

Supporting Information for

Carboxylesterase-2-Selective Two-Photon Ratiometric Probe
Reveals Decreased Carboxylesterase-2 Activity in Breast Cancer
Cells

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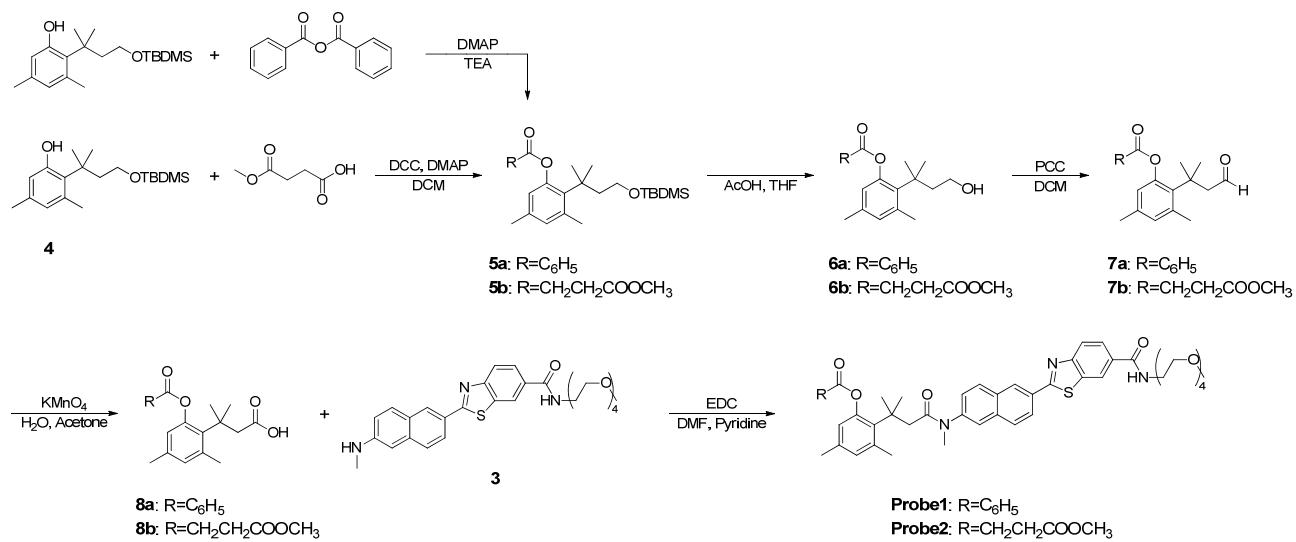
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Synthesis of Probe1 and Probe2. Compound **3** and **4** were prepared by the literature methods^{1,2} and synthesis of **Probe1** and **Probe2** are described below.



Scheme S1. Synthesis of **Probe1** and **Probe2**.

2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenyl benzoate (5a**).** A mixture of **4** (500 mg, 1.55 mmol), benzoic anhydride (1753 mg, 7.75 mmol) and 4-dimethylaminopyridine (189 mg, 1.55 mmol) in triethylamine (2 mL) was stirred overnight under nitrogen atmosphere at. Then reaction mixture was extracted with dichloromethane (10 mL), and washed with water (5 mL) and brine (5 mL × 2). The organic phase was concentrated under reduced pressure and purified with column chromatography (hexane/ethyl acetate=20:1) to obtain **5a** (443 mg) as a colorless oil. Yield: 67 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 8.21 (d, *J* = 7.6 Hz, 2H), 7.64 (t, *J* = 7.6 Hz, 1H), 7.52 (t, *J* = 7.6 Hz, 2H), 6.87 (s, 1H), 6.67 (s, 1H), 3.56 (t, *J* = 7.6 Hz, 2H), 2.57 (s, 3H), 2.26 (s, 3H), 2.08 (t, *J* = 7.6 Hz, 2H), 1.50 (s, 6H), 0.86 (s, 9H), -0.01 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 165.7, 150.0, 138.4, 136.0, 134.5, 133.4, 132.4, 130.3, 130.1, 128.6, 123.2, 61.0, 46.2, 39.3, 32.2, 26.2, 25.6, 20.5, 18.5, -5.0.

2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenyl methyl succinate (5b**).** A mixture of mono-Methyl hydrogen succinate (82 mg, 0.620 mmol) and *N,N*-dicyclohexylcarbodiimide (DCC, 154 mg, 0.744 mmol) in dichloromethane (5 mL) was stirred at 0 °C for 30 min. Then a solution **4** (200 mg, 0.620 mmol) and 4-(dimethylamino)pyridine (DMAP, 154 mg, 0.744 mmol) in dichloromethane (5 mL) was added dropwise into the above solution then stirred overnight under nitrogen atmosphere at room temperature. Precipitate dicyclohexylurea was removed by filtration and the filtrate was washed with brine (10 mL × 3). The organic phase was concentrated under reduced pressure and purified with column chromatography (hexane/ethyl

acetate=9:1) to obtain **5b** (236 mg) as a colorless oil. Yield: 87 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 6.81 (s, 1H), 6.57 (s, 1H), 3.72 (s, 3H), 3.49 (t, *J* = 7.2 Hz, 2H), 2.86 (t, *J* = 6.8 Hz, 2H), 2.74 (t, *J* = 6.8 Hz, 2H), 2.52 (s, 3H), 2.23 (s, 3H), 2.04 (t, *J* = 7.2 Hz, 2H), 1.48 (s, 6H), 0.86 (s, 9H), -0.01 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 172.4, 171.3, 149.6, 138.2, 135.9, 133.9, 132.2, 122.9, 60.8, 52.0, 46.1, 39.2, 32.0, 30.1, 28.9, 26.1, 25.4, 20.4, 18.4, -5.1.

2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenyl benzoate (6a). A compound **5a** (440 mg, 1.03 mmol) was dissolved in a co-solvent of THF (10 mL) and deionized water (5 mL) then stirred in ice bath for 30 min. Glacial acetic acid (10 mL) was added into the above solution and stirred overnight at 0 °C. After evaporation of solvent, the residue was dissolved ethyl acetate (50 mL) and washed with water (30 mL), 10 % sodium bicarbonate (30 mL) and brine (30 mL × 2). The organic phase was dried over anhydrous sodium sulfate and solvent was evaporated, to obtain **6a** (257 mg) as a colorless oil. Yield: 92 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 8.21 (d, *J* = 7.6 Hz, 2H), 7.65 (t, *J* = 7.6 Hz, 1H), 7.52 (t, *J* = 7.6 Hz, 2H), 6.88 (s, 1H), 6.67 (s, 1H), 3.58 (t, *J* = 7.2 Hz, 2H), 2.58 (s, 3H), 2.26 (s, 3H), 2.07 (t, *J* = 7.2 Hz, 2H), 1.84 (brs, 1H), 1.52 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 166.3, 149.9, 138.6, 136.3, 134.2, 133.6, 132.5, 130.3, 129.9, 128.7, 123.3, 60.6, 45.9, 39.3, 32.3, 25.6, 20.5.

2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenyl methyl succinate (6b). By following synthetic procedure for **6a**. **6b** was obtained from **5b** as a colorless oil. Yield: 97 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 6.80 (s, 1H), 6.56 (s, 1H), 3.70 (s, 3H), 3.49 (t, *J* = 7.6 Hz, 2H), 2.87 (t, *J* = 6.8 Hz, 2H), 2.73 (t, *J* = 6.8 Hz, 2H), 2.51 (s, 3H), 2.22 (s, 3H), 2.04 (t, *J* = 7.6 Hz, 2H), 1.85 (brs, 1H), 1.49 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 172.7, 171.7, 149.6, 138.2, 136.0, 133.8, 132.4, 123.0, 60.4, 52.1, 45.9, 39.2, 32.1, 30.0, 28.8, 25.6, 20.4.

3,5-dimethyl-2-(2-methyl-4-oxobutan-2-yl)phenyl benzoate (7a). A compound **6a** (420 mg, 1.34 mmol) was dissolved in dichloromethane (10 mL). Then pyridine chlorochromate (PCC, 580 mg, 2.69 mmol) was added into above solution then stirred for 3 hours under nitrogen atmosphere at room temperature. The reaction mixture was filtered to collect the eluent, filtrate was evaporated under reduced pressure. After evaporation of solvent, the residue was purified with column chromatography (hexane/ethyl acetate=4:1) to obtain **6a** (233 mg) as a colorless oil. Yield: 75 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 9.62 (s, 1H), 8.20 (d, *J* = 7.6 Hz, 2H), 7.65 (t, *J* = 7.6 Hz, 1H), 7.53 (t, *J* = 7.6 Hz, 2H), 6.91 (s, 1H), 6.73 (s, 1H), 2.86 (s, 2H), 2.59 (s, 3H), 2.27 (s, 3H), 1.60 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 202.5, 165.6, 149.6, 137.7, 136.8, 133.6, 132.9, 132.6, 130.1, 129.5, 128.6, 123.3, 56.7, 38.3, 31.8, 25.5, 20.3

3,5-dimethyl-2-(2-methyl-4-oxobutan-2-yl)phenyl methyl succinate (**7b**). By following synthetic procedure for **7a**. **7b** was obtained from **6b** as a colorless oil. Yield: 89 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 9.53 (s, 1H), 6.84 (s, 1H), 6.61 (s, 1H), 3.71 (s, 3H), 2.86 (t, *J* = 6.8 Hz, 2H), 2.83 (s, 2H), 2.74 (t, *J* = 6.8 Hz, 2H), 2.53 (s, 3H), 2.23 (s, 3H), 1.56 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 202.8, 172.4, 171.3, 149.2, 137.8, 136.8, 132.7, 132.5, 123.2, 56.7, 52.1, 38.3, 31.8, 30.1, 28.9, 25.6, 20.5.

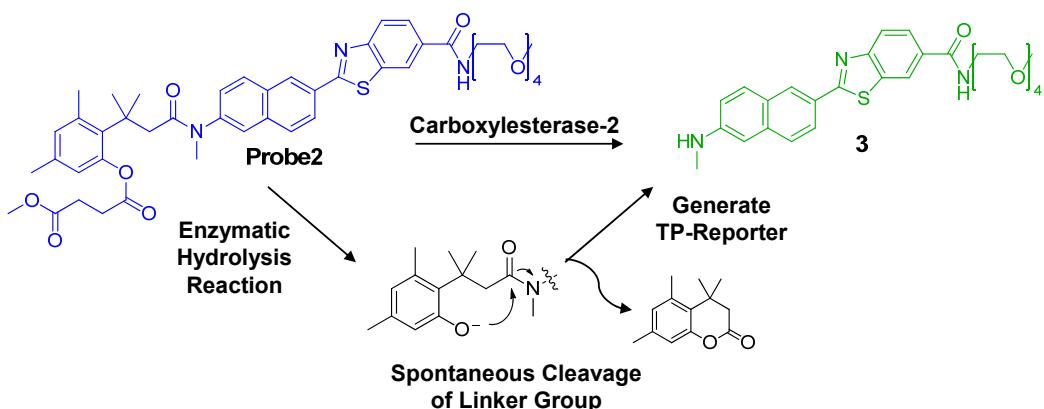
3-(2-(benzoyloxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (**8a**). A compound **7a** (200 mg, 0.644 mmol) was dissolved in a co-solvent of acetone (5 mL) and deionized water (5 mL). Then a solution of potassium permanganate (VII) (102 mg, 0.644 mmol) in a co-solvent of acetone (5 mL) and deionized water (5 mL) was added dropwise into the above solution then stirred overnight under room temperature. After evaporation of solvent, the residue was dissolved dichloromethane (30 mL) and washed with water (10 mL) and brine (10 mL × 2). The organic phase was dried over anhydrous sodium sulfate and solvent was evaporated, to obtain **8a** (191 mg) as a colorless oil. Yield: 91 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 8.19 (d, *J* = 7.6 Hz, 2H), 7.64 (t, *J* = 7.6 Hz, 1H), 7.51 (t, *J* = 7.6 Hz, 2H), 6.86 (s, 1H), 6.68 (s, 1H), 2.88 (s, 2H), 2.58 (s, 3H), 2.25 (s, 3H), 1.59 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 176.9, 165.9, 149.8, 138.2, 136.5, 133.7, 133.6, 132.6, 130.3, 129.8, 128.7, 123.2, 47.6, 39.1, 31.8, 25.6, 20.6.

3-(2-((4-methoxy-4-oxobutanoyl)oxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (**8b**). By following synthetic procedure for **8a**. **8b** was obtained from **7b** as a colorless oil. Yield: 98 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 6.81 (s, 1H), 6.59 (s, 1H), 3.71 (s, 3H), 2.88 (t, *J* = 6.8 Hz, 2H), 2.84 (s, 2H), 2.74 (t, *J* = 6.8 Hz, 2H), 2.53 (s, 3H), 2.22 (s, 3H), 1.57 (s, 6H); ¹³C NMR (CDCl₃, 100 MHz): δ (ppm) 177.3, 172.5, 171.4, 149.2, 138.0, 136.2, 133.3, 132.4, 122.9, 52.1, 47.7, 38.8, 31.4, 30.1, 28.9, 25.5, 20.4.

2-(4-((6-(6-(2,5,8,11-tetraoxatridecan-13-ylcarbamoyl)benzo[d]thiazol-2-yl)naphthalen-2-yl)(methyl)amino)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenyl benzoate (**Probe1**). A compound **7a** (200 mg, 0.613 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 235 mg, 1.23 mmol) was dissolved in a co-solvent of *N,N*-dimethylformamide (5 mL) and pyridine (5 mL) then stirred for 1 hour at room temperature. Then, **3** (160 mg, 0.306 mmol) was added into the above solution then stirred 2 days under nitrogen atmosphere at room temperature. After evaporation of solvent, the residue was dissolved ethyl acetate (30 mL) and washed with water (10 mL) and brine (10 mL × 2). The organic phase was concentrated under reduced pressure and purified with column chromatography (chloroform/methanol=10:1) to obtain **Probe1** (46 mg) as a white semi-solid. Yield: 18 %; ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 8.53 (s, 1H), 8.51 (d, *J* =

1.6 Hz, 1H), 8.22 (dd, J = 8.8 Hz, 1.6 Hz, 1H), 8.12 (d, J = 8.8 Hz, 1H), 7.96 (dd, J = 8.8 Hz, 1.6 Hz, 1H), 7.90 (d, J = 7.6 Hz, 2H), 7.88 (d, J = 8.8 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.53 (t, J = 7.6 Hz, 1H), 7.39 (s, 1H), 7.33 (t, J = 7.6 Hz, 2H), 7.25 (brs, 1H), 7.18 (d, J = 8.8, 1H), 6.76 (s, 1H), 6.62 (s, 1H), 3.72-3.64 (m, 12H), 3.62-3.59 (m, 2H), 3.52-3.49 (m, 2H), 3.32 (s, 3H), 3.24 (s, 3H), 2.79 (s, 2H), 2.41 (s, 3H), 2.25 (s, 3H), 1.54 (s, 6H); ^{13}C NMR (CDCl_3 , 100 MHz): δ (ppm) 170.8, 169.8, 166.6, 165.5, 155.8, 149.6, 143.2, 138.4, 135.7, 135.1, 134.9, 134.3, 133.3, 132.2, 131.7, 131.1, 130.4, 129.9, 129.8, 128.8, 128.4, 127.4, 126.7, 125.6, 125.3, 125.0, 123.0, 122.8, 121.5, 71.9, 70.6, 70.5, 70.3, 69.9, 59.0, 46.3, 40.2, 40.0, 37.8, 32.2, 29.8, 25.5, 20.5; HRMS (ESI $^+$): m/z calculated for $[\text{C}_{48}\text{H}_{54}\text{O}_8\text{N}_3\text{S}]^+$: 832.3626, found: 832.3660.

