SUPPORTING INFORMATION

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Materials and Methods

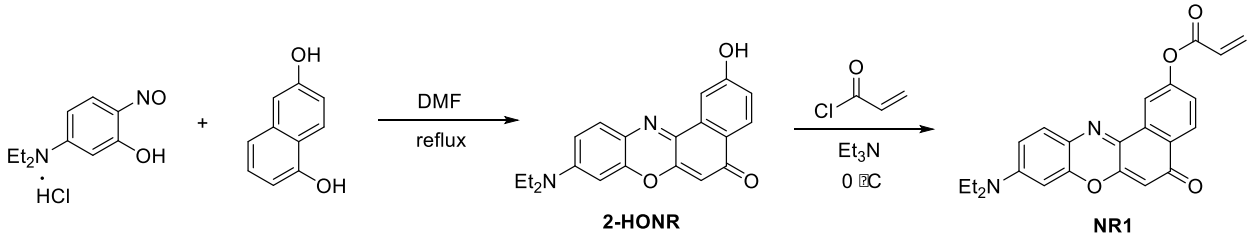
Materials

The thiol-reactive acrylodan and thrombin were purchased from Invitrogen. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids. 1,2-dipalmitoyl derivatives of phosphatidylinositol-(4,5)-bisphosphate (PIP_2) and phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) were from Cayman Chemical. Insulin was from Calbiochem.

Synthesis of Nile Red Derivatives

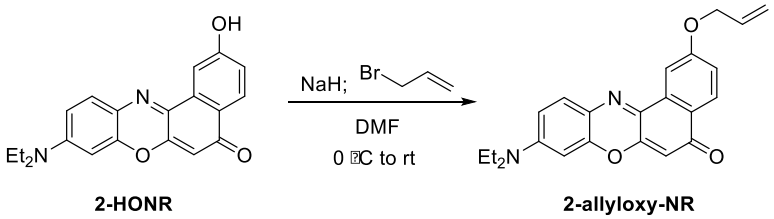
General Information: Reactions were carried out in oven or flame-dried glassware under inert gas unless otherwise noted. Starting materials were purchased from Aldrich, Acros or TCI America unless otherwise noted. Tetrahydrofuran (THF) was distilled over sodium and benzophenone under nitrogen atmosphere. Flash chromatography was performed using silica gel 60 Å (32−63 mesh) purchased from Silicycle Inc. Analytical thin layer chromatography (TLC) was performed on glass 0.25 mm Silicycle pre-coated silica gel 60 (particle size 0.040−0.063 mm). Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. 1H NMR and 13C NMR spectra were recorded on a Bruker DRX-500 spectrometer and DPX-400 spectrometers. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet), sext (sextet), m (multiplet), b (broad), and app (apparent). 1H NMR signals that fall within a ca. 0.3 ppm range are generally reported as a multiplet, with a single chemical shift value corresponding to the center of the peak. Coupling constants, J, are reported in Hz (Hertz). Electrospray ionization (ESI) mass spectra were recorded on a Waters Micromass Q-Tof Ultima in the University of Illinois at Urbana-Champaign. Electron impact (EI) mass spectra and Chemical Ionization (CI) mass spectra were obtained using a Micromass 70-VSE in the University of Illinois at Urbana– Champaign.

Preparation of NR1



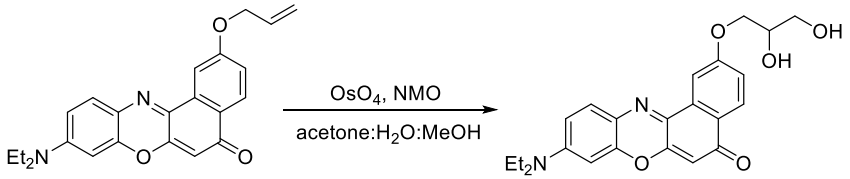
Preparation of **2-HONR** was performed utilizing a slight modification of the reported procedure. [1] 5-Diethylamino-2-nitrosophenol hydrochloride (2.3 g, 10 mmol) and 1,6-hydroxynaphthalene (1.6 g, 10 mmol) were heated under reflux in DMF (50 mL) for 4 h. After cooling to room temperature, SiO_2 (6 g) was added, and the DMF was removed under reduced pressure. The crude solid was flashed through an SiO_2 column (ethyl acetate–isopropanol, 100–50%), and the major purple band was collected as a single fraction to yield a dark purple solid whose characterization data matched those previously reported. **2-HONR** (334 mg, 1 mmol) was suspended in 10 mL dichloromethane (CH_2Cl_2) and triethylamine was added (210 µL, 1.5 mmol); the homogenous solution was cooled to 0 °C, and 1.2 equivalents of acryloyl chloride (97 µL, 1.2 mmol) was slowly added. Progress of the reaction was followed by TLC, and after completion (~30 min), 0.5 g SiO_2 was added and the volatiles were removed by rotary evaporation. Flash chromatography (hexanes:EtOAc, 4:1) of the resulting solid gave **NR1** as a purple solid (85%). 1H NMR (500 MHz, $CDCl_3$) δ 8.35 (s, 1H), 8.31 (d, J = 8.40 Hz, 1H), 7.50 (d, J = 8.92 Hz, 1H), 7.38 (d, J = 8.07 Hz, 1H), 6.66 (d, J = 17.34 Hz, 1H), 6.40-6.31(m, 3H), 6.07 (d, J = 10.31 Hz, 1H), 3.40 (q, J = 6.83 Hz, 1H), 1.22 (t, J = 6.78, 6.78 Hz, 1H); 13C NMR (125 MHz, $CDCl_3$) δ 182.8, 164.2, 153.1, 152.4, 151.0, 146.9, 139.0, 133.7, 133.1, 131.3, 129.5, 127.7, 127.6, 125.0, 123.4, 116.3, 109.9, 105.5, 96.2, 45.2, 12.7; IR (neat): 2928, 1740, 1620, 1584, 1493, 1402.

Preparation of 2-allyloxy-NR



To an ice cold solution of **2-HONR** (500 mg, 1.5 mmol) in 15 mL dimethylformamide was added NaH (66 mg, 1.65 mmol of a 60% dispersion in mineral oil). The suspension is stirred at 0 °C until gas evolution has ceased (~30 min) and allyl bromide (400 µL, 4.5 mmol) is added dropwise over 1 minute. The solution is allowed to warm to room temperature and stirred for 3 hours until TLC showed consumption of the starting material. The reaction mixture was quenched with saturated ammonium chloride (1 mL), diluted with water (45 mL) and extracted with chloroform (5 x 10 mL). The combined organic extracts were then washed with water (5 x 15 mL), brine (15 mL) and then dried (Mg_2SO_4). After filtration, the volatiles were removed by rotary evaporation, and flash chromatography (hexanes:EtOAc, 4:1) yielded **2-allyloxy-NR** as a purple solid (72%). 1H NMR (500 MHz, $CDCl_3$) δ 8.14 (d, J = 8.64 Hz, 1H), 7.95 (s, 1H), 7.45 (d, J = 8.95 Hz, 1H), 7.11 (d, J = 7.07 Hz, 1H), 6.53 (d, J = 7.51 Hz, 1H), 6.31 (s, 1H), 6.19 (s, 1H), 6.10 (m, 1H), 5.48 (d, J = 17.20 Hz, 1H), 5.33 (d, J = 10.46 Hz, 1H), 5.28 (s, 2H), 4.68 (d, J = 4.46 Hz, 2H), 3.37 (q, J = 6.64 Hz, 4H), 1.21 (t, J = 6.92 Hz, 6H); 13C NMR (125 MHz, CDCl3) δ 183.1, 161.1, 152.0, 150.7, 146.7, 139.6, 134.0, 132.8, 131.0, 127.6, 125.7, 124.6, 118.3, 118.1, 109.5, 106.7, 105.1, 96.1, 69.0, 45.0, 12.6; IR (neat): 1721, 1464, 1370; HRMS (ESI) calcd for $C_{23}H_{23}N_2O$ $[M+H]^+$ 375.1709, found 375.1707.

