Supporting Information

Photoinducible oncometabolite detection

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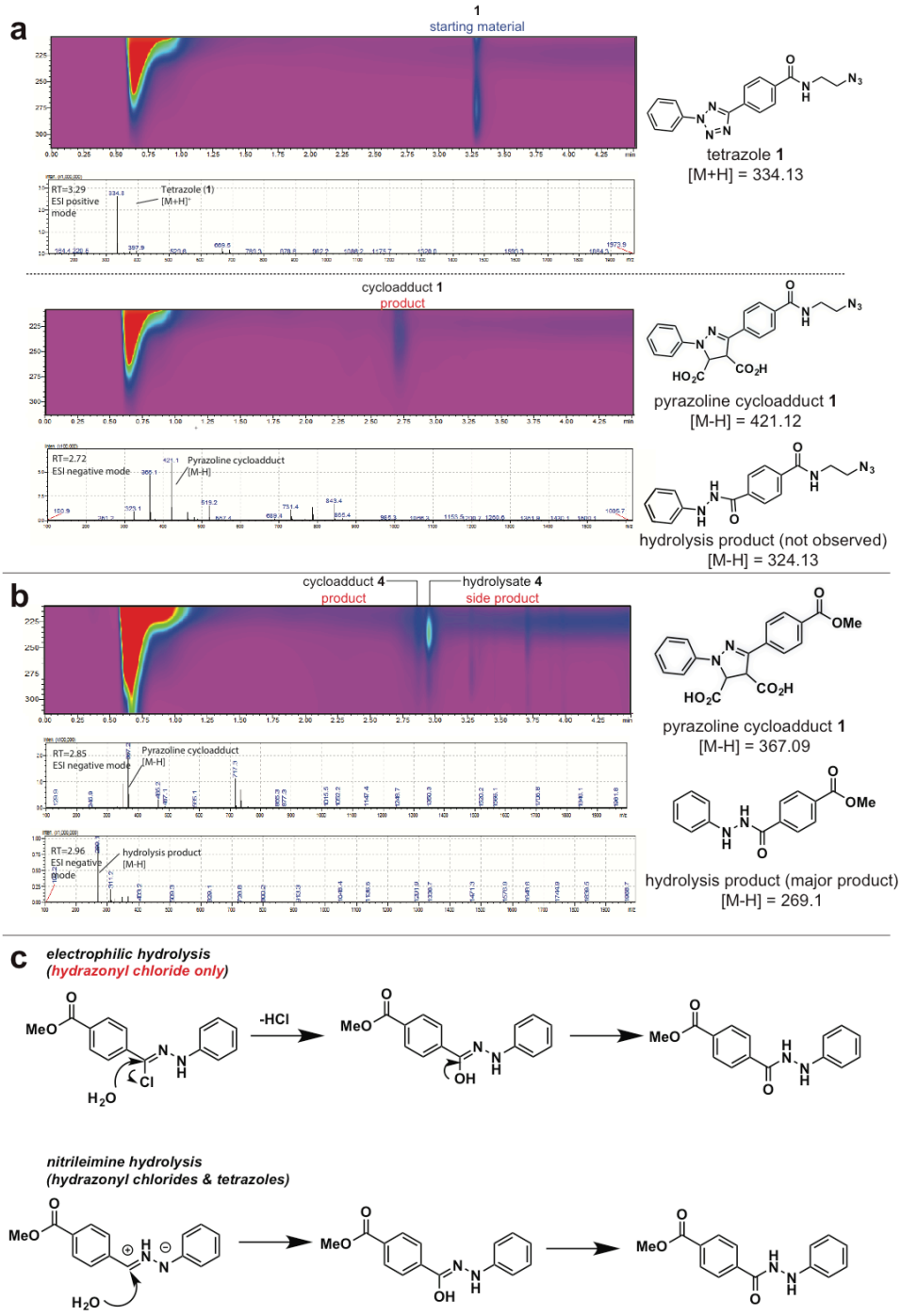


Figure S1. LC-MS analysis of reaction of diaryl tetrazole **1** with fumarate. Compounds were dissolved in CH_3CN:100 mM sodium phosphate buffer pH 7.0 (1:1). (a) LC-MS analysis of **1** (100 μM) in presence of fumarate (10 mM) before (top) and after (bottom) photoirradiation (302 nm, 2 min). (b) LC-MS analysis of **4** (100 μM) incubated with fumarate (10 mM) for 1 h showing the formation of substantial hydrolysis product. (c) Increased formation of hydrolysis products by hydrazonyl chloride nitrileimine precursors may be due to their ability to directly react with water as electrophiles.

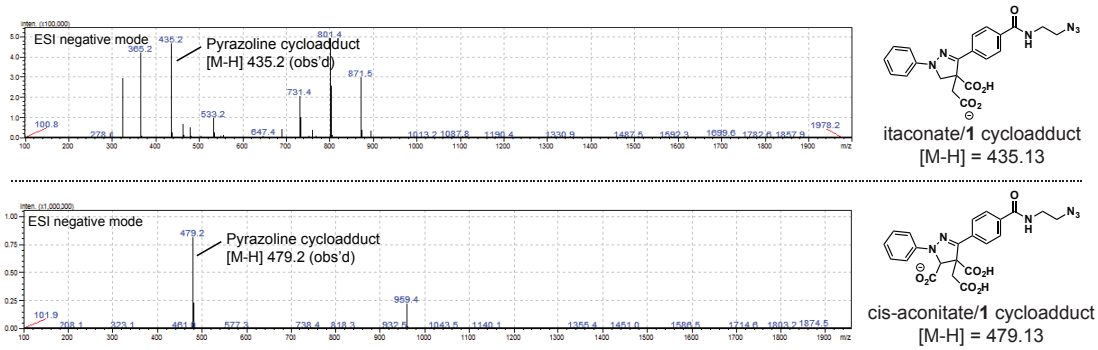


Figure S2. Itaconate (top) and cis-aconitate (bottom) readily form pyrazoline adducts when irradiated in the presence of **1**. Metabolites (200 μM) and tetrazole 1 (100 μM) were dissolved in CH3CN:100 mM sodium phosphate buffer pH 7.0 (1:1) and photoirradiated at 302 nm for 2 min prior to ESI MS analysis.

