

Synthesis of novel antibiofilm kojic acid-based bis-pyridinium salts

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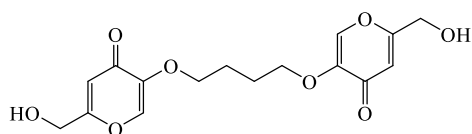
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1. Chemical experimental details

Reagents were purchased and used without further purification. Reaction progress was monitored by TLC on silica gel 60 F254 aluminum sheets under UV light. All products were analyzed by NMR spectroscopy on Bruker AM300, Bruker XWIND or Bruker AV600 spectrometers at ambient temperature in DMSO- d_6 or $CDCl_3$. HR-ESI-MS were measured on a Bruker microTOF II instrument (Bruker Daltonik GmbH, Bremen, Germany). All melting points were determined on a Gallenkamp melting point apparatus in open capillaries and are uncorrected. IR spectra were recorded with a Bruker ALPHA-T FT-IR spectrometer in KBr pellets. The ESI contains detailed experimental protocols, summaries of spectral data, and spectra.

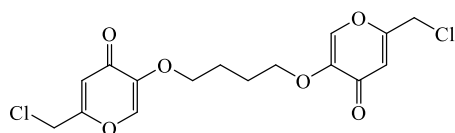
2. Synthesis and characterization of substrates and products

Synthesis of 5,5'-(butane-1,4-diylbis(oxy))bis(2-(hydroxymethyl)-4H-pyran-4-one) (1)



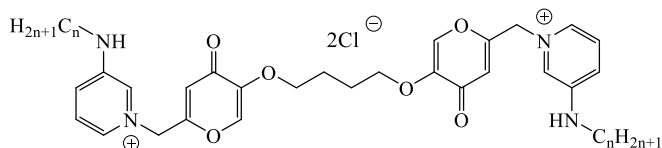
To a round-bottom flask, kojic acid (1.848 g, 13.006 mmol), K_2CO_3 (1.795 g, 13.006 mmol), 1,4-dibromobutane (0.70 mL, 5.911 mmol) in 25 ml of dry DMF were added. The mixture was heated under reflux with stirring for 24 hours. The reaction was monitored by TLC (MeOH: $CHCl_3$, 1:10). After completion, the reaction mixture was concentrated using a rotary evaporator. The residue was mixed with 20 ml of water, and the resulting precipitate was collected by filtration through a Schott filter and washed with hexane. The product was obtained as a beige powder (1.495 g, 4.419 mmol) in 75% yield. $C_{16}H_{18}O_8$: M.w. 338.312; beige solid; m.p. 154–156°C. 1H NMR (300 MHz, DMSO- d_6): δ 8.12 (s, 2H, CH), 6.31 (s, 2H, CH), 5.69 (br s, 2H, OH), 4.48–4.16 (m, 4H, 2CH₂), 4.04 – 3.72 (m, 4H, 2CH₂), 1.97 – 1.59 (m, 4H, 2CH₂). ^{13}C NMR (75 MHz, DMSO- d_6): δ 173.1, 167.9, 145.0, 140.3, 111.0, 68.5, 59.3, 25.1. HRMS (ESI) m/z [M + H] calc. for $C_{16}H_{18}O_8$: 339.1074; found: 339.1071.

Synthesis of 5,5'-(butane-1,4-diylbis(oxy))bis(2-(chloromethyl)-4H-pyran-4-one) (2)



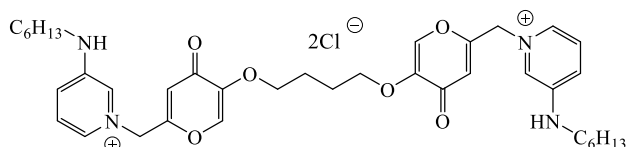
To a round-bottom flask compound **1** (1.495 g, 4.419 mmol) was dissolved in 25 ml of chloroform. The resulting suspension was cooled in an ice bath, and a solution of thionyl chloride ($SOCl_2$, 0.806 mL, 11.05 mmol) in 7 mL of $CHCl_3$ was added dropwise under stirring. The reaction mixture was then heated under reflux with stirring for 24 hours. Progress of the reaction was monitored by TLC (MeOH: $CHCl_3$, 1:10). Upon completion, the reaction mixture was concentrated and dried using a rotary evaporator. The residue was resuspended in 5 mL of chloroform and filtered through a Schott filter. After solvent evaporation, the product was obtained as a brown powder (1.558 g, 4.154 mmol), corresponding to a 94% yield. $C_{16}H_{18}Cl_2O_6$: M.w. 375.198; brown solid; m.p. 143–144°C. 1H NMR (300 MHz, DMSO- d_6): δ 7.69 (s, 2H, CH), 6.49 (s, 2H, CH), 4.33 (s, 4H, 2CH₂), 4.11 – 3.88 (m, 4H, 2CH₂), 2.15 – 1.90 (m, 4H, 2CH₂). ^{13}C NMR (75 MHz, DMSO- d_6): δ 174.1, 161.5, 148.1, 139.9, 114.8, 69.4, 41.0, 25.8. HRMS (ESI) m/z [M + H] calc. for $C_{16}H_{18}Cl_2O_6$: 375.0397; found: 375.0402.

General method of synthesis of 1,1'-(((butane-1,4-diylbis(oxy))bis(4-oxo-4H-pyran-5,2-diyl))bis(methylene))bis(3-(alkylamino)pyridin-1-ium) dichlorides (4a-f)



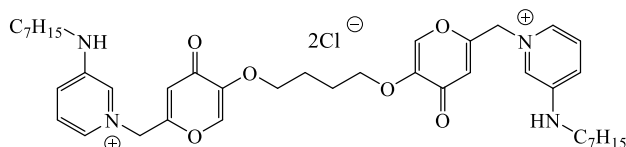
To a round-bottom flask, compound **2** (0.200 g, 0.533 mmol) and 3-alkylaminopyridine (1.173 mmol) were dissolved in 7 ml of acetonitrile. The resulting mixture was heated under reflux with stirring for 24 hours. Upon completion, the formed precipitate was collected by filtration through a Schott filter and washed with 15 ml of hot acetone.

1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4H-pyran-5,2-diyl))bis(methylene))bis(3-(hexylamino)pyridin-1-ium) dichloride (4a)



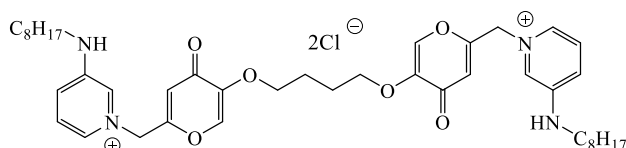
$C_{38}H_{52}Cl_2N_4O_6$; M.w. 731.756; beige solid (yield 72%); m.p. 97–99°C; 1H NMR (300 MHz, DMSO- d_6): δ 8.32 (s, 2H, 2CH_{ar}), 8.29 – 8.20 (m, 2H, 2CH_{ar}), 8.17 (s, 2H, 2CH_{koj}), 7.85 – 7.67 (m, 4H, 4CH_{ar}), 7.60 – 7.47 (m, 2H, 2NH), 6.66 (s, 2H, 2CH_{koj}), 5.74 (s, 4H, 2CH₂), 3.85 (m, 4H, 2CH₂), 3.18 – 3.04 (m, 4H, 2CH₂), 1.83 – 1.70 (m, 4H, 2CH₂), 1.64 – 1.50 (m, 4H, 2CH₂), 1.45 – 1.20 (m, 12H, 6CH₂), 0.95 – 0.77 (m, 6H, 2CH₃). ^{13}C NMR (75 MHz, DMSO- d_6): δ 173.0, 159.4, 148.7, 147.8, 141.3, 131.8, 128.3, 127.6, 127.2, 115.8, 69.0, 60.1, 42.8, 31.4, 28.3, 26.6, 25.5, 22.5, 14.4. HRMS (ESI) m/z [M/2 – 2Cl] calc. for $C_{19}H_{26}N_2O_3^+$: 330.1938; found: 330.1939.

1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4H-pyran-5,2-diyl))bis(methylene))bis(3-(heptylamino)pyridin-1-ium) dichloride (4b)



$C_{40}H_{56}Cl_2N_4O_6$; M.w. 759.810; beige solid (yield 83%); m.p. 110–111°C. 1H NMR (300 MHz, DMSO- d_6): δ 8.26 – 8.19 (m, 4H, 4CH_{ar}), 8.16 (s, 2H, 2CH_{koj}), 7.83 – 7.66 (m, 4H, 4SH_{ar}), 7.43 – 7.32 (m, 2H, 2NH), 6.63 (s, 2H, 2CH_{koj}), 5.68 (s, 4H, 2CH₂), 3.85 (m, 4H, 2CH₂), 3.17 – 3.05 (m, 4H, 2CH₂), 1.80 – 1.72 (m, 4H, 2CH₂), 1.62 – 1.53 (m, 4H, 2CH₂), 1.39 – 1.21 (m, 16H, 8CH₂), 0.92 – 0.82 (m, 6H, 2CH₃). ^{13}C NMR (75 MHz, DMSO- d_6): δ 173.0, 159.4, 148.7, 147.8, 141.2, 131.8, 128.3, 127.6, 127.1, 115.8, 69.0, 60.1, 42.8, 31.7, 28.9, 28.3, 26.9, 25.5, 22.5, 14.4. HRMS (ESI) m/z [M/2 – 2Cl] calc. for $C_{20}H_{28}N_2O_3^+$: 344.2094; found: 344.2091.

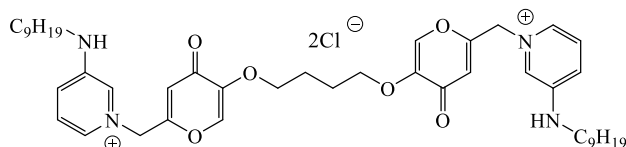
1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4H-pyran-5,2-diyl))bis(methylene))bis(3-(octylamino)pyridin-1-ium) dichloride (4c)



$C_{42}H_{60}Cl_2N_4O_6$; M.w. 787.864; beige solid (yield 78%); m.p. 129–130°C. 1H NMR (300 MHz, DMSO- d_6): δ 8.30 (s, 2H, 2CH_{ar}), 8.27 – 8.20 (m, 2H, 2CH_{ar}), 8.17 (s, 2H, 2CH_{koj}), 7.85 – 7.67 (m, 4H, 4SH_{ar}), 7.58 – 7.44 (m, 2H, 2NH), 6.66 (s, 2H, 2CH_{koj}), 5.73 (s, 4H, 2CH₂), 3.84 (m, 4H, 2CH₂), 3.17 – 3.02

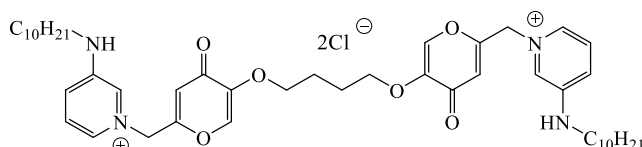
(m, 4H, 2CH₂), 1.82 – 1.70 (m, 4H, 2CH₂), 1.63 – 1.50 (m, 4H, 2CH₂), 1.42 – 1.17 (m, 20H, 10CH₂), 0.92 – 0.77 (m, 6H, 2CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 173.0, 159.4, 148.7, 147.8, 141.2, 131.8, 128.3, 127.6, 127.2, 115.8, 69.0, 60.1, 42.8, 31.7, 29.2, 29.1, 28.3, 26.9, 25.5, 22.5, 14.4. HRMS (ESI) *m/z* [M/2 – 2Cl] calc. for C₂₁H₃₀N₂O₃⁺: 358.2251; found: 358.2253.

1,1'-(((Butane-1,4-diylbis(oxy)))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(nonylamino)pyridin-1-ium) dichloride (4d)



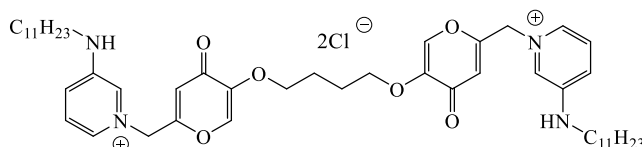
C₄₄H₆₄Cl₂N₄O₆: M.w. 815.918; beige solid (yield 80%); m.p. 148–150°C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.34 – 8.19 (m, 4H, 4CH_{ar}), 8.16 (s, 2H, 2CH_{koj}), 7.85 – 7.66 (m, 4H, 4SH_{ar}), 7.57 – 7.42 (m, 2H, 2NH), 6.65 (s, 2H, 2CH_{koj}), 5.73 (s, 4H, 2CH₂), 3.85 (m, 4H, 2CH₂), 3.18 – 3.02 (m, 4H, 2CH₂), 1.83 – 1.70 (m, 4H, 2CH₂), 1.63 – 1.49 (m, 4H, 2CH₂), 1.43 – 1.16 (m, 24H, 12CH₂), 0.94 – 0.74 (m, 6H, 2CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 173.0, 159.4, 148.7, 147.8, 141.2, 131.8, 128.3, 127.5, 127.19, 115.8, 69.0, 60.1, 42.8, 31.7, 29.4, 29.2, 29.1, 28.3, 26.9, 25.5, 22.5, 14.4. HRMS (ESI) *m/z* [M/2 – 2Cl] calc. for C₂₂H₃₂N₂O₃⁺: 372.2407; found: 372.2403.

