Supplementary Material

A Novel Near-infrared Fluorescent Probe for Detecting Intracellular Alkaline Phosphatase and Imaging of Living Cells

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Experimental Section

Instrumentation and Chemicals

Mass spectra were recorded on a TSQ Quantum Access MAX triple-quadrupole mass spectrometer (Thermo Fisher Scientific, USA). Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, and ^{31}P NMR) spectra were measured on a Mercury 300BB NMR spectrometer (Varian Inc., USA) and an Avance III500 NMR spectrometer (Bruker Inc., Germany). UV-Vis absorption and fluorescence spectra were recorded on a Cary 60 UV-spectrophotometer (Agilent Technologies, USA) and an F-7000 fluorescence spectrometer (Hitachi Co., Ltd. Japan), respectively. The pH values were measured using an INESA Scientific PHS-3C pH meter (INESA Scientific Inc., China). Cell imaging was carried out on an FV1000 laser scanning confocal microscope (Olympus Corporation, Japan).

Chemicals including 4-methoxysalicylaldehyde, 2-methylbenzothiazole, PBr_3, Cs_2CO_3, BBr_3, $POCl_3$, and pyridine were purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). Alkaline phosphatase (ALP), acid phosphatase (ACP), cysteine (Cys), glutathione (GSH), acetylcholinesterase (AChE) bovine serum albumin (BSA), and horse IgG were purchased from Shanghai Yuanye Biotechnology Co., Ltd. All other chemicals were of analytical grade and were used as received without further purification. All aqueous solutions were prepared with

ultrapure water obtained from a Milli-Q water purification system (18.2 MΩ cm).

Synthesis procedures

Synthesis of **Compound 1**

DMF (4.48 mL) and CH_2Cl_2 (20 mL) were mixed and then cooled down to 0 °C. PBr_3 (5 mL) was then added dropwise to the mixture under vigorous stirring. After 30 min, cyclohexanone (4.9 mL) was added, and the mixture was stirred overnight at room temperature. After that, the reaction solution was poured into 30 mL of water, and then neutralized with solid $NaHCO_3$. Subsequently, the aqueous solution was extracted three times with CH_2Cl_2 (60 mL), and the organic layer was dried over Na_2SO_4 and evaporated to give a yellow oil product (2.6 g).

Synthesis of **Compound 2**

Freshly prepared compound **1** (0.204 g, 1.08 mmol) was dissolved in DMF (6 mL) and 4-methoxysalicylaldehyde (0.137 g, 0.9 mmol), and Cs_2CO_3 (0.88 g, 2.7 mmol) were added into the mixture under vigorous stirring. The mixture was stirred for 24 h at room temperature. The reaction solution was poured into CH_2Cl_2 and then washed three times with water. The organic layer was then dried over Na_2SO_4 and evaporated to give crude product. The compound **2** (bright yellow crystal) was purified by silica gel column chromatography using CH_2Cl_2 as the eluent. Yield: 0.147 g, 66.7 %; ^{1}H NMR (300 MHz, $CDCl_3$): δ10.30 (1H, s), 7.08 (1H, d), 6.81–6.49

(3H, m), 3.84 (3H, s), 2.59–2.52 (2H, m), 2.44 (2H, t), 1.71 (2 H, p); ^{13}C NMR (75 MHz, $CDCl_3$): δ 187.39, 161.37, 160.62, 153.35, 127.36, 126.68, 126.57, 114.66, 112.58, 110.77, 100.50, 55.57, 29.89, 21.49, 20.38; MS (ESI, m/z) calcd for $[C_{15}H_{14}NO_3+H^+]^+$: 243.16, found: 243.41.

Synthesis of **Compound 3**

2-methylbenzothiazole (0.5 g, 3.35 mmol) and ethyl iodide (0.78 g, 5 mmol) were dissolved in acetonitrile, and then heated at reflux for 24 h. The reaction solution was filtered and washed three times with diethyl ether to obtain white crystals of 3-ethyl-2-methylbenzo[d]thiazol-3-ium (0.52 g, 87 %). ^{1}H NMR (300 MHz, CD_3OD): δ 8.33 (1H, ddd), 8.28 (1H, dd), 7.92 (1H, ddd), 7.82 (1H, ddd), 4.85 (2H, q), 3.25 (3H, s), 1.60 (3H, t); ^{13}C NMR (75 MHz, DMSO-d6): δ 176.85, 140.48, 129.39, 129.15, 128.06, 124.75, 116.73, 44.83, 16.97, 13.31; MS (ESI, m/z) calcd for $[C_{10}H_{12}NS]^+$: 178.07, found: 178.41.

Synthesis of **Compound 4**

3-ethyl-2-methylbenzo[d]thiazol-3-ium (0.088 g, 0.5 mmol) and compound 2 (0.1 g, 0.41 mmol) were dissolved in Ac_2O, and then heated at reflux at 70 °C overnight. The reaction solution was filtered, redissolved in CH_2Cl_2, and washed three times with water. The organic layer was dried over Na_2SO_4 and evaporated to give the crude product, which was further purified by silica gel column chromatography, using CH_2Cl_2:CH_3OH (50:1-20:1 v/v) as the eluent; as a result, a blue solid product was obtained.

Yield: 0.148 g (89.5 %); ^{1}H NMR (300 MHz, DMSO-d6): δ 8.24 (2H, dd), 8.07 (1H, d), 7.78 (1H, m), 7.59 (1H, t), 7.38 (1H, d), 7.21 (1H, s), 7.00 (1H, d), 6.89 (2H, m), 4.73 (2H, q), 3.87 (3H, s), 2.62 (4H, dd), 1.87 (2H, m), 1.39 (3 H, t); ^{13}C NMR (75 MHz, DMSO-d6): δ 169.26, 161.75, 157.36, 153.53, 142.03, 140.88, 130.21, 128.88, 128.25, 127.10, 126.87, 126.53, 123.69, 115.33, 114.97, 112.51, 111.97, 105.15, 100.20, 55.96, 43.06, 28.48, 24.17, 19.90, 13.50; MS (ESI, m/z) calcd for $[C_{25}H_{24}NO_2S]^+$: 402.15, found: 402.41.

Synthesis of **MTR**

Compound 4 (0.1 g, 0.25 mmol) was dissolved in CH_2Cl_2 at 0 °C, and BBr_3 (0.62 g, 0.25 mmol) was slowly added under vigorous stirring. The mixture was then stirred for 24 h at room temperature. The reaction solution was poured into ice water, and then neutralized with solid $NaHCO_3$. The aqueous layer was extracted with CH_2Cl_2, after which was washed three times with water. The organic layer was dried over Na_2SO_4 and evaporated to give the crude product, which was subsequently purified by silica gel column chromatography using CH_2Cl_2:CH_3OH (15:1 v/v) as the eluent; and a blue solid product was obtained. Yield: 0.074 g (76.5 %); ^{1}H NMR (300 MHz, DMSO-d6): δ 10.52 (1H, s), 8.26 (2H, dd), 8.07 (1H, d), 7.81 (1H, m), 7.63 (1H, t), 7.33 (1H, d), 7.23 (1H, s), 6.84 (1H, d), 6.81 (2H, m), 4.73 (2H, q), 2.64 (4H, d), 1.89 (2H, m), 1.40 (3H, t). ^{13}C NMR (75 MHz, DMSO-d6): δ 169.21, 160.73, 157.88, 153.70, 142.18, 140.93,

131.00, 128.85, 128.60, 127.01, 126.77, 125.60, 123.71, 115.22, 113.99, 113.45, 111.83, 104.60, 101.81, 48.53, 28.44, 24.25, 20.02, 13.37. MS (ESI, m/z) calcd for $[C_{24}H_{22}NO_2S]^+$: 388.14, found: 388.41.

Synthesis of **CyR**

CyR was prepared according to the procedure reported in the literature. [1] Firstly, 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium was synthesized by the procedures reported in the literature. [2] Then, 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium (0.093 g, 0.5 mmol) and compound 2 (0.1 g, 0.4 mmol) were dissolved in Ac_2O, and the mixture was then refluxed at 70 °C overnight. After the reaction solution was filtered, it was redissolved in CH_2Cl_2 and then washed three times with water. The organic layer was dried over Na_2SO_4 and then evaporated to afford the crude product containing Compound 5. After that, the crude product (0.1 g) was dissolved in CH_2Cl_2 at 0 °C, and BBr_3 (0.62 g, 2.5 mmol) was slowly added under vigorous stirring. After the mixture was stirred at room temperature for 24 h, it was poured into ice water, and then neutralized with solid $NaHCO_3$. After the aqueous layer was extracted with CH_2Cl_2, it was washed three times with water. The organic layer was dried over Na_2SO_4 and then evaporated to afford the crude product, which was subsequently purified by silica gel column chromatography using CH_2Cl_2:CH_3OH (15:1 v/v) as the eluent. As a result, a blue solid product was obtained with a yield of 0.092 g (92.8 %). ^{1}H NMR (300 MHz, DMSO-d6): δ 10.76 (1H, s), 8.58

(1H, d), 7.76 (1H, d), 7.66 (1H, d), 7.59 – 7.50 (2H, m), 7.45 (2H, dd), 6.96 – 6.80 (2H, m), 6.51 (1H, d), 4.41 (2H, q), 2.69 (4H, dd), 1.89 – 1.79 (2H, m), 1.74 (6H, s), 1.37 (3H, t). ^{13}C NMR (75 MHz, DMSO-d6): δ 176.44, 161.64, 161.05, 154.14, 144.75, 141.98, 141.12, 134.33, 129.24, 128.87, 126.74, 125.87, 122.78, 114.61, 114.43, 113.79, 112.74, 103.29, 101.97, 50.21, 40.04, 28.34, 27.43, 23.64, 20.02, 12.55. MS (ESI, m/z) calcd for $[C_{27}H_{28}NO_2]^+$: 398.21, found: 398.13.

