ELECTRONIC SUPPLEMENTARY INFORMATION

Dynamic reactions of liposomes

Sebastian A. Berg and Bart Jan Ravoo*

Organic Chemistry Institute, Westfälische Wilhelms-Universität Münster, Corrensstrasse 40, 48149 Münster, Germany

Email: b.j.ravoo@uni-muenster.de

Table of contents

Additional measurements and figures

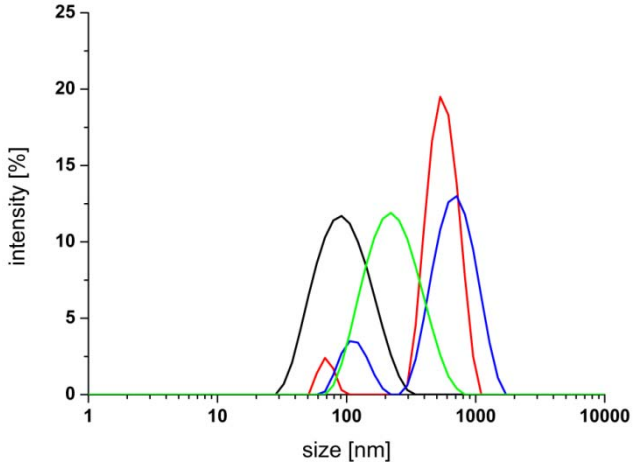


Figure S1: DLS measurement of liposomes with amphiphilic thioester **(1)** before and 16.5 h after aggregation with dithiol. (**black:** before aggregation; **red:** 10 mM dithiol; **blue:** 15 mM dithiol; **green:** 20 mM dithiol.

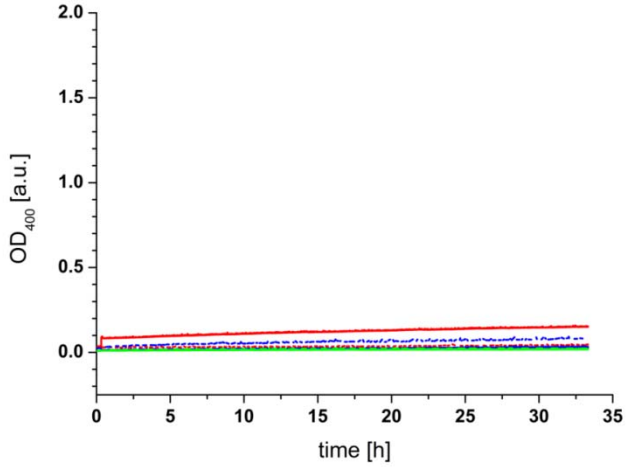


Figure S2: OD_{400} measurement of liposomes with amphiphilic thioester (**1)** and added thioglycerol (**4**) Conditions: 0.1 mM lecithin; 0.025 mM (**1)**; 100 mM phosphate buffer at pH 8.00; T = 50 °C; 200 rpm; initial liposome size: 100 nm; thioglycerol addition after 10 min; **red:** 10 mM thioglycerol, **blue:** 15 mM thioglycerol, **green:** 20 mM thioglycerol.

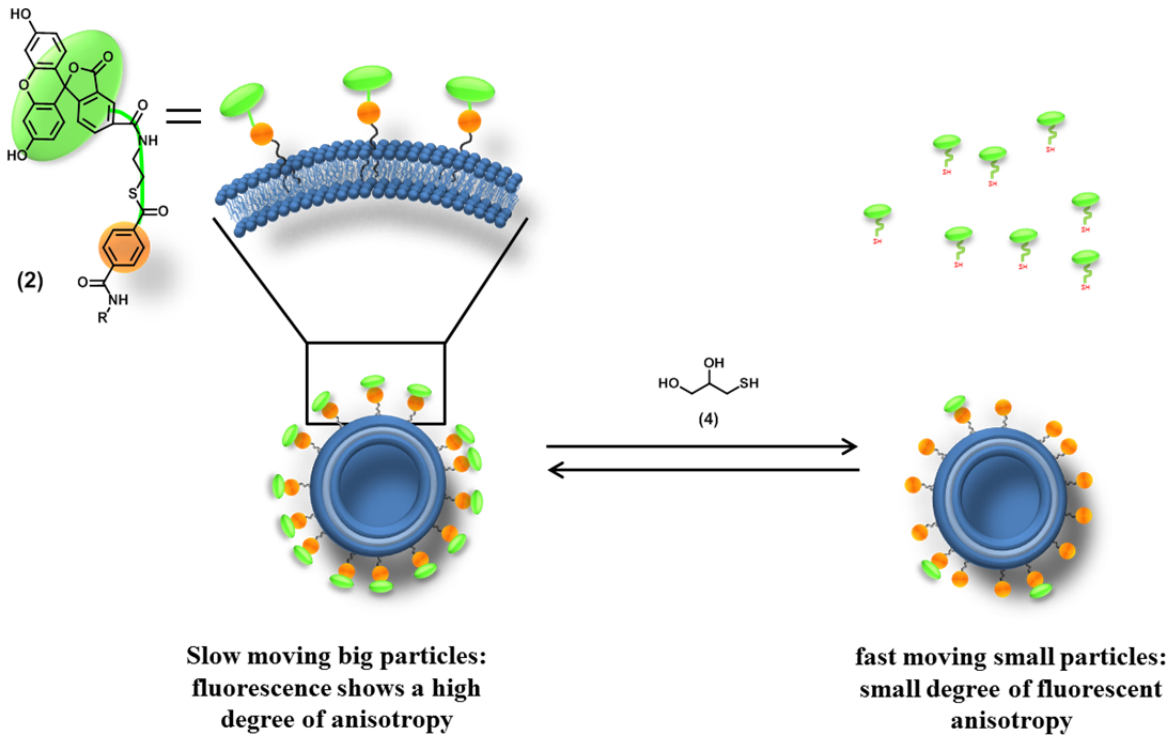


Figure S3: Schematic representation of the fluorescence polarization experiments. On the left side of the equilibrium, fluorescein thioester (**2**) is embedded in the liposomes, so that it diffuses relatively slowly in solution. The fluorescence anisotropy is substantial. As a result of a thioester exchange reaction with thioglycerol (**4**), fluorescein thiol (**14**) dissociates from the liposomes and diffuses into the solution. Hence, on the right side of the equilibrium, the fluorescence anisotropy is negligible.

Formulas

The calculation of the ratio of small to large liposomes is based on the assumption that all liposomes present in solution are perfectly shaped spheres with a single bilayer of phospholipids and that the change in liposome size does not affect the ratio of lipids between the inner and the outer layer. Furthermore the concentrations of amphiphiles in both solutions are the same.

$$\frac{c(liposomes_{small})}{c(liposomes_{large})} = \frac{A(liposomes_{large})}{A(liposomes_{small})} = \frac{\pi d_{large}^2}{\pi d_{small}^2} = \frac{\pi(124\ nm)^2}{\pi(74\ nm)^2} = \frac{48305\ nm^2}{17203\ nm^2} \approx 2.8$$

Formula S1: Calculation of the ratio of small to large liposomes at the same concentration of amphiphiles in both solutions. c = concentration, A = surface of a liposome, d = diameter of a liposome.

