Conjugation of Spermine Facilitates Cellular Uptake and Enhances Antitumor and Antibiotic Properties of Highly Lipophilic Porphyrins

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[**] Financial support from the DFG (CFN, TH Karlsruhe) is gratefully acknowledged.

Keywords: Molecular Transporters, Polyamines, PDT, Antibiotics, Amphiphilic Porphyrins, Solid Phase Synthesis

Cellular uptake of spermine-porphyrin and fluorescence microscopy. Beside the monitoring of cellular uptake of 0.01, 0.1, 1, 5, 20, 50, and 100 µM spermine-porphyrin 6 and 5 µM carboxy-porphyrin 4 by either fluorescence microscopy and fluorescence confocal microscopy the route of cellular uptake was visualized by co-staining of the cells with several fluorescent marker proteins probing for the endosomal and lysosomal compartments, the nucleus, and the mitochondria (Figures 1, S1a and S1b, S2, S3). Already after 30 min, a vesicular distribution of the conjugate was observed (data not shown), and also a longer incubation of cells with the spermine-porphyrin 6 revealed a vesicular staining that was distinct from the endosomal/lysosomal compartment (Figure 1). A significant amount was found in the perinuclear region, but not within the nucleus (Figure S2). First experiments at low doses (1µM/4h) show a mitochondrial localization of the amphiphilic porphyrin (Figure S2) and a concentration at the perinuclear region, which would indicate a swelling and perinuclear translocation of the mitochondria containing high levels of reactive oxygen species (ROS).[1] The perinuclear area has been proposed to become the site of ROS production. Photo-irradiation then resulted in nuclear fragmentation and probably in apoptotic cell death, which has to be further determined (Figure S3 B). In our experiments we also observe severe necrosis (Figures S1 A and S3 A).
Supporting Information

Figure S1a: Treatment of COS-7, HeLa-cells and human fibroblasts with carboxy porphyrin 4 and spermine-porphyrin 6: A, after a 4h treatment with high doses (100 uM) spermine-porphyrin 6 in the presence of light the majority of cells is killed. B, 24h treatment with carboxy porphyrin 4 in the absence of light, C-D, 24h treatment with different concentrations of spermine-porphyrin 6 in the absence of light, E, 24h treatment with spermine-porphyrin 6 in the absence of light and washing with trypsin to remove the surface bound compound.
Figure S1b. Fluorescence confocal microscopy of living COS7 cells (A-C) and human primary fibroblasts (D-F). A and D show the treatment with 4 (5 µM), B and E the treatment with 6 (5 µM). The cells were co-incubated for 1h with 150 nM Lysotracker green to label the endosomal-lysosomal compartment. In C and F cells were stained 4h after porphyrin treatment for 1h with Lysotracker green. In all cases the Nomarski images were merged with the fluorescent images of the Lysotracker labeling (green) and the porphyrin labeling (red).
Supporting Information

Figure S2: Preliminary fluorescence microscopy studies revealed a mitochondrial staining in living HeLa-cells treated with 1µM spermine-porphyrin 6 for 4h. The cells show a typical mitochondrial staining in the periphery and a strong perinuclear accumulation. M= mitochondria, N= nucleus, PN= perinuclear region.

Figure S3: Live imaging of primary fibroblasts treated with 1µM of 6 after photo-irradiation with visible light. The cells show severe necrosis already after 2 min after visualization in the combined fluorescence and transmission light mode of the microscope. A) The membrane structure of the cells seems to be disrupted and a release of the fluorescent material is observed indicating necrosis. B) After a 24h incubation post photo-irradiation, many cells display nuclear fragmentation indicating apoptotic cell death. In both cases the Nomarski images were merged with the fluorescent images of the porphyrin 6 labelling. NF= nuclear fragmentation
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**Solubility of spermine-porphyrin in aqueous media.** In order to probe whether unspecific aggregation occurs with the transporter porphyrin we studied solutions of different concentrations both in freshly distilled methylene chloride (from calcium hydride) and in deionized water. The aqueous solution was prepared by dissolving 2.0 mg of construct 6 in 200±10 µL 2-propanol and diluting it into 10 ml water. Short sonication yielded a transparent yellow-orange solution.

