A Novel Visible Range FRET Probe for Monitoring Acid Sphingomyelinase Activity in Living Cells

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Experimental Details

All solvents and reagents employed were obtained from Acros (Geel, Belgium), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (St. Gallen, Switzerland), TCI (Zwijndrecht, Belgium), ABCR (Karlsruhe, Germany) or Alfa Aesar (Ward Hill USA). The BODIPY dye was obtained from SETAREH BIOTECH (Eugene, USA). Technical gases (Argon 5.0, H₂ and food grade CO₂) were obtained from Air Liquide (Paris, France). Deuterated solvents were obtained from Armar (Doettingen, Switzerland). Solvents were dried using appropriate drying agents. Unless otherwise stated, reactions were performed under air. NMR spectra (¹H, ¹³C, and ³¹P) were recorded in CDCl₃, CD₃OD, pyridine-d₅, (CD₃)₄Si or mixtures of those on Bruker (Rheinstetten, Germany) AvanceDPX-300, AvanceII-300, Avance400 or AvanceIII-500 at 25°C. Chemical shifts are given in ppm with respect to TMS as external standard (¹H, ¹³C, δ = 0.00) or with calibration against the residual solvent signal. UPLC was performed on a 1290 Infinity series system (Agilent Technologies, Santa Clara, US) using Zorbax RRHD C18 Eclipse Plus 2.1x50 mm, 1.8 Micron column, UV/Vis detector and either FLD or Agilent 6120 quadrupole LC/MS (ESI+/−). UPLC-MS was carried out using mixtures of solvent A (99.9% H₂O + 0.1% FA) and B (99.9% MeOH + 0.1% FA) with gradient (5% B in A to 95% B in A in 1 min) and a flow of 0.6 mL/min at 20°C. Fluorescence Spectra were recorded on Cary Eclipse, absorption spectra were recorded on Cary 100Bio UV/Vis (both Varian, Palo Alto, US), both at 37°C.

Abbreviations

ASM = acid sphingomyelinase, aq. = aqueous, Boc₂O = di-tert-butyldicarbonate, CH = cyclohexane, DCC = N,N′-dicyclohexylcarbodiimide, DIPEA = diisopropylethylamine, DMF = N,N-dimethylformamide, DMSO = Me₂SO, EA = ethyl acetate, eq. = equivalents, FA = formic acid, rt = room temperature, sat. = saturated, SE = succinimidyl ester, THF = tetrahydrofuran, TMS = tetramethylsilane, Tris = Tris(hydroxymethyl)aminomethane.

Chromatography

Thin-layer chromatography (TLC) was performed using silica gel 60 F254 (Merck (Darmstadt, Germany)) and the compounds were detected with UV light (254 nm/366 nm) or by spraying the plates with ninhydrin solution (3 w% in EtOH), followed by heating. Preparative TLC was performed on preparative silica gel 60 plates 20 cm x 20 cm with concentrating zone (Merck (Darmstadt, Germany)). After scraping off product bands, the silica was removed by extraction with the development solvent followed by 0.45 μm filtration. Binder was removed by dissolution of the product in pure MeOH and washing it several times with 2,2,4-trimethylpentane. Flash chromatography was performed on silica gel 60 (0.040–0.063 mm) obtained from Macherey-Nagel (Dueren, Germany). Solvents were evaporated under reduced pressure while maintaining the water bath temperature at 40°C.
Buffer

ASM buffer: 0.1 M NaOAc pH (4.5, 5.0, 5.5 or 7.0) 150 mM NaCl, 0.1 mM ZnSO₄, 0.1% TritonX-100. Elution buffer: 50 mM Tris/HCl pH 7.6, 1 M NaCl, 0.1 mM ZnCl₂, 0.4% β-octylglucopyranoside (w/v)

Preparation of recombinant ASM

Human acid sphingomyelinase (ASM) was expressed in insect Sf9 cells and purified to homogeneity similar to method described by LANSMANN et al.[1] The recombinant enzyme purified in the presence of Zn²⁺ (0.1 mM) had an activity of 23 nmol h⁻¹ ml⁻¹ as determined in a micellar assay system.

