Supplementary Information

Synthesis and activity of biomimetic biofilm disruptors

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Materials

Chemicals and solvents were purchased from Sigma Aldrich or EMD chemicals. Solvents were anhydrous or HPLC grade and chemicals were of reagent grade or better and used without further purification. Temperatures were measured externally.

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Varian 400 MHz NMR-System and referenced to the residual proton and carbon signals of the deuterated solvent, respectively. Spectra in D_2O were referenced to 3-(trimethylsilyl)propionic-*2,2,3,3-d_4* acid sodium salt (0.05 wt %). Splitting patterns that appear different from their actual spin systems are denoted as peseudo-plets (Ψ).

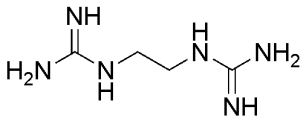
Low resolution mass spectra were obtained by LC/MS (ESI) with an Agilent 6130 Quadrupole LC/MS coupled to an Agilent 1200 series HPLC system. High resolution mass spectrometry was carried out by the mass spectrometry laboratory of the University of Illinois by Q-TOF MS in positive mode or by the WM Keck Foundation Biotechnology Resource Laboratory of Yale University using a 9.4T Bruker Qe FT-ICR MS in negative mode.

Infrared (IR) spectra were measured using an ALPHA FT-IR Spectrometer (Bruker).

1) Synthesis

Guanidines were prepared via different routes.

Preparation of 1,1'-(ethane-1,2-diyl)diguanidine sulfate (1)



Ethylenediamine (1.67 mL, 25 mmol) was added to a solution of *S*-methylisothiourea · ½ H_2SO_4 (1.39 g, 10 mmol) in 8 mL of 1:3 H_2O/EtOH and stirred for 24 h at room

temperature. A white viscous precipitate had formed. The supernatant was decanted and the residue washed with ethanol. Crystallization from ethanol/H_2O afforded transparent crystals which were sucked off and washed with 50% ethanol in water and ethanol (yield: 214 mg, 18%).

^{1}H NMR (400 MHz, D_2O): δ 3.44 (s, 4H, C$\underline{H_2}$-C$\underline{H_2}$).

^{13}C NMR (400 MHz, D_2O): δ 159.95 (2x $\underline{C}$=NH), 42.95 ($\underline{C}H_2$-$\underline{C}H_2$).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

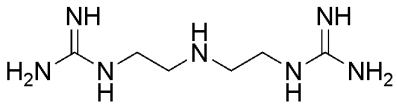
IR (solid): ν (cm^{-1}) 3319, 3160, 3111, 3034, 1686, 1627, 1153, 1078, 1029, 970, 596, 529, 472, 437.

LC-MS (LR) m/z: 145.2 $[M+H]^+$ (calc.: 145.1).

HR ESI-MS, m/z: 145.1200 $[M+H]^+$ (calc.: 145.1202).

Anal. calcd for $C_4H_{14}N_6SO_4$ ($C_4H_{12}N_6 \cdot H_2SO_4$): C, 19.83; H, 5.82; N, 34.69; S, 13.24. Found: C, 19.81; H, 5.74; N, 33.96; S, 13.23.

Preparation of *N,N*-di(2-guanidinylethyl)amine sulfate hydrate (2a)



Diethylenetriamine (540 µL, 5 mmol) was added to a solution of *S*-methylisothiourea · ½ H_2SO_4 (1392 mg, 10 mmol) in 5 mL of 1:1 H_2O/EtOH and stirred for 16 h at room temperature. A white crystalline precipitate formed, which was sucked off and washed with ethanol. The raw crystals were dissolved in the minimum volume H_2O at 30°C and isopropanol was carefully layered on top of the solution. The pure product crystallized over night in at 4°C as fine white needles (910 mg, 60%).

^{1}H NMR (400 MHz, D_2O): δ 3.34 (t, *J* = 6.0 Hz, 4H, 2x ^{Gua}NH-C$\underline{H_2}$), 2.83 (t, *J* = 6.0 Hz, 4H, C$\underline{H_2}$-NH-C$\underline{H_2}$).

^{13}C NMR (400 MHz, D_2O): δ 159.92 (2x $\underline{C}$=NH), 49.43 ($\underline{C}H_2$-NH-$\underline{C}H_2$), 43.47 (2x ^{Gua}NH-$\underline{C}H_2$).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3334, 3282, 3151, 2852, 1626, 1459, 1345, 1046, 749, 601, 508.

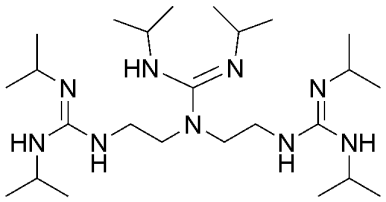
HR ESI-MS, m/z: 188.1626 $[M+H]^+$ (calc.: 188.1624).

Anal. calcd for $C_6H_{21}N_7SO_5$ ($C_6H_{17}N_7 \cdot H_2SO_4 \cdot H_2O$): C, 23.76; H, 6.98; N, 32.32; S, 10.57. Found: C, 23.75; H, 6.87; N, 32.12; S, 10.59.

Free base (**2b**):

The sulfate salt (200 mg, 0.659 mmol) was dissolved in 10 mL H_2O and a solution of $Ba(OH)_2$ (113 mg, 0.659 mmol) in 6 mL H_2O was added. Precipitation was completed at 4°C, the suspension was filtered through celite and lyophilized. The residue was extracted with hot methanol and filtered through celite giving the free base in quantitative yield. The free base was well soluble in hot methanol, the sulfate salt and barium salts were insoluble.

Preparation of 1-[2-*N*^{2},*N*^{3}-diisopropylguanidinylethane-1-yl]-1,1'-(ethane-1,2-diyl)-bis-(*N*^{2},*N*^{3}-diisopropylguanidine) trihydrochloride dihydrate (3)



Diethylenetriamine (216 µL, 2.0 mmol) was dissolved in 4 mL acetonitril, *N*,*N'*-diisopropylcarbodiimide (1174 µL, 7.5 mmol) was added and the solution was stirred at 70°C for 24 h. The solvent was removed, the oily residue was taken up in 6 mL ethanol and 4 mL of a 1:1 mixture of ethanol and 37% HCl aq. were added. After evaporation of the solvent, the solid residue was taken up in a small volume of ethanol and acetone was added until a precipitate appeared. The liquid phase was decanted and the solid material taken up in ethanol. After filtration the solution was evaporated until a highly viscous oil was obtained. Isopropanol was layered atop the oil and after standing for three days at room temperature, crystalline material had formed. The crystals were collected, washed with ice cold ethanol and isopropanol and re-crystallized twice from isopropanol/ethanol and isopropanol/H_2O yielding 58.6 mg (4.7 %) of the product as the trihydrochloride dihydrate.

^{1}H NMR (400 MHz, D_2O): δ 3.82-3.72 (m, 6H, 2x C<u>H</u>$(CH_3)_2$, 4x C<u>H</u>$(CH_3)_2$), 3.62 (t, *J* = 4.8 Hz, 4H, C<u>H</u>$_2$-N-C<u>H</u>$_2$), 3.53 (t, *J* = 4.7 Hz, 4H, 2x C<u>H</u>$_2$-NH), 1.29 (d, *J* = 6.5 Hz, 12H, 2x CH(C<u>H</u>$_3)_2$), 1.25 (d, *J* = 6.4 Hz, 24H, 4x CH(C<u>H</u>$_3)_2$).

^{13}C NMR (400 MHz, D_2O): δ 161.95 (<u>C</u>=NH), 155.60 (2x <u>C</u>=NH), 50.56 (2x <u>C</u>H$(CH_3)_2$), 49.71 (<u>C</u>H$_2$-N-<u>C</u>H$_2$), 47.07 (4x <u>C</u>H$(CH_3)_2$), 40.78 (2x <u>C</u>H$_2$-NH), 25.03 (2x CH(<u>C</u>H$_3)_2$), 24.45 (4x CH(<u>C</u>H$_3)_2$).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

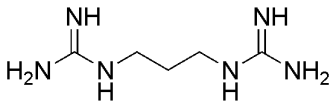
IR (solid): ν (cm^{-1}) 3199, 2973, 1615, 1430, 1388, 1368, 1335, 1268, 1167, 1147, 1133, 1073, 734, 591.

LC-MS (LR) m/z: 161.6 $[M+3H]^{3+}$ (calc.: 161.5), 241.8 $[M+2H]^{2+}$ (calc.: 241.7), 482.4 $[M+H]^{+}$ (calc.: 482.5)

HR ESI-MS, m/z: 482.4653 $[M+H]^{+}$ (calc.: 482.4659)

Anal. calcd for $C_{25}H_{62}O_2N_9Cl_3$ ($C_{25}H_{55}N_9 \cdot 3HCl \cdot 2H_2O$): C, 47.88; H, 9.96; N, 20.10; Cl, 16.96. Found: C, 48.01; H, 10.35; N, 19.73; Cl, 17.44.

Preparation of 1,1'-(propane-1,3-diyl)diguanidine sulfate (4)



Compound **9** (740 mg, 3.19 mmol) and *S*-methylisothiourea · ½ H_2SO_4 (481 mg, 3.45 mmol) were dissolved in 5 mL H_2O and the solution was stirred under reflux. 5 M sodium hydroxide solution (690 µL, 3.45 mmol) was added dropwise and stirred for 4 h. Upon cooling to 4°C a mixture of **4** and **9** crystallized which was collected, dissolved in 5 mL H_2O, and again treated with *S*-methylisothiourea · ½ H_2SO_4 (481 mg, 3.45 mmol) and sodium hydroxide solution (690 µL, 3.45 mmol) under reflux for 4 h. Large white crystals formed at 4°C which were sucked off and re-crystallized from H_2O, yielding 206.5 mg (25%) of the product as sulfate salt.

^{1}H NMR (400 MHz, D_2O): δ 3.29 (t, *J* = 6.8 Hz, 4H, 2x CH_2-NH^{Gua}), 1.9 (p, *J* = 6.8 Hz, 2H, CH_2).

^{13}C NMR (400 MHz, D_2O): δ 159.71 (2x C=NH), 41.22 (2x CH_2-NH^{Gua}), 29.85 (CH_2).

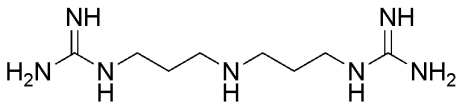
The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3304, 3131, 3026, 1690, 1667, 1636, 1465, 1223, 1085, 1060, 729, 628, 603, 556, 452.

HR ESI-MS, m/z: 159.1362 $[M+H]^{+}$ (calc.: 159.1358).

Anal. calcd for $C_5H_{16}N_6SO_4$ ($C_5H_{14}N_6 \cdot H_2SO_4$): C, 23.43; H, 6.29; N, 32.79; S, 12.51. Found: C, 23.47; H, 6.58; N, 32.36; S, 12.53.

Preparation of *N*,*N*-di-(3-guanidinylpropyl)amine sesquisulfate hydrate (5a)



Norspermidine (700 µL, 5 mmol) was added to a solution of *S*-Methylisothiourea · ½ H_2SO_4 (2505 mg, 18 mmol) in 7 mL H_2O and stirred for 3 days at 105°C. A white precipitate formed after standing overnight at 4°C. The precipitate was sucked off and washed with ice cold H_2O followed by ethanol. Recrystallization from H_2O afforded the sesquisulfate hydrate of the product (1275 mg, 67%) as white solid.

^{1}H NMR (400 MHz, D_2O): δ 3.33 (t, *J* = 6.8 Hz, 4H, 2x HN=C-NH-C$\underline{H}_2$), 3.15 (Ψt, *J* = 7.9 Hz, 4H, C$\underline{H}_2$-NH-C$\underline{H}_2$), 2.02 (Ψq, *J* = 7.4 Hz, 4H, 2x CH_2-C$\underline{H}_2$-CH_2).

^{13}C NMR (400 MHz, D_2O): δ 159.73 (2x $\underline{C}$=NH), 47.85 (2x $\underline{C}H_2$-NH-$\underline{C}H_2$), 41.04 (2x HN=C-NH-$\underline{C}H_2$), 27.83 (2x CH_2-$\underline{C}H_2$-CH_2).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD

IR (solid): ν (cm^{-1}) 1682, 1638, 1479, 1392, 1049, 969, 770, 617, 595, 555, 462.

LC-MS (LR) m/z: 108.8 $[M+2H]^{2+}$ (calc.: 108.6), 216.2 $[M+H]^+$ (calc.: 216.2).

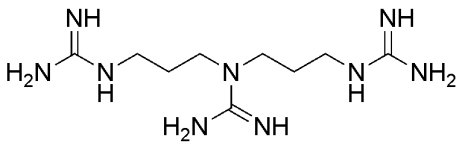
HR ESI-MS, m/z: 216.1930 $[M+H]^+$ (calc.: 216.1937).

Anal. calcd for $C_8H_{26}N_7S_{1.5}O_7$ ($C_8H_{21}N_7$ · 1.5H_2SO_4 · H_2O): C, 25.26; H, 6.89; N, 25.77; S, 12.64. Found: C, 25.65; H, 7.06; N, 25.42; S, 12.72.