![Absorption spectra](image)

**Figure S4** Absorption spectra in 2% propanol in H$_2$O (v/v)(dilution was prepared as described above). Black trace was recorded in a 1 mm quartz cuvette, while the red traces present the same solution in a 1 cm quartz cuvette. The green and blue traces were obtained after approximately five time dilutions successively, respectively. Note that there are no changes in the blue shoulder of the Soret band or in the Q bands upon dilution indicating absence of aggregation. Absorption spectra were measured on a Cary 500 UV-NIR spectrometer at room temperature.
Figure S5. Stationary emission spectra in 2% 2-propanol in H$_2$O (v/v). The same solutions as in Figure S3 were measured with the excitation wavelength fixed at the Soret maximum at 420 nm. Measurements were performed on a Cary Eclipse fluorimeter in a right angle geometry. Note that some concentration quenching occurs in the more concentrated solution (#2, red trace) in comparison to the more dilute solutions.
Figure S6. Comparison of the absorption spectrum of solution #2 from Figure S3 and the fluorescence excitation spectrum with emission monitored at 662 nm. Note that due to the absorption in the somewhat split Soret band, very little light passes through the sample to the detector. However in the Q band region, a perfect superposition of the absorption and fluorescence excitation spectrum is observed indicating a normal behaviour of the transporter in this solution. We assume that under physiological conditions, and that by using methanol instead of 2-propanol, a similar behaviour is encountered.
Figure S7. Absorption spectra in dry methylene chloride at three different concentrations. In the inset is presented the comparison between the absorption and the fluorescence excitation spectrum. As the emission was monitored at 652 nm, this explains the very intense last Q band. Note that apart from a slight blue shoulder of the Soret band, which also is less pronounced than in aqueous solution, there are no abnormal behaviours encountered.
Figure S8: Emission spectra in dry methylene chloride with the excitation wavelength set at the Soret and the \( Q_x \) maxima, respectively.
Experimental Section

Synthesis

General. $^1$H NMR: Bruker DP 300 (300 MHz), Bruker AM 400 (400 MHz), Bruker DRX 500 (500 MHz); $\delta = 7.26$ ppm for CHCl$_3$. Description of signals: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, ddd = doublet of dd, dt = doublet of triplets, dq = doublet of quartets, tt = triplet of triplets. The spectra were analyzed according to first order. All coupling constants are absolute values. $^{13}$C NMR: Bruker DP 300 (75 MHz), Bruker AM 400 (100 MHz), Bruker DRX 500 (125 MHz); $\delta 77.00$ ppm for CHCl$_3$. IR (infrared-spectroscopy): KBr pellets on a Bruker IFS88 IR; MS (mass spectroscopy): ESI-HRMS: Bruker ESI-Micro-Q-TOF. TLC (Thin layer chromatography): Silica gel coated aluminium plates (Merck, silica gel 60, F$_{254}$). Detection under UV light at 254 nm. Chemicals, solvents, reagents, and chemicals were purchased from Acros, Aldrich, Fluka, and Merck.

General conditions. All working steps are carried out at room temperature in glass frits. During the resin loading step humidity was avoided by keeping the resin under argon.

General washing procedure. Unless stated the resin was alternately washed with THF: methanol(4:1) / methanol (3x), CH$_2$Cl$_2$ / methanol (3x) and CH$_2$Cl$_2$ (dry) (3x). Per 100mg of resin, approximately 2 ml of the solvent were used.

Cleavage from resin. The target molecule is removed from the resin by addition of 10% TFA in CH$_2$Cl$_2$. Meanwhile the colour of the resin turned to red. The drained cleavage mixture was combined with washings of CH$_2$Cl$_2$ and MeOH respectively and the solvent was removed in vacuum.

