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Supporting Information

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A small molecule FRET probe to monitor PLA_2 activity in cells and organisms

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Materials and methods

General Methods

Unless otherwise noted, materials were obtained from commercial suppliers in the highest purity available and were used without further purification. Dry solvents were purchased from Fluka, stored over molecular sieves, and used as supplied. Flash chromatography was carried out using Merck silica gel 60 (63-200 mesh), (TLC) was performed on Merck silica 60 WF_{254s} analytical plates with ethyl acetate/cyclohexane or methanol/dichloromethane mixtures as eluents. Spots were detected by a UV hand lamp @ 254 nm or 366 nm or staining with either A) phosphomolybdic acid / cerium(IV) sulfate reagent or B) ninhydrin solution.

NMR spectra were recorded using a Bruker UltraShield^{TM} Advance 400 (400 MHz, ^{1}H; 100 MHz, ^{13}C; 80 MHz, ^{31}P) spectrometer and calibrated using residual undeuterated solvent as an internal reference.

High-resolution mass spectra were recorded at University of Heidelberg using fast atom bombardment (FAB) mass spectrometry on a Jeol JMS 700 mass spectrometer.

Fluorescence spectra were recorded with a Quantamaster QM4/2000SE (Photon Technology International) fluorimeter using 1 ml optical quartz cuvettes. Assays were performed in a Tris-HCl buffered Triton X-100 mixed micelle system at pH 7.5 and 37 °C (100 mM KCl, 10 mM $CaCl_2$, 25 mM Tris-HCl, 3 mM Triton X-100). Excitation wavelength was 458 nm. Emission spectra were recorded from 470 nm to 750 nm with 2 nm step size.

Quantum yields were measured by the method of Williams *et al*. (Analyst **1983**, *108*, 1067) using NBD-undecanoic acid as the standard (Φ_{NBD}(ethanol) = 0.36, ref. 21). The fluorescence spectra were recorded at five different concentrations with a defined absorbance at 458 nm between 0 and 10% of each sample in ethanol. The integrated fluorescence intensity from 470 to 750 nm was plotted against the absorbance.
The gradient of the resulting graph gave the relative quantum yield.
Absolute quantum yields were calculated with the formula:

$$\Phi_{sample}=\Phi_{NBD}(Grad_{Sample}/Grad_{NBD})$$

Fluorescence microscopy was carried out on a Zeiss Axiovert 200M. Filter setup for NBD fluorescence was 460/50x excitation, 500DCLP dichroic mirror and D535/40 m emission; for Nile Red fluorescence settings were 560/40x excitation, 595DCLP dichroic mirror, D630/60m emission. Assay were performed in CO_2 independent HEPES buffer adjusted to pH 7.4 (115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4, 20 mM HEPES).

Products were characterized by NMR (^{1}H, ^{13}C, and if appropriate ^{31}P) and high resolution MS except PENN, which did not yield sufficient material for reliable ^{13}C-NMR data, in this case the ^{13}C data were extracted from an HMBC.

1-O-(12-Bromododecyl)-*sn*-glycerol (3)

Sodium Hydride (NaH, 1.5 eq; 15 mmol; 360 mg) was added to a stirred mixture of *sn*-2,3-*O*-isopropylidene-glycerol (10 mmol; 1.32 g) and 1,12-dibromododecane (3 eq; 30 mmol; 9.84 g) in 100 ml dry DMF under argon in six portions (approx 60 mg each) over a period of one hour. After further stirring under argon at 25 °C for 14 hours, TLC (cyclohexane/ethyl acetate 8:2; staining with A) showed complete consumption of the glycerol. The mixture was poured into 600 ml of a saturated NH_4Cl solution and extracted four times with 150 ml ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to yield a crude mixture of the desired product and 1,12-dibromododecane.
This mixture was dissolved in 100 ml acetonitrile under heating and 5 ml of trifluoroacetic acid (TFA) and 1 ml of water was added. The resulting solution was stirred for 30 min until TLC (dichloromethane/methanol 95:5; R_f= 0.11; staining with A) showed now further conversion of the starting material. The solution was evaporated to dryness and this procedure was repeated twice until TLC showed complete cleavage of the ketal.
The dried crude product was then taken up in dichloromethane and put on a short silica column (250 g). The column was washed with dichloromethane until all remaining 1,12-dibromododecane had eluted. The pure diol was then eluted by washing with dichloromethane/methanol 97:3. After removal of the solvents under reduced pressure **3** was received as colourless oil (2.52 g; 7.4 mmol, 74%).

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 3.91-3.84 (m, 1H, *sn*-2 C*H*), 3.72 (dd, ^{2}J = 11.4 Hz, ^{3}J = 3.8 Hz, 1H, *sn*-3 C*H*H'), 3.64 (dd, ^{2}J = 11.5 Hz, ^{3}J = 5.4 Hz, 1H, *sn*-3 CH*H'*), 3.57-3.44 (m, 4H, *sn*-1 CH_2, *sn*-1 OCH_2), 3.41 (t, ^{3}J = 6.9 Hz, 2H, BrCH_2), 2.70 (s, 1H, O*H*), 1.86 (qi, ^{3}J= 7.3 Hz, 2H, $BrCH_2CH_2$), 1.58 (qi, ^{3}J= 6.9 Hz, 2H, OCH_2CH_2), 1.43 (qi, ^{3}J= 7.2 Hz, 2H, $BrCH_2CH_2CH_2$), 1.38-1.24 (m, 14H, CH_2 chain).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 72.42, 71.83, 70.51, 64.23, 34.01, 32.2, 29.55, 29.52, 29.51, 29.48, 29.41, 29.39, 28.74, 28.15, 26.05.

MS HR-ESI (positive mode):
$[M+H^+]$ calculated: 339.1535
$[M+H^+]$ found: 339.1537

1-O-(12-Azidododecyl)-*sn*-glycerol

4 mmol of **3** (1.357 g) was dissolved in 30 ml dimethylsulfoxide (DMSO). After addition of an excess of NaN_3 (5 eq.; 20 mmol; 1.3 g) the solution was stirred at 85°C for 3 hours. After this time reaction control (^{1}H NMR) showed now further progress of the reaction. The mixture was poured into water and was extracted three times with ethyl acetate. The combined organic layers were washed with brine dried over Na_2SO_4 and evaporated under reduced pressure. The yielded crude product was about 90 % pure according to the NMR. To further convert the remaining 10% of the bromide, it was redissolved in DMSO, NaN_3 was added (10 mmol, 650 mg) and the mixture was heated to 85°C for another 3 hours. After this time no starting material could be detected by ^{1}H NMR. The solution was poured in water and extracted three times with ethyl acetate. The organic fractions were combined, washed with brine, and dried over Na_2SO_4. The solvent was removed under reduced pressure to give the azide as colorless oil (1.17 g; 3.88 mmol; 98%).

