

# Chemical synthesis of oligodeoxyribonucleotides containing the Dewar valence isomer of the (6–4) photoproduct and their use in (6–4) photolyase studies

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## ABSTRACT

The pyrimidine(6–4)pyrimidone photoproduct, a major UV lesion formed between adjacent pyrimidine bases, is transformed to its Dewar valence isomer upon exposure to UVA/UVB light. We have synthesized a phosphoramidite building block of the Dewar photoproduct formed at the thymidylyl (3′–5′)thymidine site and incorporated it into oligodeoxyribonucleotides. The diastereoisomers of the partially protected dinucleoside monophosphate bearing the (6–4) photoproduct, which were caused by the chirality of the phosphorus atom, were separated by reversed-phase chromatography, and the (6–4) photoproduct was converted to the Dewar photoproduct by irradiation of each isomer with Pyrex-filtered light from a high-pressure mercury lamp. The Dewar photoproduct was stable under both acidic and alkaline conditions at room temperature. After characterization of the isomerized base moiety by NMR spectroscopy, a phosphoramidite building block was synthesized in three steps. Although the ordinary method could be used for the oligonucleotide synthesis, benzimidazolium triflate as an alternative activator yielded better results. The oligonucleotides were used for the analysis of the reaction and the binding of *Xenopus* (6–4) photolyase. Although the affinity of this enzyme for the Dewar photoproduct-containing duplex was reportedly similar to that for the (6–4) photoproduct-containing substrate, the results suggested a difference in the binding mode.

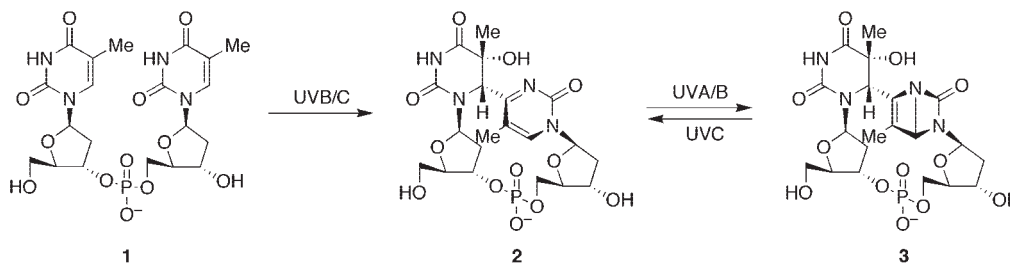
## INTRODUCTION

Ultraviolet (UV) light causes two major types of photoleisions, namely *cis*–*syn* cyclobutane pyrimidine dimers (CPDs)

and pyrimidine(6–4)pyrimidone photoproducts [(6–4) photoproducts, 2], at dipyrimidine sites (1) in DNA. The (6–4) photoproduct is converted to another product, originally designated as TpT3, by irradiation at 313 nm (1). A structural analysis of TpT3 revealed that the 2-pyrimidone ring of the (6–4) photoproduct was photoisomerized to a Dewar-type structure (3) (2), as shown in Scheme 1, and since then, the isomerized product has been called the Dewar valence isomer or the Dewar photoproduct. In addition to the isomerization of the isolated (6–4) photoproduct (3,4), the Dewar photoproducts are formed by the UVB irradiation of the original pyrimidine dinucleoside monophosphates (5,6) or DNA (7). They are reportedly detected in the DNA of mammalian cells exposed to simulated or natural sunlight (8–10). Reversion of the Dewar valence isomer to the (6–4) photoproduct by far-UV irradiation was also described previously (1,11).

The conformation of the Dewar photoproduct of thymidylyl(3′–5′)thymidine was determined by NMR spectroscopy and molecular modeling, and was found to be similar to that of the (6–4) photoproduct. However, the 3′ component of the Dewar photoproduct, i.e. 5-methyl-2-oxo-1,3-diazabicyclo[2.2.0]hex-5-ene, is not planar, unlike the 3′ base of the (6–4) photoproduct (3). This difference affects the thermodynamic properties of the base pair formation and the mutation spectra. While duplexes with guanine opposite the 3′ component of the (6–4) photoproduct are thermodynamically more stable than those with adenine, this stabilization is reduced by the isomerization to the Dewar photoproduct, although the functional groups are the same in these two photoproducts (12,13). The (6–4) photoproduct formed at the TT sequence causes a T→C transition mutation at the 3′ pyrimidone with an extremely high frequency in SOS-induced *Escherichia coli* cells, but its Dewar valence isomer shows lower mutation frequency and specificity (14,15). The human damaged DNA-binding protein, which recognizes DNA containing the (6–4) photoproduct (16) and initiates global genome nucleotide excision repair (17), binds DNA containing the Dewar valence isomer with high affinity (18), and it was demonstrated that the Dewar photoproduct

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**Scheme 1.** The (6-4) photoproduct (2) and its Dewar valence isomer (3) formed at the TT site (1).

was repaired as efficiently as the (6-4) photoproduct in human cells, probably via the nucleotide excision repair pathway (19). Another repair enzyme, the (6-4) photolyase (20), also binds DNA containing the Dewar photoproduct, although its affinity for this damaged DNA is slightly lower than that for the (6-4) photoproduct-containing DNA. The dissociation constants reported for the (6-4) and Dewar photoproducts are  $5.0 \times 10^{-10}$  and  $1.4 \times 10^{-9}$  M, respectively (21). However, the repair of the Dewar isomer to the original pyrimidines by the (6-4) photolyase is extremely slow, and the reported quantum yield is 0.5% of that obtained for the repair of the (6-4) photoproduct (21).

For biochemical studies, oligonucleotides containing the Dewar photoproduct have been prepared by two steps. Short oligonucleotides containing a single TT sequence were first irradiated at 254 nm, and the resultant products containing the (6-4) photoproduct were purified by high-performance liquid chromatography (HPLC). Subsequently, the (6-4) photoproduct in these oligonucleotides was converted to its Dewar valence isomer by a second irradiation step at longer wavelengths (12,14,22). A problem in this method, however, is that the HPLC separation of the oligonucleotide containing the Dewar photoproduct from the starting material containing the (6-4) photoproduct is very difficult, even though the oligonucleotide is short (12,22). Although the Dewar photoproduct is photoequilibrated with the (6-4) photoproduct in irradiated cells, oligonucleotides free from contamination with the isomerized form are required for *in vitro* studies, and the 100% purity is not guaranteed for the samples prepared by the post-synthetic irradiation method. For the CPD and the (6-4) photoproduct, methods for the chemical synthesis of oligonucleotides using the dinucleotide-type phosphoramidite building blocks have been developed previously (23-26), and the photolesion-containing oligonucleotides synthesized by this method have been used for various biochemical studies including translesion replication (27-30), which requires extremely pure template oligonucleotides. Here we describe the extension of this synthetic method to the Dewar photoproduct, which enables us to obtain the Dewar photoproduct-containing oligonucleotides without any contamination with the (6-4) photoproduct, and its application to the analysis of a repair enzyme, (6-4) photolyase.

