Supporting Information for "Effects of Structural Modification on Gene Transfection and Assembly Properties of Amphiphilic Dendrimers"

Marine Guillot,[a] Sara Eisler,[a] Kathrin Weller,[b] Hans P. Merkle,[b] Jean-Louis Gallani[c] and François Diederich*[a]

[a] *Laboratorium für Organische Chemie, ETH-Zürich, 8093 Zürich, Switzerland. Fax: 41 44 63 21109; Tel: 41 44 63 22992; E-mail: diederich@org.chem.ethz.ch*

[b] *Institut für Pharmazeutische Wissenschaften, ETH-Zürich, 8093 Zürich, Switzerland. Fax: 41 44 63 31314; Tel: 41 44 63 37310; E-mail: hmerkle@pharma.ethz.ch*

[c] *Institut de Physique et Chimie des Matériaux de Strasbourg, 67034 Strasbourg Cedex, France. Fax: 33 388 107246; Tel: 33 388 107164; E-mail: gallani@ipcms.u-strasbg.fr*

Cell Biological Studies

Cell Culture. HeLa cells, a human cervix epithelial adenocarcinoma cell line, were used throughout this study and were obtained from American Type Culture Collection ATCC (Rockville, MD, USA). Cell culture was performed under standard conditions in humidified atmosphere with 5% CO_2. For cultivation, trypsin-EDTA 0.25% (Gibco, Paisley, UK), phosphate buffered saline, pH 7.4 (PBS) (Gibco, Paisley, UK) and cell culturing flasks (25 cm^2) (TPP, Fischer Scientific, Wohlen, Switzerland) were utilized. Cells were used within 35 passages and cultured as exponentially growing subconfluent monolayers in Dulbecco's modified Eagles medium with GlutaMAX, sodium pyruvate, 4500 $mg.L^{-1}$ glucose (DMEM) (Gibco, Paisley, UK), supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) and penicillin/streptomycin (100 $units.mL^{-1}$ and 100 $\mu g.mL^{-1}$ respectively) (Gibco, Paisley, UK).

Plasmid DNA amplification. Plasmid DNA encoding green fluorescent protein (pGFP) was used throughout the study. pGFP was generated by cloning the green fluorescent protein (GFP) into the VR1012 vector which carries a kanamycin-resistance gene. pGFP was incorporated in competent *Escherichia coli* XL1 Blue cells with tetracycline resistance (Stratagene, La Jolla, USA). Cells were plated on agar plates containing kanamycin and tetracycline. A single clone was picked after 20 h and incubated in 300 mL LB (Luria-Bertani) medium (10 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 1L) containing tetracycline at 10 $\mu g.mL^{-1}$ and kanamycin at 50 $\mu g.mL^{-1}$ for 10 h before transfer into LB medium containing kanamycin only for additional 20 h according to a standard protocol. pGFP was purified using Quiagen Maxikit (Quiagen, Hilden, Germany) according to the manufacturer's instructions and re-suspended in 10 mM Tris buffer pH 8.5 (Quiagen plasmid purification handbook, September 2000). Quality of the plasmid was checked using an agarose gel with Smart Ladder (Eurogentec, Seraing, Belgium). In addition, quantity was calculated by measuring the electronic absorption at 260 nm and 280 nm.

Formation of dendrimer/pGFP complexes. Dendrimers and plasmid DNA encoding green fluorescent protein (pGFP) were complexed by electrostatic interactions. A given quantity of the respective dendrimer solution (1-2 $mg.mL^{-1}$ in deionised water, with 0-3% Me_2SO) was pipetted with pGFP solution (0.1 $mg.mL^{-1}$ in 10 mM Tris buffer) to obtain a dispersion of the complex with the respective charge excess (CE) ratio. The dimensionless CE ratio is defined as the number of positive charges on the dendrimer divided by the number of negative charges present on pGFP. Based on an average molecular weight 660 $g.mol^{-1}$ per base pair (bp), 1 μg of DNA is assumed to carry 3 nmol negative charges. To ensure efficient mixing, the resulting dispersions containing dendrimer and pGFP were pipetted up and down, and incubated for 30 min at 37 ˚C to complete complex formation.

Transfection in HeLa cells. Transfection experiments were performed in HeLa cells. All experiments were performed in triplicate and repeated at least twice independently. Cells were seeded in 24 well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 40 000 cells/cm^2 and were used 24 h after seeding as exponentially growing subconfluent monolayers. Dendrimer/pGFP complexes were made as described above. For transfection, the dendrimer/pGFP complex dispersions were diluted with serum free medium, the mixtures were added to the cells at a concentration of 1.0 μg pGFP/well, and then incubated. After 2 h, the complex dispersion was removed and replaced with medium containing 10% FCS. After incubation for 24 h, GFP expression was inspected by fluorescence microscopy. The cells were washed with PBS (100 μL) and trypsinised (100 μL) for 5 min in order to detach them from the surface. Trypsinisation was stopped by the addition of cold medium containing serum (400 μL). Cells were then transferred into FACS tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and stored on ice. Cells were analysed by FACS on a FacsCalibur (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) within 2 h. Data were analysed using Cytomation Summit software (Cytomation Inc., Fort Collins, USA). A total of 6 000 events per sample were counted. The number of cells with fluorescence intensity higher than untransfected control cells was used to calculate the percentage of transfected cells. Lipofectamine 2000 (LPF) was used as a positive control following the manufacturer's instructions. For graphical representation, results were normalised relative to LPF expression.

Cell Viability. The viability of HeLa cells after incubation with the dendrimer/pGFP complexes was evaluated by measuring the overall activity of mitochondrial dehydrogenase (MTT assay) according to the instructions of ATCC (MTT Cell Proliferation Assay Instructions). Briefly, HeLa cells were seeded in 96 well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 40 000 cells/cm^2 and were incubated for 24 h. Dendrimer/pGFP complexes were added as described above at a concentration of 0.5 μg pGFP per well. After 2 h, the complex dispersions were discarded, MTT reagent (0.5 mg.ml^{-1} solution of 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide in phosphate buffered saline (PBS) and medium) added and the cells left at 37 ˚C for 2-3 h. After removing the MTT solution, the purple precipitate in the cells was dissolved by the addition of detergent (81 ml isopropanol, 15 mL SDS 20%, 4 ml (1 M) HCl). After 12 h at room temperature in the dark, the overall activity of mitochondrial dehydrogenase in each well was measured spectrophotometrically at 570 nm using a ThermoMaz microplate reader (Molecular Devices, Sunnyvale, CA, USA). Untreated cells and cells treated with methanol for 10 min were used as controls.

Graphs with error bars for transfection and cytotoxicity studies:

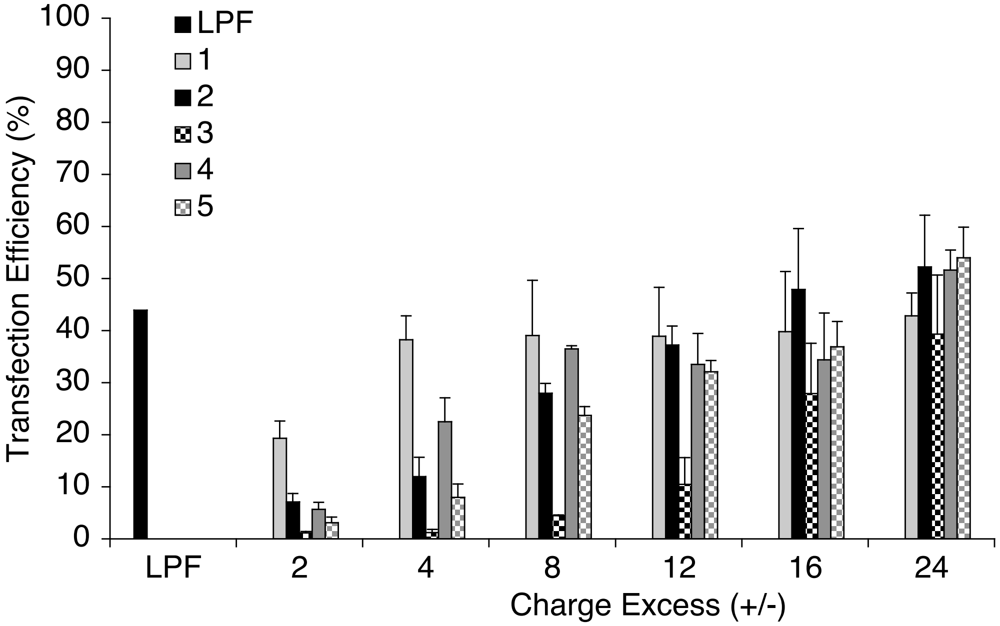


Figure 5 Transfection activity of compounds **1**-**5**.

