

Relative Performance of Alkynes in Copper-Catalyzed Azide–Alkyn Cycloaddition

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I. General

Reagents and solvents were purchased from commercial sources and used without further purification. NMR spectra were obtained on Brüker AMX-400, DRX-500, and DRX-600 instruments and referenced to the signals of residual protium in the NMR solvent. ¹³C NMR spectra were referenced to the solvent signal.¹ Routine mass spectra were obtained on an Agilent 1100 (G1946D) ESI-MSD instrument, using direct sample injection followed with 9:1 CH₃CN:H₂O containing 0.1% CF₃CO₂H as a running solvent. Flash chromatography was performed on 230–400 mesh silica or on Biotage Flash instruments. Dye-containing materials were protected from light by wrapping the reaction and storage glassware in aluminum foil. Absorbance and fluorescence spectra were recorded on Varioskan Flash plate reader (ThermoFisher).

II. Synthetic Procedures

II.1. Safety notes concerning azides

In the preparation of azides by reaction of azide ion with organic electrophile, care should be taken to keep the aqueous reaction or workup solutions non-acidic to avoid the generation of volatile, toxic, and explosive HN₃. Also, chlorinated solvents such as dichloromethane or 1,2-dichloroethane should be avoided to eliminate the chance for azide ion to displace chloride and produce dangerous small-molecule azides such as CH₂(N₃)₂. These compounds are low boiling liquids that condense in cold traps and may detonate upon warming. Unfortunately, several procedures in the literature call for dichloromethane as a solvent in reactions with sodium azide: such procedures should be avoided. (When used to separate organic azides from azide ion in aqueous extractions, chlorinated solvents are fine.) Small organic azides such as the compound prepared below should only be handled away from solvent when absolutely necessary, and should never be stored neat.

Azide ion should be quenched by nitrosation,² as follows (with thanks to Prof. Valery V. Fokin).

¹ Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, 62, 7512–7515.

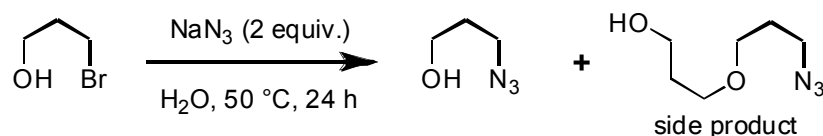
². (a) Clusius, K.; Effenberger, E. *Helv. Chim. Acta.* **1955**, 38, 1843. (b) Stedman, G. *J. Chem. Soc.* **1960**, 1702, and references therein.



In a fume hood, a stirred aqueous solution containing no more than 5% azide ion is treated with sodium nitrite (approximately 7 mL of 20% aqueous NaNO_2 per gram NaN_3 , representing a 40% excess). Aqueous sulfuric acid (20% solution) is then added slowly with stirring until the reaction mixture is acidic. Note that it is important for sodium nitrite to be introduced first. When the evolution of nitrogen oxides ceases (toxic – keep this reaction in the hood), the acidic solution is tested with starch iodide paper. If it turns blue, excess nitrite is present, and the decomposition is complete.

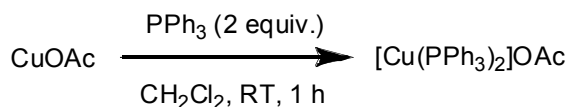
Sodium azide can be detected in solution as follows. A drop of the solution is placed on a plate and is treated with 1-2 drops of dilute HCl . A drop of ferric chloride (FeCl_3) solution is added and the spot plate gently heated. A red color indicates the presence of hydrazoic acid and thus the presence of sodium azide in the solution. A piece of filter paper wetted with dilute FeCl_3 solution can be used to detect hydrazoic acid in the head space above a reaction.

II.2. Synthesis of THPTA ligand.



Caution: the use of a blast shield is recommended while handling neat alkyl azides. No complications have been encountered when working on the indicated scale.

