Electronic Supplementary Material (ESI) for ChemComm.

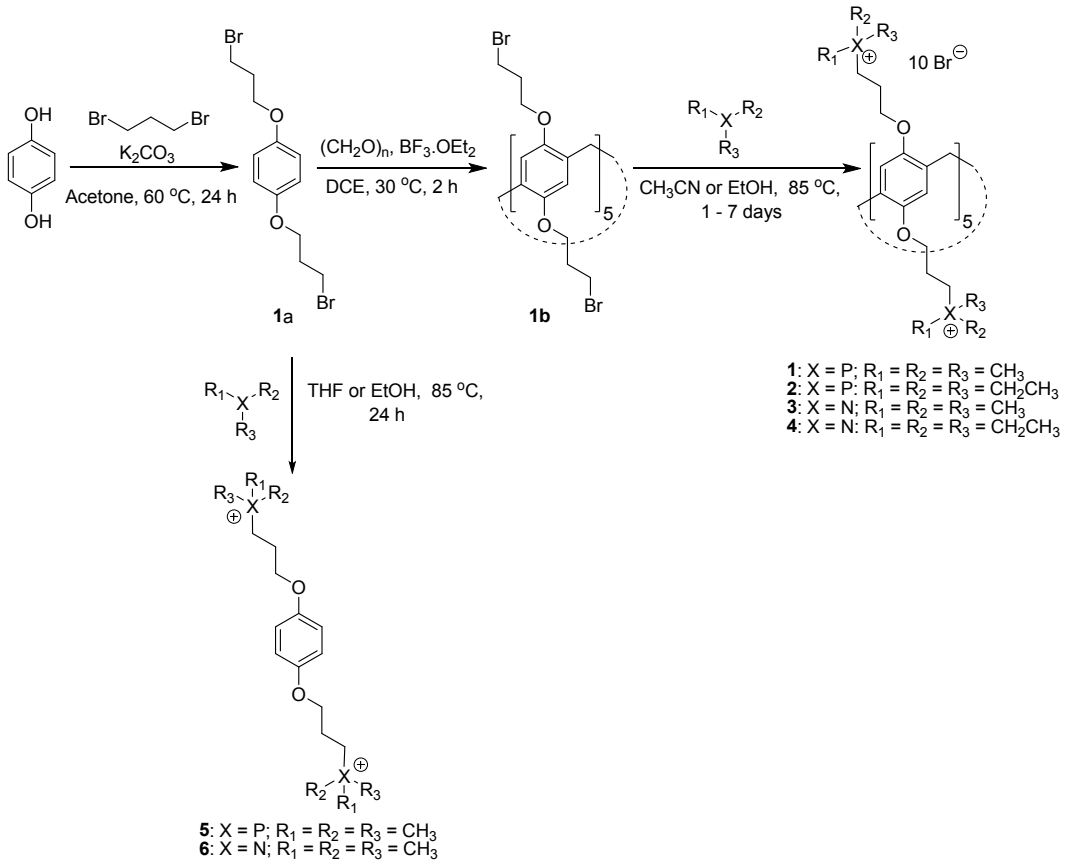
Supporting information

Table of Contents

1. General methods

Starting materials were purchased from Sigma-Aldrich, Alfa Aesar, TCI, Cambridge Isotope Laboratories, and Bio-Lab. Chemical reactions were monitored by TLC (Merck, silica gel 60 F254), and the compounds were purified by SiO_2 flash chromatography (Merck Kieselgel 60). ^1H and ^{13}C NMR spectra were recorded on 400 and 500 MHz Bruker Avance NMR spectrometers at 25 °C. Chemical shifts (δ) are given in parts per million (ppm) and spin-spin coupling (*J*) is given in Hz. The chemical shifts are relative to residual HDO signal (at δ 4.80 ppm for the ^1H NMR) when the solvent is D_2O, to residual $CHCl_3$ signal (at δ 7.26 ppm for the ^1H NMR and 77.2 ppm for the ^{13}C NMR) when the solvent is $CDCl_3$, or to residual DMSO (at δ 2.50 ppm for the ^1H NMR and 39.5 ppm for the ^{13}C NMR) when the solvent is DMSO. Determination of C, H, and N compositions were performed using a Perkin-Elmer 2400 series II Analyzer. High-resolution electrospray mass spectra were recorded on a Waters Synapt instrument.

2. Synthesis and characterization of cationic pillar[n]arene conjugates



Scheme S1: Scheme of the general synthesis of the pillararene conjugates (**1**-**4**) and monomers **5** and **6**.

Synthesis of 1a[1]: A mixture of hydroquinone (8.0 g, 73 mmol), 1,3-dibromopropane (44 g, 0.22 mol), and potassium carbonate (45 g, 0.33 mol) were refluxed in acetone (0.13 L) for 24 h under argon atmosphere. The reaction mixture was cooled to 25 °C and filtered through celite, and the solvent was evaporated under vacuum. The residue was dissolved in dichloromethane (0.1 L), washed with water (2 × 50 mL), 3 N HCl (2 × 50 mL), and brine (2 × 50 mL), dried with sodium sulphate, and concentrated *in vacuo*. The product was purified by column chromatography (silica gel; eluent: hexane/ethyl acetate). Further purification by recrystallization in ethyl acetate/hexane afforded **2a** as a white solid (9.5 g, 37%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.84 (s, Ar*H*, 4H), 4.05 (t, *J* = 5.9 Hz, $ArOC\mathit{H}_2CH_2CH_2Br$, 4H), 3.60 (t, *J* = 6.7 Hz, $ArOCH_2CH_2C\mathit{H}_2Br$, 4H), 2.29 (m, $ArOCH_2C\mathit{H}_2CH_2Br$, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.0, 115.5, 65.9, 32.5, 30.2 ppm.

Synthesis of 1b[2]: To a solution of **1a** (2.5 g, 7.1 mmol) in 1,2-dichloroethane (60 mL) was added paraformaldehyde (0.67 g, 22 mmol) followed by $BF_3{\cdot}OEt_2$ (1.1 g, 7.8 mmol). The reaction mixture was kept at 30 °C for 30 min under argon atmosphere. The resulting mixture was cooled to 25 °C, and the crude product was precipitated by addition of

methanol (0.2 L). The product was purified by chromatography (silica gel; hexane:dichloromethane) to afford the title compound as a white solid (1.1 g, 43%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.74 (s, Ar*H*, 10H), 3.99 (t, *J* = 6.3 Hz, ArOC*H*$_2$CH$_2$CH$_2$Br, 20H), 3.75 (s, ArC*H*$_2$Ar, 10H), 3.52 (t, *J* = 6.5 Hz, ArOCH$_2$CH$_2$C*H*$_2$Br, 20H), 2.21 (m, ArOCH$_2$C*H*$_2$CH$_2$Br, 20H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 149.8, 128.5, 115.3, 66.3, 32.7, 30.5, 29.9 ppm.

Synthesis of 1: Trimethylphosphine (1.0 M in THF, 2.2 mL, 2.2 mmol) was added to a solution of **1b** (0.10 g, 0.05 mmol) in acetonitrile (6.0 mL). The resulting mixture was refluxed in a pressure tube for 96 h. After cooling to 25 °C, the precipitate was filtered, washed with diethyl ether, and dried under vacuum to afford **1** as white solid (0.12 g, 86%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.85 (s, Ar*H*, 10H), 4.07 & 3.90 (br, ArOC*H*$_2$CH$_2$CH$_2$P, 20H), 3.78 (s, ArC*H*$_2$Ar, 10H), 2.69-2.60 (m, C*H*$_2$P(CH$_3$)$_3$, 20H), 2.00 (d, *J* = 14.7 Hz, CH$_2$P(C*H*$_3$)$_3$ & ArOCH$_2$C*H*$_2$CH$_2$P, 110H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ 148.7, 127.8, 114.1, 67.5 (d, *J* = 17 Hz), 28.7, 21.7, 19.7 (d, *J* = 56 Hz), 7.5 (d, *J* = 55 Hz) ppm. ^{31}P NMR (162 MHz, DMSO-d_6, H_3PO_4 reference): δ 30.0 ppm. HRMS: m/z calcd. for $C_{95}H_{170}O_{10}P_{10}Br_{11}$ $[M+Br]^-$ 2662.1098, found 2662.1116.