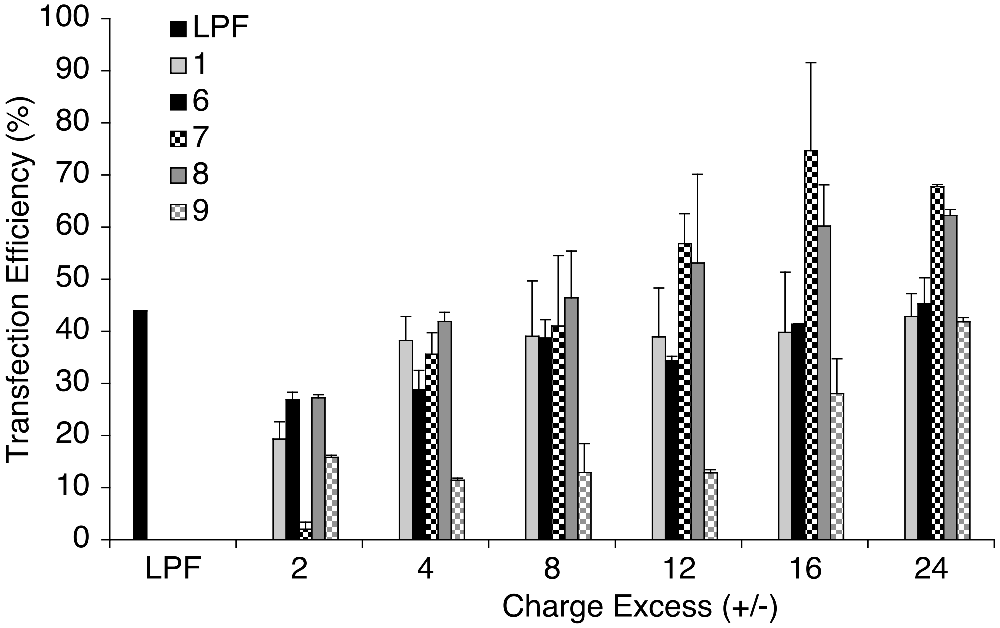


Figure 6 Transfection activity of compounds **6**-**9**.

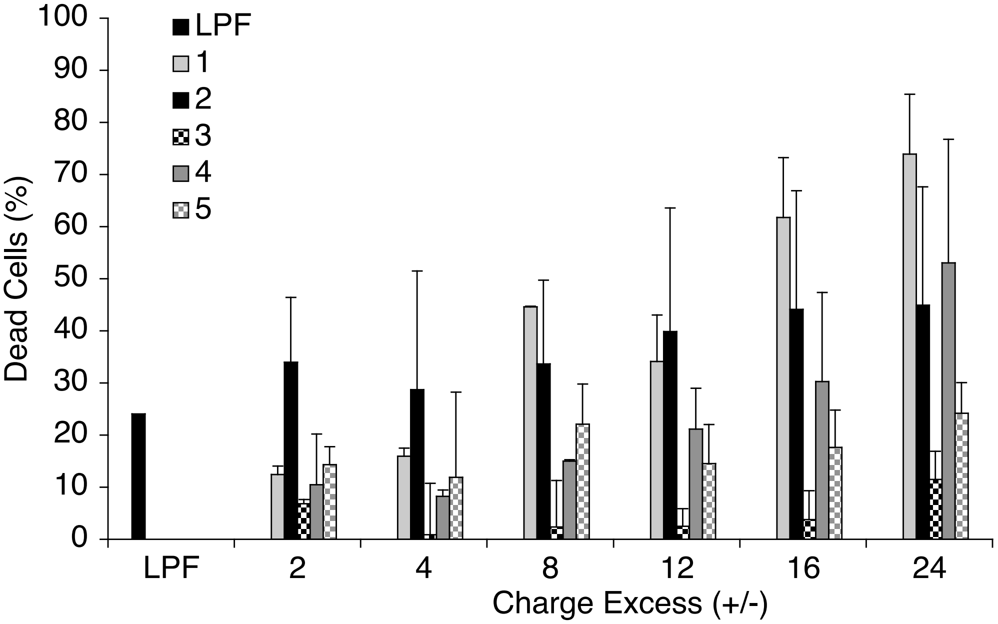


Figure 7 Toxicity of compounds **1**-**5**.

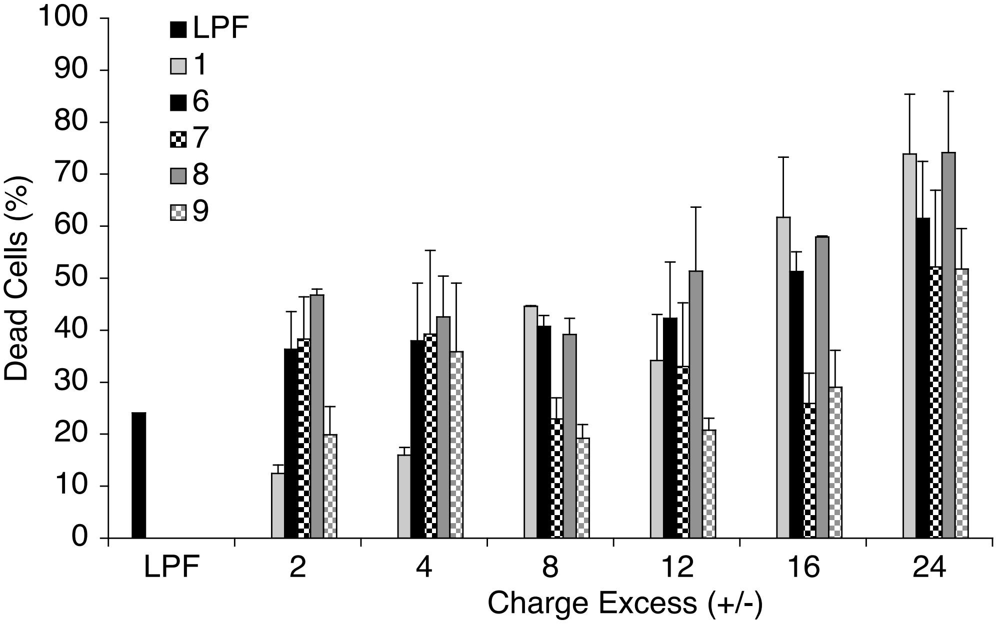


Figure 8 Toxicity of compounds **6**-**9**.

Langmuir Studies

Langmuir film data presented here were collected on pure water subphase (pH = 6.5) by compression of symmetrical hydrophilic barriers on a Teflon trough. The surface pressure was measured by means of a platinum Wilhelmy plate. The ultrapure water (ρ = 18 MΩ.cm, pH = 6.5) was purified through a Milli-RO3Plus/Milli-Q185 Ultra Purification system from Millipore. All isotherms were recorded at room temperature. Brewster-angle microscopy (BAM) pictures were recorded on a BAM2Plus from NFT. The observations are made at Brewster angle incidence. The images presented typically show a 500 μm x 500 μm area.

Spreading solutions at ~ 0.5 $mg.ml^{-1}$ concentration were prepared by dissolving the analytes in $CHCl_3$. In a typical experiment, 100 μl of fresh solution were spread on the aqueous subphase, the films left for ca. 5 min to equilibrate and the monolayers were then compressed with a speed of 7 $cm^2.min^{-1}$. Compression/expansion cycles were performed by compressing the monolayers under 32 $mN.m^1$. All measurements were repeated at least three times to ensure reproducibility. Extrapolation of the isotherms at the steepest point to zero surface pressure provided the limiting molecular areas.

