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Cationic amphiphiles based on diethanolamine esters with amino acids in the polar block

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General Materials and Procedure for Synthesis

All chemicals and reagents were used as purchased. Glycine (Gly), β-alanine (βAla), GABA (GABA) (Acros Organics), L-lysine monohydrochloride (L-Lys) L-ornithine monohydrochloride (L-Orn) (Merck), *p*-toluenesulfonic acid (HOTs), di-*tert*-butyl dicarbonate (Boc_2O), dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) (Sigma Aldrich), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Merck) *N*,*N*-diisopropylethylamine (DIPEA) (Across Organics), potassium carbonate, sodium carbonic acid, sodium sulfate anhydrous (Khimmed), trifluoroacetic acid (TFA) (Biochem).

^{1}H NMR spectra were recorded in $CDCL_3$ on a Bruker DPX-300 pulsed NMR spectrometer (operating frequency 300 MHz). The internal standard is hexamethyldisiloxane. The mass spectra were obtained using the VISION 2000 time-of-flight MALDI mass spectrometer (UK), matrix 2,5-dihydroxybenzoic acid (2,5-DHB). Thin-layer chromatography was carried out on Sorbfil plates, column chromatography was carried out on 0.040-0.063 mm Merck silica gel. To identify substances containing amino groups, a 5% solution of ninhydrin was used, followed by heating to 50 °C.

***O,O'*-(Dioctanoyl)diethanolamine 1** (see Z. G. Denieva *et al.*, *Mendeleev Commun.*, 2021, **31**, 509). A mixture of diethanolamine (1.5 g, 14 mmol), octanoic acid (9 ml, 57 mmol) and *p*-toluenesulfonic acid (10.86 g, 57 mmol) was heated in an oil bath at 130 °C for 1.5 h under vigorous stirring. After completion, the reaction mixture was cooled to room temperature, recrystallized from acetone and dried for 24 hours. The substance thus obtained was dissolved in ethyl acetate (60 ml), washed with a 5% water solution of sodium chloride (4 × 25 ml), water to pH 7 and dried with sodium sulfate. The solvent was evaporated on a rotary evaporator to afford 4.5 g (90%) of compound **1**.

***N*-(*tert*-Butoxycarbonyl)glycyl-*O,O'*-(dioctanoyl)diethanolamine 2.** Catalytic amount of DMAP and BocGly (0.84 g, 4.8 mmol) were added to a solution of compound **1** (1.43 g, 4.8 mmol) in anhydrous dichloromethane (15 ml), and this was cooled to 0 °C with stirring. After 15

min, a solution of DCC (1.65 g, 8 mmol) in dichloromethane (10 ml) was added, and stirring was continued for 24 h. The precipitate was filtered out, the solution was washed with water (3 × 50 ml), dried over sodium sulfate, the solvent was removed in vacuum. Column chromatography in the system toluene/ethyl acetate 3:1 afforded 1.11 g (53%) of compound **2**. ^{1}H NMR ($CDCl_3$,δ, ppm): 0,88 (t, 6H, $CH_2C\underline{H}_3$); 1,27 (s, 16H, $CH_2C\underline{H}_2CH_2$); 1,45 (s, 9H, $CC\underline{H}_3$); 1,58 (p, 4H, OC(O) $CH_2C\underline{H}_2$); 2,30 (k, 4H, OC(O)$C\underline{H}_2$ CH_2); 3,58 (dt, 4H, $NC\underline{H}_2CH_2$); 4,03 (d, 2H, $NHC\underline{H}_2$); 4,20 (k, 4H, $NCH_2C\underline{H}_2$); 5,43(s, 1H, N$\underline{H}$).

***N*-Glycyl-*O,O′*-(dioctanoyl)diethanolamine hydrotrifluoroacetate 3**·CF_3CO_2H**.** A solution of compound **2** (1.11 g, 2 mmol) in anhydrous dichloromethane (20 ml) was cooled to 0 °C, trifluoroacetic acid (1 ml) was added, and this was stirred for 2 h. The mixture was evaporated on a rotary evaporator to leave 1.07 g (97%) of product **3**·CF_3CO_2H.

***N*-Glycyl-*O,O′*-(dioctanoyl)diethanolamine 3**. The above hydrotrifluoroacetate (1.07 g, 2.02 mmol) solution in dichloromethane was washed with a 5% water solution of sodium bicarbonate (3×50 ml) and then with water to pH 7, dried with sodium sulfate and evaporated on a rotary evaporator to afford 0.78 g (73%) of product **3**.

***N*-Glycylglycyl-*O,O′*-(dioctanoyl)diethanolamine hydrotrifluoroacetate 5a**.

Step 1. ***N*-(*tert*-Butoxycarbonylglycylglycyl)-*O,O′*-(dioctanoyl)diethanolamine**. DIPEA (0.05 ml, 0.28 mmol) was added to HBTU (0.1 g, 0.28 mmol) solution in anhydrous MeCN (5 ml), and this was stirred at 0 °C for 15 min. A solution of BocGly **4a** (0.05 g, 0.28 mmol) in duchloromethane (5 ml) was added, and stirring was continued for another 30 min. A solution of compound **3** (0.1 g, 0.23 mmol) in dichloromethane (10 ml) was added, and the mixture was left at room temperature for 24 h. The product was isolated by column chromatography in the system toluene/ethyl acetate 1:5 to afford 0.12 g (92%) of the title compound. ^{1}H NMR: ($CDCl_3$, δ,ppm): 1.2 (t, 6 H, $CH_2C\underline{H}_3$); 1.6 (s, 16 H, $C\underline{H}_2CH_3$); 1.8 (s, 9 H, $CC\underline{H}_3$); 1.98 (p, 4 H,OC(O) $CH_2C\underline{H}_2$); 2.56 (s, 1 H, N$\underline{H}CH_2C(O)(Gly_1)$); 2.68 (dt, 4 H, OC(O)$C\underline{H}_2$ CH_2); 3.97 (dt, 4 H, $NC\underline{H}_2CH_2$); 4.2 (d, 2 H, $NHC\underline{H}_2(Gly_1)$); 4.5 (d, 2 H, $NHC\underline{H}_2$ (Gly_2)); 4.58 (dt, 4 H, $NCH_2C\underline{H}_2$); 5.57 (t, 1 H, N$\underline{H}CH_2$ (Gly_2)).

Step 2. Compound **5a**. *N*-Deprotection was performed similarly as in the preparation of compound **3**·CF_3CO_2H. Starting from the above Boc-intermediate (0.12 g, 0.12 mmol), 0.11 g (95%) of product **5a** was obtained. Mass spectrum: MS (MALDI), *m/z*: 494.142 [M+Na]+, 510.114 [M+K]+

Compounds **5b-c** and **7a,b** were synthesized in a similar way.

