Studies on the Chemical Synthesis of Oligonucleotides Containing the (6−4) Photoproduct of Thymine−Cytosine and Its Repair by (6−4) Photolyase

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Abstract: To prepare oligonucleotides containing the pyrimidine(6−4)pyrimidone photoproduct between thymine and cytosine, which is formed more efficiently than any counterpart at other dipyrimidine sites, a dimer building block of this photoproduct was synthesized. Irradiation of thymidylyl(3′−5′)deoxycytidine, with protecting groups at the phosphate and the 3′-hydroxyl function, gave a hydrate of cytosine as the main product, and the formation of the (6−4) photoproduct reached a plateau faster than that from the thymidylyl-(3′−5′)thymidine derivative. After chromatographic purification of the desired (6−4) photoproduct, a phosphoramidite building block was synthesized in three steps. When this compound was used for the usual oligonucleotide assembly on a solid support, it was found that acylation of the amino group of the 5′-pyrimidine component occurred at the capping step. Oligonucleotides were synthesized successfully by omitting the capping steps after coupling of the (6−4) photoproduct unit. These synthetic oligonucleotides were applied to the characterization of the (6−4) photolyase. By HPLC analyses of the photoreactivation product and its nucleoside composition, it was demonstrated that this (6−4) photoproduct was repaired to its original pyrimidine components by this enzyme.

Introduction

Base moieties of nucleic acids are subject to modification by ionizing radiation, ultraviolet (UV) light, and various chemicals. Among these damages, those caused by UV radiation at dipyrimidine sites in DNA, namely cis−syn cyclobutane pyrimidine dimers and pyrimidine(6−4)pyrimidone photoproducts (abbreviated as (6−4) photoproducts), have been studied most intensively.[1] The (6−4) photoproduct is produced by ring opening of an oxetane or azetidine intermediate formed by a $2\pi + 2\pi$ photocycloaddition of the excited carbonyl or iminyl group of the 3′-pyrimidine onto the C5−C6 double bond of the 5′ component. Since the two rings constituting the (6−4) photoproduct are perpendicular to each other,[2] this damage causes a large bend in the DNA helix.[3] The (6−4) photoproduct blocks replication, and translesion synthesis induces mutations at extremely high frequencies, especially on the 3′ side of the photoproduct.[4] This DNA damage is repaired by the nucleotide excision repair pathway in human cells,[5] and an enzyme to photoreactivate this damage, (6−4) photolyase, was discovered.[6] The (6−4) photoproduct is converted to its Dewar valence isomer by absorbing UVB light with wavelengths between 280 and 320 nm.[7]

In our previous work, we synthesized a phosphoramidite building block of the (6−4) photoproduct of thymidylyl(3′−5′)thymidine (T(6−4)T; Figure 1) and incorporated it into oligonucleotides,[8] which have been applied to studies of the molecular biology of mutation[9] and repair.[10] Synthetic oligonucleotides containing such specific damage at a single site in a defined sequence are useful tools for biochemical studies on various subjects, including structure,[3] mutagenesis,[4,9] and repair.[10,11] In most cases, the (6−4) photoproduct used in these experiments is T(6−4)T, but it has been reported that the (6−4) photoproduct is formed most efficiently at the TC sequence. For example, in vivo mapping of (6−4) photoproducts at single-nucleotide resolution revealed that the major site was TC (followed by CC),[12] and in quantitative experiments using

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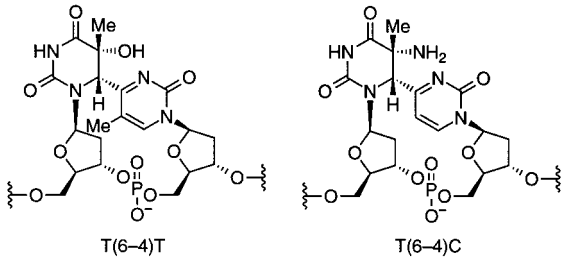

Figure 1. (6−4) Photoproducts formed at TT and TC sequences.

dinucleoside monophosphates, the (6−4) photoproduct of thymidylyl(3′−5′)deoxycytidine (T(6−4)C; Figure 1) was formed about 3 times more efficiently than T(6−4)T.[13]

Consequently, the synthesis of oligonucleotides containing T(6−4)C at a defined site has great significance. Such oligonucleotides have been prepared by UV irradiation of undamaged dodecamers containing a single TC site.[9a,11c] Since the chain length and the sequence are limited in this method, direct solid-phase oligonucleotide synthesis using a phosphoramidite building block appears to be much more efficient, as demonstrated in the T(6−4)T case.[8] However, due to the different chemical structures of thymine and cytosine, there are several problems to be solved. (1) The amino function of the cytosine must be protected to prepare the dinucleoside monophosphate, but this protecting group must be removed prior to UV irradiation. (2) Although the T(6−4)T derivative was the major product in our previous work,[8] there may be side reactions during UV irradiation in the T(6−4)C case. (3) The chemical stability of T(6−4)C may be different from that of T(6−4)T. (4) The amino group of T(6−4)C may undergo side reactions during oligonucleotide assembly. In this article, we describe an approach to the synthesis of oligonucleotides containing T(6−4)C via a dimer building block, as well as our solutions to the above problems.

In the last part of this article, the application of the T(6−4)C-containing oligonucleotides to the characterization of the (6−4) photolyase is described. The (6−4) photolyase was first discovered in a cell extract of *Drosophila melanogaster*,[6] and subsequently was found in *Xenopus laevis* and *Arabidopsis thaliana*.[14] This enzyme contains flavin adenine dinucleotide (FAD) as a chromophore,[14a] and analyses of its reaction using several enzymes strongly suggested that this enzyme repairs the (6−4) photoproducts to their original pyrimidines by utilizing blue to near-UV light.[6,11b] Recently we demonstrated that this enzyme restored the pyrimidines of T(6−4)T to their normal form.[10] Here we present direct evidence that T(6−4)C is also repaired to its intact components, thymine and cytosine.

Results and Discussion

Synthesis of the Building Block. A phosphoramidite building block of the T(6−4)C photoproduct (**7**), which would be prepared by a route similar to that for T(6−4)T,[8] was designed, and its synthesis was the first purpose of this study (Scheme 1). Since the amino functions of nucleobases react with a tetrazole-activated phosphoramidite,[15] the amino function of 2′-deoxycytidine, as well as its 3′-hydroxyl function, was protected to prepare thymidylyl(3′−5′)deoxycytidine. The protecting group for this amino function should be removed without detaching the other base-labile protecting groups because the base moiety must be deprotected before the formation of the (6−4) photoproduct by UV irradiation. The 4,4′-dimethoxytrityl (DMT) group was chosen for this purpose, and 4-*N*-DMT-3′-*O*-levulinyl-2′-deoxycytidine (**2**) was prepared in three steps from 2′-deoxycytidine (**1**). After coupling with 5′-*O*-DMT-thymidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite, the two DMT groups were removed with trichloroacetic acid. It took 25 h at room temperature to deprotect the amino group completely. The resulting dinucleoside monophosphate **3** had a 2-cyanoethyl protecting group, which is used generally in oligonucleotide synthesis, and a levulinyl group, which was used successfully in the T(6−4)T case,[8] at the internucleoside phosphate and the 3′-hydroxyl function, respectively. The diastereomers of **3**, due to the chiral phosphorus atom, could not be separated even by high-performance liquid chromatography (HPLC).

