Supporting Information

Selective Cell Death by Photochemically Induced pH Imbalance in Cancer Cells

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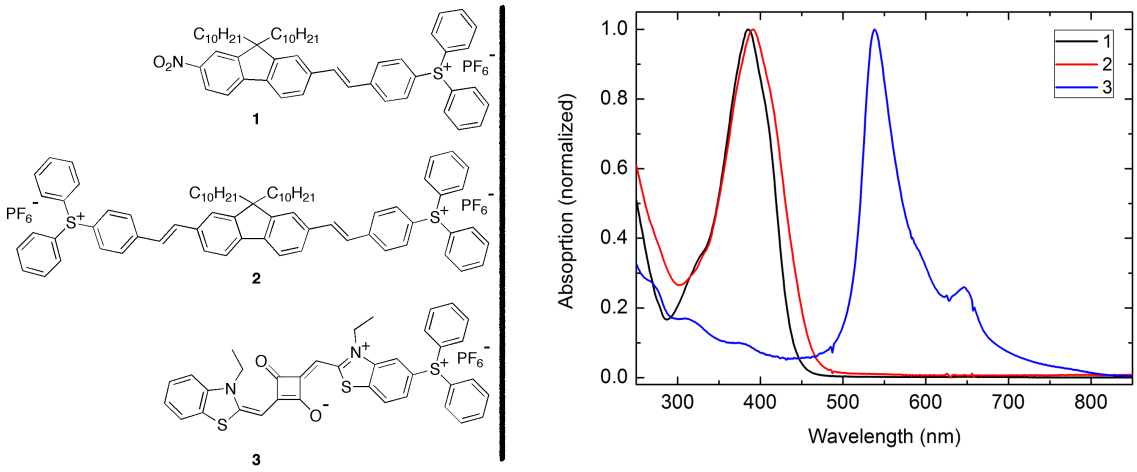


Figure S1. Sulfonium salt 2PA PAGs structures. One-photon absorption spectra of PAGs **1**-**3** in PBS following PL-127® encapsulation.

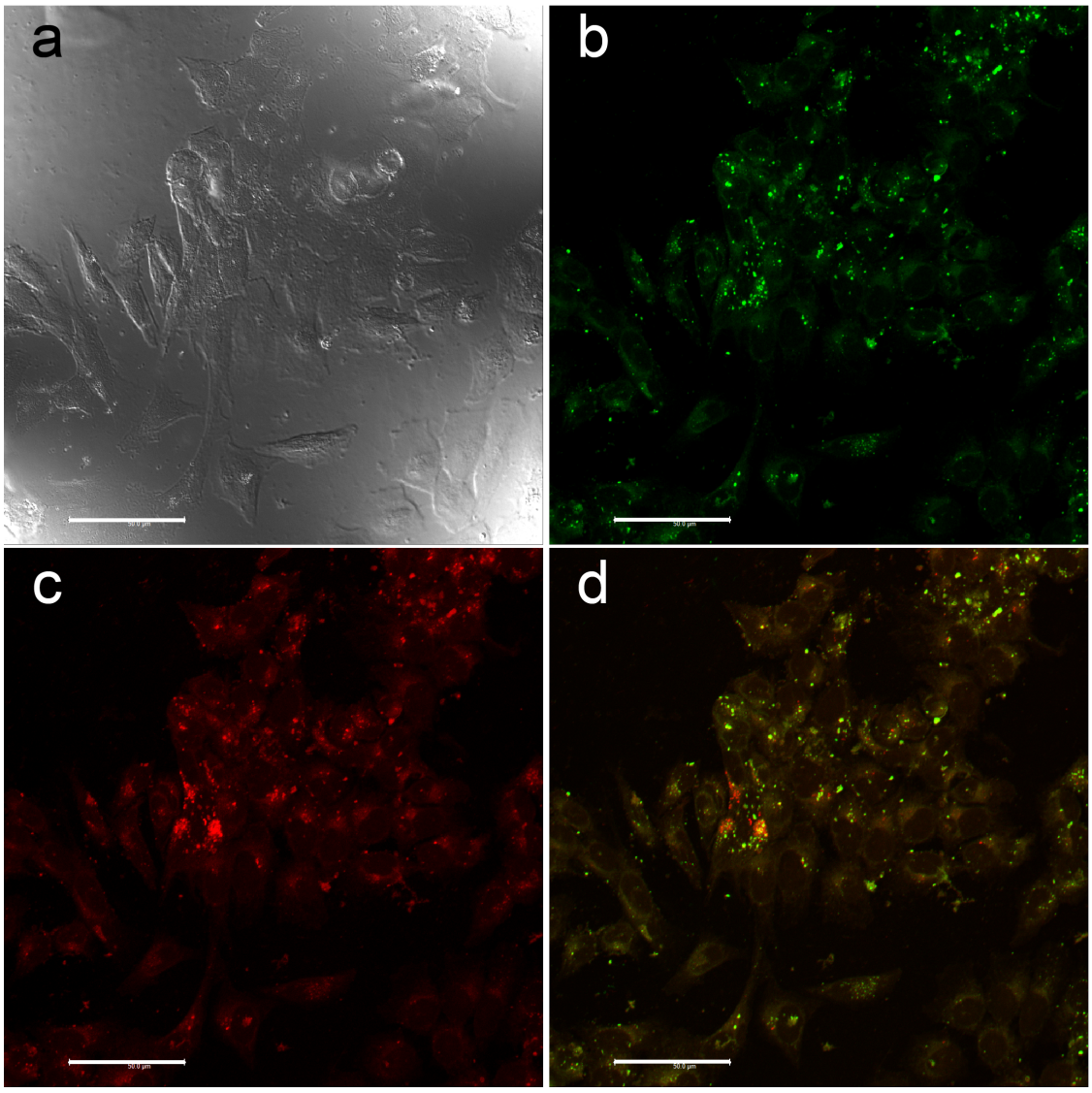


Figure S2. DIC (**a**) and confocal fluorescence micrographs of HCT 116 cells coincubated with PL PAG (10 μM) and Lysotracker Green. Uptake of the PL PAG (10 μM) in cells (**b**, green) and colocalization in **d** (overlay of **b** and **c**) with Lysotracker Red (**c**) shows PL PAG mainly built up in lysosomes and endosomes. Scale bar 50 μm.

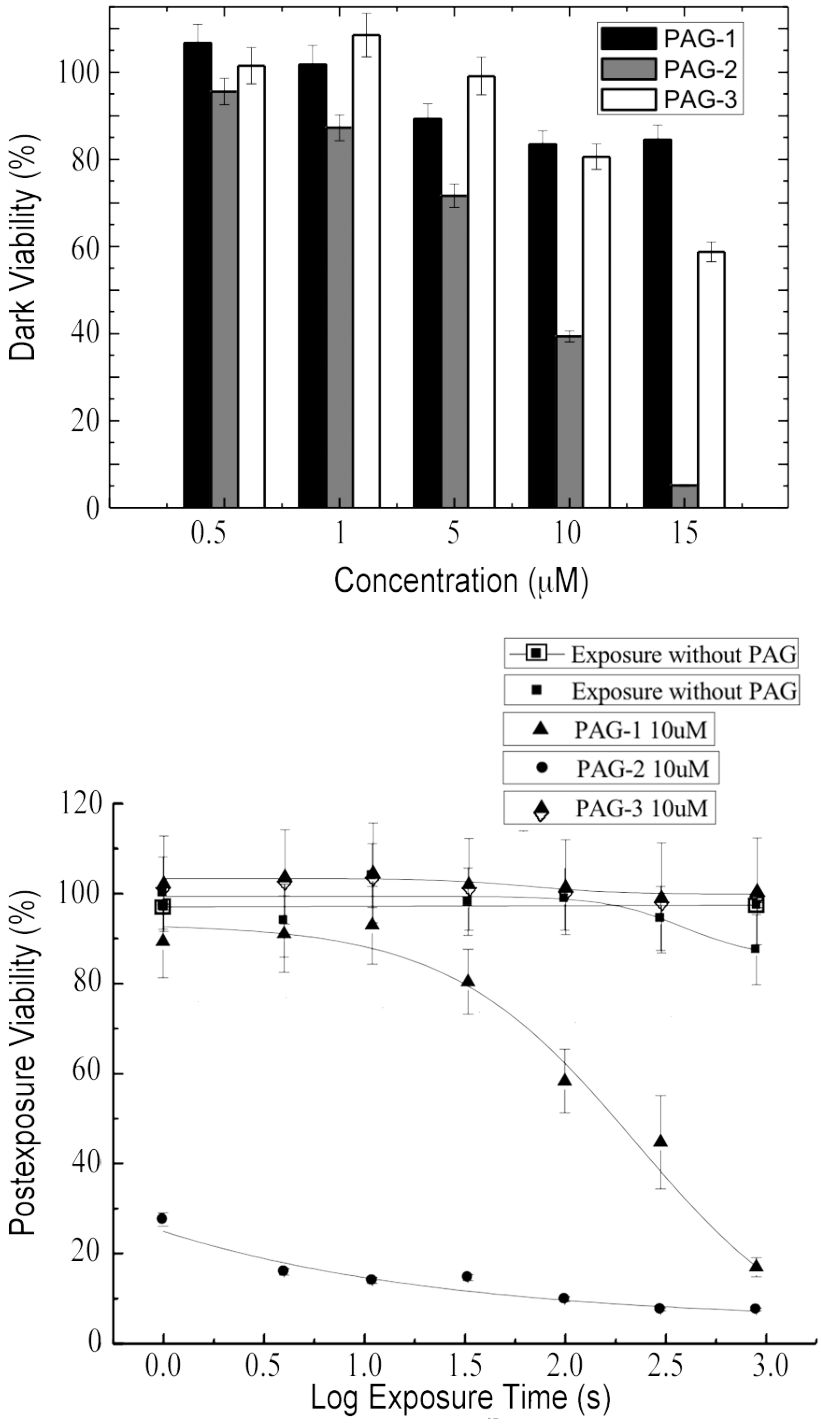


Figure S3. Dark viability and post-exposure viability of HCT 116 cells incubated with PAGs **1**-**3**. PAG **1** shows the lowest intrinsic (dark) cytotoxicity and highest post-exposure cytotoxicity. Solid square shows toxicity of light at wavelengths and doses used for PAGs **1** and **2**. Encased square shows toxicity of light at wavelengths and doses used for PAGs **3**.

