Supporting Information

Phosphinate-Containing Rhodol and Fluorescein Scaffolds for the Development of Bioprobes

Yuan Fang,[a] Gillian N. Good,[a,b] Xinqi Zhou,[a] and Cliff I. Stains[a,c,d,*]

[a]Department of Chemistry, University of Nebraska - Lincoln, Lincoln, Nebraska 68588, United States

[b]Department of Chemistry, Millersville University, Millersville, Pennsylvania 17551, United States

[c]Nebraska Center for Integrated Biomolecular Communication, University of Nebraska-Lincoln, Lincoln NE 68588

[d]Cancer Genes and Molecular Regulation Program, Fred & Pamela Buffet Cancer Center, University of Nebraska Medical Center, Omaha NE 68198

E-Mail: cstains@unl.edu

Table of Contents:

General Experimental Details

Reagents and Instrumentation

Unless otherwise noted, all reactions were done in flame-dried glassware under N_2 protection. All reagents and solvents were used as commercially supplied. Tetrahydrofuran (THF) and ethyl ether were dried using 3 Å molecular sieves.[1] Reaction progress was monitored by using thin layer chromatography (TLC) and products were purified by flash chromatography using Merck silica gel 60 (230 - 400 mesh). High-performance liquid chromatography (HPLC) purification was conducted using a Waters 1525 Binary HPLC pump with a 2489 UV/Vis detector. Large scale purification was done with a semiprep column (YMC-Pack 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. Mass spectra were obtained using electrospray ionization mass spectrometry (ESI, Thermo Finnigan LCQ Advantage) in HPLC buffer (50% acetonitrile in water with 0.1% trifluoroacetic acid). High-resolution mass spectrometry (HRMS) was performed by the Nebraska Center for Mass Spectrometry. Mass data are reported in units of m/z for $[M+H]^+$, $[M+Na]^+$ or $[M]^+$. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR experiments were performed in $CDCl_3$ or CD_3OD at room temperature and the spectra were recorded on Bruker-DRX-Avance 300 or 400 MHz instruments. UV-Vis spectra were measured using a BioMate 3S UV-Visible spectrophotometer (Thermo Scientific) with a 100 µL quartz cuvette. Fluorescence spectra were recorded using a FluoroMax-4 Spectrofluorometer (Horiba Scientific) with either 100 µL or 3.5 mL quartz cuvette. Absorbance data for determination of pK_as as well as luminescence data for cell toxicity assays were obtained on a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments) with non-treated clear flat bottom 96-well assay plates (Corning® 3370) and tissue culture treated white flat bottom 96-well assay plates (Corning® 3917), respectively. Photographs of dyes were taken using a Canon EOS 5D Mark III. NIR fluorescence photographs were taken using a home-made full-spectrum DSLR camera (Canon Rebel XSi) with a 720 nm NIR filter (Hoya 67 mm RM72 Infrared Filter). Confocal fluorescence imaging was performed using a Nikon A1R-Ti2 live-cell imaging confocal system. Laser lines were 488 nm for Cell Tracker Green (green channel, emission filter: 500 - 530 nm), 640 nm for **diAM-NR$_{600}$** (red channel, emission filter: 663 - 738 nm). Fiji software was used for data analysis.

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.

Quantum Yield Measurement

Fluorescence quantum yield was determined by the following equation:

$$\Phi_x = (\Phi_{ST} * A_{ST} * F_X * \eta_X^2) / (A_X * F_{ST} * \eta_{ST}^2)$$

Where Φ is the quantum yield; A is the absorbance at the excitation wavelength (A was kept at ≤ 0.05 during fluorescence measurements to avoid self-quenching); F is the fluorescence intensity at the emission wavelength; η is the refractive index of the solvent; and the subscripts ST and X refer to the standard and unknown, respectively. All the data was recorded in PBS buffer (10 mM, pH = 7.4, with 1% DMSO). For **NR_{675}** and **NR_{669}**, Alexa Fluor® 660 NHS Ester (Molecular Probes, A20007) was used as the standard (excitation: 668 nm, emission: 698 nm), which has a quantum yield of 0.37 in PBS.[2] For **NR_{638}**, **NR_{632}**, **NR_{604}** and **NR_{600}**, Oxazine 170 perchlorate (Sigma, 372056) was used as the standard (excitation: 627 nm, emission: 645 nm), which has a quantum yield of 0.579 in ethanol.[3]

Photostability

All the samples were kept at the same absorbance (A = 0.1) at their maximal excitation wavelength. Cy 5.5 (excitation: 678 nm, emission: 694 nm) was chosen for comparison. Fluorescence intensity at the respective emission maximum was recorded every 3 seconds with continuous exposure to excitation light for 1 hour using slit widths of 20 nm for both excitation and emission for each sample.

pK_a Determination

All **NR** family dyes were prepared at 5 μM in Britton-Robinson buffer at different pHs (containing 1% DMSO). Clear bottom, 96-well plates were used for the assay (final volume of 200 μL). Full absorption spectra were scanned from 300 nm to 700 nm.

***In vitro* Assay with PLE**

Samples containing **diAM-NR_{600}** (5 μM) in PBS (10 mM, pH = 7.4 containing 1% DMSO) and either 0.2 unit bovine serum albumin (BSA, Sigma, A1470), 0.2 unit porcine liver esterase

(PLE, Sigma, E3019), or DMEM with 10% FBS were assayed in the 100 µL quartz cuvettes. Fluorescent intensity at 619 nm (excitation: 600 nm, slit width: 1 nm) was recorded every 3 min over 1 h for each sample.

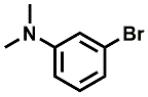
Cell Toxicity Assay

HeLa cells (ATCC, CCL-2) were seeded at 2×10^4 and were incubated with the indicated concentrations (0, 1, 5, or 10 µM) of **diAM-NR$_{600}$** (containing 1% DMSO) for 30 min. Cells were then washed once with cell culture media (DMEM with 10% FBS, and 1 × Anti-Anti) and further incubated for 3 h, 12 h, and 24 h. After washing, cell viability was assessed using the CellTiter-Glo 2.0 Assay (Promega).

Cell Imaging

HeLa (ATCC, CCL-2), NIH-3T3 (ATCC, CRL-1658), or RAW 264.7 (ATCC, TIB-71) cells were grown to 80% confluency in DMEM (Life Tech, 11965092) with 10% FBS (Life Tech, 16000036), and 1 × Anti-Anti (Life Tech, 15240062). The media was removed and washed with the pre-warmed (37 °C) DPBS (Life Tech, 14040133). Cells were then incubated with 0.6 µM CellTracker™ Green CMFDA (Invitrogen™, C2925) in DPBS (with 1% DMSO) for 20 min. After washing with pre-warmed DPBS three times, cells were further incubated with 0.6 µM **diAM-NR$_{600}$** in DPBS (1% DMSO final) for 30 min. Where indicated, media was removed, and cells were washed with pre-warmed DPBS three times before imaging. Otherwise cells were imaged without washing.

Synthesis of Nebraska Red Family Members and diAM-NR$_{600}$ Esterase Sensor

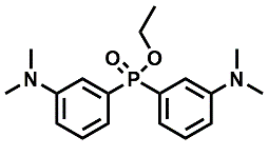


1

3-bromo-*N,N*-dimethylaniline (1). 1 was synthesized following a previously reported method.[4]

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.17-7.06 (m, 1H), 6.86 (dd, *J* = 7.2, 1.2 Hz, 2H), 6.72-6.60 (m, 1H), 2.98 (s, 6H).

MS (ESI) m/z calculated for $C_8H_{10}BrN$ $[M+H]^+$ 201.1, found 201.4.



2

ethyl bis(3-(dimethylamino)phenyl)phosphinate (2). In a flame-dried 100 mL round bottom flask, 3-bromo-N,N-dimethylaniline (5 g, 25 mmol) was dissolved in 40 mL anhydrous diethyl ether, and the solution was cooled to -78 °C using an acetone/dry ice bath for 10 min, sec-butyllithium (1.4 M in cyclohexane, 19.6 mL, 27.5 mmol) was then added dropwise. After 1 h, the resulting mixture was transferred into another flame-dried 250 mL round bottom flask containing 50 mL anhydrous diethyl ether and ethyl dichlorophosphite (1.57 mL, 13.75 mmol) using a metal canula over 15 min. The reaction was kept at -78 °C for 3 h, and then moved to an ice bath and slowly warmed to room temperature overnight. After cooling to 0 °C, hydrogen peroxide (50 wt% in H_2O, 10.5 mL) was added dropwise along with 5 mL ethanol, and the mixture was stirred in an ice/water bath for 1 h. Then the reaction was quenched by saturated sodium sulfite solution and extracted with ethyl acetate (3 × 200 mL). The organic layer was combined and dried with sodium sulfate. After removing the solvent, the resulting oil was purified by flash column chromatography (50% - 100% ethyl acetate in hexane) to afford **2** as the light-yellow oil (2.99 g, 72%).

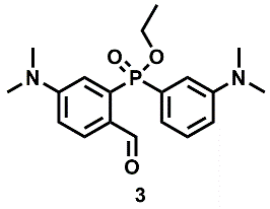
^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.34-7.29 (m, 1H), 7.28-7.24 (m, 2H), 7.22 (dd, J = 2.8, 1.3 Hz, 1H), 7.10 (ddt, J = 11.8, 7.4, 1.2 Hz, 2H), 6.91-6.78 (m, 2H), 4.12 (p, J = 7.1 Hz, 2H), 2.99 (s, 12H), 1.39 (t, J = 7.1 Hz, 3H).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 150.34, 150.14, 133.12, 131.32, 129.20, 129.00, 119.28, 119.15,

115.64, 115.60, 115.23, 115.07, 60.97, 60.90, 40.45, 16.61, 16.52.

^{31}P-NMR (121 MHz, $CDCl_3$): δ 33.98.

HRMS (ESI) m/z calculated for $C_{18}H_{25}N_2O_2P$ $[M+H]^+$ 333.1732, found 333.1722.



3

Ethyl (5-(dimethylamino)-2-formylphenyl)(3-(dimethylamino)phenyl)phosphinate (3). A flame-dried 50 mL round bottom flask was charged with 15 mL anhydrous DMF, and cooled to 0 °C in an ice bath. $POCl_3$ (839 µL, 9 mmol) was added dropwise within 5 min and the mixture was stirred in the ice bath for 30 min. Ethyl bis(3-(dimethylamino)phenyl)phosphinate (**2**) (2.72 g, 8.18 mmol) was dissolved in 10 mL anhydrous DMF, and added to the flask dropwise over 10 min. The reaction was then stirred at room temperature for 1 h, and then at 50 °C for another 3 h. After cooling to room temperature, the mixture was poured into ice water containing $NaHCO_3$ to obtain a basic pH, and extracted with DCM (3 × 200 mL). The combined organic layer was washed with saturated NaCl solution, and dried over sodium sulfate. After evaporating the solvent, flash column chromatography (10% - 95% ethyl acetate in hexane with 5% triethylamine, after presoaking the silica gel with 5% triethylamine in hexane overnight) was performed to give **3** as the light-yellow solid (1.83 g, 62%).

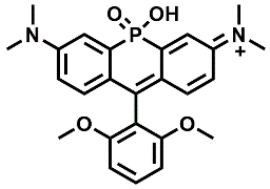
^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.42 (s, 1H), 8.02 (dd, J = 8.9, 5.5 Hz, 1H), 7.38-7.29 (m, 1H), 7.26 (dd, J = 7.5, 4.2 Hz, 1H), 7.16 (ddd, J = 14.9, 2.8, 1.3 Hz, 1H), 6.98 (dd, J = 12.6, 7.3 Hz, 1H), 6.82 (td, J = 9.0, 2.7 Hz, 2H), 4.19 (pd, J = 7.2, 1.4 Hz, 2H), 3.12 (s, 6H), 2.97 (s, 6H), 1.39 (t, J = 7.1 Hz, 3H).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 189.96, 189.90, 152.92, 152.74, 150.42, 150.22, 136.27, 134.61, 133.41, 131.59, 130.55, 130.39, 129.48, 129.27, 125.93, 125.80, 118.74, 118.59, 116.28, 116.14, 115.93, 115.89, 114.45, 114.29, 113.57, 113.54, 61.48, 61.40, 40.37, 40.05, 16.56, 16.47.

^{31}P-NMR (121 MHz, $CDCl_3$): δ 33.96, 32.83.

HRMS (ESI) m/z calculated for $C_{19}H_{25}N_2O_3P$ $[M+Na]^+$ 383.1500, found 383.1483.

General method for preparation of Nebraska Red rhodamines. In a flame-dried 100 mL round bottom flask, 1,3-dimethoxy-2-bromobenzene (236 mg, 1.09 mmol) was dissolved in 15 mL anhydrous THF, and the temperature was lowered to -78 °C using the acetone/dry ice bath for 10 min, followed by dropwise addition of sec-butyllithium (1.4 M in cyclohexane, 934 µL, 1.31 mmol) over 5 min. The reaction was then stirred at -78 °C for 1 h. Ethyl (5-(dimethylamino)-2-formylphenyl)(3-(dimethylamino)phenyl)phosphinate (**3**) (327 mg, 0.91 mmol) was dissolved in 10 mL anhydrous THF, and was then added to the reaction dropwise within 5 min. The mixture was kept in -78 °C for 0.5 h and then stirred at room temperature for another 2 h. After adding 40 mL 2 M HCl, the resulting solution was refluxed at 70 °C overnight. The pH of the water phase was adjusted to 3-4 and a mixture of chloroform/2-propanol (85/15, v/v) was used for extraction. The organic layer was collected, dried over sodium sulfate, and the solvent was removed. The resulting solid was dissolved in 10 mL DCM, and p-chloranil (671 mg, 2.73 mmol) was add. After 1 h reaction at room temperature, the mixture was purified by flash column chromatography (0% - 80% methanol in DCM with 5% acetic acid). The purified blue-green solid was added to HPLC buffer (50% acetonitrile in water with 0.1% trifluoroacetic acid), and re-purified by HPLC, monitoring at 650 nm. The product peak from HPLC was lyophilized to afford **NR_{675}** as a blue-green solid (180 mg, 44%). The synthesis of **NR_{669}** followed the same procedure as **NR_{675}** using the 2-bromo-1,3-dimethylbenzene as the starting material to yield **NR_{669}** as a blue-green solid (213 mg, 56%).



NR_{675}

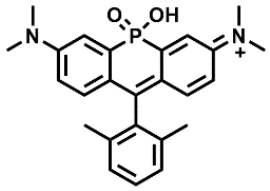
NR_{675}

^{1}H-NMR (400 MHz, CD_3OD): δ 7.61-7.48 (m, 3H), 7.18 (dd, *J* = 9.5, 5.8 Hz, 2H), 6.85 (d, *J* = 8.4 Hz, 2H), 6.79 (dd, *J* = 9.6, 2.6 Hz, 2H), 3.67 (s, 6H), 3.39 (s, 12H).

^{13}C-NMR (101 MHz, CD_3OD): δ 169.55, 157.76, 155.81, 155.69, 144.27, 140.13, 139.30, 139.21, 131.34, 128.56, 124.73, 124.61, 124.53, 120.19, 115.37, 115.32, 114.00, 113.69, 103.72, 55.05, 39.72.

^{31}P-NMR (162 MHz, CD_3OD): δ 4.17.

HRMS (ESI) m/z calculated for $C_{25}H_{28}N_2O_4P^+$ $[M]^+$ 451.1781, found 451.1782.



NR_{669}

NR_{669}

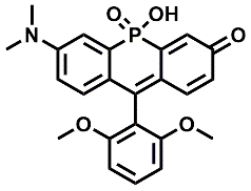
^{1}H-NMR (400 MHz, CD_3OD): δ 7.62 (dd, *J* = 15.3, 2.8 Hz, 2H), 7.39 (dd, *J* = 8.2, 7.1 Hz, 1H), 7.26 (d, *J* = 7.6 Hz, 2H), 7.05 (dd, *J* = 9.6, 6.1 Hz, 2H), 6.84 (dd, *J* = 9.6, 2.8 Hz, 2H), 3.41 (s, 12H), 2.04 (s, 6H).

^{13}C NMR (101 MHz, CD_3OD) δ 165.13, 165.04, 159.00, 158.61, 155.90, 155.77, 144.69, 143.48, 138.54, 138.44, 136.41, 135.78, 128.93, 127.45, 123.29, 123.22, 116.09, 116.03, 114.46, 114.44, 39.89, 18.48.

^{31}P-NMR (162 MHz, CD_3OD): δ 4.74.

HRMS (ESI) m/z calculated for $C_{25}H_{28}N_2O_2P^+$ $[M]^+$ 419.1883, found 419.1885.

General method for preparation of Nebraska Red rhodols. In a 50 mL round bottom flask, 20 mg **NR_{675}** was dissolved in a 20 mL 0.1 M NaOH aqueous solution. The mixture was stirred at room temperature for 4 h. 2 M HCl was used to adjust the pH to ~3-4 and a mixture of chloroform/2-propanol (85/15, v/v) was used for extraction until the water layer was colorless. The organic layer was combined, dried over sodium sulfate, and the solvent was evaporated. The resulting purple solid was dissolved in HPLC buffer (50% acetonitrile in water with 0.1% trifluoroacetic acid), and purified by HPLC, monitoring at 280 and 365 nm. The peak corresponding to **NR** rhodol was collected and lyophilized to afford **NR_{638}** as a purple solid (15.9 mg, 85%). **NR_{632}** was synthesized and purified using the same procedure as **NR_{638}**, yielding a purple solid (14.5 mg, 78%).



NR_{638}

NR_{638}

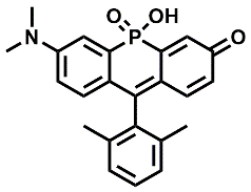
^{1}H-NMR (400 MHz, CD_3OD): δ 7.71-7.64 (m, 1H), 7.58 (s, 1H), 7.54-7.46 (m, 1H), 7.32 (dd, *J*

= 9.9, 4.9 Hz, 1H), 7.16 (dd, *J* = 8.9, 5.6 Hz, 1H), 6.97 (dd, *J* = 9.9, 2.1 Hz, 1H), 6.78 (dd, *J* = 9.0, 2.2 Hz, 1H), 3.67 (s, 6H), 3.57 (s, 6H).

