Supporting Information for:

Mild and scalable synthesis of phosphonorhodamines

Authors:

Joshua L. Turnbull,[‡] Ryan P. Golden,[‡] Brittany R. Benlian,[§] Katherine Henn,[†] Soren Lipman,[‡] and Evan W. Miller[‡§†]*

Affiliations:

Department of [‡]Chemistry, University of California, Berkeley, California 94720, United States of America.

Department of [§]Molecular & Cell Biology, University of California, Berkeley, California 94720, United States of America.

[†]Helen Wills Neuroscience Institute. University of California, Berkeley, California 94720, United States of America.

DOI: Placeholder

Table of Contents

Contents

Chemical Synthesis and Characterization

Chemical reagents and anhydrous solvents were purchased from commercial suppliers and used without further purification. For best results 3-aminophenols were purified by flash silica chromatography (100% DCM or 100% EtOAc) immediately prior to use. Molecular wire and aldehyde **27**, were synthesized according to previously published procedures.[1,2] Thin layer chromatography (TLC) (silica gel, F254, 250 μm) and preparative thin layer chromatography (PTLC) (Silicycle, F254, 1000 μm) were performed on precoated TLC glass plates and were visualized by fluorescence quenching under UV light. Flash column chromatography was performed on Silicycle Silica Flash F60 (230–400 Mesh) for normal phase or 60 RP-18 (200-400 mesh) for reverse phase, using a forced flow of air at 0.5–1.0 bar. NMR spectra were recorded on Bruker AV-300 MHz, Bruker AVB-400 MHz, Bruker AVQ-400 MhZ, Bruker NEO-500 MHz and Bruker AV-600 NMR spectrometers. Chemical shifts (δ) are expressed in parts per million (ppm) and are referenced to CDCl3 (7.26 ppm), DMSO (2.50 ppm), CD_3OD (3.31 ppm) or D_2O (4.79 ppm). Coupling constants are reported as Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; td, triplet of doublets; dt, doublet of triplets; tt, triplet of triplets; ddd, doublet of doublet of doublets; tdd, triplet of doublet of doublets; q, quartet; m, multiplet. High resolution mass spectra (ESI EI) were measured by the QB3/Chemistry mass spectrometry service at University of California, Berkeley. Analytical high performance liquid chromatography (HPLC) and low resolution ESI Mass Spectrometry were performed on an Agilent Infinity 1200 analytical instrument coupled to an Advion CMS-L ESI mass spectrometer. The column used was Phenomenex Luna 5 μm C18(2) (4.6 mm I.D. × 150 mm) with a flow rate of 1.0 mL/min. The mobile phase was MQ-H2O with 0.05% trifluoroacetic acid (eluent A) and HPLC grade MeCN with 0.05% trifluoroacetic acid (eluent B). Signals were monitored at 210, 254, 350, 460 and 520, 560 and 660 nm over 10 min, with a gradient of 10 to 100% eluent B for 8 min, then held at 100% B for 2 min.

Spectroscopic Studies

General Considerations

UV-Vis absorbance and fluorescence spectra were recorded using a 2501 Spectrophotometer (Shimadzu) and a Quantamaster Master 4 L-format scanning spectrofluorometer (Photon Technologies International). The fluorometer is equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples were measured in 1-cm path length quartz cuvettes (Starna Cells).

Absorption and emission spectra were taken at a final concentration of 2 μM (rhodamines) in PBS using 1000x dilutions from stock solutions of dyes in DMSO. pH titrations were conducted in buffers containing 150 mM NaCl and 10 mM buffer. The following buffer systems were used: phosphate (pH 2.3 – 3.1 and 5.5 – 7.5), acetate (pH 3.8 – 5.3), tris (pH 8.2 – 9.2) and carbonate (pH 9.8). pKa's were determined by recording absorbance values or maximum absorbance wavelength and fitting to sigmoidal dose response curves using GraphPad Prism software. Quantum yields are relative measurements compared to Rhodamine B taken in PBS.[3]

Photostability (Figure S4) was determined by continuous irradiation of dyes for 10 min in 1 cm path length quartz cuvettes in the fluorimeter (as above). Dyes were diluted from 5 mM (DMSO) to 500 μM (DMSO) and then diluted 200× to a final concentration of 250 nM in PBS. Excitation was provided at the λ_{max} and the fluorescence emission intensity at the λ_{max} was monitored every second for 10 minutes.

Water solubility

Rhodamines were flushed through a reverse phase silica column (100% MeOH) prior to the experiment to remove any salts. Saturated rhodamine solutions were prepared by adding solid rhodamines portion wise to 2 mL solutions of PBS, with stirring and sonication, until there was an excess of clearly visible solid particulates. The suspensions were filtered through 0.2 μm PTFE filters to remove all solid particulates and the resulting solutions were diluted between 10 to 1000-fold in PBS to ensure a final maximum absorbance reading between 0.1 and 0.8 AU. Absorbance

readings at λ_{max} were compared to standard stock solutions of known concentrations and concentrations of each unknown rhodamine solution were calculated. Factoring in the dilution factors, relative solubilities of each rhodamine in PBS were calculated and normalized to 3-carboxytetramethylrhodamine. Solubility measurements were performed in duplicate (phosTMR) and triplicate (carboxy- and sulfono-TMR).

In vitro AM ester hydrolysis reactions and characterization.

For the enzymatic reactions, commercially purified pig liver esterase (PLE, MW = 168 kDa) as a suspension in 3.2 M $(NH_4)_2SO_4$ was used as a stock solution of 28.1 mg/mL. 2 µl AM dyes (2 mM in DMSO) were incubated with or without 1 µl PLE (final concentration 0.7025 mg/mL) in HBSS (pH 7.4, final volume 40 µl) for 2 hrs at 37°C. Solutions were diluted with 200 µl MeCN, filtered through a 0.22 µm PTFE filter and characterized by HPLC comparing to standards.

Cell Culture

HEK cell culture

Human embryonic kidney 293T (HEK) cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L D-glucose, 10% fetal bovine serum (FBS; Thermo Scientific) and 1% GlutaMax (Invitrogen) at 37 °C in a humidified incubator with 5% CO2. Cells were passaged and plated in DMEM (as above) onto 12 mm glass coverslips pre-coated with Poly-D-Lysine (PDL; 1 mg/mL: Sigma-Aldrich) at a density of 50,000 cells per coverslip. Imaging was performed 16–48 hours after plating.

For loading experiments, HEK cells were incubated with a 500 nM to 1 µM solution of dye (1 mL) in HBSS at 37 °C for 20 min, prior to transfer to 3 mL fresh HBSS (no dye) for imaging. For dye wash out experiments, this imaging solution was replaced with 3 mL fresh HBSS after each subsequent round of imaging (3 washes). For electrophysiology experiments, HEK cells were passaged and plated in DMEM (as above) onto 25 mm glass coverslips pre-coated with PDL at a density of 200,000 cells per coverslip. Imaging was performed 12-18 hours after plating. All voltage dyes were loaded as solutions in HBSS at 37 °C for 20 min

Fluorescence microscopy

Epifluorescence microscopy

Imaging was performed on an AxioExaminer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor), controlled with Slidebook (v6, Intelligent Imaging Innovations). Images were acquired with a W-Plan-Apo 63x/1.0 objective (63x; Zeiss) and images were focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu). The excitation light was delivered from an LED at 542/33 nm and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP).

For plasma membrane retention imaging (Figure S12), experiments were performed as follows. Imaging was performed on an AxioExaminer Z-1 (Zeiss) equipped with a Spectra-X Light engine LED light (Lumencor) and controlled with Slidebook (v6, Intelligent Imaging Innovations). Images were acquired with a W-Plan-Apo 20x/1.0 water objective (20x; Zeiss) and focused onto an OrcaFlash4.0 sCMOS camera (sCMOS; Hamamatsu). The excitation light was delivered from an LED at either 542/33 nm (phosRhoVR, RhoVR) or 475/34 nm (di-4-ANEPPS) at 100 ms exposure. Emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). For tracked images, stage positions were saved within Slidebook at t=0 and revisited at subsequent timepoints without adjustment; for untracked images, new stage positions were visited at each timepoint.

Image analysis

Image analyses were performed in ImageJ (FIJI, NIH). For image intensity measurements, regions of interested were created by thresholding images to create binary masks (cells and background). Background subtracted mean fluorescence intensities were calculated and averaged across five images (each containing at least 15 cells) per coverslip and at least 3 coverslips were examined for each condition.

For analysis of voltage sensitivity briefly, a region of interest (ROI) was manually drawn around the cell periphery. Fluorescence intensity values were then background subtracted by an area without cells. Changes in fluorescence values (ΔF) with each voltage step were calculated by subtracting baseline fluorescence value (F) at -60 mV. The voltage response is reported as a ratio of change relative to baseline (ΔF/F). All voltage traces are single trial recordings with no averaging.

For analysis of plasma membrane retention (Figure S12), image analyses were performed in ImageJ (FIJI, NIH). For tracked imaging, each set of images at a stage position were stacked together and the display histogram set to optimal at t=0, these settings were propagated to images at t=15 and t=30. Untracked images were adjusted for visibility individually at each timepoint.

Electrophysiology

For electrophysiological experiments, pipettes were pulled from borosilicate glass (Sutter Instruments, BF150-86-10), with a resistance of 5–7 MΩ, and were filled with an internal solution; 125 mM potassium gluconate, 1 mM EGTA tetrasodium salt, 10 mM HEPES, 5 mM NaCl, 10 mM KCl, 2 mM ATP disodium salt, 0.3 mM GTP trisodium salt (pH 7.25, 275 mOsm). Electrophysiology recordings were made with an Axopatch 200B amplifier and digitized with a Digidata 1550B (Molecular Devices), sampled at 50 kHz and recorded with pCLAMP 10 software (Molecular Devices) on a PC. Fast capacitance was compensated in the on-cell configuration. Recordings of single cells were only included if they maintained a 10:1 ratio of membrane resistance (R_m) to access resistance (R_a) and an R_a value below 30 MΩ. Once in whole-cell, cells were held at -60 mV resting potential. Depolarizing steps were then applied from -100 to +100 mV in 20 mV increments. Optical traces were captured on an OrcaFlash4.0 sCMOS camera, with 4x4 binning at a framerate of 500 Hz using μManager. Excitation light was applied using an LED at 475/34 nm and 542/33 nm and emission collected with using a 540/50 BP emission filter and a 510 LP dichroic.

Supporting Tables

Table S1. Summary of 2-carboxy and 2-phosphonobenzaldehyde NMR analyses

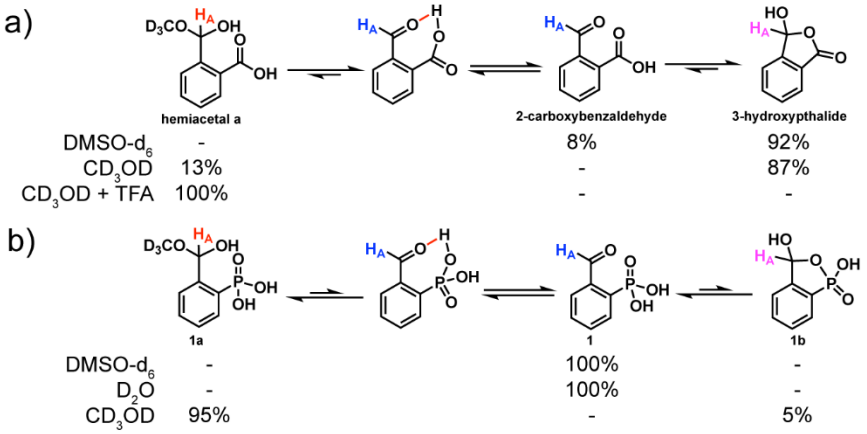
Aldehyde	Solvent	$^{1}H_A$ chemical shift (ppm)	$^{13}C_A$ chemical shift (ppm)	^{31}P chemical shift (ppm)	Ratio
2-carboxy benzaldehyde	DMSO-d_6	6.64 (d, J = 8.1 Hz) 10.45 (s)	98.32 -	- -	0.92 0.08
	CD_3OD	6.62 (s) 6.42 (s)	99.88 105.07	- -	0.87 0.13
	CD_3OD + TFA	6.42 (s)	105.09	-	1.00
2-phosphono benzaldehyde **1**	DMSO-d_6	10.72 (s)	193.39 (d, J = 3.8 Hz)	9.29	1.00
	D_2O	10.58 (s)	195.71 (d, J = 4.1 Hz)	10.65	1.00
	CD_3OD	6.02 (s) 6.29 (d, J = 12.9 Hz)	102.36 (d, J = 3.6 Hz) -	14.44 29.47	0.95 0.05

Table S2. Conditions for deallylation of **19** to **21**.

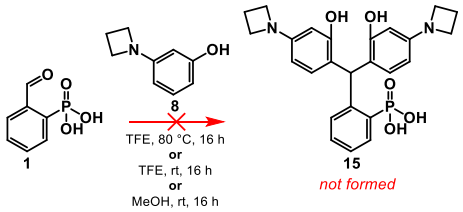
Entry	Conditions	Yield **21**	Notes
A	NDMBA (6 equiv.) $Pd(PPh_3)_4$ (4%) 3:1 DMF:NEt_3 80 °C, 16 h	52%	-isolated yield
B	^tBuOK DMSO, 100 °C, 10 min then 1 M HCl, 100 °C, 5 min	71%	-isolated yield

Supporting Schemes

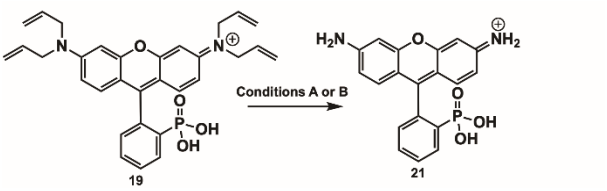
Scheme S1. *ortho*-substituted benzaldehyde equilibria.



Scheme S2. Attempted Friedel-Crafts condensation of azetidine-containing aniline **8**.



Scheme S3. De-allylation of tetraallylrhodamine **19** to phosRho110, **21**.



Supporting Figures

Figure S1. Relative solubility of tetramethylrhodamines.

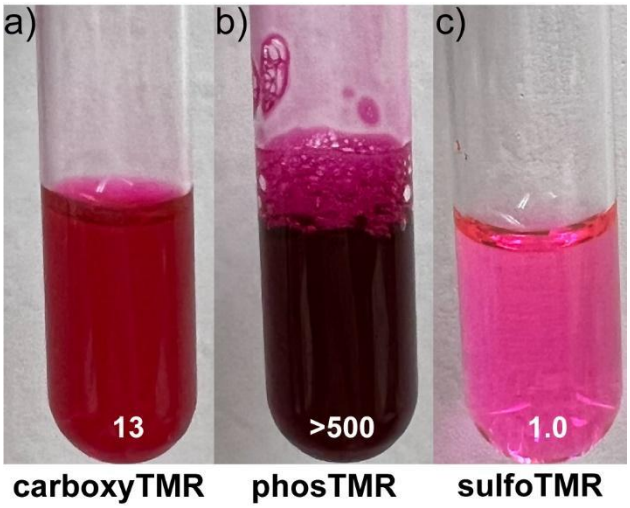


Figure S1. Relative solubility of tetramethylrhodamines. Images of saturated solutions of carboxy- (a), phos- (b) and sulfo- (c) tetramethylrhodamines in PBS after filtration through a 0.2 μm PTFE filter. Relative concentrations (normalized to sulfoTMR) as determined by absorbance spectroscopy are indicated in white.

Figure S2. Stability of carboxy TMR.

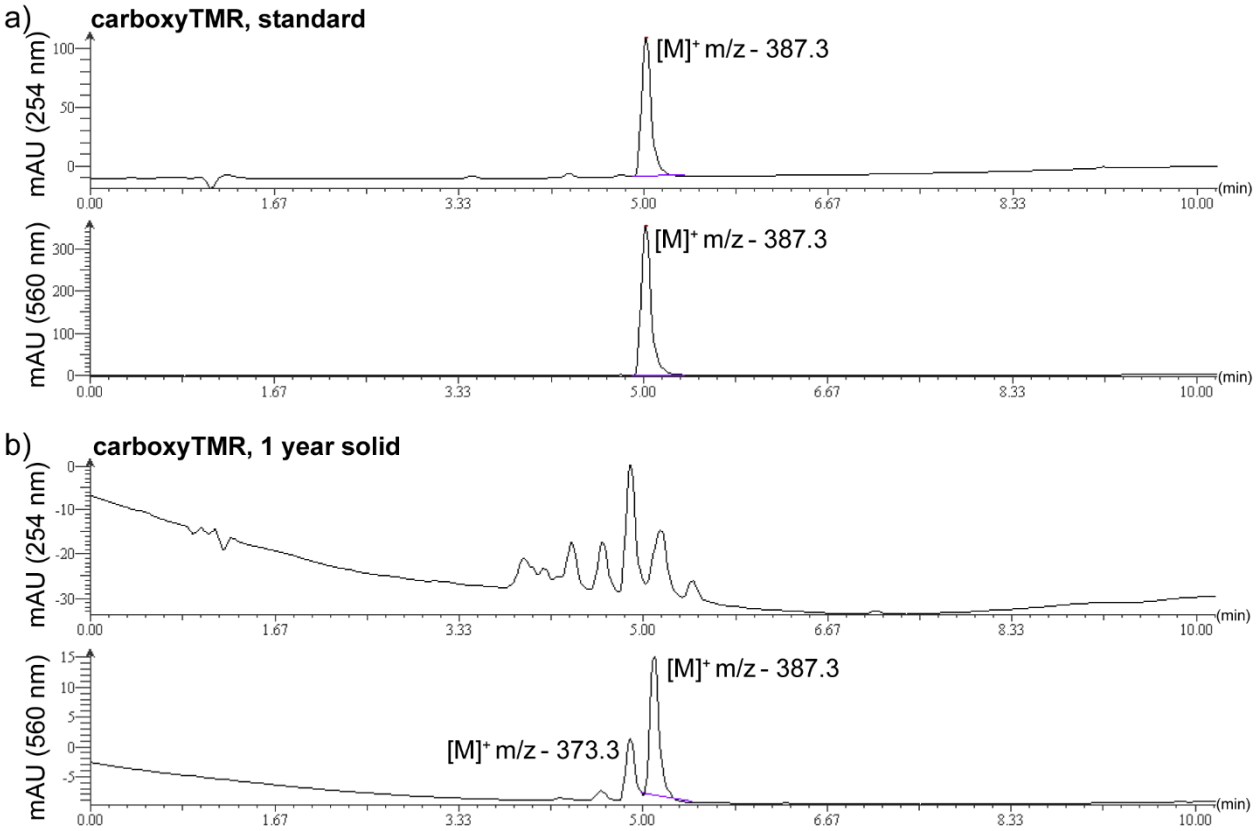


Figure S2. Stability of carboxy TMR. Analytical HPLC traces of a freshly synthesized carboxyTMR standard (a) and sample of carboxyTMR that had been sorted as a solid powder for one year at room temperature in the dark (b). Traces are 254 nm and 560 nm are depicted and where applicable corresponding m/z values are annotated. Note: decomposition of carboxyTMR in 560 nm channel showed demethylation (Δm/z -14).

Figure S3. Stability of phosTMR.

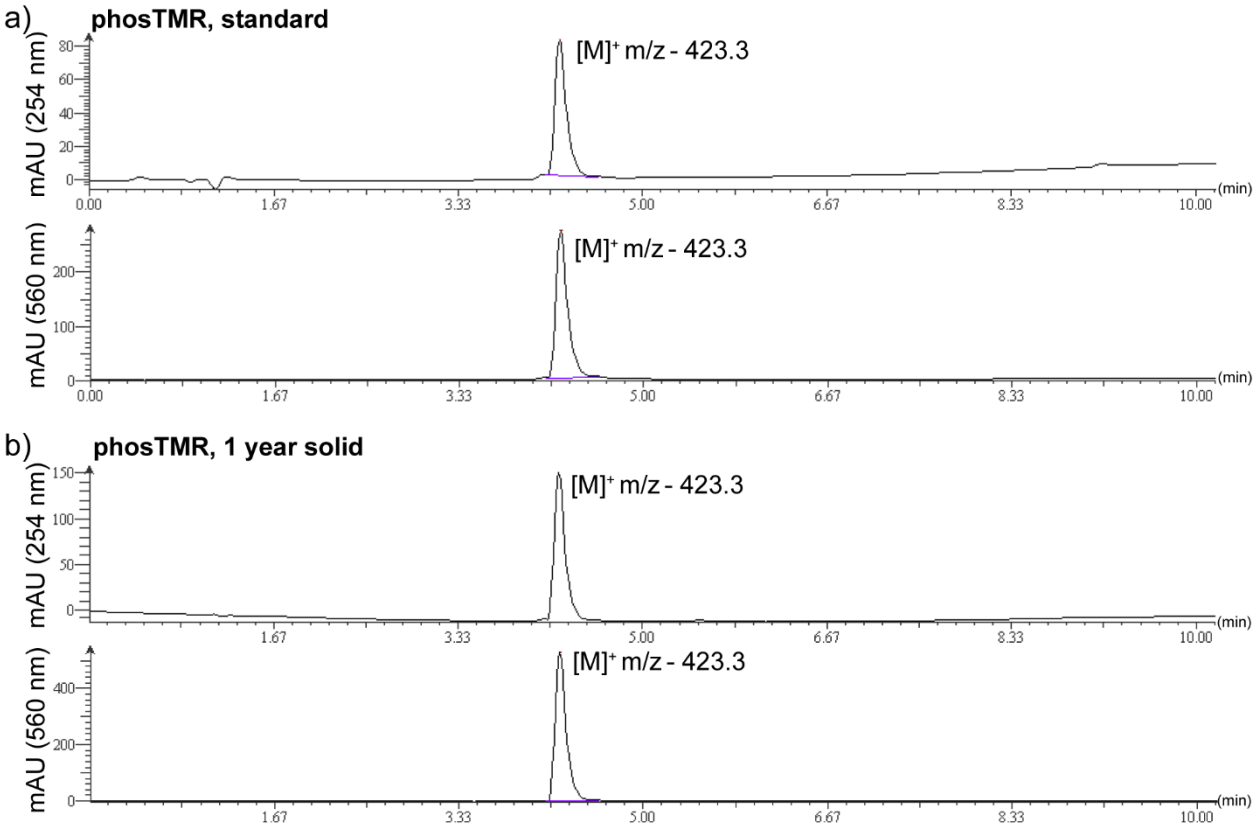


Figure S3. Stability of phosTMR. Analytical HPLC traces of a freshly synthesized phosTMR standard (a) and sample of phosTMR that had been sorted as a solid powder for one year at room temperature in the dark (b). Traces are 254 nm and 560 nm are depicted and corresponding m/z values are annotated. Note: no decomposition was observed.

Supplemental Note: Figures S2 and S3 compare relative stabilities of carboxy TMR and phos TMR. Both rhodamines were synthesized as described (pages S31 and S25). Carboxy TMR required purification by silica gel flash chromatography, whereas phos TMR was deemed to be >99% pure after filtration of the crude reaction mixture. Both fluorophores were triturated with EtOAc to yield powders. Samples were stored, together, in the dark at room temperature (15 to 22 °C) for 1 year.

Figure S4. Spectroscopic characterization of 3-phosphonorhodamines.

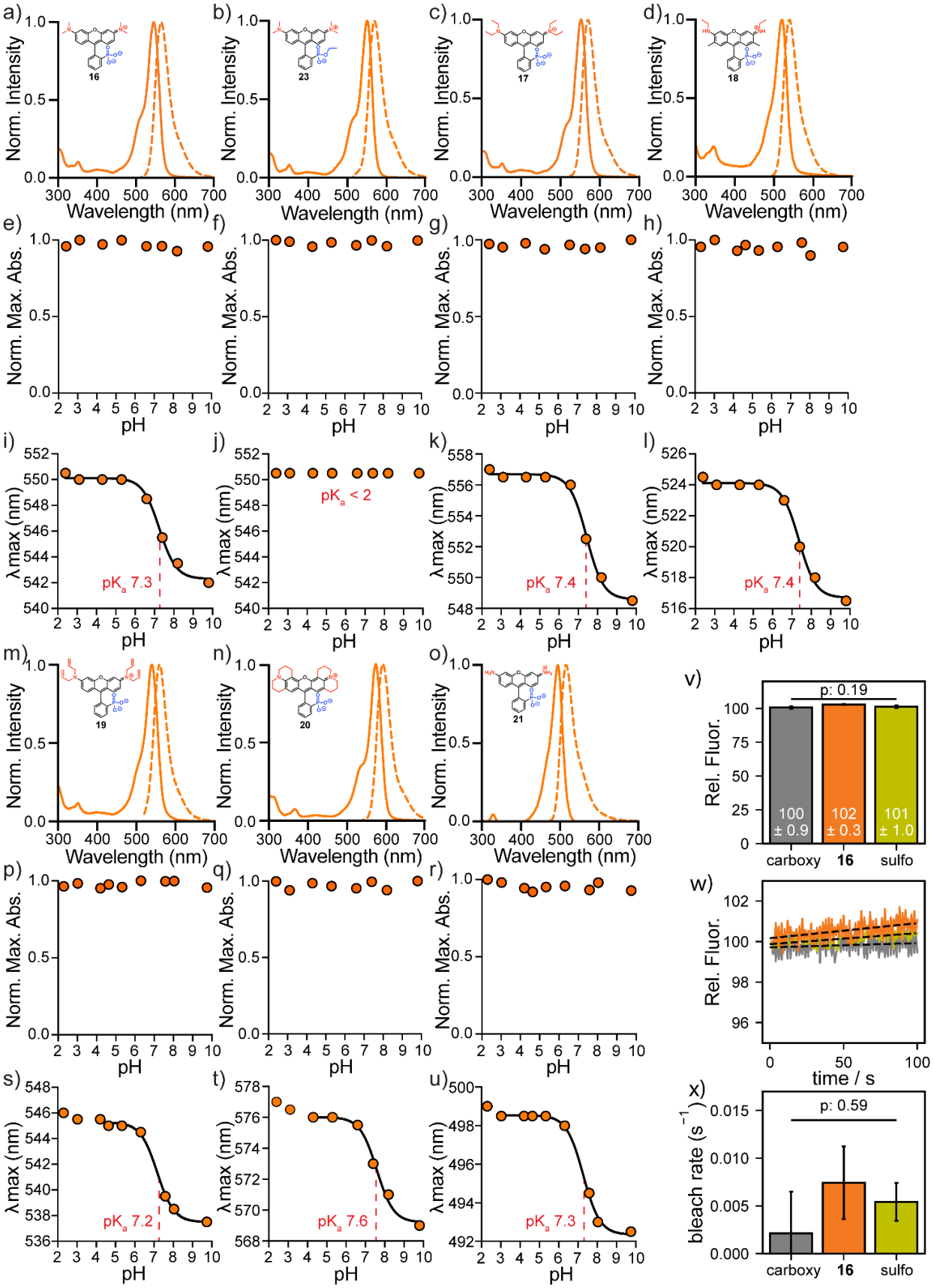


Figure S4. Spectroscopic characterization of 3-phosphonorhodamines. Normalized absorbance and fluorescence spectra (a-d, m-o) in PBS with labeled chemical structures, plots of maximum absorbance vs pH (e-h, p-r), and plots of λ_{max} vs pH (i-l, s-u) for **16** (a, e, i), **23** (b, f, j), **17** (c, g, k), **18** (d, h, l), **19** (m, p ,s), **20** (n, q t), **21** (o, r, u). pH titrations were performed in 10 mM buffered solutions containing 150 mM NaCl ranging from pH 2.3 to 9.8 at a final dye concentration of 2 μM. Titration curves were fit to sigmoidal dose response curves (solid black) to enable pK_a determination (dashed red) and phosphonate pK_{a2} values are indicated in red.

v) Plot of relative fluorescence intensity (normalized to t = 0) for carboxyTMR (grey), phosphonoTMR (**16**, orange), or sulfoTMR (yellow) under constant illumination in PBS for 10 minutes. Captions indicate the percentage fluorescence remaining ± the standard error of the mean (n = 3 experiments). The differences in remaining fluorescence after 10 min are not statistically significant (p = 0.19, ANOVA). w) Plot of relative fluorescence intensity vs. time for carboxyTMR (grey), phosphonoTMR (**16**, orange), or sulfoTMR (yellow). Data are the mean fluorescence intensity, normalized to the mean at t = 0, for 3 separate experiments. Black, dashed line is line of best fit for each data set. x) Plot of magnitude observed initial bleach rate constant for each dye. Data are for n = 3 experiments per dye. Differences in observed rate constants are not statistically significant (p = 0.59, ANOVA).

Figure S5. In vitro characterization of carboxyTMR AM hydrolysis.

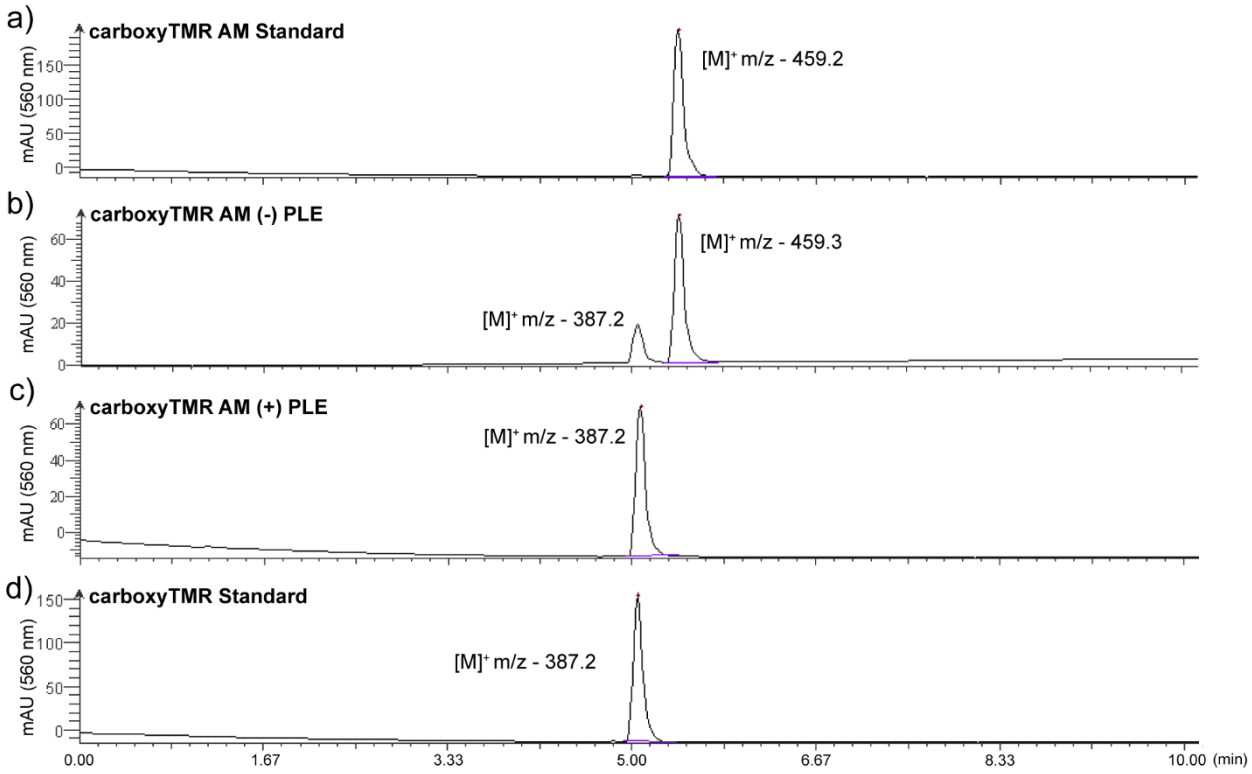


Figure S5. In vitro characterization of carboxyTMR AM hydrolysis.

CarboxyTMR AM was incubated with or without PLE in HBSS for 2 hrs at 37 °C. HPLC traces were then compared to those of **carboxyTMR AM** (not incubated in HBSS) and **carboxyTMR** standards. Masses are indicated next to the peaks.

Figure S6. In vitro characterization of phosTMR AM, **25** hydrolysis.

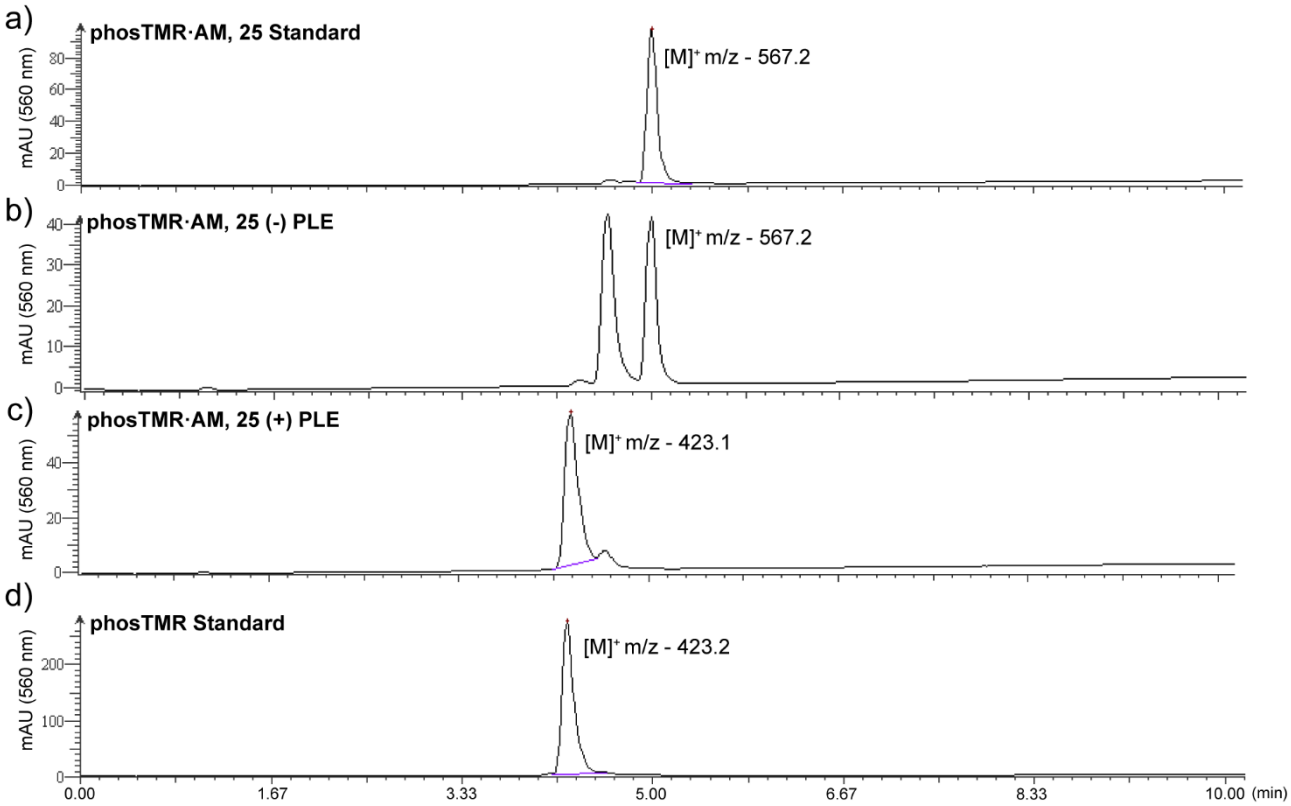


Figure S6. In vitro characterization of phosTMR AM, **25** hydrolysis.

