Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Acridinium benzoates for ratiometric fluorescence imaging of cellular Viscosity

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Experimental Section

Materials and Methods

All solvents and reagents were used as received without further purification. Reactions were performed under standard atmosphere condition in oven-dried glassware or were done in flame-dried glassware under N_2 protection. Reactions progress were monitored by using thin layer chromatography (TLC) and products were purified by flash chromatography using silica gel AR (200 - 300 mesh). High-performance liquid chromatography (HPLC) purification was conducted using LC-20AR pump with a SPD-20A UV/Vis detector. Purification was done with a semiprep column (YMCPack ODS-A, 5 μm, 250 × 20 mm) using a gradient of 5 - 95% acetonitrile containing 0.1% trifluoroacetic acid (TFA) over 30 mins in water containing 0.1% TFA. High resolution mass spectrometry (HRMS) was performed by Waters GCT Premier. ^{1}H-NMR,^{13}C-NMR experiments were performed by Bruker AM-500 in $CDCl_3$ or CD_3OD. All molecular biology grade reagents for cell culture were purchased from Sangon Biotech (Shanghai). UV-Vis spectra were measured using a UV-2600 UV-Visible spectrophotometer (SHIMADZU). Fluorescence spectra were recorded using a RF-6000 Spectrofluorometer (SHIMADZU). Confocal fluorescence imaging was performed using a Leica TCS-SP8 confocal system.

Molar Extinction Coefficient Measurement

All stock solutions were prepared at 10 mM in DMSO. Dilutions of each sample were prepared at 0, 1, 2, 3, 4, and 5 μM with 1% DMSO in PBS buffer (10 mM, pH = 7.4). The molar extinction coefficient was determined by a linear fit of the maximum absorbance value versus sample concentration.

Fluorescence Quantum yield

The fluorescence quantum yields (Φ_{fl}) for **Acr-A**, **Acr-B**, **Acr-C**, **Acr-D** were calculated by using Rhodamine 101 (sigma) as the standard (Φ_r = 0.915, in ethanol) at 450 nm. The following equation was used for calculation.

$\Phi_s = \Phi_r (A_r F_s / A_s F_r) (\eta_s^2 / \eta_r^2)$

where, *s* and *r* denote sample and reference, respectively, *A* is the absorbance, *F* is the relative integrated fluorescence intensity, and η is the refractive index of the solvent.

pKa Determination

Acr-A were prepared at 10 μM in PBS at different pHs (containing 1% DMSO).

Viscosity determination, fluorescence spectral measurement, and fluorescence lifetime detection

The solvents were obtained by mixing methanol-glycerol systems in different proportions. Measurements were carried out with a DV-79 rotational viscometer, and each viscosity value was recorded. The solutions of **Acr-A-D** of different viscosity were prepared by adding the stock solution (1 mM) to 2 mL of solvent mixture (methanol-glycerol solvent systems) to obtain the final concentration of the dye (10 μM). These solutions were sonicated for 5 min to eliminate air bubbles. After standing for 1 h at a constant temperature, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer. A fluorescence lifetime measuring equipment was used to obtain the fluorescence lifetimes of **Acr-A**, with the excitation wavelength at 405 nm and detection at 474 nm.

Cells culture

HeLa, MCF-7 and RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium media (DMEM, Hyclone) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Sijiqing) at 37 °C and 5 %

CO_2. Before the imaging experiments, 1 mL of HeLa cells were subcultured and seeded in the glass bottom culture dishes at a density of 1×10^5. About 36 hours later, the cells reached about 70 % confluence for the further experiments.

Cytotoxicity assay

The cytotoxicity of the **Acr-A** was evaluated by the standard MTT assay. The cells were inoculated into a 96-well plate, each well containing approximately 10000 cells. Increasing volumes of CNBE in 99% MEM and 1% DMSO were added to the cells after one day of culture, and the final concentrations of the probe were 0, 0.1, 2, 5, 10 and 20 μM (five parallel tests). After incubation at a constant temperature for 24 hours, 10 μL of MTT (5 mg/mL in PBS) was added to the cells and incubated for another 4 hours. Then, the culture medium was removed and 100 μL of DMSO was added into each well. The absorbance of each well at 565 nm was measured by a microplate reader (five parallel tests). The cell viability was measured by calculating $(OD - OD_{blank})/(OD_{control} - OD_{blank})$ wherein OD, $OD_{control}$ and OD_{blank} denote the optical density of cells in the presence or absence of the probe and the optical density of the culture medium, respectively.

Fluorescence life imaging

HeLa cells internalized with **Acr-A** (10 μM) were washed with PBS. Fluorescence images were recorded at different temperatures: 4, 30 ^{0}C. The fluorescence life imaging usesTCSPC FLIM equipment: SPC150, excited at 405 nm.

Distinguish cancer cells from normal cells

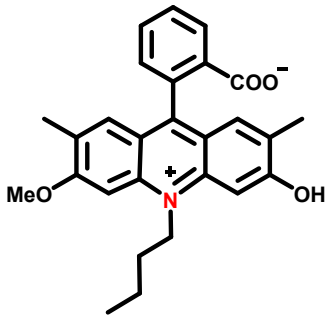
HeLa, MCF-7 and RAW264.7 cells internalized with **Acr-A** (10 μM) were washed with PBS. Fluorescence images were recorded. The confocal microscopic imaging uses Leica TCS-SP8 confocal microscope with an excitation filter of 405 nm and the collection wavelength range is from 420-490 nm (green channel) and 550-650 nm (yellow channel).

Tracking of viscosity changes during apoptosis

HeLa cells internalized with **Acr-A** (10 μM) were washed with PBS and then etoposide (2 μM) was added. Fluorescence images were recorded at different time points: 5, 15, 30, 45, 60 and 75 min. The confocal microscopic imaging uses Leica TCS-SP8 confocal microscope with an excitation filter of 405 nm and the collection wavelength range is from 420-490 nm (green channel) and 550-650 nm (yellow channel).

Synthesis of Acr-A-D

Synthesis of 3,3-bis(2-bromo-4-methoxy-5-methylphenyl) isobenzofuran-1(3H)-one (1). 4-bromo-2-methoxy-1-methylbenzene (1.0 g, 5.0 mmol) and phthalic anhydride (370.5 mg, 2.5 mmol) were added to a 50 mL round-bottom flask. The mixture was dissolved in 3 mL trifluoromethanesulfonic acid at RT overnight. Then, the mixture was dissolved in saturated sodium bicarbonate solution and extracted with CH_2Cl_2. The collected organic layer was dried over anhydrous sodium sulfate. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by column chromatography (silica gel, 1/100 AcOEt / Hexane) and product **(1)** (1.06 g) was obtained as a white solid in 81.5 % yield.
^{1}H NMR (500 MHz, Methanol-d_4) δ 7.93 (d, J = 7.7 Hz, 1H), 7.84 – 7.78 (m, 2H), 7.68 (d, J = 1.2 Hz, 1H), 7.15 (s, 2H), 6.96 (s, 2H), 3.83 (s, 6H), 2.04 (s, 6H).
^{13}C NMR (125 MHz, Methanol-d_4) δ 170.4, 158.17, 151.58, 134.29, 129.74, 125.91, 125.55, 125.30, 124.87, 116.70, 92.23, 54.88, 14.65.
HRMS (ESI) calcd for $C_{24}H_{20}Br_2O_4$ $[M+Na]^+$ 552.9626, found 552.9623.