In our previous study, the solubility of the protected thymidylyl(3′−5′)thymidine (the counterpart of **3**) in water was low, and thus, acetonitrile was added to make a solution for UV irradiation.[8] In the T(6−4)C case, compound **3** could be dissolved in water at least up to 4 mM. As shown in Figure 2A, irradiation of **3** on a commercially available UV cross-linker equipped with six 15-W germicidal lamps gave two peaks with an absorption maximum at 314 nm, which were eluted much earlier than the starting material, in an HPLC analysis using a reversed phase column. In addition, several peaks with an absorption maximum at about 240 nm (peaks 3−5 in Figure 2A) were detected, which were not found in the T(6−4)T case (Figure 2B; the two large peaks in the elution profile monitored at 254 nm are the diastereomers of TpT). The UV absorption spectra and the molecular weights determined by mass spectrometry of these products showed that they were photohydrates of cytosine (diastereomers of the dinucleoside monophosphate containing one of the two stereoisomers of 6-hydroxy-5,6-dihydrocytosine).[16] Probably due to the formation of these photohydrates as byproducts, the formation of the T(6−4)C photoproduct reached a plateau earlier than that of T(6−4)T, as shown in Figure 3, although the quantum yield of T(6−4)C at the dinucleoside monophosphate level was reported to be higher.[13] For preparative UV irradiation, a 2 mM aqueous solution of **3** was used, and the two diastereomers of **4** (peaks 1 and 2 in Figure 2A) were separated by chromatography on alkylated silica gel. The major isomer (peak 2) was purified further by anion-exchange chromatography to remove an impurity, which was close to this product in reversed phase chromatography and was probably a byproduct without the 2-cyanoethyl group protecting the internucleoside linkage. Since the minor isomer (peak 1) could not be purified satisfactorily, this major isomer, which was purified to the level shown in Figure 2C, was used to synthesize the phosphoramidite building block.

The structure of the photoproduct **4** was confirmed by ^{1}H and ^{13}C NMR spectroscopy including HMQC and HMBC techniques. The stereochemistry of the pyrimidine−pyrimidone structure was determined using ROESY spectra measured at a mixing time of 300 ms. The two cross-peaks between the proton signals of the base and sugar moieties in the 3′ component, H6−H3′ and H6−H2′, indicated that the pyrimidone base had an

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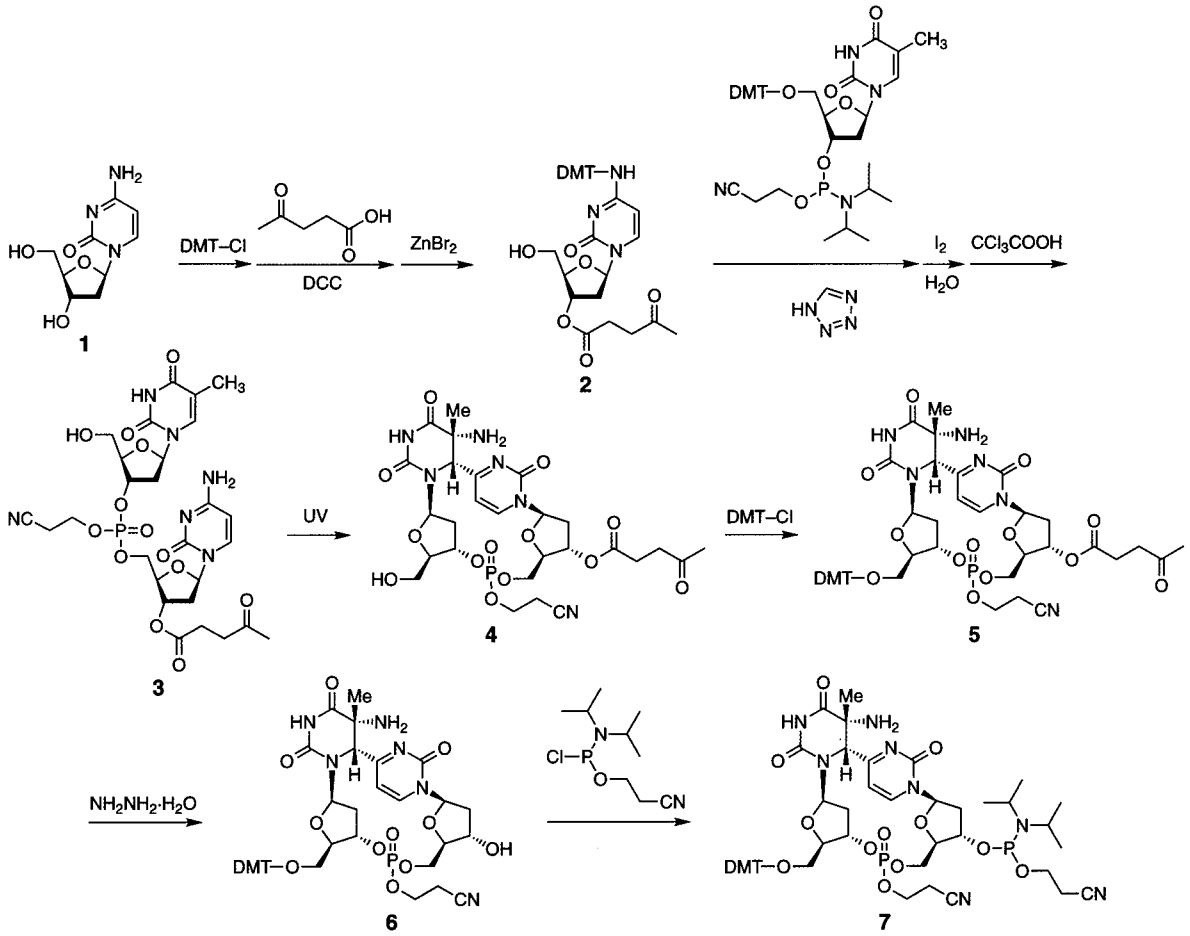
Scheme 1



anti to high-anti conformation. Taking this into account, the cross-peaks between H6 and H3′ of the 5′ component and between H6 of the 5′ component and H5 of the 3′ component demonstrated that the configuration at the C6 position of the pyrimidine was *R*. The configuration at the C5 position of the pyrimidine was determined as *R*, by using the cross-peak between H6 and CH_3 of the 5′ component.

The isolation yield of **4** was low, and about 10 mmol of the starting material **3** was required to obtain an amount sufficient for the following steps. The 5′-hydroxyl function of **4** was protected with the DMT group again, and the levulinyl group at the 3′-position was removed with hydrazine monohydrate to give **6**. Finally, the 3′-hydroxyl function was phosphitylated with (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite in the presence of diisopropylethylamine.[17] In our previous study on T(6−4)T, pyridine was used as a solvent for this phosphitylation reaction, due to a solubility problem;[8] however, the formation of a byproduct, which had an R_f value lower than that of the desired phosphoramidite, was observed frequently. In the present study, pyridine was again the only solvent in which compound **6** could be dissolved, and the formation of a similar byproduct reduced the yield of **7** to a great extent. Separation and analysis of this byproduct revealed that it contained a phosphoramidate residue produced by oxidation of the obtained 3′-phosphoramidite. Therefore, tetrahydrofuran was tested although compound **6** was precipitated when it was mixed with this solvent. As the reaction with the phosphitylating reagent proceeded, the precipitate disappeared, and the phosphoramidite **7** was obtained in good yield without the byproduct formation. Tetrahydrofuran also gave a satisfactory result in the phosphitylation reaction to prepare the T(6−4)T phosphoramidite.

Synthesis of Oligonucleotides Containing T(6−4)C. The phosphoramidite of T(6−4)C (**7**) was used as a building block to synthesize oligonucleotides containing this photoproduct at a specific site in defined sequences. This compound was dissolved in anhydrous acetonitrile at a concentration slightly higher than that of the other phosphoramidites, and the reaction time for its coupling on the synthesizer was extended to 20 min to ensure chain elongation. For adenine, guanine, and cytosine, nucleoside phosphoramidite units in which the exocyclic amino function was protected with the (4-*tert*-butylphenoxy)acetyl (tBPA) group[18] were used because the (6−4) photoproduct is labile in hot alkali.[19] Our analysis of the stability of (6−4) photoproduct-containing oligonucleotides revealed that they decomposed when heated at 55 °C in concentrated aqueous ammonia but were quite stable at room temperature, i.e., under conditions for the removal of the tBPA groups with simultaneous deprotection of the phosphates and cleavage from the solid support. As a capping reagent for termination of failed sequences, (4-*tert*-butylphenoxy)acetic anhydride was used to avoid the replacement of the tBPA group of guanines by the acetyl group which is more stable in the ammonia treatment.[18]

As a first step, a 30-mer, d(GTATGATTAATGTAT(6−4)-CATGTAATTAGTATG), was synthesized to examine whether