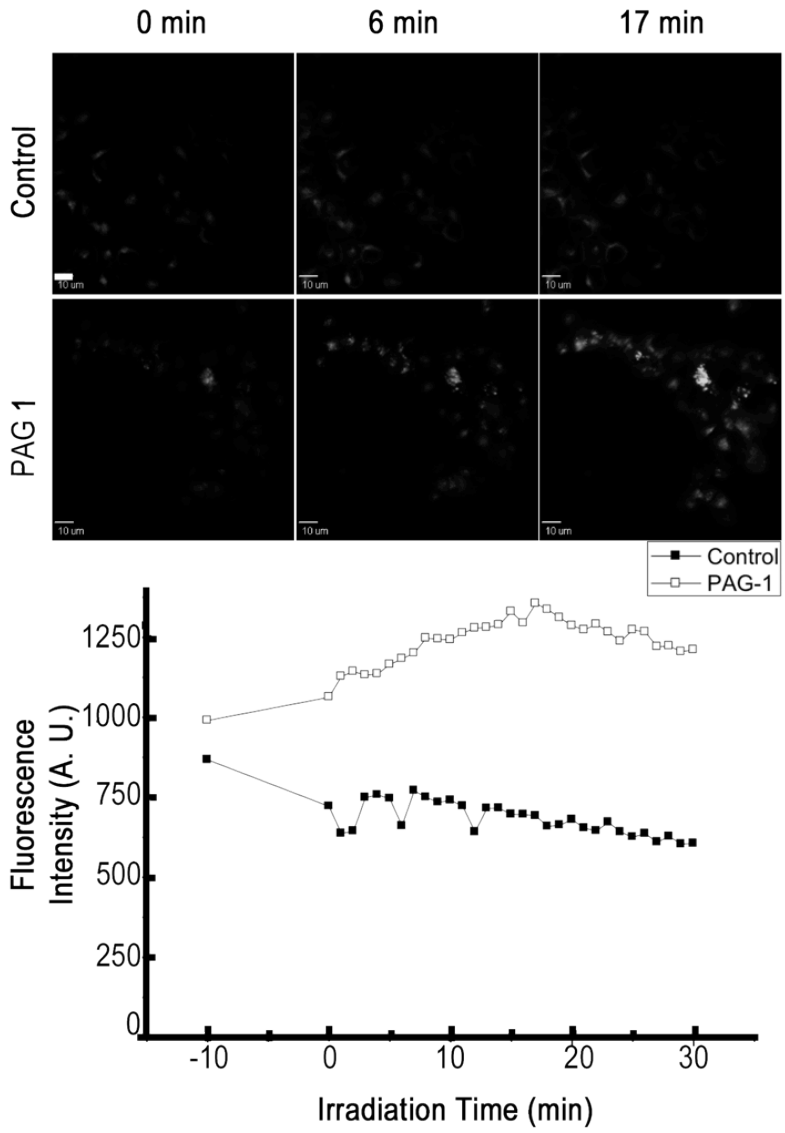


Figure S4. Increase of acidic content in cell lysosomes as a function of irradiation dose in cells incubated with PAG **1**. HCT 116 cells were coincubated with PAG **1** (10 mM, 24 h) and LysoSensor Green (1 mM, Invitrogen, USA, 2 h). After irradiation (100 s, 0.72 J/ cm^2), cells were imaged at 1 min intervals for 30 min with FITC channel. Fluorescence intensities at different time points were calculated with SlideBook.

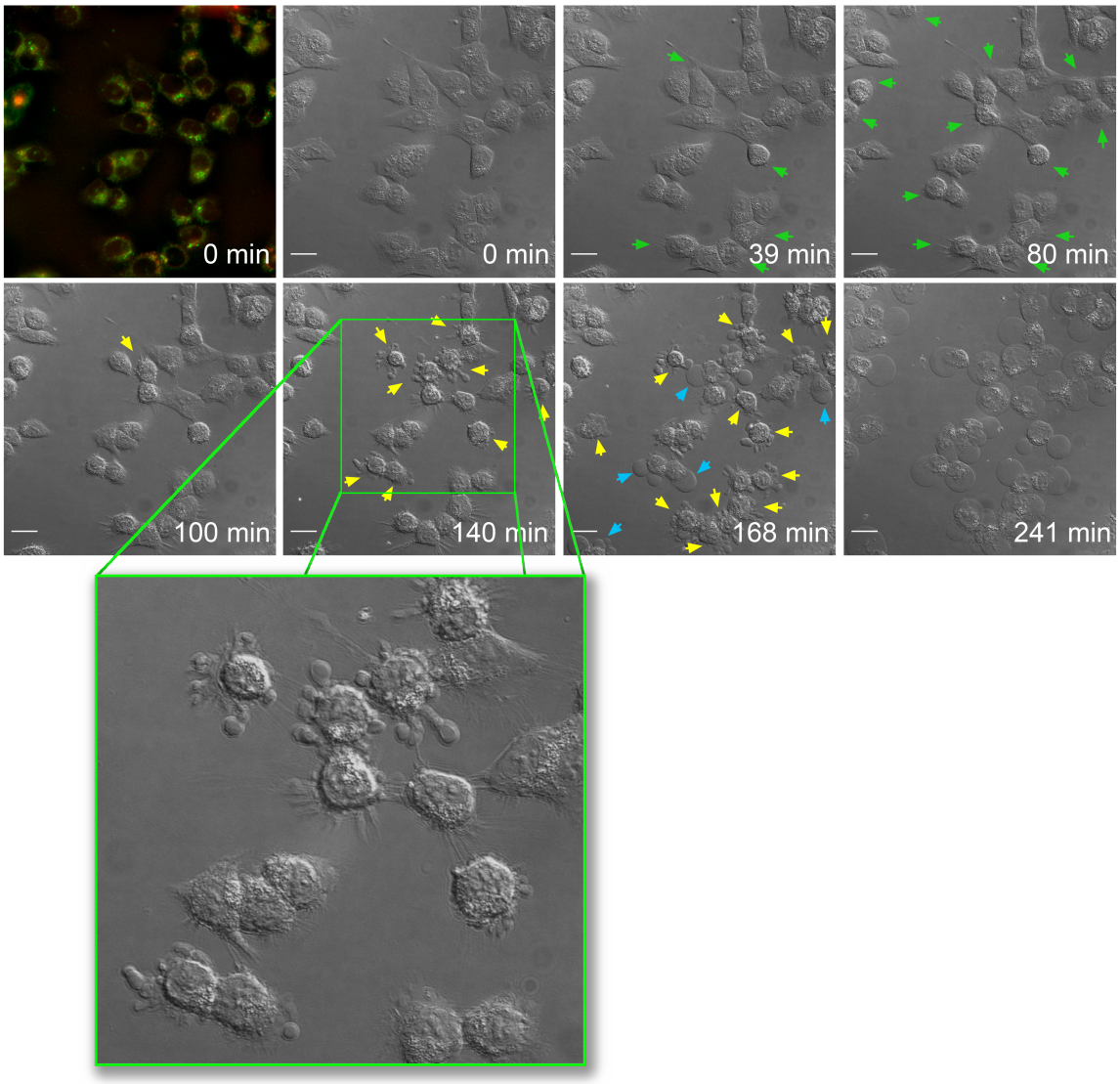


Figure S5. HCT 116 human colorectal carcinoma cells incubated for 30 minutes with PL PAG **1** (1 μM). After eliminating the PL PAG solution cells we placed in warm culture media and, irradiated (1200 ms) imaged by DIC. Widefield fluorescence shows uptake of the PL PAG in cells (green) colocalization with Lysotracker Red shows PL PAG mainly built up in lysosomes and endosomes. Green arrows show loss of cell adhesion, yellow arrows "blebbing"-like activity (shown in enlargement, lower frame), and blue arrows cell swelling. Scale bar 20 μm.

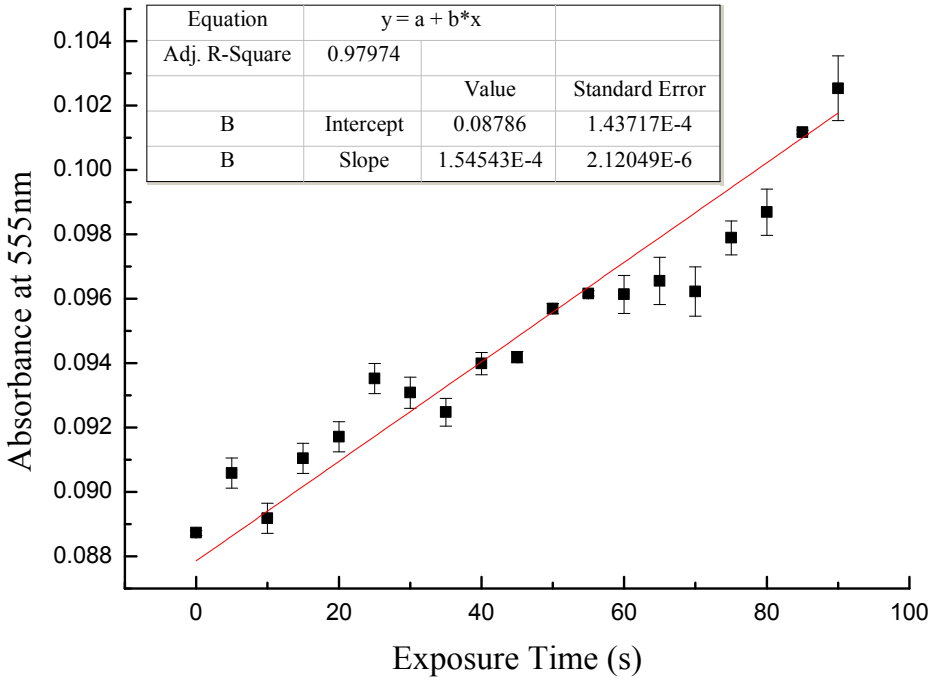
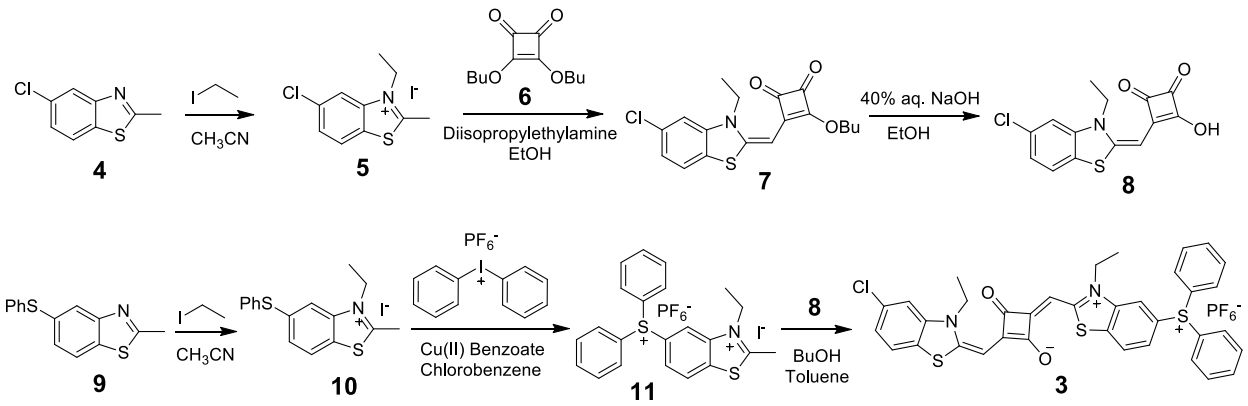


Figure S6. A solution of PAG **1** (10 μM) and Rh B (0.1 mM) was exposed was (7.4 mW/cm^2) to prepare a dose dependent calibration curve. H^+ generation was estimated to be 2.1072 x 10^{-6} M via extrapolation of the calibration curve. Lysosomal pH would, at least, be reduced in 0.2 (to approximately pH ≤ 4.5) within the lysosomes.

Experimental Methods

Materials

Dichloromethane (CH_2Cl_2) was obtained from macron chemicals. Pluronic® F-127 was obtained from BASF. The synthesis and characterization data for PAG **1** and PAG **2** has been reported elsewhere.[1] 2-Methyl-5-(phenylthio)benzo[d]thiazole (**9**) was synthesized according to literature methods.[2] All other reagents and solvents were used as received from commercial suppliers. Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at either 300 or 500 MHz and at 75 or 125 MHz, respectively.



Scheme S1. Synthesis of PAG **3**.

