Characterization and Chemical Stability of Photooxidized Oligonucleotides that Contain 2,2-Diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(2*H*)-oxazolone

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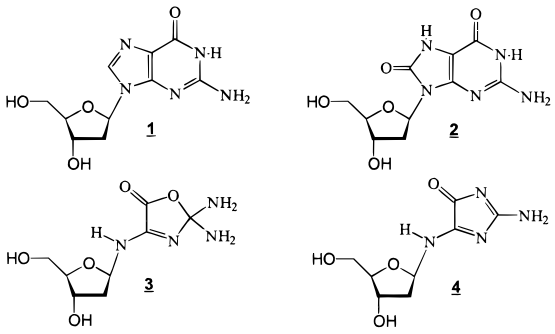
Abstract: The chemical insertion of 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(*2H*)-oxazolone (oxazolone), a major oxidation compound of 2′-deoxyguanosine, into oligonucleotides is a challenging issue, due to the high alkali lability of the lesion. This difficulty was overcome by the specific riboflavin-mediated photosensitization of a central guanine residue in DNA fragments. The modified 9-mer thus prepared, was purified by reverse HPLC and characterized by electrospray ionization mass spectrometry. Additional structural information was gained from the assignment of enzymatic hydrolysates of the oxidized DNA fragment. The high alkali lability of the oxazolone-containing DNA fragment was inferred from sequence gel analysis. In contrast, a similar 9-mer that contains 8-oxo-7,8-dihydro-2′-deoxyguanosine in place of the oxazolone was fully stable under the conditions of piperidine treatment.

Introduction

Oxidative damage to DNA may be induced by both exogenous and endogenous processes including ionizing radiation, UVA-mediated photosensitization, xenobiotic metabolism ...[1,2] During the past decade, many efforts have been devoted to characterizing the main oxidative DNA lesions with emphasis on base damage.[3] Guanine is known to be the preferential DNA target[4,5] of the direct effect of ionizing radiation and the type I photosensitized reaction,[6] both processes of which give rise to the transient formation of the guanine radical cation.[7] This is explained by the fact that guanine exhibits the lowest ionization potential among the four DNA bases. It is assumed that positive holes created in DNA through one-electron oxidation ultimately end up at guanine residues.[8] The overwhelming final product which arises from the hydration of the guanine radical cation within DNA, one of the two main competitive decomposition pathways of the latter reactive intermediate (the second being the deprotonation of the guanine radical cation), was found to be 8-oxo-7,8-dihydro-2′-deoxyguanosine (**2**) (8-oxodGuo).[9] It



should be added that 8-oxodGuo (**2**) is the predominant guanine decomposition product upon exposure of DNA to either the •OH radical or singlet oxygen ($^1O_2$). More recently it was found that 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(*2H*)-oxazolone (**3**) (oxazolone or dZ) together with its precursor, 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)-amino]-*4H*-imidazol-4-one (**4**) (imidazolone or dIz) are the main •OH-mediated oxidation products of the base of 2′-deoxyguanosine (**1**) (dGuo). The lactone structure of **3** was initially inferred from extensive ^1H and ^{15}N NMR studies which were indicative of the presence of two equivalent NH_2 in the modified nucleoside. In addition, there was no signal in the low-field region of the ^1H NMR spectrum in DMSO-d_6 which would have been assigned to the exchangeable proton of the carboxylic group of the related open-ring guanidino acid form (Scheme 1). However, our attention was recently drawn to the fact that the ^{17}O NMR data of **3**[7b] cannot be accounted for by the closed-

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(1) For a review, see: Cadet, J. In *DNA Adducts: Identification and Biological Significance*; Hemminki, K., Dipple, A., Shuker, D. E. G., Kadublar, F. F., Segerbäck, D., Bartsch, H., Eds.; IARC Scientific Publications: Lyon, 1994; pp 245−276.

(2) Marnett, L. J.; Burcham, P. C. *Chem. Res. Toxicol.* **1993**, *6*, 771−785.

