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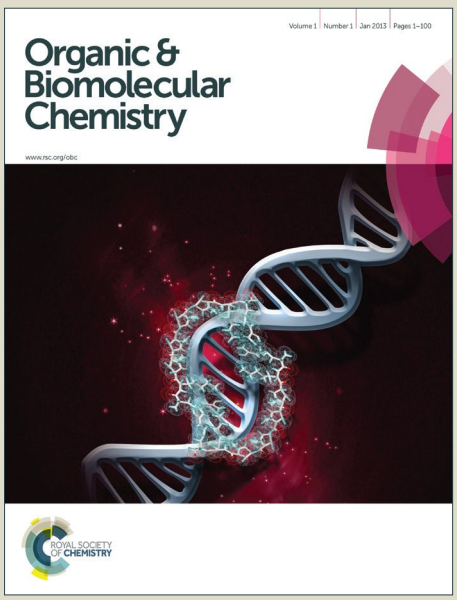

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Synthesis of the copper chelator TGTA and evaluation of its ability to protect biomolecules from copper induced degradation during copper catalyzed azide-alkyne bioconjugation reactions

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F. S. Ekholm,*[a,b] H. Pynnönen,[b] A. Vilkman,[b] J. Koponen,[b] J. Helin[b] and T. Satomaa*[b]

One of the most successful bioconjugation strategies to date is the copper(I)-catalyzed cycloaddition reaction (CuAAC), however, the typically applied reaction conditions have been found to degrade sensitive biomolecules. Herein, we present a water soluble copper chelator which can be utilized to protect biomolecules from copper induced degradation.

Bioconjugates are compounds consisting of a chemical agent covalently linked to a biomolecule. The structure of the conjugates may vary depending on their intended use, e.g. 1) fluorescently labelled biomolecules can be utilized to study the location of biomolecules in cells and animals[1]; 2) drug-biomolecule conjugates (*e.g.* antibody-drug conjugates) may be applied in selective drug-delivery applications[2] and 3) chemically modified biomolecules may have interesting material properties[3]. Clearly, bioconjugates are important tools in numerous multidisciplinary research areas. The construction of bioconjugates require high-yielding and specific conjugation protocols. In recent years, a number of protocols have been developed and applied to the synthesis of bioconjugates. These protocols include conjugation strategies based on maleimide/thiol chemistry,[4] Staudinger ligation techniques,[5] azide-alkyne cycloaddition reactions[6] and ketone/aldehyde-amine (oxime) chemistry[7]. Azide-alkyne cycloaddition chemistry has been viewed as one of the most promising techniques for bioconjugation reactions as these functionalities are rare in naturally occurring biomolecules in contrast to e.g. amines and thiols. The azide-alkyne cycloaddition reaction is generally divided into two separate categories; 1) copper catalyzed cycloaddition reactions (CuAAC)[8] and 2) copper-free strain-induced cycloaddition reactions[9]. While both reactions have their merits, the CuAAC reaction results in a single relatively hydrophilic triazole ring upon conjugation in contrast to the bicyclic/tetracyclic connection sites (which may also decrease the aqueous solubility) usually obtained by the use of cyclooctynes. The CuAAC-reaction should therefore be preferred when targeting of biological receptors is intended as the larger bicyclic/tetracyclic rings may have restraining effects on the biological activity due to e.g. an increased aggregation possibility and a lowered bio-availability. In addition, the strained alkynes are costly compared to other alkyne reagents.

The CuAAC reaction suffers from certain drawbacks e.g. a source of copper and a reducing agent are usually needed for the reaction to proceed.[10] In bioconjugation reactions these reagents may generate reactive oxy species and electrophiles which may cause degradation of/oxidative damage to sensitive biomolecules.[11] Initially, ligands such as the copper chelating and stabilizing molecule tris(benzyltriazolylmethyl)amine (TBTA) (see Figure 1) was developed in order to reduce the oxidative damage[11a,12] but the poor water solubility of TBTA was later reported to have a limiting effect on its ability to protect labile biomolecules from the generally applied CuAAC-reaction conditions[13].

