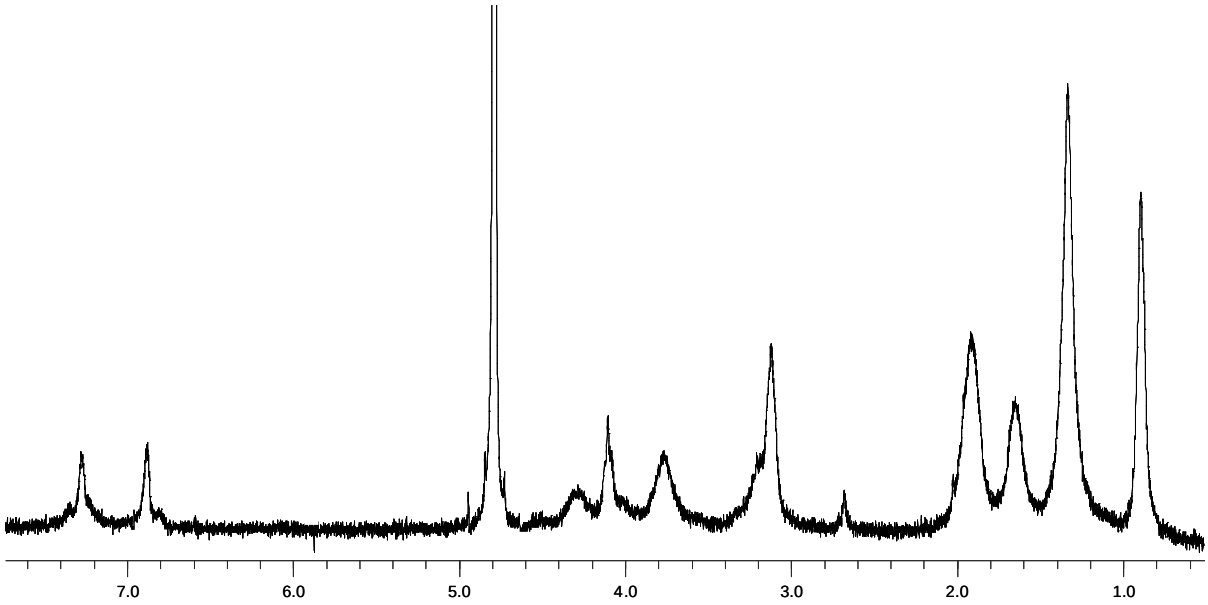
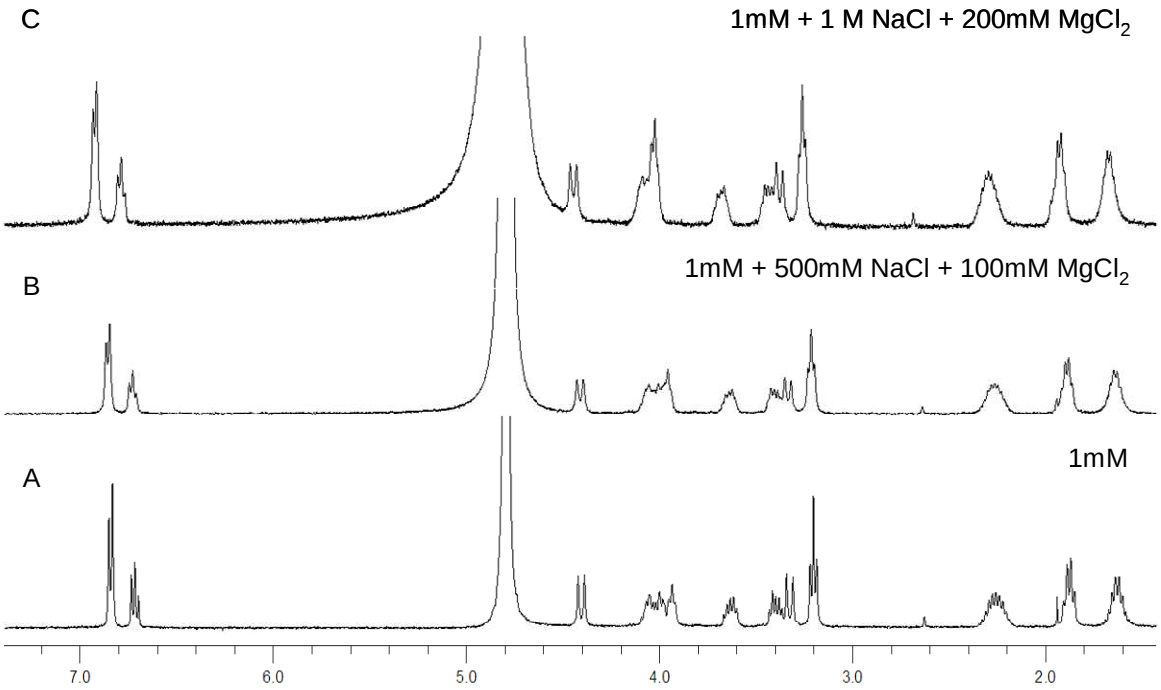
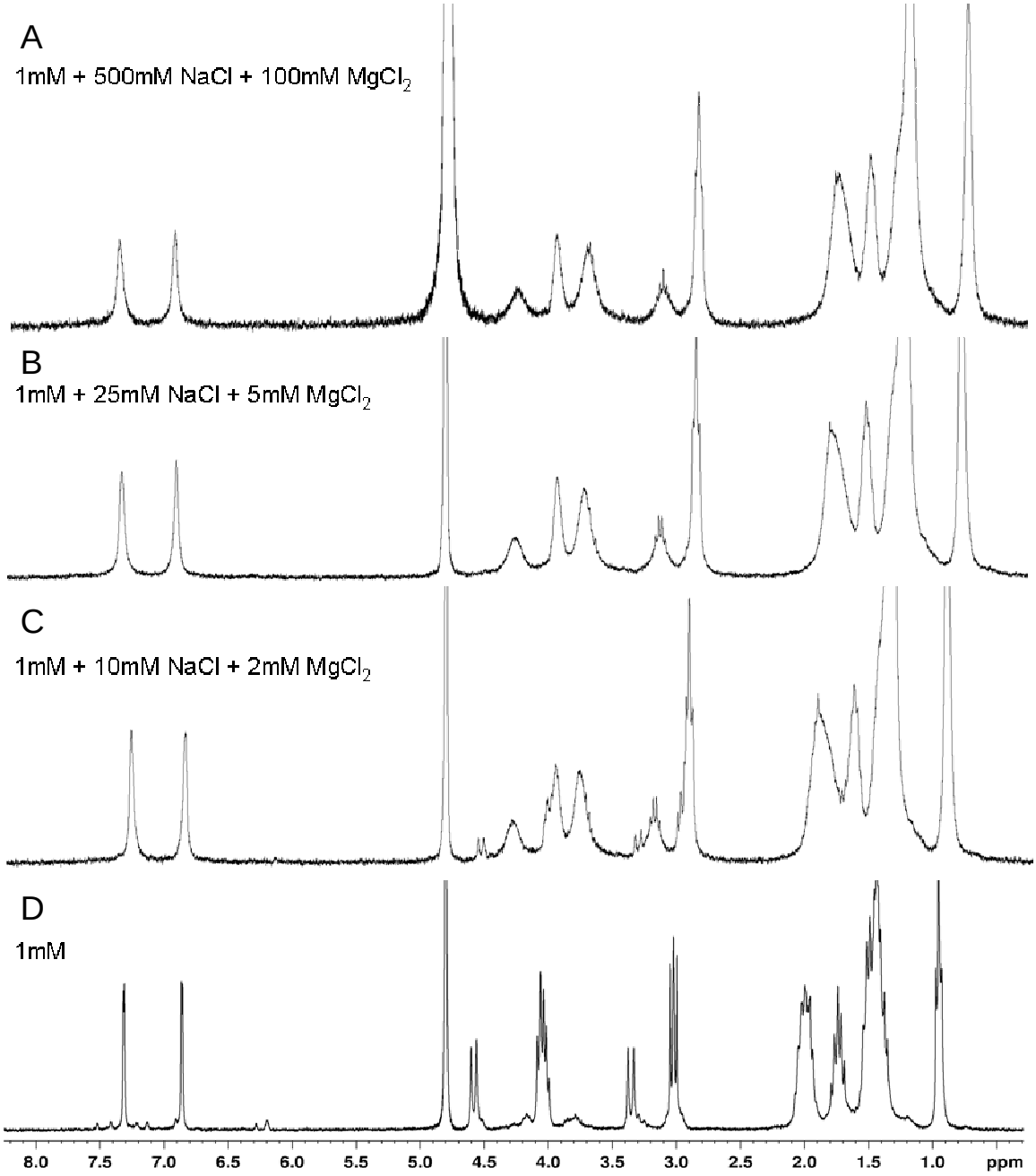


