Supporting information for

A ratiometric fluorescent molecular probe for visualization of mitochondrial temperature in living cell

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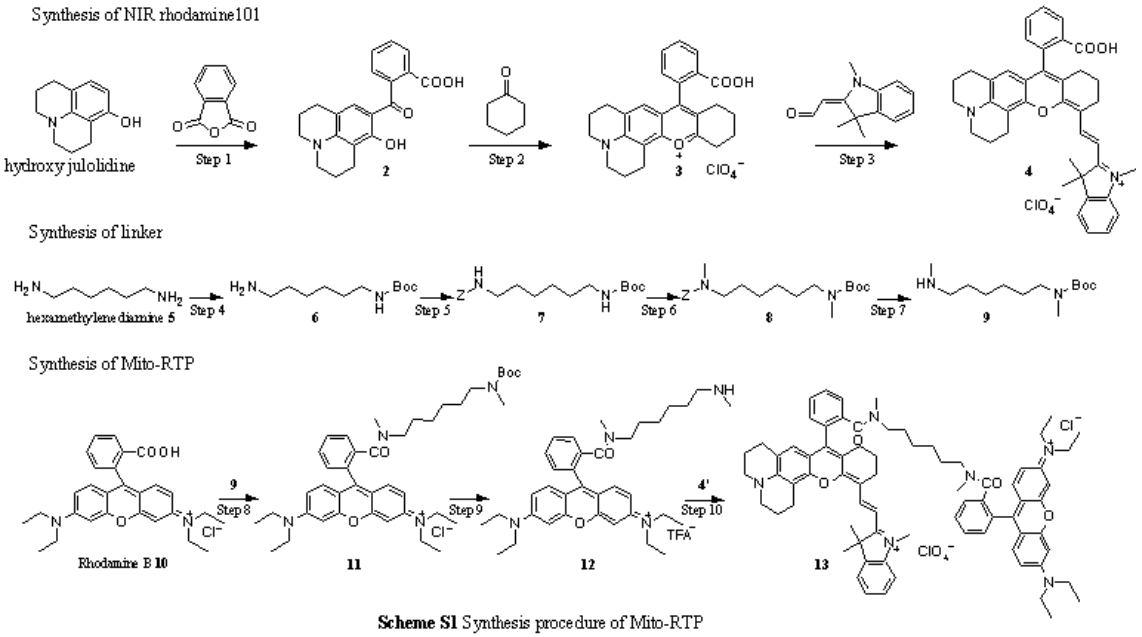
1. Synthesis and characterization of Mito-RTP

Material

Phthalic anhydride, Cyclohexanone, $(Boc)_2O$, MeI were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Fisher aldehyde, Z-chloride and Silver oxide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). hydroxyjulolidine, Rhodamine B, 1.6.hexametylene diamine were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan). Other reagents and solvents were purchased from Kanto Chemistry Industry Co. (Tokyo, Japan). All reagents were used as received.

Synthesis and characterization of Mito-RTP

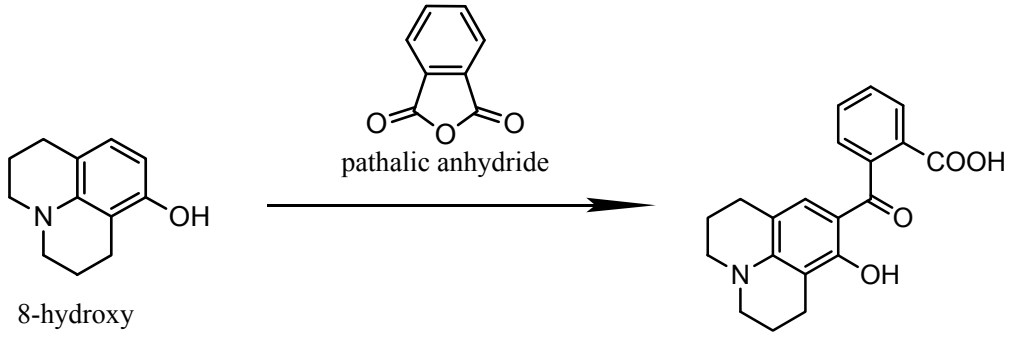
Mito-RTP **13** was synthesized from hydroxyjulolidine **1** with 10 steps (**Scheme S1**). Full description of each reaction steps are described below. All compounds were characterized by ^1H NMR, ^{13}C NMR and ESI-MS. The NMR spectra of compounds were obtained using samples prepared in a deuterated solvent and were recording using OXFORD NMR AS400 (400MHz) spectrometer. Electrospray ionization mass spectra (ESI-MS) were recorded using LCQ Fleet (Thermo Fisher Scientific, USA) mass spectrometer. Plastic sheets coated with 0.2mm silica gel 60 (Merck & Co., Germany) were used for Thin Layer chromatography (TLC) to monitor all reactions. For silica gel chromatography, silica gel 60 (Kanto Chemical Industry Co.) were used with appropriate solvents indicated in each step.



Scheme S1 Synthesis procedure of Mito-RTP

Scheme S1 Synthesis procedure of Mito-RTP

Step 1: Hydroxyjulolidine **1** to **2**



Hydroxy jurolidine (500.0 mg, 2.65 mmol) and pathalic anhydride (469.8 mg, 3.18 mmol) were dissolved in toluene and refluxed 3 h at 100 °C. After removing toluene under reduced pressure, NaOHaq (35%, 100ml) was added and refluxed 6h at 90°C. The reaction mixture was acidified with 1M HCl aq and precipitate was filtered. The crude compound was purified by recrystallization (MeOH/H_2O) and compound **2** was obtained as a yellow powder (679.6 mg, 2.01 mmol, **76%**). **TLC**: R_f = 0.7 (CH_3Cl / MeOH = 8/1). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.88 (dd, 4H, -C**H**$_2$-), 2.44 (d, 2H, -C**H**$_2$-), 2.66 (d, 2H, -C**H**$_2$-), 3.19 (d, 4H, -C**H**$_2$-), 6.45 (s, 1H, Ar**H**), 7.27 (t, 1H, Ar**H**), 7.56 (dd, 2H, Ar**H**), 8.02 (t, 1H, Ar**H**), 9.51 (s, 1H, O**H**), 12.9 (s, 1H, COO**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 20.0, 20.8, 21.8, 27.4, 49.9, 50.3 (-**C**H_3-), 105.7, 109.3, 113.1, 128.3, 129.1, 130.5, 131.1, 132.5, 141.5, 149.5, 160.9, 170.1 (Ar**C**), 198.2 (**C**OOH). **ESI-MS** (m/z): 336.42 ($[M-H]^-$, found), 337.42(calcd).

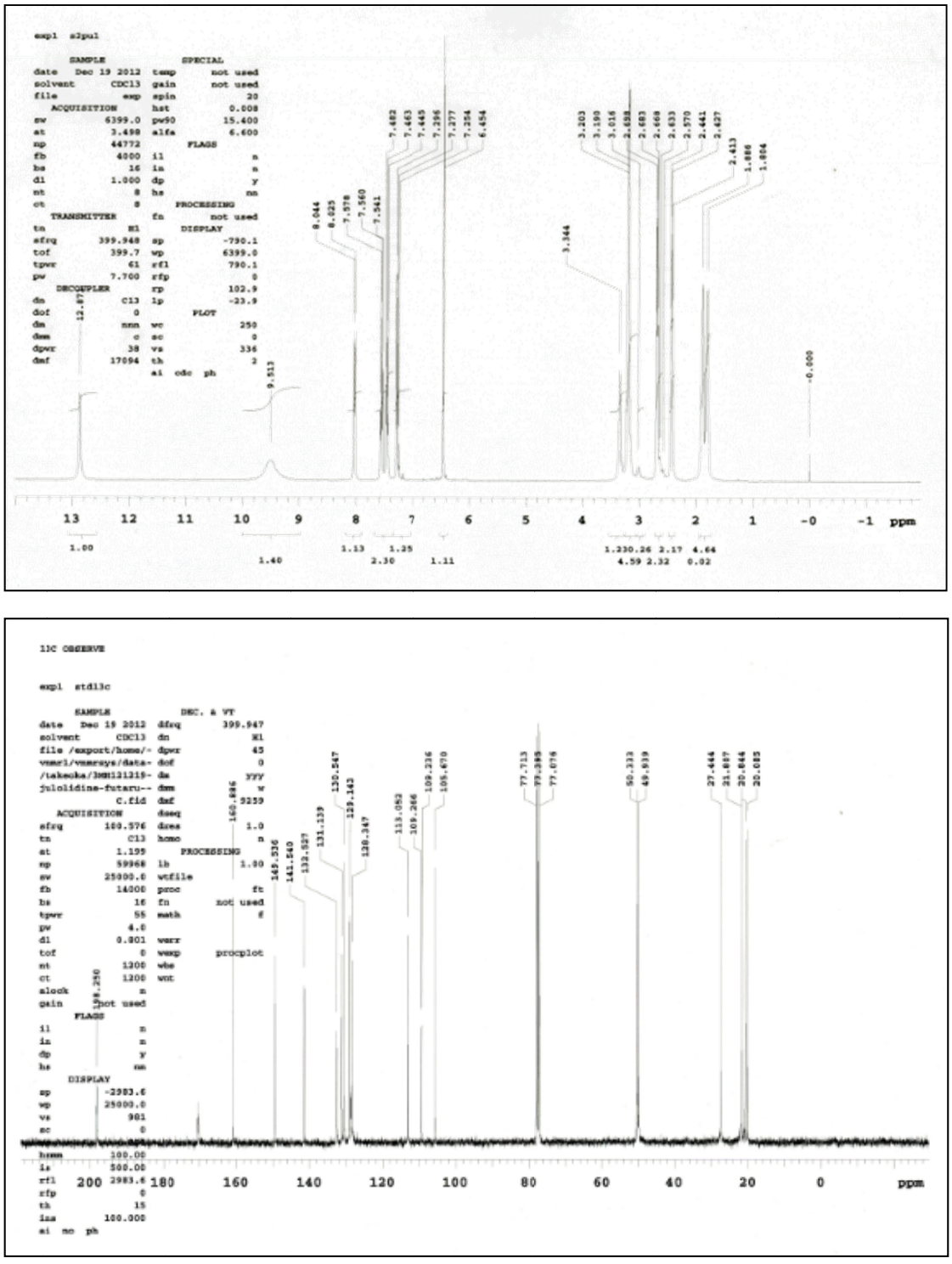
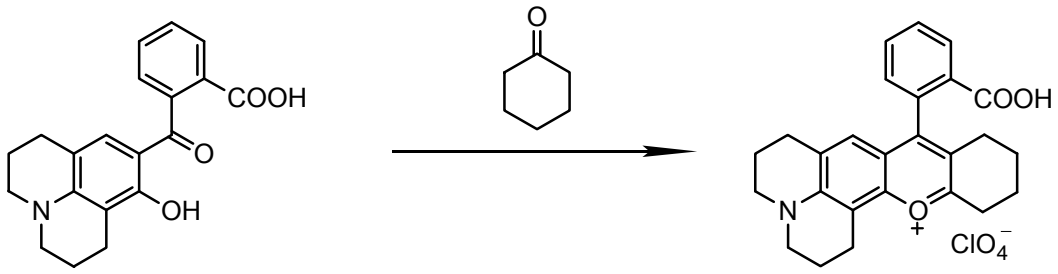


Fig. S2. (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **2**.

Step 2: **2** to **3**



Compound 2 **Compound 3**

Compound **2** (253.6 mg, 0.821 mmol) and cyclohexanone (168.0 µl, 1.62 mmol) were added to concentrated H_2SO_4 (1.75 ml) at 4°C. The reaction mixture was heated and stirred for 1.5 h. After the reaction, perchloric acid (175 ul) was added and the resulting precipitate was filtered. The filter cake was washed with cold water. Compound **3** as a red solid was used for next step without further purification. Yield to Compound **3** (309.4 mg, 0.79mmol, 96%). **TLC**: R_f = 0.9 (CH_3Cl / MeOH = 8/1). **ESI-MS** (m/z): 400.42($[M]^+$, found), 400.42(calcd).

