Published in final edited form as:
Bioconjug Chem. 2011 July 20; 22(7): 1259–1263. doi:10.1021/bc1003668.

Strain-promoted "click" chemistry for terminal labeling of DNA

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Abstract

1,3-Dipolar [3+2] cycloaddition between azides and alkynes—an archetypal "click" chemistry—has been used increasingly for the functionalization of nucleic acids. Copper(I)-catalyzed 1,3-dipolar cycloaddition reactions between alkyne-tagged DNA molecules and azides work well, but they require optimization of multiple reagents, and Cu ions are known to mediate DNA cleavage. For many applications, it would be preferable to eliminate the Cu(I) catalyst from these reactions. Here we describe the solid-phase synthesis and characterization of 5'-dibenzocyclooctyne (DIBO)-modified oligonucleotides, using a new DIBO phosphoramidite, which react with azides via copper-free, strain-promoted alkyne-azide cycloaddition (SPAAC). We found that the DIBO group not only survived the standard acidic and oxidative reactions of solid-phase oligonucleotide synthesis SPOS, but that it also survived the thermal cycling and standard conditions of the polymerase chain reaction (PCR). As a result, PCR with DIBO-modified primers yielded "clickable" amplicons that could be tagged with azide-modified fluorophores or immobilized on azide-modified surfaces. Given its simplicity, SPAAC on DNA could streamline the bioconjugate chemistry of nucleic acids in a number of modern biotechnologies.

The recent, rapid development of new DNA sequencing and sequence detection technologies[1–3] has created a demand for new bioconjugate chemistries for oligodeoxynucleotides (ODNs) and DNA.[4] In the case of ODNs, synthetic modifications are typically introduced during solid-phase oligonucleotide synthesis (SPOS) via modifier phosphoramidites or appropriately derivatized solid supports.[5] A great many synthetic modifiers have been developed for ODN functionalization, with the only critical requirement being that the modifier survives the conditions of SPOS. When these modified ODNs are used as primers in PCR, the functional groups must also be compatible with the thermal cycling and chemistry of PCR.[6] 5'-Terminal modifiers intended for PCR are typically appended to primers via SPOS prior to PCR amplification, and 5'-fluorophore-, -biotin-, -digoxigenin-, -amine-, -thiol- and -acrylate-tagged PCR products are commonly made in this way. Some of these functional groups can subsequently be used for post-PCR reactions, including attachment of the DNA to microarrays, beads or other surfaces.[7,8]

Despite this assortment of techniques for modifying DNA, there is still a need for new covalent DNA functionalization methods with improved kinetics, selectivity, and simplicity. Towards this end, a number of research groups have investigated 1,3-dipolar cycloaddition —a prototypical "click" reaction[9]—as a means of functionalizing ODNs and PCR-amplified DNA.[10] In particular, the copper-catalyzed azide-alkyne cycloaddition (CuAAC)[11] has been used to fluorescently tag ODNs[12–16] and PCR amplicons,[17] to attach DNA to surfaces,[18–21]

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Supporting Information Available. Synthesis, purification and characterization of organic compounds and oligonucleotides; kinetics data; details of PCR and post-PCR modification. This material is available free of charge via the Internet at http://pubs.acs.org.

to crosslink,[22] ligate[23,24] and circularize[25,26] DNA, and to conjugate ODNs to carbohydrates,[27,28] peptides,[29] and proteins.[30] These reactions give high yields, and the bio-orthogonality of the azide-alkyne reaction ensures selectivity. However, CuAAC requires multiple reagents in addition to the azide-alkyne pair, and Cu(II) is known to mediate oxidative cleavage of DNA,[31,32] although Cu-mediated DNA cleavage can be minimized by optimizing click reaction conditions and by appropriate choice of ligand.[23] As an alternative to copper-catalyzed click chemistry, various copper-free click reactions have been developed for bioconjugate applications.[33] Heaney and coworkers have recently applied copper-free alkyne-nitrone cycloadditions, in which nitrones are used in place of azides, to ODN chemistry,[34,35] and Carell and coworkers have used similar nitrone-alkene cycloadditions for modification of modified nucleosides.[36,37] These reactions are fast, but limited by the instability of many nitrones to hydrolysis and dimerization.[38] Bertozzi and coworkers[39–42] and others[43,44] have developed a catalyst-free, strain-promoted alkyne-azide cycloaddition (SPAAC) reaction between azides and cyclooctynes[45] for bioconjugate chemistry. However, strain-promoted click chemistry has not yet been applied to bioconjugation of DNA.

