Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 2318

www.rsc.org/obc

PAPER

Mechanism of the alkali degradation of (6–4) photoproduct-containing DNA†

Norihito Arichi,[a] Aki Inase,‡[a] Sachise Eto,[b] Toshimi Mizukoshi,[b] Junpei Yamamoto[a] and Shigenori Iwai*[a]

Received 28th November 2011, Accepted 19th December 2011
DOI: 10.1039/c2ob06966k

The (6–4) photoproduct is one of the major damaged bases produced by ultraviolet light in DNA. This lesion is known to be alkali-labile, and strand breaks occur at its sites when UV-irradiated DNA is treated with hot alkali. We have analyzed the product obtained by the alkali treatment of a dinucleoside monophosphate containing the (6–4) photoproduct, by HPLC, NMR spectroscopy, and mass spectrometry. We previously found that the N3–C4 bond of the 5′ component was hydrolyzed by a mild alkali treatment, and the present study revealed that the following reaction was the hydrolysis of the glycosidic bond at the 3′ component. The sugar moiety of this component was lost, even when a 3′-flanking nucleotide was not present. Glycosidic bond hydrolysis was also observed for a dimer and a trimer containing 5-methyl-2-pyrimidinone, which was used as an analog of the 3′ component of the (6–4) photoproduct, and its mechanism was elucidated. Finally, the alkali treatment of a tetramer, d(GT(6–4)TC), yielded 2′-deoxycytidine 5′-monophosphate, while 2′-deoxyguanosine 3′-monophosphate was not detected. This result demonstrated the hydrolysis of the glycosidic bond at the 3′ component of the (6–4) photoproduct and the subsequent strand break by β-elimination. It was also shown that the glycosidic bond at the 3′ component of the Dewar valence isomer was more alkali-labile than that of the (6–4) photoproduct.

Introduction

DNA is subjected to various reactions with endogenous and exogenous factors in cells, and the alteration of its chemical structure, referred to as DNA damage, may induce carcinogenesis or cell death unless cellular repair systems properly function.[1] Ultraviolet (UV) light is one of the factors that induce DNA damage, and produces two major types of lesions, the cyclobutane pyrimidine dimer (CPD) and the pyrimidine(6–4) pyrimidone photoproduct ((6–4) photoproduct), between two adjacent pyrimidine bases.[2] While a cyclobutane ring is formed between the two C5–C6 double bonds by the [2 + 2] cycloaddition in the CPD case, the (6–4) photoproduct (**1**) has a covalent bond between the C6 and C4 positions of the 5′ and 3′ components, respectively, and the oxygen/nitrogen atom at the C4 of the 3′ thymine/cytosine is transferred to the C5 of the 5′ base.[3–5] The first reaction in the formation of the (6–4) photoproduct is the same photocycloaddition as that for the CPD, but it occurs between the C5–C6 double bond and the C4 carbonyl/imino group of the 5′ and 3′ pyrimidine bases, respectively. This reaction produces an oxetane/azetidine intermediate, and then the C4–O/C4–N bond in this intermediate is cleaved to yield a stable structure (**1**).[6]

The (6–4) photoproduct is reportedly more mutagenic than the CPD.[7–11] A remarkable feature is that a T → C or C → T transition occurs with high frequency at the 3′ component of the (6–4) photoproduct. Moreover, the (6–4) photoproduct blocks replication by translesion DNA polymerases that can bypass a CPD.[12,13] This is probably due to the alterations in the chemical structure and the orientation of the 3′ base. The intramolecular hydrogen bond suggested in our previous study[14] may also exert some influence. To maintain genetic integrity, the (6–4) photoproduct must be repaired by the nucleotide excision repair pathway in human cells,[15,16] while some other organisms have an enzyme, (6–4) photolyase, that directly converts this photoproduct to the original pyrimidine bases, using blue light energy.[17]

From the viewpoint of chemistry, the formation of the (6–4) photoproduct, its isomerization to the Dewar valence isomer by exposure to longer wavelength UV light, and the light-dependent repair by (6–4) photolyase have been studied and discussed in detail.[17–20] On the other hand, the chemical reactions of the (6–4) photoproduct have not been investigated. UV irradiation of DNA produces alkali-labile sites,[21] and the product formed at

[a]*Division of Chemistry, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka560-8531, Japan. E-mail: iwai@chem.es.osaka-u.ac.jp*
[b]*Fundamental Technology Labs., Institute for Innovation, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa210-8681, Japan*

†Electronic supplementary information (ESI) available: Synthetic procedures and characterization data. See DOI: 10.1039/c2ob06966k

‡Present address: National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

these sites was identified as the (6–4) photoproduct.[22] In other words, the (6–4) photoproduct undergoes a reaction with a hydroxide ion, which leads to a strand break at this site. This property has been used to detect the formation of this type of lesion in DNA.[23–27] Terminally-labeled DNA strands were irradiated with UV light and treated with hot piperidine. The products were separated by polyacrylamide gel electrophoresis under denaturing conditions, and bands were detected at pyrimidine–pyrimidine sites. This method revealed that the frequency of (6–4) photoproduct formation was higher at TC and CC sites,[21] although this lesion can be formed at all four types of dipyrimidine sites.[22] This alkali treatment is a well established method, but very few studies on its reaction mechanism have been reported. We previously found that the first reaction was the hydrolysis of the N3–C4 bond of the 5′ component, by HPLC and NMR analyses of alkali-treated short oligonucleotides containing the (6–4) photoproduct formed at TT.[28] The biochemical properties of this hydrolyzed photoproduct were also analyzed.[28] Since the ring opening at the 5′ pyrimidine site did not seem to cause a strand break, we analyzed the following reactions occurring at a higher hydroxide concentration to reveal the mechanism of the strand break. The hydrolysis of the glycosidic bond of a nucleoside bearing 5-methyl-2-pyrimidinone, which was used as an analog of the 3′ component of the (6–4) photoproduct, was also analyzed, and its mechanism was elucidated.