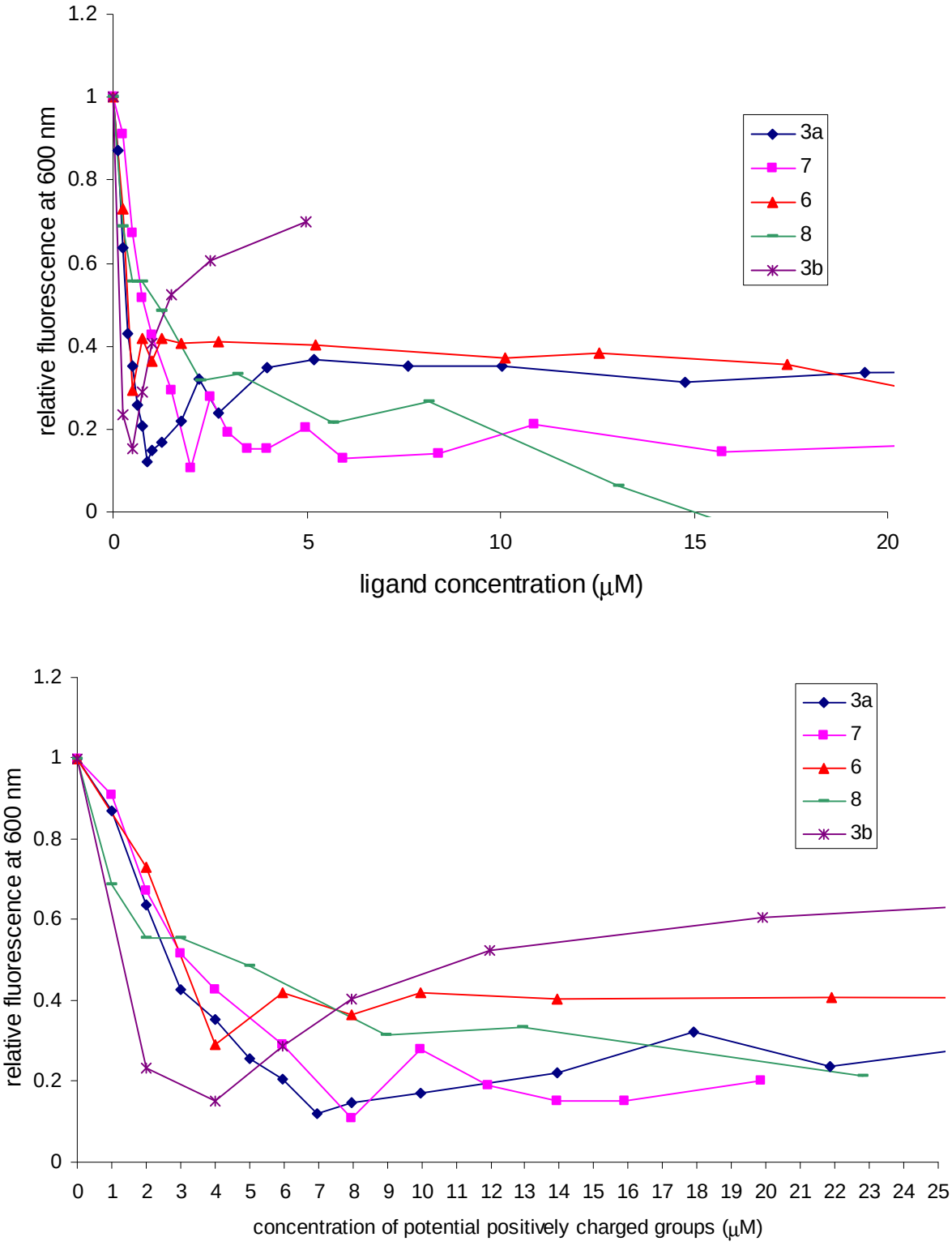
Supplementary Figure S1. **Ligand aggregation properties.** ^{1}H NMR spectrum (D_2O, 300 MHz, 298 K) of compound **3a**.



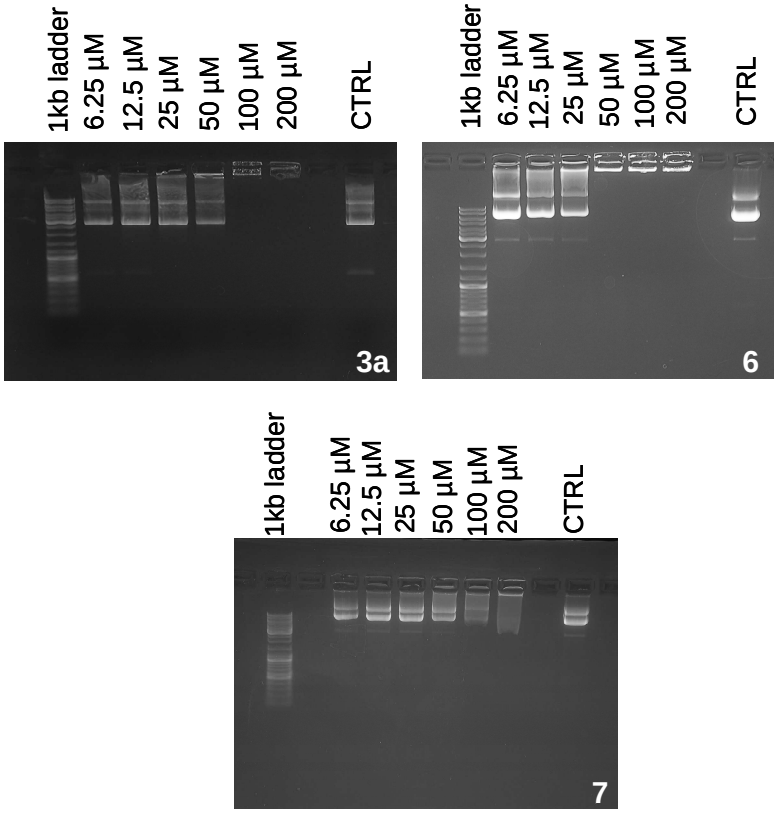
Supplementary Figure S2. **Ligand aggregation properties.** ^{1}H NMR spectra (D_2O, 300 MHz, 298 K) of 1 mM compound **6** (A) alone and (B and C) in the presence of increasing amounts of inorganic salts.



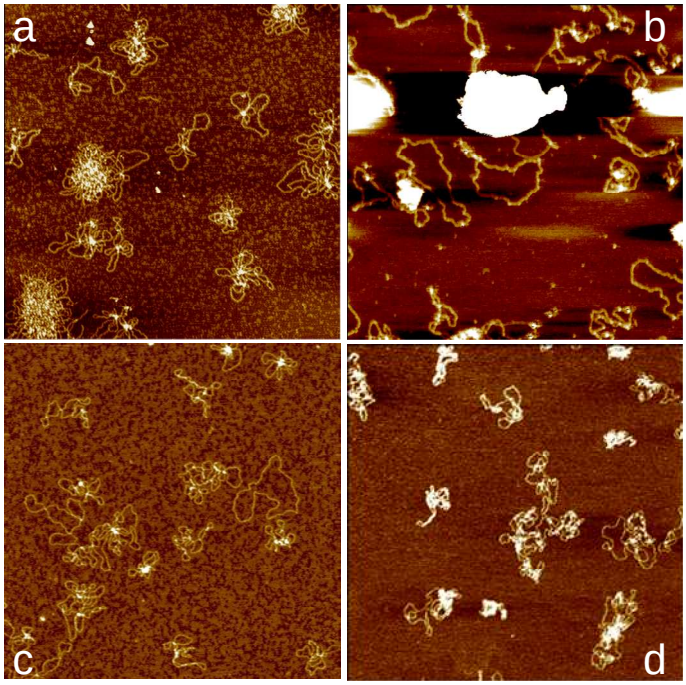
Supplementary Figure S3. **Ligand aggregation properties.** ^{1}H NMR spectra (D_2O, 300 MHz, 298 K) of 1 mM compound **3b** (A) alone and (B, C and D) in the presence of increasing amounts of inorganic salts.



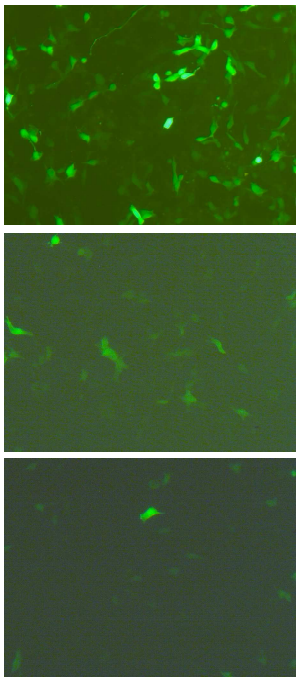
Supplementary Figure S4. **Ethidium bromide displacement assays**. Relative fluorescence *vs* (top) ligand or (bottom) charged groups concentration. Fluorescence studies (excitation at 530 nm, emission at 600 nm) were performed collecting the emission spectra of buffer solutions (4 mM Hepes, 10 mM NaCl) of 50 mM ethidium bromide (relative fluorescence = 0), mixture of 0.5 nM plasmid DNA (pEGFP-C1) and 50 mM ethidium bromide (relative fluorescence = 1) and after addition of increasing amounts of ligand to the DNA/ethidium bromide mixture.



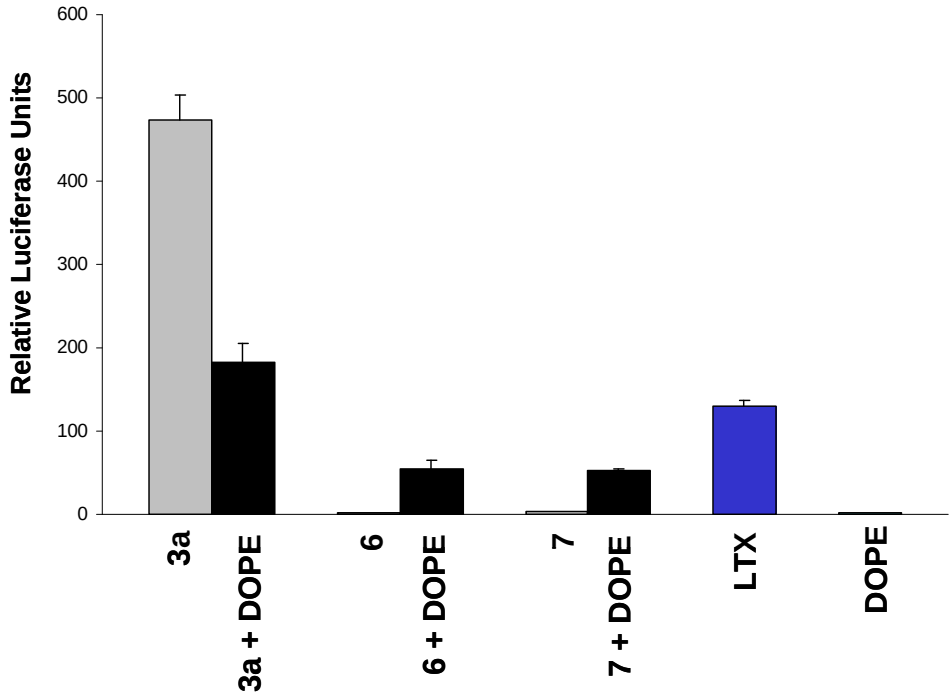
Supplementary Figure S5. **EMSA binding studies.** EMSA of pEGFP-C1 DNA (1 µg in 14 µl) in presence of increasing concentration of ligands **3**, **6** and **7**.