In this report, we describe the solid-phase synthesis, purification and characterization of cyclooctyne-modified ODNs that react with azides via SPAAC. The ODN modifications were introduced via SPOS using the new dibenzocyclooctyne (DIBO) phosphoramidite **2**, which was synthesized from the strained hydroxydibenzocyclooctyne **1** reported by Boons and coworkers (Scheme 1).[43] This phosphoramidite was then used in SPOS, using standard chemistries for synthesis, base deprotection and cleavage from solid support, to generate 5'-DIBO-modified ODNs **3a/b** (Scheme 2). These ODNs were purified by reverse-phase (RP) gradient HPLC, where the hydrophobic 5'-cyclooctyne resulted in longer retention times on C18 RP media (Figure 1A,B). The attachment of the DIBO group to each purified ODN was confirmed by ESI-MS. The 5'-DIBO group was also characterized by UV-vis absorbance spectroscopy (Figure 2A). By itself, hydroxy-dibenzocyclooctyne **1** absorbs UV light in the $\lambda = 305$ nm region of the UV-vis spectrum, with extinction coefficient $\varepsilon_{305} = 9.97 \times 10^3$ M^{-1} cm^{-1} in $CHCl_3$. When attached to an ODN, the cyclooctyne group adds a peak in the same $\lambda = 305$ nm region to the typical $\lambda = 260$ nm absorbance peak of the ssDNA, with $\varepsilon_{305} = 2 \times 10^4$ M^{-1} cm^{-1} in H_2O—larger than, but comparable to, the extinction coefficient of **1** in $CHCl_3$. These data indicate that the strained DIBO group survives reaction conditions in SPOS that we imagined might alter a strained alkyne,[46] including phosphorus oxidation with I_2, exposure to acidic tetrazole in the coupling step, and incubation in concentrated NH_4OH at 60 °C during deprotection and cleavage from solid support.

As expected, DIBO-modified ODNs **3a/b** reacted quantitatively with organic azides to produce clicked bioconjugates (Scheme 3). These reactions could be conducted either on solid support at the end of SPOS, or in aqueous solution after the cyclooctyne-modified oligonucleotide is released from support. To evaluate the efficiency and rate of the solution-phase reaction, we combined the sulforhodamine azide **5** with ODNs **3a/b** at room temperature in H_2O to generate clicked oligonucleotide products **6a/b** that were readily identified and purified by HPLC (Figure 1C). The identities of the purified products were confirmed by ESI-MS, which showed the added mass of the azide. UV-vis spectroscopy of **6a/b** indicated that the characteristic UV absorbance peak at $\lambda = 305$ nm from the starting DIBO group had disappeared, and that a new absorbance at $\lambda = 595$ nm appeared corresponding to the newly attached fluorophore (Figure 2B). Polyacrylamide gel electrophoresis (PAGE) of **3a/b, 6a/b**, and unmodified ODNs **4a/b** (Figure 3) showed that both the 5'-DIBO group and the clicked fluorophore retarded the electrophoretic mobility of each ODN slightly, and confirmed the purity of the products.

Kinetic analysis of the reaction between **3a** and **6** in phosphate-buffered saline yielded a second-order rate constant $k = 0.9\ M^{-1}\ sec^{-1}$, which is comparable to the value of $2.3\ M^{-1}\ sec^{-1}$ previously measured by Ning et al. for the reaction between **1** and benzyl azide.[43] Consistent with this result, solution-phase click reactions between even dilute (μM) concentrations of DIBO-modified ODNs and excess azides were quantitatively complete in minutes to hours. The same reaction could be conducted during SPOS, before the ODN was deprotected or cleaved from the controlled-pore glass (CPG) support. Incubation of **CPG-3a** with 270 mM **5** in H_2O overnight, followed by standard deprotection, cleavage from the CPG, and purification via RP HPLC, yielded the same clicked ODN **7a** as synthesized in solution. Morvan and coworkers previously reported that copper-catalyzed azide-alkyne click chemistry of ODNs on solid support required the use of microwaves to drive the reaction to completion;[26] we find that strain-promoted, copper-free click reactions on solid support go to completion without the need for microwave irradiation. Despite this reactivity, we found that cyclooctyne ODNs **3a/b** were indefinitely stable under typical solution and storage conditions used for DNA (and in the absence of azides).

