*ChemComm* **RSC***Publishing*

***Electronic Supplementary Information for***

# Cyanobenzo[*a*]phenoxazine-based near infrared lysosomes-tracker for *in cellulo* imaging

Ru Sun,[a] Wu Liu,[a] Yu-Jie Xu,[b] Jian-Mei Lu,*[a] Jian-Feng Ge *[a] and Masataka Ihara [c]

[a] College of Chemistry, Chemical Engineering and Material Science, Soochow University, 199 Ren'Ai Road, Suzhou 215123, China. E-mail: lujm@suda.edu.cn; ge_jianfeng@hotmail.com.

[b] School of Radiation Medicine and Protection, Medicine College of Soochow University, Suzhou 215123, China.

[c] Drug Discovery Science Research Centre, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan. Email: m-ihara@hoshi.ac.jp.

# Index

# 1. Experimental

## 1.1. Materials

Starting materials and solvents (analytical grade) were purchased from TCI Development Co., Ltd. (Shanghai branch, China) or Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used directly. Compound **1a** and **1b** were kindly gifted by the Synstar Japan Co., Ltd. (Kanagawa, Japan). Flash chromatography was performed with silica gel (300-400 mesh). Disodium hydrogen phosphate-citric acid buffer (pH = 3.4, 4.2, 4.6, 5.0, 5.4, 5.8, 6.2, 6.6, 7.0, 7.4, 7.8) were used, their theoretical pH values of the buffers were used for the buffer solution containing 1.0% DMSO.

## 1.2. Apparatus

Absorption spectra were obtained with a Shimadzu UV-3600 spectrophotometer. Emission spectra were measured on a Horiba Jobin Yvon FluoroMax-4 fluorescence spectrometer, the excitation wavelengths are 600 nm with 5 nm for the excitation and emission slits width. $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on a Varian 400 MHz spectrometer, and tetramethylsilane (TMS) or the solvent peaks were used for internal standard.[1] High resolution mass spectra were recorded on a Finnigan MAT95 mass spectrometer in ESI$^{+}$ mode. Infrared (IR) spectra were recorded on a Nicolet 5200 IR spectrometer using solid samples dispersed in KBr pellets. Melting points were determined on an X-4 microscope electron thermal apparatus (Taike, China). The pH values were measured with a Lei-Ci (pH-3C) digital pH-meter (Shanghai, China) using a combined glass-calomel electrode. All experiments were carried out at 25 $^{o}$C.

## 1.3. Synthesis and Characterization

*3-Cyano-9-(diethylamino)-5-(pyridin-3-ylimino)-5H-benzo[a]phenoxazine* (**2a**). A solution of **1a** (3.80 mmol, 1.50 g) and 3-aminopyridine (11.5 mmol, 1.10 g) in methanol (30.0 mL) was refluxed 12 h and then the

[1] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw and K. I. Goldberg, *Organometallics.*, 2010, **29**, 2176-2179.

reaction was stirred for 1 day at room temperature. The solvent was removed by evaporation, the residue was purified by column chromatograph on silica gel eluting with dichloromethane and acetone (4 : 1, v/v), the crude product was further purified by recrystallization with ethyl acetate to give **2a** (494 mg, 31%) as pure green solid. mp > 250 $^{o}$C. IR $\nu$ (KBr, cm$^{-1}$): 2970, 2223, 2026, 1639, 1582, 1485, 1444, 1408, 1385, 1272, 1184, 1132, 1115, 1075, 1015, 816, 696, 621, 561. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H, Ar-*H*), 8.63 (d, *J* = 8.1, 1H, Ar-*H*), 8.37 (br, 1H, Ar-*H*), 8.25 (s, 1H, Ar-*H*), 7.80 (d, *J* = 8.1, 1H, Ar-*H*), 7.44 (d, *J* = 8.9, 1H, Ar-*H*), 7.33 - 7.24 (m, *2*H, 2×Ar-*H*), 6.55 (d, *J* = 8.8, 1H, Ar-*H*), 6.23(s, 1H, Ar-*H*), 6.20 (s, 1H, Ar-*H*), 3.41 (q, *J* = 6.5, 4H, 2×C$H_2$ ), 1.21 (t, *J* = 6.8, 6H, 2×C$H_3$). $^{13}$C NMR (75 MHz, $CDCl_3$) δ 156.1, 150.8, 149.4, 147.5, 146.7, 144.8, 142.1, 139.3, 134.5, 132.6, 131.9, 131.0, 129.8, 128.1, 124.9, 124.5, 123.7, 118.7, 112.7, 109.3, 97.5, 96.2, 45.1, 12.6. HRMS-ESI$^{+}$: *m/z* = 420.1838 (calcd for [M + H]$^{+}$, 420.1824).

*9-(Diethylamino)-2-hydroxy-5-(pyridin-3-ylimino)-5H-benzo[a]phenoxazine* (**2b**). A solution of **1b** (2.60 mmol, 1.00 g) and 3-aminopyridine (7.80 mmol, 0.70 g) in methanol (20.0 mL) was refluxed 12 h and then the reaction was stirred for 1 day at room temperature. The solvent was removed by evaporation, the residue was purified by column chromatograph on silica gel eluting with dichloromethane and methanol (30 : 1, v/v), the crude product was further purified by recrystallization with ethyl acetate to give **2b** (123 mg, 11%) as pure green solid. mp > 250 $^{o}$C. IR $\nu$ (KBr, cm$^{-1}$): 3243, 2927, 2026, 1639, 1574, 1405, 1385, 1345, 1188, 1134, 1114, 1044, 814, 695, 623, 560. $^{1}$H NMR (400 MHz, DMSO) δ 10.22 (s, 1H, O-*H*), 8.31 (s, 1H, Ar-*H*), 8.30 (d, *J* = 8.7, 1H, Ar-*H*), 8.15 (s, 1H, Ar-*H*), 7.85 (s, 1H, Ar-*H*), 7.43 (d, *J* = 9.3, 1H, Ar-*H*), 7.39 (t, *J* = 8.3, 1H, Ar-*H*), 7.31 (d, *J* = 5.7, 1H, Ar-*H*), 7.09 (d, *J* = 7.5, 1H, Ar-*H*), 6.64 (d, *J* = 7.9, 1H, Ar-*H*), 6.41 (s, 1H, Ar-*H*), 5.96 (s, 1H, Ar-*H*), 3.40 (q, *J* = 6.1, 4H, 2×C$H_2$), 1.11 (t, J = 7.6, 6H, 2×C$H_3$).$^{13}$C NMR (75 MHz, DMSO) δ 159.5, 156.6, 149.9, 148.6, 147.7, 146.2, 144.2,142.0, 140.4, 136.9, 136.3, 132.8, 130.1, 127.9, 126.9, 124.0, 123.8, 118.7, 109.1, 108.0, 96.2, 44.3, 12.4. HRMS-ESI$^{+}$: *m/z* = 411.1817 (calcd for [M + H]$^{+}$, 411.1821).