R_f (dichloromethane/methanol 95:5) = 0.11

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 3.92-3.84 (m, 1H, *sn*-2 C*H*), 3.74 (dd, ^{2}J = 11.5 Hz, ^{3}J = 3.9 Hz, 1H, *sn*-3 C*H*H'), 3.67 (dd, ^{2}J = 11.4 Hz, ^{3}J = 5.3 Hz, 1H, *sn*-3 CH*H'*), 3.56 (dd, ^{2}J = 9.6 Hz, ^{3}J = 4.0 Hz, 1H, *sn*-1 CH*H'*), 3.52 (dd, ^{2}J = 9.3 Hz, ^{3}J = 5.8 Hz 1H, *sn*-1 C*H*H'), 3.41 (dt, ^{3}J = 6.6, 2.7 Hz, 2H, *sn*-1 OCH_2), 3.27 (t, ^{3}J = 6.9 Hz, 2H, N_3CH_2), 2.36 (s, 2H, O*H*), 1.66-1.55 (m, 4H, $N_3CH_2CH_2$, OCH_2CH_2), 1.43-1.24 (m, 16H, CH_2 chain).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 72.20, 71.14, 70.70, 64.07, 51.42, 29.51, 29.45, 29.41, 29.09, 28.77, 26.66, 25.99.

MS HR-FAB (NBA, positive mode):
$[M+H^+]$ calculated: 302.2444
$[M+H^+]$ found: 302.2447

1-O-(12-Azidododecyl)-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol (4)

1.07 g of the azide (3.56 mmol) was dissolved in 20 ml of DMF. After addition of a catalytic amount of 4-dimethylaminopyridine as well as 1.1 equivalents of 4,4'-dimethoxytrityl chloride (DMT-Cl) (4.66 mmol; 1.32 g) and triethylamine (1.5 eq; 5.34 mmol, 72.7 µl), the reaction mixture was stirred at room temperature for 14 hours, until TLC (cyclohexane/ethyl acetate 3:2, R_f= 0.51; detection with staining solution A, UV and heating) showed no remaining starting material. The mixture was diluted with 300 ml ethyl acetate and was subsequently washed three times with 10% $NaHCO_3$ solution and brine. After drying the organic layer over Na_2SO_4 the solvent was evaporated under

reduced pressure to yield the crude product as a clear oil. The latter was subjected to silica column chromatography (cyclohexane/ethyl acetate 9:1). The product-containing fractions were pooled and evaporated under reduced pressure to yield **4** as a colourless oil (1.85 g, 3.06 mmol, 86%).

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 7.47 (d, ^{3}J = 7.1 Hz, 2H, DMT), 7.36 (d, ^{3}J = 8.8 Hz, 4H, DMT), 7.32 (t, ^{3}J = 7.7 Hz, 1H, DMT), 7.27-7.18 (m, 2H DMT), 6.86 (d, ^{3}J = 9.1 Hz, 4H, DMT), 3.94-3.81 (m, 1H, *sn*-2 C*H*), 3.81 (s, 6H, CH_3 DMT), 3.57 (dd, ^{2}J = 9.7 Hz, ^{3}J = 4.2 Hz 1H, *sn*-1 CH*H'*), 3.51 (dd, ^{2}J = 9.7 Hz, ^{3}J = 6.7 Hz, 1H, *sn*-1 C*H*H'), 3.47 (dt, ^{3}J = 6.8, 2.7 Hz, 2H, *sn*-1 OCH_2), 3.28 (t, ^{3}J = 6.9 Hz, 2H, N_3CH_2), 3.24 (dd, ^{2}J = 9.7, ^{3}J = 5.9 Hz, 1H, *sn*-3 C*H*H'), 3.21 (dd, ^{2}J = 9.3, ^{3}J = 5.3 Hz, 1H, *sn*-3 CH*H'*), 2.52 (s, 1H, O*H*), 1.68-1.54 (m, 4H, $N_3CH_2CH_2$, OCH_2CH_2), 1.44-1.26 (m, 16H, CH_2 chain).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 158.48, 144.91, 136.07, 130.06, 128.16, 127.78, 126.76, 113.10, 86.07, 72.17, 71.61, 69.99, 64.52, 55.20, 29.64, 29.51, 29.49, 29.44, 29.21, 28.45, 26.94, 26.09.

MS HR-FAB (NBA, positive mode):
[M+Na^+] calculated: 626.3570
[M+Na^+] found: 626.3603

1-O-(12-Aminododecyl)-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol

Under an atmosphere of argon, 1.51 g **4** (2.52 mmol) was dissolved in 25 ml dry THF. The mixture was cooled to 0°C in an ice bath and LAH was added (2 eq.; 5.04 mmol; 189 mg). After stirring for 3 hours TLC (cyclohexane/ethylacetate 3:2) showed a single ninhydrin-sensitive spot sticking to the baseline. The reaction was quenched by slow addition of 1 ml methanol and further stirring for 15 minutes. Subsequently, the reaction mixture was diluted with 150 ml ethyl acetate and washed three times with 10% $NaHCO_3$ solution in a separation funnel. After drying the organic layer over Na_2SO_4, the solvent was removed under reduced pressure to yield a yellow crude product. The latter was dissolved in dichloromethane and subjected to column chromatography (dichloromethane/methanol/dimethylethylamine; 95:4:1). Product-containing fractions were pooled and the solvents were removed under reduced pressure to yield the amine as a slightly yellowish oil (1.34 g; 2.32 mmol, 92%).

R_f (dichloromethane/methanol/Me_2NEt 95:5:1) = 0.04

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 7.45 (d, ^{3}J = 7.3 Hz, 2H, DMT), 7.42-7.27 (m, 6H, DMT), 7.23 (t, ^{3}J = 7.2 Hz, 1H, DMT), 6.84 (d, ^{3}J = 8.8 Hz, 4H, DMT), 4.02-3.93 (m, 1H, *sn*-2 C*H*), 3.81 (s, 6H, CH_3 DMT), 3.55 (dd, ^{2}J = 9.6 Hz, ^{3}J = 4.3 Hz, 1H, *sn*-1 CH*H'*), 3.49 (dd, ^{2}J = 9.5 Hz, ^{3}J = 7.7 Hz, 1H, *sn*-1 C*H*H'), 3.48-3.40 (m, 2H, *sn*-1 OCH_2), 3.30-3.15 (m, 2H, *sn*-3 CH_2), 3.15-2.72 (t, ^{3}J = 7.1 Hz, 2H, NH_2CH_2), 2.24 (s, 2H, NH_2), 1.74-1.20 (m, 20H, CH_2 chain).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C):
δ = 158.47, 144.88, 136.09, 130.05, 128.15, 127.78, 126.74, 113.09, 86.03, 72.17, 71.64, 69.88, 64.50, 55.19, 53.40, 42.07, 33.36, 29.65, 29.56, 29.47, 29.44, 26.86, 26.09.

