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# A water soluble Cu<sup>I</sup>-NHC for CuAAC ligation of unprotected peptides under open air conditions†

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A reducing agent-free version of CuAAC able to operate under open air conditions is reported. A readily-synthesizable, hydrophilic and highly stable Cu<sup>I</sup>-NHC allows the clean ligations of unprotected peptides comprising sensitive side chains, at millimolar concentrations.

The copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction<sup>1</sup> is considered as a standard technique for bioconjugation.<sup>2</sup> This reaction requires that the catalytically active copper species, often prepared from Cu<sup>II</sup>, is brought or maintained in its air-sensitive +I oxidation state. Most CuAAC bioconjugation protocols<sup>3</sup> combine an excess of an *in situ* reducing agent (ascorbic acid,<sup>3a</sup> TCEP† or electrochemical reduction<sup>3b</sup>) with a Cu<sup>I</sup> nitrogen ligand<sup>3a-c</sup> (Fig. 1) and/or a drastic exclusion of oxygen under glovebox-like conditions.<sup>3d</sup> In practice, the triazole ligation of unprotected peptides or proteins can be hampered by an exacerbated vulnerability to oxidation of some side chains under standard CuAAC conditions. In this respect methionine<sup>3d</sup> and histidine<sup>3a</sup> are particularly sensitive presumably due to a sequence-dependent binding of copper to the sulfide or imidazole, prior to the oxidation into sulfoxide and imidazolone, respectively. Traces of O<sub>2</sub> lead to reactive oxygen species, a problem most pregnant under dilute conditions.<sup>3c</sup> This calls for demanding experimental conditions that bring the experimenter quite far away from some fundamental aspects of the “click” concept associated with this popular reaction. Moreover, ascorbic acid degradation products can undergo deleterious reactions

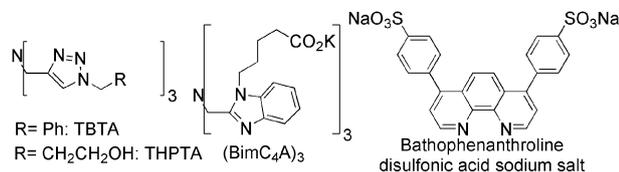


Fig. 1 Classical ligands used for CuAAC.

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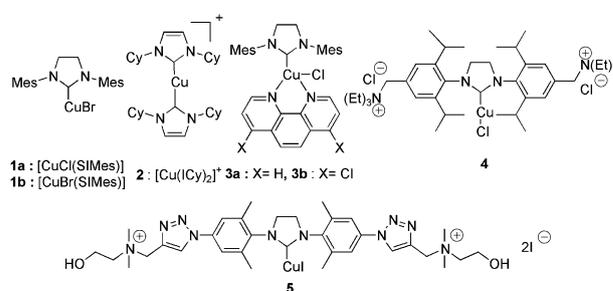
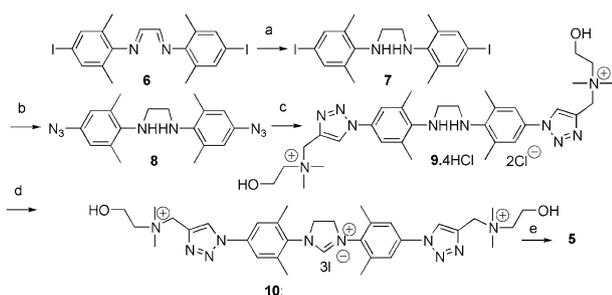


Fig. 2 Stable Cu<sup>I</sup>-NHCs catalysts.

