Supramolecular neuromuscular blocker inhibition by a pillar[5]arene through aqueous inclusion of rocuronium bromide

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Electronic Supplementary Information (16 pages)

1. Materials and methods

^{1}H NMR, ^{13}C and 2D NOESY NMR spectra were obtained on a Bruker Avance-400 spectrometer (^{13}C{^{1}H} - 100 MHz and ^{1}H and 2D NOESY - 400 MHz).Chemical shifts were determined against the signals of residual protons of deuterated solvent ($CDCl_3$-d_6, D_2O). The concentration of sample solutions was 3-5 %.

Attenuated total internal reflectance IR spectra were recorded with a Spectrum 400 (Perkin Elmer) Fourier spectrometer.

Elemental analysis was performed with a Perkin Elmer 2400 Series II instrument.

Mass spectra (MALDI-TOF) were recorded on an Ultraflex III mass spectrometer in a 4-nitroaniline matrix. Melting points were determined using a Boetius Block apparatus.

Additional control of the purity of compounds and monitoring of the reaction were carried out by thin-layer chromatography using Silica G, 200 μm plates, UV 254.

Most chemicals were purchased from Aldrich and used as received without additional purification. Organic solvents were purified in accordance with standard procedures.

^{1}H diffusion ordered spectroscopy (DOSY) spectra were recorded on a Bruker Avance 400 spectrometer at 9.4 tesla at a resonating frequency of 400.17 MHz for ^{1}H using a BBO Bruker 5 mm gradient probe. The temperature was regulated at 298 K and no spinning was applied to the NMR tube. DOSY experiments were performed using the STE bipolar gradient pulse pair (stebpgp1s) pulse sequence with16 scans of 16 data points collected. The maximum gradient strength produced in the z direction was 5.35 G mm^{-1}. The duration of the magnetic field pulse gradients (δ) was optimized for each diffusion time (Δ) in order to obtain a 2% residual signal with the maximum gradient strength. The values of δ and Δ were 1.800 μs and 100 ms, respectively. The pulse gradients were incremented from 2 to 95% of the maximum gradient strength in a linear ramp[S1]

Electrospray ionization mass spectra (ESI) were obtained on an AmazonX mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The measurements were carried out in the regime of positive ions registration in the range of *m/z* from 100 to 2800. The voltage on the capillary was -4500 V. Nitrogen was used as the gas-drier with a temperature of 300 °C and a flow rate of 10 L·min^{-1}. The compounds were dissolved in acetonitrile to a concentration of 10^{-6} g/L. Data was processed using DataAnalysis 4.0 (Bruker Daltonik GmbH, Bremen, Germany).

Fluorescence spectra were recorded on a Fluorolog 3 luminescent spectrometer (Horiba Jobin Yvon). The excitation wavelength was selected as 285 nm. The emission scan range was 300-540 nm. Excitation and emission slits were 4 nm. Quartz cuvettes with optical path length of 10 mm were used. Fluorescence spectra were automatically corrected by the Fluoressence program. The spectra were recorded in water solutions with concentration of pillar[5]arenes 10 μM. The obtained molar ratio of pillar[5]arenes **3** to rocuronium bromide was 1:200. The experiment was carried out at 293 K. The temperature dependences of fluorescence for all compounds were determined at 278 and 308 K.

UV-vis spectra were recorded using the Shimadzu UV-3600 spectrometer; the cell thickness was 1 cm, slit width 1 nm. Deionized water with a resistivity >18.0 MΩ cm was used to prepare the solutions. Deionized water was obtained from a Millipore-Q purification system. Recording of the absorption spectra of the mixtures of rocuronium bromide with pillar[5]arenes **3** (1×10^{-5} M) and model compound **4** (5×10^{-5} M) was carried out after mixing the solutions at 293 K.

The particle sizes were determined in the deionized H_2O by the Zetasizer Nano ZS instrument at 20 °C .

Calculations were undertaken using the Spartan '18 Parallel Suite[S2] running on a Mac Pro with 3.5 GHz 6-Core Intel Xenon E5 processors and two threads per core.

In vitro cytotoxicity assay for rocuronium bromide, Sugammadex and **3** were performed towards two human normal cell lines (WI-38 VA-13, human embryonic lung) from the Type Culture Collection of the Institute of Cytology (Russian Academy of Sciences) and Chang liver cells (human liver cells) from N. F. Gamaleya Research Center of Epidemiology and Microbiology. Both cell lines were cultivated in Eagle's nutrient medium with addition of 10 % fetal bovine serum. 96-well plates were used for seeding of cells (the density of seeding was kept about 100 000 cells per well). After that system was equilibrated in atmosphere of 5% CO_2 during 24 h. Twenty-four hours after seeding the cells the tested compounds were added. Estimation of cytotoxic activity was carried out through calculation of viable cells using Cytell Cell Imaging (GE Helthcare Life Science) multifunctional system using Cell Viability BioApp software application allowing of estimating the number of cells and evaluating their viability on the basis of ratio DAPI/Propidium iodide fluorescence intensity. Data represented as mean IC_{50} ± SD from three independent experiments made in three replicates. Intact cells cultured in parallel with experimental cells were used as a control [S4]. To test the ability of Sugammadex and **3** to shift cytotoxicity of rocuronium bromide, rocuronium bromide was applied alone or co-applied together with the equal concentration of Sugammadex or **3.** After that percentage of viable cells were estimated as described above.

To study the effect of **3** on the muscle contractility after the neuromuscular blockade caused with non-depolarizing muscle relaxant rocuronium bromide, isolated mouse hemidiaphragm nerve-muscle preparation was used.

Experiments were performed on neuromuscular preparations from diaphragms excised from white laboratory mice of the ICR line (CD-1) of both sexes (weighting 22–25 g) in strict accordance with European Communities Council Directive 86/609 / EEC; protocol No. 9 - 2013, approved by the ethical committee of the Kazan Federal University.

Animals were deeply anesthetized by isoflurane inhalation until the tail-pinch reflex disappeared. Hemidiaphragm muscles with phrenic nerves were isolated from mice killed by dislocation of the cervical vertebrae. Preparations were mounted in a temperature-controlled chamber filled with oxygenated (O_2 95%, CO_2 5%) Ringer's-Krebs' solution (in mM): 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 23 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 11 mM glucose. pH was adjusted to 7.2-7.4; experiments were conducted at room temperature of 20±2 °C.

For twitch tension measurements, the central tendon of the hemidiaphragm muscle was tied by a stainless steel rod via a linen thread hook to a force sensor TRI201AD (ADInstruments, serial №1762414) and the ribs were immobilized with two long hooks. The responses were evoked by stimulation of the phrenic nerve using Electronic stimulator 1001 (AD Instruments) by supramaximal current pulses 0.1 ms duration. Signals from the force sensor were acquired and analyzed digitally, using PowerLab system and LabChart 8 software (ADInstruments). The force of the contractions was measured in grams. Four contractions (Train-of-four) were evoked by phrenic nerve stimulation at 2 Hz four times, with 15 s intervals between TOFs. The drugs were delivered to diaphragm muscle by a bath application. TOFs were recorded in intact muscle preparation. Then 10 μM rocuronium bromide containing solution was applied until the muscle contractions completely disappeared, and then either **3** or Sugammadex at final concentration 10 μM were added.

Data are presented as mean +/- SEM. Statistical analysis was performed using Origin 8.1. Paired Wilcoxon test was used for statistical analysis of experimental data. The differences were considered significant at $p < 0.05$.

Decamethoxypillar[5]arene and pillar[5]arene containing 10 carboxylate groups (DCP[5]A) were synthesized according to the literature procedures[S3,5].

2. Characterization of compounds 2-4

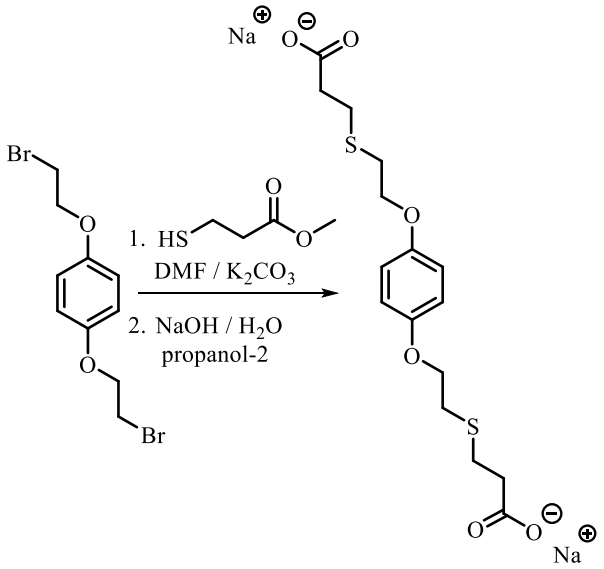
4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2)

Yield 0.31 g (84%). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): 2.55 (20H, t, $^{3}J_{HH}$= 7.3 Hz, -S-CH_2-$\underline{CH_2}$-C(O)), 2.80 (20H, t, $^{3}J_{HH}$= 7.3 Hz, -S-$\underline{CH_2}$-CH_2-C(O)), 2.94 (20H, t, $^{3}J_{HH}$= 5.7 Hz, -O-CH_2-$\underline{CH_2}$-S-), 3.62 (30H, s, -C(O)O-CH_3), 3.75 (10H, s, -CH_2-), 4.08 (20H, m, -O-$\underline{CH_2}$-CH_2-S-), 6.86 (10H, s, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): 27.58, 29.49, 31.86, 34.83, 51.87, 68.14, 115.33, 128.56, 149.64, 172.32. IR (ν/см$^{-1}$): 2942, 2887 (-CH_2-, -CH_3), 1643 (-C(O)O-CH_3), 1232 (Ar-O-CH_2-). MS (MALDI-TOF): calc. [M^+] *m/z* = 2071.6, found [M+Na]$^+$ *m/z* = 2094.6. Found (%): C, 54.88; H, 6.12; S, 15.21. Calc. for $C_{95}H_{130}O_{30}S_{10}$. (%): C, 55.05; H, 6.32; S, 15.47.

