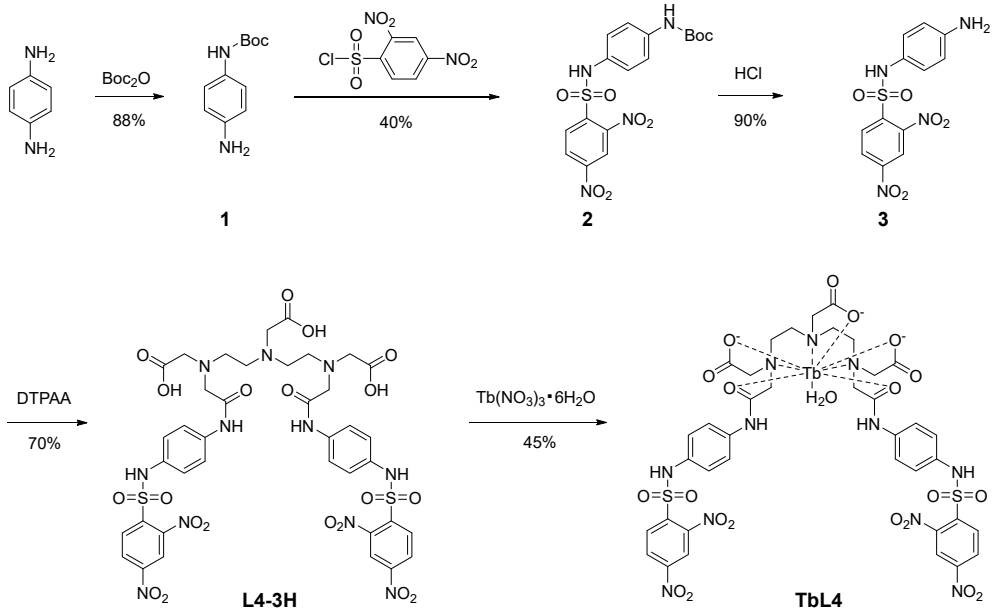
The cytotoxicity was performed by MTT assay with human ovarian cancer cell line A2780 and non-cancerous ovarian epithelial cell line IOSE80, *via* the cleavage of MTT to purple formazan crystals by cell mitochondrial dehydrogenases. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified incubator at 37 ℃ with 5% CO_2. The cells were seeded in a 96-well flat bottomed microplate at a density of 2000

cells per well and incubated overnight at 37 ℃ in a humidified atmosphere with 5% CO_2. The cells were treated with different concentration of TbL4 (0, 1.56, 3.12, 6.25, 12.5, 25, and 50 μM, final DMSO concentration 1‰) for 72 h at 37 ℃ in a humidified atmosphere with 5% CO_2. Then 5% MTT (5 mg mL^{-1}, PBS) was added to each well and incubated for 4 h at 37 ℃, 5% CO_2. Then the supernatants were removed, and the formazan crystals were dissolved in 200 μL of DMSO. The absorbance at 490 nm was determined using a microplate reader. The data were normalized and calculated as a percentage of untreated cells only containing 1‰ DMSO as control. All experiments were conducted three times to ensure reproducibility of the results.

1.8. Fluorescence imaging

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used as ROS probe to determine the capability of TbL4 to induce intracellular ROS. A2780, MDA-MB-231 and L02 cells were seeded at a density of 5-10 × 10^4/dish (35 mm dish) and cultured overnight. TbL4 (final concentration 50 μM, 1‰ DMSO) was added and treated for 24 h and 48 h at 37 ℃. Then DCFH-DA was added in serum-free medium and stained following manufacturer protocol (10 μM, 30 min). After washing with cell media, the images were acquired with Zeiss LSM 710 confocal laser scanning microscope (λ_{ex} = 488 nm, λ_{em} = 500-550 nm). The images were analyzed by ZEN software.

2. Supplementary Scheme and Figures



Scheme S1. Synthetic route to TbL4.

