Highly-Efficient and Versatile Fluorous-Tagged Cu(I)-Catalyzed Azide-Alkyne Cycloaddition Ligand for Preparation of Bioconjugates

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Supporting information:

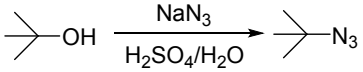
Experimental Procedures:

Materials:

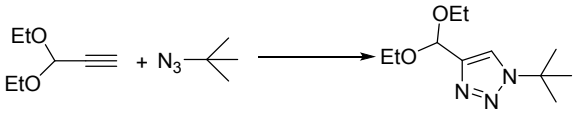
3-Azido-7-hydroxycoumarin and Sulfo-Cyanine3 azide were purchased from AK scientific and Lumiprobe respectively. All other chemicals and solvents were purchased from Sigma-Aldrich.

1. Synthesis of FBTTBE ligand:

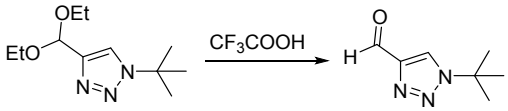
Reported compounds **2**[1], **3**[2], **4**[2], and **5**[2] were prepared according to literature.



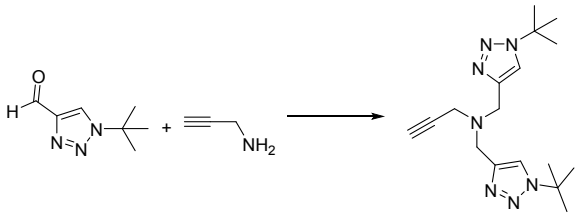
t-butyl azide (2): A solution of H_2SO_4 (55 g, 0.56 mmol, 5.6 eq.) in 55 g H_2O was prepared by the addition of 55 g H_2SO_4 to 55 g H_2O over 10 min, with vigorously stirring in an ice cooled 250 mL flask. The solution was cooled to ≤ 5 °C, and sodium azide (7.2 g. 0.11 mol) was slowly added over 10 min (maintaining the temperature ≤ 20°C in order to preclude accidental volatilization of HN_3). When all of the NaN_3 has dissolved, *t*-butyl alcohol (7.4 g. 0.1 mol) was added, and the resulting solution was stirred for 5 min and allowed to stand at room temperature for 24 h. *t*-Butyl azide floated to the top of the acid mixture was collected in a separatory funnel, washed with 50 ml of 2 M NaOH to remove all traces of HN_3, dried over Na_2SO_4, and 600mg clear liquid was obtained as the product, yield 60%. ^{1}H NMR complies with the reported value[1].



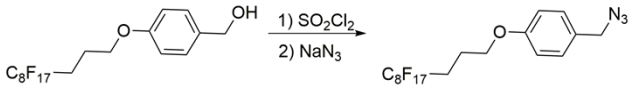
***1-tert-butyl-4-(diethoxymethyl)-1H-1,2,3-triazole (3)*:** To a 20-mL screw-capped scintillation vial equipped with a stirring bar were added 3,3-diethoxy-1-propyne (1.50 g, 11.8 mmol, 1.0 eq) and tert-butyl azide (1.34 g, 13.5 mmol, 1.15 eq) in 10 mL 1:1 mixture of tertbutyl alcohol and water. Sodium bicarbonate (1.40 g, 16.7 mmol, 1.41 eq), copper(II) sulfate pentahydrate (0.143 g, 0.57 mmol, 5.0 mol%), and sodium ascorbate (0.47 g, 2.35 mmol, 20 mol%) were added to the mixture. The reaction was stirred overnight, and then EDTA (2 mL, 0.5 M, pH = 8) was added. The resulting mixture was diluted with EtOAc (90 mL), washed with sat. aq $NaHCO_3$ (100 mL), water (50 mL), and brine (50 mL). The combined organic phases were dried over anhydrous $MgSO_4$. Solvent was removed and the 2.1g residue was used in the next step without purification, yield 79%. ^{1}H NMR complies with the reported value[2].