Free base (**5b**):

The sulfate salt (200 mg, 0.526 mmol) was dissolved in 10 mL H_2O and a solution of $Ba(OH)_2$ (135 mg, 0.789 mmol) in 6 mL H_2O was added. Precipitation was completed at 4°C, the suspension was filtered through celite and lyophilized. The residue was extracted with hot methanol giving the free base in quantitative yield. The free base was well soluble in hot methanol, the sulfate salt and barium salts were insoluble.

Preparation of 1-(3-guanidinylpropane-1-yl)-1,1'-(propane-1,3-diyl)-diguanidine trihydrochloride hydrate (6a)



Norspermidine (1399 µL, 10 mmol) was dissolved in 7 mL ethanol, 1.5 mL of concentrated hydrochloric acid were added whereupon a white precipitate formed. Cyanamide (1640 mg, 39 mmol) was added and the reaction mixture was refluxed for 36.5 h and let to cool. The solvent was removed resulting in a highly viscous mass which crystallized after 8 days at room temperature. The crystalline material was washed with isopropanol and ice cold ethanol. It was recrystallized from hot methanol/isopropanol, yielding the trihydrochloride hydrate of the product (910 mg, 23.7 %) as white crystals.

^{1}H NMR (400 MHz, D_2O): δ 3.46 (Ψt, *J* = 7.6 Hz, 4H, C<u>H</u>$_2$-N-C<u>H</u>$_2$), 3.27 (t, *J* = 6.9 Hz, 4H, 2x C<u>H</u>$_2$NH), 1.98 (Ψq, *J* = 7.2 Hz, 4H, 2x CH_2-C<u>H</u>$_2$-CH_2).

^{13}C NMR (400 MHz, D_2O): δ 159.62 (2x <u>C</u>=NH), 158.91 (<u>C</u>=NH), 48.79 (<u>C</u>H$_2$-N-<u>C</u>H$_2$), 41.18 (2x <u>C</u>H$_2$NH), 28.35 (2x CH_2-<u>C</u>H$_2$-CH_2).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD

IR (solid): ν (cm^{-1}) 3305, 3128, 1640, 1610, 1534, 1464, 1391, 1165, 1095, 1064, 464.

LC-MS (LR) m/z: 129.7 $[M+2H]^{2+}$ (calc.: 129.6), 258.2 $[M+H]^{+}$ (calc.: 258.2).

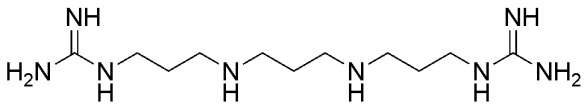
HR ESI-MS, m/z: 258.2147 $[M+H]^{+}$ (calc.: 258.2155).

Anal. calcd for $C_9H_{28}N_9Cl_3O$ ($C_9H_{23}N_9 \cdot 3HCl \cdot H_2O$): C, 28.10; H, 7.34; N, 32.77; Cl, 27.64.21. Found: C, 28.34; H, 7.54; N, 32.02; Cl, 27.14.

Free base (**<u>6b</u>**):

The chloride salt (350 mg, 0.91 mmol) was dissolved in 10 mL H_2O and a suspension of Ag_2SO_4 (423 mg, 1.36 mmol) was added in 6 mL H_2O. After sonication, precipitation was completed at 4°C, the suspension was filtered through celite and a solution of $Ba(OH)_2$ (234 mg, 1.36 mmol) in 5 mL H_2O was added. Precipitation was completed at 4°C and the suspension was filtered through celite and lyophilized. The residue was extracted with hot methanol and filtered through celite giving the free base in quantitative yield. The free base was well soluble in hot methanol, the sulfate salt, the silver and barium salts were insoluble.

Preparation of *N,N'*-di-(3-guanidinylpropyl)-1,3-propanediamine disulfate hydrate (<u>7a</u>)



Norspermine (512 µL, 2.5 mmol) was added to a solution of *S*-methylisothiourea · ½ H_2SO_4 (1670 mg, 12 mmol) in 4 mL H_2O and stirred for 3 days at 105°C. The solvent was largely removed under reduced pressure and the residue dissolved by refluxing in ethanol/H_2O. A

white precipitate formed upon cooling which was sucked off, washed with ethanol and re-crystallized from ethanol/H_2O. The disulfate hydrate salt of the product (560 mg, 46%) was obtained as white solid.

^{1}H NMR (400 MHz, D_2O): δ 3.33 (t, *J* = 6.8 Hz, 4H, 2x HN=C-NH-C$\underline{H}_2$), 3.21-3.17 (m, 4H, 2x HN=C-NH-$(CH_2)_3$-NH-C$\underline{H}_2$), 3.19-3.15 (m, 4H, 2x HN=C-NH-$(CH_2)_2$-C$\underline{H}_2$), 2.22-2.14 (m, 2H, HN-CH_2-C$\underline{H}_2$-CH_2-NH), 2.07-2.00 (m, 4H, 2x HN=C-NH-CH_2-$\underline{C}H_2$)

^{13}C NMR (400 MHz, D_2O): δ 159.72 (2x $\underline{C}$=NH), 47.88 (2x HN=C-NH-$(CH_2)_2$-$\underline{C}H_2$), 47.42 (2x HN=C-NH-$(CH_2)_3$-NH-$\underline{C}H_2$), 41.02 (2x HN=C-$\underline{C}H_2$), 27.80 (2x HN=C-NH-CH_2-$\underline{C}H_2$), 25.52 (HN-CH_2-$\underline{C}H_2$-CH_2-NH).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3118, 1685, 1638, 1478, 1060, 601, 557.

LC-MS (LR) m/z: 137.2 $[M+2H]^{2+}$ (calc.: 137.1), 273.3 $[M+H]^+$ (calc.: 273.3).

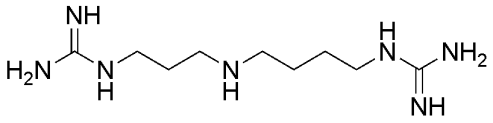
HR ESI-MS, m/z: 273.2514 $[M+H]^+$ (calc.: 273.2515).

Anal. calcd for $C_{11}H_{34}N_8S_2O_9$ ($C_{11}H_{28}N_8 \cdot 2H_2SO_4 \cdot H_2O$): C, 27.15; H, 7.04; N, 23.03; S, 13.18. Found: C, 27.27; H, 7.13; N, 23.23; S, 12.98.

Free base (**7b**):

The sulfate salt (200 mg, 0.411 mmol) was dissolved in 10 mL H_2O and a solution of $Ba(OH)_2$ (141 mg, 0.822 mmol) in 6 mL H_2O was added. Precipitation was completed at 4°C, the suspension was filtered through celite and lyophilized. The residue was extracted with hot methanol and filtered through celite giving the free base in quantitative yield. The free base was well soluble in hot methanol, the sulfate salt and barium salts were insoluble.

Preparation of *N*-(4-guanidinylbutyl)-*N*-(3-guanidinylpropyl)amine sesquisulfate dihydrate (8a)



Spermidine (393 µL, 2.5 mmol) was added to a solution of *S*-Methylisothiourea · ½ H_2SO_4 (1253 mg, 9.0 mmol) in 3.5 mL H_2O and stirred for 3 days at 105°C. After cooling to room temperature a white precipitate was sucked off and washed with ice cold ethanol followed by ethanol. Crystallization from ethanol/H_2O afforded the sesquisulfate dihydrate of the product (557 mg, 54%) as white solid.

^{1}H NMR (400 MHz, D_2O): δ 3.33 (t, *J* = 6.8 Hz, 2H, NH-C<u>H</u>$_2$-$(CH_2)_2$-NH-CH_2), 3.25 (t, *J* = 6.7 Hz, 2H, NH-C<u>H</u>$_2$-$(CH_2)_3$-NH-CH_2), 3.15-3.12 (m, 2H, NH-$(CH_2)_2$-C<u>H</u>$_2$-NH-CH_2), 3.12-3.08 (m, 2H, NH-$(CH_2)_3$-C<u>H</u>$_2$-NH-CH_2), 2.02 (tt, *J* = 7.9, 6.8 Hz, 2H, NH-CH_2-C<u>H</u>$_2$-CH_2-NH), 1.82-1.75 (m, 2H, NH-$(CH_2)_2$-C<u>H</u>$_2$-CH_2-NH-CH_2), 1.72-1.65 (m, 2H, NH-CH_2-C<u>H</u>$_2$-$(CH_2)_2$-NH-CH_2),

^{13}C NMR (400 MHz, D_2O): δ 159.72 (<u>C</u>=NH), 159.67 (<u>C</u>=NH), 50.05 (NH-$(CH_2)_3$-<u>C</u>H_2-NH-CH_2), 47.67 (NH-$(CH_2)_2$-<u>C</u>H_2-NH-CH_2), 43.26 (NH-<u>C</u>H_2-$(CH_2)_3$-NH-CH_2), 41.07 (NH-<u>C</u>H_2-$(CH_2)_2$-NH-CH_2), 27.87 (NH-CH_2-<u>C</u>H_2-$(CH_2)_2$-NH-CH_2), 27.81 (NH-CH_2-<u>C</u>H_2- CH_2-NH), 25.66 (NH-$(CH_2)_2$-<u>C</u>H_2-CH_2-NH-CH_2).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3118, 1629, 1553, 1478, 1044, 971, 748, 607.

LC-MS (LR) m/z: 115.7 $[M+2H]^{2+}$ (calc.: 115.6), 230.2 $[M+H]^{+}$ (calc.: 230.2).

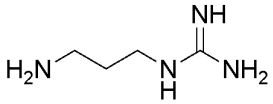
HR ESI-MS, m/z: 230.2094 $[M+H]^{+}$ (calc.: 230.2093).

Anal. calcd for $C_9H_{30}N_7S_{1.5}O_8$ ($C_9H_{23}N_7 \cdot 1.5H_2SO_4 \cdot 2H_2O$): C, 26.21; H, 7.33; N, 23.77; S, 11.66. Found: C, 26.22; H, 7.23; N, 23.45; S, 10.93.

Free base (**<u>8b</u>**):

The sulfate salt (200 mg, 0.485 mmol) was dissolved in 10 mL H_2O and a solution of $Ba(OH)_2$ (125 mg, 0.727 mmol) in 6 mL H_2O was added. Precipitation was completed at 4°C, the suspension was filtered through celite and lyophilized. The residue was extracted with hot methanol and filtered through celite giving the free base in quantitative yield. The free base was well soluble in hot methanol, the sulfate salt and barium salts were insoluble.

Preparation of (3-Aminopropyl)guanidine sulfate hydrate (<u>9</u>)



S-Methylisothiourea · ½ H_2SO_4 (696 mg, 5 mmol) was added to a solution of 1,3-diaminopropane (1043 µL, 12.5 mmol) in 5 mL of 1:3 H_2O/EtOH and stirred for a few minutes at room temperature until precipitation occurred. Another 5 mL of 1:3 H_2O/EtOH were added and stirring continued for 30 min. The precipitate was sucked off and crystallized from hot H_2O (yield: 416 mg, 36%).

^{1}H NMR (400 MHz, D_2O): δ 3.32 (t, *J* = 6.9 Hz, 2H, ^{Gua}NH-C<u>H</u>$_2$), 3.09 (Ψt, J = 7.8 Hz, 2H, C<u>H</u>$_2$-NH_2, 2.03-1.96 (m, 2H, ^{Gua}NH-CH_2-C<u>H</u>$_2$-CH_2-NH_2)

^{13}C NMR (400 MHz, D_2O): δ 159.74 (C=NH), 41.05 (^{Gua}NH-CH_2), 39.73 (CH_2-NH_2), 28.95 (CH_2).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

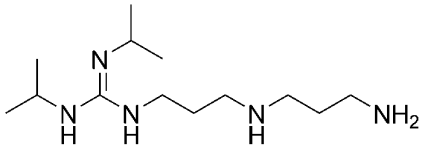
IR (solid): ν (cm^{-1}) 2884, 2805, 1684, 1638, 1079, 863, 738, 618, 601, 552, 469.

LC-MS (LR) m/z: 117.2 $[M+H]^+$ (calc.: 117.1).

HR ESI-MS, m/z: 117.1140 $[M+H]^+$ (calc.: 117.1149).

Anal. calcd for $C_4H_{16}N_4SO_5$ ($C_4H_{12}N_4 \cdot H_2SO_4 \cdot H_2O$): C, 20.69; H, 6.94; N, 24.12; S, 13.81. Found: C, 21.05; H, 6.95; N, 23.87; S, 13.89.

