

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

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

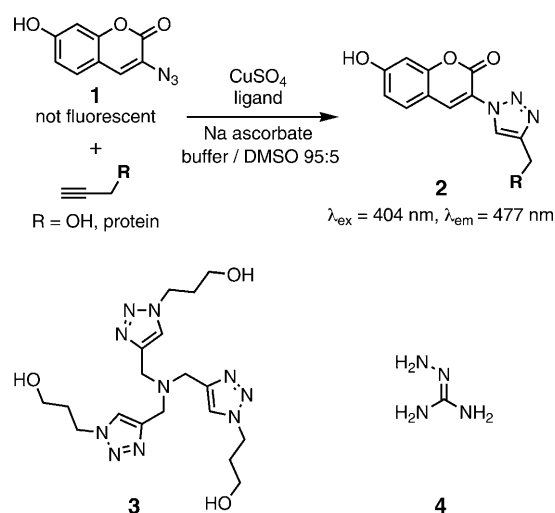
Since its discovery in 2002, the copper-catalyzed azide-alkyne cycloaddition (CuAAC)^[1] reaction—the most widely recognized example of click chemistry^[2]—has been rapidly embraced for applications in myriad fields.^[3] The attractiveness of this procedure (and its copper-free strained-alkyne variant^[4]) stems from the selective reactivity of azides and alkynes only with each other. Because of the fragile nature and low concentrations at which biomolecules are often manipulated, bioconjugation presents significant challenges for any ligation methodology. Several different CuAAC procedures have been reported to address specific cases involving peptides, proteins, polynucleotides, and fixed cells, often with excellent results,^[5] but also occasionally with somewhat less satisfying outcomes.^[6] We describe here a generally applicable procedure that solves the most vexing click bioconjugation problems in our laboratory, and therefore should be of use in many other situations.

The CuAAC reaction requires the copper catalyst, usually prepared with an appropriate chelating ligand,^[7] to be maintained in the Cu^I oxidation state. Several years ago we developed a system featuring a sulfonated bathophenanthroline ligand,^[8] which was optimized into a useful bioconjugation protocol.^[9] A significant drawback was the catalyst's acute oxygen sensitivity, requiring air-free techniques which can be difficult to execute when an inert-atmosphere glove box is unavailable or when sensitive biomolecules are used in small volumes of aqueous solution. We also introduced an electrochemical method to generate and protect catalytically active Cu^I-ligand species for CuAAC bioconjugation and synthetic coupling reactions with minimal effort to exclude air.^[10] Under these conditions, no hydrogen peroxide was produced in the oxygen-scrubbing process, resulting in protein conjugates that were uncontaminated with oxidative byproducts. However, this solution is also practical only for the specialist with access to the proper equipment. Other protocols have employed copper(I) sources such as CuBr for labeling fixed cells^[11] and synthesizing glycoproteins.^[12] In these cases, the instability of Cu^I in air imposes a requirement

for large excesses of Cu (greater than 4 mM) and ligand for efficient reactions, which raises concerns about protein damage or precipitation, plus the presence of residual metal after purification.

The most convenient CuAAC procedure involves the use of an in situ reducing agent. Sodium ascorbate is the reductant of choice for CuAAC reactions in organic and materials synthesis, but is avoided in bioconjugation with a few exceptions.^[13] Copper and sodium ascorbate have been shown to be detrimental to biological^[14] and synthetic^[15] polymers due to copper-mediated generation of reactive oxygen species.^[16] Moreover, dehydroascorbate and other ascorbate byproducts can react with lysine amine and arginine guanidine groups, leading to covalent modification and potential aggregation of proteins.^[6a,17] We hoped that solutions to these problems would allow ascorbate to be used in fast and efficient CuAAC reactions using micromolar concentration of copper in the presence of atmospheric oxygen. This has now been achieved, allowing demanding reactions to be performed with biomolecules of all types by the non-specialist.

