Supplementary Information

Cyclodextrin Facial Amphiphiles: Assessing the Impact of the Hydrophilic-Lipophilic Balance in the Self-Assembling, DNA Complexation and Gene Delivery Capabilities

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Content

General methods

Reagents and solvents were purchased from commercial sources and used without further purification. NMR spectra were recorded at 500 MHz. 2D COSY, 1D TOCSY experiments were used to assist on NMR assignments. Electrospray mass spectra (ESIMS) were obtained with a Bruker Esquire6000 instrument. Compounds **1**,[1] **2**,[2] **5**,[1] **6**,[2] **10**,[1] **11**,[2] **14**,[1] **15**[2] and **18**[1] were synthesized as previously reported.

pH buffering capacity. pK_a and pH buffering capacities were determined by ^1H NMR-pH titration and potentiometric titration, respectively. For the ^1H NMR-pH titration an acidic solution (**A**) containing 60 µL compound stock solution (10-70 mM in H_2O), 60 µL D_2O, 600 µL stock solution NaCl (0.2 M in H_2O-D_2O 9:1, 1% MeCN), 120 µL stock solution HCl (100 mM in H_2O) and 360 µL H_2O, as well as a basic solution (**B**) consisting of 125 µL compound stock solution, 125 µL D_2O, 1.25 mL stock solution NaCl, 250 µL stock solution NaOH (100 mM in H_2O) and 750 µL H_2O were prepared. Determination of the pK_a values of the compounds was performed by titrating the acidic solution **A** (500 µL, compound concentration 0.5 mM in 9:1 H_2O-D_2O) with the basic solution **B** and monitoring the induced chemical shifts of certain proton nuclei adjacent to pH-active groups ($\Delta\delta_{obs}$) by ^1H NMR spectroscopy at 25 ºC. The MeCN in solutions **A** and **B** was used as internal reference. The dissociation constants of PEI were also determined as indicated for paCDs, starting from a 200 mM stock solution. The pH value of the solution was measured after every addition of solution **B** and just before the spectrum acquisition. To record the spectra, the residual HDO peak was suppressed using a phase-shift presaturation technique. The ionization equilibrium of a protonated amine is given by equation (1),

$$AH^+ \; A + H^+ \rightleftharpoons \qquad (1)$$

whose equilibrium constant K_a is expressed as

$$K_a = \frac{[H^+][A]}{[AH^+]} \qquad (2)$$

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[1] A. Díaz-Moscoso, L. Le Gourriérec, M. Gómez-García, J. M. Benito, P. Balbuena, F. Ortega-Caballero, N. Guilloteau, C. Di Giorgio, P. Vierling, J. Defaye, C. Ortiz Mellet, J. M. García Fernández, *Chem. Eur. J.* **2009**, *15*, 12871-12888.

[2] A. Díaz-Moscoso, A. Méndez-Ardoy, F. Ortega-Caballero, J. M. Benito, C. Ortiz Mellet, J. Defaye, T. M. Robinson, A. Yohannes, V. A. Karginov, J. M. García Fernández, *ChemMedChem* **2011**, 6, 181-192.

or $$pK_a = pH + log\frac{[AH^+]}{[A]}$$ (3)

Plotting δ_{obs} as a function of pH yielded sigmoidal curves for each compound. The pK_a values were obtained by plotting according to the Henderson-Hasselbalch equation (4),

$$pH = pK_a + log\frac{\delta_{acidic} - \delta_{obs}}{\delta_{obs} - \delta_{basic}}$$ (4)

in which δ_{acidic}, δ_{basic} and δ_{obs} are the chemical shifts of the observed signal of the fully protonated and deprotonated compound and the chemical shift of the observed signal at a particular pH, respectively, and linear fitting.

To determine the buffering capacity of cationic compounds, acid-base titration studies were conducted over a pH range of 2-9. Each compound (0.05 mmol of amino groups) was dissolved in 1 mL of 150 mM NaCl aqueous solution, and 0.088 N aq. NaOH (standardized using 2% w/v aq. potassium hydrogen phthalate) was added to adjust pH to 9. Aliquots (10 µL for each) of 0.084 N aq. HCl were added, and the solution pH was measured with a conventional pH-meter after each addition. A blank (150 mM aq. NaCl) was used under the same experimental conditions. Buffering capacity is graphically determined from the HCl meq consumed to switch pH from 7 to 5. Results are collected in table S1 and Figures S32-33.

Preparation of CD:DNA CDplexes. Two different nucleic acids were used: DNA sodium salt from calf thymus (ctDNA, *Sigma-Aldrich*, ref. D3664*)* and the luciferase-encoding plasmid pTG11236 (pCMVSV40-luciferase-SV40pA),[3] a 5739 bp plasmid. The latter was utilized for transfection assays, whereas studies of physicochemical properties were carried out using commercially available ctDNA. The quantities of compound used were calculated according to the desired DNA concentration, the N/P ratio, the molecular weight and the number of protonable nitrogen atoms in the corresponding cationic CD or cationic polymeric reference (JetPEI). In particular, DNA concentrations applied were 0.06 mg·mL^{-1} (i.e. 180 µM phosphate) for gel electrophoresis, 0.02 mg·mL^{-1} (i.e. 60 µM phosphate) for nanoparticle size, polydispersity index (PDI) and ζ potential measurements, 2 µg·mL^{-1} (i.e. 6 µM phosphate) for fluorimetric ctDNA-paCD-binding studies, 4

[3] J. Gaucheron, C. Boulanger, C. Santaella, N. Sbirrazzouli, O. Boussif, P. Vierling, *Bioconjugate Chem*. **2001**, 12, 949-963.

$\mu g \cdot mL^{-1}$ (i.e. 12 µM phosphate) for heparin competitive displacement assays and 5 $\mu g \cdot mL^{-1}$ (i.e. 15 µM phosphate) for in vitro transfection experiments.

For the preparation of CDplexes, typically DNA was diluted in HEPES (20 mM, pH 7.4) to the desired final concentration as specified above, and then the desired amount of CD derivative was added from a stock solution in DMSO (typically 1-10 mM). The resulting mixture was vortexed thoroughly and the complexes were incubated for one hour prior to subjecting them to characterization or transfection experiments.

CDplex characterization. Nanoparticle size, polydispersity index (PDI) and ζ potential were determined by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern) with the following settings: automatic sampling time; 3 measurements per sample; medium viscosity, 1.054 cP; refractive index, 1.33; scattering angle, 173°; λ 633 nm; medium dielectric constant, 80; temperature, 25 °C; beam mode F(Ka)) 1.5 (Smoluchowsky). Data were analyzed making use of the multimodal number distribution software included in the instrument. Results are presented as volume distribution of the major population by the mean diameter and its standard deviation. Before each series of experiments, the performance of the instrument was calibrated with either 90 nm monodisperse latex beads (*Coulter*) for DLS or with DTS 50 standard solution (*Malvern*) for ζ potentials. Experiments were run by in triplicate.

Agarose gel electrophoresis was run in 0.8% (w/w) agarose gel in TAE buffer (1:1:1 Tris/acetate/EDTA) and stained with GelRed™ (*Biotium*, 7.5 µL 10000x in 160 mL TAE). CDplexes were prepared as previously described according to the desired ctDNA concentration (180 µM phosphate) at N/P ratios ranging from 0.5 to 20. The samples were prepared by mixing 18 µL of each CDplex formulation and 2 µL of loading buffer (6x; 5 mL glycerol, 250 µL TAE 40x, 1 mL bromophenol blue and 2.75 mL H_2O). Bromophenol blue is a negatively charged dye and thus moves in the same direction as DNA during electrophoresis. This color marker is used to monitor the process on the gel, although DNA may move more rapidly than bromophenol blue. The samples were submitted to electrophoresis for approximately 20 min under 150 V. Finally, DNA was visualized after photographing (λ 302 nm) using an *Alphaimager Mini* UV transilluminator (*Cell Biosciences*). CDplexes were prepared as previously described at N/P ratios 5 and 10.

CDplex formation and dissociation dynamics. CDplex formation and dissociation was monitored by fluorescence quenching of an intercalating agent. Experiments were performed on a *Varian*

Cary Eclipse Fluorescence Spectrophotometer using ctDNA (2 μg·mL^{-1}, [bp] = 3.0 μM). For CDplex formation studies a solution of intercalating agent RedSafe™ (*iNtRON Biotechnology*, 2.5 μL 20000x in 50 mL HEPES 20 mM, pH 7.4) was prepared. Fluorescence emission was measured in a range from 450 to 570 nm, exhibiting RedSafe™ an emission maximum at 525 nm (λ_{ex} 295 nm, emission and excitation slits 5 nm). Fluorescence emission of the buffer solution was taken as reference.

To assess CDplex dissociation and to compare the relative stability of CDplexes, competitive displacement assays were performed on CDplexes at an N/P 5 using heparin. The effect of heparin on the stability of CDplexes was evaluated by means of the change in relative fluorescence intensity obtained with the fluorescence probe RedSafe™. Starting from a CDplex solution in HEPES (20 mM, pH 7.4, RedSafe™ 1x) containing a DNA concentration of 3.0 μM (base pairs) and the corresponding volume of a CD stock solution in DMSO (50-1000 μM), a volume of 150 μL of this solution was added to the wells of a 96-well plate. Then, different volumes of heparin stock solutions (100-10000 μg·mL^{-1} in HEPES 20 mM, pH 7.4) were added to each well and after taking to a final volume of 250 μL, final heparin concentrations in the range of 5-2000 μg·mL^{-1} were obtained. The samples were incubated for 10 min at rt prior to fluorescence measurement. Finally, time-dependent fluorescence intensity was measured with a *Varian Cary Eclipse Fluorescence Spectrophotometer* equipped with a microplate reader as indicated above. Naked DNA and bPEI as references were processed in a similar manner as indicated for the CDplexes.

***In vitro* transfection**. Twenty-four hours before transfection, COS-7 cells were grown at a density of 2 x 10^4 cells/well in a 96-well plates in Dulbecco modified Eagle culture medium (DMEM; Gibco-BRL) containing 10% foetal calf serum (FCS; Sigma) in a wet (37 ºC) and 5% CO_2/95% air atmosphere. The complexes CD derivative/DNA or PEI/DNA were diluted to 100 μL in DMEM in order to have 0.5 μg of DNA in the preparation. The culture medium was removed and replaced by these 100 μL of complexes in DMEM. After 4 h and 24 h, 50 and 100 μL of DMEM supplemented with 30% and 10% FCS, respectively, were added. After 48 h, the transfection was stopped, the culture medium was discarded, and the cells washed twice with 100 μL of PBS and lysed with 50 μL of lysis buffer (Promega, Charbonnières, France). The lysates were frozen at -32 ºC, before the analysis of luciferase activity. This measurement was performed in a LB96P luminometer (BERTHOLD, France) in dynamic mode, for 10 s on 10 mL on the lysis mixture and using the "luciferase" determination system (Promega) in 96-well plates. The total protein concentration per

well was determined by the BCA test (Pierce, MontluÇon, France). Luciferase activity was calculated as femtograms (fg) of luciferase per mg of protein. The percentage of cell viability of the nanocomplexes was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells x 100%.

***In vitro* transfection in the presence of endosomolytic agent**. After twenty-four hours of cell culture, the complexes CD derivative/DNA or PEI/DNA were diluted to 100 µL in DMEM containing 100 µM chloroquine (Sigma-Aldrich). The culture medium was removed from cells and replaced by this solution of complexes. After 4 h of transfection in the presence of endosomolytic agent, the culture medium was aspirated, and the cells washed with 100 µL of PBS and covered by 150 µL of DMEM supplemented with 10% FCS. Another 100 µL of 10% FCS DMEM were added after 24 h of transfection. After 48 h, the transfection was stopped and the protocol was continued as described above.

FACS experiments (fluorescence assisted cell sorting, FACS). Flow cytometry was performed on FACScalibur (BD Biosciences) equipped with an argon laser (λ_{ex} 488 nm- λ_{em} 520 nm for GFP, λ_{ex} 520 nm- λ_{em} 590 nm for EB). EGFP plasmid DNA was labeled with ethidium bromide (1 EB per 150 bp, 38 molecules/plasmid) prior to its condensation with the CDs at an N/P ratio of 5, as described above. Twenty-four hours before transfection, COS-7 cells were seeded at a density of 10^5 cells per well in 24-well plates. The culture medium was then removed and replaced by 500 µL of EB-labelled CDplexes in DMEM supplemented with 10% FCS so as to have 2.5 µg of pDNA in the well (15 µM phosphate). After 24 h of incubation, the culture medium was discarded, and the cells were incubated for 15 min with 500 µL of a calf thymus DNA solution (200 $mg \cdot L^{-1}$) in non-supplemented DMEM and in a wet (37 ºC) and 5% CO_2/95% air atmosphere. The cells were then washed twice with PBS (500 µL) and detached with trypsin, and re-suspended in 1 mL of DMEM supplemented with FCS. Cells were then centrifuged (5 min, 1200 rpm), washed with ice-cold PBS (500 µL) then re-suspended in PBS containing 1% FCS. The samples were then analyzed with the flow cytometer. The software WinMDI 2.8 was used for the data analysis.

