Supporting Information for

When the Strategies for Cellular Selectivity Fail. Challenges and Surprises in the Design and Application of Fluorescent Benzothiadiazole Derivatives for Mitochondrial Staining

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Summary

Experimental section

General. ESI-MS and ESI-MS/MS measurements were performed in the positive ion mode (*m/z* 50-2000 range) on a HDMS instrument. This instrument has a hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight (oa-TOF) geometry and was used in the TOF V+ mode. All samples were dissolved in methanol to form 50 μM solutions and were directly infused into the ESI source. ESI source conditions were as follows: capillary voltage 3.0 kV, sample cone 20 V, extraction cone 3 V. NMR spectra were recorded on a NMR instrument using a 5-mm internal diameter probe operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts were expressed in parts per million (ppm) and referenced by the signals of the residual hydrogen atoms of the deuterated solvent, as indicated in the legends. All reagents and solvents were purchased from commercial sources.

Synthesis of BTD-4AP (*N*-4-(pyridinyl)-2,1,3-benzothiadiazole-4-amine). 4-bromo-2,1,3-benzothiadiazole (100 mg, 0.4 mmol), 1,4-aminopyridine (65.0 mg, 0.7 mmol), ($Pd(OAc)_2$ (5.0 mg, 5 mol%), DPEPhos (12 mg, 5 mol%), and ^{t}BuOK (100 mg, 0.9 mmol) were mixed in anhydrous toluene (5 mL) in a sealed Schlenk tube. The reaction mixture was stirred at 120 °C for 72 h. The reaction mixture was cooled and filtered through Celite. The Celite was rinsed with ethyl acetate and the mixture solution was concentrated. Purification by column chromatography (EtOAc/hexane 5:95 *v/v*) afforded the desired product in 80% yield (0.3 mmol, 73 mg). Quantum yield of fluorescence (MeCN): 0.27. Melting point: 140.5-142.0 °C. IR (cm^{-1}) 3418, 2925, 1594, 1420, 1150, 990. ^{1}H NMR (400 MHz, CCl_3D/CD_3OD) δ (ppm) 8.37-8.35 (m, 2H, H-a), 7.64-7.57 (m, 2H, H-d, e), 7.47-7.45 (m, 1H, H-c), 7.21- 7.10 (m, 2H, H-b), 3.69 (br, 1H, H-f). $^{13}C-\{^{1}H\}$ NMR (100 MHz, CD_3OD) δ (ppm) 155.4, 149.8, 148.8, 148.7, 132.4, 130.4, 114.2, 111.3, 111.0. HRMS (ESI-Q-TOF) calcd. for $[C_{10}H_9N_4S+H]^+$ 229.0548, found, 229.0581.

Synthesis of BTD-4APBu (*N*-(1-butyl-4-pyridinium)-2,1,3-benzothiadiazole-4-amine bromide). 1-Bromobutane (300.0 mg, 2.0 mmol) and **BTD-4AP** (50 mg, 0.2 mmol) were mixed together in a sealed Schlenk tube with 5 mL of anhydrous MeCN. The reaction mixture was stirred at 90 °C for 24 h. The Schlenk was cooled, the solvent was evaporated, the remaining oil was washed several times with ethyl acetate to remove untreated reagents. A yellowish oil was obtained in 50% yield (0.1 mmol, 37 mg). Quantum yield of fluorescence (MeCN): 0.04. IR (cm^{-1}): 3420, 3020, 2850, 1649, 1537, 1461, 1190. ^{1}H NMR (400 MHz, CD_3OD) δ (ppm) 8.34 (d, J = 7.6 Hz, 2H, H-a), 8.03-8.01 (m, 1H, H-c), 7.78-7.71 (m, 2H, H-d,e), 7.24 (d, J = 6.8 Hz, 2H, H-b), 4.28 (t, J = 7.2 Hz, 2H, H-g), 1.90 (q, J = 7.6 Hz, 2H, H-h), 1.41

(sex, *J* = 7.6 Hz, 2H, H-i), 1.01 (t, *J* = 7.2 Hz, 3H, H-j). ^{13}C-{^{1}H} NMR (100 MHz, $CDCl_3$) δ (ppm) 155.7, 155.6, 149.4, 142.3, 129.7, 128.8, 123.4, 120.0, 109.9, 58.5, 33.0, 19.2, 13.4. HRMS (ESI-Q-TOF) calcd. for $[C_{15}H_{17}N_4S]^+$, 285.1174, found, 285.1127.

Synthesis of BTD-4APOc (*N*-(1-octyl-4-pyridinium)-2,1,3-benzothiadiazole-4-amine bromide). 1-Bromooctane (300.0 mg, 2.0 mmol) and **BTD-4AP** (50 mg, 0.2 mmol) were mixed together in a sealed Schlenk tube with 5 mL of anhydrous MeCN. The reaction mixture was stirred at 90 °C for 24 h. The Schlenk was cooled, the solvent was evaporated, the remaining oil was washed several times with ethyl acetate to remove untreated reagents. A yellowish oil was obtained in 60% yield (0.1 mmol, 51 mg). Quantum yield of fluorescence (MeCN): 0.04. IR (cm^{-1}): 3130, 2936, 1680, 1517, 1460, 1124, 841. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1:1 *v/v*) δ (ppm) 8.12 (d, *J* = 7.2 Hz, 2H, H-a), 7.82-7.79 (m, 1H. H-c), 7.55-7.53 (m, 2H, H-d, e), 7.18 (m, 2H, H-b), 4.15 (t, *J* = 7.2 Hz, 2H, H-g), 1.87-1.60 (m 2H, H-h), 1.29-1.00 (m, 10H, H-i), 0.72 (t, *J* = 6.4 Hz, 3H, H-j). ^{13}C-{^{1}H} NMR (100 MHz, $CDCl_3$/CD_3OD 1:1 *v/v*) δ (ppm) 155.5, 155.4, 149.3, 142.2, 129.5, 128.6 (2C), 122.4, 119.8, 58.7, 31.3, 30.9, 28.65, 28.62, 25.7, 22.2, 13.6. HRMS (ESI-Q-TOF) calcd. for $[C_{19}H_{25}N_4S]^+$, 341.1800, found, 341.1863.

Synthesis of BTD-4APBuP (*N*-(1-(4-(triphenylphosphonium)butyl)-4-pyridinium)-2,1,3-benzothiadiazole-4-amine di-bromide). BTD-4AP (11.5 mg, 0.05 mmol) and (4-bromobutyl)-triphenylphosphonium bromide (238 mg, 0.5 mmol) were mixed in anhydrous DMF (5 mL) in a sealed Schlenk tube. The reaction mixture was stirred at 70 °C for 24 h. The Schlenk was cooled and the reaction mixture was washed several times with ethyl acetate to remove unreacted reagents. A yellowish oil was obtained in 27% yield (0.01 mmol, 8 mg). Quantum yield of fluorescence (MeCN): 0.01. IR (cm^{-1}): 3060, 3051, 984, 2925, 1667, 1450, 1114, 808. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1:1 *v/v*) δ (ppm) 8.82 (d, *J* = 7.2 Hz, 2H, H-a), 7.78-7.42 (m, 20H, H-b, c, d, e, k), 4.62 (t, *J* = 7.6 Hz, 2H, H-g), 3.85-3.78(m, 2H, H-h), 2.45-2.37 (m, 2H, H-i), 1.87-1.79 (m, 2H, H-j). ^{13}C-{^{1}H} NMR (100 MHz, $CDCl_3$/CD_3OD 1:1 *v/v*) δ (ppm) 155.6, 155.4, 149.5, 143.3, 135.1-135.0 (d, J = 10 Hz, 3C), 133.3-133.2 (d, *J* = 10.0 Hz, 6C), 130.4-130.3 (d, *J* = 20.0 Hz, 6C), 129.5, 129.0, 121.7, 119.7, 118.0-117.2 (d, *J* = 90.0 Hz, 3C), 43.7, 32.0-31.9 (d, *J* = 10.0 Hz), 21.6-21.0 (d, *J* = 50.0 Hz), 19.37-19.34 (d, *J* = 3.0 Hz) ^{31}P-{^{13}C} NMR (161 MHz, $CDCl_3$/CD_3OD 1:1 *v/v*) δ (ppm) 24.4. HRMS (ESI-Q-TOF) calcd. For $[C_{33}H_{31}N_4PS]^{2+}$, 273.1004, found, 273.1049.

Theoretical Calculations. All DFT calculations were performed using the Gaussian 09 suite of programs.[123] Geometry optimizations were carried out with the long-range corrected density functional

CAM-B3LYP with 6-31G(d) Pople's split basis set. Harmonic frequency calculations were performed to verify that a genuine energetic minimum was achieved. Solvent effects on the BTDs geometries were assessed using the Polarizable continuum model (PCM) in which the solute molecule is enclosed in a cavity embedded in a dielectric medium. No significant alterations were however observed, hence we refrain from presenting the optimized structures in solvent to avoid unnecessary proliferation of results, so that only isolated BTDs were displayed. The optimized geometries of the ground state (S_0) in vacuum were used for the single point TD-DFT calculation using density functionals of different flavors to assess the performance of different density functionals: B3LYP, CAM-B3LYP, LC-ωPBE, M06, M06-2X, and PBE1PBE. It was employed the 6-311+G(2d,p) basis set to simulate the excitation spectra of the BTDs. To include the solvent effects, the implicit PCM treatment was included in the TD-DFT calculations, selecting the same solvents as in the experimental measurements.

Biological experiments. All BTDs were diluted in water in the presence of DMSO (down to 0.1%) in the cell medium supplemented with 10% of fecal calf serum. It was used the cells lineages: A2780 (human ovarian cancer cell line), MCF-7 (human breast adenocarcinoma cell line), T47D (breast adenocarcinoma cell line) and HUVEC (Human umbilical vein endothelial cells). The cells samples were maintained according to ATCC (American Type Culture Collection) recommendations at 37 °C in an atmosphere with 5% CO_2.

Viability of the cells. To the cells' viability tests, it was applied two different solution concentrations, 10 and 100 μM of the designed BTDs. The cells were incubated with the synthesized BTDs at for 24 h and analyzed by standard MTT assays, following the manufacturer's recommendations (R&D System Inc, MN, USA). Briefly, 3×10^3 of each cells line was seeded in a 96 well plate and maintained overnight a 37 °C. The cells were also incubated with the solvent DMSO (down to 0.1%) at the same as the positive control. The samples were incubated with 150 μL of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution (0.5 mg mL^{-1}) in the cell culture medium for 4 h in the dark at 37 °C. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals that were accumulated inside the cells' cytoplasm. The MTT solution was removed and 200 μL of DMSO was added in all samples to solubilize the formazan dye crystals. The plate was read in spectrophotometer and the optimal wavelength for absorbance was 570 nm. The MTT assay was performed in triplicate and also made three independent assays. The cell viability inhibition was determined by evaluation of MTT result obtained for test samples

compared with the control samples in the same conditions, following the expression: {survival % = [(tested sample-blank)/(control sample-blank)] × 100}.

