

Supporting information for

Lithocholic Acid's Ionic Compounds as Promising Antitumor Agents: Synthesis and Evaluation of the Production of Reactive Oxygen Species (ROS) in Mitochondria

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Table of contents

1. ^1H and ^{13}C NMR and mass spectra of the compounds **2, 3a, 3b, 4a, 4b, 5a, 5b**.
2. Biological studies.

Experimental Section

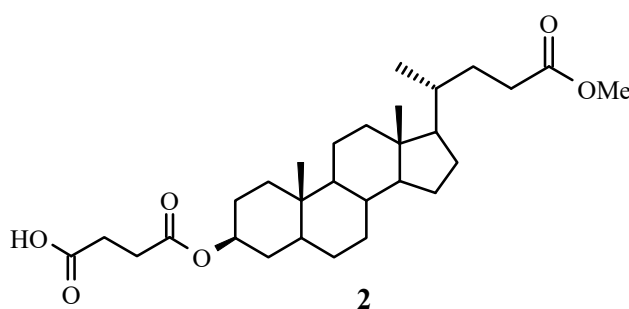
General

All commercial reagents were purchased from Sigma-Aldrich and Acros organics. Betulinic acid was prepared from commercially-available betulin by a reported procedure [1]. All commercially-available solvents and reagents used were of analytical grade and without further purification. Reactions were monitored by TLC on Sorbfil plates. Column chromatography was carried out on Acrus silica gel (0.060–0.200 mm). Optical rotations were measured on a Perkin–Elmer 341 polarimeter. Melting points were recorded on Stuart SMP3. IR spectra were recorded on Bruker VERTEX 70V using KBr discs over the range of 400–4000 cm^{-1} . ^1H and ^{13}C NMR spectra were obtained using a Bruker Ascend 500 spectrometer in CDCl_3 operating at 500 MHz for ^1H and 125 MHz for ^{13}C and a Bruker AVANCE 400 spectrometer in CDCl_3 operating at 400 MHz for ^1H and 100 MHz for ^{13}C . High-resolution mass spectra with electrospray ionization were measured with a Bruker MicroOTOF II instrument; external calibration of the mass spectrometer was performed using the Electrospray Tuning mix (Agilent).

General procedure for the synthesis of compound 2.

To a stirred solution of **1** (390.6 mg, 1 mmol) and DMAP (148.06 mg, 1.2 mmol) in dry CH_2Cl_2 (10 ml), succinic anhydride (400.97 mg, 4 mmol) was added at room temperature. The resulting reaction mixture was refluxed for 10 h. After completion of the reaction, the solvent was evaporated under reduced pressure. The residue was diluted with EtOAc (20 ml) and washed with water (2x20 ml) and brine (2x20 ml). The organic layer was dried over MgSO_4 and concentrated.

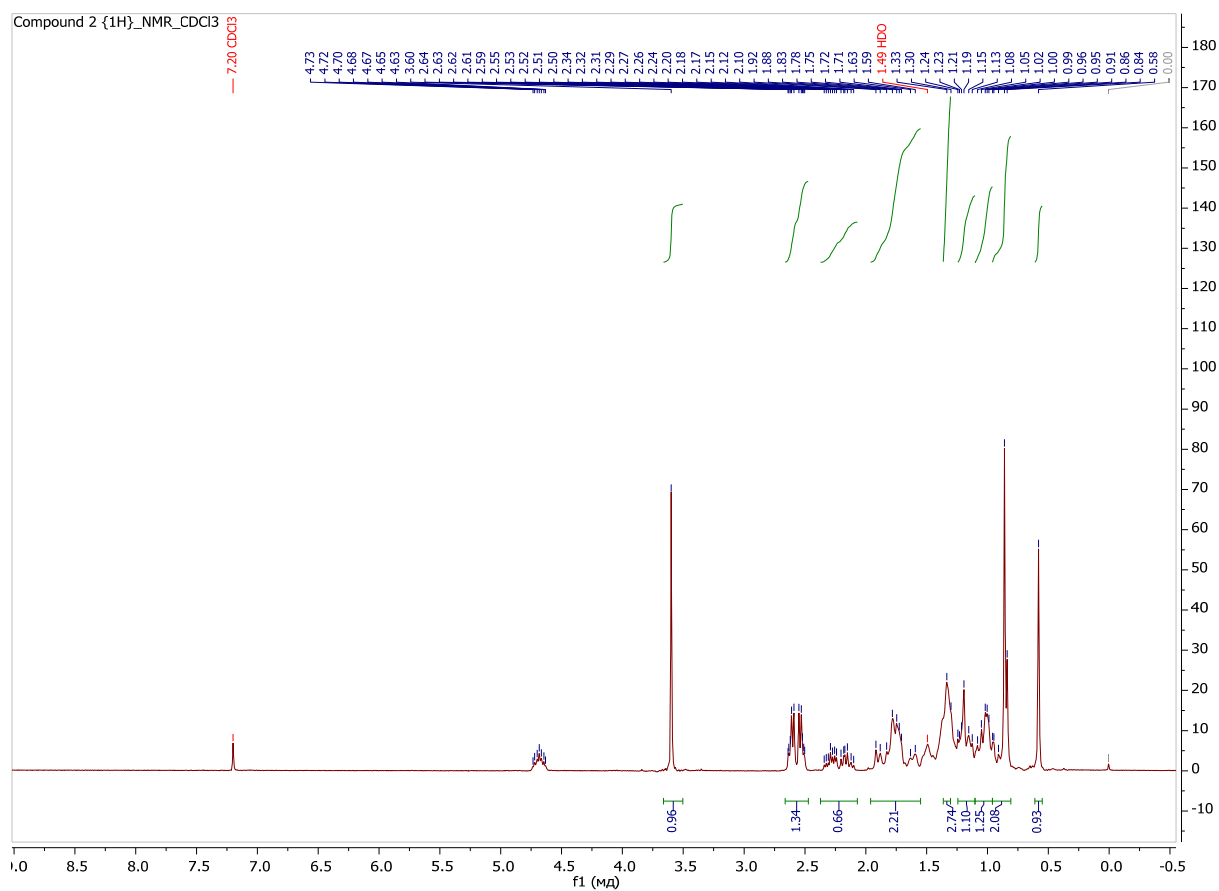
The crude product was purified by column chromatography (silica gel) using petroleum ether/EtOAc = 4/1 as the elution solvent to afford compound **2**.



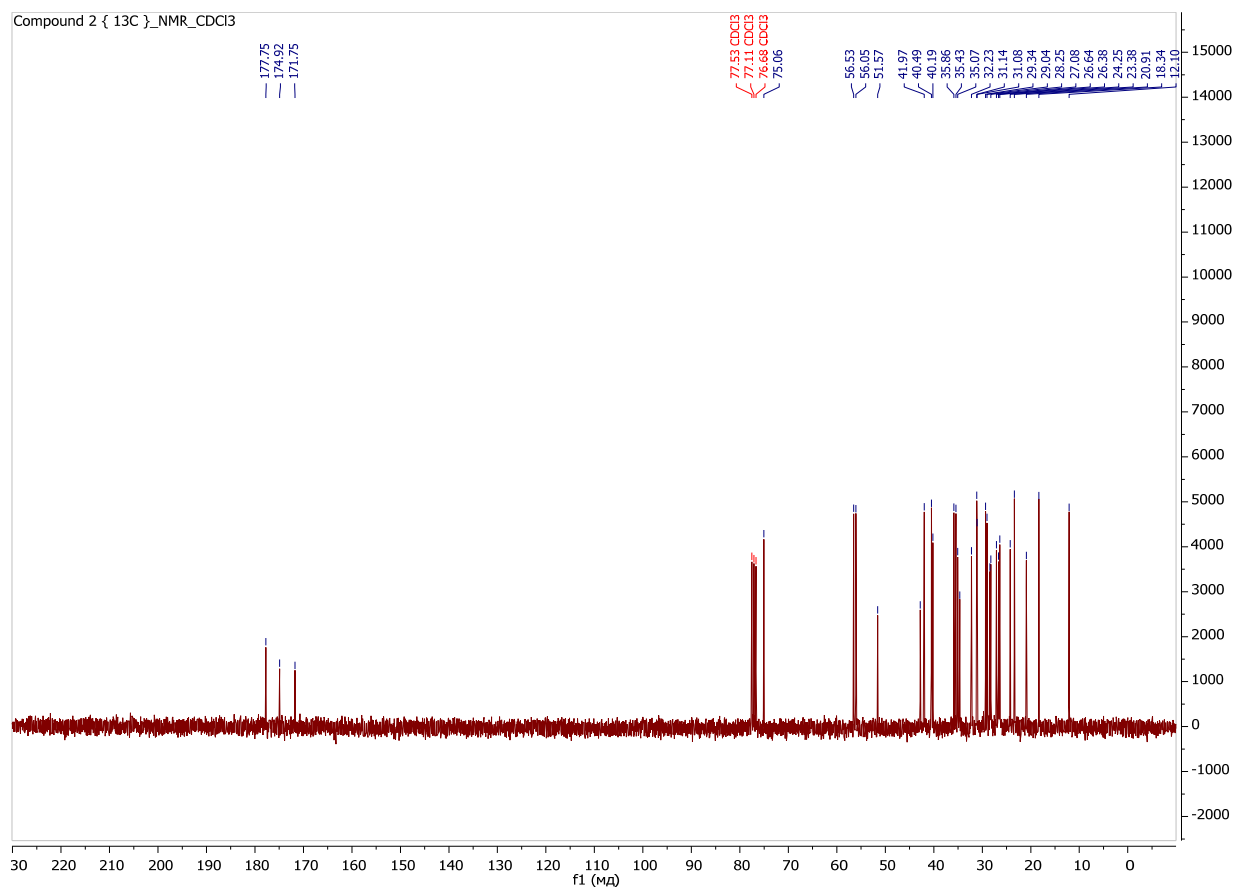
^1H NMR (300 MHz, Chloroform-*d*) δ 3.60 (s, 1H), 2.66 – 2.47 (m, 2H), 2.37 – 2.07 (m, 1H), 1.96 – 1.55 (m, 8H), 1.33 (s, 3H), 1.24 – 1.10 (m, 1H), 1.10 – 0.96 (m, 1H), 0.96 – 0.81 (m, 1H), 0.58 (s, 1H).

^{13}C NMR (75 MHz, Chloroform-*d*) δ 177.75, 174.92, 171.75, 75.06, 56.53, 56.05, 51.57, 42.81, 41.97, 40.49, 40.19, 35.86, 35.43, 35.07, 34.65, 32.23, 31.14, 31.08, 29.34, 29.04, 28.44, 28.25, 27.08, 26.64, 26.38, 24.25, 23.38, 20.91, 18.34, 12.10.

MS (ESI): 491.35 [M+H]. Calculated for C₂₉H₄₆O₆: 490.33.



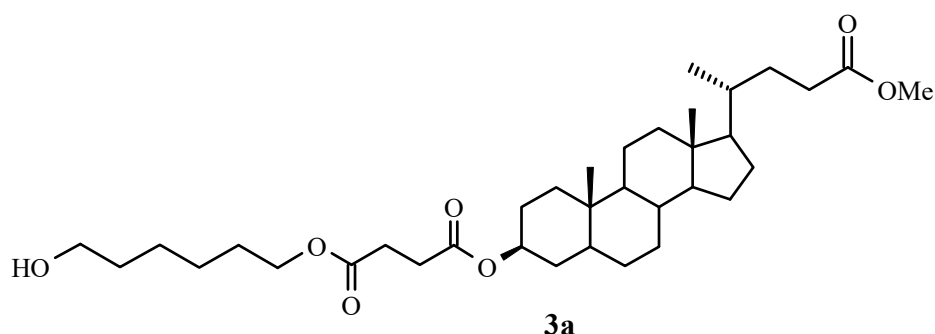
S1. ¹H NMR spectrum of compound 2.



S1. ^{13}C NMR spectrum of compound **2**.

General procedure for the synthesis of derivatives 3a, 3b.

To a solution of 3 α -((hydroxysuccinyl)oxy-5 β -cholan-24-oate **2** (466 mg, 0.95 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 °C, oxalyl chloride (1.62 mL, 17.1 mmol) was added. After stirring at room temperature overnight, the mixture was evaporated, and co-evaporated with dry hexane (3 \times 10 mL). The residue was dissolved in dry CH_2Cl_2 (20 mL), and then DIPEA (0.494 mL, 2.85 mmol) and 1,6-hexanediol (228 mg, 1.9 mmol) or 1,8-octanediol (277.4 mg, 1.9 mmol) were added at 0 °C. After stirring at room temperature for 24 h, the solvent was evaporated. The crude product was purified by column chromatography (silica gel) using petroleum ether/EtOAc = 1/1 as the elution solvent to afford lithocholic derivatives **3a, 3b**.