^{13}C-NMR (101 MHz, CD_3OD): δ 164.81, 164.68, 162.81, 162.74, 160.88, 158.43, 158.30, 157.67, 142.35, 142.27, 137.63, 137.53, 131.45, 131.09, 128.56, 127.56, 127.48, 126.90, 126.84, 118.23, 118.17, 117.94, 117.25, 117.22, 115.85, 113.64, 103.79, 103.72, 103.30, 55.10, 40.98.

^{31}P-NMR (162 MHz, CD_3OD): δ 2.12.

HRMS (ESI) m/z calculated for $C_{23}H_{22}NO_5P$ $[M+H]^+$ 424.1314, found 424.1431.



NR$_{632}$

NR$_{632}$

^{1}H-NMR (400 MHz, CD_3OD): δ 7.75 (dd, *J* = 14.9, 2.6 Hz, 1H), 7.58 (dd, *J* = 14.0, 2.6 Hz, 1H), 7.41 (t, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 7.6 Hz, 2H), 7.16 (dd, *J* = 9.9, 5.2 Hz, 1H), 7.09-6.97 (m, 2H), 6.81 (dd, *J* = 9.0, 2.4 Hz, 1H), 3.58 (s, 6H), 2.03 (s, 6H).

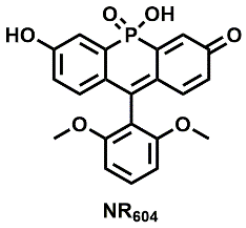
^{13}C-NMR (101 MHz, CD_3OD) δ 165.72, 165.63, 165.47, 165.33, 158.41, 158.29, 141.09, 141.01, 137.26, 137.16, 136.24, 135.76, 129.15, 127.54, 126.42, 126.34, 125.77, 125.70, 118.77, 118.71, 118.43, 118.01, 116.67, 41.14, 18.56.

^{31}P-NMR (162 MHz, CD_3OD): δ 1.25.

HRMS (ESI) m/z calculated for $C_{23}H_{22}NO_3P$ $[M+H]^+$ 392.1416, found 392.1408.

General method for preparation of Nebraska Red fluoresceins. In a 50 mL round bottom flask, 20 mg **NR$_{675}$** was dissolved in 30 mL 1 M NaOH solution (methanol/water, 1:1, v/v). The reaction flask was covered with aluminum foil to avoid exposure to ambient light and stirred at room temperature for 3 days. The pH was adjusted to ~3-4 with 2 M HCl and a mixture of chloroform/2-propanol (85/15, v/v) was used for extraction until the water layer was colorless. The organic layer was combined, and dried over sodium sulfate. After removing the solvent, the resulting brown solid was added to HPLC buffer (50% acetonitrile in water with 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 280 and 365 nm. The peak corresponding to **NR** fluorescein was lyophilized to afford **NR$_{604}$** as a brick-red solid (12.6 mg,

72%). **NR_{600}** was synthesized and purified using the same procedure as **NR_{604}**, yielding a brick-red solid (13.2 mg, 76%).



NR_{604}

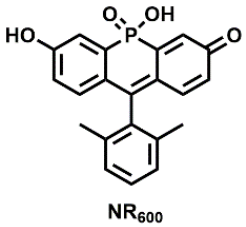
NR_{604}

^{1}H-NMR (400 MHz, CD_3OD): δ 7.57-7.51 (m, 1H), 7.33-7.21 (m, 2H), 7.06 (dd, *J* = 9.4, 6.7 Hz, 2H), 6.84 (d, *J* = 8.5 Hz, 1H), 6.55 (dd, *J* = 9.6, 2.2 Hz, 2H), 3.70 (s, 6H).

^{13}C-NMR (101 MHz, CD_3OD): δ 173.43, 157.72, 152.29, 140.28, 139.06, 138.36, 138.25, 131.19, 130.98, 127.21, 127.12, 123.28, 122.38, 119.56, 113.82, 103.76, 103.52, 55.09.

^{31}P-NMR (162 MHz, CD_3OD): δ 8.21.

HRMS (ESI) m/z calculated for $C_{21}H_{17}O_6P$ $[M+H]^+$ 397.0841, found 397.0834.



NR_{600}

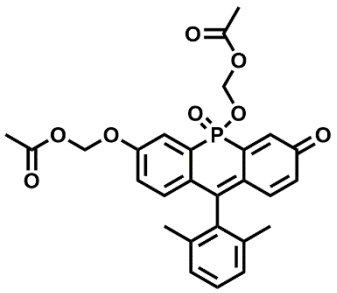
NR_{600}

^{1}H-NMR (400 MHz, CD_3OD): δ 7.40-7.26 (m, 3H), 7.24 (d, *J* = 7.6 Hz, 2H), 6.89 (dd, *J* = 9.6, 5.0 Hz, 2H), 6.50 (d, *J* = 9.4 Hz, 2H), 2.05 (s, 6H).

^{13}C-NMR (101 MHz, CD_3OD): δ 173.81, 168.03, 155.43, 137.16, 137.07, 136.83, 135.98, 135.94, 132.30, 131.09, 128.61, 128.57, 127.47, 127.42, 126.05, 126.01, 123.28, 123.25, 122.40, 18.61.

^{31}P-NMR (162 MHz, CD_3OD): δ 4.47.

HRMS (ESI) m/z calculated for $C_{21}H_{17}O_4P$ $[M+H]^+$ 365.0943, found 365.0947.



diAM-NR$_{600}$

diAM-NR$_{600}$. In an 8 mL amber glass vial equipped with a pressure relief cap, **NR$_{600}$** (10 mg, 0.027 mmol) was dissolved in 1.5 mL anhydrous DMF, followed by addition of DIPEA (26 µL, 0.15 mmol). The mixture was stirred at room temperature for 10 min, bromomethyl acetate (29 µL, 0.3 mmol) was then added dropwise over 5 min. The reaction was stirred at 70 °C overnight. The solvent was removed, and the crude product was dissolved in HPLC buffer (50 % acetonitrile in water containing 0.1% trifluoroacetic acid) and purified by HPLC, monitoring at 280 and 365 nm. The peak corresponding to the product was lyophilized to afford **diAM-NR$_{600}$** as an orange solid (6.1 mg, 44%).

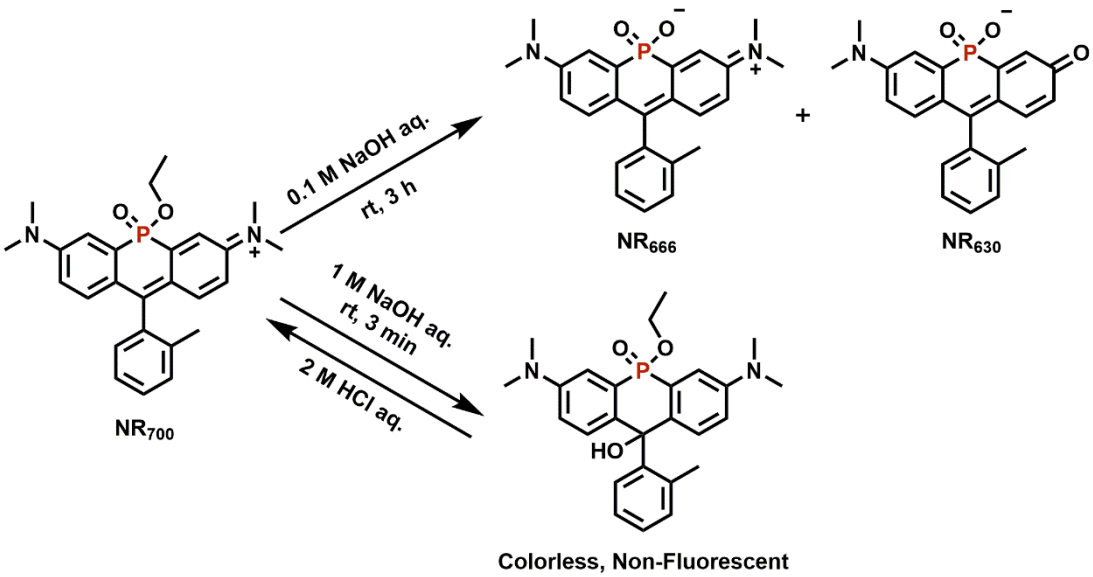
^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.76 (dd, *J* = 15.4, 2.6 Hz, 1H), 7.48 (dd, *J* = 9.4, 7.2 Hz, 1H), 7.32 (dd, *J* = 18.5, 2.1 Hz, 1H), 7.11-6.99 (m, 3H), 6.71 (dd, *J* = 8.5, 3.4 Hz, 2H), 6.36 (d, *J* = 10.1 Hz, 1H), 5.84 (q, *J* = 6.7 Hz, 2H), 5.79-5.73 (m, 2H), 2.35 (d, *J* = 1.4 Hz, 6H), 2.15 (s, 3H), 2.00 (s, 3H).

^{13}C-NMR (101 MHz, $CDCl_3$): δ 184.65, 184.51, 169.69, 169.20, 168.59, 158.49, 158.34, 157.91, 157.69, 146.55, 146.46, 140.48, 140.37, 136.60, 135.39, 135.14, 135.12, 134.87, 134.85, 133.92, 133.79, 132.68, 132.60, 131.52, 131.43, 130.05, 128.92, 128.76, 128.60, 128.59, 127.64, 127.56, 126.81, 124.19, 124.14, 120.94, 117.67, 113.10, 112.88, 104.06, 103.89, 20.92, 20.63, 18.53.

^{31}P-NMR (162 MHz, $CDCl_3$): δ 17.06, 16.50.

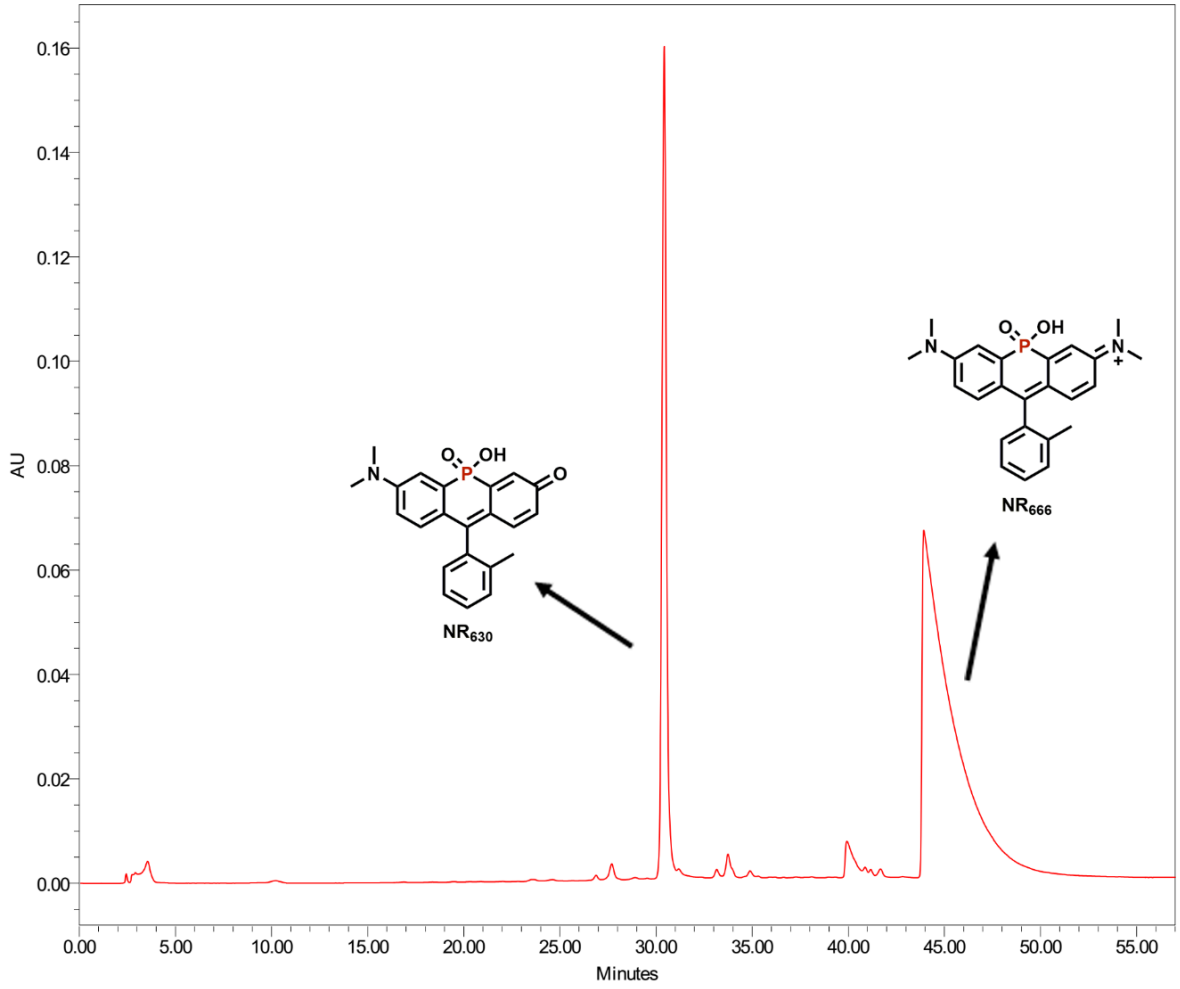
HRMS (ESI) m/z calculated for $C_{27}H_{25}O_8P$ $[M+H]^+$ 508.1287, found 508.1272.

Scheme S1



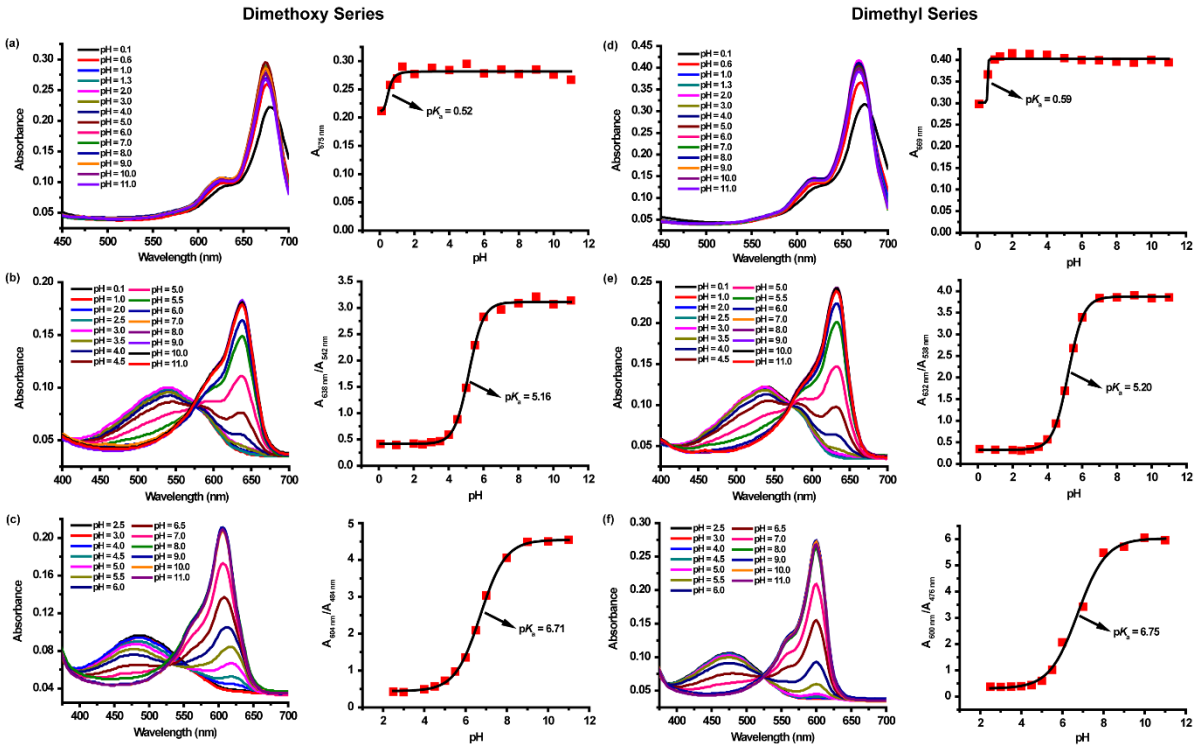
Reaction of **NR$_{700}$** with 0.1 M or 1 M aqueous NaOH for the indicated times.

