Site-Specific Insertion of the (5*R**) and (5*S**) Diastereoisomers of 1-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin into Oligodeoxyribonucleotides

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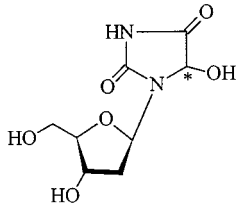
The insertion of the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (**1**) – a major oxidation product of 2′-deoxycytidine upon exposure to ·OH radicals, excited photosensitizers, or ozone – into oligonucleotides is reported. It was achieved by means of phosphoramidite chemistry, using the solid-phase synthesis approach. The synthesis of the phosphoramidite synthon **7** required 6 steps from 3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxycytidine and involved protection of the secondary hydroxy group (5-OH) of the modified base by the nonstandard levulinyl group. The modified phosphoramidite synthon **7** was efficiently incorporated into several oligonucleotides (3-*mer*, 14-*mer*, 22-*mer*) by solid-support assembling. The presence and the integrity of the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin in the synthetic oligomers was confirmed by electrospray ionization mass spectrometry, together with HPLC and MALDI-TOF mass-spectrometric analyses of enzymatic digestions. The use of exonucleases (calf spleen phosphodiesterase and bovine intestinal mucosa phosphodiesterase) clearly showed that enzymatic hydrolysis of the phosphodiester bonds between the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin and normal 2′-deoxyribonucleosides is prevented, while endonuclease (nuclease P_1) is able to cleave the lesion residue from the oligonucleotides.

Introduction

Deoxyribonucleic acid is a critical cellular target for several oxidation reactions associated either with aerobic cellular metabolism or with exposure to physical and chemical agents. The resulting oxidative damage may contribute to cancer and aging processes; it may also be implicated in a number of neurological disorders.[1–4] Thus, one-electron oxidation of nucleobases is a major reaction associated with the direct effect of ionizing radiation and the type I photosensitization mechanism. It has been shown that various reactive oxygen and nitrogen species, including hydroxyl radicals (·OH), ozone (O_3), and peroxynitrite ($ONOO^-$), are able to react with both pyrimidine and purine bases to produce a wide set of lesions.[5–9] Of the four normal nucleosides, 2′-deoxycytidine has been the target of studies aimed at characterizing the oxidative processes. Information on the structure and the mechanism of the latter nucleoside modifications is now available.[10–14] Among these modifications, the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (5-OH-dHyd, **1**) (Scheme 1) have been shown to be generated upon exposure to several oxidative processes, including those involving ·OH radicals (γ irradiation),[11] excited photosensitizers (UVA/menadione photosensitization),[11] and ozone.[12] In particular, 5-OH-dHyd (**1**) has been found to be the major decomposition product of ozone-mediated oxidation of 2′-deoxycytidine.[12]



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Scheme 1. Chemical structures of the (5*R**) and (5*S**) diastereoisomers of 5-OH-dHyd (**1**)

In order to assess the mutagenic features of (5*R**)- and (5*S**)-1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (**1**), it is necessary to prepare oligonucleotides that contain 5-OH-dHyd (**1**) at defined sites. This will also allow investigation of the possible recognition of these lesions by purified repair enzymes in a comparative study that should include the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxy-5-methylhydantoin (5-OH-5-Me-dHyd).[15]

In this paper, we report the first site-specific incorporation of 5-OH-dHyd (**1**) (Scheme 2) into oligonucleotides through application of solid-phase phosphoramidite chemistry. For this, it was necessary to protect the secondary hydroxy group with the nonstandard levulinyl moiety. The modified DNA oligomers were fully characterized by sev-