*3-Cyano-9-(diethylamino)-5-((1-(2-hydroxyethyl)pyridine-1-ium-3-yl)imino)-5H-benzo[a]phenoxazine chloride* (**3a**). To a solution of **2a** (0.23 mmol, 0.10 g) in acetonitrile (10 mL), iodoethanol (2.3 mmol, 0.41 g) was added. The mixture was refluxed for 12 h and then the solvent was removed. The residue was purified

by column chromatograph on silica gel eluting with dichloromethane and methanol (20 : 1 , v/v). The crude product was further purified by recrystallization with ethanol to give the iodide form of **3a**, then the methanol solution of the obtained iodide salt was passed through 717 anion exchange resin; the solvent was evaporated to obtain **3a** as a dark blue solid (58 mg, 54%). mp > 250 $^{o}$C. IR $\nu$ (KBr, cm$^{-1}$): 3421, 2924, 2225, 2027, 1636, 1585,1485, 1445, 1407, 1383, 1337, 1274, 1182, 1117, 1077, 1016, 818, 697, 568. $^{1}$H NMR (400 MHz, $CD_3OD$) δ 8.67 (d, *J* = 5.3, 1H, Ar-*H*), 8.62 (s, 1H, Ar-*H*), 8.50 (d, *J* = 9.0, 1H, Ar-*H*), 8.51 (s, 1H, Ar-*H*), 8.18 (d, *J* = 8.2, 1H, Ar-*H*), 8.09 (t, *J* = 6.8, 1H, Ar-*H*), 7.84 (d, *J* = 8.3, 1H, Ar-*H*), 7.41 (d, *J* = 9.0, 1H, Ar-*H*), 6.75 (d, *J* = 9.1, 1H, Ar-*H*), 6.32 (s, 1H, Ar-*H*), 6.20 (s, 1H, Ar-*H*), 4.74 (br, 2H, C$H_2$), 4.06 (br, 2H, C$H_2$), 3.50 (q, *J* = 7.0, 4H, 2×C$H_2$), 1.22 (t, *J* = 6.9, 6H, 2×C$H_3$). $^{13}$C NMR (75 MHz, $CD_3OD$) δ 158.4, 152.2, 151.9, 150.8, 146.9, 138.8, 138.0, 137.6, 136.9, 134.4, 132.4, 131.5, 131.3, 129.5, 128.4, 128.3, 125.6, 124.3, 118.3, 112.2, 111.0, 95.9, 64.1, 60.7, 45.1, 11.8. HRMS-ESI$^{+}$: *m/z* = 464.2069 (calcd for [M - Cl]$^{+}$, 464.2087).

*9-(Diethylamino)-2-hydroxy-5-((1-(2-hydroxyethyl)pyridine-1-ium-3-yl)imino)-5H-benzo[a]phenoxazine chloride* (**3b**). To a solution of **2b** (0.49 mmol, 0.20 g) in acetonitrile (10 mL), iodoethanol (4.90 mmol, 0.83 g) was added. The mixture was refluxed for 12 h and then the solvent was removed. The residue was purified by column chromatograph on silica gel eluting with dichloromethane and methanol (20 : 1 , v/v). The crude product was further purified by recrystallization with ethanol to give the iodide form of **3b**, then the methanol solution of the obtained iodide salt was passed through 717 anion exchange resin; the solvent was evaporated to obtain **3b** as a dark blue solid (92 mg, 40%). mp > 250 $^{o}$C. IR $\nu$ (KBr, cm$^{-1}$): 3476, 2928, 2363, 2026, 1635, 1593, 1568, 1411, 1384, 1346, 1273, 1187, 1114, 1084, 1014, 816, 692, 621, 561.$^{1}$H NMR (400 MHz, $CD_3OD$) δ 8.58 (d, *J* = 8.7, 1H, Ar-*H* ), 8.56 (s, 1H, Ar-*H*), 8.31 (d, *J* = 8.0, 1H, Ar-*H*), 8.11 (d, *J* = 6.8, 1H, Ar-*H*), 8.03 (t, *J* = 7.0, 1H, Ar-*H*), 7.91 (s, 1H, Ar-*H*), 7.50 (d, *J* = 8.3, 1H, Ar-*H*), 7.06 (d, *J* = 8.0, 1H, Ar-*H*), 6.74 (d, *J* = 8.3, 1H, Ar-*H*), 6.40 (s, 1H, Ar-*H* ), 6.18 (s, 1H,Ar-*H*), 4.69 (br, 2H, C$H_2$), 4.04 (br, 2H, C$H_2$), 3.48 (q, *J* = 6.7, 4H, 2×C$H_2$), 1.20 (t, *J* = 6.6, 6H, 2×C$H_3$). $^{13}$C NMR (75 MHz, $CD_3OD$) δ 165.2, 164.0, 156.3, 155.7, 154.8, 151.5, 144.4, 144.0, 142.7, 142.4, 138.3, 135.9, 133.2, 132.4, 129.5, 128.3, 124.0, 115.2, 113.2, 101.3, 101.0, 68.4, 65.4, 49.9, 17.6. HRMS-ESI$^{+}$: *m/z* = 455.2093 (calcd for [M - Cl]$^{+}$, 455.2083).

## 1.4. Absorption and Fluorescence titration

Stock solutions (100 μM) were prepared in a volumetric flask (100 mL) with DMSO (10.0 mL) and doubly distilled water. Each test solution (10 μM) was prepared in a volumetric flask (10 mL) with 1 mL stock solution and corresponding buffer solution to give a total volume of 10.0 mL. Absorption and fluorescence spectra were obtained with 1.0-cm quartz cells.

## 1.5. Selectivity experiment

Stock solutions of probes (100 μM) were prepared in a volumetric flask (100 mL) with DMSO (10.0 mL) and doubly distilled water. Stock solutions of various ions were prepared in volumetric flasks (10 mL) with concentrations of NaCl (1 M), KCl (1 M), $MgSO_4$ (5 mM), CaCl (5 mM), $CoCl_2 \cdot 6H_2O$ (3 mM), $CuCl_2 \cdot 2H_2O$ (3 mM), $NiCl_2$ (3 mM) , $CdCl_2 \cdot 2.5H_2O$ (3 mM), $HgCl_2$ (3 mM) in doubly distilled water. Stock solutions of all kinds of amino acids were all prepared in volumetric flasks (100 mL) with concentrations of 1 mM in doubly distilled water. Each test solution was prepared in a volumetric flask (10 mL) with 1 mL stock solution of probes and 1 mL stock solution of corresponding ions or amino acids solutions, diluted with disodium hydrogen phosphate-citric acid buffer solution (pH=7.0) or 0.1 M citric acid solution (pH = 1.6) in competition assay to give a total volume of 10.0 mL.

