

# Design and synthesis of a photocleavable biotinylated nucleotide for DNA analysis by mass spectrometry

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## ABSTRACT

We report here the design, synthesis and evaluation of a novel photocleavable (PC) biotinylated nucleotide analog, dUTP-PC-Biotin, for DNA polymerase extension reaction to isolate DNA products for mass spectrometry (MS) analysis. This nucleotide analog has a biotin moiety attached to the 5-position of 2'-deoxyribouridine 5'-triphosphate via a photocleavable 2-nitrobenzyl linker. We have demonstrated that dUTP-PC-Biotin can be faithfully incorporated by the DNA polymerase Thermo Sequenase into the growing DNA strand in a DNA polymerase extension reaction and that its incorporation does not hinder the addition of the subsequent nucleotide. Therefore, the DNA extension fragments generated by using the dUTP-PC-Biotin can be efficiently isolated by a streptavidin-coated surface and recovered by near-UV light irradiation at room temperature in mild condition for further analysis without using any chemicals or heat. Single and multiple primer extension reactions were performed using the dUTP-PC-Biotin to generate DNA products for MALDI-TOF MS analysis. Such nucleotide analogs that carry a biotin and a photocleavable linker will allow the isolation and purification of DNA products under mild conditions for MS-based genetic analysis by DNA sequencing or multiplex single nucleotide polymorphism (SNP) detection. Furthermore, these nucleotide analogs should also be useful in isolating DNA-protein complexes under non-denaturing conditions.

## INTRODUCTION

With the completion of the human genome project (1,2), many regions in the genome have become available for systematic studies on gene function and regulation. These studies will contribute greatly to the understanding of the molecular basis of disease and the development of new therapeutics. The

development of robust and efficient genomic analytical methods is critical for such studies as we explore the potential of the complete human genome sequence map.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been widely used for rapid and accurate analysis of DNA fragments. For instance, DNA sequencing by MS has been reported by several groups (3,4) with the potential to eliminate the difficulties typically encountered when using fluorescence capillary electrophoresis sequencing techniques, such as inaccurate data in the GC-rich region due to compressions (5,6) and ambiguous heterozygote detection. Additionally, multiplex genotyping of single nucleotide polymorphisms (SNPs) using MS has been widely used (7,8). In MS-based analysis of DNA, it is critical to isolate and purify DNA fragments from other components in the reaction mixture such as excess primers and falsely terminated DNA fragments, as they can produce extra peaks in the mass spectra, preventing accurate base identification (9). Therefore, for the isolation of DNA extension products, we have previously explored the use of biotinylated dideoxynucleotides to generate DNA fragments that can be captured on a streptavidin-coated surface for accurate DNA sequencing and genotyping (10–12).

The biotin-streptavidin interaction, one of the strongest known non-covalent bonds, is specific and fairly stable, and therefore has been commonly used for biological purification. Nonetheless, denaturation of the biotin-streptavidin bond that is subsequently required in sample recovery involves the use of ammonium hydroxide at 60°C, or formamide at 94°C. In addition, after cleavage, the biotin moiety remains with the purified DNA fragments. This is disadvantageous for the higher resolution analysis of DNA fragments using MS since biotin contains sulfur that has four major stable isotopes. These problems can be avoided by means of biotinylation reagents with cleavable linker arms. For example, a disulfide bond has been used to link the biotin with the nucleotide, forming a biotinylated nucleoside triphosphate analog to facilitate the release of the DNA products that incorporate the nucleotide analog (13). In addition, the biotin moiety has been attached to peptides and oligonucleotides through a photocleavable linker for sample isolation and purification (14–16). These studies have proven that the light-induced release of

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biomolecules from solid phase is simple and effective, circumventing the undesirable use of strong bases or other denaturing reagents. However, in these approaches, the photocleavable biotin moiety was introduced into the target biomolecules separately either during the solid phase synthesis or in post-synthetic modifications. Direct introduction of a photocleavable biotin moiety during the DNA extension reaction would significantly facilitate the utilization of such linkers. Yet, a nucleotide analog containing a photocleavable biotin which can be introduced to the growing DNA strand in PCR and polymerase extension reaction has not been reported.

