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Supporting Information

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Analysis and Optimization of Copper-Catalyzed Azide–Alkyne Cycloaddition for Bioconjugation**

*Vu Hong, Stanislav I. Presolski, Celia Ma, and M. G. Finn**

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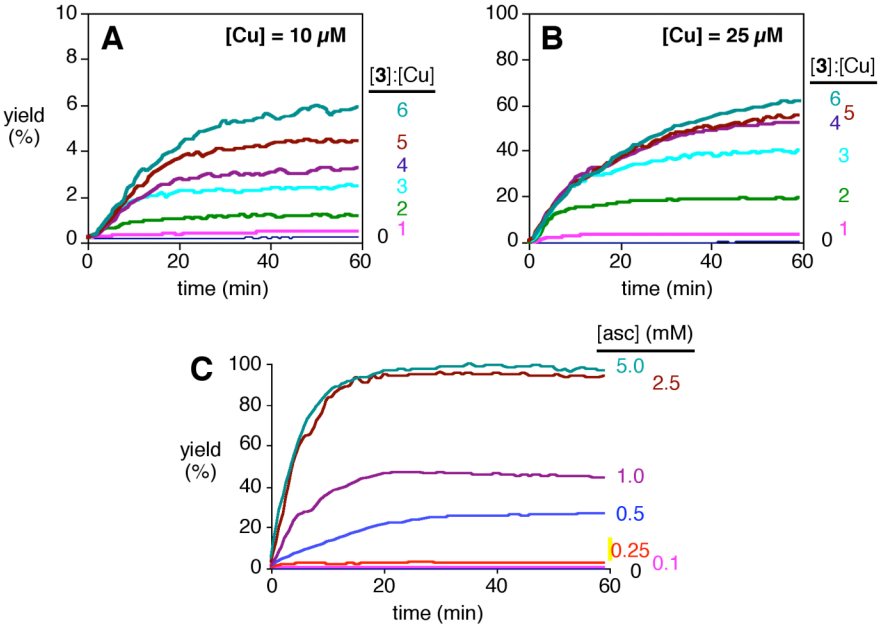
A. Comments on TCEP

We first recommended tris(carboxyethyl)phosphine (TCEP) for CuAAC bioconjugations,[1] and the method has been widely adopted. However, we have found that TCEP slows the reaction rate in some cases and is inferior in small organic molecule, peptide, and protein conjugation reactions.[2] Accordingly, we now discourage the use of TCEP in the great majority of CuAAC applications.

B. Additional data regarding Cu and ascorbate concentrations

Reactions using propargyl alcohol as a prototypical small molecule were optimized using a 96–well fluorescence plate reader (Thermo Scientific Varioskan Flash), which enabled convenient kinetic characterization. Examples of the data produced in this way are shown in Figure 2 and in Figure S1, supporting the discussion in the paper.

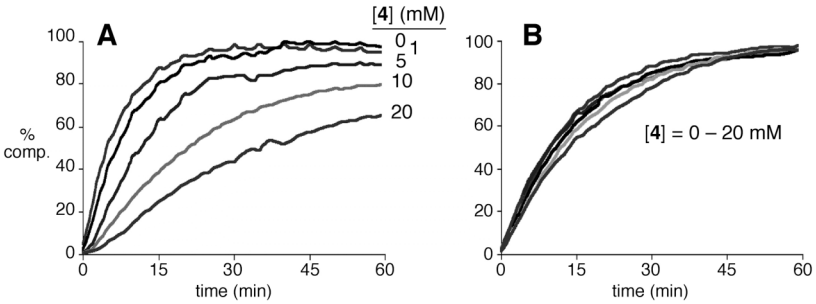
Figure S1.
(A,B) Conversion-*vs*.-time profiles as a function of ligand:Cu ratio and concentration. Conditions: propargyl alcohol (100 µM), **1** (50 µM), $CuSO_4$ and ligand **3** (indicated concentrations), 95:5 0.1 M potassium phosphate buffer (pH 7.0):DMSO, sodium ascorbate (5.0 mM), room temperature. Figure 2 of the main paper continues these plots at higher copper concentrations.
(C) Conversion-*vs*-time as a function of ascorbate concentration, using propargyl alcohol (200 µM), **1** (100 µM), $CuSO_4$ (100 µM), and **3** (500 µM), in 95:5 buffer:DMSO at room temperature.