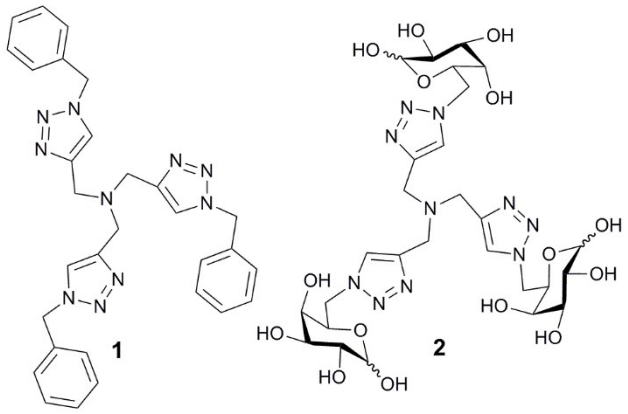

Figure 1. Chemical structures of TBTA (**1**) and TGTA (**2**).

[a] *Department of Chemistry, University of Helsinki, PO Box 55, A. I. Virtasen aukio 1, FI-00014 Helsinki, Finland. E-mail: filip.ekholm@helsinki.fi*
[b] *Glykos Finland Ltd, Viikinkaari 6, FI-00790 Helsinki, Finland. E-mail: tero.satomaa@glykos.fi*

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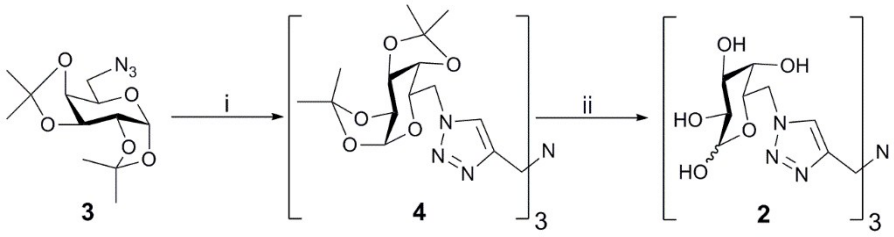
To date a number of TBTA-inspired ligands have been synthesized and screened for their applicability in bioconjugation reactions.[11a,13,14] In this paper, our recent efforts related to the synthesis of a simple water-soluble copper(I) stabilizing and chelating molecule, tris(6-galactosyltriazomethyl)amine (TGTA), for use in CuAAC bioconjugation reactions are presented. In addition, its ability to protect labile proteins under aqueous CuAAC reaction conditions is evaluated.

As mentioned, the limited aqueous solubility of TBTA was shown to limit its ability to protect sensitive biomolecules from copper induced oxidative damage during CuAAC reactions.[13] These results indicate that factors related to the solubility of the ligand should be taken into consideration when developing ligands for aqueous CuAAC bioconjugation reactions. While TBTA analogues with an increased water solubility were shown by Cai *et. al.* to be capable of protecting sensitive biomolecules, they required the addition of both $CuSO_4$ and sodium ascorbate to the reaction medium.[13] Therefore, we believe that there is still room for further improvement and optimization of the ligand structures. We envisioned that TGTA would be a good alternative to the previously reported ligands, for a number of reasons: 1) the monosaccharide pendant groups increase the water solubility of the ligand and 2) reducing sugars, such as the galactosyl moiety, have previously been shown to be able to reduce metal species as exemplified by the reduction of Ag(I) to (Ag) by similar compounds in the work of Burley *et. al.*[15] and the reduction of Cu(II) to Cu(I) by D-glucose in the work of Misra and Biswas *et. al.*[16] Based on the literature observations, TGTA should be close to an optimal compound for CuAAC bioconjugation reactions in aqueous solutions as it contains the copper chelating/stabilizing properties of TBTA (identical core), increased water solubility due to the monosaccharide pendant groups and three equivalents of the reducing ability of a reducing sugar (D-galactose) which in the best case scenario would eliminate the need for utilizing other reducing agents such as sodium ascorbate. The synthesis of TGTA commenced through the use of standard synthetic protocols and is summarized in Scheme 1. In short, commercially available tripropargylamine and azide-containing galactopyranoside **3** were subjected to a CuAAC reaction thus providing the protected form of TGTA (**4**) in excellent yield. The isopropylidene groups were removed under acidic conditions to give TGTA (**2**) in quantitative yield.

