

Design, synthesis and biological characterization of novel mitochondria targeted dichloroacetate-loaded compounds with antileukemic activity

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Corresponding authors:

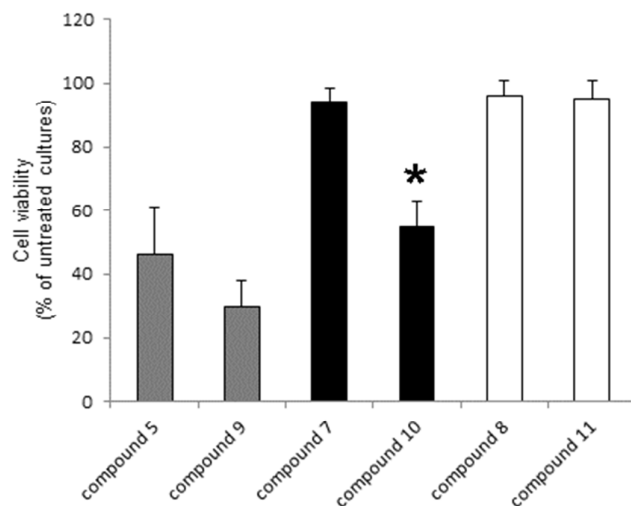
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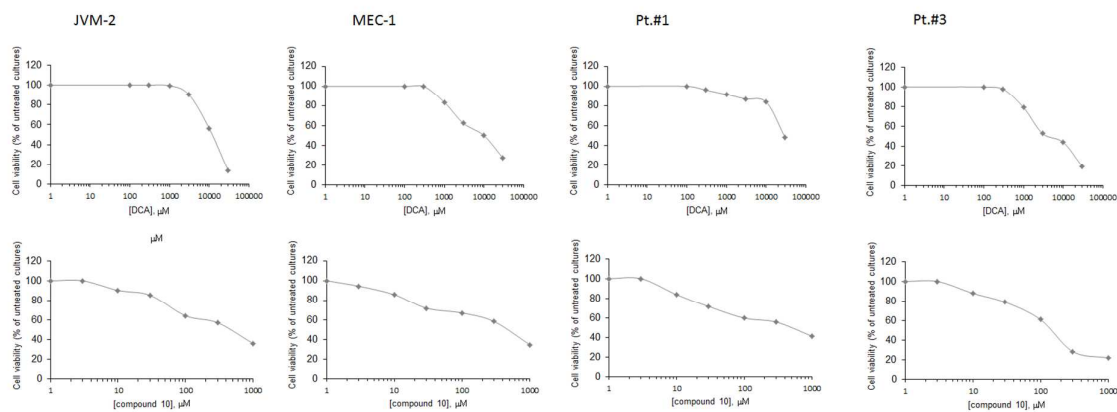
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Supplementary Figure 1



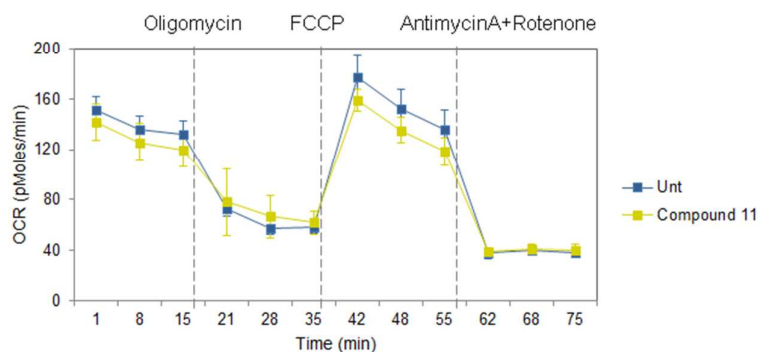
Evaluation of cytotoxic effects on cell viability of DCA-loaded compounds and corresponding backbone carriers. Leukemic cell cultures were exposed to compounds **9**, **10** and **11** and to the corresponding backbone carriers (compounds **5**, **7** and **8**) (all used at the concentration of 300 μ M) and cell viability was assessed at 48 hours of treatment. The analysis of cell viability in response to each DCA-loaded compounds is shown in comparison with respect to the corresponding carrier (bars with same colors); the asterisk indicates $p < 0.05$.

Supplementary Figure 2



Determination of IC₅₀ for DCA and compound 10. Representative dose-response curves for the determination of IC₅₀ values of the anti-leukemic activity exhibited either by DCA (upper graphs) and by compound 10 (lower graphs), assessed in leukemic cell lines and primary B-CLL patient derived cells. As shown by the graphs in log scale, compound 10 was able to exhibit the anti-leukemic effect of DCA at significantly lower concentrations (as reported also in **Table 1**).

Supplementary Figure 3



Characterization of the effects of the DCA-loaded compound 11 on cellular bioenergetics functions. Leukemic cells were left untreated or treated for 3h with 300 μ M of compound **11** and run on the Seahorse analyzer. Oxygen consumption rate (OCR) measurements were recorded after the sequential addition of oligomycin, FCCP (Carbonyl cyanide-ptrifluoromethoxyphenylhydrazine) and the combination of antimycin A and rotenone. OCR traces are expressed as pMoles O_2 per minute and normalized to cell number. Each data point represents mean \pm SD of six independent experiments.

Supplementary table 1. IC₅₀ values for DCA and compound **10** in tumor cell lines of epithelial origin

<i>Cells</i>	<i>IC₅₀ (mM)</i>	
	<i>DCA</i>	<i>compound 10</i>
HCT116	20.00±1.88	0.33±0.02
Hep3B	18.61±1.67	0.45±0.04
MCF7	16.84±1.12	0.35±0.03
MCF10A	16.11±1.49	0.48±0.03

Values are mean±SD calculated after 48 hours of culture treatment of experiments carried out at least in triplicate for each cell culture.

Supplementary Experimental Section

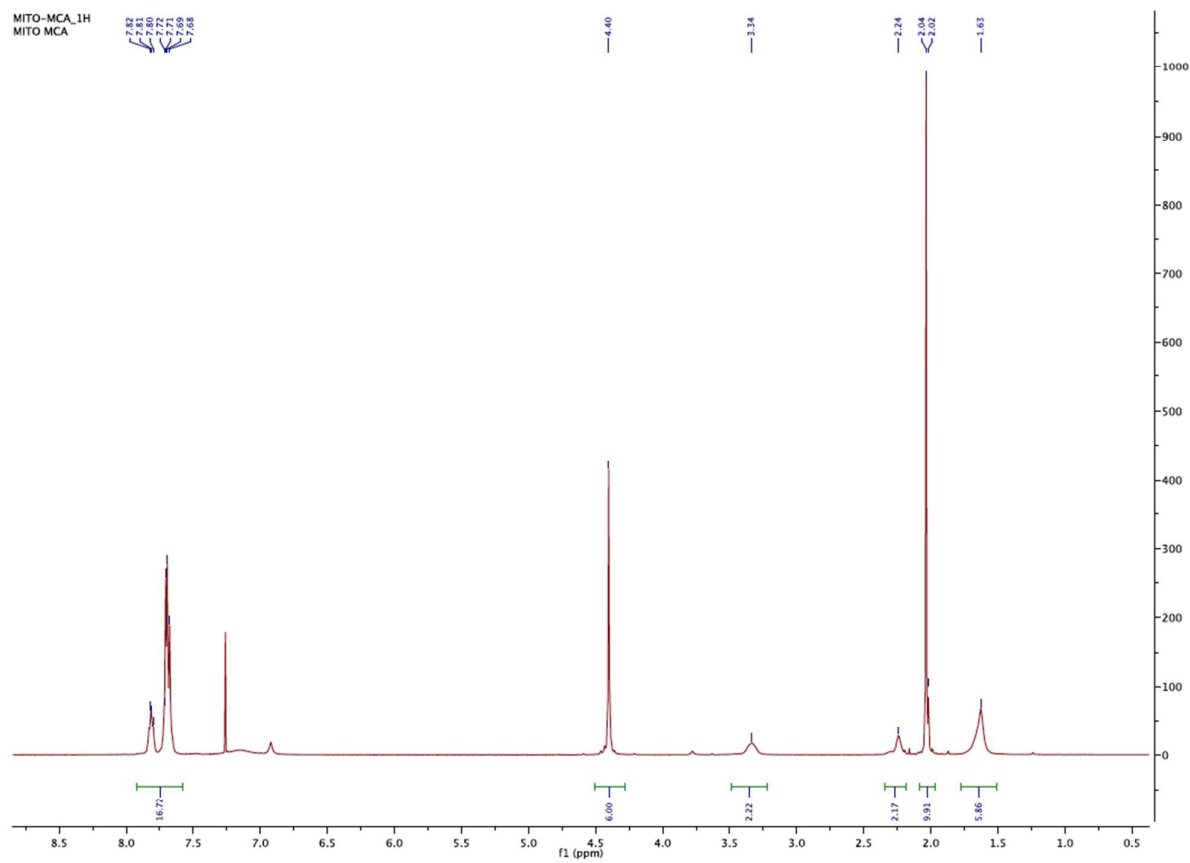
The epithelial-derived cell lines HCT116, Hep3B, MCF7 and MCF10A were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The p53^{wild-type} HCT116 human colon cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, L-glutamine and Penicillin/streptomycin (all from Gibco). The Hep3B cells and the MCF7 cells were cultured in DMEM and RPMI-1640 media respectively, all supplemented with 10% FBS, L-glutamine and Penicillin/streptomycin. MCF10A cells were maintained in DMEM/F12 medium supplemented with 10% FBS, L-glutamine and Penicillin/streptomycin, EGF (20 ng/mL), Hydrocortisone (0.5 µg/mL), Insulin (10 µg/mL) (all from Gibco). Cells were cultured in a 6-well flat-bottomed multi-well tissue culture plates under normal conditions (ambient air; 5% CO₂) and grown to subconfluence before treatments with DCA (Sigma-Aldrich, St Louis, MO; used in the range of 1-30 mM) and DCA-loaded compounds and carries (used in the range of 3-300 µM).