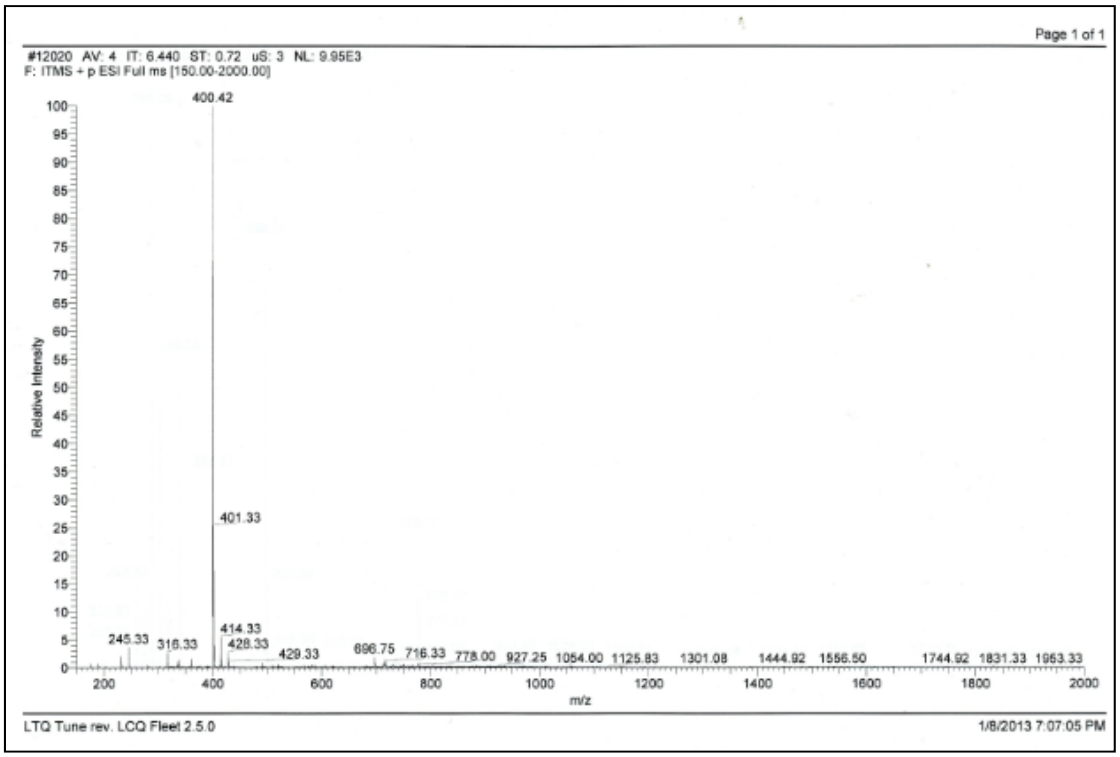
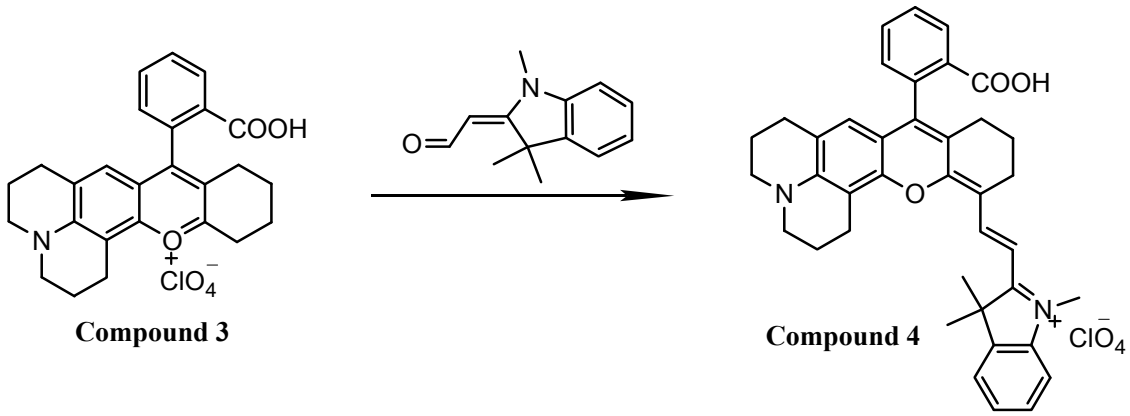


Fig. S3 ESI-MS spectra of **3**.

Step 3: **3** to **4**



Compound 3 **Compound 4**

Compound **3** (309.4 mg, 0.79 mmol) and fisher aldehyde (166.7 mg, 0,829 mmol) were dissolved in acetic anhydride (32 ml). The reaction mixture was heated to 50°C and stirred for 30 min. The reaction was quenched with water and the solvent was removed under reduced pressure. The crude product was purified by column chromatography using $CHCl_3$/MeOH = 9/1. Compound **4** was obtained as a green solid (89 mg, 0.13 mmol, **16%**). **TLC**: R_f = 0.4 (CH_3Cl / MeOH = 8/1). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.24 (t, 6H, -C**H**$_2$-), 1.83 (s, 3H, -C**H**$_3$), 1.83 (m, 4H, -C**H**$_2$-), 2.42 (m, 4H, -C**H**$_2$-), 3.02 (s, 3H, -C**H**$_3$), 3.37 (t, 3H, -C**H**$_3$), 3.47 (q, 4H, -C**H**$_2$-), 5.67 (d, 1H, -C**H**=CH-), 6.47 (d, 1H, Ar**H**), 6.56 (dd, 1H, Ar**H**), 6.87 (t, 2H, Ar**H**), 7.04 (m, 1H, Ar**H**), 7.29 (t, 1H, Ar**H**), 7.49 (d, 2H, Ar**H**), 8.15 (d, 1H, -C**H**=CH-), 8.20 (dd, 1H, Ar**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 20.5, 20.7, 21.1 (-**C**H$_3$), 24.7, 26.1, 27.8, 27.9, 28.6, 28.8 (-**C**H$_2$-), 47.6, 49.9, 50.6 (-**C**H$_2$-), 95.4, 104.3, 108.4, 114.6, 118.1,120,3, 122.0, 122.7, 123.2, 125.9, 127.0, 128.5, 128.9, 129.9, 131.1, 134.7, 139.9, 143.2, 148.6, 151.9, 163.2, 166.7 (Ar**C**). 169.7, 169.8 (-CH=**C**H-). **ESI-MS** (m/z): 583.50 ($[M]^+$, found), 583.50 (calcd).

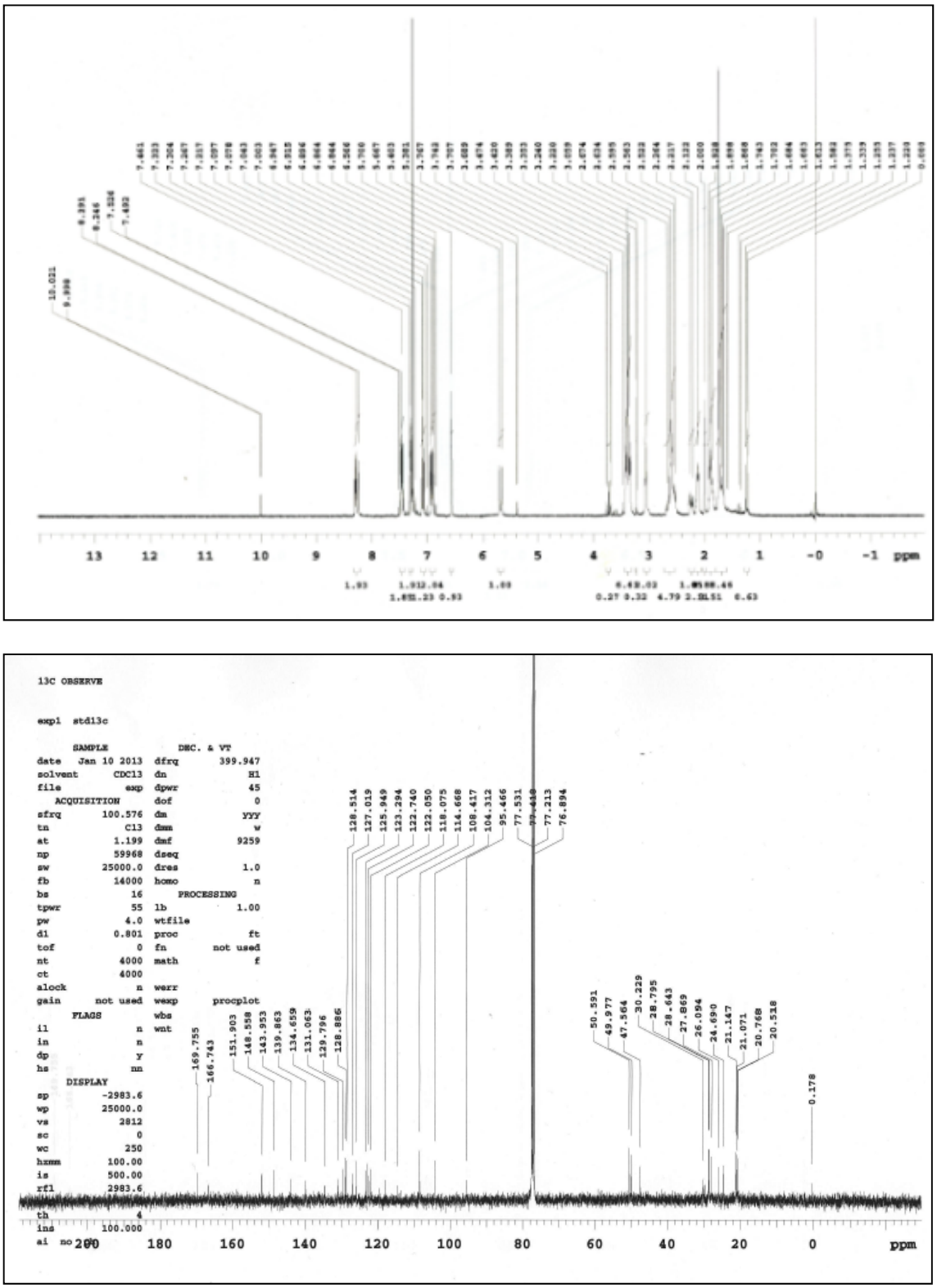
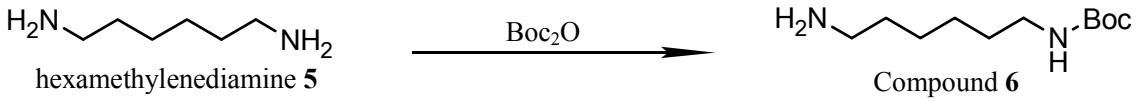


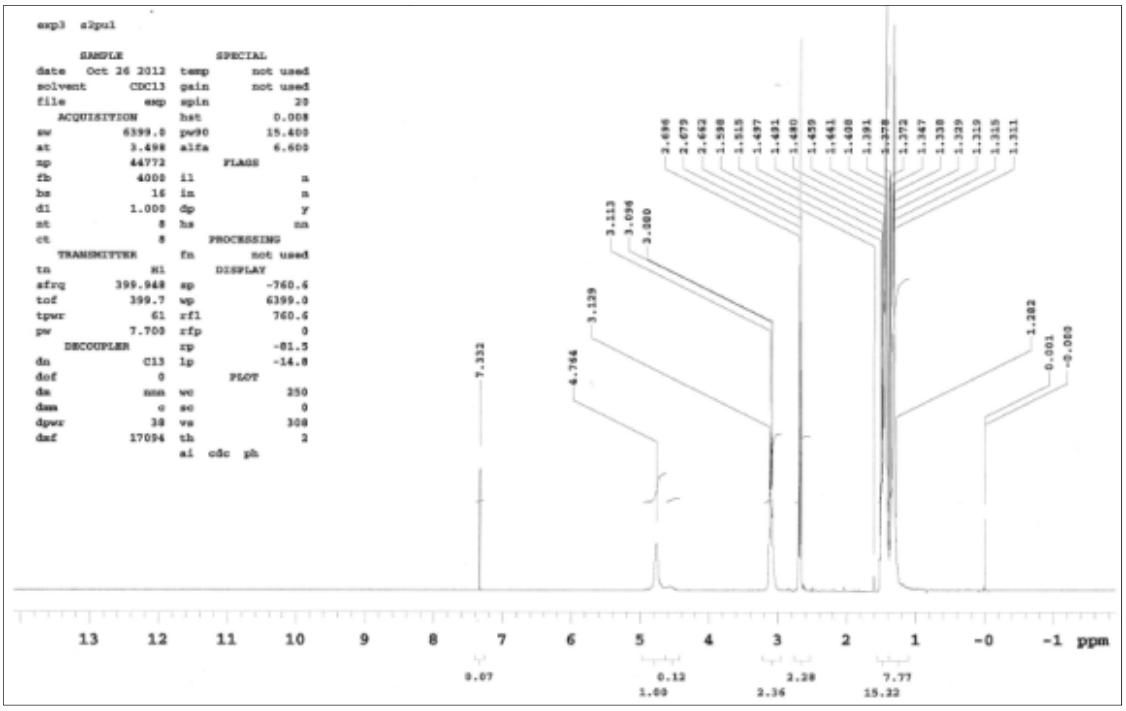
Fig. S4 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **4**.