*Synthesis of 5-chloro-3-ethyl-2-methylbenzothiazol-3-ium (**5**)*
Benzothiazole **4** (0.92 g, 5.01 mmol) and iodoethane (0.91 g, 5.83 mmol, 150%) in acetonitrile in a closed vessel were purged with Ar for 300 min. The reaction was run in a single mode microwave reactor. The microwave reaction conditions were the following: ramp time 1 min, 130 ^{o}C, 150 psi, 100W, Pressure 60-65 psi. Upon cooling, crystals were observed in the solution. The crystals were collected by filtration and washed with acetonitrile to give 0.58 g solid; yield 34%; m.p. 221-222 ^{o}C. ^{1}H NMR (500 MHz, CDCl3) δ (ppm): 8.60 (d, J = 2 Hz, 1 H), 8.48 (d, J = 9 Hz, 1H), 7.88 (dd, J = 2 Hz, J = 9 Hz, 1H), 4.76 (q, J = 5 Hz, 2H), 3.22 (s, 3H), 1.43 (t, J = 7 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 179.33, 141.95, 134.96, 128.89, 128.82, 128.60, 126.77, 126.63, 117.22, 177.07.

*Synthesis of (E)-3-butoxy-4-((5-chloro-3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione (**7**).*
A mixture of methylbenzothiazolium **5** (1.05 g, 3.0 mmol), dibutoxy-3-cyclobutene-1, 2-dione **6** (0.72g, 3.2 mmol) and diisopropylethylamine (0.47g, 3.6 mmol) in ethanol (10 mL) was stirred at room temperature for 20 h. The ethanol was removed by rotary evaporation. The solid product was purified by column chromatography (silica gel, eluent CH_2Cl_2:Ethyl Ether, 13:1) yielding 0.76g product **7** as an oil, 68% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.38 (d, J = 9 Hz, 1 H), 7.10 (dd, J = 2 Hz, J = 9 Hz, 1H), 7.03 (d, J = 2 Hz, 1H), 5.47 (s, 1H), 4.80 (t, J = 6 Hz, 2H), 4.00 (q, J = 6 Hz, 2H), 1.80 (m, 2H), 1.48 (m, 2H), 1.37 (t, J = 7 Hz, 3H), 0.99 (t, J = 7, 3H). ^{13}C NMR (125 MHz, CDCl3) δ (ppm): 192.89, 186.46, 186.34, 172.99, 159.11, 141.98, 133.34, 125.38, 123.59, 122.73, 111.06, 79.77, 73.90, 40.92, 32.30, 18.90, 13.92, 11.92. HRMS (ESI, $[M+H]^+$) calcd for $C_{14}H_{10}ClNO_3S$ 364.0696, found 364.0767.

*Synthesis of (E)-3-hydroxy-4-((5-chloro-3-ethylbenzothiazol-2(3H)-ylidene)methyl)cyclobut-3-ene-1,2-dione (**8**).*
Dione **7** (0.75 g, 2.0 mmol) was suspended in boiling EtOH and 40 % NaOH (0.24 mL, 2.4 mmol) aqueous solution was injected into the mixture, which was heated at boiling for 5 min. The mixture was cooled to room temperature. Subsequently, 1mL of 2N HCl was added, the mixture was concentrated under vacuum and the precipitate was collected

by filtration, washed with ethyl acetate:hexane (1:1) several times to give 0.49 g of the deprotected hydroxyl dione product **8** as orange solid (76% yield of crude product). This crude was used without further purification for the next condensation.

*Synthesis of 3-ethyl-2-methyl-5-(phenylthio)benzothiazol-3-ium (**10**)*
Benzothiazole **9** (1.0 g, 3.89mmol) and iodoethane (1.25 g, 0.801 mmol, 150%) in acetonitrile were refluxed in a pressure tube under Ar for 60 hours. The solvent was removed and the crude product was passed through a short column packed with silica gel using methanol as eluent. Recrystallized from isopropanol to afford 1.22 g dark green crystals (yield 76%). m.p. 166.0 ^{o}C–167.5 ^{o}C. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.39 (d, J = 10 Hz, 1 H), 8.31 (d, J = 5 Hz, 1H), 7.54 (dd, J = 5 Hz, J = 5 Hz, 1H), 4.80 (m, 5H), 4,72 (dd, J = 5 Hz, J = 7 Hz, 2H), 3.21 (s, 3H), 1.40 (t, J = 7 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 178.34, 141.67, 138.97, 133.28, 129.50, 129.22, 128.31, 126.09, 126.00, 117.54, 117.45, 45.26, 17.43, 13.71, 13.60. HRMS (ESI, $[M+H]^+$) calcd for $C_{16}H_{16}NS_2^+$ 286.0719, found 286.0713.

*Synthesis of 5-(triphenylsulfonio hexafluorophosphate(V))-3-ethyl-2-methylbenzothiazol-3-ium) (**11**)*
In a 2 mL glass reaction vessel, benzothiazole **10** (0.065g, 0.157 mmol), diphenyliodonium hexafluoro phosphate (V) (0.135g, 0.317 mmol), copper(II) benzoate (5% molar, 2.4mg, 0.0077 mmol) were mixed in the dark in 1 mL of chlorobenzene while being purged with Ar. The microwave was set to closed vessel standard mode; maximum pressure 40 psi; maximum temperature 125 ºC, maximum power 100 W, high speed stirring. The ramp time was set for 30 s and the hold time for 30 min. The product was insoluble in chlorobenzene. Isopropanol was added and the solid was collected by filtration, washed with hot water, isopropanol, methylene chloride and hexane to give 0.06g product (60% yield). m.p. 244.5^{o}C with decomposition according to TGA and DSC. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 9.18 (s, 1 H), 8.88 (d, J = 9, 1H), 8.21 (d, J = 9 Hz, 1H), 7.98 (m, 10H), 5.01 (q, J = 6 Hz, 2H), 3.48 (s, 3H), 1.61 (t, J = 7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 206.72, 142.18, 135.95, 135.12, 131.61, 128.01, 124.56, 121.24, 121.15, 46.08, 16.77, 16.67, 12.74, 12.64. HRMS (ESI, $[M+H]^+$) calcd for $C_{22}H_{21}NS_2PF_6^+$ 508.0752, found 508.0740.

*Synthesis of (E)-2-((E)-(5-chloro-3-ethylbenzothiazol-2(3H)-ylidene)methyl)-4-((5-(diphenylsulfonio)-3-ethylbenzothiazol-3-ium-2-yl)methylene)-3-oxocyclobut-1-enolate hexafluorophosphate(V) (**3**)*
Sulfonium salt **11** (0.20 g, 0.31 mmol) and the hydroxyl dione crude **8** (0.10 g, 0.31 mmol) in butanol:toluene (2:1) were refluxed with in a round-bottom flask that was connected to Dean-Stark trap for 5 hrs. The precipitate was collected by filtration, washed with butanol:toluene (2:1) and hexane to give 0.18 g pure product, 73% yield. Decomposed before melting as determined by TGA and DSC. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.10 (d, J = 9 Hz, 1H), 7.93 (m, 10H), 7.75 (s, 1H), 7.52 (d, J = 8 Hz, 1H), 7.41(d, J = 8 Hz, 1H), 5.99 (s, 1H), 5.77 (s, 1H), 4.50 (q, J = 7 Hz, 2H), 4.21 (q, J = 7 Hz, 2H), 1.48 (t, J = 7 Hz, 3H), 1.30 (t, J = 7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm):1.80, 179.87, 161.79, 156.21, 143.47, 142.40, 134.99, 131.98, 131.85, 126.07,

125.13, 124.77, 123.82, 114.65, 113.64, 104.99, 87.45, 86.52, 13.09, 12.41, 6.99. HRMS (ESI, $[M]^+$) calcd for $C_{36}H_{28}ClN_2O_2S_3^+$ 651.1001, found 651.0915.

Encapsulation of PAGs

A solution containing 25 mg of Pluronic® F-127 in 10 mL of PBS buffer (pH= 7.4) was mixed with solutions containing PAGs in CH_2Cl_2 (10 mL), respectively. The organic solvent was allowed to evaporate at room temperature overnight. The mixtures were filtered through 2 μm pore size disposable filters and used as stock solutions. Concentrations of stock solutions were determined by molar absorption coefficient.

Cell Culture

HCT-116 cells (ATCC, USA) were cultured in RPMI-1640, supplemented with 10% FBS, 1% penicillin-streptomycin, at 37°C in a 95% humidified atmosphere containing 5% CO_2.

Photocytotoxicity Assay

HCT-116 cells were seeded in 96-well black wall clear bottom plates (Corning, USA) at the concentration of 5×10^3 cells/well and incubated for 48 hours. For dark experiments, PAGs were diluted into 0.5 mM, 1 mM, 5 mM, 10 mM and 15 mM from stock solutions. Cells were then incubated with diluted PAGs for additional 24 hours. Viability was then determined with CellTiter 96® AQueous One Solution Reagent (Promega, USA). For Photocytotoxicity experiments, PAGs were diluted to 10 mM solutions and added into cells. For PAG-1 and PAG-2, plates were then placed on an inverted microscope (Olympus IX70) coupled with a 100W mercury lamp. The distance between the bottoms of plates and objective was 1cm to make sure the whole well can be irradiated by the UV light. A customized filter cube (Ex 377/50, DM 409, Em 525/40) was used to match the excitation wavelength of PAGs. The final power reached the plates was 7.4 mW/cm2. For PAG-3, plates were placed on an inverted microscope (Olympus Fluoview FV300) coupled with Coherent Mira 900F Ti:sapphire laser. Cells were irradiated at 700 nm at the CW mode. The final power reached the plates was 5.3 mW/cm^2. Different irradiation times were used to reach the power of 0.03 J/ cm^2, 0.08 J/ cm^2, 0.24 J/ cm^2, 0.72 J/ cm^2, and 2.16 J/ cm^2. After irradiation, cells were incubated for another 24 hours before measuring the viability.

Live Cell Imaging of PAG-1

Cells were cultured on 40 mm poly-D-lysine functionalized coverslips for 48 hours. PAG-1 was then added into cells at a concentration of 10 mM. After 24 hours, coverslips were washed with PBS three times and mounted onto a bioptics live cell imaging chamber. After irradiated with UV lamp for 100s (0.72 J/ cm^2), cells were imaged with Olympus IX-81confocal microscope at 1 min intervals for 3 hours with DIC channel.

For in vivo pH indicator, cells were co-incubated with 10 mM PAG-1 (24 hours incubation) and 1 mM LysoSensor Green (Invitrogen, USA) for additional 2 hours. After irradiation, cells were imaged at 1 min intervals for 30 min with FITC channel. Fluorescence intensities at different time points were calculated with SlideBook.

During irradiation and imaging, cells were kept in 37°C RPMI-1640 whole culture medium with 5% CO_2.