General Information

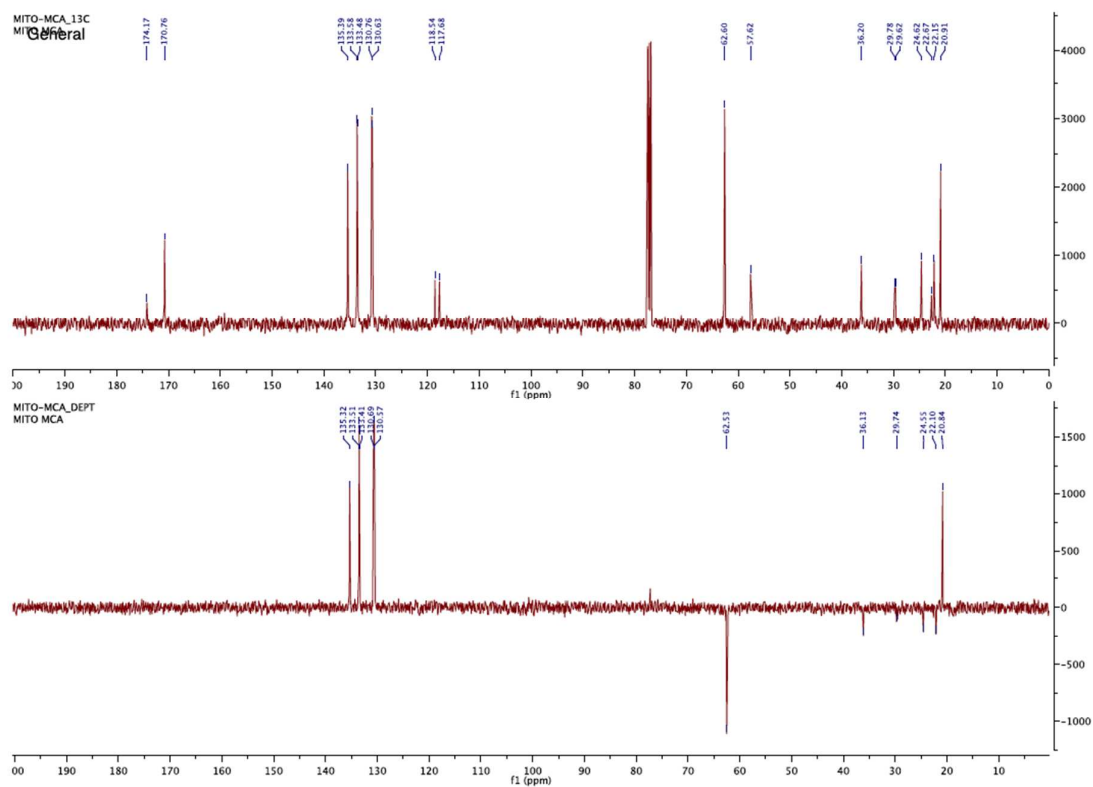
All the NMR spectra were elaborated using Mestre Nova 6.0.2 software and FID data are available on request. Analytical thin layer chromatography (TLC) was performed on silica gel Macherey-Nagel poligram SIL/UV 254 of 0.25 mm, visualization was achieved using UV light (254) and potassium permanganate (KMnO₄) 2% in water. Flash column chromatography was undertaken on silica gel Merck 60-200 mesh using Isolera Biotage (Sweden). Products were dried using sodium sulfate anhydrous (Carlo Erba). Proton nuclear magnetic resonance (¹H-NMR) and carbon nuclear magnetic resonance (¹³C-NMR) were recorded using VARIAN 400 MHz. All the spectra were recorded using as solvent CDCl₃ otherwise the solvent was specified. Chemical shifts (δ) were quoted in ppm relative to residual solvent and coupling constants (J) were quoted in Hertz (Hz). Multiplicity was reported with the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet, bs = broad signal. Molecular weights were measured with a mass spectrometer ESI MICROMASS ZMD 2000 (Waters, UK) and high resolution spectra with an Agilent ESI-Q-TOF LC/MS 6520 System (Agilent Technologies, USA). Solvents and chemicals used for TLC, chromatographic purification, crystallizations and reactions were reported with the following abbreviations: Et₂O for diethyl ether, THF for tetrahydrofuran, AcOEt for ethyl acetate, DCM for methylene chloride, CH₃CN for acetonitrile, TFA for trifluoro acetic acid. Analytical HPLC were recorded with Beckmann system Gold HPLC using a linear gradient from 0% to 100% solvent B (60% acetonitrile/40% water/0.1% TFA) using a Kinetex C₁₈ Coloumn (250mmX mm, 5μm).

Synthesis of (6-((1,3-diacetoxy-2-(acetoxymethyl)propan-2-yl)amino)-6-oxohexyl)triphenylphosphonium bromide (5)

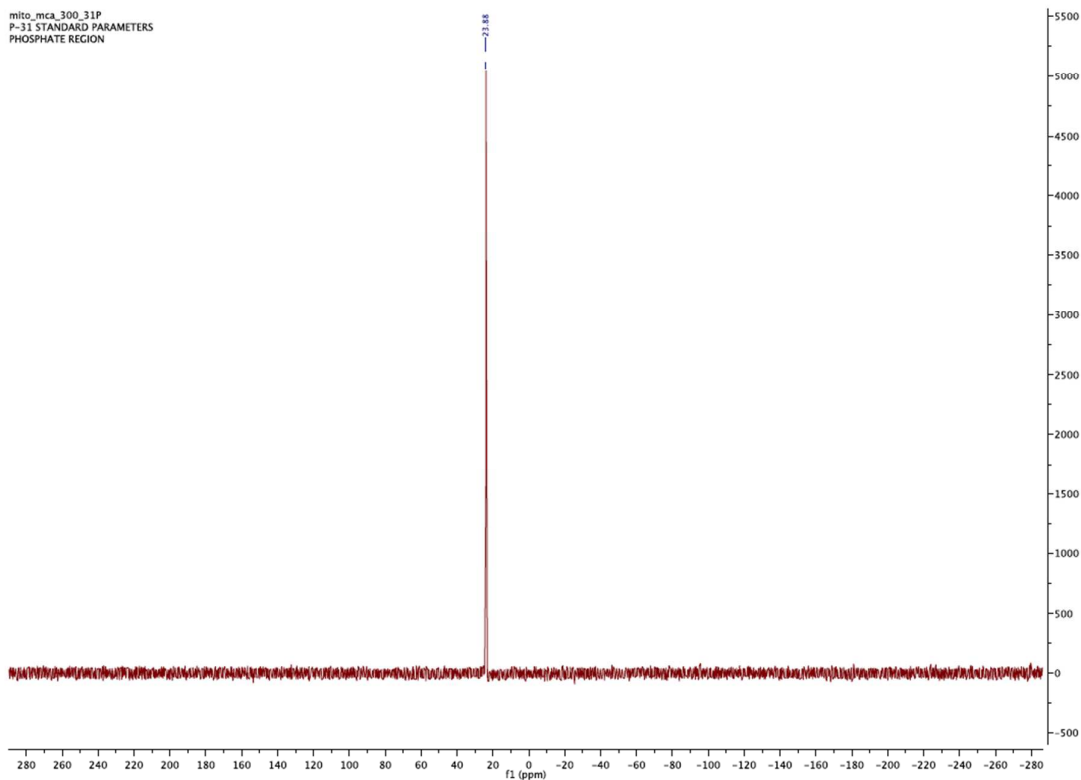
¹H-NMR compound **5**:



