

Supporting Information

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**Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine
Cycloadditions****

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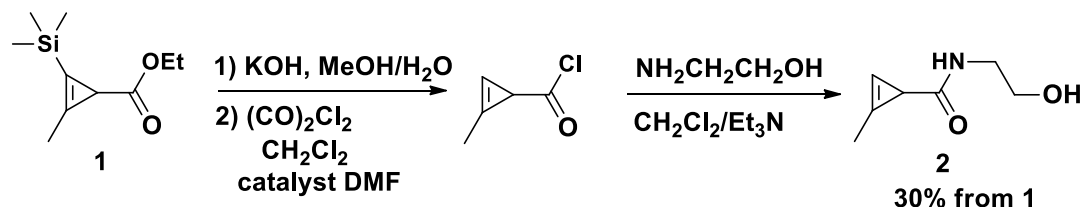
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Starting materials

All chemicals were received from commercial sources and used without further purification. Thin layer chromatography (TLC) was performed on silica gel. Chromatographic purifications were conducted using 40-63 μm silica gel. All mixtures of solvents are given in v/v ratio. ^1H and ^{13}C NMR spectroscopy was performed on a Varian NMR at 400 (^1H) or 100 (^{13}C) MHz and a Jeol NMR at 500 (^1H) or 125 (^{13}C) MHz. All ^{13}C NMR spectra were proton decoupled.

Synthesis of cyclopropene 2



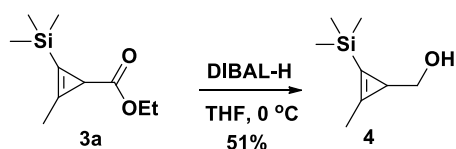
Compound **1** was synthesized according to a previously reported method (Cho, Suk H. and Liebeskind, Lanny S., *J. Org. Chem.*, **52**, 2631-4, 1987) and was obtained in 70% yield. To a stirred solution of compound **1** (213.0 mg, 1.0 mmol) in MeOH (4.0 mL) at 0 °C was slowly added a solution of KOH (140.0 mg, 2.5 mmol) in H₂O (1.0 mL) dropwise. After all the KOH was added, the reaction mixture was stirred overnight at room temperature before it was diluted with 10 mL H₂O and extracted with EtOAc (10 mL \times 2). The pH value of the aqueous layer was adjusted to 3 by addition of 1M HCl, then extracted with EtOAc (10.0 mL \times 3), the combined organic layer was dried over Na₂SO₄ and evaporated to afford 72 mg of the crude methyl cyclopropene acid as a colorless oil.

The crude acid was dissolved in 5 mL CH₂Cl₂ and one drop of DMF was added, followed by (CO)₂Cl₂ (126 mg, 1.0mmol). The resulting solution was stirred for 2 hours at room temperature and then evaporated to afford the crude methyl cyclopropene acid chloride.

To a stirred solution of ethanolamine (61 mg, 1.0 mmol) and Et₃N (101 mg, 1.0 mmol) in CH₂Cl₂ (2 mL) was added a solution of the above methyl cyclopropene acid chloride in CH₂Cl₂ (2 mL). The resulting reaction solution was stirred for 1 hour at room temperature and then evaporated to afford the crude product. The residue was purified using preparative TLC (EtOAc/Hexane = 1.5:1) to afford 42 mg of compound **2**. The overall yield is 30% from compound **1**.

^1H NMR (500 MHz, CDCl₃) δ 2.03 (1H, d, J = 5 Hz), 2.18 (3H, d, J = 5 Hz), 2.98 (1H, bs), 3.43 (2H, t, J = 5 Hz), 3.71 (2H, t, J = 5 Hz), 5.90 (1H, bs), 6.44 (1H, s); ^{13}C NMR (125 MHz, CDCl₃) 10.70, 22.35, 42.63, 62.41, 95.91, 113.57, 177.71; HRMS [M+Na]⁺ m/z calcd. for [C₇H₁₁NO₂Na]⁺ 164.0682, found 164.0684.

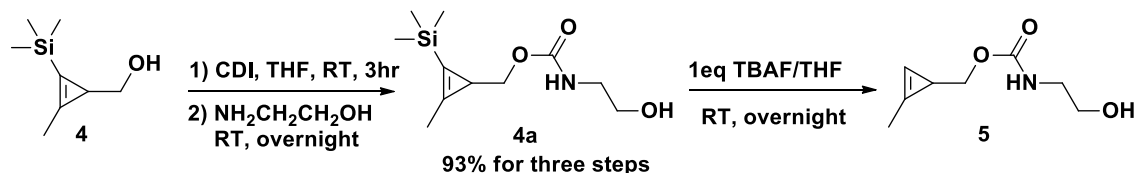
Synthesis of (2-methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl)methanol 4



To a stirred solution of compound **1** (2.0 g, 10.0 mmol) in dry THF (25.0 mL) at 0 °C was slowly added a solution of 1.0 M LiAlH₄ in THF (25.0 mL, 25.0 mmol) dropwise. After addition of LiAlH₄, the reaction mixture was stirred 3 hours at 0 °C before it was quenched with H₂O carefully. The precipitate was filtered and the filtrate was concentrated to give the crude product. The residue was purified by flash silica column chromatography (Hexane/EtOAc = 5:1) to afford 0.8 g compound **4** as a colorless oil in 51% yield. ^1H NMR (500 MHz, CDCl₃) δ 0.17 (9H, s), 1.57 (1H, t, J = 5 Hz), 2.22

(3H, s), 3.48 (2H, d, $J = 5$ Hz); ^{13}C NMR (100 MHz, CDCl_3) 0.99, 13.56, 22.25, 69.60, 111.48, 135.86.

