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Fixed-charge labels for simplified reaction analysis: 5-hydroxy-1,2,3-triazoles as byproducts of a copper(I)-catalyzed click reaction

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ABSTRACT

High-resolution multistage mass spectrometric studies of isotope-labelled derivatives of a fixed-charge labelled sugar triazole assisted the identification of 5-hydroxy-1,2,3-triazoles as byproducts of the copper(I)-catalyzed cycloaddition of azides and terminal alkynes. Reaction optimization with inclusion of the auxiliary ligand, tris(benzyltriazolylmethyl)amine furnished an improved ligation protocol in which formation of the 5-hydroxytriazole is mitigated.

The copper(I)-catalyzed azide and terminal alkyne cycloaddition (CuAAC) reaction is a powerful and remarkably general conjugation reaction (Fig. 1A).[1–4] Since its introduction less than 10 years ago, it has grown to become one of the preferred ligation methods for high-yielding bioconjugations.[5,6] The rapid acceptance and utilization of the reaction in a range of fields[7–9] point to the unmet need for high-yielding, operationally simple and efficient conjugation methods.[10] However, the reaction does not always proceed without complication—Sharpless and co-workers noted that in some cases side products were formed, and without reporting any characterization, cited the formation of diacetylenes, 5,5′-bis-triazoles and 5-hydroxytriazoles (Fig. 1B).[1,5] Angell and Burgess reported that 5,5′-bis-triazoles can constitute the major products formed in the presence of Cu/$CuSO_4$, potassium carbonate and air.[11] 1,4,5-Trisubstituted-triazoles bearing a 5-alkynyl group are the major products formed in the presence of $Cu(CH_3CN)_4PF_6$, trimethylethylenediamine, molecular oxygen and *N*-methylmorpholine *N*-oxide,[12] and are also significant byproducts in the Angell and Burgess procedure.[11] Here we report on the formation and characterization of 5-hydroxytriazoles as byproducts in the CuAAC reaction and report conditions to prevent their formation.

In our laboratory, attempts to use fixed-charge labels to assist in mass spectrometric analysis of the product of the CuAAC reaction between the alkyne-bearing carbohydrate **1** and the azide-functionalized phosphonium salt **2a**, namely the fixed-charge labelled sugar **3a**, were complicated by the regular occurrence of an oxidized product (i.e., $[M+16]^+$ vs the target triazole; Scheme 1). Fixed charge tags labels have three important benefits: (i) they can be readily used to monitor the formation of products of metal-mediated reactions using electrospray ionization mass spectrometry (ESI-MS);[13–16] (ii) the fixed-charge tag provides ionization enhancement leading to improved sensitivity of detection;[17–19] and (iii) the localization of the charge onto a known centre in a

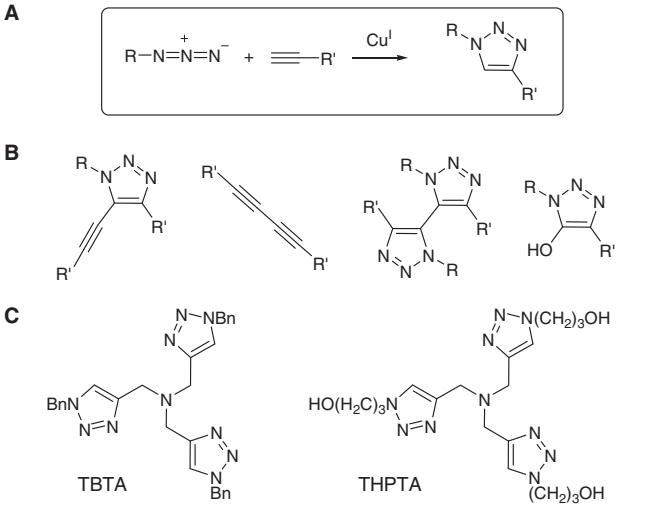

Figure 1. (A) Copper(I)-catalyzed azide and terminal alkyne cycloaddition (CuAAC) reaction. (B) Byproducts of the CuAAC reaction. (C) Auxiliary ligands for the CuAAC reaction.