Bioimaging experiments. Cells were seeded on 13 mm round glass coverslips on the bottom of a 24-well plate, allowed to adhere overnight and washed three times with serum-free medium for removal of non-adherent cells. After reaching confluence, the cells were separated in two samples (live samples and fixed samples). The live samples were incubated for 30 minutes with each compound solution (1 μM) at 37 ºC. These live samples were washed three times with PBS 1X (pH 7.4) at room temperature and fixed in formaldehyde 3.7% for 30 minutes. The samples were washed again three times in PBS 1X (pH 7.4) at room temperature and the coverslips were mounted over glass slides using ProLong Gold Antifade (Invitrogen, OR, USA) according to the manufacturer's recommendations. The fixed samples were first washed three times in PBS 1X (pH 7.4) and then fixed in formaldehyde 3.7% for 30 minutes. After fixative procedure the samples were washed three times in PBS 1X (pH 7.4) at room temperature and incubated for 30 minutes with each compound solution (1 μM) at room temperature. The samples were washed three times in PBS 1X (pH 7.4) at room temperature and the coverslips were mounted over glass slides using ProLong Gold Antifade (Invitrogen, OR, USA) according to the manufacturer's recommendations. The negative control was performed by incubation of the samples in 0.1% of DMSO, which was the diluent solution used. The samples were analyzed using a Confocal Microscopy and excited using 405 nm wavelength laser emission and the fluorescence images were acquired at 520-550 wavelength range. All assays were performed in triplicate and it was done three repetitions for each experimental condition.

Mitochondrial co-staining experiments. The cells mitochondria staining procedures were performed with MitoTracker Red i.e. a specific fluorescent commercial marker indicated to mitochondria staining. Briefly, live and pre-fixed cells (processed as described above) were incubated with MitoTracker solution (prepared according the manufacturer's instructions) or with **BTD-4APOc** during 30 minutes at room temperature. MitoTracker does not work in fixed samples but it was necessary to perform this assay in order to show that **BTD-4APOc** stains mitochondria even in fixed samples. After samples incubation, the cells were washed three times in PBS and the samples were mounted over glass slides by using antifade agent Prolong Gold (Invitrogen, OR, USA) according to the manufacture's recommendations. The samples were analyzed using a Confocal Microscopy and MitoTracker was excited using 633 nm wavelength and the fluorescence images were acquired at 630-700 wavelength range. **BTD-4APOc** was excited at 405 nm wavelength and

the fluorescence images were acquired at 520-550 wavelength range. All assays were performed in triplicate and it was done three repetitions for each cell sample and experimental condition.

Plasma membrane co-staining. The cell membrane staining procedures were performed with CellMask, a specific fluorescent commercial marker indicated to membrane staining. Briefly, live and pre-fixed cells (processed as described above) were incubated with CellMask solution (prepared according the manufacturer's instructions) or with **BTD-4APBuP** during 30 minutes at room temperature. After samples incubation, the cells were washed three times in PBS and the samples were mounted over glass slides by using antifade agent Prolong Gold (Invitrogen, OR, USA) according to the manufacture's recommendations. The samples were analyzed using a Confocal Microscopy. CellMask was excited at 633 nm wavelength and the fluorescent images were acquired at 680-720 wavelength range. **BTD-4APBuP** was excited at 405 nm wavelength and the fluorescence images were acquired at 520-550 wavelength range. All assays were performed in triplicate and it was done three repetitions for each cell sample and experimental condition.

EPR analyses and ROS quantification. To perform this assay was seeded $1.5x10^5$ of MCF-7 cells per well in 24 well plate and maintained for 24 h allowing cells adhesion in the plate bottom. After 24 h, the samples were incubated for 1 h with *N*-acetylcysteine (NAC, 20 μM) 5mM or Menadione (100 μM) or with **BTD-4APOc** (10 or 100 μM). The negative control was performed incubating the samples with complete DMEM cellular culture medium. After the treatments was add to the samples 20 μL de 1-hydroxy-3methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH). After 25 minutes of samples incubation with CMH, 400 μL of supernatant were collected from all samples. Then the ROS sensitive spin probe CMH [(1-hydroxy-3-methoxycarbonyl -2,2,5,5-tetramethylpyrrolidine, stock solution of 10 mM prepared in KHB (Krebs HEPES buffer) containing 25 μM DF (deferoxamine methanesulfonate salt) and 5 μM DETC (diethyldithiocarbamic acid sodium salt) to minimize the oxidation of CMH by any transition metal)] was added to a final concentration of 200 μM in 1000 μL cell culture medium and allowed to stand at 37 °C for 25 min. After that, 450 μL of supernatant was transferred to a 1 mL de-capped syringe and frozen in liquid nitrogen. EPR measurements were then performed in duplicate. EPR measurements were performed in spectrometer equipped with an X-band (9 GHz) high sensitivity cavity. The instrumental settings were 2 mW microwave power, 5G amplitude modulation, 100 kHz modulation frequency and 200 G sweep width. The peak height, meaning the distance between the lowest and the highest points in the first derivative

spectrum, was used for detection of the signal. A calibration curve was obtained using the nitroxide radical (CP•) diluted in KHB to the following concentrations: 0, 5, 10, 50, and 100 μM. In this concentration range, a linear calibration curve was obtained and all the recorded data were within this calibration range. All the results were expressed as the mean ± SEM. Statistical analyses were carried out with the GraphPad Prism 5 software. The statistical tests used in this study are noted in the figure legends. $P < 0.05$ was considered statistically significant.

Table S1. Experimental and theoretical electronic absorption wavelengths for **BTD-4AP**, **BTD-4APBu**, **BTD-4APBuP**, and **BTD-4APOc** as obtained from TD-DFT calculations using the 6-311+G(2d,p) level of theory

		Exp.	B3LYP	CAM-B3LYP	LC-ωPBE	M06	M06-2X	PBE1PBE
BTD-4AP	**Solvent**				λ_{max}			
	CH_2Cl_2	404	448.58	382.95	345.10	428.63	375.66	429.48
	DMSO	417	448.63	383.02	345.10	428.45	375.48	429.46
	MeCN	403	447.90	382.32	344.41	427.75	374.81	428.75
	MeOH	402	447.74	382.16	344.25	427.59	374.66	428.59
	Toluene	404	448.84	383.22	345.51	429.58	376.52	429.93
	Water	363	447.82	382.24	344.31	427.61	374.68	428.65
BTD-4APOc	**Solvent**				λ_{max}			
	CH_2Cl_2	367	388.96	342.82	315.24	377.28	338.09	375.05
	DMSO	374	394.12	345.49	316.84	380.93	340.37	379.49
	MeCN	363	393.10	344.67	316.11	380.00	339.56	378.53
	MeOH	364	392.80	344.45	315.92	379.74	339.35	378.26
	Toluene	379	378.95	337.68	312.31	370.87	334.09	366.45
	Water	366	393.99	345.09	316.33	380.61	339.91	379.29
BTD-4APBu	**Solvent**				λ_{max}			
	CH_2Cl_2	365	388.57	342.59	315.06	377.02	337.90	374.71
	DMSO	375	393.74	345.25	316.66	380.66	340.17	379.15
	MeCN	363	392.72	344.43	315.93	379.73	339.36	378.19
	MeOH	365	392.41	344.21	315.74	379.46	339.15	377.91
	Toluene	385	378.53	337.45	312.14	370.57	333.89	366.06
	Water	365	393.61	344.85	316.14	380.34	339.71	378.95
BTD-4APBuP	**Solvent**				λ_{max}			
	CH_2Cl_2	371	383.42	339.10	312.43	372.70	334.63	369.65
	DMSO	372	390.07	342.38	314.35	377.28	337.45	375.65
	MeCN	369	388.93	341.52	313.60	376.28	336.61	374.60
	MeOH	362	388.58	341.28	313.40	375.99	336.38	374.28
	Toluene	377	374.07	334.01	309.60	367.61	331.15	361.78
	Water	367	388.02	340.92	313.79	376.45	336.51	374.83

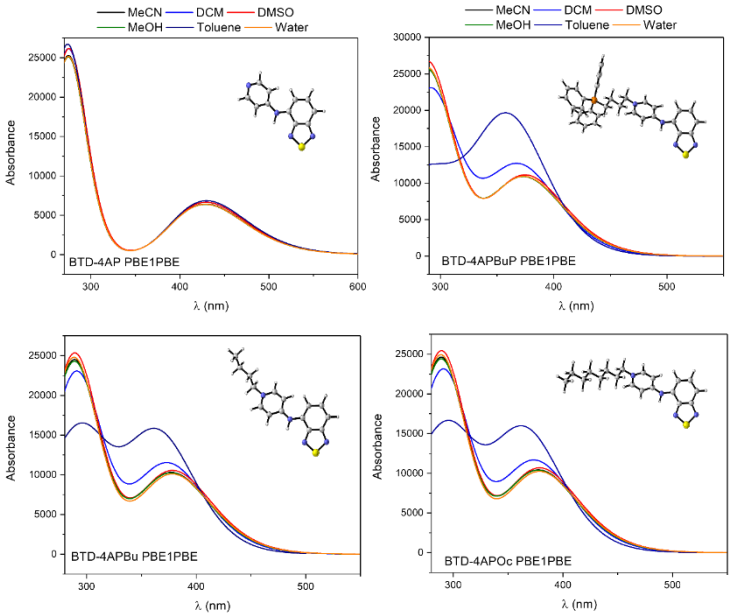


Figure S1. Simulated absorption spectra of the four BTDs in different solvents calculated at PBE1PBE/6-311+G(2d,p)//CAM-B3LYP/6-31G(d) level of theory.

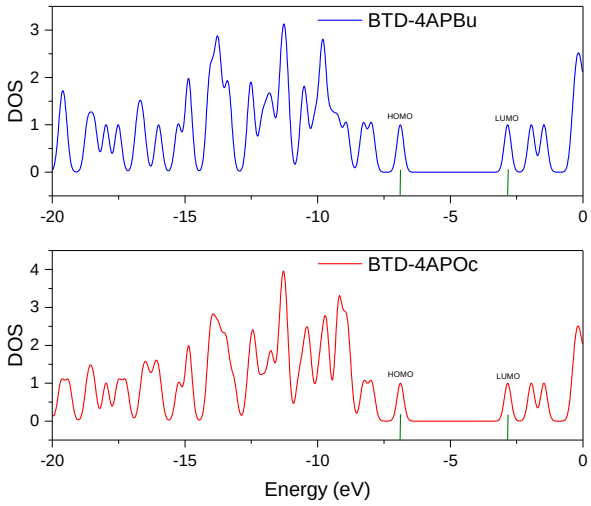


Figure S2. Density of states spectra of **BTD-4APBu** (top) and **BTD-4APOc** (bottom) calculated at the PBE1PBE/6-311+G(2d,p)//CAM-B3LYP/6-31G(d) level of theory. The lengthening of the alkyl chain from a butyl to an octyl group introduces only σ and σ* states but do not modify the energy distribution of the frontier states.

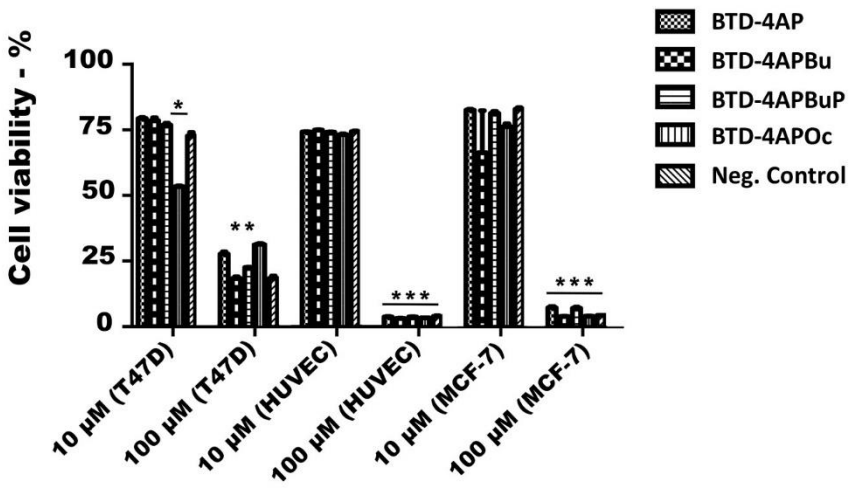


Figure S3. Cellular viability determination by MTT analysis after 24 hours for the developed dyes. The graphic bar shows no statistically significant cytotoxic effect after 24 hours of incubation for the majority of tested compounds in solution at 10 μM. All compounds tested induced strong cytotoxic effects in all tested cells in solution at 100 μM. P value *p< 0.05, ** p<0.01, ***p<0.001.