Preparation of NR-Diol

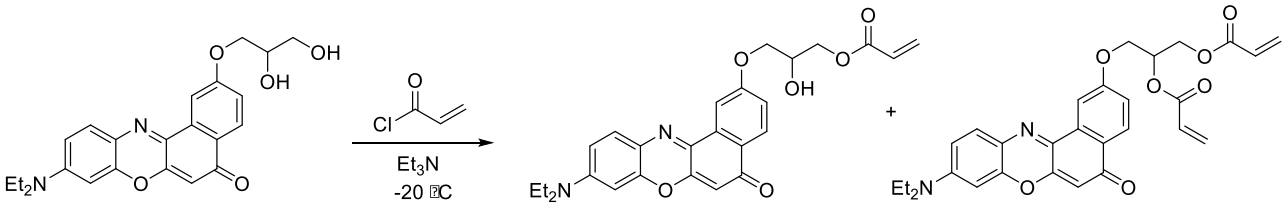


2-allyloxy-NR **NR-Diol**

To a solution of **2-allyloxy-NR** (500 mg, 1.34 mmol) in 22 mL acetone:H_2O:MeOH (8:2:1) was added NMO (313 mg, 2.68 mmol) followed by 6 drops of a 5% aqueous OsO_4 solution. The resulting mixture is allowed to stir at room temperature until TLC showed full consumption of the starting material (~36 h). After completion of the reaction, $Na_2S_2O_4$ (0.5 g) and magnesium silicate (0.5 g) were added, and the suspension was stirred for one hour at room temperature. Additional SiO_2 (1 g) was added, and the volatiles were removed by rotary evaporation. The crude solid was chromatographed on silica gel (EtOAc:CH_2Cl_2:MeOH, 2:1:2%) providing **NR-Diol** (65%). 1H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, J = 8.71 Hz, 1H), 7.92 (d, J = 2.31 Hz, 1H),

7.48 (d, J = 9.04 Hz, 1H), 7.08 (dd, J = 8.72, 2.37 Hz, 1H), 6.58 (dd, J = 9.14, 2.39 Hz, 1H), 6.39 (d, J = 2.40 Hz, 1H), 6.23 (s, 1H), 4.24-4.19 (m, 3H), 3.89 (ddd, J = 16.36, 11.54, 4.23 Hz, 1H), 3.44 (q, J = 7.00 Hz, 4H), 1.26 (t, J = 7.04 Hz, 6H); 13C NMR (125 MHz, CDCl3) δ 183.3, 161.2, 152.1, 150.7, 147.0, 139.4, 133.9, 131.2, 124.9, 118.3, 109.7, 106.7, 105.1, 96.2, 70.3, 69.3, 63.5, 45.1, 12.7; IR (neat): 3360, 3182, 2971, 2922, 2607, 1721, 1643, 1584, 1568, 1428, 1400; HRMS (ESI) calcd for $C_{23}H_{25}N_2O_5$ $[M+H]^+$ 409.1763, found 409.1767.

Acrylation of NR-Diol



NR-Diol **NR3** **NR2-*trace***

To a suspension of **NR-diol** (54 mg, 0.13 mmol) in 8 mL dichloromethane (CH_2Cl_2) was added triethylamine (100 µL); the mixture is stirred until a homogenous solution was obtained (about 10 min). After cooling to –20 °C, acryloyl chloride (4 µL, 0.33 equiv) was added slowly. After 10 minutes, SiO_2 (250 mg) was added, and the volatiles were removed. The resulting solid was chromatographed on silica gel, affording **NR3** (25%) and recovered **NR-diol** (60%).

1H NMR (500 MHz, $CDCl_3$) δ 8.21 (d, J = 8.71 Hz, 1H), 8.05 (d, J = 2.52 Hz, 1H), 7.58 (d, J = 9.03 Hz, 1H), 7.17 (dd, J = 8.73, 2.59 Hz, 1H), 6.65 (dd, J = 9.07, 2.66 Hz, 1H), 6.49 (dd, J = 17.31, 1.02 Hz, 1H), 6.45 (d, J = 2.62 Hz, 1H), 6.29 (s, 1H), 6.20 (dd, J = 17.32, 10.44 Hz, 1H), 5.90 (dd, J = 10.45, 1.04 Hz, 1H), 4.45 (ddd, J = 17.27, 11.52, 5.17 Hz, 2H), 4.38 (td, J = 10.31, 5.05, 5.05 Hz, 1H), 4.31-4.23 (m, 2H), 3.47 (q, J = 7.10, 7.08, 7.08 Hz, 4H), 1.26 (t, J = 7.09 Hz, 6H); 13C NMR (125 MHz, CDCl3) δ 183.3, 161.1, 152.1, 150.9, 146.8, 139.8, 134.2, 131.7, 131.1, 127.9, 126.2, 124.8, 118.1, 109.6, 106.8, 105.3, 96.3, 69.0, 68.6, 65.5, 45.1, 12.6; IR (neat): 3305, 2922, 2854, 1717, 1591, 1549, 1400; HRMS (ESI) calcd for $C_{26}H_{27}N_2O_6$ $[M+H]^+$ 463.1869, found 463.1872.

Preparation of Lipid Vesicles

Large unilamellar vesicles were prepared by extrusion using a 100 nm-pore membrane. Giant unilamellar vesicles (GUVs) were prepared by electroformation. The lipid mixture were prepared in chloroform/methanol (3:1) at a total concentration of 0.4 mg/ml, then the lipid solution was spread onto the indium-tin oxide electrode surface and the lipid was dried under vacuum to form a uniform lipid film. Vesicles were grown in a sucrose solution (350 mM) while an electric field (3V, 20Hz frequency) was applied for 5 hour at room temperature. After 1 to 2 ml of sucrose-loaded GUV solution was added into a well glued onto a coverslip that was placed on the microscope stage. The well contained 300 µl of 20 mM Tris-HCl buffer, pH 7.4, with 0.16 M KCl solution. The diameter of GUVs ranged from 5 to 30 µm.