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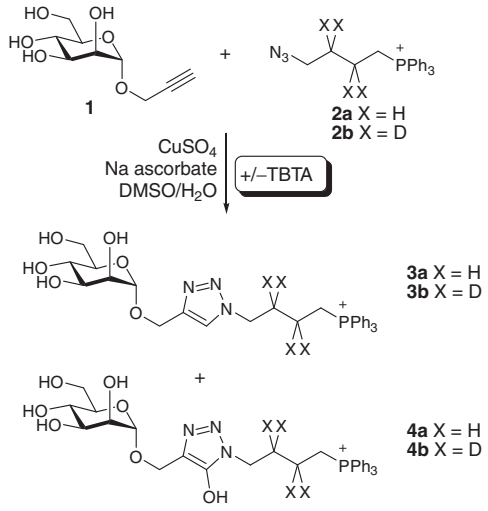


Scheme 1. Copper(I)-catalyzed azide–alkyne cycloaddition of sugar alkyne **1** and fixed-charge tags **2a** and **2b**.

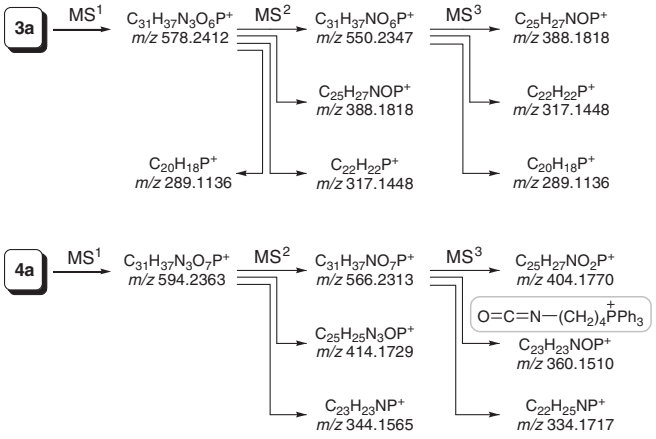
molecule can lead to well-defined fragmentation pathways, thereby aiding in structural characterization.[20] Here we use these properties to our advantage to characterize structurally this oxidation product, which was finally unequivocally identified as the 5-hydroxytriazole **4a**. Fixed-charge labels were then used to facilitate the rapid screening of reaction conditions leading to a new protocol that minimized formation of the oxidation product.

Screening of a range of reaction conditions identified that at low copper concentrations the $[M+16]^+$ product could constitute the major product formed when using $CuSO_4$/sodium ascorbate in DMSO/water. As a first step to elucidating the location of the new oxygen, the mixture of products was subjected to deuterium exchange of acidic protons by performing the reaction in D_2O and then exchanging back to protons by dissolving the products in H_2O. D/H exchange of the triazole **3a** resulted in a shift of four mass units due to the presence of the four hydroxy groups of the sugar moiety, whereas D/H exchange of the putative 5-hydroxytriazole **4a** caused a shift by five mass units, consistent with the presence of a new acidic proton.

Collision-induced dissociation (CID) of **3a** afforded a series of peaks resulting from: loss of N_2 ($[M-N_2]^+$ m/z 550); loss of the mannosyl group ($[M-C_6H_{10}O_5]^+$ m/z 388); loss of the sugar moiety and the triazole ring ($[M-C_9H_{15}O_6N_3]^+$ m/z 317); and a fragment corresponding to $CH_2CHPPh_3^+$ (m/z 289) (Scheme 2). MS^3 of $[M-N_2]^+$ yielded the same three fragments. Further evidence supporting the assignment of these fragments was obtained from CID-MS spectra of derivatives labelled at the sugar hydroxys (by D_2O exchange), at C5 of the triazole (by performing the CuAAC reaction in D_2O and D/H exchange into H_2O) and in the butylene linker (**3b**, from commercial 2,2,3,3-d_4-1,4-dibromobutane).