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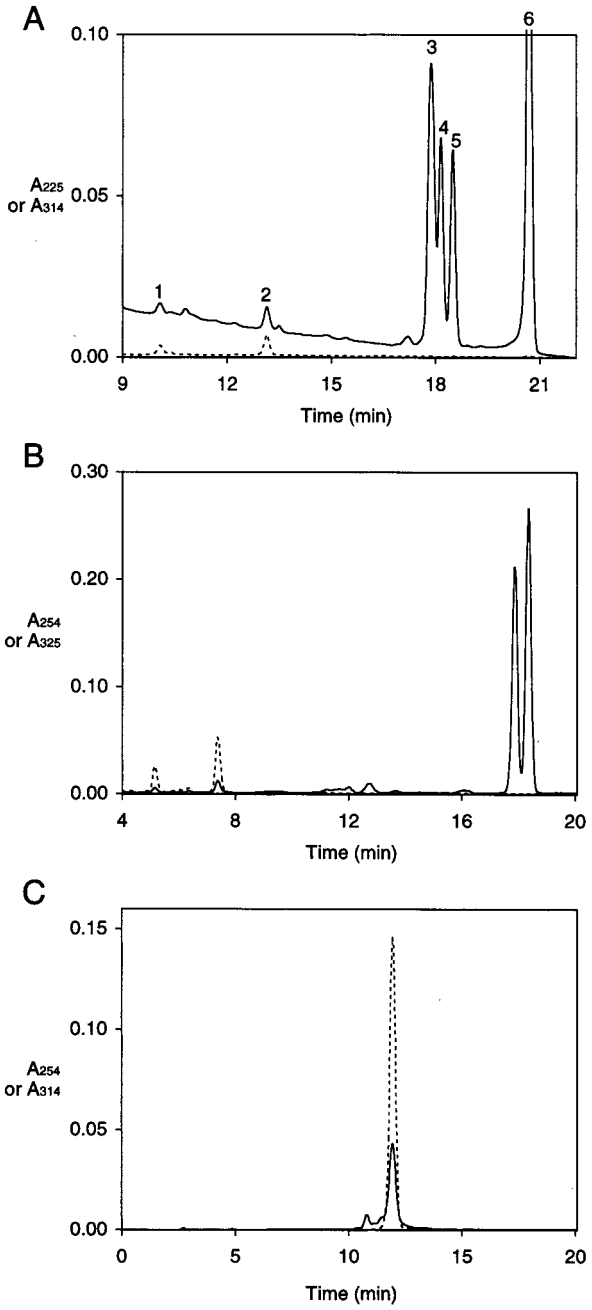


Figure 2. (A) HPLC analysis of a UV-irradiated solution of protected TpdC, monitored at 225 nm (solid line) and 314 nm (broken line). The total UV dose was about 40 J/cm^2, and the gradient was 7 to 19% acetonitrile in 0.1 M TEAA. Peaks 1 and 2, diastereoisomers of the protected T(6−4)C; peaks 3, 4, and 5, cytosine hydrates; peak 6, the starting material. (B) Formation of T(6−4)T. Elution from the HPLC column was monitored at 254 nm (solid line) and 325 nm (broken line), and the gradient was 11 to 19% acetonitrile in 0.1 M TEAA. (C) HPLC analysis of the purified T(6−4)C (peak 2 in panel A), monitored at 254 nm (solid line) and 314 nm (broken line).

the T(6−4)C building block could be used successfully in the same way as the T(6−4)T phosphoramidite. This synthesis was carried out on a synthesizer using the reagents and the reaction time described above, and after deprotection of the hydroxyl function at the 5′ end with trichloroacetic acid on the synthesizer, cleavage from the support and removal of the 2-cyanoethyl and tBPA groups were performed by treatment with 28% ammonia water at room temperature for 2 h. An aliquot of the deprotected oligonucleotide was analyzed by reversed phase HPLC, as shown in Figure 4A, which revealed that this sample contained

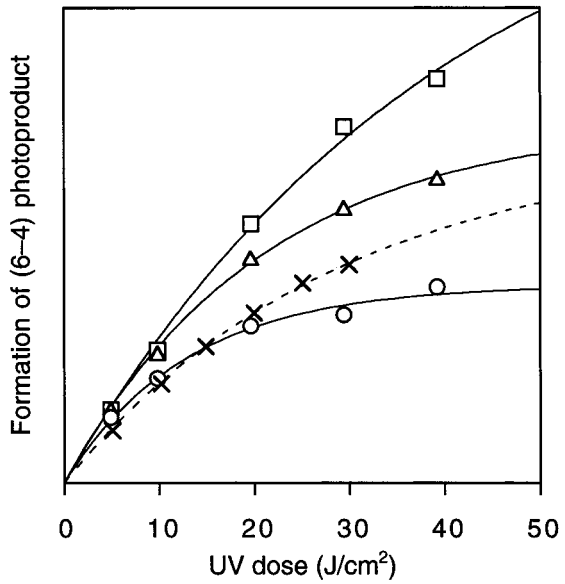


Figure 3. Formation of (6−4) photoproducts at varying UV doses at 254 nm. HPLC peak areas of the major diastereomers of protected T(6−4)C (solid line) and T(6−4)T (broken line) were plotted after normalization with each ϵ value at the detector wavelength. Concentrations of the initial dinucleoside monophosphates were 1 mM (crosses and circles), 2 mM (triangles), and 4 mM (squares).

a significant impurity, that gave a large peak eluted at a high acetonitrile concentration (peak 2 in Figure 4A). The relative size of this peak was not reduced by heating the mixture at 55 °C in aqueous ammonia or by treating it with methanolic ammonia. To identify this impurity, HPLC was used to purify it, and nuclease P1 and alkaline phosphatase were used to degrade it to its nucleoside components. HPLC analysis of the degradation products revealed that the impurity in question contained a highly hydrophobic substance (Figure 4B), which had a UV absorption maximum at 316 nm and smaller absorption peaks between 260 and 290 nm. These results strongly suggested that this component was a T(6−4)C derivative modified with the tBPA group. Therefore, the partially protected T(6−4)C derivative **4** was treated with (4-*tert*-butylphenoxy)acetic anhydride in the presence of 1-methylimidazole, and the acylation sites were determined by NMR analysis of the product. In the NOESY spectra, a cross-peak was found between H5 of the 3′-pyrimidone and the methylene proton in the tBPA group, which showed that the amino group of the 5′-pyrimidine, but not the N3 position, was acylated with the capping reagents, as shown in Scheme 2. This fully protected compound **8** was deprotected with ammonia, and it was demonstrated that this modified T(6−4)C **9** had a UV absorption spectrum identical to that of the unknown component in the impurity and coeluted with it from an HPLC column (Figure 4C).

Since this side reaction was found at the capping step in oligonucleotide synthesis, the synthesizer program was changed to omit this step in each cycle after coupling of the T(6−4)C unit. A short oligonucleotide, d(CAT(6−4)CAGCACGAC), which was prepared previously by UV irradiation of the undamaged 12-mer,[9a] was synthesized by this method. After deprotection with aqueous ammonia, this crude mixture was analyzed by reversed phase HPLC, which gave the elution profile shown in Figure 5A. The highest peak had UV absorption maxima at 256 and 314 nm and coeluted with the authentic 12-mer containing T(6−4)C.[9a] Following the successful synthesis of this 12-mer, one 14-mer, d(AAAAAAAAT(6−4)-