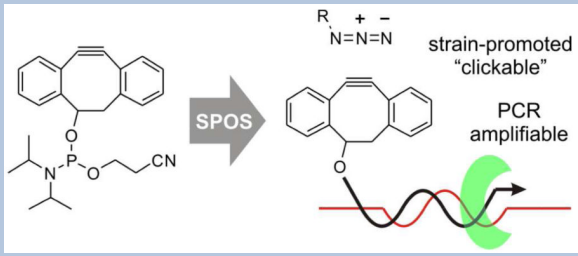
An important goal of this research was to use 5'-DIBO-modified ODNs as primers in PCR reactions, such that the resulting cyclooctyne-modified amplicons could be tagged with fluorophores or attached to surfaces via SPAAC. In the specific case of surface attachment, PCR products are often attached to streptavidin-conjugated surfaces noncovalently via a 5'-biotin group;[47] a simple, covalent alternative to the biotin-streptavidin pair could be useful for applications in which the surface-bound DNA is exposed to conditions (e.g., heat or organic co-solvents) that would release biotin.[48] To test the stability of the 5'-DIBO group to the conditions of PCR, we amplified a 500 base-pair sequence from bacteriophage λ DNA using ODN **3b** as a primer. The crude PCR reaction mixture was then incubated with Texas Red azide **5** to fluorescently label the 5'-terminus of the DNA. Agarose gel electrophoresis of the PCR product (Figure 4) demonstrated that amplification with primer **3b** had been successful, and that the DIBO group remained reactive to fluorescent azide **5** after 30 cycles of PCR, as indicated by the observation of Texas Red-labeled product bands in the gel (Figure 4, lanes 4,5). Control experiments, also monitored by gel electrophoresis, showed that no nonspecific labeling occurred in the absence of the cycloalkyne group (Figure 4, lane 2). Similarly, DIBO-modified PCR products were attached to azide-functionalized Sepharose gel by simply combining the two in Tris·HCl (pH 8) buffer overnight (Figure 5A,B). Control experiments showed no attachment for unmodified DNA or for ordinary Sepharose (Figure 5C,D). From these observations, we conclude that post-PCR, strain-promoted click reactions between DIBO-modified DNA and both solution- and solid-phase azides were efficient, selective, and simple, and serve as a potential alternative to terminal biotin-streptavidin association for surface attachment of ODNs.

As cyclooctyne reagents like **1** become commercially available, we anticipate that they will find increasing use in a variety of bioconjugate chemistries. The stability of cyclooctynes under storage, the specificity of their reactions with azides, and the overall ease of SPAAC reactions make them ideal for biomolecule tagging. In this communication, we have demonstrated the broad utility of SPAAC in the bioconjugate chemistry of nucleic acids. Our hope is that this chemistry could be useful for DNA biotechnologies—such as solid-phase and emulsion PCR, in-situ PCR labeling, or bioconjugation of extremely dilute or complex samples—where selectivity, simplicity and speed are at a premium.

Synopsis

Oligonucleotides with copper-free, strain-promoted "click" functionality at their 5'-ends can be prepared using a new dibenzocyclooctyne (DIBO) phosphoramidite and standard solid-phase synthesis methods. The 5'-DIBO group not only survives the acidic and

oxidative conditions of oligonucleotide synthesis, but it also endures PCR thermocycling, and 5'-DIBO-tagged primers generate PCR amplicons that can be fluorescently labeled or attached to azide-modified surfaces via strain-promoted click chemistry.



Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (CA122603).

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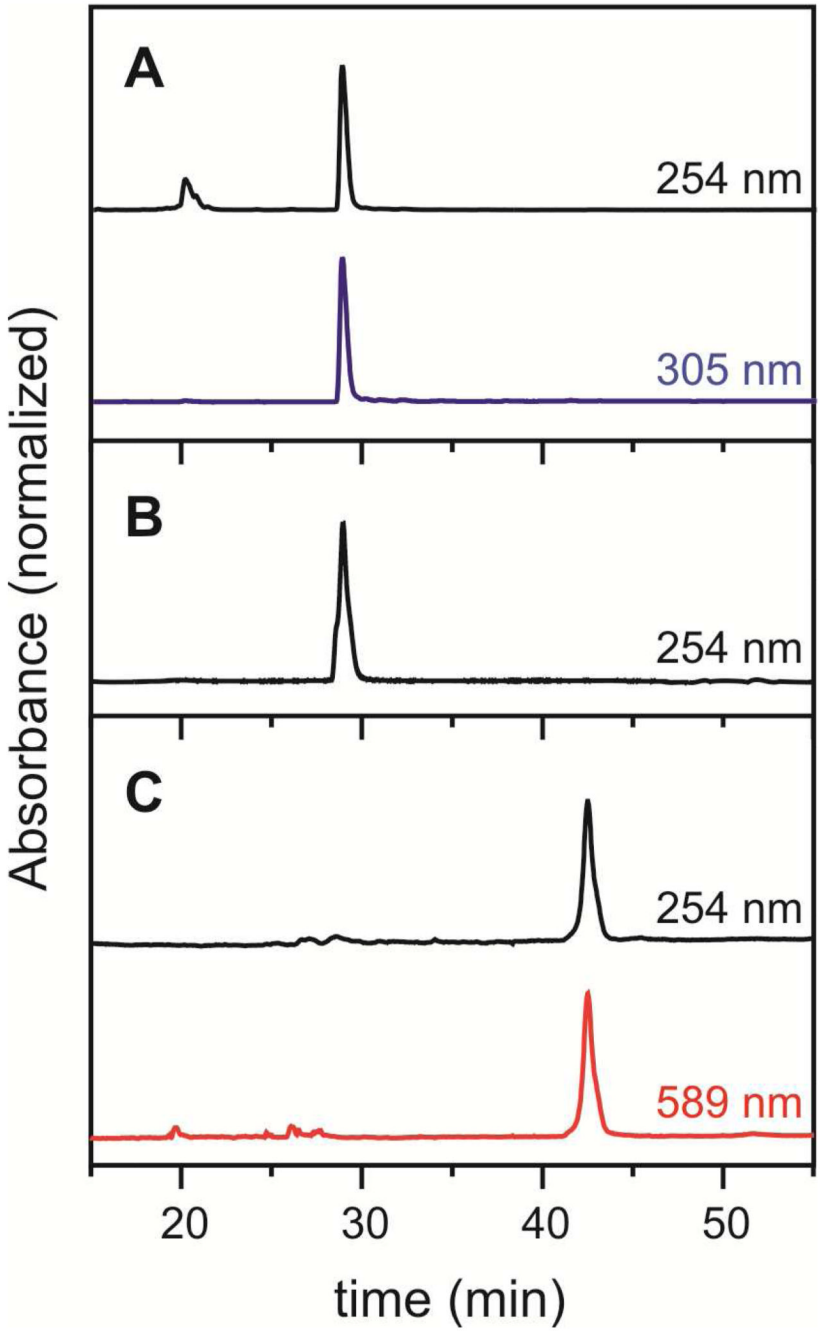


