Electronic Supplementary Material (ESI) for RSC Advances

Supporting Information

A Click Chemistry Approach to Identify Protein Targets of Cancer Chemopreventive Phenethyl Isothiocyanate

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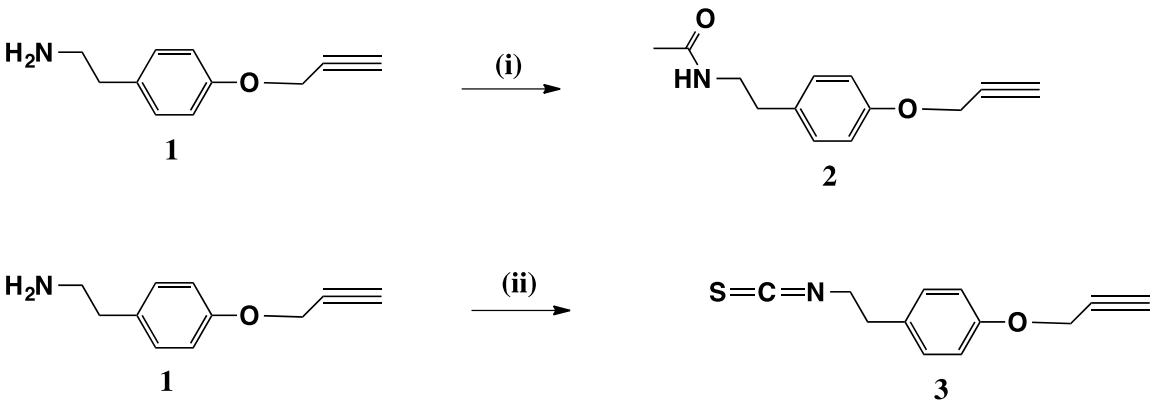
Contents:

Materials and Experimental

Materials. Tubulin (Cat. # T240-B) from porcine brain (>99% pure) was purchased from Cytoskeleton. Inc (USA); Biotin-TEG azide (Cat. #BT 1085) was purchased from Berry & Associates (USA); Dynabeads M-280 streptavidin bead (Cat. 112.05D) was purchased from Invitrogen (USA); Biotin-HRP was purchased from Invitrogen (USA); The A549 human lung cell line was purchased from the American Type Culture Collection (ATCC) (USA), RPMI-1640 media and trypsin were purchased from Invitrogen, bovine serum from Biosera, penicillin, streptomycin, sodium ascorbate, Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) and copper sulfate from Sigma-Aldrich.

Synthesis



Scheme S1. *Reagents and conditions*: (i) Acetyl chloride, triethylamine, dichloromethane, 80%; (ii) dicyclohexylcarbodiimide, CS_2, tetrahydrofuran, 75%.

General

Tetrahydrofuran (THF) and dichloromethane (anhydrous grade) were purchased from Sigma-Aldrich and used without further purification. Purification of reaction products was carried out by column chromatography using EM Reagents silica gel 60 (230-400 mesh). Analytical thin

layer chromatography was performed on EM Reagent 250 μm silica gel 60-F254 plates. ^{1}H NMR spectra were recorded on Avance 400 (400 MHz) spectrometers and are reported in ppm using solvent as the internal standard ($CDCl_3$ at 7.26 ppm). Data are reported as: (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constant(s) in Hz, integration). ^{13}C NMR spectra were recorded on Avance 400 (100 MHz) spectrometers. Chemical shifts are reported in ppm from tetramethylsilane, with the solvent resonance employed as the internal standard ($CDCl_3$ at 77.1 ppm). ESI - High Resolution Mass Spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine.

Compound **1** (PEITC) was synthesized by following the literature procedure.[1]

Preparation of N-(2-(4-methoxyalkyne)phenylethyl)acetamide (NPA) (2): Compound **1** (400 mg, 1.38 mmol) was dissolved in dichloromethane (5 mL) followed by the addition of triethylamine (0.41 ml, 2.97 mmol) and cooled to 0 ^{o}C. Acetyl chloride (0.11 ml, 1.59 mmol) was added drop-wise and the reaction mixture was stirred for 12 h at RT. Reaction mixture was washed with $NaHCO_3$ (5 mL) and brine (5 mL), dried (Na_2SO_4) and filtered. Crude product was purified by silica get column chromatography using 0-2 % MeOH in CH_2Cl_2 to give a white solid compound **2** (240 mg, 80%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.03 (d, J = 8.6, 2H), 6.81 (d, J = 8.6, 2H), 6.61 (s, 1H), 4.55 (d, J = 2.4, 2H), 3.34 (q, J = 6.9, 2H), 2.67 (t, J = 7.3 Hz, 2H), 2.49 (t, J = 2.3 Hz, 1H), 1.85 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5, 156.1, 132.0, 129.6, 114.9, 78.6, 75.7, 55.8, 40.9, 34.6, 23.0. ESI-MS *m/z* calculated for $C_{13}H_{16}NO_2$ $[M + H]^+$ 218.11, found 218.11.

Preparation of N-(2-(4-methoxyalkyne)phenethyl)isothiocyanate (NIPPEITC) (3): A mixture of dicyclohexylcarbodiimide (285 mg, 1.38 mmol) and carbondisulfide (0.83 mL, 13.83 mmol) in tetrahydrofuran (2 mL) was cooled to –10 ^{o}C and treated drop-wise with a solution of the compound **1** (400 mg, 1.38 mmol) in THF (1 mL). The reaction mixture was allowed to reach room temperature and was stirred for 5 h. The reaction was monitored by TLC. Removal of the solvent under reduced pressure produced a white solid, which was triturated with diethylether (20 mL) and the dicyclohexylthiourea was removed by filtration. The filtrate was evaporated to afford crude compound, which was purified by silica gel column chromatography using 0-10% EtOAc in hexane to give **3** as an oil (225 mg, 75%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.14 (d, J = 8.5, 2H), 6.94 (d, J = 8.5, 2H), 4.66 (d, J = 2.3, 2H), 3.66 (t, J = 6.8, 2H), 2.91 (t, J = 6.8 Hz, 2H), 2.52 (t, J = 2.3 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.7, 130.0, 129.9, 115.2, 78.6, 75.7, 55.8, 46.5, 35.6. ESI-MS *m/z* calculated for $C_{12}H_{12}NOS$ $[M + H]^+$ 218.06, found 218.06.

Instrumentation and methods

Cell Cultures. A549 human lung cancer cells (ATCC USA) were cultured in RPMI 1640 cell culture medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum (all from Sigma).