Synthesis of cyclopropene 5



Carbonyldiimidazole (CDI; 88 mg, 0.55 mmol) was added to a stirred solution of compound 4 (70 mg, 0.45 mmol) in dry THF (3.0 mL) at room temperature. The resulting solution was stirred for 3 hours and then ethanolamine (34 mg, 0.55 mmol) was added. The reaction solution was stirred overnight at room temperature and then evaporated to afford the crude product. The residue was purified by preparative TLC (Hexane/EtOAc = 2:1) to afford 0.08 g compound 4a as colorless oil. The above compound 4a was dissolved in dry THF (3.0 mL), followed by addition of 1.0 M TBAF in THF (0.5 mL, 0.5 mmol). The reaction solution was stirred at room temperature overnight until no starting material could be observed by TLC. The reaction solution was evaporated and purified by preparative TLC (Hexane/EtOAc = 1/1) to afford 71 mg of compound 5 as a colorless oil. Overall yield is 93% starting from compound 4.

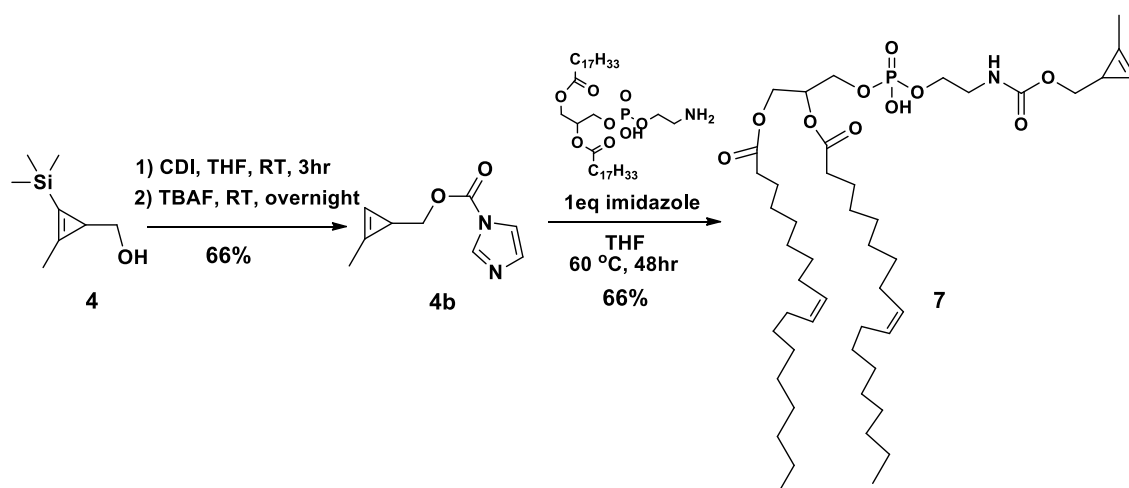
Compound 4a:

^1H NMR (500 MHz, CDCl_3) δ 0.11 (9H, s), 1.50 (1H, t, $J = 5$ Hz), 2.15 (3H, d, $J = 5$ Hz), 3.21 (1H, bs), 3.30 (2H, dd, $J = 10\text{Hz}, 5$ Hz), 3.66 (2H, bs), 3.81 (1H, bs), 3.90 (1H, dd, $J = 10$ Hz, 5 Hz), 5.29 (1H, bs); ^{13}C NMR (125 Hz, CDCl_3) 14.29, 25.87, 30.10, 50.03, 64.99, 74.28, 104.07, 122.91, 141.60.

Cyclopropene 5:

^1H NMR (500 MHz, CDCl_3) 1.61 (1H, t, $J = 5$ Hz), 2.10 (3H, d, $J = 5$ Hz), 3.16 (1H, bs), 3.30 (2H, dd, $J = 10\text{Hz}, 5$ Hz), 3.67 (2H, d, $J = 5$ Hz), 3.90 (2H, d, $J = 5$ Hz), 5.35 (1H, bs), 6.54 (1H, s); ^{13}C NMR (125 Hz, CDCl_3) 24.65, 29.05, 50.09, 65.13, 73.43, 97.04, 111.85, 141.58; HRMS $[\text{M}+\text{Na}]^+$ m/z calcd. for $[\text{C}_8\text{H}_{13}\text{NO}_3\text{Na}]^+$ 194.0788, found 194.0789.

Synthesis of lipid cyclopropene 7



To a stirred solution of compound **4** (200 mg, 1.03 mmol) in dry THF (10.0 mL) at room temperature was added CDI (176 mg, 1.10 mmol). The resulting solution was stirred for 3 hours and then 1M TBAF in THF (1.1 mL, 1.10 mmol) was added. The reaction solution was stirred overnight at room temperature and then evaporated to afford the crude product. The residue was purified by flash silica column chromatography (Hexane/EtOAc = 1:1) to afford 110 mg compound **4b** as a colorless oil in 66% yield. The above compound **4b** was dissolved in dry THF (5.0 mL) and followed by addition of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipid (200 mg, 0.27 mmol) and imidazole (45 mg, 0.66 mmol). The reaction solution was stirred at 60 °C for 48 hours. The reaction solution was evaporated and the residue dissolved with 20 mL EtOAc and washed with 1 M HCl (20 mL×2), dried over the organic layer with Na₂SO₄ and evaporated to afford the crude product. The residue was purified by flash silica column chromatography (CH₂Cl₂/MeOH = 10/1) to afford 150 mg compound **7** as colorless oil in 66% yield.