MS HR-FAB (NBA, positive mode):
$[M+Na^+]$ calculated: 600.3665
$[M+Na^+]$ found: 600.3640

1-O-(12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecyl)-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol (5)

Under an atmosphere of dry argon 470 mg 7-nitro-2-1,3-benzoxadiazol-4-yl chloride (NBD-chloride; 1.1 eq.; 2.35 mmol) was dissolved in 40 ml of dry methanol. The solution was cooled to 0°C and Huenig's base (5 eq.; 10.7 mmol, 1.8 ml) was added. Subsequently, the amine (2.14 mmol, 1.25 g) was added in small portions over a period of one hour followed by stirring for three more hours at 0°C and 12 hours at 25 °C. The color of the solution turned from slightly yellow over orange to dark brown during the reaction. At this time TLC (cyclohexane/ethylacetate 3:2; detection by ninhydrin and UV @ 366 nm) indicated completion of the reaction. The mixture was concentrated under reduced pressure and the resulting dark brown oil was subjected to column chromatography on silica (cyclohexane/ethylacetate 3:2). Product containing fractions were pooled and evaporated to give **5** as a dark orange waxy solid (1.24 g; 1.67 mmol, 78%).

R_f (cyclohexane/ethyl acetate, 3:2) = 0.44

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 8.39 (d, ^{3}J = 8.8 Hz, 1H, NBD-*H*), 7.39 (d, ^{3}J = 7.8 Hz, 2H, DMT), 7.27 (d, ^{3}J = 8.8 Hz, 4H, DMT), 7.22 (d, ^{3}J = 7.7 Hz, 2H, DMT), 7.14 (t, ^{3}J = 7.3 Hz, 1H, DMT), 6.76 (d, ^{3}J = 9.1 Hz, 4H, DMT), 6.09 (d, ^{3}J = 8.8 Hz, 1H, NBD-*H*), 3.92 (tt, ^{3}J = 5.5, 5.1 Hz, 1H, *sn*-2 C*H*), 3.71 (s, 6H, CH_3 DMT), 3.51 (dd, ^{2}J = 9.9, ^{3}J = 4.3 Hz, 1H, *sn*-1 CH*H'*), 3.45 (dd, ^{2}J = 9.9 Hz, ^{3}J = 6.6 Hz, 1H, *sn*-1 C*H*H'), 3.44-3.35 (m, 4H, *sn*-1 OCH_2, NBD-NH-CH_2), 3.15 (dd, ^{3}J = 5.3, 2.3 Hz, 2H, *sn*-3 CH_2), 1.74-1.20 (m, 20H, CH_2 chain).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 158.48, 144.91, 144.25, 143.98, 136.70, 136.07, 130.06, 128.16, 127.78, 126.76, 123.31, 113.10, 86.07, 72.17, 71.61, 69.99, 64.52, 55.20, 29.64, 29.51, 29.49, 29.44, 29.21, 28.45, 26.94, 26.09.

MS HR-FAB (NBA, positive mode):
$[M+H^+]$ calculated: 740.3785
$[M+H^+]$ found: 740.3777

1-O-(12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecyl)-2-O-(4-(9-diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)butanoyl-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol (6)

Three flame dried, argon flushed, rubber septum sealed 50 ml flasks were prepared, containing:

a) methylimidazole (3 eq.; 119µl)
b) TPSNT (1.5 eq., 285 mg)
c) **5** (1 eq.; 370 mg; 0.5 mmol) and stirring bar

1.5 Equivalents of 2-O-Nile Red-labelled butyric acid (0.75 mmol; 315 mg) were dissolved in 7 ml dry dichloromethane. The solution was taken up by a syringe and injected into flask a). After shaking this mixture for 30 seconds it was taken up with the same syringe and injected into flask b) to generate the activated carboxylic acid. This solution was then immediately transferred to flask c) and stirred over night. After 11 hours, TLC (cyclohexane/ethyl acetate 3:2) showed complete consumption of the starting material. Dichloromethane was removed in vacuo and the resulting red slurry was subjected to column chromatography on silica (dichloromethane/methanol 99.5:0.5 → 99:1). Product containing fractions were pooled and evaporated to give **6** as a dark red amorphous solid (480 mg; 0.42 mmol; 84%).

R_f (cyclohexane/ethylacetate, 3:2) = 0.16

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 8.41 (d, ^{3}J = 8.6 Hz, 1H, NBD-*H*), 8.18 (d, ^{3}J = 8.6 Hz, 1H,Nile Red H^4), 8.00 (d, ^{4}J = 2.5 Hz, 1H, Nile Red H^1), 7.54 (d, ^{3}J = 9.1 Hz, 1H, Nile Red H^{11}), 7.44 (d, ^{3}J = 7.6 Hz, 2H, DMT), 7.32 (d, ^{3}J = 8.8 Hz, 4H, DMT), 7.28 (d, ^{3}J = 8.2 Hz, 2H, DMT), 7.19 (t, ^{3}J = 7.3 Hz, 1H, DMT), 7.11 (dt, ^{3}J = 8.7 Hz, ^{4}J = 2.4, 1H, Nile Red H^3), 6.81 (d, ^{3}J = 8.8 Hz, 4H, DMT), 6.63 (dt, ^{3}J = 9.3 Hz, ^{4}J = 2.5, 1H, Nile Red H^{10}), 6.40 (d, ^{4}J = 2.5 Hz, 1H, Nile Red H^8), 6.28 (s, 1H, Nile Red H^6), 6.09 (d, ^{3}J = 8.6 Hz, 1H, NBD-*H*), 5.29 (qi, ^{3}J = 5.2 Hz, 1H, *sn*-2 C*H*), 4.19 (t, ^{3}J = 4.2 Hz, Nile Red OCH_2, 3.77 (s, 6H, CH_3 DMT), 3.70-3.57 (m, *sn*-1 CH_2), 3.52-3.33 (m, 8H, *sn*-1 OCH_2, NBD-NH-CH_2, Nile Red NCH_2 (2x), 3.31-3.21 (m, 2H, *sn*-3 CH_2), 2.73-2.59 (m, 2H, OOCCH_2), 2.23 (qi, ^{3}J = 6.4 Hz, 2H, Nile Red O-CH_2CH_2), 1.74 (qi, ^{3}J = 7.2 Hz, 2H, NBDNH-CH2CH_2), 1.45-1.20 (m, 26H, Nile Red NCH2CH_3 (2x) CH_2 chain (10x)).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 183.15, 172.57, 161.52, 158.48, 152.05, 150.84, 146.77, 144.78, 144.23, 139.58, 136.51, 135.98, 135.93, 134.06, 131.11, 130.89, 130.04, 129.99, 128.79, 128.13, 127.77, 127.66, 126.76, 125.63, 124.70, 118.17, 113.08, 109.65, 106.60, 105.08, 96.19, 85.96, 72.11, 71.47, 69.61, 68.15, 67.13, 62.45, 60.37, 55.18, 45.07, 30.98, 29.68, 29.58, 29.51, 29.48, 29.41, 29.38, 29.19, 28.93, 28.44, 26.93, 26.02, 24.73, 14.19, 12.62.