4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3)

Yield 0.30 g (96%). ^{1}H NMR (400 MHz, D_2O, 298 K), δ (ppm), J/Hz: 2.48 (20H, t, $^{3}J_{HH}$= 7.3 Hz, -S-CH_2-$\underline{CH_2}$-C(O)-O$^-$), 2.83 (20H, t, $^{3}J_{HH}$= 7.3 Hz, -S-$\underline{CH_2}$-CH_2-C(O)), 2.87 (20H, m, -O-CH_2-$\underline{CH_2}$-S-), 3.80 (10H, s, -CH_2-), 3.89 (10H, m, -O-$\underline{CH_2}$-CH_2-S-), 4.04 (10H, m, -O-$\underline{CH_2}$-CH_2-S-), 6.87 (10H, s, ArH). ^{13}C NMR (100 MHz, D_2O, 298 K), δ (ppm): 28.46, 30.99, 37.58, 68.49, 116.11, 129.23, 149.75, 180.61. IR (ν/см$^{-1}$): 2923, 2866 (-CH_2-, -CH=), 1562, 1399 (-COO^-), 1205 (Ar-O-CH_2-). MS (ESI): calc. [M^+] *m/z* = 2150.25, found [M-10Na+6H]$^{4-}$ *m/z* = 481.60. Found (%): C, 52.25; H, 6.20; S, 15.83. Calc. for $C_{85}H_{100}O_{30}S_{10}Na_{10}$. (%):C, 53.11; H, 5.24; S, 16.68.

1,4-bis(propionatsulfanediyl)ethoxy)benzene sodium salt (4)



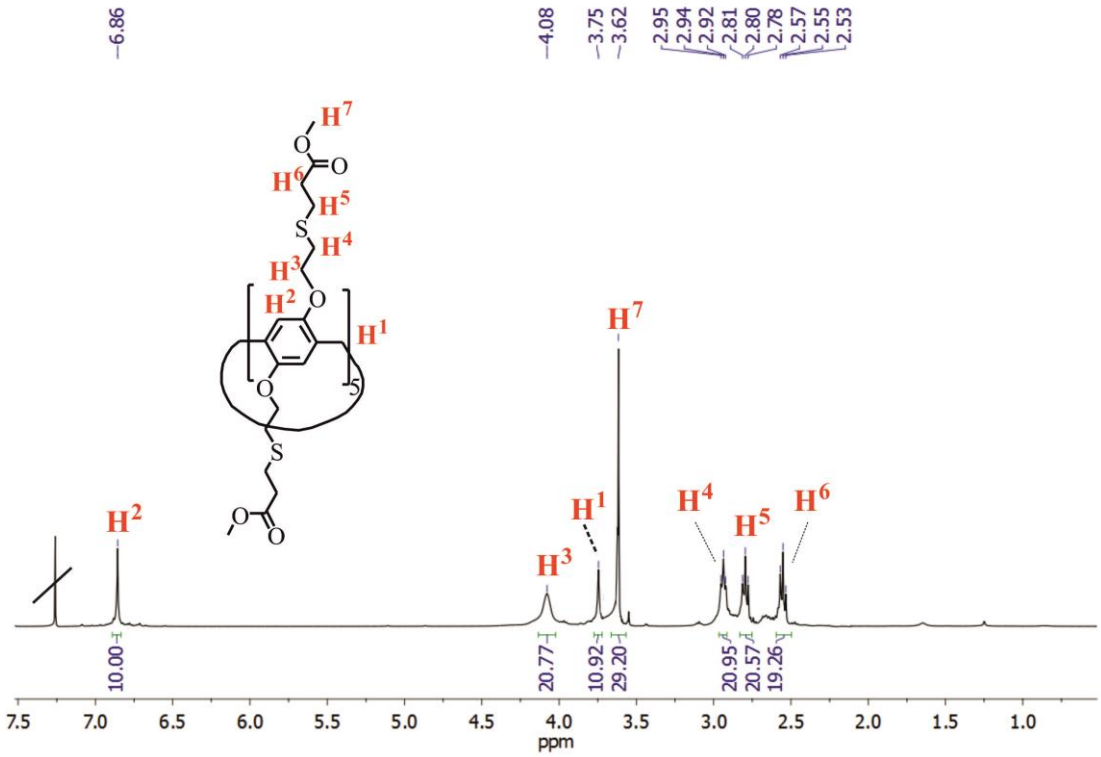
4 (89%)

Yield 1.12 g (89%). ^{1}H NMR (400 MHz, D_2O, 298 K), δ (ppm), J/Hz: 2.45 (4H, t, $^{3}J_{HH}$= 7.2 Hz, -S-CH_2-$\underline{CH_2}$-C(O)-O$^-$), 2.79 (4H, t, $^{3}J_{HH}$= 7.2 Hz, -S-$\underline{CH_2}$-CH_2-C(O)), 2.91 (4H, t, $^{3}J_{HH}$= 6.1 Hz, -O-CH_2-$\underline{CH_2}$-S-), 4.17 (4H, t, $^{3}J_{HH}$= 6.1 Hz, -O-$\underline{CH_2}$-CH_2-S-), 6.96 (4H, s, ArH). ^{13}C NMR (100 MHz, D_2O, 298 K), δ (ppm): 27.94, 30.21, 37.41, 67.99, 116.26, 116.30, 152.35, 176.38. IR (ν/см$^{-1}$):

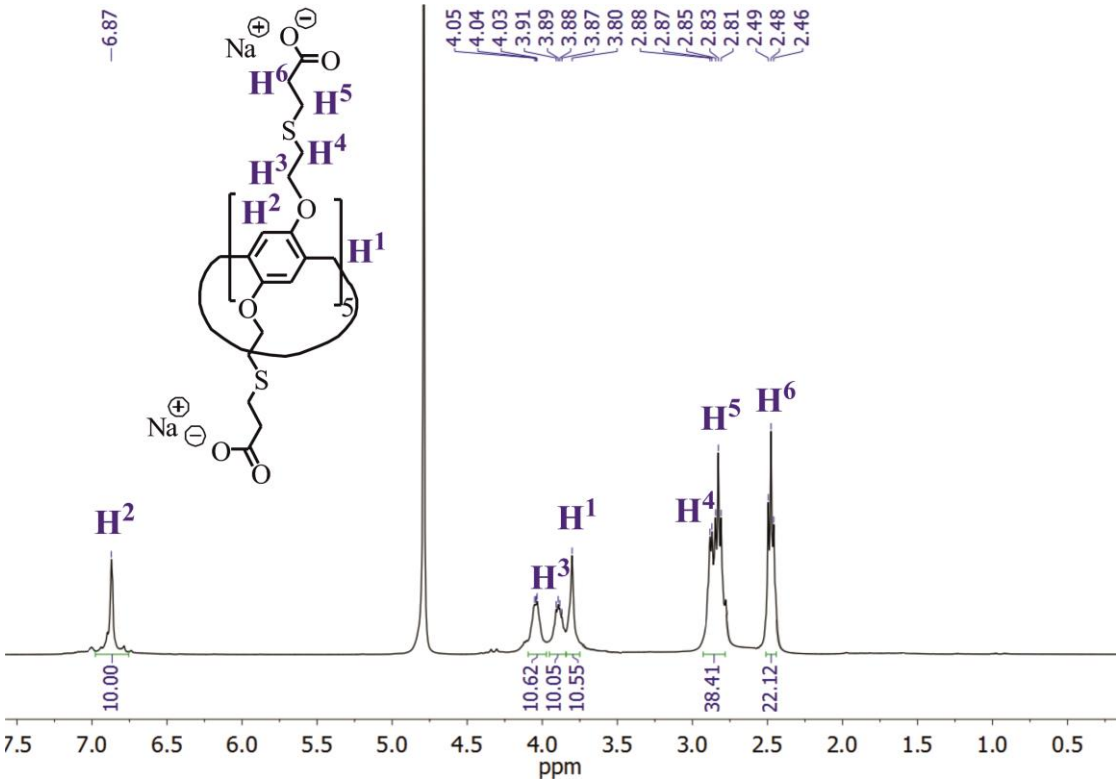
2976, 2945 (-CH_2-, -CH=), 1676 (-C=O). MS (MALDI-TOF): calc. [M^+] *m/z* = 418.1, found [M+K]$^+$ *m/z* = 457.4. Found (%): C, 50.01; H, 5.08; S, 16.83. Calc. for $C_{16}H_{20}O_6S_2Na_2$. (%):C, 51.60; H, 5.41; S, 17.22.