1,1'-(((Butane-1,4-diylbis(oxy)))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(decylamino)pyridin-1-ium) dichloride (4e)



C₄₆H₆₈Cl₂N₄O₆: M.w. 843.972; beige solid (yield 73%); m.p. 150–151°C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.29 (s, 2H, 2CH_{ar}), 8.26 – 8.19 (m, 2H, 2CH_{ar}), 8.16 (s, 2H, 2CH_{koj}), 7.83 – 7.67 (m, 4H, 4OH_{ar}), 7.52 – 7.40 (m, 2H, 2NH), 6.65 (s, 2H, 2CH_{koj}), 5.71 (s, 4H, 2CH₂), 3.85 (m, 4H, 2CH₂), 3.16 – 3.02 (m, 4H, 2CH₂), 1.80 – 1.70 (m, 4H, 2CH₂), 1.63 – 1.50 (m, 4H, 2CH₂), 1.42 – 1.17 (m, 28H, 14CH₂), 0.90 – 0.80 (m, 6H, 2CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 173.0, 159.4, 148.7, 147.8, 141.3, 131.8, 128.3, 127.6, 127.2, 115.8, 69.0, 60.1, 42.8, 31.7, 29.5, 29.4, 29.2, 29.2, 28.3, 27.0, 25.5, 22.5, 14.4. HRMS (ESI) *m/z* [M/2 – 2Cl] calc. for C₂₃H₃₄N₂O₃⁺: 386.2564; found: 386.2560.

1,1'-(((Butane-1,4-diylbis(oxy)))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(undecylamino)-pyridin-1-ium) dichloride (4f)

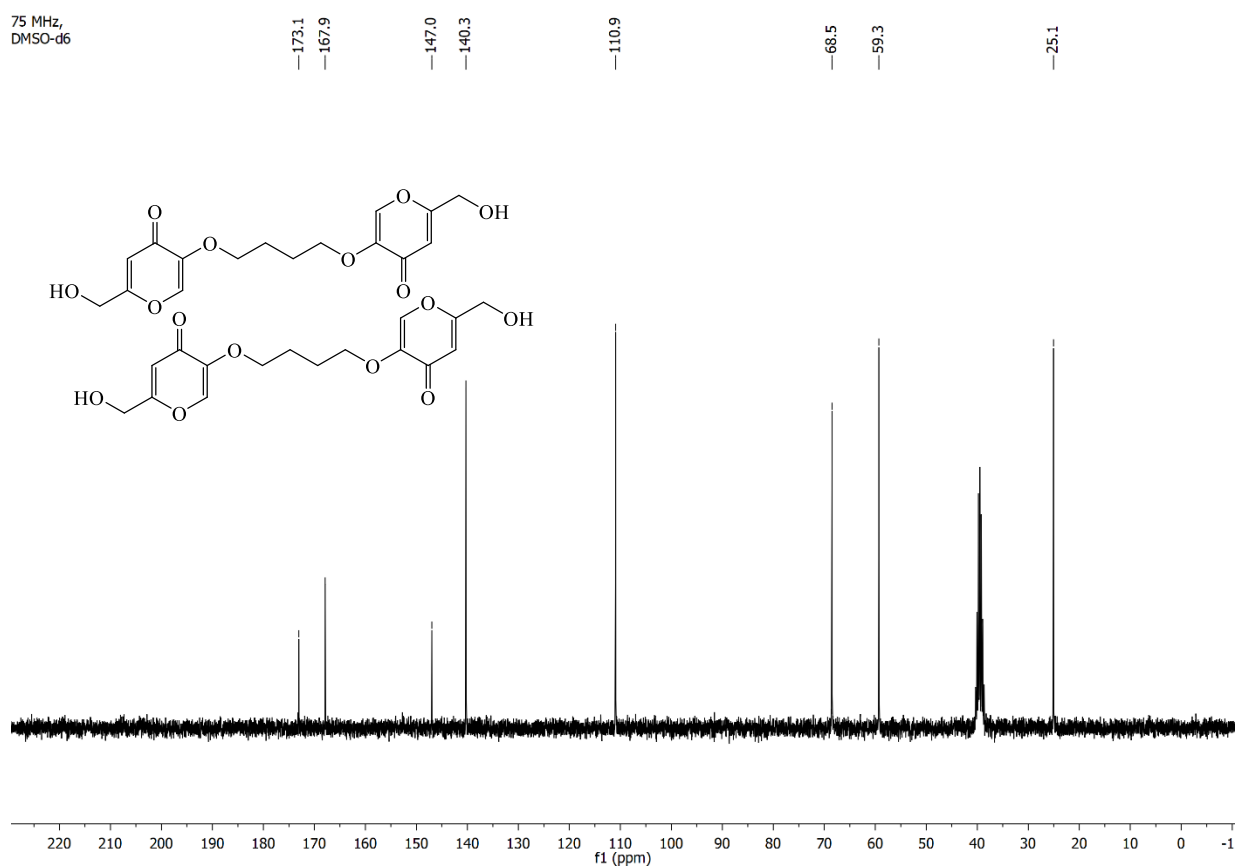


C₄₈H₇₂Cl₂N₄O₆: M.w. 872.026; beige solid (yield 75%); m.p. 155–156°C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 8.27 – 8.18 (m, 4H, 4CH_{ar}), 8.15 (s, 2H, 2CH_{koj}), 7.83 – 7.67 (m, 4H, 4SH_{ar}), 7.45 – 7.32 (m, 2H, 2NH), 6.63 (s, 2H, 2CH_{koj}), 5.69 (s, 4H, 2CH₂), 3.84 (m, 4H, 2CH₂), 3.16 – 3.03 (m, 4H, 2CH₂), 1.82 – 1.72 (m, 4H, 2CH₂), 1.62 – 1.50 (m, 4H, 2CH₂), 1.41 – 1.15 (m, 32H, 16CH₂), 0.92 – 0.77 (m, 6H, 2CH₃). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 173.0, 159.37, 148.7, 147.8, 141.2, 131.8, 128.4, 127.7, 127.1, 115.8, 69.0, 60.2, 42.8, 31.2, 31.18, 29.49, 29.23, 28.8, 27.9, 26.5, 22.1, 14.0. HRMS (ESI) *m/z* [M/2 – 2Cl] calc. for C₂₄H₃₆N₂O₃⁺: 400.2720 found: 400.2720.

3. NMR (^1H , ^{13}C) and HRMS spectra

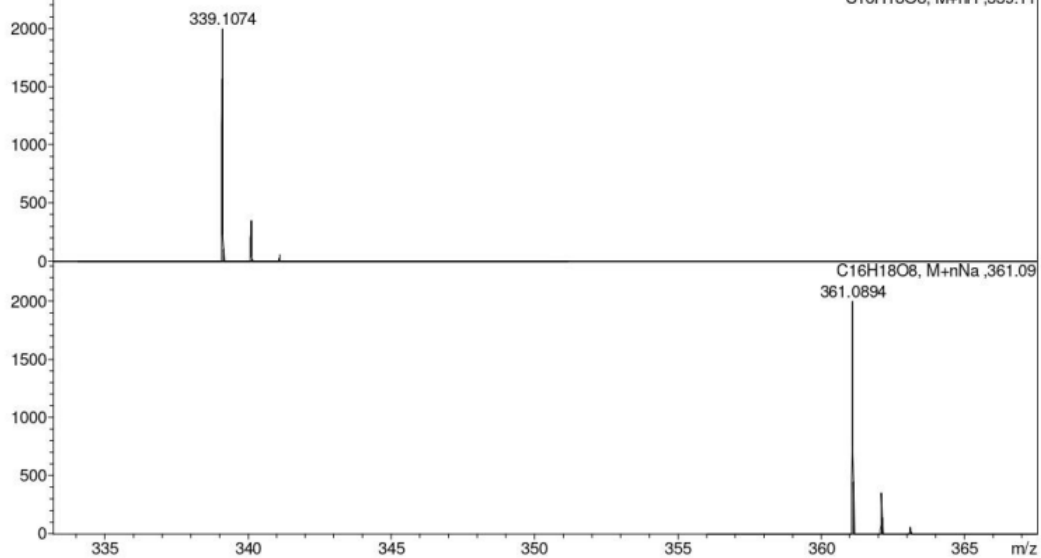
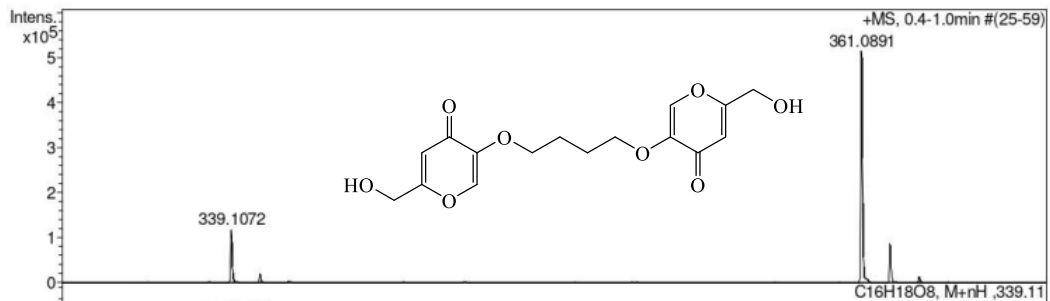
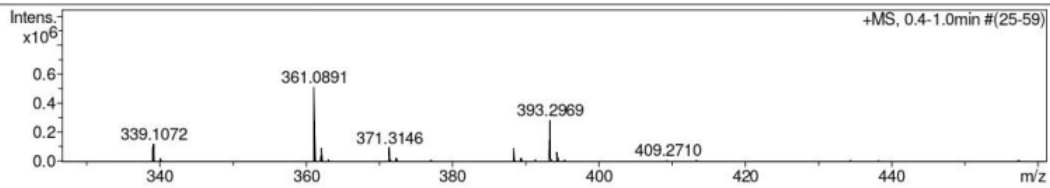
5,5'-(Butane-1,4-diylbis(oxy))bis(2-(hydroxymethyl)-4*H*-pyran-4-one) (1)

75 MHz,
DMSO- d_6



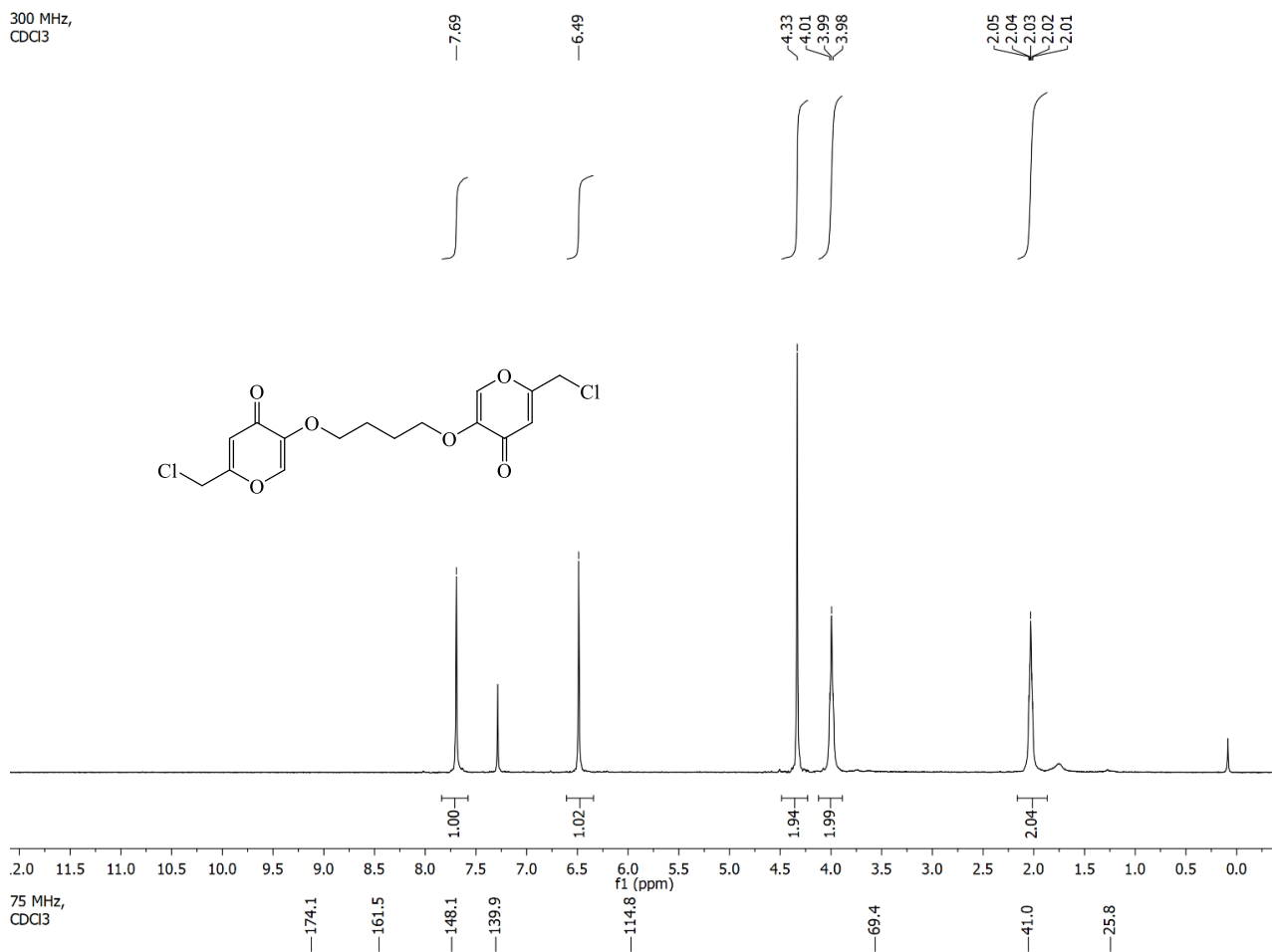
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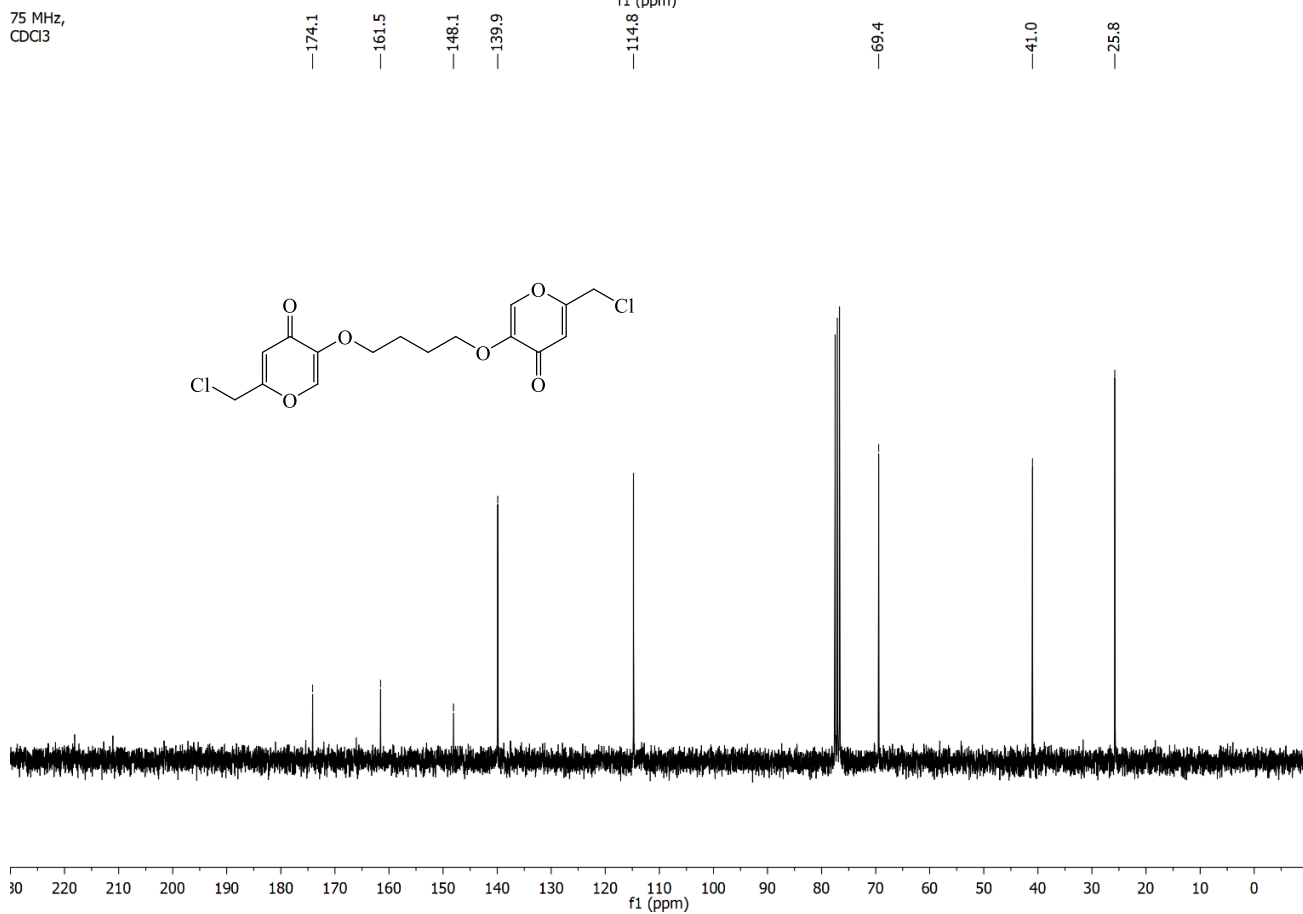


