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Copper-Chelating Azides for Efficient Click Conjugation Reactions in Complex Media**

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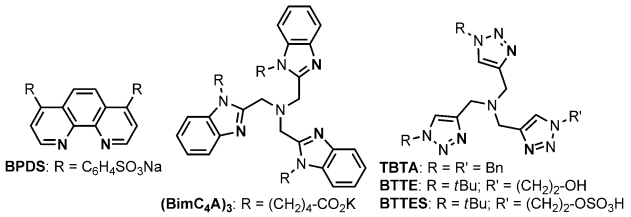
Abstract: *The concept of chelation-assisted copper catalysis was employed for the development of new azides that display unprecedented reactivity in the copper(I)-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC) reaction. Azides that bear strong copper-chelating moieties were synthesized; these functional groups allow the formation of azide copper complexes that react almost instantaneously with alkynes under diluted conditions. Efficient ligation occurred at low concentration and in complex media with only one equivalent of copper, which improves the biocompatibility of the CuAAC reaction. Furthermore, such a click reaction allowed the localization of a bioactive compound inside living cells by fluorescence measurements.*



Scheme 1. Examples of efficient N-donor ligands for CuAAC reactions. TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, Bn = benzyl, BTTE = 2-[4-({bis[1-*tert*-butyl-1*H*-1,2,3-triazol-4-yl)methyl]amino}-methyl)-1*H*-1,2,3-triazol-1-yl]ethanol.

The copper(I)-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC)[1] reaction can be considered as the archetype of click chemistry.[2] The many reviews that relate the wide variety of applications of this relatively young reaction bear witness to its huge impact.[3] In this context, the use of copper ligands that are able to stabilize and modulate the catalytic activity of the Cu^I center contributed to the success of this reaction by allowing for smoother reaction conditions and broader applicability. Some of the most representative ligands are the commercially available sulfonated bathophenantroline BPDS, the tris(benzimidazole) $(BimC_4A)_3$ and the tris(triazole) TBTA ligands (Scheme 1), which greatly accelerate the reaction and stabilize the Cu^I oxidation state.[4] Analogues of TBTA that bear bulky *tert*-butyl groups, such as BTTE and BTTES, were recently found to be the most efficient ligands for the CuAAC reaction described thus far.[5]

Despite the high reactivity of such copper complexes, the copper source, ligands, and sodium ascorbate are usually required in excess amounts to reach efficient ligation under the diluted conditions that are typically used in the fields of bioconjugation or cell labelling. To limit the damages that are caused by the copper(I)-mediated generation of reactive oxygen species (ROS) from O_2,[6] several CuAAC procedures have been reported in the literature. They are usually based on the use of water-soluble copper ligands and additives that both accelerate the reaction and act as ROS scavengers to decrease the cell toxicity of copper.[7] A second approach uses highly reactive azides designed according to the concept of chelation-assisted metal catalysis. Picolyl azides have been reported to react much faster than standard azides thanks to internal chelation of the copper catalyst, which is supposed to enhance the electrophilicity of the azido group and facilitate the formation of the metallacycle intermediate.[8] This concept was recently successfully exploited by Ting et al. to perform site-specific protein labelling on the surface of living cells at low concentrations (50 μM) of the copper tris(triazole) complex using pyridine-based copper-chelating azides as substrates.[9] Nevertheless, the use of the CuAAC reaction in the cellular context is still limited to cell-surface labelling and, aside from the above-mentioned examples, is often discouraged in favor of copper-free click reactions.[10]

Herein, we describe our efforts to improve the performance of the CuAAC reaction in complex media using new azides, which include a complete copper-chelating system in their structure (Scheme 2). These azides were designed to form strong, active copper complexes and should therefore be considered both as reactant and catalyst in the CuAAC reaction. Using this type of azides, the CuAAC should thus become a bimolecular reaction and display much faster kinetics than classic CuAAC reactions.