Fluorescent ASM assay (cuvette)

All spectra were recorded at 37°C with magnetic stirring, all slit widths were 5 nm, PMT voltage was 700 V, if not stated otherwise. ASM (usually 2970 μL) was filled into quartz cuvettes (1x1 cm, 3 mL) and blank spectra were recorded. Probe (4, 5a or 5b) (1 mM in DMSO, usually 3 μL giving final 1 μM) was added and initial static spectra were recorded (Ex/Em in nm: 485/(500-800), all slits 5 nm). Then, recombinant human ASM from insect cells (usually 24 μL 0.0625 μg/μL ASM in elution buffer) was added simultaneously to the cuvette(s), which were stoppered tightly and placed in a multicell holder. After 30 sec of preincubation, data points (485/512 + 485/624) were recorded in time intervals. After reaction completion, final spectra were recorded and the reactions were stopped by addition of 500 μL MeOH. Fluorescence data was corrected (time, blank, volume), optionally smoothed (Savitzky-Golay 40-2), normalized and plotted using OriginLab OriginPro 9.1G software.

Fluorescent ASM assay (96 well plate)

The assay was done in non-binding black clear-bottom 96-well microplate (greiner bio-one) using Victor 1420 Multilabel counter from Wallac, Perkin–Elmer Life and Analytical Sciences (Wellesley, MA). Recombinant ASM was also diluted using assay buffer to reach concentration of 2.5 μg/ml. The assay is done by adding 20 μl of the FRET probe solution and finally the addition of 80 μl of the recombinant enzyme solution containing 0.25 μg of the enzyme. The plate is then sealed using transparent plastic cover. Measurements are done at room temperature. Fluorescence is measured from the bottom (measurement height: 8 mm) using excitation filter 485 nm and emission filter 535 nm with CW-lamp energy 3096. The device is also set to mix the contents of the assay by 5 mm orbital shaking for 1 seconds before each measurement.

Cell culture
L929 cells (from ATCC) were cultured in Minimum Essential Medium (MEM) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2mM l-glutamine, 1 mM sodium pyruvate and 100 μM non-essential amino acids (all from Gibco). Cells were maintained at 37°C and 5% CO2 in a humidified incubator and were used until passage 20. Cells were grown to subconfluency before experiments.

**In-situ ASM assay with the FRET probe**

L929 cells were seeded in 96-well plates and left to rest overnight before the experiment was started. Amitriptyline hydrochloride (Sigma, A8404) was dissolved at 1 mM in PBS and was always prepared fresh. Cells were then treated with amitriptyline or PBS as a control, as indicated. At the end of the treatment, cells were incubated with the FRET probe 4 for an additional 30 min. The final concentration of the FRET probe in the culture medium was 1 μM (from 1mM stock in DMSO). After the 30 min-incubation, the medium was removed, and cells were washed ×1 with fresh medium followed by brief trypsinization. Cold fresh medium was added (3:1, v/v) to trypsin, and the plate was kept in dark on ice. The mean fluorescence intensity (MFI) on the green channel (520 nm), which correlated to the cleavage of the substrate and on the red channel (700 nm), which correlated to the uptake of the substrate was detected with Attune NxT flow cytometer (ThermoFisher). A minimum of 10,000 events per sample was acquired. Each experiment included unstained controls. To calculate ASM activity, the background fluorescence was first subtracted from all samples then the ratio of green:red fluorescence was taken to correct for differences in substrate uptake. Ratios were then normalized to the average ratio of untreated cells and expressed as % residual ASM activity. Data are representative of 3 independent experiments.