## 1.6. Cell culture

HeLa cells were cultured in Roswell Park Memorial Institute culture medium (RPMI-1640) supplemented with 10% calf serum, penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) and L-glutamine ($2.5 \times 10^{-4}$ M) at 37 °C in a 5:95 $CO_2$-air incubator. Cells with $2 \times 10^5$ density were loaded onto a glass-bottomed coverslip with a diameter of 35 mm and cultured for 48 h before use.

## 1.7. Fluorescent imaging of HeLa cells

The Hoechst 33342 (50 nM), LysoTracker Green DND-26 (50 nM) and probes **3a** or **3b** (10 μM, cells medium (1 mL) containing 1% DMSO) were used in the fluorescent imaging subsequently. In each fluorescent imaging procedure, the cells were firstly washed with PBS (pH = 7.4) three times, then

fluorescent dyes in cells medium (1 mL) were added and the cells were incubated for 20 min, fluorescence image of the stained HeLa cells was obtained with a TCS SP5 II confocal fluorescence microscope, all the images were gathered at the same confocal microscope settings and processed with attached software. Blue channel emission with Hoechst 33342 was collected in 410-480 nm upon excitation at 405 nm, green channel emission with LysoTracker Green DND-26 was detected in 505-550 nm by excitation at 488 nm, and red channel emission with probes **3a** or **3b** was measured in 650-790 nm with excitation at 633 nm respectively.

## 2. Figures

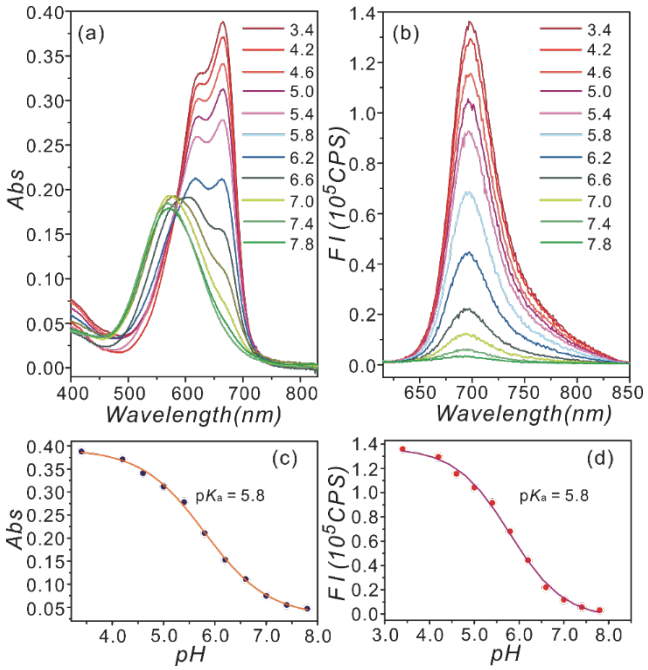


**Fig. S1** Optical responses of probe **3b** towards various pH with disodium hydrogen phosphate-citric acid buffers containing 1% DMSO. (a) Absorption spectra; (b) Emission spectra ($\lambda_{ex}$ = 600 nm); (c) Absorption changes with different pH at 665 nm; (d) Fluorescence intensity changes with different pH at 698 nm.

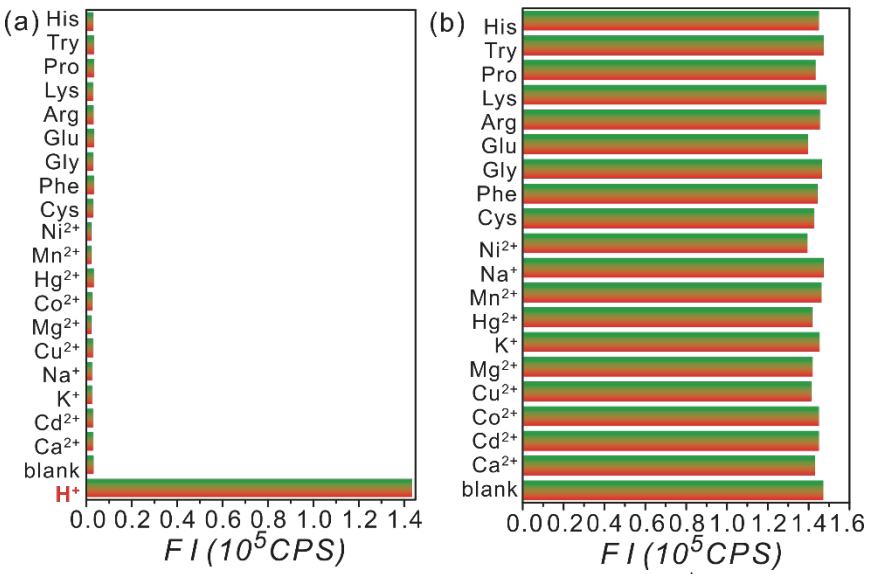


**Fig. S2** Fluorescence intensities of **3a** (10 μM) to different analytes, $K^+$ (100 mM), $Na^+$ (100 mM), $Ca^{2+}$ (0.5 mM), $Cd^{2+}$ (0.3 mM), $Cu^{2+}$ (0.3 mM), $Mg^{2+}$ (0.5 mM), $Co^{2+}$ (0.3 mM), $Hg^{2+}$ (0.3 mM), $Mn^{2+}$ (0.3 mM), $Ni^{2+}$ (0.3 mM), Cys (0.1 mM), Phe (0.1 mM), Gly (0.1 mM), Glu (0.1 mM), Arg (0.1 mM), Lys (0.1 mM), Pro (0.1 mM), Try (0.1 mM) and His (0.1 mM) were included. (a) Tested in disodium hydrogen phosphate-citric acid buffer (pH 7.0). (b) Tested in citric acid buffer (pH 1.6).