(3) (a) von Sonntag, C. *The Chemical Basis of Radiation Biology*; Taylor & Francis: London, 1987. (b) Steenken, S. *Chem. Rev.* **1989**, *89*, 503−520. (c) Cadet, J.; Berger, M.; Douki, T.; Ravanat J. L. *Rev. Physiol. Biochem. Pharmacol.* **1997**, *131*, 1−87.

(4) Candeias, L. P.; Steenken, S. *J. Am. Chem. Soc.* **1992**, *114*, 699−704.

(5) Dunn, D. A.; Lin, V. H.; Kochevar, I. E. *Biochemistry* **1992**, *31*, 11620−11625.

(6) Foote, C. S. *Photochem. Photobiol.* **1991**, *54*, 659.

(7) (a) Cadet, J.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Raoul, S.; Ravanat, J.-L. *J. Am. Chem. Soc.* **1994**, *116*, 7403−7404. (b) Raoul, S.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Morin, B.; Weinfeld, M.; Cadet, J. *J. Chem. Soc., Perkin Trans. 2* **1996**, 371−381.

(8) (a) Symons, M. C. R. *J. Chem. Soc., Faraday Trans. 1* **1987**, *83*, 1−11. (b) Candeias, L. P.; Steenken, S. *J. Am. Chem. Soc.* **1993**, *115*, 2437−2440. (c) Melvin, T.; Plumb, M. A.; Botchway, S.; O'Neill, P.; Parker, A. W. *Photochem. Photobiol.* **1995**, *61*, 584−591. (d) Hall, D. B.; Holmlin, R. E.; Barton, J. K. *Nature* **1996**, *382*, 731−735.

(9) (a) Kasai, H.; Yamaizumi, Z.; Berger, M.; Cadet, J. *J. Am. Chem. Soc.* **1992**, *114*, 9692−9694. (b) Ravanat, J.-L.; Cadet, J. *Chem. Res. Toxicol.* **1995**, *8*, 379−388.

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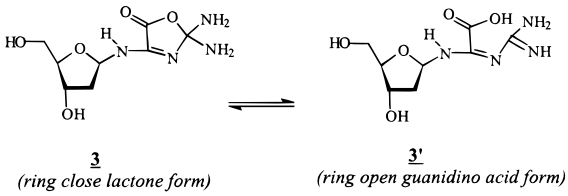
Scheme 1



3 (*ring close lactone form*) **3′** (*ring open guanidino acid form*)

ring lactone structure but rather in terms of the open-ring compound **3′**. This is based on the fact that the difference in the chemical shifts of the ^{17}O signals which were tentatively assigned as two labeled oxygen atoms of the oxazolone moiety is lower than that reported for various lactones (>100 ppm).[10] Further work is required to gain more definitive structural information on **3** which may exist in equilibrium with **3′**.[11] In addition, **3** and **4** are generated as the predominant decomposition products of dGuo **1** and guanine-containing dinucleoside monophosphate through the deprotonation pathway of the guanine radical cation. It was also shown that **3** and **4** are important $^1O_2$ side oxidation products of 8-oxodGuo. However, the characterization of **3** and **4** in isolated DNA is still required since only indirect evidence is available for the formation of the latter compounds. This was gained from the measurement of released guanidine, an alkali breakdown product of both **3** and **4**, from DNA upon oxidation by either the •OH radical or the type I photosensitizers.[12] Therefore, the present work was aimed at establishing that **3** is formed within a DNA fragment upon one-electron oxidation. The second major objective was to prepare oligonucleotides (ODNs) that contain the oxazolone **3**. In the latter respect, it has to be remembered that the site-specific incorporation of **3** in oligonucleotides is precluded due to the high lability of the latter oxidized nucleoside.[13] This particularly concerns the alkaline conditions of deprotection used at the final step of solid phase synthesis. As an answer to the two above requisites, we report herein the first photosensitized preparation of oligonucleotides which contain the highly labile 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5(*2H*)-oxazolone (**3**).