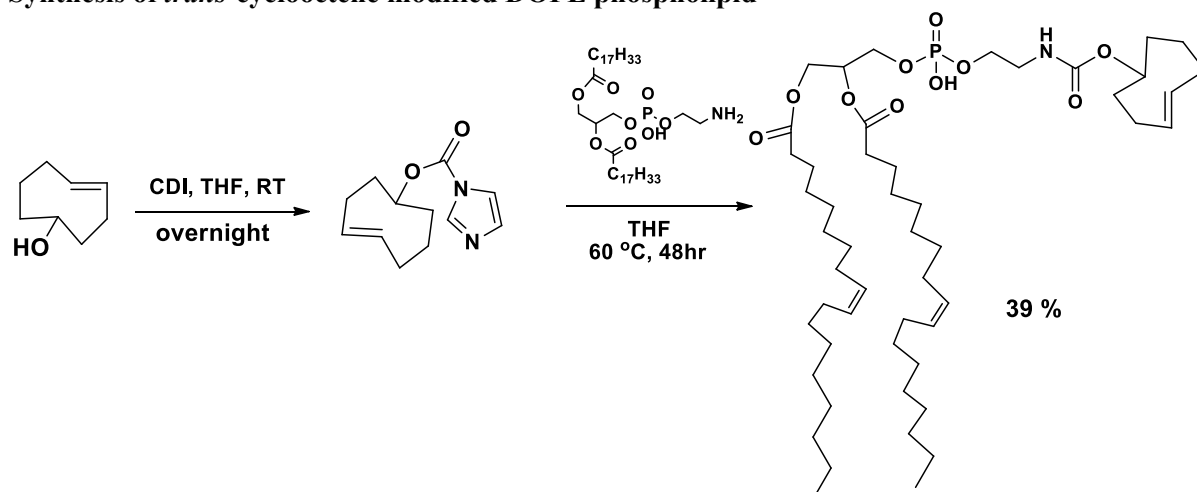
Compound **4b**:

¹H NMR (500 MHz, CDCl₃) δ 1.78 (1H, t, J = 5 Hz), 2.16 (3H, d, J = 5 Hz), 4.25 (1H, dd, J = 10Hz, 5 Hz), 4.33 (1H, dd, J = 10Hz, 5 Hz), 6.60 (1H, s), 7.07 (1H, s), 7.44 (1H, s), 8.55 (1H, s); ¹³C NMR (125 Hz, CDCl₃) 11.68, 16.72, 76.22, 101.67, 117.22, 120.11, 130.56, 137.79.

Lipid cyclopropene **7**:

¹H NMR (500 MHz, CDCl₃) δ 0.87 (6H, t, J = 10 Hz), 1.25-1.31 (40H, m), 1.57-1.59 (5H, m), 2.00 (8H, m), 2.11 (3H, s), 2.88 (4H, dd, J = 20Hz, 10Hz), 3.38 (2H, bs), 3.88 (2H, bs), 3.93 (2H, bs), 3.97 (2H, bs), 4.13 (1H, m), 4.37 (1H, m), 5.22 (1H, bs), 5.33 (4H, m), 5.96 (1H, bs), 6.55 (1H, s); ¹³C NMR (125 Hz, CDCl₃) 29.40, 31.34, 33.73, 38.21, 39.98, 41.84, 43.52, 43.88, 47.25, 53.12, 70.20, 71.50, 72.40, 76.20, 77.93, 101.90, 116.52, 123.58, 123.73, 123.82, 123.99, 145.96, 158.59, 158.75; HRMS [M-H]⁻ m/z calcd. for [C₄₇H₈₃NO₁₀P]⁻ 852.5760, found 852.5757.

Synthesis of *trans*-cyclooctene modified DOPE phospholipid



To a stirred solution of *trans*-cyclooctene (3.0 mg, 0.023 mmol) in dry THF (10.0 mL) at room temperature was added CDI (4.0 mg, 0.025 mmol). The resulting solution was stirred overnight at room temperature and then 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 15.0 mg, 0.020 mmol) was added. The reaction solution was stirred at 60 °C for 48 hours. The reaction solution was evaporated and the residue was purified by preparative TLC (DCM:MeOH=10:1) to afford 7.0 mg product as colorless oil in 39 % yield. ¹H NMR (500 MHz, CDCl₃) δ 0.87 (6H, t, J = 10 Hz), 1.25-2.31 (72H, m), 3.23-3.36 (2H, m), 3.86-3.91 (4H, m), 4.13 (1H, m), 4.30-4.38 (2H, m), 5.22 (1H, bs), 5.31-5.39 (5H, m), 5.48-5.52 (1H, m); HRMS [M-H]⁻ m/z calcd. for [C₅₀H₈₉NO₁₀P]⁻ 894.6230, found 894.6231.

Stability of cyclopropene 5

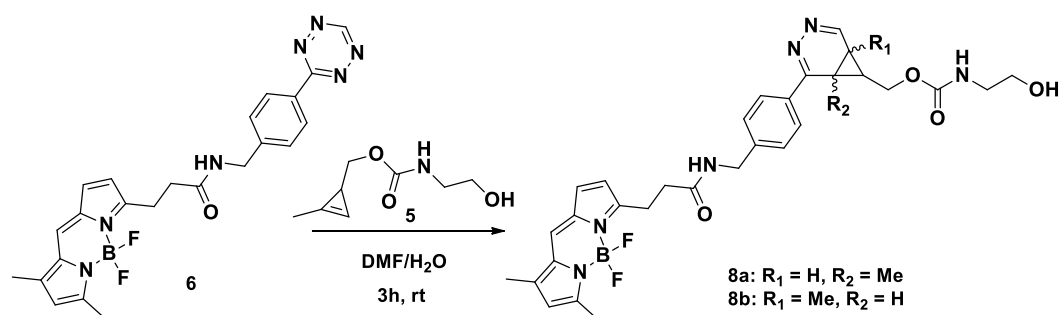
Cyclopropene **5** was kept at 37 °C in D₂O: DMSO-*d*₆ = 10:1 and ¹H NMR was taken over a period of 24 h at the following time points : 0.0, 0.5, 1.0, 1.5, 3.0, 6.0 and 24.0 h. By comparing the peak abundance of the cyclopropene alkene proton (6.21 ppm) at different time points, we determined the stability of cyclopropene **5** (Figure S2).

Stability of methyl cyclopropene 5 and *trans*-cyclooctenol in the presence of L-cysteine

Cyclopropene **5** and *trans*-cyclooctenol [*rel*-(1*R*, 4*E*, *pR*)-cyclooct-4-enol] were separately combined with 1 equivalent of L-cysteine in D₂O:DMSO-*d*₆ (10:1) and kept at room temperature. ¹H NMR were taken over a period of 4 hours. The ¹H NMR showed both dienophiles are stable to L-cysteine at room temperature.