2-(4-((6-(6-(2,5,8,11-tetraoxatridecan-13-ylcarbamoyl)benzo[d]thiazol-2-yl)naphthalen-2-yl)(methyl)amino)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenyl methyl succinate (**Probe2**). By following synthetic procedure for **Probe1**. **Probe2** was obtained from **3** and **8b** as a white semi-solid. Yield: 11 %; ^1H NMR (CDCl_3 , 400 MHz): δ (ppm) 8.59 (s, 1H), 8.53 (d, J = 1.6 Hz, 1H), 8.26 (dd, J = 8.8 Hz, 1.6 Hz, 1H), 8.13 (d, J = 8.8 Hz, 1H), 7.98 (d, J = 8.8 Hz, 1H), 7.97 (d, J = 8.8 Hz, 1H), 7.85 (d, J = 8.8 Hz, 1H), 7.39 (s, 1H), 7.29 (brs, 1H), 7.24 (d, J = 8.8 Hz, 1H), 6.66 (s, 1H), 6.55 (s, 1H), 3.73-3.65 (m, 15H), 3.63-3.60 (m, 2H), 3.53-3.50 (m, 2H), 3.33 (s, 3H), 3.24 (s, 3H), 2.75 (s, 2H), 2.61 (s, 5H), 2.24 (s, 3H), 2.20 (s, 2H), 1.51 (s, 6H); ^{13}C NMR (CDCl_3 , 100 MHz): δ (ppm) 172.4, 171.1, 171.0, 170.0, 166.7, 155.9, 149.4, 143.4, 138.4, 135.8, 135.3, 135.0, 133.8, 132.3, 131.8, 131.7, 131.3, 130.4, 128.9, 127.5, 127.0, 125.9, 125.4, 125.2, 122.9, 122.8, 121.7, 72.0, 70.7, 70.6, 70.4, 70.1, 59.1, 52.1, 45.8, 40.3, 40.0, 37.9, 32.3, 30.0, 28.9, 25.4, 20.6; HRMS (ESI $^+$): m/z calculated for $[\text{C}_{46}\text{H}_{56}\text{O}_{10}\text{N}_3\text{S}]^+$: 842.3681, found: 842.3718.



Scheme S2. Structures of **Probe2** and **3**, and the proposed mechanism of the reaction of **Probe2** with carboxylesterase 2.

Spectroscopic Measurements. Absorption spectra were recorded on a 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 9, 10-diphenylanthrancene ($\Phi = 0.93$ in cyclohexane) as the reference by the literature method.^{3,4}

Water Solubility. Small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to $6.0 \times 10^{-6} \sim 5.0 \times 10^{-8}$ M and added to a cuvette containing 3.0 mL of PBS buffer (10 mM, pH 7.4) by using a micro syringe. In all cases, the concentration of DMSO in buffer was maintained to be 0.1 %.⁵ The plot of fluorescence intensity against the dye concentration were linear at low concentration and showed downward curvature at higher concentration (Figure S1). The maximum concentration in the linear region was taken as the solubility. The solubility of **Probe1** and **Probe2** in PBS buffer was $\sim 2.0, and $\sim 1.0\mu\text{M}$ while that of **3** was $\sim 3.0\mu\text{M}$ ¹ respectively.$

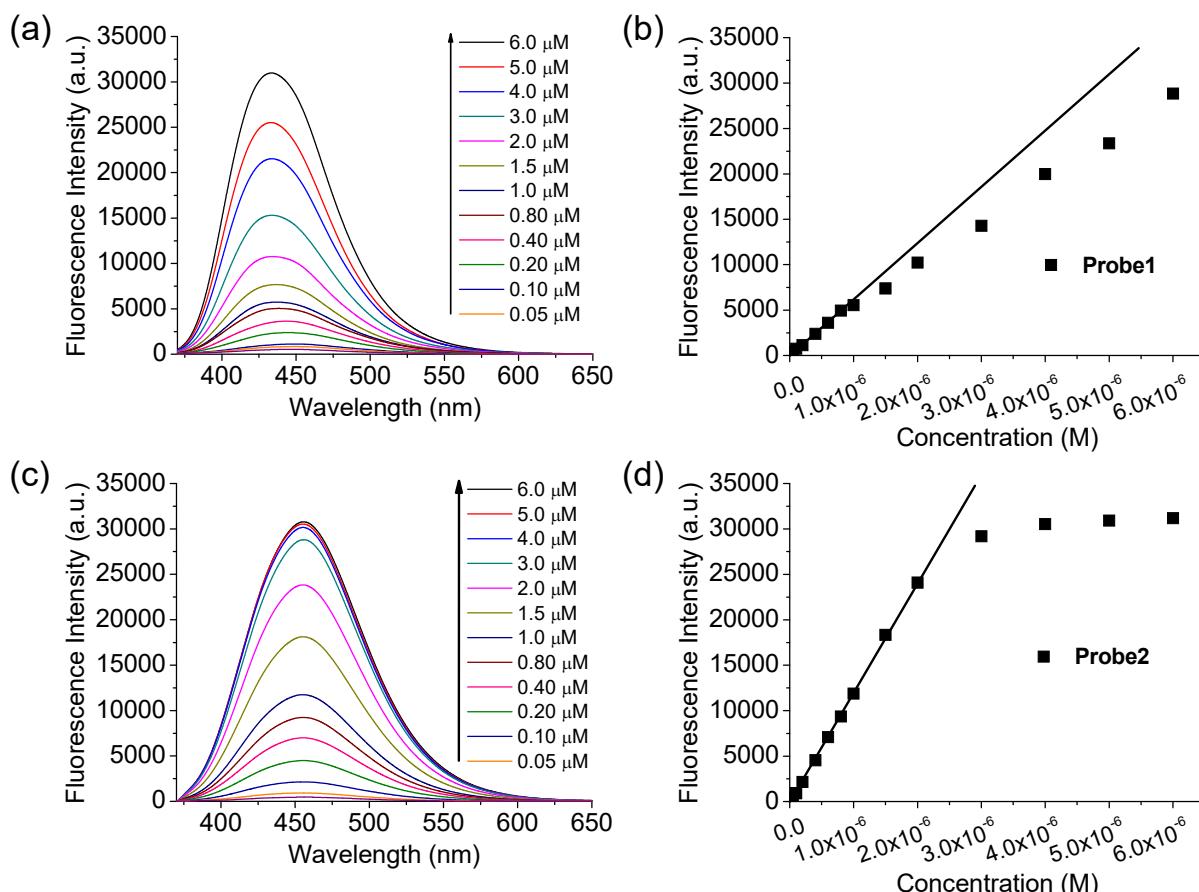


Figure S1. (a, c) One-photon fluorescence spectra and (b, d) plot of fluorescence intensity against the concentration of the dye for (a, b) **Probe1** and (c, d) **Probe2** in PBS buffer (10 mM, pH 7.4). The excitation wavelengths were 348 and 342 nm for **Probe1** and **Probe2**, respectively.

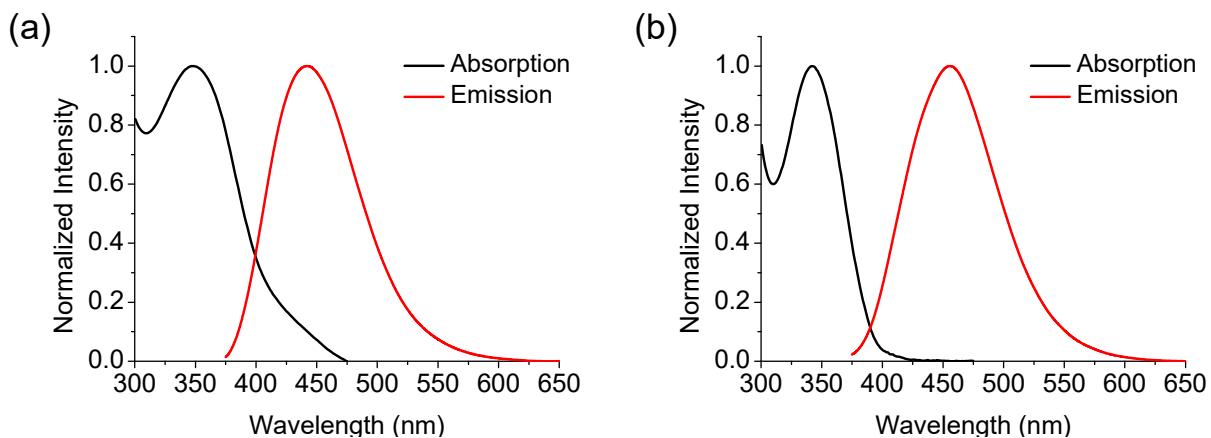


Figure S2. (a) Normalized one-photon absorption and emission spectra of (a) **Probe1** and (b) **Probe2** in PBS buffer (10 mM, pH 7.4).

Table S1. Photophysical data for **Probe1**, **Probe2** and **3** in buffer.

Compound	$\lambda_{max}^{(1)} \text{ (} 10^{-4} \varepsilon \text{)}^{\text{ac}}$	$\lambda_{max}^{fl}{}^{\text{ad}}$	Φ^{ae}	$\lambda_{max}^{(2)}{}^{\text{bf}}$	$\Phi \delta_{max}{}^{\text{bg}}$
Probe1	348 (2.16)	440	0.23	740	14
Probe2	342 (2.61)	455	0.57	740	15
3	378 (2.50)	540	0.16	750	72

Data were measured in a) PBS buffer (10 mM, pH 7.4, 37 °C) and b) PBS buffer containing 10 % DMF. c) λ_{max} of the one-photon absorption spectra in nm. The numbers in parentheses are molar extinction coefficients in $M^{-1} \text{ cm}^{-1}$. d) λ_{max} of the one-photon emission spectra in nm. e) Fluorescence quantum yield, $\pm 10\%$. f) λ_{max} of the two-photon emission spectra in nm. g) The peak two-photon action cross sections in GM (1 GM = $10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$).

Enzymatic Kinetics Assays. Enzymatic kinetic experiments were investigated using a Varioskan Flash micro plate reader (6–1536 well) with a 96-well plate. Various concentrations of **Probe1** and **Probe2** (0–60 μM) were prepared in PBS buffer solution (10 mM, pH = 7.4). Human carboxylesterase enzyme was added to a final concentration of 10 $\mu\text{g mL}^{-1}$, the fluorescence intensity was collected at 440 nm for **Probe1** and 455 nm for **Probe2**, respectively ($\lambda_{\text{ex}} = 373 \text{ nm}$) with 60 second intervals from 0 to 10 min at 37 °C. The kinetic parameters of Michaelis-Menten equation were calculated with hyperbolic function by the nonlinear fitting algorithm (OriginPro 8.0).

Table S2. Kinetic parameters for **Probe1** and **Probe2** with hCE2

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	V_{max} ($\text{nmol mg}^{-1} \text{s}^{-1}$)
Probe1	6.22 ± 0.30	0.03546	5.70×10^3	0.5911
Probe2	8.75 ± 0.37	0.03277	3.75×10^3	0.5463

Table S3. Kinetic parameters for **Probe1** and **Probe2** with hCE1

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	V_{max} ($\text{nmol mg}^{-1} \text{s}^{-1}$)
Probe1	33.01 ± 3.17	0.05297	1.605×10^3	0.8830
Probe2	15.27 ± 0.99	0.02845	1.866×10^3	0.4751

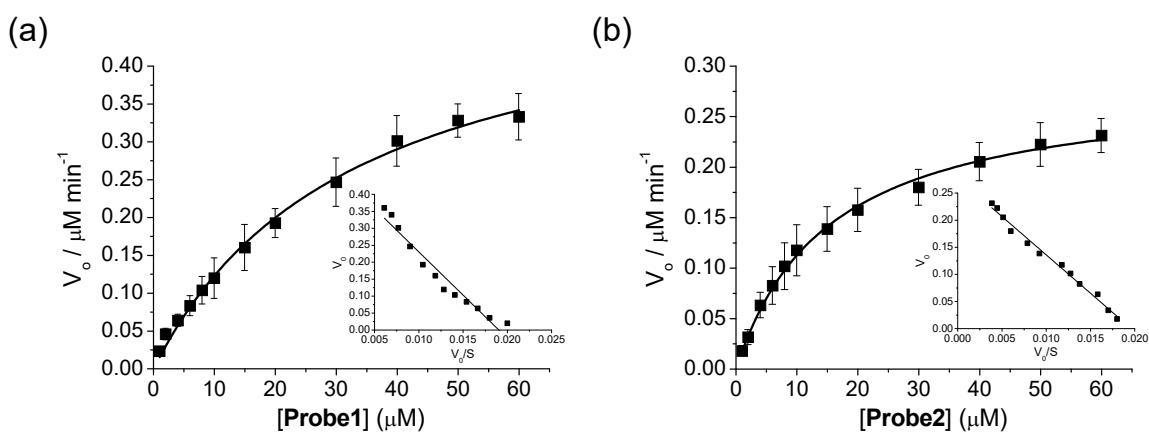


Figure S3. Plot of V_0 versus the concentration of (a) **Probe1** and (b) **Probe2** after addition of 10 $\mu\text{g mL}^{-1}$ hCE1 with 60 s intervals from 0 to 10 min. (inset) The corresponding Eadie-Hofstee plot. V_0 is the initial rate of reaction. S is the Probes concentration. The excitation wavelength was 373 nm.

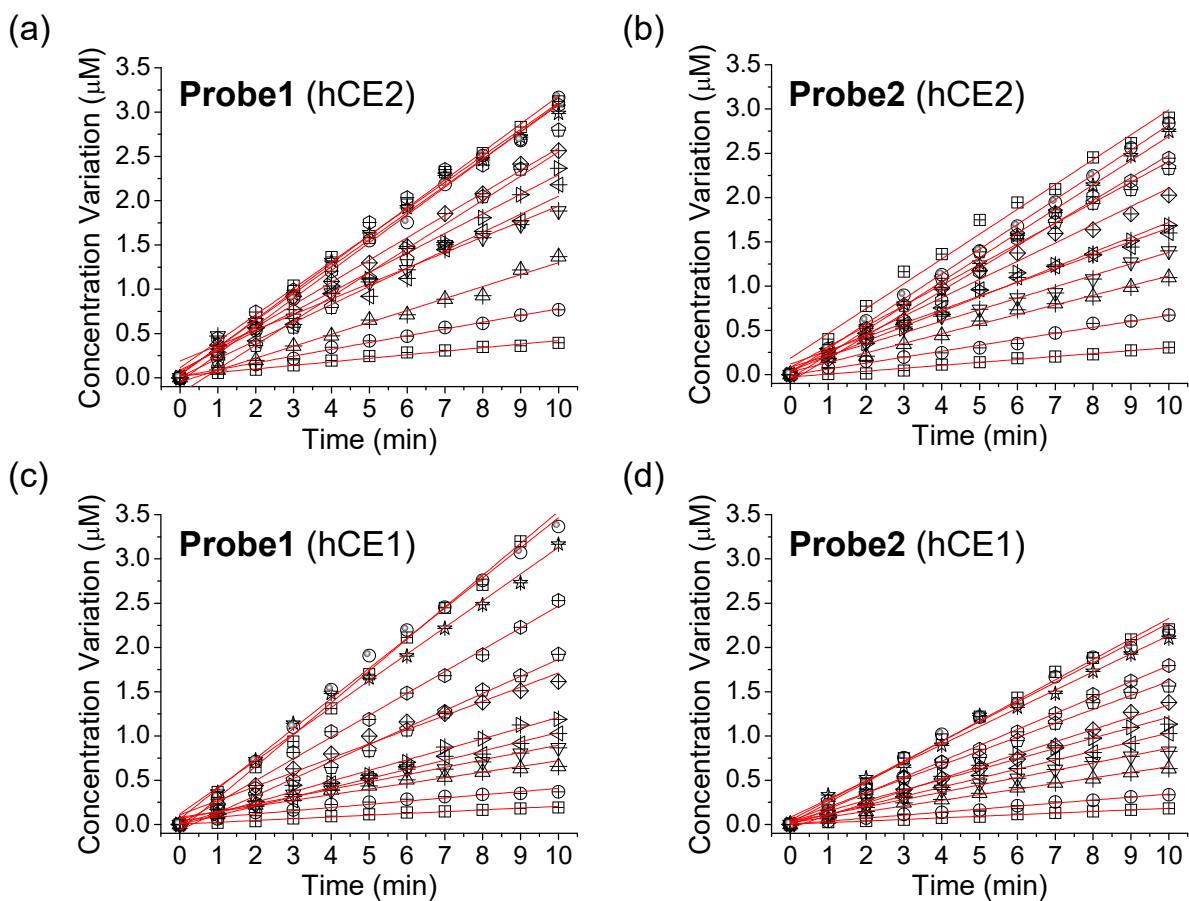


Figure S4. Concentration variation for the hydrolysis of probes after addition of $10 \mu\text{g mL}^{-1}$ (a, b) hCE2 and (c, d) hCE1 in PBS buffer (10 mM, pH 7.4) with 60 s intervals from 0 to 10 min. Initial probes concentration are 1, 2, 4, 6, 8, 10, 15, 20, 30, 40, 50 and 60 μM . The variation of the probe concentration was converted to the intensity of fluorescence that changed based on the initial fluorescence intensity according to each concentration. The excitation wavelength was 373 nm and the fluorescence intensity was collected at 440 nm for **Probe1** and 455 nm for **Probe2**, respectively.