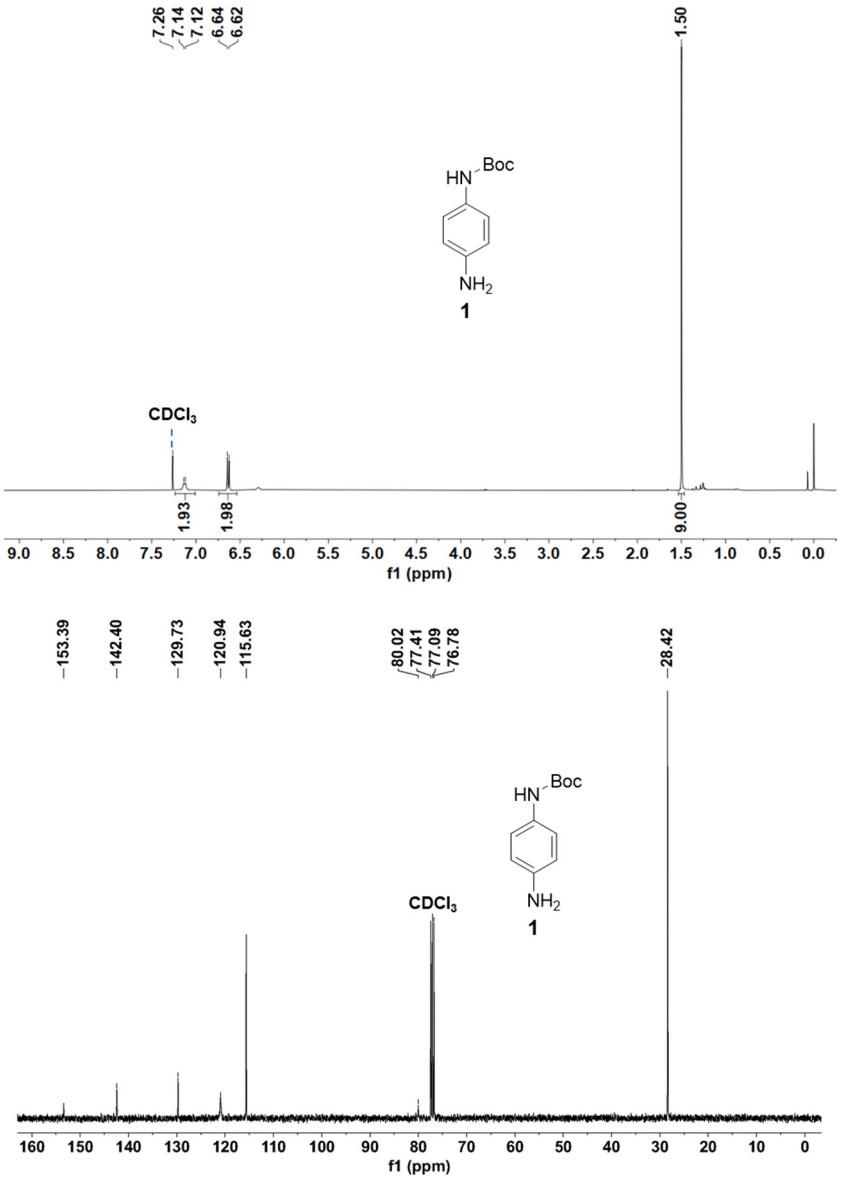


Fig. S1 ^{1}H NMR ($CDCl_3$), and ^{13}C NMR ($CDCl_3$) spectra of compound **1**.