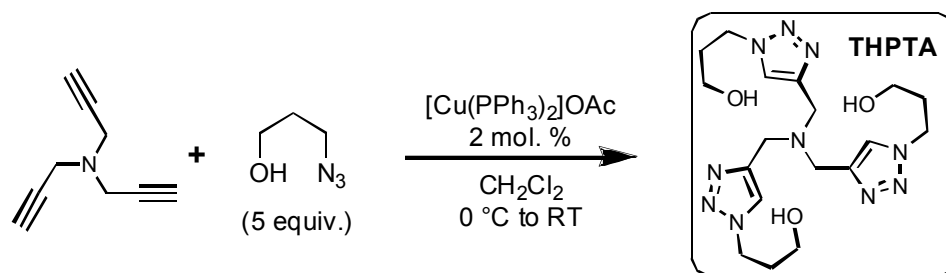
3-Bromo-1-propanol (20.0 g, 144 mmol) was dissolved in deionized water (150 mL) and sodium azide (18.7 g, 288 mmol, 2 equiv.) was added in one portion. The contents were heated to 50 °C and stirred for 24 hours. After cooling, the mixture was extracted with CH_2Cl_2 (3×150 mL). The combined organic fractions were dried (MgSO_4), filtered, and concentrated. The resulting liquid was purified by fractional distillation under vacuum (bp 62 °C at 3–4 mbar³) to yield 12.4 g (85%) of 3-azidopropan-1-ol as a clear, colorless oil. The distillation step is essential to eliminate the 3-(3-azidopropoxy)propan-1-ol side product.



Solid Cu(I) acetate (1.0 g, 8.2 mmol) and triphenylphosphine (4.3 g, 16.3 mmol, 2 equiv.) were combined in a two-necked round-bottomed flask, capped with a gas/vacuum inlet valve and a septum. (*Note: The cuprous acetate should be a yellow-brown solid; material with any shade of blue will not give good results.*) After evacuating and backfilling the flask with nitrogen, dry dichloromethane (50 mL) was injected. The contents were stirred for 1 hr at room temperature under nitrogen, and the reaction remained brown and turbid the entire time. The contents were filtered over packed Celite to give a clear and colorless filtrate. This solution was immediately poured into diethyl ether (400 mL), and the product was crystallized by gently scraping the bottom of the beaker with a spatula. The flask was stored at –20° C overnight, and the resulting product was isolated by filtration and dried under

³ Van Camp, W.; Germonpré, V.; Mespouille, L.; Dubois, P.; Goethals, E. J., Du Prez, F. E. *React. Funct. Polym.* **2007**, 67, 1168–1180.

vacuum to yield 4.0 g (76% yield) of colorless crystals.



Tripropargylamine (4.00 g, 30.5 mmol, Petra Research, Inc.) and 3-azido-1-propanol (12.3 g, 122 mmol, 4 equiv.) were combined in dry CH_2Cl_2 (75 mL). Bis(triphenylphosphine)copper(I) acetate (197 mg, 305 μmol) was added as a solid while stirring under nitrogen. The reaction was cooled using an ice-water bath and stirred under nitrogen for 6 hours. *Caution: the reaction has been observed to violently exotherm due to auto-acceleration after 2 hours if not cooled.* The mixture was then allowed to warm to room temperature with vigorous stirring overnight under nitrogen. After approximately 10 h, the biphasic reaction was charged with additional 3-azido-1-propanol (3.10 g, 30.5 mmol) and bis(triphenylphosphine)copper (I) acetate (197 mg, 305 μmol) and stirred vigorously for an additional 24 h. Dichloromethane (50 mL), water (50 mL), and saturated aqueous disodium ethylenediaminetetraacetic acid (EDTA) (50 mL) were added to the reaction and the contents were transferred to a separatory funnel. The mixture was washed four times with dichloromethane. The combined aqueous layers were frozen and dried by lyophilization overnight. The resulting blue/green solid was dissolved in a minimal amount of a 80/20 $\text{CH}_2\text{Cl}_2/\text{MeOH}$ mixture and passed over a small column of neutral alumina (>150 g) eluting with 300-500 mL of the same solvent mixture. The eluting solution was concentrated to *ca.* 50 mL, poured into diethyl ether (500 mL), and placed in the freezer for several hours. The precipitate was filtered, dried in a vacuum oven for 1 hour at 40 degrees, and subsequently further dried in a lyophilizer overnight to yield 12.8 g (94% yield) of THPTA as a white solid. The spectral properties of this material matched those reported previously;⁴ mp 82-85 $^\circ\text{C}$.