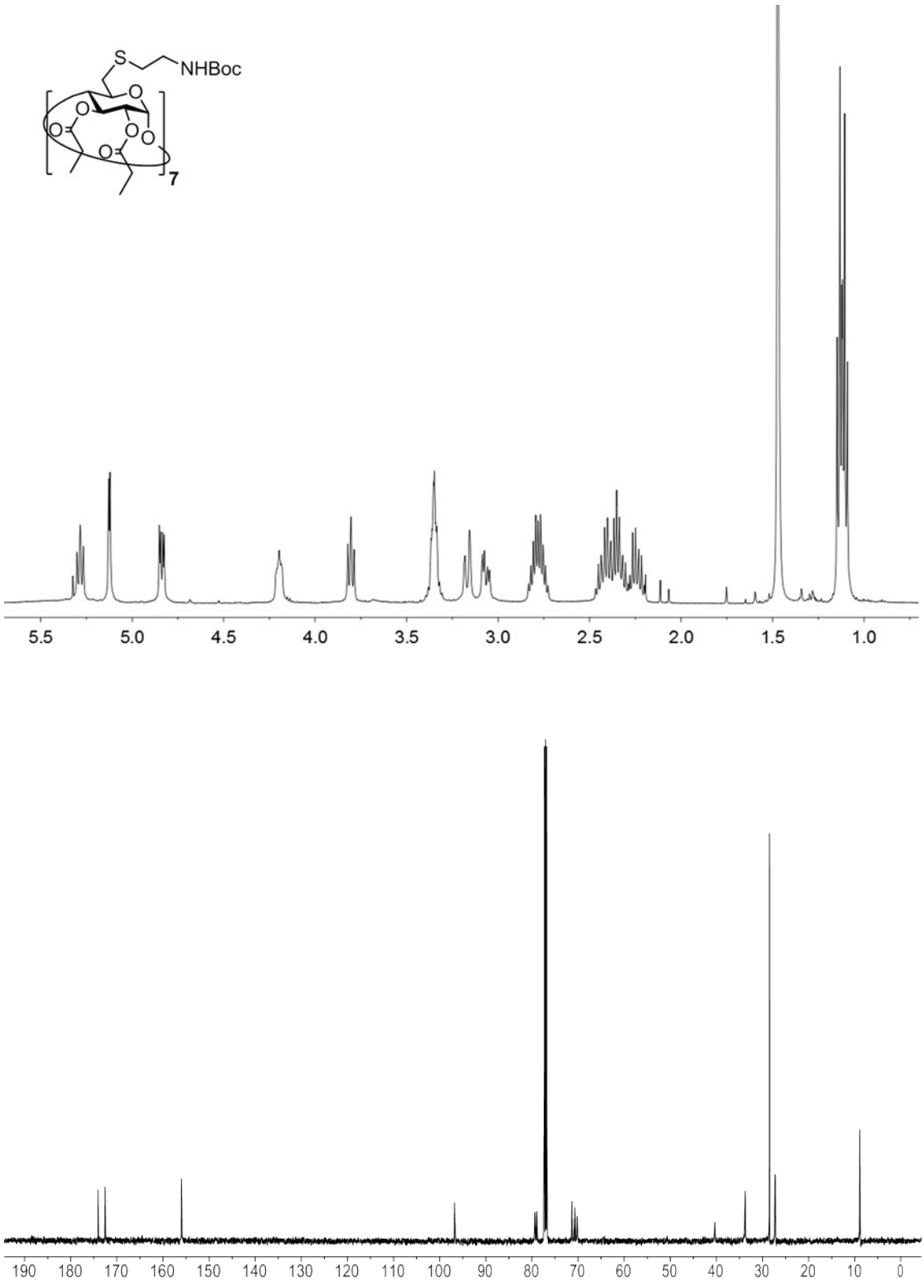

Figure S1. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, $CDCl_3$) of compound **3**.

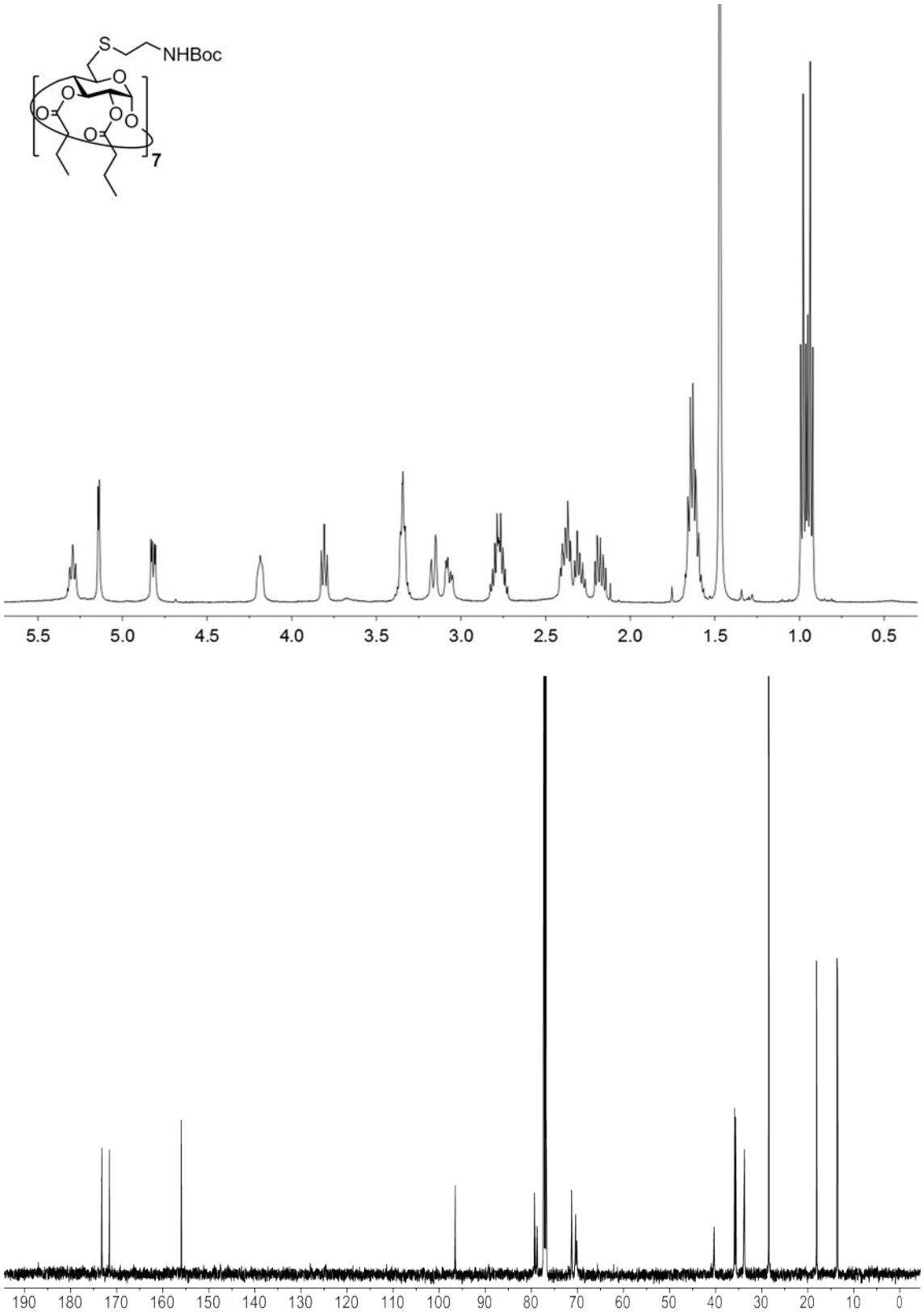

Figure S2. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, $CDCl_3$) of compound **4**.

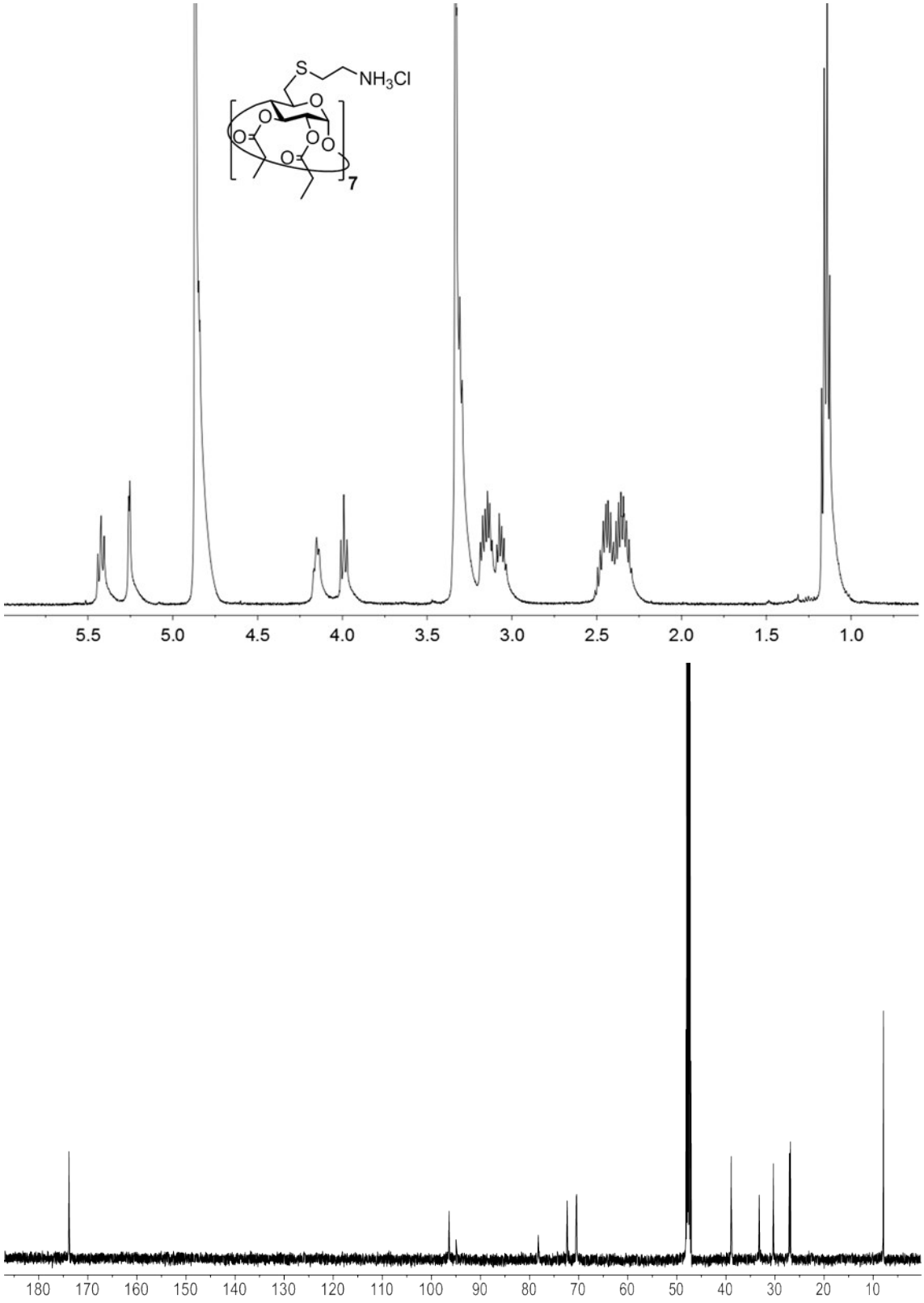

Figure S3. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, MeOD) of compound **8**.

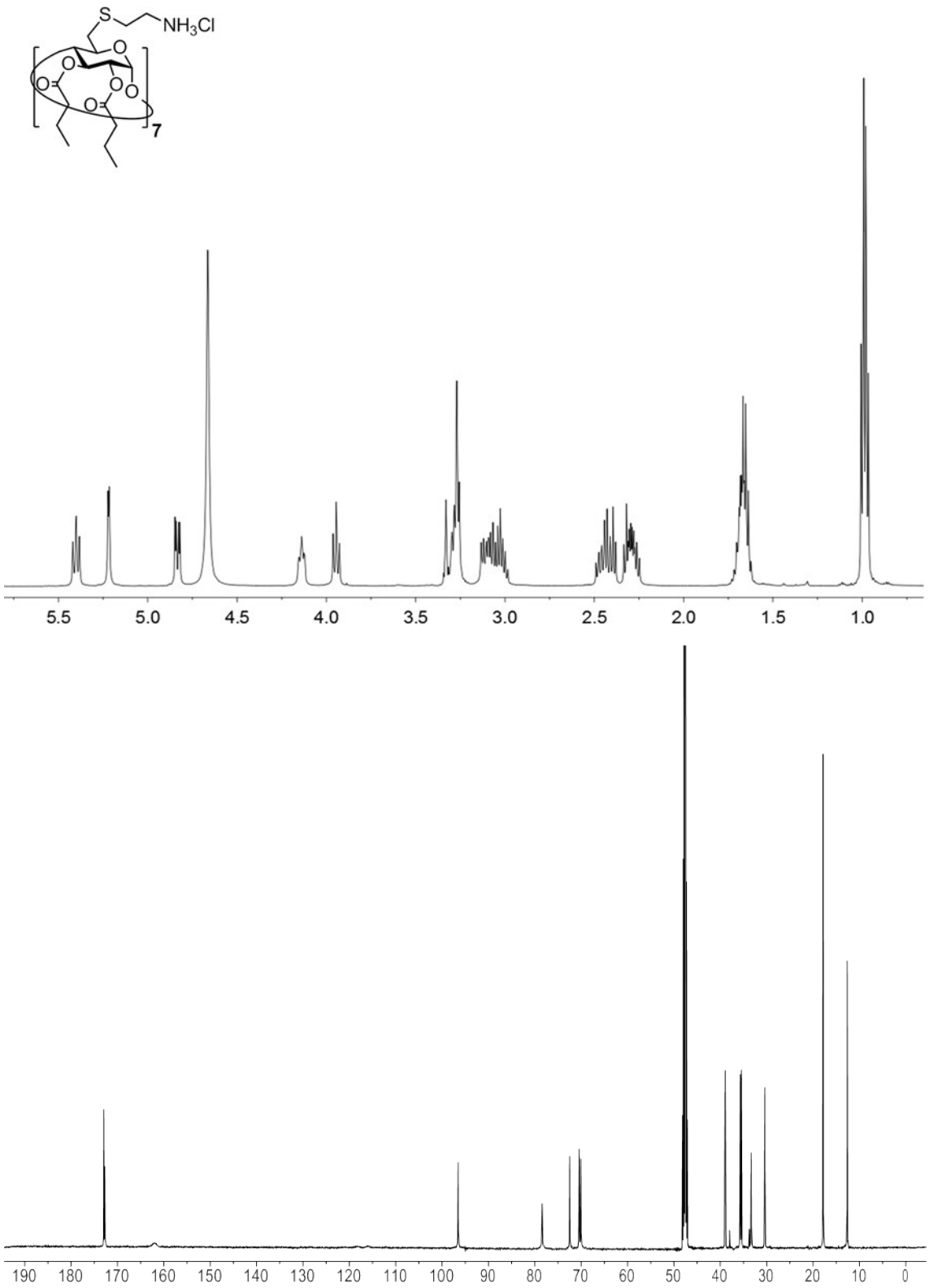

Figure S4. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, MeOD, 313 K) of compound **9**.