Synthesis of 2: Triethylphosphine (1.0 M in THF, 2.2 mL, 2.2 mmol) was added to a solution of **1b** (0.10 g, 0.05 mmol) in dry acetonitrile (8.0 mL). The resulting mixture was refluxed in a pressure tube for 72 h. After cooling to 25 °C, diethyl ether was added to give a white precipitate. The precipitate was filtered, washed with diethyl ether, and dried under vacuum to afford **2** as white solid (68 mg, 41%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.81 (s, Ar*H*, 10H), 4.15 & 3.84 (br, ArOC*H*$_2$CH$_2$CH$_2$P, 20H), 3.76 (s, ArC*H*$_2$Ar, 10H), 2.61-2.55 (m, C*H*$_2$P(CH$_2$CH$_3$)$_3$, 20H), 2.46-2.39 (m, P(C*H*$_2$CH$_3$)$_3$, 60H), 2.06 (m, OCH$_2$C*H*$_2$CH$_2$, 20H), 1.23-1.17 (m, P(CH$_2$C*H*$_3$)$_3$, 90H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ 148.7, 127.9, 113.8, 67.5 (d, *J* = 16 Hz), 28.8, 21.7, 14.1 (d, *J* = 50 Hz), 10.8 (d, *J* = 48 Hz), 5.4 (d, *J* = 5 Hz) ppm. ^{31}P NMR (162 MHz, D_2O, H_3PO_4 reference): δ 39.8 ppm. Anal. calcd. for $C_{125}H_{230}Br_{10}P_{10}O_{10}.14.45H_2O$: C, 45.77; H, 7.74. Found: C, 46.02; H, 8.00. HRMS: m/z calcd. for $C_{125}H_{230}O_{10}P_{10}Br_{11}$ $[M+Br]^-$ 3081.5760, found 3081.5796

Synthesis of 3[3]: Trimethylamine (33% in ethanol, 0.79 mL, 4.4 mmol) was added to a solution of **1b** (0.20 g, 0.11 mmol) in ethanol (10 mL). The resulting mixture was refluxed for 24 h. After cooling to 25 °C, the solvent was removed under vacuum and the residue was dissolved in water (5.0 mL). The solution was filtered, and the solvent was removed by evaporation. The residue recrystallized as light brown crystals in ethanol (0.21 g, 79%). ^{1}H NMR (400 MHz, D_2O): δ 6.78 (s, Ar*H*, 10H), 3.90 (br, ArOC*H*$_2$CH$_2$CH$_2$ & ArC*H*$_2$Ar, 30H), 3.39 (br, ArOCH$_2$CH$_2$C*H*$_2$, 20H), 3.10 (br, N(CH$_3$)$_3$, 90H), 2.07 (br, ArOCH$_2$C*H*$_2$CH$_2$, 20H) ppm. ^{13}C NMR (100 MHz, D_2O): δ 150.5, 129.9, 116.9, 66.6, 64.4, 53.6, 30.8, 23.4 ppm. Anal. calcd for $C_{95}H_{170}Br_{10}N_{10}O_{10}.21.05H_2O$: C, 40.89; H, 7.66; N, 5.02. Found: C, 40.57; H, 7.34; N, 4.87. HRMS: m/z calcd. for $C_{95}H_{170}O_{10}N_{10}Br_{11}$ $[M+Br]^-$ 2490.4050, found 2490.4045.

Synthesis of 4: An excess amount of triethyl amine (5.0 mL, 36 mmol) was added to a solution of **1b** (0.40 g, 0.22 mmol) in ethanol (5.0 mL). The resulting mixture was refluxed in a pressure tube for 7 days. After cooling to 25 °C, the product was precipitated by the addition of diethyl ether. The precipitate was filtered, and the solid was washed with diethyl ether and acetone. The product was sonicated in acetone (5 × 10 mL) to remove excess triethyl amine. Finally the solid was dissolved in water and concentrated to afford a white solid (0.42 g, 68%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.80 (s, Ar*H*, 10H), 4.19 & 3.89 (br, ArOC*H*$_2$CH$_2$CH$_2$N, 20H), 3.73 (s, ArC*H*$_2$Ar, 10H), 3.44 (br, ArOCH$_2$CH$_2$C*H*$_2$N, 20H), 3.43 (br, NC*H*$_2$CH$_3$, 60H), 2.22 (br, ArOCH$_2$C*H*$_2$CH$_2$, 20H), 1.26 (t, *J* = 7.0 Hz, NCH$_2$C*H*$_3$, 90H) ppm. ^{13}C NMR (100 MHz): δ 148.7, 127.9, 113.7, 65.0, 53.6, 52.3, 28.7, 22.4, 7.4 ppm. Anal. calcd. for $C_{125}H_{230}Br_{10}N_{10}O_{10}$.10.75$H_2O$: C, 49.62; H, 8.38; N, 4.63. Found: C, 49.31; H, 8.06; N, 4.46.

Synthesis of 5: Trimethyl phosphine (1 M in THF, 9.0 ml, 9 mmol) was added to **1a** (0.15 g, 0.43 mmol) under argon atmosphere. The solution was refluxed for 24 h in a pressure tube. The precipitate was filtered, washed with THF, and dried under vacuum to afford a white solid (0.15 g, 69%). ^{1}H NMR (400 MHz, D_2O): 7.00 (s, Ar*H*, 4H), 4.12 (t, *J* = 5.8 Hz, ArOC*H*$_2$, 4H), 2.42-2.34 (m, ArOCH$_2$C*H*$_2$, 4H), 2.09-2.04 (m, C*H*$_2$P(CH$_3$)$_3$, 4H), 1.86 (d, *J* = 14.3 Hz, P(C*H*$_3$)$_3$, 18H) ppm. ^{13}C NMR (100 MHz, D_2O): δ 153.1, 117.0, 68.6 (d, *J* = 16 Hz), 21.7, 21.2 (d, *J* = 54 Hz), 8.0 (d, *J* = 55 Hz) ppm. ^{31}P NMR (162 MHz, D_2O, H_3PO_4 reference): δ 27.3 ppm. HRMS: m/z calcd. for $C_{18}H_{34}O_2P_2Br_3$ [M+Br]$^-$ 580.9584, found 580.9599.

Synthesis of 6: Trimethylamine (33% in ethanol, 1.0 mL, 5.7 mmol) was added to a solution of **1a** (0.2 g, 0.57 mmol) in ethanol (5 mL). The resulting mixture was refluxed in a pressure tube for 24 h. After cooling to 25 °C, the precipitate was filtered, washed with ethanol, and dried under vacuum to afford white solid (0.26 g, 97%). ^{1}H NMR (400 MHz, D_2O): δ 6.96 (s, Ar*H*, 4H), 4.11 (t, *J* = 5.8 Hz, ArOC*H*$_2$, 4H), 3.52 (m, CH$_2$C*H*$_2$N, 4H), 3.12 (s, N(C*H*$_3$)$_3$, 18H), 2.24 (m, ArOCH$_2$C*H*$_2$, 4H) ppm. ^{13}C NMR (125 MHz, D_2O): δ 153.1, 117.0, 66.2, 64.7, 53.7, 23.4 ppm. HRMS: m/z calcd. for $C_{18}H_{34}O_2N_2Br_3$ [M+Br]$^-$ 547.0170, found 547.0181.

3. ^{1}H, ^{13}C and ^{31}P NMR spectra of compounds 1-6 and their precursors

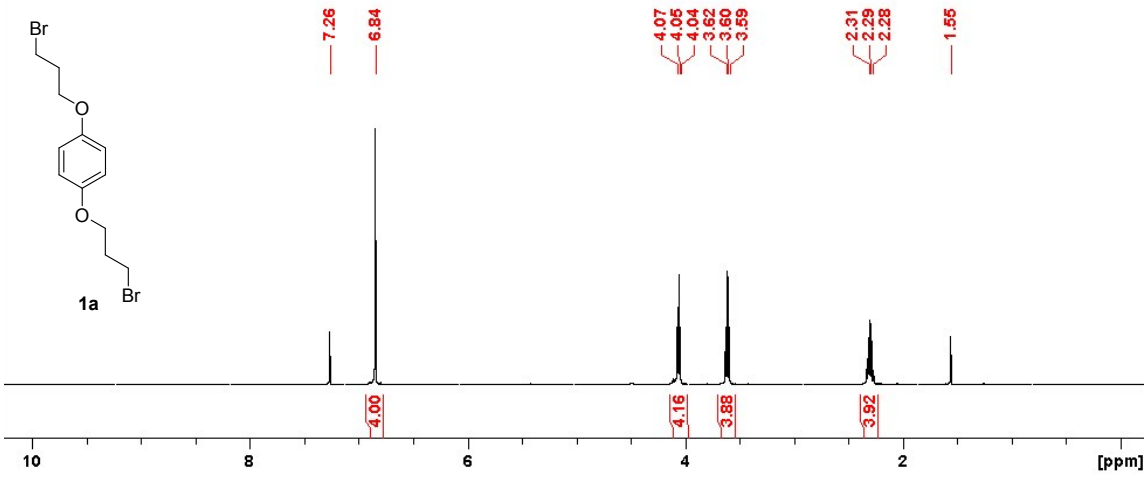


Figure S1a. ^{1}H NMR spectrum of **1a** in $CDCl_3$ (400 MHz).

