

# Mode of Action Study of Cationic Anthraquinone Analog: A New Class of Highly Potent Anti-cancer Agent

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## **Supporting Information**

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2. DNA cleaving experiment (S6-S7)
3. Alkylation experiment (S8)
4. Cyclic Voltammetry Measurements (S9-S18)

## 1. General procedures, Material and Methods

### **Materials:**

All the anticancer compounds (**1c-9c**) used for biological assay were synthesized in our laboratory with purity >95%, confirmed by HPLC analysis. Etoposide was purchased from Sigma-Aldrich Co (St Louis, MO). Cell culture medium was purchased from Gibco-Life Technologies (Grand Island, NY).

**Cell culture:** A549 and MRC-5 cells were grown in commercial DMEM 1X (Gibco) with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO<sub>2</sub>. The cells were allowed to adhere for 48 h before drug treatment.

**Cell viability assay.** The cells were seeded in 96-well microtiter plates (3000/200 µL). After 48 h of incubation in the corresponding media, cells were treated with various concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) for 48 h. 20 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Upon completion of incubation, the media was carefully removed and washed twice with 100 mL of pbs buffer. Then, 100 µL of DMSO was added to each well; agitated on orbital shaker for 15min., and the absorbance at 590 nm with 620 nm filter was determined with a microplate reader. The results were expressed as viability compared with that of control. The experiment was carried out in triplicate in three independent experiments.

**Flow cytometry analysis.** “BD Biosciences Special Order FACS Aria™ II” was used for flow cytometry analysis. The service was provided by Center for Integrated Biosystems (CIB), Utah State University.

**Procedure for cell cycle analysis.** A549 cells ( $5 \times 10^4$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0  $\mu$ M) were added and incubated for 24 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The cells were washed by 1 mL of PBS buffer and collected by centrifugation. The pelleted cells were then re-suspended in 0.3 mL of PBS buffer, fixed with 0.7 mL of ice cold ethanol and left at 4 °C for overnight. The cells were then centrifuged and collected, washed with PBS buffer and re-centrifuged. The pelleted cells were then re-suspended in 0.25 mL of PBS buffer and 5  $\mu$ L of 10 mg/mL Rnase A was added. The solution was incubated at 37 °C for 1 h. 10  $\mu$ L of 1 mg/mL PI solution was added and left in the dark for FACS analysis (488nm).

**Procedure for annexin-V/PI apoptosis assay.** A549 cells ( $5 \times 10^4$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0  $\mu$ M) were added and incubated for 24 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The cells were washed by 1 mL of PBS buffer and collected by centrifugation. The cell pellet was resuspended in 500  $\mu$ L of 1X binding buffer (Annexin V/PI assay kit, Biovision, USA.); 5  $\mu$ L of annexin V-PI was added, and incubated at room temperature for 5 min in the dark. The cells were then analyzed by flow cytometry at ex. 488 nm and em. 578nm.

**Procedure for ROS measurement assay.** A549 cells ( $5 \times 10^4$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. 2 mL solution of DCFH-DA (10  $\mu$ M) in serum free media was added and incubated for 1 h. The cells were washed with 1 mL of PBS buffer and different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0  $\mu$ M) in normal media were added and

incubated for 4 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The pelleted cells were washed once with 1 mL of PBS buffer and centrifuged. The cell pellets were then diluted with 500 µL of PBS buffer and analyzed in FACS scan at Ex/Em: ~ 492 – 495/517 – 527 nm.

**Procedure for mitochondrial membrane potential measurement.** A549 cells ( $5 \times 10^4$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 24 h. 100 mg/mL rhodamine 123 stock solution was directly added to the culture media and incubated at 37 °C for 30 min. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The pelleted cells were washed twice with 1 mL of PBS buffer and centrifuged. The cell pellets were then diluted with 500 µL of PBS buffer analyzed in FACS scan.

**Procedure for glutathione depletion assay.** A549 cells ( $5 \times 10^4$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 48 h. The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min. The level of glutathione was measured with modified Glutathione Fluorometric assay kit (Biovision, catalog number: K51-100). Fluorescence intensities were measured using 96 well plate readers (BioTek Synergy H4 reader with Gen5 software) with 360 nm excitation and 461 nm emission.

**Procedure for caspase-3 release assay.** A549 cells ( $1 \times 10^6$  cells/2ml) were cultured in 6-well plates and allowed to adhere for 48 h. Different concentrations of **2c** (0.0, 0.01, 0.10, 1.0, 10.0, and 100.0 µM) were added and incubated for 4, 12 h and 48 h . The adherent cells were removed by 0.25% Trypsin-EDTA solution and harvested by centrifugation (1200 rpm at 4 °C) for 5 min.

The level of Caspase-3/CPP32 was measured with Caspase-3/CPP32 fluorometric assay kit (Biovision, catalog number: K105-25). Fluorescence intensities were measured using 96 well plate reader (BioTek Synergy H4 reader with Gen5 software) with 400 nm excitation and 505 nm emission.

**Procedure for fluorescence imaging.** Following the same procedure as cell viability, cells are seeded in  $\mu$ -Slide 8 well plate (CVRGLS LABKITKII 8 Well#1.5). The morphological studies in cells were studied in phase contrast mode while nuclear condensation (DAPI nuclear stain) and mitochondrial morphology (Rhodamine 123) were studied by fluorescence microscopy (Olympus IX70). The images were recorded by spot RT color camera (model: 2.2.1) and processed with spot 5 software.

**Procedure for cyclic voltammetry measurement.** Cyclic Voltammetry was performed in a BASi-Epsilon EC system using 0.1 M Tetrabutylammonium hexafluorophosphate (TBAF) for electrochemical analysis (purity  $\geq 99.0\%$ ) in an anhydrous Acetonitrile as a supporting electrolyte. The 3 mm glassy carbon electrode was used as the working electrode, together with a platinum counter-electrode and a Ag/AgCl reference electrode. The working electrode was polished with 0.05  $\mu$ M alumina, washed with dH<sub>2</sub>O, and sonicated for 5 min. before using for each sample. 2 mM samples in supporting electrolyte were prepared just before the electrochemical experiment, degassed with N<sub>2</sub> for 15 min., and readings were taking under N<sub>2</sub> blanket.