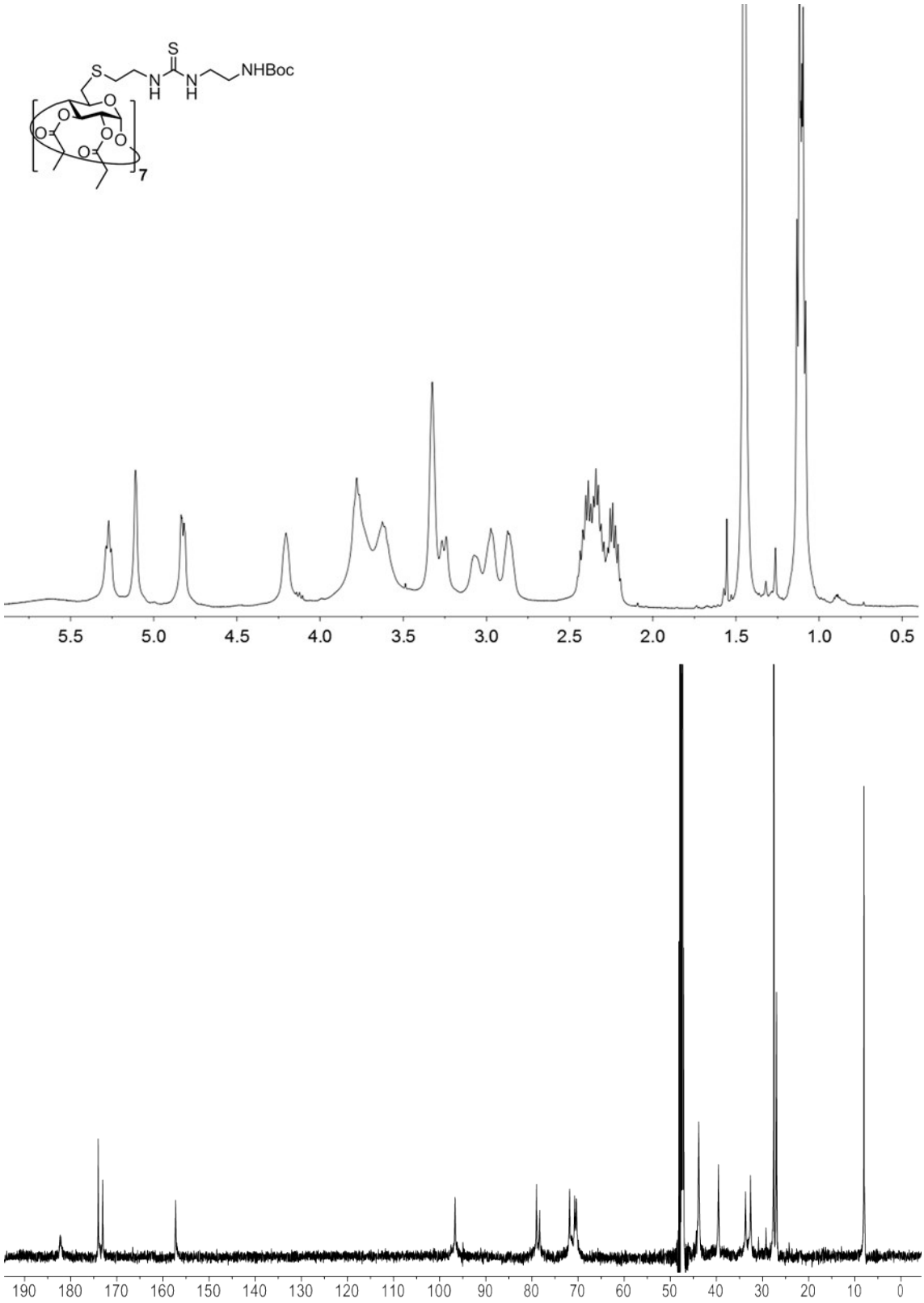

Figure S5. ^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125.7 MHz, $CDCl_3$, 313 K) spectra of compound **12**.

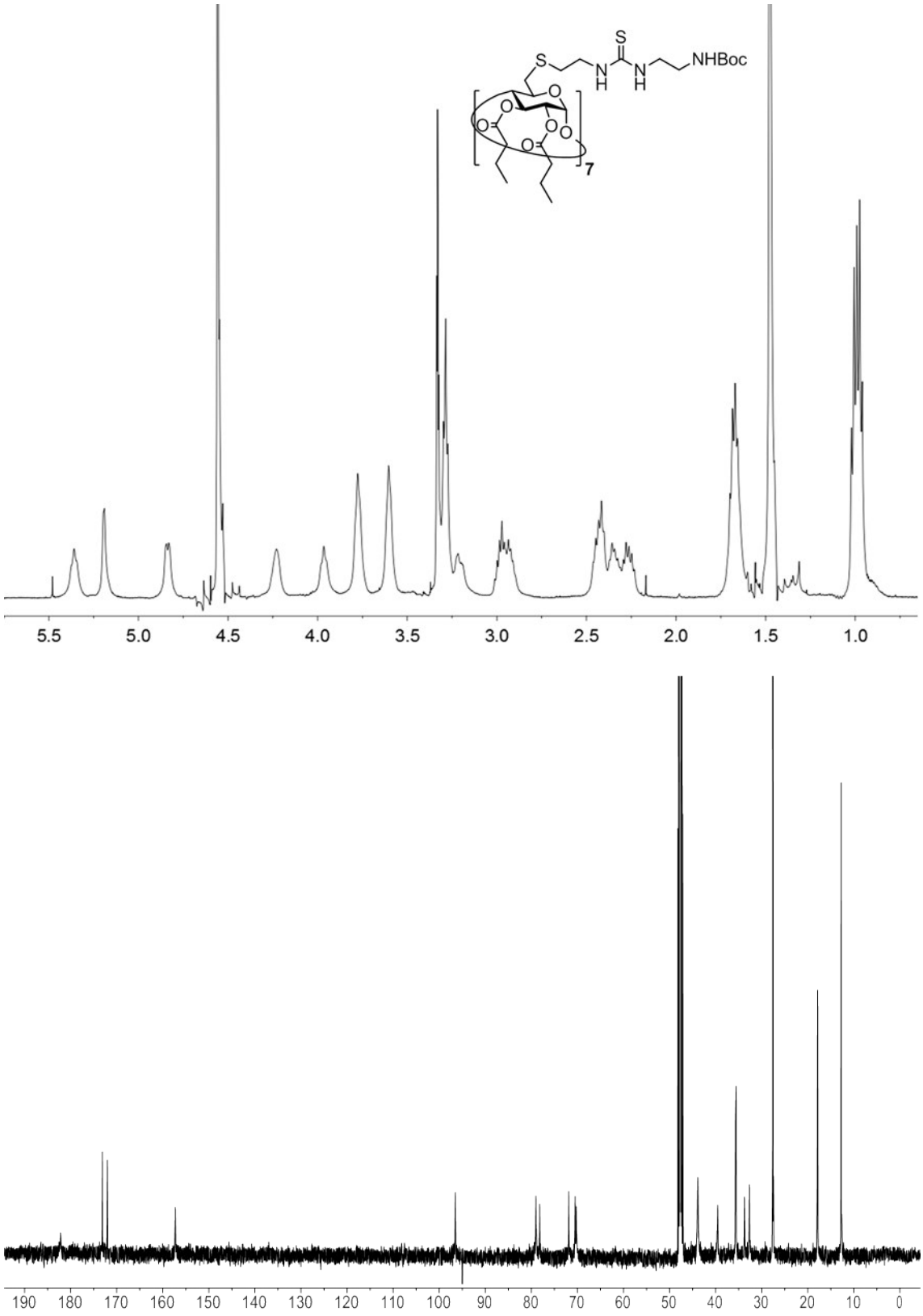

Figure S6. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, MeOD, 313 K) of compound **13**.

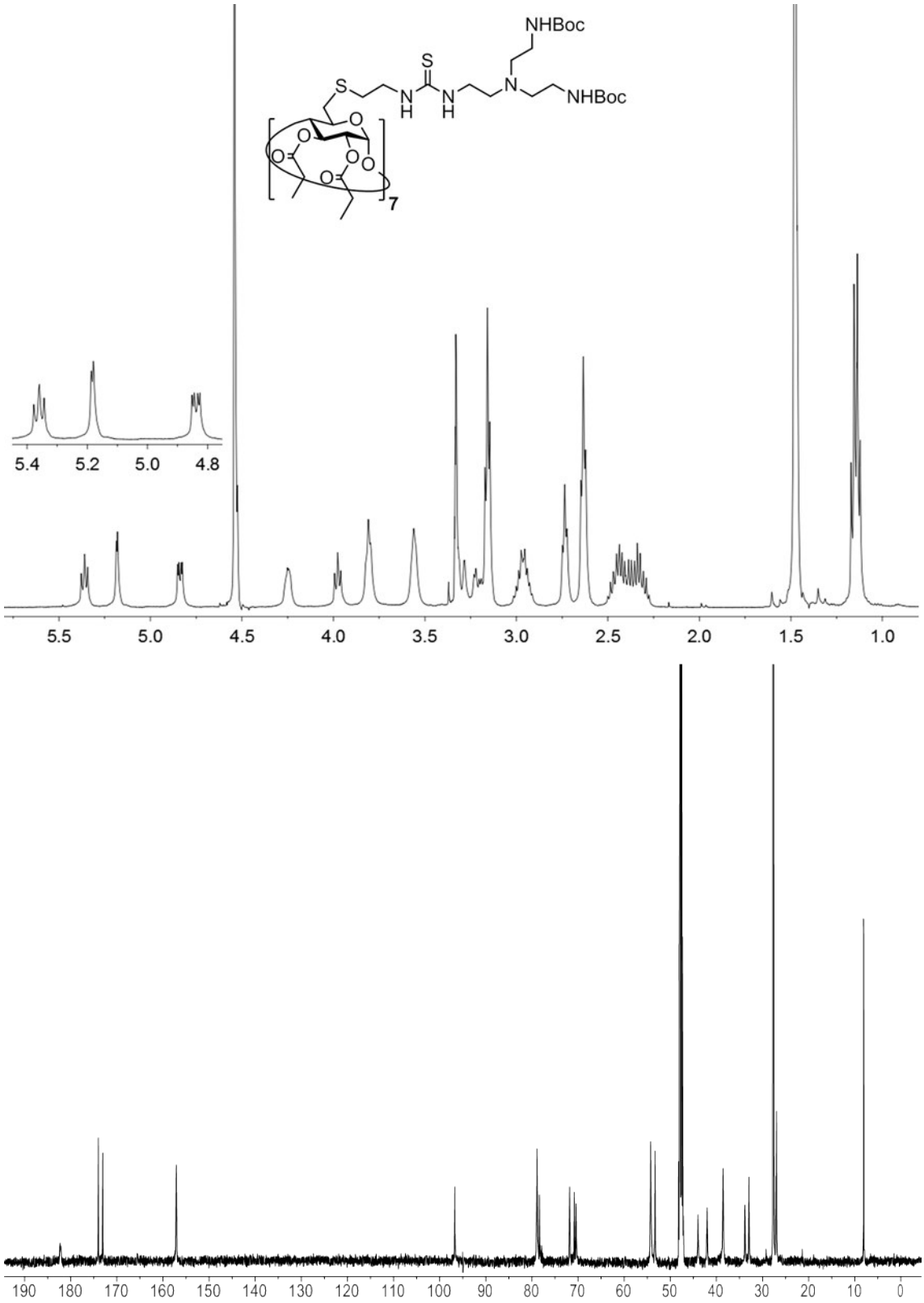

Figure S7. ^{1}H and ^{13}C NMR (500 and 125.7 MHz, respectively, MeOD, 323 and 313 K, respectively) spectra of compound **16**.