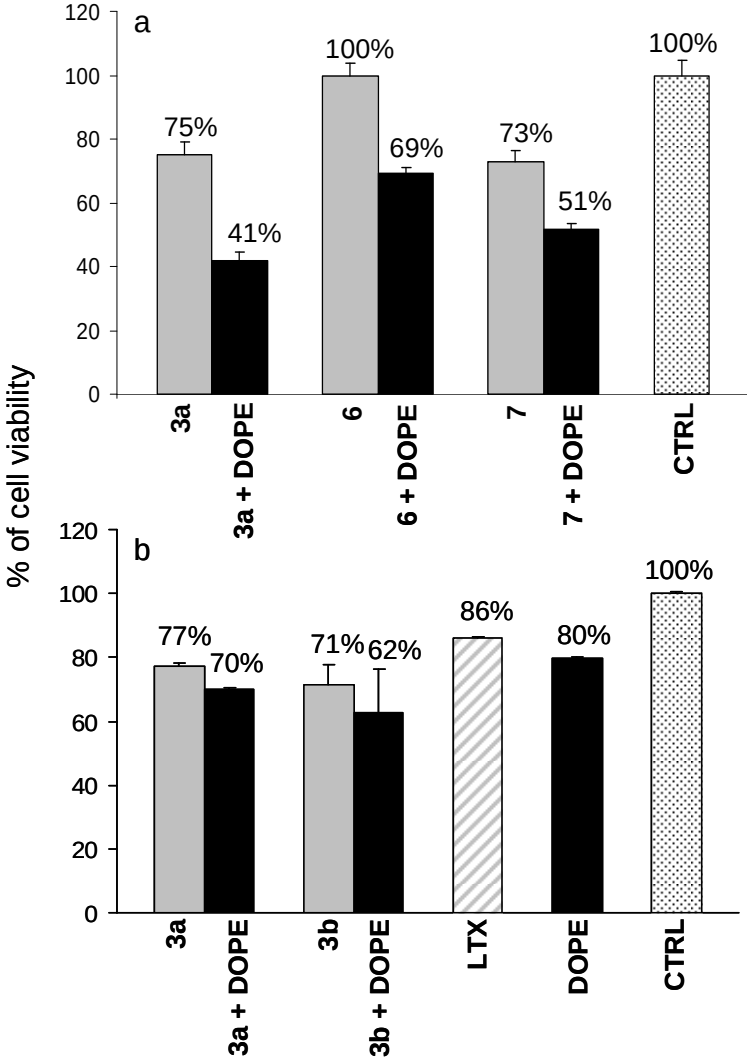
Supplementary Figure S6. AFM binding studies. AFM images (2×2 μm scan each) in tapping mode on air showing the effects induced on plasmid EGFP-C1 DNA 0.5 nM by incubation with a) 0.5 μM calixarene **6**, b) 2 μM calixarene **3b**, c) 4 μM Gemini-type derivative **7** and d) 10 μM Gemini-type derivative **8**.



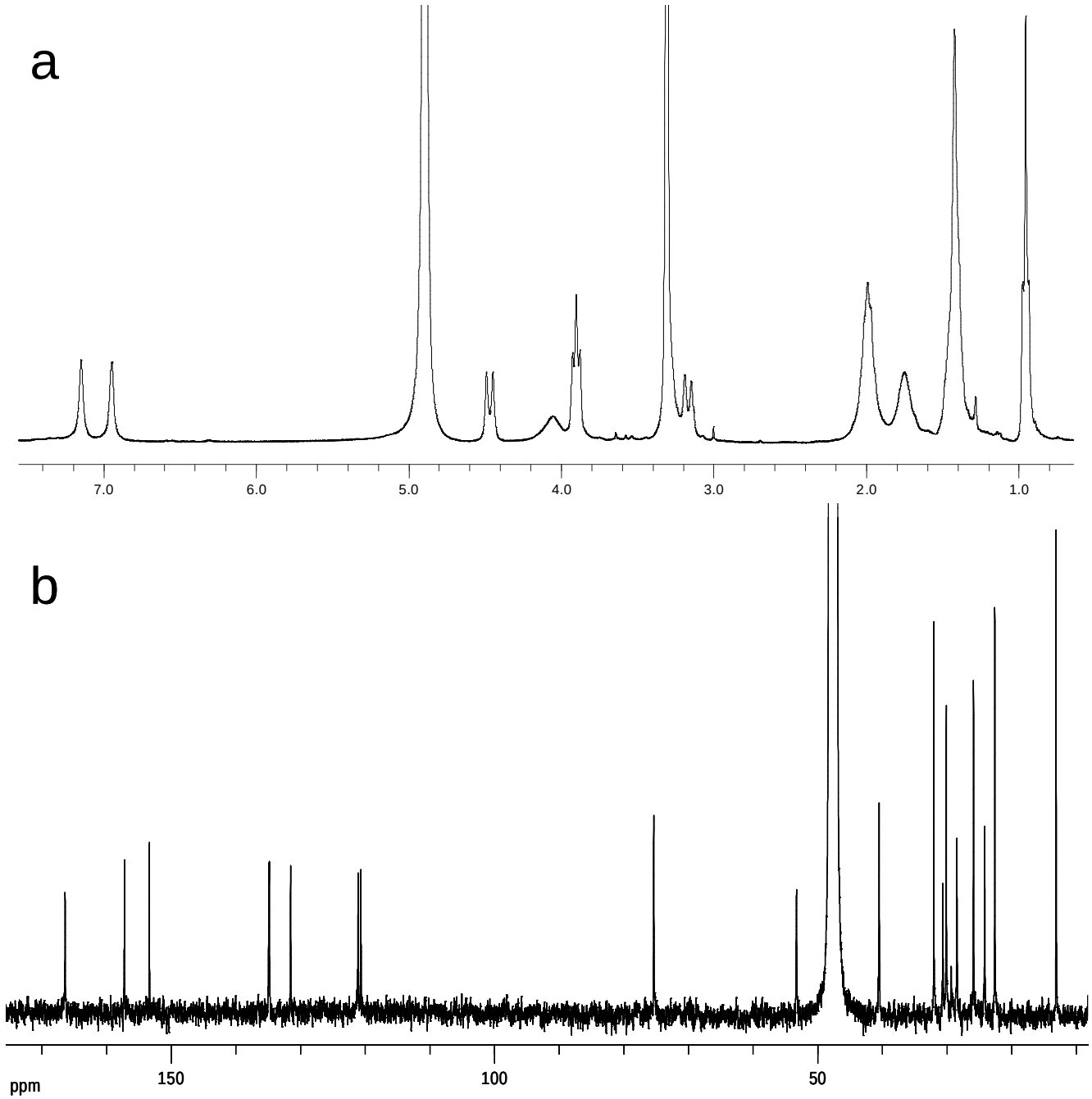
Supplementary Figure S7. **Transfection studies.** Images by fluorescence microscopy of Rhabdomyosarcoma cells transfected (in green) upon treatment with pEGFP-C1 DNA 1 nM formulated with argininocalix[4]arenes **3a**, **6** or Gemini-type ligand **7**, in the presence of DOPE (ligand/adjuvant molar ratio 1:2).



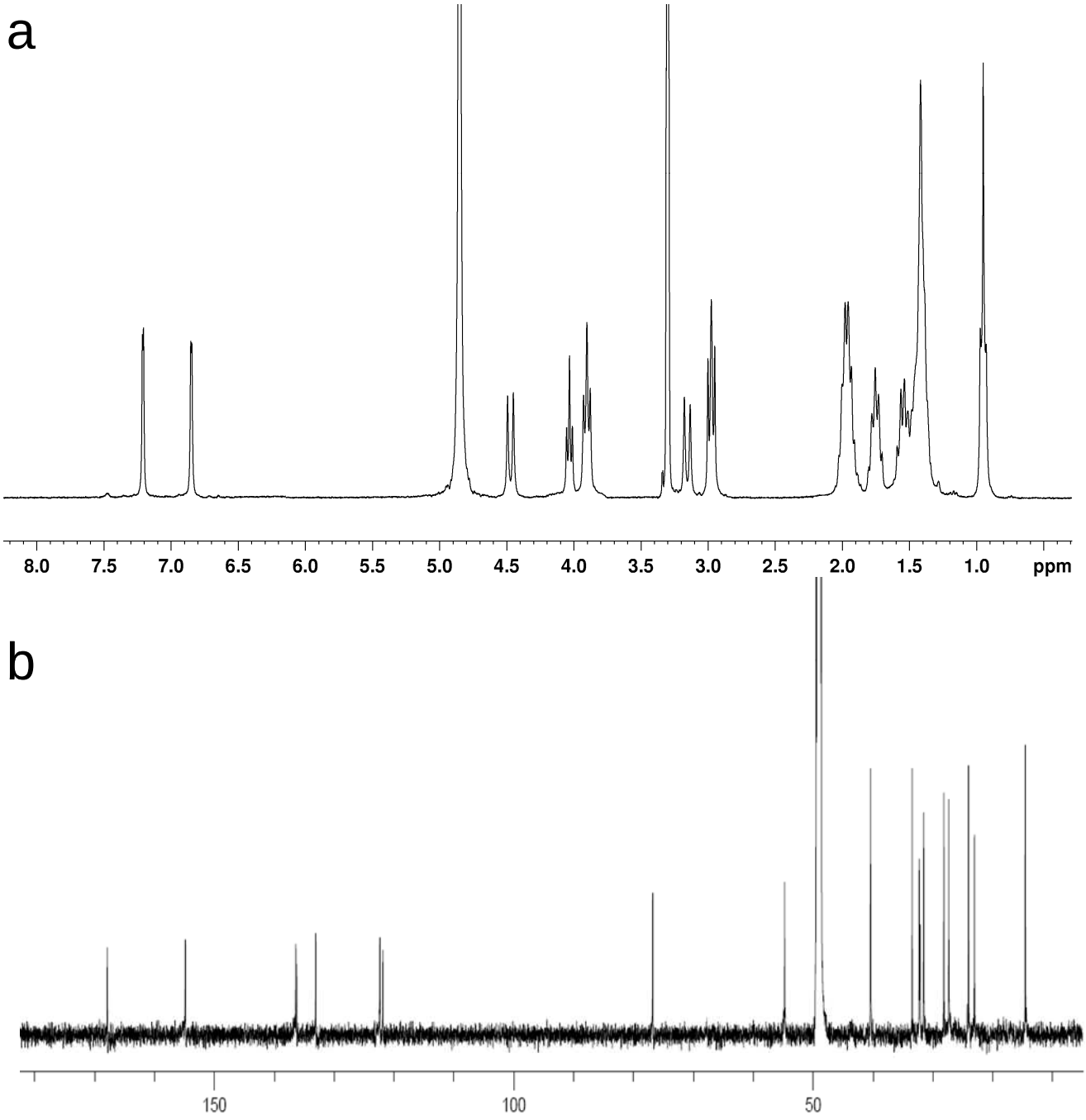
Supplementary Figure S8. **Transfection studies.** Transfection efficiency at 48h from the treatment of ligands **3a**, **6** and **7** with and without DOPE (ligand:adjuvant molar ratio 1:2) compared to LTX and DOPE, expressed as Relative Luciferase Units.