3. NMR, MALDI TOF MS, ESI MS, IR spectra of compounds 2 and 3.

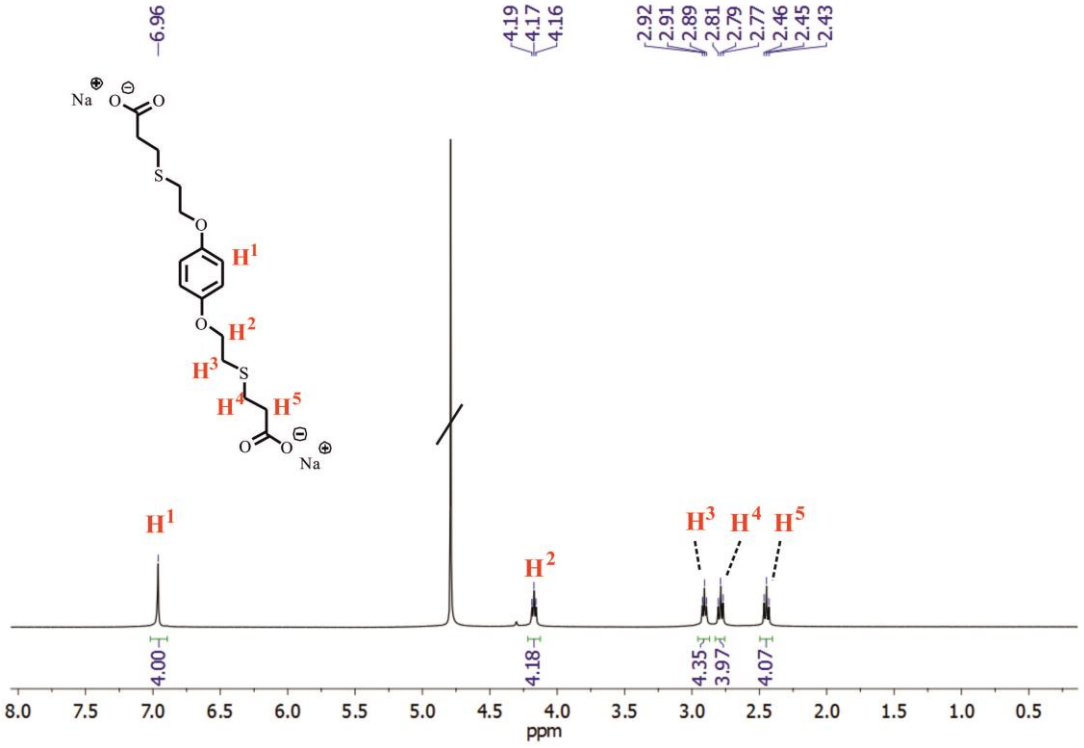
^{1}H NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2), $CDCl_3$, 298 K, 400 MHz



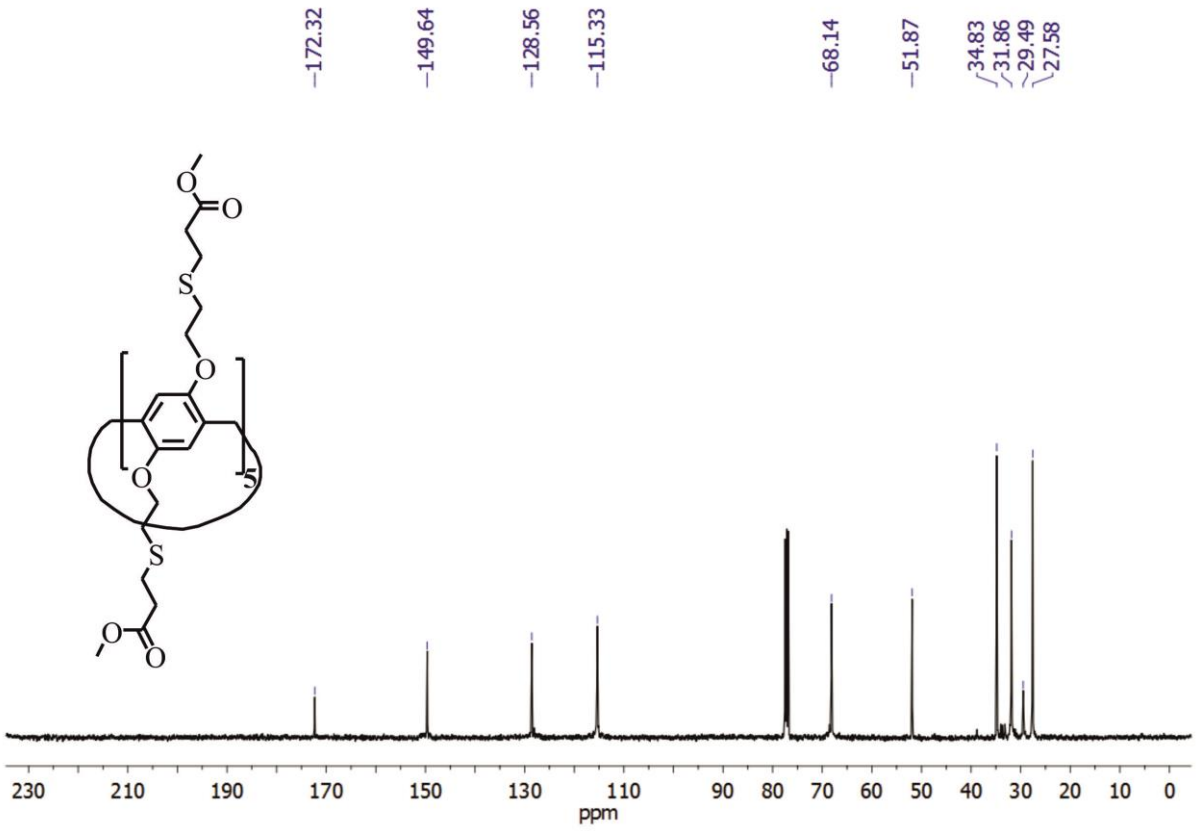
^{1}H NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3), D_2O, 298 K, 400 MHz



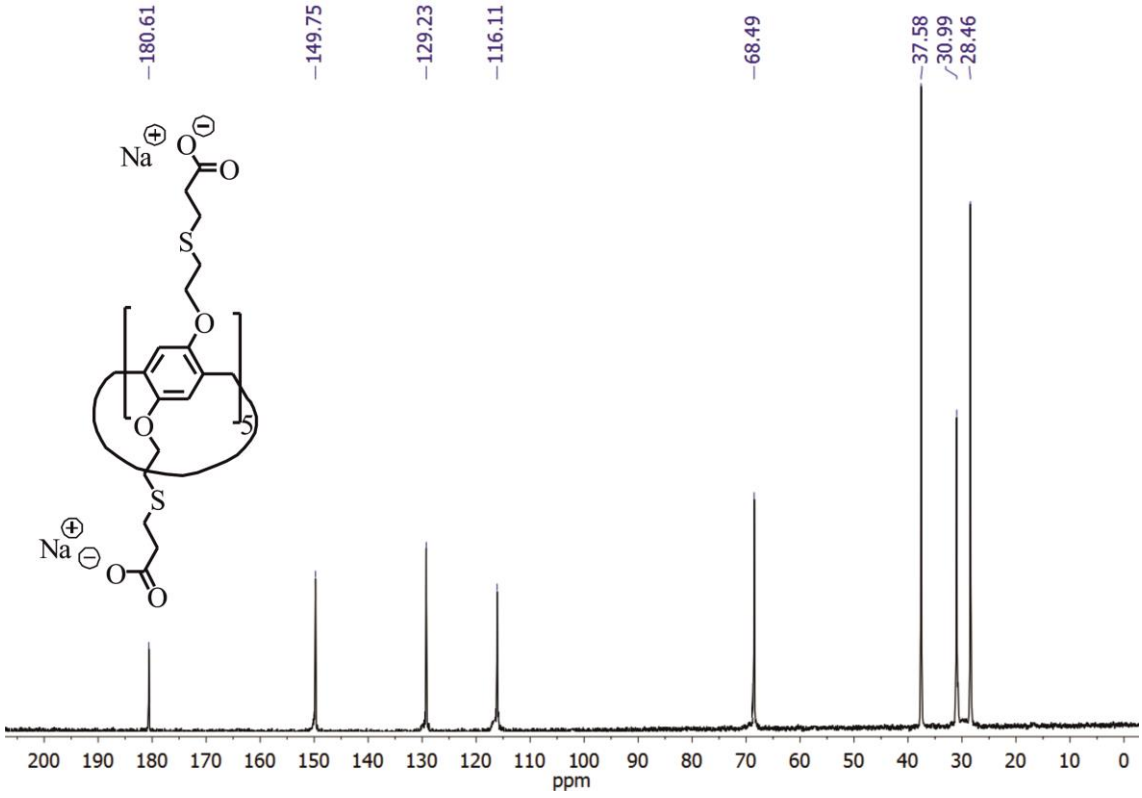
^{1}H NMR spectrum of 1,4-bis(propionatsulfanediyl)ethoxy)benzene sodium salt (4), D_2O, 298 K, 400 MHz



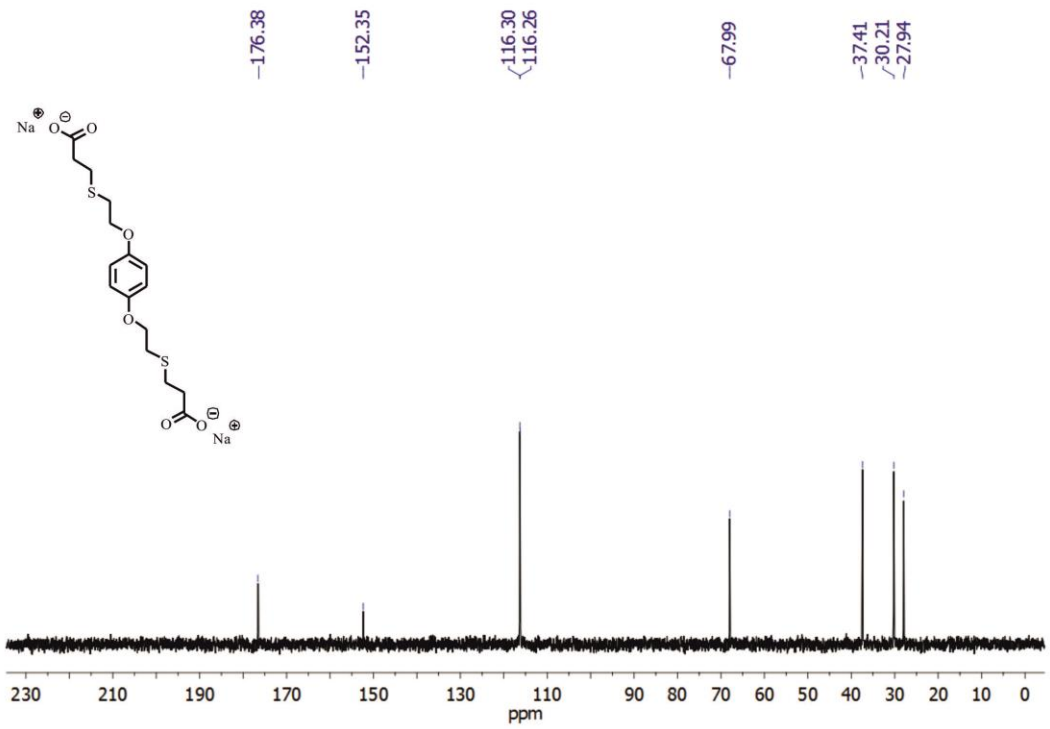
^{13}C NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2), $CDCl_3$, 298 K, 100 MHz