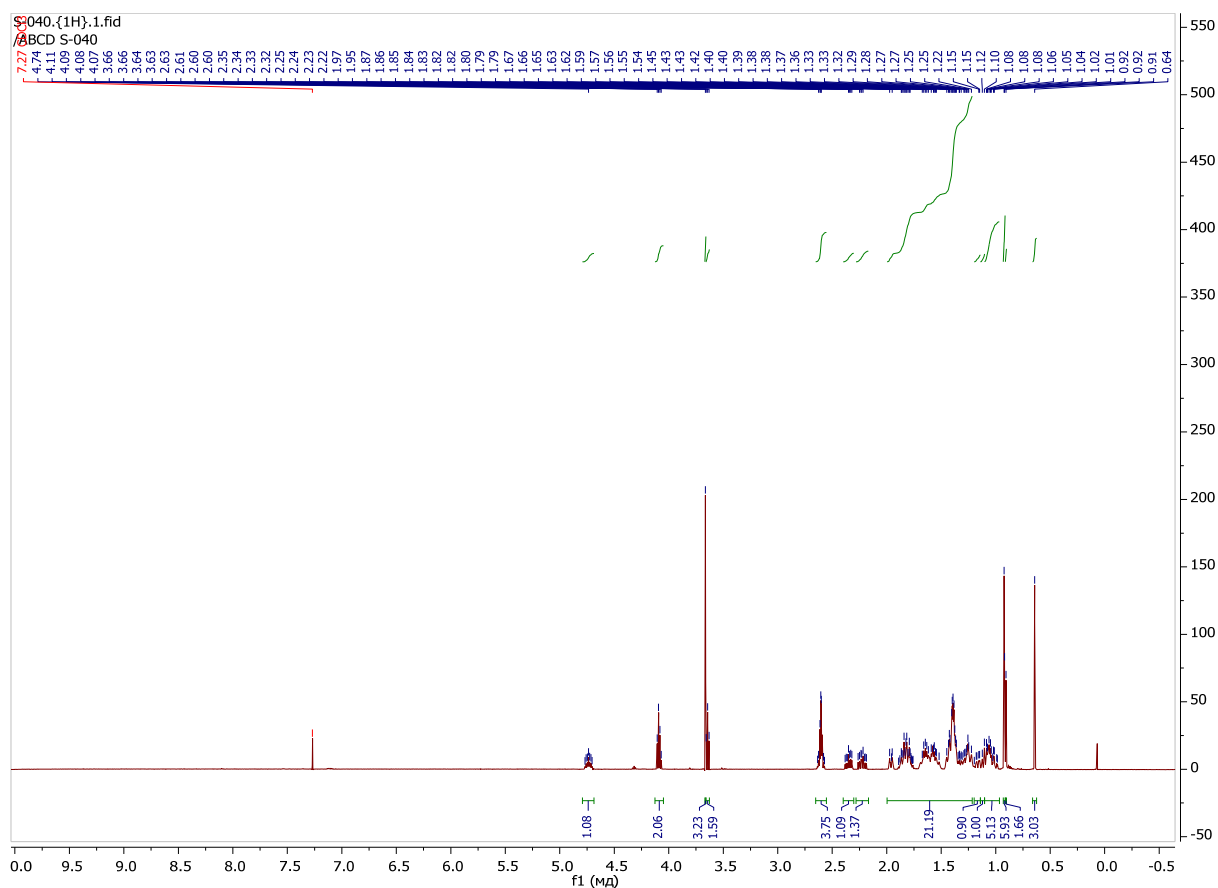


*6-Hydroxyhexyl 3 α -(hydroxysuccinyl)oxy-5 β -cholan-24-oate (**3a**).*

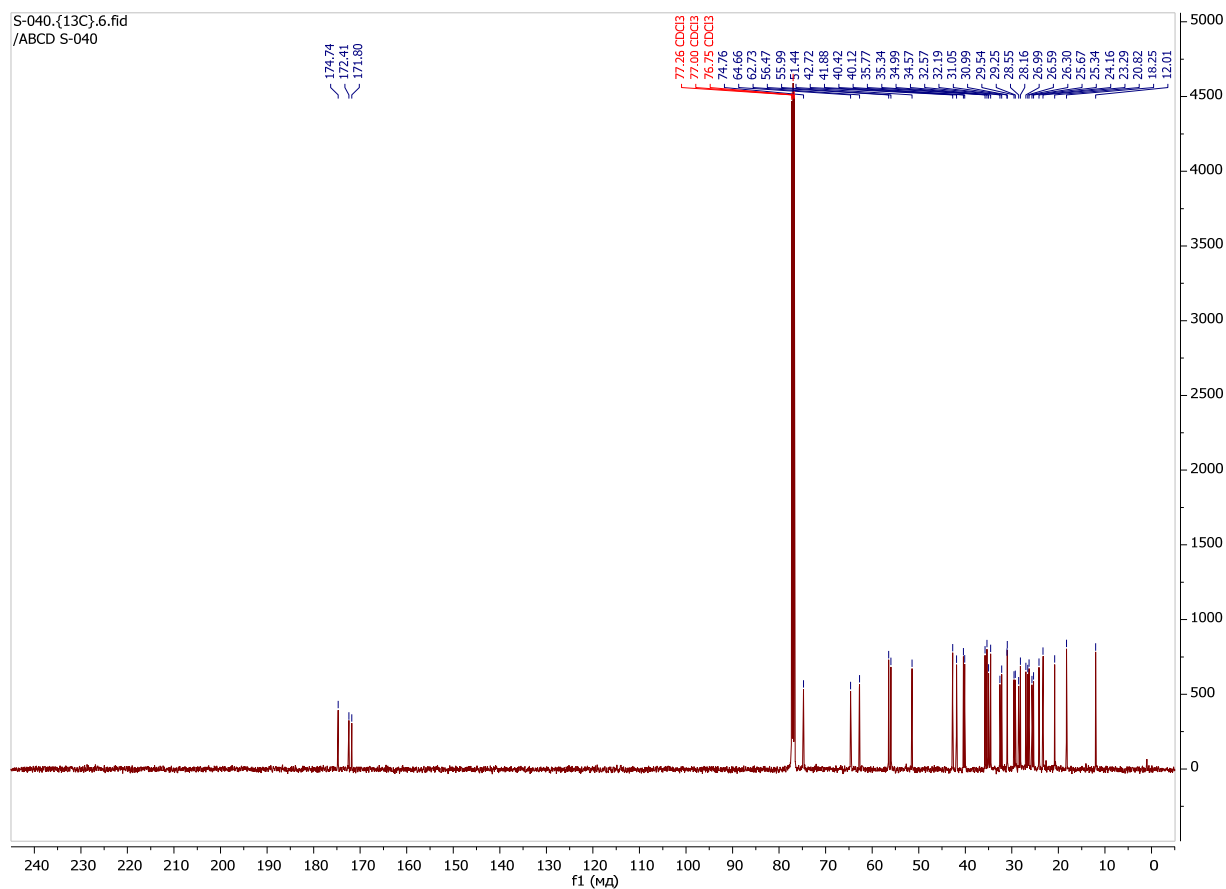
^1H NMR (500 MHz, Chloroform-*d*) δ 4.73 (t, J = 9.9, 4.9 Hz, 1H), 4.09 (t, J = 6.4 Hz, 2H), 3.66 (s, 3H), 3.64 (t, J = 6.5 Hz, 2H), 2.65 – 2.55 (m, 4H), 2.35 (t, J = 15.3, 10.2, 5.1 Hz, 1H), 2.28 – 2.17 (m, 1H), 2.00 – 1.22 (m, 21H), 1.16 (dd, J = 12.4, 3.5 Hz, 1H), 1.11 (d, J = 9.5 Hz, 1H), 1.10 – 0.97 (m, 5H), 0.92 (d, J = 2.4 Hz, 6H), 0.91 (s, 2H), 0.64 (s, 3H).

^{13}C NMR (126 MHz, Chloroform-*d*) δ 174.74, 172.41, 171.80, 77.26, 77.00, 76.75, 74.76, 64.66, 62.73, 56.47, 55.99, 51.44, 42.72, 41.88, 40.42, 40.12, 35.77, 35.34, 34.99, 34.57, 32.57, 32.19, 31.05, 30.99, 29.54, 29.25, 28.55, 28.16, 26.99, 26.59, 26.30, 25.67, 25.34, 24.16, 23.29, 20.82, 18.25, 12.01.

MS (ESI): 613.4075 [M+Na]. Calculated for $\text{C}_{35}\text{H}_{58}\text{O}_7\text{Na}$: 613.4080.



S2. ^1H NMR spectrum of compound **3a**.



S2. ^{13}C NMR spectrum of compound **3a**.

Display Report

Analysis Info

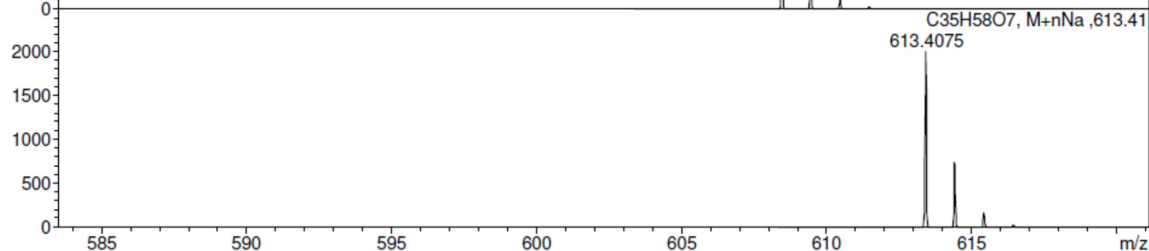
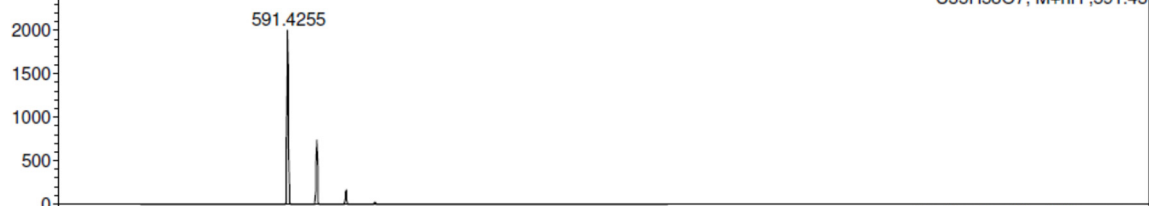
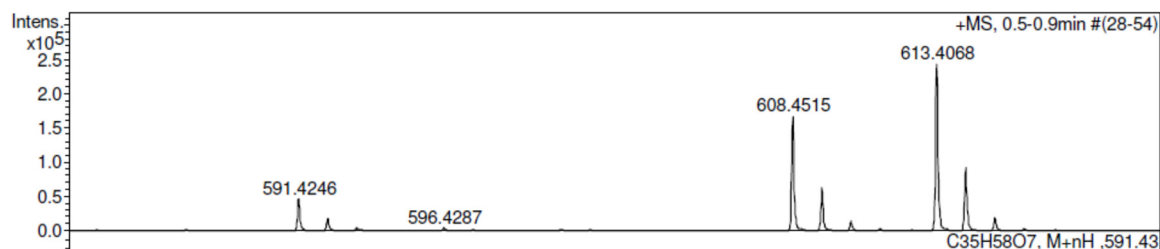
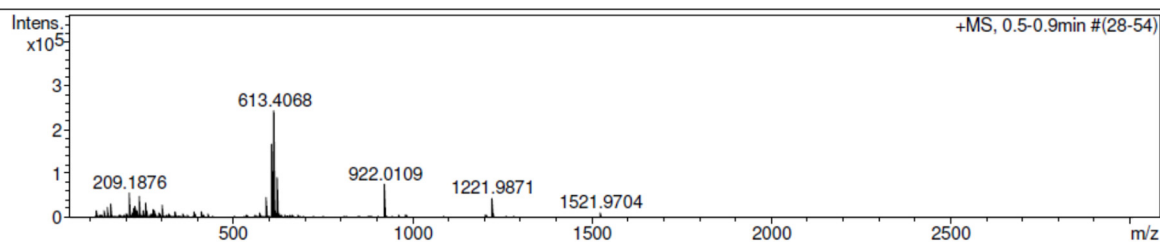
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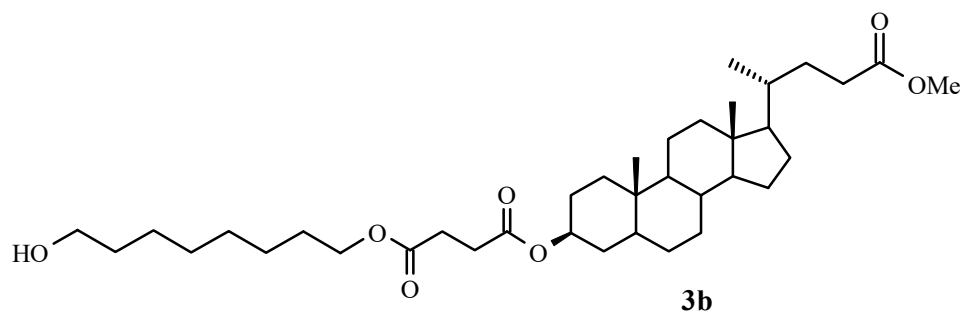
Operator BDAL@DE
Instrument / Ser# microTOF 10248

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S2. Mass spectrum of compound 3a.

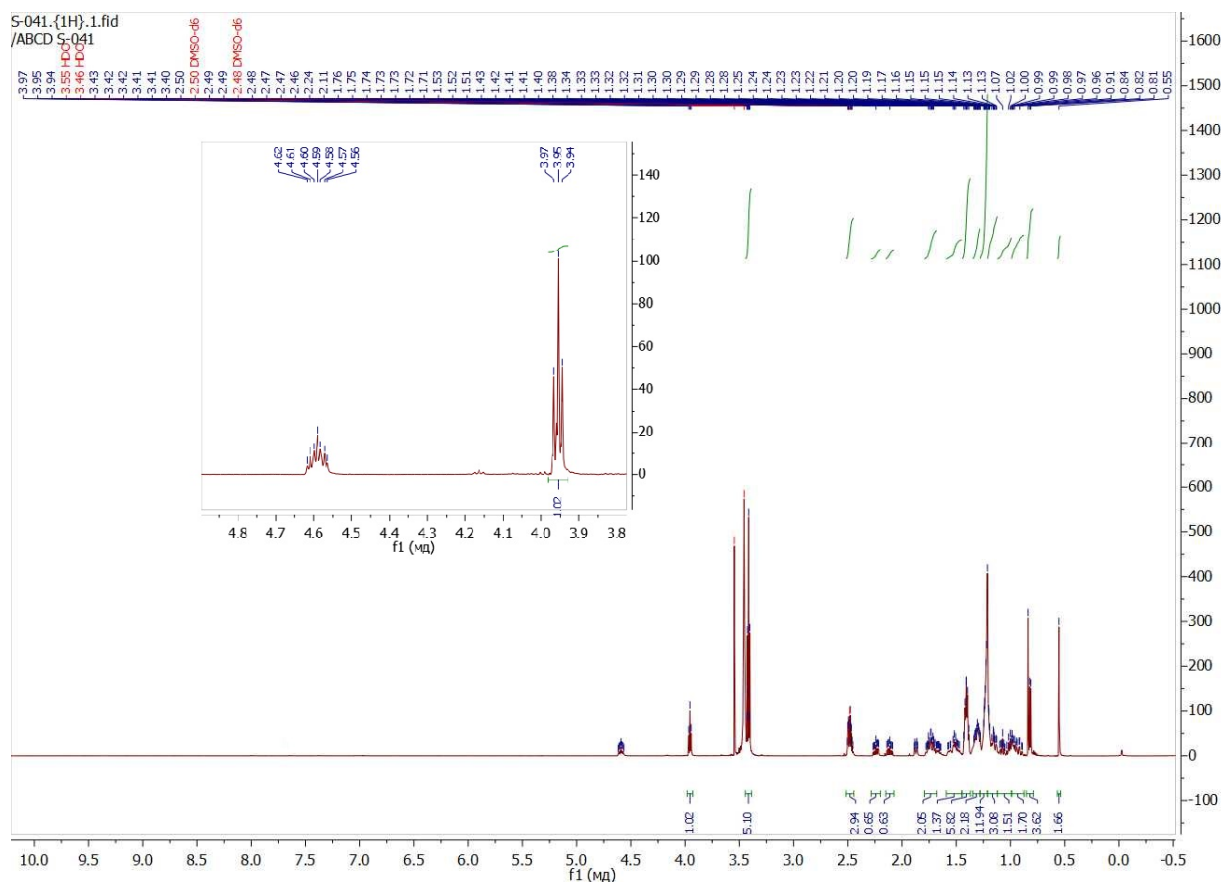


8-Hydroxyoctyl 3α-(hydroxysuccinyl)oxy-5β-cholan-24-oate (3b).