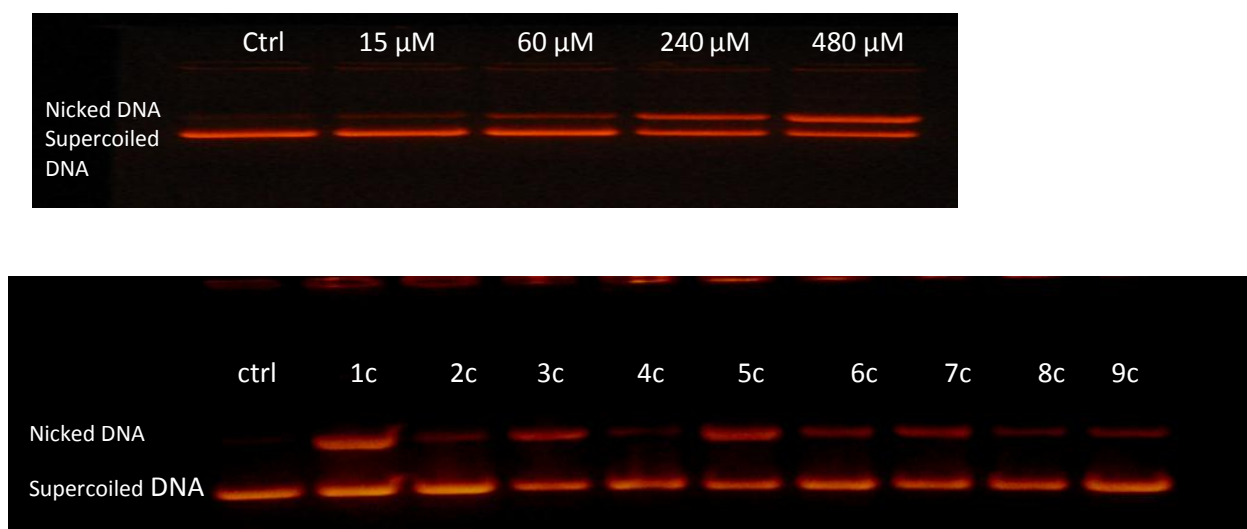
**Statistical data analyses.** All results are presented as the mean  $\pm$  standard deviation of three independent experiments using GraphPad software using an unpaired t-test. The results were considered significant at  $P < 0.05$  (\* =  $P \leq 0.05$ ; \*\* =  $P \leq 0.01$ ; \*\*\* =  $P \leq 0.001$ ; and \*\*\*\* =  $P \leq 0.0001$ ).

## 2. DNA cleaving experiment

**Procedure for DNA cleavage experiment.** The DNA cleavage experiments of supercoiled pUC19 plasmid DNA 0.75  $\mu\text{L}$  (~125 ng) by **1c** (15, 30, 60, 120, 240, and 480  $\mu\text{M}$ ) containing ~3% DMSO in (10mM Tris-HCl, 1mM EDTA Buffer and 50mM NaCl) buffer at pH 8.0 were performed by agarose gel electrophoresis (Fisher Biotech Electrophoresis FB-SB-170 System). The samples were incubated for 24 h at 37 °C in the dark. A 3.3  $\mu\text{L}$  loading dye containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol , 30% (v/v) glycerol was added and electrophoresis was carried out at 65 V for 55 min in Tris–Acetate–EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA) using 1% agarose gel containing 0.5  $\mu\text{g/mL}$  ethidium bromide. The gels were viewed in an Alpha Innotech Corporation Gel doc system at 302 nm and photographed using an 8 MP Olympus digital camera. Following the same condition, electrophoresis was carried for rest of the compounds at 240  $\mu\text{M}$  concentrations.

**Result for DNA cleavage experiment.** Numerous studies have reported that compounds bearing quinone motif are capable of causing photo-induced, transition metal complex-assisted, or oxidative DNA cleavage.<sup>21</sup> We selected compound **1c** for the initial DNA cleavage study using supercoiled pUC 19 DNA, which has been commonly used in DNA cleavage studies. The experiments were conducted using Tris buffer (pH 8). To our surprise, compound **1c** manifests a concentration dependent DNA cleavage without the presence of transition metal complex, light irradiation or oxidative reagent (**Figure 7A**). One dominant form of nicked supercoiled DNA (form II) was noted with concentrations of **1c** at 240 and 480  $\mu\text{M}$  while no or very little DNA cleavage at the lower tested concentrations (15 and 60  $\mu\text{M}$ ). We then tested the remaining compounds at 240  $\mu\text{M}$ ; and all the cationic 1,4-naphthoquinone analogs

were capable of cleaving DNA (**Figure 7B**). However, there is no obvious correlation between the anticancer activities and the extent of DNA cleavage. For example, compound **2c** has similar anticancer activity as **1c** but cause much less DNA cleavage as compared to compound **1c**. The lack of correlation and DNA cleavage only at much higher concentration than  $IC_{50}$ , can be attributed to the possibility that the direct DNA damage may not be the primary mode of its anticancer activity. However, this property is unique to these cationic 1,4-naphthoquinone analogs and is different from other reported classes of DNA-cleaving agents.

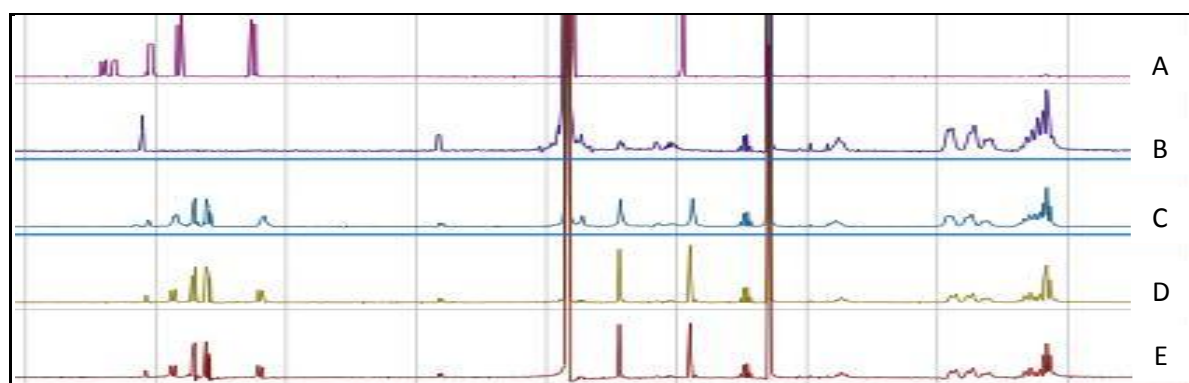


**Figure 7:** (A) Concentration dependent DNA cleavage experiment using **1c**; (B) DNA cleavage experiment for all other cationic anthraquinone analogs using 240  $\mu$ M drug concentrations.

### 3. Alkylation experiment

**Cationic 1,4-naphthoquinone analogs are not alkylating agents.** Mitomycin C, a quinone motif bearing anticancer drug is known to alkylate nucleic acid after bioreductive activation. These cationic 1,4-naphthoquinone analogs may also function as alkylating agents (methylation) via the N-3 methyl group. The alkylation experiment was carried out with guanosine mono-phosphate (GMP). GMP was ion exchange with cyclohexyl amine to increase its solubility in methanol. The mixture of **2c** and GMP were incubated at 37 °C, and analyzed by  $^1\text{H}$  NMR at different time intervals (**Figure 6**). It is expected that the methylation of GMP will result in the appearance of new methyl peak; and a change in the chemical shift of anomeric proton. The loss of methyl from **2c** will result in the formation of new compound **2c** (**scheme 1**), which has a very different pattern in the  $^1\text{H}$  NMR chemical shift. No shift in anomeric proton and no appearance of new signals in  $^1\text{H}$  NMR demonstrate that they are not an alkylating agent.