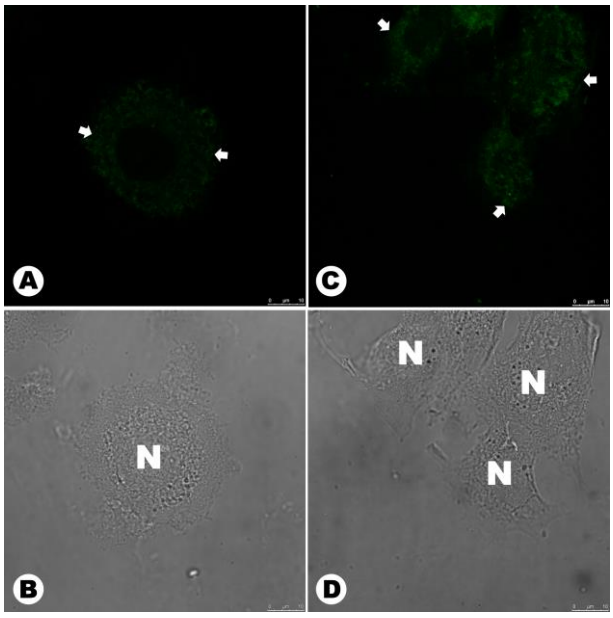


Figure S4. MCF-7 cancer cells stained with **BTD-4AP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. An unspecific fluorescent signal in the cells' cytoplasm (white arrows) is noted in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

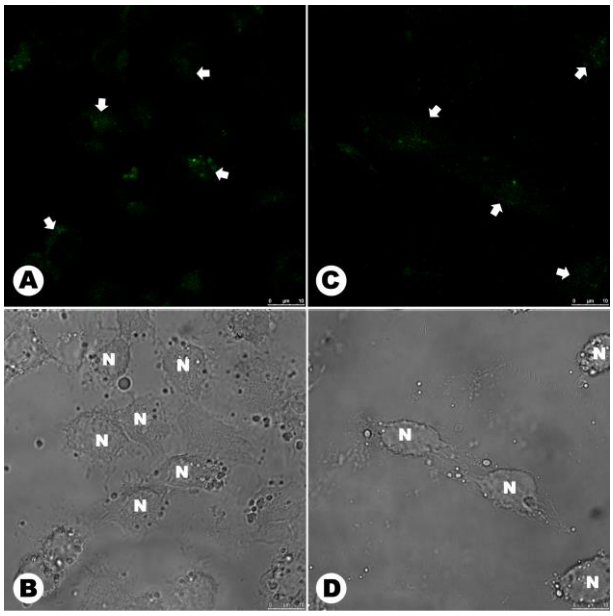


Figure S5. As above A2780 cancer cells stained with **BTD-4AP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. An unspecific fluorescent signal in the cells' cytoplasm (white arrows) is noted in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

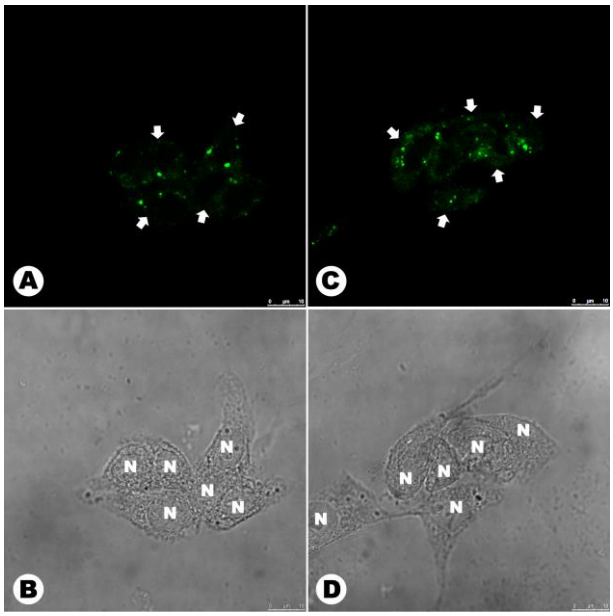


Figure S6. T47D cancer cells stained with **BTD-4AP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. An unspecific fluorescent signal in the cells' cytoplasm (white arrows) is noted in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

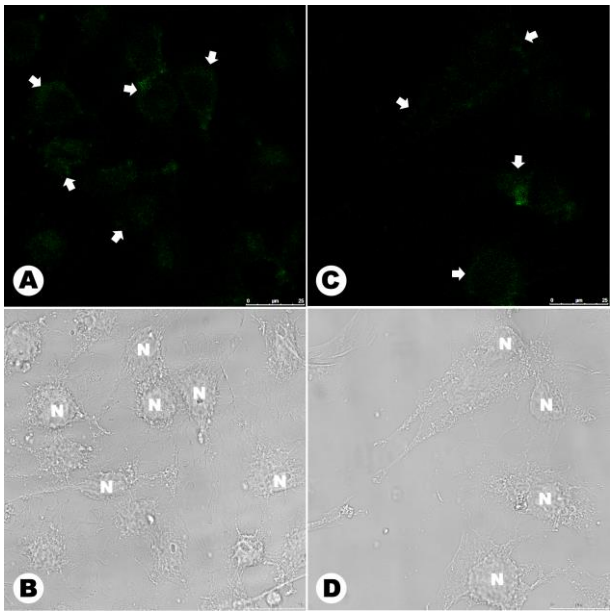


Figure S7. HUVEC cells stained with **BTD-4AP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. An unspecific fluorescent signal in the cells' cytoplasm (white arrows) is noted in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

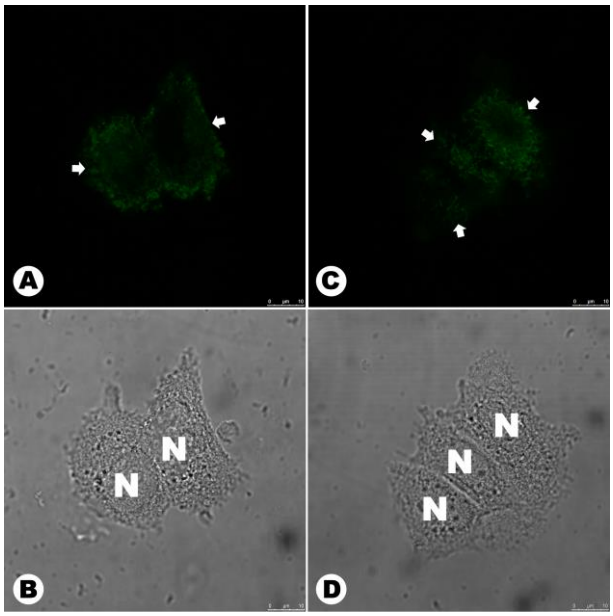


Figure S8. MCF-7 cancer cells stained with **BTD-4APBu** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. Note the accumulation in the cytoplasm (white arrows) in a region near to the cells' nuclei in both samples, a region known to be rich in mitochondria. Leakage is noted. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

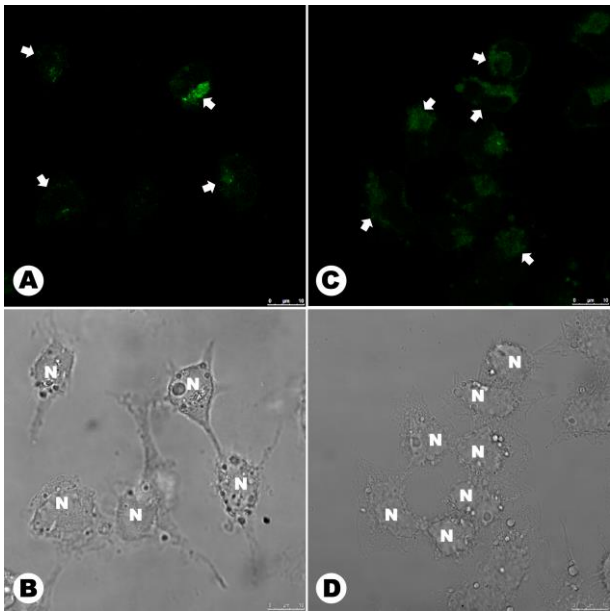


Figure S9. A2780 cancer cells stained with **BTD-4APBu** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. Note the accumulation in the cytoplasm (white arrows) in a region near to the cells' nuclei in both samples, a region known to be rich in mitochondria. Leakage is noted. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

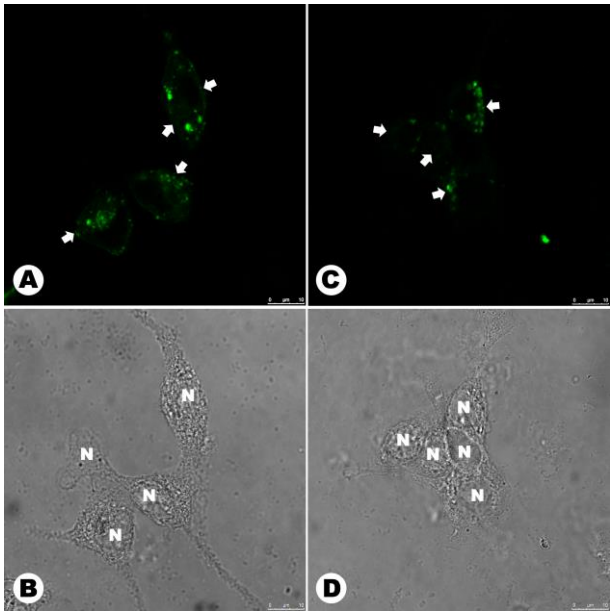


Figure S10. T47D cancer cells stained with **BTD-4APBu** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. Note the accumulation in the cytoplasm (white arrows) in a region near to the cells' nuclei in both samples, a region known to be rich in mitochondria. Leakage is noted. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

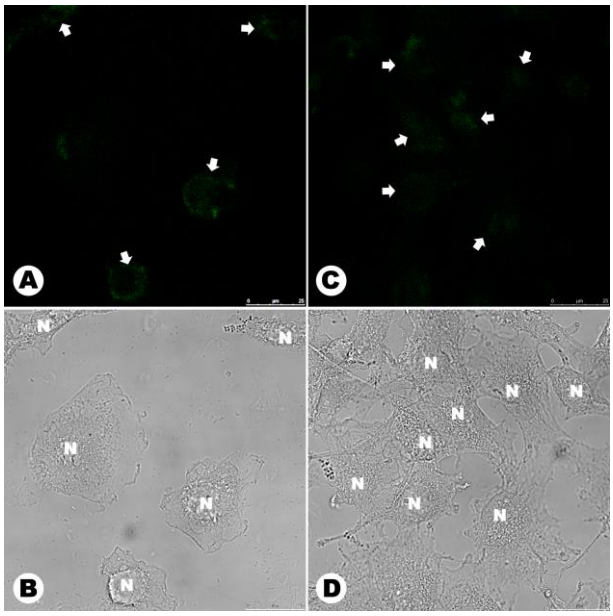


Figure S11. HUVEC cells stained with **BTD-4APBu** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. Note the accumulation in the cytoplasm (white arrows) in a region near to the cells' nuclei in both samples, a region known to be rich in mitochondria. Leakage is noted. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