Preparation of PS, PIP_2, and PIP_3 Sensor Constructs

The PIP_2 sensor, eENTH, was prepared from human epsin1 ENTH domain (1-158 amino acids) as described previously.[2] For PIP_3 sensor, human MyoX tandem PH domain (MyoXPH: a.a. 1170-1385)[3] was cloned into pGEX 4T-1 vector using the BamHI and EcoRI restriction enzyme sites. Two surface-exposed cysteine, Cys-1174 and Cys-1344, were mutated to Ala and a single cysteine for labeling was introduced by the Leu-1190 to Cys mutation. Finally, to improve membrane affinity of the protein, Met-1191 was mutated to Ala, yielding MyoXPH-C1174A/L1190C/M1191A/C1344A (eMyoXPH). The PS sensor, eLactC2, was prepared from the C2 domain region of lactadherin (LactC2; 1-158 amino acid), which was cloned into pET21 expression vector using the *Eco*RI and *Not*I restriction enzyme sites. To introduce a single cysteine labeling site in the membrane-binding region, Trp-26 was mutated to cysteine. Two endogenous cysteine residues were not removed because they form a disulfide bond and thus not accessible for labeling. All mutations were performed by polymerase chain reaction mutagenesis and verified by DNA sequencing.

Protein Expression, Purification and Labeling

eENTH and eMyoXPH were expressed as GST-tagged proteins whereas eLactC2 as a His_6-tagged protein. *E. coli* BL21 RIL codon plus (Stratagene) cells were used for protein expression. Cells were grown in Luria broth media containing 100 µg/ml of ampicillin at 37 ^{0}C. 0.1 mM of isopropyl β-D-1-thiogalactopyranoside was added to induce over-expression of recombinant

proteins when the OD_{600} reached 0.5-0.8 and cells were grown for additional 6 to 10 hours at 25 ^{0}C. Cells were harvested by centrifugation and cell pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.16 M KCl, 1 mM phenylmethanesulphonylfluoride, and 5 mM of dithiothreitol (DTT). Cells were lysed by sonication and the lysate was collected by centrifugation at 4 ^{0}C. All proteins were purified by affinity chromatography and labeled on the column before elution. For eENTH and eMyoXPH, GST-affinity resin (GenScript) was added into the cell lysate and the mixture was gently shaken for 30 min at 4 ^{0}C. The mixture was applied to a column and the column was washed once with 20 mM Tris-HCl buffer (pH 7.4) containing 0.16 M KCl and 5 mM of DTT to reduce thiol group, and washed several times with the same buffer without DTT to remove DTT. For eLactC2, cell lysates were mixed with Ni-nitrilotriacetic acid agarose (Qiagen) and the mixture was poured onto a column and washed with 50 mM Tris-HCl buffer (pH 7.4) containing 0.16 M KCl and 5 mM of DTT and with the same buffer without DTT. For labeling of the proteins, stock solutions of acrylodan or NR3 in dimethyl sulfoxide were prepared and >100 μg of acrylodan or NR3 was added to the resin and incubated at 4^{0}C overnight with gentle shaking. The excess reagent was removed by washing the resin with the buffer several times. For eENTH and eMyoXPH, the resin was incubated with thrombin (Life Technologies) at room temperature for 2 h to cleave the labeled protein from the GST tag. The labeled protein was collected from the resin by centrifugation. The labeled eLactC2 was eluted from the resin with 50 mM Tris buffer, pH 7.4, containing 300 mM imidazole and the sensor solution was dialyzed against 50 mM Tris buffer, pH 7.4, containing 0.16 M KCl. Purity and the concentration of the recombinant proteins were determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis and Bradford (BioRad) assay, respectively. The labeling yield of each sensor was then calculated spectrophotometrically as described previously.[2] The labeling step was repeated until >70% labeling yield was achieved.

Spectrofluorometric Measurements

Horiba Flurolog-3 spectrofluorometer was used for all cuvette-based fluorescence measurements. Lipid sensors (typically 500 nM) were added to large vesicles with various lipid compositions and the emission spectra of DAN and NR3 were measured with excitation wavelength set at 392 nm and 520 nm, respectively, in a single-photon excitation mode.

Calibration of Lipid Sensors by Fluorescence Microscopy

All fluorescence microscopy measurements were carried out at 37 ºC in a two-photon excitation mode using a custom-built multi-photon, four-channel microscope equipped with two femtosecond-pulsed laser sources (Newport) [4]. Both instrument control and data analysis were performed by PrairieView (Bruker). *In vitro* calibration of DAN-eLactC2 and NR3-eLactC2 was performed using GUVs composed of POPC/POPS (100-*x*:*x*) (*x* = 0-30 mole%). These GUV were mixed with DAN-eLactC2 (or NR3-eLactC2) in the concentration range of 0-500 nM. DAN-eLactC2 and NR3-eLactC2 were two-photon excited at 780 nm and 1000 nm, respectively. 436 ± 10 and 525 ± 25 band pass filters were employed for the blue channel and the green channel, respectively, whereas 600 ± 19 and 635 ± 20 band pass filters were used for the orange channel and the red channel, respectively. For DAN-eLactC2, blue channel fluorescence signals derive from membrane-bound sensors only whereas green channel signals are from both membrane-bound and sensors. Likewise, orange channel fluorescence signals derive from membrane-bound sensors only whereas red channel signals are from both membrane-bound and sensors for NR3-eLactC2. At each time point, an image of 512 x 512 pixels was collected with the pixel dwell time of 20 millisecond using Peltier-cooled 1477P style Hamamatsu photomultiplier tubes. For each PS concentration, 10 GUVs were selected for image analysis by MATLAB (MathWorks, Inc) or Image-Pro Plus (Media Cybernetics, Inc). For data analysis, each cross-sectional two-dimensional vesicle image was read into a M x N (typically 256 x 256) matrix. The region of interest (the membrane in our case) was selected by setting a threshold intensity (or brightness) value on the basis of the intensity distribution profile of the image obtained using a virtual line drawn over the vesicle and rotated 360 degree around it. Typically, 15% of the maximal brightness (in the center) was set as a threshold to distinguish the membrane region from the non-membrane region (by graythresh function in MATLAB or by the automatic intensity cutoff function in Image-Pro Plus) and a virtual mask was drawn to cover the selected membrane region. Since the orange channel (or blue channel for DAN-eLactC2) always gives stronger membrane signals than the red channel (or green channel for DAN-eLactC2) for NR3-eLactC2, we first selected the mask from the orange (or blue) channel and superimposed it onto the same image in the red (or green) channel. The estimated membrane region of the vesicle was validated by comparing it with the membrane region in the differential interference contrast image of the vesicle. The total photon counts of GUV ($F_{B(total)}$ and $F_{G(total)}$ for DAN-eLactC2 and $F_{O(total)}$ and

$F_{R(total)}$ for NR3-eLactC2) were divided by the total area of the pixels that constitute each GUV to yield the average intensities, F_B and F_G for DAN-eLactC2 and F_O and F_R for NR3-eLactC2 (counts/m^2), which were then used to prepare the calibration curves for ratiometric analyses. For DAN-eLactC2, K_d and $(F_B/F_G)_{max}$ values were calculated from non-linear least-squares analysis of the (F_B/F_G) versus PS plot using the equation; $(F_B/F_G) = (F_B/F_G)_{max}/(1 + K_d/[PS]) + C$ where $(F_B/F_G)_{max}$, K_d, and C indicate the maximal F_B/F_G value, the equilibrium dissociation constant (in terms of mole%), and the arbitrary instrumental parameter, respectively. The theoretical calibration curve was then constructed using these values (see Figure 1c) and [PS] from an unknown sample was calculated using the calibration curve. The same calibration was performed for NR3-eLactC2. Essentially the same protocol was used for the calibration of the PIP_2 and PIP_3 sensors except that POPC/POPS/PIP_2 (80-*x*:20:*x*, where *x* = 0-4 mole%) and POPC/POPS/PIP_3 (80-*x*:20 *x*, where *x* = 0-3 mole%) GUVs were employed for the PIP_2 and PIP_3 sensors, respectively.