Preparation of *N*-(3-aminopropyl)-*N*-(3-N^2,N^3-diisopropylguanidinylpropyl)amine triformate (10)



N,*N*-Diisopropylcarbodiimide (313 µL, 2.0 mmol) was dissolved in 1 mL dichloromethane and norspermidine (140 µL, 1.0 mmol) was added under vigorous stirring. After stirring over night, the solvent was evaporated and the residue dissolved in a mixture of 1.1 mL water and 0.4 mL methanol. 50 µL of the solution were purified by HPLC with on a Hypercarb column (250 x 10 mm, 5 µm particle size, Thermo Scientific). Mobile phase A = water, B = acetonitril, gradient (T_{min}) T_0: B = 0%, T_{30}: B = 100%. The product eluted with a retention time of 8 min (yield 1.78 mg, 14%).

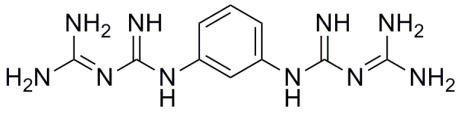
^{1}H NMR (400 MHz, CD_3OD): δ 3.82 (sept, *J* = 6.6 Hz, 2H, 2x C$H(CH_3)_2$), 3.36 (t, *J* = 7.0 Hz, 2 H, NH^{Gua}-CH_2), 3.03 (t, *J* = 7.6, 2H, CH_2-NH_2), 2.99 (Ψt, *J* = 7.4 Hz, 2H, CH_2-$(CH_2)_2$-NH_2), 2.95 (Ψt, *J* = 7.4 Hz, 2H, NH^{Gua}-$(CH_2)_2$-CH_2-NH), 2.01 (Ψq, *J* = 7.5 Hz, 2H, CH_2-CH_2-NH_2), 1.94 (Ψq, *J* = 7.1 Hz, 2H, CH_2-CH_2-NH), 1.27 (d, *J* = 6.4, 12H, 4x CH_3).

^{13}C NMR (400 MHz, CD_3OD): δ 154.50 (C=N), 46.61 (NH^{Gua}-$(CH_2)_2$-CH_2-NH), 46.50 (CH_2-$(CH_2)_2$-NH_2), 45.52 ($CH(CH_3)_2$), 40.09 (NH^{Gua}-CH_2), 38.37 (CH_2-NH_2), 27.86 (CH_2-CH_2-NH), 26.53 (CH_2-CH_2-NH_2), 22.76 (CH_3).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

HR ESI-MS, m/z: 258.2662 $[M+H]^+$ (calc.: 258.2658).

Preparation of *m*-Phenylenedibiguanide dihydrochloride (<u>11a</u>)



1,3-Diaminobenzene (1081 mg, 10 mmol) was dissolved in 7 mL H_2O, concentrated hydrochloric acid was added until pH = 1 and dicyandiamide (2186 mg, 26 mmol) was added. The reaction was refluxed for 4 h, let to cool and acetone was added until a white precipitate formed. After standing over night the fine suspended material had settled and was washed with two times 20 mL acetone. After drying, the material was crystallized from hot ethanol/H_2O. The white crystalline material was sucked off and washed with cold ethanol (yield: 1.956 g, 56%).

^{1}H NMR (400 MHz, D_2O): δ 7.45 (t, *J* = 8.1 Hz, 1H, aromate: CH=C<u>H</u>-CH), 7.33 (t, *J* = 2.0 Hz, 1H, aromate: N-C=C<u>H</u>-C-N), 7.15 (dd, *J* = 8.1, 2.1 Hz, 2H, aromate: C<u>H</u>=CH-C<u>H</u>).

^{13}C NMR (400 MHz, D_2O): δ 163.83 (2x <u>C</u>=NH), 160.34 (2x <u>C</u>=NH), 140.16 (N-<u>C</u>=CH-<u>C</u>-N), 133.12 (CH=<u>C</u>H-CH), 123.68 (<u>C</u>H=CH-<u>C</u>H), 121.68 (N-C=<u>C</u>H-C-N).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD

IR (solid): ν (cm^{-1}) 3292, 3136, 1628, 1575, 1541, 1525, 1448, 1394, 1283, 781, 600, 468.

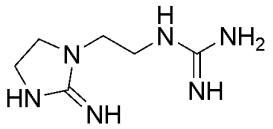
HR ESI-MS, m/z: 277.1632 $[M+H]^+$ (calc.: 277.1638).

Anal. calcd for $C_{10}H_{18}N_{10}Cl_2$ ($C_{10}H_{16}N_{10} \cdot 2HCl$): C, 34.39; H, 5.20; N, 40.11; Cl, 20.30. Found: C, 34.52; H, 5.05; N, 38.94; Cl, 20.85.

Free base (**<u>11b</u>**):

The chloride salt (350 mg, 1.00 mmol) was dissolved in 10 mL H_2O and a suspension of Ag_2SO_4 (313 mg, 1.00 mmol) was added in 10 mL H_2O. After sonication, precipitation was completed at 4°C and a solution of $Ba(OH)_2$ (172 mg, 1.00 mmol) in 5 mL H_2O was added. Precipitation was completed at 4°C, 5 mL ethanol were added and the suspension was filtered through celite and lyophilized. The residue was extracted with hot ethanol and filtered through celite giving the free base in 33% yield. The free base was well soluble in hot ethanol, the sulfate salt, the silver and barium salts were insoluble.

Preparation of 1-(2-(2-Iminoimidazolidin-1-yl)ethyl)guanidine sulfate (12)



Diethylenetriamine (540 µL, 5.0 mmol) was added to a solution of *S*-methylisothiourea (1392 mg, 10 mmol) in 2.5 mL H_2O and refluxed for 3 days. After cooling to room temperature, 10 mL isopropanol were added followed by 1.3 mL of a 10% solution of sulfuric acid in isopropanol. The solvent was removed and the residue crystallized from hot ethanol/H_2O. The crystals were sucked off, washed with ice cold ethanol and re-crystallized from hot ethanol/H_2O affording 794 mg (59 %) of white crystalline material.

^{1}H NMR (400 MHz, D_2O): δ 3.81-3.76 (m, 2H, cyclic NH-CH_2-C$\underline{H}_2$-N), 3.68-3.64 (m, 2H, cyclic NH-C$\underline{H}_2$-CH_2-N), 3.55-3.49 (m, 4H, N-C$\underline{H}_2$-CH_2-NH^{Gua} and N-CH_2-C$\underline{H}_2$-NH^{Gua}).

^{13}C NMR (400 MHz, D_2O): δ 161.93 (ring $\underline{C}$=NH), 159.91 ($\underline{C}$=NH), 50.70 (cyclic NH-CH_2-$\underline{C}H_2$-N), 46.49 (N-$\underline{C}H_2$-CH_2-NH^{Gua}), 43.59 (cyclic NH-$\underline{C}H_2$-CH_2-N), 41.38 (N-CH_2-$\underline{C}H_2$-NH^{Gua}).

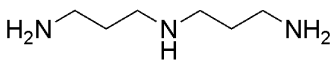
The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3097, 2996, 2899, 1674, 1646, 1631, 1587, 1462, 1312, 1074, 797, 647, 614, 593, 549.

HR ESI-MS, m/z: 171.1367 $[M+H]^+$ (calc.: 171.1358)

Anal. calcd for $C_6H_{16}N_6SO_4$ ($C_6H_{14}N_6 \cdot H_2SO_4$): C, 26.86; H, 6.01; N, 31.32; S, 11.95. Found: C, 26.85; H, 6.27; N, 31.06; S, 11.92.

Preparation of Norspermidine trihydrochloride



Norspermidine (2.80 mL, 20 mmol) was dissolved in 40 mL ethanol and 12 mL of a 1:1 (vol.) mixture of 37% aqueous HCl and ethanol was added. The precipitate was sucked off, washed with ethanol and the product crystallized from hot ethanol/ H_2O (yield 3.73 g, 78%).

^{1}H NMR (400 MHz, D_2O): δ 3.20 (Ψt, *J* = 8.0 Hz, 4H, C$\underline{H}_2$-N-C$\underline{H}_2$), 3.13 (Ψt, *J* = 7.9 Hz, 4H, 2x C$\underline{H}_2$$NH_2$), 2.16-2.08 (m, 4H, 2x CH_2-C$\underline{H}_2$-CH_2).

^{13}C NMR (400 MHz, D_2O): δ 47.55 ($\underline{C}H_2$-N-$\underline{C}H_2$), 39.40 (2x $\underline{C}H_2NH_2$), 26.60 (2x CH_2-$\underline{C}H_2$-CH_2).

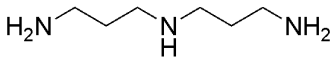
The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 2954, 2894, 2841, 2751, 2702, 2502, 2415, 2025, 1607, 1526, 1489, 1462, 1404, 1356, 1281, 1165, 1055, 1027, 994, 930, 779, 436.

HR ESI-MS, m/z: 132.1504 $[M+H]^+$ (calc.: 132.1501)

Anal. calcd for $C_6H_{20}N_3Cl_3$ ($C_6H_{17}N_3 \cdot 3HCl$): C, 29.95; H, 8.38; N, 17.46; Cl, 44.21. Found: C, 30.12; H, 8.55; N, 17.18; Cl, 44.16.

Preparation of Norspermidine disulfate hydrate



Norspermidine (700 µL, 5.0 mmol) was dissolved in 10 mL ethanol and 8 mL of 20% sulfuric acid in ethanol was added slowly under ice cooling. The precipitate was sucked off and dissolved in hot ethanol/H_2O. Addition of isopropanol precipitated amorphous material which was crystallized from H_2O/acetone (yield 510 mg, 30%).

^{1}H NMR (400 MHz, D_2O): δ 3.18 (Ψt, *J* = 7.8 Hz, 4H, $C\underline{H}_2$-N-$C\underline{H}_2$), 3.12 (Ψt, *J* = 7.9 Hz, 4H, 2x $C\underline{H}_2NH_2$), 2.16-2.08 (m, 4H, 2x CH_2-$C\underline{H}_2$-CH_2).

^{13}C NMR (400 MHz, D_2O): δ 47.63 ($\underline{C}H_2$-N-$\underline{C}H_2$), 39.50 (2x $\underline{C}H_2NH_2$), 26.68 (2x CH_2-$\underline{C}H_2$-CH_2).

The structure was confirmed via gCOSY, HSQCAD and gHMBCAD.

IR (solid): ν (cm^{-1}) 3448, 2923, 1633, 1526, 1475, 1407, 1372, 1213, 1085, 996, 967, 895, 760, 595, 524, 420.

HR ESI-MS, m/z: 132.1505 $[M+H]^+$ (calc.: 132.1501).

Anal. calcd for $C_6H_{23}N_3S_2O_9$ ($C_6H_{17}N_3 \cdot 2H_2SO_4 \cdot H_2O$): C, 20.86; H, 6.71; N, 12.17; S, 18.57. Found: C, 21.02; H, 6.96; N, 12.16; S, 18.43.

2) Bacterial strains

B. subtilis strain NCBI 3610: a wild strain of *B. subtilis*, which is capable of forming robust biofilms.[1]

Staphylococcus aureus SC01 was obtained from the Kolter lab collection.[2]

B. subtilis strain NCBI 3610 and *S. aureus* SC01 were isolated as a single colonies growing overnight at 37°C on solid LB medium.

3) Biofilm inhibition assay

B. subtilis strain NCBI 3610 was grown for 2 h with shaking at 37°C, then diluted 1:1000 in either 3 ml/well (12 well plates, polystyrene, VWR) or 1.5 ml/well (24 wells plates, polystyrene, VWR) of defined biofilm medium MSgg (pH 7.0).[1] Cells were grown without shaking for 3 days at 25°C. For pH experiments bacteria were additionally grown in MSgg at pH 6.0 and 8.0. Floating biofilms (pellicles) of *B. subtilis* were examined optically.

S. aureus strain SC01 was grown overnight with shaking at 37°C, then diluted 1:100 in 1ml/well of TSB medium, 0.5% glucose, 3% NaCl (pH 7.0). Cells were grown without shaking for 24 h at 37°C. For pH experiments bacteria were additionally grown in TSB at pH 6.0 and 8.0. Submerged biofilms of *S. aureus* were observed and quantified by crystal violet staining as blow.

Crystal violet staining. Crystal Violet (CV) staining was done as described previously except that the cells were grown in 24-well plates.[3] Wells were stained with 300 µl of 1.0 % Crystal-violet dye, rinsed twice with 2 ml deionized water and thoroughly dried. For quantification, 0.5 ml of 95 % ethanol were added to each well. Plates were incubated for one hour at room temperature with shaking. CV solution was diluted and the OD at 595 nm was measured using an Ultraspec 2000 (Pharmacia Biotech).

4) Growth data

B. subtilis was diluted from overnight LB cultures to MSgg, with and without the test compounds at 200 µM and *S. aureus* was diluted from overnight LB culture to TSB medium, 0.5% glucose, 3% NaCl (pH 7.0). with and without the test compounds at 200 µM. The strains were grown under shaking at 37°C and OD_{600} was measured at various times. Each compound was tested with 3-4 replicates. Growth curves are given in figure S20.

5) Solubility data (K_s values)

Typically 20-30 mg of each compound was incubated with a volume of H_2O that was insufficient to fully dissolve the entire amount (typically 100-300 µL) and incubated for at least 24 h at 20°C. The undissolved material was centrifuged off and a defined volume of the saturated was removed, lyophilized and the weight of was determined.