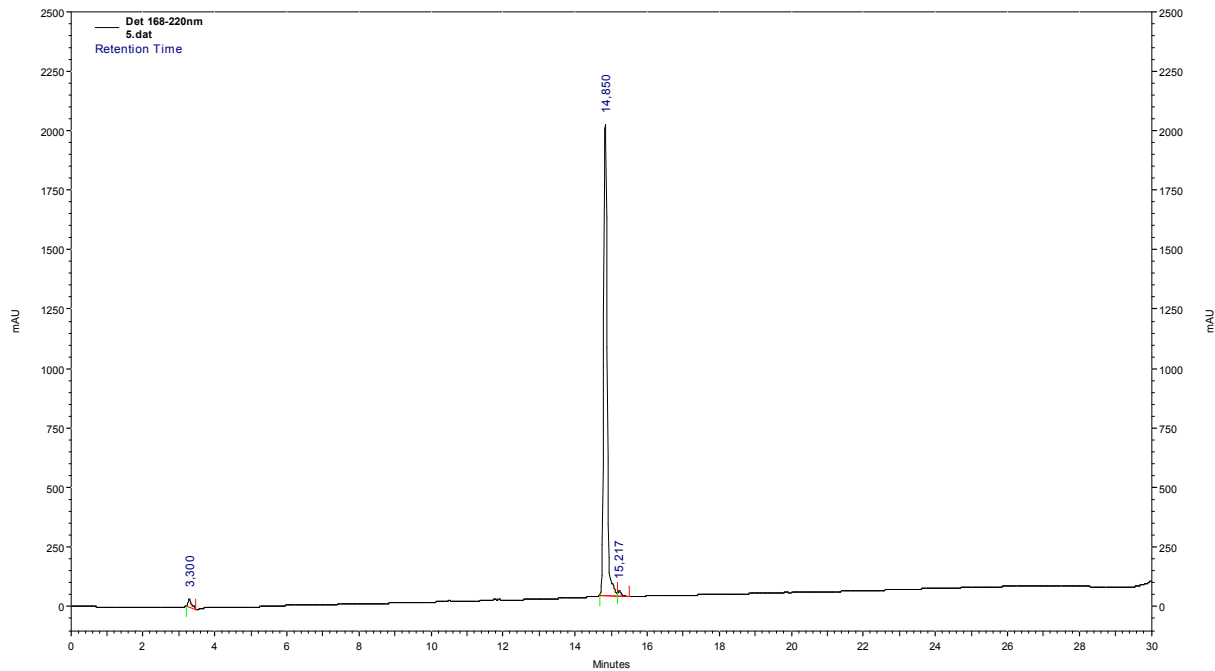
^{13}C -NMR and DEPT compound **5**:



^{31}P -NMR of compound **5**



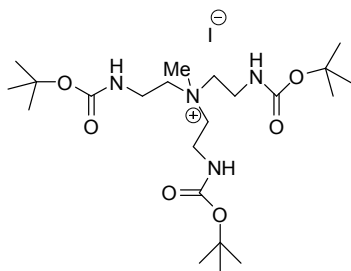
Purity grade by HPLC of compound **5**:



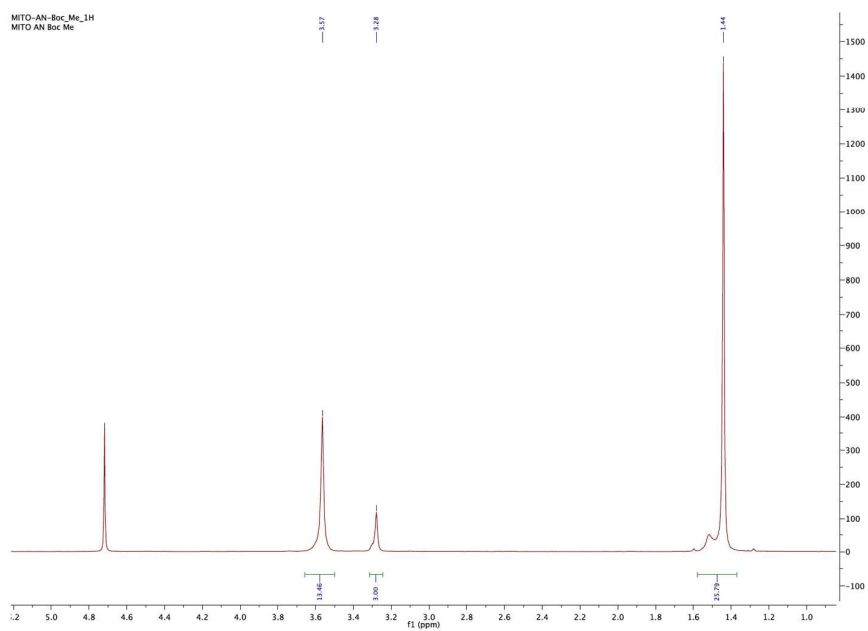
Det 168-220nm
Results

Time	Area	Area %	Height	Height %
3,300	260722	1,83	35642	1,75
14,850	13877492	97,27	1980168	97,27
15,217	129154	0,91	19969	0,98
Totals	14267368	100,00	2035779	100,00

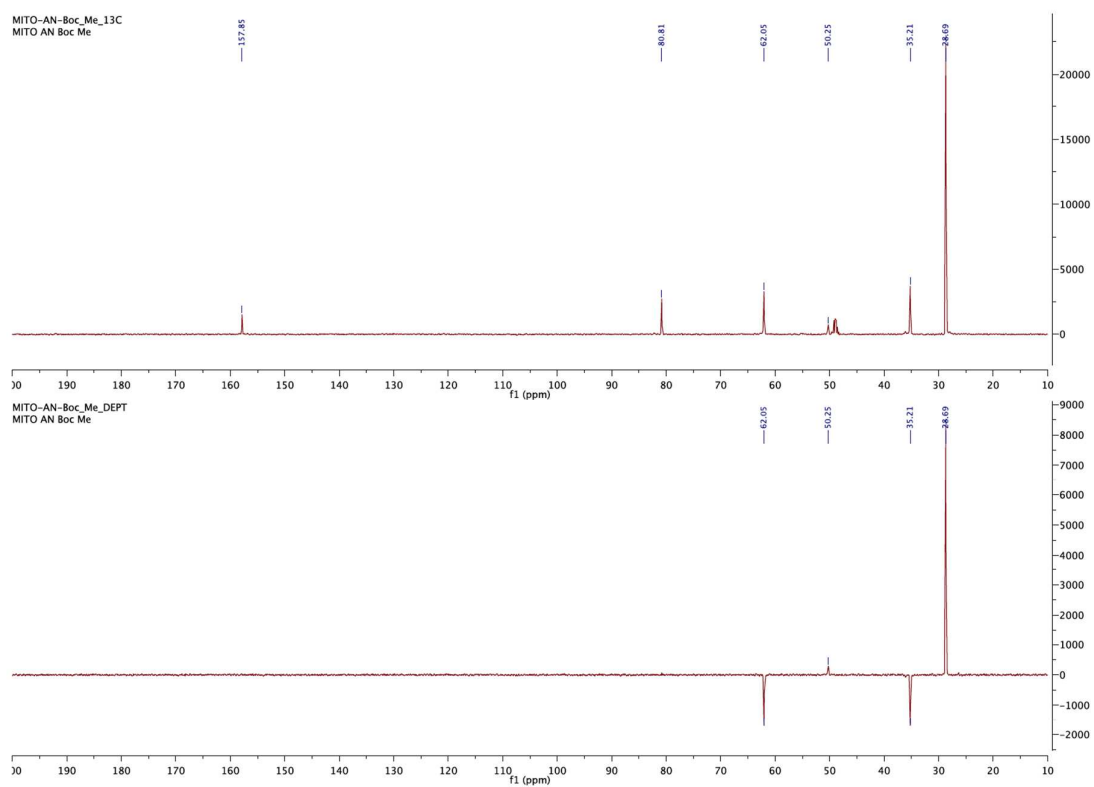
Synthesis of 2-((tert-butoxycarbonyl)amino)-N,N-bis((2-((tert-butoxycarbonyl)amino)ethyl)-N-methylthalaminium iodide (8)



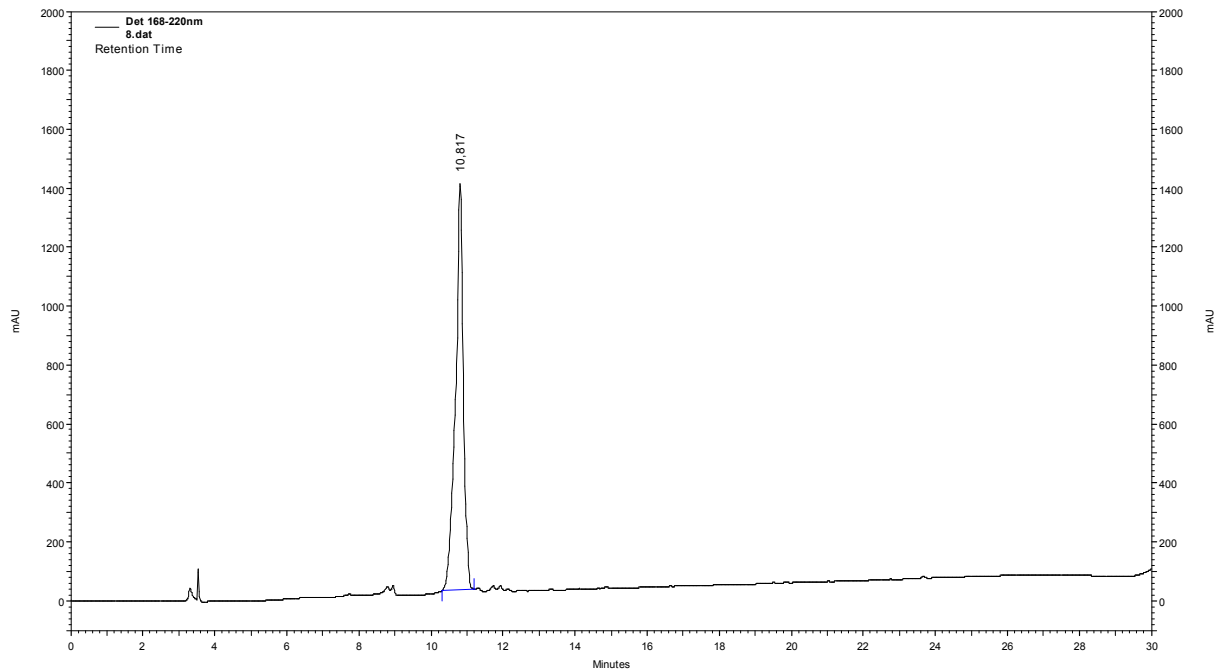
^1H -NMR compound **8**:



¹³C-NMR and DEPT compound **8**:



Purity grade by HPLC of compound **8**:



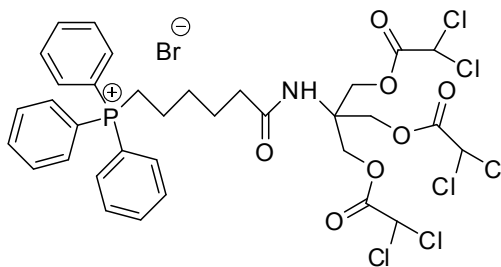
Det 168-220nm

Results

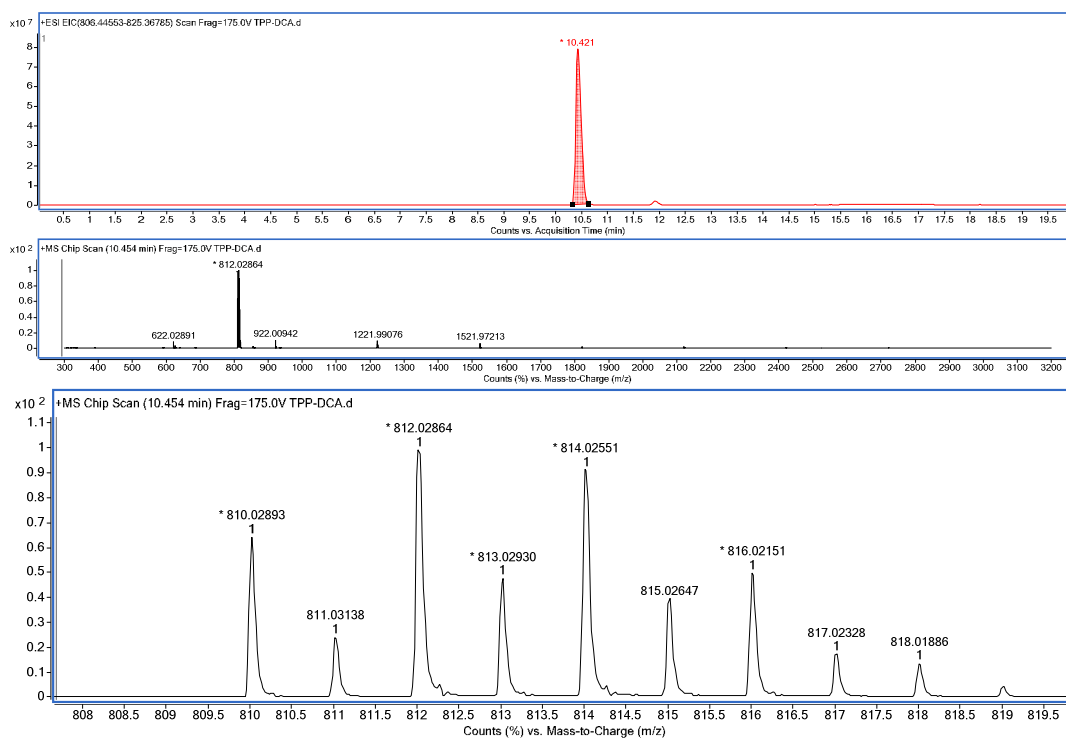
Time	Area	Area %	Height	Height %
10,817	21154899	100,00	1377744	100,00

Totals	21154899	100,00	1377744	100,00
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Synthesis of (6-((1,3-bis(2,2-dichloroacetoxy)-2-((2,dichloroacetoxy)methyl)propan-2-yl)amino)-6-oxohexyl)triphenylphosphonium (9)