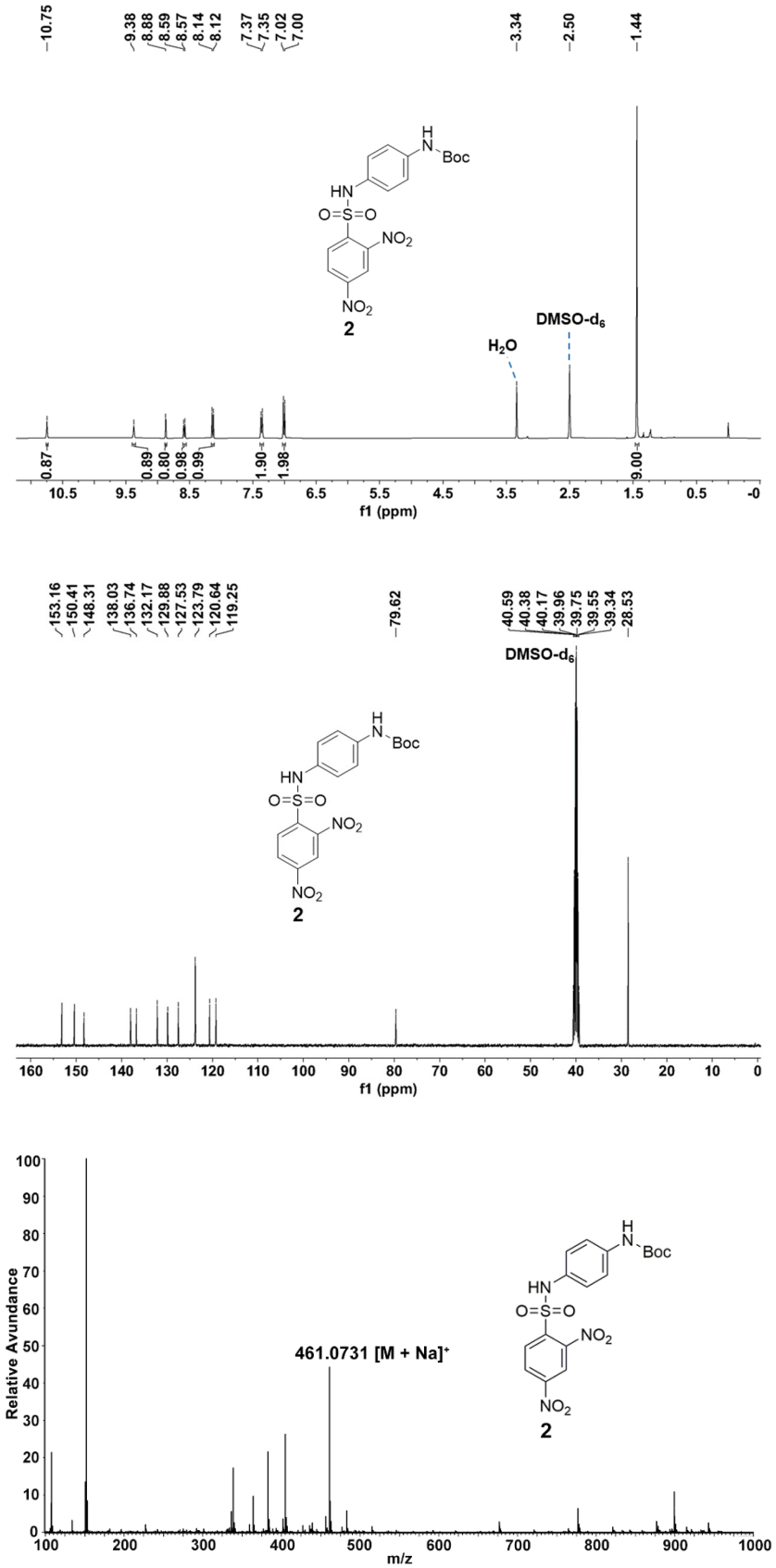


Fig. S2 ^{1}H NMR (DMSO-d_6), ^{13}C NMR (DMSO-d_6), and ESI-MS spectra of compound **2**.