Determination of IC_{50} Values. The concentrations of the osmium complexes inhibiting 50% of the proliferation of human lung A549 cancer cells were determined using the WST-1 ([2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]) assay. A2780 cells were seeded in 96-well plate (Falcon) at 10,000 cells/well, after the incubation for 24 h. The complexes were solubilised in DMSO (Sigma) to provide 10 mM stock solutions. These were serially diluted by cell culture media to give concentrations four-fold greater than the final concentrations for the assay. The complexes diluted in cell culture media were added to the 96-well plate with cells in triplicate. The final DMSO concentration in each well was no more than 1% (v/v). 20 µL of reconstituted WST-1 was added to each well, then the plate was incubated for 2 hrs at 37˚C in a CO_2 incubator. The absorbance of each well was determined using a GLOMA

MULTI plate reader (Promega) at 450 nm. The absorbance in each well is directly proportional to the cell number. Then the absorbance was plotted against concentration and the IC_{50} determined by using Origin software.

Apoptosis Analysis by Flow Cytometry. A549 cells were incubated with PEITC, NPEITC or NPA of various concentrations for 24 h and then harvested. Detached and adherent cells were washed with PBS and fixed in 70% ethanol. The fixed cells were centrifuged and washed once with PBS, followed by resuspension in PBS containing 7.5 μM Propidium Iodide (PI) and 10 μg/ml RNase A. After the cells had stood at room temperature for 30 min, they were analysed by flow cytometry (FACS-Calibur, Beckton Dickenson). The cell cycle distribution was evaluated using the Cellquest software (Beckton Dickenson).

Purification of Biotin-Tagged Proteins with Magnetic Streptavidin Beads

Purification of biotin-tagged proteins from the click reaction was done with 100 μL of cell lysates (100 μg of total protein) and 45 μL of Dynabeads M-280 Streptavdin (10mg/mL, in PBS, pH=7.4, containing 0.1% BSA and 0.02% NaN_3) with end-over-end rotation for binding for 1 h. The tubes containing the beads were placed on a magnet for 1-2 min. The supernatant was removed by aspiration with a pipette while the tube was on the magnet. The beads were washed three times by adding PBS along the inside of the tube where the beads are collected and resuspended (washing buffer is collected for detection of protein). Biotin-tagged proteins for both western blot and protein analysis were released from the beads by heating at 95 °C for 20 min in water.

Click Chemistry of Reactions with Tubulin *in vitro*. An aliquot of tubulin solution (0.8 mg/ml) was incubated with 180 μM PEITC, NPEITC and NPA (at the ratio of ITC to tubulin cysteine were 1:1) at 37 °C in the dark for 60 min. The click reaction conditions and purification of biotin-labeled tubulin were carried under the same conditions as mentioned above.

Click Chemistry. A549 cells were seeded in 10 cm petri dish at 1.0×10^6 cells/dish in RPMI1640 media with 10% FBS. After incubation for 24 h, cells were washed with 10 mL of PBS twice then PEITC, NPEITC or NPA was added. The cells were then incubated for 4 h at 37 °C. Cells were washed twice with PBS, scraped into 5 mL of PBS, and collected after centrifugation (1,000 rpms, 3 min, 4 °C). The cells were lysed in RIPA buffer on ice for 20 min. Click chemistry was performed with cellular lysates containing 1.0 mg/mL of protein with 1 mM of the azide-TEG biotin TBTA (1 mM), sodium ascorbate (10 mM) and $CuSO_4$ (1 mM) for 2 h at room temperature.

Mass Spectrometry Analysis. Eluted proteins were precipitated by 80% acetone overnight at -20°C. Proteins were dissolved in 20µl of 25mM ammonium bicarbonate buffer, reduced by 5Mm DTT for one hour at 60°C, alkylated by 15mM IAA for 20min at RT and digested by 0.2µg trypsine (Promega, Madison, WI) for one hour using barocycler NEP2320 (Pressure BioSciences, South Easton, MA) at 37°C. Peptide analysis was achieved by RP chromatography (Tempo Capillary Chromatography, Eksigent, Framingham, MA) coupled with HiPLC™-nanoflex (Eksigent, Framingham, MA) using Nano cHiPLC Trap column 200 µm x 0.5 mm ChromXP C18-CL 3 µm 300 Å and 15 cm x 75 µm nano cHiPLC columns packed with ChromXP C18-CL 3 µm 300Å. (Eksigent, Framingham, MA) interfaced with a 5600 TripleTOF mass spectrometer (AB Sciex, Framingham, MA). Tryptic digest was separated with a 10 min trapping/washing step using 2% ACN, 0.1% formic acid at 3 µl/min flow rate followed by a 60 min elution gradient of 0.1% formic acid in ACN. For all runs, we have injected 10 µl of sample directly after enzymatic digestion. Analysis used an Information Dependent Acquisition workflow with one full scan (400-1800 m/z) followed by 20 MS/MS fragmentation experiments of major multiply-charged precursor ions with rolling collision energy. Mass spectra were recorded with accuracy up to 2ppm in high sensitivity mode. The experimental parameters were set as follows: declustering potential 80V, curtain gas 15, ion spray voltage 2300V, ion source gas1 20, interface heater

180°C, entrance potential 10V, collision exit potential 10V. Data were processed using ProteinPilot 4.0 software (AB Sciex, Framingham, MA)

Measurement of Free Thiols in Tubulin. The number of free thiols in tubulin was determined by Ellman assay.[2] Lyophilized porcine tubulin (Cytoskeleton Inc., Denver, CO) was dissolved in 100 mM phosphate buffer (pH = 7.4). An aliquot of tubulin solution (0.8 mg/ml) was incubated with 180, 360 and 720 μM PEITC, NPEITC and NPA at 310 K in the dark for 60 min. After incubation, tubulin samples were diluted in 4 M guanidine HCl and 1 mM 5,5′-dithiobis-2-nitrobenzoic acid, and the absorbance at 412 nm was measured by a UV-1700 UV/visible spectrophotometer (Shimadzu, Columbia, MD). A standard curve was generated using glutathione (Sigma).