6) pK_a determination and ion speciation

For pH and pD measurements, a PHR-146B Micro combination pH electrode (Cole Parmer) was used. The electrode was calibrated for pH using commercial standard solutions of pH 4.0, 7.0 and 10.0 and for pD applying the relation[4] pD = pH + 0.40 to convert pD to pH which was additionally verified by two pD calibration standards of K_2DPO_4/ KD_2PO_4 (0.025 M K_2DPO_4 and 0.025 M KD_2PO_4 in D2O) and K_2CO_3/$KDCO_3$ (0.025 M K_2CO_3 and 0.025 M $KDCO_3$) at 25°C.[5]

To measure pK_a values in the range of 2≤pH≤12 the strategy of Blagbrough et al. was applied. Titrations were conducted in D_2O using ^1H or ^{13}C NMR as described before without correction for ionic strength.[5] Solutions of 25 mM of the compounds in D_2O were prepared (~1 mL to compensate for losses during titration steps). Small amounts of DCl (35 wt.% in D_2O, 99 atom% D, Sigma Aldrich) and KOD (40 wt.% in D_2O, 98 atom% D, Sigma Aldrich) were added successively and pD values were measured followed by ^1H or ^{13}C NMR spectroscopy at 25°C.

Higher pK_a values were determined by the method recommended by Popov et al. for 12≤pH≤14 in H_2O by ^{13}C NMR at fixed ionic strength of I = 1 M with KOH/KCl using calculated pH values.[4] Compounds were dissolved in the pre-adjusted KOH/KCl solutions to give about 500 µL of 25 mM concentration. Corrections for the deprotonation of compound were applied as suggested. Close below pH 12.5, the calculated values were checked and if necessary corrected by pH measurements. In some cases, pH values below 12 were added to expand the pH range and directly measured by the microelectrode after compound addition.

Chemical shifts were plotted as function of pH or pD (Figure S2-S11) and cumulative association constants were calculated by HypNMR2008 (Protonic Software)[6, 7] with the setting pK_w = 13.80[8] and using the relation pD = pH + 0.40 to finally convert pK_a (D_2O) to pK_a (H_2O).[4]

Ion speciations were calculated from pK_a values using the program HySS2009 - Hyperquad Simulation and Speciation (Protonic Software) and are given in Figure S12-S14.

7) Crystal structures

Compounds **2a**, **5a** and **11a** were re-crystallized from deionized water and structures were determined by X-ray diffraction at the Harvard University Center for Crystallographic Studies. Data were collected at 100 K from a crystal mounted on a Bruker APEX II CCD diffractometer ($Mo_{K\alpha}$ radiation, λ = 0.71073 Å) equipped with an Oxford Cryosystems nitrogen flow apparatus. The collection method involved 0.5° scans in ω at 28° in 2θ. Data

integration down to 0.78 Å resolution was carried out using SAINT V7.46 A (Bruker diffractometer, 2009) with reflection spot size optimization. Absorption corrections were made with the program SADABS (Bruker diffractometer, 2009). The structure was solved by the direct methods procedure and refined by least-squares methods again F^2 using SHELXS-97 and SHELXL-97[9] with OLEX 2 interface.[10] Non-hydrogen atoms were refined anisotropically, and hydrogen atoms were allowed to ride on the respective atoms. Crystal data as well as details of data collection and refinement are summarized in Table S4 and Figures S17-S19.

Table S1. Spacing of the repeating units of potential polysaccharides in the EPS. Distances of energy minimized structures of Chem3D Ultra 10.0.were measured in PyMOL as half medium linear distance across three sugar units in the secondary structure corresponding to the binding motif of norspermidine. For polyamines, shortest NH to NH bond distances are given.

Structure	Medium distance
Norspermidine	4.9 Å
Diethylenetriamine	3.7 Å
Spermidine (butyldiamine distance)	6.3 Å
Poly-α-1,4-D-glucuronic acid	5.3 Å
Poly-α-1,2-D-glucuronic acid	4.9 Å
Poly-α-1,6-D-glucose-2-sulfate	5.0 Å
Poly-α-1,6-D-glucose-β-1,3-D-glucuronic acid	4.6 Å
Poly-β-1,6-N-acetyl-d-glucosamine (PGA)	5.2 Å
Helical poly(γ-D-glutamic acid)	~ 5 Å

Table S2. Minimal biofilm inhibitory concentrations (MBIC) for all compounds.

Compound			MBIC (μM) at pH 7.0	
			B. subtilis	*S. aureus* [a]
Norspermidine		base	37.5±12.5	175±25
		sulfate	500	250
		chloride	150	500
Norspermine		base	200	200
Diethylenetriamine		base	>1000	>1000
Biguanide		chloride	>1000	>1000
Spermidine		base	1000	1000
1		sulfate	>1000	>1000
2	**a**	sulfate	>1000	750
	b	base	1000	1000 (50*)
3		chloride	>1000	750
4		sulfate	>1000	50
5	**a**	sulfate	500	75
	b	base	375±125	400
6	**a**	chloride	10*	>1000 (500*)
	b	base	10	50
7	**a**	sulfate	5	55±15
	b	base	2	250
8	**a**	sulfate	600	300
	b	base	>1000	100
9		sulfate	100	500
10		formate	30	20±10
11	**a**	chloride	30	300
	b	base	7±3	750±250
12		sulfate	>1000	>1000

[a]MBICs for *S. aureus* are based on 75% reduction of biofilm formation, *incomplete inhibition.

Table S3. Solubility data as K_s values for the compounds.

Compound	K_s (M)
5a	$(3.1 \pm 0.1)\cdot 10^{-2}$
6a	1.58 ± 0.01
8a	$(6.3 \pm 0.3)\cdot 10^{-2}$
2a	$(5.34 \pm 0.03)\cdot 10^{-2}$
4	$(4.6 \pm 0.1)\cdot 10^{-2}$
9	$(4.1 \pm 0.1)\cdot 10^{-2}$
3	$(2.75 \pm 0.02)\cdot 10^{-1}$
1	$(6.4 \pm 0.2)\cdot 10^{-2}$
Norspermidine chloride	2.84 ± 0.03
Norspermidine sulfate	2.12 ± 0.08
11a	$(3.9 \pm 0.1)\cdot 10^{-1}$
7a	$(8.4 \pm 0.7)\cdot 10^{-2}$
12	$(3.0 \pm 0.2)\cdot 10^{-1}$

Table S4. Crystal data, and data on collection and refinement.

	Compound **2**	Compound **5**	Compound **11**
Crystal data			
Crystal system, space group	Monoclinic, $P2_1/n$	Monoclinic, $C2/c$	Triclinic, P-1
a, b, c (Å)	14.6138 (10), 12.5995 (9), 14.617 (1)	9.0449 (13), 10.9984 (16), 36.497 (5)	9.3527 (11), 9.5924 (11), 11.4378 (14)
α, β, γ (°)	93.700 (1)	94.140 (2)	82.994 (2), 71.145 (2), 73.734 (2)
V (Å^3)	2685.8 (3)	3621.2 (9)	931.65 (19)
Z	8	8	2
Radiation type	Mo $K\alpha$	Mo $K\alpha$	Mo $K\alpha$
μ (mm^{-1})	0.27	0.29	0.38
Crystal size (mm)	0.24 × 0.16 × 0.14	0.18 × 0.10 × 0.05	0.20 × 0.16 × 0.12
Data collection			
Absorption correction	Multi-scan *SADABS*	Multi-scan *SADABS*	Multi-scan *SADABS*
T_{min}, T_{max}	0.938, 0.963	0.950, 0.986	0.928, 0.956
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	40375, 5934, 5362	26494, 3993, 3252	12063, 4113, 3599
R_{int}	0.046	0.053	0.026
$(\sin\theta/\lambda)_{max}$ (Å^{-1})	0.641	0.641	0.641
Refinement			
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.035, 0.090, 1.03	0.061, 0.167, 1.06	0.033, 0.086, 1.04
No. of reflections	5934	3993	4113
No. of parameters	446	305	305
No. of restraints	26	26	20
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement		
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.66, -0.42	0.46, -0.52	0.37, -0.30

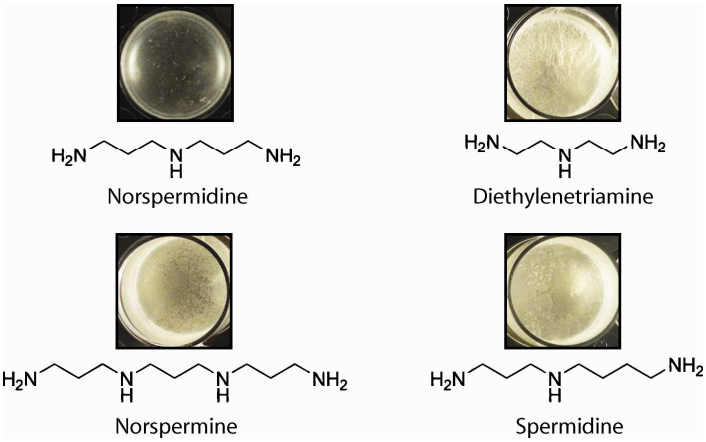


Figure S1. Strcutures of norspermidine and other polyamines and biofilm inhibition in *B. subtilis*.

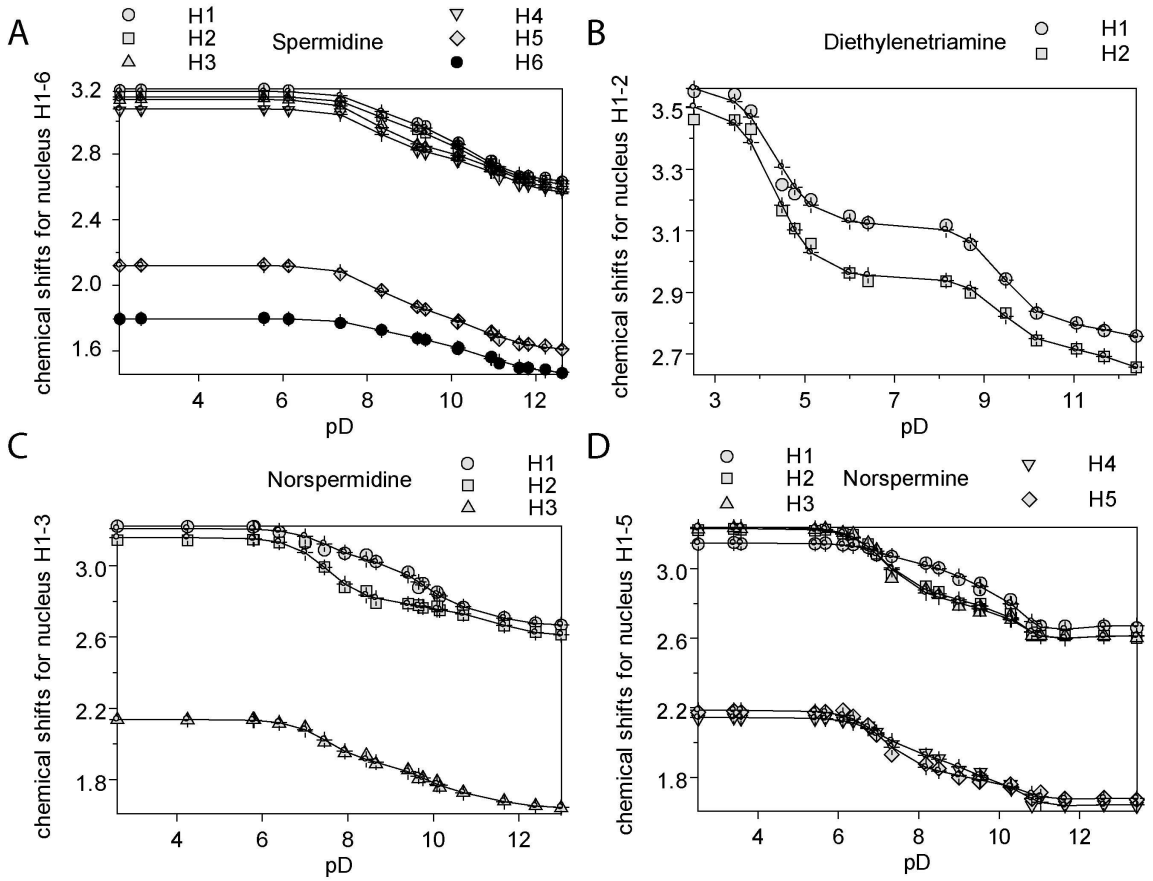


Figure S2. ^{1}H-NMR-titration curves of polyamines for A) spermidine, B) diethylenetriamine, C) norspermidine, D) norspermine.