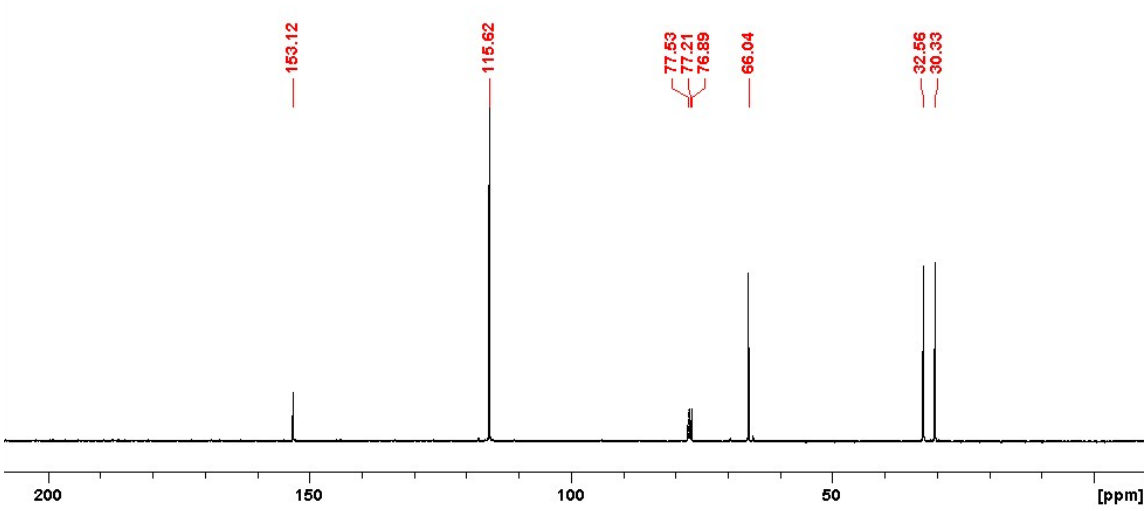


Figure S1b. ^{13}C NMR spectrum of **1a** in $CDCl_3$ (100 MHz).

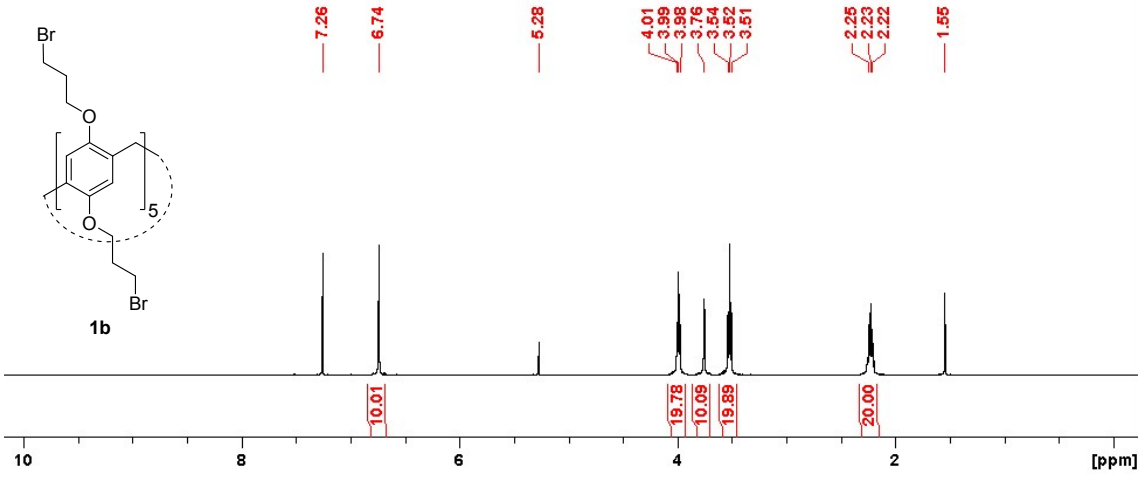


Figure S2a. ^{1}H NMR spectrum of **1b** in $CDCl_3$ (400 MHz).

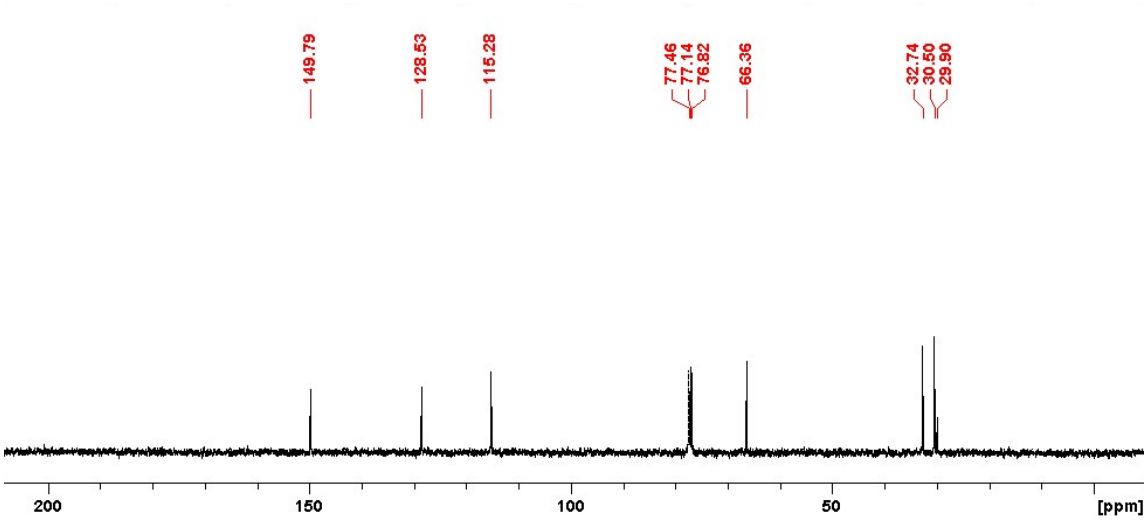


Figure S2b. ^{13}C NMR spectrum of **1b** in $CDCl_3$ (100 MHz).

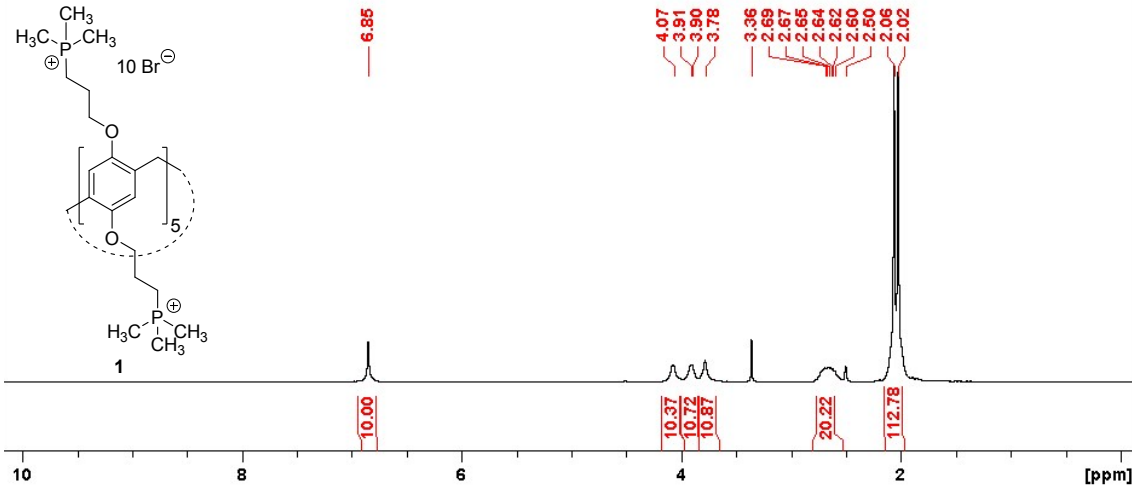


Figure S3a. ^{1}H NMR spectrum of **1** in DMSO-d_6 (400 MHz).

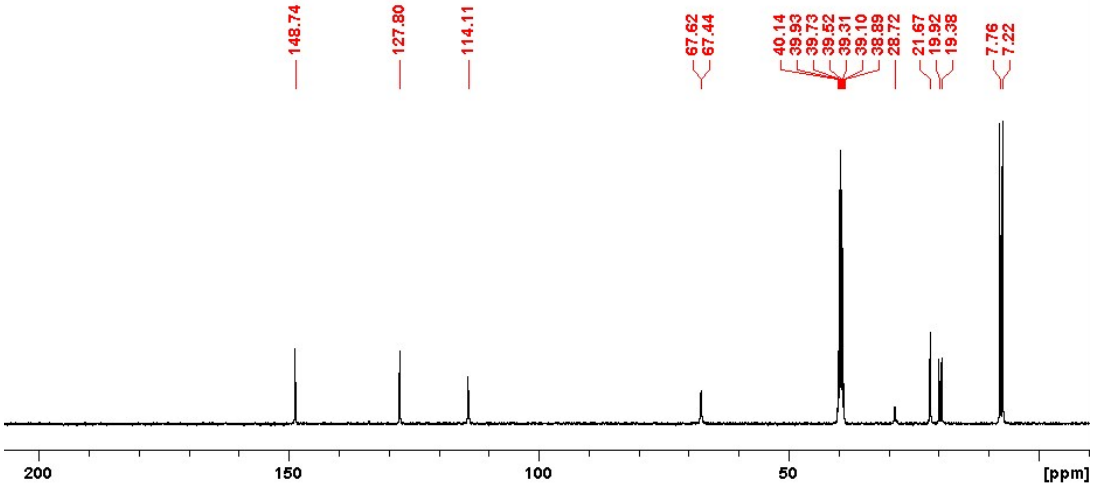


Figure S3b. ^{13}C NMR spectrum of **1** in DMSO-d_6 (100 MHz).