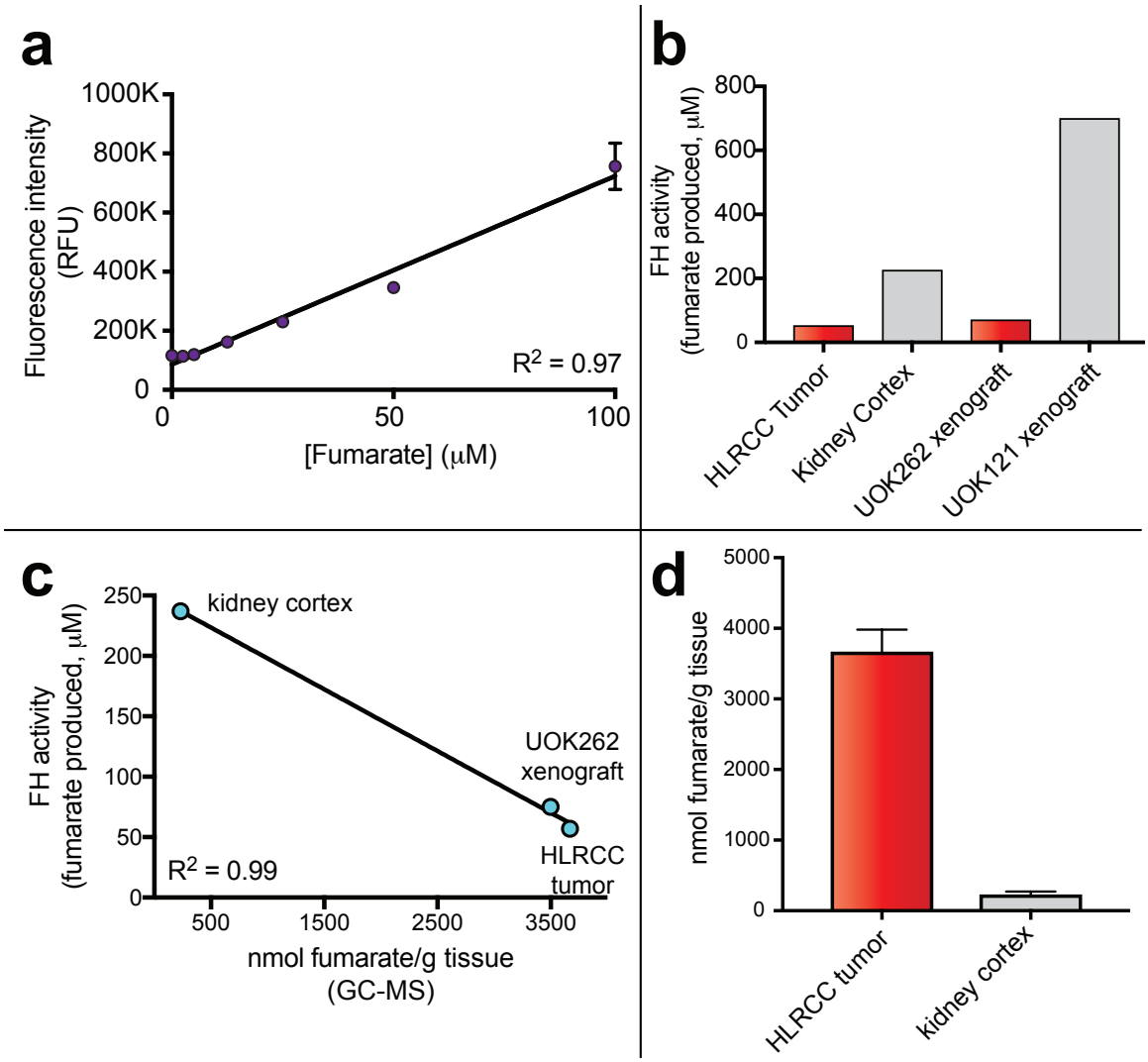


Figure S3. (a) Standard curve measuring fluorescence intensity as a function of fumarate concentration after photoirradiation (302 nm, 2 min) in the presence of **1**. Conditions: CH_3CN:100 mM sodium phosphate buffer pH 7.0 (1:1), malate (25 mM), tetrazole **1** (150 μM), λ_{ex} = 380 nm, λ_{em} = 540 nm. (b) Calculated concenration of fumarate produced (μM) by addition of malate to HLRCC tumor and xenograft samples. Source data is identical to Figure 3b. (c) Correlation of FH activity (as measured with fluorescent tetrazole **1**, Fig. 3) with levels of fumarate measured by GC-MS. (d) GC-MS comparisons of fumarate in HLRCC patient samples and adjacent kidney cortex show greater differences (~10-fold) compared to direct analysis by probe **1** (~1.5-fold, data in Fig. 3c). For panels b-d, analyses were performed from 3 different HLRCC tumors and kidney cortex samples from same patient, and 3 separate xenografts from 3 separate mice.

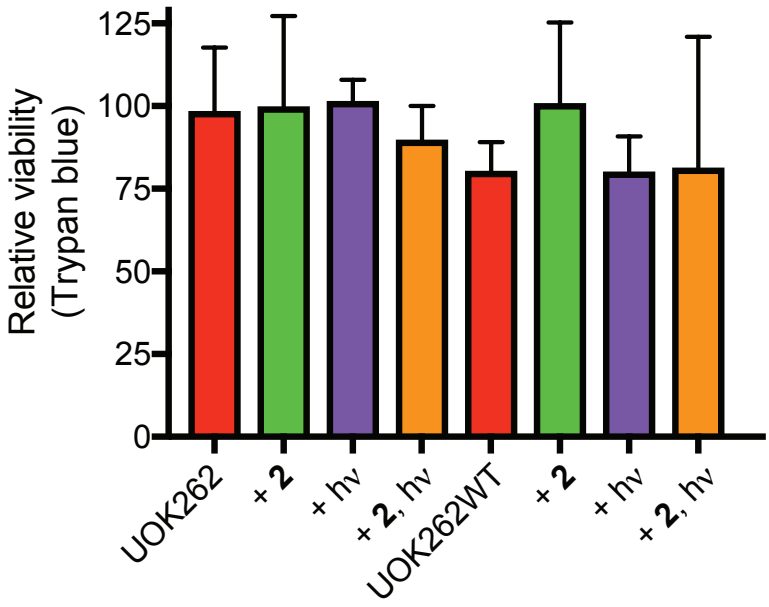


Figure S4. Effect of diaryl tetrazole **2** and photoirradiation on cell viability. UOK262 (*FH* -/-) or UOK262WT (*FH* +/+) cells were plated identically as in microscopy experiments and treated with tetrazole **2** (100 μM, 2 h), washed, and subjected to photoirradiation (302 nm, 2 min) as indicated. Cells were trypsinized and assessed for viability using Trypan Blue staining and a TC20 automated cell counter (Biorad).

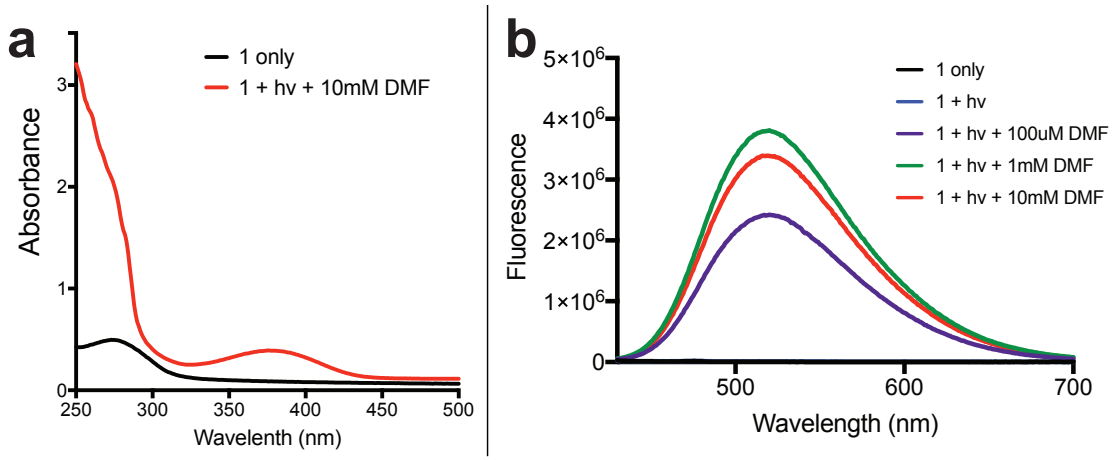


Figure S5. Optical data for detection of dimethyl fumarate (DMF) by probe **1**. Compounds were dissolved in 5% DMSO in PBS (pH 7.2). Final concentration of tetrazole **1** used was 100 μM and photoirradiation was carried out at 302 nm for 2 min prior to recording spectra. (a) Absorbance spectra of **1** photoirradiated in presence (red trace) or absence (black trace) of DMF (10 mM). (b) Fluorescence spectra of **1** photoirradiated in absence (blue trace) or presence varying concentrations of DMF (10 mM DMF- red trace, 1 mM DMF- green trace, 100 μM DMF- purple trace). Fluorescence spectra of **1** prior to photoirradiation is shown as black trace. For all fluorescence spectra, λ_{ex} = 410 nm.

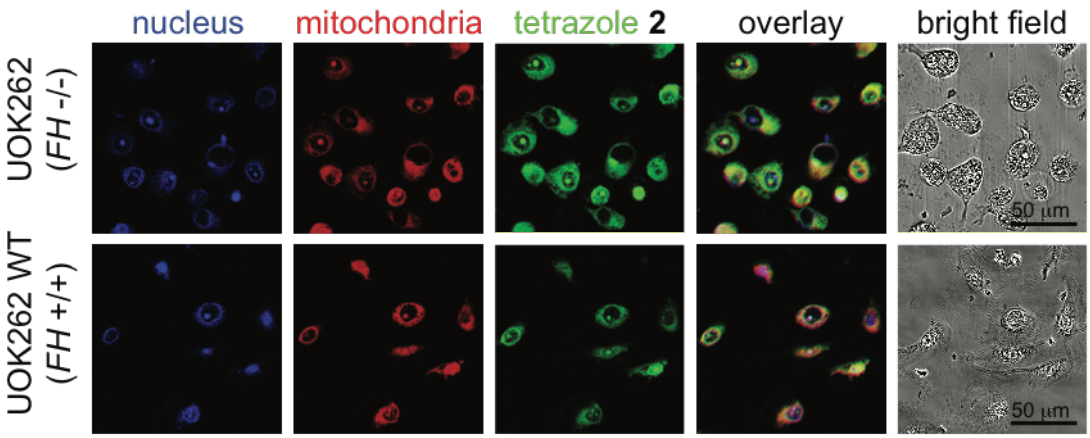


Figure S6. Fluorescence imaging data for UOK262 cells with mitochondrial tetrazole **2**. UOK262 (*FH* -/- top row) and UOK262WT (*FH* +/+ bottom row) cells were treated with tetrazole **2** (100 μM, 2 h), MitoTracker Green FM (100 nM, 30 min) and DRAQ5 (200 nM, 30 min), washed, and, photoirradiated (302 nm, 2 min). Confocal images displayed are obtained for DRAQ5 (633 nm HeNe633 laser, false-colored blue, 1st column), MitoTracker Green (488 nm argon laser, false-colored red, 2nd column), and tetrazole (820 nm two-photon laser, green, 3rd column). Column 4 represents overlay of confocal images and column 5 represents differential interference contrast (DIC) microscopy images with a 50 μm scale bar.