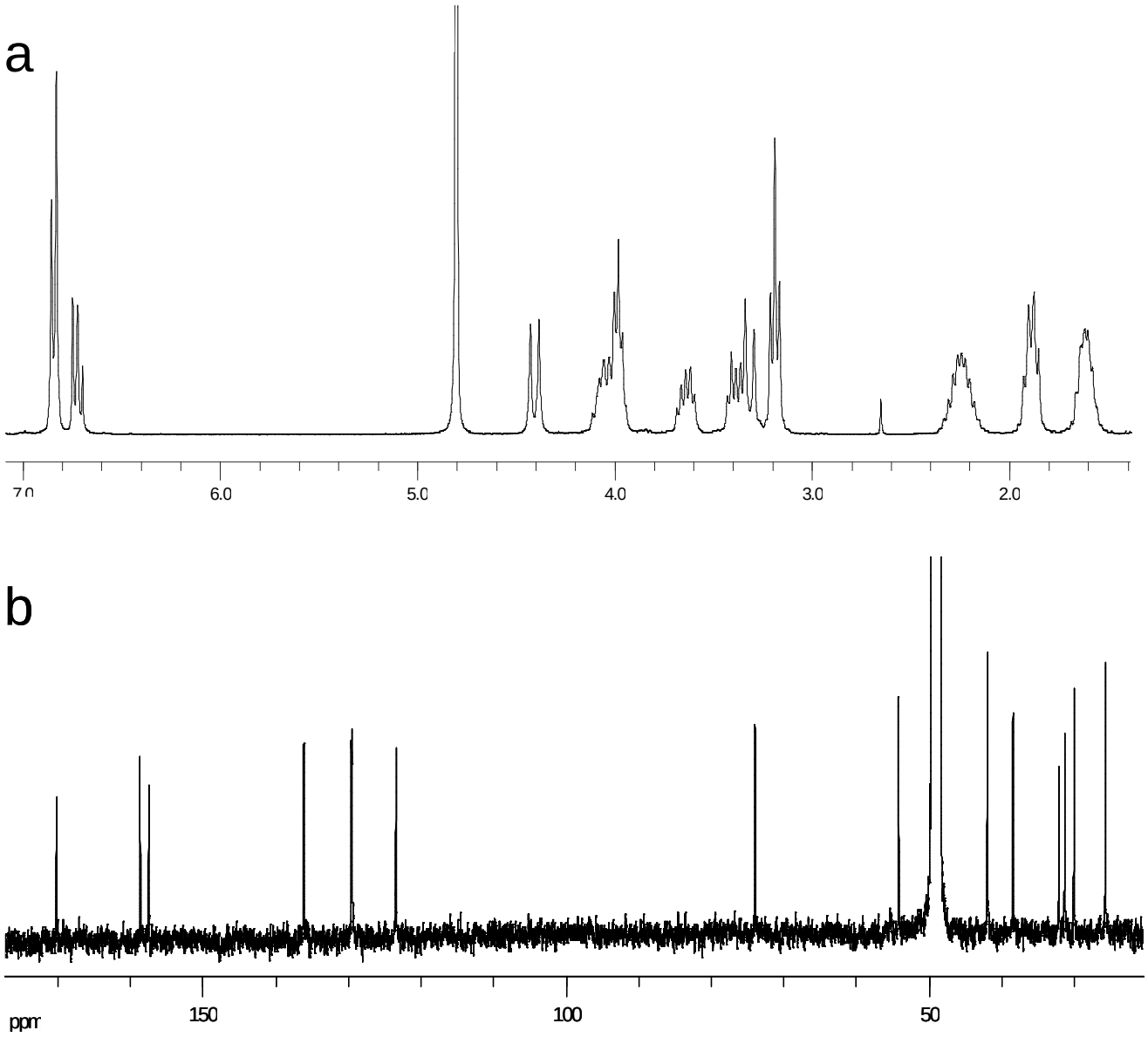
Supplementary Figure S9. Cytotoxicity studies. Cell viability (RD-4 human Rhabdomyosarcoma cells) at 48 h from the treatment a) after simple incubation with ligands **3a**, **6** and **7**, alone and together with adjuvant, and b) in transfection conditions with argininocalix[4]arene **3a** or lysinocalix[4]arene **3b**, alone and with adjuvant, compared to LTX and DOPE. CTRL is relative to untreated cells.



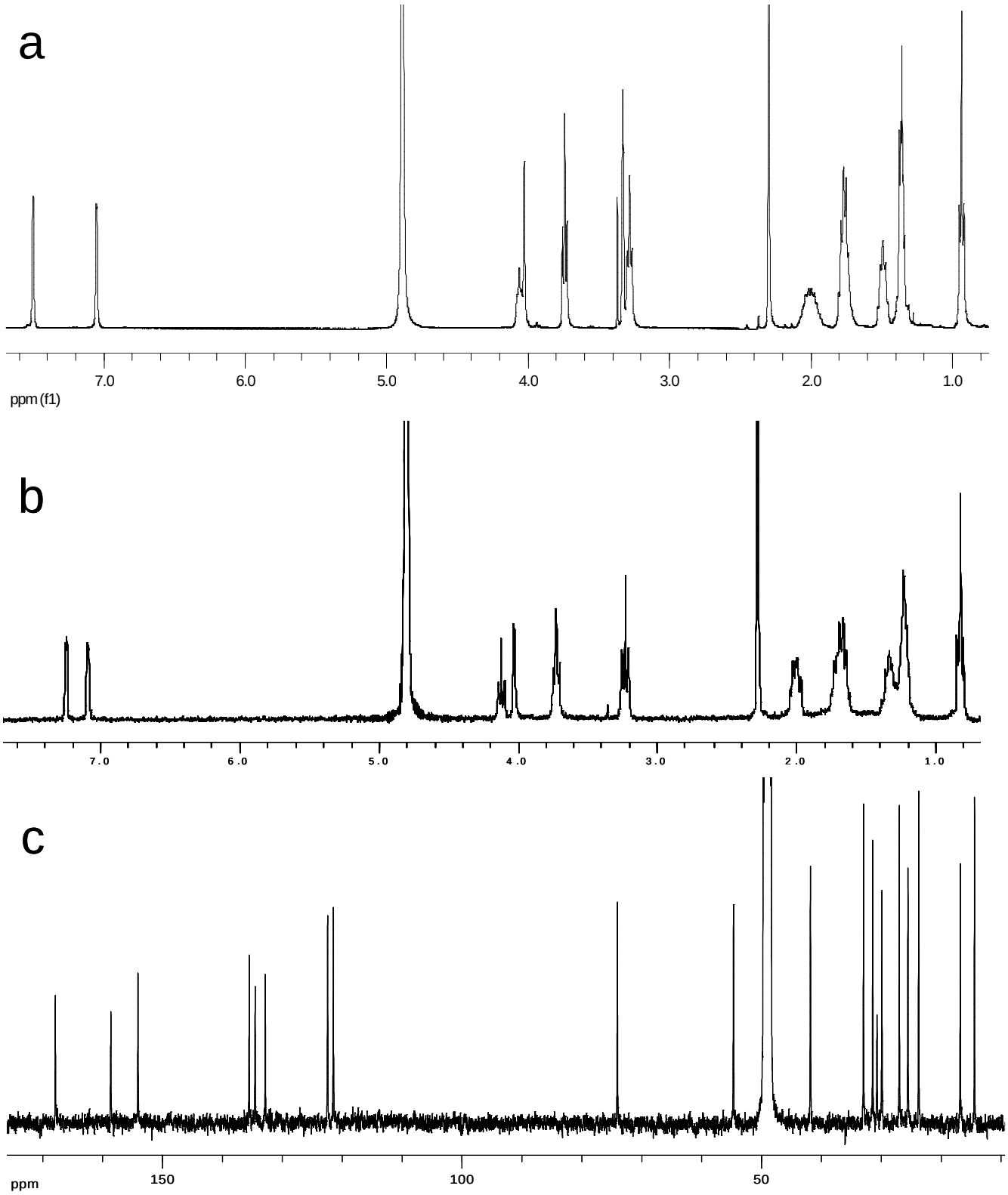
Supplementary Figure S10. NMR spectra of argininocalix[4]arene 3a. a) ^{1}H NMR spectrum (CD_3OD, 300 MHz, 298 K) and b) ^{13}C NMR spectrum (CD_3OD, 100 MHz, 298 K).