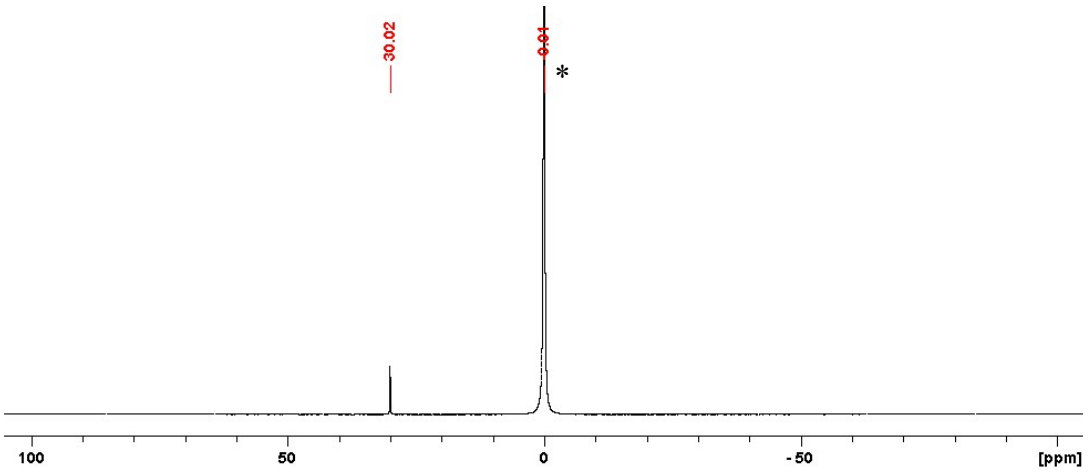


Figure S3c. ^{31}P NMR spectrum of **1** in DMSO-d_6 (162 MHz); reference used for ^{31}P NMR is H_3PO_4 (*).

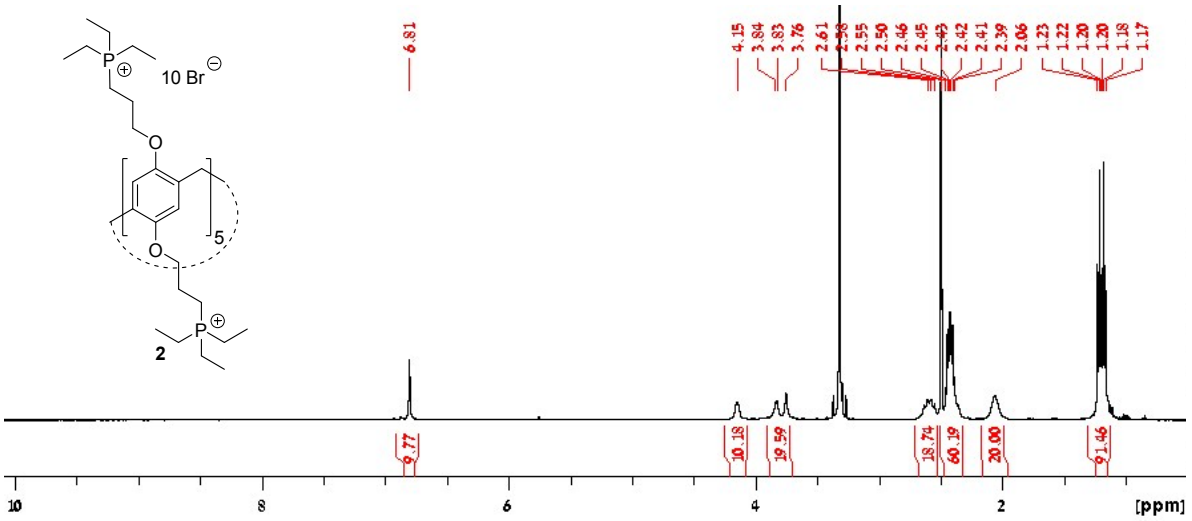


Figure S4a. ^{1}H NMR spectrum of **2** in DMSO-d_6 (400 MHz).

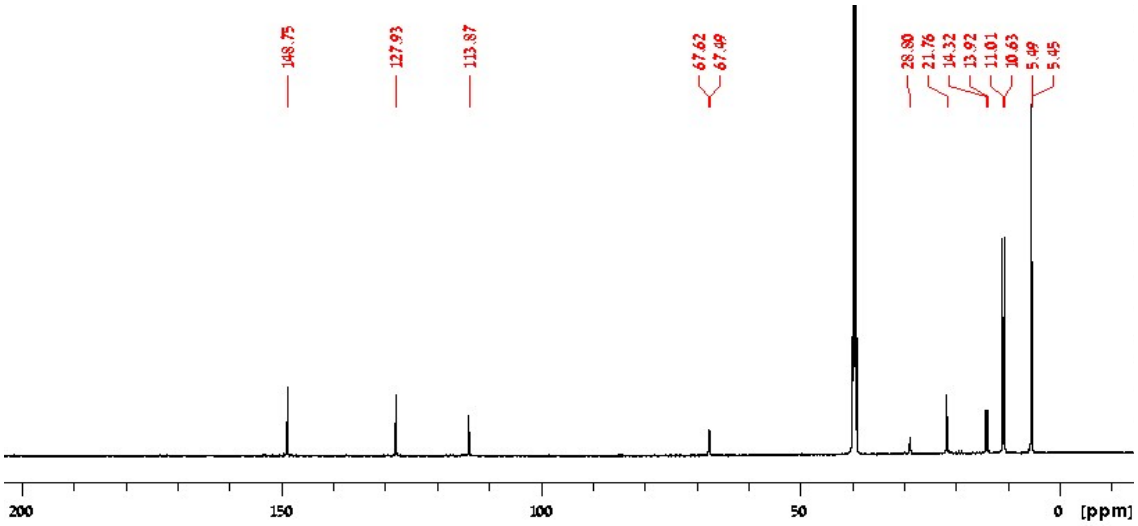


Figure S4b. ^{13}C NMR spectrum of **2** in DMSO-d_6 (100 MHz).

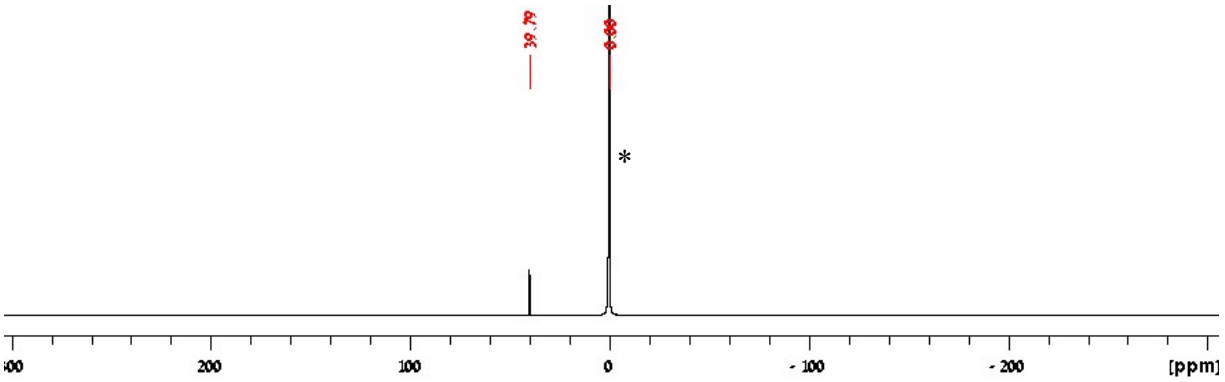


Figure S4c. ^{31}P NMR spectrum of **2** in D_2O (162 MHz); reference used for ^{31}P NMR is H_3PO_4 (*).

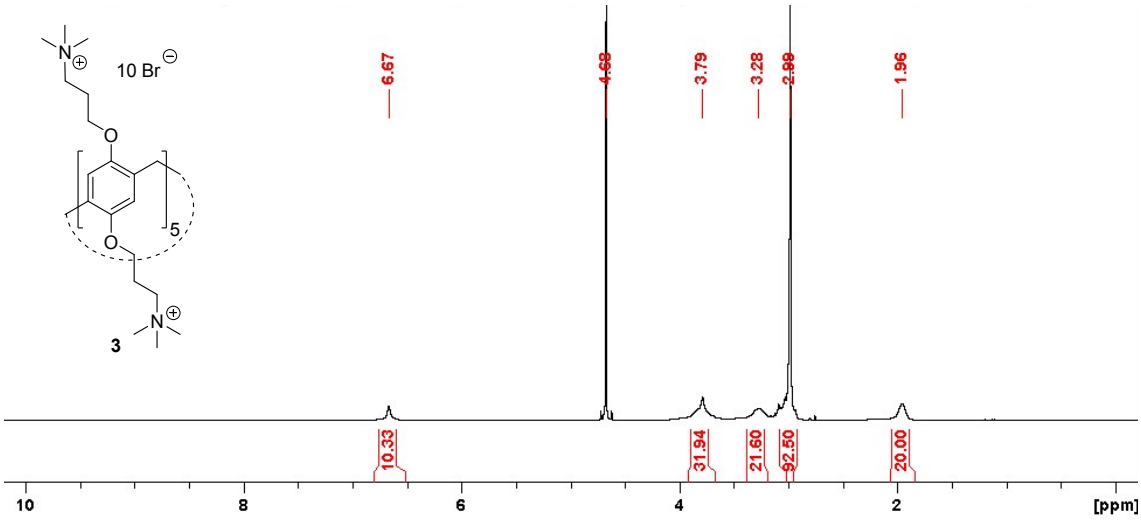


Figure S5a. ^{1}H NMR spectrum of **3** in D_2O (400 MHz).