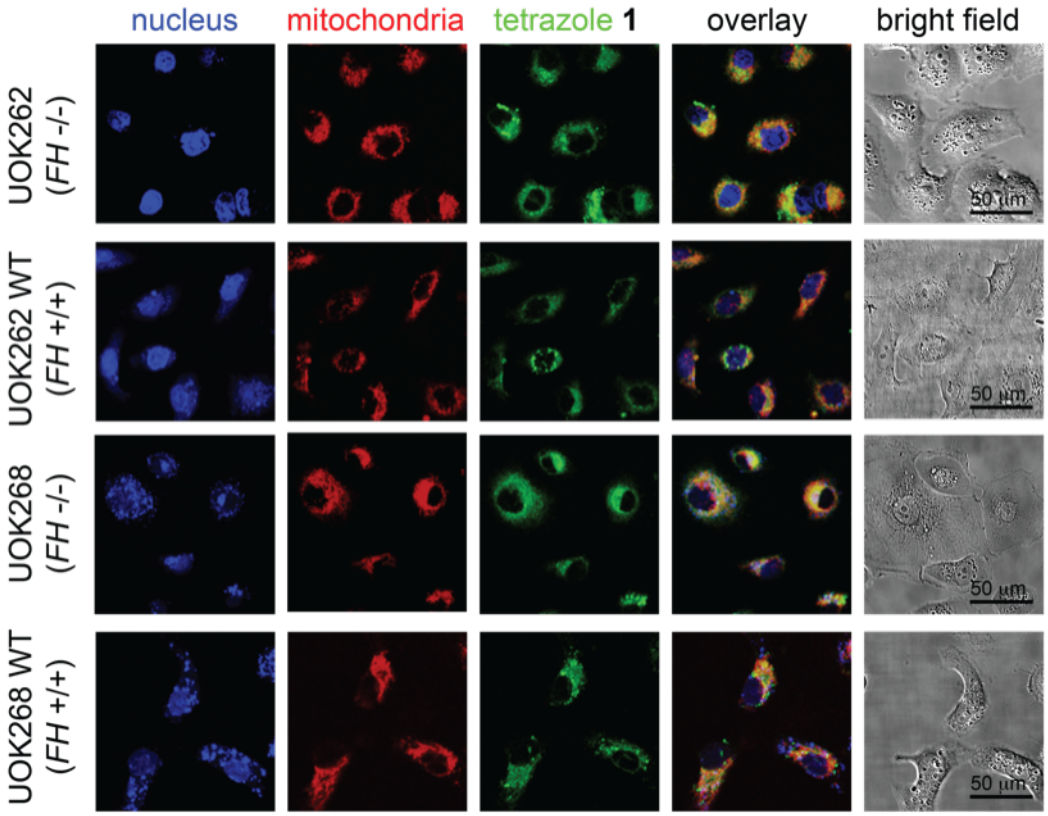


Figure S7. Fluorescence imaging data for UOK262 and 268 cells with untargeted tetrazole **1**. UOK262 (*FH -/-* top row), UOK262WT (*FH +/+* second row), UOK268 (*FH -/-* third row) and UOK268WT (*FH +/+* bottom row) cells were treated with tetrazole **1** (100 μM, 2 h), MitoTracker Green FM (100 nM, 30 min) and DRAQ5 (200 nM, 30 min), washed, and, photoirradiated (302 nm, 2 min). Confocal images displayed are obtained for DRAQ5 (633 nm HeNe633 laser, false-colored blue, 1st column), MitoTracker Green (488 nm argon laser, false-colored red, 2nd column), and tetrazole (820 nm two-photon laser, green, 3rd column). Column 4 represents overlay of confocal images and column 5 represents differential interference contrast (DIC) microscopy images with a 50 μm scale bar.

General materials and methods

HK2, UOK262 (*FH-/-*), UOK262WT (*FH* +/+ rescue), UOK268 (*FH-/-*) and UOK268WT (*FH* +/+ rescue) cells were obtained from W. Marston Linehan laboratory (Urologic Oncology Branch, National Cancer Institute).[1] HK2, UOK262 and UOK268 cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate. UOK262WT and UOK268WT cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate and either 0.3 mg/mL of G418 (UOK262WT) or 3 μg/mL blasticidin (UOK268WT). Fumaric acid (A10976) and dimethyl fumarate (242926) were purchased from Alfa Aesar and Sigma. MitoTracker Green FM (M7514), DRAQ5 (62251), and 8-well Lab-Tek chambered #1.0 borosilicate coverglass slides (Nunc) (155411) were purchased from ThermoFisher Scientific. Qubit Protein Assay kit was purchased from Life Technologies (Q33212). Fluorimetric analysis of fumarate using hydrazonyl chloride **4** was performed on Photon Technology International QuantMaster fluorimeter using 1-cm path length, 0.13 mL quartz microcuvettes (Helma #101-015-40) at ambient temperature (22 ± 2 °C), using an excitation wavelength of 390 nm, slit width of 3.5 nm, and monitoring emission from 410 nm to 615 nm. Primary FH-deficient HLRCC tumor and normal kidney cortex specimens were obtained intraoperatively from a patient undergoing radical nephrectomy for metastatic HLRCC kidney cancer. Tissues were procured with the assistance of a pathologist using rapid procurement procedures and were snap-frozen in liquid nitrogen. Frozen tissue specimens were pulverized in liquid nitrogen using a SPEX Freezer/Mill and extracted using acetonitrile:methanol:water:chloroform as previously described.[2] Metabolite derivatization for GC/MS analysis was performed with N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) and analyses of standards and samples were performed with a Thermo GC-ion trap MS system as described previously.[2] Spectra were processed and analyzed using Thermo Xcalibur software. Fumarate measurements were normalized to wet tissue weight and reported as nmol fumarate per gram tissue. Tissue specimens were procured at the Urologic Oncology Branch, National Cancer Institute (NCI) under a protocol approved by the NCI Institutional Review Board, and patient gave written informed consent for participation in the study.

Protocol for in vitro reaction of fumarate with diaryl tetrazoles 1-2

For determining absorbance and fluorescence spectra, tetrazole **1** or **2** (100 μM) with fumarate (10 mM) or vehicle (DMSO) were first dissolved in 100 μL of 5% DMSO in PBS (pH 7.2), and photoirradiated at 302 nm for 2 min. Absorbance spectra were then recorded from 300-700 nm using a Biotek Synergy plate reader and Greiner UV-Star 96-well microplates (half area # 655801). Fluorescence emission spectra were recorded as described in the general methods using an excitation wavelength of 410 nm, slit width of 3.5 nm, and monitoring emission from 430 nm to 630 nm. For limit of detection and fold-fluorescence determination, tetrazole **1** and metabolites were dissolved in 100 μL of 1:1 CH_3CN/aqueous sodium phosphate (100 mM, pH 7) and the fluorescence emission spectra were recorded as described above using an excitation wavelength of 410 nm. Limit of detection and fold-fluorescence were determined using the fluorescence values at 540 nm. LC-MS studies were performed from the tetrazole **1**-metabolite reactions carried out in 1:1 CH_3CN/aqueous sodium phosphate (100 mM, pH 7) using a Shimadzu LCMS-2020 Single Quadrupole utilizing a Kinetex 2.6 μm C18 100 Å (2.1 x 50 mm) column obtained from Phenomenex Inc. with runs employing a gradient of 0→90% MeCN/0.1% aqueous formic acid over 4 minutes at a flow rate of 0.2 mL/min.

