Supporting Information for

Trehalose-Polyamine/DNA Nanocomplexes: Impact of Vector Architecture on Cell and Organ Transfection Selectivity

Fernando Ortega-Caballero,[a,b] María L. Santana-Armas,[c] Conchita Tros de Ilarduya,[c] Christophe Di Giorgio,[d] Raphäel Tripier,[e] Nathalie Le Bris,[e] Cedric Ollier,[e] Carmen Ortiz Mellet,[a] José M. García Fernández,[f] José L. Jiménez Blanco,[a*] Alejandro Méndez-Ardoy[a,f*]

General Methods.

Thin layer chromatography (TLC) was performed on aluminum sheets coated with Silica Gel 60 F_{245} (layer thickness 0.25 mm, *E. Merck*), with visualization by UV light (λ 254 nm) and by charring with 10% ethanolic H_2SO_4, 0.1% ethanolic ninhydrin and heating at 100 ºC. With preparative purposes**, column chromatography** was carried out on *Silice 60 A.C.C. Chromagel* (SDS 70-200 and 35-70 μm). **Optical rotations** were measured with a *JASCO P-2000 polarimeter*, using a sodium lamp (λ 589 nm) at 22 ºC in 1 cm or 1 dm tubes. **Elemental analyses** were performed at the Servicio de Microanálisis del Instituto de Investigaciones Químicas de Sevilla, Spain, with a *Leco CHNS-932* elemental analyzer. **IR spectra** were recorded on a *JASCO FTIR-410 instrument* and were processed using the Jasco Spectra Manager ™ software. It has been recorded both in solid and in solution using an ATR MIRacleTM, presenting data indicating the numbers corresponding to the maximum absorption wavelength. **NMR experiments** were performed at 300 (75.5), and 500 (125.7) MHz using *Bruker DMX300*, and *DRX500* spectrometers. 1D TOCSY as well as 2D COSY and HMQC experiments were carried out to assist on signal assignment. The chemical shift values (δ) are given in ppm (parts per million), using the solvent as internal standard, tetramethylsilane (for $CDCl_3$). The values of the coupling constants (*J*) are measured in Hz abbreviations to indicate the multiplicity of the signals are: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), q (quintet) and m (multiplet). **Mass spectra** were performed on a *Bruker Daltonics Esquire6000* ™. The samples were

introduced via a solid probe heated from 30 to 280 °C. ESI as ionization source (Electrospray Ionization) was used to which methanol was used as solvent. The samples were introduced via direct injection using a Cole-Parmer syringe at a flow of 120 $\mu L \cdot h^{-1}$.

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 plasmid pCMV100-IL-12 (5500 bp) encoding interleukin-12 (IL-12) was provided by Dr. C. Tros de Ilarduya's research group (University of Navarra). 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), COS-7 (American green monkey kidney), RAW264.7 (murine macrophage), HeLa (human cervical cancer) 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. 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 trehalose and CT derivative or cationic polymer (bPEI, 25 kDa). pDNA complexes were prepared with plasmid DNA

and the corresponding trehalose and CT polycationic supramolecules 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 trehalose 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.

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. 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 paTrehalose/CTplexes were prepared in BHG (HEPES 10 mM, pH 7.4, glucose 5% w/v) at N/P ratio 10 to a final concentration of 50 µg/mL. Then, samples were electrophoresed for 30 min under 150 mV in 0.8% agarose gel. 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.

Particle size and zeta-potential measurements. The size of the paTrehalose/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 BHG (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.

Transmission Electron Microscopy (TEM). Formvar-carbon coated grids previously made hydrophilic by glow discharge were placed on top of small drops of the paTrehalose/CTplexes (HEPES 20 mM, pH 7.4, DNA 303 µM phosphate) prepared as describe above using N/P 20 ratios. After 1-3 min, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grids were then dried and observed with a Philips CM12 electron microscope working under standard conditions. All the experiments were reproduced twice on each formulation.

In vitro transfection activity. The procedure for *in vitro* transfection assays was the same for four 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 picograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein. Citokine levels were obtained using the kit BD OptEIA ELISA Set (Pharmingen, San Diego,

CA, U.S.A.) for IL-12 p70 following the manufacturer's instructions. Values were calculated based on a standard curve. Samples were analized in a plate spectrophotometer Power Wave XS and a data processor KC junior, BioTek®.

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.

***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. Individual mice in groups of eight were injected via the tail vein with 200 µL of complexes containing 60 µg of pCMV-Luc VR1216 plasmid DNA at N/P 10 in BHG (HEPES 10 mM, pH 7.4, glucose 5% w/v). 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. The data were expressed as picograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein.

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

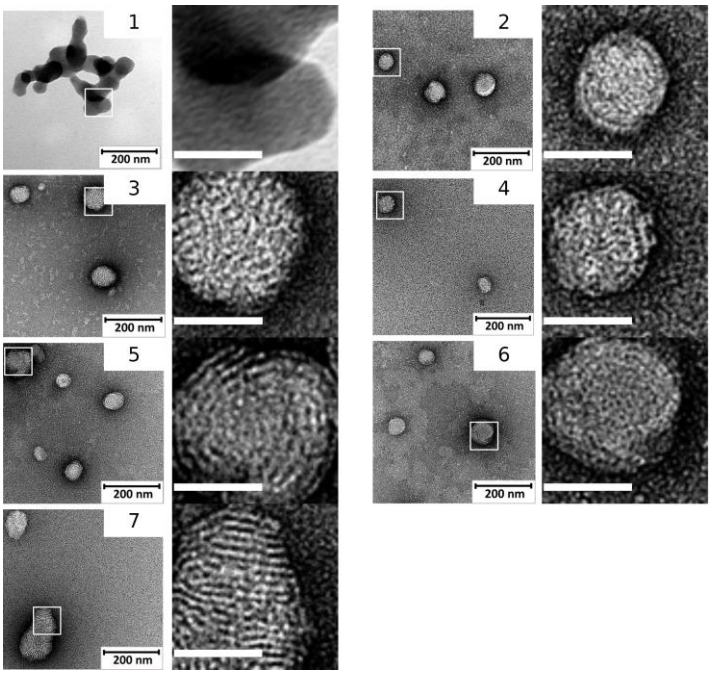


Figure S1: Representative TEM micrographs of the DNA-vector complexes at N/P 20 (HEPES 20 mM, pH 7.4, DNA 303 µM phosphate) and magnifications, shown to the right of each micrograph. Scale bar in magnifications denote 50 nm.

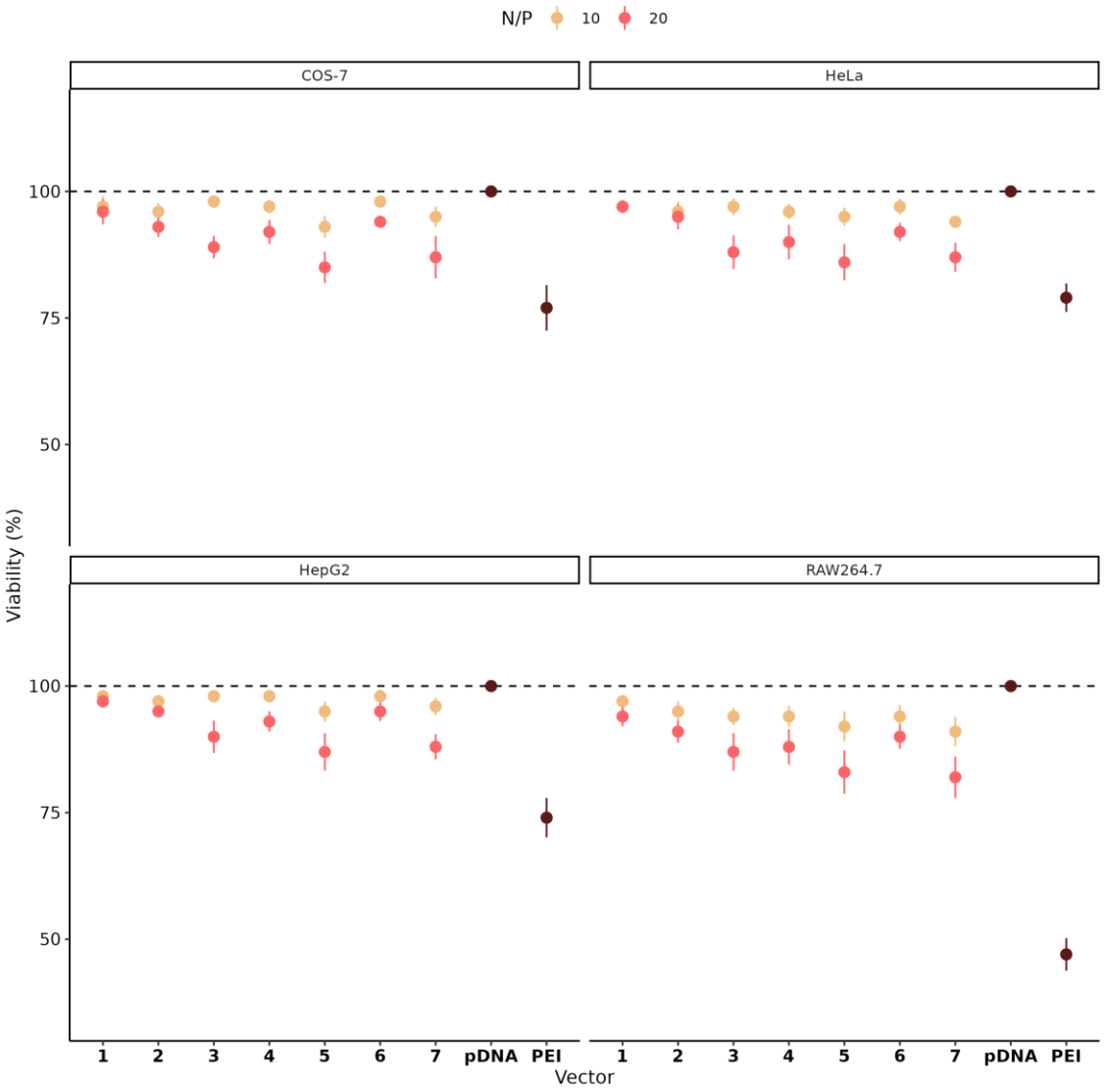


Figure S2. Cell viability determined by MTT experiments with nanocomplexes formulated at N/P 10 (yellow dots) and N/P 20 (red dots). Vertical lines denote the standard deviation. pDNA and PEI were used as negative and positive toxicity controls, respectively.

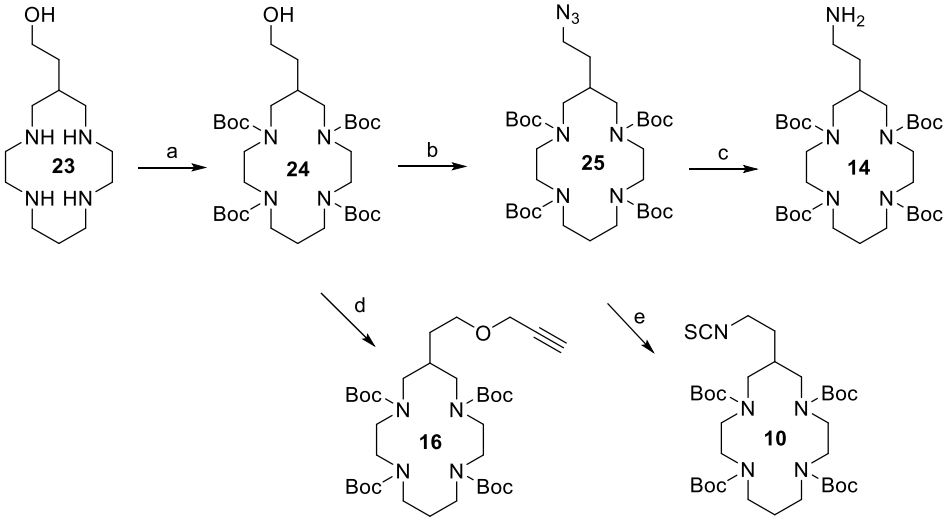
EXPERIMENTAL

Materials. All reagents and solvents used in this work were purchased from commercial sources and used without further purification, unless otherwise specified. TEI CT2 (**1**),[i] [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 (**8**),[ii] 2-{N^{I},

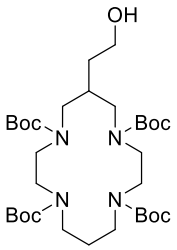
N^{II}, N^{III}, N^{IV}-[triethylenetetra(*tert*-butoxycarbonyl)amino]}ethaneisothiocyanate (**9**),[i] 6,6'-dideoxy-2,2',3,3',4,4'-hexa-*O*-hexanoyl-6,6'-diisothiocyanate-α,α'-trehalose (**13**),[ii] 6,6'-Diazido-6,6'-dideoxy-2,2',3,3',4,4'-hexa-*O*-hexanoyl-α,α'-trehalose (**17**),[iii] 1,4,8-tris(*tert*-butyloxycarbonyl)-11-propargyl-1,4,8,11-tetraazacyclotetradecane (**18**),[iv] 1,4,7-tris(*tert*-butyloxycarbonyl)-10-propargyl-1,4,7,10-tetraazacyclododecane (**19**)[v] and 3-(2-hydroxyethyl)-1,5,8,12-tetraaza-cyclotetradecane (**23**)[vi], were obtained according to literature procedures.

Synthesis.

Preparation of 3-ethyl-cyclam derivatives

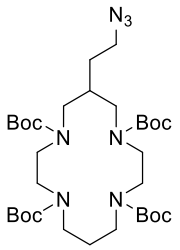


Scheme S1. Synthesis of cyclam derivatives. Reagents and conditions: a) Boc_2O, DCM, rt, overnight, 96%; b) i. MsCl, DCM, Et_3N, 0 ºC→rt, overnight, ii. NaN_3, DMF, Ar, 65 ºC, overnight, 92%; c) Pd/C, MeOH, H_2, rt, overnight, quantitative; d) Propargyl bromide, NaH, DMF, Ar, 0 ºC→rt, overnight, 35%.; e) CS_2, TPP, dioxane, Ar, rt, overnight, 63%.



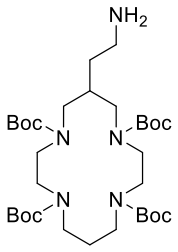
1,5,8,12-Tetrakis(*tert*-butoxycarbonyl)-3-(2-hydroxyethyl)-1,5,8,12-tetraazacyclotetradecane (24). To a solution of 3-(2-hydroxyethyl)-1,5,8,12-tetraazacyclotetradecane (**23**)[vi] (203 mg, 0.83

mmol) in DCM (60 mL) di-*tert*-butyl dicarbonate (Boc_2O, 796 mg, 3.65 mmol) was added. The reaction mixture was stirred at room temperature overnight. The solvent was removed, the oily residue dissolved in diethyl ether (10 mL) and rapid stripping of all solvent under vacuum yielded an amorphous solid. The residue was purified by column chromatography (1:1→2:1→4:1 EtOAc-petroleum ether) to give **24.** Yield: 516 mg (96%); R_f = 0.27 (2:1 EtOAc-petroleum ether); IR: ν_{max} = 2976, 2933, 1683, 1156; ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ = 4.22 (bt, 1 H, OH), 3.64 (dt, 2 H, $^2J_{H,H}$ = 14.4, $^3J_{H,H}$ = 6.9 Hz, NCH_2-cyclam), 3.47 (m, 2 H, CH_2OH), 3.43 (m, 2 H, NCH_2-cyclam), 3.29-3.14 (m, 10 H, NCH_2-cyclam), 3.01 (dt, 2 H, $^3J_{H,H}$ = 5.9 Hz, NCH_2-cyclam), 2.01 (m, 1 H, CH-cyclam), 1.77 (m, 1 H, CHa-cyclam), 1.64 (m, 1 H, CHb-cyclam), 1.45 (s, 36 H, Boc), 1.38 (m, 2 H, CH_2CH_2OH); ^{13}C NMR (125.7 MHz, DMSO-d_6, 343 K): δ = 155.7, 155.3 (CO), 79.3, 79.2 (*C*Me_3), 58.9 (*C*H_2OH), 51.2, 48.0, 47.7, 46.8 (NCH_2-cyclam), 34.3 (CH-cyclam), 33.5 (*C*H_2CH_2OH), 28.6 (Me), 28.4 (CH_2-cyclam). ESIMS: *m/z* = 667,5 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{60}N_4O_9$: calcd. C, 59.60; H, 9.38; N, 8.69; found: C, 59.67; H, 9.47; N, 8.56.



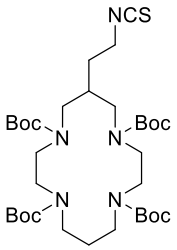
3-(2-Azidoethyl)-1,5,8,12-tetrakis(*tert*-butoxycarbonyl)-1,5,8,12-tetraazacyclotetradecane (25). To a solution of **24** (362 mg, 0.56 mmol) and Et_3N (389 μL, 2.81 mmol) in DCM (18 mL) at 0 ºC methanosulfonyl chloride (130 μL, 1.68 mmol) was added and the reaction mixture was stirred at room temperature overnight. The organic phase was then washed with saturated aqueous $NaHCO_3$ (2 x 25 mL), 5% HCl (2 x 25 mL) and dried ($MgSO_4$), filtered and concentrated. The crude residue was dissolved in DMF (6 mL) and then, NaN_3 (109 mg, 1.68 mmol) was added. The reaction mixture was stirred at 65 ºC under Ar overnight. The solvent was removed under vacuum and the residue was suspended in water (15 mL), extracted with Et_2O (3 x 15 mL), dried

($MgSO_4$), filtered, concentrated and purified by column chromatography (1:2 EtOAc-petroleum ether) to give **25.** Yield: 344 mg (92%); R_f = 0.56 (1:2 EtOAc-petroleum ether); IR: ν_{max} = 2977, 2928, 2096, 1683, 1156; ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ = 3.67 (dt, 2 H, $^2J_{H,H}$ = 14.7, $^3J_{H,H}$ = 5.9 Hz, NCH_2-cyclam), 3.45 (m, 2 H, NCH_2-cyclam), 3.39 (t, 2 H, $^3J_{H,H}$ = 7.4 Hz, CH_2N_3), 3.33-3.15 (m, 10 H, NCH_2-cyclam), 3.00 (dt, 2 H, $^3J_{H,H}$ = 5.1 Hz, NCH_2-cyclam), 1.97 (m, 1 H, CH-cyclam), 1.76 (m, 1 H, CHa-cyclam), 1.66 (m, 1 H, CHb-cyclam), 1.52 (q, 2 H, $CH_2CH_2N_3$), 1.43, 1.42 (s, 36 H, Boc); ^{13}C NMR (125.7 MHz, DMSO-d_6, 343 K): δ = 155.7, 155.3 (CO), 79.5, 79.2 (CMe_3), 50.7 (NCH_2-cyclam), 48.7 (CH_2N_3), 47.7, 46.8 (NCH_2-cyclam), 35.3 (CH-cyclam), 29.4 ($CH_2CH_2N_3$), 28.6 (Me), 28.5 (CH_2-cyclam). ESIMS: *m/z* = 692,5 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{59}N_7O_8$: calcd. C, 57.38; H, 8.88; N, 14.64; found: C, 57.26; H, 8.66; N, 14.42.