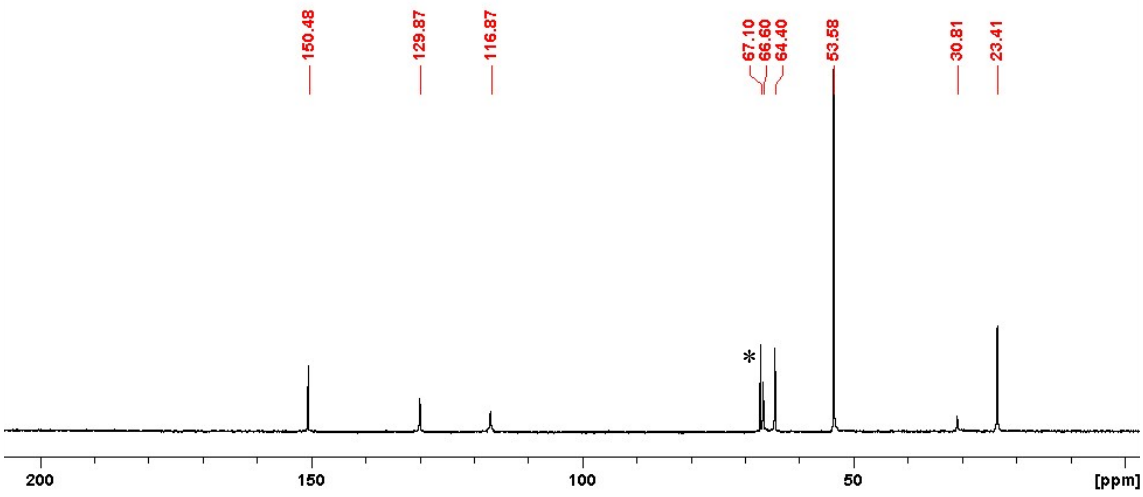


Figure S5b. ^{13}C NMR spectrum of **3** in D_2O (100 MHz); reference used for ^{13}C NMR is 1,4-dioxane (*), δ 67.1 ppm.

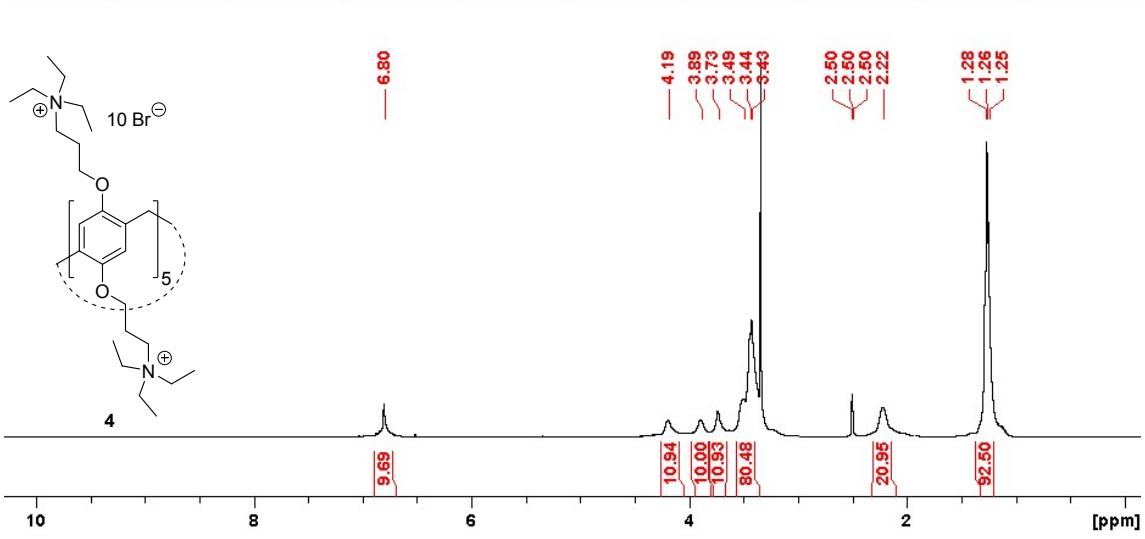


Figure S6a. ^{1}H NMR spectrum of **4** in DMSO-d_6 (400 MHz).

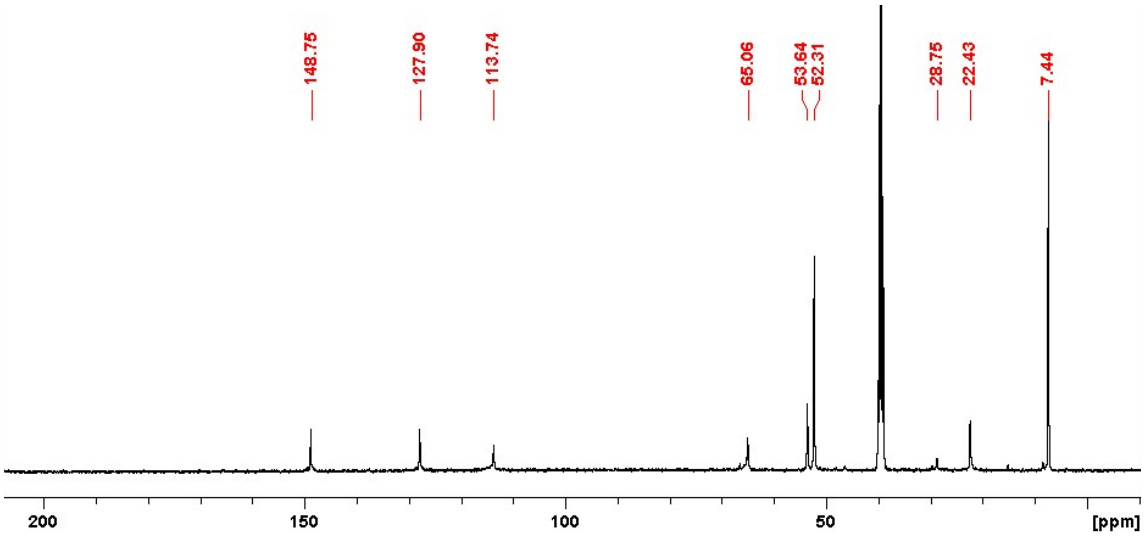


Figure S6b. ^{13}C NMR spectrum of **4** in DMSO-d_6 (100 MHz).

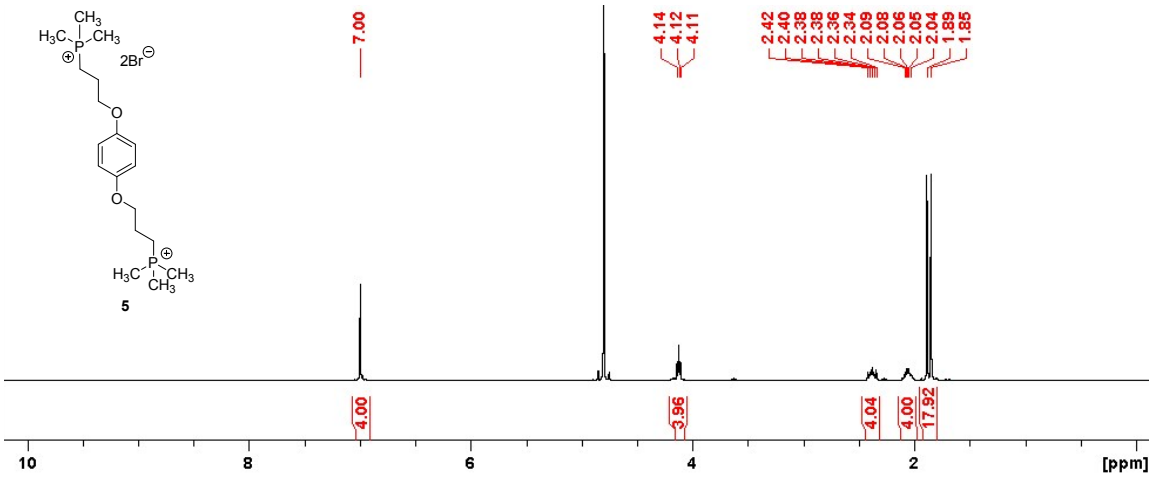


Figure S7a. ^{1}H NMR spectrum of **5** in D_2O (400 MHz)

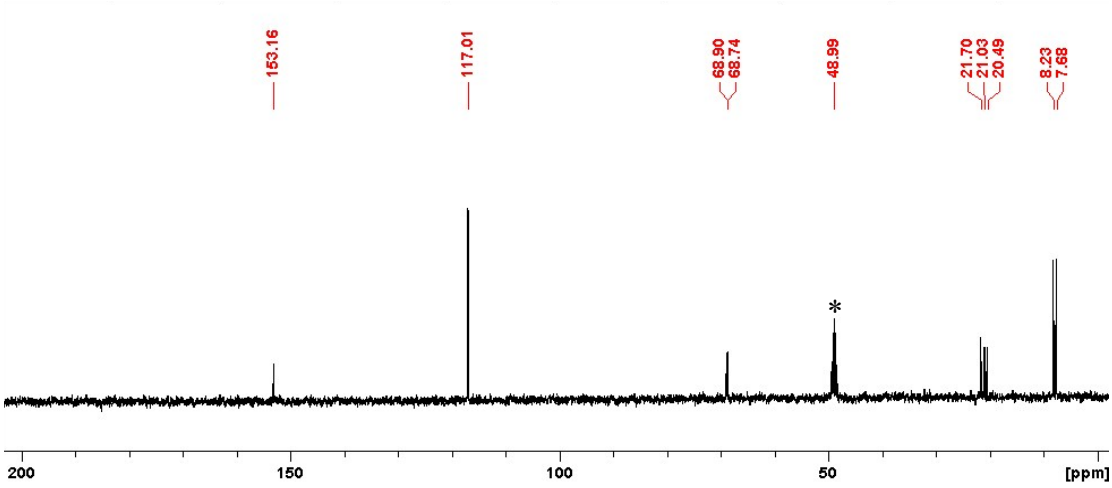


Figure S7b. ^{13}C NMR spectrum of **5** in D_2O (100 MHz); reference used for ^{13}C NMR is CD_3OD (*), δ 49.0 ppm.