Cellular Culture

NIH 3T3 cells were seeded into 50 mm round glass-bottom plates and grown at 37^oC in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fatal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Life Technologies) and passaged every 2-3 days. PC3 cells were cultured in F-12 medium with 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate and also passaged every 2-3 days. Transient transfection of PC-3 cells with PTEN was performed using Lipofectamine (Life Technologies). NIH 3T3 cells were treated with 1 μM doxorubicin and 1 μM insulin in the media to induce apoptosis and to activate PI3K, respectively. The cells were cultured in the plates for about 24 h before lipid quantification.

Cellular Lipid Quantification

For PS quantification in the inner and outer PM of NIH 3T3 cells, DAN-eLactC2 was delivered into NIH 3T3 cells by microinjection and NR3-eLactC2 was added to the growth media. For simultaneous quantification of PIP_2 and PIP_3, both sensors were microinjected into the same cell. Microinjection was performed using the Eppendorf InjectMan NI 2 system as described

previously.[2] P-97 Pipette Puller was used for microinjection micropipette preparation. To minimize cell damages by microinjection, we used the calcium-free media and gave enough time for cells to recover after injection, and selected only healthy cells for further fluorescence measurements. Lipid sensor concentrations were adjusted to give strong enough fluorescence signals for robust data analysis. Typically, 20-30 femtoliter of 0.5-1 μM sensor solution was microinjected into the cell. All microscopy measurements and imaging data analysis were performed as described above. The local lipid concentration on the cell membrane was determined from the observed local (F_B/F_G) or (F_O/F_R) values using the *in vitro* calibration curves determined using GUVs with corresponding lipid compositions. The three-dimensional display of local lipid concentration was obtained using the surf function in MATLAB. The angular profile of photon counts in the cellular PM was calculated by MATLAB using the algorithm described previously.[2] Briefly, two binary image masks were created: the first one was around the PM as described above and the second one was a triangular mask that connects the center of the cell, any position on the PM and the second position on the PM that is advanced from the first position by 2 (or any discrete number) degree. The average local intensity was calculated by multiplying the two masks and the cell's image matrix. The same operation was repeated for every 2 degrees around the PM in a counter-clockwise manner.

[1] M. S. J. Briggs, I. Bruce, J. N. Miller, C. J. Moody, A. C. Simmonds, E. Swann, *J Chem Soc-Perkin Trans 1* **1997**, 1051-1058.

[2] Y. Yoon, P. J. Lee, S. Kurilova, W. Cho, *Nat Chem* **2011**, *3*, 868-874.

[3] Q. Lu, J. Yu, J. Yan, Z. Wei, M. Zhang, *Mol Biol Cell* **2011**, *22*, 4268-4278.

[4] R. V. Stahelin, M. A. Digman, M. Medkova, B. Ananthanarayanan, J. D. Rafter, H. R. Melowic, W. Cho, *J. Biol. Chem.* **2004**, *279*, 29501-29512.

Supplementary Figures

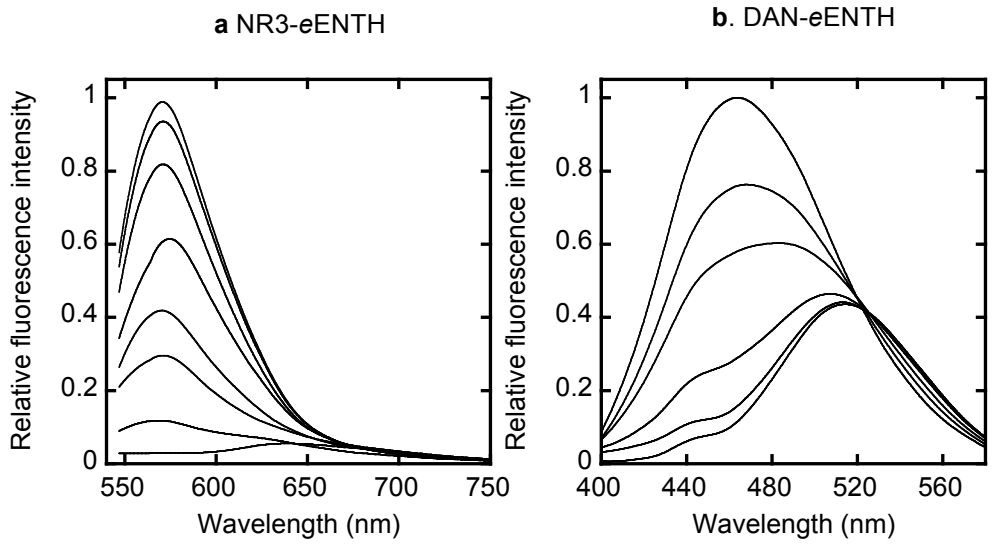


Figure S1. Fluorescence emission spectra of NR3-eENTH (a) and DAN-eENTH (b) in the presence of PC/PS/PIP_2 (80-*x*:20:*x*) large vesicles. Numbers indicate mole% of PIP_2. For NR3-eENTH, PIP_2 concentrations varied from 0, 0.5, 1, 2, 3, 4, and 5 mole% from bottom to top. For DAN-eENTH, PIP_2 concentrations varied from 0, 0.5, 1, 2, and 3 mole% from bottom to top. For both sensors, the bottom spectra are from the sensor alone. Excitation wavelengths were 392 nm and 520 nm for DAN-eENTH and NR3-eENTH, respectively. 500 nM of PS sensor was allowed to interact with vesicles in 20 mM Tris, pH 7.4 containing 0.16 M KCl.