Protocol for fumarate hydratase activity analyses using probe 1

For collecting unfractionated proteomes from the tumor samples, 100 µL of lysis buffer containing ice cold PBS, protease inhibitor cocktail (1x, EDTA-free, Cell Signaling Technology # 5871S) and PMSF (1 mM, Sigma # 78830) was first added. These samples were then lysed by sonication using a 100 W QSonica XL2000 sonicator (10 x 1s pulse, amplitude 1, 60s resting on ice between pulses). Lysates were pelleted by centrifugation (14,000 rcf x 30 min, 4 °C) and quantified on a Qubit 2.0 Fluorometer using a Qubit Protein Assay Kit. Quantified proteomes were diluted to 2 mg/mL and stored in 1 mg aliquots at -80 °C for FH enzyme activity analyses.

For fumarate hydratase activity determination, proteomes (5 µg) were incubated with neutralized *L*-malic acid (25 mM) in PBS in a final reaction volume of 100 µL for 1 h. The reactions were then developed by adding 100 µL of tetrazole **1** (300 µM in CH_3CN, final reaction concentration 150 µM) and irradiating with 302 nm light for 2 min. Fluorescence determinations were performed on a Photon Technology International QuantMaster fluorimeter using 1-cm path length, 0.13 mL quartz microcuvettes (Helma #101-015-40) at ambient temperature (22 ± 2 °C), using an excitation wavelength of 380 nm, slit width of 5 nm, and monitoring emission at 540 nm. Values represent the average of three replicates. For determining the absolute FH activity from samples, standard curve was generated using 2.5, 1, 0.5, 0.25, 0 mU fumarase from porcine heart (Sigma F1757). Different activities of fumarase was first incubated with neutralized *L*-malic acid (25 mM) in PBS (100 µL) for 1 h. Fumarate produced was then measured by addition of 100 µL of tetrazole **1** (300 µM in CH_3CN, final reaction concentration 150 µM) and photoirradiation (302 nm, 2 min).

Protocol for tissue dipolarophile analyses using probe 1

For extraction of the metabolites from the tumor samples, 20 µL of ice-cold acetonitrile-water (4:1) mixture was added per mg of the tumor followed by sonication using a 100 W QSonica XL2000 sonicator (10 x 1s pulse, amplitude 1, 60s resting on ice between pulses). Insoluble fraction was pelleted by centrifugation (14,000 rcf x 30 min, 4 °C) and the supernatant was collected. To 20 µL of the supernatant, 60 µL of acetonitrile-water (2:3), 10 µL of sodium phosphate buffer pH 7.0 (0.5 M) and 10 µL of tetrazole **1** (2.5 mM in CH_3CN, final reaction concentration 250 µM) were added and irradiated with 302 nm light for 2 min. Fluorescence determinations were performed on a Photon Technology International QuantMaster fluorimeter using 1-cm path length, 0.13 mL quartz microcuvettes (Helma #101-015-40) at ambient temperature (22 ± 2 °C), using an excitation wavelength of 410 nm, slit width of 5 nm, and monitoring emission at 540 nm. Values represent the average of three replicates.

Protocol for endogenous dipolarophile imaging using probes 1 and 2

HK2, UOK262 (*FH-/-*), UOK262WT (*FH* +/+ rescue), UOK268 (*FH-/-*) and UOK268WT (*FH* +/+ rescue) cells were plated on 8-well Lab-Tek chambered #1.0 borosilicate coverglass slides (1.25×10^4 cells/well in 400 µL media/well), and allowed to adhere and grow for 24 h. Cells were dosed with tetrazole **1** or **2** (500 µM in 100 µL media; 100 µM final concentration) and allowed to incubate for 90 mins. Cells were further treated with MitoTracker Green FM and DRAQ5 (0.6 µM and 1.2 µM in 100 µL media, respectively; 100 nM and 200 nM final concentration, respectively) for 30 min. At this point, the media was removed and cells were gently washed once with PBS (500 µL). Fresh phenol red

free DMEM media supplemented with 2 mM glutamine (500 µL) was added to each well. The chamber was photoirradiated (302 nm, 2 min) and imaged.

Confocal fluorescence imaging studies were performed with a Zeiss laser scanning microscope 780 with a 40x oil immersion objective lens using Zen 2009 software (Carl Zeiss). Pyrazoline cycloadduct was excited using 820 nm two photon laser (laser power 2.4 and 1.2 mW for tetrazole **1** and **2**, respectively) and emission was collected from 515 nm to 570 nm. MitoTracker Green was excited using 488 nm argon laser and emission was collected between 495 nm to 543 nm. DRAQ5 was excited using 633 nm HeNe633 laser and emission was collected between 650 to 741 nm. The cells were imaged at 37 °C throughout the course of experiment using live-cell imaging settings. Image analysis and quantification was performed using Fiji. For quantification, a total of 10 cells were analyzed by creating a region of interest (ROI) around each image and determining mean fluorescence intensity using the "Measure" function. Fluorescence from MitoTracker Green was false colored red and fluorescence from DRAQ5 was false-colored blue.

Protocol for detection of dipolarophiles by flow cytometry using probe 1

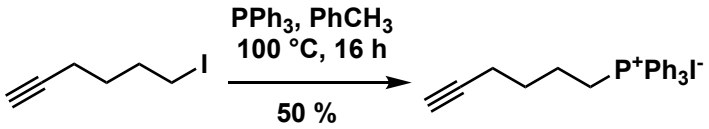
UOK262 (*FH-/-*), UOK262WT (*FH* +/+ rescue), UOK268 (*FH-/-*) and UOK268WT (*FH* +/+ rescue) cells were plated in 6-well plates (4 x10^5 cells/well in 2.5 mL media/well), and allowed to adhere and grow for 24 h. Cells were dosed with tetrazole **2** (500 µM in 500 µL media; 100 µM final concentration) and incubated for 2 h. The media was removed and cells were gently washed once with PBS (3 mL). Additional PBS (3 mL) was added to the cells and the 6-well plate was photoirradiated (302 nm, 2 min). PBS was then removed, and cells were trypsinized and transferred to flow cytometry tubes using 2 mL of media. Cells were then pelleted by centrifugation (500 rcf x 5 min) and media was carefully removed by aspiration. Cells were then thoroughly resuspended in PBS supplemented with 2% FBS (500 µL) and the fluorescence was analyzed by flow cytometry on BD LSRII Fortessa flow cytometer. Pyrazoline cycloadduct was excited using 405 nm diode laser (100 mW) and fluorescence emission was collected using standard AmCyan filter (525/50 nm bandpass filter with 475 nm longpass filter). Doublets and debris were gated out before quantification of median fluorescence intensities using FlowJo.

General synthetic procedures and materials

Chemicals were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, and TCI America) and used without further purification unless otherwise noted. Flash column chromatography was performed using normal phase on a CombiFlash® Rf 200i (Teledyne Isco Inc). ^{1}H NMR spectra were recorded at 400 or 500 MHz, and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz), and integration. ^{13}C NMR spectra were recorded at 100 or 125 MHz. Data for ^{13}C NMR spectra are reported in terms of chemical shift.

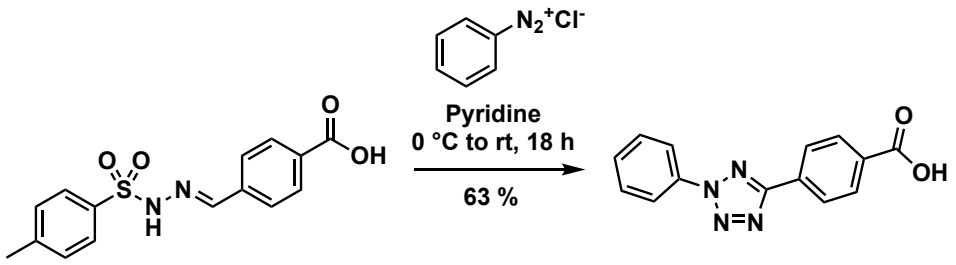
Synthesis and characterization data for diaryl tetrazoles 1-2

(a) 5-Hexynyltriphenylphosphonium iodide



6-iodohexyne (0.5 g, 2.4 mmol) was added to a solution of triphenylphosphine (0.63 g, 2.4 mmol) in toluene (10 ml) and the reaction was heated to 100 °C for 16 h. Cooling the reaction mixture to room temperature resulted in precipitation of the product. The product was then collected by filtration, washed with toluene, and dried under vacuum to yield white solid (0.56 g, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.94-7.88 (m, 4H), 7.86-7.74 (m, 11H), 3.60 (dd, *J*= 13.7, 7.6 Hz, 2H), 2.76 (t, *J*= 2.6 Hz, 1H), 2.25 (m, 2H), 1.63 (d, *J*= 7.1 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d6) δ 134.83,134.80, 133.50, 133.40, 130.20, 130.08, 118.77, 117.92, 83.62, 71.64, 28.47, 28.30, 20.64, 20.59, 19.76-19.25, 16.69. TOF MS: [M+H]+ calculated: 343.1616, [M]+ found: 343.1615.

