Nucleic Acids Research, 1998, Vol. 26, No. 15 **3599–3607**

Neighboring base damage induced by permanganate oxidation of 8-oxoguanine in DNA

Shiro Koizume, Hideo Inoue*, Hiroyuki Kamiya[1] and Eiko Ohtsuka

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan and [1]Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Received December 22, 1997; Revised March 16, 1998; Accepted June 16, 1998

ABSTRACT

We found that single-stranded DNA oligomers containing a 7,8-dihydro-8-oxoguanine (8-oxo-G) residue have high reactivity toward $KMnO_4$; the oxidation of 8-oxo-G induces damage to the neighboring nucleotide residues. This paper describes the novel reaction in detail, including experiments that demonstrate the mechanism involved in the induction of DNA damage. The results using DNAs of various base compositions indicated that damaged G, T and C (but not A) sites caused strand scissions after hot piperidine treatment and that the damage around the 8-oxo-G occurred at G sites in both single and double strands with high frequency. The latter substrates were less sensitive to damage. Further, kinetic studies of the $KMnO_4$ reaction of single-stranded oligomers suggested that the reactivity of the DNA bases at the site 5′-adjacent to the 8-oxo-G was in the order G > A > T, C. This preference correlates with the electron donating abilities of the bases. In addition, we found that the DNA damage at the G site, which was connected with the 8-oxo-G by a long abasic chain, was inhibited in the above order by the addition of dG, dA or dC. On the other hand, the damage reactions proceeded even after the addition of scavengers for active oxygen species. This study suggests the involvement of a redox process in the unique DNA damage initiated by the oxidation of the 8-oxo-G.

INTRODUCTION

One of the most common forms of oxidative damage to DNA *in vivo* is the generation of 7,8-dihydro-8-oxoguanine (8-oxo-G; also referred to as 8-hydroxyguanine, Fig. 1) from guanine. This damage may lead to mutations (1,2), which play a significant role in cancer (1) and aging (3), as well as some other human diseases (4,5). 8-Oxo-G is a better electron donor than any of the natural nucleosides (6), which allows its presence in DNA to be selectively detected with an electrochemical detector (7). The high reactivity of 8-oxo-G towards oxidation *in vitro* suggests that it may be also be sensitive to *in vivo* oxidation. Our investigation with permanganate was designed to explore some of the potential consequences of further oxidation. We found that an 8-oxo-G residue in DNA is more sensitive to oxidation by permanganate than thymine and the other common bases (8). The sensitivity of 8-oxo-G, as the nucleoside or in DNA, to singlet oxygen (9,10) or triplet-excited carbonyl (11,12) has been reported, but there have been no reports on the fate of the nucleotide residues next to or near the 8-oxo-G after oxidation of the modified base. Here we report full details of the damage to the bases of neighboring nucleotides that occurs when the single 8-oxo-G residue in single- or double-stranded oligodeoxynucleotides is oxidized with permanganate. Also, we describe several experiments, including the use of oligomers with abasic site analogue(s) or hexa(ethylene glycol) linker(s) (Fig. 1), to examine the nature of the damage around the 8-oxo-G. The data suggest that a redox (electron transfer) reaction participates in the induction of the DNA damage.

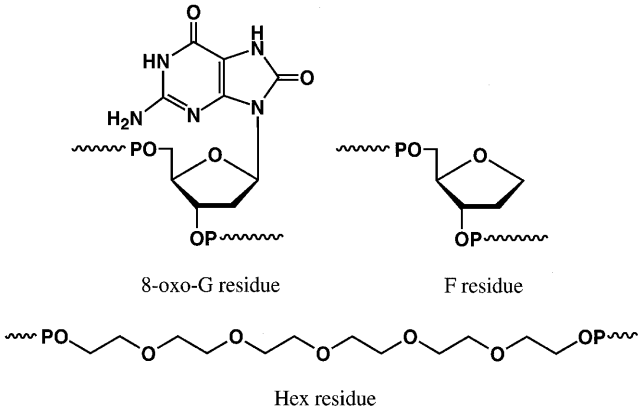

Figure 1. Modified residues in the DNA used in this study. **P** designates PO_3^-.

*To whom correspondence should be addressed. Tel: +81 11 706 3750; Fax: +81 11 706 4989; Email: inoue@pharm.hokudai.ac.jp

Table 1. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled single strands[a]

Compound	Sequence	Cleavage yield (%)							
1	5′GC**G**CC**GG**CGGTG3′	4.8	73(29)	(47)					
2	5′GCGCC**GG**C**G**GTG3′	53	32(80)	(4.8)					
3	5′GC**G**C**GGG**C**GG**TG3′	2.7	13	26	28(58)	(9.8)	(3.4)		
4	5′GC**G**CCA**GCGG**TG3′	7.9	63(57)	(8.0)	(10)	(4.1)			
5	5′GC**G**CC**TGCG**GTG3′	4.3	20	47(62)	(11)	(9.7)			
6	5′GCGCC**CG**CGGTG3′	15	54						
7	5′AT**G**AC**GG**AATAT3′	16	44	18(64)					
8	5′TT**TTCGTT**CCTT3′	15	18	6.6	28(56)	(2.7)	(0.4)		
9	5′TT**TTTTGTTTT**TT3′	2.0	9.0	7.7	18	20(39)	(5.5)	(2.5)	(2.4) (1.3)
3[b]	5′GC**G**C**GGG**CGGTG3′	2.7	13	26	32				
8[b]	5′TT**TTCG**TTCCTT3′	14	19	5.9	26				

[a]All reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiments with 3′-end-labeled oligomers. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left and the underlined percentages are for 8-oxo-G residues. Cleavage yields at the Ts of the 8-oxo-G-containing strands after the $KMnO_4$ oxidation were corrected by subtraction of the cleavage yields at the Ts of the control strands. Also, the cleavage yields (4–8%) at the 8-oxo-G site upon hot piperidine treatment alone (see Text) were subtracted from those for the 8-oxo-G-containing strands after the oxidation.
[b]The cleavage yields were obtained from the experiment with an oxygen-free buffered solution of 5′-end-labeled **3** or **7**.

RESULTS

Damage of single-stranded DNA detected by strand cleavage analysis

Various single-stranded oligodeoxynucleotides containing a single 8-oxo-G residue in the middle of a 12mer (13mer for **9**) were prepared (Table 1) according to the reported method (13). We first examined the susceptibility of the 8-oxo-G to permanganate oxidation using oligonucleotides **1** and **2** (5′-end-labeled), which correspond to a part of the human c-Ha-*ras* gene with a G-rich sequence and a modified codon 12. Since the results showed that the reaction occurred not only at the 8-oxo-G site but also, unexpectedly, at the 5′-upstream or 5′-next G position in the strands (Table 1), we next investigated the base- and region-specificities of this reaction. Strand **3** is an analog of **2** with the substitution of a cytosine base (C) 5′-adjacent to the G-(8-oxo-G) site with G. Strands **4**, **5** and **6** are also analogs of **2** and differ only by a single base [adenine (A), thymine (T) and C, respectively] 5′-adjacent to the 8-oxo-G. Strand **7** corresponds to the sequence of codons 1–4 of the c-Ha-*ras* gene, which is A, T-rich as compared with **1**–**6**. Strands **8** and **9** have pyrimidine sequences (T-tracts for **9**) on both sides of the 8-oxo-G.

