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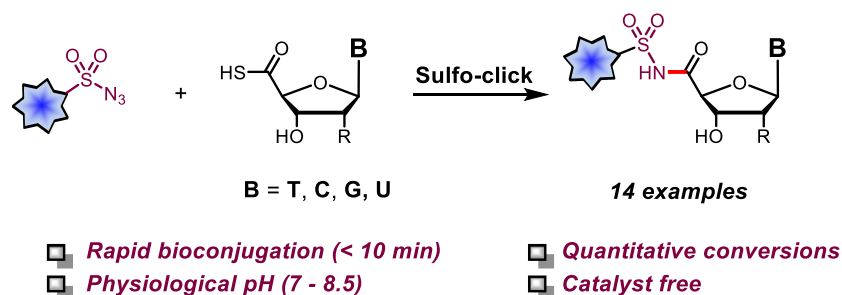
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An Entry of the Chemoselective Sulfo-Click reaction into the Sphere of Nucleic Acids

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Supporting Information Placeholder

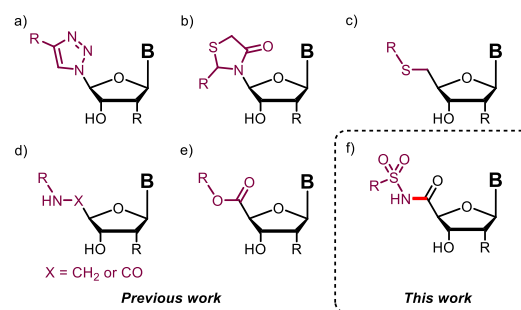


ABSTRACT: We report here the first example of a sulfo-click conjugation reaction to be applied to modified nucleosides. The reaction, which proceeds rapidly ($k \sim 4.9 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) under aqueous biocompatible conditions in both the ribo- and the deoxyribonucleoside series, affords the corresponding conjugated products in excellent yields. Furthermore, we demonstrate the orthogonality of the reaction with the copper-catalyzed azide-alkyne click reaction (CuAAC) by performing a one-pot dual labeling of a nucleoside carrying two orthogonal azido groups.

Site-specific bioconjugation and labeling of biomolecules to different partners (i.e. fluorophore, vectors, affinity tags, ...) has become an essential tool for understanding and imaging cellular processes.¹ In particular, the unique advantages of biocompatible click reactions that combine bioorthogonality, efficiency and chemoselectivity have provided important advances for the development of new diagnostic and therapeutic applications. While many bioorthogonal ligations have been applied to proteins,² lipids,³ saccharides,⁴ and DNA,⁵ the site-specific 5' modification of ribo- and deoxyribonucleosides is rather limited to classical coupling reactions⁶ or click derived methodologies⁷ that cannot support biocompatible conditions (Figure 1). The selective coupling of different labels to nucleosides is however important to increase their biological activity, drug resistance or their bioavailability but also for imaging purposes.⁸

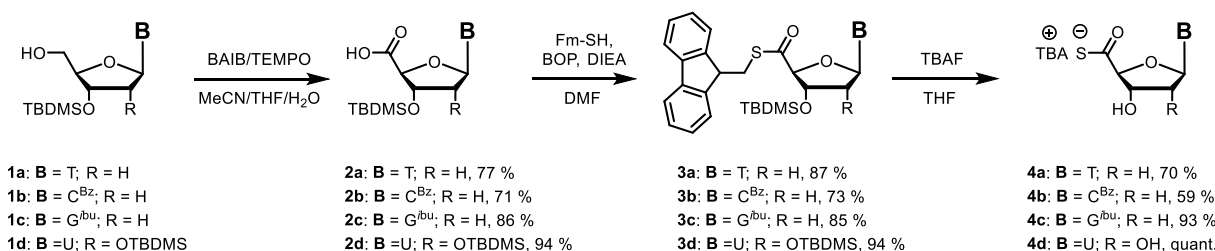
The reaction between azides and thioacids also known as thioacid-azide ligation (TAL) is an emergent surrogate click reaction that upon a stepwise addition-cycloaddition-retro [3+2] cycloaddition sequence leads to an amide linkage generating elemental sulfur and dinitrogen as the sole by-products. First described as a side reaction in 1980,⁹ it was only in 2003 that Williams and co-workers highlighted its potential as an original amidation reaction in a neutral or basic medium.¹⁰ While the use of aliphatic azides requires heating at 60 °C for several hours, it has been demonstrated that electron-deficient azides¹¹ readily react at rt. In 2010, in their study pertaining to the site-specific conjugation of peptides,