## MATERIALS AND METHODS

### General methods

All solvents and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Reagents for the

DNA synthesizer were purchased from Applied Biosystems Japan (Tokyo, Japan) and Glen Research (Sterling, VA). TLC analyses were carried out on Merck Silica gel 60 F<sub>254</sub> plates, which were visualized by UV illumination at 254 nm. For column chromatography, Wakogel C-200 (Wako Pure Chemical Industries) was used. For reversed-phase chromatography, Prep C18 55-105  $\mu$ m 125 Å resin (Waters Corporation, Milford, MA) was used on a Bio-Rad Econo System. UV and fluorescence spectra were recorded on a Shimadzu BioSpec-mini spectrophotometer and a Shimadzu RF-5300PC spectrofluorophotometer, respectively. <sup>1</sup>H and <sup>31</sup>P NMR spectra were measured on Varian INOVA 600 and JEOL GSX270 spectrometers, respectively. Tetramethylsilane and trimethyl phosphate were used as internal standards. Proton signals were assigned by using homonuclear 2D NMR techniques, correlation spectroscopy (COSY) and rotating frame Overhauser effect spectroscopy (ROESY). The ROESY spectra were measured with a mixing time of 800 ms. Mass spectra were obtained on a Hitachi M-4000H spectrometer. HPLC analyses were carried out on a Gilson gradient-type analytical system equipped with a Waters 2996 photodiode array detector, and a Waters  $\mu$ Bondasphere C18 5 $\mu$ m 300 Å column (3.9 mm  $\times$  150 mm) was used at a flow rate of 1.0 ml/min with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (pH 7.0). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of oligonucleotides were measured in the negative ion mode on an Applied Biosystems Voyager DE PRO spectrometer, using 3-hydroxypicolinic acid as a matrix.

### Conversion of the (6-4) photoproduct (4a, 4b) to its Dewar valence isomer (5a, 5b)

The dinucleoside monophosphate of the (6-4) photoproduct (4a, 4b) was purified on a column of alkylated silica gel (1.5 cm  $\times$  48 cm) with a linear gradient of 10-25% aqueous acetonitrile for 400 min, after the UV irradiation described previously (25). The separated compounds (4a, 191 mg, 273  $\mu$ mol; 4b, 371 mg, 532  $\mu$ mol) were dissolved in 20% aqueous acetonitrile (1.2 l), and after nitrogen bubbling for 10 min, each solution was irradiated in a Pyrex immersion well apparatus fitted with a 450 W high-pressure mercury lamp (UM-452; Ushio, Tokyo, Japan) in a 5°C water bath (NCB-2200; EYELA, Tokyo, Japan) for 1 h. The products (5a and 5b) were purified separately on the same column with an acetonitrile gradient of 10-17.5% (5a) or 10-25% (5b) for 400 min, and the fractions were analyzed by reversed-phase HPLC with an acetonitrile gradient from

11 to 17% for 15 min. The fractions containing the product without UV absorption in the long wavelength region were collected, and a glassy solid was obtained after evaporation. **5a**: Yield 94 mg (134  $\mu$ mol, 49%).  $^1\text{H-NMR}$  (pyridine- $d_5$ )  $\delta$  (p.p.m.) 12.64 (s, 1H, -NH-), 7.02 (dd,  $J = 1.9, 9.5$  Hz, 1H, Tp H1'), 5.62 (m, 2H, pT H1', Tp H6), 5.50 (m, 1H, Tp H3'), 5.32 (m, 1H, pT H3'), 4.94 (s, 1H, pT H6), 4.60 (m, 3H, pT H5', -OCH<sub>2</sub>CH<sub>2</sub>CN), 4.44 (m, 1H, pT H5''), 4.33 (dd,  $J = 2.0, 12.2$  Hz, 1H, Tp H5'), 4.12 (m, 2H, pT H4', Tp H5''), 4.01 (m, 1H, Tp H4'), 3.28 (m, 1H, Tp H2'), 3.12 (m, 2H, -OCH<sub>2</sub>CH<sub>2</sub>CN), 2.84–2.56 (m, 5H, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-, Tp H2''), 2.38 (s, 3H, pT, -CH<sub>3</sub>), 2.19 (m, 2H, pT H2', pT H2''), 2.07 (s, 3H, -COCH<sub>3</sub>), 1.84 (s, 3H, Tp, -CH<sub>3</sub>).  $^{31}\text{P-NMR}$  (pyridine- $d_5$ )  $\delta$  -6.39 p.p.m. HRMS (SI)  $m/z$  720.1889 ([M+Na]<sup>+</sup>; calcd for C<sub>28</sub>H<sub>36</sub>N<sub>5</sub>O<sub>14</sub>PNa, 720.1891). **5b**: Yield 247 mg (339  $\mu$ mol, 67%).  $^1\text{H-NMR}$  (pyridine- $d_5$ )  $\delta$  (p.p.m.) 12.61 (s, 1H, -NH-), 7.06 (d,  $J = 9.1$  Hz, 1H, Tp H1'), 5.72 (s, 1H, Tp H6), 5.70 (t,  $J = 8.6$  Hz, 1H, Tp H3'), 5.65 (dd,  $J = 5.6, 9.5$  Hz, 1H, pT H1'), 5.17 (m, 1H, pT H3'), 4.96 (s, 1H, pT H6), 4.60 (m, 1H, pT H5'), 4.54 (m, 1H, pT H5''), 4.44 (m, 2H, -OCH<sub>2</sub>CH<sub>2</sub>CN), 4.32 (d,  $J = 10.7$  Hz, 1H, Tp H5'), 4.22 (d,  $J = 10.9$  Hz, 1H, Tp H5''), 4.12 (m, 1H, pT H4'), 4.03 (d,  $J = 7.9$  Hz, 1H, Tp H4'), 3.26 (dd,  $J = 8.6, 14.2$  Hz, 1H, Tp H2'), 3.02 (t,  $J = 6.1$  Hz, 2H, -OCH<sub>2</sub>CH<sub>2</sub>CN), 2.77 (m, 2H, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-), 2.68–2.58 (m, 3H, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-, Tp H2''), 2.36 (s, 3H, pT, -CH<sub>3</sub>), 2.13 (m, 2H, pT H2', pT H2''), 2.07 (s, 3H, -COCH<sub>3</sub>), 1.91 (s, 3H, Tp, -CH<sub>3</sub>).  $^{31}\text{P-NMR}$  (pyridine- $d_5$ )  $\delta$  -3.27 p.p.m. HRMS (SI)  $m/z$  720.1896 ([M+Na]<sup>+</sup>; calcd for C<sub>28</sub>H<sub>36</sub>N<sub>5</sub>O<sub>14</sub>PNa, 720.1891).