***N*-(β-Alanylglycyl)-*O,O′*-(dioctanoyl)diethanolamine hydrotrifluoroacetate 5b**.

Step 1. ***N*-[(*tert*-Butoxycarbonyl-β-alanyl)glycyl]-*O,O′*-(dioctanoyl)diethanolamine**. ^{1}H NMR: ($CDCl_3$, δ,ppm): 0.9 (t, 6 H, $CH_2C\underline{H}_3$); 1.3 (d, 16 H, $C\underline{H}_2CH_3$); 1.4 (s, 9 H, $CC\underline{H}_3$); 1.6 (m, 4 H, OC(O) $CH_2C\underline{H}_2$); 2.3 (dt, 4 H, $OC(O)C\underline{H}_2CH_2$); 2.48 (t, 2 H, $NHC\underline{H}_2CH_2$(bAla)); 3.4(t, 2 H, $NHCH_2C\underline{H}_2$(bAla)); 3.5 (s, 1 H, $N\underline{H}CH_2$ (bAla)); 3.6 (dt, 4 H, $NC\underline{H}_2CH_2$); 4.16 (s, 2 H, $NHC\underline{H}_2$ (Gly)); 4.24 (dt, 4 H, $NCH_2C\underline{H}_2$); 5.25 (t, 1 H, $N\underline{H}CH_2C(O)$(Gly)).

Step 2. Compound **5b**. Mass spectrum: MS (MALDI), m/z: 508.141 $[M+Na]^+$, 524.133 $[M+K]^+$

***N*-(GABA-glycyl)-*O,O′*-(dioctanoyl)diethanolamine hydrotrifluoroacetate 5c.**

Step 1. ***N*-[(*tert*-Butoxycarbonyl-GABA)-glycyl]-*O,O′*-(dioctanoyl)diethanolamine.** ^{1}H NMR: ($CDCl_3$, δ,ppm): 1.2 (t, 6 H, $CH_2C\underline{H}_3$); 1.6 (d, 16 H, $C\underline{H}_2CH_3$); 1.78 (s, 9 H, $CC\underline{H}_3$); 1.9(m, 4 H, $OC(O)CH_2C\underline{H}_2$); 2.2 (p, 2 H, $NHC\underline{H}_2CH_2CH_2$ (GABA)); 2.67 (m, 2 H, $NHCH_2C\underline{H}_2CH_2$ (GABA)); 2.67 (m, 4 H, $OC(O)C\underline{H}_2CH_2$); 3.5 (d, 2 H, $NHCH_2CH_2C\underline{H}_2$ (GABA)); 3.82 (s, 1 H, $N\underline{H}CH_2$ (GABA)); 3.98 (dt, 4 H, $NC\underline{H}_2CH_2$); 4.5 (s, 2 H, $NHC\underline{H}_2$ (Gly)); 4.58 (dt, 4 H, $NCH_2C\underline{H}_2$); 5.2 (t, 1 H, $N\underline{H}CH_2C(O)$ (Gly)).

Step 2. Compound **5c.** Mass spectrum: MS (MALDI), m/z: 522.187 $[M+Na]^+$, 538.464 $[M+K]^+$

***N*-(L-Lysylglycyl)-*O,O′*-(dioctanoyl)diethanolamine bis-hydrotrifluoroacetate 7a.**

Step 1. ***N*-[(*N,N′*-Di-*tert*-butoxycarbonyl-L-lysyl)glycyl]-*O,O′*-(dioctanoyl)diethanolamine.** ^{1}H NMR: ($CDCl_3$, δ,ppm): 0.87 (t, 6 H, $CH_2C\underline{H}_3$); 1.1 (k, 2 H, $NHC\underline{H}_2$ (Lys)); 1.26 (s, 16 H, $C\underline{H}_2CH_3$); 1.4 (s, 18 H, $CC\underline{H}_3$); 1.49 (d, 2 H, $NHC\underline{H}_2$ (Lys)); 1.68 (d, 2 H, $NHC\underline{H}_2$ (Lys)); 1.95(m, 4 H, $OC(O)CH_2C\underline{H}_2$); 2.3 (k, 4 H, $OC(O)C\underline{H}_2CH_2$); 3.12 (d, 2 H, $NHC\underline{H}_2$ (Lys)); 3.47 (s, 1 H, $N\underline{H}CH_2$ (Lys)); 3.6 (dt, 4 H, $NC\underline{H}_2CH_2$); 4.19 (d, 2 H, $NHC\underline{H}_2$ (Gly)); 4.21 (m, 4 H, $NCH_2C\underline{H}_2$); 4.64 (s, 1 H, $NHC\underline{H}$ (Lys)); 5.13 (s, 1 H, $N\underline{H}CH_2C(O)$ (Gly)); 6.94 (s, 1 H, $N\underline{H}CH_2$ (Lys)).

