Design and synthesis of a 3′-*O*-allyl photocleavable fluorescent nucleotide as a reversible terminator for DNA sequencing by synthesis

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DNA sequencing by synthesis (SBS) offers an approach for potential high-throughput sequencing applications. In this method, the ability of an incoming nucleotide to act as a reversible terminator for a DNA polymerase reaction is an important requirement to unambiguously determine the identity of the incorporated nucleotide before the next nucleotide is added. A free 3′-OH group on the terminal nucleotide of the primer is necessary for the DNA polymerase to incorporate an incoming nucleotide. Therefore, if the 3′-OH group of an incoming nucleotide is capped by a chemical moiety, it will cause the polymerase reaction to terminate after the nucleotide is incorporated into the DNA strand. If the capping group is subsequently removed to generate a free 3′-OH, the polymerase reaction will reinitialize. We report here the design and synthesis of a 3′-modified photocleavable fluorescent nucleotide, 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 (PC-Bodipy, photocleavable 4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene), as a reversible terminator for SBS. This nucleotide analogue contains an allyl moiety capping the 3′-OH group and a fluorophore Bodipy-FL-510 linked to the 5 position of the uracil through a photocleavable 2-nitrobenzyl linker. Here, we have shown that this nucleotide is a good substrate for a DNA polymerase. After the nucleotide was successfully incorporated into a growing DNA strand and the fluorophore was photocleaved, the allyl group was removed by using a Pd-catalyzed reaction to reinitiate the polymerase reaction, thereby establishing the feasibility of using such nucleotide analogues as reversible terminators for SBS.

2-nitrobenzyl linker | photocleavage

The completion of the Human Genome Project (1, 2) has led to an increased demand for high-throughput and rapid DNA sequencing methods to identify genetic variants for applications in pharmacogenomics (3), disease gene discovery (4, 5), and gene function studies (6). Current state-of-the-art DNA sequencing technologies (7–11) to some extent address the accuracy and throughput requirements but suffer limitations with respect to cost and data quality. Thus, a new DNA sequencing approach is required to broaden the applications of genomic information in medical research and health care. In this regard, DNA sequencing by synthesis (SBS) offers an alternative approach to possibly address the limitations of current DNA sequencing techniques. We have previously described the design of a parallel chip-based SBS system, which uses a self-priming DNA template covalently linked to the glass surface of a chip and four modified nucleotides (12–14). The nucleotides are modified such that they have a photocleavable fluorescent moiety attached to the base (5 position of pyrimidines, 7 position of purines) and a chemically cleavable group to cap the 3′-OH. When the correct nucleotide is incorporated in a DNA polymerase reaction, specific to the template sequence, the reaction is temporarily terminated because of the lack of a free 3′-OH group. After the fluorescent signal is detected and the nucleotide identified, the 3′-OH needs to be regenerated to continue incorporating the next nucleotide. In the accompanying report, we have demonstrated that four photocleavable fluorescent nucleotides can be efficiently incorporated by DNA polymerase into a growing DNA strand base specifically in a polymerase extension reaction, and that the fluorophores can be completely removed by photocleavage under near-UV irradiation (≈355 nm) with high efficiency (15). Using this system in a four-color sequencing assay, we were able to accurately identify multiple bases in a self-priming DNA template covalently attached to a glass surface.

Another important requirement for this approach to sequence DNA unambiguously is a suitable chemical moiety to cap the 3′-OH of the nucleotide such that it terminates the polymerase reaction to allow the identification of the incorporated nucleotide. The capping group then needs to be efficiently removed to regenerate the 3′-OH, thereby allowing the polymerase reaction to continue. Thus, the photocleavable fluorescent nucleotides used in SBS must be reversible terminators of the DNA polymerase reaction to allow the detection of the fluorescent signal such that the complementary DNA synthesis and sequence identification can be efficiently performed in tandem. The principal challenge posed by this requirement is the incorporation ability of the 3′-modified nucleotide by DNA polymerase into the growing DNA strand. The 3′ position on the sugar ring of a nucleotide is very close to the amino acid residues in the active site of the DNA polymerase. This is supported by the 3D structure of the previously determined ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (16). Thus, any bulky modification at this position provides steric hindrance to the DNA polymerase and prevents the nucleotide from being incorporated. A second challenge is the efficient removal of the capping group once the fluorescence signal is detected. Thus, it is important to use a functional group small enough to present no hindrance to DNA polymerase, stable enough to withstand DNA extension reaction conditions, and able to be removed easily and rapidly to regenerate a free 3′-OH under specific conditions.