PhosTMR AM, 25 was incubated with or without PLE in HBSS for 2 hrs at 37 °C. HPLC traces were then compared to those of **phosTMR AM, 25** (not incubated in HBSS) and **phosTMR, 19** standards. Masses are indicated next to the peaks unless no mass was observed (b).

Figure S7. In vitro characterization of phosTMR·2OEt, **24** hydrolysis.

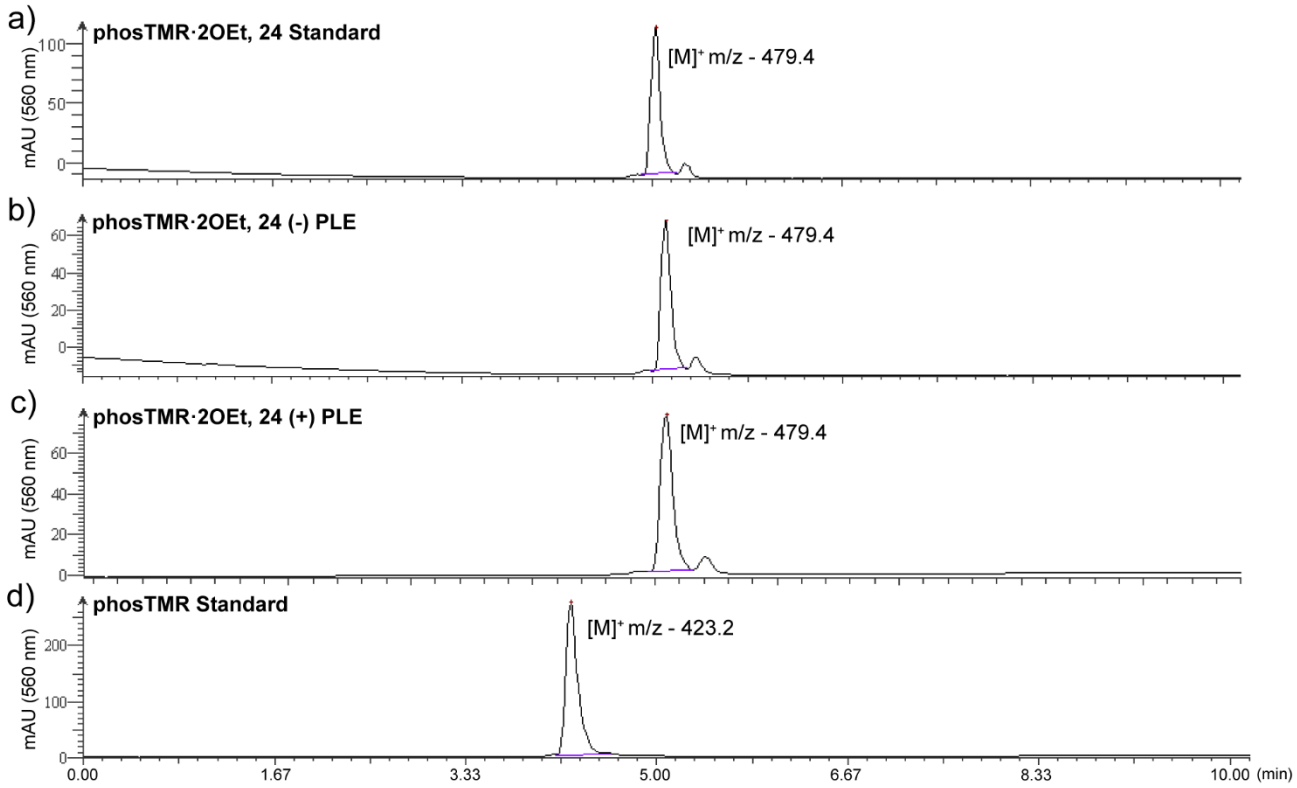


Figure S7. In vitro characterization of phosTMR·2OEt, **24** hydrolysis.

PhosTMR·2OEt, 24 was incubated with or without PLE in HBSS for 2 hrs at 37 °C. HPLC traces were then compared to those of **phosTMR·2OEt, 24** (not incubated in HBSS) and **phosTMR, 19** standards. Masses are indicated next to the peaks.

Figure S8. Localization of phosTMR·2OEt, **24** and phosTMR AM, **25**

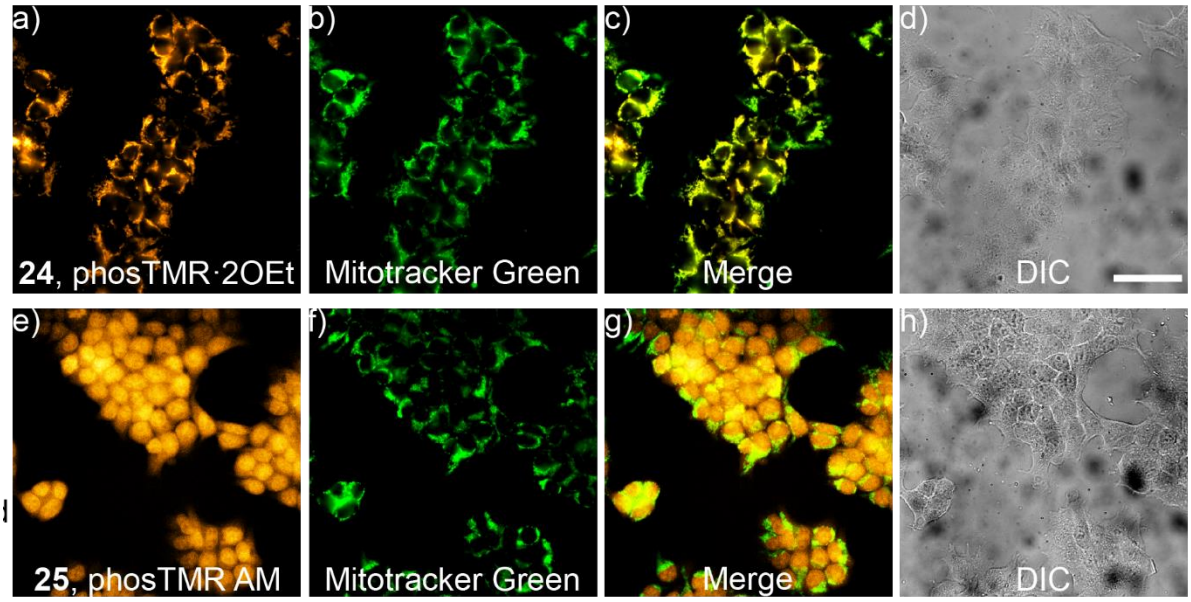


Figure S8. Localization of phosTMR·2OEt, **24** and phosTMR AM, **25**. Widefield fluorescence images in the orange (a/e) and green (b/f) channels, a merge of images a and b (c/g) and corresponding DIC image (d/h) of HEK293T cells stained with 500 nM **24** (a-d) or 500 nM **25** (e-h) and 100 nM Mitotracker Green FM (ThermoFisher, M7514) in HBSS for 20 minutes at 37 °C. The coverslip was transferred into fresh HBSS prior to imaging. Scale bar is 50 μm.

Figure S9. Intracellular localization of 3-carboxytetramethylrhodamines.

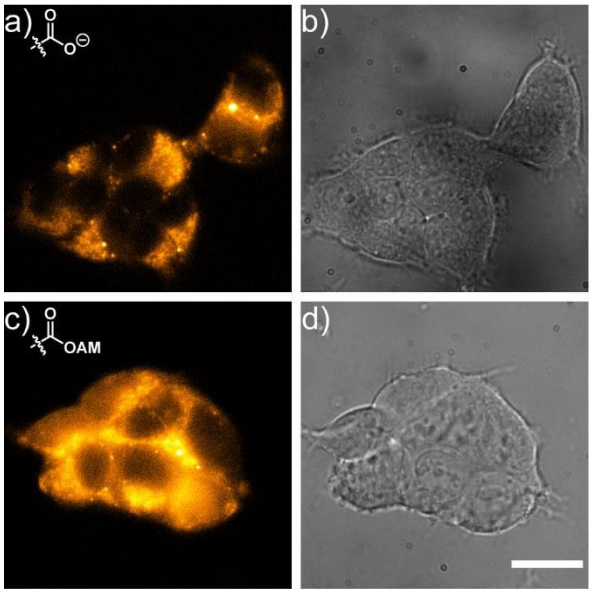


Figure S9. Intracellular localization of 3-carboxytetramethylrhodamines. Widefield fluorescence (a, c) and DIC (b, d) images of HEK293T cells stained with 500 nM carboxyTMR (a, b) and carboxyTMR acetoxymethyl ester (c, d) for 20 minutes at 37 °C. Coverslips were transferred into fresh HBSS prior to imaging. Florescence images are normalized to image c. Scale bar is 20 μm.

Figure S10. Cellular retention of TMR acetoxymethyl esters.

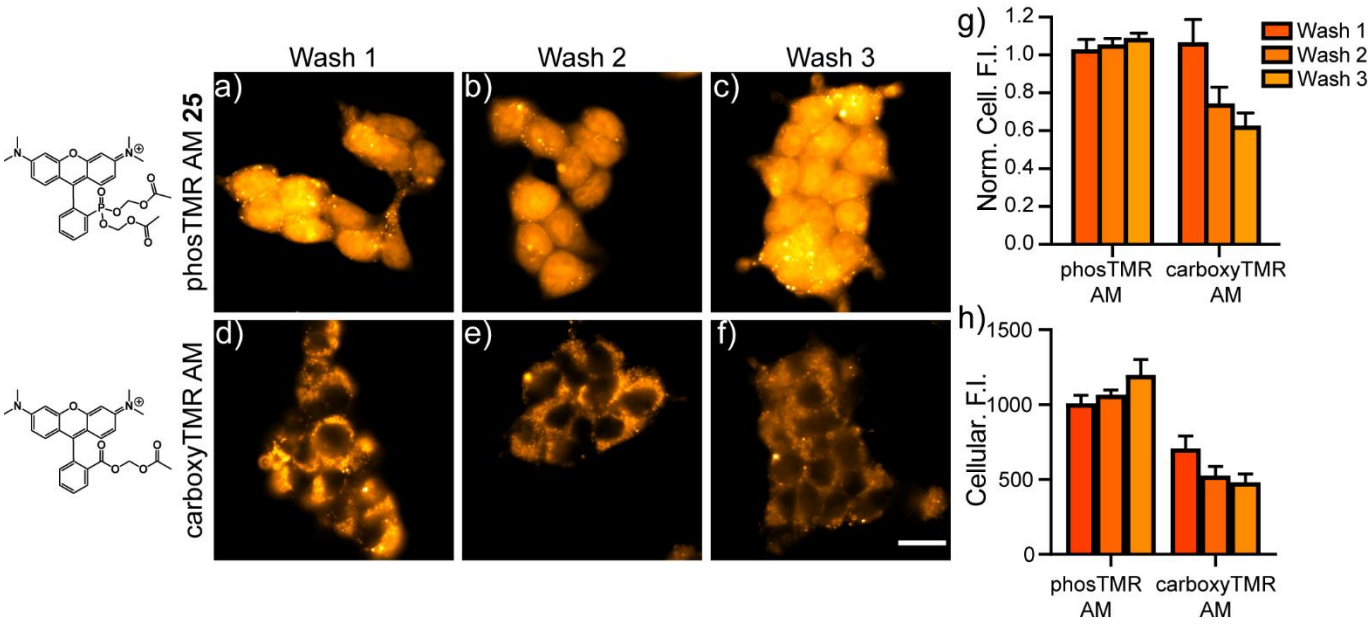


Figure S10. Cellular retention of TMR acetoxymethyl esters. Widefield fluorescence images of HEK293T cells stained with 1 μM phosTMR AM, **25** (a-c) and carboxyTMR (d-f) for 20 minutes at 30 °C. Coverslips were placed into fresh HBSS (1 wash, t = 26 min) prior to imaging and coverslips were washed by replacement with fresh HBSS (washes 2, t = 32 min, and wash 3, t = 36 min). Scale bar is 20 μm. Quantification of associated changes in fluorescence intensity (g) through serial washing. Images and quantification for each dye are normalized to the wash 1 condition. (h) The same data as in (g), but showing the raw cellular fluorescence intensities ("Cellular F.I."). Error bars represent + SEM for n = 15 images across 3 coverslips.

Figure S11. Relative cell brightness of RhoVR 1 and phosRhoVR 1.

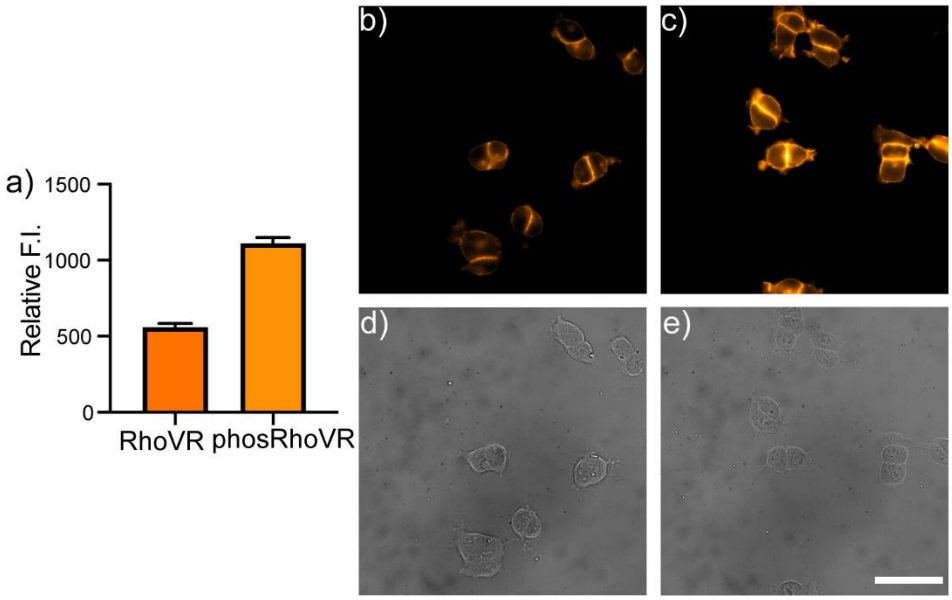


Figure S11. Relative cell brightness of RhoVR 1 and phosRhoVR 1.

Quantification (a), widefield fluorescence (b, c) and DIC (d, e) images of HEK cells stained with 5000 nM RhoVR 1(b, d) and phosRhoVR 1. Fluorescence images are normalized to phosRhoVR1 (c). Scale bar is 50 μm. Error bars are ± SEM for n = 10-15 cellular regions of interest from 2 (RhoVR) or 3 (phosRhoVR) coverslips.

Figure S12. Membrane retention of phosRhoVR 1, RhoVR 1, and di-4-ANEPPS

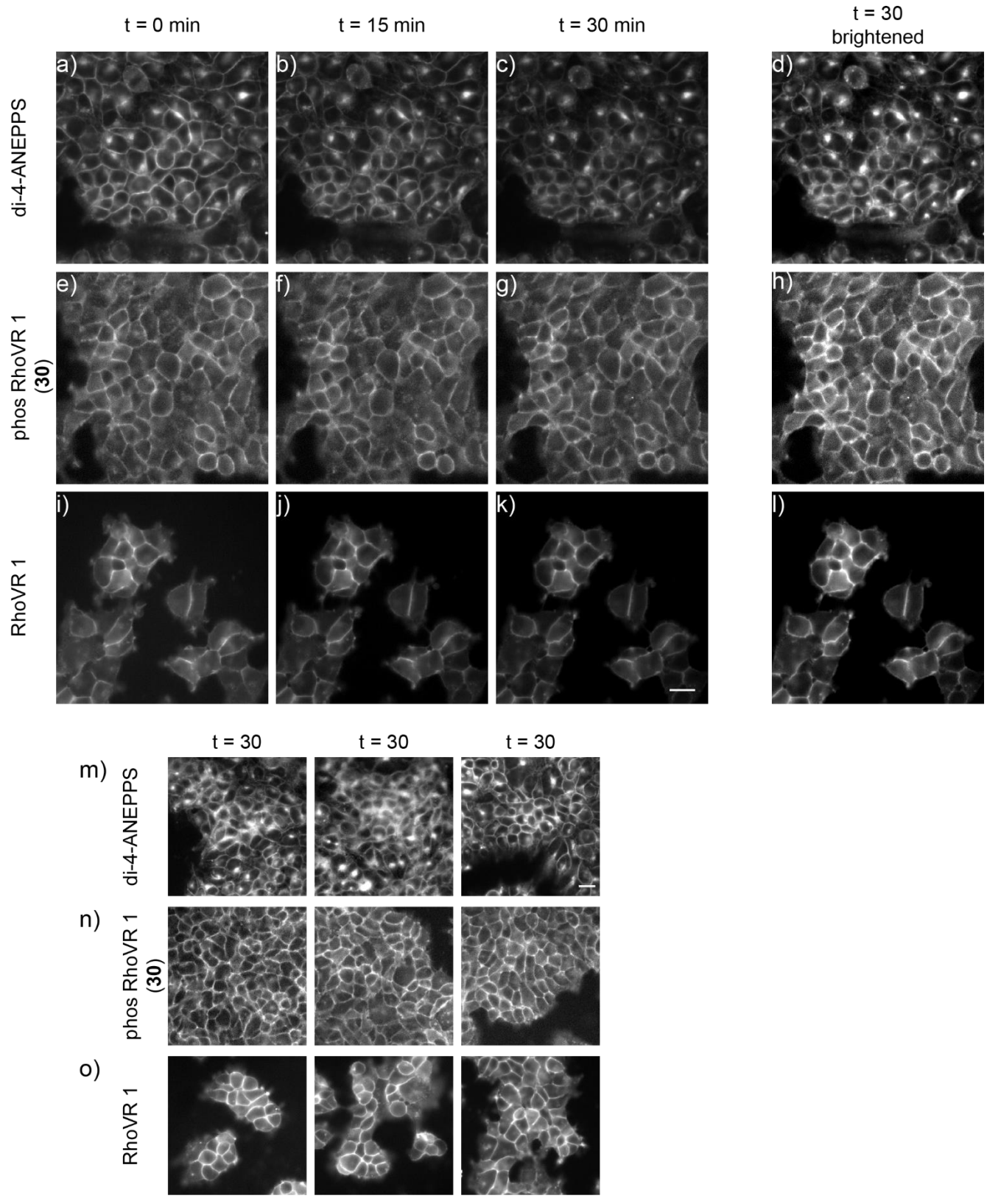


Figure S12. Membrane retention of phosRhoVR 1, RhoVR 1, and di-4-ANEPPS.

Epifluorescence images of HEK cells stained with di-4-ANEPPS (a-d), phosRhoVR 1 (30, e-h), or RhoVR 1 (i-l). Cells were loaded with the indicated dye in HBSS for 37 °C for 30 min. Dye concentration was 500 nM (0.1% DMSO). Cells were transferred into fresh HBSS solution containing no dye and imaged immediately (t = 0 min) and at 15 min intervals. For each dye, image grey values are scaled to min/max value for t = 0 to allow for comparison of brightness within a dye series. In the final column (panels **d**, **h**, **l**), the t = 30 min has been brightened to allow examination of dye localization. Scale bar is 20 μm.

m, n, o) Additional images of m) di-4-ANEPPS, n) phosRhoVR 1 (**30**), and o) RhoVR 1, all at t = 30 min. Scale bar is 20 μm.

General Synthetic Procedures

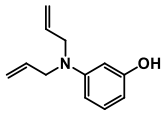
General procedure A for synthesis of triarylmethane intermediates (A):

1 (1 mmol, 1 equiv.) and 3-aminophenols (2.25 mmol, 2 equiv.) were charged into a reaction flask that was subsequently evacuated and backfilled with N_2 (x3). 2,2,2-trifluoroethanol was added, and the reaction stirred at 80 °C overnight. Upon cooling to temperature, trituration with either EtOAc or cold MeOH yielded triarylmethane intermediates.

General procedure B for the synthesis of 3-phosphonorhodamines (B):

Triarylmethane intermediates (0.5 mmol, 1 equiv.) and p-chloranil (1 mmol, 2 equiv.) were refluxed in MeOH for 2 to 24 hours, or until all particulates had dissolved. Upon cooling to room temperature, any remaining solids were removed by vacuum filtration and the filtrate concentrated *in vacuo*. Trituration with EtOAc yielded 3-phosphonorhodamines.

Detailed Synthetic Procedures

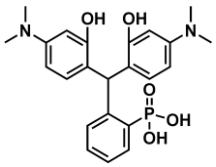


*Synthesis of Compound **5***

3-(diallylamino)phenol

3-aminophenol, **7** (3.0 g, 27.5 mmol, 1 equiv.) and K_2CO_3 (7.6 g, 5 mmol, 2 equiv.) were suspended in ethanol (30 mL). While stirring, allyl bromide (5.9 mL, 69 mmol, 2.5 equiv.) was added and the solution was refluxed for 90 minutes. Upon cooling to room temperature, the suspension was diluted with water and extracted into EtOAc (x3), dried over Na_2SO_4 and concentrated *in vacuo*. The crude oil was purified by flash silica chromatography to attain # as a golden oil (4.05 g, 21.4 mmol, 78%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 (t, *J* = 8.1 Hz, 1H), 6.30 (d, *J* = 7.0 Hz, 1H), 6.23 – 6.09 (m, 2H), 5.84 (ddt, *J* = 17.1, 10.0, 4.9 Hz, 2H), 5.21 – 5.12 (m, 4H), 3.89 (dt, *J* = 4.4, 1.4 Hz, 4H).



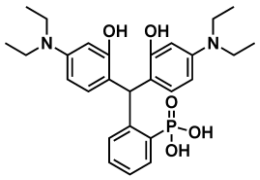
*Synthesis of Compound **9***

1 (300 mg, 1.61 mmol, 1 equiv.) and 3-(dimethylamino)phenol, **2** (496 mg, 3.62 mmol, 2.25 equiv.) were charged into a round bottom flask that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of TFE (12.5 mL), the solution was stirred at 80 °C for 16 hours. The resulting suspension was concentrated *in vacuo* and triturated with cold methanol, yielding **9** as a white solid (698 mg, 1.58 mmol, 98%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.67 (dd, *J* = 13.7, 7.5 Hz, 1H), 7.43 (t, *J* = 7.6 Hz, 1H), 7.25 (td, *J* = 7.5, 2.7 Hz, 1H), 7.20 (dd, *J* = 7.4, 5.5 Hz, 1H), 6.61 – 6.53 (m, 3H), 6.08 (m, 4H), 2.78 (s, 12H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 14.97

HR-ESI-MS m/z for $C_{23}H_{36}O_5N_2P^-$ $[M-H]^-$ calcd: 451.2362 found: 451.2364



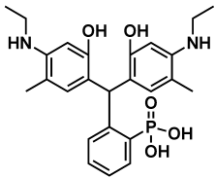
*Synthesis of Compound **10***

1 (50 mg, 0.28 mmol, 1 equiv.) and 3-(diethylamino)phenol, **3** (100 mg, 0.60 mmol, 2.25 equiv.) were stirred in TFE (7 mL) at 80 °C. The resulting suspension was cooled in an ice bath, filtered and washed with cold methanol, yielding **10** as a white/pale pink solid (128 mg, 0.26 mmol, 96%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.71 – 7.60 (m, 1H), 7.41 (t, *J* = 7.5 Hz, 1H), 7.27 – 7.18 (m, 2H), 6.63 (d, *J* = 8.0 Hz, 2H), 6.54 (s, 1H), 6.06 (s, 4H), 3.23 (q, *J* = 6.9 Hz, 8H), 1.03 (t, *J* = 7.0 Hz, 12H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 14.53.

HR-ESI-MS m/z for $C_{27}H_{34}O_5N_2P_1^-$ $[M-H]^-$ calcd: 497.2211 found: 497.2213



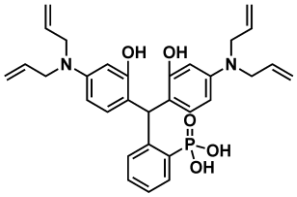
*Synthesis of Compound **11**.*

1 (75 mg, 0.40 mmol, 1 equiv.) and 3-(ethylamino)-p-cresol, **4** (137 mg, 0.91 mmol, 2.25 equiv.) were stirred in TFE (7.5 mL) at 80 °C for 16 hours. The resulting suspension was cooled to room temperature, diluted with methanol (10 mL) and filtered, yielding an off-white solid (136 mg). The filtrate was concentrated *in vacuo*, and trituration with EtOAc yielded a further 55mg of solid. Combined, **11** was isolated as a white/pale purple powder (188 mg, 0.40 mmol, 99%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.66 (ddd, J = 13.8, 7.9, 1.3 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.26 – 7.17 (m, 2H), 6.51 (s, 1H), 6.40 (s, 2H), 5.97 (s, 2H), 3.03 – 2.97 (m, 4H), 1.87 (s, 6H), 1.16 (t, J = 7.1 Hz, 6H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 14.84.

HR-ESI-MS m/z for $C_{25}H_{30}O_5N_2P^+$ $[M]^+$ calcd: 469.1898 found: 469.1890



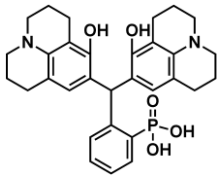
*Synthesis of Compound **12.***

1 (200 mg, 1.08 mmol, 1 equiv.) and **5** (448 mg, 2.37 mmol, 2.2 equiv.) were added to a round bottom flask that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of TFE (6 mL) the solution was stirred at 80 °C for 16 hours. Upon cooling to room temperature, the suspension was concentrated *in vacuo* and triturated with EtOAc to yield **12** as a pale pink powder (525 mg, 0.96 mmol, 89%)

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.66 (dd, J = 13.7, 7.6 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.23 (ddt, J = 12.9, 8.0, 4.1 Hz, 2H), 6.54 (d, J = 8.5 Hz, 2H), 6.51 (s, 1H), 6.08 – 5.99 (m, 4H), 5.81 (ddt, J = 15.4, 10.2, 5.1 Hz, 4H), 5.15 – 5.08 (m, 8H), 3.80 (d, J = 5.0 Hz, 8H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 15.03.

HR-ESI-MS m/z for $C_{31}H_{36}O_5N_2P^+$ $[M]^+$ calcd: 547.2356 found: 547.2354



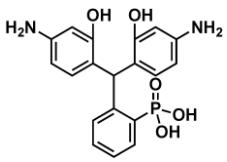
*Synthesis of Compound **13**.*

1 (150 mg, 0.81 mmol, 1 equiv.) and 8-hydroxyjulolidine, **6** (344 mg, 1.82 mmol, 2.25 equiv.) were charged into a round bottom flask that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of TFE (5 mL), the solution was stirred at 80 °C for 16 hours. The resulting suspension was concentrated *in vacuo*, triturated with cold methanol, and filtered, yielding **13** as a white solid (405 mg, 0.74 mmol, 92%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.65 (dd, J = 13.7, 7.8 Hz, 1H), 7.45 (t, J = 7.5 Hz, 1H), 7.33 – 7.18 (m, 2H), 6.56 (s, 1H), 6.22 (s, 2H), 3.05 – 2.85 (m, 8H), 2.49 – 2.38 (m, 8H), 1.92 – 1.70 (m, 8H).

^{31}P NMR (162 MHz, DMSO-d_6) δ 14.86.

HR-ESI-MS m/z for $C_{31}H_{34}O_5N_2P_1^-$ [M-H]$^-$ calcd: 545.2211 found: 545.2205



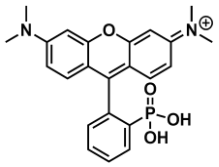
*Synthesis of Compound **14***

1 (150 mg, 0.81 mmol, 1 equiv.) and 3-aminophenol, **7** (220 mg, 2.01 mmol, 2.5 equiv.) were stirred in TFE (5 mL) at 80 °C for 16 hours. Upon cooling to room temperature, the suspension was filtered and washed with cold methanol to yield **14** as a beige solid (303 mg, 0.79 mmol, 97%).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.65 (dd, J = 13.0, 7.7 Hz, 1H), 7.38 (q, J = 7.4 Hz, 1H), 7.19 (dt, J = 18.3, 6.2 Hz, 2H), 6.51 (d, J = 7.5 Hz, 2H), 6.14 – 5.88 (m, 5H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 12.91.

HR-ESI-MS m/z for $C_{19}H_{18}O_5N_2P_1^-$ [M-H]$^-$ calcd: 385.0959 found: 385.0954



*Synthesis of Compound **16.***

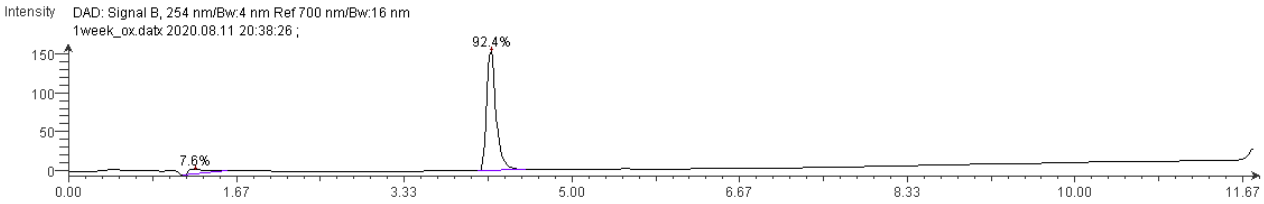
9 (350 mg, 0.8 mmol, 1 equiv.) and p-chloranil (394 mg, 1.6 mmol, 2 equiv.) were refluxed in MeOH (50 mL) for 48 hours (or until all solids had dissolved). Upon cooling to room temperature, the solution was filtered, and the filtrate was concentrated *in vacuo*. Trituration with EtOAc yielded **16** as a dark purple solid (335 mg, 0.8 mmol, >99%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (ddd, J = 13.3, 6.0, 3.5 Hz, 1H), 7.80 – 7.69 (m, 2H), 7.36 (dd, J = 5.3, 4.7 Hz, 1H), 7.08 (dd, J = 9.4, 1.7 Hz, 2H), 6.98 (d, J = 9.5 Hz, 2H), 6.93 (d, J = 1.7 Hz, 2H), 3.27 (s, 12H).

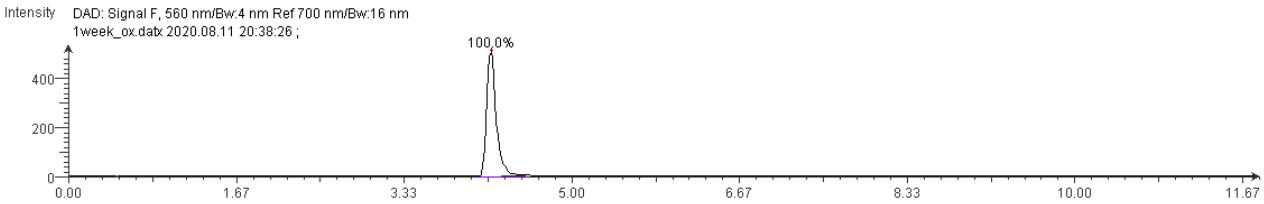
^{31}P NMR (162 MHz, DMSO-d_6) δ 9.70.

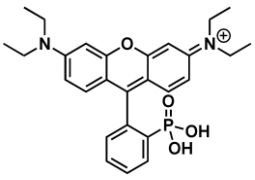
HR-ESI-MS m/z for $C_{23}H_{24}O_4N_2P^+$ $[M]^+$ calcd: 423.1468 found: 423.1470

254 nm



560 nm





*Synthesis of Compound **17.***

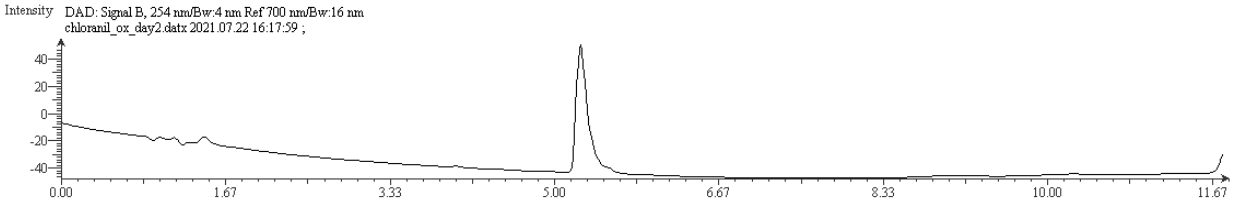
10 (54 mg, 0.11 mmol, 1 equiv.) and p-chloranil (58 mg, 0.24 mmol, 2 equiv.) were refluxed in MeOH (15 mL) for 16 hours. Upon cooling to room temperature, the solution was filtered, and the filtrate was concentrated *in vacuo*. Trituration with EtOAc yielded **17** as a dark purple powder (42 mg, 0.09 mmol, 80%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.29 – 7.98 (m, 1H), 7.71 (m, 2H), 7.34 – 7.28 (m, 1H), 7.16 (d, J = 9.5 Hz, 2H), 7.00 (dd, J = 9.5, 2.3 Hz, 2H), 6.93 (d, J = 2.3 Hz, 2H), 3.67 (q, J = 7.2 Hz, 8H), 1.30 (t, J = 7.1 Hz, 12H).

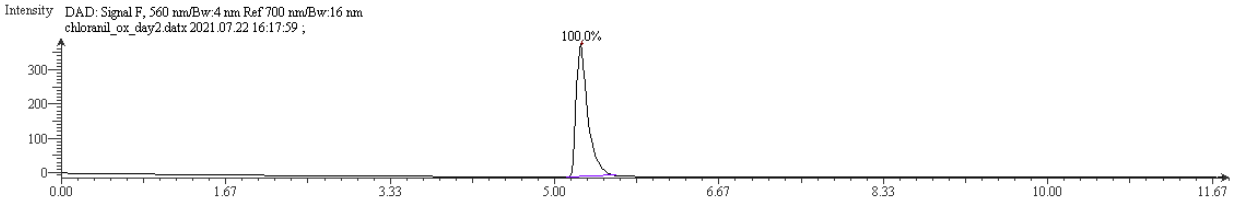
^{31}P NMR (202 MHz, Methanol-d_4) δ 10.34.

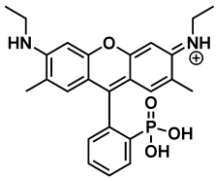
HR-ESI-MS m/z for $C_{27}H_{32}O_4N_2P_1^+$ $[M]^+$ calcd: 479.2094 found: 479.2100

254 nm



560 nm





*Synthesis of Compound **18.***

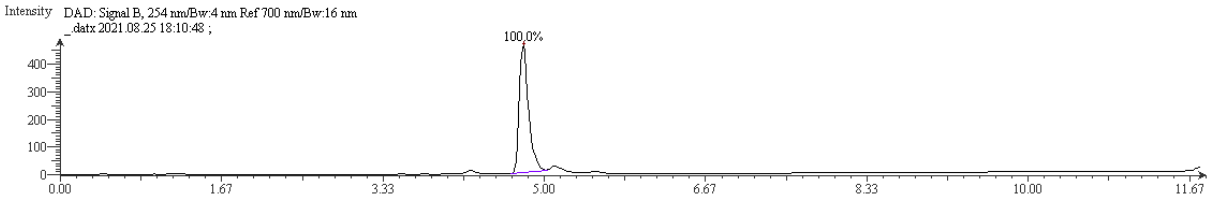
11 (126 mg, 0.27 mmol, 1 equiv.) and p-chloranil (86 mg, 0.35 mmol, 1.3 equiv.) were refluxed in MeOH (10 mL) for 20 hours. Upon cooling to room temperature, the solution was filtered, and the filtrate was concentrated *in vacuo*. Trituration with EtOAc yielded **18** as a dark red powder (100 mg, 0.22 mmol, 82%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.19 (dd, J = 13.8, 7.3 Hz, 1H), 7.74 – 7.66 (m, 2H), 7.28 – 7.22 (m, 1H), 6.94 (s, 2H), 6.87 (s, 2H), 3.52 (q, J = 7.2 Hz, 4H), 2.12 (s, 6H), 1.36 (t, J = 7.2 Hz, 6H).