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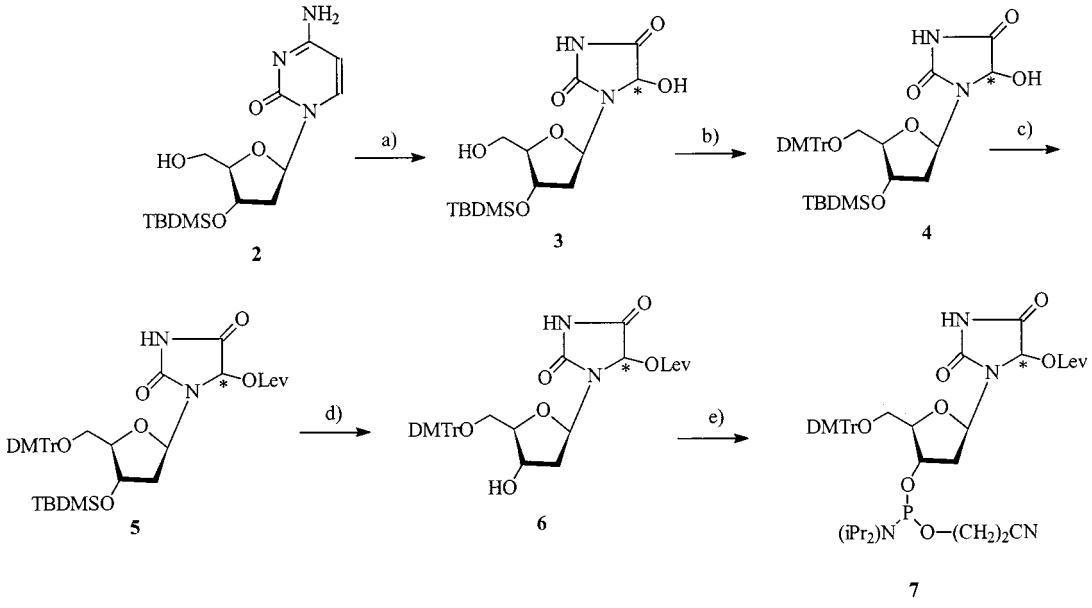


Scheme 2. Synthetic pathway used for the preparation of the phosphoramidite synthon **7** of 5-OH-dHyd; reagents: a) A. O_3, CH_3CN/H_2O, 4 h, room temperature; b) DMTr-Cl, DMAP, pyridine, 20 h, room temperature; c) levulinic acid, DCC, DMAP, tetrahydrofuran, 20 h, room temperature; d) TBAF, THF, 2 h, room temperature; e) chloro-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite, DIPEA, CH_2Cl_2, 2 h, room temperature

eral methods (HPLC, ESI-MS, and MALDI-TOF-MS). They were also used to determinate the ability of several exonucleases to cleave the modified nucleoside.

Results and Discussion

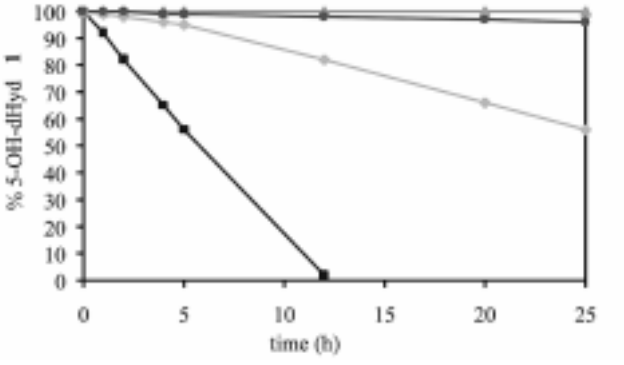
Synthesis of the Modified Phosphoramidite Building Block and Its Insertion into Oligonucleotides of Defined Sequence

Stability Studies on the (5*R**) and (5*S**) Diastereoisomers of the 5-OH-Hydantoin Nucleoside 1

Prior to the preparation of the phosphoramidite synthon **7**, the stability of the 5-OH-hydantoin nucleoside **1** was studied under the main experimental conditions used during the course of solid-support synthesis (Scheme 3). Authentic samples of (5*S**)- and (5*R**)-5-OH-hydantoin, obtained by ozonolysis of an aqueous solution of 2′-deoxycytidine at room temperature,[12] were used for the stability studies. Thus, they were treated variously with 80% acidic acetic, a commercial oxidizing solution of iodine at room temperature, or with ammonia, under two sets of temperature conditions, namely 25 °C and 55 °C. Aliquots of the reaction mixtures were taken over increasing periods of time and analyzed by reversed-phase HPLC on a Hypercarb graphitized column. It was clear that neither degradation nor isomerization occurred either under the applied acidic conditions, or under the oxidizing conditions. However, **1** was found to be unstable under the alkaline conditions. Indeed, after 4 h at room temperature, approximately 3% of **1** was degraded, while the yield of decomposition reached 30% after 24 h at room temperature. Moreover, isomerization between the two diastereoisomers of **1** was observed under the basic conditions used. Such an epimerization at C-5 prevents the single chemical insertion of each of the purified diastereoisomers into DNA fragments. The main consequence of the instability of **1** under alkaline conditions was the substitution of the usual amino protecting groups (benzoyl for dAdo, isobutyryl for dCyd and dGuo) with those used in Pac-protected phosphoramidite chemistry. Thus, the phenoxyacetyl, the isopropylphenoxyacetyl, and the acetyl groups were used for dAdo, dGuo, and dCyd, respectively.[16]