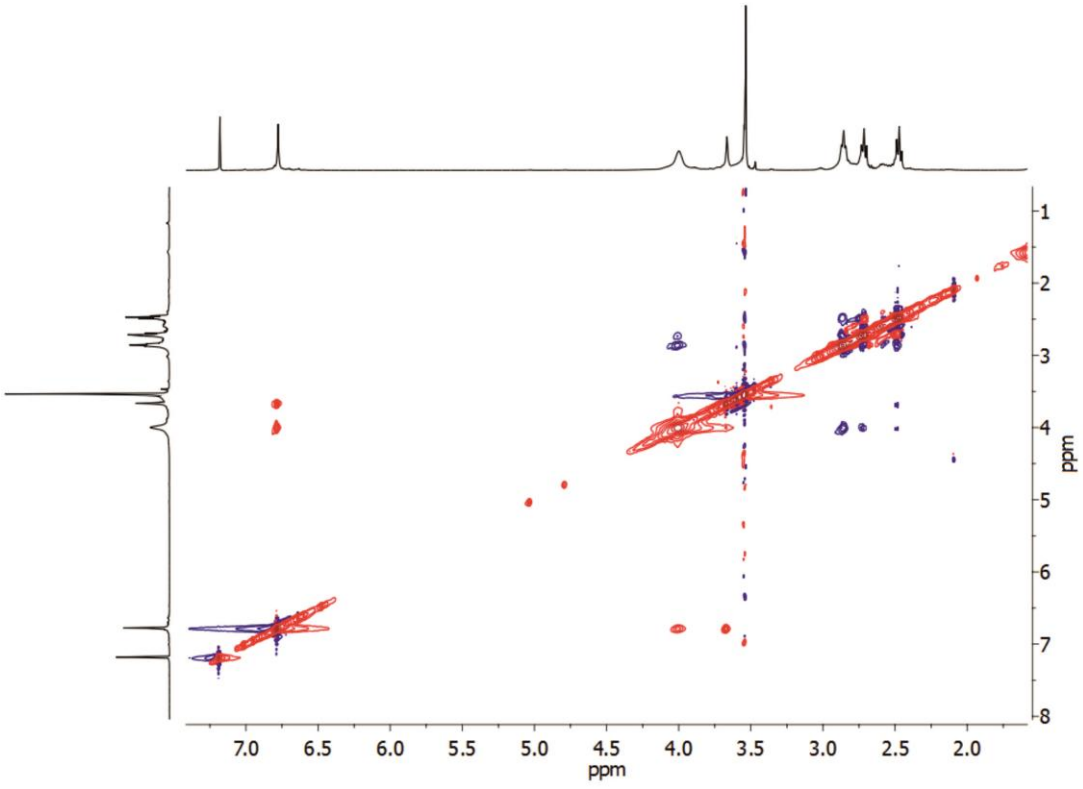
^{13}C NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3), D_2O, 298 K, 100 MHz



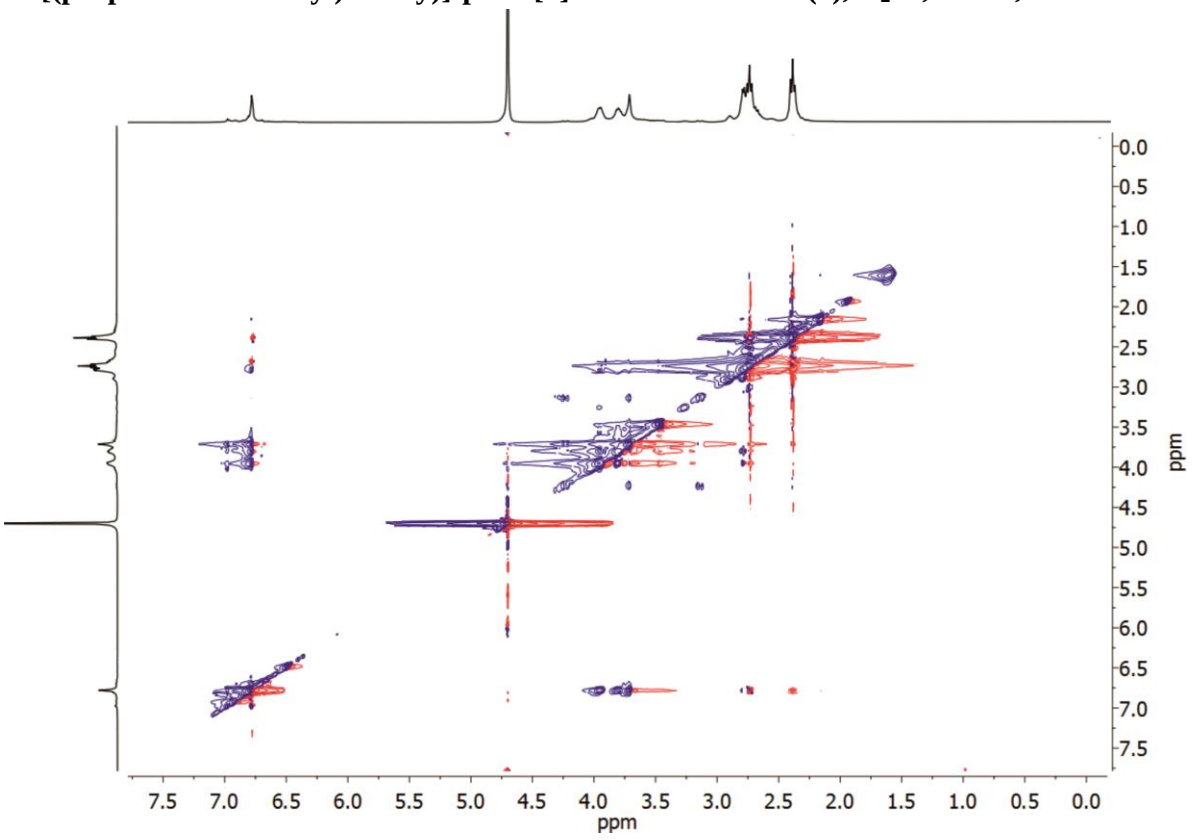
^{13}C NMR spectrum of 1,4-bis(propionatsulfanediyl)ethoxy)benzene sodium salt (4), D_2O, 298 K, 100 MHz



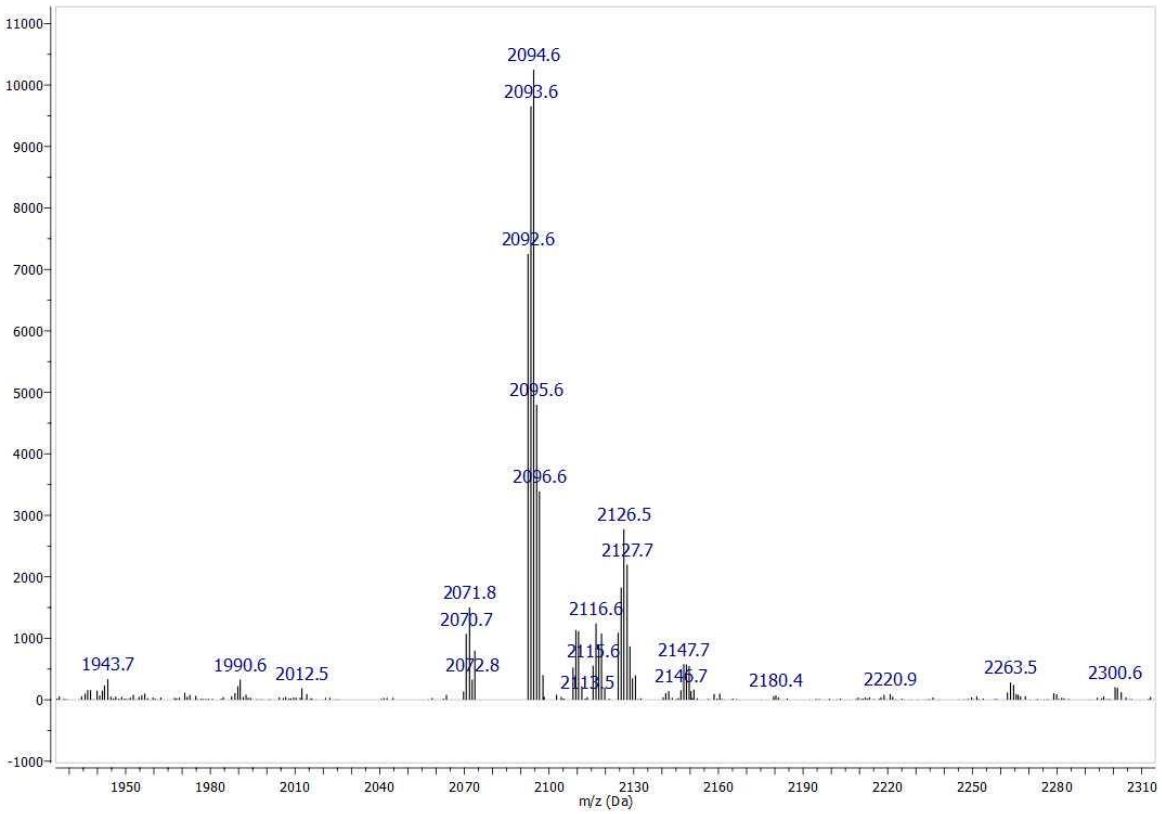
^{1}H-^{1}H NOESY NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2), $CDCl_3$, 298 K, 400 MHz



