# Supporting Information

## Superresolution Imaging of Targeted Proteins in Fixed and Living Cells Using Photoactivatable Organic Fluorophores

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# I. Plasmids for HaloEnz Fusion Construction

### Construction of HaloEnz–α-tubulin Plasmid for Mammalian Cell Expression

The gene encoding α-tubulin (Clontech) was amplified by PCR using primers that incorporate *Nhe*I and *BamH*I restriction sites at the 5' and 3' ends, respectively, digested with *Nhe*I and *BamH*I, and ligated into HaloEnz pHT2 (Promega) to make the plasmid HaloEnz–α-tubulin that enables C-terminal HaloTagging of human α-tubulin for mammalian cell expression.

### Construction of FtsZ-HaloEnz and AmiC-HaloEnz Plasmid for *Caulobacter crescentus*

The gene encoding the HaloTag enzyme (Promega) was amplified by PCR using primers that incorporate *Eco*RI and *Nhe*I restriction sites at the 5' and 3' ends, respectively, digested with *Eco*RI and *Nhe*I, and ligated into pXYFPC-2 [1] that was similarly digested to remove the gene encoding EYFP and make the plasmid pXHALOC-2. pXHALOC-2 is an integration vector that enables C-terminal Halo tagging of a gene of interest. The gene encoding *Caulobacter ftsZ* was amplified by PCR using primers that incorporate *Nde*I and *Eco*RI restriction sites at the 5' and 3' ends, respectively, digested with *Nde*I and *Eco*RI, and ligated into pXHALOC-2 that was similarly digested to make plasmid pEG515. The gene encoding *Caulobacter amiC* was amplified by PCR using primers that incorporate *Kpn*I and *Sac*I restriction sites at the 5' and 3' ends, respectively, digested with *Kpn*I and *Sac*I, and ligated into pXHALOC-2 that was similarly digested to make plasmid pEG254. pEG515 and pEG254 were introduced into *Caulobacter* strain CB15N by electroporation and integrated at the *xylX* locus to make strains EG603 and EG152 for xylose-inducible expression of *ftsZ-HaloEnz* and *amiC-HaloEnz*, respectively.

### Construction of HaloEnz-PopZ Plasmid for *Caulobacter crescentus*

To make the *Caulobacter* strain for producing HaloEnz-PopZ, the HaloTag enzyme (Promega) was inserted as an N-terminal tag between the *popZ* promoter and coding sequence. The P*popZ-haloEnz-popZ* sequence was cloned into the pNPTS138 vector (MR Alley, unpublished) and subsequently integrated into the *popZ* locus in the CB15N *Caulobacter* genome. This resulted in a merodiploid strain in which *halo-popZ* and untagged *popZ* are expressed from tandem P*popZ* promoters.

To construct the P*popZ-haloEnZ- popZ* sequence, the *popZ* promoter region and halo tag were amplified using primer sets "1319 leftflank 5'KpnINheI" / "1319 HALO at N olp Left NdeI" and "1319 HALO at N olp Right NdeI" / "3' HALO EcoRI nostop", respectively. The PCR products were stitched together by overlap PCR, then cloned into pNPTS138 using NheI and EcoRI restriction sites. Subsequently, the *popZ* coding sequence was amplified using the primer set "1319 5' EcoRI" / "1319 3' EcoRV", then cloned into the vector using EcoRI and EcoRV restriction sites.

**Primer sequences:**

1319 leftflank 5'KpnINheI: aaaaggtaccgctagcGACGGTCTCGGCGCGCGCTT
1319 HALO at N olp Left NdeI: TGGCTCGAGcataTGCGGGGCCGTCGTAAAGAG
1319 HALO at N olp Right NdeI:
ACGGCCCCGCAtatgCTCGAGCCAACCACTGAGGA
3' HALO EcoRI nostop: aaaagaattcaccATGTCCGATCAGTCTCAAGA
1319 5' EcoRI: aaaagaattcaccATGTCCGATCAGTCTCAAGA
1319 3' EcoRV: ttttgatatcGGCGCCGCGTCCCCGAGAGA

## II. Cell Labeling Protocol

### HeLa Cell Labeling

HaloEnz–α-tubulin DNA was transfected into HeLa cells along with HaloTag pHT2 as the control. Transfected cells were split at 1:8 ratio 16 hours later and plated on ploy L-lysine coated 12 mm glass cover slips. Cellular morphology was not perturbed by the transfection. Cells were then treated with 1 μM, 5 μM and 10 μM Azido DCDHF-V-P fluorophore **3** respectively, at 37 °C for 30 min, followed by 3 washings with DMEM medium at 37 °C (10 min for each wash) and subsequent fixation with 4% paraformaldehyde. Fixed cells went through 0.1% Triton X-100 permeabilization in PBS with 5% Bovine Serum Albumin. Monoclonal anti-α-tubulin antibody was applied onto permeabilized fixed cells at 1: 2,000 dilution and Alexa488-conjugated goat-anti-mouse secondary antibody was added to label expressed human α-tubulin. Cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) to localize nuclei before mounting and imaging.

### CHO Cell Labeling

1000ng DNA/1mL HaloEnz–α-tubulin DNA was transfected into CHO cells along with α-tubulin–eGFP as the control using Lipofectamine 2000 and Opti-MEM l. Transfected cells were split at 1:9 ratio 24 hours later and plated on poly L-lysine coated #1 borosilicate glass cover slips. Cellular morphology was not perturbed by the transfection. Cells were then treated with 1 μM DCDHF-V-P fluorophore **4** at 37 °C for 30 min, followed by 3 washings by DMEM medium at 37 °C (10 min for each wash).

### BS-C-1 Cell Labeling

HaloEnz–α-tubulin DNA was transfected into BS-C-1 cells on a 24 well plate at 1000ng DNA/1mL using Lipofectamine 2000 and Opti-MEM. Cellular morphology was not perturbed by the transfection. Transfected cells were split at 1:12 ratio 12 hours later and plated on chambered #1 borosilicate glass cover slips. 24 Hours later, cells were fixed with 4% PFA, followed by 3 times PBS wash, permeabilization with 0.15% Triton X-100, and pre-block with 1mg/mL BSA. Cells were then treated with 12 nM HaloTag DCDHF-V-P fluorophore **3,** at room temperature for 90 min, followed by 3 washings by 0.15% Triton X-100 containing PBS.

### *Caulobacter crescentus* Cell Labeling

The protocol for labeling *Caulobacter crecentus* cells expressing HaloEnz fusions with **3** or **4** is as follows. Cells were first grown in PYE growth medium overnight and then diluted to M2G minimal-fluorescence buffer, to which was added 0.03% xylose to induce expression of the HaloEnz fusions of FtsZ and AmiC. HaloEnz-PopZ is not on an inducible promoter and as such, no xylose is needed for expression of the fusion protein. After inducing for 3 h and the cell suspension has reached OD 0.3, the cells were

centrifuged at 8 krpm for 90 s to pellet and rinsed with clean M2 buffer. To label with the fluorescent probe, HaloTag DCDHF **3** in DMSO was added very slowly to a final concentration of 1 nM of DCDHF and 8% DMSO v/v. After 90 min incubation, cells were pelleted and resuspended with clean M2 to wash 3 times, followed by another incubation for 30 min in clean M2. Finally, the cells were then pelleted and resuspended 6 more times in M2 buffer for final wash. Cells were then placed on a 1.5% agarose in M2 pad and imaged on an inverted Olympus IX71 microscope as described below. All steps involving **3** were performed in a dark room under dim red lights.

## III. Cell Imaging Protocol

### Diffraction-Limited Imaging of HeLa Cells

Phase contrast images of HeLa cell shape and location were recorded with a Cool Snap HQ charge-coupled device (CCD) camera (Roper Scientific), with focused illumination from a 12 volt Halogen lamp (Zeiss). Fluorescence images were acquired with a Zeiss Axiovert 200M microscope under the following conditions: Zeiss 20× objective, excitation under a Xenon Lamp, green, red and DAPI fluorescence filters. Samples were photo-activated by 2-hours Halogen lamp illumination or 1 min UV illumination at 365 nm.

### Diffraction-Limited Imaging of CHO Cells

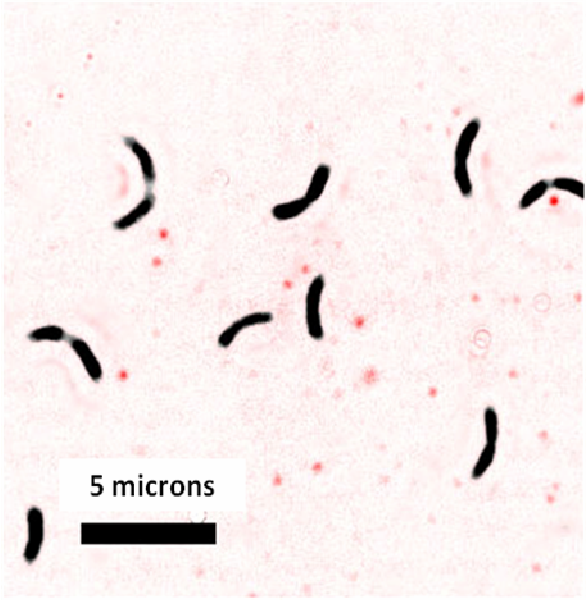
The fluorescence imaging of the cells was performed with wide-field epi-illumination using an inverted microscope (Olympus IX71). Laser illumination at 594 nm provided an intensity of ~1 kW/cm$^2$ at the sample plane. The epifluorescence was collected with a 100× magnification, 1.4 NA, oil-immersion objective (PlanApo, Nikon) and an infinity-corrected objective adaptor. A 594 nm dichroic beamsplitter and 610 nm long-pass filter were used to filter the emission. The emission was collected using an 512×512 pixel Andor Ixon EMCCD at the frame rate of 100ms/frame.

### Diffraction-Limited and Superresolution Imaging of *Caulobacter crescentus* Cells

For bulk DL imaging, we labeled the HaloEnz fusion proteins in living *Caulobacter crescentus* cells by incubating with **4** as described above. In the cells expressing HaloEnz-FtsZ, approximately 25% of the cells exhibited the expected protein localization; the remaining were either unhealthy, or were in a part of the cell cycle in which targeted protein does not assemble at the division plane. [2]

In the cells expressing HaloEnz-PopZ, about 50% exhibited the expected protein localization; the remaining 50% exhibited no labeling, probe aggregates within the cells, or unhealthy morphology. Cells that were unlabeled may have been new daughter cells that reproduced during the washing steps of the labeling. A certain degree of aggregation is to be expected as a result of the imperfect solubility of the probe in buffer. Unhealthy

looking cells might have been the result of overexpression of the HaloEnzyme, disrupting the essential functions of the targeted proteins. The fluorescence imaging of the cells was performed with wide-field epi-illumination using an inverted microscope (Olympus IX71). Laser illumination at 594 nm provided an intensity of ~1 kW/cm$^2$ at the sample plane. The epifluorescence was collected with a 100x magnification, 1.4 NA, oil-immersion objective (PlanApo, Nikon) and an infinity-corrected objective adaptor. A 594 nm dichroic beamsplitter and 610 nm long-pass filter were used to filter the emission. The emission was collected using a 512×512 pixel Andor Ixon EMCCD at the frame rate of 100ms/frame.