Figure S1



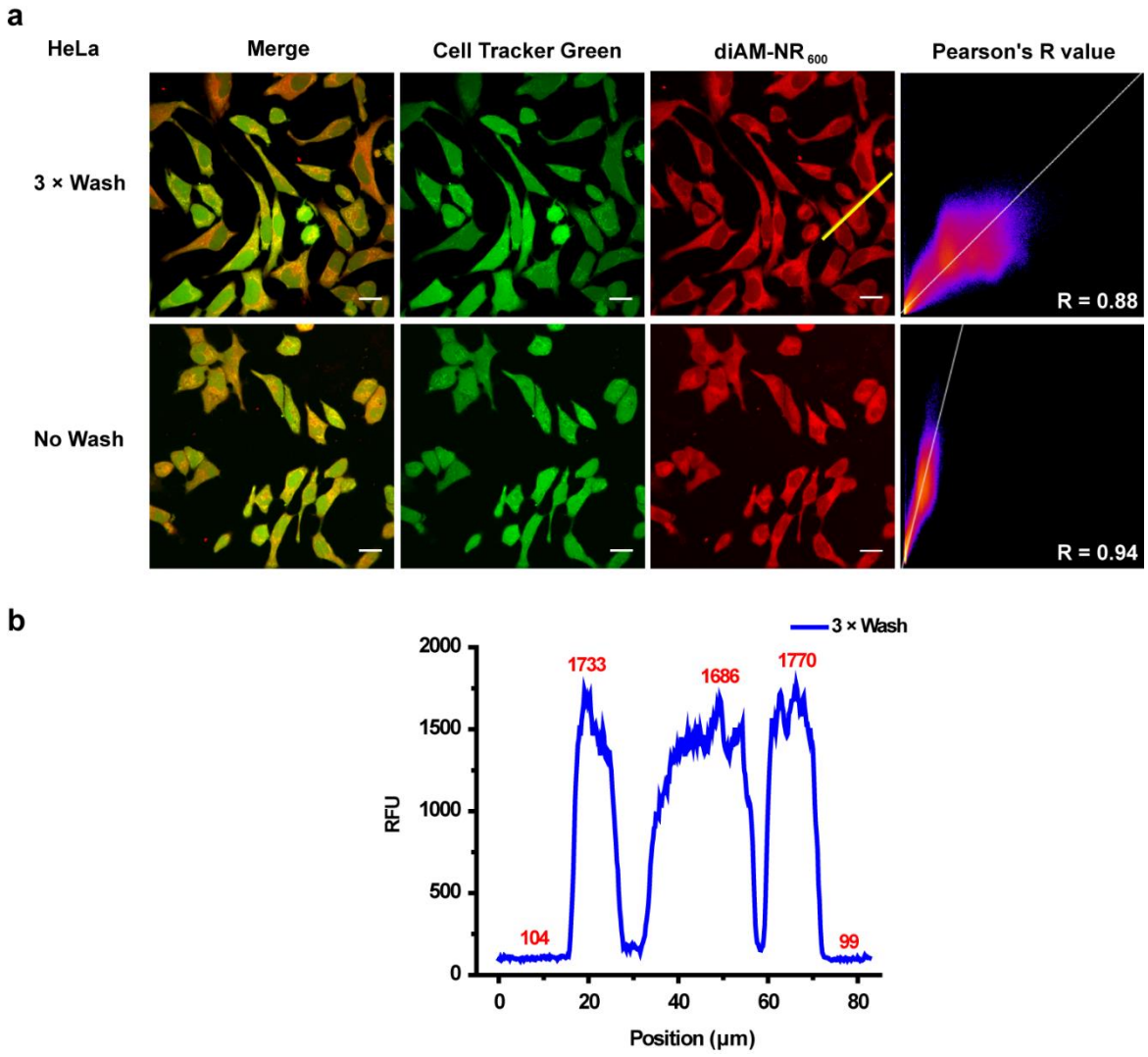
HPLC purification of a reaction containing **NR_{700}** stired in 0.1 M NaOH aqueous solution for 3 h.

Figure S2



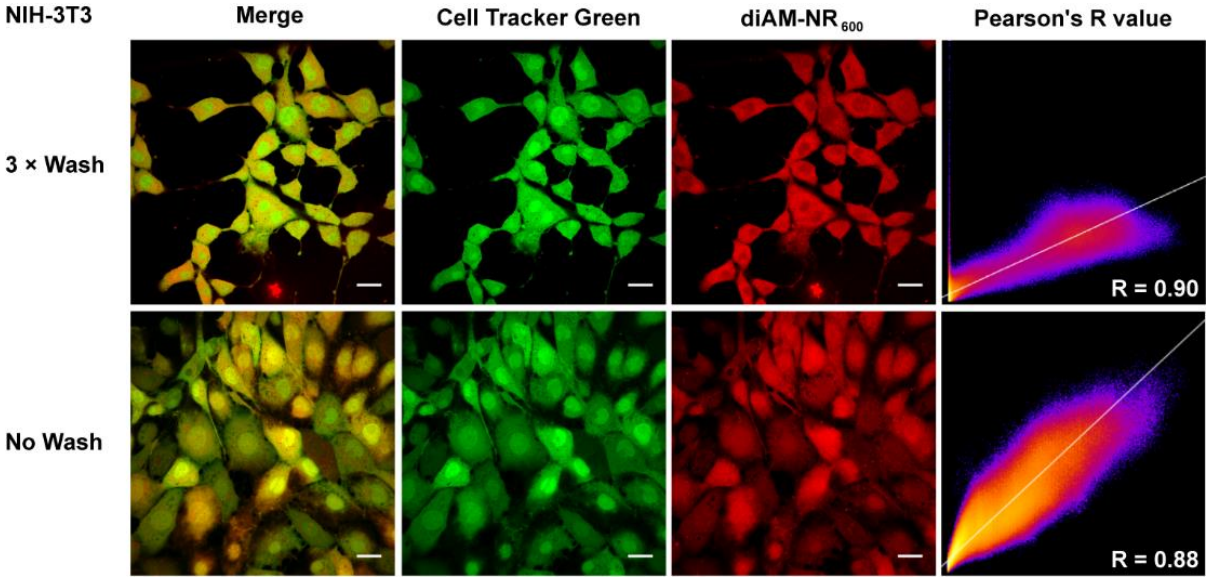
Absorbance of **NR$_{675}$** (a), **NR$_{638}$** (b), **NR$_{604}$** (c), **NR$_{669}$** (d), **NR$_{632}$** (e), **NR$_{600}$** (f) at varying pHs.

Figure S3



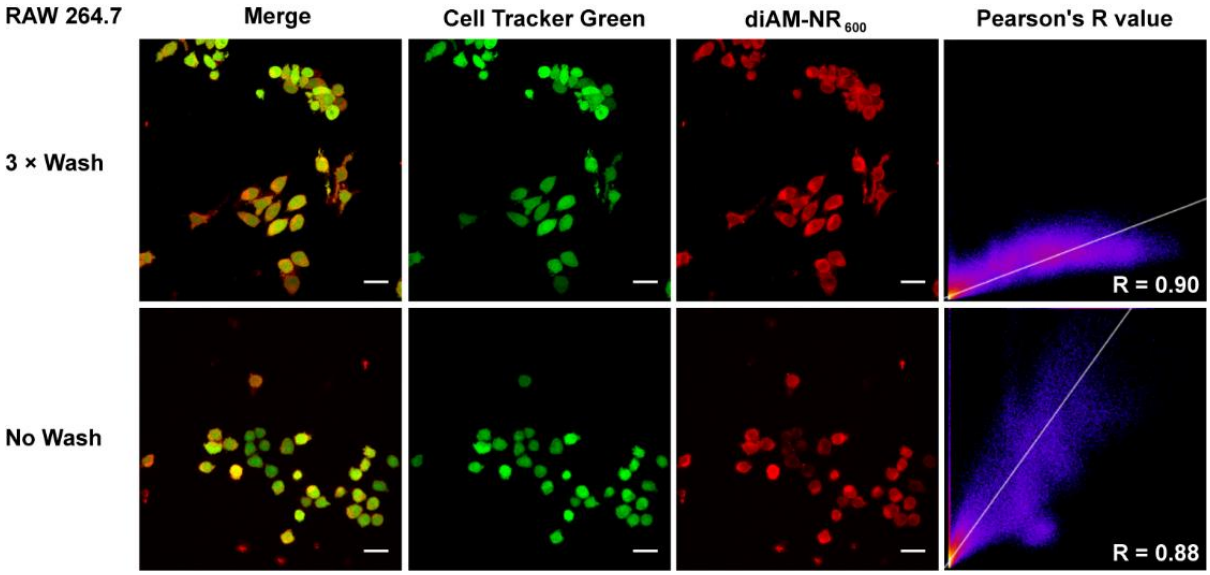
(a) Confocal imaging of HeLa cells incubated with 0.6 μM Cell Tracker Green CMFDA for 20 min followed by 0.6 μM **diAM-NR$_{600}$** for 30 min (Scale bar: 20 μm). (b) Quantification of fluorescence intensity from panel a (yellow line in 3 × wash red channel).

Figure S4



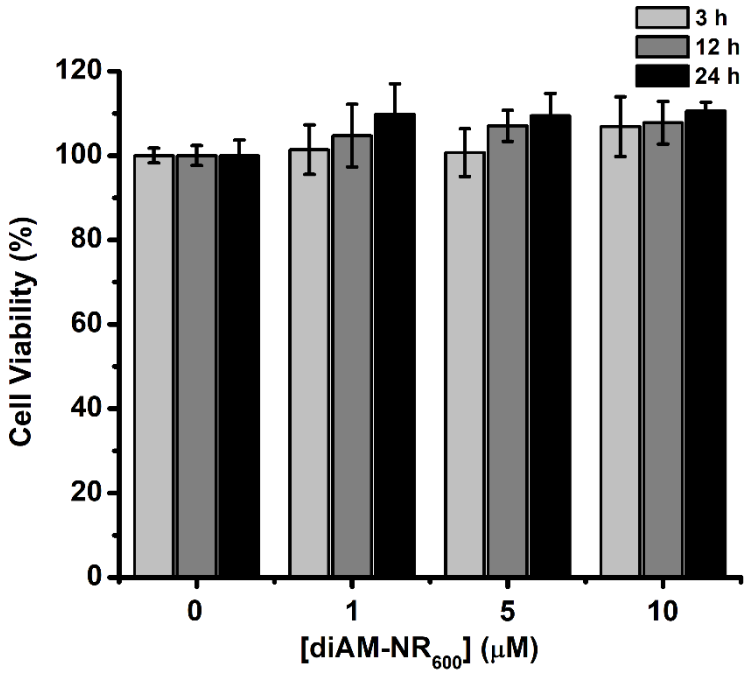
Confocal imaging of NIH-3T3 cells incubated with 0.6 μM Cell Tracker Green CMFDA for 20 min followed by 0.6 μM **diAM-NR$_{600}$** for 30 min (Scale bar: 20 μm).

Figure S5



Confocal imaging of RAW 264.7 cells incubated with 0.6 μM Cell Tracker Green CMFDA for 20 min followed by 0.6 μM **diAM-NR$_{600}$** for 30 min (Scale bar: 20 μm).

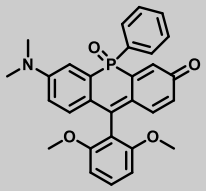
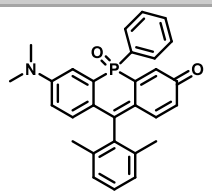
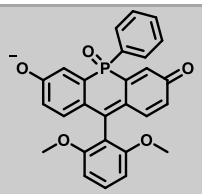
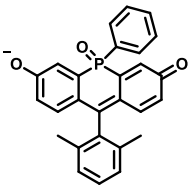
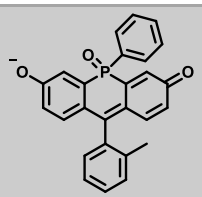
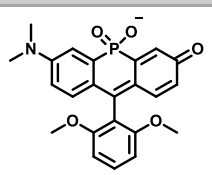
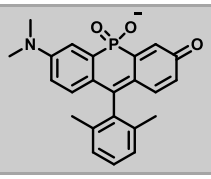
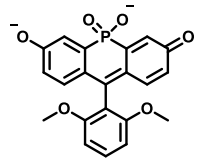
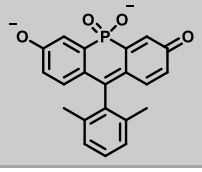
Figure S6



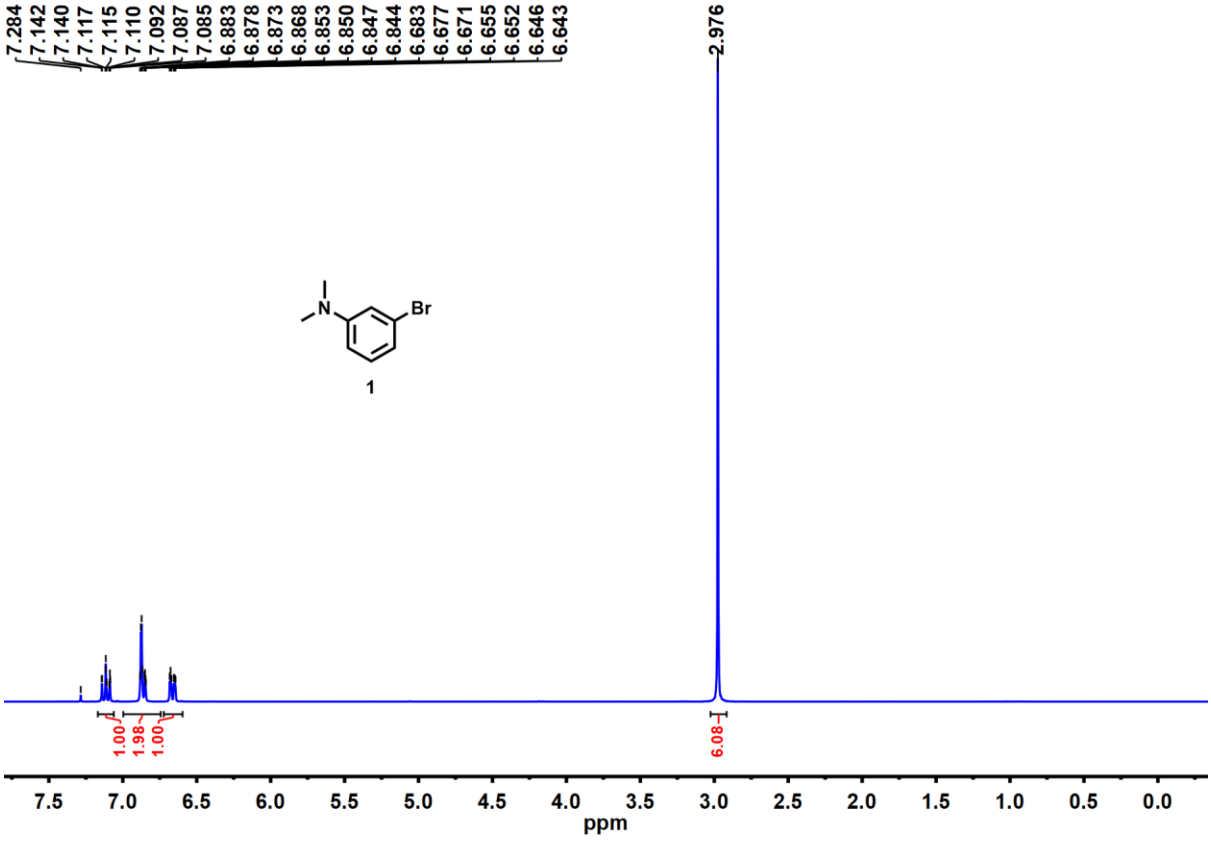
HeLa cells were incubated with the indicated concentration of **diAM-NR$_{600}$** (containing 1% DMSO) for 3, 12, or 24 h, respectively. Cell viability was assessed using the CellTiter-Glo 2.0 Assay (Promega). Error bars represent the standard deviation of triplicate experiments.

Table S1

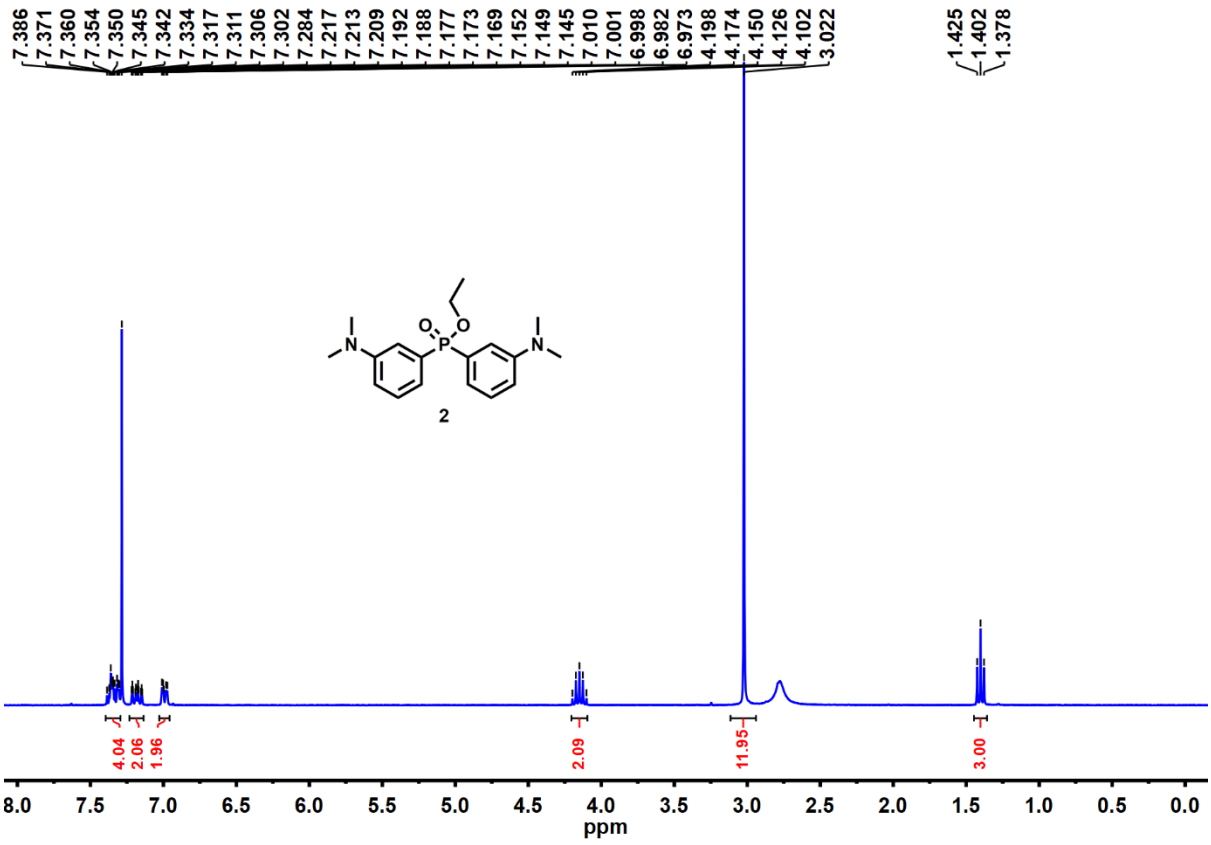
Photophysical properties of phosphine oxide- and phosphinate-containing xanthenes.