Numerous studies have previously been undertaken to identify a 3′-modified nucleotide as a substrate for DNA polymerase. 3′-*O*-methyl nucleotides have been shown to be good substrates for several polymerases (17). However, the procedure to chemically cleave the methyl group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methyl group to cap the 3′-OH group for SBS. It has been reported that nucleotides with ether linkages at the 3′ position can be incorporated by some DNA polymerases, whereas those with ester linkages are not generally accepted by most of the polymerases tested (18). Significant efforts have been dedicated to evaluating a wide

Abbreviations: PC-Bodipy, photocleavable 4,4-difluoro-4-bora-3α,4α,4-diaza-*s*-indacene; SBS, sequencing by synthesis; TPPTS, triphenylphosphinetrisulfonate.

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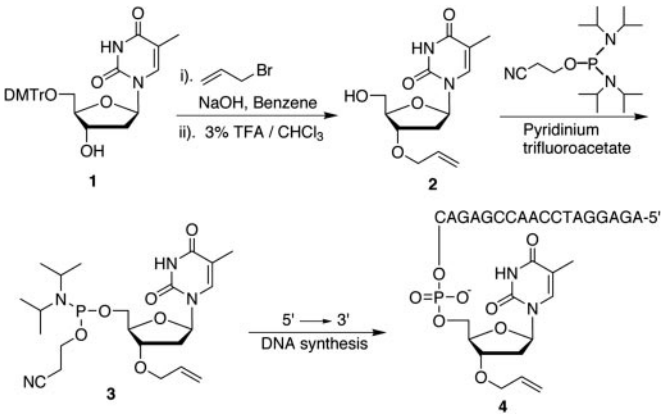
variety of 3′-modified nucleotides to be used as terminators for various DNA polymerases and reverse transcriptases, but none of the functional groups tested have had established methods to regenerate a free 3′-OH (19–22).

It is known that stable chemical functionalities such as allyl (—CH_2—CH═CH_2) and methoxymethyl (—CH_2—O—CH_3) groups can be used to cap an OH group, and can be cleaved chemically with high yield (23, 24). We therefore proposed the use of such groups as reversible caps for the 3′-OH of the nucleotide for SBS (12). We report here our efforts in establishing the allyl group as a 3′-OH capping moiety for the nucleotide analogues that can be used in SBS. The choice of this group was based on the fact that the allyl moiety, being relatively small, would not provide significant hindrance for the polymerase reaction and would therefore allow the incoming 3′-*O*-allyl-modified nucleotide analogue to be accepted by DNA polymerase. Furthermore, it would be possible to remove this group by using catalytic deallylation. Here, we report the design and synthesis of a photocleavable fluorescent nucleotide analogue, 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 (PC-Bodipy, photocleavable 4,4-difluoro-4-bora-3α,4α-diaza-*s*-indacene), that can be efficiently incorporated by DNA polymerase into a growing DNA strand. The allyl group can be rapidly and completely removed by a Pd-catalyzed reaction to regenerate a 3′-OH group, and the deallylated DNA can then allow reinitiation of the polymerase reaction to incorporate the subsequent nucleotide analogue.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. Oligonucleotides used as primers or templates were synthesized on an Expedite nucleic acid synthesizer (Applied Biosystems). ^{1}H NMR spectra were recorded on a Bruker 400 spectrometer, and ^{13}C and ^{31}P NMR spectra were recorded on a Bruker 300 spectrometer. High-resolution MS data were obtained by using a JEOL JMS HX 110A mass spectrometer. Mass measurement of DNA was made on a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems). Photolysis was performed by using a Spectra Physics GCR-150-30 Nd-yttrium/aluminum garnet laser that generates light pulses at 355 nm (≈50 mJ per pulse; pulse length of ≈7 ns) at a frequency of 30 Hz with a light intensity at ≈1.5 W/cm^2. Thermo Sequenase DNA polymerase, HIV-1, and RAV2 reverse transcriptases were obtained from Amersham Biosciences. Therminator, Vent (exo-), Deep Vent (exo-), Bst, and Klenow (exo-) fragment DNA polymerases were obtained from New England Biolabs. 9°N polymerase (exo-) A485L/Y409V was generously provided by New England Biolabs. Sequenase V2 DNA polymerase, M-MulV, and AMV reverse transcriptases were obtained from United States Biochemical. Tfl and Tth DNA polymerases were obtained from Promega. Pfu (exo-) DNA polymerase was obtained from Stratagene. Phosphoramidites and columns for nucleic acid synthesis were obtained from Glen Research (Sterling, VA).