MS HR-FAB (NBA, positive mode):
[M+H$^+$] calculated: 1142.5365
[M+H$^+$] found: 1142.5363

Deprotection of 6 1-O-(12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecyl)-2-O-(4-(9-diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)-butanoyl-*sn*-glycerol

400 mg (0.35 mmol) of the DMT protected alcohol **6** was dissolved in dichloromethane and treated with excess Dowex 50 WX8 ion exchange resin (1.2 g) for 3 to 4 hours. After this time TLC (dichloromethane/methanol 95:5) showed almost complete conversion of the starting material to a slower migrating red spot (R_f= 0.19). To remove the resin as well as the DMT, the reaction mixture was concentrated in vacuo and directly applied to a short silica column. The remaining traces of starting material and the DMT-OH were washed off the column by dichloromethane/methanol 99:1. Subsequently, the eluent was changed to 5% methanol in dichloromethane to obtain 223 mg (0.27 mmol) of the desired *sn*-3 alcohol, containing traces (<5%) of the migration product. This crude alcohol was phosphorylated in the next step without further purification.
For analytical purposes a small portion of the alcohol was further purified by silica column chromatography (dichloromethane/methanol 99:1 → 98:2).

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 8.41 (d, ^{3}J = 8.6 Hz, 1H, NBD-*H*), 8.16 (d, ^{3}J = 8.6 Hz, 1H,Nile Red H^4), 7.96 (d, ^{4}J = 2.3 Hz, 1H, Nile Red H^1), 7.54 (d, ^{3}J = 9.1 Hz, 1H, Nile Red H^{11}), 7.11 (dt, ^{3}J = 8.8 Hz, ^{4}J = 2.0, 1H, Nile Red H^3), 6.65 (dt, ^{3}J = 9.1 Hz, ^{4}J = 2.4, 1H, Nile Red H^{10}), 6.41 (d, ^{4}J = 2.0 Hz, 1H, Nile Red H^8), 6.29 (s, 1H, Nile Red H^6), 6.10 (d, ^{3}J = 8.3 Hz, 1H, NBD-*H*), 5.10 (qi, ^{3}J = 4.8 Hz, 1H, *sn*-2 C*H*), 4.22 (t, ^{3}J = 5.8 Hz, Nile Red OCH_2,),3.88 (dd, ^{2}J = 12.1 Hz, ^{3}J = 4.0 Hz, 1H, *sn*-3 C*H*H'), 3.84 (dd, ^{2}J = 11.9 Hz, ^{3}J = 5.4 Hz, 1H, *sn*-3 CH*H'*), 3.67 (dd, ^{2}J = 10.7, ^{3}J = 5.2 Hz, 1H, *sn*-1 CH*H'*), 3.63 (dd, ^{2}J = 10.7 Hz, ^{3}J = 5.2 Hz 1H, *sn*-1 C*H*H'), 3.52-3.37 (m, 8H, *sn*-1 OCH_2, NBD-NH-CH_2, Nile Red NCH_2 (2x)), 2.67 (t, 2H, ^{3}J = 7.1 Hz, OOCCH_2), 2.22 (qi, ^{3}J = 6.4 Hz, 2H, Nile Red O-CH2CH_2), 1.75 (qi, ^{3}J = 7.2 Hz, 2H, NBDNH-CH_2CH_2), 1.53-1.20 (m, 26H, Nile Red NCH_2CH_3 (2x) CH_2 chain (10x)).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 182.83, 173.01, 161.44, 151.95, 150.93, 146.72, 144.17, 139.14, 136.53, 133.93, 131.11, 127.55, 125.35, 124.87, 118.17, 109.93, 106.38, 104.78, 96.14, 73.38, 71.75, 69.83, 67.11, 62.57, 45.14, 30.94, 29.66, 29.48, 29.43, 29.39, 29.35, 29.27, 29.14, 28.39, 26.90, 25.97, 24.63, 12.61.

MS HR-ESI (positive mode):
[M+H$^+$] calculated: 841.4136
[M+H$^+$] found: 841.4111

General phosphorylation procedure

One equivalent of the alcohol and 4 equivalents of 4,5-dicyanoimidazole were placed into a Schlenk flask and the flask was sealed with a rubber stopper. Dry acetonitrile was added and evaporated *in vacuo* three times to remove traces of water azeotropically. Then

DMF was added under an atmosphere of dry Argon and the mixture was cooled to 0°C. Two equivalents of the phosphoramidite were added and the mixture was stirred over night. After 12 hours TLC (dichloromethane/methanol 95:5) showed a faster migrating spot of the phosphorous (III) triester which could not be isolated. Instead the whole reaction mixture was cooled to 0°C and treated with an excess of tBuOOH for one hour to oxidize to the stable phosphorous (V) triester. The products were either isolated (in case of PENN/SATE) or further reacted (PENN).

PENN/SATE:
1-O-(12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecyl)-2-O-(4-(9-diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)butanoyl-3-O-((2-(Acetylthio)ethoxy)(2-((2-(acetylthio)ethoxy)carbonyl)aminoethoxy)-phosphoryl)-*sn*-glycerol (1)

According to the general procedure 40 mg (0.048 mmol) of the *sn*-3 alcohol was reacted with 22.6 mg (0.186 mmol) dicyanoimidazole and 43.7 mg (0.096 mmol) of the SATE protected phosphoramidite **8**. The oxidation was performed by addition of 60 µl of 5.5 M tBuOOH solution in nonane.
The resulting product (R_f= 0.24, dichloromethane/methanol 95:5) was purified by column chromatography on silica (dichloromethane/methanol 98:2). Product containing fractions were pooled to obtain PENN/SATE (**1**) as deep red oil (30.6 mg, 0.025 mmol, 52.7%).

