Synthesis of a Phosphoramidite Coupling Unit of the Pyrimidine (6−4) Pyrimidone Photoproduct and Its Incorporation into Oligodeoxynucleotides

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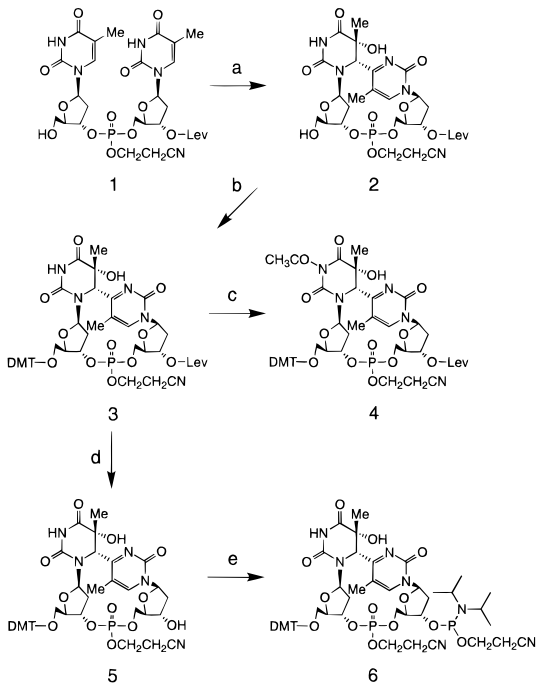
Ultraviolet (UV) light causes lesions in DNA, which induce mutations, cellular transformation, and cell death. At adjacent pyrimidine sites, two major types of photoproducts, namely the cis-syn cyclobutane pyrimidine dimer and the pyrimidine (6−4) pyrimidone photoproduct, are formed.[1] It appears that the (6−4) photoproduct, which induces 3′ thymine-to-cytosine or cytosine-to-thymine transitions,[2] is much more mutagenic than the cis-syn dimer.[3] The (6−4) photoproduct is also important in studies of DNA repair, because it has been suggested that this lesion is removed in human cells via the nucleotide excision repair pathway.[4] For these studies, oligonucleotides containing the damaged base at a single site are of great use, as demonstrated previously.[5] Smith and Taylor reported the preparation of an oligonucleotide containing the (6−4) photoproduct at a single site, in which a hexamer was irradiated with UV and then purified by HPLC.[6] Although this hexamer was elongated by ligation to a 49-mer, which was used for analyses of protein binding[7] and enzyme reactions,[8] this procedure suffers from limitations in chain length, sequence, and yield. One way to solve this problem is to use a dinucleotide building block, as developed for the cis-syn thymine dimer.[9] In this communication, we describe the synthesis of a phosphoramidite coupling unit of the (6−4) photoproduct of thymidylyl(3′−5′)thymidine and its incorporation into 8-mer and 30-mer oligodeoxynucleotides.

The phosphoramidite coupling unit of the (6−4) photoproduct (**6**) was designed in order that it would be used generally on DNA synthesizers. The 4,4′-dimethoxytrityl and 2-cyanoethyl groups were used for the protection of the 5′-hydroxyl group and the internucleoside phosphate, respectively. At the beginning, protection of the hydroxyl group generated by photoproduct formation was planned, but it was found that the reactivity of this hydroxyl was extremely low, as described below. A levulinyl group, which was used successfully in the preparation of the coupling unit of the cis-syn thymine dimer,[10] was chosen for the transient protection of the 3′-hydroxyl group.

Scheme 1[a]



[a] Reagents and yields: (a) UV (254 nm), 16%; (b) 4,4′-dimethoxytrityl chloride, pyridine, 84%; (c) $(CH_3CO)_2O$, DMAP, 49%; (d) $NH_2NH_2{\cdot}H_2O$, pyridine−AcOH, 85%; (e) $NCCH_2CH_2OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, 85%. Abbreviations: DMT, 4,4′-dimethoxytrityl; Lev, levulinyl.