Results

Alkali treatment of the (6–4) photoproduct (1)

We previously used a tetramer containing the (6–4) photoproduct to detect an intermediate in the alkali degradation reactions, and then analyzed its structure by NMR spectroscopy, using a dinucleoside monophosphate in which the base moiety was the (6–4) photoproduct. In the present study, we started with the same dinucleoside monophosphate (**1**) shown in Fig. 1, to facilitate the analysis of the chemical structure of the product. The starting material (**1**) was prepared by deprotection, with ammonium hydroxide, of the compound obtained in the course of the synthesis of the oligonucleotide building block of the (6–4) photoproduct.[29] This dinucleoside monophosphate was treated with 1 M NaOH at 60 °C, and the reaction mixture was analyzed by reversed-phase HPLC (Fig. 2). Two products (peaks ii and iii in Fig. 2b) were detected, while the same treatment of thymidylyl(3′–5′)thymidine did not cause any reaction. Since peak ii diminished over time, this was presumed to be the intermediate (**2**) found in our previous study.[28] The final product (peak iii) was prepared and purified on a larger scale, and its structure was analyzed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy, including measurements of COSY, NOESY, HMQC, and HMBC spectra, as shown in the ESI.† We discovered a missing set of signals that should be assigned to one of the two sugars, and the NOESY spectra (Fig. S3†) indicated that the lost sugar moiety belonged to the 3′-side nucleoside. The ^{31}P spectra revealed that the product contained a phosphate group, and the result that the coupling pattern of the H3′ was changed by the ^{31}P decoupling (Fig. S7†) indicated the position of this phosphate. These results strongly suggested that the reaction following the intermediate formation was the hydrolysis of the glycosidic bond at the 3′ component of the (6–4) photoproduct. This hydrolysis produces an abasic site, which leads to a strand break by the β-elimination reaction under alkaline conditions when the (6–4) photoproduct resides in DNA.

Subsequently, the product yielding peak iii was analyzed by mass spectrometry (Fig. S8†). An *m*/*z* value of 467.19, which corresponded to $[M + H]^{+}$ of the sugar-lost form, was obtained for **3** by nano-electrospray ionization mass spectrometry, as expected. Another strong signal was detected at the *m*/*z* value of 450.17, and this signal was observed exclusively when fast atom bombardment was used for ionization. These results suggested that at the ionization step in the mass analysis, a 1,3-oxazine-2,6-dione derivative was formed at the 5′ base by a cyclization reaction, accompanied with ammonia release. The identity of the chemical structure of the 5′ base between **2** and the obtained product (**3**) is supported by the ^{13}C-NMR data shown in Table 1 in the ESI.†

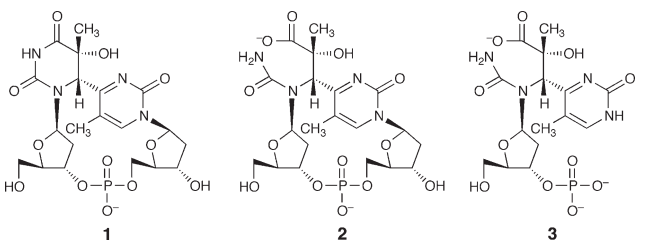


Fig. 1 Structures of the (6–4) photoproduct (**1**), the intermediate found in our previous study (**2**), and the product obtained in the present study (**3**).

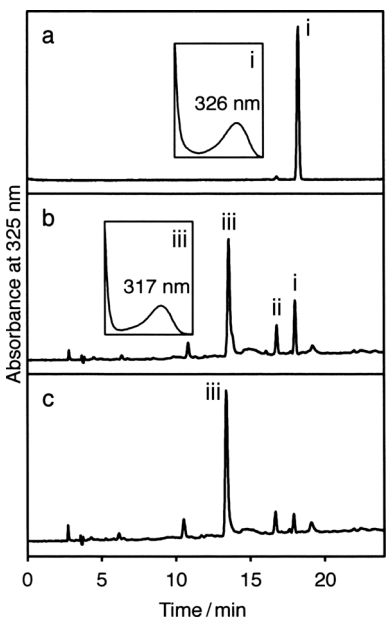


Fig. 2 HPLC analysis of the alkali treatment of the (6–4) photoproduct. Compound **1** was treated with 1 M NaOH at 60 °C for 0 h (a), 6 h (b), or 8 h (c). The insets are UV absorption spectra (220–365 nm) of the major peaks, and the absorption maxima are shown.

Alkali treatment of a dinucleoside monophosphate containing 5-methyl-2-pyrimidinone (4)

In order to confirm the hydrolysis of the glycosidic bond at the 3′ component of the (6–4) photoproduct and the subsequent loss of the sugar moiety at this site, we prepared a dinucleoside monophosphate containing 5-methyl-2-pyrimidinone[30] as the 3′-side base (**4** in Fig. 3), as shown in Scheme 1 in the ESI.† This base analog resembles the 3′ component of the (6–4) photoproduct, but has no linkage between the two bases. After the treatment of this compound with 1 M NaOH at 60 °C for 6 h, two products (peaks v and vi) were detected by HPLC (Fig. 4). One of the products (peak vi) was identified with thymidine 3′-phosphate (**5**) by a coinjection experiment (Fig. 4c), as expected. The other product, which yielded peak v, was isolated. This product did not have a sugar, and the H4 and the H6 were found to be equivalent in the NMR spectrum. Although a similar spectrum was expected for 5-methyl-2-pyrimidinone (**6**),[31] the UV absorption spectra (Fig. 4b) strongly suggested that the product was 2-hydroxy-5-methylpyrimidine (**7**), which is a tautomer of **6**. This product (**7**) was converted to **6** by acid treatment, as shown in Fig. S11 in the ESI.†