Materials and methods

Chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), ABCR (Karlsruhe, Germany), Acros Organics (Nidderau, Germany), Alfa Aesar (Karlsruhe, Germany), Iris Biotech GmbH (Marktredwitz, Germany) or Carl Roth (Karlsruhe, Germany) and were used without further purification. Thin layer chromatography was performed on Merck (Darmstadt, Germany) analytical TCL plates (60 F_{254} silica gel plates). Visualization of compounds was achieved either by UV light irradiation at 254 or 366 nm or by dipping in basic permanganate solution. Silica gel with a grain size of 40 – 65 µm (Merck, Darmstadt, Germany) was used for preparative silica gel chromatographie. For all measurements in aqueous solutions MilliQ water prepared by a PureLab UHQ purification unit (ELGA, High Wycombe, UK) with a resistance greater than 18 MΩ was used. All pH values were adjusted with a freshly calibrated S220 SevenCompact™ pH/ion meter (Mettler Toledo GmbH, Gießen, Germany). UV/Vis-spectroscopy was carried out in PMMA cuvettes (Brand GmbH & Co. KG, Wertheim, Germany) with a V-650 spectrophotometer from JASCO (Gross-Umstadt, Germany) equipped with a temperature controlled PAC-743 automatic 6-position cell changer from JASCO. Fluorescence anisotropy measurements were performed in semi-micro quartz-glass cuvettes (Hellma Analytics, Müllheim, Germany) with a FP-6500 spectrofluorimeter (JASCO, Gross-Umstadt, Germany) and a manual FDP-223 polarization equipment from JASCO. DLS measurements were carried out with a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). All data was plotted and analyzed using Origin Pro (version 8.5.0G, OriginLab Corporation, Northampton, USA). Mass spectrometry measurements were either recorded on a LTQ Orbitrap XL (Thermo-Fisher Scientific, Bremen, Germany) or on a MicroTof (Bruker Daltronics, Bremen, Germany) system. NMR spectroscopic measurements were recorded using a Bruker AV 300, DPX300 or AV400 (Bruker Analytische Messtechnik, Karlsruhe, Germany) or a DD2 600 from Agilent Technologies (Böblingen, Germany). Chemical shifts were referenced to the residual solvent peak and the data was analyzed using MestReNova (version 8.1.0-11315, Mestrelab Research S.L., Santiago de Compostela, Spain).

Preparation of phosphate buffer

Phosphate buffer (PB) was prepared by mixing 100 mM stock solutions of $NaH_2PO_4 * 2H_2O$ and $Na_2HPO_4 * 12H_2O$ until the desired pH of 8.00 was reached.

Liposome preparation

Stock solutions of soy bean lecithin and thioester **(1)** in organic solvents were mixed to achieve the desired ratio. After drying the solution for at least 1 h in a gentle argon stream 3 ml of PB were added and the solution was subjected to an ultra-sonication bath for 10 s. Afterwards the solution was transferred to a manual extruder equipped with two 2.5 ml Hamilton® syringes (Hamilton Messtechnik GmbH, Höchst, Germany) and two polycarbonate membranes with a poresize of 100 nm. The system was heated to 60 °C and extruded 11 times

at this temperature. The resulting liposome solution was diluted with 100 mM PB pH 8.00 to the desired concentration and size of the liposomes was measured with DLS. For the preparation of 50 nm liposomes a solution of freshly extruded 100 nm liposomes was again subjected to 19 extrusion cycles through a 50 nm polycarbonate membrane and the size of the liposomes was measured with DLS.

Aggregation experiments

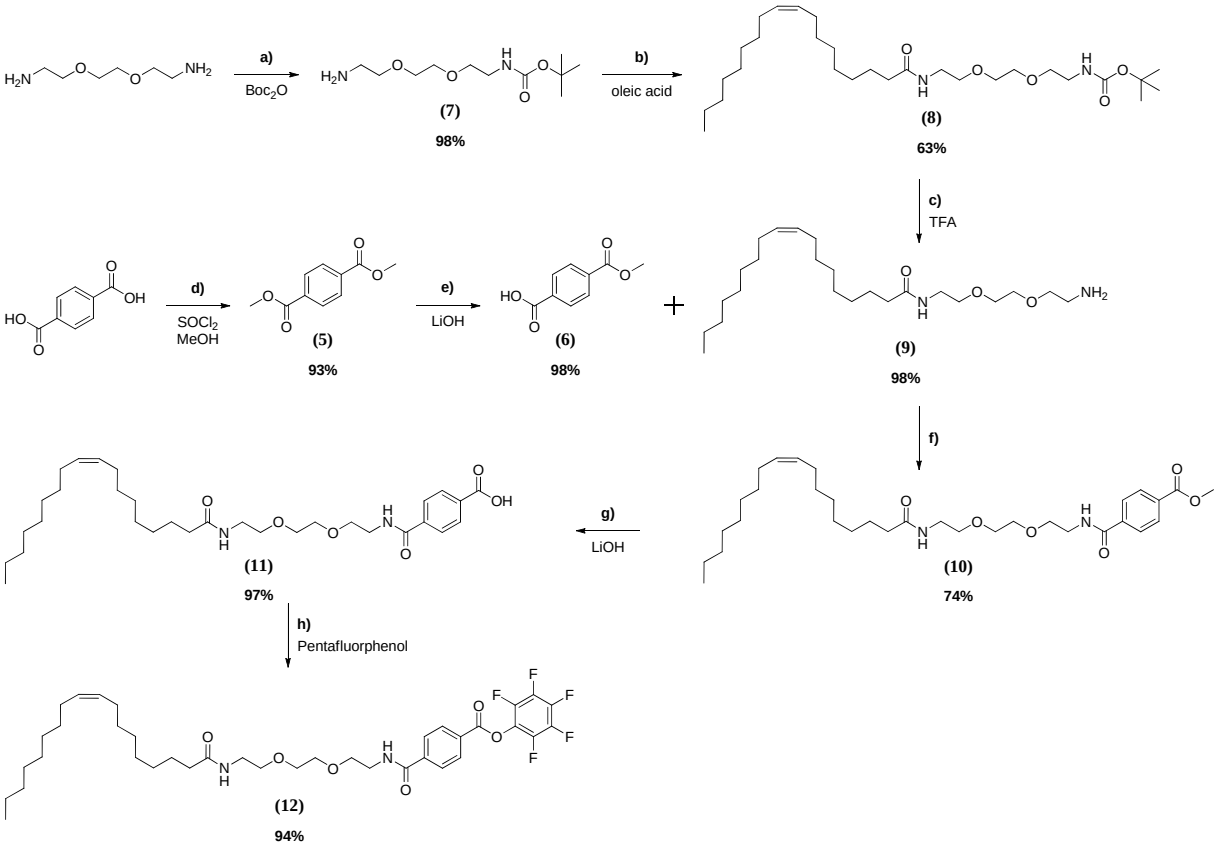
For aggregation experiments 2.5 ml of extruded liposome solution was transferred to semi-micro PMMA cuvettes with a volume of 3 ml equipped with a small stirring bar. After capping the cuvettes they were placed in the pre heated cell changer of the UV device at 50 °C and were equilibrated to the temperature for 30 min while stirring with 200 rpm. The optical density measurements were conducted at the same temperature and stirring rate measuring at a wavelength of 400 nm. Approximately 10 min after starting the measurement stock solutions of dithiol (in ethanol absolute) or respectively thioglycerol (in 100 mM PB pH 8.00) were added with an eppendorf® pipette agitating and mixing the solution thoroughly in the process to avoid any gradients of reactant due to the addition. The cuvettes were again capped and additionally sealed with parafilm® and the OD_{400} measurement was continued as stated in the measurements. After each measurement the size of the liposomes or aggregates was measured with DLS.