To evaluate this strategy, we first synthesized various chelating azides with increasing copper-chelation capabilities (Scheme 3; see the Supporting Information for detailed synthetic procedures). Azides **A1**, **A3**, and **A4** were designed for negative-control experiments; all other azides were chosen for their potential ability to coordinate copper as bi- (**A2**, **A5**, **A6**–**A10**, and **A12**), tri- (**A11** and **A13**), or

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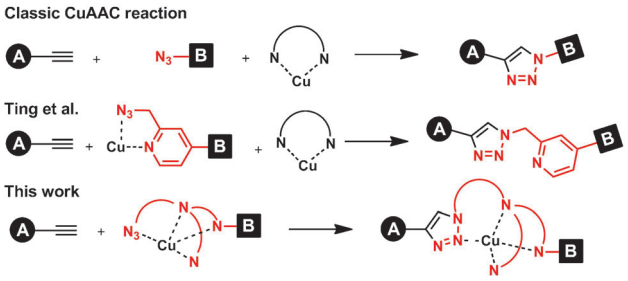
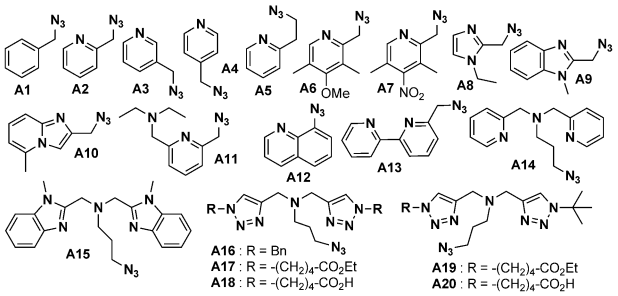


Scheme 2. Strategies for the development of fast CuAAC reactions.



Scheme 3. Structures of azides **A1**–**A20**.

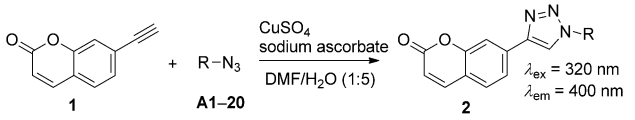
tetradentate (**A14**–**A20**) N-heterocyclic ligands. The structures of most of these azides were inspired by ligands that are known to be active for the CuAAC reaction. Bis(triazole) azides that bear carboxylic groups (**A18** and **A20**) were designed to allow post-functionalization for bioconjugation applications.

The reactivity of these chelating azides for the CuAAC reaction was evaluated at low concentrations using the coumarin-based fluorogenic probe **1**, which was inspired by previous studies.[11] This probe enabled us to monitor the CuAAC reaction of all azides **A1**–**A20** by simple fluorescence measurements.

All of the corresponding triazole products **2** were synthesized and found to display similar fluorescence properties ($\lambda_{ex} = 320$ nm; $\lambda_{em} = 400$ nm; Supporting Information, Table S1). Standard curves that correlate the fluorescence signal to the triazole concentration were systematically determined before the kinetic experiments.

Comparative kinetic measurements for the CuAAC reaction (Table 1) were performed with $CuSO_4$ (17.5 μM) in the presence of one equivalent of alkyne **1** and azides **A1**–**A20** in phosphate buffer (0.1M, pH 7.4) containing 25% of DMF. Experiments were carried out in duplicate in microtiter plates. Kinetic parameters were monitored for five minutes for the fastest reaction and overnight for the slowest reaction (Table S2). As expected, non-chelating azides **A1**, **A3**, and **A4** gave very low yields even after 16 hours, and the kinetics were greatly improved by the addition of Cu^I ligands. For example, the reaction was approximately 1500 times faster when **A1** was used in the presence of BTTE (Table 1, entry 3). Overall, picolyl and other bidentate azides induced increases in the rate of the reaction that are similar to those that were

Table 1: Comparative kinetic studies with azides **A1**–**A20**.[a]