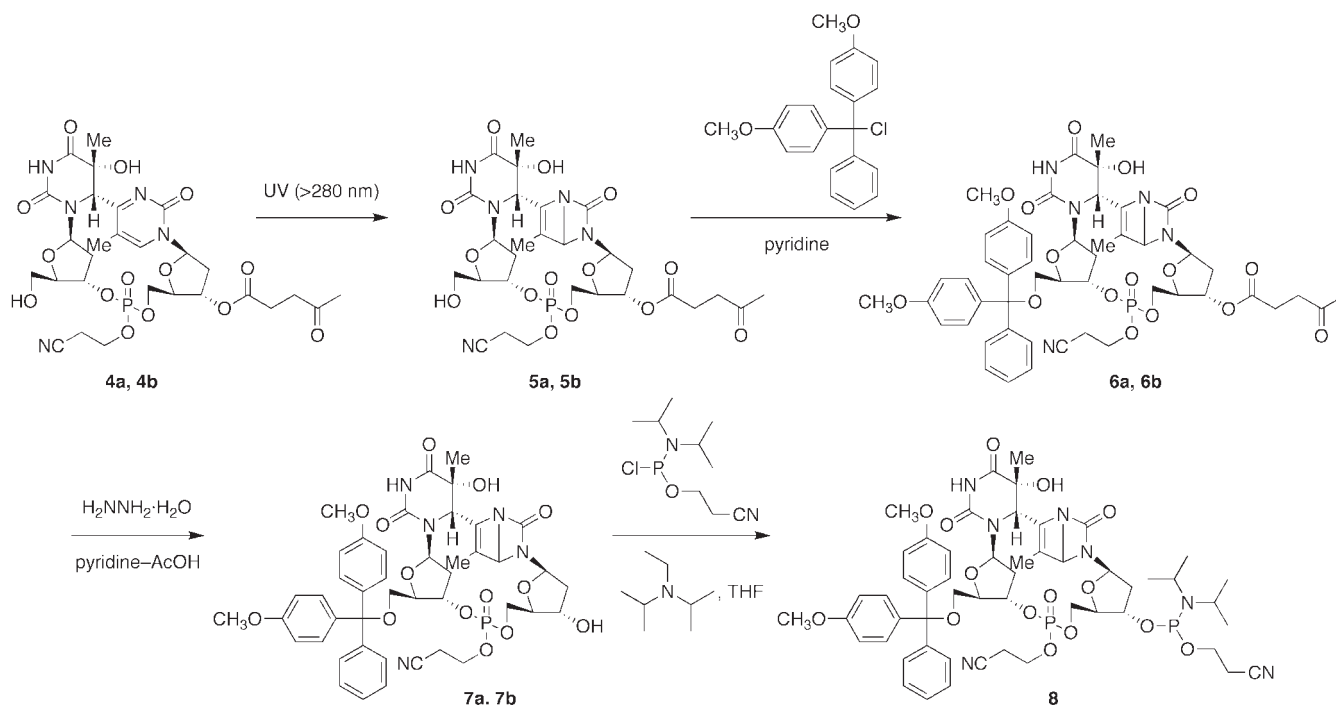
### Protection of the 5'-OH function (6a and 6b)

The partially protected dinucleoside monophosphate of the Dewar photoproduct (**5a**, 171 mg, 245  $\mu$ mol) was dissolved in pyridine (3 ml) and was mixed with 4,4'-dimethoxytrityl (DMT) chloride (211 mg, 623  $\mu$ mol). After stirring at room temperature for 3 h, methanol (1.0 ml) was added and the mixture was concentrated. The residue was dissolved in chloroform (30 ml), washed with water and was chromatographed, after concentration and coevaporation with toluene, on silica gel with a step gradient of 0–4% methanol in chloroform. The fractions containing the tritylated product were collected, and **6a** was obtained as a foam after evaporation. The other isomer (**5b**, 218 mg, 313  $\mu$ mol) was treated in the same way. **6a**: Yield 204 mg (204  $\mu$ mol, 83%).  $^1\text{H-NMR}$  (CDCl<sub>3</sub>)  $\delta$  (p.p.m.) 7.67 (br, 1H, -NH-), 7.43 (d,  $J = 4.1$  Hz, 2H, aromatic), 7.30 (m, 6H, aromatic), 7.24 (t,  $J = 7.3$  Hz, 1H, aromatic), 6.84 (d,  $J = 4.4$  Hz, 4H, aromatic), 6.45 (dd,  $J = 2.8, 9.7$  Hz, 1H, Tp H1'), 5.56 (dd,  $J = 6.1, 8.5$  Hz, 1H, pT H1'), 5.08 (m, 1H, pT H3'), 5.00 (s, 1H, pT H6), 4.73 (m, 1H, Tp H3'), 4.65 (s, 1H, Tp H6), 4.37 (d,  $J = 11.0$  Hz, 1H, pT H5'), 4.09 (m, 2H, pT H5'', -OCH<sub>2</sub>CH<sub>2</sub>CN), 3.95 (m, 1H, pT H4'), 3.82 (m, 2H, Tp H4', -OCH<sub>2</sub>CH<sub>2</sub>CN), 3.79 (s, 6H, -OCH<sub>3</sub>), 3.72 (dd,  $J = 2.6, 11.0$  Hz, 1H, Tp H5'), 3.48 (br, 1H, -OH), 3.24 (dd,  $J = 2.9, 11.0$  Hz, 1H, Tp H5''), 2.86 (m, 1H, Tp H2'), 2.81–2.71 (m, 2H, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-), 2.65–2.49 (m, 3H, -OCH<sub>2</sub>CH<sub>2</sub>CN, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-), 2.40–2.28 (m, 3H, Tp H2'', pT H2', -OCH<sub>2</sub>CH<sub>2</sub>CN), 2.19 (s, 3H, -COCH<sub>3</sub>), 2.09 (m, 1H, pT H2''), 2.01 (s, 3H, pT -CH<sub>3</sub>), 1.51 (s, 3H,

Tp -CH<sub>3</sub>).  $^{31}\text{P-NMR}$  (CDCl<sub>3</sub>)  $\delta$  -6.85 p.p.m. HRMS (SI)  $m/z$  1022.3202 ([M+Na]<sup>+</sup>; calcd for C<sub>49</sub>H<sub>54</sub>N<sub>5</sub>O<sub>16</sub>PNa, 1022.3197). **6b**: Yield 297 mg (297  $\mu$ mol, 95%).  $^1\text{H-NMR}$  (CDCl<sub>3</sub>)  $\delta$  (p.p.m.) 7.79 (br, 1H, -NH-), 7.47 (d,  $J = 8.1$  Hz, 2H, aromatic), 7.38 (dd,  $J = 2.0, 8.9$  Hz, 4H, aromatic), 7.30 (t,  $J = 7.8$  Hz, 2H, aromatic), 7.20 (t,  $J = 7.4$  Hz, 1H, aromatic), 6.84 (d,  $J = 8.8$  Hz, 4H, aromatic), 6.43 (dd,  $J = 2.6, 9.7$  Hz, 1H, Tp H1'), 5.53 (dd,  $J = 4.7, 10.3$  Hz, 1H, pT H1'), 5.01 (m, 1H, Tp H3'), 4.96 (s, 1H, pT H6), 4.83 (m, 2H, Tp H6, pT H3'), 4.31 (m, 1H, pT H5'), 4.21 (m, 1H, pT H5''), 4.04–3.89 (m, 3H, pT H4', -OCH<sub>2</sub>CH<sub>2</sub>CN), 3.78 (s, 6H, -OCH<sub>3</sub>), 3.72 (d,  $J = 8.3$  Hz, 1H, Tp H4'), 3.69 (dd,  $J = 2.4, 11.1$  Hz, 1H, Tp H5'), 3.64 (br, 1H, -OH), 3.11 (dd,  $J = 2.6, 11.2$  Hz, 1H, Tp H5''), 2.75 (m, 3H, Tp H2', -OCOCH<sub>2</sub>CH<sub>2</sub>CO-), 2.64–2.49 (m, 4H, -OCH<sub>2</sub>CH<sub>2</sub>CN, -OCOCH<sub>2</sub>CH<sub>2</sub>CO-), 2.30–2.20 (m, 2H, Tp H2'', pT H2'), 2.18 (s, 3H, -COCH<sub>3</sub>), 1.94 (m, 1H, pT H2''), 1.90 (s, 3H, pT -CH<sub>3</sub>), 1.62 (s, 3H, Tp -CH<sub>3</sub>).  $^{31}\text{P-NMR}$  (CDCl<sub>3</sub>)  $\delta$  -3.44 p.p.m. HRMS (SI)  $m/z$  1022.3190 ([M+Na]<sup>+</sup>; calcd for C<sub>49</sub>H<sub>54</sub>N<sub>5</sub>O<sub>16</sub>PNa, 1022.3197).