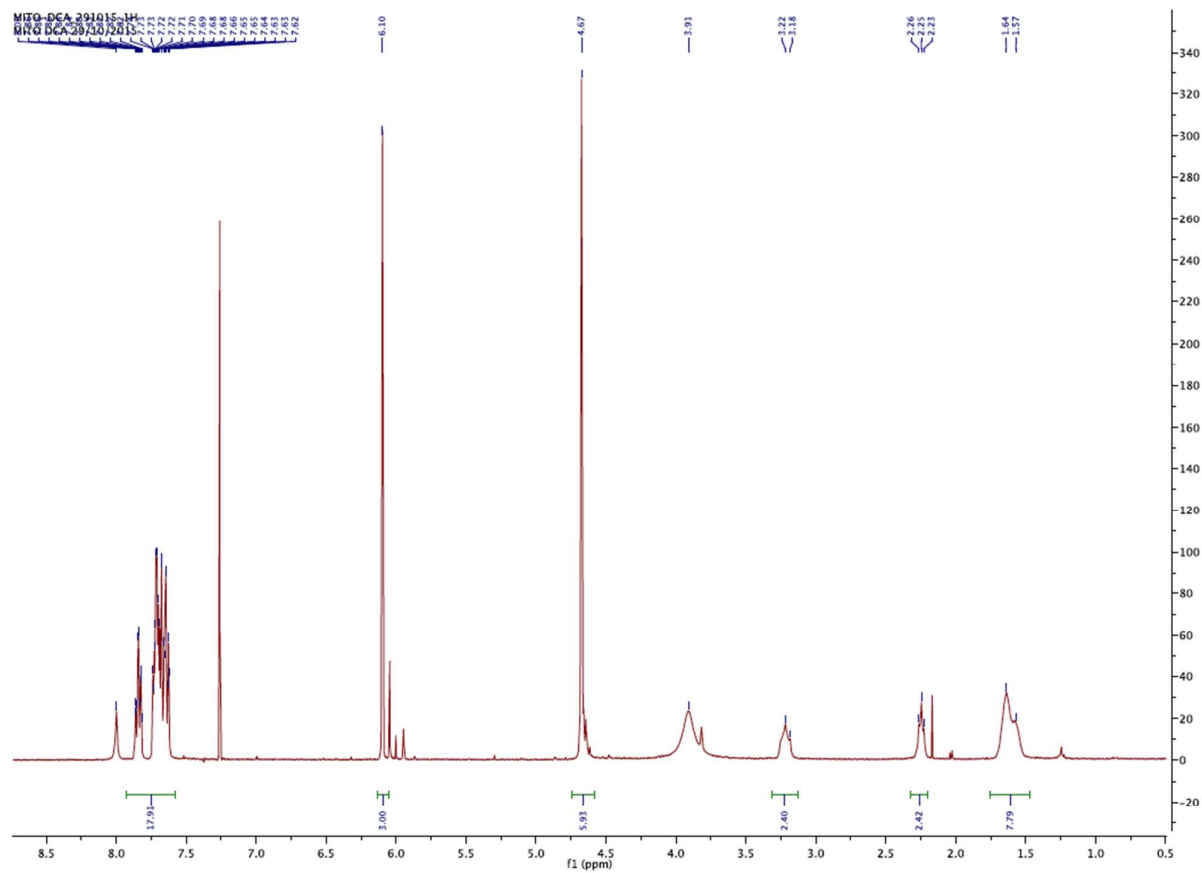


HRMS (ESI) of compound **9**: $[M+H]^+ = 812.028640$

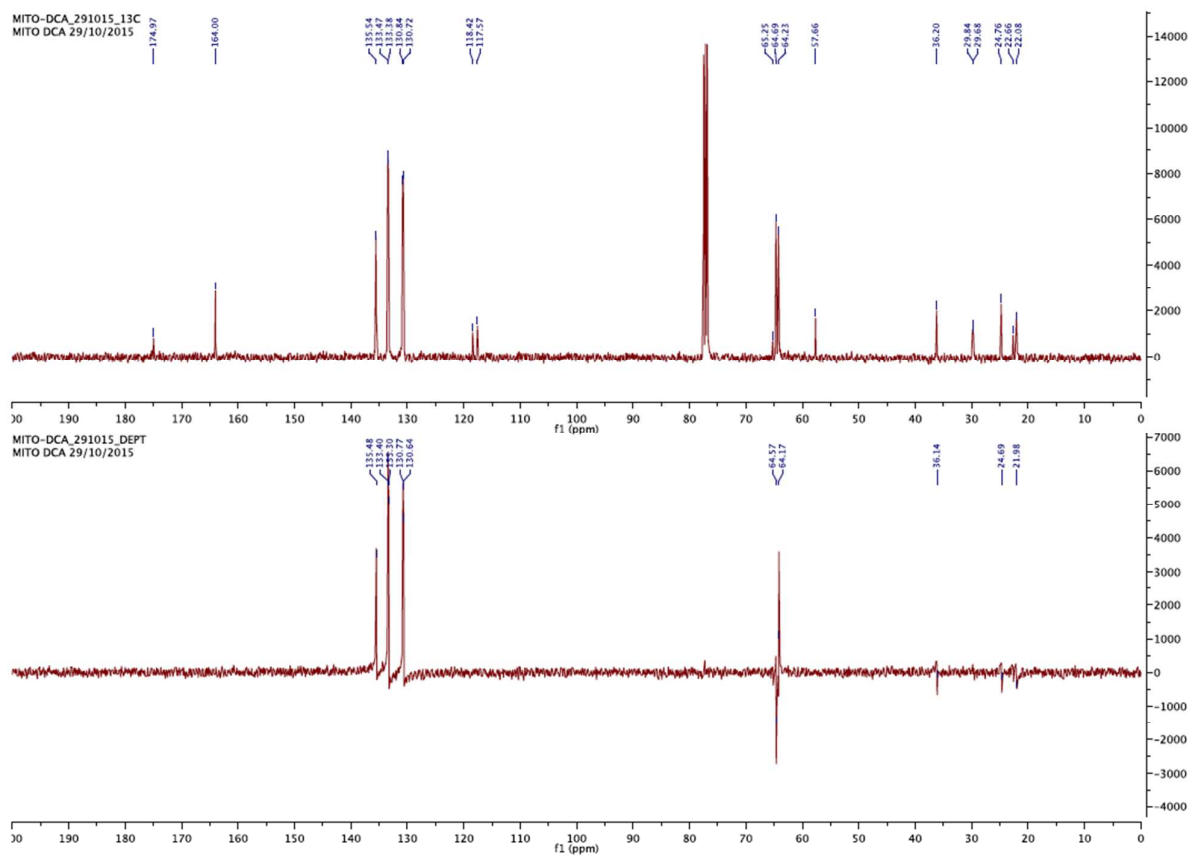


	Calc. Mass	Mis. Mass	Error ppm
	812.0257	812.028640	3.620575

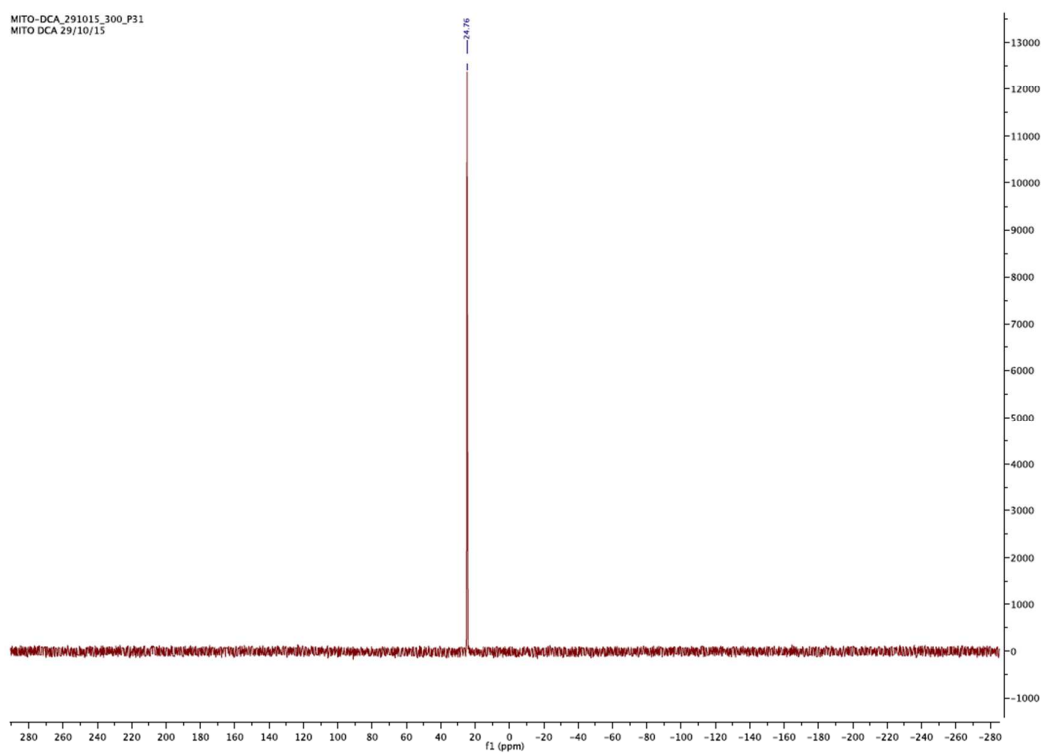
^1H -NMR compound **9**:



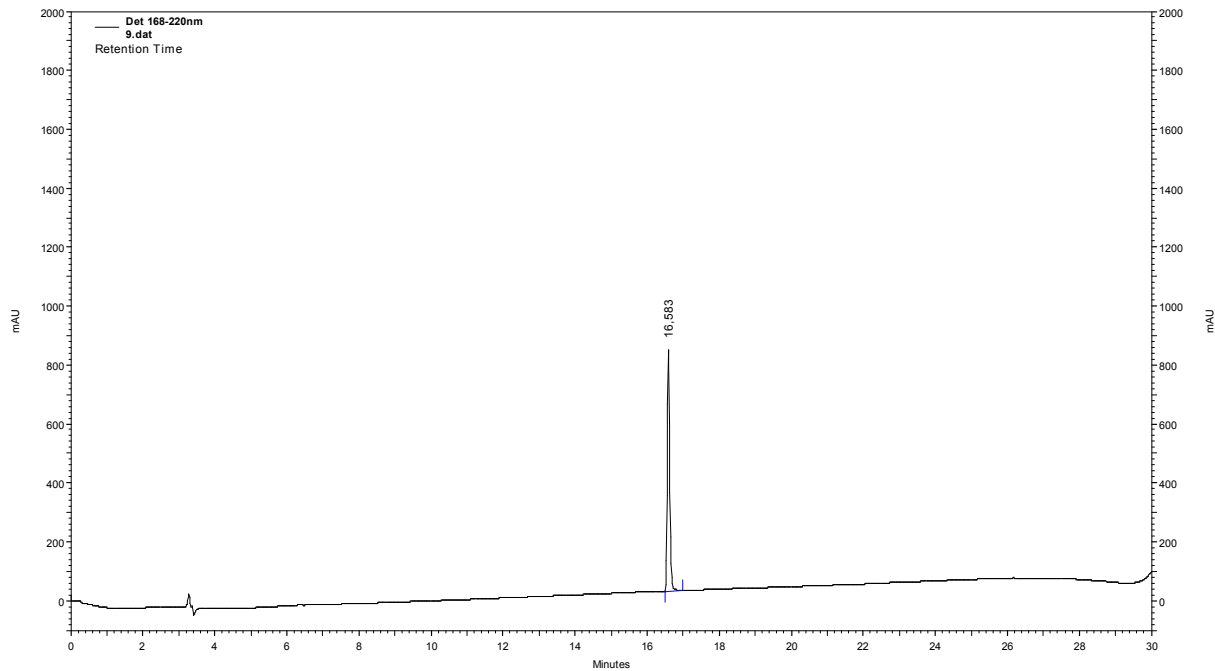
¹³C-NMR and DEPT compound 9:



³¹P-NMR of compound 9



Purity grade by HPLC of compound **9**:



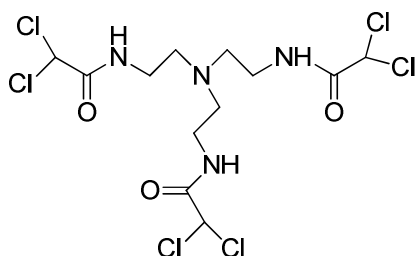
Det 168-220nm

Results

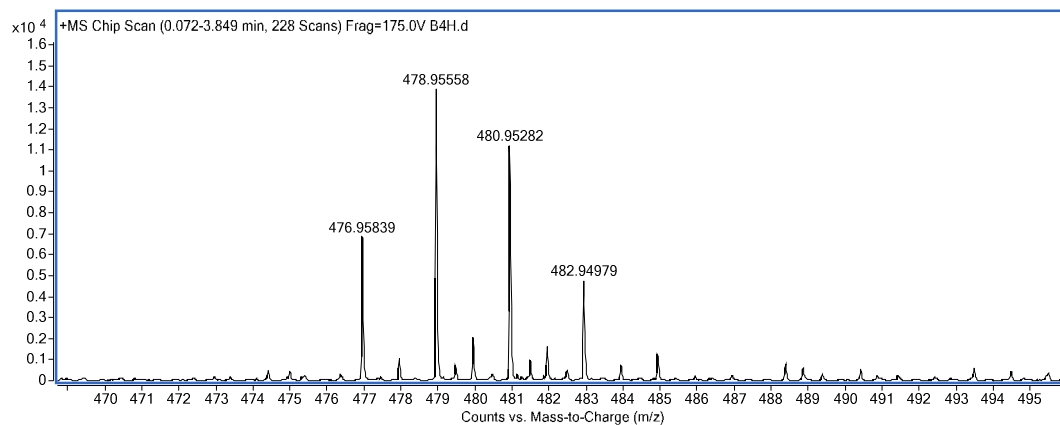
Time	Area	Area %	Height	Height %
16,583	3959035	100,00	819455	100,00