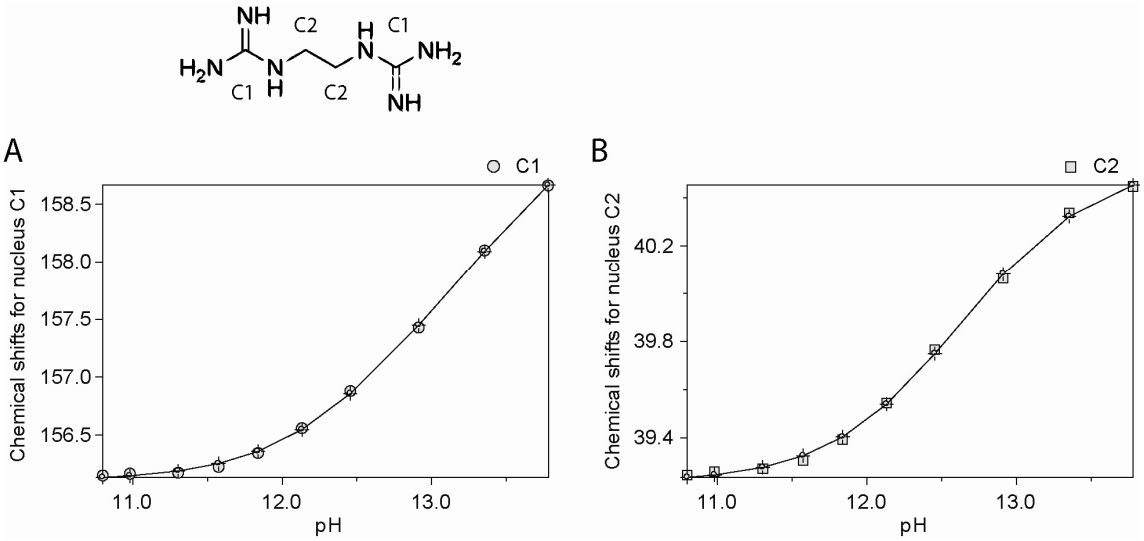


Figure S3. A) and B) ^{13}C-NMR-titration curves of compound **1**.

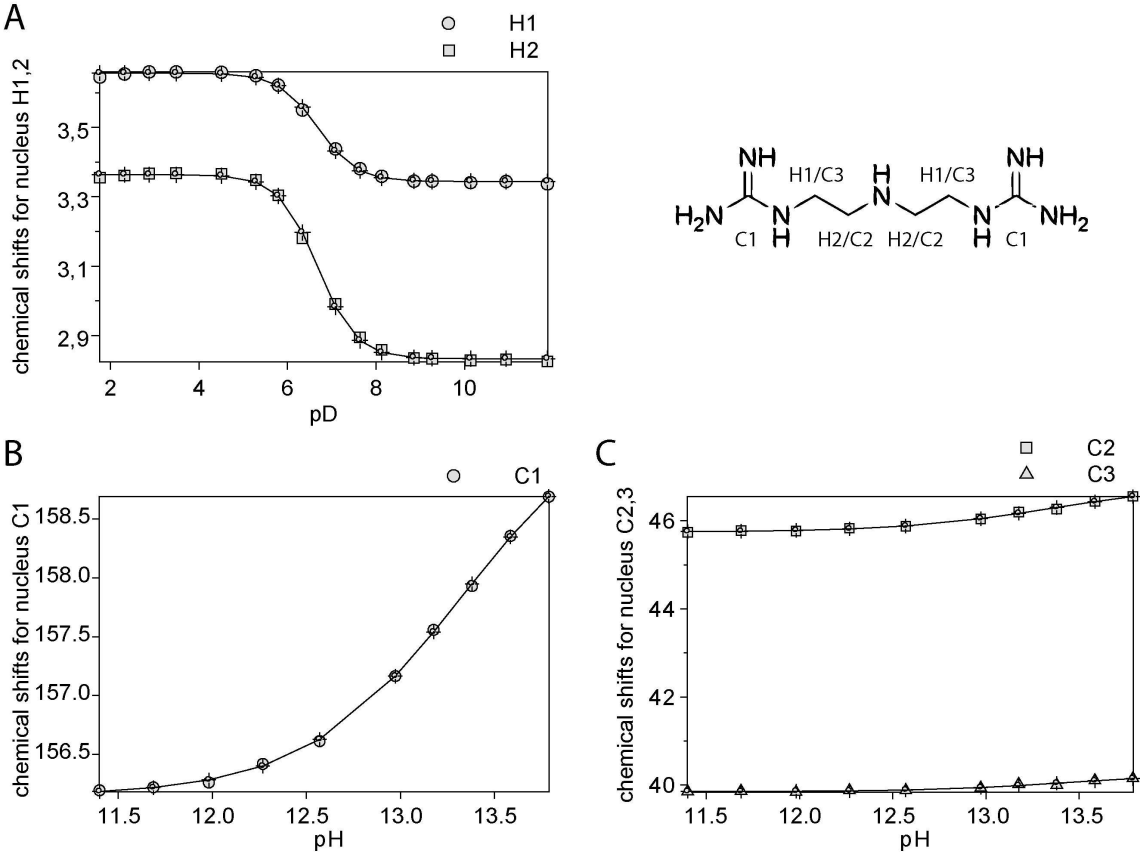


Figure S4. NMR-titration curves of compound **2**. A) ^{1}H-NMR titration in D_2O. B) and C) ^{13}C-NMR titration in H_2O for the upper pH range.

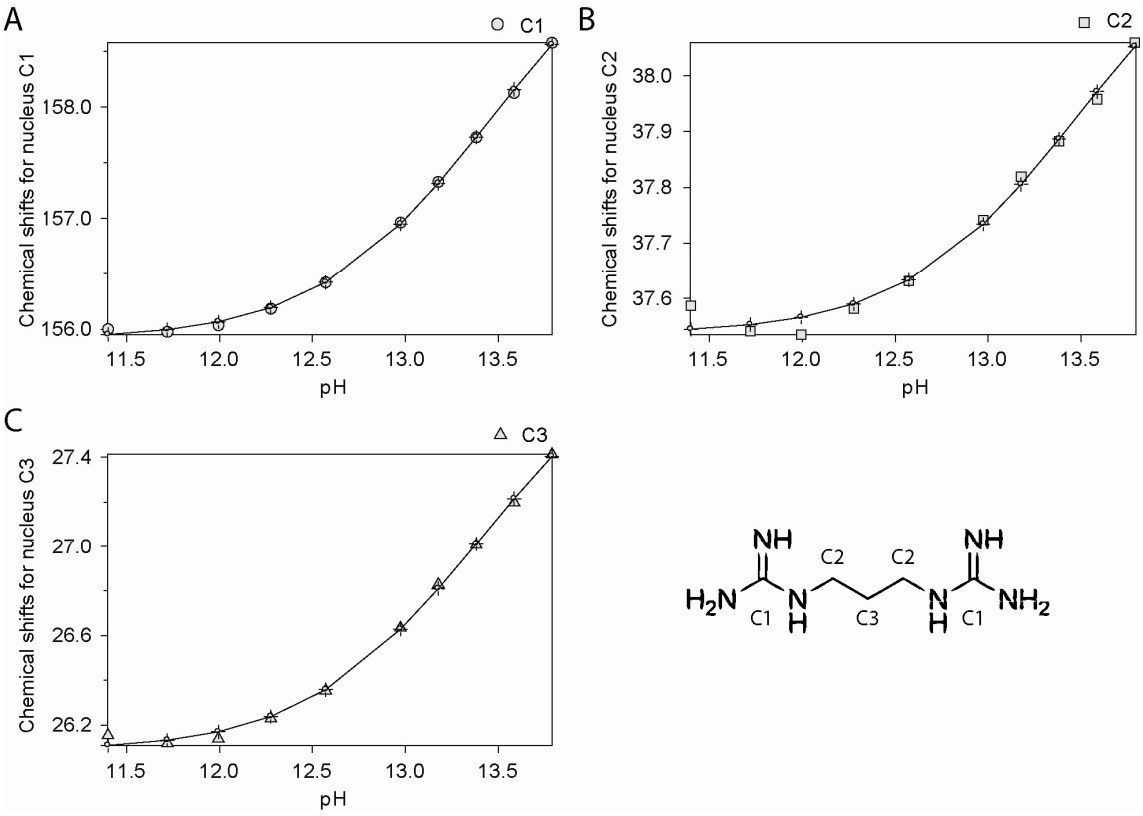


Figure S5. NMR-titration curves of compound **4**. A), B) and C) ^{13}C-NMR titration in H_2O.

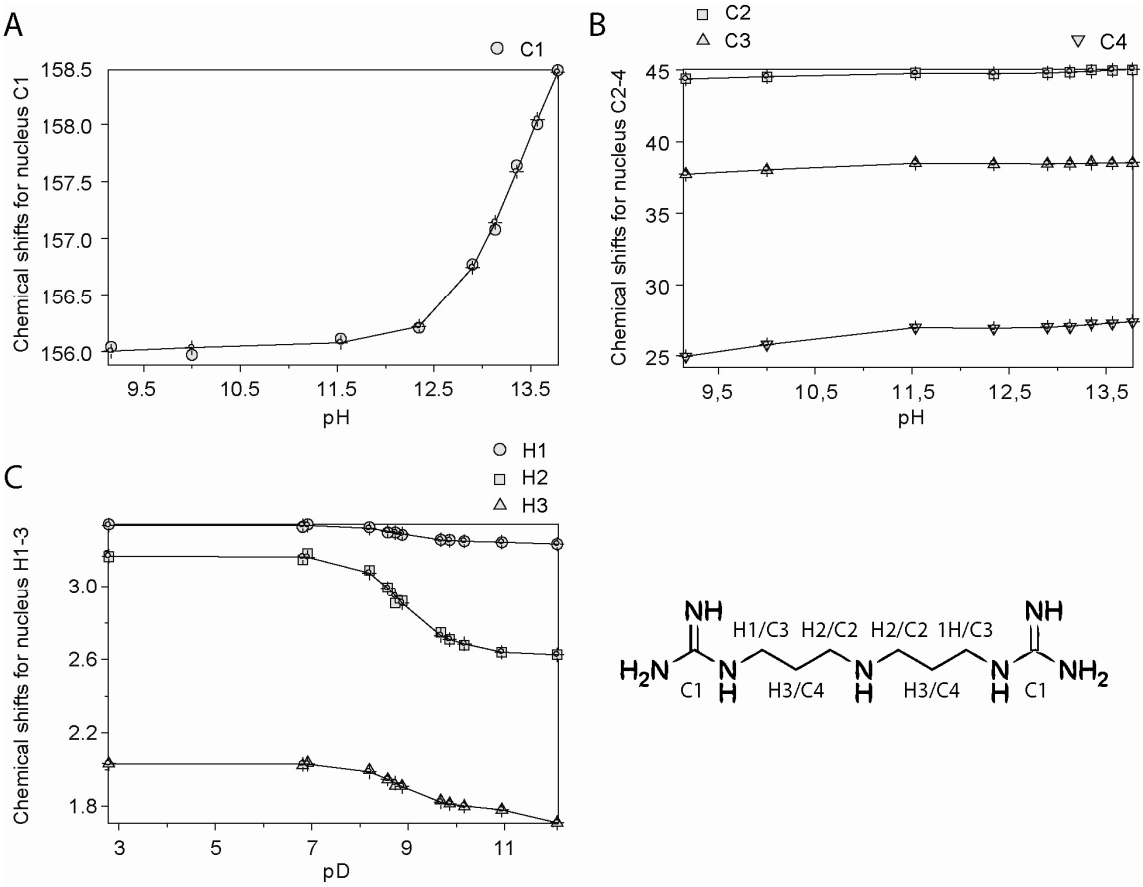


Figure S6. NMR-titration curves of compound **5**. A) and B) ^{13}C-NMR for the upper pH range in H_2O and C) ^{1}H-NMR titration in D_2O.

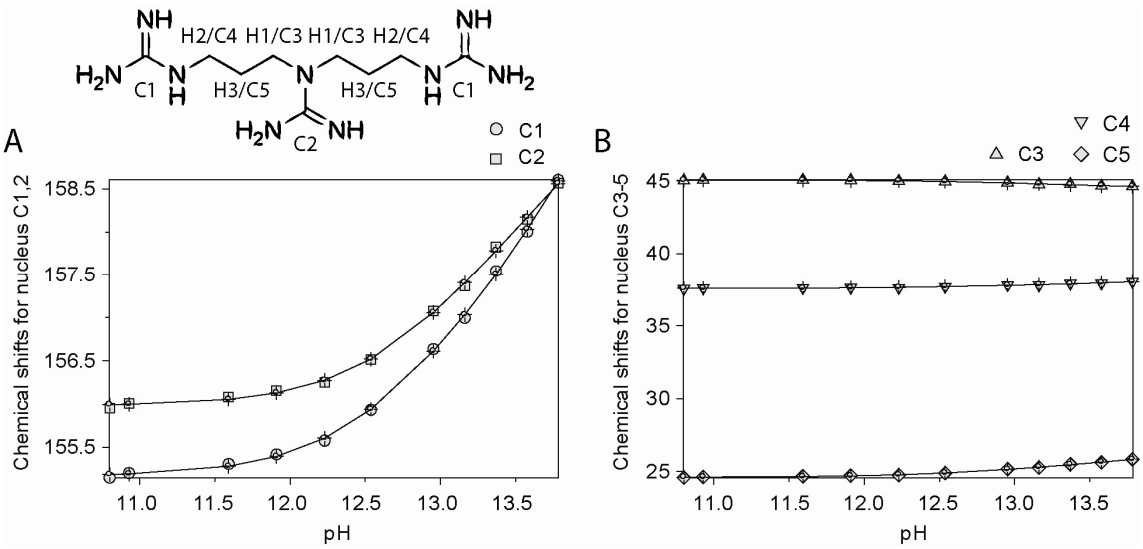


Figure S7. A) and B) ^{13}C-NMR titration curves of compound **6**.