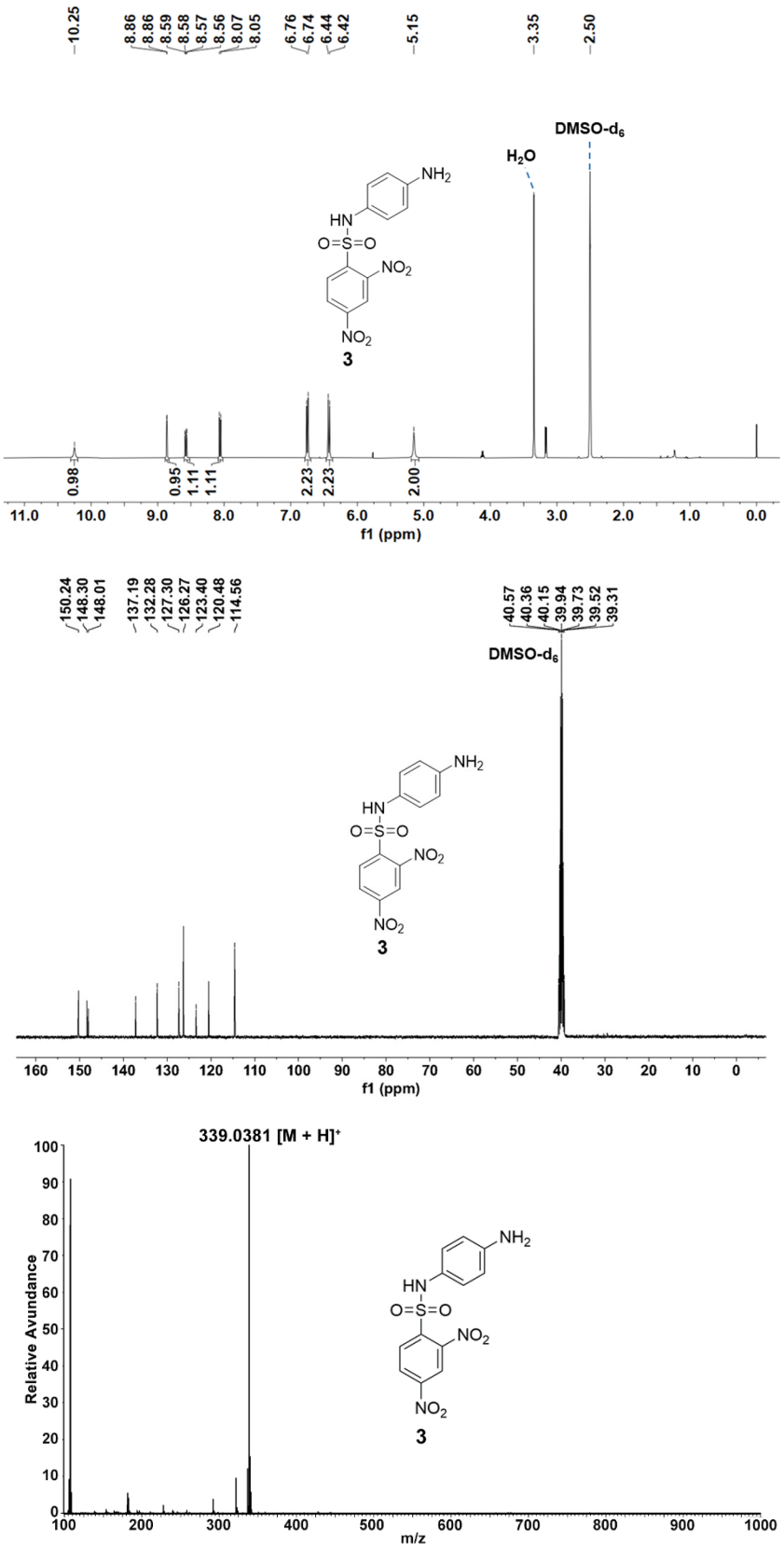


Fig. S3 ^{1}H NMR (DMSO-d_6), ^{13}C NMR (DMSO-d_6), and ESI-MS spectra of compound **3**.