Fluorescence polarization measurements

For fluorescence polarization measurements liposomes consisting purely of soy bean lecithin were prepared and extruded before the fluorescein thioester **(2)** was added as a concentrated stock solution in MeOH resulting in a concentration of 0.1 mM lecithin and 0.0025 mM thioester **(2)**. Next a thioglycerol stock solution (in 100 mM PB pH 8.00) was added to the samples resulting in sample concentrations of 1 mM, 0.5 mM, 0.2 mM and 0.15 mM thioglycerol **(4)**. Directly after addition of the thioglycerol **(4)** the anisotropy of each sample was measured by transferring approx. 1 ml of labeled liposome solution to a semi-micro quartz glass cuvette, placing it the fluorescence device and measuring the anisotropy by manually adjusting the polarization filters to the appropriate values. All measurements were performed three times and the average value was calculated from these. Subsequently the samples were placed in an oil bath at 50 °C and stirred at 200 rpm. For all following measurements they were taken out of the oil bath, cooled to room temperature for 5 min and the anisotropy was measured again.

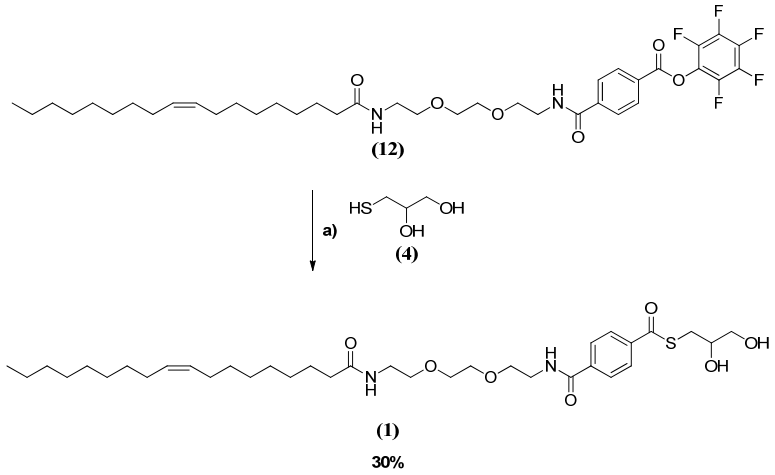
Synthesis

The synthesized amphiphilic thioesters consist of a hydrophobic chain to facilitate the incorporation into the membrane of liposomes. Therefore oleic acid was chosen that was coupled via peptide coupling to an ethylene glycol spacer. The hydrophobic ethylene glycol unit is incorporated to enhance the accessibility of the thioesters when they are incorporated into the phospholipid bilayer. As aromatic unit a terephthalic acid was used that was coupled to the second end of the ethylene glycol spacer and transformed to the active pentafluorophenol ester (**Scheme S1**).



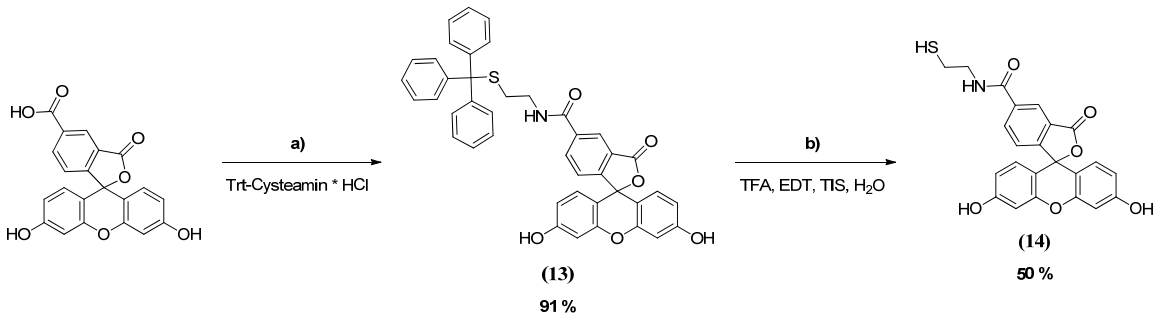
Scheme S1: Synthesis of the activated terephthalic amphiphile **(**12**)**. Reaction conditions: **a)** Boc_2O, Dioxane, 16 h, 98 %; **b)** oleic acid, NMM, HOBt, EDC*HCl, CH_2Cl_2, 16 h, 63 %; **c)** TFA/DCM 1:1, 2 h, 92 %; **d)** $SOCl_2$, MeOH, 12 h reflux, 93 %; **e)** LiOH, MeOH, 3.5 h reflux, 98 %; **f)** NMM, HOBt, EDC*HCl, CH_2Cl_2, 16 h, 74 %; **g)** LiOH, H_2O/THF, 9 h, 97 %; **h)** pentafluorophenol, NMM, EDC*HCl, CH_2Cl_2, 16 h, 94 %.

The conversion of the active pentafluorophenol ester **(12)** to the amphiphilic thioglycerol thioester **(1)** was conducted under concentrated conditions in peptide grade DMF (**Scheme S2**).



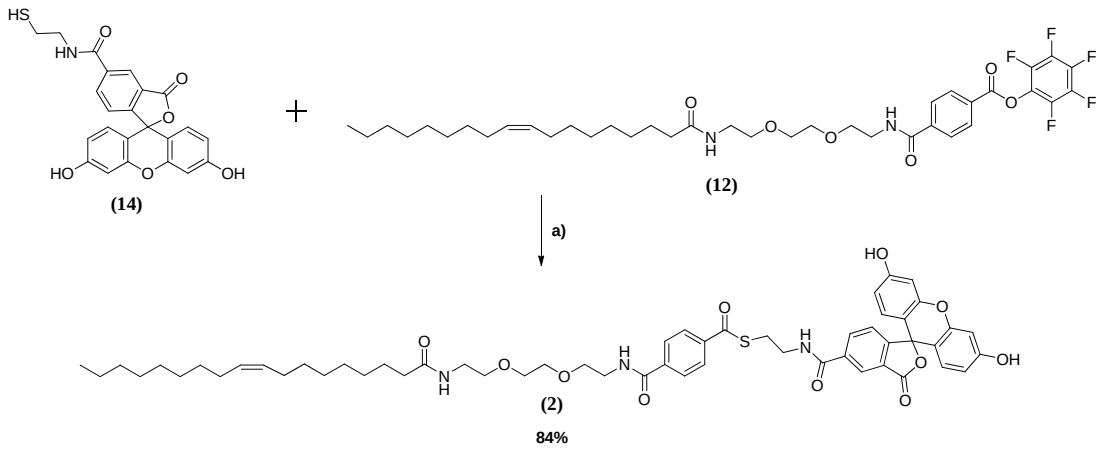
Scheme S2: Synthesis of the amphiphilic thioglycerol thioester **(1)**. Reaction conditions: **a)** Thioglycerol **(4)**, DIPEA, peptide grade DMF, 16 h, 32 %.