For purposes of catalyst optimization and reaction screening, the fluorogenic coumarin azide **1** developed by Wang et al. has proven to be invaluable (Scheme 1).^[18] The progress of cycloaddition reactions between mid-micromolar concentrations of azide and alkyne in aqueous buffers was followed by the increase in fluorescence at 470 nm upon formation of the triazole **2**.



Scheme 1. Top: Reaction used for screening CuAAC catalysts and conditions. Below: Accelerating ligand **3** and additive **4** used in these studies. DMSO = dimethylsulfoxide.

[*] V. Hong, S. I. Presolski, C. Ma, Prof. M. G. Finn
Department of Chemistry and The Skaggs Institute for Chemical
Biology, The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Fax: (+1) 858-784-8850
E-mail: mgfinn@scripps.edu

[**] This work was supported by The Skaggs Institute for Chemical
Biology, Pfizer, Inc., and the NIH (RR021886). We are grateful to
Matthias Park for assistance with the synthesis of **3**.

Supporting information for this article is available on the WWW
under <http://dx.doi.org/10.1002/anie.200905087>.

Ligand/Cu ratio: The results of a survey of known and new tris(heterocycle)methylamine accelerating ligands under conditions appropriate for bioconjugation will appear elsewhere.^[19] For the reasons discussed below, we focused on the catalyst incorporating varying amounts of ligand **3** [tris(3-hydroxypropyltriazolylmethyl)amine, THPTA], a water-soluble member of the tris(triazolylmethyl)amine family.^[7a] The performance of this system was found to be sensitive to the nature of the solvent and the overall copper concentration. At less than 50 μM in metal, the number of turnovers was poor and depended on the concentration of ligand, but initial reaction rates were similar (see Supporting Information). A copper concentration of 50 μM marked a transition point at which the use of ligand in any ratio greater than 1:1 with respect to metal gave rise to complete reaction in less than 10 min (Figure 1 A). At 100 μM Cu, the reaction was very fast

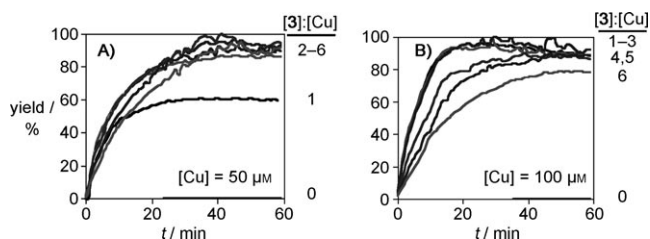


Figure 1. Conversion–time profiles as a function of ligand/Cu ratio. Conditions: propargyl alcohol (100 μM), **1** (50 μM), CuSO_4 , and ligand **3** (indicated concentrations), 0.1 M potassium phosphate buffer (pH 7.0)/DMSO 95:5, sodium ascorbate (5.0 mM), room temperature.

(Figure 1 B), and the rate decreased modestly as more than 1 equivalent of ligand was used. At a ligand/Cu ratio of 5:1, the overall reaction rate was only reduced by half. This striking tolerance of excess ligand such as **3** has been previously noted,^[19,20] and is crucial to the practical bioconjugation protocol described below.

Ascorbate concentration: The amount of ascorbate required to keep the active copper(I) catalyst available was similarly determined (Supporting Information). Reactions involving 100 μM Cu and 500 μM **3** in air, initiated by the addition of different concentrations of sodium ascorbate, were found to stop before completion in the presence of 1 mM or less reducing agent. The next highest concentration tested, 2.5 mM, proved to be sufficient; further increases did not enhance the rate. This is consistent with the need to remove oxygen from the aqueous solution (approximately 0.27 mM at room temperature, plus whatever diffuses in during the reaction) in order to maintain copper in the active +1 oxidation state.