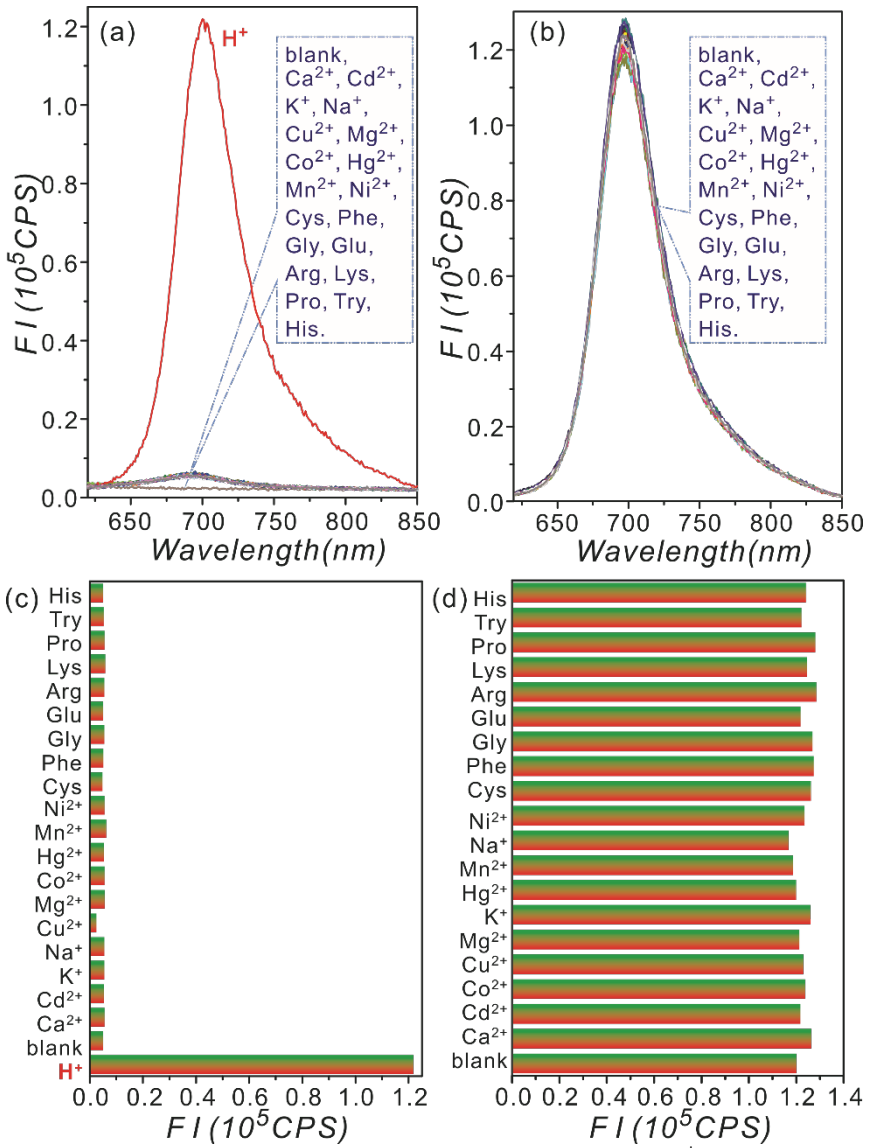


**Fig. S3** Fluorescence responses of **3b** (10 μM) to different analytes, $K^+$ (100 mM), $Na^+$ (100 mM), $Ca^{2+}$ (0.5 mM), $Cd^{2+}$ (0.3 mM), $Cu^{2+}$ (0.3 mM), $Mg^{2+}$ (0.5 mM), $Co^{2+}$ (0.3 mM), $Hg^{2+}$ (0.3 mM), $Mn^{2+}$ (0.3 mM), $Ni^{2+}$ (0.3 mM), Cys (0.1 mM), Phe (0.1 mM), Gly (0.1 mM), Glu (0.1 mM), Arg (0.1 mM), Lys (0.1 mM), Pro (0.1 mM), Try (0.1 mM) and His (0.1 mM) were included. (a, c) Tested in disodium hydrogen phosphate-citric acid buffer (pH 7.0). (b, d) Tested in citric acid buffer (pH 1.6).

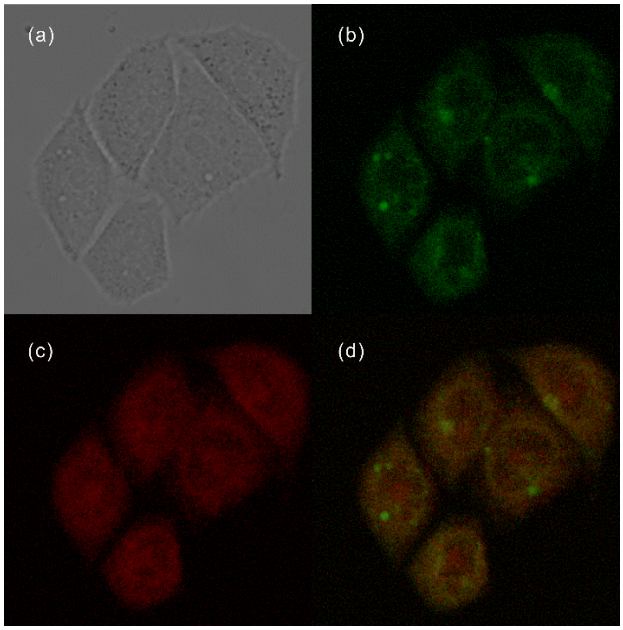


**Fig. S4** Fluorescence images of HeLa cells (Green channel emission was collected in 505–550 nm upon excitation at 488 nm, and red channel emission was collected in 650–790 nm upon excitation at 633 nm.). (a) The bright–field image; (b) Image with LysoTracker Green DND-26 (50 nM); (c) Image with probe **3b** (10 μm); (d) Overlay of (b) and (c).