Alkali treatment of a trimer containing 5-methyl-2-pyrimidinone (8)

The mechanism of the unexpected loss of the sugar moiety from **1** and **4** is described in the Discussion. On the other hand, the hydrolysis of a glycosidic bond in DNA causes a strand break under alkaline conditions by the β-elimination reaction at the resulting abasic site. This reaction was demonstrated with a trimer containing 5-methyl-2-pyrimidinone (**8**), which was prepared with a phosphoramidite building block.[30] Alkali treatment of this compound yielded three peaks (peaks viii, ix, and x), as shown in Fig. 5, and the two products yielding peaks viii and x were coeluted with authentic **7** and **5**, respectively. The other product that yielded peak ix was coeluted with 2′-deoxycytidine 5′-phosphate (**9**), as expected. The small peak with a retention time of 11.3 min was 2′-deoxyuridine 5′-phosphate, which was derived from **9** by deamination.

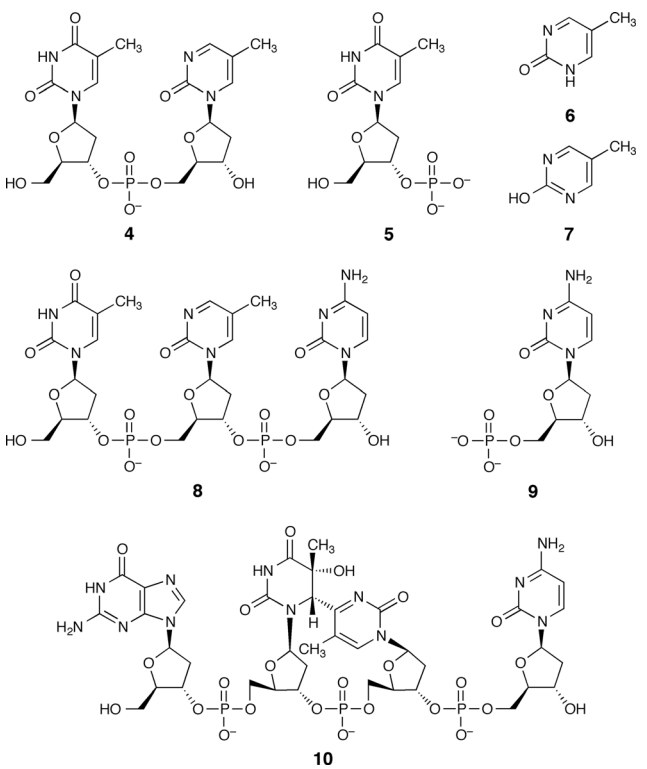


Fig. 3 Structures of the compounds used in this study (**4**, **8**, and **10**) and the products obtained by alkali treatment (**5**, **6**, **7**, and **9**).

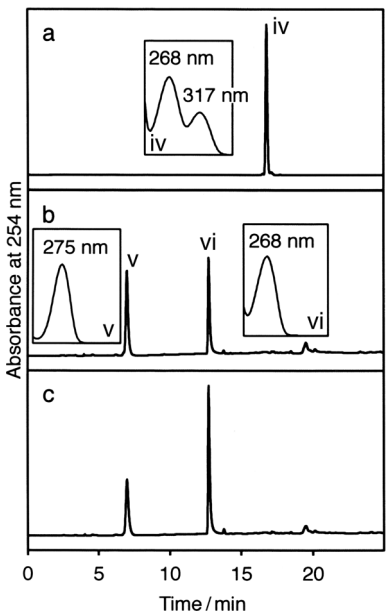


Fig. 4 HPLC analysis of the alkali treatment of a dimer containing 5-methyl-2-pyrimidinone. Compound **4** was treated with 1 M NaOH at 60 °C for 0 h (a) or 6 h (b and c). In panel c, thymidine 3′-phosphate was coinjected. The insets are UV absorption spectra (220–365 nm) of the major peaks, and the absorption maxima are shown.

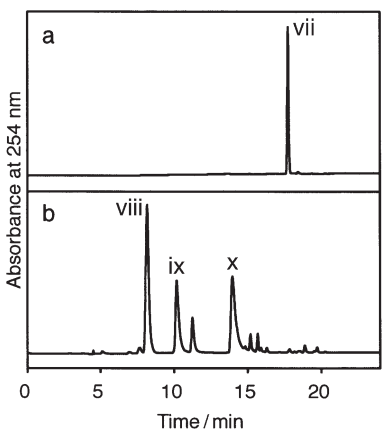


Fig. 5 HPLC analysis of the alkali treatment of a trimer containing 5-methyl-2-pyrimidinone. Compound **8** was treated with 1 M NaOH at 60 °C for 0 h (a) or 6 h (b).