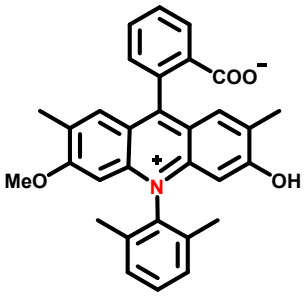


Acr-A

Synthesis of Acr-A. To a degassed mixture of 3,3-bis(2-bromo-4-methoxy-5-methylphenyl) isobenzofuran-1(3H)-one **(1)** (319.7 mg, 0.6 mmol), $Pd_2(dba)_3$ (34.0 mg, 0.037 mmol), Xantphos (43.5 mg, 0.043 mmol), butylamine (150 µl, 1.5 mmol) and sodium tert-butoxide (175 mg, 2.8 mmol) was added toluene (7 ml) at RT and stirred 24 h at 115 °C. After cooling to room temperature, the mixture was extracted with CH_2Cl_2.The organic layer was dried over Na_2SO_4 and evaporated to dryness. The solvent was evaporated, and the crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford 2-(10-butyl-3,6-dimethoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate. An oven-dried 50 mL round bottom flask equipped with a stir bar was charged with 2-(10-butyl-3,6-dimethoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate (4.9 mg, 0.011 mmol) and dry CH_2Cl_2 (1 mL) under nitrogen system. And then the mixture was kept at -78 °C. BBr_3 in methylene chloride (1.0 M, 0.5 mL, 0.5 mmol) was added slowly. The temperature was slowly raised to 25 °C and the mixture was stirred overnight. The reaction was quenched with water at 0°C and extracted with CH_2Cl_2. The collected organic fraction was washed with brine, dried over Na_2SO_4, and filtered over a pad of celite. The pad was rinsed with CH_2Cl_2, and the combined solutions were concentrated in vacuo. The crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford **Acr-A** (2.8 mg) as an orange solid in 58.9% yield.

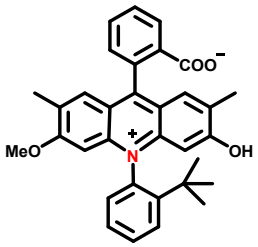
^{1}H NMR (500 MHz, Methanol-d_4) δ 8.43 (s, 1H), 7.89 (d, J = 19.0 Hz, 2H), 7.57 (s, 1H), 7.50 (s, 1H), 7.41 (s, 1H), 7.34 (d, J = 10.2 Hz, 2H), 4.25 (s, 3H), 2.32 – 2.24 (m, 6H), 2.18 (s, 2H), 1.79 (s, 2H), 1.33 (d, J = 26.2 Hz, 2H), 1.20 (s, 3H).
^{13}C NMR (125 MHz, Methanol-d_4) δ 166.68, 166.03, 165.86, 157.82, 135.72, 132.61, 131.32, 130.78, 130.59, 130.18, 130.08, 130.00, 129.32, 119.97, 97.94, 94.92, 49.02, 19.71, 14.97.
HRMS (ESI) calcd for $C_{27}H_{28}NO_4$ $[M]^+$ 430.2013, found 430.2011.

Synthesis of Acr-B. Acr-B was synthesized following a previously reported method [1].



Acr-C

Synthesis of Acr-C. To a degassed mixture of 3,3-bis(2-bromo-4-methoxy-5-methylphenyl) isobenzofuran-1(3H)-one **(1)** (319.7 mg, 0.6 mmol), $Pd_2(dba)_3$ (34.0 mg, 0.037 mmol), Xantphos (43.5 mg, 0.043 mmol), 2,6-dimethylaniline (188 µl, 1.5 mmol) and sodium tert-butoxide (175 mg, 2.8 mmol) was added toluene (7 ml) at RT and stirred 24 h at 115 °C. After cooling to room temperature, the mixture was extracted with CH_2Cl_2.The organic layer was dried over Na_2SO_4 and evaporated to dryness. The solvent was evaporated, and the crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford 2-(10-(2,6-dimethylphenyl)-3-hydroxy-6-methoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate. An oven-dried 50 mL round bottom flask equipped with a stir bar was charged with 2-(10-(2,6-dimethylphenyl)-3-hydroxy-6-methoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate (5.4 mg, 0.011 mmol) and dry CH_2Cl_2 (1 mL) under nitrogen system. And then the mixture was kept at -78 °C. BBr_3 in methylene chloride (1.0 M, 0.5 mL, 0.5 mmol) was added slowly. The temperature was slowly raised to 25 °C and the mixture was stirred overnight. The reaction was quenched with water at 0°C and extracted with CH_2Cl_2. The collected organic fraction was washed with brine, dried over Na_2SO_4, and filtered over a pad of celite. The pad was rinsed with CH_2Cl_2, and the combined solutions were concentrated in vacuo. The crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford **Acr-C** (3.2 mg) as an orange solid in 60.9% yield.
^{1}H NMR (500 MHz, Methanol-d_4) δ 8.46 (d, J = 8.8 Hz, 1H), 7.98 (d, J = 7.5 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.62 (dd, J = 19.6, 7.7 Hz, 4H), 7.44 (s, 1H), 6.60 (s, 1H), 6.48 (s, 1H), 3.80 (s, 3H), 2.28 (s, 6H), 1.96 (s, 3H), 1.84 (s, 3H).
^{13}C NMR (125 MHz, Methanol-d_4) δ 135.23, 133.15, 132.64, 132.06, 131.48, 131.27, 130.34, 130.19, 130.09, 129.87, 129.30, 127.18, 119.63, 98.26, 94.23, 54.97, 15.63, 15.42, 14.98.
HRMS (ESI) calcd for $C_{31}H_{28}NO_4$ $[M]^+$ 478.2013, found 478.2015.