Fluorophores	E_x (nm)	E_m (nm)	ε ($M^{-1}\cdot cm^{-1}$)	Φ	ε·Φ ($M^{-1}\cdot cm^{-1}$)	Ref.
	670	698	66,100	0.11	7,271	5
	666	692	49,900	0.11	5,489	5
	632	661	54,500	0.24	13,080	5
	628	656	53,900	0.32	17,248	5
	627	656	51,300	0.33	16,929	6
	638	662	55,630	0.21	11,682	This work
	632	655	63,970	0.26	16,632	This work
	604	627	45,740	0.48	21,955	This work
	600	619	67,570	0.68	45,948	This work

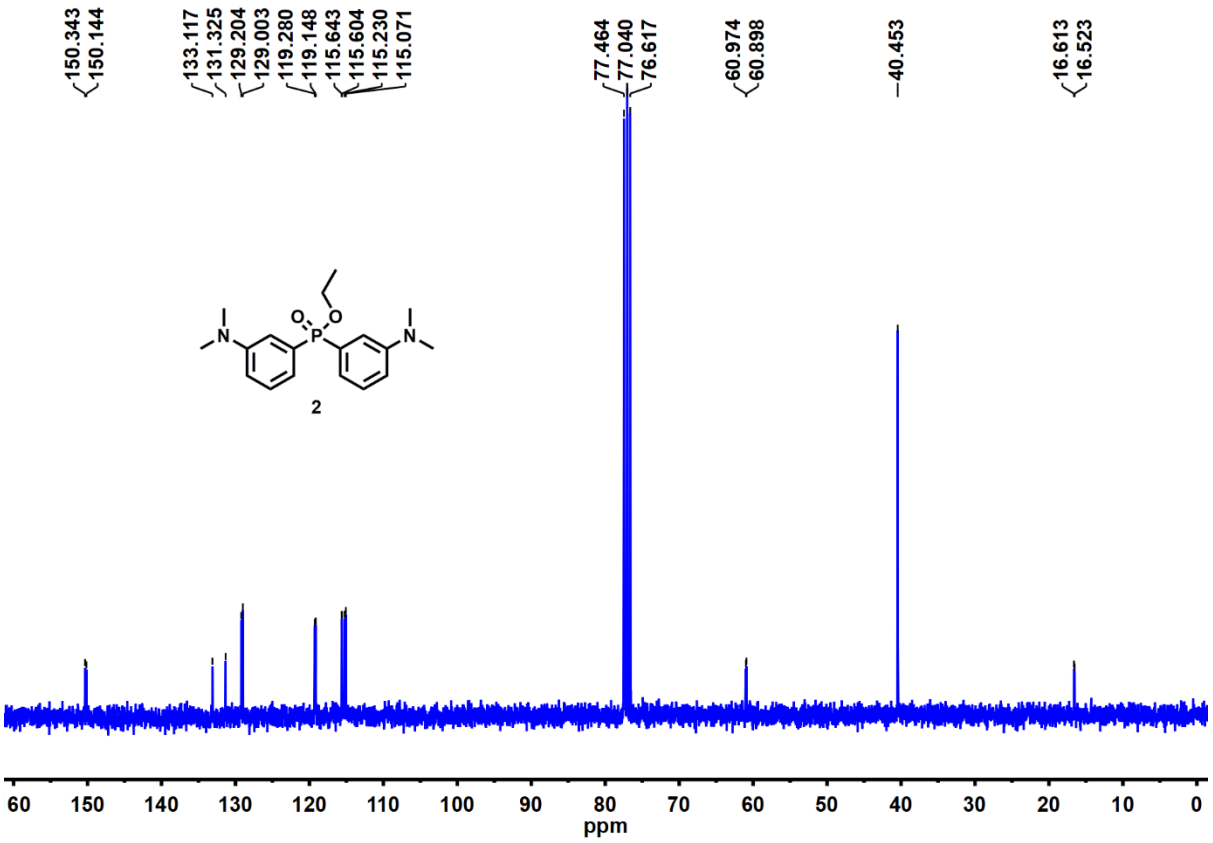
Copies of ^{1}H, ^{13}C, and ^{31}P NMR Spectra



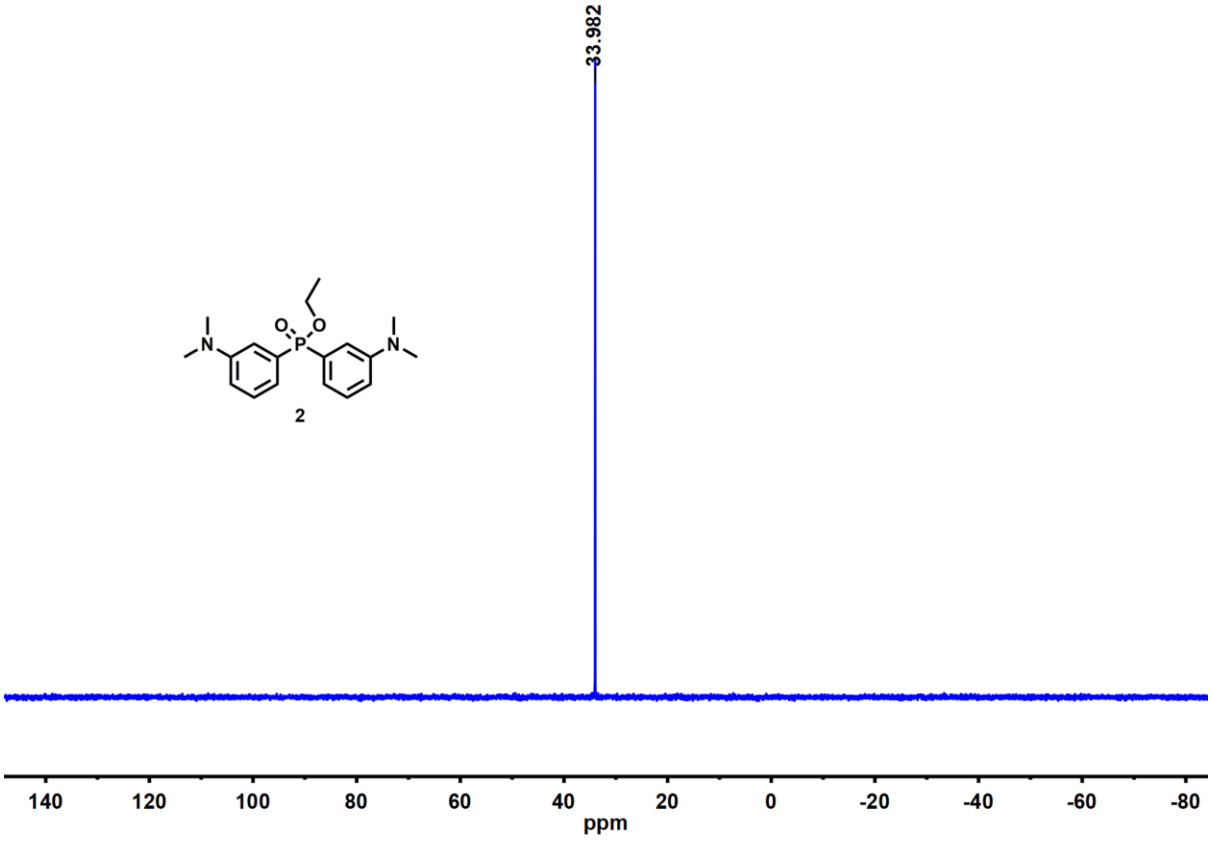
^{1}H-NMR of **3-bromo-*N,N*-dimethylaniline (1)** in $CDCl_3$ (300 MHz)



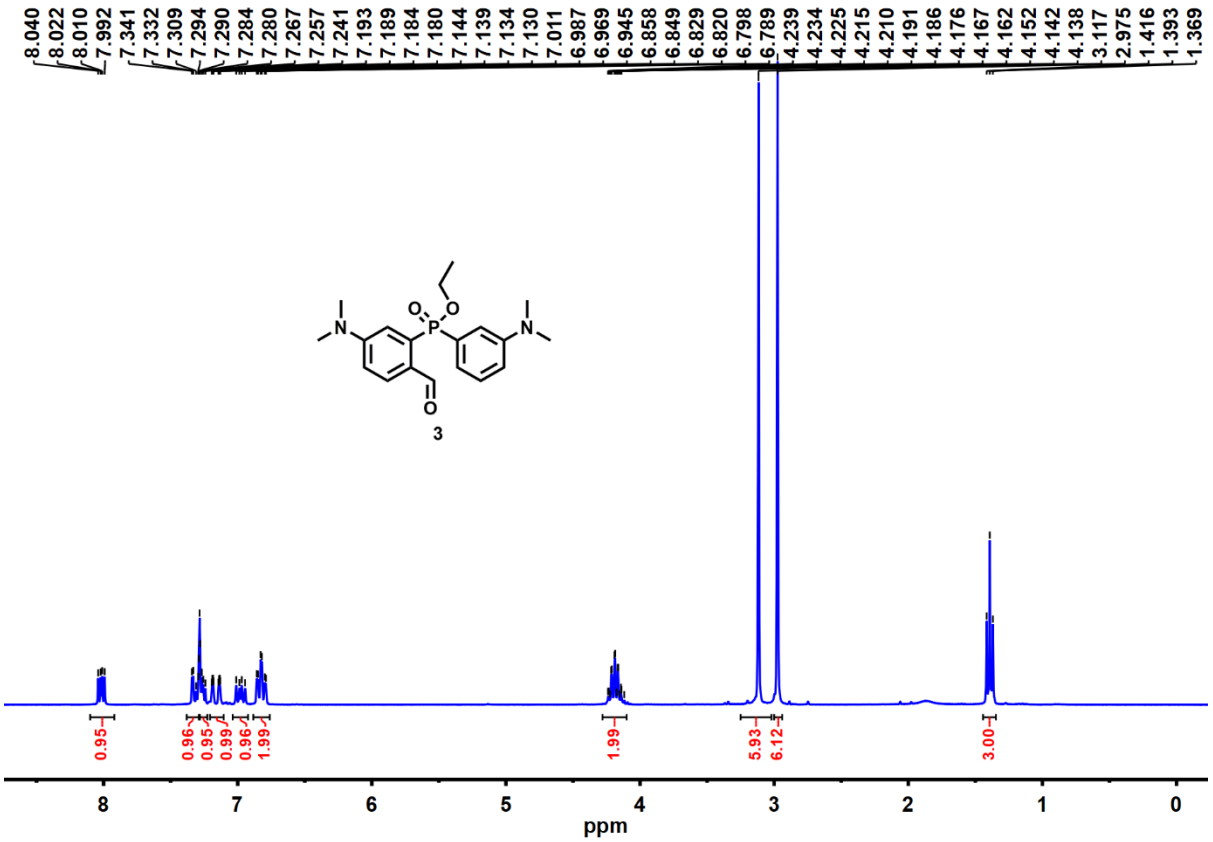
^{1}H-NMR of **ethyl bis(3-(dimethylamino)phenyl)phosphinate (2)** in $CDCl_3$ (300 MHz)



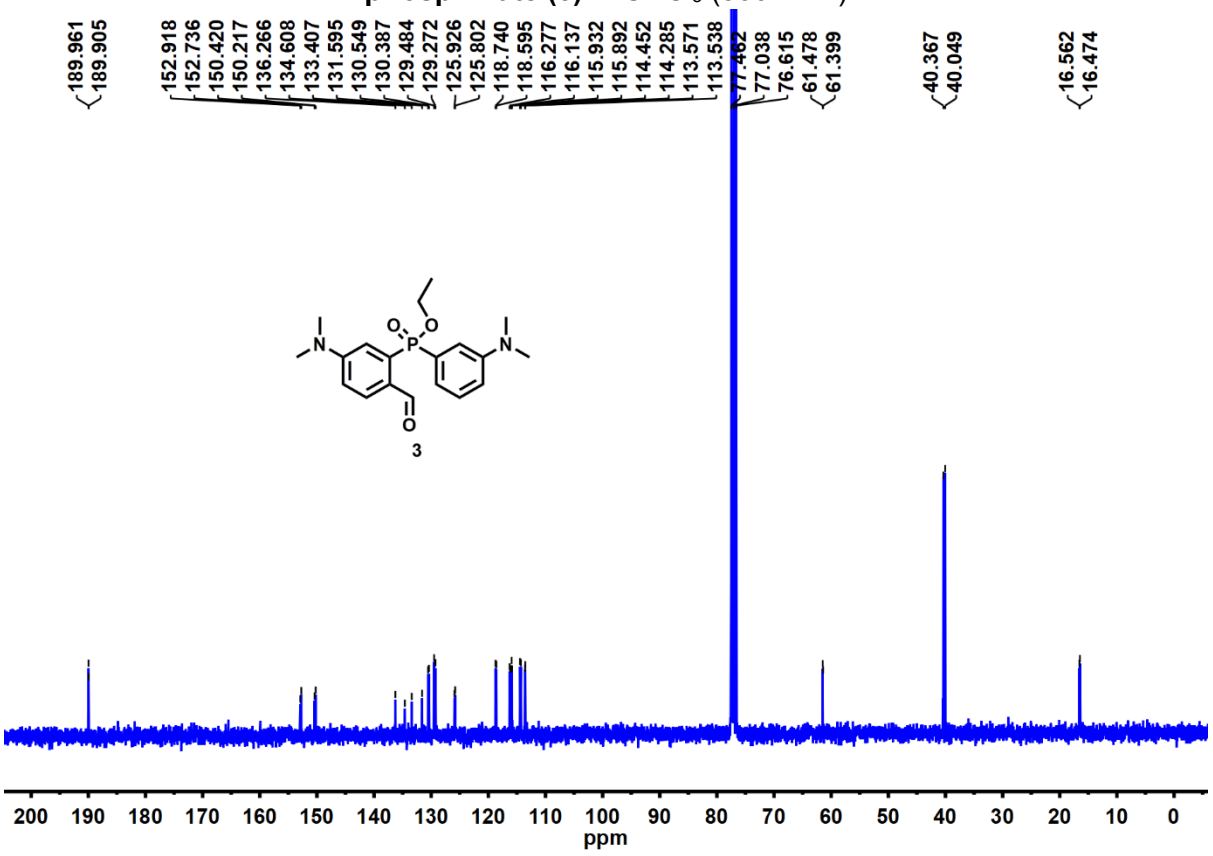
^{13}C-NMR of **ethyl bis(3-(dimethylamino)phenyl)phosphinate (2)** in $CDCl_3$ (75 MHz)



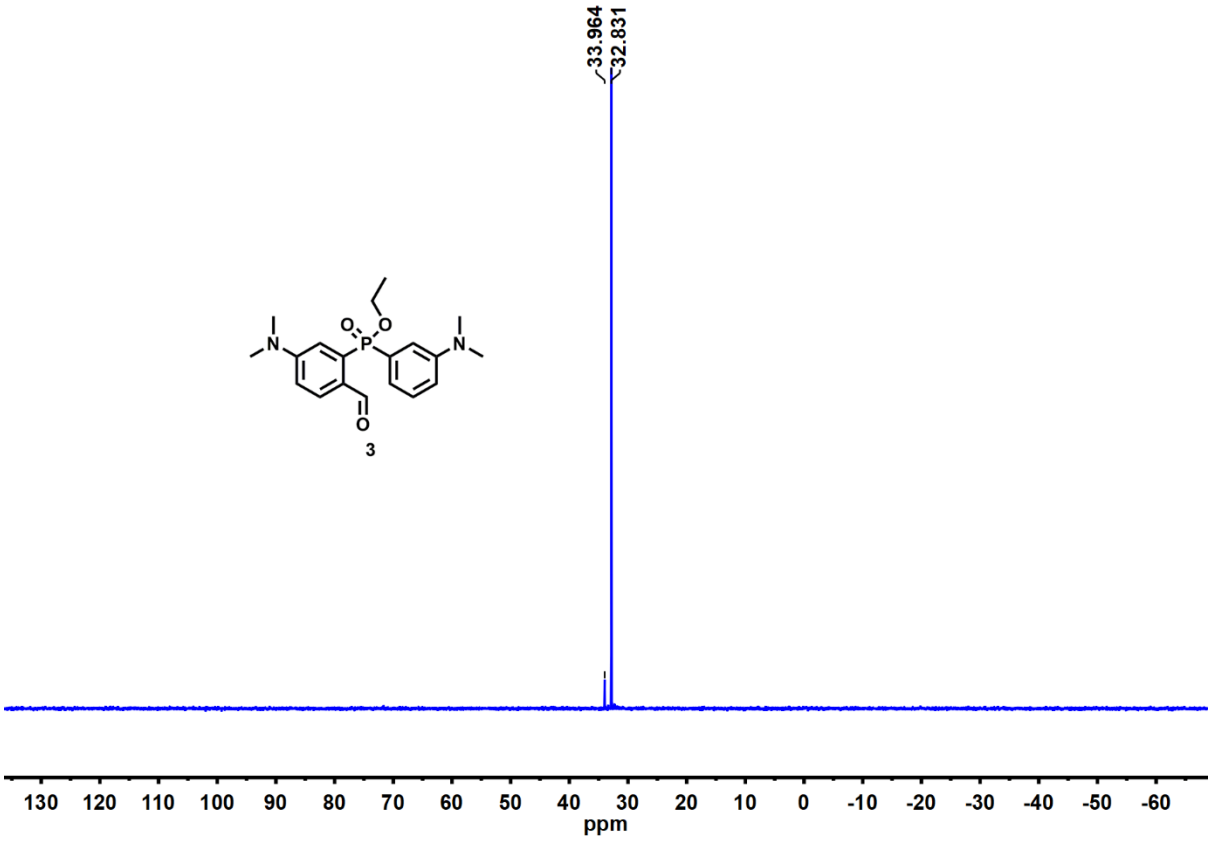
^{31}P-NMR of **ethyl bis(3-(dimethylamino)phenyl)phosphinate (2)** in $CDCl_3$ (121 MHz)