For the synthesis of the fluorescein thioester **(2)** cysteamin was coupled to 5(6)-carboxyfluorescein via peptide coupling using HATU (**Scheme S3**).



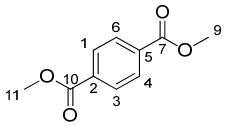
Scheme S3: Synthesis of the fluorescein thiol **(14)**. Reaction conditions: **a)** DIPEA, HATU, peptide grade DMF, Trt-cysteamine*HCl, 16 h, 91 %; **b)** TFA, EDT, TIS, H_2O, 9 h, 50 %.

The synthesis of the fluorescein thioester **(2)** was performed in a concentrated solution of peptide grade DMF (**Scheme S4**).



Scheme S4: Synthesis of the fluorescein thioester **(2)**. Reaction conditions: **a)** DIPEA, peptide grade DMF, 16 h, 84%.

Dimethyl terephthalate (5)



According to literature[1] terephthalic acid (2.00 g, 12.0 mmol, 1.00 eq.) was solubilized in MeOH p.a. (80 ml) and under vigorous stirring thionyl chloride (36.0 ml, 59.0 g, 496 mmol, 41.3 eq.) was added drop wise. After refluxing for 12 h the solvent was removed under reduced pressure and the residual was extracted with EtOAc (2x 50 ml). The combined organic layers were washed with saturated sodium bicarbonate solution (2x 100 ml) and dried with $MgSO_4$. After removing the solvent under reduced pressure and drying in high vacuum the product was obtained as a white solid.

Molecular formula: $C_{10}H_{10}O_4$ (194.18 g/mol)

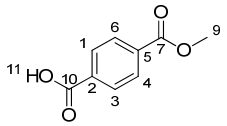
Yield: 93 % (2.16 g, 11.1 mmol, white solid).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{10}H_{10}O_4Na]^+$: 217.0471; found: 217.0476.

^{1}H-NMR(300 MHz, $CDCl_3$, 297 K): δ = 8.01 (s, 4H, H-1, H-3, H-4, H-6), 3.86 (s, 6H, H-9, H-11) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 298 K): δ = 166.31 (C-7, C-10), 133.97 (C-2, C-5), 129.61 (C-1, C-3, C-4, C-6), 52.48 (C-9, C-11) ppm.

Terephthalic monomethyl ester (6)



Following the literature[1] **(5)** (2.18 g, 11.2 mmol, 1.00 eq.) and potassium hydroxide (0.630 g, 11.2 mmol, 1.00 eq.) were dissolved in MeOH and refluxed for 3.5 h. The solvent was removed under reduced pressure and the residual was taken up in water and extracted with EtOAc (2x 50 ml). After adjusting the pH to 2-3 with HCl the resulting precipitate was extracted with EtOAc (3x 50 ml). The combined organic layers were dried with $MgSO_4$, the solvent was removed under reduced pressure and after drying under high vacuum the product was obtained as a white solid.

Molecular formula: $C_9H_8O_4$ (180.16 g/mol).

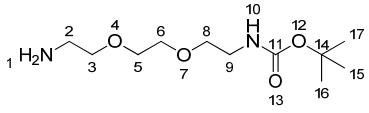
Yield: 98 % (1.99 g, 11.1 mmol, white solid).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_9H_8O_4Na]^+$: 203.0315; found: 203.0329.

^{1}H-NMR(300 MHz, DMSO-d_6, 293 K): δ = 8.07 – 8.01 (m, 4H, H-1, H-3, H-4, H-6), 3.87 (s, 3H, H-9) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 294 K): δ = 166.63 (C-7), 165.67(C-10), 134.85(C-2), 129.65 (C-4, C-6), 129.54 (C-5), 129.40 (C-1, C-3), 52.52 (C-9) ppm.

tert-Butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (7)



As described in literature[2] a solution of Boc anhydride (2.20 g, 10,1 mmol, 1.00 eq.) in dioxane (15 ml) was added drop wise over a period of 2 h to a solution of 2,2′-(Ethylenedioxy)bis(ethylamine) (8.90 ml, 9.03 g, 61.0 mmol, 6,04 eq.) in Dioxane (60 ml). After stirring overnight and removal of the solvent under reduced pressure the residual yellow oil was solubilized in water (20 ml) and extracted with DCM (4x 25 ml). The combined organic layers were washed with brine (50 ml), dried over $MgSO_4$, and after removal of the solvent under reduced pressure the product was dried under high vacuum yielding a colorless oil.

Molecular formula: $C_{11}H_{24}N_2O_4$ (248.32 g/mol).

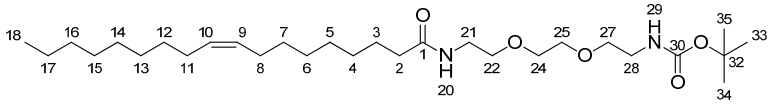
Yield: 98 % (2.459 g, 9.90 mmol, colorless oil).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{11}H_{24}N_2O_4H]^+$: 249.1809; found: 249.1814.

^{1}H-NMR(300 MHz, DMSO-d_6, 293 K): δ = 6.80 (t, J = 6.0 Hz, 1H, H-10), 3.49 (d, J = 2.3 Hz, 4H, H-5, H-6), 3.43 – 3.29 (m, 4H, H-3, H-8), 3.06 (q, J = 6.0 Hz, 2H, H-9), 2.63 (t, J = 5.8 Hz, 2H, H-2), 1.37 (s, 9H, H-15, H-16, H-17)

^{13}C-NMR (75 MHz, DMSO-d_6, 293 K): δ = 155.58 (C-11), 77.54 (C-14), 73.17 (C-3), 69.62-69.49 (C-5,6), 69.20 (C-8), 41.41 (C-2), 39.70 (C-9) 28.21 (C-15 – C-17) ppm.

(Z)-tert-Butyl (2-(2-(2-oleamidoethoxy)ethoxy)ethyl)carbamate (8)



Referring to literature[3] **(7)** (175 mg, 0.705 mmol, 1.99 eq.) and oleic acid (112 µl, 100 mg, 0.354 mmol, 1.00 eq.) were solubilized in DCM (45 ml) and NMM (400 µl, 362 mg, 3.58 mmol, 10.1 eq.), HOBt (60.0 mg, 0.389 mmol, 1.10 eq.), and EDC*HCl (75.05 mg, 0.389 mmol, 1.10 eq.) were added consecutively. After stirring overnight the reaction mixture was washed with $KHSO_4$ solution (2x 100ml, 10%wt), saturated sodium bicarbonate (2x

100 ml) and brine (1x 100 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using Ethylacetate as eluent.

Molecular formula: $C_{29}H_{56}N_2O_5$ (512.77 g/mol).

Yield: 63 % (114 mg, 0.222 mmol, yellow oil).