Results and Discussion

Preparation and Characterization of the Oligonucleotide-Containing Oxazolone. As an alternative to the usual phase condensation method which involves a final deprotection step incompatible with the instability of **3**, guanine-specific one-electron oxidation of a small DNA fragment was carried out. Typically, the unmodified synthetic ODN 5′-d(ATCTGTACT) (**5**) which exhibits one central guanine residue in its sequence was exposed in aerated aqueous solutions to photoexcited riboflavin, a typical type I photodynamic agent.[14] The reverse phase HPLC profile obtained immediately after the irradiation

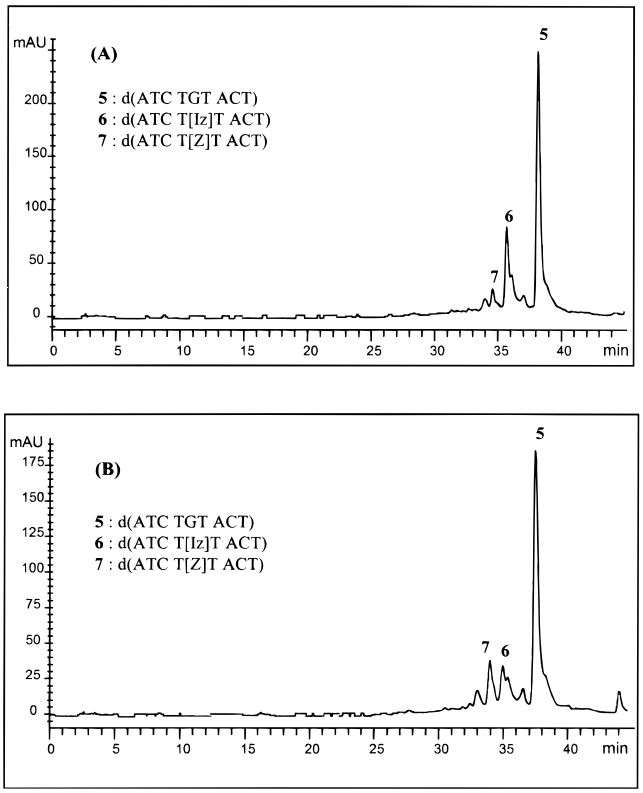


Figure 1. RP-HPLC elution profile of the riboflavin photosensitized oxidation products of ODN **5**. (A) Analysis performed immediately after irradiation and (B) 20 h after irradiation. The conditions of the analysis are described in the Experimental Section.

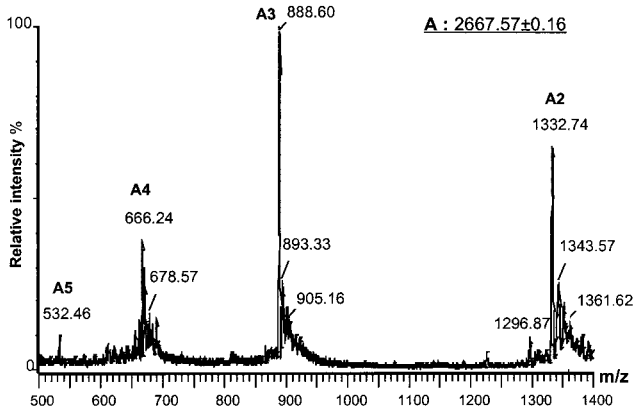


Figure 2. ESI-MS (negative mode) of the modified ODN **7**.

of **5** is reported in Figure 1(A). This shows the formation of a major photoproduct corresponding to 5′-d(ATCT[Iz]TACT) (**6**) that eluted at 35.5 min. After conversion of **6** into 5′-d(ATCT-[Z]TACT) (**7**) by spontaneous hydrolysis in neutral aqueous solution in 20 h at room temperature,[7,13] the modified ODN **7**, which is eluted faster than the parent molecule **5** and the precursor **6** (retention times of **5**, **6**, and **7** are 37.5, 35, and 33.9 min, respectively), is formed as the main photoproduct in a 15% yield (Figure 1B). Isolation of modified ODN **7** was then efficiently performed by HPLC on an ODS column. Relevant structural information on the oxidized ODN **7** was inferred from electrospray ionization mass spectrometry (ESI/MS) analysis. Interestingly, the ESI mass spectrum of ODN **7** obtained in the negative mode (Figure 2) revealed the presence of ions at *m*/*z* 1332.74, 888.60, 666.24, and 532.46, corresponding to 2-, 3-, 4- and 5-charged ions, respectively. This is indicative of an observed molecular weight of 2667.57 for **7**