We report here the design, synthesis and evaluation of a photocleavable (PC) biotinylated nucleotide analog, dUTP-PC-Biotin, for the DNA polymerase extension reaction to isolate DNA products for MS analysis. This nucleotide analog was designed to have a biotin moiety attached to the 5-position of 2'-deoxyuridine 5'-triphosphate via a photocleavable 2-nitrobenzyl linker. Previously, we reported that a photocleavable fluorescent nucleotide using the 2-nitrobenzyl linker could be used successfully for DNA sequencing by synthesis, and the fluorophore was shown to be completely cleaved by near-UV irradiation ( $\lambda \sim 340$  nm) after its incorporation into a growing DNA strand (17). The 2-nitrobenzyl linker is thus chosen as the photocleavable moiety to link a biotin to dUTP in the current study, because it can be selectively cleaved by irradiation with near-UV light at  $\sim 340$  nm without using any chemicals or heat, whereas chemically cleavable linkers such as a disulfide bond can be reversible and destabilized under basic conditions (18,19). We demonstrate here that dUTP-PC-Biotin can be faithfully incorporated into a growing DNA strand in a base-specific manner during the polymerase extension reaction, producing DNA fragments with a photocleavable biotin. The DNA fragments generated can be efficiently isolated by a streptavidin-coated surface and the DNA fragments can be regenerated for MS analysis with near-UV light irradiation at 340 nm onto the surface. Single and multiple primer extension reactions were performed using dUTP-PC-Biotin to generate DNA products for light-induced recovery of the DNA products from the solid surface. Such nucleotide analogs that carry a biotin and a photocleavable linker as shown in this study will allow the isolation and purification of DNA products under mild condition for MS-based genetic analysis by DNA sequencing or multiplex SNP detection. Furthermore, these nucleotide analogs should also be useful in isolating DNA-protein complexes under non-denaturing conditions.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.  $^1\text{H-NMR}$  spectra were recorded on a Bruker 400 spectrometer. High-resolution MS (HRMS) data were obtained by using a JEOL (Tokyo) JMS HX 110A mass spectrometer. Mass measurements were made on a Voyager DE<sup>TM</sup> MALDI-TOF mass spectrometer (Applied Biosystems). Electrospray ionization (ESI) MS was recorded on a Micromass (Manchester, UK) quadrupole-TOF mass spectrometer. HPLC was performed on a Waters system (Milford, MA) consisting of a Rheodyne 7725i injector, 600 controller and a 996 photodiode array detector.

