Electronic Supplementary Material (ESI) for ChemComm.

Trehalose-based Janus cyclooligosaccharides: "Click" synthesis and DNA-directed assembly into pH-sensitive transfectious nanoparticles

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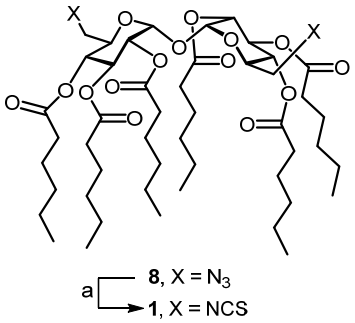
General methods. 6,6'-Diazido-6,6'-dideoxy-2,2',3,3',4,4'-hexa-*O*-hexanoyl-α,α'-trehalose (**8**)[1] 6,6'-di-*O*-trityl-α,α'-trehalose (**9**)[2] and 2-[*N*,*N*-bis(2-(*N*-*tert*-butoxyaminocarbonyl)ethylamino]ethyl isothiocyanate (**5**)[3] were obtained according to literature procedures. Optical rotations were measured at 20 ± 2 °C in 1-dm tubes on a Jasco P-2000 polarimeter. Ultraviolet-visible (UV) spectra were recorded in 1-cm tubes on a Jasco V-630 spectrophotometer. Infrared (IR) spectra were recorded on a Jasco ATR MIRacle™ spectrophotometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 300 (75.5), 500 (125.7) MHz with Bruker 300 ADVANCE and 500 DRX. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminium sheets coated with Sílica Gel 60 F_{254} Merck with visualization by UV light and by charring with 10% H_2SO_4. With preparative purposes, column chromatography was carried out on Silica Gel 60 F_{254} Merck. Electrospray mass spectra were obtained for samples dissolved in MeOH or H_2O-MeOH mixtures at low μm concentrations. Elemental analyses were carried out at the Instituto de Investigaciones Químicas (Sevilla, Spain) using an elemental analyser Leco CHNS-932 or Leco TruSpec CHN.

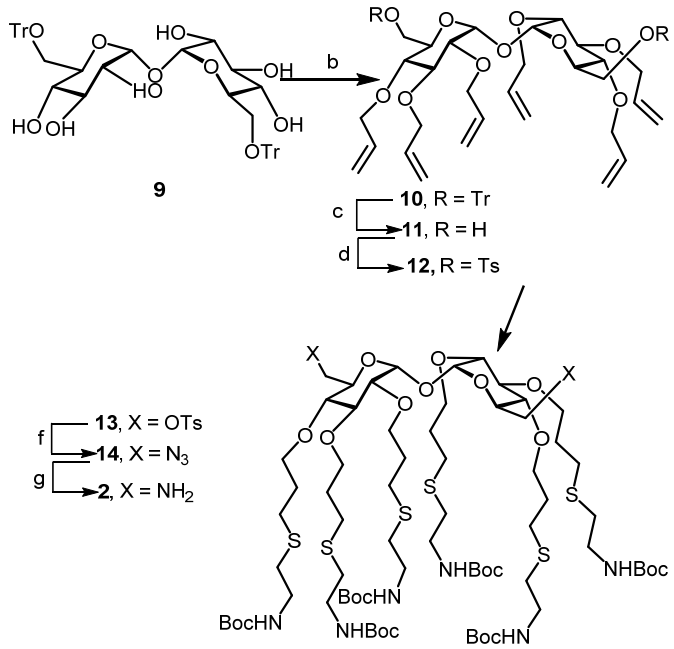
Synthesis of new compounds.



Scheme 1. Synthesis of diisothiocyanate **1**. Reagents and conditions: (a) CS_2, TPP, DCM, rt, 24 h, 96%.

6,6'-Dideoxy-2,2',3,3',4,4'-hexa-*O*-hexanoyl-6,6'-diisothiocyanato-α,α'-trehalose (1). To a solution of **8** (0.625 g, 0.617 mmol) in dry dioxane (15 mL) TPP (356 mg, 1.357 mmol) and CS_2 (0.92 mL, 12.34 mmol) were added under Ar atmosphere. The solution was stirred at rt for 24 h. Then the solvents were eliminated under vacuum and the residue was purified by column chromatography (1:15→1:10 EtOAc-cyclohexane). Yield: 0.597 g (96%); R_f = 0.48 (1:8 EtOAc-cyclohexane); $[\alpha]_D$ = +68.8 (*c* 1.0, DCM); IR (ATR): ν_{max} =, 2071, 1751 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$,): δ = 5.49 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.7 Hz, H-3), 5.37 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 5.12 (dd, 2 H, H-2), 4.99 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.05 (ddd, 2 H, $J_{5,6a}$ = 6.5 Hz, $J_{5,6b}$ = 3.0 Hz, H-5), 3.61 (dd, 2

H, $J_{6a,6b}$ = 14.7 Hz, H-6a), 3.46 (dd, 2 H, H-6b), 2.33-2.14 (m, 12 H, H-2_{Hex}), 1.68-1.49 (m, 12 H, H-3_{Hex}),1.31-1.24 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.88 (m, 18 H, H-6_{Hex}).^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 172.4, 172.3, 172.1 (CO), 136.3 (NCS), 92.7 (C-1), 69.5, 69.3 (C-3, C-2, C-4), 68.8 (C-5), 46.1 (C-6), 33.9 (C-2_{Hex}), 31.2 (C-4_{Hex}), 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}); ESIMS: *m/z* = 1035.8 [M + Na]$^+$. Anal. Calcd for $C_{50}H_{78}N_2O_{15}S_2$: calcd. C, 59.27; H, 7.96; N, 2.76; S, 6.33; found: C, 59.41; H, 8.11; N, 2.57; S, 6.12.



Scheme 2. Synthesis of diamine derivative **2**. Reagents and conditions: (b) Allyl bromide, NaH, DMF, overnight, 76%; (c) PTSA, 1:1 DCM-MeOH, rt, 3 h, 83% (d) TsCl, DMAP, DCM, rt, 24 h, 70%; (e) $HS(CH_2)_2NHBoc$, MeOH, h*ν*, 89%; (f) NaN_3, DMF, 56 ºC, Ar, 80%; (g) TPP, THF, rt, Ar, 30 min, addition of NH_4OH, overnight, 98%.

2,3,4,2',3',4'-Hexa-*O*-allyl-6,6'-di-*O*-trityl-α,α'-trehalose (10). To a solution of 6,6'-di-*O*-trityl-α,α'-trehalose (**9**, 1 g, 1.2 mmol) in dry DMF (18 mL), NaH (1.74 g, 43.3 mmol) was added at 0 ºC and the mixture was stirred for 15 min. Then, allyl bromide (3.68 mL, 43.3 mmol) was added dropwise, and the reaction mixture was stirred overnight under Ar atmosphere at rt. MeOH (5 mL) was added, the solvents were evaporated and the residue diluted in DCM (15 mL) and washed with water (2 × 15 mL). The organic layer was dried ($MgSO_4$), filtered, and the residue was purified by column chromatography (1:30 → 1:20 EtOAc-cyclohexane). Yield: 965 mg (76%). R_f = 0.33 (1:6 EtOAc-cyclohexane); $[\alpha]_D$ = +92.6 (*c* 1.0, DCM); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.41-7.12 (m, 30 H, aromatics), 5.84, 5.54 (m, 6 H, =CH=), 5.22 (d, 2 H, $J_{1,2}$ = 3.8 Hz, H-1), 5.18-4.83 (m, 12 H, $=CH_2$), 4.30-3.74 (m, 14 H, OCH_2, H-5), 3.60 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.48 (t, 2 H, $J_{4,5}$ = 9.2 Hz, H-4), 3.39 (dd, 2 H, H-2), 3.34 (dd, 2 H, $J_{6a,6b}$ = 10.2 Hz, $J_{5,6a}$ = 1.8 Hz, H-6a), 3.03 (dd, 2

H, $J_{5,6b}$ = 3.7 Hz, H-6b); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.0, 128.8, 127.8, 126.9 (Ph), 135.4, 134.9, 134.8 (=CH), 116.6 (=CH_2), 94.1 (C-1), 86.3 (Ph_3C), 81.5 (C-3), 79.6 (C-2), 77.8 (C-4), 74.6 (OCH_2), 71.8 (C-5), 70.4 (OCH_2), 62.1 (C-6); ESIMS: *m/z* = 1089.6 $[M + Na]^+$. Anal. Calcd for $C_{68}H_{74}O_{11}$: calcd. C, 76.52; H, 6.99; found: C, 76.33; H, 7.12.

2,3,4,2',3',4'-Hexa-*O*-allyl-α,α'-trehalose (11). To a solution of **10** (860 mg, 0.8 mmol) in 1:1 MeOH-DCM (20 mL), PTSA·H_2O (153 mg, 0.64 mmol) was added and the mixture was stirred at rt for 3 h. A satd. solution of $NaHCO_3$ (20 mL) was added and the organic layer was dried ($MgSO_4$), filtered, concentrated and the residue was purified by column chromatography (1:6 → 1:3 → 2:1 EtOAc-cyclohexane). Yield: 400 mg (85%). R_f = 0.35 (2:1 EtOAc-cyclohexane); $[\alpha]_D$ = +146.2 (*c* 1.0, DCM); ^{1}H NMR (300 MHz, CD_3OD): δ = 6.05, 5.83 (m, 6 H, =CH=), 5.35-5.13 (m, 12 H, =CH_2), 5.19 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.42 - 3.87 (m, 14 H, OCH_2, H-5), 3.75 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.4 Hz, H-3), 3.67 (m, 4 H, H-6a, H-6b), 3.35 (m, 4 H, H-2, H-4); ^{13}C NMR (75.5 MHz, CD_3OD): δ = 136.9, 136.5, 136.2 (=CH), 117.0, 116.6, 116.4 (=CH_2), 94.3 (C-1), 82.2 (C-3), 80.7 (C-2), 78.4 (C-4), 75.1, 74.6, 72.8 (OCH_2), 72.9 (C-5), 61.9 (C-6); ESIMS: *m/z* = 605.3 $[M + Na]^+$. Anal. Calcd for $C_{30}H_{46}O_{11}$: C, 61.84; H, 7.96; found: C, 61.76; H, 7.89.

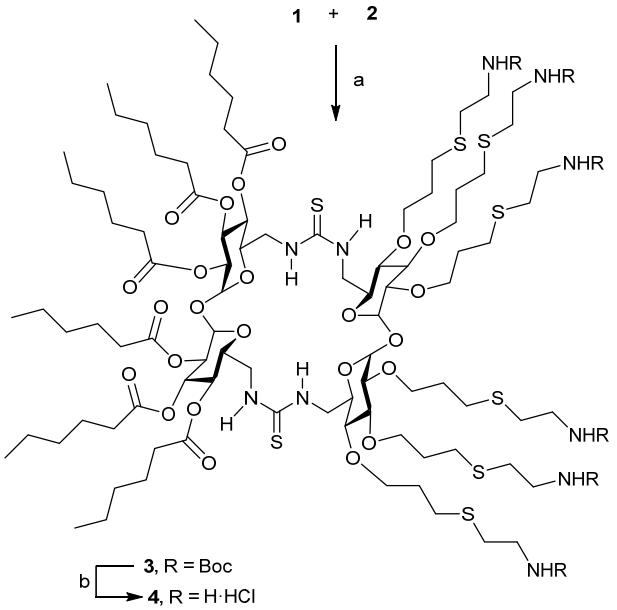
2,3,4,2',3',4'-Hexa-*O*-allyl-6,6'-di-*O*-*p*-toluensulfonyl-α,α'-trehalose (12). To a solution of **11** (454 mg, 0.78 mmol) and DMAP (287 mg, 2.34 mmol) in dry DCM (8 mL), *p*-toluensulfonyl chloride (446 mg, 2.34 mmol) was added dropwise and the mixture was stirred at rt for 24 h. The reaction mixture was diluted in DCM (10 mL), satd. solution of $NaHCO_3$ (20 mL) was added and the organic layer was dried ($MgSO_4$), filtered, concentrated and the residue was purified by column chromatography (1:2 EtOAc-cyclohexane). Yield: 485 mg (85%). R_f = 0.62 (1:2 EtOAc-cyclohexane); $[\alpha]_D$ = +106.8 (*c* 1.0, DCM); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.77, 7.33 (2 d, 8 H, $^3J_{H,H}$ = 8.5 Hz, A_2X_2, aromatics), 5.99-5.70 (m, 6 H, =CH), 5.29-5.08 (m, 12 H, =CH_2), 4.88 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 4.34-3.92 (m, 12 H, OCH_2), 4.21 (m, 2 H, H-6a), 4.09 (dd, 2 H, $J_{6a,6b}$ = 10.5 Hz, $J_{5,6b}$ = 2.0 Hz, H-6b), 4.04 (m, 2 H, H-5), 3.62 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.23 (t, 2 H, $J_{4,5}$ = 9.3 Hz, H-4), 3.22 (dd, 2 H, H-2), 2.44 (s, 6 H, CH_3Ph); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 144.8 (Ph), 135.1, 134.5 (=CH), 133.0, 129.7, 127.9 (Ph), 117.2, 116.9, 116.4 (=CH_2), 93.8 (C-1), 80.9 (C-3), 78.8 (C-2), 76.8 (C-4), 74.2, 73.9, 71.9 (OCH_2), 68.8 (C-5), 68.5 (C-6), 21.6 (CH_3); ESIMS: *m/z* = 913.6 $[M + Na]^+$. Anal. Calcd for $C_{44}H_{58}O_{15}S_2$: C, 59,31; H, 6,56; S, 7.20; found: C, 59.45; H, 6.63; S, 6.97.

2,3,4,2',3',4'-Hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonylaminoethylthio)propyl)-6,6'-di-*O*-*p*-toluensulfonyl-α,α'-trehalose (13). To a solution of **12** (457 mg, 0.51 mmol) in degassed MeOH (2 mL), 2-(terc-butoxycarbonylamino)ethanethiol (2.7 mL, 15.38 mmol) was added and the mixture was irradiated at 254 nm and stirred at rt for 1 h. Solvent was evaporated and the residue purified by column chromatography (1:2 → 1:1 EtOAc-cyclohexane). Yield: 890 mg (89%). R_f = 0.48 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +47.6 (*c* 1.0, DCM); IR (ATR): ν_{max} = 1707 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 7.77, 7.33 (2 d, 8 H, $^3J_{H,H}$ = 7.9 Hz, A_2X_2, aromatics), 5.07 (bs, 6 H, NH), 4.86 (d, 2 H, $J_{1,2}$ = 3.2 Hz, H-1), 4.14 (bs, 4 H, H-6a, H-6b), 3.87 (m, 2 H, H-5), 3.83-3.67 (m, 6 H, CH_2O), 3.60-3.43 (m, 6 H, CH_2O), 3.43 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.27 (m, 12 H, CH_2N), 3.11 (dd, 2 H, H-2), 3.06 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4), 2.64-2.48 (m, 24 H, S*C*$H_2$$CH_2$NHBoc, CH_2S), 2.44 (s, 6 H, CH_3Ph), 1.87-1.68 (m, 12 H, C$H_2$$CH_2O$), 1.42 (bs, 54 H, CMe_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.8 (CO carbamate),144.9, 132.9, 129.8, 127.9 (Ph), 92.5 (C-1), 80.9 (C-3), 79.8 (C-2), 79.3 (*C*Me_3), 77.2 (C-4), 71.6, 71.3, 69.7 (OCH_2), 69.0 (C-5), 68.7 (C-6), 39.8 (CH_2NHBoc), 32.1 (S*C*$H_2$$CH_2$NHBoc), 30.5, 30.1, 30.0 (*C*$H_2$$CH_2O$), 28.4 (C*Me*$_3$), 28.0 ($CH_2S$), 21.6 ($CH_3Ph$); ESIMS: *m/z* = 1977.1 $[M + Na]^+$, 999.5 $[M + 2Na]^{2+}$. Anal. Calcd for $C_{86}H_{148}N_6O_{27}S_8$: C, 52.85; H, 7.63; N, 4.30; S, 13.12; found: C, 52.93; H, 7.57; N, 4.11; S, 12.81.