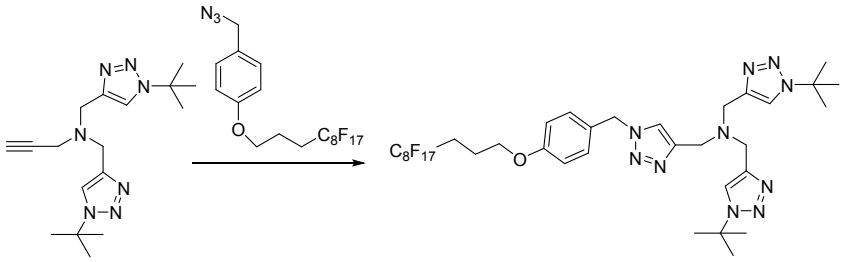
***1-tert-butyl-1H-1,2,3-triazole-4-carbaldehyde (4)*:** To a 50-mL round bottom flask was added a solution of 1-*tert*-butyl-4-(diethoxymethyl)-1*H*-1,2,3-triazole (1.28 g, 5.63 mmol) in dichloromethane (6.0 mL), followed by addition of water (3.0 mL) and trifluoroacetic acid (1.0 mL). The reaction mixture was stirred vigorously under nitrogen for 3 h and then was diluted with EtOAc (100 mL), washed with sat aq $NaHCO_3$ (3 × 40 mL) and brine (40 mL). The combined organic phases were dried over anhydrous $MgSO_4$, filtered. Solvent was removed and the 0.65g residue was used in the next step without purification, yield 75%. ^1H NMR complies with the reported value[2].



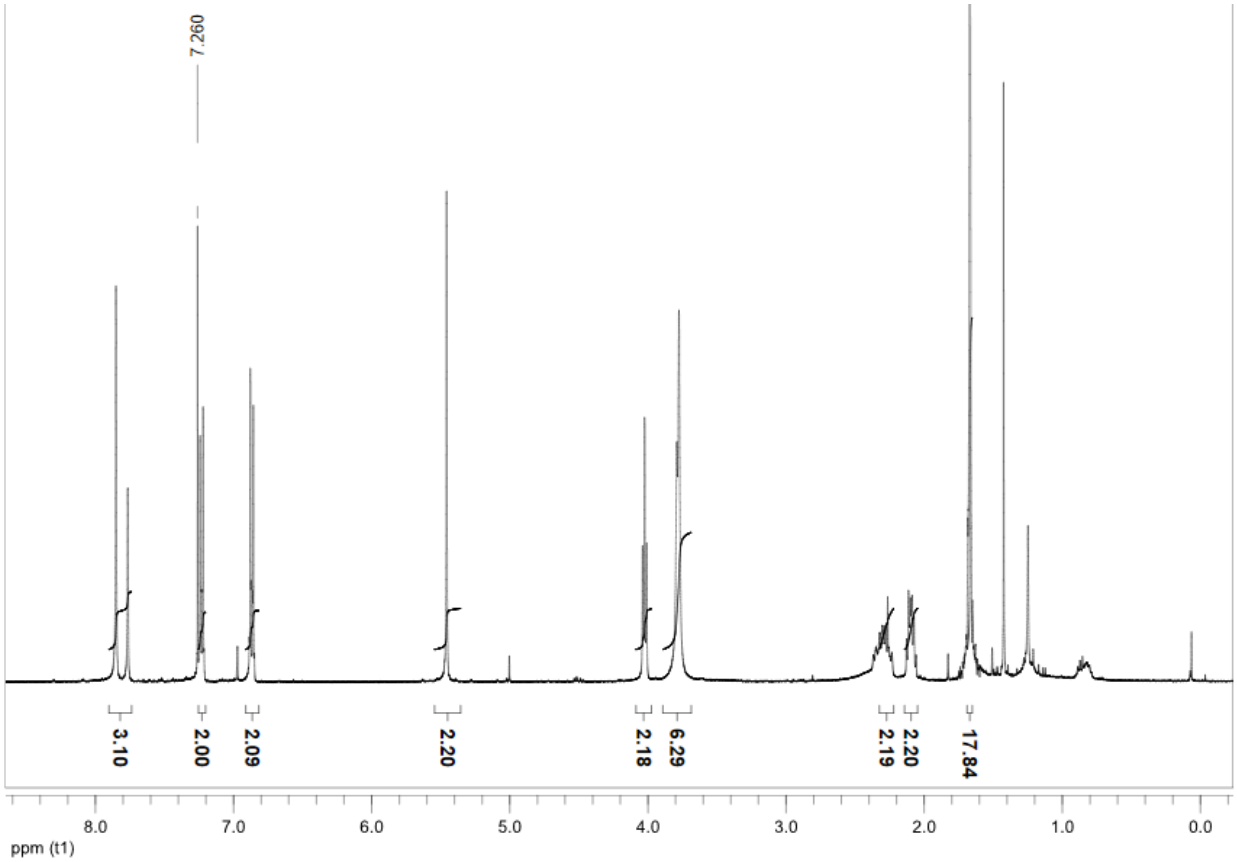
***N,N-bis((1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl)prop-2-yn-1-amine (5)*:** To a 250-mL round bottom flask was added a solution of 1-*tert*-butyl-1*H*-1,2,3-triazole-4-carbaldehyde (2.47 g, 16.1 mmol, 2.2 eq) in dichloroethane, followed by addition of propargyl amine (361 mg, 7.2 mmol, 1.0 eq). To this mixture sodium triacetoxyborohydride (3.8 g, 17.9 mmol, 2.5 eq) was added in one portion with vigorous stirring. The reaction mixture was stirred at room temperature for 40 h. 1N H_2SO_4 (86 mL) was added to the reaction, and the mixture was stirred for 15 min. The pH was adjusted to >10 by addition of potassium carbonate. The reaction mixture was diluted with water (100 mL) and extracted with dichloromethane (3 × 300 mL). The organic layers were combined, dried over anhydrous $MgSO_4$, and filtered. Solvent was removed and the residue was purified on column to afford 1.47g product as a white powder, yield 62%. ^1H NMR complies with the reported value[2].