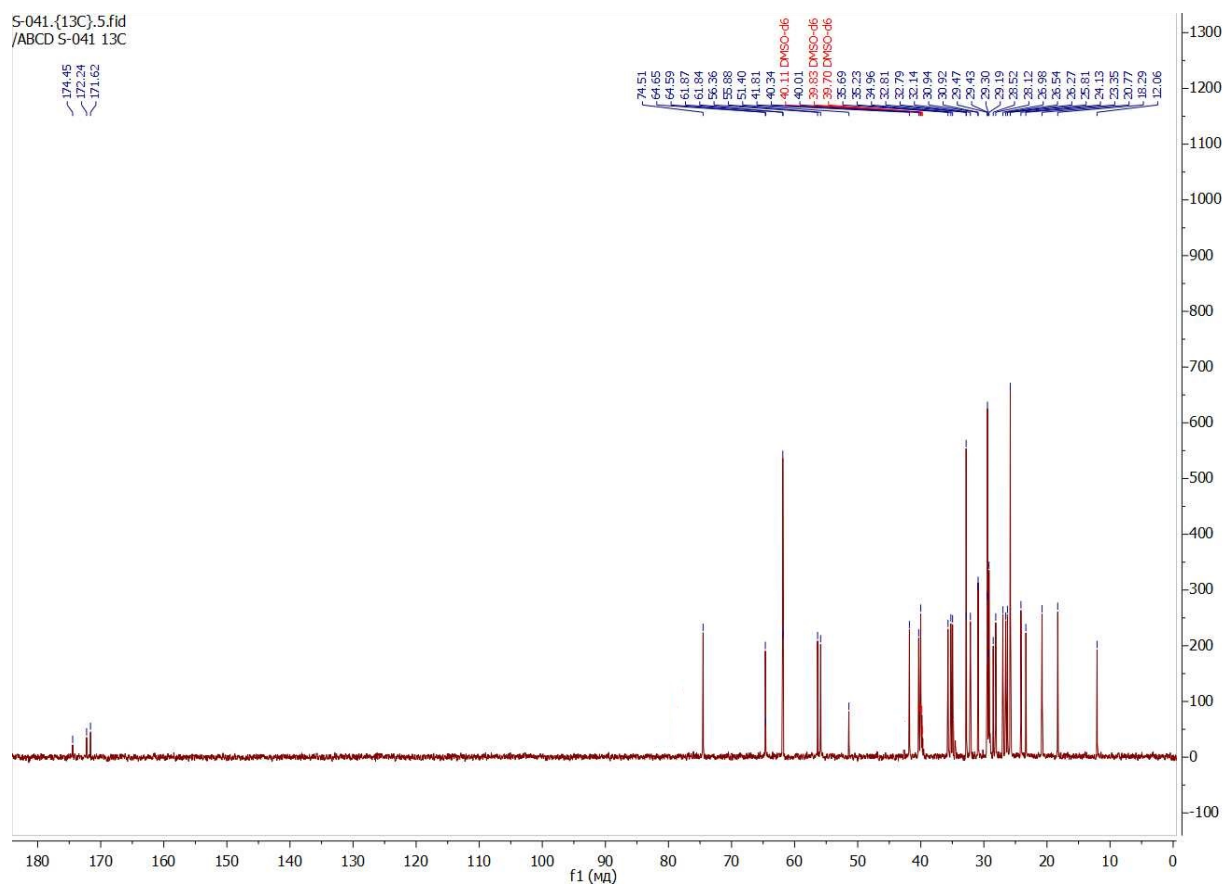
^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ 3.95 (t, $J = 6.7$ Hz, 1H), 3.44 – 3.39 (m, 6H), 2.53 – 2.43 (m, 4H), 2.28 – 2.20 (m, 1H), 2.17 – 2.07 (m, 1H), 1.87 (d, $J = 12.1$ Hz, 1H), 1.80 – 1.63 (m, 4H), 1.57 – 1.46 (m, 1H), 1.45 – 1.37 (m, 10H), 1.35 – 1.19 (m, 18H), 1.19 – 0.95 (m, 6H), 0.95 – 0.88 (m, 1H), 0.85 – 0.80 (m, 5H), 0.55 (s, 3H).

^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 174.45, 172.24, 171.62, 74.51, 64.65, 64.59, 61.87, 61.84, 56.36, 55.88, 51.40, 41.81, 40.34, 40.01, 35.69, 35.23, 34.96, 32.81, 32.79, 32.14, 30.94, 30.92, 29.47, 29.43, 29.30, 29.19, 28.52, 28.12, 26.98, 26.54, 26.27, 25.81, 24.13, 23.35, 20.77, 18.29, 12.06.

MS (ESI): 641.4396 $[\text{M}+\text{Na}]$. Calculated for $\text{C}_{37}\text{H}_{62}\text{O}_7\text{Na}$: 641.4393.



S3. ^1H NMR spectrum of compound **3b**.



S3. ^{13}C NMR spectrum of compound **3b**.

Display Report

Analysis Info

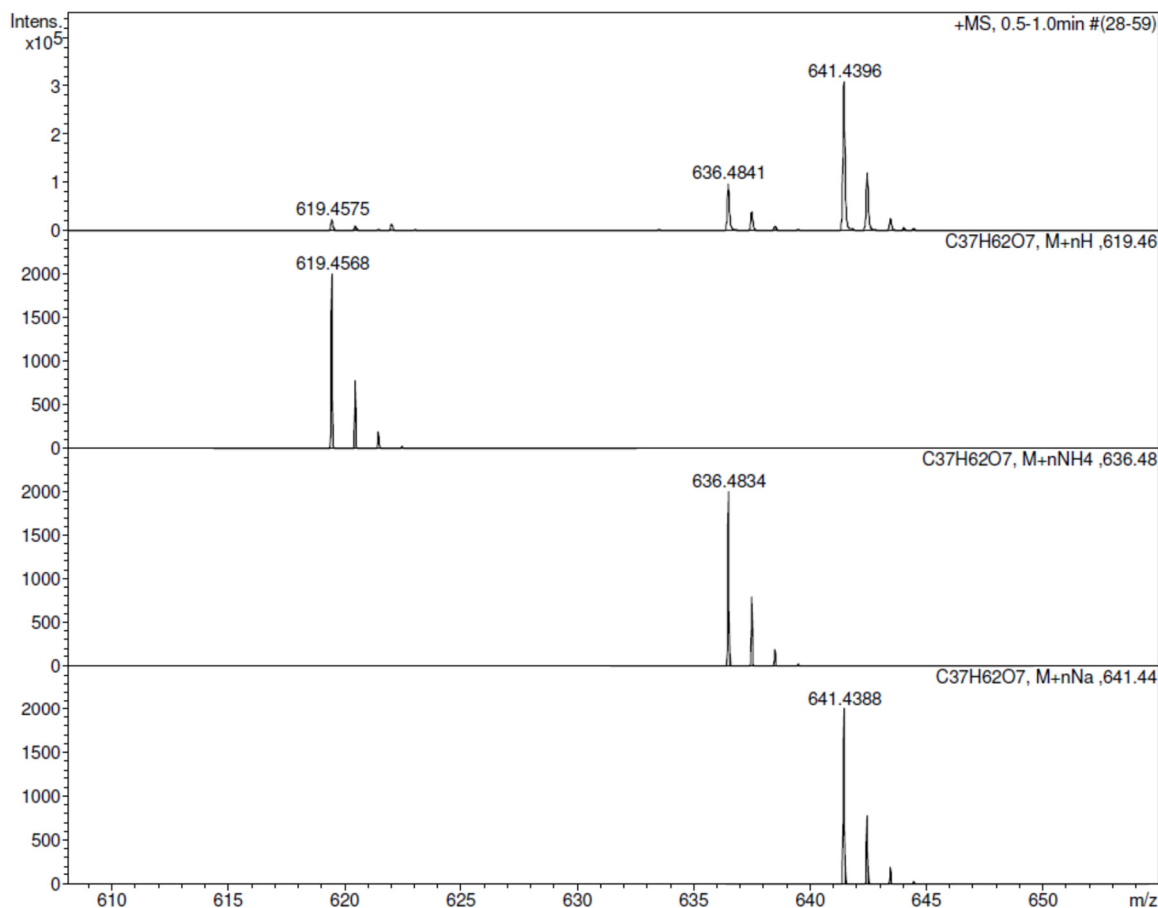
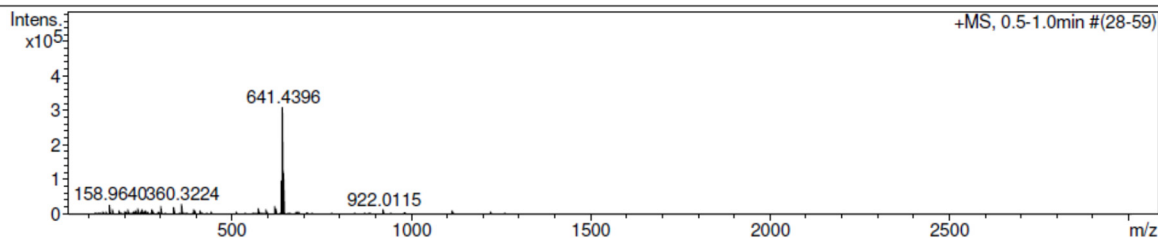
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Operator BDAL@DE
Instrument / Ser# microTOF 10248

Acquisition Parameter

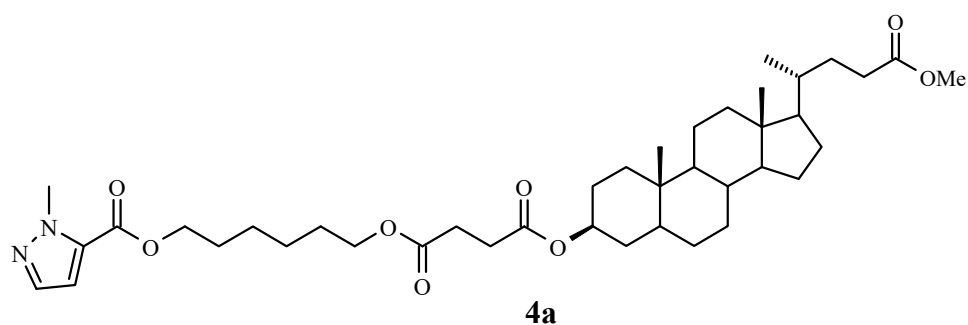
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S3. Mass spectrum of compound **3b**.

*General procedure for the synthesis of pyrazole derivatives of lithocholic acid **4a**, **4b**.*

To a solution of 6-hydroxyhexyl 3 α -(hydroxysuccinyl)oxy-5 β -cholan-24-oate **3a** (414 mg, 0.7 mmol) or 8-hydroxyoctyl 3 α -(hydroxysuccinyl)oxy-5 β -cholan-24-oate **3b** (433 mg, 0.7 mmol) in anhydrous dichloromethane (20 ml), 1-methyl-1*H*-pyrazole-5-carboxylic acid (130 mg, 1.1 mmol) was added, followed by *N*-[3-(methylamino)propyl]-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) (210 mg, 1.1 mmol) and 4-dimethylaminopyridine (DMAP) (43 mg, 0.35 mmol) under argon. After stirring at room temperature overnight, the mixture was diluted with H₂O (10 ml) and the CH₂Cl₂ layer was separated, dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (silica gel) using petroleum ether/EtOAc = 1/2 as the elution solvent to afford the pyrazole derivatives of lithocholic acid **4a**, **4b**.

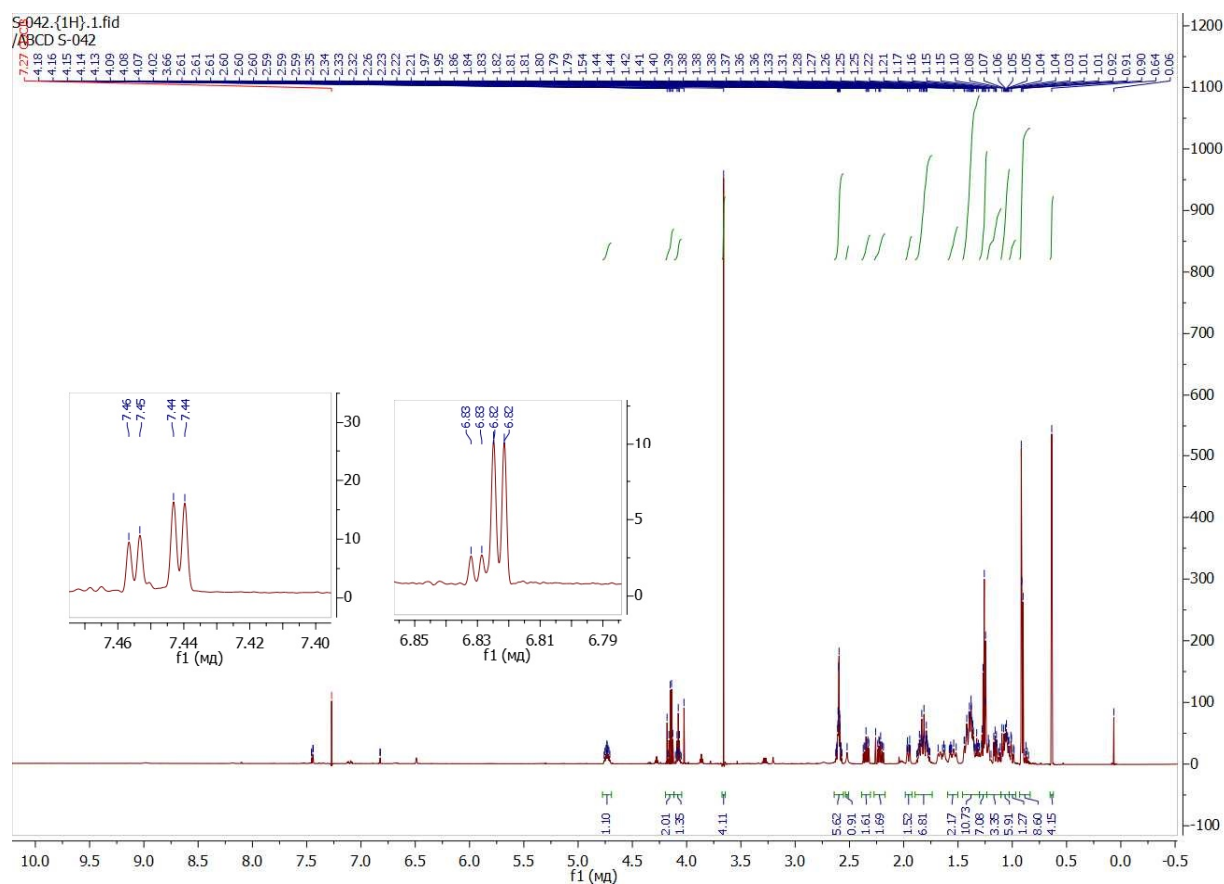


*6-{[(1-Methyl-1*H*-pyrazol-5-yl)carbonyl]oxy}hexyl(3 α)-(succinyl)oxy-5 β -cholan-24-oate (**4a**).*

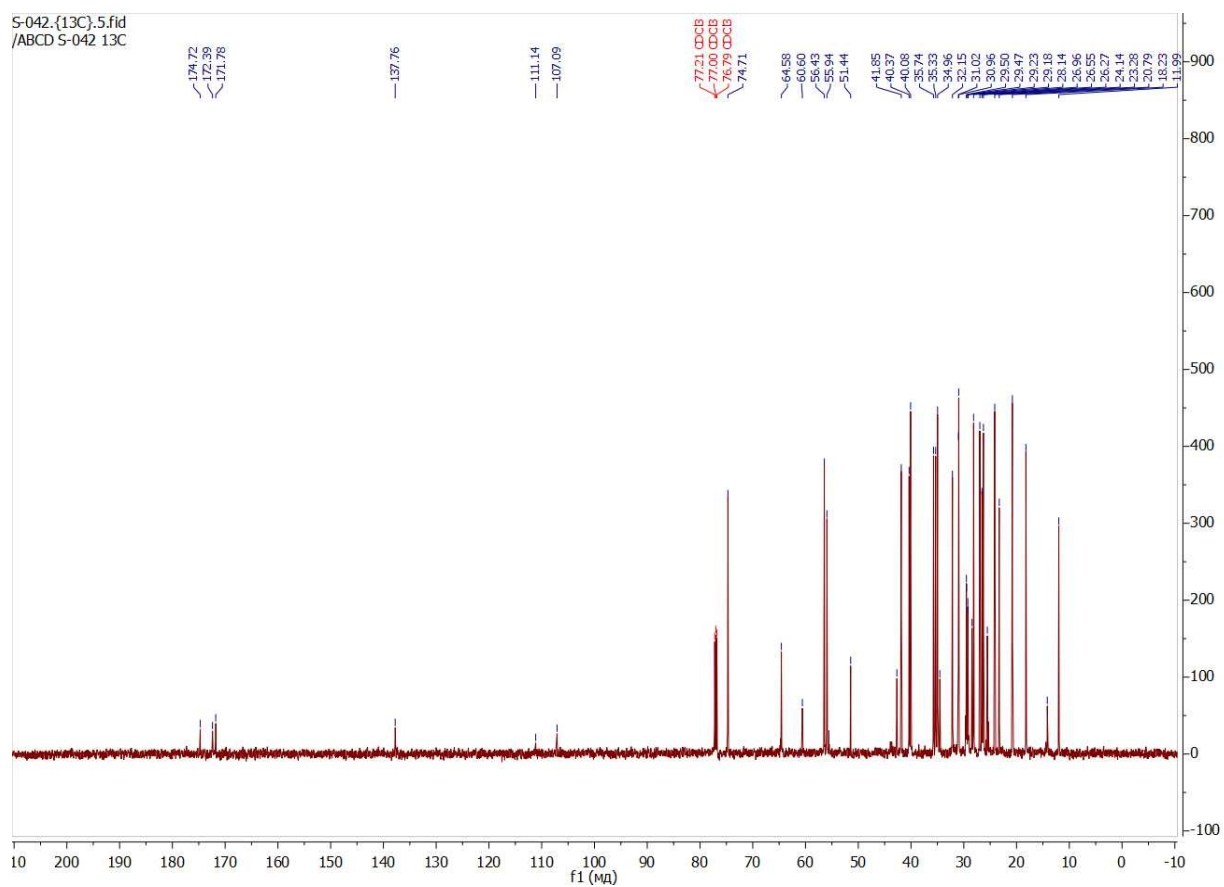
¹H NMR (600 MHz, Chloroform-*d*) δ 4.19 – 4.04 (m, 3H), 3.66 (s, 4H), 2.64 – 2.56 (m, 2H), 2.39 – 2.31 (m, 1H), 2.27 – 2.17 (m, 2H), 1.99 – 1.92 (m, 2H), 1.90 – 1.74 (m, 7H), 1.60 – 1.50 (m, 1H), 1.46 – 1.30 (m, 11H), 1.30 – 1.23 (m, 7H), 1.23 – 1.10 (m, 3H), 1.10 – 1.03 (m, 6H), 1.03 – 0.97 (m, 1H), 0.93 – 0.83 (m, 9H), 0.64 (s, 3H).