Acr-D

Synthesis of Acr-D. To a degassed mixture of 3,3-bis(2-bromo-4-methoxy-5-methylphenyl) isobenzofuran-1(3H)-one **(1)** (319.7 mg, 0.6 mmol), $Pd_2(dba)_3$ (34.0 mg, 0.037 mmol), Xantphos (43.5 mg, 0.043 mmol), 2 - (tert butyl) aniline (238 µl, 1.5 mmol) and sodium tert-butoxide (175 mg, 2.8 mmol) was added toluene (7 ml) at RT and stirred 24 h at 115 °C. After cooling to room temperature, the mixture was extracted with CH_2Cl_2.The organic layer was dried over Na_2SO_4 and evaporated to dryness. The solvent was evaporated, and the crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford 2-(10-(2-(tert-butyl) phenyl)-3-hydroxy-6-methoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate. An oven-dried 50 mL round bottom flask equipped with a stir bar was charged with 2-(10-(2-(tert-butyl) phenyl)-3-hydroxy-6-methoxy-2,7-dimethylacridin-10-ium-9-yl) benzoate (5.7 mg, 0.011 mmol) and dry CH_2Cl_2 (1 mL) under nitrogen system. And then the mixture was kept at -78 °C. BBr_3 in methylene chloride (1.0 M, 0.5 mL, 0.5 mmol) was added slowly. The temperature was slowly raised to 25 °C and the mixture was stirred overnight. The reaction was quenched with water at 0°C and extracted with CH_2Cl_2. The collected organic fraction was washed with brine, dried over Na_2SO_4, and filtered over a pad of celite. The pad was rinsed with CH_2Cl_2, and the combined solutions were concentrated in vacuo. The crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 254 and 365 nm. The peak corresponding to the product was lyophilized to afford **Acr-D** (3.3 mg) as an orange solid in 59.8 % yield.
^{1}H NMR (500 MHz, Methanol-d_4) δ 8.49 (d, J = 7.9 Hz, 1H), 8.15 (d, J = 8.3 Hz, 1H), 7.97 – 7.86 (m, 3H), 7.73 (t, J = 8.2 Hz, 1H), 7.45 – 7.35 (m, 4H), 6.66 (s, 1H), 6.52 (s, 1H), 3.81 (d, J = 6.9 Hz, 3H), 2.28 (s, 6H), 1.03 (s, 9H).
^{13}C NMR (125 MHz, Methanol-d_4) δ 166.85, 165.84, 165.62, 159.45, 134.49, 132.87, 131.47, 130.89, 130.67, 130.62, 130.48, 130.26, 129.70, 129.51, 129.42, 128.95, 119.45, 119.16, 100.16, 96.78, 55.59, 36.30, 30.39, 14.96.
HRMS (ESI) calcd for $C_{33}H_{32}NO_4$ $[M]^+$ 506.2326, found 506.2324.

Table S1 Spectroscopic data of **Acr**s in different solvents.

	Properties	Toluene	Dioxane	Acetone	MeCN	THF	DMF	MeOH	PBS
Acr-A	λ_{ex} /nm	368 / 478	371 /479	369 / 477	396 / 468	400 / 483	397 / 473	394 / 464	390 / 457
	λ_{em}/nm	543	548	543	538	545	538	522	526
	Φ	0.74	0.73	0.63	0.75	0.53	0.65	0.25	0.11
	ε($M^{-1}cm^{-1}$)	1.6×10^4	1.6×10^4	1.7×10^4	1.5×10^4	1.7×10^4	1.4×10^4	2.4×10^4	1.3×10^4
Acr-B	λ_{ex}/nm	369 / 483	374 / 481	368 / 479	397 / 470	399 / 485	398 / 475	398 / 466	395 / 458
	λ_{em}/nm	550	550	545	540	547	540	524	528
	Φ	0.78	0.75	0.67	0.78	0.58	0.69	0.29	0.16
	ε($M^{-1}cm^{-1}$)	1.5×10^4	1.6×10^4	1.8×10^4	1.6×10^4	1.9×10^4	1.3×10^4	2.8×10^4	1.4×10^4
Acr-C	λ_{ex}/nm	371 / 480	373 / 483	371 / 475	395 / 467	396 / 481	397 / 473	395 / 461	392 / 459
	λ_{em}/nm	542	541	539	537	541	536	520	524
	Φ	0.81	0.78	0.72	0.81	0.62	0.71	0.31	0.18
	ε($M^{-1}cm^{-1}$)	1.7×10^4	1.6×10^4	1.6×10^4	1.5×10^4	2.2×10^4	1.4×10^4	2.3×10^4	1.4×10^4
Acr-D	λ_{ex}/nm	369 / 471	374 / 473	372 / 468	397 / 465	399 / 469	395 / 464	399 / 460	398 / 456
	λ_{em}/nm	543	544	541	536	541	531	522	520
	Φ	0.87	0.84	0.74	0.88	0.61	0.63	0.4	0.27
	ε($M^{-1}cm^{-1}$)	1.7×10^4	1.4×10^4	1.6×10^4	1.3×10^4	1.6×10^4	1.5×10^4	2.1×10^4	1.2×10^4

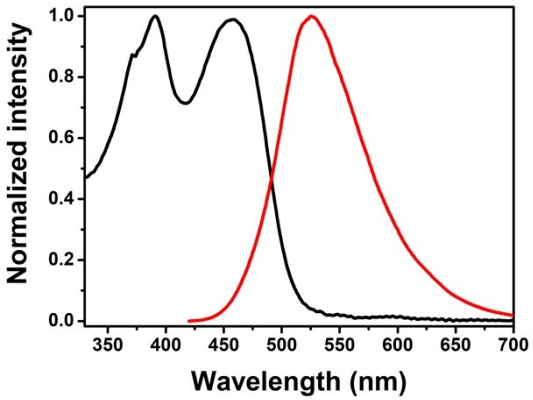


Fig. S1 Absorption spectrum (black) and emission spectrum (red) of **Acr-A** in aqueous solution (25 mM PBS buffer, pH 7.4).

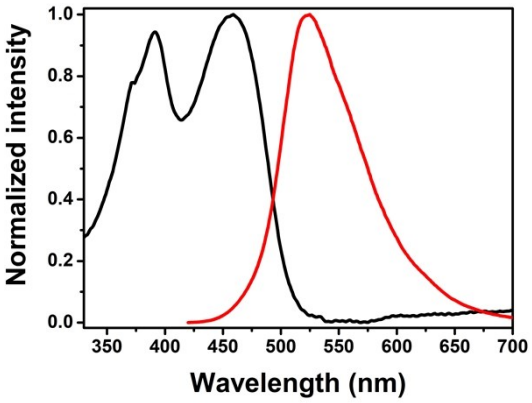


Fig. S2 Absorption spectrum (black) and emission spectrum (red) of **Acr-C** in aqueous solution (25 mM PBS buffer, pH 7.4).

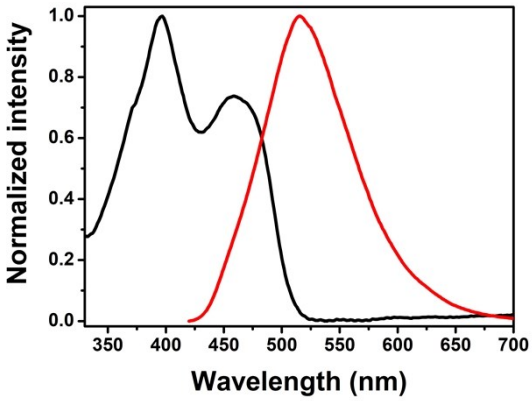


Fig. S3 Absorption spectrum (black) and emission spectrum (red) of **Acr-D** in aqueous solution (25 mM PBS buffer, pH 7.4).