Cytotoxicity Assay

The cytotoxic effect of MTR-P was examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. BEL-7402 cells (obtained from the Life Sciences College of Jilin University, Jilin, China) were seeded in a 96-well plate and then incubated at 37 °C for 24 h in an incubator saturated with 5% CO_2. After that, the cells were treated or untreated with different concentrations of MTR-P. After incubation at 37 °C for 24 h, MTT solution was added into each well and then removed after 4 h. Subsequently, DMSO was added into each well to dissolve formazan crystals. After shaking for 10 min, the absorbance at 490 nm was recorded on a microplate reader.

Figures

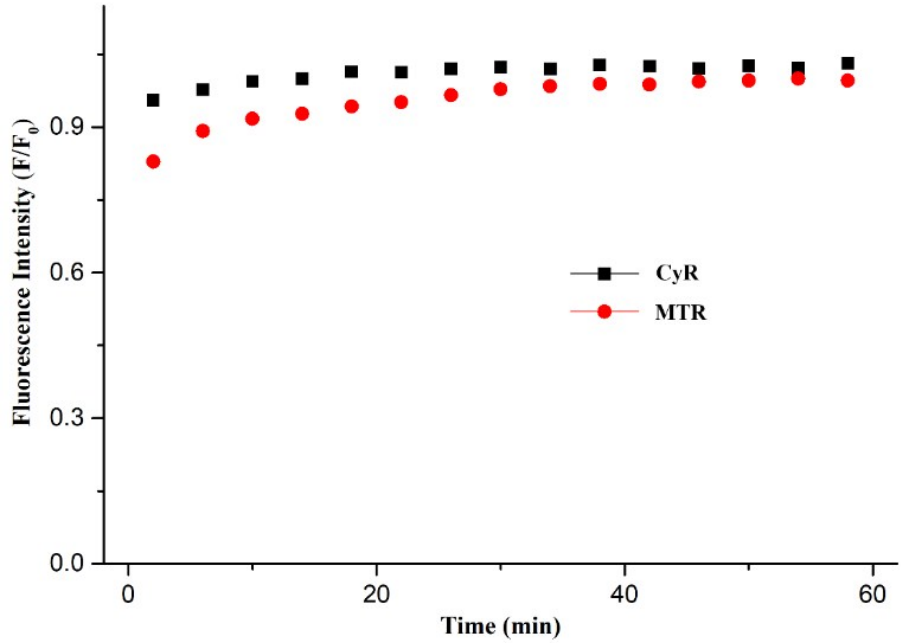


Figure S1. Fluorescence intensity of MTR (10 μM) at 723 and CyR (10 μM) at 712 nm (CH_3OH:H_2O, 1:99 v/v, 25 mM Tris-HCl buffer solution, pH=8.0) as a function of time (0-60 min) under excitation at 680 nm.

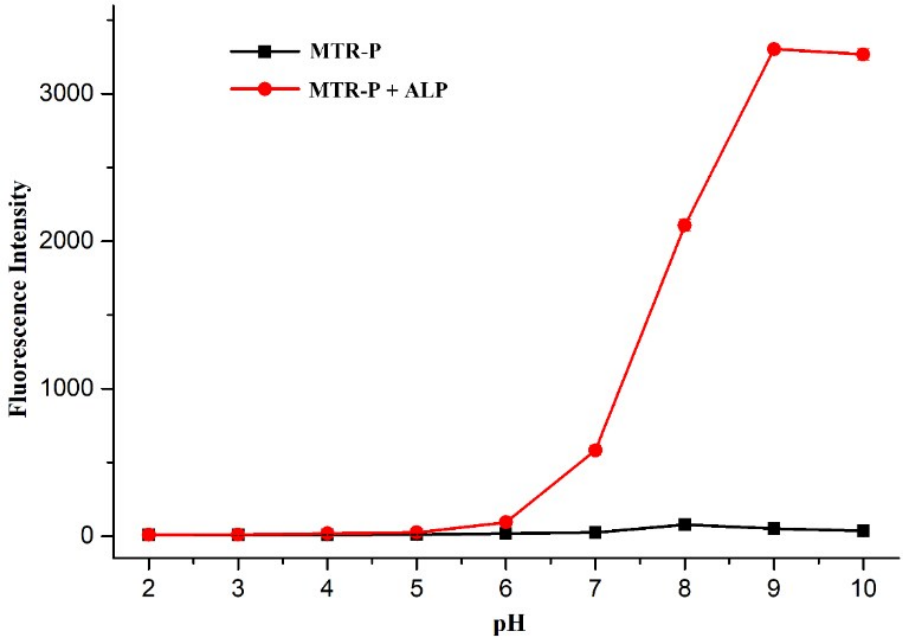


Figure S2. Effect of pH on the fluorescence response changes of MTR-P (10 μM) in the absence of ALP and in the presence of ALP (10 U L^{-1}).

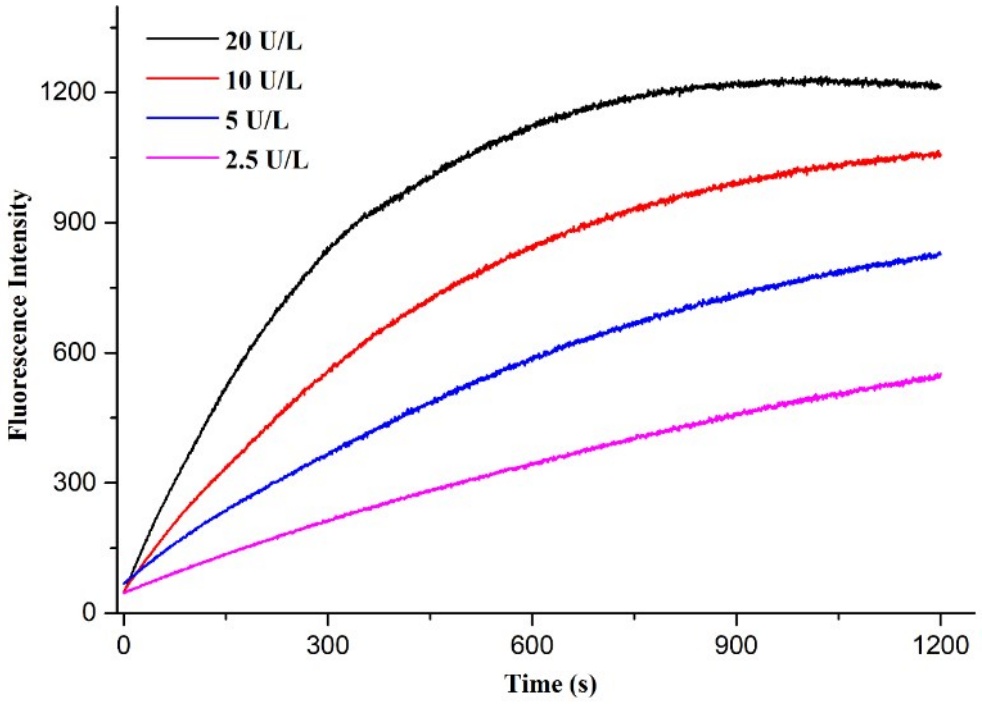


Figure S3. Time Fluorescence spectra of MTR-P upon addition of ALP (2.5, 5, 10, 20 U L^{-1}) in Tris-HCl buffer solution.

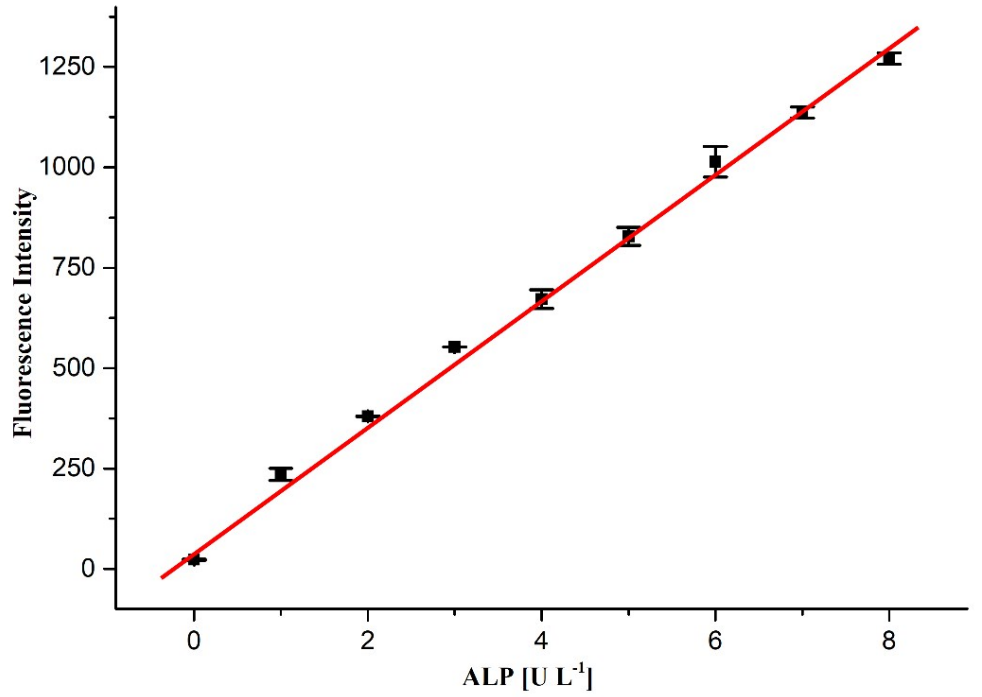


Figure S4. A plot of fluorescence intensity versus ALP concentration.