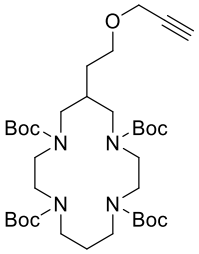


3-(2-Aminoethyl)-1,5,8,12-tetrakis(*tert*-butoxycarbonyl)-1,5,8,12-tetraazacyclotetradecane (14). To a solution of **25** (249 mg, 0.37 mmol) in MeOH (29 mL) at room temperature and under H_2 (1atm), 10% wet Pd/C (38 mg) was added, and the reaction mixture was stirred at room temperature overnight. The mixture was filtered over celite and concentrated to give **14** quantitatively. Yield: 239 mg (100%); R_f = 0.15 (40:10:1 DCM-MeOH-H_2O); IR: ν_{max} = 3423, 2977, 2927, 1682, 1160; ^{1}H NMR (500 MHz, DMSO-d_6, 343 K): δ = 3.69 (dt, 2 H, $^2J_{H,H}$ = 14.1, $^3J_{H,H}$ = 6.3 Hz, NCH_2-cyclam), 3.44-3.16 (m, 12 H, NCH_2-cyclam), 2.99 (dt, 2 H, $^3J_{H,H}$ = 5.5 Hz, NCH_2-cyclam), 3.39 (t, 2 H, $^3J_{H,H}$ = 8.0 Hz, CH_2NH_2), 1.96 (m, 1 H, CH-cyclam), 1.74 (m, 1 H, CHa-cyclam), 1.67 (m, 1 H, CHb-cyclam), 1.49 (q, 2 H, $CH_2CH_2N_3$), 1.43, 1.42 (s, 36 H, Boc); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 156.3, 155.6 (CO), 80.6, 79.9 (CMe_3), 51.9-46.7 (NCH_2-cyclam), 48.9 (CH_2NH_2), 36.1 (CH-

cyclam), 29.7 (*C*$H_2CH_2NH_2$), 28.5 (Me, CH_2-cyclam). ESIMS: *m/z* = 644.6 $[M + H]^+$, 666.6 $[M + Na]^+$. Anal. Calcd for $C_{32}H_{61}N_5O_8$: calcd. C, 59.69; H, 9.55; N, 10.88; found: C, 59.32; H, 9.36; N, 10.51.

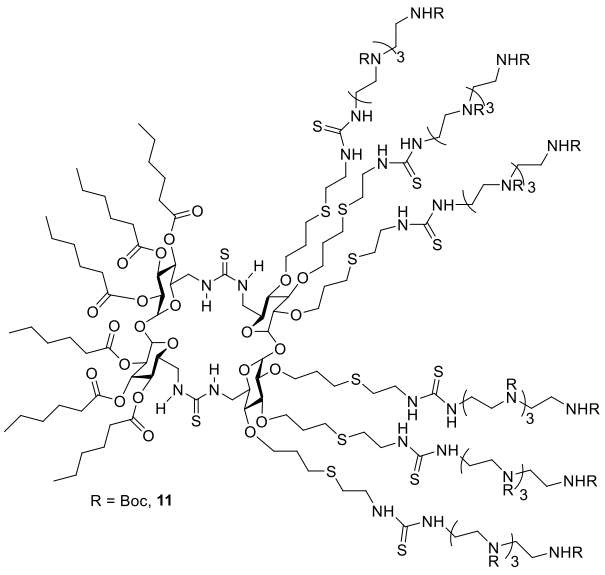


3-(2-Isothiocyanatoethyl)-1,5,8,12-tetrakis(*tert*-butoxycarbonyl)-1,5,8,12-tetraazacyclotetradecane (10). To a solution of azide **25** (0.401 g, 0.60 mmol) in dry dioxane (14 mL) TPP (173 mg, 0.66 mmol) and CS_2 (0.44 mL, 5.99 mmol) were added under Ar atmosphere. The solution was stirred at room temperature for 24 h. Then the solvents were removed, and the residue was purified by column chromatography using 1:2 EtOAc-cyclohexane as eluent. Yield: 260 mg (63%); R_f = 0.29 (1:2 EtOAc-cyclohexane); IR: ν_{max} = 3370, 2974, 2931, 2058, 1682, 1158; ^{1}H NMR (400 MHz, DMSO-d_6, 343 K): δ = 3.73 (t, 2 H, $^3J_{H,H}$ = 6.9 Hz, CH_2NCS), 3.47 (dt, 2 H, $^2J_{H,H}$ = 14.8, $^3J_{H,H}$ = 5.3 Hz, NCH_2-cyclam), 3.45 (m, 2 H, NCH_2-cyclam), 3.35-3.16 (m, 12 H, NCH_2-cyclam), 3.00 (dt, 2 H, $^3J_{H,H}$ = 5.3 Hz, NCH_2-cyclam), 1.99 (m, 1 H, CH-cyclam), 1.73 (m, 1 H, CHa-cyclam), 1.64 (q, 2 H, CH_2CH_2NCS), 1.63 (m, 1 H, CHb-cyclam), 1.44, 1.43 (s, 36 H, Boc); ^{13}C NMR (100.6 MHz, DMSO-d_6, 343 K): δ = 155.7, 155.3 (CO), 79.6, 79.3 (*C*Me_3), 50.5, 48.1, 47.9, 46.9 (NCH_2-cyclam), 43.2 (*C*H_2NCS), 35.2 (CH-cyclam), 30.9 (*C*H_2CH_2NCS), 28.6 (Me, CH_2-cyclam). ESIMS: *m/z* = 708.5 $[M + Na]^+$. Anal. Calcd for $C_{33}H_{59}N_5O_8S$: calcd. C, 57.79; H, 8.67; N, 10.21; S, 4.67 found: C, 57.87; H, 8.71; N, 10.13.

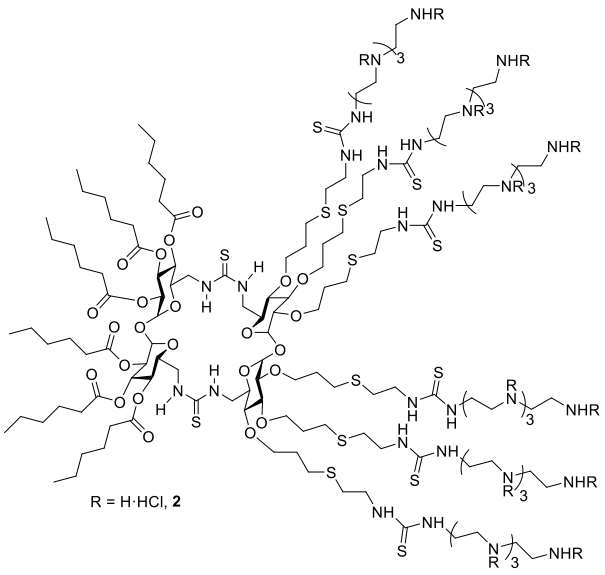


1,5,8,12-Tetrakis(*tert*-butoxycarbonyl)-3-(2-propargyloxyethyl)-1,5,8,12-tetraazacyclotetra-decane (16). To a solution of **24** (300 mg, 0.46 mmol) in dry DMF (10 mL) and NaH (796 mg, 3.65 mmol) at 0 ºC under Ar atmosphere, propargyl bromide (120 μL, 1.40 mmol) was added. The reaction mixture was stirred at room temperature overnight and quenched by addition of MeOH (5 mL). The solvent was removed under vacuum, the residue was suspended in EtOAc (15 mL) and H_2O (15 mL), the organic phase was separated and dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:2→1:1 EtOAc-petroleum ether) to give **16.** Yield: 110 mg (35%); R_f = 0.83 (2:1 EtOAc-petroleum ether); IR: ν_{max} = 2975, 2930, 1683, 1158; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.07 (d, 2 H, $^4J_{H,H}$ = 2.4 Hz, CH_2C≡CH), 3.67-2.99 (m, 14 H, NCH_2-cyclam), 3.53 (t, 2 H, $^3J_{H,H}$ = 6.1 Hz, CH_2O), 2.38 (t, 1 H, C≡CH), 1.99 (m, 1 H, CH-cyclam), 1.77 (m, 4 H, CH_2-cyclam), 1.43 (s, 36 H, Boc), 1.22 (m, 2 H, CH_2CH_2O); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 155.9, 155.5 (CO), 79.8 (*C*Me_3), 79.7 (*C*≡CH), 74.4 (*C*H_2C≡CH), 67.3 (*C*H_2O), 74.4 (C≡*C*H), 51.7-46.8 (NCH_2-cyclam), 34.6 (CH-cyclam), 29.7 (*C*H_2CH_2O), 28.4 (Me, CH_2-cyclam). ESIMS: *m/z* = 709.7 [M + Na]$^+$. Anal. Calcd for $C_5H_{62}N_4O_9$: calcd. C, 61.56; H, 9.15; N, 8.20; found: C, 61.63; H, 9.19; N, 8.13.

Synthesis of Janus amphiphilic trehalose-derived vectors

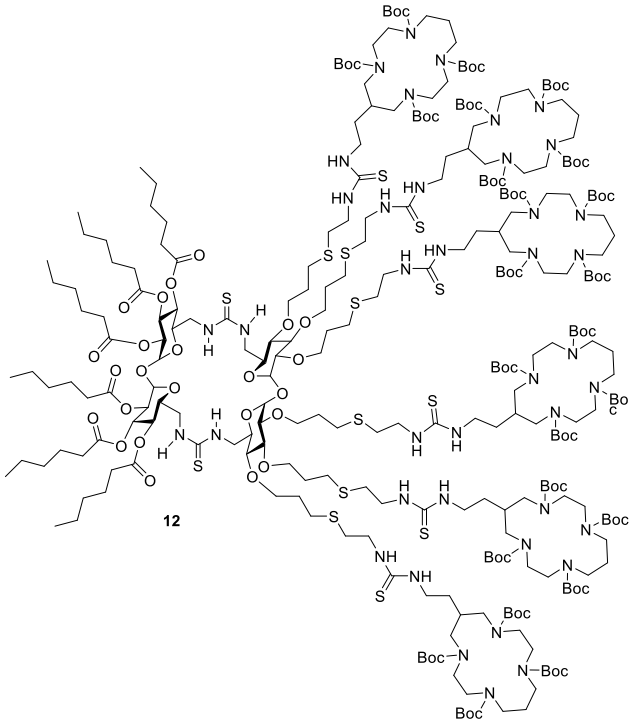


{*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-[3-(2-(2-(N^{I},N^{II},N^{III},N^{IV}-triethylenetetra(*tert*-butoxy-carbonylamino)ethyl)thioureido)ethylthio)propyl]-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-di-deoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]} thiourea (11). To a solution of **8**^{ii} (146 mg, 0.064 mmol) and DMAP (94 mg, 0.77 mmol) in pyridine (15 mL), a solution of **9**^{i} (267 mg, 0.42 mmol) in pyridine (10 mL) was slowly added and the mixture was stirred at 40 ºC for 24 h. The mixture was concentrated, and the resulting residue purified by column chromatography (1:1 → 4:1 EtOAc-cyclohexane). Yield: 276 mg (74%). R_f = 0.69 (4:1 EtOAc-cyclohexane); $[\alpha]_D$ = +46.1 (*c* 1.0, DCM); IR (ATR): ν_{max} = 3347, 2973, 2930, 1749, 1695 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, 323 K): δ = 7.25-6.66 (m, 16 H, NHCS), 5.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3'), 5.47 (bs, 2 H, H-1'), 5.07 (bs, 14 H, NHBoc, H-1), 5.04 (t, 2 H, $J_{4,5}$ = 9.5 Hz, H-4'), 4.91 (bd, 2 H, H-2'), 3.91-3.54 (m, 32 H, CH_2O, SCH_2CH_2NHCS, H-6ab, H-6'ab), 3.88 (m, 2 H, H-5), 3.80 (m, 2 H, H-5'), 3.59 (m, 2 H, H-3), 3.48 (bs, 2 H, H-2), 3.42 (m, 12 H, CH_2NHCS), 3.34 (bs, 72 H, CH_2NBoc), 3.27 (bt, 12 H, CH_2NHBoc), 3.16 (bs, 2 H, H-4), 2.77 (m, 12 H, SCH_2CH_2NHCS), 2.67 (m, 12 H, CH_2S), 2.36-2.23 (m, 12 H, H-2_{Hex}), 1.94-1.87 (m, 12 H, CH_2CH_2O), 1.63-1.55 (m, 12 H, H-3_{Hex}), 1.48, 1.44 (2 x s, 216 H, CMe_3), 1.36-1.27 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.95-0.89 (m, 18 H, H-6_{Hex}); ^{13}CNMR (100.6 MHz, $CDCl_3$, 323 K): δ = 182.7 (CS), 172.8, 172.1 (CO ester), 156.6, 155.9, 155.4 (CO carbamate), 92.5 (C-1), 91.2 (C-1'), 81.1 (C-3), 80.1 (C-2, CMe_3), 79.6 (C-4), 79.1 (CMe_3), 71.8 (C-5, C-5', OCH_2), 70.4 (OCH_2), 70.1 (C-2'), 69.1 (C-3'), 68.2 (C-4'), 59.7 (C-6, C-6'), 47.3, 47.0, 45.6 (CH_2NBoc), 43.7 (SCH_2CH_2NHCS), 39.6 (CH_2NHBoc), 33.9 (C-2_{Hex}), 31.2 (C-4_{Hex}), 30.7 (SCH_2CH_2NHCS), 30.2, 29.6 (CH_2CH_2O), 28.5, 28.4 (CMe_3), 28.0 (CH_2S,), 24.5, 24.4 (C-3_{Hex}), 22.2, 22.1 (C-5_{Hex}), 13.8, 13.7 (C-6_{Hex}). Anal. Calcd for $C_{266}H_{488}N_{40}O_{72}S_{14}$: C, 54.63; H, 8.41; N, 9.58; S, 7.68; found: C, 54.79; H, 8.70; N, 9.21; 7.34.



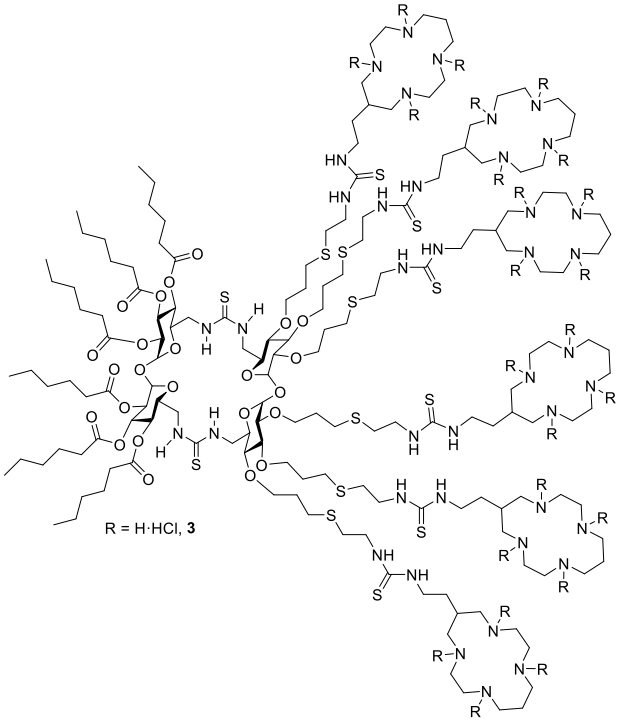
{*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-[3-(2-(2-(N^{I},N^{II},N^{III},N^{IV}-triethylenetetra-(ammonium)ethyl)thioureido)ethylthio)propyl]-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]} thiourea tetraeicosahydrochloride (2). Compound **11** (226 mg, 0.039 mmol) was achieved by treatment with 1:1 DCM-TFA (3 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 H_2O-HCl 0.1 N and freeze-dried to yield the product as hydrochloride. Yield: 167 mg (100%). $[\alpha]_D$ = +43.3 (*c* 1.0, MeOH); IR (ATR): ν_{max} = 3280, 2955, 2927, 2858, 1671 cm^{-1}; ^{1}H NMR (400 MHz, D_2O, 353 K): δ = 5.95 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz,H-3'), 5.86 (bs, 2 H, H-1'), 5.67 (bs, 2 H, H-1), 4.62-4.56 (m, 4 H, H-2', H-4'), 4.41 (bt, 14 H, H-5, H-5', CH_2N), 4.39-4.19 (m, 20 H, CH_2O, H-6ab, H-6'ab), 4.17 (bt, 14 H, H-3, C*H*$_2$NHCS), 4.10-3.89 (m, 60 H, CH_2N), 3.95 (m, 2 H, H-2), 3.75 (bs, 2 H, H-4), 3.28 (m, 12 H, SC*H*$_2$CH$_2$NHCS), 3.18 (t, 12 H, $^{3}J_{H,H}$ = 6.9 Hz, CH_2S) 2.92-2.70 (m, 12 H, H-2$_{Hex}$), 2.45-2.35 (m, 12 H, C*H*$_2$CH$_2$O), 2.12-2.00 (m, 12 H, H-3$_{Hex}$), 1.83-1.73 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 1.41-1.36 (m, 18 H, H-6$_{Hex}$); ^{13}CNMR (100.6 MHz, D_2O, 353 K): δ = 183.0, 182.8 (CS), 165.9, 165.6 (CO), 91.6 (C-1), 91.2 (C-1'), 80.8 (C-3), 79.8 (C-2, C-4), 71.9, 71.5, 71.0, 70.7 (C-3', OCH_2, C-2'), 70.5 (C-5), 70.0 (C-5'), 69.5 (C-4'), 48.5, 45.4, 44.3, 44.1 (CH_2N), 40.7 (CH_2NHCS), 36.2 (CH_2N), 34.7, 34.2 (C-2$_{Hex}$), 31.6, 31.5 (C-4$_{Hex}$), 31.0 (SCH_2CH$_2$NHCS), 30.7, 30.4 (C*H*$_2$CH$_2$O), 28.8 (CH_2S,), 24.7, 24.5 (C-3$_{Hex}$), 22.4 (C-5$_{Hex}$), 13.9 (C-6$_{Hex}$);

ESIMS: *m/z* = 1723 $[M + 2H]^{2+}$. Anal. Calcd for $C_{146}H_{278}N_{40}O_{24}S_{14}\cdot 24HCl\cdot 12H_2O$: C, 38.81; H, 7.27; N, 12.40; S, 9.93; found: C, 38.90; H, 7.38; N, 12.35; S, 9.68.