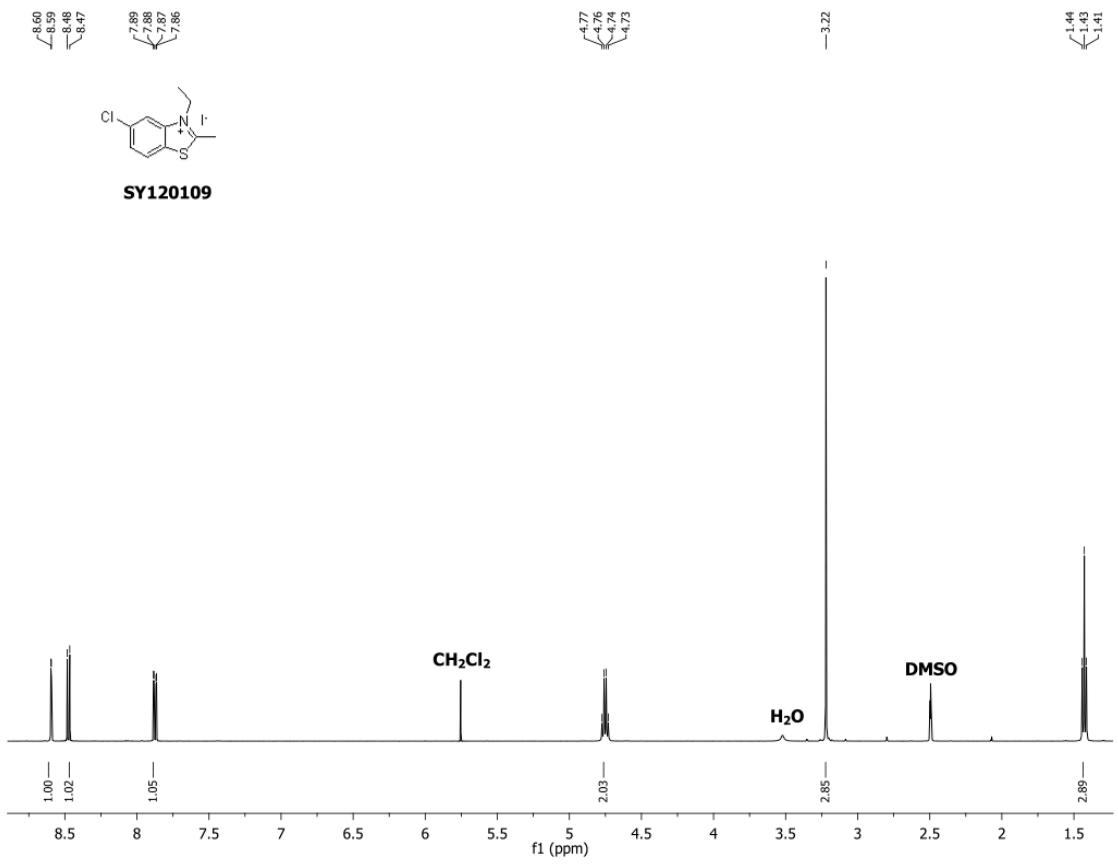
Two-Photon Irradiation and Determination of the Type of Cell Death

Cells were cultured on 12 mm poly-D-lysine functionalized coverslips for 48 hours. PAG-1 was then added into cells at a concentration of 10 mM. After 24 hours, cells were irradiated with a Coherence two photon laser at 710 nm for 13 min. Cells were incubated for additional 4 hours before stained with Propidium Iodide (BD Biosciences, USA) and fixed with 4% Formaldehyde. The coverslips were washed with PBS three times and mounted with ProLong® Gold antifade reagent (Invitrogen, USA). Images were taken with Olympus IX-81confocal microscope.

Estimation of Lysosomal pH Drop

Though there are reports of measuring lysosomal pH via ratiometric analysis (i.e. Lysosensor Green, FITC conjugates, Oregon Green 488 conjugates), this is rather complex and controversial, e.g., Haggie and Verkman[3] and Lackowicz et al.[4] report difficulties in using commercial Lysosensor probes to quantitatively measure lysosomal pH, concluding previous reports using these dyes for pH measurement were either invalid or semi-quantitative at best. Our situation is further complicated due to the presence of another absorbing molecule (e.g., PAG-1) with overlapping absorption and/or emission spectra with the pH probe. Thus, to provide an estimate of pH change, we used an approach previously found to quantify the concentration of photoacid molecules generated/photons absorbed by PAG-1 has consisted using Rhodamine B Base as an indicator.[1] By means of this method the number of acid molecules generated was assumed to be the same as the number of Rhodamine B Base (Rh B) molecules converted to Rhodamine B+ (Rh B+). A solution of PAG 1 (10 μM) and Rh B (0.1 mM) was exposed to make a dose dependent calibration curve. Acid molecule generation was estimated to be 2.1072×10^{-6} M via extrapolation of the calibration curve (Figure S6). Using this as an estimate of H^+ concentration in lysosomes, the lysosomal pH would conservatively be reduced by at least 0.2 pH units (to approximately $pH \leq 4.5$) within the lysosomes. It is undoubtedly a catastrophic event as most cells underwent necrosis.

^{1}H NMR of 5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7.86
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4.76
4.74
4.73
3.22
1.44
1.43
1.41
Cl
N
I-
S
SY120109
CH2Cl2
H2O
DMSO
1.00
1.02
1.05
2.03
2.85
2.89
8.5
8
7.5
7
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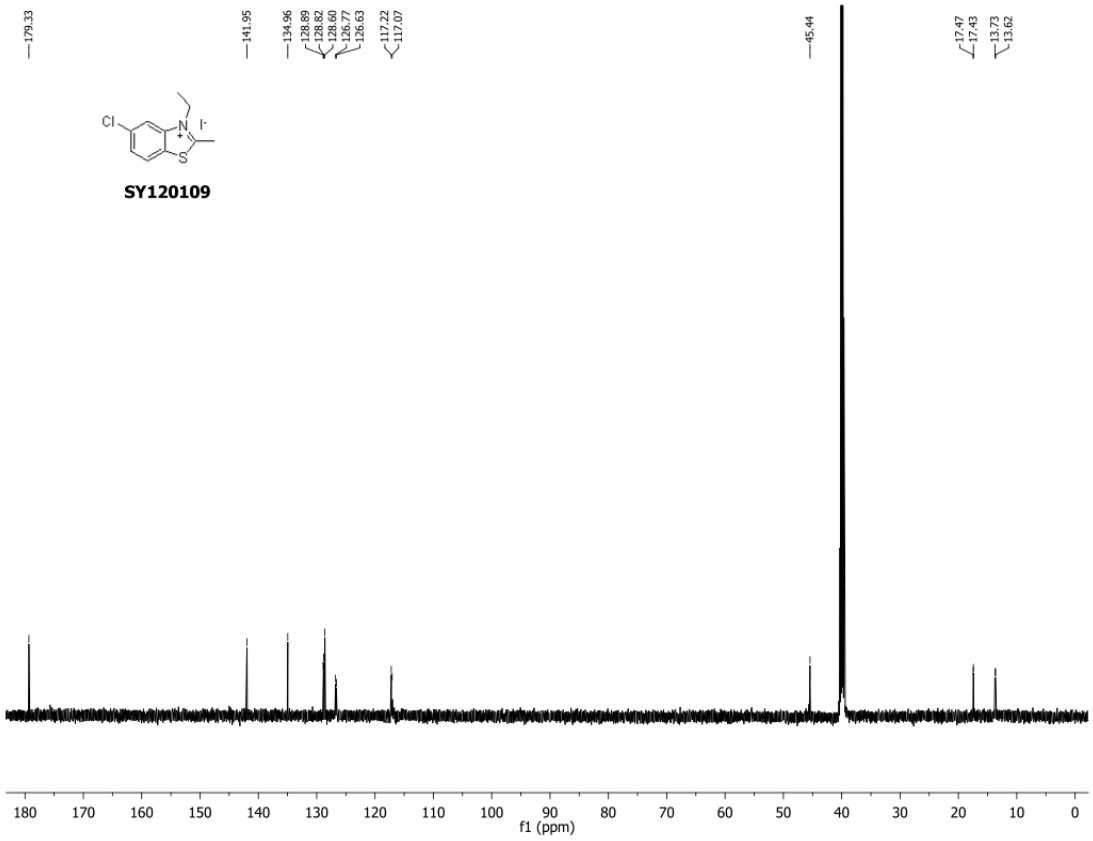
2.5
2
1.5
f1 (ppm)

^{13}C NMR of 5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160
150
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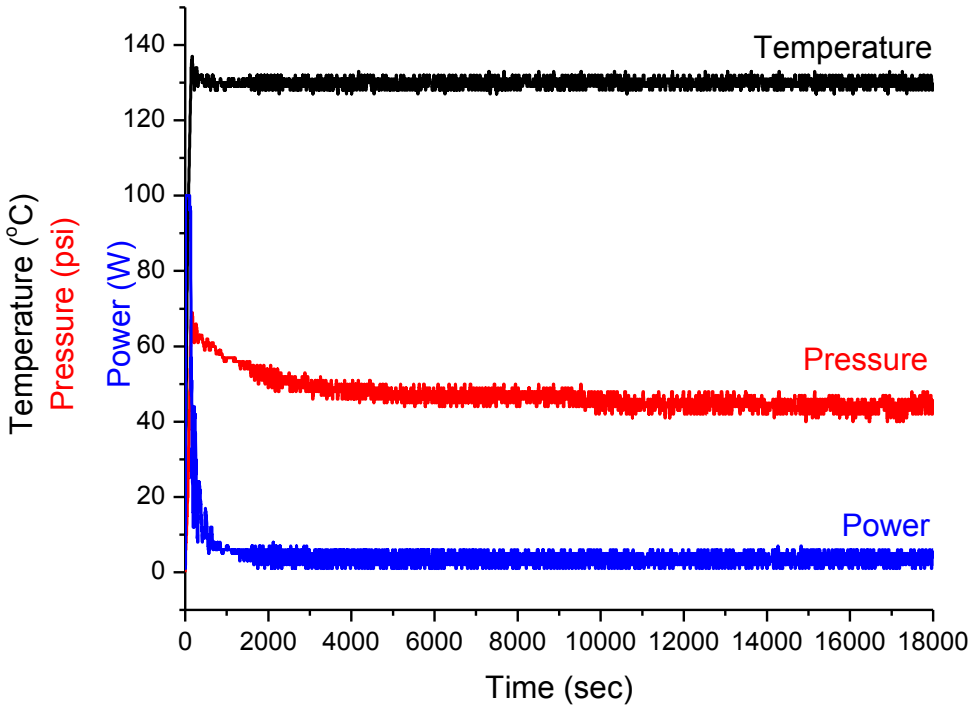
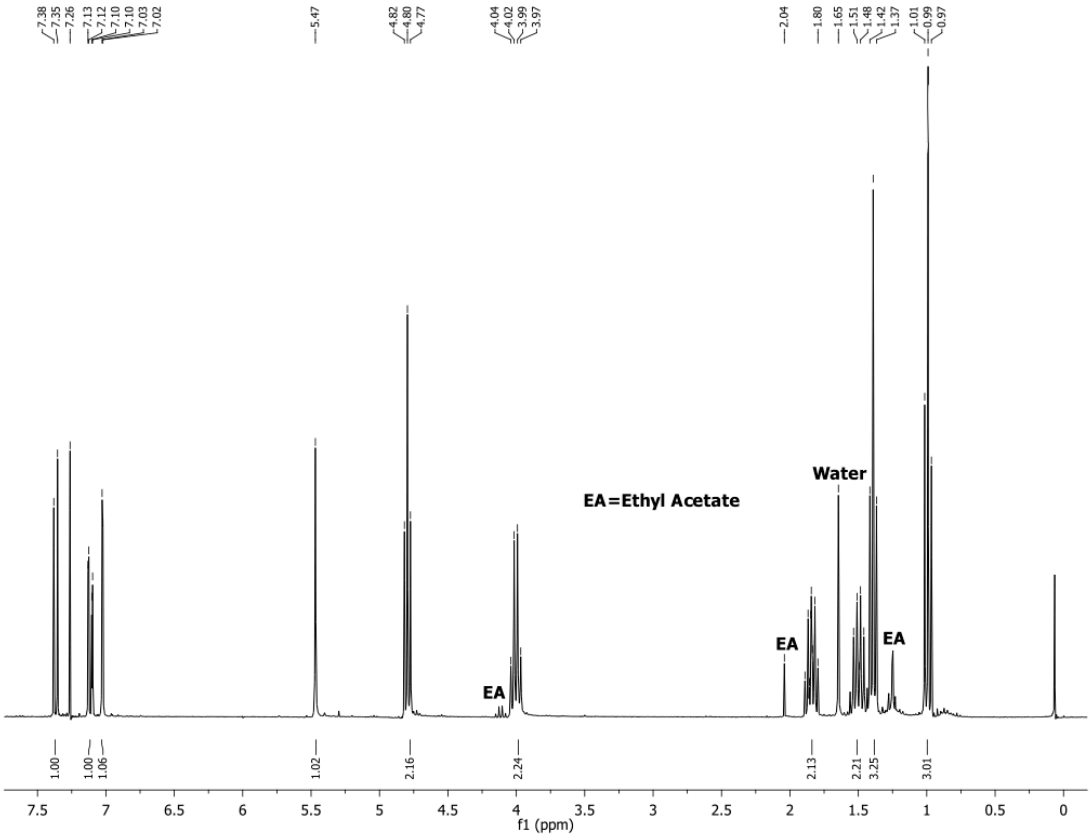
f1 (ppm)