Figure 1. Known 5' nucleoside coupling reactions



Liskamp *et al.* unveiled the 'sulfo-click' between a thioacid and a sulfonyl azide,¹² which was later used in various applications, including protein and carbohydrate modification by several research groups.¹³ The reactions are generally complete within 10 min at room temperature either in organic or aqueous media in the presence of a base. Interestingly, the sulfo-click is one of the few click reactions that can generate a native amide linkage,¹⁴ and does not require obtrusive bulky or hydrophobic reactive moieties compared to other click reactions (e.g. tetrazine, norbornene, cyclooctyne...). Despite these interesting properties, the sulfo-click reaction has surprisingly never been applied to nucleic acid derivatives if we exclude the thioacid-azide

Scheme 1. Synthesis of 4'-thioacid nucleoside derivatives



ligation performed on a non-activated 3'-azido-3'-deoxythymidine with thioacetic and thiobenzoic acids at 60 °C for 36 h reported by Shangguan and co-workers.^{10a}

Considering our interest in the development of 5'-modified nucleoside derivatives for the formation of new internucleoside linkages¹⁵ and the potential of the sulfo-click reaction to generate functional bioconjugates, we decided to explore its compatibility with nucleic acids by focusing on the introduction of the thioacid moiety at the 5' position. We describe herein our endeavor and demonstrate that 4'-thioacid-modified deoxyribo- and ribonucleosides can be readily conjugated to various small molecules in water at very low concentrations.

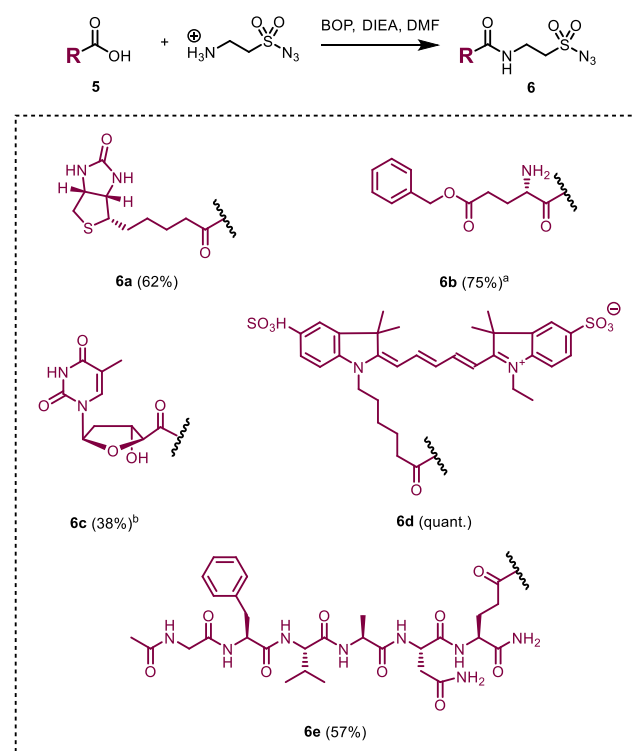
The synthesis of 4'-thioacid nucleosides was developed through an efficient three-step sequence from 3'-O-TBDMS-nucleosides (Scheme 1). The 5'-hydroxyl nucleosides **1a-d** were oxidized to the corresponding carboxylic acids **2a-d** using TEMPO in the presence of BAIB as co-oxidant.¹⁶ A coupling reaction with 9-fluorenylmethylthiol (Fm) generated the corresponding thioesters **3a-d** in high yields. Previous works have reported the *in situ* generation of thioacids in the context of the sulfo-click reaction from base-sensitive thioesters.^{13b, 17} However, Fm thioesters have been shown to be stable in aqueous media at physiological pH. Therefore, we decided to generate the unprotected 4'-thioacid nucleoside derivatives **4a-d** by treatment with TBAF in THF leading to the desired compounds in the form of tetrabutylammonium (TBA) thiocarboxylate salts. To prevent possible hydrolysis of the thioacids under acidic¹⁸ or basic¹⁹ aqueous media, the thiocarboxylate nucleosides were purified as such by normal phase chromatography. Noteworthy, all thiocarboxylate nucleosides salts were completely stable at -20 °C for months but are likely to degrade at rt.