Bacterial controls: In this image, the white light transmission image of the cells is in the black channel and the fluorescence from activated DCDHF fluorophores is in the red channel. *Caulobacter crescentus* expressing HaloEnz-PopZ incubated with Azido DCDHF-V-P **1** (no HaloTag functionality) shows no labeling inside the cells. Additional control experiments in which cells not expressing any HaloEnz fusions were incubated with HaloTag Azido DCDHF-V-P **3** also showed no labeling inside the cells.

**Superresolution Imaging of *BS-C-1* Cells**

Samples were prepared as described in **II.** The fluorescence imaging of the cells was performed with wide-field epi-illumination using an inverted microscope (Olympus IX71). Laser illumination at 594 nm provided an intensity of ~1 kW/cm$^2$ at the sample plane. The epifluorescence was collected with a 100× magnification, 1.4 NA, oil-immersion objective (PlanApo, Nikon). A 594 nm dichroic beamsplitter and 610 nm long-pass filter were used to filter the emission. The emission was collected using an 512×512 pixel Andor Ixon EMCCD at the frame rate of 150ms/frame.

## IV. Superresolution Image Processing

From raw image stacks, super-resolution images were obtained using previously published image processing techniques as previously described [3, 4]. Briefly, for each imaging frame, the position of the a single emitter was determined by fitting the signal above background in a small region of interest containing the single-molecule spot to a 2-D Gaussian with nonlinear least squares regression analysis (**nlinfit**, in MATLAB). Each single-molecule point spread function was fit to determine the following parameters: **background, amplitude, width, center(x) and center(y)**. Finally, each single-molecule position was re-plotted using a macro written in the image processing software program ImageJ as a 2-D Gaussian profile defined by the measured integrated intensity and a width given by the average statistical error in localization of the center (95% confidence interval, averaged over all single-molecule localizations). The resulting mean localization precision was 32 ± 12nm.

## V. High Photoactivation Quantum Yield

Bulk solution absorption and emission spectra were acquired on a Cary 6000i UV–vis spectrometer and a Horiba Fluorolog-3 fluorimeter using standard 1-cm path length, quartz cuvettes. Molar absorption coefficients were measured from dilutions of solutions with known concentrations.

The overall chemical reaction yields to fluorescent product listed in Table 1 were measured from the absorbance values in the photoactivation spectra. Yield was defined by $[\text{amino}]_f/[\text{azido}]_i = (A_{\text{amino}}/\varepsilon_{\text{amino}})_f/(A_{\text{azido}}/\varepsilon_{\text{azido}})_i$, where A is the absorption, ε is the molar absorptivity, *i* and *f* refer to initial and final values, respectively.

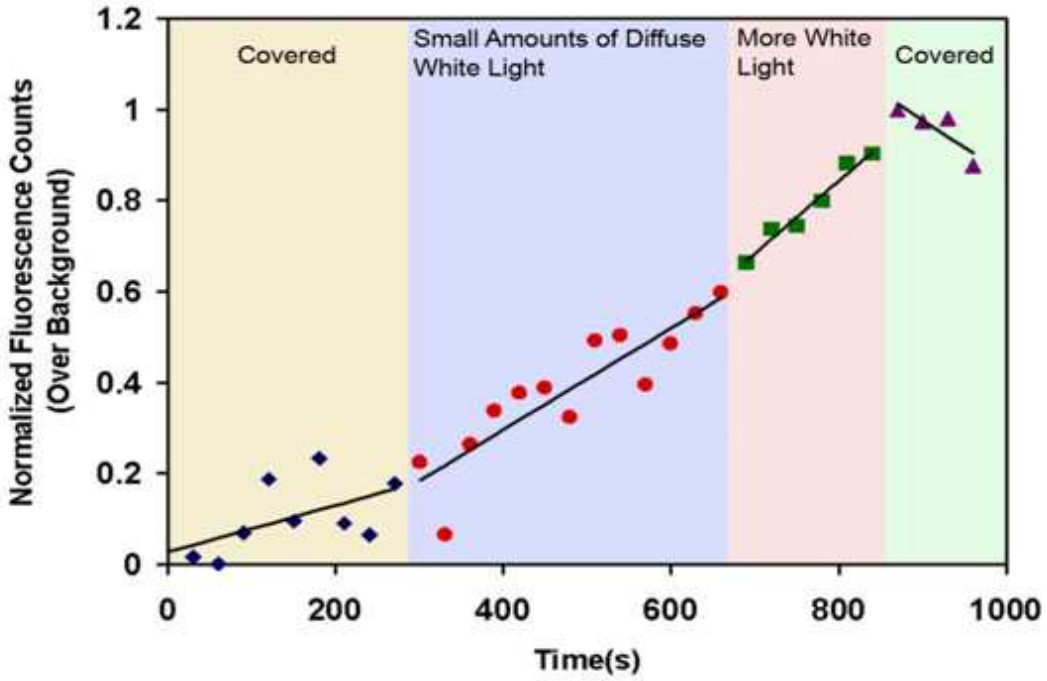
Photoconversion of compound **1** in ethanol was performed using a 385-nm diode flashlight (1.1 mW $cm^{-2}$). The probability of photoconverting an azido fluorogen to any product per photon absorbed is the photoconversion quantum yield ($\Phi_P$), listed in Table 1. The higher the value of $\Phi_P$, the more the sensitive the fluorogen is to the activating light, so less potentially cell-damaging blue or UV irradiation is required to activate fluorescence.

Photoconversion was measured by monitoring changes over time in absorbance values of the reactant and photoproduct of interest in ethanol (such as the absorption spectra in Figure 1). The photoconversion quantum yield $\Phi_P$ is defined in the following equation:

$$\Phi_P = \frac{R_P}{R_{abs}} = \frac{1}{\tau_P R_{abs}} = \frac{1}{\tau_P \sigma_\lambda I_\lambda \left(\frac{\lambda}{hc}\right)},$$

where $R_P$ is the rate of photoconversion; $R_{abs}$ is the rate of photon absorption; $\tau_P$ as the average decay constant from the exponential fit of the decaying absorption values for the starting material; the absorption cross-section is related to the molar absorption coefficient by the equation $\sigma_\lambda = (1000)2.303\varepsilon_\lambda/N_A \approx 10^{-16}$ $cm^2$; $I_\lambda$ is the irradiance at the sample; $\lambda$ is the excitation wavelength; $h$ is Planck's constant; and $c$ is the speed of light. Note that $\Phi_P$ is the probability that the starting material will photoconvert for each photon absorbed; a fraction of those photoconverted molecules become fluorescent, because the photoreaction chemical yield is less than unity (see Table 1 for overall chemical yield). For further details and discussion, see Ref. [5]

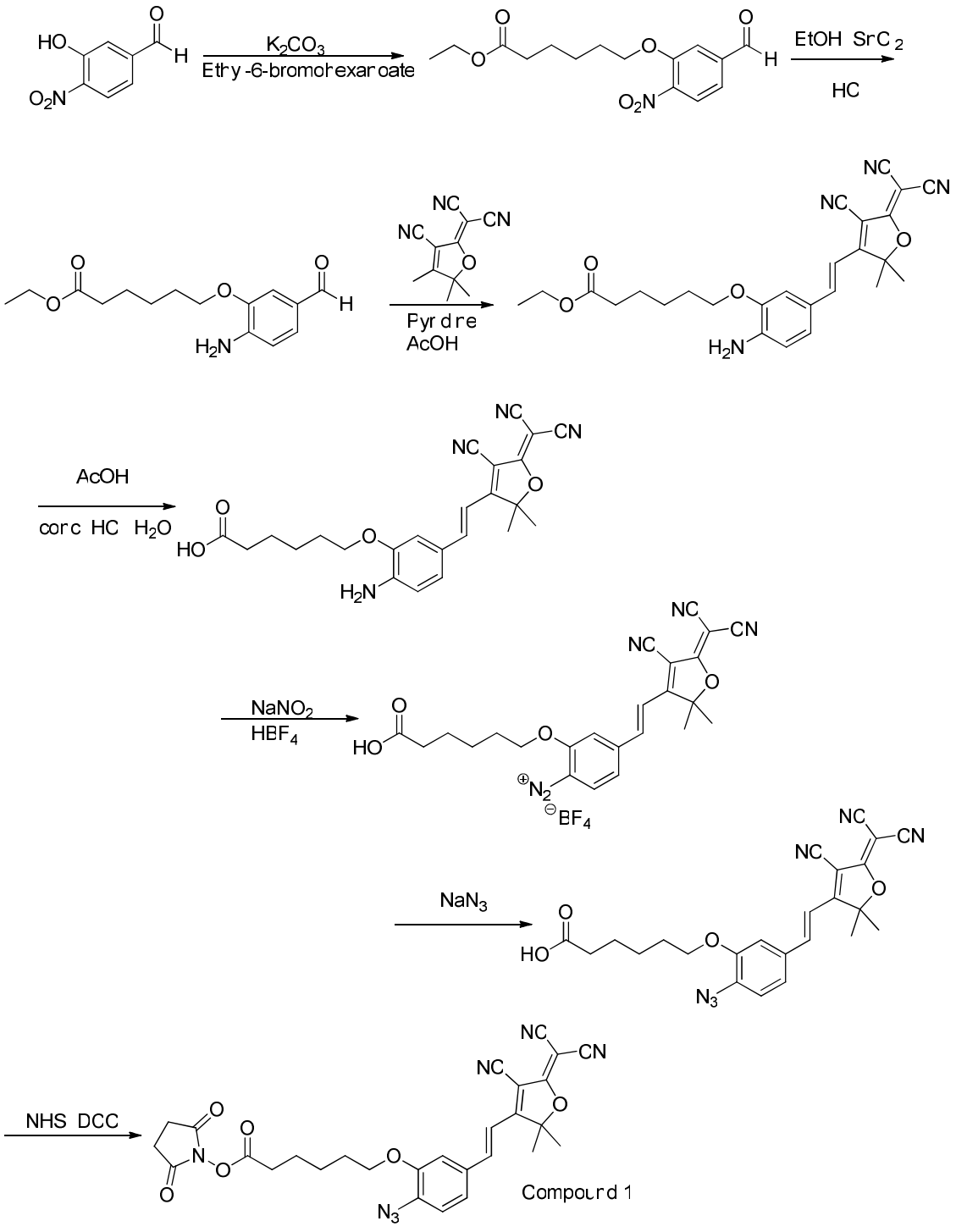
## VI. Effects of Diffuse White Light



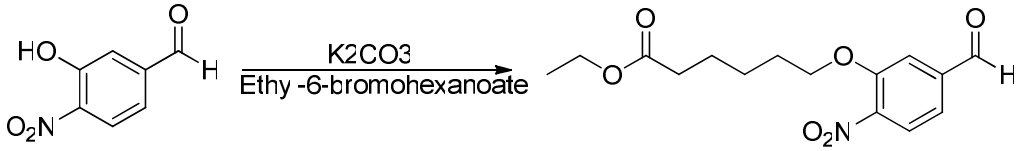

The effects of diffuse white light on molecule **1** fluorescence were characterized. In dim red lights, a sample of **1** was prepared on a polylysine coated borosilicate cover glass at 1 μM concentration in pH7.4 PBS buffer. Laser illumination at 594 nm provided an intensity of ~1 kW/cm$^2$ at the sample plane. The epifluorescence was collected with a 100x magnification, 1.4 NA, oil-immersion objective (PlanApo, Nikon) and an infinity-corrected objective adaptor. A 594 nm dichroic beamsplitter and 610 nm long-pass filter were used to reject Rayleigh scattered light and pass the emission. The emission was recorded using a 512×512 pixel Andor Ixon EMCCD. 500 ms of illumination and recording were used to sample the fluorescence above background in the images (dots in the figure). A small amount of fluorescence appeared when the sample was covered and not exposed to the ambient diffuse white light from the nearby computer monitor (tan strip) – this degree of activation was presumably due to the reading light. As the sample was exposed to additional weak diffuse light from a small handheld single LED flashlight, more molecules were photoactivated to fluoresce (blue strip). As the amount of diffuse light was increased, the number of photoactivated molecules increased, contributing to a higher fluorescence signal (red strip). Finally, when the sample was covered again (green strip), we saw a decrease in the fluorescence signal illustrating the attenuation of photoactivation and photobleaching.