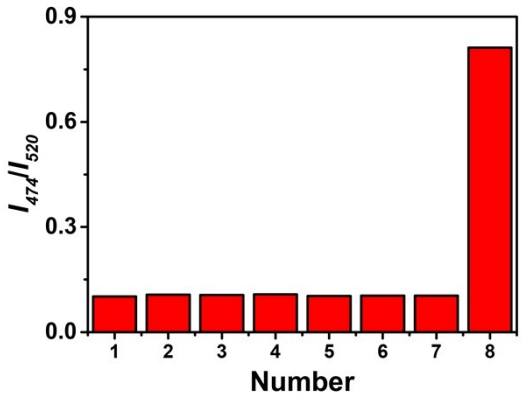


Fig. S4 The ratio values of fluorescence intensity (I_{474}/I_{520}) of **Acr-A** (10 μM) in the presence of various analytes in aqueous solution (25 mM PBS buffer, pH 7.4). (1) blank, (2) HSA, (3) concanavalin A, (4) thrombin, (5) bovine hemoglobin, (6) trypsin, (7) lysozyme, (8) 70% glycerol. 100 μM for species (2) – (7).

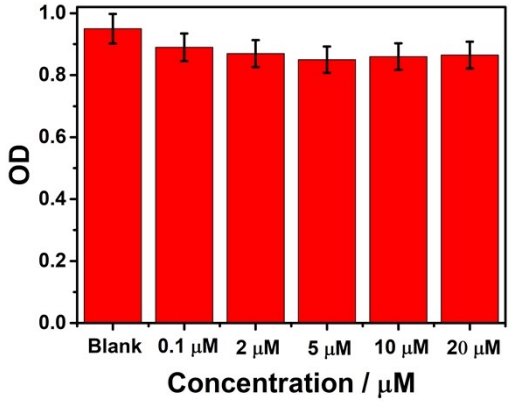


Fig. S5 Cell viability of HeLa cells incubated with **Acr-A** of different concentration (0, 0.1, 2, 5, 10, and 20 μM) for 24 h.

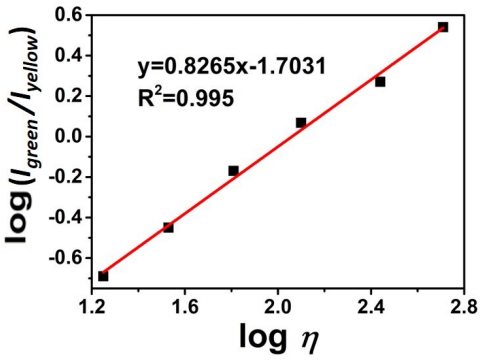


Fig. S6. A set of viscosity solutions (8, 18, 34, 64, 126, 274, 416, 960 cP) were prepared in an 8-well dish. The fluorescence images were obtained by confocal microscopy under the same conditions as cell imaging. The image was obtained by focusing on an area 5 μm above the bottom of the dish. The calibration curve was obtained by the linear relationship between the ratio of emission intensity in green channel (I_{green}, λ_{ex} = 405 nm and λ_{em} = 420 - 490 nm) to yellow channel (I_{yellow}, λ_{ex} = 405 nm and λ_{em} = 550 - 650 nm) and log η, and the ratio was converted into viscosity.

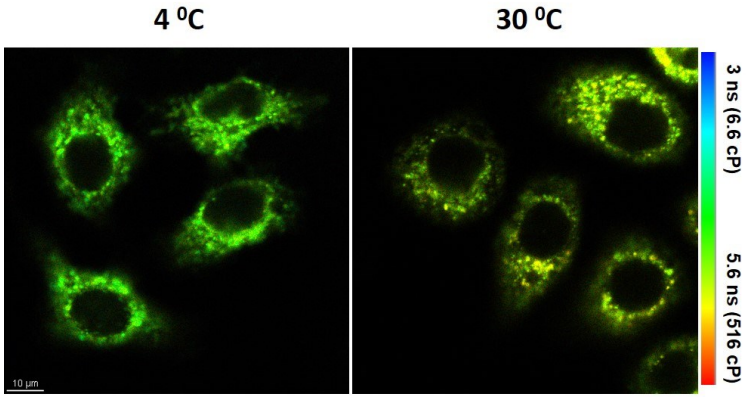


Fig. S7. FLIM investigation of Hela cells stained with 10 µM **Acr-A**, at 4 °C and 30 °C. Fluorescence lifetime imaging using fluorescence detection at 474 ± 15 nm after pulsed excitation at 405 nm.

