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Probing Phospholipase A₂ with Fluorescent Phospholipid Substrates

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Abstract

The Foerster resonance energy transfer-based sensor, PENN, measures intracellular phospholipase A₂ (PLA₂) activity in living cells and small organisms. In an attempt to modify the probe for the detection of particular isoforms, we altered the *sn*-2 fatty acid in such a way that either one or three of the Z double bonds in arachidonic acid were present in the sensor molecule. Arachidonic-acid-mimicking fatty acids were prepared by copper-mediated coupling reactions. Probes with a single double bond in the 5-position exhibited favorable substrate properties for secretory PLA₂s. In vitro experiments with the novel unsaturated doubly labeled phosphatidylethanolamine derivatives showed preferred cleavage of the sensor PENN2 (one double bond) by the physiologically important group V sPLA₂, while the *O*-methyl-derivative PMNN2 was accepted best by the isoform from hog pancreas. For experiments in living cells, we demonstrated that bio-activation via *S*-acetylthioethyl (SATE) groups is essential for probe performance. Surprisingly, membrane-permeant versions of the new sensors that contained double bonds, PENN2 and PENN3, were only cleaved to a minor extent in HeLa cells while the saturated form, PENN, was well accepted.

Keywords

alkynes; bioorganic chemistry; fluorescent probes; FRET; phospholipase; phospholipids

Introduction

PLA₂ isoenzymes are broadly classified in three groups, secretory (sPLA₂), cytosolic (cPLA₂) and Ca₂⁺-independent isoforms (iPLA₂). [1] All isoforms cleave the *sn*-2 acyl chain of phospholipids to generate a fatty acid and a lysophospholipid. Both molecules can have signaling functions. [2] In particular, the release of arachidonic acid by cPLA₂ leads to early inflammatory responses. Therefore PLA₂s are important mediators in cell function and their activity has implications in various diseases. [3,4] The physiological roles of many PLA₂ isoforms, in particular the secreted ones, are not clearly understood to date. There is evidence that, after re-internalization, some sPLA₂ isoforms, most notably group V PLA₂, participate in the release of arachidonic in a cPLA₂-dependent as well as an independent way. [5–8] To further elucidate the distinct roles of the different isoforms, it would be helpful to have access to probes that are selectively hydrolyzed by only one particular isoform.

Intracellular enzyme activities, including those of PLA₂s, can be measured by fluorescent probes based on FRET between a donor fluorophore and a FRET acceptor. [9] The latter could be a quenching dye or a fluorophore. In the second case, sensitized emission after FRET can

be used for ratiometric measurements. Because they are bound in the same molecule, the two corresponding fluorophores are present in a defined ratio, typically 1:1. This ensures that ratio measurements of the two emission intensities can be performed nearly independently of the probe's concentration.

The vast majority of FRET probes that are available today are based on fluorescent proteins from jellyfish or corals and are genetically encoded.[9–11] Unfortunately, most of these probes exhibit FRET changes in the range of only 10–100 %, although there are exceptions.[12] This is large enough to be very useful for studying cell biology in living cells, but is limiting when the probes need to be developed into assays that are suitable for high-throughput screening (HTS). The latter is shifting to a significant degree towards cell-based assays making FRET probes with large ratio changes very desirable. Larger FRET changes should be easier to achieve by probes that are based on small molecules, simply because the fluorophores are usually in closer proximity; this permits a higher initial FRET efficiency and therefore results in a large dynamic range. Furthermore, the outcome of the manipulation is more predictable. In addition, small fluorophores usually have the advantage of being able to rotate freely; this results in FRET changes that depend solely on distance alterations. In pioneering work, Zlokarnik et al. used this principle together with doubly labeled cephalosporin derivatives (CFF-2) that functioned as a sensor for β -lactamase activity.[13] This system found wide application in reporter gene assays for the screening industry. The change in the emission ratio was about 70-fold, which is very suitable for applications that involve cell-sorter experiments.

Generally, all hydrolytic enzymes are attractive targets for small-molecule sensors because, after enzymatic hydrolysis, the fragments separate infinitely, thereby eliminating energy transfer. Up to now, most work has been done on substrates for proteinases and lipases. For phospholipases, including those for phospholipase A₂ activity, fluorogenic probes were introduced some time ago.[14–18] However, these probes were used for in vitro experiments, a few cell experiments,[18,19] and were injected into fish-embryos[20] or administered to the gastrointestinal tract of animals, which is readily accessible.[21] Another attempt to deliver a FRET sensors into cells employed liposomes.[17] Very recently, we developed the small-molecule FRET probe PENN (**1**) that monitored phospholipase A₂ activity in living cells when used as a membrane-permeant derivative.[22] The probe was based on a phosphatidylethanolamine derivative with the FRET donor (NBD) and the FRET acceptor (Nile Red) attached to the ω -CH₂ groups of the lipid chains (Scheme 1). Upon hydrolytic cleavage by bee venom sPLA₂ the probe exhibited a ~20-fold emission-ratio change. We also introduced a membrane-permeant bioactivatable derivative of the probe (**2**) in which charged moieties were masked with *S*-acylthioethyl (SATE)-groups.[23,24] This reporter performed well in cells and small organisms. Cell entry was very rapid and led to a localization of the probe in microsomal membranes. Although the probe was fully hydrolyzed in HeLa cells in about 30 min, PENN was not a substrate for the physiologically important cytosolic PLA₂ (cPLA₂ α) in vitro. Because cPLA₂ α prefers substrates with a *sn*-2 arachidonyl chain,[25,26] we attributed the poor activity of PENN to a lack of double bonds in the *sn*-2 acyl chain. We therefore intended to alter the *sn*-2-fatty acid into a better mimic of arachidonic acid, while maintaining the fluorophore composition. Our target molecules were derivatives of PENN that bore the first (PENN2, **3**), or the first three double bonds (PENN3, **7**) of arachidonic acid as well as their respective membrane-permeant bis(SATE) esters **4** and **8** (Scheme 1). For synthetic reasons, the fluorophores in PENN3 were attached in a way that NBD was coupled to the arachidonic acid mimic, while in PENN and PENN2 the cleavable fatty acid carried Nile Red.

Results

Synthesis

One of the main difficulties in synthesizing arachidonic acid derivatives is the all-*Z* configuration of the double bonds. Because separations of *Z/E* isomer mixtures are often very tedious or even impossible, reactions are needed that produce none of the undesired isomer. For instance, Wittig reactions were not suitable in our hands, although the major products showed the desired *Z* configuration. For the synthesis of PENN3 (**7**), we therefore employed copper-mediated coupling reactions between terminal alkynes and propargyl halides,[27] as was previously described for the synthesis of anandamide (arachidonyl-ethanolamide) analogues.[28] Reduction with Lindlar catalyst was ultimately successful to generate exclusively *Z* trienes (Schemes 2 and 3).

For preparing a labeled fatty acid with one *Z* double bond, we decided to attach Nile Red as a fluorophore, as in PENN (**1**), because we expected a NBD-tagged heptenoic acid to be too hydrophilic to stay in membranes. Furthermore, our anticipated synthetic pathway (Scheme 2) would involve a catalytic hydrogenation in the presence of the fluorophore, which is better tolerated by Nile Red than NBD. 4-Bromobutyric acid (**9**) was coupled with tetrahydropyran (THP)-protected propargyl alcohol in the presence of butyllithium in a standard substitution reaction. The resulting THP-protected hept-5-ynoic acid **10** was quantitatively deprotected by acidic catalysis in methanol. Simultaneously, the carboxyl function was esterified to the methyl ester to give hydroxyheptenoic acid methyl ester **11**.

The hydroxyl group was substituted by bromide in an Appel reaction to yield **12**, and subsequently Nile Red was coupled to form fatty acid ester **13**. Hydrogenation over Lindlar catalyst in the presence of quinoline afforded heptenoic acid ester **14**. It is known from previous unpublished work and other groups that hydrolysis of esters under basic conditions can damage Nile Red.[29] Thus, we successfully removed the ester by treatment with pig liver esterase in phosphate-buffered saline (PBS) buffer at pH 7.4 without harming the dye.[30] Fatty acid **15** was then coupled to the already described *sn*-2 alcohol **16** to generate the protected glycerol derivative **17**. Removal of the DMT group with DOWEX WX8 resin as a mild solid-phase-bound acid gave alcohol **18**, which was phosphorylated with reagents **PI**, **PII**, or **PIII** (Scheme 4) in the presence of 4,5-dicyanoimidazol (DCI) as a mediator, respectively,[31] followed by in situ oxidation with *tert*-butylhydroperoxide and, if necessary, deprotection to yield the charged probe PENN2 (**3**), or PENN2/SATE (**4**) and PMNN2/SATE (**6**). All probes were purified by HPLC or by preparative thin layer chromatography. For in vitro experiments, a small sample of PMNN2/SATE (**6**) was deprotected by treatment with lipase from *Candida cylindracea* to yield PMNN2 (**5**).

For the preparation of the labeled and triple-unsaturated fatty acid **27** (Scheme 3), the use of NBD as the fluorophore was required (see below). The reaction sequence started from 5-hexynoic acid **19** which was protected by esterification with trimethylsilylethanol in the presence of diisopropylcarbodiimide (DIC). The resulting ester **20** was treated with an excess of 1,4-dichlorobut-2-yne in the presence of two equivalents of CuI to furnish diyne **21**. The latter was subsequently coupled to but-3-yn-1-ol under identical conditions. Both reactions gave only modest yields, as was described previously.[20] Reduction of triyne **22** with Lindlar catalyst gave exclusively triene **23** in good yield. Substitution of the alcohol group to the azide **24** under Mitsunobu conditions and subsequent reduction afforded amine **25**,[32,33] which was successfully labeled with NBD in 72 % yield. Deprotection of fluorescent compound **26** by TFA gave tetradecyltrieneic acid derivative **27**. All efforts to prepare Nile-Red-labeled trieneic acids starting from **23** resulted in decomposition of the triene.

The phosphatidylethanolamine backbone was prepared from the known *sn*-3-bromododecylether **28** (Scheme 4).[14] Direct alkylation of Nile Red gave the 2'-*O*-Nile-Red-labeled compound **29**, which was protected at the primary alcohol group by forming the dimethoxytrityl (DMT) ether **30**. The latter was successfully coupled to **27** with 1-(2,4,6-triisopropyl-benzenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (TPSNT) and methylimidazole (MeIm) in 79 % yield.

The doubly labeled glycerol derivative **31** was deprotected and the resulting alcohol **32** was phosphitylated either with reagent **PI** or the SATE-protected reagent **PII**, this was followed by mild in situ oxidation. In case of PENN3 (**7**) subsequent removal of the protecting groups by 10 % TFA in dichloromethane gave the deprotected phosphoethanolamine derivative. The SATE protected derivative **8** and **7** were purified as previously described for PENN2 and PENN2/SATE, respectively.

Biochemistry

To compare the performance of the new sensors with PENN, we examined their sensitivity to various sPLA₂s in vitro. We chose a set of commercially available enzymes from different subfamilies; these ranged from PLA₂s derived from animal poisons, pancreatic juices, and bacterial origin to human group V sPLA₂. From a pharmacological point of view, the latter is the most interesting and important because it is known to be involved in inflammatory processes by releasing arachidonic acid extra- as well as intracellularly.[8] Group V sPLA₂-induced arachidonic acid release is known to occur in concert[5,7] as well as independently [6] from cytosolic PLA₂ (group IV), and is therefore considered to be another important key player in inflammation; this finding is further supported by studies with gene knock-out mice. [34]

Micelles of Triton X-100 (3 mM in Tris-HCl, pH 8.6) were prepared by sonication and were spiked with the sensor of interest. The dye concentration was kept at 1 μM, which corresponds to one molecule per ~20 micelles, to avoid intermolecular energy transfer. Addition of the sPLA₂ started the reaction, which was monitored by an increase in NBD- and a decrease in sensitized Nile Red fluorescence (Figures 1A–D). All four sensors exhibited similar behavior with respect to the increase in NBD fluorescence, but they differed with respect to kinetic parameters. PENN2 was the most widely accepted sensor for sPLA₂s and exhibited, in most cases, the fastest kinetics. As is shown for the example of human group V PLA₂ in Figures 2 and 3, PENN2 (**3**) is by far the favored substrate of this enzyme. Hog pancreatic PLA₂ predominantly accepts PMNN2 (**5**). These selectivities might be useful to monitor isoenzyme-specific catalysis in the future. As expected, bee venom PLA₂ was the most potent enzyme; cleaving all substrates without preference. The next most potent enzyme was *Naja mossambica mossambica* venom PLA₂.