## VII. Synthesis and Characterization of Compound 1

6-{2-Azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester



6-(5-Formyl-2-nitro-phenoxy)-hexanoic acid ethyl ester




To a solution of 3-hydroxy-4-nitrobenzaldehyde (0.75 g, 4.48 mmol), in DMF (15 ml) was added ethyl 6-bromohexanoate (1.20 g, 5.37 mmol) and potassium carbonate (1.83 g, 13.26 mmol). The mixture was stirred under an inert atmosphere at 70ºC for 12 hours. After cooling to room temperature the mixture was poured into water and extracted twice with ethyl acetate. The combined organic fractions were washed with water, dried over anhydrous magnesium sulfate and filtered. After the solvent was removed under reduced pressure, a red oil remained which was purified by column chromatography (7/3 hexane/ethyl acetate) to give 6-(5-formyl-2-nitro-phenoxy)-hexanoic acid ethyl ester (0.80 g, 57%) as a yellow oil. IR (neat, $cm^{-1}$): 3396, 3110, 3080, 3047, 2944, 2869, 2735, 1730, 1705, 1607, 1531, 1489, 1465, 1434, 1381, 1351, 1311, 1272, 1161, 1093, 1063, 1030, 961, 863; $^{1}H$ NMR (400 MHz, $CDCl_3$, δ): 10.06 (s, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.58 (d, *J* = 1.2 Hz, 1H), 7.54 (dd, *J* = 8 Hz, J=1.6Hz, 1H), 4.20 (q, *J* = 6.8 Hz, 2H), 4.14 (t, *J* = 7.2 Hz, 2H,), 2.36 (t, *J* = 8.0 Hz, 2H), 1.90 (m, 2H), 1.73 (m, 2H), 1.57 (m, 2H), 1.29 (t, *J* = 6.8 Hz, 3H); $^{13}C$ NMR (400 MHz, $CDCl_3$, δ): 173.79, 169.46, 151.87, 143.23, 133.66, 125.32, 121.93, 115.88, 69.65, 60.42, 34.12, 28.54, 25.40, 24.52, 14.25.

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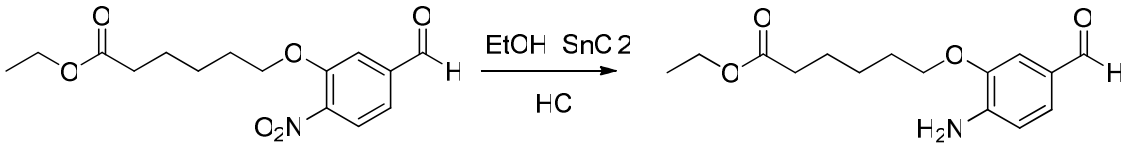
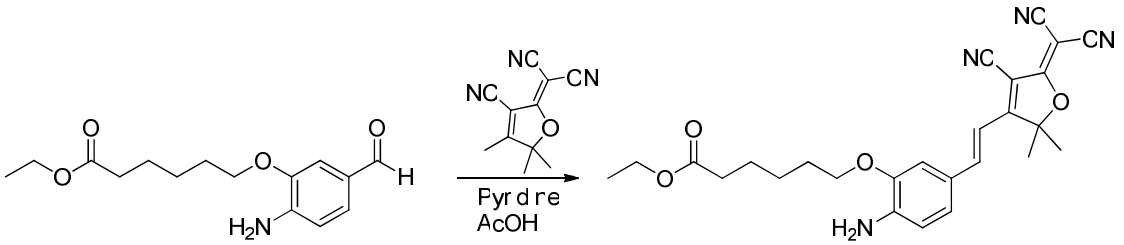
6-(2-Amino-5-formyl-phenoxy)-hexanoic acid ethyl ester



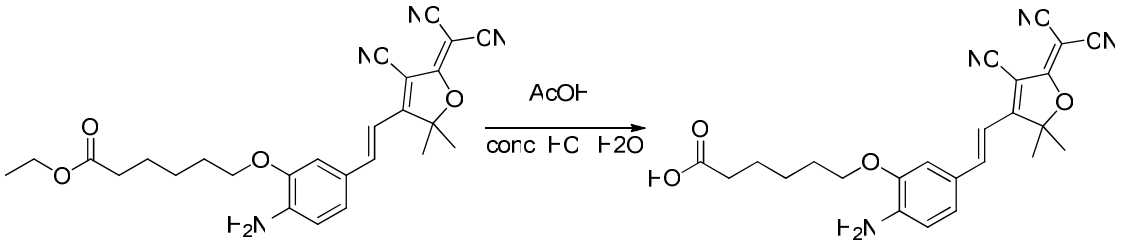

To a solution of 6-(2-nitro-5-formyl-phenoxy)-hexanoic acid ethyl ester (1.0 g, 3.23 mmol) in ethanol (20 mL) and concentrated HCl (5 mL) was added stannous chloride (2.17 g, 11.63 mmol). The mixture was stirred at 70°C for 1.5 hours and then allowed to cool to room temperature. The reaction mixture was poured into water (100 mL) and neutralized with a solution of saturated sodium bicarbonate and extracted twice with ethyl acetate (150 mL). The combined organic fractions were dried over magnesium sulfate and vacuum filtered. Purification by column chromatography ($CH_2Cl_2$ 45%, hexanes 45%, $Et_3N$ 10%) gave 6-(2-amino-5-formyl-phenoxy)-hexanoic acid ethyl ester as a red oil (0.33 g, 36% yield). IR (neat, $cm^{-1}$): 3481, 3362, 3197, 2936, 2868, 2722, 2360, 2341, 2229, 2156, 1964, 1727, 1671, 1612, 1587, 1572, 1517, 1444, 1394, 1368, 1313, 1243, 1164, 1140, 1095, 1029, 864, 817; $^1$H NMR (400 MHz, $CDCl_3$, δ):9.74 (s, 1H), 7.32 (m, 2H), 6.75 (d, *J* = 8.4 Hz, 1H), 4.47 (br s, 2H), 4.16 (t, *J* = 7.2 Hz, 2H), 4.09 (t, *J* = 6.8 Hz), 2.36 (t, *J* = 7.2 Hz, 2H), 1.88 (m, 2H), 1.74 (m, 2H), 1.55 (m, 2H), 1.30 (t, *J* = 7.2 Hz), 1.30 (t, *J* = 7.2 Hz); $^{13}$C NMR (400 MHz, $CDCl_3$, δ): 190.6, 173.6, 146.0, 143.3, 127.8, 127.6, 112.7, 109.1, 68.2, 60.4, 34.2, 28.8, 25.7, 24.6, 14.2.

6-{2-Amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid ethyl ester



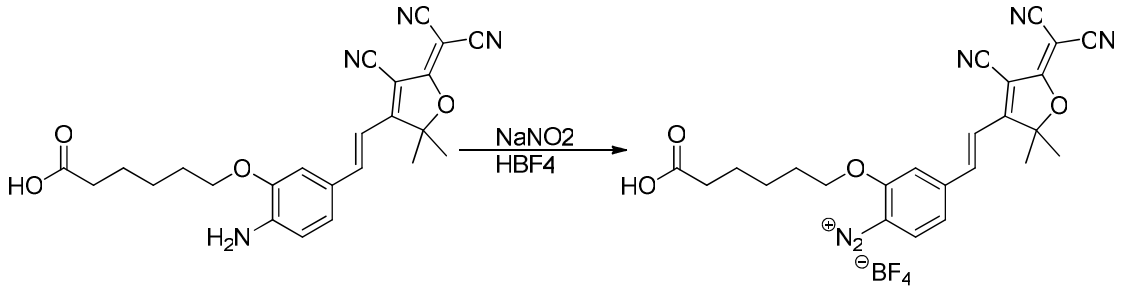

To a solution of 6-(2-amino-5-formyl-phenoxy)-hexanoic acid ethyl ester (0.31 g, 1.10 mmol) in pyridine (10 mL) was added 2-(3-cyano-4,5,5-trimethyl-5H-furan-2-ylidene)-malononitrile (0.26 g, 1.33 mmol, synthesized as described in Ref. [6]) and 5 drops of acetic acid. After stirring at room temperature for one day water (100 mL) was added and after stirring the mixture was filtered giving pure 6-{2-amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid ethyl ester as a dark solid (0.35 g). The filtrate was stripped of solvent under reduced pressure and purified by column chromatography giving an additional 0.03 g of 6-{2-amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid ethyl ester (0.38 g, 74.5% yield). IR (neat, $cm^{-1}$): 3416, 3341, 3251, 3215, 2987, 2946, 2837, 2360, 2219, 2208, 1728, 1630, 1587, 1549, 1521, 1482, 1448, 1415, 1357, 1335, 1273, 1235, 1211, 1187, 1166, 1145, 1108, 1028, 1013, 961, 946, 906, 871; $^1$H NMR (400 MHz, $CDCl_3$, δ):7.58 (d, *J* = 16.0 Hz, 1H), 7.18 (dd, *J* = 1.6 Hz, 8.0 Hz, 1H), 7.01 (d, *J* = 1.6 Hz, 1H), 6.77 (d, J = 16.0 Hz, 1H), 6.73 (d, *J* = 8.0 Hz, 1H), 4.70 (br s, 2H), 4.16 (q, *J* = 7.2 Hz, 2H), 4.10 (t, *J* = 6 Hz, 2H), 2.38 (t, *J* = 7.2 Hz), 1.92 (m, 2H), 1.78 (s, 6H), 1.76 (m, 2H), 1.62 (m, 2H), 1.44 (t, *J* = 7.2 Hz).; $^{13}$C NMR (400 MHz, $CDCl_3$, δ): 176.2, 174.3, 173.6, 148.8, 146.4, 143.2, 127.0, 123.9, 113.9, 112.4, 111.7, 111.2, 110.2, 109.7, 97.0, 95.4, 68.4, 60.4, 34.1, 28.8, 26.7, 25.7, 24.6, 14.3. HRMS m/z Calcd. for $C_{26}H_{28}N_4O_4$(M+Na):483.2008. Found: 483.2016.