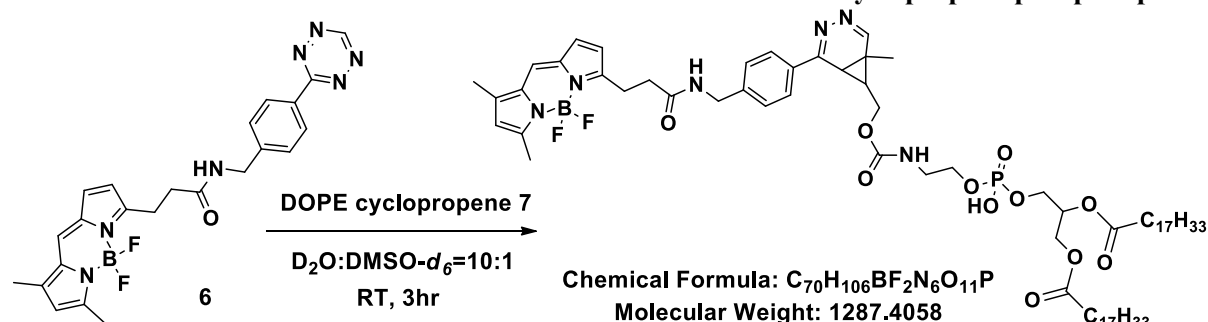
We next heated both mixtures to 60 °C for 4 hours. ¹H NMR showed cyclopropene **5** remained stable at this temperature (Figure S3 shows the ¹H NMR for cyclopropene **5** after 4 hours incubation with L-cysteine at room temperature and after 4 hours at room temperature followed by 4 hours at 60 °C). However we observed that *trans*-cyclooctenol was being converted to *cis*-cyclooctenol under these conditions. After heating 4 hours, 34% of the *trans*-cyclooctenol was converted to *cis*-cyclooctenol. We studied the conversion of *trans*-cyclooctene to *cis*-cyclooctene over a period of 21 hours at 60 °C in the presence of L-cysteine. The ¹H NMR showed that after 21 hours, 88% of the *trans*-cyclooctenol was converted to *cis*-cyclooctenol (Figure S4).

HPLC characterization of the reaction between tetrazine-BODIPY FL 6 with cyclopropene 5



Tetrazine-BODIPY FL **6** (1.0 mM in dry DMF, 20 μ L) and Cyclopropene **5** (1.0 mM in H₂O, 20 μ L) were combined in 210 μ L of H₂O at a final concentration of 0.08 mM for tetrazine-BODIPY FL **6**. The reaction solution was agitated for 3 hours at room temperature and then analyzed by LC-MS. Multiple peaks were identified with molecular mass corresponding to diazanorcaradiene adducts (m/z 605 [M+H]⁺). The multiple peaks are expected given the previously demonstrated potential to form several isomeric products. The reaction, based on the remaining signal from the tetrazine-BODIPY FL **6**, went to completion (Figure S5).

Characterization of reaction between tetrazine-BODIPY-FL 6 and cyclopropene phospholipid 7



Tetrazine-BODIPY FL **6** (1.0 mM in dry DMF, 20 μ L) and DOPE cyclopropene **7** (1.0 mM in DMSO, 20 μ L) were combined in 60 μ L of H₂O at a final concentration of 0.2 mM for tetrazine-BODIPY FL **6**. The reaction solution was agitated for 3 hours at room temperature and then analyzed by negative ion electrospray mass spectrometry in order to identify the molecular mass corresponding to diazanorcaradiene adducts (m/z 1286 [M-H]⁻, Figure S6).

Fluorescence unquenching measurements

Freshly-purified tetrazine-BODIPY FL **6** was dissolved in DMF and reacted with 10-fold excess cyclopropene **5** at the final concentrations of 10 μ M tetrazine and 100 μ M cyclopropene in 1% v/v DMF/dH₂O. The reaction mixture was kept at room temperature (20 $^{\circ}$ C). Emission scans were recorded using a Perkin Elmer LD-45 spectrometer, with the excitation wavelength of 470 nm (2.5-nm slit width), and emission signal was tracked over the 485-640 nm range (5.0-nm slit width). Emission was measured over time and compared against a control sample lacking cyclopropene. There was no emission change observed over the initial 2 h timeframe for the control sample. The resulting unquenching of the BODIPY FL fluorescence increased as measured at 30, 90, and 120 min intervals (Figure S7). The measurements were stopped after 2 h, as the rate of change in fluorescence peak intensity was decreasing.

Reaction rate determination

A tetrazine **3** stock solution was prepared in DMSO and used to prepare tetrazine solutions at 0.6 mM final concentration in 12% v/v DMSO in ddH₂O. Reactions were initiated with excess cyclopropene at final concentrations of 6.0, 8.0, and 10.0 mM. The disappearance of the tetrazine absorption peak at 520 nm was tracked over the reaction timeframe by measuring the absorption spectra using a NanoDrop 2000c spectrophotometer (Thermo Scientific). Samples were placed in a quartz cuvette with 10-mm pathlength and stirred at the maximum speed setting of the instrument. The temperature was uncontrolled by the instrument for room temperature (20 °C) measurements, and set and equilibrated at 37 °C for the higher temperature experiments.

Absorption spectra were measured manually over time. Absorption peak signal was taken as the average of measurements at 519-521 nm. Baseline signal was determined as the sloping line connecting the measured background levels preceding and following the tetrazine peak (410-430 nm and 590-610 nm, respectively). Final peak intensity value was taken as the signal above the baseline (Figure S8).

Reaction rates were obtained by fitting the exponential decays of tetrazine peak absorption intensity as a pseudo first order reaction. Nonlinear data fits were performed with GraphPad Prism. Tetrazine reactions with cyclopropene carbamate **5** were carried out at 20 and 37 °C (data points and corresponding fitted curves at 20 °C in Figure S1, 37 °C in Figure 2c). Tetrazine reactions with cyclopropene carboxamide **2** were done at 37 °C (Figure 2f). A representative comparison fit is shown for cyclopropenes **5** and **2** at 8.0 mM concentration reacting at 37 °C with 0.6 mM tetrazine (Figure S9).