Substrate oxidation: Copper ions mediate the catalytic oxidation of sodium ascorbate by molecular oxygen, producing hydrogen peroxide in a two-step process involving the superoxide radical anion as an intermediate.^[14b,21] If this reaction occurs in the presence of polypeptides, oxidation (such as of cysteine, methionine, and histidine imidazole groups)^[22] or cleavage of the biomolecule can occur. We tested the ability of CuAAC-accelerating ligands to affect this type of process in a model reaction with *N*-benzoylhistidine

(**5**). The compound was stable in the presence of CuSO_4 or ascorbate alone in pH 7 buffer, but the combination of the two induced the oxidation of approximately 16% of **5** to **6** in 90 min, increasing to 65% after 20 h (Figure 2 A).

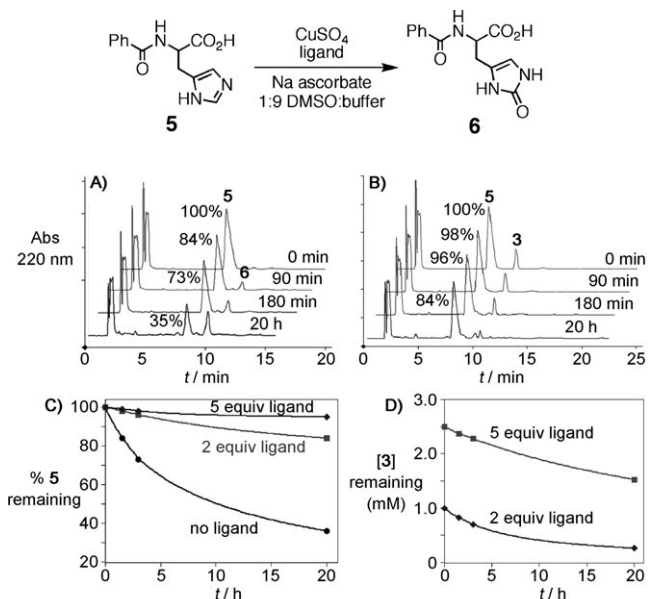


Figure 2. A) HPLC profiles for the oxidation of **5** (2 mM) in the presence of CuSO_4 (0.5 mM) and ascorbate (5 mM) in 10% DMSO/0.1 M phosphate buffer pH 7. B) The same analysis as in (A) in the presence of ligand **3** (1 mM). C, D) Summary of data showing oxidative loss of **5** and **3** in the presence of different amounts of **3**.

Ligand **3** protected the histidine moiety in a manner proportional to the ligand concentration. At a ligand/Cu ratio of 2:1, no histidine oxidation was observed after 90 min, and only approximately 15% of **5** was lost after 20 h (Figure 2 B). At 5:1, less than 5% of histidine was oxidized after 20 h (Figure 2 C). Ligand **3** was also found to be consumed over the same period, with approximately the same amount lost (0.7–1 mM) when two or five equivalents was used relative to Cu (Figure 2 D). We therefore suggest that the ligand protects against histidine oxidation as a sacrificial reductant, intercepting reactive oxygen species in the coordination sphere of the metal as they are generated.^[23] Thus, an excess of ligand is required, and the unusual nature of this class of ligand, outlined in Figure 1 and explored more fully elsewhere,^[19,20] allows such an excess to be used without sacrificing much in the way of CuAAC rate.

We also measured H_2O_2 concentrations under various CuAAC conditions by the standard amplex red–horseradish peroxidase assay, with the results shown in Figure 3. The initial production of peroxide took place in a Cu-ascorbate dependent manner, with slightly greater activity at lower ascorbate concentrations in the presence of 5 equivalents of ligand **3** per metal (Figure 3 A,C). However, after 60 min, the highest levels of hydrogen peroxide were accumulated in the presence of the lowest concentration of copper (Figure 3 B), showing that the metal mediates the decomposition of H_2O_2 as well as its formation. The presence of ligand **3** strongly