Synthesis of a 3′-*O*-allyl-Modified 19-mer Oligonucleotide. 3′-*O*-allylthymidine phosphoramidite **3**, prepared according to Scheme 1 (also see the supporting information, which is published on the PNAS web site), was used to synthesize a 19-mer oligonucleotide, 5′-AGA-GGA-TCC-AAC-CGA-GAC-**T**(**allyl**)-3′ **4** (molecular weight of 5,871). The synthesis was carried out in the 5′-to-3′ direction by using **3** along with dA-5′-CE, dC-5′-CE, dG-5′-CE, and dT-5′-CE phosphoramidites and a dA-5′-CPG column. The oligonucleotide was purified by HPLC, using an Xterra MS C18 (4.6 × 50 mm) column (Waters). The elution was performed over 90 min at a flow rate of 0.5 ml/min and a fixed temperature of 50°C, using a linear gradient (12–34.5%) of methanol in a buffer containing 8.6 mM triethylamine and 100 mM hexafluoroisopropyl alcohol (pH 8.1). The product was characterized by using MALDI-TOF MS.



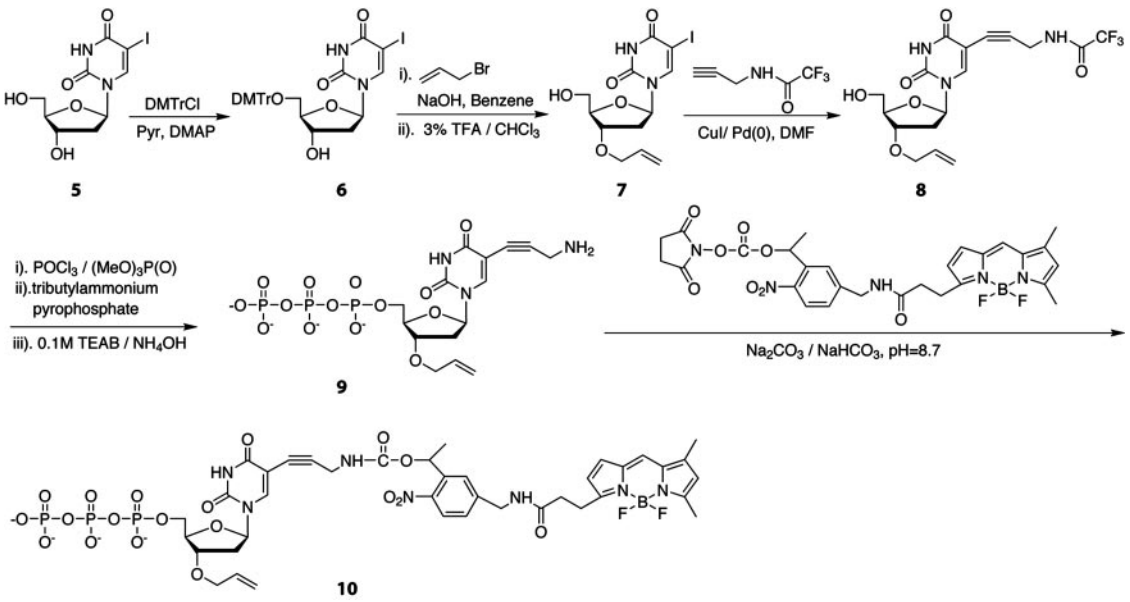
Scheme 1. Synthesis of a 3′-*O*-allyl-modified 19-mer oligonucleotide.

Deallylation Reaction Performed by Using the 3′-*O*-allyl-Modified 19-mer Oligonucleotide. For the deallylation reaction, we used 55 eq of Na_2PdCl_4 and 440 eq of a trisodium triphenylphosphinetrisulfonate (TPPTS) ligand in water at 70°C. Na_2PdCl_4 in degassed water (0.7 μl, 2.2 nmol) was added to a solution of TPPTS in degassed water (1 μl, 17.6 nmol) and mixed well. After 5 min, a solution of 3′-*O*-allyl-modified oligonucleotide **4** (1 μl, 40 pmol) was added. The reaction mixture was then placed in a heating block at 70°C and incubated for 30 sec. The resulting deallylated product was desalted by ZipTip (Millipore) and analyzed by using MALDI-TOF MS.