Alkali treatment of a tetramer containing the (6–4) photoproduct (10)

In order to confirm that the nucleotide 3′-flanking to the (6–4) photoproduct is similarly released as a nucleoside 5′-phosphate upon cleavage of the glycosidic bond of the 3′ component in this photoproduct, a tetramer containing the (6–4) photoproduct (**10**), which was prepared by using the oligonucleotide building block,[29] was treated with alkali. In this case, a lower concentration (0.1 M) of NaOH was used to confirm the first reaction at the 5′ base to form **2**, as observed in our previous study.[28] After incubation at 60 °C for 4 h, the tetramer was completely converted to this type of intermediate, as shown in Fig. 6b. Peaks xi and xii were separately eluted in a coinjection experiment (data not shown). As shown in Fig. 6c, this intermediate was primarily converted into two products, which yielded peaks xiii and xiv, after prolonged incubation. A coinjection experiment revealed that the product that yielded peak xiii was 2′-deoxycytidine 5′-phosphate (**9**). We confirmed that this type of strand break occurred by the treatment with 0.1 M KOH at 90 °C for 30 min, which was used to test the alkaline lability of UV-irradiated DNA,[22] but the amount of the released nucleotide was small (Fig. S14†).

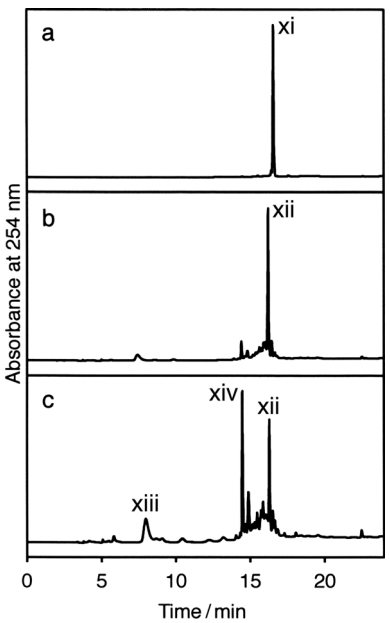


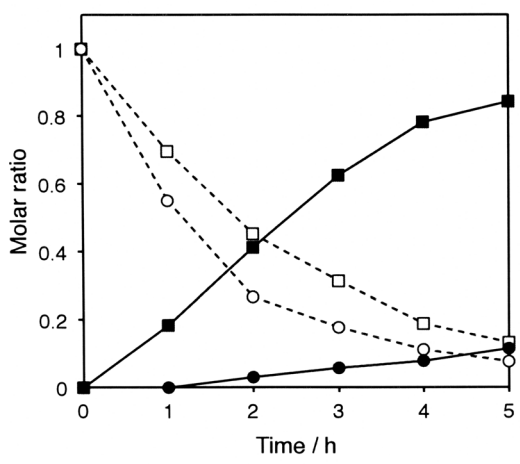
Fig. 6 HPLC analysis of the alkali treatment of a tetramer containing the (6–4) photoproduct. Compound **10** was treated with 0.1 M NaOH at 60 °C for 0 h (a), 4 h (b), or 8 h (c).

Comparison between the (6–4) photoproduct and its Dewar valence isomer

The (6–4) photoproduct is isomerized to its Dewar valence isomer by exposure to the UVA/B light,[32] and this isomer is reportedly more labile than the (6–4) photoproduct under alkaline conditions.[33] We confirmed this property by analyzing the release of the 3′-flanking nucleotide. Another tetramer containing the (6–4) photoproduct (**11**) was prepared by UV irradiation of d(ATTG),[34] and it was converted to the Dewar isomer-containing tetramer (**12**) by exposure to the Pyrex-filtered light.[35] Each tetramer was treated with 0.1 M NaOH at 60 °C, and the products were analyzed by HPLC at 1-hour intervals. In both cases, 2′-deoxyguanosine 5′-phosphate was detected, and as shown in Fig. 7, its release from **12** was much faster than that from **11**. In the case of the (6–4) photoproduct, the intermediate in which the N3–C4 bond of the 5′ component was hydrolyzed was accumulated in the same way as shown in Fig. 6b. These results indicate that the isomerization to the Dewar isomer renders the glycosidic bond at the 3′ component more labile.

Discussion

In this study, we investigated the mechanism of the strand breaks at the (6–4) photoproduct sites in UV-irradiated DNA, caused by the hot alkali treatment. In our previous study, we found that the



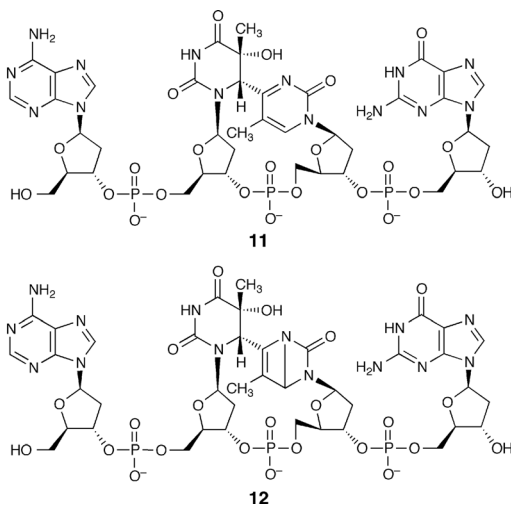


Fig. 7 Comparison of the strand breaks at the (6–4) photoproduct and its Dewar valence isomer. The starting materials **11** and **12** (open circles and open squares, respectively) and 2′-deoxyguanosine 5′-phosphate released from **11** and **12** (filled circles and filled squares, respectively) after the treatment with 0.1 M NaOH at 60 °C are shown as molar ratios.