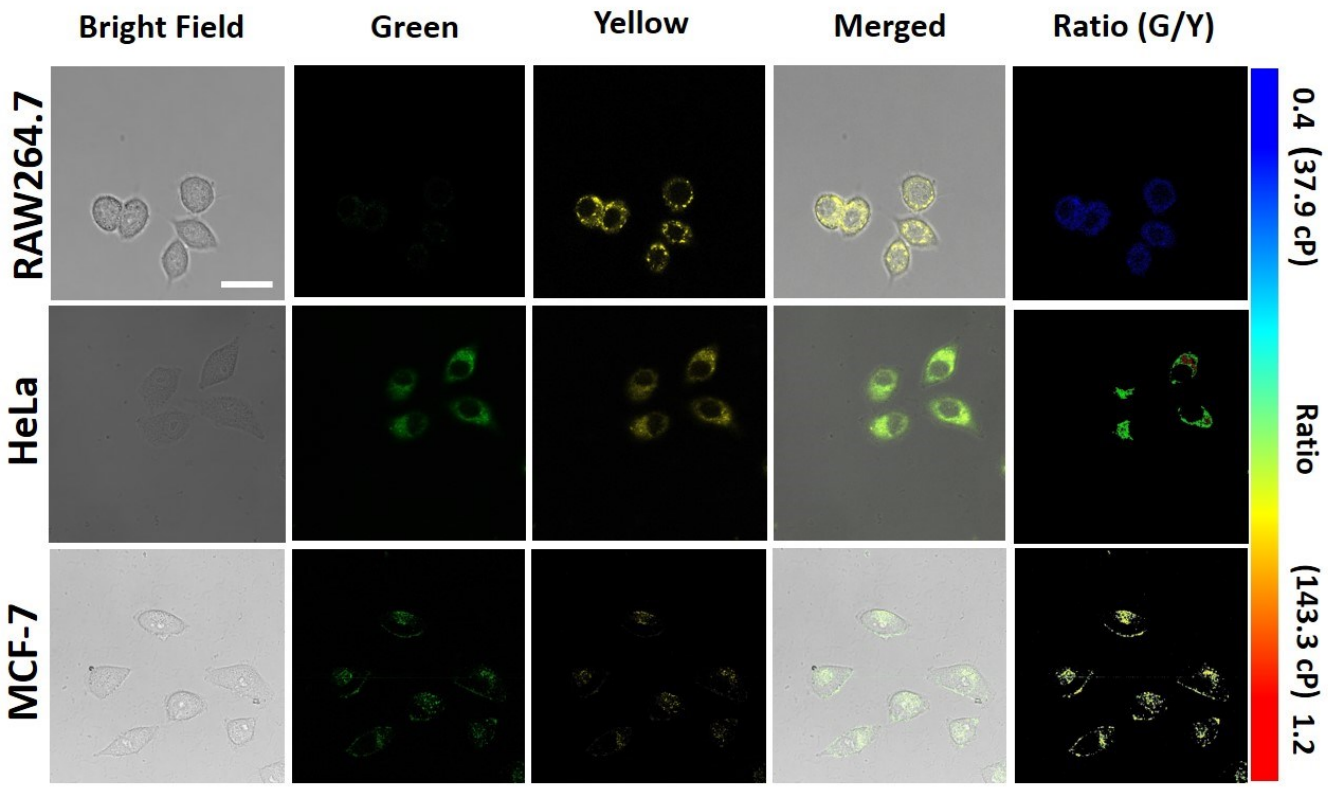


Fig. S8. Confocal images of HeLa, MCF-7 and RAW264.7 cells incubated with probe **Acr-A** (10 μM). First column: bright field images, second column: fluorescence images at the green channel (420–490 nm), third column: fluorescence images at the yellow channel (550–650 nm), fourth column: overlay images of the first, second and third columns, fifth column: ratiometric images of the second and third columns. λ_{ex} = 405 nm, Scale bar: 25 μm. Ratio from green channel to yellow channel are measured in random selection of three different cells in each image, values are the mean ± SD for three different cells in each image.

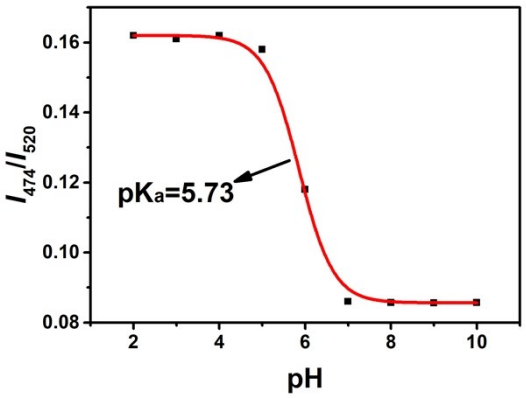
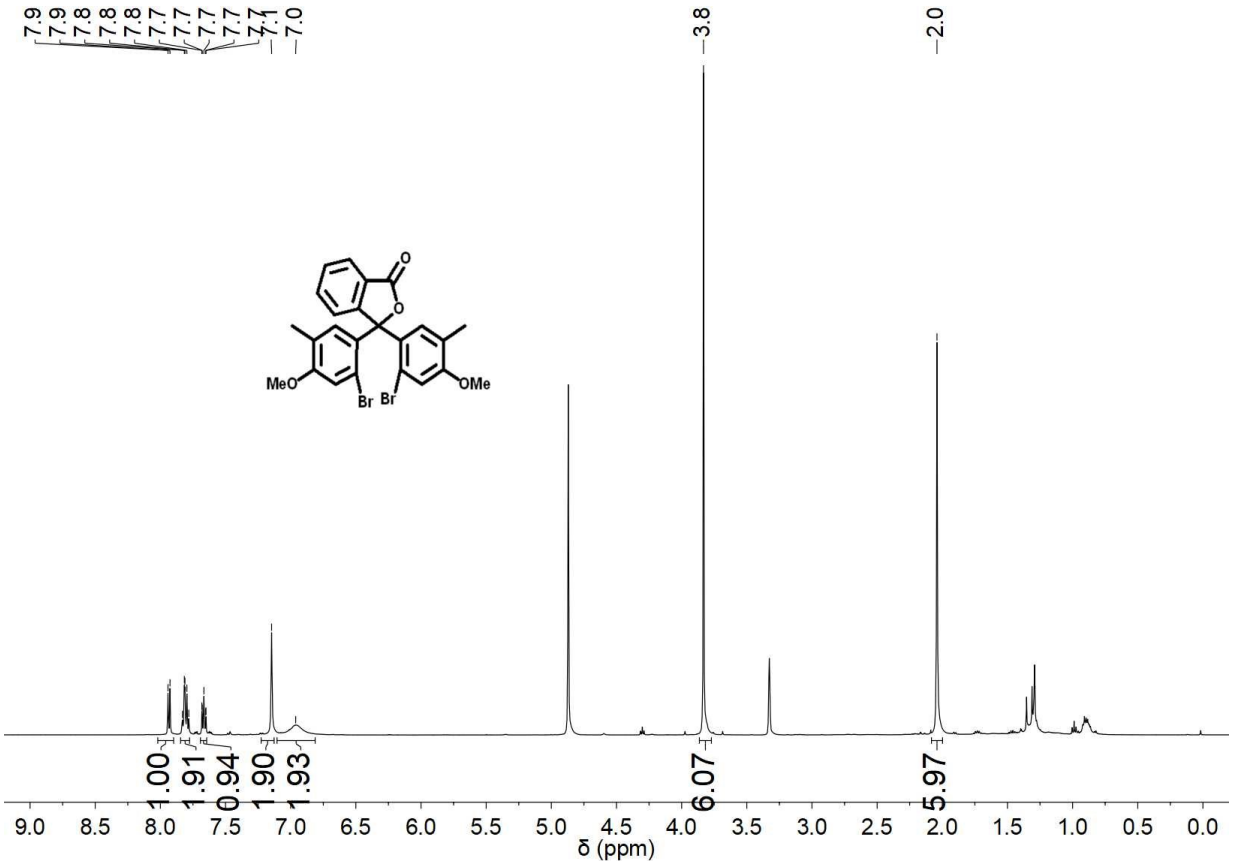
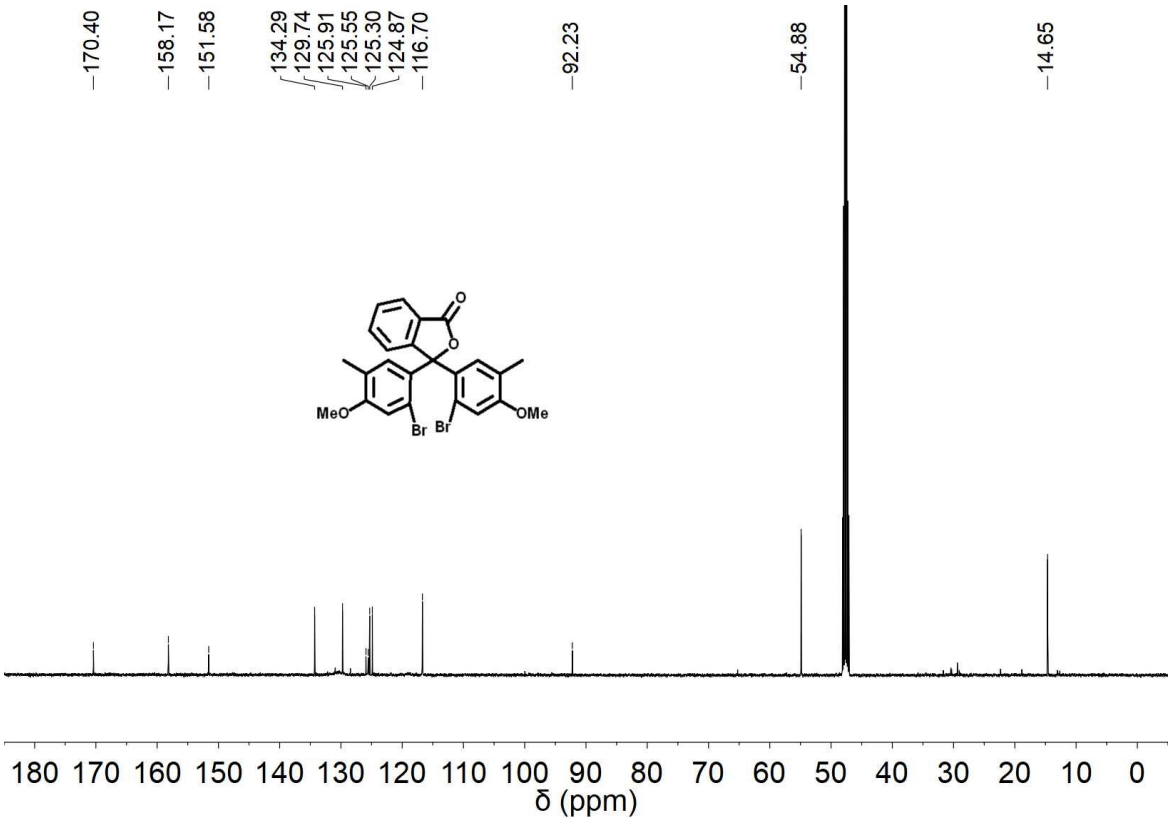


