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Copper-Free "Click" Modification of DNA via Nitrile Oxide−Norbornene 1,3-Dipolar Cycloaddition

Katrin Gutsmiedl, Christian T. Wirges, Veronika Ehmke, and Thomas Carell*

Center for Integrative Protein Science at the Department of Chemistry and Biochemistry, Ludwig Maximilians University, Butenandtstr 5-13, 81377 Munich

thomas.carell@cup.uni-muenchen.de

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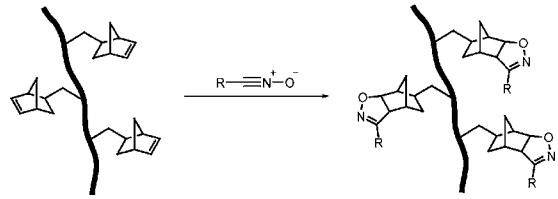
ABSTRACT



Nitrile oxides react smoothly and rapidly with norbornene-modified DNA in a copper-free click reaction. The reaction allows high density functionalization of oligodeoxyribonucleotides (ODNs) with a large variety of molecules directly on solid supports and even in synthesizers without the need for an additional catalyst.

Oligonucleotides are important molecules for the construction of functional nanosystems[1−3] and the development of molecular diagnostic tools[4] and are currently being developed as new therapeutically active entities.[5] For many applications, oligonucleotides must be modified with non-natural groups such as fluorescent markers,[6] biotin, thiols for attachment to gold surfaces,[7] or positively charged peptides (octaarginines) for improved cell permeability and specificity.[8,9] Today most oligonucleotide producers introduce either thiols or amino groups into DNA/RNA and then attach modifications using maleimic chemistry or reactive esters.[10] Currently, the Cu(I)-catalyzed reaction of alkynes and azides is increasingly used to attach modifications to alkyne-modified oligonucleotides.[11,12] This click reaction,[13] discovered by Meldal[14] and Sharpless,[15] typically provides high yields, particularly for difficult to solubilize modifiers, and requires short reaction times.[16,17]

However, most of these methods require that labeling reaction is performed outside the oligonucleotide synthesizer which is a drawback in today's world of online ordered, fully automated DNA and RNA synthesis. Particularly for commercial oligonucleotide labeling, there is a need for a fast and high yielding reaction that allows the modification of DNA and RNA directly in the synthesizer during or after

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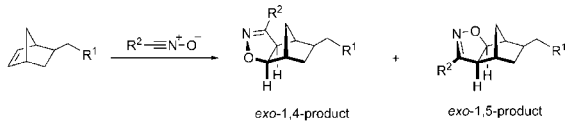
complete DNA/RNA assembly. Ideally, this reaction should be orthogonal to the azide/alkyne click reaction so that multiple labeling of DNA can be achieved by combining both methods.[18] If the new reaction proceeds without a catalyst, the reaction may even complement the fast growing repertoire of copper-free click reactions which so far involve: (1) The reaction of azides with strained cycloalkynes pioneered by the Bertozzi group[19−24] and used for glycoconjugate labeling by Boons;[25] (2) the reaction of strained alkenes with tetrazines developed by Fox[26] and Hilderbrand;[27] (3) the reaction of oxanorbornadienes with alkynes used, for example, for the synthesis of RGD peptide conjugates;[28,29] and (4) a photoclick reaction discovered by Lin.[30]

With the aim of developing a high-yielding reaction that meets the above-described criteria, we investigated the Huisgen 1,3-dipolar cycloaddition reaction of norbornenes (as strained alkenes) with nitrile oxides,[31−36] bearing in mind that nitrile oxides are strong electrophiles, which may cross react with DNA bases. The reaction provides typically exoselective substituted 2-isoxazolines as depicted in Scheme 1.[37] Here we report that this reaction enables labeling of

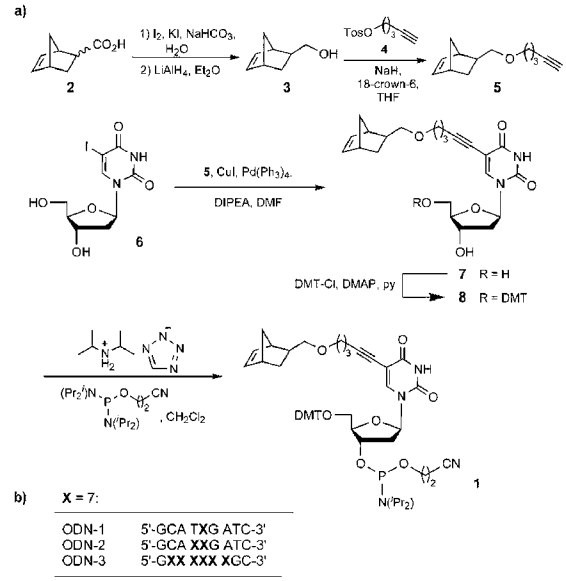
Scheme 1. Depiction of the Nitrile Oxide−Norbornene Click Reaction Giving 1,4- and 1,5-*exo*-Products



oligonucleotides in solution and, more importantly, on solid supports directly in oligonucleotide synthesizers, with high efficiencies associated with the release of the angular strain of the norbornene ring system of about 25.1 kJ/mol.[37]