Primer Extension Reaction Performed with the Deallylated DNA Product. The 10-μl extension reaction mixture consisted of 45 pmol of the deallylated DNA product as a primer, 100 pmol of a single-stranded synthetic 60-mer DNA template (sequence shown in ref. 15) corresponding to a portion of exon 7 of the *p53* gene, 100 pmol of biotin-11–2′,3′-dideoxyguanosine-5′-triphosphate (Biotin-11-ddGTP) terminator (PerkinElmer), 1× Thermo Sequenase reaction buffer, and 4 units of Thermo Sequenase DNA polymerase. The extension reaction consisted of 15 cycles at 94°C for 20 sec, 48°C for 30 sec, and 60°C for 60 sec. The product was purified by using solid-phase capture on streptavidin-coated magnetic beads (25), desalted by using ZipTip, and analyzed by using MALDI-TOF MS.

Synthesis of 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510. 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 **10** was synthesized as shown in Scheme 2. Detailed synthesis procedures and characterization data for all intermediate compounds (**6**-**9**) are described in the supporting information.

PC-Bodipy-FL-510 NHS ester (13) (7.2 mg, 12 mol) in 300 μl of acetonitrile was added to a solution of 3′-*O*-allyl-5-(3-aminoprop-1-ynyl)-2′-deoxyuridine-5′-triphosphate **9** (2 mg, 4 mol) in 300 μl of Na_2CO_3-$NaHCO_3$ buffer (0.1 M, pH 8.7). The reaction mixture was stirred at room temperature for 3 h. A preparative silica-gel TLC plate was used to separate the unreacted PC-Bodipy-FL-510 NHS ester from the fractions containing **10** ($CHCl_3$/CH_3OH, 85/15). The product was concentrated further under vacuum and purified with reverse-phase HPLC on a 150 × 4.6-mm C18 column to obtain the pure product **10** (retention time of 35 min). Mobile phase: A, 8.6 mM triethylamine/100 mM hexafluoroisopropyl alcohol in water (pH 8.1); B, methanol. Elution was performed with 100% A isocratic over 10 min, followed by a linear gradient of 0–50% B for 20 min and then 50% B isocratic over another 20 min. 3′-*O*-allyl-dUTP-PC-



Scheme 2. Synthesis of 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510.

Bodipy-FL-510 **10** was characterized by the following single-base extension reaction and MALDI-TOF MS.

Primer Extension by Using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 and Photocleavage of the Extension Product. An 18-mer oligonucleotide, 5′-AGA-GGA-TCC-AAC-CGA-GAC-3′ (molecular weight of 5,907), was synthesized by using dA-CE, dC-CE, dG-CE, and biotin-dT phosphoramidites. A primer extension reaction was performed by using a 15-μl reaction mixture consisting of 50 pmol of primer, 100 pmol of single-stranded synthetic 60-mer DNA template corresponding to a portion of exon 7 of the *p53* gene (15), 200 pmol of 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510, 1× Thermopol reaction buffer (New England Biolabs), and 15 units of 9°N polymerase (exo-) A485L/Y409V. The extension reaction consisted of 15 cycles of 94°C for 20 sec, 48°C for 30 sec, and 60°C for 60 sec. A small portion of the DNA extension product **11** was desalted by using ZipTip and analyzed by using MALDI-TOF MS. The rest of the product was freeze-dried, resuspended in 200 μl of deionized water, and irradiated for 10 sec in a quartz cell with path lengths of 1.0 cm employing a Nd-yttrium/aluminum garnet laser (≈355 nm) to cleave the fluorophore from the DNA, yielding product **12**.

Deallylation of the DNA Extension Product Generated by the Incorporation of 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510. The above photocleaved 3′-*O*-allyl-modified DNA product **12** (180 pmol produced in multiple reactions) was dried and resuspended in 1 μl of deionized H_2O. Na_2PdCl_4 in degassed H_2O (4.1 μl, 72 nmol) was added to a solution of TPPTS in degassed H_2O (2.7 μl, 9 nmol) and mixed well. After 5 min, the above DNA product (1 μl, 180 pmol) was added. The reaction mixture was then placed in a heating block, incubated at 70°C for 90 sec to yield deallylated product **13**, and cooled to room temperature for analysis by MALDI-TOF MS.