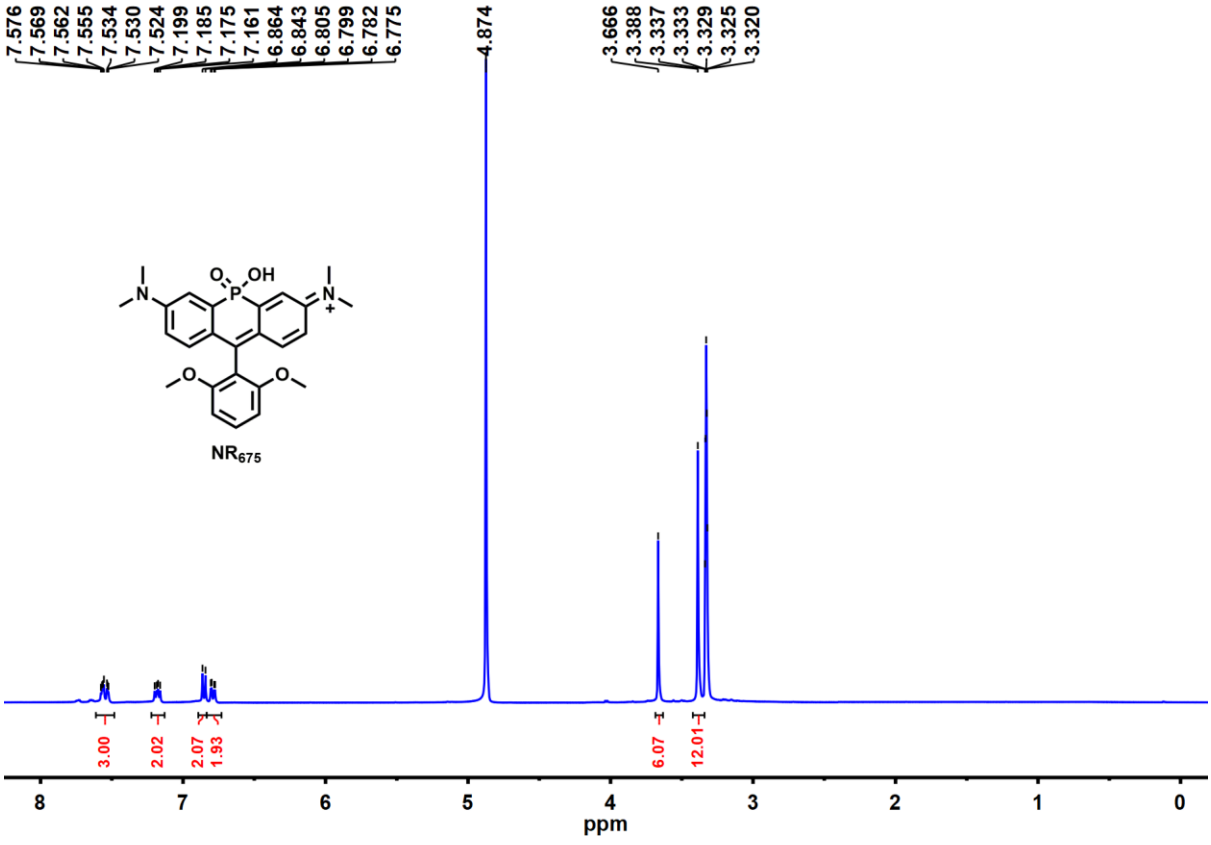
^{1}H-NMR of **ethyl (5-(dimethylamino)-2-formylphenyl)(3-(dimethylamino)phenyl) phosphinate (3)** in $CDCl_3$ (300 MHz)



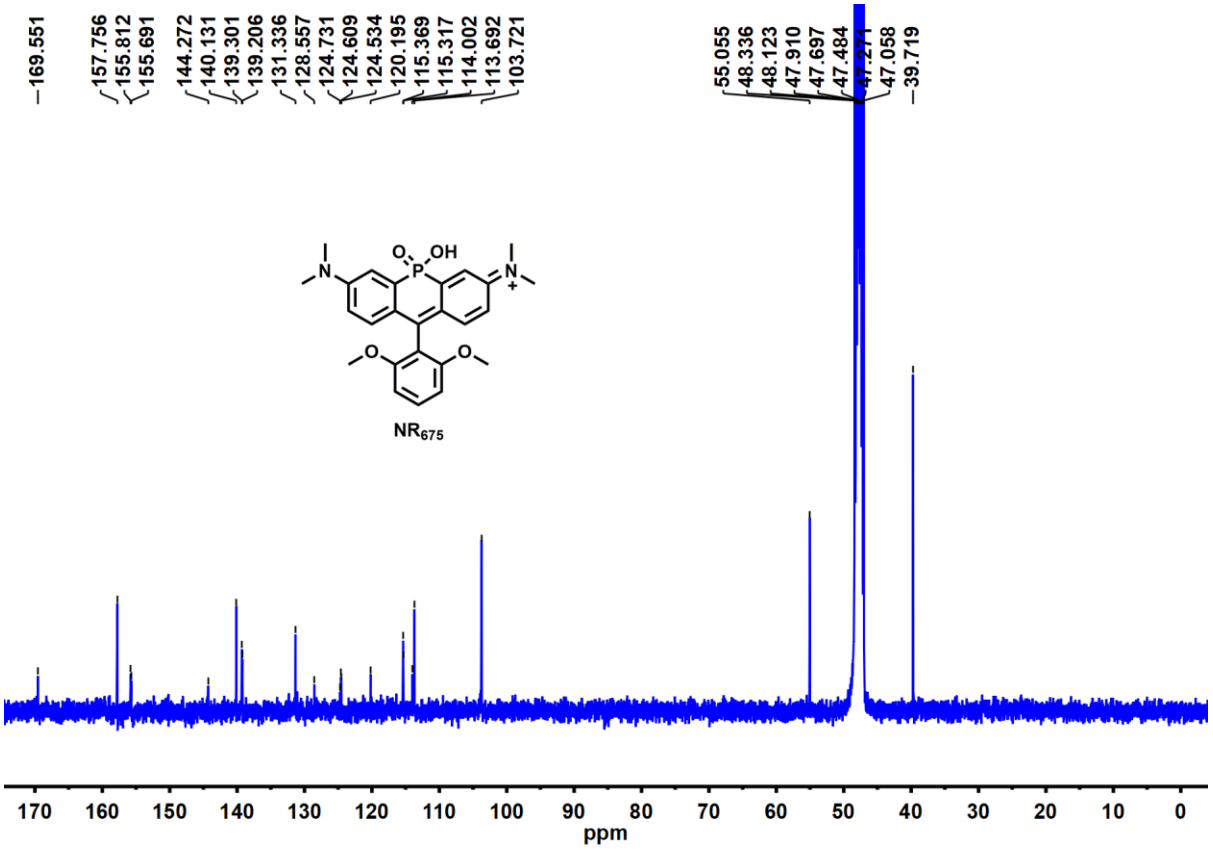
^{13}C-NMR of **ethyl (5-(dimethylamino)-2-formylphenyl)(3-(dimethylamino)phenyl) phosphinate (3)** in $CDCl_3$ (75 MHz)



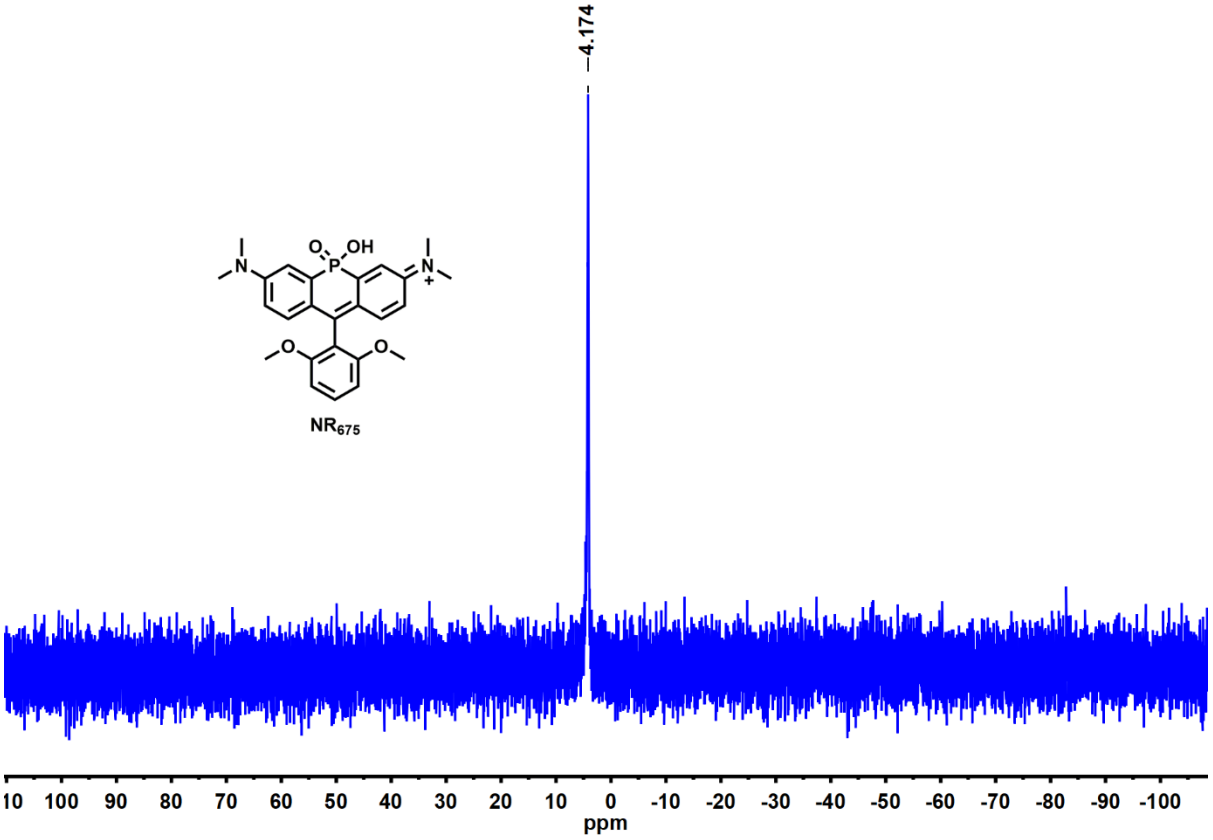
^{31}P-NMR of **ethyl (5-(dimethylamino)-2-formylphenyl)(3-(dimethylamino)phenyl) phosphinate (3)** in $CDCl_3$ (121 MHz)



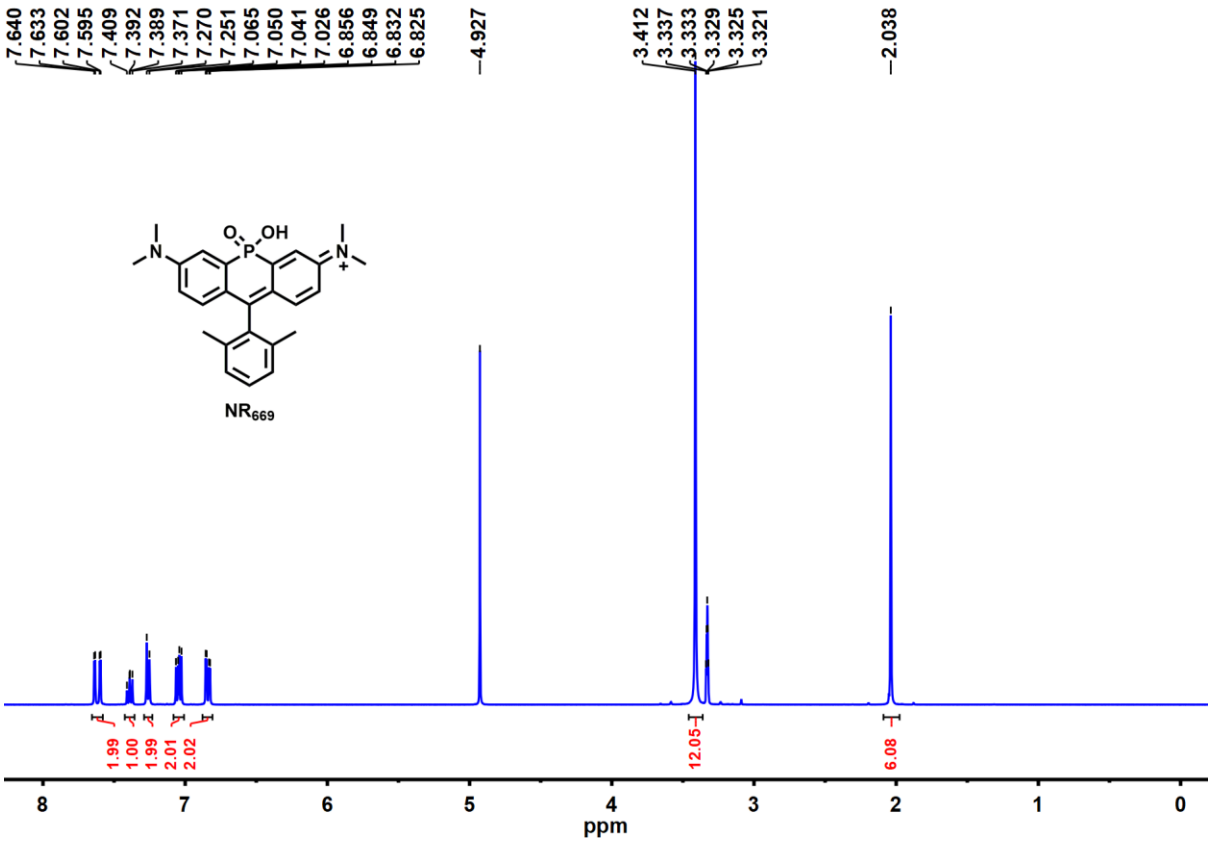
^{1}H-NMR of **NR_{675}** in CD_3OD (400 MHz)



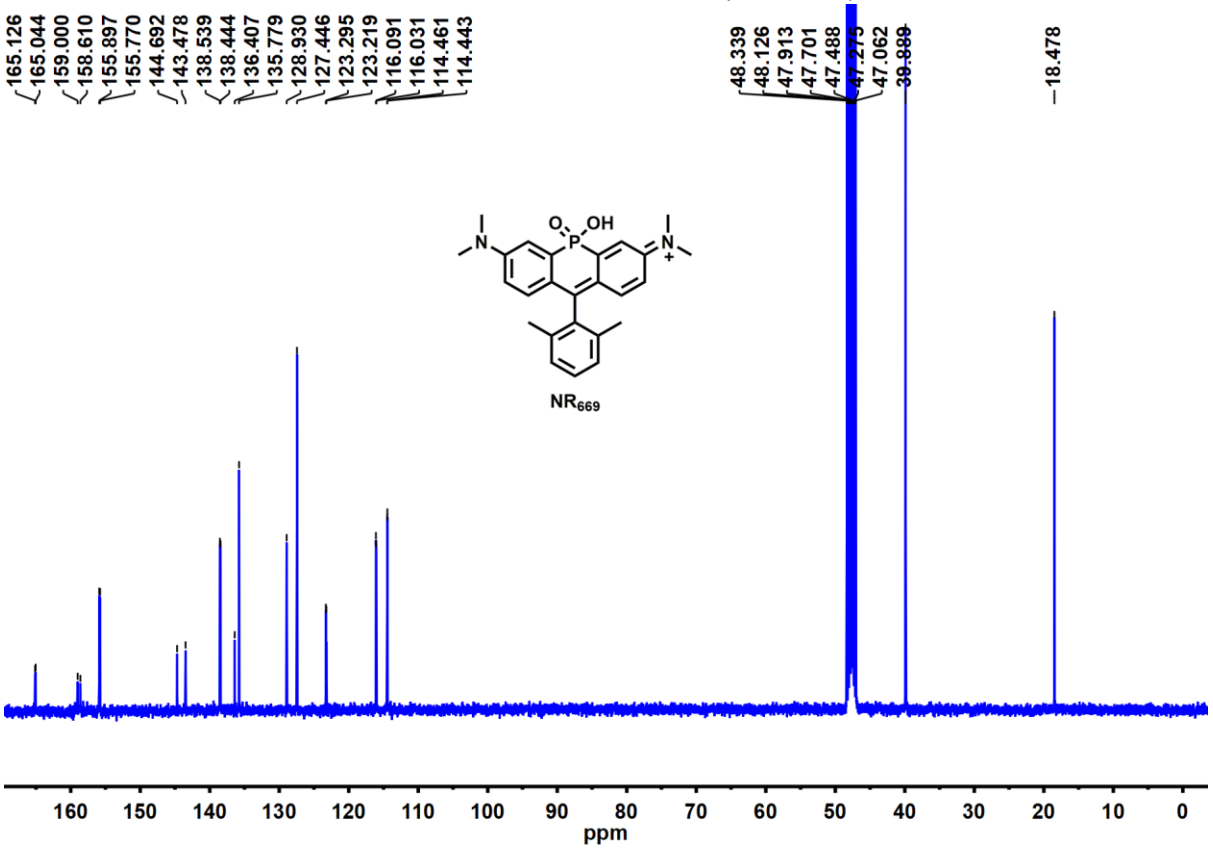
^{13}C-NMR of **NR$_{675}$** in CD_3OD (101 MHz)