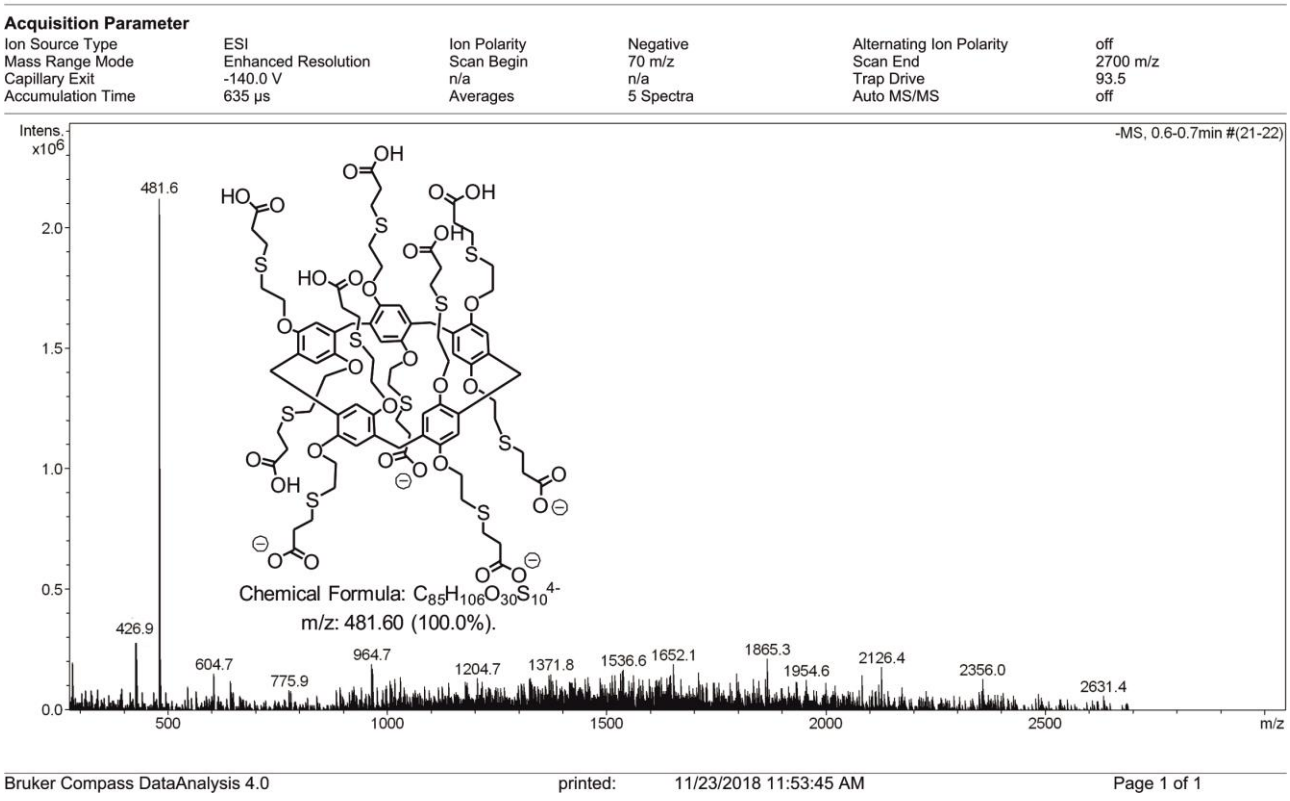
^{1}H-^{1}H NOESY NMR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3), D_2O , 298 K, 400 MHz



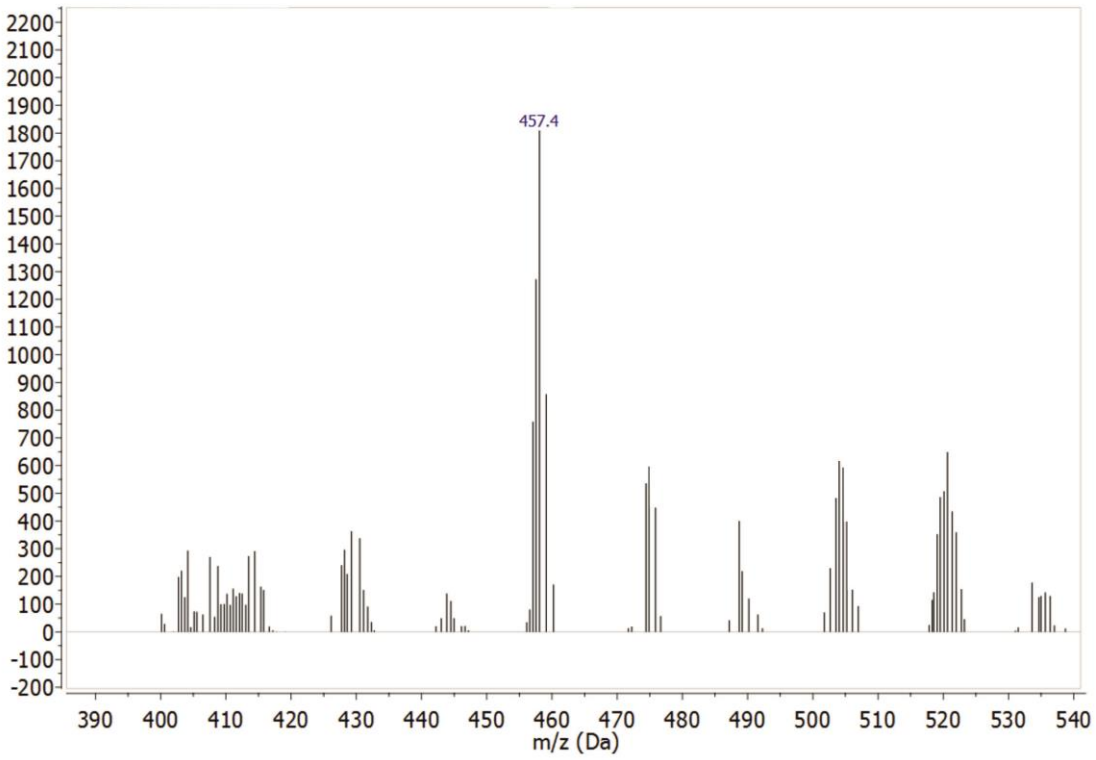
Mass spectrum (MALDI-TOF, 4-nitroaniline matrix) of 4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2)



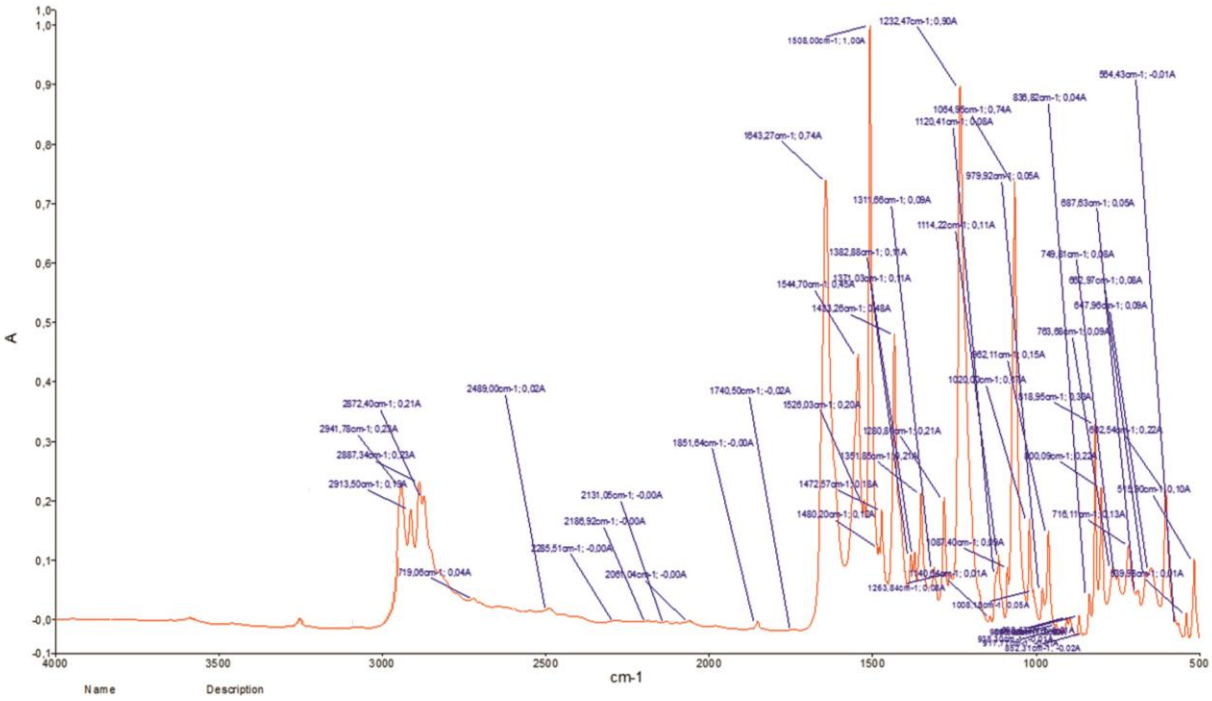
Mass spectrum (ESI) of 4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3)