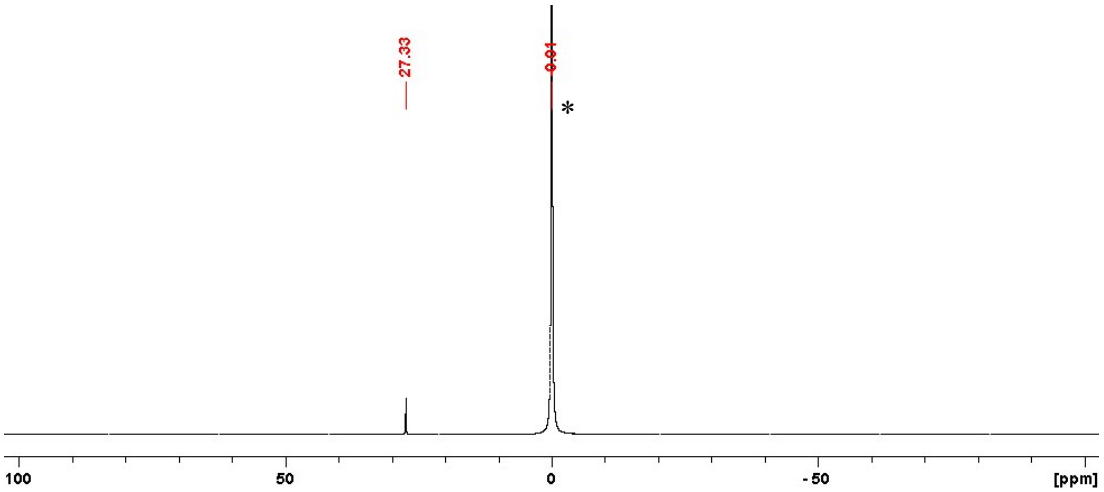


Figure S7c. ^{31}P NMR spectrum of **2** in D_2O (162 MHz); reference used for ^{31}P NMR is H_3PO_4 (*).

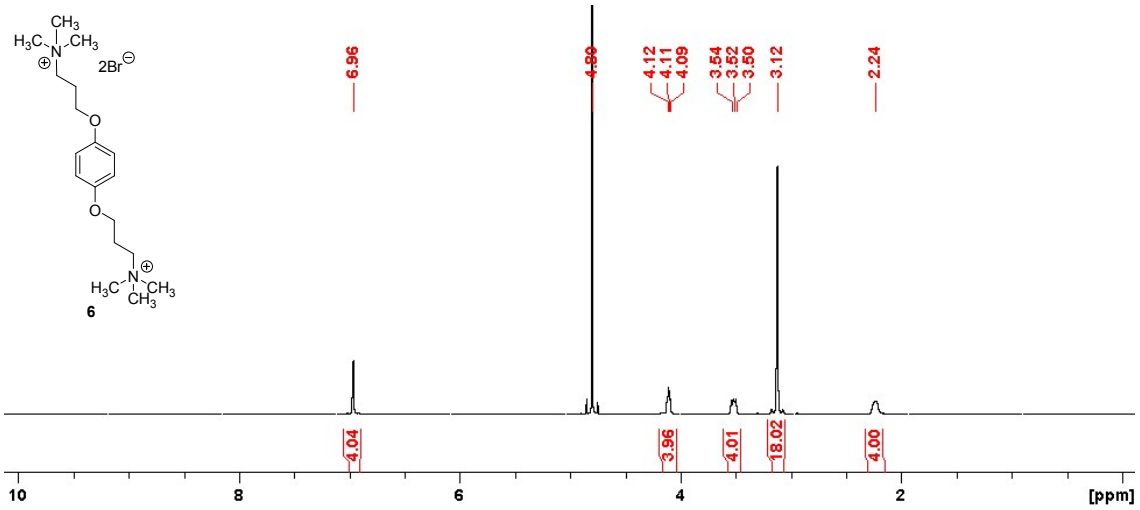


Figure S8a. ^{1}H NMR spectrum of **6** in D_2O (400 MHz).

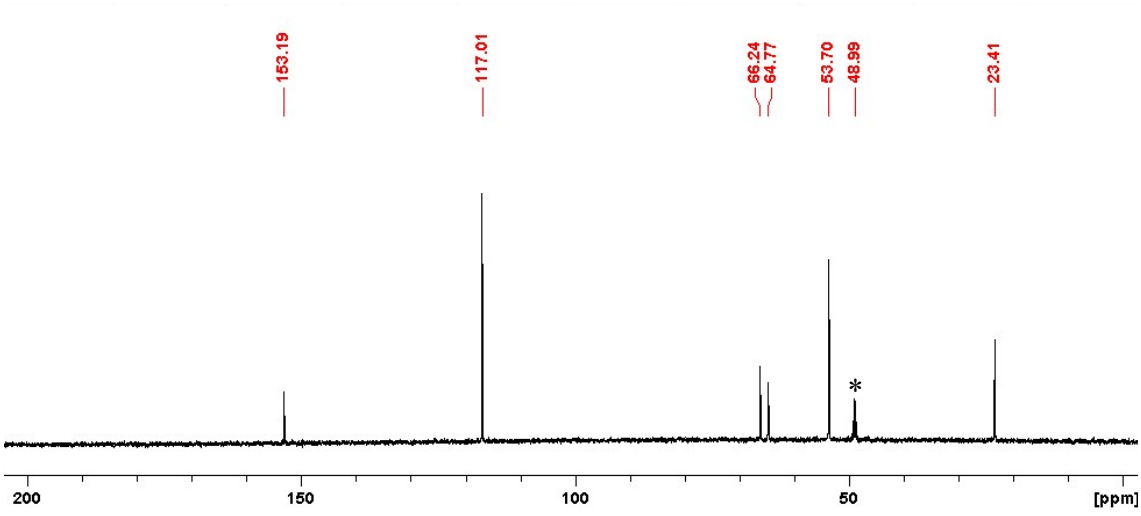


Figure S8b. ^{13}C NMR spectrum of **6** in D_2O (100 MHz); reference used for ^{13}C NMR is CD_3OD (*), δ 49.0 ppm.

4. Biological assays

(a) Analysis of biofilm inhibition

The assay was performed as described previously[3,4] with minor modifications. Briefly, all tested bacterial strains were grown from the frozen stock in BHI broth for 24 h at 37 °C in 5% CO_2. Next, 100 µl of serial 1:2 dilutions of compounds in TSB + 1% glucose (32, 16, 8, 4, 2, 1, and 0.5 µg/ml) were prepared in flat-bottomed 96-well microplates (Costar, Corning). Control wells with no compounds and wells without bacteria containing each tested concentration of the compounds (blanks) were also prepared. An equal volume (100 µl) of bacterial suspensions diluted 1:100 (OD_{600}=0.01) in TSB + 1% glucose was added to each well. After incubation for 24 h at 37 °C in 5% CO_2 under aerobic conditions, spent media and free-floating bacteria were removed by turning over the plates. The wells were vigorously rinsed at least four times with doubly distilled water (DDW). Next, 0.4% crystal violet (200 µl) was added to each well. After 45 min, wells were vigorously rinsed three times with DDW to remove unbound dye. After adding 200 µl of 30% acetic acid to each well, the plate was shaken for 15 min to release the dye. Biofilm formation was quantified by measuring the difference between absorbance of untreated and treated bacterial samples for each tested concentration of the compounds and the absorbance of appropriate blank well at 600 nm (A_{600}) using Tecan plate reader. The $MBIC_{50}$ was defined as the lowest concentration at which at least 50% reduction in biofilm formation was measured compared to untreated cells. Each concentration of compound was tested in five replicates, and three to six independent experiments were performed.