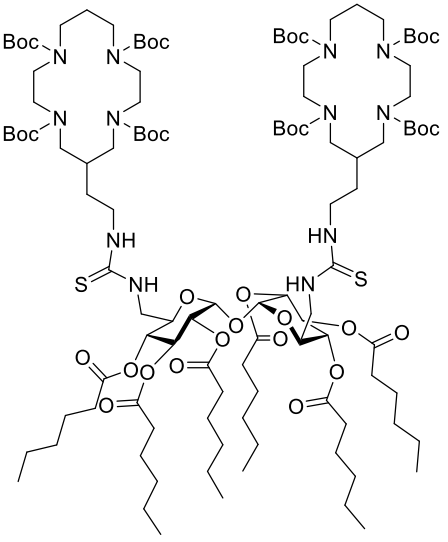
[*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-(3,6,10,13-tetrakis(*tert*-butoxycarbonyl)-3,6,10,13-tetraazacyclotetradecyl)ethylthioureido)ethylthio)propyl)-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]] thiourea (12). To a solution of **8**^{ii} (130 mg, 0.057 mmol) and DMAP (84 mg, 0.69 mmol) in pyridine (5 mL), a solution of **10** (260 mg, 0.38 mmol) in pyridine (5 mL) 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 (20:1 DCM-H_2O). Yield: 124 mg (35%). R_f = 0.43 (20:1 DCM-H_2O); $[\alpha]_D$ = +47.0 (*c* 1.0, DCM); IR (ATR): ν_{max} = 3324, 2975, 2932, 1688, 1159 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 6.94-6.69 (m, 16 H, NHCS), 5.52 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.2 Hz, H-3'), 5.46 (bs, 2 H, H-1'), 5.06 (d, 2 H, H-1), 5.02 (bt, 2 H, $J_{4,5}$ = 10.1 Hz, H-4'), 4.90 (bd, 2 H, H-2'), 3.91-3.50 (m, 20 H, CH_2O, H-6ab, H-6'ab), 3.88 (m, 2 H, H-5), 3.76 (m, 2 H, H-5'), 3.76-3.50 (m, 24 H, C*H*$_2$NHCS), 3.62 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.1 Hz, H-3), 3.56 (m, 2 H, H-2), 3.50-2.98 (m, 96 H, CH_2Ncyclam), 3.15 (bs, 2 H, H-4), 2.77 (m, 12 H, SC*H*$_2$CH$_2$NHCS), 2.66 (m, 12 H, CH_2S), 2.36-2.22 (m, 12 H, H-2$_{Hex}$), 2.03-1.84 (m, 12 H,

C*H*$_2$CH$_2$O), 1.77 (m, 12 H, CH$_2$cyclam), 1.61-1.58 (m, 12 H, H-3$_{Hex}$), 1.47, 1.46 (m, 222 H, CHcyclam, CMe$_3$), 1.29-1.26 (m, 24 H, H-4$_{Hex}$, H-5$_{Hex}$), 0.94-0.87 (m, 18 H, H-6$_{Hex}$); ^{13}C NMR (100.6 MHz, CDCl$_3$): δ = 182.6, 182.4 (CS), 172.8, 172.6, 172.1 (CO ester), 156.2, 155.9, 155.6 (CO carbamate), 92.7 (C-1), 91.3 (C-1'), 81.1 (C-3), 80.2, 80.1, 80.0, 79.8 (*C*Me$_3$, C-2, C-4), 71.8 (OCH$_2$, C-5), 70.6-68.0 (C-5', C-3', C-2', C-4'), 70.4 (OCH$_2$), 51.2, 48.7, 47.9, 46.9, 46.7 (CH$_2$Ncyclam), 44.8 (C-6, C-6'), 43.8, 43.5, 42.8, 41.6 (CH$_2$NHCS), 34.0 (C-2$_{Hex}$), 31.3, 31.2 (C-4$_{Hex}$), 31.0, 30.7, 30.6 (S*C*H$_2$CH$_2$NHCS), 30.2 (C*H*$_2$CH$_2$O), 29.6, 29.2 (CH$_2$cyclam), 28.5 (CMe$_3$, CH$_2$S), 24.5, 24.4 (C-3$_{Hex}$), 22.1 (C-5$_{Hex}$), 13.7 (C-6$_{Hex}$). Anal. Calcd for $C_{290}H_{524}N_{40}O_{72}S_{14}$: C, 56.43; H, 8.56; N, 9.08; S, 7.27; found: C, 56.11; H, 8.30; N, 8.74; 6.90.



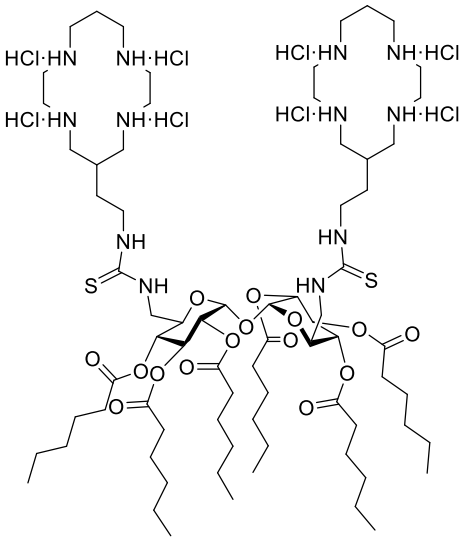
[*N*^{I},*N*^{III}-[6,6'-Dideoxy-2,3,4,2',3',4'-hexa-*O*-(3-(2-(3,6,10,13-tetraazacyclotetradecyl)ethyl-thioureido)ethylthio)propyl)-α,α'-trehalos-6,6'-diyl]-*N*^{II},*N*^{IV}-[6,6'-dideoxy-2,3,4,2',3',4'-hexa-*O*-hexanoyl-α,α'-trehalos-6,6'-diyl]] thiourea tetraeicosahydrochloride (3). Compound **11** (100 mg, 0.016 mmol) was achieved by treatment 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: 84 mg (100%). $[\alpha]_D$ = +39.3 (*c* 1.0, MeOH); IR (ATR): ν_{max} = 3280, 2927, 1740, 1670 cm^{-1}; ^1H NMR (500 MHz, CD_3OD, 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'), 5.20 (bd, 2 H, H-1), 5.08 (m, 4 H, H-2', H-4'), 3.95 (m, 2 H, H-5'), 4.03-3.66 (m, 14 H, CH_2O, H-5), 3.72 (m, 12 H, SCH_2CH_2NHCS), 3.63 (m, 12 H, CH_2NHCS), 3.26-2.90 (m, 110 H, H-2, H-3, H-4, H-6ab, H-6'ab, CH_2N), 2.79 (m, 12 H, SCH_2CH_2NHCS), 2.74 (m, 12 H, CH_2S), 2.46-2.27 (m, 12 H, $H\text{-}2_{Hex}$), 2.14 (m, 6 H, CHcyclam), 1.96 (m, 24 H, CH_2CH_2O, CH_2cyclam), 1.69-1.57 (m, 24 H, CH_2-cyclam, $H\text{-}3_{Hex}$), 1.38-1.30 (m, 24 H, $H\text{-}4_{Hex}$, $H\text{-}5_{Hex}$), 0.96-0.91 (m, 18 H, $H\text{-}6_{Hex}$); ^{13}CNMR (100.6 MHz, CD_3OD, 333 K): δ = 182.6 (CS), 172.7 (CO), 91.8 (C-1), 90.9 (C-1'), 81.2 (C-3), 79.7 (C-2, C-4), 71.4 (C-5 ,OCH_2), 71.0 (C-3'), 70.1 (OCH_2), 69.9 (C-2'), 69.3 (C-5'), 68.8 (C-4'), 53.6, 48.8, 46.3, 46.1 (CH_2N), 43.7 (SCH_2CH_2NHCS), 41.2 (CH_2NHCS), 33.7 ($C\text{-}2_{Hex}$), 33.0, 32.8 (CH_2cyclam), 31.1, 31.0, 30.8, 30.5 (SCH_2CH_2NHCS, $C\text{-}4_{Hex}$, CH_2CH_2O), 28.3 (CH_2S), 24.5 (CH_2-cyclam), 24.2 ($C\text{-}3_{Hex}$), 21.8 ($C\text{-}5_{Hex}$), 12.7 ($C\text{-}6_{Hex}$); ESIMS: *m/z* = 1885.7 $[M + 2H]^{2+}$. Anal. Calcd for $C_{170}H_{332}N_{40}O_{24}S_{14}\cdot 24HCl\cdot 6H_2O$: C, 42.96; H, 7.80; N, 11.79; S, 9.44; found: C, 43.29; H, 7.40; N, 11.54; S, 9.20.

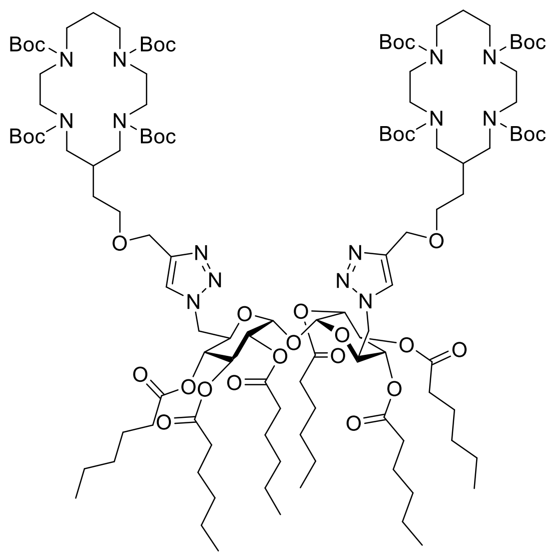


6,6'-Bis[2-(3,6,10,13-tetrakis(*tert*-butoxycarbonyl)-3,6,10,13-tetraaza-cyclotetradecanyl)-ethylthioureido]-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexanoyl]-α,α'-trehalose (15). To a solution of

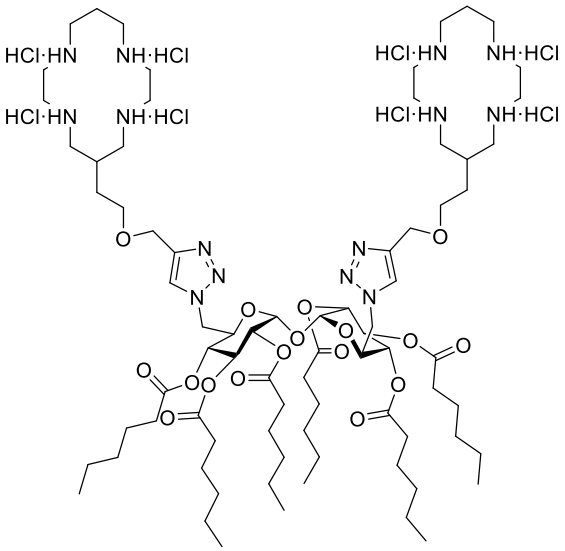
14 (239 mg, 0.37 mmol) in pyridine (15 mL), a solution of **13**[ii] (169 mg, 0.17 mmol) in DCM (5 mL) was added and the mixture was stirred at room temperature overnight. The mixture was concentrated, and the resulting residue purified by column chromatography (1:2→2:1 EtOAc-cyclohexane). Yield: 200 mg (61%). R_f = 0.31 (1:1 EtOAc-cyclohexane); $[\alpha]_D$ = +52.2 (*c* 1.0, DCM); IR (ATR): ν_{max} = 2933, 2875, 1750, 1682, 1161 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, 323 K): δ = 6.68 (bs, 2 H, NH), 6.42 (bs, 2 H, NH), 5.52 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3), 5.29 (d, 2 H, $J_{1,2}$ = 3.7 Hz, H-1), 4.97 (dd, 2 H, H-2), 4.96 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.05 (m, 2 H, H6a), 3.91 (m, 2 H, H-5), 3.77-3.05 (m, 32 H, cyclam), 3.54 (m, 4 H, CH_2NH), 3.47 (m, 2 H, H-6b), 2.44-2.21 (m, 12 H, H-2_{Hex}), 1.90 (m, 2 H, cyclam), 1.77 (m, 4 H, cyclam), 1.66-1.54 (m, 16 H, CH_2CH_2NH, H-3_{Hex}), 1.48, 1.47 (bs, 72 H, CMe_3), 1.36-1.27 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.93-0.89 (m, 18 H, H-6_{Hex}); ^{13}CNMR (100.6 MHz, $CDCl_3$, 323 K): δ = 183.7 (CS), 173.0, 172.5, 172.1 (CO ester), 156.2, 155.7 (CO carbamate), 90.9 (C-1), 80.3, 80.2, 80.0, 79.9 (*C*Me_3), 69.9 (C-3), 69.7 (C-2), 69.4 (C-4), 69.3 (C-5), 51.2, 48.5, 47.8 46.7 (cyclam), 44.7 (C-6), 41.9 (CH_2NH), 36.1 (cyclam), 34.0 (C-2_{Hex}), 31.2 (C-4_{Hex}), 29.2 (*C*H_2CH_2NH), 28.5 (CMe_3), 28.3, 26.7 (cyclam), 24.5, 24.4 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8, 13.7 (C-6_{Hex}); ESIMS: *m/z* = 2323.5 $[M + Na]^+$, 1173.2 $[M + 2Na]^{2+}$. Anal. Calcd for $C_{114}H_{202}N_{12}O_{31}S_2$: C, 59.51; H, 8.85; N, 7.30; S, 2.79; found: C, 59.43; H, 8.59; N, 6.97; 2.49.



6,6'-Bis[2-(3,6,10,13-tetraazacyclotetradecanyl)ethyl-thioureido]-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexanoyl]-α,α'-trehalose octahydrochloride (4). Compound **15** (198 mg, 0.047 mmol) was treated with 1:1 DCM-TFA (5 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 H_2O-HCl 0.1 N and freeze-dried to yield quantitatively **4** as octahydrochloride. Yield: 154 mg (100%). $[\alpha]_D$ = +48.4 (*c* 1.0, DCM); IR (ATR): ν_{max} = 3275, 2958, 2931, 2860, 1751, 1678, 1174 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 313 K): δ = 5.53 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 5.36 (d, 2 H, $J_{1,2}$ = 3.9 Hz, H-1), 4.97 (dd, 2 H, H-2), 4.96 (t, 2 H, $J_{4,5}$ = 9.8 Hz, H-4), 4.14 (bd, 2 H, $J_{6a,6b}$ = 14.5 Hz, H6a), 3.91 (ddd, 2 H, $J_{5,6b}$ = 7.7 Hz, $J_{5,6a}$ = 2.4 Hz, H-5), 3.70-3.53 (m, 4 H, CH_2NH), 3.42 (dd, 2 H, H-6b), 3.23-2.89 (m, 32 H, cyclam), 2.46-2.24 (m, 12 H, H-2_{Hex}), 2.09 (m, 2 H, cyclam), 1.94 (m, 4 H, cyclam), 1.67-1.54 (m, 16 H, C$H_2$$CH_2$NH, H-$3_{Hex}$), 1.40-1.30 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.96-0.92 (m, 18 H, H-6_{Hex}); ^{13}CNMR (125.7 MHz, CD_3OD, 298 K): δ = 183.9 (CS), 173.0 (CO ester), 90.9 (C-1), 70.4 (C-3), 69.9 (C-2), 69.8 (C-4), 69.6 (C-5), 53.9, 53.8, 49.1 46.5, 46.3 (cyclam), 44.6 (C-6), 41.5 (CH_2NH), 34.2, 34.0 (C-2_{Hex}), 33.2 (cyclam), 31.5, 31.4, 31.3 (C-4_{Hex}), 30.4 (cyclam), 29.7 (*C*H_2CH_2NH), 24.8 (cyclam), 24.7, 24.6, 24.5 (C-3_{Hex}), 22.4 (C-5_{Hex}), 13.5, 13.3 (C-6_{Hex}); ESIMS: *m/z* = 1791.9 $[M + H]^+$. Anal. Calcd for $C_{74}H_{138}N_{12}O_{15}S_2 \cdot 8HCl$: C, 49.61; H, 8.21; N, 9.38; S, 3.58; found: C, 49.32; H, 8.02; N, 9.11; 3.23.

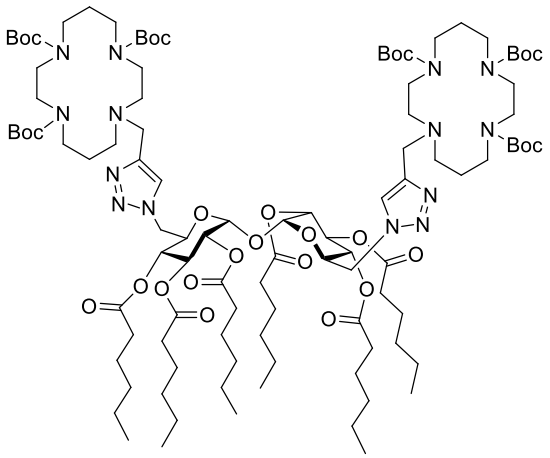


6,6'-Bis[*O*-[2-(3,6,10,13-tetrakis(*tert*-butoxycarbonyl)-3,6,10,13-tetraaza-cyclotetradecanyl)-ethyl]methyl-1*H*-1,2,3-triazol-1-yl]-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexanoyl]-α,α'-trehalose (20). To a solution of **17**[ii] (78 mg, 0.080 mmol) and **16** (120 mg, 0.18 mmol) in H_2O-^{t}BuOH (3:1, 8 mL) the Cu-supported catalyst Si-BPA·Cu^{+} [vii] (5.4 mg) was added and the reaction mixture was refluxed at 110 ºC overnight. The reaction mixture was diluted with DCM (8 mL), the catalyst was filtered, the organic phase was separated, dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (1:2 → 4:1 EtOAc-cyclohexane). Yield: 72 mg (38%). R_f = 0.57 (2:1 EtOAc-cyclohexane). $[\alpha]_D$ = +40.9 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2932, 2864, 1746, 1687, 1156 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$, 298 K): δ = 7.57 (s, 2 H, =CH), 5.47 (t, 2 H, $J_{2,3}$ = $J_{3,4}$ = 10.1 Hz, H-3), 4.95 (dd, 2 H, $J_{1,2}$= 3.8 Hz, H-2), 4.86 (t, 2 H, $J_{4,5}$ = 10.1 Hz, H-4), 4.85 (d, 2 H, H-1), 4.56 (s, 4 H, CH_2 triazole), 4.46 (bd, 2 H, $J_{6a,6b}$ = 12.6 Hz, H-6a), 4.30 (dd, 2 H, $J_{5,6b}$ = 8.2 Hz, H-6b), 4.22 (dd, 2 H, H-5), 3.76-2.88 (m, 32 H, cyclam), 3.53 (t, 4 H, $^3J_{H,H}$ = 6.3 Hz, CH_2O), 2.39-2.16 (m, 12 H, H-2_{Hex}), 1.93 (m, 2 H, cyclam), 1.71 (m, 4 H, cyclam), 1.65-1.50 (m, 16 H, C$H_2$$CH_2O$, H-$3_{Hex}$), 1.44 (bs, 72 H, C$Me_3$), 1.32-1.23 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.91-0.86 (m, 18 H, H-6_{Hex}); ^{13}C NMR (75.5 MHz, $CDCl_3$, 298 K): δ = 172.4, 172.2, 172.1 (CO ester), 155.9, 155.6 (CO carbamate), 145.3 (C-4 triazole), 123.8 (C-5 triazole), 91.5 (C-1), 79.8, 79.7 ($C$$Me_3$), 69.3 (C-3, C-4), 68.9 (C-2), 68.7 (C-5), 68.2 (CH_2O), 64.0 ($C$$H_2$ triazole), 53.4, 52.1 (cyclam), 50.4 (C-6), 48.2, 46.7, 35.4 (cyclam), 33.9, 33.7 (C-2_{Hex}), 31.2 (C-4_{Hex}), 29.9 ($C$$H_2$$CH_2O$), 29.6, 29.3 (cyclam), 28.4 (CMe_3), 28.2 (cyclam), 24.4, 24.3 (C-3_{Hex}), 22.2 (C-5_{Hex}), 13.8 (C-6_{Hex}); ESIMS: *m/z* = 2369.3 $[M + Na]^+$, 1196.3 $[M + 2Na]^{2+}$. Anal. Calcd for $C_{118}H_{204}N_{14}O_{33}$: C, 60.39; H, 8.76; N, 8.36; found: C, 60.46; H, 8.83; N, 8.09.



