Supporting Information to Accompany

Cysteamine-selective two-photon fluorescent probe for ratiometric bioimaging

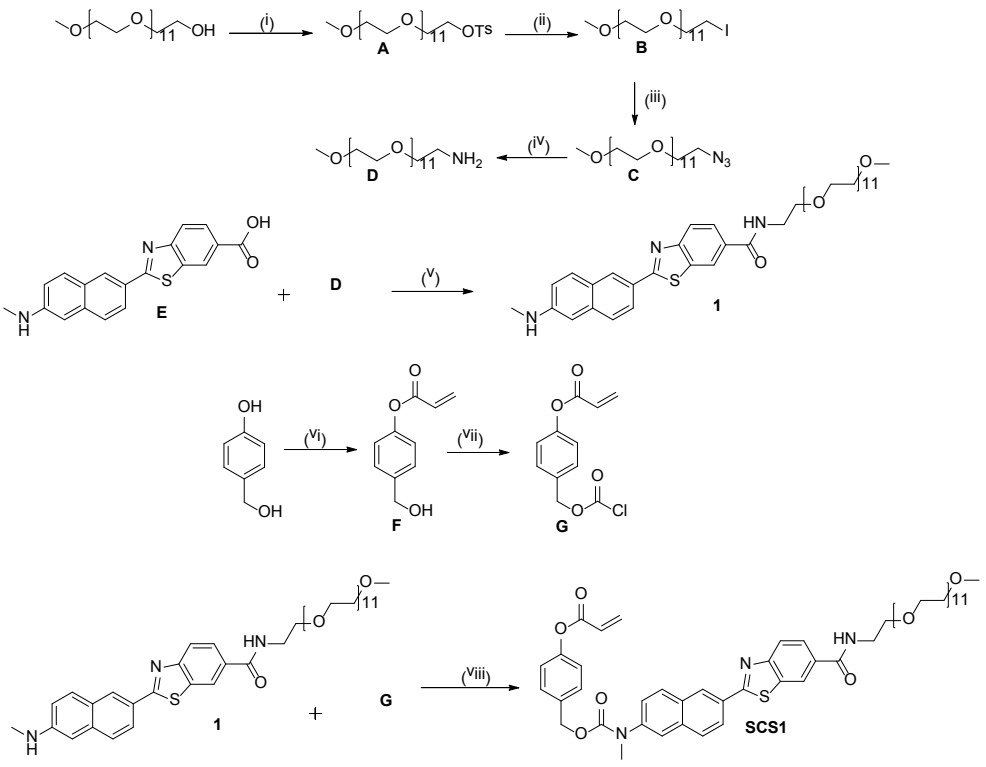
Avik R. Sarkar,[a] Cheol Ho Heo,[a] Eunjin Kim,[b] Hyo Won Lee,[a] Hardev Singh,[a] Jeong Jin Kim,[a] Hyuk Kang,[a] Chulhun Kang,*[b] and Hwan Myung Kim*[a]

[a]*Department of Chemistry and Department of Energy Systems Research, Ajou University, Suwon 443-749, Korea.*

[b]*East-West Medical Science, Kyung Hee University, Yongin 446-701, Korea.*

Table of Contents

Synthesis of SCS1. A, **B**, **C**, **D** and **E** were prepared by the literature methods.[1-3] Syntheses of other compounds are described below.



Scheme S1. Synthesis of **SCS1**. (i) *p*-Toluenesulfonyl chloride, triethylamine, 0°C; (ii) NaI, butanone, 60°C, 12h; (iii) Sodium azide (NaN_3), DMF, 85°C, 12h, (iv) Triphenylphosphine (PPh_3), THF, H_2O, RT, 12h, (v) *N,N'*-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), DCM, RT, 12h, (vi) Acryloyl Chloride, Acetone: H_2O (1:1,v/v), K_2CO_3, 3h, (vii) Triphosgene, Na_2CO_3, THF, -5°C, (vii) Pyridine, DCM, RT, 12h.

Synthesis of **1**. **E** (0.30 g, 0.90 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC, 0.220 g, 1.07 mmol) and 1-hydroxybenzotriazole (HOBt, 0.145 g, 1.07 mmol) were dissolved in dry CH_2Cl_2 (50 mL) and the reaction mixture was stirred at room temperature for 30 min under nitrogen atmosphere. To this mixture, **D** (0.604 g, 1.08 mmol) was added and whole the reaction mixture was stirred for 12 h. The solvent was evaporated and the crude product was purified by column chromatography using 5 % methanol in $CHCl_3$ as the eluent to give **1** as brown oil. Yield: 0.353 g (45 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.43 (s, 1H), 8.37 (s, 1H), 8.50 – 7.99 (m, 3H), 7.91 (dd, *J* = 8.4, 2.0 Hz 1H), 7.74 – 7.92 (m, 2H), 7.21 (s, 1H), 6.91 (dd, *J* = 8.8, 1.6 Hz, 1H), 6.76 (d, *J* = 2.4 Hz, 1H), 4.17 (br s, 1H), 3.71-3.52 (m, 48H), 3.36 (s, 3H), 2.97 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/*d*$_6$-DMSO): δ 170.9, 166.9, 166.8, 155.8, 148.5, 136.9, 134.6, 130.7, 129.6, 127.6, 126.3, 126.1, 125.1, 124.5, 121.9, 121.2, 118.7, 102.8, 71.7, 70.3, 70.0, 69.9, 58.9, 30.3. HRMS (FAB$^+$): m/z calcd for $[C_{44}H_{66}N_3O_{13}S_1]^+$: 876.4316, found: 876.4316.

Synthesis of **F**. To a stirred suspension of potassium carbonate (2.50 g, 18.12 mmol) in acetone / H_2O (4:1 v/v), acryloyl chloride was added at 0°C under argon atmosphere. To this reaction mixture, the solution of 4-(hydroxymethyl) phenol (1.50 g, 12.08 mmol) in acetone was added dropwise over a period of 10 min and then whole reaction mixture allowed to stirring for 3h at 0°C. After completion of reaction, the reaction mixture was filtered to remove the solid. The solvent was removed in *vacuo* and extract with CH_2Cl_2 (3 x 30 mL). The organic layer was dried over Na_2SO_4, filters and distilled out under reduced pressure to obtain the crude. The crude product was purified by flash column chromatography using 30 % ethyl acetate in hexane as the eluent to give **F** as colorless oil. Yield: 1.16 g (51 %); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (d, *J* = 8.4 Hz, 2H), 7.10-7.06 (m, 2H), 6.61 (dd, *J* = 17.2, 1.6 Hz, 1H), 6.31 (dd, *J* = 17.2, 10.4 Hz, 1H), 6.02 (dd, *J* = 10.8, 0.8 Hz, 1H), 4.59 (s, 2H), 2.65 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.5, 149.4, 138.5, 132.6, 127.8, 127.5, 121.2, 64.0. HRMS (FAB$^+$): m/z calcd for $[C_{10}H_{10}O_3]^+$: 178.0627, found: 178.0630.

Synthesis of **G**. Na_2CO_3 (0.48 g, 4.48 mmol) and triphosgene (0.332 g, 1.12 mmol) were *vacuo* dried in a round-bottom flask. The round-bottom flask was cooled in an ice bath and THF (15 mL) was added. After stirring under nitrogen in the dark for 1 h at -5°C, a solution of **F** (0.20 g, 1.12 mmol) in dry THF (15 mL) was added dropwise and the stirring was continued for additional 6 h at room temperature. After completion of the reaction, the solvent was removed under reduced pressure and used without further purification for the next step; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.39 (d, *J* = 8.4 Hz, 2H), 7.15 (d, *J* = 8.4 Hz, 2H), 6.59 (d, *J* = 17.6 Hz, 1H), 6.30 (dd, *J* = 16.8, 10.0 Hz, 1H), 6.01 (d, *J* = 10.4 Hz, 1H), 5.25 (s, 2H).

Synthesis of **SCS1**. To a stirred solution of **G** (0.071 g, 0.0.27 mmol) and **1** (0.235 mg, 0.27 mmol) in 25 mL dry CH_2Cl_2, dry pyridine (0.20 mL, 2.48 mmol) was added slowly. The reaction mixture was stirred at room temperature for 12 h under nitrogen atmosphere. After completion of the reaction, the solvent was evaporated and the crude product was purified by silica gel column chromatography using 3 % CH_3OH in $CHCl_3$ as the eluent to afford **SCS1** as a yellow solid. Yield: 0.18 g (62 %); m.p. 108-110 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.55 (s, 1H), 8.52 (s, 1H), 8.21 (dd, *J* = 8.8, 1.6 Hz, 1H), 8.11 (d, *J* = 8.8 Hz, 1H), 7.97-7.88 (m, 3H), 7.71 (s, 1H), 7.52 (d, *J* = 7.2 Hz, 1H), 7.39-7.35 (m, 3H), 7.12 (dd, *J* = 8.4, 3.2 Hz, 2H), 6.62 (dd, *J* = 17.2, 1.6 Hz, 1H), 6.34 (dd, *J* = 17.2, 10.4 Hz, 1H), 6.02 (dd, *J* = 10.8, 1.2 Hz, 1H), 5.20 (s, 2H), 3.72-3.58 (m, 46H), 3.54-3.51 (m, 2H), 3.45 (s, 3H), 3.36 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.1, 166.7, 164.2, 155.7, 155.1, 150.1, 142.2, 134.9, 134.8, 133.9, 132.6, 131.4, 130.9, 130.5, 129.3, 129.1, 128.5, 127.6, 127.4, 125.6, 125.3, 124.7, 122.6, 121.5, 119.7, 103.9, 71.9, 70.5, 70.2, 69.9, 61.2, 59.0, 40.1. HRMS (FAB^+): m/z calcd for $[C_{55}H_{74}N_3O_{17}S_1]^+$: 1080.4739, found: 1080.4739.