Step 4: **1.6-hexamethylenediamine 5** to **6**



hexamethylenediamine **5** Compound **6**

Boc_2O (634 mg, 2.38 mmol) was dissolved in 40 ml of DCM. The Boc_2O solution was added dropwisely to hexamethylene diamine (1.00 ml, 14.28 mmol) 40 ml DCM solution and stirring was continued for 18 h at room temperature. The solvent was removed under reduced pressure. Crude product was purified by silica gel chromatography using $CHCl_3$ / MeOH = 12/1. Compound **6** was obtained as oily solid (157.3 mg, 0.98 mmol, **41%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.37 (m, 9**H**, -C**H**$_3$), 1.40 (m, 10**H**, -C**H**$_2$-), 2.68 (t, 2**H,** -N**H**$_2$-), 3,11 (q, 2**H**, NH-C**H**$_2$-), 4.77 (s, 1**H**, -N**H**-). **^{13}C NMR** (σ ppm in $CDCl_3$): 26.7, 28.6, 30.2, 33.8,(-**C**H$_2$-), 40.6, (N**H**$_2$-**C**H$_2$), 42.2 (-NH-**C**H$_2$-), 77.0, 77.3, 77.6 (C-**C**H$_3$), 79.0 (O-**C**-CH$_3$). **ESI-MS** (m/z): 217.33 ($[M+H]^+$, found), 216.33 (calcd).



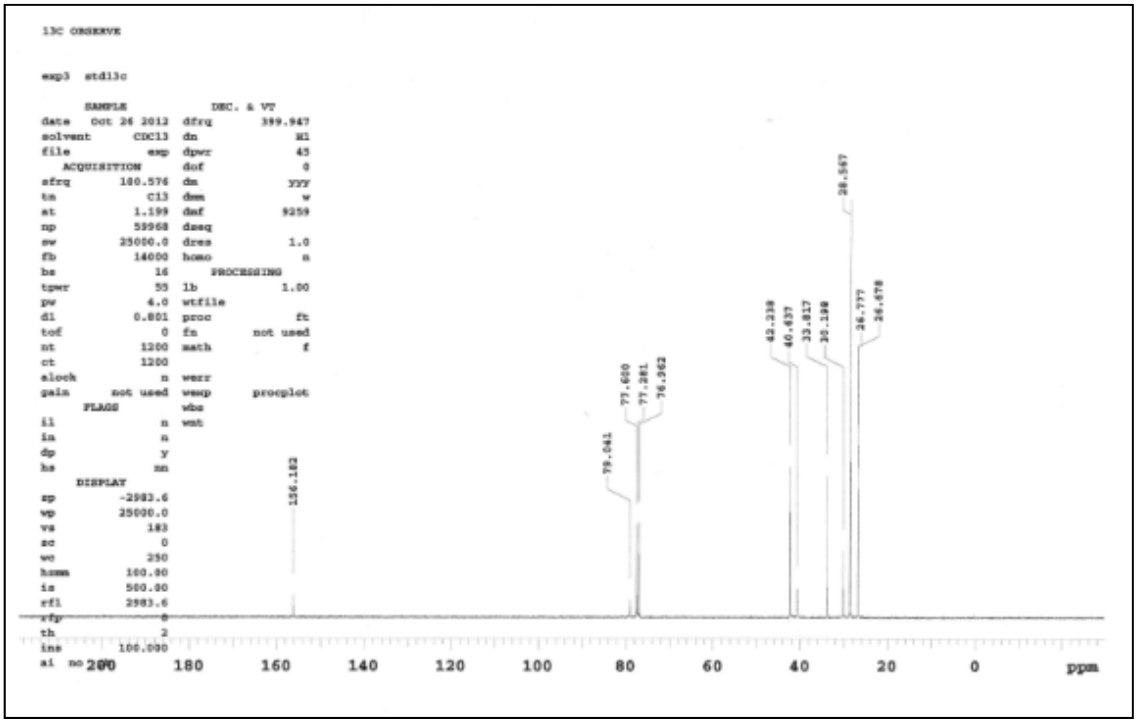
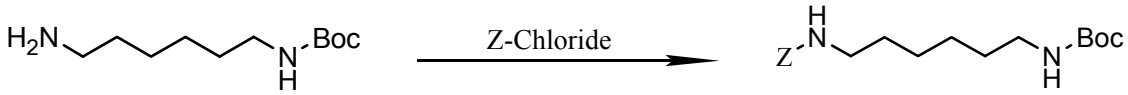


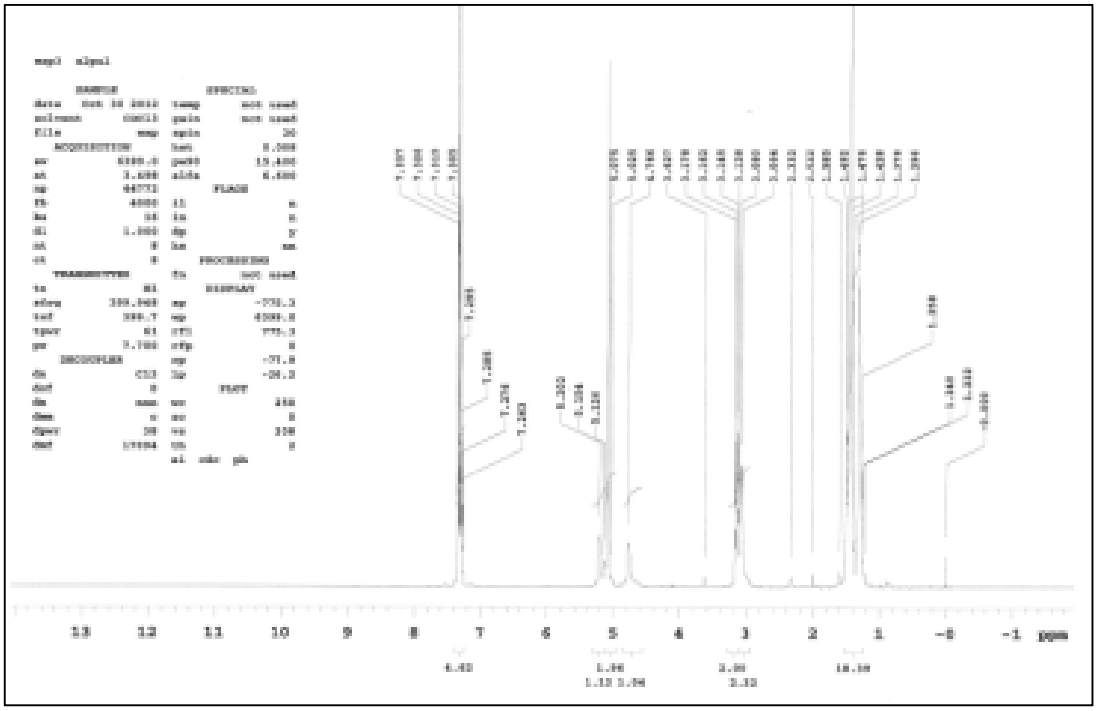
Fig. S5 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **6**.

Step 5: **6** to **7**



Compound **6** Compound **7**

Benzyl chloroformate (Z-chloride) (595 µl, 4.25 mmol) and TEA (597 µl, 4.25 mmol) were added dropwisely to Compound **6** (680.8 mg, 4.25 mmol) in THF (4ml) solution. The reaction mixuture was stirred for 4.5 h. The solvent was removed under reduced pressure and the residue was taken up in EtOAc. This solution was washed with water. The crude product was purified by recrystallization (EtOAc/Hexane). Compound **7** was obtained as a white solid (874 mg, 2.97 mmol, **70%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.26 (m, 9**H**, -C**H**$_3$), 1.48 (m, 8**H**, -C**H**$_2$-), 3.01 (t, 2**H, -N**H-C**H**$_2$-), 3,13 (q, 2**H**, NH-C**H**$_2$-), 4.77 (s, 1**H**, -N**H**-),.5.96 (m, 2**H**, O-C**H**$_2$-C), 5.15 (s, 1**H**, -N**H**-), 7.30 (m, 5**H,** Ar**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 26.5, 28.6, 30.0, 33.2,(-**C**H$_2$-), 40.5, 41.0 (-NH-**C**H$_2$-), 66.9 (O-**C**H$_2$-C), 34.3 (C-**C**H$_3$), 77.1, 77.4, 77.7 (C-**C**H$_3$), 79.0 (O-**C**-CH$_3$) , 128.2, 128.6, 129.0, 136.9, 156.3, 156.7 (Ar**C**). **ESI-MS** (m/z): 373.33 ($[M+Na]^+$, found), 350.33 (calcd).



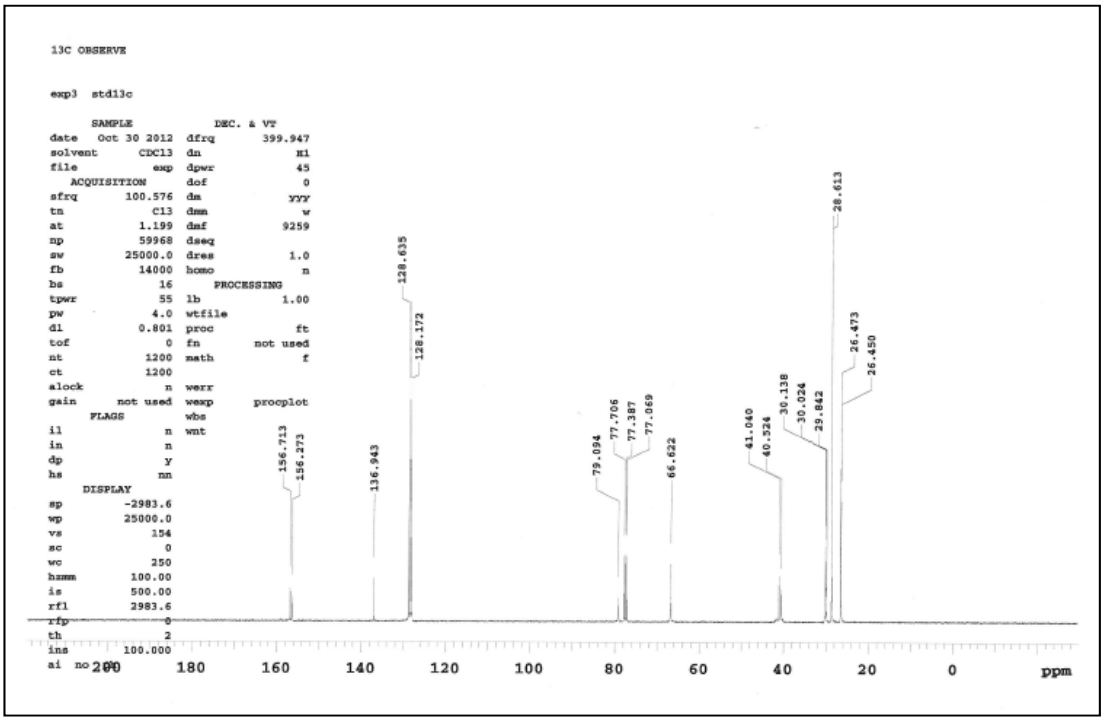
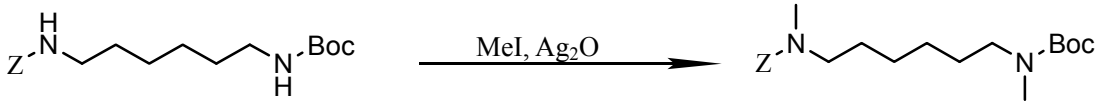


Fig. S6 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **7**.

