Bioconjugate Chemistry

Communication

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CBTF: New Amine-to-Thiol Coupling Reagent for Preparation of Antibody Conjugates with Increased Plasma Stability

Sergii Kolodych,[†] Oleksandr Koniev,[†] Zoljargal Baatarkhuu,[†] Jean-Yves Bonnefoy,[§] François Debaene,[‡,⊥] Sarah Cianférani,[‡,⊥] Alain Van Dorsselaer,[‡,⊥] and Alain Wagner*[,†]

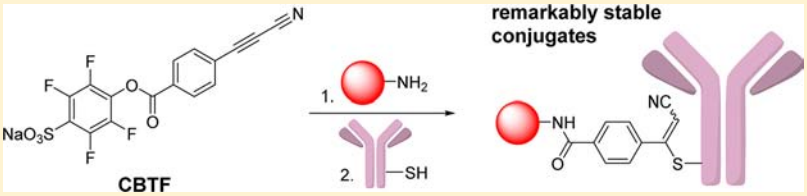
[†]Laboratory of Functional ChemoSystems (UMR 7199), Labex Medalis, University of Strasbourg, 67081 Strasbourg, France

[§]Syndivia SAS, 4 Rue Boussingault, 67000 Strasbourg, France

[‡]BioOrganic Mass Spectrometry Laboratory (LSMBO), IPHC, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg, France

[⊥]IPHC, CNRS, UMR7178, 67087 Strasbourg, France

Supporting Information



ABSTRACT: Amine-to-thiol coupling is the most common route for the preparation of antibody−drug conjugates (ADC). It is usually achieved by using heterobifunctional reagents possessing an activated ester at one end and a maleimide group at the other. However, maleimide-based conjugates were recently revealed to have limited stability in blood circulation, which can compromise therapeutic efficacy of the conjugate. To address this issue, we have developed a heterobifunctional reagent, sodium 4-((4-(cyanoethynyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (CBTF), for amine-to-thiol coupling. It comprises a recently described 3-arylpropionitrile (APN) function in replacement of maleimide and allows for the preparation of remarkably stable conjugates. A series of antibody−dye conjugates have been prepared using this reagent and shown superior stability in human blood plasma compared to maleimide-derived conjugates.

INTRODUCTION

Heterobifunctional reagents are thoroughly used in biological research for binding biological molecules to the functional entities of interest.[1] In the variety of functional groups present in biological media, amines and thiols are among the most important targets for conjugation.[2] Their high reactivity allows for chemical modification even at low concentrations in aqueous media. At the same time, they have a substantially different nucleophilicity,[3] which allows for creation of selective electrophilic reagents that target only cysteines or lysines.

The most common way of performing lysine modification is through creation of amide bonds in a reaction with activated esters. Examples of such esters include *N*-hydroxysuccinimide (NHS),[4] pentafluorophenol,[5] or *p*-nitrophenol esters. Efforts to increase water solubility of the activated esters have led to the development of sulfo-NHS and sulfotetrafluorophenol esters[6] that can be used in biological media without the risk of precipitation.

Among functional groups suitable for thiol conjugation, maleimide derivatives are the most commonly applied.[1] While offering fast reaction kinetics and being widely used, maleimides still suffer from some drawbacks. For instance, the reagent itself tends to hydrolyze when dissolved in aqueous media.[7] In addition, some recent reports have shown that maleimide-derived bioconjugates have limited *in vivo* stability, thus compromising their therapeutical applications.[8]

Indeed, stability of the adducts is a crucial parameter in the development of antibody−drug conjugates (ADCs)s—efficient biopharmaceutical drugs that combine the high selectivity of therapeutic antibodies with the high potency of small molecules.[9−11] Nevertheless, insufficient stability of the linker was recently shown to be a critical issue, since premature release of cytotoxic payload contributes to off-target toxicity.[12−14]