Analysis. The shown NMR and mass spectroscopy-data are taken from the free product after cleavage from resin. The course of the reactions was qualitatively followed by the Kaiser-Test.
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Cell culture techniques for mammalian cells. All procedures with mammalian cells are carried out under sterile conditions. $10^4$-$10^5$ HeLa (human cervix carcinoma) cells, COS7 cells, human primary fibroblast were plated into each well of a μslide 8-well ibiTreat slide (ibidi, Martinsried, Germany) and cultured in 200 µl of Dulbecco’s modified Eagle’s medium, high glucose, (DMEM, Sigma Taufkirchen) supplemented with 10% fetal calf serum (FCS, PAA), and 1 u/ml Penicillin/Streptomycin at 37° C, 5% CO₂.

Treatment of adherent cells with porphyrin derivatives. The spermine-porphyrin 6 was dissolved in methanol to yield a 20 mM stock solution and further diluted with 10% DMEM to yield the respective incubation media. The carboxy-porphyrin 4 was dissolved in methanol to yield a stock solution of 18 µM and further diluted with 10% DMEM to yield the respective incubation media. To ensure equal incubation conditions all dilutions contain equal amounts of methanol. $10^4$-$10^5$ HeLa (human cervix carcinoma) cells, COS-1 cells, human primary fibroblast were plated into each well of a μ-slide 8-well ibiTreat (ibidi, Martinsried, Germany) and cultured in 200 µl of Dulbecco’s modified Eagle’s medium, high glucose, (DMEM, Sigma Taufkirchen) supplemented with 10% fetal calf serum (FCS, PAA), and 1% Penicillin/Streptomycin at 37° C, 5% CO₂. After the cells reach a confluency of about 80% the culture medium was removed and replaced by 0.2 ml of the respective porphyrin supplemented media. Spermine-porphyrin (6) was added to the medium up to a final concentration of 0.1, 1, 5, 1, 20, 50, 100 µM, respectively and carboxy porphyrin (4) was added to the medium up to a final concentration of 0.01, 0.1, 1, 5 µM, respectively. It should be mentioned that the methanol concentration of the 5µM dilution of 4 is about 25-30 %, which is not suitable for a further in vivo treatment. Cellular uptake of both compounds, 6 and 4, was measured by live-cell imaging since fixation would alter the intracellular distribution as described for other polycationic species.

Subcellular Localization. For the intracellular localization of spermine-porphyrin 6 the cells were co-incubated with fluorescent probes (Molecular Probes, Karlsruhe) for different intracellular compartments: For endosomal/lysosomal labeling: 150 nM Lysotracker® Green DND 26 for 30 min, 4h, and 24h prior to examination; for mitochondria labeling: 1 µM Mitotracker® Green FM for 1 h prior to examination; For Golgi apparatus: BSA complex of NBD-C6-ceramide (5 µM), For nucleus labeling: Hoechst 33342 (Invitrogen, Karlsruhe) according to the manufacturers manual.

Fluorescence and confocal microscopy. Cellular uptake of 6 and 4 was measured after incubation for 30 min, 1 h, 2 h, and 4 h, and 24h, respectively by live imaging using a fluorescence microscope (Zeiss Axiovert 35, filter: Bp 546 FT 580, LP 590). For confocal microscopy cellular uptake was visualized using a confocal microscope (Zeiss Axiovert 200) fitted with a Zeiss LSM 510 Laser Module (argon, 458/488/514 nm; HeNe, 543/633 nm). Images were collected with the AxioCam MRc and the images were visualized with the AxioVision 3.1 and the LSM imaging software as well as the LSM Image Browser software version 3.1.0.99.
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Fluorometric cellular uptake measurements: Hela cells were plated in black flat bottom 96 well plates at a density of $10^4$ cells/well and incubated for 24h at cell culture conditions. Eventually, the cells were treated with 0.5, 1, 5, and 10 µM of spermine-porphyrine 6 and 5 µM of carboxy-porphyrin 4 (final concentration in DMEM medium) and incubated for 4, 16, 24, and 48h. After incubation, the medium was removed and the cells were washed with phosphate buffered saline (PBS). The cells were lysed with 100 µl of 1x RIPA buffer (including Complete protease inhibitors, Roche, Mannheim) and the fluorescence was measured using a Thermofisher Scientific Varioskan plate reader at an excitation/emission wavelength of 420/650 nm. After the measurement, the protein concentration was quantified.