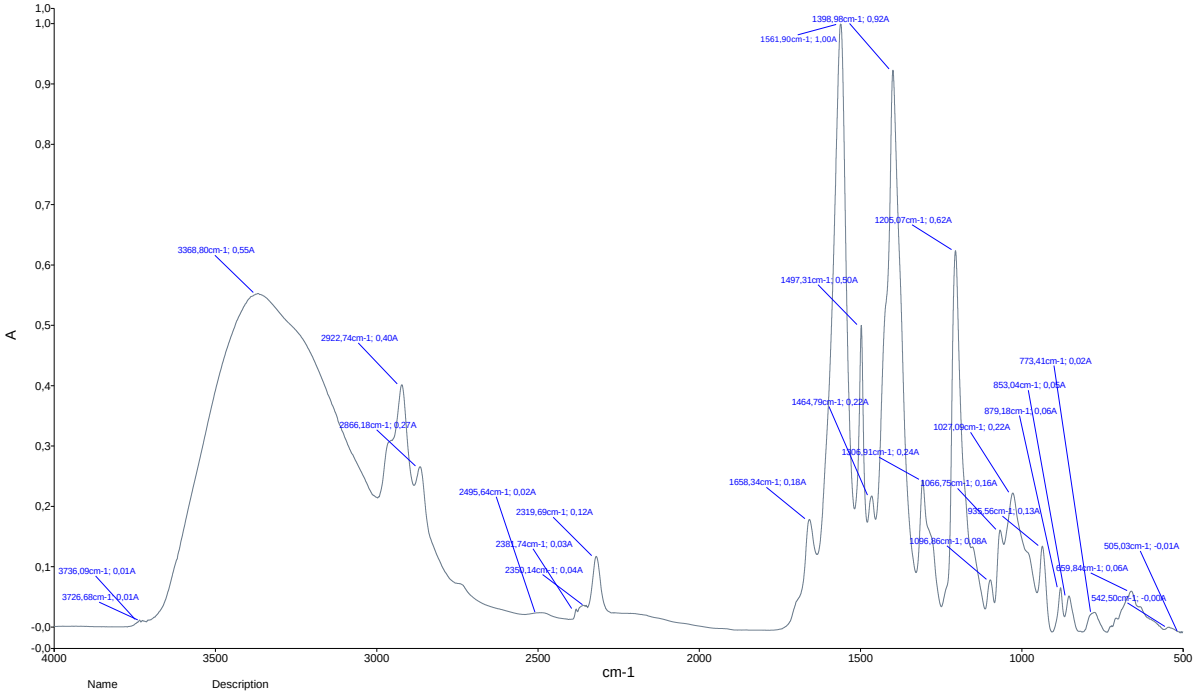
Mass spectrum (MALDI-TOF, 4-nitroaniline matrix) of 1,4-bis(propionatsulfanediyl)ethoxy)benzene sodium salt (4)



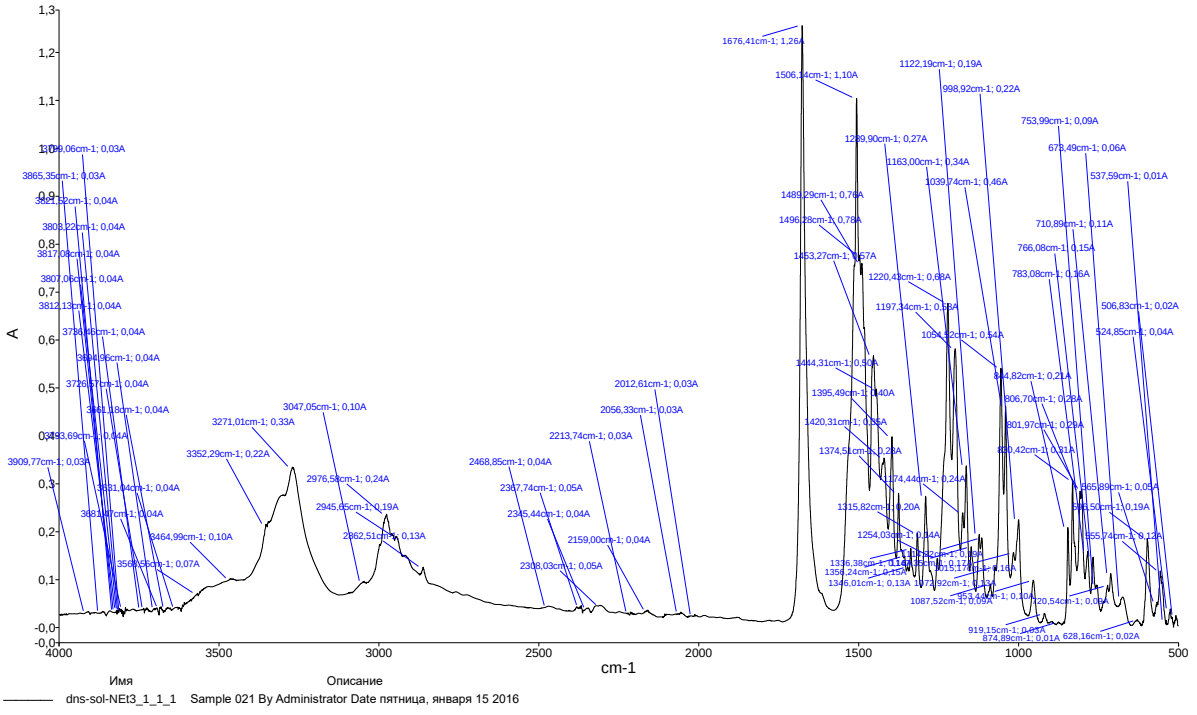
IR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[methyl-(propionatsulfanediyl)ethoxy)]-pillar[5]arene (2)



IR spectrum of 4,8,14,18,23,26,28,31,32,35-deca-[(propionatsulfanediyl)ethoxy)]-pillar[5]arene sodium salt (3)



IR spectrum of 1,4-bis(propionatsulfanediyl)ethoxy)benzene sodium salt (4)



4. Bindfit (Fit data to 1:1, 1:2 and 2:1 Host-Guest equilibria)

Screenshots taken from the summary window of the website supramolecular.org. This screenshots shows the raw data for UV-vis titration of 3 with rocuronium bromide, the data fitted to 1:1 binding model (A), 1:2 binding model (B) and 2:1 binding model (C).

(a)

Fitter: UV 1:1 | Fit | Summary | Save

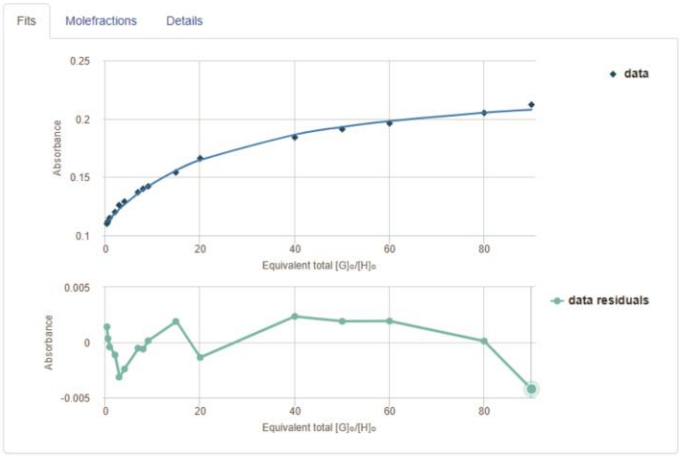
Details

Time to fit	0.2196 s
SSR	2.0526e-4
Fitted datapoints	16
Fitted params	2

Parameters

Parameter (bounds)	Optimised	Error	Initial
K (0 → ∞)	4506.32 M⁻¹	± 5.3399 %	100.00 M⁻¹

Back | Next



(b)

Fitter: NMR 1:2 | Fit | Summary | Save

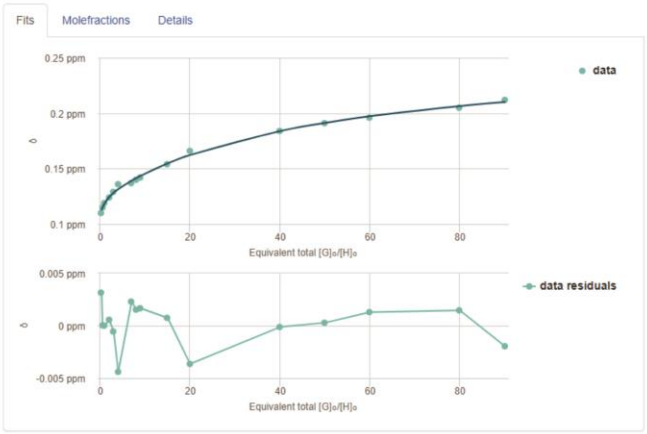
Details

Time to fit	1.5148 s
SSR	6.1830e-5
Fitted datapoints	16
Fitted params	4

Parameters

Parameter (bounds)	Optimised	Error	Initial
K_{11} (0 → ∞)	166943.10 M⁻¹	± 70.8094 %	1000.00 M⁻¹
K_{12} (0 → ∞)	2084.34 M⁻¹	± 7.0054 %	100.00 M⁻¹

Back | Next



(c)

Fitter: UV 2:1 | Fit | Summary | Save

Details

Time to fit	1.1709 s
SSR	7.4270e-5
Fitted datapoints	16
Fitted params	4

Parameters

Parameter (bounds)	Optimised	Error	Initial
K_{11} (0 → ∞)	14753.68 M⁻¹	± 192.8592 %	1000.00 M⁻¹
K_{21} (0 → ∞)	2201996.58 M⁻¹	± 207.6653 %	100.00 M⁻¹