Spectroscopic measurements. Absorption spectra were recorded on S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1

cm standard quartz cell. The fluorescence quantum yield was determined by using coumarine 307 (Φ = 0.95 in MeOH) as the reference by the literature method.[4]

Water solubility. Small amount of **SCS1** and **1** were dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to $1.0 \times 10^{-7} \sim 8.0 \times 10^{-5}$ M and added to a cuvette containing 3.0 mL of phosphate buffered saline (PBS, 10 mM, pH = 7.4), Dulbecco's Modified Eagle's Medium (DMEM) and artificial cerebrospinal fluid (ACSF), respectively, by using a micro syringe. In all cases, the concentration of DMSO in buffer, DMEM and ACSF was maintained to be 0.1 %.[5] The plots of fluorescence intensity against the dye concentration were linear at low concentration and showed curvature at higher concentration (Figure S1). The maximum concentration in the linear region was taken as the solubility. The solubility values of **SCS1** and **1** in PBS buffer were ~2.0 and 6.0 μM, respectively, and the values of **SCS1** in DMEM and ACSF were ~ 6.0 μM.

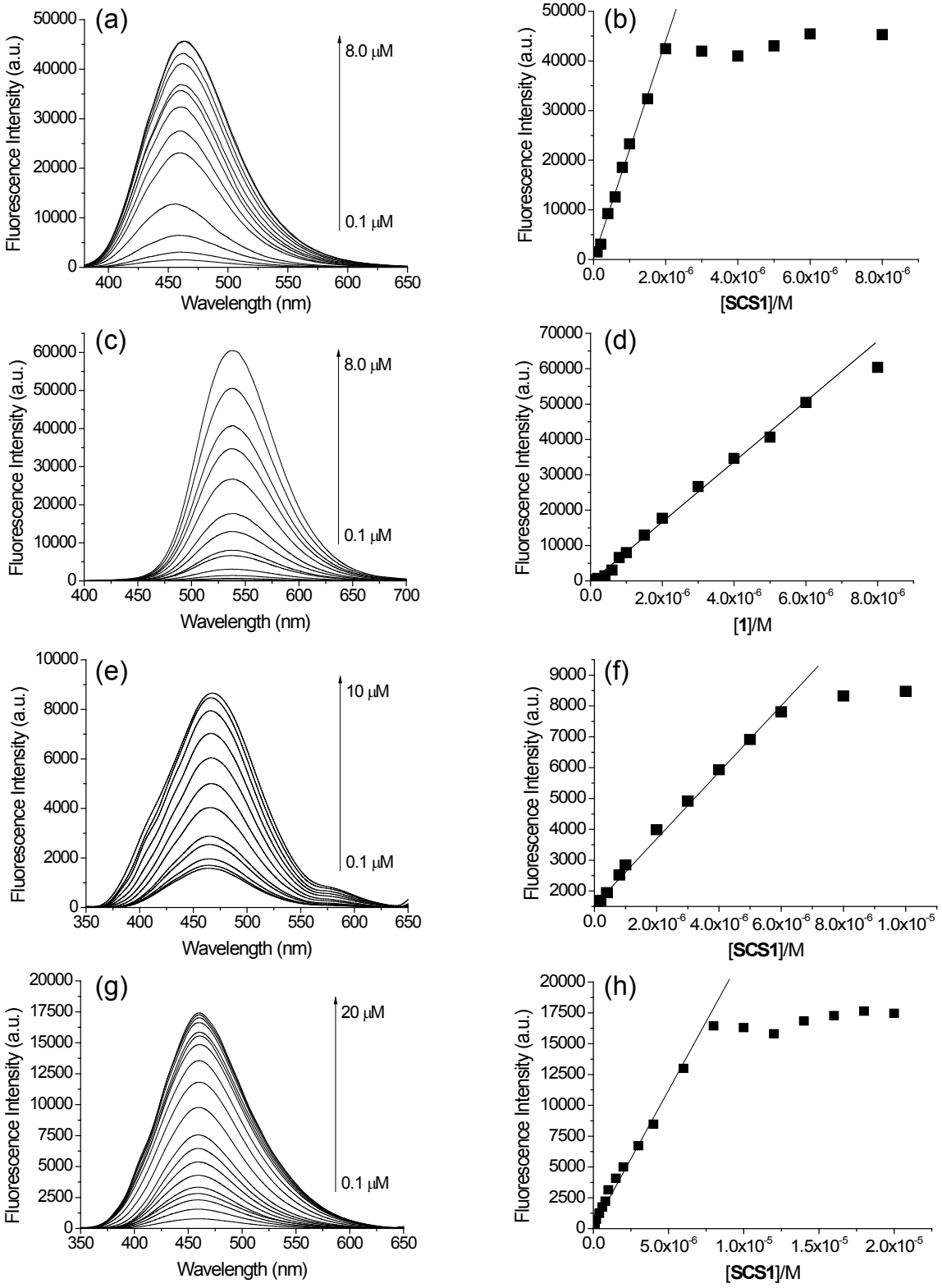


Figure S1. (a,c,e,g) One-photon fluorescence spectra and (b,d,f,h) plot of fluorescence intensity against the concentration for **SCS1** (a,b) in PBS buffer (10 mM, pH 7.4), (e,f) in DMEM and (g,h) in ACSF, respectively, and for **1** (c,d) in PBS buffer (10 mM, pH 7.4). The excitation wavelengths were 335 nm for **SCS1** and 376 nm for **1**, respectively.

Product analysis. The reaction of **SCS1** (200 μM) with cysteamine (200 mM) was carried out for 1 hr at 25°C in PBS buffer (10 mM, pH = 7.4). The HPLC traces of **SCS1**, **1**, and the reaction are shown below (Figure S2). The result shows that **1** is the only product.

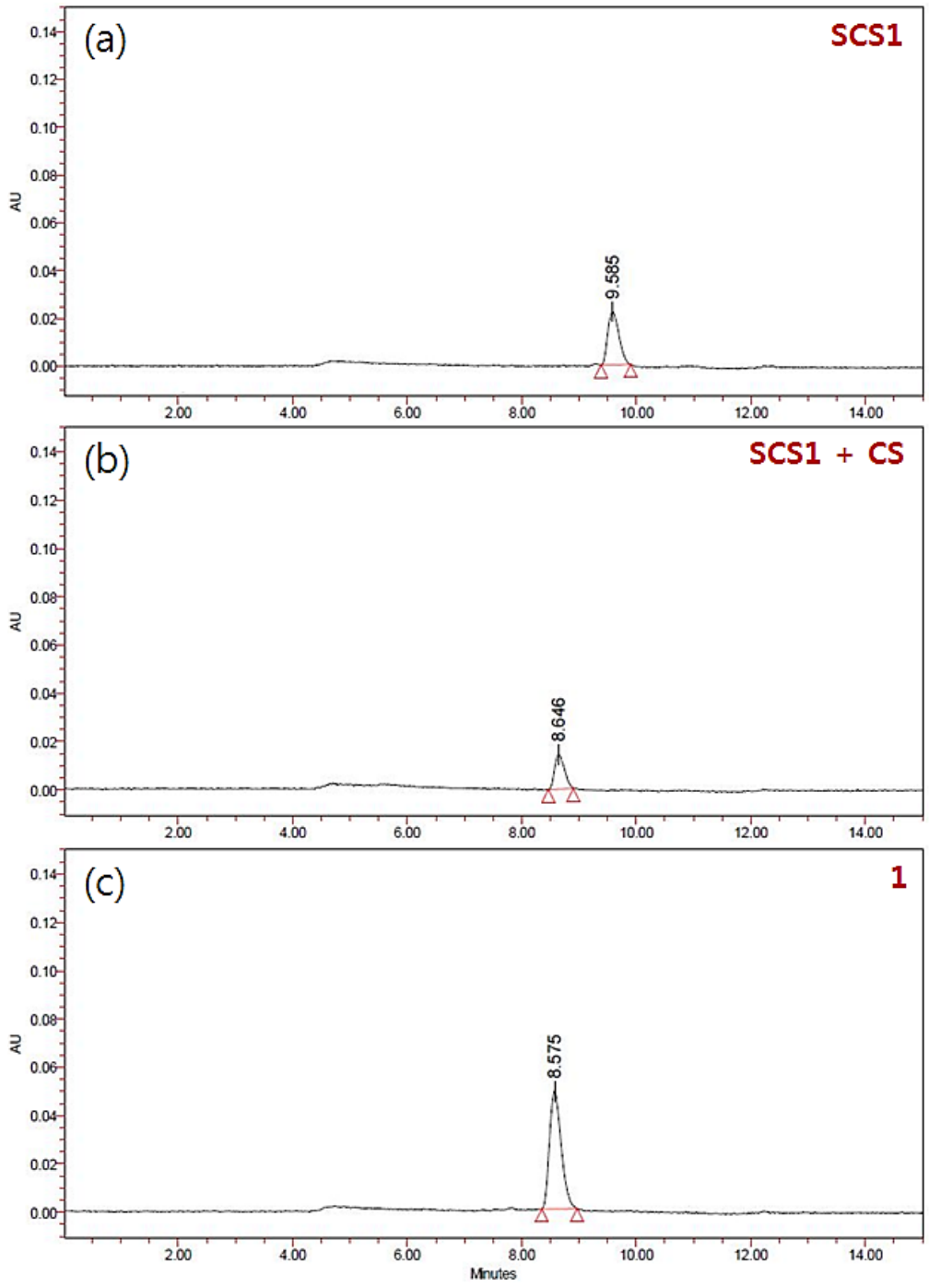


Figure S2. HPLC traces of (a) **SCS1**, (b) the reaction product between **SCS1** and cysteamine (CS), and (c) **1**. HPLC conditions: 0.3 mL/min flow rate, 5 % B to 100 % B over 20 min, detected at 370 nm. Solvent A is water and solvent B is acetonitrile. Peaks at 8.6 min and 9.6 min correspond to **1** and **SCS1**, respectively.