TGTA was analysed and characterised by the use of NMR-spectroscopy and mass spectrometry. Due to the symmetric nature of TGTA, the NMR-spectra were simple to solve. As expected, mutarotation occurring in the unprotected monosaccharide residues give rise to a number of stereoisomers. TGTA was found to exist as a 2:3 alpha:beta mixture of galactopyranoses (H-1α at 5.19 ppm and H-1β at 4.48 ppm) with smaller amounts of furanoses (d at 5.28 ($J_{1,2}$ = 4.7 Hz) and d at 5.21 ($J_{1,2}$ = 3.3 Hz) visible close to the baseline. The area where the H-6α and H-6β protons occur (4.75 – 4.66 ppm) was challenging to solve due to overlapping signals, however, the accurate chemical shifts and coupling constants could be obtained by the use of the spectral simulation software PERCH.[17] In the past, we have utilized this software extensively in order to obtain accurate chemical shifts and coupling constants in more demanding structures such as oligosaccharides and glycoconjugates.[18] The accurate chemical shifts and coupling constants for TGTA can be found in the supplementary information.

With the characterized ligand available, our intention was to investigate if TGTA could be utilized as a protective agent under generally applied CuAAC reaction conditions with sensitive biomolecules. Basic human fibroblast growth factor (FGF) is a small protein of the FGF-family involved in a wide range of cell-cell communication events.[19] Previously, we have worked with the modification of FGF and found that the protein decomposes under CuAAC reaction conditions in the absence of a copper(I) chelating/stabilizing agent. It was therefore selected as a suitable biomolecule for the evaluation of TGTA's protective features. Lysine residues in FGF were modified with NHS-PEG_4-N_3 by the use of conventional synthetic protocols[20] thus providing suitable linking sites for the conjugation reaction. According to MALDI-TOF mass spectrometric analysis, modification of the lysine residues resulted in the incorporation of on average 5.1 PEG_4-N_3 in FGF. In the stained SDS-PAGE gel, the FGF-PEG_4-N_3-conjugate appeared as an 18.4 KDa sized band indicating that the reaction had proceeded as expected. With the azido-functionalized FGF available, a water soluble counterpart bearing a terminal alkyne was needed for the bioconjugation reaction. 6-*O*-propargyl-D-galactose was chosen as a suitable model substrate as it is water soluble and contains an alkyne functionality required for the CuAAC reaction.



Scheme 1. Reaction conditions: i) Tripropargylamine, $CuSO_4$, sodium L-ascorbate, DMF:H_2O 3:1, RT, 40 h, quant; ii) 60 % TFA (in H_2O), 60 °C, 2.5 h, quant.

