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Discovery and Characterization of Catalysts for Azide-Alkyne Cycloaddition by Fluorescence Quenching

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The 1,3-dipolar cycloaddition of alkynes and azides[1] has emerged as a highly useful connection method in both uncatalyzed[2] and copper(I)-catalyzed[3] forms. The practical importance of the process derives from the fact that it is the only facile 1,3-dipolar reaction which uses chemically stable components: others generally employ at least one reactant that is highly energetic, water-sensitive, or transient in nature.[4] This feature makes the azide–alkyne process highly tolerant of diverse functional groups and reaction conditions. Copper(I) catalysis of this process, discovered in 2002,[3] has been adopted by many groups for bioconjugation applications,[5] in which chemoselectivity and high reaction rate are crucial requirements. To extend these potential applications we have focused on catalysis of the azide-alkyne cycloaddition with water-soluble copper(I) systems.[3a] Here we describe two new families of catalysts discovered by a screening method that allows for measurements of relative and absolute rates in a convenient microtiter-plate format.

Catalysis of the cycloaddition reaction of dansyl azide fluorophore **1** and dabsyl alkyne **2** in aqueous-organic mixtures induces a dramatic disappearance of fluorescence, since the triazole product **3** exhibits intramolecular quenching of the dansyl unit by the dabsyl (Figure 1).[6] The effect is easily visualized by eye under long-wavelength ultraviolet illumination or quantitated by fluorescence spectroscopy. Screening of candidate ligands in parallel format was done first by simple visual imaging, which offers a rapid readout with simultaneous evaluation of control reactions. Interesting candidates were then analyzed more carefully using a 96-well fluorescence plate reader, which enabled kinetic characterization.[7]

The use of **1** and **2** at mid-micromolar concentrations in DMSO/water provided emission intensities in a convenient range for visual and plate reader detection; these compounds are completely unreactive in the absence of Cu^{I}. The organic co-solvent was used to improve both the fluorescence intensity of **1** and the solubility of several classes of ligands tested. Compound **4**, with a propyl group in place of the reactive propargyl of **2**, served as an unreactive quencher to control for false positive readings caused by intermolecular quenching, absorbance, or catalyst precipitation, as was observed for certain highly-colored copper complexes. A typical experiment involved side-by-side comparisons of the reaction of interest (**1** + **2** + catalyst) and a control set of reactions (**1** + **4** + catalyst), at room temperature over the same range of copper and/or ligand concentrations with constant concentrations of fluorophore and quenchers. An example is shown in Figure 1, with a positive indication of catalysis being the disappearance of fluorescence only in the top half of the plate. Ligand-accelerated catalysis[8] was suspected when the apparent rate of reaction in the presence of ligand was greater than in its absence. A large excess of copper relative to ligand was used to boost the rate of the ligand-free process and thus make the screen for ligand acceleration more demanding.

Figure 1. Fluorescence quenching assay. The microtiter plate was visualized under long-wavelength ultraviolet irradiation after a reaction time of 20 minutes. For all reactions, [Na ascorbate] = 16 mM, [**1**] = [**2**] = [**4**] = 0.031 mM, [ligand] = 77 μM, solvent = 38% DMSO + 62% Tris buffer (pH 7.5). SDS (sodium dodecyl sulfate) was included to verify that surfactants do not influence fluorescence intensity.

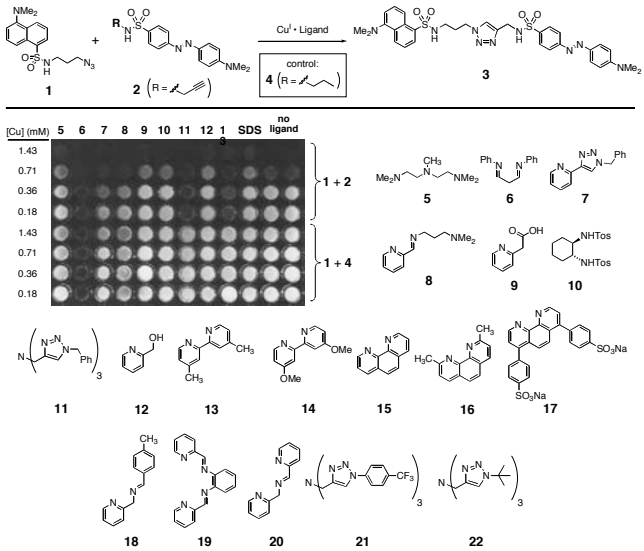


Figure 1 shows one example of fluorescence quenching in the control wells (ligand **6**), thereby comprising a case that cannot be evaluated by this assay. Among the others, **5**, **9**, **10**, and **12** were ineffective, **7** and **8** marginally so. Tris(triazolyl)amine **11** was previously identified as a useful ligand for bioconjugation reactions,[10] and the assay identified bipyridine copper complexes such as those formed by **13** to be comparably potent catalysts. Thus, catalytic activity persisted upon dilution of copper to 180

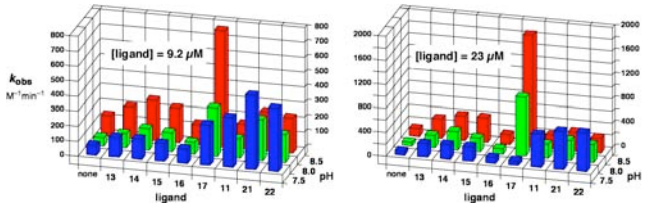
μM, a level at which much slower cycloaddition was observed in the absence of ligand.[9]

The same rapid assay was used to compare variations in the bipyridine theme (data not shown). Under otherwise identical conditions, the relatively electron-rich ligands **13** and **14**, as well as phenanthroline **15,** were found to give two to three-fold more rapid rates of cycloaddition than bipyridine itself. The copper complex of neocuproin **16** is a less active catalyst than unsubstituted phenanthroline, presumably because of steric factors. Bathophenanthrolinedisulfonic acid, **17**, used in the specific colorimetric detection of copper(I),[10] provided an excellent and very water-soluble catalyst (see below).