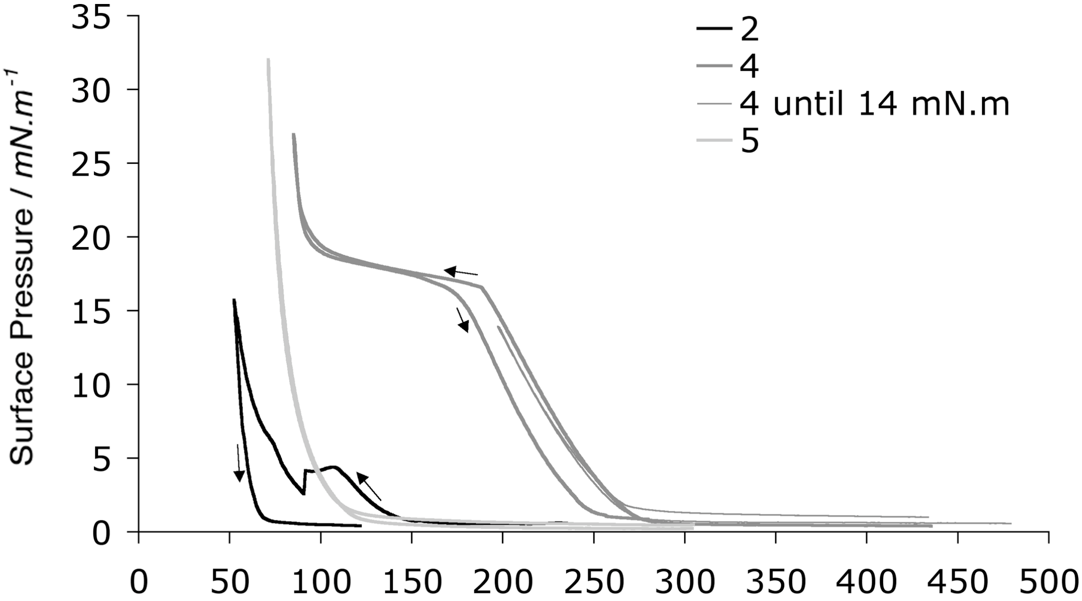


Figure 9 Hysteresis cycles recorded for compounds **2**, **4** and **5**. For compound **2**, the drop occurring at 80 Å^2 during compression is due to a pause in order to take BAM pictures. In the case of compound **4**, a reversible hysteresis was only recorded when surface pressure did not exceed 14 $mN.m^{-1}$.

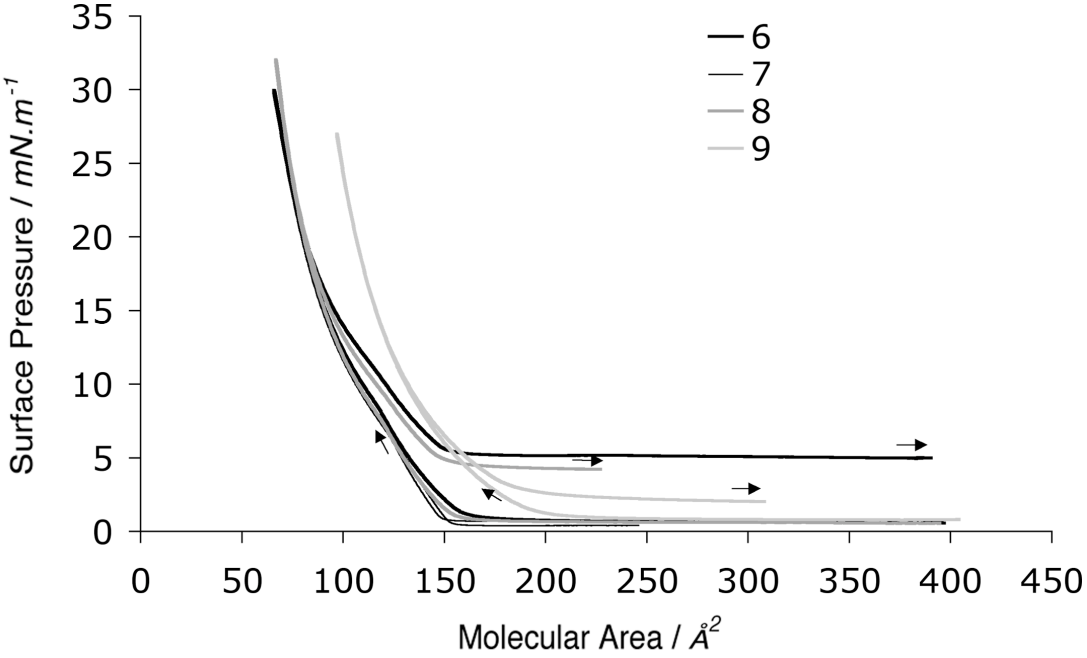
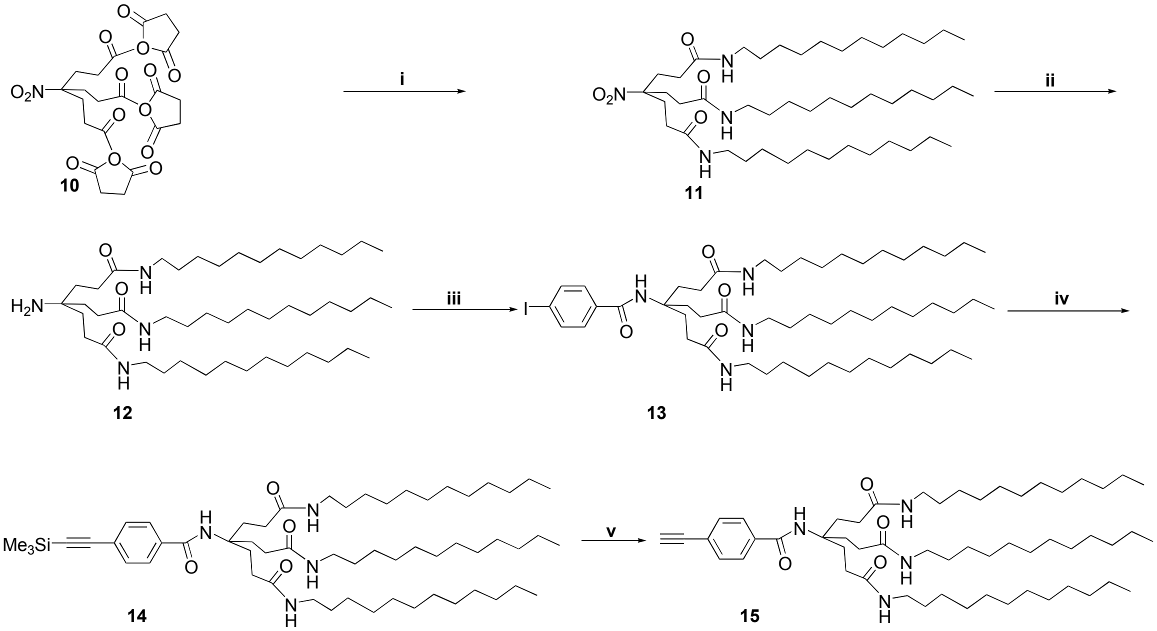


Figure 10 Hysteresis cycles recorded for compounds **6-9**. Only compound **7** shows a fully reversible hysteresis cycle.

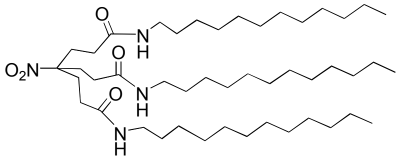
Synthesis and Compound Characterisation

Characterisation: Melting points (M.p.) were determined using a *Büchi 510* apparatus in open capillaries and are not corrected. IR Spectroscopy was performed neat on a Perkin Elmer Spectrum BX FT-IR system. NMR Spectra were recorded at room temperature on *Varian Gemini 300* (^{1}H 300 MHz, ^{13}C 75 MHz) or *Bruker AMX-500* (^{1}H 500 MHz, ^{13}C 125 MHz). The chemical shifts (δ) are given in ppm. Coupling constants (J) are given in Hz. For the signals, the following abbreviations are used: s (singlet), d (doublet), t (triplet), m (multiplet or unresolved signal) and br (broad). ^{13}C NMR spectra taken at 125 MHz are reported to two decimal places while those taken at 75 MHz are reported to one decimal place. Mass Spectra were recorded on an *Ion Spec Ultima FT-ICR* mass spectrometer in a matrix (HR-MALDI) or by Electronspray Ionisation (ESI). The matrix is either DHB (2,5-dihydroxybenzoic. acid) or 3-HPA (3-hydroxypicolinic acid). The detected masses are given in *m/z* and the molecular ion peak is written as *M.*

Synthesis: The synthesis of the new vectors is illustrated with the preparation of **8** in Scheme 1 and Scheme 2. Characterisation for vectors **1**-**7** and **9** follows and the synthesis of these compounds will be reported elsewhere after completion of the entire study dealing with structure-activity relationships.