6,6'-Diazido-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonylamino-ethylthio)propyl)-α,α'-trehalose (14). To a solution of **13** (0.74 g, 0.379 mmol) in dry DMF (5 mL), NaN_3 (70 mg, 1.061 mmol) was added. The reaction mixture was stirred overnight at 56 ºC under Ar atmosphere. The mixture was poured into ice-water (10 mL), and the product was extracted with DCM (4 x 10 mL). The organic phase was dried ($MgSO_4$), filtered, concentrated and the residue was purified by column chromatography (1:1 EtOAc-cyclohexane). Yield: 520 mg (80%). R_f = 0.67 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +59.2 (*c* 1.0, DCM); IR (ATR): ν_{max} = 2102 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ = 5.11 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 5.04 (bs, 6 H, NH), 3.92 (m, 2 H, H-5), 3.88-3.58 (m, 12 H, CH_2O), 3.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.1 Hz, H-3), 3.44 (dd, 2 H, $J_{6a,6b}$ = 13.2 Hz, $J_{5,6a}$ = 2.6 Hz, H6a), 3.37 (dd, 2 H, $J_{5,6b}$ = 5.2 Hz, H-6b), 3.28 (m, 12 H, CH_2N), 3.25 (dd, 2 H, H-2), 3.14 (t, 2 H, $J_{4,5}$ = 9.1 Hz, H-4), 2.66-2.54 (m, 24 H, S*C*$H_2$$CH_2$NHBoc, CH_2S), 1.90-1.77 (m, 12 H, C$H_2$$CH_2O$), 1.43 (bs, 54 H, CMe_3); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO carbamate), 92.9 (C-1), 81.0 (C-3), 80.2 (C-2), 79.3 (*C*Me_3), 78.8 (C-4), 71.6, 71.2 (OCH_2), 70.7 (C-5), 69.9 (OCH_2), 51.4 (C-6), 39.8 (CH_2NHBoc), 32.2, 32.1 (S*C*$H_2$$CH_2$NHBoc), 30.6, 30.1, 30.0 (C*H*$_2$$CH_2O$), 28.4 (C*Me*$_3$), 28.2 ($CH_2S$); ESIMS: *m/z* = 1781.1 $[M + Na]^+$. Anal. Calcd for $C_{72}H_{134}N_{12}O_{21}S_6$: C, 50.98; H, 7.96; N, 9.91; S, 11.34; found: C, 51.23; H, 7.68; N, 9.75; S, 10.97.

6,6'-Diamino-6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonylamino-ethylthio)propyl)-α,α'-trehalose (15). To a solution of **14** (180 mg, 0.106 mmol) in THF (15 mL), TPP (126 mg, 0.48 mmol) was added and the mixture was stirred at rt for 30 min. Then NH_4OH (1.5 mL) was added and the solution was stirred overnight. The mixture was concentrated and the resulting residue purified by column chromatography (EtOAc →1:9 DCM-MeOH). Yield: 171 mg (98%). R_f = 0.15 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +40.4 (*c* 1.0, 9:1 DCM-MeOH); ^{1}H NMR (300 MHz, 9:1 $CDCl_3$-CD_3OD): δ = 5.45, 5.35 (bs, 6 H, NH), 4.97 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 3.79-3.44 (m, 12 H, CH_2O), 3.66 (m, 2 H, H-5), 3.47 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.6 Hz, H-3), 3.15 (m, 12 H, CH_2N), 3.12 (dd, 2 H, H-2), 2.96 (t, 2 H, $J_{4,5}$ = 9.6 Hz, H-4), 2.82 (dd, 2 H, $J_{6a,6b}$ = 13.7 Hz, $J_{5,6a}$ = 2.6 Hz, H6a), 2.64 (dd, 2 H, $J_{5,6b}$ = 5.9 Hz, H-6b), 2.54-2.46 (m, 24 H, S*C*$H_2$$CH_2$NHBoc, CH_2S), 1.80-1.67 (m, 12 H, *C*$H_2$$CH_2O$), 1.32 (bs, 54 H, CMe_3); ^{13}C NMR (75.5 MHz, 9:1 $CDCl_3$-CD_3OD): δ = 156.0 (CO carbamate), 92.0 (C-1), 81.0 (C-3), 80.1 (C-2), 79.3 (*C*Me_3), 79.0 (C-4), 71.4 (OCH_2), 70.9 (C-5), 69.5 (OCH_2), 42.0 (C-6), 39.5 (CH_2NHBoc), 31.8 (S*C*$H_2$$CH_2$NHBoc), 30.4, 29.9, 29.8 (*C*$H_2$$CH_2O$), 28.3 ($CH_2$S), 28.1 (C*Me*$_3$), 27.9 ($CH_2$S); ESIMS: *m/z* = 1666.2 $[M + Na]^+$, 1644.2 $[M]^+$. Anal. Calcd for $C_{72}H_{238}N_8O_{21}S_6$: C, 52.59; H, 8.46; N, 6.81; S, 11.70; found: C, 52.24; H, 8.30; N, 6.47; S, 11.23.

6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonylaminoethylthio)-propyl)-6,6'-diisothiocyanato-α,α'-trehalose (EMARev11). To a solution of **EMARev3** (250 mg, 0.147 mmol) in dry dioxane (6 mL) TPP (85 mg, 0.324 mmol) and CS_2 (0.217 mL, 2.947 mmol) were added under Ar atmosphere. The solution was stirred at rt for 24 h. Then the solvents were evaporated and the residue was purified by column chromatography using 1:2→1:1 EtOAc-cyclohexane as eluent. Yield: 202 mg (80%). R_f = 0.69 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +41.0 (*c* 1.0, DCM); IR (ATR): ν_{max} = 2090 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.14 (d, 2 H, $J_{1,2}$ = 3.5 Hz, H-1), 5.06 (bs, 6 H, NH), 3.94-3.57 (m, 18 H, CH_2O, H-5, H-6a, H-6b), 3.54 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.2 Hz, H-3), 3.27 (m, 14 H, CH_2N, H-2), 3.08 (t, 2 H, $J_{4,5}$ = 9.2 Hz, H-4), 2.65-2.54 (m, 24 H, S*C*$H_2$$CH_2$NHBoc, CH_2S), 1.90-1.76 (m, 12 H, *C*$H_2$$CH_2O$), 1.42 (bs, 54 H, CMe_3); ^{13}CNMR (75.5 MHz, $CDCl_3$): δ = 155.7 (CO carbamate), 133.6 (NCS), 93.0 (C-1), 81.1 (C-3), 80.0 (C-2), 79.3 (*C*Me_3), 79.0 (C-4), 71.7, 71.3 (OCH_2), 70.1 (C-5), 69.5 (OCH_2), 46.4 (C-6), 39.8 (CH_2NHBoc), 32.1, 32.0 (S*C*$H_2$$CH_2$NHBoc), 30.5, 30.2, 30.0 (*C*$H_2$$CH_2O$), 28.4 (C*Me*$_3$), 28.1 ($CH_2$S); ESIMS: *m/z* = 1749.9 $[M + Na]^+$, 886.3 $[M + 2Na]^{2+}$. Anal. Calcd for $C_{74}H_{134}N_8O_{21}S_8$: C, 51.42; H, 7.81; N, 6.48; S, 14.84; found: C, 51.51; H, 7.90; N, 6.17; S, 14.55.

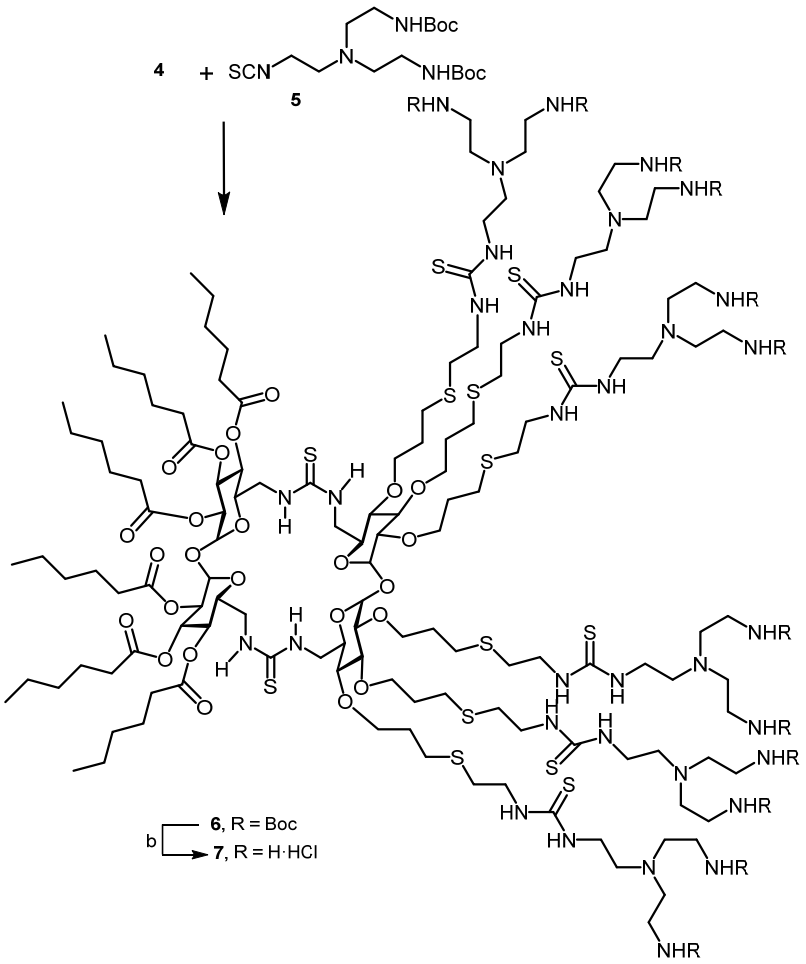


Scheme 3. Synthesis of paCT **4**. Reagents and conditions: (a) Pyridine, 40 ºC, overnight, 81%; (b) 1:1 TFA-DCM, rt, 30 min, 100%.

[N^{I},N^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-*N*-*tert*-butoxycarbonylaminoethylthio)-propyl)-α,α'-trehalos-6,6'-diyl]-N^{II},N^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]] thiourea (3). To a solution of **2** (157 mg, 0.093 mmol) in pyridine (15 mL), a solution of **1** (93 mg, 0.093 mmol) in pyridine (10 mL) was slowly added and the mixture was stirred at 40 ºC overnight. The mixture was evaporated under vacuum and the resulting residue was purified by column chromatography (1:1 EtOAc-cyclohexane). Yield: 200 mg (81%). R_f = 0.50 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +101.9 (*c* 1.0, DCM); ^{1}H NMR (500 MHz, CD_3OD, 333 K): δ = 5.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.9 Hz, H-3'), 5.40 (bs, 2 H, H-1'), 5.19 (d, 2 H, $J_{1,2}$ = 3.6 Hz, H-1), 5.08 (dd, 2 H, $J_{1,2}$ = 3.9 Hz, H-2'), 5.07 (t, 2 H, $J_{4,5}$ = 9.9 Hz, H-4'), 3.99-3.71 (m, 20 H, CH_2O, H-6ab, H-6'ab), 3.99-3.92 (m, 4 H, H-5, H-5'), 3.68 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 3.48 (dd, 2 H, H-2), 3.27 (m, 12 H, CH_2N), 3.20 (t, 2 H, $J_{4,5}$ = 9.6 Hz, H-4), 2.72-2.65 (m, 24 H, SC$H_2$$CH_2$NHBoc, CH_2S), 2.47-2.28 (m, 12 H, H-2_{Hex}), 1.97-1.88 (m, 12 H, C$H_2$$CH_2O$), 1.70-1.58 (m, 12 H, H-3_{Hex}), 1.47 (bs, 54 H, CMe_3), 1.39-1.31 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.97-0.92 (m, 18 H, H-6_{Hex}); ^{13}C NMR (100.6 MHz, CD_3OD, 323 K): δ = 184.8 (CS), 172.7 (CO ester), 156.8 (CO carbamate), 92.0 (C-1), 90.6 (C-1'), 81.1 (C-3), 79.7 (C-2), 78.9 (C-4, *C*Me_3), 71.4 (C-5), 71.4, 71.0, 70.1 (OCH_2), 70.1 (C-3'), 70.0 (C-2'), 69.3 (C-5'), 68.6 (C-4'), 40.1 (CH_2NHBoc, C-6), 33.7 (C-2_{Hex}), 31.5 (S*C*$H_2$$CH_2$NHBoc), 31.1, 31.0 (C-$4_{Hex}$), 30.5, 30.2, 30.1 (C$H_2$$CH_2O$), 28.3, 28.0 ($CH_2S$), 27.5 (C*Me*$_3$), 24.2, 24.1 (C-$3_{Hex}$), 22.0, 21.9

(C-5_{Hex}), 12.9, 12.8 (C-6_{Hex}); ESIMS: *m/z* = 2680 $[M + Na]^+$, 1351.2 $[M + 2Na]^{2+}$. Anal. Calcd for $C_{122}H_{218}N_{10}O_{36}S_8$: C, 55.14; H, 8.27; N, 5.27; S, 9.65; found: C, 55.33; H, 8.30; N, 5.13; 9.28.

[*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-aminoethylthio)-propyl)-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]] thiourea (4). Compound **4** (125 mg, 0.047 mmol) was obtained by treatment of **3** with 1:1 DCM-TFA (2 mL) at rt for 30 min. Then the solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in 10:1 water-HCl 0.1 N and freeze-dried to yield the product as hydrochloride. Yield: 106 mg (100%). $[\alpha]_D$ = +106.5 (*c* 1.0, DCM; ^{1}H NMR (500 MHz, 6:1 CD_3OD-$CDCl_3$, 333 K): δ = 5.52 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3'), 5.39 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1'), 5.17 (d, 2 H, $J_{1,2}$ = 3.4 Hz, H-1), 5.05 (dd, 2 H, H-2'), 5.04 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4'), 3.96-3.67 (m, 20 H, CH_2O, H-6ab, H-6'ab), 3.96-3.90 (m, 4 H, H-5, H-5'), 3.65 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3), 3.46 (dd, 2 H, H-2), 3.17 (m, 14 H, CH_2N, H-4), 2.87 (m, 12 H, SC$H_2$$CH_2$NHBoc), 2.72 (m, 12 H, CH_2S), 2.44-2.26 (m, 12 H, H-2_{Hex}), 1.96-1.88 (m, 12 H, C$H_2$$CH_2O$), 1.68-1.56 (m, 12 H, H-3_{Hex}), 1.37-1.31 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.95-0.88 (m, 18 H, H-6_{Hex}); ^{13}C NMR (100.6 MHz, 6:1 CD_3OD-$CDCl_3$, 333 K): δ = 184.9 (CS), 172.9, 172.6 (CO ester), 91.8 (C-1), 90.9 (C-1'), 81.1 (C-3), 79.7 (C-2), 79.0 (C-4), 71.2 (OCH_2), 71.0 (C-5), 70.0 (C-3', C-2', OCH_2), 69.4 (C-5'), 68.8 (C-4'), 45.4 (C-6), 44.0 (C-6'), 38.9 (CH_2NH_2HCl), 33.8, 33.7 (C-2_{Hex}), 31.1, 31.0 (C-4_{Hex}), 30.3, 30.1, 29.9 (C$H_2$$CH_2O$), 28.6 (S$C$$H_2$$CH_2$ NH_2HCl), 28.3, 28.1 (CH_2S), 24.2, 24.1 (C-3_{Hex}), 21.9 (C-5_{Hex}), 13.0, 12.9 (C-6_{Hex}); ESIMS: *m/z* = 2056.0 $[M + H]^+$, 1059.4 $[M + H + Cu]^{2+}$. Anal. Calcd for $C_{92}H_{176}Cl_6N_{10}O_{24}S_8$: C, 48.56; H, 7.80; N, 6.16; S, 11.27; found: C, 48.20; H, 7.70; N, 5.78; S, 10.90.