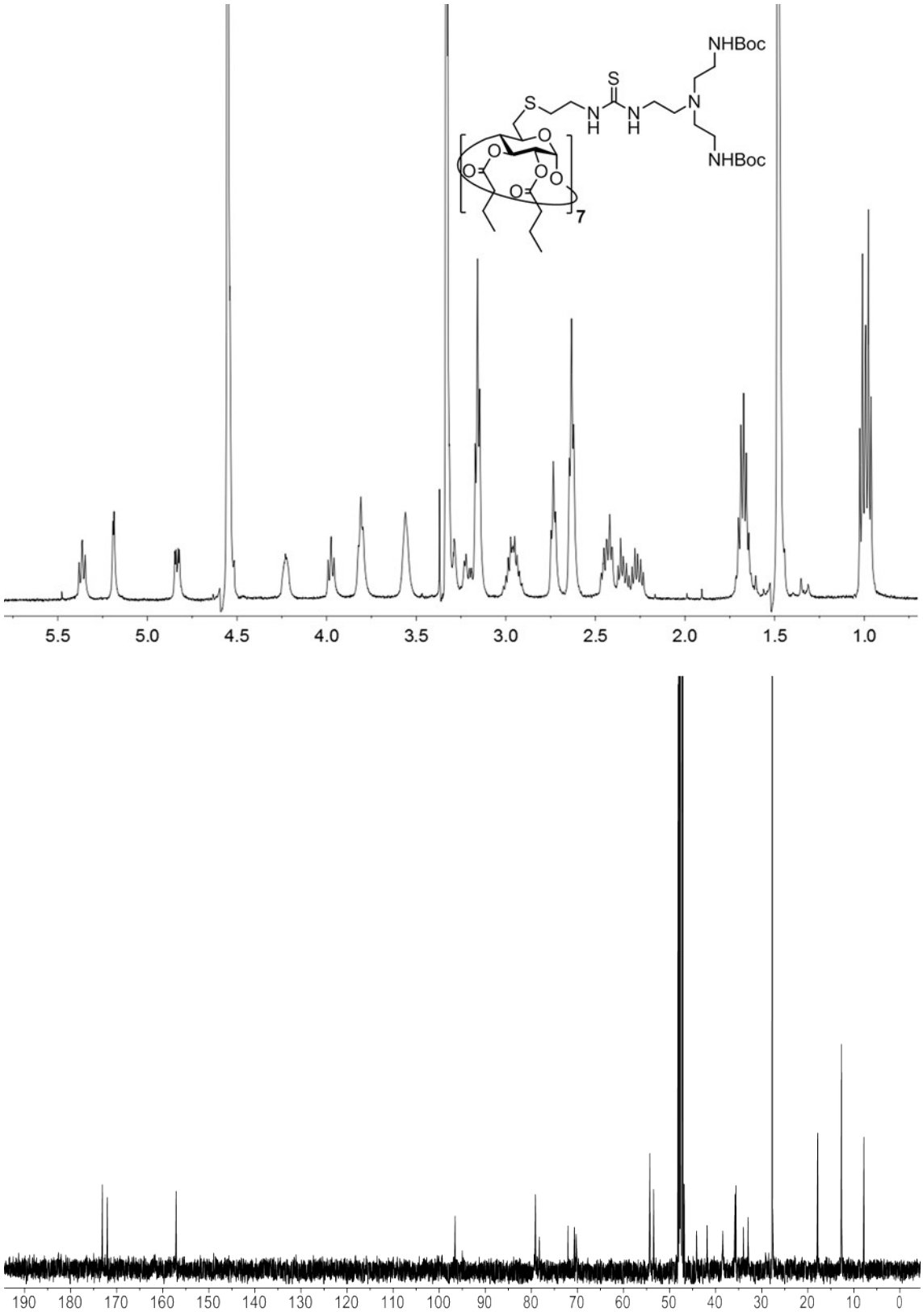

Figure S8. ^{1}H and ^{13}C NMR (500 and 125.7 MHz, respectively, MeOD, 323 and 313 K, respectively) spectra of compound **17**.

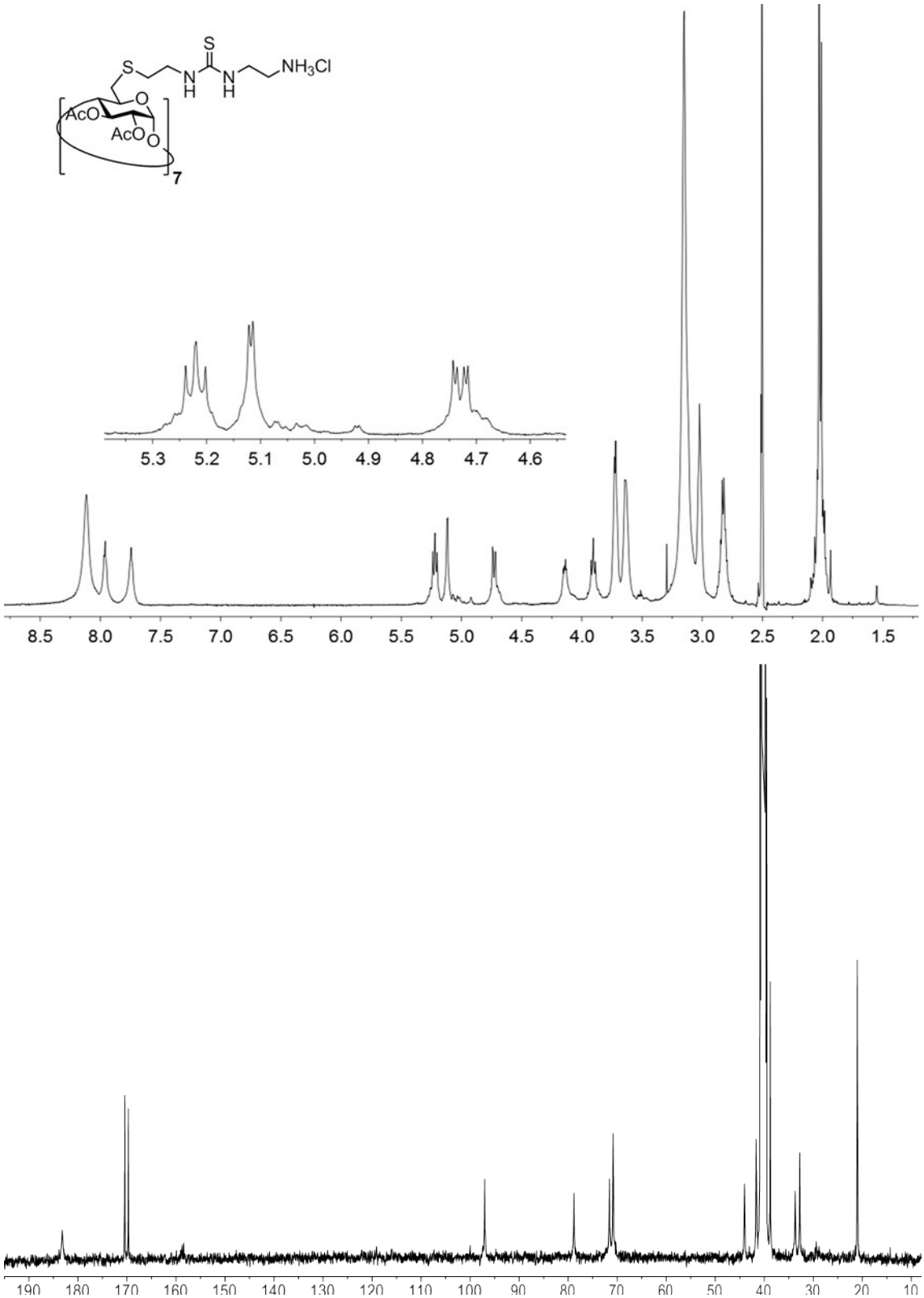

Figure S9. ^{1}H and ^{13}C NMR spectra (500 MHz and 100.6 MHz, respectively, DMSO-d_6, 333 K and 323 K, respectively) of compound ***7***$_{Ac}$.

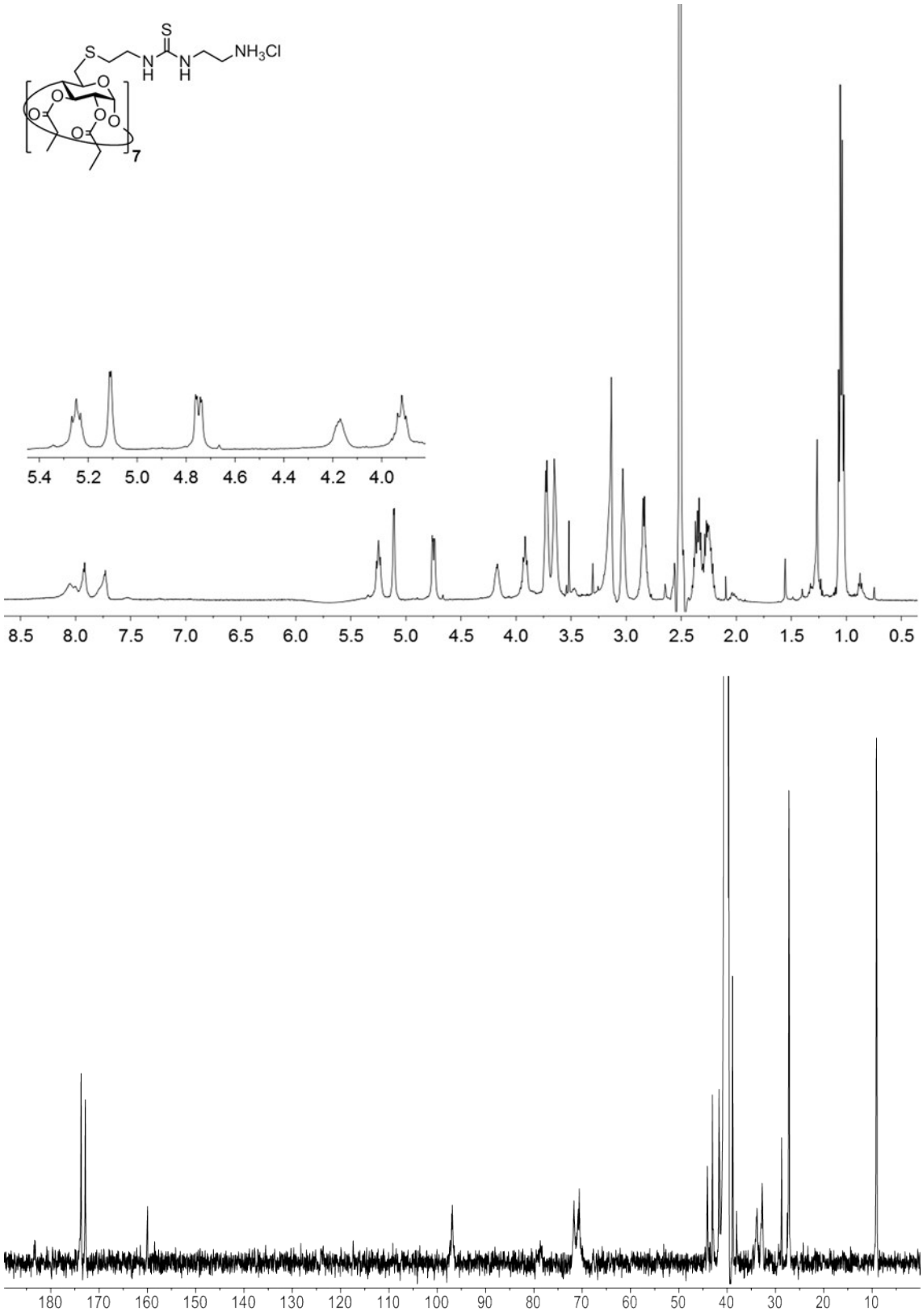

Figure S10. ^{1}H and ^{13}C NMR (500 MHz and 125.7 MHz, respectively, DMSO-d_6, 333 and 323 K, respectively) spectra of compound ***7***$_{Pr}$.

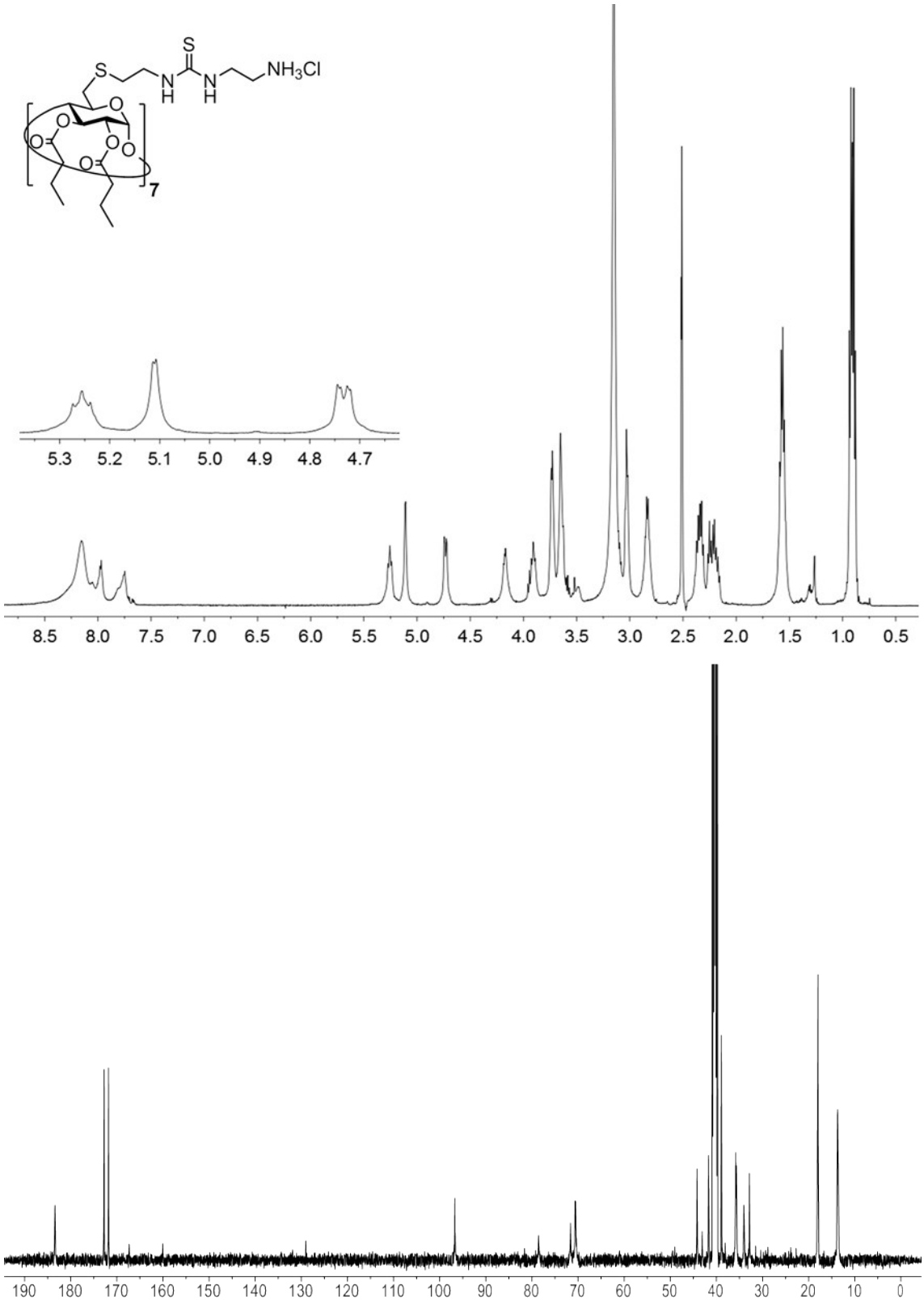

Figure S11. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, DMSO-d_6, 333 K) of compound ***7**$_{But}$*.