first reaction in the alkali treatment of the (6–4) photoproduct was the hydrolysis of the N3–C4 bond of the 5′ component, and characterized the biochemical properties of this hydrolyzed photoproduct.[28] However, this reaction did not seem to lead directly to the strand break. In the present study, the analysis of the products obtained by treatment with a higher concentration of NaOH revealed that the glycosidic bond was hydrolyzed at the 3′ component of the (6–4) photoproduct in a dinucleoside monophosphate. NMR spectroscopy and mass spectrometry analyses revealed that the 2-deoxyribose moiety at the resultant abasic site was lost by this alkali treatment, which resulted in the formation of **3** as a product. In DNA, β-elimination to cleave the internucleotide linkage occurs under alkaline conditions at the abasic site formed by hydrolysis of the glycosidic bond, but the loss of the sugar moiety was not expected for **1**, because there was no 3′-flanking nucleotide to function as a leaving group. When another dinucleoside monophosphate (**4**) containing 5-methyl-2-pyrimidinone, an analog of the 3′ component of the (6–4) photoproduct, was treated in the same way, the 3′-side sugar was lost again. We propose a reaction mechanism for the removal of the 3′-end sugar moiety, as shown in Fig. 8. To our knowledge, this reaction has not been reported previously, because the properties of the abasic site have been studied within the context of oligonucleotides. We used dinucleoside monophosphates to study the chemical properties of the damaged base, but the biological significance of the reaction that occurs only at the 3′ end, without the 3′-phosphate, may be limited. Therefore, we analyzed the reactions using the trimer (**8**) and the tetramer (**10**) to demonstrate the strand break by β-elimination.

The most important finding in this study was that the glycosidic bond at the 3′ component of the (6–4) photoproduct was hydrolyzed by the hot alkali treatment, because this hydrolysis causes a strand break at this site by the β-elimination reaction, as shown in Fig. 9. It was shown that the strand break by the same mechanism occurred in a tetramer containing the Dewar valence isomer of this photoproduct at a reaction rate higher than that obtained for the (6–4) precursor (Fig. 7). This observation agrees with the previous reports showing higher alkaline lability of the Dewar isomer.[33,36,37] When we tested the conditions of 0.1 M KOH at 90 °C for 30 min, which were used to detect the (6–4) photoproduct sites in the past,[22] the strand break was not completed (Fig. S14†). These results strongly suggest that the strand

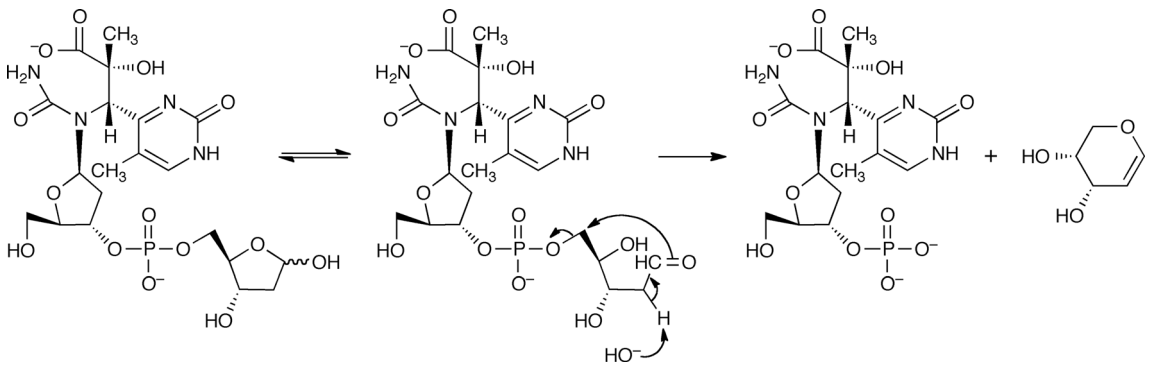


Fig. 8 Proposed mechanism for the loss of the 3′ sugar after hydrolysis of the glycosidic bond.

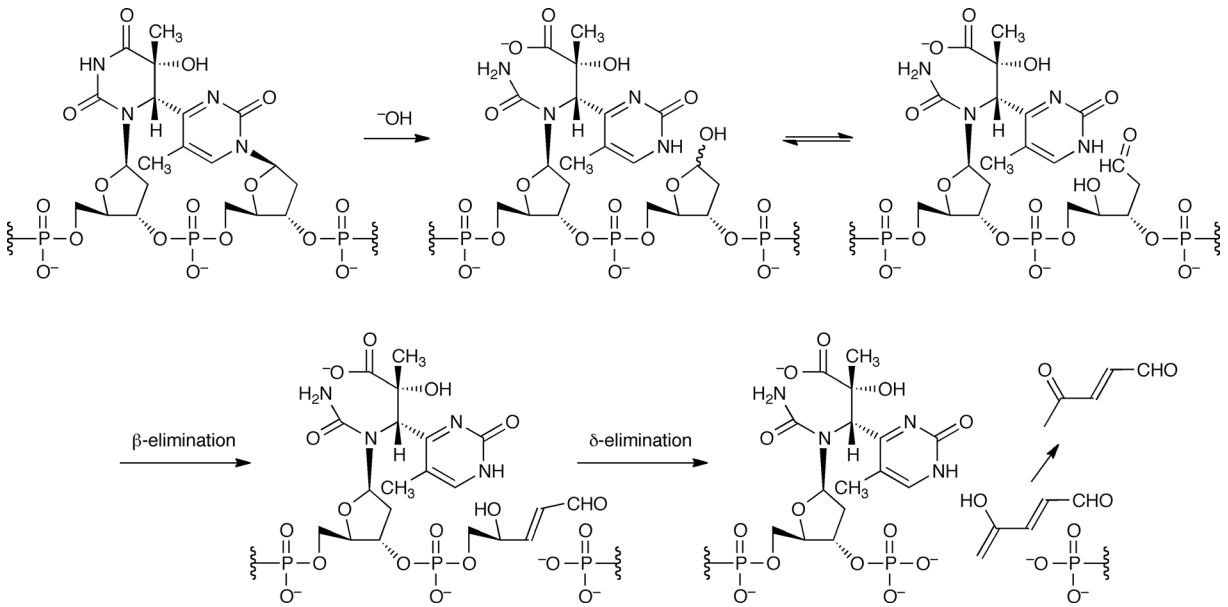


Fig. 9 Mechanism of alkali degradation at the (6–4) photoproduct site.

breaks detected under such conditions were caused mainly at the sites of the Dewar photoproduct. However, it should be noted that the (6–4) photoproduct is not intact after this treatment because the hydrolytic ring opening of its 5′ component[28] occurs even at the lower alkali concentration, as shown in Fig. S14.†.