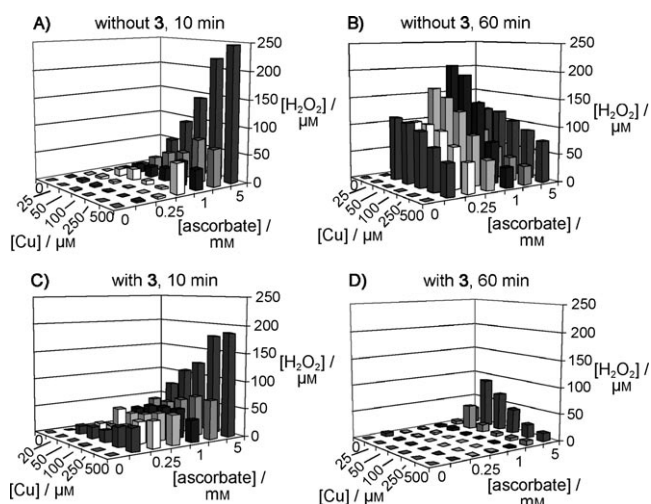


Figure 3. Hydrogen peroxide formation in the presence of various concentrations of CuSO_4 (0–500 μM) and sodium ascorbate (0–5 mM), monitored by fluorescence of amplex red ($\lambda_{\text{ex}} = 570 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$) in the presence of horseradish peroxidase at 10 and 60 min after addition of ascorbate: A,B) In the absence of ligand **3**; C,D) in the presence of 5 equivalents of ligand **3** with respect to Cu.

accelerated the peroxide decomposition reaction (Figure 3D). For these reasons, we recommend that five equivalents of tris(triazolyl)methylamine ligands such as **3** be used in most cases, and especially when substrate oxidation is a danger.

Ascorbate byproducts: Early applications of CuAAC to bioconjugation using sodium ascorbate led to protein adduct formation, crosslinking, and precipitation.^[6a] The initial oxidation product, dehydroascorbate, is a potent electrophile, and can also hydrolyze to form reactive aldehydes such as 2,3-diketogulonate and presumably glyoxal.^[24] These species can make connections with arginine, N-terminal cysteine, and lysine side-chains.^[25] To avoid such unwanted side-reactions, we require an additive to efficiently capture reactive carbonyl compounds while not inhibiting the CuAAC reaction. Aminoguanidine (**4**) and pyridoxamine are known to alleviate glyoxal toxicity in mammalian cells,^[24] so we investigated the properties of the former molecule. The rate of the CuAAC reaction mediated by 100 μM Cu was unaffected by **4** at 1 mM, but was noticeably lowered when **4** was present at 5 mM and higher (Supporting Information). At a higher Cu concentration (0.5 mM), additive **4** had very little inhibitory effect even up to 20 mM. These results show that aminoguanidine is only a modest inhibitor with fairly weak binding affinity for Cu^{I} .

The ability of **4** to prevent protein crosslinking was assayed using cowpea mosaic virus (CPMV), which we have found previously to be unstable in the presence of CuSO_4 and sodium ascorbate due to aggregation-dependent decomposition.^[14a] As shown in the Supporting Information, ligand **3** and aminoguanidine (**4**) were both helpful in protecting the protein while allowing for rapid CuAAC coupling.

Tests of the refined bioconjugation protocol: The use of excess amounts of ligand **3** in CuAAC bioconjugation was tested on a 21-mer siRNA strand, as a chemically sensitive biomolecule used in low concentration. Oligonucleotide **7**,

obtained from a commercial supplier as a 3'-amine derivative, was condensed with an excess of NHS ester **8** to give the alkyne **9** after ethanol precipitation. A click reaction of **9** (10 μM) was then performed with coumarin azide **1** (50 μM) mediated by CuSO_4 (100 μM) and **3** (500 μM) in 0.1 M phosphate buffer at pH 7, to give **10**. Fluorescence measurements showed the reaction to be complete within 1 h, and HPLC analysis showed the single peak of the starting material **7** to be converted to a single product (Supporting Information). Gel electrophoresis revealed only one fluorescent band (Figure 4, lane 3), which shifted after binding to its complementary strand (lane 7), suggesting that no strand breaks occurred. In addition, MALDI-TOF mass spectrometry showed the expected molecular weight for the corresponding cycloadduct.