*An alkylation experiment was carried in NMR tube for upto 48 h.*



**Figure S2:** *2c* alkylation experiment on GMP. A = **2c**, B = GMP complexed with cyclohexyl ammonium ion, C = 0 min., D = 24 h, E = 48 h.



## 4. Cyclic Voltammetry Measurements

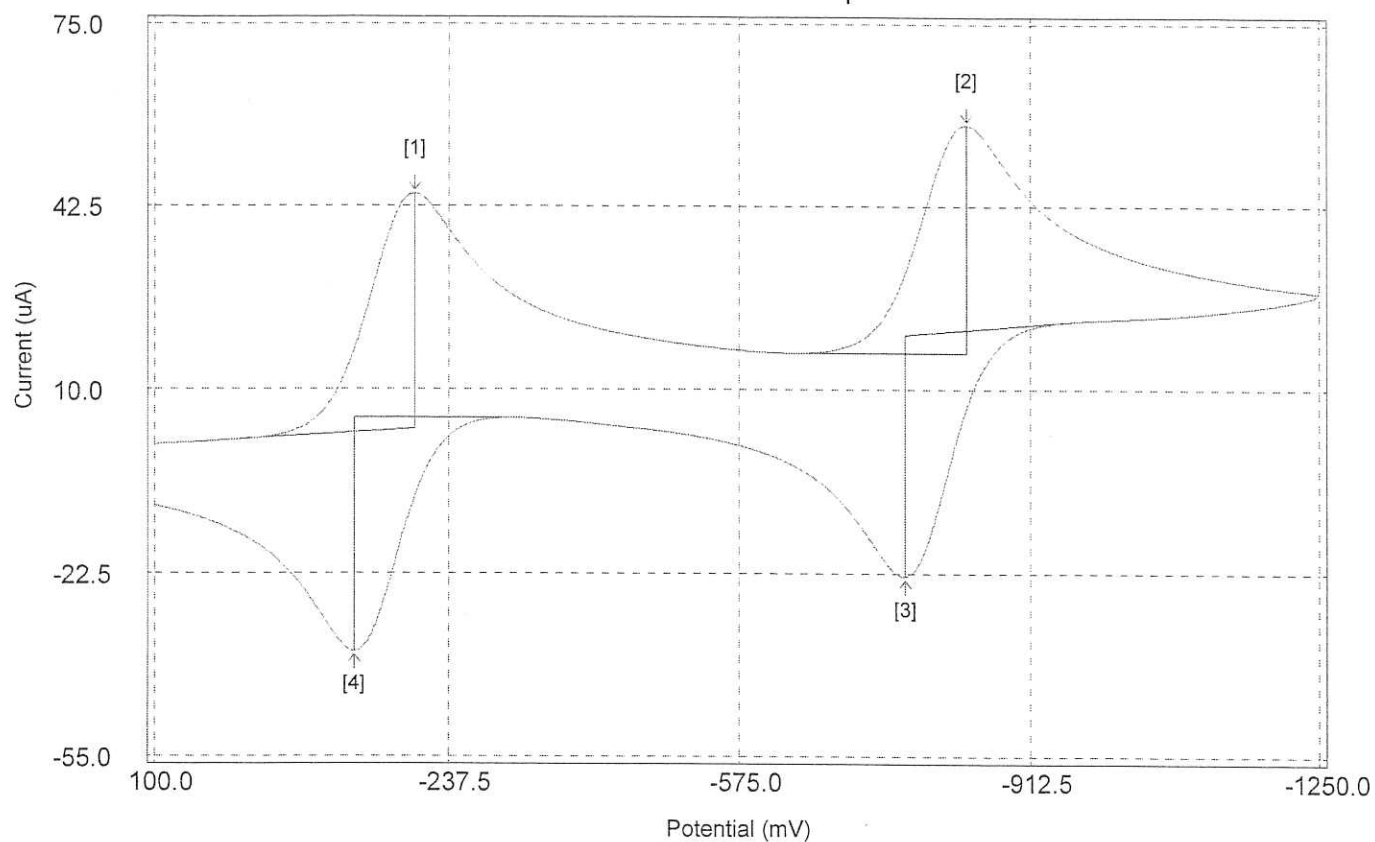
### 1. Summary of Cyclic Voltammetry

Compound	first wave					Second wave				
	Pc	Pa	$\Delta ep$	Electron	E1/2	Pc	Pa	$\Delta ep$	Electron	E1/2
<b>1c</b>	-198	-129	-69	-0.86	-163.5	-901	-832	-69	-0.86	-866.5
<b>2c</b>	-217	-148	-69	-0.86	-182.5	-921	-849	-72	-0.82	-885
<b>4c</b>	-211	-141	-70	-0.84	-176	-848	-775	-73	-0.81	-811.5
<b>5c</b>	-165	-96	-69	-0.86	-130.5	-858	-789	-69	-0.86	-823.5
<b>3c</b>	-182	-115	-67	-0.88	-148.5	-878	-806	-72	-0.82	-842
<b>6c</b>	-191	-122	-69	-0.86	-156.5	-900	-832	-68	-0.87	-866
<b>8c</b>	-202	-134	-68	-0.87	-168	-902	-832	-70	-0.84	-867
<b>7c</b>	-217	-148	-69	-0.86	-182.5	-857	-667	-190	-0.31	-762
<b>9c</b>	-217	-147	-70	-0.84	-182	-923	-848	-75	-0.79	-885.5

### 2. Cyclic Voltammetry for the synthesized compounds

Compound	Page
<b>1c</b>	S10
<b>2c</b>	S11
<b>4c</b>	S12
<b>5c</b>	S13
<b>3c</b>	S14
<b>6c</b>	S15
<b>8c</b>	S16
<b>7c</b>	S17
<b>9c</b>	S18