Polymerase Extension and Photocleavage by Using the Deallylated DNA Product as a Primer. The above deallylated DNA product **13** was used as a primer in a single-base extension reaction. The 10 μl reaction mixture consisted of 50 pmol of the above deallylated product **13**, 100 pmol of the 60-mer template (15), 125 pmol of dGTP-PC-Bodipy-FL-510 (14), 4 units of Thermo Sequenase DNA polymerase, and 1× reaction buffer. The extension reaction consisted of 15 cycles of 94°C for 20 sec, 48°C for 30 sec, and 60°C for 60 sec. The DNA extension product **14** was desalted by using the ZipTip protocol, and a small portion was analyzed by using MALDI-TOF MS. The remaining product was then irradiated with near-UV light for 10 sec to cleave the fluorophore from the extended DNA product. The resulting photocleavage product **15** was desalted and analyzed by using MALDI-TOF MS.

Results and Discussion

In this article, we have shown that an allyl moiety can be successfully used as a blocking group for the 3′-OH of a photocleavable fluorescent nucleotide analogue in SBS to prevent the DNA polymerase reaction from continuing after the incorporation of the 3′-*O*-allyl-modified nucleotide analogue. Furthermore, we have demonstrated that the allyl group can be efficiently removed to generate a free 3′-OH group and allow the DNA polymerase reaction to continue to the subsequent cycle.

Conventional methods for cleavage of the allyl group combine a transition metal-catalyzed isomerization of the double bond to the enol ether and subsequent hydrolysis of the latter to produce the corresponding alcohol (26, 27). For application in SBS, it is important to ensure that complete chemical cleavage of the 3′-*O*-allyl group can be rapidly and specifically carried out while leaving the DNA intact. TPPTS has been widely used as a ligand for Pd-mediated deallylation under aqueous conditions (28–30), whereas an active Pd catalyst can be generated from Na_2PdCl_4 and an appropriate ligand (31, 32). Thus, we investigated a water-soluble Pd catalyst system generated from Na_2PdCl_4 and TPPTS for deallylation of the 3′-*O*-allyl-modified DNA product.

To evaluate the cleavage conditions of the allyl group capping the 3′-OH of DNA, we first synthesized a 19-mer oligonucleotide [5′-AGAGGATCCAACCGAGAC-**T**(**allyl**)-3′] using 3′-*O*-allyl-thymidine phosphoramidite (Scheme 1). The identity of the purified oligonucleotide was established by using MALDI-TOF MS. We then tested the above Na_2PdCl_4/TPPTS catalyst system for the deallylation of the oligonucleotide. In Fig. 1*A*, the mass peak at m/z 5,871 corresponds to the mass of the purified oligonucleotide bearing the allyl group. Fig. 1*B* shows a single mass peak at m/z 5,831, corresponding to the deallylated DNA product, indicating that we were able to achieve near-complete deallylation with a DNA/Na_2PdCl_4/TPPTS ratio of 1/55/440 in