[1] Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192.
[2] A. Vila, K. A. Tallman, A. T. Jacobs, D. C. Liebler, N. A. Porter, L. J. Marnett, *Chem. Res. Toxicol.* **2008**, *21*, 432.

C. Aminoguanidine (4) does not affect the CuAAC rate very much.

Figure S2. Effect of aminoguanidine (**4**) on the kinetics of the CuAAC reaction of 1 (100 μM) and propargyl alcohol (200 μM). (A) [$CuSO_4$] = 100 μM, [**3**] = 500 μM; (B) [$CuSO_4$] = 500 μM, [**3**] = 2.5 mM, room temperature. For both, solvent = 1:9 DMSO:buffer.



D. Protection against protein crosslinking and aggregation using 3 and aminoguanidine (4)

The ability of **4** to prevent protein crosslinking was assayed using cowpea mosaic virus (CPMV), which we have shown previously to be unstable in the presence of $CuSO_4$ and sodium ascorbate.[1] Size-exclusion chromatography (SEC) was used to detect aggregation or decomposition of the virus particle, as shown in Figure S3. While the particles were stable after 24 hours of incubation with $CuSO_4$ (0.5 mM, not shown) or ascorbate (5 mM) alone (Figure S3A), they were decomposed into component subunits in the presence of both copper and ascorbate (Figure S3B), a process that is induced by irreversible aggregation of the particles. The addition of ligand **3** at a 5:1 ligand:Cu diminished but did not eliminate this decomposition. SEC analysis showed broadening of the protein peak, an indication of partial aggregation and virus disassembly (Figure S3C). Aminoguanidine (5 mM) completely rescued the particles from aggregation, presumably by preventing crosslinking of protein subunits (Figure S3D).

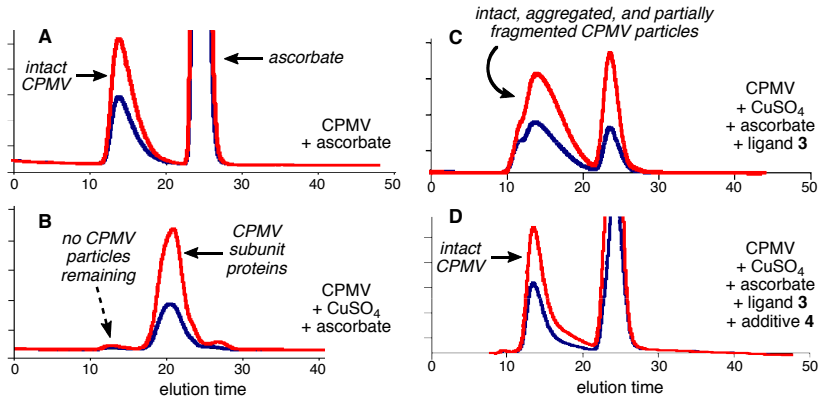


Figure S3. Size-exclusion fast protein liquid chromatography (Superose 6) on CPMV (1 mg/mL) samples incubated with the indicated reagents for 24 h at room temperature in 0.1 M potassium phosphate buffer, pH 7. Concentrations: $CuSO_4$, 0.5 mM; sodium ascorbate, 5 mM; ligand **3**, 2.5 mM; additive **4**, 5 mM. Red traces show absorbance at 260 nm, blue show absorbance at 280 nm.