Then, a series of sulfonyl azide partners were synthesized in one step upon classical peptide coupling conditions by using the 2-aminoethanesulfonyl azide as a short linker.²⁰ Different classes of labels were selected to demonstrate the versatility of the sulfo-click reaction applied to nucleoside derivatives; these include biotin **6a**, an amino acid **6b**, a modified nucleoside **6c**, a fluorophore **6d** and a peptide **6e** (Scheme 2).

A preliminary kinetic study run with 4'-thiocarboxylate uridine **4d** and sulfonyl azide thymidine **6c** showed that the reaction reached complete conversion within 10 min at rt under aqueous conditions (10 mM of sulfonyl azide in aq. 0.1 M NaHCO₃ buffer pH 8.5/NMP 25:75 mixture) (see ESI Figure S2). Under the aforementioned conditions, the sulfonyl azides previously synthesized (Scheme 2) were successfully conjugated to all thioacetate **4a-d** leading to various 5'-labeled nucleo-

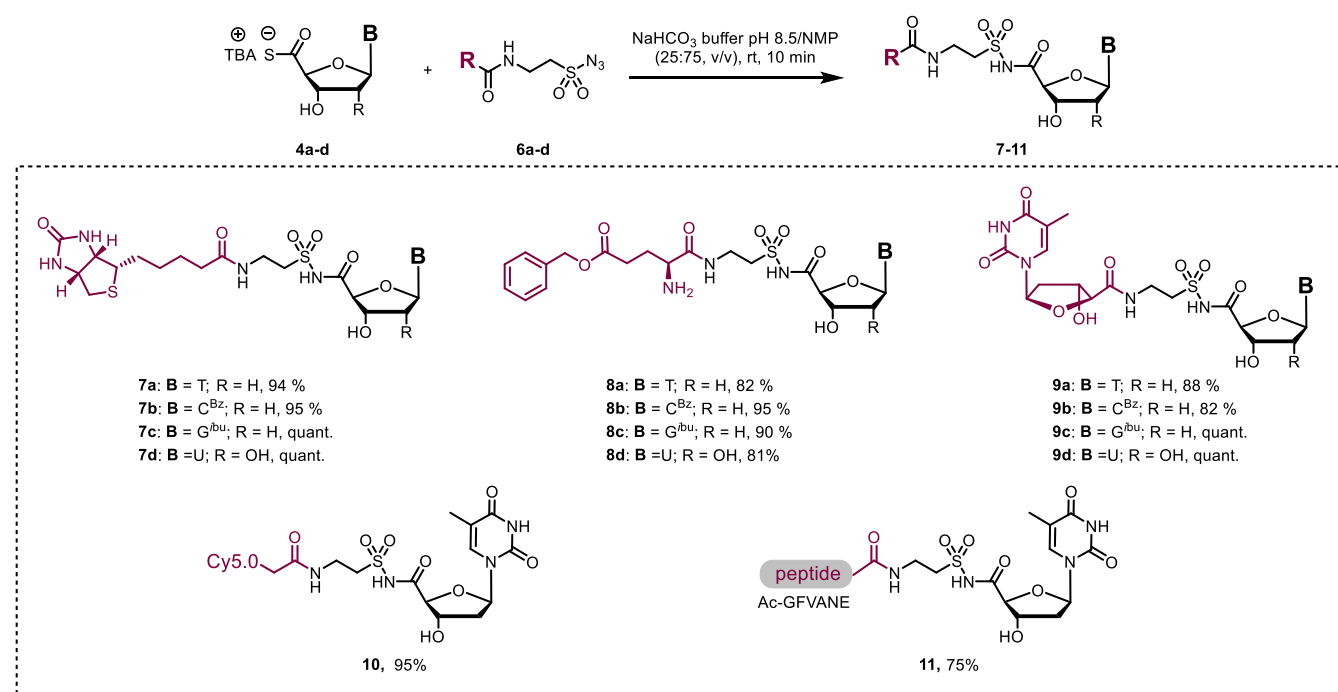
sides in excellent yields including biotin, which is often coupled to biomolecules as a tool for biological applications,²¹ and the hydro-soluble fluorophore Cy 5.0, used for *in vivo* imaging (Scheme 3).²² Similarly, 5',5'-dinucleotides bearing a non-natural *N*-acyl sulfonamide linkage were also obtained in good to excellent yields. Remarkably, the sulfo-click reaction proceeded efficiently with sulfonyl azide **6b** demonstrating the orthogonality of the reaction in the presence of a free primary amine. Ultimately, we were able to implement the sulfo-click on the hexapeptide Ac-GFVANE-NH₂, which underlined the potential of the reaction on larger molecules. All reactions were monitored by RP-HPLC analysis, which confirmed the complete conversion of the sulfonyl azide within the ten first minutes. Following these results, we next evaluated the rate of the sulfo-click reaction at low concentrations by monitoring the formation of the *N*-acyl sulfonamide **10** (Table 1).