Scheme 3. Synthesis of paCT **7**. Reagents and conditions: (a) Pyridine, DMAP, 40 ºC, overnight, 75%; (b) 1:1 TFA-DCM, rt, 30 min, 100%.

[N^{I},N^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(*N'*-(2-(*N*,*N*-di-(2-(*N*-*tert*-butoxycarbonylamino)ethyl)amino)ethyl)thioureido)-propyl)-α,α'-trehalos-6,6'-diyl]-N^{II},N^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]] thiourea (6). To a solution of **4** (108 mg, 0.044 mmol) and DMAP (35 mg, 0.29 mmol) in pyridine (5 mL), a solution of **5** (113 mg, 0.29 mmol) in pyridine was slowly added and the mixture was stirred at 45 ºC 24 h. The mixture was concentrated and the resulting residue purified by column chromatography (1:1 EtOAc-cyclohexane→EtOAc→45:5:3 EtOAc-EtOH-H_2O). Yield: 144 mg (75%). R_f = 0.62 (45:5:3 EtOAc-EtOH-H_2O); $[\alpha]_D$ = +62.6 (*c* 1.0, DCM); ^{1}H NMR (400 MHz, $CDCl_3$, 323 K): δ = 7.27-6.60 (m, 6 H, NHCS), 5.52 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.9 Hz, H-3'), 5.45 (bd, 2 H, H-1'), 5.07 (bs, 12 H, NHBoc), 5.07 (bd, 2 H, H-1), 5.03 (t, 2 H, $J_{4,5}$ = 9.9 Hz, H-4'), 4.91 (dd, 2 H, $J_{1,2}$ = 3.5 Hz, H-2'), 3.91-3.43 (m, 20 H, CH_2O, H-6ab, H-6'ab), 3.89 (m, 2 H, H-5), 3.79 (m, 14 H, CH_2NHCS, H-5'), 3.57 (m, 14 H, CH_2NHCS, H-3), 3.38 (bd, 2 H, H-2), 3.16 (bs, 14 H, CH_2NHBoc, H-4), 2.80-2.65 (m, 24 H, SC$H_2$$CH_2NHCS$, CH_2S), 2.60 (m, 36 H, CH_2N, NC$H_2$$CH_2NHCS$) 2.36-2.23 (m, 12 H, H-$2_{Hex}$), 1.94-

1.87 (m, 12 H, CH_2CH$_2$O), 1.63-1.55 (m, 12 H, H-3$_{Hex}$), 1.46 (bs, 108 H, CMe$_3$), 1.36-1.26 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.94-0.89 (m, 18 H, H-6$_{Hex}$); ^{13}C NMR (100.6 MHz, CDCl$_3$, 323 K): δ = 183.1 (CS), 172.8, 172,6, 172.1 (CO ester), 156.5 (CO carbamate), 92.5 (C-1), 91.2 (C-1'), 81.6 (C-3), 79.5 (C-2, *C*Me$_3$), 78.1 (C-4), 72.0 (C-5), 72.0, 70.7 (OCH$_2$), 70.1 (C-2'), 69.6, 69.3 (C-3', C-5'), 68.0 (C-4'), 55.1, 54.2 (CH$_2$N, NCH_2CH$_2$NHCS), 45.1, 43.3 (CH$_2$NHCS), 39.9 (CH$_2$NHBoc), 35.1 (C-2$_{Hex}$), 31.4 (SCH_2CH$_2$NHCS), 30.7 (C-4$_{Hex}$), 30.4 (CH_2CH$_2$O), 28.3, 28.2 (CH$_2$S, C*Me*$_3$), 24.6 (C-3$_{Hex}$), 21.9 (C-5$_{Hex}$), 13.5 (C-6$_{Hex}$); ESIMS: *m/z* = 2258.0 [M + 2Cu]$^{2+}$. Anal. Calcd for C$_{194}$H$_{362}$N$_{34}$O$_{48}$S$_{14}$: C, 53.10; H, 8.32; N, 10.85; S, 10.23; found: C, 52.87; H, 8.12; N, 10.58; 10.01.

[*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(*N*'-(2-(*N*,*N*-di-(2-amino ethyl)amino)ethyl)thioureido)propyl)-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]]thiourea (7). Compound **7** (74 mg, 0.017 mmol) was obtained by treatment of **6** with 1:1 DCM-TFA (2 mL) at rt for 30 min. Then the solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in 10:1 water-HCl 0.1 N and freeze-dried to yield the product as hydrochloride. Yield: 61 mg (100%). [α]$_D$ = +48.1 (*c* 1.0, MeOH); ^{1}H NMR (400 MHz, CD$_3$OD, 333 K): δ = 5.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3'), 5.42 (bs, 2 H, H-1'), 5.21 (bd, 2 H, H-1), 5.09 (dd, 2 H, $J_{1,2}$ = 3.3 Hz, H-2'), 5.07 (m, 2 H, H-4'), 3.99-3.94 (m, 4 H, H-5, H-5'), 3.99-3.68 (m, 48 H, CH$_2$O, H-6ab, H-6'ab, CH$_2$NHCS), 3.69 (m, 2 H, H-3), 3.50 (bd, 2 H, H-2), 3.20 (bs, 2 H, H-4), 3.15 (bt, 24 H, $^3J_{H,H}$ = 5.3 Hz, CH_2NH$_2$·HCl), 2.90 (bt, 36 H, CH$_2$N), 2.80 (bt, 12 H, $^3J_{H,H}$ = 5.9 Hz, SCH_2CH$_2$NHCS), 2.75 (m, 12 H, CH$_2$S), 2.47-2.81 (m, 12 H, H-2$_{Hex}$), 1.98-1.93 (m, 12 H, CH_2CH$_2$O), 1.71-1.57 (m, 12 H, H-3$_{Hex}$), 1.39-1.31 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.97-0.92 (m, 18 H, H-6$_{Hex}$); ^{13}C NMR (100.6 MHz, CD$_3$OD, 333 K): δ = 182.8 (CS), 172.7 (CO), 91.9 (C-1), 90.9 (C-1'), 81.0 (C-3), 79.6 (C-2, C-4), 71.5 (C-5), 71.2 (OCH$_2$), 70.2 (C-3', OCH$_2$), 69.9 (C-2'), 69.0 (C-5'), 68.8 (C-4'), 52.6, 51.4 (CH$_2$N), 43.8, 41.4 (CH$_2$NHCS), 37.6 (CH$_2$NH$_2$·HCl), 33.8 (C-2$_{Hex}$), 31.1, 30.9, 30.7, 30.4 (SCH_2CH$_2$NHCS, C-4$_{Hex}$), 30.4 (CH_2CH$_2$O), 28.3, 28.2 (CH$_2$S), 24.2 (C-3$_{Hex}$), 22.0 (C-5$_{Hex}$), 12.8 (C-6$_{Hex}$); ESIMS: *m/z* = 3616.4 [M + H]$^+$, 3638.3 [M + Na]$^+$. Anal. Calcd for C$_{134}$H$_{278}$Cl$_{12}$N$_{34}$O$_{24}$S$_{14}$: C, 44.41; H, 7.73; N, 13.14; S, 12.38; found: C, 44.03; H, 7.46; N, 12.80; S, 11.93.

NMR Spectra.

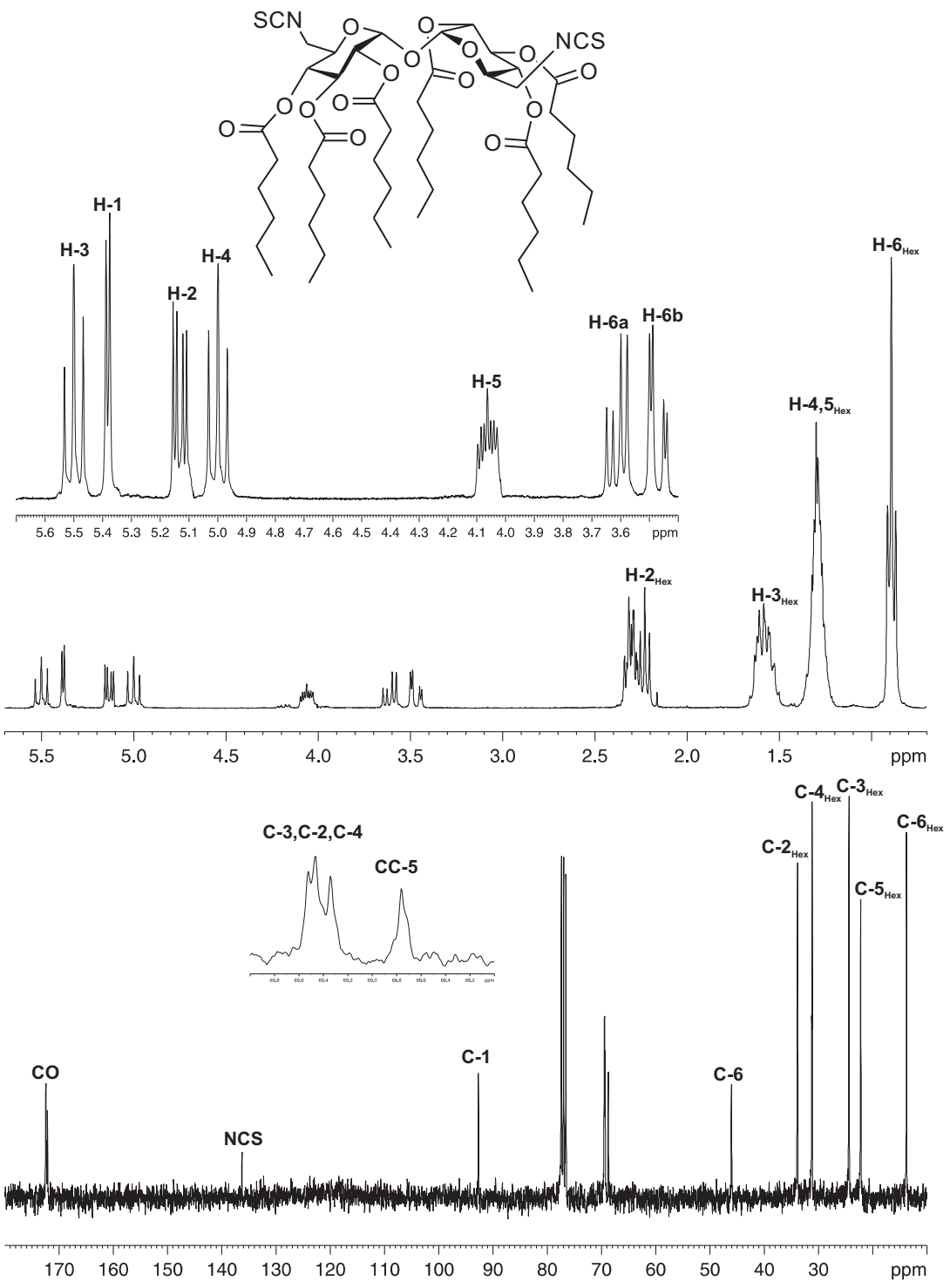


Figure S1. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **1.**

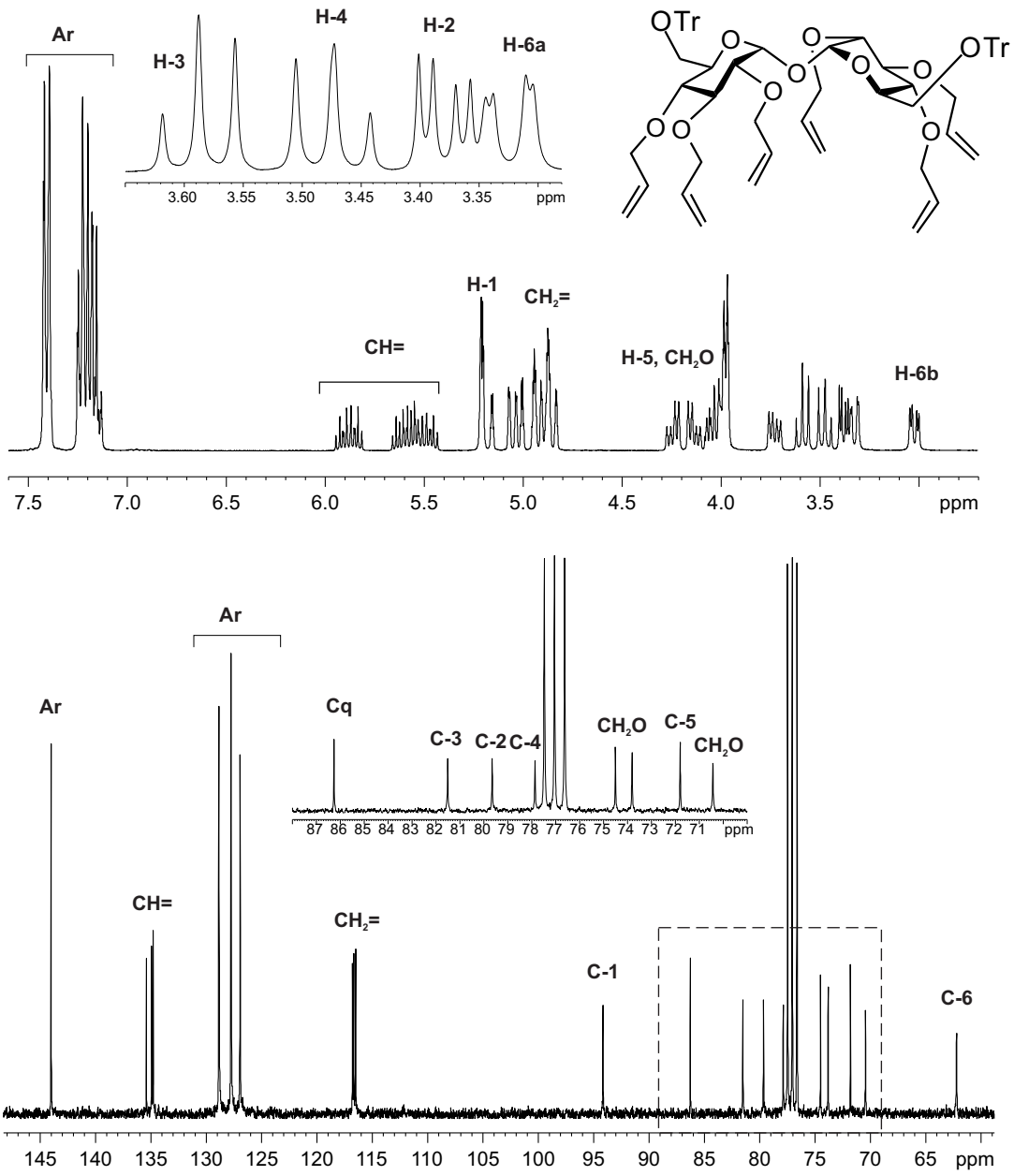


Figure S2. ^{1}H and ^{13}C NMR spectra (300 and 75.5 Mhz, $CDCl_3$) of **10**.