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 8.48 (d, ^{3}J = 8.6 Hz, 1H, NBD-*H*), 8.25 (d, ^{3}J = 8.8 Hz, 1H, Nile Red H^{4}), 8.06 (d, ^{4}J = 2.5 Hz, 1H, Nile Red H^{1}), 7.62 (d, ^{3}J = 9.1 Hz, 1H, Nile Red H^{11}), 7.19 (dd, ^{3}J = 8.6 Hz, ^{4}J = 2.5, 1H, Nile Red H^{3}), 6.70 (dd, ^{3}J = 9.1 Hz, ^{4}J = 2.5, 1H, Nile Red H^{10}), 6.48 (d, ^{4}J = 2.5 Hz, 1H, Nile Red H^{8}), 6.35 (s, 1H, Nile Red H^{6}), 6.16 (d, ^{3}J = 8.3 Hz, 1H, NBD-*H*), 5.53 (t, ^{3}J = 4.5 Hz, 0.5H, N*H* carbamate),5.48 (t, ^{3}J = 4.5 Hz, 0.5H, N*H* carbamate), 5.30-5.20 (m, 1H, *sn*-2 C*H*), 4.37-4.10 (m, 12H, Nile Red OCH_2, POCH_2 (3x), NHCOOCH_2), 3.61 (d, ^{3}J = 5.1 Hz, 1H, *sn*-1 CH_2), 3.56-3.40 (m, 10H, *sn*-1 OCH_2, NBD-NH-CH_2, Nile Red NCH_2 (2x), OOCNHCH_2), 3.20 (t, 2.67 , ^{3}J = 6.3 Hz, 2H, COSCH_2),3.15 (t, ^{3}J = 6.4 Hz, 2H, COSCH_2),2.69 (t, 2H, ^{3}J = 7.2 Hz, OOCCH_2), 2.38 (s, 3H, CH_3COS), 2.37 (s, 3H, CH_3COS), 2.26 (qi, ^{3}J = 6.7 Hz, 2H, Nile Red O-CH_2CH_2), 1.79 (qi, ^{3}J = 7.2 Hz, 2H, NBDNH-CH2CH_2), 1.60-1.19 (m, 26H, Nile Red NCH_2CH_3 (2x) CH_2 chain (10x)).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 183.21, 161.50, 152.13, 150.89, 146.88, 136.49, 134.10, 131.10, 130.87, 128.80, 127.80, 125.74, 124.74, 118.22, 109.68, 106.61, 105.21, 96.28, 71.79, 70.96, 68.34, 67.03, 66.34, 66.18, 63.35, 45.10, 44.07, 41.40, 38.74, 31.92, 30.80, 30.56, 30.37, 29.69, 29.48, 29.43, 29.37, 29.32, 29.15, 29.06, 28.93, 28.45, 28.25, 26.91, 25.99, 12.61.

^{31}P-NMR (MHz, $CDCl_3$, 25°C, H_3PO_4):
δ = -1.1

MS HR-FAB (NBA, positive mode):

[M+H$^+$] calculated: 1212.4398
[M+H$^+$] found: 1212.4366

PENN:
1-O-(12-[(7-Nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecyl)-2-O-(4-(9-diethylamino)-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)-butanoyl-*sn*-glycero-3-Phosphoethanolamine (7)

According to the general procedure 40 mg (48 µmol) of the *sn*-3 alcohol was reacted with 22.6 mg (0.186 mmol) dicyanoimidazole and 43.7 mg (0.096 mmol) of the *t*-butyl/Boc protected phosphoramidite **9**. The oxidation was performed by addition of 60 µl of 5.5 M *t*BuOOH solution in nonane. Excess of oxidizing agent was quenched by addition of 10 ml 5% aqueous sodium thiosulfate solution. The resulting aqueous mixture was then extracted with ethyl acetate until the organic phase remained clear. The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure. The resulting *t*-butyl/Boc protected phosphatidylethanolamine was not stable under flash chromatography conditions and was therefore deprotected to the desired final product by treatment with 5 ml dichloromethane/TFA 90:10 for 40 minutes until TLC showed no further conversion (the product spot did not move in dichloromethane/methanol 95:5, but with 80:20 (R_f= 0.56)). The dichloromethane/TFA mixture was removed under reduced pressure and the resulting crude product was subjected to column chromatography (dichloromethane/methanol 90:10 → dichloromethane/methanol/water 90:10:1). Solvents were removed under reduced pressure to yield 12 mg PENN (12.4 µmol, 26%). In case the product contained some silica, the product was re-dissolved in dichloromethane/methanol and filtered through Celite^{TM} to remove the silica.

R_f (dichloromethane/methanol 80:20) = 0.56

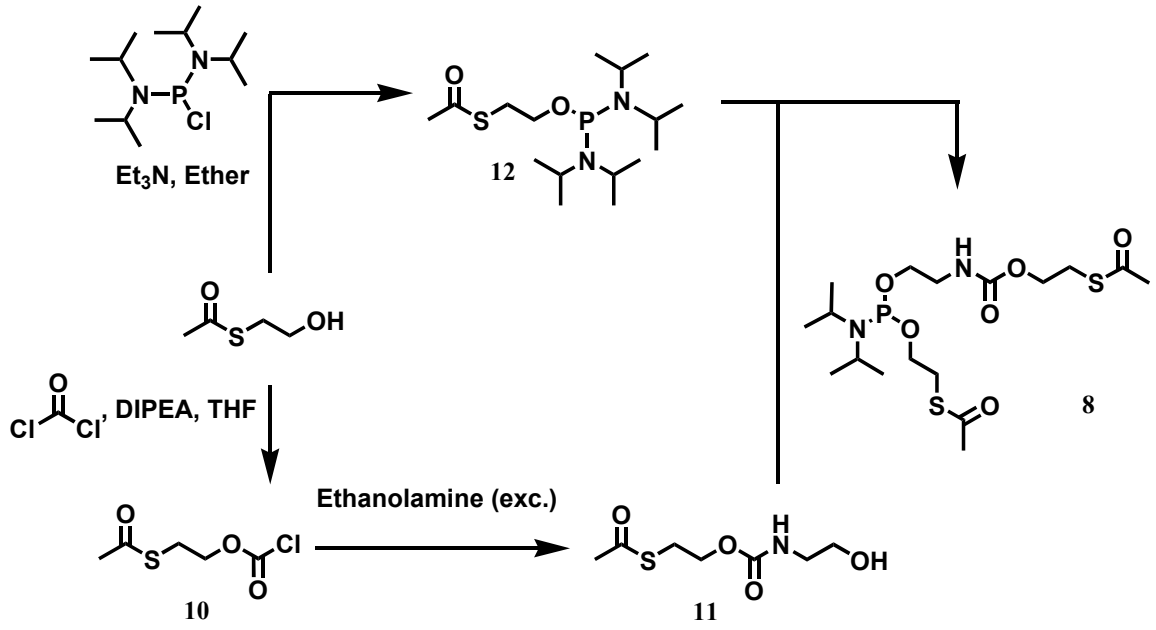
^{1}H-NMR (400 MHz, $CDCl_3$/MeOD (1:1),25°C, TMS):
δ = 8.12 (d, ^{3}J = 8.8 Hz, 1H, NBD-*H*), 7.83 (d, ^{3}J = 8.8 Hz, 1H,Nile Red H^4), 7.74(d, ^{4}J = 2.7 Hz, 1H, Nile Red H^1), 7.41 (d, ^{3}J = 9.1 Hz, 1H, Nile Red H^{11}), 6.87 (dd, ^{3}J = 8.7 Hz, ^{4}J = 2.5, 1H, Nile Red H^3), 6.46 (dd, ^{3}J = 9.2 Hz, ^{4}J = 2.7, 1H, Nile Red H^{10}), 6.19 (d, ^{4}J = 2.5 Hz, 1H, Nile Red H^8), 5.94 (s, 1H, Nile Red H^6), 5.84 (d, ^{3}J = 7.6 Hz, 1H, NBD-*H*),5.08 (s, 3H, NH_3),4.92 (qi, 1H, *sn*-2 C*H*), 4.00-3.64 (m, 6H, Nile Red OCH_2, POCH_2 (2x), NHCOOCH_2), 3.40-2.9 (m, 12H, *sn*-1 OCH_2, *sn*-1 CH_2, NBD-NH-CH_2, Nile Red NCH_2 (2x),CH_2NH_3),2.39-2.30 (m, 2H, ^{3}J = 7.2 Hz, OOCCH_2), 1.97-1.84 (m, 2H, ^{3}J = 6.7 Hz, Nile Red O-CH_2CH_2), 1.42-0.79 (m, 28H, Nile Red NCH_2CH_3 (2x) CH_2 chain (11x)).