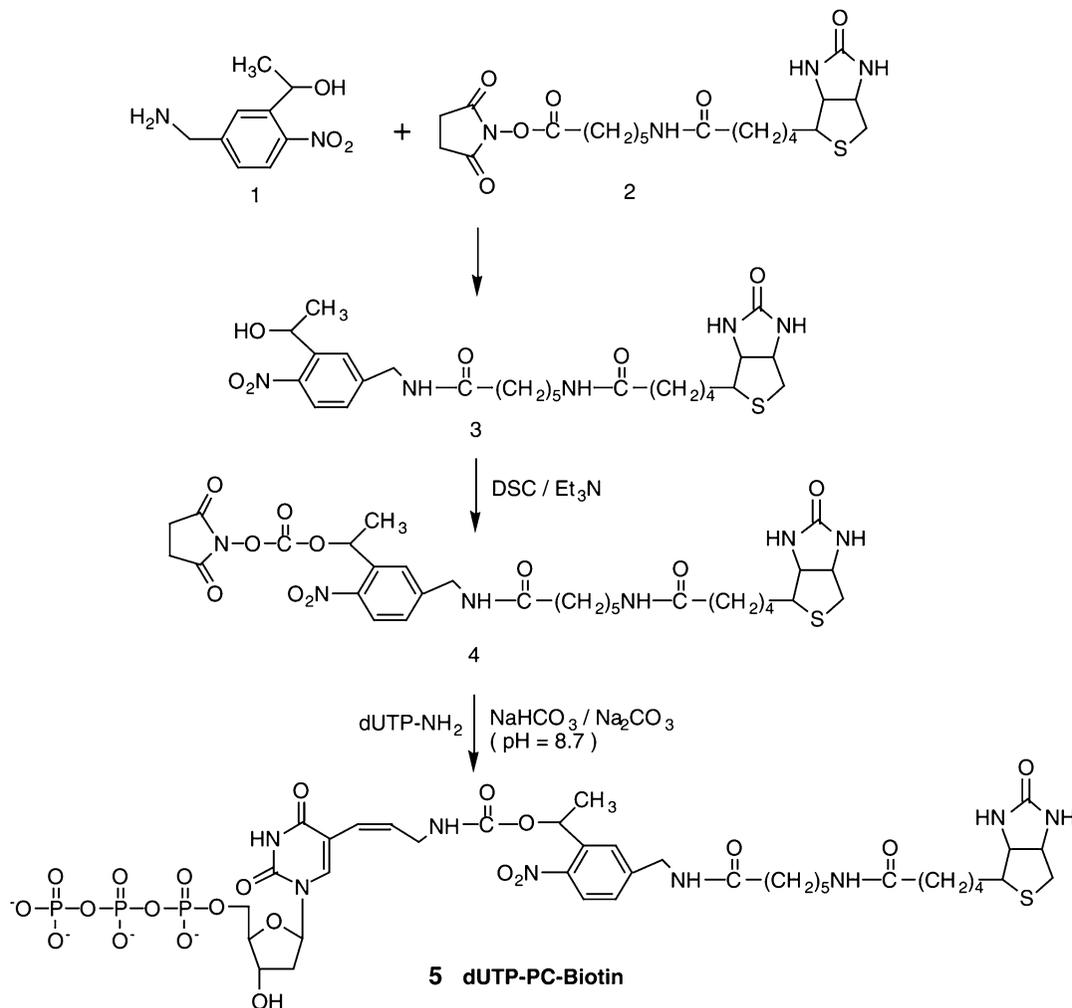
## Synthesis of dUTP-PC-Biotin

dUTP-PC-Biotin was synthesized as shown in Figure 1, involving the following steps.

**Biotin-PC linker 3.** 1-[5-(Aminomethyl)-2-nitro]phenyl ethanol **1** was synthesized as reported in the literature (14). A 9 mg aliquot (47  $\mu\text{mol}$ ) of **1** was dissolved in 450  $\mu\text{l}$  of DMF and then mixed with 100  $\mu\text{l}$  of 1 M  $\text{NaHCO}_3$  aqueous solution. To this solution was slowly added 9 mg (20  $\mu\text{mol}$ ) of Biotin NHS ester **2** in 450  $\mu\text{l}$  of DMF. The resulting solution was stirred for 5 h at room temperature and TLC analysis showed that all NHS ester was completely consumed. The crude products were separated on a preparative TLC plate ( $\text{CHCl}_3/\text{CH}_3\text{OH} = 80/20$ ) to yield 8.6 mg of the pure Biotin-PC linker **3** with 80% yield.  $^1\text{H-NMR}$  (400 MHz,  $d_6$ -DMSO)  $\delta$  8.48(t, 1H), 7.85(d, 1H), 7.75(t, 1H), 7.71(s, 1H), 7.31(d, 1H), 6.41(s, 1H), 6.36(s, 1H), 5.76(s, 1H), 5.52(q, 1H), 4.35(d, 2H), 4.32–4.29(m, 1H), 4.15–4.11(m, 1H), 3.13–3.08(m, 1H), 2.99(q, 2H), 2.85–2.80(m, 1H), 2.57(d, 1H), 2.14(t, 2H), 2.03(t, 2H), 1.66–1.24(m, 15H). HRMS (FAB<sup>+</sup>)  $m/z$ : anal. calculated for  $\text{C}_{25}\text{H}_{38}\text{O}_6\text{N}_5\text{S}$  ( $\text{M}+\text{H}^+$ ), 536.2543; found, 536.2530.