To address the stability issues, researchers developed a way of stabilizing maleimide-based adducts consisting of hydrolysis of the resulting thiosuccinimide ring.[15−17] Another way of producing stable ADCs consists of site-specific introduction of non-natural amino acids followed by conjugation of the payload using click chemistry.[18] At the same time, extensive research has led to the development of thiol-specific reactive groups, which prevent or retard the premature release, including sulfones,[19] allenamides,[20] and phenyloxadiazole sulfone derivatives.[21]

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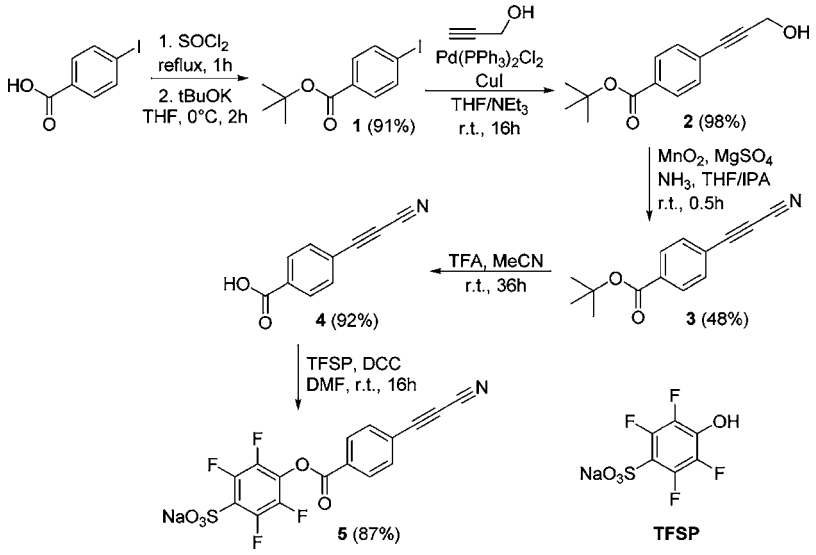
Scheme 1. Synthesis of CBTF



In this context, our group has recently reported a new class of chemical reagents, 3-arylpropiolonitriles (APN), that enable stable and specific thiol conjugation.[7] Herein we disclose the development of a new heterobifunctional APN-based reagent for amine-to-thiol conjugation and its application for preparation of stable antibody conjugates.

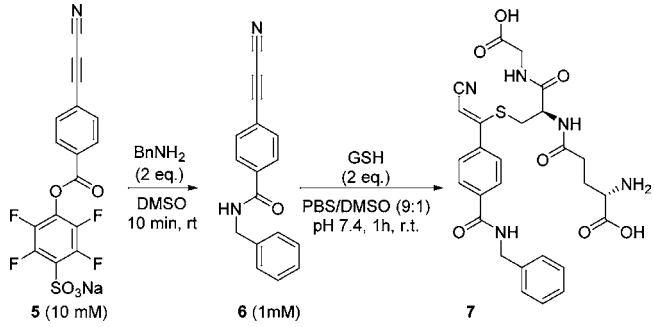
RESULTS AND DISCUSSION

The reagent for thiol-to-amine coupling **5** (CBTF, sodium (cyanoethynyl)benzoyloxy-tetrafluorobenzenesulfonate) was prepared from 4-iodobenzoic acid in 5 steps (Scheme 1). First, carboxylic group was protected by transforming it into the *tert*-butyl ester **1**. The latter was reacted with propargylic alcohol under standard Sonogashira coupling conditions to give intermediate **2**, which was oxidized with MnO_2 in the presence of ammonia according to the previously published procedure.[22] The resulting ester **3** was hydrolyzed with TFA to give the carboxylic acid **4**.