### Removal of the levulinyl group (7a and 7b)

The protected dinucleoside monophosphate of the Dewar photoproduct (**6a**, 181 mg, 181  $\mu$ mol) was dissolved in pyridine (2.3 ml), and a solution (2.3 ml) of hydrazine monohydrate (88  $\mu$ l, 1.8 mmol) in pyridine-acetic acid (3:2, v/v) was added. After stirring at room temperature for 5 min, the mixture was cooled in an ice bath, and acetone (2.0 ml) was added. The mixture was diluted with chloroform (30 ml), washed with 2% aqueous NaHCO<sub>3</sub> and with water, dried with Na<sub>2</sub>SO<sub>4</sub> and concentrated. After coevaporation with toluene, the residue was chromatographed on silica gel (4.8 g) with a step gradient of 0–5% methanol in chloroform and a product (**7a**) was obtained as a glassy solid after evaporation. The other isomer (**6b**, 454 mg, 454  $\mu$ mol) was treated in the same way. **7a**: Yield 151 mg (167  $\mu$ mol, 92%).  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  (p.p.m.) 10.46 (s, 1H, -NH-), 7.40 (d,  $J = 7.7$  Hz, 2H, aromatic), 7.26 (m, 7H, aromatic), 6.88 (dd,  $J = 2.6, 9.2$  Hz, 4H, aromatic), 6.29 (dd,  $J = 2.8, 9.8$  Hz, 1H, Tp H1'), 5.92 (br, 1H, Tp -OH), 5.41 (m, 2H, pT H1', 3'-OH), 5.21 (s, 1H, pT H6), 4.46 (m, 1H, Tp H3'), 4.25 (s, 1H, Tp H6), 4.18 (m, 1H, pT H5'), 4.10 (m, 1H, pT H3'), 3.94 (m, 2H, pT H5'', -OCH<sub>2</sub>CH<sub>2</sub>CN), 3.81 (m, 1H, Tp H4'), 3.74 (m, 7H, -OCH<sub>2</sub>CH<sub>2</sub>CN, -OCH<sub>3</sub>), 3.65 (m, 1H, pT H4'), 2.77 (m, 1H, Tp H2'), 2.69 (m, 2H, -OCH<sub>2</sub>CH<sub>2</sub>CN), 2.11 (m, 2H, Tp H2'', pT H2'), 2.00 (m, 1H, pT H2''), 1.88 (s, 3H, pT -CH<sub>3</sub>), 1.39 (s, 3H, Tp -CH<sub>3</sub>).  $^{31}\text{P-NMR}$  (DMSO- $d_6$ )  $\delta$  -6.75 p.p.m. HRMS (SI)  $m/z$  924.2824 ([M+Na]<sup>+</sup>; calcd for C<sub>44</sub>H<sub>48</sub>N<sub>5</sub>O<sub>14</sub>PNa, 924.2829). **7b**: Yield 392 mg (435  $\mu$ mol, 96%).  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  (p.p.m.) 10.46 (s, 1H, -NH-), 7.42 (d,  $J = 8.3$  Hz, 2H, aromatic), 7.30 (m, 6H, aromatic), 7.22 (t,  $J = 7.3$  Hz, 1H, aromatic), 6.87 (dd,  $J = 2.8, 9.2$  Hz, 4H, aromatic), 6.25 (dd,  $J = 2.8, 9.7$  Hz, 1H, Tp H1'), 6.00 (br, 1H, Tp -OH), 5.40 (m, 2H, pT H1', 3'-OH), 5.21 (s, 1H, pT H6), 4.93 (m, 1H, Tp H3'), 4.42 (s, 1H, Tp H6), 4.19 (d,  $J = 10.8$  Hz, 1H, pT H5'), 4.00 (m, 4H, pT H3', pT H5'', -OCH<sub>2</sub>CH<sub>2</sub>CN), 3.74 (m, 8H, Tp H4', pT H4', -OCH<sub>3</sub>), 3.40 (d,  $J = 10.7$  Hz, 1H, Tp H5'), 3.06 (dd,  $J = 2.9, 11.3$  Hz, 1H, Tp H5''), 2.83 (m, 2H, -OCH<sub>2</sub>CH<sub>2</sub>CN),



**Scheme 2.** Synthesis of the building block of the Dewar photoproduct.

2.64 (m, 1H, Tp H2'), 2.15 (m, 1H, Tp H2''), 1.97 (m, 2H, pT H2', pT H2''), 1.84 (s, 3H, pT -CH<sub>3</sub>), 1.38 (s, 3H, Tp -CH<sub>3</sub>). <sup>31</sup>P-NMR (DMSO-*d*<sub>6</sub>) δ -3.29 p.p.m. HRMS (SI) *m/z* 924.2831 ([M+Na]<sup>+</sup>; calcd for C<sub>44</sub>H<sub>48</sub>N<sub>5</sub>O<sub>14</sub>PNa, 924.2829).

### Phosphoramidite building block of the Dewar photoproduct (8)

An aliquot of **7b** (165 mg, 183 μmol) was mixed with tetrahydrofuran (1.6 ml) and *N,N*-diisopropylethylamine (127 μl, 732 μmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (82 μl, 336 μmol) was added. This mixture was stirred at room temperature for 30 min and diluted with ethyl acetate (40 ml). The resulting solution was washed with 2% aqueous NaHCO<sub>3</sub> and with water, dried with Na<sub>2</sub>SO<sub>4</sub>, concentrated to a gum and coevaporated with toluene. The residue was chromatographed on silica gel (5 g) with a step gradient of 0–2% methanol in ice-cooled chloroform containing 0.5% pyridine. The appropriate fractions were collected and concentrated. The residue was dissolved in chloroform (1 ml) and was precipitated in pentane (20 ml), and the precipitate was washed with pentane. Yield 166 mg (151 μmol, 83%). <sup>31</sup>P-NMR (CDCl<sub>3</sub>) δ (p.p.m.) 147.04, 146.69, -3.29, -3.53. HRMS (SI) *m/z* 1124.3919 ([M+Na]<sup>+</sup>; calcd for C<sub>53</sub>H<sub>65</sub>N<sub>7</sub>O<sub>15</sub>P<sub>2</sub>Na, 1124.3908).