**Biotin-PC-NHS ester 4.** To a solution of 5 mg (9  $\mu\text{mol}$ ) of the Biotin-PC linker **3** in 200  $\mu\text{l}$  of dry DMF was added 3.4 mg (14  $\mu\text{mol}$ , 1.5 equivalents) of  $N,N'$ -disuccinimidyl carbonate and 3.8  $\mu\text{l}$  of triethylamine (27  $\mu\text{mol}$ , 3 equivalents) and then the solution was stirred under argon at room temperature for 6 h. The solvent was removed and 1 ml of 1 M  $\text{NaHCO}_3$  aqueous solution was added into the residual mixture. The solution was extracted with ethyl acetate three times and the combined extracts were washed with brine and dried over  $\text{Na}_2\text{SO}_4$  and then concentrated under vacuum. A preparative TLC plate was used to isolate 2.1 mg of the desired Biotin-PC-NHS **4** with 35% yield ( $\text{CHCl}_3/\text{CH}_3\text{OH} = 80/20$ ). The compound **4** was characterized by  $^1\text{H-NMR}$  (400 MHz,  $d_6$ -DMSO)  $\delta$  8.50(t, 1H), 8.03(d, 1H), 7.75(t, 1H), 7.67(s, 1H), 7.45(d, 1H), 6.45(s, 1H), 6.38(s, 1H), 6.25(q, 1H), 4.40(d, 2H), 4.32–4.29(m, 1H), 4.14–4.11(m, 1H), 3.12–3.06(m, 1H), 2.98(q, 2H), 2.84–2.79(m, 5H), 2.56(d, 1H), 2.16(t, 2H), 2.02(t, 2H), 1.71(d, 3H), 1.67–1.25(m, 12H). HRMS (FAB<sup>+</sup>)  $m/z$ : anal. calculated for  $\text{C}_{30}\text{H}_{41}\text{O}_{10}\text{N}_6\text{S}$  ( $\text{M}+\text{H}^+$ ), 677.2605; found, 677.2643.

**dUTP-PC-Biotin 5.** To a solution of 1 mg (2  $\mu\text{mol}$ ) of 5-(3-aminoallyl)-2'-deoxyuridine 5'-triphosphate (dUTP-NH<sub>2</sub>) in 300  $\mu\text{l}$  of 0.1 M  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer (pH 8.7) was added 2.5 mg (4  $\mu\text{mol}$ ) of Biotin-PC-NHS **4** in 500  $\mu\text{l}$  of DMF, and the resulting solution was stirred at room temperature for 5 h. TLC was used to remove the unreacted Biotin-PC-NHS ( $\text{CHCl}_3/\text{CH}_3\text{OH} = 60/40$ ) and the fractions containing dUTP-PC-Biotin were pulled and then purified by reverse-phase HPLC on a  $150 \times 4.6$  C18 column (Supelco, PA) to yield  $\sim 0.6$  mg of pure product dUTP-PC-Biotin ( $R_t = 28$  min) with an isolation yield of 30%. 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. The compound **5**, dUTP-PC-Biotin, was characterized by the TOF MS ES<sup>+</sup>  $m/z$ : anal. calculated for  $\text{C}_{38}\text{H}_{56}\text{O}_{21}\text{N}_8\text{P}_3\text{S}$  ( $\text{M}+\text{H}^+$ ), 1085.88; found, 1085.31.



DSC : N,N'-disuccinimidyl carbonate

dUTP-NH<sub>2</sub> : 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate

**Figure 1.** Synthesis of dUTP-PC-Biotin.

### DNA extension reaction using dUTP-PC-Biotin

A polymerase extension reaction was conducted using dUTP-PC-Biotin, a primer, and a synthetic template containing sequences related to a portion of exon 7 or 8 of the *p53* gene. The sequences of primers and templates are listed in Table 1. Templates and primers were chosen such that the annealing site of the primer was immediately followed by an 'A' and a 'C', so that by using dUTP-PC-Biotin and ddGTP, the reaction was necessarily terminated after a two-base extension. The extension reaction mixture for a single primer extension included 80 pmol of template **1**, 160 pmol of the primer **2**, 320 pmol of dUTP-PC-Biotin, 160 pmol of ddGTP, 8  $\mu$ l of 10 $\times$  reaction buffer and 8 U of DNA polymerase Thermo Sequenase in a total volume of 80  $\mu$ l. The extension reaction consisted of 25 cycles of 94°C for 20 s, 48°C for 40 s and 72°C for 20 s. For the multiplex extension reaction, 60 pmol of template **1**, 20 pmol of template **2** and 50 pmol each of four