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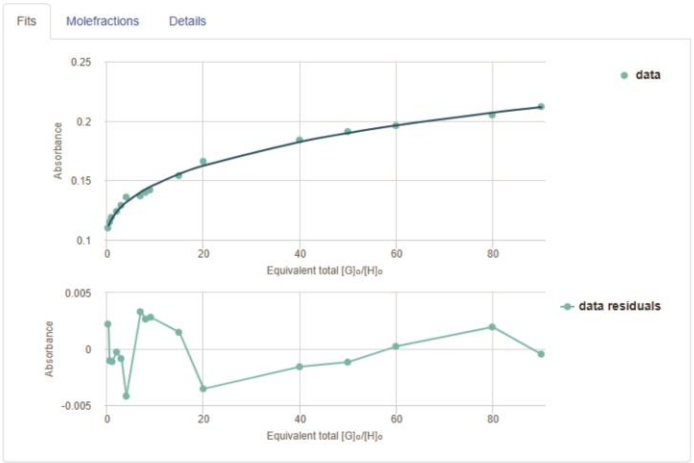
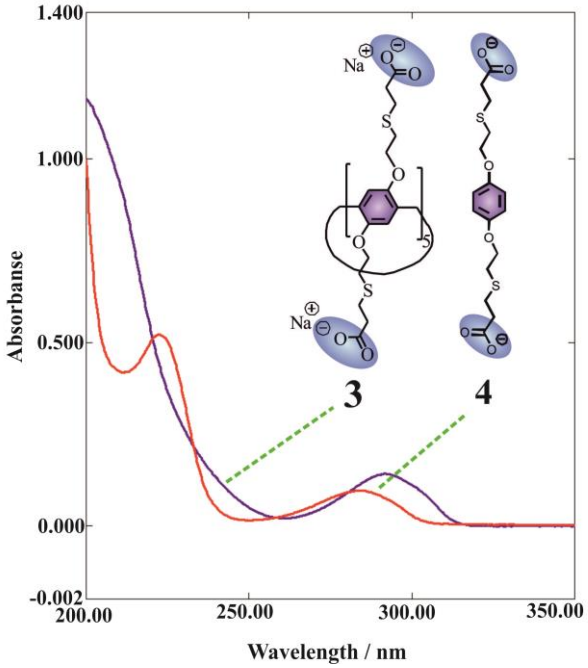
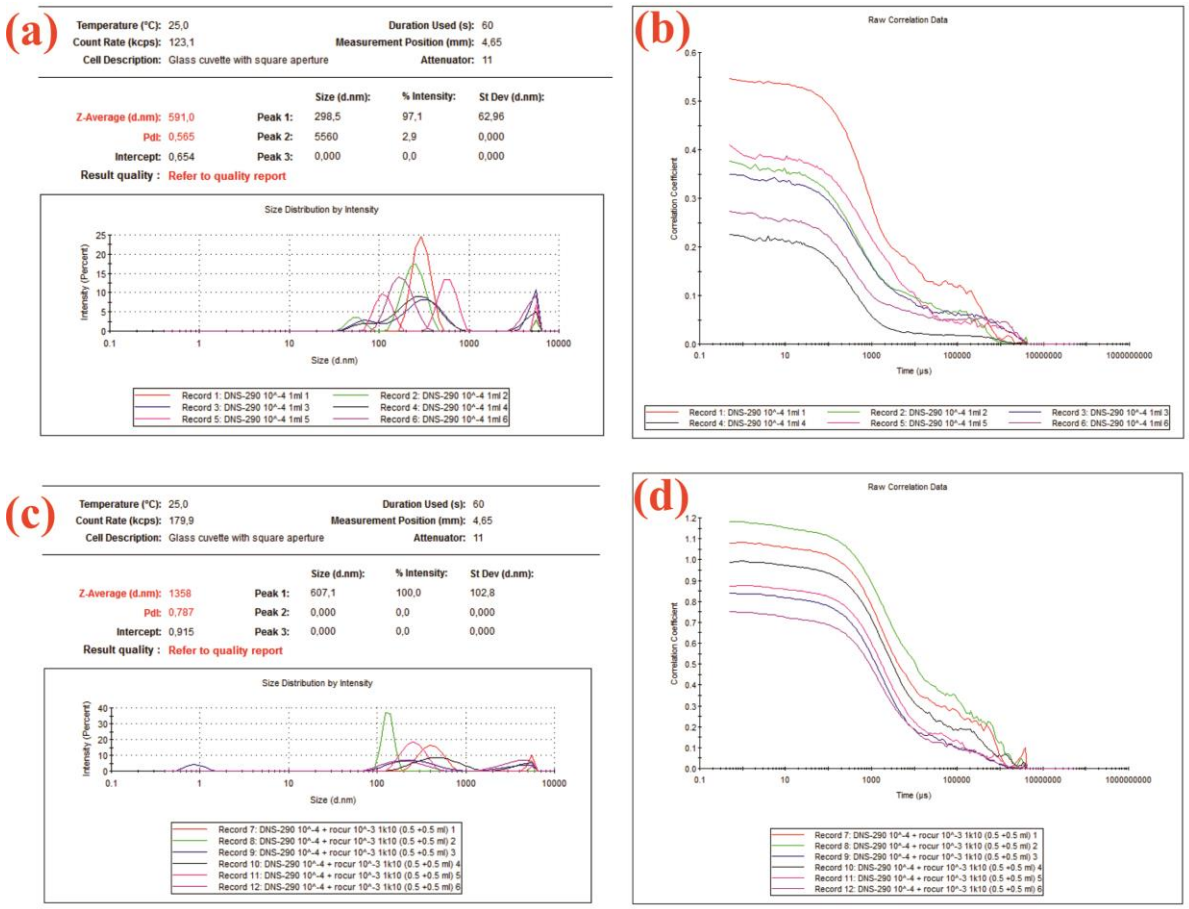


5. UV-vis, Fluorescence and DLS study.

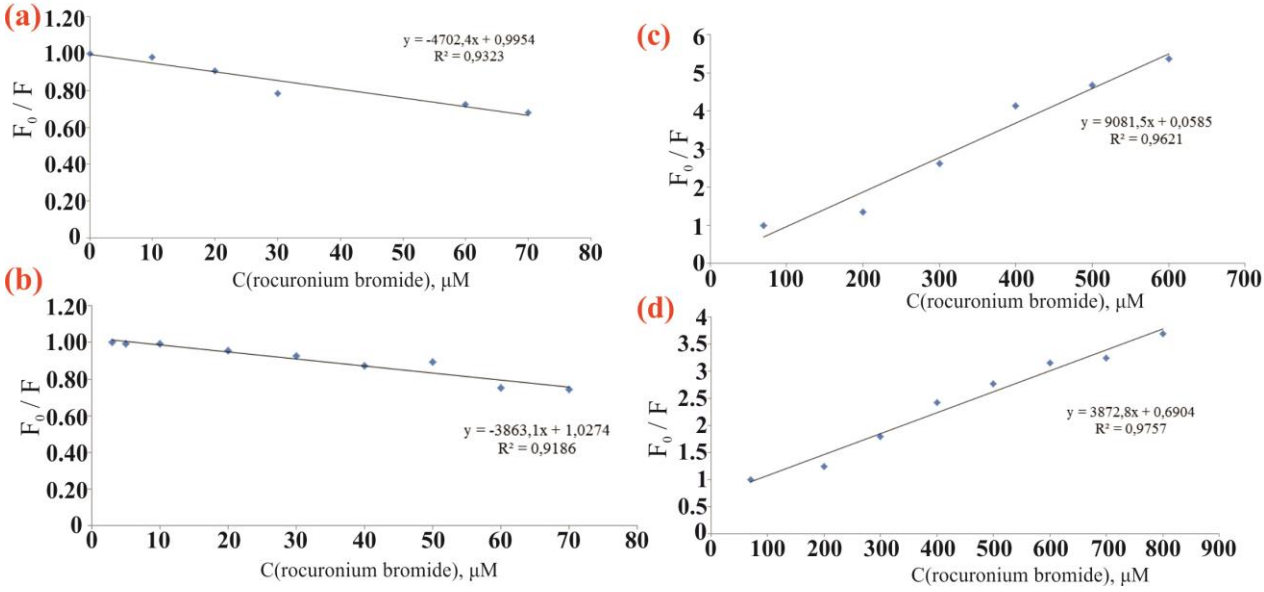
UV/Vis absorption spectra of pillar[5]arene 3 (10^{-5}M) and model compound 4 (5×10^{-5}M) at 293 K.



DLS studies of the pllar[5]arene system (10^{-4} M) (a and b) and the pillar[5]arene 3 (10^{-4} M) / rocuronium bromide (10^{-3} M) system (c and d).

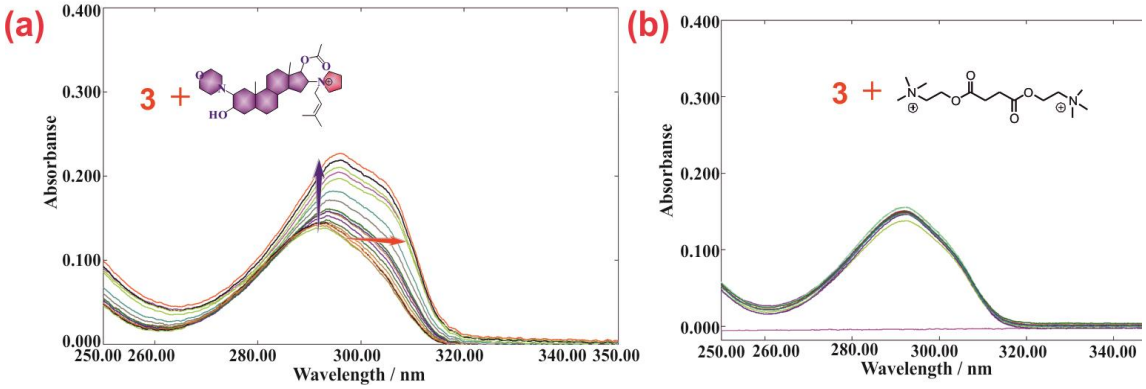


Stern – Volmer plots in the area of ignition (0-60 µM rocuronium bromide) and quenching (70-800 µM rocuronium bromide) of fluorescence of mixtures 3 / rocuronium bromide at different temperatures of 5 °C (a, c) and 35 °C (b, d).