**Conventional ASM assay**

L929 Cells were seeded in 24-well plates in parallel to cells for the in-situ assay and left to rest overnight before the experiment was started. The same batch and solution of amitriptyline (see above) was used to treat cells in parallel for both FRET in-situ and conventional assay as indicated. After treatment, cells were lysed on ice in ASM lysis buffer (250 mM sodium acetate (Sigma), 1% NP-40 (IGEPAL, Sigma) pH 5.0) for 10 min. Protein concentration was determined using the Bradford Protein Reagent (BioRad). Bodipy FL C12-sphingomyelin (Invitrogen) was dissolved at a final concentration of 0.5 μM corresponding to 100 pmol/sample in ASM assay buffer (250 mM sodium acetate, 0.1% NP-40, pH 5.0). Substrate solutions and samples for 10 min. The reaction mixtures consisted of 20 μL lysate and 180 μL assay buffer and were incubated for 30 min at 37°C with 300 rpm. The reaction was terminated by adding chloroform:methanol (2:1, v/v). Phases were separated by centrifugation at 20,000 xg for 5 min and the organic phase was collected and dried in SpeedVac (ThermoFisher). Dried lipids were resuspended in chloroform:methanol (2:1, v/v) and spotted onto a thin layer chromatography plate (Macherrey-Nagel, Germany). Chromatography was conducted with chloroform:methanol (80:20, v/v). The plates were scanned using Typhoon FLA 9500 (GE Healthcare) and spots were quantified with
ImageQuant software (GE Healthcare). The values were normalized to the protein concentration. The average values of untreated samples were used express data as % residual ASM activity. Data are representative of 3 independent experiments.

Synthesis

tert-butyl((2S,3R,E)-1-(((2-aminoethoxy)(hydroxy)phosphoryl)oxy)-3-(methoxymethoxy)-octadec-4-en-2-yl)carbamate 2

[Image: a chemical structure diagram]

Compound 1 was synthesized in 11 steps from L-Serine, as described previously.[3] 1 (0.184 g, 0.264 mmol, 1.00 eq.) was dissolved in MeOH (5.2 mL). Hydrazine hydrate (64%, 78 μL, 1.587 mmol, 6.00 eq.) was added and the mixture stirred at rt for 5.5 h when TLC indicated complete consumption of the starting material. The solvent was evaporated and the crude material was dried under high vacuum. The material was dissolved in CHCl₃, Celite® was added and the volatiles removed in vacuo. The powder thus obtained was subjected to short flash column chromatography (CHCl₃:MeOH:H₂O = 100:15:1→65:25:3). The title compound was isolated as a colorless glassy solid (0.125 g, 0.221 mmol, 84 %).

Rₖ (CHCl₃:MeOH:H₂O = 65:25:3) = 0.43;

¹H NMR (500 MHz, pyridine-d₅) δ = 9.54 (bs, 2H, Hₓ), 7.75 (bs, 1H, Hₓ), 5.86 – 5.78 (m, 1H, =CHCH₂), 5.70 (dd, J = 15.2, 8.3 Hz, 1H, =CHCH), 4.95 (d, J = 6.4 Hz, 1H, OCHHO), 4.76 (d, J = 6.3 Hz, 1H, OCHHO). 4.61 – 4.46 (m, 5H, 2 x CH₂OP, CHO), 4.38 (bs, 1H, CHNH), 3.50 – 3.45 (m, 5H, CH₂NH₂, OCH₂), 2.09 (s, 2H, CH₂), 1.58 (s, 9H, OC(CH₃)₃), 1.41 (s, 2H, CH₂), 1.30 (m, 20H, 10 x CH₂), 0.90 (t, J = 6.9 Hz, 3H, CH₃);
13C NMR (126 MHz, pyridine-d5) δ = 157.2 (COO), 137.5 (=CHCH2), 128.3 (=CHCH), 94.5(OCH2O), 78.9 (OC(CH3)3), 77.6 (CHO), 65.9 (CH2OP), 63.3 (CH2OP), 56.2 (OCH3), 55.9 (NCH2CH2OP), 41.5 (CH2NH2), 33.3 (CH2CH=), 32.7 (CH2), 30.6 (CH2), 30.5 (CH2), 30.5 (CH2), 30.4 (CH2), 30.2 (CH2), 30.2 (CH2), 30.0 (CH2), 29.3 (CH2), 29.2 (CH2), 23.5 (CH2CH3), 14.9 (CH3);

31P NMR (202 MHz, pyridine-d5) δ = 2.30.