primers were mixed with 320 pmol of dUTP-PC-Biotin, 200 pmol of ddGTP, 10  $\mu$ l of 10 $\times$  reaction buffer and 8 U of DNA polymerase Thermo Sequenase in a total volume of 100  $\mu$ l. The concentrations of all four primers and the two templates were chosen experimentally to generate four extension products that give relatively even peaks in the mass spectrum. The reaction cycles were the same as the single primer extension reaction. Prior to photolysis, the extension reaction products were purified and analyzed by MALDI-TOF MS to verify the incorporation of dUTP-PC-Biotin. A portion of the reaction product (20  $\mu$ l) was mixed with 15  $\mu$ l of streptavidin-coated magnetic beads (Seradyn, Indianapolis) that had been pre-washed and resuspended in a modified binding and washing (B/W) buffer (0.5 mM Tris-HCl buffer, 2 M NH<sub>4</sub>Cl, 1 mM EDTA, pH 7.0), and incubated for 30 min as described (20). Then the beads were washed with 200  $\mu$ l each of a modified B/W buffer, 0.1 M TEAA buffer and water. Subsequently, the extension products were recovered by

**Table 1.** Oligonucleotide primers and synthetic DNA templates for the DNA polymerase reaction using dUTP-PC-Biotin

Primers	Primer sequences	Mass (Da)	Mass of extension products (Da)	Mass of photocleaved extension products (Da)
1	5'-CTCTCTGGCCGCG-3'	3903	5122	4561
2	5'-AGAGGATCCAACCGAGAC-3'	5526	6744	6183
3	5'-AGACTGACATGGTGGTAGGTGA-3'	6879	8099	7538
4	5'-AAAGGATAGGACTCATCACCATTAGA-3'	7996	9215	8654
Templates	Template sequences			
1	5'-TACCCGGAGGCCAAGTACGGCGGGTACGTCCTTGACAATGTGTACATCAACATCACCTACCACCATGTCAGTCTCGGTGGATCCTCTATTGTGTCCGGG-3'			
2	5'-GAAGGAGACACGGGCCAGAGAGGGTCTGTCCGTGTTGTGCGTGGAGTTTCGACAAGGCAGGGTCATCTAATGGTGATGAGTCCTATCCTTTTCTCTCCGT-3'			

For single primer extension reaction, template **1** and primer **2** were used whereas all four primers and the two templates were mixed together in a single tube for multiplex extension reaction.

treatment with 5  $\mu$ l of 98% formamide solution containing 0.2 M EDTA at 94°C for 5 min. In general, the recovery efficiency of oligonucleotides immobilized on a magnetic bead by formamide treatment at 94°C is >90%. The released primer extension products were precipitated with 100% ethanol at 4°C for 1 h, centrifuged at 4°C and 14 000 r.p.m. for 45 min and dried for MALDI-TOF MS as described before (12).

#### Photocleavage of DNA extension products generated using dUTP-PC-Biotin

For the photocleavage reaction, the products of either the single (40  $\mu$ l) or multiplex (60  $\mu$ l) DNA extension reaction were mixed with 60  $\mu$ l of pre-washed streptavidin-coated magnetic beads in a modified B/W buffer. After 30 min of incubation, the beads were washed with 200  $\mu$ l each of modified B/W buffer, 0.1 M TEAA buffer and water, successively. After washing, the beads were resuspended in 500  $\mu$ l of water and irradiated by an LX300UV xenon lamp (ILC Tech., Sunnyvale, CA) in conjunction with a monochromator at 340 nm (light intensity 3 mW/cm<sup>2</sup>), for 10 min with gentle stirring. Subsequently, the liquid phase containing the released DNA fragments was separated from the beads, dried, and analyzed by MALDI-TOF MS.