Figure 1.
HPLC chromatograms of 5'-DIBO-modified ODN **3a** (A) before purification, (B) after HPLC purification, and (C) after reaction with azide **5** to form ODN **7a**.

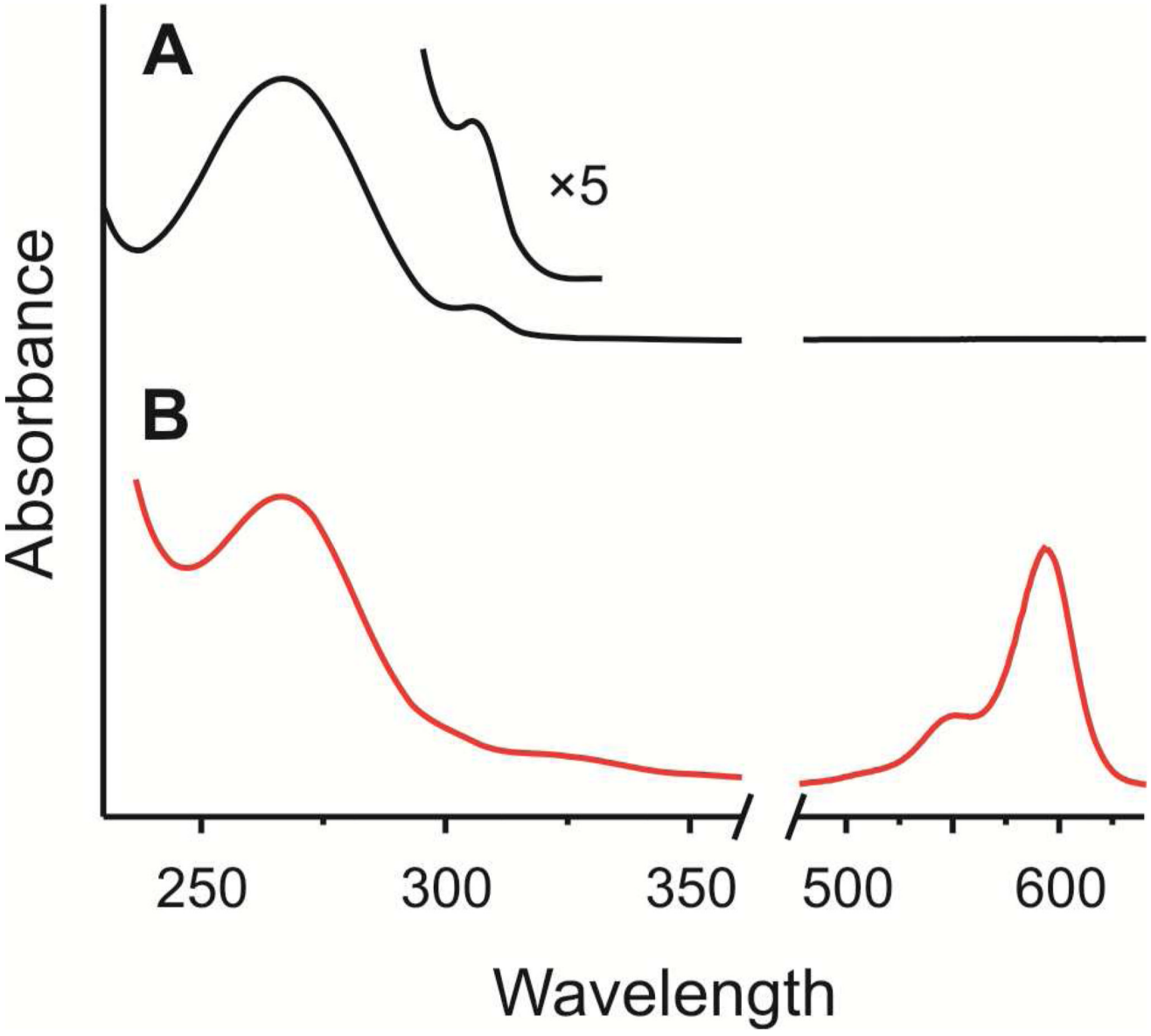


Figure 2.
UV-vis spectra of ODN **3a** (A) and clicked Texas Red-ODN **7a** (B).

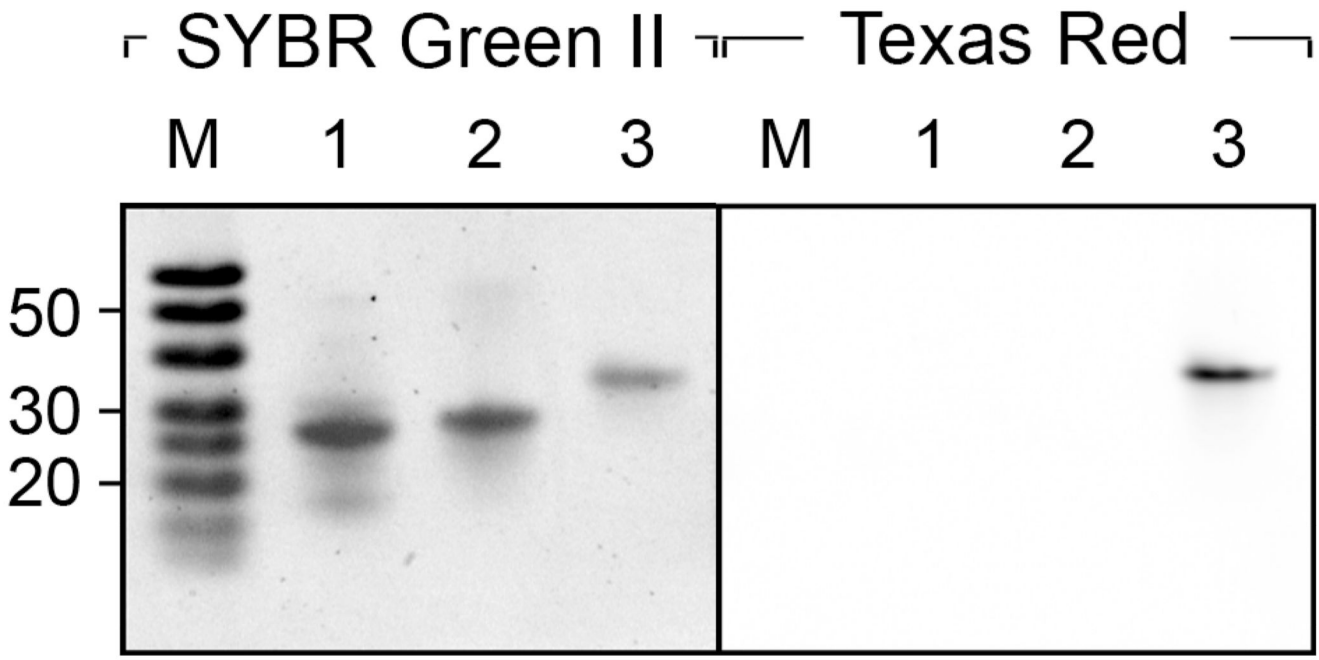


Figure 3.
Gel electrophoresis (16% denaturing PAGE) of unmodified ODN **4b** (lane 1), DIBO-modified ODN **3b** (lane 2), and clicked Texas Red-ODN **7b** (lane 3). After electrophoresis, the gel was scanned in the Texas Red channel to generate the image on the right, stained with SYBR Green II, and then scanned in the SYBR Green channel to generate the image on the left.