Live-cell microscopy

Human breast cancer SKBR3 cells were received from Professor Jered Haun (University of California, Irvine). The cells were incubated overnight on a Lab-Tek chamber slide maintained in cDMEM medium (10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin). Cells were washed with phosphate-buffered saline (PBS) and incubated in cDMEM with 100 μM of cyclopropene **5** for 1 hour at 37 °C. The media was aspirated, and cells were washed twice with PBS. Cells were then incubated in cDMEM and 10 μM tetrazine-BODIPY FL **6** probe for 1-2 hours at 37 °C. In the last 30 min of incubation 300 nM DAPI was added to the incubation media. Cells were washed twice with PBS before imaging. All photos were collected with an Olympus FV1000 confocal microscope using ImageJ 1.45j software package.

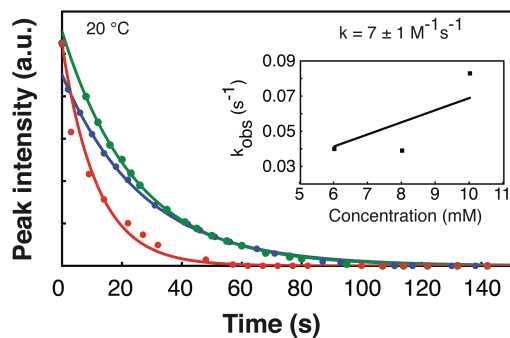


Figure S1. Kinetics of the tetrazine **3** reaction with cyclopropene **5** at 20 °C. Experiment was done with 0.6 mM tetrazine **3** and increasing excess of cyclopropene **5**: 6.0 mM (green), 8.0 mM (blue), 10.0 mM (red). Insert shows the corresponding observed reaction rates (k_{obs}) from the fitted data (individual fits shown as lines in the main graph) plotted against cyclopropene **5** concentrations. The slope of the resulting line was used to determine the second-order rate constant, indicated in the upper right corner.

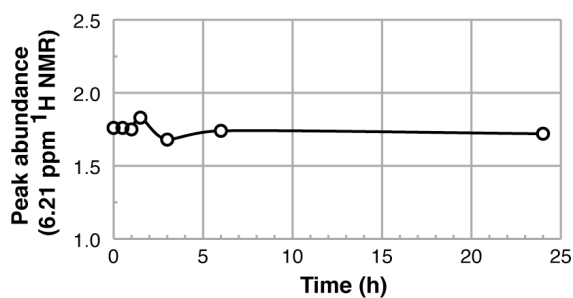


Figure S2. Time course of stability of cyclopropene carbamate **5** by ^1H NMR peak abundance at 6.21 ppm over 24 h.

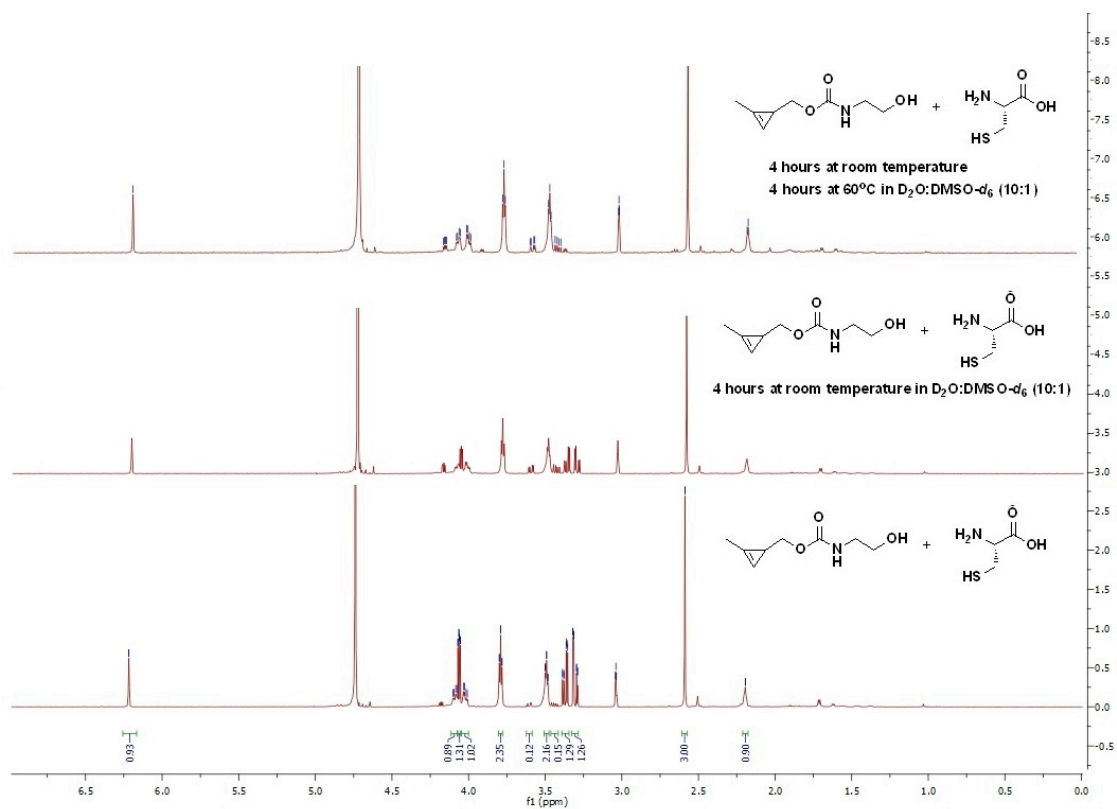


Figure S3. ^1H NMR spectrum of methyl cyclopropene **5** and L-cysteine after 4 hours at room temperature; 4 hours at room temperature and 4 hours at 60°C in $\text{D}_2\text{O}:\text{DMSO-}d_6$ (10:1). L-cysteine slowly oxidizes to cystine over the time-course of the experiment.