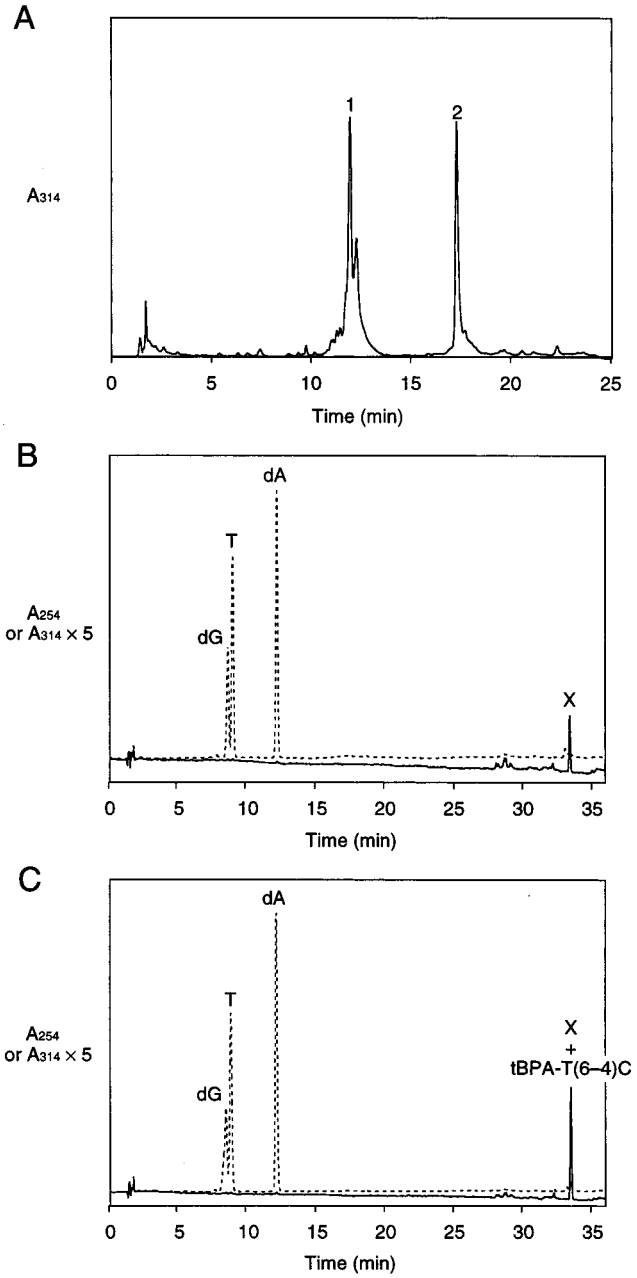
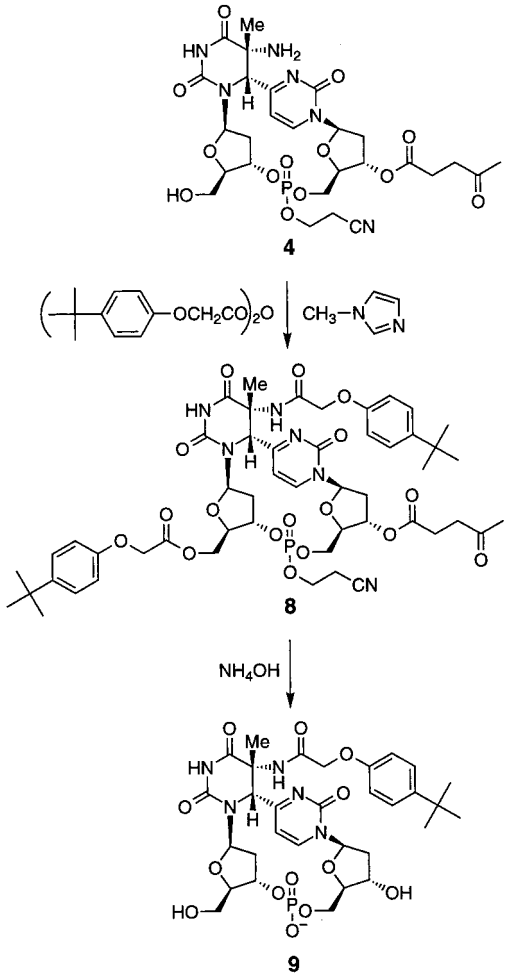


Figure 4. Detection and analysis of the byproduct produced in the oligonucleotide synthesis. (A) A 30-mer, d(GTATGATTAATGTAT-(6−4)CATGTAATTAGTATG), was synthesized on a synthesizer by the usual method, and the deprotected mixture was analyzed by HPLC using a 5−25% acetonitrile gradient in 0.1 M TEAA. Peaks 1 and 2 are the desired 30-mer and the byproduct discussed in the text, respectively. (B) After purification by HPLC, the byproduct (peak 2 in panel A) was treated with nuclease P1 and alkaline phosphatase, and the degraded products were analyzed by HPLC. The acetonitrile gradient was 0−10% during the first 20 min and 10−50% during the following 15 min, and the elution was monitored at 254 nm (broken line) and 314 nm (solid line, magnified by a factor of 5). X represents the unknown component discussed in the text. (C) Prior to injection, the T(6−4)C bearing the *tert*-butylphenoxyacetyl group at the amino function (compound **9**) was added to the degradation products.

CAAAA), and five 30-mers, d(GCACGACCAACGCAT(6−4)CACGCAACCAGCACG), d(GTATGATTAATGTAT(6−4)CATGTAATTAGTATG), d(AAAGTCAGCCTTGTGCT(6−4)CGCCTACGGCCAT), d(CCTGGTTAGTACAT(6−4)-CGATTTAGGTGACACT), and d(CTCGTCAGCATCT(6−4)-CCATCATACAGTCAGTG), were synthesized on a 0.2 μmol scale. An HPLC analysis of the deprotected mixture of d(CCTGGTTAGTACAT(6−4)CGATTTAGGTGACACT) is shown in Figure 5B, as an example. In addition to the highest peak with the largest 314-nm absorption, which eluted at 17.0 min, several other peaks were detected. From the elution profile monitored at 314 nm, the impurity eluted before the highest peak is supposed to be a 28-mer lacking T(6−4)C, which was produced due to the omission of the capping steps. The peaks with longer retention times, which were also found in the synthesis of T(6−4)T-containing oligonucleotides, may be attributed to the coupling of phosphoramidites with the imino function of the 5′-pyrimidine in the (6−4) photoproduct, as discussed previously.[8] Purification was performed under the same HPLC conditions, and the yield of this 30-mer was 4.8 A_{260} units, which means an overall yield of ca. 9% from the 3′-terminal nucleoside on the solid support. The oligonucleotides purified by reversed phase HPLC were analyzed further by anion-exchange HPLC, which confirmed extremely high purity (Figure 5C).

To confirm that the desired oligonucleotides were obtained, the purified 30-mers were degraded with nuclease P1 and alkaline phosphatase, and the components were separated by HPLC. A typical result is shown in Figure 6. The peak detected at 314 nm coeluted with the authentic T(6−4)C, prepared by UV irradiation of deprotected TpdC, and had the same UV absorption spectrum as T(6−4)C. The nucleoside composition obtained from the peak areas was dA:dG:dC = 9.3:5.9:12.8 (the calculated ratio is 10:6:12) in this case.

Scheme 2



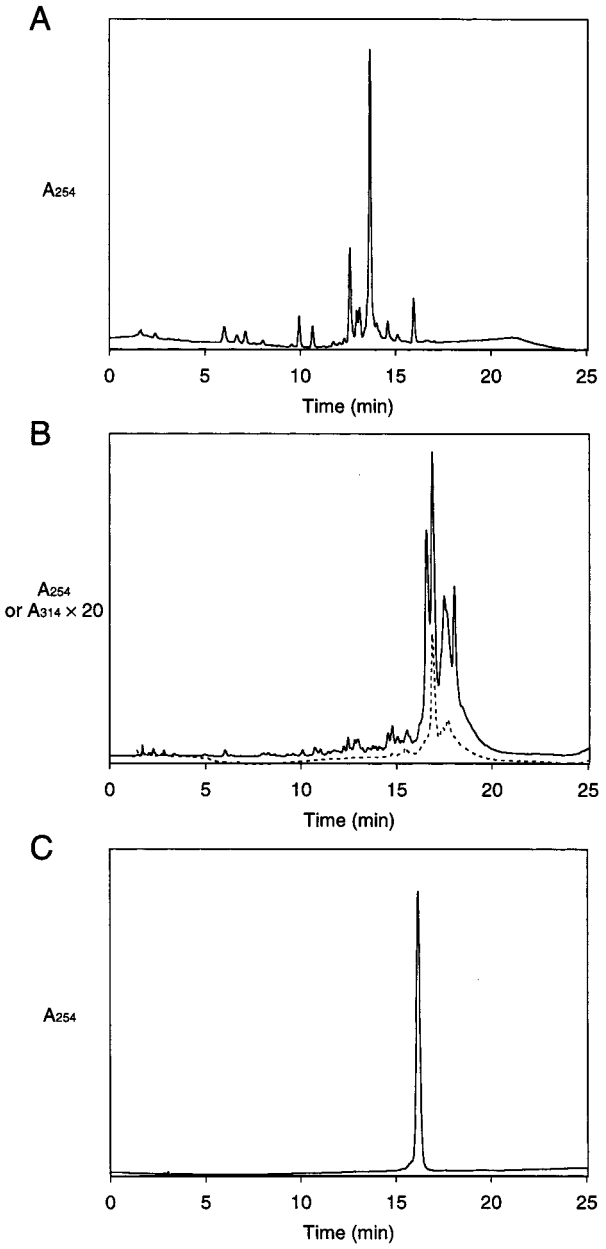


Figure 5. (A) HPLC analysis of a deprotected mixture of the 12-mer, d(CAT(6−4)CAGCACGAC). The gradient was 5−15% acetonitrile in 0.1 M TEAA during 20 min. (B) HPLC analysis of a deprotected mixture of the 30-mer, d(CCTGGTTAGTACAT(6−4)CGATTTAGGTGACACT). The gradient was the same as above, and the elution was monitored at 254 nm (solid line) and 314 nm (broken line, magnified by a factor of 20). (C) Anion-exchange HPLC analysis of the purified 30-mer. A TSK-gel DEAE-2SW column was used with a linear gradient of ammonium formate (0.4−1.2 M during 20 min) in 20% aqueous acetonitrile.