Oligonucleotides **1**–**9** and their control strands with G instead of 8-oxo-G were 5′- or 3′-end-labeled with ^{32}P, treated with $KMnO_4$, and subjected to hot alkaline treatment followed by denaturing 20% polyacrylamide gel electrophoresis (PAGE). Autoradiograms of the reaction products from **3** and **8** are shown in Figure 2A and B. Lanes 3 and 12 show the piperidine-induced scission without oxidation at the 8-oxo-G position (14,15) of strands **3** and **8**, which can be compared with the results for their control strands (no scission, lanes 7 and 17). The minor cleavage at the 8-oxo-G by the direct piperidine treatment may be due to the decomposition of the 8-oxo-G by aerobic oxidation under basic conditions, because the cleavage was inhibited in the presence of 2-mercaptoethanol (15 and our independent result). Lanes 4, 13 and 14 represent the permanganate-induced damage at the 8-oxo-G and its neighboring G and T residues, which can be detected by piperidine-induced scission; no cleavage was observed without piperidine treatment (lanes 5 and 15). The damage of the control strands was found only as specific damage at T sites (lanes 8 and 19) (16,17). It should be noted that $KMnO_4$ is a classic reagent for the oxidation of double bonds in organic molecules and the primary products from the oxidation of pyrimidine bases are 5,6-dihydroxy-5,6-dihydro derivatives (18). The reactivity of the common bases in DNA toward permanganate oxidation (37°C and pH 9 for 19 h) occurs in the order T > C > G, and A is inert toward the oxidation (19). Figure 2C shows the time-course of the reaction of **3**, which indicates early induction and an increase in the damage of the neighboring nucleotide residues. Table 1 represents the damaged positions of **1**–**9**, as analyzed by the strand scission and the cleavage yields. According to the above observations, the cleavage yields at the 8-oxo-G and T positions were normalized.

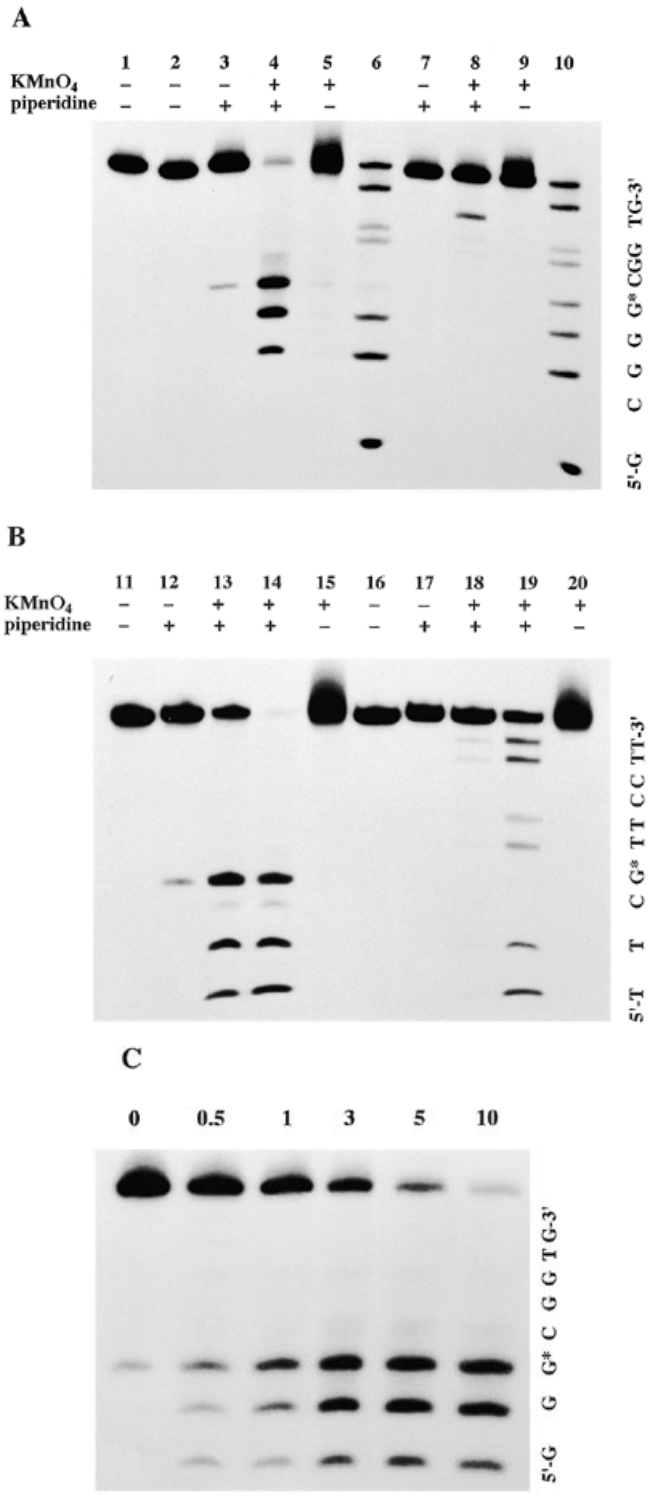

Figure 2. Autoradiograms of reaction products from 5′-end-labeled **3** (**A**) and **8** (**B**), and their control strands after denaturing 20% PAGE. The $KMnO_4$ oxidation of **3**, **8** and the control strands, followed by the strand cleavage analysis of the products, was performed under the conditions described in Materials and Methods. Lanes 1, 3–5: **3**; lanes 2, 7–9: the control strand for **3**; lanes 11–15: **8**; lanes 16–20: the control strand for **8**; lanes 6, 10: Maxam–Gilbert A+G lanes for **3** and the control strand, respectively. Lanes 13 and 18, and 14 and 19 have 23 μM and 120 μM permanganate concentrations, respectively. (**C**) A time course of the strand cleavage of **3** after oxidation and the following piperidine treatment. The numbers above each lane refer to the reaction time (min). G* designates G or 8-oxo-G.