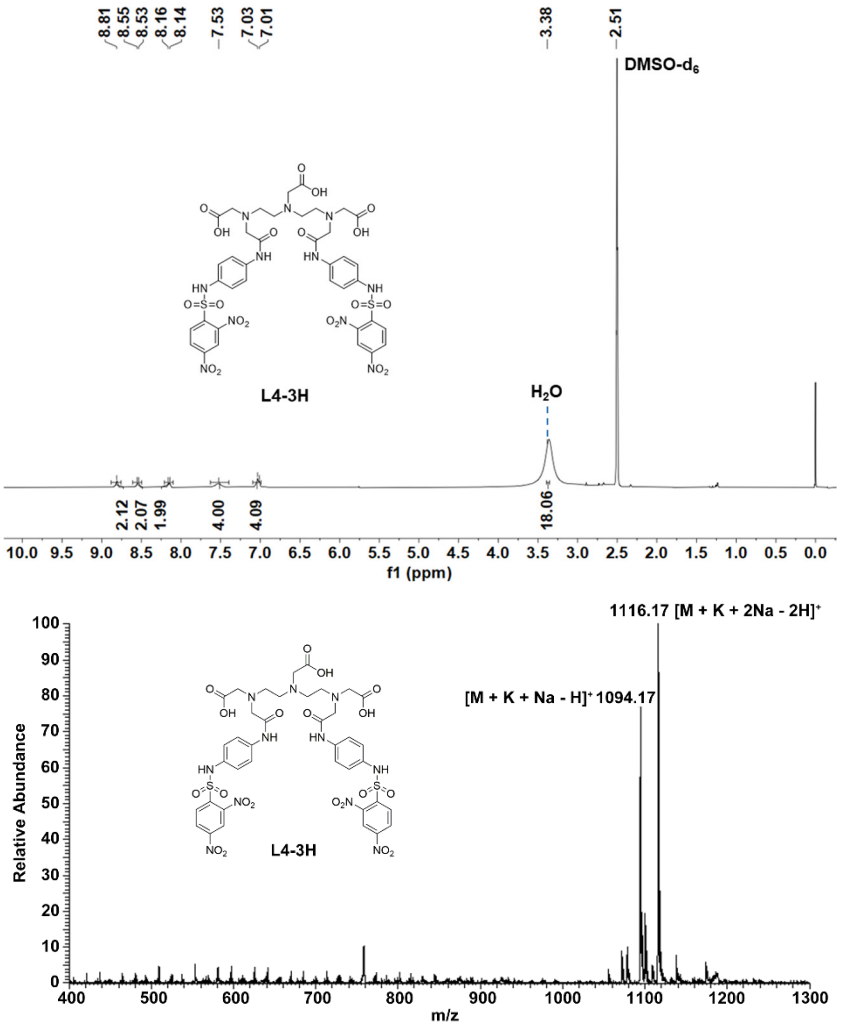


Fig. S4 ^{1}H NMR (DMSO-d_6), and ESI-MS spectra of compound **L4-3H**.

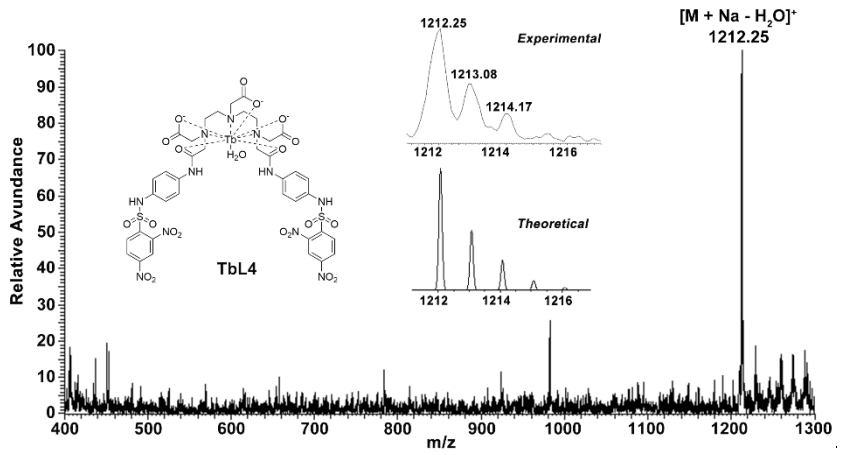


Fig. S5 ESI-MS spectra of compound **TbL4**.