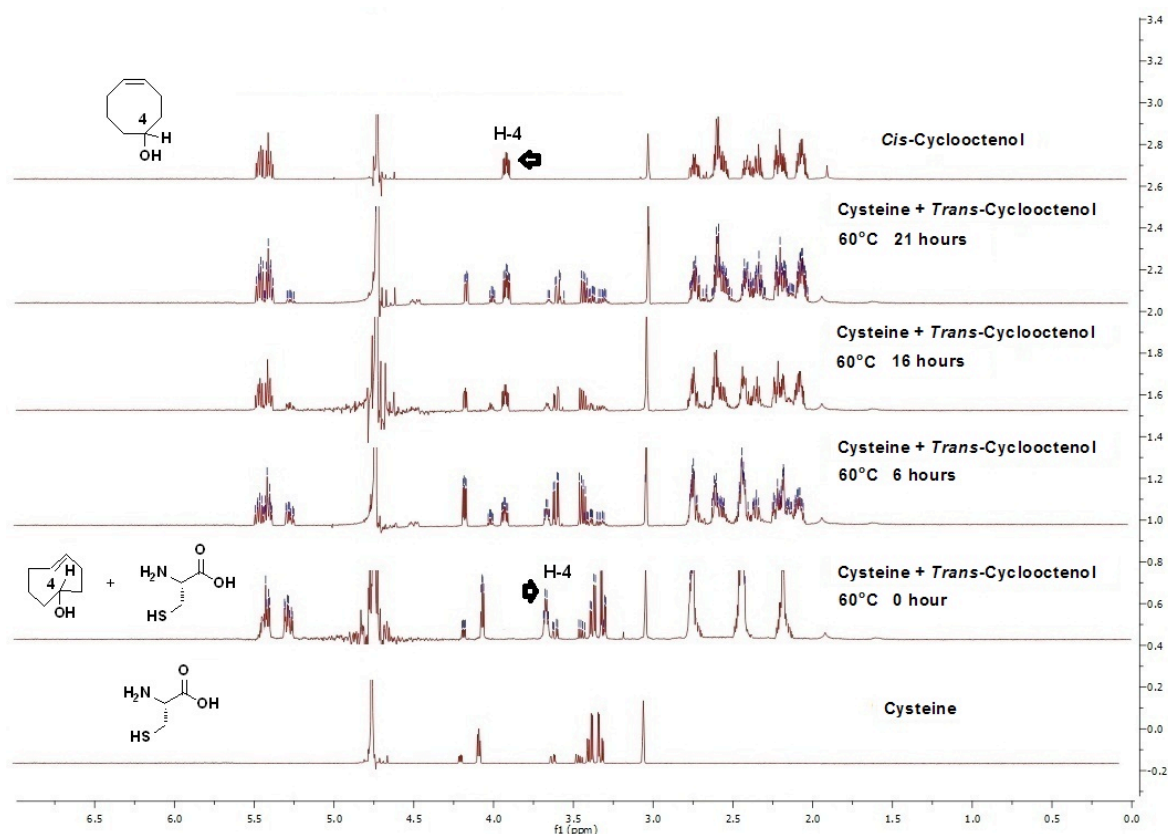


Figure S4. ^1H NMR spectra demonstrating the conversion of *trans*-cyclooctenol to *cis*-cyclooctenol in the presence of L-cysteine at 60°C . L-cysteine slowly oxidizes to cystine over the time-course of the experiment.

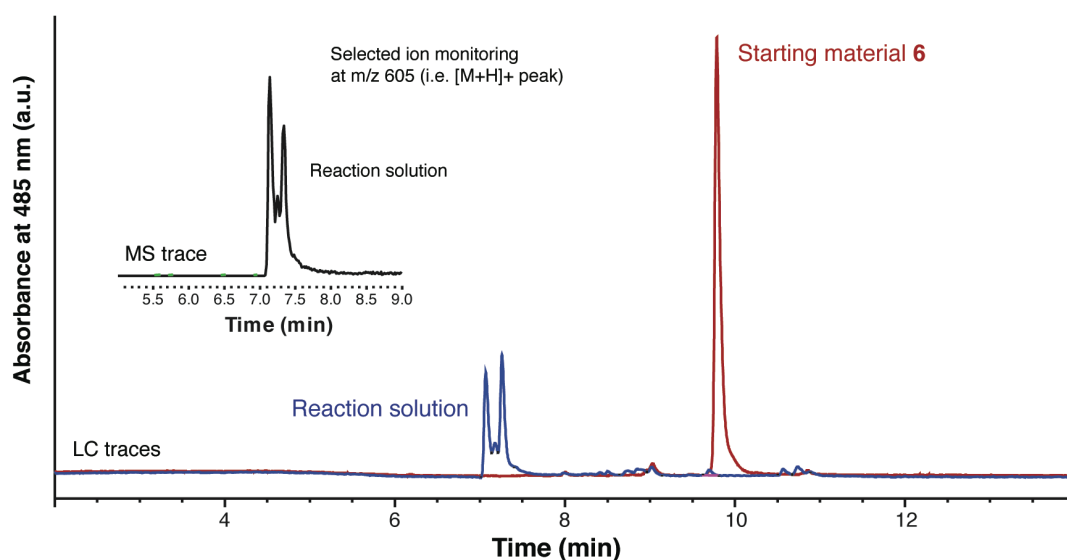


Figure S5. Reaction between tetrazine-BODIPY FL **6** with cyclopropene **5**. HPLC trace of purified tetrazine-BODIPY FL **6** (0.08 mM in 8% DMF/ H_2O) (red line). HPLC trace of the reaction products from addition of cyclopropene **5** to tetrazine-BODIPY FL **6** (blue line). MS trace of the reaction solution, selected ion monitoring at m/z 605 (i.e. $[\text{M}+\text{H}]^+$ peak) (black insert line).