Totals	3959035	100,00	819455	100,00
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Synthesis of N,N',N''-(nitrilotris(ethane-2,1-diyl))tris(2,2-dichloroacetamide) (**10**)

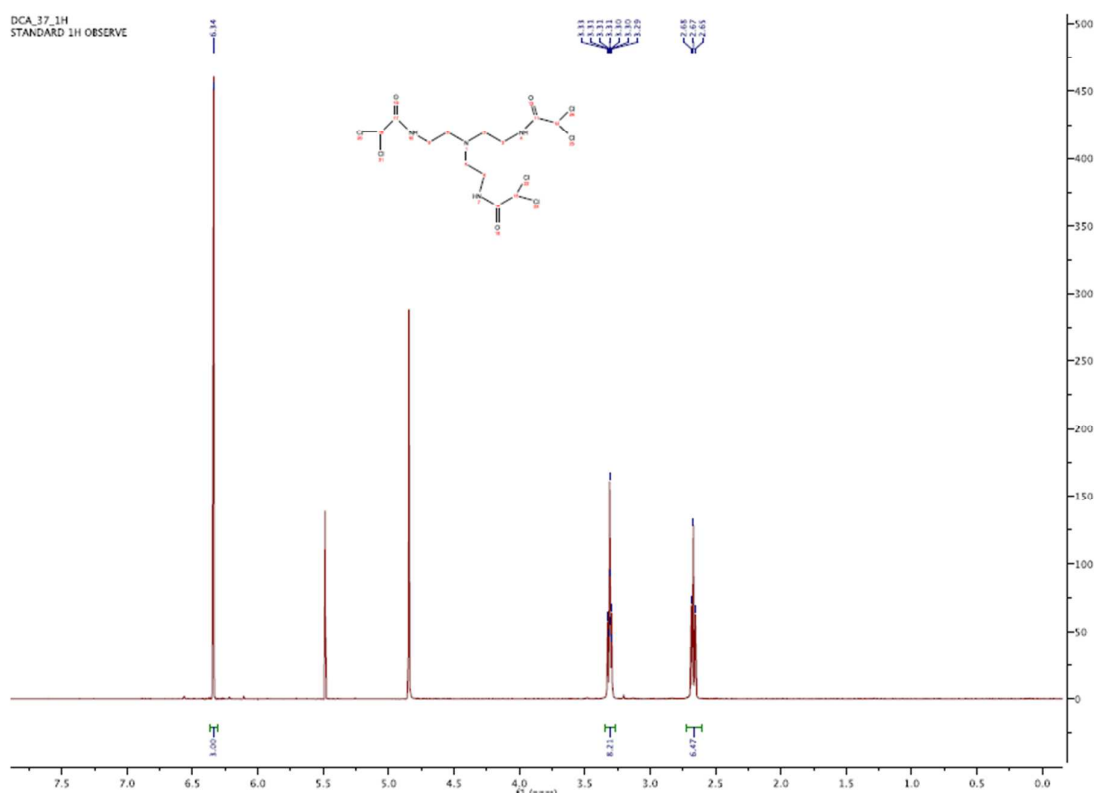


HRMS (ESI) of compound **10**: $[M+H]^+ = 476.95839$

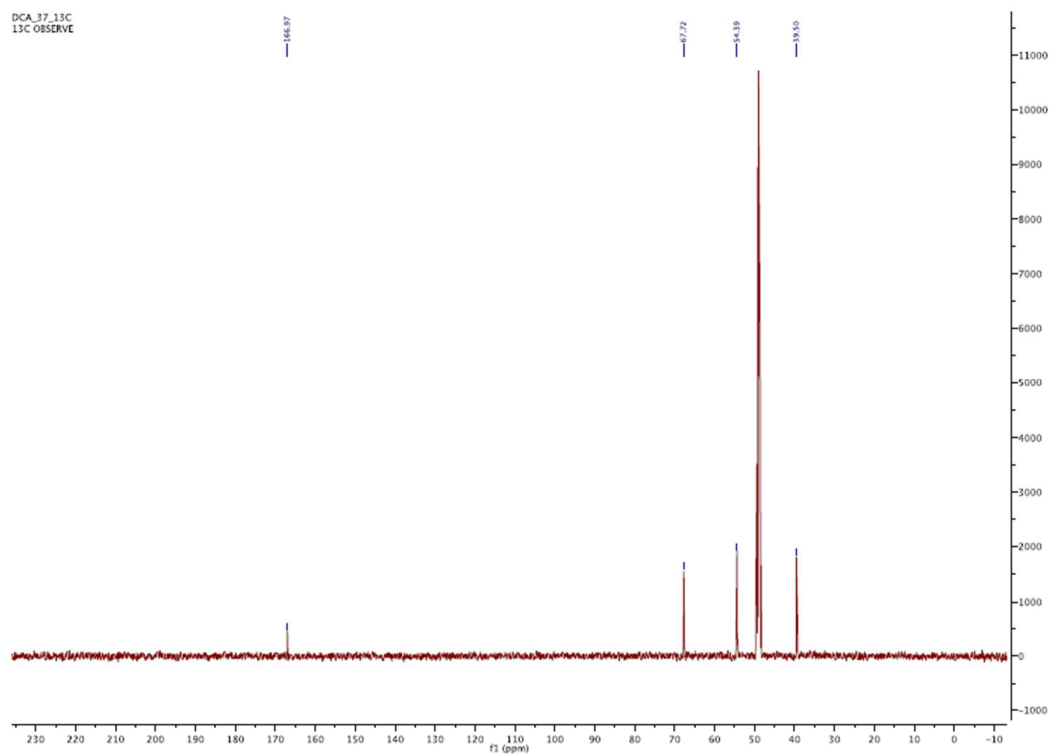


Calc. Mass	Mis. Mass	Error ppm
476.958283	476.95839	0.224338

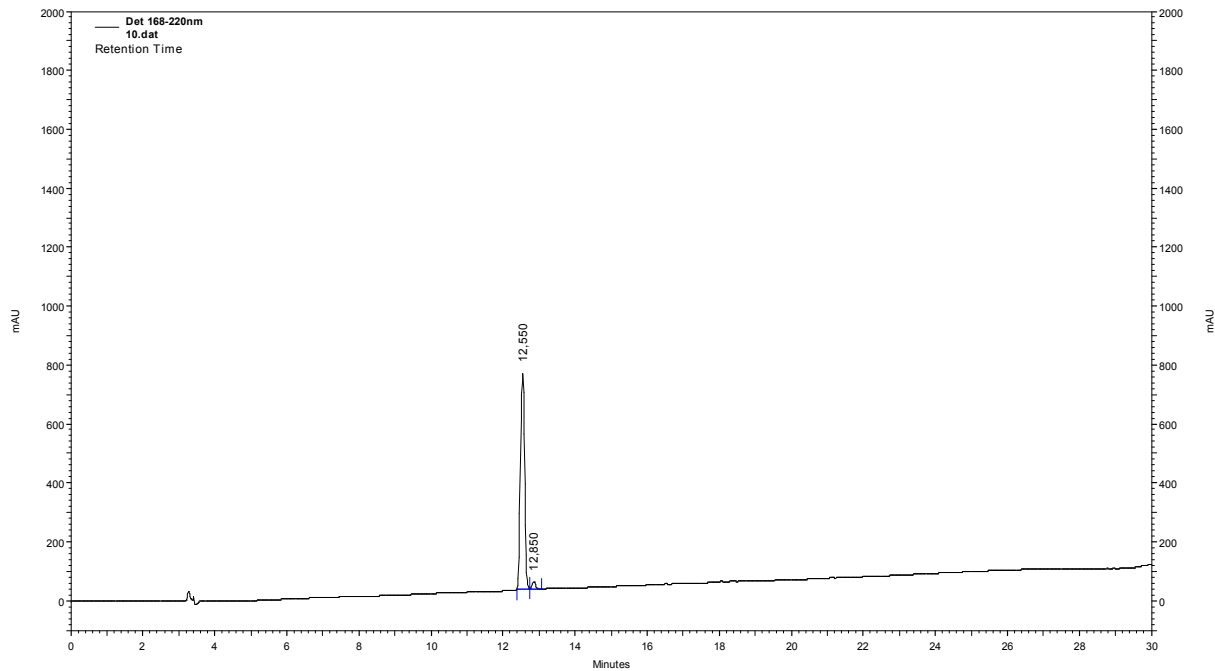
¹H-NMR compound **10**:



^{13}C -NMR compound **10**:



Purity grade by HPLC of compound **10**:



Det 168-220nm

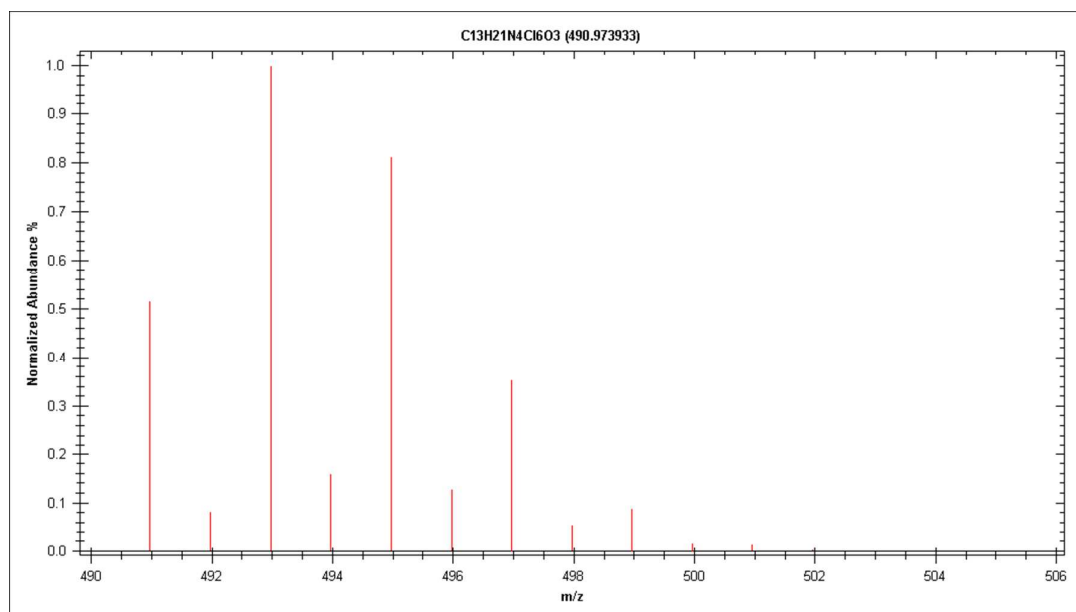
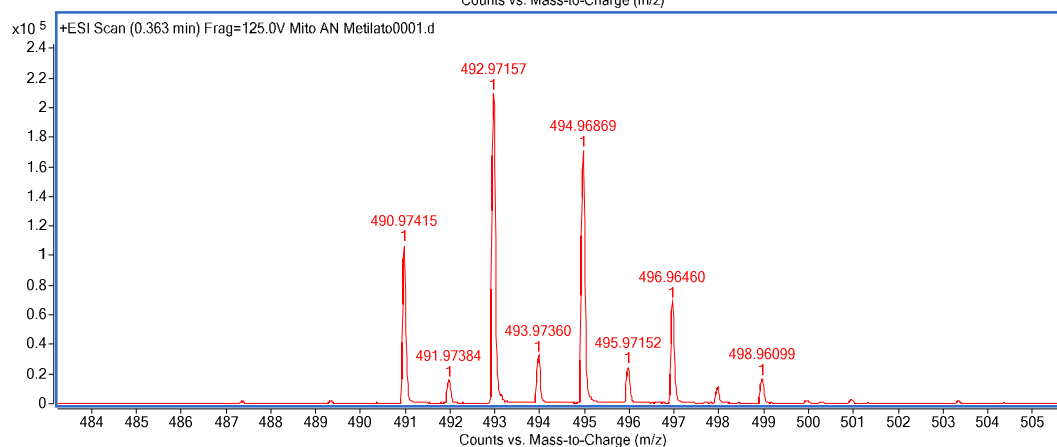
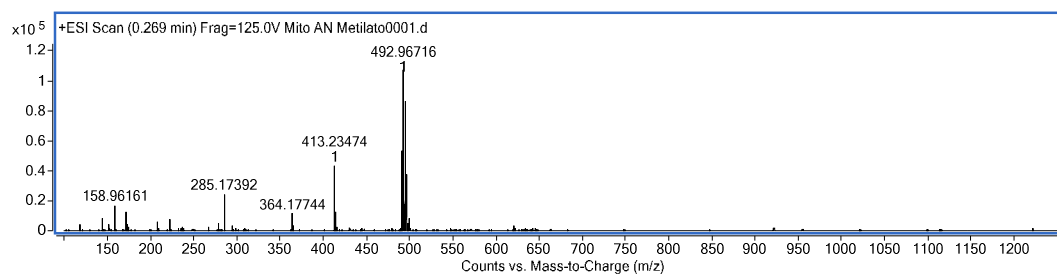
Results

Time	Area	Area %	Height	Height %
12,550	5561952	96,64	731875	96,57
12,850	193209	3,36	26007	3,43

Totals	5755161	100,00	757882	100,00
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Synthesis of 2-(2,2-dichloroacetamido)-N,N-bis(2-(2,2-dichloroacetamido)ethyl)-N-methylethanaminium trifluoroacetate (**11**)

HRMS (ESI) of compound **11**: $[M+H]^+ = 490.974115$



Calc. Mass	Mis. Mass	Error ppm
490.973933	490.974115	0.370692

MITO-AN-DCA_lifilizzato_1H

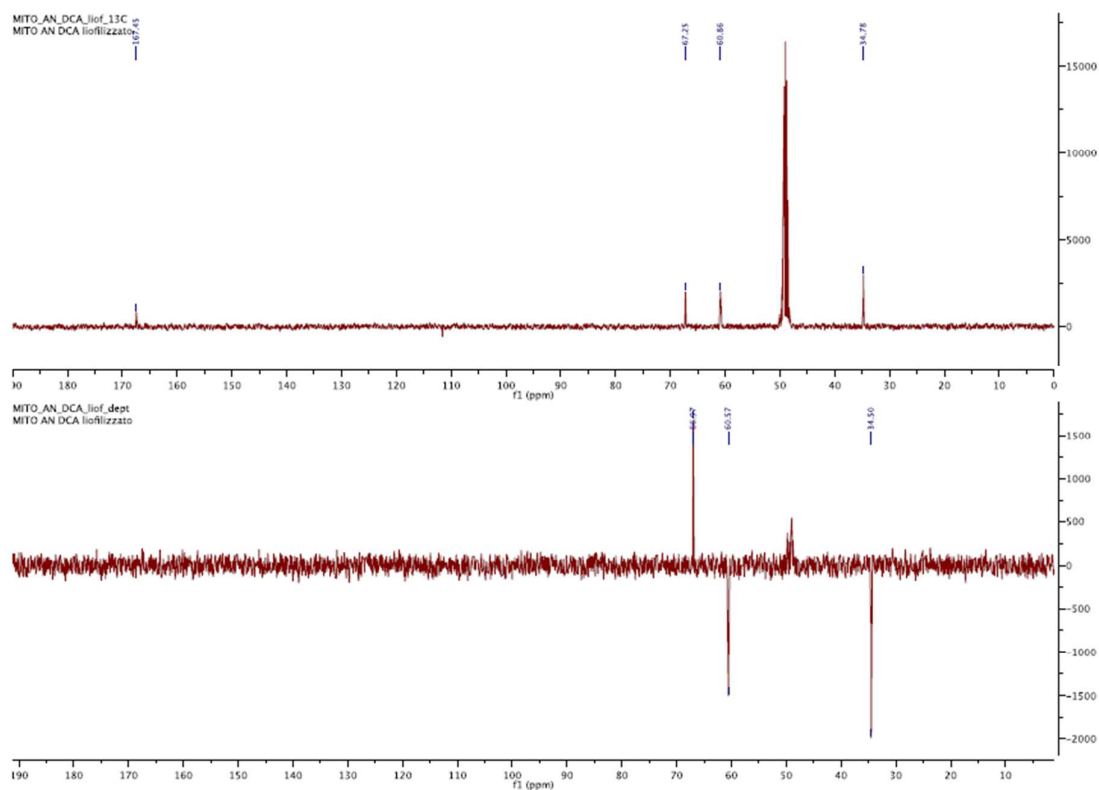
MITO-AN-DCA_lifilizzato

Chemical structure of MITO-AN-DCA is shown in the top left corner.