¹³C NMR (151 MHz, CDCl₃) δ 174.72, 172.39, 171.78, 137.76, 111.14, 107.09, 74.71, 64.58, 60.60, 56.43, 55.94, 51.44, 42.68, 41.85, 40.37, 40.08, 35.74, 35.33, 34.96, 34.54, 32.15, 31.02, 30.96, 29.50, 29.47, 29.23, 29.18, 28.45, 28.14, 26.96, 26.55, 26.27, 25.54, 24.14, 23.28, 20.79, 18.23, 14.18, 11.99.

MS (ESI): 721.4405 [M+Na]. Calculated for C₄₀H₆₂N₂O₈Na: 721.4404.



S4. ^1H NMR spectrum of compound **4a**.



S4. ^{13}C NMR spectrum of compound **4a**.

Display Report

Analysis Info

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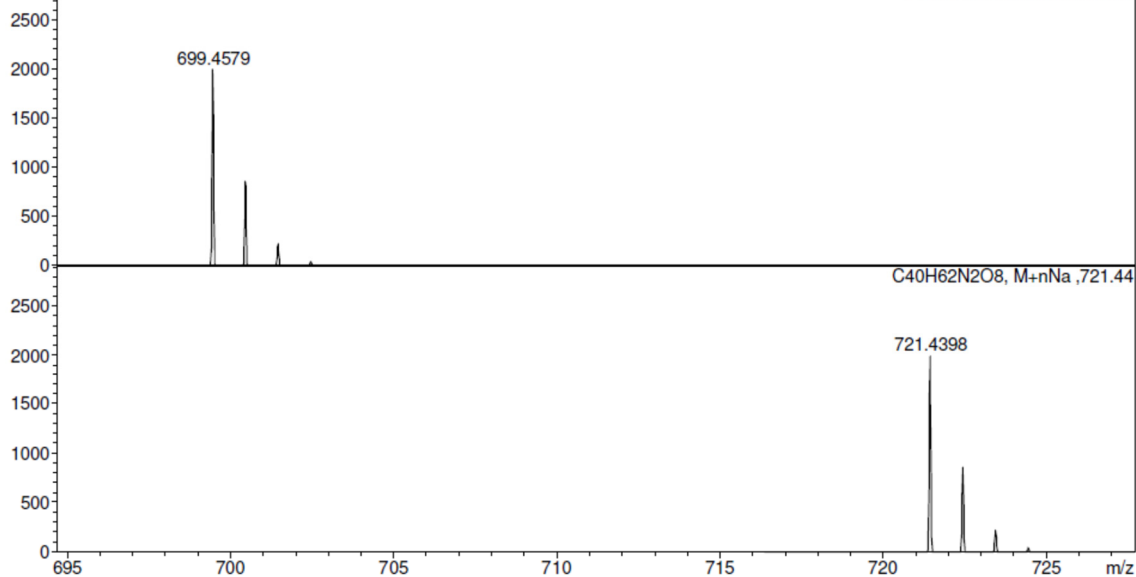
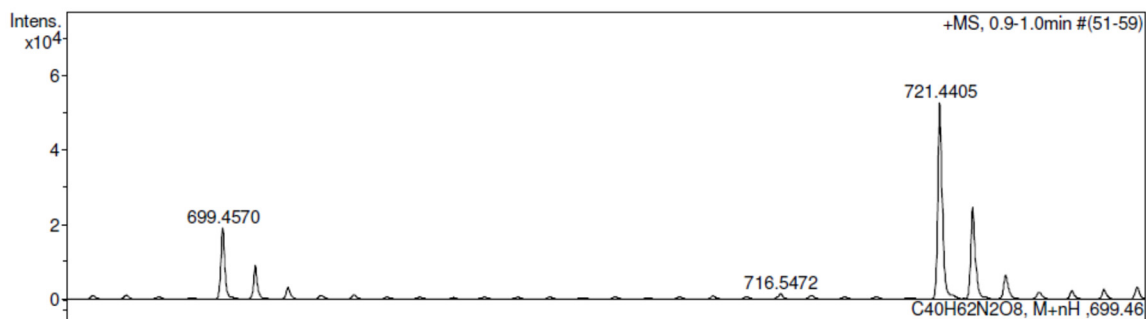
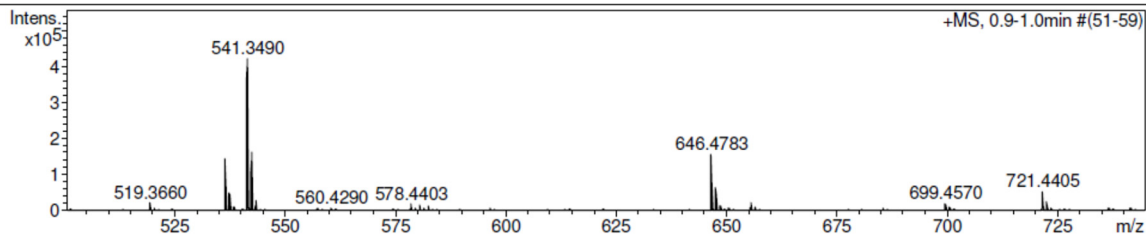
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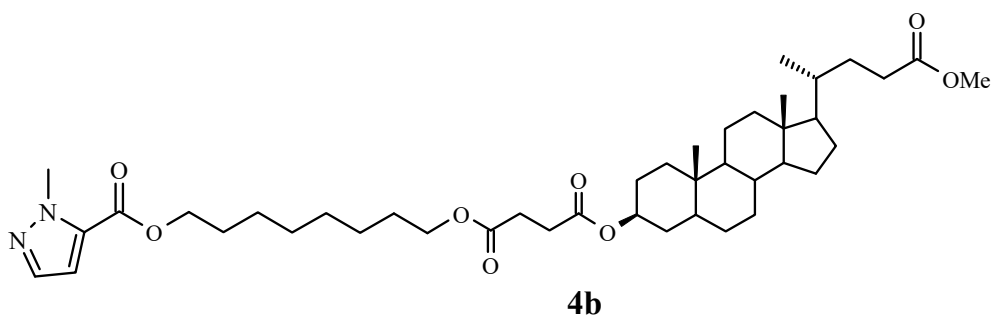
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S4. Mass spectrum of compound 4a.

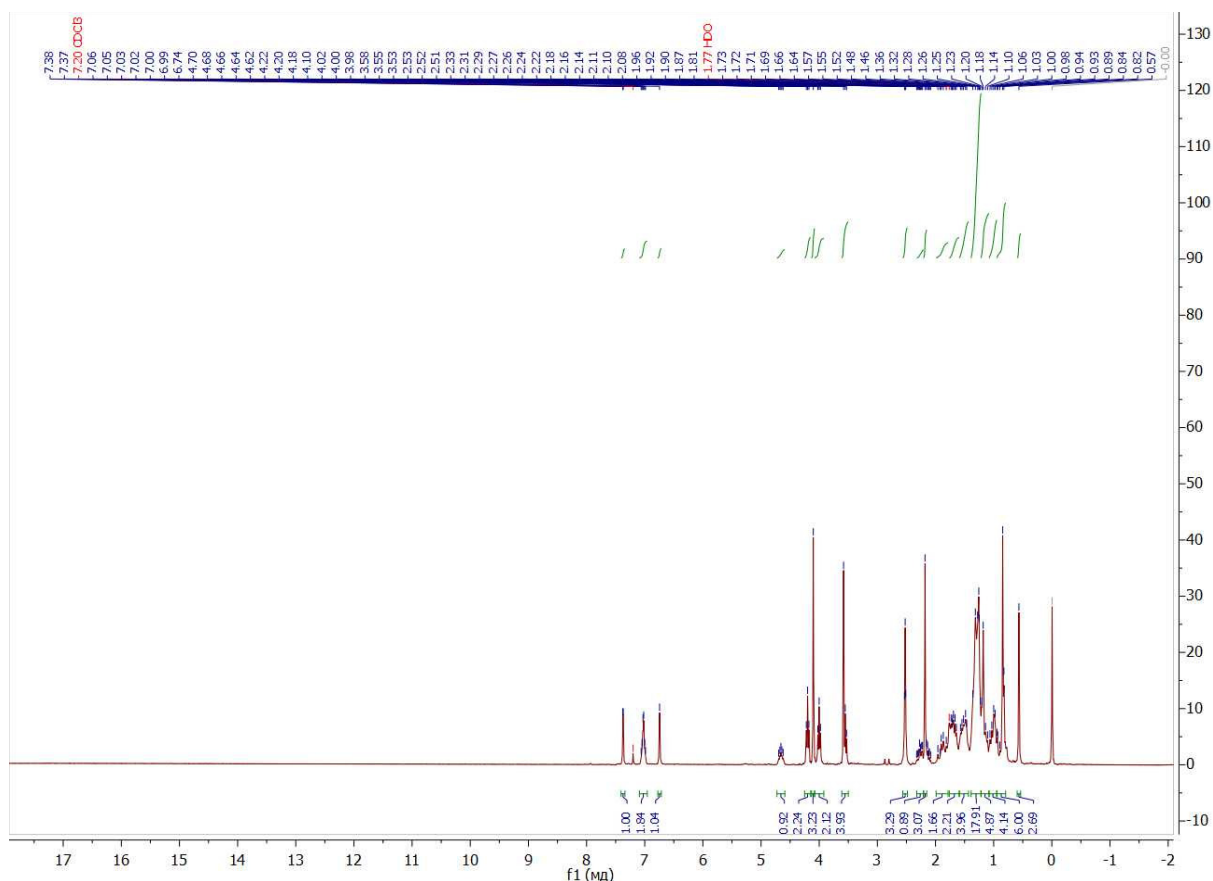


8- $\{[(1\text{-Methyl-1H-pyrazol-5-yl})\text{carbonyl}]\text{oxy}\}\text{octyl}(3\alpha)\text{-(succinyl)oxy-5}\beta\text{-cholan-24-oate (4b)}$.

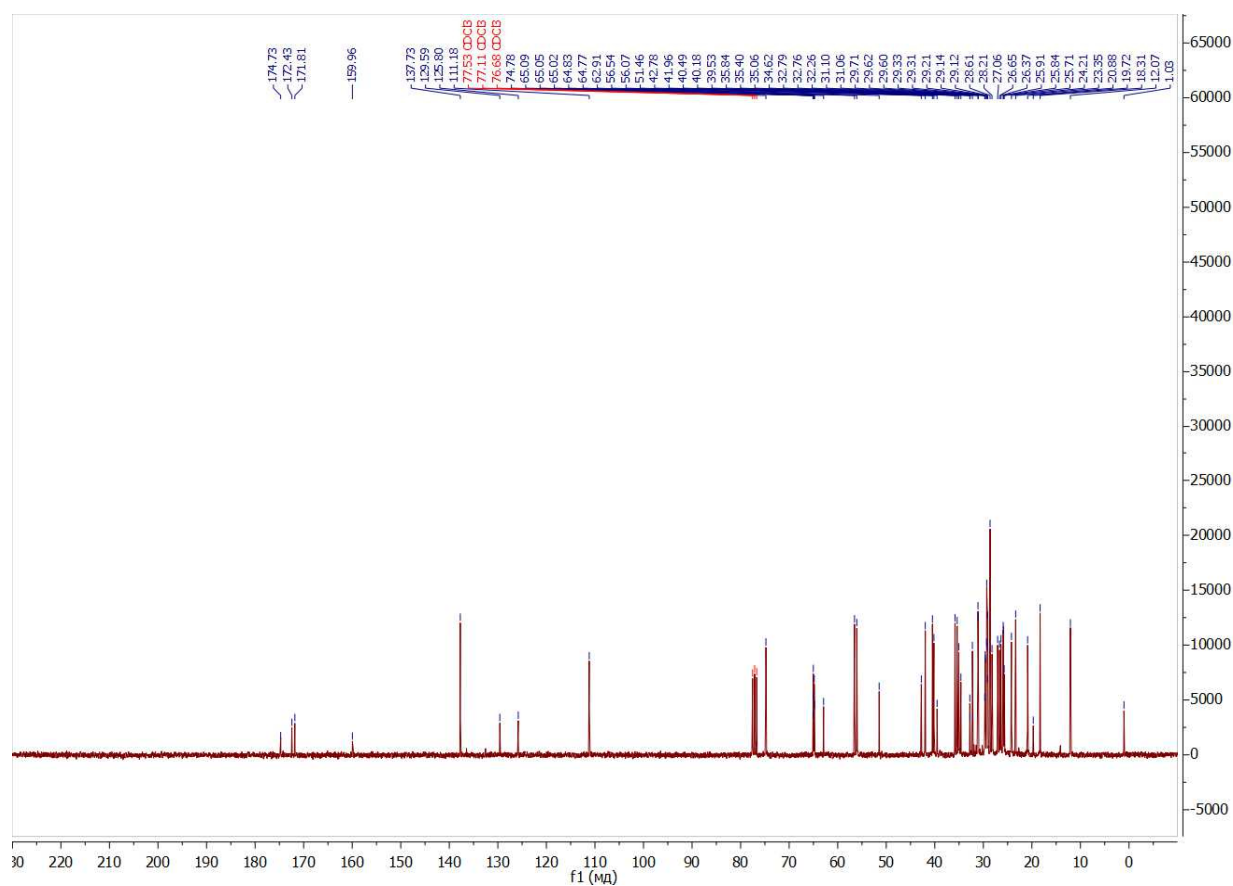
^1H NMR (300 MHz, Chloroform-*d*) δ 7.37 (d, J = 2.1 Hz, 1H), 7.09 – 6.96 (m, 2H), 6.74 (s, 1H), 4.73 – 4.59 (m, 1H), 4.20 (t, J = 6.7 Hz, 2H), 4.10 (s, 3H), 4.00 (t, J = 6.8 Hz, 2H), 3.61 – 3.50 (m, 4H), 2.53 (s, 3H), 2.32 – 2.21 (m, 1H), 2.18 (s, 3H), 1.99 – 1.78 (m, 2H), 1.76 – 1.59 (m, 2H), 1.59 – 1.43 (m, 4H), 1.39 – 1.22 (m, 18H), 1.16 (q, J = 10.9, 8.7 Hz, 5H), 1.08 – 0.95 (m, 4H), 0.95 – 0.79 (m, 6H), 0.57 (s, 3H).