Dark toxicity tests: The dark toxicity was estimated indirectly by determination of the viability of the cells. Therefore HeLa cells and human primary fibroblasts were plated in a Greiner 96 well plate at a density of 3000 cells/well. The cells were incubated with increasing concentrations (1-100 µM) of 6 at 37°C and the viability was determined after 24, 48, and 72h using the CellTiter96 viability test (Promega) according to the manufacturers instructions. The viability was measured by quantification of the absorbance at 570 nm using a Thermofisher Scientific Varioskan plate reader. The signal was normalized to 100% from untreated cells and 0% from lysed cells.

Measurement of cell death rate after illumination at 650-660 nm. Beside measuring the uptake rate, determination of the cellular toxicity of the spermine-porphyrin 6 is important. Therefore, HeLa (human cervix carcinoma) cells, COS-1 cells, and human primary fibroblast were plated into an µslide 8-well ibiTreat ($10^4$-$10^5$ cells/well) and incubated with 0,01, 0.1, 1, 5, 20, 50, 100 µM spermine-porphyrin 6 and 5 µM carboxyporphyrin 4 in 200µl DMEM, high glucose, supplemented with 10% fetal calf serum (FCS, PAA), and 1% Penicillin/ Streptomycin at 37°C, 5% CO$_2$ for 4h and 24h, respectively. Eventually, the cells were washed with PBS and the medium was replaced by DMEM, high glucose without phenol red (Sigma, Taufkirchen), supplemented with 10% fetal calf serum (FCS, PAA). To induce photodynamic destruction the cells were irradiated with light of a 650 +/- 10 nm diode laser at a dose rate of 30-42 mW/cm$^2$ giving total fluence rate of 10-13 J/cm$^2$. After exposure with light for 5 min, 10 min, 15 min, 30 min, 2 h, 4 h, respectively the cells were stained with trypan blue and propidium iodide and visualized using a Zeiss Axiovert 35 (filter: Bp 546 FT 580, LP 590) microscope. Necrotic and dead cells were counted in comparison to intact cells.

Colony-Forming Assay. The colony-forming assay was performed as it has been previously reported. After photoirradiation or illumination of human primary fibroblasts co-incubated with gram(-) and gram (+) bacteria 100 µl aliquots were removed from the medium for the colony-forming assay, serially diluted, and streaked on agar plates as described in [2]. The plates were incubated in the dark to avoid further photoirradiation. Controls were bacteria treated with 6 without illumination. The treatment with 1 µM of 6 and illumination for 15 min at 650 nm revealed no colonies after 24h. Likewise, 100 µl of fresh DMEM
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supplemented with 10% fetal calf serum in absence of antibiotics were added to the cells to investigate the re-growth of bacteria overnight. After 24h –72h of co-incubation no re-growth of the bacteria could be observed, while the cells were proliferating.