Fig. S9. The ratio of I_{474}/I_{520} of **Acr-A** (10 μM) with varied pHs.

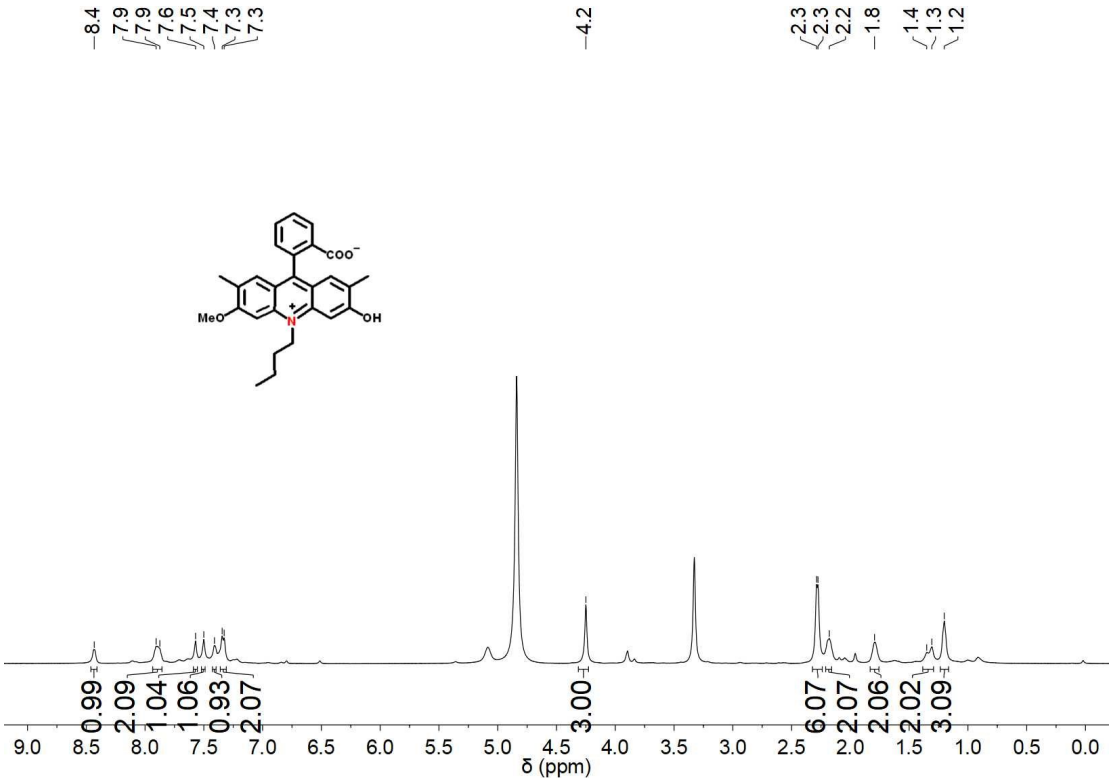
Copies of ^{1}H and ^{13}C NMR Spectra



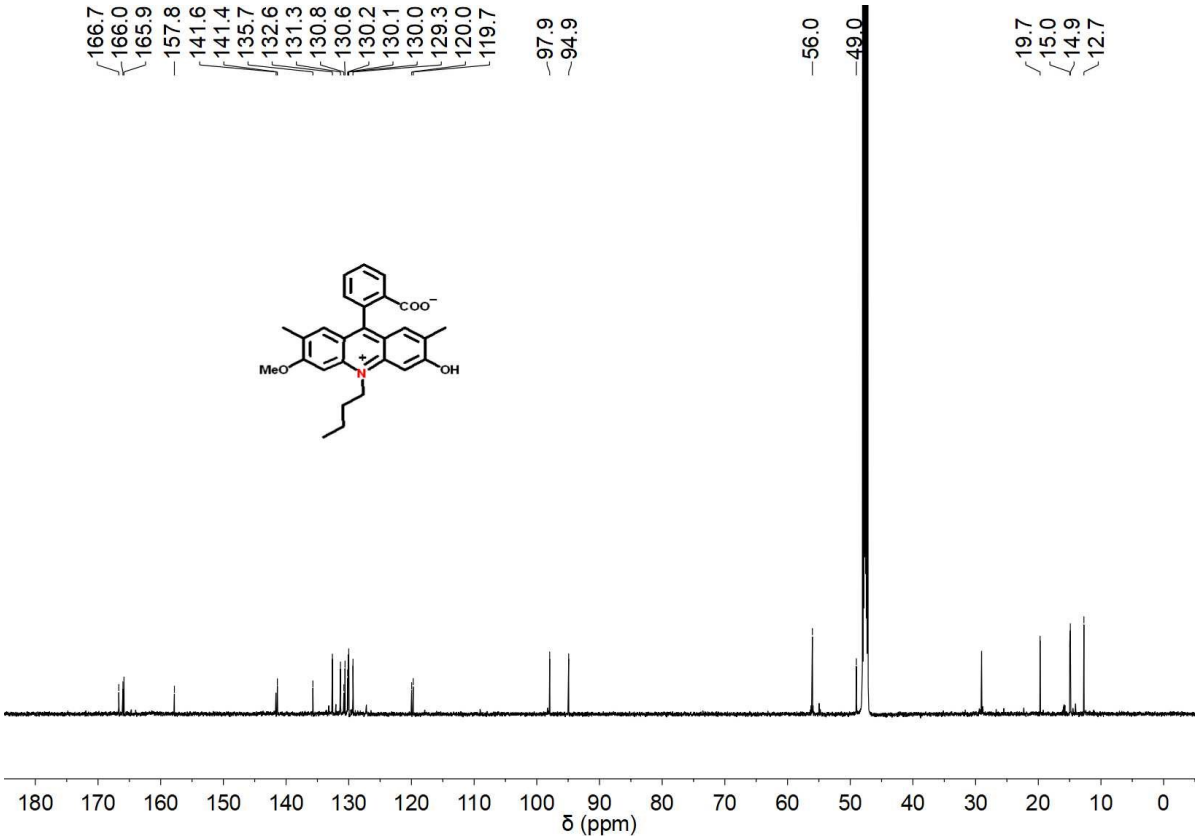
^{1}H-NMR of **(1)** in Methanol-d_4 (500 MHz)