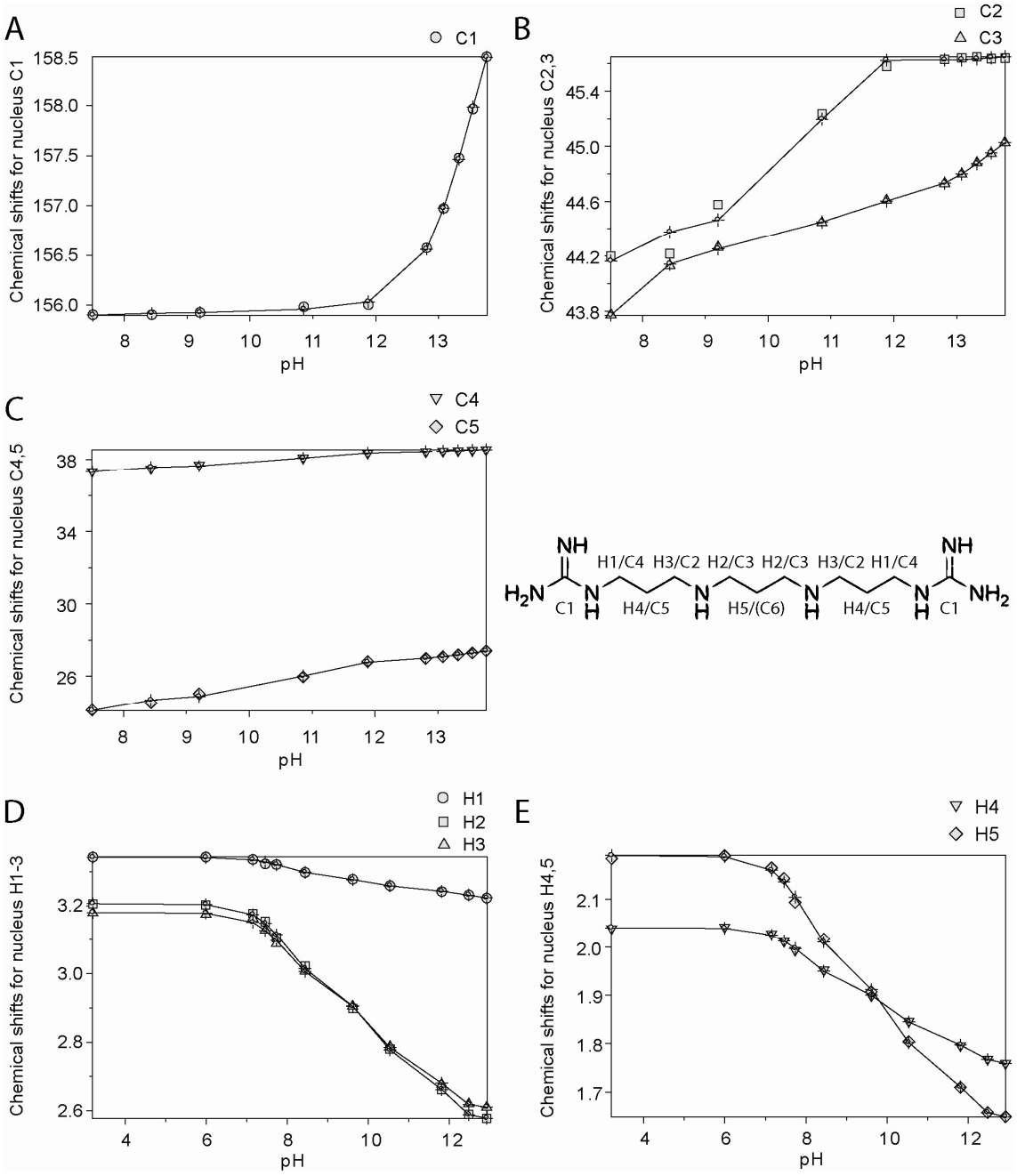


Figure S8. NMR-titration curves of compound **7**. A) - C) ^{13}C-NMR for the upper pH range in H_2O and D) - E) ^{1}H-NMR titration in D_2O.

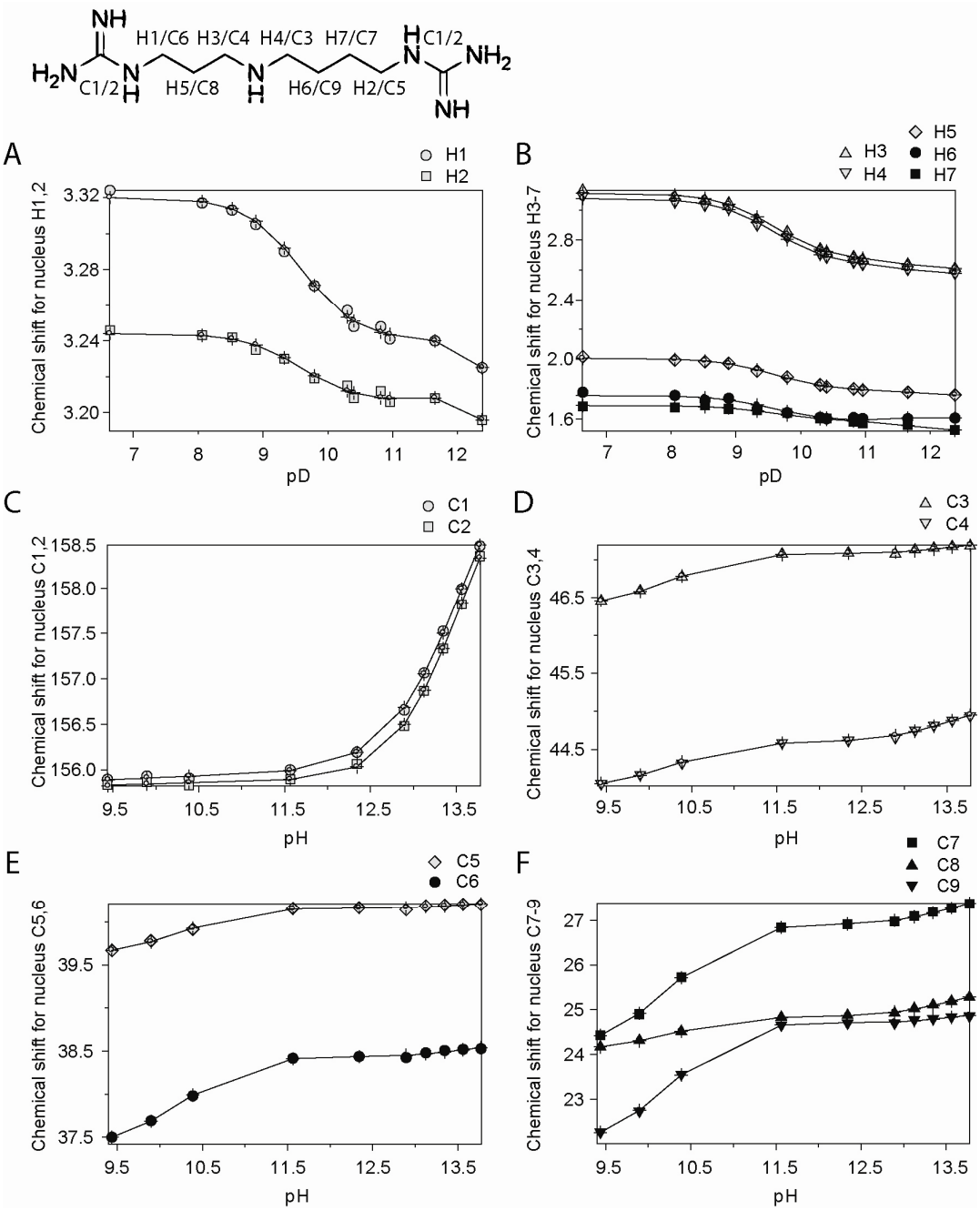


Figure S9. NMR-titration curves of compound **8**. A) and B) ^{1}H-NMR titration in D_2O. C)-F) ^{13}C-NMR for the upper pH range in H_2O.

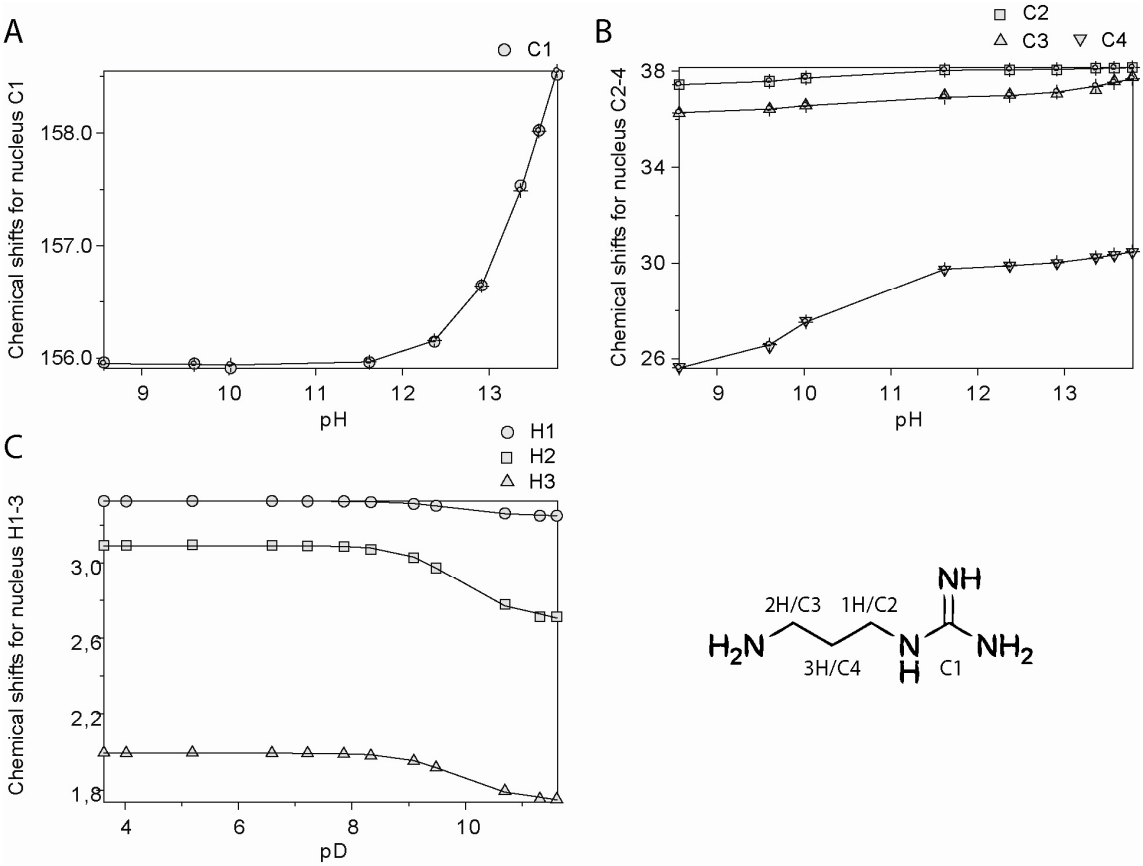


Figure S10. NMR-titration curves of compound **9**. A) and B) ^{13}C-NMR for the upper pH range in H_2O and C) ^{1}H-NMR titration in D_2O.