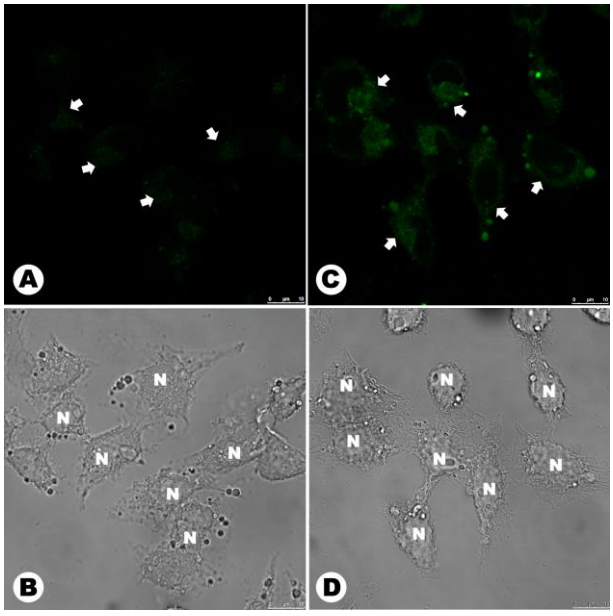


Figure S12. A2780 cancer cells stained with **BTD-4APBuP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. The dye was found accumulated in the peripheral region of the cellular membrane (white arrows) in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

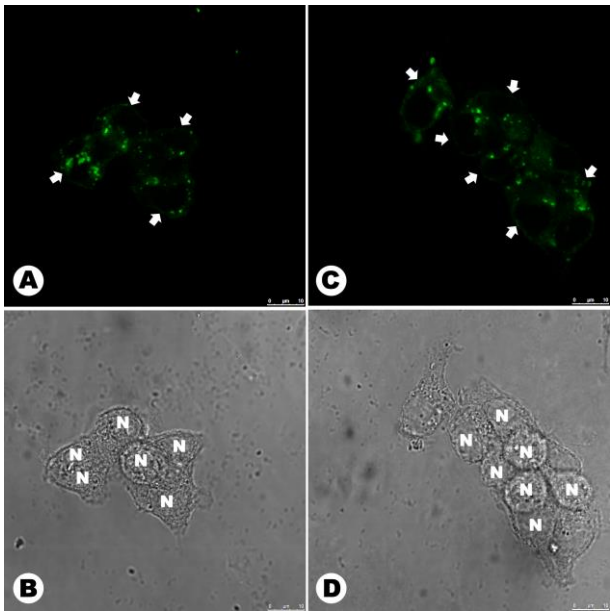


Figure S13. T47D cancer cells stained with **BTD-4APBuP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. The dye was found accumulated in the peripheral region of the cellular membrane (white arrows) in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

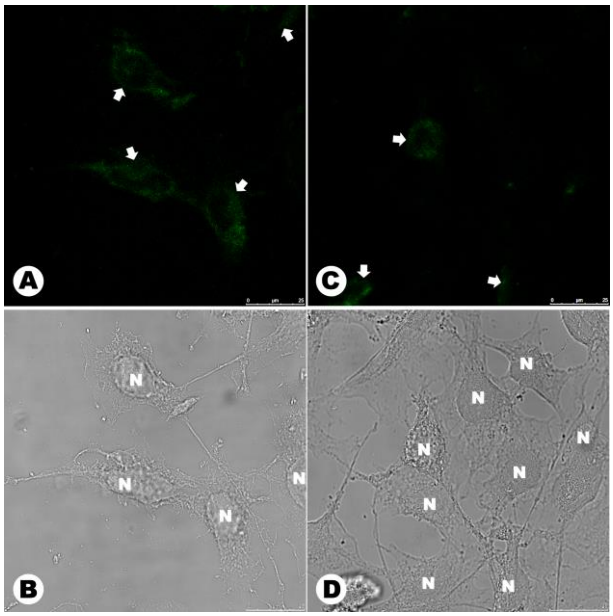


Figure S14. HUVEC cells stained with **BTD-4APBuP** (1 μM). (A) and (B) live cells. (C) and (D) Fixed cells. The dye was found accumulated in the peripheral region of the cellular membrane (white arrows) in both samples. The letter N indicates the nuclei of the cells. Scale bar of 10 μm.

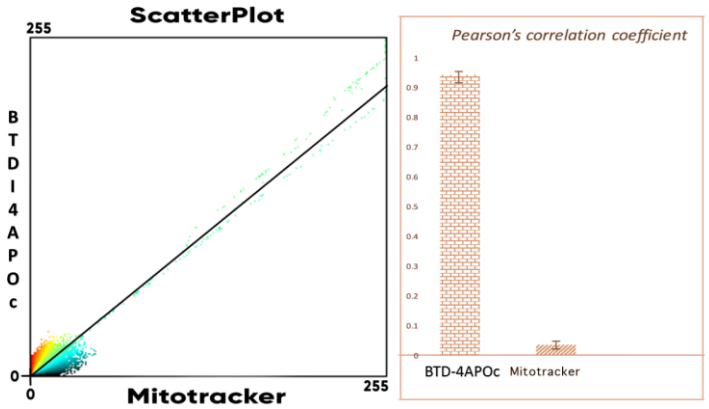


IMAGE NUMBER	NORMAL	90 DEGREES ROTATE
1	R = 0.9563	R = 0.0382
2	R = 0.9282	R = 0.0233
3	R = 0.9477	R = 0.0265
4	R = 0.9615	R = 0.0557
5	R = 0.9242	R = 0.0423
6	R = 0.9818	R = 0.0612
7	R = 0.9753	R = 0.0236
8	R = 0.9221	R = 0.0323
9	R = 0.9362	R = 0.0474
10	R = 0.9347	R = 0.0268
	R = 0.95	

Figure S15. PCC from MCF-7 live cells samples (from ten different images) stained with **BTD-4APOc** (green emission) and MitoTracker red (red emission). (Left) Scatterplot from **BTD4APO** and red (MitoTracker) intensities. The black line in scatterplot is according to the results of ordinary least squares regression. (Center) Pearson's correlation coefficient of the images with respectively negative controls. (Right) Normal PCC values (average highlighted in yellow) and 90° counterclockwise rotation.

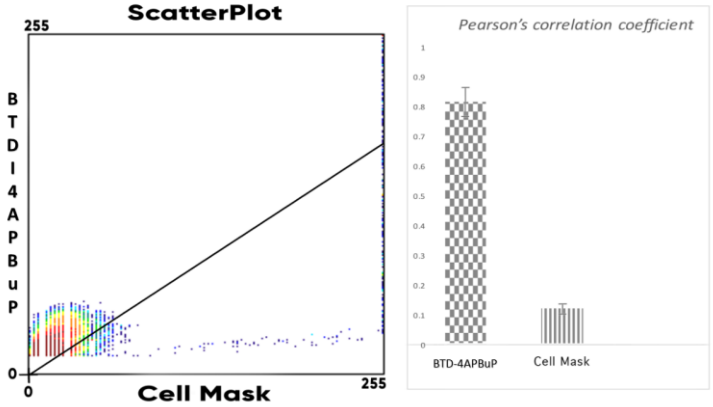
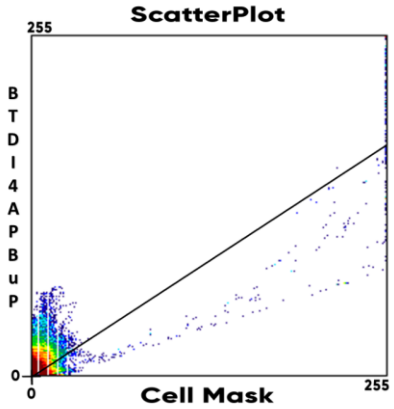


IMAGE NUMBER	NORMAL	90 DEGREES ROTATE
1	R = 0.8683	R = 0.1053
2	R = 0.8342	R = 0.1218
3	R = 0.7895	R = 0.1008
4	R = 0.8834	R = 0.1312
5	R = 0.9342	R = 0.1034
6	R = 0.8215	R = 0.1065
7	R = 0.8738	R = 0.1141
8	R = 0.8821	R = 0.1021
9	R = 0.8265	R = 0.1142
10	R = 0.9629	R = 0.1012
	R = 0.87	

Figure S16. PCC from MCF-7 live cells samples (from ten different images) stained with **BTD-4APBuP** (green emission) and CellMask (red emission). (Left) Scatterplot from **BTD-4APBuP** and red (CellMask) intensities. The black line in scatterplot is according to the results of ordinary least squares regression. (Center) Pearson's correlation coefficient of the images with respectively negative controls. (Right) Normal PCC values (average highlighted in yellow) and 90° counterclockwise rotation.



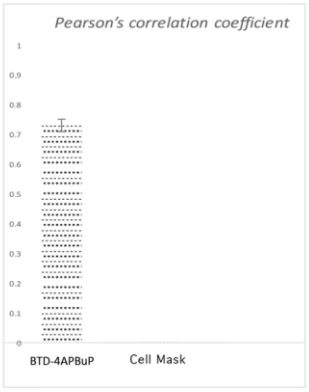


IMAGE NUMBER	NORMAL	90 DEGREES ROTATE
1	R = 0.7891	R = 0.0036
2	R = 0.7343	R = 0.0022
3	R = 0.6514	R = 0.0038
4	R = 0.8429	R = 0.0025
5	R = 0.7565	R = 0.0042
6	R = 0.6984	R = 0.0013
7	R = 0.8232	R = 0.0034
8	R = 0.6881	R = 0.0020
9	R = 0.7930	R = 0.0071
10	R = 0.6943	R = 0.0063
	R = 0.75	

Figure S17. PCC from MCF-7 fixed cells samples (from ten different images) stained with **BTD-4APBuP** (green emission) and CellMask (red emission). (Left) Scatterplot from **BTD-4APBuP** and red (CellMask) intensities. The black line in scatterplot is according to the results of ordinary least squares regression. (Center) Pearson's correlation coefficient of the images with respectively negative controls. (Right) Normal PCC values (average highlighted in yellow) and 90° counterclockwise rotation.