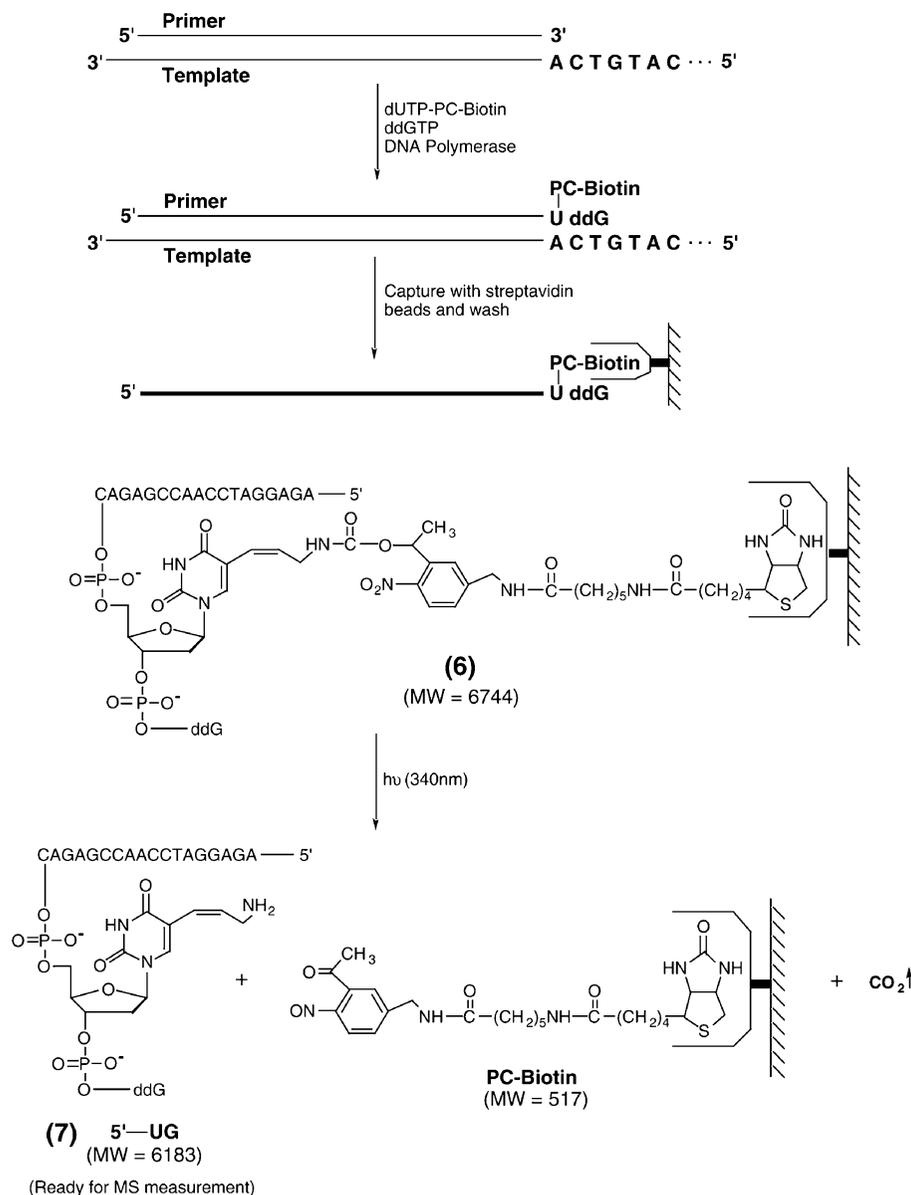
## RESULTS AND DISCUSSION

A photocleavable biotinylated nucleotide, dUTP-PC-Biotin, was designed for direct use in DNA polymerase reactions to introduce a photocleavable biotin moiety into the DNA strand. The dUTP-PC-Biotin, synthesized as shown in Figure 1, bears a biotin moiety on its 5-position via a photocleavable 2-nitrobenzyl linker. The 2-nitrobenzyl moiety has been previously shown to be a stable linker that is also highly efficient in photocleavage under UV irradiation ( $\lambda$  ~340 nm) and was thus chosen as the photocleavable linker in this study (17,21,22).

In DNA analysis utilizing dUTP-PC-Biotin, it is essential that the nucleotide analog be incorporated faithfully and efficiently into a growing DNA strand in a polymerase reaction. To verify this, we conducted a DNA extension reaction first with a single primer. The extension reaction was performed using dUTP-PC-Biotin, template **1** and primer **2**. The two nucleotides in the template immediately adjacent to the annealing site of the primer **2** were 5'—CA—3'.