6-{2-Amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid



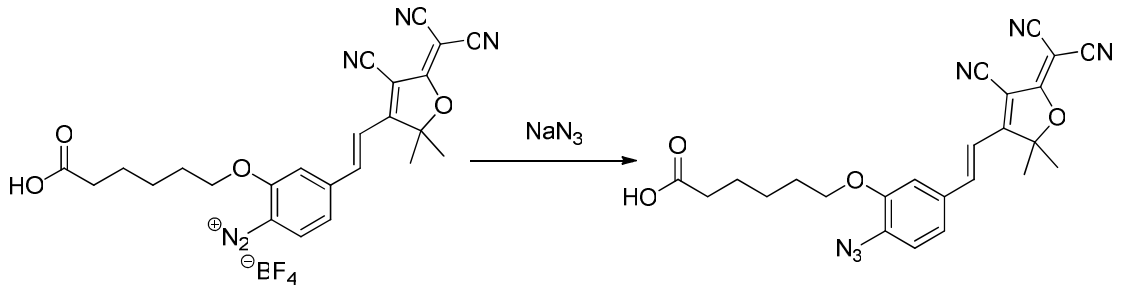

To a mixture of acetic acid (6 mL) and 6M HCl (5 mL) was added 6-{2-amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid ethyl ester (0.33 g, 0.72 mmol). After stirring at room temperature for 16 hours, the solution was poured into water (50 mL), neutralized with sodium bicarbonate and extracted with ethyl acetate (2 × 100 mL). The combined organic fractions were dried over magnesium sulfate and vacuum filtered. Removal of the solvent under reduced pressure gave a sticky dark solid which was purified by column chromatography (EtOAc 60%, EtOH 5%, hexanes 20%) giving 6-{2-amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid as a shiny green solid (0.23 g, 74%). IR (neat, $cm^{-1}$): 3487, 3354, 3207, 2933, 2866, 2360, 2342, 2222, 1705, 1623, 1555, 1496, 1448, 1333, 1268, 1230, 1187, 1144, 1104, 1015, 958; $^{1}H$ NMR (400 MHz, $CDCl_3$, δ): 7.57 (d, *J* = 16.0 Hz, 1H), 7.17 (dd, *J* = 8.4 Hz, 1.6 Hz, 1H), 7.04 (d, *J* = 1.6 Hz, 1H), 6.78 (d, *J* = 16.0 Hz, 1H), 6.73 (d, *J* = 8.4 Hz, 1H), 4.12 (t, *J* = 6.4 Hz, 2H), 2.45 (t, *J* = 7.2 Hz, 2H), 1.93 (m, 2H), 1.79 (m, 8H), 1.62 (m, 2H) ; $^{13}C$ NMR (400 MHz, $CDCl_3$, δ): 174.3, 148.8, 146.5, 143.2, 127.2, 124.2, 113.8, 112.4, 112.3, 111.6, 111.1, 110.2, 109.8, 97.4, 68.6, 33.3, 30.9, 29.7, 29.3, 28.8, 26.8. HRMS m/z Calcd. for $C_{24}H_{24}N_4O_4$(M+Na):455.1695. Found: 455.1678.

2-(5-Carboxy-pentyloxy)-4-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-benzenediazonium tetrafluoroborate




To a suspension of 6-{2-amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid (0.30 g, 0.69 mmol) at 5°C in 3.2 ml of fluoroboric acid (50%) was added a solution of sodium nitrite (0.29 g, 4.27 mmol) in 2ml of water. The cooling bath was removed and the mixture was allowed to stir at room temperature for 2 hours. The resulting yellow solid were removed by vacuum filtration and air dried giving 2-(5-carboxy-pentyloxy)-4-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-benzenediazonium tetrafluoroborate as a dark solid (0.35 g, 97%). IR (neat, $cm^{-1}$): 2360, 2341, 2264, 2230, 1730, 1586, 1481, 1307, 1106; $^1H$ NMR (400 MHz, $CDCl_3$, δ): 8.66 (d, *J* = 8.4 Hz, 1H), 8.07 (s, 1H), 7.97 (d, *J* = 9.2 Hz 1H), 7.86 (d, *J* = 16.4 Hz,1H), 7.55 (d, *J* = 16.4 Hz, 1H), 4.52 (t, J = 6.4Hz, 2H), 2.27 (t, *J* = 7.2Hz, 2H), 1.86 (m, 2H), 1.83 (s, 6H), 1.61 (m, 2H), 1.53 (m, 2H).

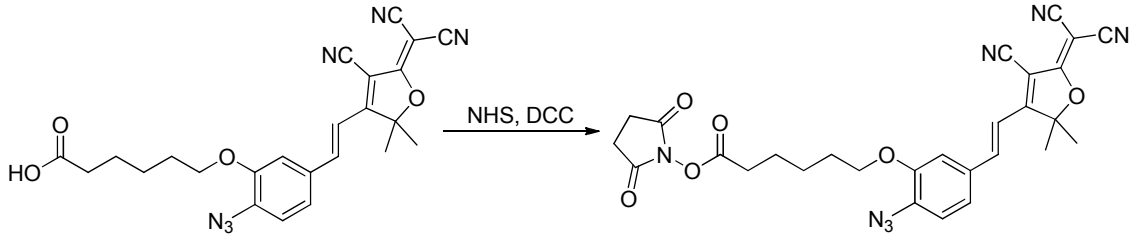
6-{2-Azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid




DARK ROOM CONDITIONS! The starting material and product are sensitive to ambient light. Lighting for this preparation was provided by the red LEDs of a headbeam flashlight (e.g., Energizer HD33A1EN).

To a mixture of 2-(5-carboxypentyloxy)-4-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-benzenediazonium tetrafluoroborate (0.34 g, 0.64 mmol) in DMF (1.5mls) was added a solution of azidotrimethylsilane (0.088 g, 0.76 mmol) in DMF (1.5ml). After stirring for 2 hours at room temperature, the mixture was poured into 50 mls of water and extracted twice with equal volumes of ethyl acetate. The combined organic fractions were washed twice with water, dried over magnesium sulfate and vacuum filtered. Removal of the solvent under reduced pressure gave 0.260 g of a brown oil that was purified by column chromatography (77% EtOAc, 4.5% EtOH, 18.5% hexanes) to give 6-{2-azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid as a dark orange solid (0.20 gm, 68%). IR (neat, $cm^{-1}$): 2937, 2869, 2360, 2341, 2227, 2130, 2093, 1707, 1572, 1530, 1498, 1432, 1314, 1247, 1100, 971, 814; $^1H$ NMR (400 MHz, DMSO, δ): 7.86 (d, $J$ = 16.4 Hz, 1H), 7.60 (d, $J$ = 1.6 Hz, 1H), 7.53 (dd, $J$ = 8.4 Hz, 1.6 Hz, 1H), 7.19 (d, $J$ = 16.4 Hz, 1H), 7.11 (d, $J$ = 8.4 Hz, 1H), 4.15 (t, $J$ = 6.4 Hz, 2H), 2.24 (t, $J$ = 7.2 Hz, 2H), 1.77 (m, 8H), 1.58 (m, 2H), 1.49 (m, 2H); $^{13}C$ NMR (400 MHz, $CDCl_3$, δ): 177.5, 175.5, 174.8, 152.4, 147.2, 132.7, 131.7, 113.2, 112.3, 111.3, 99.8, 99.5, 68.4, 54.7, 34.0, 28.6, 25.6, 24.7. HRMS m/z Calcd. for $C_{24}H_{22}N_6O_4$(M+Na):481.1600. Found: 481.1589.

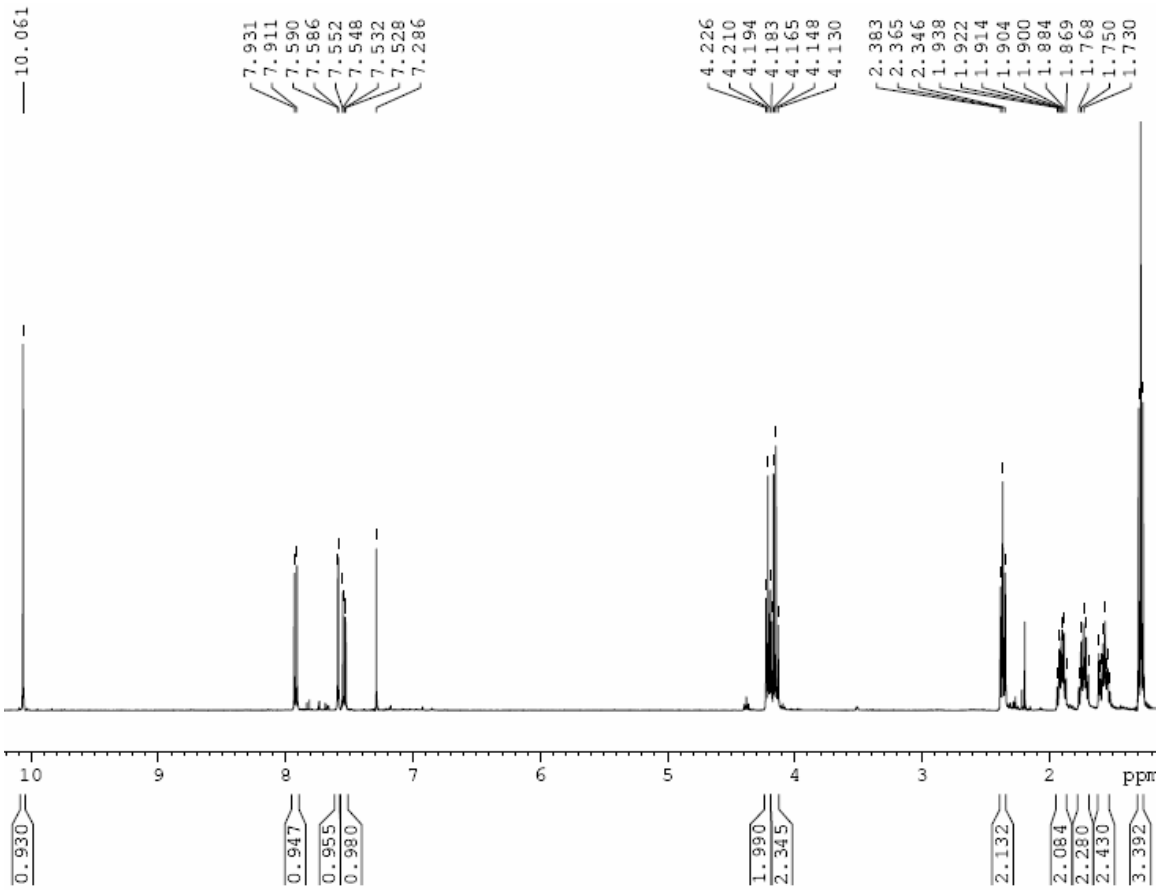
6-{2-Azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester **1**