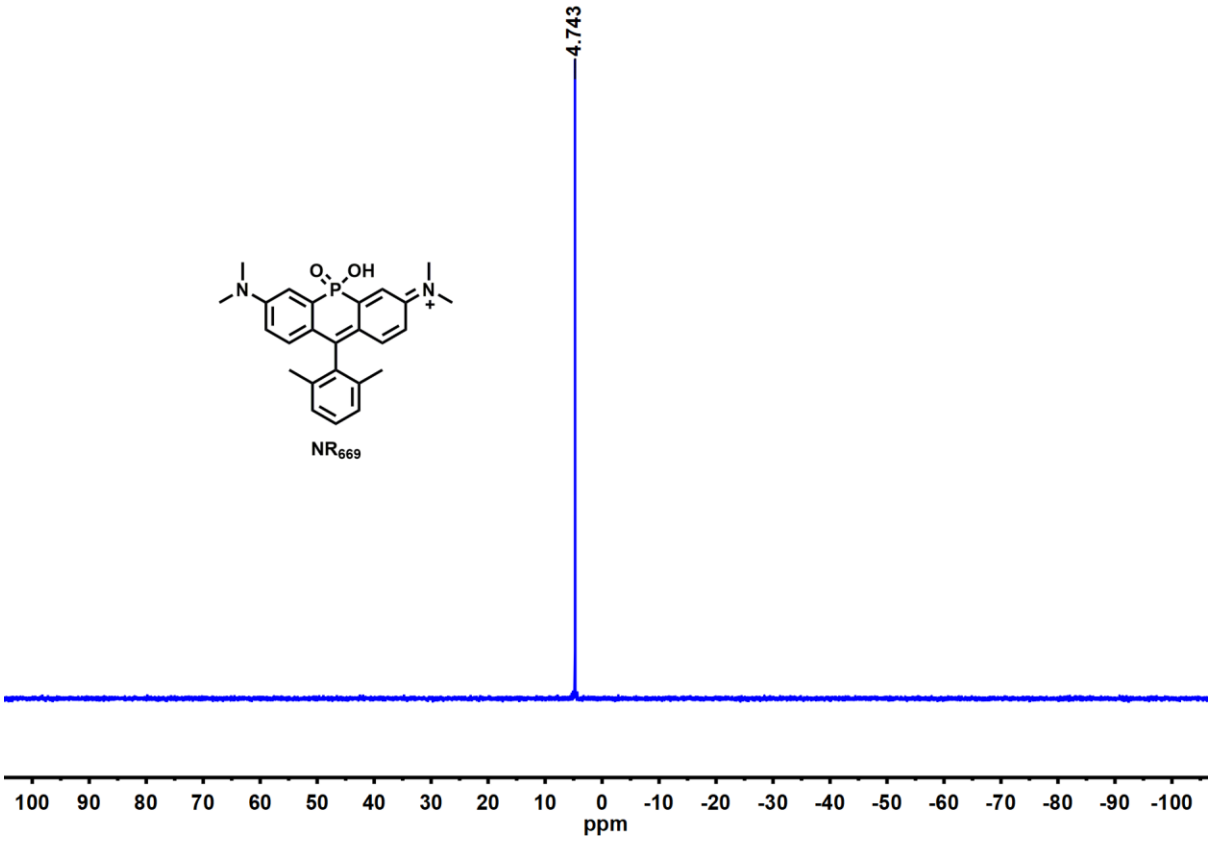
^{31}P-NMR of **NR$_{675}$** in CD_3OD (162 MHz)



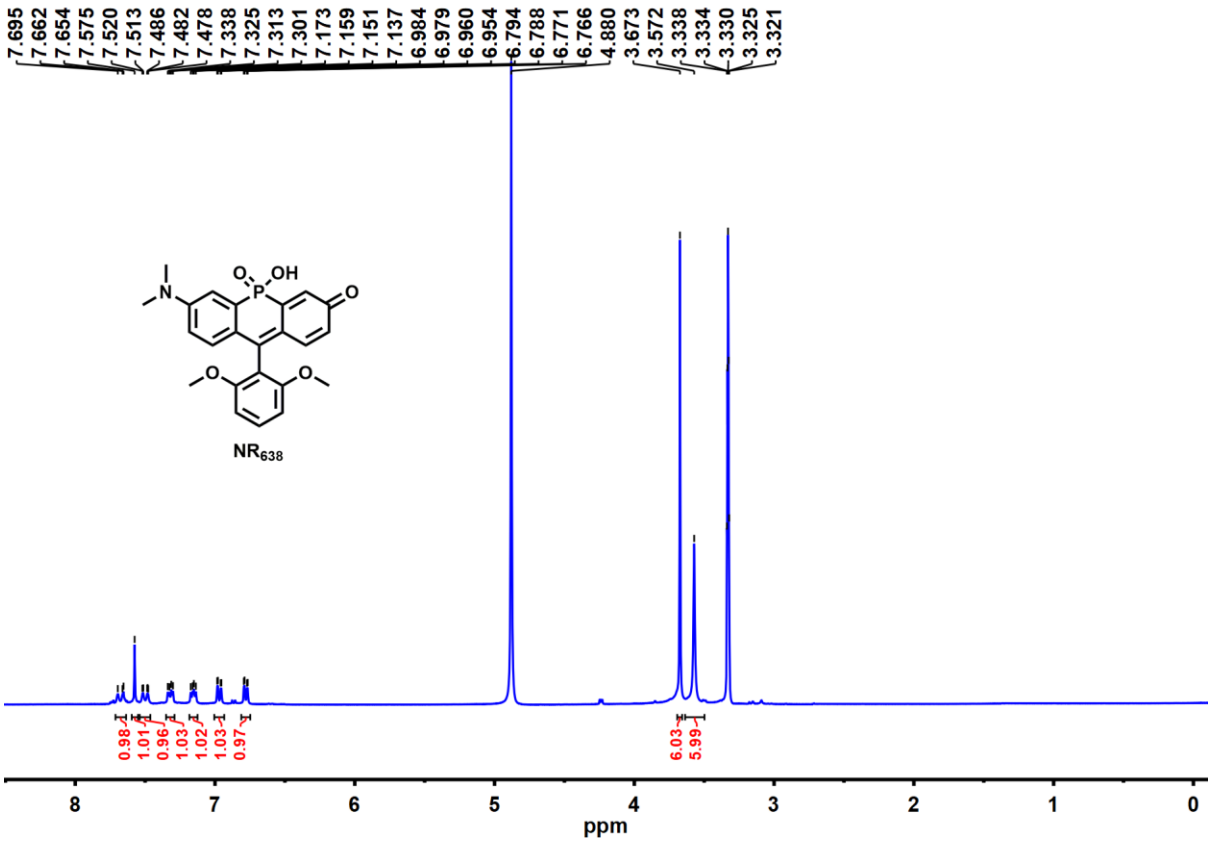
^{1}H-NMR of **NR$_{669}$** in CD_3OD (400 MHz)



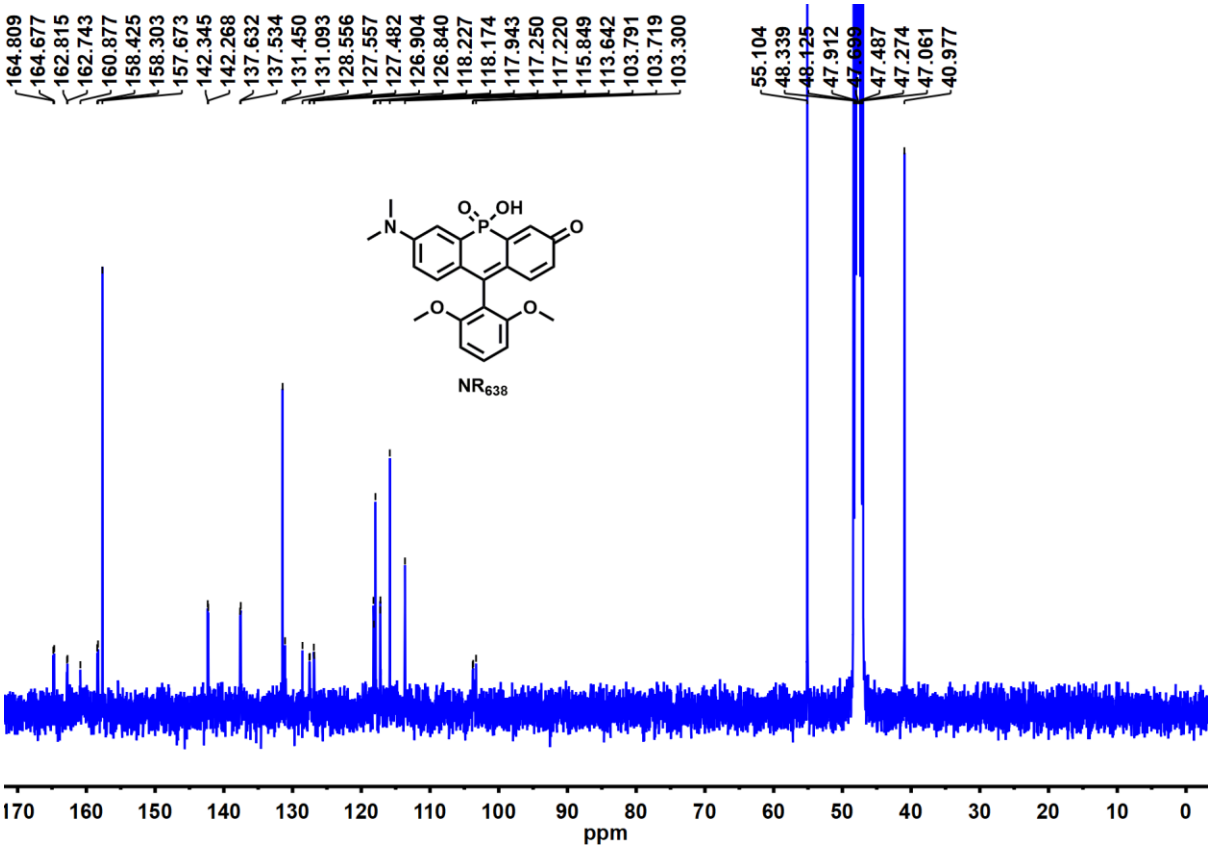
^{13}C-NMR of **NR$_{669}$** in CD_3OD (101 MHz)



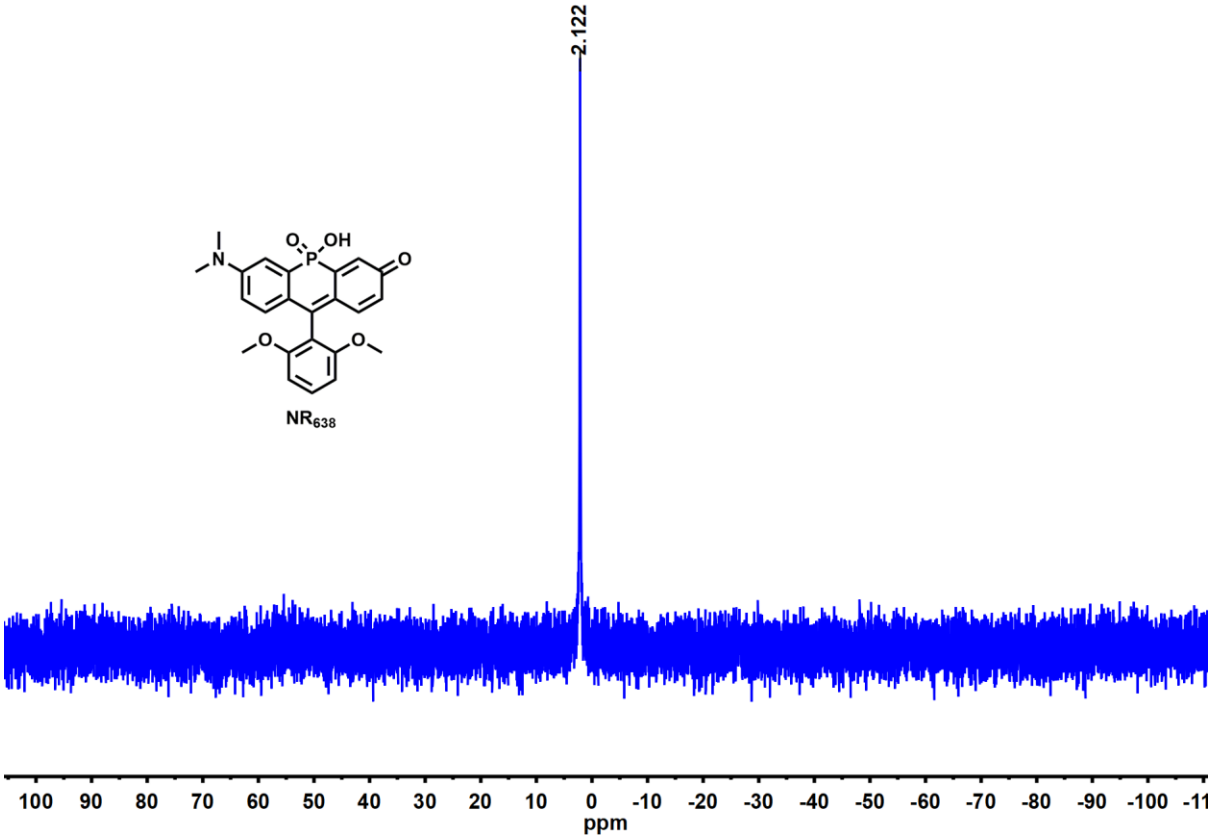
^{31}P-NMR of **NR$_{669}$** in CD_3OD (162 MHz)



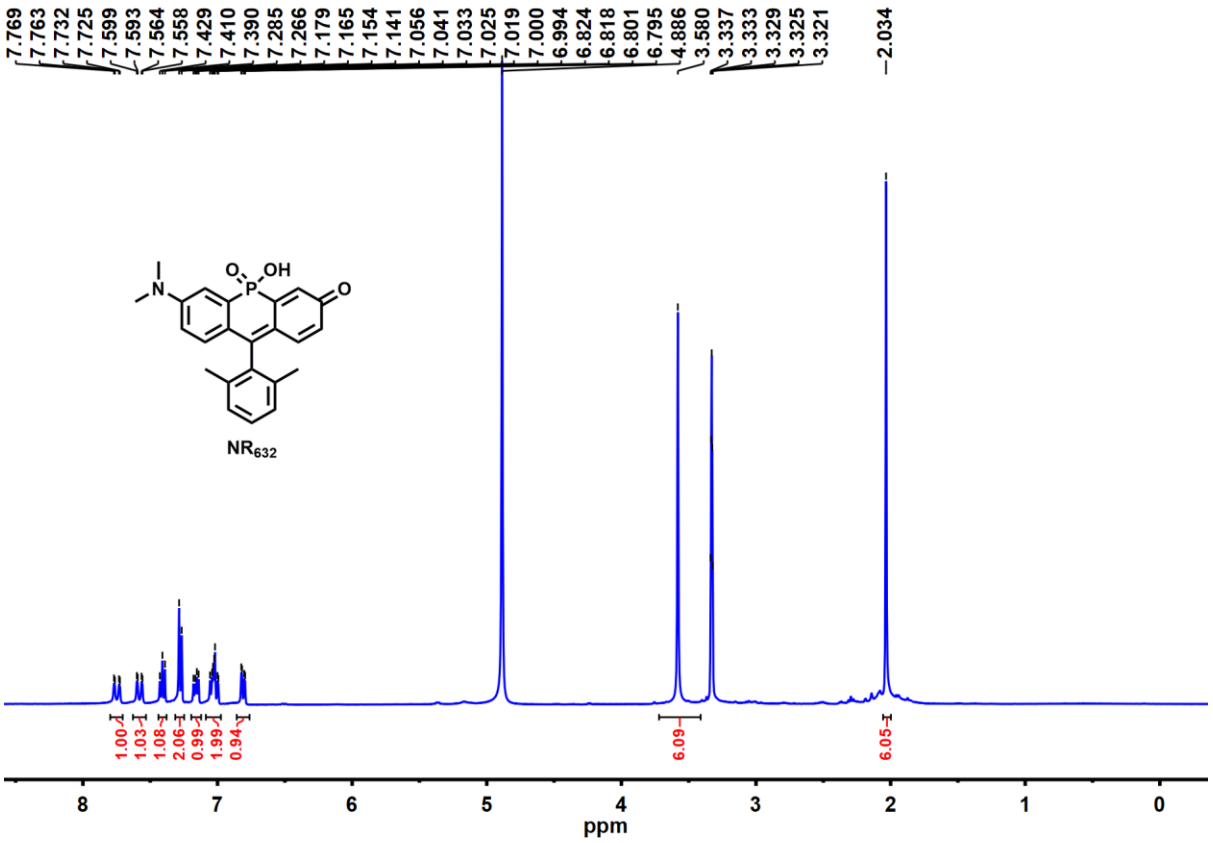
^{1}H-NMR of **NR$_{638}$** in CD_3OD (400 MHz)



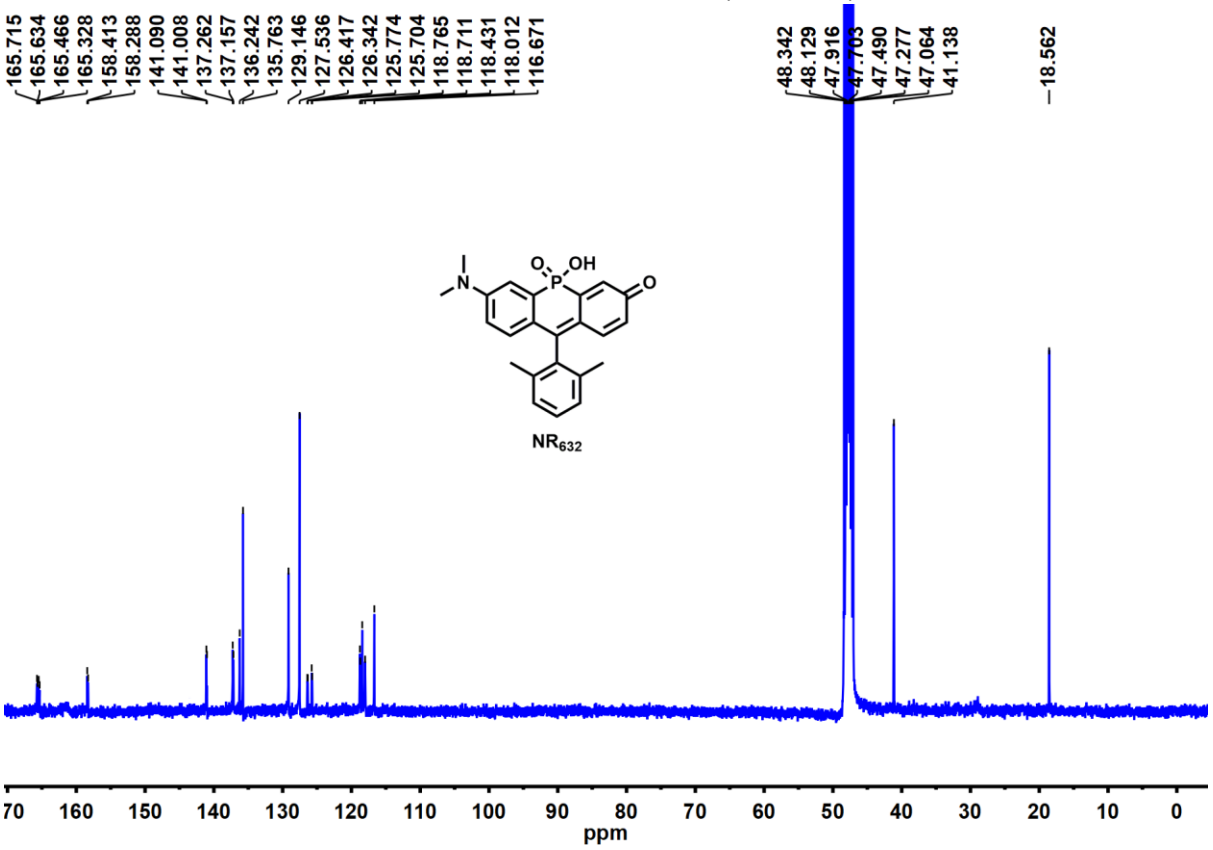
^{13}C-NMR of **NR$_{638}$** in CD_3OD (101 MHz)