Step 2. Compound **7a.** Mass spectrum:: MS (MALDI), m/z: 567.194 $[M+Na]^+$

***N*-(L-Ornithylglycyl)-*O,O′*-(dioctanoyl)diethanolamine bis-hydrotrifluoroacetate 7b.**

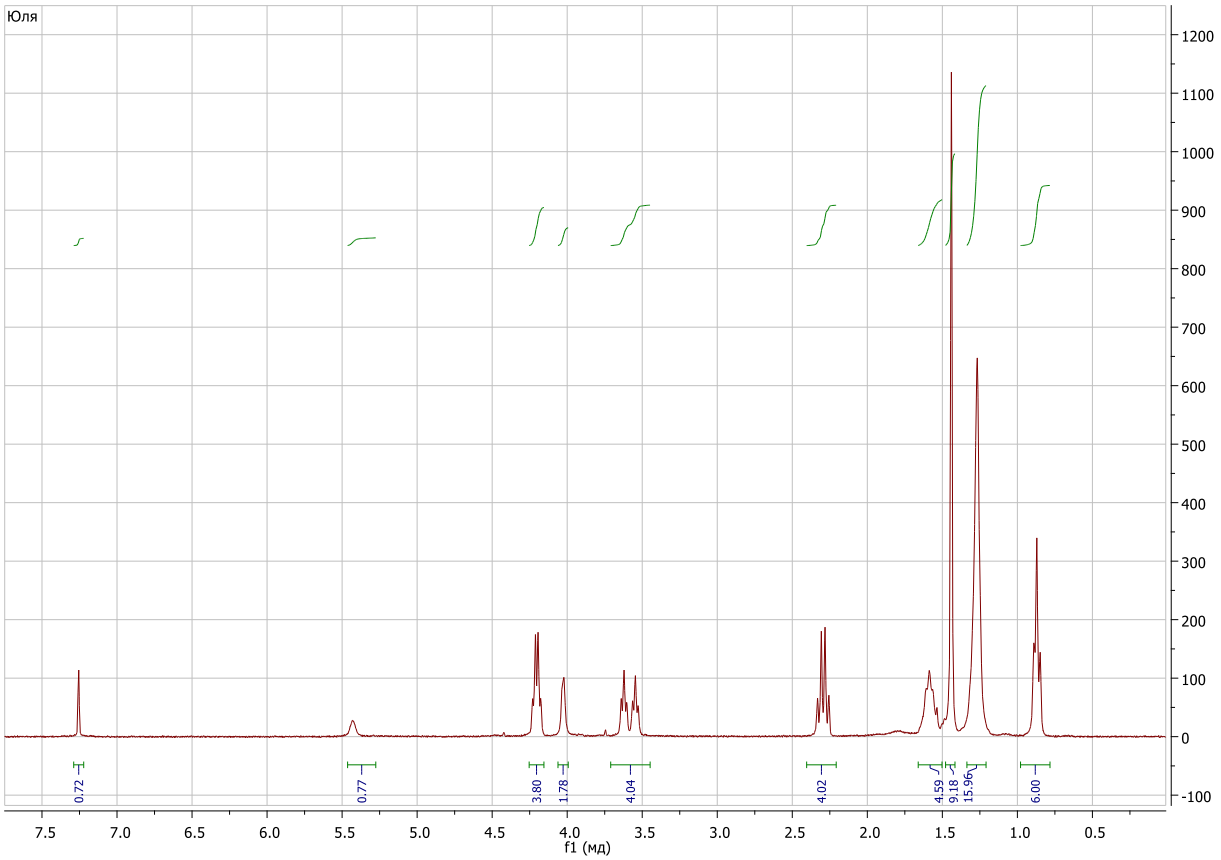
Step 1. ***N*-[(*N,N′*-Di-*tert*-butoxycarbonyl-L-ornithyl)glycyl]-*O,O′*-(dioctanoyl)-diethanolamine.** ^{1}H NMR: ($CDCl_3$, δ,ppm): 0.86 (t, 6 H, $CH_2C\underline{H}_3$); 1.27 (s, 16 H, $C\underline{H}_2CH_3$); 1.41 (s, 18 H, $CC\underline{H}_3$); 1.58 (m, 2 H, $NHC\underline{H}_2$ (Orn)); 1.58 (m, 4 H, $OC(O)CH_2C\underline{H}_2$); 1.85 (k, 2 H, $NHC\underline{H}_2$ (Orn)); 2.29 (k, 4 H, $OC(O)C\underline{H}_2CH_2$); 3.1 (k, 2 H, $NHC\underline{H}_2$ (Orn)); 3.45 (s, 1 H, $N\underline{H}CH_2$ (Orn)); 3.59 (dt, 4 H, $NC\underline{H}_2CH_2$); 4.1 (d, 2 H, $NHC\underline{H}_2$ (Gly)); 4.2 (t, 4 H, $NCH_2C\underline{H}_2$); 4.7 (s, 1 H, $N\underline{H}CH_2C(O)$ (Gly)); 5.15 (d, 1 H, $NHC\underline{H}$ (Orn)); 7.04 (s, 1 H, $N\underline{H}CH_2$ (Orn)).

Step 2. Compound **7b.** Mass spectrum: MS (MALDI), m/z: 565.171 $[M+Na]^+$, 581.148 $[M+K]^+$

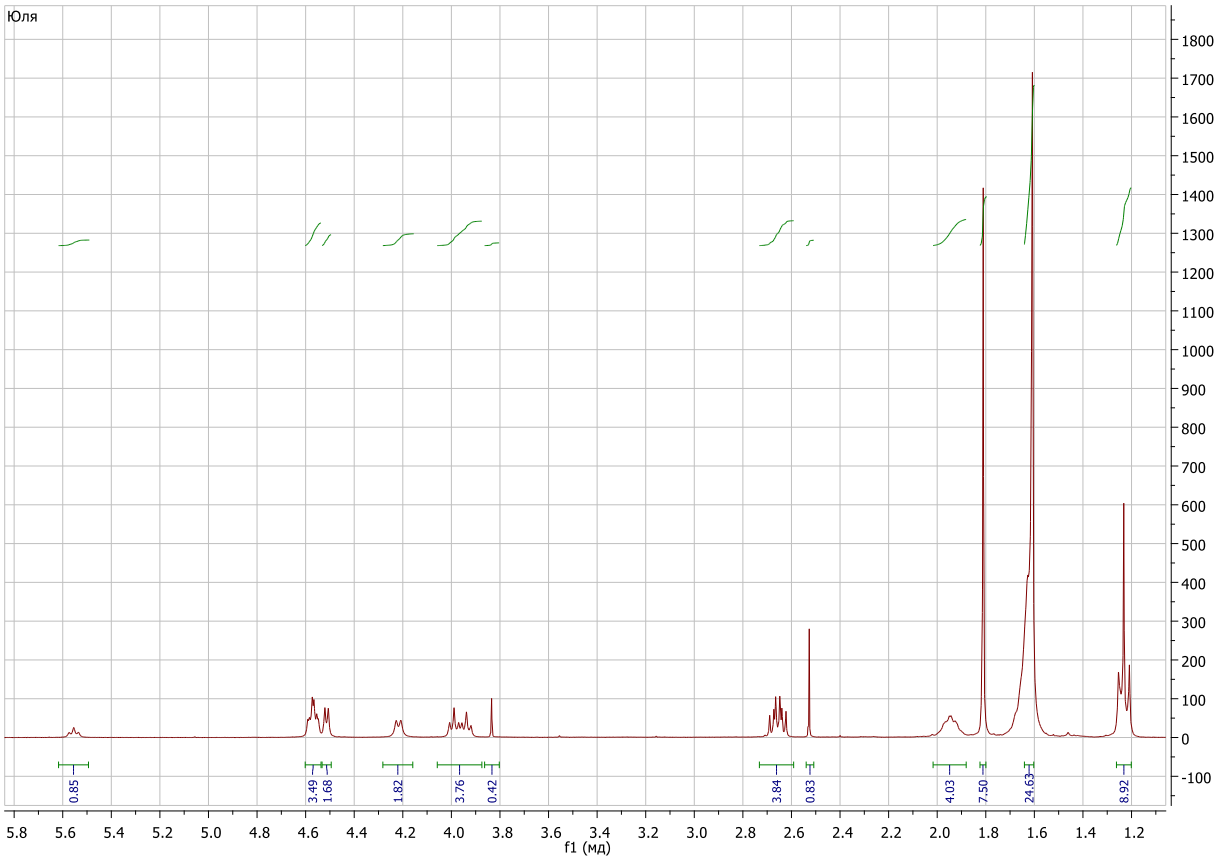
Evaluation of antibacterial activity in a liquid nutrient medium. Suspensions of microorganisms (*Bacillus subtilis 534* and *Escherichia coli M17*) were prepared so that the optical density was 0.5 McFarland units, which corresponded to a concentration of 1.5×10^8 CFU per 1 ml. The final titer of the inoculum in the nutrient broth contained 10^5 CFU per 1 ml.