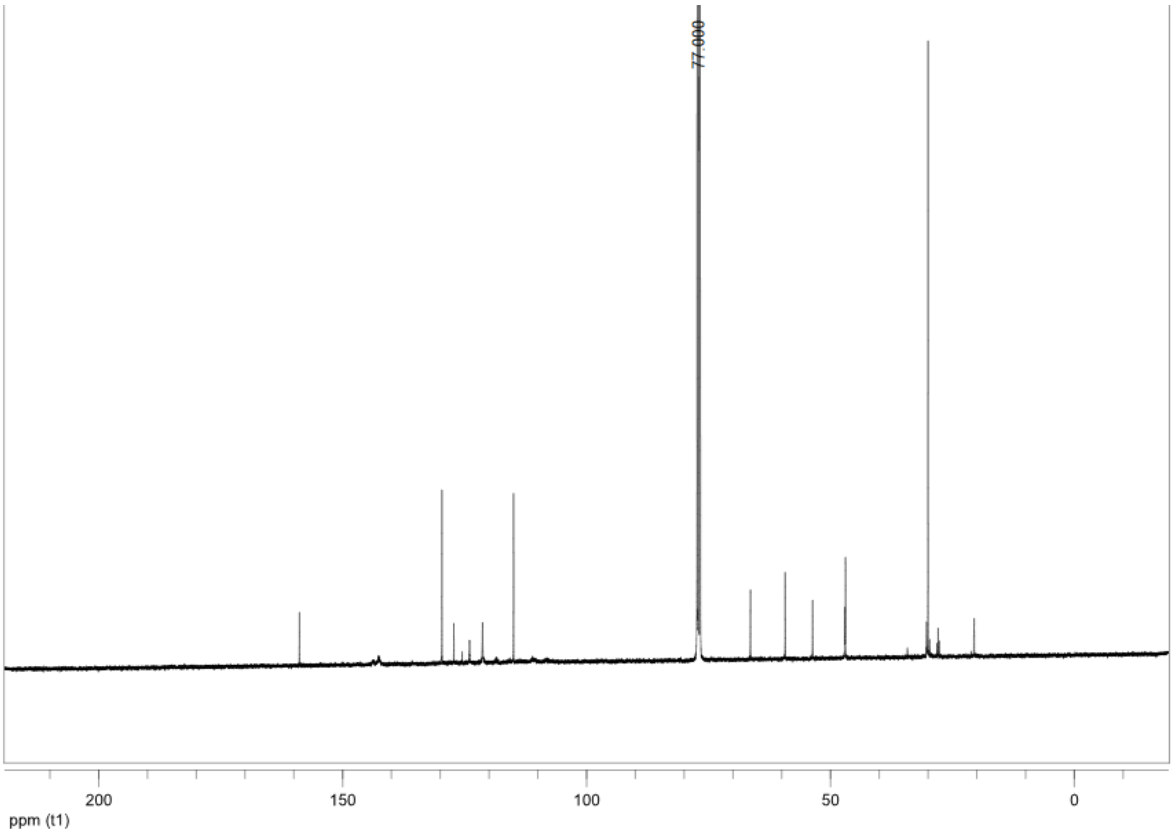


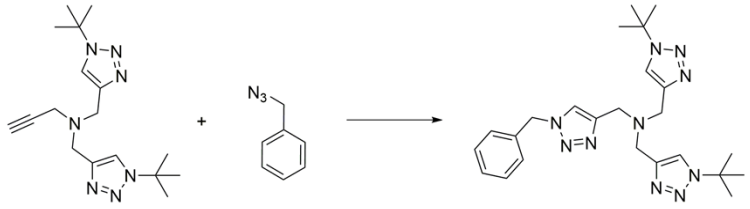
4-[3-(Perfluorooctyl)-1-propyloxy]benzyl azide (7): 4-[3-(Perfluorooctyl)-1-propyloxy]benzyl alcohol (290mg, 0.5 mmol) was dissolved in 1.0 mL DCM. Thionyl chloride (110 µl) and DMF (10 µl) were then added to the mixture. After stirred for 1 hour, the solvent was removed under reduced pressure. After lyophilization for 1 hour, the residue was dissolved in 20 mL THF/DMF (1:1) and NaN_3 (325 mg, 0.5 mmol) was added to the mixture. The mixture was stirred for 3 days at 60 °C. Solvent was removed under reduced pressure. Product was extracted by DCM (100 ml) and washed with 60 mL $NaHCO_3$:Brine (1:1). Solvent was removed under reduced pressure and the residue was purified on column with EA:HEX (1:4) to afford 160mg target product, yield 53%. The product was used in next step without further purification, . ESI-MS, M/Z $(M+H)^+$ = 610.52.



FBTTBE *(**F**luorous **T**agged -2-[4-{(**B**is[(1-**T**ert-butyl-1H-1,2,3-**T**riazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl])* ***(8)***: 4-[3-(Perfluorooctyl)-1-propyloxy]benzyl azide (100 mg, 0.16 mmol), *N,N*-bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)prop-2-yn-1-amine (53 mg, 0.16 mmol), $CuSO_4.5H_2O$ (12.5 mg, 0.05 mmol) and sodium ascorbate (40 mg, 0.20 mmol) were dissolved in *t*-BuOH/H_2O (1:1) in 2.0 mL and stirred overnight. Solvent was removed