We used 5-methyl-2-pyrimidinone as a model compound for the 3′ component of the (6–4) photoproduct. When this base analog is incorporated into oligonucleotides, its glycosidic bond is reportedly labile under both acidic[38] and alkaline[39] conditions, although the mechanism has not been investigated. In our present study, 2-hydroxy-5-methylpyrimidine (**7**), a tautomer of 5-methyl-2-pyrimidinone (**6**), was obtained by the hot alkali treatment. The tautomerism between 2-hydroxypyrimidine and 2-pyrimidinone has been studied mainly by theoretical methods.[40–42] The two tautomers can be distinguished easily by UV-absorption, whereas two singlet signals are observed for both of the tautomers by ^{1}H-NMR spectroscopy, due to the symmetry of these molecules. In this study, the enol form, with a UV absorption maximum at a shorter wavelength, was obtained as an isolated product (**7**), and was also detected immediately after the reaction (peak v in Fig. 4b). In our previous study, acid hydrolysis of 1-(2-deoxy-β-D-ribofuranosyl)-5-methyl-2-pyrimidinone yielded **6**, which had an absorption maximum at 311 nm.[14] These results indicate that the acid and alkali hydrolyses of the glycosidic bond of this modified base exclusively produce the keto and enol forms, respectively. This observation corresponds to each reaction mechanism. In an acidic solution, protonation occurs at N3,[14] which induces glycosidic bond cleavage in an S_N1 reaction with a water molecule at the C1′ position. By contrast, the attack of ammonia at C6, leading to saturation of the C5–C6 bond, was proposed as the mechanism of glycosidic bond cleavage during the deprotection of synthetic oligonucleotides containing this base analog,[39] and in our study, hydration products were detected by LC-MS after the hot alkali treatment, as shown in Fig. S12 in the ESI.† Since the resonance stabilization of the base moiety is lost, due to the saturation of the C5–C6 bond, an S_N2 reaction with a hydroxide ion occurs at C1′. In this case, the enol form is produced by the displacement of the electron pair from the double bond in the C2 carbonyl group to the oxygen atom, and the aromatic pyrimidine ring is reproduced by the following dehydration (Fig. S13†). Although the mechanism could be the same, there was a difference in the product obtained after the glycosidic bond hydrolysis between 5-methyl-2-pyrimidinone and the (6–4) photoproduct. The product obtained from the compound containing the (6–4) photoproduct had an absorption maximum at 317 nm, as shown in Fig. 2b, which indicated that the structure of the 3′ base released from the sugar moiety was the keto form (Fig. 9). The 5′ base linked at the C4 position may have some influence on the tautomerism of the 3′ component.

Conclusion

We have elucidated the mechanism for the strand breaks caused at (6–4) photoproduct sites in UV-irradiated DNA by the hot alkali treatment. Independently of the ring opening at the 5′ component, which we reported previously, the glycosidic bond is cleaved at the 3′ component, and strand breakage by the β-elimination reaction occurs at the resultant abasic site. The mechanism for the alkali hydrolysis of the glycosidic bond of 1-(2-deoxy-β-D-ribofuranosyl)-5-methyl-2-pyrimidinone, which resembles the 3′ component of the (6–4) photoproduct, was also elucidated. The chemical basis of the alkali lability of the (6–4) photoproduct-containing DNA obtained in this study will contribute to molecular and cellular biology. For example, the (6–4) photoproduct in cellular DNA may be degraded artificially, by targeting the glycosidic bond of the 3′ component.

Experimental

Materials

All solvents and reagents were obtained from Wako Pure Chemical Industries, except for 2′-deoxycytidine 5′-phosphate, which was obtained from Sigma. Reagents for the DNA synthesizer (Applied Biosystems 3400) were purchased from Glen Research. For column chromatography, Wakogel C-200 (Wako Pure Chemical Industries) was used. Cation exchange was performed using AG 50W-X2 resin (Bio-Rad Laboratories).

HPLC analysis and purification

HPLC analysis was performed on a Gilson gradient-type analytical system equipped with a Waters 2996 photodiode array detector. HPLC purification was performed on the same system equipped with a Gilson 151 UV/Vis detector. An Inertsil ODS-3 5 μm column (4.6 × 250 mm, GL Sciences) was used at a flow rate of 1.0 ml min^{-1} for analysis, and a μ-Bondasphere C18 15 μm 300A column (300 × 7.8 mm, Waters) was used at a flow rate of 2.0 ml min^{-1} for purification. A linear gradient of acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) was used for both analysis and purification.

NMR spectroscopy

^{1}H NMR spectra were measured at 30 °C on a Varian Unity-INOVA 500 or JEOL JNM-AL400 spectrometer. The ^{1}H chemical shift was calibrated with internal TMS (0 ppm) in deuterated organic solvents or with HDO (4.70 ppm) in D_2O. ^{13}C and ^{31}P NMR spectra were measured on a Varian Unity-INOVA 500 spectrometer. In the ^{13}C measurement in D_2O, the ^{13}C reference frequency was obtained by calculation from the ^{1}H reference frequency, as reported previously.[43] Briefly, after adjustment of the ^{1}H chemical shift, the resonance frequency at 0 ppm for ^{1}H was converted to that for ^{13}C by multiplying by the frequency ratio of ^{13}C/^{1}H. The ^{31}P chemical shift was calibrated with external trimethyl phosphate. Two-dimensional NMR spectra were recorded on a 5 mm pulse field gradient probe for indirect detection on the Varian Unity-INOVA 500 spectrometer. For the NOESY measurement, the mixing time was set to 700 ms.