The importance of 3-azidopropanol distillation is evident from the ESI-MS monitoring of the click reaction to generate the THPTA ligand. Using pure 3-azidopropanol, only THPTA peaks are observed (Fig. S1A: $[\text{M}+\text{H}]^+$, 435.3; $[\text{M}+\text{Na}]^+$, 457.3). Without distillation of the azide component, 3-(3-azidopropoxy)propan-1-ol is incorporated into the *tris*-triazole product (Fig. S1B). The resulting *trsi*-triazole is quite competent as an accelerating ligand, but of course it is more difficult to determine precise molar quantities of the mixture. Still, when used in excess as recommended for bioconjugation,⁴ such precision is not usually required.

⁴ Hong, V.; Presolski, S.I.; Ma, C.; Finn, M.G. *Angew. Chem. Int. Ed.* **2009**, 48, 9879-9883.

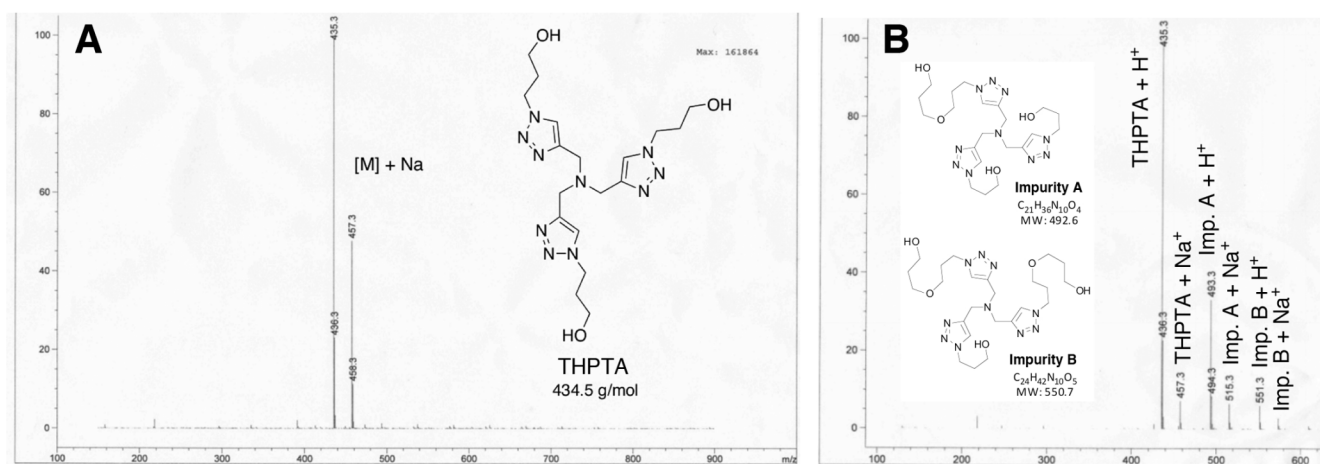
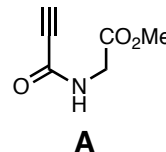


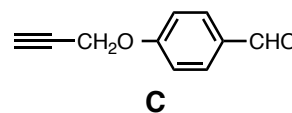
Figure S1. ESI-MS spectra of THPTA obtained using distilled (A) and undistilled (B) 3-azidopropanol.