Consequently, the primer extension reaction was carried out in the presence of dUTP-PC-Biotin and ddGTP. Should the dUTP-PC-Biotin be an effective substrate for the DNA polymerase, the extension reaction would be terminated after extension by two bases ('U' and then 'G'), generating DNA fragment **6** as shown in Figure 2. The extension product was purified with streptavidin-coated magnetic beads, released using formamide at 94°C, and subsequently analyzed by MALDI-TOF MS. The result is shown in Figure 3A, where a strong signal corresponding to **6** (6744 *m/z*, found; 6745 *m/z* calculated) is observed. This result indicates that dUTP-PC-Biotin is incorporated efficiently by the Thermo Sequenase in the polymerase reaction. The resulting mass spectrum (Fig. 3A) had two peaks. The major peak corresponded to the reaction product **6**. The smaller peak corresponds to the DNA fragment **7** that is a product of photocleavage reaction. The observed difference in mass between the two peaks matched perfectly with the theoretically calculated value for the cleaved moiety, PC-Biotin. As solid phase capture procedures performed prior to photolysis isolate only biotinylated DNA fragments free from other reaction components, DNA fragment **7** could only be produced by photocleavage of the extension product **6**. This photocleavage occurred in the ionization process of the MALDI-TOF MS measurement where a nitrogen laser at 337 nm is used, which cleaves the photocleavable linker between dUTP and biotin in a small fraction of product **6** (17,21). In addition, peaks corresponding to either a false stop, a primer extended only with dUTP-PC-Biotin (mol. wt 6431 Da) or its photocleaved product (mol. wt 5856 Da) were not detected. This suggests that the incorporation of the dUTP-PC-Biotin did not arrest the extension reaction and was followed immediately by the successive incorporation of another nucleotide. These results demonstrate that the dUTP-PC-Biotin is a good substrate for the DNA polymerase, Thermo Sequenase.

Efficient photocleavage for a prompt recovery of the DNA fragment captured on a streptavidin-coated surface is another critical requirement for successful application of photocleavable nucleotide analogs in DNA analysis. As a proof-of-principle experiment for testing the light-induced release of DNA fragments from a surface, DNA extension fragments carrying biotin were immobilized on streptavidin-coated magnetic beads, and a photocleavage reaction was performed according to the scheme in Figure 2. After 10 min of near-UV

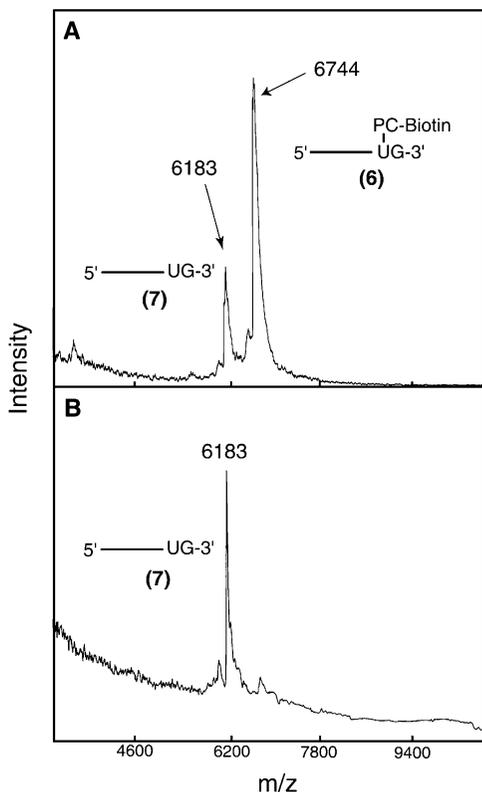


**Figure 2.** Polymerase extension reaction using dUTP-PC-Biotin as a substrate and photocleavage of DNA fragments containing dU-PC-Biotin on a solid surface. DNA polymerase incorporates dUTP-PC-Biotin and ddGTP to generate the DNA fragment **6**. Photocleavage by near-UV light (340 nm) of the DNA fragment **6** captured on streptavidin-coated beads produces DNA fragment **7**, while the PC-Biotin moiety stays on the solid surface of the beads.

irradiation of the bead surface at 340 nm, the aqueous phase was analyzed by MALDI-TOF MS for the released DNA fragments. Figure 3B showed that the MALDI-TOF MS spectrum of the solution produced a single peak, whose mass corresponded to the photocleaved DNA product **7** shown in Figure 2. This experiment indicates that near-UV illumination of the photocleavable linker in DNA fragments immobilized on a solid surface effectively breaks the linker without causing fragmentation of DNA strands and releases DNA molecules that are free of biotin.