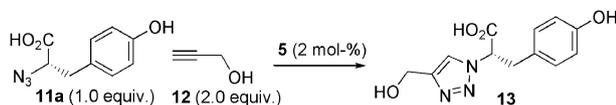
with some side chains if no precaution such as addition of aminoguanidine is taken.<sup>3a</sup>

These limitations led us to hypothesize that a reducing agent-free catalytic system based on a water-soluble catalyst that strongly stabilizes Cu<sup>I</sup> should be envisaged. We were attracted by the high stability of the copper(I) *N*-heterocyclic carbenes (NHCs) [CuX(SIMes)] (1)† and [Cu(ICy)<sub>2</sub>]<sup>+</sup> (2)† (Fig. 2).<sup>4a,b</sup>

Unfortunately, they are efficient for catalyzing azide-alkyne cycloaddition only under neat or “on water” conditions. The scope of the [CuCl(SIMes)] catalyst was increased by the addition of aromatic *N*-donors, but **3a,b** are still restricted to alcoholic solvents.<sup>4c,d</sup> Recently Wang *et al.* reported the water soluble catalyst **4**, but it was still used “on water”.<sup>4e</sup> In this communication we report the scope and the limitations of the new functionalized aqueous-soluble Cu<sup>I</sup>-NHC, **5**, allowing clean CuAAC reactions in water with peptides bearing sensitive side chains. To this end, a modification of the SIMes core by two triazolyl-choline arms as hydrophilic and cationic surrogates would fit the desired requirements of both stability and aqueous solubility (Scheme 1).<sup>5</sup> **9** was obtained from **8**<sup>7</sup> using the stable catalyst **3b** which allows a clean completion at room temperature in an open flask. Therefore, **9**·4HCl was refluxed in ethanol-triethyl orthoformate, then in water, affording **10a**. A chloride-to-iodide exchange ensues to deliver the filterable solid **10b**. Finally, the copper atom was efficiently introduced by the reaction with a stoichiometric amount of CuI in the presence of NaOH in methanol to afford the heteroleptic complex **5**.<sup>§</sup> The choice of iodide anion was dictated by the poor stability toward air oxidation of the corresponding chloride complex whereas **5** was indefinitely stable upon storage on the bench. The whole process is very practical



**Scheme 1** Synthesis of  $\text{Cu}^{\text{I}}\text{-NHC } \mathbf{5}$ . *Reagents and conditions:* (a) Ref. 6, 93%. (b)  $\text{NaN}_3$ ,  $\text{CuI}$  (20 mol%),  $\text{MeNH}(\text{CH}_2)_2\text{NHMe}$  (30 mol%), sodium ascorbate (20 mol%),  $\text{DMSO} : \text{H}_2\text{O}$  (v/v) 9 : 1, 70 °C, 81%. (c)  $\text{HO}(\text{CH}_2)_2\text{N}(\text{Me})_2\text{CH}_2\text{C}\equiv\text{CH}$ ,  $\text{Cl}$ ,  $\mathbf{3b}$  (0.6 mol%),  $\text{MeOH}$ , then  $\text{HCl}$ , 81%. (d)  $\text{EtOH}/\text{HC}(\text{OEt})_3$ , reflux, then water reflux,  $\mathbf{10a}$ : 74%, then halogen exchange,  $\mathbf{10b}$ : 71%. (e)  $\mathbf{5}$ :  $\text{CuI}$ ,  $\text{NaOH}$ ,  $\text{MeOH}$ , 98%.



**Scheme 2**  $\text{CuAAC}$  reaction between  $\mathbf{11a}$  and  $\mathbf{12}$ .

as it requires only simple filtrations and it is achievable on the gram scale ( $\text{ESI}^{\dagger}$ ).

We examined the ability of  $\mathbf{5}$  to catalyze the formation of  $\mathbf{13}$  from (*S*)-2-azido-3-(4-hydroxyphenyl) propanoic acid ( $\text{N}_3\text{Tyr-OH}$ ,  $\mathbf{11a}$ )<sup>8</sup> and propargyl alcohol ( $\mathbf{12}$ ) (Scheme 2) in common aqueous buffers (pH: 10 to 5, Table 1) under challenging conditions: low concentrations, air atmosphere, low catalyst loading.