Entry	Azide	Ligand	V [nM sec^{-1}]	Yield of **2**[b] [%]	t[c]
1	**A1**	–	0.01 ± 0.02	0.9	16 h
2	**A1**	TBTA	1.78 ± 0.01	28	2 h
3	**A1**	BTTE	15.59 ± 0.02	53	20 min
4	**A2**	–	13.35 ± 0.03	29	13 min
5	**A2**	BTTE	38.26 ± 0.01	39	7 min
6	**A3**	–	0.02 ± 0.05	1	16 h
7	**A4**	–	0.59 ± 0.06	1	5 min
8	**A5**	–	19.87 ± 0.02	39	12 min
9	**A6**	–	14.79 ± 0.03	35	13 min
10	**A7**	–	11.49 ± 0.02	27	10 min
11	**A8**	–	12.02 ± 0.02	30	13 min
12	**A9**	–	17.30 ± 0.03	43	12 min
13	**A10**	–	8.44 ± 0.01	28	19 min
14	**A11**	–	20.17 ± 0.05	34	6 min
15	**A12**	–	0.22 ± 0.03	0.4	10 min
16	**A13**	–	0.98 ± 0.05	7	23 min
17	**A14**	–	3.00 ± 0.01	2	10 min
18	**A15**	–	**171.25** ± 0.15	**60**	**2 min**
19	**A16**	–	41.13 ± 0.05	45	3 min
20	**A17**	–	46.14 ± 0.06	67	4 min
21	**A18**	–	37.29 ± 0.07	75	6 min
22	**A19**	–	**356.26** ± 0.23	**53**	**50 s**
23	**A20**	–	**402.54** ± 0.11	**59**	**40 s**
24	**A20**	BTTE	41.07 ± 0.05	48	20 min

[a] Kinetic measurements were conducted with **1**, azides **A1**–**A20**, $CuSO_4$ (17.5 μM each), sodium ascorbate (437.5 μM, 25 equiv) in a mixture of phosphate buffer and DMF (75:25, 0.1 M). When used, the ligand concentration was 17.5 μM. [b] Relative yields were determined by fluorescence measurements using standard curves. [c] Time to reach the plateau.

observed on addition of the ligand BTTE (entries 8–14), except for aromatic azide **A12**, which appeared inactive. Bipyridine azide **A13** was also inactive, probably because of geometric constraints (entry 16). The most promising results were obtained with the bis(benzimidazole) and bis(triazole) azides **A15**–**A20**, for which substantial rate enhancements were observed. The presence of a pendent *tert*-butyl group in azides **A19** and **A20** seemed to enhance their ability to undergo ultra-fast click reactions (compare entries 20 and 21 with entries 22 and 23, Table 1). For example, the reaction with **A20** displayed a rate constant of $K_{obs} = 1300\,M^{-1}s^{-1}$ under our reaction conditions and is more than 200 times faster than standard reactions with strained cyclooctynes, which makes this azide more than 4×10^4 times more reactive than standard azides. Unlike for picolyl azide **A2**, the addition of BTTE significantly decreased the reaction rate with **A20** (compare entries 4 and 5 with entries 23 and 24 in Table 1), indicating that the copper complex of **A2** is probably the active form that undergoes cycloaddition.

This unprecedented reactivity is illustrated in Figure 1 A, which shows the conversion–time profiles of our most reactive chelating azides. After only 30 seconds, yields above 50% were obtained when the chelating azides **A15**

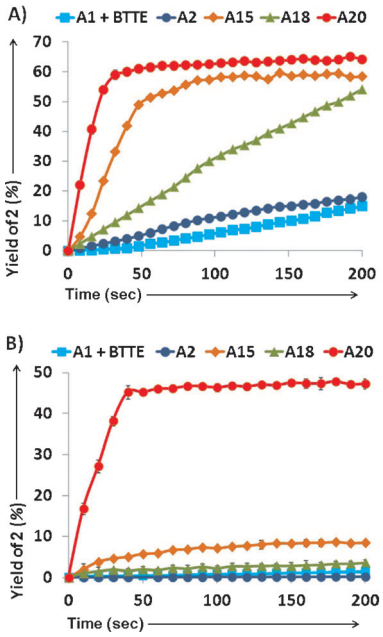


Figure 1. Comparison of CuAAC kinetics: A) In phosphate buffer (0.1 M, pH 7.4); B) in cell lysate (K562, 3,4 $\mu g\,\mu L^{-1}$, pH 7.4). Kinetic measurements were carried out with a concentration of 17.5 μM for the reactants, $CuSO_4$ (1 equiv), and sodium ascorbate (25 equiv).