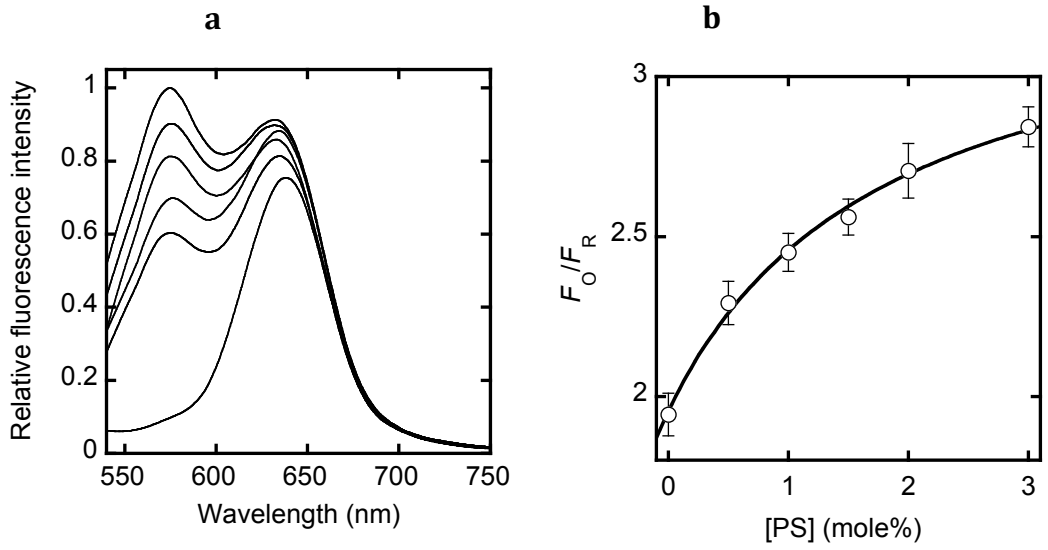


Figure S2. a) Fluorescence emission spectra of NR3-eMyoXPH in the presence of PC/PS/PIP_3 (80-*x*:20:*x*) large vesicles. Numbers indicate mole% of PIP_3. Excitation wavelength was 520 nm. 500 nM of PS sensor was allowed to interact with vesicles in 20 mM Tris, pH 7.4 containing 0.16 M KCl. b) The ratiometric calibration curve of NR3-eMyoXPH for PIP_3 quantification. The PIP_3 sensor was monitored by a two-photon microscope in the presence of PC/PS/PIP_3 (80-*x*:20:*x*) giant vesicles. F_o and F_R indicate orange and red channel fluorescence intensity, respectively. Non-linear least-squares analysis of the plot using the equation: $F_O/F_R = (F_O/F_R)_{max}/(1 + K_d/[PIP_3]) + C$ where $(F_O/F_R)_{max}$, K_d, and C indicate the maximal F_O/F_R value, the equilibrium dissociation constant (in terms of mole%), and the arbitrary instrumental parameter, respectively. K_d, $(F_O/F_R)_{max}$ and C values and the calibration curves are constructed using these parameters. Error bars indicate standard deviations calculated from >3 independent sets of measurements. 20 mM Tris buffer at pH 7.4 containing 0.16 M KCl was used for all measurements.

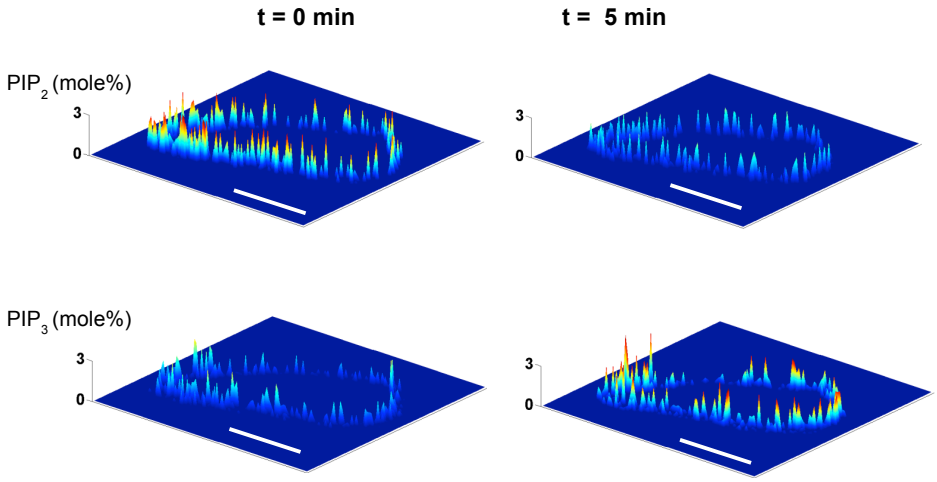
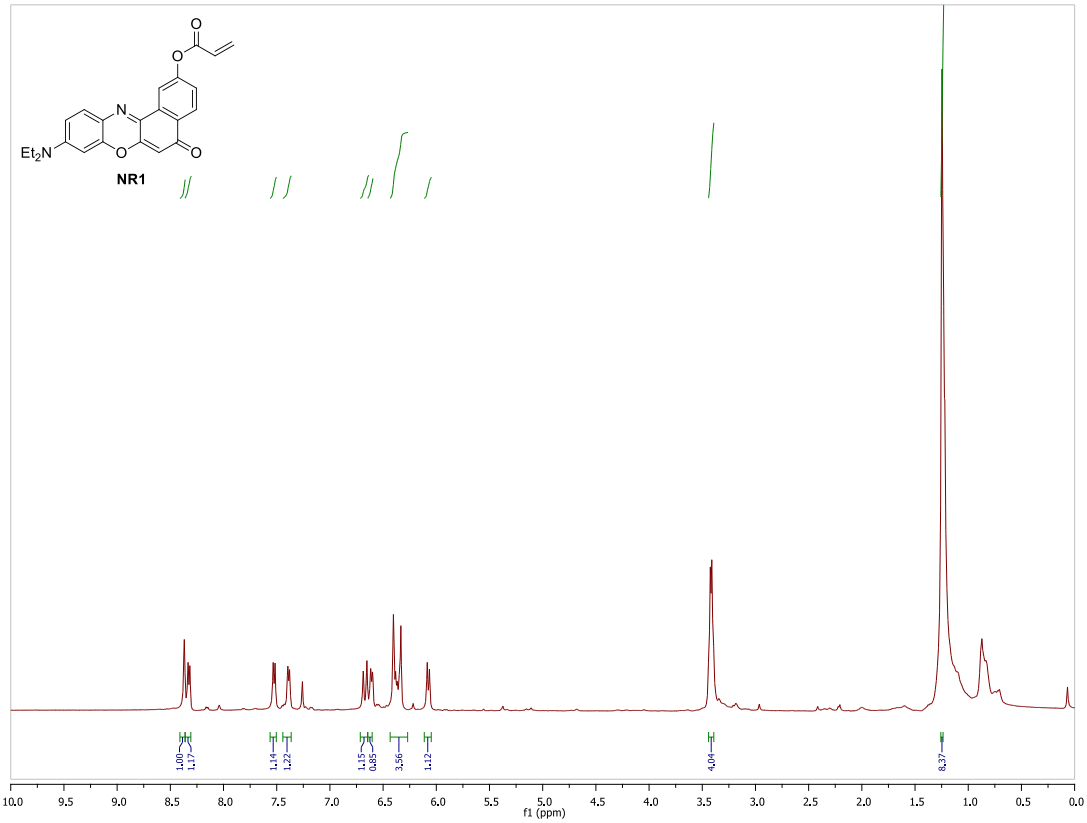


Figure S3. Simultaneous *in situ* quantification of PIP_2 and PIP_3 in the inner PM of PC3 cells. PIP_2 and PIP_3 were quantified using microinjected DAN-eENTH and NR3-eMyoXPH, respectively, through ratiometric calibration (see Figure S2). Notice that at 5 min PIP_3 was enriched over PIP_2 in the PM. White bars indicate 5 μm.