^{13}C-NMR (100 MHz, $CDCl_3$/MeOD (1:1) 25°C, TMS) *derived from HMBC:*
δ = 181.34, 173.11, 161.84, 156.21, 152.09, 147.54143.42, 143,34, 137.36, 134.11, 131.08, 127.18, 123.71, 118.51, 112.23, 105.95, 102.70, 95.76, 71.91, 68.03, 66.81, 60.67, 48.75, 47.02, 41.82, 38.57, 29.46, 24.05, 22.53, 18.63, 12.13.

^{31}P-NMR (MHz, $CDCl_3$, 25°C, H_3PO_4):

$\delta = 4.2$

MS HR-FAB (NBA, positive mode):
$[M+H^+]$ calculated: 964.4221
$[M+H^+]$ found: 964.4207



Scheme S1: Synthesis of phosphitylation agent **8**. The synthesis of reagent **9** was previously described (ref. 16).

S-2-(Chlorocarbonyloxy)-ethyl ethanethioate (10)

2.4 g (20 mmol) S-Acetyl-thioethanol and 1.1 equivalent triethylamine (22 mmol, 3.1 ml) were taken up in 30 ml of dry tetrahydrofurane and cooled to 0°C. To the stirred solution an excess of phosgene (3 equivalents, 60 mmol, 31 ml of a 20% solution in toluene) was added. The mixture was stirred for 3 hours until TLC (cyclohexane/ethyl acetate 3:2) showed complete conversion of the starting material (R_f = 0.27) to a faster travelling product (R_f = 0.47). All volatile components were removed under reduced pressure into a liquid nitrogen cooling trap, doted with 30% aqueous ammonia solution. The resulting yellow slurry was taken up in diethyl ether and filtered under argon to remove the salts. Solvents were removed *in vacuo* to quantitatively yield a light yellow oil which was pure enough to proceed, according to ^{1}H- and ^{13}C-NMR.

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
$\delta = 4.41$ (t, $^3J = 6.4$ Hz, 2H, CH_2OCO), 3.21 (t, $^3J = 6.6$ Hz, 2H, CH_2SCO), 2.38 (s, 3H, CH_3CO).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS)
δ = 195.00, 151.13, 70.17, 31.05, 27.77.

MS HR-EI (positive mode):
$[M(^{37}Cl)]^{+\cdot}$ calculated: 183.9775
$[M(^{37}Cl)]^{+\cdot}$ found: 183.9774

S-2-((2-Hydroxyethyl)carbamoyloxy)-ethyl ethanethioate (11)

To a stirred mixture of 305 mg ethanolamine (5mmol) and triethylamine (10 mmol, 950 mg) in 15 ml dry THF the chloroformate (10 mmol, 1.81 g) was added under an argon atmosphere. After 14 hours stirring at room temperature, TLC (cyclohexane/ethyl acetate 3:2) indicated complete consumption of the amine and showed a new spot (R_f = 0.23) that stained weakly with ninhydrin. The solvent was removed under reduced pressure and the resulting oil was taken up in 100 ml ethyl acetate and washed three times with phosphate buffer pH 7 and brine. Solvents were evaporated and the resulting crude product was subjected to silica column chromatography (cyclohexane/ethyl acetate 7:3). Product-containing fractions were pooled and evaporated to dryness to give 787 mg (3.8 mmol, 76%) of the desired alcohol as colourless oil.

^{1}H-NMR (400 MHz, $CDCl_3$, 25°C, TMS):
δ = 5.20 (s, 1H, N*H*), 4.23 (t, ^{3}J = 6.3 Hz, 2H, CH_2OCON), 3.73 (t, ^{3}J = 4.9 Hz, 2H, CH_2OH), 3.34 (t, ^{3}J = 4.8 Hz, 2H, NHCH_2), 3.14 (t, ^{3}J = 6.3 Hz, 2H, COSCH_2), 2.37 (s, 3H, CH_3CO).

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C, TMS):
δ = 195.17, 156.68, 63.38, 62.04, 43.50, 30.51, 28.31.

MS HR-FAB (NBA, positive mode):
$[M+H]^+$ calculated: 208.0644
$[M+H]^+$ found: 208.0645

S-2-(Bis(diisopropylamino)phosphinooxy)ethyl ethanethioate (12)

5.2 g of bis(diisopropylamino)chlorophosphin (19.4 mmol) was dissolved in 40 ml of dry diethyl ether under an argon atmosphere. The flask was sealed with a rubber septum and cooled to 0°C. To this solution a mixture of S-acetoxythioethanol (1 eq., 2.33 g) and triethylamine (1.1 eq., 3.01 ml) in 20 ml of dry diethyl ether was added slowly *via syringe*. After complete addition, the mixture was stirred for another three hours at 0°C. After this, TLC (cyclohexane/ethyl acetate/dimethylethylamine 60:40:1) showed no further progress of the reaction. The precipitated salts were removed by filtration under argon and the solvent was removed under reduced pressure, to yield as clear oil which was 90% pure (according to ^{31}P-NMR). For further purification the crude product was subjected to column chromatography on silica de-activated by dimethylethylamine with cyclohexane/ethyl acetate/dimethylethylamine 90:10:1 as the eluent. Product-containing

fractions were pooled and evaporated to dryness to yield 4.48 g (13.4 mmol, 69%) of the desired phosphorousdiamidite as a amorphous, colourless solid.