5,5'-(Butane-1,4-diylbis(oxy))bis(2-(chloromethyl)-4H-pyran-4-one) (2)

300 MHz,
CDCl₃

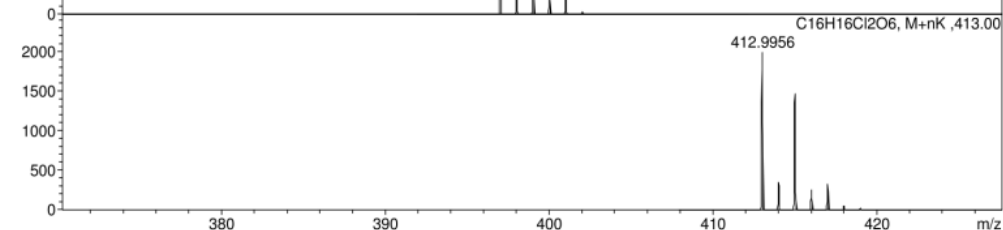
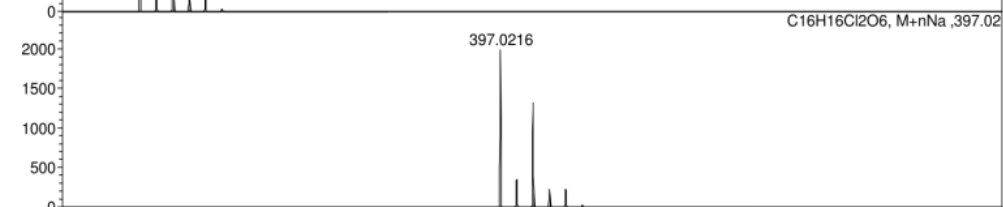
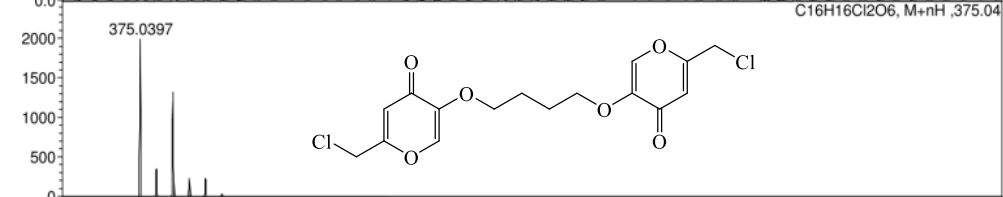
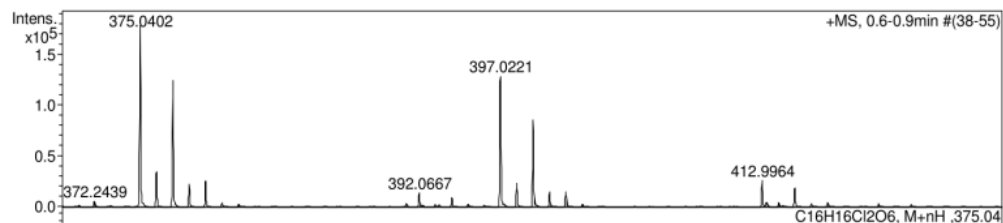
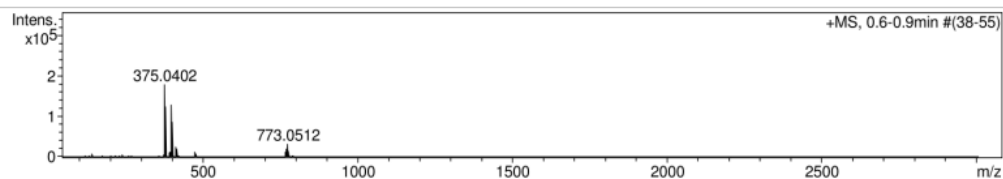


75 MHz,
CDCl₃



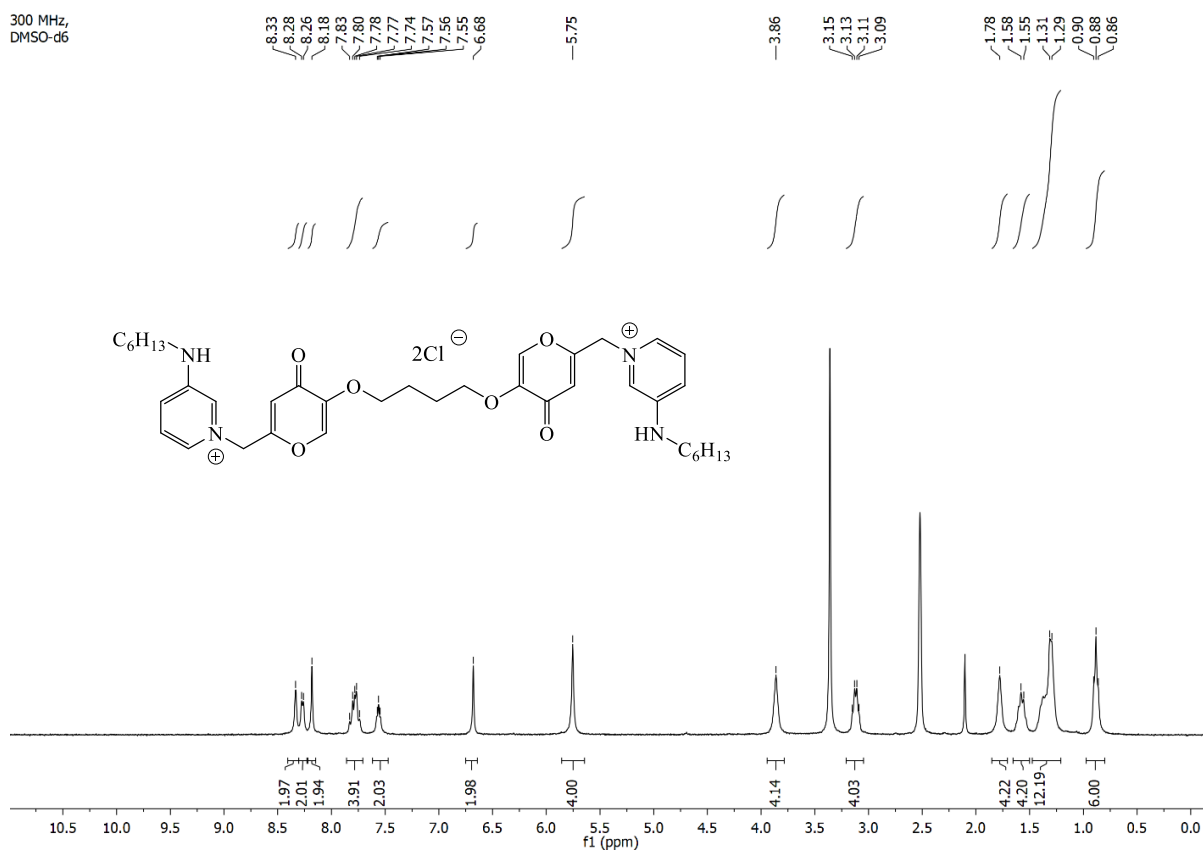
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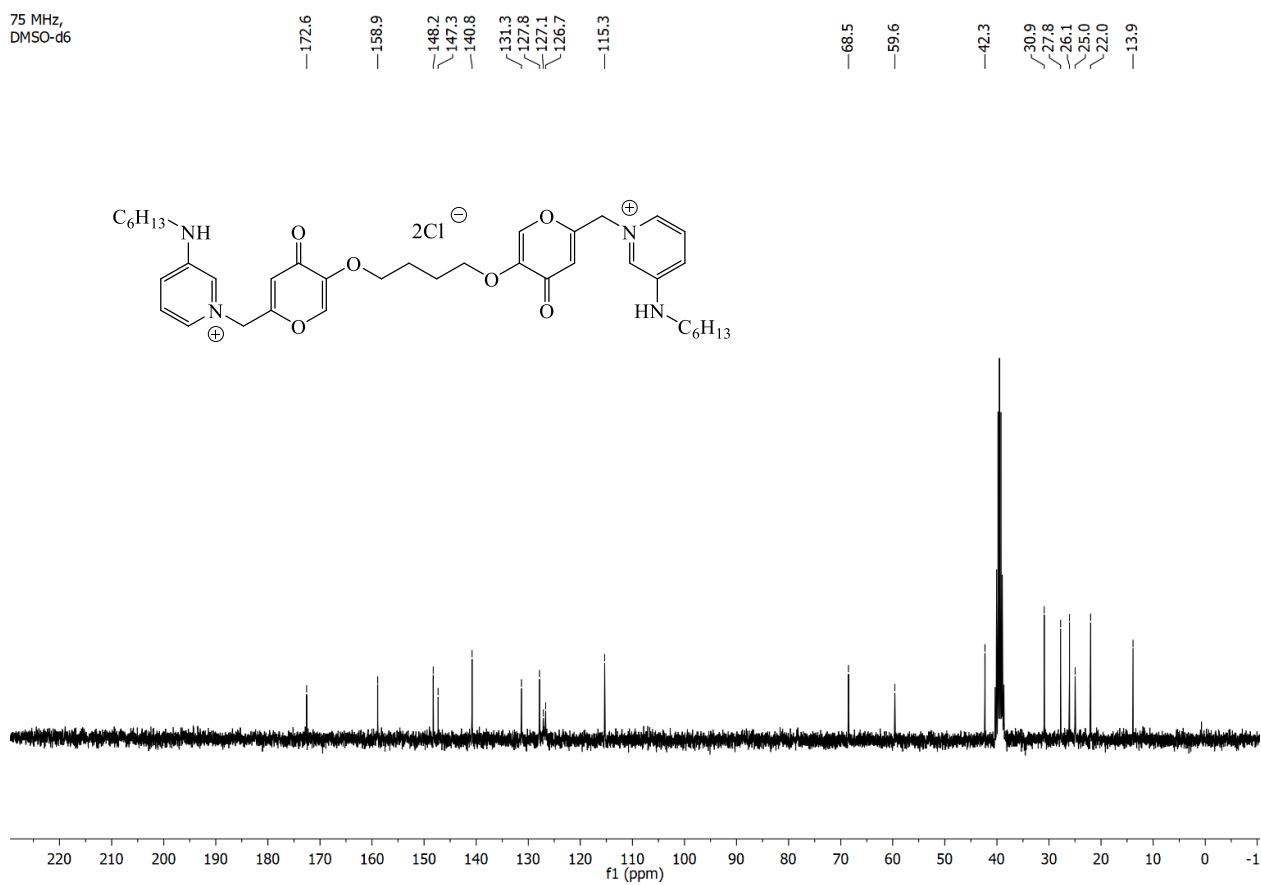


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4H-pyran-5,2-diyl))bis(methylene))bis(3-(hexylamino)pyridin-1-ium) dichloride (4a)