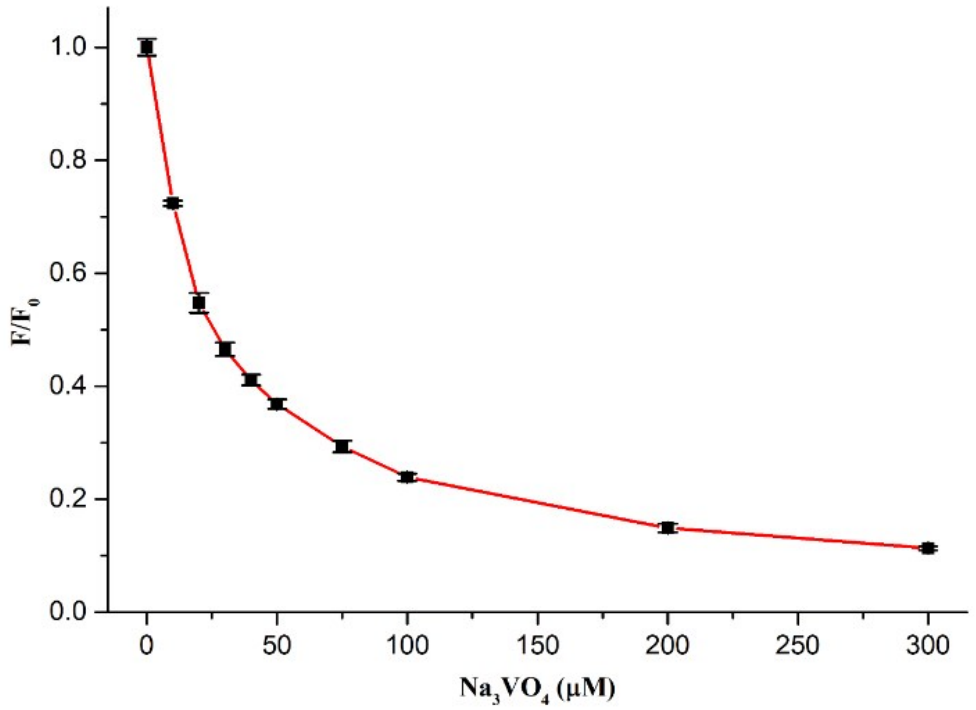


Figure S5. Fluorescence spectra of MTR-P (10 μM) in the presence of ALP at different Na_3VO_4 (0-300 μM) concentrations in Tris-HCl buffer solution (25 mM, pH = 8.0).

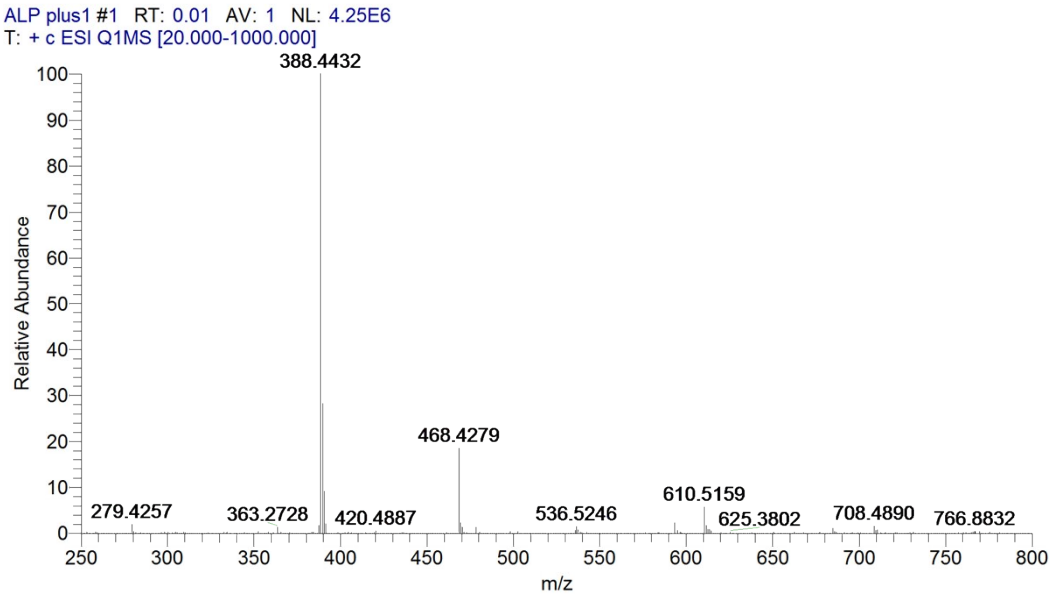


Figure S6. ESI-MS spectrum of MTR-P with the addition of ALP (20 U L^{-1}).

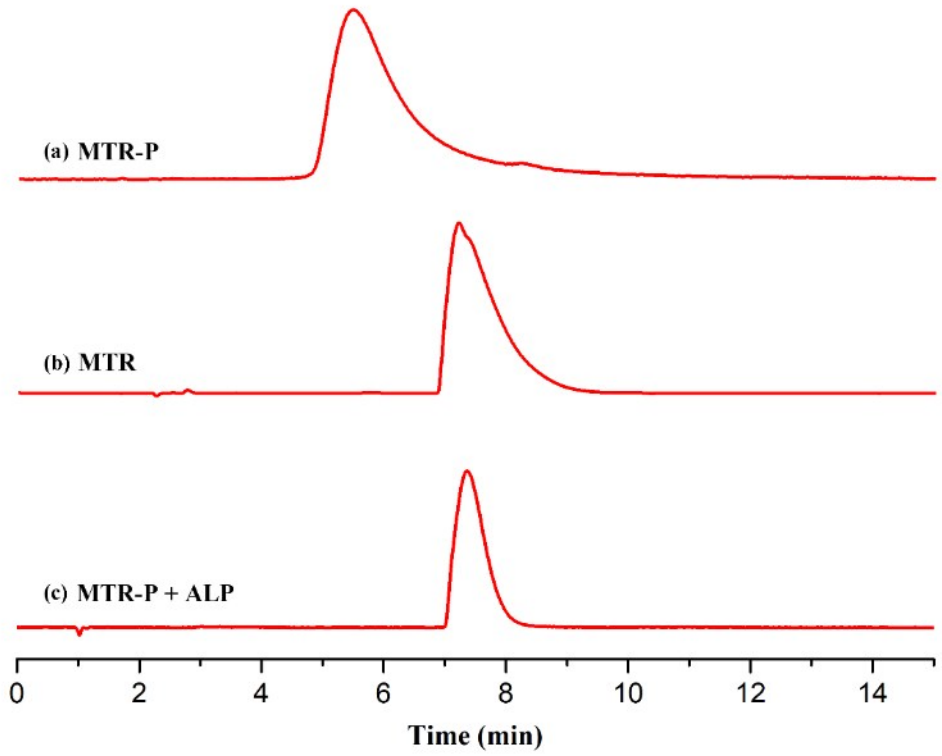


Figure S7. HPLC chromatograms of (a) MTR-P, (b) MTR and (c) MTR-P reacted with ALP.

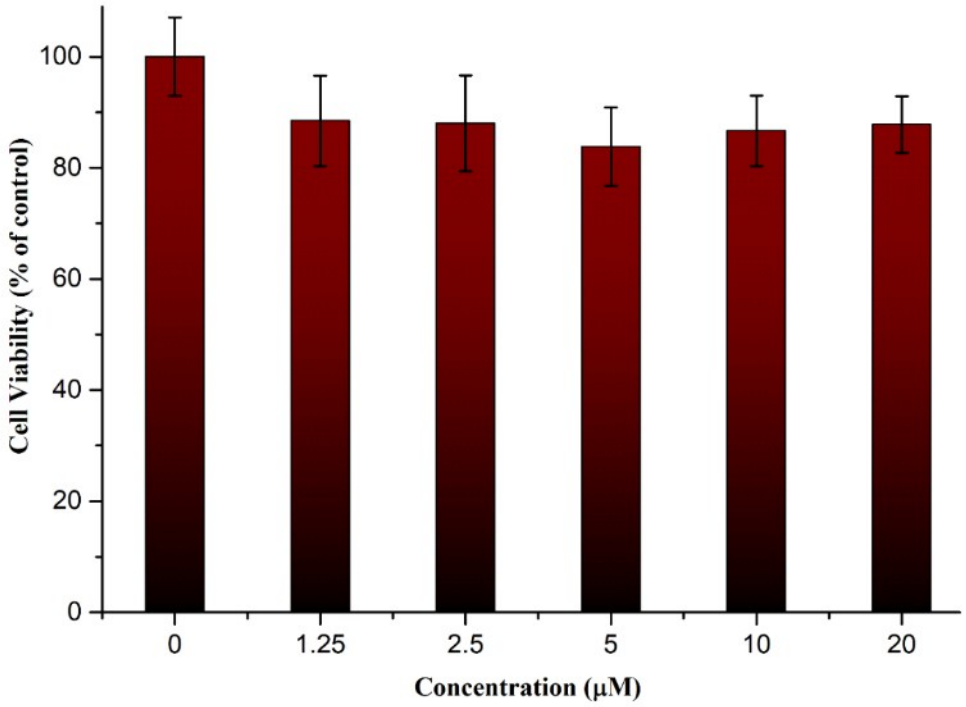


Figure S8. Cell viability of BEL 7402 treated with different concentrations.

of MTR-P over 24 h.

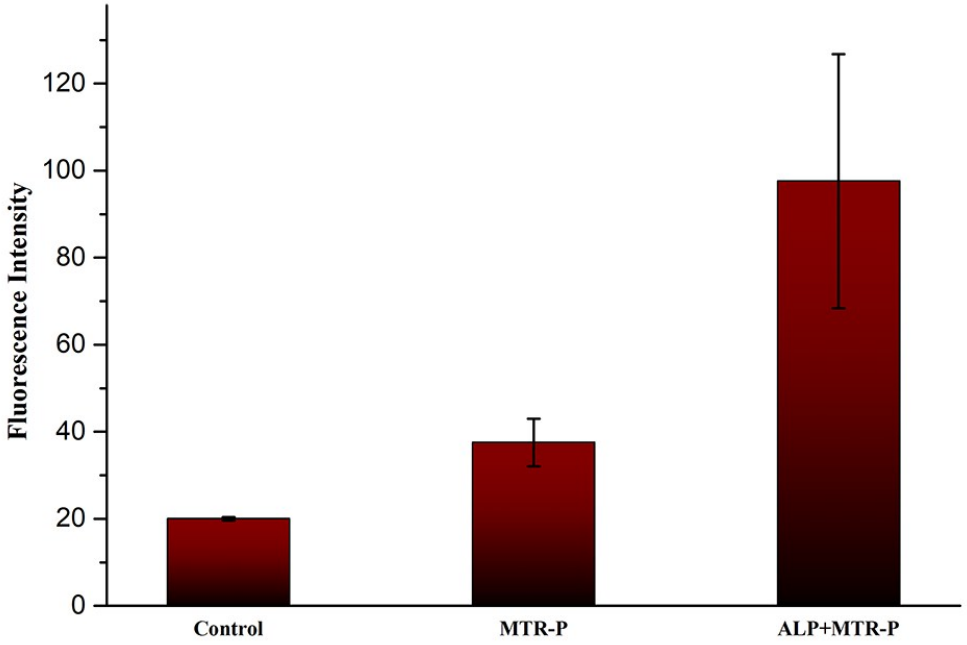


Figure S9. Bars represent the fluorescence intensities from the corresponding HEK 293T cells.