4(5)-((2-(((2S,3R,E)-2-((tert-butoxycarbonyl)amino)-3-(methoxymethoxy)octadec-4-en-1-yl)oxy)(hydroxy)phosphoryl)oxy)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid 3

In an argon atmosphere, 5(6)-carboxyfluorescein (76.6 mg, 0.204 mmol, 1.50 eq.), N-hydroxysuccinimide (28.1 mg, 0.244 mmol, 1.80 eq.) and DCC (47.6 mg, 0.231 mmol, 1.70 eq.) were dissolved in dry DMF (1.5 mL). The mixture was stirred for 4 h at rt and protected from light. The mixture then was added to a solution of 2 (76.9 mg, 0.136 mmol, 1.00 eq.) in dry CH2Cl2 (2.5 mL) and NEt3 (189 µL, 1.36 mmol, 10 eq.). The mixture was stirred for 19.5 h at rt. The reaction was quenched by the addition of MeOH (2 mL) and stirred for 30 min at rt. The mixture was evaporated to dryness and dissolved in CHCl3 (60 mL) and washed with 0.1 M HCl (2 x 15 mL). The organic layer was separated, dried over Na2SO4 and evaporated to dryness. The crude product was purified by column chromatography (CHCl3:MeOH:H2O = 100:15:1→ 65:25:3). The product contained fraction were combined and purified again by column chromatography (CHCl3:MeOH:H2O:NEt3 = 100:15:1:0.5 → 80:20:2:0.5). The title compound was obtained as orange solid (101.7 mg, 90.2 µmol, 66 %).

Rf (CHCl3:MeOH:H2O:NEt3 = 65:25:3:0.5) = 0.46;

1H NMR (500 MHz, CDCl3, CD3OD, TMS) δ = 8.56 (s, 0.5H, 0.5x CHar), 8.22 – 8.11 (m, 1.5H, 0.5x CHar, CHar), 7.73 (s, 0.5H, 0.5x CHar), 7.29 (d, J = 8.0 Hz, 0.5H, 0.5x CHar), 6.87 (d, J = 9.0 Hz, 1H, CHar), 6.79 (d, J = 8.9 Hz, 1H, CHar), 6.73 – 6.70 (m, 2H, 2x CHar), 6.60 (td, J = 9.2, 2.2 Hz, 2H, 2x CHar), 5.76 – 5.66 (m, 1H, =CHCH2), 5.35 – 5.28 (m, 1H, =CHCH), 4.69 (d, J = 6.6 Hz, 0.5H, 0.5x OCHHO), 4.66 (d, J = 6.6 Hz, 0.5H, 0.5x OCHHO), 4.51 (d, J = 6.6 Hz, 0.5H, 0.5x OCHHO), 4.48 (d, J = 6.5 Hz, 0.5H, 0.5x OCHHO), 4.14 – 3.98 (m, 5H, 2x CH2O, CHO), 3.79 – 3.68 (m, 2H, CHNH, 0.5x CH2N), 3.64 – 3.61 (m, 1H, 0.5x CH2N), 3.36 (s, 1.5H, 0.5x OCH3), 3.34 (s, 1.5H, 0.5x OCH3), 2.99 (q, J = 7.3 Hz, 12H, 2x N(CH2CH3)3), 2.04 (d, J = 6.1 Hz, 2H, CH2CH3), 1.41 (s, 4.5H, 0.5x
OC(CH₃)₃, 1.40 (s, 4.5H, 0.5x OC(CH₃)₃), 1.33 – 1.24 (m, 22H, 11x CH₂), 1.21 (t, J = 7.3 Hz, 18H, 2x N(CH₂CH₃));

³¹P NMR (202 MHz, CDCl₃, CD₃OD, TMS) δ = 0.66 (s), 0.47 (s).