NR1
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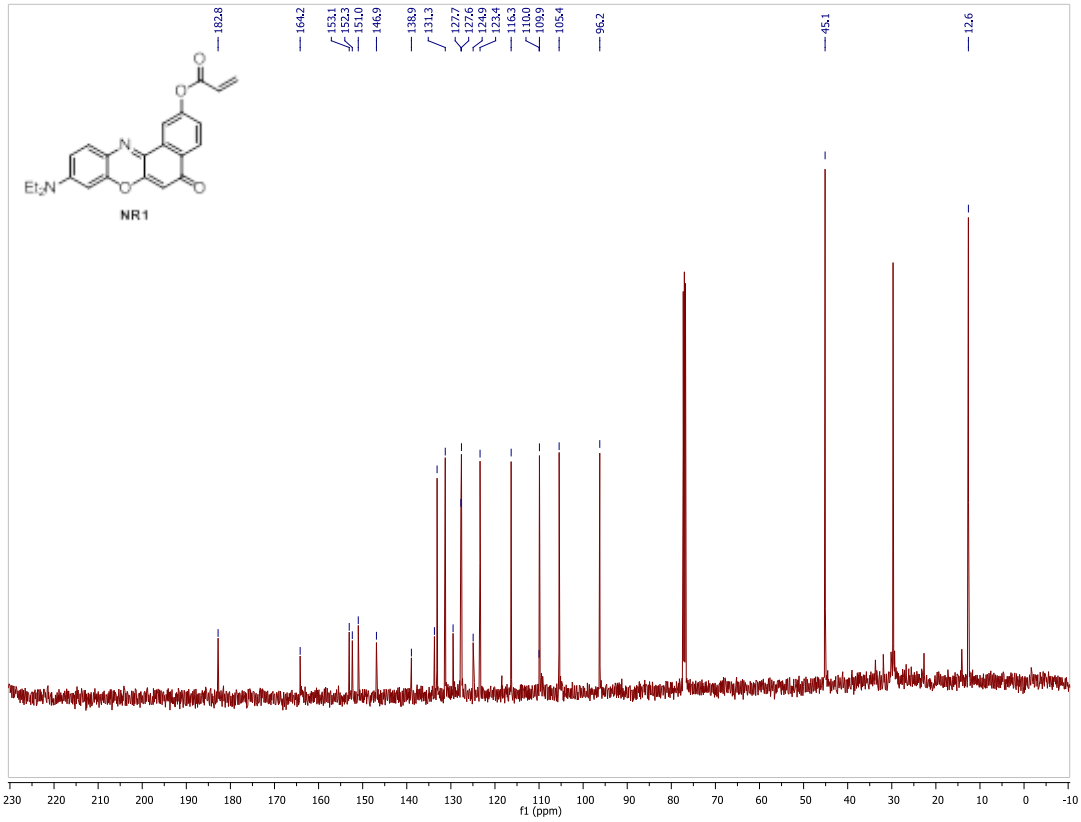
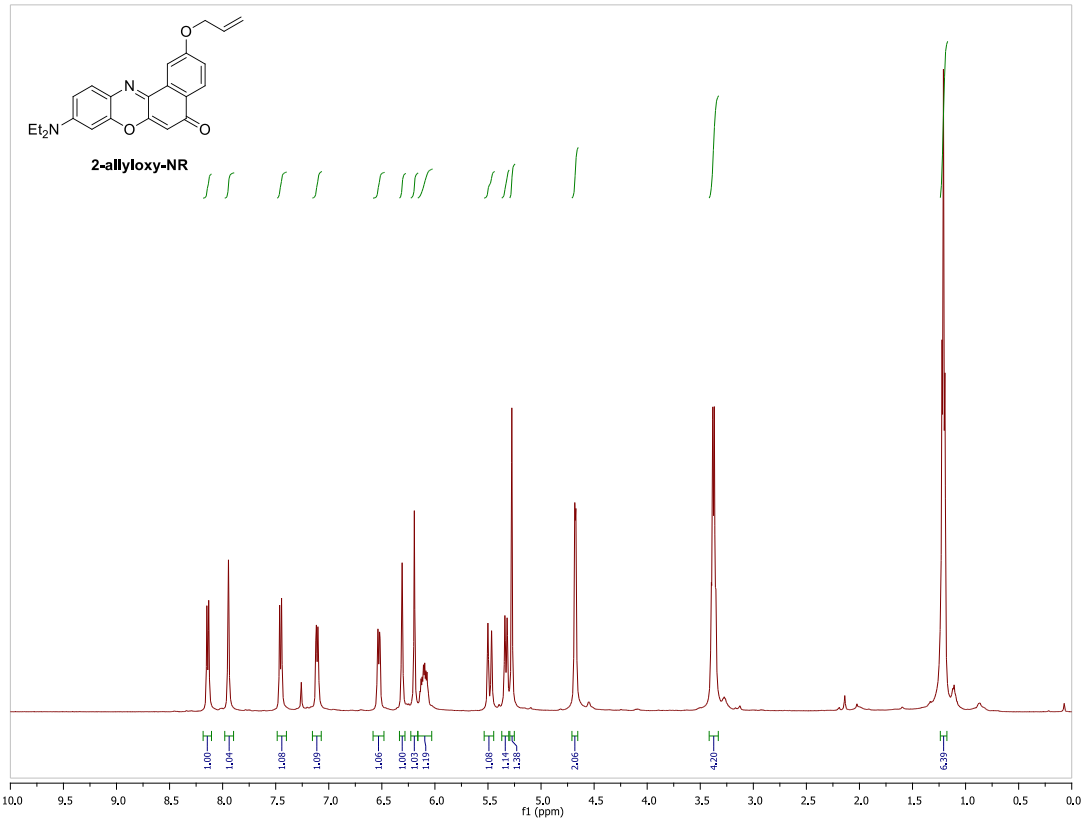