Infected broth (50 μl) was introduced into the wells of the 96-well tablet. Next, sequential double dilutions of the studied substances and positive control were prepared from a solution in DMSO with a concentration of 1 μg ml^{-1} in the wells of the tablet. Wells with infected broth that did not contain the studied substances (negative control) served as a control of the growth of microorganisms. The glycopeptide antibiotic vancomycin was used as a positive control. Then the tablets were incubated at (36 ± 1) °C for 16-18 h. The minimum suppressive concentration (MIC) was determined by the lowest concentration of the test substance, which suppressed the visible growth of microorganisms when compared with growth control.

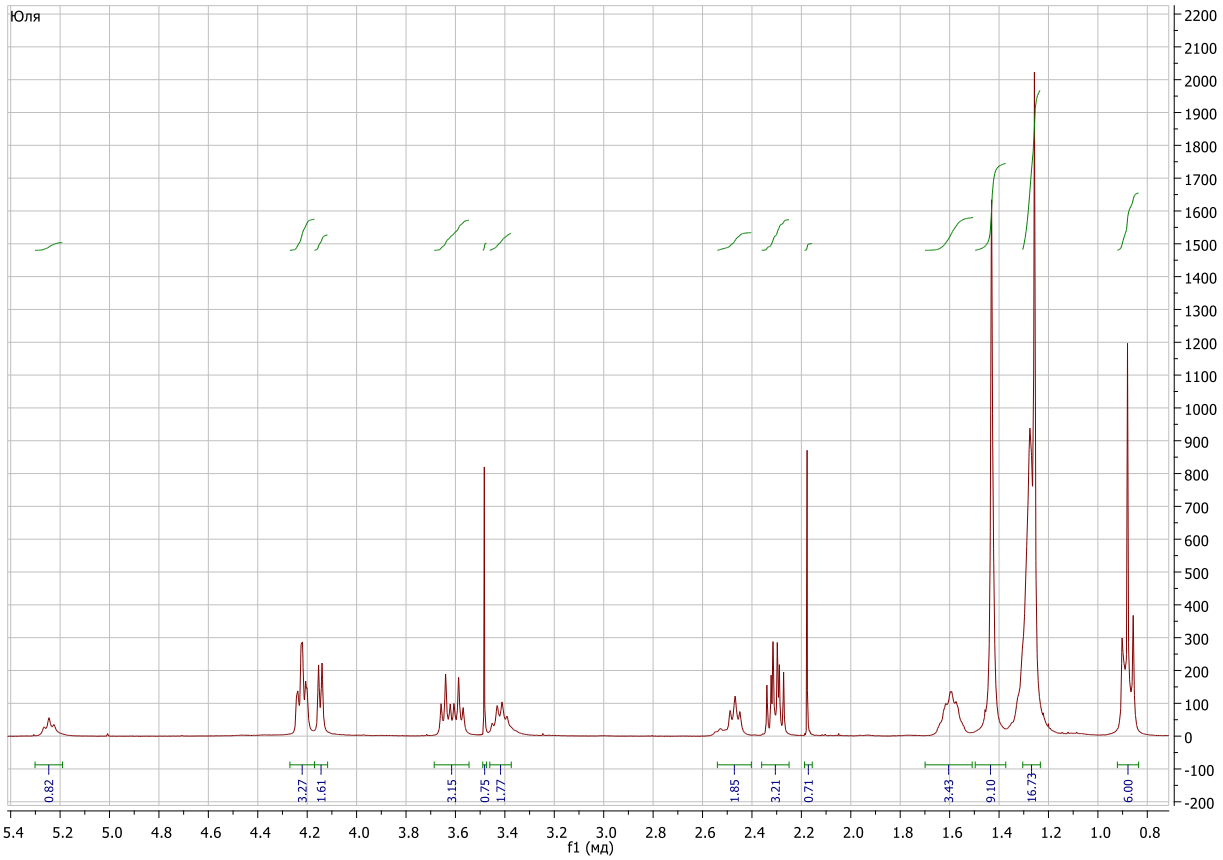
Formation of flat lipid membranes (BLM) and measurement of their electrical conductivity: Flat lipid membranes (BLM) were formed on holes of a copper mesh (SPI-Grids, 200 mesh, 3 mm). 0.4 μl of a lipid mixture in a solution of *n*-octane: *n*-decane (1:1 v/v) (Acros Organics, USA) at a concentration of 15 mg ml^{-1} was previously applied to the mesh and dried under a jet of argon for 5-10 sec until the solvent completely evaporated. Then the mesh was fixed in a Petri dish at a distance of 1-2 mm from the bottom and filled with 4 ml of a working buffer solution of 100 mM KCl (Sigma Aldrich, Saint-Louis, MO, USA) and 10 mM HEPES (Helicon, Russia) at pH 7.5. The Milli-Q water purification system (MilliPore, Direct-Q 3UV system, USA) was used to prepare the buffer. The lipid mixture of the same composition in squalane (Sigma Aldrich, Saint-Louis, MO, USA) at a concentration of 25 mg ml^{-1} was used to form BLM. The membranes were formed by the 'brush drawing' method, the bristles of which were previously washed with Milli-Q water, ethanol (1 ml) and chloroform (1 ml), and then dried under a jet of argon. When applying the lipid mixture to the mesh, BLMs were spontaneously formed on the mesh openings, and the process was controlled by an image in a microscope (magnification 40x). For the preparation of lipid mixtures, stock solutions of 1,2-diolenoyl-*sn*-glycerol-3-phosphatidylcholine (DOPC), 1,2-diolenoyl-*sn*-glycerol-3-phosphatidyl-(1'-*rac*-glycerol) (sodium salt) (DOPG), 1,2-diolenoyl-*sn*-glycerol-3-were used. Phosphatidylethanolamine (DOPE) (all lipids from Avanti Polar Lipids, Alabaster, AL, USA) dissolved in $CHCl_3$ (Sigma Aldrich, Saint-Louis, MO, USA) at a concentration of 10 mg ml^{-1} each were taken. In the work was used BLM with the following DOPC lipid compositions: DOPG:DOPE 60:20:20 mol%, DOPC:DOPG:DOPE 40:40:20 mol% and DOPC:DOPE 80:20 mol%. The test sample was dissolved in a working buffer solution of 100 mM KCl, 10 mM HEPES at pH 7.5 to a molar concentration of 2 nM (corresponding to MIC 1.5625 μg ml^{-1}) before the experiment and stored at a temperature of +4 °C. To add the test substance directly to the lipid bilayer, the solution was injected onto a membrane from a glass patch pipette and changes in membrane conductivity were observed in real time using patch-clamp technology (HEKA EPC 8). Electrical measurements were carried out at a fixed voltage (100 mV) using a pair of chlorine-silver electrodes.