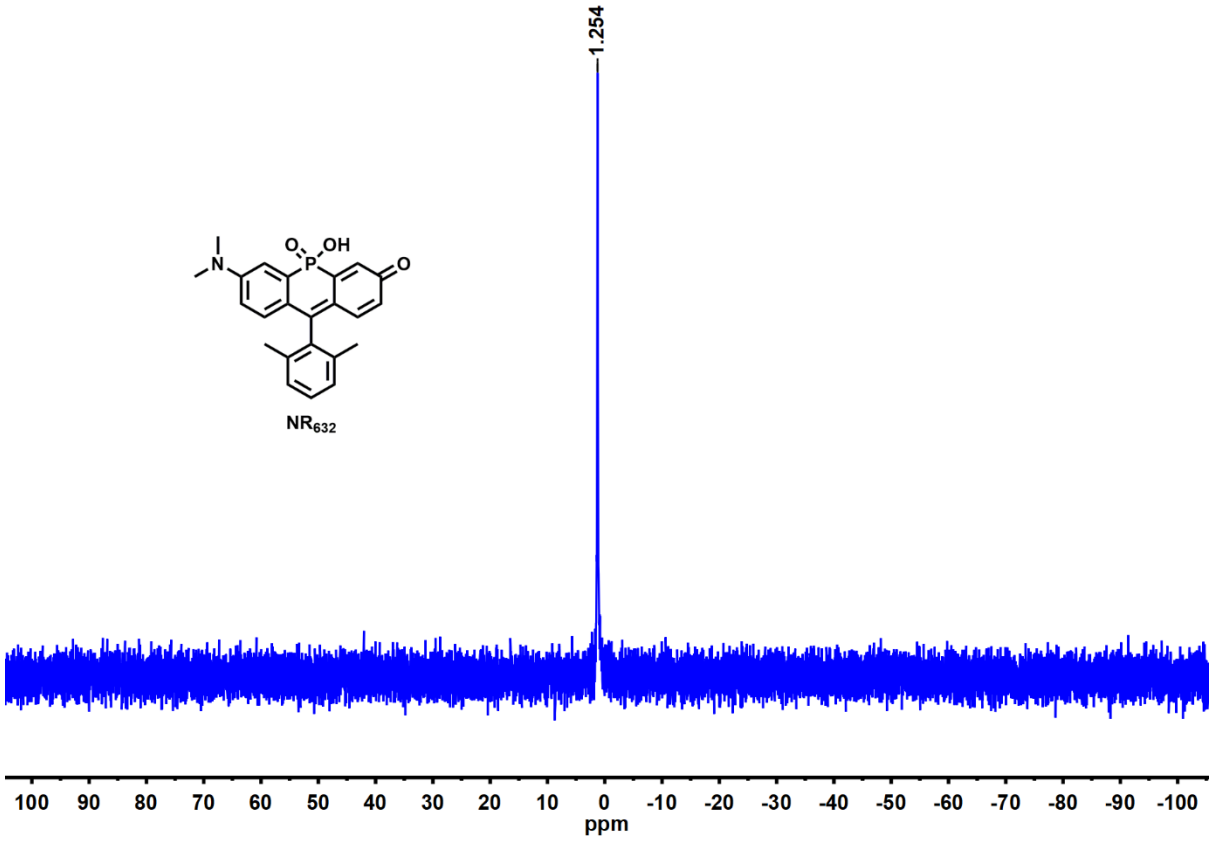
^{31}P-NMR of **NR$_{638}$** in CD_3OD (162 MHz)



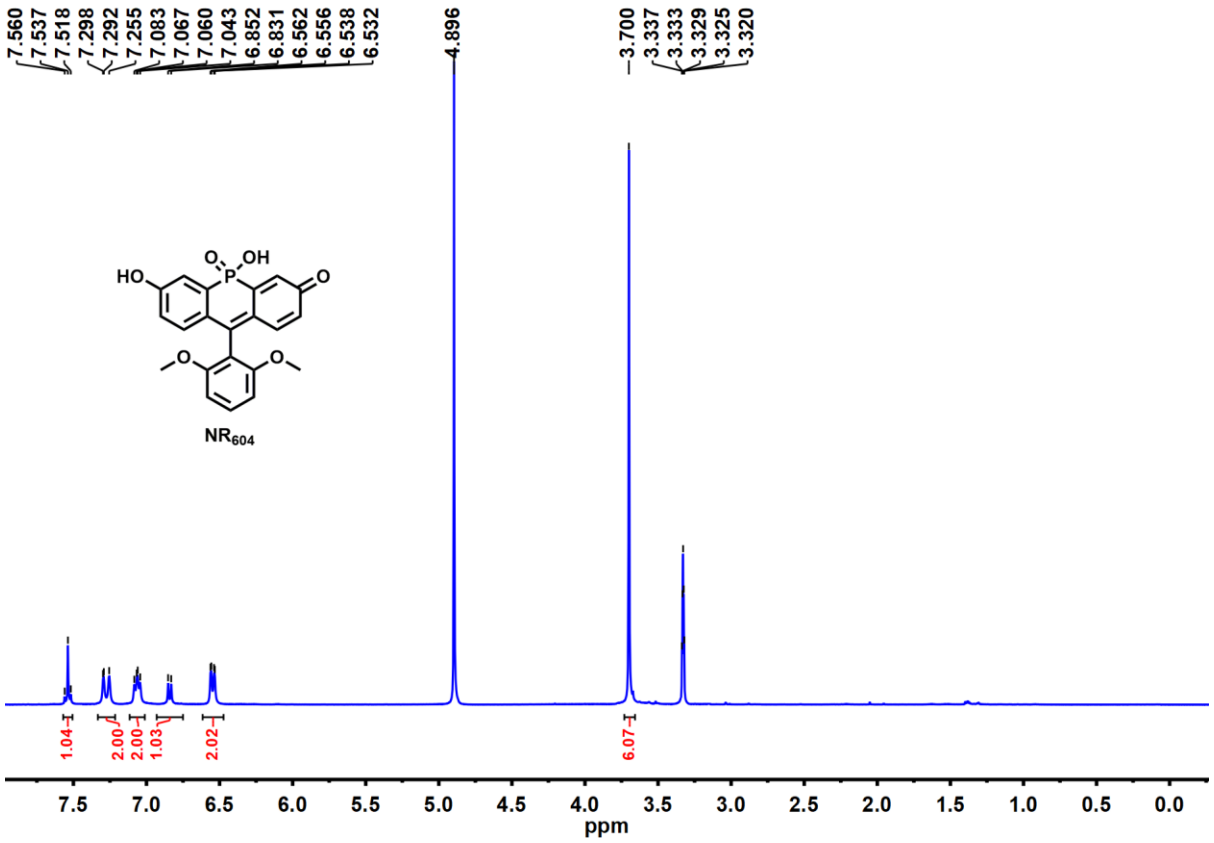
^{1}H-NMR of **NR$_{632}$** in CD_3OD (400 MHz)



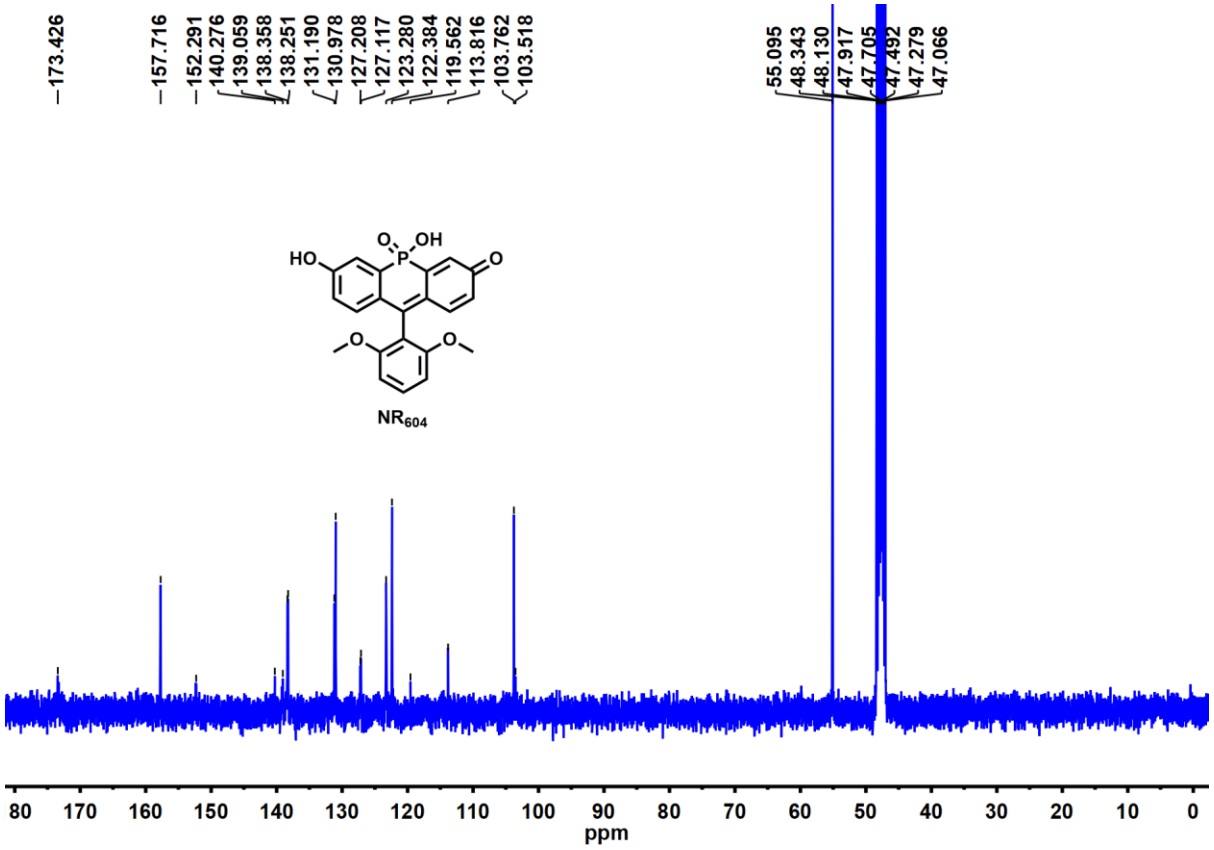
^{13}C-NMR of **NR$_{632}$** in CD_3OD (101 MHz)



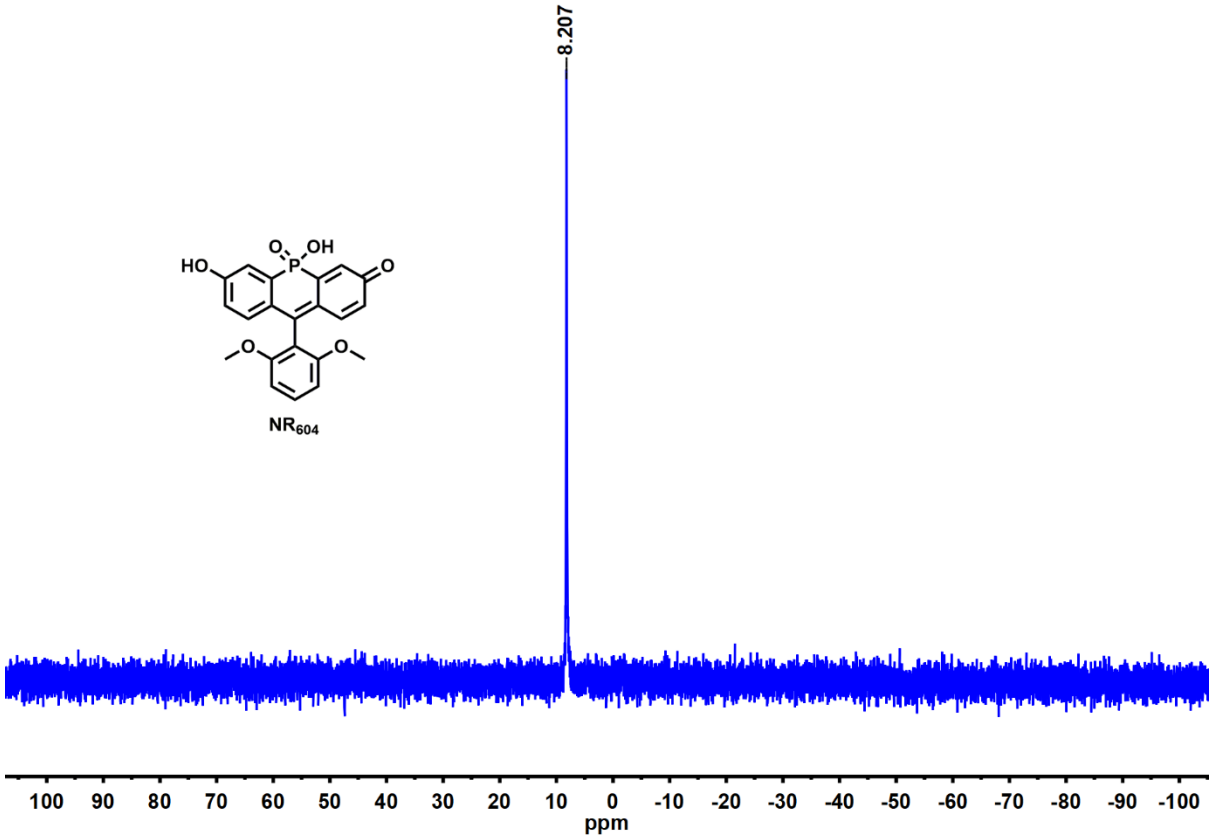
^{31}P-NMR of **NR$_{632}$** in CD_3OD (162 MHz)



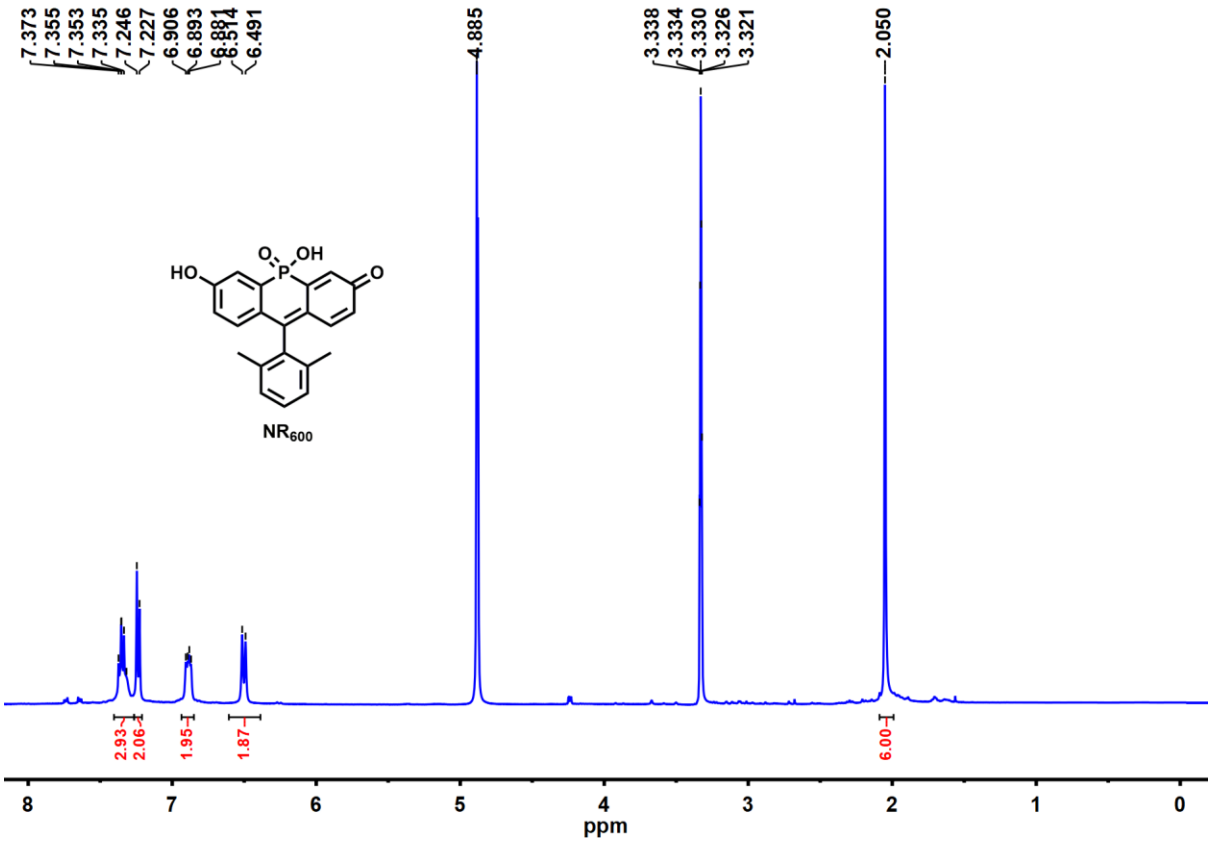
^{1}H-NMR of **NR$_{604}$** in CD_3OD (400 MHz)