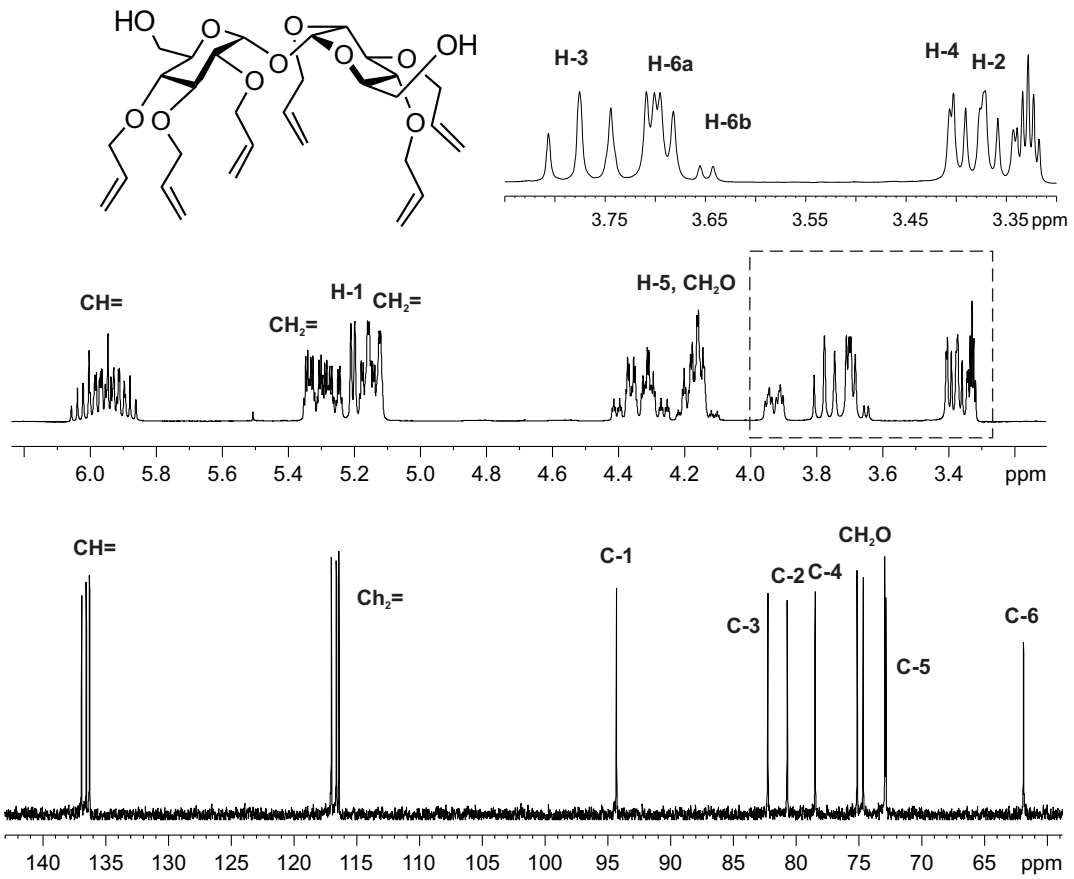


Figure S3. ^{1}H and ^{13}C NMR spectra (300 and 75.5 Mhz, $CDCl_3$) of **11**.