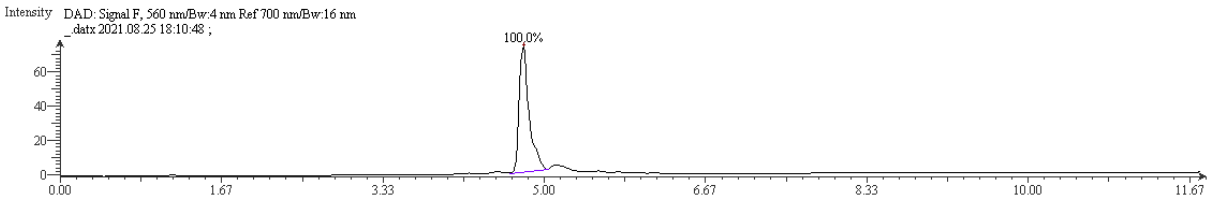
^{31}P NMR (202 MHz, Methanol-d_4) δ 9.68.

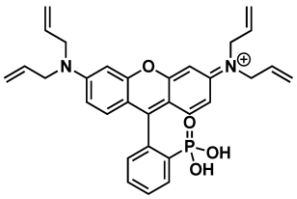
HR-ESI-MS m/z for $C_{25}H_{28}O_4N_2P^+$ $[M]^+$ calcd: 451.1781 found: 451.1774

254 nm



560 nm





*Synthesis of Compound **19.***

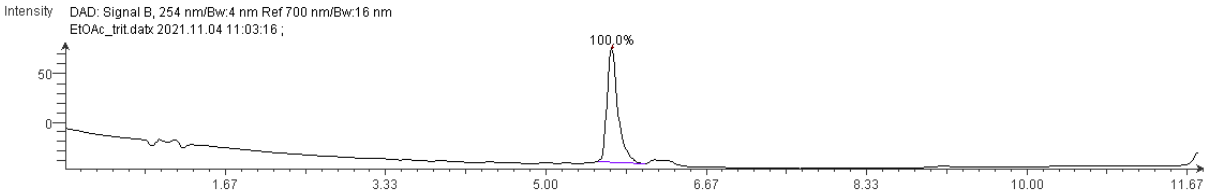
12 (440 mg, 0.81 mmol, 1 equiv.) and p-chloranil (297 mg, 1.21 mmol, 1.5 equiv.) were refluxed in MeOH (25 mL) for 12 hours. Upon cooling to room temperature, the solution was filtered, and the filtrate concentrated *in vacuo*. Trituration with EtOAc yielded **19** as a purple powder (397 mg, 0.75 mmol, 93%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.21 – 8.13 (m, 1H), 7.68 (pt, J = 7.6, 1.8 Hz, 2H), 7.28 – 7.24 (m, 1H), 7.21 (d, J = 9.4 Hz, 2H), 7.01 (dd, J = 9.5, 2.5 Hz, 2H), 6.97 (d, J = 2.4 Hz, 2H), 5.95 (ddt, J = 17.2, 10.1, 4.9 Hz, 4H), 5.32 – 5.16 (m, 8H), 4.26 (dd, J = 4.8, 2.0 Hz, 8H).

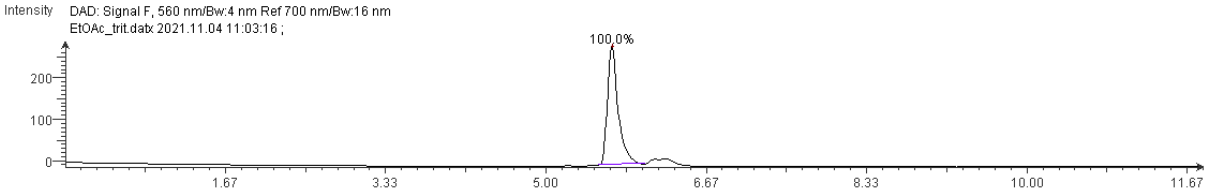
^{31}P NMR (202 MHz, Methanol-d_4) δ 9.29.

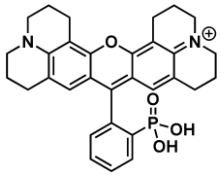
HR-ESI-MS m/z for $C_{31}H_{32}O_4N_2P^+$ $[M]^+$ calcd: 527.2094 found: 527.2094

254 nm



560 nm





*Synthesis of Compound **20**.*

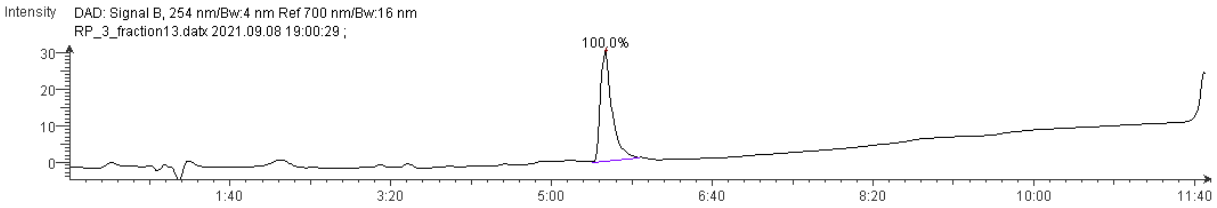
13 (50 mg, 0.09 mmol, 1 equiv.) was suspended in MeOH (20 mL) and refluxed for 48 hours whilst bubbling compressed air through the suspension. The solution was then cooled in an ice bath, filtered to remove unoxidized starting material, and the filtrate was concentrated *in vacuo*. Trituration with EtOAc yielded a crude solid that was purified by reverse phase silica chromatography (100% MeOH). **20** was isolated as a purple-pink solid (23 mg, 0.04 mmol, 48%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.08 (dd, J = 12.4, 8.0 Hz, 1H), 7.47 (dt, J = 23.8, 8.0 Hz, 2H), 7.00 (dd, J = 7.1, 3.9 Hz, 1H), 6.64 (s, 2H), 3.42 (t, J = 5.8 Hz, 4H), 3.37 (t, J = 5.7 Hz, 4H), 3.02 – 2.88 (m, 4H), 2.72 – 2.56 (m, 2H), 2.57 – 2.48 (m, 2H), 1.99 (p, J = 6.3 Hz, 4H), 1.83 (p, J = 5.5 Hz, 4H).

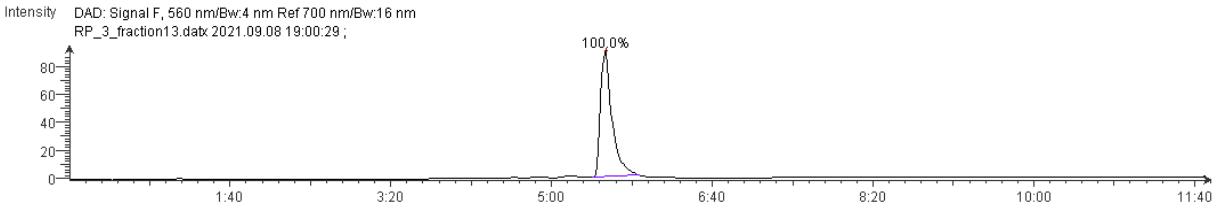
^{31}P NMR (202 MHz, Methanol-d_4) δ 7.76.

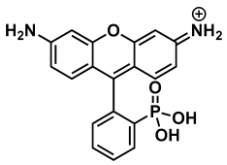
HR-ESI-MS m/z for $C_{31}H_{32}O_4N_2P_1^+$ $[M]^+$ calcd: 527.2094 found: 527.2104

254 nm



560 nm





*Synthesis of Compound **21.***

By air oxidation:

14 (100 mg, 0.26 mmol, 1 equiv.) was dissolved in DMSO (1 mL) and stirred at 100 °C for 12 hours. Upon cooling to room temperature, the crude solution was purified by reverse phase silica chromatography (60% MeOH/ H_2O) affording **21** as a red solid (21 mg, 0.05 mmol, 21%).

By deallylation of **19**: Method A (**Table S2**, entry A): **19** (50 mg, 0.095 mmol, 1 equiv.), 1,3-dimethylbarbituric acid (90 mg, 0.576 mmol, 6 equiv.) and $Pd(PPh_3)_4$ (4.5 mg, 0.004 mmol, 0.04 equiv.) were charged into a flame dried Schlenk flask, that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of DMF (0.75 mL) and triethylamine (0.25 mL), the solution was stirred at 80 °C overnight. Upon cooling to room temperature, the solution was diluted with EtOAc and filtered through a pad of celite. The celite was flushed several times with EtOAc to remove excess NDMBA, before elution of the fluorophore by washing with MeOH. Purification by reverse phase silica chromatography (60% MeOH/ H_2O) afforded **21** as an orange/red solid (18 mg, 0.049 mmol, 52 %).

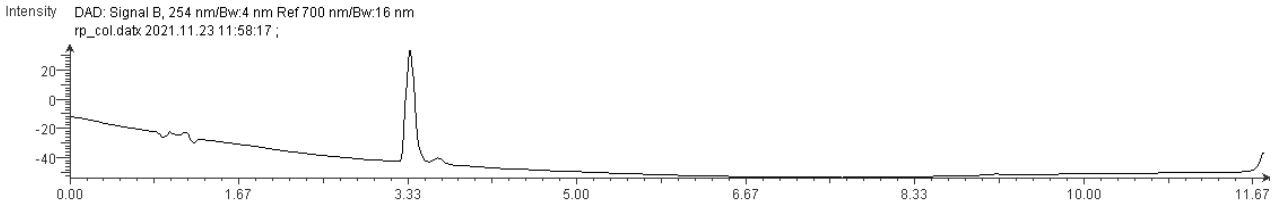
By deallylation of **19**: Method B (**Table S2**, entry B): **19** (65 mg, 0.12 mmol, 1 equiv.) and potassium tert-butoxide (95 mg, 0.85 mmol, 7 equiv.) were dissolved in DMSO (2.5 mL) and stirred at 100 °C for 5 minutes. Upon a color change to a deep brown solution water (7.5 mL) was added. This was followed by acidification with 1 M HCl (1 mL) and continued stirring at 100 °C for 15 minutes. The solution was cooled to room temperature and concentrated to a small volume of liquid < 3 mL. Purification by reverse phase silica chromatography (60% MeOH/ H_2O) afforded **21** as an orange/ red solid (31 mg, 0.085 mmol, 71%).

^{1}H NMR (500 MHz, Methanol-d_4 + 1 drop NaOD) δ 7.86 (dd, J = 10.0, 7.7 Hz, 1H), 7.40 (dtt, J = 7.5, 3.6, 1.9 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.10 (d, J = 8.7 Hz, 2H), 6.81 (dd, J = 7.7, 2.6 Hz, 1H), 6.53 (d, J = 2.2 Hz, 2H), 6.49 (dd, J = 8.7, 2.2 Hz, 2H).

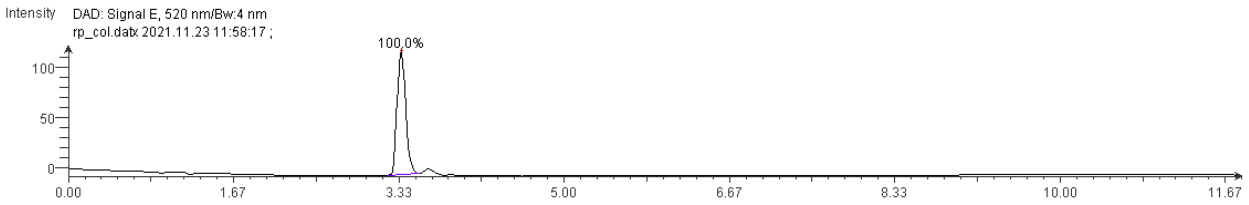
^{1}P NMR (202 MHz, Methanol-d_4 + 1 drop NaOD) δ 21.52.

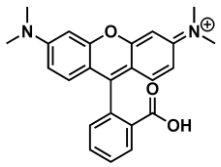
HR-ESI-MS m/z for $C_{19}H_{16}O_4N_2P^+$ $[M]^+$ calcd: 367.0842 found: 367.0844

254 nm



520 nm



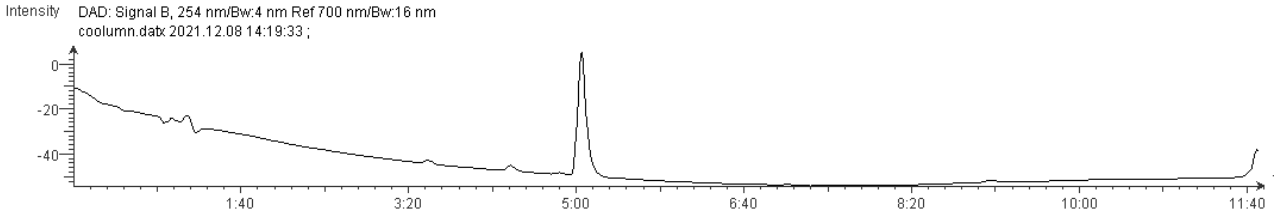


Synthesis of 3-carboxyTMR.

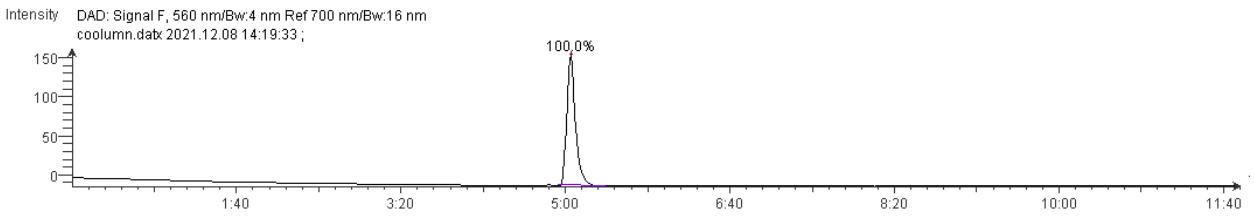
2-carboxybenzaldehyde (159 mg, 1.2 mmol, 1 equiv.) and 3-dimethylaminophenol, **2** (363 mg, 2.9 mmol, 2.5 equiv.) were charged into a round bottom flask that was subsequently evacuated and backfilled with N_2 (x3). TFE (10 mL) was added, and the solution was stirred at 80 °C for 3 days. Upon cooling to room temperature, and concentration *in vacuo* the residue was triturated with EtOAc to afford a pale purple solid (237 mg). The solid was combined with chloranil (286 mg, 1.2 mmol, 1 equiv.) and refluxed in MeOH (15 mL) for 12 hours. The solution was cooled, concentrated *in vacuo*, and purified by flash silica chromatography to afford **3-carboxytetramethylrhodamine** as a pink solid (147 mg, 0.36 mmol, 31%).

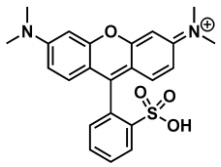
^{1}H NMR (400 MHz, Methanol-d_4) δ 8.23 – 8.12 (m, 1H), 7.73 – 7.67 (m, 2H), 7.33 – 7.27 (m, 1H), 7.24 (d, J = 9.5 Hz, 2H), 7.02 (dd, J = 9.4, 2.3 Hz, 2H), 6.93 (d, J = 2.0 Hz, 2H), 3.28 (s, 12H).

254 nm



560 nm





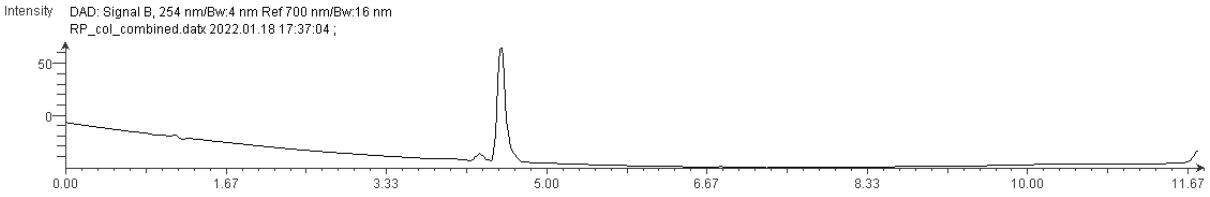
*Synthesis of **3-sulfoTMR**.*

2-sulfonobenzaldehyde (139 mg, 0.75 mmol, 1 equiv.) and 3-dimethylaminophenol, **2** (257 mg, 1.88 mmol. 2.5 equiv.) were dissolved in methanesulfonic acid (2 mL) and stirred at 130 °C for 72 hours. Upon cooling to room temperature, the solution was diluted with water (20 mL) and made basic with addition of 10 M KOH. Extraction into 3:1 DCM: ^{i}PrOH (x4), drying over Na_2SO_4 and concentration *in vacuo* afforded a deep purple solid which was subsequently purified by reverse phase silica chromatography (60% MeOH/ H_2O). **3-sulfoTMR** was isolated as a dark purple solid (36 mg, 0.085 mmol, 12%).

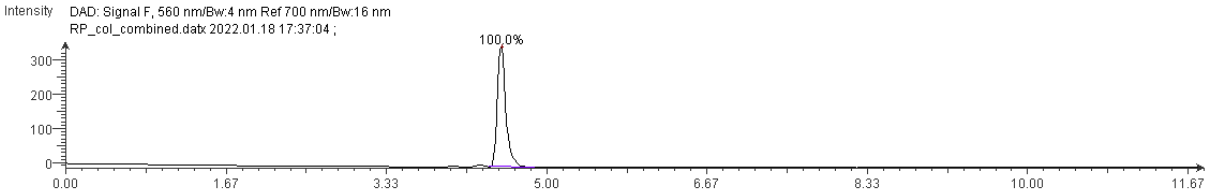
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 – 7.95 (m, 1H), 7.67 – 7.60 (m, 1H), 7.56 (t, J = 7.5 Hz, 1H), 7.18 (d, J = 8.1 Hz, 1H), 7.07 – 6.99 (m, 4H), 6.90 (d, J = 1.4 Hz, 2H), 3.24 (s, 12H).

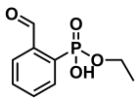
HR-ESI-MS m/z for $C_{23}H_{23}O_4N_2S^+$ $[M]^+$ calcd: 423.1373 found: 423.1376

254 nm



560 nm



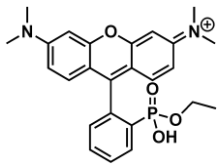


*Synthesis of Compound **22.***

diethyl (2-formylphenyl)phosphonate (685 mg, 2.8 mmol, 1 equiv.) was dissolved in DCM (3 mL) and added to a solution of 1 M NaOH (3 mL) with tetrabutylammonium bromide (273 mg, 0.9 mmol. 0.3 equiv.). The biphasic mixture was stirred vigorously at 40 °C overnight. Upon cooling to room temperature the mixture was diluted with water and the aqueous layer was washed with EtOAc (x3). The aqueous layer was then acidified with 1 M HCl and extracted into EtOAc (x3). Drying of the pooled organics with Na_2SO_4 and concentration *in vacuo* afforded **22** as a golden viscous oil (415 mg, 1.9 mmol, 70%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 10.59 (s, 1H), 8.21 – 7.99 (m, 2H), 7.77 – 7.62 (m, 2H), 4.20 – 4.12 (m, 2H), 1.32 (t, *J* = 7.1 Hz, 3H).

^{31}P NMR (202 MHz, Chloroform-*d*) δ 18.45.



*Synthesis of Compound **23.***

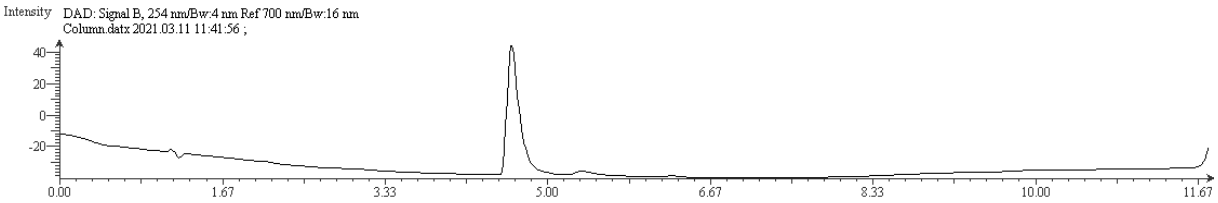
22 (75 mg, 0.35 mmol, 1 equiv.) and 3-dimethylaminophenol, **2** (112 mg, 0.88 mmol, 2.5 equiv.) were stirred in TFE (7 mL) at 80 °C overnight. Upon cooling to room temperature, the solution was concentrated *in vacuo* and the residue was triturated with EtOAc to afford a pale purple solid. The solid was combined with chloranil (90 mg, 0.37 mmol, 1.05 equiv.) and refluxed in MeOH (5 mL) for 3 hours. Purification by flash silica chromatography afforded **23** as a purple solid (62 mg, 0.14 mmol, 39%).

^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.25 (dd, *J* = 12.1, 7.8 Hz, 1H), 7.58 (t, *J* = 7.4 Hz, 1H), 7.47 (t, *J* = 7.4 Hz, 1H), 7.39 (d, *J* = 9.4 Hz, 2H), 7.05 (dd, *J* = 7.8, 3.2 Hz, 1H), 6.83 (dd, *J* = 9.4, 2.0 Hz, 2H), 6.73 – 6.67 (m, 2H), 3.72 (p, *J* = 6.9 Hz, 2H), 3.23 (s, 12H), 1.02 (t, *J* = 7.0 Hz, 3H).

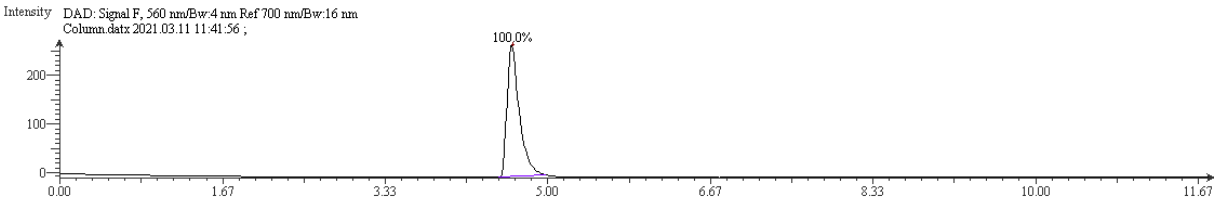
^{31}P NMR (202 MHz, Chloroform-*d*) δ 6.17.

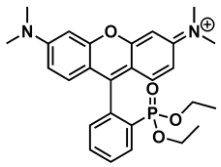
HR-ESI-MS m/z for $C_{25}H_{28}O_4N_2P^+$ $[M]^+$ calcd: 451.1781 found: 451.1782

254 nm



560 nm





*Synthesis of Compound **24.***

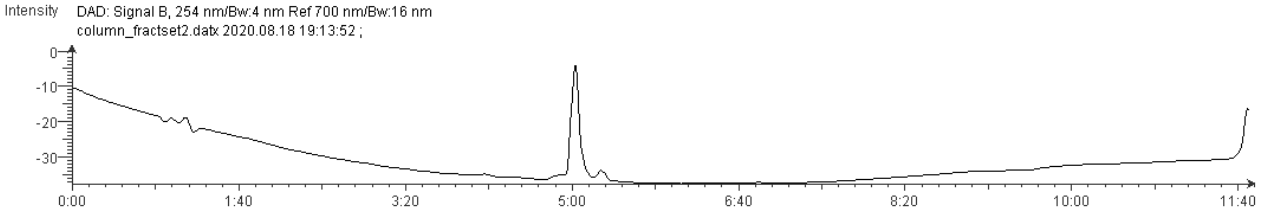
16 (200 mg, 0.47 mmol, 1 equiv.), Ag_2O (237 mg, 1.18 mmol, 2.5eq) and 4 Å molecular sieves (200 mg) were charged into a flame dried round bottom flask that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of MeCN (12 mL), the suspension was stirred at room temperature for 30 minutes. Bromoethane (0.11 mL, 1.41 mmol, 3.5 equiv.) was added, and the reaction stirred at 50 °C overnight. A second addition of bromoethane (0.2 mL, 2.1 mmol, 4.5 equiv.) was added and the reaction stirred for a further 6 hours. Upon cooling to room temperature, the suspension was filtered through a pad of celite and washed with methanol, and the filtrate was concentrated *in vacuo*. Purification by flash silica chromatography (5 to 10% MeOH/DCM) yielded **24** as a purple solid (127 mg, 0.27 mmol, 22%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.11 (dd, *J* = 14.0, 7.8 Hz, 1H), 7.79 (t, *J* = 7.2 Hz, 1H), 7.72 (td, *J* = 7.6, 3.4 Hz, 1H), 7.45 – 7.33 (m, 1H), 7.07 (d, *J* = 9.4 Hz, 2H), 6.99 – 6.75 (m, 4H), 3.91 – 3.72 (m, 4H), 3.34 (s, 12H), 1.01 (t, *J* = 7.1 Hz, 6H).

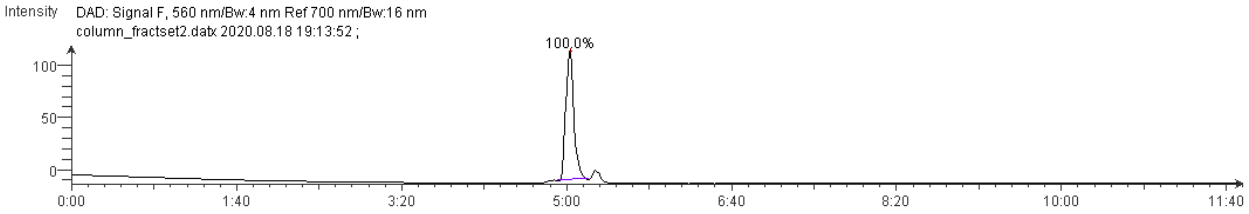
^{31}P NMR (162 MHz, Chloroform-*d*) δ 15.16.

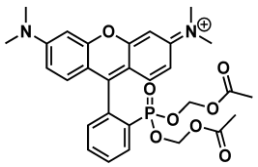
HR-ESI-MS m/z for $C_{27}H_{32}O_4N_2P^+$ $[M]^+$ calcd: 479.2094 found: 479.2093

254 nm



560 nm





*Synthesis of Compound **25***

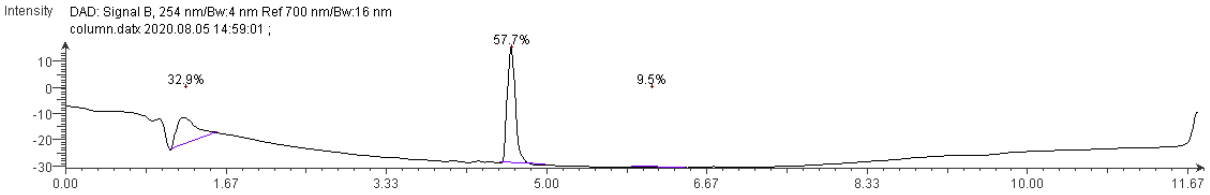
16 (46 mg, 0.11 mmol, 1 equiv.), Ag_2O (63 mg, 0.27 mmol, 2.5 equiv.) and 4 Å molecular sieves (50 mg) were charged into a flame dried round bottom flask, that was subsequently evacuated and backfilled with N_2 (x3). Upon addition of MeCN (4 mL), the suspension was stirred at room temperature for 1 hour and then bromomethyl acetate (0.04 mL, 0.039 mmol, 3.5 equiv.) was added. The suspension was stirred at 50 °C overnight, before cooling to room temperature, and filtering through a pad of celite and washed with DCM. The filtrate was concentrated *in vacuo* and purified by flash silica chromatography (8% MeOH/ DCM) to afford **25** as a purple solid (15 mg, 0.03 mmol, 25%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.10 (dd, *J* = 14.6, 7.7 Hz, 1H), 7.86 (t, *J* = 7.8 Hz, 1H), 7.82 – 7.68 (m, 1H), 7.41 (t, *J* = 6.3 Hz, 1H), 7.05 (d, *J* = 9.4 Hz, 2H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.91 (s, 2H), 5.51 – 5.12 (m, 4H), 3.37 (s, 12H), 1.99 (s, 6H).

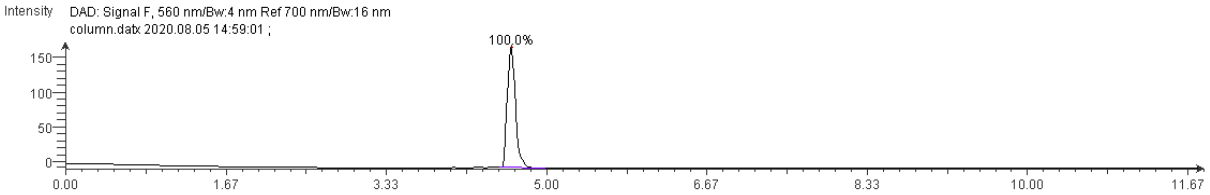
^{31}P NMR (162 MHz, Chloroform-*d*) δ 14.34.

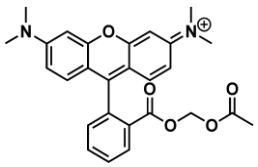
HR-ESI-MS m/z for $C_{29}H_{32}O_8N_2P^+$ $[M]^+$ calcd: 567.1891 found: 467.1886

254 nm



560 nm





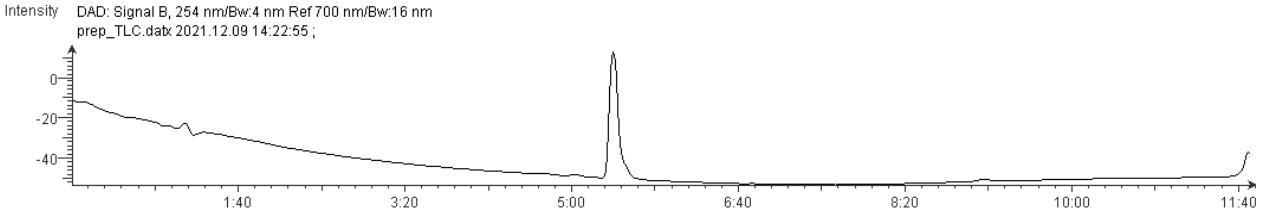
*Synthesis of Compound **26***

(50 mg, 0.13 mmol, 1 equiv.), Ag_2O (45 mg, 0.19 mmol, 1.5 equiv.) and 4 Å molecular sieves (50 mg) were charged into a flame dried reaction vial that was subsequently evacuated and backfilled with N_2 (x3). DMF (1.5 mL) was added, and the reaction warmed to 60 °C. Bromomethyl acetate (0.03 mL, 0.32 mmol, 2.5 equiv.) was added and the suspension stirred for 16 hours. Upon cooling to room temperature, the mixture was diluted with DCM and filtered through a plug of celite. Concentration *in vacuo*, followed by flash silica chromatography (7.5% MeOH/DCM) afforded **26** as a dark purple solid (19 mg, 0.04 mmol, 32%).

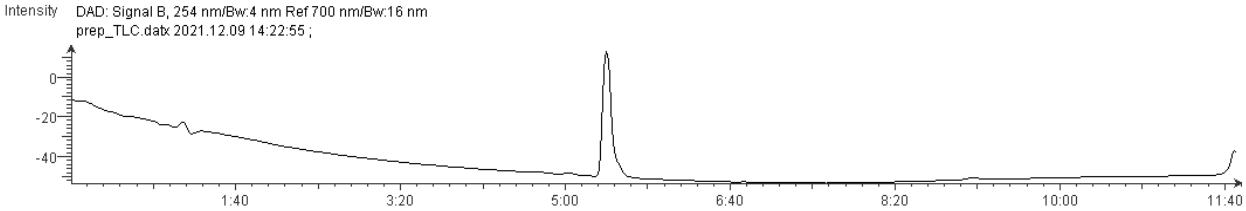
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.26 (dd, J = 7.9, 1.3 Hz, 1H), 7.95 (td, J = 7.5, 1.4 Hz, 1H), 7.87 (td, J = 7.7, 1.4 Hz, 1H), 7.54 (dd, J = 7.5, 1.3 Hz, 1H), 7.10 (dd, J = 9.5, 2.4 Hz, 2H), 7.01 (d, J = 9.5 Hz, 2H), 6.99 (d, J = 2.5 Hz, 2H), 5.55 (s, 2H), 3.28 (s, 12H), 1.83 (s, 3H).

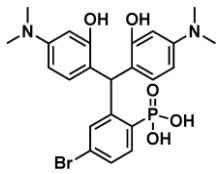
HR-ESI-MS m/z for $C_{27}H_{27}O_5N_2^+$ $[M]^+$ calcd: 459.1914 found: 459.1912

254 nm



560 nm





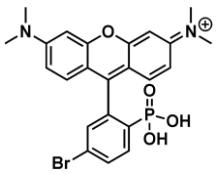
*Synthesis of Compound **28***

5-bromo-2-phosphonobenzaldehyde, **27** (400 mg, 1.51 mmol, 1 equiv.) and 3-(dimethylamino)phenol (517 mg, 3.77 mmol, 2.5 equiv.) were stirred in TFE (30 mL) at 80 ºC for 16 hrs. The resulting suspension was concentrated *in vacuo*, and triturated with EtOAc, yielding **28** as a pale purple powder (786 mg, 1.51 mmol, >99%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.61 (dd, J = 13.2, 8.2 Hz, 1H), 7.51 – 7.45 (m, 1H), 7.23 (dd, J = 4.0, 1.7 Hz, 1H), 6.57 – 6.48 (m, 3H), 6.18 – 6.06 (m, 4H), 2.80 (s, 12H).

^{31}P NMR (202 MHz, DMSO-d_6) δ 13.11.

HR-ESI-MS m/z for $C_{23}H_{25}O_5N_2Br_1P_1^-$ [M-H]$^-$ calcd: 519.0690 found: 519.0678



*Synthesis of Compound **29***

6-bromo-3-phosphono-tetramethylrhodamine

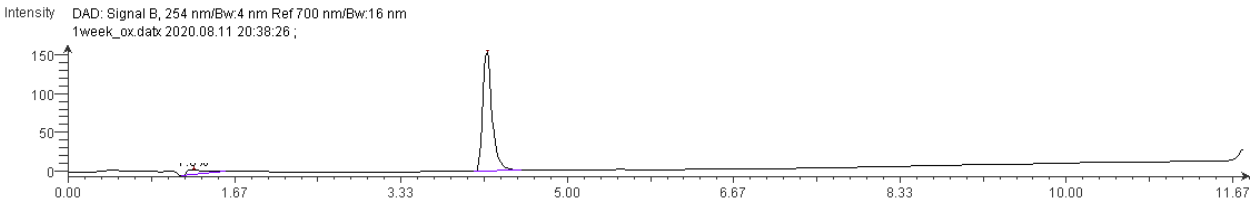
28 (780 mg, 1.49 mmol, 1 equiv.) and p-chloranil (445 mg, 1.81 mmol, 1.2 equiv.) were refluxed in MeOH (50 mL) for 24 hours. Upon cooling to room temperature, the solution was filtered, and the filtrate was concentrated *in vacuo*. Trituration with EtOAc yielded **29** as a dark purple powder (748 mg, 1.49 mmol, >99%).

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.06 (dd, J = 12.9, 8.3 Hz, 1H), 7.89 (dt, J = 8.3, 2.0 Hz, 1H), 7.54 (dd, J = 3.6, 1.9 Hz, 1H), 7.17 (d, J = 9.5 Hz, 2H), 7.04 (dd, J = 9.5, 2.4 Hz, 2H), 6.92 (d, J = 2.4 Hz, 2H), 3.30 (s, 12H).