Acid hydrolysis of porphyrin-substituted methyl benzoate

B-COOH: 5-(4-Carboxyphenyl)-10,15,20-triundecyl-21H-23H-porphyrin (4). Porphyrin ester B-COOCH₃[3] (200 mg, 0.22 mmol) was hydrolyzed with a mixture consisting of 20 ml of TFA, 10 ml of aq. HCl (36% ) and 10 ml of water by heating for four days under gentle reflux (bath temperature 120 °C). After cooling the upper oily coloured drops were collected, dissolved in diethylether : chloroform (1:1) and washed with aqueous sodium hydrogen carbonate until the porphyrin free base purple colour returns. Evaporation of the solvent leaved violet plates with metallic lustre (190 mg, 98% yield), which were sufficiently pure for further transformations. Column chromatography on silica gel followed by recrystallization from chloroform: n-hexane (1:5) and cooling at -18 °C overnight gave an analytically pure sample. Shorter or longer reaction times decrease the yield. The proton NMR spectra are very dependent upon the concentration, indicating stacking at higher concentration as well as cooperative, multiple hydrogen bonding and/ or salt bridges between the carboxyl groups and pyrrolic NH. Rᵣ = 0.18 on SiO₂ eluted with 1% MeOH in CH₂Cl₂. ¹H NMR (200 MHz, CDCl₃, dilute solution): 9.50 (4 H, quartet, 12-,13-, 17-, 18-H₄), 9.36 (2 H, broad d, J = 4.3 Hz, 2-, 8-H₂), 8.76 (2 H, d, J = 4.9 Hz, 3-, 7-H₂), 8.57 (2 H, d, J = 8.5 Hz, 3', 5'-H), 8.27 (2 H, d, J = 7.9 Hz, 2', 6'-H), 4.88 (6 H, t, 1"- and 1"'- CH₂), 2.48 (6 H, quintet, 2"-and 2"'-CH₃), 1.76 (6 H, quintet, 3"- and 3"'-CH₃), 1.52 (6 H, m, 4"- and 4"'-CH₂), 1.29 (36 H, s, 5"-10"- and 5"'-10"'-CH₂), 0.88 (9H, t, 11"- and 11"'-CH₃), -2.62 (2 H, s, NH); ¹³C NMR (50 MHz, CDCl₃): 172.41 (COO), 148.56 (1'), ~146.5 (very broad C-pyrrole), 134.59 (2'-, 6'-C ), ~130.8 (broad, 3-, 7-C), 128.45 (4'-, 3', 5'-C ), ~128.05 (broad, 2 -, 8-, 12-, 13-, 17-, 18-C), 119.85 (15-C), 119.37 (10-, 20-C), 116.14 (5-C), 38.92 (2"'-CH₂), 38.74 (2"'-CH₂), 35.73 (1"'-CH₂), 35.40 (1"'-CH₂), 31.98 (9"'-, 9"'- CH₂), 30.61 (3"'-, 3"'-CH₂), 29.73 and 29.40 (4"'-8"'- and 4"'-8"'-CH₂), 22.71 (10"- and 10"'-CH₂), 14.15 (11"- and 11"'-CH₂); FAB-MS: 893.5 (M + H)*, 751.3 (M - C₁₀H₁₈) *, 611.2 (M + H - 2 C₁₀H₁₈) *; IR (KBr): 2921 s, 2849 s, 1605 w, 1278 w, 787 s; UV-Vis (CH₂Cl₂), λmax (lg εmax): 655 (3.73), 597 (3.51), 554 (3.93), 517.5 (4.15), 418 (5.60). C₆₁H₅₆N₄O₂: Calc.: C: 80.67; H: 9.48; N: 6.27; found: C: 80.90; H: 9.45; N: 6.24.
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**bis-Aloc-nosyl-spermine resin (2).** 1 g of an activated alkoxytrityl resin (0.46 mmol; 1 eq) were swollen in CH$_2$Cl$_2$(dry) for 5 min. The solvent was removed and 2.56 g (4.6 mmol; 10 eq) of 1 and 200 µl (1.150 mmol; 2.5 eq) DiPEA in 20 ml CH$_2$Cl$_2$ were added. The suspension was agitated for 8 h. Fifteen minutes prior termination of the reaction 4 ml of MeOH$_2$(dry) were added to block the remaining binding sites on the resin. The resin was washed with CH$_2$Cl$_2$: MeOH (4 : 1) / MeOH (3x) and CH$_2$Cl$_2$(dry). After drying on high vacuum the loading of the resin with bis-aloc-nosyl-spermine was calculated by mass difference (0.40 mmol / g resin). $^1$H NMR (400MHz, CDCl$_3$): δ: 8.00 – 8.25 (m, 4 H), 7.81 (m, 1 H), 7.73 (m, 2 H), 5.90 (ddt, $J_1$ = 17.2 Hz, $J_2$ = 10.5 Hz, $J_3$ = 5.4 Hz, 2 H), 5.27 (ddt, $J_1$ = 17.1 Hz, $J_2$ = 1.4 Hz, $J_3$ = 1.4 Hz, 4 H), 5.19 (ddt, $J_1$ = 10.5 Hz, $J_2$ = 1.4 Hz, $J_2$ = 1.4 Hz, 4 H), 4.57 (dddd, $J_1$ = 5.5 Hz, $J_2$ = 1.5 Hz, $J_3$ = 1.5 Hz, 4 H), 3.37 (m, 2 H), 3.30 (m, 2 H), 3.20 (m, 4 H), 3.08 (t, $J$ = 6.7 Hz, 2 H), 2.98 (m, 2 H), 1.93 (m, 2 H), 1.74 (m, 4 H), 1.50 (m, 4 H); HRMS (EI): calc. 555.2363, found 555.2354; FAB-MS: 556.3 (100) [M + H]$^+$, 540.2 (5) [M – NH]$^+$, 393.1 (49) [M – oNosyl + Na]$^+$.