E. HPLC analysis of RNA derivatives.

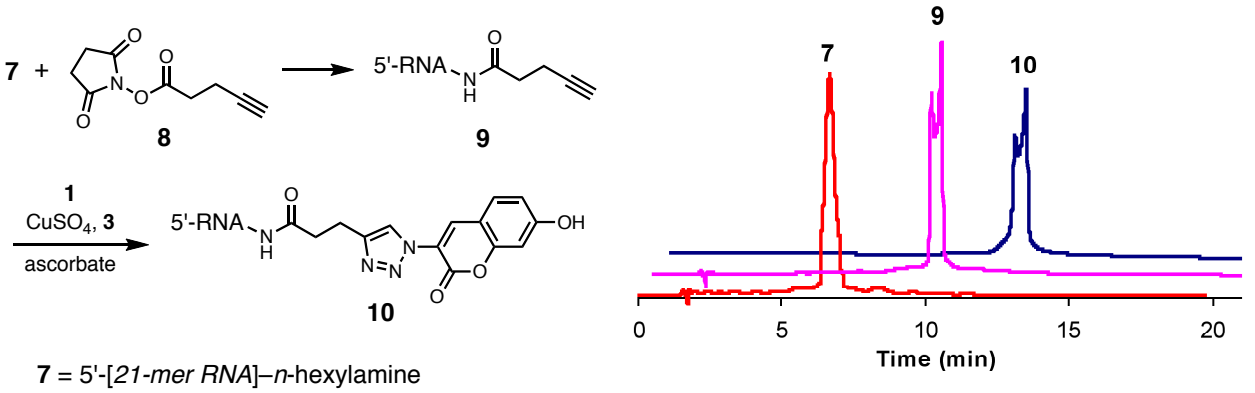
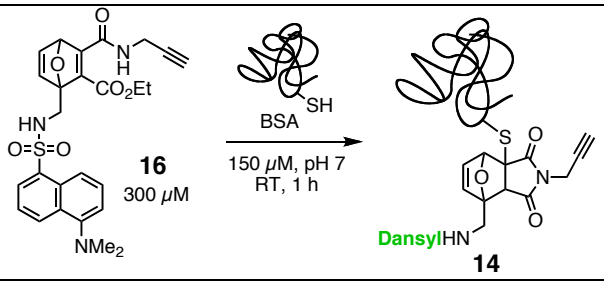


Figure S4. HPLC traces for the RNA modification by CuAAC reaction. Agilent Zorbax Bio Series Oligo column eluted with 20% B to 50% B in 40 min (A: 20% Acetonitrile, 80% 0.02 M sodium phosphate monobasic pH 7.0; B: A + 1.0 M NaCl).

F. Preparation of 14.

Figure S5. Schematic representation of the condensation reaction performed with BSA and oxanorbornadiene electrophile **16** in 0.1 M phosphate buffer, containing 10% DMSO. The dansyl group becomes fluorescent upon conjugation.[3] The product was purified by size-exclusion chromatography to remove excess **16**.



G. Notes concerning competitive Cu binders in solution or on the substrates

To investigate cases in which the protein substrate contains a copper-binding motif, we expressed the 25-kD N-terminal aspartate dipeptidase PepE[4] with a hexahistidine sequence fused to the C-terminus. This protein at 70 μM was found to inhibit the reaction of **1** with propargyl alcohol in the presence of 100 μM Cu and 500 μM ligand **3**. Increasing the concentration of the Cu•**3** mixture to 400 μM overcame this problem (Figure S6A). An additional adjustment can be the addition of Ni or Zn salts to occupy the metal-binding motif, freeing up more of the cuprous catalyst, as shown in Figures S6B and S6C. In each case, the CuAAC reaction rate in the presence of 100 μM Cu increased with concentration of added Ni^{II} or Zn^{II}, with nickel being the more effective. Note that, in the absence of copper, neither Ni nor Zn accelerates the cycloaddition reaction at all.

[3] V. Hong, A. A. Kislukhin, M. G. Finn, *J. Am. Chem. Soc.* **2009**, *131*, 9986
[4] Lassy, R.A.; Miller, C.G. *J. Bacter.* **2000**, *182*, 2536-2543.

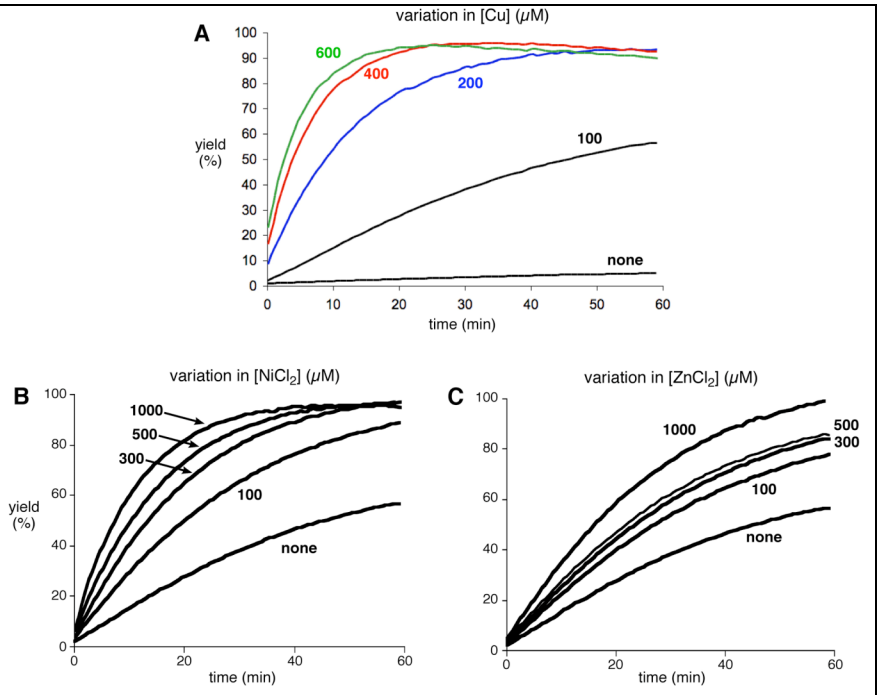
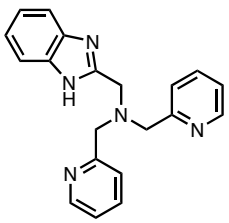