Photoreactivation of T(6−4)C with (6−4) Photolyase. As the first application of the chemically synthesized oligonucleotides containing the T(6−4)C photoproduct, we analyzed the photoreactivation of this photoproduct with the (6−4) photolyase.[6] This enzyme contains FAD as a chromophore[14a] and specifically repairs the (6−4) photoproduct in DNA using blue to near-UV light. Enzyme activities from *D. melanogaster*,[6,11b,c] *X. laevis*,[10,14a] and *A. thaliana*[14b] have been characterized, and several model studies toward the elucidation of its reaction mechanism have been published.[20] In our previous work, we

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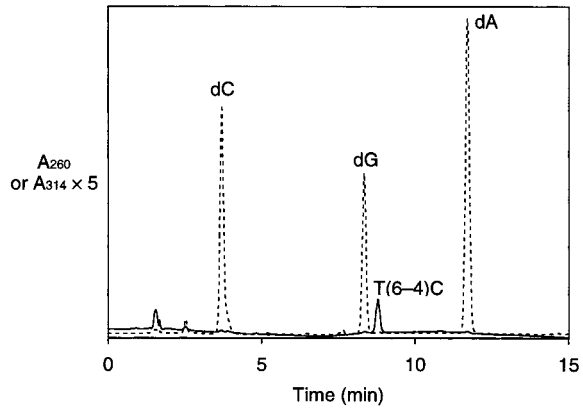

Figure 6. HPLC analysis of the components of the 30-mer, d(GCACGACCAACGCAT(6−4)CACGCAACCAGCACG), produced by degradation with nuclease P1 and alkaline phosphatase. The gradient was 0−10% acetonitrile in 0.1 M TEAA during 20 min, and the elution was monitored at 260 nm (broken line) and 314 nm (solid line, magnified by a factor of 5).

demonstrated that the T(6−4)T photoproduct is repaired to normal thymines by *Xenopus* (6−4) photolyase, by direct HPLC analysis of a photoreactivation product.[10] An important problem remaining to be solved is whether this enzyme repairs other types of (6−4) photoproducts, especially the most abundant T(6−4)C. On this subject, only a primer extension assay has been reported previously.[11c]

For the direct analysis of the photoreactivation product with this enzyme, an oligonucleotide in which the T(6−4)C photoproduct was incorporated into an oligo(deoxyadenylate), d(AAAAAAAAT(6−4)CAAAA), was synthesized. The chain length and the site of the photoproduct were chosen on the basis of the results of a footprinting experiment reported previously,[10] to give a minimal DNA for recognition by the (6−4) photolyase. This 14-mer was treated with *Xenopus* (6−4) photolyase, which was produced in *Escherichia coli* and purified as described previously,[14a] and the reaction mixture was subjected to HPLC analysis on a reversed phase column. As shown in Figure 7A, two large peaks were detected. Peak 1 was determined to be FAD contained by the enzyme, from its retention time and absorption spectrum, and peak 2 appeared to be a photoreactivation product because it had no absorption in the long wavelength region, which is characteristic of the (6−4) photoproduct. To confirm this, the starting 14-mer containing T(6−4)C was co-injected into the column, and it was shown that this photoproduct-containing oligonucleotide was separated from peak 2, as shown in Figure 7B. By the addition of the undamaged 14-mer, d(AAAAAAAATCAAAA), to the photoreactivation mixture prior to injection, it was demonstrated that this undamaged oligonucleotide coeluted with peak 2 (Figure 7C).

The photoreactivation product (peak 2 in Figure 7A) was purified under the same HPLC conditions. This oligonucleotide had a UV absorption spectrum identical to that of the undamaged 14-mer. To confirm this identity, the nucleoside components of both oligonucleotides were analyzed by HPLC. As shown in Figure 8, two peaks, corresponding to deoxycytidine and thymidine, were detected in the sample obtained by degradation of the photoreactivation product with nuclease P1 and alkaline phosphatase. These two components had UV absorption spectra, as well as retention times, identical to those of deoxycytidine and thymidine, and the ratio calculated from the peak areas was dC:T:dA = 1.00:1.08:11.6 (The actual ratio for d(AAAAAAAATCAAAA) is 1:1:12). These results clearly demonstrate that the

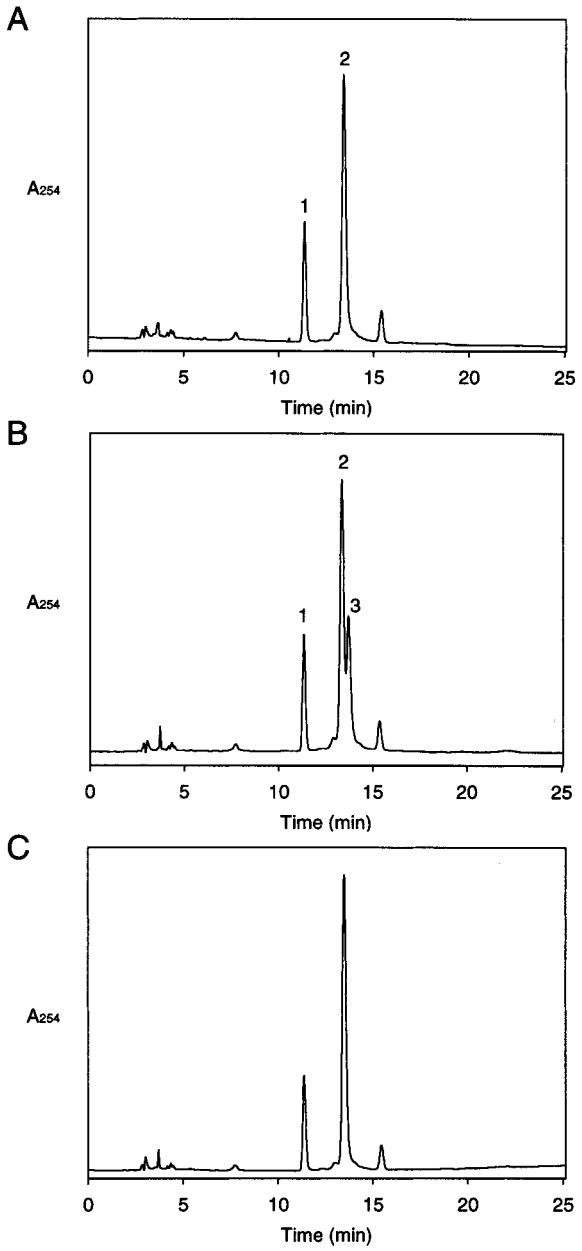

Figure 7. HPLC analysis of the photoreactivation product. (A) The 14-mer, d(AAAAAAAAT(6−4)CAAAA), was mixed with *Xenopus* (6−4) photolyase, and the mixture was illuminated with fluorescent light for 4 h. An aliquot was analyzed by reversed phase HPLC, using an Inertsil ODS-2 column with a linear gradient of acetonitrile (9−12% during 20 min) in 0.1 M TEAA. (B) The T(6−4)C-containing 14-mer was added to the reaction mixture prior to injection. Peak 1, FAD contained by the enzyme; peak 2, the photoreactivation product; peak 3, the T(6−4)C-containing 14-mer. (C) The undamaged 14-mer, d(AAAAAAAATCAAAA), was added to the reaction mixture.