300 MHz,
DMSO-d₆

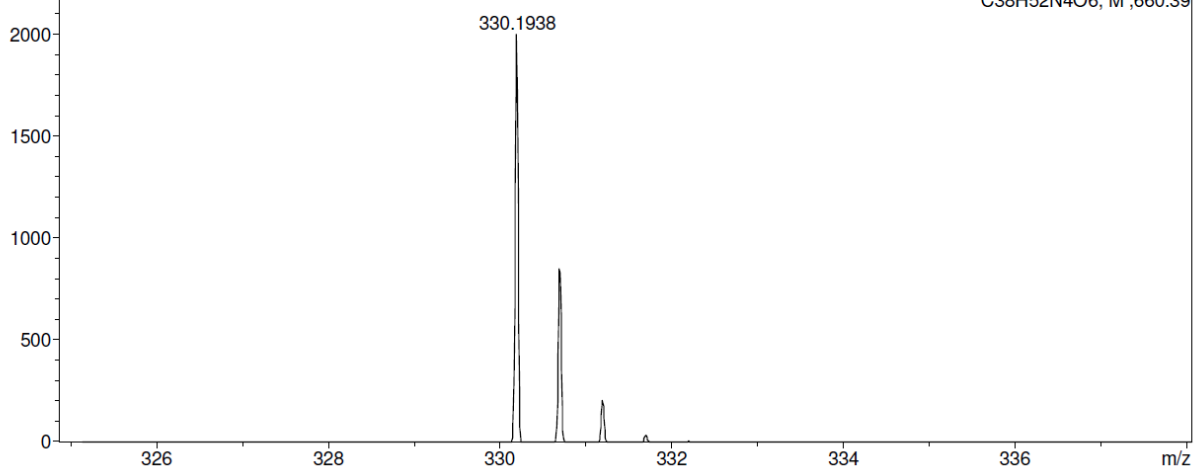
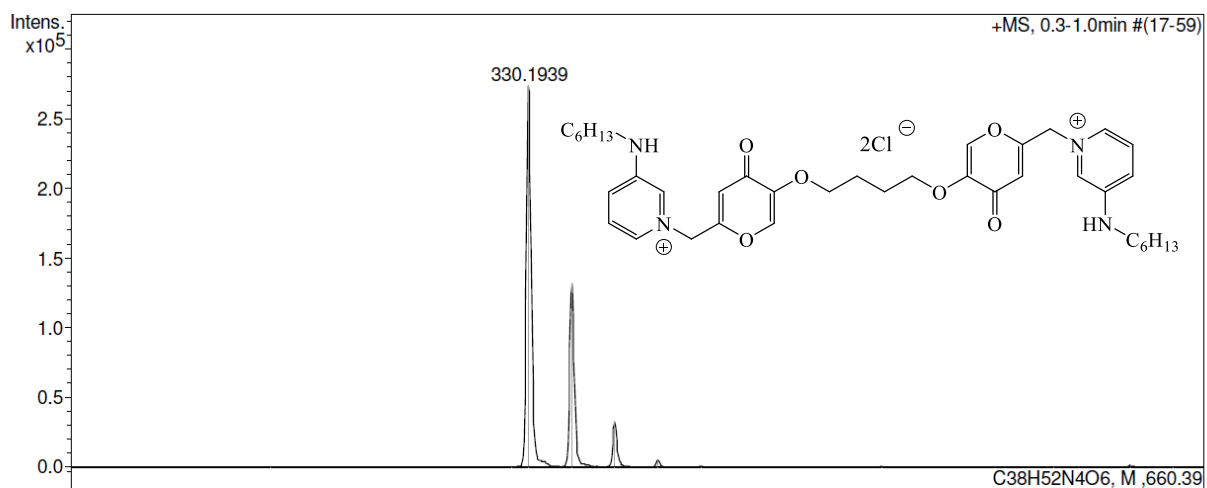
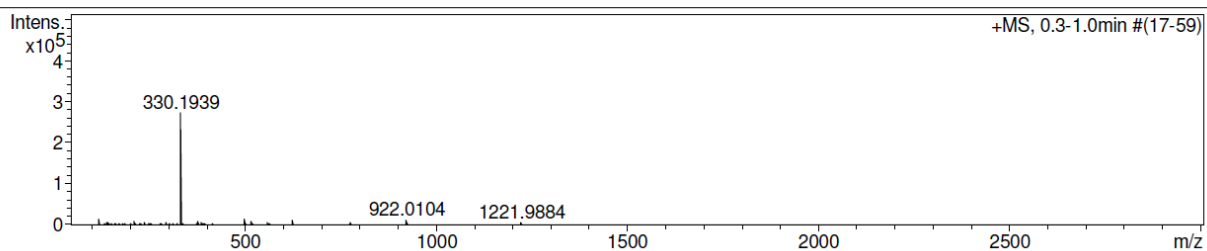


75 MHz,
DMSO-d₆



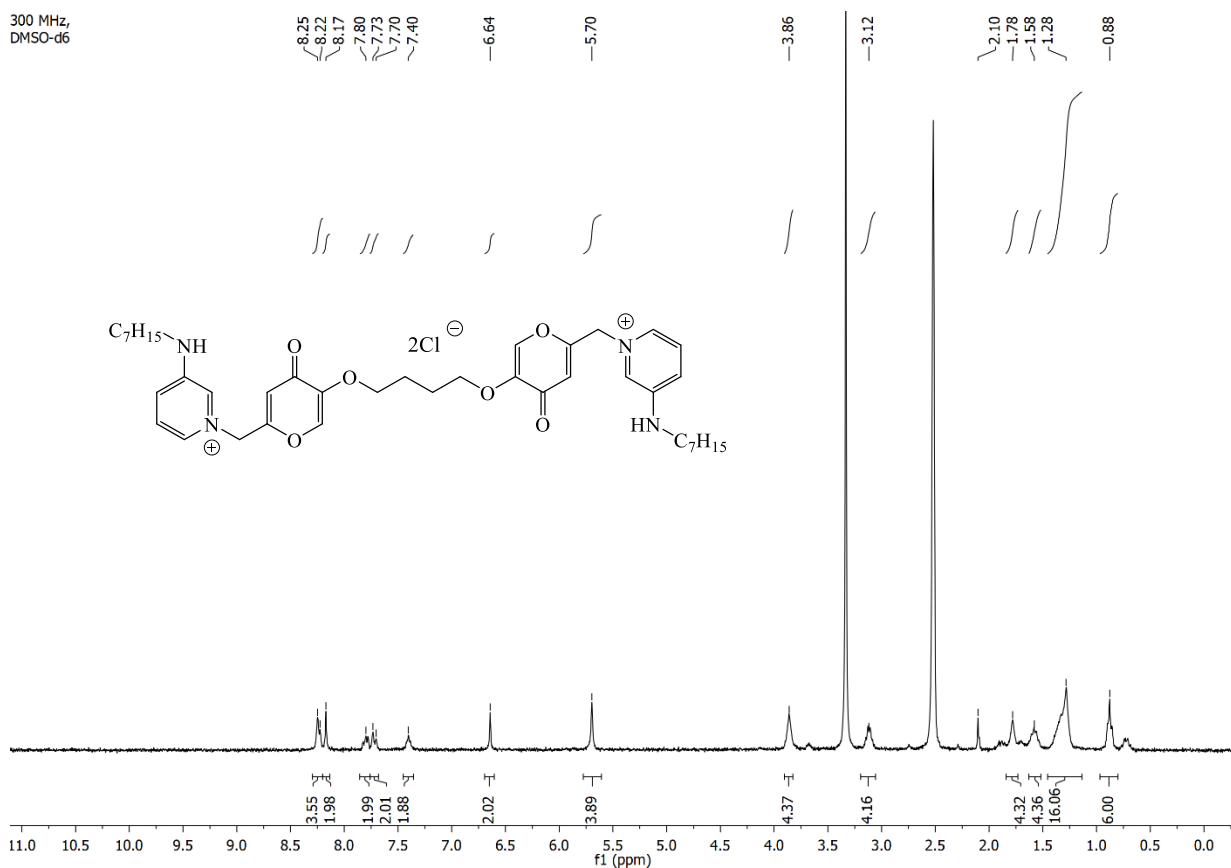
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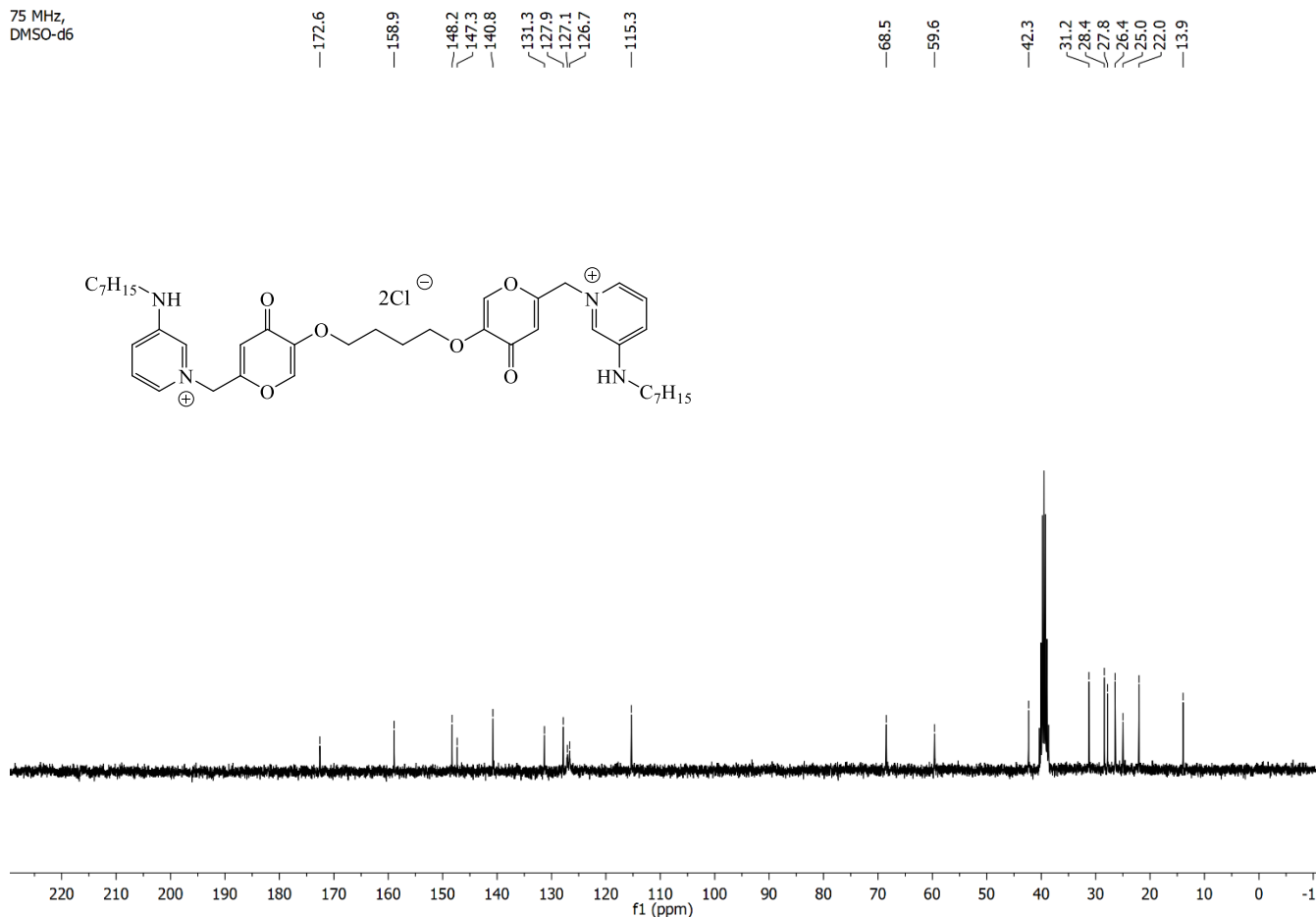


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(heptylamino)pyridin-1-ium) dichloride (4b)