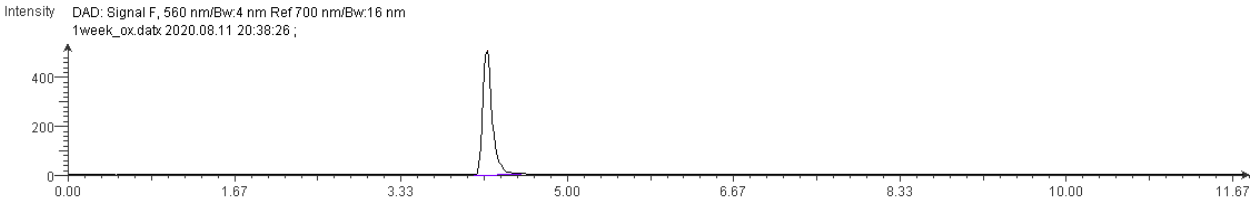
^{31}P NMR (202 MHz, Methanol-d_4) δ 8.70.

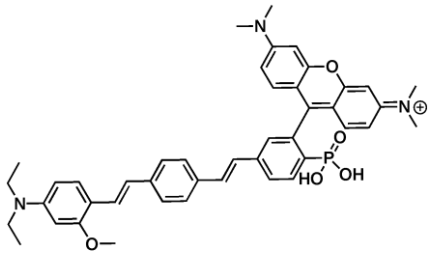
HR-ESI-MS m/z for $C_{23}H_{23}O_4N_2Br_1P_1^+$ $[M]^+$ calcd: 501.0573 found: 501.0572

254 nm



560 nm





*Synthesis of Compound **30***

phosRhoVR 1

29 (357 mg, 0.71 mmol, 1 equiv.), molecular wire (240 mg, 0.78 mmol, 1.1 equiv.), $Pd(OAc)_2$ (16 mg, 0.07 mmol, 0.1 equiv.), P(*o*-tol)$_3$ (43 mg, 0.14 mmol, 0.2 equiv.) were charged into a flame dried round bottom flask and was subsequently evacuated and backfilled with N_2 (x3). Upon addition of DMF (10.5 mL) and NEt_3 (5.25 mL), the solution was stirred at 110 ºC under static N_2 for 16 hours. After cooling to room temperature, the solution was diluted with DCM and flushed through a celite plug. The filtrate was concentrated *in vacuo* and triturated with EtOAc to yield a crude red solid (658).* **30** could be purified by reverse phase silica chromatography (80% to 100% MeOH/H_2O) (432 mg, 0.59 mmol, 76%).

*Crude solid was a relatively clean NEt_3 salt.

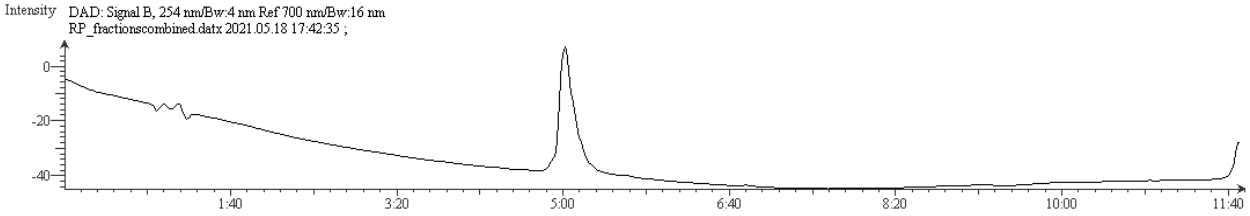
^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 – 7.43 (m, 3H), 7.45 – 7.32 (m, 4H), 7.31 – 7.22 (m, 3H), 7.07 (d, *J* = 16.6 Hz, 1H), 7.01 (d, *J* = 16.3 Hz, 1H), 6.89 (d, *J* = 16.5 Hz, 1H), 6.62 (s, 1H), 6.44 (dd, *J* = 8.8, 2.4 Hz, 2H), 6.40 (d, *J* = 2.4 Hz, 2H), 6.27 (dd, *J* = 8.7, 2.1 Hz, 1H), 6.19 (d, *J* = 2.0 Hz, 1H), 3.82 (s, 3H), 2.90 (s, 12H), 1.11 (t, *J* = 7.0 Hz, 6H).

Ethyl CH_2's hidden under H_2O peak

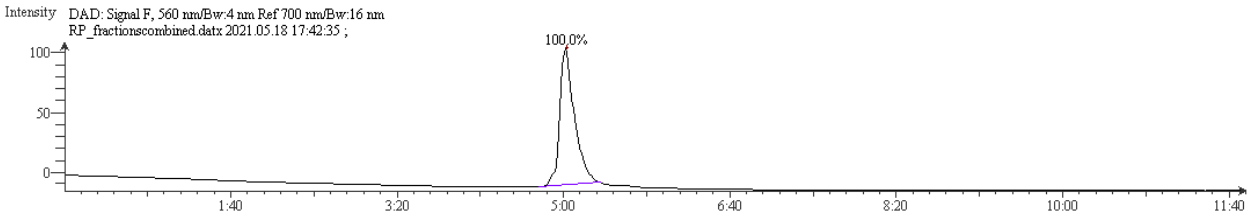
^{31}P NMR (202 MHz, DMSO-d_6) δ 22.57.

HR-ESI-MS m/z for $C_{44}H_{47}O_5N_3P_1^+$ $[M]^+$ calcd: 728.3248 found: 728.3236

254 nm

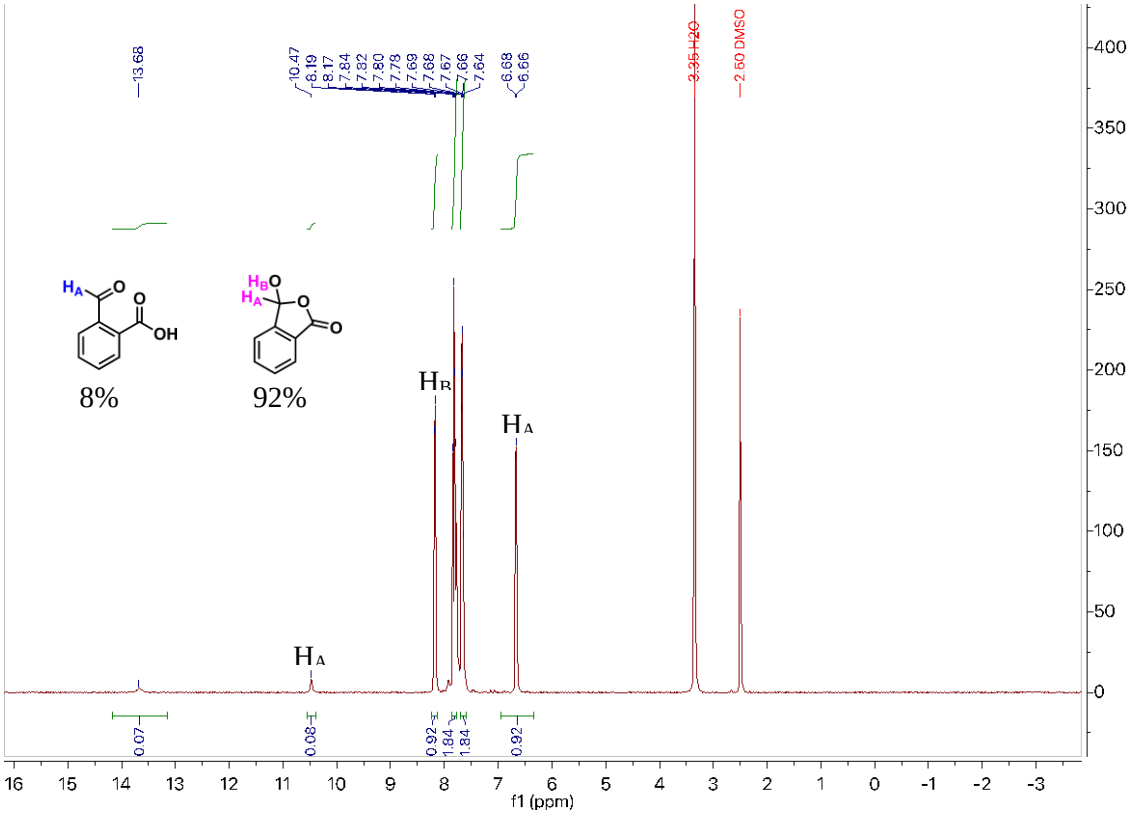


560 nm

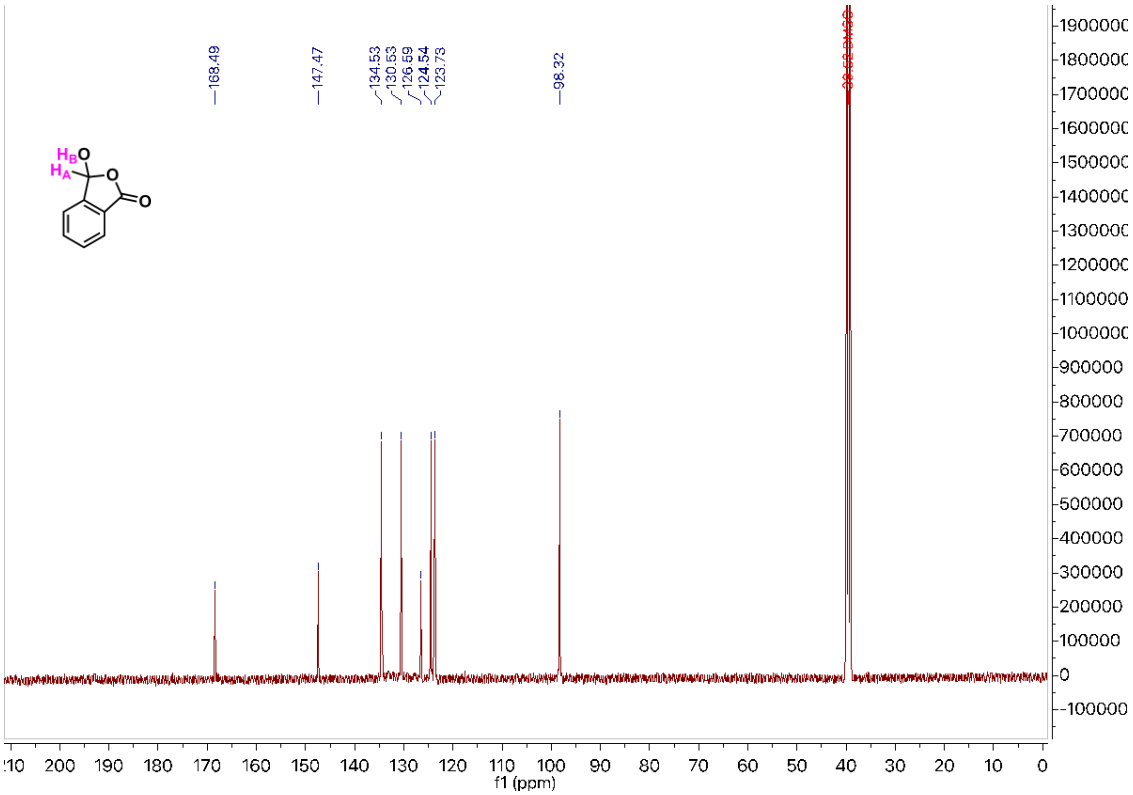


Supporting Spectra

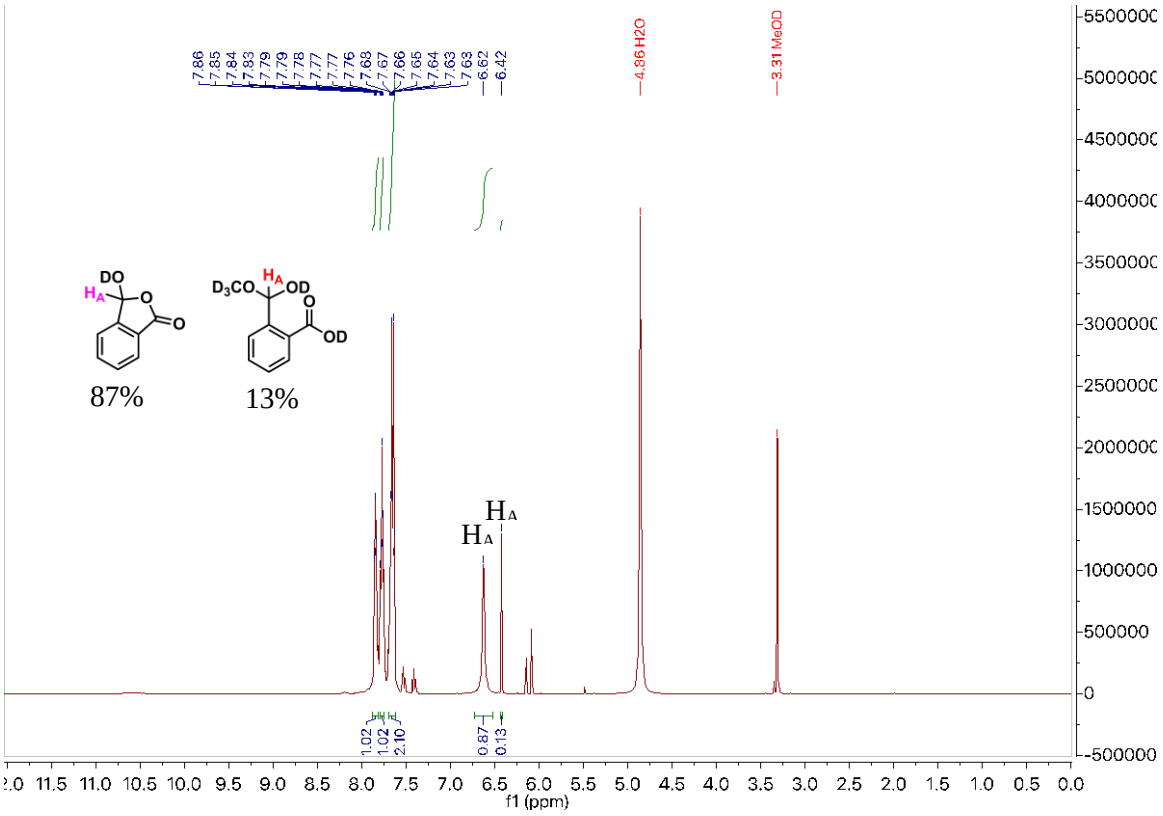
Spectrum S1. ^{1}H NMR of 2-carboxybenzaldehyde in DMSO-d_6



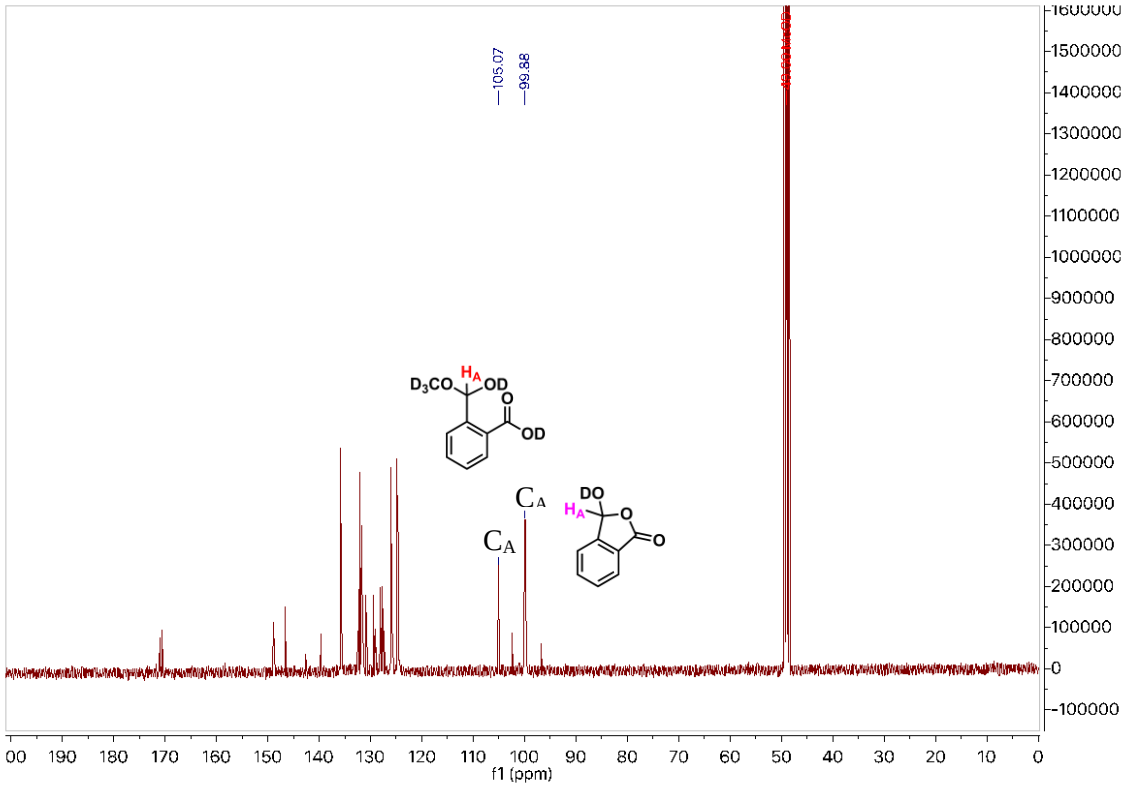
Spectrum S2. ^{13}C NMR of 2-carboxybenzaldehyde in DMSO-d_6



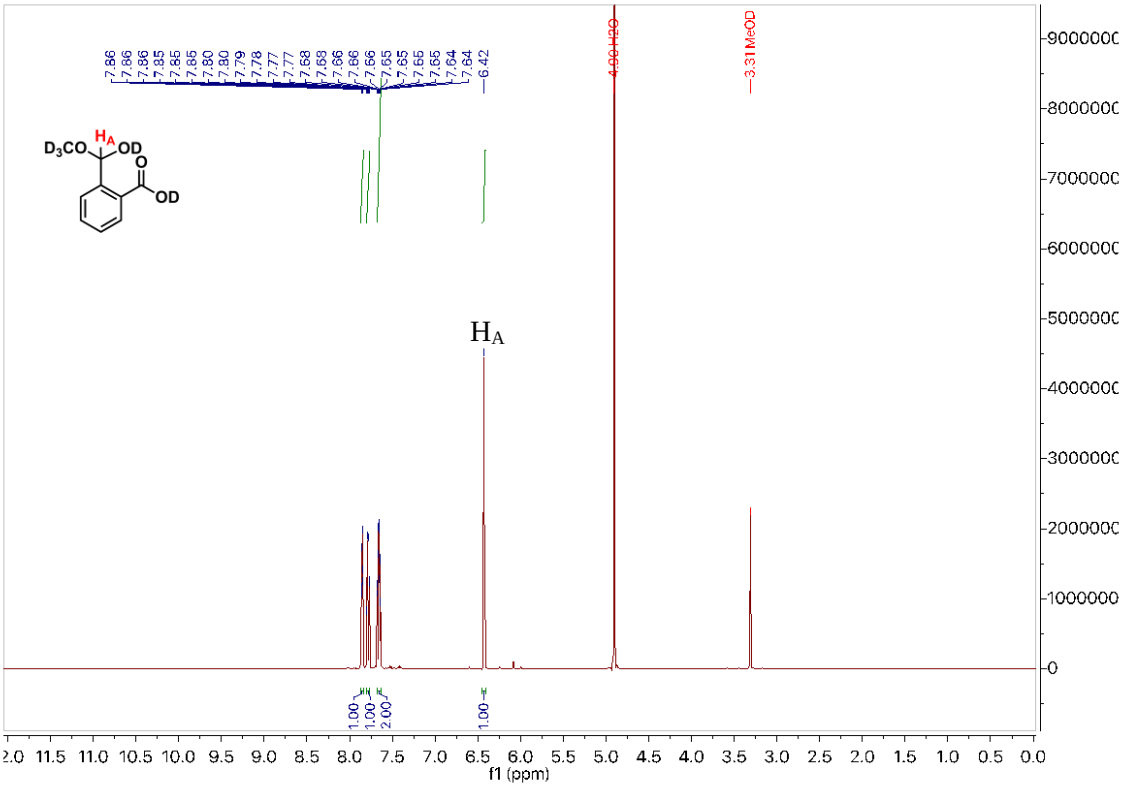
Spectrum S3. ^{1}H NMR of 2-carboxybenzaldehyde in CD_3OD



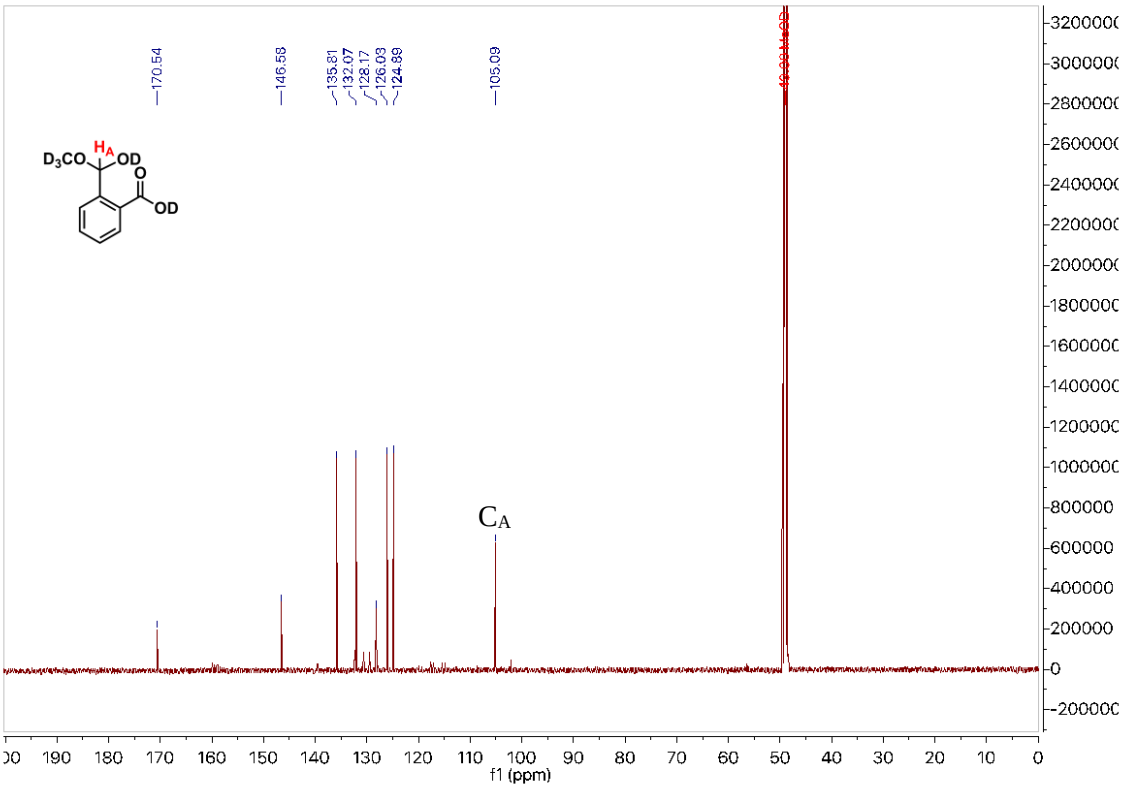
Spectrum S4. ^{13}C NMR of 2-carboxybenzaldehyde in CD_3OD



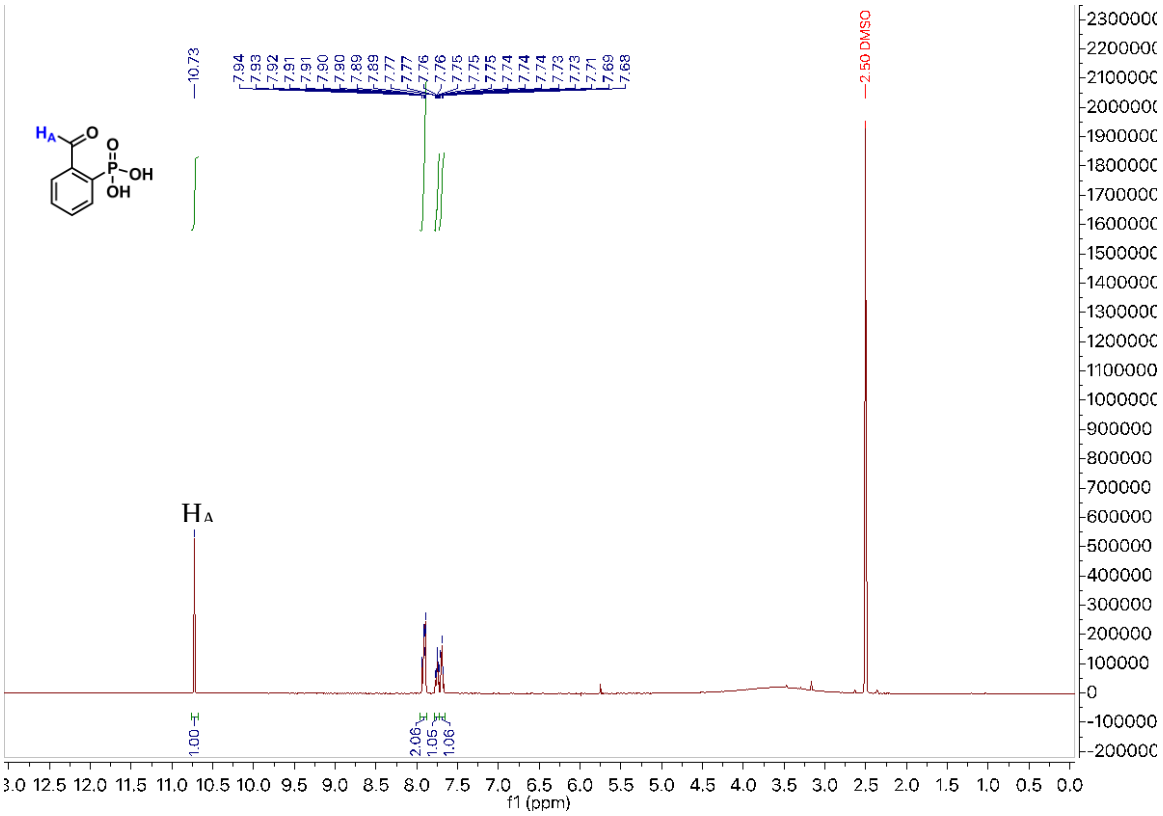
Spectrum S5. ^{1}H NMR of 2-carboxybenzaldehyde in CD_3OD + TFA



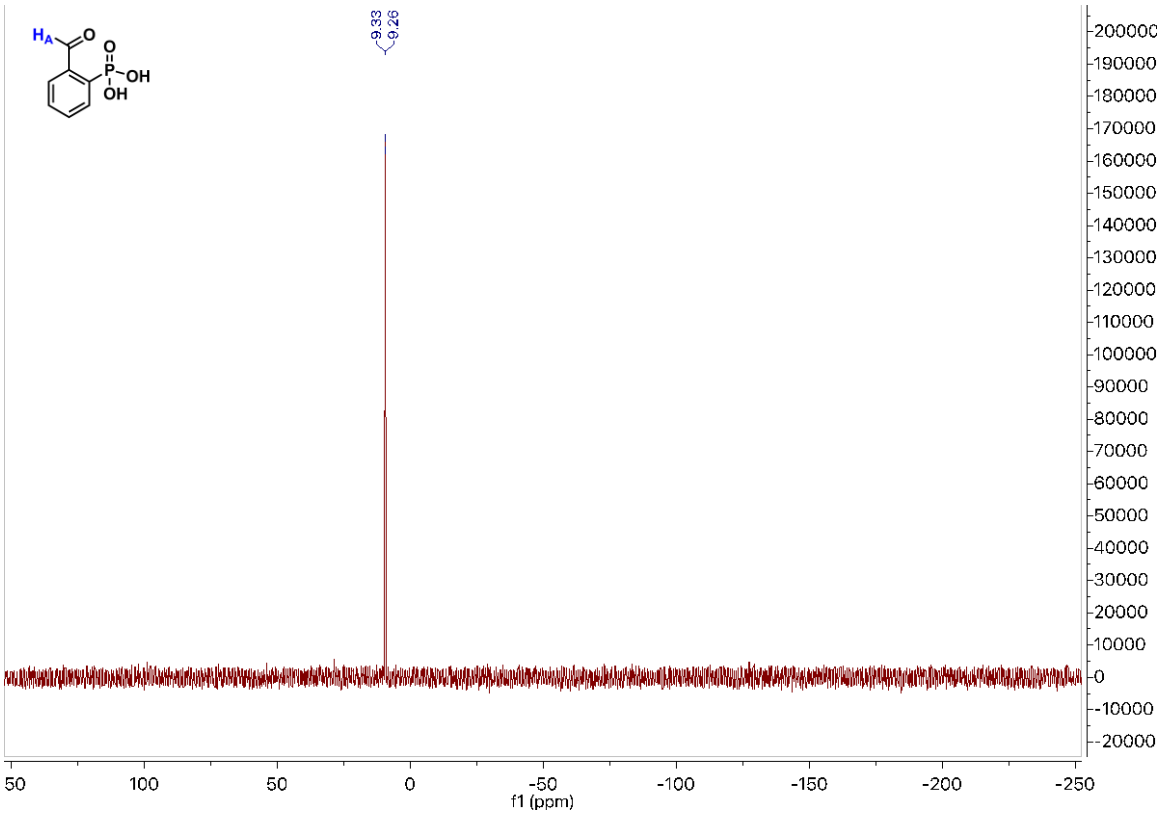
Spectrum S6. ^{13}C NMR of 2-carboxybenzaldehyde in CD_3OD + TFA



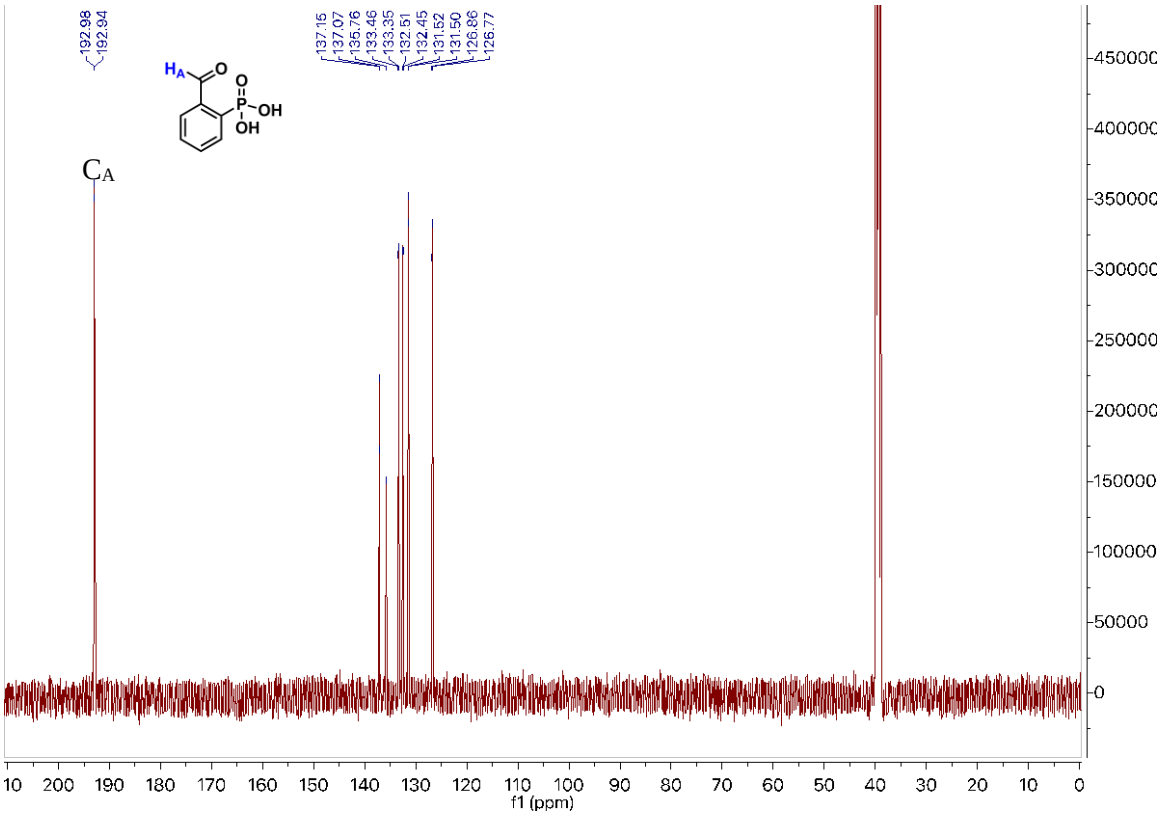
Spectrum S7. ^{1}H NMR of Compound **1** in DMSO-6_6



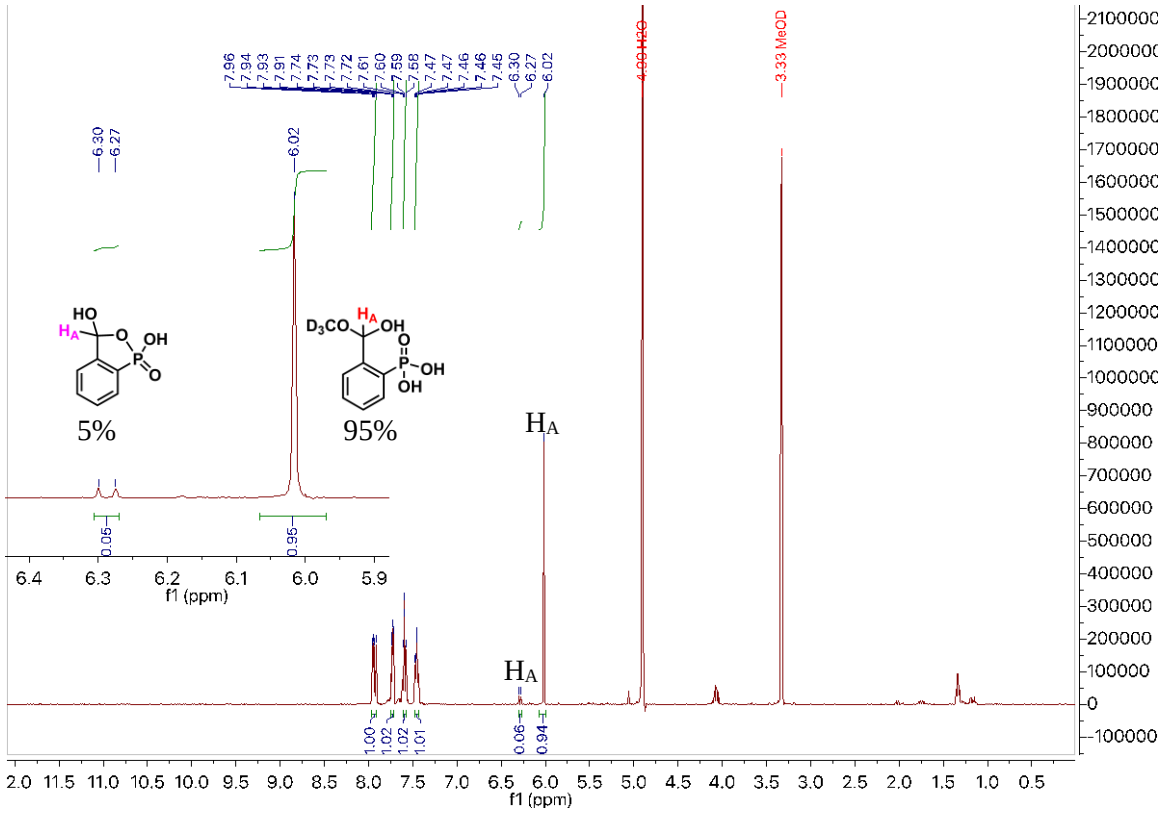
Spectrum S8. ^{31}P NMR of Compound **1** in DMSO-6_6