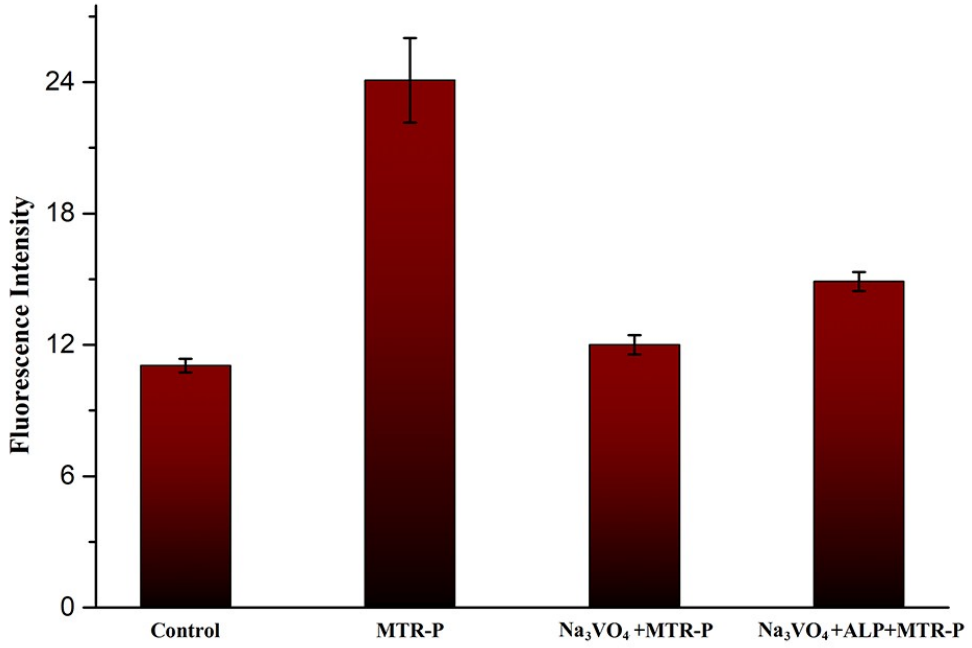


Figure S10. Bars represent the fluorescence intensities from the corresponding BEL 7402 cells.

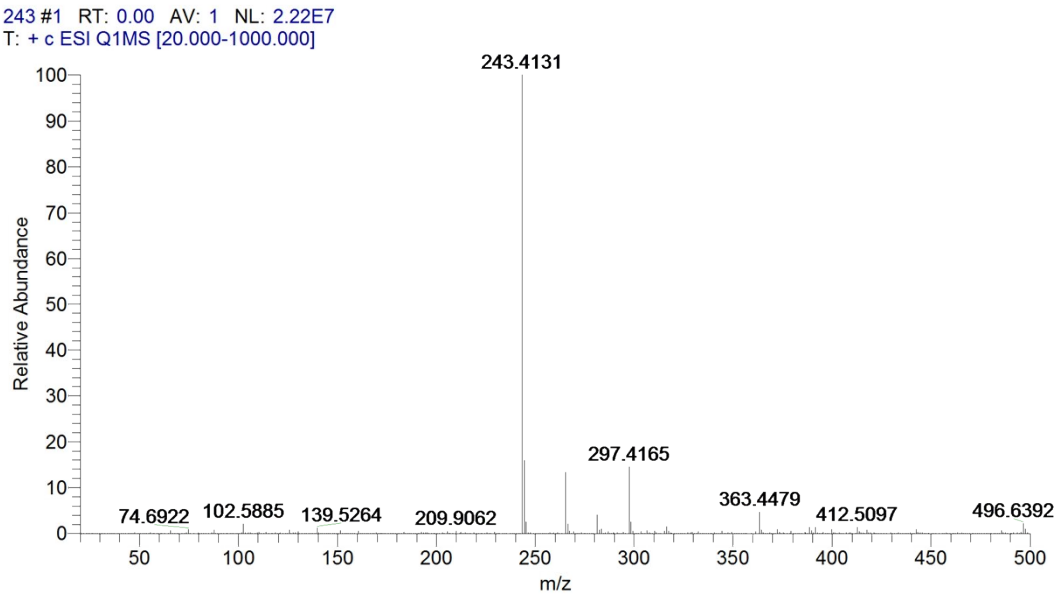


Figure S11. ESI-MS spectrum of Compound 2.

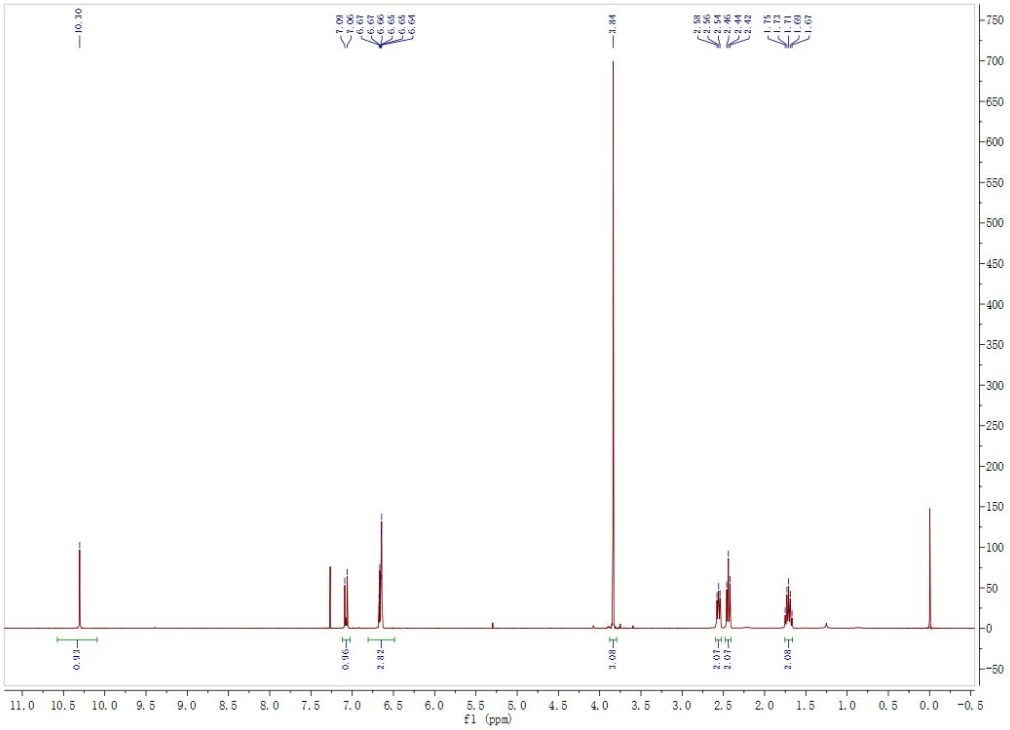


Figure S12. ^{1}H NMR (300 MHz, $CDCl_3$) of Compound 2.

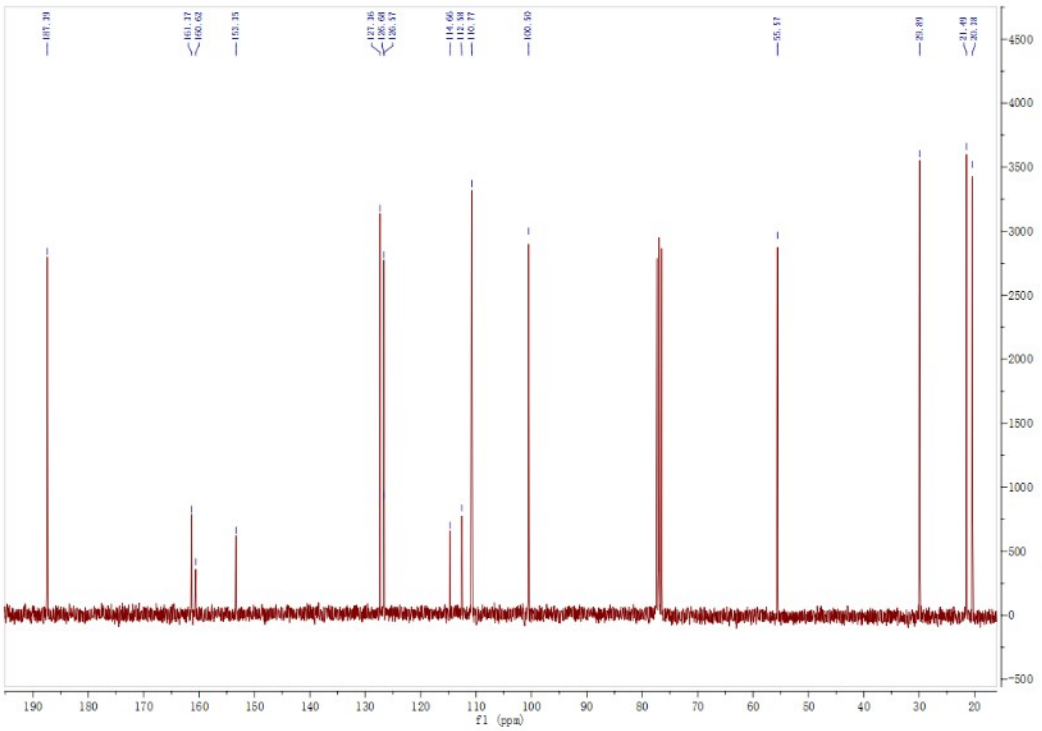


Figure S13. ^{13}C NMR (75 MHz, $CDCl_3$) of Compound 2.