Step 6: **7** to **8**



Compound **7** Compound **8**

Compound **7** (874 mg, 2.97 mmol) in 6ml of DMF was added Ag_2O (2.61g, 11.29 mmol). The solution was added MeI (2.28 ml, 42.8 mmol) dissolved in 6ml of DMF. The reaction mixture was stirred vigorously for 3 days at room temperature. After the reaction, 20 ml of DCM was added to the mixture and the resulting precipitate was filtered. The filter cake was washed with DCM and organic layer was washed with water. Compound **8** was obtained as oily solid (671.7 mg, 2.08 mmol, **72%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.28 (m, 9**H**, -C**H**$_3$), 1.40 (m, 8**H**, -C**H**$_2$-), 2.08 (s, 3**H,** C-C**H**$_3$), 2.95 (s, 3**H**, C-C**H**$_3$), 3.24 (m, 4**H**, -N-C**H**$_2$),.5.12 (s, 2**H**, O-C**H**$_2$-C), 7.30 (m, 5**H,** Ar**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 26.3, 26.5, 28.5, 29.9,(-**C**H$_2$-), 31.3, 34.3 (-N-**C**H$_2$), 36.4, 48.6 (N-**C**H$_3$), 66.9 (O-**C**H$_2$-C), 77.3, 77.6, 77.7 (C-**C**H$_3$), 79.0 (O-**C**-CH$_3$) , 127.8, 129.0, 137.1, 155.7, 156.3, 162.5 (Ar**C**). **ESI-MS** (m/z): 379.51 ($[M+H]^+$, found), 378.51 (calcd).

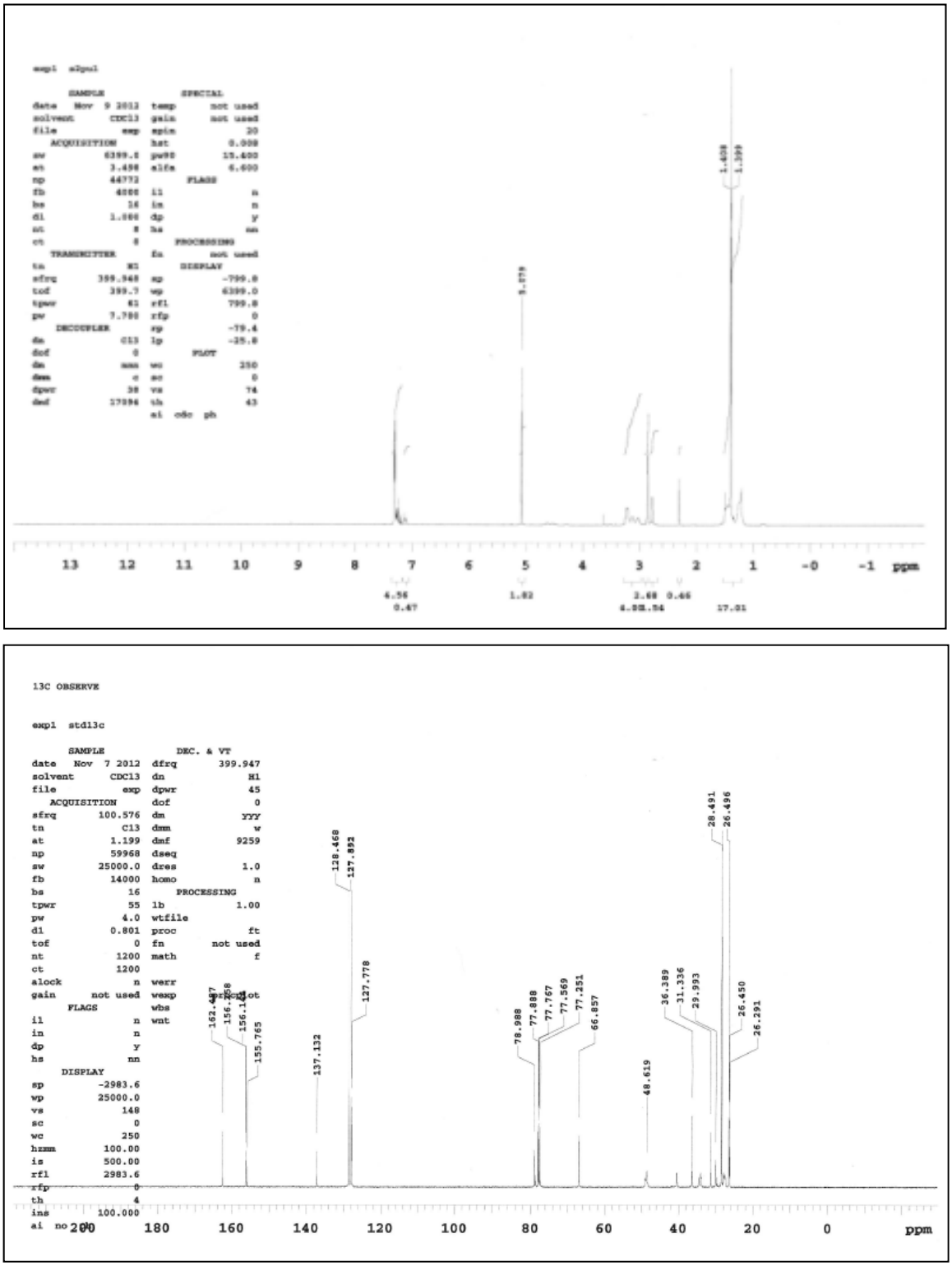
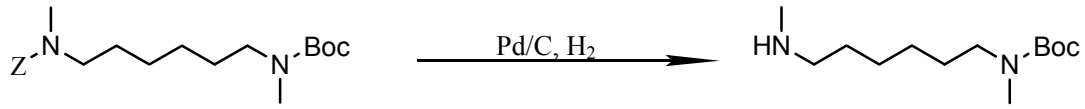


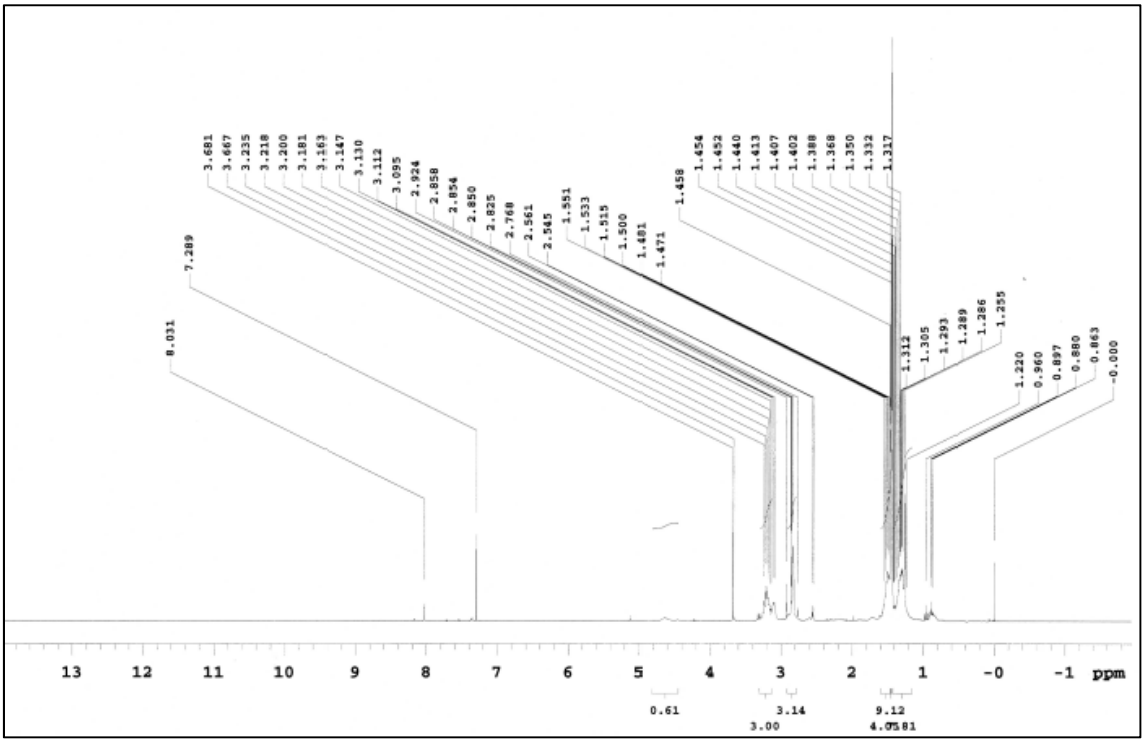
Fig. S7 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **8**.

Step 7: **8** to **9**



Compound **8** Compound **9**

A solution of Compound **8** (671.7 mg, 2.08 mmol) dissolved in 20 ml of EtOAc was vigorously stirred with excess Pd/C (300mg). The mixture was flushed with H_2 and stirred 5 h at room temperature. After the reaction, Pd/C was filtered through celite and filter-cake was washed with EtOAc. The solvent was removed under reduced pressure to give a compound **9** (201 mg, 2.08 mmol, **52%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.26 (m, 8**H**, -C**H**$_2$), 1.45 (m, 9**H**, -C**H**$_3$), 1.50 (m, 4**H**, -C**H**$_2$-), 2.85 (t, 3**H,** C-C**H**$_3$), 3.15 (m, 3**H**, C-C**H**$_3$), 4.76 (s, 1**H**, -N**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 26.6, 26.7, 28.1, 28.2 (-**C**H$_2$-), 28.6 (C-**C**H$_3$), 30.1 (-NH-**C**H$_2$-), 34.2 (N-**C**H$_2$), 41.5, 48.3 (N-**C**H$_3$). **ESI-MS** (m/z): 245.37 ($[M+H]^+$, found), 244.37 (calcd).