Microwave Vessel Conditions in Synthesis of 5



^{1}H NMR of 7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7.02
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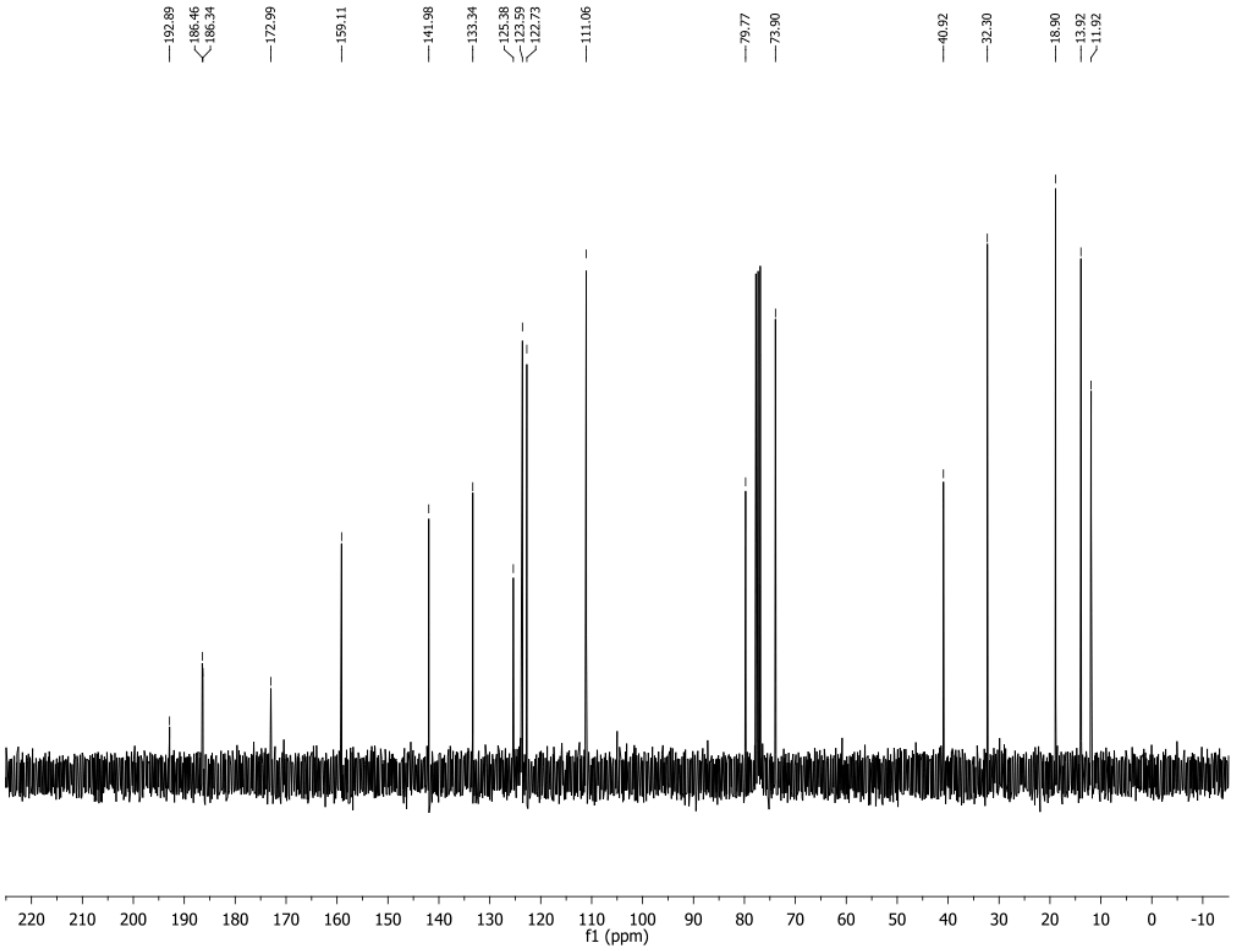
4.04
4.02
3.99
3.97
2.04
1.80
1.65
1.51
1.48
1.42
1.37
1.01
0.99
0.97
EA=Ethyl Acetate
Water
EA
EA
EA
1.00
1.00
1.06
1.02
2.16
2.24
2.13
2.21
3.25
3.01
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
f1 (ppm)

^{13}C NMR of 7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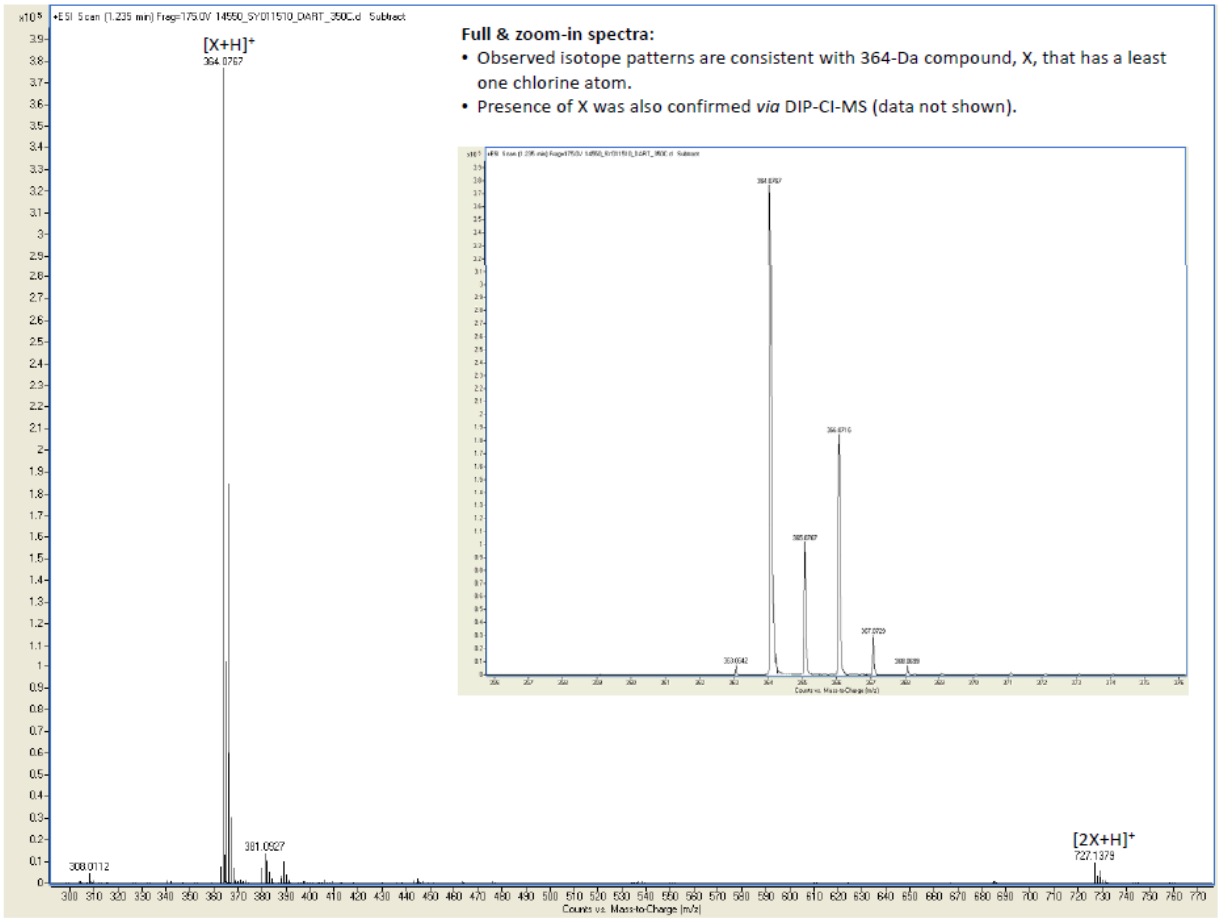
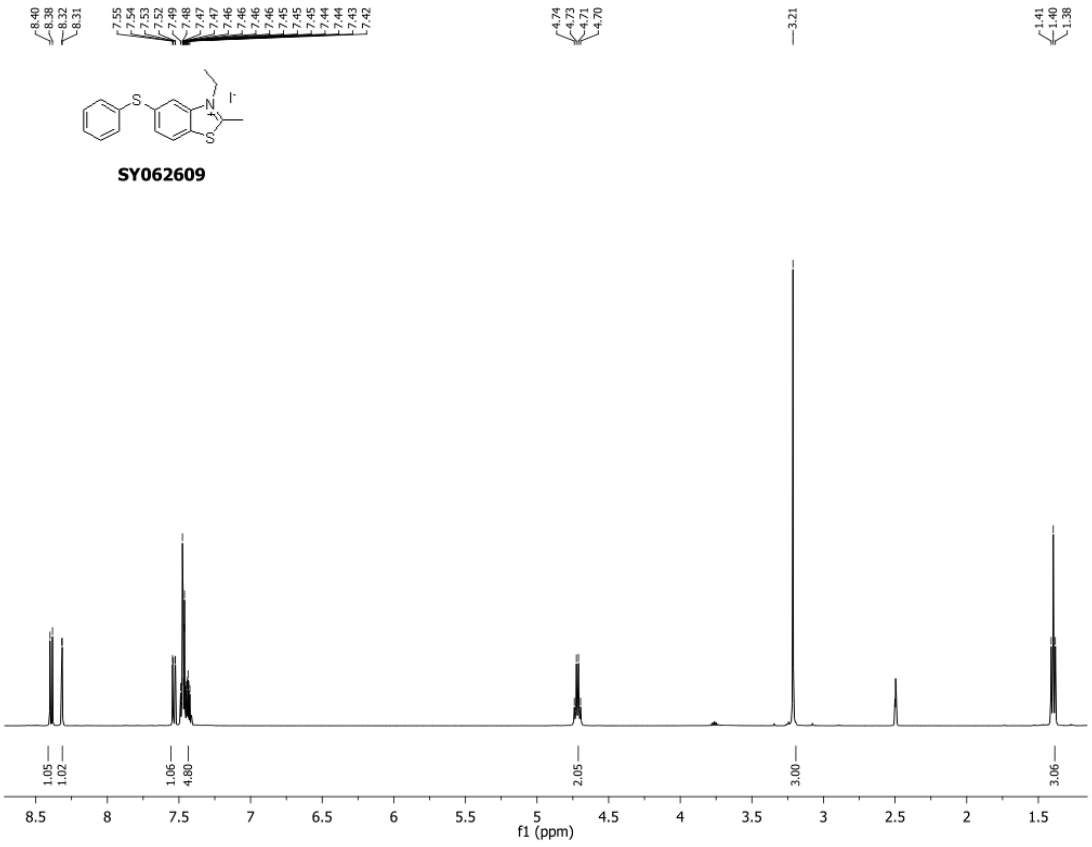
f1 (ppm)