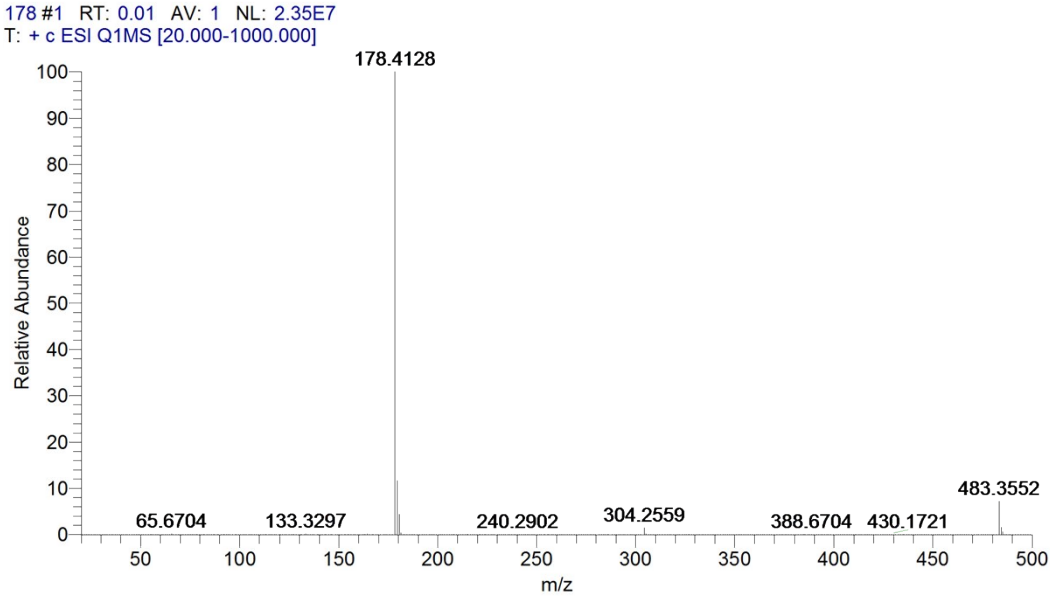


Figure S14. ESI-MS spectrum of Compound 3.

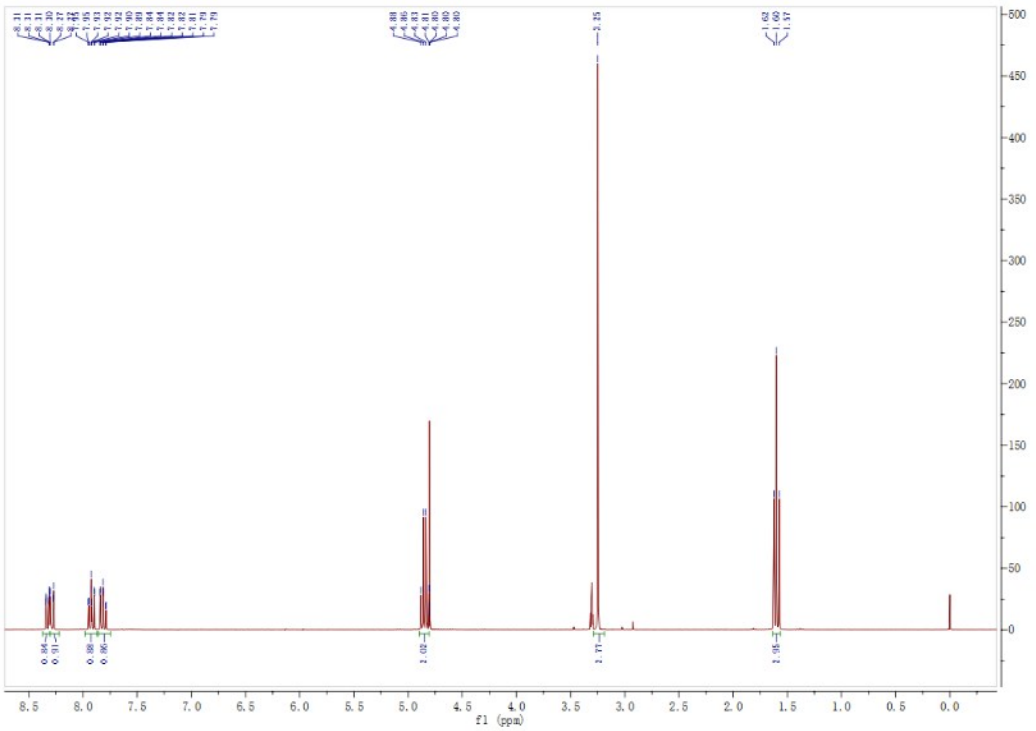


Figure S15. ^{1}H NMR (300 MHz, CD_3OD) of Compound 3.

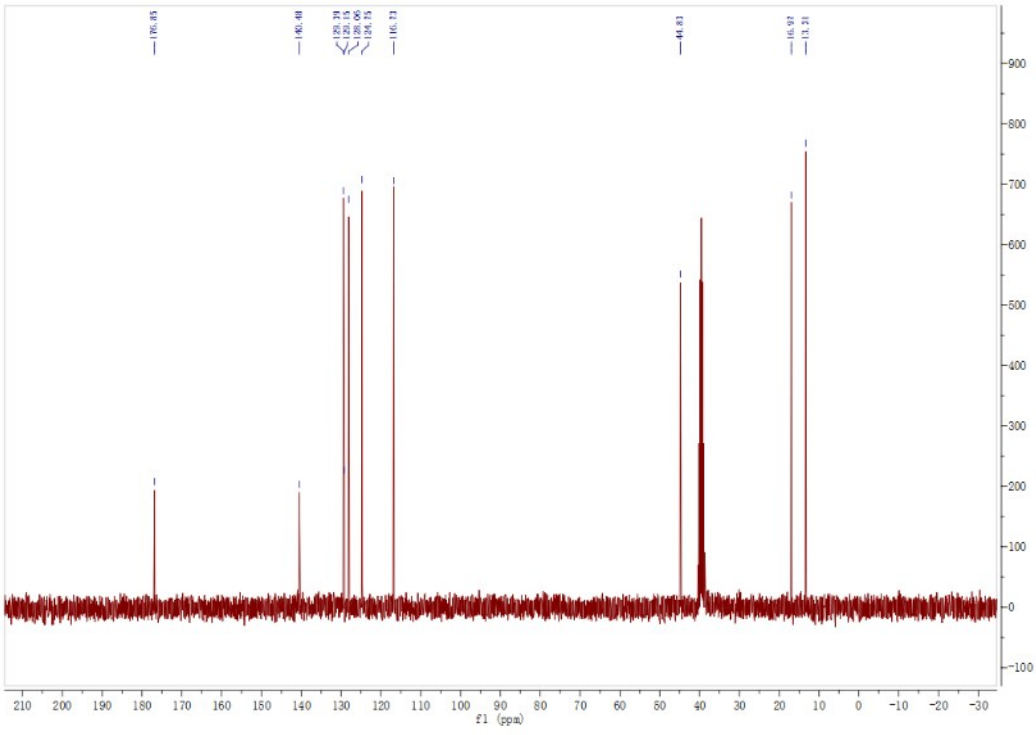


Figure S16. ^{13}C NMR (75 MHz, DMSO-d6) of Compound 3.

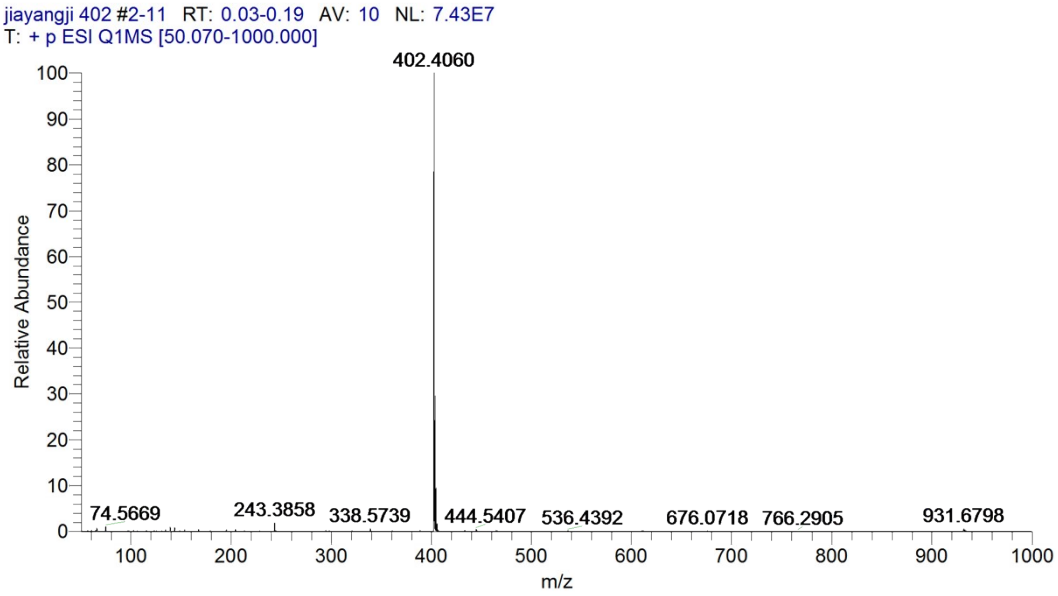


Figure S17. ESI-MS spectrum of Compound 4.