Western Blotting. A549 cells were treated with PEITC, NPEITC and NPA for 4 h. Treated cells were lysed in RIPA buffer on ice for 20 min. Twenty micrograms of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed by incubating the membrane with a mouse α-tubulin antibody (1:2000, clone DM1A; Sigma) followed by a horseradish peroxidase-labeled goat anti-mouse secondary antibody (1:10,000; GE Healthcare) or streptavidin-HRP (1: 500,000, Invitrogen). The targeted proteins were detected by chemiluminescence (Pierce). Proteins are visualized with the method based on Bio-Rad stain-free V3 System.

entry	reagents[a]	Na ascorbate/mM	$CuSO_4$/mM	TBTA/mM	Solvents	yield[b]
1	NPEITC and Biotin-TEG azide	10	1	1	acetonitrile:water=4:1	0%
2	NPEITC and Biotin-TEG azide	10	1	1	ethanol:water=4:1	100%
3	NPEITC and Biotin-TEG azide		1	1	ethanol:water=4:1	0%
4	NPEITC and Biotin-TEG azide	10		1	ethanol:water=4:1	0%
5	NPEITC and Biotin-TEG azide	10	1		ethanol:water=4:1	0%
6	NPAand Biotin-TEG azide	10	1	1	acetonitrile:water=4:1	0%
7	NPAand Biotin-TEG azide	10	1	1	ethanol:water=4:1	100%
8	NPAand Biotin-TEG azide		1	1	ethanol:water=4:1	0%
9	NPAand Biotin-TEG azide	10		1	ethanol:water=4:1	0%
10	NPAand Biotin-TEG azide	10	1		ethanol:water=4:1	0%

Table S1. [a] NPEITC, Biotin-TEG azide and NPA were used at a final concentration of 1 mM. [b] Yields were calculated according to the disappearances of reactants by HPLC after incubation of the reactions for 2 h at ambient temperature.

(A)

N	%Cov	Accession	Name	Peptides(95%)
1	35.2	sp|P60709|ACTB_HUMAN	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1	6
2	35.2	sp|P63261|ACTG_HUMAN	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1	6
3	22.71	sp|P07355|ANXA2_HUMAN	Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2	2
4	13.2	sp|P68104|EF1A1_HUMAN	Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 PE=1 SV=1	2
5	29.55	sp|P04406|G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=1 SV=3	4
6	12.43	sp|P07900|HS90A_HUMAN	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5	2
7	15.65	sp|P06748|NPM_HUMAN	Nucleophosmin OS=Homo sapiens GN=NPM1 PE=1 SV=2	2
8	17.4	sp|A6NMY6|AXA2L_HUMAN	Putative annexin A2-like protein OS=Homo sapiens GN=ANXA2P2 PE=5 SV=2	2
9	13.2	sp|Q5VTE0|EF1A3_HUMAN	Putative elongation factor 1-alpha-like 3 OS=Homo sapiens GN=EEF1A1P5 PE=5 SV=1	2
10	14.12	sp|P14618|KPYM_HUMAN	Pyruvate kinase isozymes M1/M2 OS=Homo sapiens GN=PKM PE=1 SV=4	2
11	15.27	sp|P02768|ALBU_HUMAN	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	6
12	23.87	sp|P07437|TBB5_HUMAN	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2	2
13	26.52	sp|Q13885|TBB2A_HUMAN	Tubulin beta-2A chain OS=Homo sapiens GN=TUBB2A PE=1 SV=1	2
14	23.82	sp|Q9BVA1|TBB2B_HUMAN	Tubulin beta-2B chain OS=Homo sapiens GN=TUBB2B PE=1 SV=1	2
15	17.56	sp|Q13509|TBB3_HUMAN	Tubulin beta-3 chain OS=Homo sapiens GN=TUBB3 PE=1 SV=2	2
16	25.32	sp|P08670|VIME_HUMAN	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	6

(B)

Proteins identified by click chemistry	Functions
Actin	major constituent of actin microfilaments, involved with cell mechanics and cell motility
Annexin A2	involved in diverse cellular processes such as cell motility
Elongation factor 1-alpha 1	play a role in translation and nuclear export of proteins
Vimentin	a member of intermediate filament family, supports and anchors the position of organelles
Glyceraldehyde-3-phosphate dehydrogenase	catalyzes the sixth step of glycolysis
Heat shock protein HSP 90-alpha	a chaperone protein, assists other proteins to fold properly, stabilizes proteins against heat stress
Tubulin	major constituent of microtubules, involed in mitosis, cytokinesis and vesicular transport

Table S2. Full list of Protein Targets identified through click chemistry method of NPEITC (A) and their biological functions (B).

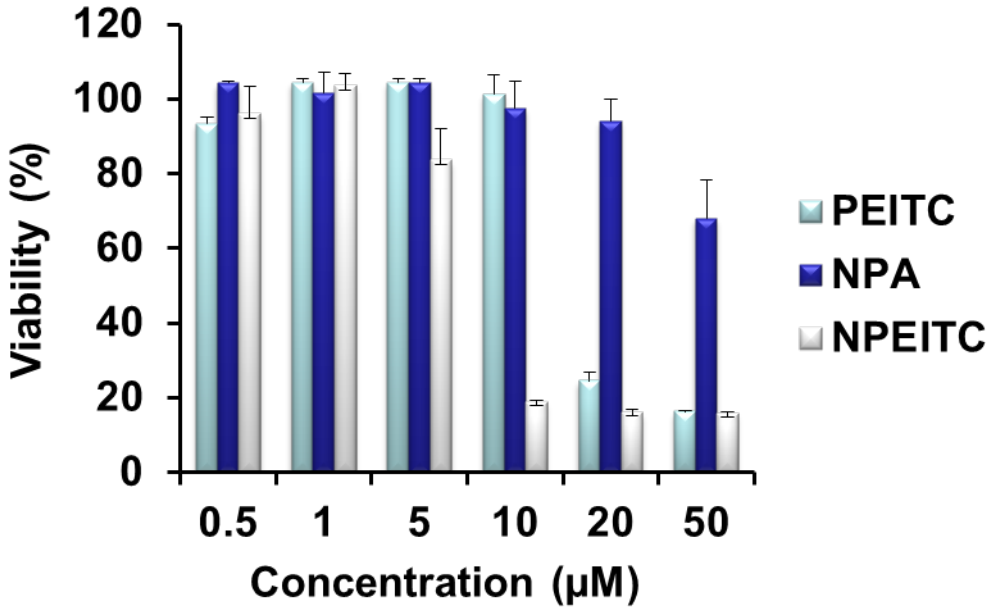


Figure S1. Cell viability of A549 human lung cancer cells after the treatment of PEITC, NPA and NPEITC for 24 h.