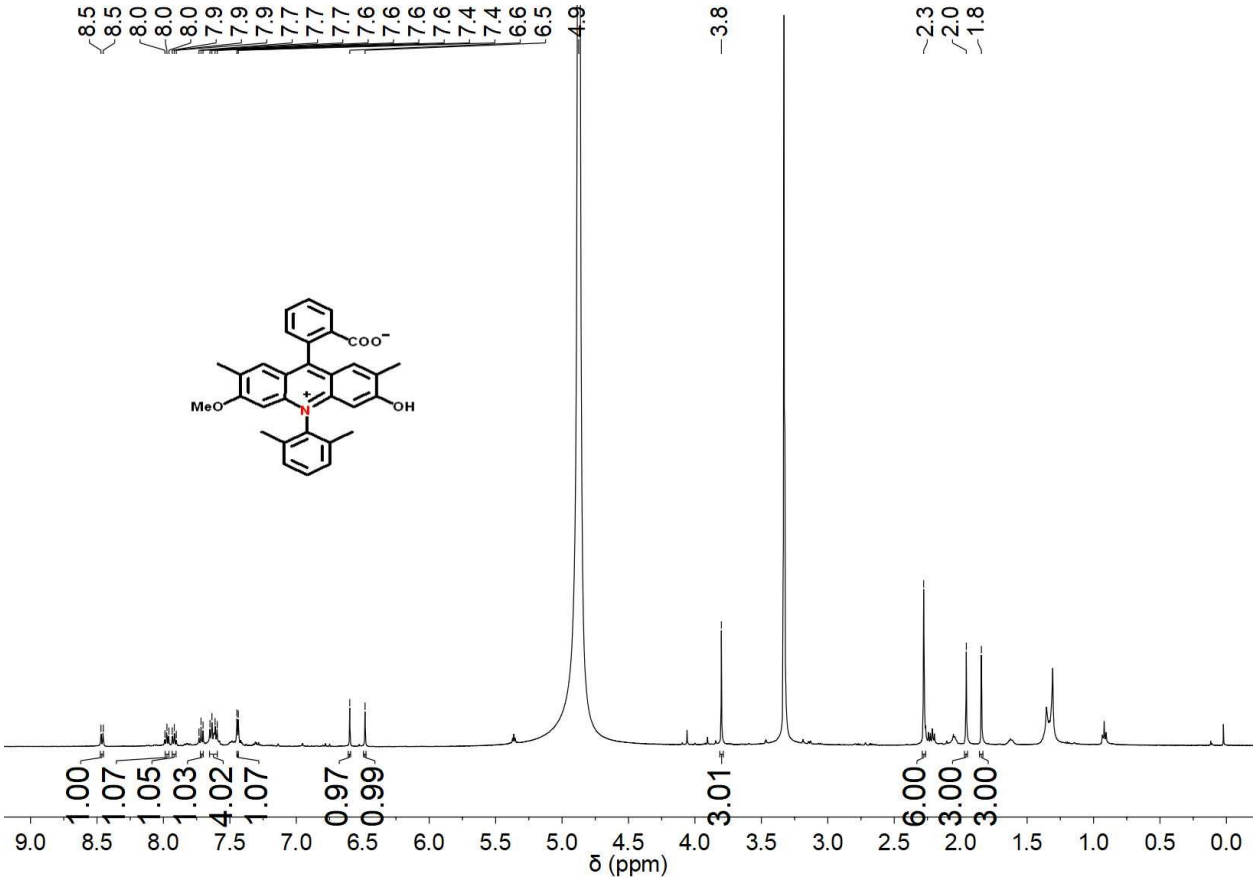
^{13}C-NMR of **(1)** in Methanol-d_4 (125 MHz)



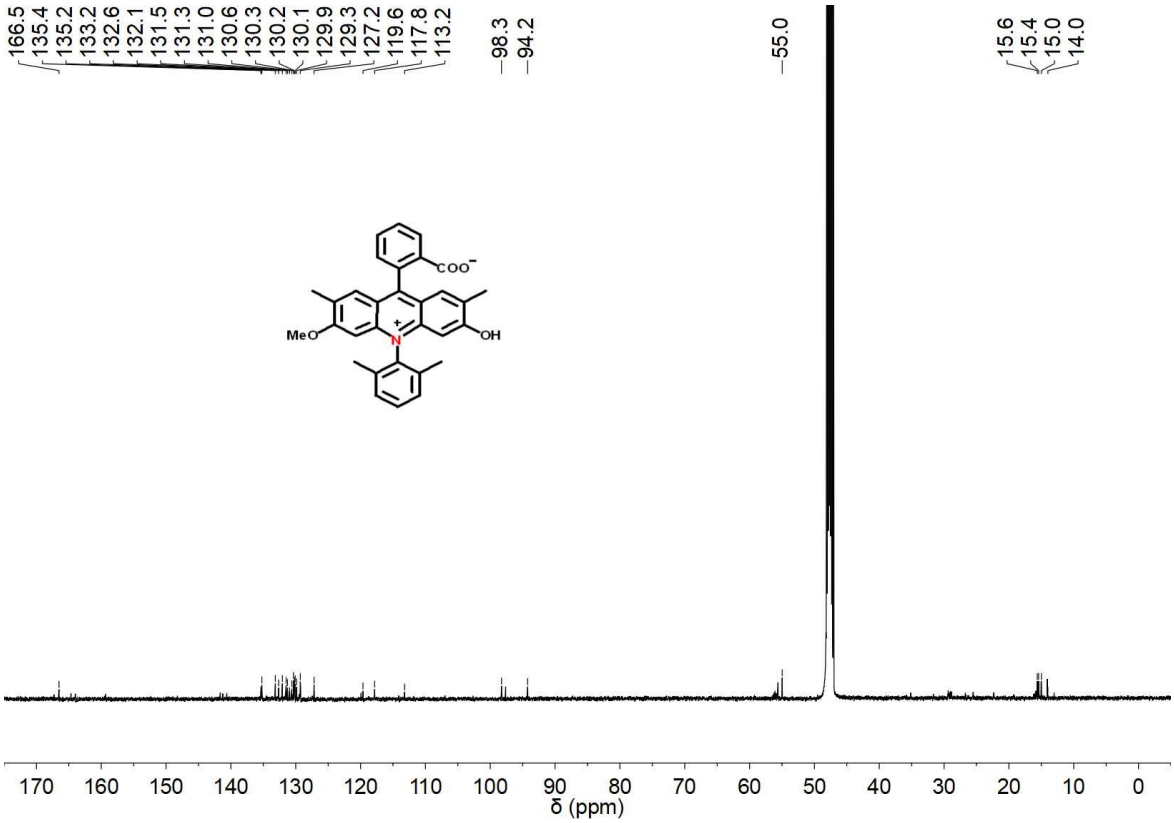
^{1}H-NMR of **Acr-A** in Methanol-d_4 (500 MHz)