Figure S6. Reaction of azide **1** (100 μM) with propargyl alcohol (200 μM) in the presence of His_6-tagged peptidase E (70 μM) under standard conditions (95:5 buffer:DMSO, 2.5 mM sodium ascorbate, pH 7.0, room temperature). Yields were determined by taking the ratio of the fluorescence of product **2** produced during the reaction relative to the fluorescence of 100 μM **2** under identical conditions. (A) Variation in catalyst concentration. The concentration of $CuSO_4$ is shown; the [THPTA]:[Cu] ratio was kept at 5 for all reactions.
(B) and (C) Addition of the indicated concentrations of $NiCl_2$ or $ZnCl_2$ as competing His_6-binding metal ions, in the presence of 100 μM $CuSO_4$ and 500 μM **3**.

If more than 40% DMSO is used in the aqueous mixture, or if the alkyne concentration is greater than 5 mM, a different ligand should be used. We currently recommend ligand **BimPy$_2$** shown here, [5] but others are being developed. In reaction mixtures dominated by water, the ligand:Cu ratio should be 0.5; with increasing concentrations of donor solvents such as DMSO, the ratio should be increased to a maximum of 2:1. This type of ligand will probably be effective for at least some cases in which the substrate binds Cu, as in the histidine tag example above, but we have not yet fully explored this option. We do not recommend **BimPy$_2$** for routine use in bioconjugation because it cannot be employed in large excess relative to copper, and so is less efficient at suppressing oxidative damage.



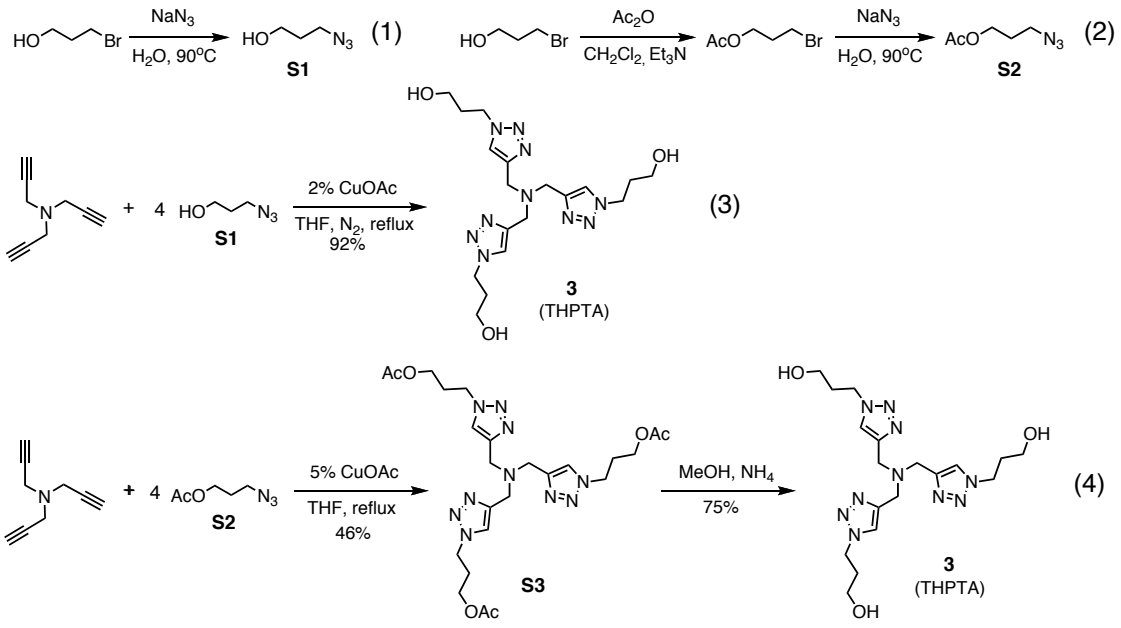
BimPy$_2$

[5] Presolski, S.I.; Hong, V.; Finn, M.G., "Mixed Heterocyclic Ligands for Cu-Catalyzed Azide-Alkyne Cycloaddition," manuscript in preparation.