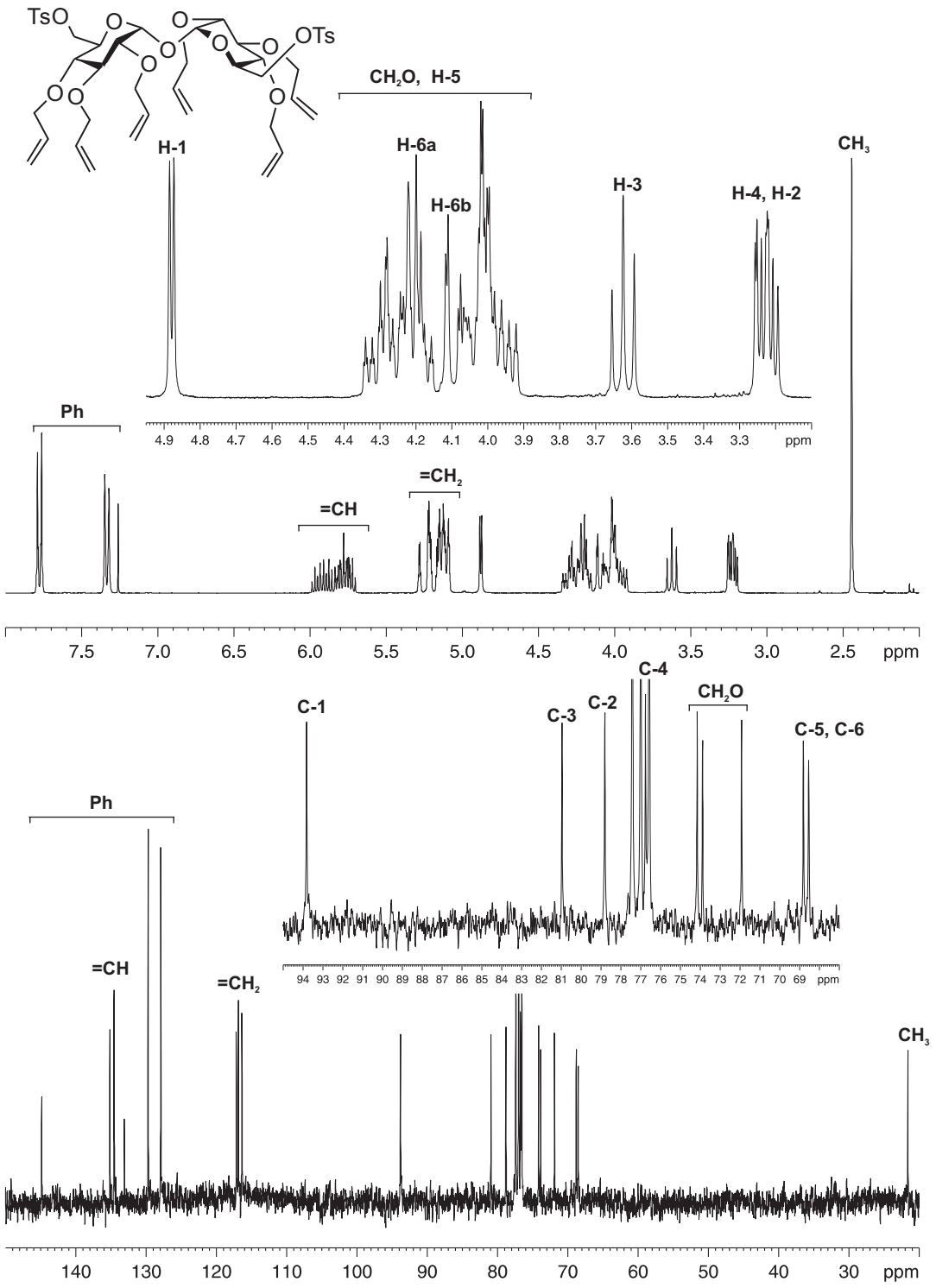


Figure S4. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **12.**

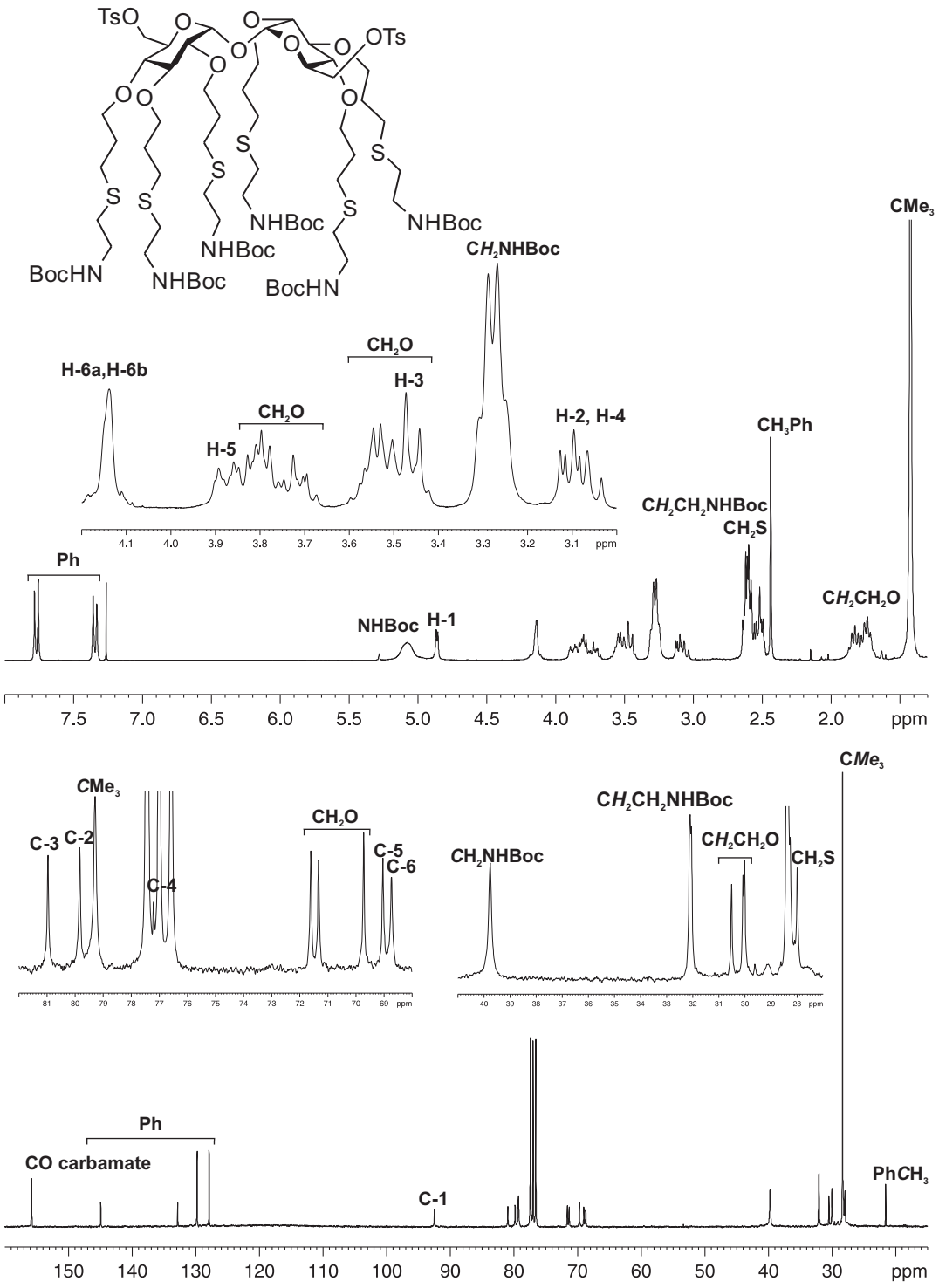


Figure S5. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **13.**

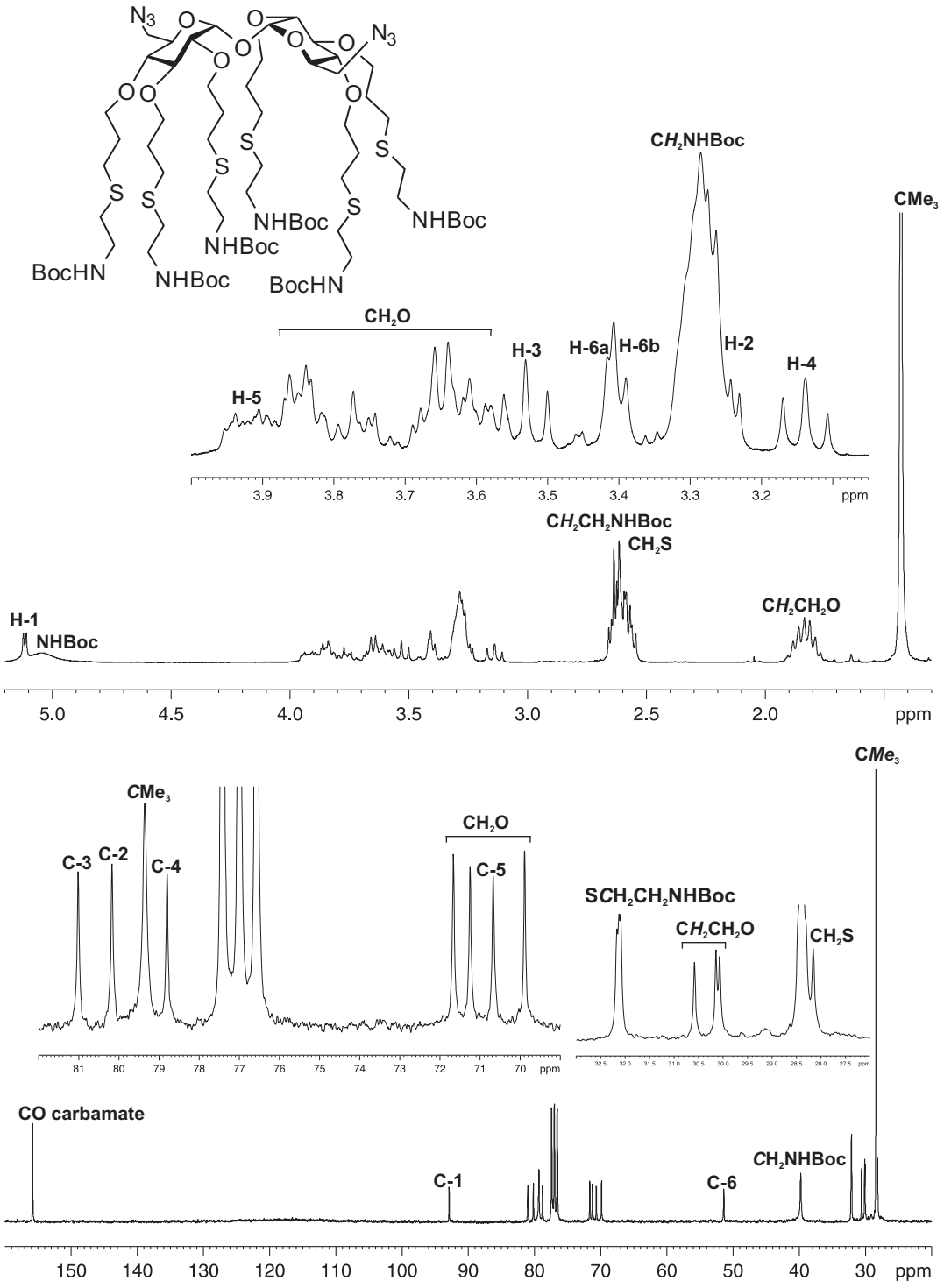


Figure S6. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **14.**

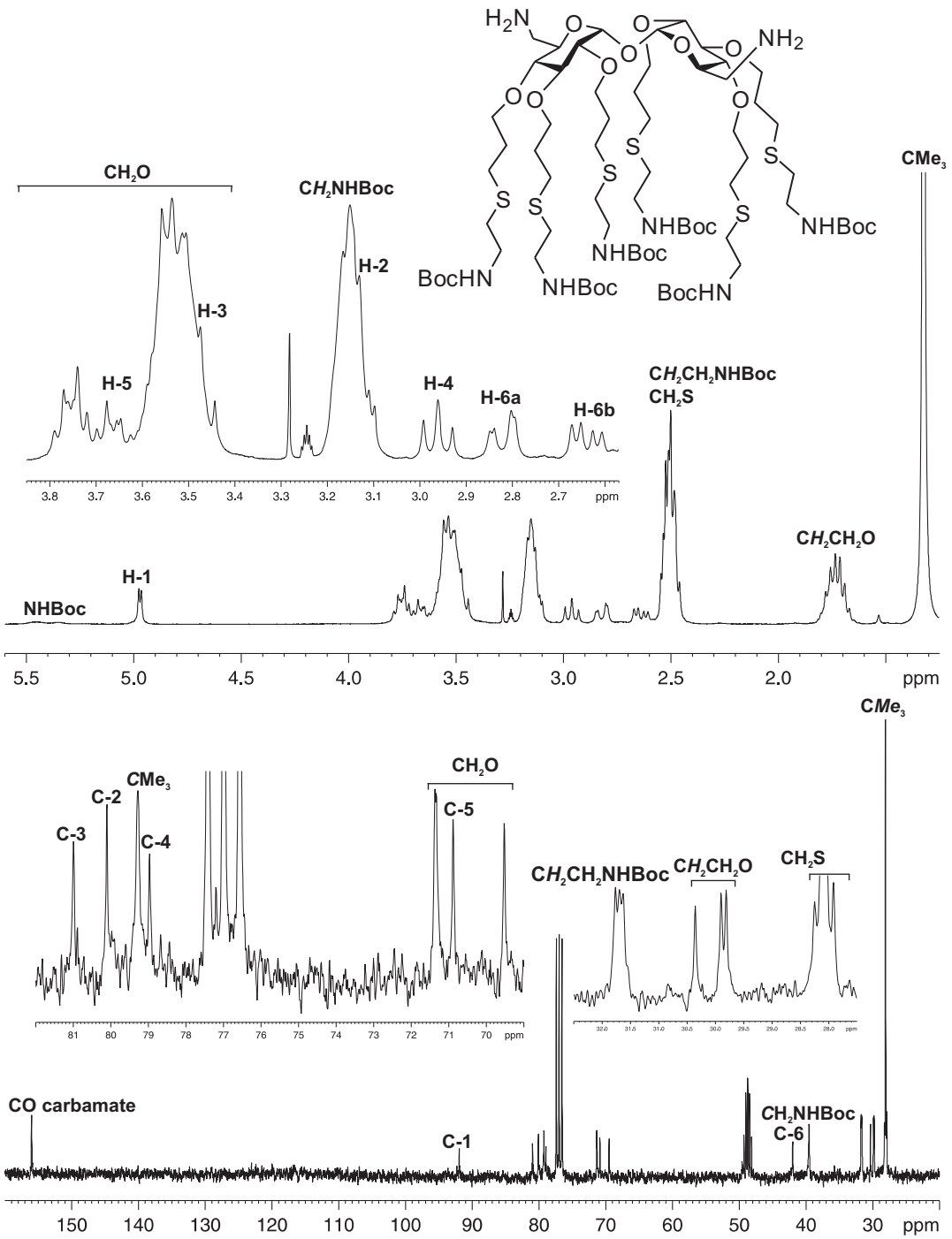


Figure S7. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, 9:1 $CDCl_3$-CD_3OD) of **2**.

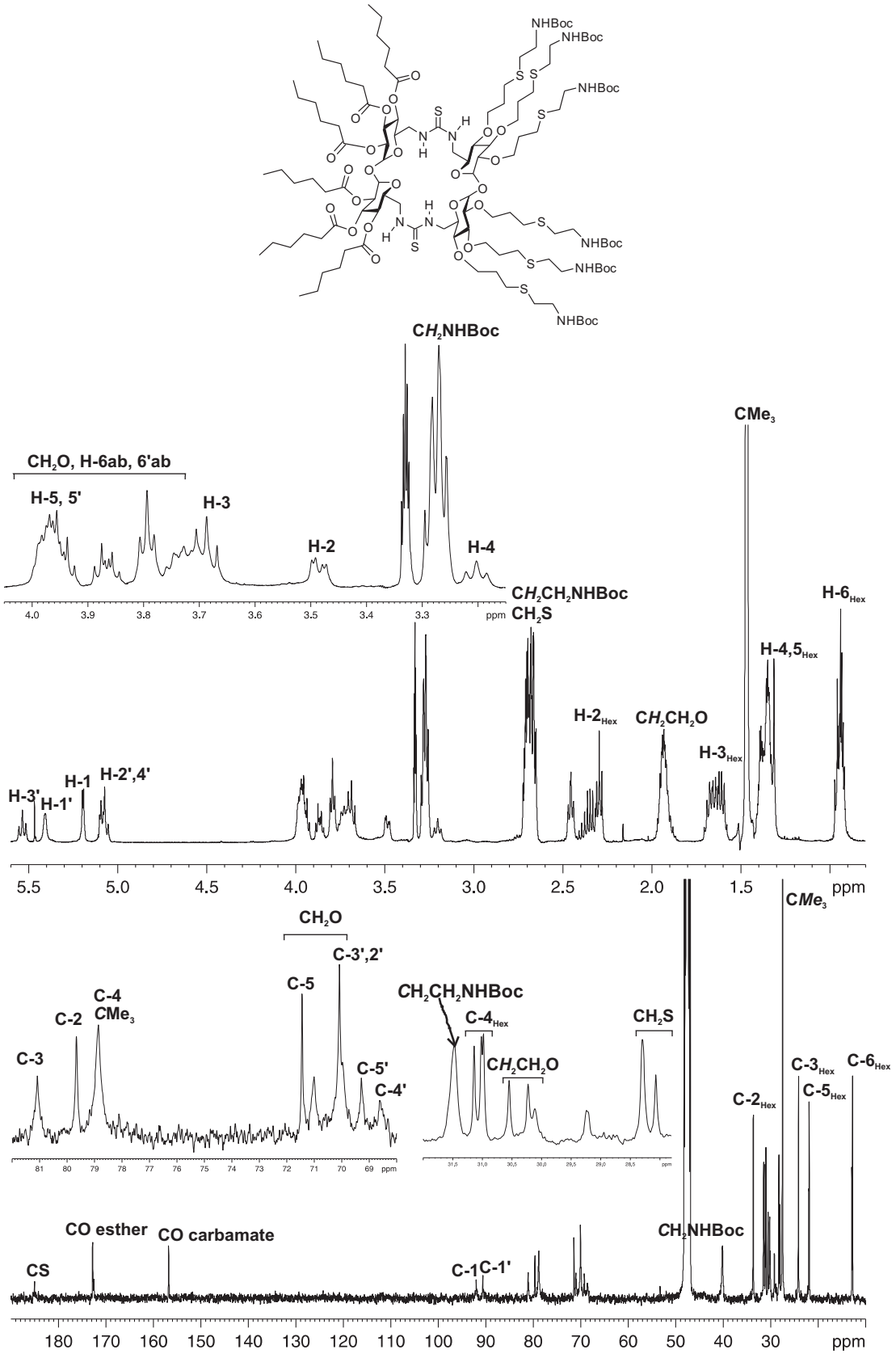


Figure S8. ^{1}H and ^{13}C NMR spectra (500 and 100.6 MHz, CD_3OD, 333 K and 323 K) of **3.**

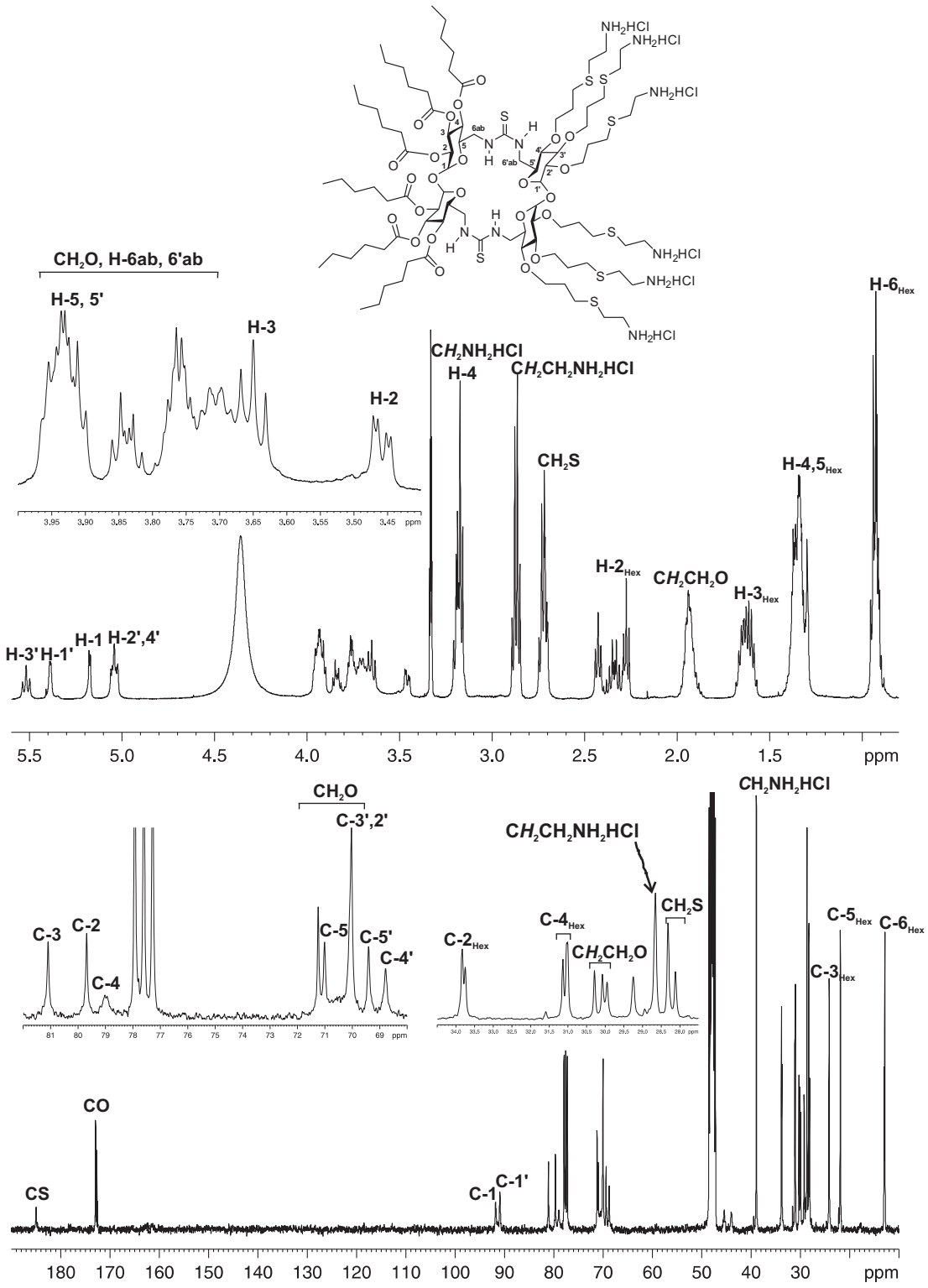


Figure S9. ^{1}H and ^{13}C NMR spectra (500 and 100.6 MHz, 6:1 CD_3OD-$CDCl_3$, 333 K) of **4.**

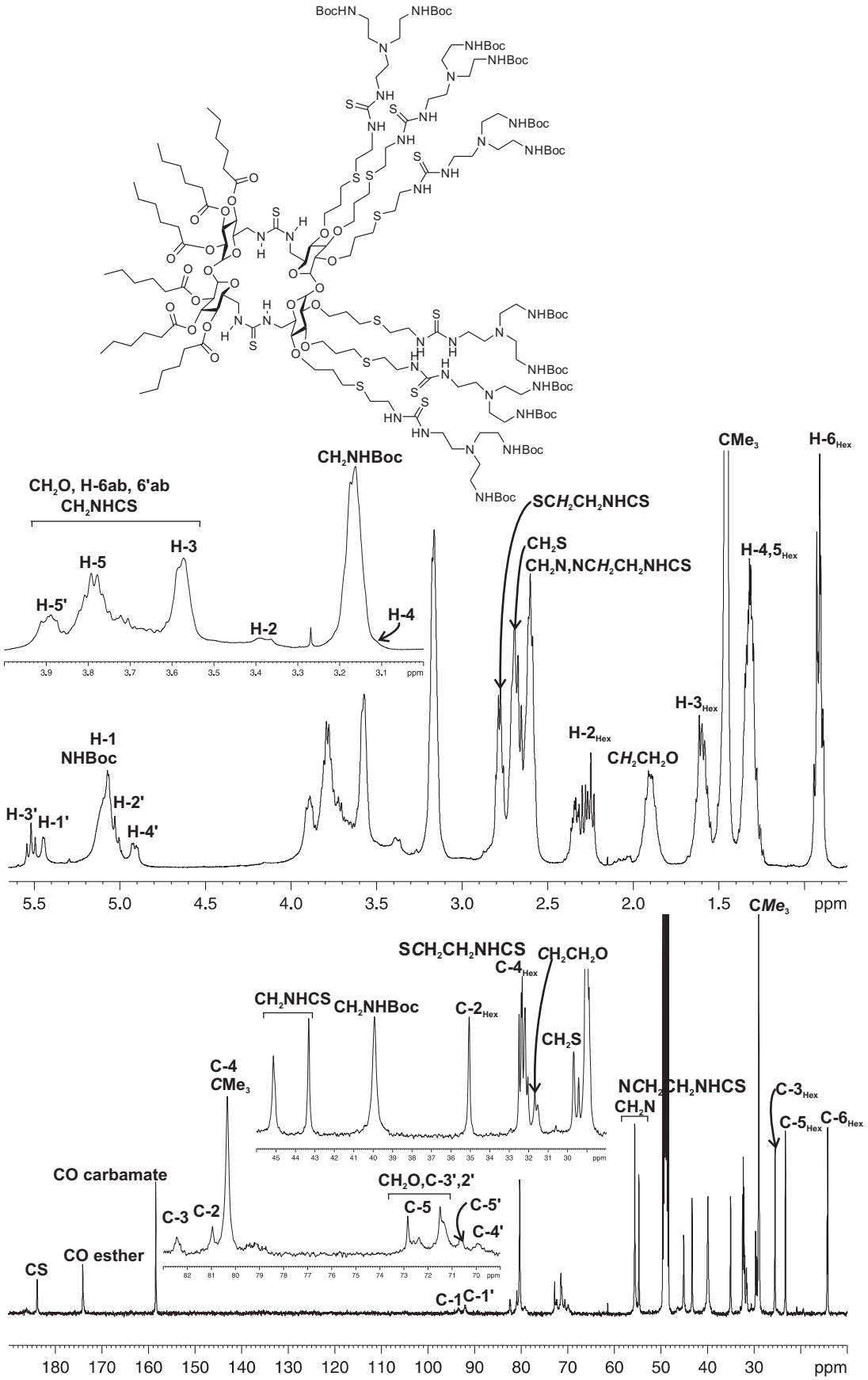


Figure S10. ^{1}H and ^{13}C NMR spectra (400 MHz, $CDCl_3$, 333 K and 100.6 MHz, CD_3OD, 323 K, respectively) of **6.**