The 8-oxo-G residue was highly reactive to the oxidation, as expected, and the other damage was observed near the 8-oxo-G (at both the 5′ and 3′ sides of the 8-oxo-G). The results indicated that the reactivity of the common base residues was apparently in the order G > T > C. For example, the G residue 5′-adjacent to the 8-oxo-G in strand **2** was cleaved efficiently (53% yield) after oxidation and piperidine treatment, whereas the corresponding T site in **5** and the corresponding C site in **6** gave cleavage yields of 20 and 15%, respectively. In addition, it appears that as the position of G (or T) moves further away from the 8-oxo-G, the cleavage at the G (or T) position becomes less efficient. The tendency can clearly be seen in the case of the T-dominant oligonucleotide **9**. On the other hand, a comparison between the data for **9** and its control strand with G instead of 8-oxo-G indicates that their patterns of strand cleavage were quite different and, in the latter case, the Ts of the middle part of the strand were rather insensitive to the oxidation (20): the intrinsic T cleavage (%) of a part of the 5′-end-labeled control strand (5′-TTT^{1}T^{2}T^{3}T^{4}GTTTTTT-3′): T^1, 12; T^2, 10; T^3, 8.3; T^4, 4.6. The results described here demonstrate that this novel damage is initiated by the oxidative modification of 8-oxo-G (also see the next section) and that it has base-selectivity.

Damage of single-stranded DNA detected by nucleoside composition analysis

Although the strand cleavage analysis showed the presence of damage to G, T and C, the damage to adenine residues could not be elucidated by this analysis. To test the possibility that the damaged A site is inert to piperidine-induced strand cleavage and that for other bases there might also be types of damage not leading to strand scission, the nucleoside composition of the oxidized oligomers was analyzed after the permanganate oxidation. The analysis was carried out by enzymic digestion of the oxidation products of **4**, **7**, **8** and their control strands with snake venom phosphodiesterase and alkaline phosphatase, followed by detection of the resulting nucleosides by reversed-phase HPLC. The analysis showed that the 8-oxo-2′-deoxyguanosine (8-oxo-dG) completely disappeared and that a large amount (~70%) of the 2′-deoxyadenosine (dA) also disappeared after the oxidation of **4** (Fig. 3), although dA was intact in the control experiments with the control strand. On the other hand, the reduction in the amounts of dG, dC and dT did not exceed the degree of their damage, as determined by the strand scission analyses of **4**, **7** and **8**. For example, 21% of the entire amount of dG was found to be damaged after the oxidation and this was comparable to the degree (30%) of damaged G elucidated by the strand cleavage analysis. In addition, new products detectable with a UV (254 nm) monitor were not observed for **4**, **7** and **8** under the same HPLC conditions. These results, as well as the strand cleavage analysis, indicated that the damage of the common nucleosides, except for the dA residues, in 8-oxo-G-containing single-stranded DNAs would exclusively result in strand cleavage by the piperidine treatment. Also, the data that the strand scissions of 5′-end-labeled oligomers were observed only at the 5′-side region of the 8-oxo-G (for the 3′-end-labeled, at the 3′-side region) (Table 1) mean that the 8-oxo-G residue was always damaged whenever the neighboring residues were damaged.

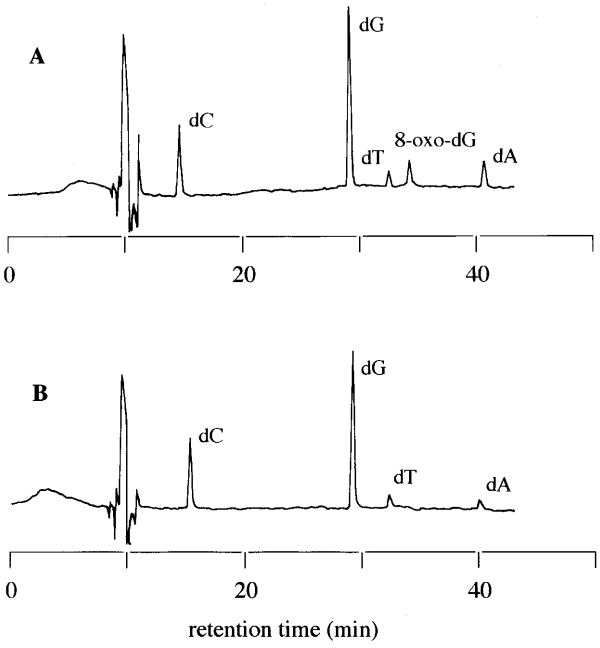

Figure 3. Reversed-phase HPLC chromatogram of the snake phosphodiesterase and alkaline phosphatase digests of single strand **4** before (**A**) and after (**B**) the oxidation. Each nucleoside component was separated under the conditions described in Materials and Methods.

Possible mechanisms for neighboring nucleotide damage

To investigate the mechanisms of this reaction, a mixture of a non-labeled strand (**2**, **7** or **8**) and an excess of its control 5′-end-labeled strand was treated with $KMnO_4$ and then with piperidine. The control strand was not cleaved at any of the G positions, and the resulting scissions at the T sites of the control strand were unchanged in their positions and frequencies, as compared with the scissions in the absence of the 8-oxo-G-containing strand. Similarly, the permanganate oxidation of the control strand in the presence of a large excess (300-fold to the oligomer) of 8-oxo-dG did not induce oligomer damage (data not shown). These results suggest that inter-molecular participation (diffusional contact) by the oxidation product(s) of the 8-oxo-G residue is not involved in the induction of the damage. The participation of diffusible oxidants (hydroxyl radicals and singlet oxygens), which might be generated by the oxidation of the 8-oxo-G residue, would be eliminated based on the results that the induction of the DNA damage was not inhibited when the permanganate oxidation of the 8-oxo-G-oligomers **3** and **8** was carried out with a reaction buffer containing a large excess (500-fold to oligomer) of sodium formate (a hydroxyl radical scavenger) or sodium azide and histidine (singlet oxygen quenchers) (data not shown). The superoxide anion is relatively unreactive with DNA (21) (but it can cause DNA damage via the conversion to a hydroxyl radical). Also, the 8-oxo-G-containing DNAs were insensitive to hydrogen peroxide, as described later.

Kinetic studies of DNA damage

The base-selective DNA damage is induced by the oxidation of an 8-oxo-G residue and would be caused by an intra-molecular reaction within the DNA chain. If a redox mechanism, which may contribute to the damage migration, is involved in the reaction, the entire reactivity of the 8-oxo-G-containing oligomers should depend on the electron donating activities of the DNA bases (G > A > T > C) (22,23) around the 8-oxo-G residue [redox potentials of DNA bases are as follows: guanine, +1.29; adenine, +1.39; thymine, +1.49; cytosine, +1.64 (in V versus saturated calomel electrode) (22)]. In other words, the reactivities of the oligomers would be higher when Gs exist near the 8-oxo-G, than when pyrimidines are located near the modified base. In order to test this hypothesis, we determined the pseudo-first-order rate constants (k_{obs}) for the oxidation of seven oligomers **1**–**6** and **8** (Table 2).