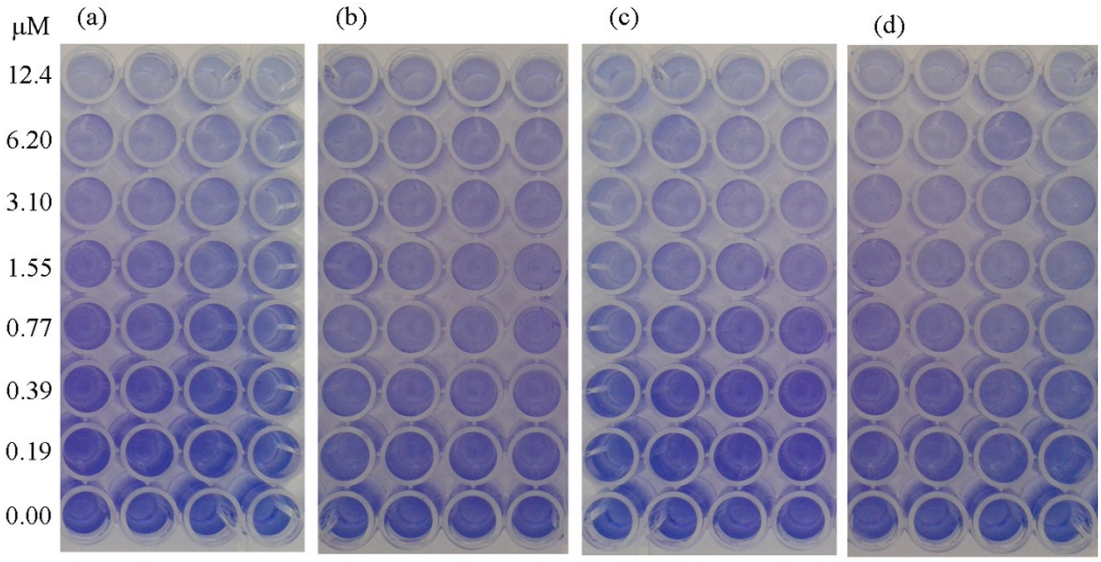


Figure S9. Biofilms of *S. aureus* subsp. aureus Rosenbach ATCC 33592 in the presence of increasing concentrations (μM) of pillar[5]arene derivatives (a) Compound **1**, (b) Compound **2**, (c) Compound **3**, and (d) Compound **4** stained with crystal violet.

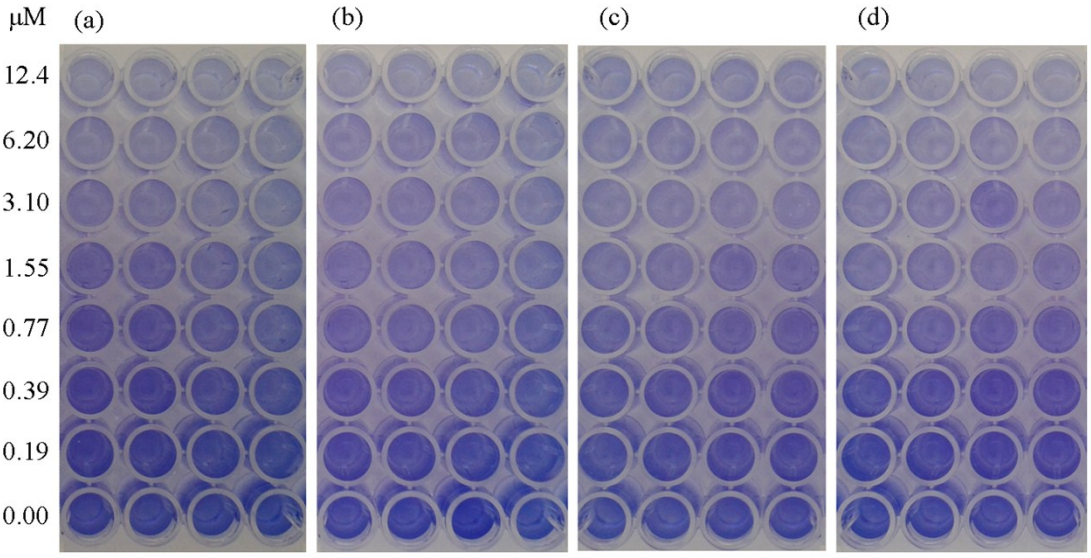


Figure S10. Biofilms formed by *E. faecalis* ATCC 29212 in the presence of increasing concentrations (μM) of pillar[5]arene derivatives (a) Compound **1**, (b) Compound **2**, (c) Compound **3**, and (d) Compound **4** stained with crystal violet.

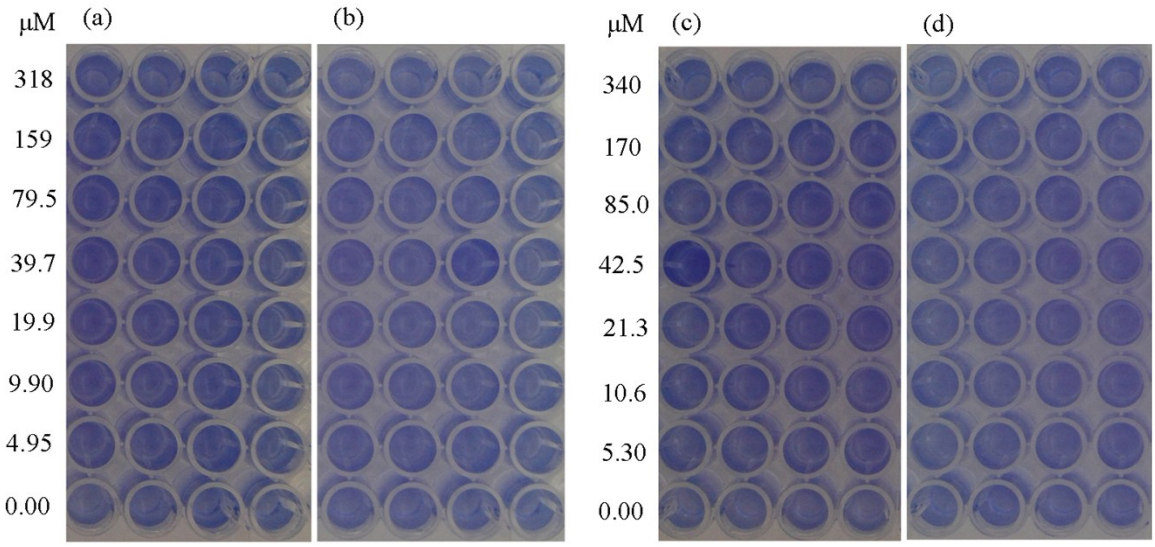


Figure S11. Biofilms formed by *S. aureus* subsp. aureus Rosenbach ATCC 33592 in the presence of increasing concentrations of (a) Compound **5** and (c) Compound **6**. Biofilms formed by *E. faecalis* ATCC 29212 in the presence of increasing concentrations of (b) Compound **5** and (d) Compound **6**. All the wells were stained with crystal violet.

(b) Rat red blood cell haemolysis assay

A sample of rat red blood cells (2% w/w) were incubated with each of the tested compounds for 1 h at 37 °C in 5% CO2 using the double dilution method starting at a concentration of 256 μg/mL. The negative control was PBS, and the positive control was 1% w/v solution of Triton X-100 (which induced 100% haemolysis). Following centrifugation (2000 rpm, 10 min, ambient temperature), the supernatant was removed and absorbance at 550 nm was measured using a microplate reader (SpectraMax-M2). The results are expressed as percentage of haemoglobin released relative to the positive control (Triton X-100). Experiments were performed in triplicate, and the results are an average of experiments in blood samples taken from at least two rats. No measurable red blood cell haemolysis was detected at any of the tested concentrations for the compounds **1-6**.

(c) pH stability assay

Aqueous solutions of compounds **2** and **4** were treated with dilute HCl to obtain a pH of 2.3. Basic pH values of 10.7 and 10.2 of solutions of the compounds **2** and **4**, respectively, were obtained by adding dilute ammonia solution. The solutions were kept at room temperature for 4 h and then freeze-dried. The ^{1}H spectra recorded for all samples matched the ^{1}H HMR spectra of the respective compounds at pH 7.4. Biofilm inhibition studies were conducted with these compounds according to the procedure reported above and the results are presented in Figure S12 and Table S1.

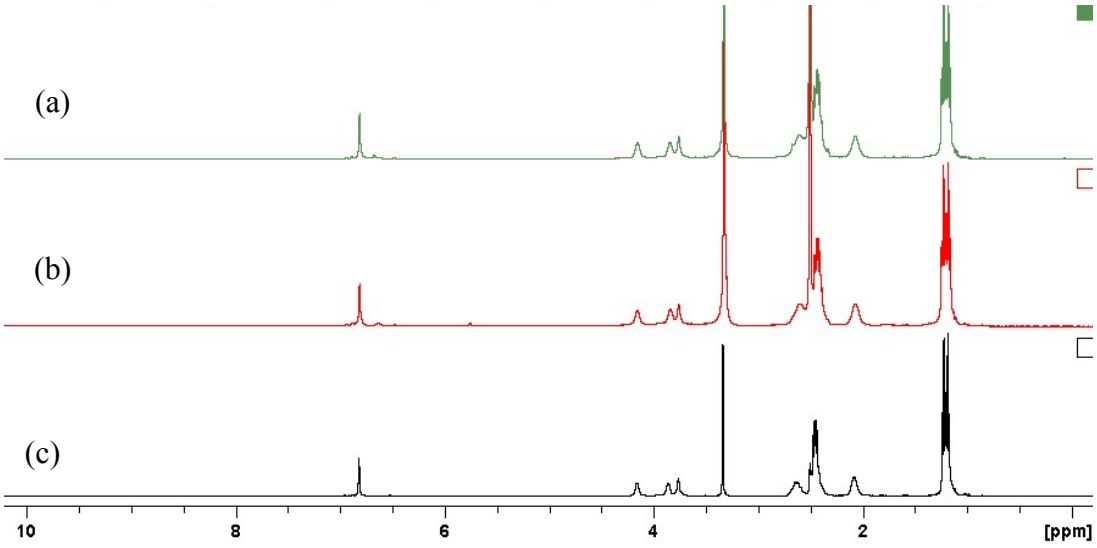


Figure S11. ^{1}H NMR spectra of compound **2** after incubation at pH (a) 7.4, (b) 2.3, and (c) 10.7 for 4 hours.