T(6−4)C photoproduct is repaired to its original pyrimidine components by the photoreactivation with *Xenopus* (6−4) photolyase in the same way as T(6−4)T.

Conclusions

In this article, we described the synthesis of oligonucleotides containing the (6−4) photoproduct formed between thymine and cytosine. Although the procedure was basically the same as that for oligonucleotides containing the thymine−thymine (6−4) photoproduct,[8] two different points were found in this study. The formation of cytosine hydrates reduced the actual yield of

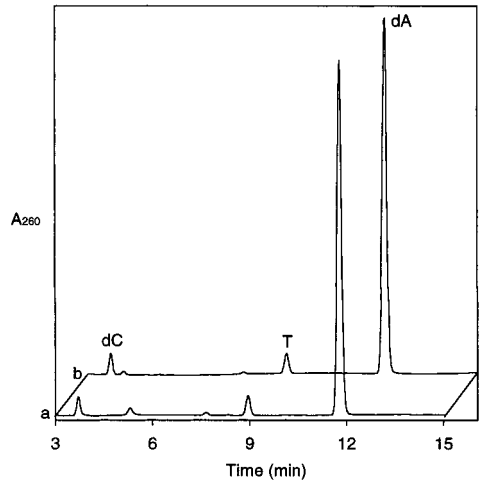

Figure 8. HPLC separation of the nucleoside components of the photoreactivation product from d(AAAAAAAAT(6−4)CAAAA) (a) and those of the undamaged 14-mer, d(AAAAAAAATCAAAA) (b).

T(6−4)C to a great extent in the UV irradiation of partially protected thymidylyl(3′−5′)deoxycytidine, whereas the quantum yield of T(6−4)C is reported to be higher than that of T(6−4)T.[13] The other problem was the acylation of the amino function in the T(6−4)C photoproduct with the capping reagents, and thus, the capping steps were omitted to improve the yields of the desired oligonucleotides. Despite these difficulties, we believe that T(6−4)C-containing oligonucleotides are worth synthesizing because this photoproduct is formed more efficiently than T(6−4)T in human cells and is more important in terms of DNA repair.

One of the oligonucleotides synthesized in this study was used to characterize a repair enzyme, (6−4) photolyase. Direct HPLC analysis of the photoreactivation product demonstrated that this enzyme restored the pyrimidines in the T(6−4)C photoproduct to their original structures. This result also proves that the oligonucleotides synthesized by this method contain the naturally occurring T(6−4)C photoproduct.

Experimental Section

General Methods. All solvents and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), except for *N*,*N*-diisopropylethylamine and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite, which were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI) and Sigma Chemical Company (St. Louis, MO), respectively. Nucleic acid synthesis grade solvents were used for reactions. Reagents for the DNA/RNA synthesizer were purchased from Perkin-Elmer Japan (Chiba, Japan) and PerSeptive Biosystems, Inc. (Framingham, MA). TLC analyses were carried out on Merck silica gel 60 F_{254} plates, which were visualized by UV illumination at 254 nm and spraying of an anisaldehyde/sulfuric acid solution, followed by heating. Merck silica gel 60 F_{254} 0.5 mm plates were used for preparative TLC. For column chromatography, either Wakogel C-200, C-300 (Wako Pure Chemical Industries), or Merck silica gel 60 was used. Preparative C18 125 Å (Waters Corporation, Milford, MA) was used on a Bio-rad Econo System for reversed phase chromatography.

UV and fluorescence spectra were recorded on a Beckman DU-64 spectrophotometer and a Hitachi F-4500 fluorescence spectrophotometer, respectively. NMR spectra were measured on either a Bruker DPX 300 or DMX 750 spectrometer. Proton signals were assigned by using homonuclear 2D NMR techniques, COSY and NOESY, measured on the DPX 300 spectrometer. For dinucleoside monophosphates, DQF-COSY, ROESY, HMQC, and HMBC spectra were used for the signal assignments. Carbon assignments were carried out by heteronuclear 2D NMR, HMQC, and HMBC. ^{31}P NMR spectra were measured on a Bruker DPX 300 spectrometer using trimethyl phosphate

as an internal standard. Mass spectra were obtained on a JEOL HX-110 spectrometer.

HPLC analyses were carried out on a Gilson gradient-type analytical system equipped with a Waters 996 photodiode array detector. A μBondasphere 5μ C18 300 Å column (3.9 mm × 150 mm; Waters) was used at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA, pH 7.0). An Inertsil ODS-2 column (4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan) was used in the case mentioned in the figure legend. For anion-exchange HPLC, a TSK-gel DEAE-2SW column (4.6 mm × 250 mm; Tosoh Corporation, Tokyo, Japan) was used at a flow rate of 1.0 mL/min with a linear gradient of ammonium formate in 20% aqueous acetonitrile.

4-*N*-(4,4′-Dimethoxytrityl)-3′-*O*-levulinyl-2′-deoxycytidine (2). To 2′-deoxycytidine hydrochloride (6.00 g, 22.8 mmol), which was dried three times by coevaporation with pyridine and was suspended in anhydrous pyridine (115 mL), were added 4,4′-dimethoxytrityl chloride (19.3 g, 57.0 mmol), triethylamine (9.45 mL, 68.0 mmol), and (dimethylamino)pyridine (117 mg, 95.8 μmol).[21] After stirring at room temperature for 40 min, the mixture was diluted with ethyl acetate (400 mL) and washed with saturated aqueous $NaHCO_3$ (600 mL) and then with water (400 mL). The organic layer was dried with Na_2SO_4 and concentrated, and the residue was subjected to silica gel column chromatography eluted with a step gradient of 0−3% methanol in chloroform. The product was precipitated with hexane (2 L) from a chloroform solution to give the 4-*N*,5′-*O*-ditrityl derivative as a white powder (16.3 g, 19.6 mmol, 86%). This compound (16.3 g, 19.6 mmol) was dissolved in 1,4-dioxane (160 mL) containing pyridine (4 mL) and mixed with 4-(dimethylamino)pyridine (191 mg, 1.57 mmol) and 1,3-dicyclohexylcarbodiimide (10.1 g, 49.0 mmol). To this solution, levulinic acid (4.01 mL, 39.2 mmol) was added. After stirring at room temperature for 4 h, the mixture was diluted with chloroform (700 mL) and washed with saturated aqueous $NaHCO_3$ (300 mL), water (300 mL), and brine (300 mL). The organic layer was dried with Na_2SO_4 and concentrated, and the residue was subjected to silica gel column chromatography eluted with a step gradient of 0−2% methanol in chloroform. The product was precipitated with hexane from a chloroform solution to give the 3′-levulinyl derivative as a white powder (16.3 g, 17.5 mmol, 77% from 2′-deoxycytidine). This compound (16.1 g, 17.3 mmol) was treated with 1 M $ZnBr_2$ in dichloromethane containing 2-propanol (158 mL, 158 mmol) at room temperature for 6 h. The reaction mixture was diluted with chloroform (800 mL) and washed with 1.2 M aqueous ammonium acetate (1000 mL), water (800 mL), and brine (800 mL). The organic layer was dried with Na_2SO_4 and concentrated, and the residue was subjected to silica gel column chromatography eluted with a step gradient of 0−4% methanol in chloroform. The product was precipitated with hexane from a chloroform solution to give **2** as a white powder (10.4 g, 16.6 mmol, 73% from 2′-deoxycytidine). TLC (methanol−chloroform, 1:10) R_f 0.32. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.37−6.78, (13H, m, aromatic), 7.32 (1H, d, J = 7.5 Hz, H6), 6.03 (1H, t, J = 7.5, 6.0 Hz, H1′), 5.34−5.28 (1H, m, H3′), 5.08 (1H, d, J = 7.5 Hz, H5), 4.10−4.04 (1H, m, H4′), 3.91−3.73 (2H, m, H5′,5″), 3.80 (6H, s, -OCH_3 × 2), 3.18 (1H, br s, -OH), 2.75, 2.56 (2H each, t, J = 6.0 Hz, -OCOCH_2CH_2CO-), 2.58−2.37 (2H, m, H2′,2″), 2.18 (3H, s, -$COCH_3$). HRMS (FAB) calcd for $C_{35}H_{38}N_3O_8$ (M + H^+) 628.2659, found 628.2668.