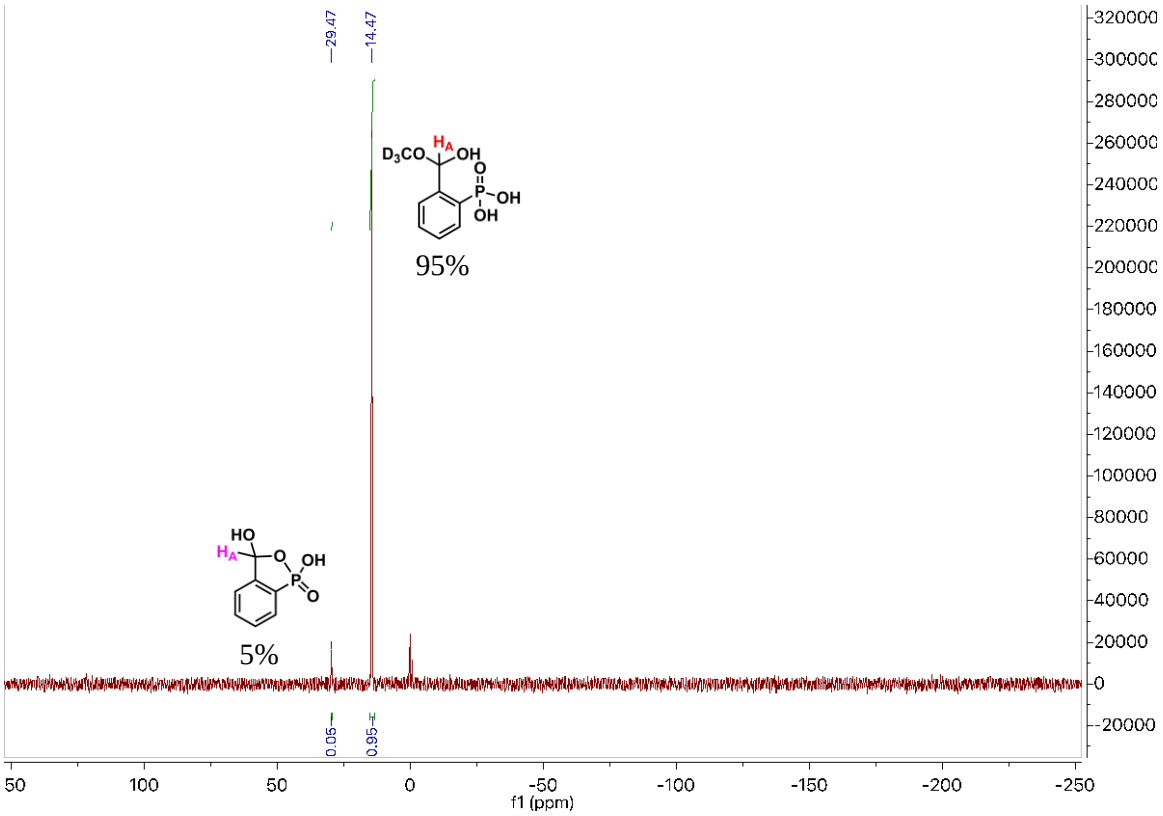
Spectrum S9. ^{13}C NMR of Compound **1** in DMSO-6_6



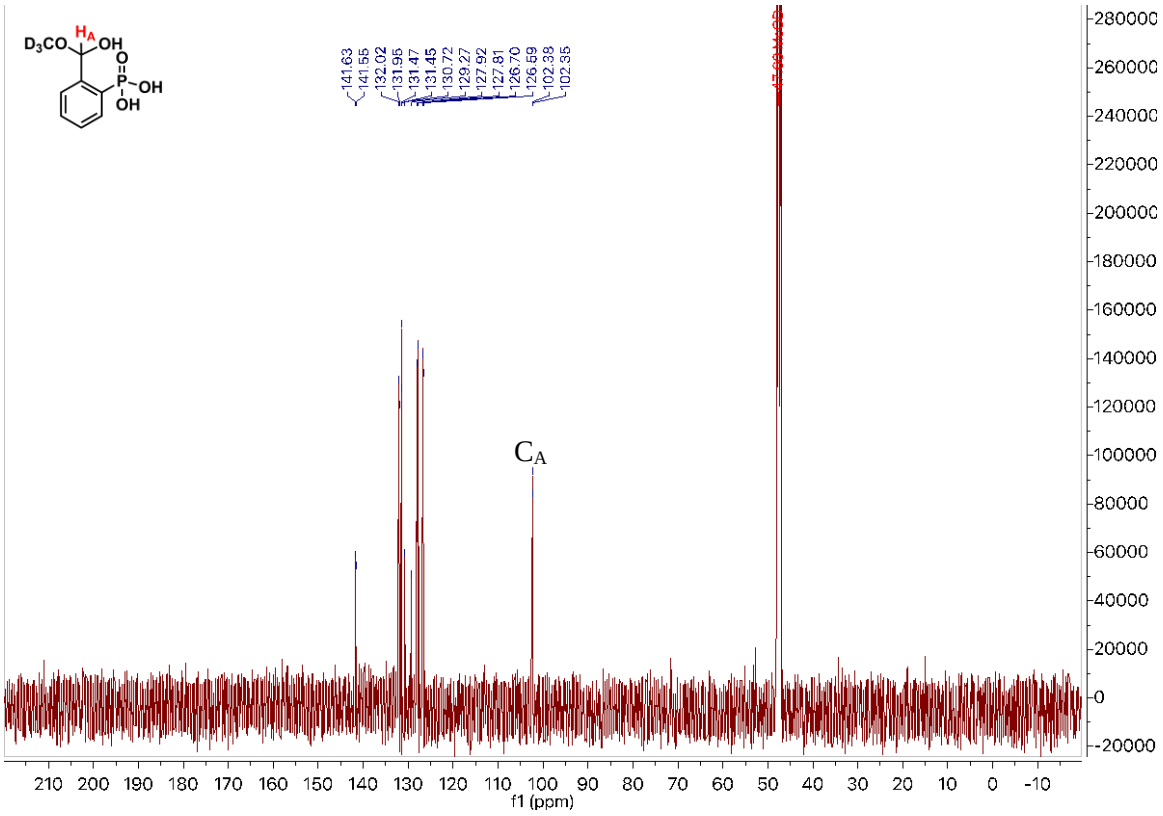
Spectrum S10. ^{1}H NMR of Compound **1** in CD_3OD



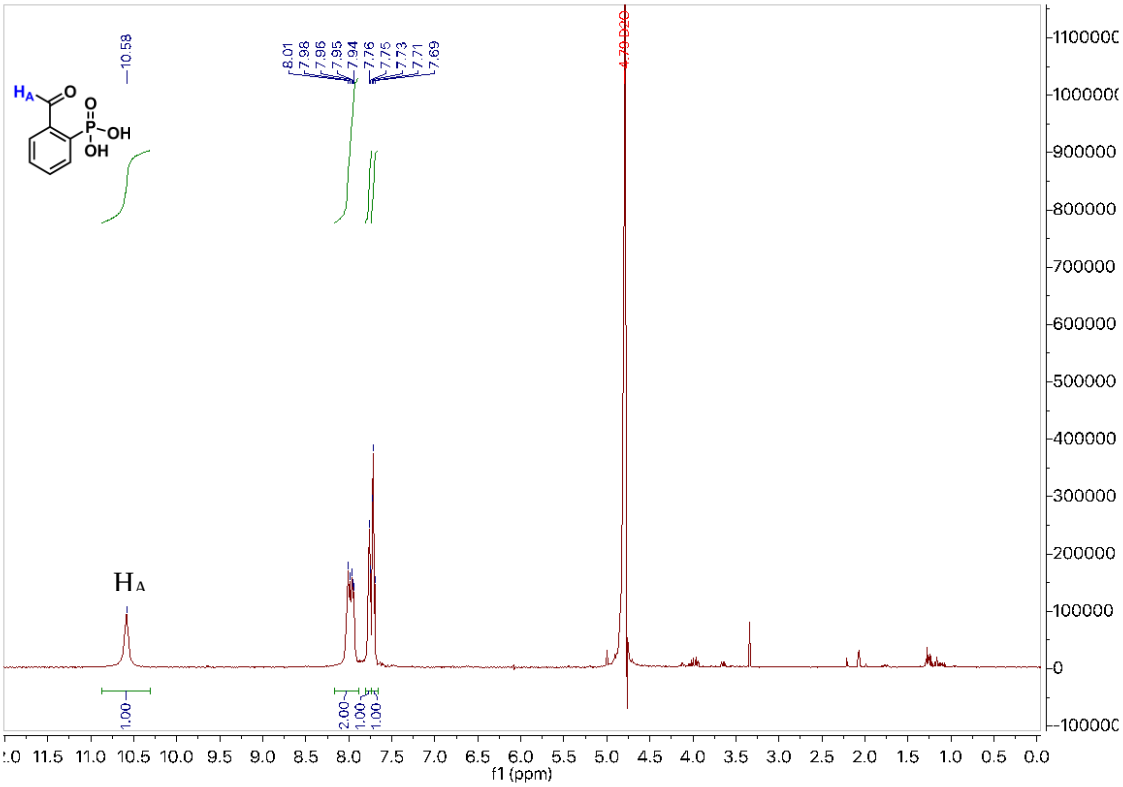
Spectrum S11. ^{31}P NMR of Compound **1** in CD_3OD



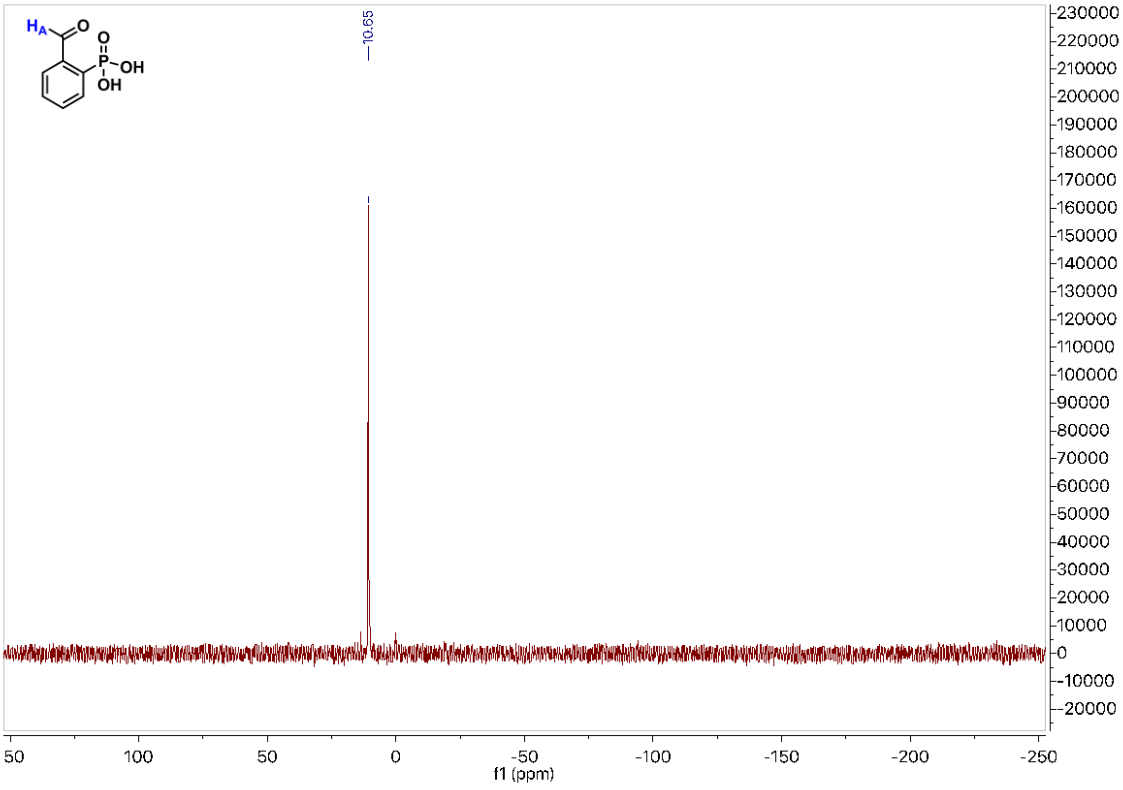
Spectrum S12. ^{13}C NMR of Compound **1** in CD_3OD



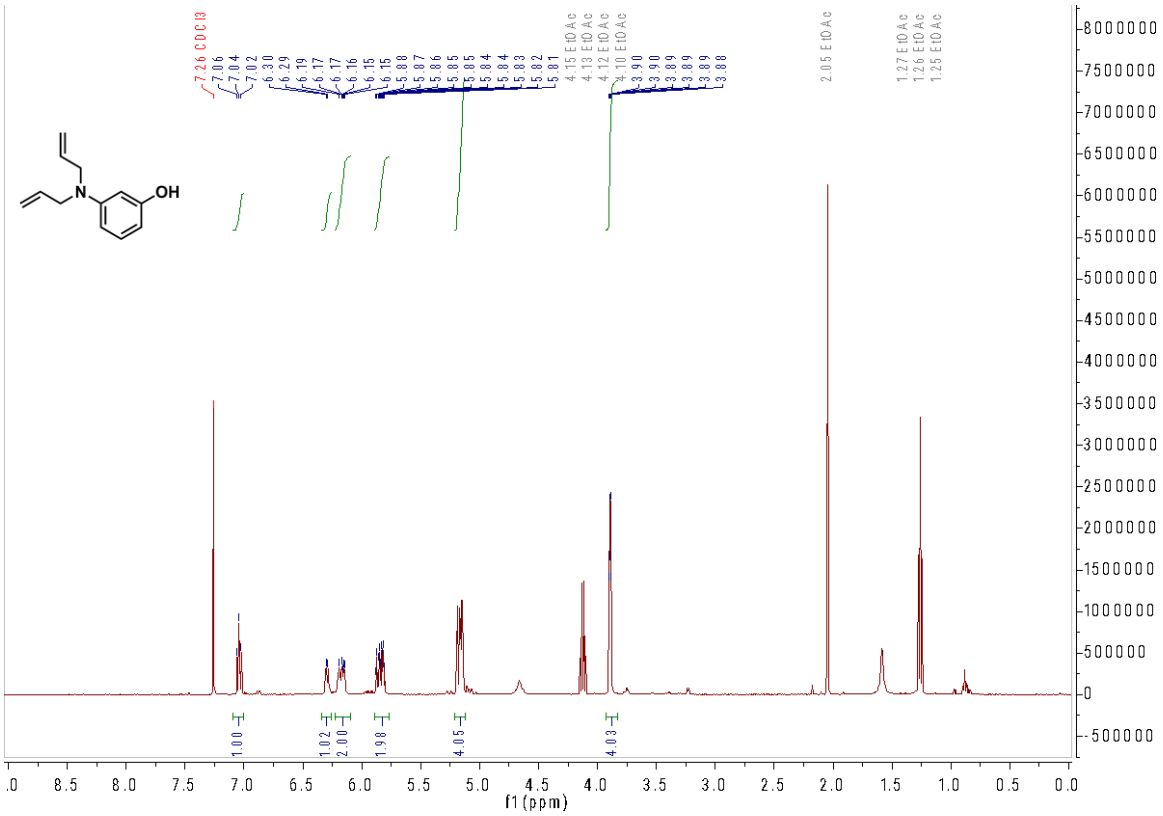
Spectrum S13. ^{1}H NMR of Compound **1** in D_2O



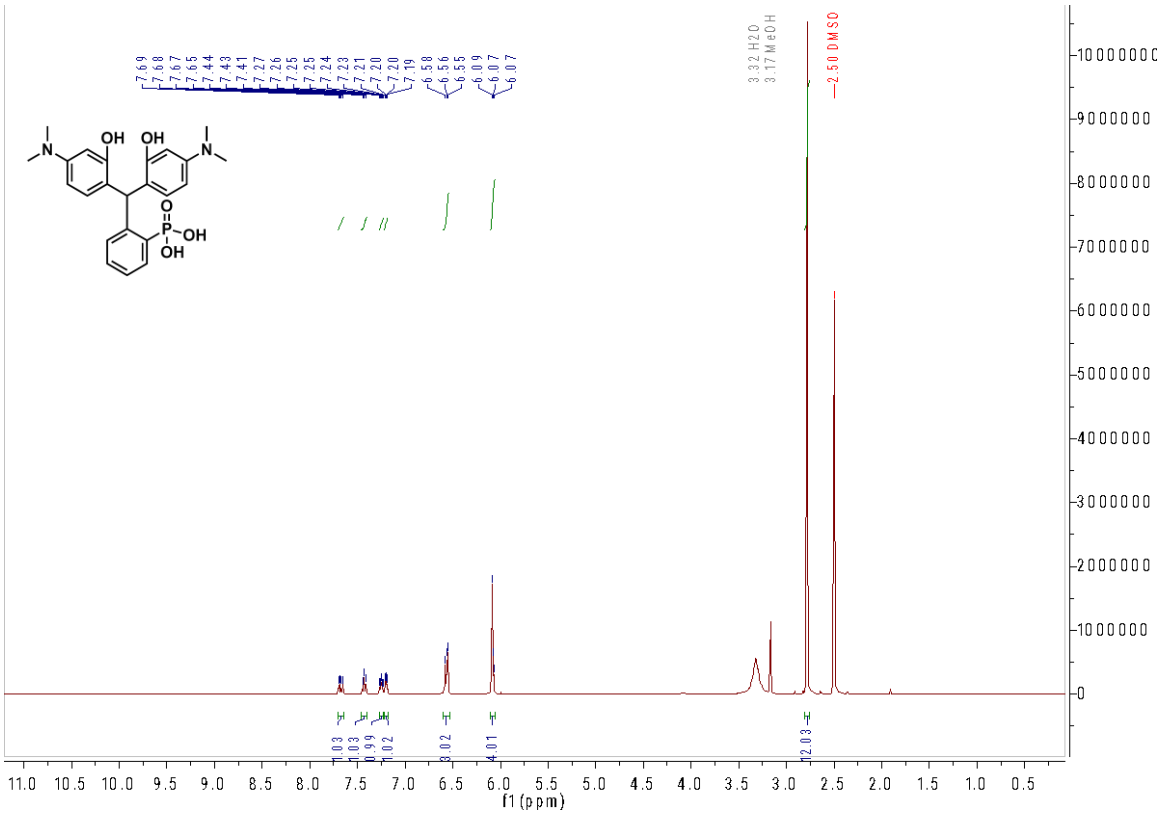
Spectrum S14. ^{31}P NMR of Compound **1** in D_2O



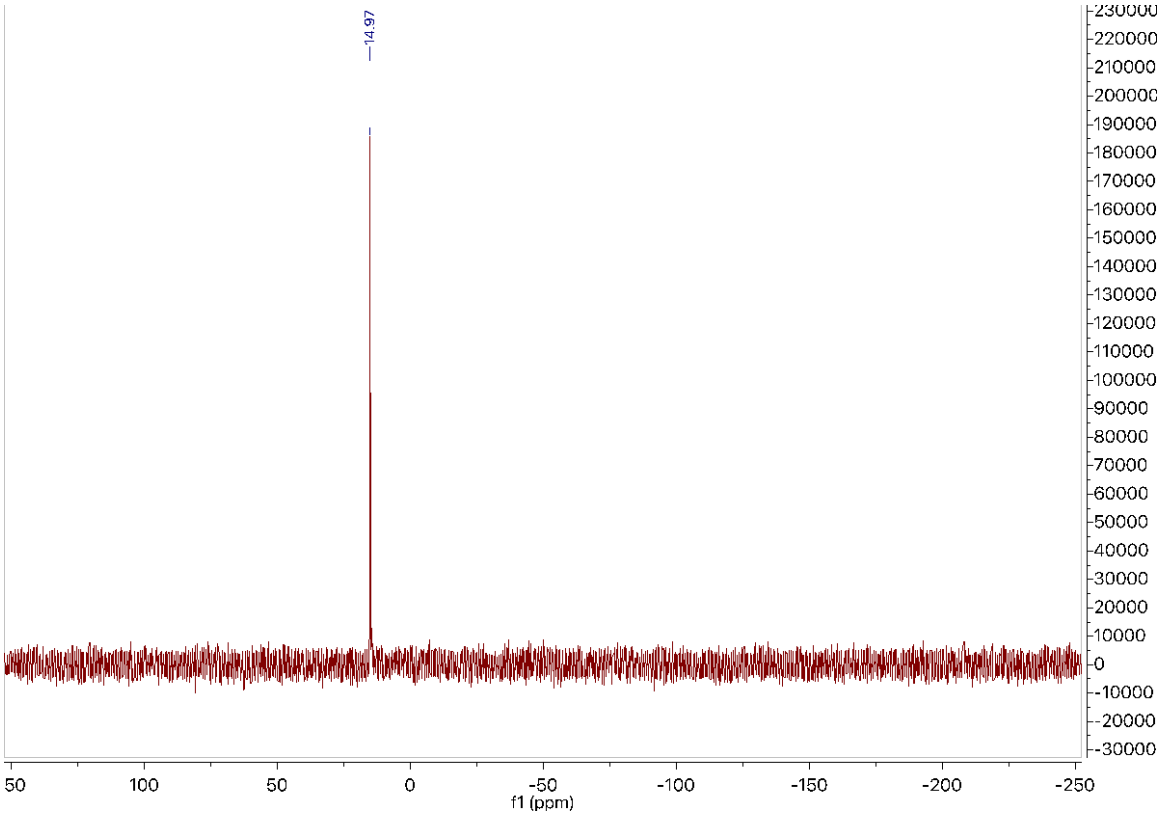
Spectrum S15. ^{1}H NMR of Compound **5**.



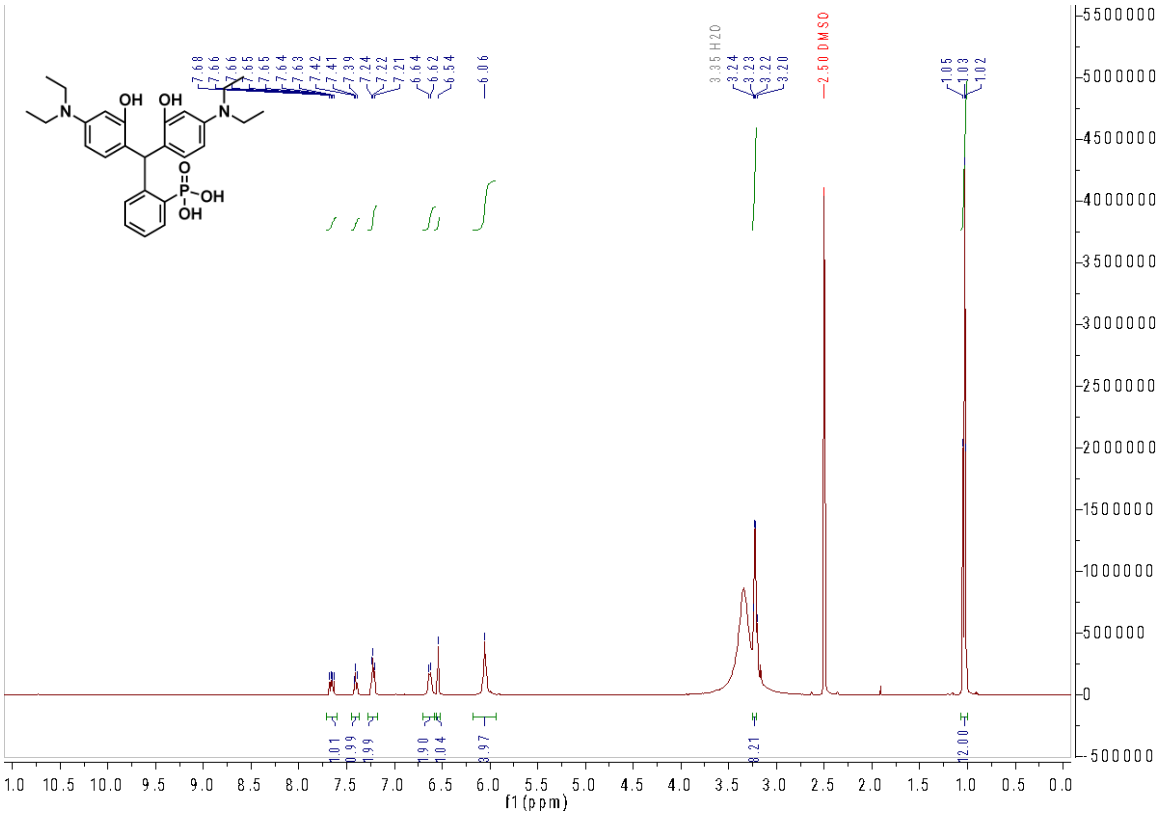
Spectrum S16. ^{1}H NMR of Compound **9**.



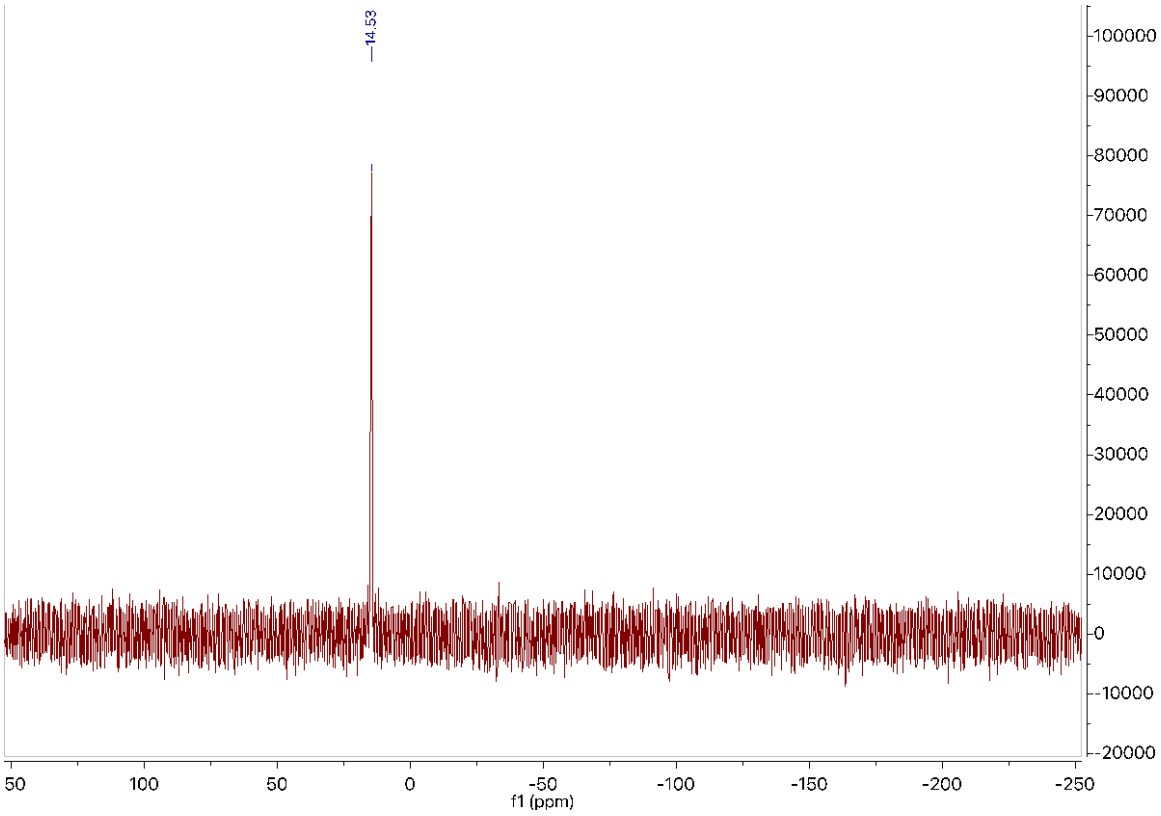
Spectrum S17. ^{31}P NMR of Compound **9**.



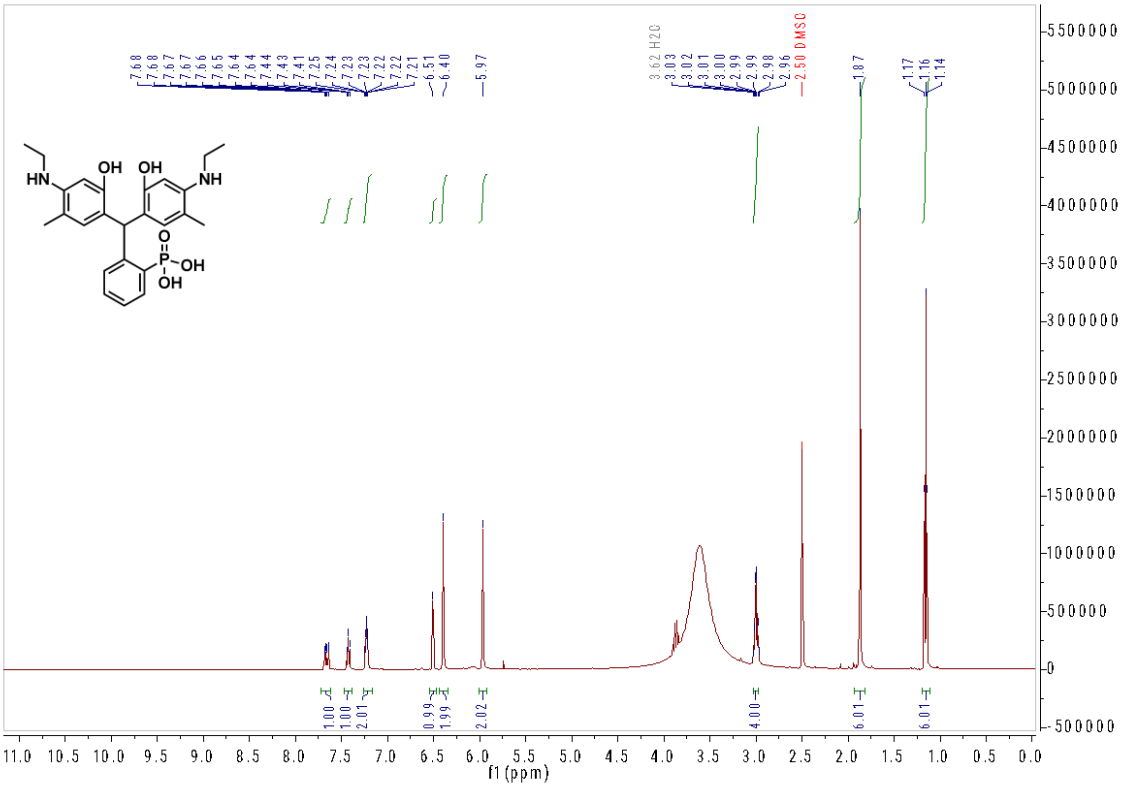
Spectrum S18. ^{1}H NMR of Compound **10**.



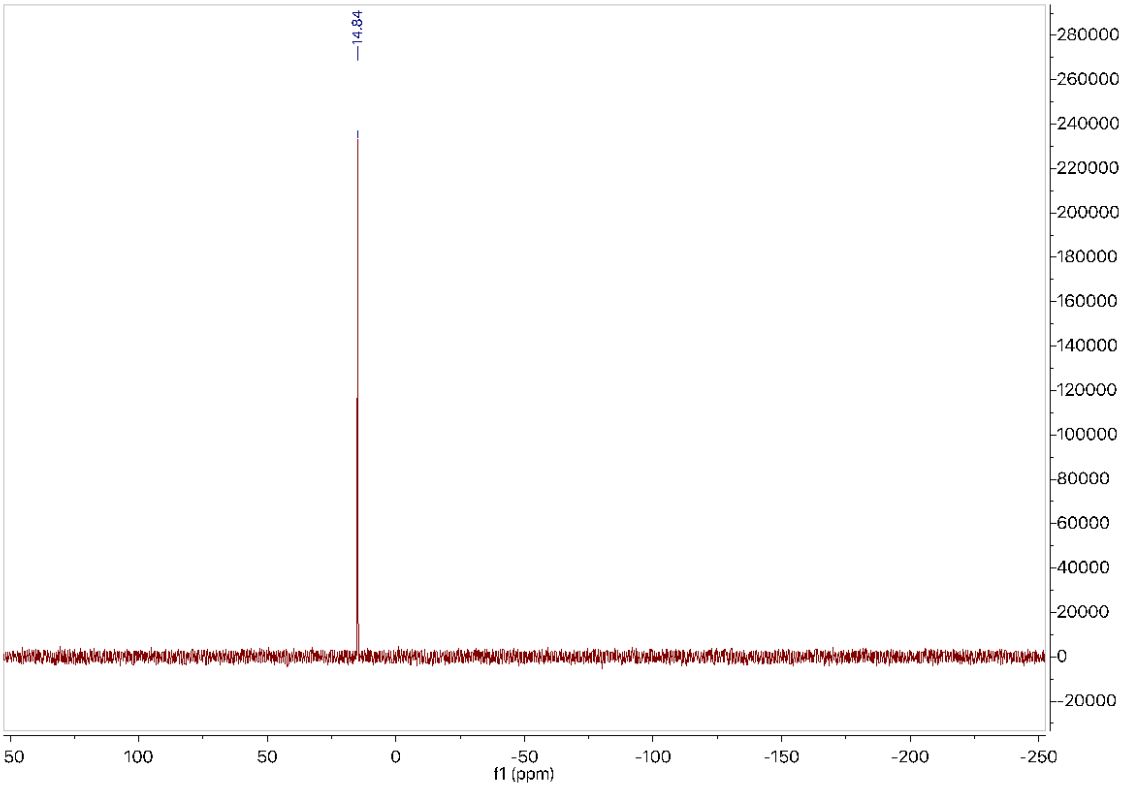
Spectrum S19. ^{31}P NMR of Compound **10**.