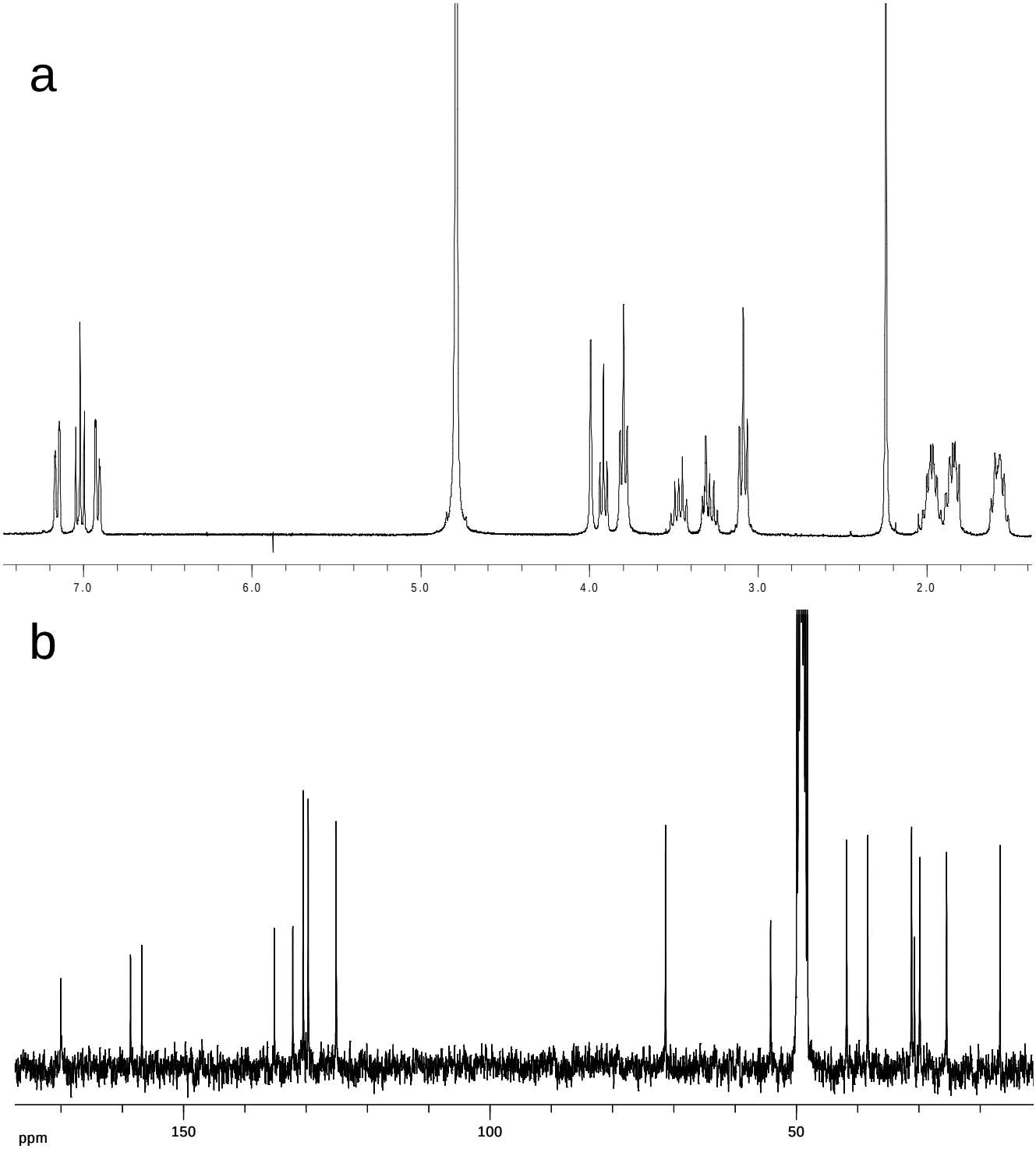
Supplementary Figure S11. NMR spectra of lysinocalix[4]arene 3b. a) ^{1}H NMR spectrum (CD_3OD, 300 MHz, 298 K) and b) ^{13}C NMR spectrum (CD_3OD, 150 MHz, 298 K).



Supplementary Figure S12. NMR spectra of argininocalix[4]arene 6. a) ^{1}H NMR spectrum (D_2O, 300 MHz, 298 K) b) ^{13}C NMR spectrum (CD_3OD, 100 MHz, 298 K).



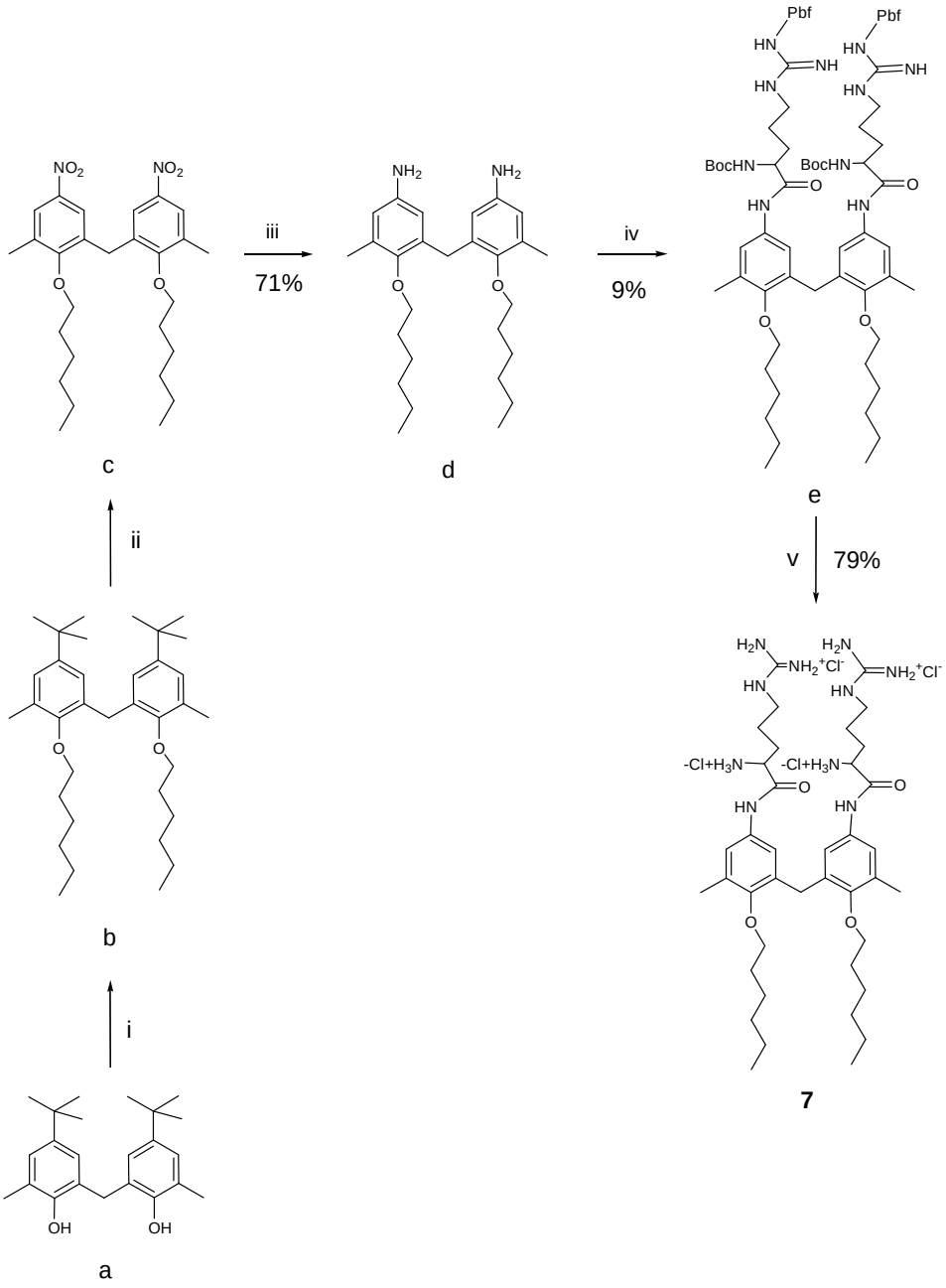
Supplementary Figure S13. NMR spectra of Gemini-type arginino derivative 7. a) ^{1}H NMR spectrum in CD_3OD (400 MHz, 298 K), b) ^{1}H NMR spectrum in D_2O (400 MHz, 298 K) and c) ^{13}C NMR spectrum (CD_3OD, 100 MHz, 298 K).



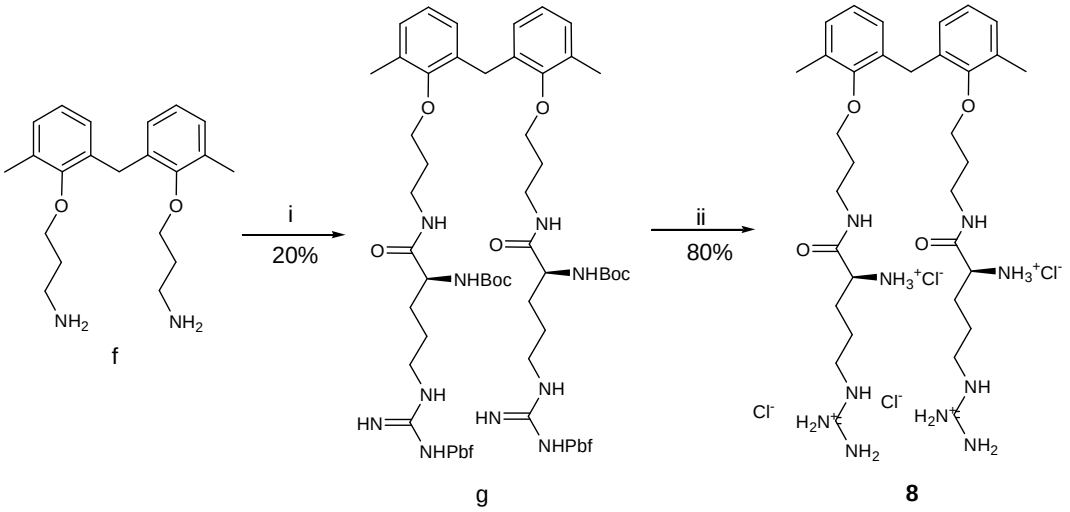
Supplementary Figure S14. NMR spectra of Gemini-type arginino derivative 8. a) ^{1}H NMR spectrum (D_2O, 300 MHz, 298 K) and b) ^{13}C NMR spectrum (CD_3OD, 75 MHz, 298 K).

Supplementary Methods

Abbreviations. DMF: dimethylformamide, TFA: trifluoroacetic acid, HOBt: hydroxybenzotriazole, DCC: N,N'-dicyclohexylcarbodiimide, TIS: triispopropylsilane, Boc: *t*-Butoxycarbonyl, Pbf: 2,2,4,6,7-Pentamethyl-dihydrobenzofuran-5-sulfonyl, DMAP: dimethylaminopyridine, EDAC: N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride, DIPEA: N,N-diisopropylethylamine, HBTU: O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, Cbz: carbobenzyloxy, DCU: N,N'-dicyclohexylurea, PTFE: polytetrafluoroethylene (Teflon), TLC: Thin Layer Chromatography.



Synthesis of compound 7. i) NaH, iodohexane, dry DMF, N_2, rt; ii) $NaNO_3$, TFA, rt; iii) $NH_2NH_2 \cdot H_2O$, Pd/C, N_2, abs ethanol, reflux, N_2; iv) Boc-Arg(Pbf)-OH, HOBt, DCC, dry DMF, N_2, rt; v) TFA/TIS/H_2O (95/2.5/2.5), dil. HCl in methanol, pH 4, rt.



Synthesis of compound 8. i) Boc-Arg(Pbf)-OH, HOBt, DCC, dry DMF, N_2, rt; ii) TFA/TIS/H_2O (95/2.5/2.5), dil. HCl in methanol, pH 4, rt.