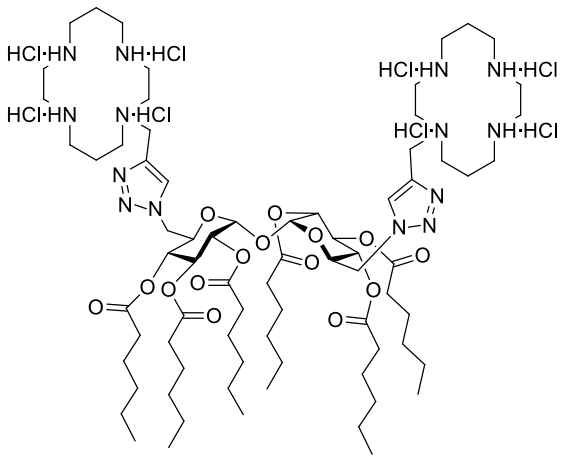
6,6'-Bis[*O*-[2-(3,6,10,13-tetraazacyclotetradecanyl)ethyl]-methyl-1*H*-1,2,3-triazol-1-yl]-6,6'-dideoxy-2,3,2',3'-tetra-*O*-hexanoyl]-α,α'-trehalose octahydrochloride (5). Compound **20** (70 mg, 0.030 mmol) was treated with 1:1 DCM-TFA (4 mL) at rt for 30 min. Then the solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried to yield quantitatively **5** as octahydrochloride. Yield: 55 mg (100%). $[\alpha]_D$ = +382.3 (*c* 1.0, DCM); IR: ν_{max} = 2962, 2932, 2859, 1751, 1673, 1126 cm^{-1}; ^1H NMR (400 MHz, CD_3OD, 298 K): δ = 7.96 (s, 2 H, =CH), 5.50 (t, 2 H, $J_{2,3} = J_{3,4}$ = 9.9 Hz, H-3), 5.05 (dd, 2 H, $J_{1,2}$= 3.7 Hz, H-2), 4.99 (t, 2 H, $J_{4,5}$ = 9.9 Hz, H-4), 4.92 (d, 2 H, H-1), 4.63 (dd, 2 H, $J_{6a,6b}$ = 14.9 Hz, $J_{5,6a}$ = 2.8 Hz, H-6a), 4.60 (s, 4 H, CH_2 triazole) 4.56 (dd, 2 H, $J_{5,6b}$ = 7.8 Hz, H-6b), 4.30 (ddd, 2 H, H-5), 3.61 (t, 4 H, $^3J_{H,H}$ = 5.9 Hz, CH_2O), 3.19-2.87 (m, 32 H, cyclam), 2.46-2.24 (m, 12 H, H-2_{Hex}), 2.19 (m, 2 H, cyclam), 1.94 (m, 4 H, cyclam), 1.67-1.54 (m, 16 H, CH_2CH_2O, H-3_{Hex}), 1.42-1.29 (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, 298 K): δ = 174.0, 173.7, 173.4 (CO ester), 145.9 (C-4 triazole), 126.4 (C-5 triazole), 92.3 (C-1), 71.4 (C-3), 70.6 (C-4), 70.2 (C-2, C-5), 68.6 (CH_2O), 64.5 (CH_2 triazole), 54.9 (cyclam), 51.4 (C-6), 50.1, 47.5, 47.3 (cyclam), 34.9, 34.8 (C-2_{Hex}), 34.4 (cyclam), 32.5, 32.4 (C-4_{Hex}), 31.7 (CH_2CH_2O), 26.0 (cyclam), 25.6, 25.5, 25.4 (C-3_{Hex}), 23.4 (C-5_{Hex}), 14.3, 14.2 (C-6_{Hex}); ESIMS: *m/z* = 773.9 [M - 6H -

8Cl]$^{2+}$. Anal. Calcd for $C_{78}H_{140}N_{14}O_{17}$·8HCl: C, 50.98; H, 8.12; N, 10.67; found: C, 50.81; H, 7.93; N, 10.32.



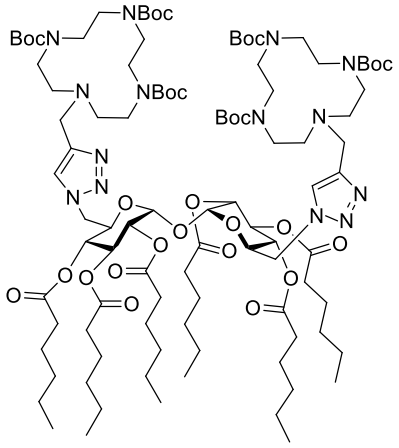
6,6´-Bis-[4-(4,8,11-tris(*tert*-butoxycarbonyl)-1,4,8,11-tetraazacyclotetradecyl-1-methyl)-1*H*-1,2,3-triazol-1-yl]-6,6´-dideoxy-2,3,4,2´,3´,4´-hexa-*O*-hexanoyl-α,α´-trehalose (21). To a solution of **17**[iii] (140 mg, 0.14 mmol) and **18**[iv] (170 mg, 0.32 mmol) in ^{t}BuOH-H_2O (3:1, 8 mL) the silica-supported Cu(I) catalyst Si-BPA·Cu^{+}[vii] (9.7 mg) was added and the reaction mixture was refluxed for 16h. Then, the solvent was removed under reduced pressure, the residue was taken in DCM (4 mL), and the catalyst was filtered. After evaporation of solvent, the residue was purified by column chromatography (1:1→3:1 AcOEt-cyclohexane) to yield compound **21** (208 mg, 71%). R_f = 0.50 (1:1 EtOAc-cyclohexane). $[\alpha]_D$ = +32.4 (*c* 1.0, DCM); IR: ν_{max} = 2959, 2104, 1755, 1689, 1163 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.50 (bs, 2 H, =CH), 5.45 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.64 Hz, H-3), 4.95 (dd, 2 H, $J_{1,2}$ = 3.4 Hz, H-2), 4.86 (t, 2 H, $J_{4,5}$ = 9.90 Hz, H-4), 4.83 (m, 2 H, H-1), 4.45 (d, 2 H, $J_{6a,6b}$ = 12.95 Hz, H-6a), 4.26 (dd, 2 H, $J_{5,6b}$ = 8.0 Hz, H-6b), 4.08 (m, 2 H, H-5), 3.78 (bs, 4 H, CH_2 triazol) , 3.31 (bs, 28 H, CH_2 cyclam), 2.58 (bs, 4 H, CH_2 cyclam), 2.26 (m, 12 H, H-2_{Hex}), 1.90 (bs, 4 H, CH_2 cyclam), 1.70 (bs, 4 H, CH_2 cyclam), 1.55 (m, 12 H, H-4_{Hex}), 1.44, 1.43 (2 x s, 54 H, $C(CH_3)_3$), 1.27 (m, 24 H, H-3_{Hex}, H-5_{Hex}), 0.87 (m, 18 H, H-6_{Hex});. ^{13}C NMR (75.5 MHz, CDCl3): δ = 172.4-172.0 (CO ester), 155.8-155.5 (CO carbamate), 143.3 (C-4 triazole), 124.0 (C-5 triazole), 91.3 (C-1), 79.4 (*C*Me_3), 69.4 (C-3, C-4), 69.1 (C-5), 68.7 (C-2), 50.4 (C-6), 50.6-45.2 (CH_2 cyclam,

CH_2 triazole), 33.9-33.7 (C-2_{Hex}), 31.2 (C-3_{Hex}), 29.6 (CH_2 cyclam), 28.5 (CMe_3), 24.4-24.3 (C-4_{Hex}), 22.2 (C-5_{Hex}), 14.0-13.8 (C-6_{Hex}); ESIMS: *m/z* = 2081.4 $[M + Na]^+$. Anal. Calcd for $C_{104}H_{180}N_{14}O_{27}$: calcd. C, 60.68; H, 8.81; N, 9.53; found: C, 60.46; H, 8.501; N, 9.27.



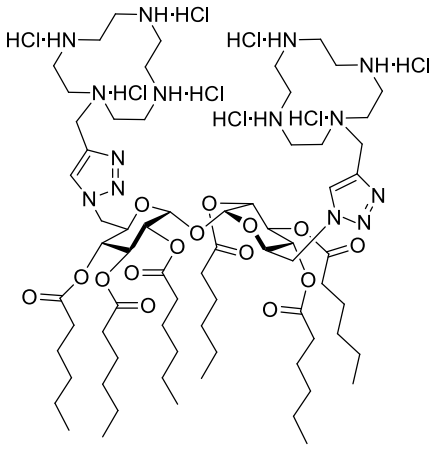
6,6´-Bis-[1,4,8,11-tetraaza-yclotetradecyl-1-methyl)-1*H*-1,2,3-triazol-1-yl]-6,6´-dideoxy-2,3,4,2´,3´,4´-hexa-*O*-hexanoyl-α,α´-trehalose octahydrochloride (6). Compound **21** (130 mg, 0.063 mmol) was treated with 1:1 DCM-TFA (5 mL) at rt for 30 min. Then the solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in 10:1 H_2O-HCl 0.1 N and freeze-dried to yield quantitatively **6** as octahydrochloride. Yield: 111 mg. $[\alpha]_D$ = +48.1 (*c* 1.0, DCM); IR: ν_{max} = 2957, 2932, 1756, 1673, 1023, 801, cm^{-1}; ^1H NMR (500 MHz, 323K, CD_3OD): δ = 7.89 (bs, 2 H, =CH), 5.54 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.7 Hz, H-3), 5.08 (dd, 2 H, $J_{1,2}$ = 3.6 Hz, H-2), 4.98 (m, 2 H, H-1), 4.92 (t, 2 H, $J_{4,5}$ = 9.9 Hz, H-4), 4.69 (bs, 4 H, CH_2 triazole), 4.57 (m, 2 H, H-5), 3.93 (d, 2 H, $J_{6a,6b}$ = 15.0 Hz, H-6a), 3.76 (d, 2 H, H-6b), 3.27-2.83 (m, 32 H, CH_2 cyclam), 2.36 (m, 12 H, H-2_{Hex}), 2.11-1.90 (bs, 8 H, CH_2 cyclam), 1.64 (m, 12 H, H-4_{Hex}), 1.37 (m, 24 H, H-3_{Hex}, H-5_{Hex}), 0.94 (m, 18 H, H-6_{Hex}); ^{13}C NMR (125.7 MHz, 323K, CD_3OD): δ = 173.0-172.7 (CO ester), 144.7 (C-4 triazole), 125.5 (C-5 triazole), 91.0 (C-1), 70.5 (C-3), 69.7 (C-2), 69.5 (C-4), 68.6 (C-5), 50.3-44.9 (CH_2 cyclam, CH_2 triazole), 47.3 (C-6), 34.2-33.9 (C-2_{Hex}), 31.5-31.3 (C-3_{Hex}), 25.2 (CH_2 cyclam), 24.7-24.4 (C-4_{Hex}), 23.2-23.0 (CH_2 cyclam), 22.3-22.2 (C-5_{Hex}),

13.2-13.1 (C-6_{Hex}); ESIMS: *m/z* = 1558.2 [M - 6 H - 8 Cl]$^{2+}$. Anal. Calcd for $C_{74}H_{132}N_{14}O_{15}$·8HCl: calcd. C, 50.80; H, 8.07; N, 11.21; found: C, 50.49; H, 7.78; N, 10.85.



6,6´-Bis-[4-(4,7,10-tris(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecyl-1-methyl)-1*H*-1,2,3-triazol-1-yl]-6,6´-dideoxy-2,3,4,2´,3´,4´-hexa-*O*-hexanoyl-α,α´-trehalose (22). To a solution of **17**[iii] (147 mg, 0.15 mmol) and **19**[v] (169 mg, 0.33 mmol) in ^{t}BuOH-H_2O (3:1, 8 mL) the silica-supported Cu(I) catalyst Si-BPA·Cu^{+}[vii] (9.7 mg) was added and the reaction mixture was refluxed for 16h. Then, the solvent was removed under reduced pressure, the residue was taken in DCM (4 ml), and the catalyst was filtered. After evaporation of solvent, the residue was purified by column chromatography (1:1→3:1 AcOEt-cyclohexane) to yield compound **22** (142 mg, 47%). R_f = 0.62 (2:1 EtOAc-cyclohexane). $[\alpha]_D$ = +31.5 (*c* 1.0, DCM); IR: ν_{max} = 2962, 2932, 1754, 1682, 1028, 767 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.62 (bs, 2 H, =CH), 5.44 (t, 2 H, $J_{3,4}$ = $J_{2,3}$ = 9.9 Hz, H-3), 4.91 (dd, 2 H, $J_{1,2}$ = 3.4 Hz, H-2), 4.87 (t, 2 H, $J_{4,5}$ = 9.7 Hz, H-4), 4.65 (bs, 2 H, H-1), 4.45 (d, 2 H, $J_{6a,6b}$ = 13.5 Hz, H-6a), 4.25 (dd, 2 H, $J_{5,6b}$ = 8.7 Hz, H-6b), 4.07 (m, 2 H, H-5), 3.93 (m, 4 H, CH_2 triazol) , 3.58-3.28 (m, 24 H, CH_2 cyclen), 2.69-2.52 (m, 8 H, CH_2 cyclen), 2.35-2.15 (m, 12 H, H-2_{Hex}), 1.64-1.49 (m, 12 H, H-3_{Hex}), 1.46, 1.43, 1.42 (3 x s, 54 H, $C(CH_3)_3$), 1.33-1.22 (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 = 172.5-172.0 (CO ester), 156.1-155.3 (CO carbamate), 141.5 (C-4 triazole), 124.8 (C-5 triazole), 90.9 (C-1), 79.5-79.2 (*C*Me_3), 69.5 (C-4), 69.3 (C-3, C-5), 68.6 (C-2), 54.1-52.8 (CH_2 cyclen), 50.4 (C-6), 49.9-47.1

(CH_2 cyclen), 44.1 (CH_2 triazole), 33.9-33.7 (C-2_{Hex}), 28.7 (C-4_{Hex}), 28.5 (CMe_3), 24.4 (C-4_{Hex}), 22.2 (C-5_{Hex}), 13.9-13.8 (C-6_{Hex}); ESIMS: *m/z* = 2025.2 $[M + Na]^+$. Anal. Calcd for $C_{100}H_{172}N_{14}O_{27}$: calcd. C, 59.98; H, 8.66; N, 9.79; found: C, 60.12; H, 8.56; N, 9.40.



6,6´-Bis-[1,4,7,10-tetraazacyclododecyl-1-methyl)-1*H*-1,2,3-triazol-1-yl]-6,6´-dideoxy-2,3,4,2´,3´,4´-hexa-*O*-hexanoyl-α,α´-trehalose octahydrochloride (7). Compound **22** (120 mg, 0.060 mmol) was treated with 1:1 DCM-TFA (5 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 H_2O-HCl 0.1 N and freeze-dried to yield quantitatively **7** as octahydrochloride. Yield: 101.5 mg. $[\alpha]_D$ = +46.68 (*c* 1.0, DCM); IR: ν_{max} = 2957, 1756, 1678, 1156, 796 cm^{-1}; ^1H NMR (300 MHz, DMSO-d6): δ = 8.03 (s, 2 H, =CH), 5.33 (t, 2 H, $J_{3,4} = J_{2,3}$ = 9.9 Hz, H-3), 5.01 (dd, 2 H, $J_{1,2}$ = 3.4 Hz, H-2), 5.00 (t, 2 H, $J_{4,5}$ = 9.4 Hz, H-4), 4.73 (d, 2 H, H-1), 4.57 (bd, 2 H, $J_{6a,6b}$ = 12.4 Hz, H-6a), 4.47 (dd, 2 H, $J_{5,6b}$ = 7.9 Hz, H-6b), 4.10 (m, 2 H, H-5), 3.81 (bs, 4 H, CH_2 triazol) , 3.13-2.69 (m, 32 H, CH_2 cyclen), 2.37-2.14 (m, 12 H, H-2_{Hex}), 1.54-1.40 (m, 12 H, H-3_{Hex}), 1.31-1.17 (m, 24 H, H-4_{Hex}, H-5_{Hex}), 0.84 (t, 18 H, $^3J_{H,H}$ = 6.7 Hz, H-6_{Hex});. ^{13}C NMR (75.5 MHz, DMSO-d6): δ = 171.8-171.4 (CO ester), 140.5 (C-4 triazole), 125.5 (C-5 triazole), 90.0 (C-1), 69.4 (C-3), 68.8, 68.6 (C-2, C-5), 68.1 (C-4), 49.6 (C-6), 46.8, 44.5, 42.1 (CH_2 cyclen), 44.9 (CH_2 triazole), 33.2-33.1 (C-2_{Hex}), 30.6-30.5 (C-4_{Hex}), 23.8 (C-3_{Hex}), 21.7 (C-5_{Hex}), 13.6 (C-6_{Hex}); ESIMS: *m/z* = 1509.9 $[M - 6HCl]^+$.

Anal. Calcd for $C_{70}H_{124}N_{14}O_{15}\cdot 8HCl$: calcd. C, 49.65; H, 7.86; N, 11.58; found: C, 49.34; H, 7.57; N, 11.22.