In an argon atmosphere, 3 (6.83 mg, 6.06 µmol, 1.00 eq.) was dissolved in dry iso-propanol (1.5 mL). HCl (4 M in 1,4-dioxan, 76 µL, 303 µmol, 50 eq.) was added and the mixture was stirred at 70°C for 3 h with exclusion of light. Then, UPLC-MS and TLC indicated complete conversion of the starting material. The solvent was removed and the residue was dried under high vacuum. The orange solid was dissolved in dry pyridine (1.00 mL) and dry NEt₃ (16.8 µL, 121 µmol, 20 eq.). Then, EverFluor TR-X SE (5.00 mg, 7.88 µmol, 1.30 eq.), dissolved in dry DMF (0.80 mL) was added dropwise to the solution. After 10 h, MeOH (1.00 mL) was added and the mixture was stirred for 1 h. The mixture was evaporated to dryness by coevaporation with toluene (2 x 10 mL). The crude product was dissolved in EA (40 mL) and MeOH (2.00 mL) and washed with 0.1 M HCl (3 x 10 mL). The organic phase was dried over Na₂SO₄ and evaporated to dryness. The residue was purified by short column chromatography (CHCl₃:MeOH:H₂O = 100:15:1 → 65:25:3). The product contained fraction were combined and purified by preparative TLC (CHCl₃:MeOH:H₂O = 65:25:3). The title compound was obtained as violet solid (4.62 mg, 3.55 µmol, 59%).

Rₜ (CHCl₃:MeOH:H₂O = 65:25:3) = 0.28;

ESI MS C₆₈H₇₆BF₂N₅O₁₄PS [M-H]⁻ m/z calc.: 1298.5 found: 1298.3.

¹H NMR (400 MHz, CDCl₃, CD₃OD, TMS) δ = 8.52 (s, 0.5H, 0.5x CHar), 8.23 (d, J = 8.0 Hz, 0.5H, 0.5x CHar), 8.15 (d, J = 7.9 Hz, 0.5H, 0.5x CHar), 8.09 – 8.06 (m, 1.5H, 0.5x CHar, CHar), 7.95 (dd, J = 8.9 Hz, 3.6 Hz, 2H, 2x CHar), 7.72 (dd, J = 5.7 Hz, 3.3 Hz, 0.5H, 0.5x CHar), 7.68 (s, 0.5H, 0.5x CHar), 7.58 (dd, J = 5.7 Hz, 3.3 Hz, 0.5H, 0.5x CHar), 7.51 – 7.49 (m, 1H, 1x CHar), 7.38 (d, J = 7.7
Hz, 0.5H, 0.5x CHar), 7.27 – 7.19 (m, 2H, 2x CHar), 7.15 – 7.10 (m, 2H, 2x CHar), 7.03 (d, J = 8.8 Hz, 2H, 2x CHar), 6.83 (dd, J = 4.3 Hz, 2.0 Hz, 1H, CHar), 6.76 (m, 2H, 2 x CHar), 6.68 – 6.63 (m, 2H, 2 x CHar), 6.61 – 6.56 (m, 2H, 2x CHar), 5.73 – 5.63 (m, 1H, =CHCH₂), 5.47 – 5.38 (m, 1H, =CHCH), 4.56 (s, 1H, 0.5x OCCH₂COO), 4.54 (s, 1H, 0.5x OCCH₂COO), 4.14 – 4.10 (m, 3H, 2x CH, NH), 3.98 (bs, 2H, 2x NH), 3.88 (bs, 1H, NH), 3.70 – 3.64 (m, 2H, CH₂), 3.57 (bs, 1H, OH), 3.33 – 3.29 (m, 2H, CH₂), 2.23 – 2.14 (m, 2H, CH₂), 2.01 - 1.96 (m, 2H, CH₂), 1.67 – 1.51 (m, 4H, 2x CH₂), 1.34 – 1.23 (m, 26H, 13x CH₂), 0.87 (t, J = 6.5 Hz, 3H, CH₃);