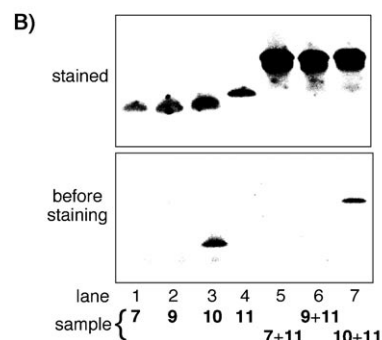
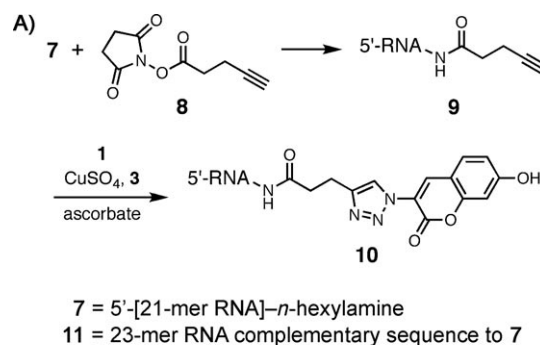


Figure 4. Demonstration of RNA modification by the CuAAC reaction: A) Reaction scheme. B) RNA gel visualized under long-wavelength UV light before (bottom) or after (top) staining with SYBR Green. The duplexes analyzed in lanes 5–7 were formed by annealing equimolar amounts of the two strands at room temperature for 30 min.

The bioconjugation method described here was further verified in reactions involving the capsid derived from bacteriophage Q β , an icosahedral particle comprised of 180 copies of a 14 kDa coat protein. We have previously attached gadolinium complexes, carbohydrates, and other species to this particle using $[\text{Cu}(\text{MeCN})_4](\text{OTf})$ and sulfonated bathophenanthroline ligand under oxygen-free conditions in a glovebox.^[26] The polyvalent azide-decorated capsid **12** was prepared by acylation of surface lysine and N-terminal amine groups (4 per subunit; 720 per particle) with a large excess of 5-(3-azidopropylamino)-5-oxopentanoic acid NHS ester (Figure 5). Subsequent click reaction of **12** (1 mg mL^{-1}

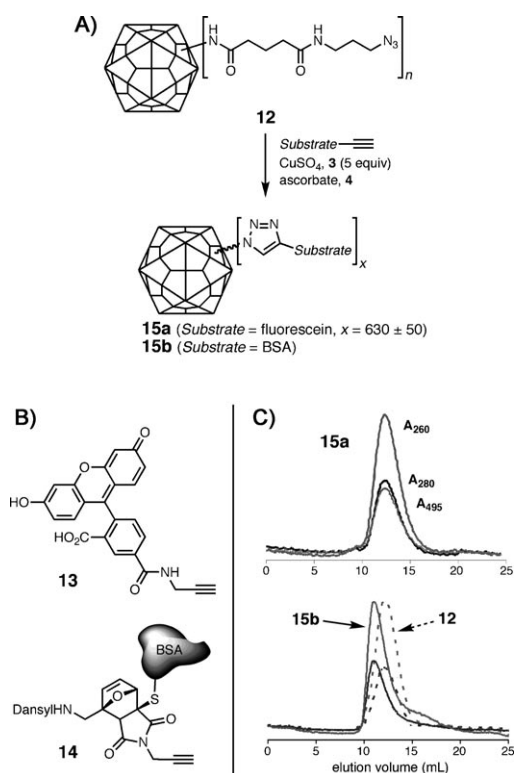


Figure 5. CuAAC reactions on the Q β virus-like particle using an excess of **3** as a ligand. A) Reaction scheme. B) Substrate alkynes. **14** was prepared by reaction of highly purified BSA (10 mg mL⁻¹) with two equivalents of an oxanorbornadiene electrophile, followed by size-exclusion purification. C) Size-exclusion chromatography of the products.

protein, 0.4 μ M in particles, approximately 280 μ M in azide) with only 2 equivalents of fluorescein alkyne **13** per azide (250 μ M CuSO₄, 1.25 mM **3**, 5 mM aminoguanidine **4**, 5 mM sodium ascorbate, pH 7 phosphate buffer) for 1 h gave an excellent yield of particles (**15a**) bearing an average of 630 dyes per capsid, determined by MALDI-TOF. No effort was made to exclude air other than to cap the Eppendorf tube containing the reaction mixture after initiation of the CuAAC reaction by addition of sodium ascorbate.