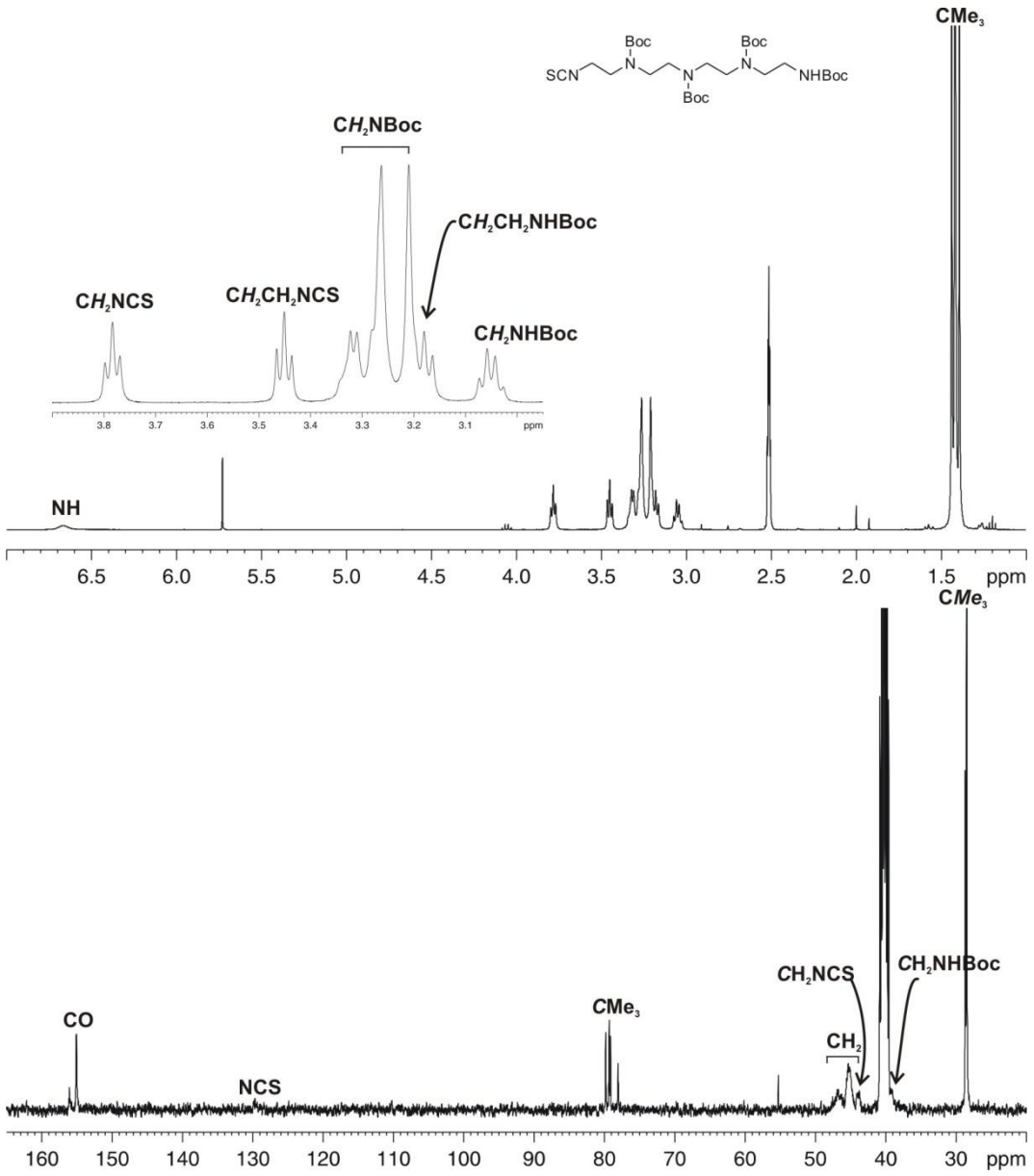


Figure S2. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **9**.

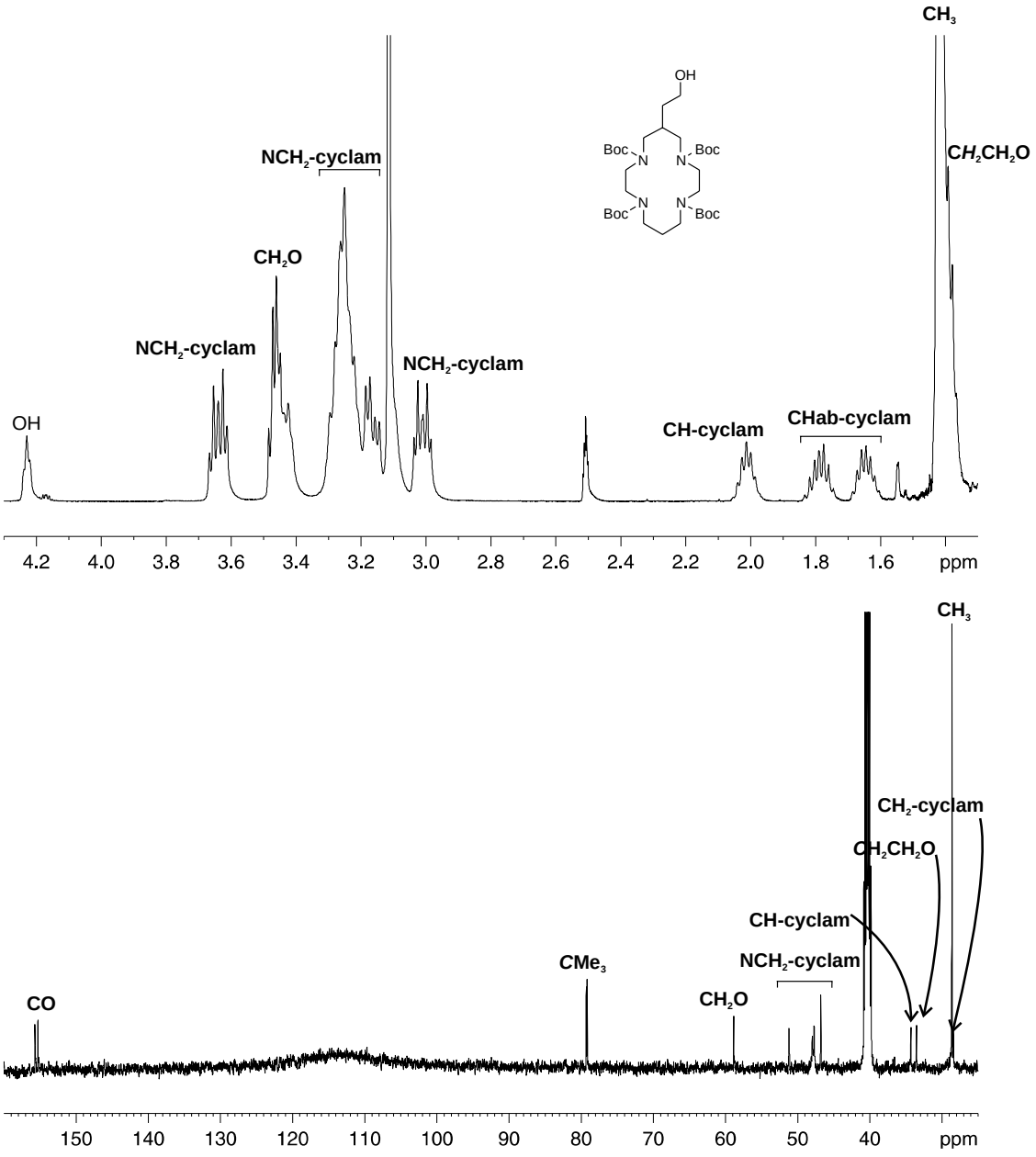


Figure S3. ^{1}H and ^{13}C NMR spectra (500 and 125.7 MHz, DMSO-d_6, 343 K) of **24.**

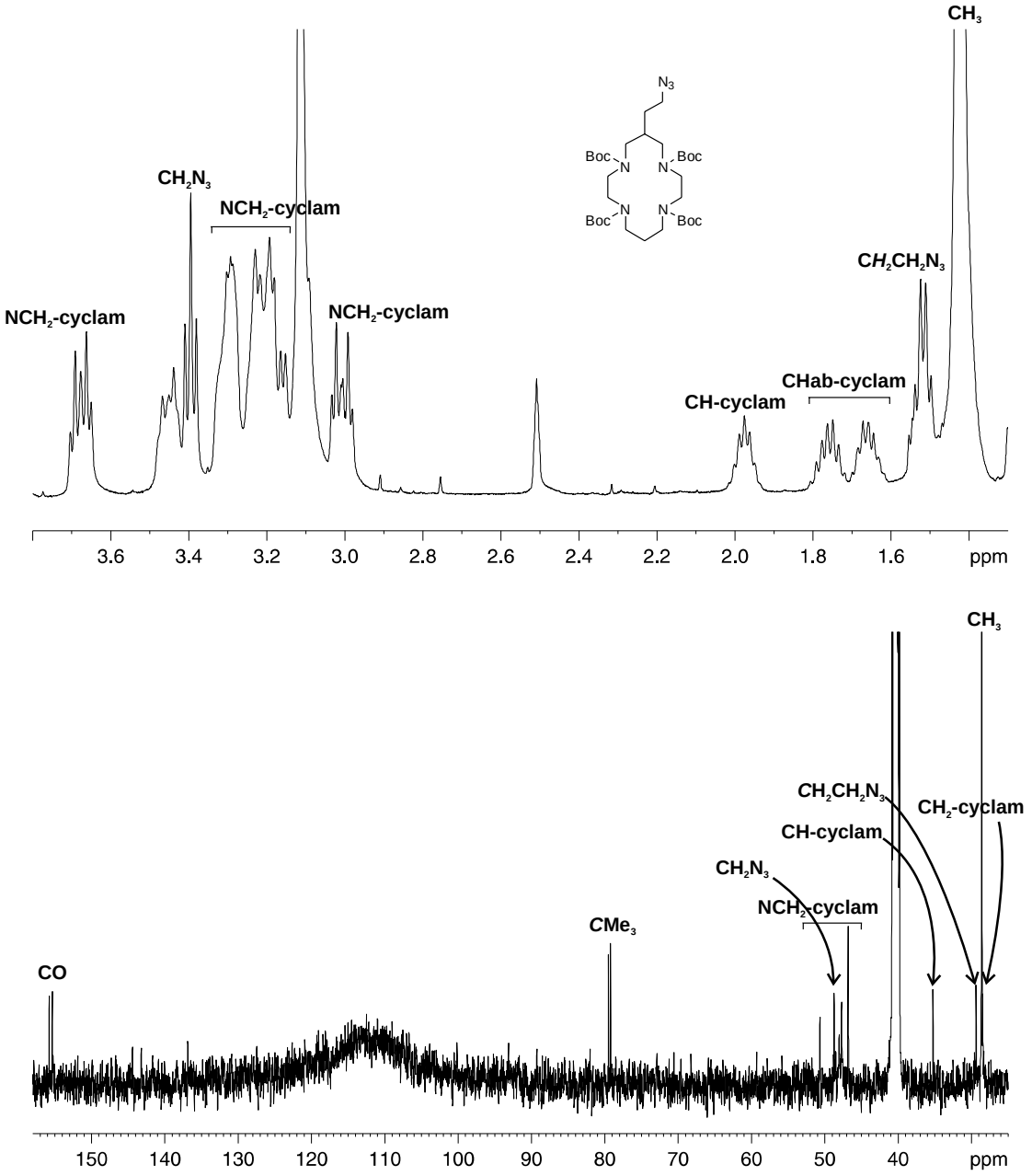


Figure S4. ^{1}H and ^{13}C NMR spectra (500 and 125.7 MHz, DMSO-d_6, 343 K) of **25.**

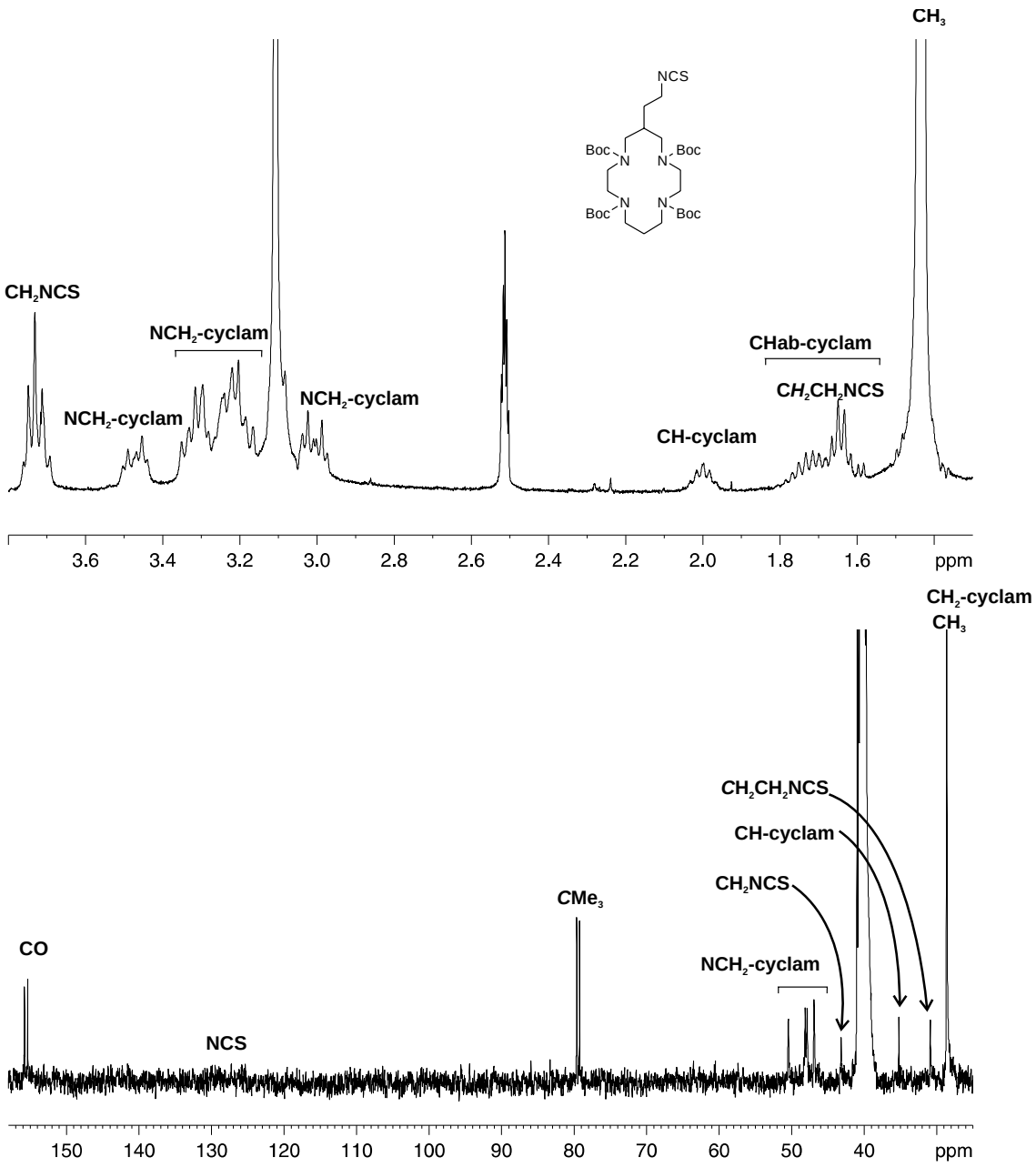


Figure S5. ^{1}H and ^{13}C NMR spectra (400 and 100.6 MHz, DMSO-d_6, 343 K) of **10.**

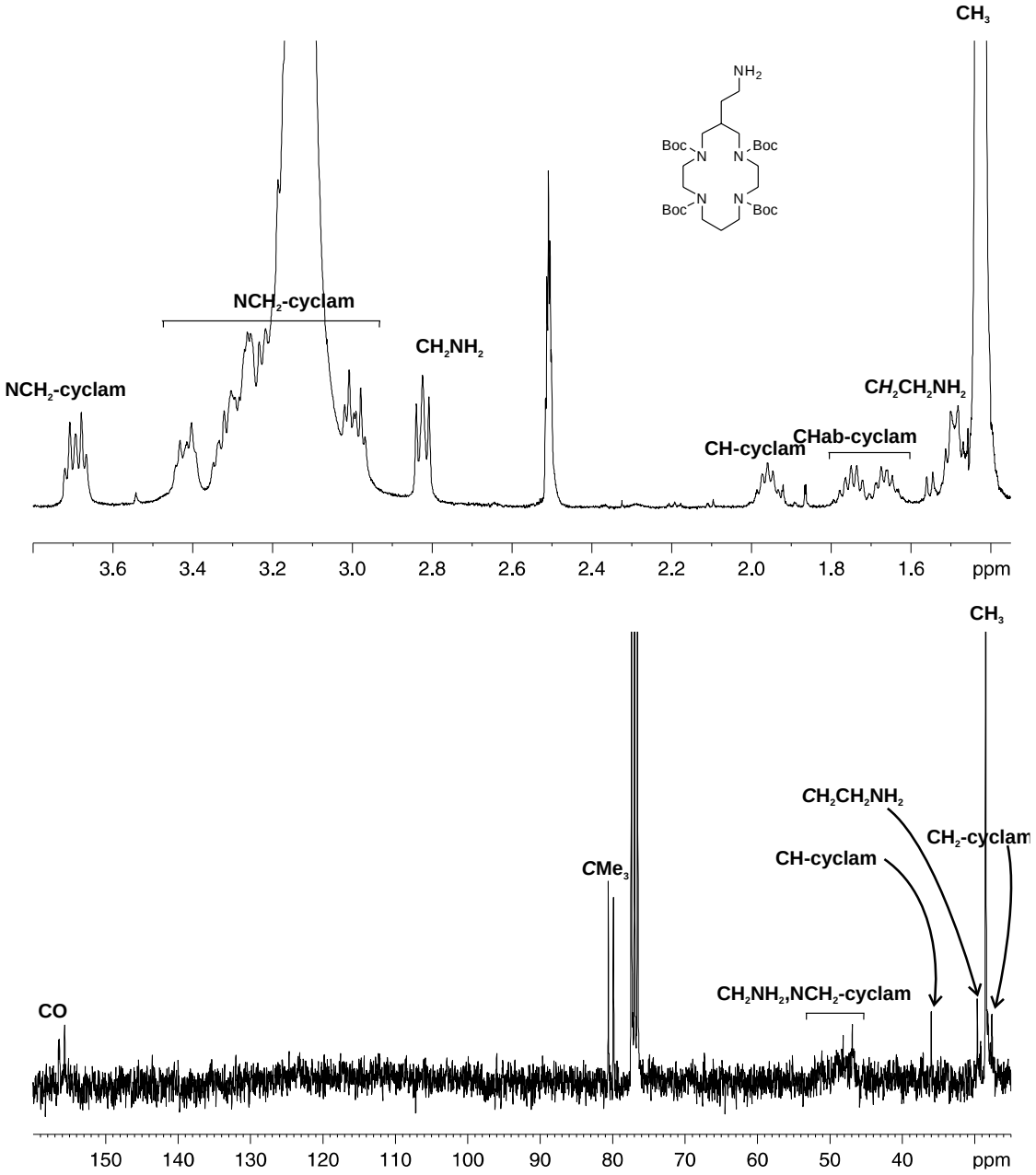


Figure S6. ^{1}H and ^{13}C NMR spectra (500 MHz, DMSO-d_6, 343 K and 75.5 MHz, $CDCl_3$, 298K) of **14.**