# 3. Appendix

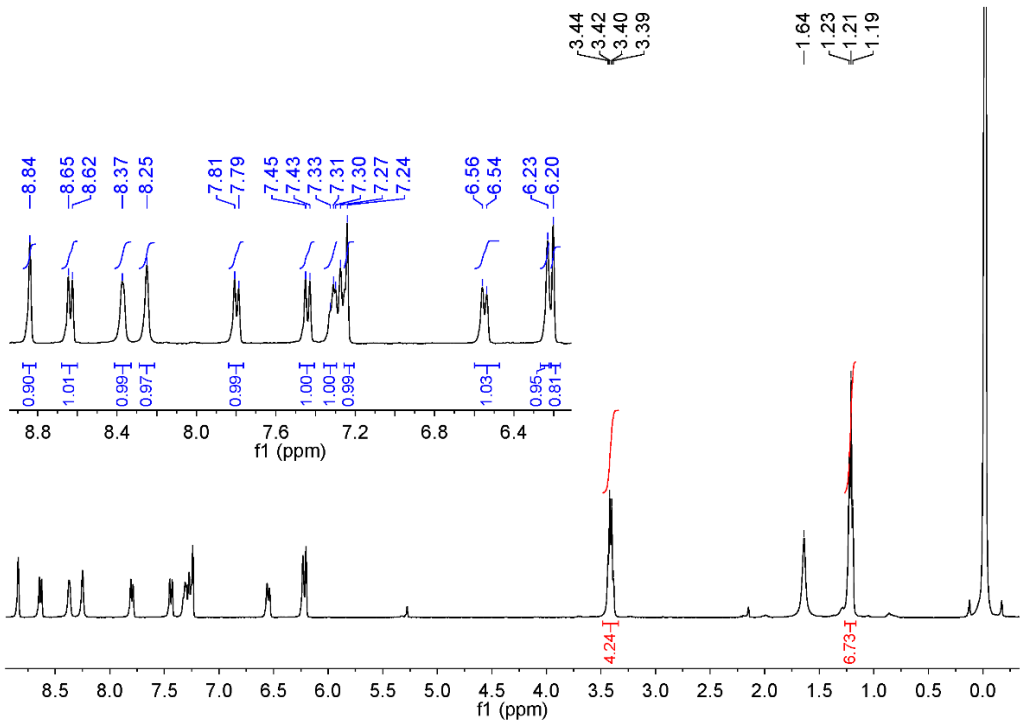


**Fig. S5** $^{1}$H NMR of **2a**.

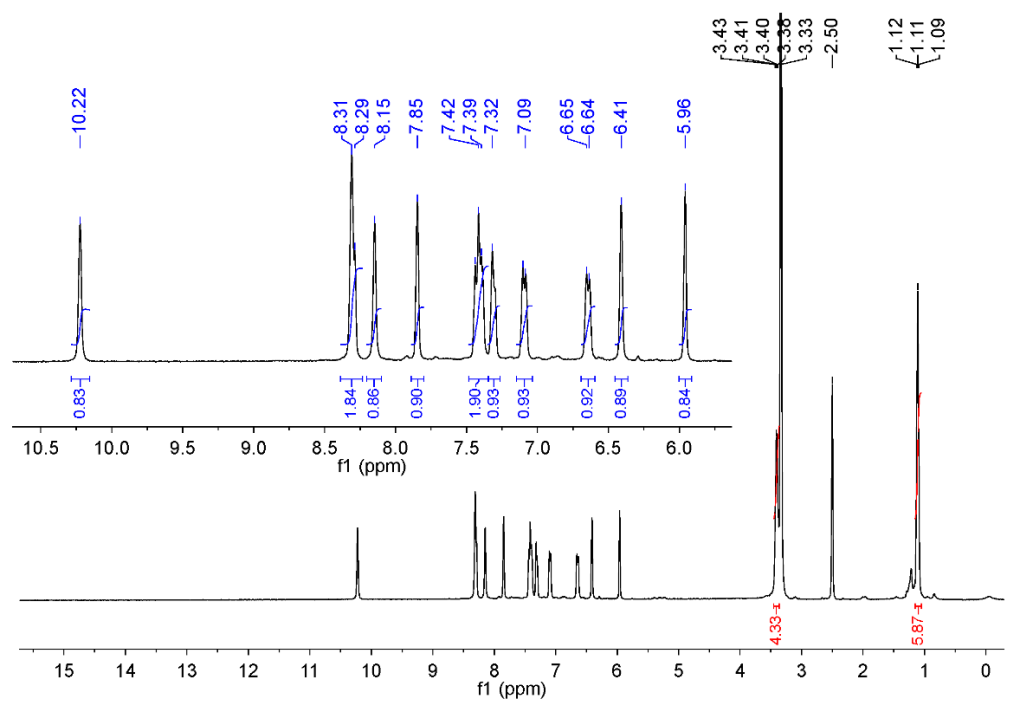


**Fig. S6** $^{1}$H NMR of **2b**.

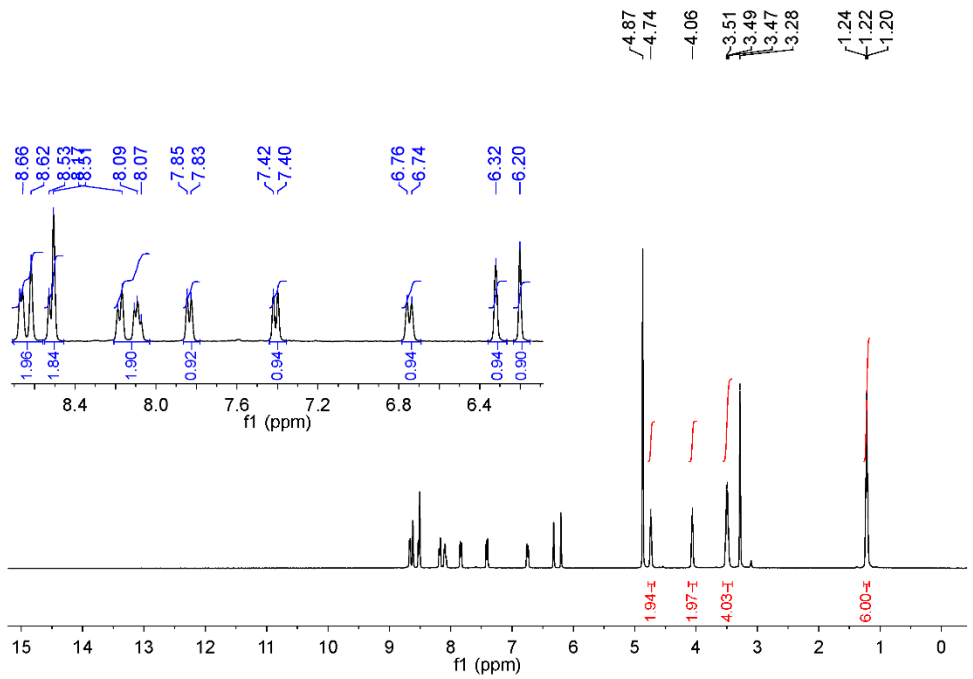