To introduce norbornene linkers into oligonucleotides (ODNs), we prepared the norbornene-modified uridine nucleoside **1** and incorporated the phosphoramidite into a series of 9-mer ODNs depicted in Scheme 2.[38] The synthesis

Scheme 2. (a) Synthesis of Norbornene Bearing Phosphoramidite **1** and (b) a List of the ODN Series Containing **7**



of **1** was achieved in six steps as described in Scheme 2. A mixture of *exo*/*endo*-norbornenylcarboxylic acid **2** was converted into the known alcohol **3** in two steps involving *exo*/*endo* separation via iodolactonization followed by reduction of the *exo*-isomer.[39] Subsequent Williamson ether synthesis with tosylate **4**[40] furnished the alkyne-bearing norbornenyl derivative **5** in 74% yield which was subsequently coupled to 5-iododeoxyuridine **6** via Sonogashira coupling to produce the nucleoside **7** (with 39% yield). The phosphoramidite building block **1** was prepared using standard DMT protection of **7** in 70% yield and phosphitylation of **8** to **1** using 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (yield of 79%). Incorporation of the norbornene phosphoramidite **1** into 9-mer oligonucleotides via solid phase synthesis proceeded smoothly. However, an

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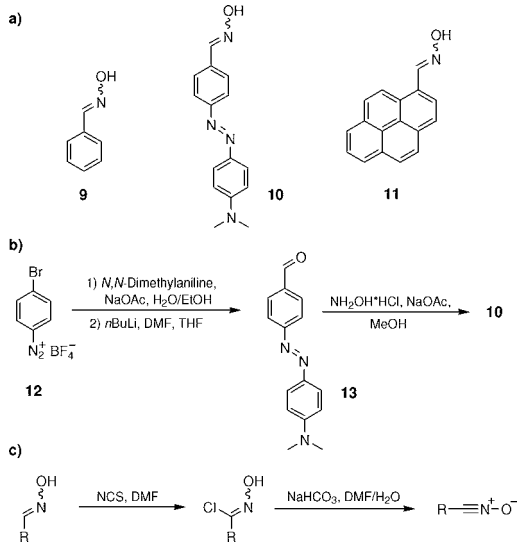
ultramild DNA synthesis protocol[38,41] was required as standard cleavage conditions yielded isoxazoline derived byproduct.

The DNA strands shown in Scheme 2 were reacted with the nitrile oxides derived from **9**−**11** depicted in Scheme 3.

Scheme 3. (a) Nitrile Oxide Precursors **9**−**11**, (b) Synthesis of the Dabcyl-Oxime **10**, and (c) Depiction of the Nitrile Oxide Preparation via Hydroximoyl Chlorides



Oxime **10**, an often used fluorescence quencher, was prepared as described in Scheme 3b.[38] For the cycloaddition, we either mixed a solution of *N*-chlorosuccinimide and aldoxime **9**−**11** in DMF in the presence of a weak base to generate the hydroximoyl chloride in situ (Huisgen's in situ method)[37] or directly used the prepared hydroximoyl chloride without base (Scheme 3c). The nitrile oxide solution was added directly either to the support attached DNA or to an unbuffered solution containing the purified, deprotected DNA in 10-fold excess.

The reaction mixture was then shaken for 10−20 min at room temperature. For the reaction performed directly on the solid support, the material was thoroughly washed with DMF and CH_2Cl_2, dried, and the DNA was cleaved from the support with a solution of potassium carbonate in MeOH over three hours. Purification of the labeled DNA was possible by either reversed-phase HPLC or PAGE.[38]

Alternatively, the click reaction can be performed after complete DNA assembly by pumping the nitrile oxide solution directly through the cartridge on the synthesizer.[38]

Figure 1a shows the MALDI-TOF spectra of the unpurified click products ODN-**1a** (ODN-**1** + **9**), ODN-**1b** (ODN-**1** + **10**), and ODN-**1c** (ODN-**1** + **11**) obtained by click reaction directly on solid support and subsequent cleavage of the DNA from the support (see also Figure S1, Supporting Information). It is evident that all three nitrile oxides reacted quantitatively with the norbornene functionality on the DNA. No remaining starting material (ODN-**1**) could be detected