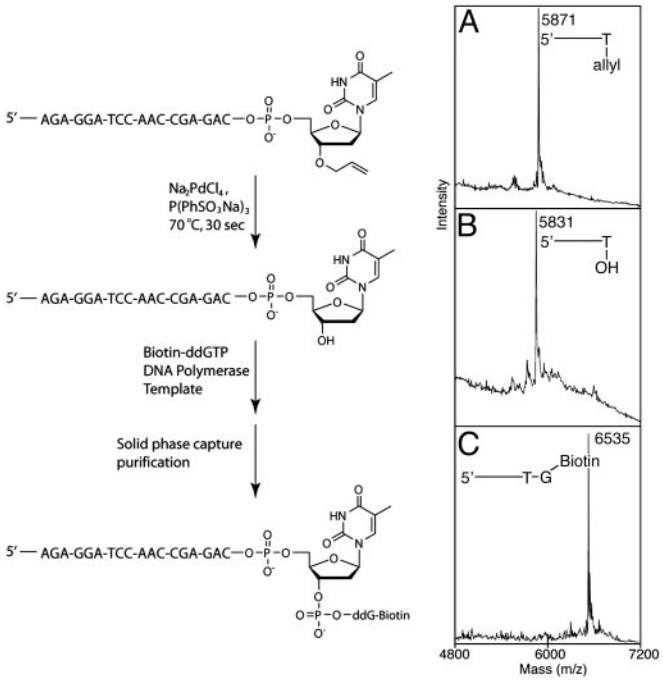


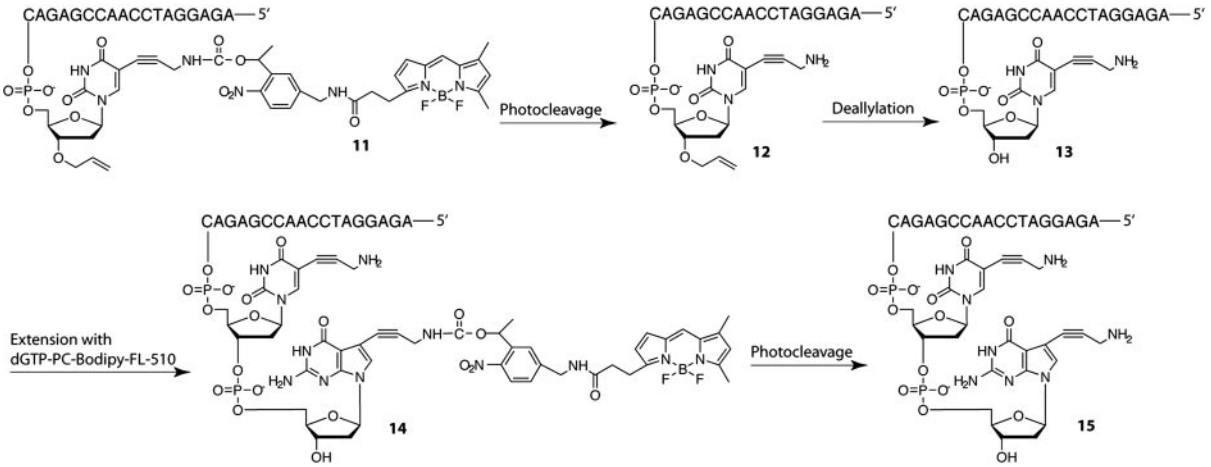
Fig. 1. Schematic representation (*Left*) and step-by-step MALDI-TOF MS results (*Right*) for the deallylation of a 3′-*O*-allyl-modified oligonucleotide and the use of the deallylated oligonucleotide as a primer in a polymerase extension reaction. (*A*) Peak at m/z 5,871 corresponding to the HPLC-purified 3′-*O*-allyl-modified 19-mer oligonucleotide. (*B*) Peak at m/z 5,831 corresponding to the above oligonucleotide without the allyl group, obtained after 30 sec of incubation with Na_2PdCl_4 and TPPTS [$P(PhSO_3Na)_3$] at 70°C. (*C*) Peak at m/z 6,535 corresponding to the extension of the deallylated oligonucleotide by Biotin-11-ddGTP using Thermo Sequenase DNA polymerase.

a reaction time of 30 sec. The next step was to prove that the above deallylated DNA product could be used as a primer in a polymerase extension reaction. We therefore carried out a single-base extension reaction using the deallylated DNA product as a primer, a synthetic template, and a biotin-11-ddGTP nucleotide terminator that was complementary to the base immediately adjacent to the priming site on the template. The DNA extension product was isolated by using solid-phase capture purification and analyzed by using MALDI-TOF MS (25). The mass spectrum in Fig. 1*C* shows a clear peak at m/z 6,535 corresponding to the extension product, indicating that the deallylated product can be successfully used as a primer in a polymerase reaction.

The above experiments established that Na_2PdCl_4 and TPPTS could be used to efficiently carry out deallylation on DNA in an aqueous environment. Our next step was to investigate whether a 3′-*O*-allyl-modified nucleotide could be incorporated in a DNA polymerase reaction. For this purpose, we synthesized a nucleotide analogue 3′-*O*-allyl-thymidine triphosphate (3′-*O*-allyl-dTTP) (supporting information), which was tested with 15 different polymerases for incorporation. The tested enzymes included Therminator, Thermo Sequenase, Vent (exo-), Deep Vent (exo-), Tth, Tfl, Bst, Pfu (exo-), Klenow (exo-) fragment and Sequenase DNA polymerases, AMV, RAV2, M-MulV, HIV reverse transcriptases, and a 9°N polymerase (exo-) bearing the mutations A485L and Y409V. Our preliminary results showed that 9°N DNA polymerase (exo-) A485L/Y409V could efficiently incorporate 3′-*O*-allyl-dTTP in an extension reaction, consistent with results reported recently (31).