***P*-(2-Cyanoethyl)thymidylyl-(3′→5′)-(2′-deoxycytidine) 3′-levulinate (3).** Compound **2** (3.51 g, 5.60 mmol) and 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (5.00 g, 6.72 mmol) were dissolved in anhydrous acetonitrile (37.0 mL), and to this solution was added 0.86 M tetrazole in acetonitrile (31.3 mL, 26.9 mmol). After stirring at room temperature for 10 min, the mixture was diluted with chloroform (300 mL) and washed with saturated aqueous $NaHCO_3$ (250 mL) and with brine (250 mL). The organic layer was dried with Na_2SO_4 and evaporated in vacuo. To this residue was added 0.12 M iodine in tetrahydrofuran−pyridine−water (7:2:1, v/v/v; 150 mL), and the solution was stirred at room temperature for 60 min. This mixture was diluted with chloroform (250 mL) and was washed with 1 M aqueous $Na_2S_2O_3$ (180 mL). After being dried with Na_2SO_4 and after the organic layer was concentrated, the product was purified by silica gel column chromatography eluted with a step gradient of 0−4% methanol in chloroform. The fully protected dinucleoside monophosphate was obtained as a foam by evaporation. This compound was treated with 3.5% trichloroacetic acid in dichloromethane (270 mL) at room temperature for 25 h, and the mixture was neutralized and extracted with 0.33 M aqueous $NaHCO_3$ (150 mL) and with water (80 mL). The combined aqueous layer was concentrated to 40 mL and desalted on a column of alkylated silica gel (2.5 cm × 7 cm) with a linear gradient of 9−25% acetonitrile in water. The product was purified on a column of alkylated silica gel (1.5 cm × 48 cm) with a linear gradient of 5−22.5% acetonitrile. The fractions were analyzed by reversed phase HPLC, and those containing the desired product were collected. Compound **3** was obtained as a white powder by lyophilization (2.47 g, 65%). ^{1}H NMR (750 MHz, pyridine-d_5) δ 13.23 (1H, br s, -NH-/T), 8.54−8.20 (2H, m, -NH_2/C), 8.02 (1H, s, H6/T), 7.99 (1H, d, J = 7.4 Hz, H6/C), 6.93 (1H, dd, J = 8.2, 6.0 Hz, H1′/T), 6.88−6.78 (1H, m, H1′/C), 6.22−6.12 (1H, m, H5/C), 5.75−5.67 (1H, m, H3′/T), 5.65−5.57 (1H, m, H3′/C), 4.75−4.64 (2H, m, H5′,5″/C), 4.65−4.57 (1H, m, H4′/T), 4.52, 3.08 (2H each, t, J = 5.9 Hz, -CH_2CH_2-CN), 4.52−4.44 (1H, m, H4′/C), 4.23−4.13 (2H, m, H5′,5″/T), 2.94−2.72 (2H, m, H2′,2″/T), 2.77, 2.66 (2H each, t, J = 5.8 Hz, -OCOCH_2CH_2CO-), 2.73−2.44 (2H, m, H2′,2″/C), 2.09, 2.08 (3H in total (two diastereomers), s, -$COCH_3$), 1.84, 1.83 (3H in total (two diastereomers), s, -CH_3/T). ^{31}P NMR (121.5 MHz, pyridine-d_5) δ −4.43, −4.53. HRMS (FAB) calcd for $C_{27}H_{36}N_6O_{13}P$ (M + H^+) 683.2078, found 683.2087.

Formation of the photoproduct 4. A 2 mM solution of compound **3** in water (150 mL) was irradiated for 2 h in an ice-cooled aluminum tray (23 × 32 cm) on a Spectrolinker XL-1500 UV cross-linker (Spectronics Corp., Westbury, NY) equipped with six 15-W germicidal lamps (the total UV dose was ca. 40 J/cm^2). After this irradiation was repeated 36 times, using a total of 5.4 L of the solution, the mixture was concentrated and applied to a column of alkylated silica gel (1.5 cm × 48 cm) equilibrated with 5% aqueous acetonitrile. Elution was performed with a linear gradient of acetonitrile (5−17%), and the fractions were analyzed by reversed phase HPLC using a μBondasphere 5μ C18 300 Å column with an acetonitrile gradient (7−19% during 20 min) in 0.1 M TEAA. The major diastereomer of the photoproduct **4** was separated, and the starting material **3** was recovered (4.79 g, 67%). The recovered starting material was used for reirradiation in the same manner as described above, and the desired photoproduct was combined with that from the first preparation. The product was passed through a column of DEAE-Toyopearl 650 M (1.0 cm × 17 cm; Tosoh Corporation), equilibrated and eluted with water, and was obtained as a glassy solid by evaporation (280 mg, 3.9%). UV (H_2O) λ_{max} 314 nm (ϵ = 7.8 × 10^3), λ_{min} 265 nm. Fluorescence (H_2O) λ_{em} 384 nm (at λ_{ex} 313 nm). ^{1}H NMR (750 MHz, D_2O) δ 8.25 (1H, d, J = 6.9 Hz, H6/C), 6.75 (1H, d, J = 6.9 Hz, H5/C), 6.40 (1H, dd, J = 7.5, 0.7 Hz, H1′/C), 6.05 (1H, d, J = 8.7, 1.4 Hz, H1′/T), 5.42−5.38 (1H, m, H3′/C), 4.93 (1H, s, H6/T), 4.31−4.24 (3H, m, H4′/C and -OCH_2CH$_2$CN), 4.22−4.18 and 4.15−4.11 (1H each, m, H5′,5″/C), 4.02−3.97 (1H, m, H3′/T), 3.90 (1H, dd, J = 13.2, 2.1 Hz, H5′ or H5″/T), 3.84−3.81 (1H, m, H4′/T), 3.73 (1H, dd, J = 13.2, 3.3 Hz, H5′ or H5″/T), 3.06 (1H, br dd, H2′ or H2″/C), 2.88−2.84 (4H, m, -OCOCH$_2$CH_2CO- and -OCH$_2$CH_2CN), 2.76 (1H, ddd, J = 15.4, 9.1, 7.5 Hz, H2′ or H2″/C), 2.57 (2H, t, J = 6.1 Hz, -OCOCH_2CH$_2$CO-), 2.38 (1H, m, H2′ or H2″/T), 2.16 (s, 3H, -$COCH_3$), 1.82 (br dd, 1H, H2′ or H2″/T), 1.51 (3H, s, -CH_3/T). ^{31}P NMR (121.5 MHz, D_2O) δ −2.93. HRMS (FAB) calcd for $C_{27}H_{36}N_6O_{13}P$ (M + H^+) 683.2078, found 683.2054.

5′-*O*-(4,4′-Dimethoxytrityl)-*P*-(2-cyanoethyl)-T(6−4)C 3′-levulinate (5). To a solution of compound **4** (168 mg, 246 μmol) in dry pyridine (2.7 mL), was added 4,4′-dimethoxytrityl chloride (162 mg, 479 μmol). After the mixture stirred at room temperature for 3.5 h, methanol (1.0 mL) was added, and the mixture was concentrated. The residue was dissolved in chloroform (20 mL) and was washed with water. The organic layer was dried with Na_2SO_4 and concentrated, and the product was purified by preparative TLC, using a developing solvent of chloroform−methanol (8:1, v/v). The tritylated derivative

(21) Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316−1319.