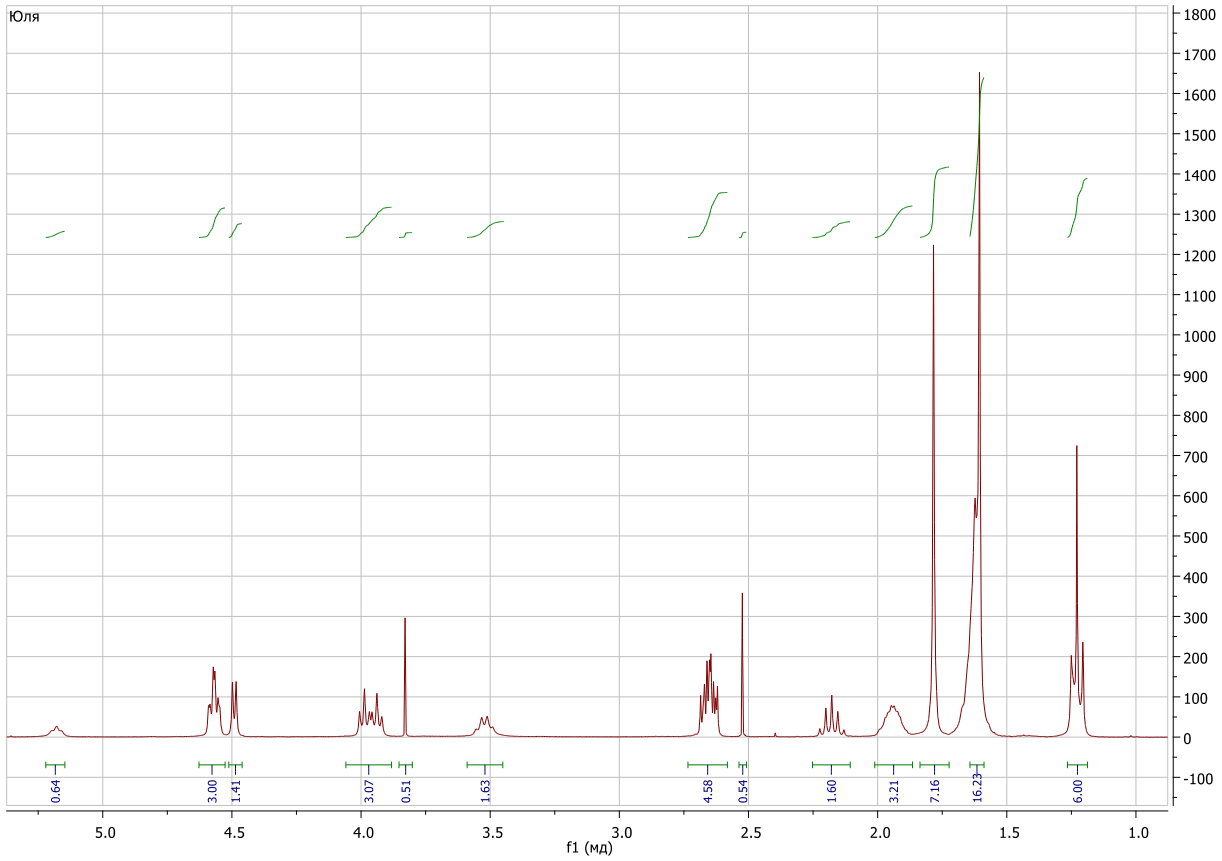
^{1}H NMR of compound **2**



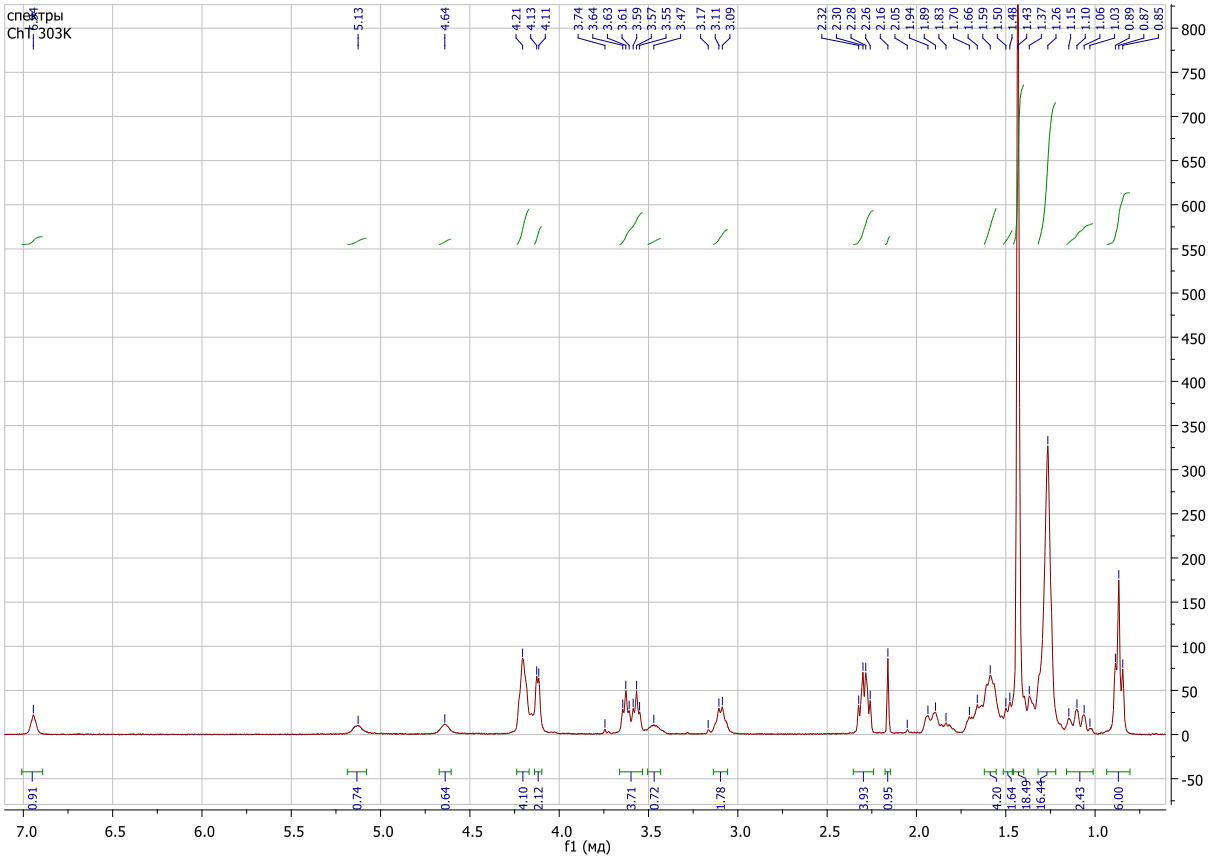
^{1}H NMR of Boc-precursor of **5a**



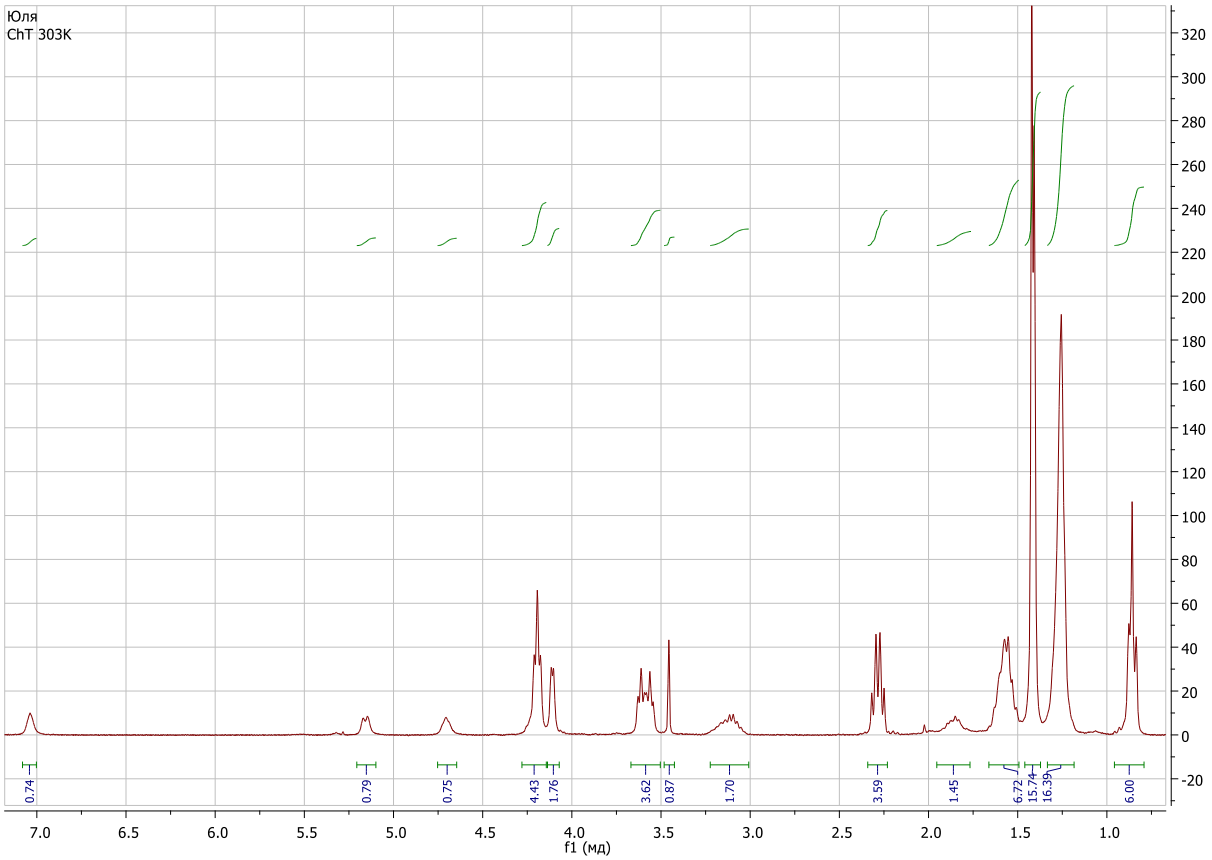