II.3. Synthesis of alkynes. Alkynes **D**, **G–M** are commercially available. Alkyne **F** was obtained from a known procedure.⁵

Methyl 2-propiolamidoacetate (alkyne A). A solution of dicyclohexyl carbodiimide (1M in THF, 5 mL, 5 mmol) was added dropwise over 1 minute to a chilled (acetone–ice, external temperature -5 ± 0 °C) solution of propiolic acid (350 mg, 5.0 mmol) in THF (10 mL). The resulting suspension was stirred for 5 minutes. In a separate vial, methyl glycinate hydrochloride (628 mg, 5.0 mmol) was dissolved in aqueous Na_2CO_3 (1 M, 5 mL) and extracted with EtOAc (2×5 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and added to the solution of activated acid dropwise over 5 minutes. Once the addition of amine was complete, the cooling is discontinued and the reaction mixture was allowed to warm to 23 °C over 30 minutes. The precipitated dicyclohexylurea (0.6 g) was filtered, washed with EtOAc (2×5 mL), and the combined filtrates were concentrated to *ca.* 5 mL. This solution was placed in a freezer (-20 °C) overnight. Additional dicyclohexylurea was removed by filtration, washed with EtOAc (2×3 mL), and concentrated. The resulting orange-brown oil was purified by flash chromatography (25 to 60% EtOAc/hexanes) to yield 170 mg (24%) of **A**. Yellow oil, solidifies on storage at 4 °C. R_f 0.19 (50% EtOAc/hexanes). ESI-MS: $[\text{C}_6\text{H}_7\text{NO}_3 + \text{Na}]^+$ 164.0. ^1H NMR (500 MHz, CDCl_3) δ 6.51 (s, 1H), 4.09 (d, $J = 5.3$ Hz, 2H), 3.78 (s, 3H), 2.86 (s, 1H). ^{13}C NMR (126 MHz, CDCl_3) δ 169.5, 152.1, 99.7, 77.2, 74.4, 52.8, 41.5.



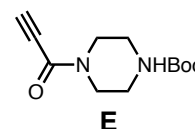
4-Propargyloxybenzaldehyde (alkyne C). A suspension of 4-hydroxybenzaldehyde (7.98 g, 65.3 mmol) and K_2CO_3 (16.2 g, 117 mmol) in DMF (65 mL) was treated with propargyl bromide (80 wt% in PhMe, 9.35 g, 62.8 mmol) and stirred at 60 °C for 3 hours. Water (260 mL) was added in one portion. The precipitated product was washed with water (50 mL) and 50% aqueous EtOH (20 mL) and dried in air, followed by drying under high vacuum to a constant mass to yield **C** (8.75 g, 87%). Colorless solid, R_f 0.56 (25% EtOAc/hexanes). ESI-MS: $[\text{C}_{10}\text{H}_8\text{O}_2 + \text{K}]^+$ 199.1. Alkyne **B** was obtained under similar conditions as an oil (after extraction with ether and solvent evaporation). The NMR spectra matched those reported previously.⁶



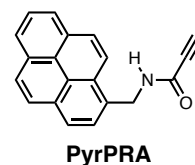
⁵ Abedin, M. J.; Liepold, L.; Suci, P.; Young, M.; Douglas, T. *J. Am. Chem. Soc.* **2009**, *131*, 4346–4354

⁶ Bateman, S. A.; Kelly, D. P.; Martin, R. F.; White, J. M. *Aust. J. Chem.* **1999**, *52*, 291–301