and the residue was purified on column with EA:HEX (1:4) to afford 60mg target product, yield 39%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.68 (18H, s, *tert*-butyl CH_3), 2.05-2.15 (2H, m, CH_2), 2.22-2.38 (2H, m, CH_2), 3.76 (6H, s, broad, NCH_2), 4.03 (2H, t, J = 6Hz, OCH_2), 5.46 (2H, s, NCH_2), 6.87 (2H, d, J = 8.4Hz, CH), 7.25 (2H, d, J = 8.4Hz, CH), 7.93 (3H, s, broad, CH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.5, 27.9, 30.0, 46.9, 47.0, 53.6, 59.3, 66.4, 115.0, 121.3, 124.0, 125.5, 127.2, 129.6, 158.8. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{35}H_{39}F_{17}N_{10}NaO$ 961.2929, found 961.2938.







BTTBE *(2-[4-{(**B**is[(1-**T**ert-butyl-1H-1,2,3-**T**riazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl])*: benzyl azide (108 mg, 0.8 mmol), *N,N*-bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)prop-2-yn-1-amine (53 mg, 0.16 mmol), $CuSO_4.5H_2O$ (12.5 mg, 0.05 mmol) and sodium ascorbate (40 mg, 0.20 mmol) were dissolved in *t*-BuOH/H_2O (1:1) in 2.0 mL and stirred overnight. Solvent was removed and the residue was purified on column with MeOH:DCM (1: 10, 0.5%Et_3N) to afford 30mg target product, yield 41%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.67 (18H, s, *tert*-butyl CH_3), 3.74 (4H, s, NCH_2), 3.76 (2H, s, NCH_2), 5.53 (2H, s, NCH_2), 7.25 – 7.28 (2H, m, CH), 7.33 – 7.37 (3H, m, CH), 7.82 (1H, s, CH), 7.83 (2H, s, CH).