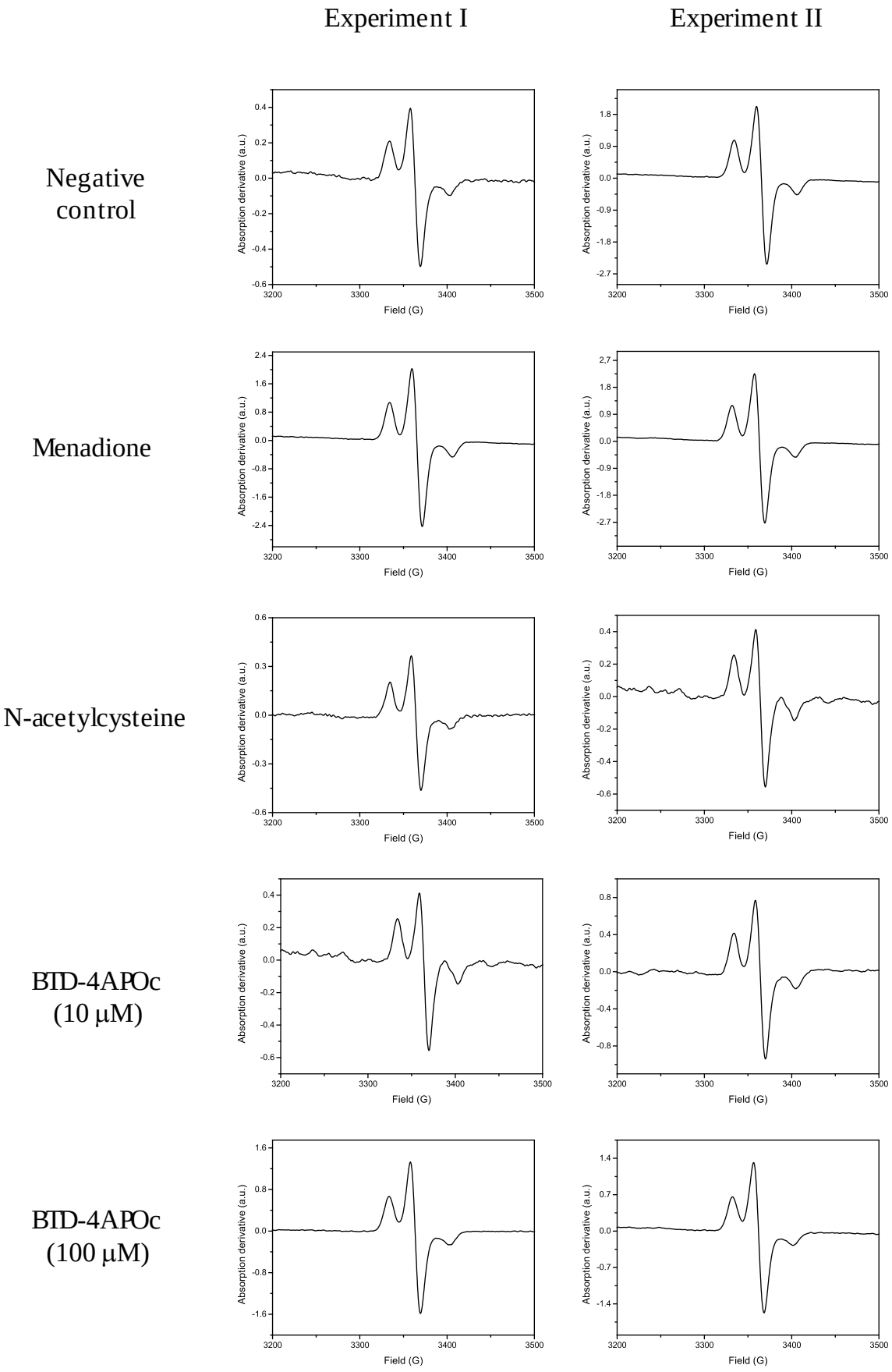


Figure S18. EPR experiments to evaluate the intracellular ROS generation of the developed probe.

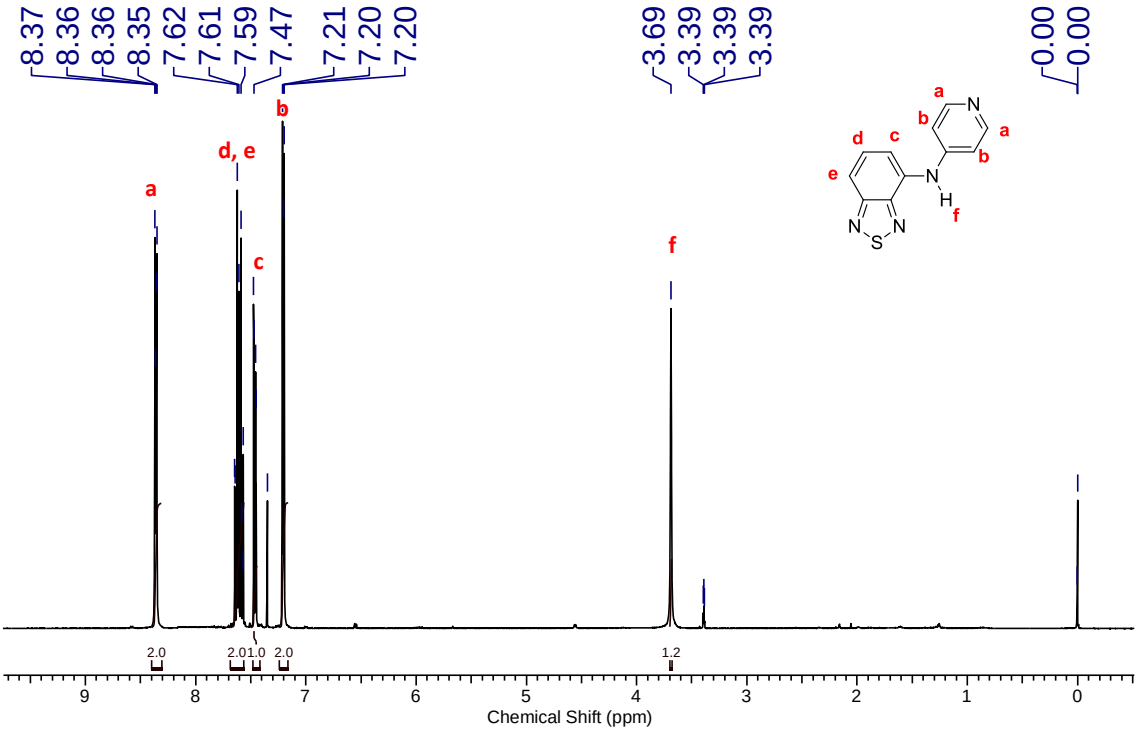


Figure S19. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) of **BTD-4AP**.

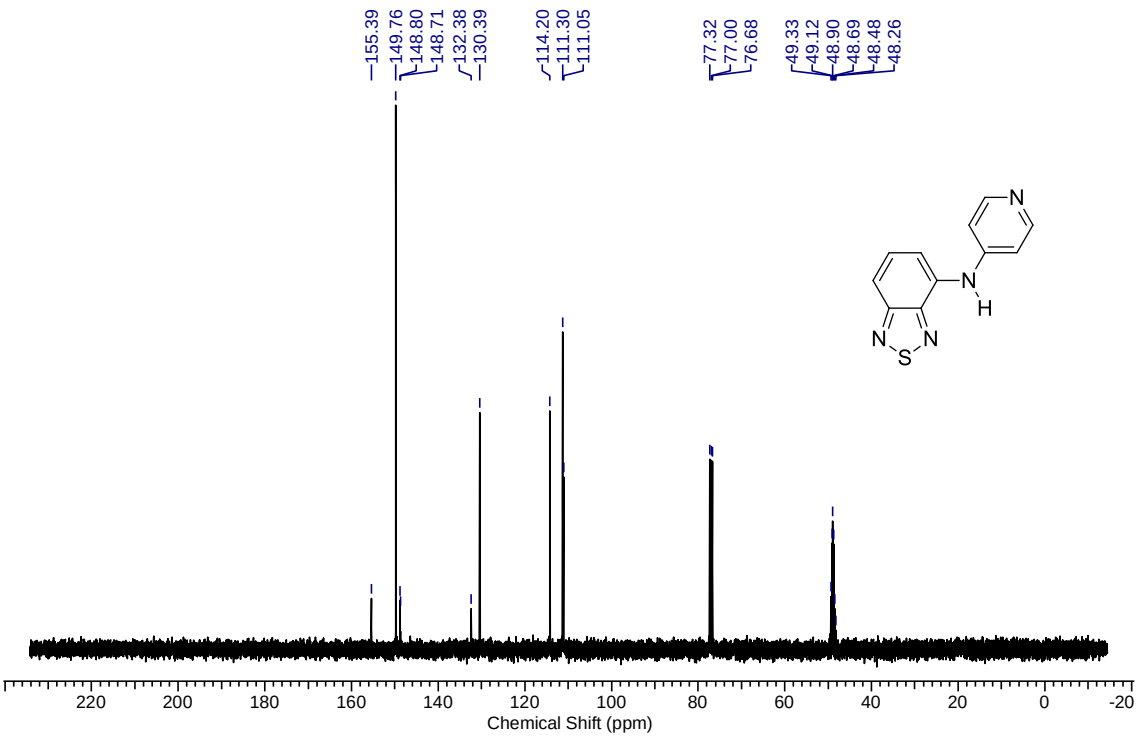


Figure S20. ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$) of **BTD-4AP**.

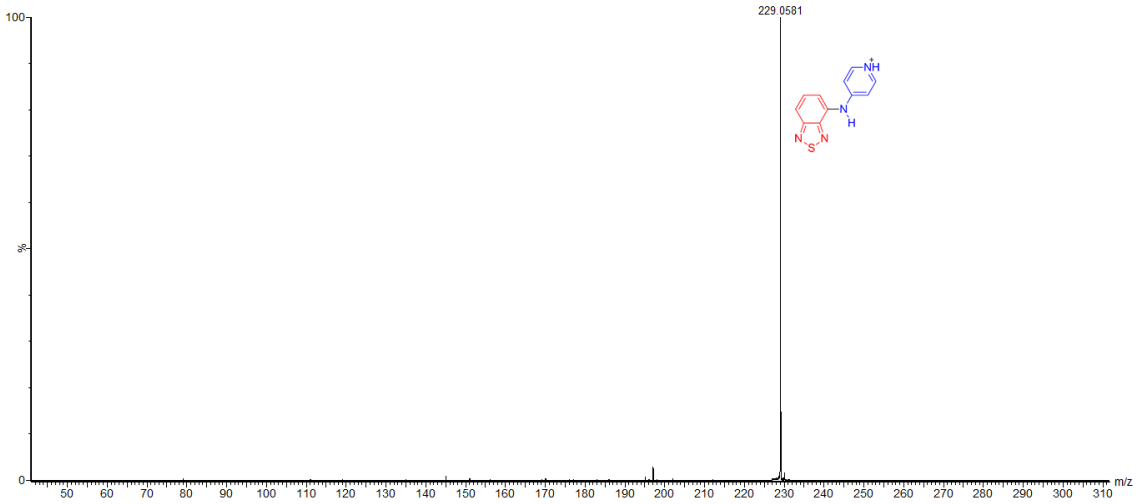


Figure S21. ESI(+)-QTOF product ion spectrum of protonated **[BTD-4AP + H]$^+$.**

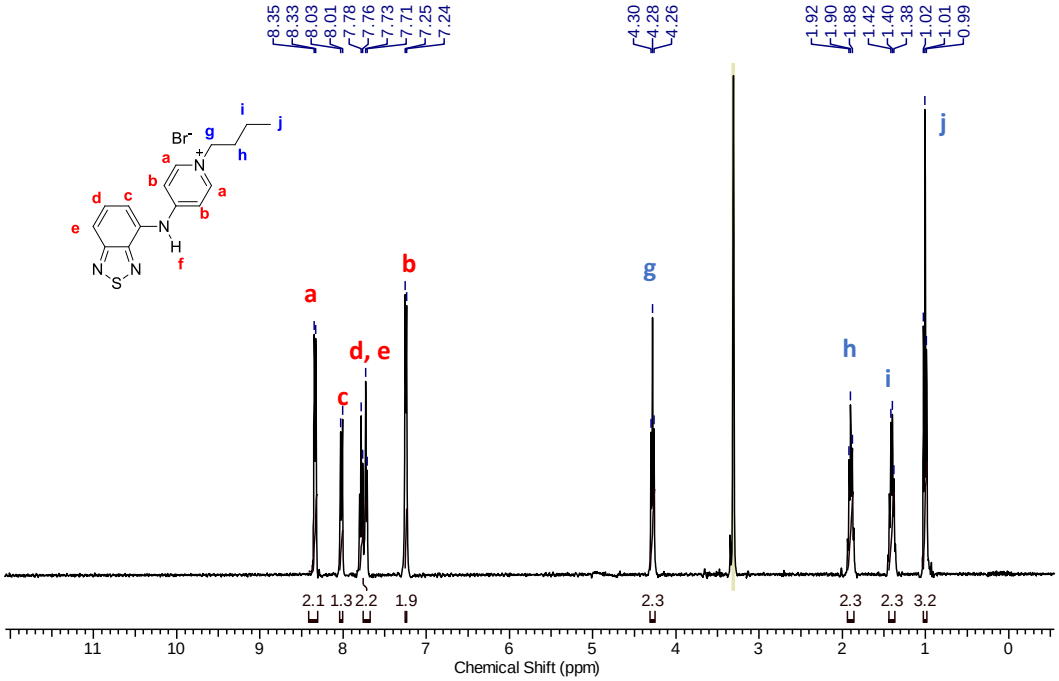


Figure S22. ^{1}H NMR (400 MHz, CD_3OD) of **BTD-4APBu**.