This screening allowed selecting HEPES and  $\text{MES}^9$  (entries 5 and 9) as the most suited buffers for our challenging conditions. Also, pure water can be used (entry 1), which could be advantageous for some synthetic purposes. We decided to keep 0.2 M HEPES, pH 7.6 (entry 5) as standard conditions, and investigated the effect of the addition of co-solvents or salts (Table 2).

Catalyst  $\mathbf{5}$  was found to be compatible with methanol, DMSO, NMP and HFIP (entries 1–4) whereas the presence of chloride ions slightly decreased the conversion (entry 5). Acetonitrile displays a stronger detrimental effect (entry 6). It is noteworthy that chloride and MeCN have been reported as inhibitors of  $\text{THPTA}/\text{Cu}^{\text{I}}$ -catalyzed  $\text{CuAAC}$ .<sup>3c</sup> As  $\alpha$ -amino acids usually chelate copper cations, we tested the influence of

**Table 1** Formation of  $\mathbf{13}$  in buffered media<sup>a</sup>

Entry	Buffer <sup>a</sup>	pH	Conversion <sup>b</sup> (%)
1	None <sup>c</sup>	N.D. <sup>d</sup>	95
2	Carbonate	10.2	0
3	TEABC <sup>†</sup>	8.8	45
4	Borate	7.9	10
5	HEPES <sup>‡</sup>	7.6	100
6	TRIS-maleate <sup>‡</sup>	7.6	0
7	TRIS-HCl <sup>‡</sup>	7.4	0
8	Phosphate	7.4	0
9	MES <sup>‡</sup>	6.2	100
10	Acetate	4.7	0

<sup>a</sup> [Buffer] = 0.2 M; [ $\mathbf{11}$ ] = 24 mM; [ $\mathbf{12}$ ] = 48 mM; [ $\mathbf{5}$ ] = 0.48 mM; reaction time: 16 h; reactions were run in air. <sup>b</sup> By  $^1\text{H NMR}$ . <sup>c</sup> Using the monosodium salt of  $\mathbf{11a}$ . <sup>d</sup> Not determined.

**Table 2** Influence of additives during the formation of  $\mathbf{13}$

Entry	Additive <sup>a</sup>	Conversion <sup>b</sup> (%)
1	$\text{MeOH}$ (25% v/v)	100
2	$\text{DMSO}$ (25% v/v)	100
3	$\text{NMP}^{\ddagger}$ (25% v/v)	100
4	$\text{HFIP}^{\ddagger}$ (25% v/v)	100
5	$\text{NaCl}$ (0.1 M)	80
6	$\text{MeCN}$ (25% v/v)	50

<sup>a</sup> In 0.2 M HEPES pH 7.6; [ $\mathbf{11}$ ] = 24 mM; [ $\mathbf{12}$ ] = 48 mM; [ $\mathbf{5}$ ] = 0.48 mM; reaction time: 16 h; reactions were run in air. <sup>b</sup> By  $^1\text{H NMR}$ .

**Table 3** Formation of  $\mathbf{13}$  in the presence of L-amino acids

Entry	L-Amino acid <sup>a</sup>	$\mathbf{5}^b$	Buffer (pH) <sup>c</sup>	Conversion <sup>d</sup> (%)
1	Alanine	2	HEPES (7.6)	0
2	Cysteine	2	HEPES (7.6)	0
3	Glutathione	2	HEPES (7.6)	0
4	<i>N</i> -Acetyl alanine	2	HEPES (7.6)	100
5	<i>N</i> -Acetyl cysteine	2	HEPES (7.6)	100
6	<i>N</i> -Acetyl methionine	2	HEPES (7.6)	100
7	<i>N</i> -Acetyl histidine	2	HEPES (7.6)	~5
8	<i>N</i> -Acetyl histidine	2	MES (6.2)	40
9	<i>N</i> -Acetyl histidine	2	MES (5.7)	70
10	<i>N</i> -Acetyl histidine	5	MES (6.2)	100