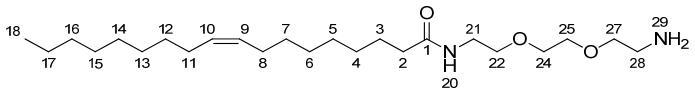
R_F-value (Ethyl acetate): 0.33.

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{29}H_{56}N_2O_5Na]^+$: 535.4081; found: 535.4082.

^{1}H-NMR(300 MHz, $CDCl_3$, 294 K): δ = 5.40 – 5.22 (m, 2H, H-9, H-10), 3.65 – 3.56 (m, 4H, H-22, H-27), 3.53 (t, J = 5.1 Hz, 4H, H-24, H-25), 3.43 (q, J = 5.2 Hz,, 2H, H-21), 3.30 (q, J = 5.4 Hz, 2H, H-28), 2.15 (t, J = 7.6 Hz, 2H, H-2), 1.98 (q, J = 6.2 Hz, 4H, 4H, H-8, H-11), 1.60 (t, J = 7.3 Hz, 2H, H-3), 1.42 (s, 9H, H-33, H-34, H-35), 1.25 (dd, J = 10.7, 3.2 Hz, 20H, H-4 – H-7, H-12 – H-17), 0.85 (t, J = 6.7 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 293 K): δ = 173.37 (C-1), 156.06 (C-30), 130.04-129.83 (C-9, C-10), 79.44 (C-32), 70.27 (C-22, C-24, C-25, C-27), 40.40 (C-28), 39.22 (C-21), 36.80 (C-2), 31.99 (C-16), 29.85-27.27 (C-4 – C-8, C-11 – C-15, C-33 – C-35), 25.85 (C-3), 22.77 (C-17), 14.22 (C-18) ppm.

N-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)oleamide (TFA Salt) (9)



Based on literature procedures[4] a 1:1 mixture of DCM and TFA (10.0 ml each) were added to **(8)** (0.112 g, 0.218 mmol, 1.00 eq.) and the solution was stirred for 6 h at RT. Afterwards the solvent was removed under reduced pressure. The crude product was three times solubilized in MeOH (20 ml) and dried under reduced before the product was dried under high vacuum yielding a yellow oil.

Molecular formula: $C_{24}H_{48}N_2O_3$ * TFA (526.67 g/mol).

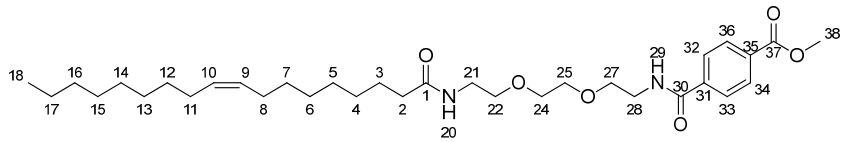
Yield: 98 % (0.112 g, 0.213 mmol, yellow oil).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{24}H_{48}N_2O_3H]^+$: 413.3738; found: 413.3750.

^{1}H-NMR(300 MHz, $CDCl_3$, 296 K): δ = 8.09 (s, 3H, H-29), 6.84 (s, 1H, H-20), 5.45 – 5.21 (m, 2H, H-9, H-10), 3.78 – 3.51 (m, 8H, H-22, H-24, H-25, H-27), 3.48 – 3.30 (m, 2H, H-21), 3.15 (s, 2H, H-28), 2.19 (t, *J* = 7.9, 7.3 Hz, 2H, H-2), 1.99 (d, *J* = 5.7 Hz, 4H, H-8, H-11), 1.69 – 1.46 (m, 2H, H-3), 1.26 (d, *J* = 5.2 Hz, 20H, H-4-H-7, H-12-H-17), 0.86 (t, *J* = 6.7 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 297 K): δ = 175.16 (C-1), 130.12-129.81 (C-9, C-10), 70.29 (C-27), 70.21 (C-24), 70.01 (C-25), 66.78 (C-22), 39.70 (C-28), 39.45 (C-21), 36.46 (C-2), 32.01 (C-16), 29.88-29.30 (C-4-C7, C-12-C15), 27.33-27.30(C-8, C-11), 25.94 (C-3), 22.79 (C-17), 14.22 (C-18) ppm.

(Z)-Methyl 4-((2-(2-(2-oleamidoethoxy)ethoxy)ethyl)amino)benzoate (10)



Based on literature[3] **(TFA** Salt) (9) (4.73 g, 8.98 mmol, 1.10 eq.) and **(6)** (1.47 g, 8.16 mmol, 1.00 eq) were dissolved in 180 ml DCM and NMM (3.30 ml, 3.30 g, 32.6 mmol, 4.00 eq.), HOBt (1.37 g, 8.98 mmol, 1.10 eq.) and EDC*HCl (1.72 g, 8.98 mmol, 1.10 eq.) were added. After stirring the solution overnight at RT the organic phase was washed with saturated sodium bicarbonate solution (2x 100 ml), $KHSO_4$ solution (10%wt, 2x 100 ml), saturated sodium bicarbonate solution (2x 100 ml) and brine (2x 100 ml), dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography using ethyl acetate / MeOH 10:0.5 as eluent.

Molecular formula: $C_{33}H_{54}N_2O_6$ (574,79 g/mol).

Yield: 74 % (0.769 g, 1.34 mmol, white solid).

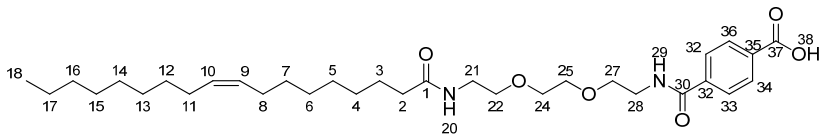
R_F-value (EtOAc/MeOH 10:0.5): 0.35.

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{33}H_{54}N_2O_6Na]^+$: 597.3874; found: 597.3872.

^{1}H-NMR(300 MHz, $CDCl_3$, 293 K): δ = 8.13 – 8.00 (m, 2H, H-32, H-33), 7.91 – 7.77 (m, 2H, H-34, H-36), 6.91 (s, 1H, H-20), 5.99 (s, 1H, H-29), 5.40 – 5.22 (m, 2H, H-9, H-10), 3.91 (s, 3H, H-38), 3.70 – 3.57 (m, 8H, H-22, H-24, H-25, H-27), 3.52 (t, *J* = 5.2 Hz, 2H, H-21), 3.40 (q, *J* = 5.2 Hz, 2H, H-28), 2.13 (t, *J* = 7.6 Hz, 2H, H-2), 1.97 (q, *J* = 6.6, 6.0, 5.9 Hz, 4H, H-8, H-11), 1.57 (p, *J* = 7.3 Hz, 2H, H-3), 1.25 (d, *J* = 4.2 Hz, 20H, H-4-H-7, H-12-H-17), 0.85 (t, *J* = 6.5 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 293 K): δ = 173.46 (C-1), 166.76 (C-30), 166.35 (C-37), 138.46 (C-31), 132.76 (C-9, C-10), 129.86 (C-34, C-36), 129.80 (C-35), 127.18 (C-32, C-33), 70.35 (C-27), 70.33 (C-24), 70.03 (C-25), 69.74 (C-22), 52.48 (C-38), 39.96 (C-28), 39.18 (C-21), 36.77 (C-2), 31.98 (C-16), 29.84-29.24 (C-4-C7, C-12-C15), 27.29-27.25 (C-8, C-11), 25.81 (C-3), 22.76 (C-17), 14.21 (C-18) ppm.