R_f (cyclohexane/ethylacetate/dimethylethylamine 60:40:1) = 0.68

^{1}H-NMR (400 MHz, C_6D_6, 25°C, TMS):
δ = 3.80 (dt, $^3J_{P-H}$ = 6.91 Hz, ^3J = 6.76 Hz, 2H, C*H*$_2$OP), 3.61 (dhep, $^3J_{P-H}$ = 10.71 Hz, ^3J = 6.73 Hz, 2H, C*H* *i*PR), 3.24 (t, ^3J = 6.44 Hz, 2H, C*H*$_2$SCO), 1.95 (s, 3H, *CH*$_3$CO), 1.34 (d, ^3J = 6.82 Hz, 2H, C*H*$_3$ *i*Pr), 1.29 (d, ^3J = 6.82 Hz, 2H, C*H*$_3$ *i*Pr).

^{13}C-NMR (100 MHz, C_6D_6, 25°C, TMS):
δ = 194.00, 63.44, 44.73, 31.28, 30.02, 27.19, 24.65, 24.05.

^{31}P-NMR (162 MHz, C_6D_6, 25°C, H_3PO_4):
δ = 122.91

MS HR-FAB (NBA, positive mode):
$[M+H]^+$ calculated: 351.2235
$[M+H]^+$ found: 351.2213

S-2-((Diisopropylamino)(2-((2-(acetoxythio)ethoxy)carbonyl)aminoethoxy)-phosphinooxy)ethyl ethanethioate (8)

2.5 mmol of the SATE-oxycarbonyl-protected ethanolamine (502 mg), 1.5 mmol tetrazole (105 mg) and 1.5 mmol diisopropylamine (211 µl) were dried by repeated (three times) suspending in dry acetonitrile followed by evaporation in high vacuum. After the last cycle the mixture was dissolved in 15 ml dry dichloromethane under an argon atmosphere and cooled to 0 °C in an ice bath. To the stirred solution, 3 mmol (1.05 g) of the SATE protected phosphorous diamidite in 8 ml dry dichloromethane was added drop wise via syringe. After complete addition, the mixture stirred for another four hours until TLC (cyclohexane/ethyl acetate/dimethylethylamine 60:40:1) showed no further progress. The solvents were removed under reduced pressure and the crude product was purified by column chromatography on deactivated silica with cyclohexane/ethyl acetate/ dimethylethylamine 80:20:1 as running solvent. Product-containing fractions were pooled and evaporated to dryness to yield 712 mg (1.56 mmol, 62%) of the phosphorous amidite as colourless oil.

R_f (cyclohexane/ethyl acetate/dimethylethylamine 60:40:1) = 0.47

^{1}H-NMR (400 MHz, C_6D_6, 25°C, TMS):
δ = 5.18 (t, ^3J = 5.4 Hz, 1H, N*H*), 4.27 (t, ^3J = 6.2 Hz, 2H, C*H*$_2$OCON), 3.91-3.81 (m, 1H, POC*H*$_2$ [SATE]), 3.80-3.73 (m, 1H, POC*H*$_2$ [SATE]), 3.73-3.56 (m, 4H, C*H* *i*Pr, POCH$_2$ [ethanolamine]), 3.37 (q, ^3J = 5.3 Hz, 2H, OCNHC*H*$_2$),), 3.20 (t, ^3J = 6.57 Hz, 2H, C*H*$_2$SCO). 3.11 (t, ^3J = 6.44 Hz, 2H, C*H*$_2$SCO), 37 (s, 3H, *CH*$_3$CO), 1.98 (s, 3H,

CH$_3$CO), 1.92 (s, 3H, *CH*$_3$CO), 1.24 (d, ^{3}J = 6.82 Hz, 2H, C*H*$_3$ *i*Pr), 1.23 (d, ^{3}J = 7.32 Hz, 2H, C*H*$_3$ *i*Pr).

^{13}C-NMR (100 MHz, C_6D_6, 25°C:)
δ = 194.06, 193.53, 155.87, 62.98, 62.59 (d, $^2J_{P-C}$ = 16.1 Hz), 62.18 (d, $^2J_{P-C}$ = 16.8 Hz), 42.99 (d, $^2J_{P-C}$ = 12.4 Hz), 42.31 (d, $^3J_{P-C}$ = 6.6 Hz), 30.56 (d, $^3J_{P-C}$ = 6.6 Hz), 29.91, 29.81, 28.40, 24.47, 24.45, 24.40, 24.38.

^{31}P-NMR (162 MHz, C_6D_6, 25°C, H_3PO_4):
δ = 148.21

MS HR-FAB (NBA, positive mode):
$[M+H]^+$ calculated: 457.1596
$[M+H]^+$ found: 457.1582

Photostability:

Photostability of PENN was first tested in ethanol. No significant bleaching was found after continuous exposure of a 1 µM solution for 10 hours. We then tested PENN under assay conditions in Triton X-100 micelles. Again no bleaching was observed within 250 minutes (Figure S1).