For applications in living cells, we tested if the hydrolysis of bioactivatable protecting groups is required to produce PLA₂ substrates. PENN/SATE (**2**) was added to Triton X-100 micelles. When incubated with bee venom PLA₂, no significant hydrolysis was monitored until an unspecific lipase from *C. cylindracea* was added. The latter hydrolyzed the SATE group off of the phosphate; this made the probe a substrate for the PLA₂, which was indicated by the onset of ratio change (Figure 4). The other sensors exhibited similar properties. The lipase itself had no effect on the ester at the *sn*-2 position, but subsequent addition of PLA₂ gave rapid cleavage. This suggested that the deprotection of bioactivatable protecting groups might be the rate-limiting step when endogenous PLA₂ levels are monitored in living cells.

It was previously shown that HeLa cells that have been stimulated with fetal calf serum have an endogenous PLA₂ activity that can be measured with PENN/SATE (**2**).[22] For example, **2** was cleaved within 30 min when administered to the cells in the presence of serum. In order

to compare our novel probes to PENN/SATE, HeLa cells were incubated for 30 min in the presence or absence of 5 % fetal calf serum (FCS) and the amount of hydrolysis was shown by the overlay of the NBD and the Nile Red fluorescence. Stimulated cells that were incubated with PENN/SATE showed a stronger green signal than those that were treated with PENN2/SATE or PENN3/SATE. The latter gave only a very weak signal (Figure 5).

This result supports previous findings that led to the hypothesis that platelet-activating factor acetyl hydrolase 2 (PAF-AH2) is responsible for probe turnover in HeLa cells.[22] This enzyme predominantly cleaves shorter fatty acids (preferably acetate) as well as oxidatively damaged chains from the *sn*-2 position.[35,36] Here PENN has advantages over the other probes, which could explain the lack of hydrolysis of PENN3 and the reduced cleavage of PENN2. As expected from the in vitro experiments, stimulation with reagents that elevate intra-cellular calcium levels like ionomycin and bromo-A23187 were not able to activate probe hydrolysis. This confirms the lack of substrate properties of all probes for calcium-activated cytosolic phospholipases like cPLA₂α.

Discussion

Copper-mediated coupling reactions proved to be very useful for preparing fluorescently labeled arachidonic acid mimics. Incorporation of these fatty acids readily gave doubly labeled phosphatidylethanolamine derivatives that exhibited sensitized emission in a comparable range. Charged sensors (**1**, **3**, **5**, **7**) gave similar endpoint spectra after complete hydrolysis. Only PENN3 (**7**) with NBD attached to the fatty acid showed a twofold lower NBD emission, potentially due to the higher solubility in water and therefore reduced quantum yield.[37] All SATE esters (**2**, **4**, **6**, and **8**) readily entered cells. Furthermore, we demonstrated the release of PLA₂ substrates from the SATE precursors by the action of unspecific lipases.

The new PLA₂ substrates presented in this paper might serve as valuable sensors for pharmacologically important members of the PLA₂ family in vitro. In particular the group V PLA₂ is interesting in this respect, because it is of physiological relevance, it shows a strong preference for only one of the reporters (PENN2), and it is known to act inside living cells. [8] It seems to be feasible to expose a set of FRET sensors to a given biological sample to determine the isoform activity by comparing substrate cleavage patterns. Inside living cells, it remains to be shown whether group V PLA₂ activity can be monitored, for instance after overexpressing the enzyme. Unfortunately, none of the probes showed improved properties towards the pharmacologically interesting cPLA₂α in vitro. Most likely, the fluorophores are interfering with the catalytically productive binding to the active site of cPLA₂α. Potentially, the tolerance of the enzyme might be stretched by adding bulky or partially polar fluorophores to the fatty acids. Future experiments might require having all four double bonds of arachidonic acid available. Furthermore, the replacement of the PENN head group by phosphatidylglycerol might improve the acceptance of the sensors by this enzyme.[38] It cannot be excluded that the ether linkage in the *sn*-1 position is preventing enzymatic hydrolysis by the enzyme variant that we were probing, although the acceptance of *sn*-1 ethers was previously described.[12]

In living cells, the shorter probe, PENN, is still the best-accepted substrate. This suggests that an alignment of in vitro and in vivo results so far gives misleading answers. Apparently, it is necessary to generate more specific fluorogenic substrates and to identify so-far-uncharacterized phospholipase activities in target cells.

Experimental Section

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. Purified enzymes were purchased from Sigma (PLA₂ from *Streptomyces violaceoruba* and *Naja mossambica mossambica*), Fluka (Lipase from *C. cylindracea* and PLA₂ from Hog pancreas as well as honey bee venom or Cayman Chemicals (human Group V sPLA₂; Ann Arbor, MI). Flash chromatography was carried out by using Merck silica gel 60 (63–200 mesh). Analytical TLC was performed on Merck silica 60 WF_{254s} analytical plates with ethyl acetate/cyclohexane or methanol/methylene chloride mixtures as eluents. Spots were detected by a UV hand lamp at 254 nm or 366 nm or by staining with either phosphomolybdic acid/cerium(IV) sulfate reagent, ninhydrin solution, or anisaldehyde/sulfuric acid-reagent. Preparative TLC was carried out on 20 cm × 20 cm × 2 mm Merck silica glass plates using the same eluent mixtures.

NMR spectra were recorded using a Bruker UltraShield™ Advance 400 spectrometer (400 MHz, ¹H; 100 MHz, ¹³C; 162 MHz, ³¹P) and calibrated by using residual non-deuterated solvent as an internal reference.

High-resolution mass spectra were recorded at University of Heidelberg by using either fast atom bombardment (FAB) or Electron Impact (EI) mass spectrometry on a Jeol JMS 700 mass spectrometer. Electrospray mass spectra were recorded on an ESI-Q-TOF Ultima API (Micromass/Waters) mass spectrometer.

Fluorescence spectra were recorded with a Quantamaster QM4/2000SE (Photon Technology International, Birmingham, NJ) fluorimeter by using 1 mL disposable plastics cuvettes. Assays were performed in a Tris–HCl-buffered Triton X-100 mixed micelle system at pH 8.6 and 37 °C (100 mM KCl, 10 mM CaCl₂, 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.

Kinetics were measured in 384-well clear-bottom plates (Costar) in an EnVision (Perkin–Elmer) plate reader at room temperature. For the assay, the same Triton-based buffer mentioned above was used. Excitation was at 458 nm (30 nm bandwidth), NBD emission was measured at 535/35 nm, Nile red emission with a 630/40 nm filter.

Fluorescence microscopy was carried out on a Zeiss Axiovert 200M. Filter setup for NBD fluorescence was 460/50× excitation, 500DCLP dichroic mirror, D535/40 m emission; for Nile red fluorescence settings were 560/40× excitation, 595DCLP dichroic mirror, D630/60 m emission. Assays were performed in CO₂-independent HEPES buffer that had been adjusted to pH 7.4 (115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 2.4 mM K₂HPO₄, 20 mM HEPES).

Products were characterized by NMR spectroscopy (¹H, ¹³C and if appropriate ³¹P) and high resolution MS except PENN3, which did not yield sufficient material for reliable ¹³C NMR data.

7-(Tetrahydro-pyran-2-yloxy)hept-5-ynoic acid (10)

In a 100 mL Schlenk flask tetrahydropyran (THP)-protected propargylic alcohol (1.4 g, 10 mmol) was dissolved in dry THF (30 mL) under an atmosphere of dry argon. The mixture was cooled to –80 °C in an ethanol/liquid nitrogen bath and was treated with 2.2 equiv of *n*-butyllithium. After stirring for 20 min the solution was allowed to warm up to –20 °C and one equivalent of 4-bromobutyric acid (**9**) in DMSO (10 mL) was added via syringe. The mixture

was stirred for another 6 h at this temperature until TLC (cyclohexane/ethyl acetate 3:2) showed no further progress of the reaction. Subsequently the reaction was quenched by the addition of methanol (5 mL), and was stirred for 15 min longer. The mixture was poured into phosphate buffer (pH 7), and was extracted three times with ethyl acetate. The combined organic layers were washed with brine, dried over Na₂SO₄ and evaporated to dryness. The resulting brownish oil was subjected to silica column chromatography (cyclohexane/ethyl acetate 7:3). Product-containing fractions were pooled and evaporated under reduced pressure to yield the THP-protected heptynoic acid as a clear oil (859 mg, 3.8 mmol, 38%). *R*_f = 0.24 (cyclohexane/ethyl acetate 3:2); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 4.74 (t, ³*J* = 3.4 Hz, 1 H; OCHO), 4.23 (t, AB, ³*J* = 2.1 Hz, 2 H; THPOCH₂), 3.77 (ddd, ²*J* = 11.4 Hz, ³*J* = 8.8, 3.0 Hz, OCHH THP), 3.51–3.43 (m, 1H; OCHH THP), 2.41 (t, ³*J* = 7.4 Hz, OCOCH₂), 2.25 (tt, ³*J* = 6.9 Hz, ⁴*J* = 2.1 Hz, 2 H; CCCH₂), 1.77 (qi + m, ³*J* = 7.1 Hz, 3H; CCCH₂CH₂ + CHH THP), 1.71–1.62 (m, 1 H; CHH THP), 1.60–1.41 (m, 4H; 2 CH₂ THP); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 178.88, 96.67, 85.08, 76.87, 61.93, 54.54, 32.72, 30.22, 25.32, 23.43, 19.01 18.17; MS HR-ESI (positive mode): [*M*+H]⁺ calcd: 249.1103, found: 249.1132.

7-Hydroxy-hept-5-ynoic acid methyl ester (11)

The acid **10** (791 mg, 3.5 mmol) was dissolved in methanol (40 mL). Concentrated HCl (3 drops) were added, and the resulting reaction mixture was heated to reflux. To monitor the reaction, samples (100 μL) were taken, evaporated and the ¹H NMR spectrum of the residue was measured. After three hours, the THP signals disappeared completely, and the reaction was stopped by the addition of phosphate buffer (200 mL, pH 7). The mixture was transferred into a separation funnel and was extracted with ethyl acetate (3×50 mL). The combined organic layers were washed with brine and dried over Na₂SO₄. After evaporation under reduced pressure, the deprotected alcohol was isolated quantitatively (544 mg, 3.5 mmol) as a colorless oil. *R*_f = 0.23 (cyclohexane/ethyl acetate 3:2); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 4.19–4.16 (m, 2H; HOCH₂), 3.61 (s, OCH₃), 2.83 (s, 1 H; OH), 2.38 (t, ³*J* = 7.4 Hz, OCOCH₂), 2.22 (tt, ³*J* = 6.8 Hz, ⁴*J* = 2.0 Hz, 2 H; CCCH₂), 1.77 (qi, ³*J* = 6.9 Hz, 2 H; CCCH₂CH₂); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 173.78, 84.54, 79.44, 51.57, 32.74, 23.65, 18.10; EI HRMS: [*M*]⁺ calcd: 156.0787, found: 156.0768.

7-Bromo-hept-5-ynoic acid methyl ester (12)

The hydroxyl-heptynoic acid **11** (484 mg, 3.1 mmol) was dissolved in dichloromethane (30 mL). Triphenylphosphine (839 mg, 3.2 mmol) and tetrabromo-methane (1.06 g, 3.2 mmol) were added and the mixture was stirred for 20 min. After this time, TLC (cyclohexane/ethyl acetate 3:2) indicated complete conversion of the starting material to a further-migrating product. The solvent was removed under reduced pressure and the resulting oil was subjected to silica column chromatography (cyclohexane/ethyl acetate 8:2). The fractions that contained product were pooled and evaporated to dryness to yield the bromide (638 mg, 94%) as a colorless oil. *R*_f = 0.77 (cyclohexane/ethyl acetate 3:2); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 3.90 (m, 2H; BrCH₂), 3.67 (s, OCH₃), 2.43 (t, ³*J* = 7.3 Hz, OCOCH₂), 2.32 (tt, ³*J* = 6.9 Hz, ⁴*J* = 2.4 Hz, 2H; CCCH₂), 1.82 (qi, ³*J* = 7.2 Hz, 2 H; CCCH₂CH₂); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 173.36, 86.69, 76.20, 51.57, 32.68, 23.50, 18.10, 15.32; MS HR-EI: [*M*-HBr]⁺ calcd: 139.0759, found: 139.0759.