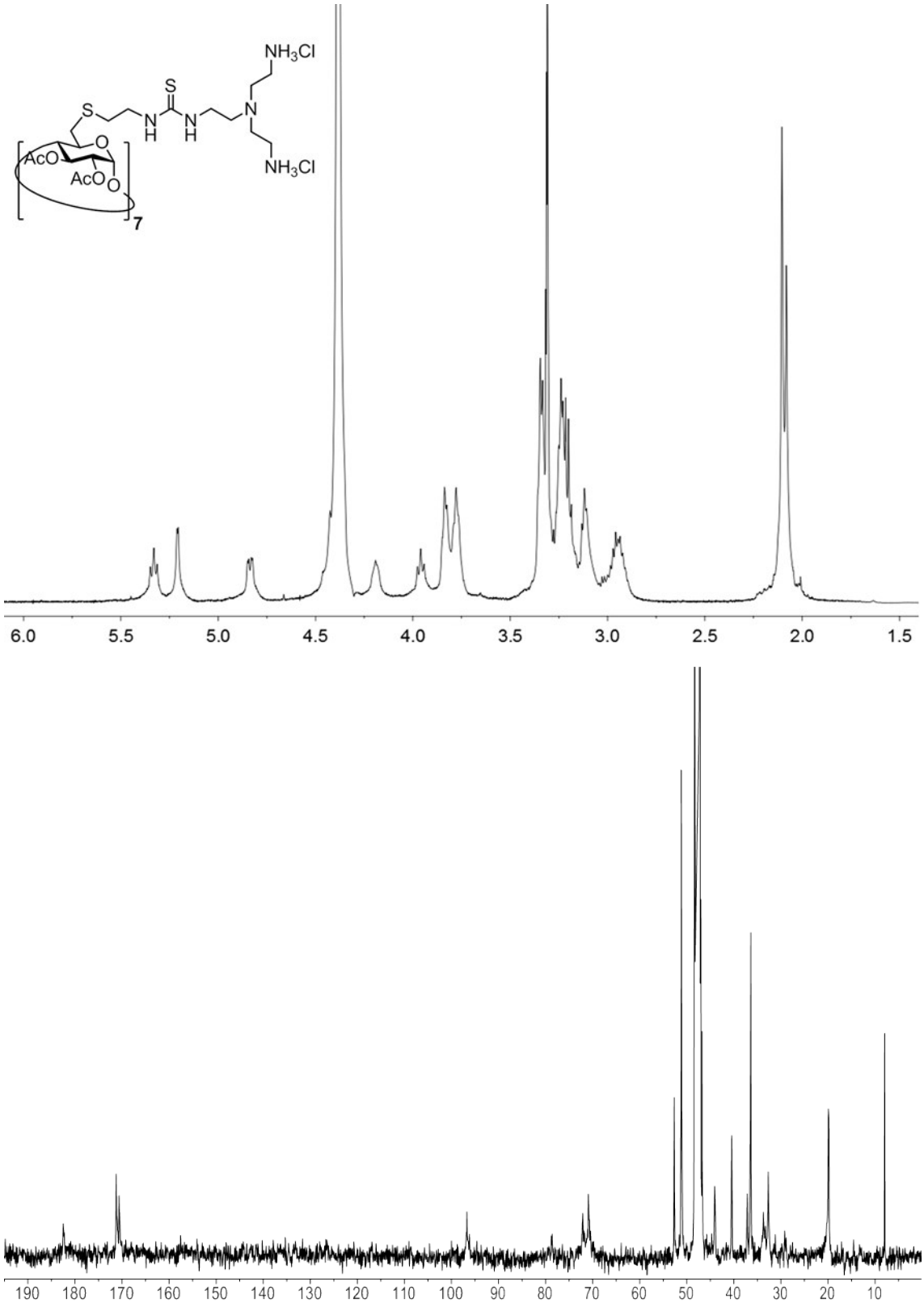

Figure S12. ^{1}H and ^{13}C NMR spectra (500 MHz and 100.6 MHz, respectively, 10:1 MeOD-D_2O, 333 K and 323 K, respectively) of compound ***21***$_{Ac}$.

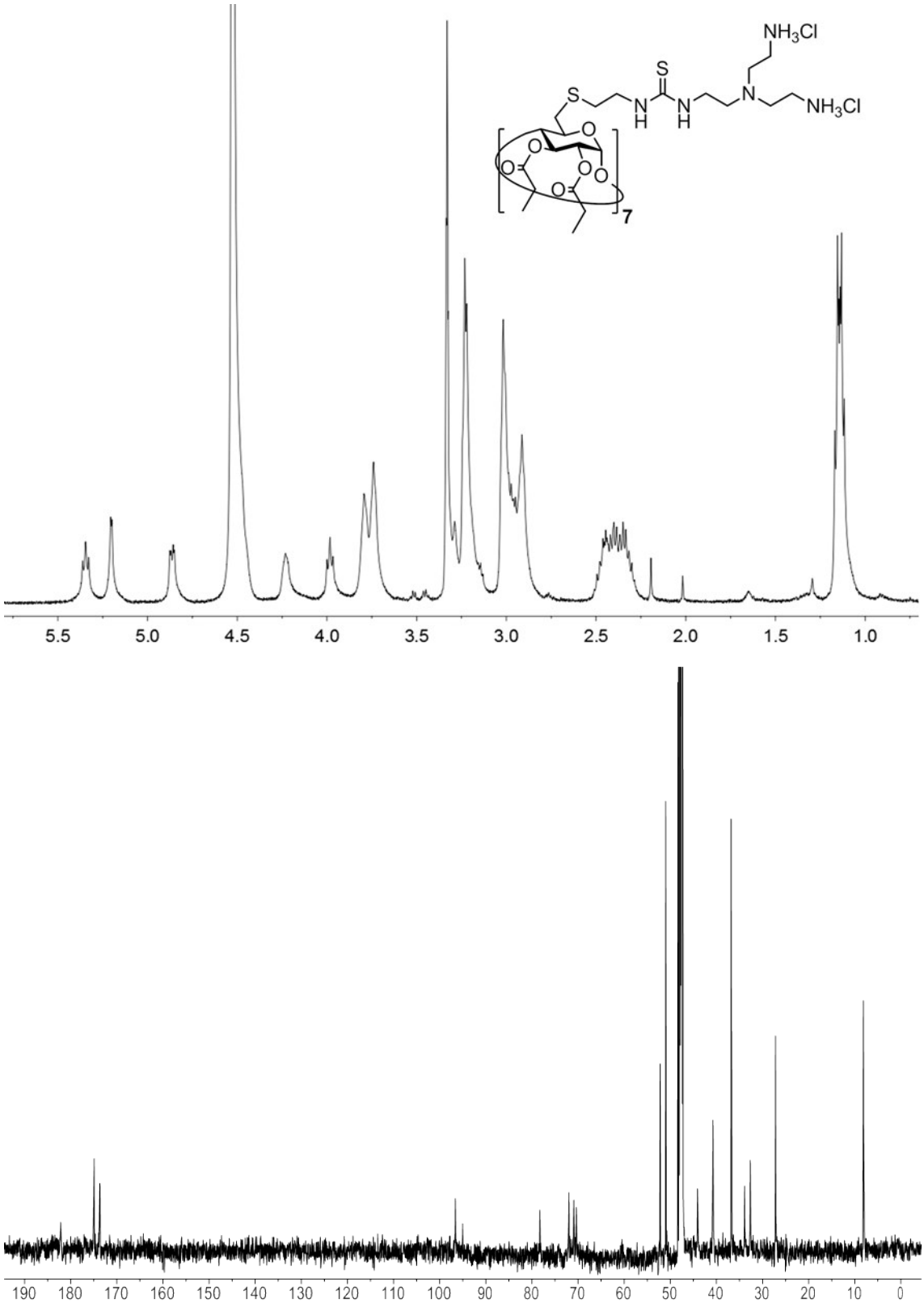

Figure S13. ^{1}H and ^{13}C NMR spectra (500 MHz and 125.7 MHz, respectively, MeOD-D_2O, 323 K) of compound ***21***$_{Pr}$.

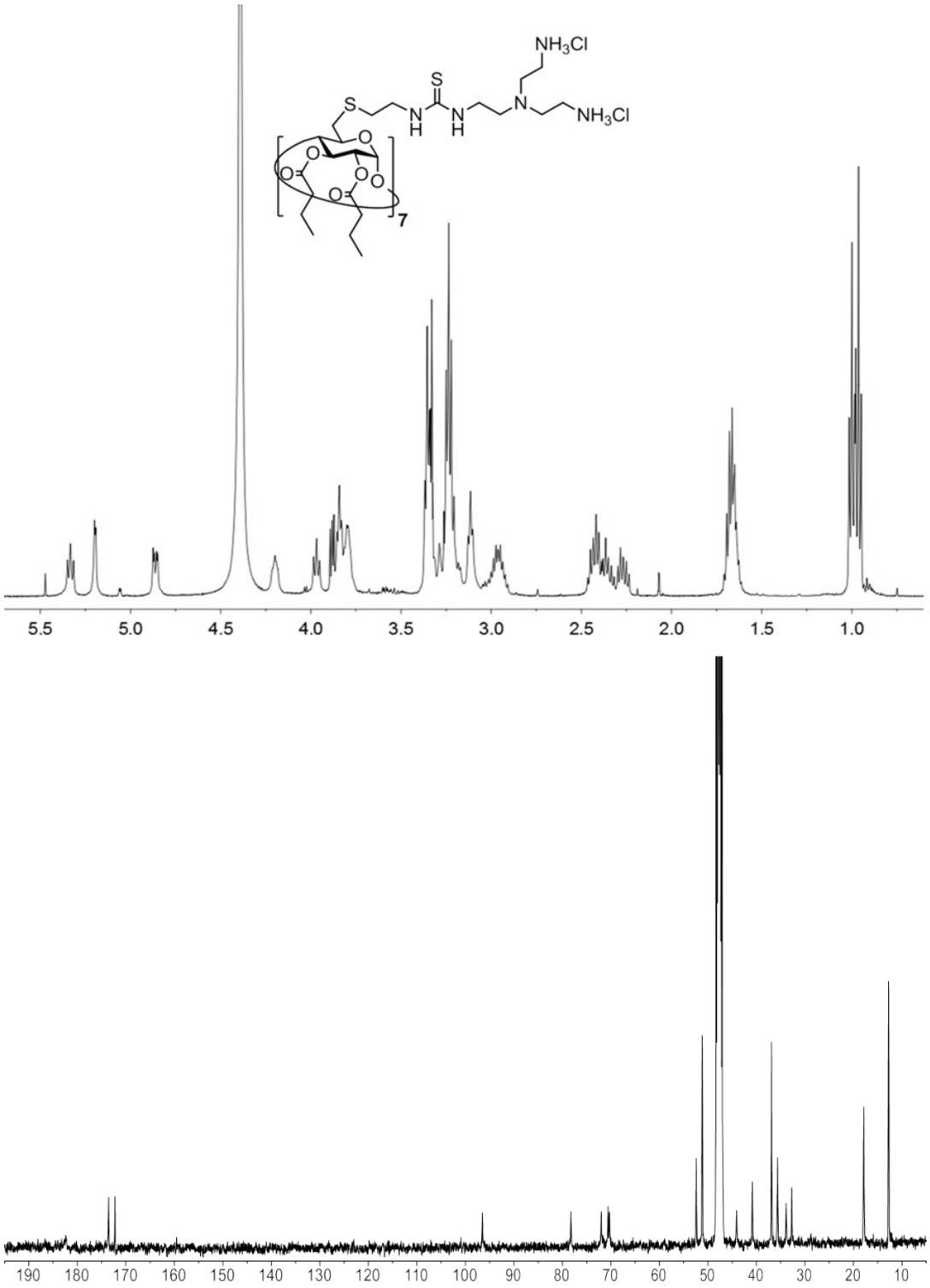

Figure S14. ^{1}H and ^{13}C NMR spectra (500 MHz and 100.6 MHz, respectively, MeOD-D_2O, 333 K) of compound ***21***$_{But}$.

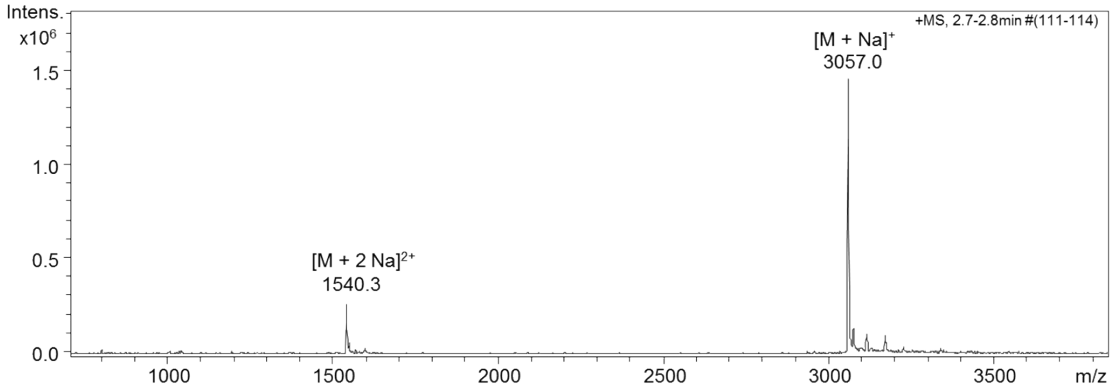

Figure S15. ESI-MS spectrum of compound **4**.