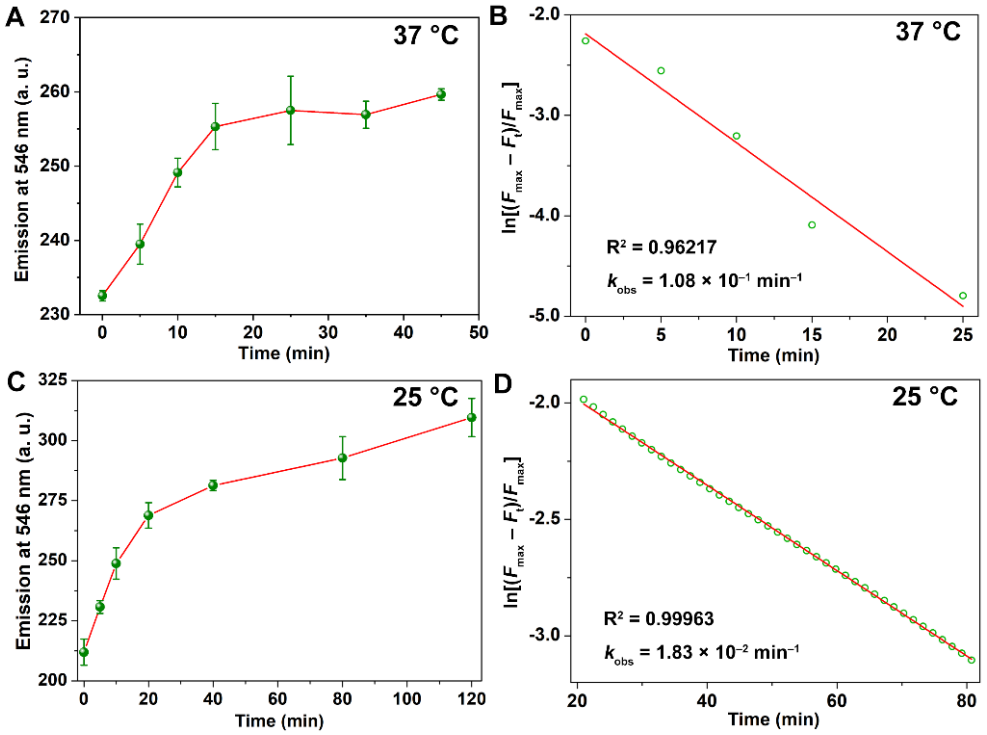
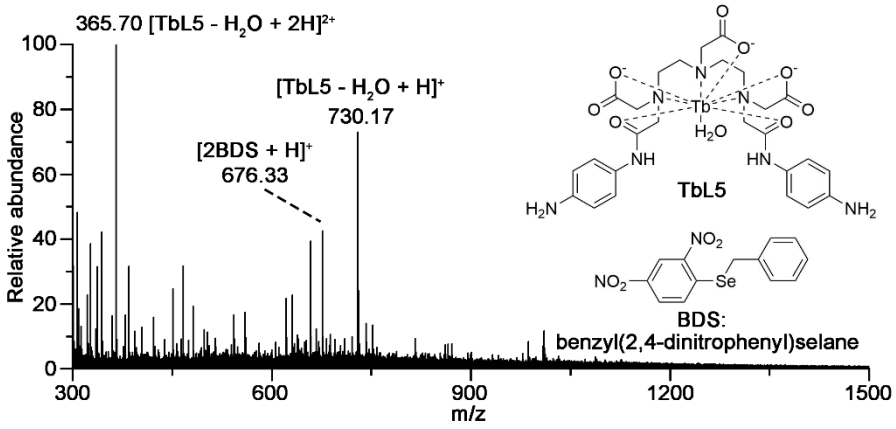


Fig. S6 Kinetics of time-dependent luminescence response of TbL4 (20 μM, λ_{ex} = 260 nm) to BzSeH at 37 °C (A and B) and 25 °C (C and D) in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). Error bars indicate ± s.d. (n = 3).



Species	Formula	Obsd m/z	Calcd m/z
[2BDS + H]⁺	$C_{26}H_{21}N_4O_8Se_2$	676.33	676.96
[TbL5 - H_2O + 2H]²⁺	$C_{26}H_{34}N_7O_8Tb$	365.70	365.58
[TbL5 - H_2O + H]⁺	$C_{26}H_{33}N_7O_8Tb$	730.17	730.16

Fig. S7 Mass spectroscopic analysis of the reaction between TbL4 and BzSeH.

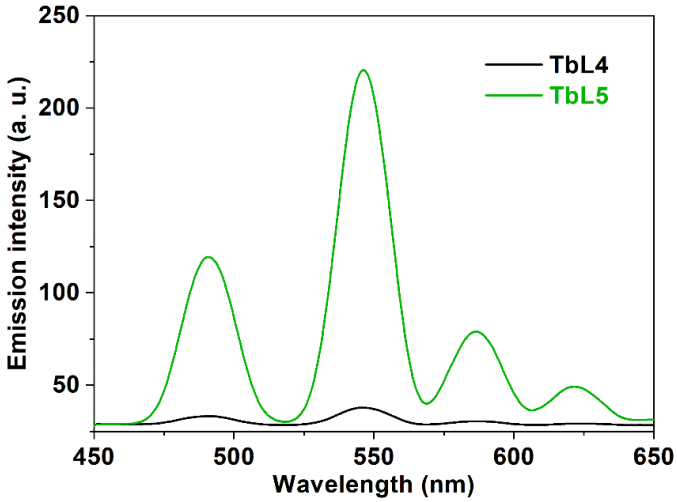


Fig. S8 Time-dependent emission spectra of TbL4 and TbL5 (20 μM, λ_{ex} = 260 nm) in a Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4).