¹⁹F NMR (282 MHz, CDCl₃, CD₃OD, TMS) δ = -133.51 (q, J = 32.9 Hz);

³¹P NMR (162 MHz, CDCl₃, CD₃OD, TMS) δ = -0.42 (s).
4(5)-((2-(((2S,3R,E)-2-dodecanamido-3-hydroxyoctadec-4-en-1-yl)oxy)(hydroxy)phosphoryl)oxy)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid 5a

In an argon atmosphere, 3 (37.53 mg, 33.29 µmol, 1.00 eq.) was dissolved in dry iso-propanol (4.0 mL). HCl (4 M in 1,4-dioxan, 998 µL, 250 µmol, 30 eq.) was added and the mixture was stirred at 70°C for 3.5 h with exclusion of light. Then, UPLC-MS and TLC indicated complete conversion of the starting material. The solvent was evaporated and the crude material was dried under high vacuum. The yellow residue was dissolved in dry iso-propanol (1.5 mL) and dry NEt₃ (69.2 µL, 0.500 mmol, 15 eq.). Then, lauric acid N-hydroxysuccinimide (14.12 mg, 46.6 µmol, 1.40 eq.), dissolved in dry THF (1.0 mL) was added dropwise to the solution at 0°C. After addition the mixture was stirred at rt for 30 h. The reaction was quenched by addition of MeOH (2.0 mL) and stirred at rt for 2 h. The reaction mixture was evaporated to dryness. The residue was dissolved in CHCl₃ (45 mL), washed with 1 M HCl (2 x 6 mL), dried over Na₂SO₄ and evaporated to dryness. The obtained material was purified by column chromatography (CHCl₃:MeOH:H₂O = 100:15:1 → 80:20:2 → 65:25:3). Preparative TLC (CHCl₃:MeOH:H₂O = 65:25:3) of the product containing fraction yielded an orange solid (25.0 mg, 25.96 µmol, 78 %).

Rₜ (CHCl₃:MeOH:H₂O = 65:25:3) = 0.40;

^1H NMR (500 MHz, CDCl₃, CD₃OD, TMS) δ = 8.53 (s, 0.5H, 0.5x CHar), 8.25 (d, J = 7.9 Hz, 0.5H, 0.5x CHar), 8.19 (d, J = 7.3 Hz, 0.5H, 0.5x CHar), 8.07 (d, J = 7.7 Hz, 0.5H, 0.5x CHar), 7.62 (s, 0.5H, 0.5x CHar), 7.30 (d, J = 6.9 Hz, 0.5H, 0.5x CHar), 6.72 (s, 2H, 2x CHar), 6.61 (d, J = 8.5 Hz, 2H, 2x CHar), 6.55 (d, J = 8.5 Hz, 2H, 2x CHar), 5.69 (td, J = 15.3, 6.9 Hz, 1H, =CHCH₂), 5.48 – 5.36 (m, 1H, =CHCH), 4.28 – 4.04 (m, 3H, 3x CH), 3.94 (m, 2.5H, CH₂O 0.5x CHNH), 3.82 (s, 0.5H, 0.5x CHNH), 3.69 (m, 1H, 0.5x CH₂N), 3.58 (m, 1H, 0.5x CH₂N), 2.22 – 2.13 (m, 2H, CH₂), 2.00 (q, J = 6.8 Hz, 2H, CH₂), 1.57 (bs, 2H, CH₂), 1.40 – 1.22 (m, 38H, 19x CH₂), 0.91 – 0.84 (m, 6H, 2x CH₃);