H. Improved syntheses of ligand 3

Ligand **3** is difficult to cleanly assemble by CuAAC reaction of tripropargylamine with 3-azidopropanol in solvent systems containing a substantial amount of water because the bis(triazole) intermediate is a potent inhibitor of the reaction under these conditions. We describe this and related phenomena elsewhere,[5] but note here that such inhibition does not occur in organic solvents. Accordingly, reactions using Cu^{I} salts in organic solvents offer a practical route to this class of ligands. We are grateful to Dr. Jason Hein and Prof. Valery V. Fokin of The Scripps Research Institute for suggesting cuprous acetate and THF as a felicitous combination.

Two practical routes to the ligand are illustrated below. 3-Azido-1-propanol (**S1**) was prepared by azide displacement on the precursor bromide (Eq. 1) and was isolated by extraction into organic solvent and concentrated by rotary evaporation of a portion of the solvent at room temperature. **[*Caution!* Small-molecule azides should not be isolated away from solvent, as by distillation, precipitation, or recrystallization.** In this case, compounds S1 and S2 were freed of solvent by rotary evaporation at room temperature or below, weighed to determine the approximate yield, and immediately redissolved in solvent for storage. One can also determine approximate concentrations in many solvents by quantitative NMR against a known amount of added internal standard. ***Caution!* Workup of reactions involving inorganic azide should avoid acid, as HN_3 is volatile, explosive, and highly toxic.]** CuAAC reaction with tripropargylamine, using a slight excess of **S1** in THF provided **3** in excellent yield (Eq. 3). However, the material was slightly contaminated with impurities that were very difficult to remove by recrystallization or chromatography. The solid obtained from this procedure is fully active as a ligand for all of the reactions described in this paper. If highly pure material is desired, the acetate **S2** can be used. Reaction with tripropargylamine in THF provided the organic-soluble **S3**, which can be readily purified by column chromatography. Acetate hydrolysis by methanolic ammonia, followed by evaporation of all of the volatile components, provided highly pure **3** directly.



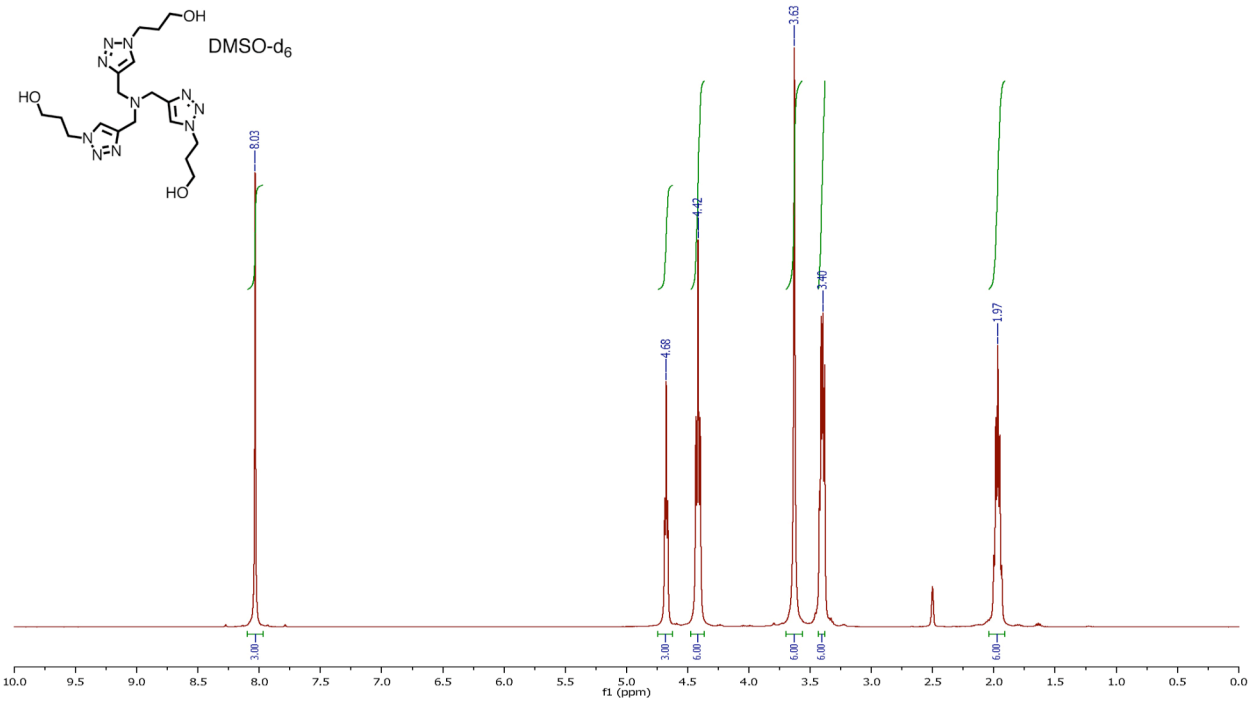
*3-Azido-1-propanol (**S1**).* 3-Bromo-1-propanol (20 g, 144 mmol) and sodium azide (18.7 g, 288 mmol) were dissolved in water (150 mL) and the resulting solution was stirred at 90°C overnight. The mixture was extracted with dichloromethane (3 x 150 mL). The combined organic layers were dried over $MgSO_4$ and concentrated by rotary evaporation under reduced pressure using a bath kept at room temperature, to obtain 3-azido-1-propanol **S1** as pale yellow oil (11.9 g, 82%). After weighing, the oil was immediately dissolved in toluene to make a concentrated stock solution. In this and the synthesis of **S2** described below, heating at 90°C is almost certainly more strenuous than necessary; stirring at room temperature or with only mild heating is probably sufficient, but this was not tested at the reported scale.

