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Design and Synthesis of a Chemically Cleavable Fluorescent Nucleotide, 3′-O-Allyl-dGTP-allyl-Bodipy-FL-510, as a Reversible Terminator for DNA Sequencing by Synthesis

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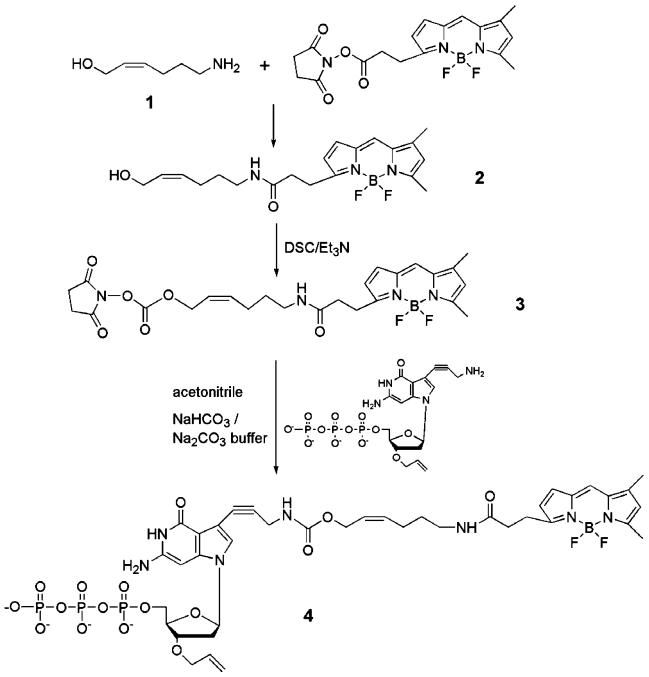
With the completion of the human genome project, there is now a focus on developing new DNA sequencing technology that will dramatically reduce the cost of sequencing without sacrificing accuracy. This will ultimately enable personalized medicine in healthcare.[1] Current state-of-the-art DNA sequencing technologies face limitation in terms of cost, read length, and throughput. DNA sequencing by synthesis (SBS), where the identity of each nucleotide is detected immediately after its incorporation into a growing strand of DNA in a polymerase reaction, offers an alternative approach to address some of these limitations. An important requirement for the SBS approach is a 3′-OH-capped fluorescent nucleotide that can act as a reversible terminator.[2] Following the identification of the nucleotide incorporated in a DNA polymerase reaction, the 3′-OH capping group along with a fluorescent label are removed to regenerate a free 3′-OH group, thus allowing DNA chain elongation. The importance of removing the fluorescent label after each base identification is to ensure that the residual fluorescence from the previous nucleotide incorporation does not affect the identification of the next incorporated fluorescent nucleotide. We have reported the design and synthesis of a 3′-O-allyl photocleavable fluorescent nucleotide analogue, 3′-O-allyl-dGTP-PC-Bodipy-FL-510, as a reversible terminator to determine two repeated nucleotide sequences in SBS.[3] A two-step process, photocleavage and Pd-catalyzed deallylation, was used to remove the fluorophore and the 3′-O-allyl group, respectively.[3,4]

The speed and sequence read length of SBS depend on the efficiency of the fluorophore and allyl group cleavage reactions. Due to multiple steps required in the identification, removal of the fluorescent label, and regeneration of the 3′-OH group after each nucleotide incorporation in SBS, even minor losses in efficiency at each step may lead to inhibited read length. For this reason, any improvement in efficiency within each cycle of nucleotide identification, fluorophore removal, and 3′-OH regeneration can have significant impact on read length, thus tackling the physical limit in DNA sequencing by synthesis.