^31P NMR (202 MHz, CDCl₃, CD₃OD, TMS) δ = 1.31.
In an argon atmosphere, \(3\) (20.07 mg, 21.70 µmol, 1.00 eq.) was dissolved in dry 1,4-dioxane (6.0 mL). HCl (4 M in 1,4-dioxan, 245 µL, 978 µmol, 45 eq.) was added and the mixture was stirred at 70°C for 4 h with exclusion of light. Then, UPLC-MS and TLC indicated complete conversion of the starting material. The solvent was evaporated and the crude material was purified by short column chromatography (CHCl₃:MeOH:H₂O = 100:15:1 → 65:25:3). The Boc and MOM deprotected starting compound was obtained as yellow solid (11.03 mg, 14.13 µmol). After drying at high vacuum, the Boc and MOM deprotected intermediate (10.90 mg, 13.96 µmol, 1.00 eq.) was dissolved in dry DCM (1.5 mL), dry pyridine (0.7 mL) and dry DIPEA (24.3 µL, 0.140 mmol, 10 eq.). Then, palmitoyl chloride (4.03 mg, 4.45 µL, 14.66 µmol, 1.05 eq.), dissolved in dry DCM (0.8 mL) was added dropwise over 2 h to the solution at 0°C. After addition the mixture was stirred at 0°C for 3.5 h. Then, UPLC-MS and TLC indicated incomplete conversion of the starting material. Palmitoyl chloride (0.58 mg, 0.64 µL, 2.11 µmol, 0.15 eq.), dissolved in dry DCM (0.150 mL) was added dropwise over 30 min to the solution. After addition the mixture was stirred at 0°C for 2 h. The reaction was quenched by addition of MeOH (1.0 mL) and stirred at rt for 1 h. CHCl₃ (45 mL) was added and the organic layer was washed with 1 M HCl (2 x 6 mL), brine (6 mL) and dried over Na₂SO₄. The obtained material was purified by column chromatography (CHCl₃:MeOH:H₂O = 100:15:1 → 65:25:3). The title compound was isolated as a yellow solid (2.65 mg, 2.60 µmol, 12 % over two steps).

\[ R_f (\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O} = 65:25:3) = 0.72; \]

ESI MS \(C_{57}H_{83}N_2O_{12}P\) [M+H]^+ m/z calc.: 1019.6 found: 1019.4;

\(^1\)H NMR (500 MHz, CDCl₃, CD₂OD) \(\delta = 8.48\) (s, 0.5H, 0.5x CHar), 8.25 (dd, \(J = 8.0\) Hz, 1.4 Hz, 0.5H, 0.5x CHar), 8.18 (dd, \(J = 8.1\) Hz, 1.2 Hz, 0.5H, 0.5x CHar), 8.08 (d, \(J = 8.0\) Hz, 0.5H, 0.5x CHar), 7.66 (s, 0.5H, 0.5x CHar), 7.30 (d, \(J = 8.0\) Hz, 0.5H, 0.5x CHar), 6.69 (d, \(J = 2.3\) Hz, 2H, 2x CHar ), 6.59 (dd, \(J = 8.7\) Hz, 4.7 Hz, 2H, 2x CHar), 6.55 – 6.52 (m, 2H, 2x CHar), 5.87 – 5.79 (m, 1H, =CHCH₂), 5.48 – 5.40 (m, 1H, =CHCH), 4.28 – 4.21 (m, 2H, 2x CH), 4.13 – 4.08 (m, 2H, CH₂O), 4.02 – 3.96 (m,
2H, CH, CHNH), 3.68 (t, J = 5.1 Hz, 1H, 0.5x CH2N), 3.56 (t, J = 5.3 Hz, 1H, 0.5x CH2N), 2.08 – 2.02 (m, 2H, CH2), 1.71 – 1.66 (m, 2H, CH2), 1.45 – 1.26 (m, 46H, 13x CH2), 0.90 – 0.87 (m, 6H, 2x CH3);

31P NMR (202 MHz, CDCl3, CD3OD) δ = 1.22.