**bis-Aloc-(6-NH-Fmoc-caproyl)-spermine (3).** 1 g of 2 (0.04 mmol; 1 eq) were expanded in DMF$_2$(dry) for 10 min. After removal of the solvent the resin was resuspended in 2 ml DMF$_2$(dry) and 120 µl (0.80 mmol; 20 eq) DBU and 57 µl (0.81 mmol; 20 eq) β-mercaptoethanol were added. The suspension was agitated for 20 h and then washed. The Kaiser-Test was positive. 400 mg of the resin were suspended in CH$_2$Cl$_2$(dry) and 170 mg (0.48 mmol; 3 eq) N-Fmoc-aminocaproic acid, 150 mg PyPrOP (0.32 mmol; 2 eq), and 63 ml (0.64 mmol; 4 eq) DiPEA were added to the suspension. The suspension was agitated for 20 h and the resin was washed and dried on high vacuum. $^1$H NMR (400MHz, [D$_4$]-MeOH): δ: 7.79 (d, $J$ = 7.6 Hz, 1 H), 7.64 (d, $J$ = 7.3 Hz, 1 H), 7.39 (t, $J$ = 7.6 Hz, 1 H), 7.31 (t, $J$ = 7.3 Hz, 1 H), 5.94 (ddt, $J_1$ = 17.2 Hz, $J_2$ = 10.5 Hz, $J_3$ = 5.4 Hz, 2 H), 5.29 (ddt, $J_1$ = 17.1 Hz, $J_2$ = 1.4 Hz, $J_3$ = 1.4 Hz, 4 H), 5.20 (ddt, $J_1$ = 10.5 Hz, $J_2$ = 1.4 Hz, $J_3$ = 1.4 Hz, 4 H), 4.58 (dt, $J_1$ = 5.3 Hz, $J_2$ = 1.4 Hz, 4 H), 4.55 (dt, $J_1$ = 5.3 Hz, $J_2$ = 1.4 Hz, 4 H), 4.34 (m, 2 H), 4.19 (t, $J$ = 6.8 Hz, 1 H), 3.36 (t, $J$ = 7.1 Hz, 2 H), 3.27 (t, $J$ = 7.1 Hz, 4 H), 3.16 (t, $J$ = 6.8 Hz, 2 H), 3.10 (t, $J$ = 6.5, 2 H), 2.92 (t, $J$ = 7.5, 2 H), 2.29 (t, $J$ = 7.3 Hz, 2 H), 2.18 (t, $J$ = 7.3 Hz, 2 H), 1.89 (tt, $J_1$ = 7.3 Hz, $J_2$ = 7.1 Hz, 2 H), 1.73 (m, 2 H), 1.62 (m, 2 H), 1.54 (m, 4 H), 1.35 (m, 2 H).