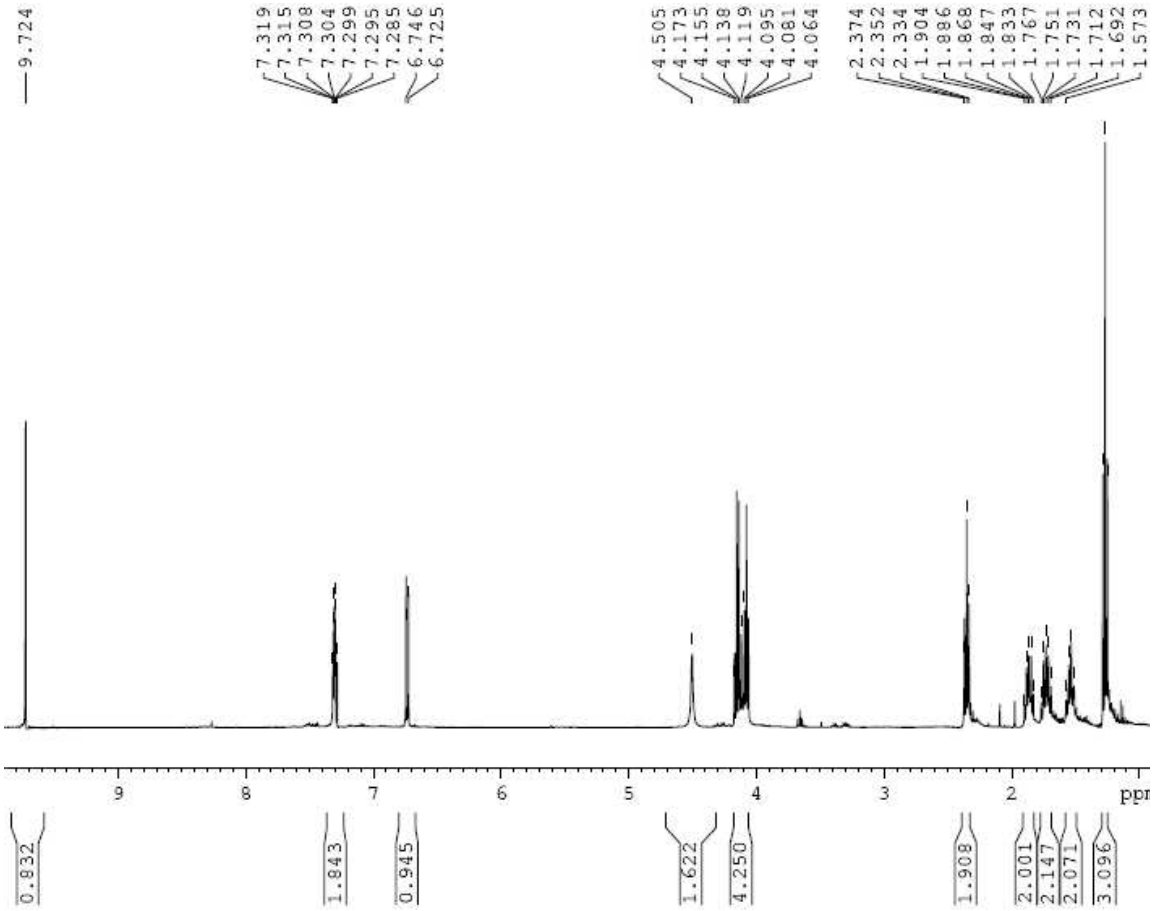

DARK ROOM CONDITIONS! The starting material and product are sensitive to ambient light. Lighting for this preparation was provided by the red LEDs of a headbeam flashlight (e.g., Energizer HD33A1EN).

To a cooled solution (5°C) of 6-{2-azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid (0.1g, 0.21 mmol) in dichloromethane (20 ml) was added N-hydroxysuccinimide (0.027 g, 0.24 mmol) and DCC (0.052 g, 0.25 mmol). The resulting solution was allowed to stir at 5°C for 2 hours and then at room temperature for 12 hours. The solvent was removed under reduced pressure and the resulting dark red solid was purified by column chromatography (70% EtOAc/30% hexanes) to give 6-{2-azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester as a dark red solid (0.084 g, – 69%). IR (neat, $cm^{-1}$): 3321, 2926, 2850, 2361, 2225, 2128, 2094, 1812, 1783, 1733, 1563, 1536, 1497, 1307, 1292, 1186, 1102, 1064, 1046, 849; ; $^{1}H$ NMR (400 MHz, $CDCl_3$, δ): 7.60 (d, *J* = 16.4 Hz, 1H), 7.24 (dd, *J* = 8.4 Hz, *J* = 1.6 Hz, 1H), 7.09 (d, *J* = 1.6 Hz 1H), 7.04 (d, *J* = 8 Hz, 1H), 6.96 (d, *J* = 16.4 Hz 1H), 4.13 (t, *J* = 6.4 Hz, 2H), 2.87 (s, br, 4H), 2.70 (t, *J* = 7.2Hz, –2H), 1.98 (m, 2H), 1.91 (m, 2H) 1.82 (s, 6H), 1.68 (m, 2H); $^{13}C$ NMR (400 MHz, $CDCl_3$, δ): 173.50, 169.25, 168.48, 152.61, 146.50, 133.66, 131.19, 123.40, 121.54, 114.10, 112.07, 111.62, 110.83, 110.29, 97.48, 69.18, 30.85, 29.71, 28.34, 26.55, 25.62, 25.22, 24.22. HRMS m/z Calcd. for $C_{28}H_{25}N_7O_6$(M+Na):578.1764. Found: 578.1726.

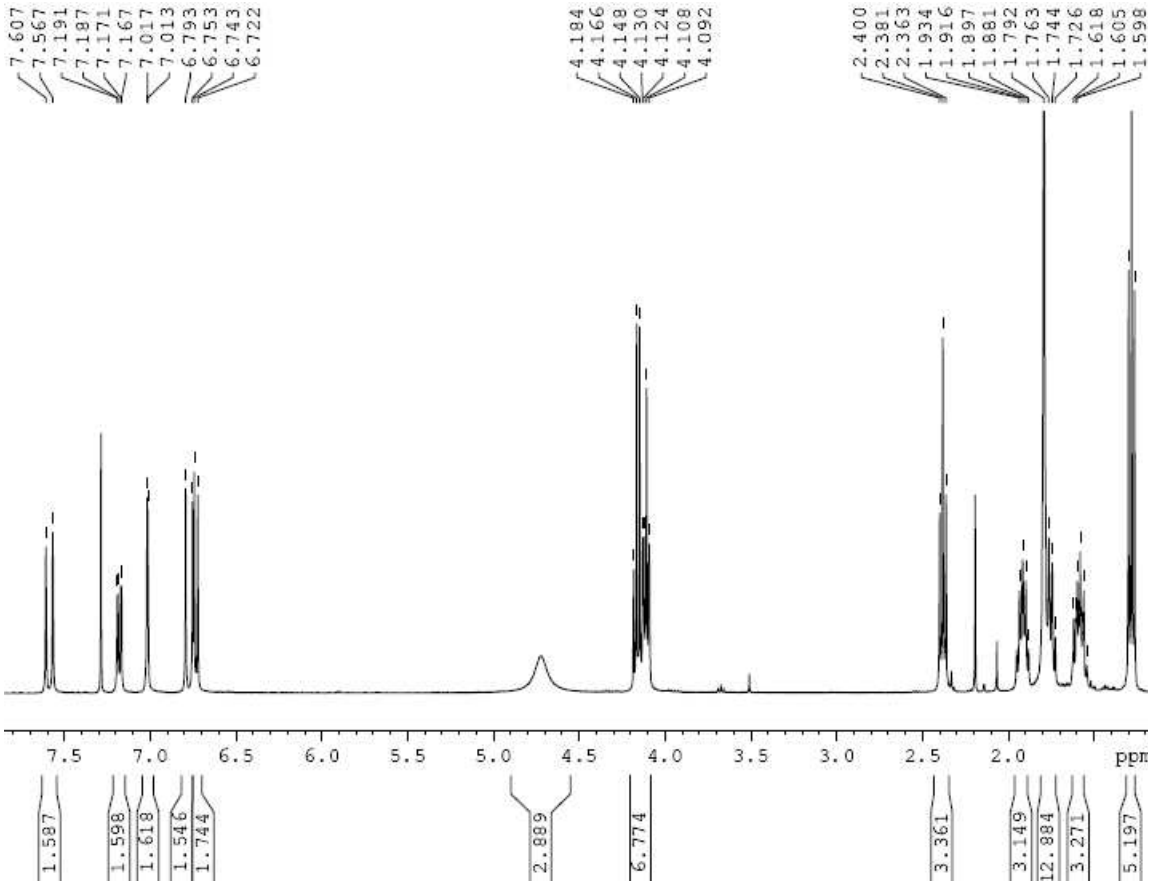
6-(5-Formyl-2-nitro-phenoxy)-hexanoic acid ethyl ester



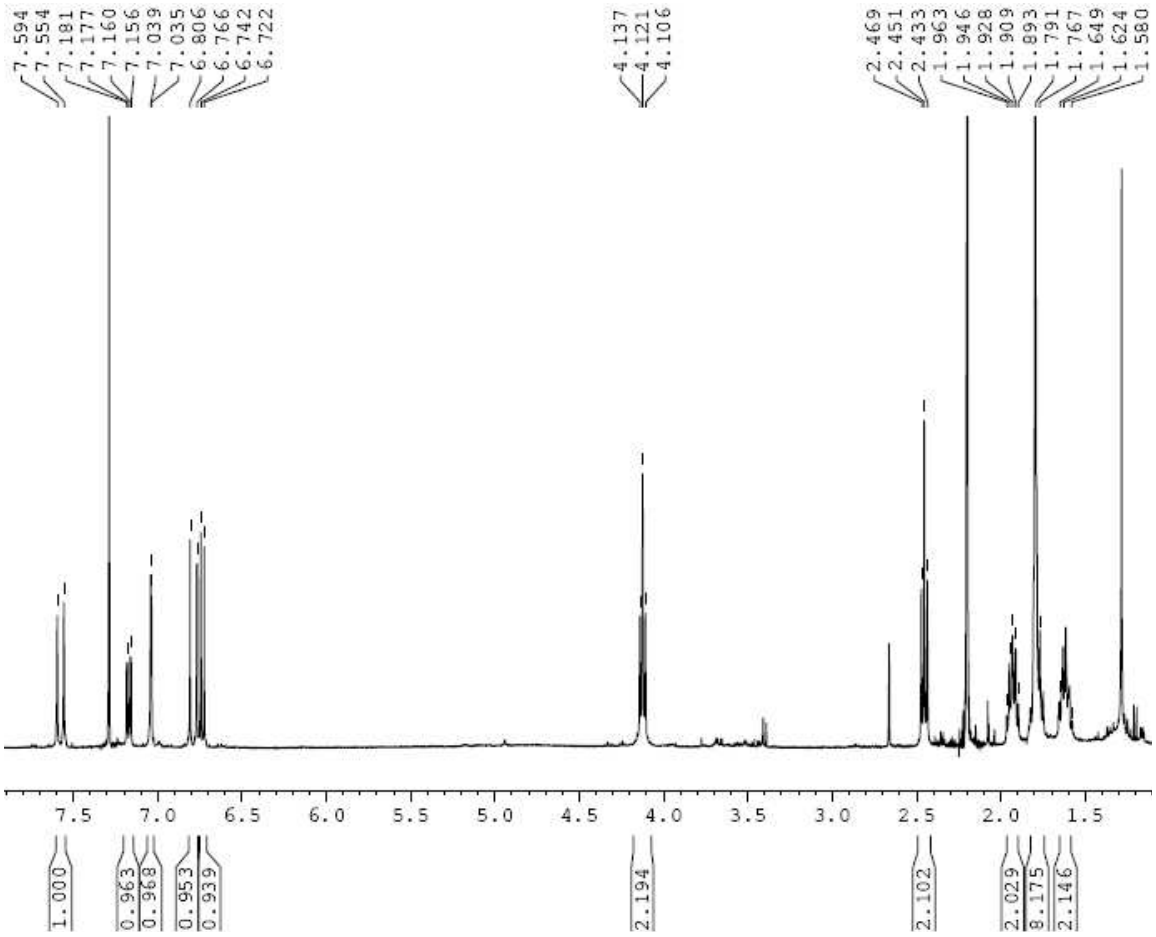
6-(2-Amino-5-formyl-phenoxy)-hexanoic acid ethyl ester