^{13}C NMR (75 MHz, CDCl_3) δ 174.73, 172.43, 171.81, 159.96, 137.73, 129.59, 125.80, 111.18, 74.78, 65.09, 65.05, 65.02, 64.83, 64.77, 62.91, 56.54, 56.07, 51.46, 42.78, 41.96, 40.49, 40.18, 39.53, 35.84, 35.40, 35.06, 34.62, 32.79, 32.76, 32.26, 31.10, 31.06, 29.71, 29.62, 29.60, 29.33, 29.31, 29.21, 29.14, 29.12, 28.61, 28.21, 27.06, 26.65, 26.37, 25.91, 25.84, 25.71, 24.21, 23.35, 20.88, 19.72, 18.31, 12.07.

MS (ESI): 749.4715 $[\text{M}+\text{Na}]$. Calculated for $\text{C}_{42}\text{H}_{66}\text{N}_2\text{O}_8\text{Na}$: 749.4716.



S5. ^1H NMR spectrum of compound **4b**.



S5. ^{13}C NMR spectrum of compound **4b**.

Display Report

Analysis Info

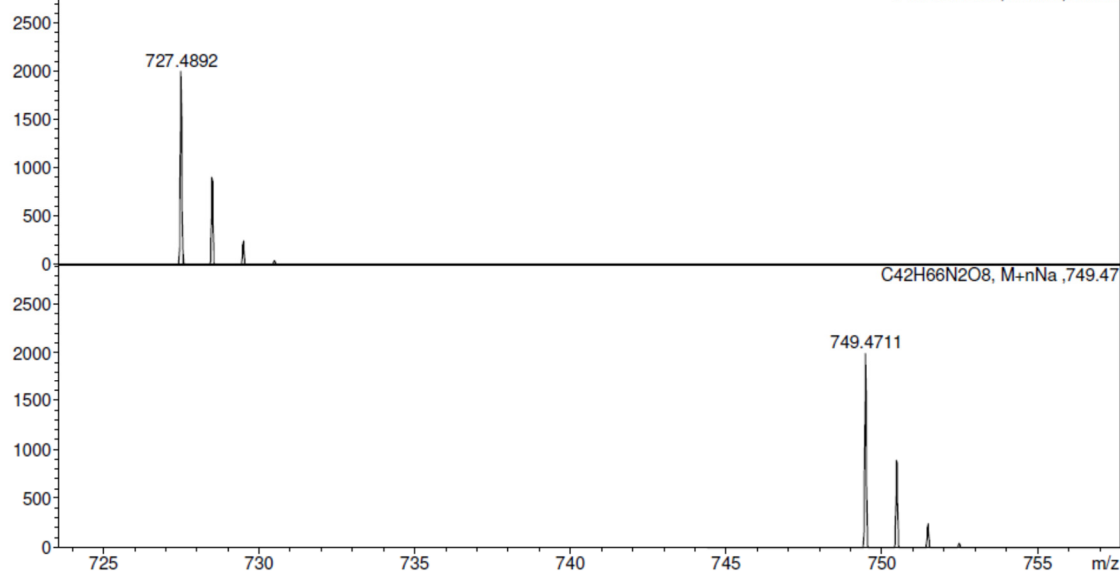
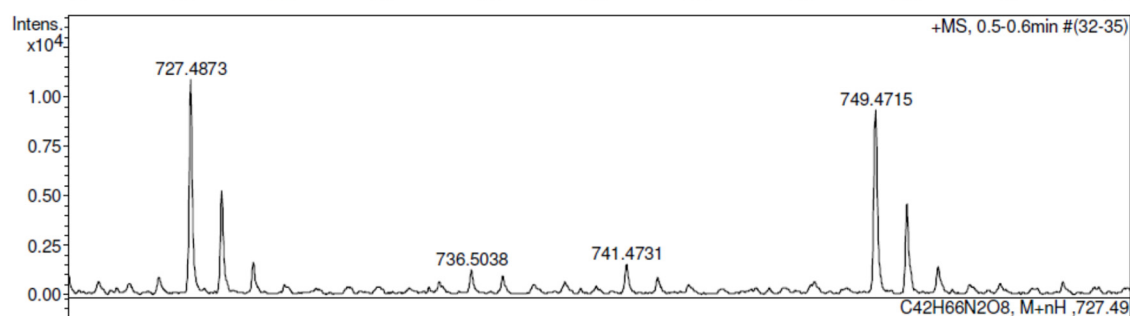
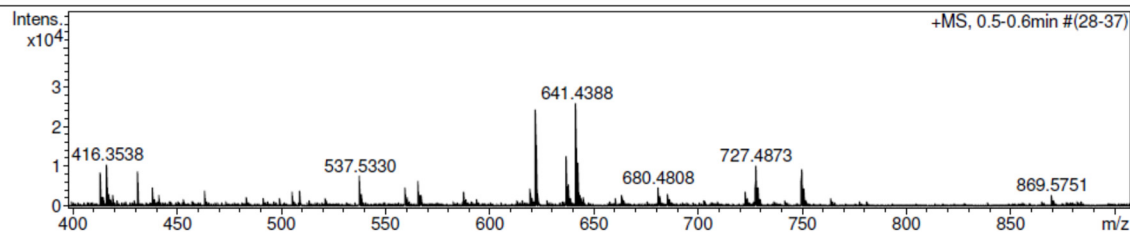
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Operator BDAL@DE
Instrument / Ser# micrOTOF 10248

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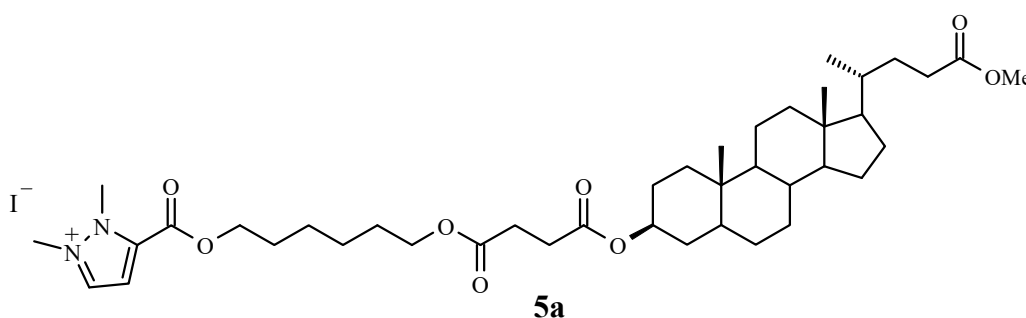


S5. Mass spectrum of compound 4b.

*General procedure for the synthesis of ionic compounds based on pyrazole derivatives of lithocholic acid **5a**, **5b**.*

To a solution of pyrazole derivatives of lithocholic acid **4a** (421 mg, 0.5 mmol) or **4b** (435 mg, 0.5 mmol), respectively) in anhydrous dichloromethane (10 ml), methyl iodide (CH₃I) (71 mg, 0.5 mmol) was added under argon. The mixture was stirred at room temperature overnight. After stirring, the solvent was evaporated. The crude product was purified by column chromatography (silica gel) using petroleum ether/EtOAc = 1/1 as the elution solvent to afford ionic compounds of pyrazole derivatives of lithocholic acid **5a**, **5b**.

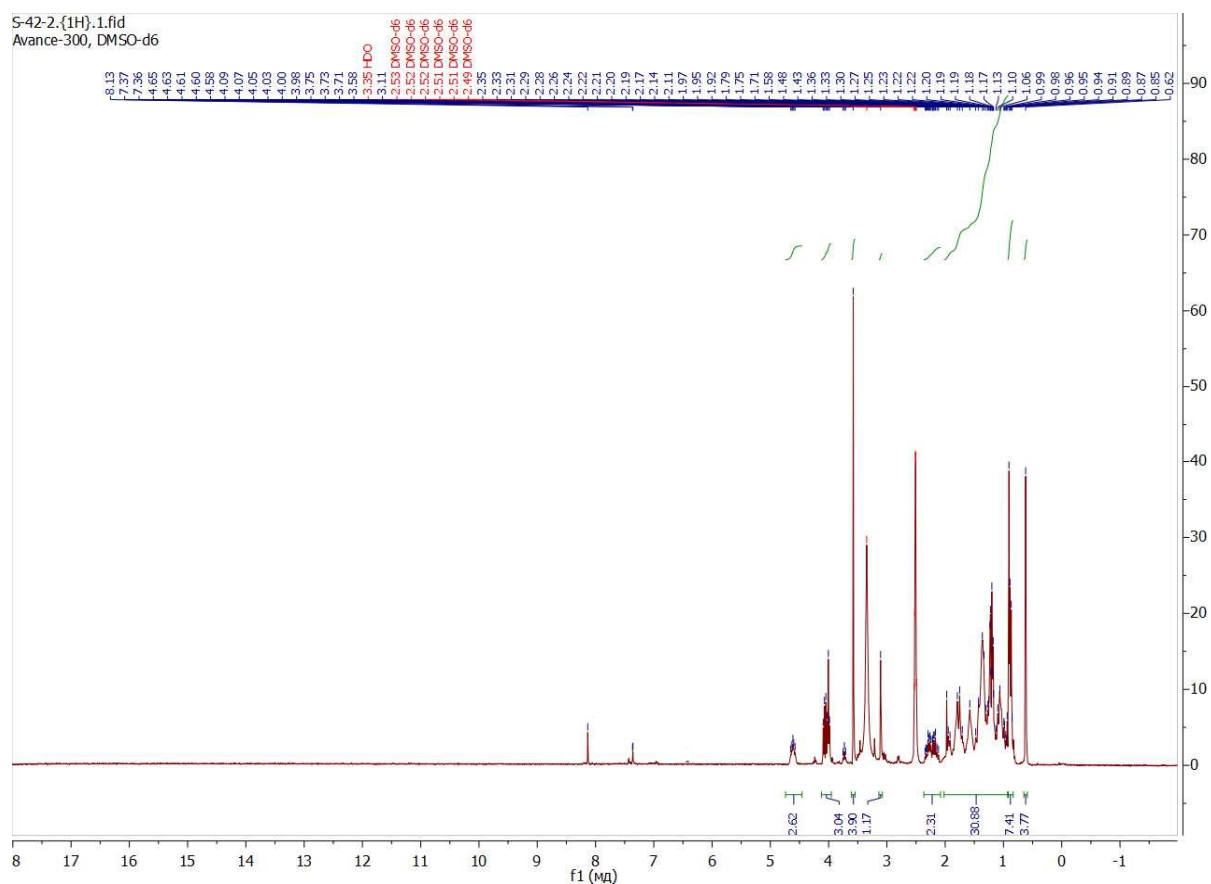
*6-{[(1,2-dimethyl-1H-pyrazolyliodide)carbonyl]oxy}hexyl(3 α)-(succinyl)oxy-5 β -cholan-24-oate (**5a**).*



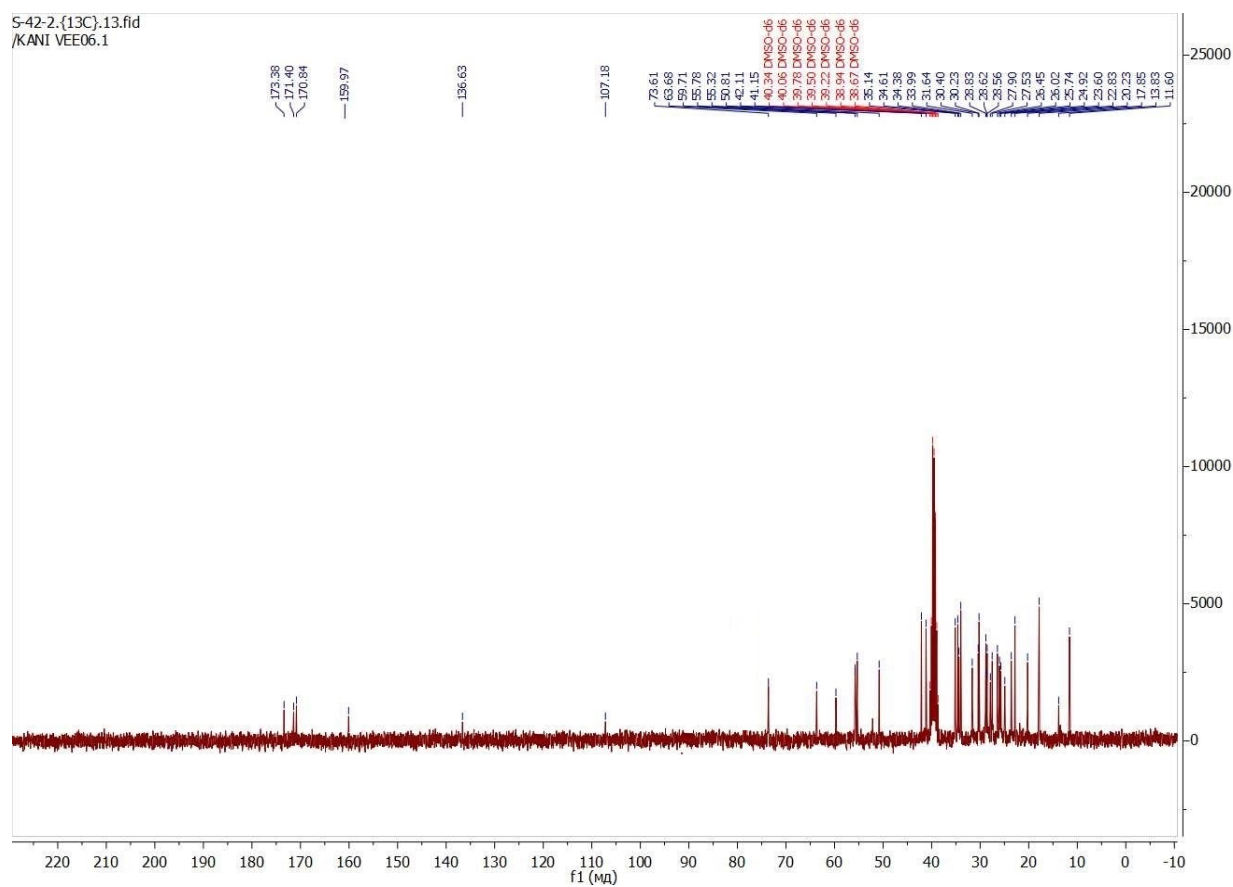
¹H NMR (300 MHz, DMSO-*d*₆) δ 4.74 – 4.46 (m, 3H), 4.12 – 3.95 (m, 3H), 3.58 (s, 4H), 3.11 (s, 1H), 2.37 – 2.08 (m, 2H), 2.02 – 0.92 (m, 31H), 0.93 – 0.83 (m, 7H), 0.62 (s, 4H).

¹³C NMR (75 MHz, DMSO-*d*₆) δ 173.38, 171.40, 170.84, 136.63, 73.61, 63.68, 59.71, 55.78, 55.32, 50.81, 42.11, 41.15, 40.34, 40.06, 39.78, 39.50, 39.22, 38.94, 38.67, 35.14, 34.61, 34.38, 33.99, 31.64, 30.40, 30.23, 28.83, 28.62, 28.56, 27.90, 27.53, 26.45, 26.02, 25.74, 24.92, 23.60, 22.83, 20.23, 17.85, 13.83, 11.60.

MS (ESI): C₄₁H₆₅N₂O₈: 713.4734 [M]. Calculated for C₄₁H₆₅N₂O₈: 713.4740.