CID-MS^2 of **4a** afforded fragments resulting from: loss of N_2 ($[M-N_2]^+$ m/z 566); loss of the mannosyloxy group ($[M-C_6H_{12}O_6]^+$ m/z 414); and fragmentation of the triazole ($[M-C_8H_{14}N_2O_7]^+$ m/z 344) (Scheme 2). These assignments were confirmed by HRMS and deuterium labelling in the sugar and 5-hydroxy group (by D_2O exchange), and in the butylene linker (**4b**, from commercial 2,2,3,3-d_4-1,4-dibromobutane).

In both cases major fragmentation channels arise from the loss of N_2 from the triazole fragment, which confirms that the oxidation of the triazole is not on nitrogen. Definitive evidence for the 5-hydroxytriazole structure was obtained through the observation of the unique fragmentation pathway m/z 594 (M^+)→566→360, which we assign to the formation of an isocyanate with N–C–O connectivity (Scheme 2 and Fig. 2).[21]



Scheme 2. Summary of the fragment ions of **3a** and **4a** observed using high resolution multistage mass spectrometry. For a graphical summary see the Supplementary data.

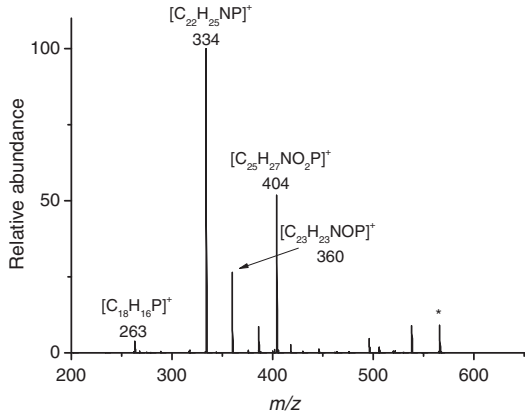


Figure 2. MS^3 spectrum showing the fragmentation of [5-hydroxytriazole **4a**–N_2] (m/z 566), designated with an asterisk. Assignments were verified using HRMS.

In order to investigate the origin of the oxygen of the 5-hydroxytriazole, we performed a CuAAC reaction in $H_2{}^{18}O$. Mass spectrometric analysis of the reaction mixture did not indicate the presence of any ^{18}O incorporation into **4**. We speculate that oxygen incorporation arises from molecular oxygen, possibly via superoxide ion formed from the reaction of oxygen with Cu^{II}-ascorbate. $CuSO_4$/sodium ascorbate affords hydrogen peroxide in amounts dependent on both $CuSO_4$ and ascorbate concentrations,[22] with peroxide or superoxide most likely effecting oxidative demetallation of the intermediate copper(I)-triazolide. Reactive oxygen species may arise through the ascorbate (AH^-)-mediated copper-dependent (Eq. 1) and copper-independent processes (Eqs. (2)–(4)).[23,24] Fenton-like processes with Cu^I and Cu^{II} may result in the formation of $HO^\bullet$ and $HOO^\bullet$ leading to oxidation reactions.

$$Cu^{II}.AH^- \rightarrow Cu^{I}.A^{-\cdot} + H^+ \quad (1)$$

$$AH^- + O_2 \rightarrow A^{-\cdot} + HO_2^\cdot \quad (2)$$

$$A^{-\cdot} + HO_2^\cdot \rightarrow A + HO_2^- \quad (3)$$

$$A^{-\cdot} + O_2 \rightarrow A + O_2^{-\cdot} \quad (4)$$

In order to assess whether the formation of the 5-hydroxytriazole byproduct is specific to the combination of substrates **1** and **2**, we investigated the CuAAC cycloaddition of other substrate combi-