Tert-butyl 4-propiolylpiperazine-1-carboxylate (alkyne **E**). A solution of dicyclohexyl carbodiimide (1M in THF, 20 mL, 20 mmol) was added dropwise over 1 minute to a chilled (acetone–ice, external temperature -5°C) solution of propiolic acid (1.57 g, 22.5 mmol) in CH_2Cl_2 (100 mL). The resulting suspension was stirred for 5 minutes, and a solution of *t*-butyl piperazine-1-carboxylate (3.70 g, 20.0 mmol) in CH_2Cl_2 (10 mL) was added dropwise over 5 minutes. Once the addition of amine was complete, the cooling was discontinued and the reaction mixture was allowed to warm to 23°C over 30 minutes. The precipitated dicyclohexylurea (2.9 g) was filtered, washed with EtOAc (2×10 mL), and the combined filtrates were concentrated to *ca.* 10 mL. This solution was placed in a freezer (-20°C) overnight. Additional dicyclohexylurea (1.2 g) was removed by filtration, washed with EtOAc (2×5 mL), and solvent was removed from the combined filtrates by rotary evaporation. The resulting orange-brown oil was purified by flash chromatography (10 to 50% EtOAc/hexanes) to yield **E** as an off-white solid (2.20 g, 46%). R_f 0.35 (50% EtOAc/hexanes). ESI-MS: $[\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_3 + \text{Na}]^+$ 261.1. ^1H NMR (400 MHz, CDCl_3) δ 3.73 – 3.67 (m, 2H), 3.61 – 3.53 (m, 2H), 3.48 – 3.36 (m, 4H), 3.16 (s, 1H), 1.43 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 154.4, 152.0, 80.6, 79.9, 75.2, 46.8, 41.4, 28.4.



N-(pyren-1-ylmethyl)propiolamide (**PyrPRA**). A suspension of 1-(aminomethyl)pyrene hydrochloride (75.2 mg, 95% purity, 0.267 mmol) and propiolic acid (20.5 mg, 0.293 mmol) in THF (2 mL) was treated sequentially with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (56 mg, 0.293 mmol) and Et_3N (81 mg, 0.8 mmol). The resulting mixture was stirred for 3 hours at room temperature and treated with 1% aqueous HCl (15 mL) to precipitate the product. The suspension was allowed to settle overnight and the solids were recovered by centrifugation. The resulting brown solid was purified by flash chromatography (0 to 25% EtOAc/ CH_2Cl_2) to give **PyrPRA** as a colorless solid (10.5 mg, 15%). R_f 0.39 (50% EtOAc/heptane). ESI-MS: $[\text{C}_{20}\text{H}_{13}\text{NO} + \text{Na}]^+$ 306.1. ^1H NMR (500 MHz, CDCl_3) δ 8.22 – 8.12 (m, 5H), 8.09 – 8.01 (m, 3H), 7.94 (d, $J = 7.8$ Hz, 1H), 6.24 (s, 1H), 5.17 (d, $J = 5.5$ Hz, 2H), 2.77 (s, 1H). ^{13}C NMR (126 MHz, CDCl_3) δ 151.9, 131.7, 131.4, 130.8, 129.7, 129.2, 128.7, 127.9, 127.4, 126.4, 125.73, 125.66, 125.2, 125.0, 124.8, 122.6, 73.8, 42.4.