A disulfide linker has been previously explored as a chemically cleavable moiety to attach a fluorophore to a deoxynucleotide, and the use of 2-mercaptoethanol to remove the fluorophore after the nucleotide incorporation and detection in SBS has also been studied.[5] However, the disulfide bond can be reversed and destabilized under certain conditions.[6,7] Encouraged by our successful application of allyl protection of the 3′-OH group of the nucleotide analogues,[3,4] we have explored the construction of a novel chemically cleavable fluorescent labeling system based on an allyl group to modify a nucleotide. The goal was to discover linker chemistry and condition to remove the fluorophore and the 3′-O-allyl group in one step to increase the SBS efficiency. We report here that an allyl moiety can be used successfully as a linker to tether a fluorophore to a 3′-O-allyl-modified nucleotide, forming a chemically cleavable reversible terminator, 3′-O-allyl-dGTP-allyl-Bodipy-FL-510 **4** (Scheme 1).[8] We have found that the fluorophore and the 3′-O-allyl group on a DNA extension product, which is generated by incorporation of **4**, are removed simultaneously in 30 s by Pd-catalyzed deallylation in aqueous solution. This one-pot dual-deallylation reaction thus allows the reinitiation of the polymerase reaction. Here, we describe the design and synthesis of a nucleotide analogue, 3′-O-allyl-dGTP-allyl-Bodipy-FL-510 **4**, with a fluorophore attached to the 7 position of a guanine base via an allyl carbamate linker, and its application as a reversible terminator for SBS.

Scheme 1. Synthesis of 3′-O-Allyl-dGTP-allyl-Bodipy-FL-510 (**4**)



Readily available allylic alcohol **1** was chosen as a starting material for the preparation of **4**. First, allylic alcohol **1** was reacted with *N*-hydroxysuccinimide (NHS) ester of the BODIPY-FL-510 to produce allylic-Bodipy-FL-510-NHS **2**, which was subsequently converted to its corresponding NHS ester **3** by reacting with *N*,*N*′-disuccinimidyl carbonate. The coupling reaction between **3** and the modified nucleotide (3′-O-allyl-dGTP-NH_2)[3] produced the chemically cleavable fluorescent nucleotide, 3′-O-allyl-dGTP-allyl-Bo-

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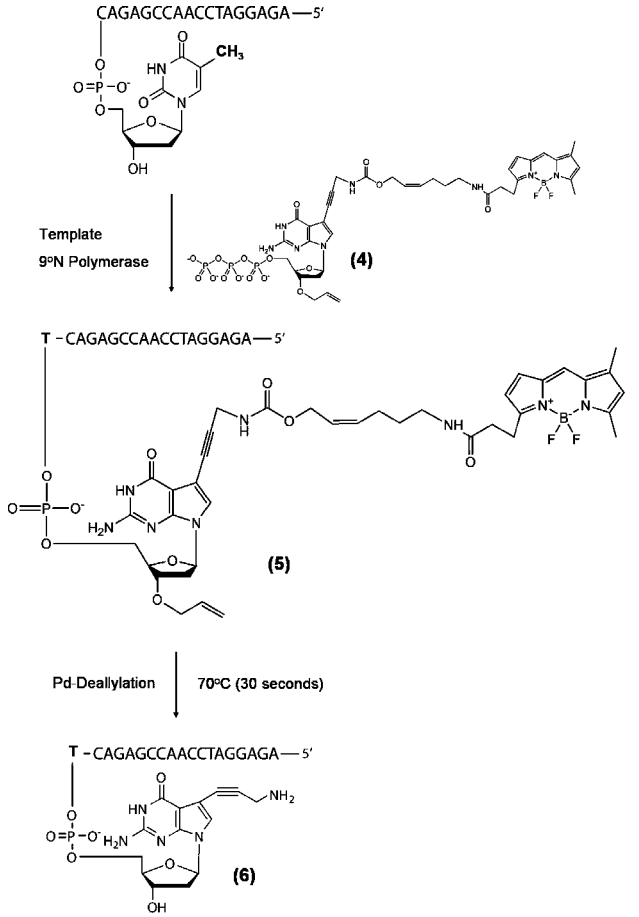
Scheme 2. Polymerase DNA Extension Reaction Using 3′-O-Allyl-dGTP-allyl-Bodipy-FL-510 as a Reversible Terminator