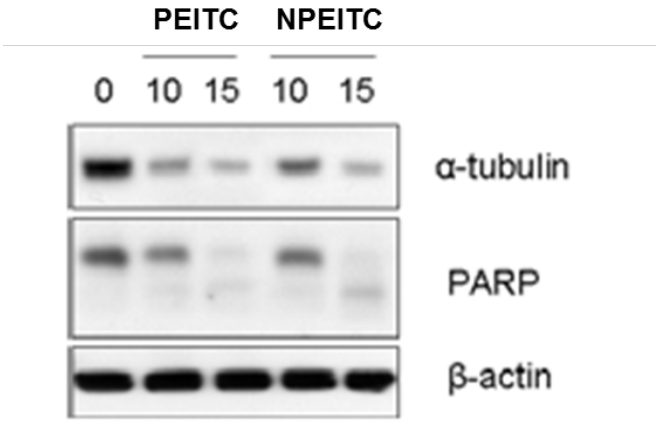


Figure S2. Effects of PEITC and NPEITC on the α-tubulin and poly(ADP-ribose) polymerase (PARP) cleavage in HT29 cells. Cells were incubated with DMSO (control), PEITC (10 and 15 μM) and NPEITC (10 and 15 μM) for 16 h.

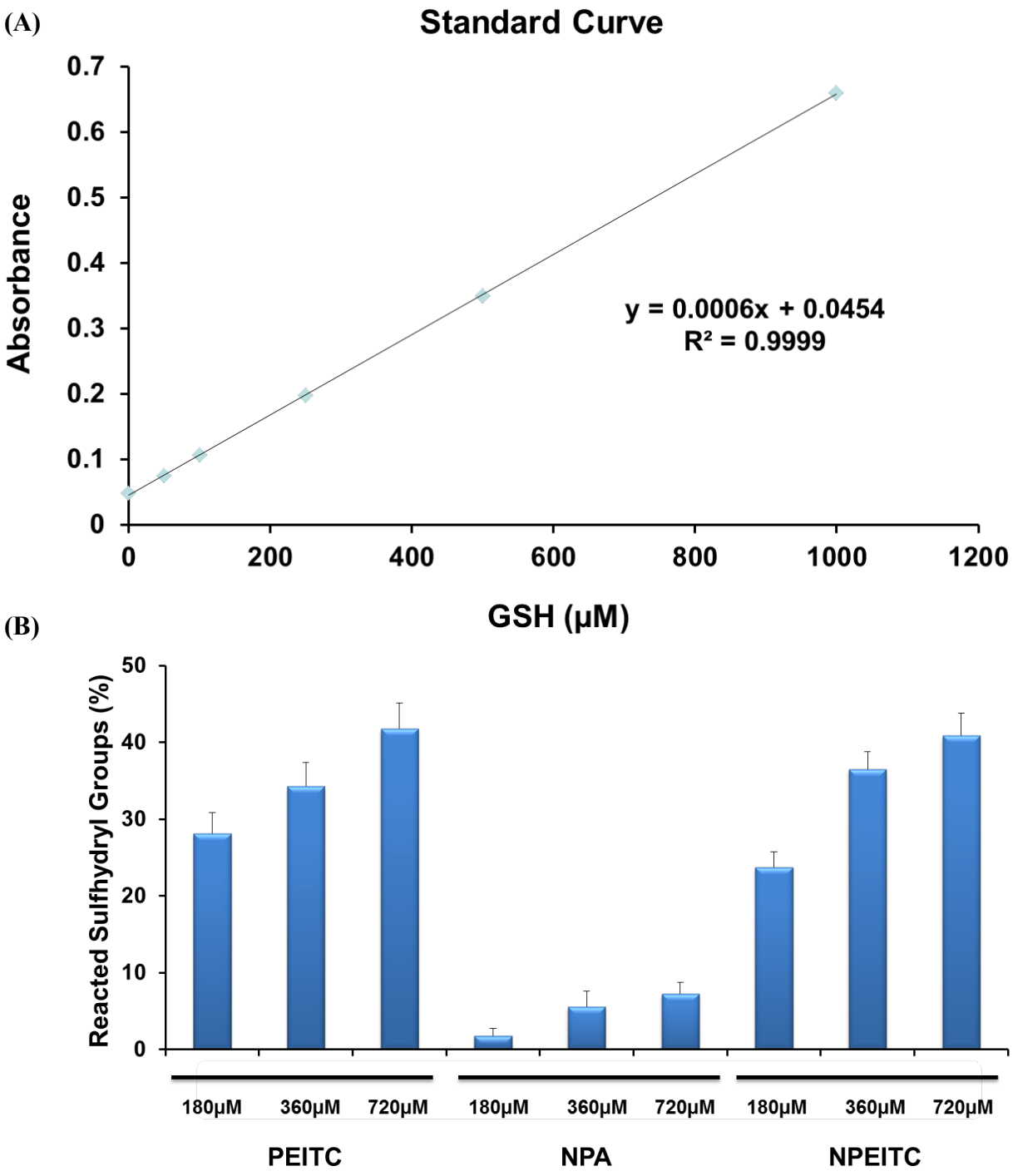
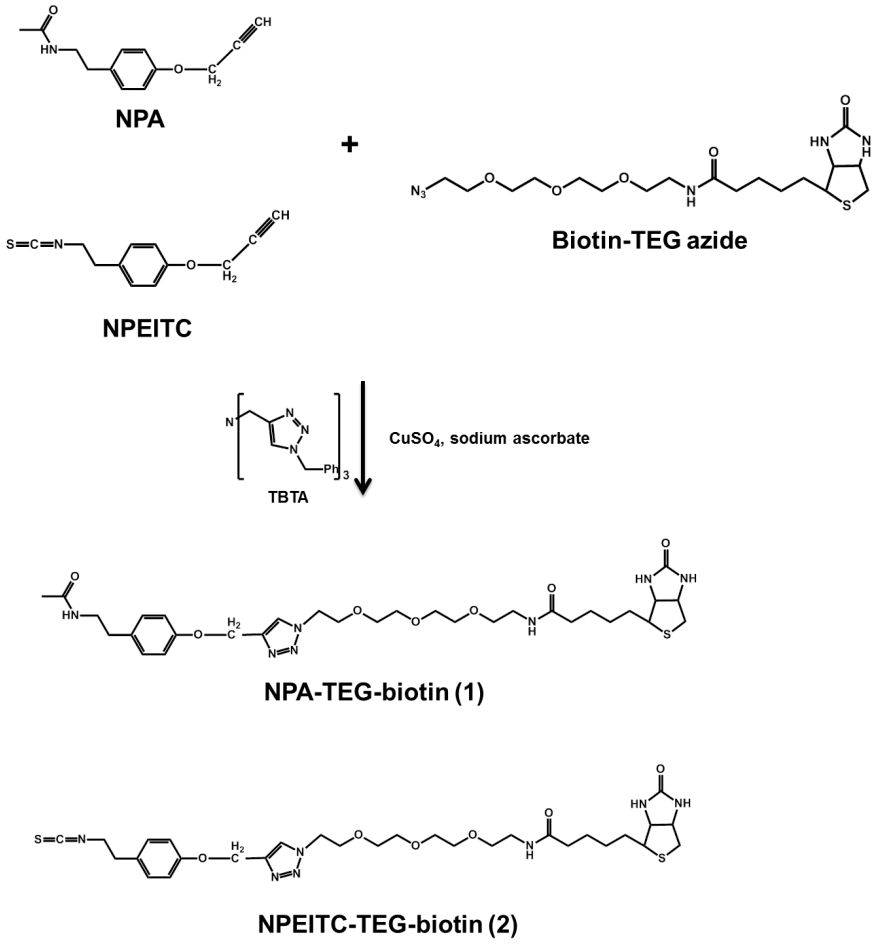