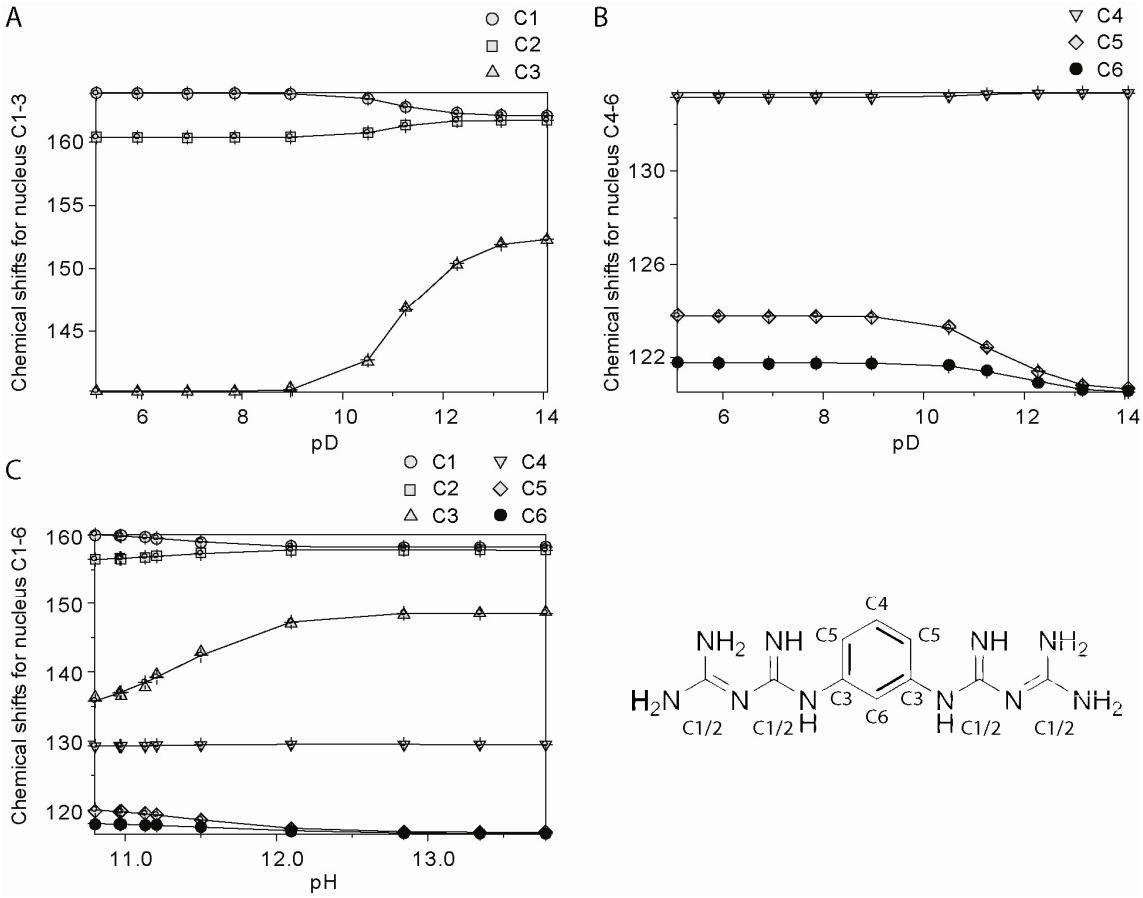


Figure S11. ^{13}C-NMR-titration curves of compound **11**. A) and B) titration in D_2O and C) refinement in H_2O for the upper pH range.

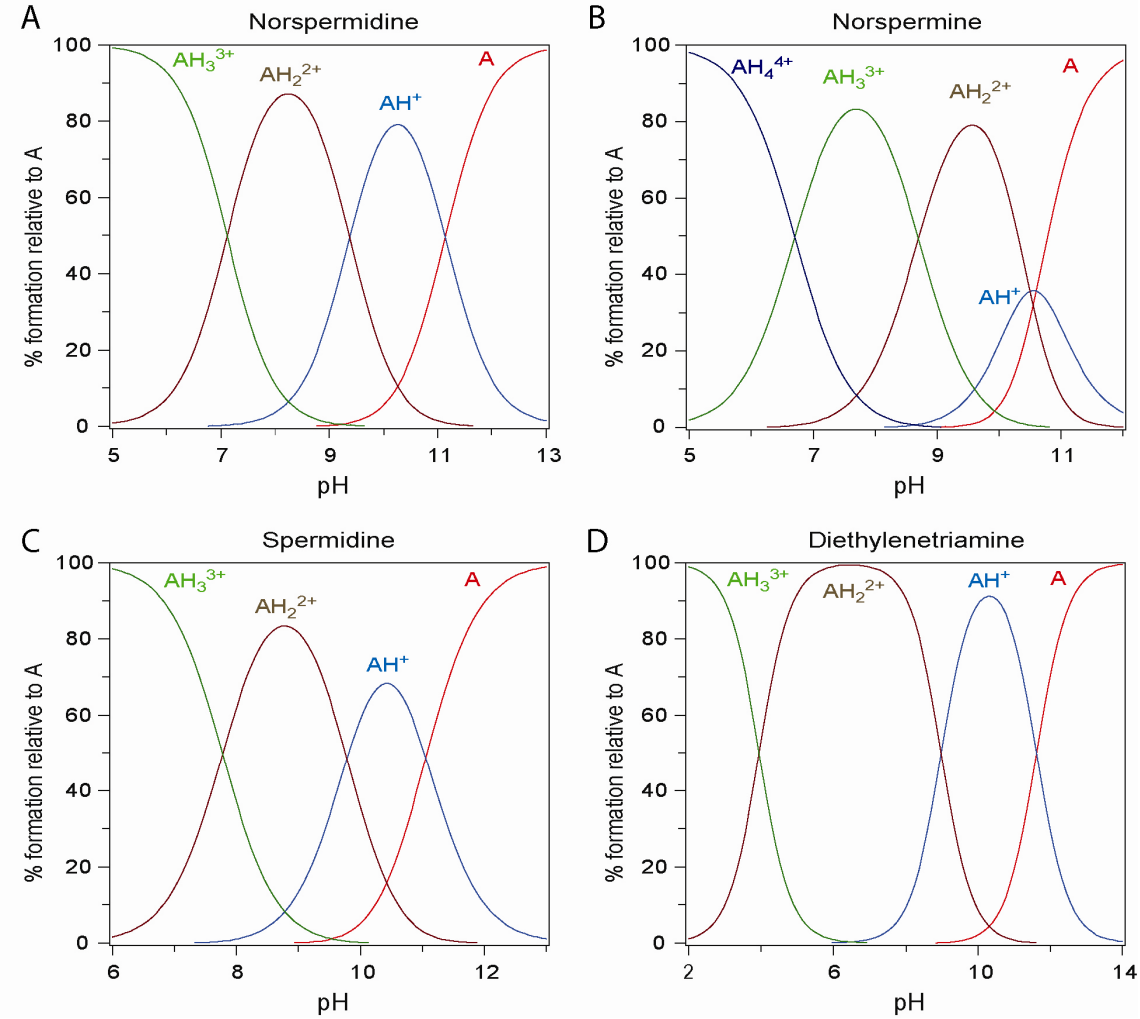


Figure S12. Ion speciation data for A) norspermidine, B) norspermine, C) spermidine, and D) diethylenetriamine.

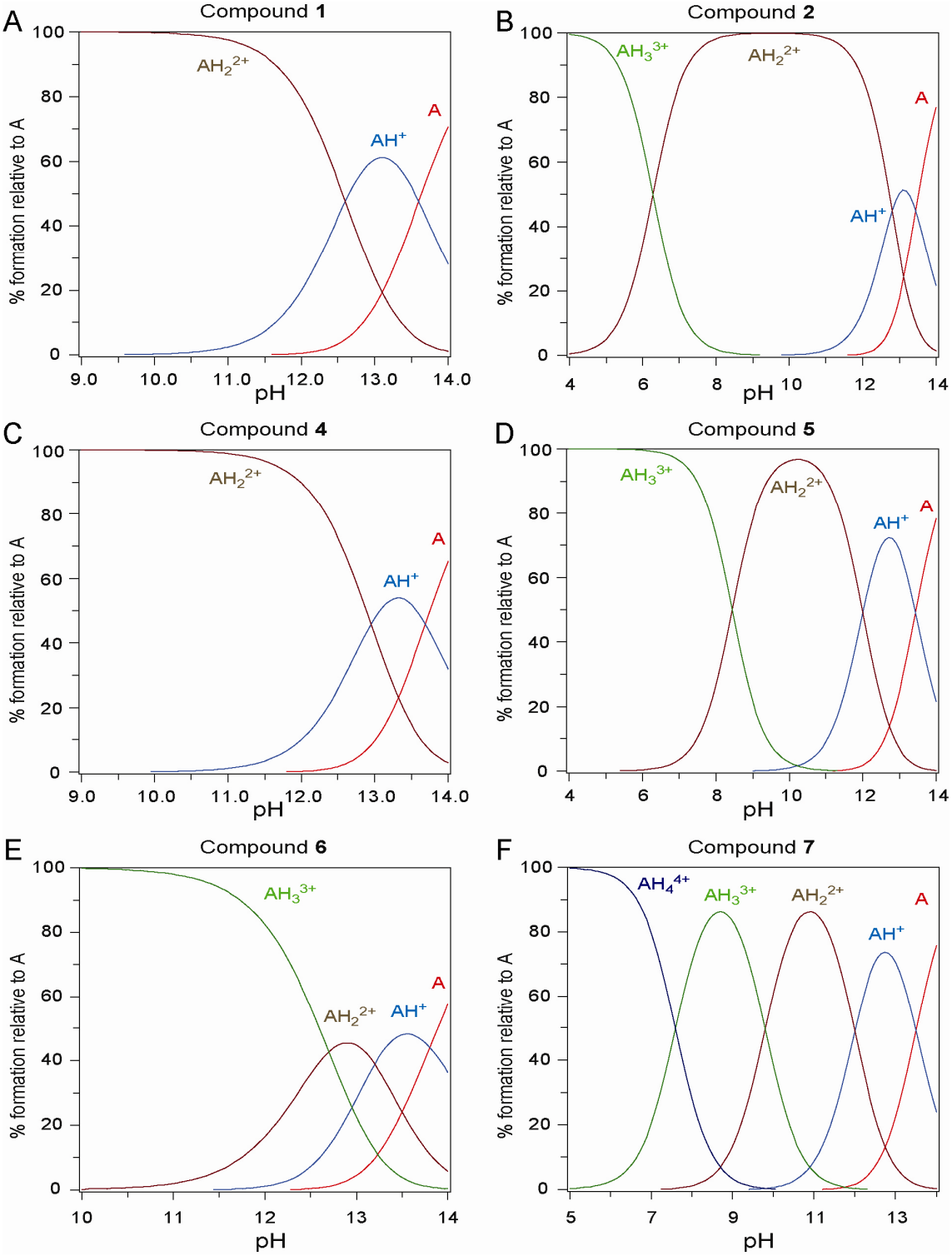


Figure S13. Ion speciation data for compounds **1** (A), **2** (B), **4** (C), **5** (D), **6** (E), and **7** (F).

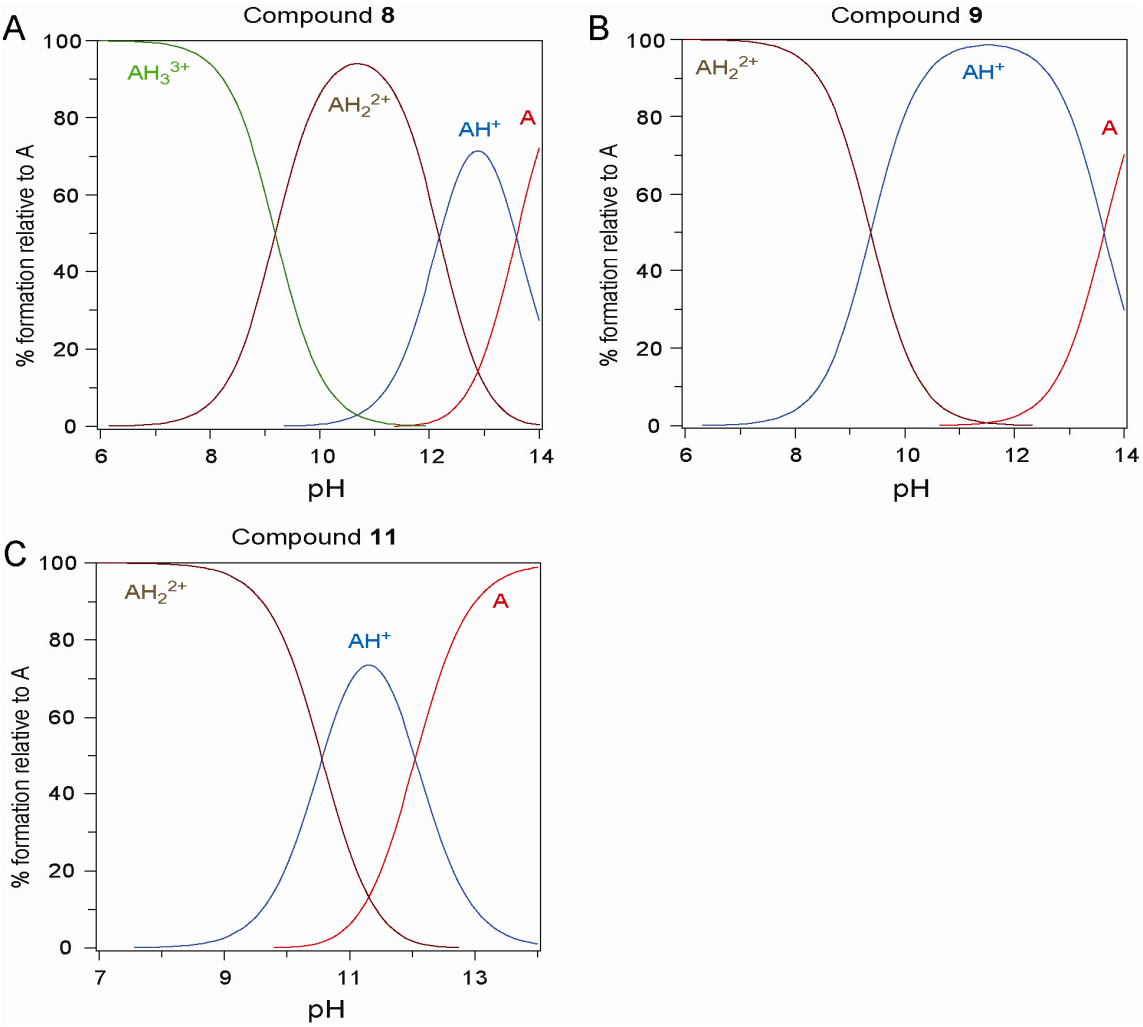


Figure S14. Ion speciation data for compounds **8** (A), **9** (B), and **11** (C).