The coupling of a protein to the outer surface of the Q β virus-like particle served as a final example of the ability of the new CuAAC conditions to accomplish efficient bioconjugation. Bovine serum albumin (BSA), which contains one free cysteine residue (C34) was labeled first with a thiol-reactive linker^[27] to afford the alkyne-derivatized protein **14**. Ligation of **14** (1 equiv per capsid subunit) to the polyvalent azide **12** provided a high yield of BSA-coated particle **15b** within 1 h. Densitometry analysis after denaturing gel electrophoresis on the purified product allowed us to estimate that an average of 50 BSA molecules were attached to each capsid. This is consistent with size-exclusion chromatography (Figure 5B) and dynamic light scattering (hydrodynamic radius increase from 14 to 22 nm) analyses of the product (Supporting Information).

The CuAAC ligation chemistry illustrated here for connecting RNA and protein to small and large molecules was

performed with the same convenient protocol in all cases, a far cry from the testing of varying methods that has often been required to achieve maximal rates in demanding settings. However, in our experience problems can still arise in two general circumstances. First, one of the substrates may contain groups that strongly bind copper ions. In the case of proteins, this is potentially problematic because the bound metal may be unavailable for CuAAC catalysis, and because the Cu ions may induce protein precipitation.^[28] For example, we tested catalase to decompose hydrogen peroxide in the studies described by Figure 2 and 3. However, copper is a noncompetitive inhibitor of catalase, and the enzyme reciprocally inhibited the CuAAC reaction by sequestering the metal. We have also found that hexahistidine-tagged proteins can have the same effect. In such cases, three adjustments are suggested. 1) The concentration of the metal–ligand complex can be increased to a maximum of 0.5 mM, or 2–3 equivalents with respect to the His₆ sequence. 2) An accelerating ligand with greater affinity for Cu ions can be employed in place of THPTA.^[19] 3) Other metal ions such as Ni^{II} or Zn^{II} can be added to occupy the metal-binding protein motif in competition with Cu (see the Supporting Information for a brief discussion of these options).

Second, the azide or alkyne group on the biomolecule may be sterically hindered or somehow inaccessible to the catalyst and the coupling partner. Such cases are more difficult to both diagnose and remedy, but increasing the reaction temperature or adding solubilizing agents such as DMSO can have a beneficial effect. We presume this is because even modest increases in temperature or in the ability of the medium to solvate hydrophobic domains can boost the conformational dynamics of large molecules so as to expose hindered sites to a potent catalyst. We therefore recommend testing difficult cases at as high a temperature (and/or in the presence of as much DMSO) as the substrates can withstand, taking care to cap the reaction vessel while heating so as to minimize exposure to oxygen.

In summary, the key elements for the use of the optimized bioconjugation procedure are the following.

- Sodium ascorbate is the preferred reducing agent for most applications, due to its convenience and effectiveness at generating the catalytically active Cu^I oxidation state.
- Cu concentrations should generally be between 50 and 100 μ M. The lower limit is necessary to achieve a sufficient concentration of the proper catalytic complex which incorporates more than one metal center, and more than 100 μ M Cu is usually not necessary to achieve high rates. A fluorogenic or colorimetric assay, such as that enabled by coumarin **1**,^[18] is strongly recommended for optimization of specific cases.
- At least five equivalents of THPTA (**3**, or other water-soluble variants) relative to Cu should be employed. The purpose is to intercept and quickly reduce reactive oxygen species generated by the ascorbate-driven reduction of dissolved O₂ without compromising the CuAAC reaction rate very much.
- Aminoguanidine is a useful additive to intercept byproducts of ascorbate oxidation that can covalently modify or crosslink proteins.