300 MHz,
DMSO-d₆

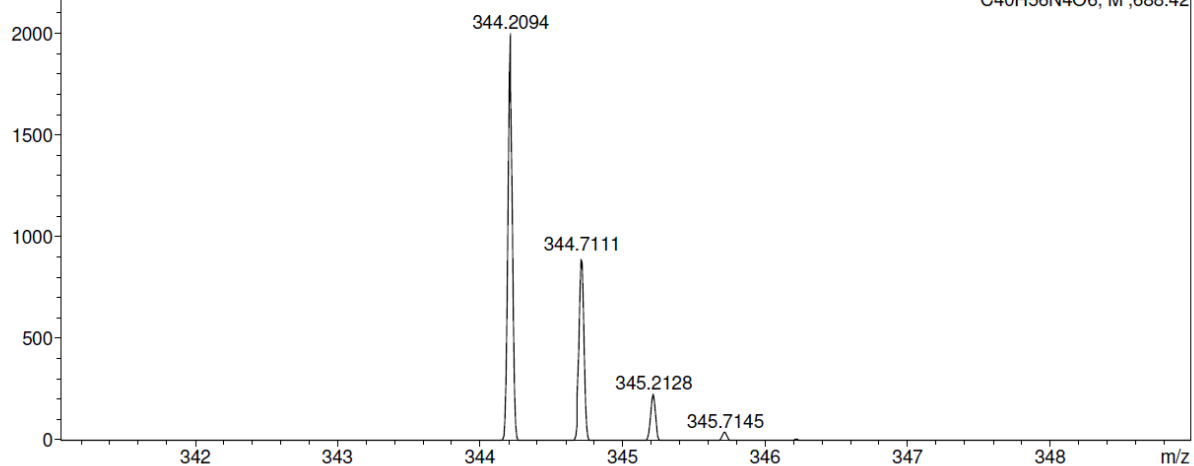
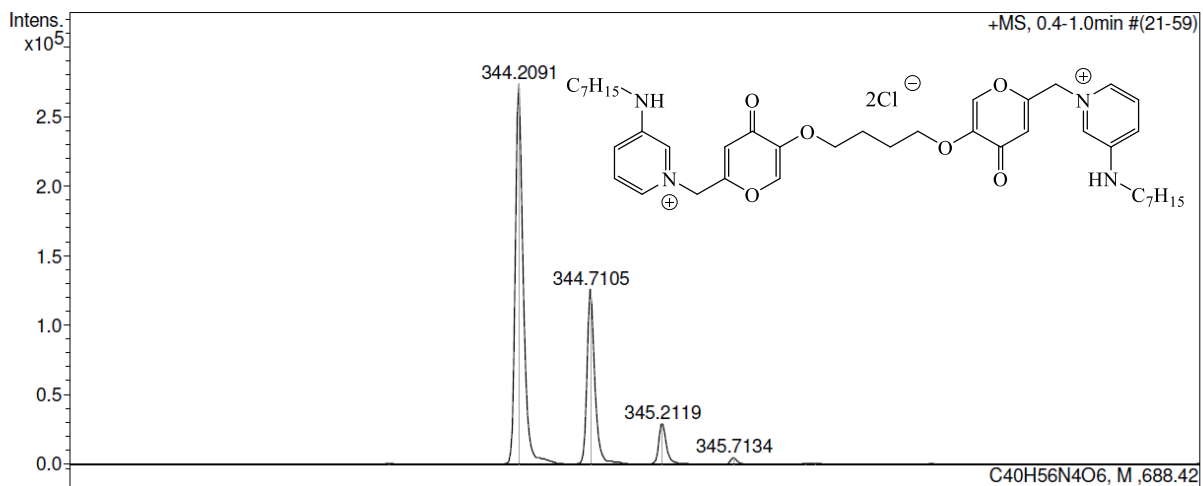
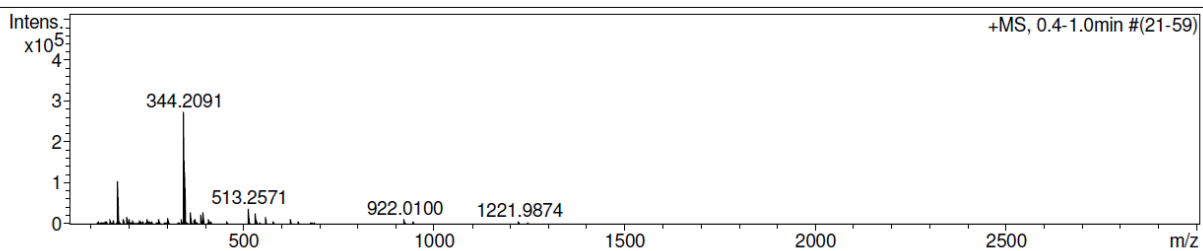


75 MHz,
DMSO-d₆



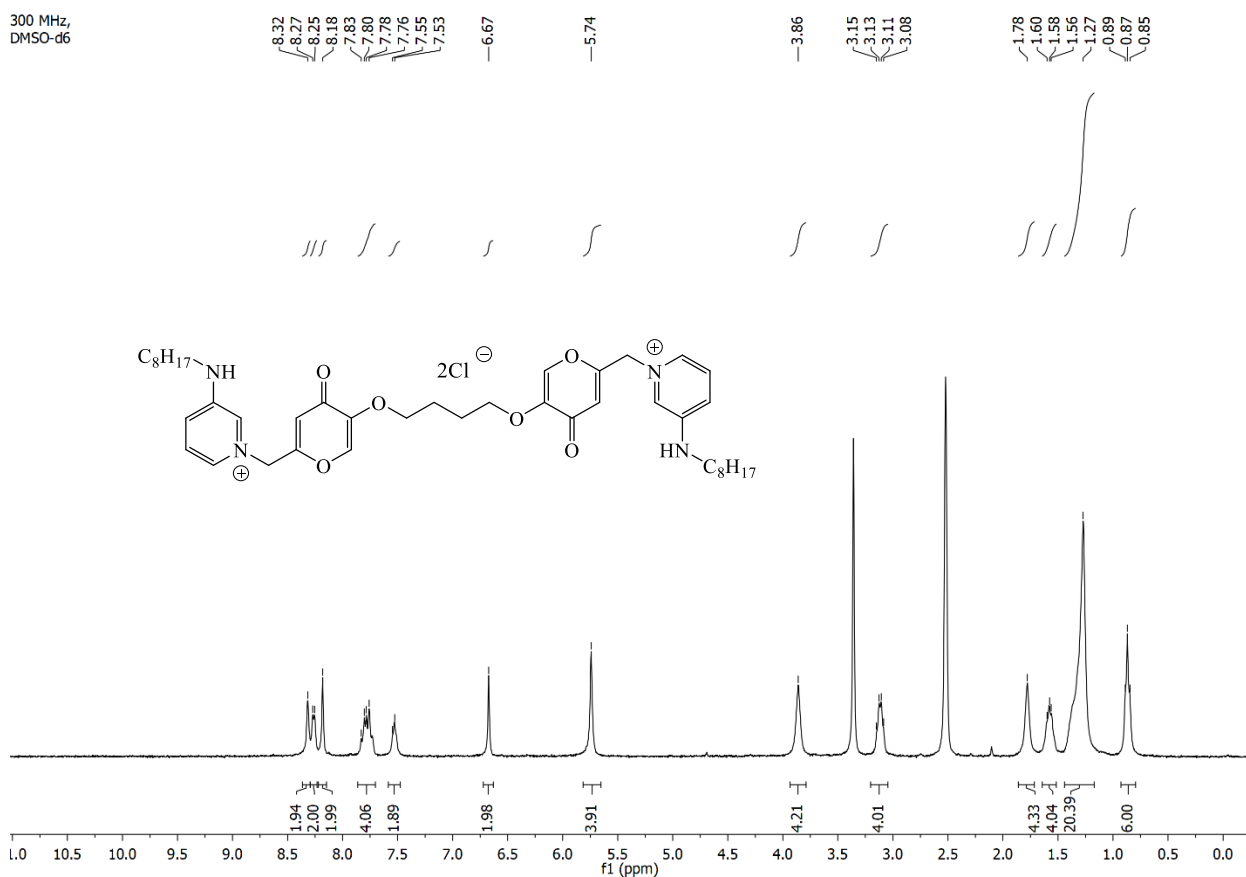
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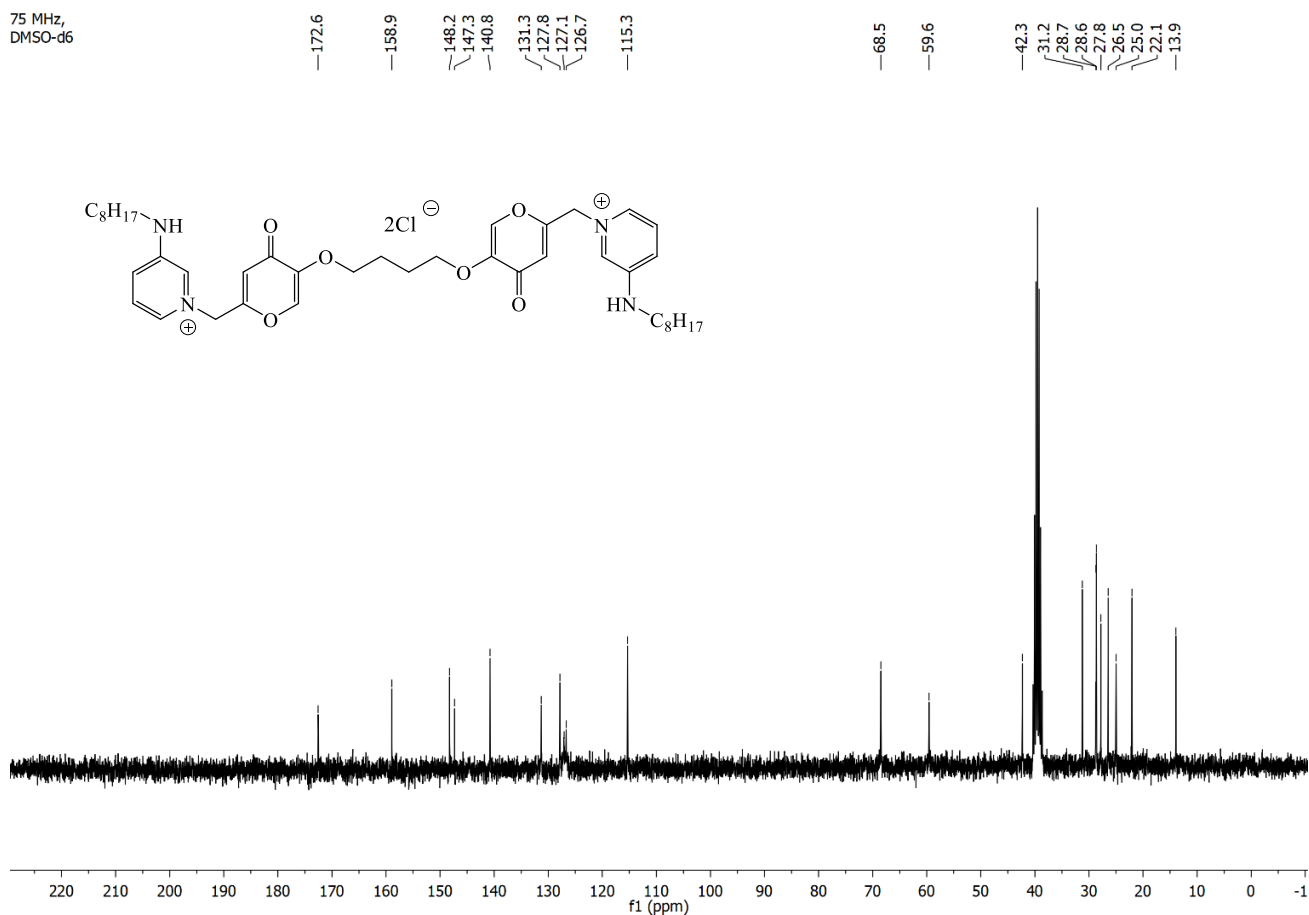


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(octylamino)pyridin-1-ium) dichloride (4c)

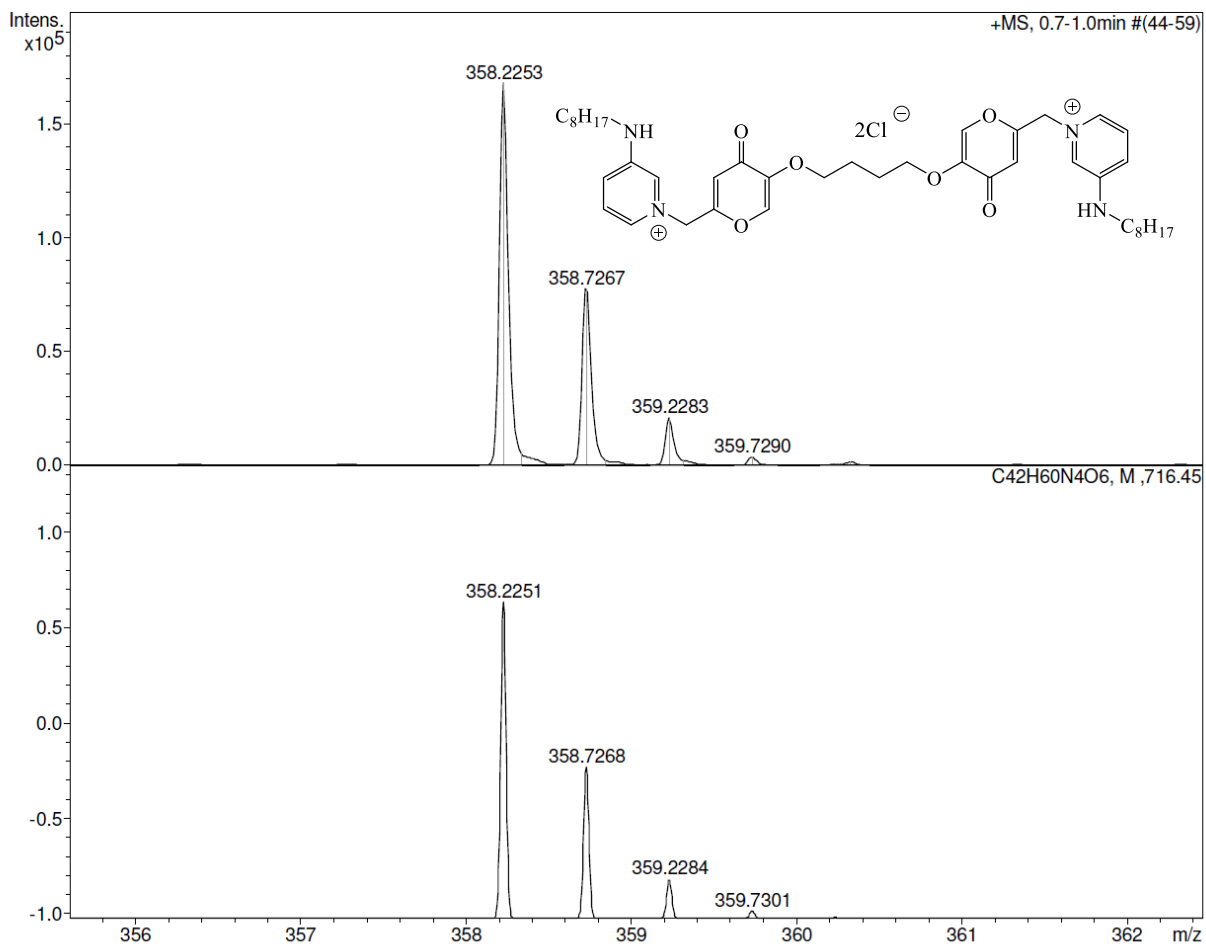
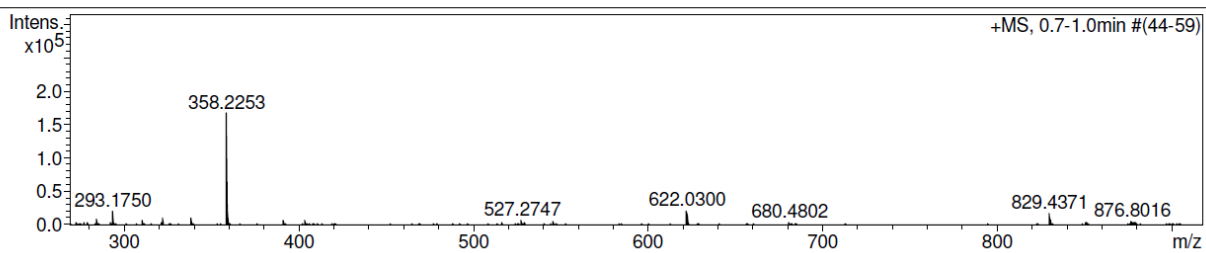
300 MHz,
DMSO-d₆