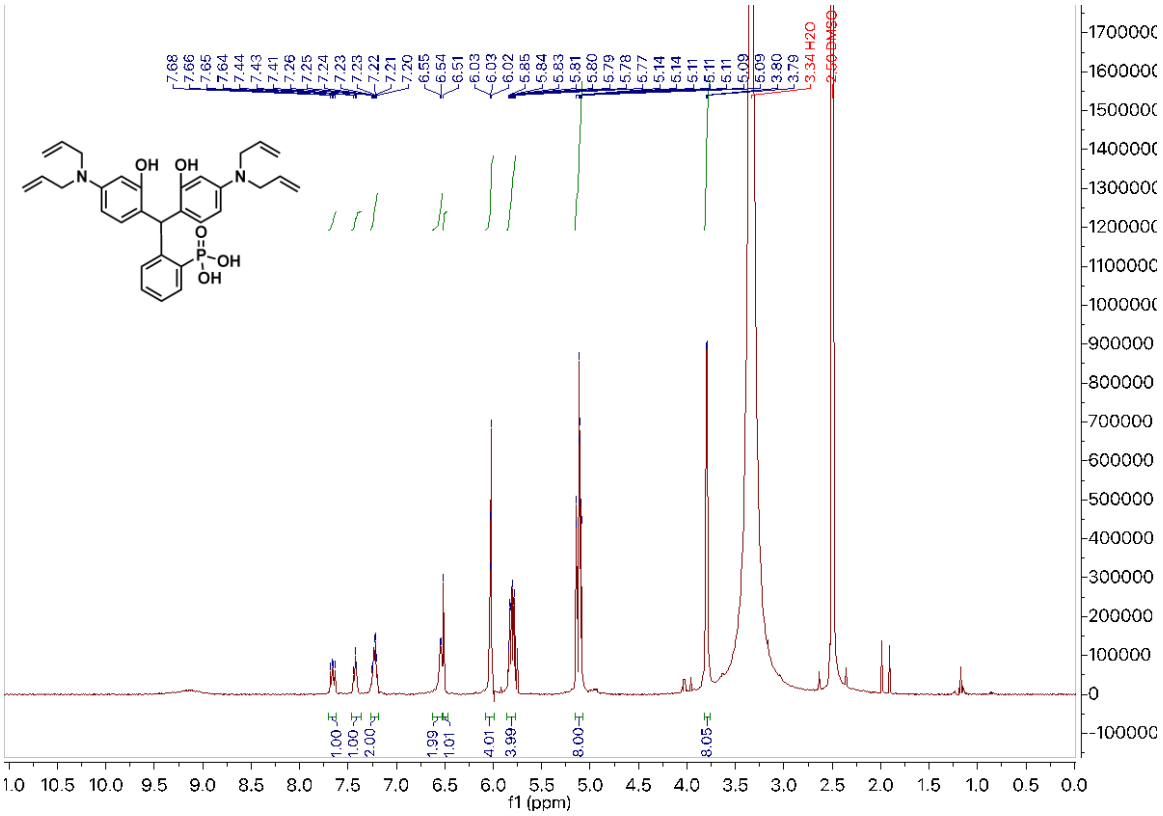
Spectrum S20. ^{1}H NMR of Compound **11**.



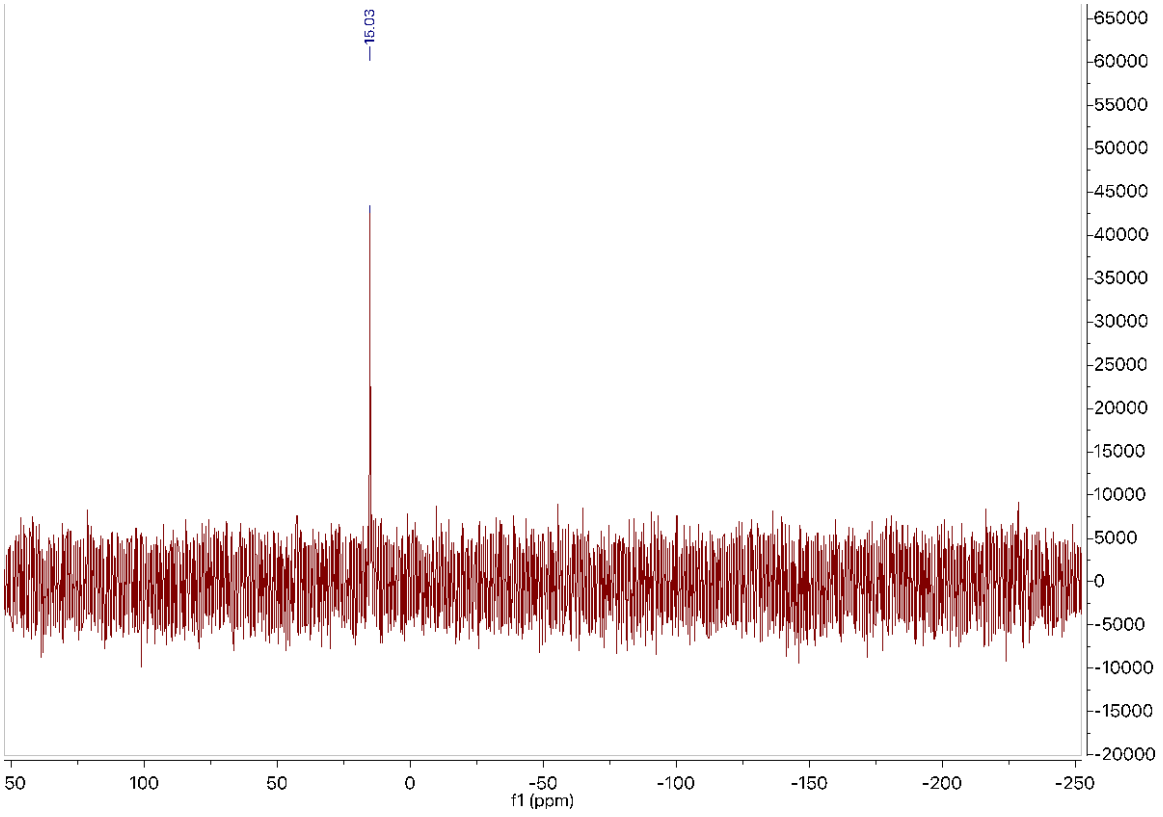
Spectrum S21. ^{31}P NMR of Compound **11**.



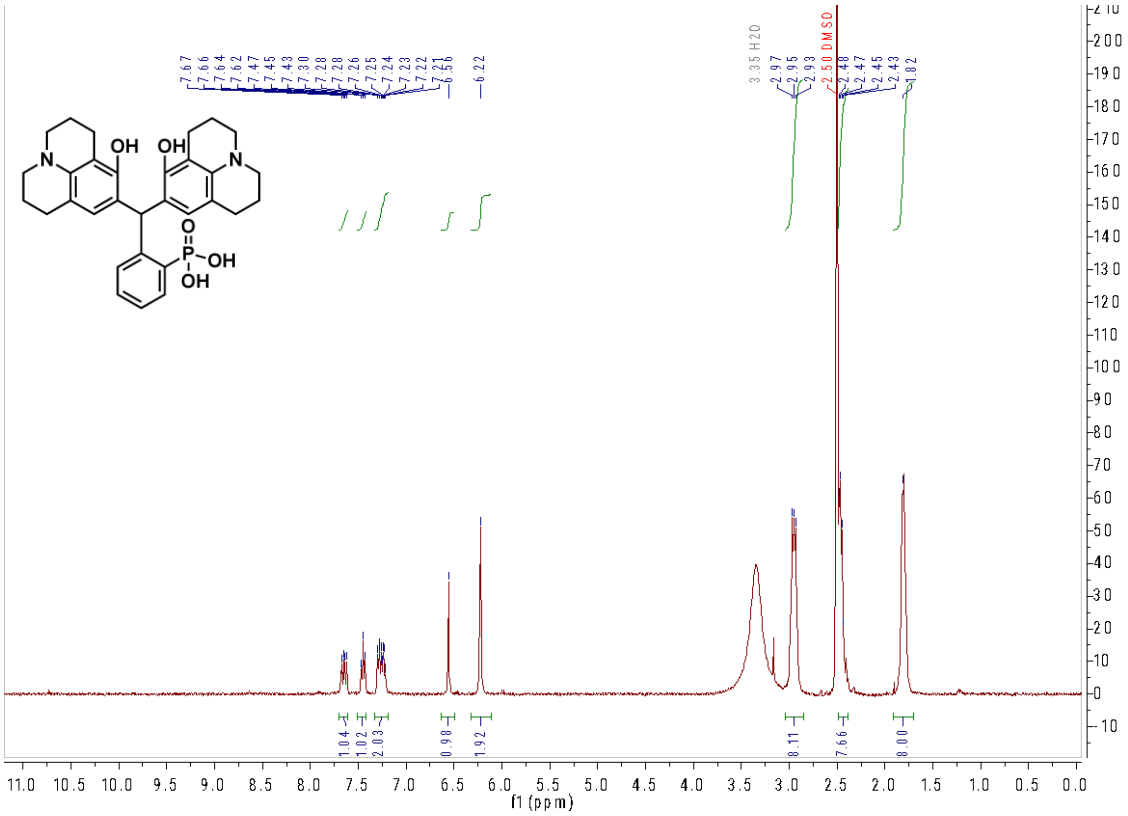
Spectrum S22. ^{1}H NMR of Compound **12**.



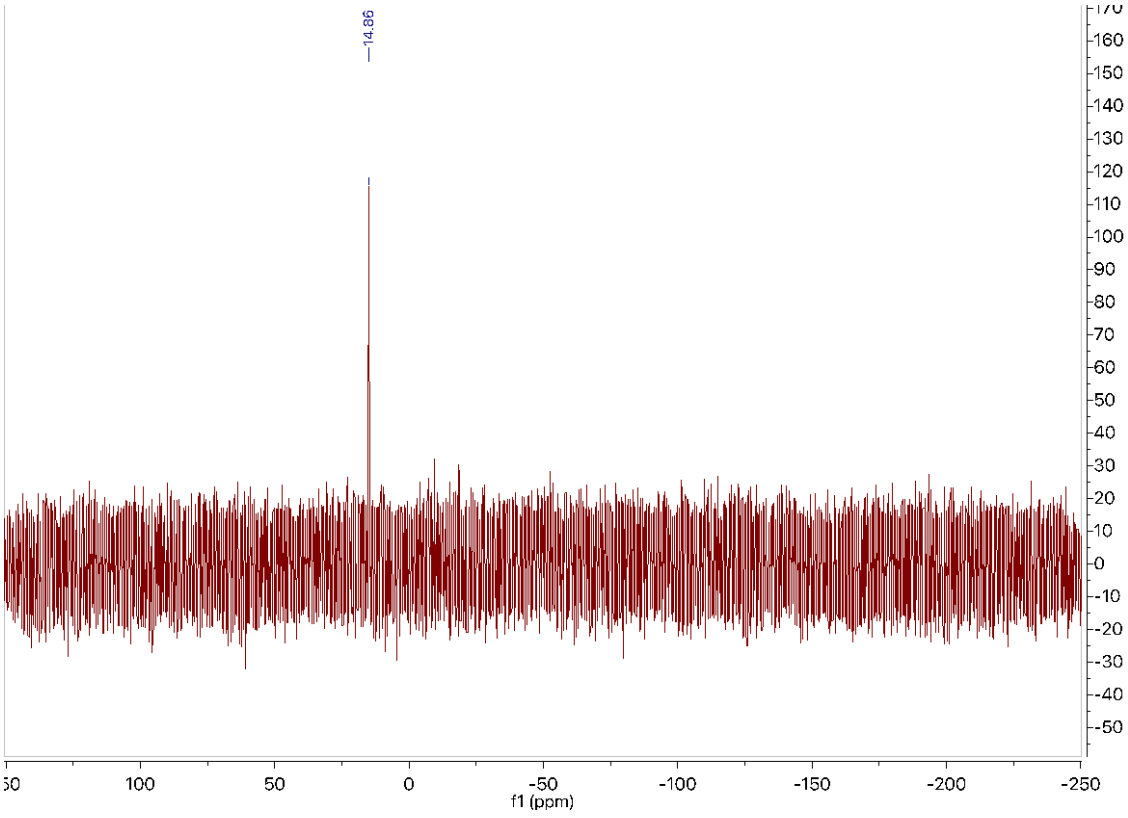
Spectrum S23. ^{31}P NMR of Compound **12**.



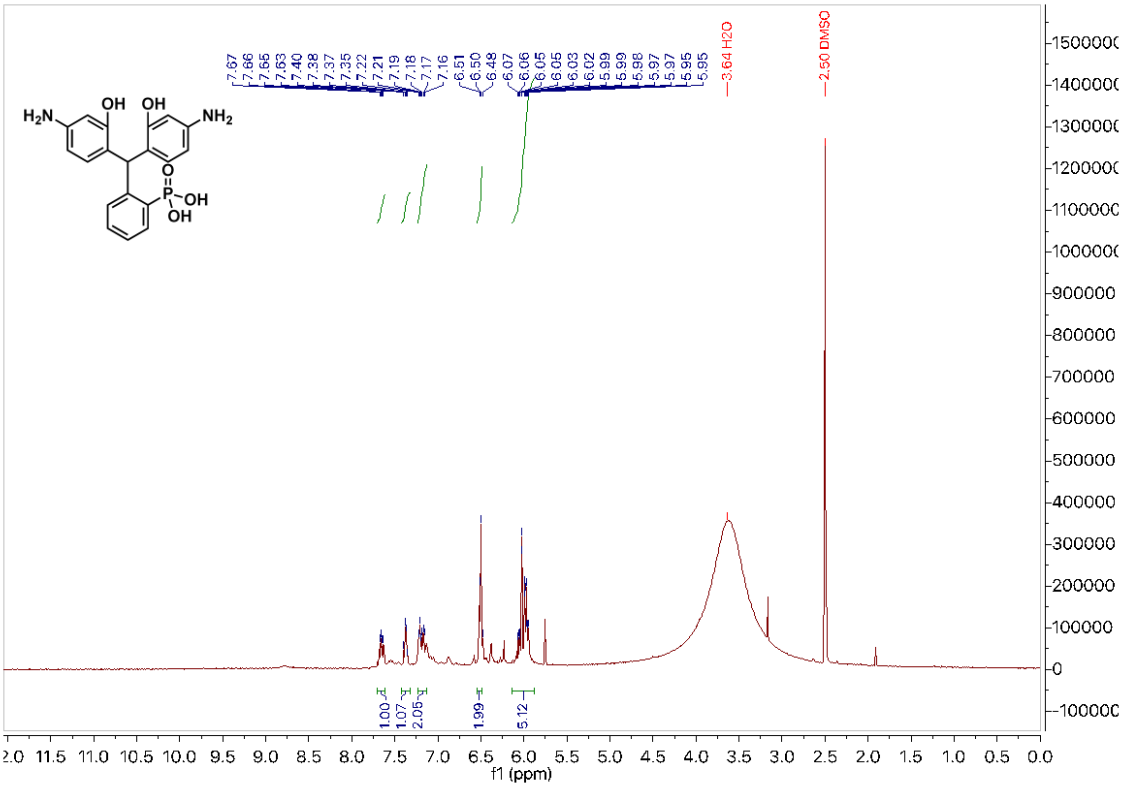
Spectrum S24. ^{1}H NMR of Compound **13**.



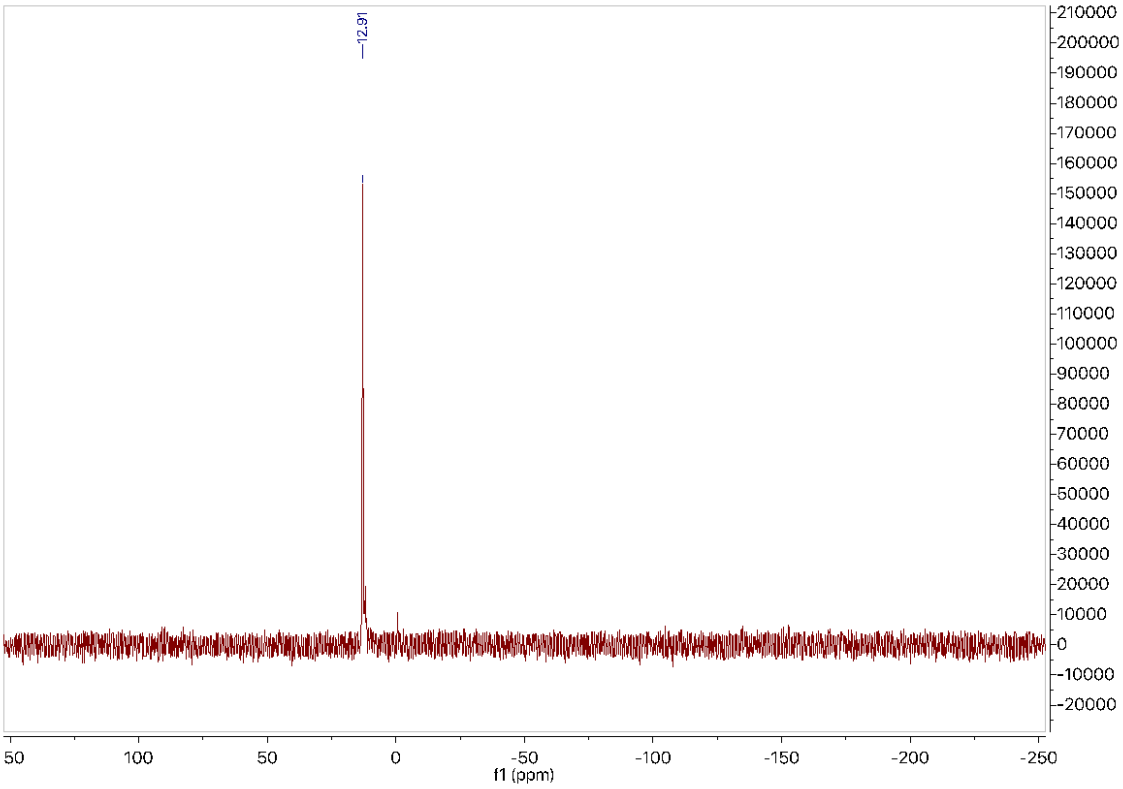
Spectrum S25. ^{31}P NMR of Compound **13**.



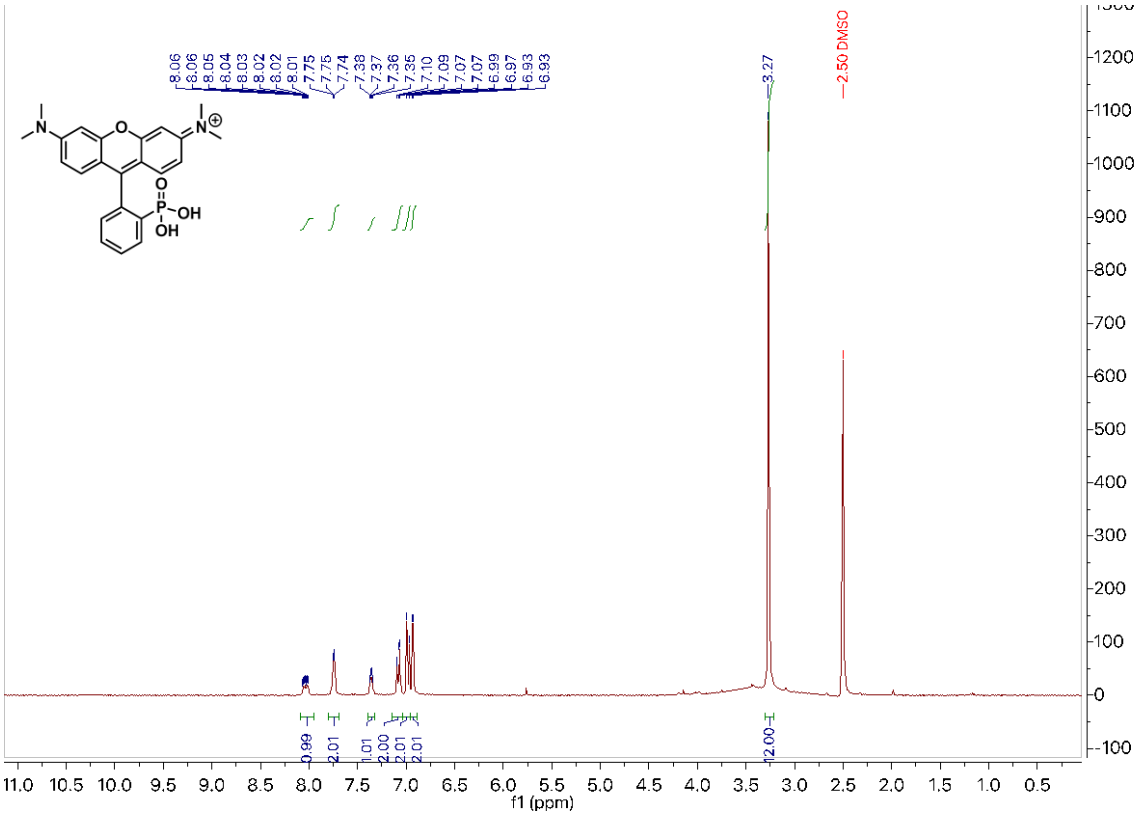
Spectrum S26. ^{1}H NMR of Compound **14**.



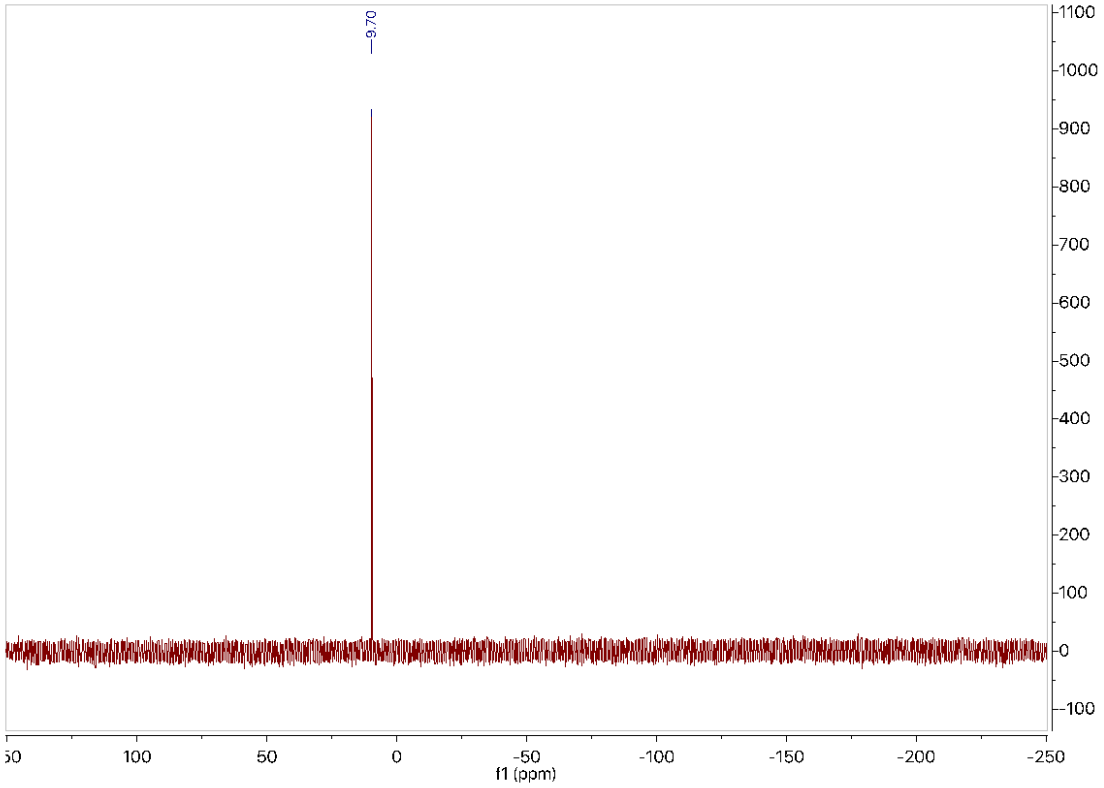
Spectrum S27. ^{31}P NMR of Compound **14**.



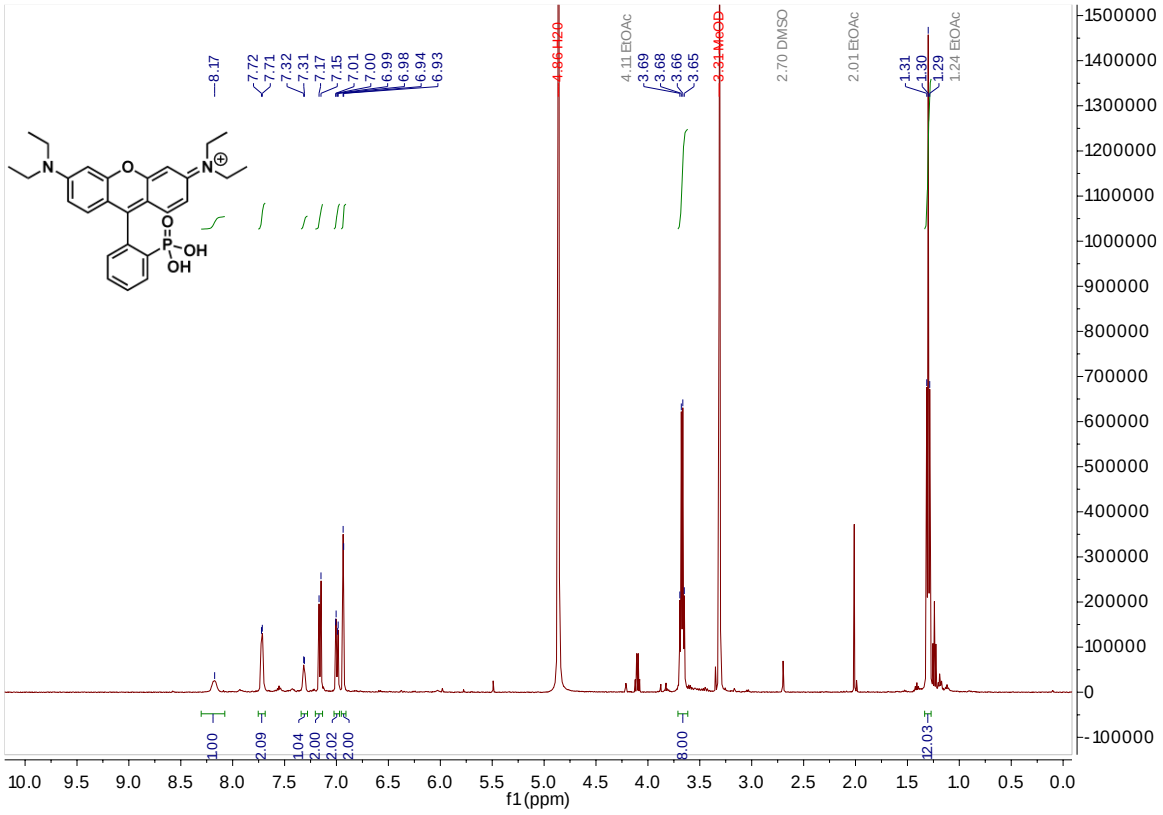
Spectrum S28. ^{1}H NMR of Compound **16**.



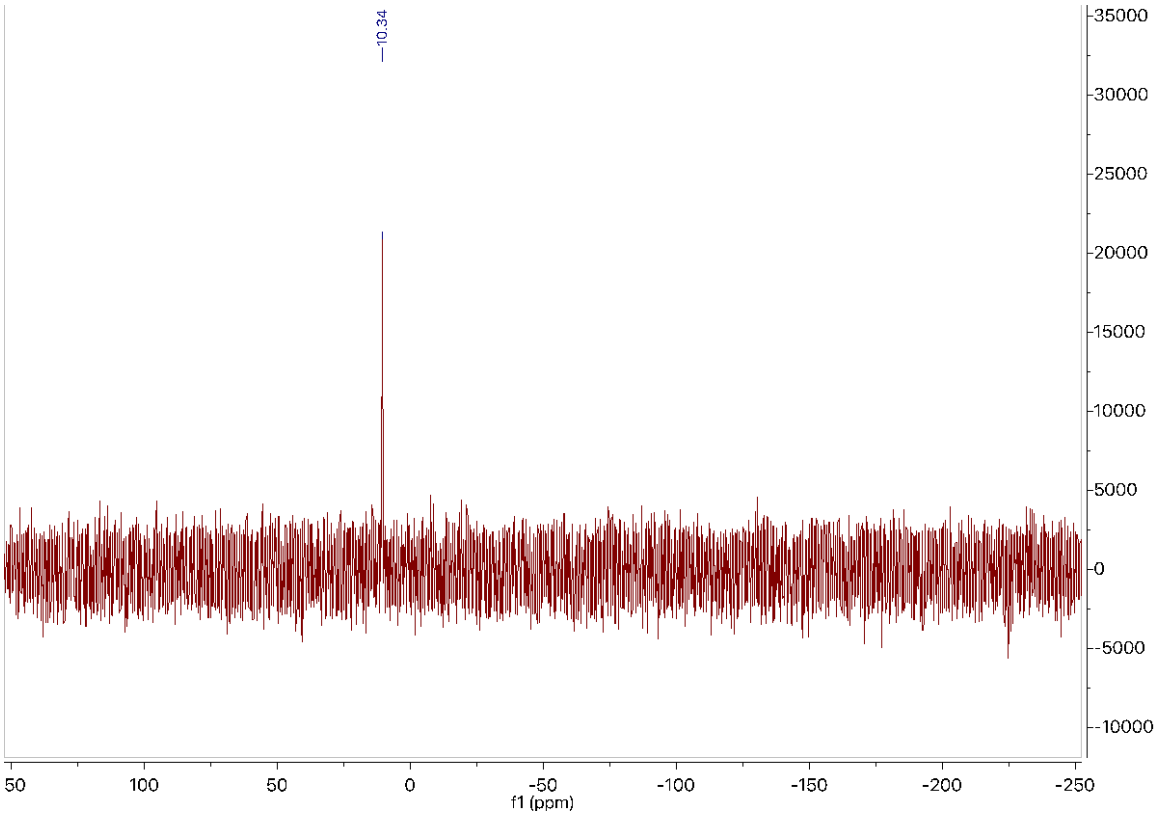
Spectrum S29. ^{31}P NMR of Compound **16**.



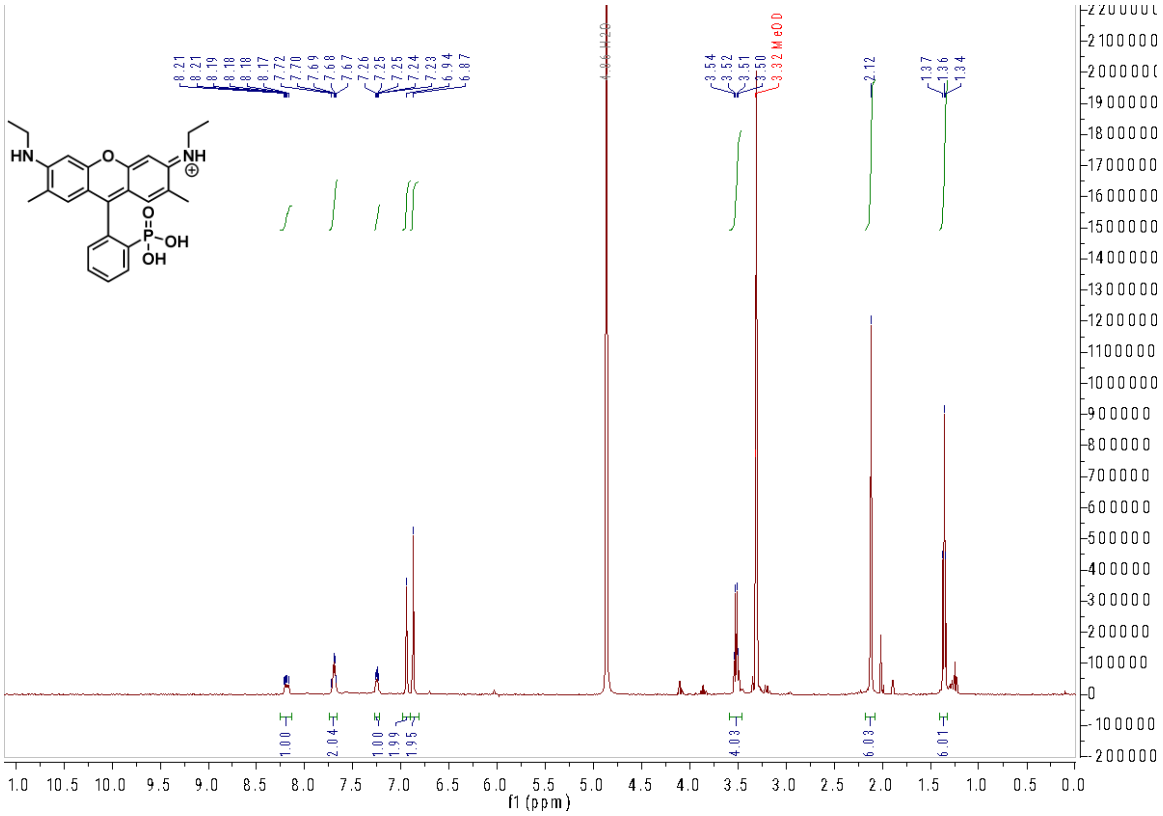
Spectrum S30. ^{1}H NMR of Compound **17** .



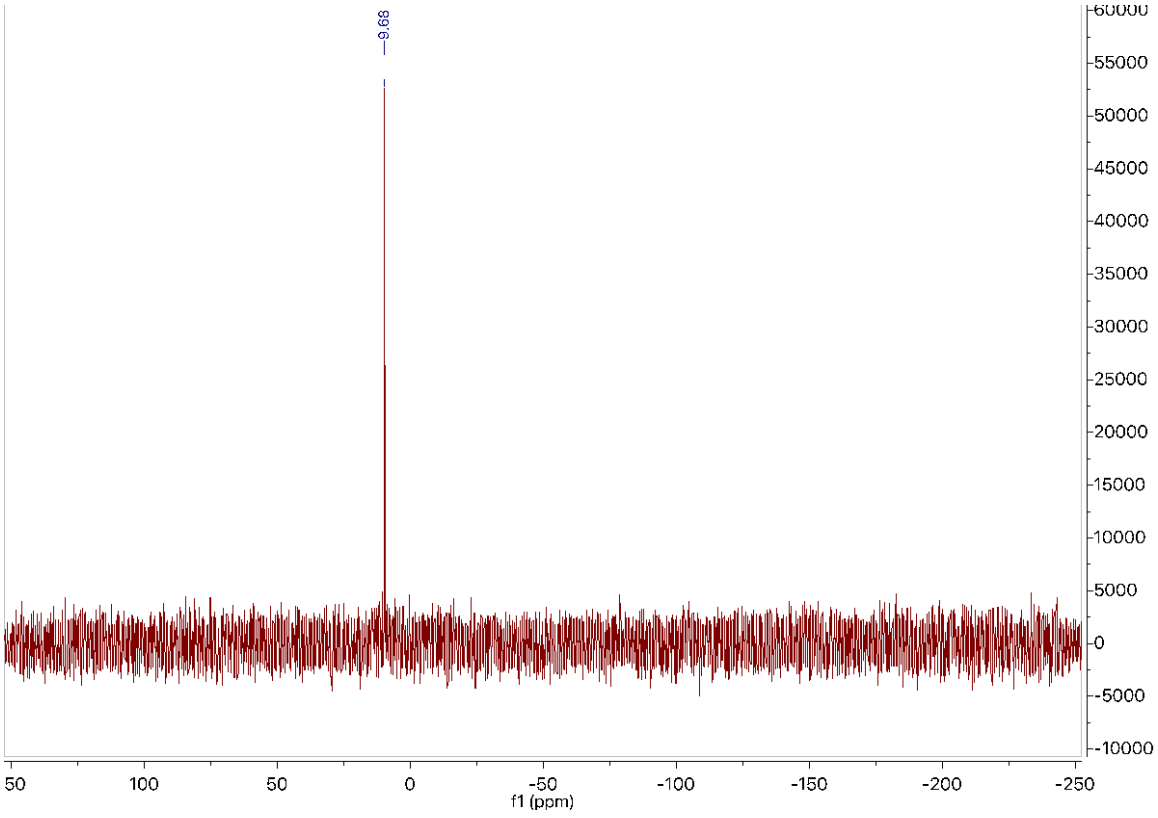
Spectrum S31. ^{31}P NMR of Compound **17**.