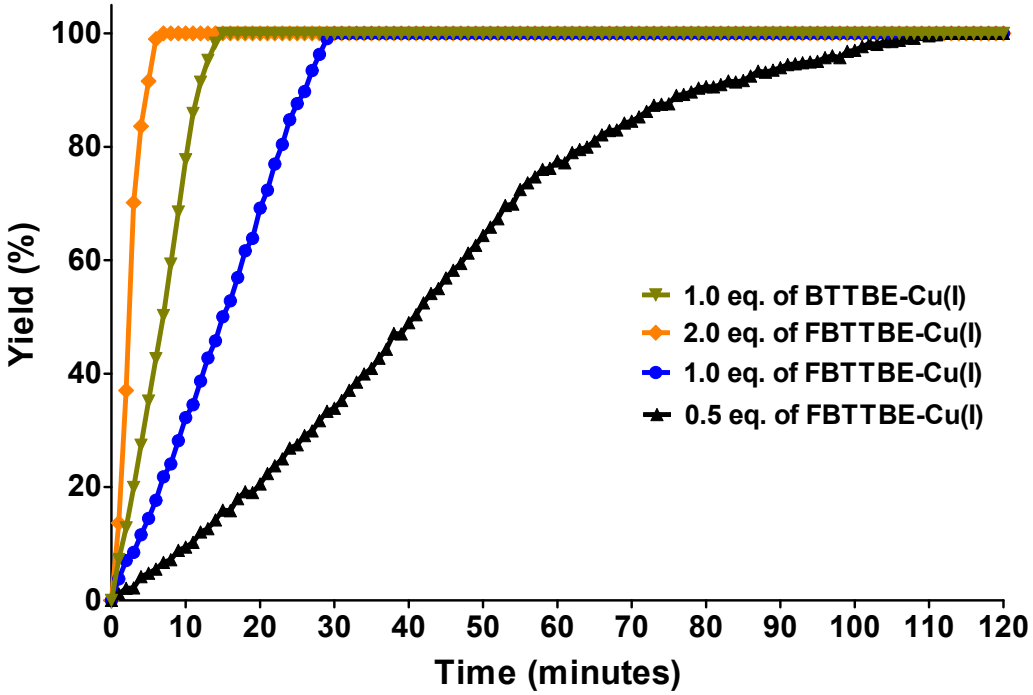
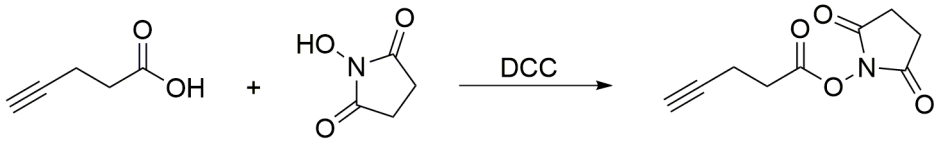


Fig. S1. Comparison of FBTTBE and BTTBE in CuAAC between propargyl amine and 3-azido-7-hydroxycoumarin.

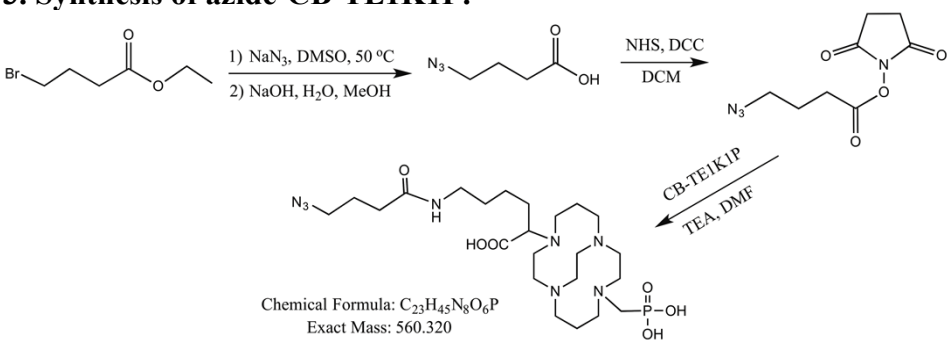
2. Synthesis of acetylene-AE105 peptide:



4-pentynoic acid succinimidyl ester:[3] Pentynoic acid (0.98 g, 10.0 mmol) and N-Hydroxysuccinimide (1.15 g, 10 mmol) were suspended in 200 mL of dry dichloromethane, and then N,N′ -Dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added. The reaction mixture was stirred at room temperature overnight, and then the white precipitate was filtered off. The filtrate was concentrated to afford the crude product. 1.25g pure 4-pentynoic acid succinimidyl ester was obtained as white solid after recrystallization from dichloromethane/diethylether, yield 64%. ^{1}H NMR complies with the reported value[3].