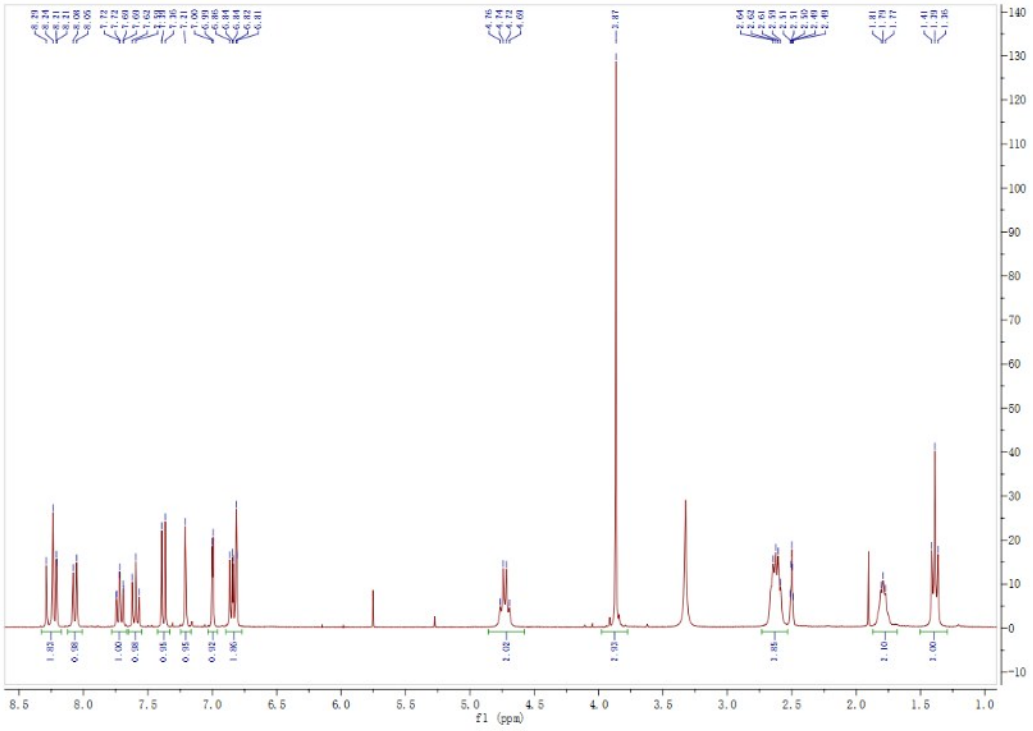


Figure S18. ^{1}H NMR (300 MHz, DMSO-d6) of Compound 4.

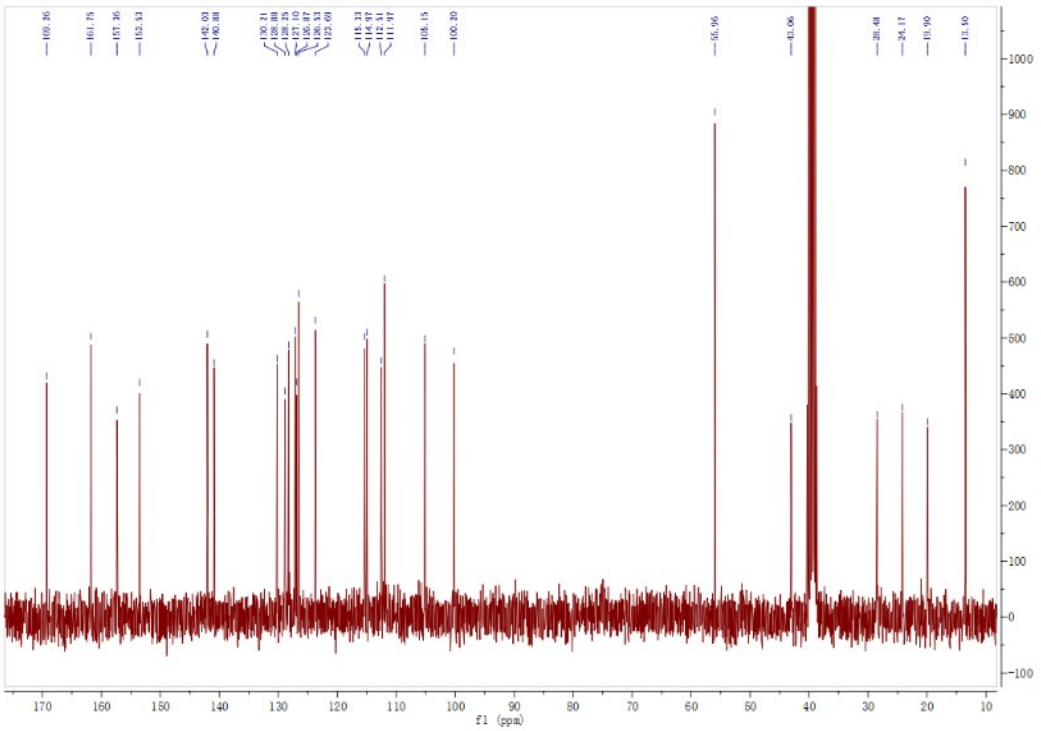


Figure S19. ^{13}C NMR (75 MHz, DMSO-d6) of Compound 4.

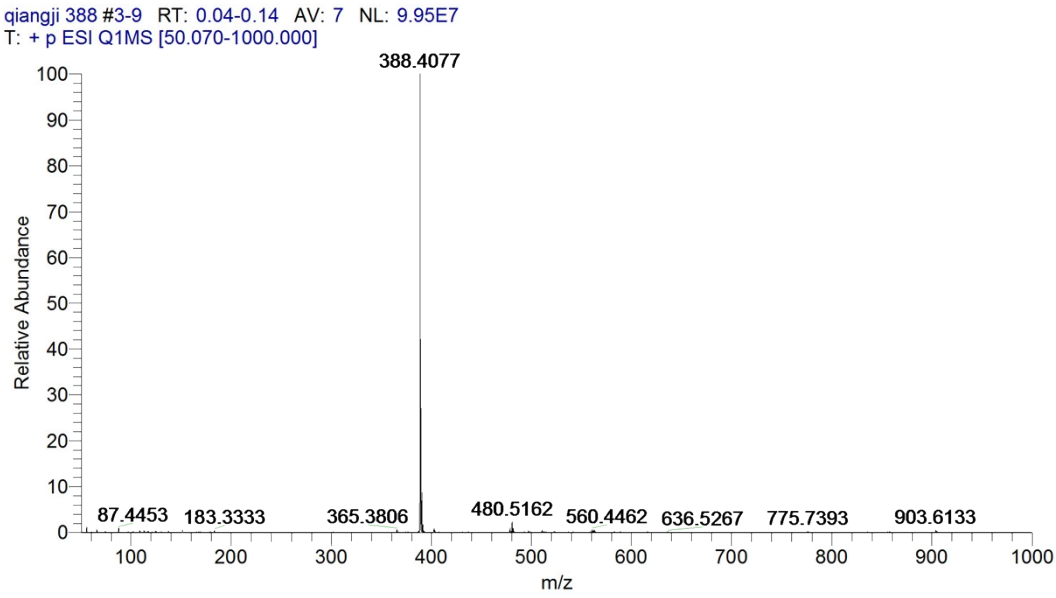


Figure S20. ESI-MS spectrum of MTR.

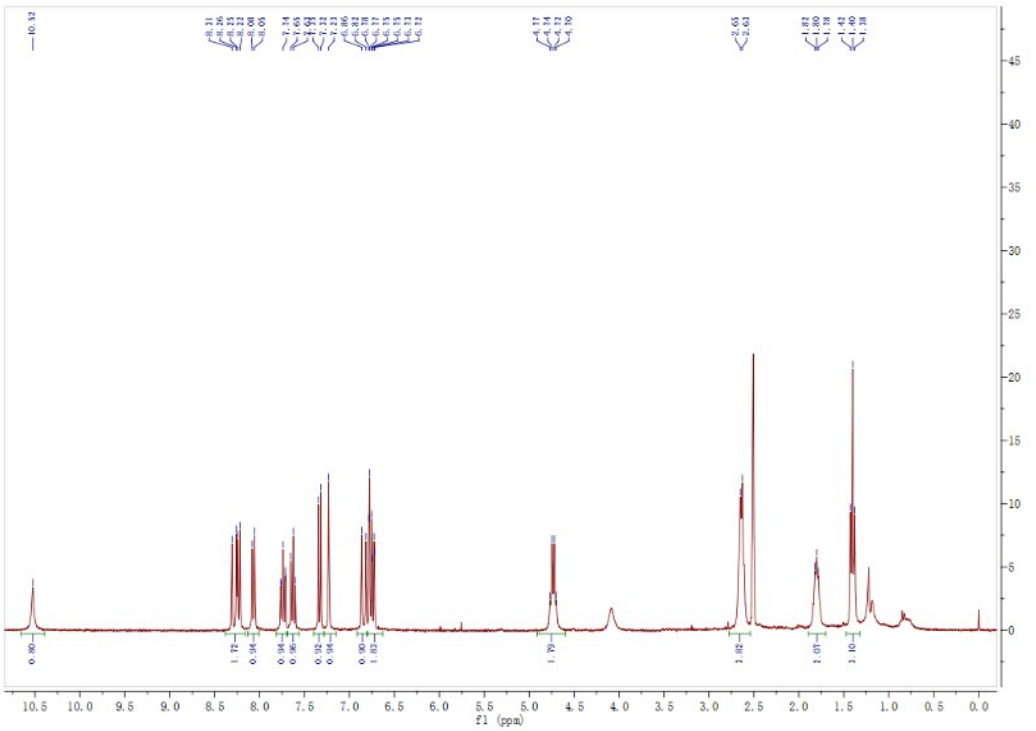


Figure S21. ^{1}H NMR (300 MHz, DMSO-d6) of MTR.