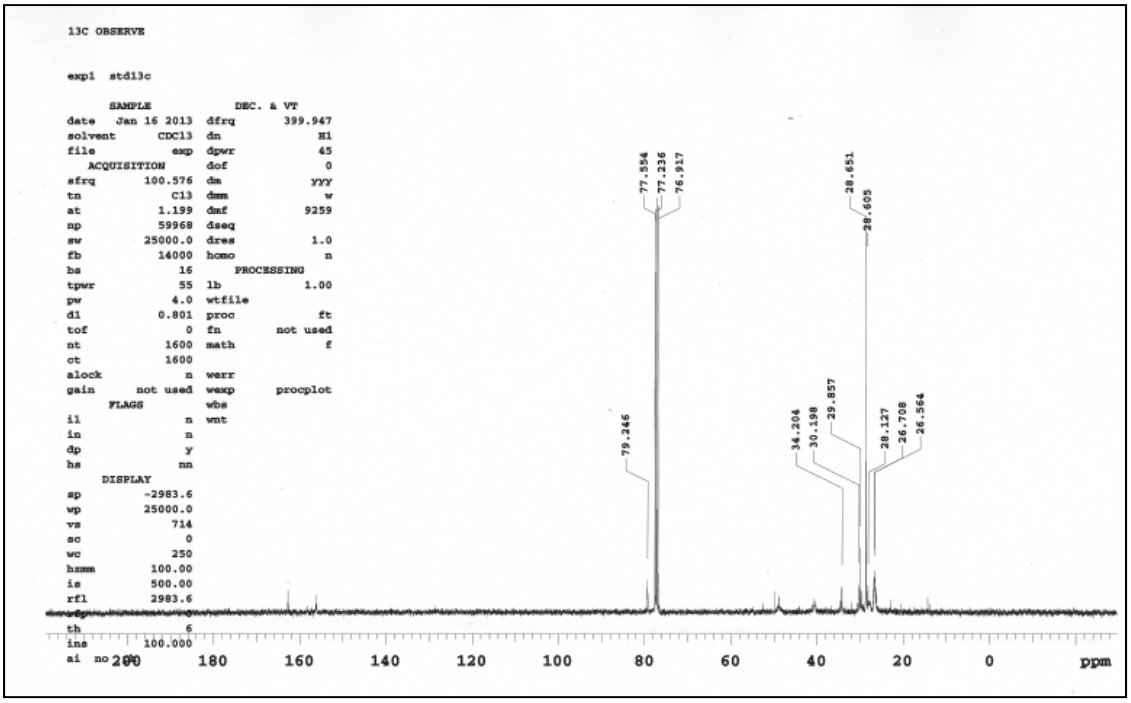
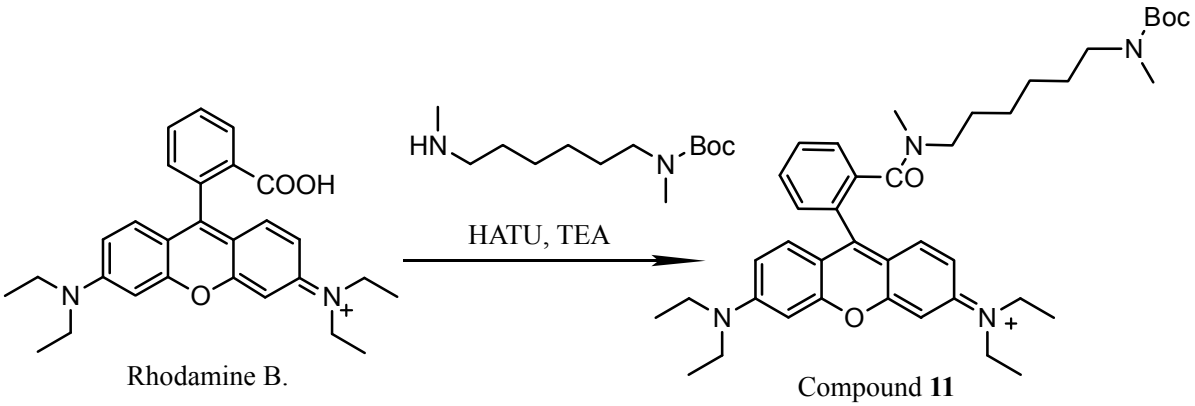


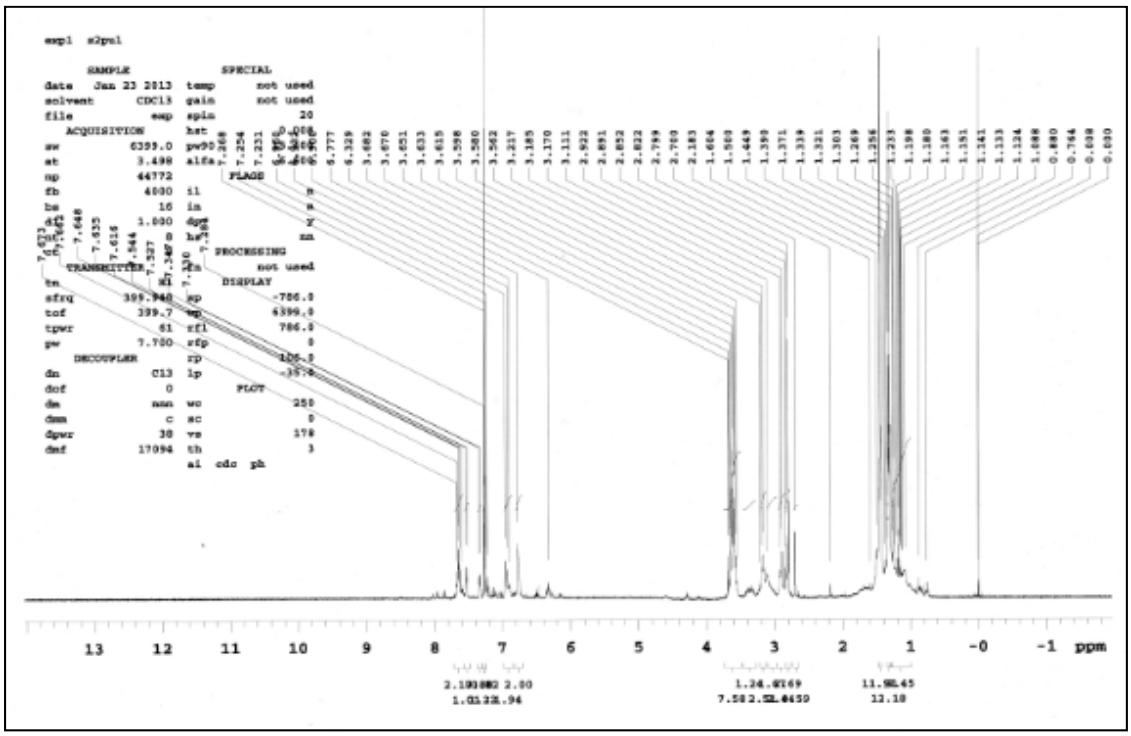
Fig. S8 (Left) ^{1}H NMR and **(Right)** ^{13}C NMR spectra of **9**.

Step 8: **RhodamineB 10** to **11**



Rhodamine B. Compound **11**

Compound **9** (32.4 mg, 0.13 mmol), rhodamine B **10** (52 mg, 0.11 mmol), HATU (50.6 mg, 0.133 mmol) and 733 mL of triethylamine were dissolved in 7.0 mL of DCM and stirred for 24 h. The reaction mixture was washed with water and crude product was purified by column chromatography using $CHCl_3$ / MeOH = 8/1. Compound **11** as a red solid was obtained (64.5 mg, 0.096 mmol, **82%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 1.16 (m, 8**H**, -C**H**$_2$-), 1.32 (t, 12**H**, -CH_2-C**H**$_3$), 1.44 (s, 9**H**, -C**H**$_3$), 2.82 (m, 3**H**, -C**H**$_3$), 2.89 (m, 3**H**, -C**H**$_3$), 3.11 (m, 2**H**, N-C**H**$_2$), 3.19 (m, 2**H**, N-C**H**$_2$), 3.61 (q, 8**H**, -C**H**$_2$-CH_3), 6.78 (m, 2**H**, Ar**H**), 6.93 (d, 2**H**, Ar**H**), 7.27 (dd, 2**H**, Ar**H**), 7.33 (d, 1**H**, Ar**H**), 7.53 (d, 1**H**, Ar**H**), 7.65 (dd, 2**H**, Ar**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 12.764 (4**C**, CH_2-**C**H_3), 26.48 (3**C**, -**C**H_3), 28.6 (4**C**, **C**H_2-CH_3), 37,8 (**C**-$(CH_3)_3$), 44.62 (N-**C**H_2-), 46.229 (4**C**, -**C**H_2-), 79.26, 96.36, 113.97, 114.01, 114.21, 114.31, 127.64, 128.34, 130.19, 132.41, 154.11, 155.87 155.99, 157.97, 158.02, 167.32, 168.33, 174.92 (Ar**C**). **ESI-MS** (m/z): 669.58 ($[M]^+$, found), 669.58 (calcd).



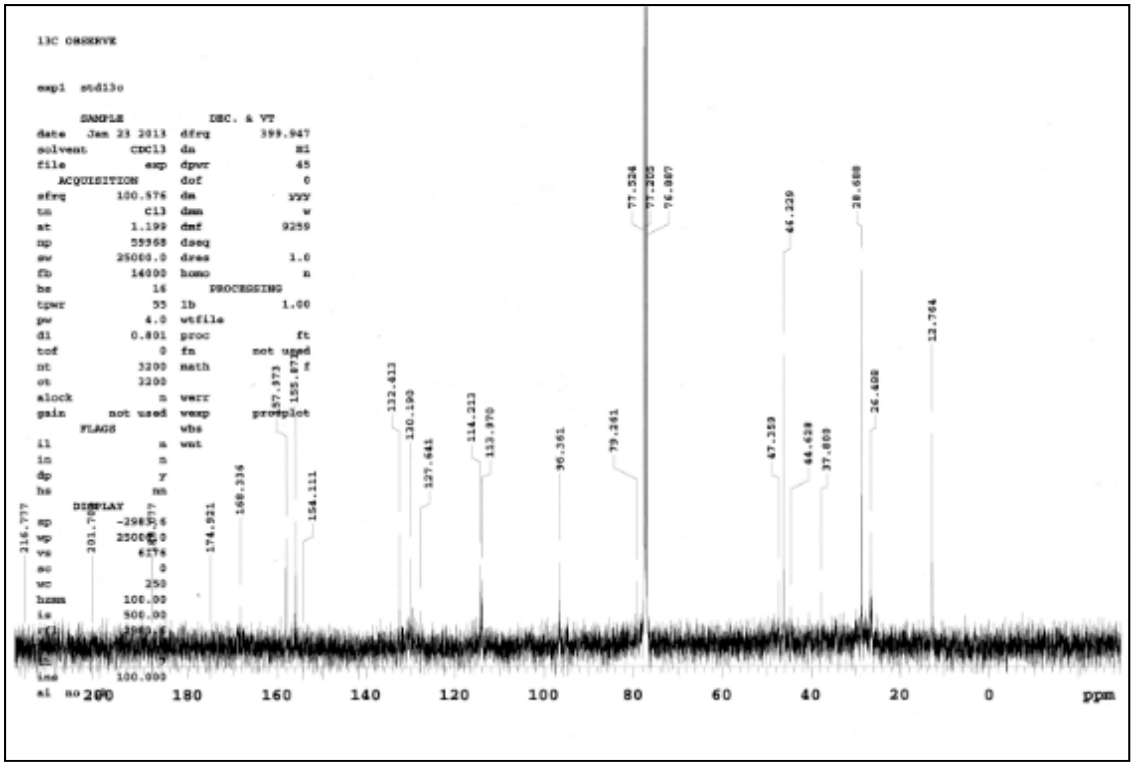
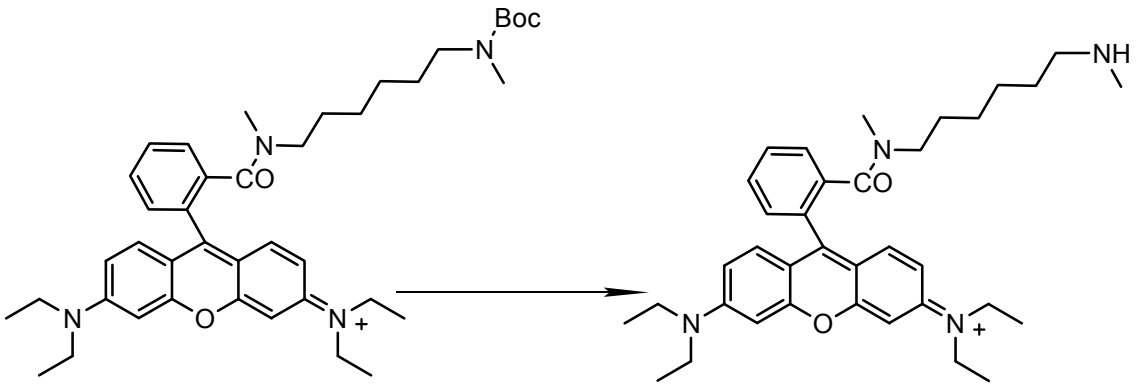


Fig. S9 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **11**.