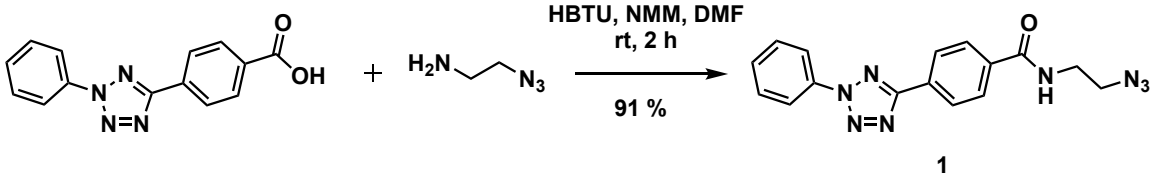
(b) 4-(2-phenyl-5-tetrazolyl)benzoic acid



To an ice-cold solution of aniline (0.86 ml, 9.44 mmol), ethanol (8 ml), water (8 ml), and concentrated hydrochloric acid (2.5 ml), a solution of sodium nitrite (0.65 g, 9.44 mmol) in water (4 ml) was added dropwise over a period of 10 min and allowed to stir for an additional 15 min at 0 °C. This resulting solution was added dropwise to a stirring solution of 4-(2-tosylhydrazineylidene)methylbenzoic acid[3] (3 g, 9.44 mmol) in pyridine (50ml) at 0 °C over 15 min and allowed to warm up to room temperature. The reaction was stirred overnight at room temperature resulting in a precipitation. The precipitate was collected by filtration, washed thoroughly with water and air-dried to yield pale yellow solid, which was used in next step without purification (1.58 g, 63%). ^{1}H NMR (400 MHz, DMSO-d_6) δ

13.26 (s, 1H), 8.34-8.28 (m, 2H), 8.21-8.15 (m, 4H), 7.75-7.69 (m, 2H), 7.68-7.62 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.57, 163.68, 136.02, 132.66, 130.31, 130.19, 130.12, 130.09, 126.71, 119.96. TOF MS: [M+H]+ calculated: 267.0877, [M+H]+ found: 267.0882.

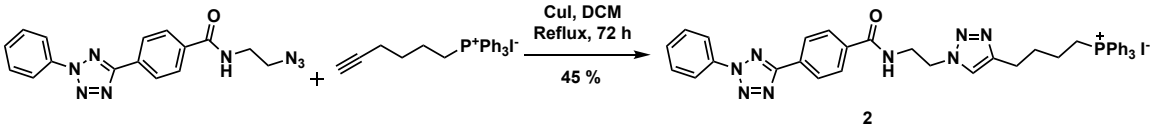
*(c) N-(2-azidoethyl)-4-(2-phenyl-5-tetrazolyl)benzamide (**1**)*



1

4-(2-phenyl-5-tetrazolyl)benzoic acid (0.5 g, 1.88 mmol), 2-azidoethanamine (0.323 g, 3.76 mmol), N-methylmorpholine (1.88 ml, 2 M solution in DMF), and HBTU (0.784 g, 2.07 mmol) were dissolved in anhydrous dimethylformamide (DMF, 8 mL). The reaction mixture was stirred for 2 h at room temperature and water (50 mL) was added at the end of 2 h. Addition of water resulted in precipitation of the product. The precipitate was collected by filtration, washed with water, air-dried, and purified by flash chromatography (25→ 100% EtOAc:Hexanes) to yield product **1** as pink solid (0.57 g, 91%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.90 (s, 1H), 8.31-8.26 (m, 2H), 8.21-8.16 (m, 2H), 8.11-8.06 (m, 2H), 7.76-7.68 (m, 2H), 7.68-7.61 (m, 1H), 3.51 (d, *J*= 2.6 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.69, 163.79, 136.06, 136.04, 130.27, 130.08, 128.79, 128.14, 126.52, 119.95, 49.65, 38.97. TOF MS: [M+H]+ calculated: 335.1363, [M+H]+ found: 335.1262.

*(d) (4-(2-phenyl-5-tetrazolyl)benzamido)ethyl-1,2,3-triazolyl)-4-butyltriphenylphosphonium iodide (**2**)*