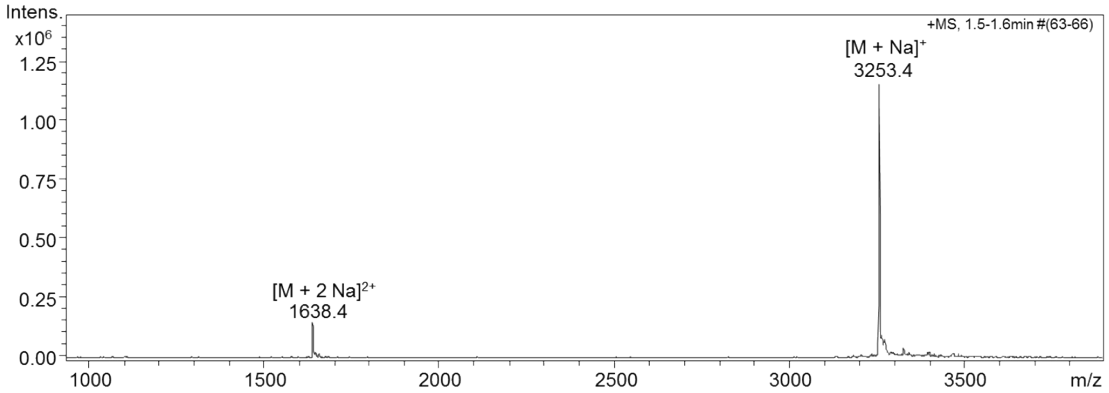

Figure S16. ESI-MS spectrum of compound **5**.

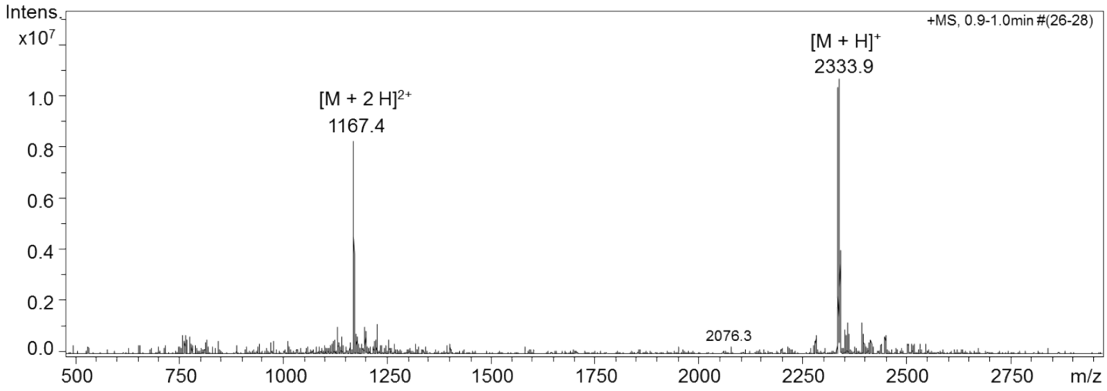

Figure S17. ESI-MS spectrum of compound **8**.

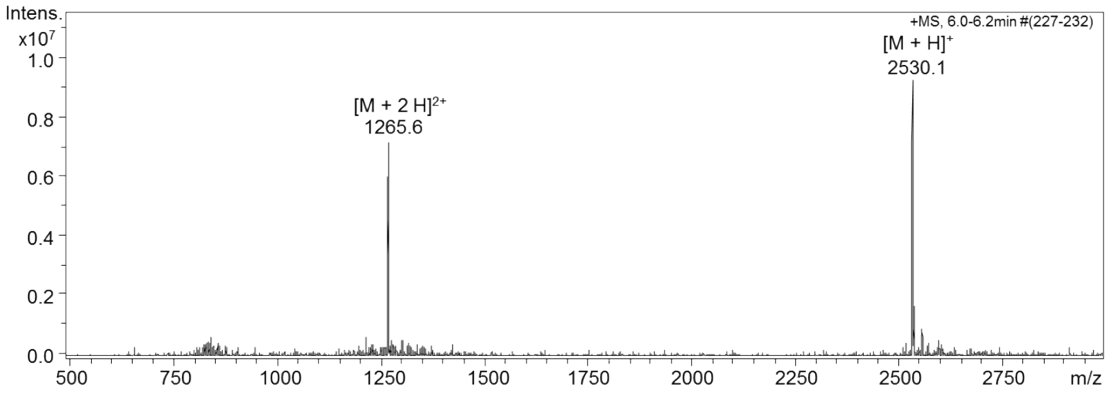

Figure S18. ESI-MS spectrum of compound **9**.

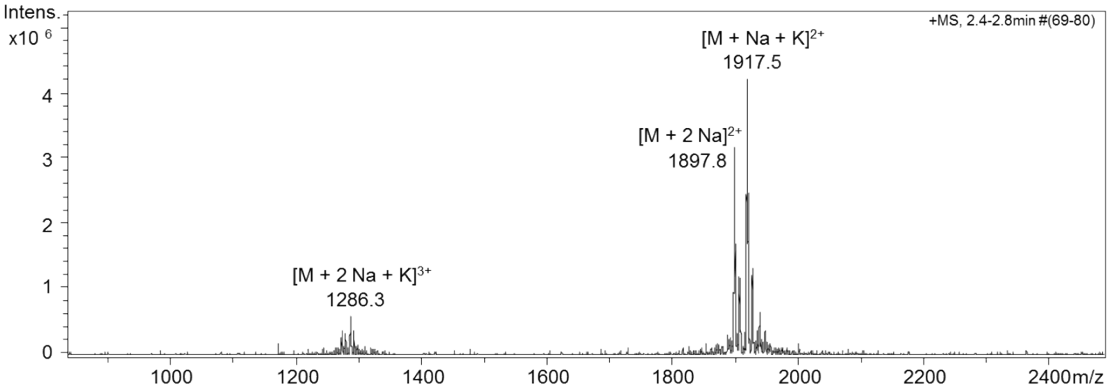

Figure S19. ESI-MS spectrum of compound **12**.

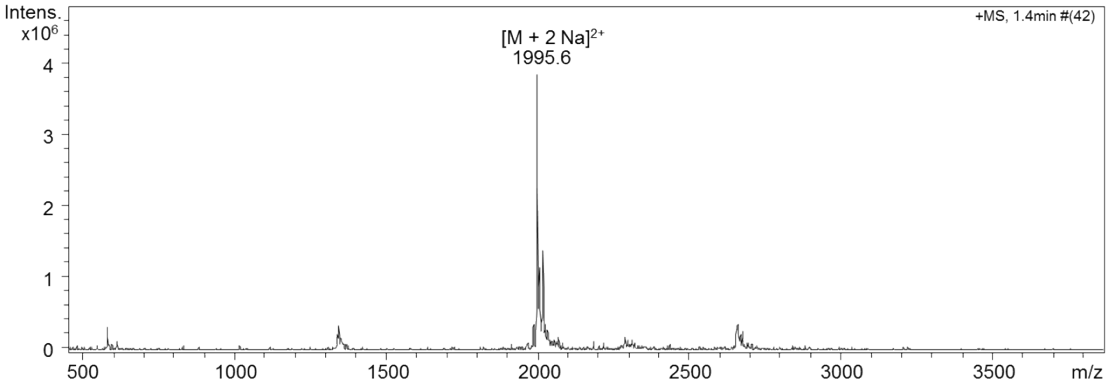

Figure S20. ESI-MS spectrum of compound **13**.

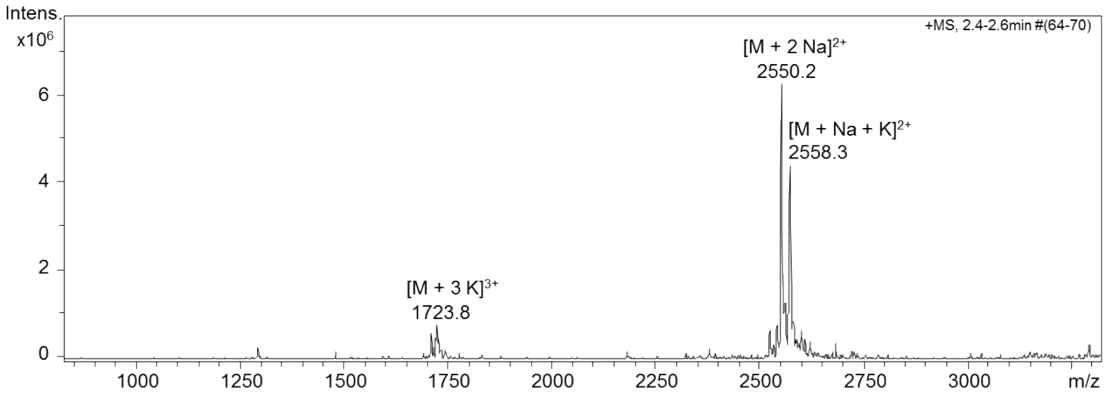

Figure S21. ESI-MS spectrum of compound **16**.

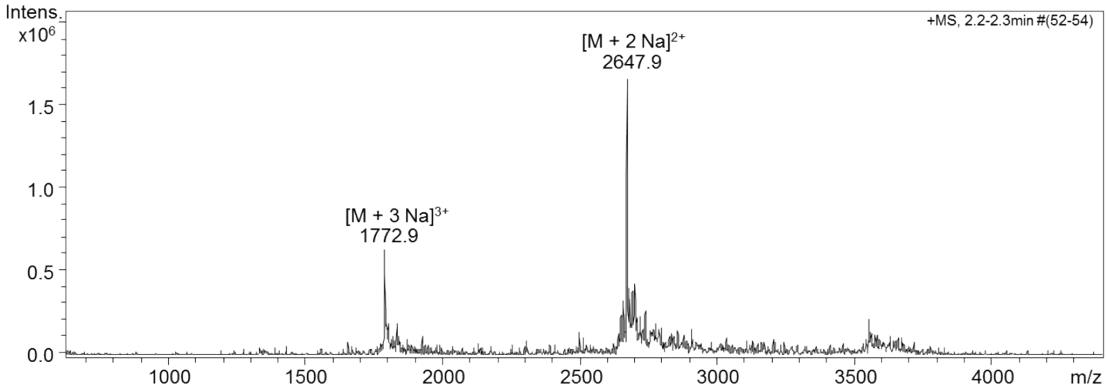

Figure S22. ESI-MS spectrum of compound **17**.

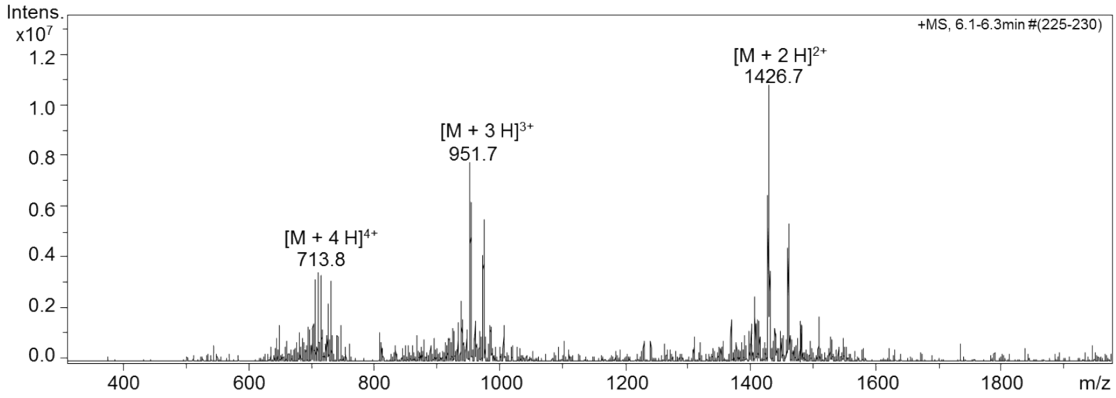

Figure S23. ESI-MS spectrum of compound $\boldsymbol{7_{Ac}}$.

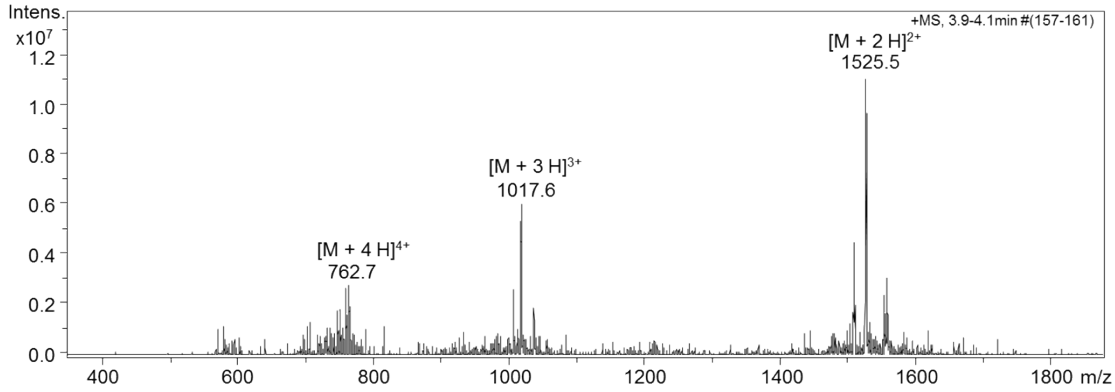

Figure S24. ESI-MS spectrum of compound $\boldsymbol{7_{Pr}}$.