Synthetic Procedure for the Preparation of the Phosphoramidite Synthon of 5-OH-Hydantoin (1)

The major sources of difficulty in the preparation of oligonucleotides containing 5-OH-dHyd (**1**) are the result of the presence of the hydroxy group at C-5 of the modified



Scheme 3. Stability studies of 5-OH-dHyd (**1**) in a 0.1 M oxidizing solution of iodine at room temperature (s); in an 80% acid acetic aqueous solution at room temperature (l); in a 30% ammonia aqueous solution at room temperature (u) and at 55 °C (n)

base. Firstly, this secondary hydroxy group (5-OH) may interfere during the solid-phase DNA synthesis, either by providing a starting point for the attachment of nucleosides, or by giving rise to other undesired by-products. Consequently, it was deemed necessary to mask the 5-OH function with a protecting group stable under the conditions of solid-phase DNA synthesis; however, this had to be easily removable during the final ammonia deprotection step at room temperature. Moreover, preparation of the phosphoramidite synthon **7** starting directly from the 5-OH-hydantoin nucleoside **1**, which can be easily prepared by ozonolysis, was not convenient. Indeed, protection of the 5-OH group was not selective; the 3′- and 5′-*O*-silylated or -acetylated compounds were also observed. It was therefore necessary to protect the 3′-OH group of the 2-deoxyribose moiety before ozonolysis, and the 3′-*O*-*tert*-butyldimethylsilyl group was chosen for this protection. Interestingly, it was found to be inert toward ozone.

Thus, the synthesis of the targeted phosphoramidite synthon **7**, required for the incorporation of compound **1** into oligonucleotides, was achieved in 6 steps as shown in Scheme 2. Firstly, 3′-*O*-(*tert*-butyldimethylsilyl)-2′-deoxycytidine (**2**), dissolved in a mixture of water/acetonitrile (1:1 v/v), was oxidized by a stream of ozone (3−4% in O_2), by the method developed by Girault et al.[12] Because of the insolubility of **2** in water, it was necessary to find another solvent, inert toward ozone; acetonitrile was shown to fulfill this requirement. The reaction was monitored by reversed-phase HPLC. After 4 h at room temperature, the reaction had gone to completion and a predominant product was observed in the HPLC elution profiles found by UV detection at 230 nm. Analyses by ESI-MS in both the positive and negative modes confirmed the structure of the desired 3′-*O*-silylated-5-OH-hydantoin **3**. In order to avoid a tedious and time-consuming semipreparative HPLC purification step, the resulting crude product was not purified further, but was directly used in the next step of the synthesis. Selective protection of the 5-OH group of the hydantoin base first required the protection of the reactive 5′-hydroxy group of the 2-deoxyribose moiety. Thus, the crude product was treated with 4,4′-dimethoxytrityl chloride (DMTr-Cl) in pyridine for 20 h at room temperature in the presence of 4-(dimethylamino)pyridine (DMAP) as the catalyst. The expected 5′-DMTr ether **4** of the 3′-*O*-silylated-5-OH-hydantoin was isolated by silica gel chromatography [yield 28% (two steps)]. Attempts to protect the 5-hydroxy group of **4** with conventional acylation reagents − including acetic anhydride, isobutyryl chloride, and benzoyl chloride − failed; indeed, numerous products were obtained and degradation of **4** was observed. Therefore, the levulinyl (Lev) group, a nonstandard hydroxy protecting group, was chosen. This group has already successfully been applied to the solid-phase synthesis of oligonucleotides by the phosphoramidite and phosphotriester approaches,[17−19] and can easily be introduced into secondary alcohol functions. Moreover, the removal of this ether group under basic conditions (30% aqueous ammonia, 10 min at room temperature) is compatible with the conditions for stability of **1**. Thus, compound **5** was obtained in 64% yield. The 3′-*O*-TBDMS ether **5** was selectively desilylated by treatment with tetrabutylammonium fluoride (TBAF) in THF at room temperature for 2 h, giving **6** in 53% yield. The desired phosphoramidite **7** was finally obtained in a 51% yield (after silica column chromatographic purification) upon treatment of the secondary alcohol group of **6** with 2-cyanoethyl *N*,*N*-diispropylphosphoramidochloridite [ClP-(N*i*Pr$_2$)O(CH$_2$)$_2$CN] in the presence of *N*,*N*-diisopropylethylamine (DIPEA). The mass spectrum of **7** is given in Figure 1.