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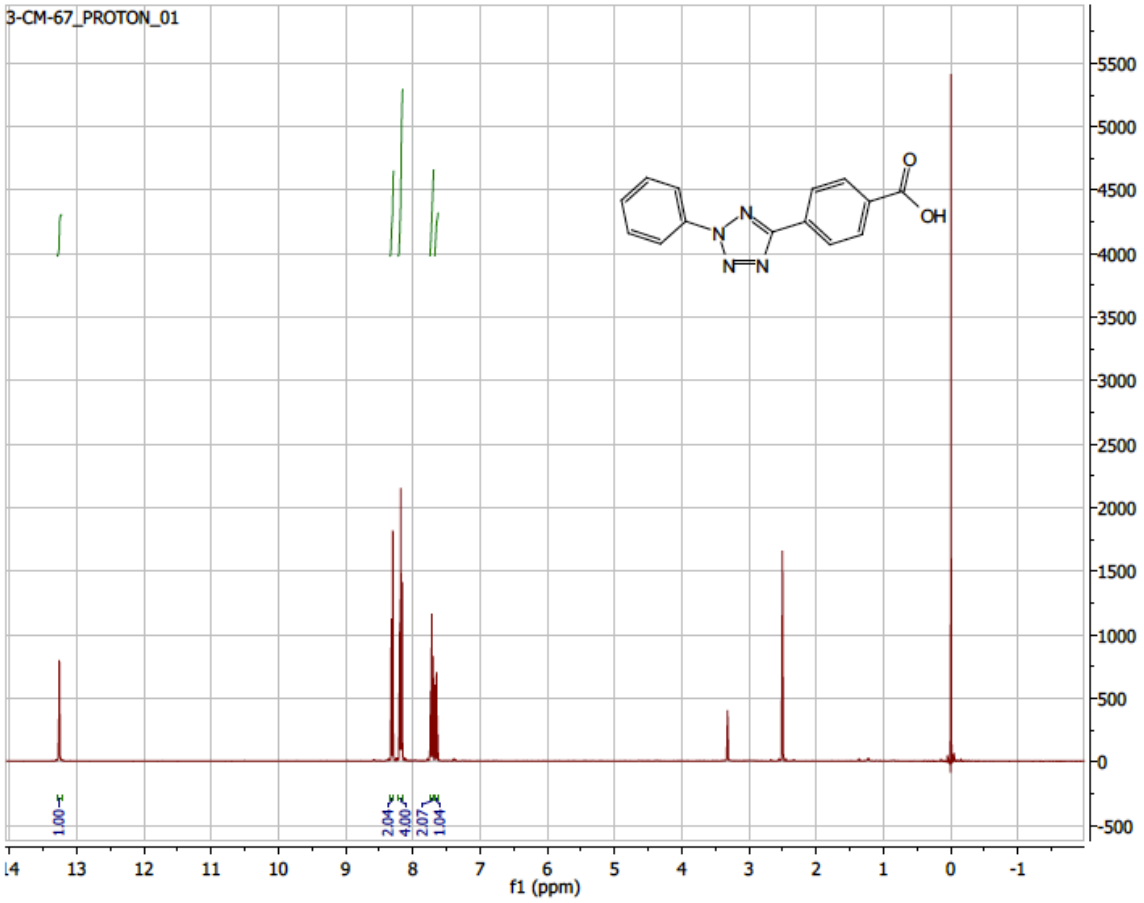
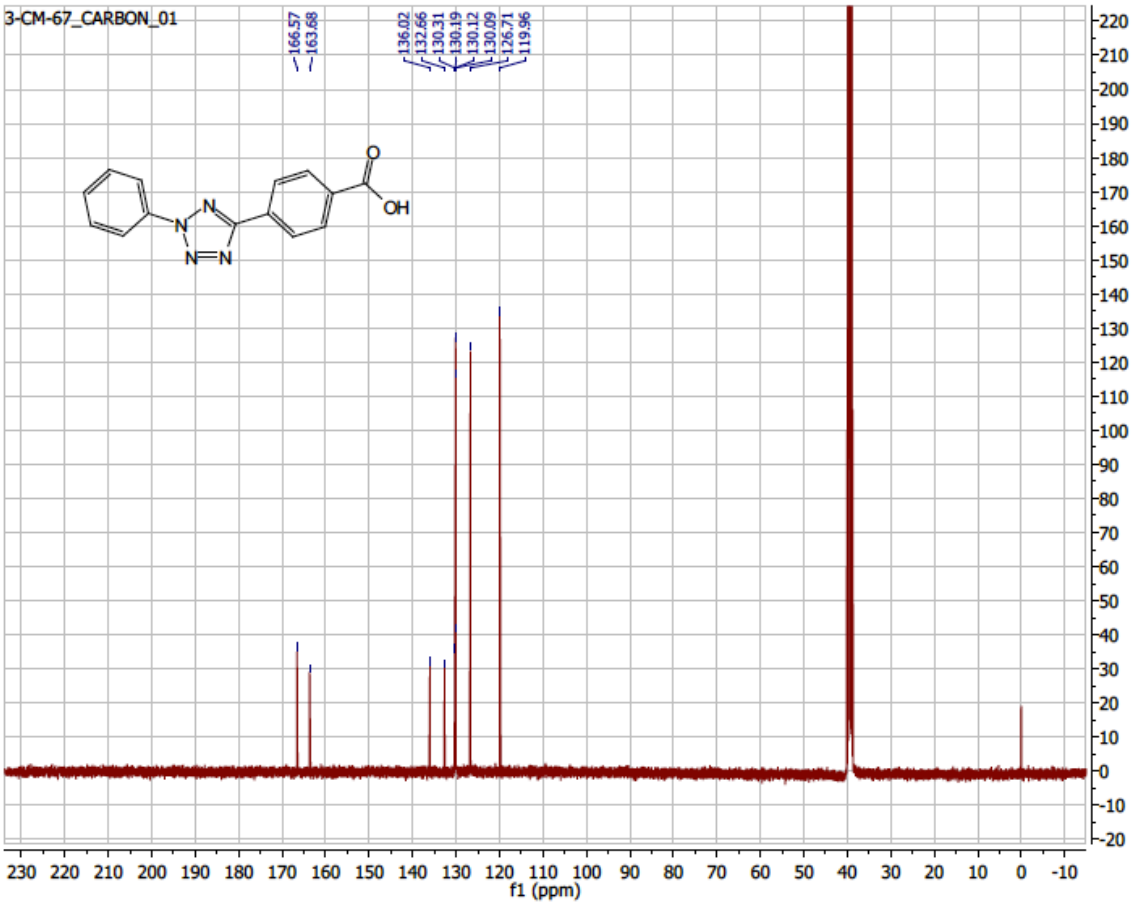
To a stirring solution of N-(2-azidoethyl)-4-(2-phenyl-5-tetrazolyl)benzamide (0.1 g, 0.299 mmol) and 5-hexynyltriphenylphosphonium iodide (0.141 g, 0.299 mmol) in dichloromethane (2 ml), cuprous iodide (3 mg, 0.015 mmol) was added and the reaction was refluxed for 72 h. The crude reaction mixture was adsorbed on silica gel and purified by flash chromatography (0→ 25% methanol:DCM) to yield product **2** as pink solid (90 mg, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28-8.14 (m, 7H), 8.08 (s, 1H), 7.75 (m, 3H), 7.72-7.60 (m, 11H), 7.61-7.54 (m, 2H), 7.53-7.47 (m, 1H), 4.77-4.68 (m, 2H), 3.98 (ddd, *J*= 7.2, 5.5, 3.6 Hz, 2H), 3.53 – 3.41 (m, 2H), 2.79 (t, *J*= 6.6 Hz, 2H), 2.04 (p, *J*= 6.9 Hz, 2H), 1.78-1.66 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 165.79, 163.85, 145.95, 136.11, 136.09, 134.88, 133.60, 133.50, 130.40, 130.26, 130.19, 130.13, 128.89, 128.21, 126.59, 122.20, 120.03, 118.92, 118.07, 54.90, 48.42, 29.67, 23.87, 21.10, 20.14, 19.65. TOF MS: [M]+ calculated: 677.2901, [M+H]+ found: 677.2906.

^{1}H- and ^{13}C-NMR spectra for synthetic intermediates

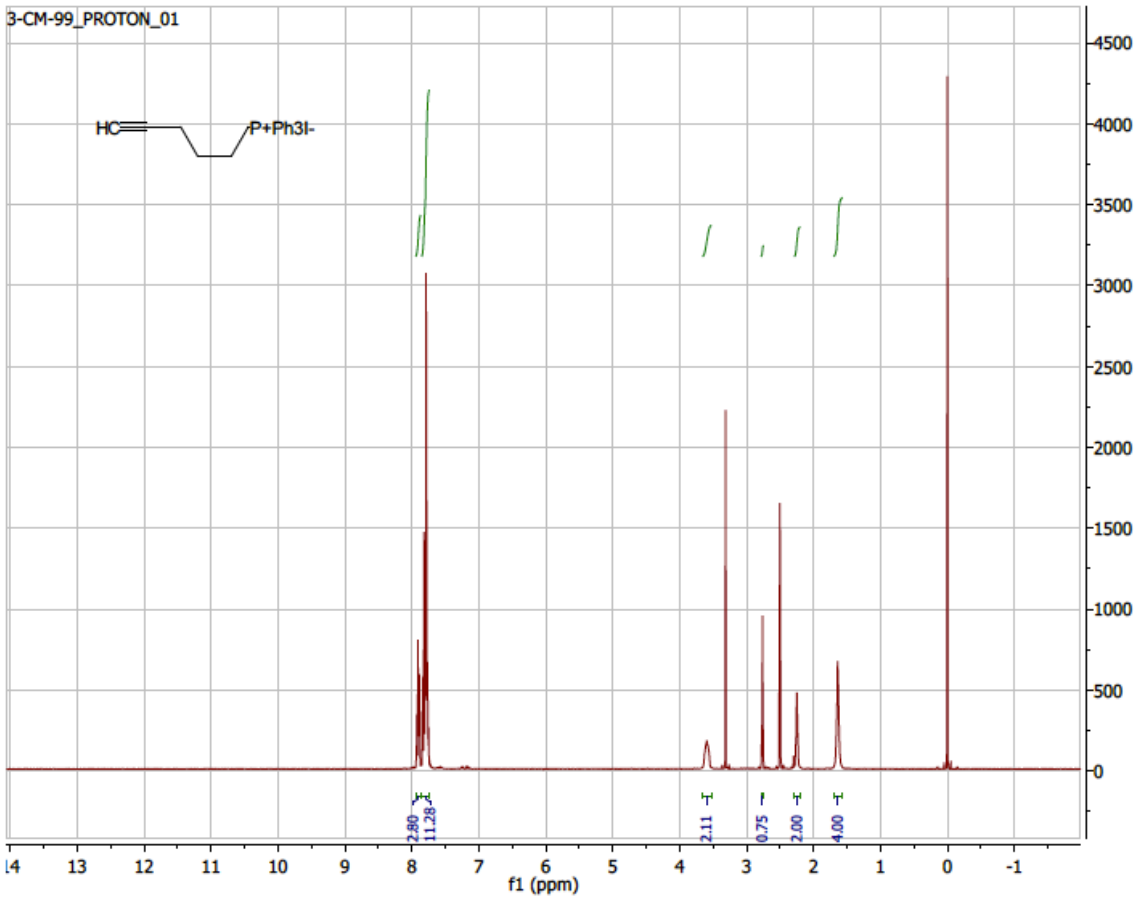
^{1}H NMR of *4-(2-phenyl-5-tetrazolyl)benzoic acid*