*Tris(3-hydroxypropyltriazolylmethyl)amine **3** (Eq. 3).* To a stirred solution of tripropargylamine (27 mmol) and **S1** (108 mmol, 4.0 equiv) in THF (90 mL) under N_2 was added CuOAc (cuprous acetate, 2 mol%) and the resulting solution was refluxed overnight under inert atmosphere. The mixture was concentrated, dissolved in 50 mL of water, and stirred with Cuprisorb resin (2-3 g) to remove the copper ions. The solution was filtered, the solid washed, and the combined solutions concentrated under vacuum to provide a yellow oil which solidified under high vacuum. The resulting yellow solid was dispersed in acetonitrile, sonicated to further break up the solid, filtered, washed with acetonitrile, and dried under vacuum to yield **3** (10.8 g, 92%) as an off-white solid. ***Note**:* CuOAc must be a white solid; old samples are often a blue-green color, which indicates the partial oxidation of the salt, rendering it inactive unless a reducing agent is also used.

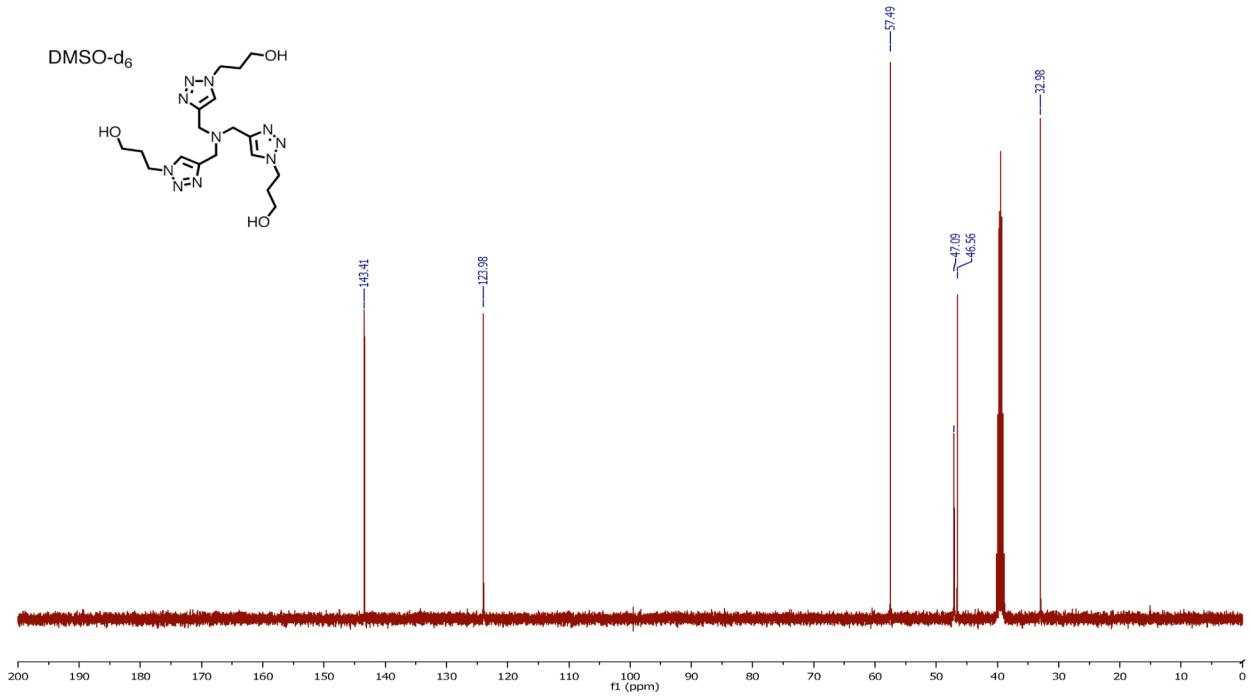
*3-Azidopropyl acetate (**S2**).* A mixture of Ac_2O (7.4 g, 72 mmol) and Et_3N (7.3 g, 72 mmol) was added to 3-bromo-propanol (10 g, 72 mmol) dissolved in CH_2Cl_2 (60 mL) and stirred at room temperature for an hour. An aqueous solution of $NaHCO_3$ was added and the phases were separated. The organic layer was washed once more with aqueous $NaHCO_3$ and twice with brine. After drying with $MgSO_4$, the solvent was removed to obtain 3-bromopropyl acetate as colorless oil. Water (100 mL) and NaN_3 (144 mmol) were added and the resulting