S6. ^1H NMR spectrum of compound **5a**.



S6. ^{13}C NMR spectrum of compound **5a**.

Display Report

Analysis Info

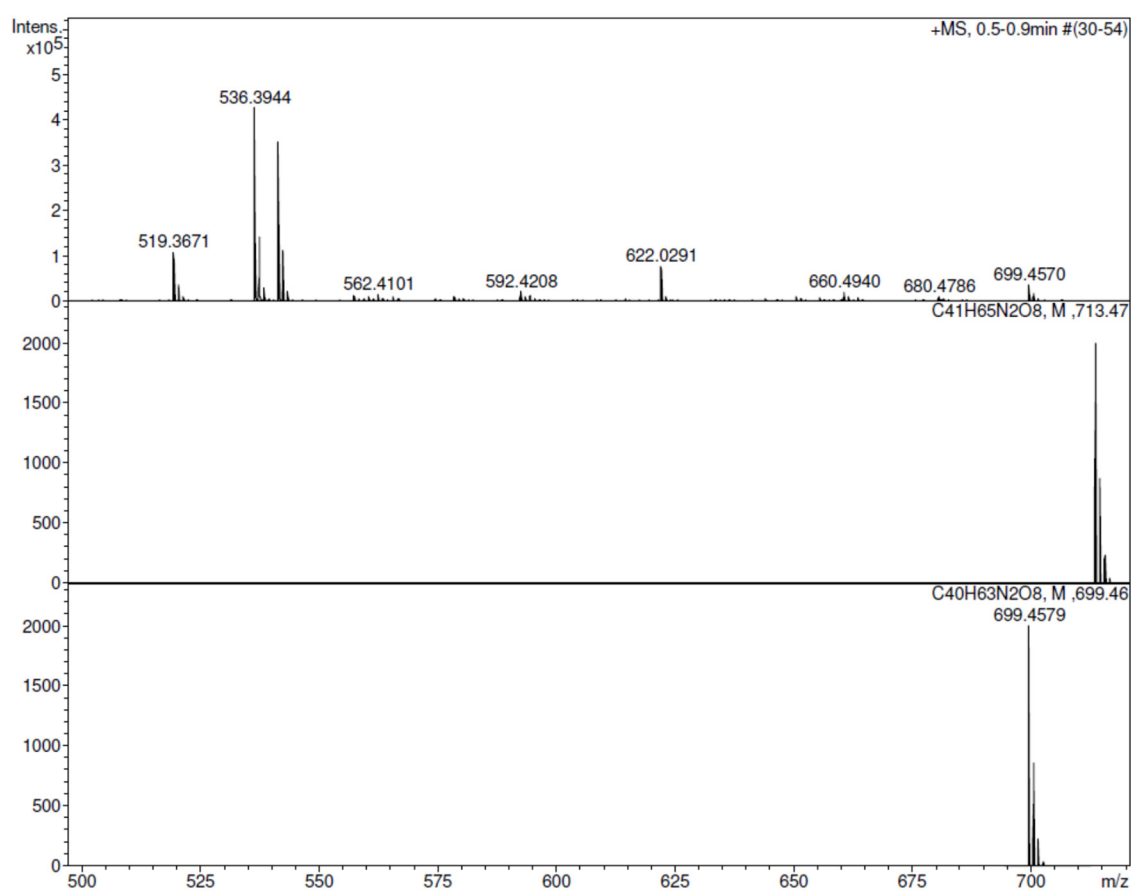
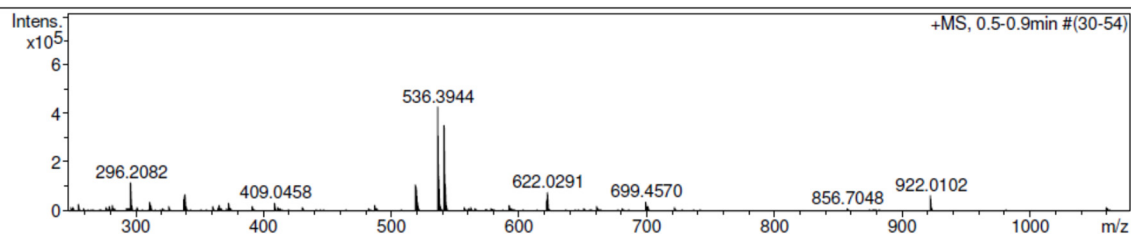
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Operator BDAL@DE
Instrument / Ser# micrOTOF 10248

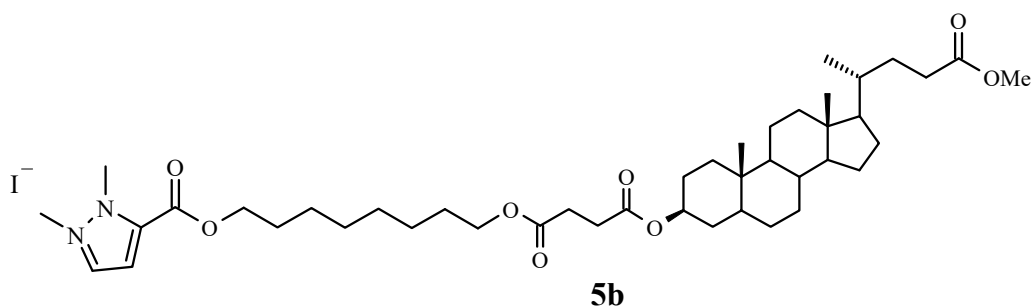
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S6. Mass spectrum of compound 5a.

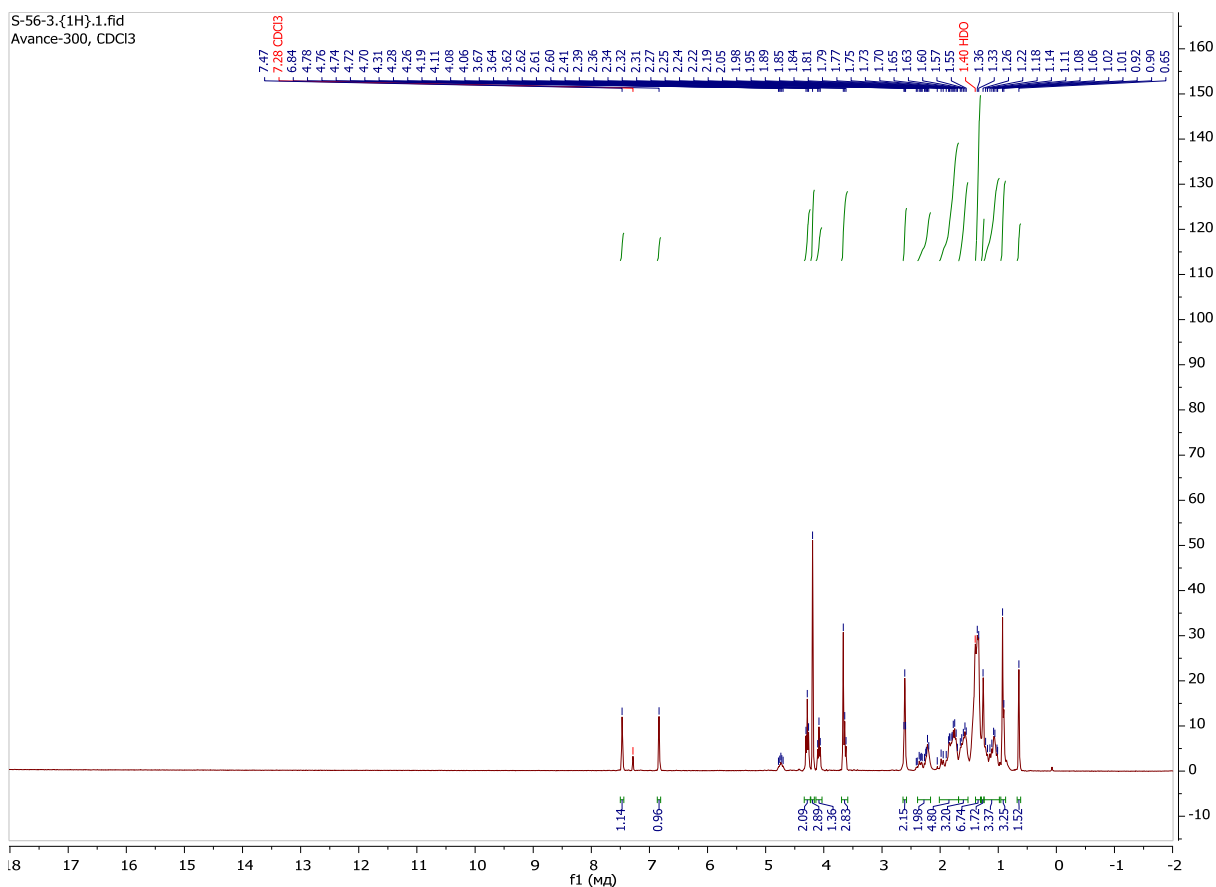
6- $\{[(1,2\text{-dimethyl-}1H\text{-pyrazolyl}iodide)carbonyl]oxy\}octyl(3\alpha)\text{-(succinyl)oxy-}5\beta\text{-cholan-}24\text{-oate}$ (**5b**).



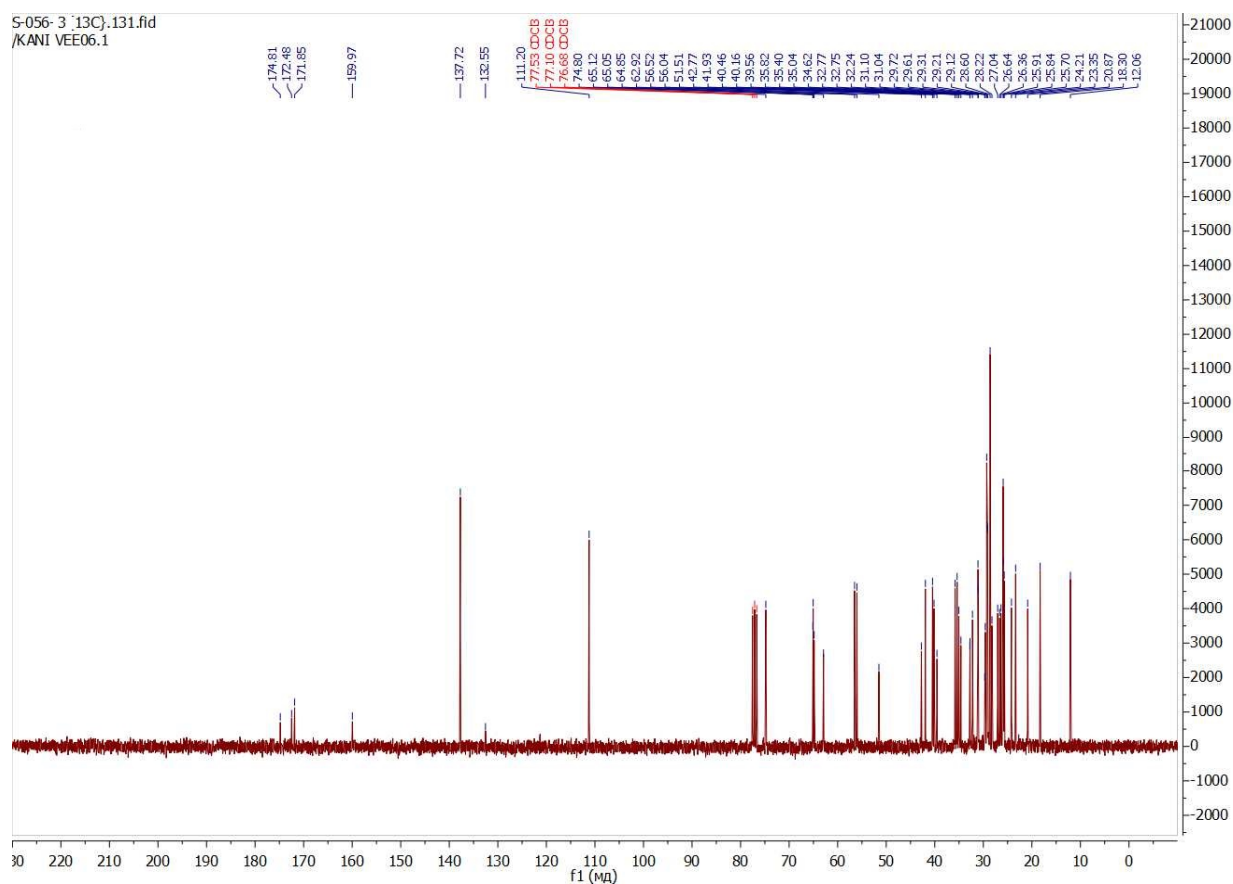
^1H NMR (300 MHz, Chloroform-*d*) δ 4.81 – 4.67 (m, 1H), 4.28 (t, J = 6.7 Hz, 4H), 4.19 (s, 6H), 4.08 (t, J = 6.8 Hz, 3H), 3.70 – 3.59 (m, 6H), 2.64 – 2.58 (m, 4H), 2.41 – 2.16 (m, 4H), 2.01 – 1.81 (m, 4H), 1.81 – 1.68 (m, 6H), 1.68 – 1.52 (m, 6H), 1.39 – 1.30 (m, 13H), 1.29 – 1.19 (m, 4H), 1.19 – 0.98 (m, 6H), 0.91 (d, J = 6.4 Hz, 6H), 0.65 (s, 3H).

^{13}C NMR (75 MHz, CDCl_3) δ 174.81, 172.49, 171.87, 159.95, 137.69, 132.55, 111.21, 74.81, 65.16, 65.09, 64.86, 62.95, 56.53, 56.05, 51.52, 42.78, 41.95, 40.47, 40.17, 39.56, 35.83, 35.41, 35.06, 34.63, 32.76, 32.25, 31.11, 31.05, 29.73, 29.62, 29.32, 29.21, 29.14, 28.61, 28.23, 27.05, 26.65, 26.37, 25.92, 25.85, 25.71, 24.22, 23.36, 20.88, 18.31, 12.08.

MS (ESI): $\text{C}_{43}\text{H}_{69}\text{N}_2\text{O}_8$: 741.5048 [M]. Calculated for $\text{C}_{43}\text{H}_{69}\text{N}_2\text{O}_8$: 741.5053.



S7. ^1H NMR spectrum of compound **5b**.



S7. ¹³C NMR spectrum of compound **5b**.