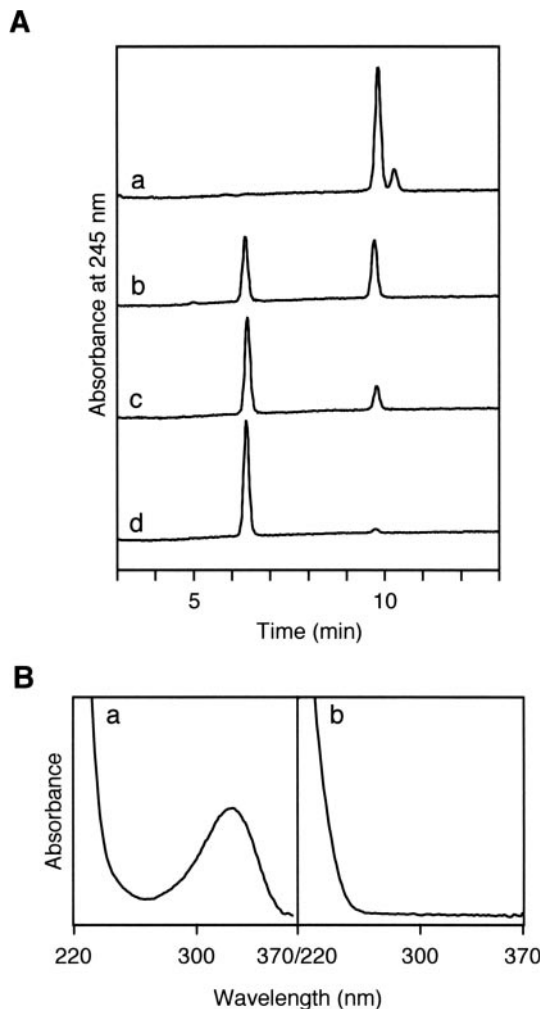
### Oligonucleotide synthesis

The phosphoramidite building block of the Dewar photoproduct (**8**) was dissolved in anhydrous acetonitrile at a concentration of 0.13 M and was installed on an Applied Biosystems 3400 DNA synthesizer. Nucleoside phosphoramidites for ultramild DNA synthesis (Glen Research), as well as the base-unprotected thymidine phosphoramidite, were also dissolved in acetonitrile to make 0.1 M solutions and were

installed on the synthesizer. Oligonucleotides were synthesized on a 0.2 μmol scale, and the reaction time for the coupling of the Dewar photoproduct building block was prolonged to 20 min. For the synthesis using benzimidazolium triflate (BIT) as an activator, a 0.2 M solution was prepared to replace the tetrazole solution, and a polystyrene support (Universal Support II PS, Glen Research) was used without changing the synthesis procedure. After chain assembly and removal of the 5'-terminal DMT group on the synthesizer, the solid supports containing the oligonucleotides were treated with 28% aqueous ammonia (2 ml) at room temperature for 2 h. The resulting ammoniac solutions were concentrated to dryness on a rotary evaporator equipped with a vacuum pump. The residues were dissolved in water (1 ml) and the oligonucleotides were analyzed and purified by HPLC. The column was heated to 50°C and the acetonitrile gradients were 5–11, 6–12 and 8–13% for 20 min in the cases of the 12mer, 20mer and 30mer, respectively. After purification, the HPLC analysis of the 12mer was performed at room temperature using an acetonitrile gradient from 7 to 13% for 20 min.

### Analysis of the (6–4) photolyase reaction

Solutions (180 μl) of *Xenopus laevis* (6–4) photolyase (31) (2 nmol), in a buffer containing 10 mM Tris-HCl (pH 8.0) and 2 mM 2-mercaptoethanol, were covered with a Pyrex lid and were irradiated on ice with an 18 W fluorescent lamp at a distance of 15 cm for 30 min. A solution (20 μl) of the 12mer containing the (6–4) or Dewar photoproduct (0.2 nmol) was added, and the mixtures were irradiated again for 3 or 24 h. After heating to 80°C for 30 min, the mixtures were passed through a NAP-5 column (Amersham Biosciences AB, Uppsala, Sweden), and the eluates were



**Figure 1.** (A) Formation of the Dewar photoproduct. The dinucleoside monophosphate of the (6-4) photoproduct (**2**) was irradiated with Pyrex-filtered light from a 100 W high-pressure mercury lamp for 0 h (a), 1 h (b), 2 h (c) and 4 h (d), and the samples were analyzed by HPLC, using an acetonitrile gradient from 0 to 5% for 16 min. (B) Absorption spectra of **2** (a) and the irradiation product (b). The absorption spectra of the two peaks of trace b in (A) were extracted with the data processing software for the photodiode array detector.

analyzed by HPLC under the same conditions as those used for the analysis of the purified 12mer.

#### Analysis of the binding of (6-4) photolyase

The 20mer containing the Dewar photoproduct (Dw), d(CCTACGCAAAT-Dw-GGCATCC), was hybridized to a complementary strand containing 2-aminopurine (Ap), d(GGATGCC-Ap-AATTTGCGTAGG), by heating a mixture (40  $\mu$ l) of the two oligonucleotides (1.2 nmol each) to 80°C for 3 min and cooling it to room temperature. A duplex without the photoproduct was prepared in the same way. These duplexes were dissolved at 0.3  $\mu$ M in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM 2-mercaptoethanol and 10% glycerol, and fluorescence spectra were measured at 5°C, in the absence or presence of 350 nM *X.laëvis* (6-4) photolyase. The excitation wavelength was 313 nm.

Experiments using the undamaged and (6-4) photoproduct-containing duplexes were performed in the same way.

## RESULTS AND DISCUSSION

### Formation and stability of the Dewar photoproduct

We intended to synthesize oligonucleotides containing the Dewar photoproduct formed at the TT sequence (**3**) using a dinucleotide building block, to extend our previous studies on the (6-4) photoproduct (25,26). Before the preparation of the building block, we analyzed the formation and the stability of the Dewar photoproduct. A solution of the dinucleoside monophosphate of the (6-4) photoproduct (**2**), which was obtained by deprotection of the intermediate in the preparation of the (6-4) photoproduct building block (a mixture of compounds **4a** and **4b** in Scheme 2), was irradiated on ice in a petri dish with a Pyrex lid using a 100 W high-pressure mercury lamp covered with a Pyrex water jacket. HPLC analysis of the irradiated solution on a reversed-phase column is shown in Figure 1A. A new peak emerged, and the (6-4) photoproduct was converted to this product almost completely in 4 h. We assigned this product as the Dewar valence isomer (**3**) because the absorption at wavelengths longer than 260 nm was lost (Figure 1B), as reported for the Dewar photoproduct (1,32).