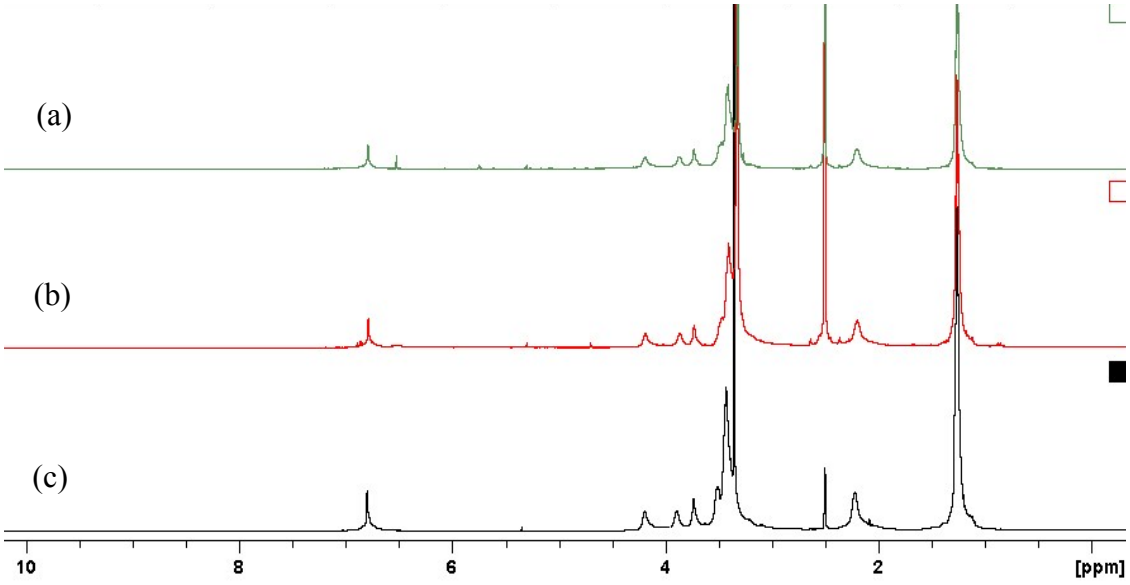


Figure S11. ^{1}H NMR spectra of compound **4** after incubation pH (a) 7.4, (b) 2.3, and (c) 10.2 for 4 hours.

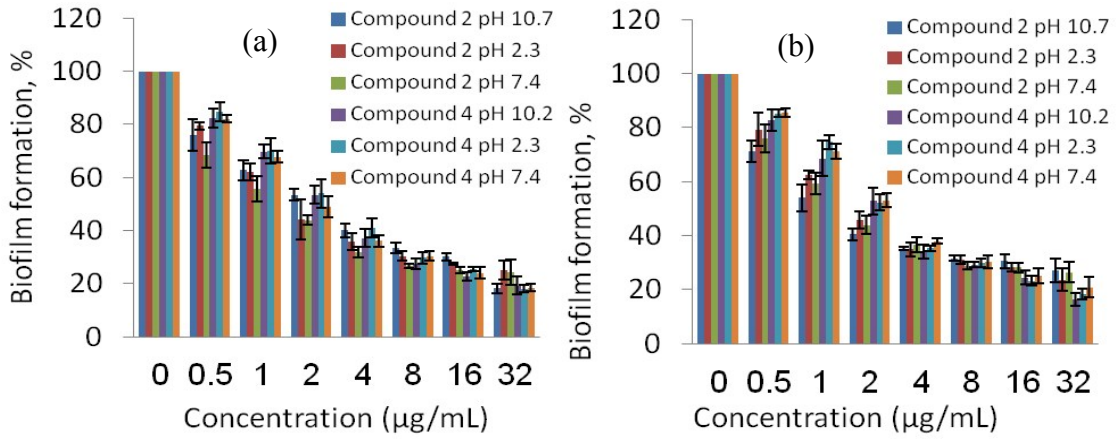


Figure S12**.** Biofilm formation by (a) *S. aureus* subsp. aureus Rosenbach ATCC 33592 and (b) *E. faecalis* ATCC 29212 in the presence of increasing concentrations (µM) **2** and **4** that had been incubated for 4 hours at different pH levels.

Table S1. Biofilm inhibitory activity against Gram-positive strains of compounds **2** and **4** after incubation at different pH levels for 4 hours.

Compound	pH	$MBIC_{50}$ in µM (µg/mL)	
		S. aureus subsp. aureus Rosenbach ATCC 33592	*E. faecalis* ATCC 29212
2	2.3	0.67 (2)	1.33 (4)
	7.4	0.67 (2)	1.33 (4)
	10.7	1.33 (4)	0.67 (2)
4	2.3	1.41 (4)	1.41 (4)
	7.4	1.41 (4)	1.41 (4)
	10.2	1.41 (4)	1.41 (4)

(d) Toxicity towards mammalian cells

Cystic fibrosis human bronchi epithelial cells IB3-1 (ATCC CRL-2777) and HaCaT human skin keratinocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 IU/mL penicillin, 100 g/mL streptomycin (all from Biological Industries, Beit HaEmek, Israel). Cells were plated in 96-well format (10000 cells/well for both cell lines) for 24 h, at 37C, 5% CO_2. Then compounds were added at final concentrations of 2, 4, 8, 16, 32, 64 and 128 µg/ml to the appropriate wells. Wells without compounds served as controls. Plates were incubated at above conditions for 24 h. Cell viability was

determined using MTT (3-[4,5-dimethylthiazoyl-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Wataha et al., 1992) as well as observed using Light microscopy. The percentage of cell death was determined relative to vehicle-treated cells. Experiments were performed in triplicate, and the results were obtained from two independent experiments.

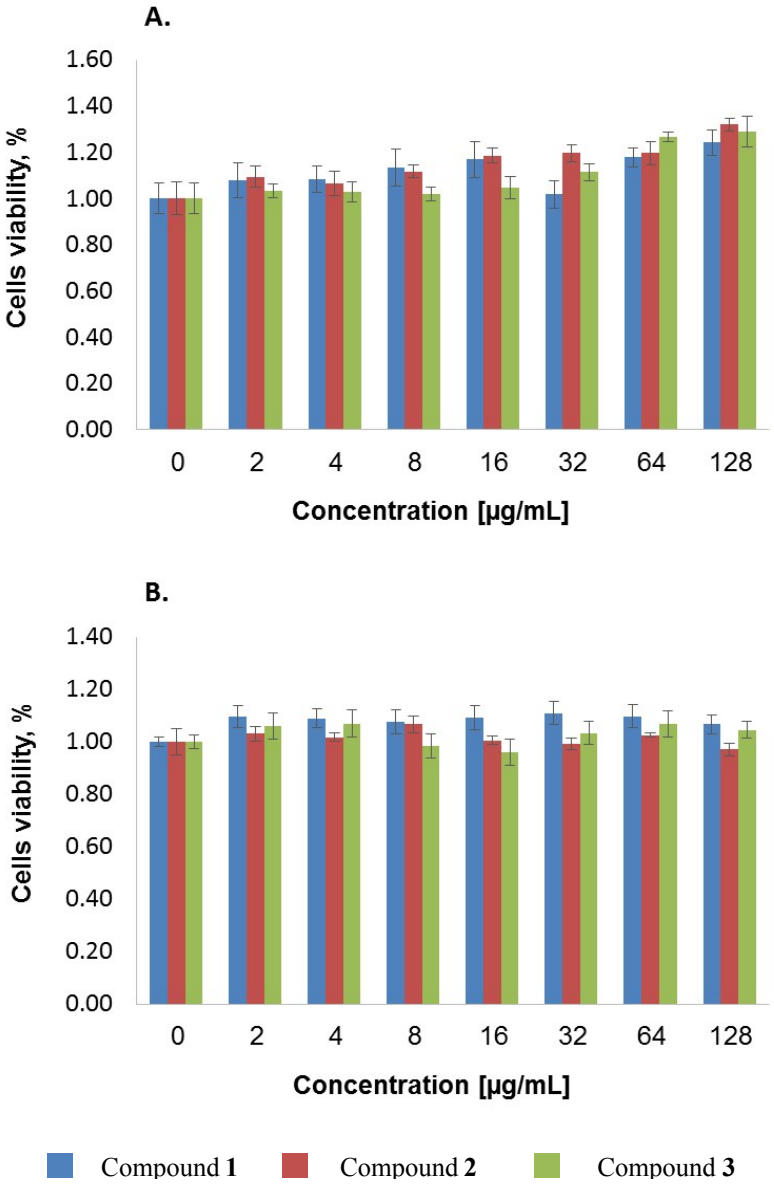


Figure S13. Mammalian cell toxicity: **A.** IB3-1 cell-line, **B.** HaCaT cell-line.

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