<sup>a</sup> 20 mol%. <sup>b</sup> mol%. <sup>c</sup> [Buffer] = 0.2 M; [ $\mathbf{11}$ ] = 24 mM; [ $\mathbf{12}$ ] = 48 mM; reaction time: 16 h; reactions were run under air. <sup>d</sup> By  $^1\text{H NMR}$ .

unprotected L-amino acids and glutathione, a tripeptide containing an  $\alpha$ -amino acid unit (Table 3). Clearly  $\alpha$ -amino acids behave as strong inhibitors (entries 1–3), whereas satisfactory results were obtained with a tenfold excess (compared to the catalyst) of *N*-acetylated derivatives: a full conversion took place in the presence of *N*-acetyl alanine, cysteine and methionine (entries 4–6). A lack of reactivity was observed at pH 7.6 with *N*-acetyl histidine (entry 7). Nevertheless, this inhibition seems pH-dependent as 40% and 70% conversions were reached by lowering the pH to 6.2 and 5.7 (0.2 M MES buffer, entries 8 and 9), respectively. The inhibition was finally suppressed using 5 mol% of  $\mathbf{5}$  (0.25 equiv./His) at pH 6.2 (entry 10).

Histidine side chain's imidazole is known to participate in the chelation of several metals through N1 binding. As the imidazole's H2 chemical shift is a good indicator of the protonation state (protonated form:  $\delta = 8.40$  ppm, basic form  $\delta = 7.45$  ppm),<sup>10</sup> we examined the aromatic  $^1\text{H NMR}$  signature of *N*<sup>z</sup>-acetylated histidine and found that H2 appears at 8.0 ppm at pH 7.6 (0.2 M HEPES) and 8.4 ppm at pH 6.2 (0.2 M MES), confirming, respectively, a partial and a total protonation ( $\text{ESI}^{\dagger}$ ). Thus, protonation appears to protect catalyst  $\mathbf{5}$  from imidazole inhibition. The pH effect on the kinetics was examined by  $^1\text{H NMR}$  (Fig. 3). Clearly,  $\mathbf{13}$  forms more rapidly in slightly basic ( $\sim 3$  h at pH 7.6) rather than acidic media ( $> 15$  h at pH 6.2).

Inspection of the initial curves also highlights the existence of an induction period indicating that the catalytic species is probably not  $\mathbf{5}$ . As the induction period decreases with increasing pH, we hypothesize that the hydrolysis of the copper-iodide bond is of importance in this process.

To prove the applicability of  $\mathbf{5}$  to peptide synthesis, the  $\text{CuAAC}$  ligation of model pentapeptides comprising residues sensitive to copper-mediated oxidation (His, Met, Cys) was examined.

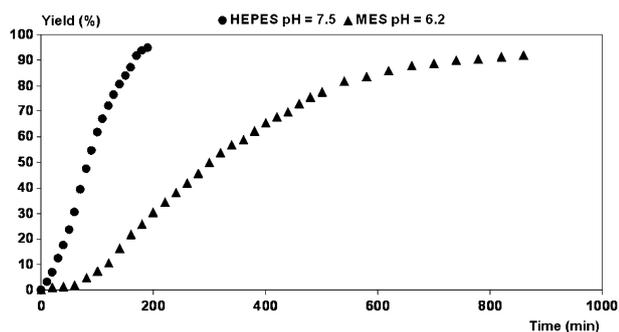
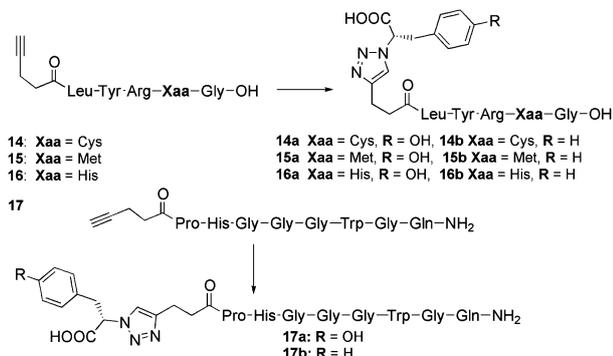


Fig. 3 Kinetics of the formation of **13** at pH 6.2 and 7.6 (ESI<sup>+</sup>).