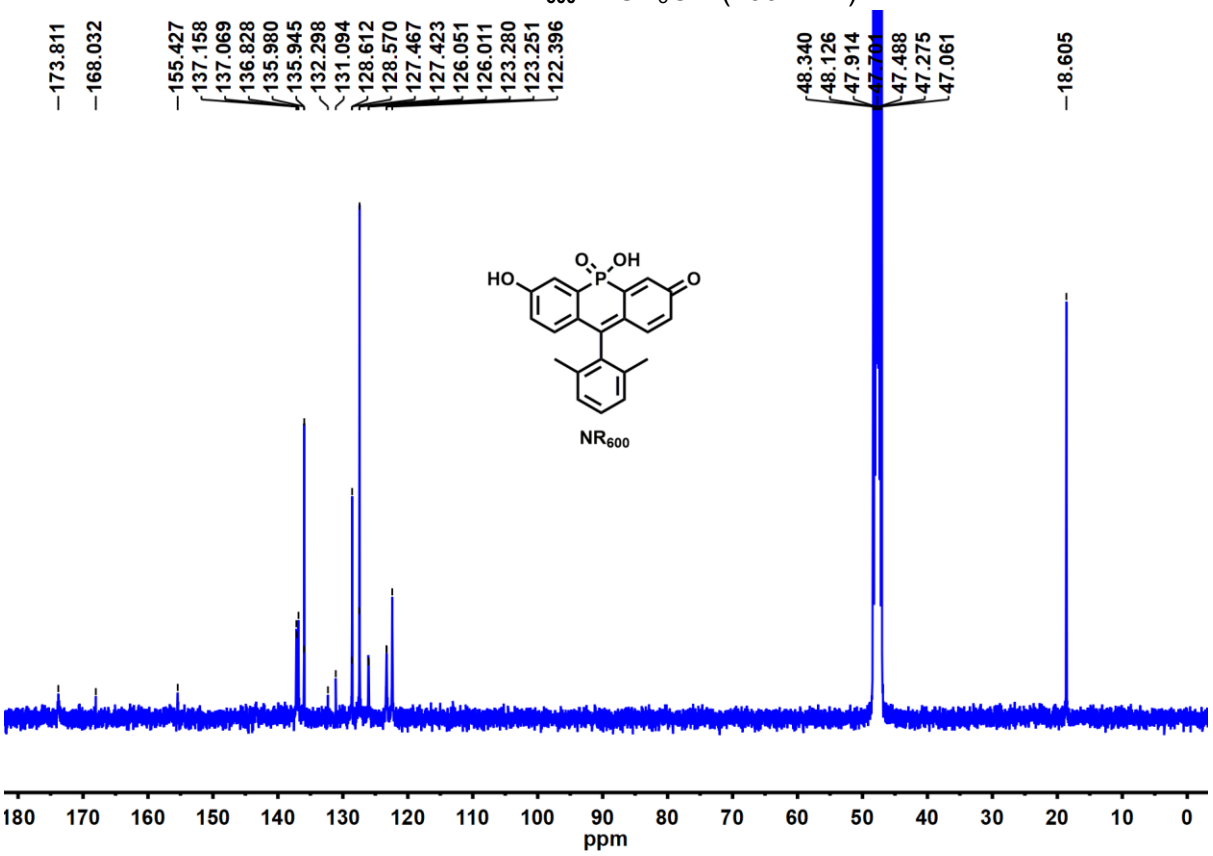
^{13}C-NMR of **NR$_{604}$** in CD_3OD (101 MHz)



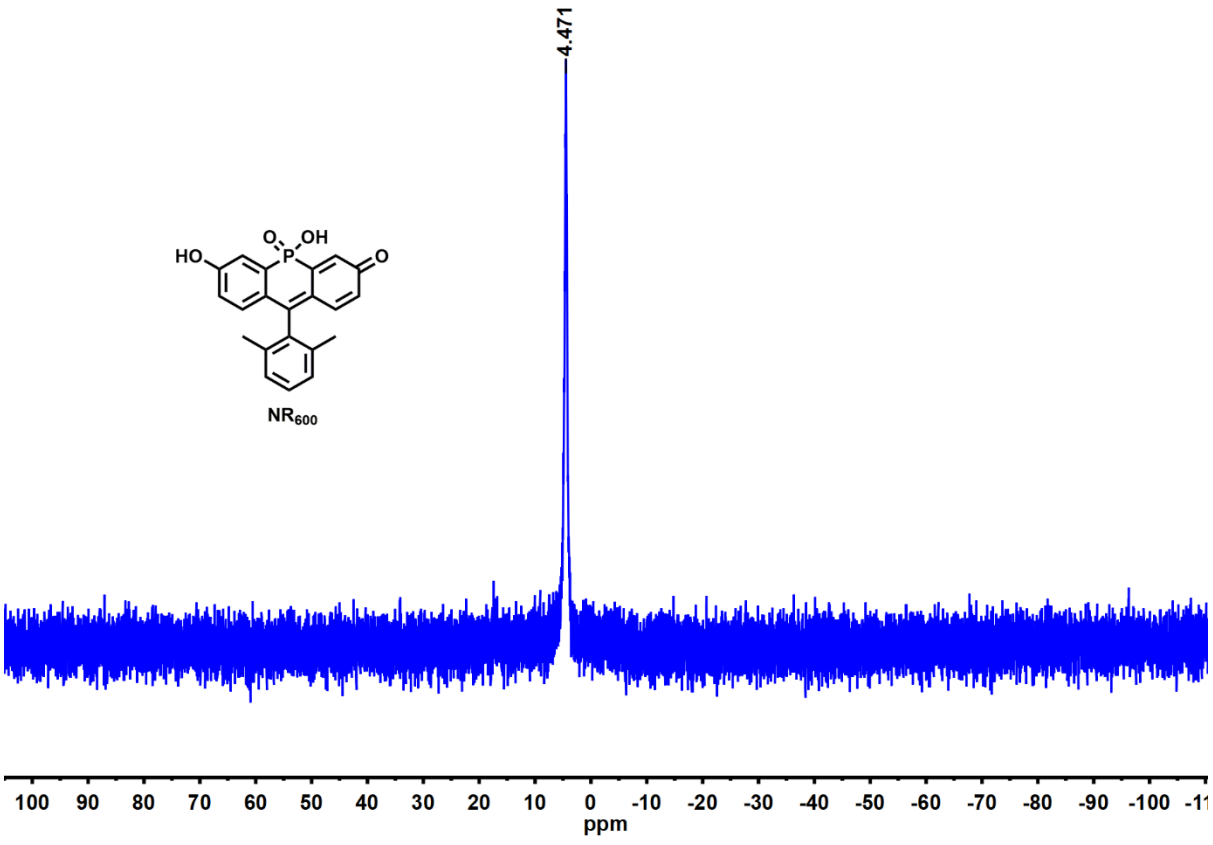
^{31}P-NMR of **NR$_{604}$** in CD_3OD (162 MHz)



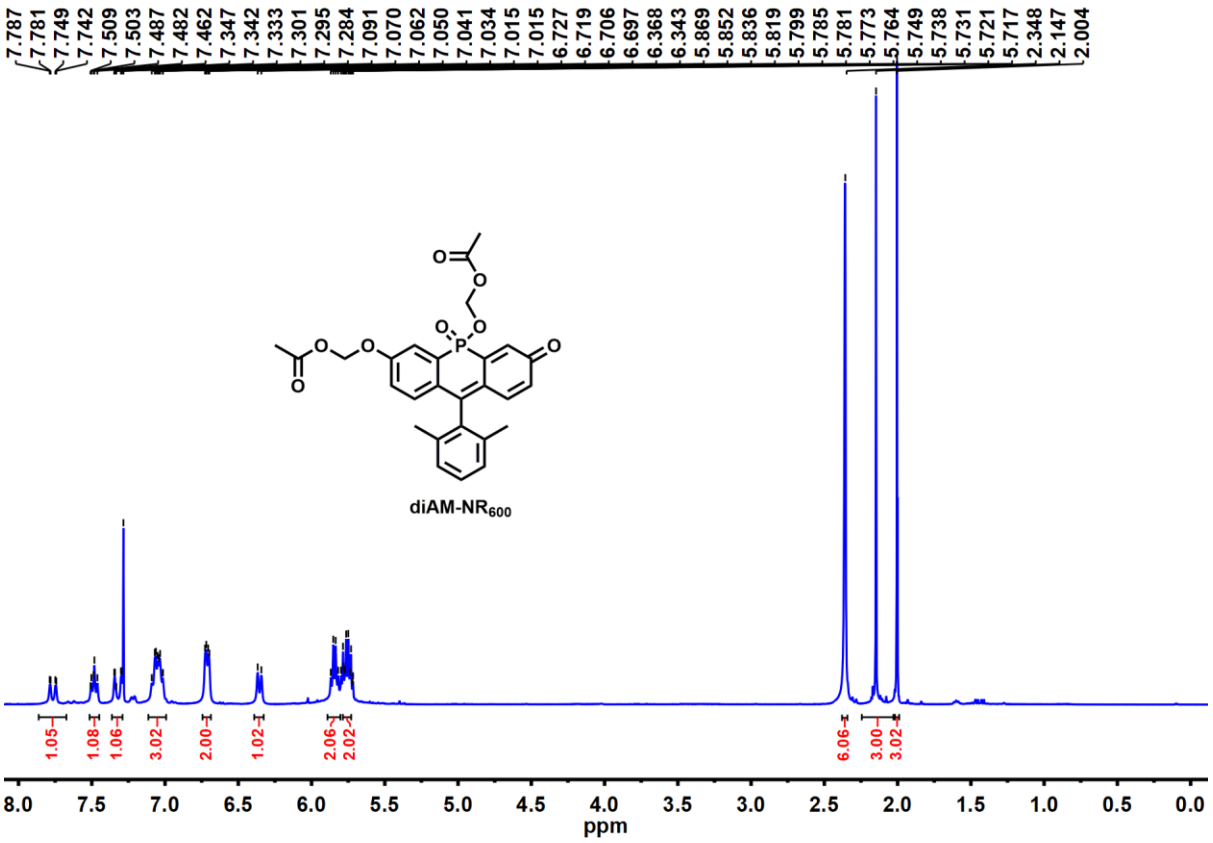
^{1}H-NMR of **NR$_{600}$** in CD_3OD (400 MHz)



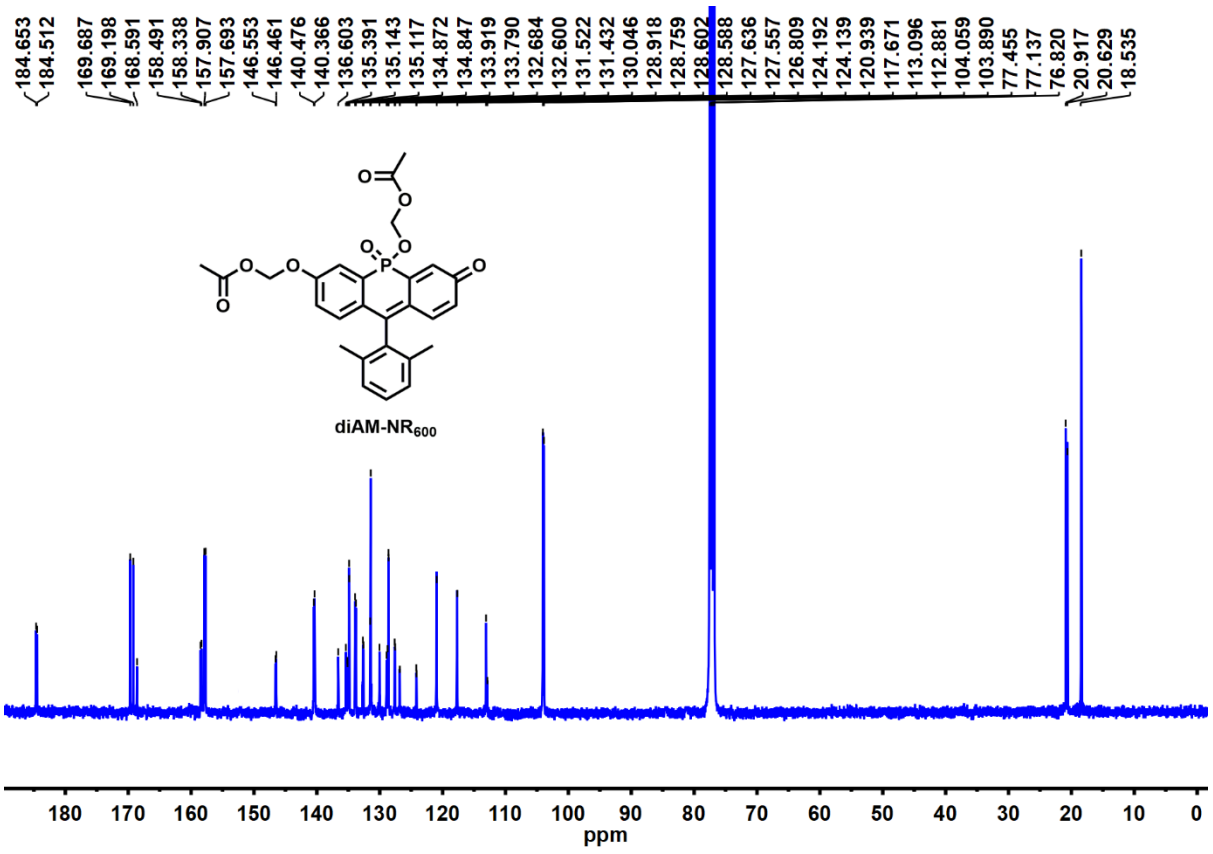
^{13}C-NMR of **NR$_{600}$** in CD_3OD (101 MHz)



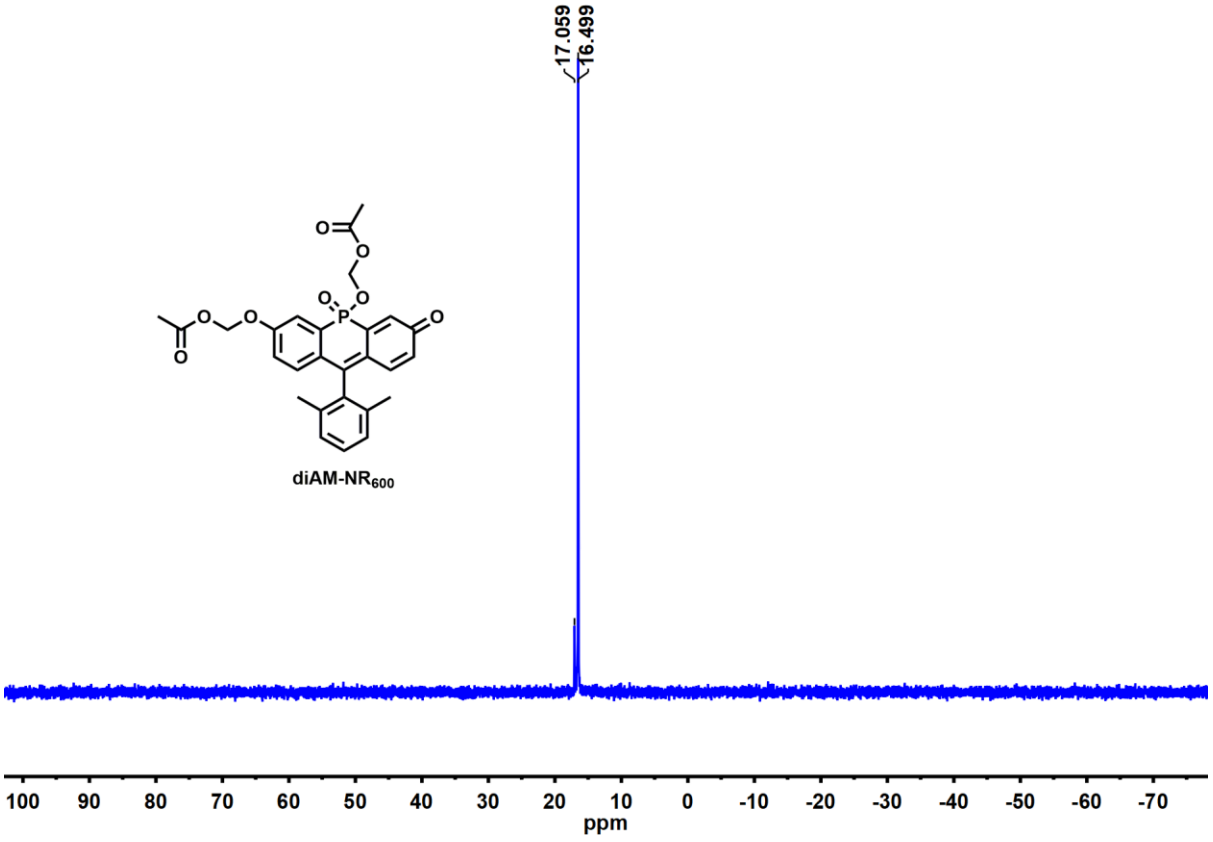
^{31}P-NMR of **NR_{600}** in CD_3OD (162 MHz)



^{1}H-NMR of **$diAM\text{-}NR_{600}$** in $CDCl_3$ (400 MHz)



^{13}C-NMR of **diAM-NR$_{600}$** in $CDCl_3$ (101 MHz)



^{31}P-NMR of **diAM-NR$_{600}$** in $CDCl_3$ (162 MHz)

References

1. D. B. Williams and M. Lawton, *J. Org. Chem.*, 2010, **75**, 8351-8354.
2. I. D. Johnson, *Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies*, Life Technologies Corporation, 2010.
3. K. Rurack and M. Spieles, *Anal. Chem.*, 2011, **83**, 1232-1242.
4. T. Pastierik, P. Sebej, J. Medalova, P. Stacko and P. Klan, *J. Org. Chem.*, 2014, **79**, 3374-3382.
5. M. Grzybowski, M. Taki and S. Yamaguchi, *Chem. Eur. J.*, 2017, **23**, 13028-13032.
6. A. Fukazawa, S. Suda, M. Taki, E. Yamaguchi, M. Grzybowski, Y. Sato, T. Higashiyama and S. Yamaguchi, *Chem. Commun.*, 2016, **52**, 1120-1123.