7-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-3-yl oxy)hept-5-ynoic acid methyl ester (13)

3-Hydroxy-Nile red (668 mg, 2 mmol) was dissolved in DMF (40 mL). Bromoheptynoic acid **12** (329 mg, 1.5 mmol) and excess K₂CO₃ (552 mg, 4 mmol) were added. The mixture was heated to 70 °C and stirred at this temperature for 4 h. After this time, TLC (cyclohexane/ethyl acetate 3:2) indicated no further conversion of the starting materials. The mixture was poured into phosphate buffer (pH 7) and was extracted with ethyl acetate until the organic layer

remained colorless. The combined organic layers were washed with phosphate buffer (2×) and brine (30), dried over Na₂SO₄ and evaporated to dryness under reduced pressure. The remaining deep-red oil was purified by silica column chromatography (dichloromethane/methanol, 100:0→98:2) Product-containing fractions were pooled and evaporated under reduced pressure to yield the protected acid as deep-red oil (530 mg, 75%). *R*_f = 0.27 (cyclohexane/ethyl acetate 3:2); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 8.60 (d, *J* = 8.6 Hz, 1 H; Nile red H1), 7.82 (d, ⁴*J* = 2.52 Hz, 1 H; Nile red H4), 7.61 (d, ³*J* = 9.1 Hz, 1H; Nile red H11), 7.34 (dd, ³*J* = 8.7 Hz, ⁴*J* = 2.7 Hz, Nile red H2), 6.70 (dd, ³*J* = 9.1 Hz, ⁴*J* = 2.5 Hz, Nile red H10), 6.51 (d, ⁴*J* = 2.5 Hz, 1H; Nile red H8), 6.41 (s, 1 H; Nile red H6), 4.84 (s, ArOCH₂), 3.67 (s, 3H; OCH₃), 3.49 (q, ³*J* = 7.1 Hz, 4 H; ArNCH₂), 2.43 (t, ³*J* = 7.5 Hz, OOCCH₂), 2.34 (tt, ³*J* = 6.9 Hz, ⁴*J* = 1.2 Hz, 2H; CCCH₂), 1.85 (qi, ³*J* = 7.2 Hz, 2H; CCCH₂CH₂), 1.28 (t, ³*J* = 7.2 Hz, 6 H; ArNCH₂CH₃); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 183.29, 173.52, 162.62, 159.56, 151.89, 150.39, 146.55, 139.75, 133.30, 130.80, 125.80, 125.03, 120.71, 109.78, 108.46, 105.57, 96.31, 87.45, 75.29, 56.72, 51.54, 45.05, 32.67, 23.53, 18.24, 12.59; FAB HRMS (NBA, positive mode): [*M*+H]⁺ calcd: 473.2076, found: 473.2100.

7-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-3-yl oxy)hept-5-enoic acid methyl ester (14)

Under an atmosphere of dry argon alkyne **13** (378 mg, 0.80 mmol) was dissolved in dry THF (10 mL). Lindlar catalyst (10 mol %) and chinolin (10 μL) were added and the argon was exchanged by hydrogen. The deep-red solution was stirred until the color changed to a greenish yellow (approximately 30 min), which indicated that the reduction of the fluorophore was complete. At that time, the hydrogen was removed. Contact with air reoxidized the Nile red immediately, and the red color returned. The catalyst was removed by filtration via Celite, and the solvent was removed under reduced pressure. The resulting crude alkene was then further purified by silica column chromatography (dichloromethane/methanol, 100:0→98:2) to yield the pure product as a red amorphous solid (367 mg, 96%). *R*_f = 0.31 (cyclohexane/ethyl acetate 3:2); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 8.53 (d, *J* = 8.8 Hz, 1H; Nile red H1), 7.72 (d, ⁴*J* = 2.5 Hz, 1 H; Nile red H4), 7.54 (d, ³*J* = 9.1 Hz, 1 H; Nile red H11), 7.26 (dd, ³*J* = 8.8 Hz, ⁴*J* = 2.8 Hz, Nile red H2), 6.62 (dd, ³*J* = 9.1 Hz, ⁴*J* = 2.5 Hz, Nile red H10), 6.42 (d, ⁴*J* = 2.5 Hz, 1 H; Nile red H8), 6.36 (s, 1 H; Nile red H6), 5.83–5.73 (m, 1H; CH olefin), 5.73–5.62 (m, 1 H; CH olefin), 4.72 (t, ³*J* = 6.0 Hz, ArOCH₂), 3.67 (s, 3H; OCH₃), 3.43 (q, ³*J* = 8.8 Hz, 4H; ArNCH₂), 2.37 (t, ³*J* = 7.5 Hz, OOCCH₂), 2.24 (q, ³*J* = 7.4 Hz, 2H; C = CCH₂), 1.78 (qi, ³*J* = 7.4 Hz, 2 H; C = CCH₂CH₂), 1.24 (t, ³*J* = 7.2 Hz, 6H; ArNCH₂CH₃); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 183.35, 173.84, 160.52, 151.79, 150.29, 146.45, 139.74, 133.52, 133.34, 130.72, 125.78, 125.43, 125.27, 125.00, 120.79, 109.71, 107.74, 105.52, 96.24, 64.27, 51.53, 45.01, 33.31, 27.16, 24.55, 12.61; MS HR-ESI (positive mode): [*M*+H]⁺ calcd: 475.2233, found: 475.2211.

7-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-3-yl oxy)hept-5-enoic acid (15)

The methyl ester **14** (204 mg, 431 μmol) was dissolved in a minimum amount of THF (approximately 1 mL) as a co-solvent. This solution was diluted with cyclohexane (300 mL). With heavy stirring, a dispersion of pig liver esterase (PLE; 250 mg) in phosphate buffer pH 8 (200 mL) was added, and the resulting mixture was stirred for five days. After this time TLC (cyclohexane/ethyl acetate 3:2) showed about 50% conversion of the ester to a slower-migrating spot. The whole mixture was transferred to a separation funnel and the cyclohexane phase was collected. The remaining aqueous layer was extracted four times with ethyl acetate/acetic acid (95:5; 150 mL) until the organic layer remained colorless. The combined organics were washed with brine, dried over Na₂SO₄, filtered and evaporated to dryness. The resulting crude mixture of product and starting material was separated by silica column chromatography. The remaining starting material eluted smoothly with cyclohexane/ethyl acetate (3:2), while the product remained at the top of the column. It was eluted with cyclohexane/ethyl acetate/acetic acid (60:40:1). The product-containing fractions were pooled, and the solvent was

removed in vacuo to yield the pure product as a red amorphous solid (101 mg, 51%). The starting-material-containing fractions were also evaporated and the re-isolated pure ester underwent the procedure for a second and a third time. The combined yield of all three cycles was 88.2 % (175 mg, 380.1 mmol). $R_f = 0.10$ (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25°C , TMS): $\delta = 8.50$ (d, $J = 8.8$ Hz, 1 H; Nile red H1), 7.64 (d, $^4J = 2.8$ Hz, 1 H; Nile red H4), 7.55 (d, $^3J = 9.1$ Hz, 1 H; Nile red H11), 7.24 (dd, $^3J = 8.8$, $^4J = 2.8$ Hz, Nile red H2), 6.65 (dd, $^3J = 9.1$ Hz, $^4J = 2.5$, Nile red H10), 6.44 (d, $^4J = 2.5$ Hz, 1H; Nile red H8), 6.34 (s, 1H; Nile red H6), 5.77–5.68 (m, 1 H; CH olefin), 5.68–5.58 (m, 1H; CH olefin), 4.68 (t, $^3J = 6.0$ Hz, ArOCH_2), 3.41 (q, $^3J = 7.1$ Hz, 4H; ArNCH_2), 2.31 (t, $^3J = 7.3$ Hz, OOCCH_2), 2.26–2.15 (m, 2H; $\text{C} = \text{CCH}_2$), 1.71 (qi, $^3J = 7.3$ Hz, 2 H; $\text{C} = \text{CCH}_2\text{CH}_2$), 1.20 (t, $^3J = 6.7$ Hz, 6H; $\text{ArNCH}_2\text{CH}_3$); ^{13}C NMR (100 MHz, CDCl_3 , 25°C): $\delta = 183.43$, 175.44, 160.02, 151.66, 150.12, 146.06, 138.58, 133.06, 132.05, 130.30, 125.24, 124.87, 124.76, 124.70, 120.52, 109.76, 107.18, 104.53, 95.64, 63.78, 44.55, 32.68, 26.51, 23.92, 11.95; MS HR-ESI (positive mode): $[M+H]^+$ calcd: 461.2076, found: 461.2047.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-3-O-(4,4'-di-methoxytrityl)-sn-glycerol (17)

Three flame-dried, argon flushed, rubber septum sealed 50 mL-flasks were prepared, containing: a) methylimidazole 3 equiv (48 μL , 600 μmol); b) TPSNT 1.5 equiv (114 mg, 300 μmol); c) NBD-labeled glycerol derivative **16** (223 mg, 200 μmol) and a stirring bar. 1.5 equiv of the Nile red labeled acid **15** (139 mg, 300 μmol) was dissolved in dry dichloromethane (10 mL). 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 get the activated acid. This solution was then immediately transferred to flask c, and was stirred for 11–14 h at room temperature. The solvent was removed in vacuo, and the resulting red slurry was subjected to silica column chromatography. The product-containing fractions were pooled and evaporated to yield (278 mg, 78%) of the pure coupled product as dark-red oil. $R_f = 0.33$ (cyclohexane/ethyl acetate 95:5); ^1H NMR (400 MHz, CDCl_3 , 25°C , TMS): $\delta = 8.56$ (d, $^3J = 8.8$ Hz, 1H; NBD-*H*) 8.45 (d, $^3J = 8.6$ Hz, 1 H; Nile red H1), 7.74 (d, $^4J = 2.78$ Hz, 1 H; Nile red H4), 7.58 (d, $^3J = 9.1$ Hz, 1H; Nile red H11), 7.43 (d, $^3J = 7.3$ Hz, 2 H; DMT), 7.32 (d, $^3J = 8.8$ Hz, 4 H; DMT), 7.23 (m, 3 H; DMT (2 H), Nile red H2), 7.15 (m, 1 H; DMT), 6.81 (d, $^3J = 8.8$ Hz, 4 H; DMT), 6.69 (dd, $^3J = 9.0$, $^4J = 2.4$, Nile red H10), 6.52 (s, 1H; *NHNBD*), 6.49 (d, $^4J = 1.8$ Hz, 1 H; Nile red H8), 6.40 (s, 1H; Nile red H6), 6.11 (d, $^3J = 8.8$ Hz, 1H; NBD-*H*), 5.83–5.74 (m, 1 H; CH olefin), 5.74–5.66 (m, 1 H; CH olefin), 5.25 (qi, $^3J = 5.4$ Hz, 1H; *sn-2 CH*), 4.73 (t, $^3J = 6.1$ Hz, ArOCH_2), 3.79 (s, 6 H; CH_3 DMT), 3.68–3.57 (m, 2 H; *sn-1 CH}_2*), 3.48 (q, $^3J = 7.0$ Hz, 4H; ArNCH_2), 3.46–3.34 (m, 4 H; *sn-1 OCH}_2*, NBD-NH- CH_2), 3.28–3.19 (m, 2H; *sn-3 CH}_2*), 2.43 (dd, $^3J = 7.9$, 6.7 Hz, 2H; OOCCH_2), 2.26 (q, $^3J = 7.3$ Hz, 2 H; $\text{C} = \text{CCH}_2$), 1.81 (qi, $^3J = 7.6$ Hz, 2 H; NBDNH- CH_2CH_2), 1.76 (qi, $^3J = 7.4$ Hz, 2 H; $\text{C} = \text{CCH}_2\text{CH}_2$), 1.59–1.20 (m, 26 H; $\text{ArNCH}_2\text{CH}_3$, 10CH_2); ^{13}C NMR (100 MHz, CDCl_3 , 25°C) [too many signals due to DMT cleavage during the overnight measurement]: $\delta = 183.23$, 173.52, 173.14, 161.92, 158.64, 158.44 152.04, 150.73, 147.32, 146.84, 144.79, 143.85, 139.46, 136.35, 135.96, 134.05, 132.33, 132.30, 131.06, 130.01, 129.12, 129.03, 128.91, 128.84, 128.53, 128.43, 128.12, 127.76, 127.71, 127.22, 127.17, 126.73, 125.46, 124.74, 124.68, 118.28, 113.16, 113.05, 109.57, 106.63, 105.24, 96.35, 85.87, 72.98, 71.91, 70.04, 69.54, 68.41, 62.99, 62.42, 55.24, 55.19, 45.10, 33.93, 33.68, 29.58, 29.51, 29.47, 29.41, 29.34, 29.23, 26.63, 26.49, 26.34, 26.07, 26.03, 25.72, 25.68, 24.84, 24.70, 12.60; FAB HRMS (NBA, positive mode): $[M+H]^+$ calcd: 1183.5756, found: 1183.5679; $[M]^+$ calcd: 1182.5678, found: 1182.5618.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-sn-glycerol (18)