Table 2. Pseudo-first-order rate constants for the $KMnO_4$ oxidation of 8-oxo-G- containing single strands[a]

Compound	Sequence	k_{obs} (x $10^{-3}s^{-1}$)
3	5'GCGCGGGCGGTG3'	3.8
2	5'GCGCCGGCGGTG3'	3.3
1	5'GCGCCGGCGGTG3'	3.2
4	5'GCGCCAGCGGTG3'	2.4
5	5'GCGCCTGCGGTG3'	1.8
6	5'GCGCCCGCGGTG3'	1.7
8	5'TTTTCGTTCCTT3'	1.1

[a]Pseudo-first-order rate constants were determined on the basis of the time course of the decrease of the substrate oligomers. The procedure for this determination is described in Materials and Methods.

The results indicate that the rate constants may depend on the electron donating activities of the bases 5′-flanking the 8-oxo-G. Strand **3**, which has a continuous sequence of Gs 5′-adjacent to the 8-oxo-G was most reactive ($k_{obs} = 3.8 \times 10^{-3}$/s) among the seven substrates, whereas strand **8**, which contains only pyrimidines except for the 8-oxo-G, had the smallest constant ($k_{obs} = 1.1 \times 10^{-3}$/s).

It should be noted that the present method for the determination of the rate constants, in principle, is not dependent on whether the damage sites are cleaved or not. The 8-oxo-G residue has been always damaged whenever the damage is induced at the neighboring residues, and the damaged 8-oxo-G site is certainly cleaved so as to lead to the consumption of the substrates. Thus, the results from Table 2 indicate that the reactivity of the common DNA bases in the present reaction would be in the order G > A > T, C. This trend is consistent with the order of the redox potentials of the bases. Such base-selectivity in the reaction, as well as the analyses of the sites and the frequencies of the damage (Table 1), suggests that redox mechanisms are involved in the generation of the DNA damage not only at the position next to the 8-oxo-G, but also at the neighboring positions.

Measurement of the damage of double-stranded DNA

We next studied the permanganate reaction of duplexes that contain 5′- or 3′-end-labeled strands with 8-oxo-G (**1**, **2**, **4**, **5** and **7**) and their complementary strands, **c1**, **c2**, **c4**, **c5** and **c7** (Table 3).

Each duplex has an 8-oxo-G•C pair, and the reaction buffer contained 100 mM NaCl to allow the oligonucleotides to form duplexes. The scission at the 8-oxo-G and the neighboring positions was diminished, as compared with the cases of the single strands (Table 3); the cleavage yields of the 8-oxo-G containing single strands were independent of the absence or presence of NaCl in the reaction buffer. However, it should be noted that whereas the degree of the damage at the T sites of the control duplexes was very low (0–5%), the damage at the T sites in their modified duplexes **5•c5** and **7•c7** was more frequent. In addition to these observations, a slight strand scission was found to occur at the C position 5′-adjacent to the 8-oxo-G in strand **1** of the duplex, although the scission was not observed at this position on the single strand **1** (Table 1). Furthermore, the reactions of the 5′-end-labeled complementary strands **c1**, **c2**, **c4**, **c5** and **c7**, without 8-oxo-G in the duplexes, were also examined. In contrast to the lack of cleavage of **c1**, a small amount of strand scission (2.3% yield) was detected at the fifth G position from the 5′-end in the case of **c2**. Similarly, **c4**, **c5** and **c7** were cleaved at the G and/or T sites close to the 8-oxo-G residue in **4**, **5** and **7.** On the other hand, strand scission at the A sites was not observed, as in the case of the single strands.

Table 3. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled double strands[a]

Compound	Sequence	Cleavage yield (%)		
1	5'GCGC**C**G**G**CGGTG3'	4.9	41(20)	(17)
c1	3'CGCGGCCGCCAC5'			
2	5'GCGCC**G**GCGGTG3'	3.8	39(48)	
c2	3'CGCGGCC**G**CCAC5'	2.3		
4	5'GCGCCA**G**C**G**GTG3'	41 (45)	(3.0)	
c4	3'CGCGGTC**G**CCAC5'	5.6		
5	5'GCGCC**T**G**C**GGTG3'	6.4	39(45)	(3.2)
c5	3'CGCGGAC**G**CCAC5'	5.2		
7	5'ATGAC**G**GAA**T**AT3'	4.1	22	26(37) (7.1)
c7	3'TACTGCC**T**TATA5'	10	12	
2	5'GCGCC**G**GCGGTG3'	15	28	
c2A	3'CGCGGCA**G**CCAC5'	8.2		
2	5'GCGCC**G**GCGGTG3'	7.0	23	
c2T	3'CGCGGC**T**GCCAC5'	3.9	7.5	
2	5'GCGCC**G**GCGGTG3'	26	34	
c2G	3'CGCGGC**G**GCCAC5'	8.1	6.4	

[a]All reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiments with 3′-end-labeled oligomers. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left, and the underlined percentages are for 8-oxo-G residues. The percentages for the damage of the T and 8-oxo-G sites are corrected values, as described in Table 1.

To examine the possibility that some damage that does not cause strand scission occurred in these duplexes, the nucleoside compositions of **2**, **4** and the control strands were analyzed after the oxidation of the duplexes. The decrease in the amounts of the nucleosides, dG, dT and dC, was comparable with those values obtained from the strand cleavage analysis (data not shown). These results suggest that almost all of the damage of the G, T and C residues leads to strand scission upon piperidine treatment, as in the case of the single-stranded DNAs. On the other hand, although no intact 8-oxo-dG was detected after the oxidation in the duplexes, as well as in the single-strands, ~50% of the substrate was not cleaved by the piperidine treatment (Table 3). These results indicate the existence of uncleavable damage at the 8-oxo-dG site. Similarly, the decrease of dA in duplex **4•c4** was examined, each strand had a dA residue. If the reactivity of the 8-oxo-G-containing strand was similar to that of the single-stranded **4**, and the dA near the 5′-end of **c4** was insensitive to the permanganate treatment, a decrease of ~35% in the amounts of dA would be expected, because an ~70% decrease has been observed for the single-stranded **4**. The fact that the amounts of dA were less diminished (12% decrease) than in the case of the single strand suggests that the damage of the A residue is suppressed in the duplex form, as in the cases of the other DNA bases.

We oxidized the duplexes containing strands **c2A**, **c2T** and **c2G**, which have A, T and G bases, respectively, opposite the 8-oxo-G contained in the complementary **2** (Table 3), and examined the G damage of the 8-oxo-G (= G)-core (5′-GGC-3′ • 3′-CNG-5′) in the duplexes. The damage of **2** at the G (5′-) next to the 8-oxo-G, which was in a ‘mismatched base pair (N = A, T, G)’, was higher than that for the parent duplex (**2•c2**) with a ‘matched 8-oxo-G•C base pair (N = C)’. The reactivity of the G site seemed to correlate with the electron donating activity of the N bases: the reactivity increased according to the order G > A > T > C. On the other hand, the damage in the **c2** series occurred at the site opposite to the 8-oxo-G and/or at its 5′-adjacent site. Comparison with the strand cleavage efficiency at the G site 5′-adjacent to the N base in the **c2** series indicated that the N bases opposite the 8-oxo-G may also affect the reactivity of the G site: the reactivity increased according to the order G, A > T, C.