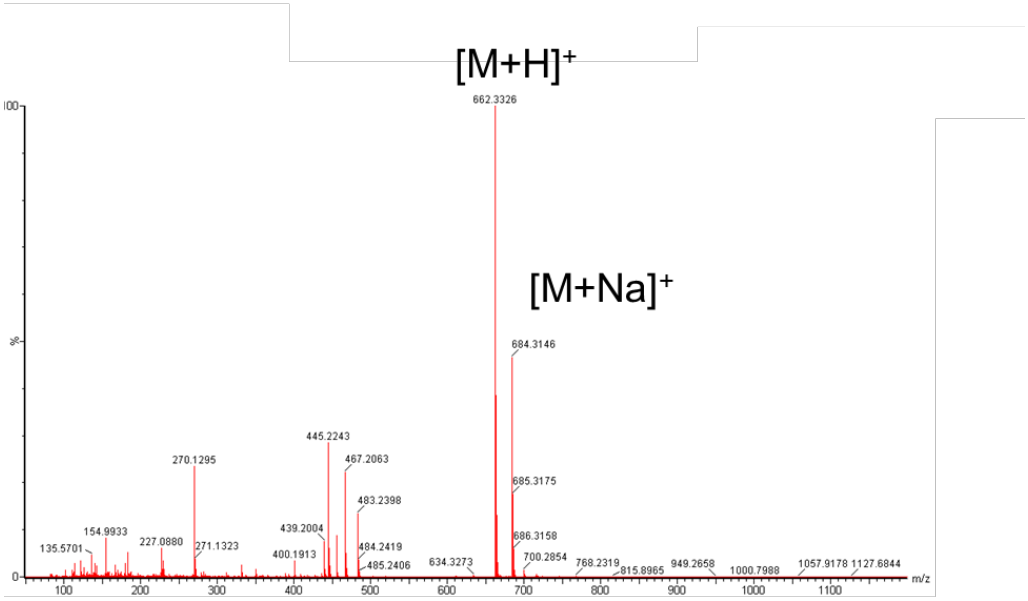
Figure S3. Reacted sulfhydryl groups on tubulin, Ellman assay was used for quantitative measurements of sulfhydryl groups on tubulin. (A) Standard curve of Ellman assay; (B) incubation for 1 h at 310 K of PEITC, NPA and NPEITC with tubulin (1mg/mL).

(A)



(B)

Theoretical $[MH]^+$ = 662.3336
Found $[MH]^+$ = 662.3326



Mass spectrometry m/z

(C)