(Z)-4-((2-(2-(2-Oleamidoethoxy)ethoxy)ethyl)amino)benzoic acid (11)



Referring to literature[5] **(10)** (1.03 g, 1.80 mmol, 1.00 eq.) was solubilized in 30 ml THF and a solution of LiOH*H_2O (0.754 g, 18.0 mmol, 10.0 eq.) in H_2O (10 ml) was added. The reaction mixture was stirred for 9 h at RT before H_2O (150 ml) was added and the pH was adjusted with solid $KHSO_4$ to 2-3. The resulting precipitate was extracted with EtOAc (3x 100 ml) and the combined organic layers were washed with brine (100 ml). After drying with MgSO4 and removal of the solvent under reduced pressure the product was dried under high vacuum to obtain a white solid.

Molecular formula: $C_{32}H_{52}N_2O_6$ (560,77 g/mol).

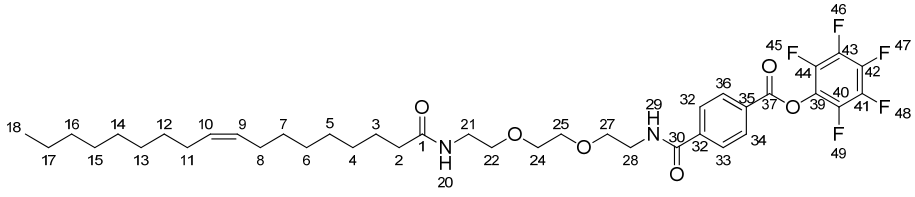
Yield: 97 % (0.981 g, 1.75 mmol, white solid).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{32}H_{52}N_2O_6Na]^+$: 583.3718; found: 583.3701.

^{1}H-NMR(300 MHz, DMSO-d_6, 293 K): δ = 8.70 (t, *J* = 5.4 Hz, 1H, H-29), 8.00 (d, *J* = 8.5 Hz, 2H, H-32, H-33), 7.98 – 7.88 (m, 2H, H-34, H-36), 7.81 (t, *J* = 5.5 Hz, 1H, H-20), 5.38 – 5.21 (m, 2H, H-9, H-10), 3.59 – 3.41 (m, 8H, H-22, H-24, H-25, H-27), 3.38 (t, *J* = 5.9 Hz, 2H, H-21), 3.16 (q, *J* = 5.7 Hz, 2H, H-28), 2.02 (t, *J* = 7.4 Hz, 2H, H-2), 1.95 (q, *J* = 6.1, 5.8, 5.3 Hz, 4H, H-8, H-11), 1.44 (p, *J* = 7.1 Hz, 2H, H-3), 1.21 (s, 20H, H-4-H-7, H-12-H-17), 0.83 (t, *J* = 6.9, 6.4 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, DMSO-d_6, 293 K): δ = 172.27 (C-1), 166.84 (C-30), 165.63 (C-37), 138.17 (C-30), 133.01 (C-37), 129.64(C-9, C-10), 129.27 (C-34, C-36), 127.45 (C-32, C-33), 69.63 (C-24, C-25), 69.24 (C-27), 68.86 (C-22), 39.34 (C-28), 38.48 (C-21), 35.36 (C-2), 31.36 (C-16), 29.20-28.64 (C-4-C7, C-12-C15), 26.67-26.63 (C-8, C-11), 25.34 (C-3), 22.17 (C-17), 13.98 (C-18) ppm.

(Z)-Pentafluorophenyl-4-((2-(2-(2-oleamidoethoxy)ethoxy)ethyl)amino)benzoate (12)



Pentafluorophenol (32.8 mg, 0.178 mmol, 1.00 eq.) was added under argon atmosphere to a solution of **(11)** (100 mg, 0.178 mmol, 1.00 eq.) in dry DCM (30 ml). Under argon NMM (1.70 µl, 1.50 mg, 14.8 µmol, 0.0830 eq.) and EDC*HCl (34.0 mg, 0.178 mmol, 1.00 eq.) were added to the solution and after stirring overnight under argon it was concentrated under reduced pressure. After purification of the crude product by silica gel column chromatography using ethyl acetate / MeOH 10:0.5 as eluent a beige solid was obtained.

Molecular formula: $C_{38}H_{51}F_5N_2O_6$ (726,81 g/mol).

Yield: 94 % (121 mg, 0.166 mmol, beige solid).

R_F-value (EtOAc/MeOH 10:0.5): 0.42.

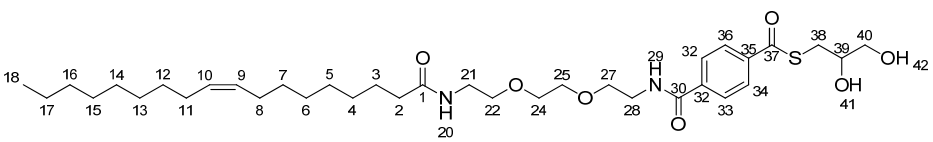
ESI-HRMS (MeOH) (m/z): Calculated for $[C_{38}H_{51}F_5N_2O_6Na]^+$: 749.3559; found: 749.3547.

^{1}H-NMR(300 MHz, $CDCl_3$, 293 K): δ = 8.26 (d, *J* = 8.4 Hz, 2H, H-32, H-34), 7.98 (d, *J* = 8.6 Hz, 2H, H34, H36), 6.97 (s, 1H, H-29), 5.96 – 5.83 (m, 1H, H-20), 5.42 – 5.23 (m, 2H, H-9, H-10), 3.79 – 3.61 (m, 8H, H-22, H-24, H-25, H-27), 3.57 (t, *J* = 5.2 Hz, 2H, H-21), 3.44 (q, *J* = 5.3 Hz, 2H, H-28), 2.16 (t, *J* = 7.6 Hz, 2H, H-2), 1.99 (q, *J* = 6.5 Hz, 4H, H-8, H-11), 1.60 (p, *J* = 7.5, 6.7, 6.6 Hz, 2H, H-3), 1.37 – 1.18 (m, 20H, H-4-H-7, H-12-H17), 0.87 (t, *J* = 6.6 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 293 K): δ = 173.60 (C-1), 166.31 (C-30), 161.98 (C-37), 140.10 (C-32), 131.07 (C-34, C-36), 130.14 (C-35), 129.82 (C-39), 127.72 (C-32, C-33), 70.47 (C-27), 70.35 (C-24), 70.10 (C-25), 69.81 (C-22), 40.15 (C-28), 39.25 (C-21), 36.90 (C-2), 32.04 (C-16), 29.90-29.29 (C-4-C7, C-12-C15), 27.35-27.30 (C-8, C-11), 25.85 (C-3), 22.83 (C-17), 14.26 (C-18) ppm.

^{19}F-NMR (282 MHz, $CDCl_3$, 293 K): δ = 152.25 – -152.49 (m, F-45, F-49), -157.40 (td, *J* = 21.8, 9.3 Hz, F-47), -161.82 – -162.16 (m, F-46, F-48).