The dinucleoside monophosphate of the Dewar photoproduct formed in the above experiment was used to analyze its stability. Compound **3** was treated with acetic acid at room temperature for 1 h to confirm that the damaged base would not be degraded during the DMT removal step in every cycle of oligonucleotide synthesis. Another experiment was its treatment with aqueous ammonia at room temperature for 2 h, which is used for the deprotection of oligonucleotides synthesized by the 'ultramild' method. The latter analysis is very important, because the Dewar isomer is reportedly more labile than the (6-4) photoproduct under alkaline conditions (11,22). HPLC analysis of the reaction mixtures revealed that the Dewar photoproduct was stable under both of these conditions, although a very small peak was detected at a short retention time after the ammonia treatment (Supplementary Figure 1).

### The phosphoramidite building block of the Dewar photoproduct

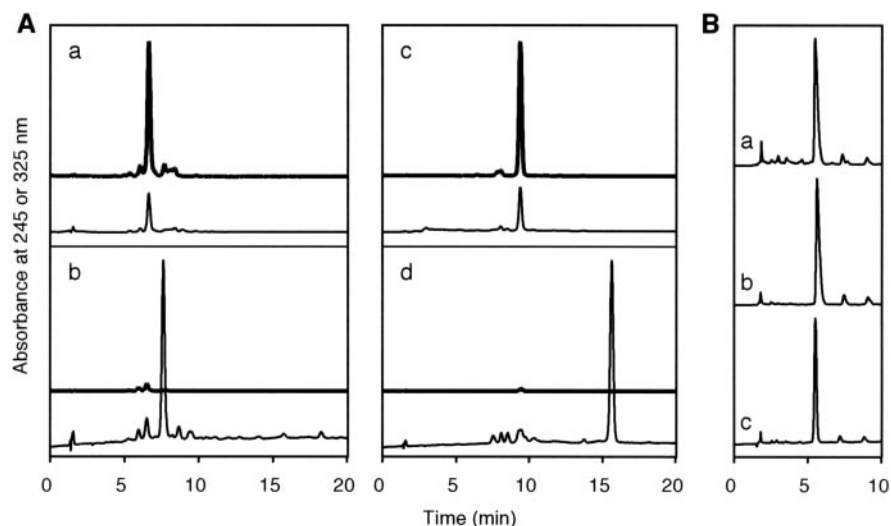
Although the Dewar photoproduct can be obtained directly by irradiation of thymidylyl(3'-5')thymidine at wavelengths longer than 280 nm, its yield by this method is very low (2). Therefore, the (6-4) photoproduct prepared by our previous method (25) was isomerized in this study. After the formation of the (6-4) photoproduct by irradiation of the protected thymidylyl(3'-5')thymidine with germicidal lamps, the diastereomers caused by the chirality of the phosphorus atom were separated by reversed-phase chromatography on alkylated silica gel, as described previously (25). The isomers with shorter and longer retention times in reversed-phase HPLC analysis were designated as **4a** and **4b**, respectively. Each diastereomer of the dinucleoside monophosphate was dissolved in 20% aqueous acetonitrile and was irradiated at 5°C in a Pyrex immersion well apparatus fitted with a

450 W high-pressure mercury lamp. At intervals of 10 min, aliquots of the solution were analyzed by reversed-phase HPLC, and the decrease of the starting material, as determined from the peak areas of the chromatograms at 325 nm, was monitored (Supplementary Figure 2). After 70 min, the (6-4) photoproduct was consumed almost completely and was converted to a single product, which did not absorb UV light with wavelengths longer than 280 nm (Figure 2A). The same results were obtained regardless of the stereochemistry of the phosphotriester linkage. Aliquots of the products were treated with aqueous ammonia at room temperature for 2 h for deprotection and were subjected to HPLC analysis. As shown in Figure 2B, the deprotected products were identical to the authentic sample of the Dewar photoproduct obtained by irradiation of the deprotected (6-4) photoproduct (trace d in Figure 1A).

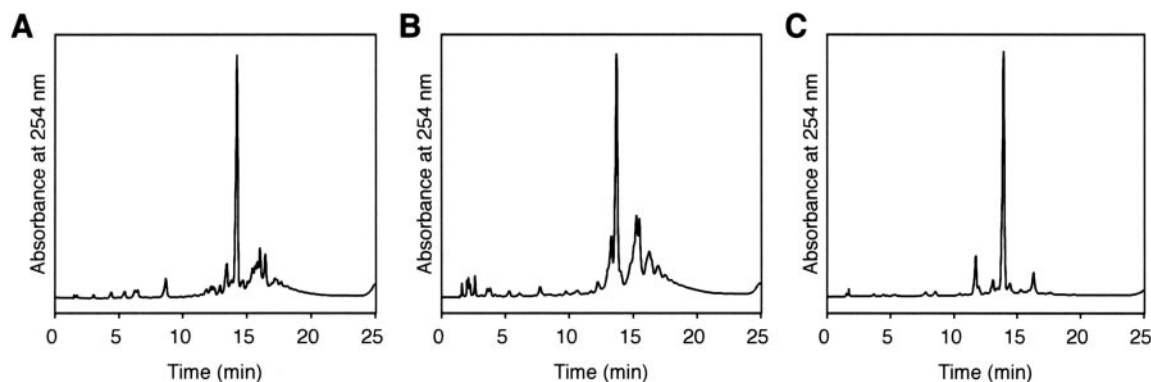
The putative Dewar-containing dinucleoside monophosphates (**5a** and **5b**) were purified separately by reversed-phase chromatography and were analyzed by  $^1\text{H-NMR}$

spectroscopy. The proton signals were assigned using COSY and ROESY spectra, and as reported for the Dewar photoproduct without the protecting groups (3), ROESY crosspeaks were observed between pT CH<sub>3</sub> and Tp H3' and between pT CH<sub>3</sub> and pT H3' for both **5a** and **5b**. It was also reported that the conversion of the (6-4) photoproduct to its Dewar valence isomer resulted in an up-field shift of the H6 signal of the 3' base moiety (3). In the present study, the chemical shifts of H6 were 7.84, 8.01, 4.94 and 4.96 for **4a**, **4b**, **5a** and **5b**, respectively. This observation, together with the HPLC analysis shown in Figure 2B, the UV absorption spectra and the molecular weight determined by high-resolution mass spectrometry, demonstrated that the obtained products were the dinucleoside monophosphates of the Dewar photoproduct.

The dinucleoside monophosphates bearing the Dewar photoproduct (**5a** and **5b**) were converted to the 5'-DMT derivatives (**6a** and **6b**) before the removal of the 3'-protecting group to yield **7a** and **7b**, in the same way as described for



**Figure 2.** (A) Conversion of **4a** and **4b** to **5a** and **5b**. Compounds **4a** (a) and **4b** (c) were irradiated with a 450 W high-pressure mercury lamp for 1 h, and the starting materials (a and c) and the reaction mixtures (b and d) were analyzed by HPLC, using an acetonitrile gradient from 11 to 17% for 20 min. The thick and thin lines show the chromatograms monitored at 325 and 245 nm, respectively. (B) Deprotection of **5a** and **5b**. Aliquots of **5a** and **5b** were treated with aqueous ammonia at room temperature for 2 h, and were analyzed by HPLC after evaporation (a and b, respectively). (c) A mixture of the deprotected compounds was co-injected with the authentic Dewar photoproduct (trace d in Figure 1A). The acetonitrile gradient was the same as that described in the legend to Figure 1A.