The DMT-protected glycerol **17** (81 mg, 86 μ mol) was dissolved in dichloromethane (10 mL). Dried DOWEX-50 WX-8 (350 mg) resin was added and the flask was mounted on a rocking platform and shaken for 4 h at room temperature. After this time, the resin was filtered off and the solvent was removed under reduced pressure. The remaining crude mixture was then purified by silica column chromatography with a stepwise gradient (100 % $\rightarrow$ 98%, 0.5 % steps) of dichloromethane/methanol to yield of the pure deprotected glycerol as a red wax (41 mg, 69%). R_f = 0.06 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$, TMS): δ = 8.53 (d, 3J = 8.8 Hz, 1 H; NBD-*H*) 8.43 (d, 3J = 8.6 Hz, 1 H; Nile red H1), 7.71 (d, 4J = 2.8 Hz, 1 H; Nile red H4), 7.56 (d, 3J = 9.1 Hz, 1H; Nile red H11), 7.27 (dd, 3J = 9.2, 4J = 2.1 Hz, 1 H; Nile red H2), 6.68 (dd, 3J = 9.1 Hz, 4J = 2.8 Hz, Nile red H10), 6.45 (d, 4J = 2.3 Hz, 1H; Nile red H8), 6.39 (s, 1H; Nile red H6), 6.11 (d, 3J = 8.6 Hz, 1 H; NBD-*H*), 5.80–5.72 (m, 1H; CH olefin), 5.72–5.63 (m, 1 H; CH olefin), 5.09 (qi, 3J = 4.7 Hz, 1 H; *sn*-2 CH), 4.75 (t, 3J = 5.7 Hz, ArOCH_2), 3.88 (dd, 2J = 12.2 Hz, 3J = 4.0 Hz, 1H; *sn*-3 CHH'), 3.83 (dd, 2J = 12.1, 3J = 5.1 Hz, 1H; *sn*-3 CHH'), 3.65 (dd, 2J = 10.5 Hz, 3J = 5.0 Hz, 1 H; *sn*-1 CHH'), 3.62 (dd, 2J = 10.6, 3J = 5.0 Hz, 1H; *sn*-1 CHH'), 3.46 (q, 3J = 7.2 Hz, 4H; ArNCH_2), 3.44–3.36 (m, 4H; *sn*-1 OCH_2 , NBD-NH- CH_2), 2.53–2.38 (m, 2 H; OOCCH_2), 2.27 (q, 3J = 7.2 Hz, 2 H; C = CCH_2), 1.71–1.59 (m, 4 H; C = CH_2CH_2 , NBDNH- CH_2CH_2), 1.59–1.20 (m, 26H; $2\times \text{ArNCH}_2\text{CH}_3$, 10CH_2); ^{13}C NMR (100 MHz, CDCl_3 , 25 $^\circ\text{C}$): δ = 184.18, 173.94, 161.17, 152.55, 151.12, 147.16, 144.90, 144.73, 144.60, 140.08, 137.18, 133.96, 133.83, 131.52, 126.48, 126.29, 126.09, 125.91, 124.22, 121.80, 121.69, 110.79, 108.32, 105.96, 99.17, 96.96, 74.02, 72.39, 70.42, 65.09, 63.18, 54.12, 45.82, 44.75, 34.38, 31.59, 30.37, 30.19, 30.11, 30.08, 29.98, 29.84, 29.15, 27.89, 27.60, 26.67, 25.20, 13.27; FAB HRMS (NBA, positive mode): $[M+H]^+$ calcd: 881.4449; $[M+H]^+$ found: 881.4412.

Hex-5-ynoic acid 2-trimethylsilanylethyl ester (20)

Under an atmosphere of dry argon, 5-hexynoic acid **19** (2.8 g, 25 mmol) was dissolved in dichloromethane (70 mL). 2-Trimethylsilylethanol (3.55 g, 30 mmol) and DIC (5.04 g, 40 mmol) were added and the mixture was stirred at room temperature overnight for 15 h. After this time, the TLC showed complete consumption of the acid. The precipitated diisopropylurea was filtered off. The filtrate was poured into saturated Na_2CO_3 solution and was extracted three times with dichloromethane. The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness at the rotary evaporator under reduced pressure. The remaining oil was purified by silica column chromatography (cyclohexane/ethyl acetate 8:2). Product-containing fractions were pooled and evaporated under reduced pressure to yield the protected acid as a clear oil (5.04 g, 23.8 mmol, 95 %). R_f = 0.64 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$, TMS): δ = 4.18 (m, 2H; COOCH_2), 2.44 (t, 3J = 7.4 Hz, 2 H; CH_2COO), 2.28 (dt, 3J = 7.4 Hz, 4J = 2.7 Hz, 2H; CH_2CC), 1.98 (t, 4J = 2.5 Hz, 1H; CCH), 1.87 (qi, 3J = 7.2 Hz, 2 H; $\text{OOCCH}_2\text{CH}_2$), 1.00 (m, 2H; CH_2Si), 0.06 (s, 9H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 $^\circ\text{C}$): δ = 174.68, 70.53, 64.08, 34.60, 25.17, 19.37, 18.83, 0.00; MS EI: $[M]^+$ calcd: 212.12, $[M]^+$ found: 211.2.

10-Chloro-deca-5,8-diyne acid 2-trimethylsilanylethyl ester (21)

A mixture of K_2CO_3 (20 mmol, 2.76 g), CuI (20 mmol, 3.81 g), NaI (20 mmol, 3.00 g), the TMSE protected hexynoic acid **20** (20 mmol, 4.2 g) and 1,4-dichlorobutylene (80 mmol, 7.4 g) in DMF (60 mL) was stirred overnight at room temperature. The mixture was diluted with ethyl acetate and filtered through a pad of Celite to remove the copper salts. The resulting solution was washed with NH_4Cl (4 $\times$ 100 mL) and brine. The organic layer was dried over Na_2SO_4 and all volatile compounds were removed in vacuo after filtration. The residue was purified by silica column chromatography (cyclohexane/ethyl acetate 9:1 $\rightarrow$ 7:3) to give the diyne as

slightly yellowish oil (3.11 g, 52%). $R_f = 0.54$ (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): $\delta = 4.19$ (m, 2H; COOCH_2), 4.16 (t, $^5J = 2.4$ Hz, 2 H; CH_2Cl), 3.22 (qi, 2.44 Hz, $^5J = 2.3$ Hz, 2H; CCCH_2CC), 2.41 (t, $^3J = 7.5$ Hz, 2H; CH_2COO), 2.26 (tt, $^3J = 7.0$ Hz, $^5J = 2.4$ Hz, 2 H; CH_2CC), 1.84 (qi, $^3J = 7.2$ Hz, 2H; $\text{OOCCH}_2\text{CH}_2$), 1.00 (m, 2H; CH_2Si), 0.06 (s, 9H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): $\delta = 174.80$, 83.01, 81.60, 76.55, 75.27, 64.08, 34.77, 32.17, 25.36, 19.66, 18.83, 11.45, 0.00; MS ESI (positive mode): $[M(^{35}\text{Cl}) + \text{K}]^+$ calcd: 337.1, $[M(^{35}\text{Cl}) + \text{K}]^+$ found: 337.0.

14-Hydroxy-tetradeca-5,8,11-triynoic acid 2-trimethylsilanylethyl ester (22)

A mixture of K_2CO_3 (1.24 g, 9 mmol), CuI (1.71 g, 9 mmol), NaI (1.35 g, 9 mmol), the TMSE protected hexynoic acid **21** (2.69 g, 9 mmol) and 4-butynol (700 mg, 10 mmol) in DMF (30 mL) was stirred overnight at room temperature. The mixture was diluted with ethyl acetate and filtered through a pad of Celite to remove the copper salts. The resulting solution was washed with NH_4Cl (4 $\times$ 50 mL) and brine. The organic layer was dried over Na_2SO_4 and all volatile compounds were removed in vacuo after filtration. The residue was purified by silica column chromatography (cyclohexane/ethyl acetate, 8:2 $\rightarrow$ 6:4) to give the triyne as yellow oil (1.62 g, 54%). $R_f = 0.24$ (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): $\delta = 4.17$ (m, 2 H; COOCH_2), 3.70 (t, $^3J = 6.3$ Hz, 2 H; CH_2OH), 3.16 (q, $^5J = 2.3$ Hz, 2H; CCCH_2CC), 3.13 (q, $^5J = 2.3$ Hz, 2 H; CCCH_2CC), 2.45 (tt, $^3J = 6.2$ Hz, $^5J = 2.4$ Hz, 2H; $\text{HOCH}_2\text{CH}_2\text{CC}$), 2.40 (t, $^3J = 7.5$ Hz, 2 H; CH_2CO_2), 2.23 (tt, $^3J = 6.9$ Hz, $^5J = 2.3$ Hz, 2H; CH_2CC), 1.82 (qi, $^3J = 7.2$ Hz, 2H; $\text{O}_2\text{CCH}_2\text{CH}_2$), 1.00 (m, 2H; CH_2Si), 0.06 (s, 9 H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): $\delta = 174.87$, 81.11, 78.75, 77.59, 76.48, 76.01, 64.08, 62.58, 34.82, 25.42, 24.59, 19.70, 18.84, 11.30, 11.24, 0.00; MS-ESI (positive mode): $[M + \text{Na}]^+$ calcd: 355.2, found: 355.1.

14-Hydroxy-tetradeca-5,8,11-trienoic acid 2-trimethylsilanylethyl ester (23)

Under an atmosphere of dry argon, triyne **22** (2.78 mmol, 922 mg) was dissolved in dry THF (20 mL). Lindlar catalyst (2.13 g, 13 mol %), chinolin (113 μL) and dihydroxybenzaldehyde (600 mg, 4.3 mmol) as a scavenger were added, and the argon was exchanged by hydrogen. The deep-red solution was stirred at room temperature until RP-TLC (100% methanol) showed complete conversion to the triene after approximately 90 min. At this time the hydrogen was removed, and the catalyst was filtered off through a small pad of Celite. Solvents were removed under reduced pressure, and the resulting crude product was then further purified by silica column chromatography to yield the pure product as colorless oil (805.8 mg, 86 %). R_f (cyclohexane/ethyl acetate, 3:2) = 0.42; ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): $\delta = 5.56$ –5.44 (m, 1 H; CH olefin), 5.41–5.24 (m, 5H; CH olefin), 4.12 (m, 2H; COOCH_2), 3.61 (t, $^3J = 6.6$ Hz, 2 H; CH_2OH), 2.81 (t, $^3J = 6.2$ Hz, 2H; = $\text{CHCH}_2\text{CH} =$), 2.77 (t, $^3J = 4.9$ Hz, 2H; = $\text{CHCH}_2\text{CH} =$), 2.33 (q, $^3J = 6.9$ Hz, 2H; $\text{HOCH}_2\text{CH}_2\text{CH} =$), 2.26 (t, $^3J = 7.5$ Hz, 2H; CH_2COO), 2.06 (qi, $^3J = 6.9$ Hz, 2 H; $\text{CH}_2\text{CH} =$), 1.65 (qi, $^3J = 7.4$ Hz, 2H; $\text{OOCCH}_2\text{CH}_2$), 0.94 (m, 2 H; CH_2Si), 0.00 (s, 9 H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): $\delta = 175.37$, 132.53, 130.54, 129.88, 129.43, 127.15, 64.00, 63.70, 35.40, 32.39, 28.10, 27.26, 26.31, 18.82, 0.00; MS HR-ESI (positive mode): $[M + \text{Na}]^+$ calcd: 361.2175, found: 361.2162.

14-Azido-tetradeca-5,8,11-trienoic acid 2-trimethylsilanylethyl ester (24)

A solution of DPPA (3.8 mmol, 863.4 mg) in THF (1.5 mL) was added to a stirred solution of alcohol **23** (1.9 mmol, 667 mg), Ph_3P (3.8 mmol, 996.7 mg) and diisopropyl-azo-dicarboxylic acid DIAD (3.8 mmol, 768.4 mg) in dry THF (20 mL) at room temperature. The reaction mixture was stirred for 2 h. After this time, TLC (cyclohexane/ethyl acetate 3:2) indicated complete consumption of the alcohol, and showed a new, further-migrating spot that corresponded to the azide. The solvent was removed in vacuo, and the crude product was purified by silica column chromatography (cyclohexane/ethyl acetate 8:2) to yield the pure

azide as colorless oil (649 mg, 94%). R_f = 0.62 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 5.63–5.49 (m, 1H; CH olefin), 5.48–5.32 (m, 5 H; CH olefin), 4.18 (m, 2 H; COOCH_2), 3.31 (t, 3J = 6.9 Hz, 2H; CH_2N_3), 2.86 (t, 3J = 6.3 Hz, 2 H; = $\text{CHCH}_2\text{CH=}$), 2.83 (t, 3J = 5.4 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.40 (q, 3J = 7.0 Hz, 2H; $\text{N}_3\text{CH}_2\text{CH}_2\text{CH=}$), 2.32 (t, 3J = 7.5 Hz, 2H; CH_2COO), 2.13 (dt, 3J = 6.7, 6.2 Hz, 2H; $\text{CH}_2\text{CH=}$), 1.72 (qi, 3J = 7.5 Hz, 2H; $\text{OOCCH}_2\text{CH}_2$), 1.00 (m, 2H; CH_2Si), 0.07 (s, 9 H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 175.37, 132.55, 130.71, 130.21, 129.15, 126.83, 64.00, 52.50, 35.40, 28.56, 28.38, 27.11, 26.30, 18.83, 0.01; ESI HRMS (positive mode): $[M+H]^+$ calcd: 346.2420, found: 346.2430.