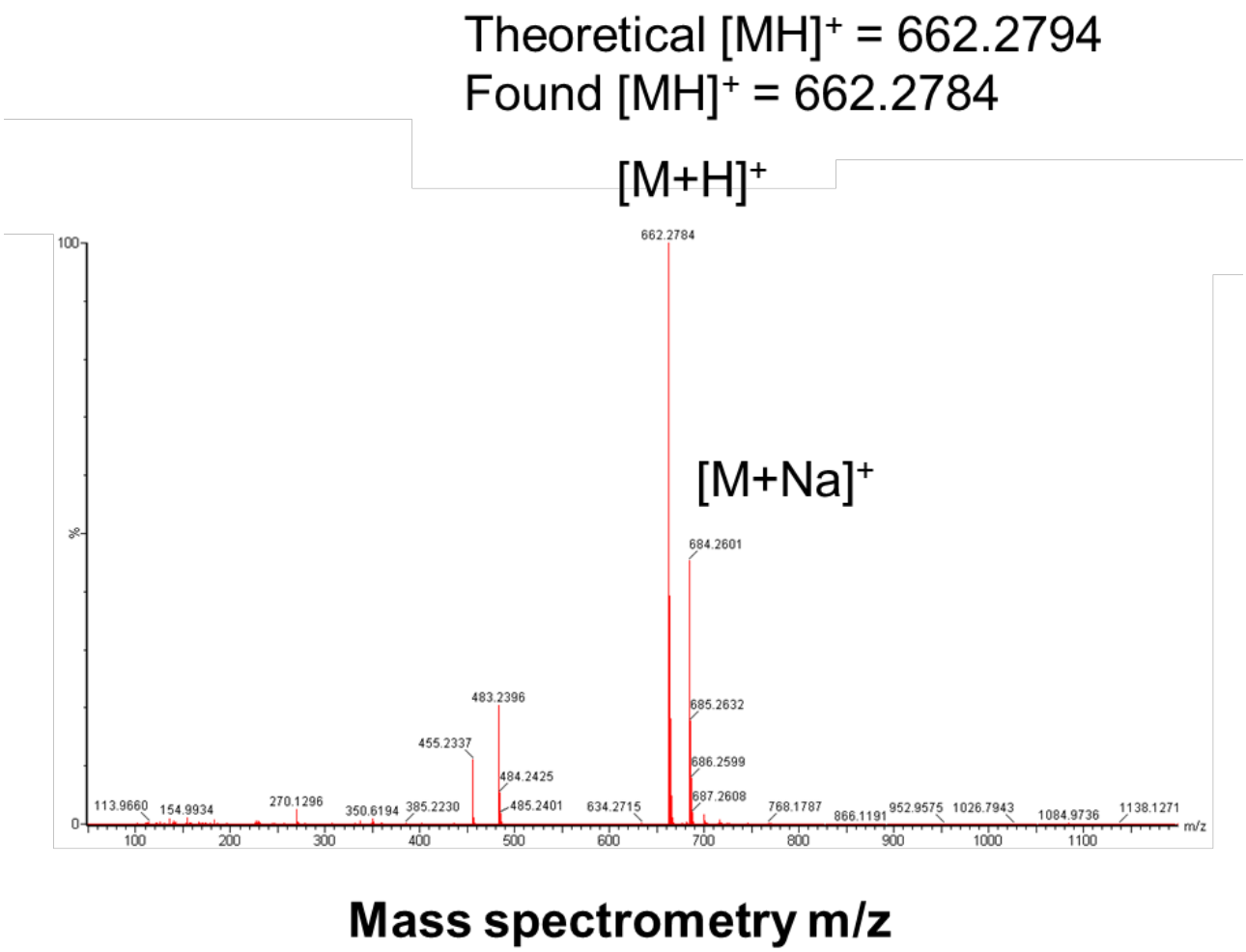
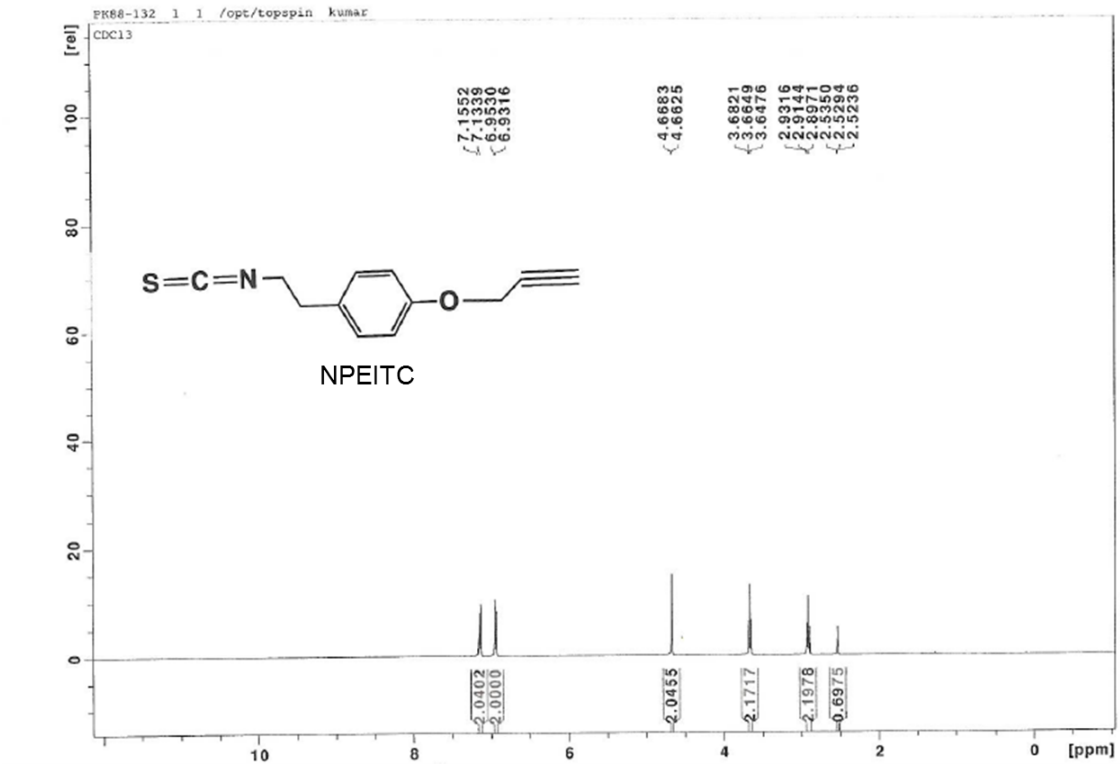
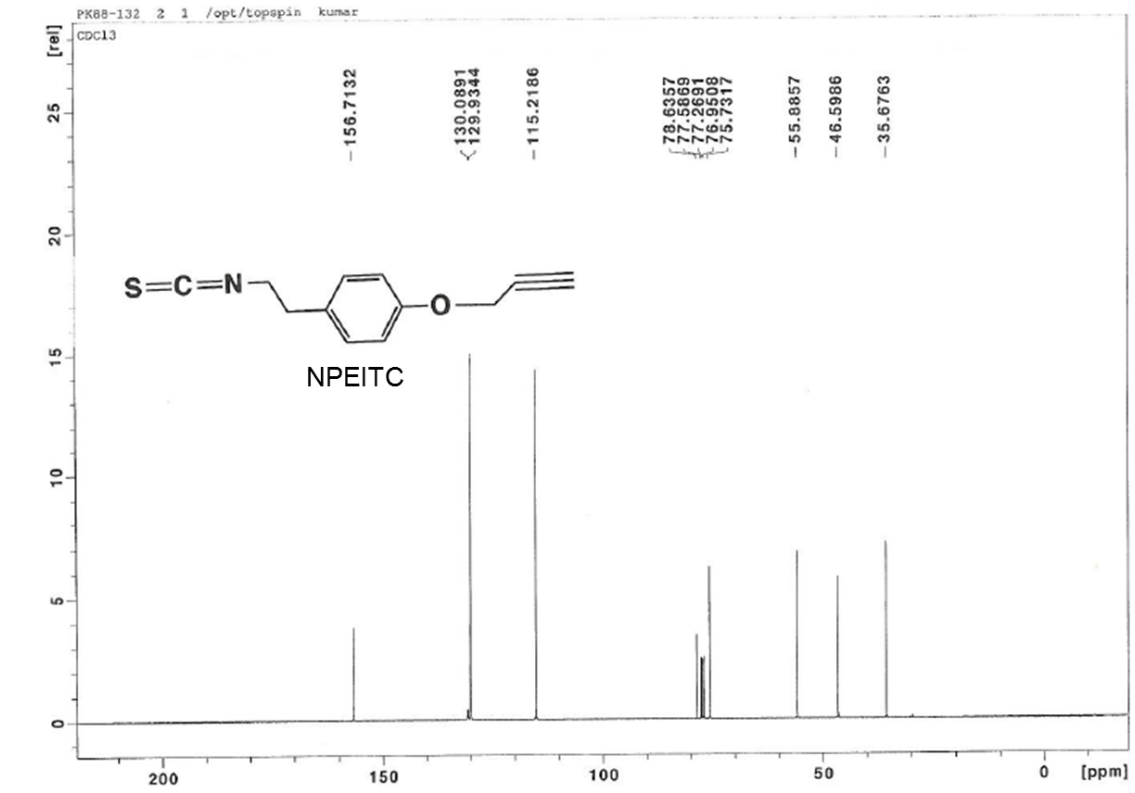


Figure S4. Mass-spec (MS) results of the product formed after click reactions. (A) Click reaction scheme; (B) reaction of NPEITC with biotin-TEG azide; (C) reaction of NPA with biotin-TEG azide.