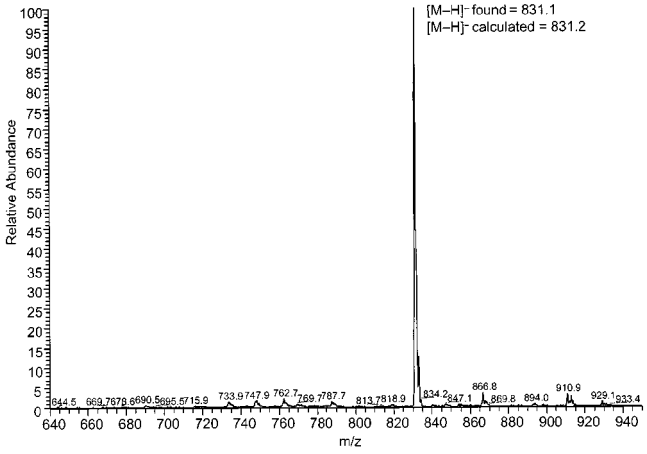


Figure 1. ESI mass spectrum (negative mode) of the phosphoramidite synthon **7**

Solid-Phase Synthesis and Characterization of the Oligonucleotides Containing the (5*R**) and (5*S**) Diastereoisomers of 5-OH-Hydantoin 1

Several oligonucleotides bearing a 5-OH-dHyd (**1**) residue were synthesized on solid supports using "Pac phosphoramidite" chemistry, with the modifications described in the Exp. Sect. This resulted in a coupling efficiency of more than 90% for the modified monomer. After ammonia deprotection at room temperature for 4 h, the crude 5′-tritylated oligonucleotides were purified by RP HPLC on a polymeric support, using an on-line detritylation purification procedure.[20] The purity and homogeneity of the modified oligonucleotides were monitored first by HPLC and then by polyacrylamide gel electrophoresis of the related 5′-[^{32}P]-labeled fragments. Finally, measurements by MALDI-TOF-MS in the positive mode and ESI-MS in the negative mode were performed (Table 1 and Figure 2). The obtained data confirmed the incorporation of 5-OH-dHyd (**1**) into the modified oligomers and its integrity.

Piperidine Stability of the Modified Oligodeoxyribonucleotides Containing 5-OH-dHyd (1)

The availability of modified oligonucleotides containing the 5-OH-dHyd (**1**) lesion enabled the stability of oligonucleotide **9** in the presence of piperidine − used to reveal

Table 1. Sequences and molecular masses of the modified oligonucleotides synthesized and used in this study [X = 5-OH-dHyd (**1**)]; the oligonucleotide masses were inferred from ESI-MS measurements in the negative mode

Name	Sequences (5′-3′)	Length	Mass calcd.	Mass found
8	A**X**C	3	834.6	834.3
9	ATC GTG A**X**T GAT CC	14	4243.8	4243.5
10	CAC TTC GGA T**X**G TGA CTG ATC C	22	6691.4	6692.0