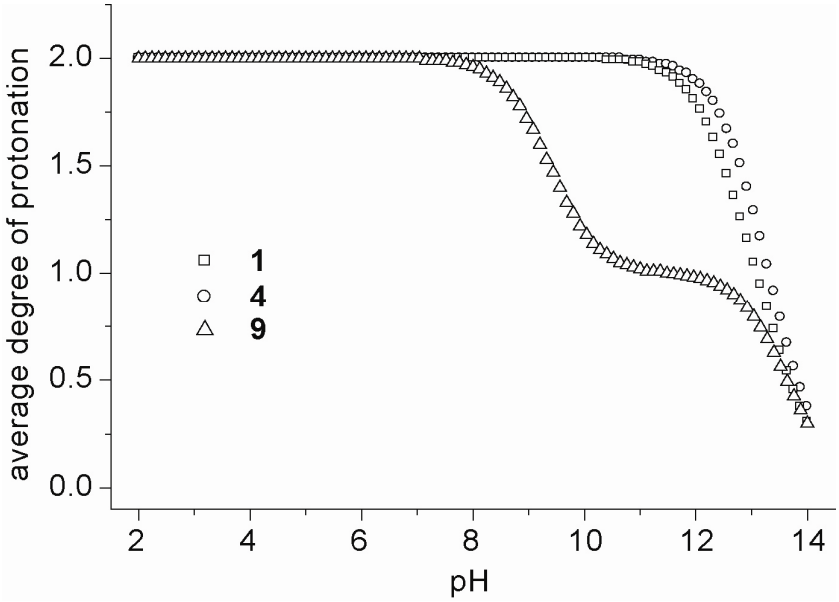


Figure S15. Average degree of protonation in dependence of pH for compounds **1**, **4** and **9**.

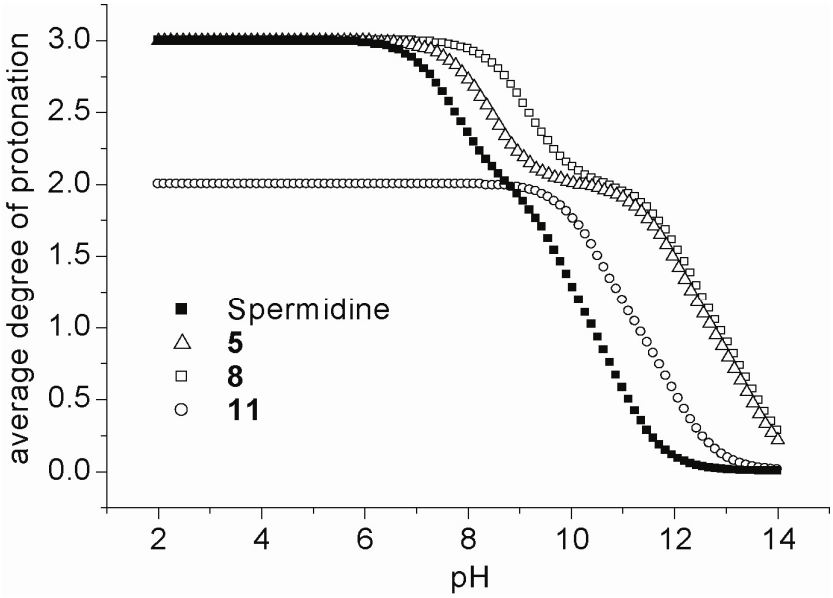


Figure S16. Average degree of protonation in dependence of pH for spermidine and compounds **5**, **8** and **11**.

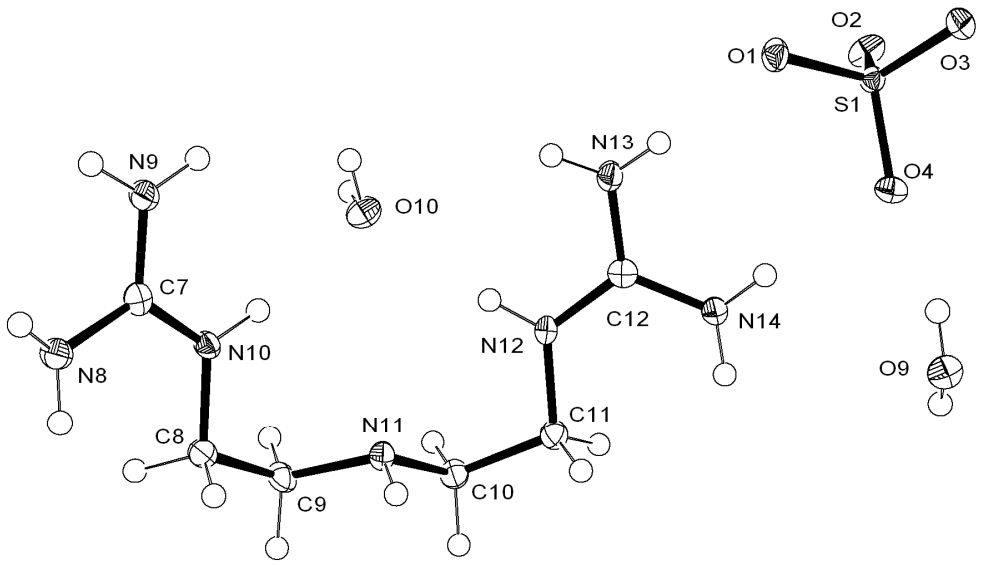


Figure S17. Crystal structure of compound **2**.

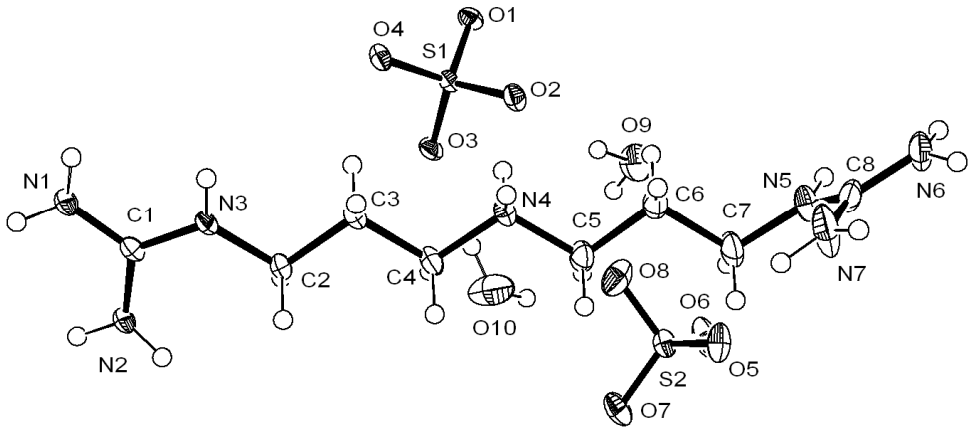


Figure S18. Crystal structure of compound **5**.

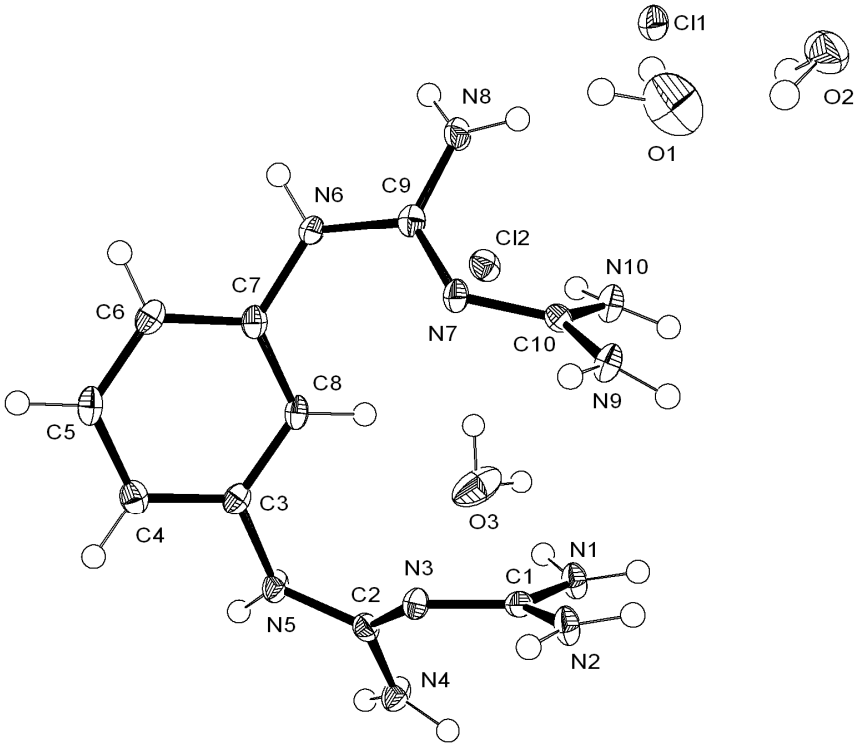


Figure S19. Crystal structure of compound **11**.

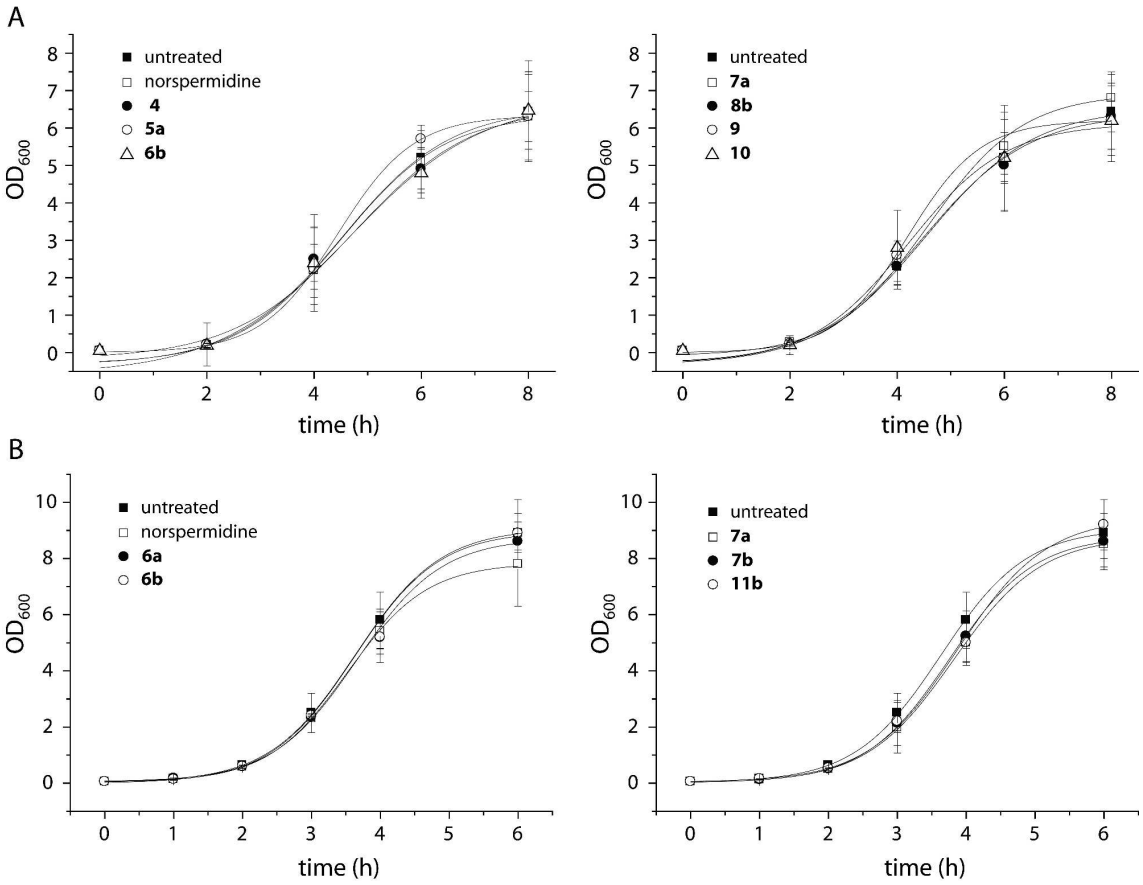


Figure S20. No significant inhibition of bacterial growth in A) *S. aureus* and B) *B. subtilis* was observed with 200 μM of selected compounds.

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