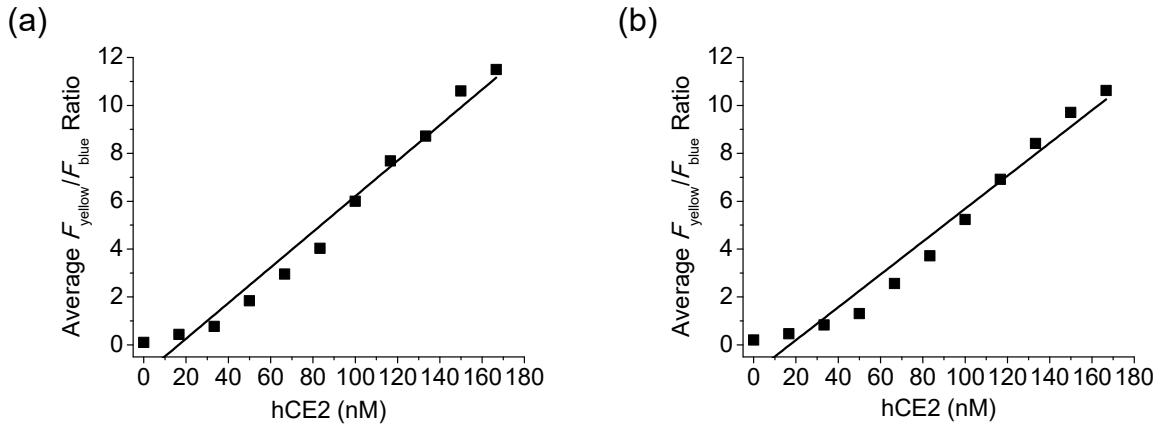


Figure S5. Plot of the average $F_{\text{yellow}}/F_{\text{blue}}$ ratios for (a) **Probe1** and (b) **Probe2** vs hCE2 in PBS buffer (10 mM, pH 7.4). Each data was acquired 40 min after hCE2 addition at 37 °C. The detection limit of **Probe1** and **Probe2** were 1.6 nM and 7.7 nM, respectively, which were calculated with $3\sigma/k$; where σ is the standard deviation of blank measurement, k is the slope. The excitation wavelength was 373 nm.

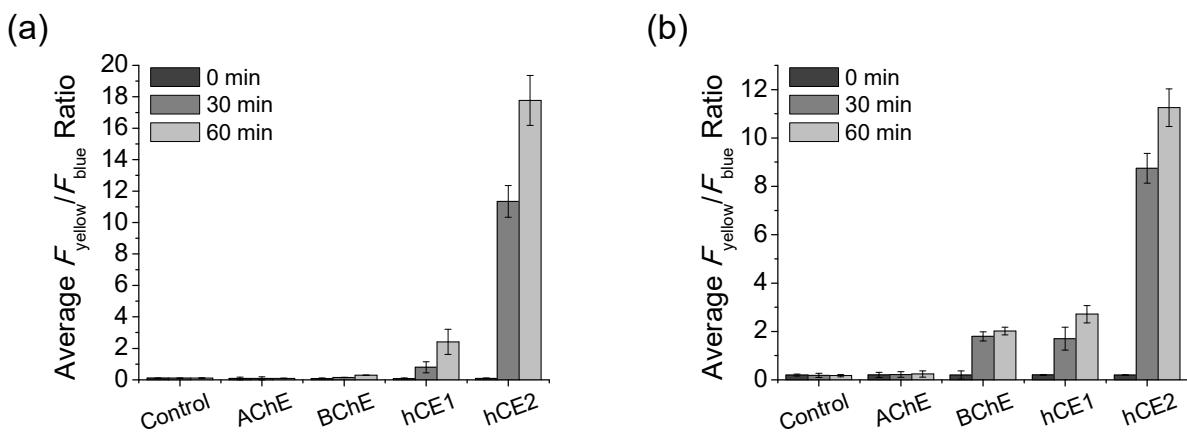


Figure S6. Fluorescence responses of (a) **Probe1** (1.0 μM) and (b) **Probe2** (1.0 μM) to 5 units mL^{-1} of AChE, BChE, hCE1, and hCE2. Bars represent the integrated fluorescence ratios ($F_{\text{yellow}}/F_{\text{blue}}$) 0 to 1 h after the addition of each species. The excitation wavelength was 373 nm.

Inhibitor Assays. MCF-10A cells were washed with PBS and homogenized using a bullet blender in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 % Triton X-100, 1 mM EDTA, and protease inhibitor cocktail (all reagents are from Sigma) for 30 min in ice. Centrifugation of the homogenate at 10,000 rpm for 15 min gave a clear supernatant which was collected into a new tube. 100 mg protein of the supernatant was incubated with 10 mM of each inhibitor (donepezil hydrochloride monohydrate for the AChE inhibitor, ethopropazine hydrochloride for the BChE inhibitor, naringenin for the PON1 inhibitor, lead acetate for the PON2 inhibitor, bis(4-nitrophenyl) phosphate (BNPP) for the CEs inhibitor and loperamide for CE2 inhibitor). The relative fluorescence intensity variation was acquire after pretreatment each inhibitor (10 μ M) for 20 min then incubation with probes (2 μ M) for 30 min. The excitation wavelength was 373 nm and the fluorescence intensity was collected at 540 nm.

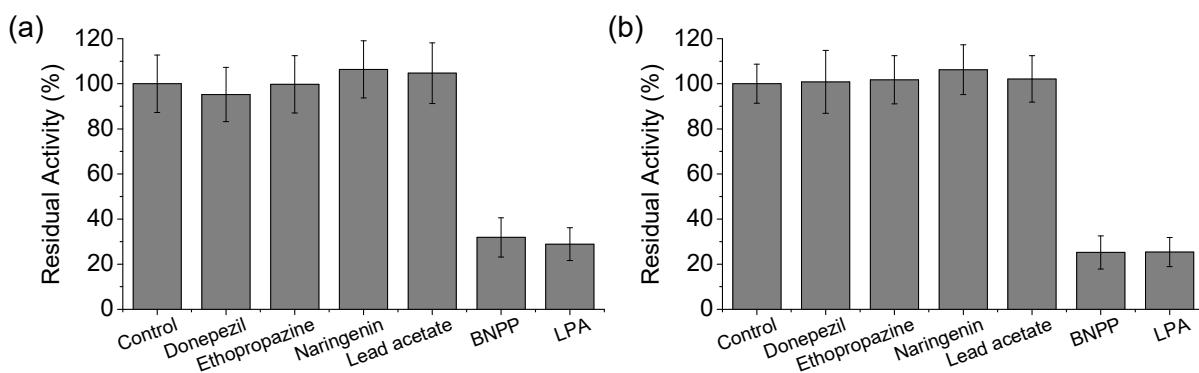


Figure S7. Fluorescence response of (a) **Probe1** and (b) **Probe2** to various inhibitors in homogenized MCF-10A cells (donepezil for AChE, ethopropazine for BChE, naringenin for PON1, lead acetate for PON2, BNPP for CEs and LPA for CE2). Bars represent the relative fluorescence intensity variation after pretreatment each inhibitor (10 μ M) for 20 min then incubation with probes (2 μ M) for 30 min. The excitation wavelength was 373 nm and the fluorescence intensity was collected at 540 nm.

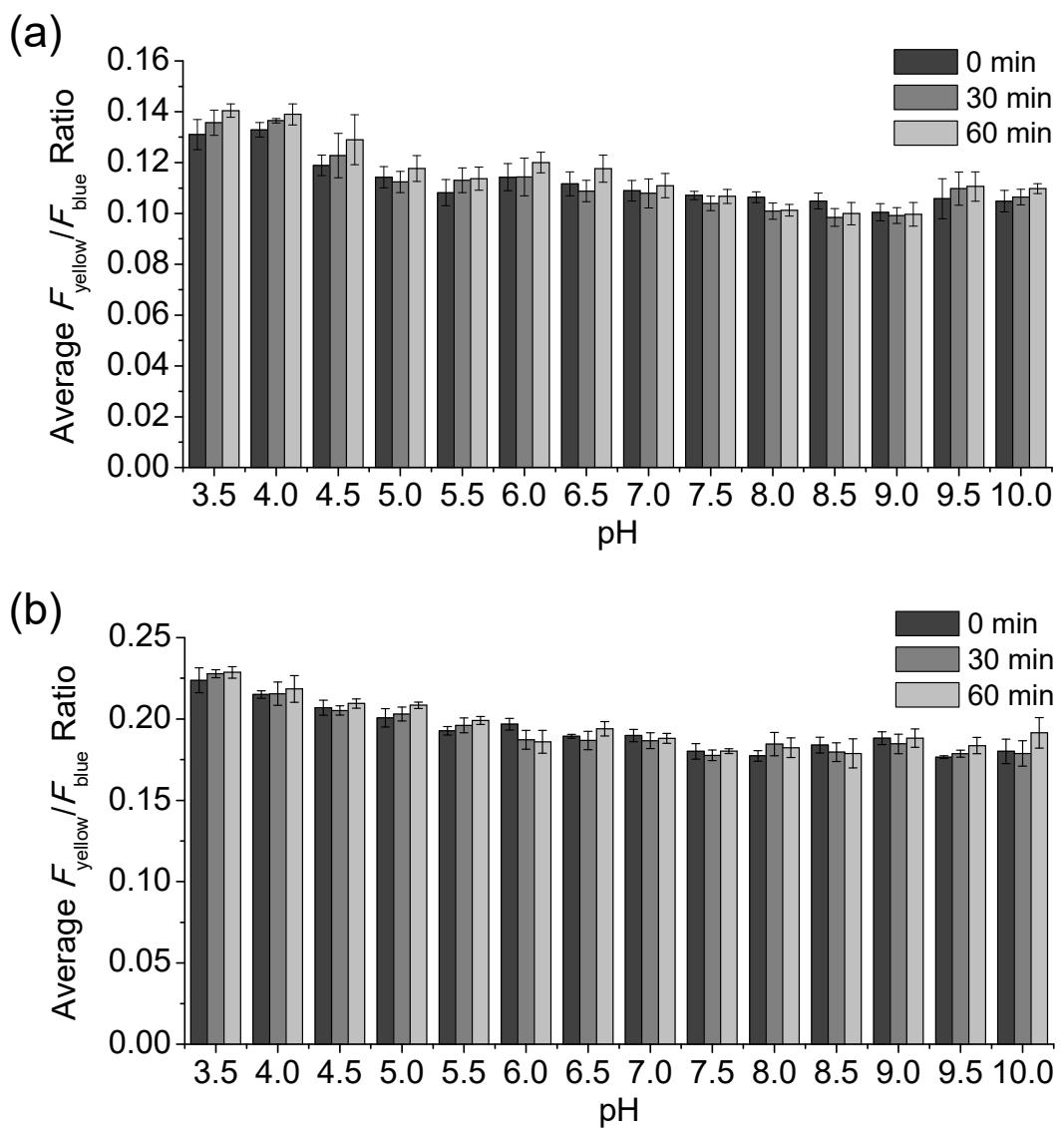


Figure S8. Effect of pH on the fluorescence intensity ratios ($F_{\text{yellow}}/F_{\text{blue}}$) for (a) **Probe1** and (b) **Probe2** in universal buffer (0.1 M citric acid, 0.1 M KH_2PO_4 , 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, 0.1 M Tris, 0.1 M KCl) at 37 °C. The excitation wavelength was 373 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.⁶ Probe (1.0×10^{-6} M) was dissolved in PBS buffer (10 mM, pH = 7.4) containing 10 % DMF and 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.⁷ 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 $\delta = \delta_r(S_s\Phi_r\varphi_r c_r)/(S_r\Phi_s\varphi_s c_s)$: 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.

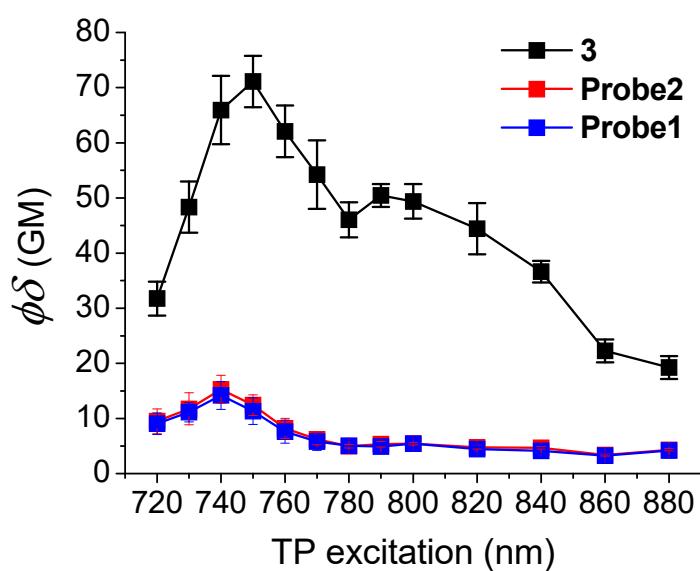


Figure S9. Two-photon action spectra of **Probe1**, **Probe2** and **3** in PBS buffer (10 mM, pH 7.4) containing 10 % DMF.

Cell viability. To evaluate the cytotoxic effect of **Probe1** and **Probe2** in RKO cells, CCK-8 kit (Cell Counting Kit-8; Dojindo, Japan) assay were performed according to the manufacture's protocol. The results are shown in Figure S10, which revealed that the **Probe1** and **Probe2** have low cytotoxicity at its different concentration in our incubation condition.

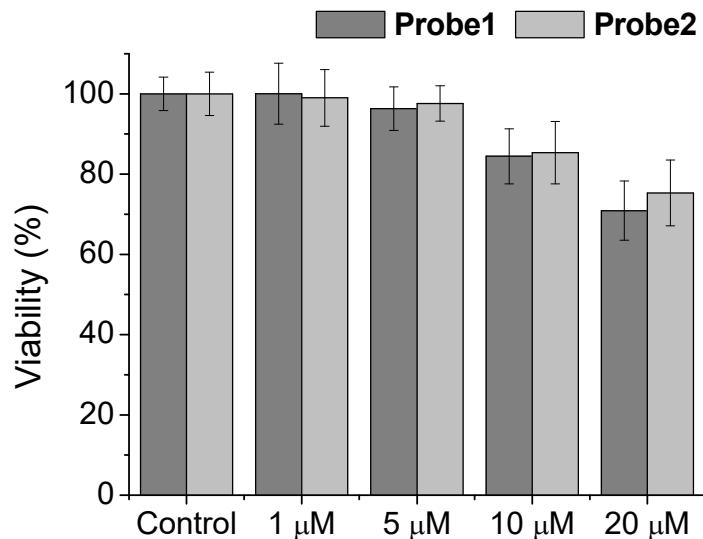


Figure S10. Viability of RKO cells in the presence of **Probe1** and **Probe2** as measured by using CCK-8 assays. The cells were incubated with 0–20 μM of probes for 2 h.

Photostability. Photostability of Probes was determined by monitoring the changes in TPEF intensity with time at three designated positions of **Probe1** and **Probe2** (2.0 μ M) in PBS buffer (10 mM, pH 7.4). The TPEF intensity of **Probe2** remained nearly the same for one hour, indicating high photostability.

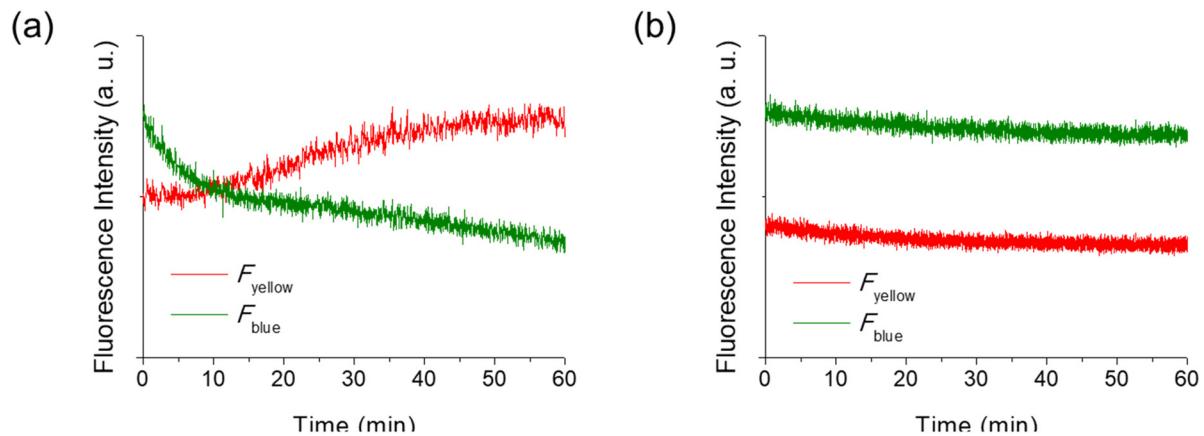


Figure S11. (a, b) The relative TPEF intensity of (a) **Probe1** and (b) **Probe2** in PBS buffer (10 mM, pH 7.4) as a function of time, respectively. The digitized intensities were recorded with 2.00 sec intervals for the duration of one hour using *xyt* mode. Data were acquired using 740 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (yellow) with femto-second pulses.