(10) Boykin, D. W.; Sullins, D. W.; Eisenbraun, E. J. *Heterocycles* **1989**, *29*, 301−306.

(11) It should be added that other structures involving the hydration product of the imidazolone ring of **4** at C5 and the related open-ring oxamide compound may be ruled out on the basis of FAB-MS measurement of ^{18}O-labeled **3**. As a striking feature, a loss of ^{18}O fragment was observed (M-46) which is in favor of a decarboxylation reaction. This analysis is compatible with structure **3** or **3′**.

(12) (a) Douki, T.; Cadet, J. *Free Radical Res.* **1996**, *25*, 369−380. (b) Cadet, J.; Berger, M.; Douki, T.; Morin, B.; Raoul, S.; Ravanat, J.-L.; Spinelli, S. *Biol. Chem.* **1997**, *378*, 1275−1286.

(13) Buchko, G. W.; Cadet, J.; Morin, B.; Weinfeld, M. *Nucleic Acids Res.* **1995**, *23*, 3954−3961.

(14) (a) Cadet, J.; Decarroz, C.; Wang, S. Y.; Midden, W. R. *Isr. J. Chem.* **1983**, *23*, 420−429. (b) Buchko, G. W.; Cadet, J. *Can. J. Chem.* **1992**, *70*, 1827−1832.

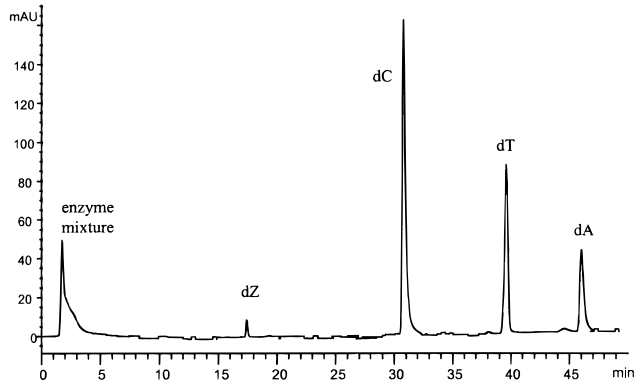


Figure 3. RP-HPLC elution profile of the enzymatic digestion mixture of the modified ODN **7**. The conditions of the analysis are reported in the Experimental Section.

which has to be compared with the expected value (MW = 2667.8). Further support for the presence of **3** in ODN **7** was provided by HPLC analysis of the mixture of nucleosides arising from the quantitative enzymatic digestion of the photooxidized DNA fragment. Then, quantitative enzymatic digestion of **7** was achieved using *Penicillium citrinum* nuclease P1 and white potato acid phosphatase. It should be noted that the latter dephosphorylating enzyme was substituted for the usual alkaline phosphatase from bovine intestinal mucus to prevent the degradation of released **3** at alkali pH. In this respect, it was found in preliminary experiments carried out on an oxazolone-containing trinucleotide that the bulk of released **3** has undergone decomposition upon incubation with alkaline phosphatase at pH 8.5. No such degradation was observed using acid phosphatase at pH 5.3. The resulting HPLC elution profile (Figure 3) showed the presence of the oxazolone nucleoside **3** at the expected retention time and in the right ratio with respect to the eight intact 2′-deoxyribonucleosides. The structure and integrity of released **3** from **7** were further confirmed by both ESI/MS and GC−MS[15] analyses.