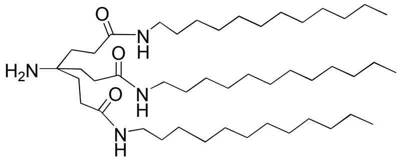


Scheme 1 i) dodecylamine, CH_2Cl_2, Et_3N, rt, 95%; ii) Raney-Ni, H_2 (6 bar), EtOH, 40 ˚C, 89%; iii) 4-iodobenzoyl chloride, CH_2Cl_2, Et_3N, 0 ˚C, 98%; iv) trimethylsilylacetylene, $[Pd(PPh_3)_2Cl_2]$, CuI, $(n\text{-}Bu)_4NBr$, $i\text{-}Pr_2NH$, THF, rt, 99%; v) tetrabutylammonium fluoride, THF, rt, 93%.



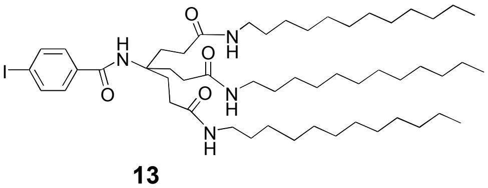
11

Synthesis of ***N,N*'-didodecyl-4-[3-(dodecylamino)-3-oxopropyl]-4-nitroheptanediamide** (**11**): A solution of **10** (8.0 g, 14 mmol) and dodecylamine (7.8 g, 42 mmol) in triethylamine (6.0 ml, 42 mmol) and CH_2Cl_2 (2 L) was stirred at room temperature under N_2 for 1 d. The organic phase was washed with aq. sat. $NaHCO_3$ and water, dried ($MgSO_4$) and evaporated. Recrystallisation (ethyl acetate) afforded **11** (10.5 g, 95 %) as a white solid; M.p. 98.5°C; ν_{max}($CHCl_3$)/cm^{-1} 3442, 3325, 2927, 2855, 1661, 1539, 1466, 1349 and 1089; δ_H(300 MHz; $CDCl_3$) 6.05 (3 H, t, *J* 8.7), 3.17 (6 H, m), 2.23 (6 H, m), 2.14 (6 H, m), 1.45 (6 H, br m), 1.25 (54 H, br s) and 0.87 (3 H, t, *J* 6.9); δ_C(75 MHz; $CDCl_3$) 171.0, 93.5, 39.8, 32.0, 30.9, 29.7, 29.6, 29.4, 27.1, 22.8 and 14.2; HR-MALDI-MS (DHB) calcd for $C_{46}H_{90}N_3O_3^+$ ([M-NO_2]$^+$): 732.6977; found: 732.6986.



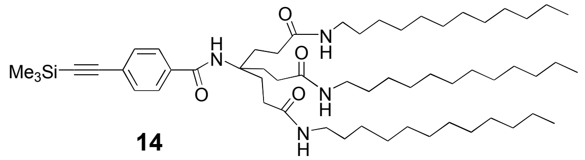
12

Synthesis of **4-amino-*N,N*'-didodecyl-4-[3-(dodecylamino)-3-oxopropyl]heptanediamide** (**12**): A suspension of **11** (5.25 g, 6.74 mmol) and Raney-Ni (1 g) in absolute ethanol (500 ml) was vigorously stirred under H_2 (6 bar) at 40 – 50 ˚C for 48 h. Filtration through a pad of celite and evaporation afforded crude **12** (4.49 g, 89 %) as a white solid; M.p. 100.9 °C. ν_{max}(KBr)/cm^{-1} 3297, 2955, 2916, 2849, 1628, 1542, 1465, 1377, 1292, 1158, 1119, 955, 855 and 719; δ_H(300 MHz; $CDCl_3$) 5.94 (3 H, t, *J* 5.5), 3.15 (6 H, m), 1.62 - 2.21 (12 H, *AA'BB'*), 1.43-1.48 (6 H, m), 1.23 - 1.43 (54 H, m), 0.86 (9 H, t, *J* 6.6); δ_C(75 MHz; $CDCl_3$) 173.0, 53.2, 39.8, 35.0, 32.0, 31.2, 29.8, 29.8, 29.7, 29.7, 29.5, 29.5, 27.1, 22.8, and 14.3, (one CH_2 signal not resolved in the 29.5 - 29.8 ppm area); HR-MALDI-MS (DHB) calcd for $C_{46}H_{92}N_4O_3Na^+$ ([M + Na]$^+$): 771.7067; found 771.7075.



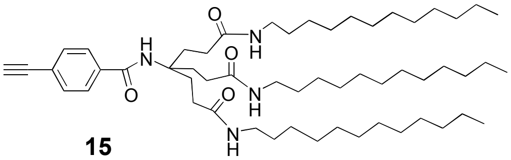
13

Synthesis of ***N*****,*****N*****'-didodecyl-4-[3-(dodecylamino)-3-oxopropyl]-4-{[(4-iodophenyl)carbonyl]amino}heptanediamide** (**13**): A solution of **12** (2.5 g, 3.34 mmol) and triethylamine (15 ml) in freshly distilled CH_2Cl_2 (150 ml) was stirred under Ar at 0 °C. To this mixture, a suspension of 4-iodo-benzoyl chloride (900 mg, 3.38 mmol) in freshly distilled CH_2Cl_2 (10 ml) was added dropwise. The mixture was then allowed to warm to room temperature. After 3 h of stirring, the mixture was poured on ice water and the aqueous phase was extracted with CH_2Cl_2. The organic phase was washed with water and (0.1 N) aq. NaOH, dried ($MgSO_4$) and concentrated to give crude **13** (3.23 g, 98 %) as a white solid; M.p. 103.1 °C; ν_{max}(KBr)/cm^{-1} 3279, 2919, 2849, 1676, 1636, 1586, 1555, 1468, 1377, 1310, 1271, 1006, 958, 911, 720 and 611; δ_H(300 MHz; $CDCl_3$) 8.23 (1 H, s), 7.74 (2 H, m), 7.59 (2 H, m), 5.88 (3 H, br), 3.15 (6 H, q, *J* 6.3), 2.3 – 2.1 (12 H, m), 1.5 – 1.0 (60 H, m) and 0.86 (9 H, t, *J* 6.9); δ_C(75 MHz; $CDCl_3$) 173.1, 166.3, 137.4, 134.2, 128.8, 98.3, 58.6, 39.9, 32.0, 31.7, 31.2, 29.8, 29.8, 29.7, 29.5, 29.5, 27.1, 22.8 and 14.3 (one CH_2 signal could not be resolved in the 29.7 - 29.8 ppm area); HR-MALDI-MS (DHB) calcd for $C_{53}H_{95}IN_4O_4Na^+$ ($[M + Na]^+$): 1001.6296; found 1001.6304.