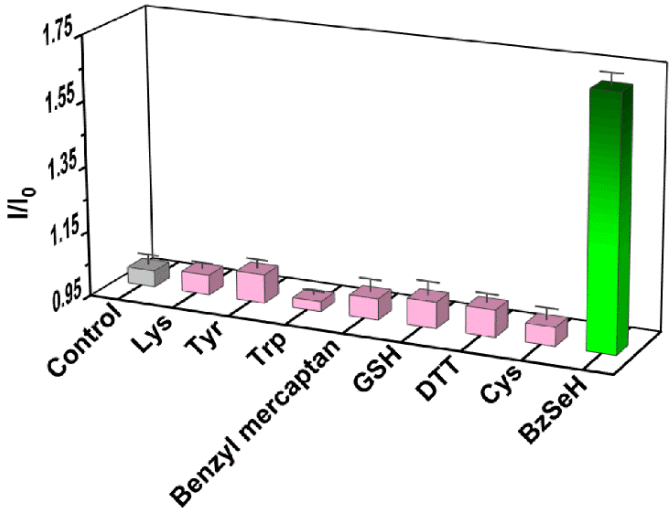


Fig. S9 Emission intensity ratio (I/I_0) of TbL4 (20 μM, λ_{ex} = 260 nm) at 546 nm in response to BzSeH (30 μM) and different biorelated molecules (30 μM) including amino acids and thiols in a Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). Error bars indicate ± s.d. (n = 3).

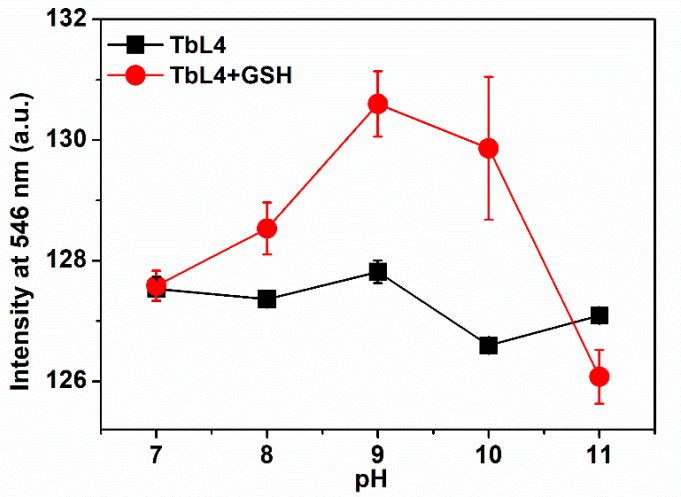


Fig. S10 Emission intensity of TbL4 at 546 nm at different pH in the absence and presence of GSH (30 μM). Error bars indicate ± s.d. (n = 3).