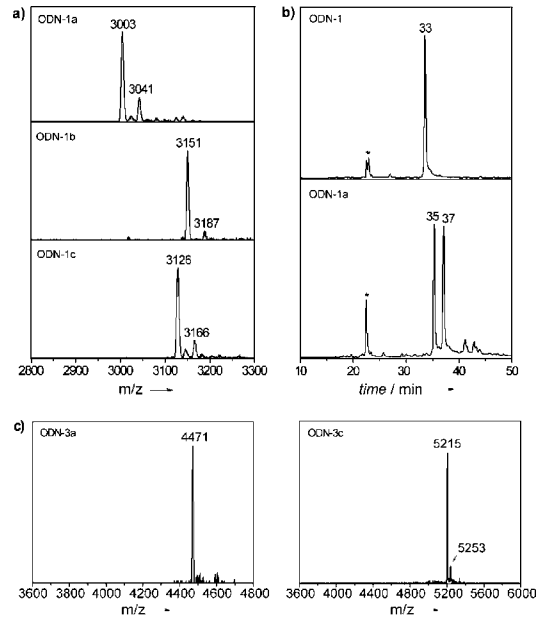


Figure 1. (a) MALDI-TOF spectra of the unpurified click reaction performed with ODN-**1** (M.W. 2881) containing one norbornene **7**, nitrile oxide precursor **9** (ODN-**1a**, M.W. 3000), nitrile oxide precursor **10** (ODN-**1b**, M.W. 3147), and nitrile oxide precursor **11** (ODN-**1c**, M.W. 3124) directly on solid support. (b) HPLC traces of unpurified ODN-**1** before (top) and after (bottom) the click reaction on solid support (* = byproduct of the DNA synthesis). (c) Raw MALDI-TOF spectra of the click products ODN-**3a** (left, M.W. 4468) and ODN-**3c** (right, M.W. 5212) produced with solid-supported ODN-**3** (M.W. 3754) and nitrile oxide precursor **9** and **11**.

even after the rather short reaction time. The obtained MALDI-TOF data are in full agreement with the expected molecular weights for the corresponding cycloadducts (plus an additional K^+-adduct). For comparison, a similar click modification with alkyne-labeled DNA and the Cu(I)-mediated reaction with azides typically takes between one and several hours depending on the azide, and we use a 100- to 500-fold excess of the azide.

Additional support for complete click transformation was obtained by enzymatic digestion of ODN-**1c** (see Figure S3, Supporting Information) which showed besides the canonical bases only the pyrene-modified base. No norbornene base starting material could be detected. Most importantly, reactions with other DNA bases were not observed. The HPLC-MS recorded after enzymatic digestion showed only the canonical bases plus the click-modified norbornene base.[38]

In addition, a gel electrophoresis analysis of the click products revealed only one band, showing that strand breaks did not occur (Figure S4, Supporting Information).[38]

Raw HPLC traces obtained for ODN-**1** before and after click reaction with the nitrile oxide precursor **9** proved the purity of the strands (Figure 1b). After the click reaction, two new peaks were detected which correspond to the expected 1,4- and 1,5-regioisomers.[38] HPLC integration revealed an equal amount of these two regioisomers in 90% yield.

To explore the robustness of the new DNA/RNA-labeling method, we next investigated the reaction of ODN-**3** containing six consecutive norbornene units with the nitrile oxide precursors **9** and **11**. As depicted in Figure 1c, the MALDI-TOF spectra obtained after the 1,3-dipolar cycloaddition reaction (10 min reaction time) were again extremely clean, and no byproducts were observed. Only the signals for the 6-fold reacted oligonucleotides (plus K^+-adduct) were detected showing that even the high density functionalization of DNA is possible using this mild cycloaddition reaction. Again, reaction with DNA bases was not oberved. Full conversion of the norbornene-modified DNA was achieved when the click reaction was performed on DNA in solution, even if the hydroximoyl chlorides were added as nitrile oxide precursors in the absence of additional base (see Figure S2, Supporting Information). The concentration of the nitrile oxide is seemingly sufficiently high in solution.[38]

Control experiments with unmodified DNA showed after one hour reaction <5% cycloaddition of nitrile oxide to natural DNA bases (data not shown). After 6 h of reacting DNA with nitrile oxides, about 50% DNA conversion is observed. Reaction of nitrile oxides with DNA is consequently far slower than with the norbornene double bond, which is the basis for the observed clean conversion.

A major advantage of this new method is that the nitrile oxides are only needed in 10-fold excess with respect to the norbornenes, which makes the reaction particularly suitable for labeling of DNA with expensive biomarkers. Other advantages of the system are the ready availability of all starting materials and most importantly that the click reaction can be performed directly in an automated synthesizer following DNA assembly by simply pumping the nitrile oxide solution through the cartridge containing the immobilized norbornene-bearing DNA. Because the nitrile oxide reacts under our conditions significantly slower with alkynes, the reaction is in principle orthogonal to the standard alkyne−azide−Cu(I) chemistry (see Figure S5, Supporting Information).

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Supporting Information Available: Information concerning the preparation of **1**−**13**, the click reaction conditions, further MALDI-TOF spectra, an enzymatic digestion, and PAGE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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