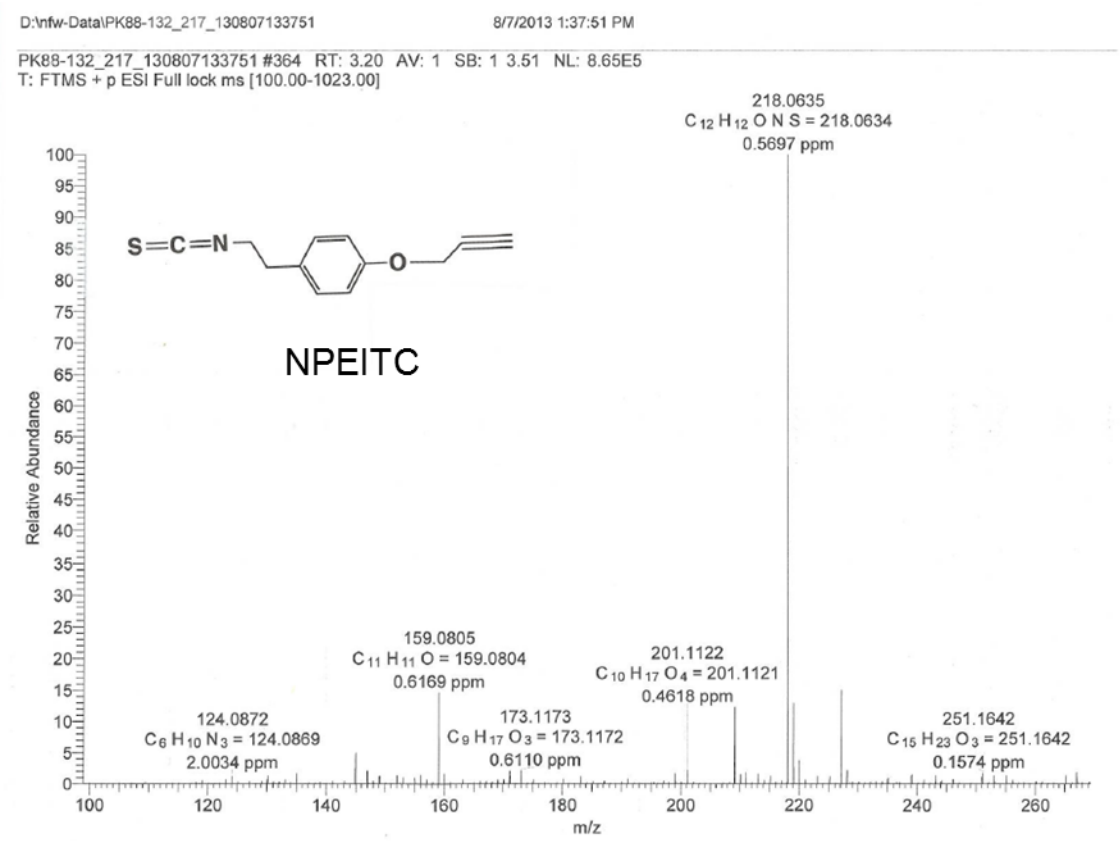
^{1}H NMR spectrum of NPEITC



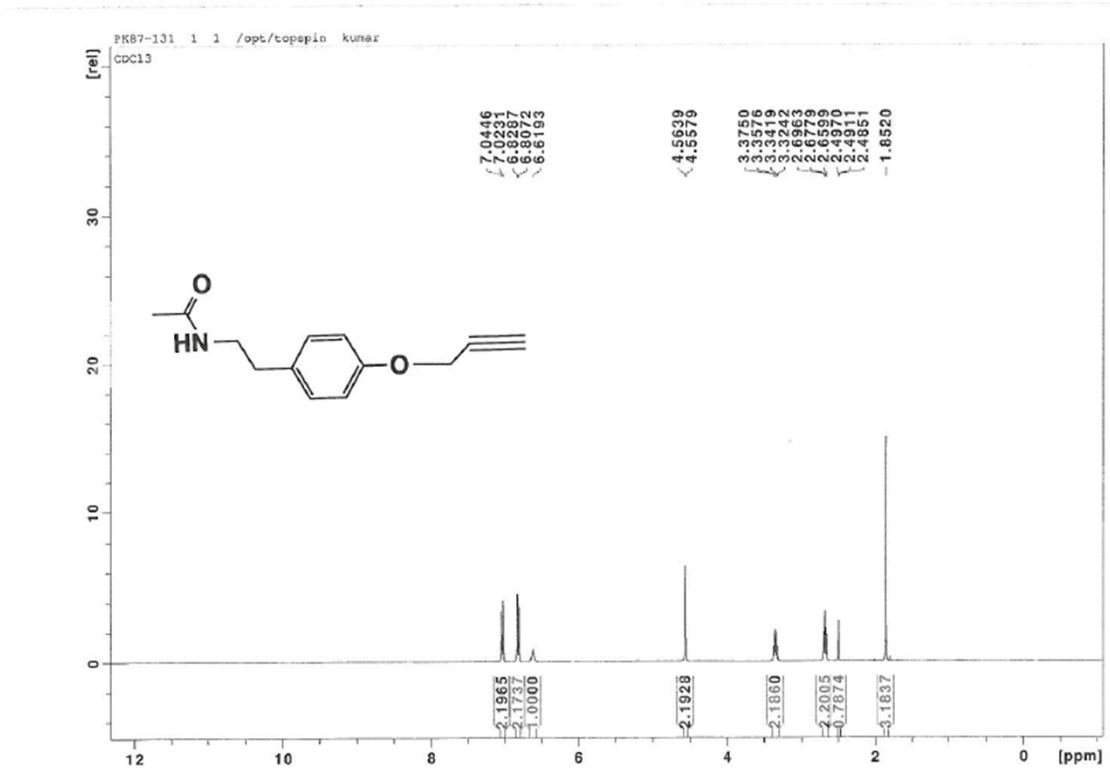
^{13}C NMR spectrum of NPEITC



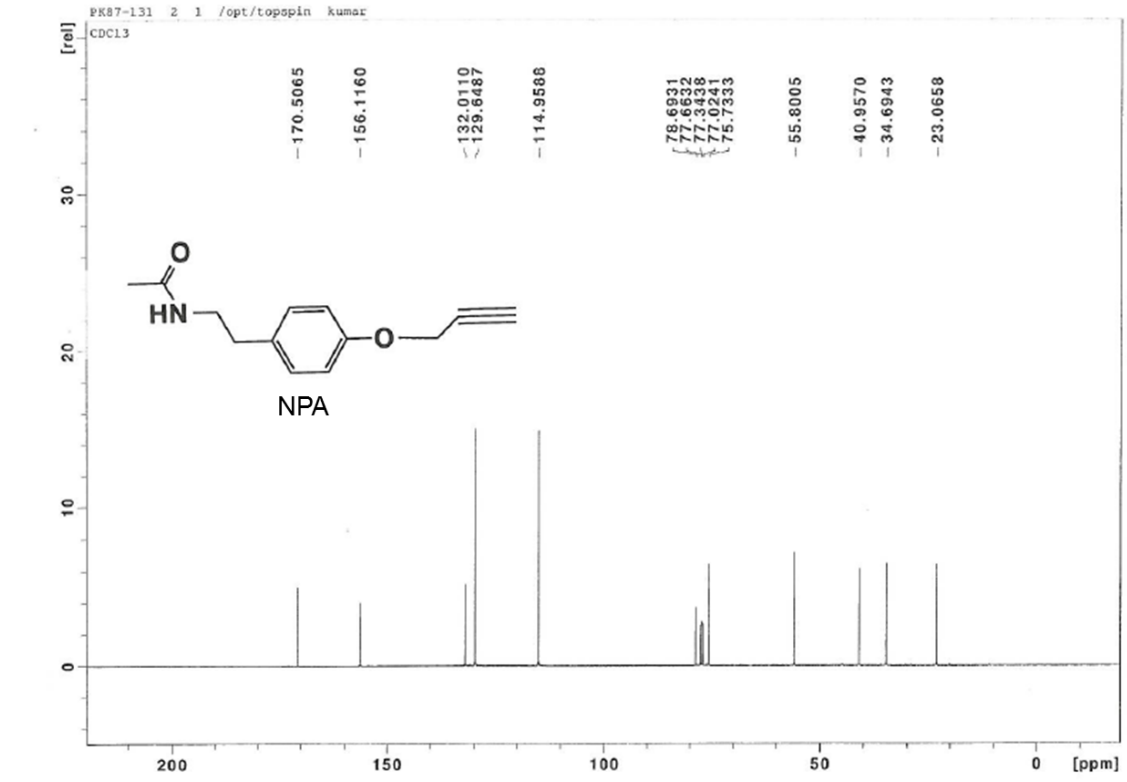
HR-MS data for NPEITC



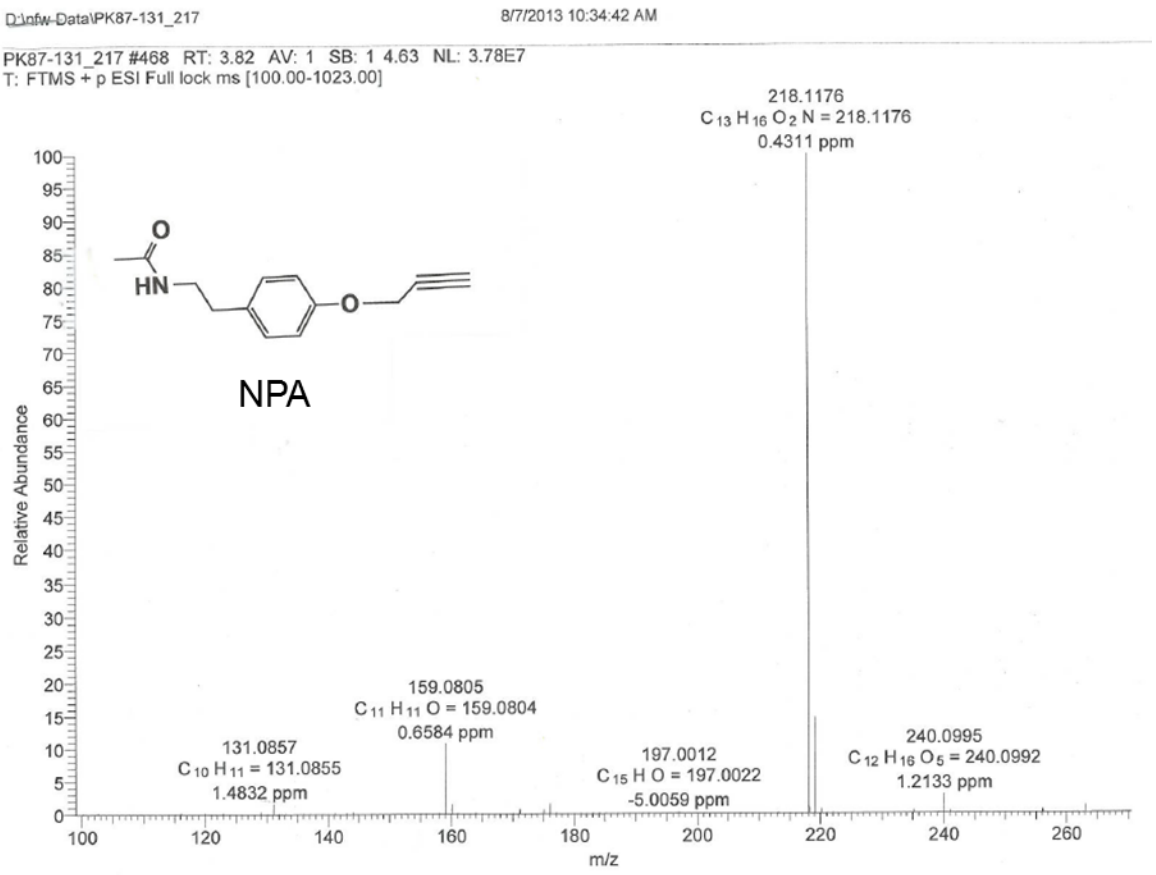
^{1}H NMR spectrum of NPA



^{13}C NMR spectrum of NPA



HR-MS data for NPA



Reference:

1. Maillard, L. T., Benohoud, M., Durand, P., and Badet, B. *J. Org. Chem.,* **2005**, 70, 6303-6312.
2. Ellman, G. L. *Arch. Biochem. Biophys.,* **1958**, 74, 443-450.