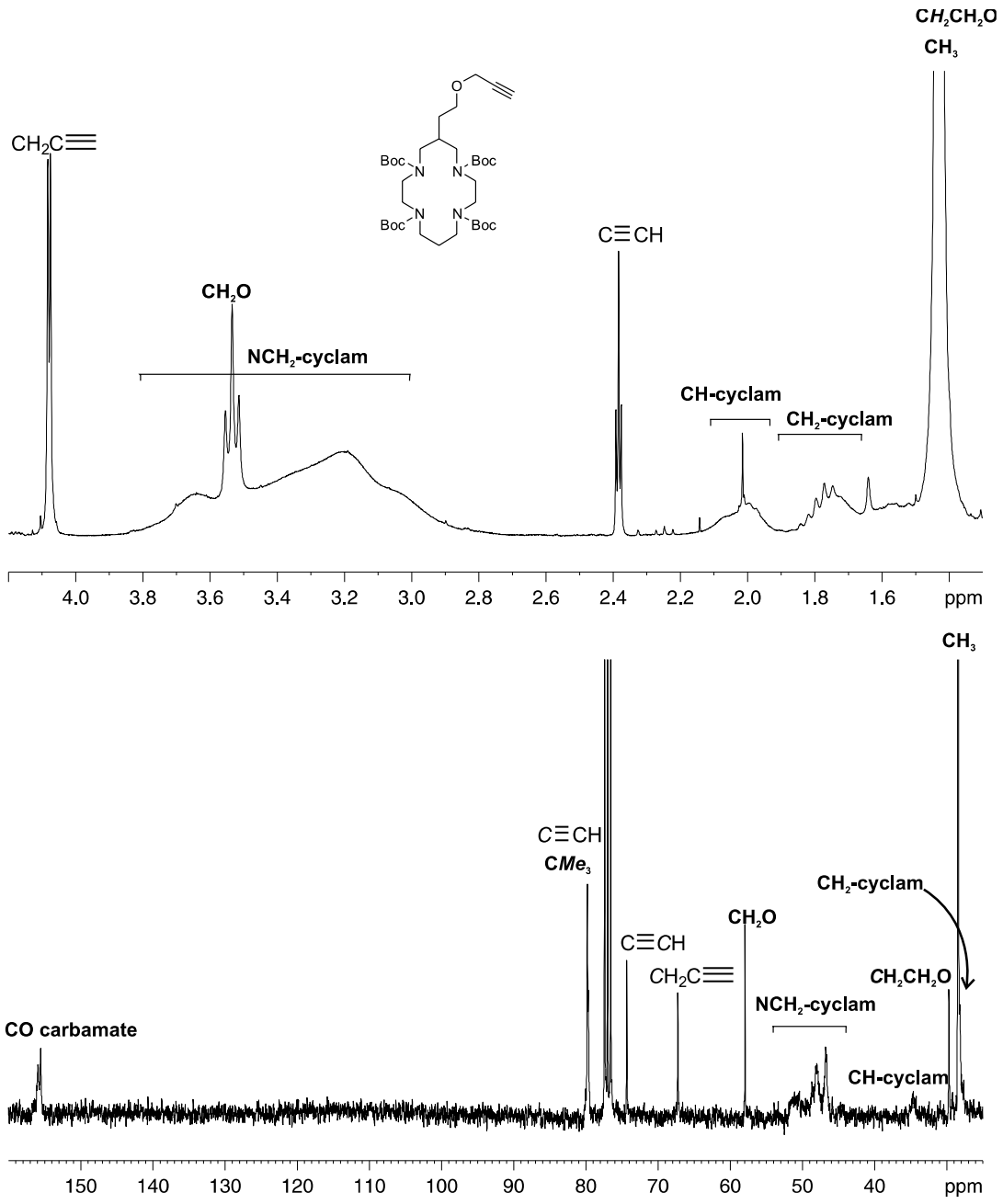


Figure S7. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **16.**

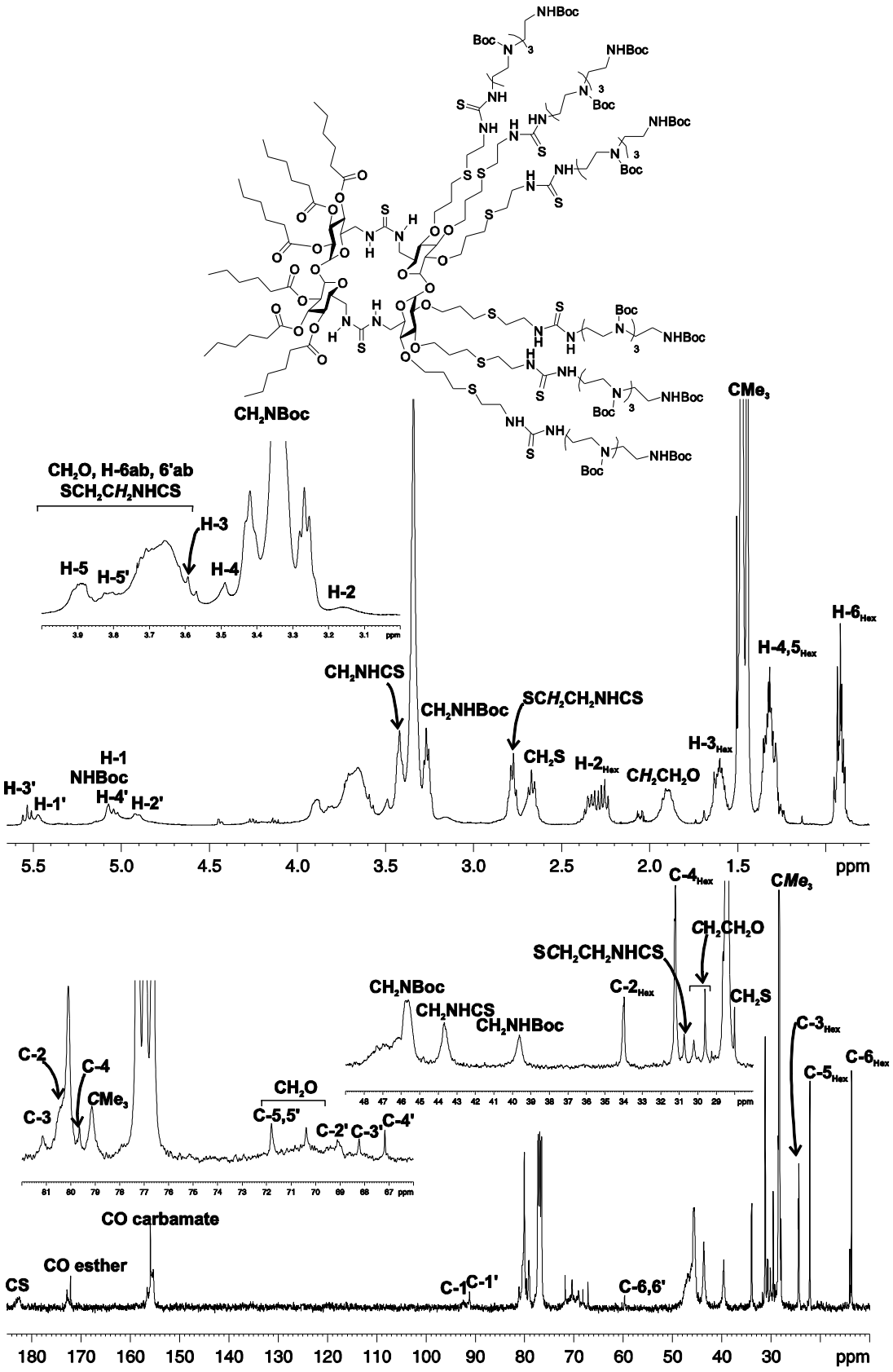


Figure S8. ^{1}H and ^{13}C NMR spectra (400 and 100.6 MHz, $CDCl_3$, 323 K) of **11**.

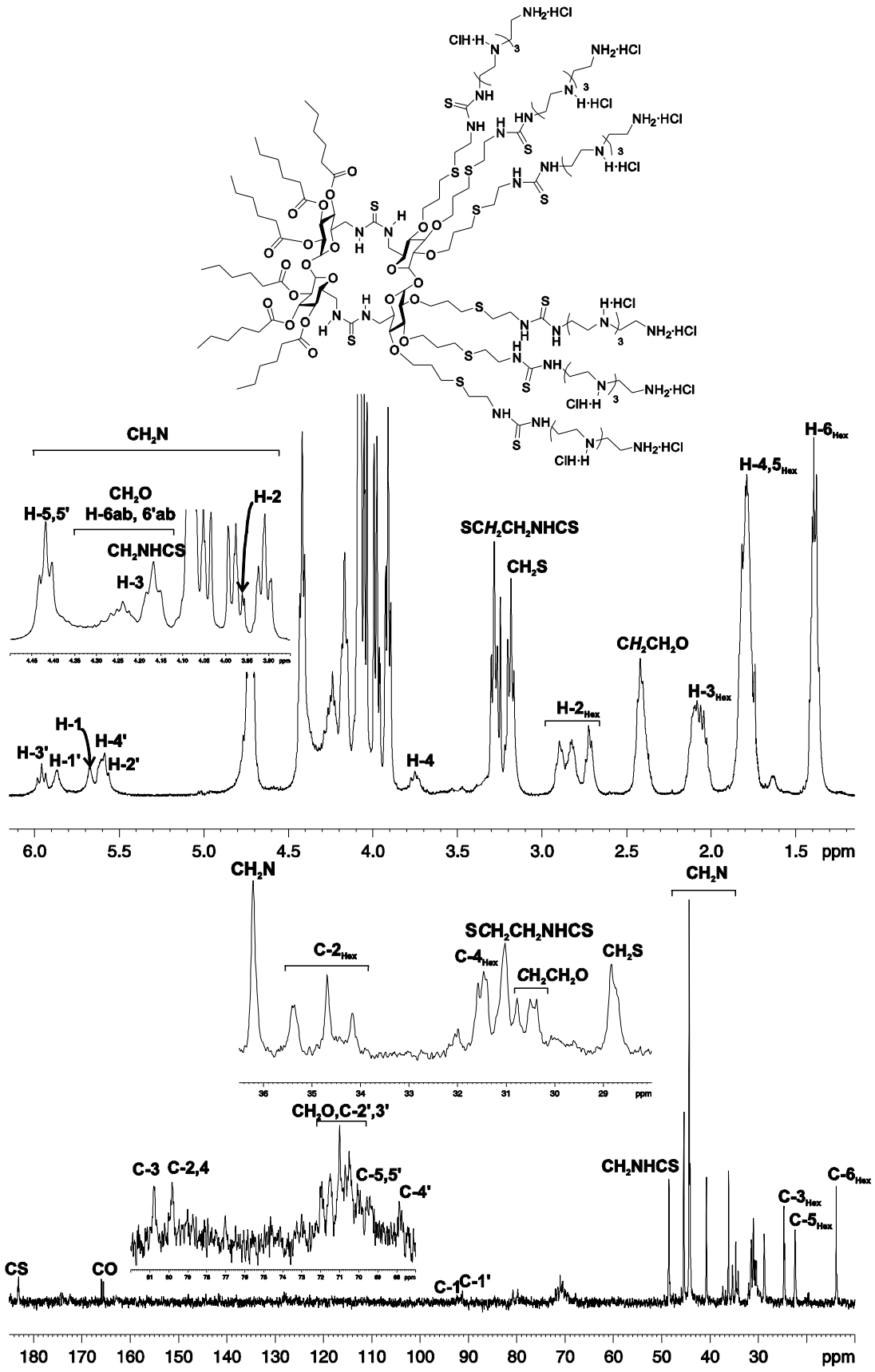


Figure S9. ^{1}H and ^{13}C NMR spectra (400 and 100.6 MHz, D_2O, 353 K) of **2**.

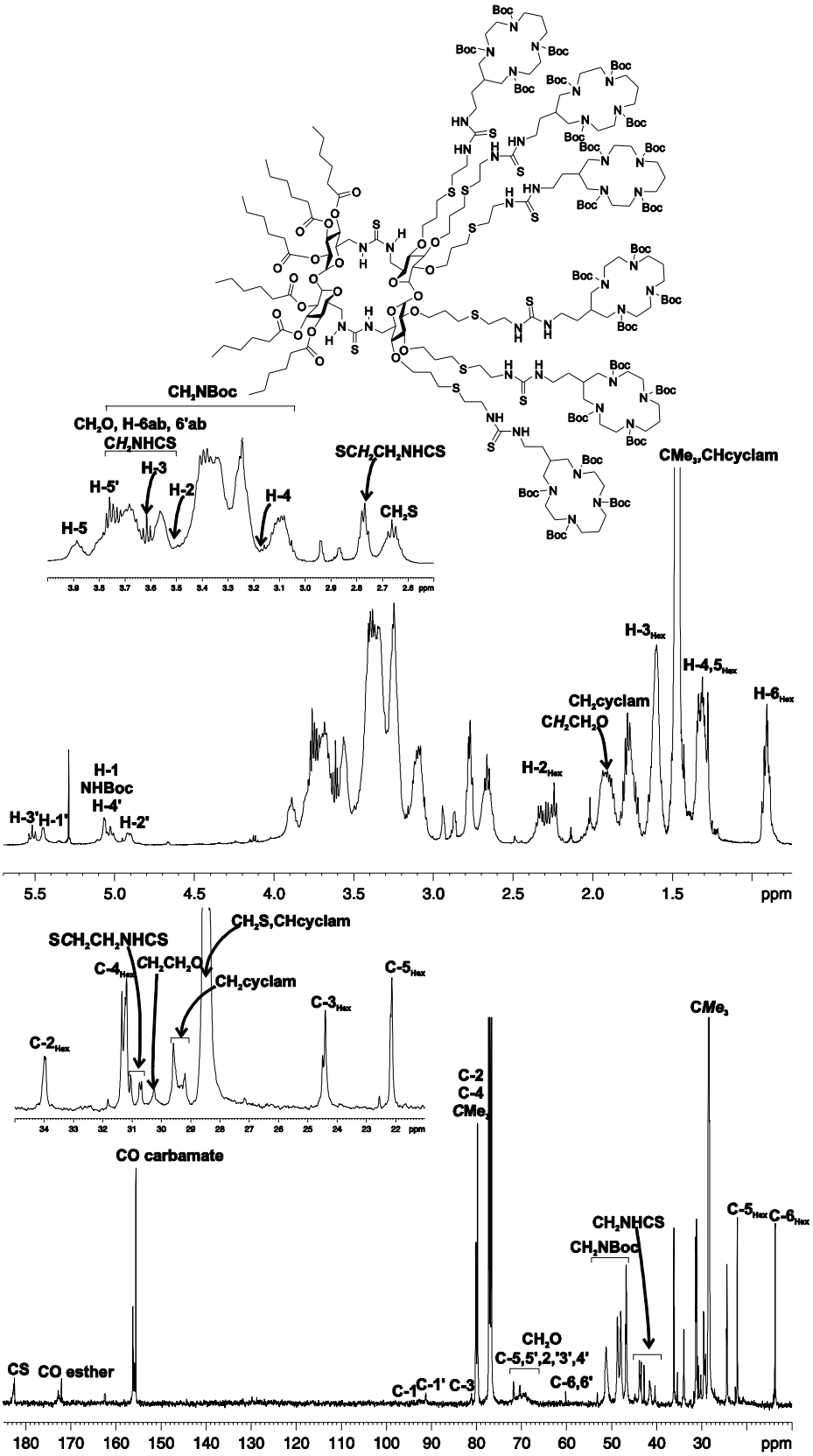


Figure S10. ^{1}H and ^{13}C NMR spectra (400 MHz and 100.6 MHz, $CDCl_3$, 333 K) of **12.**

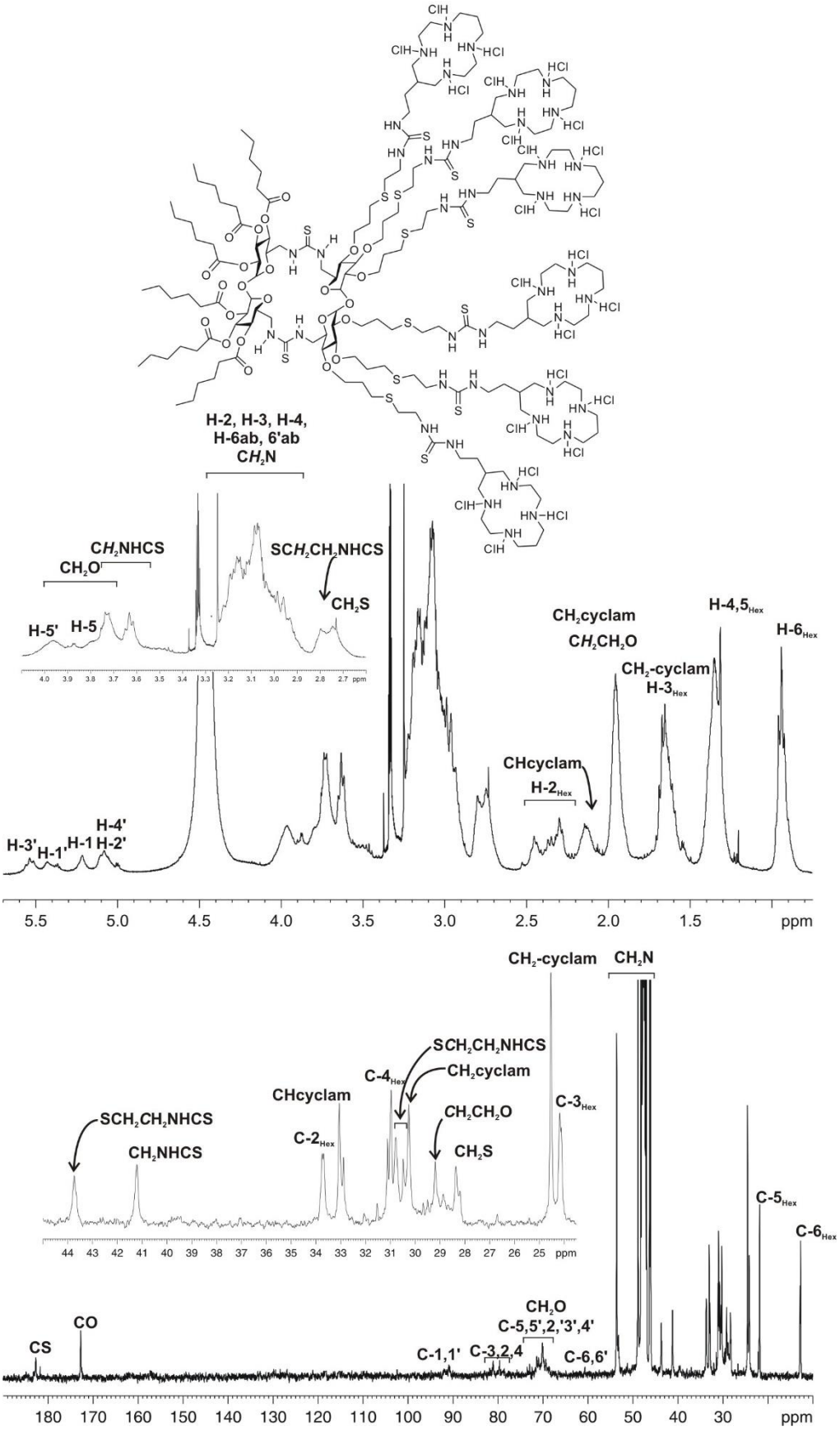


Figure S11. ^{1}H and ^{13}C NMR spectra (400 MHz and 100.6 MHz, CD_3OD, 333 K) of **3.**