***Preparation of acetylene-AE105 peptide conjugate*:** To the solution of AE105 peptide (in dimethylformamide), 4-pentynoic acid succinimidyl ester (1.5 equiv.) was added, and followed by triethylamine (5.0 equiv.). The resulting mixture was stirred overnight at room temperature in the dark. The acetylene attached peptide conjugate was purified by HPLC (high performance liquid chromatography), and pure product was obtained as white powder after lyophilization overnight. ESI-MS, M/Z $(M+H)^{+}$ = 1305.22.

3. Synthesis of azide-CB-TE1K1P:

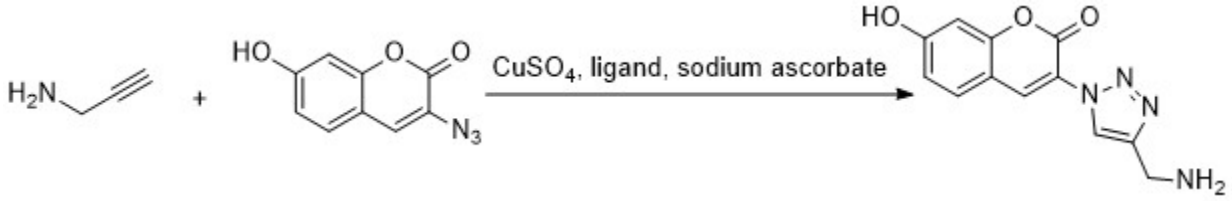


Azidobutyrate acid succinimidyl ester was prepared from ethyl 4-bromobutyrate as previously described[4], and CB-TE1K1P was synthesized following the published procedures[5]. Azidobutyrate acid succinimidyl ester (56.5 mg, 250 μmol) was added to the mixture of TEA (111 μl, 800 μmol), CB-TE1K1P (45 mg, 100 μmol) and DMF (2.5 mL). The resulting mixture was stirred overnight, and then the solvent was removed under vaccum and the residue was purified using HPLC. 31mg pure product was obtained as a white powder, yield 55%. ESI-MS, M/Z $(M+H)^+ = 561.31$.

4. General procedures for the CuAAC reaction using the stabilizing ligand:

Preparation of the ligand FBTTBE-Cu(I) stock solution: 15 mM of FBTTBE in ethanol, was mixed with 10 mM of Cu(I) in water with the volume ratio of 1 : 1. Therefore, the molar concentration of FBTTBE and Cu(I) was 7.5 mM and 5 mM respectively.

1). Preparation of the fluorescence dye labeled compounds:



Scheme S1. The click reaction between propargyl amine and 3 -azido-7- hydroxycoumarin.

2). Preparation of Cy3 attached AE105 peptide with various Cu(I) stabilizing ligands:

50 μL of azide-fluorescence dye (Sulfo-Cyanine3 azide, 5.0 mM in DMSO) and 50 μL of acetylene-AE105 (5.0 mM in 10% DMSO) were added to 375 μL of NH_4OAc buffer (0.1 M, pH ~ 8.20), followed by 75 μL of the above FBTTBE-Cu(I) stock solution. The reaction mixture was incubated for a given time, monitored by HPLC. Once all azide-fluorescence dye was converted to the product, the reaction solution was diluted with water, and then passed through the FluoroFlash SPE silica gel. The filtrate was collected as product, while the FBTTBE and FBTTBE-Cu(I) complex retained on the silica gel. ESI-MS, M/Z $(M+H)^+ = 2003.57$.