Effects of molecular oxygen and other oxidants on the oxidation

To examine the participation of molecular oxygen in the DNA damage, nitrogen gas was bubbled into the buffer solutions of the single-stranded substrates (**3**, **4** or **8**) prior to the permanganate oxidation. Analysis of the products showed that the cleavage yields at the G, C and T residues in the 5′-end-labeled strands (**3** and **8**) were unchanged, even after the removal of the O_2 (Table 1). Also, the degree of damage of the A residue in **4** was unchanged after the exclusion of O_2 (data not shown). Thus, the participation of O_2 in the generation of the DNA damage is ruled out.

We also tested oxidation using the oligomers listed in Table 1 with various oxidants that have different oxidizing abilities and either contain heavy metals (A) or not (B); (A) potassium ferricyanate, potassium perchromate, osmium tetraoxide, ferric chloride, copper sulfate and cytochrome-*c*; (B) sodium nitrate, hydrogen peroxide, methylene blue, DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) and oxone ($2KHSO_5•KHSO_4•K_2SO_4$). The reactions were performed under conditions similar to those for the permanganate treatment. However, little cleavage was found with any of these oxidants at the 8-oxo-G and the neighboring residues, after piperidine treatment.

Effect of continuous base residues on the oxidation

If the electron transfer reaction participates in the generation and the migration of the damage, and it occurs mainly through π-electrons within the continuous bases of an oligomer, the

degree and the extent of the damage around the 8-oxo-G may be decreased by the insertion of an abasic region at sites near the 8-oxo-G and the next base. To test the effects of the bases on the DNA damage, we synthesized DNA oligomers with substitutions of the various normal residues of oligomers **2**, **7** and **8** with one or more tetrahydrofuran derivatives (Fs), which mimic the structure of an abasic site and we carried out the permanganate oxidation (Table 4). The cleavage at the substituted sites was negligible (data not shown) and it seemed that the disappearance of the damage at these sites was compensated for by the appearance of damage at other sites; that is, there may be whole damage deposition. In the case of strand **10**, which has the sequence of **2** with the substitution of F for the G 5′-flanking the 8-oxo-G, the oxidation yielded new damage (6.0% yield) at the G site four residues away from the 8-oxo-G. Also, for strand **13**, enhancement of the damage (16%) was observed at the G site four residues away from the 8-oxo-G, as compared with the case of the parent **7**. For strand **18**, which lacks Gs, the damage occurred efficiently at the C site adjacent to the 8-oxo-G, and the cleavage yield at the C was 21% (for the parent **8**, 6.6%). While these results indicate that continuous base alignment is not essential for the generation of damage, they do not preclude damage migration through continuous bases.

Table 4. Sequences, cleavage positions and percentages of 5′- or 3′-end-labeled oligonucleotide derivatives[a]

Compound	Sequence	Cleavage yield (%)			
10	5'GC**G**CCF**GCGG**TG3'	6.0	45(51)	(12)	(8.6) (3.6)
11	5'GC**G**FFF**G**CGGTG3'	19	55		
12	5'GC**G**-Hex-**G**CGGTG3'	17	59		
13	5'AT**G**ACF**G**AATAT3'	32	25		
14	5'AT**G**FC**GG**AATAT3'	18	31	15	
15	5'AT**G**AFF**G**AATAT3'	44	22		
16	5'AT**G**FFF**G**AATAT3'	51	16		
17	5'TT**TT**F**G**TTCCTT3'	30	32	27	
18	5'TTTF**CG**TTCCTT3'	28	21	41	
19	5'AT**G**-Hex-**G**AATAT3'	56	15		
20	5'AT**G**-$(Hex)_2$-**G**AATAT3'	45	26		
21	5'AT**G**-$(Hex)_3$-**G**AATAT3'	28	39		

[a]All reactions were performed under the conditions described in Materials and Methods. G and bold letters refer to 8-oxo-G and cleavage sites, respectively. Cleavage yields in parentheses were obtained from the experiment with the 3′-end-labeled oligomer. Cleavage percentages correspond to those for sites with 5′ to 3′ shown at the left, and the underlined percentages are for 8-oxo-G residues. The percentages for the damage of the T and 8-oxo-G sites are corrected values, as described in Table 1.

Further studies using **11**, **15** and **16** (5′-end-labeled) showed that as the numbers of F-substitutions increase in the region between the G site to be damaged and the 8-oxo-G, the G site becomes damaged more frequently, as shown in Table 4. These results suggest that the bases intervening between the 8-oxo-G and the G suppress the damage of the G. On the other hand, the reaction of the 3′-end-labeled **10** showed an increase in the respective yields of the cleavages at the damaged sites on the 3′-side of the 8-oxo-G, as compared with the case of the parent **2**. Since the results of the oxidations with **4**, **5** and their parent **2**, which contain A, T and G, respectively, 5′-next to the 8-oxo-G can also be explained in terms of whole damage deposition, the results obtained from the F-substituted oligomers would not be due to structural changes of the substrates caused by the introduction of the F residue(s).

Effect of poly (alkylether) linkers instead of the sugar–phosphate backbone on the oxidation

We next examined the possibility of the transfer of damage (e.g. radical cation species, see Discussion) via a sugar–phosphate backbone (24,25). If the sugar–phosphate backbone is an essential medium for the damage migration, the replacement of F by a linker lacking a sugar–phosphate skeleton in the oligomer may diminish the damage of the nucleotide (dG) joined with the 8-oxo-G residue via the linker. Thus, we carried out the synthesis and the permanganate oxidation of oligomers, in which two oligomer fragments were linked with a hexa (ethylene glycol) unit (Hex) via two phosphodiesters (**12** and **19** in Table 4). Since the linker is three nucleotides long (~20 Å), the results of the strand cleavage analysis were compared with those of strands **11** and **16** with three continuous F residues. The results shown in Table 4 indicated that the cleavage yields at the 8-oxo-G and the G sites were almost equal for **12** and **11** and for **19** and **16**. All of our results argue against a direct role for the sugar–phosphate backbone in damage migration.

In order to test whether the damage migration occurs in DNA oligomers linked by a long linker, we prepared substrates **20** and **21** containing a long spacer composed of two and three continuous Hex residues, respectively. These linkers, $(Hex)_2$ and $(Hex)_3$, are ~45 and 70 Å long, respectively. The oxidation of the 5′-end-labeled substrates revealed that the damage at the G site joined with the 8-oxo-G via the linker was significant. Also, from the results, it appears that as the length of the linker increases, the damage of the G becomes less efficient (Table 4): the cleavage yields at the G site of the substrates (**19**, **20** and **21**) with $(Hex)_1$, $(Hex)_2$ and $(Hex)_3$ were 56, 45 and 28%, respectively.