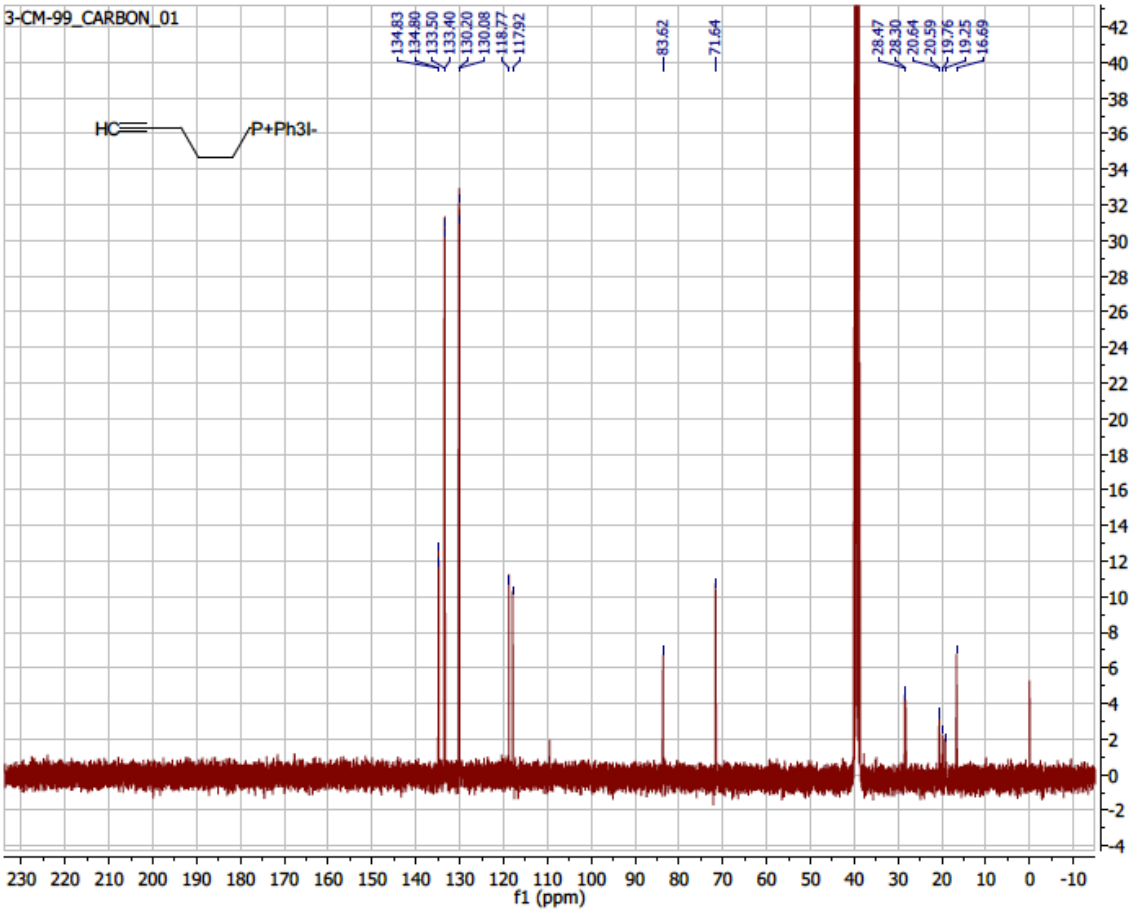
^{13}C NMR of *4-(2-phenyl-5-tetrazolyl)benzoic acid*



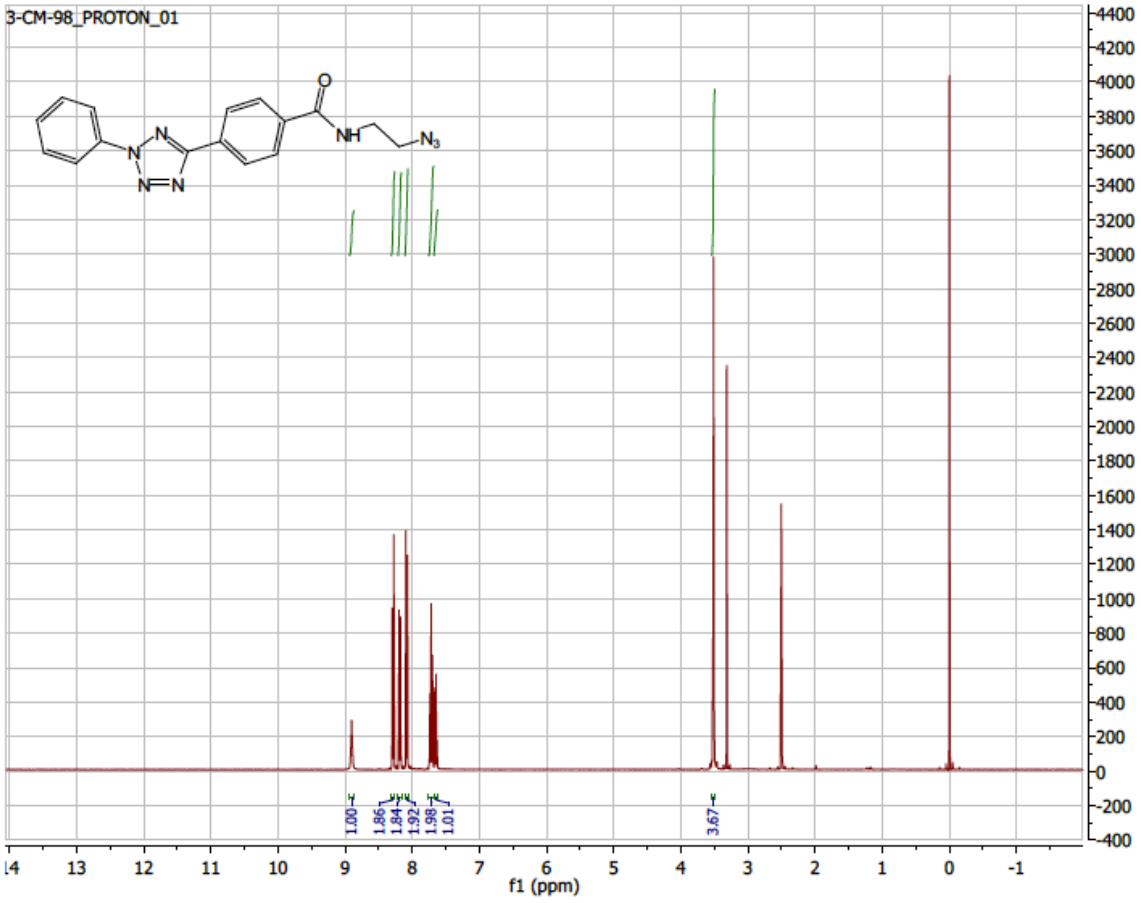
^{1}H NMR of *5-Hexynyltriphenylphosphonium iodide*



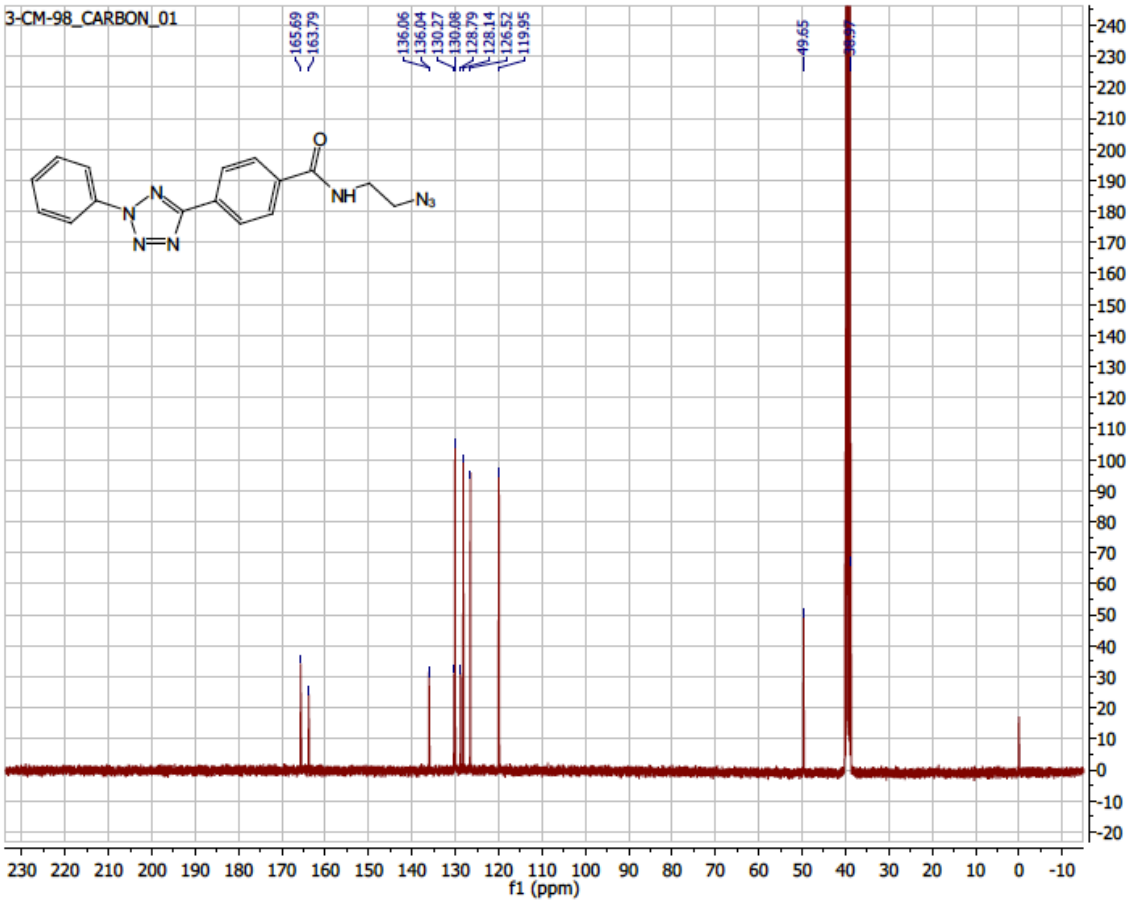
^{13}C NMR of *5-Hexynyltriphenylphosphonium iodide*