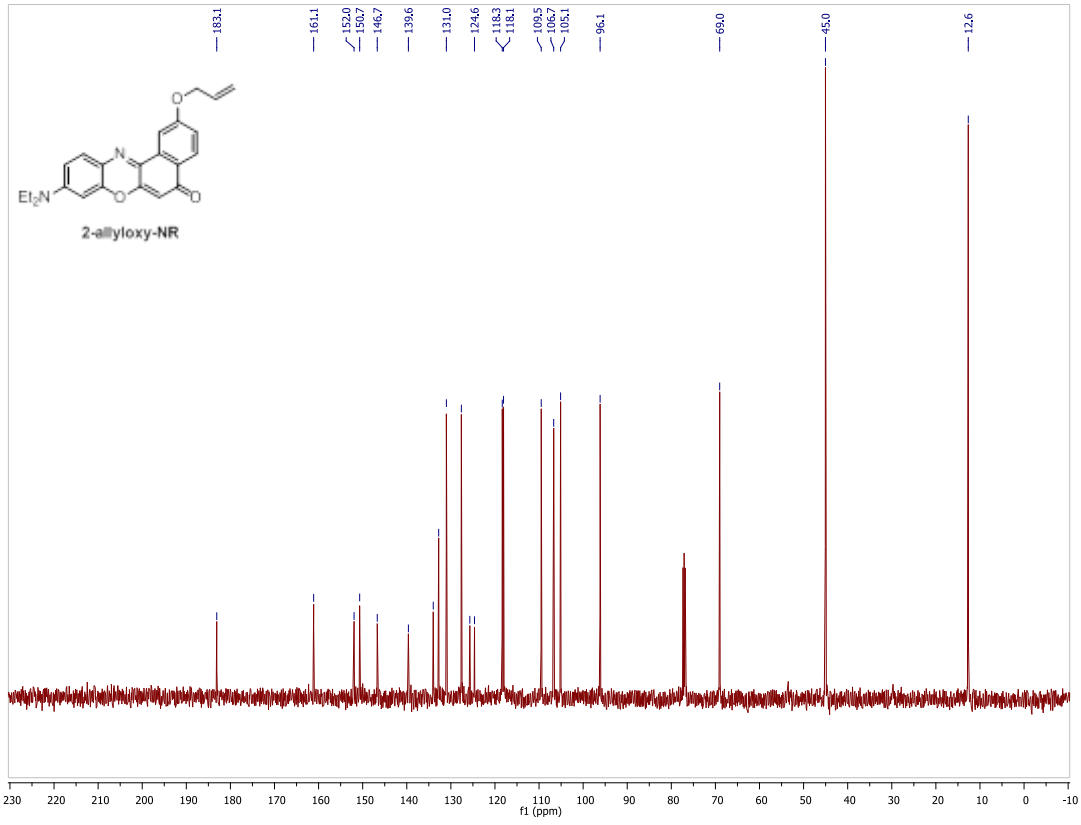
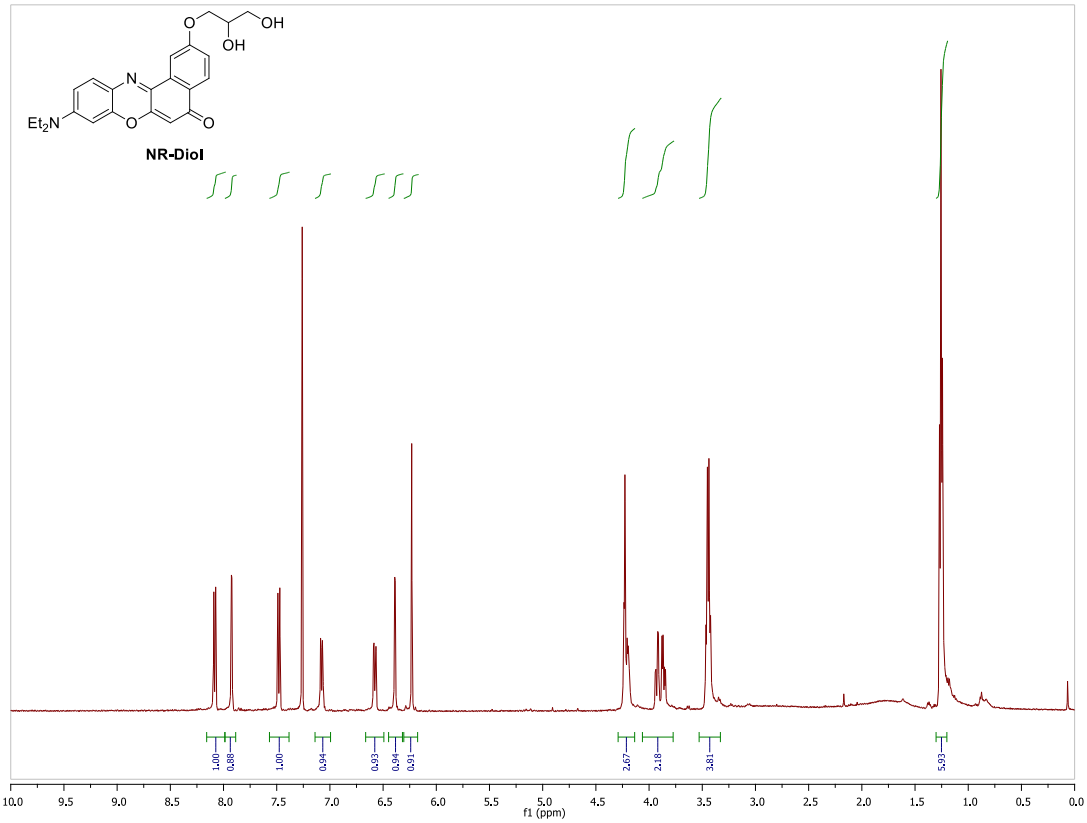
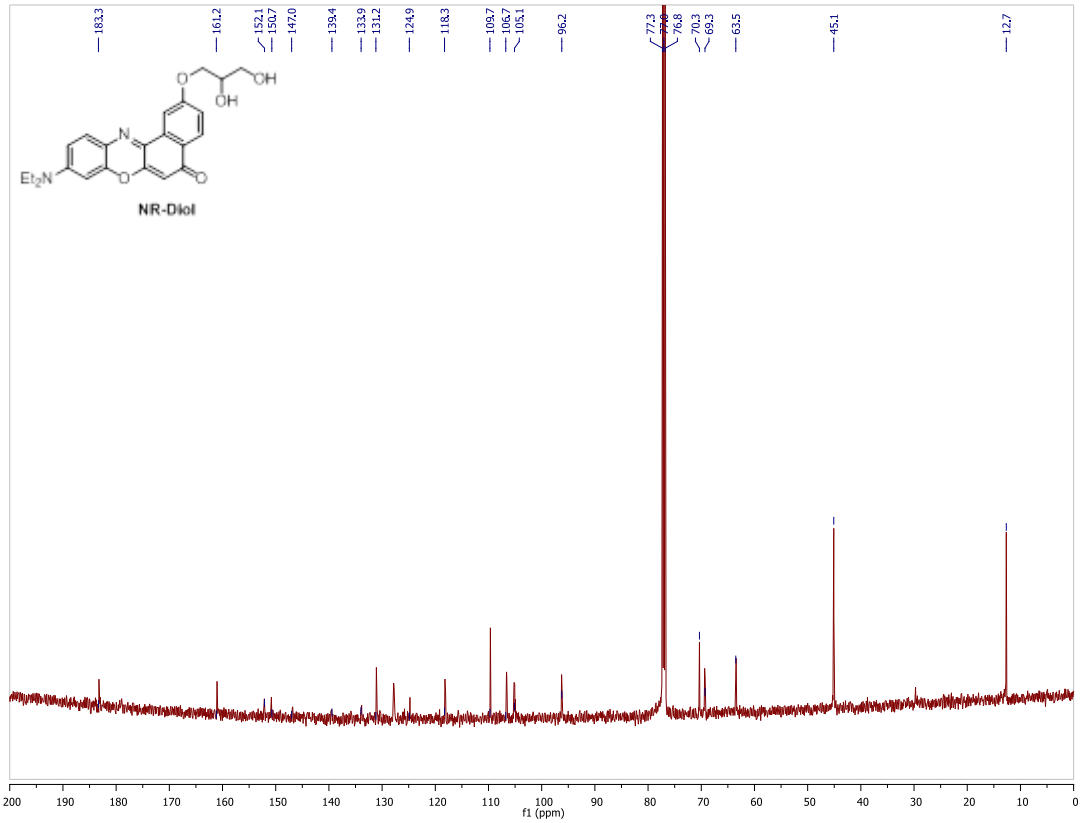
1.14
1.22
1.15
0.85
3.56
1.12
4.04
8.37
f1 (ppm)