Inhibition of the DNA damage by nucleosides: intra-molecular reaction versus inter-molecular reaction

We propose that the generation of the neighboring base damage involves an intra-molecular redox process. If the electron transfer process is truly involved in the generation of the DNA damage, then the G damage in the modified DNAs may be decreased by the addition of deoxynucleosides, and the efficiency of the inhibition would be in the order dG > dA > dC. These nucleosides are inert to permanganate oxidation, whereas dT is not, under the present conditions.

Thus, we examined the effects of the additives (dG, dA and dC) on the generation of the damage at the G sites of strands **19–21.** The permanganate oxidation of the oligomers was carried out in the presence of various concentrations of the nucleosides and then the strand cleavage analysis was performed. Experiments with strand **19** containing a Hex linker indicated that none of the nucleosides (~150-fold excess to the G residue) inhibited the generation of the damage at the G site (data not shown); that is, the intra-molecular reaction was not inhibited by the additives and the inter-molecular (between DNA molecules) reaction is unlikely. As

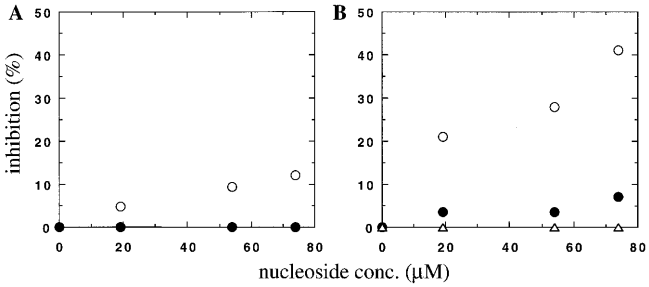

Figure 4. Inhibition of the damage migration within the 8-oxo-G-containing oligonucleotides with a long spacer, **20** (**A**) and **21** (**B**), by the deoxynucleosides, dG (○), dA (●) and dC (Δ). Each derivative was labeled at the 5′-end with ^{32}P and was used in the $KMnO_4$ reaction. The inhibition percentage was determined by quantification of the resulting G scission after hot piperidine treatment.

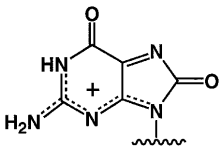

Figure 5. Putative structure of the intermediate, as an inducer of the neighboring base damage, formed by the permanganate oxidation of an 8-oxo-G residue.

shown in Figure 4, however, inhibition was observed in the examinations of **20** and **21** containing the longer spacers $(Hex)_2$ and $(Hex)_3$, respectively. The G damage in **20** was inhibited only by dG and the degree of the inhibition was 12% in the presence of 74 μM dG (150-fold excess to the G residue). Furthermore, in the case of **21**, the inhibition occurred more efficiently with dG and a slight inhibition by dA was also observed. The reductions in the damage were 41 and 7.0% in the presence of 74 μM dG and dA, respectively. As shown in Figure 4B, dC did not entirely inhibit the damage. Thus the inhibitory effects of the nucleosides on the damage production (in **20** and **21**) depended on the properties of the base moieties, and the effect was in the order G > A > C. This order is similar to that for the base-preference of the present DNA damage and for the electron transfer rate of deoxynucleosides as elucidated by fluorescence quenching experiments (26). The mechanism of the inhibition probably involves inhibition, by dG and dA, of the intra-molecular redox reaction in the DNA oligomer containing the spacer: inter-molecular electron transfer between the nucleoside and the DNA leads to the inhibition. The results also demonstrate that the inhibition of dG becomes effective as the distance (spacer) between the 8-oxo-G and the G residue is longer. In addition, using oligomer **21**, we found that sodium formate, sodium azide and histidine did not inhibit the generation of the DNA damage.

DISCUSSION

Mechanisms including redox chemistry in base damage

From the results of various experiments for single-stranded DNA, we found that the reactivity of the common bases in the permanganate oxidation of DNA oligomers with a single 8-oxo-G residue is in the order G > A > T, C (Tables 1 and 2). The tendency is consistent with the order of the electron donating activity of the bases (22,23). This was also found for the inhibitory effect of the common deoxynucleosides (dG > dA > dC) in the oxidation of the oligomer containing a long non-nucleotide spacer (Fig. 4). These results suggest that a redox mechanism participates in the generation of the damage of single-stranded DNA.

In double-stranded DNA with the 8-oxo-G in one strand, inter-strand base damage as well as intra-strand base damage was observed at the bases close to 8-oxo-G, but with lesser efficiency than that of the single strand (Table 3). The pathway involving redox chemistry for the double strand damage was suggested by the strand cleavage analysis of the damage to the 8-oxo-G-core (5′-GGC-3′ • 3′-CNG-5′) in **2•c2** and the analogs. The damage at the two G sites in the core was influenced, according to the N base residue opposite to the 8-oxo-G, and the damage of the Gs increased in the order G ≥ A > T ≥ C. The base selectivity for the damage is similar to that for the single strand.

From the above observation and results that reactive oxygen species and dioxygen may not participate in the neighboring base damage, we speculate a mechanism for the damage induction. It is well known that ionizing radiation (25,27) and photoirradiation with photosensitizers (28–32) to DNA cause oxidative damage, mainly at guanine sites. In the reactions, radical cations (often called positive holes) of the bases are formed, and they intramolecularly migrate within single-stranded DNA (33–35) and double-stranded DNA (33,36,37) according to the electron donating activities of the bases. Namely, the holes of $T^{\bullet+}$, $C^{\bullet+}$ and $A^{\bullet+}$ tend to transfer to G to produce $G^{\bullet+}$. If such a reaction has occurred in the present cases, a reactive species (oxidant) might be a transient intermediate generated from the $KMnO_4$ oxidation of the 8-oxo-G residue, and the resulting residue might oxidize the neighboring inter- and intra-strand bases to afford the radical cations, which would react with H_2O and/or $KMnO_4$. Recently, we carried out the $KMnO_4$ reaction using 5′-*O*-*tert*-butyldimethylsilyl-7,8-dihydro-8-oxo-2′-deoxyguanosine as a model. On the basis of the characterization of isolated three main products, each of which had an intact sugar residue, the products were considered to be produced via oxidation of the purine 4,5-double bond (the results will be published by M.Fukuoka *et al.* (1998) *Nucleic Acids Symp. Ser.* No.39). Similar reactivity of the double bond has been observed in the reaction of an 8-oxo-guanosine derivative with singlet oxygen (38). We envisioned the structure (or its deprotonated form) depicted in Figure 5 as the intermediate described above, which might be produced by the double bond oxidation followed by elimination of water molecule(s) and expected to have oxidizing ability. Its analogous structure has been proposed as an electrochemical oxidation intermediate of 9-methyluric acid (39). Although the reason for the apparent low reactivity of 8-oxo-G-containing duplexes to the neighboring base damage as well as mechanisms of the damage formation is unclear, we could imagine the inaccessibility of attacking agents ($KMnO_4$ or H_2O) to the bases (or holes), owing to the more anionic and more rigid character of double-stranded DNA.