We further investigated the application of the nucleotide analog with the photocleavable biotin moiety in the multiplex analysis of DNA extension products. Four primers shown in Table 1 were simultaneously extended with dUTP-PC-Biotin

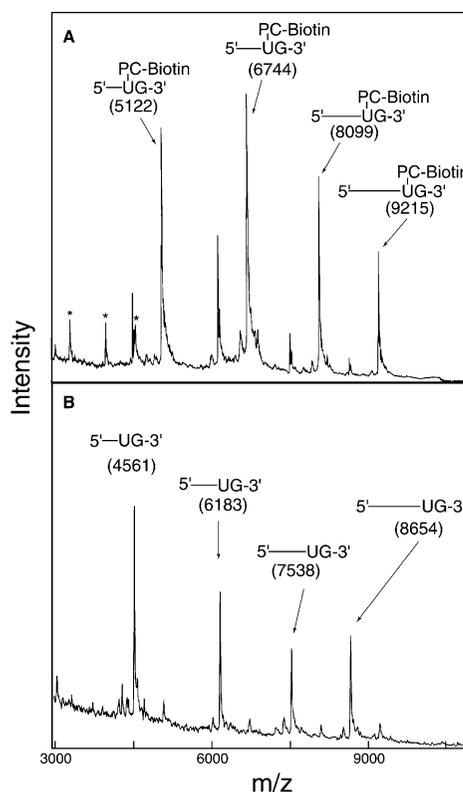
and ddGTP to produce extension products of 15–28 bp in length. Figure 4A shows a mass spectrum of the extension products released by using formamide to denature the biotin–streptavidin binding after solid phase purification. Four extension products were detected in the MALDI-TOF mass spectrum (Fig. 4A) and their masses accurately matched the calculated mass values. Partial photocleavage of the extension products due to the MALDI ionization process by the nitrogen laser was also observed, and false termination was not detected. Then the products of the simultaneous extension reaction were captured on a streptavidin-coated surface to test the photocleavage and the recovery of DNA extension fragments. After washing the beads, near-UV light was irradiated onto the DNA captured on the bead surface and



**Figure 3.** MALDI-TOF mass spectra of the DNA extension product generated from dUTP-PC-Biotin before and after photocleavage. (A) Before UV irradiation. The extension product was purified by solid phase capture and released for MS analysis by using formamide to denature the biotin-streptavidin binding. (B) After 10 min irradiation (340 nm) of the DNA fragments captured on the streptavidin-coated beads, the photocleaved DNA fragment 7 is released without biotin, and detected in the solution.

the released DNA fragments were analyzed by MALDI-TOF MS. As shown in Figure 4B, strong signals corresponding to the photocleaved fragments of all four extension products were detected, suggesting that the photolysis was successful in recovery of multiple DNA samples captured on a surface.

In conclusion, we have synthesized a photocleavable biotinylated nucleotide analog, dUTP-PC-Biotin, and evaluated its applications in rapid recovery of DNA extension products. This nucleotide analog is shown to be an excellent substrate for DNA polymerase Thermo Sequenase in DNA extension reactions. The introduction of a photocleavable biotin moiety into the 5-position of dUTP does not interfere with its recognition by DNA polymerase. DNA fragments that have been incorporated with dUTP-PC-Biotin are isolated by the biotin-streptavidin interaction and rapidly recovered by near-UV ( $\lambda \sim 340$  nm) irradiation without using any strong base and other denaturing reagents. The DNA fragments recovered by photolysis are free of the biotin moiety, making them ideal analytes for higher resolution MS analysis. It should be pointed out that photocleavable biotinylated dideoxy- and deoxynucleotide analogs for all four nucleotides (A, C, G and T) can be synthesized using a procedure similar to that described here. These nucleotide analogs will be a valuable set of reagents for many current and future genetic



**Figure 4.** MALDI-TOF mass spectra of the DNA extension products from four simultaneous primer extension reactions using dUTP-PC-Biotin before and after photocleavage. (A) Before UV irradiation. The extension products were purified by solid phase capture and released for MS analysis by using formamide to denature the biotin-streptavidin binding. The double charged ion peaks are indicated by asterisks. (B) After 10 min irradiation (340 nm) of the DNA fragments captured on the streptavidin-coated beads, photocleaved DNA products are released without biotin, and detected in the solution.

analysis techniques (23) as well as for developing new bioanalytical approaches.

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