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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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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.

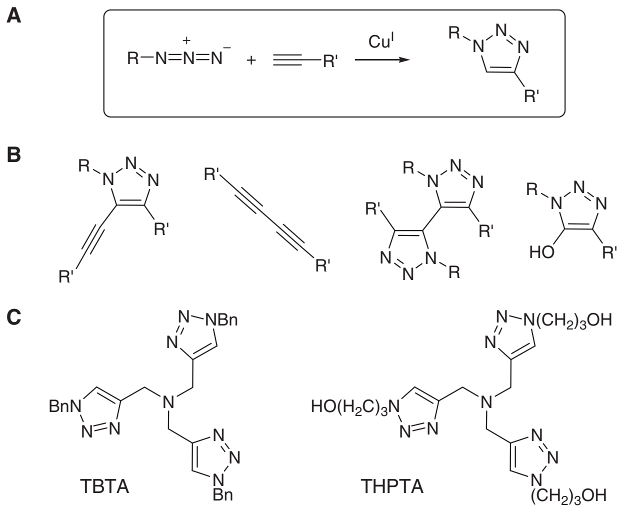

Figure 1. (A) Copper(I)-catalyzed azide and terminal alkyne cycloaddition (CuAAC) reaction. (B) Byproducts of the CuAAC reaction. (C) Auxiliary ligands for the CuAAC reaction.

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

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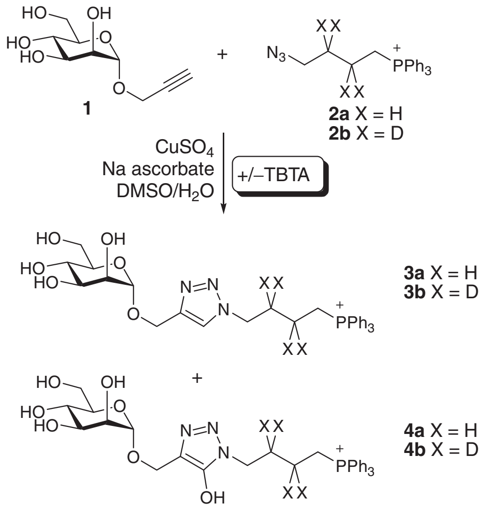


Scheme 1. Copper(I)-catalyzed azide–alkyne cycloaddition of sugar alkyne **1** and fixed-charge tags **2a** and **2b**.

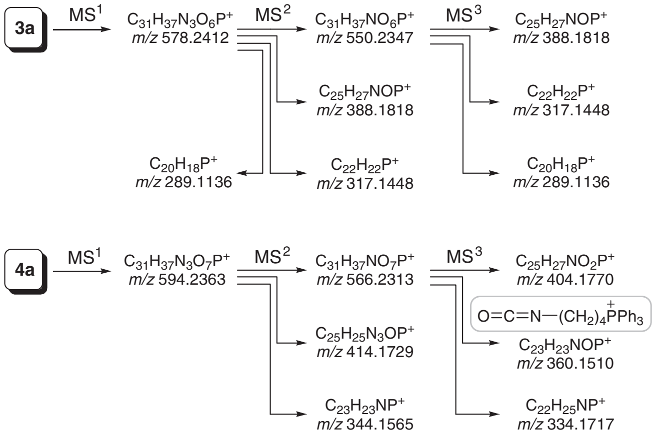
molecule can lead to well-defined fragmentation pathways, thereby aiding in structural characterization.[20] Here we use these properties to our advantage to characterize structurally this oxidation product, which was finally unequivocally identified as the 5-hydroxytriazole **4a**. Fixed-charge labels 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.

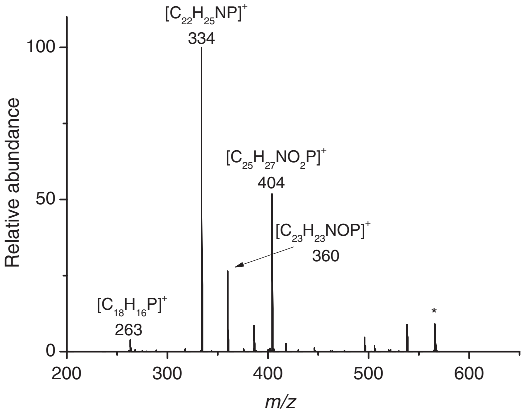


Figure 2. MS^3 spectrum showing the fragmentation of [5-hydroxytriazole **4a**$-N_2$] (m/z 566), designated with an asterisk. Assignments were verified using HRMS.

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.

$$Cu^{II}.AH^- \rightarrow Cu^{I}.A^{-\bullet} + H^+ \tag{1}$$

$$AH^- + O_2 \rightarrow A^{-\bullet} + HO_2^\bullet \tag{2}$$

$$A^{-\bullet} + HO_2^\bullet \rightarrow A + HO_2^- \tag{3}$$

$$A^{-\bullet} + O_2 \rightarrow A + O_2^{-\bullet} \tag{4}$$

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-