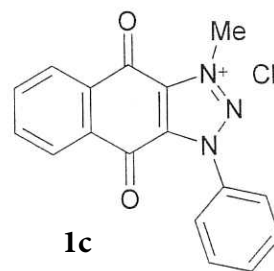
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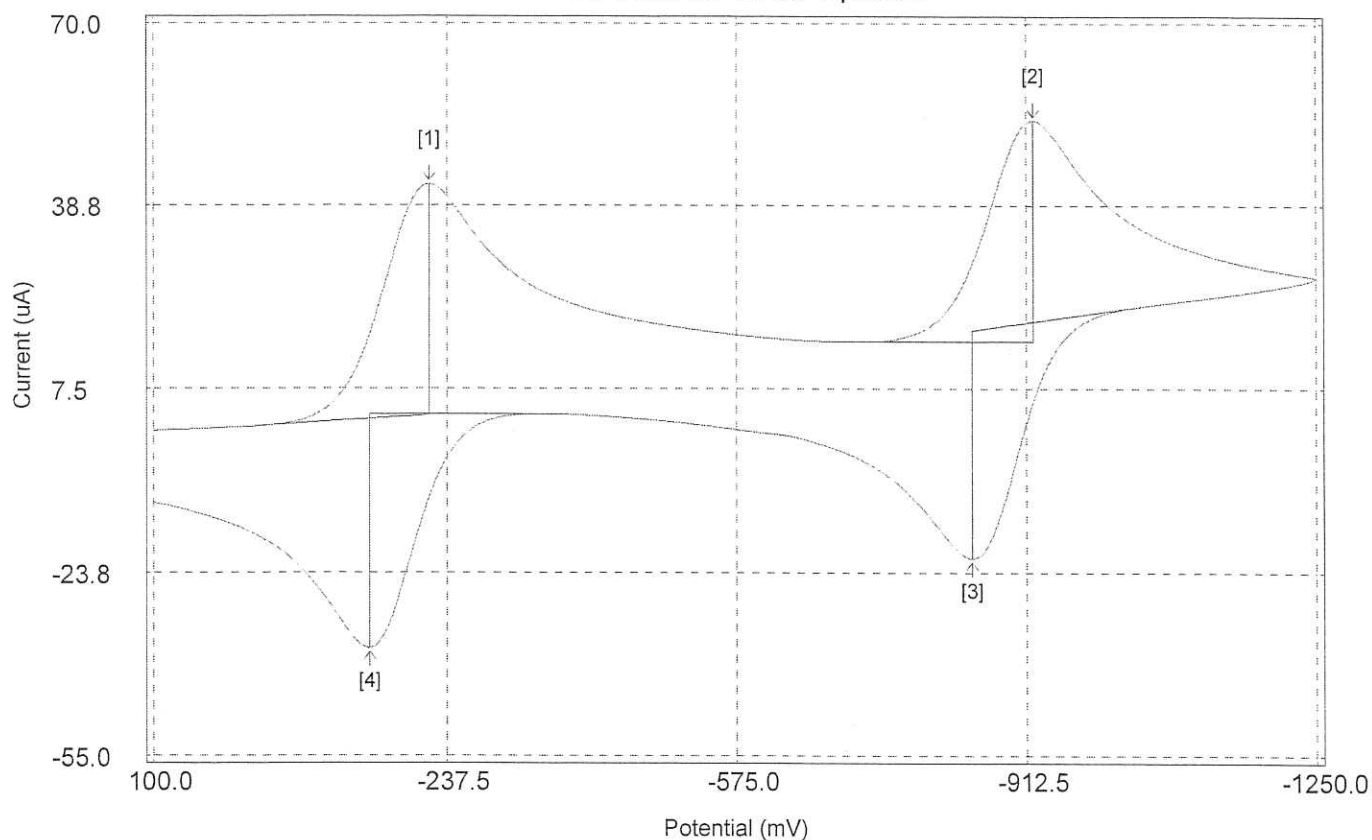
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Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -198 [mV], 41.7975 (uA), 25.8030 (uC)
peak [2]	@ -838 [mV], 40.5371 (uA), 25.4673 (uC)
peak [3]	@ -767 [mV], 42.7680 (uA), 26.6688 (uC)
peak [4]	@ -128 [mV], 41.3672 (uA), 24.9104 (uC)



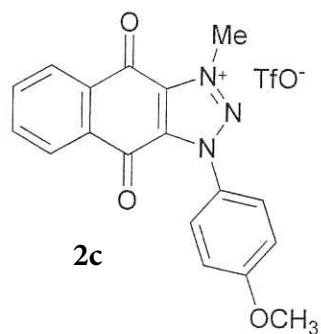
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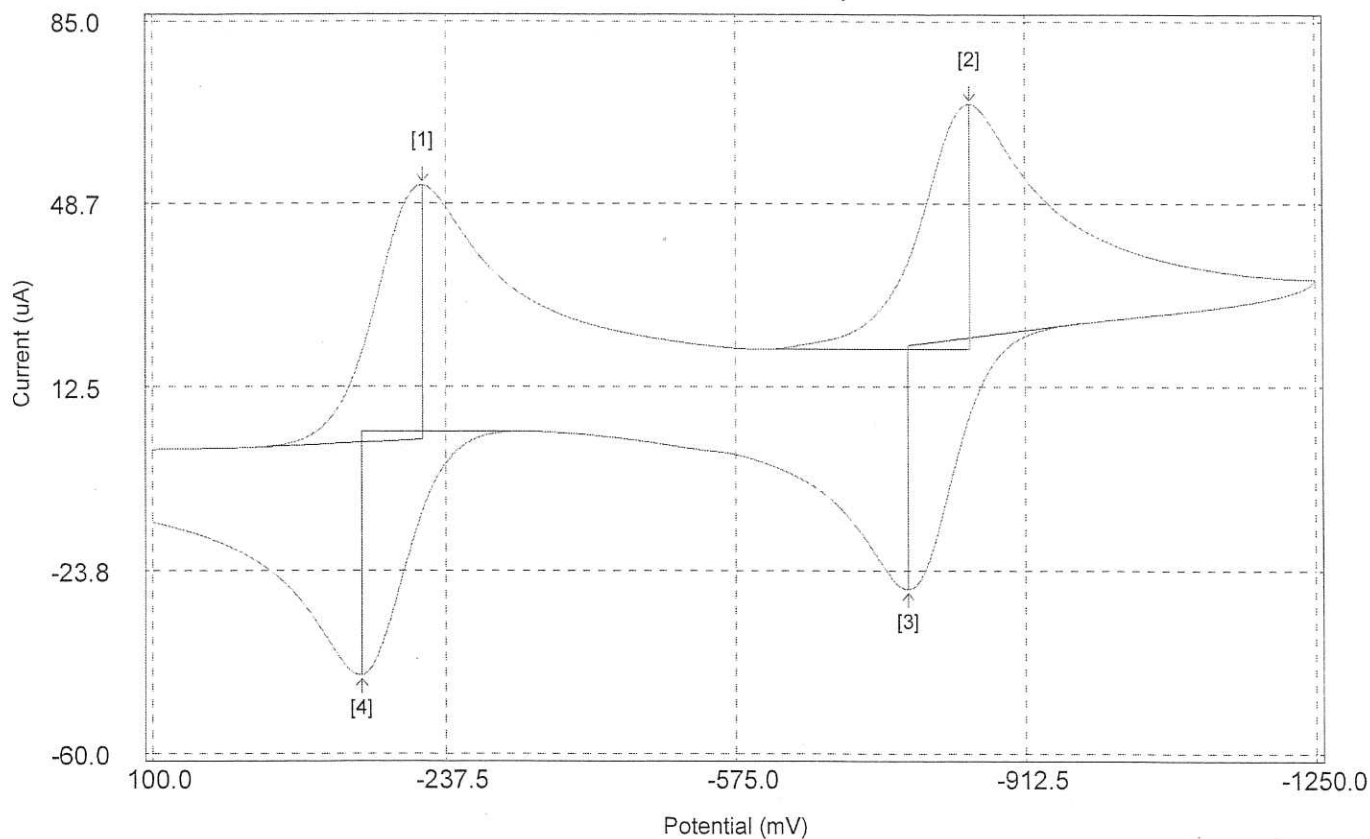
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Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -217 [mV], 39.3439 (uA), 24.2799 (uC)
peak [2]	@ -921 [mV], 37.7234 (uA), 23.8566 (uC)
peak [3]	@ -849 [mV], 38.8342 (uA), 24.7551 (uC)
peak [4]	@ -148 [mV], 39.8474 (uA), 24.7087 (uC)