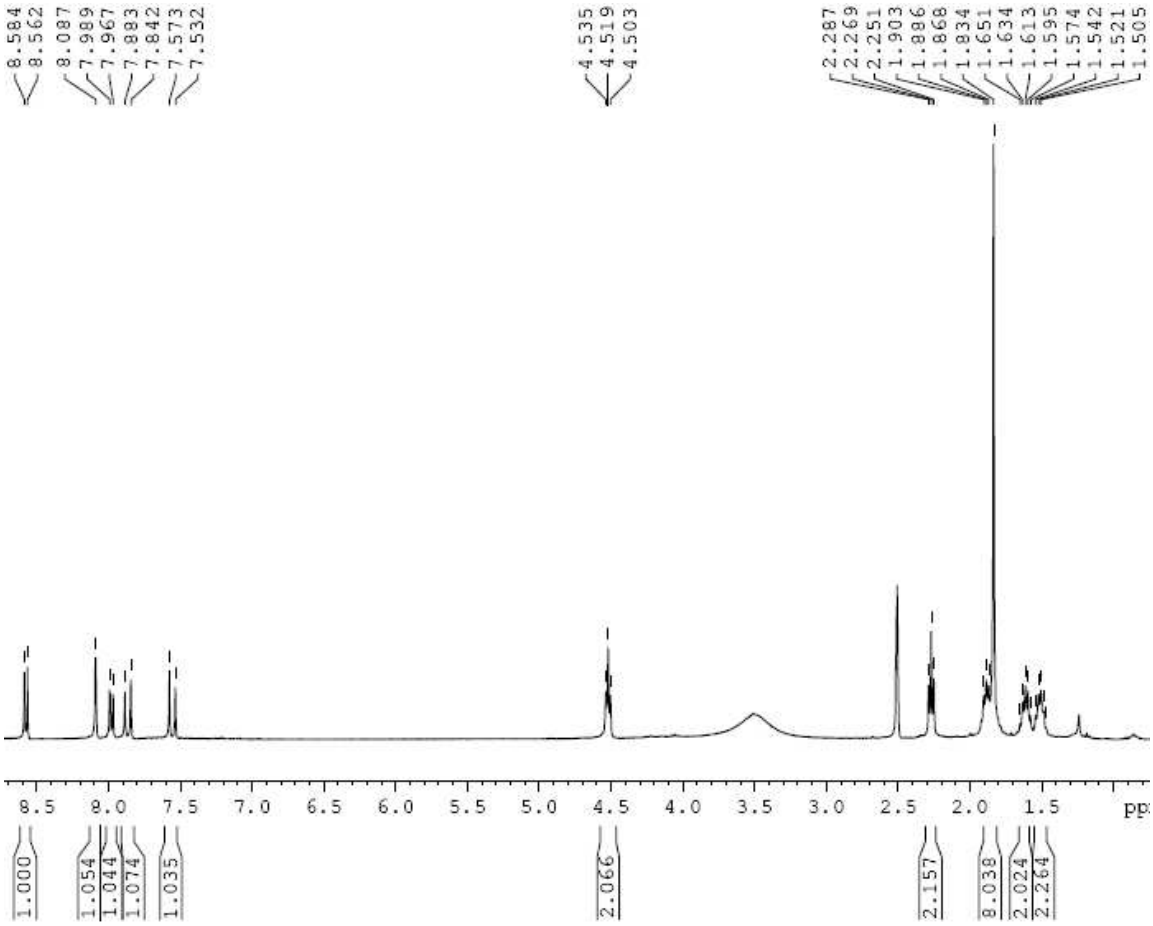
6-{2-Amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid ethyl ester



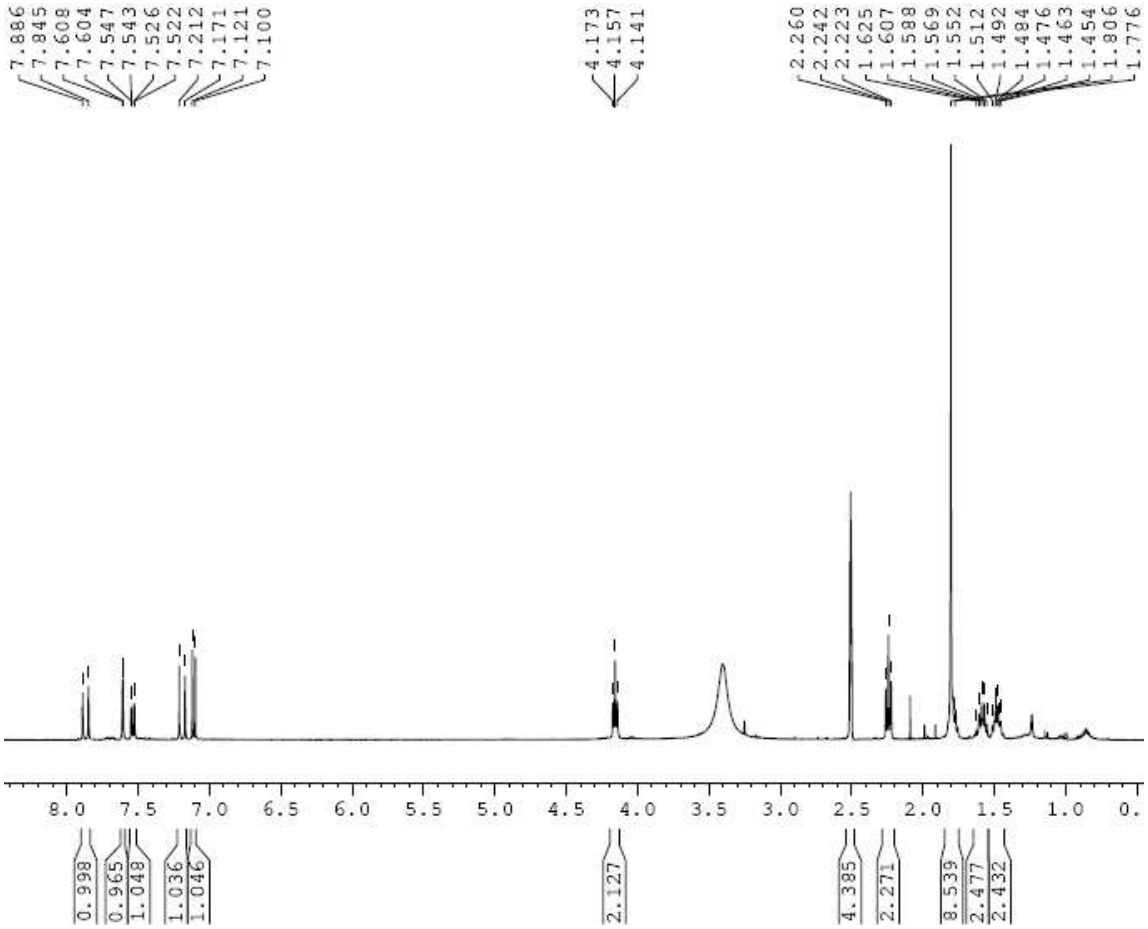
6-{2-Amino-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid



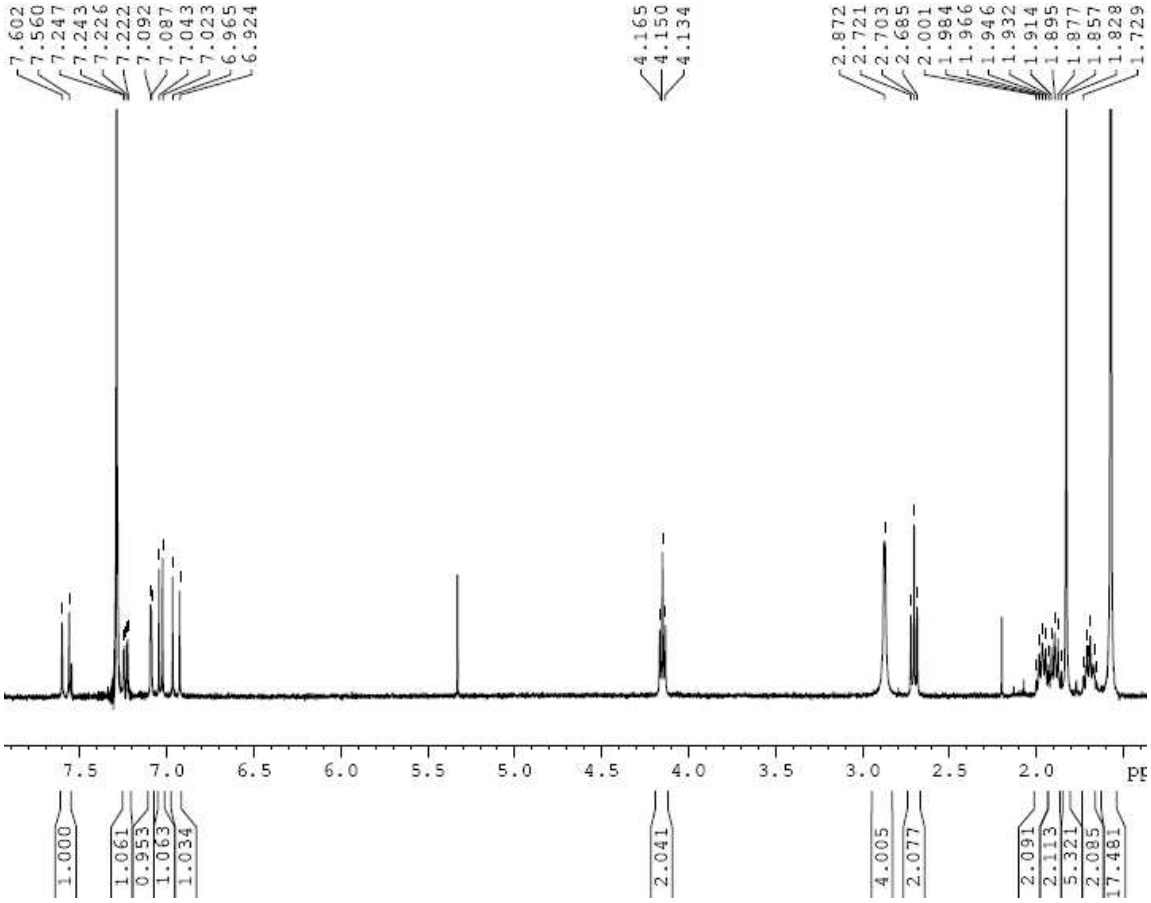
2-(5-Carboxy-pentyloxy)-4-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-benzenediazonium tetrafluoroborate