Bis(2-methylbut-3-yn-2-yl) 3'-oxo-3'H,10H-spiro[anthracene-9,1'-isobenzofuran]-3,6-diyl-dicarbamate (**Ynoc₂Rho**). Rhodamine 110 (25 mg, 0.07 mmol) and propargyl 4-nitrophenylcarbamate⁷ **5** (90 mg, 0.35 mmol) were dissolved in dry pyridine (2 mL) under argon and heated at 100°C overnight. The resulting mixture was allowed to cool to room temperature and solvent was removed. The resulting solid was purified by preparative TLC with 40% EtOAc/hexanes as the eluting solvent to obtain the desired product as a white solid (6.2 mg, 16%). ^1H -NMR (400 MHz, $\text{DMSO}-d_6$) δ (ppm) 10.07 (s, 2H), 8.00 (d, $J = 7.4$ Hz, 1H), 7.78 (td, $J = 7.4, 1.2$ Hz, 1H), 7.71 (td, $J = 7.4, 1.0$ Hz, 1H), 7.56 (d, $J = 2.0$ Hz, 2H), 7.27 (d, $J = 7.5$ Hz, 1H), 7.14 (dd, $J = 8.7, 2.1$ Hz, 2H), 6.70 (d, $J = 8.7$ Hz, 2H), 3.52 (m, 2H), 1.65 (s, 12H). ^{13}C -NMR (125 MHz, $\text{DMSO}-d_6$) δ (ppm) 168.5, 152.9, 152.3, 150.8, 141.3, 135.5, 132.9, 130.0, 128.3, 125.6, 124.6, 123.8, 117.6, 114.3, 112.3, 105.0, 81.8, 59.6, 28.8. ESI-MS: $[\text{C}_{32}\text{H}_{26}\text{N}_2\text{O}_7 + \text{H}]^+$ 551.2.



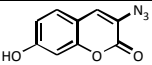
⁷ Bertrand, P.; Gesson, J. P. *J. Org. Chem.* **2007**, 72, 3596-3599.

III. Kinetic Studies

III.1. Reactions with fluorogenic coumarine azide (Figure 1).

Typical reaction conditions are detailed in **Table S1** (components listed in the order of addition). The reactions were performed in standard 96-well plates with fluorescence measurements made at regular time intervals on a Varioskan Flash plate reader (λ_{exc} 404 nm, λ_{em} 475 nm, excitation slit width 12 nm, measurement time 100 ms). All reactions progressed to >80% conversion, as evidenced by the observation of little or no increase of fluorescence (0–20%) upon the addition of superstoichiometric amounts of fresh CuSO_4 /THPTA/ascorbate. The absolute magnitude of the fluorescence intensity at completion was similar in each case to that of an equivalent concentration of the pure triazole derived from coumarin azide and propargyl alcohol.

Table S1. Conditions for “bioconjugative” LACuAAC with coumarin azide.

Stock solution (c, solvent)	V, μL	Final conc., μM
Alkyne (40 mM, DMSO)	0.4	160
5:1 THPTA: CuSO_4 (2.5 mM Cu^{2+} , H_2O),	0.4	10
 0.133 mM, 50 mM HEPES, pH 7.4 freshly prepared	75	100
Sodium ascorbate, prepared fresh immediately before the reaction (2 g/L, ~10 mM, H_2O). Added to all wells at once using a multichannel pipette.	24.2	2500
	total 100 μL	37.5 mM HEPES, pH 7.4, 0.8% DMSO