After confirming the incorporation ability of 3′-*O*-allyl-dTTP into a growing DNA strand by DNA polymerase, we then synthesized a new 3′-modified photocleavable fluorescent nucleotide analogue, 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510, according to Scheme 2, and established that it also can be efficiently incorporated by the above polymerase. The aim was to evaluate that the presence of the bulky photocleavable fluorescent moiety on the base and the allyl group on the 3′ end of the nucleotide analogue would not affect the polymerase extension reaction. Furthermore, we wanted to demonstrate an entire cycle of primer extension, photocleavage of the fluorophore, deallylation followed by extension with another photocleavable fluorescent nucleotide complementary to the next base on the template, and photocleavage once again. This experiment will thus test the feasibility of using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 as a reversible terminator for SBS.

The entire cycle of a polymerase reaction using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 as a reversible terminator is depicted in Scheme 3. The extension product **11** obtained by using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 and 9°N DNA Polymerase (exo-) A485L/Y409V was purified by using HPLC and analyzed by using MALDI-TOF MS. The base in the template immediately adjacent to the priming site was "A." Thus, if 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 was accepted by the polymerase as a terminator, the primer would extend by one base and then the reaction would terminate. Our results indicate that this was indeed the case. After confirming that the extension reaction was



Scheme 3. One entire polymerase reaction cycle using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 as a reversible terminator.

successful, we irradiated it with near-UV light at 355 nm for 10 sec to cleave the fluorophore from the DNA, generating product **12**. In an SBS system, this step would ensure that there would be no carryover of the fluorescence signal into the next incorporation cycle so as to prevent the generation of ambiguous data at each step, as shown in the companion article (15). The photocleavage product **12** was then incubated with a Na_2PdCl_4/TPPTS catalyst system at 70°C for 90 sec to perform deallylation. The deallylated DNA product **13** was purified by reverse-phase HPLC and then used as a primer in a second DNA extension reaction to prove that the regenerated 3′-OH was capable of allowing the polymerase reaction to continue. For the extension reaction, we used a photocleavable fluorescent nucleotide dGTP-PC-Bodipy-FL-510 and Thermo Sequenase DNA polymerase. The extension product **14** was irradiated as above for 10 sec to generate photocleavage product **15** and hence complete an entire reversible termination cycle.

After each step in the above cycle, a portion of the product was purified and analyzed by using MALDI-TOF MS to confirm its identity and the successful completion of that step. Each product was desalted by using the ZipTip desalting protocol to ensure the generation of sharp and well resolved data free from salt peaks. The MALDI-TOF MS data for the product in each step are shown in Fig. 2. Fig. 2*A* shows the primer extension product **11** at m/z 6,787 generated by using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510. The peak at m/z 6,292 corresponds to the photocleavage product that was generated by the partial photocleavage of the extension product due to the nitrogen laser (≈337 nm) used for ionization of the analyte in MALDI-TOF MS. Fig. 2*B* shows the photocleavage result after the 10-sec irradiation of the extension product at 355 nm. It can be seen from the data that the peak at m/z 6,787, corresponding to the extension product, has completely vanished, and only a single peak corresponding to **12** remains at m/z 6,292, which proves that photocleavage was efficiently achieved. Fig. 2*C* shows a similar single peak at m/z 6,252, which corresponds to the deallylated photocleavage product **13**. The absence of a significant peak at m/z 6,292 proves that deallylation was completed with high efficiency. Fig. 2*D* shows the MALDI-TOF MS data for the extension product obtained by using the above deallylated DNA product **13** as a primer and nucleotide analogue dGTP-PC-Bodipy-FL-510. A dominant peak is seen at m/z 7,133 corresponding to the extension product **14**. Finally, Fig. 2*E* shows a clear peak at m/z 6,637 corresponding to the photocleavage product **15** and no significant peak at m/z 7,133, indicating that complete photocleavage had occurred.

The results of the above experiments provide sufficient proof of the feasibility of using the allyl group as a reversible capping moiety for the 3′-OH of the photocleavable nucleotide analogues for SBS, validating the approach we had previously proposed (12). We have shown that a 3′-*O*-allyl-modified nucleotide bearing a photocleavable fluorophore is an excellent substrate for 9°N DNA polymerase A485L/Y409V and can be incorporated with high efficiency in a polymerase extension reaction. We have also demonstrated that complete photocleavage is achieved in ≈10 sec on these DNA products. Furthermore, we have shown that deallylation can be swiftly achieved to near completion under mild reaction conditions in an aqueous environment by using a palladium catalyst. Finally, we have established that the deallylated DNA product can be used as a primer to continue the polymerase reaction and that extension and photocleavage can be performed with high efficiency. These findings confirm that an allyl moiety protecting the 3′-OH group indeed bestows the capability of reversible terminating abilities to photocleavable nucleotide analogues, which can be used for SBS to facilitate the development of this approach for high-throughput DNA sequencing and genotyping.