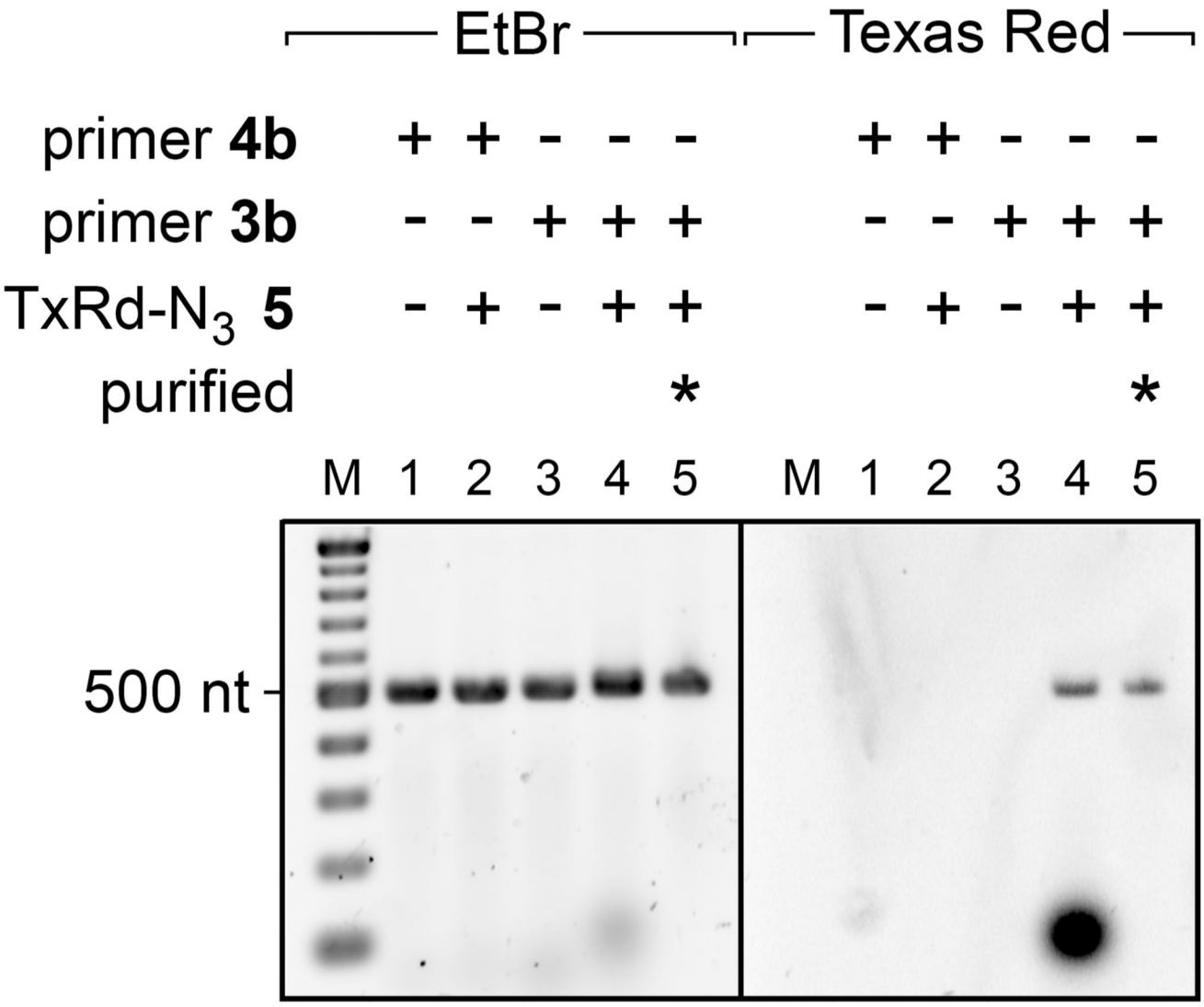


Figure 4.

Gel electrophoresis (1.5% agarose, 1X TBE) of PCR products formed from either unmodified (lanes 1–2) or DIBO-modified (lanes 3–5) upstream primers. Primers were designed to amplify nucleotides 7131–7630 (500 bp total) of the bacteriophage λ genome. Crude PCR reaction mixtures were then either loaded directly onto the gel (lanes 1,3), or incubated with 33 μM Texas Red (TxRd)-azide **5** (lanes 2,4,5) before loading onto the gel. The sample in lane 5 was also purified using a PCR purification kit (Qiagen QiaQuick) to remove excess primers and reagents prior to loading. After electrophoresis, the gel was scanned in the Texas Red channel to generate the image on the right, stained with ethidium bromide (EtBr), and then scanned in the EtBr channel to generate the image on the left.