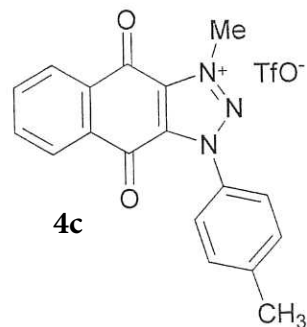
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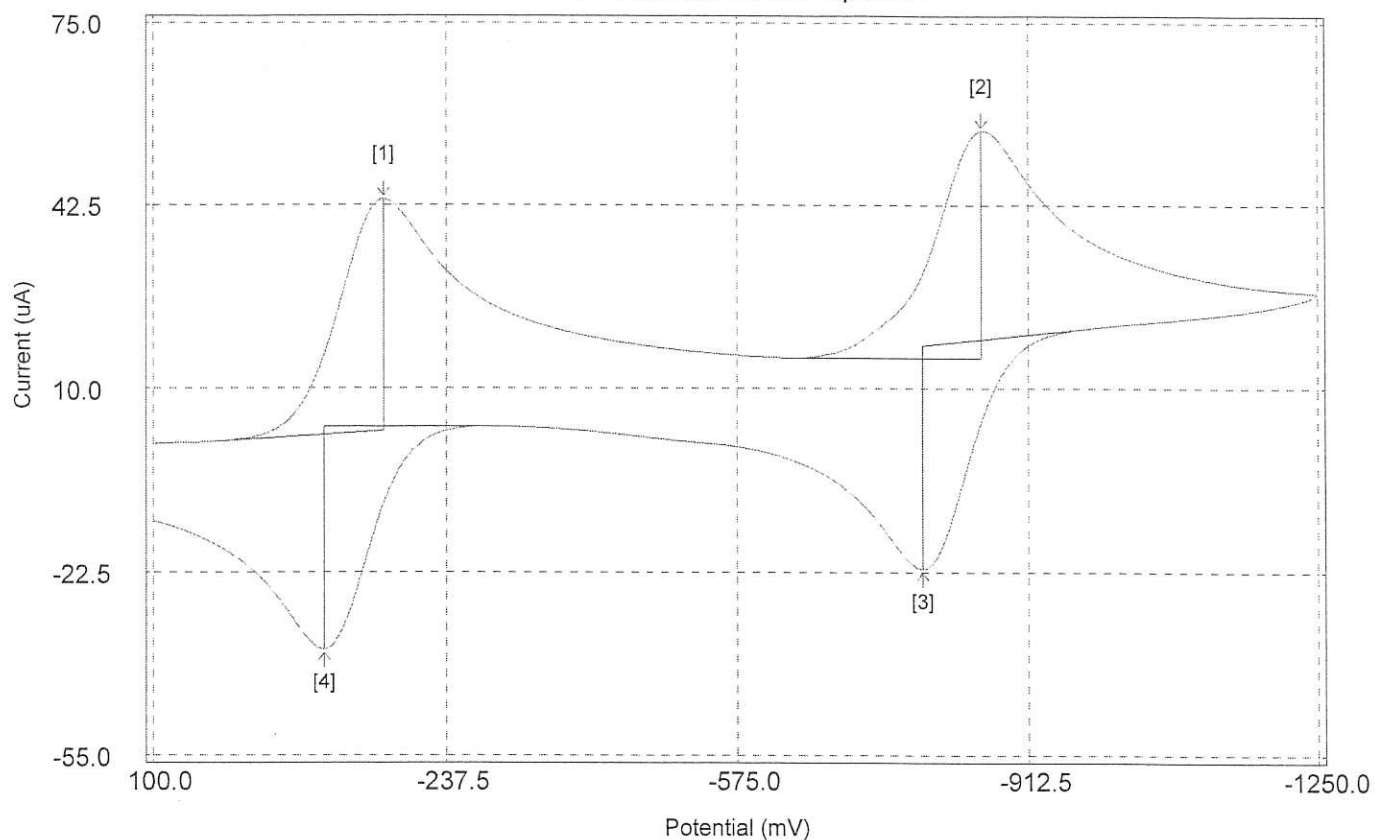
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Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -211 [mV], 50.3700 (uA), 31.1904 (uC)
peak [2]	@ -848 [mV], 48.6000 (uA), 32.6930 (uC)
peak [3]	@ -775 [mV], 48.2368 (uA), 29.8285 (uC)
peak [4]	@ -141 [mV], 48.1392 (uA), 29.2944 (uC)



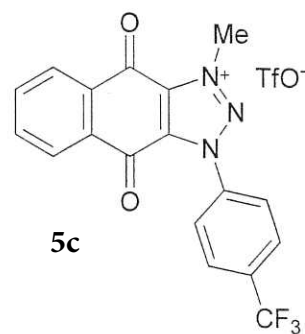
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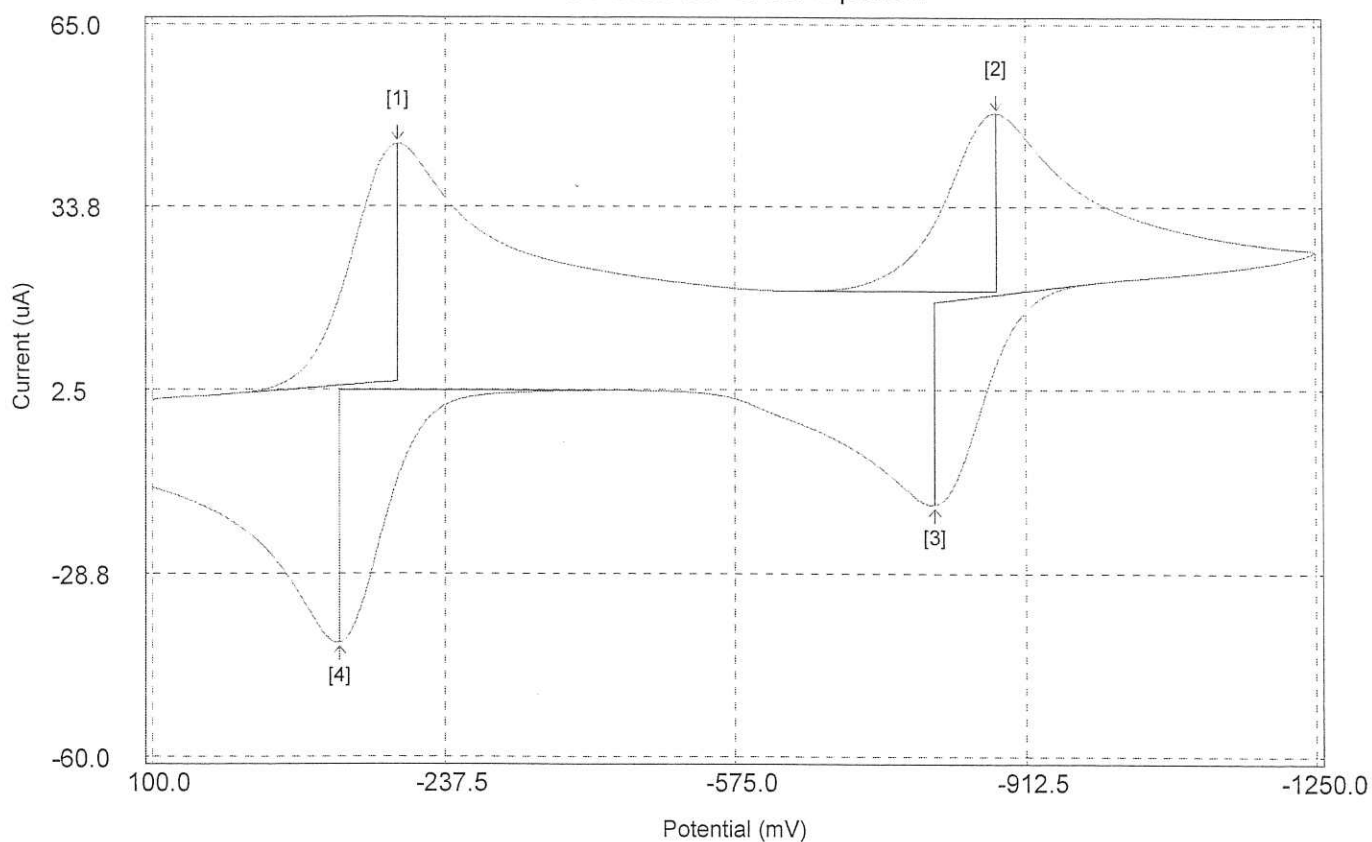
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Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