Display Report

Analysis Info

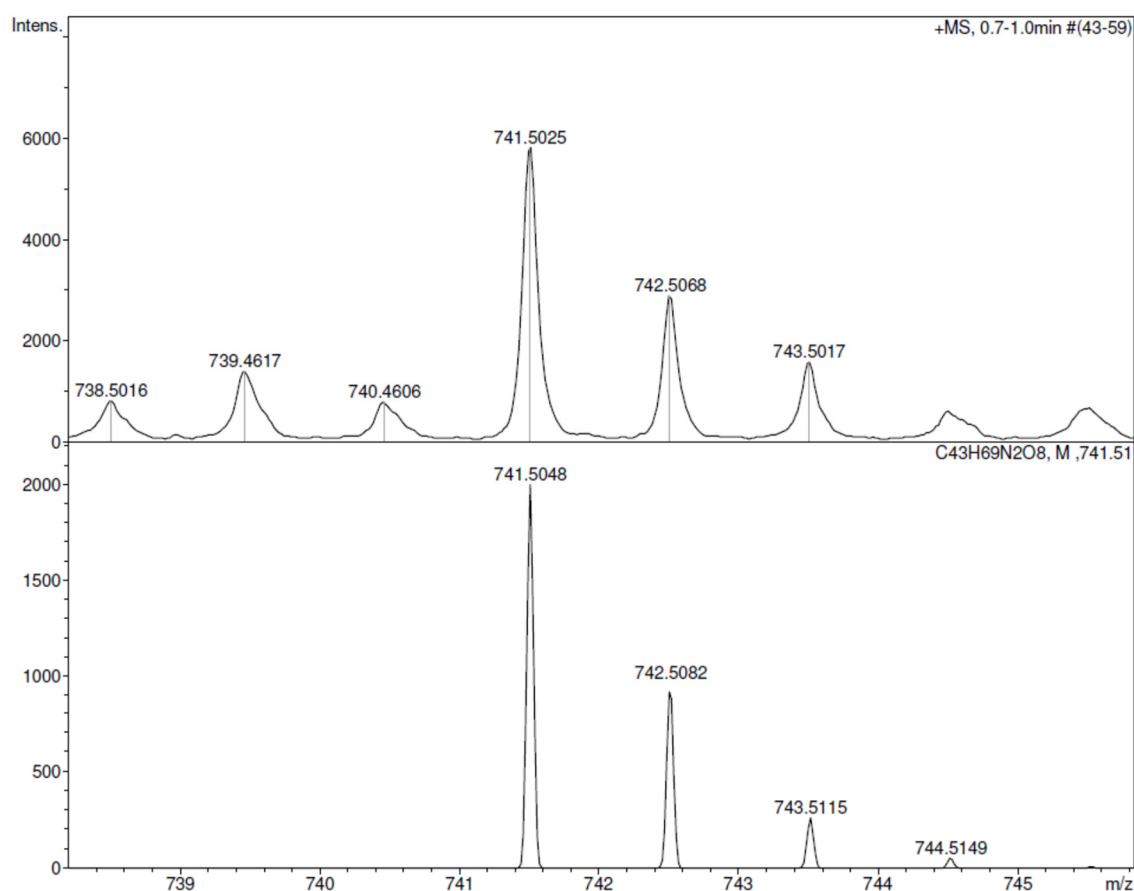
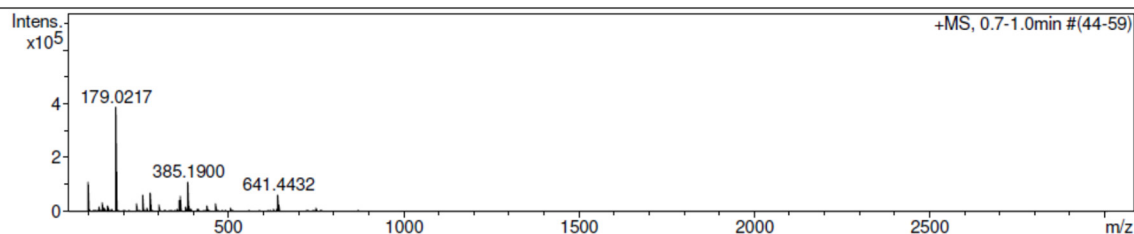
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Method tune_low.m
Sample Name /ABCD S-56-3
Comment C43H69N2O8 clb added CH3OH

Acquisition Date 11.11.2024 13:06:16

Operator BDAL@DE
Instrument / Ser# micrOTOF 10248

Acquisition Parameter

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Scan End	3000 m/z	Set End Plate Offset	-500 V	Set Divert Valve	Waste



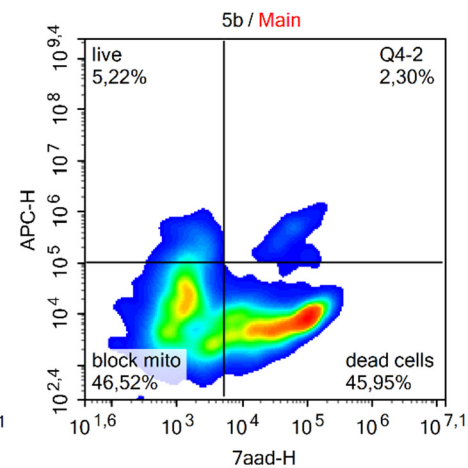
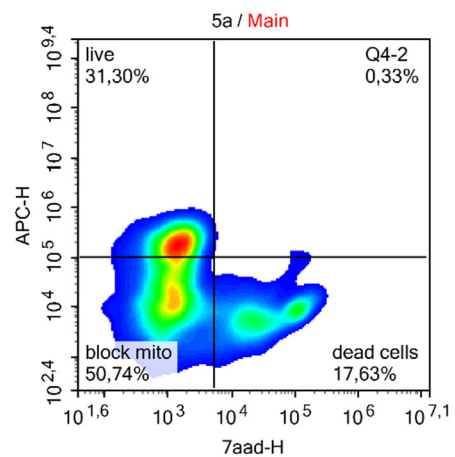
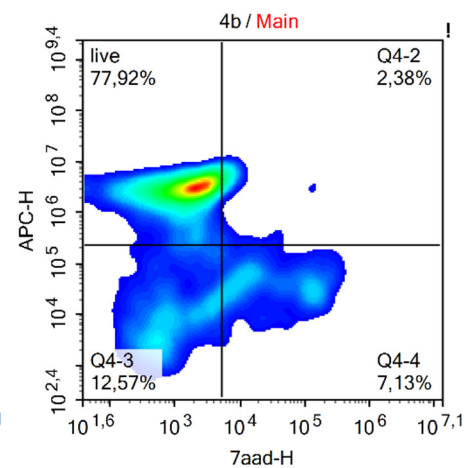
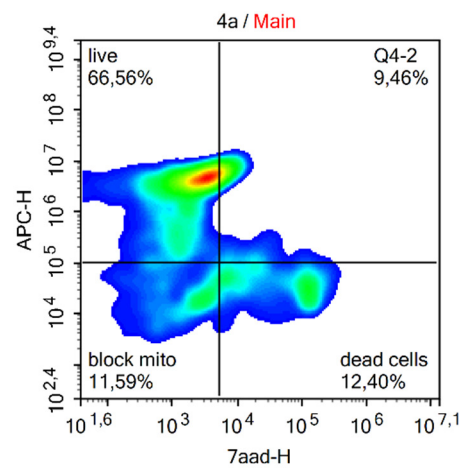
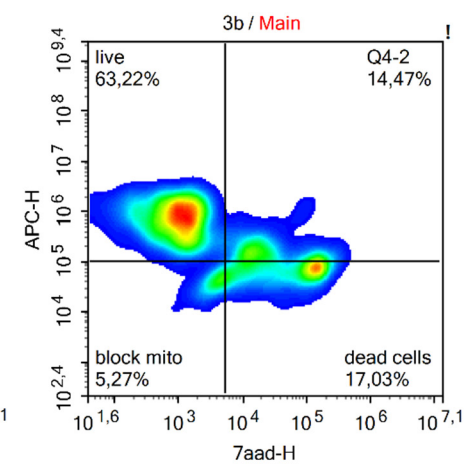
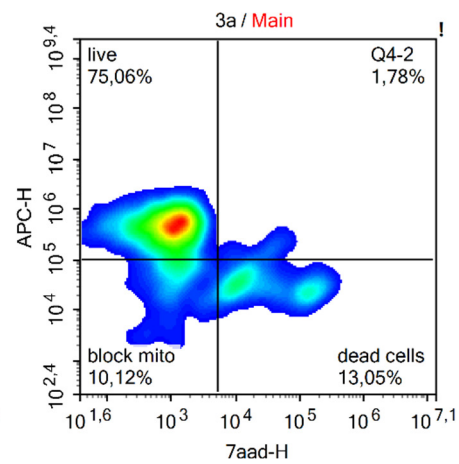
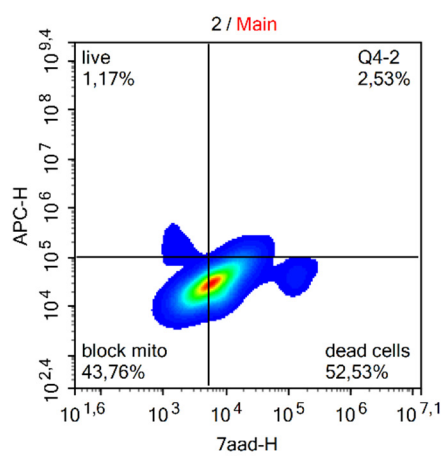
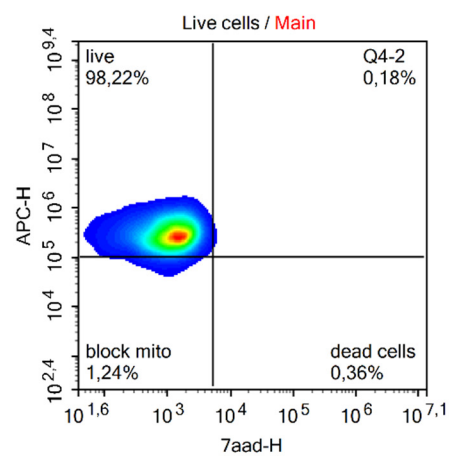
S7. Mass spectrum of compound **5b**.

Table S1. Cytotoxic activity dataset of hybrid molecules and ionic compounds measured in this study (mM).

	Jurkat	K562	A549	HEK293
Str	44±3	49±4	51±4	54±5
CPT	588±49	594±54	599±41	613±57
2 (S-030-2)	417±38	456±41	482±46	488±32
3a (S-040)	476±34	480±45	483±37	491±43
4a (S-042)	214±18	224±24	290±21	301±29
5a (S-042-2)	178±15	158±12	188±16	196±11
3b (S-041)	661±57	670±49	683±52	701±64
4b (S-056-2)	210±18	213±14	215±17	240±21
5b (S-056-3)	233±21	254±19	256±23	260±24

Table S2. The dataset comprises measurements of apoptosis induction and cell cycle phases of hybrid molecules and lithocholic acid-based ionic compounds.

Compound	Molar mass (g/mol)	Apoptosis (%)				Mitostress (%)				Cell cycle (%)				Mitopotential (%)			Cytochrome C loss (%)	
		live	early	late	necrosis	Live	Stress	Apoptosis	Apoptosis+stress	G1	S	G2/M	preG0	Live	Blocked mito	Dead	Live	Mitdead
Untreated	-	97.1	0	0	2.87	95.51	0.01	0.43	0.05	75.47	17.44	4.85	2.18	98.22	1.24	0.36	97.64	2.34
Str	466.541	88.7	0.26	3.86	7.14	n/a	n/a	n/a	n/f	n/a	n/a	n/a	n/f	n/a	n/a	n/a	n/a	n/a
CCCP	204.62	n/a	n/a	n/a	n/f	n/a	n/a	n/a	n/f	n/a	n/a	n/a	n/f	53.17	12.00	22.34	34.15	60.24
CPT	348.358	28.0	1.59	11.3	59.1	n/a	n/a	n/a	n/f	61.18	18.29	2.95	16.59	n/a	n/a	n/a	n/a	n/a
2	490.68	22.0	1.85	34.9	41.3	53.24	0.18	41.01	5.57	66.18	16.53	2.98	13.60	1.17	46.76	52.53	92.69	6.70
3a	576.82	86.0	0.69	4.30	9.06	43.52	0.05	54.17	2.25	81.33	12.89	2.75	2.78	75.06	10.12	13.05	88.90	10.00
4a	698.94	88.1	0.95	8.42	1.94	88.55	2.26	3.27	5.93	70.76	11.19	6.44	11.28	65.56	11.59	12.40	83.18	15.66
5a	841.89	92.5	0.03	0.34	7.15	31.78	0.53	43.15	24.54	83.17	11.17	3.62	1.86	31.30	50.74	17.63	77.81	21.06
3b	604.87	86.2	1.14	8.96	3.68	39.95	2.44	57.56	2.44	85.80	9.64	2.05	2.35	63.22	5.27	17.03	91.22	8.25
4b	712.97	85.9	0.15	1.6	12.3	47.31	0.97	41.82	9.91	83.71	7.10	5.57	3.37	77.92	12.57	7.13	87.26	11.66
5b	855.92	78.7	11.9	6.9	2.5	36.69	0.12	53.18	10.02	68.02	11.39	6.53	13.25	5.22	46.52	45.95	76.84	22.05