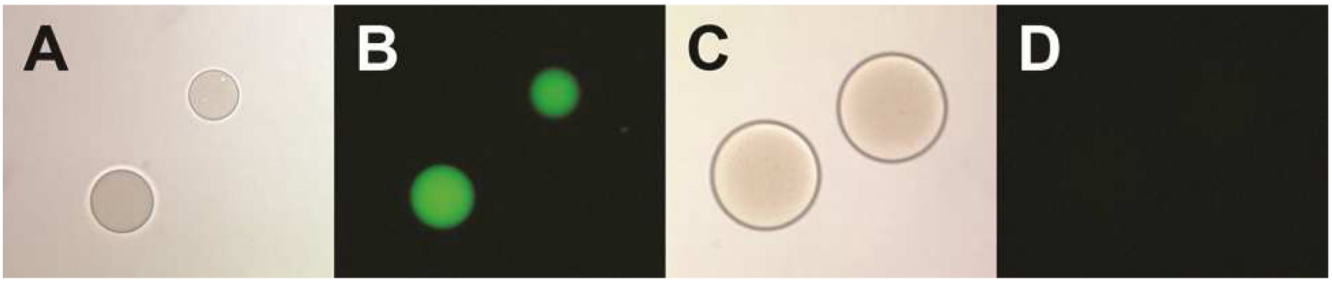
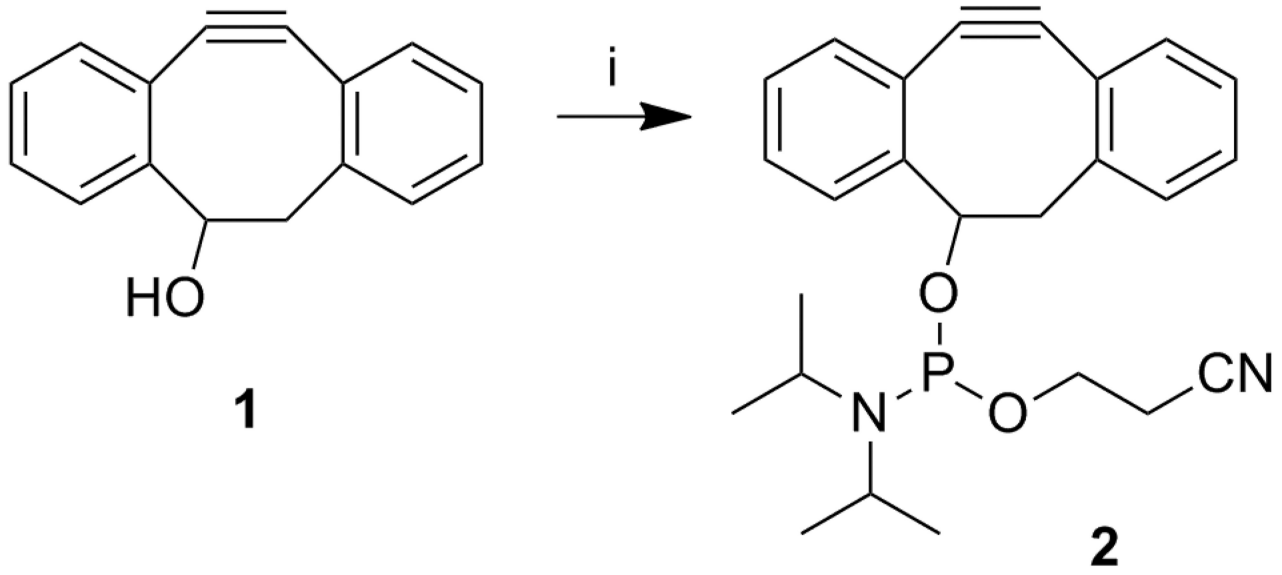


Figure 5.
Brightfield (A,C) and fluorescence (B,D) microscopy of Sepharose beads incubated successively with DIBO-modified PCR amplicon and SYBR Green I dye. The beads were either functionalized with azide groups (A,B) or not (C,D).