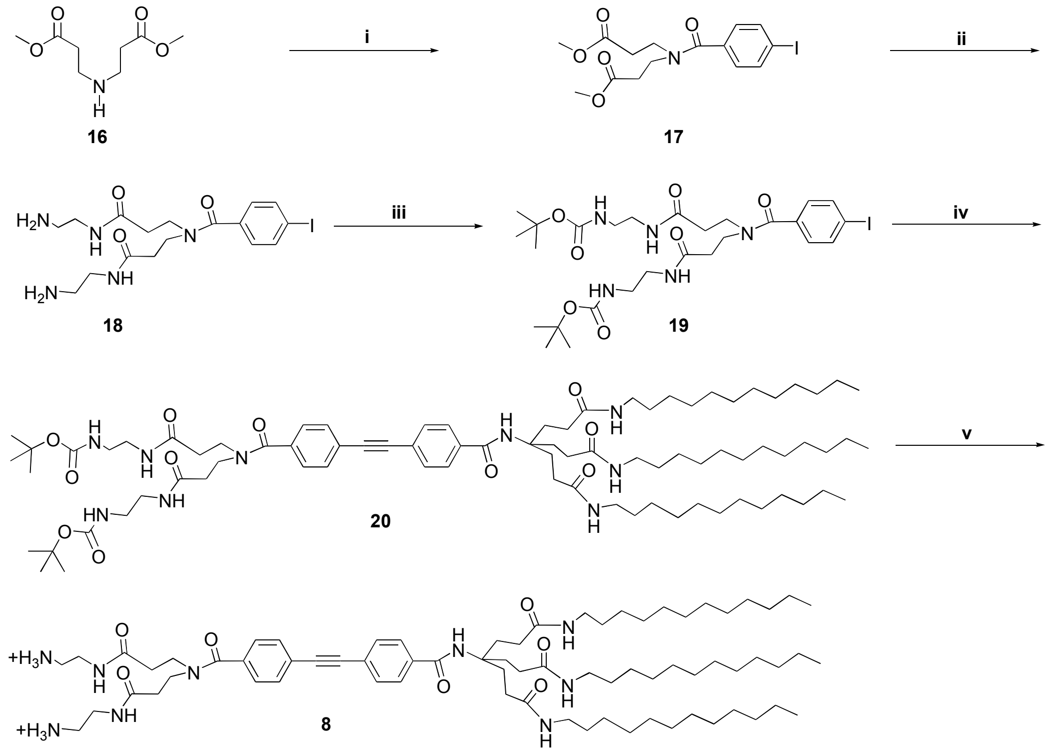
14

Synthesis of ***N*****,*****N*****'-didodecyl-4-[3-(dodecylamino)-3-oxopropyl]-4-[({4-[(trimethylsilyl)ethynyl]phenyl}carbonyl)amino]heptanediamide** (**14**): A solution of **13** (1.0 g, 1.0 mmol), trimethylsilylacetylene (430 μl, 3.05 mmol) and diisopropylamine (6 ml) in freshly distilled THF (20 ml) was degassed by freeze-pump-thaw cycles. A mixture of CuI (10 mg, 5 mol-%), $[Pd(PPh_3)_2Cl_2]$ (72 mg, 10 mol-%) and $(n\text{-}Bu)_4NBr$ (60 mg, 20 mol-%) was dried at 0.1 mbar for 1 h and added to the frozen solution. The reaction vessel was degassed by a freeze-pump-thaw cycle and allowed to warm to room temperature. After 24 h, sat. aq. NH_4Cl (150 ml) and CH_2Cl_2 (200 ml) were added. The organic phase was washed with sat. aq. NH_4Cl and water, dried ($MgSO_4$) and concentrated. Column chromatography (150 g SiO_2, CH_2Cl_2/methanol 19:1 → 15:1) gave **14** (978 mg, 99 %) as a white foam; M.p. 111.7 °C; ν_{max}(KBr)/cm^{-1} 3298, 2925, 2853, 2160, 1636, 1607, 1554, 1500, 1466, 1437, 1332, 1301, 1250, 1186, 1103, 865, 844, 760, 721 and 694; δ_H(300 MHz; $CDCl_3$) 8.14 (1 H, s), 7.77 (2 H, m), 7.44 (2 H, m), 6.09 (3 H, t, *J* 5.5), 3.12 (6 H, q, *J* 6.6), 2.22 (6H, m), 2.14 (6 H, m), 1.45 - 1.18 (60 H, m), 0.84 (9 H, t, *J* 6.6) and 0.22 (9 H, s); δ_C(75 MHz; $CDCl_3$) 173.4, 166.7, 134.6, 132.1, 127.3, 126.3, 104.4, 96.8, 58.7, 39.9, 32.1, 31.9, 31.3, 29.8, 29.8, 29.8, 29.7, 29.70, 29.5, 29.5, 27.1, 22.9, 14.3 and 0.0; HR-MALDI-MS (DHB) calcd for $C_{58}H_{104}N_4O_4SiNa^+$ ([M + Na]$^+$): 971.7725; found 971.7700.

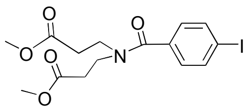


15

Synthesis of ***N*,*N*'-didodecyl-4-[3-(dodecylamino)-3-oxopropyl]-4-{[(4-ethynylphenyl)carbonyl]amino}heptanediamide** (**15**): To a solution of **14** (0.900 g, 0.948 mmol) and one drop of water in freshly distilled THF (10 ml), (1M) tetrabutylammonium fluoride in THF (1.9 ml, 1.9 mmol) was added. After stirring at room temperature for 30 min, sat. aq. NH_4Cl (50 ml) and CH_2Cl_2 (250 ml) were added. The organic phase was washed with sat. aq. NH_4Cl and water, dried ($MgSO_4$) and concentrated. Column chromatography (SiO_2, CH_2Cl_2/MeOH 30:1 → 10:1) afforded **15** (0.774 g, 93%) as a white foam; M.p. 64.1 °C. ν_{max}(KBr)/cm^{-1} 3291, 2921, 2844, 1636, 1540, 1461, 1433 and 855; δ_H(300 MHz; $CDCl_3$) 8.20 (1 H, s), 7.83 (2 H, m), 7.50 (2 H, m), 6.14 (3 H, br), 3.17 (1 H, s), 3.15 (6 H, q, J 6.5), 2.30 (6 H, m), 2.14 (6 H, m), 1.43 – 1.23 (60 H, m) and 0.84 (9 H, t, J 6.5); δ_C(75 MHz; $CDCl_3$) 173.6, 166.7, 135.1, 132.3, 127.4, 125.4, 83.1, 79.4, 58.7, 39.9, 32.0, 31.8, 31.2, 29.7, 29.7, 29.6, 29.6, 29.4, 29.4, 27.0, 22.7 and 14.1 (one CH_2 signal could not be resolved in the 29 ppm area); HR-MALDI-MS (DHB) calcd for $C_{55}H_{96}N_4O_4Na^+$ ([M + Na]$^+$): 899.7330; found 899.7300.

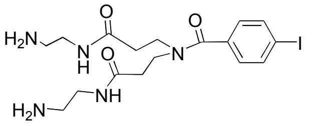


Scheme 2 Synthesis of vector **8**: i) 4-Iodobenzoyl chloride, CH_2Cl_2, Et_3N, 0 ˚C, 75%; ii) ethylenediamine, MeOH, rt, 96%; iii) Boc_2O, $NaHCO_3$, $CHCl_3$, rt, 71%; iv) $[Pd(PPh_3)_4]$, CuI, Et_3N, **15**, THF, rt, 68%; v) TFA, CH_2Cl_2, rt, 99%.



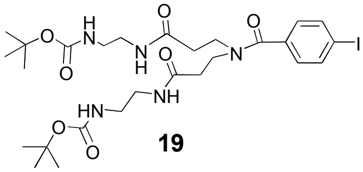
17

Synthesis of ***N,N*-bis-(2-methoxycarbonyl-ethyl)-4-iodobenzamide** (**17**): A solution of **16** (4.96 g, 26.4 mmol) and triethylamine (2 ml) in freshly distilled CH_2Cl_2 (50 ml) was stirred under Ar at 0 °C. To this mixture, a suspension of 4-iodo-benzoyl chloride (6.09 g, 27.1 mmol) in freshly distilled CH_2Cl_2 (10 ml) was added dropwise. The mixture was then allowed to warm to room temperature. After 3 h of stirring, the mixture was poured on ice water and the aqueous phase was extracted with CH_2Cl_2. The organic phase was washed with water and (0.1 N) aq. NaOH, dried ($MgSO_4$) and concentrated to give crude **17** (5.86 g, 75 %) as a yellow oil; $\nu_{max}(CHCl_3)/cm^{-1}$ 2951, 1733, 1634 and 1587; δ_H(300 MHz; $CDCl_3$) 7.73 (2 H, m), 7.08 (2 H, m), 3.65 (10 H, m), 2.60 (4 H, m); δ_C(75 MHz; $CDCl_3$) 171.9, 170.9, 170.8 137.5, 135.4, 128.1, 95.8, 51.9, 45.5, 41.5, 33.4 and 32.4 (four aliphatic Cs and both ester carbonyl Cs are observed); HR-ESI-MS calcd for $C_{15}H_{18}NNaO_5^+$ ($[M+Na]^+$): 442.0122; found 442.0122.