14-Amino-tetradeca-5,8,11-trienoic acid 2-trimethylsilanylethyl ester (25)

3 equiv Ph_3P (1.18 g) and a drop of water were added to a stirred solution of the azide **24** (545 mg, 1.5 mmol) in THF (10 mL). Immediately gas bubbles formed, which indicated the start of the reaction. The mixture was stirred for 3 h at RT, until TLC analysis showed complete consumption of the azide, and a new ninhydrin-active spot (R_f = 0) was observed. The solvent was removed under reduced pressure, and the residue was subjected to short column silica chromatography. Ph_3P and impurities were eluted with cyclohexane/ethyl acetate (7:3). Then the eluent was switched to cyclohexane/ethyl acetate/dimethylethylamine (6:4:0.1) to wash off the pure amine as a colorless oil (315 mg, 62%). ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 5.55–5.23 (m, 6H; CH olefin), 4.12 (m, 2H; COOCH_2), 2.80 (t, 3J = 6.6 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.76 (t, 3J = 4.5 Hz, 2 H; CH_2NH_2), 2.71 (t, 3J = 6.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.25 (t, 3J = 7.5 Hz, 2H; CH_2CO_2), 2.19 (q, 3J = 7.0 Hz, 2H; $\text{CH}_2\text{CH=}$), 2.07 (qi, $\text{H}_2\text{NCH}_2\text{CH}_2$), 1.65 (qi, 3J = 7.5 Hz, 2H; $\text{O}_2\text{CCH}_2\text{CH}_2$), 1.4 (s, 2 H; NH_2), 0.94 (m, 2 H; CH_2Si), 0.00 (s, 9H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 175.25, 131.495, 130.54, 130.23, 129.75, 129.61, 128.73, 63.93, 43.41, 35.38, 33.00, 28.08, 27.25, 27.11, 26.31, 18.82, –0.01; ESI HRMS (positive mode): $[M+H]^+$ calcd: 338.2515, found: 338.2545.

14-(7-Nitrobenzooxadiazol-4-ylamino)tetradeca-5,8,11-trienoic acid 2-trimethylsilanylethyl ester (26)

Amine **25** (260.2 mg, 771 μmol) in methanol (2 mL) was added dropwise to a stirred solution of NBD-chloride (160 mg, 800 μmol) and DIPEA (5 equiv) in dry methanol (10 mL), at 0 °C. After complete addition, the mixture was stirred for further 3 h at 0 °C. The solution was allowed to warm up to room temperature and stirred for another 14 h. After this period of time TLC analysis indicated consumption of the starting material and showed a new spot, which exhibited bright fluorescence when illuminated with 366 nm UV light. All volatile compounds were removed under reduced pressure, and the residual deep-brown oil was purified by silica column chromatography (cyclohexane/ethyl acetate 7:3) to yield the pure labeled ester as a deep-orange oil (280 mg, 560 μmol , 72 %). R_f = 0.40 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.51 (d, 3J = 8.6 Hz, 1H; NBD), 6.47 (t, 3J = 4.3 Hz, 1H; NHNBD), 6.21 (d, 3J = 8.6 Hz, 1H; NBD), 5.74–5.64 (m, 1H; CH olefin), 5.53–5.35 (m, 5 H; CH olefin), 4.18 (m, 2H; CO_2CH_2), 3.57 (t, 3J = 6.2 Hz, 2H; CH_2NHNBD), 2.88 (t, 3J = 6.9 Hz, 2 H; = $\text{CHCH}_2\text{CH=}$), 2.81 (t, 3J = 5.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.62 (q, 3J = 6.8 Hz, 2H; CH_2CH), 2.32 (t, 3J = 7.5 Hz, 2H; CH_2COO), 2.12 (q, 3J = 7.0 Hz, 2H; $\text{CH}_2\text{CH=}$), 1.71 (qi, 3J = 7.4 Hz, 2H; $\text{O}_2\text{CCH}_2\text{CH}_2$), 1.00 (m, 2H; CH_2Si), 0.05 (s, 9 H; SiCH_3); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 175.40, 145.79, 145.32, 137.87, 133.96, 130.70, 130.46, 129.87, 128.63, 126.16, 100.10, 64.06, 44.80, 35.39, 28.11, 27.87, 27.23, 27.18, 26.27, 18.83, –0.01; MS HR-ESI (positive mode): $[M+H]^+$ calcd: 501.2533, found: 501.2504.

14-(7-Nitrobenzooxadiazol-4-ylamino)tetradeca-5,8,11-trienoic acid (27)

The NBD-labeled ester **26** (250 mg, 0.5 mmol) was dissolved in dichloromethane (3 mL). TFA (300 μL) was added, and the mixture was stirred for 60 min. TLC analysis showed conversion

to a more slowly migrating spot. Toluene (3 mL) was added, and all volatile compounds were removed in vacuo. The resulting crude product was subjected to silica column chromatography (cyclohexane/ethyl acetate 3:2 → cyclohexane/ethyl acetate/acetic acid 2:3:0.1) to yield the pure NBD-labeled acid (152 mg, 380 μmol, 76%). R_f = 0.19 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.46 (d, 3J = 8.8 Hz 1 H; NBD), 6.75 (t, 3J = 4.9 Hz 1H; *NHNBD*), 6.21 (d, 3J = 8.8 Hz 1H; NBD), 5.69–5.59 (m, 1 H; CH olefin), 5.51–5.31 (m, 5H; CH olefin), 3.57 (t, 3J = 6.2 Hz, 2H; CH_2NHNBD), 2.86 (t, 3J = 6.4 Hz, 2 H; = $\text{CHCH}_2\text{CH=}$), 2.79 (t, 3J = 5.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.62 (q, 3J = 7.0 Hz, 2H; CH_2CH), 2.36 (t, 3J = 7.3 Hz, 2H; CH_2COO), 2.12 (q, 3J = 6.8 Hz, 2H; $\text{CH}_2\text{CH=}$), 1.69 (qi, 3J = 7.4 Hz, 2 H; $\text{OOCCH}_2\text{CH}_2$); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 179.62, 144.18, 144.00, 136.65, 132.18, 130.82, 128.86, 128.75, 128.70, 127.29, 124.74, 123.58, 98.77, 43.43, 33.28, 26.33, 25.72, 25.65, 24.41; MS HR-ESI (positive mode): $[M+H]^+$ calcd: 401.1825, found: 401.1823.

1-O-(12-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-3-yloxy)-dodecyl)-*sn*-glycerol (29)

2-Hydroxy Nile red (720 mg, 2.15 mmol) was dissolved in DMF (15 mL). Bromide 28 (720 mg, 2.15 mmol) and excess K_2CO_3 (356 mg, 2.58 mmol) were added. The mixture was heated to 70 °C and stirred at this temperature for 3 h. After this time TLC (dichloromethane/methanol 95:5) indicated no further conversion of the starting materials. The mixture was poured into phosphate buffer (pH 7) and was extracted with ethyl acetate until the organic layer remained nearly colorless. The combined organic layers were washed with brine, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The remaining deep-red solid was purified by silica column chromatography (cyclohexane/ethyl acetate, 1:1 → ethyl acetate → ethyl acetate/acetone, 1:1 → acetone). Product-containing fractions were pooled and evaporated under reduced pressure to yield the labeled glycerol ether as a black powder (1.04 g, 82%). R_f = 0.10 (dichloromethane/methanol 95:5); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.17 (d, 3J = 8.8 Hz, 1 H; Nile red H4), 8.00 (d, 4J = 2.5 Hz, 1H; Nile red H1), 7.53 (d, 3J = 9.0 Hz, 1H; Nile red H11), 7.12 (dd, 3J = 8.7 Hz, 4J = 2.4 Hz, Nile red H3), 6.59 (dd, 3J = 9.3 Hz, 4J = 2.5 Hz, Nile red H10), 6.37 (d, 4J = 2.5 Hz, 1H; Nile red H8), 6.25 (s, 1H; Nile red H6), 4.13 (t, 3J = 6.4 Hz, 2H; ArOCH_2), 3.88 (qi, 3J = 4.3 Hz, *sn*-2 CH), 3.73 (dd, 2J = 11.4, 3J = 3.9 Hz, 1H; *sn*-3 CHH'), 3.65 dd, 2J = 11.5, 3J = 5.4 Hz, 1H; *sn*-3 CHH'), 3.57–3.42 (m, 4 H; *sn*-1 CH_2 , *sn*-1 OCH_2), 3.42 (q, 3J = 7.1 Hz, 4H; ArNCH_2 (2×)), 1.85 (qi, 3J = 7.2 Hz, 2 H; OCH_2CH_2), 1.66–1.25 (m, 18H; CH_2 chain (9×)), 1.23 (t, 3J = 7.2 Hz, 6 H; NCH_2CH_3 (2×)); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 183.75, 162.23, 152.41, 147.13, 140.10, 134.40, 131.42, 128.00, 125.73, 125.06, 118.60, 109.94, 106.89, 105.46, 96.54, 72.82, 72.19, 70.93, 68.76, 64.65, 45.44, 29.95, 29.84, 26.45, 13.02; ESI HRMS (positive mode): $[M+H]^+$ calcd: 593.3591, found: 593.3619.

1-O-(12-(9-Diethylamino-5-oxo-5*H*-benzo[*a*]phenoxazin-2-yloxy)-dodecyl)-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol (30)

A catalytic amount of DMAP and Et_3N (210 μL, 1.5 mmol) in DMF (20 mL) as well as DMTCL (372 mg, 1.1 mmol) were added to a stirred solution of the Nile-red-labeled glycerol (592 mg, 1 mmol). The solution was stirred overnight until TLC analysis (dichloromethane/methanol 95:5) showed no further progress of the reaction. The solution was diluted with ethyl acetate and was washed with 10 % NaHCO_3 (3 × 60 mL) and brine (2 × 50 mL). The organic layer was dried over Na_2SO_4 , and the solvent was evaporated under reduced pressure. To remove residual DMF, the resulting deep red slurry was taken up with methanol, and the DMT-labeled raw product was precipitated by dropwise addition of water. The precipitate was collected by filtration and dried overnight in a desiccator over P_2O_5 under reduced pressure. The resulting crude product was subjected to silica column chromatography (cyclohexane/ethyl acetate 3:2 +1% Me_2NEt). The product containing fractions were pooled and evaporated to dryness in vacuo to yield the DMT protected glycerol as a deep red powder (528 mg, 0.59 mmol, 59%).

To recover the starting material, the column was washed with (dichloromethane/methanol 95:5) to furnish the unprotected glycerol (233 mg, 39.3%). The combined overall yield was 98 %. R_f = 0.23 (cyclohexane/ethyl acetate 3:2); ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.23 (d, 3J = 8.8 Hz, 1 H; Nile red H^4), 8.07 (d, 4J = 2.5 Hz, 1 H; Nile red H^1), 7.62 (d, 3J = 9.1 Hz, 1H; Nile red H^{11}), 7.45 (d, 3J = 7.3 Hz, 2 H; DMT), 7.34 (d, 3J = 8.8 Hz, 4 H; DMT), 7.25–7.21 (m, 2 H; DMT), 7.23 (t, 3J = 7.3 Hz, 1 H; DMT), 7.19 (dd, 3J = 8.7 Hz, 4J = 2.7 Hz, Nile red H^3), 6.84 (d, 3J = 8.8 Hz, 4 H; DMT), 6.68 (dd, 3J = 9.1 Hz, 4J = 2.8 Hz, Nile red H^{10}), 6.49 (d, 4J = 2.8 Hz, 1 H; Nile red H^8), 6.32 (s, 1H; Nile red H^6), 4.18 (t, 3J = 6.4 Hz 2 H; ArOCH_2), 3.92 (m, *sn*-2 CH), 3.80 (s, 6 H; CH_3 DMT), 3.58–3.42 (m, 8H; *sn*-1 CH_2 , OCH_2 , ArNCH_2 (2 $\times$)), 3.20 (dd, 3J = 5.4, 3.7 Hz, 2 H; *sn*-3 CH_2), 1.88 (qi, 3J = 7.0 Hz, 2H; OCH_2CH_2), 1.66–1.20 (m, 24H; CH_2 chain (9 $\times$), NCH_2CH_3 (2 $\times$)); ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): δ = 183.30, 161.93, 158.64, 158.46, 152.05, 150.69, 146.87, 144.88, 139.47, 136.07, 134.07, 131.05, 130.06, 128.16, 127.85, 127.80, 127.77, 126.76, 125.52, 124.73, 118.30, 113.17, 113.09, 109.49, 106.69, 105.34, 96.37, 86.04, 81.44, 72.55, 72.12, 71.85, 70.41, 69.91, 68.41, 64.46, 64.35, 55.26, 55.21, 45.09, 29.60, 29.56, 26.92, 26.12, 26.09, 12.62; FAB HRMS (NBA, positive mode): $[M+\text{Na}]^+$ calcd: 917.4717, found: 917.4750.