Scheme 2. Sulfonyl azide compounds synthesized



^aYield over two steps after *N*-Boc deprotection. ^bYield over two steps after 3'-O-TBDMS deprotection.

Scheme 3. Scope of the sulfo-click applied to nucleosides^a



^aReactions were performed with 1 eq. of sulfonamide derivative, 1.5 eq. of 4'-thioacetate nucleoside in a mixture of aq. 0.1 M NaHCO₃ buffer pH 8.5 and NMP (25:75, v/v).

The data demonstrated that the reaction still occurred rapidly even at 1 mM concentration reaching completion within 5 h. Interestingly, under more dilute conditions (100 and 10 μ M), the ligation still occurred albeit at a slower rate. All-in-all, these results demonstrated the applicability of the sulfo-click reaction at low concentrations. The longer reaction times were not detrimental as both the thioacid and the sulfonyl azide are stable in aqueous media under physiological conditions for dozens of hours. Kinetic analysis unveiled a second-order kinetic constant ($k = 4.9 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$) at 37 $^{\circ}\text{C}$, which is of the same order of magnitude than the one determined earlier in organic media ($k = 5.7 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$)^{10b} and comparable to those of the Staudinger ligation ($k \sim 10^{-4} - 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$) or the SPAAC ($k \sim 10^{-2} - 10^{-1} \text{ M}^{-1} \text{ s}^{-1}$).²³

Encouraged by these results, we decided to evaluate the possibility to carry out a one-pot sulfo-click/CuAAC sequence on a substrate carrying two azido groups. Based on the high reactivity of the electron-deficient sulfonyl azide, we assumed a complete orthogonality of the two azido groups. We thus designed a modified nucleoside bearing both a sulfonyl azide and an aliphatic azide at the 5' and 3' positions respectively. Its synthesis started with commercially available azidothymidine which was first oxidized using the TEMPO/BAIB conditions described above, leading to carboxylic acid **12**. A coupling reaction between **12** and 2-aminoethanesulfonyl azide using BOP reagent in the presence of DIEA allowed the formation of the corresponding di-azidothymidine **13**. The latter was then subjected to a one-pot double-click sulfo-click/CuAAC conjugation sequence (Scheme 4). First, the sulfo-click was performed at 37 $^{\circ}\text{C}$ with a slight excess of 4'-thioacetate-thymidine **4a** in a NaHCO₃ buffer (0.1 M, pH 8.5)/NMP mixture (1:4) resulting in a successful chemoselective and quantitative conjugation after 10 min (determined by RP-

HPLC, see Figure S1). CuI, DIEA and commercially available biotin alkyne **14** were then added leading to the quantitative formation of the desired bis-conjugate **15** after 30 min (86% isolated yield). This result demonstrated that both reactions could be run sequentially and highlighted the tunability of the azido groups for dual conjugation.