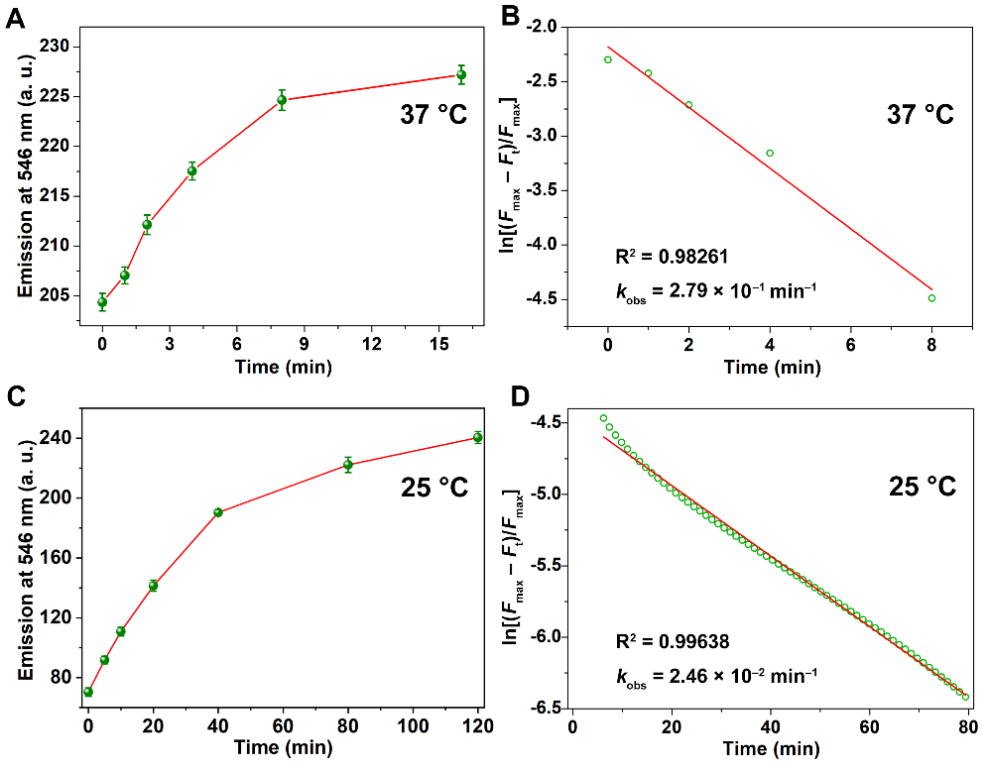
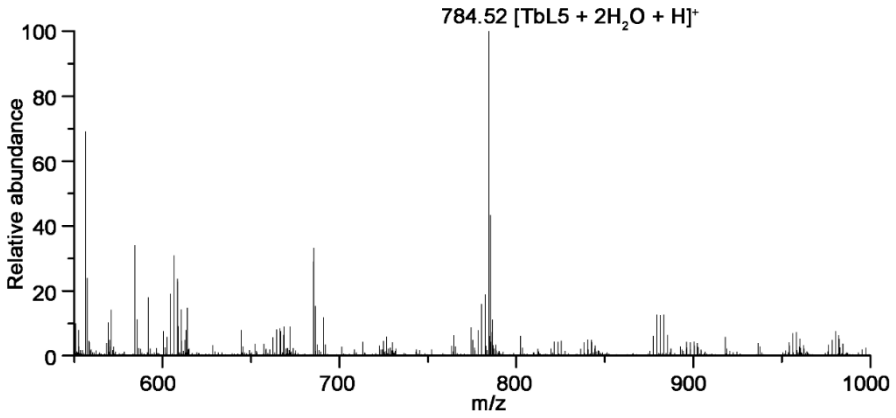


Fig. S11 Kinetics of time-dependent luminescence response of TbL4 (20 μM, λ_{ex} = 260 nm) to Sec at 37 °C (A and B) and 25 °C (C and D) in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, 5‰ v/v DMSO, pH 7.4). Error bars indicate ± s.d. (n = 3).



Species	Formula	Obsd m/z	Calcd m/z
$[TbL5 + 2H_2O + H]^+$	$C_{26}H_{39}N_7O_{11}Tb$	784.52	784.20

Fig. S12 Mass spectroscopic analysis of the reaction product of TbL4 with TrxR.

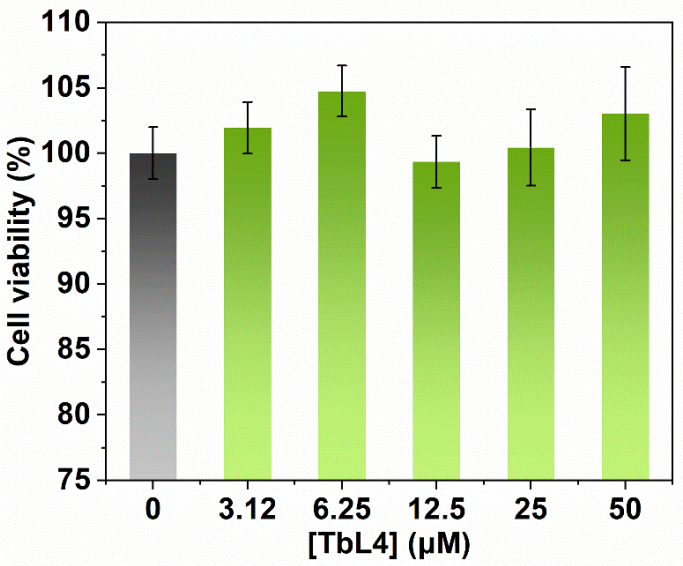


Fig. S13 Viability (%) of IOSE80 cells upon incubation with different concentrations of TbL4. The data was normalized and calculated as a percentage of untreated cells only containing 1‰ DMSO as a control. Error bars indicate ± s.d. (n = 3).

Reference

1. C. Ding, C. Li, Q. Meng, C. Qian, C. Zhang, L. Yang, X. Wang and Y. Wang, *Sens. Actuators B-Chem.*, 2021, **347**, 130607.