Mass spectrometry

Mass spectra were acquired on a JEOL JMS-700 mass spectrometer. The LC-MS analysis of the alkali-treated sample of 1-(2-deoxy-β-D-ribofuranosyl)-5-methyl-2-pyrimidinone (**11** in

ESI†) was performed with an Agilent 1200SL HPLC system equipped with a Micromass Q-Tof Premier mass spectrometer. The electrospray interface was operated in the ESI negative ion mode. The capillary voltage was maintained at 2.5 kV, and the voltages of the sample cone and the collision were set to 20 V and 4.0 V, respectively. The elution conditions were the same as described above.

Alkali treatment of the (6–4) photoproduct (1)

Compound **1** (128 μmol) was dissolved in 1 M NaOH (1.0 ml) and incubated at 60 °C. After neutralization with 1 M HCl, the mixture was analyzed by reversed-phase HPLC using a linear gradient of 0–10% acetonitrile. The product obtained by an 8 h alkali treatment of **1** was purified by reversed-phase HPLC with a linear gradient of 0–5% acetonitrile during 20 min. The cation was then exchanged for Na^+. The structure of this product was analyzed by NMR spectroscopy and mass spectrometry. ^{1}H NMR (500 MHz, D_2O): δ (ppm) 7.99 (s, 1H, pT-H6), 5.85 (dd, J = 9.9, 5.1 Hz, 1H, H1′), 5.51 (s, 1H, TpH6), 4.42 (m, 1H, H3′), 4.04 (m, 1H, H4′), 3.65 (m, 2H, H5′), 2.23 (s, 3H, pT-Me), 1.98 (m, 1H, H2′), 1.50 (m, 1H, H2′), 1.24 (s, 3H, Tp-Me). ^{13}C NMR (125 MHz, D_2O): δ (ppm) 179.9 (TpC4), 174.8 (pTC4), 161.8 (pTC2), 161.5 (TpC2), 152.7 (pTC6), 117.3 (pTC5), 87.8 (C4′), 87.0 (TpC5), 86.7 (C1′), 77.1 (C3′), 65.0 (C5′), 61.9 (TpC6), 39.4 (C2′), 21.1 (TpMe), 15.9 (pTMe). ^{31}P NMR (203 MHz, D_2O): δ (ppm) 4.06. ESI-LRMS m/z: 467.19 ($[M + H]^+$ calcd. for $C_{15}H_{24}N_4O_{11}P$: 467.12).

Alkali treatment of a dinucleoside monophosphate containing 5-methyl-2-pyrimidinone (4)

Compound **4** (1 μmol) was dissolved in 1 M NaOH (1.0 ml) and incubated at 60 °C. After neutralization with 1 M HCl, the mixture was analyzed by reversed-phase HPLC, using a linear gradient of 0–25% acetonitrile. Thymidine 3′-phosphate, which was synthesized on a DNA synthesizer using 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidite and 3′-phosphate CPG (Glen Research), was then coinjected into an HPLC column along with the sample treated with hot alkali for 6 h. The base moiety released by the alkali treatment was purified by reversed-phase HPLC with a linear gradient of 0–10% acetonitrile during 20 min. After the cation exchange for Na^+, the structure of this product was analyzed by NMR spectroscopy. ^{1}H NMR (400 MHz, D_2O): δ (ppm) 8.39 (s, 2H, H4 and H5), 1.51(s, 3H, Me).

Alkali treatment of a trimer containing 5-methyl-2-pyrimidinone (8)

Compound **8** (500 nmol) was dissolved in 1 M NaOH (1.0 ml) and incubated at 60 °C. After neutralization with 1 M HCl, the mixture was analyzed by reversed-phase HPLC, using a linear gradient of 0–25% acetonitrile. Thymidine 3′-phosphate and 2′-deoxycytidine 5′-phosphate were then coinjected into an HPLC column along with the sample treated with hot alkali for 6 h.

Alkali treatment of a tetramer containing the Dewar valence isomer of the (6–4) photoproduct (12)

Compound **12** (20 nmol) and thymine (20 nmol) were dissolved in 0.1 M NaOH (100 μl), and the mixture was incubated at 60 °C. At 1-hour intervals, 10 μl of the solution was neutralized with 0.1 M HCl, and was analyzed by reversed-phase HPLC, using a linear gradient 0–25% acetonitrile. The release of 2′-deoxyguanosine 5′-phosphate was confirmed by a coinjection experiment. For comparison, the alkali degradation of **11** was analyzed in the same way. From the peak areas, molar ratios were calculated using thymine as an internal standard.