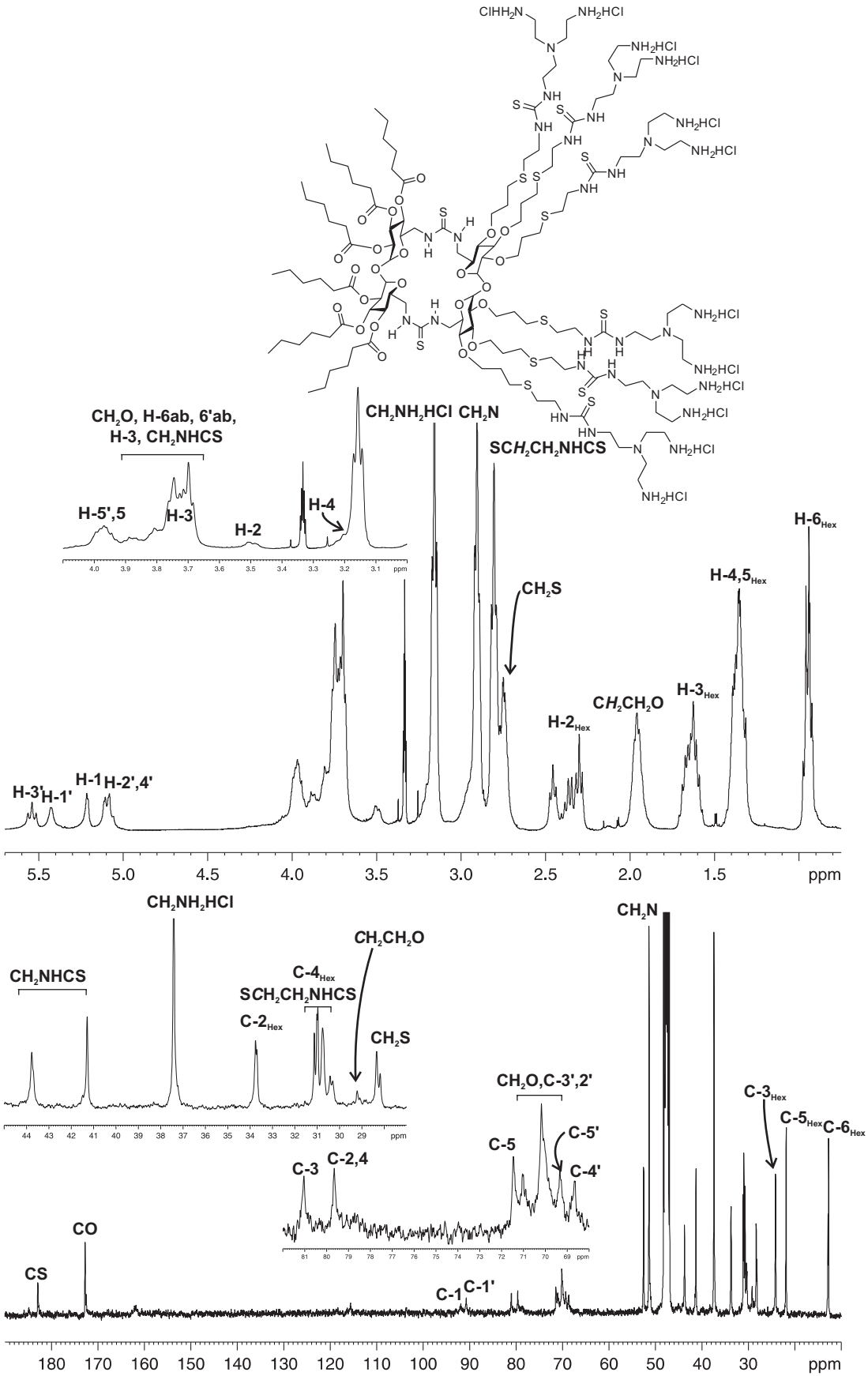


Figure S11. ^{1}H and ^{13}C NMR spectra (400 MHz and 100.6 MHz, CD_3OD, 333 K) of **7.**

Preparation of CTplexes for electrochemical and SAXS studies. For electrochemical and SAXS studies, the green fluorescent protein-encoding plasmid pEGFP-C3 was used (pEGFP-C3, 4700 base pair; kindly provided by Dr. C. Aicart-Ramos, Biochemical and Biomolecular I Department, University Complutense Madrid, Spain). The plasmid DNA (pDNA) was extracted from competent *E. Coli* bacteria previously transformed with pEGFP-C3, the extraction being carried out using GenElute HP Select plasmid Gigaprep Kit (Sigma Aldrich) following a protocol previously described.[4,5] Sodium salt of calf thymus DNA (ctDNA), provided by Sigma-Aldrich, was used as linear DNA to determine the effective charge (q^+_{CT}) of the cationic non-viral vector. CT:DNA complexes were formed by mixing the exact amounts of aqueous solutions (HEPES 20 mM, at pH = 7.4) of CTs and DNA. Solutions thus prepared were left during 20 minutes prior to run the experiments. pDNA concentrations were optimized to fit the optimum conditions for each experimental technique, as follows: 0.1 mg/mL for zeta (ζ) potential and 10 mg/mL for SAXS experiments. Composition of complexes is expressed either in terms of the mass ratio (m_{CT}/m_{DNA}), between mass of the gene vector (m_{CT}) to plasmid DNA (m_{DNA}), or the effective charge ratio ρ_{eff} between CT and pDNA effective charges.

Zeta Potential. The Phase Analysis Light Scattering technique (*Zeta PALS,* Brookhaven Instrum. Corp., USA)[6,7] was used to measure electrophoretic mobility (and from it, zeta potential). This interferometric technique uses phase analysis light scattering to determine the electrophoretic mobility of the charged colloidal suspensions. Each data point is taken as an average over 50 independent measurements. Electrophoretic mobility for CTplex solutions was measured as a function of m_{CT}/m_{DNA} ratio. In all the cases, zeta potential, ζ, has been obtained from the electrophoretic mobility, μ_e, using the well-known Henry equation:

$$\zeta = 3\eta\mu_e /(2\varepsilon_0\varepsilon_r f(\kappa_D a)) \qquad \text{(eq.1)}$$

where η is the viscosity of water; ε_0 and ε_r are the vacuum and relative permittivity, respectively; and $f(\kappa_D a)$ the Henry function, that depends on the reciprocal Debye length, κ_D, and the hydrodynamic particle radius, a. For medium-to-large particles in a medium of moderate ionic strength ($a >> \kappa_D^{-1}$), Smoluchowski limit is usually applied ($f(\kappa_D a) = 1.5$) to estimate the Henry function.[8,9]

Small-angle X-ray Scattering. SAXS experiments were carried out on the beamline NCD11 at ALBA Synchrotron Barcelona (Spain). The energy of the incident beam was 12.6 KeV (λ = 0.995 Å). The machine is run in multibunch mode with a filling pattern on 10 trains, 64 ns long and a gap of 24 ns between the trains. Samples were placed in sealed glass capillaries purchased from

Hilgenberg with an outside diameter of 1.5 mm and wall thickness of 0.01 mm. The scattered X-ray was detected on CCD detector Quantum 210r (4096 x 4096 pixels highest achievable resolution - pixel size 51 microns), converted to one-dimensional scattering by radial averaging, and represented as a function of the momentum transfer vector q (= $4\pi\sin\theta/\lambda$), in which θ is half the scattering angle and λ is the wavelength of the incident X-ray beam. The sample to detector distance was maintained at 1.4 m. Measurements on each sample were collected over 5 to 20 s each. SAXS experiments were run at different effective charge ratios (ρ_{eff}) of the CTplex.

Protocol for determining effective charges, q_{eff}, and effective charge ratios, ρ_{eff}. It is a two-step protocol, designed to obtain: i) on a first step, the effective charge of the cationic vector by characterizing the complexes formed by this vector and a double stranded linear DNA of well-established charge (-2/bp), and ii) on a second step, the effective charge of the pDNA by characterizing the complex formed by the cationic vector, already of known charge (step i), and the pDNA in the same experimental conditions.

The effective charge ratio (ρ_{eff}) between the positive charges of CT and negative DNA phosphate groups (per bp) is described by:

$$\rho_{eff} = \frac{n_+}{n_-} = \frac{q^+_{eff,CT}(m_{CT} / M_{CT})}{q^-_{eff,DNA}(m_{DNA} / \overline{M}_{bp})} \qquad \text{(eq. 2)}$$

where n^+ and n^- are the number of moles of positive and negative charges of CT and DNA; m_{CT} and m_{DNA} are the masses of the cationic CT based vector and the nucleic acid; M_{CT} and $\overline{M}_{bp}$ are the molar mass of the vector and the average molar mass of DNA per bp; and $q^+_{eff,CT}$ and $q^-_{eff,DNA}$ are the effective charges of CT and DNA per bp, respectively.

The m_{CT} / m_{DNA} ratio at which the complex reaches the electroneutrality, i.e. when n^+ balances n^- (ρ_{eff} = 1), is called the isoneutrality ratio $(m_{CT} / m_{DNA})_\phi$. It is an important parameter, characteristic of the CT:pDNA complex, since it marks the lower limit from which the CTplex becomes a net positively charged system, a crucial attribute to cross the negative cell membrane in an efficient cell transfection process. Accordingly, the effective positive charge of the vector ($q^+_{eff,CT}$) can be obtained if the isoneutrality ratio $(m_{CT} / m_{linearDNA})_\phi$ is experimentally determined for a complex consisting of a CT based vector and a commercial linear DNA ($q^-_{eff,linear\,DNA}$ = -2/bp), by following eq. 3, easily deduced from eq. 2 for ρ_{eff} = 1:

$$q^{+}_{eff,CT} = q^{-}_{eff,linear\,DNA}\left(\frac{m_{CT}}{m_{linearDNA}}\right)^{-1}_{\phi}\frac{M_{CT}}{\overline{M}_{bp}} \qquad \text{(eq. 3)}$$

With the knowledge of $q^{+}_{eff,CT}$, the negative effective charge of the plasmid ($q^{-}_{eff,pDNA}$) can be straightforwardly determined from the experimental value of $\left(m_{CD}/m_{pDNA}\right)_{\phi}$ for a complex formed by the same vector and a plasmid DNA instead of the linear DNA, on the same experimental conditions, as follows:

$$q^{-}_{eff,pDNA} = q^{+}_{eff,CD}\left(\frac{m_{CD}}{m_{pDNA}}\right)_{\phi}\frac{\overline{M}_{bp}}{M_{CD}} \qquad \text{(eq. 4)}$$

Among the different experimental techniques, the electrochemical methods, and particularly electrophoretic mobility (and, in turn, zeta potential measurements) are the most suitable ones to obtain effective charges. Figure S12 shows zeta potential of 4:pDNA vs m_{CT}/m_{DNA} mass ratio. The electroneutrality ratio $\left(m_{CD}/m_{DNA}\right)_{\phi}$ of the CTplexes can be determined as the $\left(m_{CD}/m_{DNA}\right)$ where a sign inversion on the charge occurs on the ζ-potential sigmoidal profiles. With these $\left(m_{CD}/m_{DNA}\right)_{\phi}$ values and following the procedure above explained, the effective charges of both the CT based cationic vector **4** and pDNA herein used, $q^{-}_{eff,pDNA}$ and $q^{+}_{eff,CT}$, are calculated and resumed in Table S1.

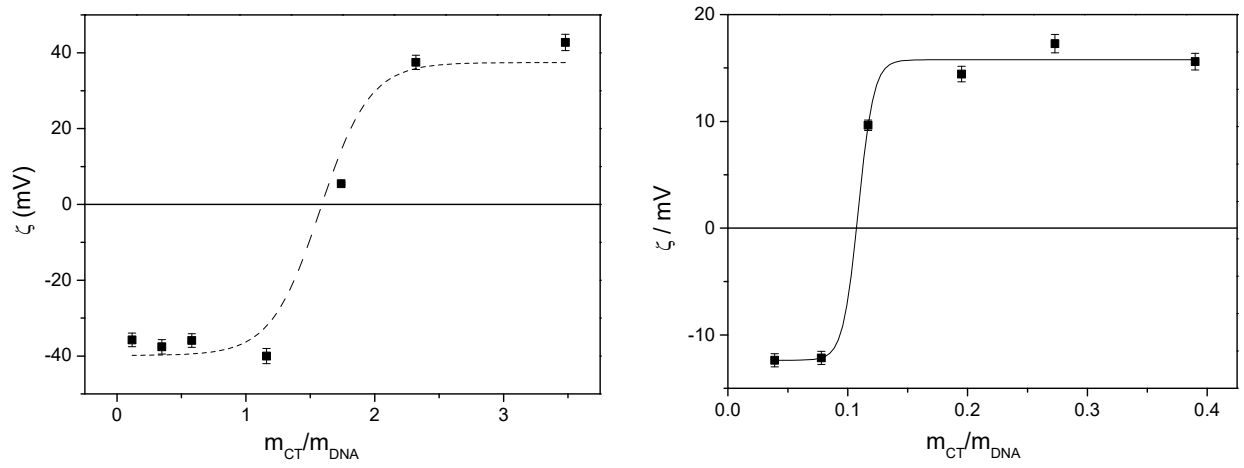


Figure S12. Plot of ζ potential *vs* **4**:DNA CTplex composition (m_{CT}/m_{DNA}) for ctDNA (dash lines) and pDNA (solid line).

Table S1. Nominal and effective charges of **4** and pDNA (values estimated with 5% error). Notice that ρ_{nom} is also known as N/P ratio.

$q^{+}_{nom,CT}$	6.0
$q^{+}_{eff,CT}$	4.5
$q^{+}_{eff,CT} / q^{+}_{nom,CT}$	0.75
$q^{-}_{nom,pDNA/bp}$	-2.0
$q^{-}_{eff,\ pDNA/bp}$	-0.14
$q^{-}_{eff,\ pDNA} / q^{-}_{nom,pDNA/}$	0.07
ρ_{eff}/ρ_{nom}	11

Table S2. Values of q (nm^{-1}), d (nm) and d_{pDNA} (nm) of the lamellar, L_α, liquid crystal phase found for **4**:pDNA CTplexes, at several effective (ρ_{eff}) and nominal (ρ_{nom} = N/P ratio) charge ratios. Values of d are calculated as an average over those results obtained with the first more intense peaks (100 and 200) of the diffractograms.

			4:pDNA
ρ_{eff}	ρ_{nom} (N/P ratio)		L_α
5	0.45	q_{100}	1.18
		q_{pDNA}	2.28
		q_{200}	2.55
		q_{300}	3.37
		d	5.1
		d_{pDNA}	2.8
10	0.91	q_{100}	1.19
		q_{pDNA}	2.26
		q_{200}	2.52
		q_{300}	3.34
		d	5.3
		d_{pDNA}	2.8
41	3.7	q_{100}	1.19
		q_{pDNA}	2.25
		q_{200}	2.45
		q_{300}	3.31
		d	5.3
		d_{pDNA}	2.8
81	7.4	q_{100}	1.21
		q_{pDNA}	2.23
		q_{200}	2.48
		q_{300}	3.30
		d	5.1
		d_{pDNA}	2.8

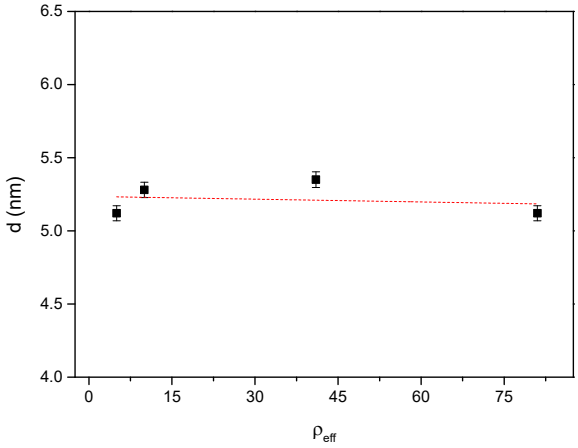


Figure S13. Plots of the periodic distance of the lamellar structure, d, for **4**:pDNA complex as a function of ρ_{eff}.