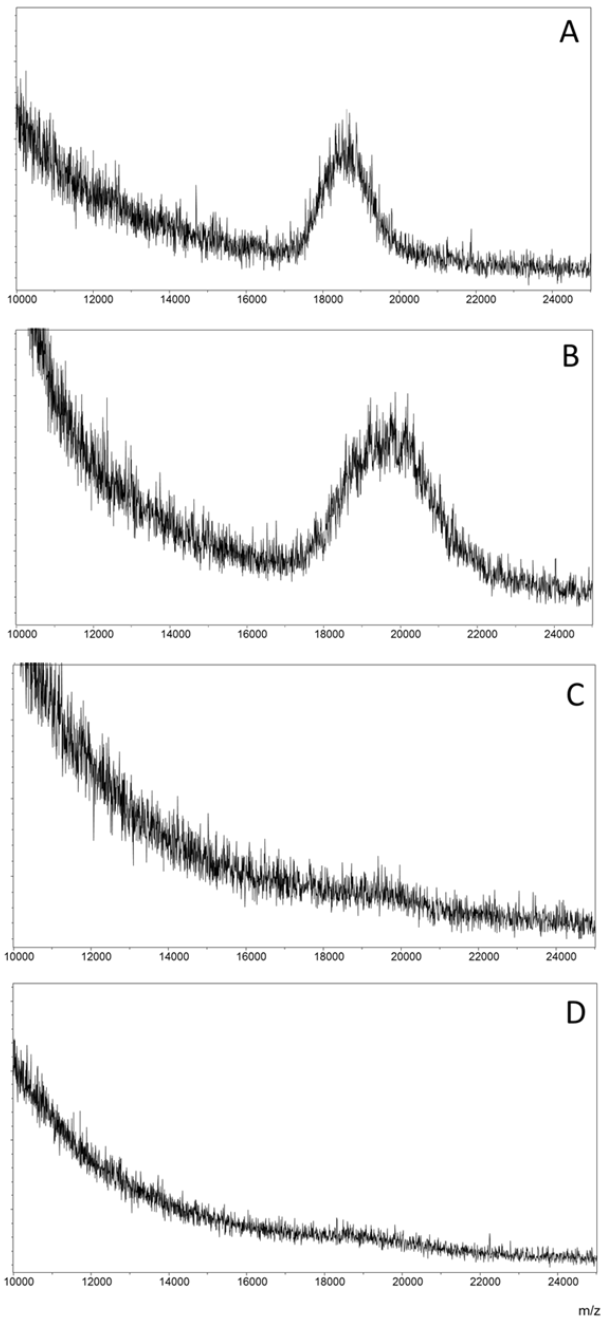


Figure 2. MALDI-TOF MS analysis of FGF-galactose conjugates. N_3-PEG_4 FGF (A), CuAAC reactions with TGTA (B), TBTA (C) and without chelator (D).

Three different bioconjugation protocols (typically applied reaction conditions featuring $CuSO_4$ and sodium ascorbate in buffered aqueous solution) were tested in order to evaluate which protocol might offer the best protection for sensitive biomolecules such as FGF in aqueous CuAAC reactions (see Figure 2).[21] The commercially available copper chelator TBTA was compared to TGTA while the reaction conditions without chelators served as a control. In the control reaction, the biomolecule was found to decompose thereby suggesting that the reaction conditions are not suitable as such for sensitive biomolecules. The limited water solubility of TBTA was circumvented by dissolving it in DMSO before addition to the reaction mixture as has been suggested in the literature.[22] Nevertheless, a precipitate formed after 30 minutes which indicates that TBTA may not be optimal for copper catalyzed bioconjugation reactions carried out in buffered aqueous solution. Despite the limited solubility of TBTA in aqueous solutions, it has been reported to retain its catalytic activity also in the heterogenous form, however, such systems were reported to use dithiothreitol as an additional copper complexation agent.[22] With TGTA as the copper chelator, the aqueous solution remained clear throughout the reaction (5-20 hour reaction time) which indicates that the TGTA-copper complex can be viewed as a homogenous one. MALDI-TOF MS analysis of the FGF conjugates revealed that with TGTA as a protective agent, an average of 4.8 6-*O*-propargyl-D-galactose-units were conjugated to the azido-functionalized FGF (see Figure 2). Clear signals could not be obtained from the reaction with TBTA or without chelator thereby suggesting that TGTA is the superior protective agent. The MALDI-analysis was in good agreement with the stained SDS-PAGE gel which revealed that the conjugation product had a 19.1 kDa sized band which corresponds to the successful incorporation of ~ four galactose residues in FGF. Smaller sized bands could not be identified which indicates that TGTA can be used as a protective agent for sensitive biomolecules such as FGF in CuAAC reactions conducted in buffered aqueous solutions.

Conclusions

Based on our observations, TGTA is a good alternative to other ligands described in the literature for CuAAC reactions with biomolecules. It is simple to prepare, water soluble and was designed to contain the copper coordinating core of TBTA and the reducing properties of D-galactose and should therefore be close to optimal for aqueous CuAAC-reactions. In this study, TGTA was shown to be compatible with reaction conditions generally applied for conjugation reactions with biomolecules. In addition, TGTA was able to protect the sensitive FGF-protein from copper induced degradation during the applied reaction conditions. While 6-*O*-propargyl-D-galactose was utilized as a model substrate in the present study, it should be noted that TGTA has been successfully applied in antibody-drug bioconjugation reactions with other substrates.[23] We are currently planning a more thorough assessment of TGTA's properties including its: catalytic properties, complexation mode and further optimization of the reaction conditions with biomolecules. Under optimal reaction conditions, a preformed TGTA-copper complex should prove to be the only needed reagent for CuAAC-reactions thus removing the need for additional reagents such as $CuSO_4$, DTT and sodium ascorbate. The results of these studies will be reported in due course.

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