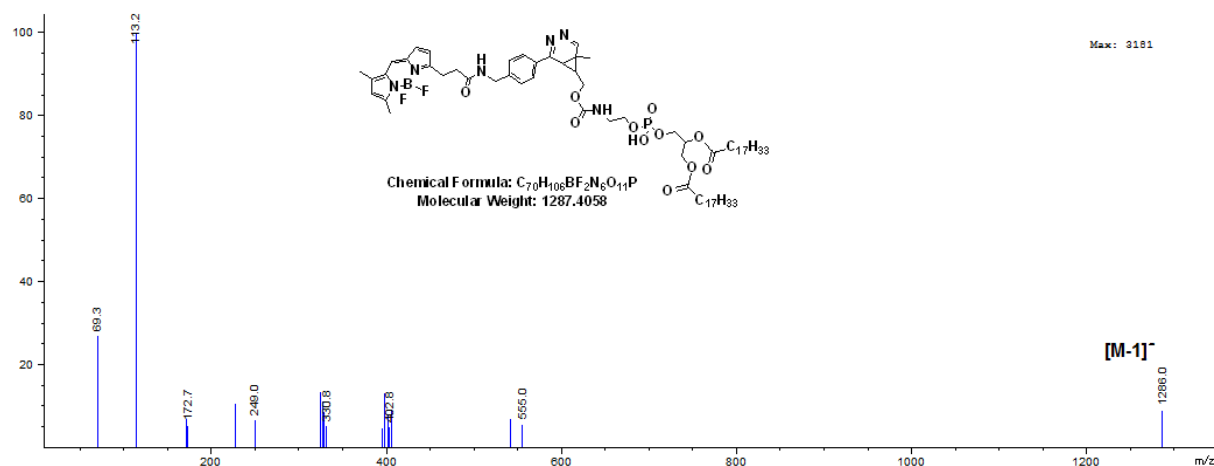


Figure S6. Negative ion electrospray mass spectra of diazanorcaradiene adducts between lipid **7** and bodipy tetrazine **6**.

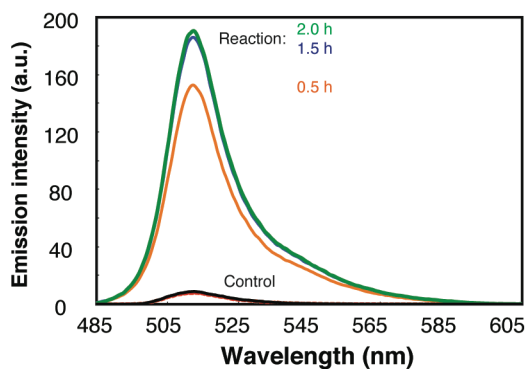


Figure S7. Emission intensity measurements of the tetrazine-BODIPY FL **6** reaction with cyclopropene **5**. Emission of the reaction mixture is shown at 30 min (orange line), 90 min (blue), and 120 min (green). Corresponding control sample lacking cyclopropene **5** was measured initially at 0 min (dashed red line) and 120 min (black line).

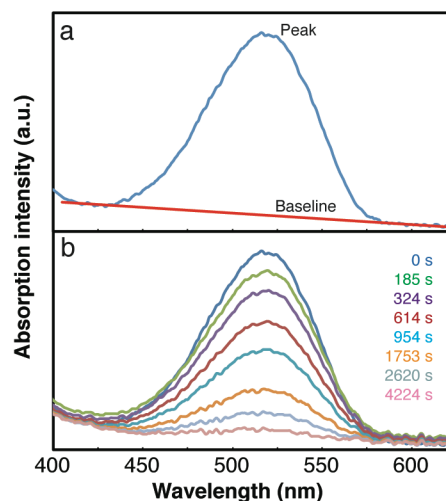


Figure S8. Tetrazine peak absorption measurements for the reaction rate determination. Representative reaction of 0.6 mM tetrazine **3** with 6.0 mM cyclopropene **2** at 37 °C is shown. (a) Initial time 0 s tetrazine **3** absorption peak was defined by the difference in the absorption trace (blue line) and the baseline slope (red line). Baseline was determined from the absorption values preceding and following the peak. (b) Measured absorption traces over time for the above reaction, shown at a few reaction timepoints (in seconds: 0 in blue; 185 in green; 324 in purple, 614 in red, 954 in teal, 1753 in orange, 2620 in pale blue, 4224 in rose).

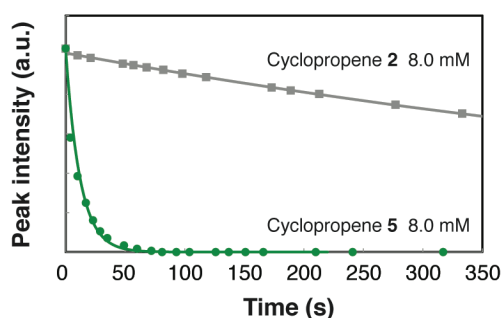


Figure S9. Tetrazine-cyclopropene reaction kinetics comparison. Tetrazine **3** absorption peak intensity was measured as a function of time while reacting with cyclopropenes: cyclopropene **5** at 37 °C (green points) and cyclopropene **2** at 37 °C (gray points), at 8.0 mM. Corresponding data fits are shown as lines. Starting tetrazine **3** concentration was 0.6 mM in both cases.