Scheme 3 Catalyzed reactions of peptides in 0.2 M HEPES, pH 7.6.

The three alkyne-containing peptides **14–16** were reacted at a dilute 1 mM concentration with five fold excess of the azides N<sub>3</sub>Tyr-OH (**11a**) or (*S*)-2-azido-3-phenyl-propanoic acid (N<sub>3</sub>Phe-OH, **11b**) in a 8–2 mixture of 0.2 M HEPES, pH 7.6 and HFIP (Scheme 3).

The reactions were run overnight in an open flask at RT, then worked up through a C18 solid phase extraction column and analyzed by standard reverse phase HPLC/HRMS (ESI<sup>+</sup>). The reaction of thiol-containing **14** gives a full conversion to the triazole products using either 40 or 100 mol% of **5**. As expected from a long stay at neutral pH under non-deoxygenated conditions, the products were obtained as the homodimeric disulfides, but no over-oxidation into disulfide oxide, sulfenic or sulfonic acids could be detected. Therefore, treatment of the crude mixture with 5-fold excess of TCEP cleanly delivers the expected products **14a,b** (ESI<sup>+</sup>). The conversion of sulfide-containing **15** into **15a,b** was also clean and complete using both 40 and 100 mol% of catalyst and no oxidation to the methionine sulfoxide was observed. Regarding imidazole-containing **16**, an incomplete reaction occurred using 40 mol% of catalyst in 0.2 M HEPES, pH 7.6 (~60% conversion). Nevertheless, a total conversion was reached with one full equivalent of **5** to afford **16a,b** (ESI<sup>+</sup>). No trace of oxidation into the imidazolone commonly observed under standard CuAAC catalytic systems was detected.<sup>3d</sup> The reaction also applies quantitatively to peptide **17** (ESI<sup>+</sup>), which corresponds to the *N*-terminal octapeptide repeat region of the human prion protein (PrP<sup>C</sup>) sequence and is known to strongly bind Cu<sup>II</sup>.<sup>11</sup> This proves that good copper(II) chelators do not inhibit CuAAC mediated by **5**.

In conclusion we report the first soluble copper(I)-NHC that can realize CuAAC reactions with peptides in buffered aqueous media in the absence of sacrificial reducing agents and

other protective additives. Ligations are performed at low concentrations under open air conditions on oxidation-sensitive peptides, *i.e.*: containing methionine, cysteine, arginine, tyrosine, tryptophan and histidine. The applicability of this methodology to other bio(macro)molecules is currently studied. We believe that our preliminary results pave the way for a new generation of CuAAC catalysts that will make this cycloaddition a user-friendly tool for the bioconjugation of peptides and proteins.

We thank Philippe Marceau for synthesizing the peptides.

## Notes and references

‡ SIMEs = 1,3-bis(2,4,6-trimethylphenyl)imidazolin-2-ylidene; Icy = 1,3-bis(cyclohexyl)imidazol-2-ylidene; TEABC: triethylammonium bicarbonate; HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; TRIS: tris(hydroxymethyl)methylamine; MES: 2-(*N*-morpholino)-ethanesulfonic acid; NMP: *N*-methyl pyrrolidone; HFIP: hexafluoroisopropanol; TCEP: tris(2-carboxyethyl)phosphine.