Step 9: **11** to **12**



Compound **11** in 2ml of DCM was added dropwisely to 50 ml of trifluoroacetic acid by at 4 °C. The solution was to be room temperature and kept stirring for 90 min. After the reaction, the solvent was removed under reduced pressure to give a compound **12** and can be used for next step without farther purification (49.4 mg, 0.087 mmol, **90%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 0.92 (m, 8**H**, -C**H**$_2$-), 1.32 (m, 12**H**, -CH_2-C**H**$_3$), 1.64 (m, 3**H**, -C**H**$_3$), 2.78 (m, 3**H**, -C**H**$_3$), 2.91 (s, 2**H**, N-C**H**$_2$), 2.91 (s, 2**H**, N-C**H**$_2$) 3.18 (s, 1**H**, N**H**-CH_2), 3.65 (q, 8H, -C**H**$_2$-CH_3), 4.26 (d, 2**H**, NH-C**H**$_2$), 6.80 (m, 2**H**, Ar**H**), 7.00 (s, 1**H**, Ar**H**), 7.34 (dd, 2**H**, Ar**H**), 7.35 (dd, 2**H**, Ar**H**), 7.64 (m, 3**H**, Ar**H**). **^{13}C NMR** (σ ppm in $CDCl_3$): 11.209, 12.779, 14.312, 14.315 (4**C**, -**C**H_2-), 26.405 (4**C**, CH_2-**C**H_3), 29.166 (-NH-**C**H_3-), 30.3 (N-**C**H_3), 38.96 (N-**C**H_2), 46.35 (4**C**, -**C**H_2-CH_3), 70.80(-NH-**C**H_2-), 96.35, 97.04, 113.63, 113.86, 127.75, 129.05, 129.46, 130.11, 130.52, 131.15, 132.25, 132.57, 132.68, 155.57, 155.94, 158.00, 161.46, 161.81 (Ar**C**). 168.03 (**C**=O). **ESI-MS** (m/z): 569.38 ($[M]^+$, found), 569.38 (calcd).

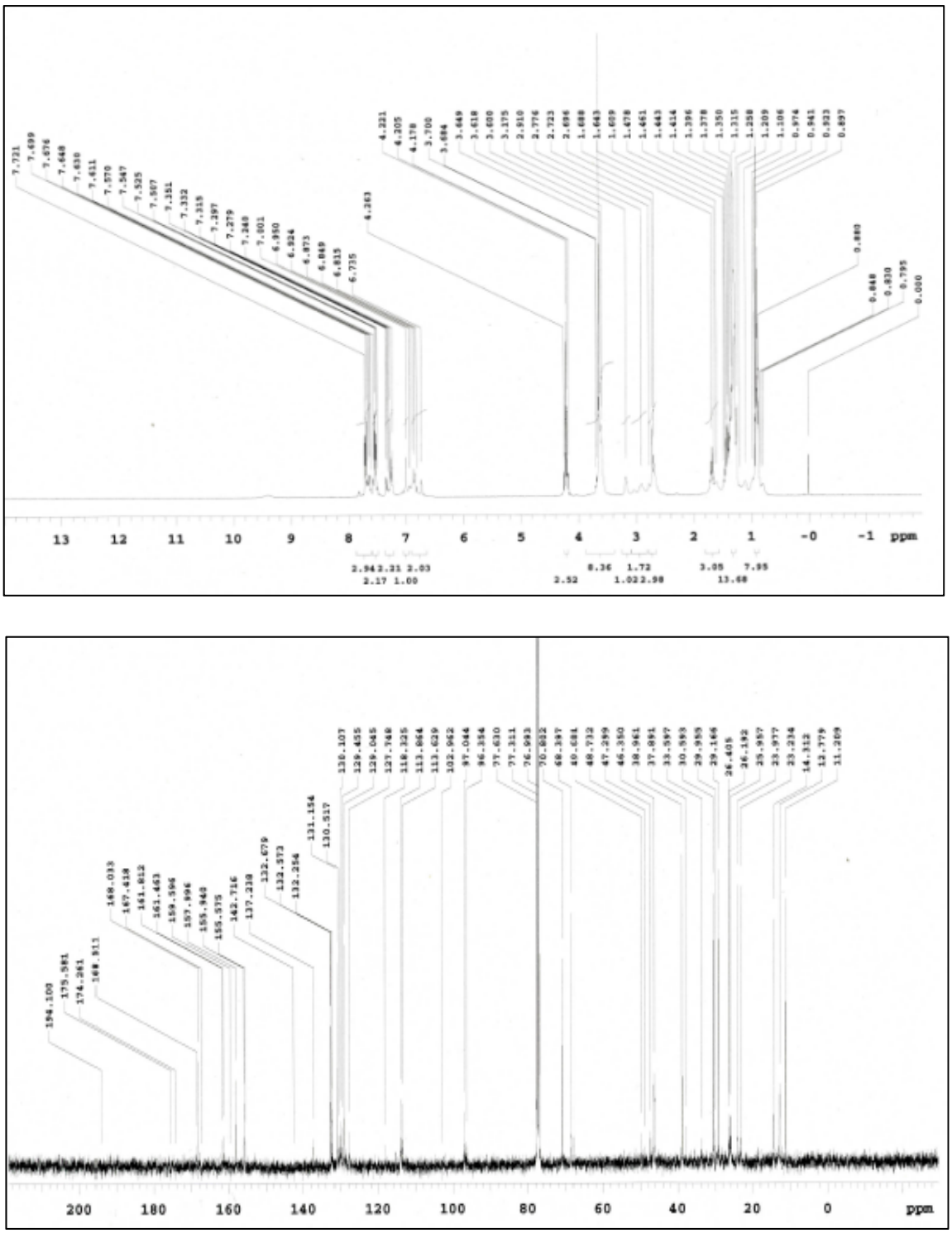
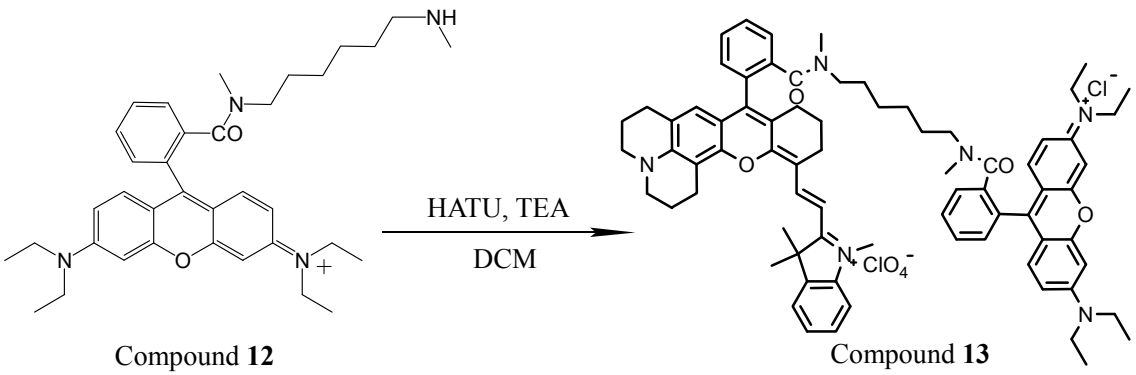


Fig. S10 (Upper) ^{1}H NMR and **(Bottom)** ^{13}C NMR spectra of **12**.

Step 10: **12** to Mito-RTP **13**



Compound **12** Compound **13**

Compound **12** (10mg, 0.016mmol) and Compound **4** (9.06mg, 0.016mmol) and HATU (50.6 mg, 0.133 mmol) were dissolved in DCM (7.0 mL) and added triethylamine (733 mL). The solution was kept stirring for 24 h and washed with water and purified by column chromatography using $CHCl_3$/MeOH = 5/1. Yield to Compound **13** (Mito-RTP) (6.2 mg, 0.0053 mmol, **33%**). **^{1}H NMR** (σ ppm in $CDCl_3$): 0.88 (m, 8**H**, -C**H**$_2$-), 1.29 (d, 12**H**, -CH$_2$-C**H**$_3$), 1.46 (s, 2**H**, N-C**H**$_2$), 1.75 (m, 9**H**, -C**H**$_3$), 2.14 (m, 3**H**, -C**H**$_3$), 2.34 (m, 4**H**, -C**H**$_2$-), 2.66 (m, 3**H**, -C**H**$_3$), 2.89 (m, 4**H**, -C**H**$_2$-), 3.12 (dd, 4**H**, -C**H**$_2$-), 3.29 (m, 2**H**, N-C**H**$_2$), 3.45 (t, 4**H**, -C**H**$_2$-), 3.64 (q, 8H, -C**H**$_2$-CH$_3$), 4.30 (m, 2**H**, N-C**H**$_2$), 5.92 (d, 1H, -C**H**=CH-), 6.44 (m, 1H, Ar**H**), 6.76 (t, 2H, Ar**H**), 6.89 (t, 2H, Ar**H**), 7.06 (m, 1H, Ar**H**), 7.20 (m, 3H, Ar**H**), 7.34 (m, 3H, Ar**H**), 7.46 (s, 1H, Ar**H**), 7.63 (m, 6H, Ar**H**), 8.46 (d, 1H, -C**H**=CH-). **^{13}C NMR** (σ ppm in $CDCl_3$): 12.79, 13.09 (4**C**, -**C**H$_2$-), 20.13, 20.53, 21.18 (-**C**H$_3$), 25.96 (N-**C**H$_2$), 26.39 (4**C**, CH$_2$-**C**H$_3$), 28.12 (N-**C**H$_2$), 29.38, 29.44 (-**C**H$_3$), 22.87, 24.43, 26.22, 26.37, 27.02, 28.54 (-**C**H$_2$-), 46.27 (4**C**, -**C**H$_2$-CH$_3$), 49.56, 49.09, 50.53 (-**C**H$_2$-), 94.35, 96.32, 96.48, 113.91, 114.15, 114.22, 114.70, 122.52, 122.61, 124.51, 124.82, 127.73, 128.60, 128.82, 129.21, 129.54, 129.84, 130.17, 130.44, 136.10, 136.23, 136.96, 137.08, 139.96, 140.13, 141.61, 155.38, 155.83, 157.94, 158.12, 160.03, 161.58, 166.48 (ArC) 168.717 (-CH=**C**H-). **ESI-MS** (m/z): 567.33 ($[M]^{2+}$, found), 1134.66 (calcd). **HR-ESI MS** (m/z): calcd for $C_{75}H_{86}N_6O_4^{2+}$,$[M]^{2+}$= 567.3350: obsd 567.3356 (error 1.1 ppm).