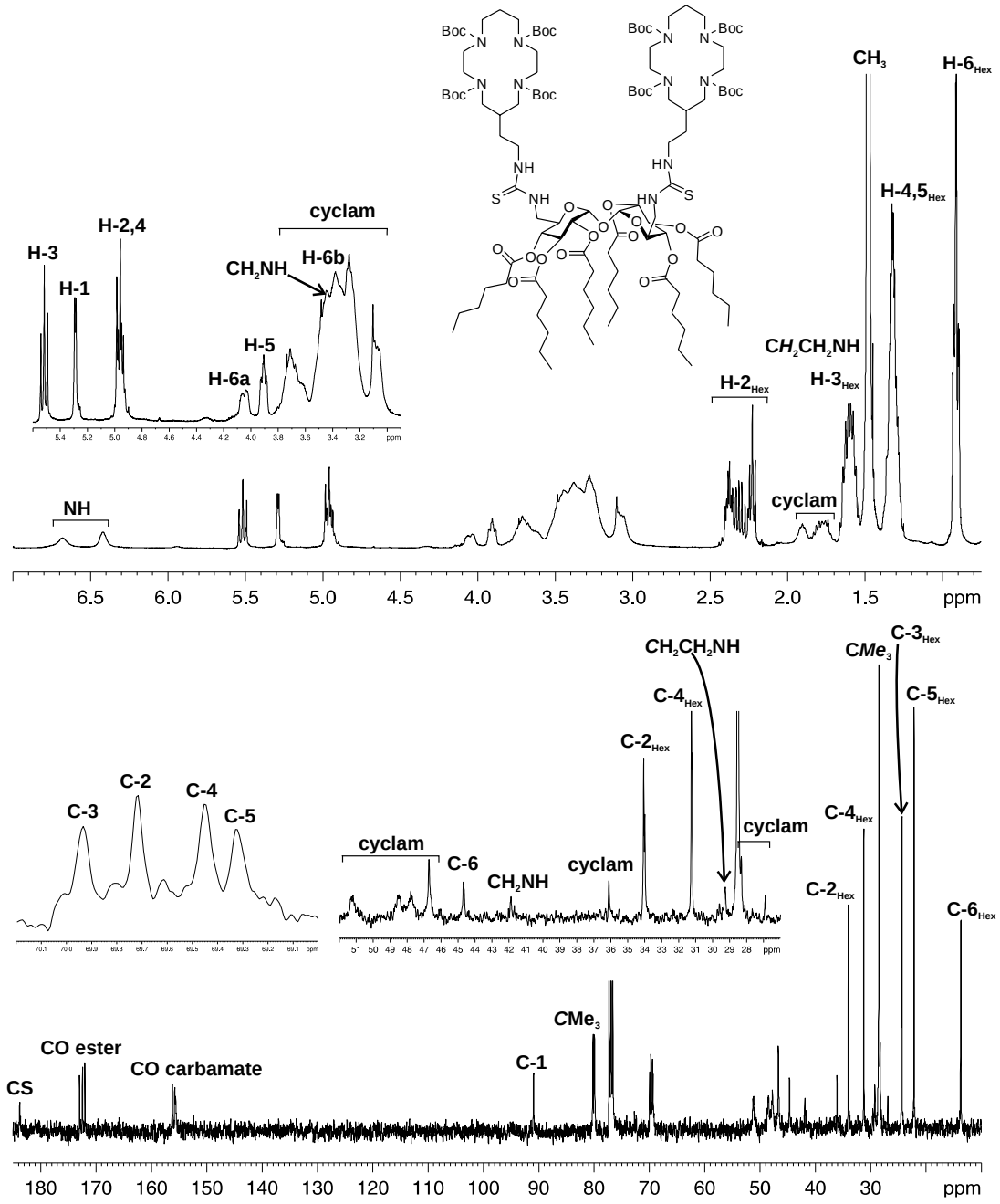


Figure S12. ^{1}H and ^{13}C NMR spectra (400 and 100.6 MHz, $CDCl_3$, 323 K) of **15.**

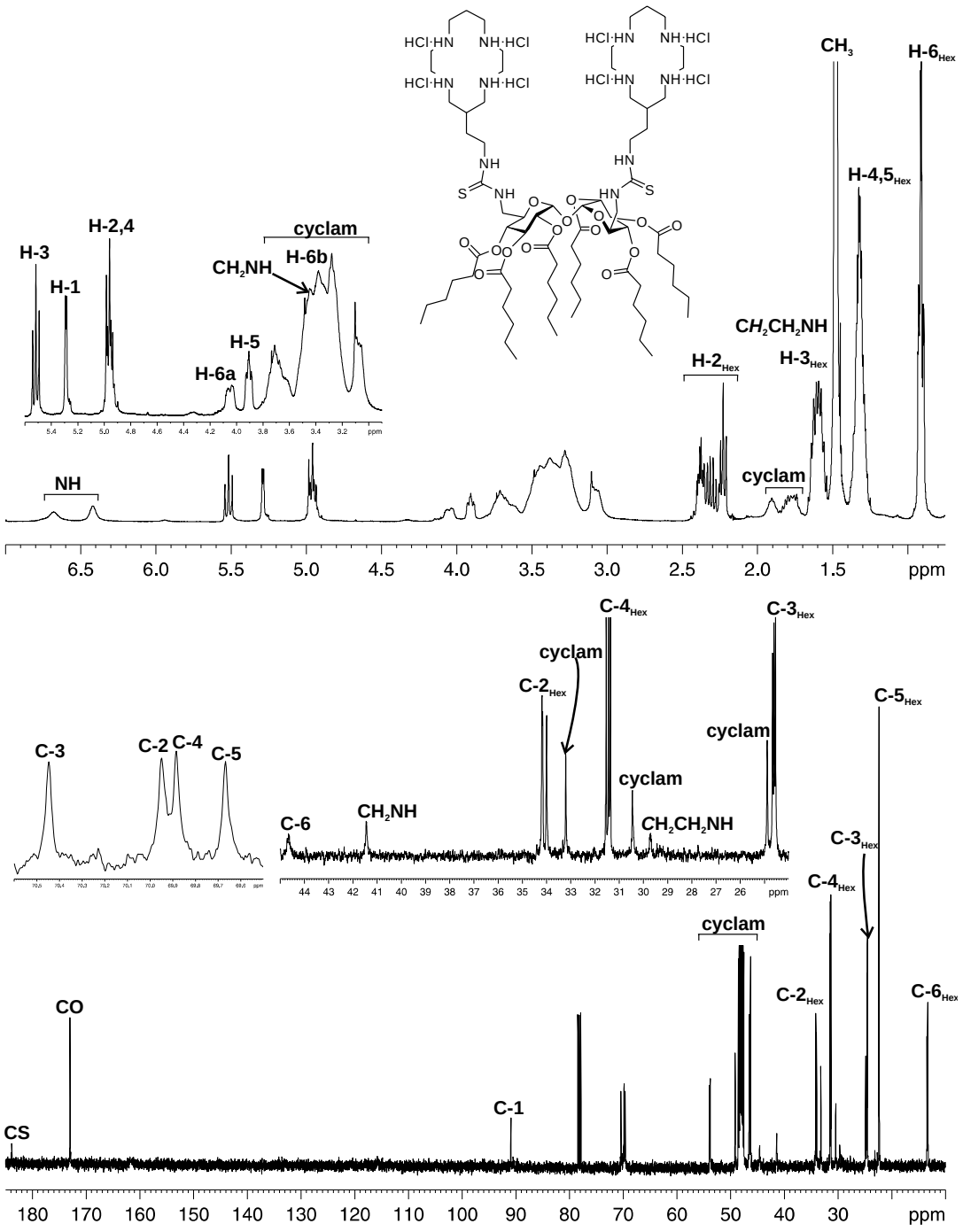


Figure S13. ^{1}H and ^{13}C NMR spectra (500 and 125.7 MHz, CD_3OD 313 K and 298 K, respectively) of **4.**

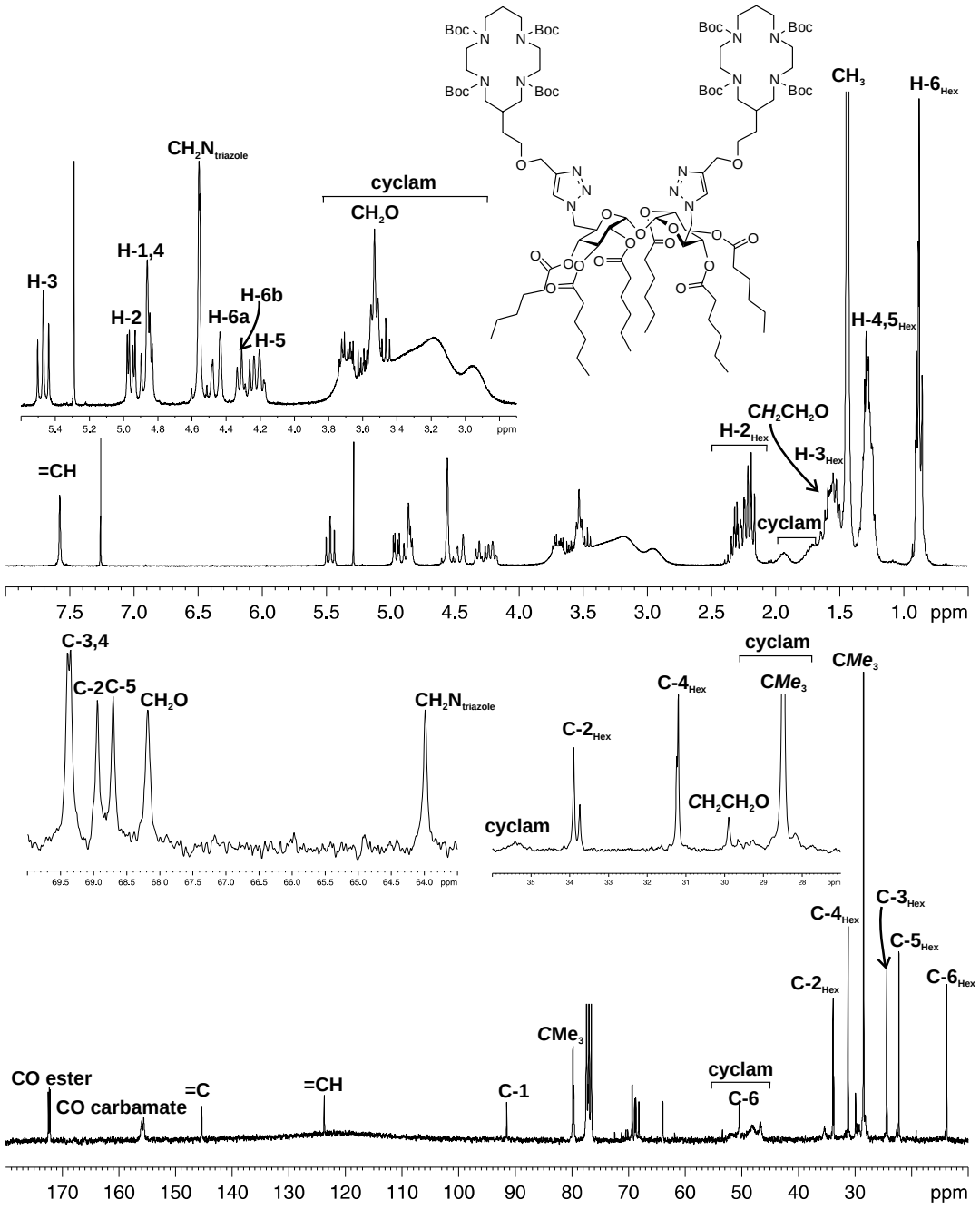


Figure S14. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$, 298 K) of **20.**

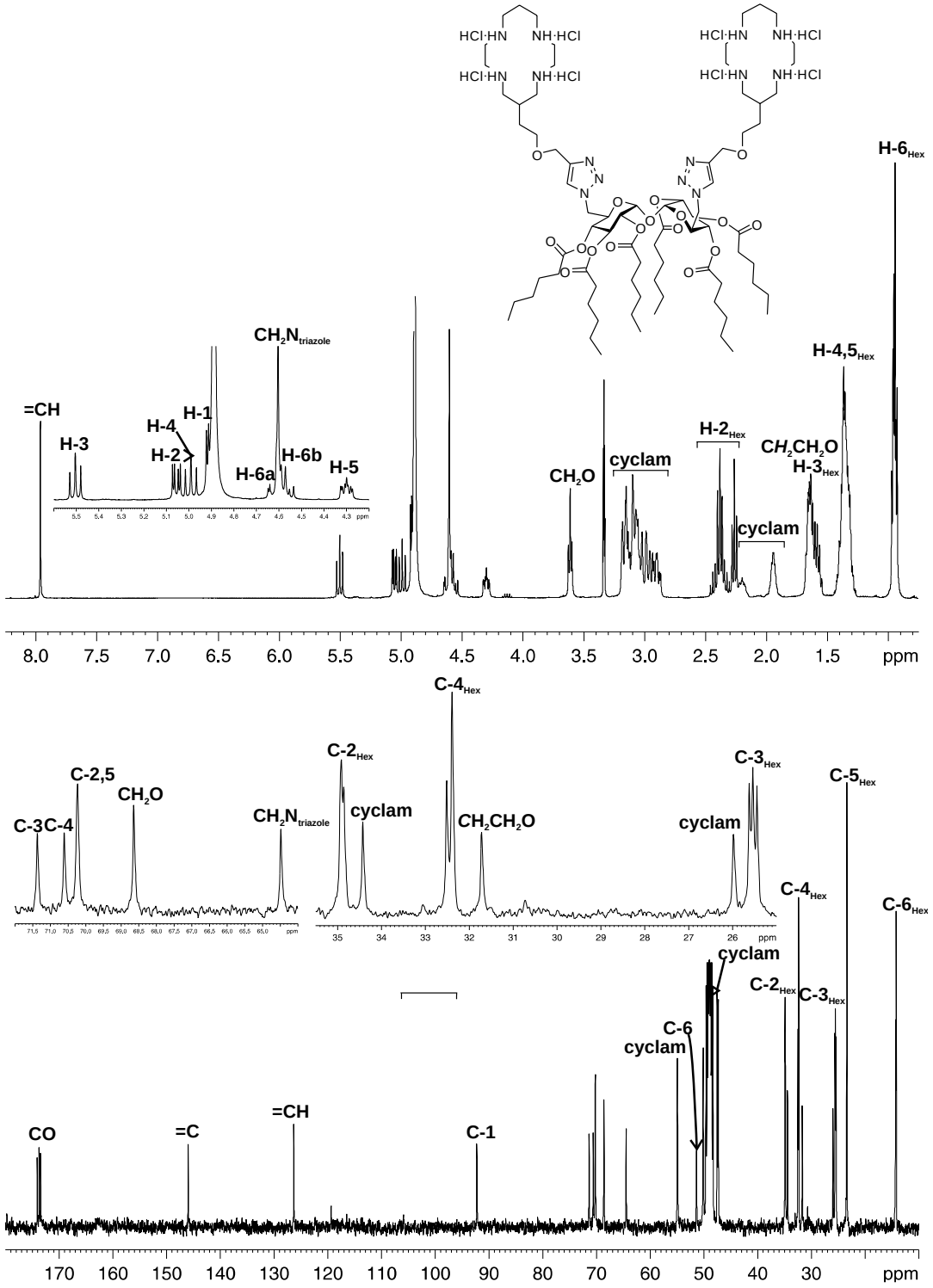


Figure S15. ^{1}H and ^{13}C NMR spectra (400 and 100.6 MHz, CD_3OD 298 K, respectively) of **5.**