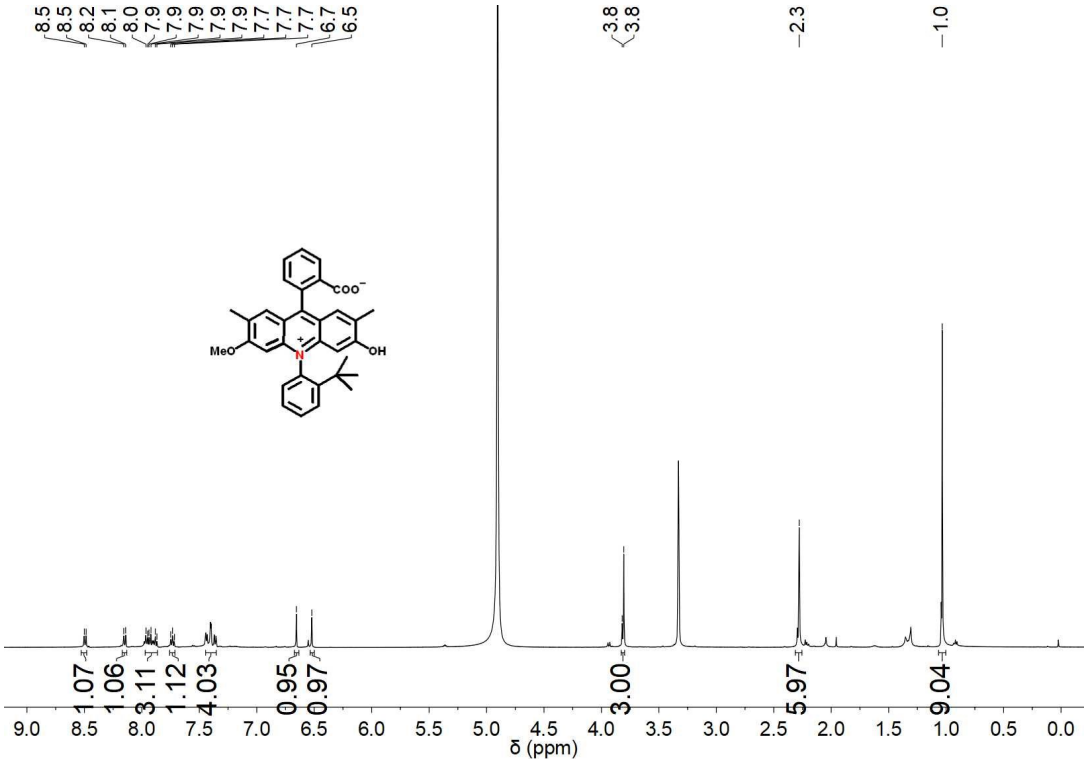
^{13}C-NMR of **Acr-A** in Methanol-d_4 (125 MHz)



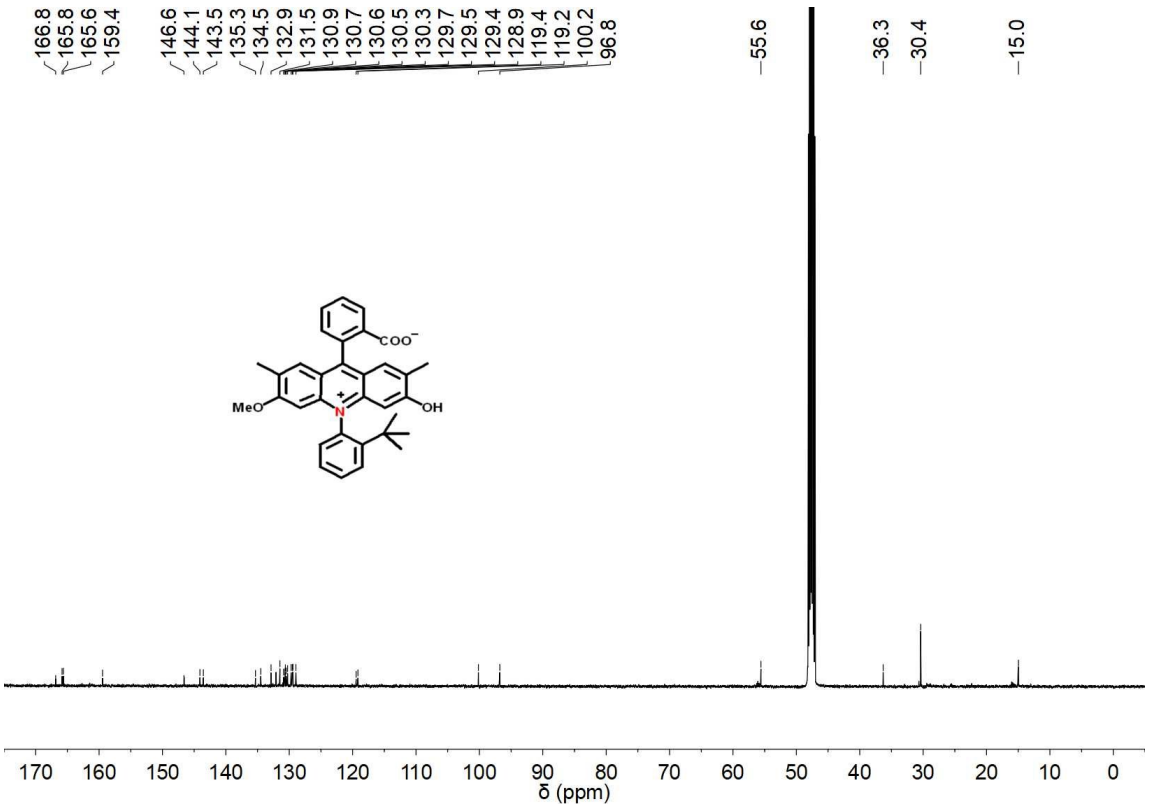
^{1}H-NMR of **Acr-C** in Methanol-d_4 (500 MHz)



^{13}C-NMR of **Acr-C** in Methanol-d_4 (125 MHz)



^{1}H-NMR of **Acr-D** in Methanol-d_4 (500 MHz)



^{13}C-NMR of **Acr-D** in Methanol-d_4 (125 MHz)

References

[1] M. Wen, X. J. Wang, T. Wang, Y. Sun, M. T. Fan, M. Li, J. R. Zhu, D. Z. Zhang, X. Y. Cui, Y. K. Shan, *Chem.-Eur. J.*, 2020, **26**, 3247-3251.