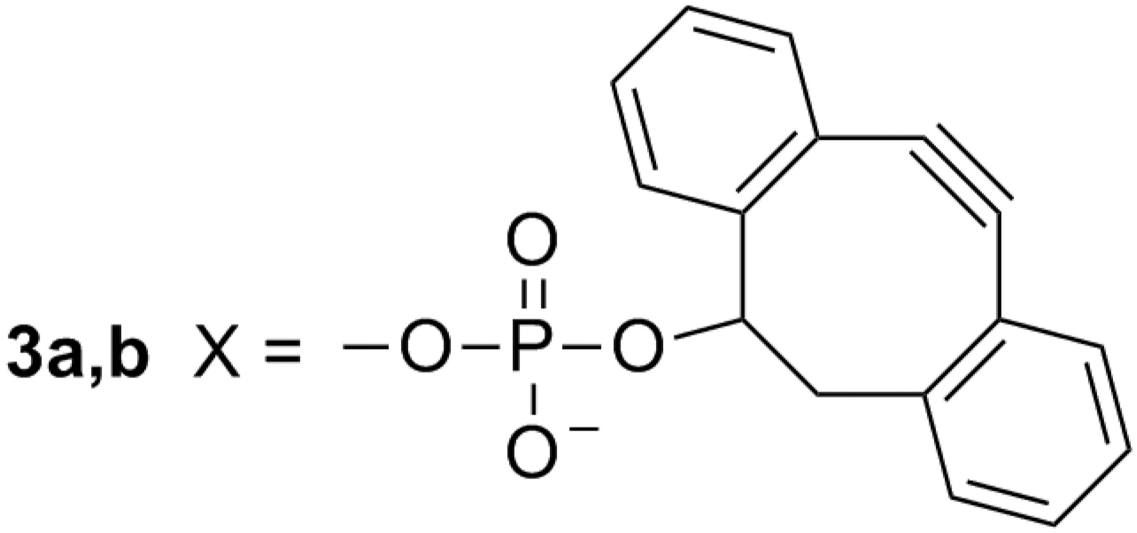


i. $ClP[N(iPr)_2](OEtCN)$, DIPEA, rt, CH_2Cl_2, 1 h

Scheme 1.

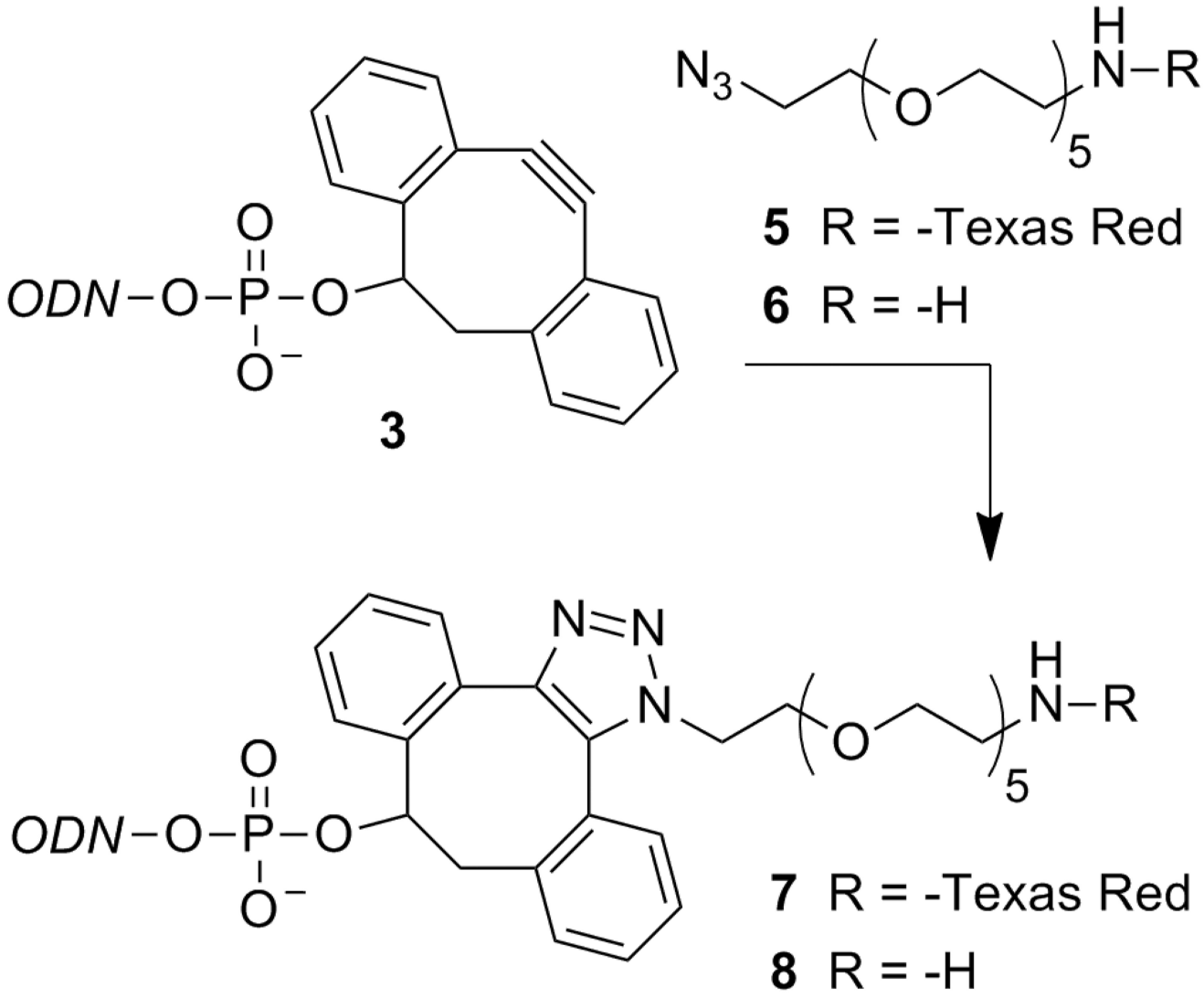
a 5′ X-T_{10}

b 5′ X-T_8CGTGATCCCACCTCATTTTC



4a,b X = −OH

Scheme 2.



Scheme 3.