Subsequently, we tried to transform the acid **4** into the NHS-activated ester by reacting it with DCC and *N*-hydroxysuccinimide. Surprisingly, we found that *N*-hydroxysuccinimide reacted with the APN group giving a product of Michael addition as side product. After screening a panel of groups we found that polyfluorophenols do not react with APN, even when put in large excess, and therefore are more suitable for the formation of the activated ester. We replaced the commonly used pentafluorophenol with sodium 2,3,5,6-tetrafluoro-4-hydroxybenzenesulfonate (TFSP) in order to increase the solubility of the final product in water, which is an important parameter for biological applications.

The reactivity of the new heterobifunctional coupling reagent was first assessed on model substrates (Scheme 2). Thus, **5** was first reacted with 2 equiv of benzylamine at 10 mM concentration. After 10 min of incubation, HPLC showed clean conversion into the corresponding amide **6**. An aliquot of the reaction mixture was added to 2 mM solution of glutathione in PBS. After 1 h of incubation the complete conversion into the product **7** was observed (Supporting Information, Figure S1). Consistent with our previous results,[7] conjugation of APN function with thiols provided predominantly Z-isomers of the addition products.

Scheme 2. Reaction of CBTF with Model Substrates



To test the toxicity of the new linker we prepared an adduct between the acid **4** and cysteine. This product is expected to be released after full proteolytic degradation of ADCs comprising the APN-derived linker. The toxicity was assessed on HeLa and HuH-7 cell lines using MTS assay. The product showed no toxicity at up to 100 μM concentration (Supporting Information, Figure S2).

With these promising results in hand, we decided to apply this reagent for the preparation of antibody conjugates (Figure 1). For comparison, the bioconjugation was also performed with the most commonly used SMCC reagent. Using classical conjugation conditions, we first carried out the reaction between CBTF and TAMRA-NH_2. After incubation for 30 min, complete conversion into the amide **9** was observed by HPLC.

At the same time, the interchain disulfide bonds of Trastuzumab were reduced by incubating with TCEP (1.1 equiv or 2.2 equiv) at 37 °C for 1 h. The reduced antibody then was reacted with **9** at 25 °C for 12 h to give the conjugate **10**. After purification by size-exclusion chromatography, the conjugation was confirmed by SDS-PAGE. The average dye-to-antibody ratio (DAR) was determined using ESI-MS analysis.[23] CBTF reagent provided conjugates with DAR 1.9 and 3.8 for antibody reduced with 1.1 and 2.2 equiv of TCEP, respectively. The experiment carried out with SMCC yielded comparable DAR of 2.3 and 3.9. Comparison of native mass spectra showed that CBTF gives more defined conjugates with

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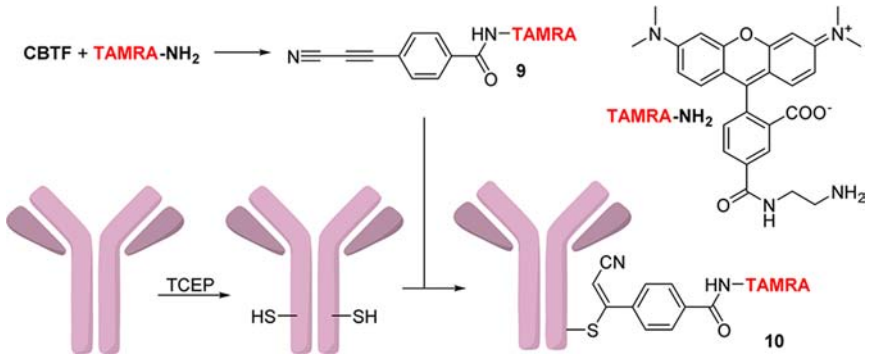

Figure 1. Conjugation of TAMRA-amine to Trastuzumab using CBTF.