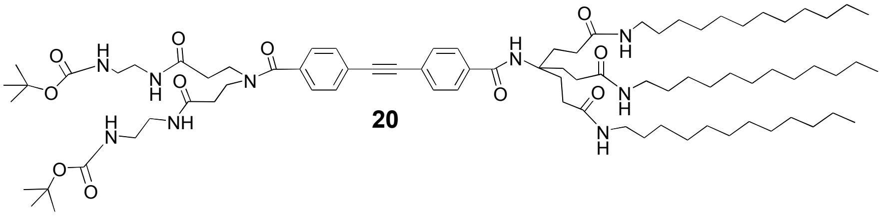
18

Synthesis of ***N*, *N*-bis-[2-(2-aminoethyl carbamoyl)-ethyl]-4-iodobenzamide (18)**: To a solution of **17** (4.01 g, 9.51 mmol) in MeOH (100 ml), ethylenediamine (34.2 g, 0.569 mol) was added. After stirring at room temperature for 5 d, solvent and excess ethylenediamine was removed *in vacuo* to afford **18** (4.32 g, 96%) as a white solid; M.p. 142-143 ˚C; ν_{max}(CHCl$_3$)/cm^{-1} 3358, 3290, 3085, 2927, 2868, 1644, 1622, 1584 and 1552; δ_H(300 MHz; CDCl$_3$) 7.72 (2 H, d, *J* 8), 7.09 (2 H, d, *J* 8), 6.72 (2 H, m), 3.64 (4 H, m), 3.23 (4 H, m), 2.78 (4 H, m) and 2.51 (4 H, m); δ_C(75 MHz; CD$_3$OD) 174.1, 173.4, 172.9, 139.0, 137.0, 129.6, 96.6, 47.4, 43.1, 42.7, 41.8, 35.9 and 35.0 (three amide Cs and six aliphatic Cs are observed); HR-ESI-MS calcd for $C_{17}H_{27}IN_5O_3^+$ ([*M*+H]$^+$): 476.1153; found 476.1147.



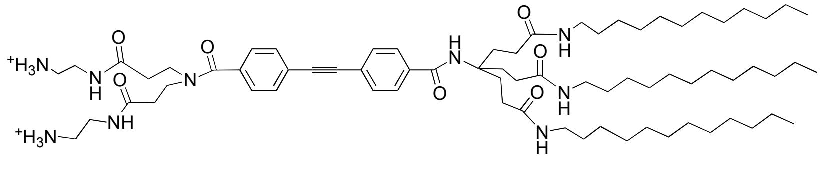
19

Synthesis of ***N*, *N*-bis-[2-(2-*tert*-butoxycarbonyl amino-ethylcarbamoyl)-ethyl]-4-iodobenzamide** (**19**): To a solution of **18** (0.977 g, 2.06 mmol) in CH$_2$Cl$_2$ (50 ml), NaHCO$_3$ (0.359 g, 4.27 mmol) in H$_2$O (8 mL) was added. NaCl (1.00 g, 17.1 mmol) and di-*t*-butyl dicarbonate (1.00 g, 4.60 mmol) were then added, and the mixture was heated to reflux for 90 min. The solution was separated and the aqueous layer was extracted with CHCl$_3$. The combined organic layers were dried (MgSO$_4$) and concentrated *in vacuo*. Column chromatography (SiO$_2$, CH$_2$Cl$_2$/MeOH 4.7/0.3) afforded **19** (0.980 g, 71%) as a white solid; M.p. 156-158 ˚C; ν_{max}(CHCl$_3$)/cm^{-1} 3333, 2979, 2939, 1684, 1639 and 1524; δ_H(300 MHz; CDCl$_3$) 7.71 (2 H, d, *J* 8.7), 7.07 (2 H, d, *J* 8.4), 6.85 (2 H, br), 5.21 (2 H, br), 3.63 (4 H, m), 3.24 (8 H, m), 2.46 (4 H, m), 1.39 (18 H, s); δ_C(75 MHz; CDCl$_3$) 171.6, 170.4, 170.2, 156.6, 137.6, 135.4, 128.2, 96.0, 79.6, 46.7, 46.3, 42.4, 42.1, 40.5, 40.3, 35.5, 35.0 and 28.5 (four amide Cs and ten aliphatic Cs are observed); HR-ESI-MS calcd for $C_{27}H_{42}IN_5NaO_7^+$ ([*M*+Na]$^+$): 698.2021; found 698.2027.



20

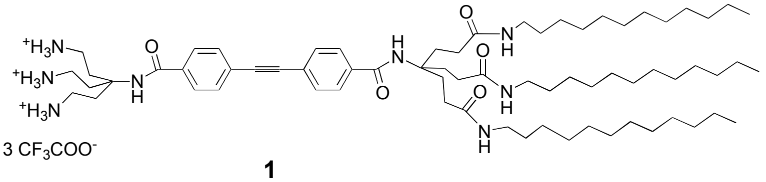
Synthesis of ***tert*-butyl [7-(4-{[4-({4-(dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}carbamoyl)phenyl]ethynyl}benzoyl)-17,17-dimethyl-4,10,15-trioxo-16-oxa-3,7,11,14-tetraazaoctadec-1-yl]carbamate** (**20**): A solution of **19** (0.067 g, 0.099 mmol), **15** (0.111 g, 0.127 mmol) and diisopropylamine (2 ml) in freshly distilled THF (12 ml) was degassed by freeze-pump-thaw cycles. CuI (3 mg, 5 mol-%) and [Pd(PPh$_3$)$_4$] (10 mg, 8 mol-%) were added to the frozen solution. The reaction vessel was degassed by a freeze-pump-thaw cycle and allowed to warm to room temperature. After 24 h, sat. aq. NH$_4$Cl (10 ml) and CH$_2$Cl$_2$ (20 ml) were added. The organic phase was washed with sat. aq. NH$_4$Cl and water, dried (MgSO$_4$) and concentrated. Purification by recycling GPC (CHCl$_3$) afforded **20** (96 mg, 68 %) as a white solid; ν_{max}(CHCl$_3$)/cm^{-1} 3297, 3085, 2924, 2360, 2343 and 1638; δ_{H}(300 MHz; CD$_3$OD) 8.07 (1 H, s), 7.81 (2 H. m), 7.49 (4 H. m), 7.28 (2 H. m), 7.01 (2 H, br), 6.22 (3 H, br s), 5.33 (2 H, br s), 3.66 (4 H, br), 3.23 (8 H, m), 3.16 (6 H, m), 2.45 (4 H, m), 2.23 (12 H, m), 1.42 (24 H, s), 1.23 (54 H, m) and 0.86 (9 H, t, *J* 6.5); δ_{C}(125 MHz; CD$_3$OD) 173.12, 171.68, 170.51, 170.28, 166.41, 156.52, 135.77, 134.36, 131.70, 131.43, 127.15, 126.56, 125.70, 124.34, 90.56, 90.20, 79.49, 58.65, 46.12 (br), 42.04 (br), 40.38, 39.82, 35.61 (br), 34.82 (br), 31.96, 31.60, 31.12, 29.69, 29.63, 29.57, 29.41, 28.47, 27.05, 22.75 and 14.22 (two aliphatic Cs in the 29.7-29.4 ppm region are unobserved and six amide Cs are observed); HR-MALDI-MS (DHB) calcd for C$_{82}$H$_{137}$N$_9$NaO$_{11}^+$ ([*M*+Na]$^+$): 1447.0330; found 1447.0358.