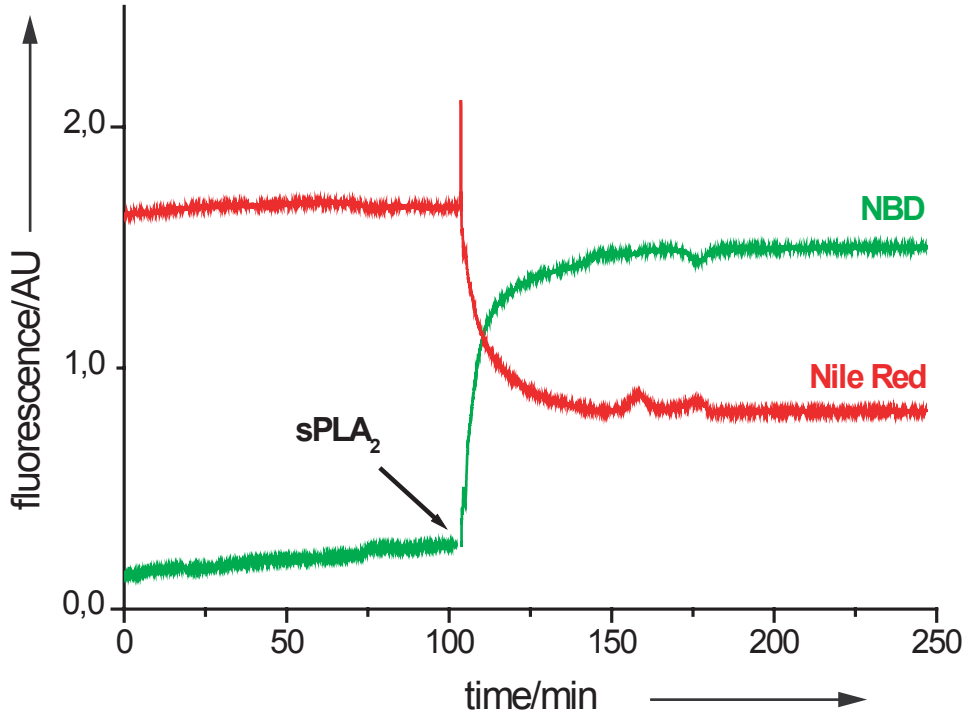
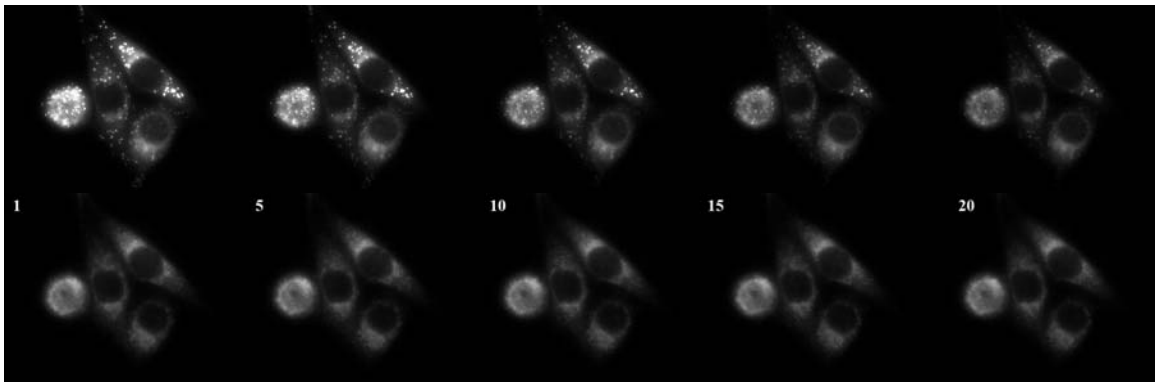


Figure S1: Photostability in vitro: Neither before nor after cleavage of PENN by bee venom sPLA$_2$ significant bleaching was observed. NBD emission (green curve) was measured at 540 nm, Nile Red emission at 640 nm. Excitation was at 458 nm.

We then investigated bleaching in living HeLa cells. Within 10 frames (Figure S2A) significant bleaching of NBD was observed, while Nile Red was fairly photostable.

A



B

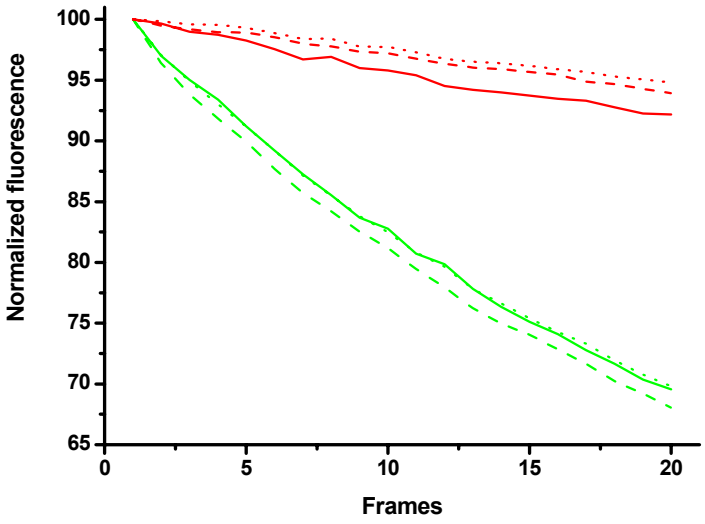


Figure S2: Photostability in vivo. HeLa cells were incubated with PENN/SATE for 40 minutes. After washing cells were observed on the microscope for 20 continuous frames (25 x, 400 ms at 20 watt). While NBD bleached quite fast (**A** upper panel, **B** green lines), Nile Red is fairly photostable (**A** lower panel, **B** red lines).
Straight lines in **B** represent measurement in the Golgi apparatus, dashed lines show measurements in the periphery of the nucleus without Golgi, and the dotted lines show whole cell fluorescence.

Biological experiments:

Medaka embryo injections

A stock solution of 100 mM PENN/SATE in DMSO/Pluronic (9:1) was prepared. In a 1 ml Eppendorf tube, 0.1 µl of the stock solution was diluted with 4.9 µl BSS (Balanced salt solution: 111 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, adjusted to pH 7.3 with 5% $NaHCO_3$). After sonication and vortexing the solution was centrifuged at 14000 rpm for 2 minutes to remove undissolved material. This procedure gave an approximately 2 mM injection solution.
Medaka eggs were collected, sorted, and placed into an agarose plate for incubation at 28°C. Embryos developed to the 64 or 128 cell stage within 60 minutes. At this stage, the PENN/SATE solution was injected inbetween the embryo and the yolk with a semi-automatic micromanipulator (Eppendorf microinjector 5242 combined with Injectman NI2). It was crucial to avoid injection into the yolk. The injection volume was about one third of the embryo volume (500-1000 pl). After injection the eggs were incubated at 28 °C again. Embryos were monitored every 9 hours with a fluorescence binocular holding a GFP/dsRed setup.

$sPLA_2$ in vitro studies

A stock solution of 10 mM PENN in DMSO was prepared. 0.5 µl of this stock was diluted with 5 ml of the assay buffer to get a final concentration of 1 µM. 1 ml of this solution was placed into a cuvette and fluorescence spectra were recorded at 37°C until the system had equilibrated. Subsequently, 0.5 units of honey bee venom phospholipase A_2 were added and spectra were recorded every two minutes.

Cell experiments

A stock solution of 2 mM PENN/SATE in DMSO/Pluronic (9:1) was prepared. This solution was diluted 1:1000 with HEPES buffer with or without 5% FCS to prepare the final incubation solutions.
A stock solution of 100 mM PENN/SATE in DMSO/PluronicTM (9:1) was prepared. This was also diluted with HEPES buffer to give final concentrations of 1, 5, 25, and 125 µM.
HeLa cells were plated in 4-chambered dishes and grown over night to a confluency of about 40%. The medium was exchanged with MAFP-containing solutions or HEPES buffer containing 0.2% DMSO as vehicle. Cells were incubated at 37°C for 20 minutes. After this time the buffer was removed, cells were washed twice with HEPES buffer and were then treated with the PENN/SATE/FCS solution for 40 min at 37°C. After this time the solution was removed again, cells were washed three times with HEPES buffer and finally monitored in the same buffer on a widefield microscope with a NBD and Texas red filter set up.