To verify that 3′-O-allyl-dGTP-allyl-Bodipy-FL-510 **4**, acting as a reversible terminator, is incorporated accurately in a base-specific manner in a polymerase reaction, we have performed a polymerase DNA extension reaction, as shown in Scheme 2.[8] This allows the isolation of the DNA product at each step for detailed molecular structure characterization by using MALDI-TOF MS, as shown in Figure 1. First, a polymerase extension reaction using **4** as a terminator along with a primer and the synthetic 100-mer DNA template corresponding to a portion of exon 7 of the human *p53* gene was performed to yield a single-base extension product **5**. After the reaction, a small portion of the extension product **5** was characterized by MALDI-TOF MS. The rest of the extended DNA product **5** was added to a deallylation cocktail [1X Thermopol reaction buffer/Na_2PdCl_4/$P(PhSO_3Na)_3$] and incubated for 30 s to yield deallylated DNA product **6**, which was characterized by MALDI-TOF MS.

The deallylated DNA product with both the fluorophore removed and a free 3′-OH group regenerated can then be used as a primer for the next nucleotide extension reaction.

Figure 1 (right panel) shows the sequential mass spectrum at each step of DNA sequencing by synthesis using 3′-O-allyl-dGTP-allyl-Bodipy-FL-510 **4** as a reversible terminator. As can be seen from Figure 1A, the MALDI-TOF MS spectrum consists of a distinct peak at *m*/*z* 6967, corresponding to the single-base DNA extension product **5** with 100% incorporation efficiency, confirming that the reversible terminator **4** can be incorporated base-specifically by DNA polymerase into a growing DNA strand. Figure 1B shows the one-pot dual-deallylation result after 30 s incubation of the DNA extension product in a deallylation cocktail solution. The peak at

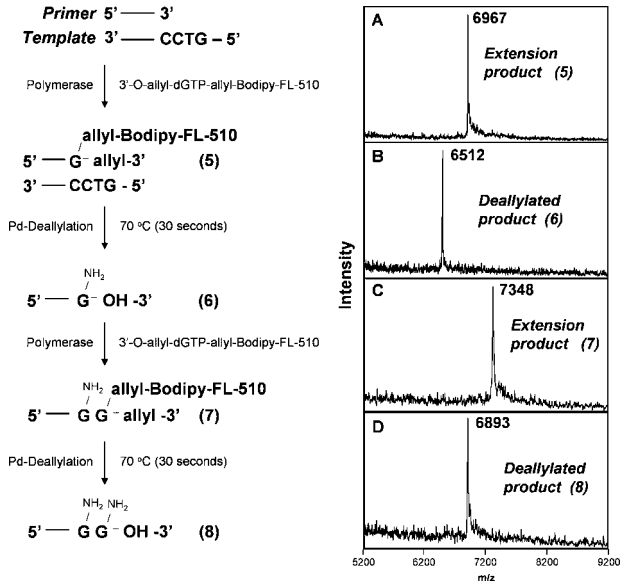


Figure 1. A polymerase extension scheme (left) and MALDI-TOF MS spectra of extension and dual-deallylation product (right).

corresponding to a DNA product **6** with both the fluorophore and 3′-O-allyl removed, appeared as the sole product. The absence of a peak at *m*/*z* 6967 confirms that the one-pot dual-deallylation reaction to remove both the fluorophore and the 3′-O-allyl group from the DNA product was very efficient. Figure 1C shows that DNA product **6** can be successfully used as a primer to continue the incorporation of another nucleotide **4** to yield product **7** (*m*/*z* 7348). Upon deallylation, both the fluorophore and the 3′-O-allyl group of **7** were removed quantitatively to yield **8** (*m*/*z* 6893).

Thus, these experimental results demonstrate that the allyl moiety on the nucleotide analogue is completely stable in a polymerase extension condition and can be selectively cleaved in a rapid and efficient manner. Furthermore, the nucleotide analogue, 3′-O-allyl-dGTP-allyl-Bodipy-FL-510, can be faithfully incorporated into a growing DNA strand in a polymerase extension reaction to act as a reversible terminator in SBS. As a result, we expect expansion of this novel linker and protection strategy to other applications that include bioconjugation and solution- and solid-phase organic synthesis.

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Supporting Information Available: Experimental procedures, characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) See the Supporting Information for experimental procedures and characterization data for all the compounds in Schemes 1 and 2.