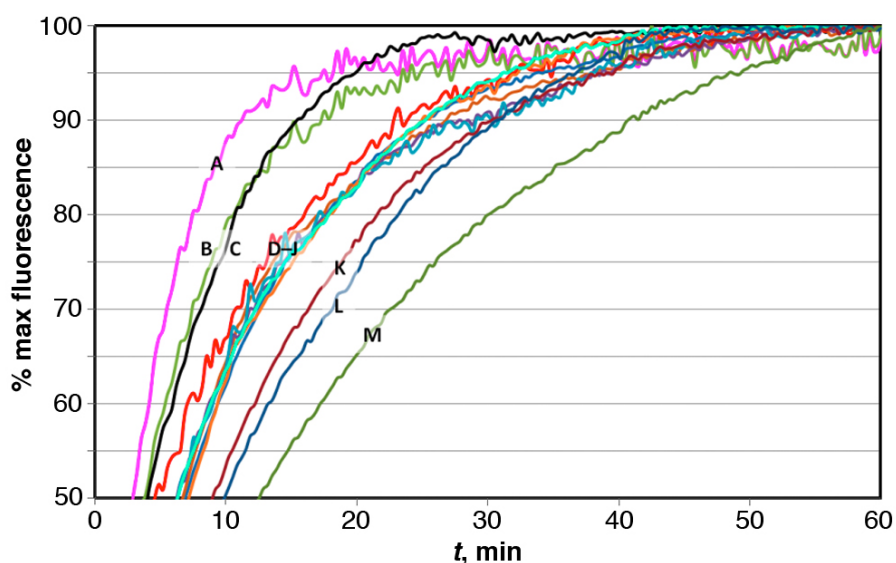


Figure S2. Kinetic traces of the reactions in Fig. 1, normalized to maximum fluorescence achieved within 1 hour of reaction with each alkyne. Conditions: (0.1 mM coumarin azide, 0.16 mM alkyne, 0.01 mM CuSO_4 , 0.05 mM THPTA, 2.5 mM NaAsc).

III.2. Competition Experiments (Figure 2).

To acetone solutions of propiolamide **E** (0.05 mL, 400 mM), a competing alkyne (0.05 mL, 400 mM), and benzyl azide (0.05 mL, 400 mM) was added a solution of copper sulfate and **BImPy**₂ ligand in DMSO (0.01 mL, 10 mM in Cu, 2:1 Cu:L), followed by a freshly prepared aqueous solution of sodium ascorbate (0.04 mL, 100 mM). The resulting homogenous solutions were allowed to stand at room temperature for 1h. The reaction mixtures were diluted with water (0.8 mL) and extracted with chloroform (0.5 mL). The chloroform extracts were concentrated on a vacuum centrifuge at room temperature, redissolved in CDCl₃ and analyzed by ¹H NMR. The triazole products are expected to partition quantitatively to the organic phase, and not expected to be volatile, ensuring that NMR analysis reflects the product distribution in the aqueous phase. No impurities were observed in the NMR spectra.

III.3. Reactions with fluorogenic alkyne **PyrPRA**.

The reactions with **PyrPRA** were performed as described in **Table S2** (components listed in the order of addition). The reactions were performed in standard 96-well plates with fluorescence measurements taken at regular time intervals on Varioskan Flash plate reader (λ_{exc} 348 nm, λ_{em} 394 and 488 nm, excitation slit width 12 nm, measurement time 100 ms). The formation of triazole product, **PyrTRZ**, was confirmed by ESI-MS ($[\text{C}_{24}\text{H}_{21}\text{N}_5\text{O}_3 + \text{H}]^+$ $m/z = 428.2$) after extracting the reaction mixtures with EtOAc. Precipitation of pyrene-containing materials (punctate blue-green fluorescence) was observed in some cases after 15–20 minutes of reaction.

Table S2. Conditions for “bioconjugative” LACuAAC with **PyrPRA** (Figure 3, main text).

Stock solution (c, solvent)	V, μL	Final conc., μM
PyrPRA (0.5 mM, DMSO)	25	100
Azide: γ -azidohomoalanine or Q β azide (1 mM, H ₂ O)	12.5	100
5:1 THPTA:CuSO ₄ (5 mM Cu ²⁺ , H ₂ O),	2.5	10
Aminoguanidine•HCl (100 mM, H ₂ O)	6.25	5000
50 mM HEPES, pH 7.4	77.15	31000
Sodium ascorbate, prepared fresh immediately before the reaction (40 g/L, ~200 mM, H ₂ O). Added to all wells at once using a multichannel pipette.	1.6	2560
	total = 125 μL	31 mM HEPES, pH 7.4, 20% DMSO

III.4. Reactions with *Ynoc*₂*Rho*

Fluorogenic alkyne **Ynoc₂Rho** is virtually non-fluorescent and stable in aqueous media for at least one month. The reactions with **Ynoc₂Rho** were performed as described in **Figure S3**. The presence of an azide did not change the rate of Ynoc group cleavage. Like CuAAC, azide-independent Ynoc cleavage was inhibited by thiols: no fluorescence increase was observed in the presence of 0.25 mM glutathione.