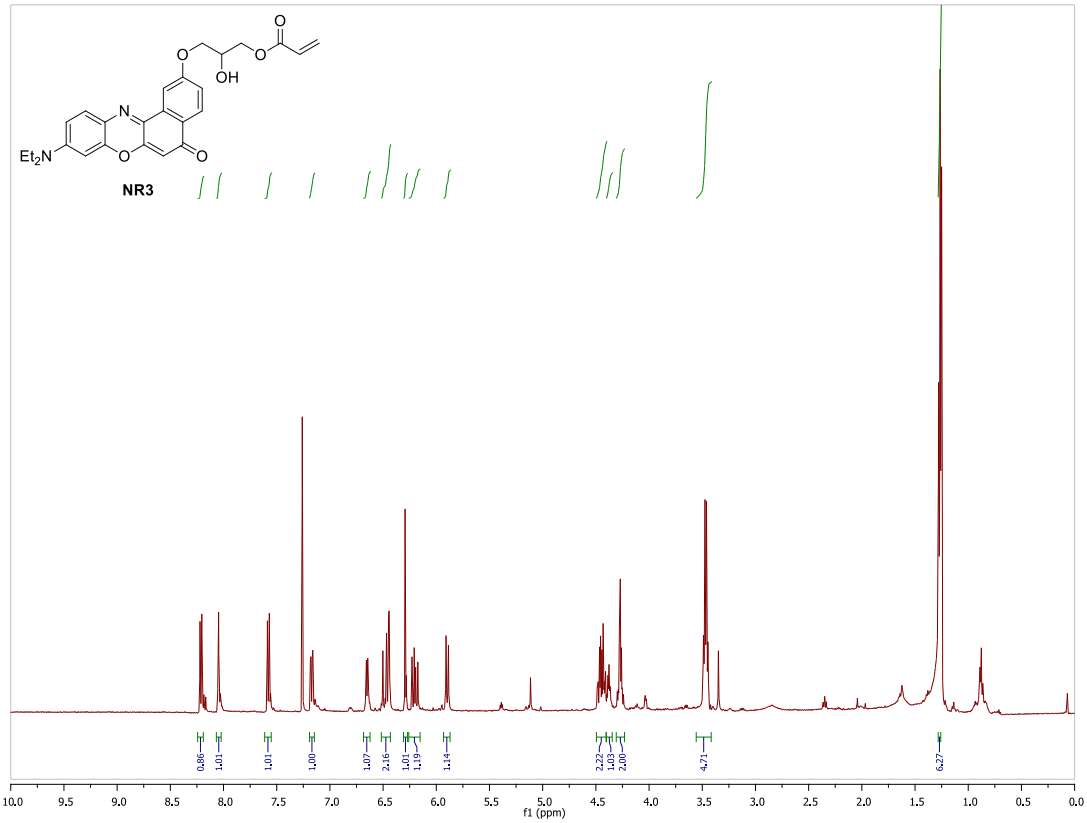

NR1
182.8
164.2
153.1
152.3
151.0
146.9
138.9
131.3
127.7
127.6
124.9
123.4
116.3
110.0
109.9
105.4
96.2
45.1
12.6
f1 (ppm)

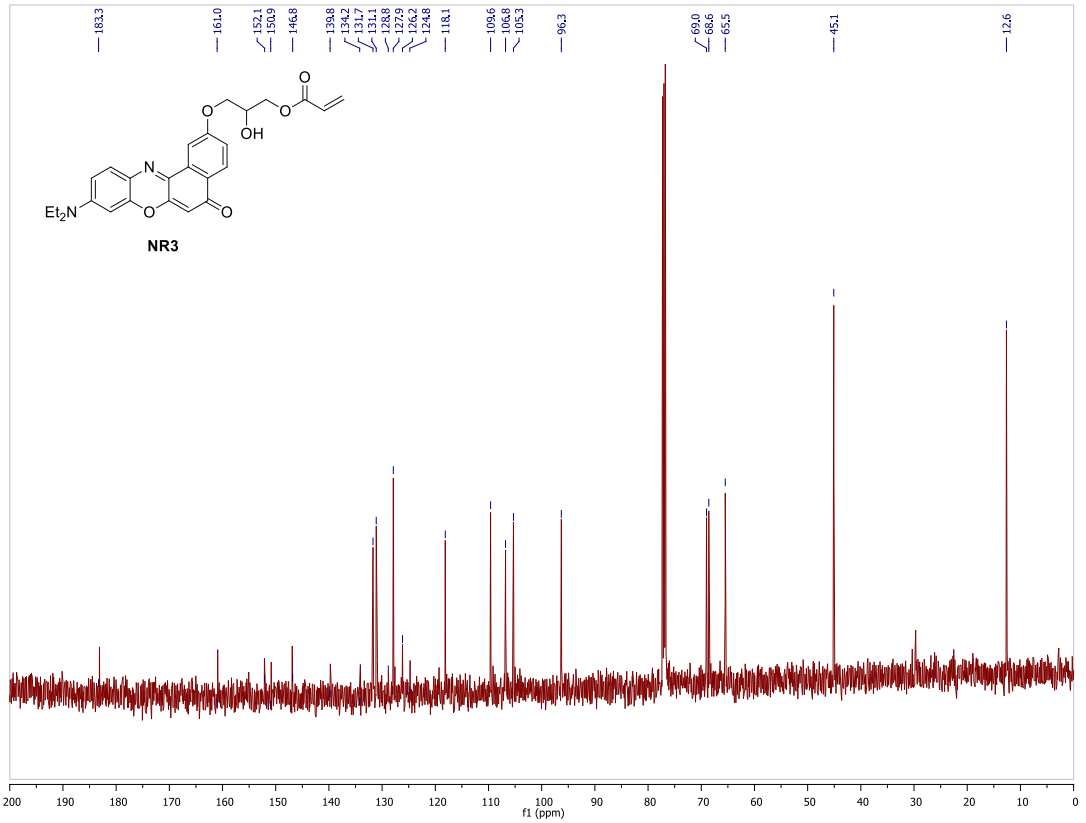

2-allyloxy-NR
1.00
1.04
1.08
1.09
1.06
1.00
1.03
1.19
1.08
1.14
1.38
2.06
4.20
6.39
f1 (ppm)


183.1
161.1
152.0
150.7
146.7
139.6
131.0
124.6
118.3
118.1
109.5
106.7
105.1
96.1
69.0
45.0
12.6
2-allyloxy-NR
f1 (ppm)


NR-Diol
1.00
0.88
1.00
0.94
0.93
0.94
0.91
2.67
2.18
3.81
5.93
f1 (ppm)


183.3
161.2
152.1
150.7
147.0
139.4
133.9
131.2
124.9
118.3
109.7
106.7
105.1
96.2
77.3
76.8
70.3
69.3
63.5
45.1
12.7
NR-Diol
f1 (ppm)


NR3
0.86
1.01
1.01
1.00
1.07
2.16
1.01
1.19
1.14
2.22
1.03
2.00
4.71
6.27
f1 (ppm)


183.3
161.0
152.1
150.9
146.8
139.8
134.2
131.7
131.1
128.8
127.9
126.2
124.8
118.1
109.6
106.8
105.3
96.3
69.0
68.6
65.5
45.1
12.6
NR3
f1 (ppm)