(Z)-S-(2,3-Dihydroxypropyl) 4-((2-(2-(2-oleamidoethoxy)ethoxy)ethyl)amino)-benzothioate (1)



Thioglycerol (240 µl, 300 mg, 2.77 mmol, 10.1 eq.) and DIPEA (952 µl, 723 mg, 5.59 mmol, 20.3 eq.) were added consecutively to a solution of **(12)** (200 mg, 0.275 mmol, 1.00 eq.) in peptide grade DMF (10 ml). The solvent was removed under reduced presser after stirring overnight at RT and the crude product was purified two consecutive times by silica gel column chromatography using ethyl acetate / DCM / MeOH 5:5:1.5 as eluent. The pure product was obtained as a white solid.

Molecular formula: $C_{35}H_{58}N_2O_7S$ (650.91 g/mol).

Yield: 30 % (56.6 mg, 82.7 µmol, white solid).

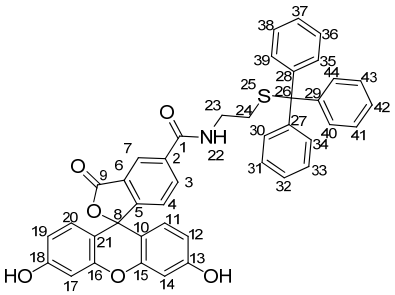
R_F-value (EtOAc/DCM/MeOH 5:5:1.5): 0.46.

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{35}H_{58}N_2O_7SNa]^+$: 673.3857; found: 673.3842.

^{1}H-NMR(300 MHz, $CDCl_3$, 293 K): δ = 8.10 (d, *J* = 8.5 Hz, 2H, H-34, H-36), 7.85 (d, *J* = 8.5 Hz, 2H, H-32, H-33), 6.93 (s, 1H, NH), 5.92 (t, *J* = 4.1 Hz, 1H, NH), 5.36 (p, *J* = 2.4 Hz, 2H, H-9, H-10), 4.47 (dd, *J* = 11.4, 4.0 Hz, 1H, H-40), 4.38 (dd, *J* = 11.4, 6.3 Hz, 1H, H-40´), 4.13 – 3.99 (m, 1H, H-39), 3.72 – 3.59 (m, 8H, H-22, H-24, H-25, H-27), 3.55 (t, *J* = 5.2 Hz, 2H, H-28), 3.41 (q, *J* = 5.2 Hz, 2H, H-21), 2.81 – 2.70 (m, 2H, H-38), 2.11 (t, J = 8.2, 7.3 Hz, 2H, H-2), 1.94 (s, 4H, H-8, H-11), 1.61 (t, *J* = 8.6 Hz, 2H, H-3), 1.31 – 1.19 (m, 29H, H-4 – H-7, H-12 – H-17), 0.87 (t, *J* = 6.6 Hz, 3H, H-18) ppm.

^{13}C-NMR (75 MHz, $CDCl_3$, 293 K): δ = 173.79 (C-37), 166.67 (C-1), 165.86 (C-30), 138.72 (C-35), 132.47 (C-32), 130.60 (C-9), 130.30 (C-10), 130.09 (C-34, C-36), 127.22 (C-32, C-33), 70.63 (C-22, C-27), 70.31 (C-24, C-25), 69.93 (C-39), 67.64 (C-40), 39.85 (C-28), 39.37 (C-21), 36.85 (C-2), 32.74-32.70 (C-38), 32.03 (C-16), 29.83-29.14 (C-4 – C-7) C-12 – C-15), 28.29 (C-8, C-11), 25.85 (C-3), 22.81 (C-17), 14.26 (C-18) ppm.

3',6'-Dihydroxy-N-(2-(tritylthio)ethanamine)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4-carboxamide (*Cysteamin(S-Trt)-Carboxyfluorescein)* (13)



5(6)-carboxyfluorescein (0.300 g, 0.797 mmol, 1.00 eq.) was solubilized under argon in dry peptide grade DMF (30 ml) and DIPEA (0.260 ml, 0.197 g, 1.52 mmol, 1.91 eq.) and HATU (0.288 g, 0.757 mmol, 0.950 eq.) were added consecutively. The solution was stirred for five min before Trt-cysteamine*HCl (0.270 g, 0.759 mmol, 0.952 eq.) and DIPEA (0.260 ml, 0.197 g, 1.52 mmol, 1.91 eq.) were added. After stirring overnight under argon the solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using chloroform / MeOH / formic acid 10:0.75:0.1 as eluent yielding a yellow to orange solid.

Molecular formula: $C_{42}H_{31}NO_6S$ (677.76 g/mol).

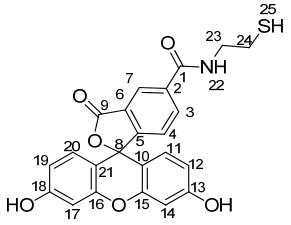
Yield: 91 % (0.494 g, 0.729 mmol, yellow to orange solid).

R_F-value ($CHCl_3$/MeOH/HCOOH 10:1:0.1): 0.45.

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{42}H_{31}NO_6SH]^+$: 678.1945; found: 678.1940.

^{1}H-NMR(300 MHz, DMSO-d_6, 293 K): δ = 8.75 (dd, J = 4.4, 1.4 Hz, 1H, H-7), 8.53 (dd, J = 8.4, 1.4 Hz, 1H, H-3), 7.51 (dd, J = 8.4, 4.4 Hz, 1H, H-4), 7.33 (d, J = 4.3 Hz, 6H, H-30, H-34, H-35, H-39, H-40, H-44), 7.29 – 7.16 (m, 9H, H-31 – H-33, H-36 – H-38, H-41 – H-43), 6.69 (dd, J = 5.0, 2.2 Hz, 2H, H-12, H-19), 6.67 – 6.51 (m, 4H, H-11, H-14, H-17, H-20), 3.23 (ddd, J = 34.3, 12.9, 6.6 Hz, 2H, H-23), 2.32 (dt, J = 35.8, 6.9 Hz, 2H, H-24) ppm.

3',6'-Dihydroxy-N-(2-mercaptoethyl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4-carboxamide (*Cysteamin(SH)-Carboxyfluorescein*) (14)



As described in the literature[6,7] a mixture of TFA (23.6 ml), H_2O (0.625 ml), 1,2-ethanedithiol (0.625 ml) and triisopropylsilane (0.250 ml) were added to **(13)** (0.516 mg, 0.761 mmol, 1.00 eq.). The red solution was stirred for 9 h at room temperature and was then concentrated under reduced pressure to approximately 5 ml. A mixture of diethyl ether and pentane (2:1, 200 ml) was added, the resulting emulsion was stored in a freezer at -18 °C for 1 h before the precipitate was collected by filtration over a pore 4 glass frit and was washed with ice cold diethyl ether (100 ml). The collected orange solid was used without further purification.

Molecular formula: $C_{23}H_{17}NO_6S$ (435.45 g/mol).