8

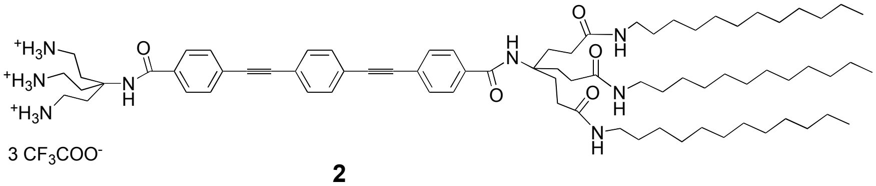
Synthesis of **2,2'-{[(4-{[4-({4-(dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}carbamoyl)phenyl]ethynyl}benzoyl)imino]bis[(1-oxopropane-3,1-diyl)imino]}diethanaminium Bis(2,2,2-trifluoroacetate)** (**8**): To a solution of **20** (0.085 g, 0.060 mmol) in CH$_2$Cl$_2$ (5 ml), trifluoroacetic acid (2 ml) was added under N$_2$. After stirring at room temperature for 1 h, the solvent was removed *in vacuo* to afford **8** (73 mg, 99 %) as a beige solid; M.p. 100–101 °C; ν_{max}(film)/cm^{-1} 3248, 3073, 2920, 2358, 2339 and 1663; δ_{H}(300 MHz;

CD_3OD) 7.84 (2 H, d, *J* 8.4), 7.62 (2 H, d, *J* 8.4), 7.58 (2 H, d, *J* 8.4), 7.42 (2 H, d, *J* 8.4), 3.72 (4 H, m), 3.42 (4 H, m), 3.12 (6 H, t, *J* 6.9), 3.01 (4 H, m), 2.52 (4 H, m), 2.19 (12 H, m), 1.44 (6 H, m), 1.26 (54 H, m) and 0.87 (9 H, t, *J* 6.5); δ_C(75 MHz; CD_3OD) 175.7, 173.9, 169.0, 137.5, 136.5, 132.9, 132.6, 128.8, 128.0, 127.1, 125.8, 91.3, 91.0, 59.9, 40.6, 38.2, 33.1, 31.9, 31.4, 30.8 (2X), 30.7, 30.5, 30.4 (2X), 28.0, 23.8 and 14.5 (one amide C and four aliphatic Cs are unobserved); ESI-MS 1224.9 ($[M + H]^+$, 100).



1

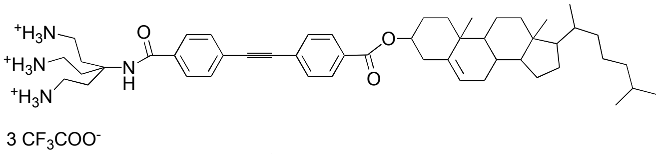
3-(2-Ammonioethyl)-3-({[4-({4-[({4-(dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]carbonyl}amino)pentane-1,5-diaminium Tris(2,2,2-trifluoroacetate) (**1**): Yellow solid; M.p. 100–155 °C; ν_{max}($CHCl_3$)/cm^{-1} 3282, 3140, 2920, 2851, 1633, 1537, 1510, 1463, 1323, 1294, 1199, 1180, 1131, 1017, 837, 798, 766, and 721; δ_H(300 MHz; CD_3OD) 7.80 (4 H, m), 7.56 (4 H, m), 3.07, 3.00 (12 H, m), 2.23, 2.18, 2.09 (18 H, m), 1.40 (6 H, m), 1.22 (54 H, m) and 0.83 (9 H, t, *J* 6.9); δ_C(125 MHz; CD_3OD) 175.70, 169.92, 168.95, 136.59, 135.57, 132.73, 132.63, 128.90, 128.79, 127.82, 127.04, 91.91, 91.29, 59.93, 57.09, 40.59, 35.96, 33.58, 33.09, 31.96, 31.46, 30.78, 30.75, 30.72, 30.68, 30.47, 30.43, 30.38, 28.03, 23.73 and 14.45; HR-MALDI-MS (DHB) calcd for $C_{69}H_{118}N_8NaO_5^+$ ($[M + Na]^+$): 1161.9117; found 1161.9135.



2

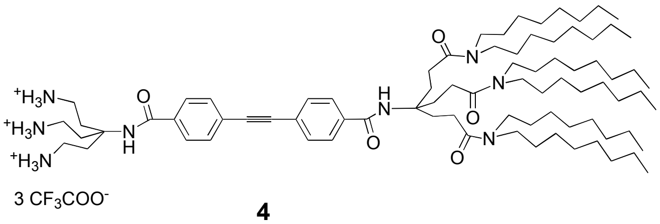
3-(2-Ammonioethyl)-3-{[(4-{[4-({4-[({4-(dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]ethynyl}phenyl)carbonyl]amino}pentane-1,5-diaminium Tris(2,2,2-trifluoroacetate) (**2**): Yellow solid; M.p. 127–190 °C; ν_{max}(neat)/cm^{-1} 3250, 3140, 2921, 2851, 1626, 1538, 1514, 1467, 1321, 1300, 1180, 1134, 1020, 835, 798, 767, 722 and 706; δ_H(300 MHz; CD_3OD) 7.85 (4 H, m), 7.60 (4 H, m), 7.56 (4 H, s), 3.13 (6 H, m), 3.07 (6 H, m), 2.26, 2.16 (18 H, m), 1.45, 1.27 (60 H, m) and 0.88 (9 H, t, *J* 6.6);

δ_C(125 MHz; CD$_3$OD) 174.49, 168.76, 167.77, 135.16, 134.27, 131.67, 131.65, 131.44, 131.35, 127.67, 127.56, 126.75, 126.12, 123.43, 123.11, 91.05, 90.65, 90.40, 90.03, 58.74, 55.91, 39.41, 34.75, 32.33, 31.90 30.75, 30.27, 29.63, 29.59, 29.57, 29.54, 29.32, 29.28, 29.20, 26.86, 22.56 and 13.27; HR-MALDI (3-HPA) calcd for $C_{77}H_{122}N_8NaO_5^+$ ([*M* + Na]$^+$): 1261.9430; found 1261.9407.



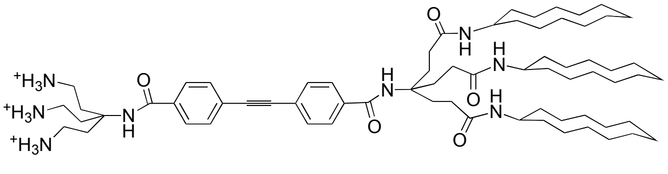
3

3-(2-Ammonioethyl)-3-({[4-({4-[(cholest-5-en-3-yloxy)carbonyl]phenyl}ethynyl)phenyl]carbonyl}amino)pentane-1,5-diaminium Tris(2,2,2-trifluoroacteate) (**3**): Yellow solid; Decomposes at 112 °C; ν_{max}(neat)/cm^{-1} 2933, 1669, 1538, 1466, 1273, 1174, 1132, 1017, 838, 797, 768, 722 and 706; δ_H(300 MHz; CD$_3$OD) 8.04 (2 H, d, *J* 8.4), 7.87 (2 H, d, *J* 8.1), 7.65 (4 H, d, *J* 8.4), 5.45 (1 H, br), H-C(6) hidden by the H$_2$O signal, 3.05 (6 H, m), 2.48 (2 H, m), 2.29 (6 H, m), 2.16–0.80 (38 H, m) and 0.73 (3 H, s); δ_C(125 MHz; CD$_3$OD) 169.93, 166.74, 140.97, 135.72, 132.76, 132.67, 131.88, 130.61, 128.88, 128.65, 127.62, 123.90, 91.98, 91.73, 76.47, 58.15, 57.57, 57.07, 51.65, 43.55, 41.18, 40.71, 39.22, 38.30, 37.83, 37.39, 37.12, 35.90, 33.53, 33.24, 33.07, 29.31, 29.14, 28.89, 25.30, 24.94, 23.18, 22.93, 22.18, 19.81, 19.27 and 12.33; HR-MALDI (3-HPA) calcd for $C_{50}H_{72}N_4NaO^+$ ([*M* + Na]$^+$): 777.5677; found 777.5663.