- e) Compatible buffers include phosphate, carbonate, or HEPES in the pH 6.5–8.0 range. Tris buffer should be avoided as the tris(hydroxymethyl)aminomethane molecule is a competitive and inhibitory ligand for Cu; sodium chloride (as in phosphate-buffered saline) up to 0.5 M can be used.
- f) Ascorbate should not be added to copper-containing solutions in the absence of the ligand. As a matter of routine, we first mix CuSO₄ with the ligand, add this mixture to a solution of the azide and alkyne substrates, and then initiate the CuAAC reaction by the addition of sodium ascorbate to the desired concentration.
- g) The Cu–THPTA catalyst in water is inhibited by excess alkyne, and so the procedure described here is useful for alkyne concentrations less than approximately 5 mM. When more concentrated solutions are used, a different ligand is suggested (Supporting Information).
- h) Free thiols such as glutathione at more than two equivalents with respect to copper are strong inhibitors of the CuAAC reaction in the form described here.

Experimental Section

A sample experimental protocol that takes into account the above factors is provided in the Supporting Information.

Received: September 10, 2009

Published online: November 26, 2009

Keywords: alkynes · azides · bioconjugation · cycloaddition · copper

- [1] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596.
- [2] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004.
- [3] a) M. D. Best, *Biochemistry* **2009**, *48*, 6571; b) W. H. Binder, R. Sachsenhofer, *Macromol. Rapid Commun.* **2008**, *29*, 952; c) A. D. Moorhouse, J. E. Moses, *ChemMedChem* **2008**, *3*, 715; d) H. Nandivada, X. Jiang, J. Lahann, *Adv. Mater.* **2007**, *19*, 2197; e) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128.
- [4] a) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046; b) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793; c) J. A. Codelli, J. M. Baskin, N. J. Agard, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 11486.
- [5] a) A. E. Speers, B. F. Cravatt, *Chem. Biol.* **2004**, *11*, 535; b) K. E. Beatty, F. Xie, Q. Wang, D. A. Tirrell, *J. Am. Chem. Soc.* **2005**, *127*, 14150; c) J. Gierlich, K. Gutsmedl, P. M. E. Gramlich, A. Schmidt, G. A. Burley, T. Carell, *Chem. Eur. J.* **2007**, *13*, 9486; d) T.-L. Hsu, S. R. Hanson, K. Kishikawa, S.-K. Wang, M. Sawa, C.-H. Wong, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2614.
- [6] a) M. R. Levengood, C. C. Kerwood, C. Chatterjee, W. A. van der Donk, *ChemBioChem* **2009**, *10*, 911; b) M. Galibert, P. Dumy, D. Boturyn, *Angew. Chem.* **2009**, *121*, 2614; *Angew. Chem. Int. Ed.* **2009**, *48*, 2576.
- [7] a) T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853; b) V. O. Rodionov, S. I. Presolski, S. Gardinier, Y. H. Lim, M. G. Finn, *J. Am. Chem. Soc.* **2007**, *129*, 12696.
- [8] W. G. Lewis, F. G. Magallon, V. V. Fokin, M. G. Finn, *J. Am. Chem. Soc.* **2004**, *126*, 9152.
- [9] S. Sen Gupta, J. Kuzelka, P. Singh, W. G. Lewis, M. Manchester, M. G. Finn, *Bioconjugate Chem.* **2005**, *16*, 1572.
- [10] V. Hong, A. K. Udit, R. A. Evans, M. G. Finn, *ChemBioChem* **2008**, *9*, 1481.