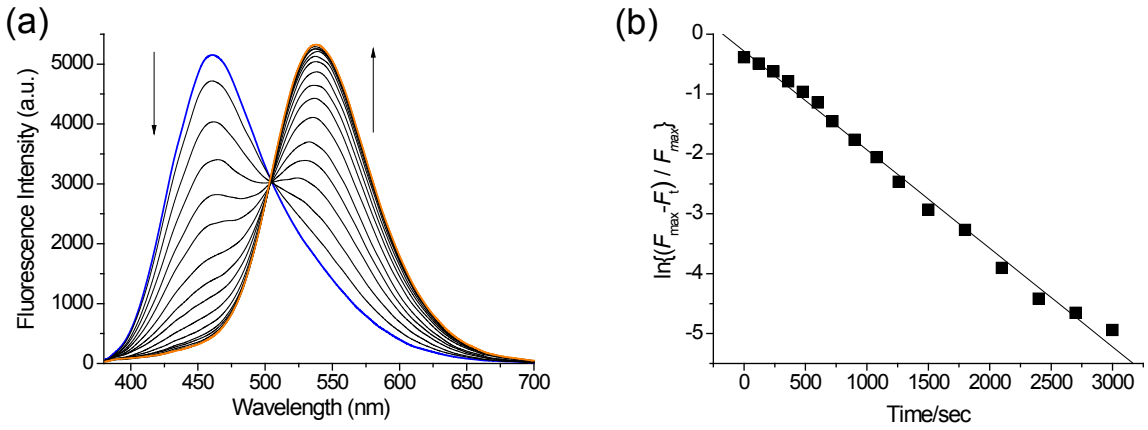


Figure S3. (a) One-photon fluorescence response with time for the reaction of **SCS1** (1μM) with cysteamine (1mM) in PBS buffer (10 mM, pH 7.4). (b) Plots of ln $[(F_{max}–F_t)/F_{max}]$ vs time (measured at 540 nm) for the reaction of **SCS1** (1μM) with cysteamine (1mM) in PBS buffer (10 mM, pH 7.4). The $k_{obs} = 1.7 \pm 0.1 \times 10^{-3}$ s^{-1} were obtained from slope of the plot (b).

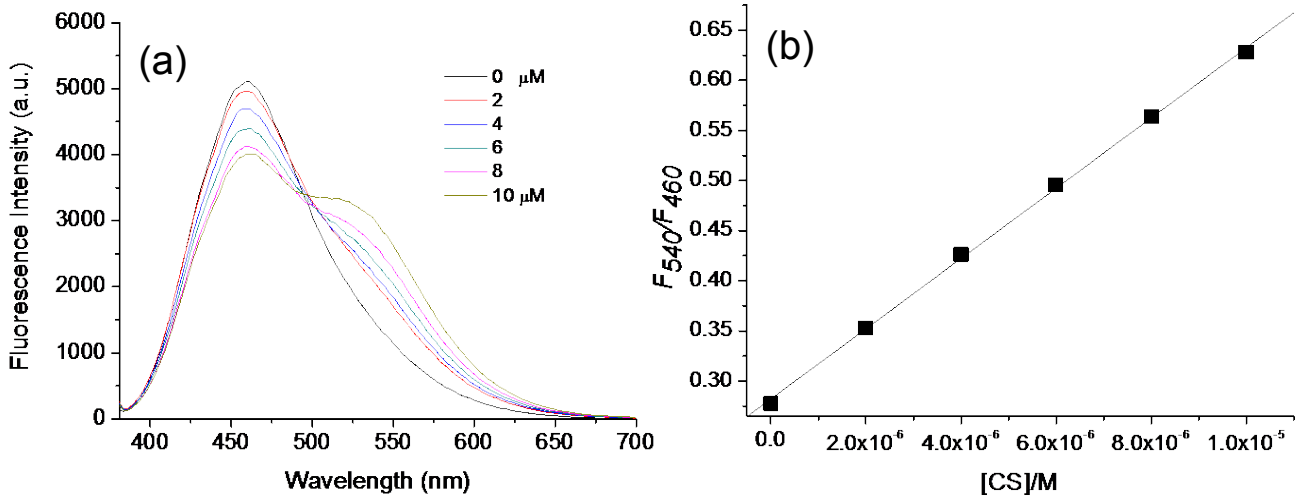


Figure S4. (a) One-photon fluorescence response with time for the reaction of **SCS1** (1 μM) with different concentration of cysteamine (CS, 0 to 10 μM) in PBS buffer (10 mM, pH 7.4). (b) Plot of the fluorescence intensity ratios for **SCS1** vs the concentration of CS in PBS buffer (10 mM pH 7.4). Each data was acquired 1 h after addition of CS at 37 °C. The detection limit (0.81 ± 0.03 μM for **SCS1**) was calculated with 3σ/k respectively; where σ is the standard deviation of blank measurement, k is the slope in Figure S4.[6] The excitation wavelength was 370 nm.

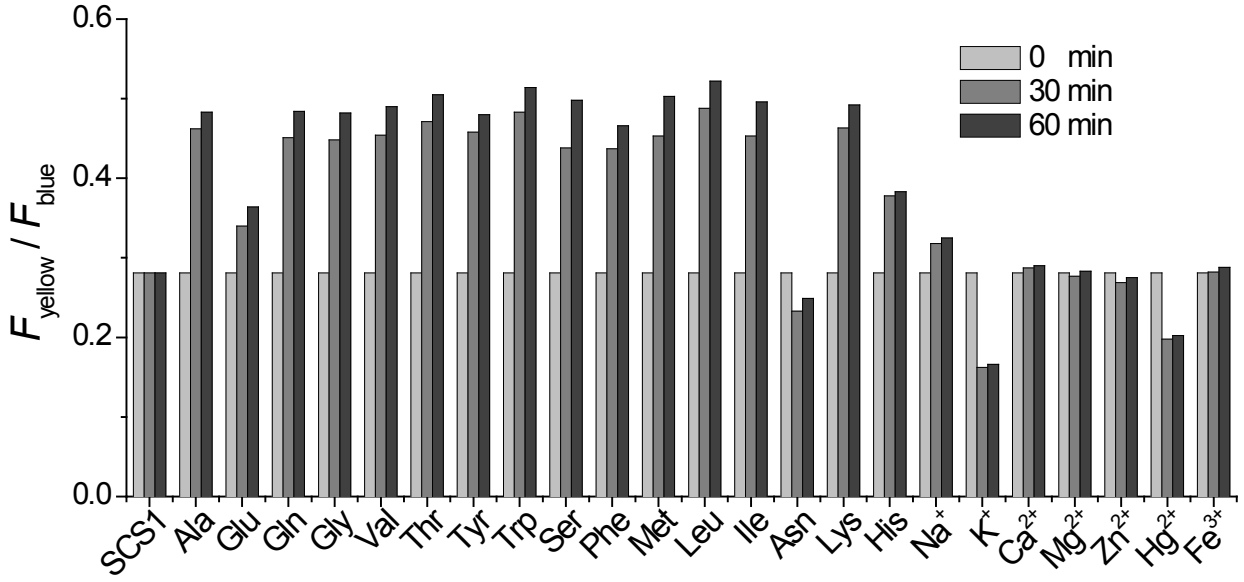


Figure S5. Fluorescence response of 1 μM **SCS1** to various amino acids and metal ions (1 mM). Bars represent the integrated fluorescence emission ratios (F_{yellow}/F_{blue}) at 0, 30 and 60 min after the addition of amino acids and metal ions. Data were acquired in PBS buffer (10 mM, pH 7.4) at 25°C. The excitation wavelength was 370 nm.

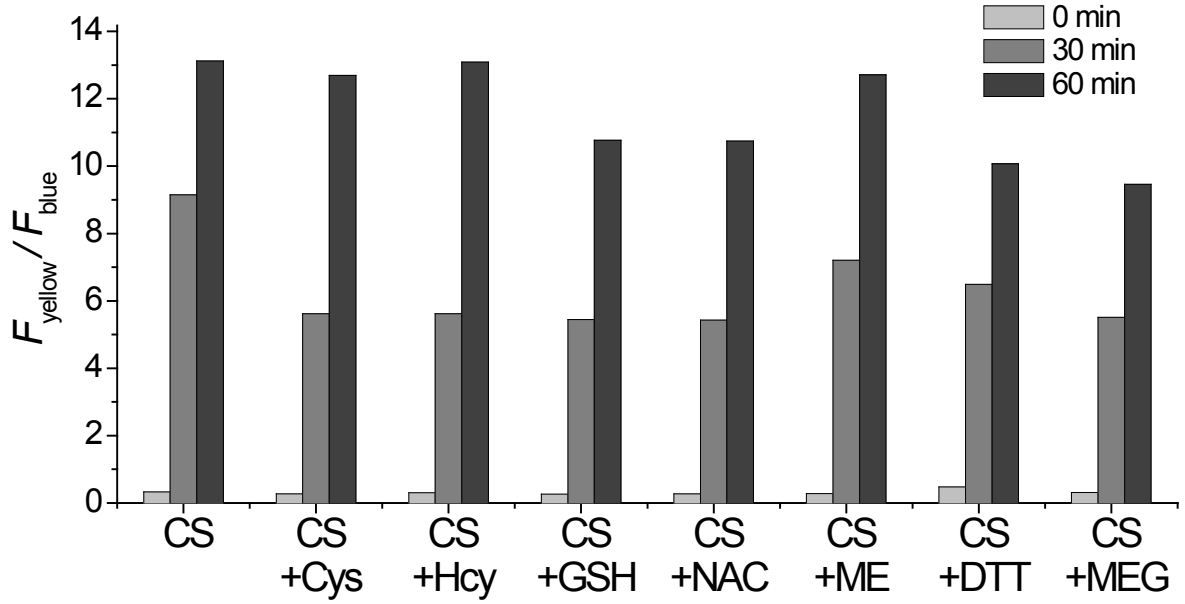


Figure S6: Fluorescence response of 1 μM **SCS1** to cysteamine in presence of 1 mM of Cys, Hcy, GSH, NAC, 2-ME, DTT and MEG. The bars represent the integrated fluorescence intensity ratios (F_{yellow}/F_{blue}) of **SCS1** at before, 30 min and 1 h after addition of the cysteamine, respectively. Data were acquired in PBS buffer (10 mM, pH 7.4). The excitation wavelength was 370 nm.