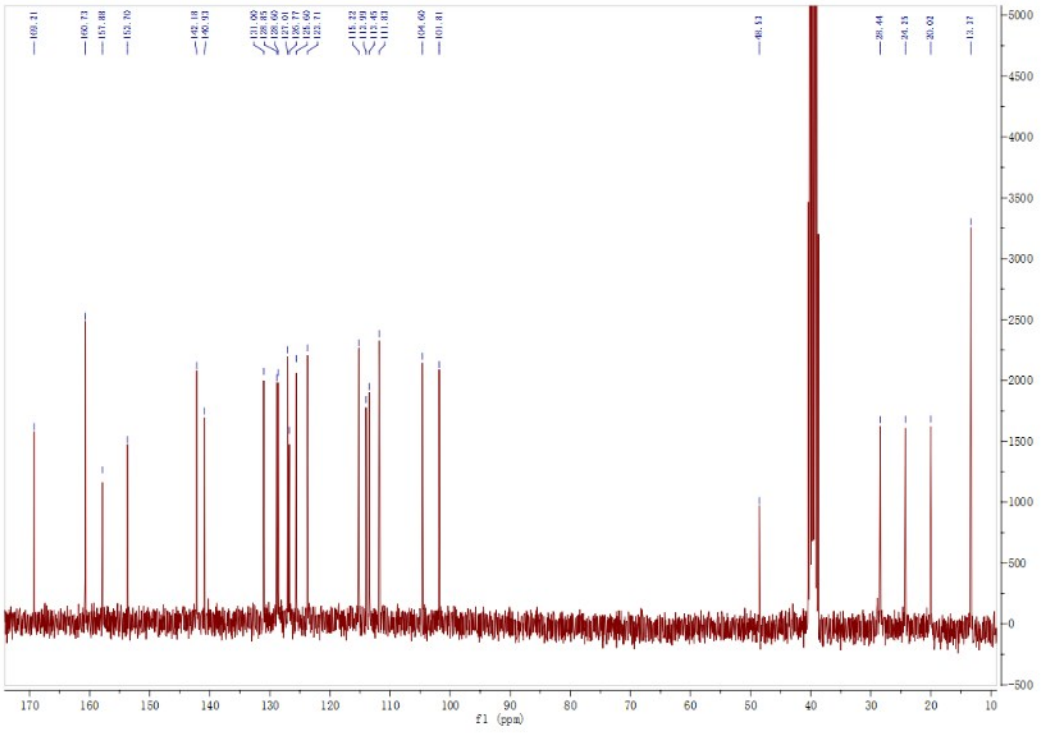


Figure S22. ^{13}C NMR (75 MHz, DMSO-d6) of MTR.

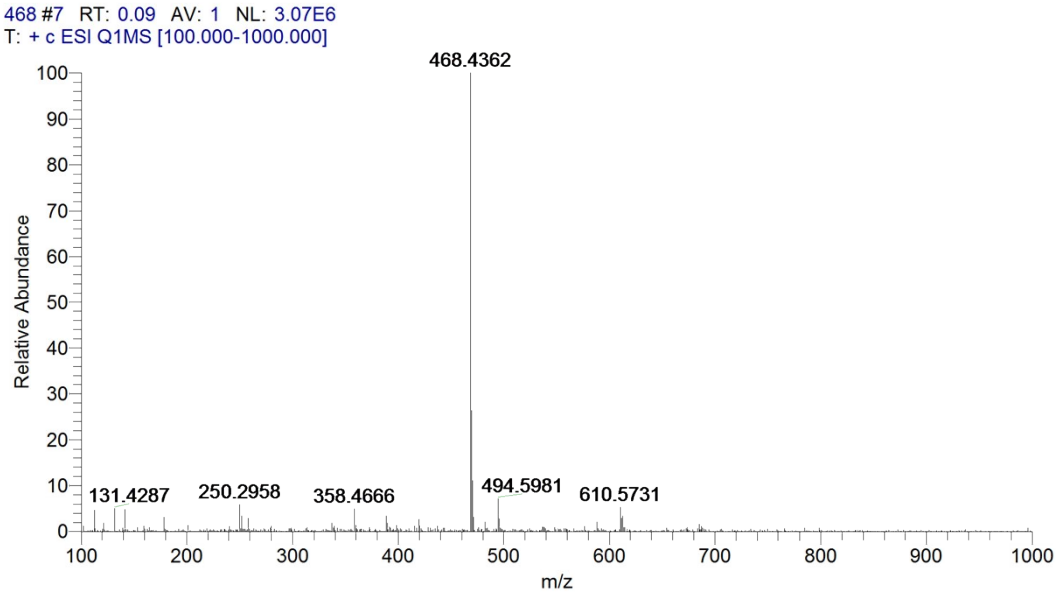


Figure S23. ESI-MS spectrum of MTR-P.

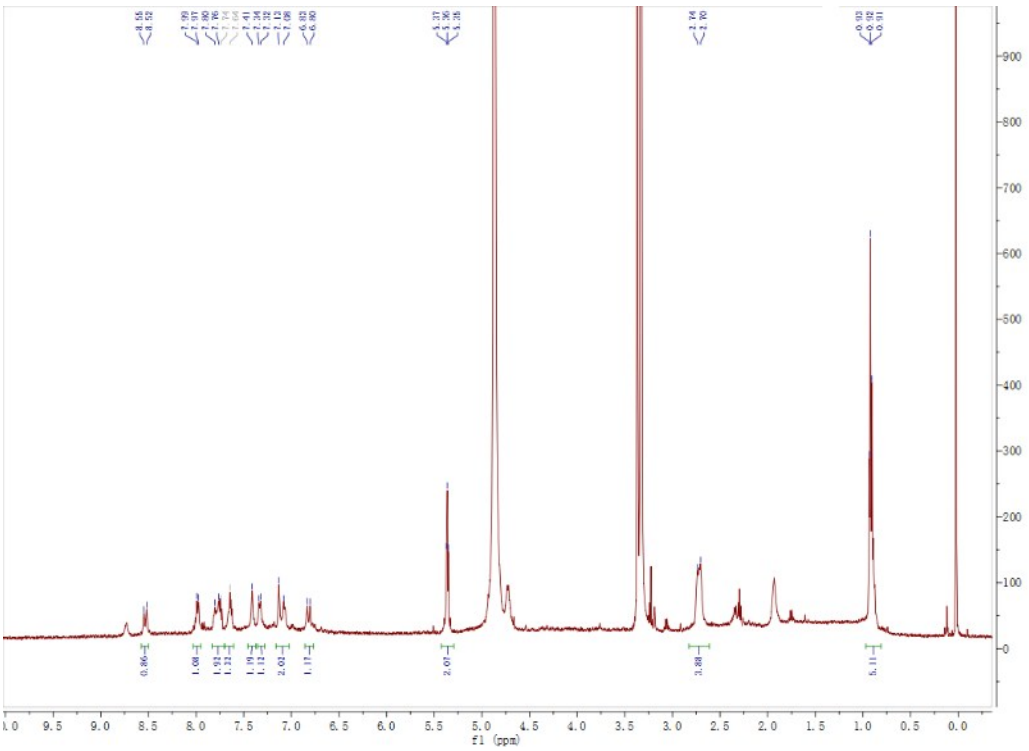


Figure S24. ^{1}H NMR (500 MHz, CD_3OD) of MTR-P.

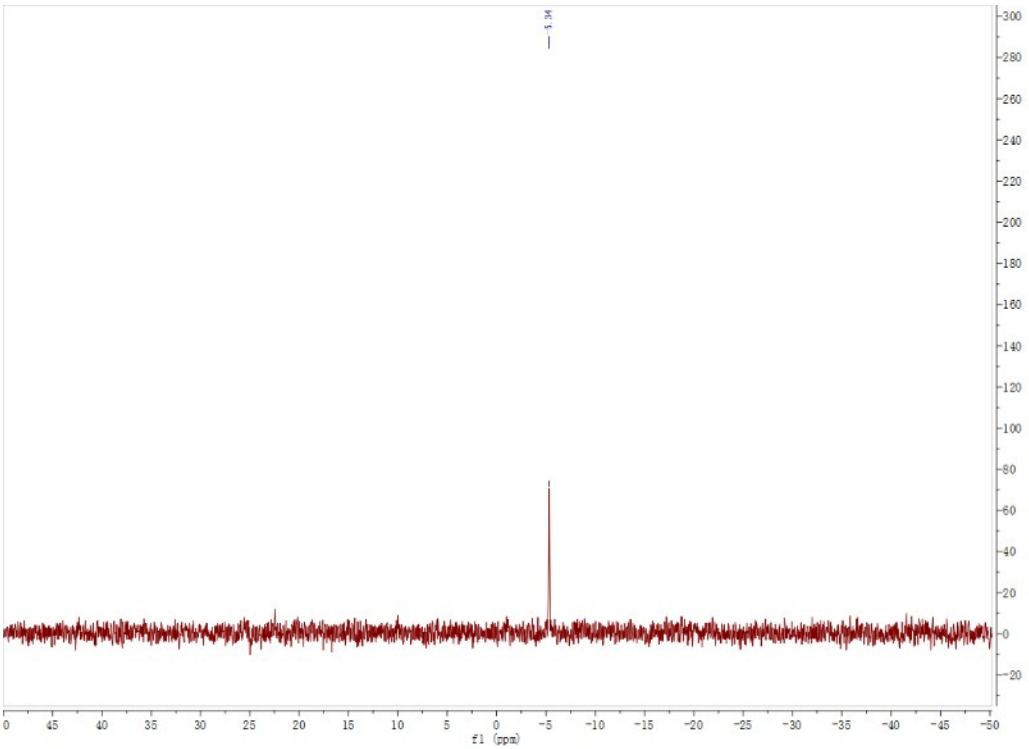


Figure S25. ^{31}P NMR (243 MHz, DMSO-d6) of MTR-P.