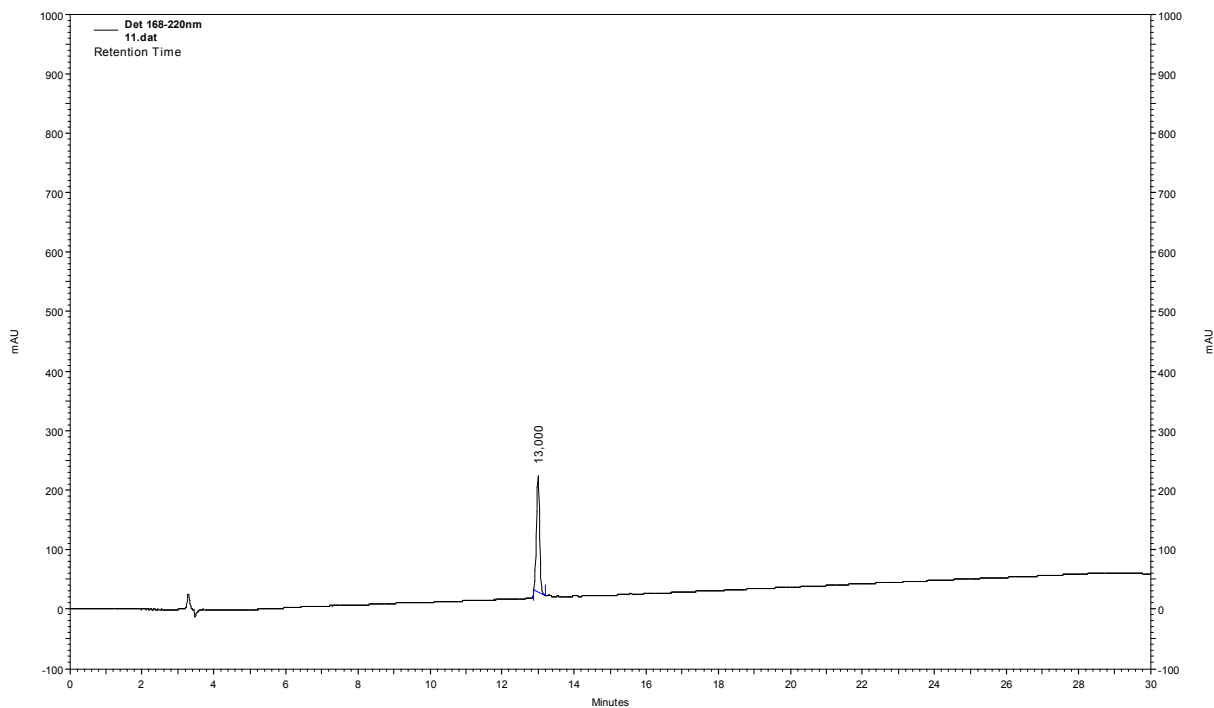
¹H NMR spectrum (ppm) showing peaks at 6.34, 4.88, 3.28, 3.61, 3.79, 3.78, 3.63, 3.61, 3.59, and 3.58 ppm. The x-axis ranges from 7.4 to 1.8 ppm. The y-axis ranges from -200 to 2800.

Integration values are provided for the peaks: 2.61, 6.01, 6.00, and 2.89.

^{13}C -NMR and DEPT compound **11**:



Purity grade by HPLC of compound **11**:

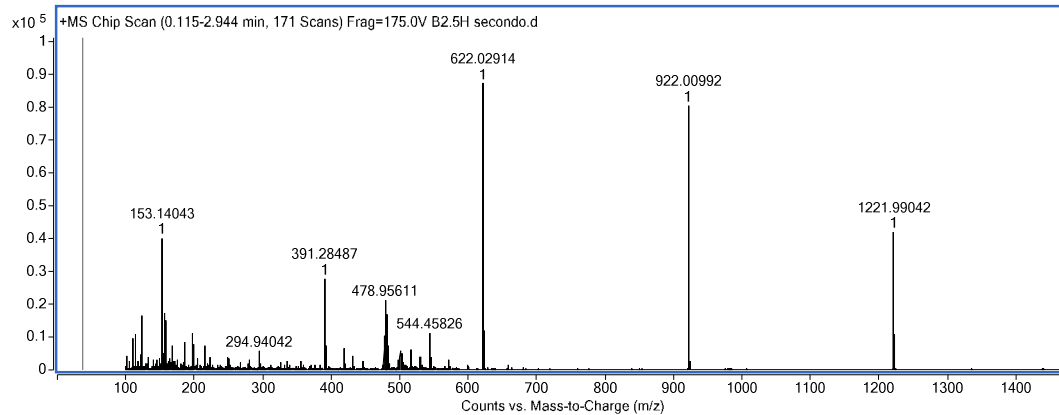
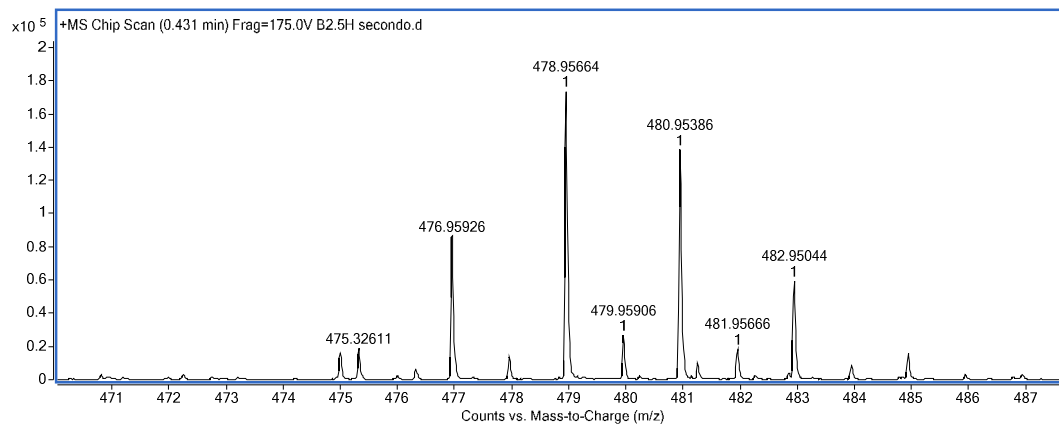
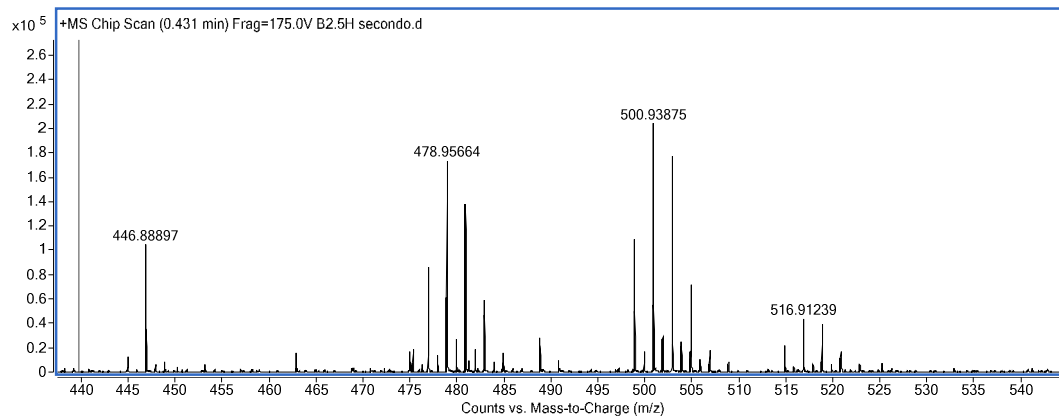
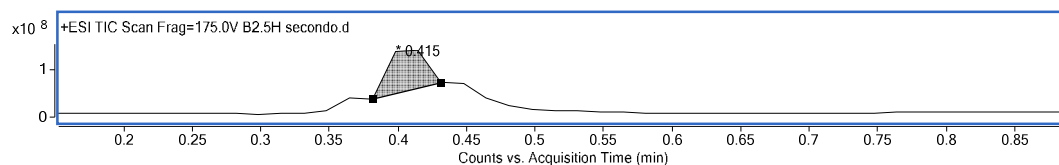


Det 168-220nm**Results**

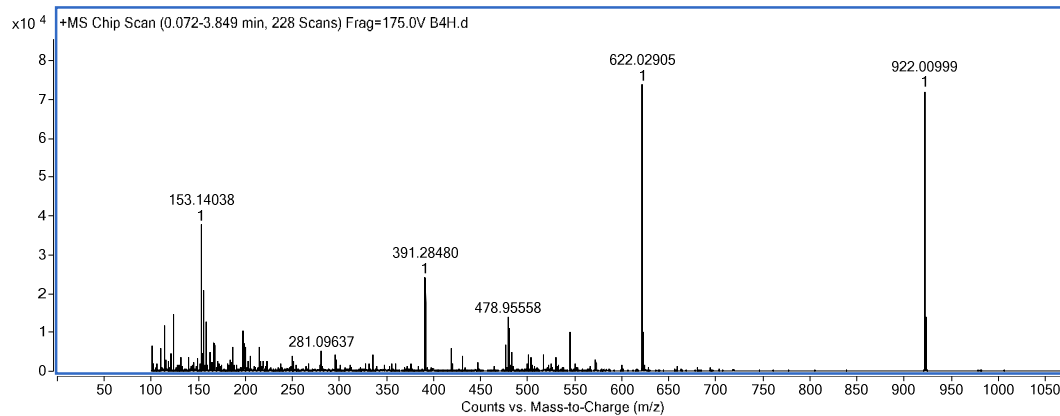
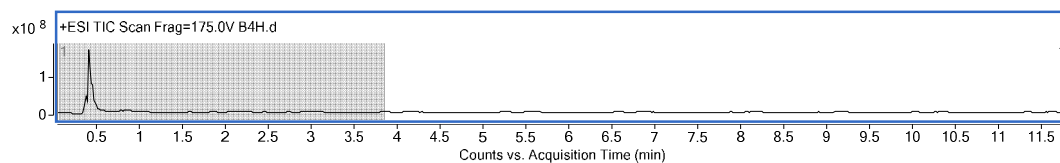
Time	Area	Area %	Height	Height %
13,000	1209324	100,00	193908	100,00

Totals	1209324	100,00	193908	100,00
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LC-MS spectra of mouse plasma at 2.5h



LC-MS spectra of mouse plasma at 4h



LC-MS spectra of mouse plasma at 7h