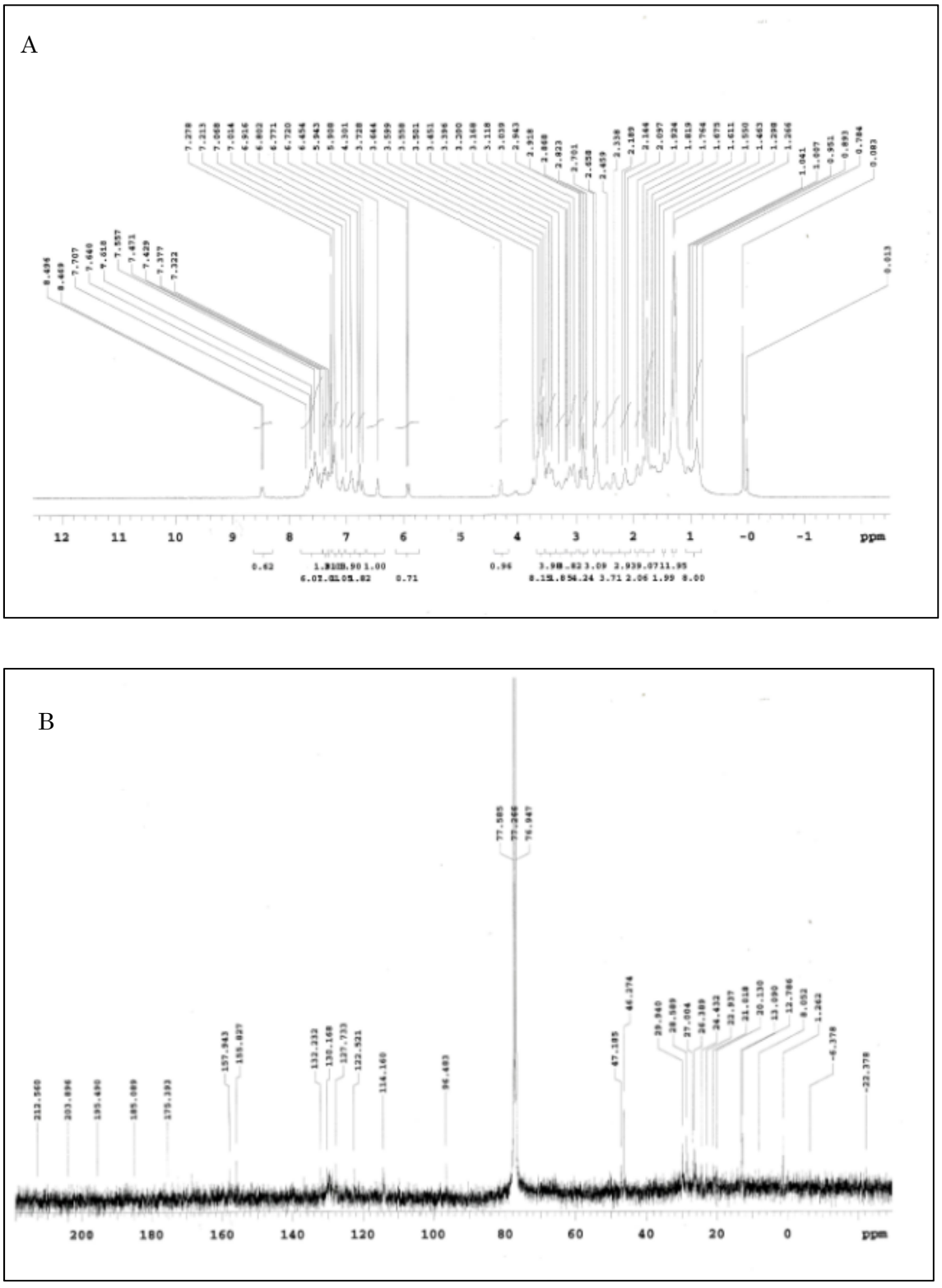


Fig. S11 (A) ^{1}H NMR, **(B)** ^{13}C NMR of **Mito-RTP**

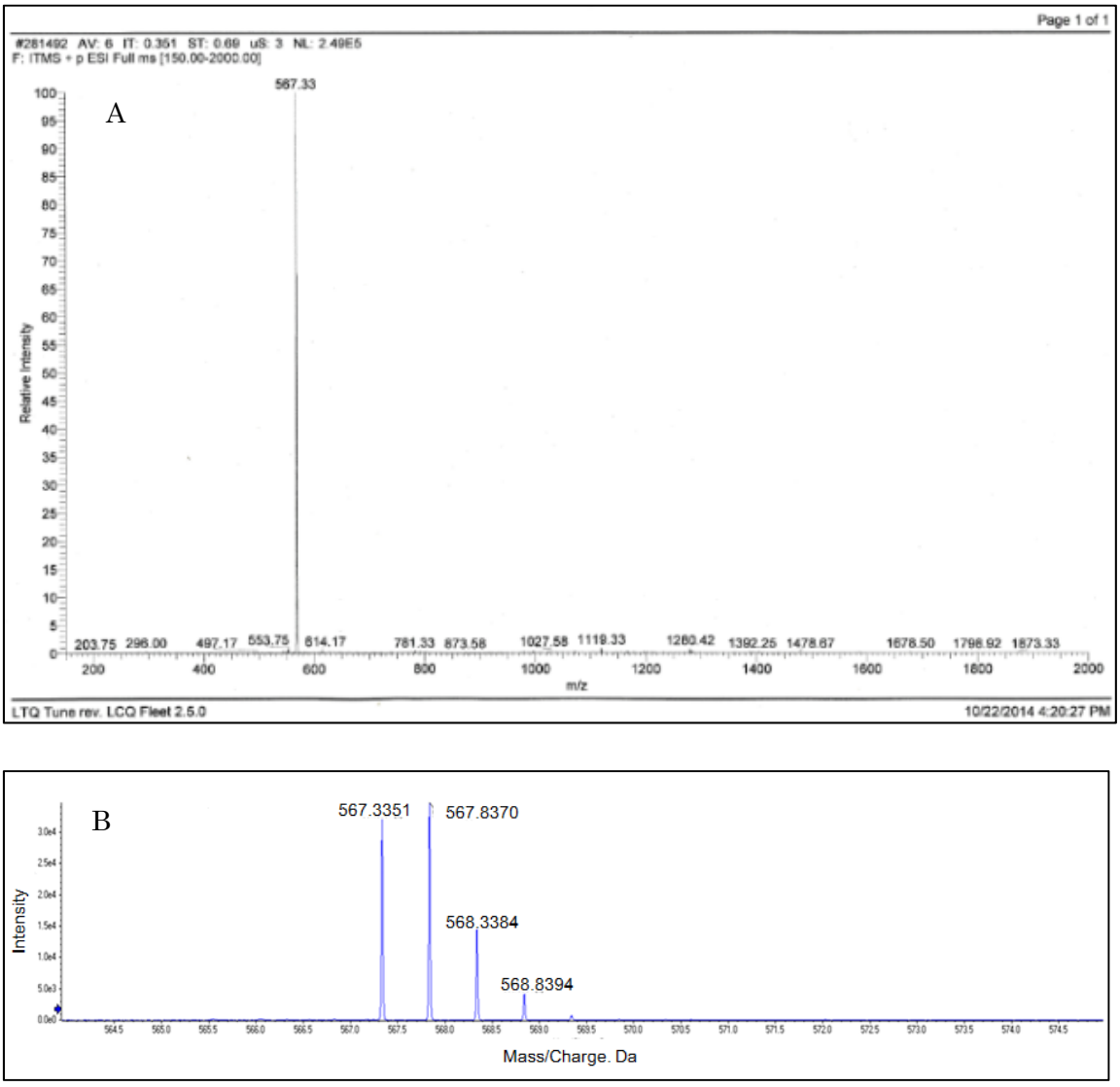


Fig. S12 (A) ESI-MS spectra, **(B)** HR-ESI MS spectra of **Mito-RTP**.

HPLC analysis

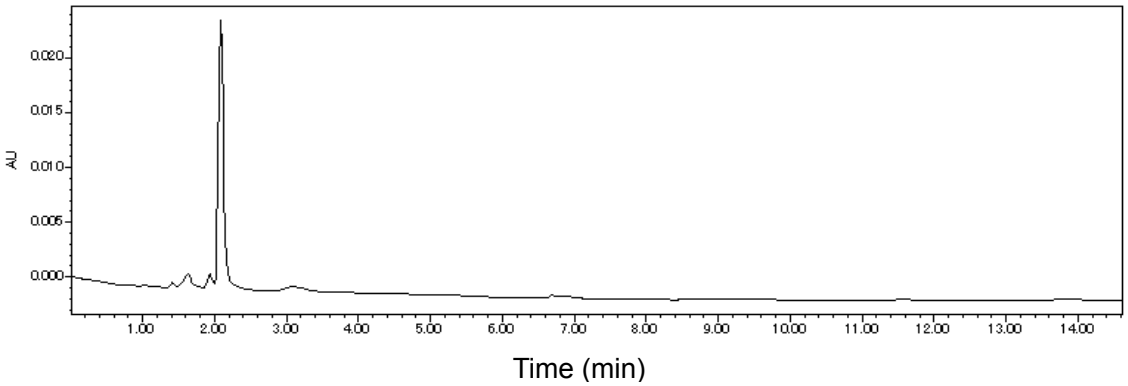


Fig. S13 To determine the purity of Mito-RTP, analytical high performance liquid chromatography (HPLC) was performed on LC-10AD VP (Simazu Corporation at) 285 nm equipped RP-18 GP column (f = 5 µm, Kanto Chemical Co.) A 20 µl sample solution in MeOH (10 µM) was injected and then chromatography was performed at 25°C with an initial flow rate of 1 ml/min of MeOH/water = 5/1 (v/v) as a moving phase. The purity of Mito-RTP was more than 94%.

2. In vitro evaluation of Mito-RTP

Temperature sensing property of Mito-RTP.

Emission spectrum of rhodamine B and CS NIR dye in Mito-RTP was measured by RF5300PC spectrophotometer (SHIMAZDU, Tokyo, Japan). 100 μM Mito-RTP EtOH solution was dissolved in PBS (EtOH/ PBS = 1:19) and added into qualtz cell (10 mm thickness, 3.5 mL volume) (Sigma). Temperature of the Mito-RTP solution was controlled by thermostat bath (BU150A, Yamato, Japan) and monitored with a digital thermocouple (ASF-250T, AS ONE, Japan) having an accuracy at ±0.1°C. Emission spectrum of rhodamine B and CS NIR dye were excited at 530 nm and 710 nm respectively and captured every 3°C step from 25 to 43°C.

Fluorescence stability of Mito-RTP against environmental conditions

Emission spectrum of rhodamine B and CS NIR dye in Mito-RTP against various pH and ionic strength were measured by spectrophotometer. The pH of PBS (pH 4 – 10) was controlled by HCl or KOH with monitoring by a pH meter (F-52, HORIBA, Tokyo, Japan) and ionic strength (KCl 0 – 500 mM) of PB was controlled by addition of each amount of KCl. 100 μM Mito-RTP EtOH solution was dissolved in each condition of the pH and ionic strength buffer (EtOH/buffer = 1:19) and added into a slit glass cuvette (2 mm thickness). Emission spectrum of rhodamine B and CS NIR dye were excited at 530 nm and 710 nm respectively and captured each condition.

3. Living cell Evaluation of Mito-RTP

Cell culture

HeLa cells (2×10^5 cells) were cultured on a φ60mm glass-bottom dish (Iwaki, Tokyo, Japan) in culture medium (89% DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen)). Cells were incubated in the presence of 5% CO_2 at 37°C.

Loading of Mito-RTP to the cells

Mito-RTP at a concentration of 1 μM in DMEM dispersed with 0.1% DMSO (Wako) was added to the cell. After 30 minutes incubation, loading solution was removed and washed twice with pre-warmed PBS. Then, pre-warmed 1 mL DMEM without phenol red (Gibco) was added for microscopic imaging.

Optical setting of fluorescence microscope

Rhodamine B and CS NIR dye were excited using a 75W xenon lamp (Ushio UXL-75XE). Excitation filter BP535-555HQ (Olymppus) and ET710/75x (Chroma), dichroic mirror DM565HQ (Olymppus) and T760lpxr (Chroma), and emission filter BA570-625HQ (Olympus) and ET810/90m (Chroma) were used for filter set of rhodamine B and CS NIR dye respectively. The filter wheel was changed automatically from the filter set of rhodamine B to that of CS NIR dye when measure the fluorescence images of HeLa cells. Extracellular temperature was kept at same degree with 5% CO_2 using a stage incubator (INUseries; 413 Tokai Hit,Shizuoka, Japan).

Localization of the Mito-RTP in HeLa cell

Mito-RTP stained HeLa cells were loaded 200 nM Mito-Tracker green FM (Invitrogen) for visualizing mitochondria. After 30 minutes incubation, wash twice with pre-warmed PBS and added 1mL of DMEM without phenol red (Gibco). Excitation filter BP465-495HQ (Olymppus), dichroic mirror DM515HQ (Olymppus), emission filter LP515HQ (Olympus) were used for filter set of Mito-Tracker green FM (Invitrogen). Both rhodamine B and CS NIR dye were excited for 200 ms and Mito-Tracker green FM were excited for 100 ms.

Z stack evaluation of Mito-RTP

The focus of Mito-RTP stained HeLa cells were changed from z = -8 to 9μm (z = 0 is in focus) by each 1 μm steps. Fluorescence intensities of both rhodamine B and CS NIR dye were captured at every 1 μm step. The fluorescence intensity ratio was calculated from the pictures.