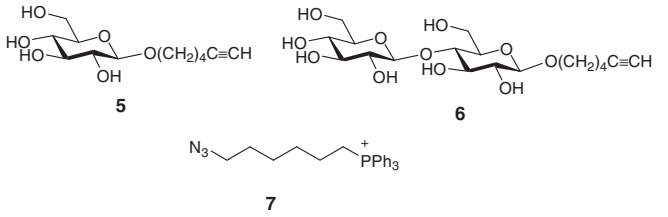


Figure 3.

nations in which the nature of the sugar, alkyne length and spacing between the azide and phosphonium groups were varied (Fig. 3). In every case, $[M+16]^+$ adducts were observed for various combinations of the sugar alkynes **1**, **5** and **6** and the phosphonium azides **2a** and **7** (Table 1).

A wide range of conditions have been reported for CuAAC conjugations with specialized protocols being required for the most challenging bioconjugation applications.[22,25–28] Many of these protocols do not allow complete analysis of reaction mixtures and we suspect that oxidation products have, in the majority of cases, been overlooked. Incompletely characterized $[M+16]^+$ adducts were identified in the original report of the CuAAC reaction by Sharpless and co-workers,[5] and more recently in the oxidation of histidine residues to 2-oxo-histidine, a problem that was resolved by the inclusion of THPTA as a sacrificial reductant (Fig. 1C).[22] 4-Hydroxytriazoles have been proposed as byproducts in the regioselective formation of 1,5-disubstituted-1,2,3-triazoles from the addition of bromomagnesium acetylides to azides.[29]

To minimize the formation of 5-hydroxytriazoles, we studied the optimization of the reaction conditions through variation of copper and sodium ascorbate concentrations, and the inclusion of the auxiliary ligands tris(benzyltriazolylmethyl)amine (TBTA) and tris(hydroxypropyltriazolylmethyl)amine (THPTA) at various concentrations. Crude reaction mixtures were analyzed by ESI-MS with the fixed-charge phosphonium tags allowing sensitive and semi-quantitative monitoring of consumption of **2a** and formation of **3a** and **4a**. Optimal reaction conditions involved the use of [$CuSO_4$] = 0.2 mM, [sodium ascorbate] = 20 mM, [TBTA] = 0.2 mM with [**2a**] = 5 mM and [**1a**] varying from 0.005 to 5 mM. Under these conditions, the cycloaddition was complete within 2 h with essentially no contamination by the 5-hydroxytriazole (Fig. 4). A noteworthy feature of the new conditions is the use of higher concentrations of both copper source and TBTA. These conditions are complementary to those of Hong et al. which were optimized for bioconjugation applications.[22,25] Taken together, these results

Table 1
5-Hydroxytriazoles formed from CuAAC reaction of alkynes **1**, **5** and **6** and phosphonium azides **2a** and **7**[a]

Entry	Alkyne	Azide	ESI-MS molecular ion[b]	
			Observed	Calculated
1	**1**	**7**	Triazole: 606.27	606.27
			5-OH: 622.27	622.27
2	**5**	**2a**	Triazole: 620.29	620.29
			5-OH: 636.28	636.28
3	**5**	**7**	Triazole: 648.32	648.32
			5-OH: 664.32	664.31
4	**6**	**2a**	Triazole: 782.34	782.34
			5-OH: 798.33	798.34
5	**6**	**7**	Triazole: 810.37	810.37
			5-OH: 826.37	826.37

[a] CuAAC reactions were performed using [azide] = 6.7 mM, [alkyne] = 6.7 mM, [$CuSO_4$] = 0.083 mM, [sodium ascorbate] = 8.3 mM in DMSO/H_2O 1:5 at 4 °C for 24 h.

[b] See Supplementary data for mass spectra.