Circular dichoism measurements on CT:DNA formulations. A buffer HEPES solution 20.07 mM was prepared by weighting 4.7831 g of HEPES (Aldrich, MW 238.3 $gmol^{-1}$), adding 1 L of deionized Milli-Q water and stirring for 4 h. before its pH was fixed at 7.4 by using a NaOH 1 M solution. 1 mg/mL of a stock ct-DNA/buffer solution was prepared by dissolving 2 mg of calf thymus DNA (ctDNA, Aldrich) in the buffer solution. The concentration per pair of bases for this stock solution was determined, by UV-Vis spectroscopy (molar absorptivity at 260 nm, ε_{260nm} = 13,200), to be 1.23 x 10^{-3} M/bp. A 1.23 x 10^{-6} M ct-DNA solution, prepared by dilution with buffer, was used in the experiments. Starting from 1 mL of a 9.36×10^{-4} M solution prepared in the buffer HEPES solvent, several **4** or **7**/DNA solutions in the approximate 0-9.85 mM concentration range for the fixed DNA concentration of 1.23 x 10^{-6} M/bp were prepared. Molar **4**/DNA ratios for these solutions were 0, 0.30, 0.41, 0.61, 0.91, 1.22, 1.52, 3.04, 4.80 and 8.00 respectively. Molar **7**/DNA ratios for these solutions were 0, 0.32, 0.63, 0.95, 1.57, 2.36 and 3.15, respectively. Circular dichroism measurements were then by using a JASCO-715 spectropolarimeter. Recorded spectra were the average of 3 scans taken at the speed of 20 $nm\cdot min^{-1}$ with a 0.125 s time response. The sensitivity and resolution were fixed at 20 mdeg and 10.0 nm respectively. All measurements were performed at 25 °C in 100 mm path cylindrical quartz cells. Data for **4** are depicted in Figure 2A and 2B in the manuscript. Data for 7 are depicted in Figure S14.

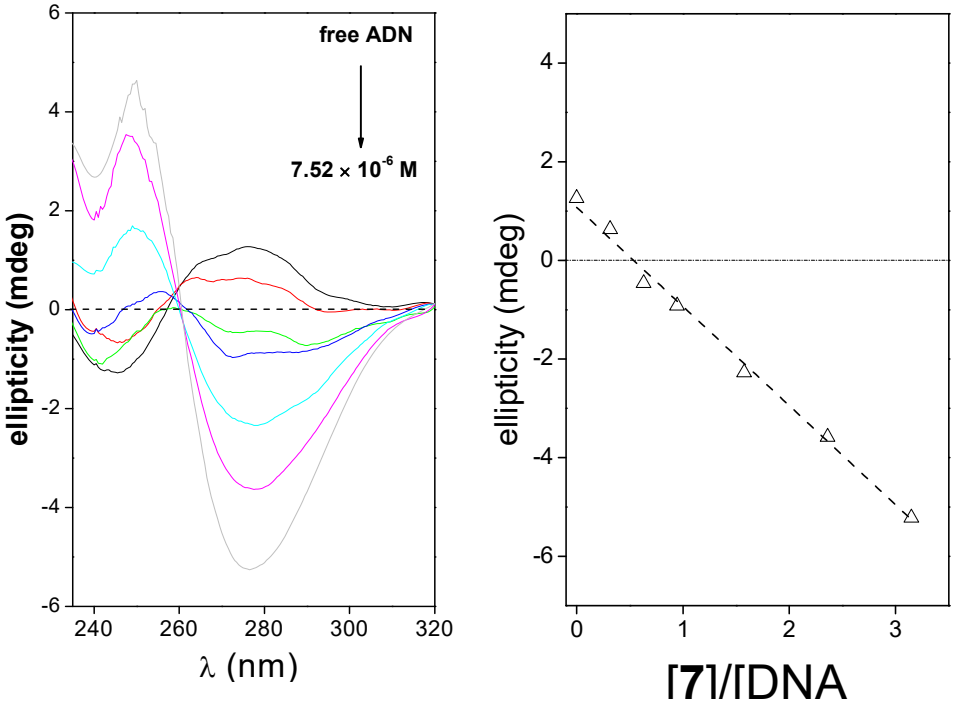


Figure S14. (Left) Circular dichroism spectra in the 230-320 nm region for **7**/DNA solutions in HEPES of 0, 0.75, 1.50, 2.25, 3.76, 5.64, and 7.52 μM sugar concentration for a fixed ctDNA concentration 2.39 μM. (Right) Ellipticity as a function of **7**/DNA molar ratios for the maxima of the band appearing near 275 nm. Spectra were performed at 25ºC.

Transmission Electron Microscopy (TEM). Formvar-carbon coated grids previously made hydrophilic by glow discharge were placed on top of small drops of the CTplexes (HEPES 20 mM, pH 7.4, DNA 303 μM phosphate) prepared as describe above using N/P 10 ratios. After 1-3 min, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grides were then dried and observed with a Philips CM12 electron microscope working under standard conditions. All these experimentes were reproduces twice on each formulation. A representative micrograph of **4**:pDNA complexes is shown in Figure 1D in the manuscript. A representative micrograph of **7**:pDNA complexes is here depicted in Figure S15.

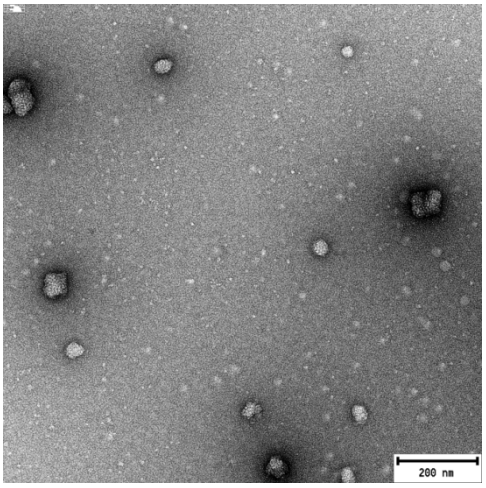


Figure S15. TEM micrograph of **7**:pDNA complexes formulated at N/P 10.

Molecular Mechanics (MM) and Molecular Dynamics (MD) calculations of 4-DNA interactions. For calculations the Sybyl X-2.0 and the Tripos Force Field [Sybyl-X 2.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA][10] were used. B-DNA fragments contained twelve nucleotides with a CGCGAATTCGCG sequence each. Charges for CTs were obtained by using the Gaussian suite of quantum chemical programs [Frisch MJ, GWT, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Scalmani G, et al. Gaussian 09, revision A. 1. ed. Wallingford, CT, USA: Gaussian, Inc.; **2009**] at the HF/6-31G(d) level, except for the charged tails that contain NH_3^+ substituents, in whose nitrogen atoms a net +1 (esu) charge was placed. The rest of the charges for the substituent where rescaled to provide a total net charge for the molecule of +6 (esu) in the absence of chloride ions. DNA fragment charges, however, were derived by using the Gasteiger and Marsili method.[11] Water solvation was performed by using Molecular Silverware algorithm (MS) and periodic boundary conditions (PBC).[12] A relative permittivity $\varepsilon = 1$ was used for electrostatic contributions in the presence of explicit water. Non-bonded cut-off distances for MM, as well as for MD were set at 12 Å. Optimizations were carried out by the simplex algorithm, and the conjugate gradient was used as a termination method with gradients of 3.0 Kcal/molÅ for the MM calculations.[13,14]

Initially the $(CT)_2$ dimer formation and stability in water was studied by MM. For this purpose a stable optimized (gradient 0.5 Kcal/molÅ) conformation (branches in the *all-trans* arrangement) CT, named CT(1), was located with its center of mass at the origin of a coordinate system and oriented, as Figure S16 depicts, with the hydrophobic tails towards the positive *y* axis side. Then, another CT(2) in the same conformation was approached along this axis by the hydrophobic side. The most

favorable CT(2) relative orientation for approaching CT(1) was previously obtained by studying the conformations in the vacuum which result from the rotation of CT(2) around the *y* axis followed by its approaching CT(1).

Structures generated by scanning *oo*' distances from 40 Å to 12Å at 1Å intervals, followed by solvation (MS and PBC) and optimization (gradient 3.0 kcal/molÅ) were analyzed. Calculations were performed starting from (*a*) the totally charged (+6 esu, net charge) CTs or (*b*) those whose charges from NH_3^+ terminal groups were neutralized by chloride contraions (of –1 esu, charge each) bounded to N by dummy bonds. The resulting minimum binding energy (MBE) structure for the dimer $(CT)_{2,min}$ (Figure S17) was used to study the $(CT)_2$-DNA_2 supramolecular complexation once chlorine ions were removed. The purpose was to investigate its stabilization in the presence of DNA moieties and the structure and interaction responsible for the stabilization of the whole supramolecular CTplex.

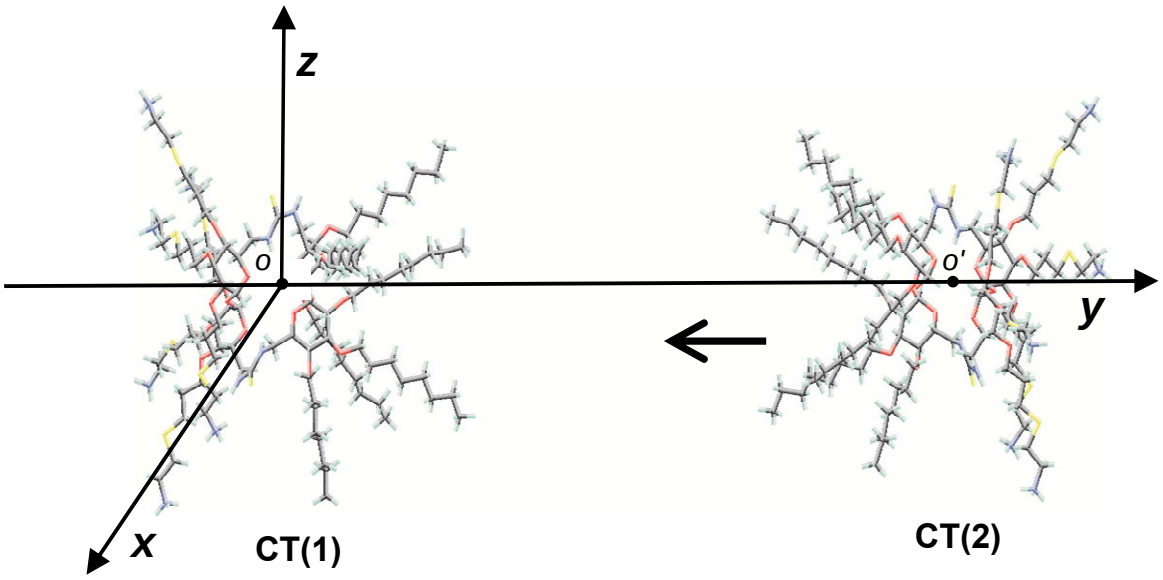


Figure S16. Coordinate system used for CT(2)-to-CT(1) approaching along the *y* coordinate.

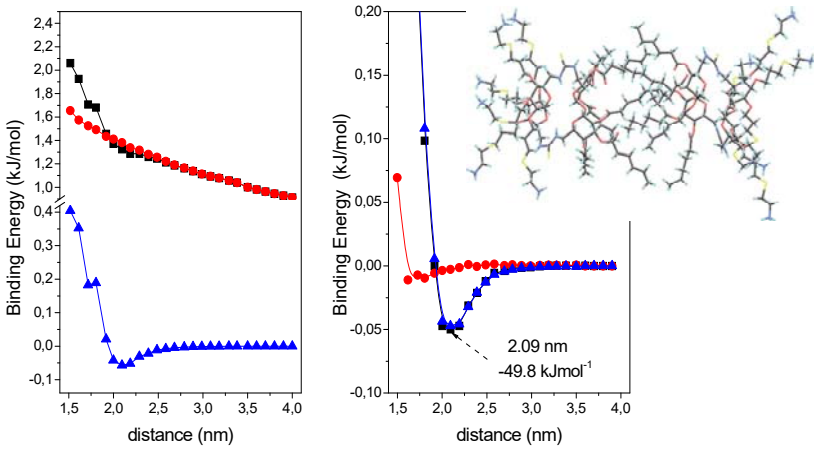


Figure S17. Total CT(1)-CT(2) binding energies (squares), electrostatics (circles) and van der Waals (triangles) contributions as a function of the CT-CT distance along the *y* coordinate, for CT(2) approaching CT(1), which was centred at the origin of the coordinate system depicted in Figure S16, for the charged CT (left) and for the uncharged one (right). The MBE structure is represented.

The $(CT)_{2,min}$ dimer was next placed with its center of mass at the origin of a coordinate system between the two symmetrically located and oriented DNA helix fragments, as Figure S18 shows. DNA fragments were initially placed at distances where they hardly interact with the dimer structure (40 Å from the origin) and both DNA1 and DNA2 fragments were simultaneously approached to the $(CT)_{2,min}$ dimer, in 0.5 Å steps along the *y* coordinate by the *major* groove and from $y = +35$ to 10 (Å) and from $y = -35$ to -10 (Å) respectively. Every structure generated was solvated (MS and PBC), optimized (gradient 1.5 kcal/molÅ) and analyzed. To avoid strong interactions of the CT branches with DNA, each solvation and minimization process starts on the dimer structure optimized in the previous step instead of on the initial $(CT)_{2,min}$ dimer structure.

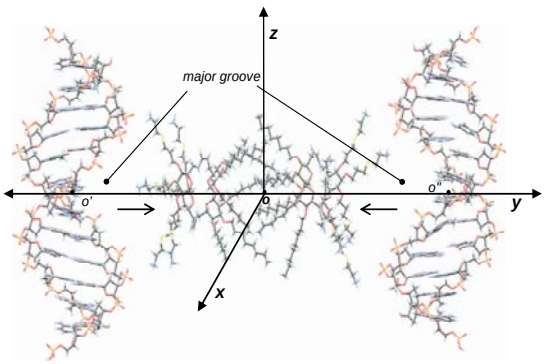


Figure S18. Coordinate system used for DNA fragments-to-$(CT)_{2,min}$ dimer approaching along the y coordinate by the major groove.

The most stable CTplex structure (minimum total energy, Figure S19) generated was optimized once again (gradient 0.5 kcal/molÅ) and used as the starting conformations for 1.0 ns MD simulations following the same strategy described earlier.[15] To maintain a regular helical structure for the pair of DNA chains and to avoid the unwinding of the end portions of this short DNA helix during MD, the simulations were performed on the MBE structures where the N···HN hydrogen bond distances for each pair of DNA nucleobases were constrained to keep them constant. For this purpose a harmonic penalty function was added to the force field equation for those atoms which were involved in the constraint. This energy function is written as $E = k\,(r\text{-}r_i)^2$, where k =200 kcal/molÅ^2, r_i and r are the initial distance and the variable distance during the MD trajectory, respectively.

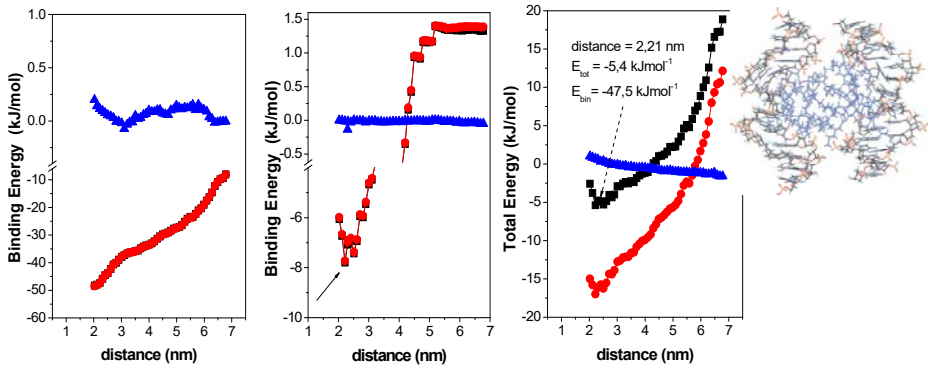


Figure S19. (Left) Total interaction (or binding) energies (■) and the electrostatics (●) and van der Waals (▲) contributions between DNA fragments and the $(CT)_2$ dimer and (middle) between DNA1CT1 and CT2DNA2 units, as well as, (right) the total energy and contributions for the $(CT)_2(DNA)_2$ complex as a function of the DNA1-DNA1 distance along the *y* coordinate, for simultaneous DNA~~s~~ fragments approaching along the y coordinate by the *major groove* to the charged dimer centred at the origin of the coordinate system, as depicted in Figure S18. The arrow indicates the most stable structure, which is also depicted.