Thermo sensitivity of Mito-RTP which localized at mitochondria

The temperature of the extra cellular buffer was gradually increased from 34 to 41°C (0.1°C / min) by a stage top incubator with four types of heaters (top cover, stage, water bath and lens heaters). The temperature of extracellular buffer was monitored by digital thermo couple (CT-800WP, AS ONE) having an accuracy of ±0.1°C. Fluorescence images of rhodamine B and CS NIR dye was detected at every 0.5±0.2°C step.

Measuring mitochondrial temperature with FCCP stimulation

Mito-RTP stained HeLa cells were kept at 37°C with 5% CO_2. Rhodamine B and CS NIR dye were excited for 200 ms. The fluorescence intensities of rhodamine B and CS NIR dye were captured every 30 seconds for 580 seconds (20 times). 10 µL of 1 mM FCCP in pre-warmed DMEM solution (1 µL of 10 mM FCCP DMSO stock was dispersed in 9 µL of DMEM) was added dropwisely (t = 0) to a 990 µL of cell culture medium to final concentration of 10 µM. As a control experiment, 10 µL of DMSO in pre-warmed DMEM solution (1 µL of DMSO was dispersed in 9 µL of DMEM) was added dropwisely (t = 0) to a 990 µL of cell culture medium.

Data analysis

ImageJ software was used for analyzing the fluorescence images which captured by the optical microscope. The fluorescence signals of both rhodamine B and CS NIR dye in Mito-RTP was determined by a circular region of interest (ROI) (2-6 ROIs per one cell) and the total fluorescence intensities were analyzed. The same size and shape of ROI was used for the fluorescence images of rhodamine B and CS NIR dye in each focus point in figure 2 B, each temperature in figure 2 C and each time in figure 3. The total intensities of the background were also analyzed and subtracted from that of rhodamine B and CS NIR dye to get the actual fluorescence intensities of Mito-RTP. The fluorescence intensity ratio was calculated from the total intensities of rhodamine B divided by that of CS NIR dye.

Supplementary Results

4. Excitation and emission spectra of Mito-RTP

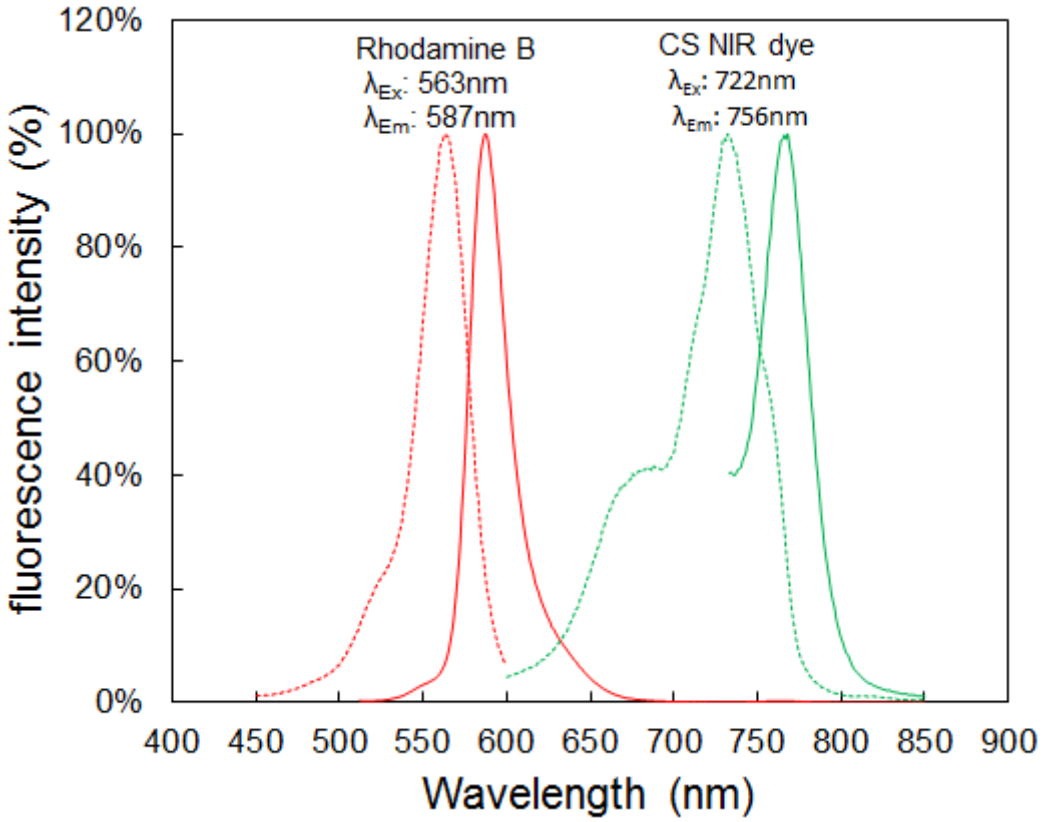


Fig. S14 Excitation and emission spectra of rhodamine B (red) and CS NIR dye (green) in Mito-RTP at 25°C (normalized at the peak of each spectrum). Excitation spectra were shown as dashed lines.

5. Fluorescence reversibility with alternative temperature changes

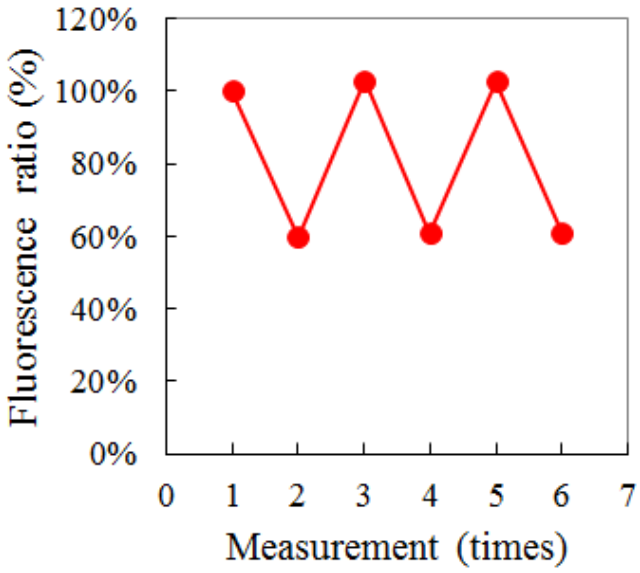


Fig. S15 The fluorescence reversibility in accordance with alternately temperature change between 30ºC and 45ºC.

6. Fluorescence stability of Mito-RTP in various pH and ionic strength

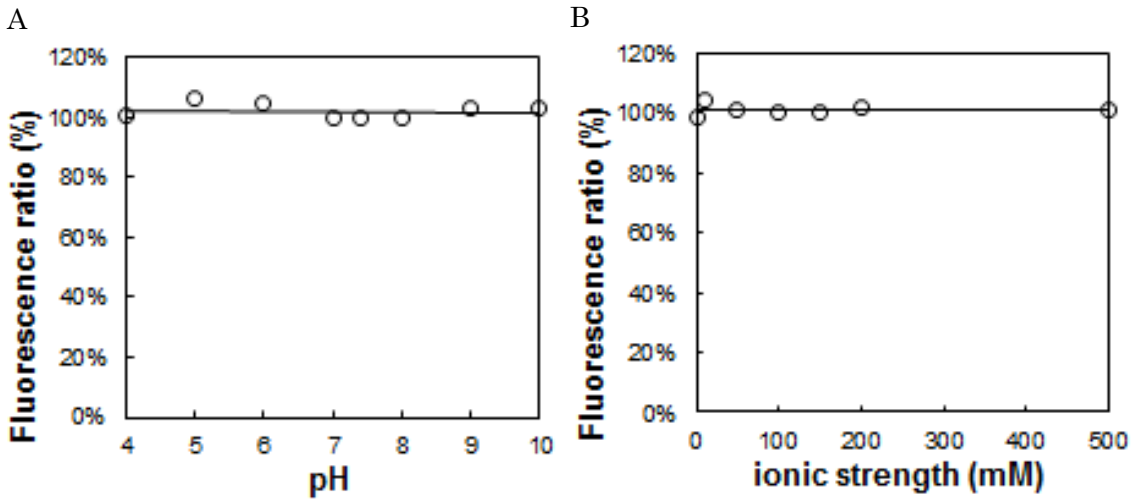


Fig. S16 Stability of Mito-RTP to various parameters in a cuvette. Fluorescence intensity ratio at 25°C in (A) phosphate buffer between pH 4 and 10 (Normalized at pH = 7.4) and (B) KCl solution between 0 and 500 mM (Normalized at KCl = 150 mM).

7. Evaluation of the photo-breaching

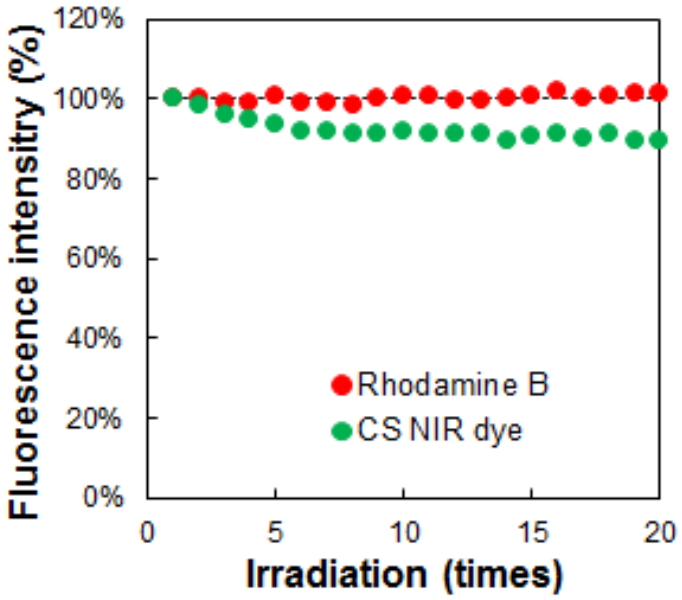


Fig. S17 Evaluation of the photo-breaching of rhodamine-B and CS NIR dye by several times of excitation. Exposure time was 200 ms for both rhodamine b and CS NIR dye. This data was used for calibration for microscopic temperature measurement.

8. Evaluation of cytotoxicity

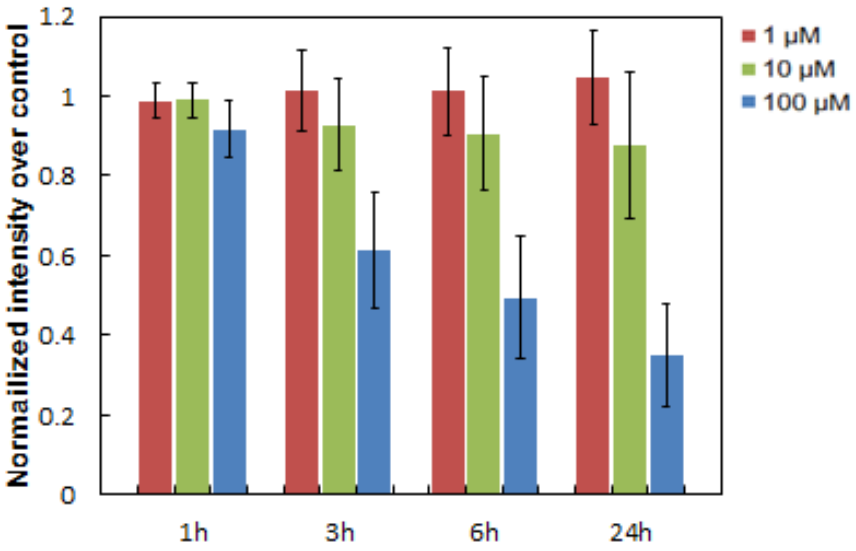


Fig. S18 Three different concentration of Mito-RTP was applied to HeLa cells and cytotoxicity was evaluated by WST-1 assay. Data was 9 times duplicated. Control: 0.1% DMSO in Medium.

References

S1. Z. Xu, J. C. DiCesare, P. W. Baures, *J. Comb. Chem.*, 2010, ***12*** (2), 248–254.

S2. J. Fçlling, S. Polyakova, V. Belov, A. van Blaaderen, M. L. Bossi, S. W. Hell, *Small*, 2008, **4**, 134-142