1-O-(12-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-dodecyl)-2-O-(14-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-5(Z),8(Z),11(Z)-tetra-decenoyl-3-O-(4,4'-dimethoxytrityl)-*sn*-glycerol (31)

As described for **17**, the NBD-labeled acid **27** (98 mg, 245 μmol) was coupled to the Nile-red-labeled glycerol **30** (242 mg, 270 μmol) in dry dichloromethane (10 mL) with TPSNT/methylimidazole (93 mg, 244 mmol/59 μL , 740 mmol) as coupling reagent. Silica column chromatography (stepwise gradient dichloromethane/methanol, 100 $\rightarrow$ 99%) yielded (238 mg, 186 μmol , 78%) of the glycerol derivative as a dark-red solid. R_f = 0.60 (dichloromethane/methanol 95:5); ^1H NMR (400 MHz, CDCl_3 , 25°C, TMS): δ = 8.45 (d, 3J = 8.6 Hz, 1H; NBD), 8.22 (d, 3J = 8.8 Hz, 1H; Nile red H^4), 8.04 (d, 4J = 2.5 Hz, 1 H; Nile red H^1), 7.0 (d, 3J = 8.8 Hz, 1H; Nile red H^{11}), 7.43 (d, 3J = 8.8 Hz, 2 H; DMT), 7.34–7.25 (m, 7H; DMT), 7.17 (dd, 3J = 8.8 Hz, 4J = 2.5 Hz, 1 H; Nile red H^3), 6.83 (d, 3J = 8.8 Hz, 4H; DMT), 6.66 (dd, 3J = 9.1 Hz, 4J = 2.5 Hz, 1H; Nile red H^{10}), 6.59 (s, 1H; *NHNBD*), 6.46 (d, 4J = 2.8 Hz, 1 H; Nile red H^8), 6.31 (s, 1H; Nile red H^6), 6.13 (d, 3J = 8.6 Hz 1 H; NBD), 5.70–5.59 (m, 1H; CH olefin), 5.50–5.33 (m, 5H; CH olefin), 5.24 (qi, 3J = 8.1 Hz, 1 H; *sn*-2 CH), 4.17 (t, 3J = 6.4 Hz, 2 H; ArOCH_2), 3.81 (t, 3J = 6.2 Hz, 2H; CH_2NHNBD), 3.79 (s, 6H; CH_3 DMT), 3.69–3.56 (m, 2H; *sn*-1 CH_2), 3.48 (q, 3J = 7.0 Hz, 4H; ArNCH_2 (2 $\times$)), 3.46–3.33 (m, 2H; OCH_2), 3.28–3.21 (m, 2H; *sn*-3 CH_2), 2.85 (t, 3J = 6.9 Hz, 2 H; = $\text{CHCH}_2\text{CH=}$), 2.79 (t, 3J = 5.9 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.66 (q, 3J = 7.0 Hz, 2H; CH_2CH), 2.39 (t, 3J = 7.3 Hz, 2H; CH_2COO), 2.13 (q, 3J = 7.2 Hz, 2H; $\text{CH}_2\text{CH=}$), 1.88 (qi, 3J = 7.2 Hz, 2 H; OCH_2CH_2), 1.74 (qi, 3J = 7.4 Hz, 2 H; $\text{OOCCH}_2\text{CH}_2$), 1.45–1.18 (m, 24H; CH_2 (9 $\times$), NCH_2CH_3 (2 $\times$)); ^{13}C NMR (100 MHz, CDCl_3 , 25°C, TMS): δ = 183.24, 173.14, 161.92, 158.64, 158.44, 152.04, 150.74, 147.33, 146.85, 144.80, 144.26, 143.92, 143.16, 139.46, 136.38, 135.96, 134.05, 132.33, 132.30, 131.06, 130.02, 129.12, 129.03, 128.86, 128.53, 128.44, 128.12, 127.84, 127.76, 127.71, 127.22, 127.17, 127.07, 126.73, 125.46, 124.74, 123.93, 118.29, 109.58, 106.64, 105.25, 98.56, 96.35, 85.88, 81.43, 72.99, 71.91, 71.53, 70.04, 69.54, 68.40, 62.99, 62.42, 55.24, 55.19, 45.10, 33.93, 33.68, 29.58, 29.51, 29.47, 29.20, 26.63, 26.49, 26.07, 26.03, 25.72, 25.67, 24.83, 24.70, 12.63; FAB HRMS (NBA, positive mode): $[M+\text{Na}]^+$ calcd: 1299.6358, found: 1299.6301.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-*sn*-glycerol (32)

According to the procedure for **18**, the DMT protected lipid **31** (127 mg, 100 μmol) was dissolved in dry dichloromethane (5 mL), of DOWEX-50 WX-8 resin (400 mg) was added, and the mixture was shaken for 4 h. After purification on silica gel (dichloromethane/methanol

99.5→98%) the deprotected glycerol was isolated as a red oil (70.3 mg, 72 μ mol, 72%). R_f = 0.30 (dichloromethane/methanol 95:5); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$, TMS): δ = 8.43 (d, 3J = 8.6 Hz, 1H; NBD), 8.19 (d, 3J = 8.8 Hz, 1H; Nile red H4), 8.00 (d, 4J = 2.5 Hz, 1H; Nile red H1), 7.56 (d, 3J = 9.1 Hz, 1H; Nile red H11), 7.14 (dd, 3J = 8.7 Hz, 4J = 2.4 Hz, Nile red H3), 6.87 (s, 1H; NHNBD), 6.64 (dd, 3J = 9.1 Hz, 4J = 2.5 Hz, Nile red H10), 6.41 (m, 1H; Nile red H8), 6.28 (s, 1H; Nile red H6), 6.14 (d, 8.6 Hz, 1H; NBD), 5.68–5.57 (m, 1H; CH olefin), 5.50–5.27 (m, 5H; CH olefin), 5.03 (qi, 3J = 4.7 Hz, 1H; *sn*-2 CH), 4.16 (t, 3J = 6.3 Hz, 2H; ArOCH_2), 3.84 (dd, 3J = 3.6, 3.2 Hz, 2H; CH_2NHNBD), 3.63 (t, 3J = 5.2 Hz, 2H; *sn*-1 CH_2), 3.55–3.39 (m, 8H; ArNCH_2 (2 $\times$), *sn*-3 CH_2 , OCH_2), 2.84 (t, 3J = 6.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.77 (t, 3J = 5.4 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.57 (q, 3J = 7.1 Hz, 2H; CH_2CH), 2.37 (t, 3J = 7.3 Hz, 2H; CH_2CO_2), 2.10 (q, = 6.2 Hz, 2H; $\text{CH}_2\text{CH=}$), 1.86 (qi, 3J = 7.2 Hz, 2H; OCH_2CH_2), 1.70 (qi, 3J = 7.3 Hz, 2H; $\text{O}_2\text{CCH}_2\text{CH}_2$), 1.45–1.18 (m, 24H; CH_2 chain (9 $\times$), NCH_2CH_3 (2 $\times$)); ^{13}C NMR (100 MHz, CDCl_3 , 25 $^\circ\text{C}$): δ = 183.18, 173.51, 161.89, 152.02, 150.79, 146.77, 144.22, 143.89, 139.72, 136.43, 134.02, 132.16, 131.06, 129.12, 129.03, 128.79, 128.53, 127.62, 127.27, 125.35, 124.80, 124.74, 118.24, 109.66, 106.55, 105.56, 96.23, 73.04, 71.87, 69.94, 68.41, 62.81, 53.43, 45.08, 43.39, 33.69, 33.47, 30.91, 29.54, 29.53, 29.41, 29.35, 29.21, 26.49, 26.34, 26.03, 26.01, 25.71, 25.65, 24.71, 12.61; ESI HRMS (positive mode): $[M+H]^+$ calcd: 975.5232, $[M+H]^+$ found: 975.5208.

General phosphorylation procedure

The alcohol (1 equiv) and 4,5-dicyanoimidazole (4 equiv) were placed into a Schlenk flask. The flask was sealed with a rubber stopper. Dry acetonitrile was added and evaporated in vacuo three times to remove traces of water azeotropically. DMF was added under an atmosphere of dry argon, and the mixture was cooled to 0 $^\circ\text{C}$. The phosphoramidite (2 equiv) was added, and the mixture was stirred overnight. After 12 h, 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 $^\circ\text{C}$ and treated with an excess of *t*BuOOH for 1 h to oxidize to the stable phosphorous(V) triester. The products were either isolated in case of SATE-protected derivatives or further treated to give the free phosphatidylethanolamine derivatives.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-*sn*-glyceryl phosphatidylethanolamine [PENN2] (3)

According to the general procedure, the *sn*-3 alcohol **18** (38 mg, 39 μ mol) was treated with 4,5-dicyanoimidazole (20 mg, 0.158 mmol) and the *tert*-butyl/Boc-protected phosphoramidite **PI** (30 mg, 0.080 mmol). The oxidation was performed by the addition of 5.5 M *t*BuOOH solution in nonane (50 μ L). After 2 h all volatile compounds of the reaction mixture were removed 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 dichloromethane/TFA (90:10, 5 mL) for 50 min until TLC showed no further conversion (the product spot did not move in dichloromethane/methanol 95:5, but with 80:20 (R_f = 0.46)). The dichloromethane/TFA mixture was removed under reduced pressure, and the resulting crude product was subjected to column chromatography (dichloromethane/methanol, 90:10→87:25). Product-containing fractions were combined, and the solvents were removed under reduced pressure to yield PENN2 (23 mg, 23 μ mol, 59%). R_f = 0.46 (dichloromethane/methanol, 80:20); ^1H NMR (400 MHz, $\text{CD}_3\text{OD}/\text{CDCl}_3$ (1:1), 25 $^\circ\text{C}$, TMS): δ = 8.42 (d, 3J = 8.4 Hz, 1H; NBD), 8.24 (d, 3J = 8.6 Hz, 1H; Nile red H4), 7.56 (d, 4J = 2.0 Hz, 1H; Nile red H1), 7.45 (d, 3J = 8.9 Hz, 1H; Nile red H11), 7.13 (dd, 3J = 8.7 Hz, 4J = 2.1 Hz, Nile red H3), 6.58 (dd, 3J = 9.1 Hz, 4J = 2.2 Hz, Nile red H10), 6.36 (d, 3J = 2.6 Hz, 1H; Nile red H8), 6.24 (s, 1H; Nile red H6), 5.95 (d, 7.8 Hz, 1H; NBD), 5.70–5.52 (m, 2H; CH olefin), 5.10–5.02 (m, 1H; *sn*-2 CH), 4.58 (d, 3J = 6.1 Hz

2H; ArOCH₂), 3.96–3.71 (m, 4 H; POCH₂ (2×)), 3.48–3.20 (m, 10H; *sn*-1 CH₂, Nile red NCH₂ (2×), *sn*-1-OCH₂, NBD-NH-CH₂), 3.01 (m, 2H; H₃NCH₂), 2.30 (t, ³J = 7.3 Hz, 2 H; CH₂COO), 2.13 (m, 2 H; CH₂CH₂), 1.68–1.55 (m, 4 H; OCH₂CH₂, OOCCH₂CH₂), 1.53–1.18 (m, 24H; CH₂ chain (9×), NCH₂CH₃ (20)); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 178.80, 165.79, 157.60, 156.18, 151.92, 144.02, 138.75, 138.30, 138.18, 131.12, 130.75, 130.59, 130.54, 126.10, 124.61, 115.79, 113.20, 110.13, 101.38, 76.98, 74.35, 69.55, 66.85, 58.11, 50.40, 47.26, 45.84, 34.93, 34.79, 34.76, 34.74, 34.71, 34.67, 34.49, 33.47, 33.24, 32.34, 32.24, 31.23, 29.83, 17.72, 14.54; ³¹P NMR (162 MHz, CD₃OD/CDCl₃ (1:1), 25 °C, H₃PO₄): δ = 4.0 ppm.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-3-O-((2-(acetyl-thio)ethoxy)(2-((2-(acetylthio)ethoxy)-carbonyl)aminoethoxy)phosphoryl)-*sn*-glycerol [PENN2/SATE] (4)