**Fig. S7** $^{1}$H NMR of **3a**.

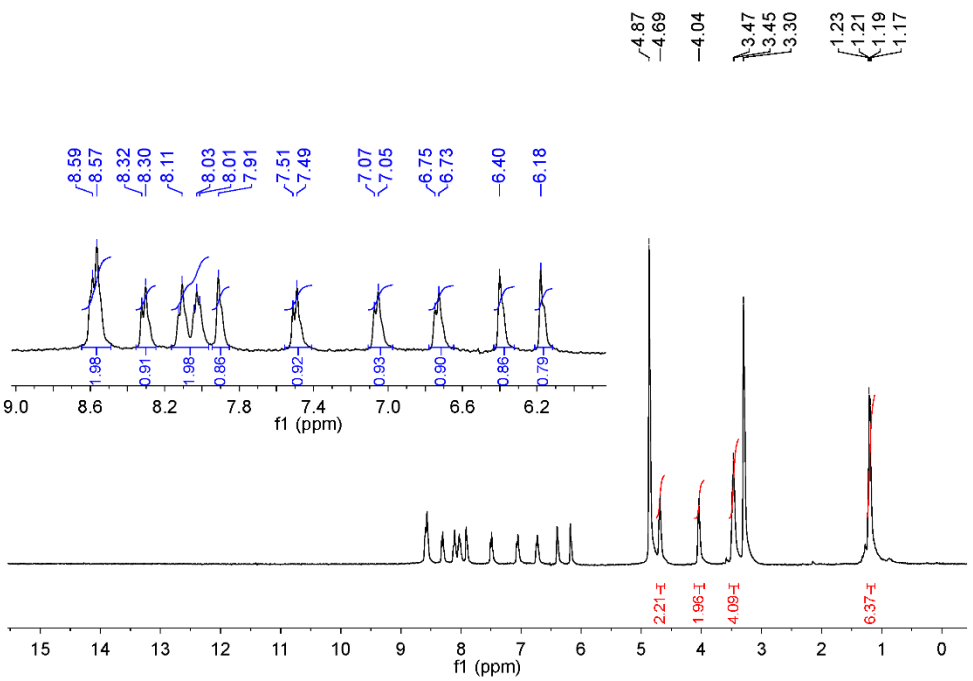


**Fig. S8** $^{1}$H NMR of **3b**.

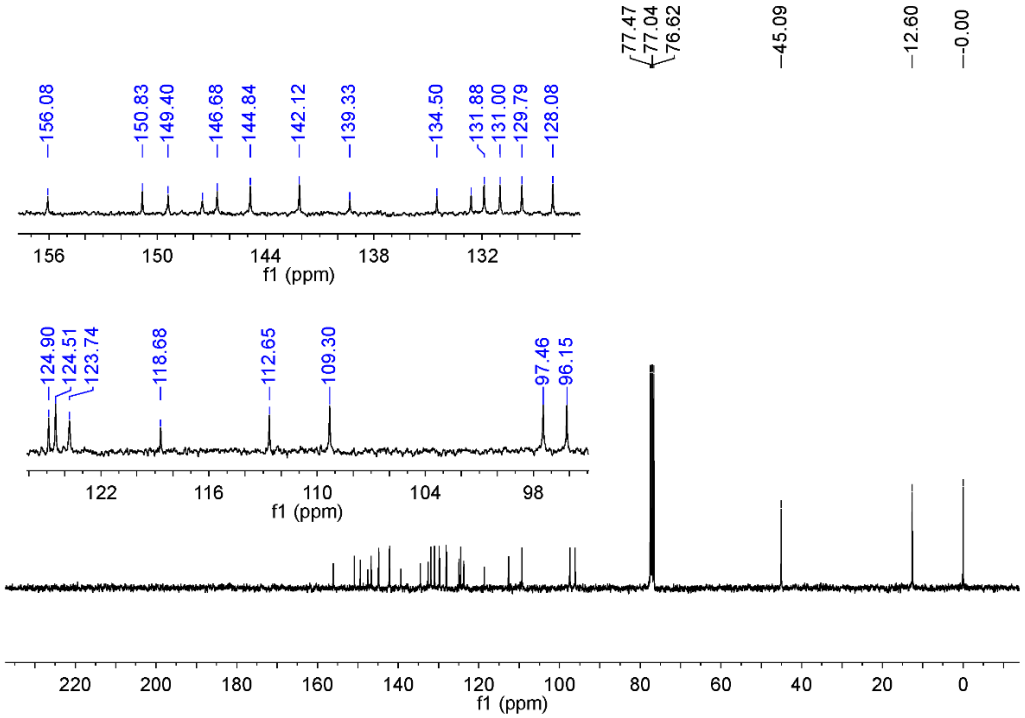


**Fig. S9** $^{13}$C NMR of **2a**.

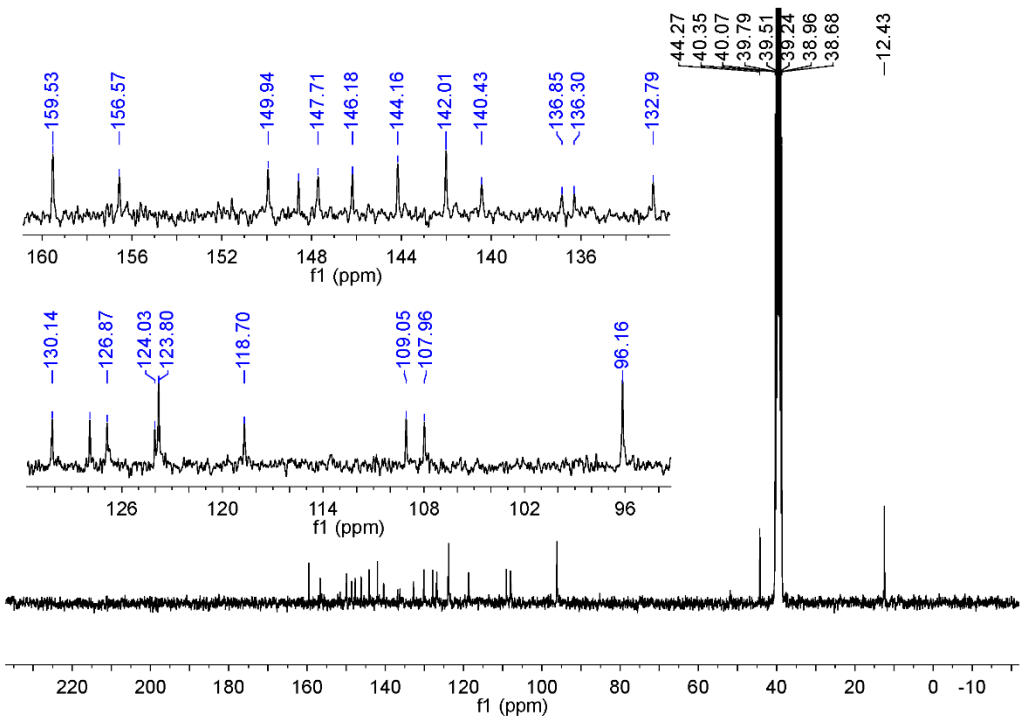


**Fig. S10** $^{13}$C NMR of **2b**.