75 MHz,
DMSO-d₆

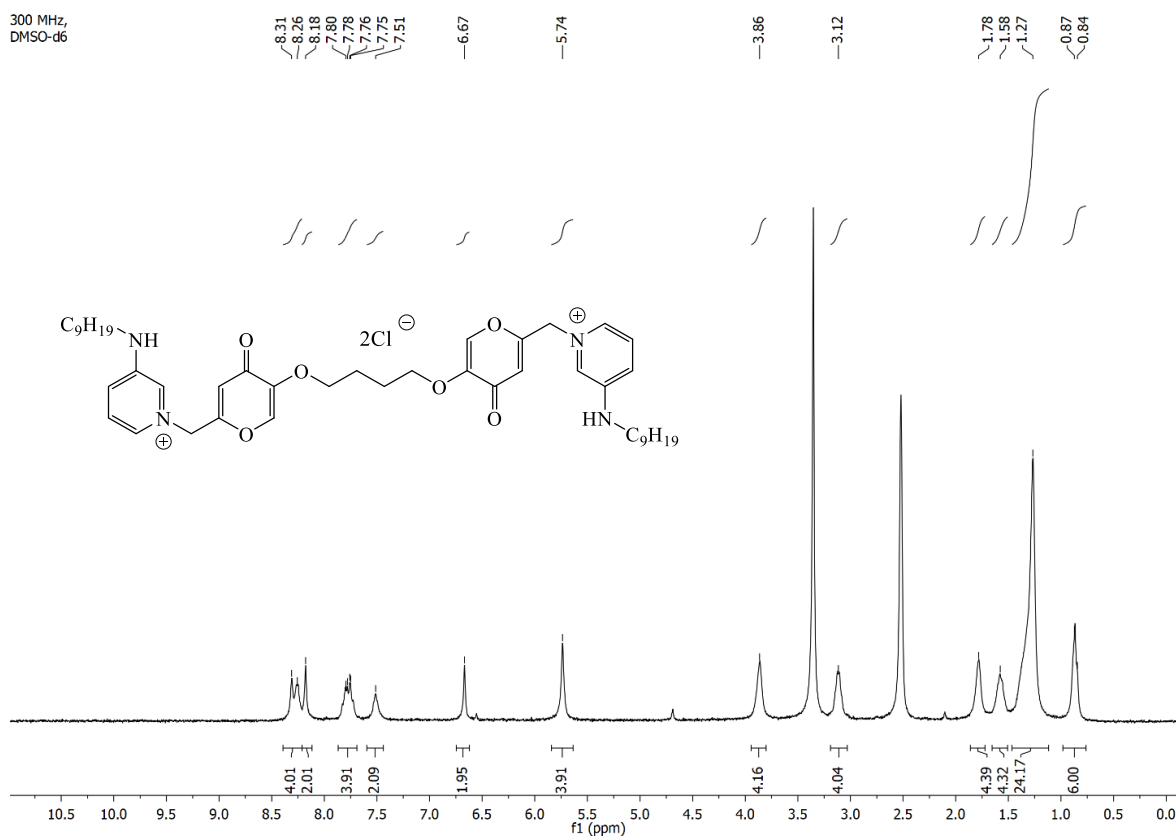


Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	50 m/z	Set Capillary	4500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Waste

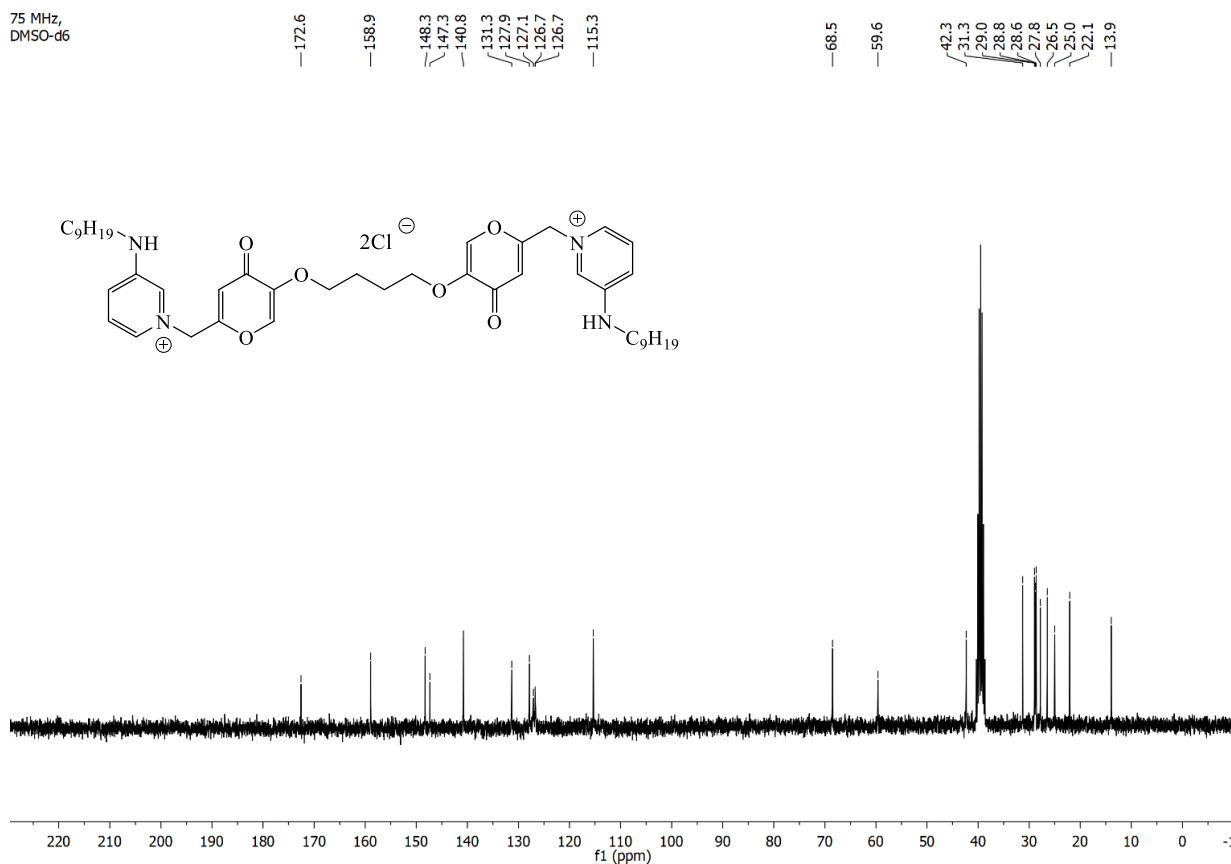


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(nonylamino)pyridin-1-ium) dichloride (4d)

300 MHz,
DMSO-d₆

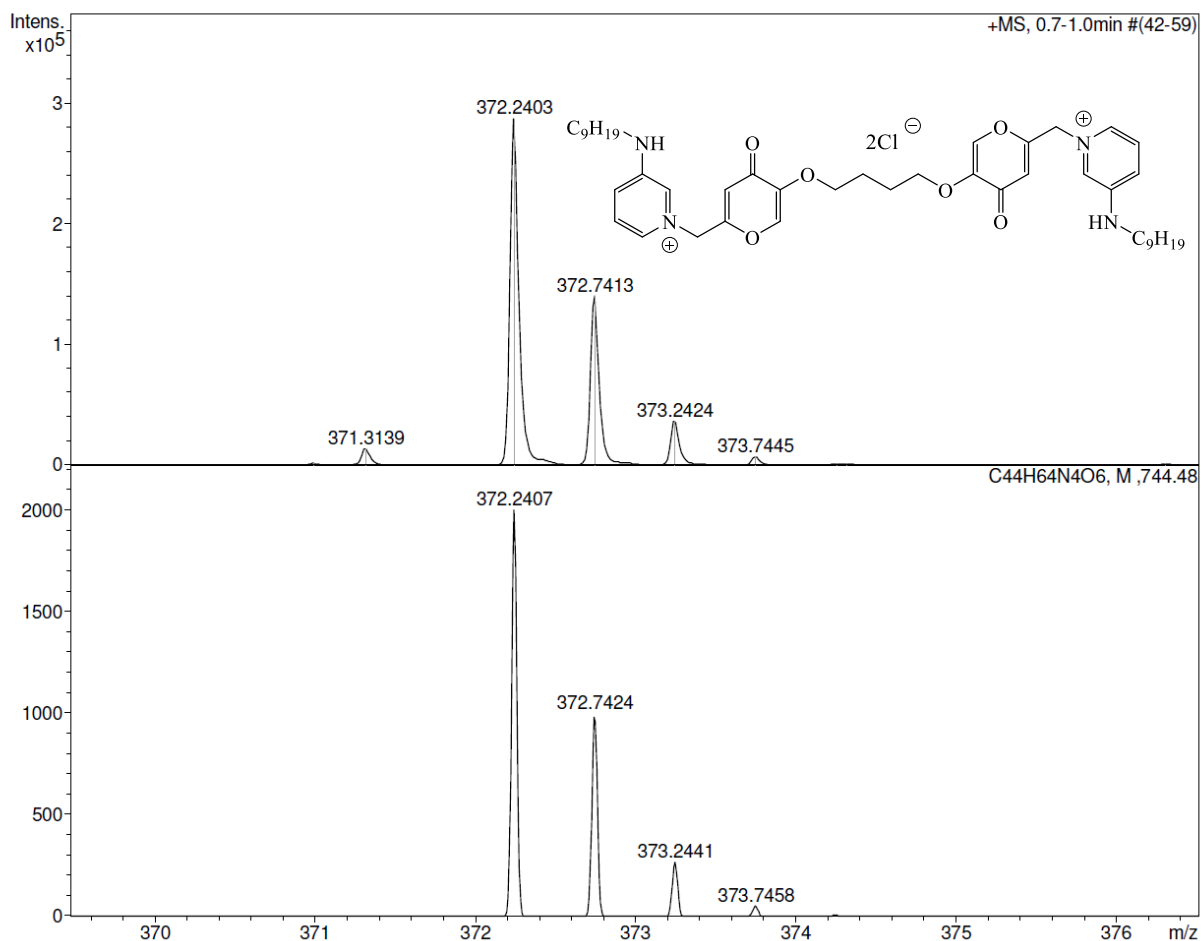
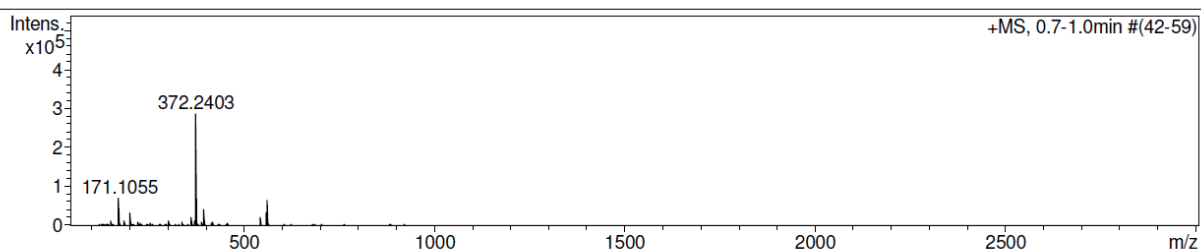