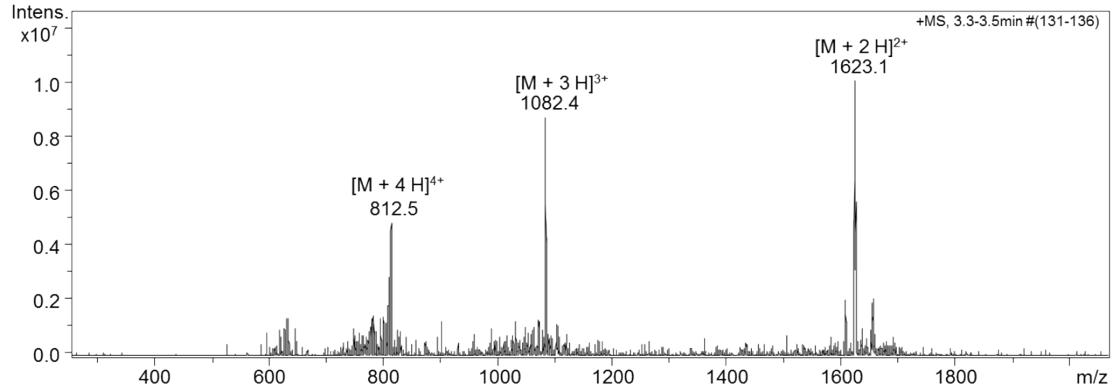

Figure S25. ESI-MS spectrum of compound $\boldsymbol{7_{But}}$.

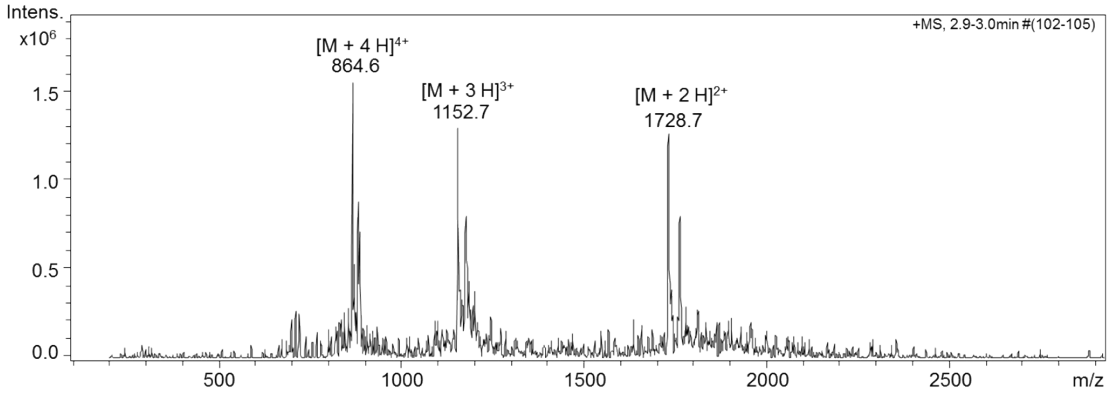

Figure S26. ESI-MS spectrum of compound $\boldsymbol{21_{Ac}}$.

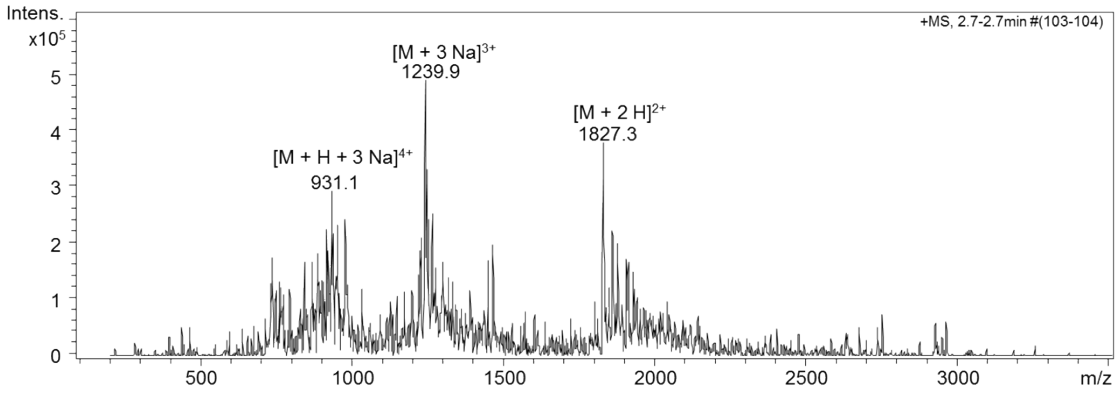

Figure S27. ESI-MS spectrum of compound ***21*$_{Pr}$**.

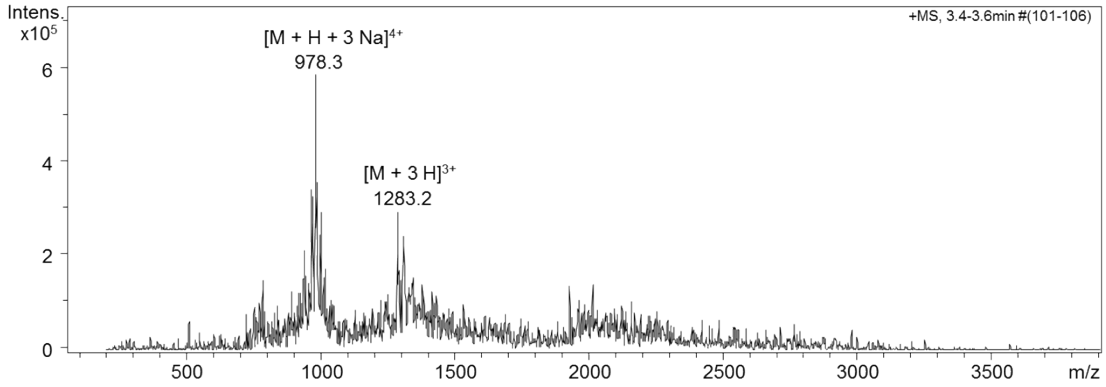

Figure S28. ESI-MS spectrum of compound ***21*$_{But}$**.

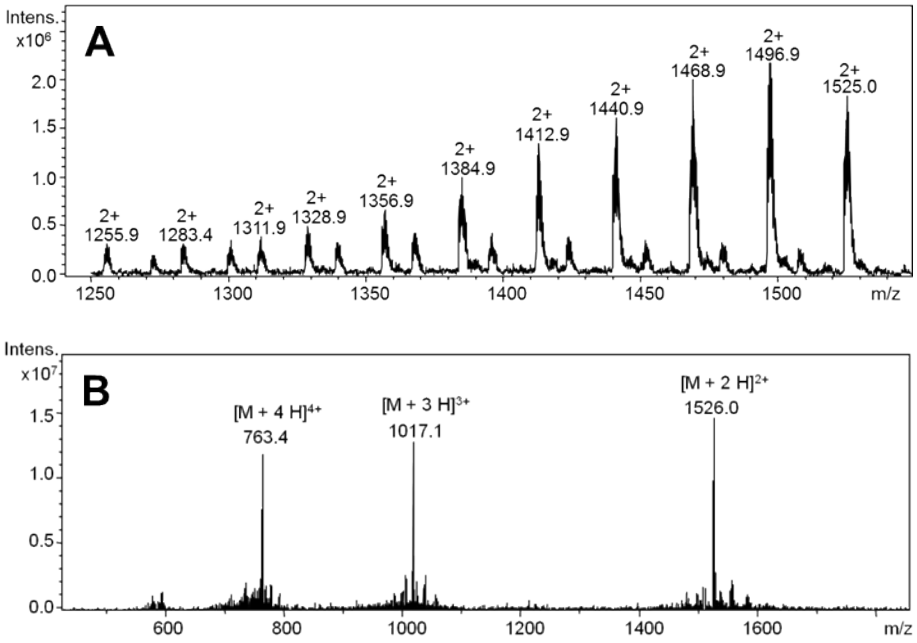

Figure S29. ESI-MS spectra of compound ***7***$_{Pr}$ obtained by carbamate hydrolysis after treatment with (A) 1:1 TFA-DCM at rt for 1.5 h, evidencing partial propanoyl ester hydrolysis (58 unit gaps), and (B) anhydrous TFA at 0 ºC.

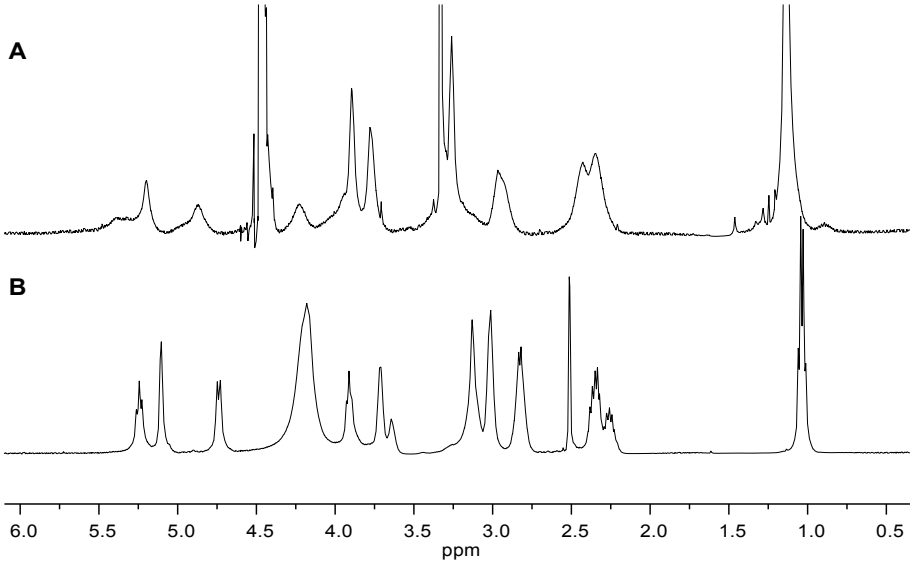

Figure S30. ^{1}H NMR spectra (500 MHz, 323 K) of compound ***7***$_{Pr}$ recorded in 2:1 D_2O-MeOD (A) and DMSO-d_6 (B), respectively, evidencing the different aggregation behavior depending of the media.

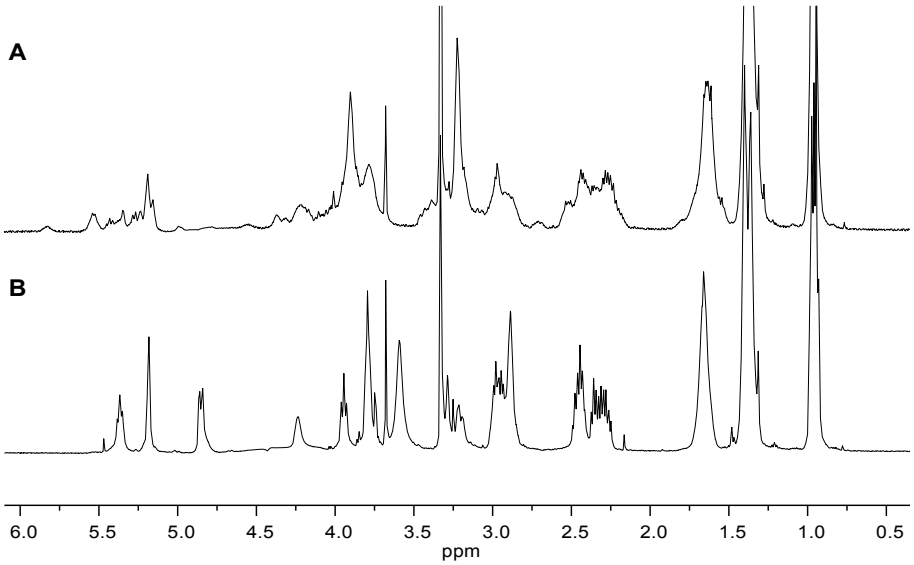

Figure S31. ^{1}H NMR spectra (500 MHz, 323 K, MeOD) of compound ***7***$_{Hex}$ before (A) and after (B) treatment with a mild basic anion exchange resin. The residual HDO peak was suppressed by presaturation.

Table S1. Buffering capacities of water solutions of cationic CDs ***7$_H$***, ***7$_{But}$***, ***21$_H$*** and ***21$_{But}$*** and of the control compounds **22** and **23** (50 µm in amino groups and 0.1 m in NaCl) determined as the meq of HCl consumed to switch from pH 7 to pH 5. Data for saline solution alone and PEI (25 kDa) are included as negative and positive reference, respectively.

compound	meq of HCl
7$_H$	17
7$_{But}$	67[c]
22	17
21$_H$	34
21$_{But}$	84[d]
23	< 17
none[a]	n. d.[b]
PEI (25 kDa)	134

[a]0.1 m NaCl solution alone. [b]Below detection limit. [c]ca. 50% of the buffering capacity of 25 kDa bPEI. [d]ca. 63% of the buffering capacity of 25 kDa bPEI.

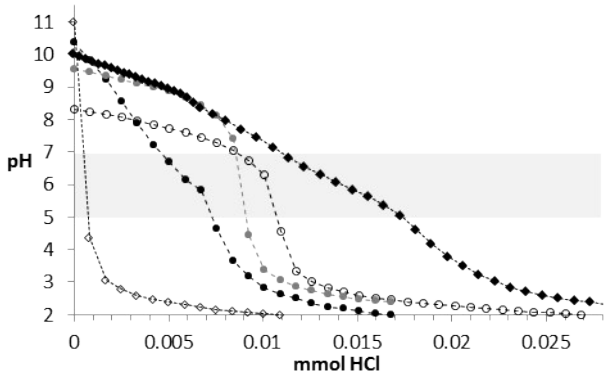

Figure S32. pH Titration curves of ***7$_H$*** (empty circles), ***7$_{But}$*** (black circles) and **22** (grey circles) at 50 µm concentration on a protonable-amine molar basis in the presence of 0.1 m NaCl. Titration plots for PEI (25 kDa, black diamonds) and NaCl alone (empty diamonds) are included as positive and negative references. The shadowed region represents the biologically relevant buffering window.