**Figure 3.** Reversed-phase HPLC analysis of crude oligonucleotides after deprotection. (A and B) The 20mer (A) and the 30mer (B) synthesized by the ordinary method using tetrazole. (C) The 20mer synthesized by the BIT-activation method. The HPLC conditions are described in Materials and Methods.

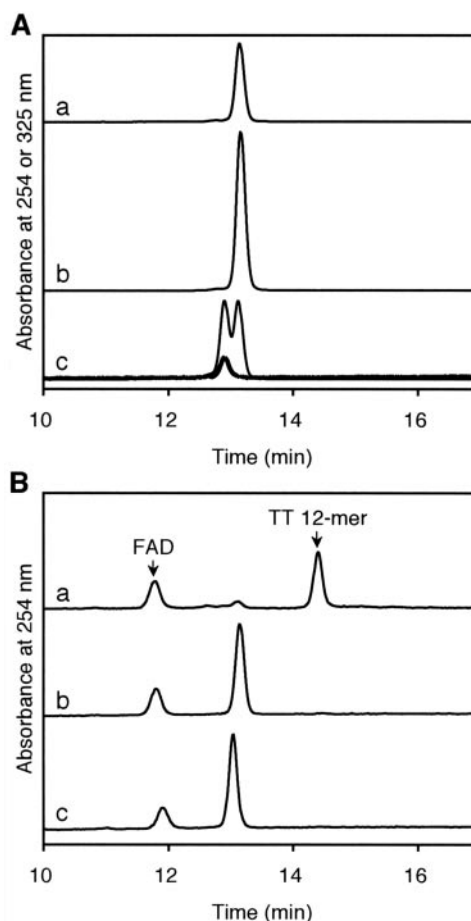
the (6–4) photoproduct (25). Since the obtained amount of **7b** was 2.6-fold larger than that of **7a**, **7b** was used for the preparation of the phosphoramidite building block. After the phosphorylation of **7b**, we tried to purify the phosphoramidite building block of the Dewar photoproduct (**8**) by chromatography on silica gel using a solvent system of chloroform–methanol containing 0.1% pyridine, as developed for the (6–4) photoproduct. However, a  $^{31}\text{P}$ -NMR measurement revealed that the phosphoramidite was degraded during this chromatographic purification. Both of the phosphoramidite signals were diminished, and a new signal emerged at 11.66 p.p.m. The degradation was reproducible and also occurred when **7a** was used. This result suggested that the phosphoramidite moiety attached to the dinucleoside monophosphate of the Dewar photoproduct is more labile than those in other building blocks. After testing several solvent systems, we found that the phosphoramidite could be obtained in an intact form by flash chromatography using chloroform containing 0.5% pyridine.

In this study, the two phosphorus diastereomers were separated at the initial stage, in order to characterize the intermediates by NMR spectroscopy, but it is possible to use a mixture of the diastereomers as we are practicing in the case of the (6–4) photoproduct.

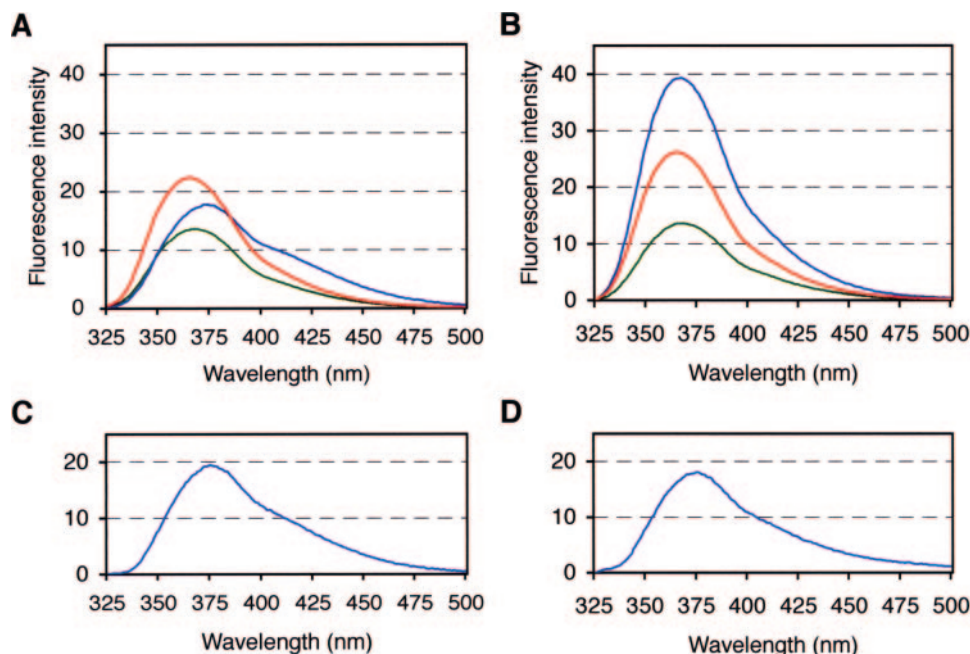
#### Synthesis of oligonucleotides containing the Dewar photoproduct

Using the phosphoramidite building block of the Dewar photoproduct (**8**) prepared as above, a 12mer, d(CA-Dw-AGCAC-GAC), a 20mer, d(CCTACGCAAAT-Dw-GGCATCC), and a 30mer, d(CTCGTCAGCATC-Dw-CATCATACAGTCAG-TG), in which Dw represents the Dewar valence isomer of the (6–4) photoproduct formed at the TT site, were synthesized on a DNA synthesizer. Since this photoproduct is labile under alkaline conditions (11,22), 2'-deoxyribonucleoside phosphoramidites of  $N^6$ -phenoxyacetyl adenine,  $N^4$ -acetyl cytosine and  $N^2$ -(4-isopropylphenoxy)acetyl guanine, which can be deprotected under mild conditions, were used together with that of unprotected thymine. After the chain assembly, the oligonucleotides were cleaved from the support and deprotected by the treatment with aqueous ammonia at room temperature for 2 h. Analysis of the crude samples of the obtained oligonucleotides was performed by reversed-phase HPLC, as shown in Figure 3A and B. A major peak was obtained in each case, but many peaks of impurities were detected at longer retention times. These impurities were also detected in our previous studies on the (6–4) photoproduct, and they were assumed to be formed by the coupling of the phosphoramidites with the N3 imino function of the 5' component of the (6–4) photoproduct (25,26,33). Since the 5' component of the Dewar isomer has the same chemical structure as that of the (6–4) photoproduct, this type of branching side reaction must have occurred. To verify this proposal, as well as to improve the yields of the desired oligonucleotides, the tetrazole was replaced with benzimidazolium triflate (BIT), which was originally used as an activator of phosphoramidites by Hayakawa's group (34,35) and prevented the by-product formation in the synthesis of (6–4) photoproduct-containing oligonucleotides (33). As shown in Figure 3C, the results were improved to a great extent using this alternative activator.