Bis[(2-hexyloxy-3-methyl-5-*tert*-butyl)phenyl]methane (b). The product was obtained according to the literature procedure relative to alkylation of calixarenes.[36] To a suspension of Gemini **a**[21] (0.8 g, 2.35 mmol) in dry DMF (24 mL) at 0 °C NaH (60 wt.-% in oil, 0.57 g, 14.1 mmol) was added. The mixture was stirred for 30 min. and then 1-iodohexane (2.13 mL, 14.1 mmol) was added. After 1 h the ice bath was removed and the reaction mixture was stirred for one day. The reaction was quenched with 1 N HCl (70 mL) and the resulting solid filtered on a Büchner funnel. The crude product was dissolved in CH_2Cl_2 (70 mL) and washed with 1 N HCl (3×50 mL). The organic phase was separated, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain a yellowish oil. The residue was purified by flash column chromatography on silica gel (eluent: CH_2Cl_2 and CH_2Cl_2/MeOH= 99:1) to obtain the pure product as a colourless oil in 90% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.12 (d, *J* = 2.3 Hz, 2H, ArH), 6.98 (d, *J* = 2.3 Hz, 2H, ArH), 4.16 (s, 2H, $ArCH_2Ar$), 3.82 (t, *J* = 6.7 Hz, 4H, OCH_2), 2.40 (s, 6H, $ArCH_3$), 1.86 (quint, *J* = 7.0 Hz, 4H, OCH_2CH_2), 1.62-1.32 (m, 12H, $O(CH_2)_2CH_2CH_2CH_2$), 1.31 (s, 18H, $C(CH_3)_3$), 0.99 (t, *J* = 6.8 Hz, 6H, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.8, 145.8, 133.1, 129.8, 125.7, 125.6, 72.8, 34.1, 31.8, 31.5, 30.5, 29.6, 25.9, 22.7, 16.7, 14.1; MS (*m/z*): $[M + Na]^+$ calcd for $C_{35}H_{56}O_2$, 531.4; found, 531.5.

Bis[(2-hexyloxy-3-methyl-5-nitro)phenyl]methane (c). The product was obtained according to the general procedure for the ipso nitration of calixarenes.[37] To a solution of **b** (1.05 g, 2.07 mmol) in trifluoroacetic acid (4.70 mL, 61.2 mmol), $NaNO_3$ (3.45 g, 40.7 mmol) was added and the mixture turned orange. After one night the reaction was stopped with addition of water (100 mL) and extracted with CH_2Cl_2 (2×50 mL). The separated organic layer was washed with water (75 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (eluent: hexane/ethyl acetate= 98:2) to obtain the pure product as a light yellow oil in 60% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.99 (d, *J* = 2.8 Hz, 2H, ArH), 7.77 (d, *J* = 2.8 Hz, 2H, ArH), 4.10 (s, 2H, $ArCH_2Ar$), 3.81 (t, *J* = 6.6 Hz, 4H, OCH_2), 2.38 (s, 6H, $ArCH_3$), 1.79 (quint, *J* = 6.6 Hz, 4H, OCH_2CH_2), 1.55-1.25 (m, 12H, $O(CH_2)_2CH_2CH_2CH_2$), 0.89 (t, *J* = 6.7 Hz, 6H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 161.4, 143.5, 134.1, 132.8, 125.3, 123.6, 73.4, 31.6, 30.3, 30.2, 25.6, 22.5, 16.8, 13.9; MS (*m/z*): $[M + Na]^+$ calcd for $C_{27}H_{38}N_2O_6$, 509.3; found, 509.4.

Bis[(2-hexyloxy-3-methyl-5-amino)phenyl]methane (d). The product was obtained according to the general procedure for the nitro reduction of calixarenes.[36] To a solution of **c** (0.22 g, 0.45 mmol) and hydrazine monohydrate (0.22 mL, 4.52 mmol) in ethanol (14 mL), a catalytic amount of Pd/C was added and the reaction mixture was stirred and refluxed overnight. The solvent was then removed under reduced pressure, the residue dissolved in CH_2Cl_2 (50 mL) and the catalyst filtered off through a paper filter. Pure amine **d** was obtained after evaporation of the solvent under reduced pressure as a light yellow oil in 78% yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.34 (d, *J* = 2.7 Hz, 2H, ArH), 6.17 (d, *J* = 2.7 Hz, 2H, ArH), 3.90 (s, 2H, $ArCH_2Ar$), 3.69 (t, *J* = 6.9 Hz, 4H, OCH_2), 3.30 (bs, 4H, NH_2), 2.23 (s, 6H, $ArCH_3$), 1.76 (quint, *J* = 6.9 Hz, 4H, OCH_2CH_2), 1.46 (bquint, *J* = 5.4 Hz, 4H, $OCH_2CH_2CH_2$), 1.42-1.28 (m, 8H, $O(CH_2)_2CH_2CH_2CH_2$), 0.91 (t, *J* = 6.9 Hz, 6H, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 148.5, 142.0, 134.6, 131.4, 115.7, 115.0, 73.2, 31.8, 30.3, 29.6, 29.0, 25.8, 22.6, 16.4, 14.0; MS (*m/z*): $[M + H]^+$ calcd for $C_{27}H_{42}N_2O_2$, 427.3; found, 427.5, $[M + Na]^+$ calcd, 449.3; found, 449.5.

Bis[(2-aminopropoxy-3 methyl)phenyl]methane (f). The compound was obtained according to literature procedure.[21]

Synthesis of 25,26,27,28-Tetrakis(3-aminopropoxy)calix[4]arene (4). It was synthesized according to literature procedure.[29]

5,11,17,23-Tetrakis[(N_α-Boc-N_ω-Pbf-L-Arg)amino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (2a). To a solution of Boc-L-Arg(Pbf)-OH (0.58 g, 1.09 mmol) and DMAP (0.27 mg, 2.19 mmol) in dry CH_2Cl_2 (15 mL), HOBt (0.17 g, 1.24 mmol) and EDAC (0.21 g, 1.09 mmol) were added and the mixture stirred for 15 min. Then, aminocalixarene **1** (0.15 g, 0.18 mmol) dissolved in CH_2Cl_2 (6 mL) was added. The reaction proceeded at room temperature for 24 h. The reaction was quenched by addition of water and the organic layer washed with water (2×25 mL) and saturated $NaHCO_3$ aqueous solution (2×25 mL). The organic solvent was removed at reduced pressure giving a crude material that was purified by flash column chromatography (gradient from CH_2Cl_2 to CH_2Cl_2/CH_3OH 95:5). The pure product was isolated as a white solid in 40% yield. mp: 156-158 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD= 19:1): δ 9.04 (bs, ArNH), 7.10-6.10 (bs, 8H, ArH and NHCNHNH), 5.90 (bs, NHBoc), 4.33 (d, *J* = 13.1 Hz, 4H, $ArCHHAr$), 4.06 (bs, 4H, $COCHNH$), 3.77 (bs, 8H, OCH_2), 3.10 (bs, 8H, CH_2NH), 3.04 (d, *J* = 13.1 Hz, 4H, $ArCHHAr$), 2.89 (s, 8H, CH_2 of Pbf), 2.49 (s, 12H, CH_3 of Pbf), 2.43 (s, 12H, CH_3 of Pbf), 2.00 (s, 12H, CH_3 of Pbf), 1.90-1.20 (m, 108H, $OCH_2CH_2CH_2CH_2CH_2$, $C(CH_3)_3$, $COCHCH_2CH_2$ and $C(CH_3)_2$ of Pbf), 0.84 (t, *J* = 6.5 Hz, 12H, CH_2CH_3); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD = 19:1): δ 171.0, 158.6, 156.3, 155.9, 153.5, 138.2, 134.9, 132.4, 132.2, 131.0, 124.6, 121.0, 117.4, 86.4, 79.9, 75.3, 54.3, 43.0, 40.2, 31.9, 31.0, 30.0, 29.5, 28.3, 28.1, 25.8, 25.1, 22.7, 19.1, 17.8, 13.9, 12.2; MS (m/z): $[M + 2Na]^{++}$ calcd for $C_{148}H_{220}N_{20}O_{28}S_4$, 1449.8; found *m/z* = 1450.6.

Synthesis of 5,11,17,23-Tetrakis[(N_α-Boc-N_ω-Boc-L-Lys)amino]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (2b). To a stirring solution of Boc-L-Lys(Boc)-OH (0.14 g, 0.36 mmol) and DIPEA (0.06 mL) in dry CH_2Cl_2 (5 mL), HBTU (0.16 g, 0.42 mmol) and aminocalixarene **1** (0.05 g, 0.06 mmol) were added. The mixture proceeded at room temperature for 24 h. Then the reaction was quenched by adding water (6 mL), the organic layer separated and washed with brine (6 mL), and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed at reduced pressure and the pure product was isolated by flash column chromatography (gradient from CH_2Cl_2 to CH_2Cl_2/CH_3OH 95:5) as white solid in 69% yield. mp: 182-185 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD 19/1): δ 7.10 (bs, 4H, ArH), 6.32 (bs, 4H, ArH), 4.34 (d, *J* = 13.2 Hz,

4H, ArCH*H*Ar), 4.05 (bs, 4H, COC*H*NHBoc), 3.87-3.60 (m, 8H, OCH_2), 3.12-2.87 (m, 12H, ArC*H*HAr and CH_2NHBoc), 1.79 (bs, 16H, OCH_2CH_2 and $COCHCH_2$), 1.59 (bs, 8H, $CH_2CH_2NH_3^+$), 1.80-1.20 (m, 104H, $(CH_3)_3$, $COCHCH_2CH_2$ and $O(CH_2)_2CH_2CH_2CH_2$), 0.85 (bs, 12H, CH_2CH_3); ^{13}C NMR (150 MHz, $CDCl_3/CD_3OD$ 19/1): δ 170.9, 156.3, 156.0, 153.5, 135.1, 131.3, 121.2, 119.8, 79.9, 79.1, 75.1, 54.7, 39.9, 32.0, 31.1, 30.0, 29.4, 29.0, 28.5, 28.3, 25.9, 22.7, 22.5, 14.1; MS (m/z): $[M + 2Na]^{++}$ calcd for $C_{116}H_{188}N_{12}O_{24}$, 1089.7; found, 1090.2.