References

1 G. C. Walker, W. Siede, R. A. Schultz, T. Ellenberger and E. C. Friedberg, in *DNA Repair and Mutagenesis 2nd Edn*, ASM Press, Washington D. C., 2006.
2 S. Iwai, in *Modified Nucleosides in Biochemistry, Biotechnology and Medicine*, ed. P. Herdewijn, Wiley-VCH, Weinheim, 2008, pp. 97–131.
3 S. Y. Wang and A. J. Varghese, *Biochem. Biophys. Res. Commun.*, 1967, **29**, 543–549.
4 A. J. Varghese and S. Y. Wang, *Science*, 1968, **160**, 186–187.
5 I. L. Karle, S. Y. Wang and A. J. Varghese, *Science*, 1969, **164**, 183–184.
6 R. O. Rahn and J. L. Hosszu, *Photochem. Photobiol.*, 1969, **10**, 131–137.
7 J. E. LeClerc, A. Borden and C. W. Lawrence, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 9685–9689.
8 C. A. Smith, M. Wang, N. Jiang, L. Che, X. Zhao and J.-S. Taylor, *Biochemistry*, 1996, **35**, 4146–4154.
9 A. Gentil, F. Le Page, A. Margot, C. W. Lawrence, A. Borden and A. Sarasin, *Nucleic Acids Res.*, 1996, **24**, 1837–1840.
10 H. Kamiya, S. Iwai and H. Kasai, *Nucleic Acids Res.*, 1998, **26**, 2611–2617.
11 M. J. Horsfall and C. W. Lawrence, *J. Mol. Biol.*, 1994, **235**, 465–471.
12 C. Masutani, M. Araki, A. Yamada, R. Kusumoto, T. Nogimori, T. Maekawa, S. Iwai and F. Hanaoka, *EMBO J.*, 1999, **18**, 3491–3501.
13 C. Masutani, R. Kusumoto, S. Iwai and F. Hanaoka, *EMBO J.*, 2000, **19**, 3100–3109.
14 J. Yamamoto, Y. Tanaka and S. Iwai, *Org. Biomol. Chem.*, 2009, **7**, 161–166.
15 K. Sugasawa, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 2010, **685**, 29–37.
16 T. Nouspikel, *Cell. Mol. Life Sci.*, 2009, **66**, 994–1009.
17 J. Li, Z. Liu, C. Tan, X. Guo, L. Wang, A. Sancar and D. Zhong, *Nature*, 2010, **466**, 887–890.
18 Z. B. Yang, R. B. Zhang and L. A. Eriksson, *Phys. Chem. Chem. Phys.*, 2011, **13**, 8961–8966.
19 Y.-J. Ai, R.-Z. Liao, S.-F. Chen, Y. Luo and W.-H. Fang, *J. Phys. Chem. B*, 2010, **114**, 14096–14102.
20 K. Sadeghian, M. Bocola, T. Merz and M. Schütz, *J. Am. Chem. Soc.*, 2010, **132**, 16285–16295.
21 J. A. Lippke, L. K. Gordon, D. E. Brash and W. A. Haseltine, *Proc. Natl. Acad. Sci. U. S. A.*, 1981, **78**, 3388–3392.
22 W. A. Franklin, K. M. Lo and W. A. Haseltine, *J. Biol. Chem.*, 1982, **257**, 13535–13543.
23 D. E. Brash and W. A. Haseltine, *Nature*, 1982, **298**, 189–192.
24 F. Bourre, G. Renault and A. Sarasin, *Nucleic Acids Res.*, 1987, **15**, 8861–8875.
25 G. P. Pfeifer, R. Drouin, A. D. Riggs and G. P. Holmquist, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 1374–1378.
26 J. Jen, D. L. Mitchell, R. P. Cunningham, C. A. Smith, J.-S. Taylor and J. E. Cleaver, *Photochem. Photobiol.*, 1997, **65**, 323–329.
27 J.-H. Yoon, C.-S. Lee, T. R. O'Connor, A. Yasui and G. P. Pfeifer, *J. Mol. Biol.*, 2000, **299**, 681–693.
28 M. Higurashi, T. Ohtsuki, A. Inase, R. Kusumoto, C. Masutani, F. Hanaoka and S. Iwai, *J. Biol. Chem.*, 2003, **278**, 51968–51973.
29 S. Iwai, M. Shimizu, H. Kamiya and E. Ohtsuka, *J. Am. Chem. Soc.*, 1996, **118**, 7642–7643.
30 S. F. Singleton, F. Shan, M. W. Kanan, C. M. McIntosh, C. J. Stearman, J. S. Helm and K. J. Webb, *Org. Lett.*, 2001, **3**, 3919–3922.

31 S. Buchini and C. J. Leumann, *Eur. J. Org. Chem.*, 2006, **2006**, 3152–3168.
32 J.-S. Taylor and M. P. Cohrs, *J. Am. Chem. Soc.*, 1987, **109**, 2834–2835.
33 L.-S. Kan, L. Voituriez and J. Cadet, *J. Photochem. Photobiol., B*, 1992, **12**, 339–357.
34 C. A. Smith and J.-S. Taylor, *J. Biol. Chem.*, 1993, **268**, 11143–11151.
35 J.-S. Taylor, D. S. Garrett and M. P. Cohrs, *Biochemistry*, 1988, **27**, 7206–7215.
36 D. L. Mitchell, *Mutat. Res. DNA Repair*, 1988, **194**, 227–237.
37 T. Douki, L. Voituriez and J. Cadet, *Photochem. Photobiol.*, 1991, **53**, 293–297.
38 J. A. Iocono, B. Gildea and L. W. McLaughlin, *Tetrahedron Lett.*, 1990, **31**, 175–178.
39 B. A. Connolly and P. C. Newman, *Nucleic Acids Res.*, 1989, **17**, 4957–4974.
40 C. Ögretir and M. Yaman, *THEOCHEM*, 1999, **458**, 217–226.
41 C. W. Bird, *Tetrahedron*, 1992, **48**, 7857–7862.
42 A. Les and L. Adamowicz, *J. Phys. Chem.*, 1990, **94**, 7021–7032.
43 D. S. Wishart, C. G. Bigam, J. Yao, F. Abildgaard, H. J. Dyson, E. Oldfield, J. L. Markley and B. D. Sykes, *J. Biomol. NMR*, 1995, **6**, 135–140.