### Peak Data:

peak [1]	@ -165 [mV], 41.0773 (uA), 25.0195 (uC)
peak [2]	@ -858 [mV], 40.3143 (uA), 27.6188 (uC)
peak [3]	@ -789 [mV], 39.5575 (uA), 24.6871 (uC)
peak [4]	@ -96 [mV], 39.5392 (uA), 24.1784 (uC)



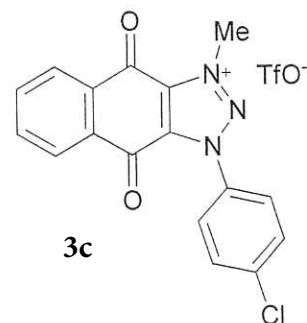
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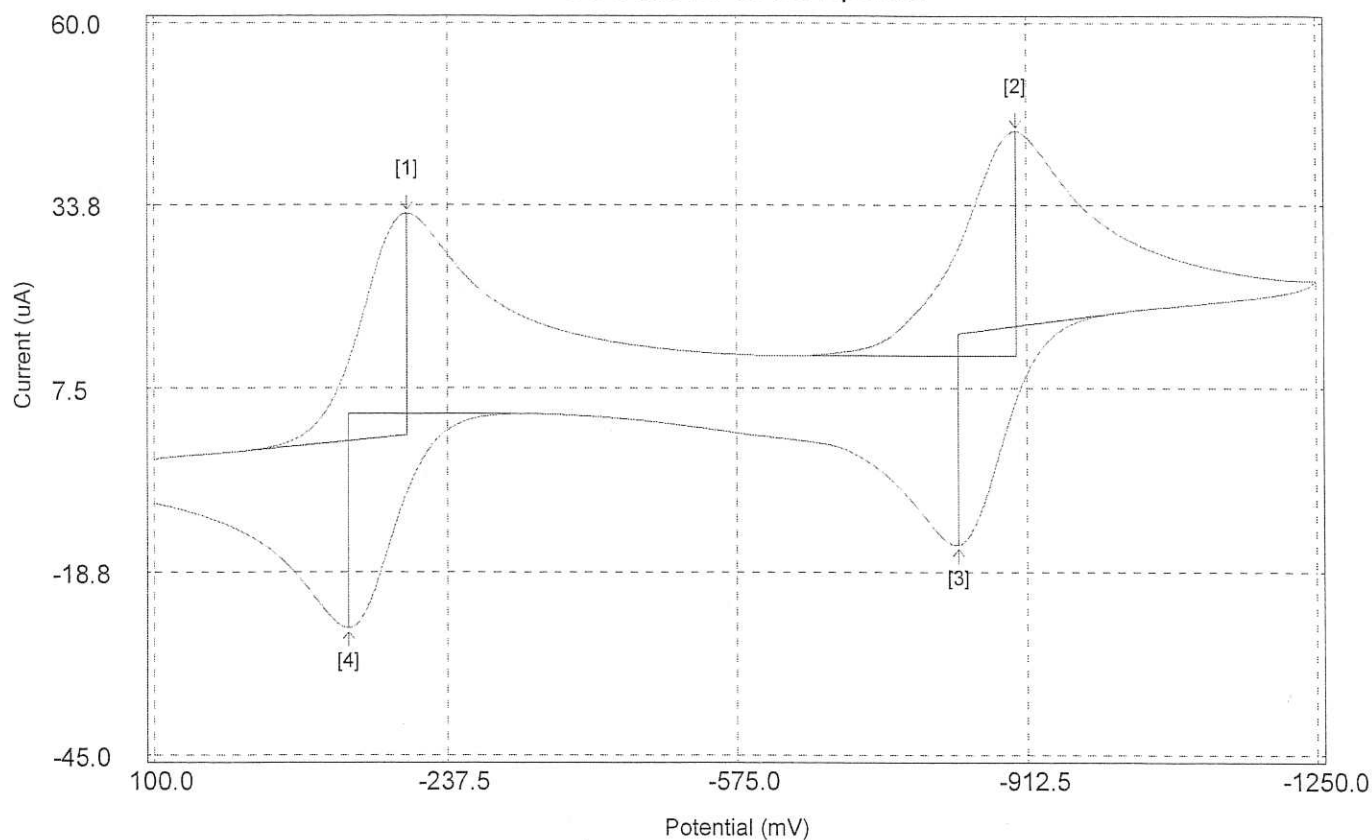
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Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

### Peak Data:

peak [1]	@ -182 [mV], 40.5127 (uA), 25.2514 (uC)
peak [2]	@ -878 [mV], 30.4387 (uA), 20.6920 (uC)
peak [3]	@ -806 [mV], 34.4762 (uA), 22.7076 (uC)
peak [4]	@ -115 [mV], 42.9847 (uA), 27.1775 (uC)