- [11] a) A. J. Link, D. A. Tirrell, *J. Am. Chem. Soc.* **2003**, *125*, 11164; b) D. C. Dieterich, J. J. Lee, A. J. Link, J. Graumann, D. A. Tirrell, E. M. Schuman, *Nat. Protoc.* **2007**, *2*, 532.
- [12] a) S. I. van Kasteren, H. B. Kramer, H. H. Jensen, S. J. Campbell, J. Kirkpatrick, N. J. Oldham, D. C. Anthony, B. G. Davis, *Nature* **2007**, *446*, 1105; b) S. I. Van Kasteren, H. B. Kramer, D. P. Gamblin, B. G. Davis, *Nat. Protoc.* **2007**, *2*, 3185.
- [13] a) M. A. Bruckman, G. Kaur, L. A. Lee, F. Xie, J. Sepulveda, R. Breitenkamp, X. Zhang, M. Joralemon, T. P. Russell, T. Emrick, Q. Wang, *ChemBioChem* **2008**, *9*, 519; b) R. Kumar, A. El-Sagheer, J. Tumpene, P. Lincoln, L. M. Wilhelmsson, T. Brown, *J. Am. Chem. Soc.* **2007**, *129*, 6859; c) P. Kocalka, A. H. El-Sagheer, T. Brown, *ChemBioChem* **2008**, *9*, 1280; d) A. H. El-Sagheer, T. Brown, *J. Am. Chem. Soc.* **2009**, *131*, 3958; e) A. J. Dirks, J. J. L. M. Cornelissen, R. J. M. Nolte, *Bioconjugate Chem.* **2009**, *20*, 1129.
- [14] a) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192; b) S. C. Fry, *Biochem. J.* **1998**, *332*, 507.
- [15] E. Lallana, E. Fernandez-Megia, R. Riguera, *J. Am. Chem. Soc.* **2009**, *131*, 5748.
- [16] P. Y. Liu, N. Jiang, J. Zhang, X. Wei, H. H. Lin, X. Q. Yu, *Chem. Biodiversity* **2006**, *3*, 958.
- [17] R. H. Nagaraj, D. R. Sell, M. Prabhakaram, B. J. Ortwerth, V. M. Monnier, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 10257.
- [18] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603.
- [19] S. I. Presolski, V. Hong, M. G. Finn, **2009**, unpublished results.
- [20] V. O. Rodionov, S. I. Presolski, D. D. Díaz, V. V. Fokin, M. G. Finn, *J. Am. Chem. Soc.* **2007**, *129*, 12705.
- [21] W. L. Baker, J. Goode, L. Cooper, *Mikrochim. Acta* **1992**, *106*, 143.
- [22] a) Y. Liu, G. Sun, A. David, L. M. Sayre, *Chem. Res. Toxicol.* **2004**, *17*, 110; b) K. Uchida, S. Kawakishi, *Biochem. Biophys. Res. Commun.* **1986**, *138*, 659.
- [23] A more polar compound derived from the ligand was observed but not characterized during these experiments. This material is presumably produced by oxidation at one of the C–H sites adjacent to the triazole ring, or at the central nitrogen atom.
- [24] N. Shangari, T. S. Chan, K. Chan, S. H. Wu, P. J. O'Brien, *Mol. Nutr. Food Res.* **2007**, *51*, 445.
- [25] P. J. Thornalley, *Chem. Biol. Interact.* **1998**, *111–112*, 137.
- [26] a) E. Strable, D. E. Prasuhn, A. K. Udit, S. Brown, A. J. Link, J. T. Ngo, G. Lander, J. Quispe, C. S. Potter, B. Carragher, D. A. Tirrell, M. G. Finn, *Bioconjugate Chem.* **2008**, *19*, 866; b) E. Kaltgrad, M. K. O'Reilly, L. Liao, S. Han, J. Paulson, M. G. Finn, *J. Am. Chem. Soc.* **2008**, *130*, 4578; c) D. E. Prasuhn, P. Singh, E. Strable, S. Brown, M. Manchester, M. G. Finn, *J. Am. Chem. Soc.* **2008**, *130*, 1328.
- [27] V. Hong, A. A. Kislukhin, M. G. Finn, *J. Am. Chem. Soc.* **2009**, *131*, 9986.
- [28] a) R. Agarwal, M. N. Gupta, *Biotechnol. Tech.* **1994**, *8*, 655; b) T. B. Osborne, C. S. Leavenworth, *J. Biol. Chem.* **1916**, *28*, 109.