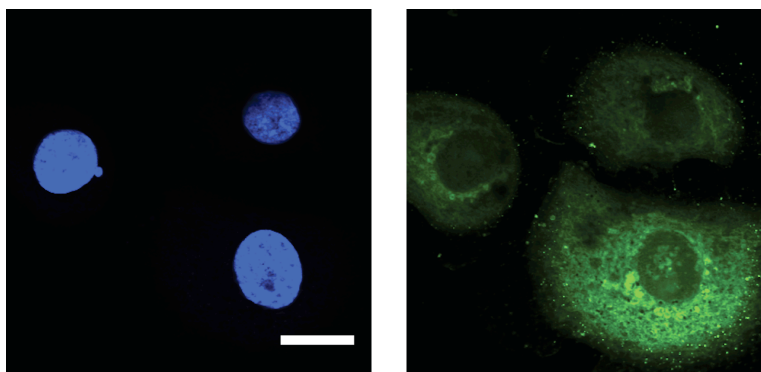


Figure S10. Staining of SKBR3 cells with cyclopropene 7 and tetrazine-BODIPY FL. DAPI fluorescence (left panel) indicates the stained nuclei. The green fluorescence (right panel) is the result of the cycloaddition of the quenched tetrazine-BODIPY FL probe with the methyl-cyclopropene carbamate. Scale bar denotes 20 μm .

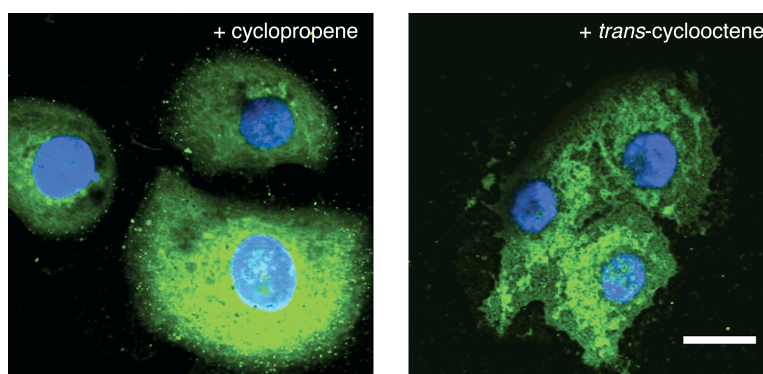
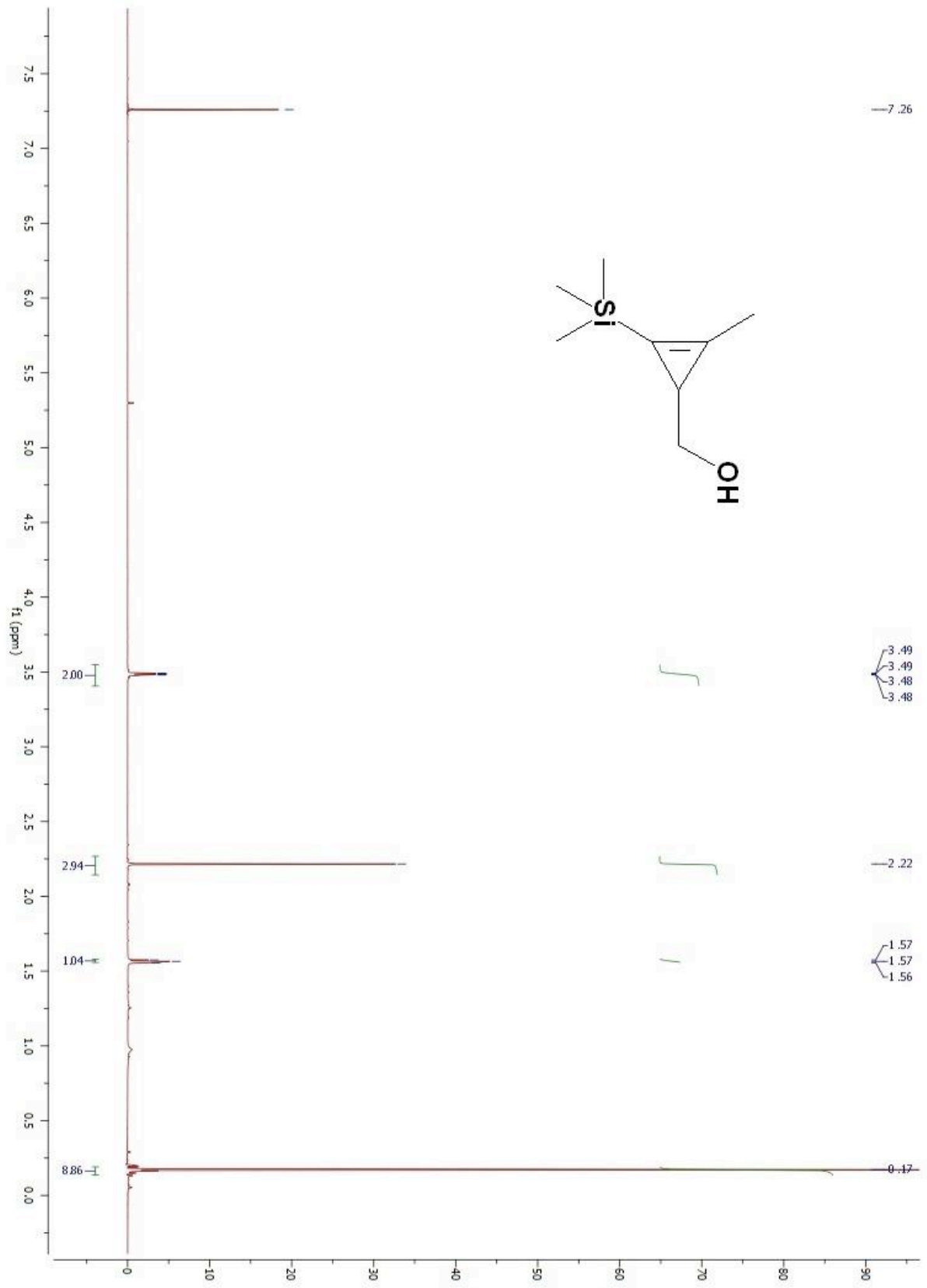


Figure S11. Staining of the cyclopropene 7 (50 μM) by tetrazine-BODIPY FL (10 μM)(left panel). Staining of *trans*-cyclooctene modified DOPE phospholipid (50 μM) by tetrazine-BODIPY FL (10 μM)(right panel) Scale bar denotes 20 μm .



→7.26