HRMS of 7

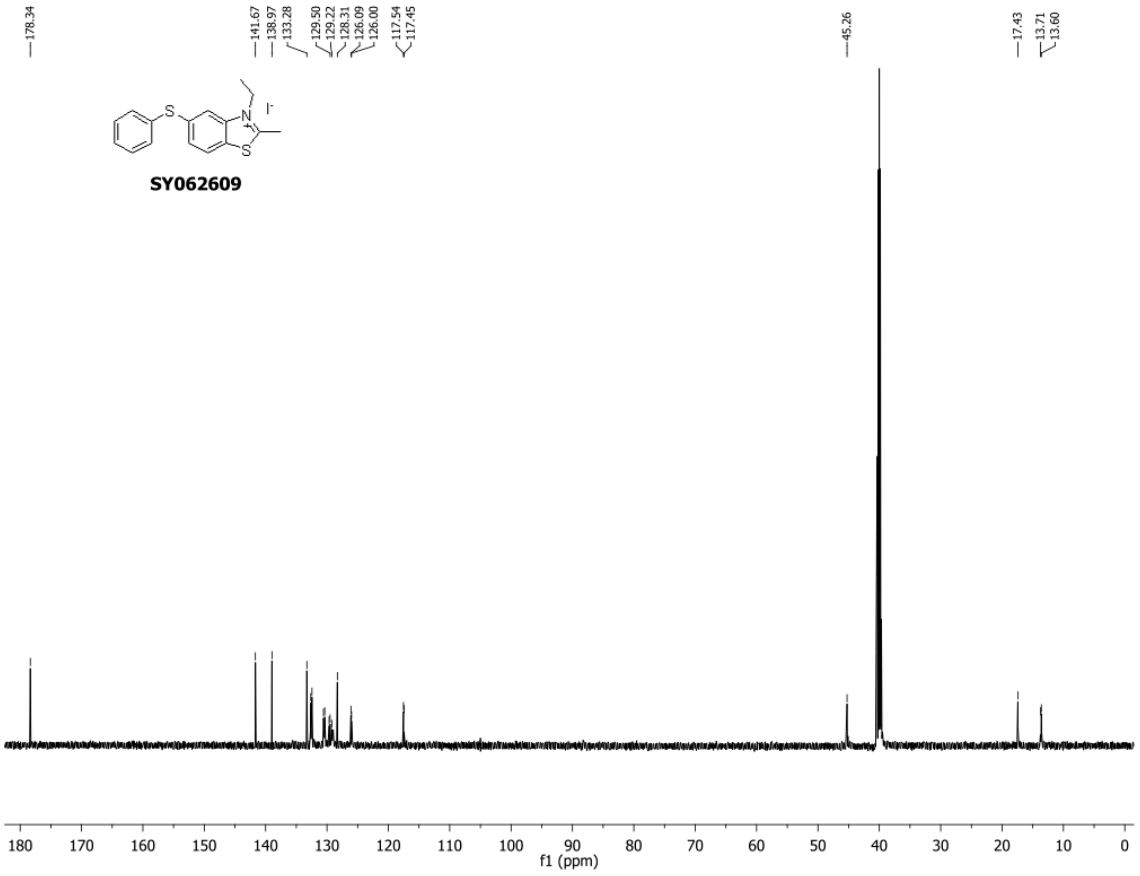

Full & zoom-in spectra:
• Observed isotope patterns are consistent with 364-Da compound, X, that has a least one chlorine atom.
• Presence of X was also confirmed *via* DIP-CI-MS (data not shown).
[X+H]+
364.0767
381.0927
308.0112
[2X+H]+
727.1379
Counts vs. Mass-to-Charge [m/z]

Spectral Data

^{1}H NMR of 10



^{13}C NMR of 10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141.67
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133.28
129.50
129.22
128.31
126.09
126.00
117.54
117.45
45.26
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13.71
13.60
SY062609
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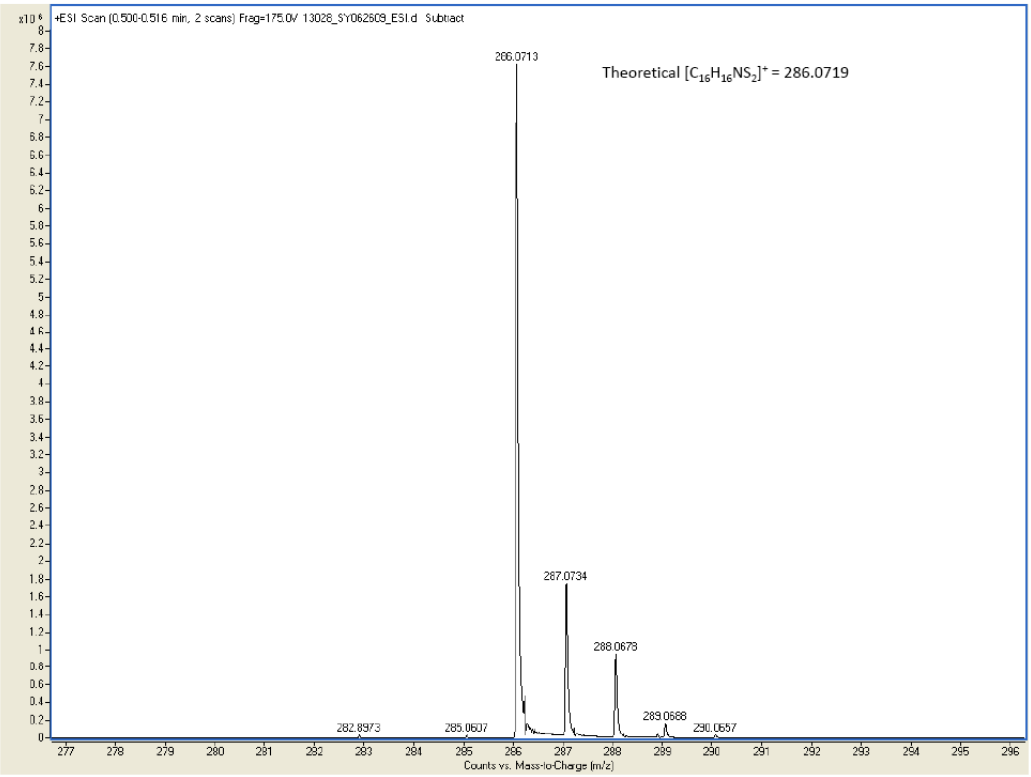
f1 (ppm)

HRMS of 10


+ESI Scan (0.500-0.516 min, 2 scans) Frag=175.0V 13028_SY062509_ESI.d Subtract
286.0713
Theoretical $[C_{16}H_{16}NS_2]^+$ = 286.0719
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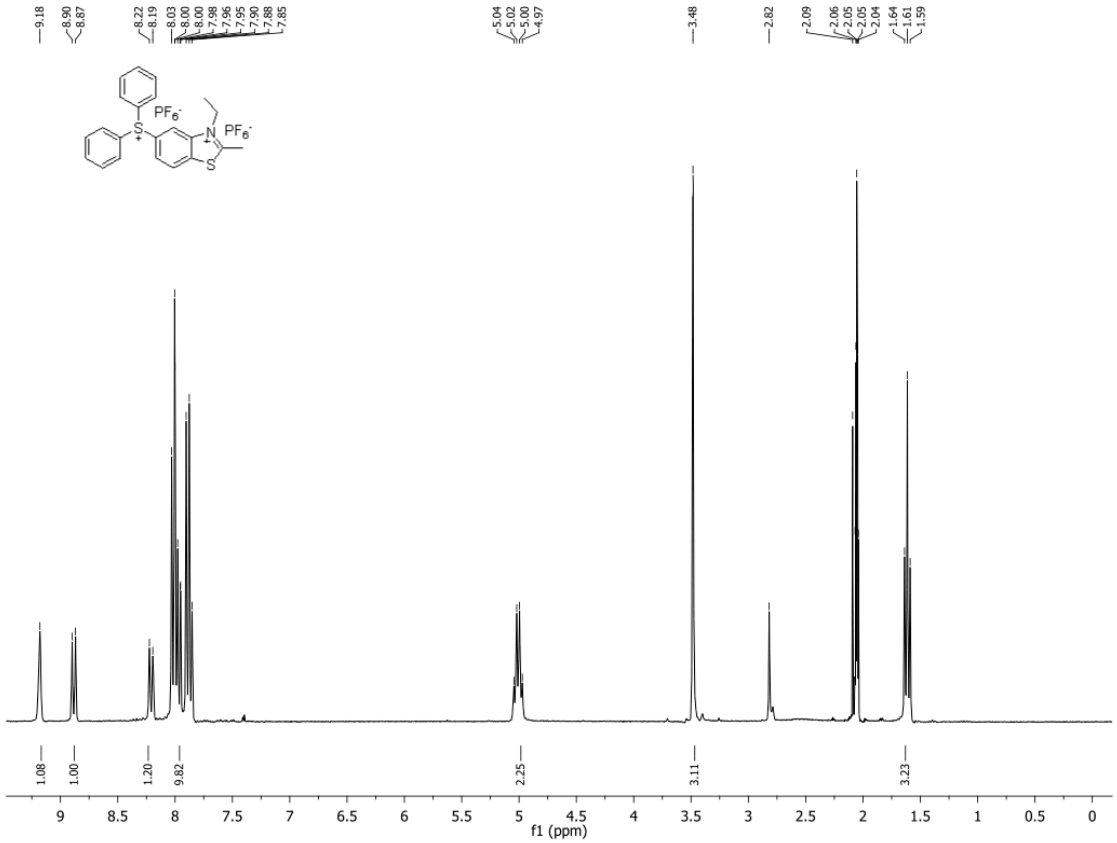
289.0668
290.0657
282.8973
285.0607
Counts vs. Mass-to-Charge (m/z)