The 3′-levulinyl thymidylyl(3′−5′)thymidine 2-cyanoethyl phosphotriester (**1**) was prepared as described previously.[10] The two diastereomers, due to the chiral phosphorus, could not be separated on silica gel. Irradiation of a 1 mM solution of **1** in 20% aqueous acetonitrile, on a UV-cross-linker equipped with six 15 W germicidal lamps, resulted in the production of two peaks with retention times shorter than that of the starting material, as well as several other peaks, as analyzed by reversed-phase HPLC. These two products each had a UV absorption spectrum with a maximum at 326 nm, which was exactly the same as that reported for the unprotected (6−4) photoproduct of thymidylyl(3′−5′)thymidine.[11] The amounts of these peaks reached a plateau at a UV dose of 30 J/cm^2, while the starting materials were still decreasing. The formation of the photoproduct was performed on a preparative scale, and the products were purified by reversed-phase chromatography on alkylated silica gel. The diastereomers mentioned above were separated at this step and obtained in a ratio of 1:2. The product showed a maximum emission at 397 nm, at an excitation of 313 nm, in its fluorescence spectrum, as reported previously for the unprotected photoproduct.[11] The pyrimidine−pyrimidone structure, including the stereochemistry, was confirmed by NMR spectroscopy, as described in the supporting information. The yield of **2** was not high (16%, in the total of both isomers), but a practical amount was obtained.

The following procedure is shown in Scheme 1. The 5′-hydroxyl group of **2** was protected with the 4,4′-dimethoxytrityl group, and then we tried protection of the hydroxyl group at the base moiety. An acetyl group was chosen to avoid steric hindrance, and the protected dimer (**3**) was treated with an excess amount of acetic anhydride in the presence of 4-(dimethylamino)pyridine. However, the reaction proceeded very

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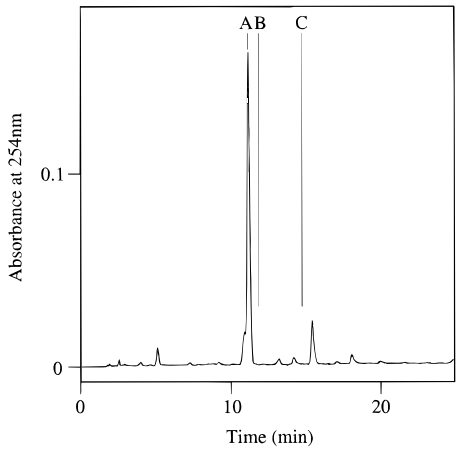

Figure 1. HPLC analysis of crude d(GTAT(6−4)TATG). Lines A, B, and C indicate the retention times of d(GTAT(6−4)TATG) prepared by UV irradiation of d(GTATTATG), d(GTAT(Dewar)TATG), and d(GTATTATG), respectively. A μBondasphere C18 300 Å column (3.9 mm × 150 mm) was used at a flow rate of 1.0 mL/min with a linear gradient of 7%−11% acetonitrile for 20 min in 0.1 M triethylammonium acetate (pH 7.0).

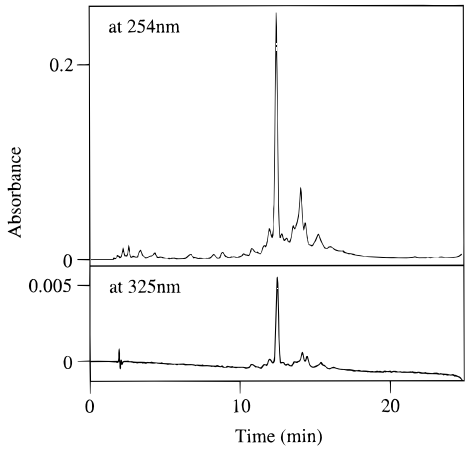

Figure 2. HPLC analysis of the crude (6−4) 30-mer, monitored at 254 and 325 nm. The elution conditions are described in the legend to Figure 1, except that the acetonitrile gradient was from 7% to 13% and the column temperature was 50 °C.

slowly, and only the *N*-acetylated product was obtained. Since the N3 position of thymidine is not protected in oligonucleotide synthesis, the base moiety was left unprotected. Finally, the levulinyl group was removed from **3**, and the resultant 3′-hydroxyl group was phosphitylated.