References

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[2] J. Folch, M. Lees, G. H. S. Stanley, J. Biol. Chem., 1957, 226, 497–509.

[3] T. Pinkert, D. Furkert, T. Korte, A. Herrmann and C. Arenz, Angew Chem Int Ed Engl, 2017, 56, 2790-2794.

Supplementary Figures

Figure S1: Flow cytometry (FC) analysis of L929 cells: Mean fluorescence intensities (MFI) of the (BODIPY) channel. Black curve = cell w/o probe; blue curve = cell with probe 4, but w/o inhibitor; red curve = cells with probe 4 plus 10 μM amitriptyline (24h pre-treatment).
**Figure S2:** FRET probe 4 is fully cleaved under the conditions applied. Thin layer chromatography (TLC) of probe 4 before (left lanes) and after (right lanes) treatment with ASM. Left: exemplary photo taken in Cy3 channel right: exemplary photo taken in the ethidium bromide channel.
Figure S3: FRET probe 4 with 2.5 µg/ml ASM: cleavage kinetics.
Figure S4: FRET probe 4 with 2.5 µg/ml ASM: determination by the initial slopes
Figure S5: FRET probe 4 calibration curve, derived from the plateau values observed in figure S3.

Figure S6: FRET probe 4 lineweaver burk plot
Figure S7: probe 5a with 2.5 µg/ml ASM: cleavage kinetics.
Figure S8: probe 5a with 2.5 µg/ml ASM: determination by the initial slopes.
Figure S9: probe 5a calibration curve, derived from the plateau values observed in figure S7.

Figure S10: FRET probe 5a lineweaver burk plot.
NMR Spectra

tert-butyl((2S,3R,E)-1-(((2-aminoethoxy)(hydroxy)phosphoryl)oxy)-3-(methoxymethoxy)-octadec-4-en-2-yl)carbamate 2

$^1$H NMR (500 MHz, pyridine-d$_5$)
$^{13}$C NMR (126 MHz, pyridine-d$_5$)

$^{31}$P NMR (202 MHz, pyridine-d$_5$)
4(5)-((2-(((2S,3R,E)-2-((tert-butoxycarbonyl)amino)-3-(methoxymethoxy)octadec-4-en-1-yl)oxy)(hydroxy)phosphoryl)oxy)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid 3

^1H NMR (500 MHz, CDCl$_3$, CD$_3$OD, TMS)
$^{31}$P NMR (202 MHz, CDCl$_3$, CD$_3$OD, TMS)

3-(4-(2-(((2S,3R,E)-1-(((2-(3(4)-carboxy-3(4)-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzamido)ethoxy)(hydroxy)phosphoryl)oxy)-3-hydroxyoctadec-4-en-2-yl)amino)-6-oxohexyl)amino)-2-oxoethoxy)phenyl)-5,5-difluoro-7-(thiophen-2-yl)-5H-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide 4
$^1$H NMR (400 MHz, CDCl$_3$, CD$_3$OD, TMS)

$^{19}$F NMR (282 MHz, CDCl$_3$, CD$_3$OD, TMS)
$^{31}$P NMR (162 MHz, CDCl$_3$, CD$_3$OD, TMS)

4(5)-((2-(((2S,3R,E)-2-dodecanamido-3-hydroxyoctadec-4-en-1-yl)oxy)(hydroxy)phosphoryl)oxy)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid 5a
$^1$H NMR (500 MHz, CDCl$_3$, CD$_3$OD, TMS)

$^{31}$P NMR (202 MHz, CDCl$_3$, CD$_3$OD, TMS)
4(5)-((2-((hydroxy((2S,3R,E)-3-hydroxy-2-palmitamidoctadec-4-en-1-yl)oxy)phosphoryl)oxy)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid 5b

$^1$H NMR (500 MHz, CDCl$_3$, CD$_2$OD)
$^{31}$P NMR (202 MHz, CDCl$_3$, CD$_3$OD)