^{1}H NMR of 11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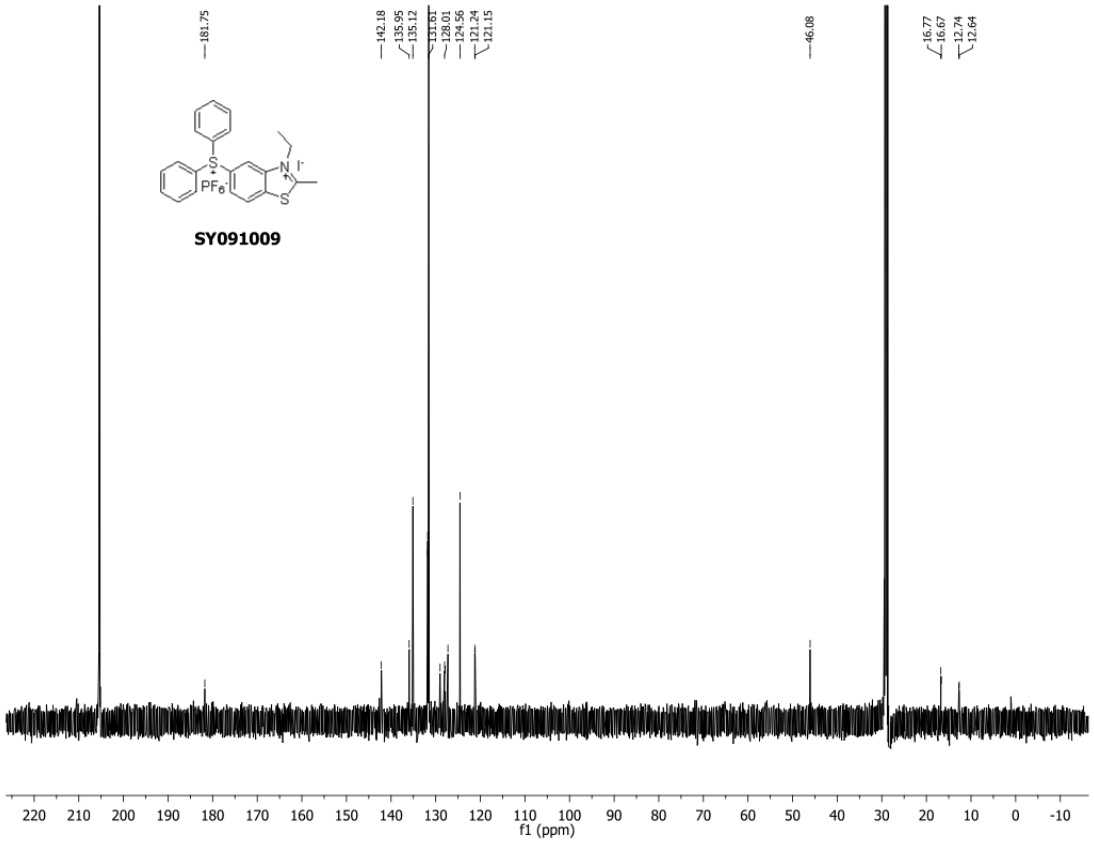
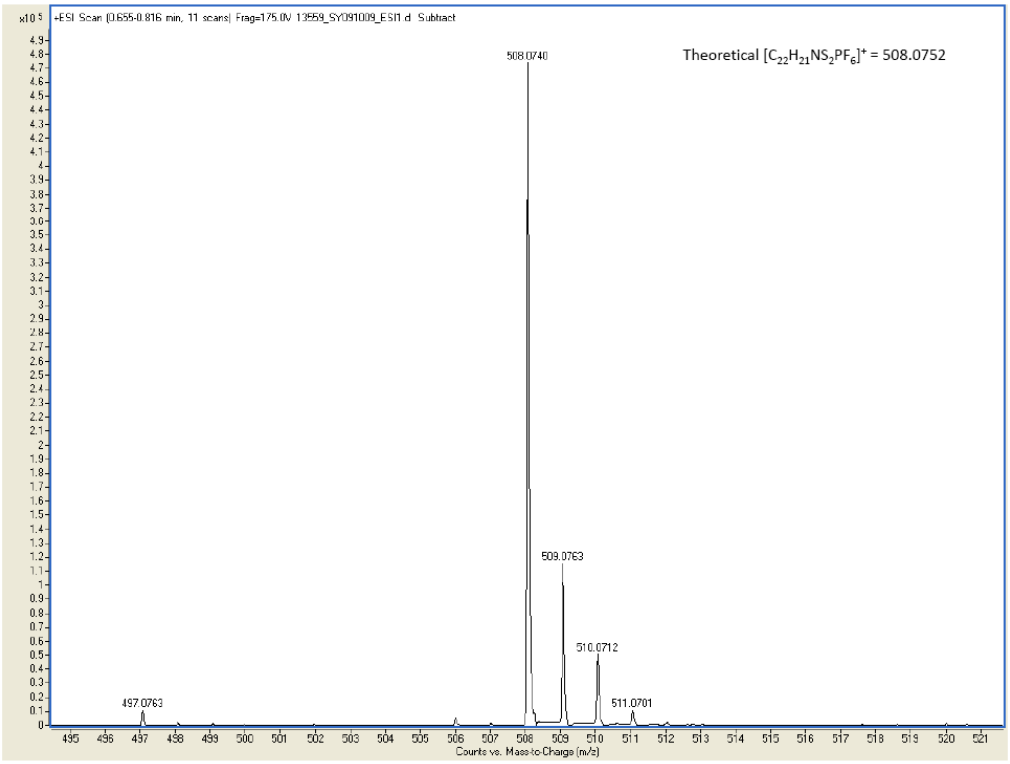
8.03
8.00
8.00
7.98
7.96
7.95
7.90
7.88
7.85
5.04
5.02
5.00
4.97
3.48
2.82
2.09
2.06
2.05
2.05
2.04
1.64
1.61
1.59
PF6-
PF6-
1.08
1.00
1.20
9.82
2.25
3.11
3.23
f1 (ppm)

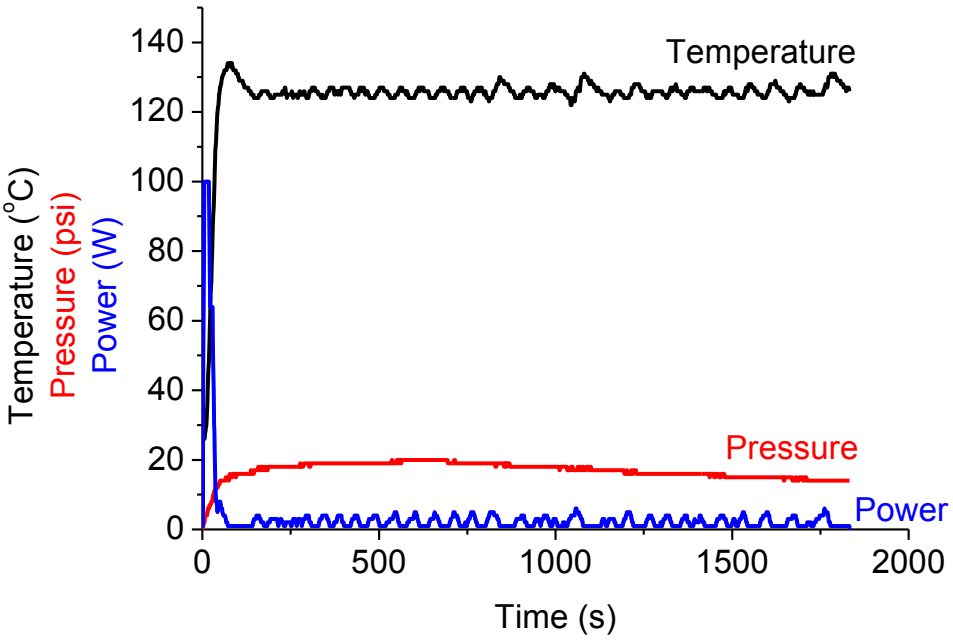
^{13}C NMR of 11


181.75
142.18
135.95
135.12
131.61
128.01
124.56
121.24
121.15
46.08
16.77
16.67
12.74
12.64
SY091009
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

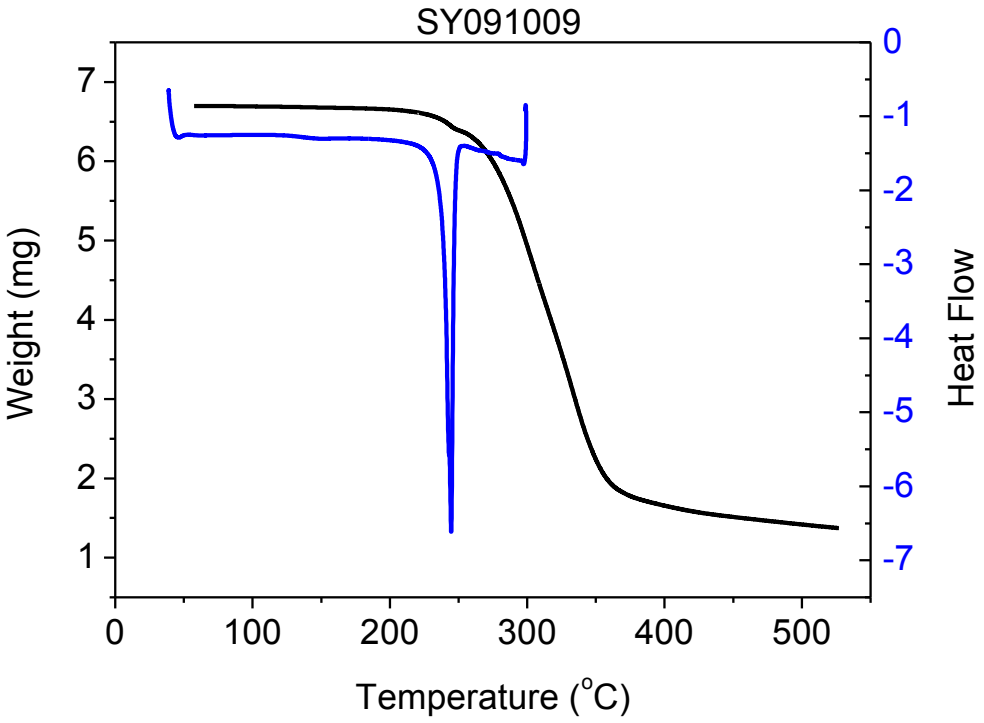
HRMS of 11


+ESI Scan (0.655-0.816 min, 11 scans) Frag=175.0V 13559_SY091009_ESI1.d Subtract
Theoretical [C22H21NS2PF6]+ = 508.0752
508.0740
509.0763
510.0712
511.0701
497.0763
Counts vs. Mass-to-Charge (m/z)

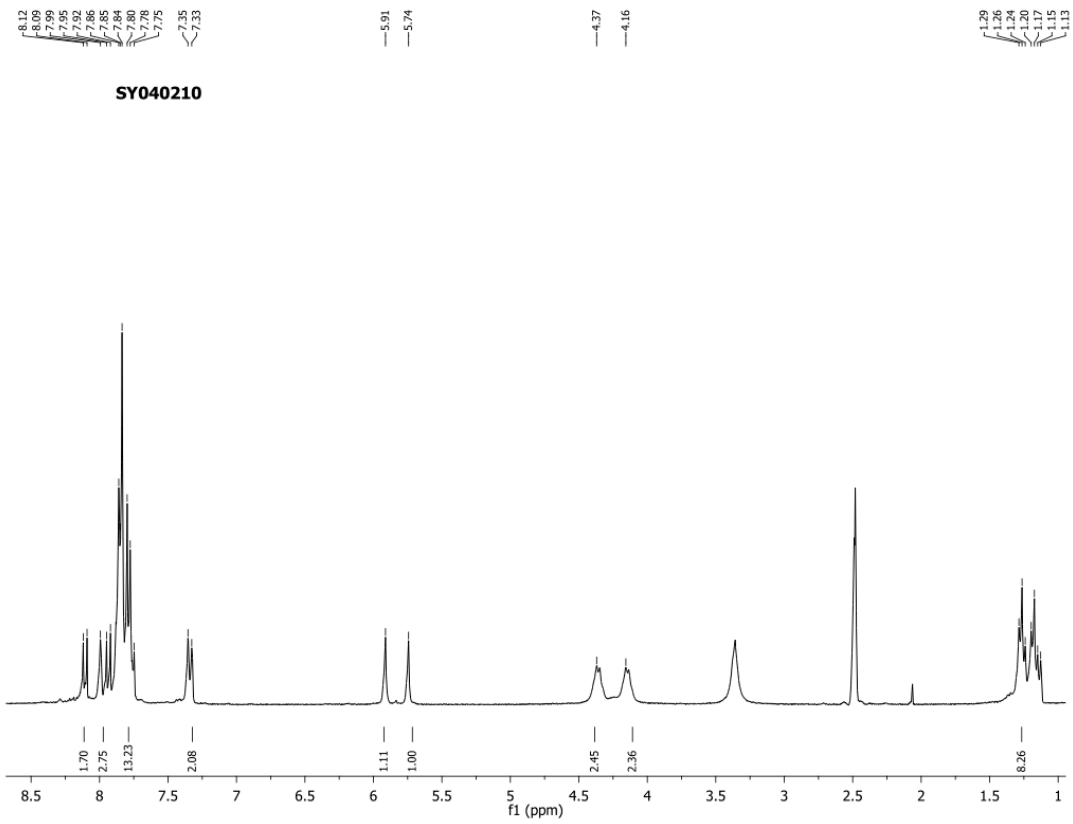
Microwave Vessel Conditions in Synthesis of 11