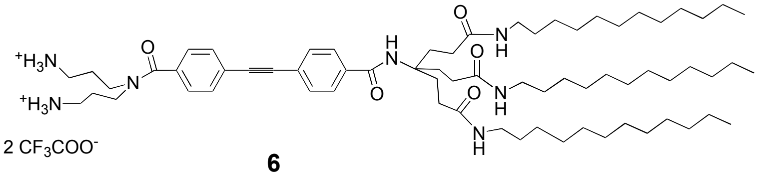
4

3-(2-Ammonioethyl)-3-({[4-({4-[({4-(dioctylamino)-1,1-bis[3-(dioctylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]carbonyl}amino)pentane-1,5-diaminium Tris(2,2,2-trifluoroacetate) (**4**): White powder; M.p. 27–67 °C; ν_{max}(neat)/cm^{-1} 3315, 2925, 2854, 1684, 1623, 1539, 1507, 1457, 1365, 1300, 1275, 1251, 1167, 1045, 1017, 855, 757 and 720; δ_H(300 MHz; CD$_3$OD) 7.90 (2H, d, *J* 8.7), 7.84 (2H, d, *J* 8.4), 7.59 (4H, m), 3.25 (12 H, m hidden by the solvent signal), 3.02 (6 H, m), 2.42 (6 H, m), 2.26, 2.20 (12 H, m), 1.51 (12 H, br), 1.26, 1.20 (60 H, br) and 0.87 (18 H, m); δ_C(75 MHz; CD_3OD) 173.56, 168.72, 167.21, 134.92, 134.47, 131.61, 131.49, 127.70, 127.59, 126.50, 126.22, 90.64, 90.43, 58.66, 55.94, 48.84, 34.75, 32.34, 31.83, 30.89, 29.37, 29.34, 29.31, 29.21, 29.12, 27.65, 26.91, 26.87, 22.60, 22.55, 13.34, 13.29 and 4 aliphatic C from separate octyl chains can be distinguished; HR-MALDI (3-HPA) calcd for $C_{81}H_{142}N_8NaO_5^+$ ($[M + Na]^+$): 1330.0995; found 1330.0970.



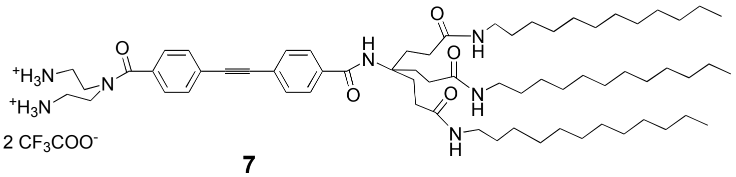
5

3-(2-Ammonioethyl)-3-({[4-({4-[({4-(cyclododecylamino)-1,1-bis[3-(cyclododecylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]carbonyl}amino)pentane-1,5-diaminium Tris(2,2,2-trifluoroacetate) (**5**): White powder; M.p. 126–144 °C; ν_{max}(neat)/cm^{-1} 3300, 3150, 2926, 2853, 1750, 1635, 1540, 1510, 1468, 1440, 1326, 1200, 1180 1130, 1017, 840, 799 and 722; δ_H(300 MHz, CD_3OD) 7.84 (4 H, m), 7.60 (4 H, m), 3.98 (3 H, br), 3.05 (6 H, m), 2.28 (12 H, m), 2.16 (6 H, m), 1.60 (6 H, m) and 1.51-1.21 (60 H, br); δ_C(125 MHz, CD_3OD) 175.01, 169.95, 168.82, 136.47, 135.61, 132.65, 132.64, 128.89, 128.76, 127.79, 127.07, 91.85, 91.35, 59.98, 57.12, 46.76, 35.95, 33.53, 32.14, 31.57, 31.35, 24.91, 24.76, 24.63, 24.39 and 22.97; HR-MALDI (3-HPA) calcd for $C_{69}H_{112}N_8NaO_5^+$ ($[M + Na]^+$): 1155.8648, found 1155.8629.



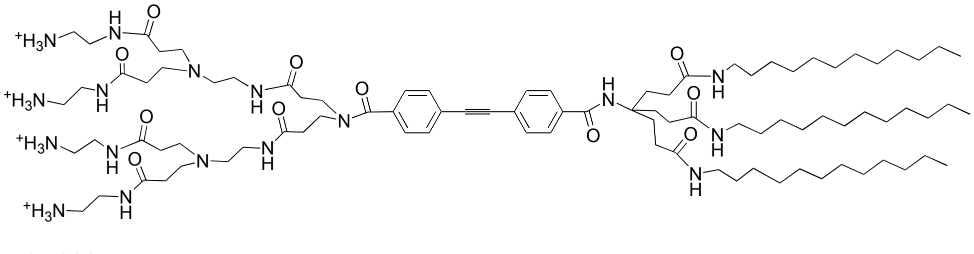
6

3,3'-({[4-({4-[({4-(Dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]carbonyl}imino)dipropan-1-aminium Bis(2,2,2-trifluoroacetate) (**6**): Oily beige solid; ν_{max}(film)/cm^{-1} 3281, 3120, 2921, 2357, 2341, 1669 and 1634; δ_H(300 MHz; CD_3OD) 8.01 (1 H, s), 7.84 (2 H, d, *J* 8.4), 7.64 (2 H, d, *J* 8.4), 7.58 (2 H, d, *J* 8.4), 7.44 (2 H, d, *J* 8.4), 3.51 (4 H, m), 3.11 (6 H, m), 2.88 (4 H, m), 2.18 (12 H, m), 1.97 (4 H, m), 1.43 (6 H, m), 1.26 (54 H, m) and 0.84 (9 H, t, *J* 7.0); δ_C(125 MHz; $(CD_3)_2SO$) 172.98, 171.17, 165.86, 137.22, 136.20, 132.31, 131.75, 128.43, 127.47, 124.98, 123.66, 90.97, 90.69, 58.68, 39.24, 37.35, 31.93, 30.85, 30.47, 29.69, 29.65, 29.63, 29.40, 29.36, 27.21, 22.74 and 14.58 (3 aliphatic Cs are unobserved)); ESI-MS 1110.9 ($[M + H]^+$, 100).



7

2,2'-({[4-({4-[({4-(Dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}amino)carbonyl]phenyl}ethynyl)phenyl]carbonyl}imino)diethanaminium Bis(2,2,2-trifluoroacetate) (**7**): Oily beige solid; ν_{max}(film)/cm^{-1} 3286, 3076, 2923, 2360, 2323 and 1634; δ_H(300 MHz; CD_3OD) 8.01 (1 H, s), 7.83 (2 H, d, *J* 7.8), 7.65 (2 H, d, *J* 7.8), 7.57 (2 H, d, *J* 8.1), 7.52 (2 H, d, *J* 8.4), 3.74 (4 H, m), 3.20 (4 H, m), 3.11 (6 H, t, *J* 7.1), 2.18 (12 H, m), 1.44 (6 H, m), 1.25 (54 H, m) and 0.87 (9 H, t, *J* 6.5); δ_C(125 MHz; CD_3OD) 174.50, 173.56, 167.75, 135.36, 135.11, 131.97, 131.39, 127.59, 126.94, 125.82, 125.16, 90.09, 89.98, 58.73, 39.37, 31.87, 30.76 (broad), 30.28 (broad), 29.58, 29.55, 29.52, 29.49, 29.27, 29.23, 29.17, 26.82, 22.53 and13.25 (2 aliphatic Cs are unobserved); ESI-MS 1082.9 ($[M + H]^+$, 100).



9

7,21-Bis{3-[(2-ammonioethyl)amino]-3-oxopropyl}-14-(4-{[4-({4-(dodecylamino)-1,1-bis[3-(dodecylamino)-3-oxopropyl]-4-oxobutyl}carbamoyl)phenyl]ethynyl}benzoyl)-4,11,17,24-tetraoxo-3,7,10,14,18,21,25-heptaazaheptacosane-1,27-diaminium Tetrakis(2,2,2-trifluoroacetate) (**9**): Oily beige solid; ν_{max}(film)/cm^{-1} 3277, 3073, 2921, 2359, 2332 and 1634; δ_H(300 MHz; CD_3OD) 7.83 (2 H, d, *J* 8.7), 7.62 (2 H, d, *J* 8.4), 7.57 (2 H, d, *J* 8.4), 7.43 (2 H, d, *J* 8.7), 3.80-3.25 (24 H, m), two protons hidden by solvent signal, 3.11 (6 H, t, *J* 6.9), 3.07 (10 H, m), 2.78 (8 H, m), 2.54 (4 H, m), 2.17 (12 H, m), 1.44 (6 H, m), 1.25 (54 H, m) and 0.86 (9 H, t, *J* 6.7); δ_C(125 MHz; CD_3OD) 175.74, 173.74, 173.53, 169.21, 137.50, 136.62, 133.04, 132.61, 128.84, 128.14, 127.10, 125.91, 91.34, 91.16, 59.98, 51.51, 40.82, 40.61, 38.23, 36.13, 36.42, 33.12, 32.83, 32.41, 30.83, 30.79, 30.77, 30.73, 30.52, 30.45, 30.42, 29.19, 28.06, 23.78 and 14.49 (one amide C and three aliphatic Cs are unobserved); ESI-MS 1681.4 ($[M + \mathrm{H}]^+$, 100).