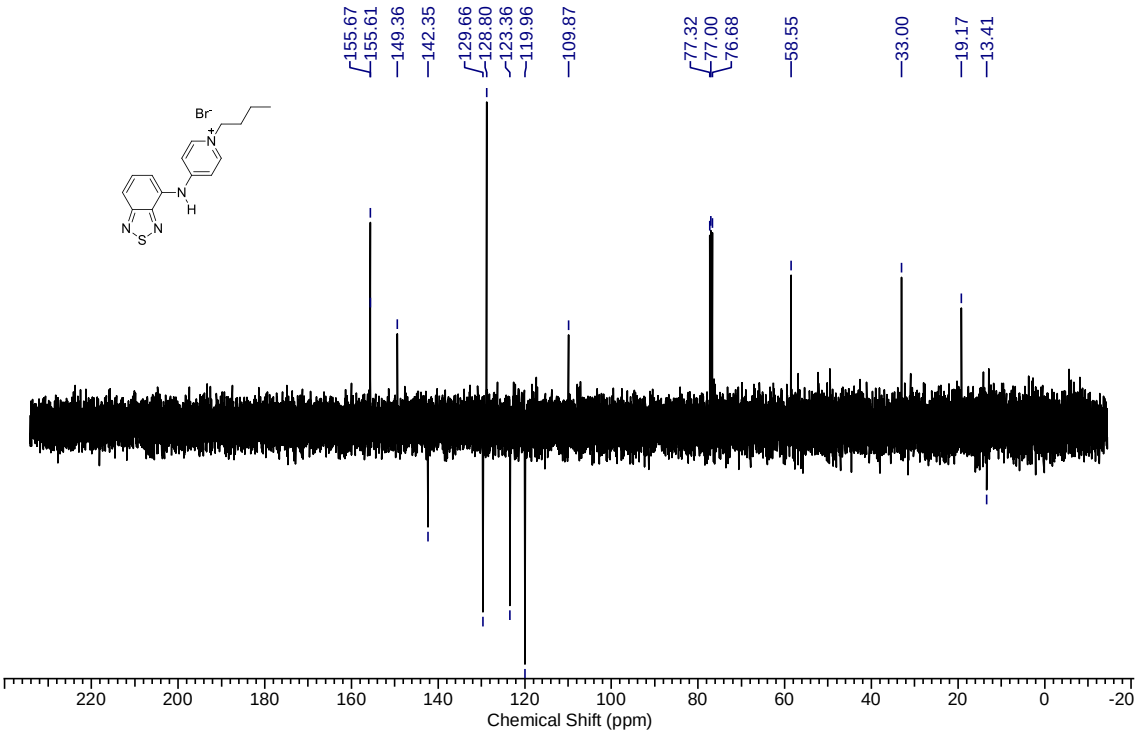


Figure S23. ^{13}C NMR (APT,100 MHz, $CDCl_3$) of **BTD-4APBu**.

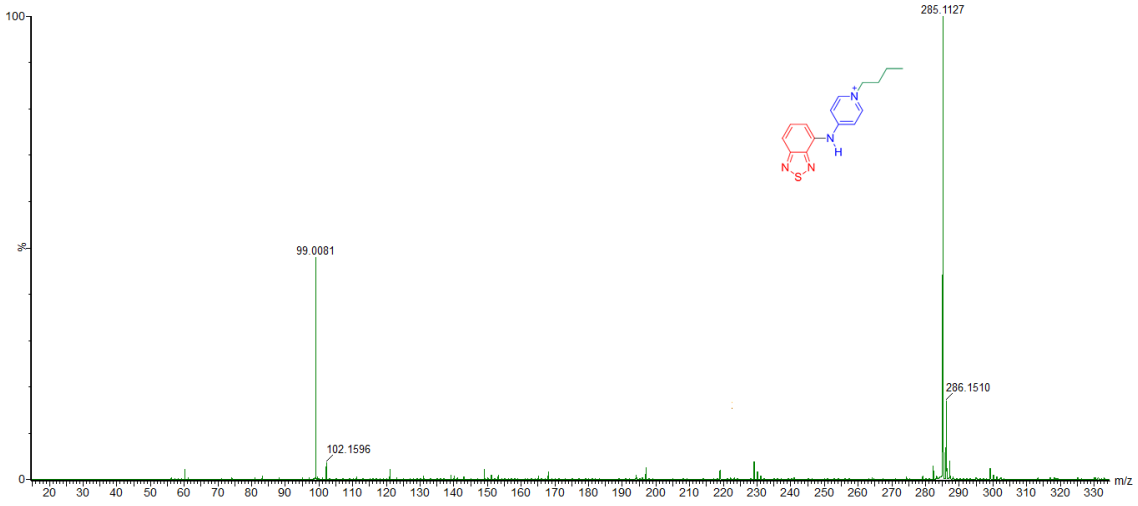


Figure S24. ESI(+)-QTOF product ion spectrum of **[BTD-4APBu]**$^+$.

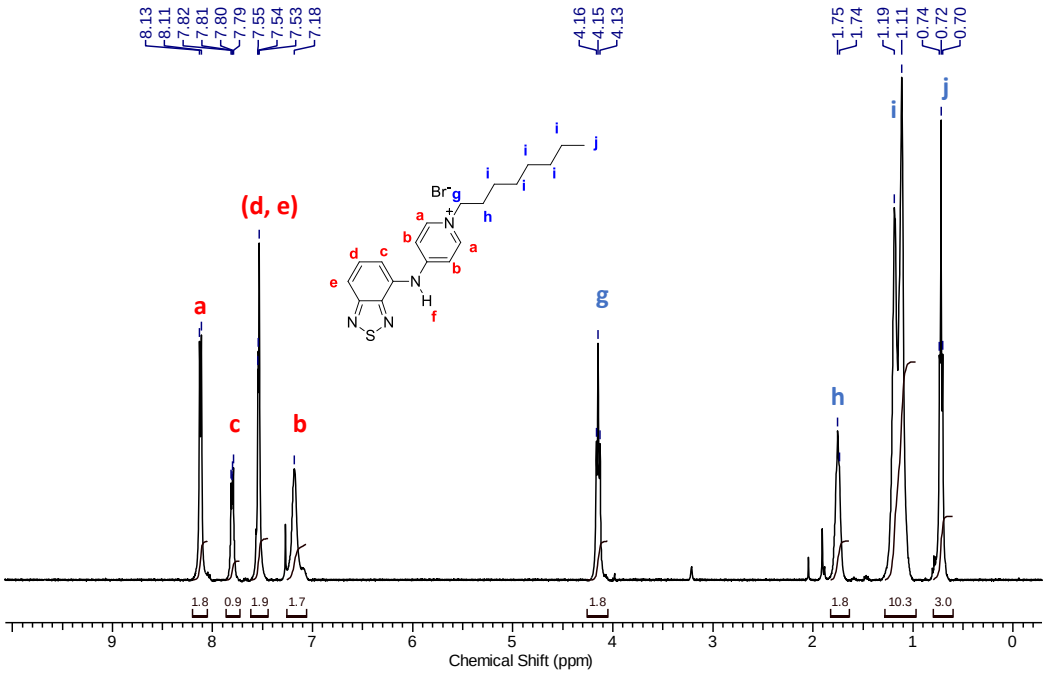


Figure S25. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) of **BTD-4APOc**.

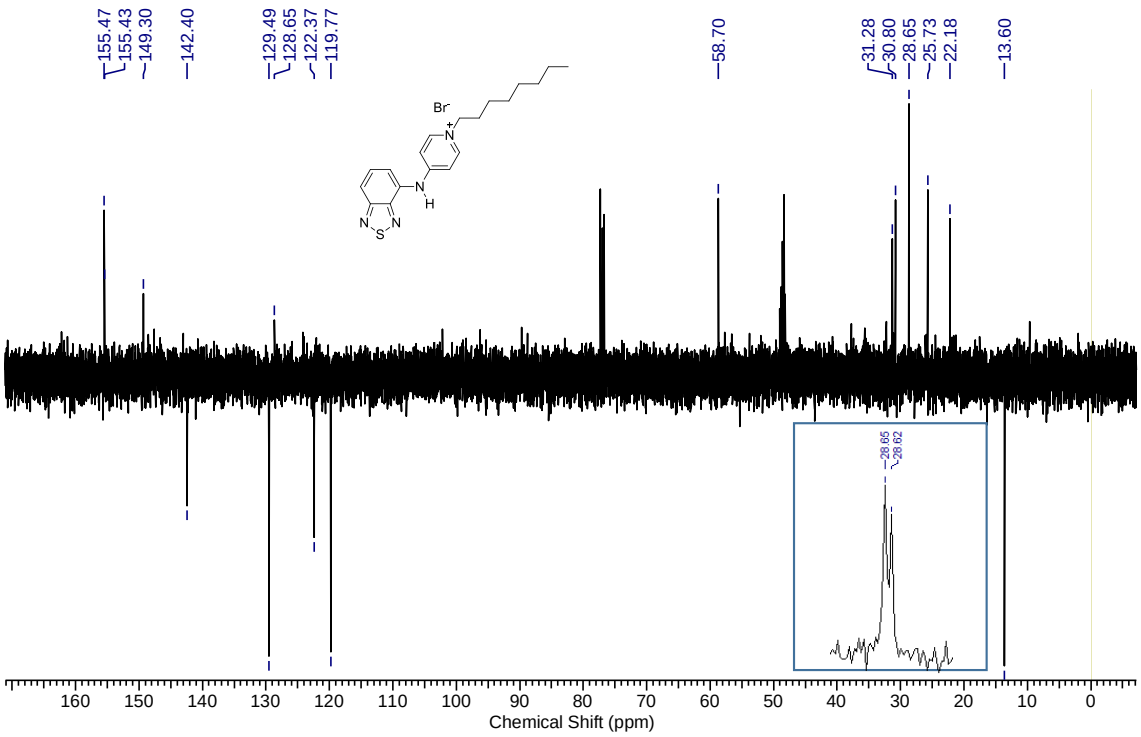


Figure S26. ^{13}C NMR (APT,100 MHz, $CDCl_3/CD_3OD$) of **BTD-4APOc**.