^{1}H NMR of Boc-precursor of **5b**



^{1}H NMR of Boc-precursor of **5c**



^{1}H NMR of di-Boc-precursor of **7a**



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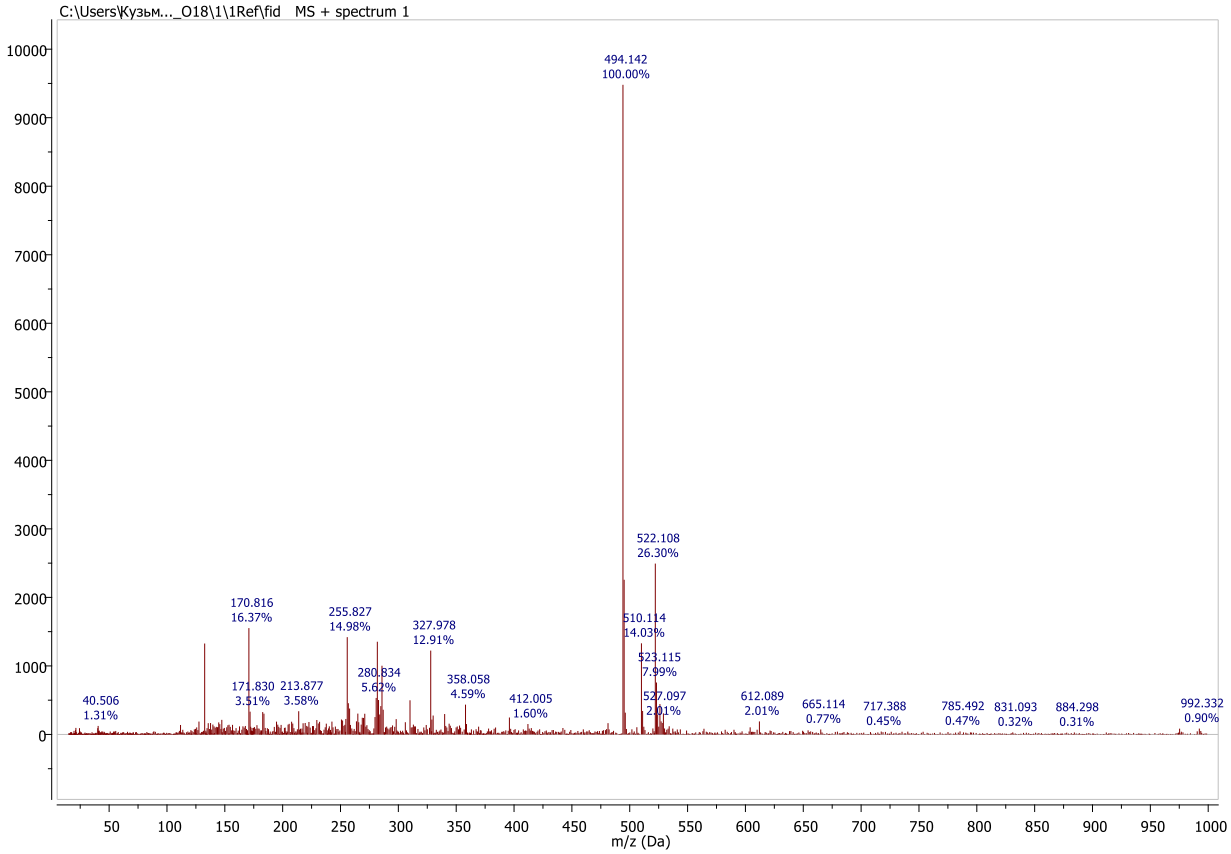
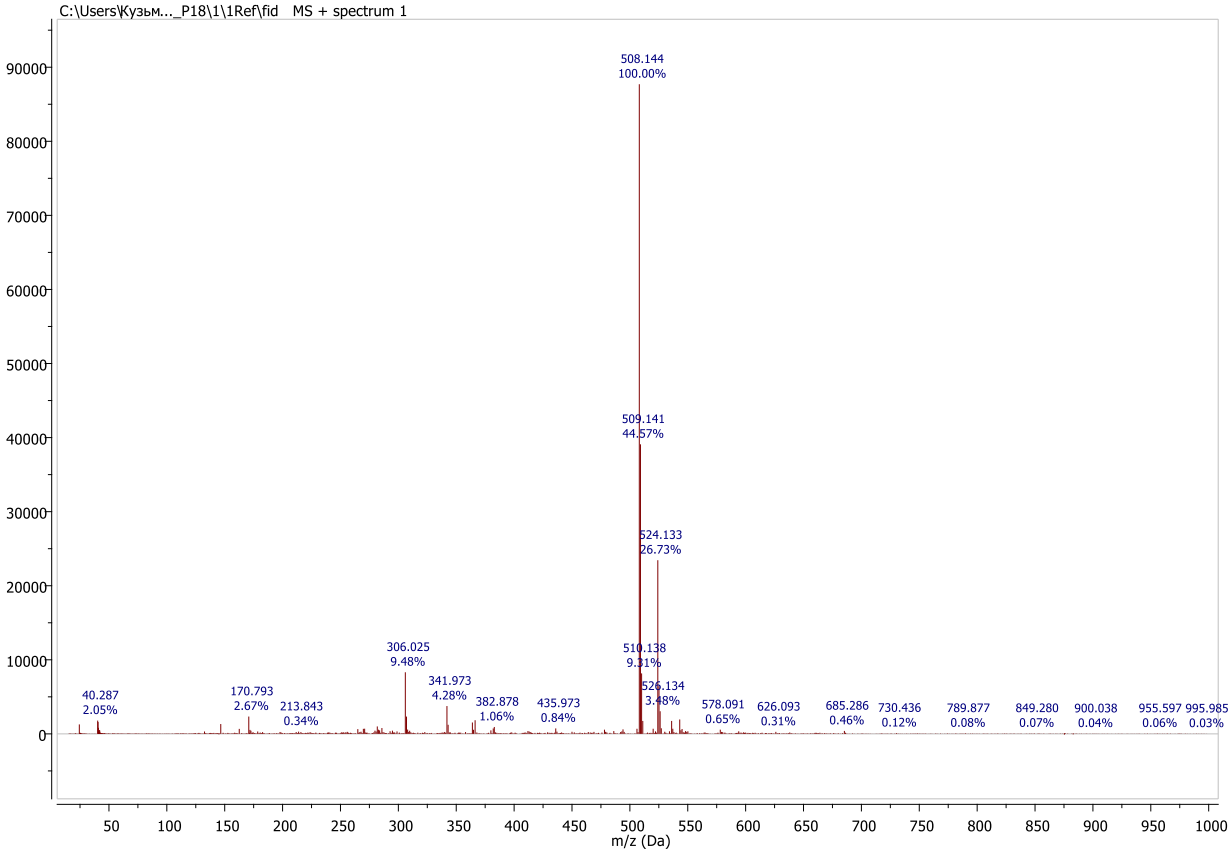


MS (MALDI) of **5a**



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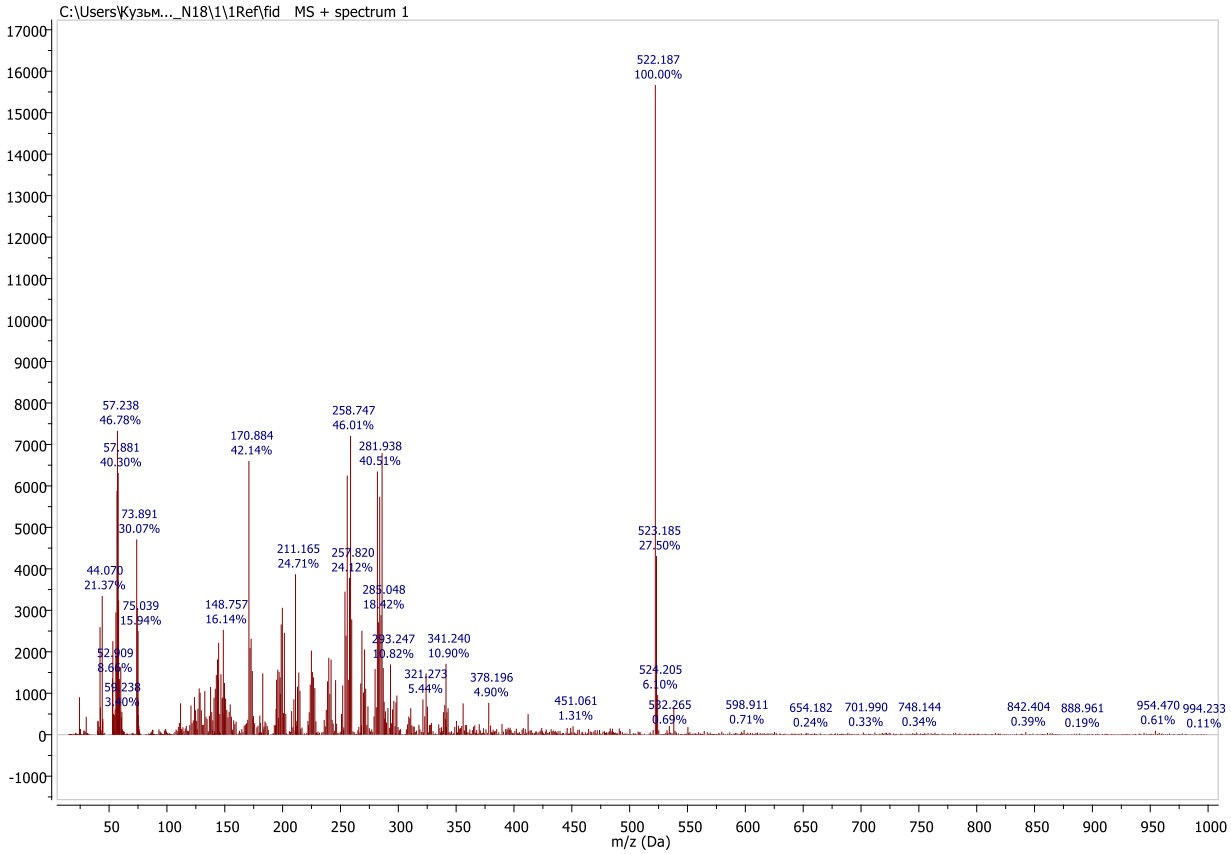