According to the general procedure, the *sn*-3 alcohol **18** (36 mg, 0.041 mmol) was treated with 4,5-dicyanoimidazole (20 mg, 0.164 mmol) and the SATE protected phosphoramidite PII (36.5 mg, 0.08 mmol). The oxidation was performed by the addition of 5.5 M *t*BuOOH solution in nonane (50 µL). The resulting product was purified by preparative TLC on silica (dichloromethane/methanol, 90:10). The product band was extracted with dichloromethane/methanol (95:5) to obtain PENN2/SATE (**4**) as a deep-red oil (14 mg, 32%). *R*_f = 0.60 (dichloromethane/methanol, 90:10); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 8.59 (d, ³J = 8.8 Hz, 1 H; NBD-*H*) 8.48 (d, = 8.6 Hz, 1H; Nile red H1), 7.76 (d, ⁴J = 2.8 Hz, 1H; Nile red H4), 7.61 (d, ³J = 9.1 Hz, 1 H; Nile red H11), 7.31 (dd, ³J = 8.7, ⁴J = 2.7 Hz, 1 H; Nile red H2), 6.71 (dd, ³J = 9.1 Hz, ⁴J = 2.5 Hz, Nile red H10), 6.51 (d, ³J = 2.8 Hz, 1 H; Nile red H8), 6.50 (s, 1 H; NBDN*H*), 6.44 (s, 1 H; Nile red H6), 6.14 (d, ³J = 8.6 Hz, 1H; NBD-*H*), 5.81 (dt, ³J = 11.2, 6.0 Hz, ⁴J = 0.4 Hz, 1 H; CH olefin), 5.58 (dt, ³J = 11.0, 7.8 Hz, ⁴J = 0.4 Hz 1 H; CH olefin), 5.61 (t, ³J = 6.1 Hz, 0.5 H; NH carbamate) 5.56 (t, ³J = 6.1 Hz, 0.5 H; NH carbamate), 5.26–5.18 (m, 1 H; *sn*-2 CH), 4.77 (d, ³J = 5.8 Hz, ArOCH₂), 4.37–4.10 (m, 8 H; POCH₂ (3×), NHCOOCH₂), 3.58 (d, ³J = 4.8 Hz, 2 H; *sn*-1 CH₂), 3.56–3.38 (m, 10 H; *sn*-1 OCH₂, NBD-NH-CH₂, Nile red NCH₂ (2×), OOCNHCH₂), 3.19 (t, ³J = 6.4 Hz, 2 H; COSCH₂) 3.13 (t, ³J = 6.4 Hz, 2 H; COSCH₂), 2.44 (t, 2 H; ³J = 7.7 Hz, OOCCH₂), 2.37 (s, 3H; CH₃COS), 2.36 (s, 3 H; CH₃COS), 2.28 (q, ³J = 7.3 Hz, 2H; C = CCH₂), 1.86–1.74 (m, 4 H; C = CH₂CH₂, NBDNH-CH₂CH₂), 1.59–1.20 (m, 26H; 2× ArNCH₂CH₃, 10 CH₂), ³¹P NMR (80 MHz, CDCl₃, 25 °C, H₃PO₄): δ = -1.13, -1.16 ppm; ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 183.06, 173.69, 163.13, 160.12, 153.21, 152.38, 148.07, 146.65, 140.76, 138.24, 137.35, 135.39, 132.32, 132.21, 131.42, 130.51, 129.94, 129.76, 129.48, 129.12, 128.70, 128.09, 127.18, 126.85, 125.64, 119.12, 114.42, 111.06, 107.96, 105.96, 97.53, 87.26, 73.09, 72.42, 70.65, 69.55, 63.95, 56.08, 46.13, 34.87, 30.99, 30.96, 30.91, 30.86, 30.80, 30.75, 30.70, 30.66, 30.60, 30.52, 30.46, 30.41, 30.22, 30.03, 29.83, 27.76, 27.40, 27.32, 26.88, 26.80, 26.27, 13.51; FAB HRMS (NBA, positive mode): [*M*+H]⁺ calcd: 1077.4408, found: 1077.4437.

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-*sn*-glyceryl phosphatidylmethanol [PMNN2] (5)

A small sample (about 1 mg) of the SATE-protected derivative **6** was dissolved in a minimum amount of DMSO (about 25 µL). This solution was diluted with the Triton X-100 assay buffer (5 mL), of lipase (20 mg) from *C. cylindracea* was added, and the mixture was stirred for 24 h at 37 °C. After this time the reaction mixture was transferred into a separation-funnel and was extracted with dichloromethane/methanol (1:1) until no further stained compound went into the organic layer. The solvents were evaporated and the crude product was purified by TLC (Merck 20 × 20 cm analytical plate; eluent dichloromethane/methanol 8:2). The product band was scraped off of the plate, and the pure PMNN2 was eluted with dichloromethane/methanol 9:1. *R*_f = 0.51 (dichloromethane/methanol, 80:20).

1-O-(12-(7-Nitrobenz-2-oxa-1,3-diazol-4-ylamino)dodecyl)-2-O-(7-(9-diethyl-amino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-5(Z)-heptenoyl-3-O-((2-(acetyl-thio)ethoxy)(2-methyl)phosphoryl)- sn-glycerol [PMNN2 SATE] (6)

According to the general procedure, the *sn*-3 alcohol **18** (28 mg, 0.032 mmol) was treated with 4,5-dicyanoimidazole (15.5 mg, 0.128 mmol) and the SATE protected phosphoramidite **PIII** (18 mg, 0.064 mmol). Oxidation was performed by the addition of *t*BuOOH solution in nonane (5.5 M, 40 μ L). The product was purified by preparative TLC on silica (dichloromethane/methanol 90:10). The product band was extracted with dichloromethane/methanol (95:5) to obtain PMNN2/SATE (**6**) as deep-red oil (18 mg, 0.017 mmol, 53%). R_f = 0.65 (dichloromethane/methanol 90:10); ^1H NMR (400 MHz, CDCl_3 , 25 $^\circ\text{C}$, TMS): δ = 8.59 (d, 3J = 8.6 Hz, 1 H; NBD-*H*) 8.47 (d, J = 8.6 Hz, 1H; Nile red H1), 7.75 (d, 4J = 2.8 Hz, 1 H; Nile red H4), 7.61 (d, 3J = 9.1 Hz, 1H; Nile red H11), 7.30 (dd, 3J = 8.7, 4J = 2.7 Hz, 1H; Nile red H2), 6.71 (dd, 3J = 9.1 Hz, 4J = 2.5 Hz, Nile red H10), 6.54 (d, 4J = 2.8 Hz, 1H; Nile red H8), 6.51 (s, 1H; NBDNH), 6.42 (s, 1 H; Nile red H6), 6.14 (d, 3J = 8.6 Hz, 1 H; NBD-*H*), 5.81 (dt, 3J = 11.2, 6.0 Hz, 4J = 0.4 Hz, 1H; CH olefin), 5.71 (dt, 3J = 11.0, 7.8 Hz, 4J = 0.4 Hz 1H; CH olefin), 5.20 (qi, 4.7 Hz, 1H; *sn*-2 CH), 4.76 (d, 3J = 6.1 Hz, ArOCH_2), 4.31–4.09 (m, 4H; POCH_2 (2)), 3.81 (d, 3J = 3.3 Hz, 1.5 H; POCH_3 (0.5 $\times$)), 3.78 (d, 3J = 3.3 Hz, 1.5 H; POCH_3 (0.5 $\times$)), 3.58 (d, 3J = 5.3 Hz, 2 H; *sn*-1 CH_2), 3.49 (q, 3J = 7.2 Hz, 4H; Nile red NCH_2 (2 $\times$)), 3.48–3.38 (m, 4H; *sn*-1 OCH_2 , NBD-NH- CH_2), 3.19 (d, 3J = 6.4 Hz, 1 H; COSCH_2 (0.5 $\times$)), 3.13 (t, 3J = 6.4 Hz, 1 H; COSCH_2 (0.5 $\times$)), 2.44 (t, 2 H; 3J = 7.5 Hz, OOCCH_2), 2.37 (s, 3 H; CH_3COS), 2.28 (q, 3J = 7.3 Hz, 2H; C = CCH_2), 1.86–1.74 (m, 4 H; C = CH_2CH_2 , NBDNH- CH_2CH_2), 1.59–1.20 (m, 26H; $2\text{ArNCH}_2\text{CH}_3$, 10CH_2); ^{31}P NMR (MHz, CDCl_3 , 25 $^\circ\text{C}$, H_3PO_4): δ = –0.22, –0.25 ppm; ^{13}C NMR (DEPT-135, 100 MHz, CDCl_3 , 25 $^\circ\text{C}$): δ = 136.45, 133.39, 130.82, 125.81, 125.39, 120.94, 107.74, 105.46, 96.23, 71.74, 68.27, 67.17, 66.39, 64.28, 63.32, 50.26, 45.08, 33.56, 33.45, 31.92, 30.53, 29.68, 29.57, 29.42, 29.35, 29.14, 28.47, 28.26, 27.10, 26.92, 25.97, 24.53, 23.11, 15.87, 14.12, 12.60; FAB HRMS (NBA, positive mode): $[M+H]^+$ calcd: 1252.4712, found: 1252.4734.

1-O-(12-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-dodecyl)-2-O-(14-(7-nitrobenz-2-oxa-1,3-diazol-4-ylamino)-5(Z),8(Z),11(Z)-tetra-decenoyl- sn-glycerol phosphatidylethanolamin [PENN3] (7)

According to the general procedure, *sn*-3 alcohol **32** (32 mg, 33 μ mol) was treated with 4,5-dicyanoimidazole (16 mg, 0.132 mmol) and the *tert*-butyl/Boc-protected phosphoramidite **PI** (25 mg, 0.067 mmol). The oxidation was performed by addition a solution of *t*BuOOH in nonane (5.5 M, 45 μ L). After 2 h, all volatile compounds of the reaction mixture were removed under reduced pressure. As in the case of PENN2, the ethanolamine was deprotected in situ to the desired final product by treatment with dichloromethane/TFA (90:10, 5 mL) for 40 min. The dichloromethane/TFA mixture was removed under reduced pressure, and the resulting crude product was subjected to column chromatography (dichloromethane/methanol 90:10→80:20). Product-containing fractions were combined, and the solvents were removed under reduced pressure to yield PENN3 (8 mg, 7.6 μ mol, 23%). R_f = 0.57(dichloromethane/methanol, 80:20); ^1H NMR (400 MHz, $\text{CD}_3\text{OD}/\text{CDCl}_3$ (1:1), 25 $^\circ\text{C}$, TMS): δ = 8.38 (d, 3J = 8.6 Hz, 1H; NBD), 8.14 (d, 3J = 8.6 Hz, 1 H; Nile red H4), 7.92 (d, 4J = 2.0 Hz, 1 H; Nile red H1), 7.50 (d, 3J = 8.8 Hz, 1 H; Nile red H11), 7.10 (dd, 3J = 8.7 Hz, 4J = 2.1 Hz, Nile red H3), 6.61 (dd, 3J = 9.1 Hz, 4J = 2.3 Hz, Nile red H10), 6.38 (d, 3J = 2.5 Hz, 1 H; Nile red H8), 6.24 (s, 1 H; Nile red H6), 6.11 (d, 7.8 Hz, 1 H; NBD), 5.63–5.50 (m, 1 H; CH olefin), 5.46–5.26 (m, 5H; CH olefin), 5.22–5.08 (m, 1 H; *sn*-2 CH), 4.36–3.81 (m, 6H; 2POCH_2 , ArOCH_2), 3.58–3.28 (m, 10H; *sn*-1 CH_2 , Nile red NCH_2 (20), *sn*-1- OCH_2 , NBD-NH- CH_2), 2.79 (m, 2H; = $\text{CHCH}_2\text{CH=}$), 2.71 (t, 3J = 5.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.53 (q, 3J = 6.7 Hz, 2 H; CH_2CH), 2.30 (m, 2H; CH_2CO_2), 2.03 (m, 2 H; $\text{CH}_2\text{CH=}$), 1.82 (qi, 3J = 7.4 Hz, 2H; OCH_2CH_2), 1.62 (qi, 3J = 7.3 Hz, 2H; $\text{OOCCH}_2\text{CH}_2$), 1.53–1.18 (m, 24H; CH_2 chain (9 $\times$), NCH_2CH_3

(2×)); ^{31}P NMR (162 MHz, $\text{CD}_3\text{OD}/\text{CDCl}_3$ (1:1), 25 °C, H_3PO_4): δ = 0.0 ppm; MS HR-FAB (NBA, positive mode): $[M+H]^+$ calcd: 1098.5317, found: 1098.5385.