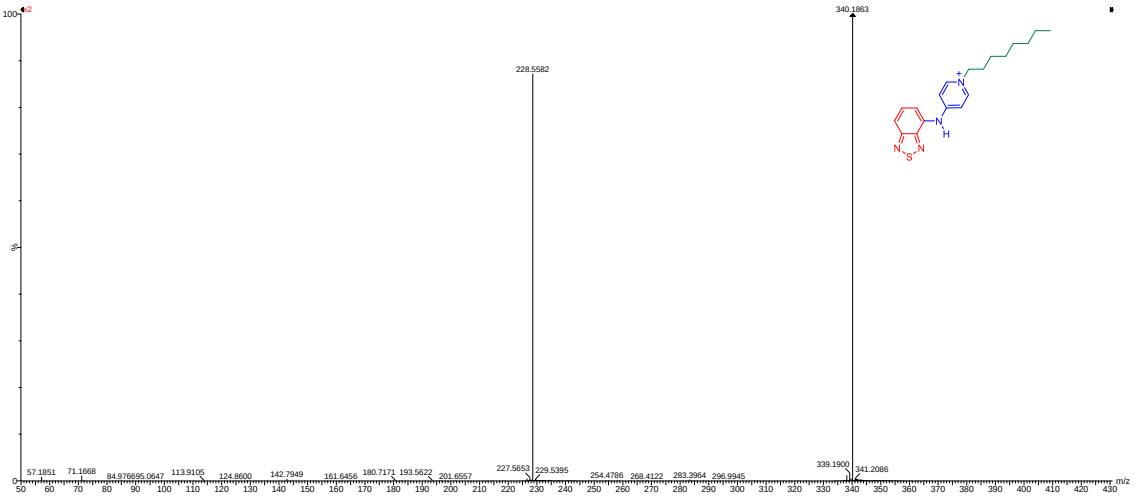


Figure S27. ESI(+)-QTOF product ion spectrum of **[BTD-4APOc]$^{+}$.**

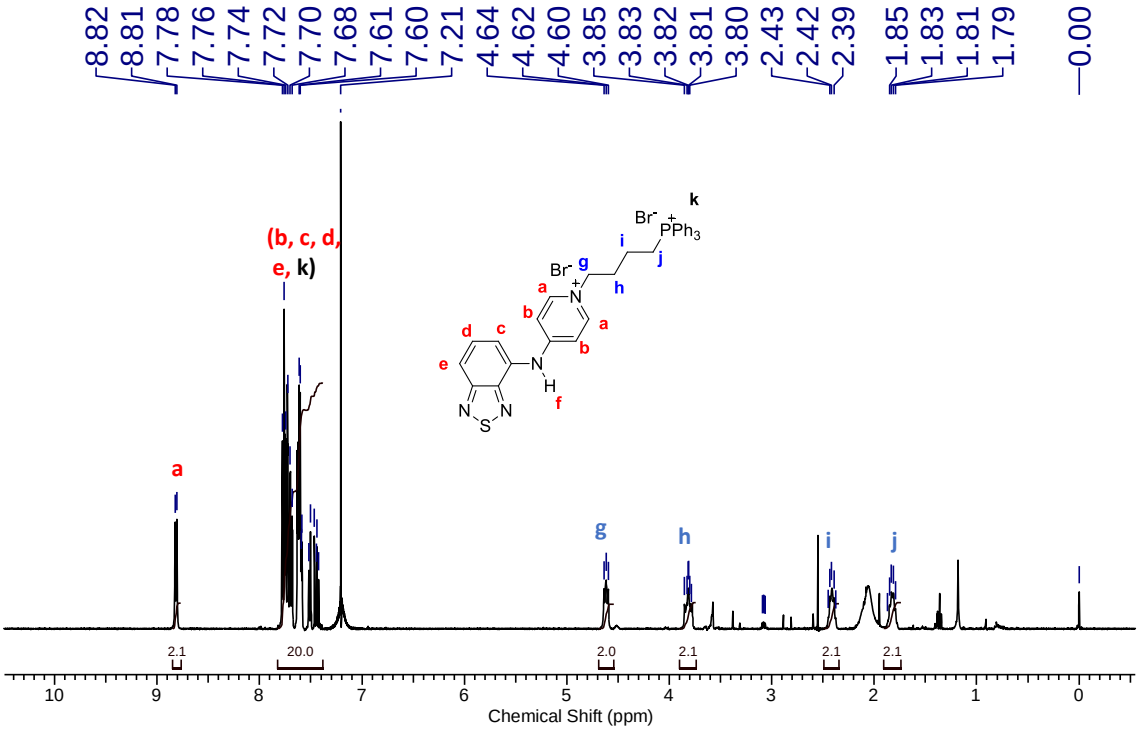


Figure S28. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) of **BTD-4APBuP**.

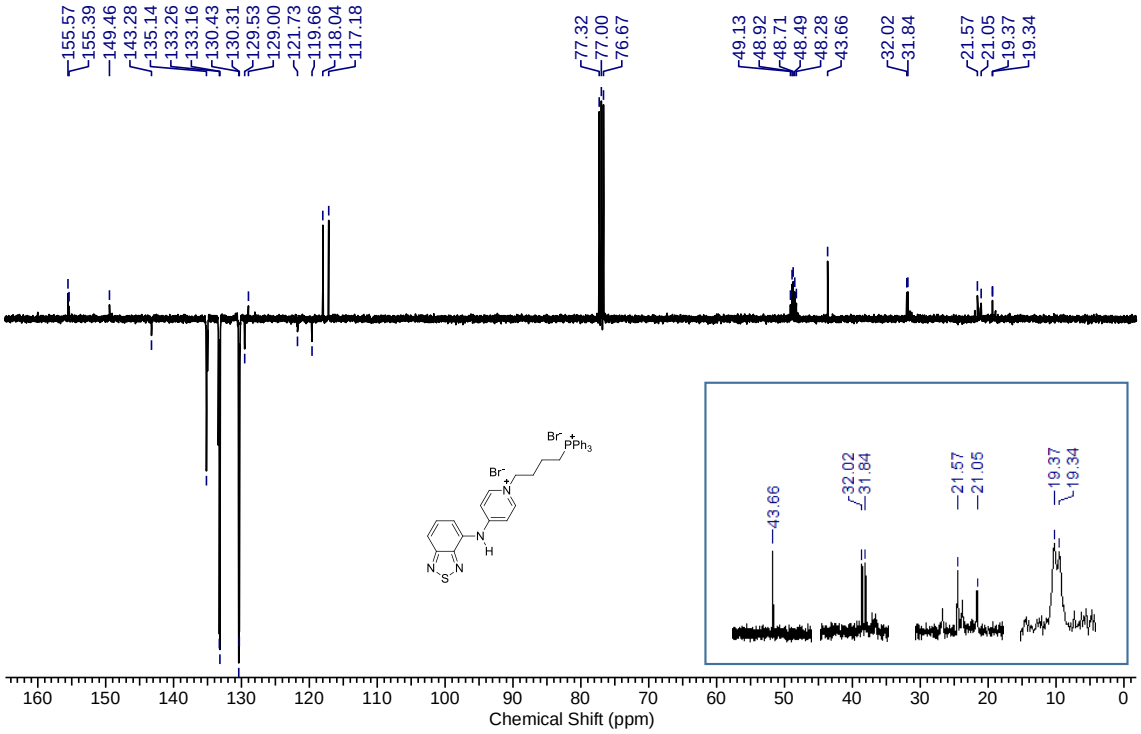


Figure S29. ^{13}C NMR (APT, 100 MHz, $CDCl_3/CD_3OD$) of **BTD-4APBuP**.

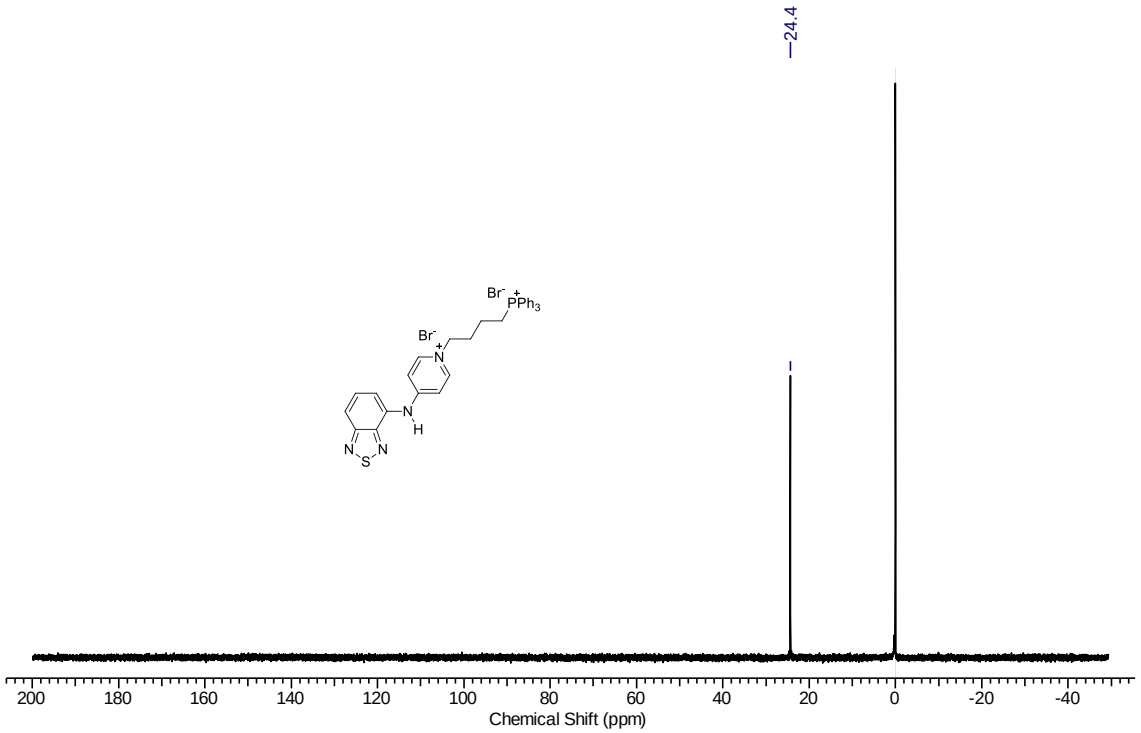


Figure S30. ^{31}P NMR (161 MHz, $CDCl_3/CD_3OD$, H_3PO_4 as the reference of 0.0 ppm) of **BTD-4APBuP**.

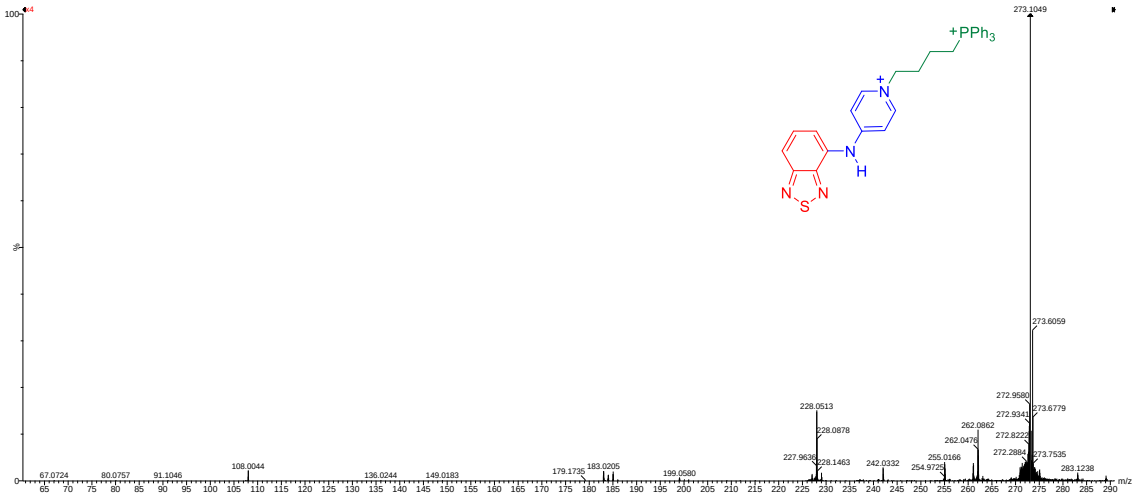
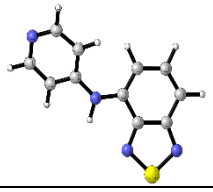


Figure S31. ESI(+)-MS/MS of the product ion spectrum of **[BTD-4APBuP]**$^{2+}$.