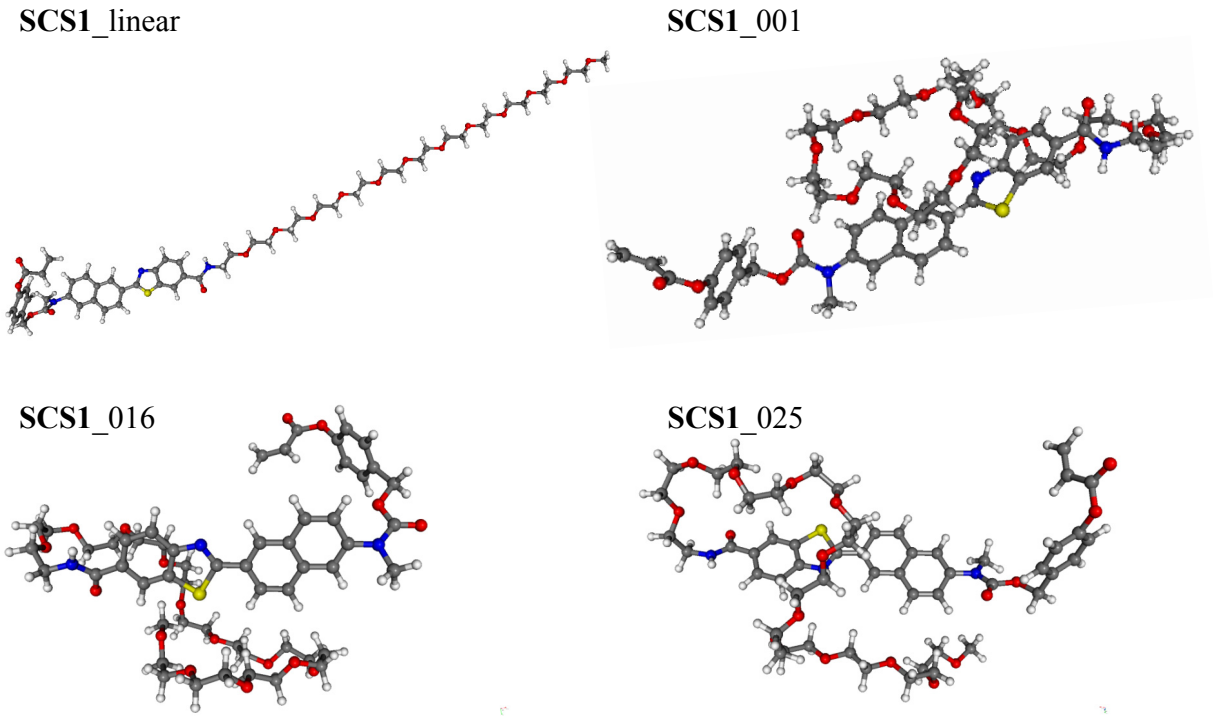


Figure S7. Optimized geometries of **SCS1**: the linear conformer (**SCS1**_linear), the most stable conformer (**SCS1**_001), and two representative folded conformers (**SCS1**_016 and **SCS1**_025). The structure of **SCS1**_linear was optimized only by Amber* force field because it converged to a folded structure at DFT level, and others were optimized by B3LYP/6-31G with polarization continuum model for solvent effect.

Table S1. Relative energies of the conformers of **SCS1**.[7]

	ΔE (kJ/mol)[a]	ΔE (kJ/mol)[b]
SCS1_linear	59.094	N.A.[c]
SCS1_001	0	0
SCS1_016	4.961	5.672
SCS1_025	7.195	1.446

[a]Relative energy calculated by Amber* force field. [b]Relative energy calculated by B3LYP/6-31G with polarization continuum model for solvent effect. [c]Not available because the structure converged to one of the folded structures.

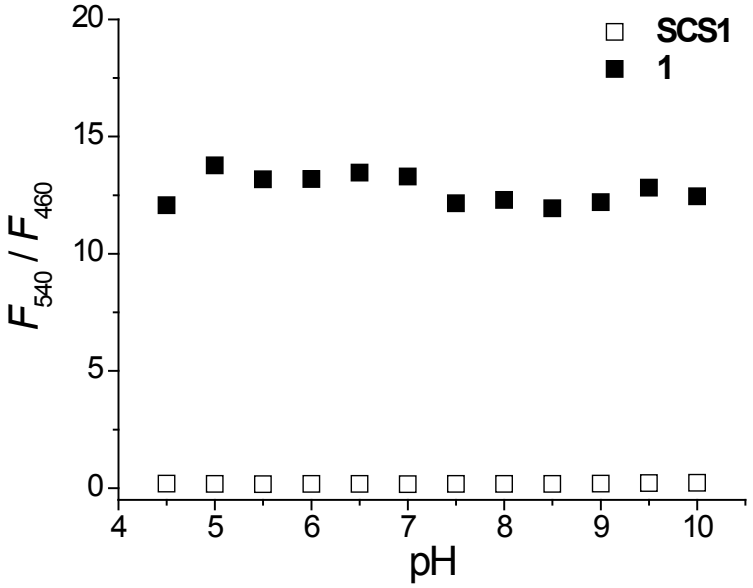


Figure S8. Effect of the pH on the one-photon fluorescence intensity ratio (F_{540}/F_{460}) of **SCS1** (□) and **1** (■) in universal buffer (30 mM, 0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, 0.1 M KCl). The excitation wavelength was 370 nm.

Measurement of Two-Photon Cross Section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[8] **SCS1** (1.0×10^{-6} M) was dissolved in PBS buffer (10 mM, pH 7.4). The two-photon induced fluorescence intensity was measured at 720−880 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[9] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

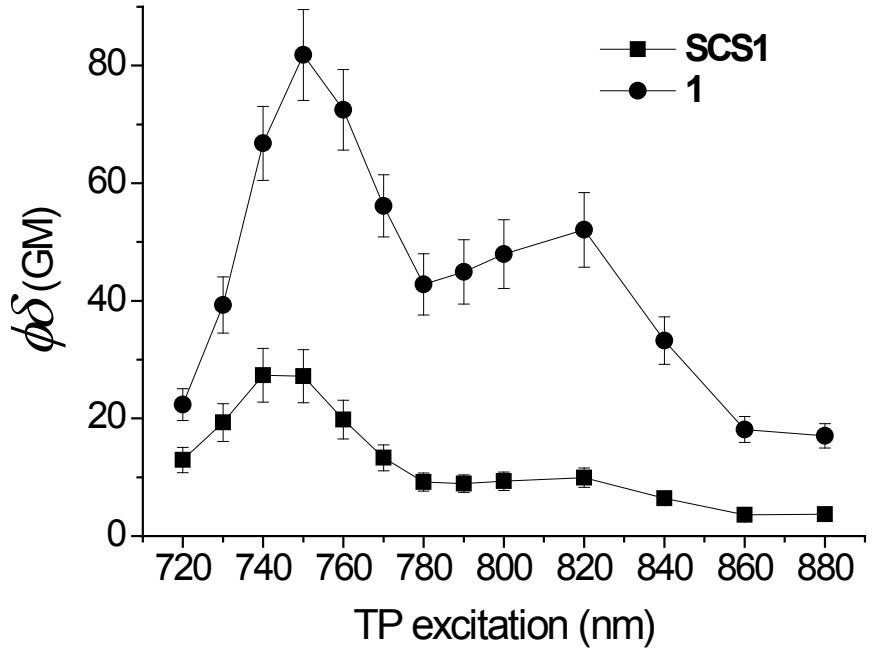


Figure S9. Two-photon action spectra of **SCS1** and **1** in PBS buffer (10 mM, pH 7.4). The estimated uncertainties for the two-photon action cross section values (δΦ) are ± 15 %.

Cell Culture. U87 human primary glioblastoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL). Two days before imaging, the cells were passed and plated on glass-bottomed dishes (NEST). All the cells were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37°C. For labeling, the growth medium was removed and replaced with serum-free DMEM. The cells were treated and incubated with 2.0 μL of 1 mM **SCS1** in DMSO stock solution (2.0 μM **SCS1**) at 37°C under 5 % CO_2 for 30 min.

Two-Photon Fluorescence Microscopy. Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with ×10 dry, ×40 oil, and ×100 oil objectives, numerical aperture (NA) = 0.30, 1.30, and 1.30, respectively. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at wavelength 750 nm and output power 2690 mW, which corresponded to approximately 2.2 mW average power in the focal plane. To obtain images at 410-460 nm (F_{blue}) and 530-580 nm (F_{yellow}) range, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 and 1024×1024 pixels at 400 Hz scan speed, respectively. Ratiometric image processing and analysis was carried out using MetaMorph software.