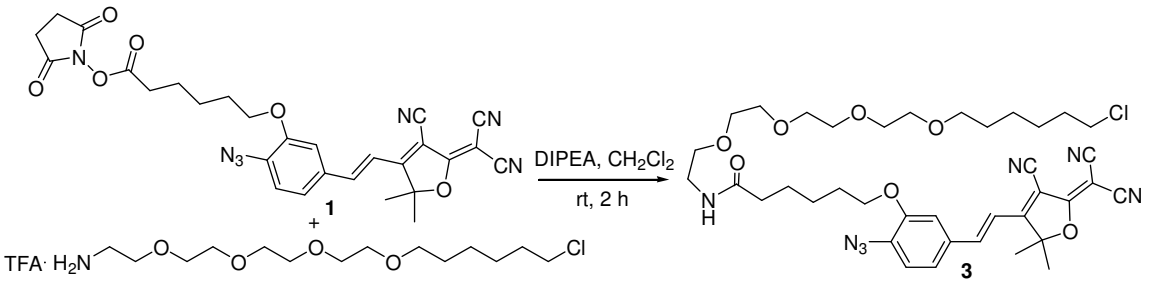
6-{2-Azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid



6-{2-Azido-5-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-phenoxy}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester **1**



## VIII. Synthesis and Characterization of Compound 3

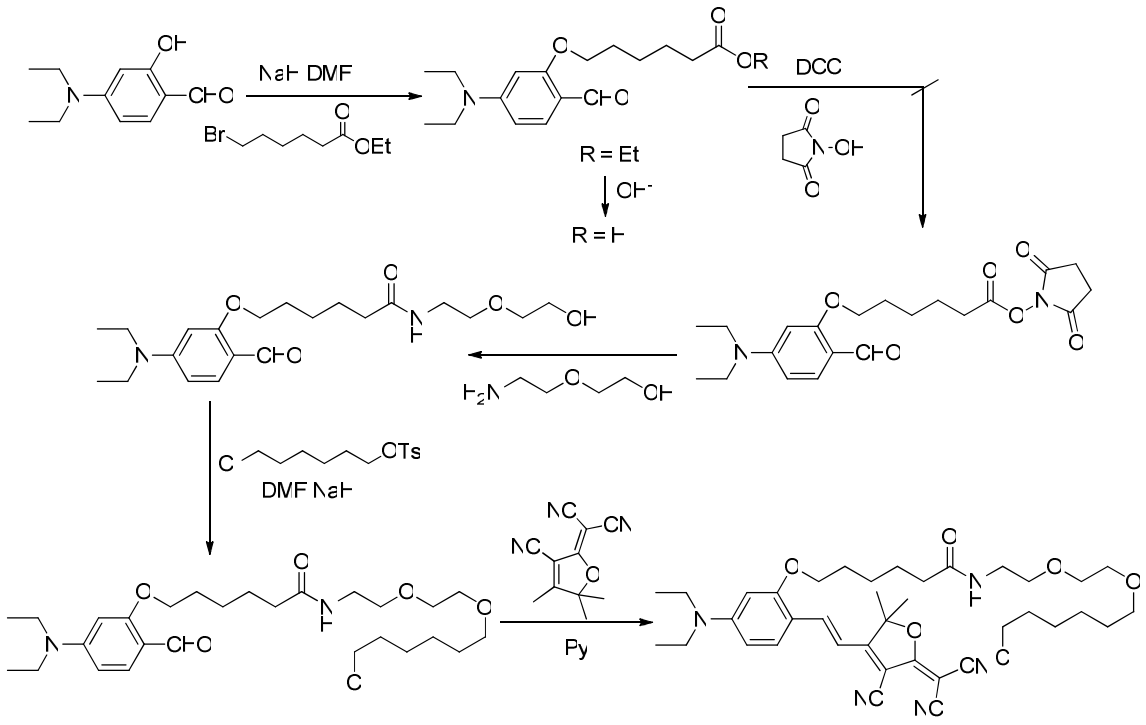



In a dark room dimly lit with red lights, the following synthesis was performed. To a mixture of azido-DCDHF **1** (1.0 mg, 1.8 μmol) and 18-chloro-3, 6, 9, 12-tetraoxaoctadecan-1-amine trifluoroacetic acid (1.1 mg, 3.6 μmol) in $CH_2Cl_2$ (100 μL) was added N, N-diisopropylethylamine (DIPEA, 1.3 μL, 7.2 μmol). The resulting reaction solution was stirred at room temperature until starting material **1** completely disappeared as monitored by thin layer chromatography (TLC). Purification with chromatography in silica gel column afforded the pure product **3**. The yield of **3** was determined to be 68% by absorption spectrophotometry. MS (ESI) observed $[M+H]^+$: 752.3, calculated: 752.3.

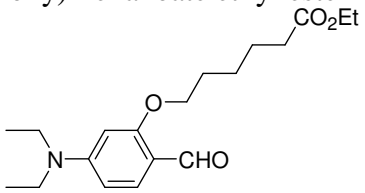
Compound **3** was stored in DMSO at -20 ℃ in the dark to avoid degradation. (Note: it was observed that DIPEA can accelerate its decomposition in the dark before photoactivation, therefore this compound should be thoroughly removed during the silica gel column purification).

## IX. Synthesis and Characterization of Compound 4

6-{2-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-5-diethylamino-phenoxy}-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide.

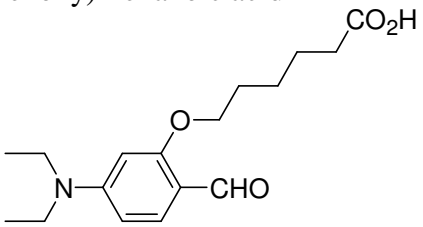


(5-diethylamino-2-formyl-phenoxy)-hexanoate ethyl ester



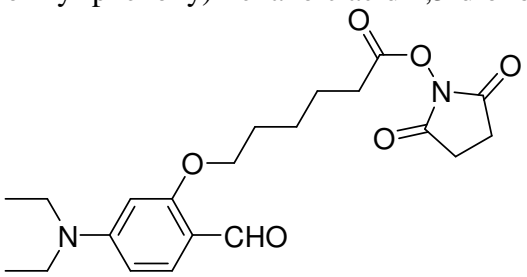

To a solution of sodium hydride (60% in mineral oil, 0.6g, 15 mmol) in anhydrous DMF (20 ml) was added 4-diethylaminosalicylaldehyde (1.93g, 10 mmol). The mixture was stirred for 1h at room temperature and ethyl 6-chlorohexanoate (2ml, 11 mmol) was added dropwise. After stirring at room temperature for 8h, the reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was collected, washed with brine and dried over magnesium sulfate. After filtration, solvent was removed by rotary evaporation and the residue was purified with flash chromatography (hexane/ethyl acetate: 4/1) to give the product as slightly yellow oil (2.6 g, 78%). $^{1}$HNMR (400MHz, $CDCl_3$): 10.2 (s, 1H), 7.72 (dd, J=0.8Hz, 9.2Hz, 1H), 6.28 (d, J=9.2Hz, 1H), 6.03 (s, 1H), 4.14 (q, J=21.6Hz, 2H), 4.05 (t, J=12.4Hz, 2H), 3.43 (q, J=21.2Hz, 4H), 2.35 (t, J=14.8Hz, 2H), 1.87 (m, 2H), 1.73 (m, 2H), 1.55 (m, 2H), 1.25 (m, 3H), 0.87 (m, 6H); $^{13}$CNMR ($CDCl_3$): 187.0, 173.6, 163.7, 153.8, 130.2, 114.3, 104.3, 93.2, 67.7, 60.3, 44.8, 34.2, 28.9, 25.7, 2407, 14.2, 12.6.

(5-diethylamino-2-formyl-phenoxy)-hexanoic acid



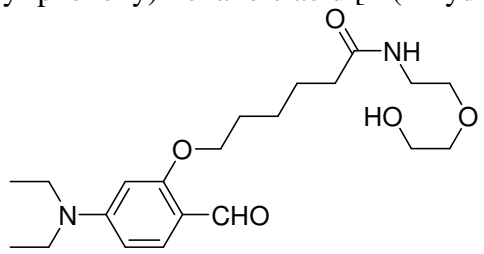

To a solution of (5-diethylamino-2-formyl-phenoxy)-hexanoate ethyl ester (0.9 g, 2.69 mmol) in methanol (10 ml) and dioxane (10 ml) was added aqueous potassium hydroxide solution (1N, 10ml). The reaction mixture was refluxed for 4h. After cooling, solvent was distilled out under vacuum and the residue was poured into water and extracted with ethyl acetate. The aqueous layer was collected and acidified with 6N hydrochloric acid. The acidic solution was then extracted with ethyl acetate. The organic layer was collected and dried over magnesium sulfate. Evaporation of the solvent gave the product as yellow solid (0.7 g, 85%). $^{1}$HNMR (400MHz, $CDCl_3$): 10.2 (s, 1H), 7.75 (d, J=8.0Hz, 1H), 6.34 (m, 1H), 6.14 (s, 1H), 4.08 (t, J=12.4Hz, 2H), 3.46 (1, J=21Hz, 4H), 2.43 (t, J=15Hz, 2H), 1.92 (m, 2H), 1.76 (m, 2H), 1.62 (m, 2H), 1.25 (t, J=14Hz, 6H), $^{13}$CNMR ($CDCl_3$): 187.3, 178.5, 163.7, 130.3, 104.8, 67.8, 45.3, 33.8, 28.8, 25.6, 24.4, 12.5. IR (neat, $cm^{-1}$): 2983, 2950, 2927, 1728, 1620, 1565, 1525. Anal. Calcd for $C_{17}H_{25}NO_4$: C, 66.43; H, 8.20; N, 4.56. Found: C, 66.54; H, 8.15; N, 4.78.