25,26,27,28-Tetrakis{3-[(N_α-Cbz-L-Arg)amino]propoxy}calix[4]arene, tetra-hydrochloride (5). To a stirring solution of N_α-Cbz-L-Arg-OH (0.79 g, 2.58 mmol) in dry DMF (10 mL), HOBt (0.4 g, 2.92 mmol) and DCC (0.54 g, 2.58 mmol) were added. After 15 min, a solution of calix[4]arene **4** (0.28 g, 0.43 mmol) in dry DMF (10 mL) was added dropwise to the mixture. The reaction proceed at room temperature for 5 days following its evolution by ESI-MS, when further HOBt (0.07 g, 0.51 mmol) and DCC (0.09 g, 0.43 mmol) were added and the reaction stirred for 2 days longer. The reaction was quenched by filtration of DCU using a Gooch 5 funnel and by evaporation of solvent at reduced pressure. The crude was dissolved in MeOH (5 mL) and eluted through anionic exchange sorbent SAX (ammonium form, Cl^-) to obtain **5** as a white solid in 76% yield. mp: 107-110 °C; ^{1}H NMR (300 MHz, CD_3OD): δ 7.42-7.20 (m, 20H, ArH of Cbz), 6.70-6.42 (m, 12H, ArH), 5.15-4.93 (m, 8H, CH_2Cbz), 4.37 (d, *J* = 13.1 Hz, 4H, ArCH*H*Ar), 4.26-4.09 (m, 4H, COC*H*NH), 4.00-3.76 (m, 8H, OCH_2), 3.50-3.31 (m, 4H, OCH_2CH_2CH), 3.25-3.03 (m, 16H, ArC*H*HAr, CH_2NHCNH and OCH_2CH_2CH), 2.28-2.00 (m, 8H, OCH_2CH_2), 2.00-1.49 (m, 16H, $COCHCH_2CH_2$); ^{13}C NMR (100 MHz, CD_3OD) δ 174.8, 174.4, 158.9, 158.7, 158.6, 157.7, 138.3, 138.2, 136.4, 129.7, 129.3, 129.2, 129.1, 129.0, 123.5, 74.1, 68.0, 67.9, 56.5, 55.2, 42.2, 42.1, 38.3, 32.1, 31.5, 30.8, 29.9, 26.9, 26.7, 26.6; MS (m/z): $[M + 3H - 4HCl]^{3+}$ calcd for $C_{96}H_{128}N_{20}O_{16}Cl_4$, 605.3; found, 605.5.

Bis{[(5-N_α-Boc-N_ω-Pbf-L-Arg)-amino]-2-hexyloxy-3-methylphenyl}methane (e). To a solution of Boc-L-Arg(Pbf)-OH (0.51 g, 0.97 mmol) in dry DMF (5 mL) were added HOBt (0.15 g, 1.09 mmol) and DDC (0.20 g, 0.96 mmol) and the mixture stirred for 10 minutes. Then compound **d** (0.12 g, 0.29 mmol) dissolved DMF (2 mL) was added. The reaction proceeded under stirring at room temperature for 24 h. Ethyl acetate was added (10 mL), DCU was filtered off by gravity on a PTFE filter, and the organic solvents were removed at reduced pressure. The crude was dissolved in ethyl acetate (10 mL) and the organic layer was washed first with a saturated $NaHCO_3$ aqueous solution (2×10 mL), then with brine (3×10 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed at rotavapor and the residue purified by preparative TLC (eluent: ethyl acetate/hexane= 2:1) to obtain the pure product **e** as a white solid in 10% yield. mp: 136 °C dec; ^{1}H NMR (300 MHz, CD_3OD): δ 7.70 (bs, 2H, ArH), 6.75 (bs, 2H, ArH), 4.35-3.50 (bm, 8H, $ArCH_2Ar$, NHCOC*H* and OCH_2), 3.30-3.02 (bm, 4H, $N{=}CNHCH_2$), 2.92 (s, 4H, CH_2 of Pbf), 2.52 (s, 6H, CH_3 of Pbf), 2.46 (s, 6H, CH_3 of Pbf), 2.23 (s, 6H, $ArCH_3$), 1.99 (s, 6H, CH_3 of Pbf), 1.90-1.10 (bm, 54H, $N{=}CNHCH_2CH_2CH_2$, $C(CH_3)_3$, $C(CH_3)_2$ of Pbf and $OCH_2CH_2CH_2CH_2CH_2$), 0.90 (bs, 6H, CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 173.5, 159.8, 158.0, 153.5, 139.4, 135.1, 134.8, 134.3, 133.5, 132.4, 125.9, 122.1, 121.0, 118.4, 87.6, 80.8, 74.1, 62.7, 56.5, 44.0, 41.6, 33.0, 31.4, 30.9, 30.8, 28.8, 27.3, 27.0, 23.7, 19.7, 18.5, 17.0, 14.5, 12.6; MS (m/z): $[M + Na]^+$ calcd for $C_{75}H_{114}N_{10}O_{14}S_2$, 1465.8; found, 1466.0.

Bis{2-[3-(N_α-Boc-N_ω-Pbf-L-Arg)-amino]propoxy-3-methylphenyl}methane (g). To a solution of Boc-L-Arg(Pbf)-OH (0.168 g, 0.32 mmol) in dry DMF (2 mL) were added HOBt (0.050 g, 0.36 mmol) and DCC (0.066 mg, 0.32 mmol) and the mixture stirred for 10 minutes. Then, compound **f** (0.032g, 0.094 mmol) dissolved in dry DMF (1 ml) was added. The reaction proceeded under stirring at room temperature for 24 h. Ethyl acetate was added (10 mL), DCU was filtered off by gravity on a PTFE filter, and the organic solvents were removed at reduced pressure. The crude was dissolved in ethyl

acetate (10 mL) and the organic layer was washed first with a saturated $NaHCO_3$ aqueous solution (2×10 mL), then with brine (3×10 mL) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed at rotavapor and the residue purified by preparative TLC (eluent: ethyl acetate/hexane= 9:1) to obtain the pure product **g** as a colourless oil in 21% yield. ^{1}H NMR (300 MHz, CD_3CN): δ 7.13 (bt, 2H, N*H*COCH), 7.02 (dd, 2H, *J* = 7.4 Hz, 1.3 Hz, ArH), 6.89 (t, 2H, *J* = 7.4 Hz, ArH), 6.82 (dd, 2H, *J* = 7.4 Hz, 1.3 Hz, ArH), 6.10 (bs, N*H*CN*H*N*H*), 5.70 (bs, 2H, NHBoc), 3.97 (bs, 4H, $ArCH_2Ar$ and NHCOC*H*), 3.73 (t, *J* = 6.3 Hz, 4H, OCH_2), 3.45-3.20 (m, 4H, $OCH_2CH_2CH_2$), 3.20-2.95 (m, 4H, $NCNHCH_2$), 2.96 (s, 4H, CH_2 of Pbf), 2.52 (s, 6H, CH_3 of Pbf), 2.45 (s, 6H, CH_3 of Pbf), 2.22 (s, 6H, $ArCH_3$), 2.03 (s, 6H, CH_3 of Pbf), 1.98-1.82 (m, 4H, OCH_2CH_2), 1.75-1.30 (m, 8H, $NCNHCH_2CH_2CH_2$), 1.41 (s, 18H, $C(CH_3)_3$), 1.34 (s, 12H, $C(CH_3)_2$ of Pbf); ^{13}C NMR (75 MHz, CD_3CN): δ 173.1, 158.9, 157.1, 156.3, 156.2, 138.6, 134.7, 134.3, 132.8, 131.7, 129.9, 128.9, 125.6, 124.4, 87.1, 79.6, 71.0, 54.8, 43.2, 40.7, 37.1, 30.6, 30.2, 29.9, 28.3, 28.2, 26.1, 19.2, 17.9, 16.3, 12.3; MS (m/z): $[M + Na]^+$ calcd for $C_{69}H_{102}N_{10}O_{14}S_2$, 1381.7; found, 1382.3.

General procedure for Boc/Pbf deprotection. A solution of calix[4]arene or Gemini-type compound (15 μmol) in TFA/TIS/H_2O (95/2.5/2.5, 5 mL) was stirred at room temperature. The progression of the reaction was followed by ESI mass spectrometry. After completion (2-24 h), the volatiles were removed under reduced pressure, the solid residue suspended in ethyl acetate (3×5 mL) and every time the organic solvent was evaporated to help in removing the exceeding TFA. The crude material was repeatedly washed with distilled diethyl ether (3×7 mL) removed after sample centrifugation.

Bis(5-L-Arg-amino-2-hexyloxy-3-methylphenyl)methane, tetra-hydrochloride (7). The trifluoroacetate anion of the TFA tetra-salt resulting from the deprotection reaction was exchanged with chloride by dissolving (4×) the solid in a methanol solution of conc HCl (pH= 3-4) followed by evaporation. The pure product **7** was obtained as a white solid in 79% yield. mp: 238 °C dec.; ^{1}H NMR (400 MHz, CD_3OD): δ 7.50 (s, 2H, ArH), 7.05 (s, 2H, ArH), 4.06 (bt, *J* = 5.6 Hz, 2H, COCH), 4.03 (s, 2H, $ArCH_2Ar$), 3.74 (t, *J* = 6.4 Hz, 4H, OCH_2), 3.28 (t, *J* = 6.8 Hz, 4H, $N=CNHCH_2$), 2.30 (s, 6H, $ArCH_3$), 2.10-1.90 (m, 4H, $COCHCH_2$), 1.82-1.67 (m, 8H, OCH_2CH_2 and $COCHCH_2CH_2$), 1.55-1.42 (m, 4H, $OCH_2CH_2CH_2$), 1.42-1.28 (m, 8H, $OCH_2CH_2CH_2CH_2CH_2$), 0.93 (t, *J* = 6.8 Hz, 6H, CH_2CH_3); ^{1}H NMR (400 MHz, D_2O): δ 7.25 (d, *J* = 2.2 Hz, 2H, ArH), 7.09 (d, *J* = 2.2 Hz, 2H, ArH), 4.12 (t, *J* = 8.8 Hz, 2H, COCH), 4.03 (s, 2H, $ArCH_2Ar$), 3.73 (t, *J* = 8.4 Hz, 4H, OCH_2), 3.23 (t, *J* = 8.8 Hz, 4H, $N=CNHCH_2$), 2.28 (s, 6H, $ArCH_3$), 2.10-1.92 (m, 4H, $COCHCH_2$), 1.80-1.55 (m, 8H, OCH_2CH_2 and $COCHCH_2CH_2$), 1.42-1.10 (m, 12H, $O(CH_2)_2CH_2CH_2CH_2CH_2$), 0.84 (t, *J* = 8.8 Hz, 6H, CH_2CH_3); ^{13}C NMR (100 MHz, CD_3OD) δ 167.9, 158.6, 154.1, 135.5, 134.5, 132.8, 122.4, 121.5, 74.0, 54.6, 41.8, 32.9, 31.4, 30.7, 29.9, 27.0, 25.5, 23.7, 16.8, 14.4; MS (m/z): $[M + 2H - 4HCl]^{++}$ calcd. for $C_{39}H_{70}Cl_4N_{10}O_4$, 370.3; found, 370.9; HRMS (m/z): $[M + 2H - 4HCl]^{++}$ calcd. for $C_{39}H_{70}Cl_4N_{10}O_4$, 370.2707; found, 370.2706.