Photostability. Photostability of **SCS1** was determined by monitoring the changes in TPEF intensity with time at three designated positions of **SCS1**-labeled (2.0 μM) U87 cells chosen without bias. The TPEF intensity remained nearly the same for one hour, indicating high photostability.

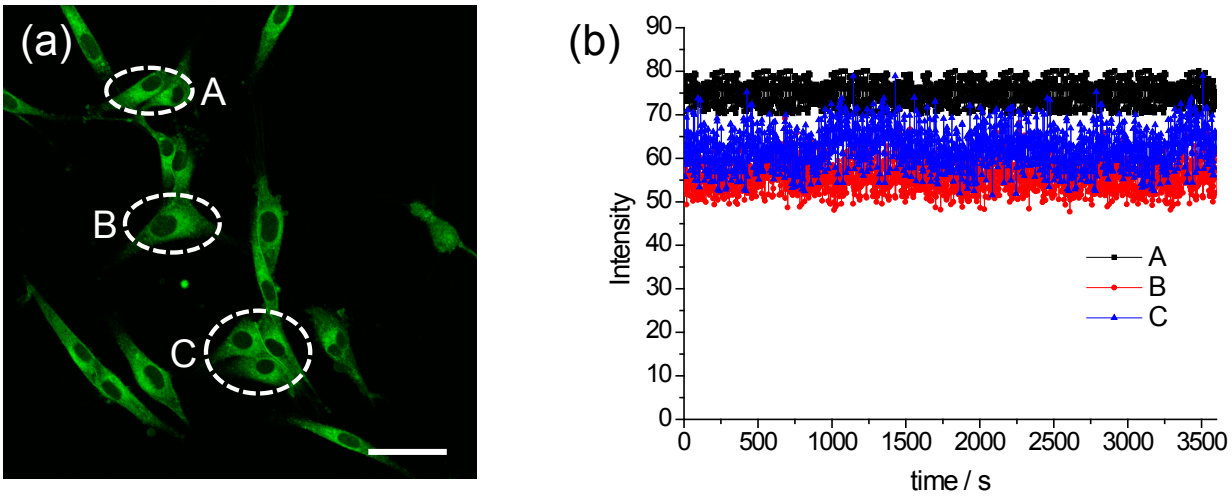


Figure S10. (a) TPM images of U87 cells labeled with **SCS1**. (b) The relative TPEF intensity from A-C in Figure (a) as a function of time. The digitized intensity was recorded with 2.00 sec intervals for the duration of one hour using *xyt* mode. The TPEF intensities were collected at 410-580 nm upon excitation at 750 nm with femto-second pulses at 2.2 mW average power at the focal point. Cells shown are representative images from replicate experiments (n = 3).

Cell viability. To confirm that the probe couldn't affect the viability of U87 cells in our incubation condition, MTS assay (Cell Titer 96H; Promega, Maidson, WI, USA) was used according to the manufacture's protocol. The results are shown in Figure S11.

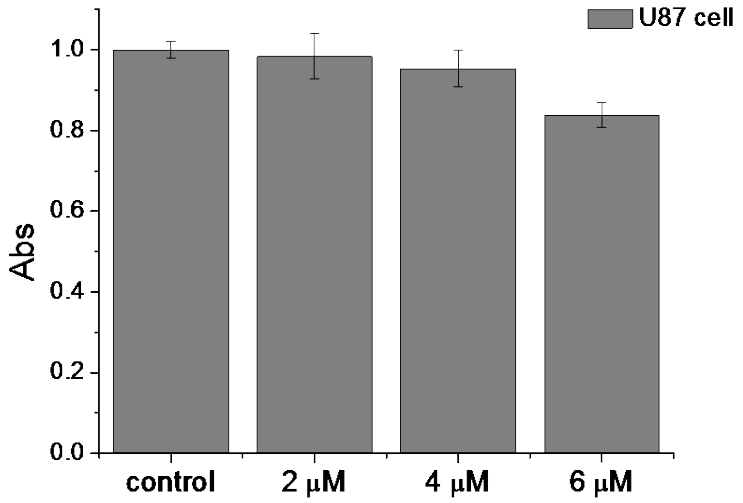


Figure S11. Viability of U87 cells in the presence of **SCS1** (0 to 6 μM) as measured by using MTS assay. The cells were incubated with probe for 24 h. Six independent experiments are performed.

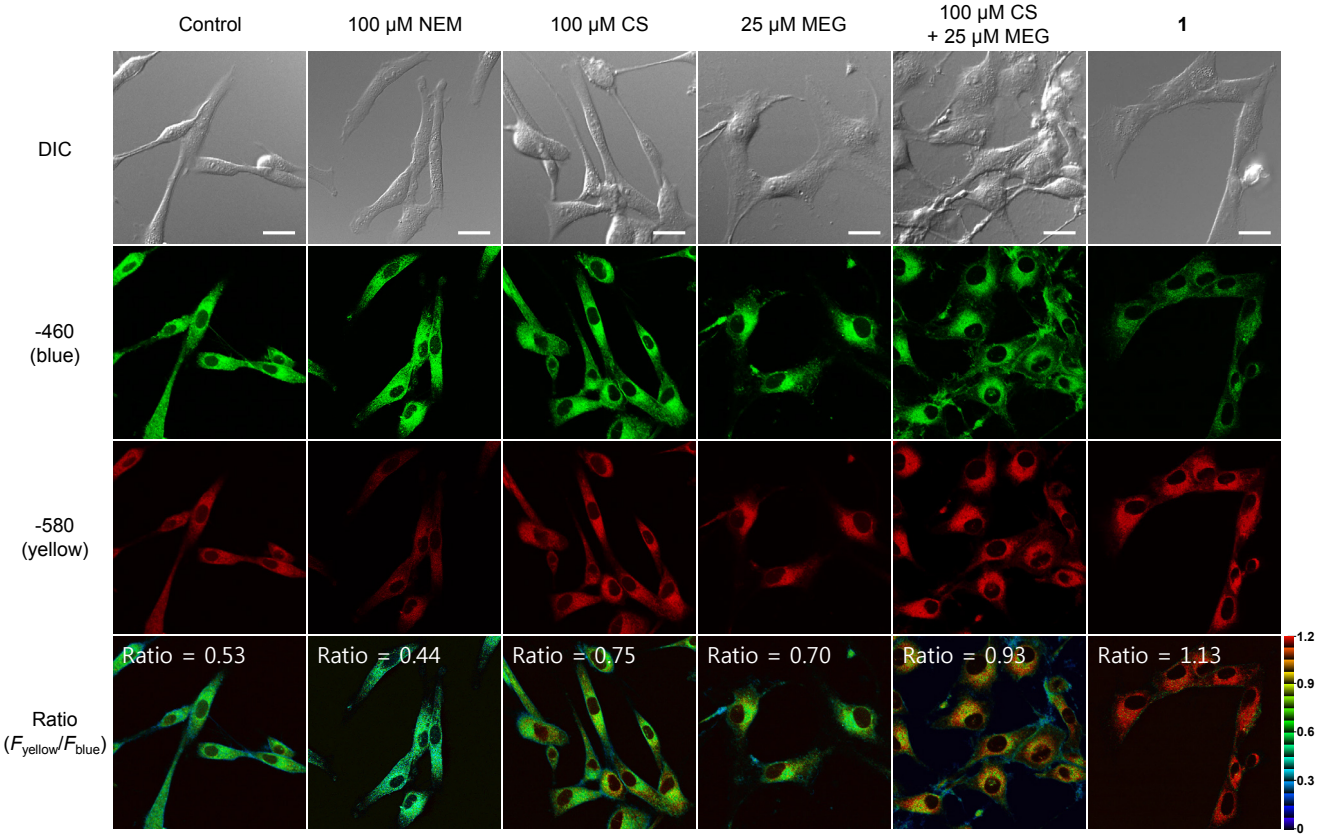


Figure S12. Pseudocolored ratiometric TPM images (F_{yellow}/F_{blue}) of U87 cells. U87 cells were pretreated with N-Ethylmaleimide (NEM, 100 μM) for 15 min, cysteamine (CS, 100 μM) for 30 min, Mercaptoethylguanidine (MEG, 25 μM) for 2 hr, and CS (100 μM) for 30 min and MEG (25 μM) for 2 h before labeling with **SCS1**. Images were acquired using 750 nm excitation and emission windows of 410-460 nm (blue) and 530-580 nm (yellow). Cells shown are representative images from replicate experiments (n = 5). Scale bars: 24 μm.

Preparation and staining of fresh rat Hippocampal slices. Slices were prepared from the hippocampi of 2-weeks-old rat (SD). Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 138.6 mM NaCl, 3.5 mM KCl, 21 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 9.9 mM D-glucose, 1 mM $CaCl_2$, and 3 mM $MgCl_2$). Slices were incubated with 20 μL from 10 mM stock solution of **SCS1** in DMSO (total 20 μM **SCS1**) in ACSF bubbled with 95% O_2 and 5% CO_2 for 30 min at 37°C. It takes a longer time to stain the living tissues, during which time they may be deformed, so an excess amount (20 μM) of **SCS1** was used to facilitate staining. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (NEST) and observed in spectral confocal and multiphoton microscopes (Leica TCS SP8 MP).