solution was stirred at 90°C overnight. The mixture was extracted with dichloromethane (3 x 100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to yield 3-azidopropyl acetate **S2** as pale yellow oil (6.5 g, 63% over two steps).

*Tris(3-hydroxypropyltriazolylmethyl)amine **3** (Eq. 4).* To a stirred solution of trispropargylamine (13 mmol), 3-azidopropyl acetate (4 equiv, 52 mmol) in THF (90 mL) under N_2 was added CuOAc (5 mol %, see the note above regarding its purity) and the resulting solution was refluxed overnight under inert atmosphere. The mixture was concentrated, dissolved in 50 mL of water, and stirred with Cuprisorb resin (2-3 g) to remove the copper ions. The solution was filtered, concentrated, and purified by flash chromatography (silica, 0-5% MeOH/CH_2Cl_2) to obtain **S3** (3.4 g, 46%) as pale yellow oil. This material was treated with ammonia in MeOH (2 M, 40 mL) and the mixture was stirred at 40°C overnight. The solution was concentrated and the residue dried under high vacuum. The resulting pale yellow solid was dispersed in acetonitrile, sonicated to further break up the solid, filtered, washed with acetonitrile, and dried under vacuum to yield **3** (2.0 g, 76%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.03 (s, 3H), 4.68 (t, J = 4.95 Hz, 3H), 4.42 (t, J = 7.00 Hz, 6H), 3.63 (s, 6H), 3.40 (m, 6H), 1.97 (m, 6H). Water contributes to the intensity of the peak at 3.4 ppm in this sample.



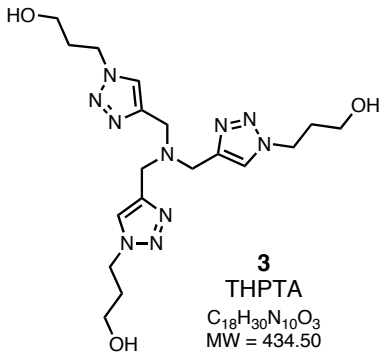
^{13}C NMR (101 MHz, DMSO-d_6) δ 143.4, 124.0, 57.5, 47.1, 46.6, 33.0.