Stability Study of the Oxazolone-Containing DNA Fragment. The isolation of **7** has allowed the determination of the stability of the latter oxazolone-containing DNA fragment under the piperidine conditions used to reveal alkali-labile DNA damage in oxidized oligonucleotides. The stability study was extended to 5′-d(ATCT[8-oxoG]TACT)-3′ (**8**), a similar 9-mer in which 8-oxodGuo **2** was inserted in place of **3**.[16] The comparative study of the stability of **2** and **3** in DNA fragments was performed by treating the 5′-[^{32}P]-labeled oligonucleotides **7** and **8** with hot piperidine for 30 min. In a subsequent step, the DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), and the relevant results are reported in Figure 4. Interestingly, the 8-oxodGuo-containing ODN **8** was stable under piperidine treatment, while the cleavage was quantitative after 30 min at the oxazolone site of modified ODN **7**. It should be added that the PAGE mobility of the released labeled DNA fragment was consistent with the cleavage of ODN

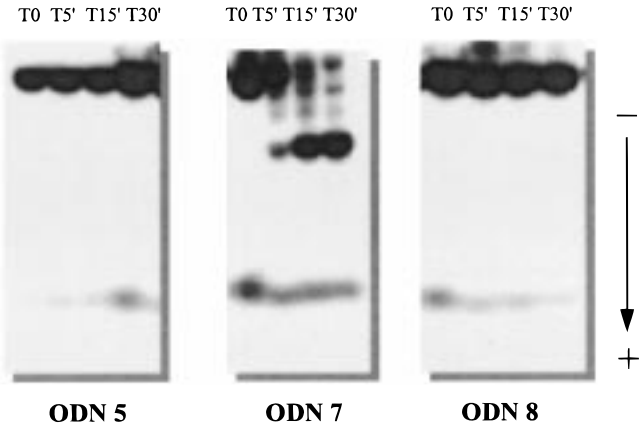


Figure 4. Sequence gel analysis (denaturing 20% PAGE) of 5′end-labeled ODNs **5**, **7**, and **8** submitted to piperidine treatments (1 M, 90 °C during 0, 5, 15, and 30 min, respectively).

7 at the oxazolone site. The latter data provide further support for the site-specific presence of **3** in the riboflavin-mediated photosensitization of the 9-mer. The stability study clearly demonstrated that the oxazolone **3**, and not 8-oxodGuo **2** as generally reported,[17] has to be considered as the main piperidine labile lesion in terms of the induction of strand cleavage at guanine residues within oxidized DNA. This should be useful to assign and measure the formation of **3** in oxidized oligonucleotides using gel sequence analysis.[18]

Conclusions and Outlook

The successful photosensitized incorporation of the alkali-labile oxazolone **3** moiety into oligonucleotides has been achieved showing for the first time that this oxidative lesion is the major product of decomposition of guanine formed in single strand DNA fragments upon the type I photooxidation conditions described herein. The resulting modified oligomers have been characterized and then successfully used to demonstrate the high differential sensitivity of 8-oxodGuo **4** and oxazolone nucleoside **3** to piperidine. Thus, it appears that this chemical stability test can be used to distinguish unambiguously these two products of oxidative base damage of 2′-deoxyguanosine that are able to be formed within DNA. Consequently, with regards to the interest focused on **4** in terms of the biomarker of the DNA oxidation and the remaining difficulties in the measurement of this adduct,[19] oxazolone **3** might be considered as a potential additional biomarker of oxidative stress to DNA. However, a major limitation to the development of assays aimed at monitoring the formation of **3** in cellular DNA and to the search for its presence in biological fluids such as urine is its instability.

Last, it should be added that the approach which was used for the photosensitized formation of **7** is currently extended in our laboratory to the preparation of various oxazolone **3**-containing oligonucleotides. This would facilitate the determination of both conformational and biological (mutagenicity, repair) features of this important oxidized DNA base lesion.