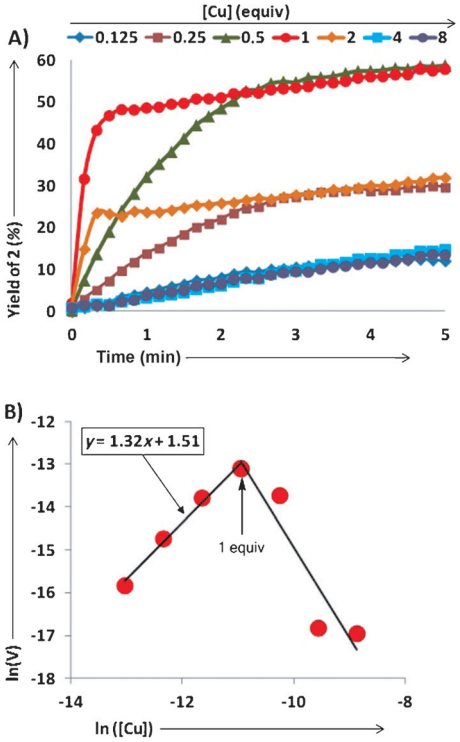
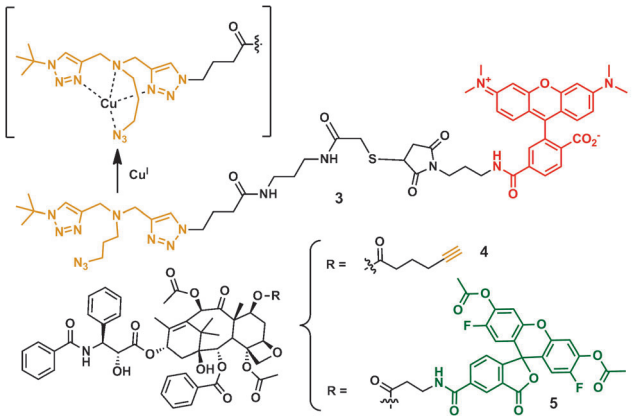


Figure 2. A) Time courses for various concentrations of $[CuSO_4{\cdot}H_2O]$. [**1**] = [**A20**] = 17.5 μM. B) Determination of the reaction order in copper.

and **A20** were used with only 1 equivalent of copper at a substrate concentration of 17.5 μM in buffer solution. We further investigated the reactivity of these azides in more complex media by repeating the CuAAC reactions in cell lysate (Figure 1 B). Interestingly, only azide **A20** kept its ability to undergo an ultra-fast click reaction under these conditions and yielded 40% of the cross-coupling product in only 30 seconds. Only poor yields were observed with **A15** and **A18**, and no reaction at all occurred with pycolyl azide **A2** or with the BTTE copper complex under these conditions. Therefore, the presence of a *tert*-butyl moiety on the chelating azide appeared to be crucial not only for the activity, but also for the stability of the corresponding copper complex in biological media.

We therefore focused our efforts on **A20**, which appeared to be the most promising chelating azide for bioconjugation. We first determined the kinetic order in copper for the reaction with **A20** under initial kinetic conditions.[12] Triazole formation was monitored by fluorescence measurements immediately after the addition of sodium ascorbate to the alkyne/azide/$CuSO_4$ mixture. Interestingly, two different behaviors in terms of copper dependence were clearly observed during these kinetic studies. The order was found to be 1.3 when the amount of copper was maintained below one equivalent; the reaction rate decreased dramatically to almost complete inhibition with excess copper (Figure 2). These kinetic results seem to indicate that a 1:1 complex of **A20** and copper is the active species, which undergoes an ultra-fast click reaction; in the presence of excess copper, a polynuclear unreactive complex might be formed.

To assess the capacity of **A20** for ligation in living cells, we decided to employ a paclitaxel-based assay in HuH-7 human liver carcinoma cells. We therefore synthesized the two required probes **3** and **4** (Scheme 4). In the first step of the cellular assay, the treatment of living cells with probe **4** localized the alkyne functional group inside the microtubule skeleton of the cell, which is due to the strong affinity of paclitaxel for tubules. After several washing steps to remove unbound alkyne, the chelating azide probe **3**, which had been preloaded with copper, was added to the cell culture medium. In the case of a clean click reaction inside the cell, imaging should reveal strong fluorescence localized at the skeleton. To confirm the localization of the clicked probes exclusively at the tubulin site, co-staining was performed with Tubulin



Scheme 4. Reagents used for the labelling experiments in living cells. The structure of the copper complex is hypothetical.