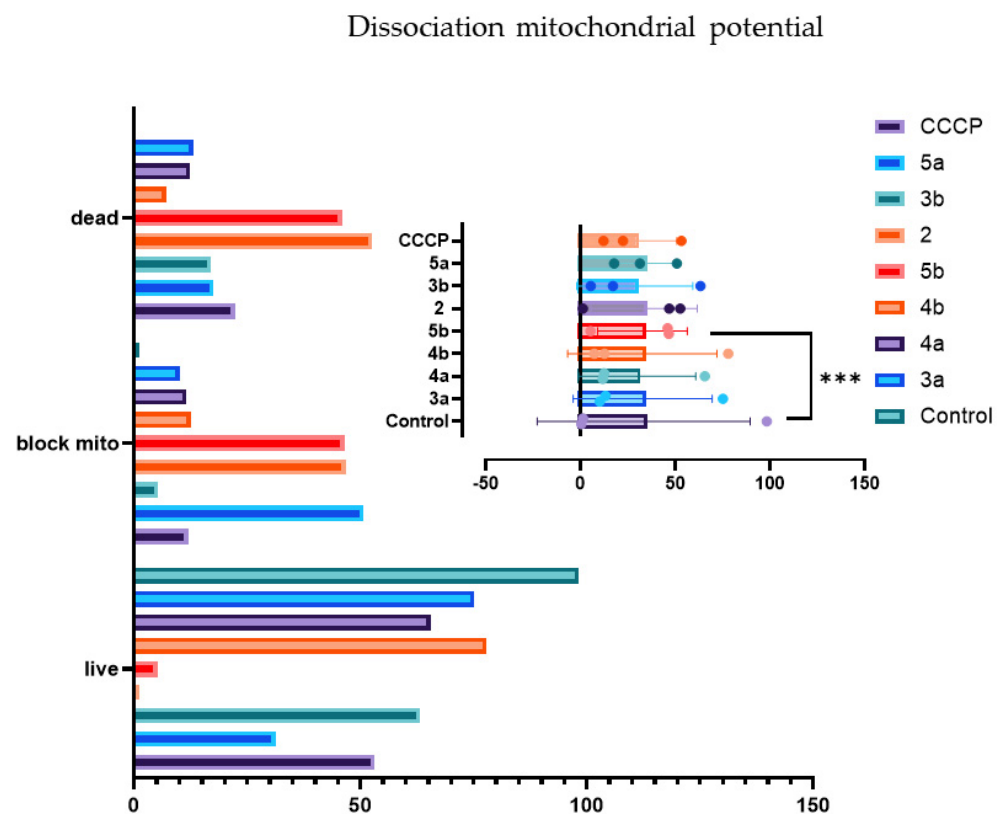
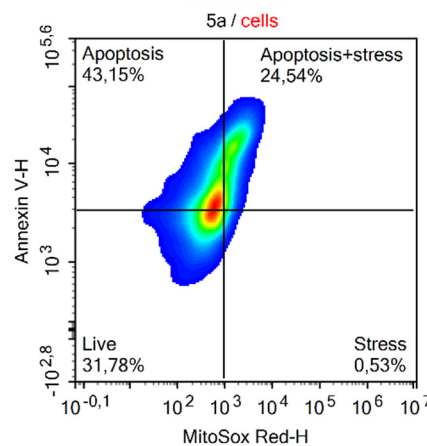
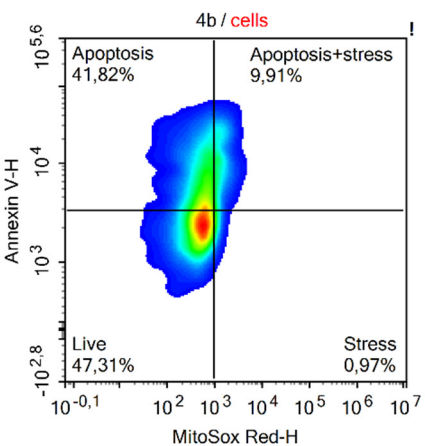
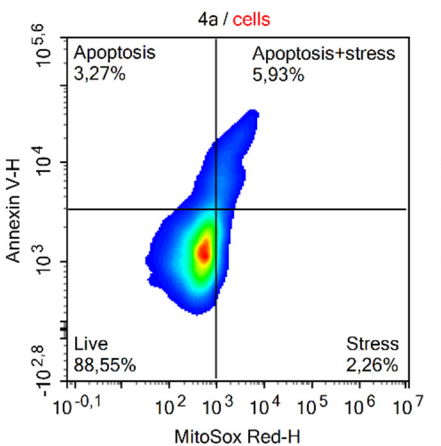
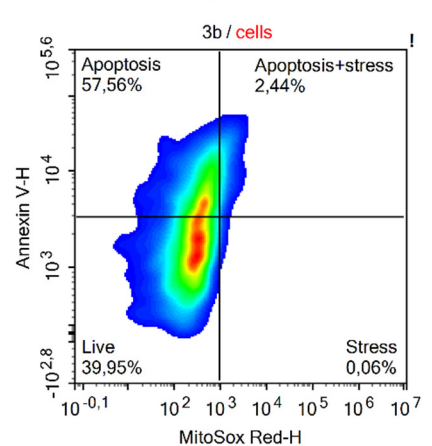
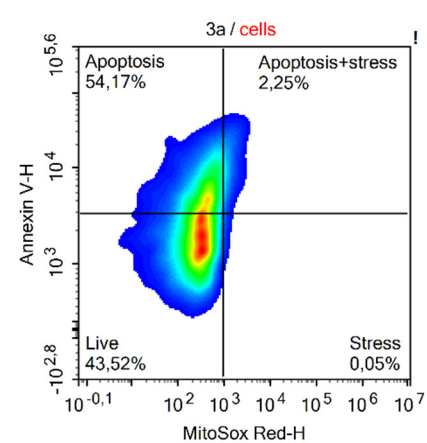
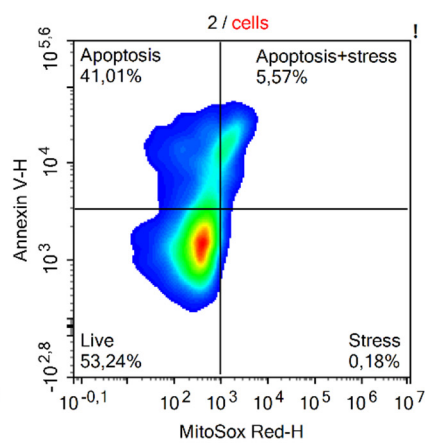
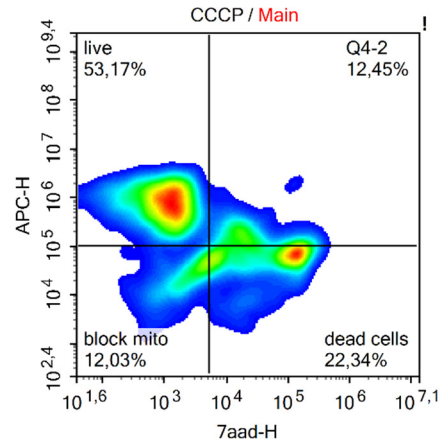
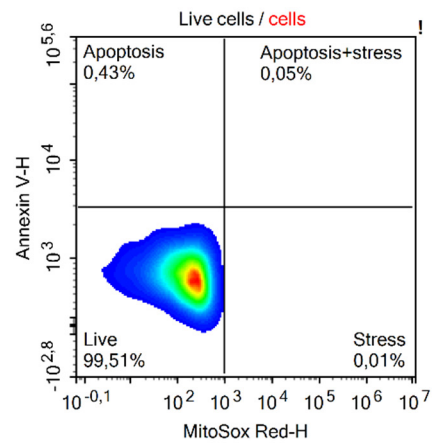
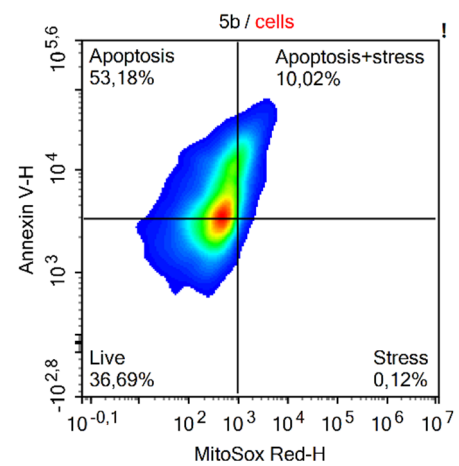


Figure S1. (4) The objective of this study is to simultaneously determine the levels of cell death markers and mitochondrial membrane potential disruption in Jurkat cells exposed to a series of synthesized hybrid molecules and lithocholic acid-based ionic compounds. (A) The number of cells with altered mitochondrial potential (negative for JC-1 and 7-AAD) and necrotic cells (negative for JC-1 and positive for 7-AAD) were observed over a 12-hour period. The concentration of the tested compounds was taken at CC_{50} (the cytotoxicity table of co-compounds is presented in SI). (B) A histogram of mitochondrial potential changes in cells in the Jurkat culture upon exposure to hybrid molecules and lithocholic acid-based ionic compounds is presented.





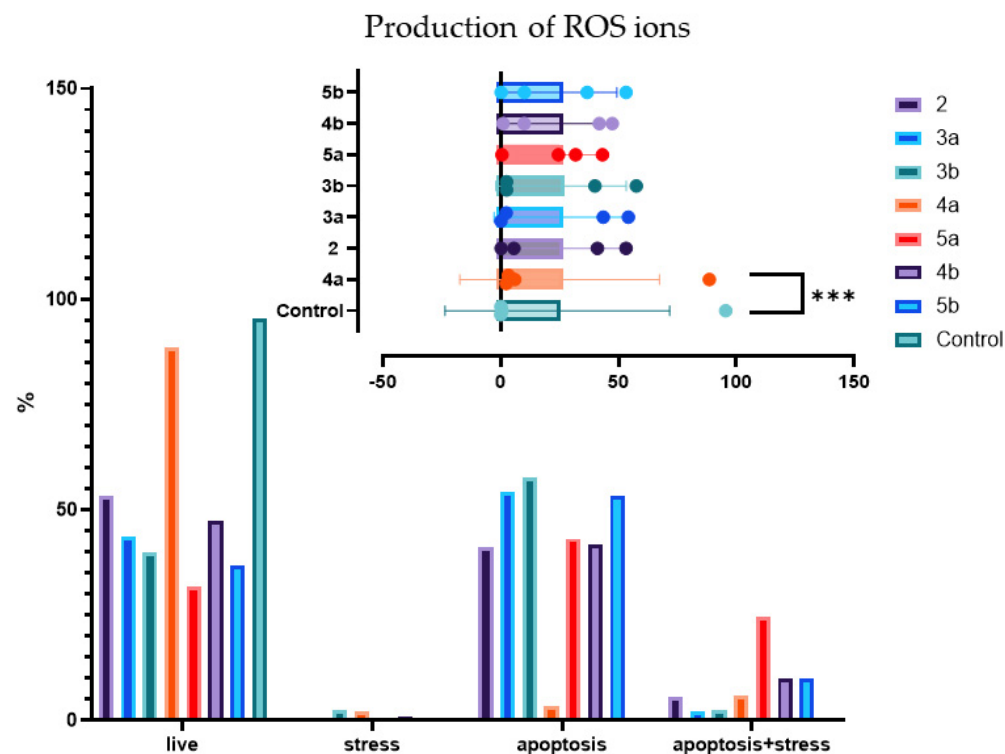
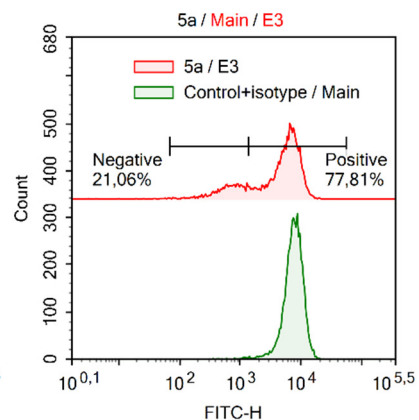
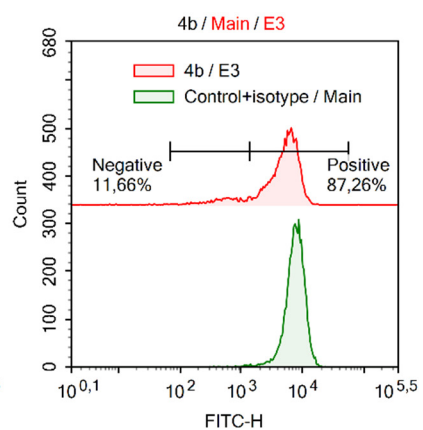
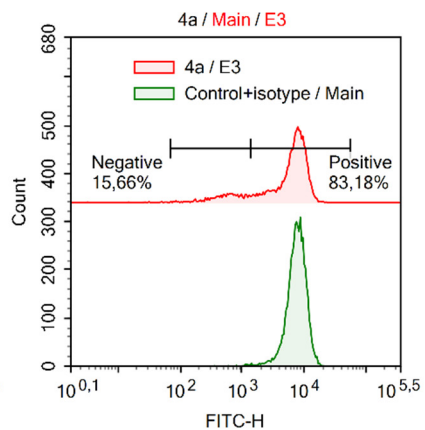
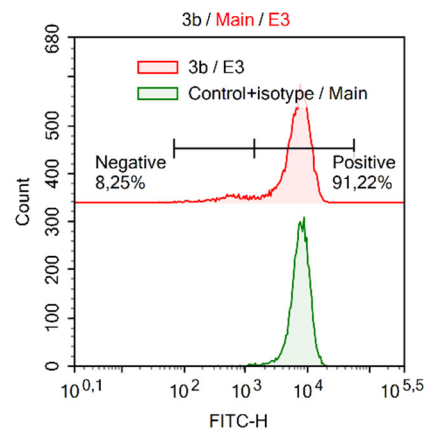
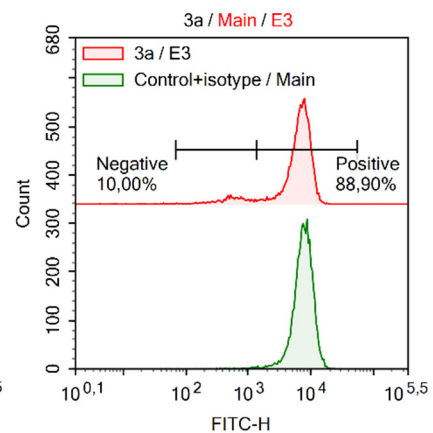
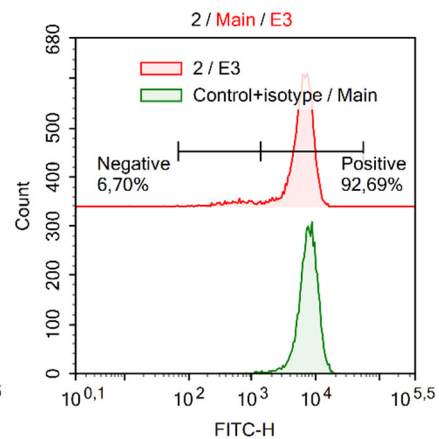
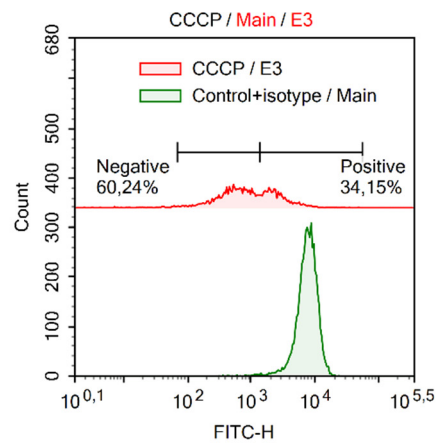
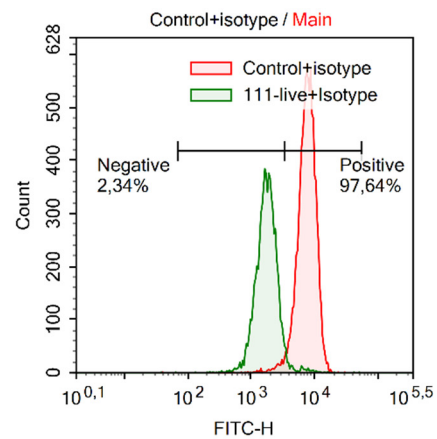


Figure S2. This study examines the impact of synthesized compounds on intracellular ROS ions levels. $\Delta\Psi_m$ and drug concentrations in Jurkat cells were treated with hybrid molecules and ionic compounds at CC50 for four hours using flow cytometry. (A) The levels of ROS ions levels were evaluated through the use of MitoSOX™ Red staining, in accordance with the instructions provided by the manufacturer (catalog number M36008, Thermo Fisher Scientific Inc.) and Annexin. (B) A histogram of the detected cellular changes is provided below. The presence of triple asterisks (***) indicates that the observed difference is statistically significant at the $p < 0.001$ level.



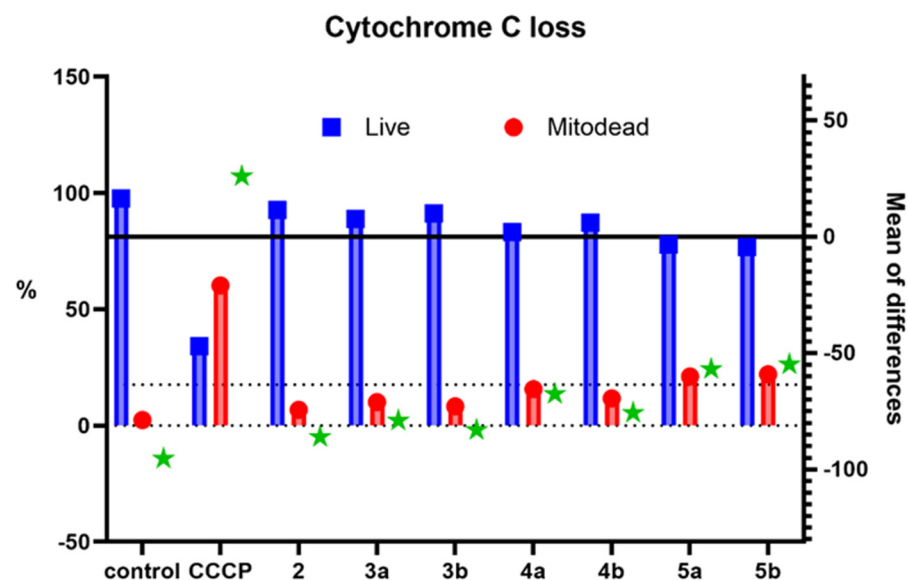


Figure S3. The figure depicts the detection of cytochrome c in Jurkat culture cells, which have been stained with the FlowCelect Cytochrome c Kit. Graph A illustrates the histogram of Jurkat cells treated with a combination of synthesized hybrid molecules and lithocholic acid-based ionic compounds at a CC50 concentration. The incubation period was four hours. Graph B illustrates the ratio of cells with damaged and undamaged mitochondria. The graphs illustrate a reduction in the fluorescence levels of cytochrome c for the Jurkat cell line. The control compound, carbonyl cyanide m-chlorophenylhydrazine (CCCP), was administered at a concentration corresponding to its IC50 value. The presence of green asterisks indicates that the observed difference is statistically significant at the $p < 0.001$ level.