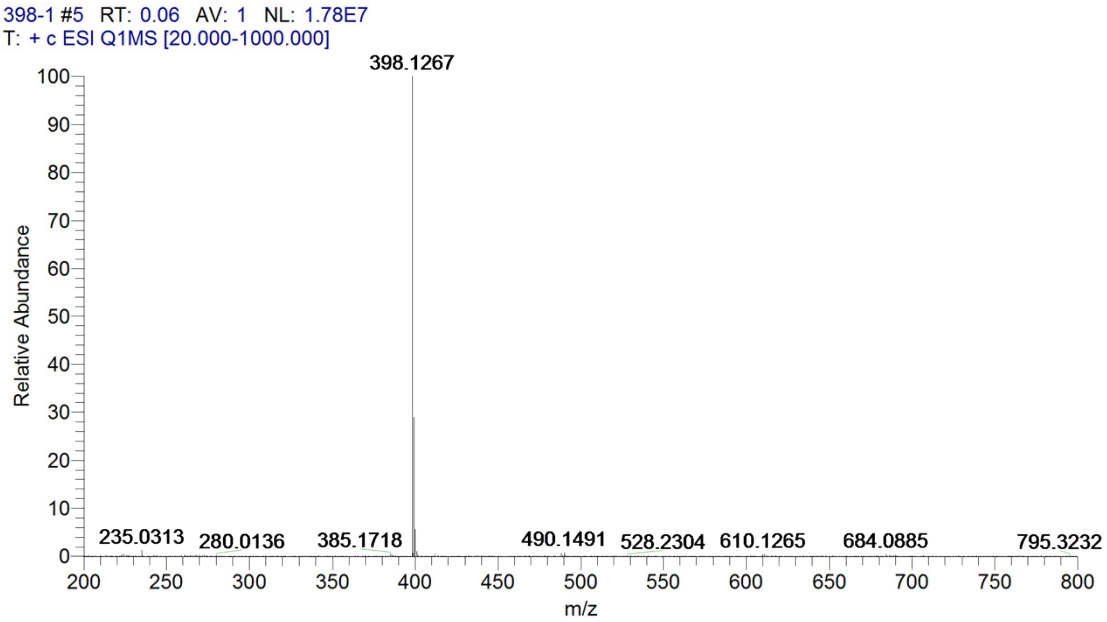


Figure S26. ESI-MS spectrum of CyR.

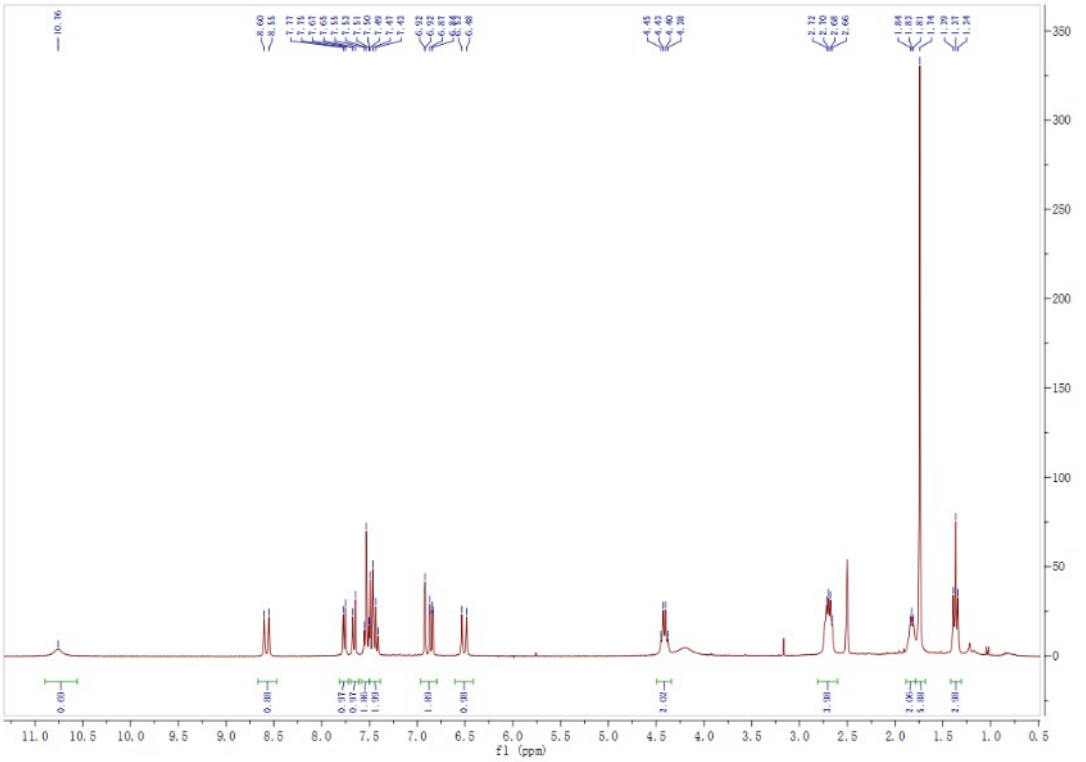


Figure S27. ^{1}H NMR (300 MHz, DMSO-d6) of CyR.

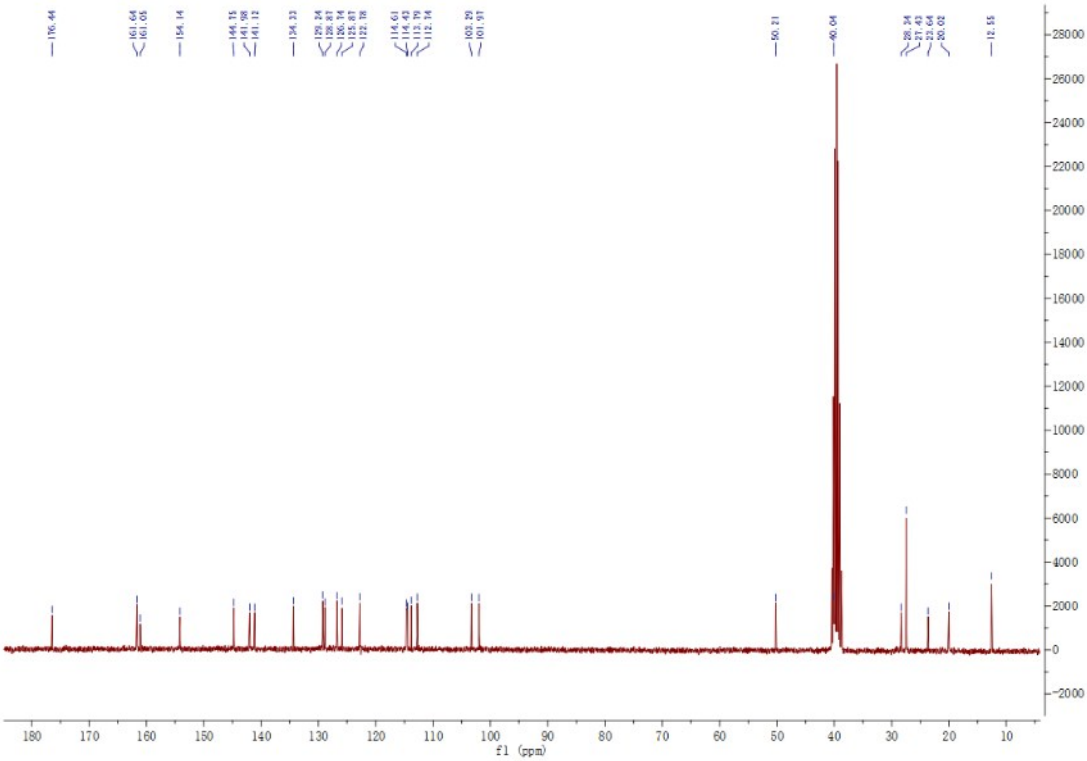
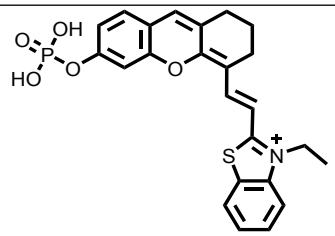
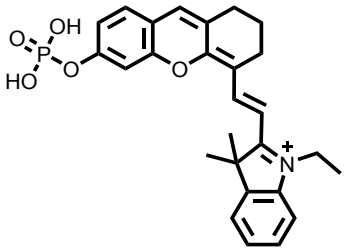
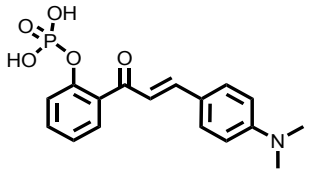
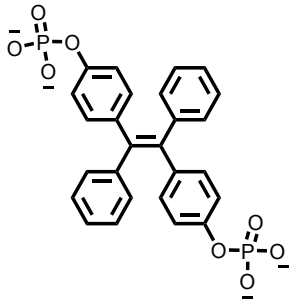
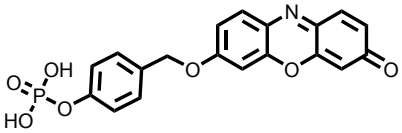
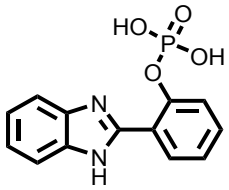
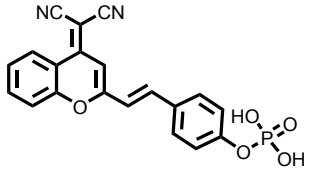
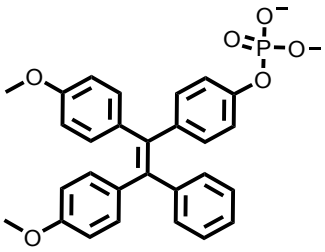
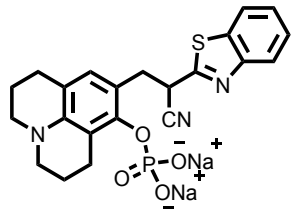


Figure S28. ^{13}C NMR (75 MHz, DMSO-d6) of CyR.

Table S1. Comparison of the performance of MTR-P with previously reported fluorescent probes for ALP.

Probe Struture	λex/λem (nm)	Detection Limit (U L^{-1})	Response Time (min)	Refernce
	680/723	0.042	15	This work
	680/700	0.07	25.2	2
	430/539, 641	0.15	45	3
	312/450	0.2	5	4
	550/585	1.09	20	5
	330/430	1.3	40	6

	488/550, 650	3.8	30	7
	350/480	18	15	8
	460/525	--	30	9

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