(15) GC−MS analysis of **3** released from the enzymatic digestion of **6** (eluting fraction at retention time = 17.2 min, using the HPLC conditions reported above) was performed after derivatization of the nucleoside following the procedure described by Ravanat, J. L.; Turesky, R. J.; Gremaud, E.; Trudel, L. J.; Stadler, R. H. *Chem. Res. Toxicol.* **1995**, *8*, 1039−1045. Due to the instability of **3**, the major peak detected during GC−MS analysis corresponds to a product which has lost a water molecule, similar to that of the imidazolone precursor.

(16) Both lesions **2** and **3** arise from the chemical decomposition of the transient guanyl radical cation. Thus, the formation of **3** is accounted for by the initial deprotonation of the radical cation,[7] while competitive hydration of the latter intermediate represents the initial step of the sequence of events leading to 8-oxodGuo.[9]

(17) (a) Chung, M. H.; Kiyosawa, H.; Ohtsuka, E.; Nishimura, S.; Kasai, H. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 1−7. (b) Ito, K.; Inoue, S.; Yamamoto, K.; Kawanishi, S. *J. Biol. Chem.* **1993**, *268*, 13221−13227. (c) Hall, D. B.; Holmlin, R. E.; Barton, J. K. *Nature* **1996**, *382*, 731−735. (d) Ito, K.; Kawanishi, S. *Biochemistry* **1997**, *36*, 1771−1781.

(18) Spassky, A.; Angelov, D. *Biochemistry* **1997**, *36*, 6571−6576.

(19) (a) Cadet, J.; Douki, T.; Ravanat, J.-L. *Environ. Health Perspect.* **1997**, *105*, 1034−1039. (b) Helbock, H. J.; Beckman, K. B.; Shigenaga, M. K.; Walter, P. B.; Woodall, A. A.; Yeo, H. C.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 288−293.

Experimental Section

General Procedures and Materials. Riboflavin was purchased from BDH Biochemicals (Poole, U.K.). Nuclease P1 (*Penicillium citrinum*), white potato acid phosphatase, and calf intestinal alkaline phosphatase were from Sigma (St Louis, MO). T4 polynucleotide kinase was from Pharmacia (Uppsala, Sweden). [γ-^{32}P]ATP was purchased from Amersham (U.K.). Acetonitrile (HPLC-grade) was obtained from Carlo Erba (Milan, Italy). Buffers for high performance liquid chromatography (HPLC) were prepared using water purified with a Milli-Q system (Milford, MA).

Oligonucleotides d(TGT), **5**, and **8** were synthesized by standard phosphoramidite chemistry[20] using an Applied Biosystems Inc. 392 DNA synthesizer. Oligonucleotide **8** was prepared by using a commercial phosphoramidite monomer of 8-oxodGuo (Glen Research, Sterling, VA) and deprotected by treatment in a concentrated aqueous ammoniac solution (32%) containing 0.25 M β-mercaptoethanol to prevent further additional oxidation of the modified base. After ammoniac deprotection, the crude 5′-tritylated oligomers were purified and detritylated online by reverse phase HPLC following the previously reported method.[21] The purity and homogeneity of modified oligodeoxynucleotides were controlled by both 20% PAGE and HPLC analyses. The presence and the integrity of 8-oxodGuo into 9-mer **8** were assessed with both mass measurements by electrospray ionization spectrometry and base composition analysis by total enzymatic digestion.

The trinucleotide d[TGT] was used successfully in preliminary studies to optimize the photoirradiation conditions of the preparation of dZ within oligonucleotides.

All irradiations of oligonucleotides were carried out in a Rayonnet photochemical reactor (The New England UV Company, Hamden, CT).

Electrospray mass spectrometry measurements were performed with a Platform 3000 model spectrophotometer from Micromass (Manchester, U.K.). Oligonucleotides were dissolved in a solution of acetonitrile and water (50/50, v/v) containing 1% triethylamine prior to being analyzed in the negative mode. Analysis of the sample **3** (resulting from the enzymatic digestion of **7**) was performed in the positive mode after dissolution in a solution of acetonitrile and water (50/50, v/v) that contained 0.2% formic acid.