75 MHz,
DMSO-d₆



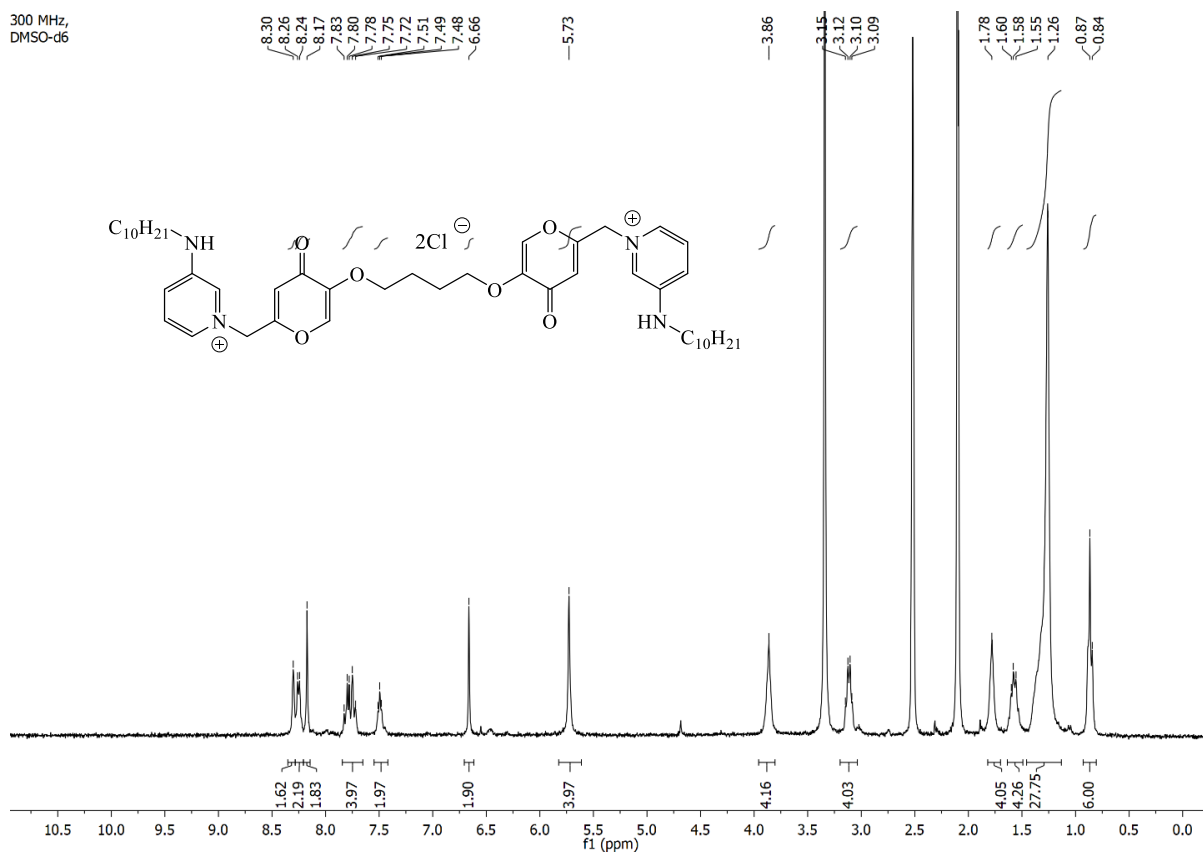
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	50 m/z	Set Capillary	4500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Waste

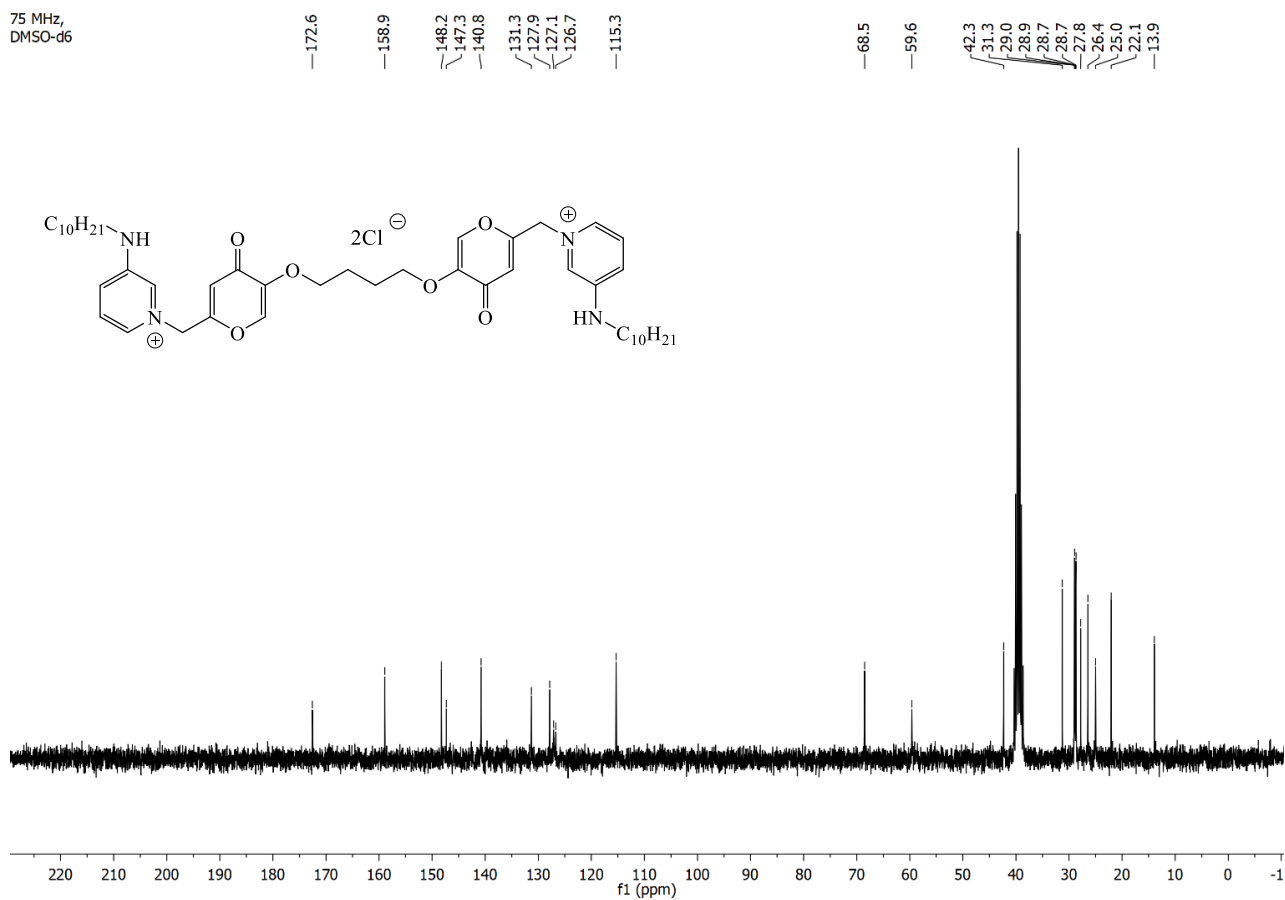


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(decylamino)pyridin-1-ium) dichloride (4e)

300 MHz,
DMSO-d₆

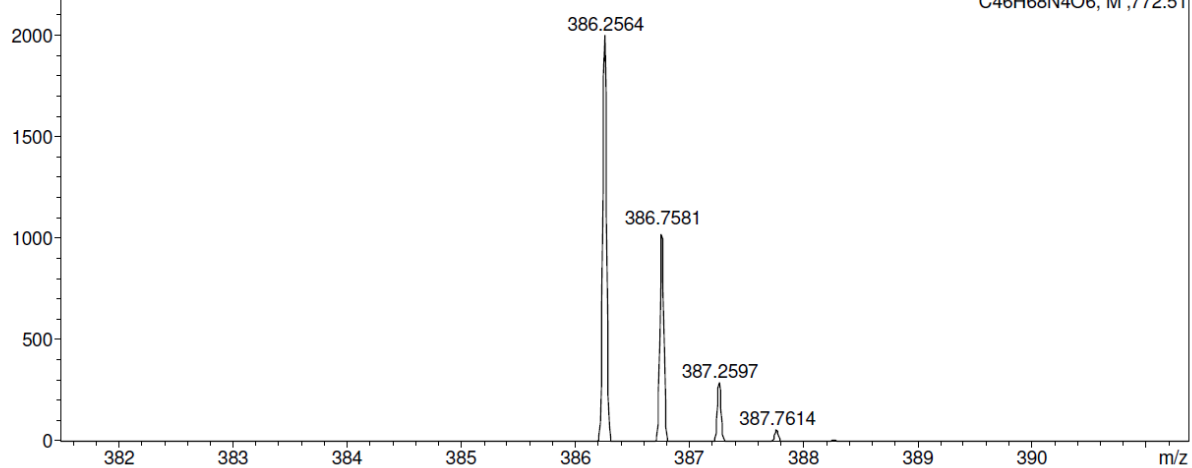
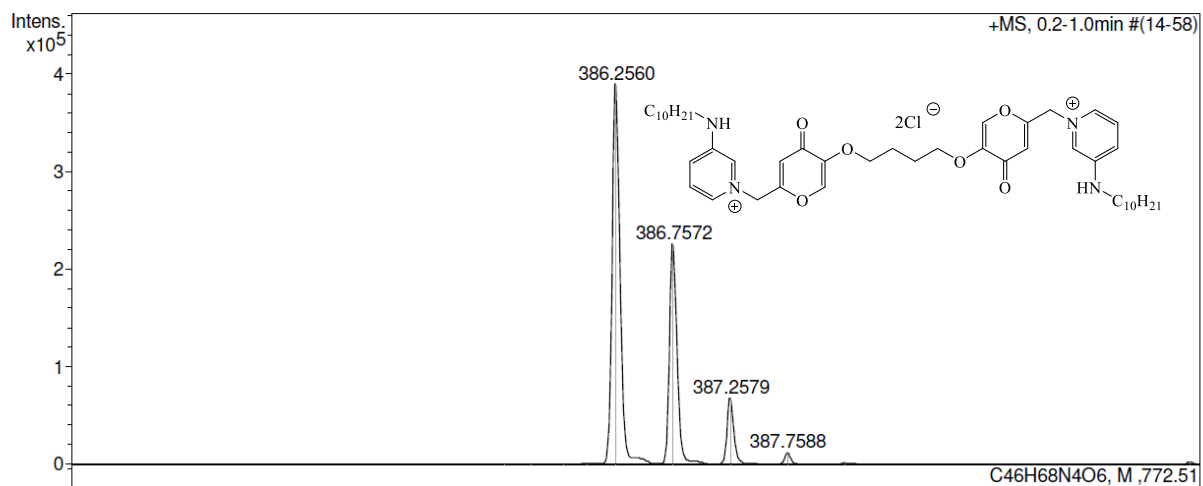
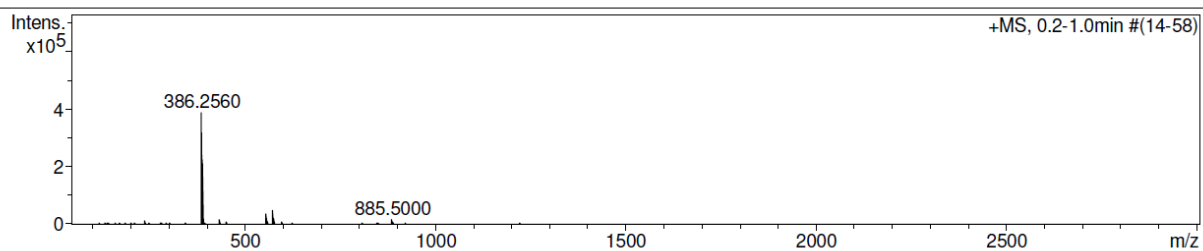


75 MHz,
DMSO-d₆



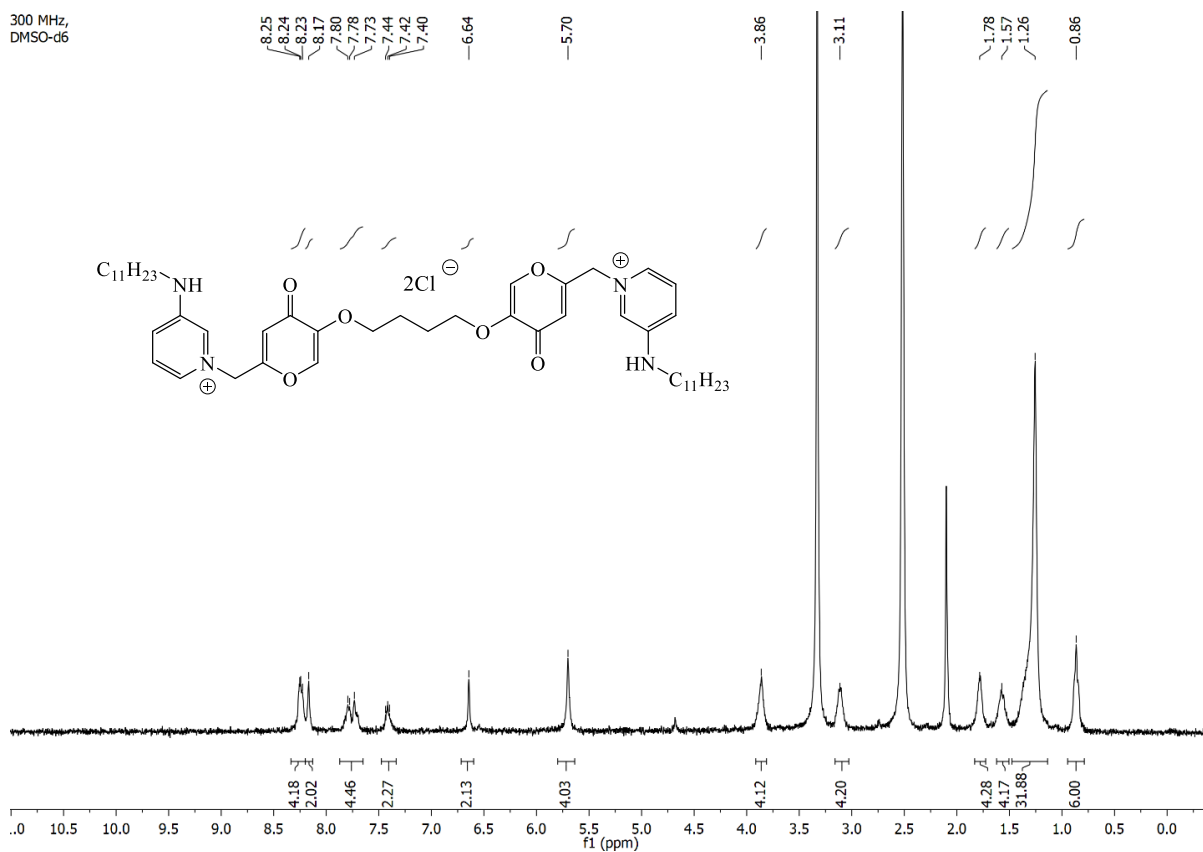
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	50 m/z	Set Capillary	4500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Waste