1-O-(12-(9-Diethylamino-5-oxo-5H-benzo[a]phenoxazin-2-yloxy)-dodecyl)-2-O-(14-(7-nitro-benz-2-oxa-1,3-diazol-4-ylamino)-5(Z),8(Z),11(Z)-tetradecenoyl-3-O-((2-(acetylthio)ethoxy)-(2-((2-(acetylthio)ethoxy)carbonyl)amino-ethoxy)phosphoryl)-sn-glycerol [PENN3/SATE] (8)

According to the general procedure the *sn*-3 alcohol **32** (32 mg, 0.033 mmol) was treated with 4,5-dicyanoimidazole (16 mg, 0.132 mmol) and the SATE protected phosphoramidite **PII** (30 mg, 0.066 mmol). The oxidation was performed by adding a solution of *t*BuOOH in nonane (5.5 M, 45 μL). The resulting product was purified by preparative TLC on silica (dichloromethane/methanol, 90:10). The product band was extracted with dichloromethane/methanol (95:5) to obtain PENN3/SATE **8** as a deep-red oil (21 mg, 0.016 mmol, 48%). R_f (dichloromethane/methanol 90:10) = 0.57; ^1H NMR (400 MHz, CDCl_3 , 25 °C, TMS): δ = 8.49 (d, 3J = 8.6 Hz, 1 H; NBD), 8.23 (d, 3J = 8.6 Hz, 1 H; Nile red H4), 8.05 (d, 4J = 2.5 Hz, 1H; Nile red H1), 7.62 (d, 3J = 9.1 Hz, 1 H; Nile red H11), 7.18 (dd, 3J = 8.6 Hz, 4J = 2.5 Hz, Nile red H3), 6.85 (s, 1H; NHNBD), 6.69 (dd, 3J = 9.1 Hz, 4J = 2.8 Hz, Nile red H10), 6.48 (d, 3J = 2.5 Hz, 1 H; Nile red H8), 6.33 (s, 1 H; Nile red H6), 6.18 (d, 8.8 Hz, 1 H; NBD), 5.69–5.60 (m, 1H; CH olefin), 5.56–5.31 (m, 6H; 5CH olefin, NH carbamate), 5.23–5.15 (m, 1H; *sn*-2 CH), 4.31–4.09 (m, 10H; 3 POCH_2 , NHCOOCH_2 , ArOCH_2), 3.56 (d, 3J = 5.3 Hz, 2H; *sn*-1 CH_2), 3.49 (q, 3J = 7.2 Hz, 4H; Nile red NCH_2 (2×)), 3.55–3.38 (m, 6H; *sn*-1- OCH_2 , NBD-NH- CH_2 , OOCNHCH_2), 3.19 (m, 2 H; COSCH_2), 3.13 (t, 3J = 6.4 Hz, 2H; COSCH_2), 2.86 (t, 3J = 6.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.79 (t, 3J = 5.7 Hz, 2H; = $\text{CHCH}_2\text{CH=}$), 2.60 (q, 3J = 7.0 Hz, 2H; CH_2CH), 2.38 (t, 3J = 6.8 Hz, 2H; CH_2CO_2), 2.37 (s, 3H; CH_3COS), 2.36 (s, 3H; CH_3COS), 2.10 (qi, 3J = 6.7 Hz, 2 H; $\text{CH}_2\text{CH=}$), 1.87 (qi, 3J = 7.4 Hz, 2 H; OCH_2CH_2), 1.71 (qi, 3J = 7.3 Hz, 2 H; $\text{OOCCH}_2\text{CH}_2$), 1.59–1.18 (m, 24H; 9 CH_2 chain, 2 NCH_2CH_3); ^{31}P NMR (162 MHz, CDCl_3 , 25 °C, H_3PO_4): δ = -1.20, -1.24 ppm; ^{13}C NMR (DEPT-135, 100 MHz, CDCl_3 , 25 °C): δ = 136.47, 132.18, 131.07, 129.01, 128.84, 128.59, 127.72, 127.28, 124.86, 118.30, 109.63, 106.63, 105.23, 96.35, 88.32, 77.23, 75.18, 71.89, 70.73, 70.66, 68.42, 68.26, 66.35, 66.17, 66.11, 63.36, 45.13, 43.39, 42.67, 41.31, 33.61, 30.58, 29.61, 29.53, 29.47, 29.40, 29.23, 29.13, 29.05, 28.23, 26.50, 26.36, 26.08, 26.02, 25.72, 25.67, 24.66, 12.62; FAB HRMS (NBA, positive mode): $[M+H]^+$ calcd: 1346.5494, found: 1346.5448.

sPLA₂ in vitro studies

A stock solution of each unprotected phosphatidylethanolamine probe (**1**, **3**, **5**, **7**) was prepared in DMSO (10 mM) and adjusted to identical absorption at 450 nm. These stocks (0.5 μL) were diluted with the assay buffer (5 mL) to get a final concentration of 1 μM . This solution (1 mL) was placed into a disposable fluorimeter cuvette and fluorescence spectra were recorded at 37 °C until the system was equilibrated. Subsequently, 0.5 units of phospholipase A₂ from the indicated source were added, and spectra or timelapses were recorded over time.

For platereader experiments the same stock solutions were used. Aliquots (50 μL) of these were transferred to the wells in a 384-well clear-bottom plate. The indicated amount of enzyme was added, and fluorescence was measured overnight in 10 min intervals at room temperature. All measurements were performed in triplicates.

Live cell microscopy

A stock solution of 2 mM SATE-protected probe (**2**, **4**, **6**, **8**) 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.

HeLa cells were plated in 4-chambered dishes and grown overnight to a confluence of about 40%. The medium was taken off, and the PENN3/SATE-containing incubation buffers (0.5

mL) were placed into each chamber. After incubation with these solutions at 37°C for 40 min, the buffer was removed, and the cells were washed with HEPES buffer (3×). Finally they were monitored in the same buffer on a widefield microscope with a DAPI, NBD or Texas Red filter setup at room temperature.

SATE deprotection studies

A solution PENN/SATE in DMSO (2 µL, 2 mM) was taken up in Triton X-100 assay buffer (4 mL) and sonicated for 5 min. Three disposable fluorescence cuvettes were filled with this solution (1 mL). The NBD and Nile red emission upon excitation at 458 nm was measured over time at 37 °C. The first cuvette was treated ($t = 0$ min) with 0.5 units of sPLA₂ from bee venom and subsequently with lipase from *C. cylindracea* (2 units, $t = 40$ min). In a second control cuvette PENN/SATE was not treated in the beginning, but after 40 min, 2 units of lipase were also added. This cuvette was further treated with 0.5 units of sPLA₂ from honey bee venom after 170 min.

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Abbreviations

PENN is an abbreviation for phosphatidylethanolamine labeled with the dyes Nilered and NBD, PMNN is the corresponding phosphatidylmethanol derivative

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Figure 1.

A) Cleavage of PENN2 after treatment with 0.2 units sPLA₂ from bee venom at pH 8.6 in a Triton X-100 mixed-micelle buffer, monitored by changes in the emission spectra. Excitation was at 458 nm. Probe concentrations were adjusted to Nile Red emission after excitation at 540 nm. For comparison: B), C), and D) show the spectra before (black) and after sPLA₂ treatment (gray) of PENN, PENN3 and PMNN2 respectively.

Figure 2.

The acceptance of PLA₂ substrates PENN2 (**3**, dotted curve), PMNN2 (**5**, dash-dot) and PENN (**1**, straight) by human PLA₂ group V differed markedly. This demonstrates that this secretory isoform is preferentially monitored by PENN2. Probes were taken up in Triton X-100 assay buffer (pH 8.6; final concentration 1 μ M), transferred to a 384-well plate (50 μ L per well) and treated with enzyme.

Figure 3.

Substrate properties of the PENN derivatives **1**, **3**, **5**, **7**: Probes were taken up in Triton X-100 assay buffer (final concentration 1 μ M), transferred to a 384-well plate (50 μ L per well) and treated with sPLA₂ from the indicated source. The change of emission ratio (632/540 nm; excitation at 458 nm) was measured every 10 min for 12 h. Relative selectivities are depicted as activities normalized to the slope over the first 50 min of the enzymatic reaction.

Figure 4.

Release of PLA₂ substrate in vitro: A) Enzymatic hydrolysis of SATE groups leads to PLA₂ substrate formation. B) PLA₂ hydrolysis of PENN/SATE requires activation by esterase. Straight curve: PENN/SATE (**2**) was incubated at 37 °C, pH 8.6 with 0.5 units sPLA₂ from honey bee venom in a Triton X-100 based mixed micelle system. After the indicated time (A) lipase from *C. cylindracea* was added and a change in FRET ratio could be detected; this indicated the release of the PLA₂ substrate. Dotted line: The lipase alone was not able to cleave the *sn*-2 ester bond. A detectable change in ratio only occurred after the addition of honey bee sPLA₂ (B). Dashed line: Control without enzymes.

Figure 5. Comparison of resting HeLa cells (left column) to FCS-induced cells (right column). A) PENN/SATE (1) is the best in vivo substrate, followed by B) PENN2/SATE (3), while C) PENN3/SATE (8) shows no sign of cleavage. Data are representative to five separate experiments. All images show the overlay of NBD (515–555 nm, green) and Nile Red (600–660 nm, red) emission. Nuclei of HeLa cells were counterstained with Hoechst 33342.

Scheme 1.

Structures of PLA₂-sensitive phospholipids PENN (1), PENN2 (3), PMNN2 (5), and PENN3 (7), as well as the membrane-permeant derivatives protected by S-acetyl thioethyl (SATE) groups.

Scheme 2.

Synthesis of PENN2 (**3**) and PENN2/SATE (**4**). Reaction conditions: a) BuLi (2 equiv.), THF, $-78 \rightarrow 0^\circ\text{C}$, THP-OCH₂-C $\equiv$ CH, DMSO; b) MeOH, HCl (cat.), reflux, 3 h; c) CBr₄, Ph₃P, DCM, RT, 20 min; d) 3-OH Nile Red, K₂CO₃, DMF, 80 $^\circ\text{C}$, 4 h; e) H₂, Lindlar catalyst (10 mol %), quinoline, RT, 30 min; f) esterase, phosphate buffer (pH 8), 37 $^\circ\text{C}$, 5 d; g) **16**, TPSNT, MeIm, DCM, RT, 13 h; h) DOWEX WX8, DCM, 25 $^\circ\text{C}$, 4 h; i) i: **PII**, DCI, DMF, RT, 16 h; ii: *t*BuOOH, DMF, RT, 2 h, (in the case of **3**: i: **PI**, DCI, DMF, RT, 16 h; ii: *t*BuOOH, DMF, RT, 2 h; iii: TFA, DCM, 25 $^\circ\text{C}$, 1 h; or in the case of **6**: i: **PIII**, DCI, DMF, RT, 16 h; ii: *t*BuOOH, DMF, RT, 2 h). For the structures of phosphitylation agents, see Scheme 3)

Scheme 3.

Synthesis of a NBD-labeled fatty acid with three Z double bonds. Reaction conditions: a) trimethylsilylethanol, EDC, DMAP, CH_2Cl_2 , 25 °C, 16 h; b) 1,4-Dichlorobutynyl, NaI, K_2CO_3 , CuI, 25 °C, 12 h; c) 3-Butynol, NaI, K_2CO_3 , CuI, 25 °C, 12 h; d) Lindlar catalyst, Quinoline, THF, 25 °C, 90 min; e) DPPA, Ph_3P , DIAD, THF, 0 °C, 2 h; f) Ph_3P , THF, water, 25 °C, 3 h; g) NBD-Cl, MeOH, DIPEA, 0→25 °C, 17 h; h) TFA, DCM, 25 °C, 1 h.

Scheme 4.

Synthesis of the FRET probe PENN3 (**7**) and its membrane-permeant derivative PENN3/SATE (**8**). Reaction conditions: a) 2-OH Nile Red, K_2CO_3 , DMF, 70 °C, 3 h; b) DMTCl, DMAP (cat.), Et_3N , DMF, 25 °C, 13 h; c) **27**, TPSNT, Melm, DCM, 25 °C, 11 h; d) DOWEX WX8, DCM, 25 °C, 4 h; e) i: **PI**, DCl, DMF, 25 °C, 16 h; ii: *t*BuOOH, DMF, 25 °C, 2 h; iii: 10 % TFA in DCM, 1 h; f) i: **PII**, DCl, DMF, 25 °C, 16 h; ii: *t*BuOOH, DMF. On the right the phosphoramidites **PI** to **PIII** used to introduce the various protected headgroups.