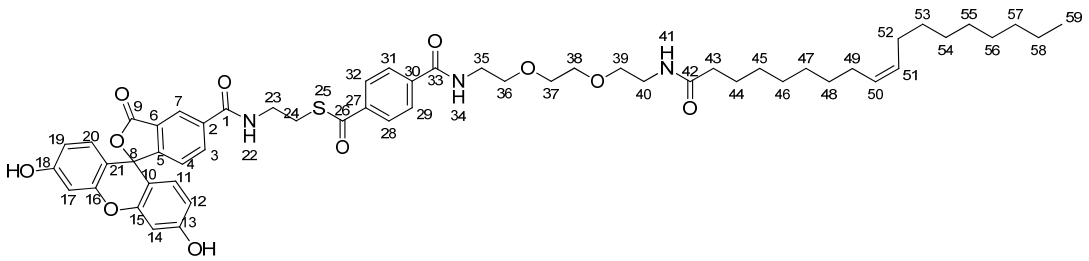
Yield: 50 % (166 mg, 0.381 mmol, orange solid).

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{23}H_{17}NO_6SNa]^+$: 458.0669; found: 458.0667.

^{1}H-NMR(300 MHz, DMSO-d_6, 293 K): δ = 8.25 (dd, J = 8.1, 1.5 Hz, 1H, H-7), 8.22 – 8.03 (m, 1H, H-3), 7.38 (d, J = 8.1 Hz, 1H, H-4), 6.69 (t, J = 2.1 Hz, 2H, H-12, H-19), 6.64 – 6.49 (m, 4H, H-11, H-14, H-17, H-20), 3.41 (ddd, J = 38.2, 12.7, 6.6 Hz, 2H, H-23), 2.82 – 2.37 (m, 2H, H-24) ppm.

^{13}C-NMR (75 MHz, DMSO-d_6, 293 K): δ = 168.12 (C-9), 164.62 (C-1), 159.56 (C-13, C-18), 151.75 (C-15, C-16), 140.43 (C-5), 134.66 (C-2), 129.22 (C-11, C-20), 126.40 (C-3), 124.23 (C-7), 123.25 (C-6), 122.19 (C-4), 112.63 (C-12, C-19), 108.99 (C-10, C-21), 102.20 (C-14, C-17), 83.32 (C-8), 42.85 (C-23), 23.18 (C-24) ppm.

(Z)-S-(2-(3',6'-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-4-ylcarboxamido)ethyl) 4-((2-(2-(2-oleamidoethoxy)ethoxy)ethyl)carbamoyl)benzothioate (*Fluorescein thioester*) (2)



To a solution of **(14)** (49.0 mg, 0.113 mmol, 3.19 eq.) in peptide grade DMF (7 ml) DIPEA (78,0 µl, 59.0 mg, 0.457 mmol, 12.9 eq.) and **(12)** (25.7 mg, 35.4 µmol, 1.00 eq.) were added. After stirring overnight at RT the solvent was removed under reduced pressure and the residual was solubilized in MeOH and again dried under reduced pressure. The pure product was obtained as a yellow solid after purification by silica gel column chromatography using ethyl acetate / MeOH / formic acid 10:0.5:0.1 as eluent.

Molecular formula: $C_{55}H_{67}N_3O_{11}S$ (978.20 g/mol).

Yield: 84 % (29.0 mg, 29.6 µmol, yellow solid).

R_F-value (EtOAc/MeOH/HCOOH 10:0.5:0.1): 0.28.

ESI-HRMS (MeOH) (m/z): Calculated for $[C_{55}H_{67}N_3O_{11}SNa]^+$: 1000.4389; found: 1000.4386.

^{1}H-NMR(400 MHz, Methanol-d_4, 295 K): δ = 8.17 (dd, J = 8.1, 1.7 Hz, 1H, H-7), 8.13 – 8.01 (m, 3H, H-3, H-28, H-32), 7.97 – 7.86 (m, 3H, H-4, H-29, H-31), 7.62 – 7.55 (m, 1H, NH), 7.29 (dd, J = 8.0, 0.7 Hz, 2H, NH), 6.69 (dd, J = 2.3, 1.4 Hz, 2H, H-14, H-17), 6.63 (dd, J = 11.1, 8.8 Hz, 2H, H-11, H-20), 6.54 (dd, J = 8.7, 2.4 Hz, 2H, H-12, H-19), 5.39 – 5.25 (m, 2H, H-50, H-51), 3.73 (t, J = 6.5 Hz, 2H, H-23), 3.73 – 3.55 (m, 10H, H-24, H-36, H-37, H-38, H-39), 3.52 (td, J = 5.5, 3.4 Hz, 2H, H-40), 3.42 (t, J = 6.6 Hz, 2H, H-35), 2.14 (t, J = 7.5 Hz, 2H, H-43), 2.00 (q, J = 6.5 Hz, 4H, H-49, H-52), 1.55 (p, *J* = 14.3, 7.1 Hz, 2H, H-44), 1.28 (d, J = 6.1 Hz, 20H, H-45 – H-48, H-53 – H-58), 0.88 (t, J = 6.8 Hz, 3H, H-59) ppm.

^{13}C-NMR (101 MHz, Methanol-d_4, 295 K): δ = 192.36 (C-26), 176.37 (C-42), 169.00 (C-9), 168.61 (C-33), 162.88 (C-1), 154.66-154.52 (C-13, C-18), 140.37-140.21 (C-15, C-17), 137.57 (C-5), 134.89 (C-30), 131.53 (C-2), 130.86-130.35 (C-28, C-32, C-50, C51), 128.88-128.21 (C-11, C-20, C-29, C-31), 126.74 (C-27), 126.21 (C-3), 125.34 (C-7), 114.80 (C-6), 114.51 (C-4), 111.54 (C-10, C-21), 111.31 (C-12, C-19), 103.66 (C-14, C-17), 82.07 (C-8), 71.34 (C-36), 71.31 (C-38), 70.63 (C-37), 70.49 (C-39), 41.04 (C-35), 40.25 (C-40), 37.06

(C-23), 33.06 (C-43), 30.84-30.23 (C-24, C-45 – C-48, C-53 – C-57), 28.14 (C-49, C-52), 27.01 (C-44), 23.74 (C-58), 14.47 (C-59) ppm.

UV/Vis-Absorbance: 497 nm (0.0025 mM in 100 mM PB, pH 8.00)

Fluorescence-Emission: 527 nm (Ex.: 494 nm, 0.0025 mM compound with liposomes (0.1 mM) in 100 mM PB, pH 8.00)

References

1 E. D. Chenot, D. Bernardi, A. Comel and G. Kirsch, *Synth. Commun.* 2007, **37**, 483–490.
2 X. Han, A. S. Achalkumar, R. J. Bushby and S. D. Evans, *Chem. Eur. J.* 2009, **15**, 6363–6370.
3 K. Molawi and A. Studer, *Chem. Commun.* 2007, 5173–5175.
4 R. K. Olsen and K. Ramasamy, *J. Org. Chem.* 1985, **50**, 2264–2271.
5 A. Guzman-Martinez, R. Lamer and M. S. VanNieuwenhze, *J. Am. Chem. Soc.* 2007, **129**, 6017–6021.
6 *Novabiochem 2004/5 Catalog,* 2004/5, p. 3.16, Merck KGaA, Darmstadt, Germany.
7 T. Bantan-Polak and K. B. Grant, *Chem. Commun.* 2002, 1444–1445.