Mechanistic proposals to explain the observed redox chemistry can be divided into two general classes: electron transfer mediated by stacked bases and an intramolecular reaction with the activated 8-oxo-G intermediate by direct contact. The former mechanism has been proposed in DNA-mediated photochemistry (36,37) and in oxidative DNA damage by ionizing radiation (34).

The relative importance of the two damage migration pathways is likely to be substrate-dependent. For example, the observation of damage at a G site four bases away from the 8-oxo-G in the double strand with **7** (Table 3) is most easily explained by electron transfer through stacked bases since the double helix would prevent direct contact between these two bases. By contrast, a direct contact mechanism offers a more plausible explanation for the damage migration observed for 8-oxo-G oligomers containing abasic spacers.

Mechanism of strand cleavage

Since the ladders observed by the strand cleavage analysis are comparable to Maxam–Gilbert sequencing ladders, the induced strand scissions may be due to the formation of an abasic site as an intermediate. However, DNA cleavage did not occur upon heating without piperidine (data not shown). Therefore, a 'regular' abasic site and a furanone site, which is also sensitive to heating without piperidine, would not be considered as proper candidates for those remnants. It is also known that most oxidation-induced sugar damage affords sites leading to frank strand scission or heat-labile sites (21): the remnants should contain degraded bases. A ureido sugar is a plausible candidate. It is known to be produced by permanganate oxidation of DNA from T, C and G residues under certain conditions, and this damage is labile to alkaline treatment and causes strand breakage (19). Also, 8-oxo-G can be considered as a precursor of a damaged G remnant, since 8-oxo-G can be generated by the reaction of $G^{+\bullet}$ with H_2O (15,28,29). However, it would not be a direct precursor for strand scission at the G sites, because 8-oxo-G in DNA yields only slight strand scission at the site upon piperidine treatment, and it is definitely permanganate-sensitive.

In a biological sense, our results about neighboring base damage have important implications. If such DNA damage involving 8-oxo-G occurs in cells, it would cause serious lesions in living organisms. Therefore, it is worthwhile to examine whether the damage migration can occur under conditions closer to those that might be observed within cells subjected to oxidative stress. This project and studies to confirm the redox process and to characterize the damaged base residues are in progress in our laboratory.

MATERIALS AND METHODS

Materials

Nucleosides dG, dA and dC were from Yuki Gosei Kogyo Co. 8-oxo-dG was prepared according to the published method (13) with a slight modification. The common phosphoramidites for DNA synthesis were obtained from Applied Biosystems, and tetrahydrofuran- and hexa(ethyleneglycol)-phosphoramidites were from Glen Research. Oligonucleotides with or without 8-oxo-G were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer using the standard solid-phase cyanoethyl phosphoramidite method, deblocked and purified as described (13,40). All solutions used in the study were made with water sterilized after prior purification by a Millipore Milli-Q water purification system.

Analysis of strand cleavage

The single-stranded oligonucleotides were labeled at the 5′- or 3′-end with T4 polynucleotide kinase (Takara) plus [γ-^{32}P]ATP (Amersham) or with terminal deoxynucleotidyl transferase (Takara) plus [α-^{32}P]ddATP (Amersham), respectively. Solutions (20 µl) buffered to pH 7.0 (10 mM sodium phosphate) and containing 0.5 µM oligonucleotide (a mixture of 10 pmol non-labeled strand and 0.1 pmol labeled strand) and 120 µM permanganate, were maintained at 25°C for 15 min and were quenched with 5 µl of allyl alcohol. Quenching of the reaction with the other oxidants, except for cytochrome-*c*, was performed by ethanol precipitation of the oligomers from 0.3 M sodium acetate solution containing 0.1 mM EDTA and 0.0025% tRNA. The cytochrome-*c* was removed by extraction with phenol/chloroform/isoamyl alcohol. Exclusion of molecular oxygen was carried out by bubbling N_2 into the reaction buffer and the permanganate solution. For duplex formation, the labeled single strands were annealed with the complementary strands by heating the buffered DNA solution (pH 7.0, 10 mM sodium phosphate, 100 mM NaCl) at 80°C for 5 min. This DNA solution was slowly cooled to room temperature and was used for the permanganate reaction; in the case of the single strands, all $KMnO_4$ reactions were performed in buffer lacking salts such as NaCl, in order to avoid the formation of higher ordered structures, such as a G tetraplex DNA (41). The samples (26 µl) were individually added to 25 µl of 2 M piperidine, heated at 90°C for 30 min, lyophilized to dryness, coevaporated with water (40 µl × 3) and dissolved in gel loading solution containing 5 M urea, 0.1% xylene cyanol and 0.1% bromophenol blue. The samples were heated to 55°C and chilled quickly for 20% polyacrylamide gel (denaturing 7 M urea) electrophoresis. The radioactivities of the fractionated, cleaved products on the gel were analyzed with a Bioimaging analyzer (Fujix BAS 2000). The cleavage yield (%) was obtained from the calculation: [radioactivity of each band/total radioactivity of the bands including the band of the remaining substrate] × 100.

Analysis of the nucleoside composition of the oxidized DNA

The single-stranded oligonucleotides were dissolved in 1.8 ml of 10 mM sodium phosphate buffer (pH 7.0) to a 0.5 µM concentration. The double-stranded solutions were prepared similarly, with 10 mM sodium phosphate buffer containing 100 mM NaCl. The permanganate reaction was carried out at a $KMnO_4$ concentration of 120 µM at 25°C for 15 min. The oxidized oligomers were desalted by gel filtration on Sephadex G-25 (NAP-5, Pharmacia) and were then digested with snake venom phosphodiesterase and alkaline phosphatase according to the reported method (40). The resultant nucleoside mixture was analyzed by reversed-phase HPLC on an Inertsil ODS 2 column (4.6 × 250 mm) and was detected by UV absorption at 254 nm; elution was performed with a linear gradient of acetonitrile (from 0 to 7.5% in 50 min) in 0.1 M triethylammonium acetate, at a flow rate of 0.5 ml/min. The decreases in the percentages of the nucleosides were calculated based on a comparison of the ratio of (peak areas on the HPLC profile/molar absorption coefficients) of each nucleoside before and after the oxidation of the oligonucleotides.