**bis-Aloc-(6-NH-porphyrinyl-caproyl)-spermine resin (5)** To 300 mg (0.12 mmol; 1 eq) of 3, 12 ml of a mixture of piperidine / DMF$_2$(dry) (1:4 (v/v)) were added and the reaction mixture was agitated (3 x 2 min). The resin was washed with DMF$_2$(dry). The Kaiser-Test was positive. 300 mg of the resin were swollen in DMF$_2$(dry). A solution of 203 mg (0.24 mmol; 2 eq) carboxyporphyrin, 56 mg (0.41 mmol; 3.4 eq) HOBt, and 124 mg (0.6 mmol; 5 eq) DCC in DMF$_2$(dry) / CH$_2$Cl$_2$(dry) (1:1) was stirred for 15 min and was then added to the resin. The suspension was agitated over night to give a black resin. Due to the color of the resin use of the Kaiser-Test was not possible. $^1$H NMR (400 MHz, [D$_4$]-MeOH), d (ppm): 9.70 (s, 4 H), 9.61 (d, $J$ = 4.8 Hz, 2 H), 8.84 (d, $J$ = 5.1 Hz, 2 H), 8.60 (d, $J$ = 8.3 Hz, 2 H), 8.46 (d, $J$ = 8.3 Hz, 2 H), 5.94 (ddt, $J_1$ = 17.2 Hz, $J_2$ = 10.5 Hz, $J_3$ = 5.4 Hz, 2 H), 5.29 (ddt, $J_1$ = 17.1 Hz, $J_2$ = 1.4 Hz, $J_3$ = 1.4 Hz, 4 H), 5.20 (ddt, $J_1$ = 10.5 Hz, $J_2$ = 1.4 Hz, $J_3$ = 1.4 Hz, 4 H), 4.58 (dt, $J_1$ = 5.3 Hz, $J_2$ = 1.4 Hz, 4 H), 4.55 (dt, $J_1$ = 5.3 Hz, $J_2$ = 1.4 Hz, 4 H), 4.34 (m, 2 H), 4.19 (t, $J$ = 6.8 Hz, 1 H), 3.36 (t, $J$ = 7.1 Hz, 2 H), 3.27 (t, $J$ = 7.1 Hz, 4 H), 3.16 (t, $J$ = 6.8 Hz, 2 H), 3.10 (t, $J$ = 6.5, 2 H), 2.92 (t, $J$ = 7.5, 2 H), 2.29 (t, $J$ = 7.3 Hz, 2 H), 2.18 (t, $J$ = 7.3 Hz, 2 H), 1.89 (tt, $J_1$ = 7.3 Hz, $J_2$ = 7.1 Hz, 2 H), 1.73 (m, 2 H), 1.62 (m, 2 H), 1.54 (m, 4 H), 1.35 (m, 2 H).
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$^{1}H$ NMR (400 MHz, [D$_4$]-MeOH), d (ppm): 9.68 (s, 4 H), 9.59 (d, J = 4.8 Hz, 2 H), 8.83 (d, J = 5.0 Hz, 2 H), 8.58 (d, J = 8.3 Hz, 2 H), 8.44 (d, J = 8.3 Hz, 2 H), 5.11 (m, 6 H), 3.59 (t, J = 7.2 Hz, 2 H), 3.35 (t, J = 6.0 Hz, 2 H), 3.02 – 3.30 (m, 16 H), 2.57 (m, 6 H), 2.34 (t, J = 7.4 Hz, 2 H), 2.10 (tt, J$_1$ = 7.8, J$_2$ = 7.6, 2 H), 1.93 (tt, J$_1$ = 6.8, J$_2$ = 7.1, 2 H), 1.72 – 1.88 (m, 10 H), 1.46 – 1.62 (m, 8 H), 1.20 – 1.40 (m, 34 H), 0.86 (t, J = 6.8 Hz, 9 H); MALDI-TOF-LD+: m/z (%): 1190.34 (100) [M + H]$^+$; ESI-MS (positive mode): 596.0 [M + 2H]$^{2+}$, 397.7 [M + 3H]$^{3+}$; HR-MS-ESI (positive mode): calc. 595.9816, found 595.9761.

References