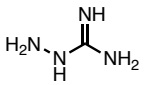
Protocol for CuAAC Coupling of "Cargo-N_3" to "Biomolecule-CCH"

Stock solutions:

$CuSO_4$: 20 mM (in water)
Ligand **3**: 50 mM (in water)
Sodium ascorbate: 100 mM (make fresh before using by adding 1 mL of water to 20 mg).
Aminoguanidine hydrochloride (**4**): 100 mM (add 1 mL of water to 11 mg.)
Cargo-azide: 20 mM
Biomolecule-alkyne: 20 mg/mL
Buffer: 100 mM phosphate pH 7



3
THPTA
$C_{18}H_{30}N_{10}O_3$
MW = 434.50



4
aminoguanidine
CH_6N_4
MW = 74.0851

Final concentrations:

$CuSO_4$: 0.10 mM (Note: can be adjusted as desired between 50 and 250 μM)
Ligand **3**: 0.50 mM (*ligand to copper ratio is 5:1*)
Sodium ascorbate: 5 mM
Aminoguanidine: 5 mM
Cargo-azide: approx. 2-fold excess with respect to alkyne groups on the biomolecule, down to 20 μM (in other words, if the alkyne concentration is very low, more than two equivalents of azide are needed for fast reaction)
Biomolecule-alkyne: successfully done with 2 μM and higher

Procedure for 0.5 mL reactions: (this example: 200 μM alkyne and 400 μM azide)
In a 2 mL eppendorf tube, *add the reagents in the following order*:

1. 25 μL of Biomolecule-alkyne
2. 407.5 μL of 100 mM phosphate buffer pH 7
3. 10 μL of Cargo-azide
4. 7.5 μL of premixed $CuSO_4$ and **3** (2.5 μL of $CuSO_4$ and 5.0 μL of **3**).
5. 25 μL of aminoguanidine.
6. 25 μL of sodium ascorbate.
7. Mix well, close the tube (to prevent more oxygen from diffusing in), and let the reaction go for an hour.
8. Workup depends on your application. Copper ions can be removed by washing (dialysis) with EDTA. Copper-adsorbing resins tend to also bind biomolecules. We routinely do no workup, but instead purify the conjugates in such a way as to leave small molecules behind.

Notes

1) The same procedure applies equally well to the reversed "polarity" (biomolecule-azide + cargo-alkyne)
2) "Biomolecule" has usually been protein (Qβ capsid, BSA, transferrin), but has also included oligoDNA, oligoRNA, and water-soluble polymer.
3) For purposes of development, "cargo" has been fluorescein, Alexa, or BODIPY dyes, a Gd(DOTA) complex, a selenomethionine derivative, or oligopeptides.
4) Aminoguanidine is included when side reactions between dehydroascorbate and protein side chains (principally arginine) are to be suppressed.
5) THPTA serves the dual purpose of protecting biomolecules from hydrolysis by Cu(II) byproducts, and sacrificially intercepting the radicals and/or peroxides derived from O_2/Cu/ascorbate that oxidize histidine and other residues. An excess of ligand does not dramatically slow the reaction, so you can use more than 5 equivalents if needed.
6) Tris(carboxyethyl)phosphine (TCEP) is not recommended. It is an inhibitor of the reaction and has the potential to react independently with azides.
7) We purify clicked oligonucleotides on NAP-10 (or NAP-25, according the amount) resins, following the protocol from the manufacturer. However, some contamination will still be present if you are trying to purify a reaction mixture containing a very large excess (e.g., greater than 50 equivalents) of a small-molecule reagent. The separation is never perfect: at least 1% of low MW molecules remains in the oligonucleotide fraction. In such cases, it is recommended to precipitate the oligonucleotide before using a NAP column, assuming that the reagent you are trying to get rid of is soluble in the 70% ethanol containing NaOAc that is used to precipitate the oligo.

   Precipitation of DNA: For 1 volume of DNA in aqueous solution, add 0.1 volume of 3M NaOAc (pH = 5.3) and 2.5 to 3 volumes (based on DNA + NaOAc total volume) of 95-100% EtOH (3 volumes for oligos smaller than 30-mers, 2.5 volumes or less for larger oligos.) Chill at -80°C for 1-4 hours. Spin at 13k-14k for 30 minutes. Pour or pipet off the supernatant. Wash with cold 70% ethanol (see below). Dry the pellet in air or in a speedvac, then redissolve in the desired buffer. If the starting DNA solution already had a lot of salt in it, the NaOAc can be omitted. (Certain monovalent cations are supposed to help precipitation, but too much is also not good.) This procedure works in our lab with oligonucleotide concentrations varying from 100 pmol/mL to 50 nmol/mL. Different sizes of pellets are obtained, but all those solutions do yield DNA. A second precipitation from water + NaOAc is often done to remove remaining traces of small molecule contaminants.