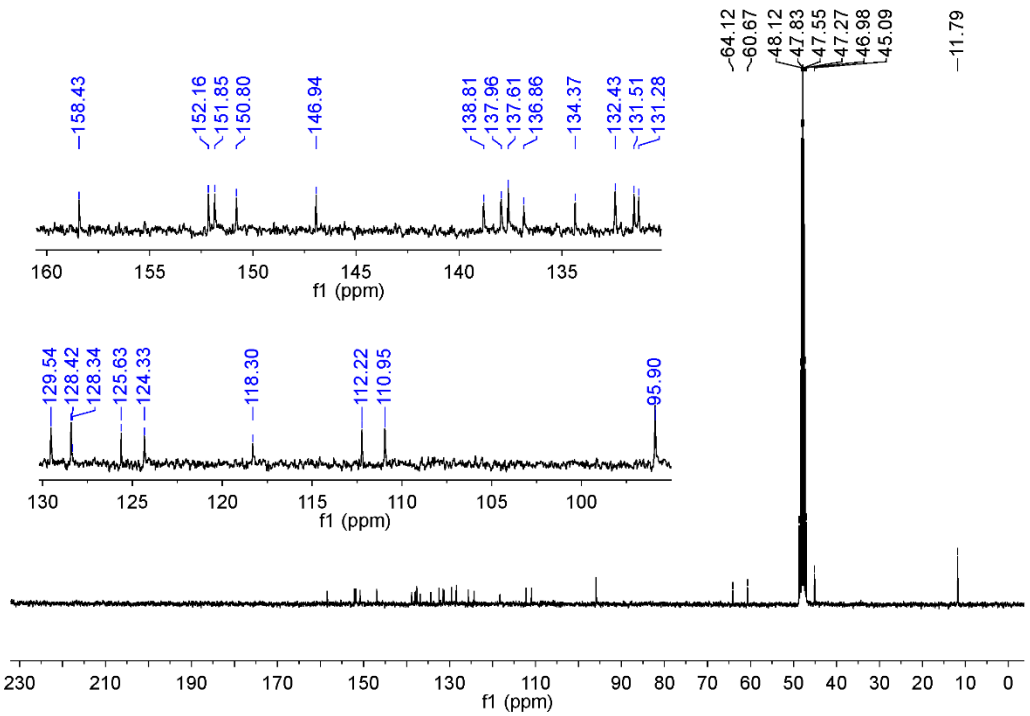


**Fig. S11** $^{13}$C NMR of **3a**.

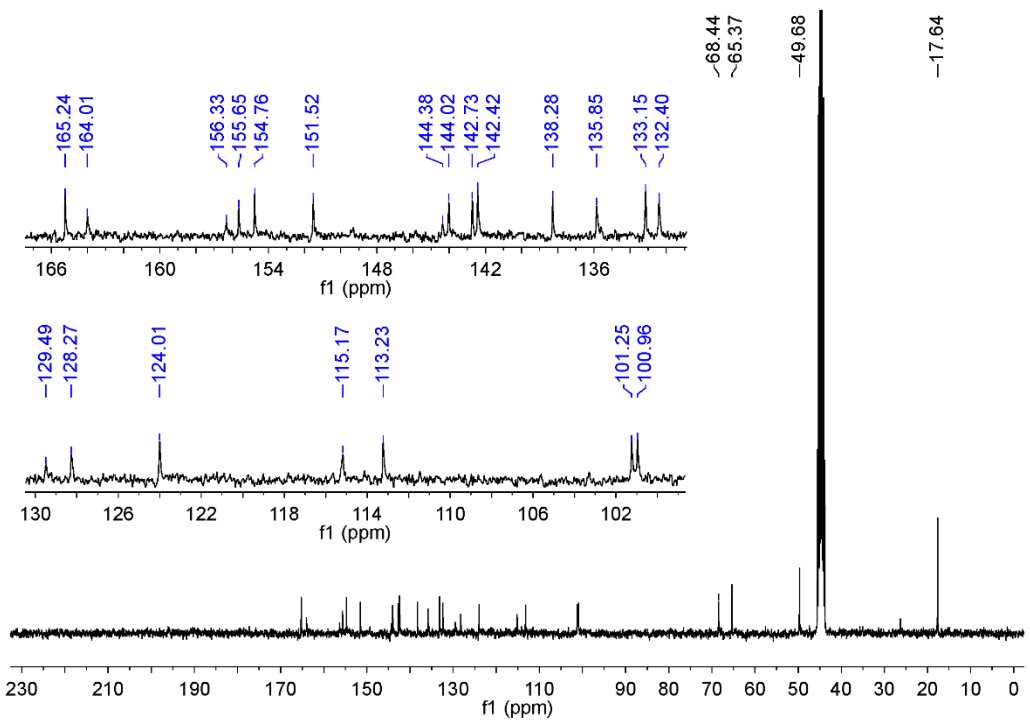


**Fig. S12** $^{13}$C NMR of **3b**.

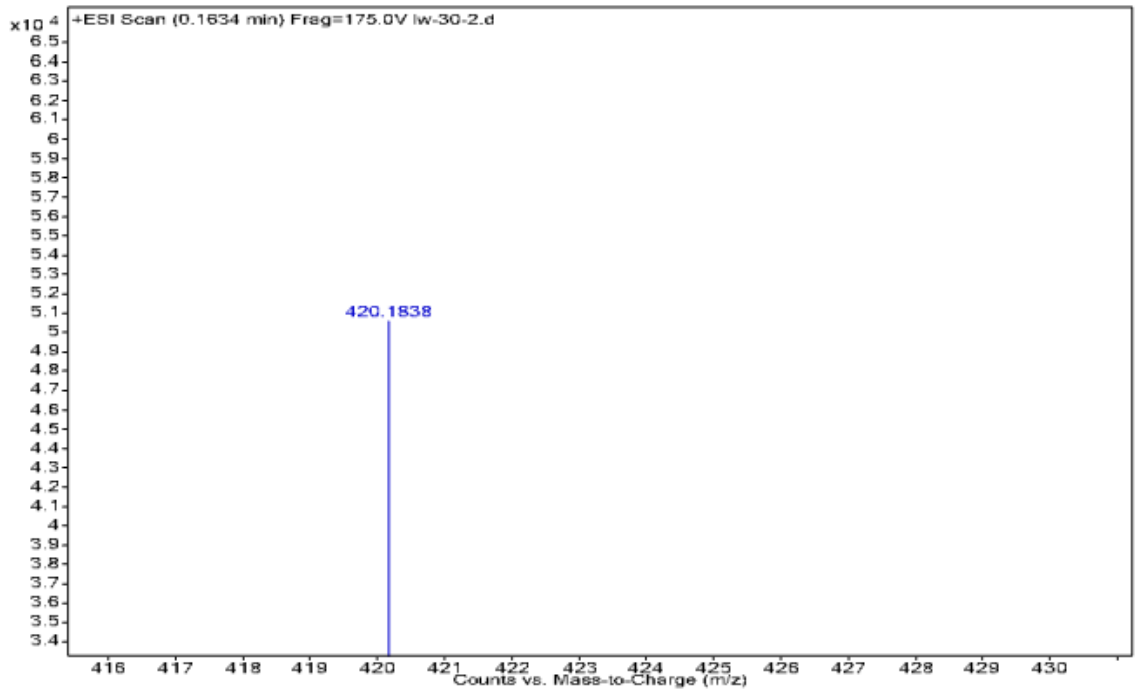


**Fig. S13** HRMS (ESI$^+$) of **2a**.