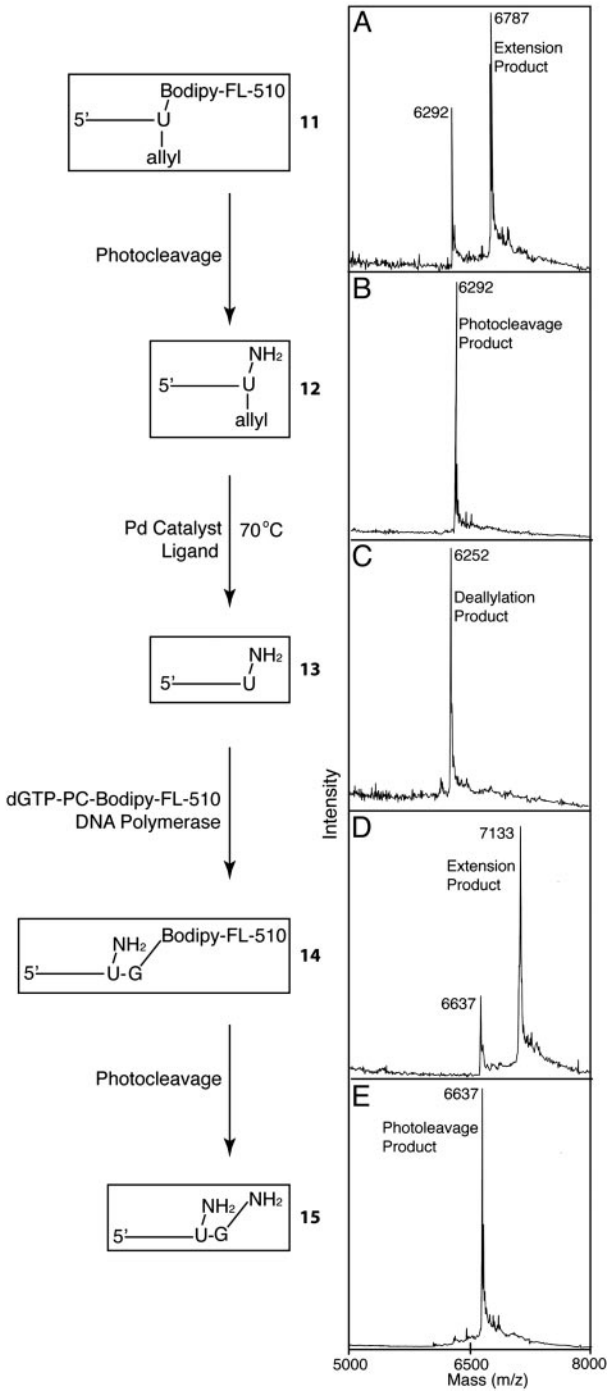


Fig. 2. MALDI-TOF MS results for each step of a polymerase reaction cycle using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 as a reversible terminator. (*A*) Peak at m/z 6,787 corresponding to the primer extension product **11** obtained by using 3′-*O*-allyl-dUTP-PC-Bodipy-FL-510 and the 9°N polymerase (exo-) A485L/Y409V. (*B*) Peak at m/z 6,292 corresponding to the photocleavage product 12. (*C*) Peak at m/z 6,252 corresponding to the photocleavage product without the allyl group **13** obtained after 90 sec of incubation with the catalyst and ligand at 70°C. (*D*) Peak at m/z 7,133 corresponding to the extension product **14** from the purified deallylated product using dGTP-PC-Bodipy-FL-510 and Thermo Sequenase DNA polymerase. (*E*) Peak at m/z 6,637 corresponding to the photocleavage product **15**.

We thank New England Biolabs for generously providing the 9°N DNA polymerase (exo-) A485L/Y409V. This work was supported by National Institutes of Health Center of Excellence in Genomic Science Grants P50 HG002806 and R01 HG003582 and the Packard Fellowship for Science and Engineering.

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