Reaction kinetics

The oxidation was performed with 5′-end-labeled single strands (0.5 μM) and $KMnO_4$ (120 μM) within 5 min of the reaction initiation under the conditions described above, and in the strand cleavage analysis, the remaining substrates (r.s.) were quantified by radiodensitometry as a function of time (t). The numbers of piperidine-induced 8-oxo-G scissions and permanganate-induced T scissions were subtracted from those induced by 8-oxo-G oxidation. The regression of the resulting net remaining substrates as a function of time was fit to a pseudo-first-order condition and rate constants were obtained from the slope of ln (r.s.) versus t plot.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1 Kasai,H. and Nishimura,S. (1991) In Sies,H. (ed.), *Oxidative Stress, Oxidants and Antioxidants*. Academic Press Inc., NY, pp. 99–116.
2 Kamiya,H., Murata-Kamiya,N., Koizume,S., Inoue,H., Nishimura,S. and Ohtsuka,E. (1995) *Carcinogenesis*, **16**, 883–889.
3 Shigenaga,M.K., Hagen,T.M. and Ames,B.N. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 10771–10778.
4 Spencer,J.P.E., Jenner,A., Aruoma,O.I., Evans,P.J., Kaur,H., Dexter,D.T., Jenner,P., Lees,A.J., Marsden,D.C. and Halliwell,B. (1994) *FEBS Lett*., **353**, 246–250.
5 Degan,P., Bonassi,S., Caterina,M.D., Korkina,L.G., Pinto,L., Scopacasa,F., Zatterale,A., Calzone,L. and Pagano,G. (1995) *Carcinogenesis*, **16**, 735–742.
6 Yanagawa,H., Ogawa,Y. and Ueno,M. (1992) *J. Biol. Chem*., **267**, 13320–13326.
7 Floyd,R.A., Watson,J.J., Wong,P.K., Altmiller,D.H. and Rickard,R.C. (1986) *Free Rad. Res. Comms*., **1**, 163–172.
8 Koizume,S., Inoue,H., Kamiya,H. and Ohtsuka,E. (1996) *Chem. Commun*., 265–266.
9 Sheu,C. and Foote,C.S. (1995) *J. Am. Chem. Soc*., **117**, 6439–6442.
10 Raoul,S. and Cadet.J. (1996) *J. Am. Chem. Soc*., **118**, 1892–1898.
11 Adam,W., Saha-Möller,C.R., Schönberger,A., Berger,M. and Cadet,J. (1995) *Photochem. Photobiol*., **62**, 231–238.
12 Adam,W., Saha-Möller,C.R. and Schönberger,A. (1996) *J. Am. Chem. Soc*., **118**, 9233–9238.
13 Koizume,S., Kamiya,H., Inoue,H. and Ohtsuka,E. (1994) *Nucleosides Nucleotides*, **13**, 1517–1534.
14 Chung,M.-H., Kiyosawa,H., Ohtsuka,E., Nishimura,S. and Kasai,H. (1992) *Biochem. Biophys. Res. Commun*., **188**, 1–7.
15 Cullis,P.M., Malone,M.E. and Merson-Davies,L.A. (1996) *J. Am. Chem. Soc*., **118,** 2775–2781.
16 Hayatsu,H. and Ukita,T. (1967) *Biochem. Biophys. Res. Commun*., **29**, 556–561.
17 McCarthy,J.G. (1989) *Nucleic Acids Res*., **18**, 7541.
18 Kochetkov,N.K. and Budovskii,E.I. (1972) *Organic Chemistry of Nucleic Acids Part B*. Plenum, New York, pp. 412–416.
19 Darby,G.K., Jones,A.S., Tittensor,J.R. and Walker,R.T. (1967) *Nature*, **216**, 793–794.
20 Hänsler,U. and Rokita,S.E. (1993) *J. Am. Chem. Soc*., **115**, 8554–8557.
21 Hutchinson,F. (1985) *Prog. Nucleic Acid Res. Mol. Biol*., **32**, 115–154.
22 Kittler,L., Löber,G., Gollmick,F.A. and Berg,H. (1980) *Bioelectrochem. Bioenerg*., **7**, 503–511.
23 Jovanovic,S.V. and Simic,M.G. (1986) *J. Phys. Chem*., **90**, 974–978.
24 Candeias,L.P. and Steenken,S. (1992) *J. Am. Chem. Soc*., **114**, 699–704.
25 Melvin,T., Plumb,M.A., Botchway,S.W., O'Neill,P. and Parker,A.W. (1995) *Photochem. Photobiol*., **61**, 584–591.
26 Shafirovich,V.Y., Courtney,S.H., Ya,N. and Geacintov,N.E. (1995) *J. Am. Chem. Soc*., **117**, 4920–4929.
27 Spassky,A. and Angelov,D. (1997) *Biochemistry*, **36**, 6571–6576.
28 Kasai,H., Yamaizumi,Z., Berger,M. and Cadet,J. (1992) *J. Am. Chem. Soc*., **114**, 9692–9694.
29 Ito,K., Inoue,S., Yamamoto,K. and Kawanishi,S. (1993) *J. Biol. Chem*., **268**, 13221–13227.
30 Saito,I., Takayama,M. and Kawanishi,S. (1995) *J. Am. Chem. Soc*., **117**, 5590–5591.
31 Sugiyama,H. and Saito,I. (1996) *J. Am. Chem. Soc*., **118**, 7063–7068.
32 Breslin,D.T., Coury,J.E., Anderson,J.R., McFail-Isom,L., Kan,Y., Williams,L.D., Bottomly,L.A. and Schuster,G.B. (1997) *J. Am. Chem. Soc*., **119**, 5043–5044.
33 Yan,M., Becker,D., Summerfield,S., Renke,P. and Sevilla,M.D. (1992) *J. Phys. Chem*., **96**, 1983–1989.
34 Candeias,L.P. and Steenken,S. (1993) *J. Am. Chem. Soc*., **115**, 2437–2440.
35 Melvin,T., Botchway,S., Parker,A.W. and O'Neill,P. (1995) *J. Chem. Soc. Chem. Commun*., 653–654.
36 Holmlin,R.E., Dandliker,P.J. and Barton,J.K. (1997) *Angew. Chem. Int. Ed. Engl*., **36**, 2715–2730.
37 Lewis,F.D., Wu,T., Zang,Y., Letsinger,R.L., Greenfield,S.R. and Wasielewski,M.R. (1997) *Science*, **277**, 673–676.
38 Sheu,C. and Foote,C.S. (1995) *J. Am. Chem. Soc*., **117**, 474–477.
39 Goyal,R.N., Jain,A.K. and Jain,N. (1995) *J. Chem. Soc. Perkin Trans. 2*, 1055–1061.
40 Kido,K., Inoue,H. and Ohtsuka,E. (1992) *Nucleic Acids Res*., **20**, 1339–1344.
41 Smith,S.S., Laayoun,A., Lingeman,R.G., Baker,D.J. and Riley,J. (1994) *J. Mol. Biol*., **243**, 143–151.