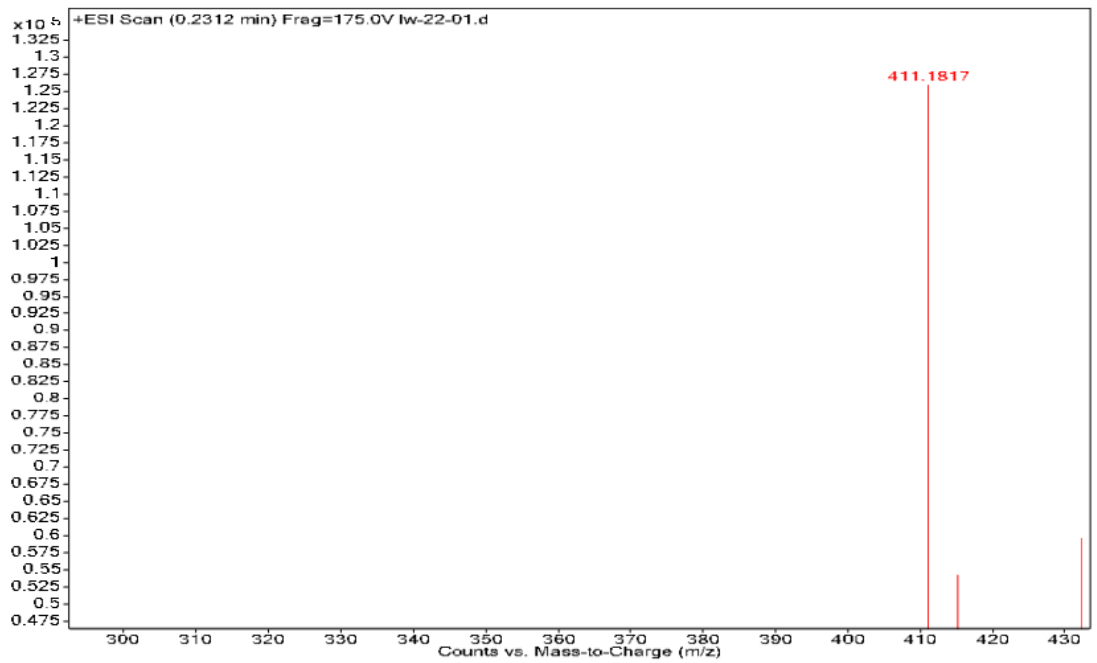


**Fig. S14** HRMS (ESI$^+$) of **2b**.

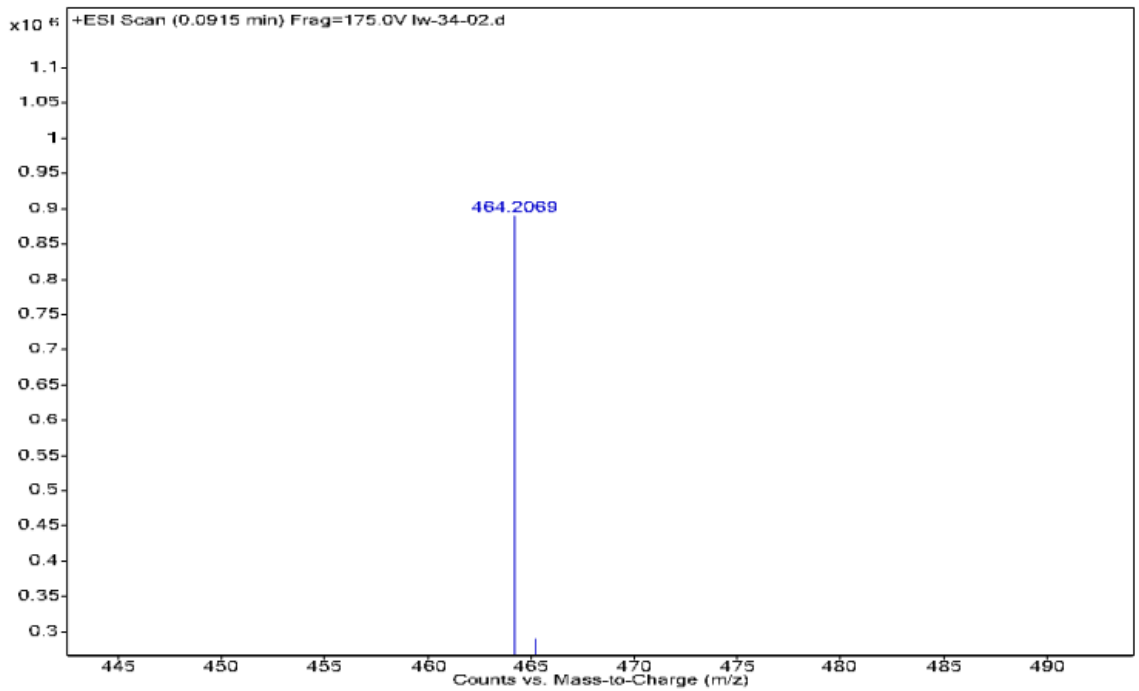


**Fig. S15** HRMS (ESI$^+$) of **3a**.

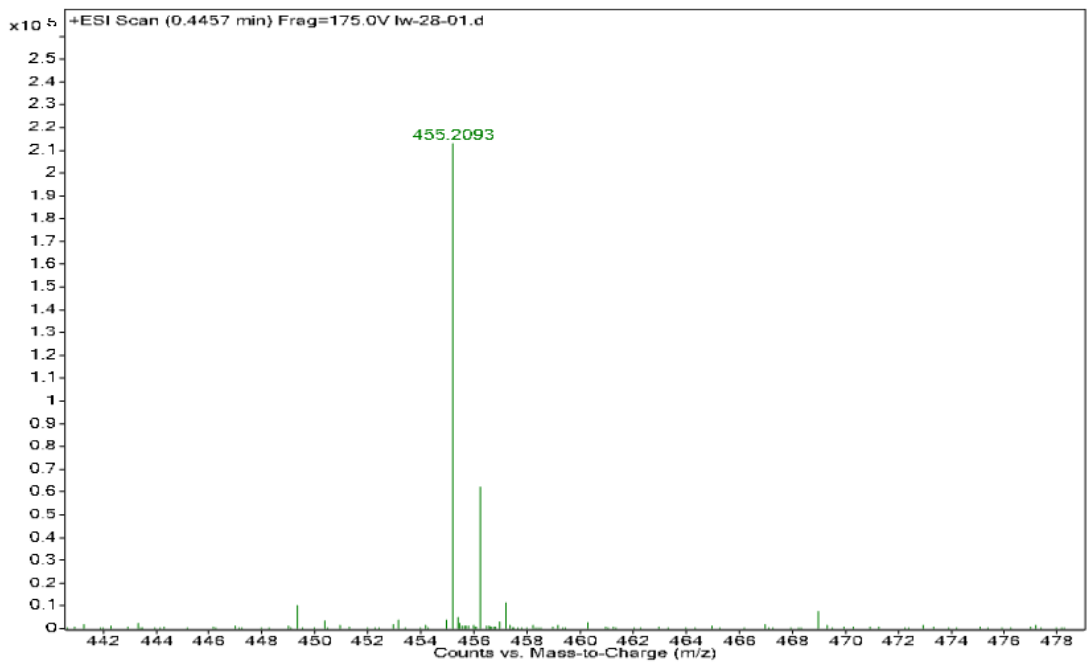


**Fig. S16** HRMS (ESI$^+$) of **3b**.