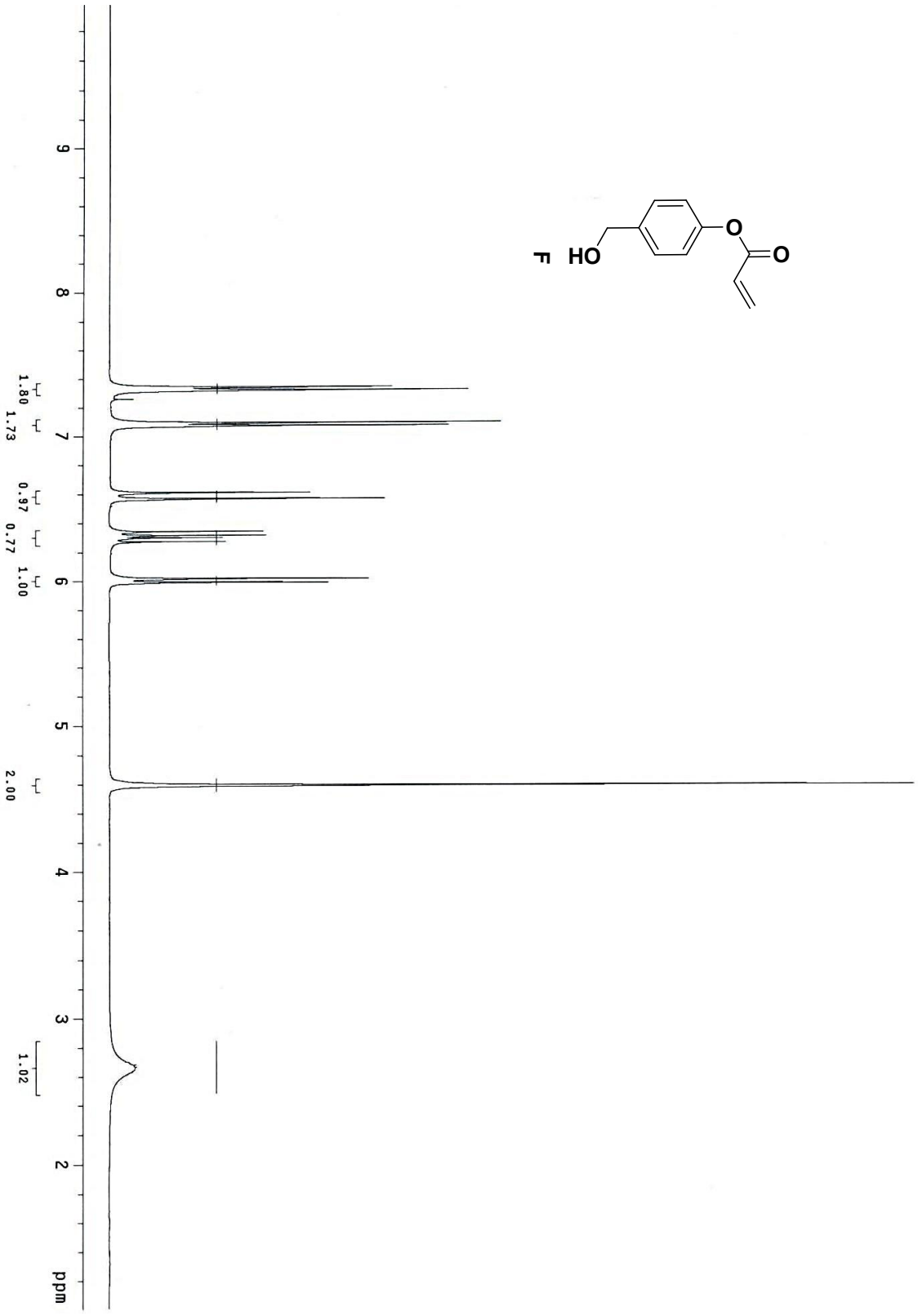


Figure S13. ^{1}H-NMR spectrum (400 MHz) of **F** in $CDCl_3$

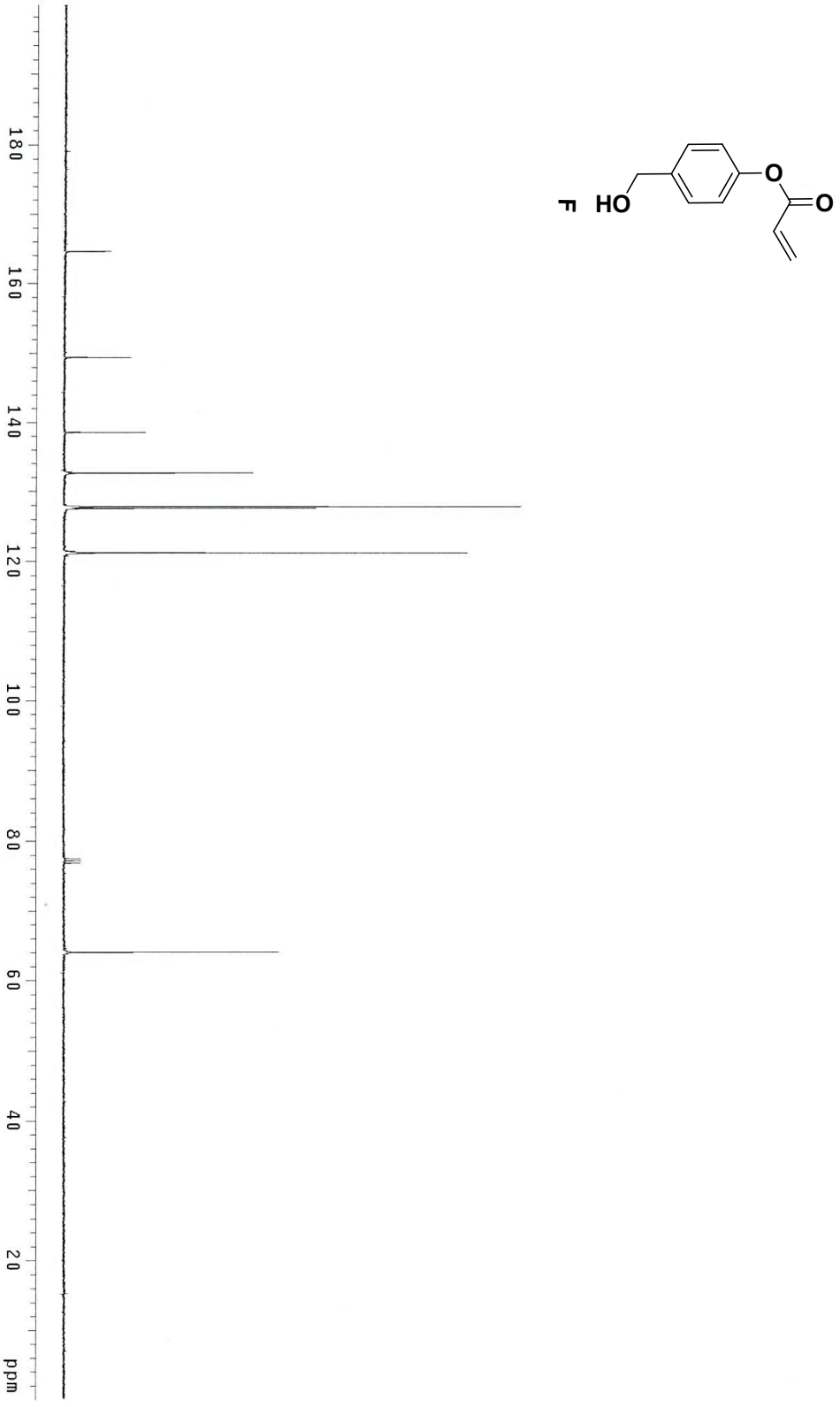


Figure S14. ^{13}C-NMR spectrum (100 MHz) of **F** in $CDCl_3$

Figure S15. HRMS spectrum of **F**

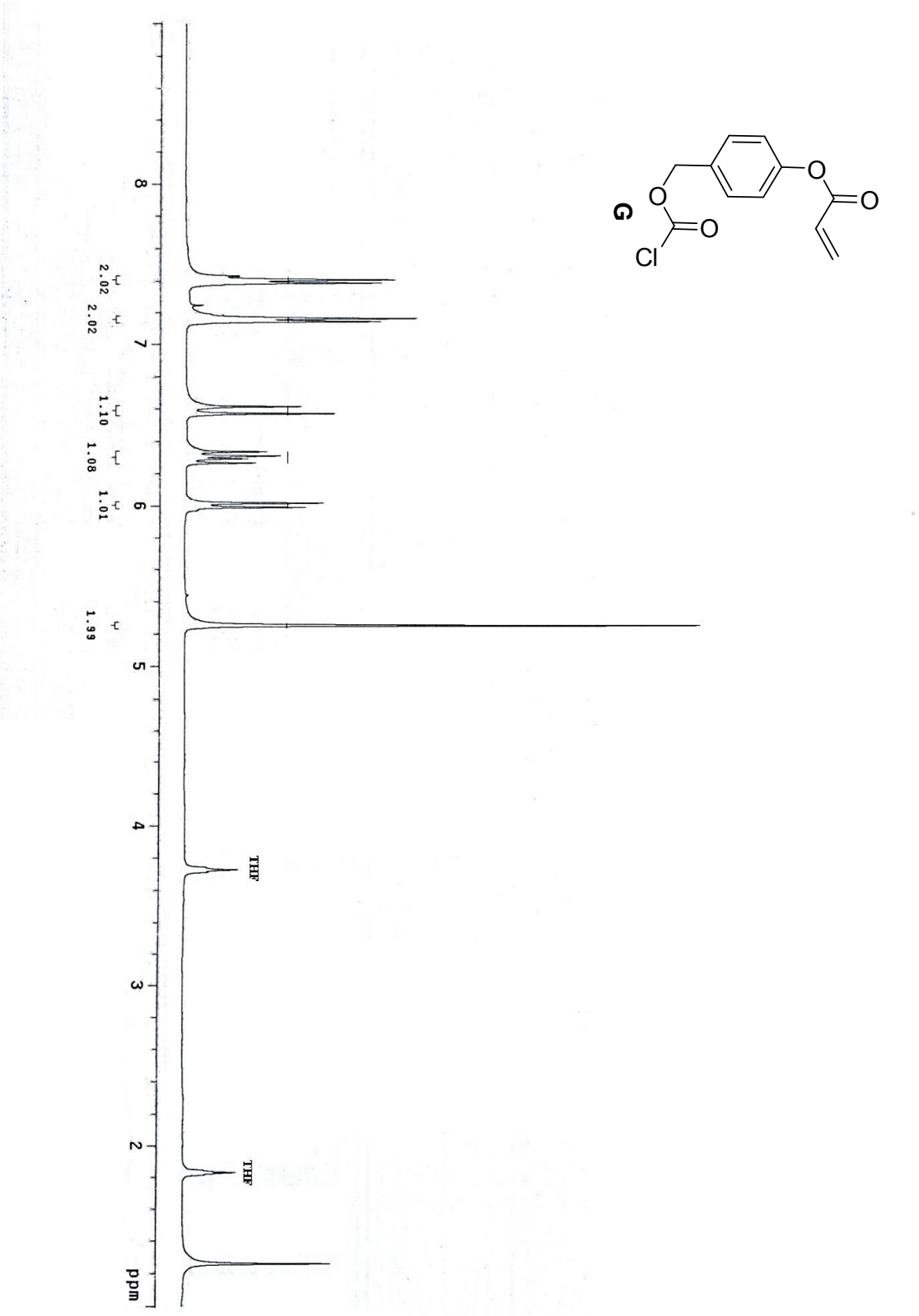


Figure S16. ^{1}H-NMR spectrum (400 MHz) of **G** in $CDCl_3$

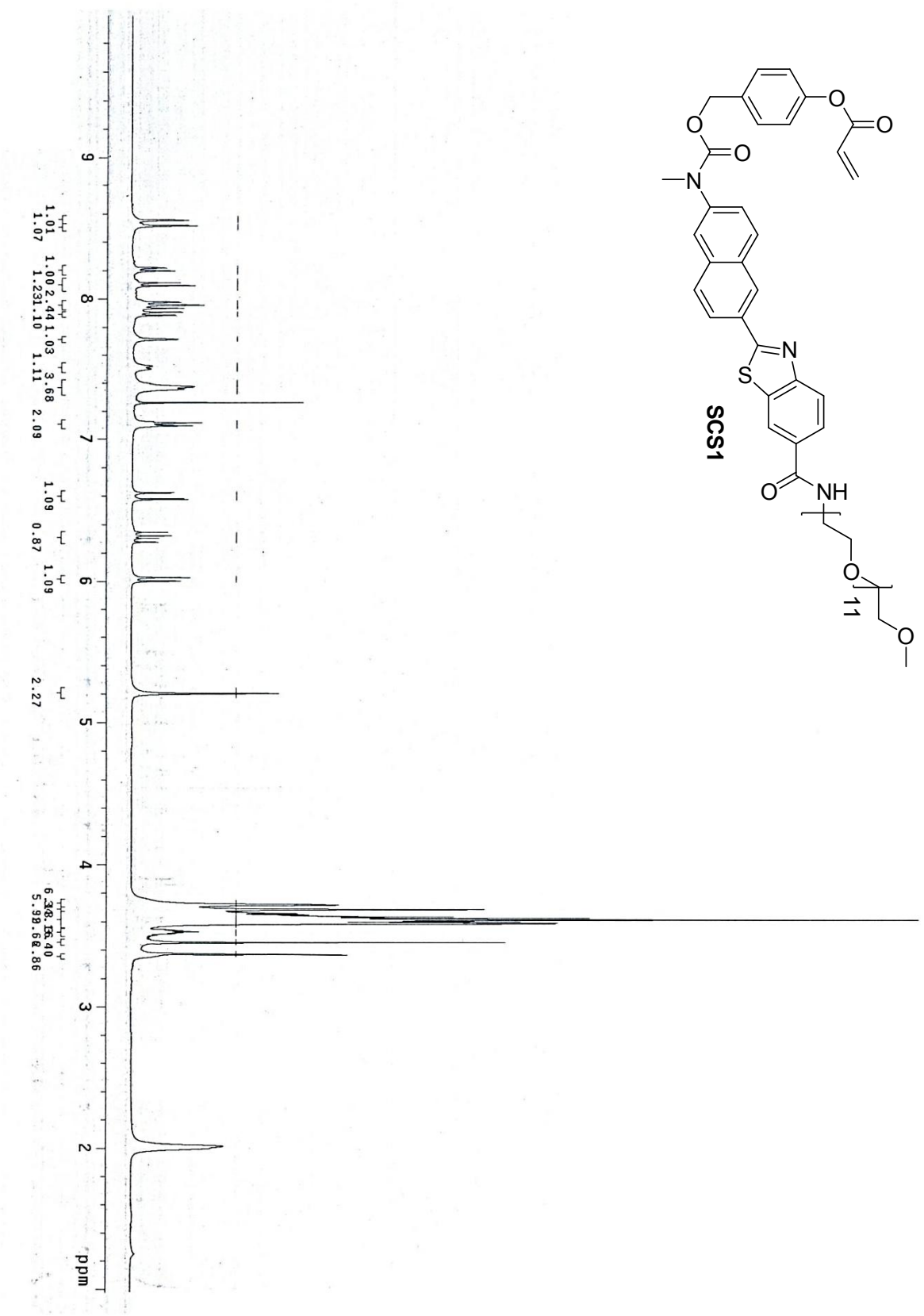


Figure S17. ^{1}H-NMR spectrum (400 MHz) of **SCS1** in $CDCl_3$

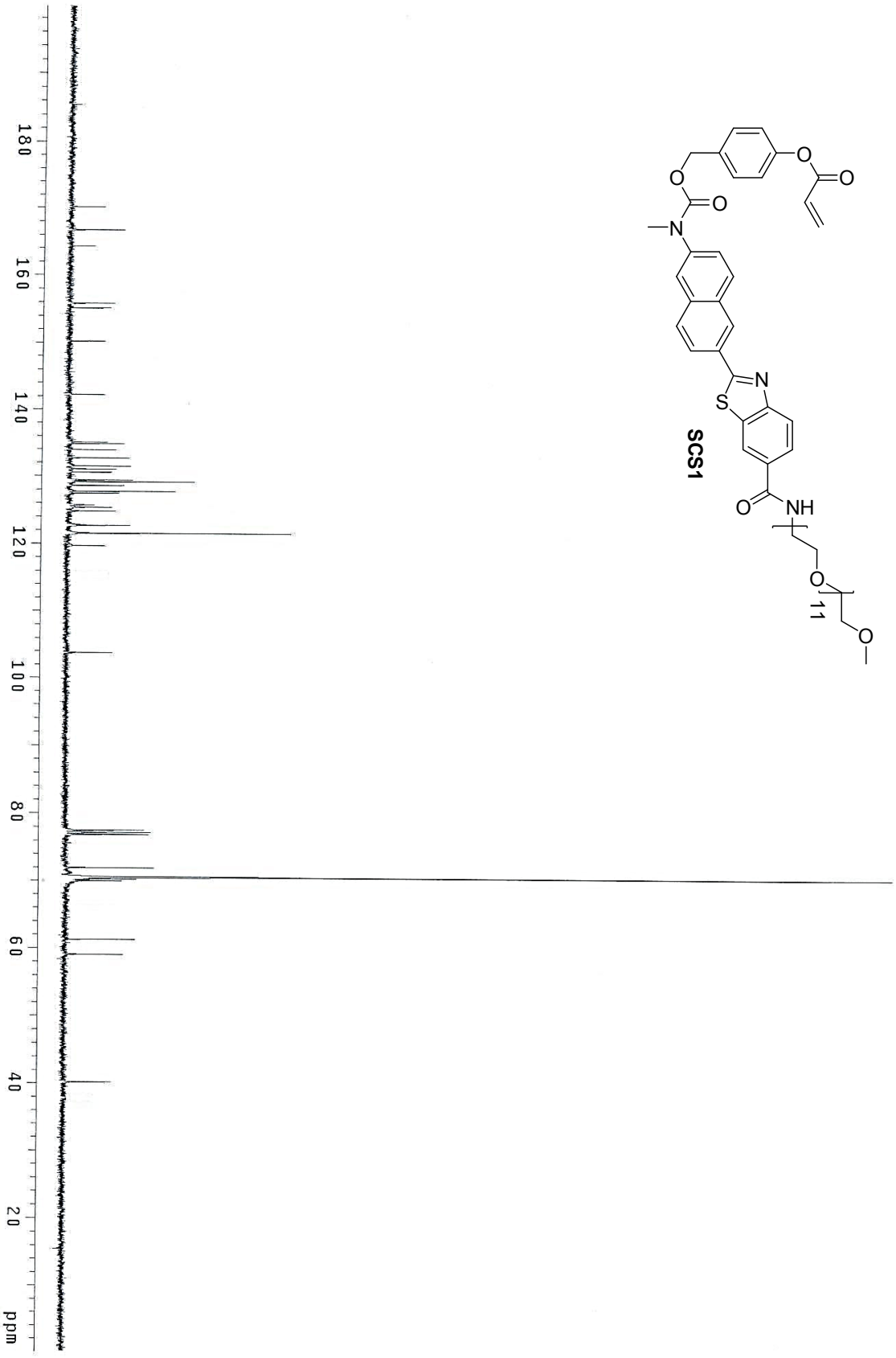


Figure S18. ^{13}C-NMR spectrum (100 MHz) of **SCS1** in $CDCl_3$

Figure S19. HRMS spectrum of **SCS1**

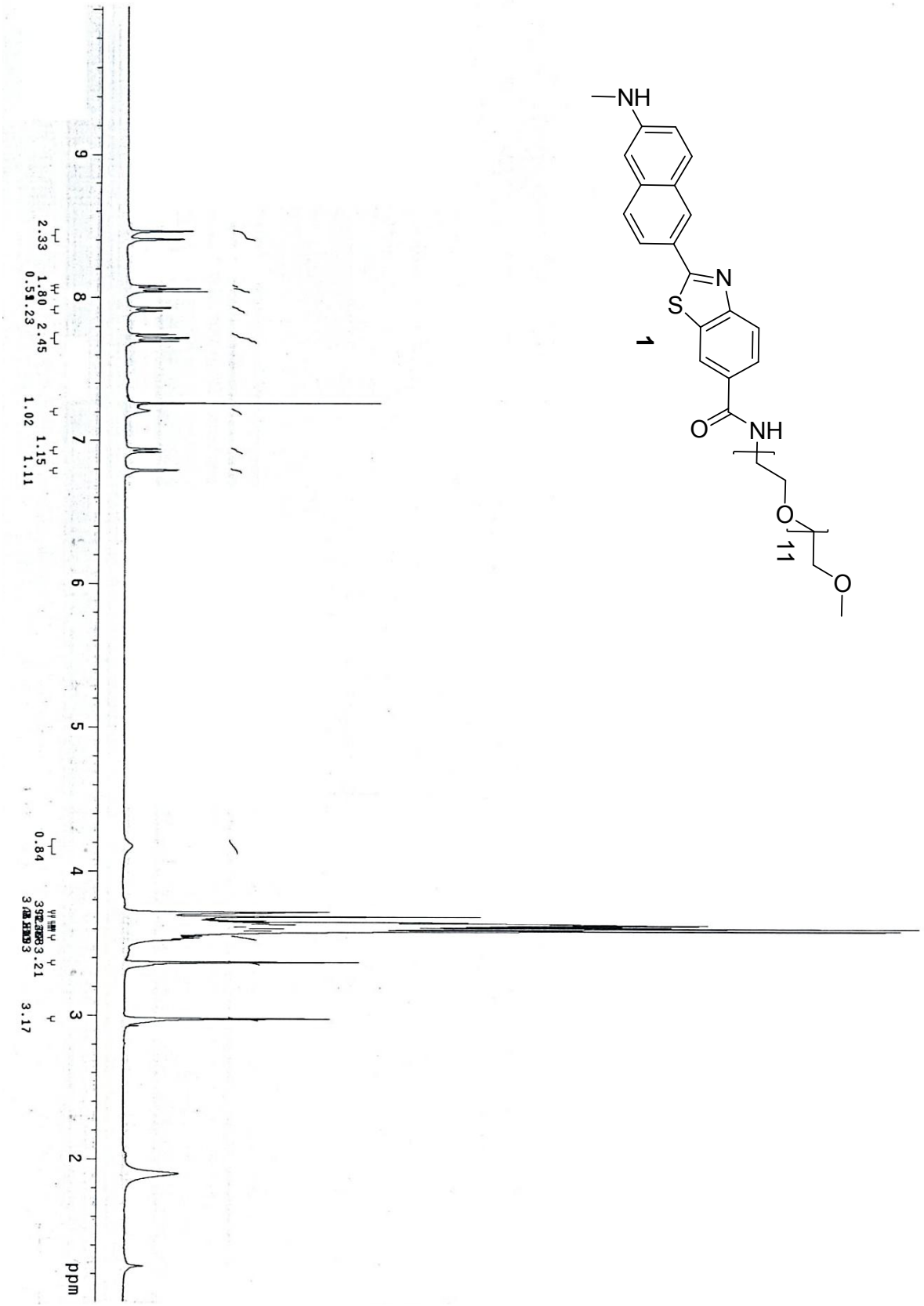