The oligonucleotides were purified by reversed-phase HPLC and the 12mer was analyzed by MALDI-TOF mass spectrometry (Supplementary Figure 3). The calculated and obtained  $m/z$  values in the negative ion mode were 3611.74 and 3611.94, respectively. The presence of the Dewar photoproduct in the obtained oligonucleotides was demonstrated using the 12mer. In our previous study (12), the same Dewar photoproduct-containing 12mer was prepared by irradiation of the (6–4) photoproduct-containing one, and these two oligonucleotides were separated from each other by reversed-phase HPLC. This experiment was performed using the 12mer synthesized in the present study. As shown in Figure 4A, the 12mer in question coeluted with the Dewar photoproduct-containing 12mer that was used in our previous study (12), whereas it was separated from the (6–4) photoproduct-containing 12mer. Further characterization of the Dewar 12mer was performed using the UV conversion to the (6–4) photoproduct and the enzymatic digestion, as shown in Supplementary Figures 4 and 5, respectively. In



**Figure 4.** (A) Reversed-phase HPLC analysis of the Dewar 12mer. (a) The 12mer synthesized in this study, (b) co-injection with the Dewar photoproduct-containing 12mer prepared by irradiation of the (6–4) photoproduct-containing 12mer and (c) co-injection with the (6–4) photoproduct-containing 12mer with the same sequence context. The thick and thin lines show the chromatograms monitored at 325 and 254 nm, respectively, and the 325 nm chromatogram is magnified by a factor of 5. (B) Analysis of the (6–4) photolyase reaction with the (6–4) 12mer (a) and the Dewar 12mer (b and c). The reaction times were 3 h (a and b) and 24 h (c). The HPLC conditions are described in Materials and Methods.



**Figure 5.** (A and B) Fluorescence emission spectra of the Dewar (red), (6-4) (blue) and TT (green) 20mers hybridized to a complementary strand containing 2-aminopurine in the absence (A) and in the presence (B) of the (6-4) photolyase. (C and D) Fluorescence emission spectra of the (6-4) 20mer hybridized to a complementary strand without 2-aminopurine in the absence (C) and in the presence (D) of the (6-4) photolyase.

the latter experiment, the phosphodiester linkage on the 3' side of the photoproduct was not cleaved. However, no difference was observed between the oligonucleotide synthesized in this study and the authentic sample prepared by irradiation of the (6-4) photoproduct-containing oligonucleotide, and the presence of the Dewar photoproduct was demonstrated by the UV conversion to the (6-4) photoproduct (trace d in Supplementary Figure 5A). These results show that the Dewar photoproduct-containing oligonucleotides, which are free from contamination with the (6-4) photoproduct-containing oligomers, can be synthesized using the method described here.

#### Reaction and binding of (6-4) photolyase with Dewar photoproduct-containing oligonucleotides

The (6-4) photolyase was first discovered in a cell extract of *Drosophila melanogaster* (20), and subsequently was found in *X.laevis* (36) and *Arabidopsis thaliana* (37). This enzyme contains flavin adenine dinucleotide (FAD) as a chromophore (36), and the analysis of its reaction strongly suggested that this enzyme repairs the (6-4) photoproducts to their original pyrimidines by utilizing blue to near-UV light (20,38). We demonstrated previously that this enzyme restored the pyrimidines of the (6-4) photoproducts formed at the TT (31) and TC (26) sites to their normal forms, and proposed the repair mechanism of this enzyme (39). Although a crystal structure of CPD photolyase, another enzyme for CPD repair, has been solved in a complex form with its substrate DNA (40), no structural information has been reported for the (6-4) photolyase thus far. In the previous studies, the (6-4) photolyase could bind DNA containing the Dewar photoproduct, but the repair rate was reduced to a great extent when the Dewar photoproduct-containing DNA was used as a

substrate (21,31). Therefore, this type of lesion may be useful in the co-crystallization experiments of the (6-4) photolyase.

To test the utility of the Dewar photoproduct-containing DNA, we verified the (6-4) photolyase reaction using the chemically synthesized oligonucleotides. The 12mer containing the Dewar photoproduct, as well as that containing the (6-4) photoproduct in the same sequence context, was treated with *X.laevis* (6-4) photolyase, and the reaction mixtures were analyzed by reversed-phase HPLC. As shown in Figure 4B, the (6-4) 12mer was converted to the undamaged 12mer (TT 12mer) by exposing the reaction mixture to fluorescent lighting for 3 h (trace a), and the repair was confirmed by co-injection of the product with the TT 12mer. In contrast, the peak of the TT 12mer was not detected in the Dewar case (trace b) and prolonged exposure (up to 24 h) did not cause any change (trace c). Since there was a possibility that the above results were obtained by the loss of the affinity of the (6-4) photolyase for the Dewar photoproduct-containing DNA, the enzyme binding was analyzed using a fluorescent base analog, 2-aminopurine. In the case of the CPD photolyase, flipping of the CPD lesion upon enzyme binding was shown by using a complementary strand containing 2-aminopurine (41), and the same mechanism was proposed for the (6-4) photolyase (39). Therefore, this type of experiment was expected to demonstrate the two phenomena, the enzyme binding and the base flipping, simultaneously. The Dewar photoproduct-containing 20mer was hybridized to d(GGATGCC-Ap-AATTTGCGTAGG), in which Ap represents 2-aminopurine. In this duplex, the 2-aminopurine was opposite the 3' component of the Dewar photoproduct. We changed the position of 2-aminopurine in this sequence context, and the fluorescence intensity observed for the duplex was sometimes close to that of the single-stranded oligonucleotide containing this fluorescent base analog (data not



shown). This type of position effect on the emission of 2-aminopurine has been reported recently for another lesion-containing DNA (42). Using the above-mentioned duplex, as well as the undamaged and (6–4) photoproduct-containing duplexes as negative and positive controls, respectively, fluorescence emission spectra were measured in the absence and presence of the (6–4) photolyase (Figure 5A and B). Since the (6–4) photoproduct is a fluorescent base, emission spectra of the (6–4) photoproduct-containing duplex without 2-aminopurine were measured under the same conditions, to confirm that its fluorescence did not affect this analysis (Figure 5C and D). Moreover, there was a possibility that fluorescence resonance energy transfer might occur between 2-aminopurine and the (6–4) photoproduct, but we confirmed that such an effect was small enough for this experiment (data not shown). An increase of the fluorescence intensity upon the addition of the enzyme was observed for the Dewar photoproduct-containing duplex, but it was much smaller than that observed for the (6–4) photoproduct-containing duplex. These results indicate that the (6–4) photolyase binds the DNA containing the Dewar photoproduct and induces a structural change in DNA to some extent. However, it is suggested that the binding mode is different between the (6–4) photoproduct-containing substrate and the Dewar isomer-containing duplex although the reported affinities of this enzyme for these two types of duplexes are similar (21,31).

We have synthesized a phosphoramidite building block of the Dewar valence isomer of the (6–4) photoproduct formed at the thymidylyl(3'–5')thymidine site and successfully incorporated it into oligodeoxyribonucleotides. Using these oligonucleotides, the reaction and the binding of the (6–4) photolyase were analyzed, and the results suggested an enzyme binding mode different from the recognition of the original substrate. It is expected that oligonucleotides synthesized by this method will contribute to molecular and structural biology in the field of mutagenesis and DNA repair.

## SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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