Tracker Green (**5**). Merging the red channel of TAMRA fluorescence (TAMRA = carboxytetramethylrhodamine) with the green channel of Tubulin Tracker thus revealed the co-localization of the covalent probe and the click reaction sites through appearance of a yellow color.

For ligation experiments, we pretreated a stock solution of **3** in DMSO with one equivalent of copper(II) and 50 equivalents of sodium ascorbate prior to the in-cell experiments. The living cells were treated with **4** (62.5 nM) at 37 °C for 30 minutes, washed with phosphate-buffered saline solution (PBS), and then incubated with the copper complex of **3** (50 μM) in the cell medium at 37 °C for two hours. Cells were then washed again, fixed with paraformaldehyde (4 %), and treated with Tubulin Tracker Green (**5**; 62.5 nM) in preparation for the co-localization experiments (Figure 3; see also Figure S6).

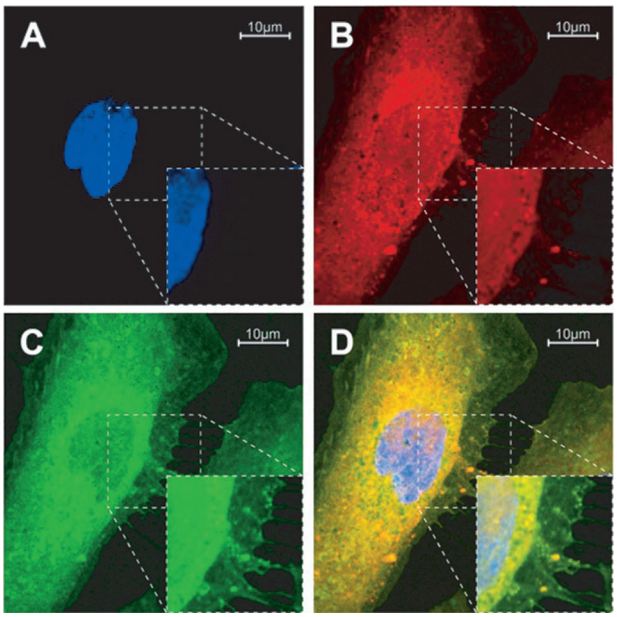


Figure 3. Confocal microscopy images of HuH-7 cells after treatment with paclitaxel–alkyne **4** and chelating azide **3**. A) Localization of the cell nucleus with 4′,6-diamidino-2-phenylindole (DAPI). B) TAMRA fluorescence of probe **3**. C) Oregon green fluorescence of probe **5**. D) Merging of the three channels to confirm co-localization.

The recorded microscopic images revealed a strong TAMRA fluorescence in the rhodamine channel (Figure 3B), whereas only a very low level of fluorescence was recorded when **3** was used without copper (Figure S4). Furthermore, TAMRA fluorescence coincided very well with the fluorescence of Oregon green, which is shown by the appearance of a yellow color (Figure 3D). This indicates that the copper complex of **3** enabled efficient CuAAC labelling of the alkyne probe **4** in living cells. Control experiments showed that no fluorescence was observed when cells were not pre-treated with paclitaxel–alkyne probe **4**, thus indicating a low level of non-specific reaction of the copper complex of probe **3** with the biological medium (Figure S3). In cell medium, click experiments with bidentate azides or under standard CuAAC conditions with BTTE as the ligand were unsuccessful (Figure S5).

In conclusion, we have used the concept of chelation-assisted copper catalysis to develop new azides that display unprecedented reactivity in the CuAAC reaction. Inspired by previous work of Ting et al.,[10] we designed and synthesized azides that bear strong copper-chelating moieties. These compounds allow the formation of azide copper complexes that react almost instantaneously with alkynes under diluted conditions. Using such azides, efficient ligation occurred at low concentration and in complex media with only one equivalent of copper, thus improving the biocompatibility of the CuAAC reaction. This increased biocompatibility was illustrated by a click reaction that allowed the localization of a bioactive compound inside living cells by fluorescence measurements.

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