Also screened was a combinatorial library of 88 Schiff bases prepared from 11 aromatic aldehydes and 8 amines.[11] Pyridine-containing compounds were found to be promising, but several candidates caused degradation of the fluorophore and alternative reducing agents were compromised by side reactions with the diazo group of the quencher. The detection of side reactions underscores the importance of negative controls in ligand screening (here represented by the use of **4**), as well as the potential diversity of copper(I) reactivity in catalyst libraries. After elimination of false positives, clear ligand-accelerated catalysis was observed for Schiff bases such as **18**–**20**, each containing a pyridine group in position to form a chelating interaction. These systems are being further refined.

Reactions in microtiter plates were followed quantitatively by measurement of emission intensity *vs*. time; linear fits for simple pseudo second-order kinetics (Cu and ligand in excess with respect to azide and alkyne) were obtained over the first hour of each reaction.[11] The apparent second-order rate constants thus obtained allowed quantitative comparisons between ligands at varying pH, as shown in Figure 2. Rate accelerations of more than two orders of magnitude were observed relative to the rate of the copper-catalyzed reaction in the absence of ligand.[12] The ligand-free process was accelerated as pH was increased from 7.5 to 8.5, as were many of the reactions in the presence of ligand. However, tris(triazolyl)amine additives[13] **11**, **21**, and **22** showed the opposite trend, with the fastest reactions observed at pH 7.5. The most active catalyst was that comprised of bathophenanthroline **17** at pH 8.5.

Figure 2. Observed econd-order rates of reaction at two different ligand concentrations. The concentrations of azide, alkyne, copper, and ascorbate were 18 μM, 18 μM, 182μM and 18 mM, respectively.



Kinetics measurements were performed as a function of the **17**:Cu ratio.[11] The second-order rate constant was observed to peak at a **17**:Cu value of 2.0 at two different copper concentrations, suggesting the involvement of two ligands per metal center and the ability of extra ligand to inhibit the reaction. Furthermore, the kinetic rate order in this complex was found to be 2.0±0.1,[11] suggesting that two copper centers may be required for catalytic turnover.

The two most promising new systems identified above were found to perform well in preparative and bioconjugation applications. Particularly important was the observation that **17** is highly effective in promoting the attachment of a dye-alkyne to a virus particle decorated with azides in analogy to previous results obtained with **11**,[5a] which is much less soluble in water. A full account of this type of application will appear elsewhere.

Ligands may assist the azide-alkyne cycloaddition process in at least two ways: stabilization of the Cu^{I} oxidation state and acceleration of the reaction itself. We did not screen for the former function, since an excess of ascorbate was maintained throughout. Thus, active ligands identified here have a direct role in promoting the reaction, but may not be useful under oxidizing conditions. Indeed, reactions using **17** without excess reducing agent are somewhat more air-sensitive than those of **11**.

In summary, the fluorescence quenching screening method has led to the discovery and quantitative kinetic characterization of ligand-accelerated catalysis of the Cu(I) azide-alkyne cycloaddition reaction under dilute aqueous conditions. Bipyridine-type compounds have been found for the first time to be components of effective catalysts, and an opposing pH dependence has been observed for bipy vs. triazolylamine compounds. Further studies directed toward the discovery and application of new ligands are underway, as are investigations of the reaction mechanism.

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Supporting Information Available. Experimental details and additional figures.

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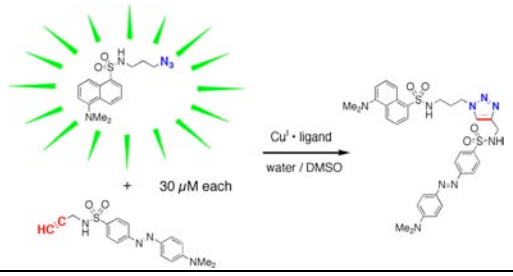
12. If used directly, the values shown in Figure 2 dramatically understate the magnitude of ligand-accelerated catalysis, since the concentration of uncomplexed Cu is much higher than that of Cu-ligand species in all cases.
13. Chan, T.R.; Hilgraf, R.; Sharpless, K.B.; Fokin, V.V., submitted.

TOC Graphic



ABSTRACT. Copper-based catalysts for the 1,3-dipolar cycloaddition of azides and alkynes were screened in parallel fashion using a fluorescence quenching assay. The method was designed to identify systems able to accelerate the coupling of reactants at micromolar concentrations in aqueous mixtures and to obtain quantitative comparisons of their activity. In addition to the tris(triazolylamines) previously reported, two types of compounds (bipy/phen and 2-pyridyl Schiff bases) were found to exhibit significant ligand-accelerated catalysis, with one complex showing especially dramatic rate enhancements. Preliminary explorations of the dependence of reaction rate on pH, ligand:Cu ratio, and Cu concentration are described.