Figure S20. ^{1}H-NMR spectrum (400 MHz) of **1** in $CDCl_3$

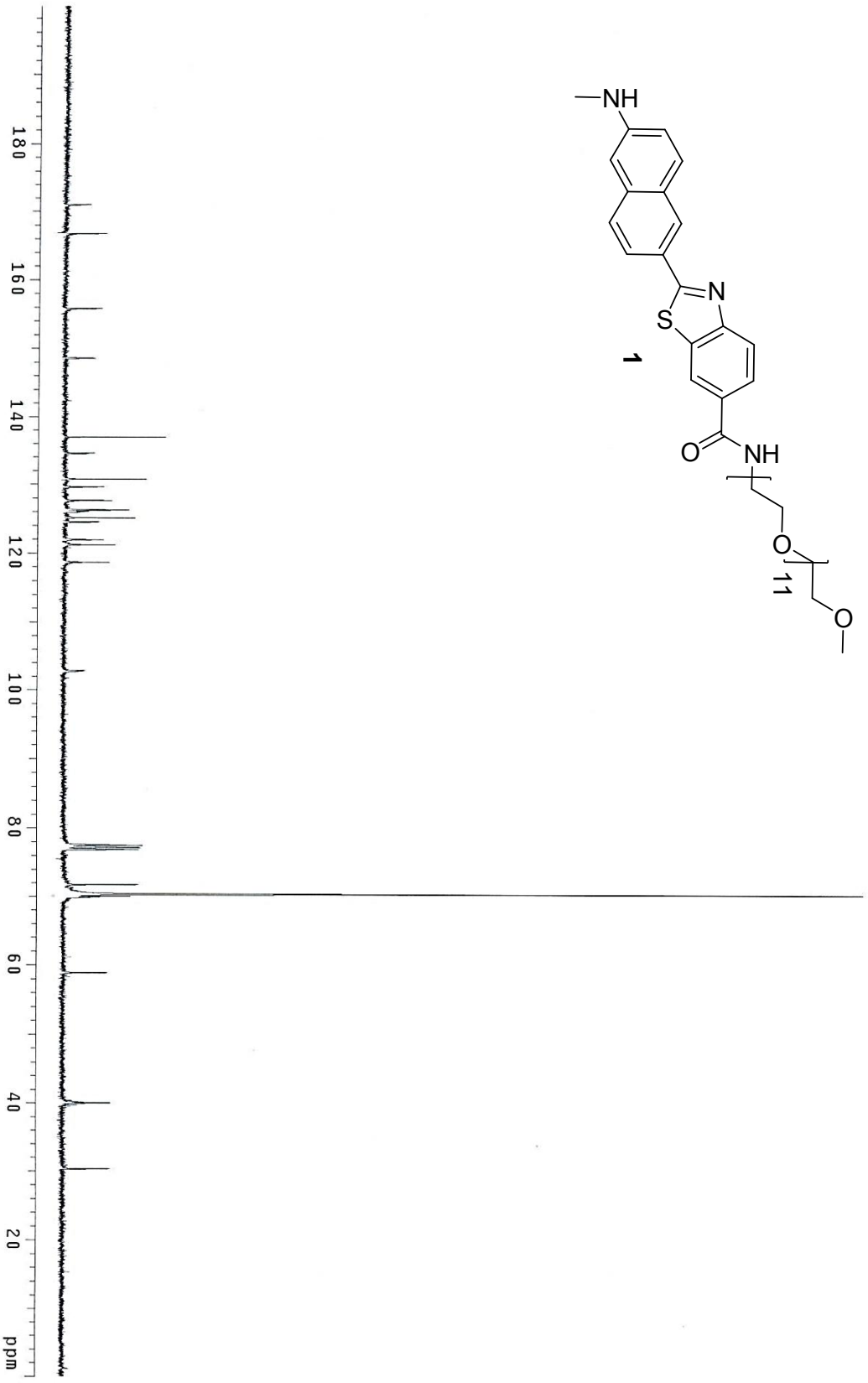


Figure S21. ^{13}C-NMR spectrum (100 MHz) of **1** in $CDCl_3/d_6$-DMSO

Figure S22. HRMS spectrum of **1**

References

1. L. V. N. R. Ganapatibhotla, J. Zheng, D. Roy and S. Krishnan, *Chem. Mater.* 2010, **22**, 6347-6360.
2. W. Liu, A. B. Greytak, J. Lee, C. R. Wong, J. Park, L. F. Marshall, W. Jiang, P. N. Curtin, A. Y. Ting, D. G. Nocera, D. Fukumura, R. K. Jain and M. G. Bawendi, *J. Am. Chem. Soc.* 2010, **132**, 472-483.
3. C. S. Lim, G. Masanta, H. J. kim, J. H. Han, H. M. Kim and B. R. Cho, *J. Am. Chem. Soc*. 2011, **133**, 11132-11135.
4. J. N. Demas and G. A. Crosby, *J. Phys*, *Chem*. 1971, **75**, 991-1024.
5. H. M. Kim, H. J. Choo, S. Y. Jung, Y. G. Ko, W.-H. Park, S. J. Jeon, C. H. Kim, T. Joo and B. R. Cho, *ChemBioChem.* 2007, **8**, 553-559.
6. B. Zhu, X. Zhang, Y. Li, P. Wang, H. Zhang and X. Zhuang, *Chem. Commun.*, 2010, **46**, 5710-5712.
7. P. K. Weiner and P. A. Kollman, *J. Comput. Chem.* 1981, **2**, 287-303.
8. S. K. Lee, W. J. Wang, J. J. Choi, C. H. Kim, S. J. Jeon and B. R. Cho, *Org. Lett*. 2005, **7**, 323-326.
9. N. S. Makarov, M. Drobizhev and A. Rebane, *Opt. Express* 2008, **16**, 4029-4047.