Figure S20 illustrates the histories for several distances obtained from the analysis of MD trajectories on the CTplex starting from the MBE structures obtained by MM. Structures seem to be relatively stable throughout the trajectory. The complex did not dissociate throughout the MD. The trajectory and the distances maintained their initial values, at least within a reasonable range.

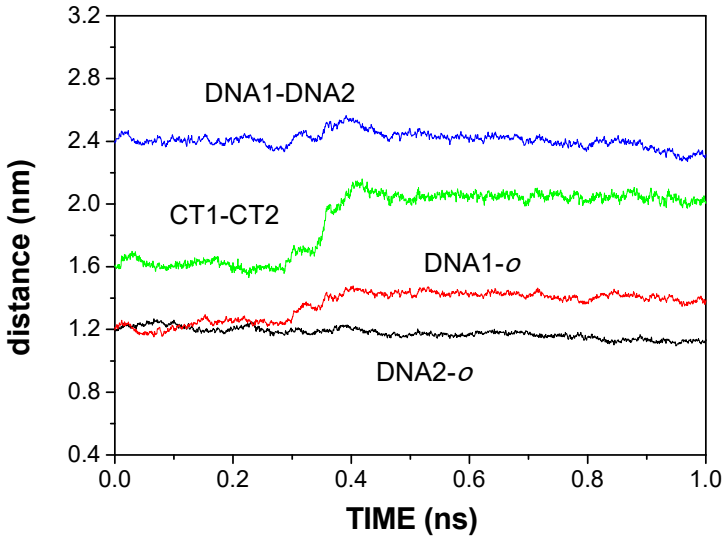


Figure S20. Histories of DNA1-DNA2, CT1-CT2, DNA1-center and DNA2-center distances obtained from the analysis of the 1ns MD trajectories in the presence of water starting from the minimized most stable structures of the CTplex obtained by MM.

Figure 21 shows the histories for DNAs–dimer interaction energies and contributions obtained from the analysis of the 1ns MD trajectories. Binding energies were initially favourable and still remained favourable at the end of their trajectories. As in MM calculations, nearly 100% of these interactions were due to electrostatics contributions. Interaction energies between CT charged units (CT1–CT2) in the CTplex were obviously unfavourable, but also remained constant (average = 2.8±0.1 kJmol^{-1}) throughout the 1ns trajectory and very close to the values for the minimized initial structure. Something similar occurred with the unfavourable interaction energies between DNA charged fragments (average = 8.7±1.9 kJmol^{-1}).

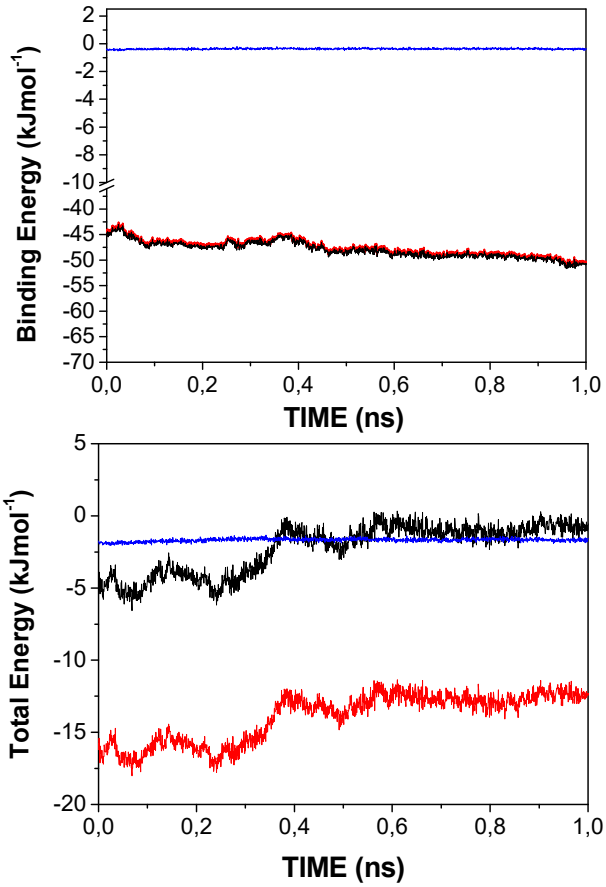


Figure S21. (upper) Histories of the total interaction energy between the $(CT)_2$ dimer and DNA fragments (black) and electrostatics (red) and van der Waals (blue) contributions in the CTplex; (bottom) total energy (black) and electrostatics (red) and van der Waals (blue) contributions. Data were obtained from the analysis of the 1ns MD trajectories in the presence of water starting from the optimized stable structures for the $(CT)_2(DNA)_2$ complex obtained by MM calculations.

The average of the distance between the centers of mass of nitrogen located at the end of the tails for each CT (CT bilayer thickness) throughout the whole MD trajectory was 23±2 Å. The DNA monolayer thickness measured as the average of distances between opposite P atoms of each helix over the whole trajectory was 23±1 Å. Both values were obtained by taking atoms as point masses and in the absence of any hydration shell.

General methods for transfection experiments. Branched polyethylenimine 25 (bPEI, MW 25 kDa, branched) was purchased from Aldrich. The plasmid pCMV-Luc VR1216 (6934 bp) encoding luciferase (Clontech, Palo Alto, CA, USA) used for transfection experiments was amplified in *E.*

coli, isolated, and purified using Qiagen Plasmid Giga Kit (Qiagen GMBH, Hilden, Germany). The following materials were used for DNAse I protection assays: agarose D-1 (Pronadisa, Madrid, Spain), Tris-boric acid-EDTA Buffer (10 x TBE Buffer) (Invitrogen, Barcelona, Spain), DNAse I and ethidium bromide (Gibco BRL, Barcelona, Spain). Sodium dodecyl sulphate (SDS) and NaCl (Roig Farma, Barcelona, Spain) were used to release DNA from the complexes. Ethylenediaminetetraacetic (EDTA) acid and DMSO Hibry-Max ® were supplied from Sigma. Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

Cell culture. HepG2 (human hepatoblastoma) and COS-7 (African green monkey kidney) cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37 ºC under 5% CO_2 in complete medium constituted by Dulbecco's modified Eagle's medium-high glucose + glutaMAX® (Gibco BRL Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were passaged by trypsinization twice a week.

Preparation of pDNA complexes and polyplexes. For *in vitro* assays, the quantities of compound used were calculated according to the desired DNA concentration of 5 µg/mL (15 µm phosphate), the molecular weight and the number of protonable nitrogens in the selected CD derivative or cationic polymer (bPEI, 25 kDa). pDNA complexes and polyplexes were prepared with plasmid DNA (luciferase-encoding plasmid pCpG-hCMV-SPECeFLuc 4640 base pairs; kindly provided by Dr. Ernst Wagner, Pharmaceutical Biotechnology, Center for NanoScience, Ludwig-Maximilians-University, Munich, Germany) and the corresponding Janus cyclotrehalan **4** or **7** and bPEI respectively, at N/P (atomic ratio) 5 and 10. Concerning the preparation of the DNA complexes, DNA was diluted in BHG (HEPES 10 mm, pH 7.4, glucose 5% w/v); then the desired amount of derivative was added from 1000 µm or 3000 µm stock solution in DMSO in order to achieve the desired concentrations of the amphiphilic derivatives for a final N/P 5 ratio. For N/P 10 formulations, the concentrations of CD derivatives were double. The preparation was orbitally stirred for 2 h and used for characterization or transfection experiments. For bPEI, a solution of bPEI 1 m (H_2O) was diluted in distilled water to a final concentration of 0.01 m. A solution of DNA (10 µg/mL) in BHG was mixed with the same volume of a bPEI solution containing the desired amount of polymer, to give a 5 µg/mL DNA solution. The preparation was briefly vortexed and kept at rt for 30 min.
The size of the CTplexes was measured by dynamic light scattering (DLS), and the overall charge by "Mixed Mode Measurement" phase analysis light scattering (M3-PALS) measurements using a Zeta Nano Series (Malvern Instruments, Spain). All measurements were performed in HEPES 10 mm, 5%

glucose, pH 7.4, in triplicate. Size results are given as volume distribution of the major population by the mean diameter with its standard deviation (Table S3).

Table S3. Hydrodynamic diameter (nm), polydispersity index, and ζ-potential (mV) of CTplexes formulated with the plasmid pCMV-Luc VR1216 and CTs **4** or **7** at N/P 5 and 10, determined by DLS and M3-PALS analysis, respectively.

N/P 5	Size (nm)	PDI	ζ-potential (mV)	N/P 10	Size (nm)	PDI	ζ-potential (mV)
4	230 ± 21	0.30	18 ± 2.8	**EMARev6**	140 ± 3.5	0.28	29 ± 7.1
7	123 ± 30	0.28	27 ± 1.4	**EMARev15**	130 ± 11.3	0.28	30 ± 0.7

For *in vivo* assays, the DNA concentration was set at 300 µg/mL. CT derivatives **4** or **7** were added from a 16.6 mm stock solution (1:2 DMSO-sterile H_2O).

Agarose gel electrophoresis. Each vector:pDNA complex (20 µL, 0.4 µg of plasmid) was submitted to electrophoresis for about 30 min under 150 V through a 0.8% agarose gel in TAE 1X (Tris-acetate-EDTA) buffer and stained by spreading GelRed Nucleic Acid Stain (Biotium). The DNA was then visualized after photographing on an *Alphaimager Mini UV* transilluminator (Figure S21a). The plasmid integrity in each sample was confirmed by electrophoresis after decomplexation with sodium dodecyl sulfate (SDS, 8%).

DNA condensation/ protection assays. 50 µL of paCTplexes were prepared in water at N/P ratio 5 and 10 to a final concentration of 50 µg/mL. Then, samples were electrophoresed for 30 min under 150 mV in 0.8% agarose gel (Figure S22a). For protection assays, DNAse I (1U/µg pDNA) was added to each sample and stirred for 30 min at 37 ºC. 20 µL of EDTA 0.25 M was added to inactivate DNAse and the sample was vortexed and incubated for 5 min. 20 µL of SDS 25% was added and further incubated for 5 min. Samples were electrophoresed as described above. Plasmid integrity was compared with free pDNA treated and untreated (Figure S22b).

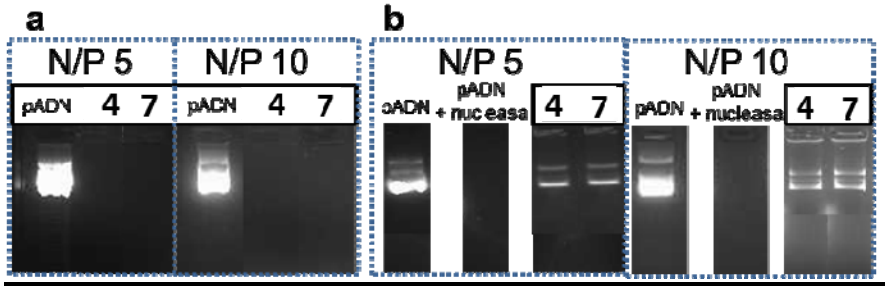


Figure S22. (a) pDNA retardation in agarose gel of CTplexes formulated with **4** and **7**; (b) Protection assays against nucleases. Naked pDNA (left lanes in each panel) is used for comparative purposes.

In vitro transfection activity. The procedure for *in vitro* transfection assays was the same for both cell lines. Cells were seeded in medium in 48-well plates (Iwaki Microplate, Japan), and incubated for 24 h at 37 ºC in 5% CO_2. After this, the medium was removed and 0.3 mL of complete medium (without serum) or serum (activated FBS) and 0.2 mL of complexes (containing 1 μg of pDNA) were added to each well. After 4 h incubation the medium was replaced for complete medium and the cells were further incubated for 48 h. Cells were washed with phosphate-buffered saline (PBS) and lysed with 100 μL of Reporter Lysis Buffer (Promega, Madison, WI, USA) at rt for 10 min, followed by a freeze-thaw cycle. 20 μL of the supernatant was assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer's protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by de DC protein Assay Reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as nanograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein.

Cell viability. Cell viability was quantified by a modified Alamar blue® assay (Invitrogen). Briefly, 1 mL of 10% (v/v) Alamar blue dye in complete medium was added to each well 48 h post-transfection. After 2.5 h of incubation at 37 ºC, 200 μL of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability (as percentage of control cells) was calculated according to the formula (A_{570} - A_{600}) of treated cells x 100/(A_{570} - A_{600}) of control cells. The data indicated cell viabilities over 90% for all CT (**4** or **7**)-based formulations (as compared with 60-70% for bPEI-based polyplexes).

In vivo transfection activity. Female Balb-c mice (6-8 weeks of age, 20-25 grams weigh) were purchased from Harlan Ibérica Laboratories. All animals were studied in accordance with guidelines established by Directive 86/609/EEC and with the approval of the Committee on Animal Research at the University of Navarra (Pamplona, 033/00). Individual mice in groups of eight were injected via the tail vein with 200 μL of CDplexes containing 60 μg of pCMV-Luc at N/P 5 and 10. Naked DNA was injected as control. Twenty four hours after injection the mice were sacrificed. The liver, heart, lungs and spleen were collected and washed with cold PBS. The organs were homogenized with 1 mL lysis buffer using a homogenizer at 5000 rpm (Mini-Beadbeater; BioSpec Products, Inc.,

Bartlesville, OK, USA) and centrifuged at 10000 rpm for 3 min. 20 µL of the supernatant were analysed for luciferase activity following the same procedure as for *in vitro* assays. Transfection data for COS-7 cells are collected in Figure 4A in the manuscript. Tranafection data in HepG2 cell are collected here in Figure S23.

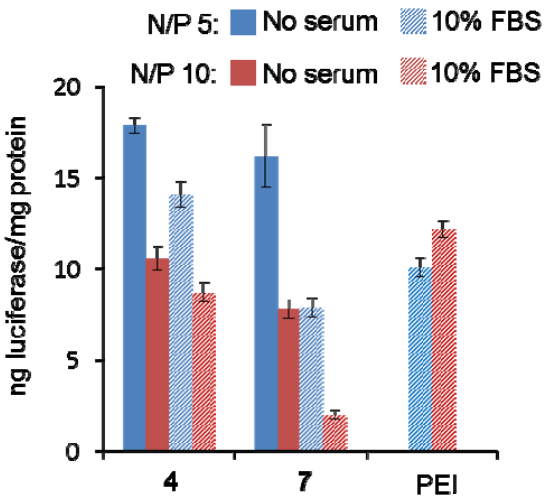


Figure S23. Transfection efficiency in HepG2 cells for CTplexes formulated with Janus CTs **4** or **7** and the luciferase-encoding reporter gene pCpG-hCMV-SPEC-eFLuc at N/P ratios 5 and 10 in the absence and presence of 10% fetal bovines serum (FBS). Data obtained with bPEI polyplexes (N/P= 5 and 10, 10% FBS) under identical conditions are included for comparison. The data represent the mean ± SD of three wells and are representative of three independent determinations.

Statistical Analysis. Statistical analyses were performed using SPSS software from SPSS Inc. (Chicago, IL, USA). The analysis of the transfection efficiency of CDplexes was performed with a two-tailed unpaired Student´s t-test. $P < 0.05$ was considered statistically significant.

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