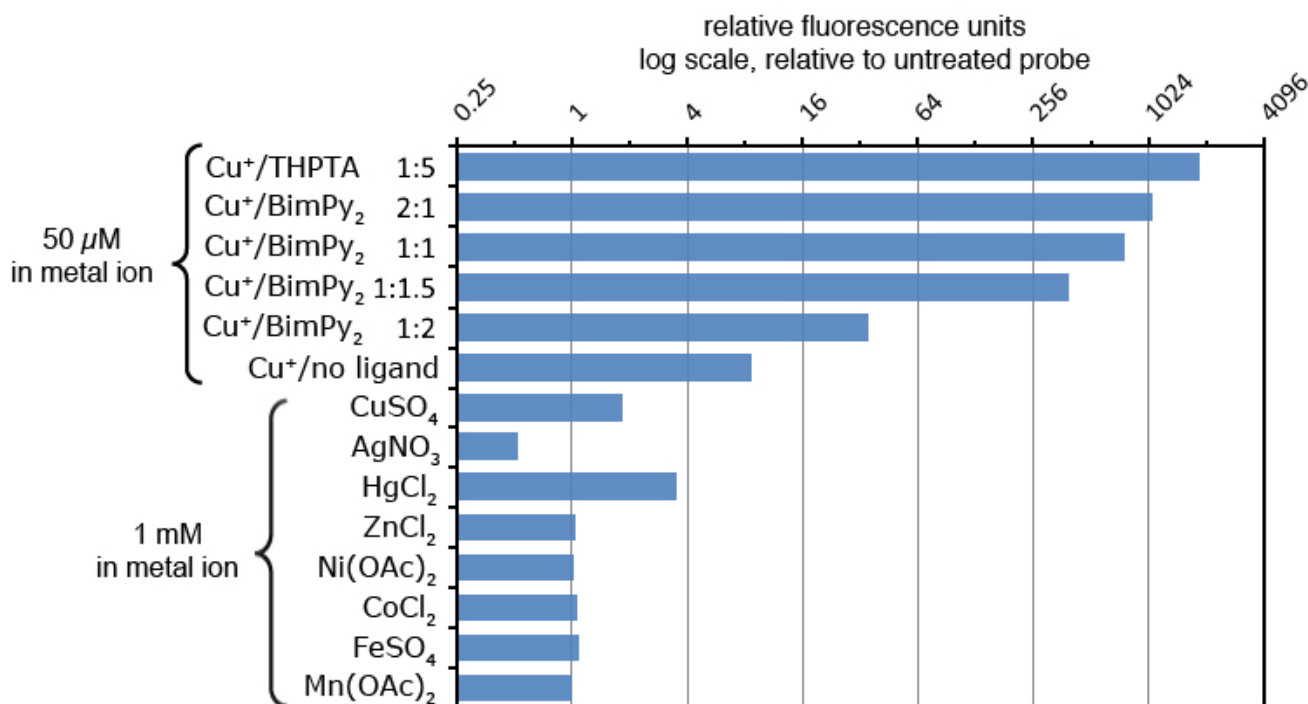


Figure S3. Fluorescence response ($\lambda_{\text{ex}} = 498$ nm, $\lambda_{\text{em}} = 520$ nm) of **Ynoc₂Rho** (50 μ M) to various metal ions in 5% DMSO PBS pH 7.4 buffer at room temperature after 1 hour. Cu(I) solutions were prepared from 50 μ M CuSO₄ + 2.5 mM sodium ascorbate, plus an amount of ligand to achieve the indicated ratio (2:1, for example, means 25 μ M ligand). The addition of 50 μ M CuSO₄ + 250 μ M THPTA + 2.5 mM ascorbate to each of the non-Cu reactions gave approximately the same level of fluorescence as Cu⁺/THPTA alone, showing that excess amounts of these metals do not inhibit the reaction or quench the fluorophore.