5 was eluted from the silica gel with 4% methanol in chloroform and was obtained as a glassy solid by evaporation (177 mg, 180 μmol, 73%). TLC (methanol−chloroform, 1:10) R_f 0.23. ^{1}H NMR (300 MHz, chloroform-*d*) δ 8.49 (1H, s, -NH-), 7.88 (1H, d, J = 6.9 Hz, H6), 7.54−6.88 (13H, m, aromatic), 6.62 (1H, dd, J = 7.5, 3.3 Hz, H1′/C), 6.51 (1H, d, J = 6.9 Hz, H5/C), 6.30 (1H, br dd, H1′/T), 5.52−5.42 (1H, m, H3′/C), 4.73 (1H, s, H6/T), 4.70−4.55 (1H, m, H3′/T), 4.30−3.97 (5H, m, H5′,5″/C, -OCH_2CH$_2$CN, and H4′/C), 3.80 (6H, s, -OCH$_3$ × 2), 3.74−3.65 (1H, m, H4′/T), 3.58 (1H, dd, J = 11.1, 1.7 Hz, H5′ or H5″/T), 3.23 (1H, dd, J = 11.1, 3.9 Hz, H5′ or H5″/T), 2.95−2.82, 2.68−2.56 (1H each, m, H2′,2″/C), 2.76 (2H, t, J = 5.9 Hz, -OCOCH$_2$CH_2CO-), 2.73−2.65 (2H, m, -OCH$_2$CH_2CN), 2.56 (2H, t, J = 5.9 Hz, -OCOCH_2CH$_2$CO-), 2.18 (3H, s, -CH$_2$COCH_3), 2.18−2.01, 1.70−1.56 (1H each, m, H2′,2″/T), 1.55 (3H, s, -CH$_3$/T). ^{31}P NMR (121.5 MHz, chloroform-*d*) δ −4.67. HRMS (FAB) calcd for $C_{48}H_{54}N_6O_{15}P$ (M + H$^+$) 985.3385, found 985.3412.

5′-*O*-(4,4′-Dimethoxytrityl)-*P*-(2-cyanoethyl)-T(6−4)C (6). The 5′- and 3′-protected T(6−4)C (**5**; 147 mg, 149 μmol) was dissolved in pyridine (2.0 mL), and a solution of hydrazine monohydrate (69 μL, 1.42 μmol) in pyridine−acetic acid (3:2, v/v; 2 mL) was added. After stirring at room temperature for 5 min, the mixture was cooled in an ice bath and mixed with acetone (1.5 mL). This mixture was diluted with chloroform (20 mL) and washed with 2% aqueous NaHCO$_3$ (12 mL) and with water (12 mL). The organic layer was dried with Na$_2$SO$_4$ and concentrated. After coevaporation with toluene, the residue was chromatographed on silica gel with a step gradient of 0−6% methanol in chloroform, and the 3′-deprotected product **6** was obtained as a glassy solid after evaporation (110 mg, 124 μmol, 83%). TLC (methanol−chloroform, 1:10) R_f 0.14. ^{1}H NMR (300 MHz, pyridine-d_5) δ 12.54 (1H, s, -NH-), 8.29 (1H, d, J = 7.0 Hz, H6/C), 7.85−7.05 (13H, m, aromatic), 6.84 (1H, br dd, H1′/C), 6.76 (1H, dd, J = 8.5, 2.0 Hz, H1′/T), 6.72 (1H, d, J = 7.0 Hz, H5/C), 5.15 (1H, s, H6/T), 5.17−5.07 (1H, m, H3′/C), 4.96−4.84 (1H, m, H3′/T), 4.53−4.43, 4.41−4.30 (1H each, m, H5′,5″/C), 4.30−4.20 (2H, m, -OCH_2CH$_2$CN), 4.28−4.15 (1H, m, H4′/C), 4.07−3.97 (1H, m, H4′/T), 3.91−3.82 (1H, m, -OH), 3.75 (6H, s, -OCH$_3$ × 2), 3.78−3.64 (1H, m, H5′ or H5″/T), 3.59 (1H, dd, J = 11.0, 4.6 Hz, H5′ or H5″/T), 2.99−2.84, 2.80−2.66 (1H each, m, H2′,2″/C), 2.88 (2H, t, J = 6.1 Hz, -OCH$_2$CH_2CN), 2.36−2.11 (2H, m, H2′,2″/T), 1.78 (3H, s, -CH$_3$/T). ^{31}P NMR (121.5 MHz, pyridine-d_5) δ −4.58. HRMS (FAB) calcd for $C_{43}H_{48}N_6O_{13}P$ (M + H$^+$) 887.3017, found 887.3007.

5′-*O*-(4,4′-Dimethoxytrityl)-*P*-(2-cyanoethyl)-T(6−4)C 3′-(2-Cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (7). A solution of compound **6** (111 mg, 125 μmol) in anhydrous tetrahydrofuran (1.8 mL) was treated with (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (56 μL, 250 μmol) in the presence of *N*,*N*-diisopropylethylamine (87 μL, 500 μmol) at room temperature for 30 min. The reaction mixture was diluted with chloroform (20 mL) and washed with 2% aqueous NaHCO$_3$ and with water. The organic layer was dried with Na$_2$SO$_4$ and evaporated in vacuo. The product was purified on a silica gel column with a step gradient of 0−3% methanol in chloroform containing 0.1% pyridine and precipitated with pentane from a chloroform solution to give **7** as a white powder (81.5 mg, 74.0 μmol, 59%). TLC (methanol−chloroform, 1:10) R_f 0.48. ^{1}H NMR (300 MHz, pyridine-d_5) δ 12.64 (1H, s, -NH-), 8.29 (1H, d, J = 6.8 Hz, H6/C), 7.92−7.07 (13H, m, aromatic), 6.92−6.69 (3H, m, H1′/C, H1′/T, and H5/C), 5.18−5.02 (1H, m, H3′/C), 5.10 (1H, s, H6/T), 4.95−4.85 (1H, m, H3′/T), 4.59−4.48 (1H, m, H5′ or H5″/C), 4.48−4.17 (4H, m, H4′/C, H5′ or H5″/C, and -CH$_2$-), 4.11−3.45 (7H, m, H4′/T, -CH$_2$-, H5′,5″/T, and -CH(CH$_3$)$_2$ × 2), 3.76 (6H, s, -OMe × 2), 3.12−2.64 (6H, m, -CH$_2$- × 2, and H2′,2″/C), 2.37−2.06 (2H, m, H2′,2″/T), 1.78 (3H, s, -CH$_3$/T), 1.26−1.08 (12H, m, -CH(CH_3)$_2$ × 2). ^{31}P NMR (121.5 MHz, pyridine-d_5) δ 145.82, 154.75, -4.54, -4.94. HRMS (FAB) calcd for $C_{52}H_{65}N_8O_{14}P_2$ (M + H$^+$) 1087.4100, found 1087.4169.

Enzymatic Digestion of Oligonucleotides. An aliquot (0.04 A_{260} unit) of each oligonucleotide was incubated with nuclease P1 (4 μg) in 30 mM ammonium acetate (pH 5.3, 20 μL) at 37 °C for 14 h. This mixture was diluted with water (18 μL) and 0.5 M Tris-HCl (pH 9.0, 10 μL), and alkaline phosphatase (from calf intestine; 2 μL, 2 units) was added. After an incubation at 37 °C for 2 h, ethanol (300 μL) was added, and the mixture was kept at −20 °C for 2 h. The proteins were pelleted by centrifugation, and the supernatant was concentrated in vacuo. The residue was dissolved in water (100 μL), and an aliquot (25 μL) was analyzed by HPLC using a μBondasphere 5μ C18 300 Å column, as shown in Figures 4, 6, and 8.

Analysis of the Oligonucleotide Photoreactivated with (6−4) Photolyase. *Xenopus* (6−4) photolyase was produced in *E. coli* and was purified as described previously.[14a] This enzyme was illuminated with fluorescent light in advance to obtain a reduced form of FAD. A 14-mer, d(AAAAAAAAT(6−4)CAAAA), (0.51 A_{260} units) was dissolved in a 1.6 μM solution of the (6−4) photolyase (1.0 mL) in 10 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, and this solution was illuminated with fluorescent light. After 4 h, the reaction mixture was heated at 75 °C for 15 min. The enzyme was pelleted by centrifugation, and aliquots of the supernatant were analyzed by HPLC using an Inertsil ODS-2 column, as shown in Figure 7. The product, to which the T(6−4)C-containing 14-mer was converted quantitatively, was purified under the same HPLC conditions (9−13% acetonitrile in 0.1 M TEAA during 20 min). This oligonucleotide and the undamaged 14-mer, d(AAAAAAAATCAAAA), were degraded to their nucleoside components, as described above, and the results of the HPLC analysis are shown in Figure 8.

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Supporting Information Available: ^{13}C NMR data for **3** and **4**, reaction of T(6−4)C with capping reagents, and a procedure for oligonucleotide synthesis (3 pages print/PDF). See any current masthead page for ordering information and Web access instructions.

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