Using the fully protected dimer, the removal of the protecting groups was tested. Treatment of **4** with 80% acetic acid and then with 28% aqueous ammonia at room temperature for 2 h gave a compound which was coeluted from the HPLC column with the authentic (6−4) photoproduct prepared by UV irradiation of unprotected thymidylyl(3′−5′)thymidine. The stability of the (6−4) photoproduct was also tested using this dimer. It was found that the (6−4) photoproduct was stable in 80% acetic acid and in 28% aqueous ammonia at room temperature but was decomposed when heated above 40 °C in aqueous ammonia.

The octamer, d(GTAT(6−4)TATG), which was previously prepared by UV irradiation of d(GTATTATG),[12] was synthesized on a 0.2 μmol scale first. Due to the instability of the (6−4) photoproduct under alkaline conditions, nucleoside 3′-phosphoramidites with the (4-*tert*-butylphenoxy)acetyl group for the protection of the exocyclic amino groups of dA, dG, and dC, which can be deprotected with ammonia at room temperature,[13] were used in combination with **6**. The reaction time for the coupling of **6** was prolonged to 20 min, and the coupling yield of **6** was 97%. After the chain assembly on a synthesizer, the solid support containing the oligonucleotide was treated with aqueous ammonia at room temperature for 2 h, and the product was analyzed by HPLC (Figure 1). The parent sequence, d(GTATTATG), the (6−4) octamer prepared by UV irradiation of d(GTATTATG), and the Dewar octamer, d(GTAT(Dewar)-TATG), prepared by irradiation of the (6−4) octamer, were eluted under the same conditions. The retention time and the UV absorption spectrum, with maxima at 256 and 327 nm, of the main peak was exactly the same as those of the authentic (6−4) octamer prepared by the irradiation, and no contamination by d(GTATTATG) or d(GTAT(Dewar)TATG) was found in the mixture. After purification by HPLC, the yield of the (6−4) octamer was 6.8 A_{260} units (0.10 μmol, 50% from the 3′-terminal dG).

In order to demonstrate that long oligonucleotides can be obtained by this method without sequence limitations, a 30-mer, d(CTCGTCAGCATCT(6−4)TCATCATACAGTCAGTG), was synthesized. In the HPLC analysis of the deprotected mixture, a main peak, which had absorption maxima at 259 and 327 nm, was detected, together with several impurities (Figure 2). The impurities with retention times longer than that of the main peak had a smaller A_{325}/A_{254} ratio, and prolonged reaction with tetrazole-activated phosphoramidites resulted in production of many peaks and increase in their relative amount. These results suggested that these impurities were branching byproducts caused by coupling with the (6−4) photoproduct. The yield after HPLC purification was 6.0 A_{260} units, and this product was coeluted with d(CTCGTCAGCATC*TT*CATCATACAGTCAGTG) by anion-exchange HPLC.

As described above, we have developed a direct method for the preparation of oligonucleotides containing the (6−4) photoproduct. Oligonucleotides prepared with the coupling unit of the cis-syn thymine dimer have been used in studies of the molecular biology of DNA repair,[14] and we have applied this method to the elucidation of the substrate recognition and the catalytic mechanism of bacteriophage T4 endonuclease V.[15] Similarly, the coupling unit of the (6−4) photoproduct synthesized in this study will contribute significantly to the molecular and cellular biology of DNA repair.

Supporting Information Available: Synthetic procedures and ^{1}H-NMR, ^{31}P-NMR, and HRMS data for all new compounds; determination of configuration at the (6−4) photoproduct (9 pages). See any current masthead page for ordering and Internet access instructions.

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