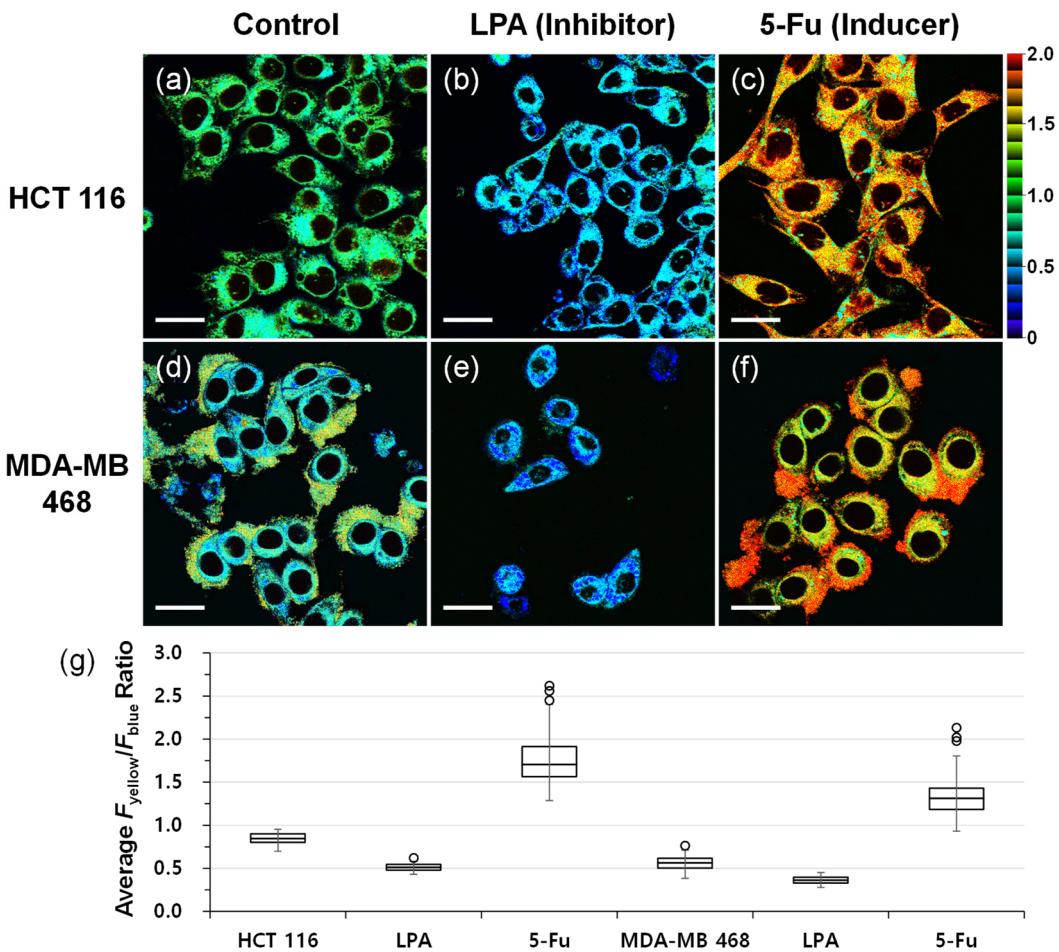


Figure S12. Pseudocolored ratiometric TPM images ($F_{\text{yellow}}/F_{\text{blue}}$) of (a-c) HCT 116 and (d-f) MDA-MB 468 cells labeled with **Probe2** (2.0 μM) for 2 h. (a, d) Control images. Cells were pretreated with (b, e) Loperamide (LPA, 100 μM) for 20 min and (c, f) 5-Fluorouracil (5-Fu, 10 μM) for 48 h before labeling with **Probe2**. (g) Average $F_{\text{yellow}}/F_{\text{blue}}$ intensity ratios in the TPM images. Images were acquired using 740 nm excitation and emission windows of 400–450 nm (blue) and 500–600 nm (yellow). Scale bars = 25 μm .