Photooxidation of Oligonucleotides in the Presence of Riboflavin. Two milliliters of a 1 mM aqueous solution of oligonucleotide **5**, to which 150 μL of an aqueous saturated solution of riboflavin was added, was exposed during 30 min to 16 black-light lamps (λ_{max} = 350 nm) of a Rayonnet photochemical reactor. Conversion of **6** to **7** was achieved by leaving the irradiation mixture at room temperature for 20 h. The ODN **7** was purified on a Hypersil (Interchim, Montluçon, France) ODS column (5 μm, 250 mm × 4.6 mm i.d.) with a gradient of acetonitrile (from 0 to 10% in 40 min) in 25 mM ammonium formiate (pH 7) at a flow rate of 1 mL/min. Detection was performed at UV absorbance at 260 nm. Under the latter conditions, retention times of **5**, **6**, and **7** are 37.5, 35, and 33.9 min, respectively.

Enzymatic Digestion and HPLC Analysis. Into 45 μL of water were taken up 0.25 $au_{260\ nm}$ of purified ODNs. Five microliters of a solution of acetate buffer (sodium acetate 300 mM, pH = 5.3, $ZnSO_4$ 1 mM) that contained the nuclease P1 (3 units) and the acid phosphatase (0.5 unit) were added. The resulting mixture was incubated at 37 °C for 2 h. Subsequently, the enzymatic hydrolysate was taken up in 50 μL of water and analyzed by reverse phase HPLC on a highly hydrophobic Hypercarb (Shandon, Runcorn, U.K.) porous carbon column (5 μm, 100 mm × 3 mm i.d.), using a linear gradient of acetonitrile (from 0 to 30% in 45 min) in water, at a flow rate of 0.4 mL/min. The use of the carbon column was found to be efficient for achieving the separation of various mixtures of normal and modified nucleobases, nucleosides, and nucleotides which were not resolved on conventional ODS columns (Gasparutto, D.; unpublished results). Detection was performed at UV absorbance at 230 nm. The retention times for oxazolone, 2′-deoxycytidine, thymidine, 2′-deoxyguanosine, and 2′-deoxyadenosine were 17.2, 30.1, 38.7, 43.6, and 45.1 min, respectively.

Piperidine Treatment and PAGE Analysis. Labeling at the 5′-end of DNA fragments was achieved by treament with [γ-^{32}P]ATP and T4 polynucleotide kinase using the standard procedure.[22] Then, 5′-[^{32}P]-labeled oligonucleotides were placed in 100 μL of freshly made 1 M piperidine solution in water and then heated at 90 °C in sealed tubes. After cooling, the samples were coevaporated twice with water and then loaded onto a 20% denaturing polyacrylamide gel. Electrophoresis was carried out at 1500 V for approximately 3 h before the gel was exposed to X-ray film.

Acknowledgment. We thank C. Bouchon for the preparation of trinucleotide d(TpGpT) used in preliminary experiments of photosensitization. We are also indebted to Professor A. Favier for providing accessibility to the electrospray mass spectrophotometer and to a reviewer for his suggestion concerning the structure of **3**. The work was partly supported by a grant from CNRS ("Programme Physique et Chimie du Vivant 1998").

Supporting Information Available: FAB mass spectrum (in the positive mode) of an ^{18}O-labeled oxazolone sample (3′,5′-di-*O*-acetylated derivative), ^{1}H NMR spectrum (in DMSO-d_6) of oxazolone, HPLC analysis of ODNs **5**, **7**, and **8**, reverse phase HPLC chromatograms of enzymatic digestion mixtures of ODNs d(TGT), d(TZT), and **5**, and ESI$^+$ and GC−MS spectra of the dZ sample resulting from the enzymatic digestion of ODN **7** (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(20) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311.
(21) Romieu, A.; Gasparutto, D.; Molko, D.; Cadet, J. *Tetrahedron Lett*. **1997**, *38*, 7531−7534.
(22) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Sring Harbor Laboratories Press: Cold Spring Harbor, NY, 1989.