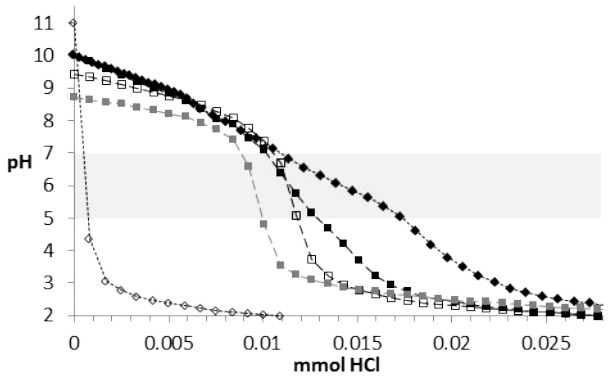

Figure S33. pH Titration curves of ***21$_H$*** (empty squares), ***21$_{But}$*** (black squares) and **23** (grey squares) at 50 µm concentration on a protonable-amine molar basis in the presence of 0.1 m NaCl). Titration plots for PEI (25 kDa, black diamonds) and NaCl alone (empty diamonds) are included as positive and negative reference, respectively. The shadowed region represents the biologically relevant buffering window.

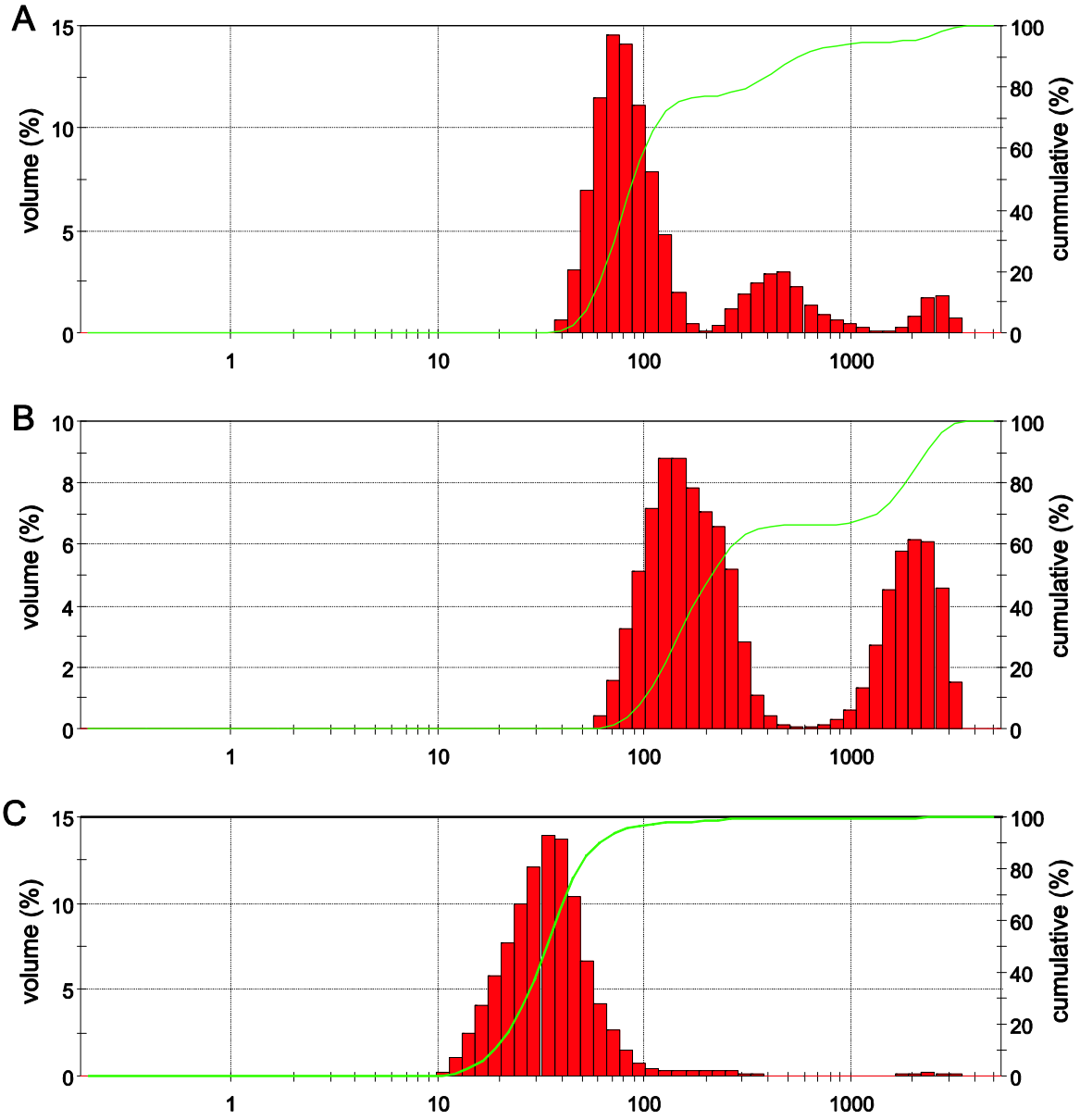

Figure S34. Comparative nanoparticle hydrodynamic ~~radius~~ diameter distribution of paCD ***21**$_{But}$* upon dispersion of a 20 mM solution in DMSO in (A) water, (B) HEPES buffer (20 mM, pH 7.4), or (C) a solution containing ctDNA at N/P 5, to a final paCD ***21**$_{But}$* concentration of 29 µM in all samples.

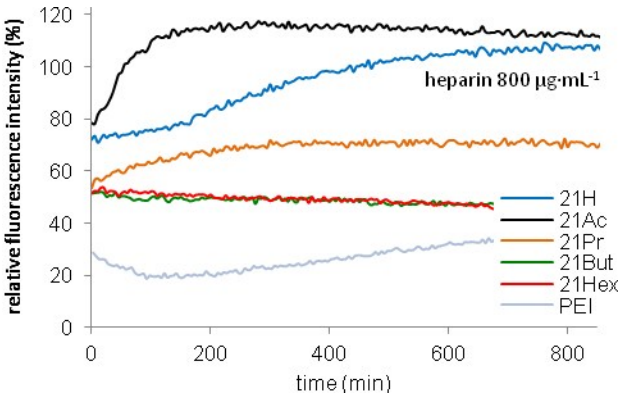

Figure S35. Time-dependent heparin-induced CDplex dissociation. RedSafe™ fluorescence recovery upon incubation of CDplexes formulated at N/P 5 with ctDNA and ***21***-cationic CDs $\boldsymbol{21_H}$ (blue), $\boldsymbol{21_{Ac}}$ (black), $\boldsymbol{21_{Pr}}$ (orange), $\boldsymbol{21_{But}}$ (green), and $\boldsymbol{21_{Hex}}$ (red) with heparin (800 µg·mL^{-1}). The behavior of ctDNA:PEI polyplexes (N/P 10) is included for comparative purposes.

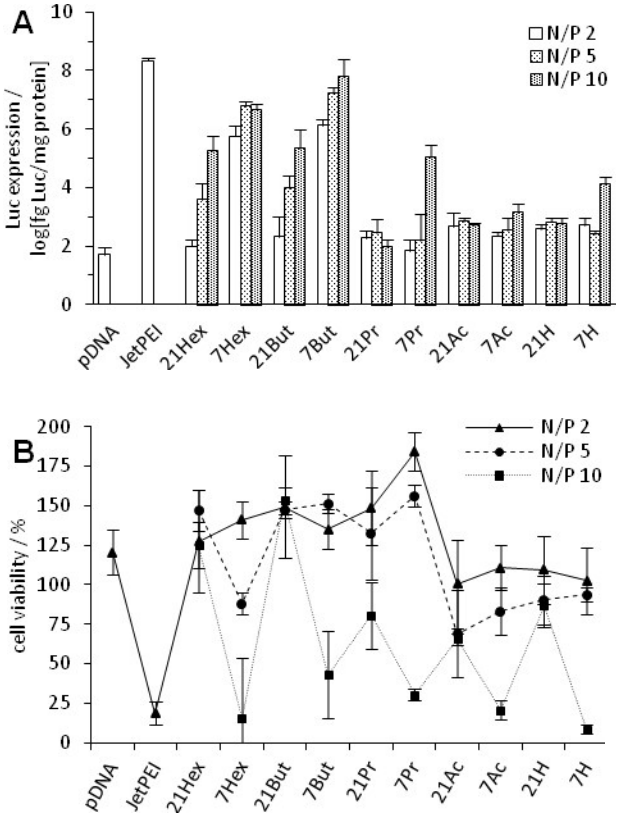

Figure S36. In vitro transfection efficiency (panel A) and cell viability (panel B) in 10% serum-containing medium in COS-7 cells of CDplexes formulated the luciferase encoding plasmid pTG11236 at N/P 2, 5 and 10. Naked pDNA and JetPEI polyplexes (N/P 10) were used as negative and positive controls, respectively.

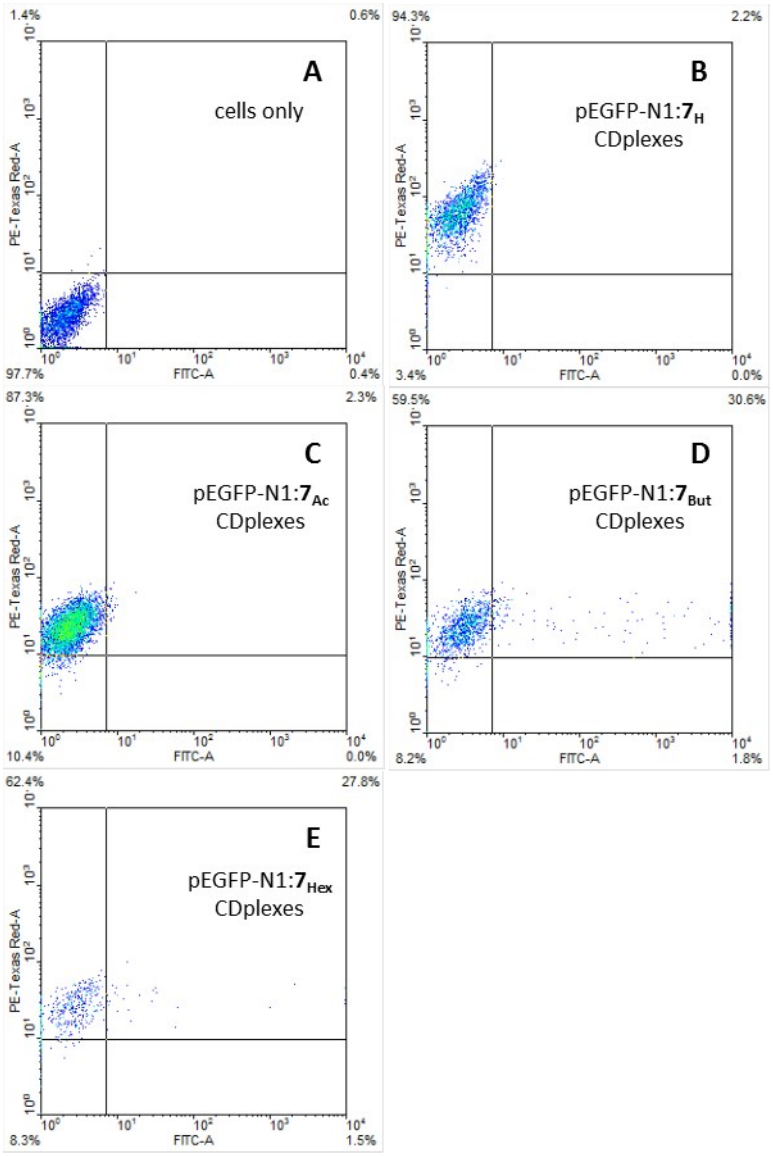

Figure S37. Fluorescence-assisted cell sorting (FACS) of COS-7 cells incubated (A) alone, in the presence of CDplexes formulated with cationic CD (B) ***7***$_{H}$, (C) ***7***$_{Ac}$, (D) ***7***$_{But}$, or (E) ***7***$_{Hex}$ at N/P 5 in 10% serum-containing medium. Spots in the upper left region represent internalized cells, while spots in the upper right region represent EGFP-expressing cells.

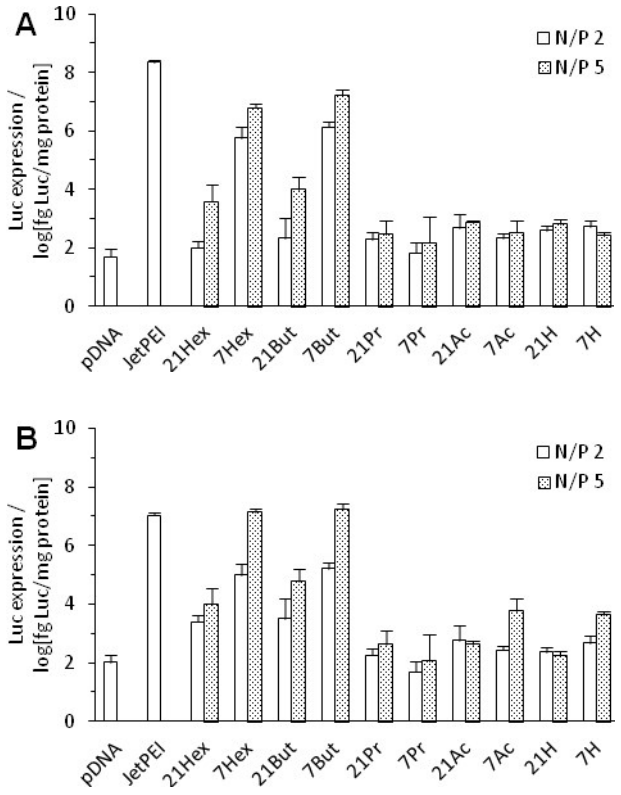

Figure S38. In vitro transfection efficiency (luciferace endosing gene in COS-7 cells) in 10% serum-containing in the absence (panel A) or presence (panel B) of the endosolomytic agent chloroquine. Naked pDNA and JetPEI polyplexes (N/P 10) were used as negative and positive controls, respectively.