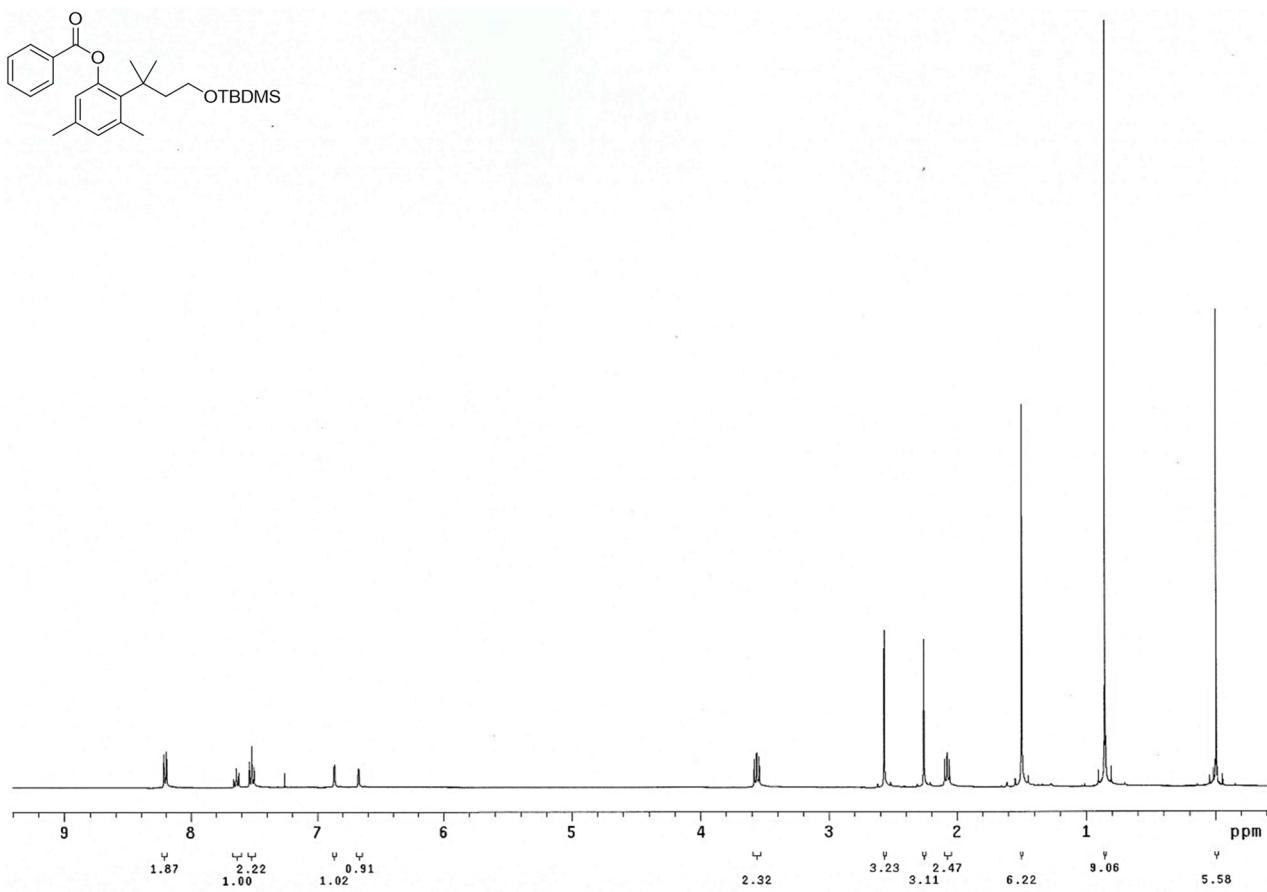


Figure S13. ¹H-NMR spectrum (400 MHz) of **5a** in CDCl₃

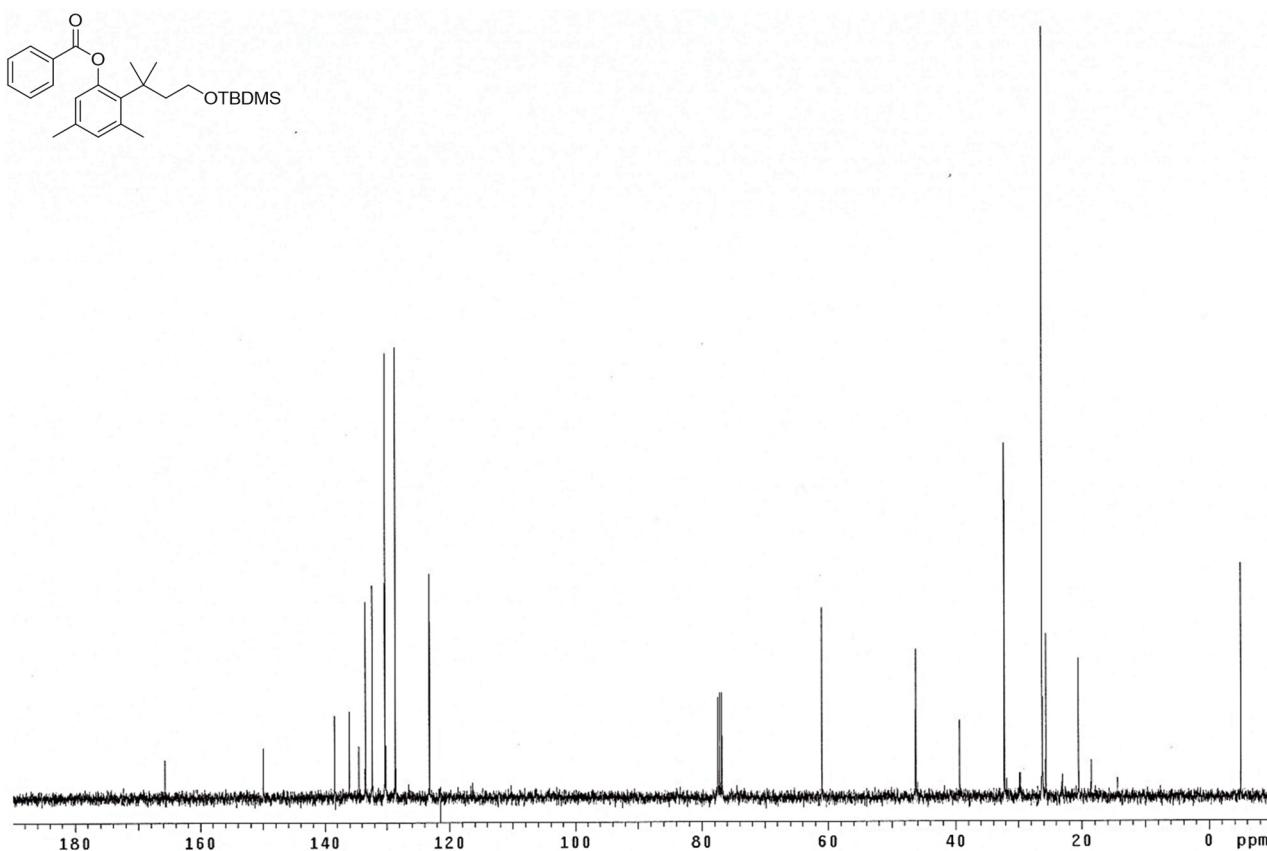


Figure S14. ¹³C-NMR spectrum (100 MHz) of **5a** in CDCl₃

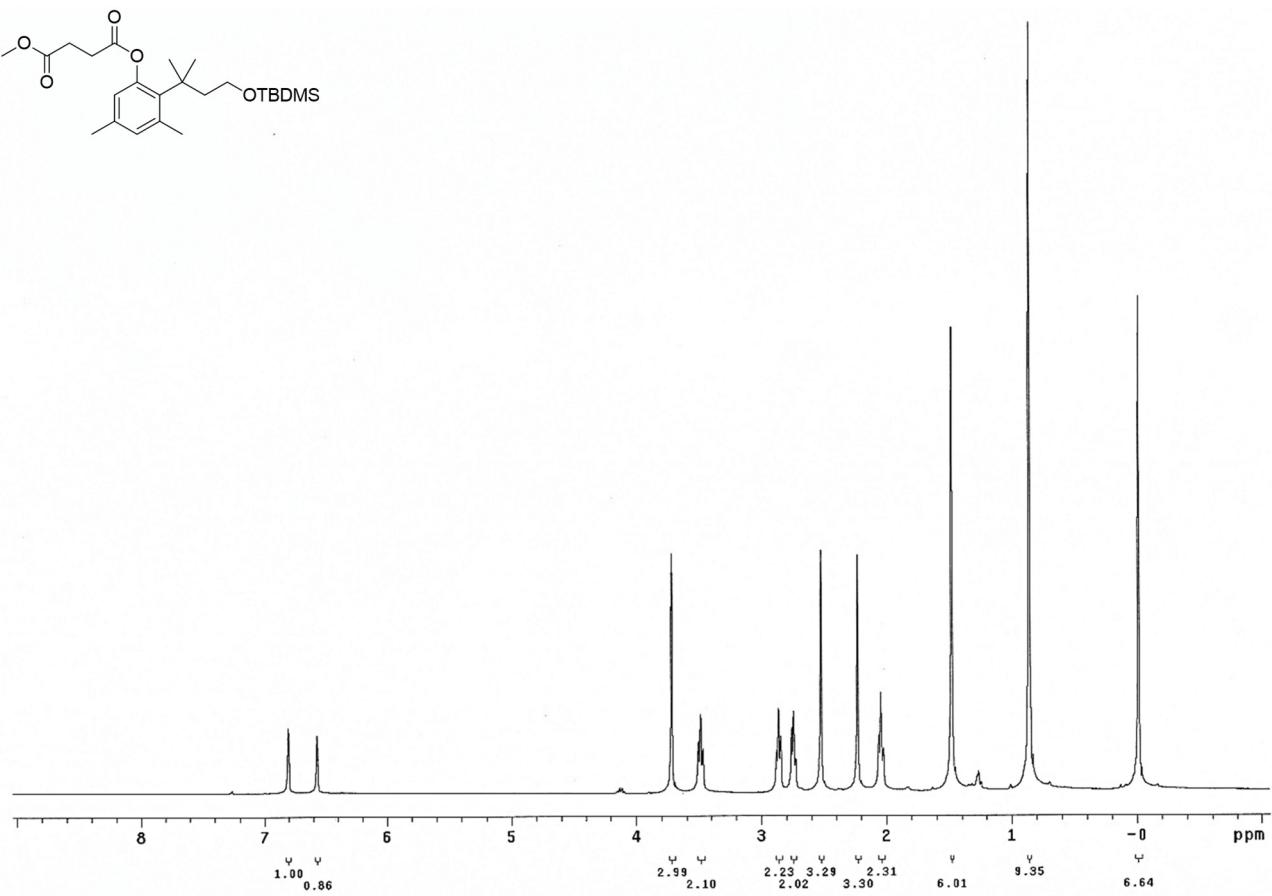


Figure S15. ¹H-NMR spectrum (400 MHz) of **5b** in CDCl₃

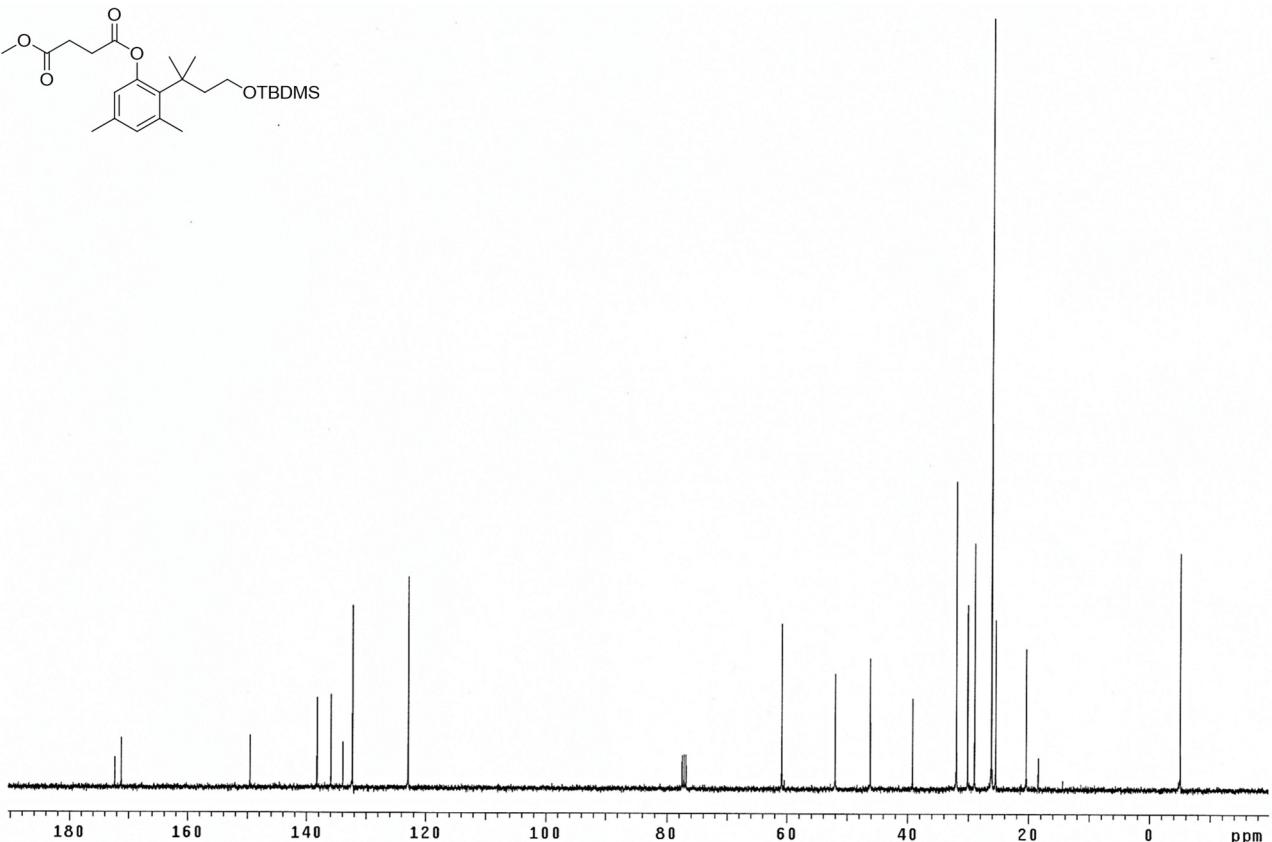


Figure S16. ¹³C-NMR spectrum (100 MHz) of **5b** in CDCl₃

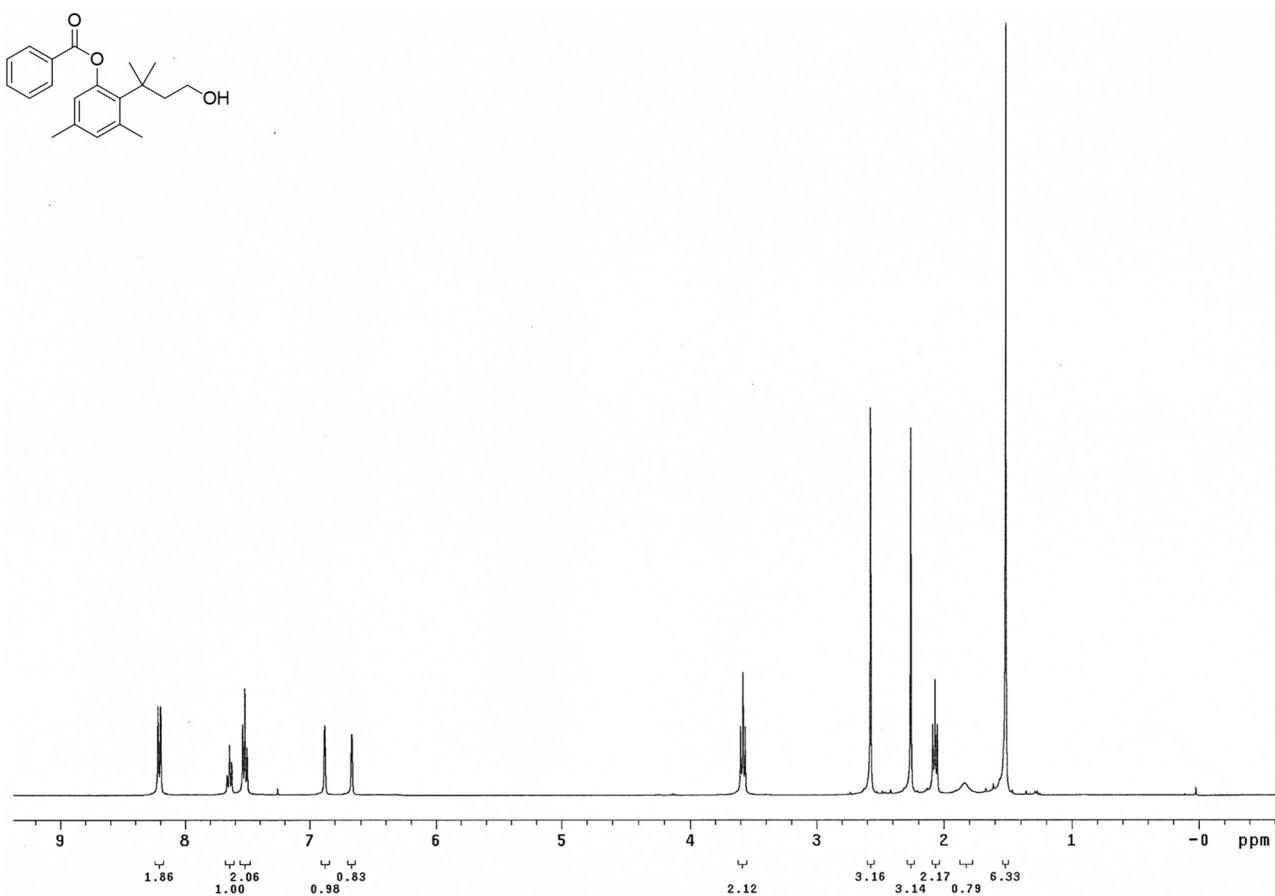


Figure S17. ¹H-NMR spectrum (400 MHz) of **6a** in CDCl₃

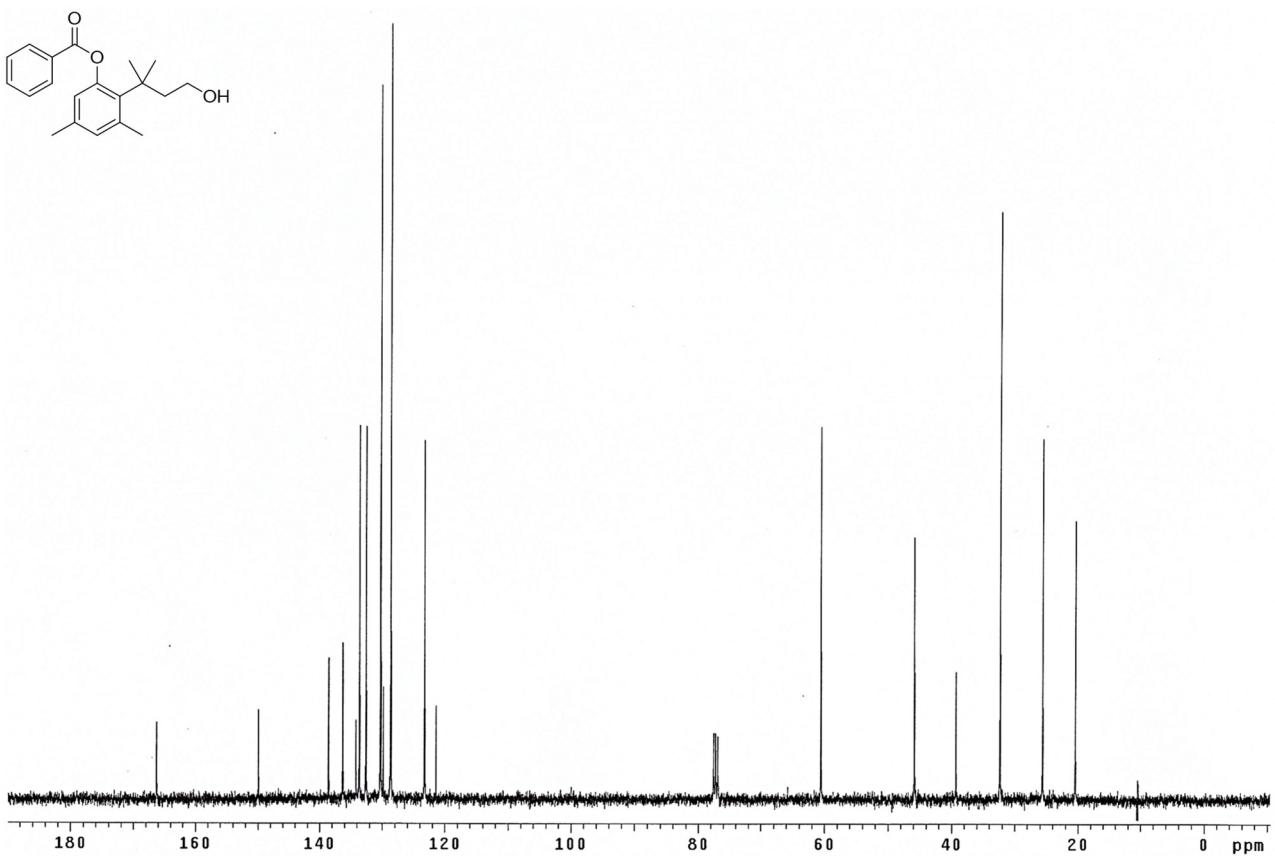