Cartesian coordinates of the optimized structures obtained at the CAM-B3LYP/6-31G(d) level of theory

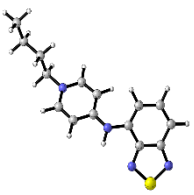
BTD-4AP



E(RCAM-B3LYP) = -1040.98505103 a.u.
Charge = 0
Multiplicity = 0

C	1.39425300	2.53793700	-0.30107700
C	2.64544000	2.03414000	-0.14131400
C	2.76232900	0.62347400	-0.00590200
C	1.58510300	-0.19870300	-0.02059000
C	0.27448600	0.37215400	-0.17429500
C	0.21529000	1.73050700	-0.32719400
N	3.87619200	-0.08527600	0.13511400
S	3.44931300	-1.64953100	0.23160400
N	1.83341200	-1.49270400	0.10257200
N	-0.76841400	-0.53136300	-0.19516800
C	-2.13375100	-0.32865600	-0.06379000
C	-3.00283100	-1.32989400	-0.50730000
C	-4.36435200	-1.15912500	-0.35214300
N	-4.92603600	-0.08376600	0.19804900
C	-4.09222700	0.85696900	0.62774500
C	-2.71088300	0.79414900	0.52975800
H	1.26496000	3.60766500	-0.42278300
H	3.52992400	2.65642300	-0.12348500
H	-0.73145000	2.21987100	-0.50406600
H	-0.47120500	-1.49152900	-0.28978500
H	-2.61486000	-2.22691200	-0.97785200
H	-5.04718600	-1.93069000	-0.69688000
H	-4.55298500	1.72453000	1.09258500
H	-2.10942800	1.58718600	0.94924900

BTD-4APBu



E(RCAM-B3LYP) = -1198.56439949 a.u.
Charge = +1
Multiplicity =1

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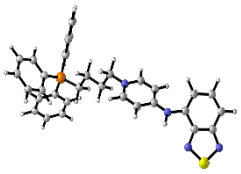
C	-4.21185400	2.15261700	0.07847500
C	-4.38151300	0.74095100	0.08299200

C	-3.25835900	-0.11718800	-0.13978800
C	-1.96457900	0.43478600	-0.37544700
C	-1.84431400	1.79092000	-0.41220100
N	-5.50850700	0.06100400	0.26866300
S	-5.15341300	-1.51172700	0.15457400
N	-3.55343300	-1.40981800	-0.12725000
N	-0.93506100	-0.49909600	-0.61945200
C	0.34809200	-0.47752900	-0.22816100
C	1.20896500	-1.53126900	-0.62319900
C	2.51123800	-1.53698700	-0.23491900
N	3.02917100	-0.55916000	0.54433600
C	2.22687800	0.44927300	0.94731500
C	0.91427200	0.52720100	0.58965700
C	4.46763200	-0.56348200	0.89403200
C	5.31912500	0.14969700	-0.14695400
C	6.80026400	0.13252000	0.22445200
C	7.66536700	0.84526800	-0.80778900
H	-2.80828300	3.71191600	-0.20058100
H	-5.06247900	2.79770300	0.25442100
H	-0.89755000	2.25232800	-0.66405000
H	-1.27305000	-1.36941500	-1.01163200
H	0.84085900	-2.34034600	-1.24137600
H	3.19235000	-2.32446500	-0.52832100
H	2.68451700	1.19364300	1.58534700
H	0.31773000	1.34069100	0.97266600
H	4.56309800	-0.09529600	1.87399600
H	4.76891300	-1.60497400	1.00892500
H	5.17658300	-0.32546800	-1.12324200
H	4.97501500	1.18390500	-0.25259000
H	6.93719500	0.60149600	1.20437200
H	7.13778600	-0.90341600	0.33301200
H	8.71746300	0.81859600	-0.52249000
H	7.58039800	0.37818200	-1.79166800
H	7.38038700	1.89467600	-0.91244900

BTD-4APBuP

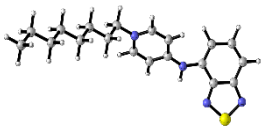
E(RCAM-B3LYP) = -2233.74332822 a.u.
Charge = +2
Multiplicity = 1



C	-8.32565200	-1.84996200	-1.49928100
C	-9.24431900	-0.86682900	-1.69519600
C	-9.09737500	0.33870800	-0.95639000
C	-7.99406100	0.50206000	-0.06095600
C	-7.04225500	-0.54547500	0.10086500
C	-7.23192300	-1.70325600	-0.59105200
N	-9.89621600	1.40148400	-0.97194600
S	-9.29336600	2.48107100	0.06603400
N	-7.98279700	1.66282200	0.58064200
N	-6.01464000	-0.30164500	1.04353500
C	-4.71642600	-0.60409200	0.98250800
C	-3.87069000	-0.27575100	2.07605400
C	-2.54925500	-0.57954900	2.03892300
N	-1.98217400	-1.19267300	0.96862000
C	-2.76161100	-1.49589300	-0.09740600
C	-4.09530500	-1.23232500	-0.12680500
C	-0.53767800	-1.49174100	0.95513800
C	0.29706300	-0.34180900	0.39716200
C	1.79057400	-0.67923200	0.39795200
C	2.61788400	0.46536100	-0.19029200
P	4.43722600	0.24521500	-0.18962800
C	5.14266300	1.74883300	-0.88509100
C	6.26688500	2.32367100	-0.29103100
C	6.82773200	3.46674700	-0.84067000
C	6.27340700	4.03788000	-1.97661400
C	5.15431500	3.46808300	-2.57170100
C	4.58758900	2.32604600	-2.03195200
C	4.88651300	-1.19028000	-1.18212000
C	4.57661800	-2.47821000	-0.72875500
C	4.90727600	-3.57881300	-1.50011300
C	5.54731700	-3.40665200	-2.72235100
C	5.86349900	-2.13350800	-3.17071200
C	5.53751000	-1.02325300	-2.40466000

C	5.00810400	0.02932300	1.50345600
C	4.46120200	0.82018600	2.51883700
C	4.94580400	0.71639200	3.81143600
C	5.97934900	-0.16767800	4.09816500
C	6.53192500	-0.94621600	3.09187100
C	6.05167100	-0.85067400	1.79373500
H	-8.42300000	-2.78975600	-2.02857300
H	-10.07872000	-0.98317200	-2.37428400
H	-6.57704900	-2.55181900	-0.43415100
H	-6.30361300	0.31431800	1.79466900
H	-4.27523700	0.20417700	2.95828300
H	-1.89060300	-0.35685900	2.86776900
H	-2.26206900	-1.96722300	-0.93351300
H	-4.65836900	-1.48611500	-1.01189800
H	-0.24559500	-1.73155200	1.97806500
H	-0.39897800	-2.39990000	0.36796500
H	-0.03371400	-0.11481800	-0.62099600
H	0.11672600	0.55981400	0.99049700
H	2.11928100	-0.89139400	1.41950200
H	1.95715700	-1.58943200	-0.18356700
H	2.33503200	0.64105900	-1.23076200
H	2.42470500	1.40223500	0.33864400
H	6.70347800	1.88940000	0.60009300
H	7.69955300	3.91198900	-0.37716200
H	6.71399300	4.93164600	-2.40202900
H	4.72327600	3.91477400	-3.45943000
H	3.72191100	1.89035700	-2.51805700
H	4.10794900	-2.62918100	0.23696000
H	4.67720900	-4.57547800	-1.14310900
H	5.80997600	-4.27123300	-3.32027700
H	6.37488300	-2.00004300	-4.11615800
H	5.80503000	-0.03502100	-2.75713200
H	3.67152400	1.53287200	2.30789400
H	4.52408000	1.33243800	4.59639600
H	6.35923800	-0.24347000	5.11006700
H	7.34316400	-1.62861200	3.31433100
H	6.49491700	-1.45784500	1.01417100

BTD-4APOc



E(RCAM-B3LYP) = -1355.75270771 a.u.
Charge = 1
Multiplicity = 1

C	-5.30026000	-1.23741800	2.04092200
C	-6.19764600	-0.22224700	1.93780400
C	-6.04258200	0.69345900	0.86114500
C	-4.95214600	0.54646200	-0.05362500
C	-4.01887500	-0.52101800	0.10116800
C	-4.21953800	-1.40338800	1.11904100
N	-6.82033800	1.72816500	0.55879200
S	-6.21326700	2.42309900	-0.76840200
N	-4.93037300	1.45330300	-1.02061900
N	-3.00404000	-0.59279400	-0.87667200
C	-1.71023600	-0.92383700	-0.74454000
C	-1.09089500	-1.24370300	0.48544900
C	0.23593200	-1.55349900	0.51494600
N	0.99768000	-1.58530300	-0.59913300
C	0.43326800	-1.26967900	-1.78760500
C	-0.88161500	-0.94240200	-1.89349800
C	2.44669100	-1.87883100	-0.51094700
C	3.27468100	-0.62607500	-0.26175800
C	4.76740100	-0.93744000	-0.18867100
C	5.61329800	0.30403700	0.07958300
C	7.10869500	0.00903600	0.13968000
C	7.95351100	1.24777500	0.41939900
C	9.45069400	0.95960700	0.47409100
C	10.28533000	2.20245600	0.75839300
H	-5.40190800	-1.96509400	2.83676300
H	-7.01932100	-0.10339800	2.63162000
H	-3.58290900	-2.27253200	1.22741800
H	-3.27584300	-0.18038700	-1.76075400
H	-1.63957600	-1.21706300	1.41401800
H	0.74186500	-1.78711200	1.44233400

H	1.08671200	-1.29908200	-2.64914300
H	-1.28785900	-0.70617900	-2.86888000
H	2.58074000	-2.61055500	0.28603200
H	2.73389900	-2.36729800	-1.44230000
H	3.08560300	0.09958600	-1.05965800
H	2.94775500	-0.15353500	0.67039100
H	4.95113100	-1.67829200	0.59770400
H	5.09164400	-1.40251600	-1.12641400
H	5.42161500	1.05032800	-0.69998700
H	5.29543000	0.76324900	1.02267800
H	7.30115000	-0.74338600	0.91344300
H	7.42895400	-0.44275100	-0.80639700
H	7.75875900	2.00340500	-0.35105100
H	7.63718900	1.69767800	1.36803600
H	9.64618600	0.20341400	1.24232300
H	9.76852900	0.51370600	-0.47472400
H	11.35118000	1.96962900	0.79204100
H	10.13987100	2.96431700	-0.01184300
H	10.01672600	2.65091500	1.71834900