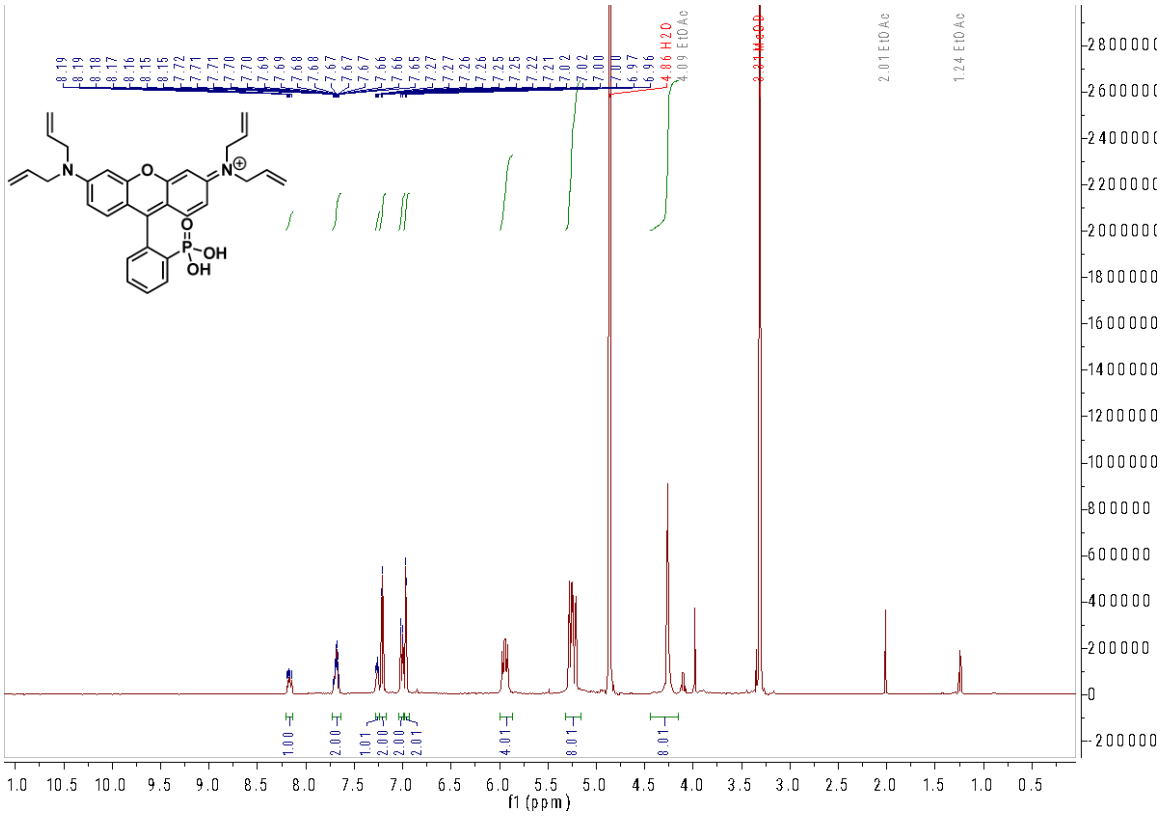
Spectrum S32. ^{1}H NMR of Compound **18**.



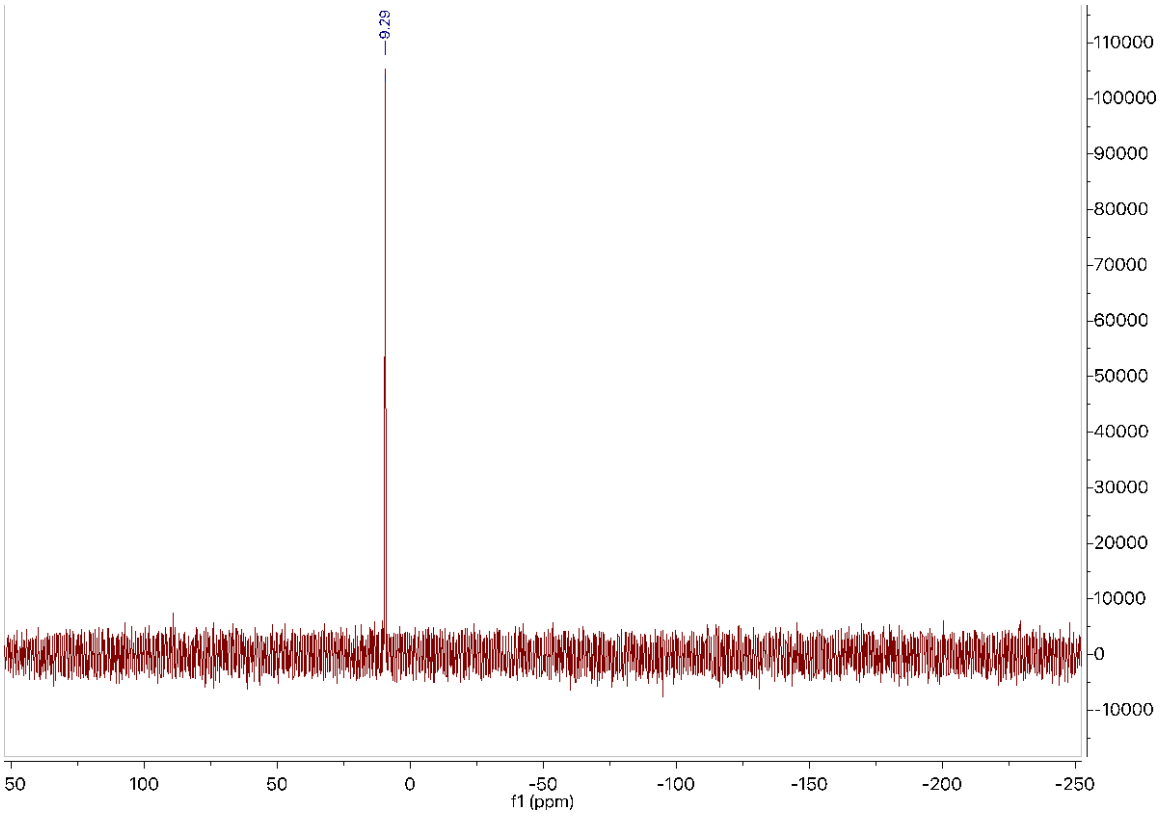
Spectrum S33. ^{31}P NMR of Compound **18**.



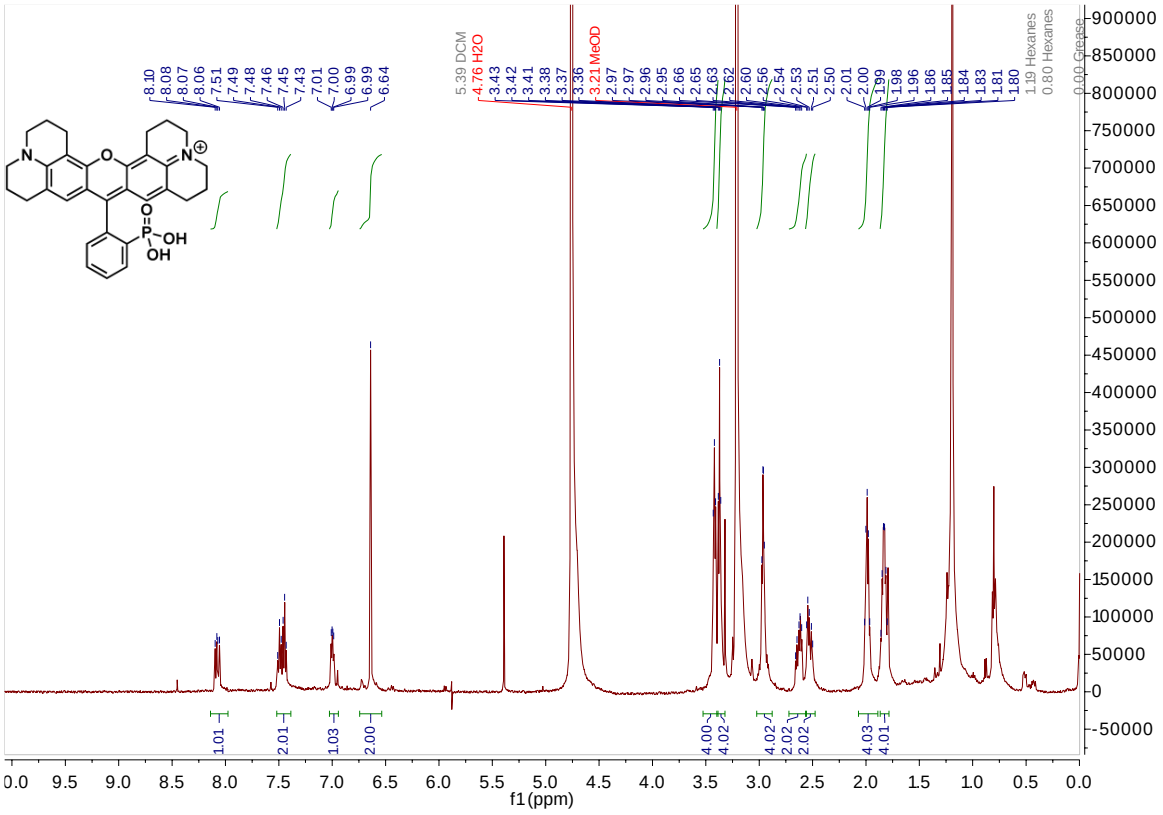
Spectrum S34. ^{1}H NMR of Compound **19**.



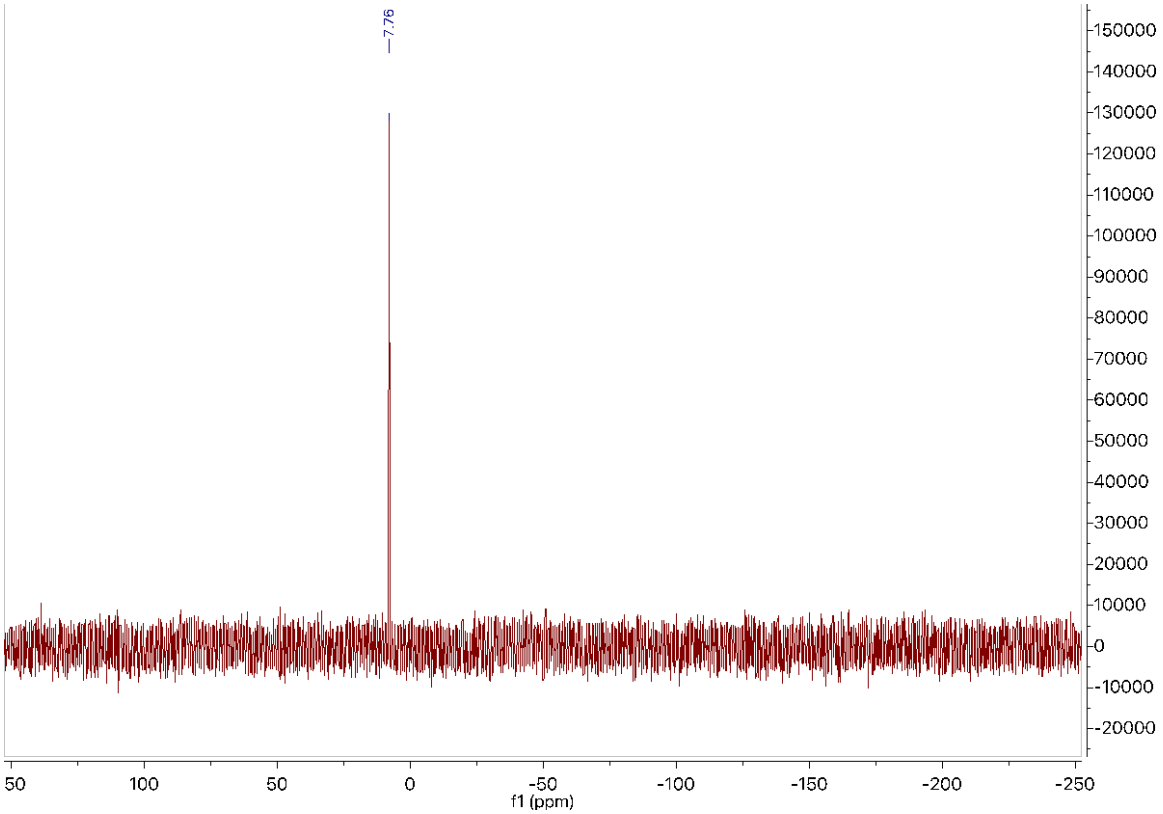
Spectrum S35. ^{31}P NMR of Compound **19**.



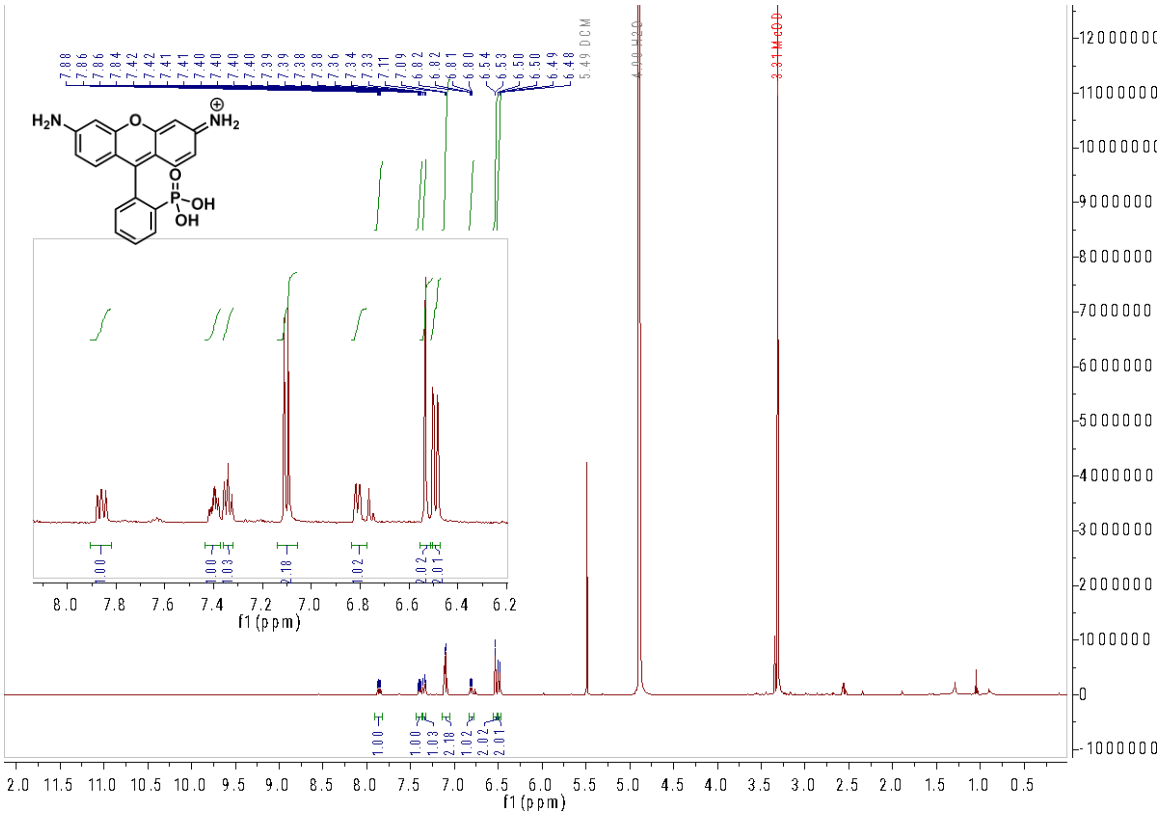
Spectrum S36. ^{1}H NMR of Compound **20**.



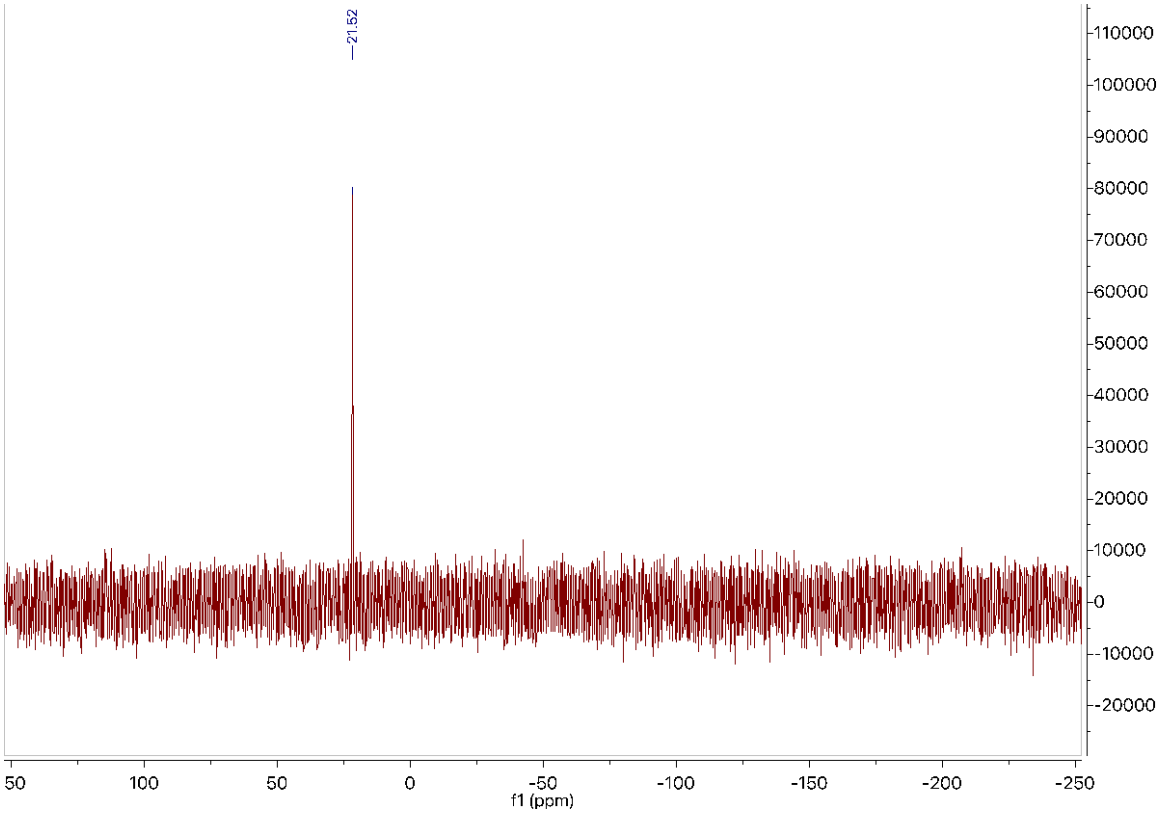
Spectrum S37. ^{31}P NMR of Compound **20**.



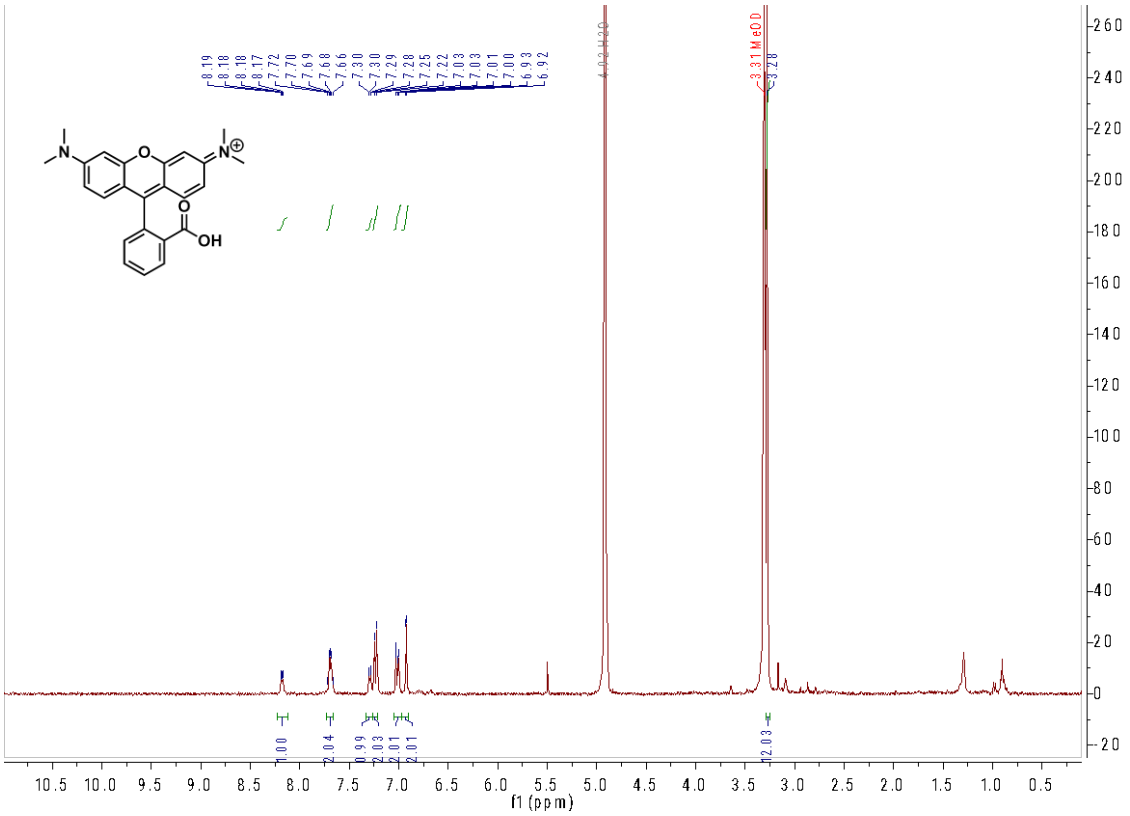
Spectrum S38. ^{1}H NMR of Compound **21**.



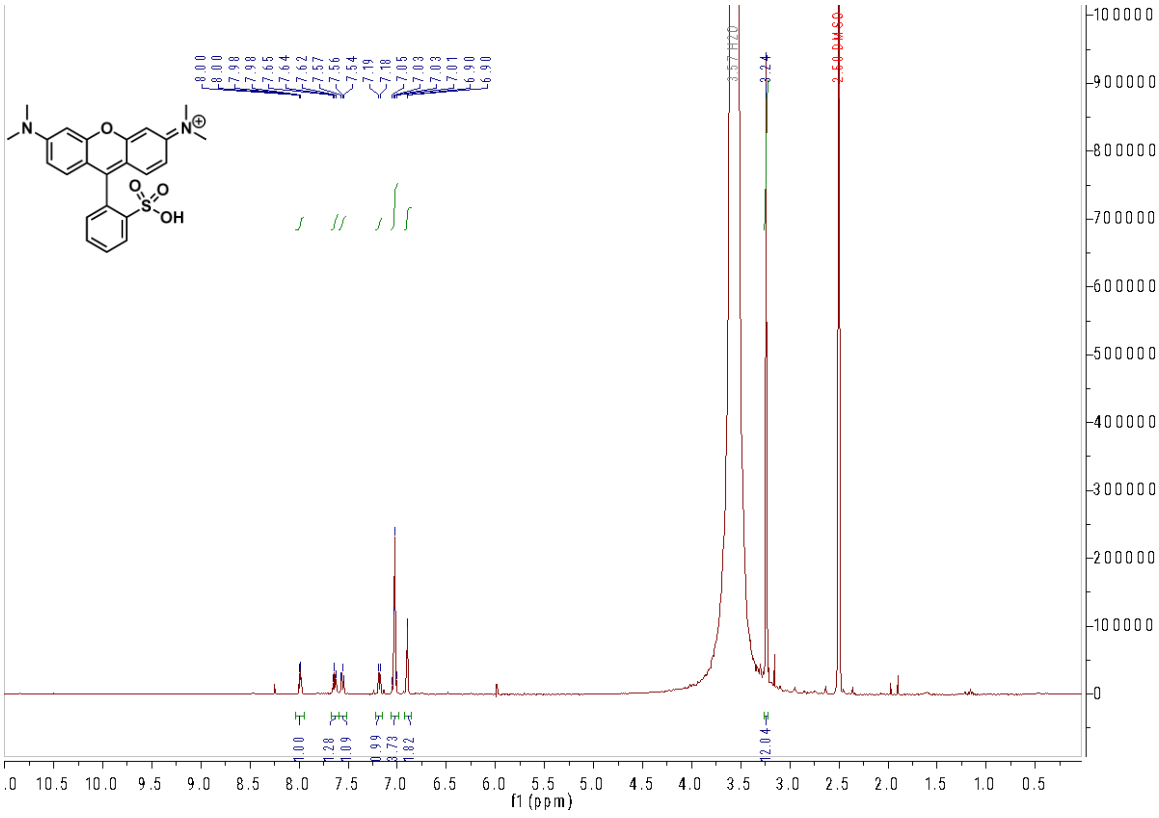
Spectrum S39. ^{31}P NMR of Compound **21**.



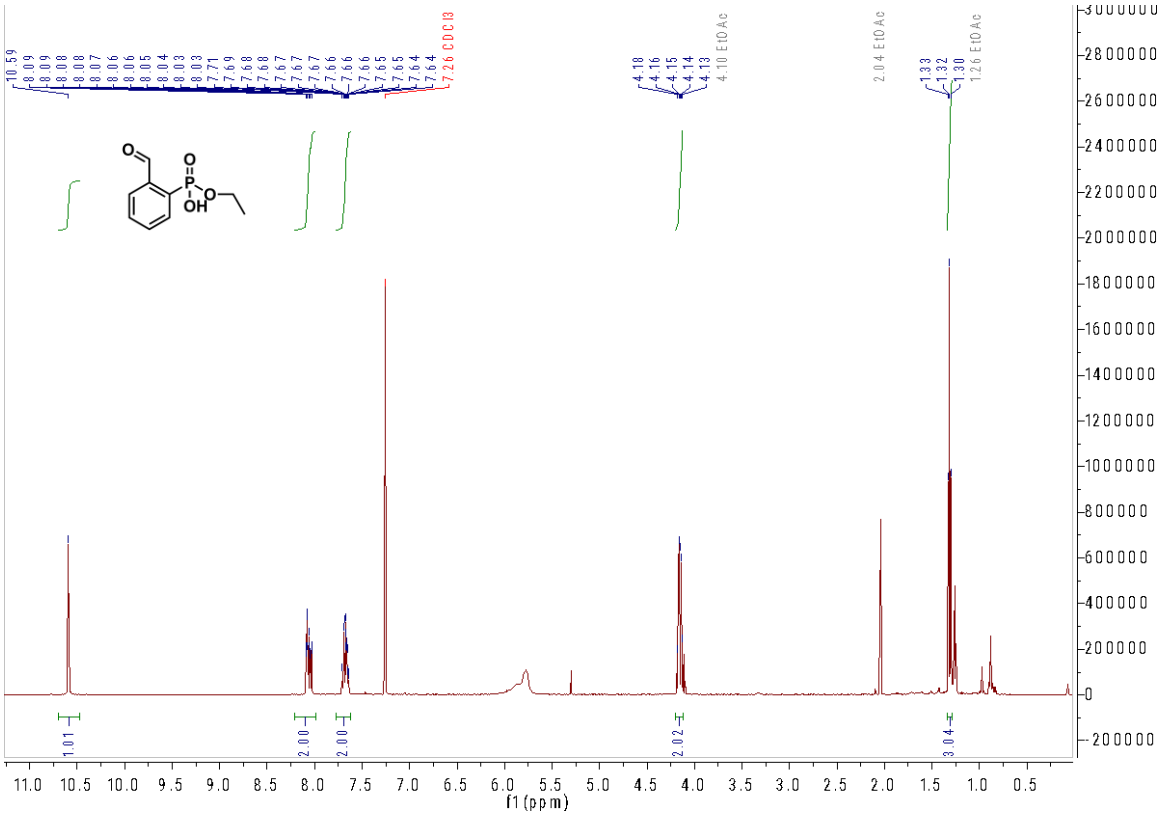
Spectrum S40. ^{1}H NMR of carboxyTMR.



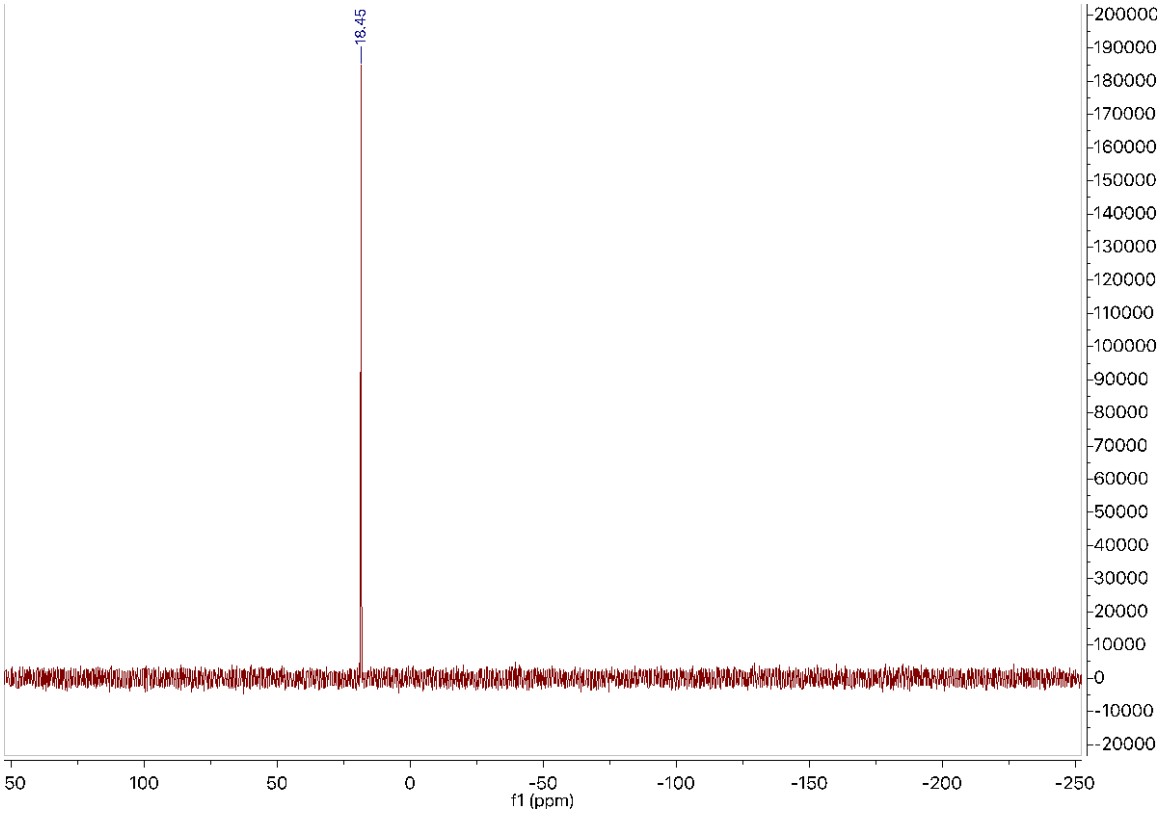
Spectrum S41. ^{1}H NMR of sulfoTMR.



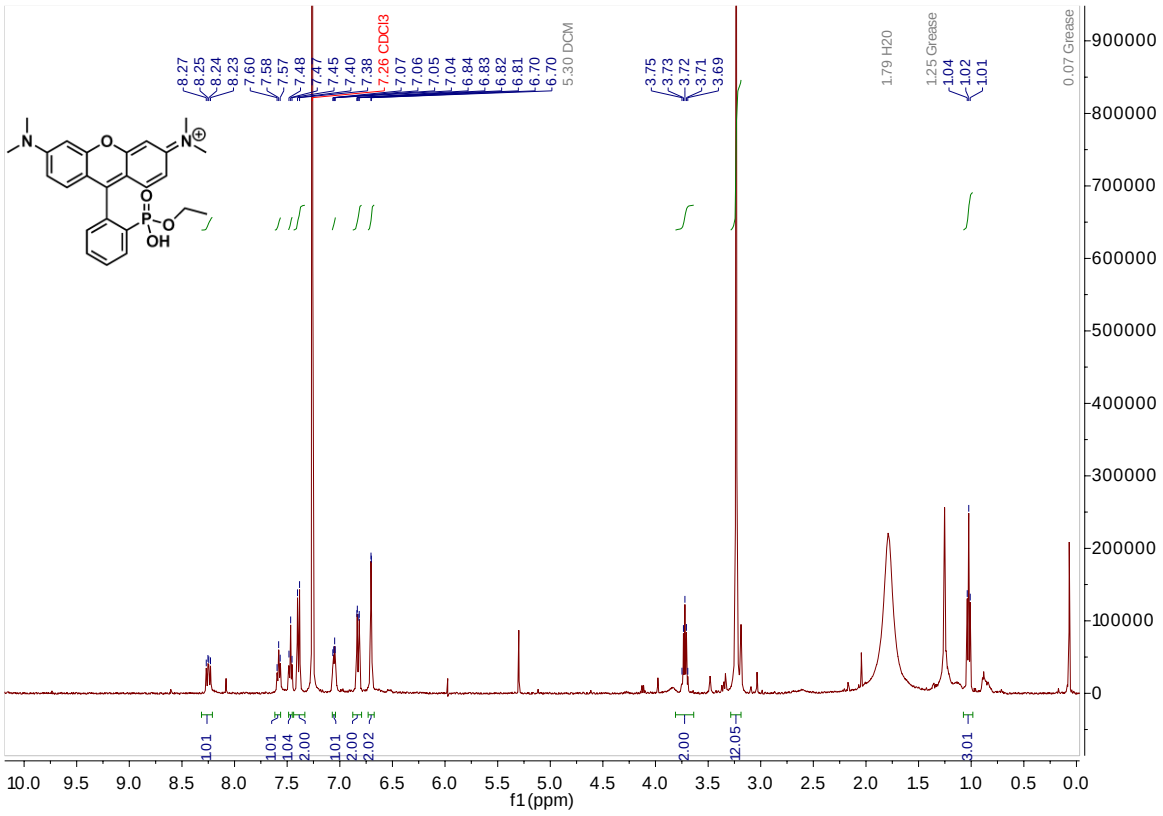
Spectrum S42. ^{1}H NMR of Compound **22**.



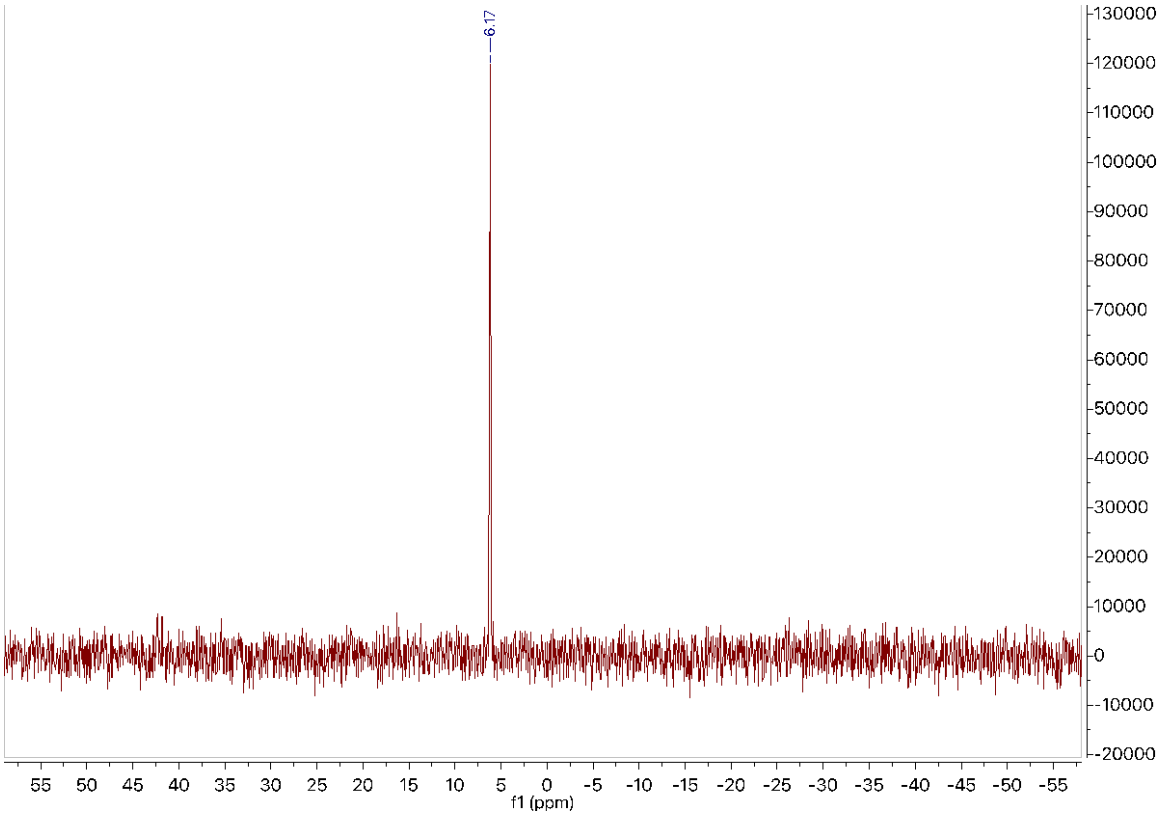
Spectrum S43. ^{31}P NMR of Compound **22**.