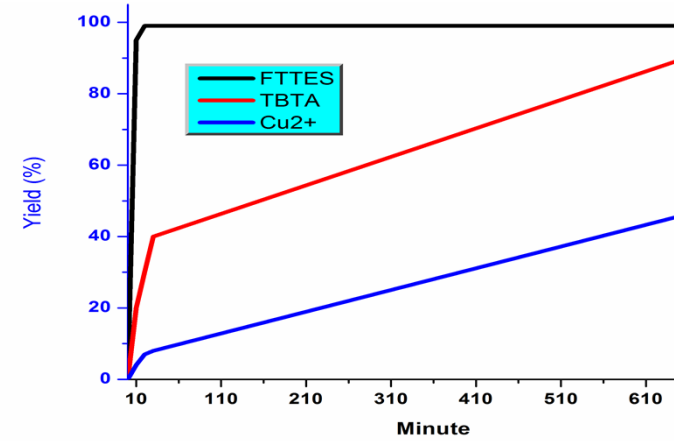
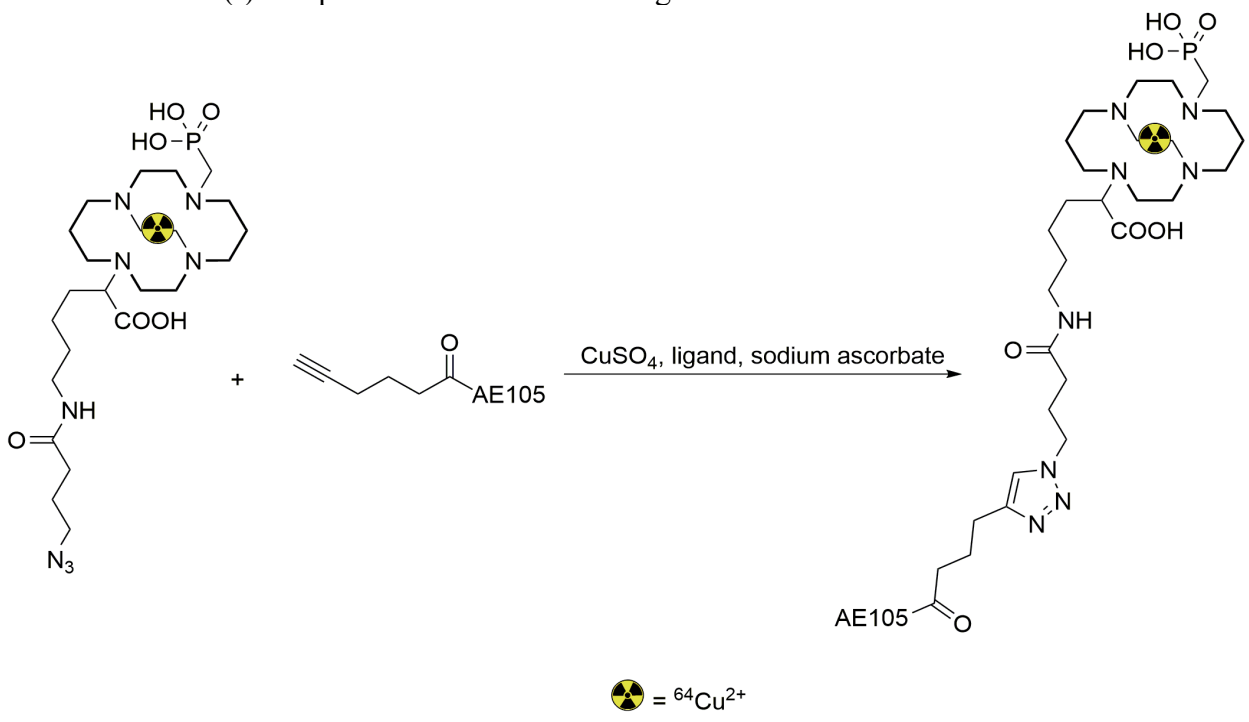


Fig. S2. Comparison of Cu(I) stabilizing ligands in CuAAC between Cy3-azide and acetylene-AE105.

3). Preparation of Cu-64 labeled AE105 peptide:

The radiometal (^{64}Cu) labeling of chelator was conducted by incubating the azide-CB-TE1K1P and ^{64}Cu in 0.1 M NH_4OAc buffer (pH ~ 8.20) at 40 °C for 15 minutes, with the specific activity (SA) of 1.0 mCi/nmole. Then, 250 μCi of the resulting ^{64}Cu labeled azide-CB-TE1K1P was added to 0.5 nmole of the prepared acetylene-AE105, followed by the addition of 1.0 μL of above FBTTBE-Cu(I) stock solution. After incubated for a given time, 10 ~ 50 μCi of the reaction mixture was loaded into radio-HPLC to determine the radiolabeling yield. Once the radiolabeling yield was over 95%, the reaction solution was diluted with water, and then passed through the FluoroFlash SPE silica gel. The filtrate was collected as product, while the FBTTBE and FBTTBE-Cu(I) complex retained on the silica gel.