Figure S18. ¹³C-NMR spectrum (100 MHz) of **6a** in CDCl₃

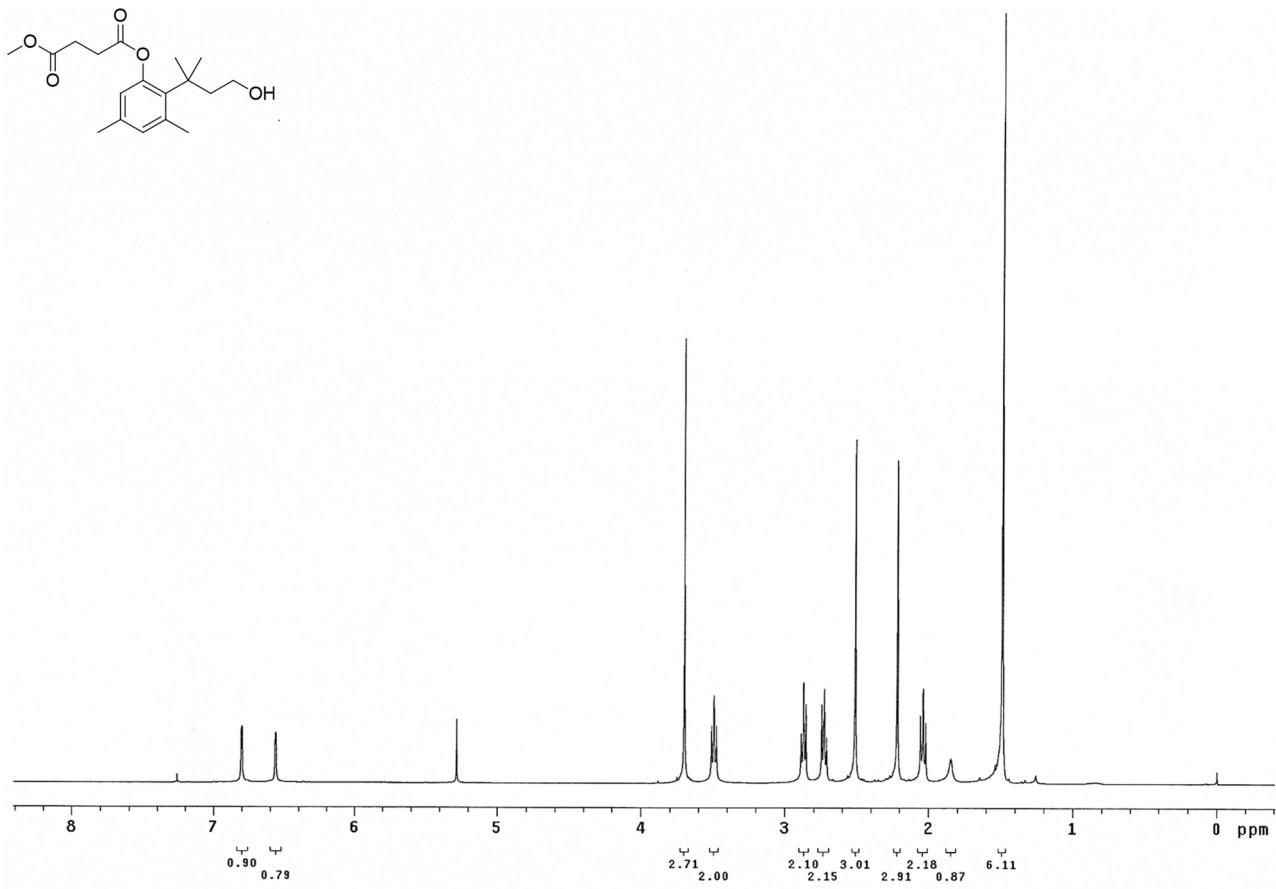


Figure S19. ¹H-NMR spectrum (400 MHz) of **6b** in CDCl₃

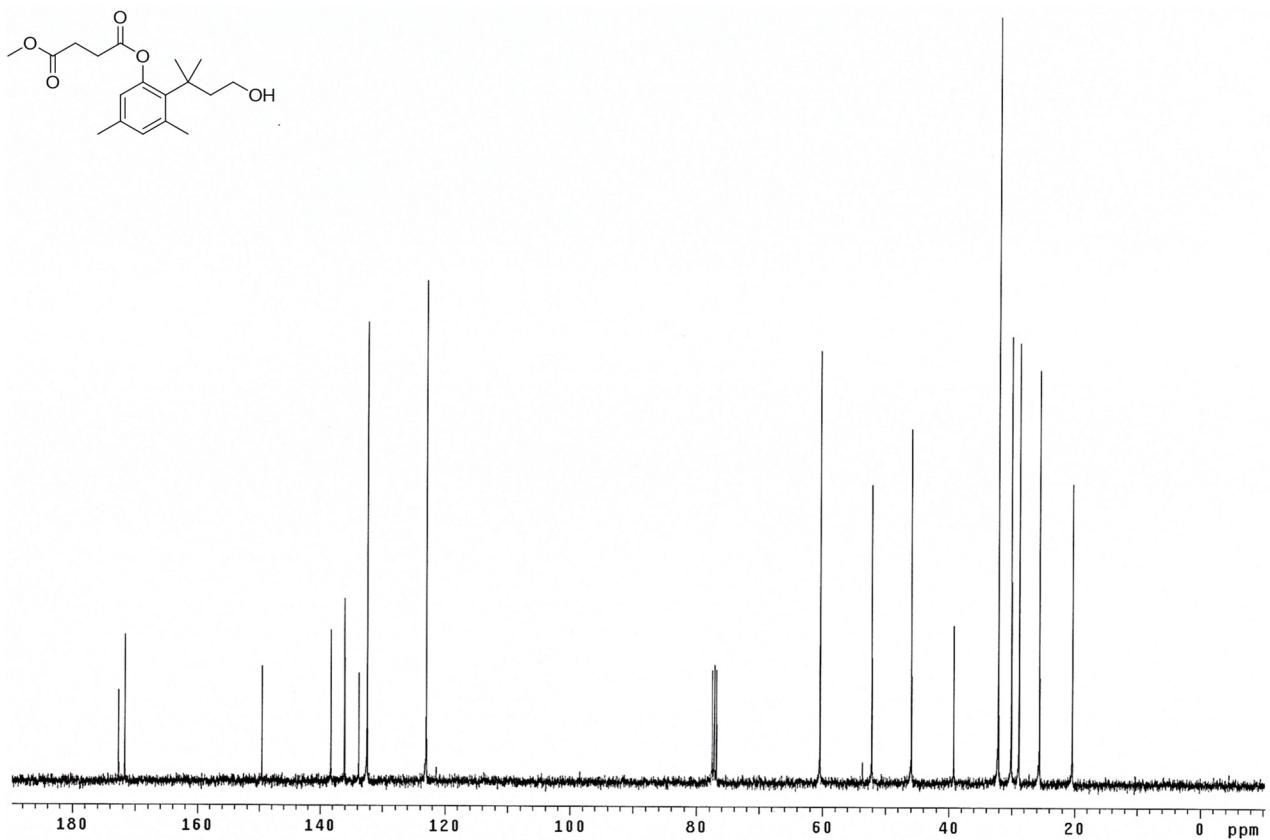


Figure S20. ¹³C-NMR spectrum (100 MHz) of **6b** in CDCl₃

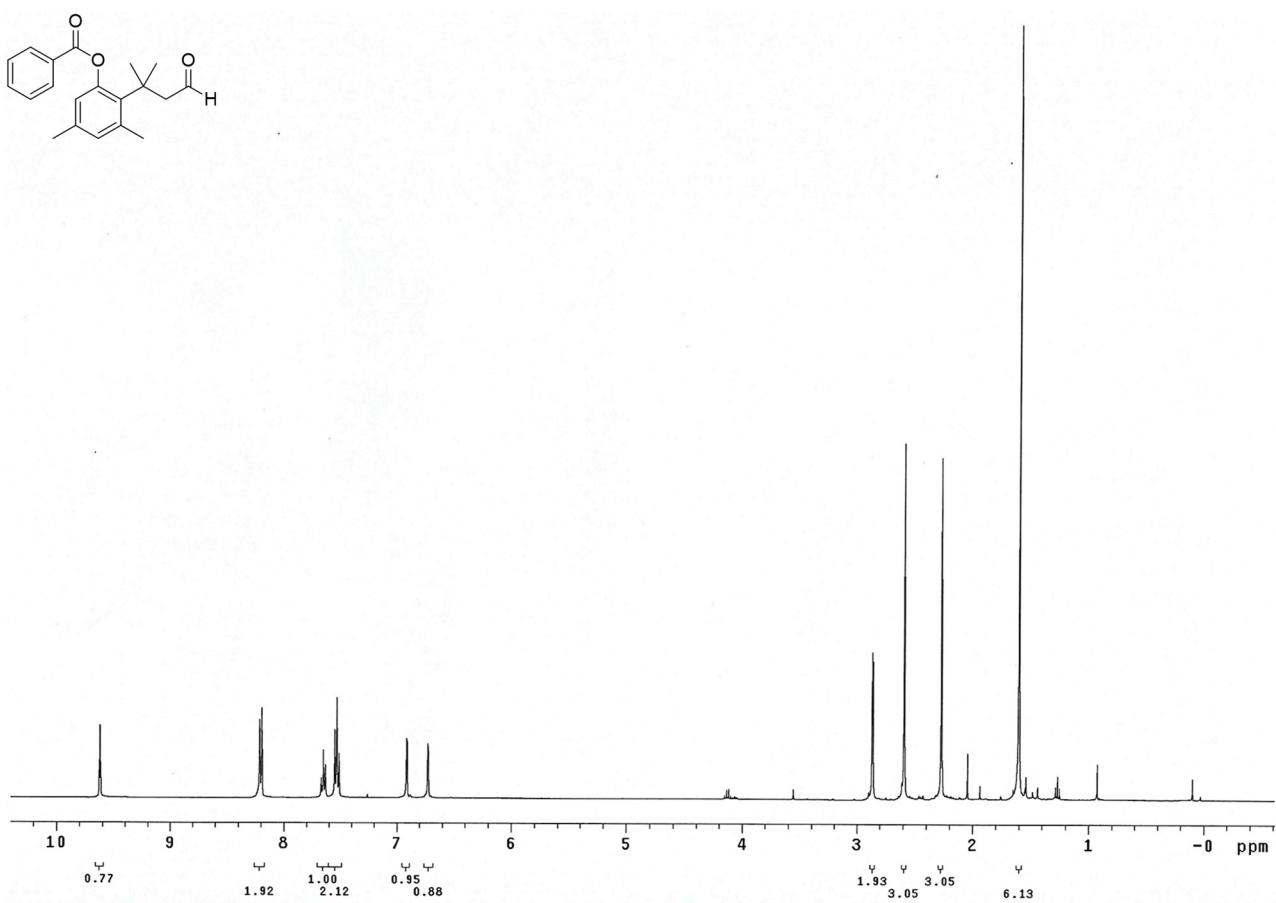


Figure S21. ¹H-NMR spectrum (400 MHz) of 7a in CDCl₃

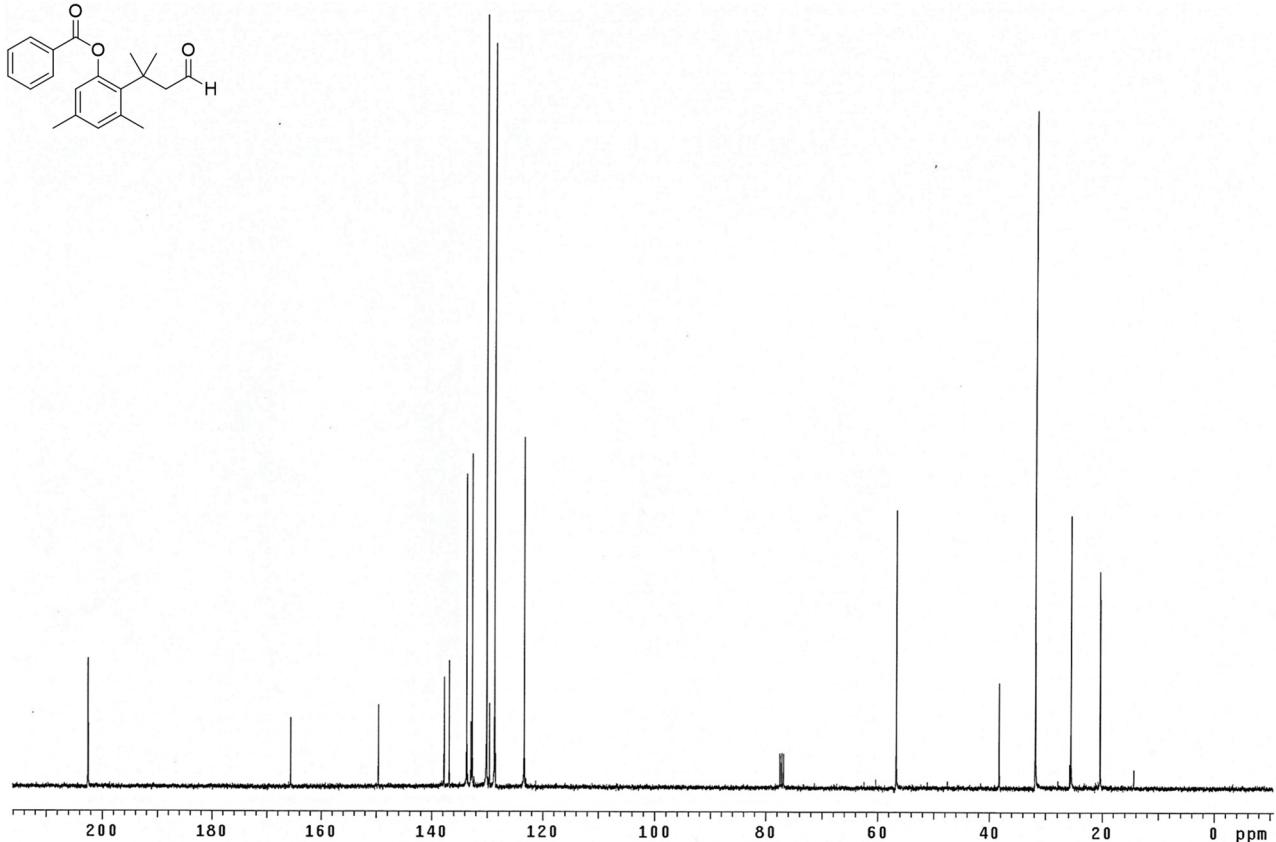


Figure S22. ¹³C-NMR spectrum (100 MHz) of 7a in CDCl₃

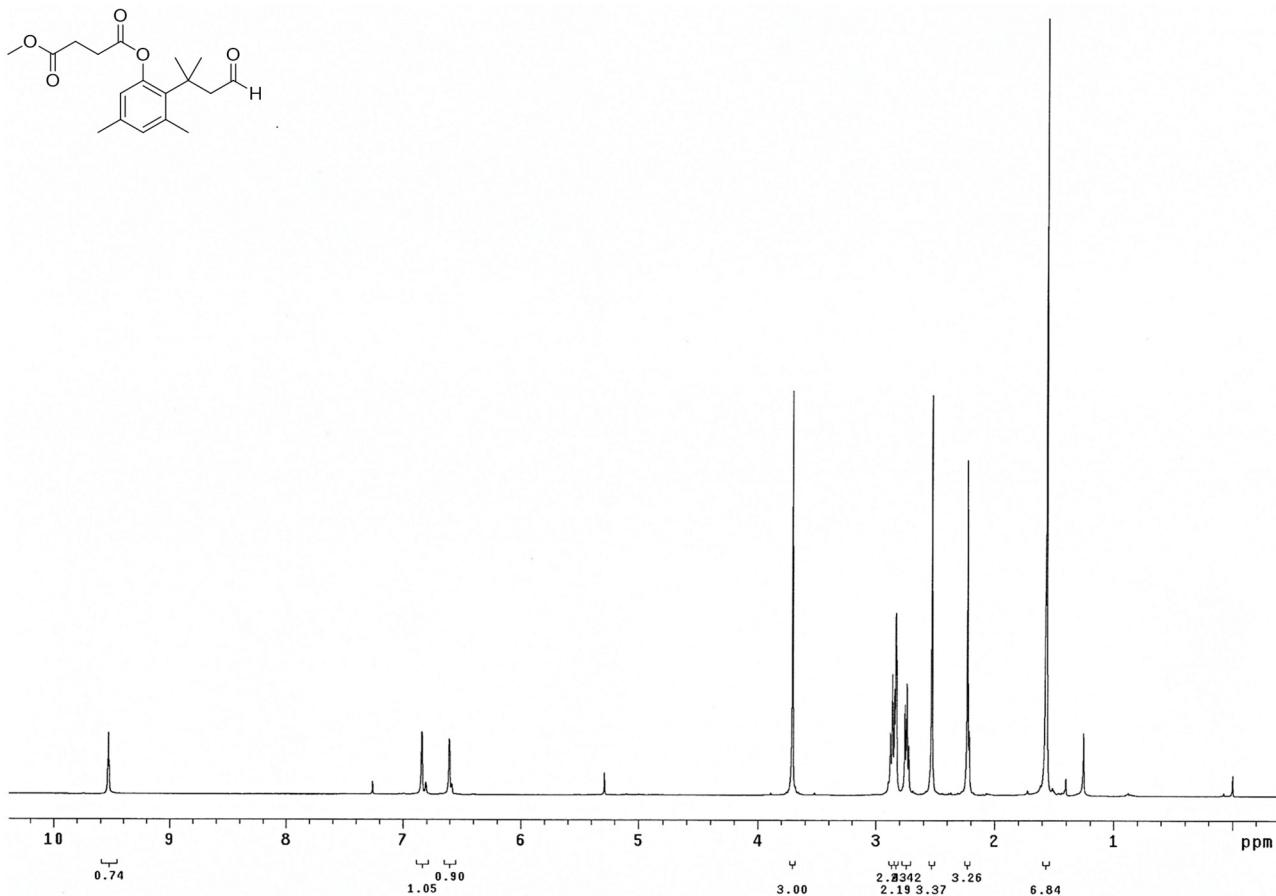


Figure S23. ¹H-NMR spectrum (400 MHz) of **7b** in CDCl₃