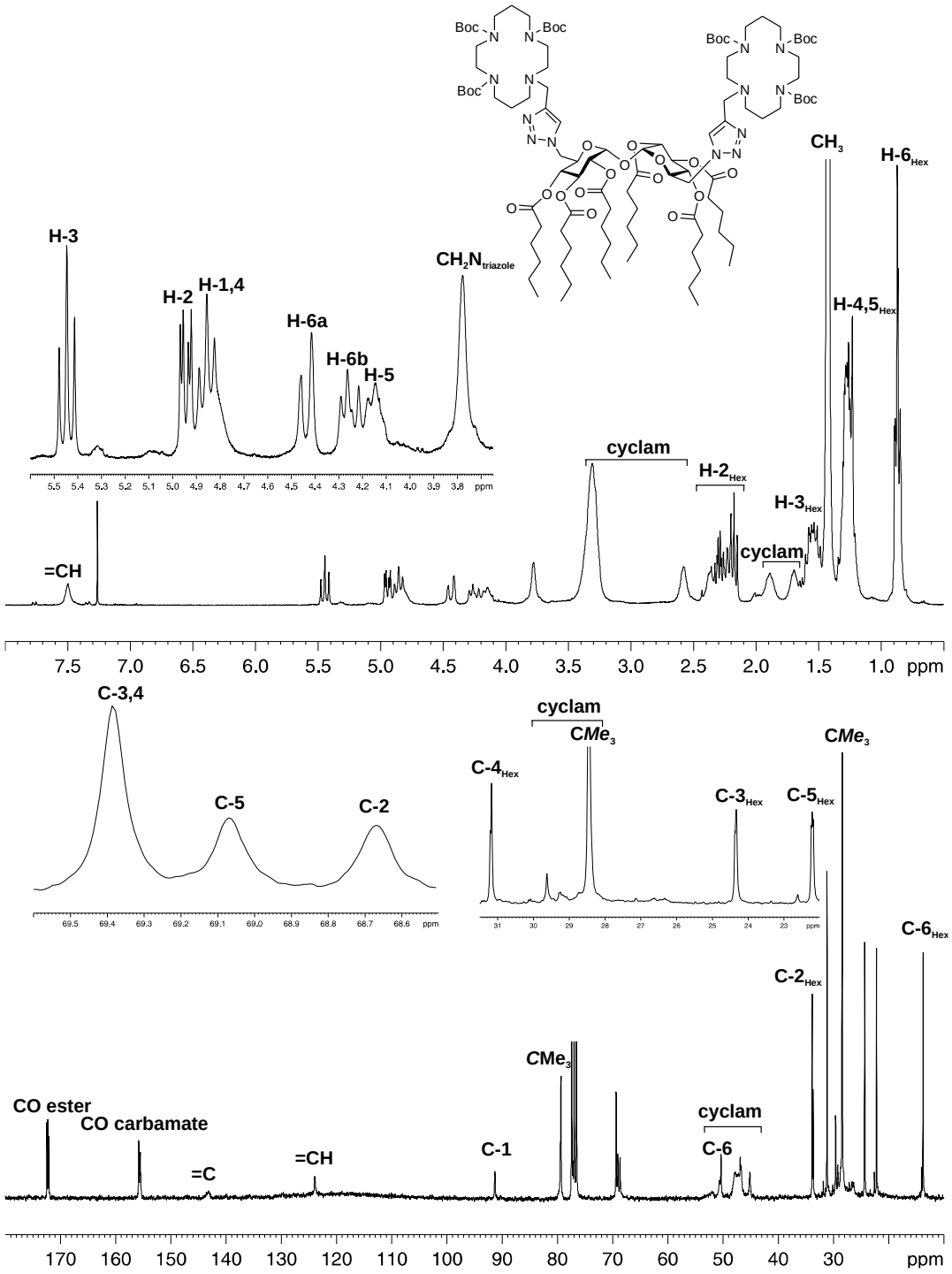


Figure S16. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **21.**

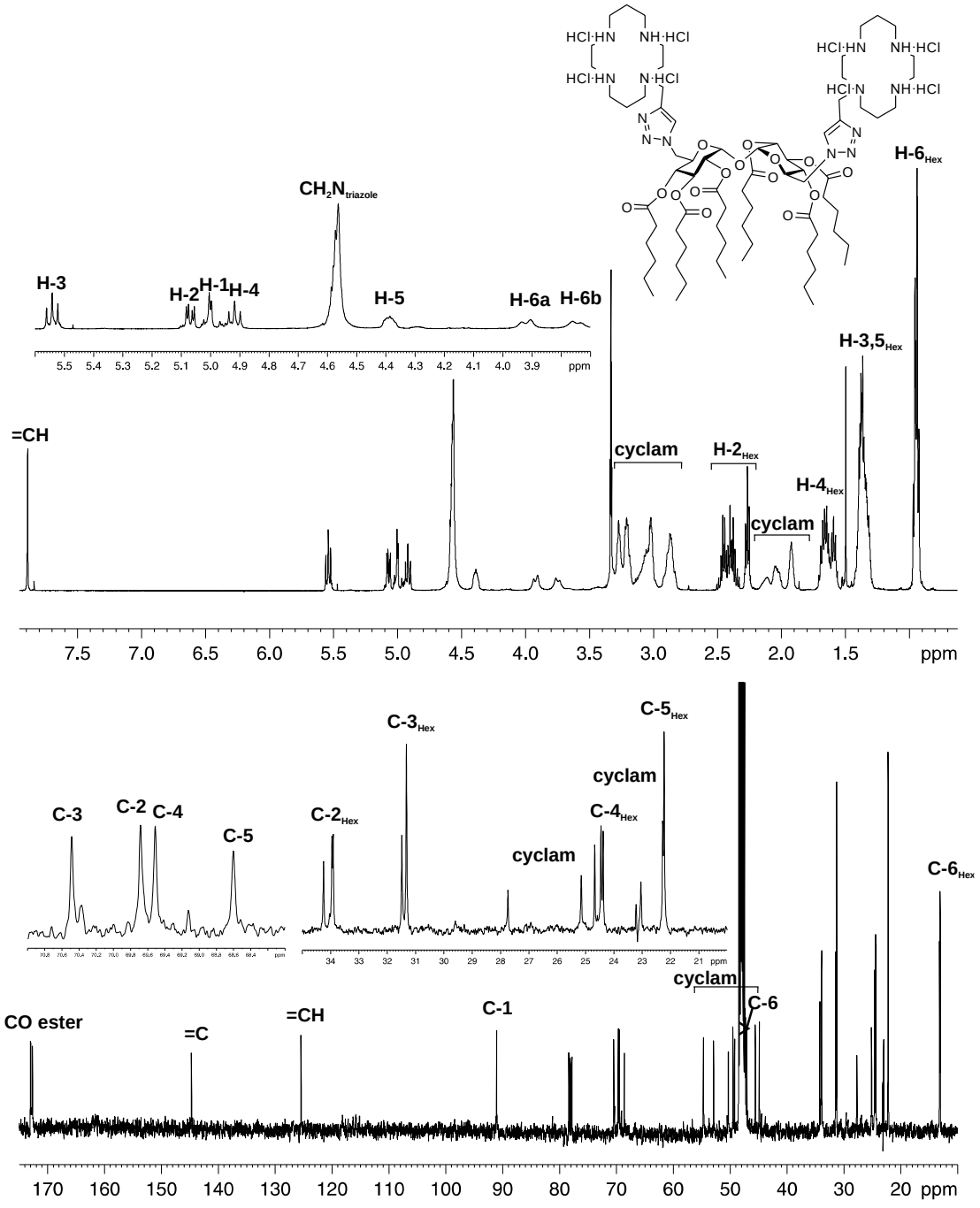
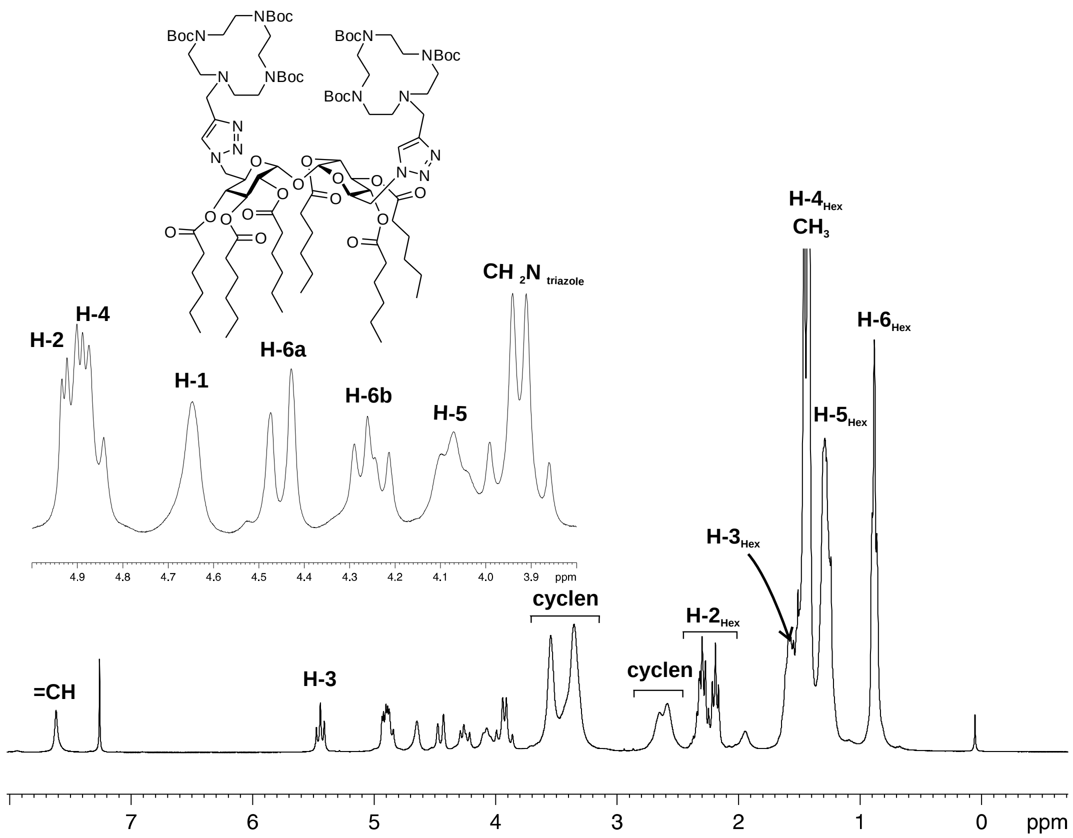


Figure S17. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, CD_3OD, 323 K) of **6.**



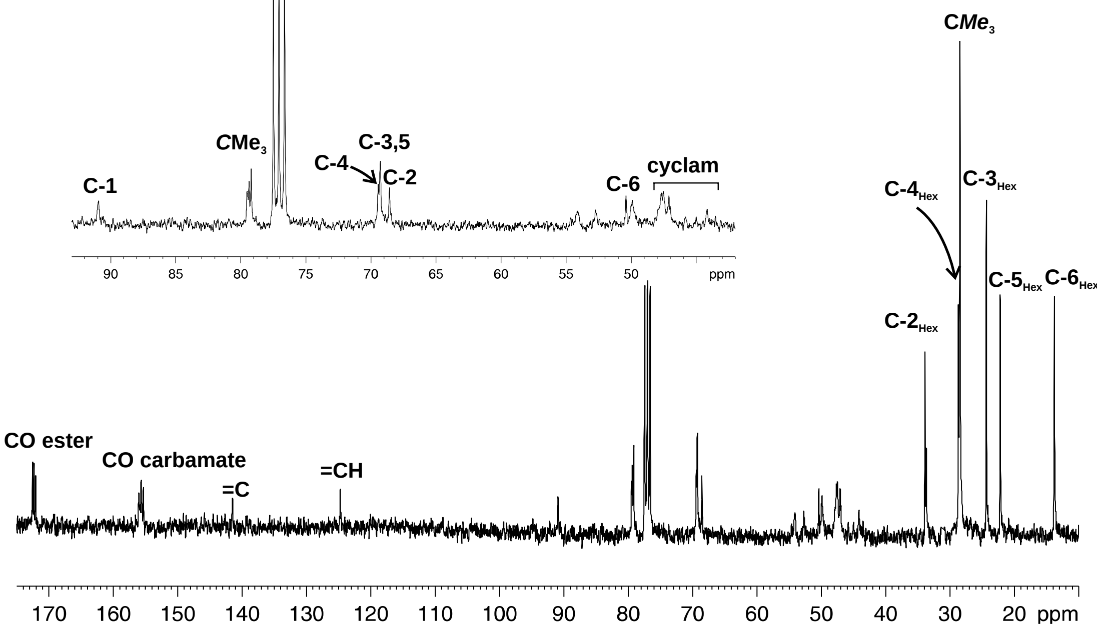


Figure S18. [1]H and [13]C NMR spectra (300 and 75.5 MHz, $CDCl_3$) of **22.**

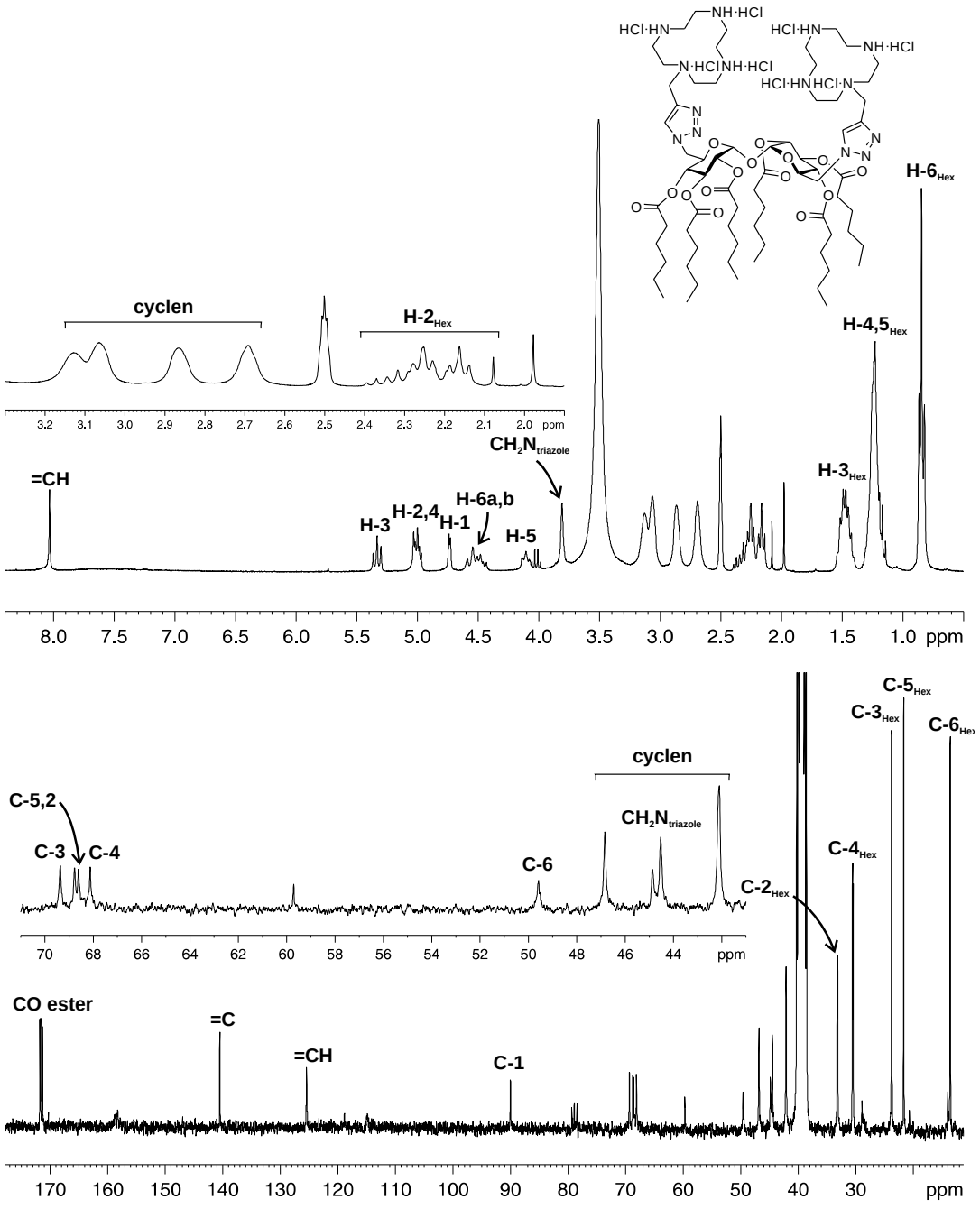


Figure S19. ^{1}H and ^{13}C NMR spectra (300 and 75.5 MHz, DMSO-d_6) of **7.**

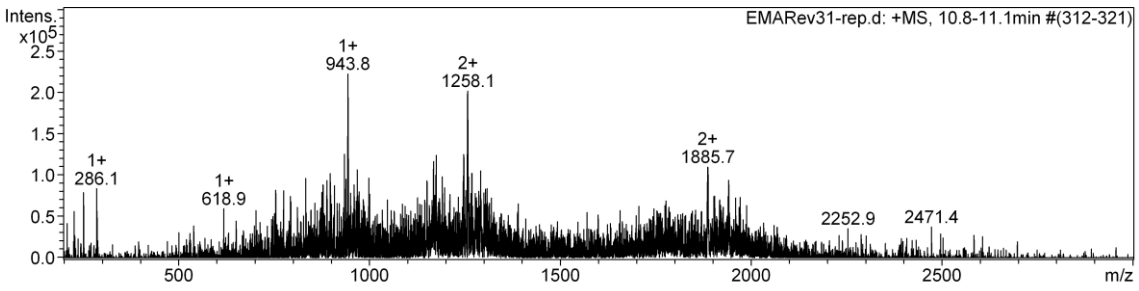


Figure S20. ESI-MS of compound **3**.

[i] A. I. Carbajo-Gordillo, J. López-Fernández, J. M. Benito, J. L. Jiménez Blanco, M. L. Santana-Armas, G. Marcelo, C. Di Giorgio, C. Przybylski, C. Ortiz Mellet, C. Tros de Ilarduya, F. Mendicuti and J. M. García Fernández*, Macromol. Rapid Commun.* **2022***,* 2200145 (1-8).

[ii] J. L. Jiménez Blanco, F. Ortega-Caballero, L. Blanco-Fernández, T. Carmona,G. Marcelo, M. Martínez-Negro, E. Aicart, E. Junquera, F. Mendicuti, C. Tros de Ilarduya, C. Ortiz Mellet and J. M. García Fernández, *Chem. Commun.*, **2016***, 52*, 10117-10120.

[iii] J. Rodriguez Lavado, S. E. Sestito, R. Cighetti, E. M. Aguilar Moncayo, A. Oblak, D. Lainscek, J. L. Jimenez Blanco, J. M. García Fernández, C. Ortiz Mellet, R. Jerala, V. Calabrese and F. Peri, *J. Med. Chem.,* **2014**, *57*, 9105.

[iv] A. Zhanaidarova, C. E. Moore, M. Gembicky and C. P. Kubiak, *Chem. Commun.*, **2018**, *54*, 4116-4119.

[v] K. Jobe, C. H. Brennan, M. Motevalli, S. M. Goldup and M. Watkinson, *Chem. Commun.*, **2011**, *47*, 6036-6038.

[vi] R. Tripier and N. Camus, Methods for preparing functional tetraazacycloalkane compounds using a specific cyclic bisaminal compound, WO2013/072491, **2013**. N. Camus, Z. Halime, N. Le Bris, H. Bernard, C. Platas-Iglesias and R. Tripier, *J. Org. Chem.* **2014**, *79*, 1885-1899. N. Camus, Z. Halime, N. Le Bris, H. Bernard, M. Beyler, C. Platas-Iglesias and R. Tripier, *RSC Adv.*, **2015**, *5*, 85898-85910. E. Lelong, J.-M. Suh, G. Kim, D. Esteban-Gómez, M. Cordier, M. H. Lim, R. Delgado, G. Royal, C. Platas- Iglesias, H. Bernard and R. Tripier, *Inorg. Chem.*, **2021**, *60*, 10857-10872

[vii] A. Megia-Fernández, M. Ortega-Muñoz, J. López-Jaramillo, F. Hernández-Mateo and F. Santoyo.González, *Adv. Synth. Catal.* **2010**, *352*, 3306-3320.