Scheme S2. Click reaction for the preparation of Cu-64 labeled AE105

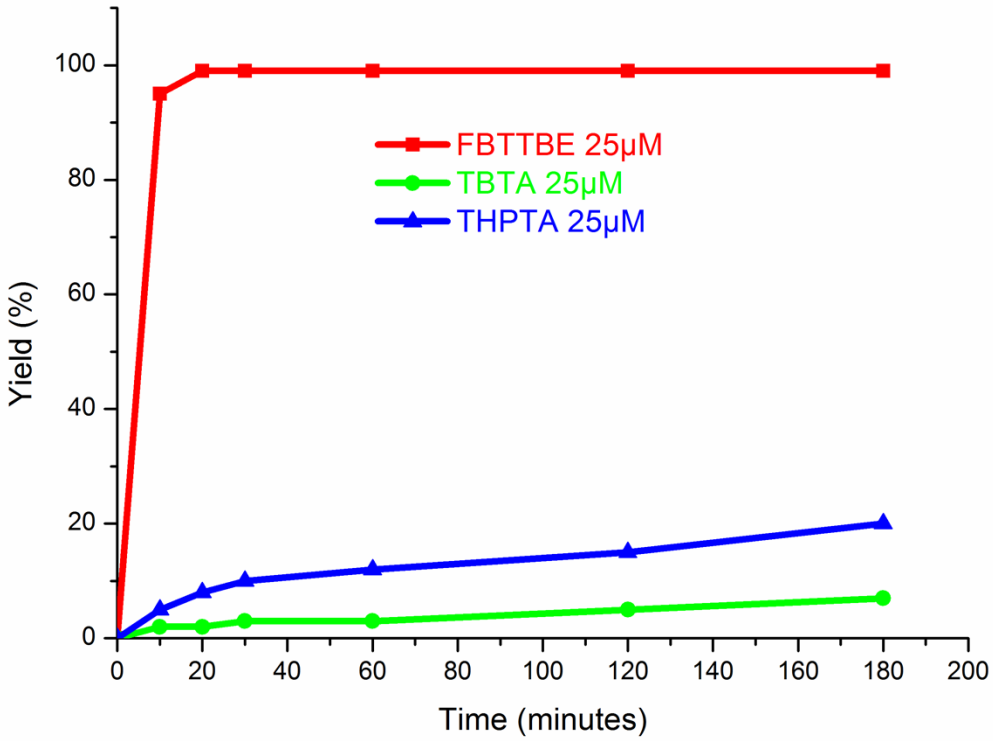


Figure S3. Comparison of catalytic efficiency (ligand:Cu:NaAA= 1.5:1:0.8) for the preparation of Cu-64 labeled peptide (SA = 0.5 mCi/nmole).

4). Preparation of Cu-64 labeled cetuximab:

Aizde-CB-TE1K1P was radiolabeled with 2.0 mCi of Cu-64 at 37 °C for 30 min, and the resulting N_3-(^{64}Cu)CB-TE1K1P (specific activity of 1.0 mCi/nmole) was then mixed with 100 ug of acetylene-cetuximab (prepared using the reported procedures[6]). The click reaction started after the catalyst FBTTBE-Cu(I) was added, and after incubating at 37 °C for 30 minutes, the FBTTBE-Cu(I) was then removed by passing through Fluorous-silica gel. In the filtrate, ~ 50% of N_3-(^{64}Cu)CB-TE1K1P was attached to cetuximab, and over 90% radiopurity was obtained after the filtrate passed over Zaba desalting column.

5. Staining of U87MG using AE105-Cy3:

Cells were seeded in an 8 well chamber slide (100,000 cells per well) 24 h prior to the experiment. Before the experiment, cells were washed twice with PBS, and added with culture medium. Then blocking agent (10 μg AE105) was added to half of the wells to determine in vitro non-specific uptake. After 1h incubation, AE105-Cy3 (10 pmol per well) was then added to each

well, and cells were incubated for another 2 h. Medium was then removed and cells were washed twice with PBS. After fixing the cells using 1% Paraformaldehyde, the nucleus was stained by DAPI. The slide was sealed and observed under fluorescence microscopy (40 X, oil).

6. Small-animal PET/CT imaging studies for AE105-^{64}Cu-CBTE1K1P:

All animal studies were conducted under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.U87MG xenograft tumor–bearing mice (n=3, 4 per group) were injected intravenously (lateral tail vein) with the prepared AE105-^{64}Cu-CBTE1K1P. Half of the mice received a dose that was premixed with AE105 (50 μg) for blocking. At 1h and 4h mice were anesthetized with 2% isoflurane, and small-animal PET/CT was performed. Static images were collected for 15 min. PET and CT images were co-registered with Inveon Research Workstation (IRW) software (Siemens Medical Solutions). PET images were reconstructed with the ordered-subsets expectation maximization 3-dimensional/maximum a posteriori probability algorithm, and the analysis of images was done using IRW.

Reference:

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