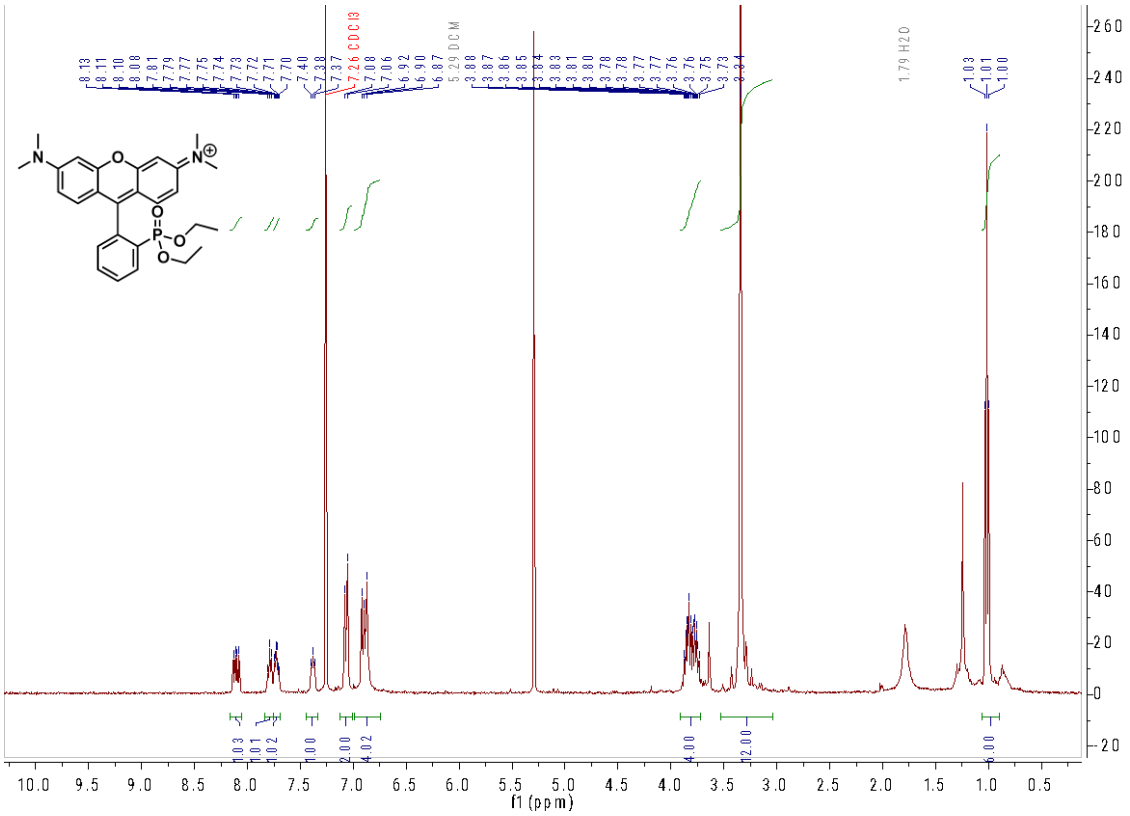
Spectrum S44. ^{1}H NMR of Compound **23**.



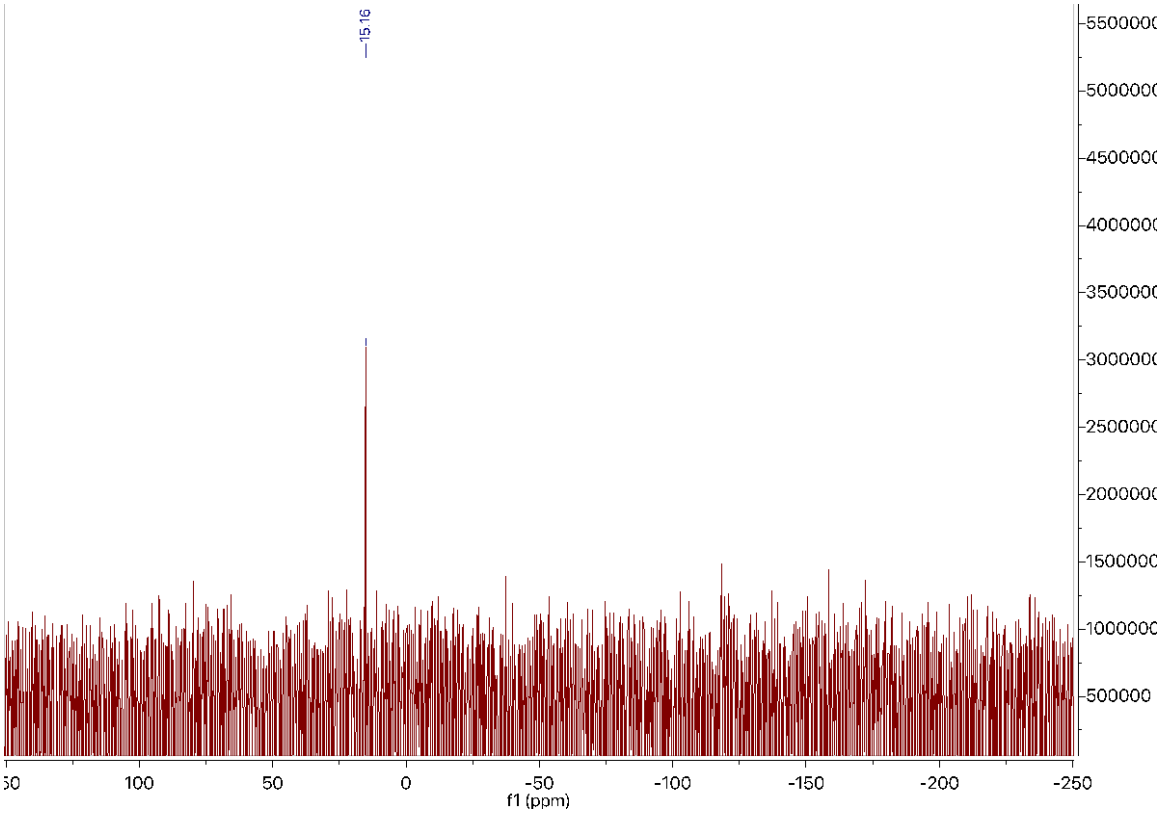
Spectrum S45. ^{31}P NMR of Compound **23**.



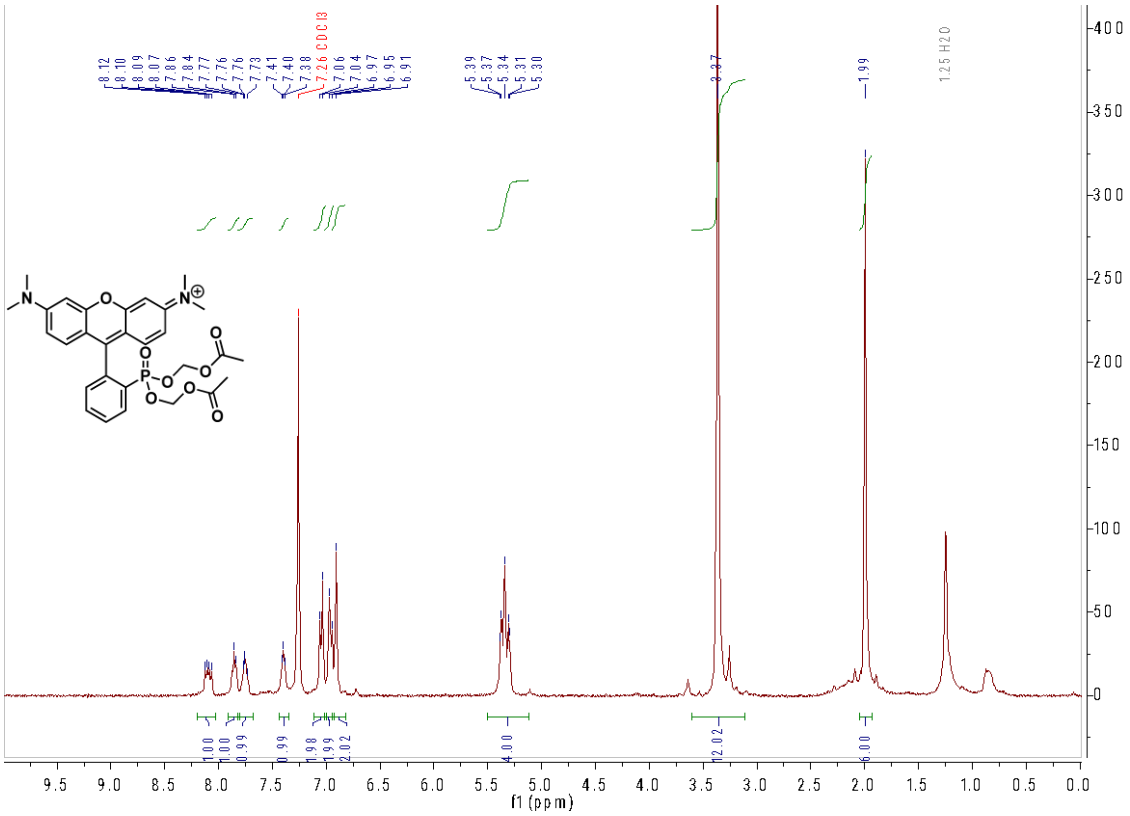
Spectrum S46. ^{1}H NMR of Compound **24**.



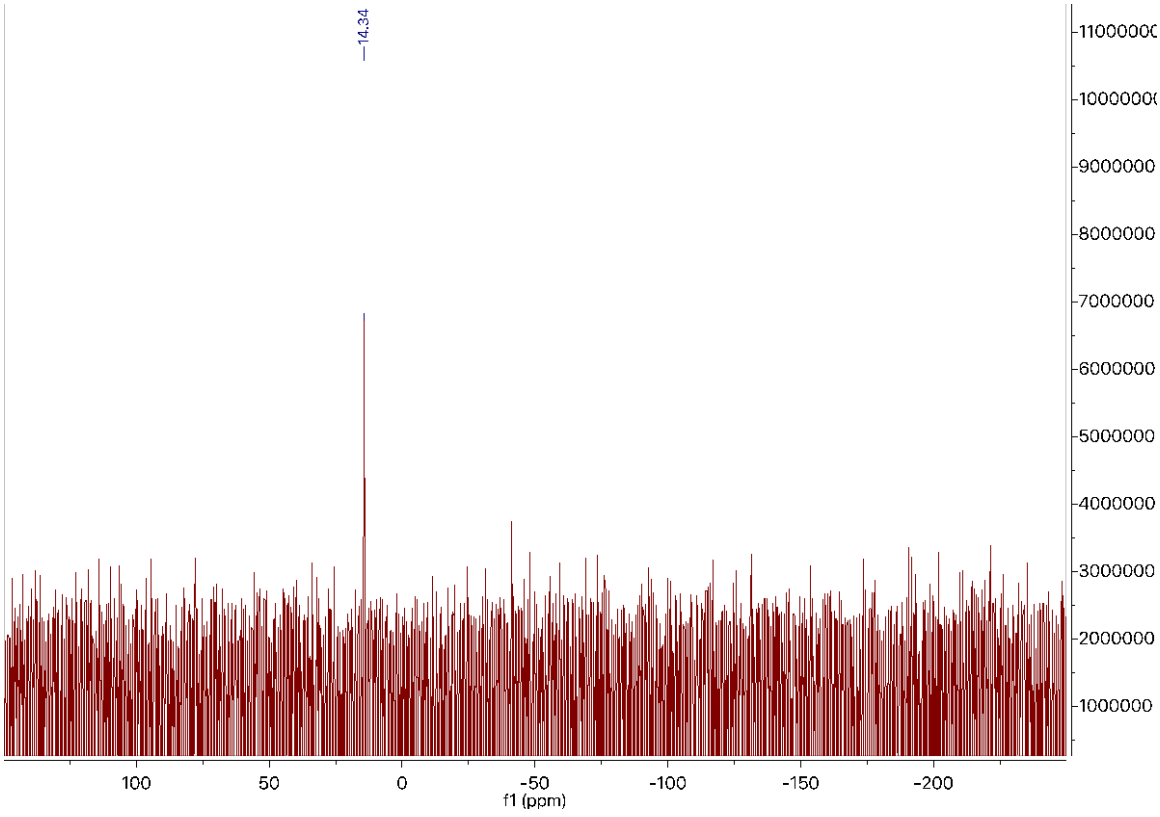
Spectrum S47. ^{31}P NMR of Compound **24**.



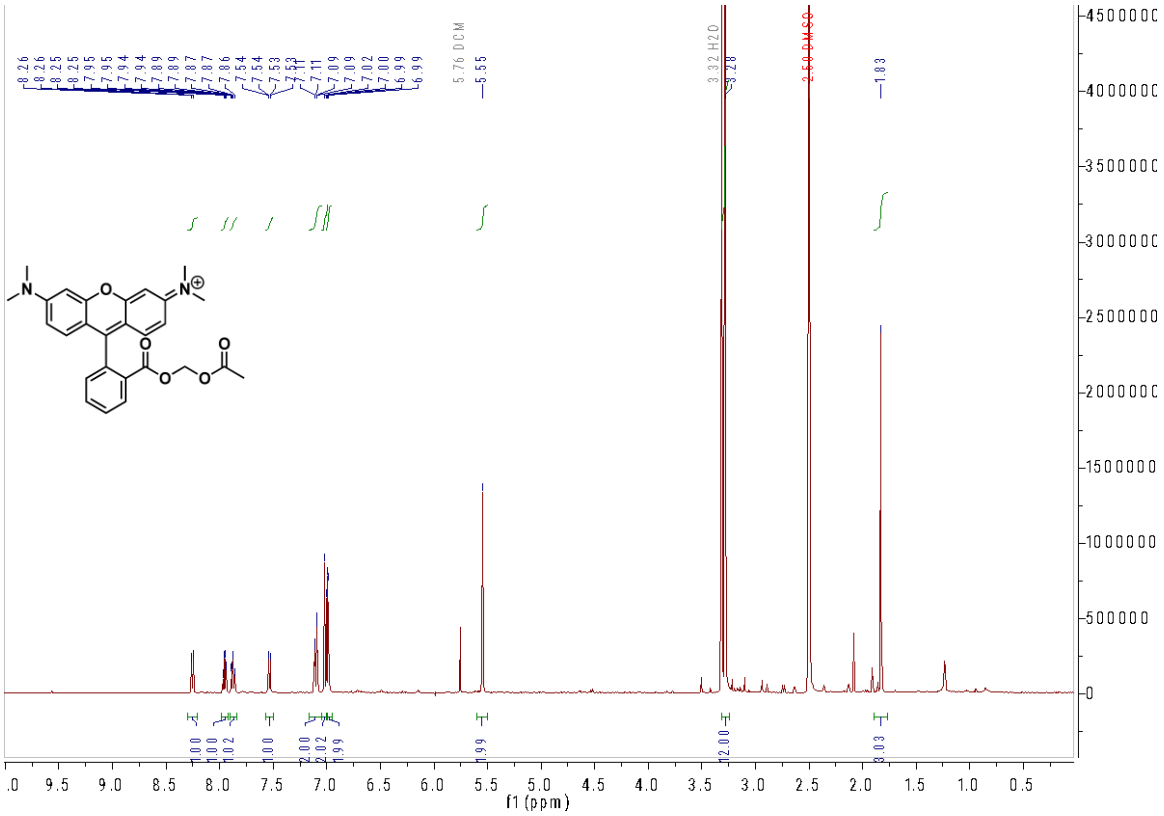
Spectrum S48. ^{1}H NMR of Compound **25**.



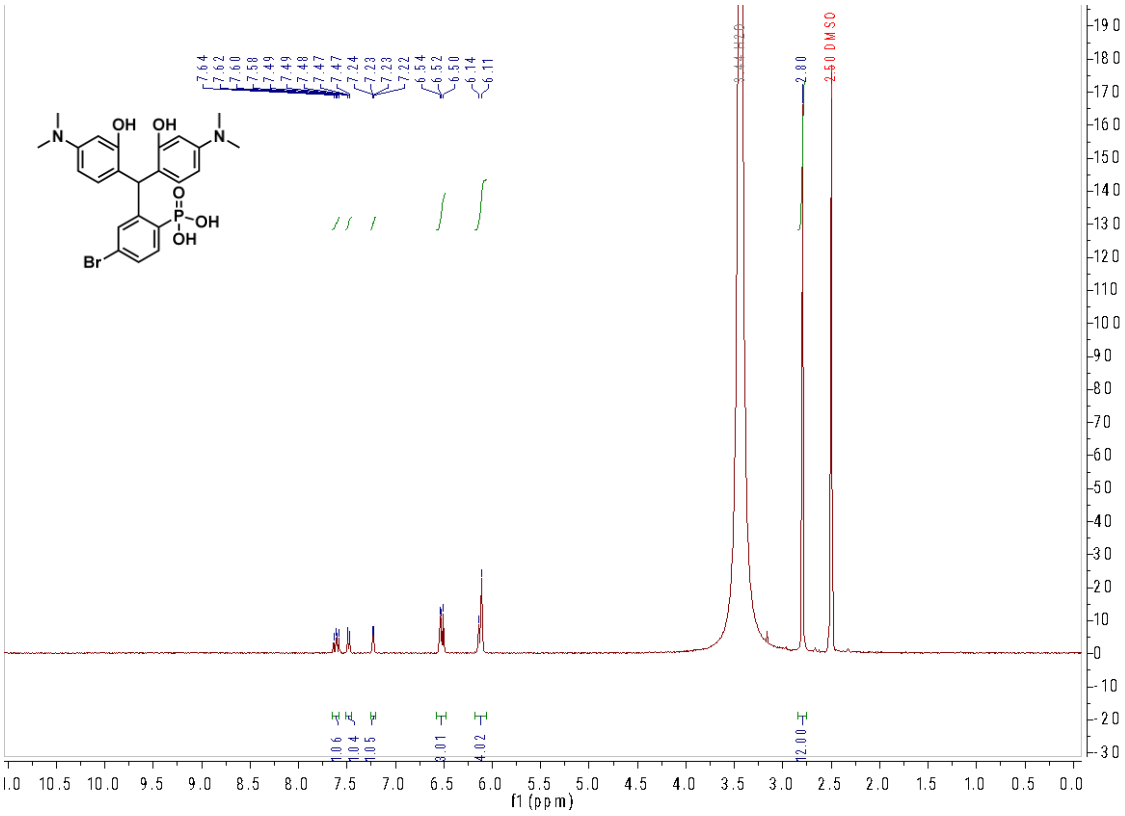
Spectrum S49. ^{31}P NMR of Compound **25**.



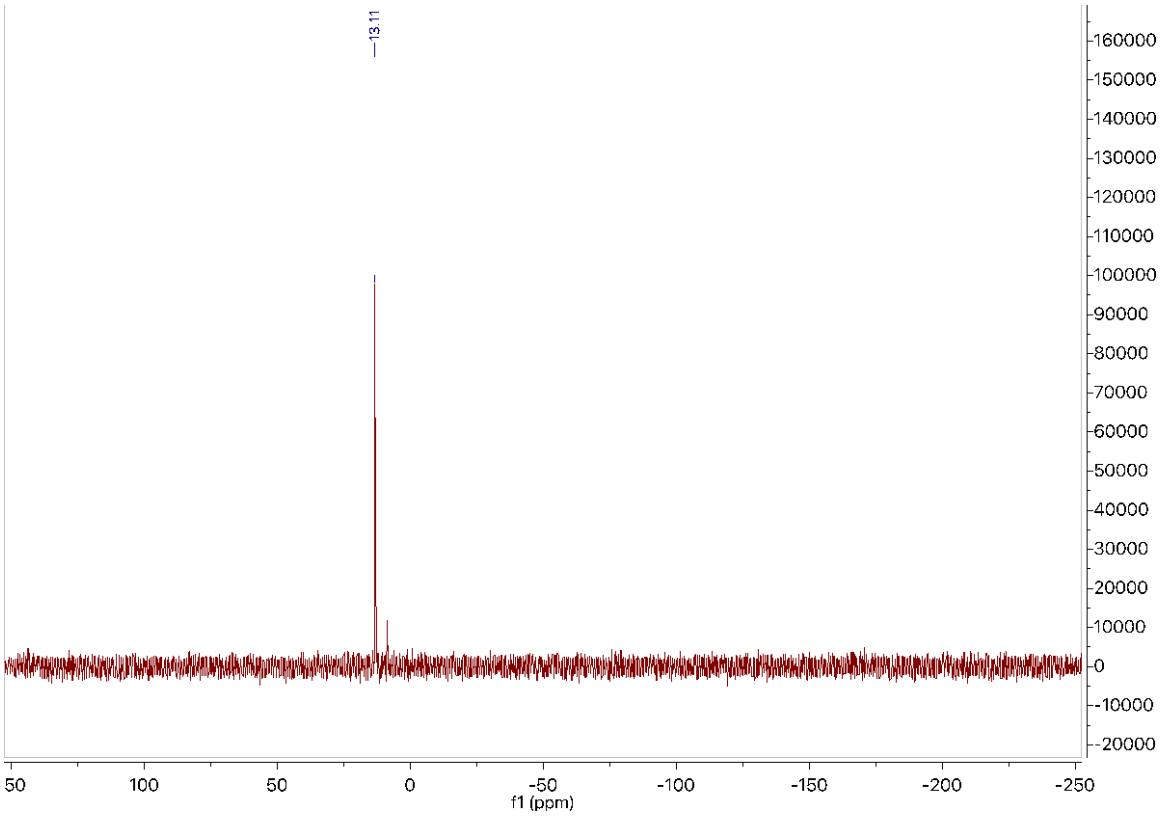
Spectrum S50. ^{1}H NMR of Compound **26**.



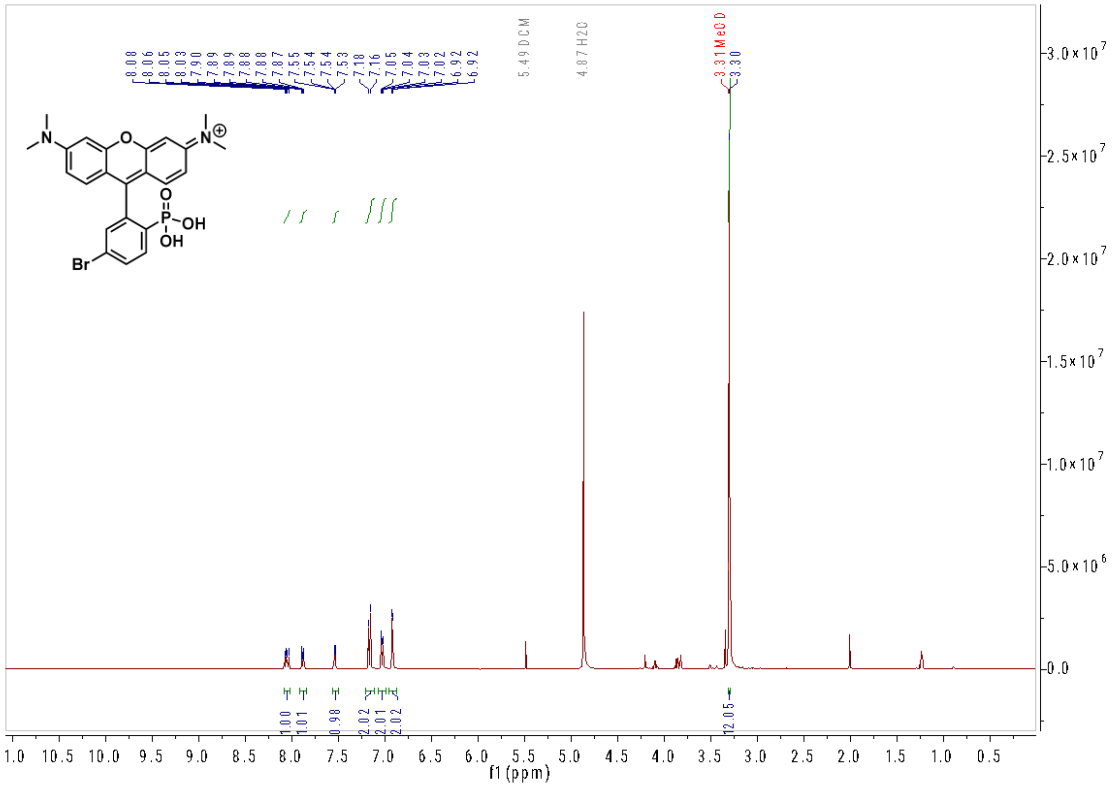
Spectrum S51. ^{1}H NMR of Compound **28.**



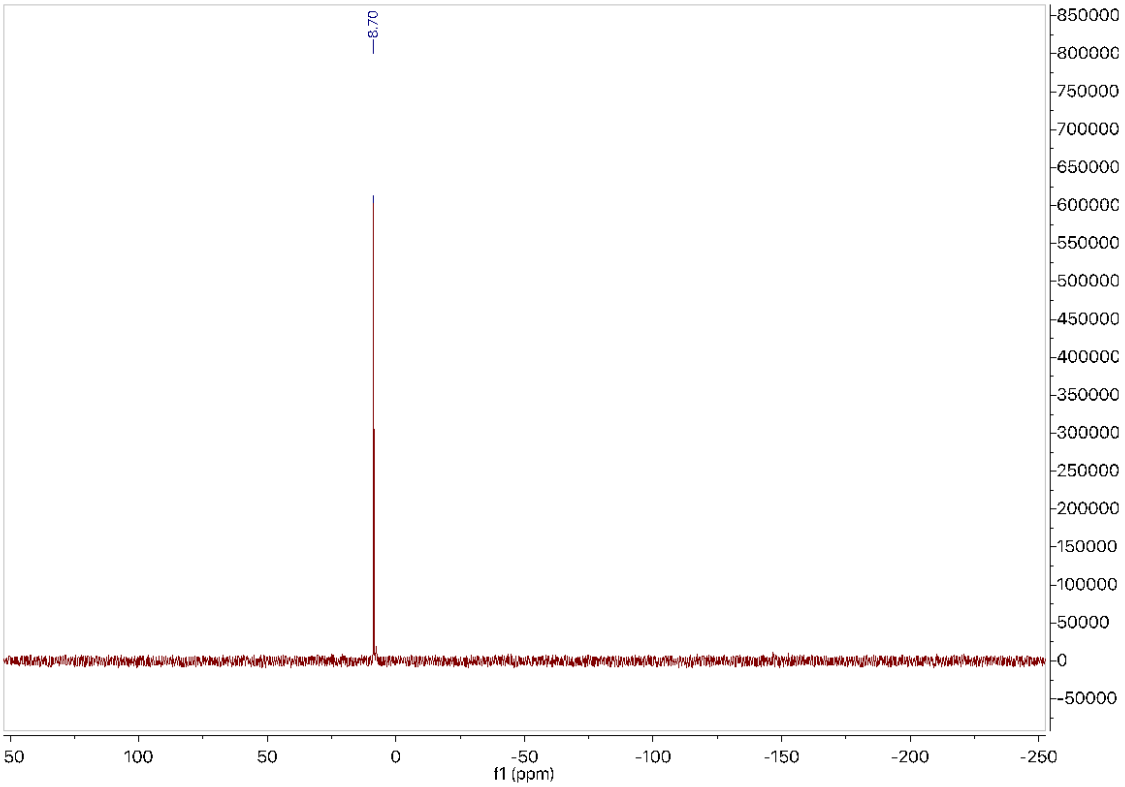
Spectrum S52. ^{31}P NMR of Compound **28.**



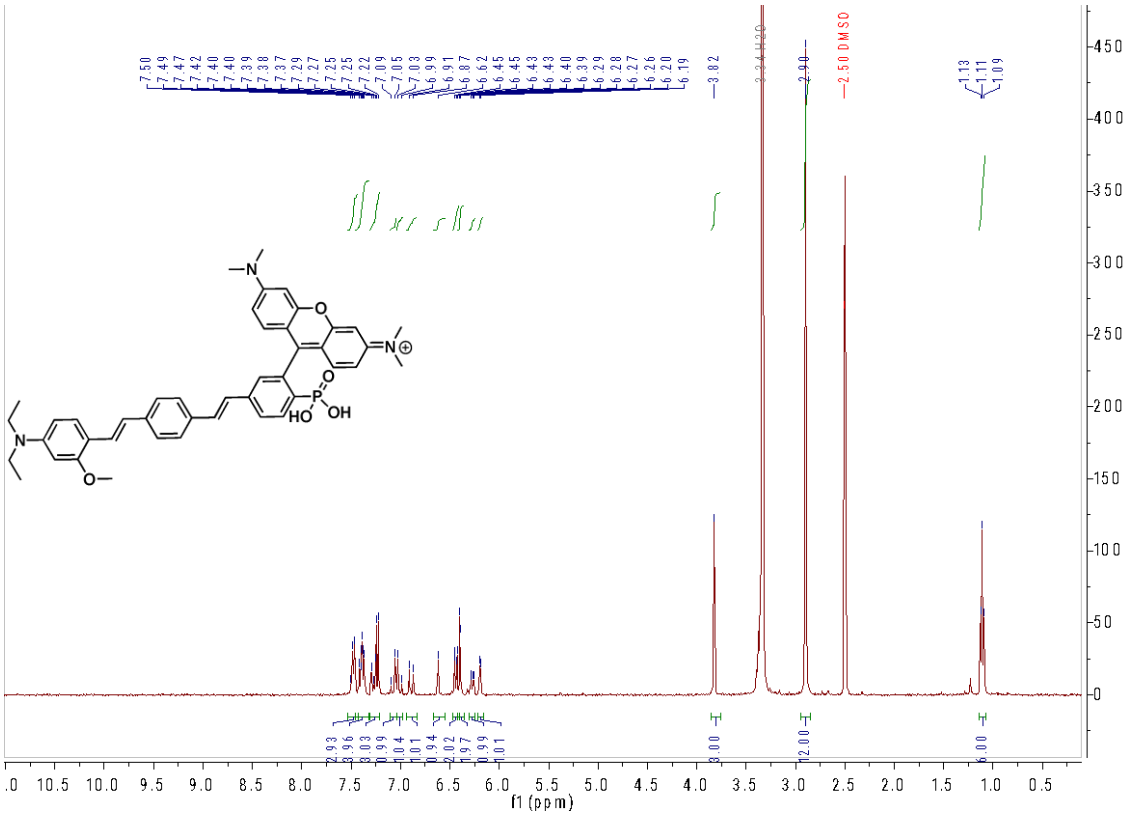
Spectrum S53. ^{1}H NMR of Compound **29.**



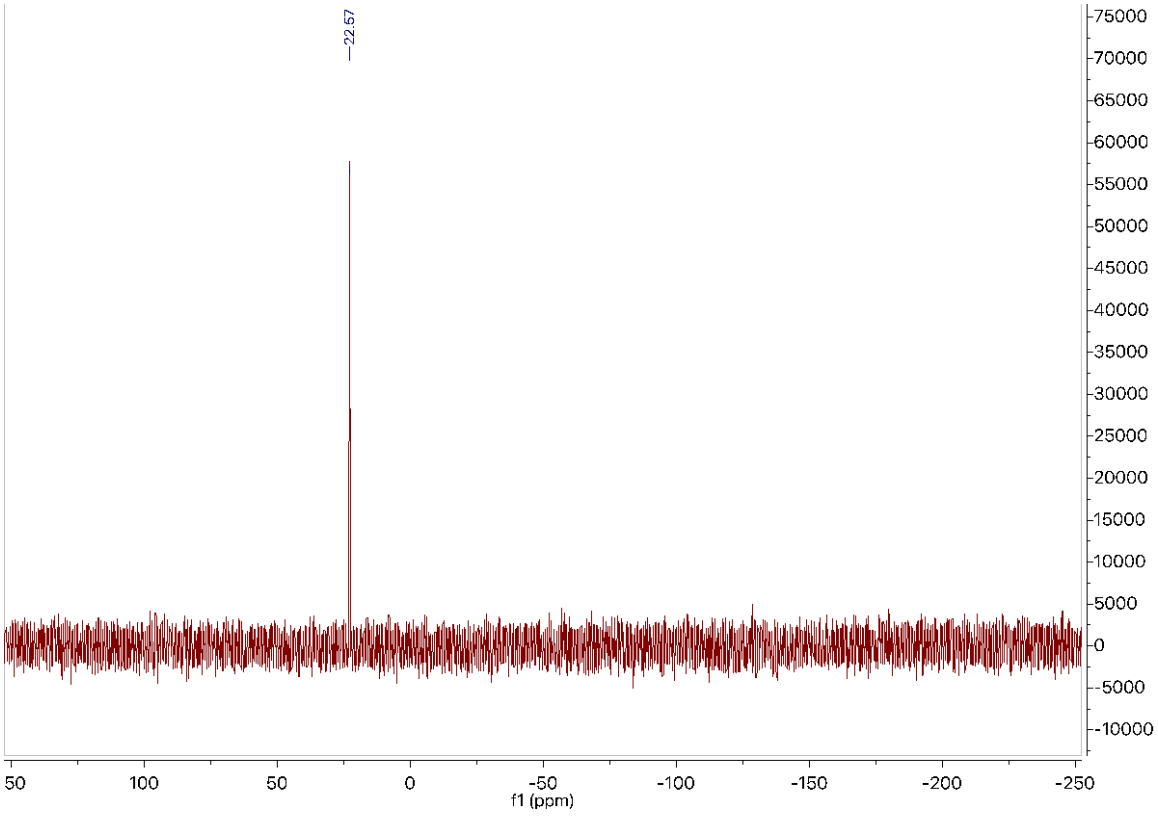
Spectrum S54. ^{31}P NMR of Compound **29.**



Spectrum S55. ^{1}H NMR of Compound **30.**



Spectrum S56. ^{31}P NMR of Compound **30.**



References

(1) Deal, P. E.; Kulkarni, R. U.; Al-Abdullatif, S. H.; Miller, E. W. Isomerically Pure Tetramethylrhodamine Voltage Reporters. *J. Am. Chem. Soc* **2016**, *138*, 29.

(2) Turnbull, J. L.; Benlian, B. R.; Golden, R. P.; Miller, E. W. Phosphonofluoresceins: Synthesis, Spectroscopy, and Applications. *J. Am. Chem. Soc*. **2021**, *143* (16), 6194–6201.

(3) AAT Bioquest, Inc. (2023, March 29). *Quest Database™ Quantum Yield [Rhodamine B]*. AAT Bioquest. https://www.aatbio.com/resources/quantum-yield/rhodamine_b.