§ Our result contrasts with what was reported with the parent SIMEs and IMES ligands which afford a mixture of homo and heteroleptic complexes (23/77).<sup>12</sup> Steric hindrance and/or charge repulsion in **5** could account for this result.

- (a) C. W. Tornøe and M. Meldal, in *Peptides—The wave of the future*, ed. M. Lebl and R. A. Houghten, Kluwer Academic Publishers, Dordrecht, 2001, pp. 263–264; (b) H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021; (c) C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064; (d) M. Meldal and C. W. Tornøe, *Chem. Rev.*, 2008, **108**, 2952–3015.
- R. K. V. Lim and Q. Lin, *Chem. Commun.*, 2010, **46**, 1589–1600.
- (a) V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew. Chem., Int. Ed.*, 2009, **48**, 9879–9883; (b) V. Hong, A. K. Udit, R. A. Evans and M. G. Finn, *ChemBioChem*, 2008, **9**, 1481–1486; (c) A. Kumar, K. Lib and C. Cai, *Chem. Commun.*, 2011, **47**, 3186–3188; (d) I. E. Valverde, F. Lecaillon, G. Lalmanach, V. Aucagne and A. F. Delmas, *Angew. Chem., Int. Ed.*, 2012, **51**, 718–722; (e) S. I. Presolski, V. Hong, S.-H. Cho and M. G. Finn, *J. Am. Chem. Soc.*, 2010, **132**, 14570–14576.
- (a) S. Diez-González, A. Correa, L. Cavallo and S. P. Nolan, *Chem.–Eur. J.*, 2006, **12**, 7558–7564; (b) S. Diez-González and S. P. Nolan, *Angew. Chem., Int. Ed.*, 2008, **47**, 8881–8884; (c) M. L. Teyssot, A. Chevry, M. Traïkia, M. El-Ghozzi, D. Avignat and A. Gautier, *Chem.–Eur. J.*, 2009, **15**, 6322–6326; (d) M.-L. Teyssot, L. Nauton, J.-L. Canet, F. Ciseti, A. Chevry and A. Gautier, *Eur. J. Org. Chem.*, 2010, 3507–3515; (e) W. Wang, J. Wu, C. Xia and F. Li, *Green Chem.*, 2011, **13**, 3440–3445.
- For a review on hydrophilic catalysts: K. H. Shaughnessy, *Chem. Rev.*, 2009, **109**, 643–710.
- S. Leuthäuser, V. Schmidts, C. M. Thiele and H. Plenio, *Chem.–Eur. J.*, 2008, **14**, 5465–5481.
- J. Andersen, U. Madsen, F. Bjorkling and X. F. Liang, *Synlett*, 2005, 2209–2213.
- (a) E. D. Goddard-Borger and R. V. Stick, *Org. Lett.*, 2007, **9**, 3797–3800; (b) N. Fischer, E. D. Goddard-Borger, R. Greiner, T. M. Klapötke, B. W. Skelton and J. Stierstorfer, *J. Org. Chem.*, 2012, **77**, 1760–1764.
- HEPES and MES were originally designed to minimize metal chelation, see: N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, *Biochemistry*, 1966, **5**, 467–477.
- E. L. Ash, J. L. Sudmeier, R. M. Day, M. Vincent, E. V. Torchilin, K. Coffman Haddad, E. M. Bradshaw, D. G. Sanford and W. W. Bachovchin, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 10371–10376.
- (a) M. Łuczowski, H. Kozłowski, M. Stawikowski, K. Rolka, E. Gaggelli, D. Valensin and G. Valensin, *J. Chem. Soc., Dalton Trans.*, 2002, 2269–2274; (b) A. P. Garnett and J. H. Viles, *J. Biol. Chem.*, 2003, **278**, 6795–6802.
- S. Diez-González, E. C. Escudero-Adán, J. Benet-Buchholz, E. D. Tevens, A. M. Z. Slawin and S. P. Nolan, *Dalton Trans.*, 2010, **39**, 7595–7606.