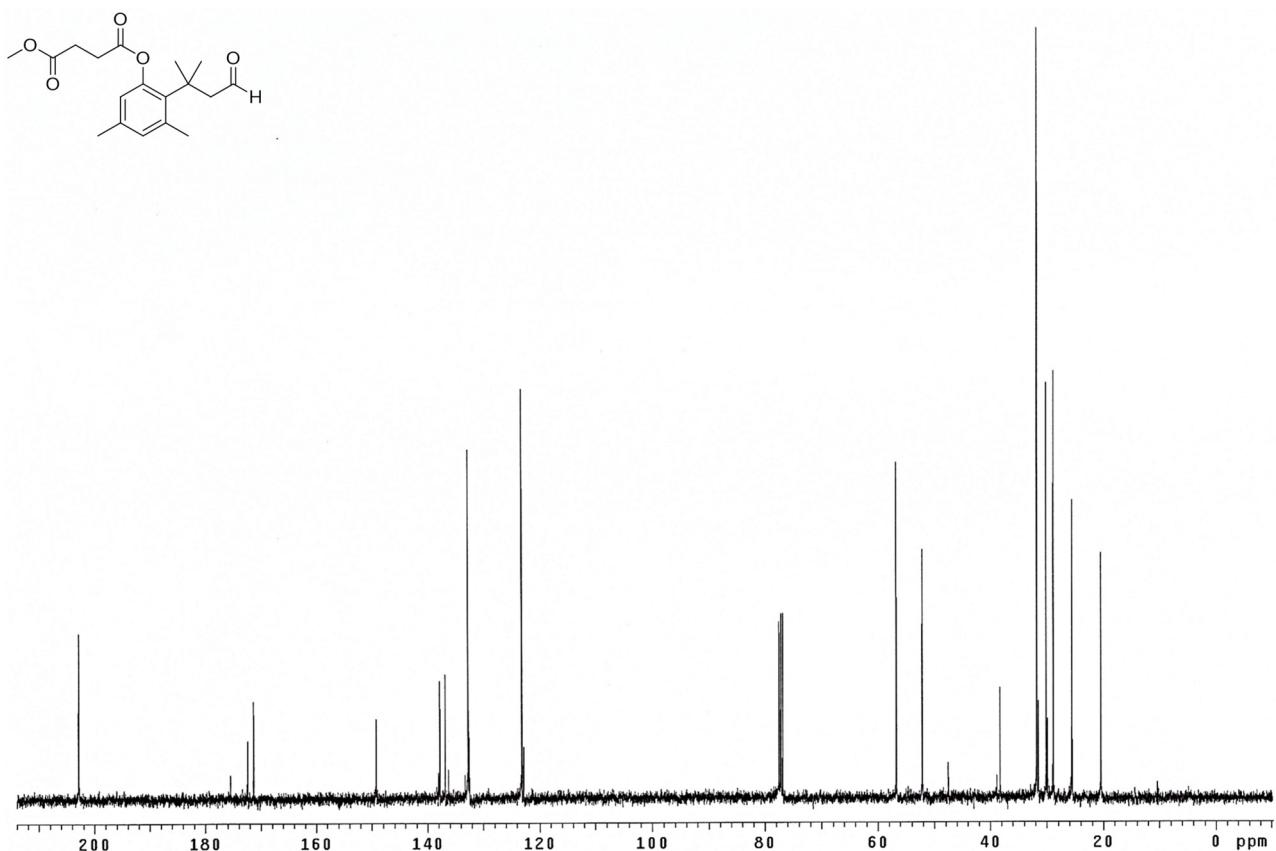


Figure S24. ¹³C-NMR spectrum (100 MHz) of **7b** in CDCl₃

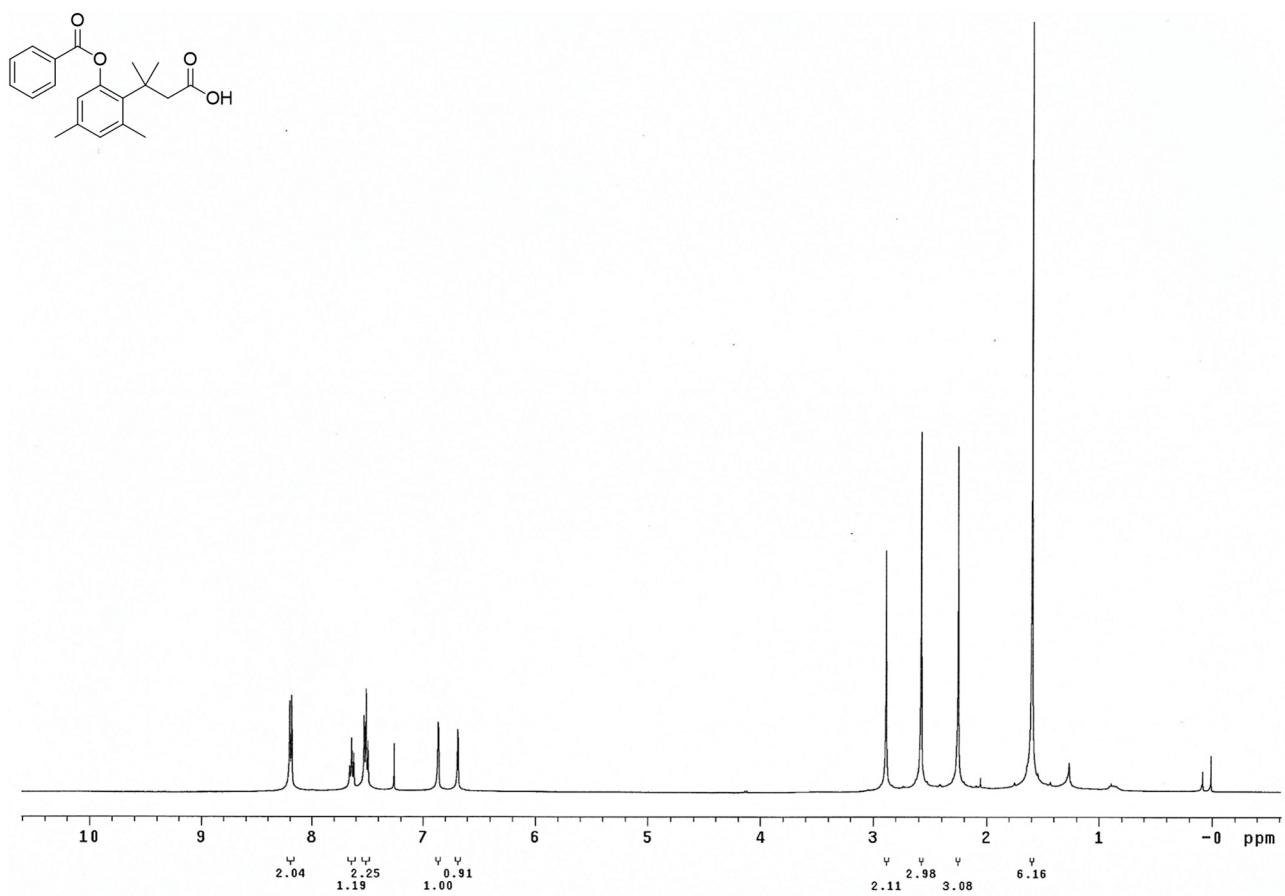


Figure S25. ¹H-NMR spectrum (400 MHz) of **8a** in CDCl₃

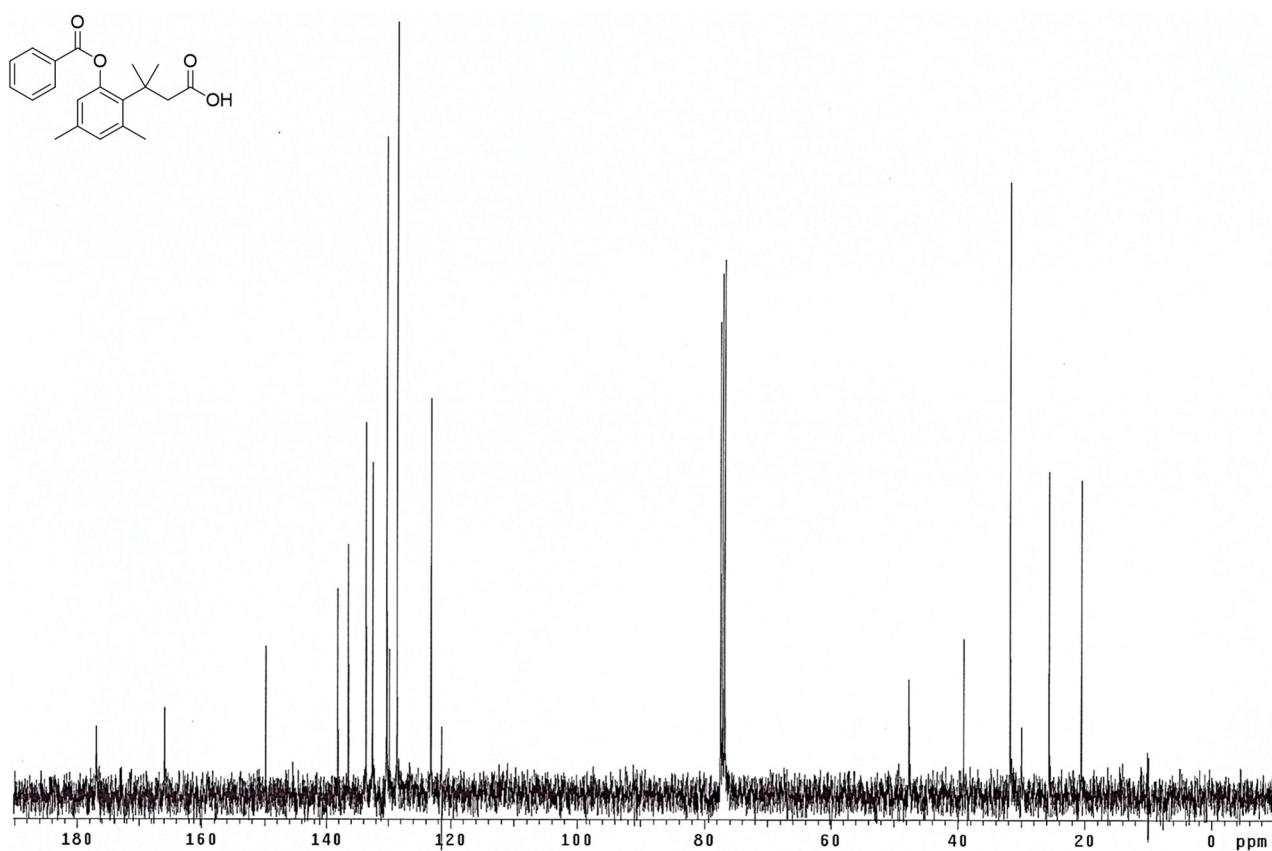


Figure S26. ¹³C-NMR spectrum (100 MHz) of **8a** in CDCl₃

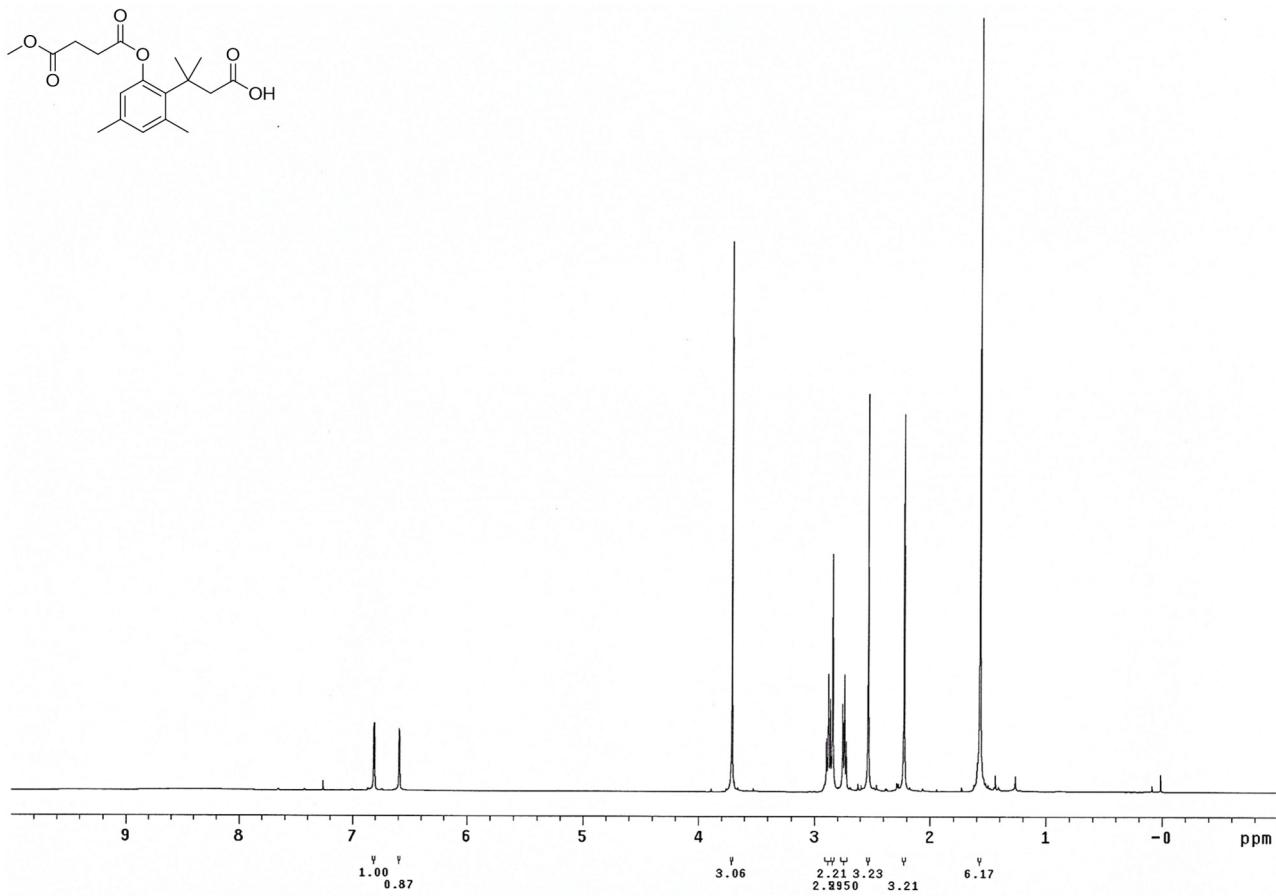


Figure S27. ¹H-NMR spectrum (400 MHz) of **8b** in CDCl₃

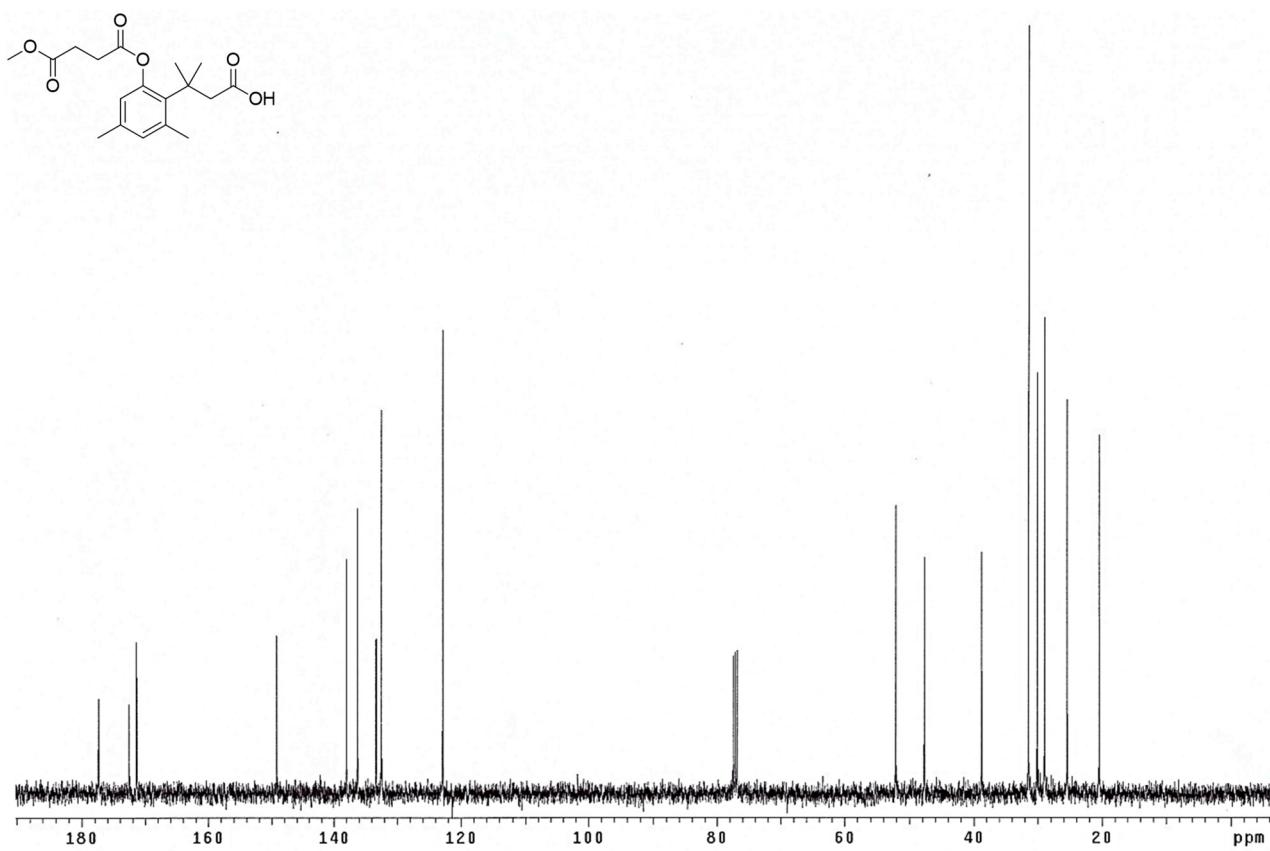


Figure S28. ¹³C-NMR spectrum (100 MHz) of **8b** in CDCl₃

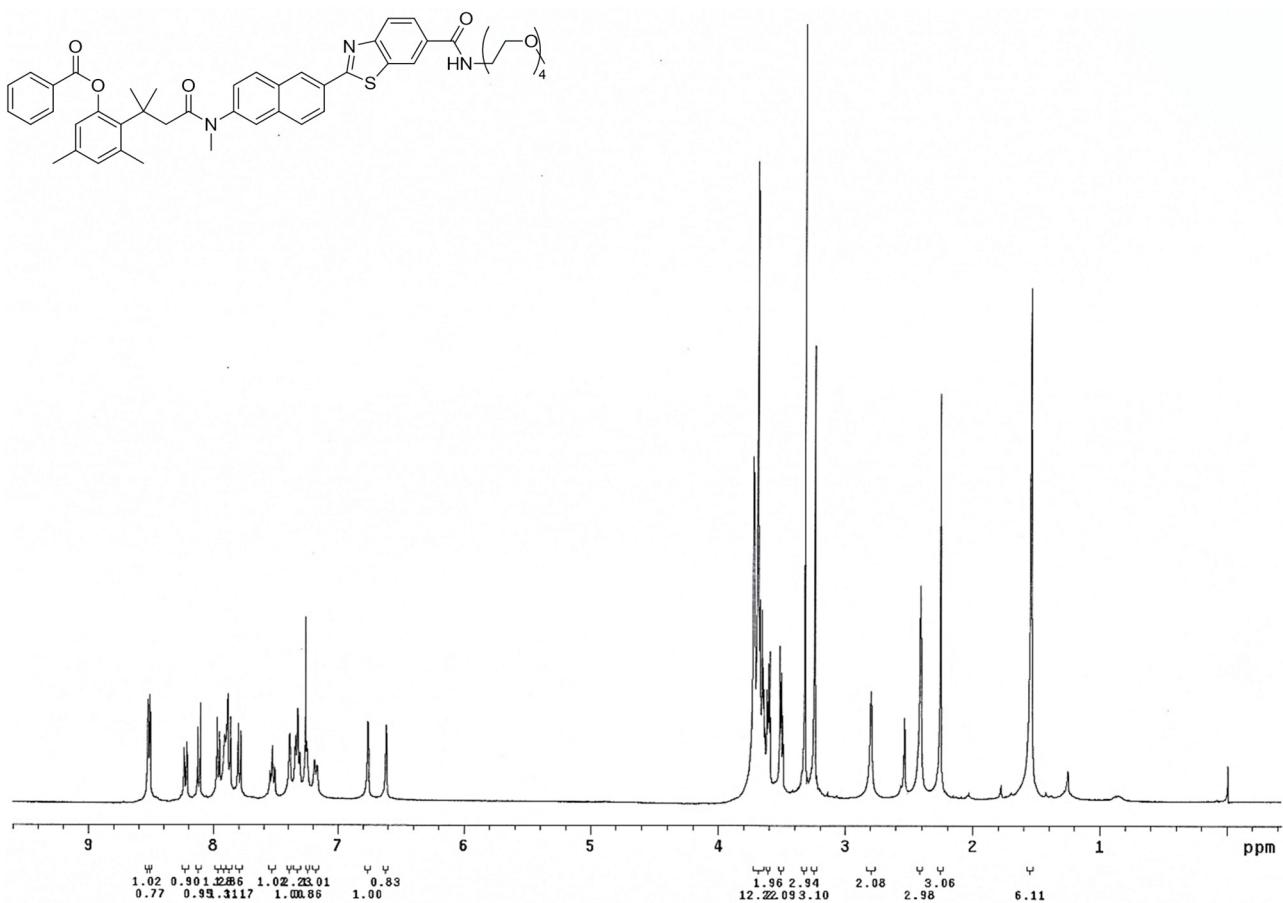