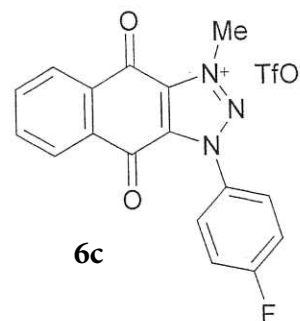
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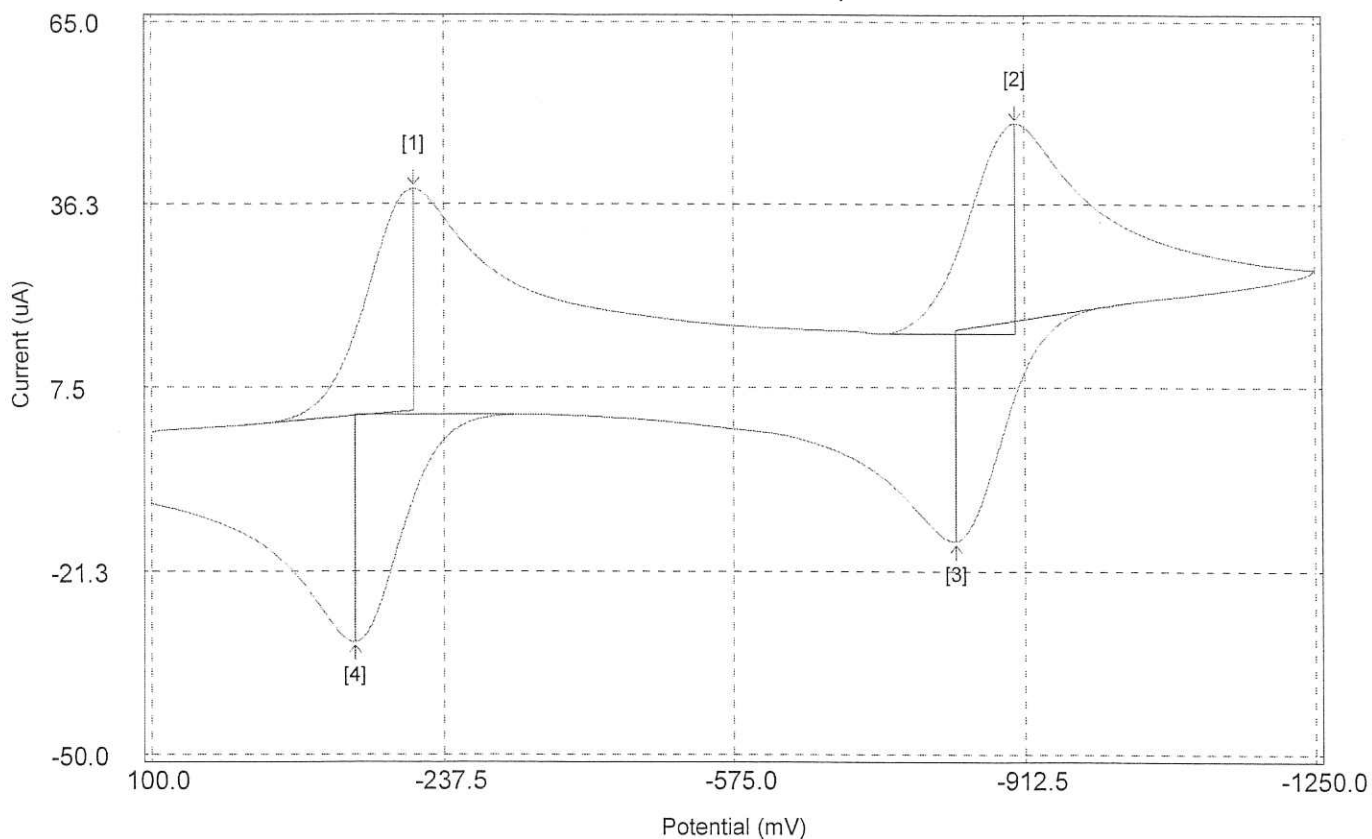
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Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -191 [mV], 31.7540 (uA), 19.5369 (uC)
peak [2]	@ -900 [mV], 32.2240 (uA), 24.3378 (uC)
peak [3]	@ -832 [mV], 30.2129 (uA), 18.7691 (uC)
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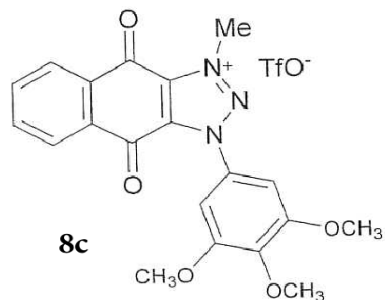
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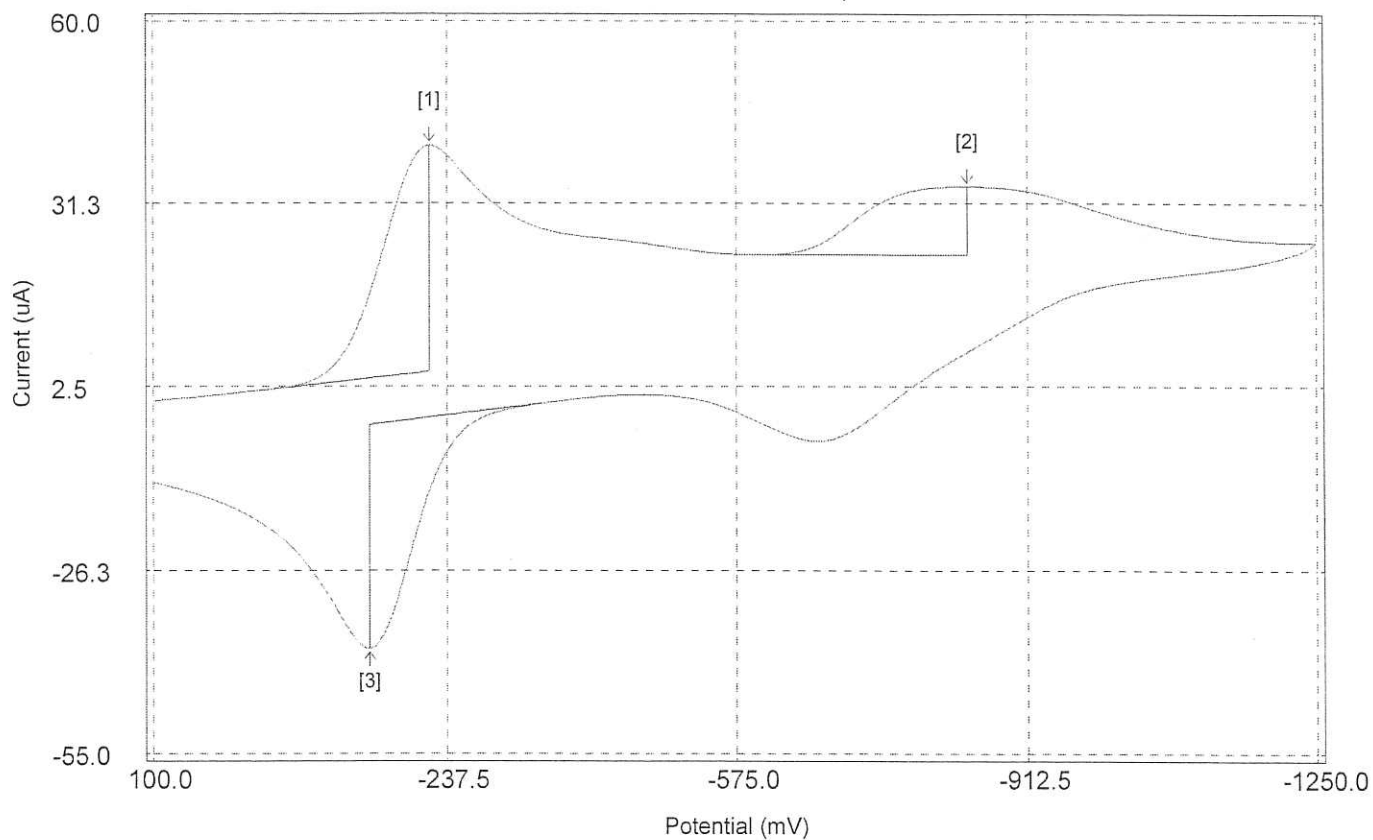
### Peak Data:

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peak [2] @ -902 [mV], 33.0877 (uA), 20.2098 (uC)
peak [3] @ -832 [mV], 33.0053 (uA), 21.0294 (uC)
peak [4] @ -134 [mV], 35.6542 (uA), 22.0838 (uC)





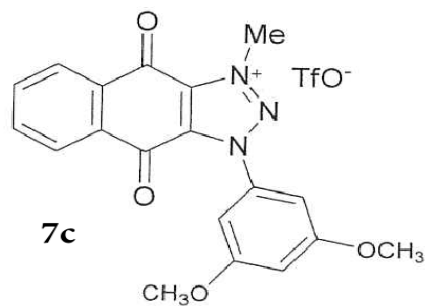
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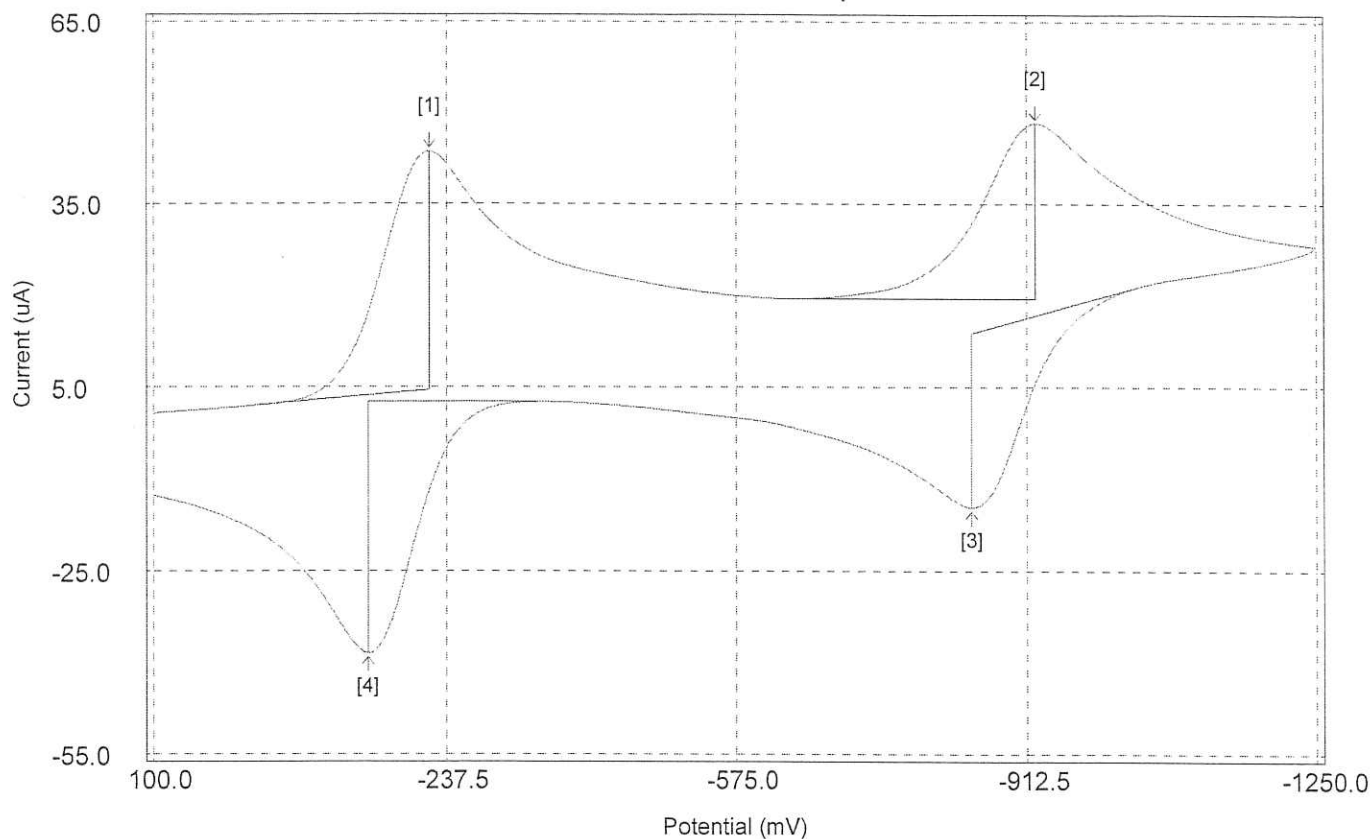
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Final Potential	: 100 (mV)
Number of segments	: 2
Scan rate	: 100 (mV/s)
Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -217 [mV], 35.4437 (uA), 22.2590 (uC)
peak [2]	@ -843 [mV], 10.7698 (uA), 13.7671 (uC)
peak [3]	@ -148 [mV], 35.0591 (uA), 21.0684 (uC)



### CV Run for BASi-Epsilon



Data File Name	: C:\Users\BASi Customer\Desktop\jayCVfinal\mqm132.CV0
Title	: CV Run for BASi-Epsilon
Experiment Type	: Cyclic Voltammetry (CV)
Printed	: 4/29/2013 6:13:50 PM
Date & Time of the run	: 4/29/2013 6:13:01 PM
Number of data points	: 2700
Display Convention	: POLAROGRAPHIC
Initial Potential	: 100 (mV)
Switching Potential 1	: -1250 (mV)
Final Potential	: 100 (mV)
Number of segments	: 2
Scan rate	: 100 (mV/s)
Current Full Scale	: 100 uA
Filter	: 10 Hz
Quiet Time	: 2 (Sec)
Sample Interval	: 1 mV

#### Peak Data:

peak [1]	@ -217 [mV], 39.0509 (uA), 23.8808 (uC)
peak [2]	@ -923 [mV], 28.7633 (uA), 22.4477 (uC)
peak [3]	@ -848 [mV], 28.4581 (uA), 20.1368 (uC)
peak [4]	@ -147 [mV], 41.1536 (uA), 25.4124 (uC)