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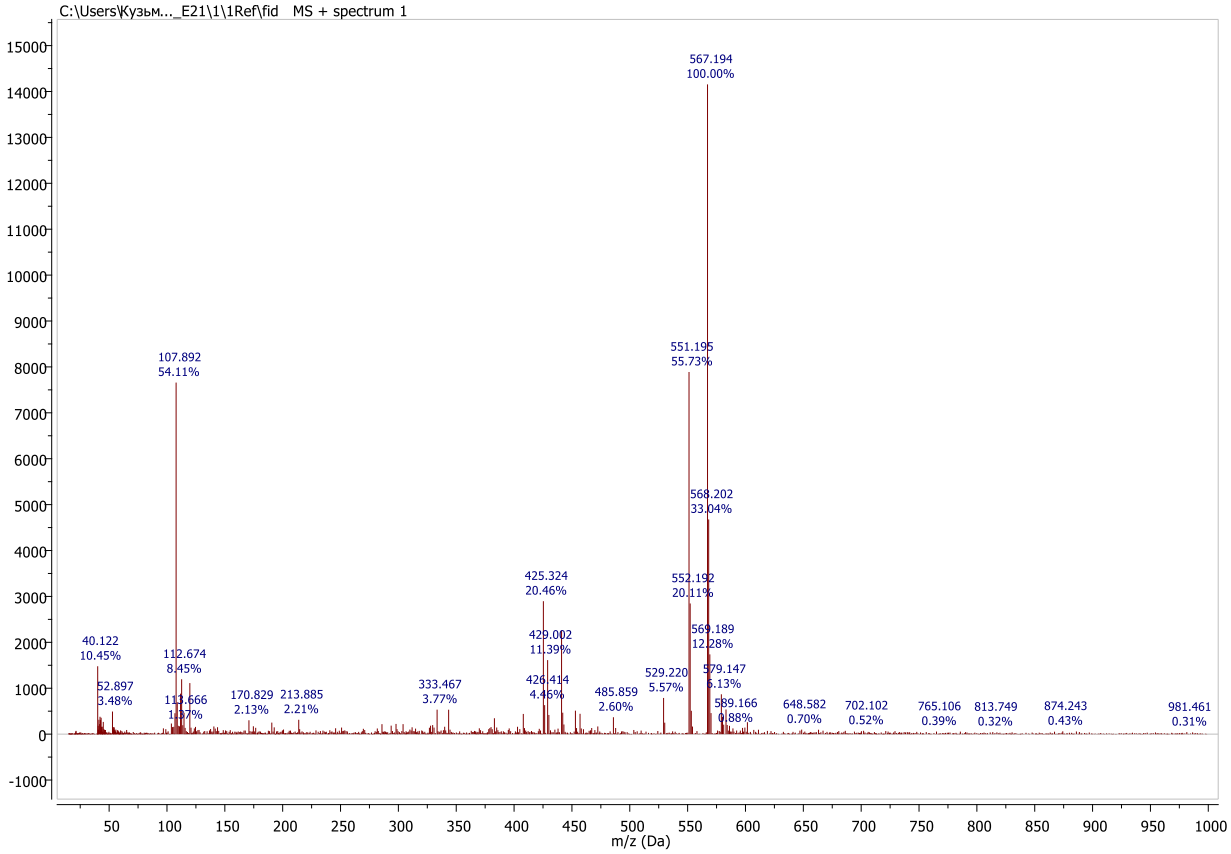

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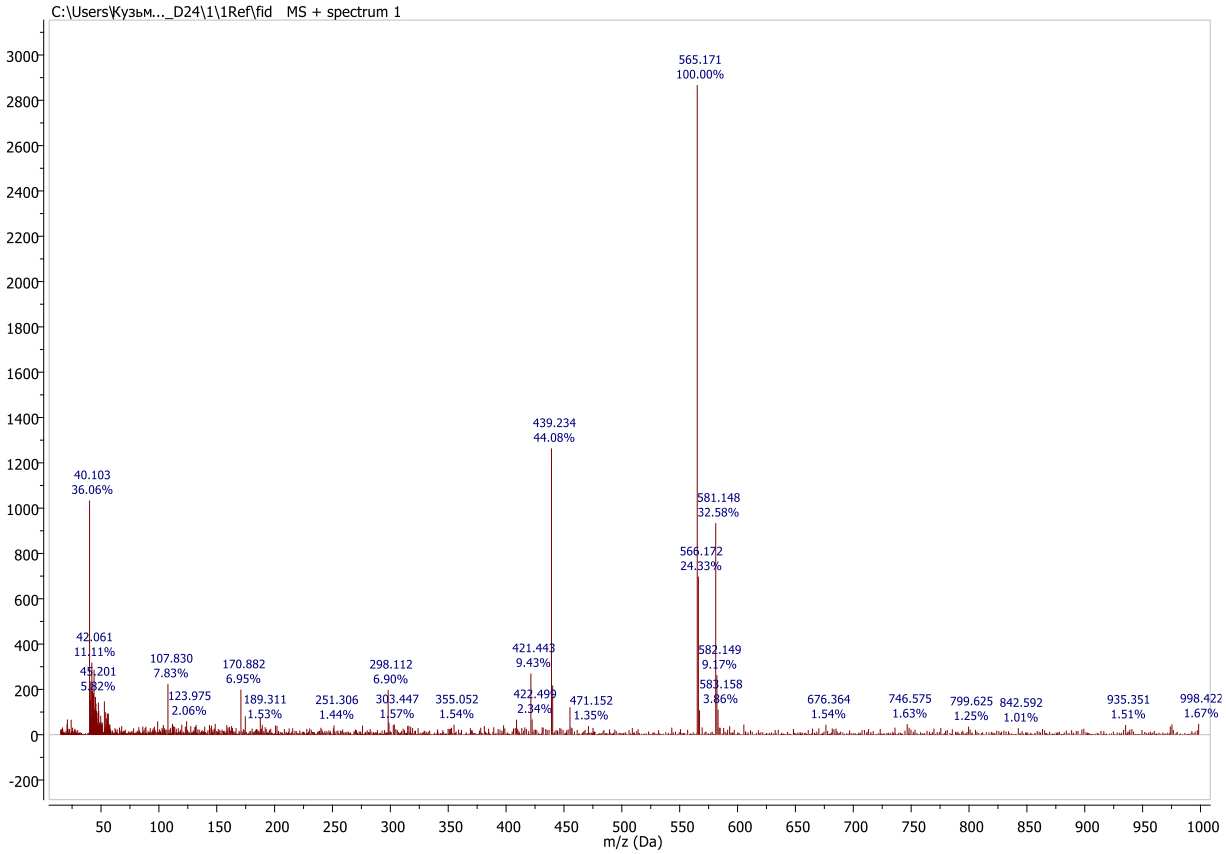

MS (MALDI) of **7b**