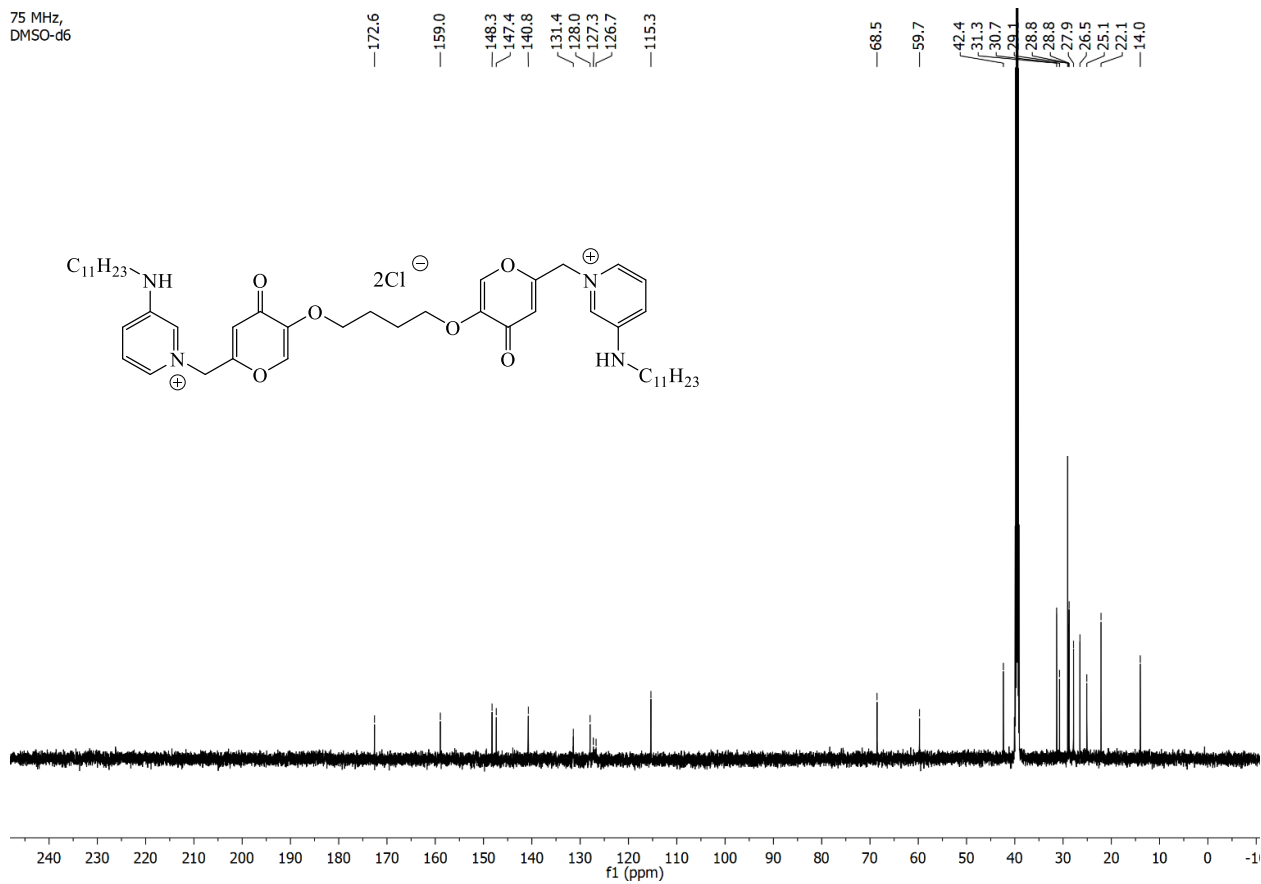


1,1'-(((Butane-1,4-diylbis(oxy))bis(4-oxo-4*H*-pyran-5,2-diyl))bis(methylene))bis(3-(undecylamino)pyridin-1-ium) dichloride (4f)

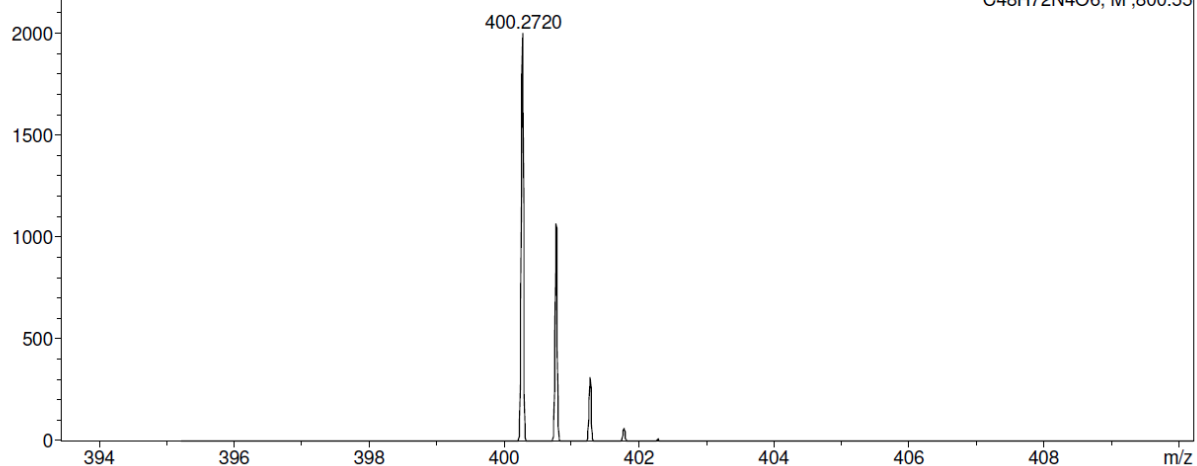
300 MHz,
DMSO-d₆



75 MHz,
DMSO-d₆



Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active			Set Dry Heater	180 °C
Scan Begin	50 m/z	Set Capillary	4500 V	Set Dry Gas	4.0 l/min
Scan End	3000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Waste



4. Biological experiments

Bacterial and Fungi Strains

Reference strains of the microorganisms *S. aureus* ATCC 43300, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 70060, *A. baumannii* ATCC 15308 and *P. aeruginosa* ATCC 27853, were obtained from the State Collection of Pathogenic Microorganisms (Obolensk, Russia). Clinical strains of *C. albicans* AV-85, *E. coli* B-3421/19, *K. pneumoniae* B-2523/18, *S. aureus* B-8648, *P. aeruginosa* B-2099/18, and *A. baumannii* B-2926/18 were isolated in the Molecular Microbiology department of the Federal Budget Institution of Science State Research Center for Applied Biotechnology and Microbiology (FBSI SRC PMB, Obolensk, Russia) from clinical samples in the investigation of infection cases in 2016–2018.

Identification of Microorganisms

Species identification of microorganisms was carried out on a MALDI-TOF Biotyper mass spectrometer (Bruker, Billerica, MA, USA) according to the standard microflex User Manual. One part of the daily bacterial culture was introduced into a 2 mL tube (Eppendorf, Hamburg, Germany) containing 300 µL of deionized water and triturated until a homogeneous suspension was obtained. Then, 900 µL of 96% ethanol was added and shaken. The tube was then centrifuged at 12,000×g for 2 min. The supernatant was discarded, and the precipitate was dried in air. After drying, the precipitate was thoroughly mixed with 30 µL of 70% formic acid, incubated for 10 min at room temperature, and then mixed with 30 µL of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA) and incubated for another 10 min. This mixture was centrifuged at 12,000×g for 2 min; then, 1 µL of the supernatant was transferred to an MSP 96 Polished Steel MALDI Target Plate (Bruker Daltonik GmbH, Bremen, Germany), air-dried, and covered with 1 µL of a saturated solution of α -cyano-4-hydroxycinnamic acid (Bruker Daltonik, Bremen, Germany). The MALDI target plate was placed in a MALDI-TOF microflex LRF instrument (Bruker Daltonik, Bremen, Germany). The data acquisition settings were as follows: ion source 1 at 19.50 kV, ion source 2 at 18.22 kV, lens at 7.01 kV, and a mass range of 2000 to 20,000 Da. Spectrum registration was performed automatically using MALDI Biotyper RTC 3.1 software (Bruker Daltonik, Bremen, Germany). The obtained spectra were compared with the reference spectra of the FFL v1.0 database. Identification scores ≥ 2.0 and 1.7–1.99 indicated recognition of the species and genus levels, respectively, while scores < 1.7 indicated a lack of reliable data.

Cultivation of Microorganisms

Microorganisms were cultivated using agar and GRM broth. Cultivation of microorganisms was carried out for 20–24 h at a temperature of 37°C.

Determination of the Biofilms

The efficiency of bacterial biofilm formation was determined using a method based on the ability of the crystal violet dye to bind with cells and biofilm matrix. The growth of biofilms of test strains of microorganisms was carried out in 96-well plates according to the standard method^{S1}. To obtain biofilms, 96-well flat-bottomed culture plates were used. In each well, 200 µL of a daily bacterial culture was inoculated at a concentration of 10⁶ CFU/mL and cultivated for 24 h at 37°C. Then, the medium with planktonic cells was carefully taken from the plate. Wells with biofilms were washed for 2–3 min using sterile PBS buffer (NaCl—8 g, KCl—0.2 g, Na₂HPO₄—1.44 g, KH₂PO₄—0.24 g per 1 L, pH = 7.4) in the same volume to remove the residual planktonic cells. After washing, PBS was completely removed by pipetting. Then, 200 µL of a filtered 0.1% solution of gentian violet was added to each well, and the biofilms were incubated with the dye for 10–15 min at room temperature. The dyes were completely removed from the well by pipetting. The unbound dye was thoroughly washed off with PBS. The plates were inverted onto filter paper and dried. After complete drying of the surface, a 200 µL mixture of ethanol–isopropanol (1:1) was added to the wells. The dye was washed off from the surface of the wells, taken, and placed in clean flat-bottomed plates. The optical density of the resulting solution was measured at a wavelength of 590 nm using an xMark™ Microplate

Absorbance Spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The measurement results were interpreted by comparing the OD₅₉₀ values of the samples with control values (pure solvent without dye added).

The absence of biofilm was recorded at OD₅₉₀ sample $\leq$ OD₅₉₀ control; weak degree of biofilm production at OD₅₉₀ (control) $<$ OD₅₉₀ (sample) $\leq$ 2*OD₅₉₀ (control); average degree of biofilm production at 2*OD₅₉₀ (control) $<$ OD₅₉₀ (sample) $\leq$ 4*OD₅₉₀ (control); high degree of biofilm production at OD₅₉₀ control $<$ 4*OD₅₉₀ sample^{S2}. All experiments were carried out in triplicate.

Antimicrobial and Antibiofilm Activity Assay

The synthesized naphthalene bis-QACs and reference compounds were dissolved in the DMSO/water system at a ratio of 1/9 with a final concentration of 1000 mg/L. For better dissolution, the samples were sonicated for 15 to 60 minutes at 40°C. The MIC and MBC of the antimicrobials was determined by the microdilution method in culture broth, as indicated by the Clinical and Laboratory Standards Institute of the United States of America^{S3}. For this, 2-fold dilutions of the tested solutions (500–0.25 mg/L) were prepared in nutrient broth. The nutrient broth with the tested QACs (0.1 mL) was added to 12 wells in the horizontal rows of the culture plate. In separate rows, nutrient broth without QACs was added for control measures. In the case of antibiofilm analysis, nutrient broth with the appropriate concentration of the tested compounds was added in 0.1 mL to 12 wells in horizontal rows of a culture plate with washed biofilms, which were prepared according to the abovementioned method. From single colonies grown on GRM medium at 37°C for 18 h, a suspension was prepared with an optical density of 0.5 according to the McFarland standard in sterile saline, which corresponds to approximately $1\text{--}2 \times 10^8$ CFU/mL. The suspension was then diluted 100-fold by adding 0.2 mL of the suspension to a flask containing 19.8 mL of Mueller–Hinton nutrient agar (MHA). The concentration of microorganisms, in this case, was 10^6 CFU/mL. An amount of 0.1 mL of the initial suspension was added to the wells with the test drug and control wells with broth. The final concentration of the microorganisms in each well was 5×10^5 CFU/mL. The plates were covered with lids and placed in a thermostat (37°C) for 20 h. The presence of bacterial growth was taken into account visually (according to the presence of turbidity in the well). The MIC or MBIC were taken to be the minimum concentration of the preparation at which bacterial growth was absent after 48 h of incubation. MBC, MFC or MBEC were determined by the results of inoculation on dense nutrient media. To do this, from all the wells in which there was no visible growth (according to the presence of turbidity), 10 μ L was sown on MHA. The results were taken into account by the presence of culture growth at the site of application after 48 h of incubation at 37°C. If there was no growth in the well, but the growth of the studied culture was observed when seeding from this well on solid nutrient medium, then this concentration was taken as the MIC or MBIC. The lowest concentration was taken as MBC, MFC or MBEC, at which cell growth was completely suppressed when seeded on a dense nutrient medium. Since the limit of detection for this technique is 10 CFU/mL, the absence of any growth on MHA plate indicated that the concentration lay below this value. The initial concentration of 10^5 CFU/mL had thus been reduced to below 10 CFU/mL. Consequently, the MBC, MFC or MBEC were effectively deemed to be the minimum concentration of antimicrobial capable of inactivating more than 99.99% of the bacteria or fungi present. Three replicates were performed for each strain and antimicrobial compound.

5. References

- S1. G. A. O'Toole, R. Kolter, *Mol. Microbiol.*, 1998, **28**, 449; <https://doi.org/10.1046/j.1365-2958.1998.00797.x>
- S2. L. B. Rodrigues, L. R. dos Santos, V. Z. Tagliari, N. N. Rizzo, G. Trenhago, A. P. de Oliveira, F. Goetz, V. P. do Nascimento, *Braz. J. Microbiol.*, 2010, **41**, 1082; <https://doi.org/10.1590/S1517-838220100004000029>.
- S3. J. Henschel, J. M. Dressler, M. Winter and S. Nowak, *Chem. Mater.*, 2019, **31**, 9970; <https://doi.org/10.1021/acs.chemmater.9b04133>.