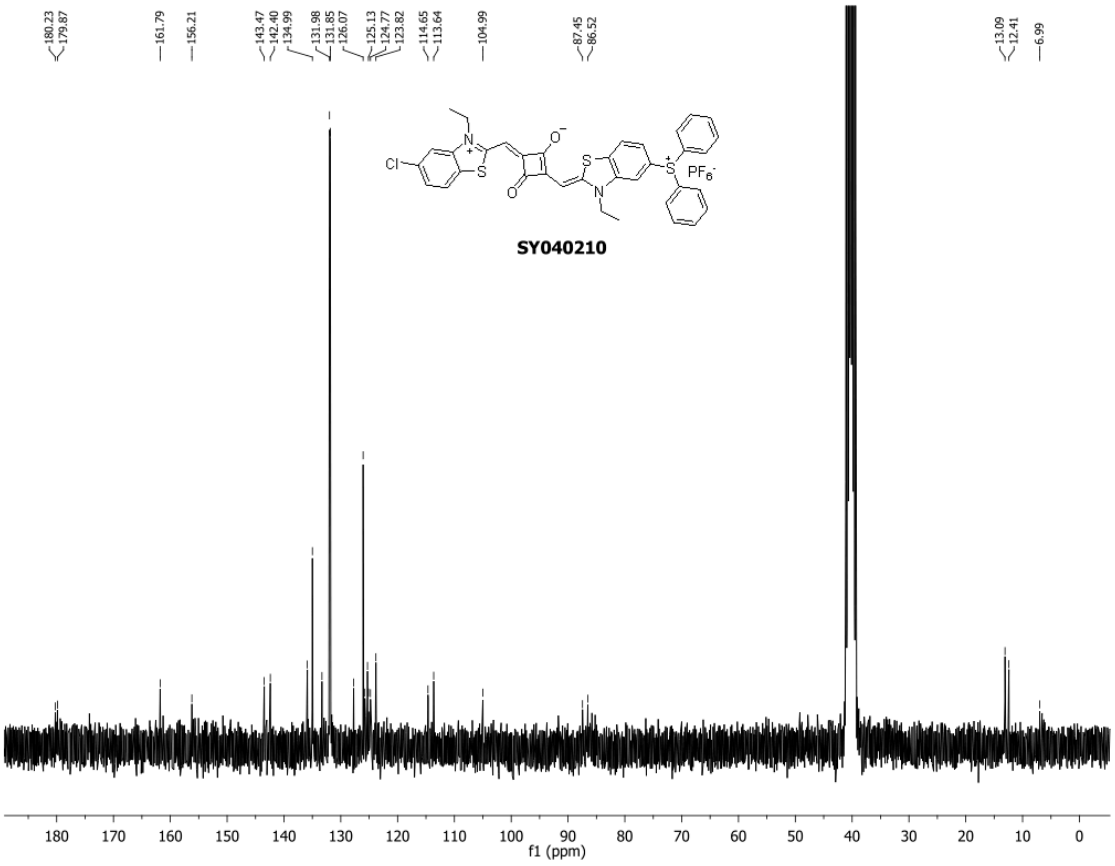
TGA and DSC of 11



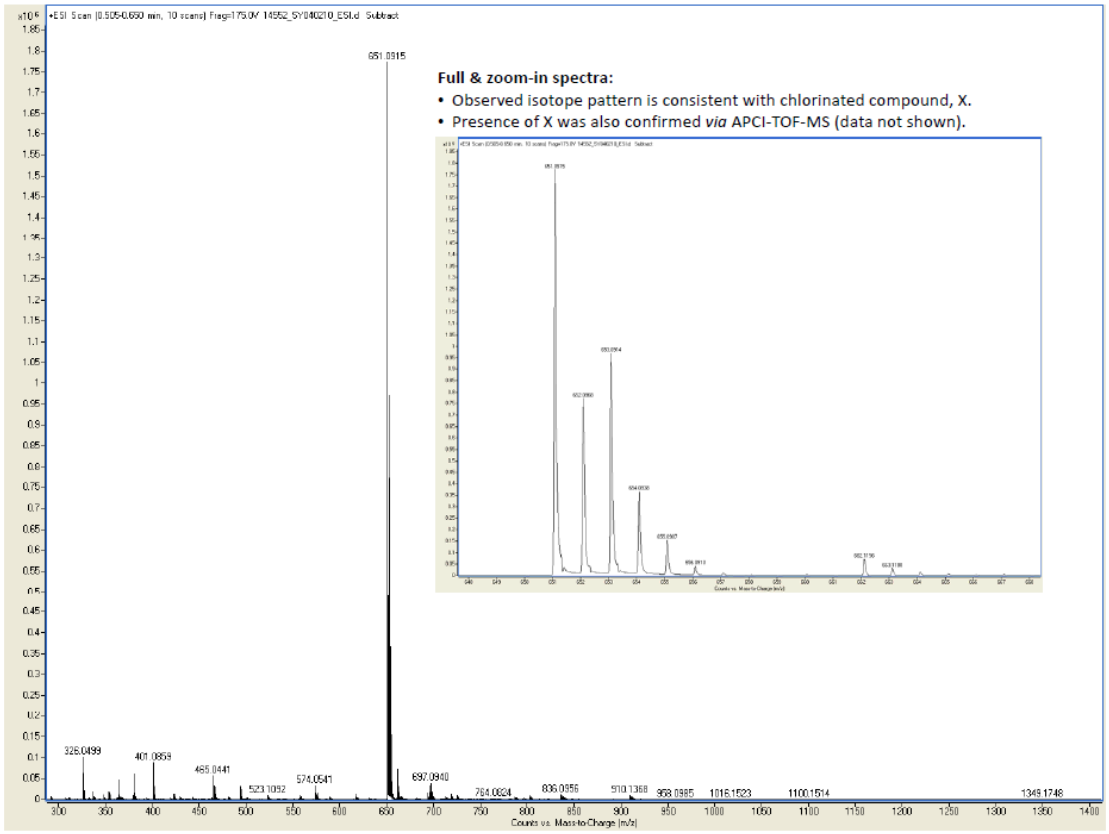
^{1}H NMR of 3


8.12
8.09
7.99
7.95
7.92
7.86
7.85
7.84
7.80
7.78
7.75
7.35
7.33
5.91
5.74
4.37
4.16
1.29
1.26
1.24
1.20
1.17
1.15
1.13
SY040210
1.70
2.75
13.23
2.08
1.11
1.00
2.45
2.36
8.26
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
f1 (ppm)

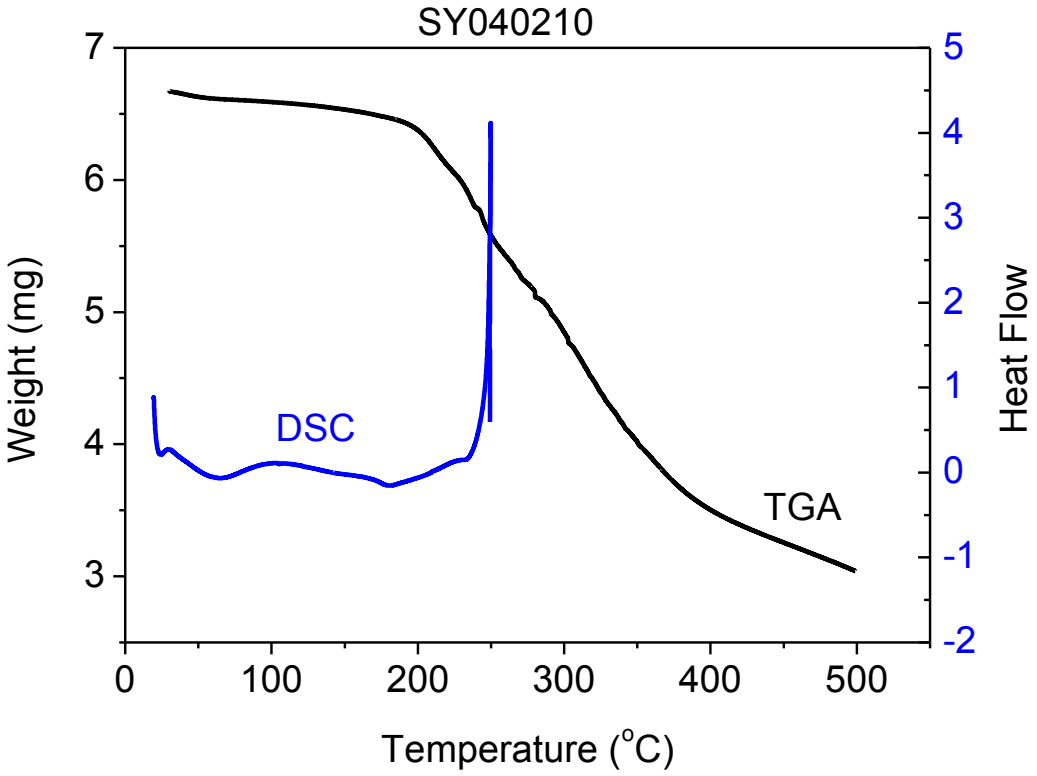
^{13}C NMR of 3


180.23
179.87
161.79
156.21
143.47
142.40
134.99
131.98
131.85
126.07
125.13
124.77
123.82
114.65
113.64
104.99
87.45
86.52
13.09
12.41
6.99
SY040210
f1 (ppm)

HRMS of 3


Full & zoom-in spectra:
• Observed isotope pattern is consistent with chlorinated compound, X.
• Presence of X was also confirmed *via* APCI-TOF-MS (data not shown).
651.0915
326.0499
401.0859
485.0441
523.1092
574.0541
697.0940
764.0824
836.0856
910.1368
968.0985
1016.1523
1100.1514
1349.1748
Counts vs. Mass-to-Charge (m/z)

TGA and DSC of 3



References:

(1) (a) Yanez, C. O.; Andrade, C. D.; Yao, S.; Luchita, G.; Bondar, M. V.; Belfield, K. D. *ACS Appl. Mater. Inter.* **2009**, *1*, 2219; (b) Yanez, C. O.; Andrade, C. D.; Belfield, K. D. *Chem. Commun.* **2009**, 827.

(2) Fry, D. J.; Burrows, R. W.; Ficken, G. E. United Kingdom Patent GB1153166, **1969**.

(3) Haggie, P.M; Verkman, A. S. *J. Biol. Chem.* **2009**, *284*, 7681.

(4) Lin, H.-J.; Herman, P.; Kang, J. S.; Lackowicz, J. R. *Analyt. Biochem.* **2001**, *294*, 118.