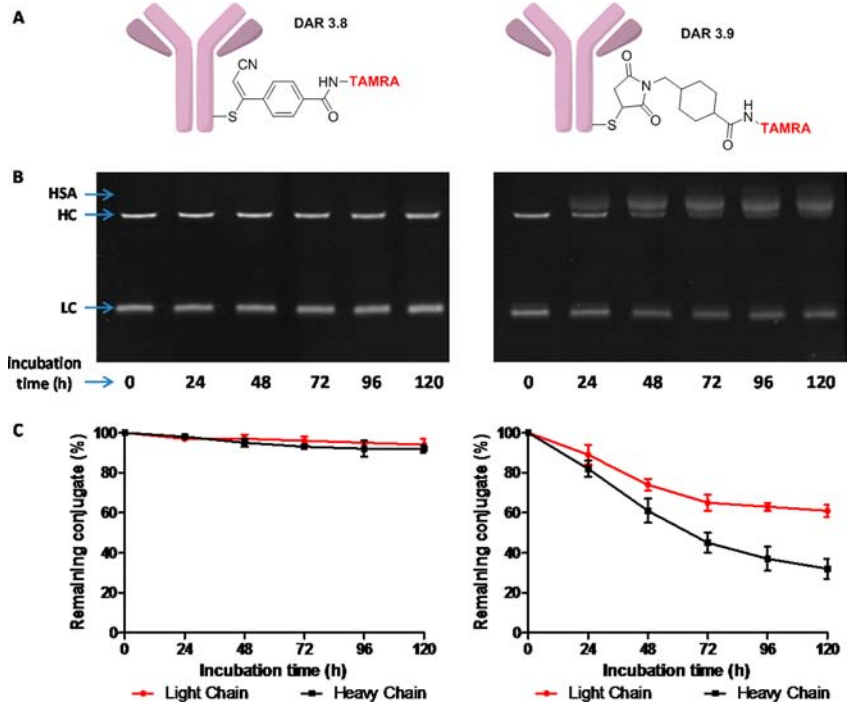

Figure 2. Comparison of plasma stability of CBTF- and SMCC-based conjugates. (A) Structure of the conjugates. (B) Fluorescent SDS-PAGE analysis of antibody−dye conjugates after incubation in human plasma. (C) Conjugate stability in human plasma.

lower numbers of species under these nonoptimized conditions (Supporting Information, Figure S3).

To test the stability in human plasma, we incubated both CBTF and SMCC conjugates with DAR 3.8 and 3.9 at 37 °C for 5 days (Figure 2). Aliquots were frozen every 24 h and analyzed by SDS PAGE. The CBTF-based conjugate showed only 2 lanes, corresponding to the labeled heavy and light chains of the antibody. In contrast, the SMCC-based conjugate showed the gradual appearance of a third lane, corresponding to the transfer of fluorophore to the HSA in accordance with the previously reported results.[13]

Quantitative analysis by integration of fluorescent lanes corresponding to the heavy chain and light chain of the antibody showed that CBTF-based conjugate had much higher stability. Indeed, in the CBTF-linked heavy chain conjugate, only 8% degradation over 120 h was observed, compared to 68% degradation of the maleimide-derived conjugate. Similarly, the CBTF-linked light chain conjugate showed only 6% degradation, compared to 39% in the case of maleimide.

CONCLUSION

In summary, we developed a new heterobifunctional reagent (CBTF) for specific amine-to-thiol conjugation. The reagent was applied for antibody conjugation. A side-by-side comparison with commercially available SMCC was performed. CBTF allowed preparation of more defined conjugates with comparable average DAR. Finally, plasma stability of CBTF and SMCC derived conjugates was studied. CBTF-based conjugates showed significantly lower level of deconjugation and payload transfer to HSA. This convenient new reagent should facilitate production of well-defined antibody−drug conjugates with superior stability in blood circulation. CBTF is also likely to find other applications where the stability of conjugates is a key requirement, such as microchip bioconjugation or long-acting therapeutics. Through an agreement with Syndivia, CBTF is now commercially available.[24]

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■ ASSOCIATED CONTENT

Supporting Information

Materials and instrumentation, experimental procedures, analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: alwag@unistra.fr.

Author Contributions

Sergii Kolodych and Oleksandr Koniev contributed equally to this work.

Notes

The authors declare no competing financial interest.

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