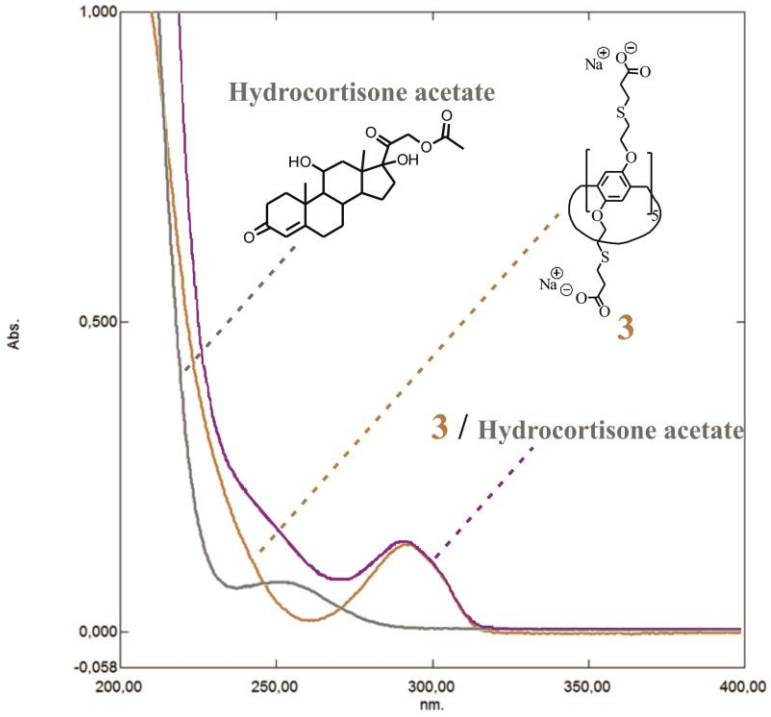


6. Interaction with succinylcholine and Hydrocortisone acetate.

a) UV-vis spectra of pillar[5]arene 3 (10^{-5} M) with different concentrations of rocuronium bromide; b) UV-vis spectra of pillar[5]arene 3 (10^{-5} M) with different concentrations of Succinylcholine (Sch).



UV-vis spectra of macrocycle 3 (10^{-5} M), Hydrocortisone acetate (10^{-4} M) and a mixture of pillar[5]arene 3 (10-5 M) / Hydrocortisone acetate (10-4 M) in H2O.



7. **Quantum chemical calculations**

Calculations on anionic and neutral 3 in acid form 3, and model compound 4.

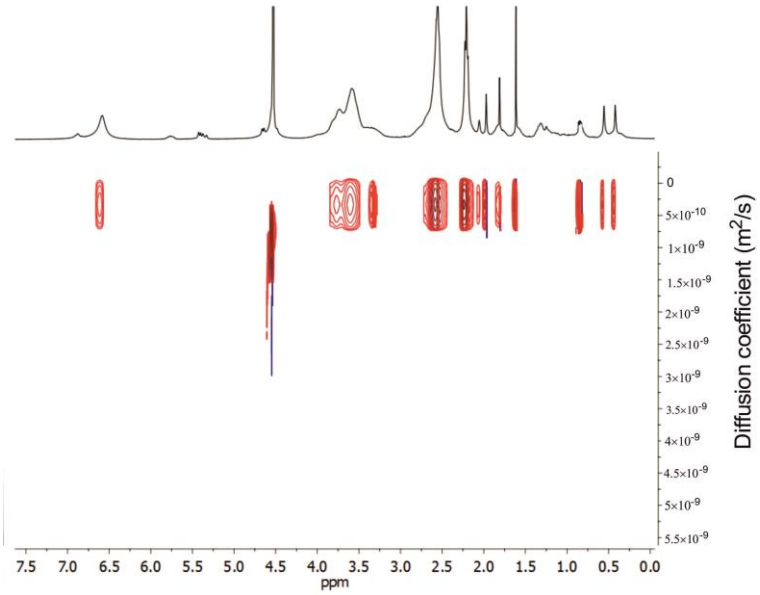
Calculations were undertaken using the Spartan '18 Parallel Suite [S2] running on a Mac Pro with 3.5 GHz 6-Core Intel Xenon E5 processors and two threads per core. 1,4-Dimethoxypillar[5]arene and model compound 4 were constructed using the Build option. Conformational analysis was used to determine the conformer with the lowest steric energy. This was used as the DFT input for gas phase geometry optimization (B3LYP/6-311G*) and calculation of frontier molecular orbital energies. The same method was used to model the pillar[5]arene host, 3, and rocuronium guest. The pillar[5]arene was assumed to be fully deprotonated and rocuronium positively charged. The effects of counterions and solvent were ignored. To construct the complexes, the rocuronium guest was manually introduced 3 in two orientations with either the morpholine group or the pyrrolidine group oriented towards the macrocyclic annulus. The geometries of the complexes were again determined by DFT (B3LYP/6-311G*).

Compound	HOMO	LUMO	Δ (eV)
3	-5.06	-1.35	3.71
3 (acid form)	-5.02	-1.41	3.61
decamethoxypillar[5]arene	-4.83	-0.04	4.79
4	-5.1	-1.6	3.6

8. **Diffusion experiments.**

Diffusion coefficients of pure **3**, **rocuronium bromide** and **3** / **rocuronium bromide** complex in D_2O (400 MHz, 298 K).

Compounds	**D (10^{-10} m^2 s^{-1})**
3	3.86
rocuronium bromide	4.83
3 / **rocuronium bromide**	2.94



9. Cytotoxicity

Table 1. Cytotoxicity of Rocuronium bromide, Sugammadex and **3** against normal human cell lines*

Compound	IC50 value, µg/ml	
	Chang liver cells	Wi38 cells
Rocuronium bromide	1563±125	1020±81
Sugammadex	>2000	>2000
3	>2000	>2000

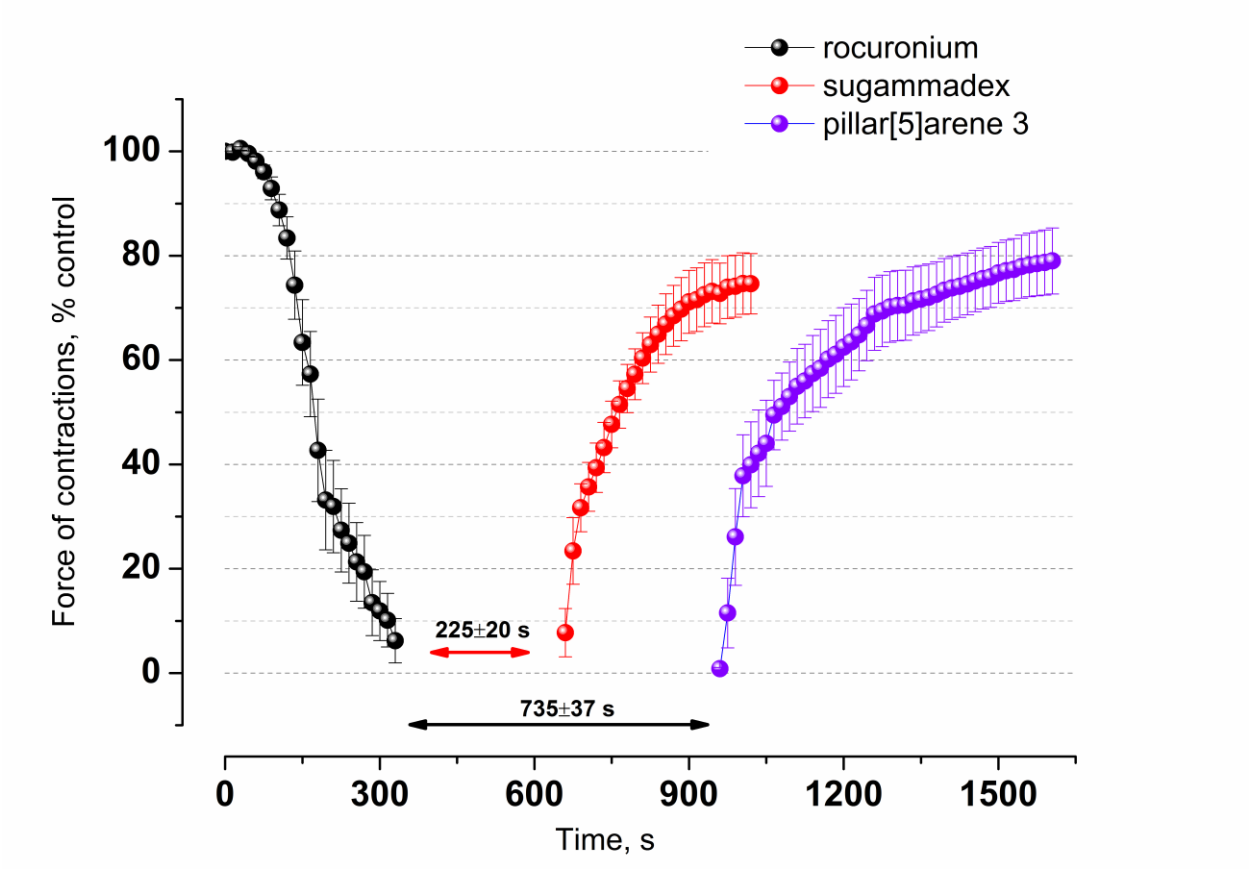
*The experiments were repeated three times and reported as the 50% inhibitory concentration (IC50 value) ± standard deviation

Table 2. Shift of Rocuronium bromide cytotoxicity by co-incubation with Sugammadex or **3***

Compounds	Concentrations of compounds, µg/ml	Chang liver cells viability, % of control	WI38 cells viability, % of control
Rocuronium bromide with the same concentration of Sugammadex	2000	75±6	80±5
	1500	97±5	100
	1000	100	100
Rocuronium bromide with the same concentration of **3**	2000	100	100
	1500	100	100
	1000	100	100
Rocuronium bromide alone	2000	1±0.5	2±0.5
	1500	44±4	72±9
	1000	95±5	96±5

*The experiments were repeated three times and reported as mean ± standard deviation. If no effect on cell viability was detected, then viability is indicated as 100% of control.

10. Relative changes of the force of first muscle contraction in TOF during bath application of rocuronium bromide (black circles) followed by recovery of contractions in presence of either Sugammadex (red circles) or pillar[5]arene 3 (violet circles). Data of 16 experiments (8 muscles in case of sugammadex and 8 muscles in case of pillar[5]arene 3) were normalized to the value prior rocuronium application and then averaged.



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