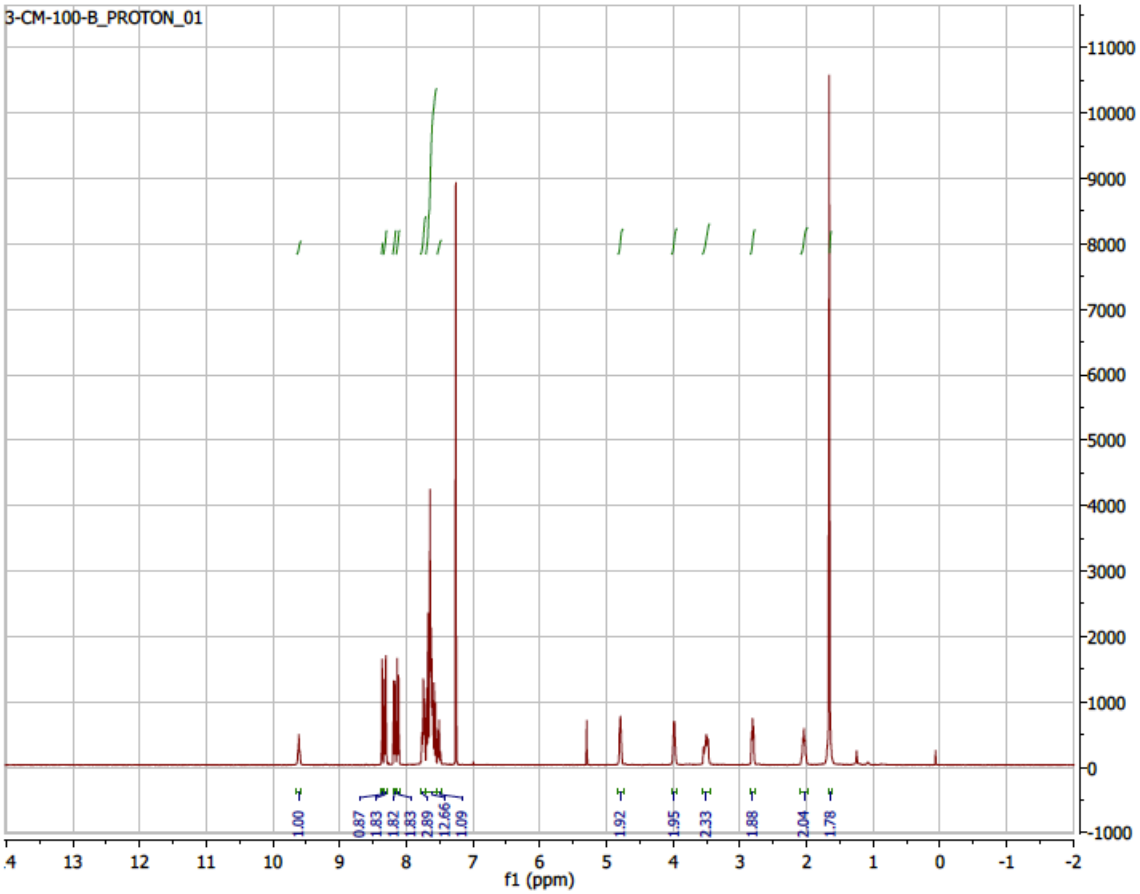
^{1}H NMR of *N-(2-azidoethyl)-4-(2-phenyl-5-tetrazolyl)benzamide (**1**)*



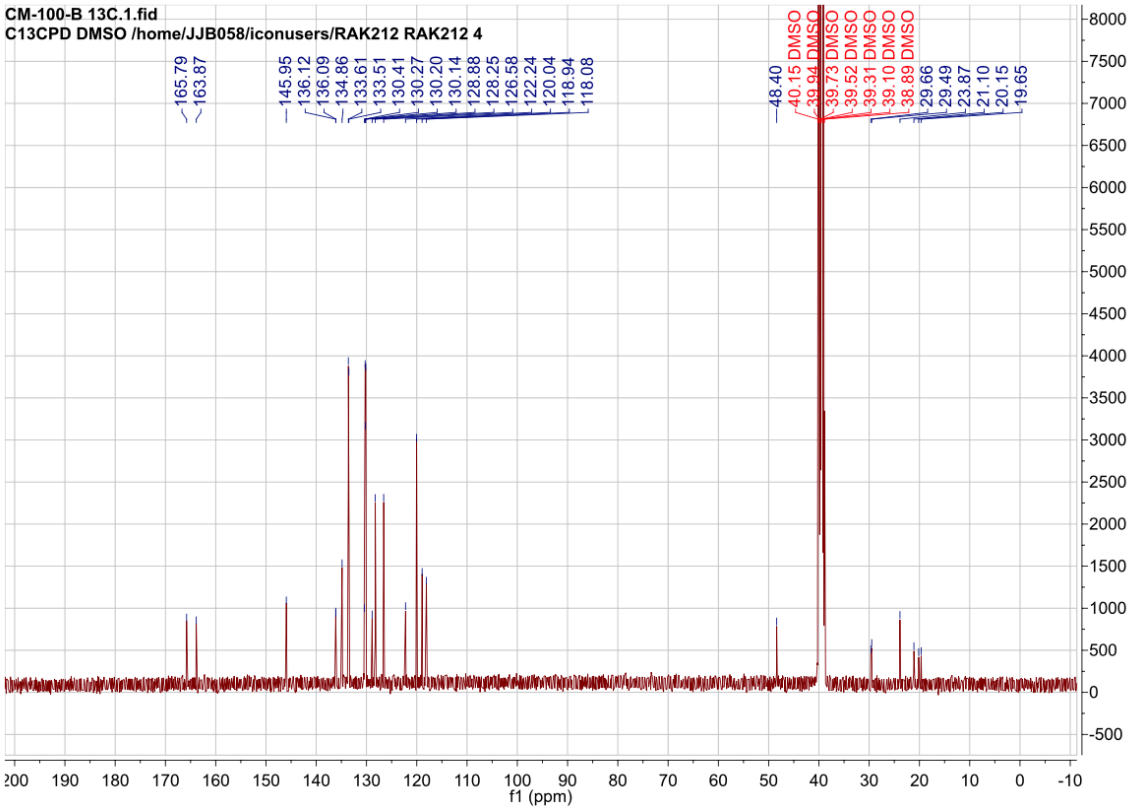
^{13}C NMR of *N-(2-azidoethyl)-4-(2-phenyl-5-tetrazolyl)benzamide* (***1***)



^{1}H NMR of *(4-(2-phenyl-5-tetrazolyl)benzamido)ethyl-1,2,3-triazolyl)-4-butyl triphenylphosphonium iodide (**2**)*



^{13}C NMR of *(4-(2-phenyl-5-tetrazolyl)benzamido)ethyl-1,2,3-triazolyl)-4-butyl triphenylphosphonium iodide (**2**)*



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