6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester



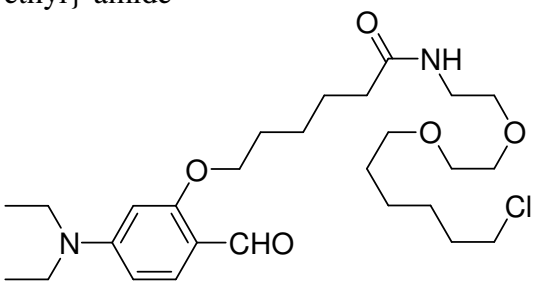

A mixture of (5-diethylamino-2-formyl-phenoxy)-hexanoic acid (0.6 g, 1.95 mmol) and *N*-hydroxysuccinimide (0.45 g, 3.91 mmol) in dichloromethane (20ml) was stirred with ice bath cooling and DCC (0.8 g, 3.8 mmol) was added portion wise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. The solvent was distilled out and the residue was purified by flash chromatography (hexane/ethyl acetate : 1/1) to give the product as a yellow solid (0.7g, 89%). $^{1}$HNMR (400MHz, $CDCl_3$): 10.14 (s, 1H), 7.70 (d, J=8.8Hz, 1H), 6.27 (dd, J=9.2Hz, 1.6Hz, 1H), 6.02 (s, 1H), 4.05 (t, J=12Hz, 2H), 3.43 (q, J=21Hz, 4H), 2.84 (s, 4H), 2.66 (t, J=14Hz, 2H), 1.88 (m, 4H), 1.67 (m, 2H), 1.22 (t, J=14Hz, 6H). $^{13}$CNMR ($CDCl_3$): 187.0, 169.2, 168.5, 163.7, 153.8, 130.1, 114.2, 104.3, 93.1, 67.5, 44.8, 33.9, 30.9, 28.7, 25.6, 25.4, 25.0, 24.3, 12.6.

6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid [2-(2-hydroxy-ethoxy)-ethyl]-amide



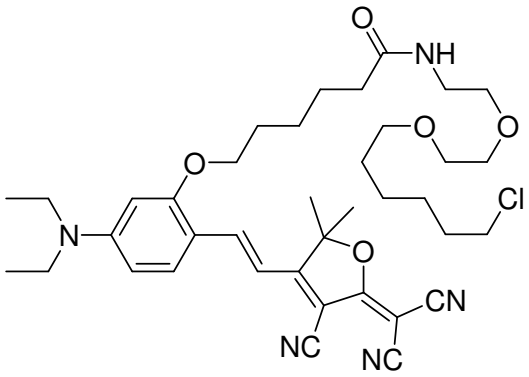

To a solution of 2-(2-aminoethoxy)ethanol (0.77 g, 7.32 mmol) in $CH_2Cl_2$ (10 ml) was added 6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (1.48 g, 3.66 mmol) in $CH_2Cl_2$ (10 ml). The reaction mixture was stirred at room temperature for 3 h and poured into water (50 ml). The organic layer was separated and washed with aqueous HCl (5%), aqueous NaOH (5%), water and dried over magnesium sulfate. After filtration, the solvent was removed to give the product as a yellow sticky oil (1.40 g, 97%). $^{1}$HNMR (400MHz, $CDCl_3$): 10.13 (s, 1H), 7.70 (s, 9.0Hz, 1H), 6.37 (t, 1H), 6.29 (dd, J=9.0Hz, 2.1Hz, 1H), 6.01 (d, J=2.2Hz, 1H), 4.04 (t, J=6.4Hz, 2H), 3.76 (t, J=4.6Hz, 2H), 3.60 (m, 4H), 3.50 (t, J=5.0Hz, 2H), 3.43 (q, J=7.2Hz, 4H), 2.24 (t, J=7.8Hz, 2H), 1.86 (m, 2H), 1.73 (m, 2H), 1.56 (m, 2H), 1.23 (t, J=7.0Hz, 6H). $^{13}$CNMR ($CDCl_3$): 187.3, 173.2, 163.8, 154.0, 130.5, 114.2, 104.3, 93.2, 72.3, 69.9, 67.8, 61.7, 44.8, 39.2, 36.6, 28.8, 25.6, 25.3, 12.6.

6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide



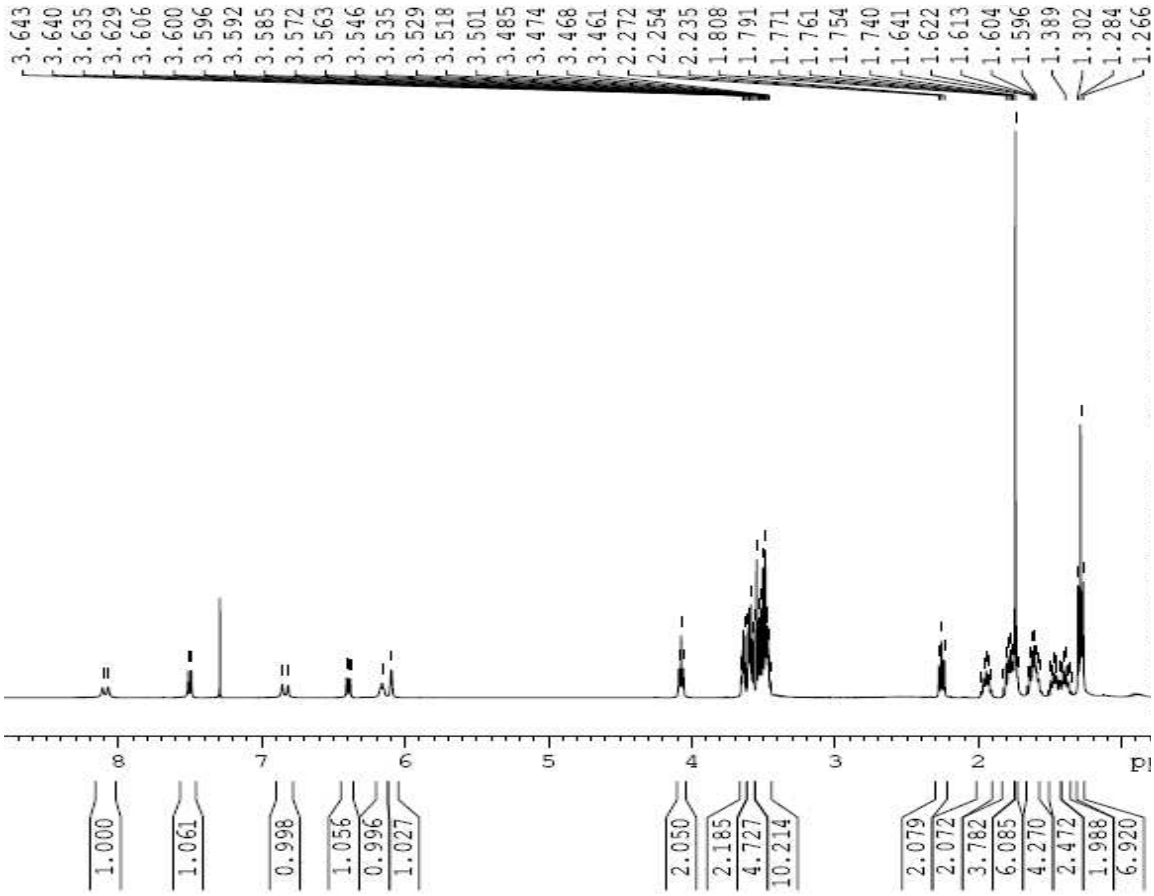

To a solution of 6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide (0.93 g, 2.36 mmol) in anhydrous DMF (10ml) was added sodium hydride (0.2 g, 60% in mineral oil, 5.0 mmol). The reaction mixture was stirred for 1h and a solution of toluene-4-sulfonic acid 6-chloro-hexyl ester (0.84 g, 2.89 mmol) in DMF (10ml) was added dropwise. The resulting solution was stirred at room temperature overnight. After that, the reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was collected, washed with water and dried over magnesium sulfate. After filtration, solvent was evaporated and the residue was purified with flash chromatography (hexane/ethyl acetate: 1/1) to give the product as a yellow oil (0.75 g, 62%). $^{1}$HNMR (400MHz, $CDCl_3$): 10.19 (s, 1H), 7.73 (d, J=9.1Hz, 1H), 6.30 (d, J=9.2Hz, 1H), 6.04 (m, 2H), 4.06 (t, J=6.4Hz, 2H), 3.64 (m, 2H), 3.59 (m, 4H), 3.55 (m, 2H), 3.50-3.41 (m, 8H), 2.24 (t, J=7.3Hz, 2H), 1.89 (m, 2H), 1.81-1.37 (m, 12H), 1.28 (t, J=7.2Hz, 6H). $^{13}$CNMR ($CDCl_3$): 187.0, 172.8, 163.8, 153.8, 130.2, 114.3, 104.3, 93.2, 71.3, 70.3, 70.0, 69.8, 67.7, 45.0, 44.8, 39.1, 36.6, 32.5, 29.7, 29.5, 28.9, 26.7, 25.8, 25.4, 25.3, 12.6. IR (neat, $cm^{-1}$): 3306, 2924, 2854, 2760, 1722, 1618.

6-{2-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-5-diethylamino-phenoxy}-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide




A mixture of 2-(3-cyano-4,5,5-trimethyl-5H-furan-2-ylidene)-malononitrile (0.25g, 1.26mmol) and 6-(5-diethylamino-2-formyl-phenoxy)-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide (0.64 g, 1.25 mmol) in 1-propanol was refluxed overnight. The solvent was evaporated and the residue was purified with flash chromatography (hexane/ethyl acetate: 4/1) to give the product as a blue solid (0.42 g, 48%). $^{1}$HNMR (400MHz, $CDCl_3$): 8.08 (d, J=15.4Hz, 1H), 7.49 (d, J=9.0Hz, 1H), 6.82 (d, J=15.4Hz, 1H), 6.37 (dd, J=8.9Hz, 2.2Hz, 1H), 6.13 (t, J=5.4Hz, 1H), 6.05 (d, J=2.3Hz, 1H), 4.06 (t, J=6.3Hz, 2H), 3.65-3.44 (m, 16H), 2.25 (t, J=7.4Hz, 2H), 1.94 (m, 2H), 1.77 (m, 4H), 1.73 (s, 6H), 1.60 (m, 4H), 1.43 (m, 4H), 1.28 (t, J=7.1Hz, 6H). $^{13}$CNMR ($CDCl_3$): 176.9, 175.0, 172.9, 162.3, 154.0, 144.4, 132.2, 113.3, 112.6, 112.3, 112.2, 107.8, 106.3, 96.4, 93.7, 71.2, 70.2, 70.0, 69.8, 68.2, 45.3, 45.1, 39.1, 36.4, 32.5, 29.7, 29.5, 28.9, 26.9, 26.7, 25.8, 25.4, 12.8. Anal. Calcd for $C_{38}H_{52}ClN_5O_5$: C, 65.74; H, 7.55; N, 10.09. Found: C, 65.58; H, 7.52; N, 10.25.

6-{2-[2-(4-cyano-5-dicyanomethylene-2,2-dimethyl-2,5-dihydro-furan-3-yl)-vinyl]-5-diethylamino-phenoxy}-hexanoic acid {2-[2-(6-chloro-hexyloxy)-ethoxy]-ethyl}-amide



## X. References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