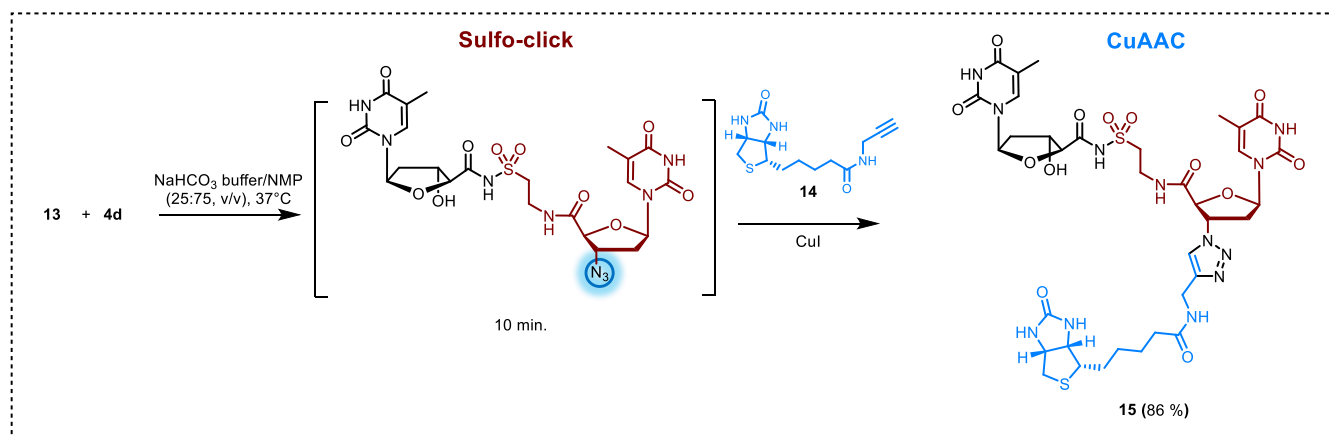
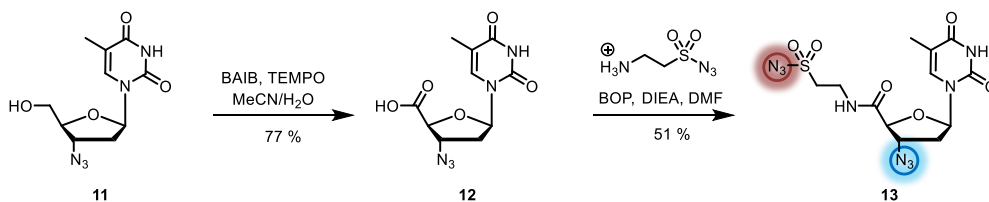
In summary, we report here the first application of the sulfo-click chemistry on nucleoside derivatives. A straightforward sequence was developed to access deoxyribo- and ribonucleosides bearing a 4'-thioacetate group, allowing the successful implementation of the sulfo-click conjugation to this particular class of biomolecules. Quantitative reactions were observed within 10 min at 10 mM concentration. The transformation is rapid ($k = 4.9 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$) under biocompatible conditions even at low micromolar concentrations. Moreover, we were able to implement the method to a one-pot sequential sulfo-click/CuAAC to selectively introduce two conjugations starting from a di-azido precursor.

Table 1. Kinetic studies of the sulfo-click reaction under physiological conditions^a

Concentration (μM)	Half conversion (h)	Quantitative conversion (h)
1000	0.6	~ 5
100	2	~ 20
10	7	~ 40

^aReactions were performed at 37 $^{\circ}\text{C}$ with 1 eq. of Cy 5.0 sulfonyl azide derivative, 2 eq. of 4'-thioacetate uridine in a mixture of aq. 50 mM TEAA buffer pH 7 and NMP (50% at 1 mM, 5% at 100 μM and 0.5% at 10 μM)

Scheme 4. Bioorthogonal of one-pot double click conjugation



ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental synthetic procedures and characterizations with detailed descriptions of TAL kinetic studies (PDF)

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Notes

The authors declare no competing financial interest.

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