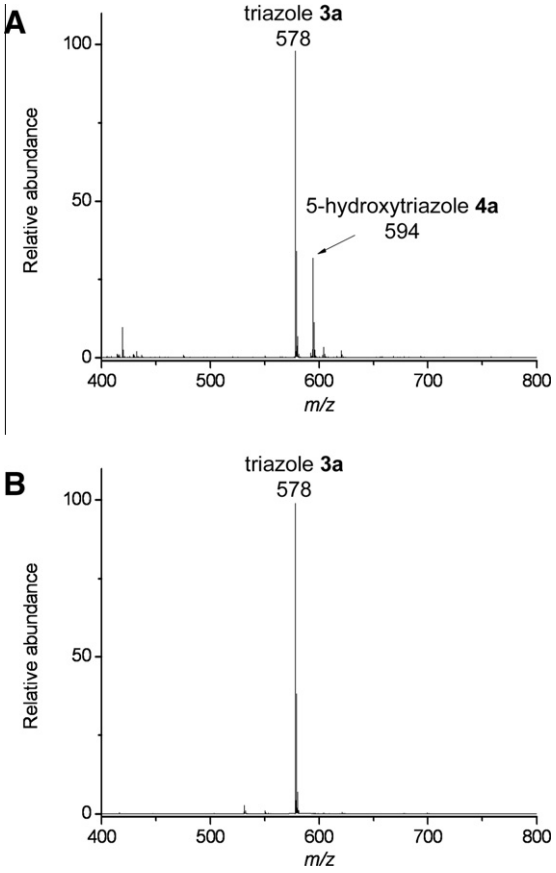


Figure 4. MS spectra of CuAAC reactions of equimolar **1** and **2a**. Reactions were run in 1:2 DMSO/H_2O. (A) Initial protocol ([$CuSO_4$] = 0.016 mM, [sodium ascorbate] = 0.16 mM, [TBTA] = 0.016 mM). (B) Optimized protocol ([$CuSO_4$] = 0.2 mM, [sodium ascorbate] = 20 mM, [TBTA] = 0.2 mM).

support the functional roles of TBTA (or THPTA)[30,31] to include: (1) controlling the coordination sphere of the Cu^I to prevent unwanted activation of molecular oxygen; (2) acting as a sacrificial reductant to prevent oxidation of the substrate by reactive oxygen species; (3) stabilizing the Cu^I oxidation state preventing disproportionation, thereby increasing the turnover number per active metal centre; and (4) increasing the thermodynamic driving force for the reduction of Cu^{II} to catalytic Cu^I.[32]

In conclusion, a fixed-charge derivative has been used for the first time to monitor and characterize the products of the CuAAC reaction via ESI-MS and to screen rapidly for new reaction conditions to minimize formation of the 5-hydroxytriazole oxidation product. A further benefit of these fixed-charge conjugated carbohydrates is that their metabolites can be readily interrogated by MS. Such studies are currently underway.

Protocol for the formation of triazole **3a** and 5-hydroxytriazole **4a**: Sodium ascorbate (40 μL of a 25 mM aqueous solution, 1 μmol) was added to an Eppendorf tube containing alkyne (**1**) (20 μL of a 40 mM aqueous solution, 800 nmol), azide (**2a**) (20 μL of a 40 mM DMSO solution, 800 nmol) and $CuSO_4$ (40 μL of a 0.25 mM aqueous solution, 10 nmol). The tube was capped and vortexed for 10 s then kept at 4 °C for 3 h before analysis by ESI-MS.

Optimized protocol for the formation of triazole **3a**: Sodium ascorbate (40 μL of an 800 mM aqueous solution, 32 μmol) was added into an Eppendorf tube containing a mixture of alkyne (**1**) (20 μL of a 40 mM aqueous solution, 800 nmol), azide (**2a**) (20 μL of a 40 mM DMSO solution, 800 nmol), $CuSO_4$ (40 μL of an 8 mM aqueous solution, 320 nmol) and TBTA (40 μL of an 8 mM DMSO solution, 320 nmol). The tube was capped and vortexed for 10 s then kept at room temperature for 2 h before analysis by ESI-MS.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.03.094.

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