Figure S29. ¹H-NMR spectrum (400 MHz) of **Probe1** in CDCl₃

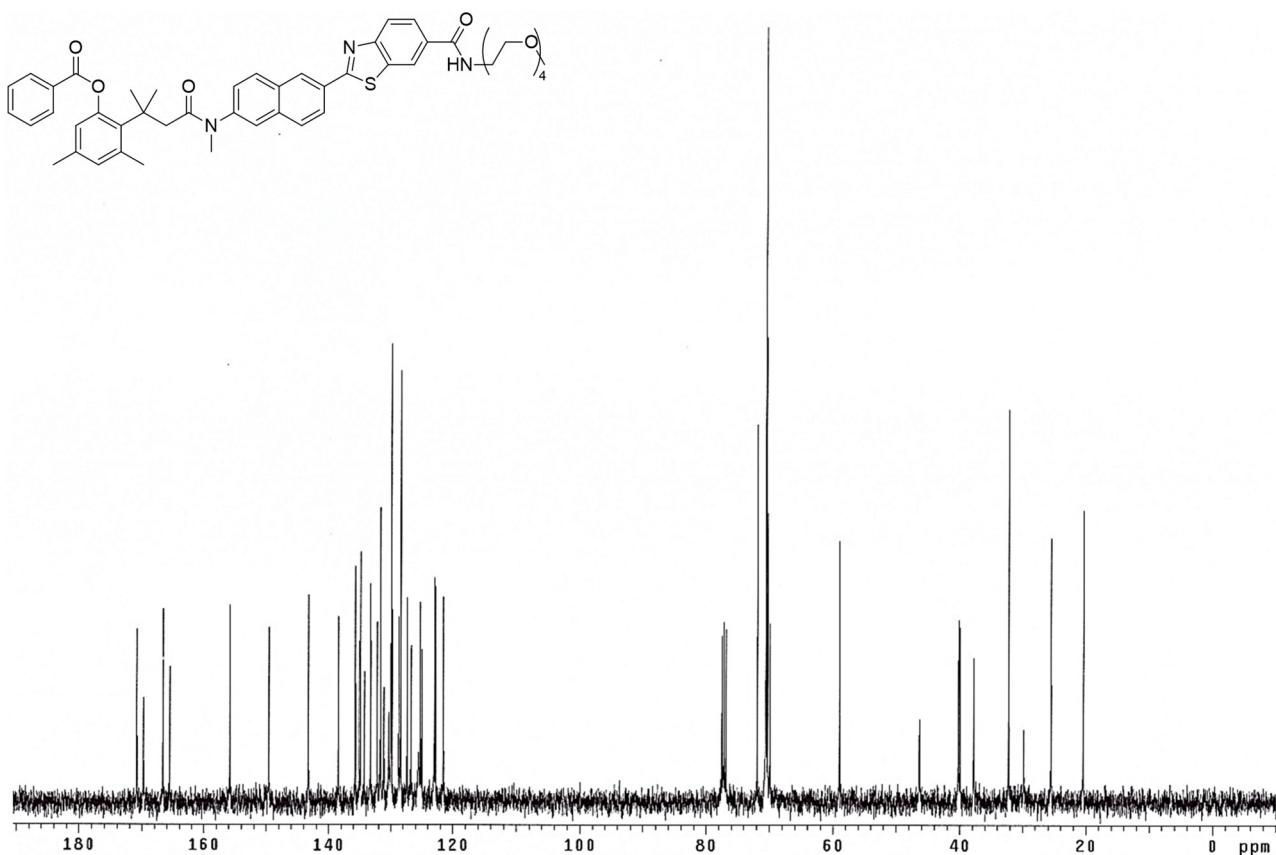


Figure S30. ¹³C-NMR spectrum (100 MHz) of **Probe1** in CDCl₃

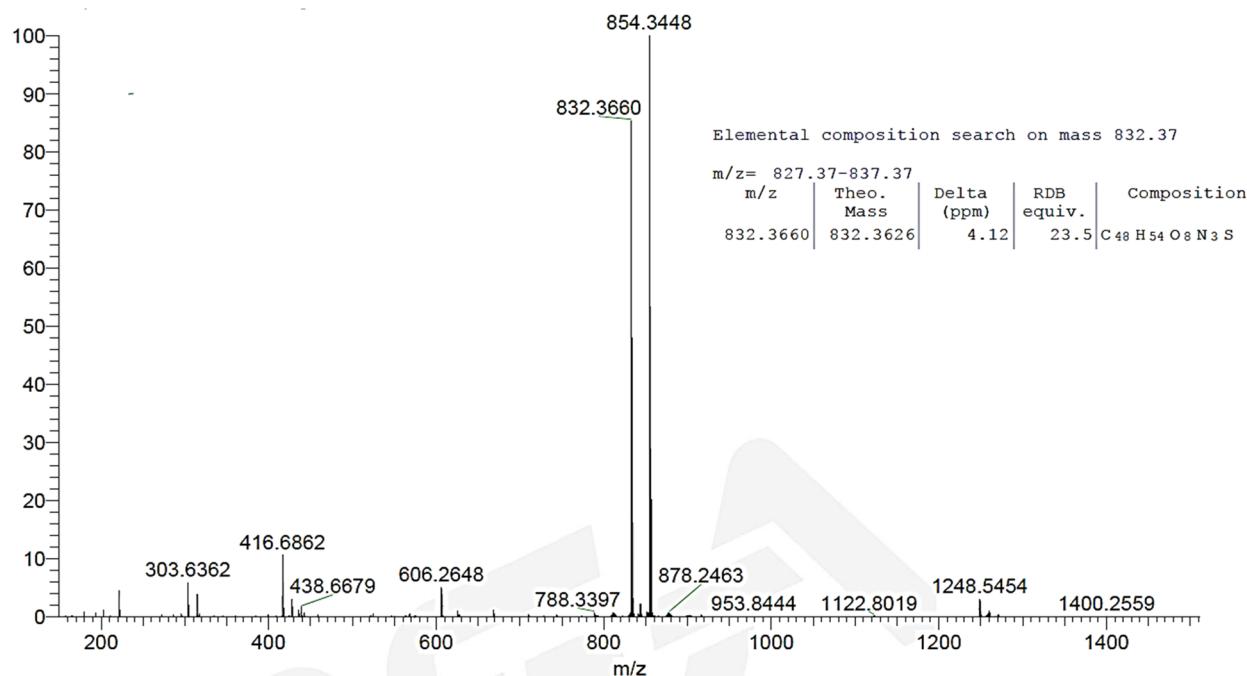


Figure S31. HRMS spectrum of **Probe1**

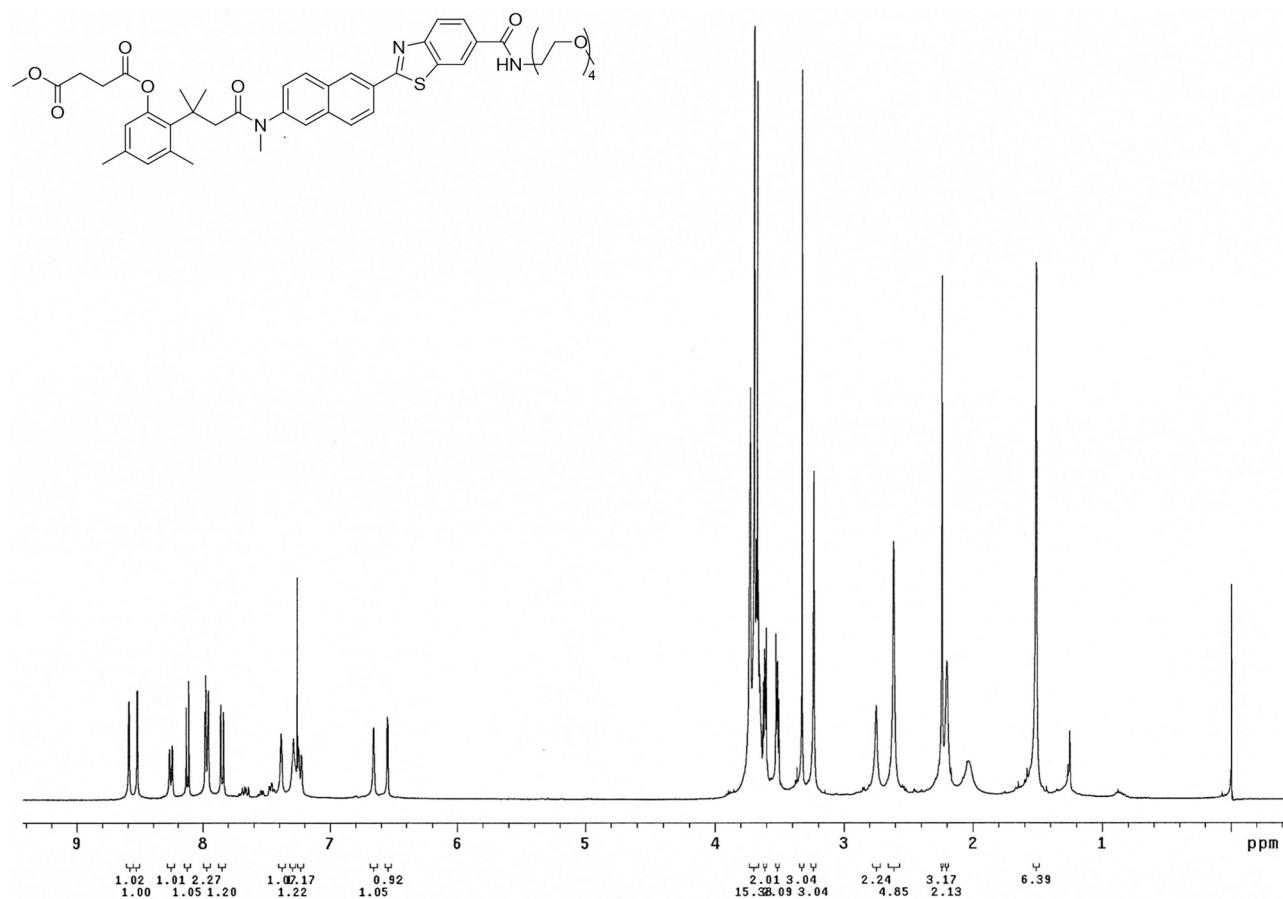


Figure S32. ¹H-NMR spectrum (400 MHz) of **Probe2** in CDCl₃

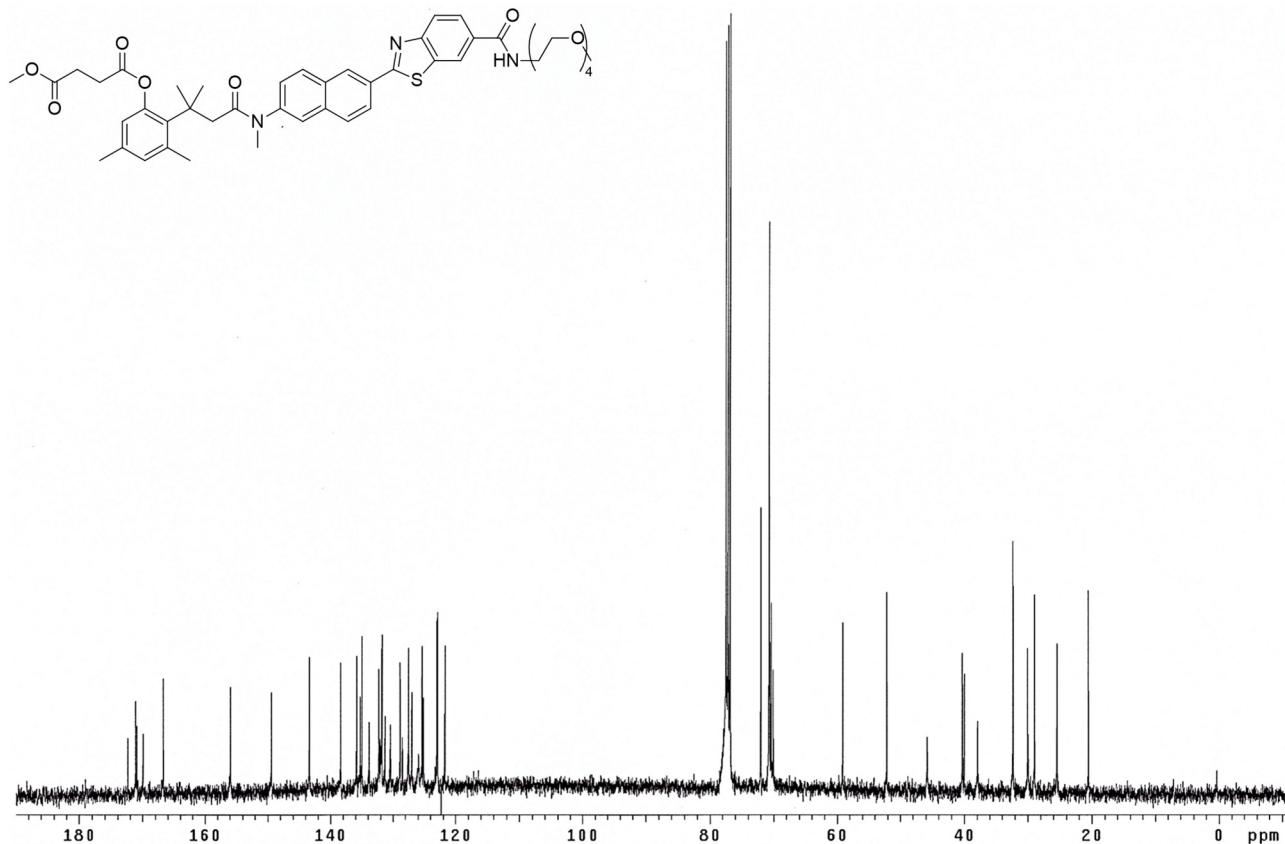


Figure S33. ¹³C-NMR spectrum (100 MHz) of Probe2 in CDCl₃

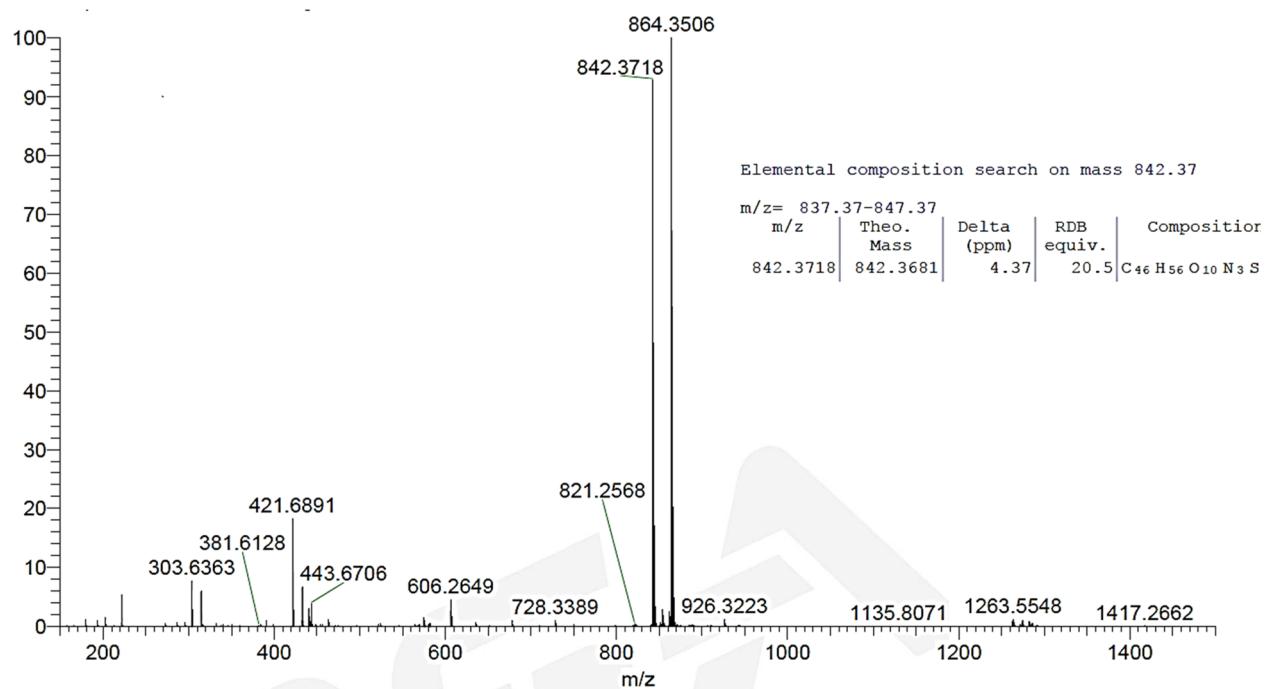


Figure S34. HRMS spectrum of Probe2

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