Bis-{2-[3-(L-Arg-amino)propoxy]-3-methylphenyl}methane, tetra-hydrochloride (8). The trifluoroacetate anion of the TFA tetra-salt resulting from the deprotection reaction was exchanged with chloride by dissolving (4×) the solid in a methanol solution of conc HCl (pH= 3-4) followed by evaporation. The pure product was obtained as a white solid after liophilization, in 85% yield. mp: 184 °C dec.; ^{1}H NMR (300 MHz, CD_3OD): δ 7.03 (d, *J* = 6.9 Hz, 2H, ArH), 6.90 (t, *J* = 6.9 Hz, 2H, ArH), 6.82 (d, *J* = 6.9 Hz, 2H, ArH), 4.02 (s, 2H, $ArCH_2Ar$), 3.94 (bs, 2H, NHCOC*H*), 3.78 (bt, 4H, OCH_2), 3.46 (bs, 4H, $OCH_2CH_2CH_2$), 3.22 (bs, 4H, $NCNHCH_2$), 2.28 (s, 6H, $ArCH_3$), 2.11-1.80 (bm, 8H, OCH_2CH_2 and $COCHCH_2$), 1.70 (bs, 4H, $COCHCH_2CH_2$); ^{1}H NMR (300 MHz, D_2O): δ 7.19 (d, *J* = 7.0 Hz, 2H, ArH), 7.05 (t, *J* = 7.0 Hz, 2H, ArH), 6.95 (d, *J* = 7.0 Hz, 2H, ArH), 4.02 (s, 2H, $ArCH_2Ar$), 3.95 (t, 2H, *J* = 6.6 Hz, COCH), 3.83 (t, 4H, *J* = 6.3 Hz,

OCH_2), 3.56-3.42 (m, 2H, $OCH_2CH_2CH\mathit{H}$), 3.40-3.26 (m, 2H, $OCH_2CH_2C\mathit{H}H$), 3.12 (t, 4H, J = 6.9 Hz, $NCNHC\mathit{H}_2$), 2.27 (s, 6H, $ArCH_3$), 2.10-1.93 (m, 4H, $OCH_2C\mathit{H}_2$), 1.93-1.82 (m, 4H, $COCHC\mathit{H}_2$), 1.68-1.52 (m, 4H, $COCHCH_2C\mathit{H}_2$); ^{13}C NMR (75 MHz, CD_3OD): δ 170.3, 158.9, 157.1, 135.4, 132.4, 130.7, 129.9, 125.4, 71.6, 54.4, 42.0, 38.6, 31.4, 30.9, 30.1, 25.7, 17.0; MS (m/z): $[M + H - 4HCl]^+$ calcd. for $C_{33}H_{58}Cl_4N_{10}O_4$, 655.4; found, 655.6; HRMS (m/z): $[M + 2H - 4HCl]^{2+}$ calcd. for $C_{33}H_{58}Cl_4N_{10}O_4$, 328.2238; found 328.2240.

DNA preparation and storage. Plasmid DNA was purified through cesium chloride gradient centrifugation.[38] A stock solution of the plasmid 0.35 μM in milliQ water (Millipore Corp., Burlington, MA) was stored at -20 °C.

Electrophoresis mobility shift assay (EMSA). Binding reactions were performed in a final volume of 14 μL with 10 μL of 20 mM Tris/HCl pH 8, 1 μL of plasmid (1 μg of pEGFP-C1) and 3 μL of compound at different final concentrations, ranging from 25 to 200 μM. Binding reaction was left to take place at room temperature for 1 h; 5 μL of 1 g/mL in H_2O of glycerol was added to each reaction mixture and loaded on a TA (40 mM Tris-Acetate) 1% agarose gel. At the end of the binding reaction 1 μL (0.01 mg) of ethidium bromide solution is added. The gel was run for 2.5 h in TA buffer at 10 V/cm. EDTA was omitted from the buffers because it competes with DNA in the reaction.

Melting curves determination. Melting curves of the linearized pEGFP-C1 DNA (0.5 nM) and of the same plasmid in the presence of ligand (16 μM) in buffer solution (4 mM Hepes, 10 mM NaCl, pH 7.4) were registered on a PerkinElmer UV-vis LAMBDA-Bio 20 spectrophotometer in the range 20-90 °C and following emission at 260 nm.

Fluorescence studies. Ethidium Bromide Displacement Assays (excitation at 530 nm, emission at 600 nm) were performed collecting on a PerkinElmer LS 55 the emission spectra of buffer solutions (4 mM Hepes, 10 mM NaCl, pH 7.4) of 50 mM ethidium bromide (relative fluorescence = 0), mixture of 0.5 nM plasmid DNA (pEGFP-C1) and 50 mM ethidium bromide (relative fluorescence = 1) and after addition of increasing amounts of ligand. Experiments with Nile Red were performed on the same instrument (excitation at 530 nm) in buffer solutions (4 mM Hepes, 10 mM NaCl, 2 mM $MgCl_2$, pH = 7.4) of the dye (0.2 μM) and mixture of the dye with ligand (2 μM) and pEGFP-C1 DNA (0.5 nM).

Sample preparation for AFM imaging. DNA samples were prepared by diluting the plasmid DNA to a final concentration of 0.5 nM in deposition buffer (4 mM Hepes, 10 mM NaCl, 2 mM $MgCl_2$, pH = 7.4) either in the presence or absence of ligands. When needed, ethanol at a defined concentration was added to the deposition buffer prior to addition of DNA and calixarenes. The mixture was incubated for 5 min at room temperature, then a 20 μL droplet was deposited onto freshly-cleaved ruby mica (Ted Pella, Redding, CA) for 1.5 min. The mica disk was rinsed with milliQ water and dried with a weak nitrogen stream. AFM imaging was performed on the dried sample with a Nanoscope IIIA Microscope (Digital Instruments Inc. Santa Barbara, CA) operating in tapping mode. Commercial diving board silicon cantilevers (NSC-15 Micromash Corp., Estonia) were used. Images of 512×512 pixels were collected with a scan size of 2 μm at a scan rate of 3-4 lines per second and were flattened after recording using Nanoscope software.

Cell culture and transient transfection assay. **Cell culture and transient transfection assay**. RD-4 [human Rhabdomyosarcoma cell line (obtained from David Derse, National Cancer Institute, Frederick, Maryland)], C2C12 [mouse myoblast; ATCC (#CRL-1772)], N2a [mouse neuroblastoma; ATCC (#CCL-131)], EADSc [Equine Adipose

Derived Stromal Cells primary culture was obtained as described in Donofrio, G. et al *Cell Biology* **11**, 73 (2010)], COS-7 [African Green Monkey Kidney cells; ATCC (#CRL-1651)], VERO [African Green Monkey Kidney Cells; ATCC (#CCL-81), HEK 293 [Human embryo Kidney cells; ATCC (#CRL-1573)], Ishikawa [Human Endometrial cancer cells; ECACC (#99040201)] and HeLa [Human Cervix Adenocarcinoma; ATCC (#CCL-2)] were grown in EMEM medium containing NEAA, 10% FBS, 2 mM l-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured to a fresh culture vessel when growth reached 70-90% confluence (i.e. every 3-5 days) and incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Transfections were performed in 24 well plates, when cells were 80% confluent (approximately 5×10^4 cells) on the day of transfection. 2.5 µg of plasmid and different concentrations of ligands were added to 1 mL of serum-free medium (DMEM, 2 mM L-glutamine and 50 µg/ml), mixed rapidly and incubated at room temperature for 20 min. When used, serum was added at this point to the transfection solution. Following the removal of the culture medium from the cells, 0.5 mL of transfection mixture were carefully added to every well. Lipoplex formulations with helper lipid were prepared adding a 2 mM ethanol solution of DOPE to plasmid-ligand mixture at 1:2 ligand:DOPE molar ratio, where ligand concentration was kept to 10 µM. These solutions administered to the cells were completely clear and homogeneus. LTX™ transfection reagent was used according to manufacturer's protocol as positive transfection control. The mixture and cells were incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2 for 5 h. Finally, transfection mixture was removed and 1 mL of growth medium added to each transfected well and left to incubate for 72 h. Five fields were randomly selected from each well without viewing the cells (one in the centre and one for each quadrant of the well) and examined. The transfected cells were observed under fluorescence microscope for EGFP expression. Each experiment was done three times. Statistical differences between treatments were calculated with Student's test and multifactorial ANOVA.

MTT survival assay for cell viability determination. 2000 cells/well (in 50 µL) were seeded in a 96 wells plate, cells were grown in complete medium (90% DMEM, 10% FBS, 2 mM l-glutamine, and 100 IU/mL penicillin, 10 mg/mL streptomycin)and incubated at 37 °C overnight. The day after 50 µL of scalar dilution of calixarenes, alone or with DOPE, were added to the cells and incubated at 37 °C overnight. Calixarenes were tested from 40 to 2.5 µM and DOPE from 80 to 5 µM. 24 hours later, 10 µL of complete medium containing MTT (5 mg/mL) was added to each well and incubated for 6 h. Then, after the addition of 100 µL per well of solubilisation solution (10% SDS in HCl 0.01 M) cells were incubated at 37 °C overnight. MTT survival assay for cell viability determination in transfection conditions was performed with the same procedure at 24 h from transfection (see transient transfection assay). Specific optical density for each well was measured at 540 nm, using 690 nm as reference wavelength in an SLT-Lab microreader (Salzburg, Austria). Each experiment was done three times and each treatment was performed with eight replicates. Statistical differences among treatments were calculated with Student's test and multifactorial ANOVA.

Luciferase reporter assay. Luciferase reporter assay was performed with a Dual Luciferase Reporter Assay System kit (Promega) with minor modifications. At 48 h from the treatment, cells were washed with PBS, lysed with 100 µl of lysis passive buffer by freeze-thawing at −80 °C. 10 µl of the cell lysate was added to 50 µl of LAR and Luciferase activity were determined with a PerkinElmer Victor Multilabel Counter, according to the manufacturer's specifications. Individual assays were normalized for *Renilla* Luciferase activity with a second reading, adding 50 µl of Stop & Glo substrate.

References

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