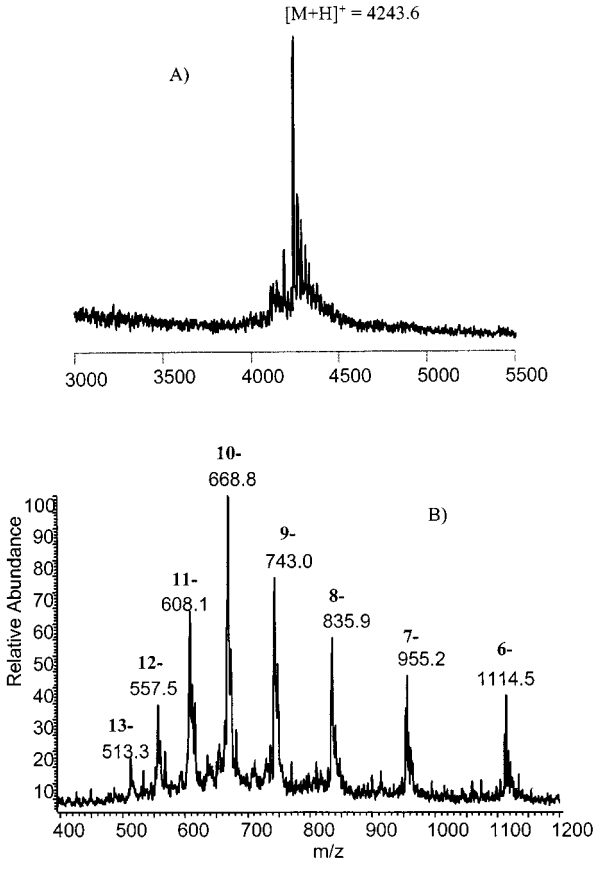


Figure 2. A) MALDI-TOF MS of the modified 14-*mer* oligonucleotide **9**; B) ESI-MS (negative mode) of the modified 22-*mer* oligonucleotide **10**

alkali-labile sites in oxidized DNA (Figure 3) − to be determined. This was achieved by treating the 5′-[^{32}P]-labeled oligonucleotide 14-*mer* **9** with piperidine at 90 °C for 15, 30, and 60 min. Subsequently, the resulting DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis. It was found that, upon insertion into an oligonucleotide, 5-OH-dHyd (**1**) was unstable to treatment with piperidine. Indeed, after a 1 h heating period, approximately 50% of the oligonucleotide **9** had been cleaved at the site of **1**. 5-OH-5-Me-dHyd was shown to be more stable than 5-OH-dHyd (**1**);[15] it may be remembered that only 20% of nicks were observed after heating oligonucleotides containing the 5-methyl residue derivative of **1** for 1 h at 90 °C. It may be concluded that 5-OH-dHyd (**1**) is a medium-range alkali-labile lesion, whereas 5-OH-5-Me-dHyd displays a weak alkali lability.

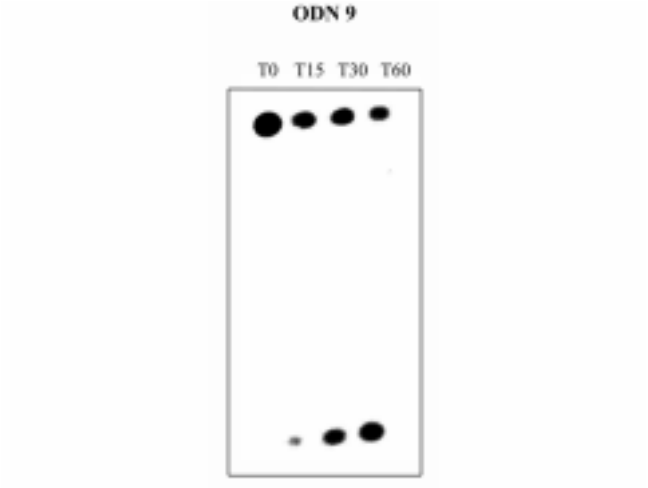


Figure 3. PAGE analysis of the 5′-end-labeled 14-*mer* ODN containing 5-OH-dHyd (**1**) (ODN **9**), after treatment with a 1 M aqueous solution of piperidine at 90 °C for 0, 15, 30, and 60 min

Nuclease-Mediated Digestion of Modified Oligonucleotides Containing 5-OH-dHyd (1)

Firstly, aliquots of the modified 14-*mer* oligonucleotide **9** were incubated with nuclease P_1 over a 2 h period, followed by digestion with bacterial alkaline phosphatase. The resulting mixture of 2′-deoxyribonucleosides arising from the two successive digestions was then analyzed by reversed-phase HPLC on a graphitized Hypercarb carbon column (Figure 4). One major peak (in addition to those corresponding to normal 2′-deoxyribonucleosides) was observed in the HPLC elution profiles (retention time: 21.8 min). The peak was found to contain the two diastereoisomers of 5-OH-dHyd (**1**), as inferred from co-chromatography with authentic samples of **1** and electrospray ionization mass-spectrometric analysis of the collected peak (data not shown). The latter observation provides additional support for the presence and the integrity of **1** in the synthetic oligomers. This clearly shows the ability of nuclease P_1 to quantitatively cleave 5-OH-dHyd (**1**) from DNA. Similar results were obtained with the modified 3-*mer* and 22-*mer* oligonucleotides **8** and **10**, respectively.

Additional enzymatic digestion experiments were performed on the modified 14-*mer* oligonucleotide **9**, using the two exonucleases bovine intestinal mucosa phosphodiesterase (3′-*exo*) and calf spleen phosphodiesterase (5′-*exo*). Thus, the course of the hydrolysis of the DNA strand by

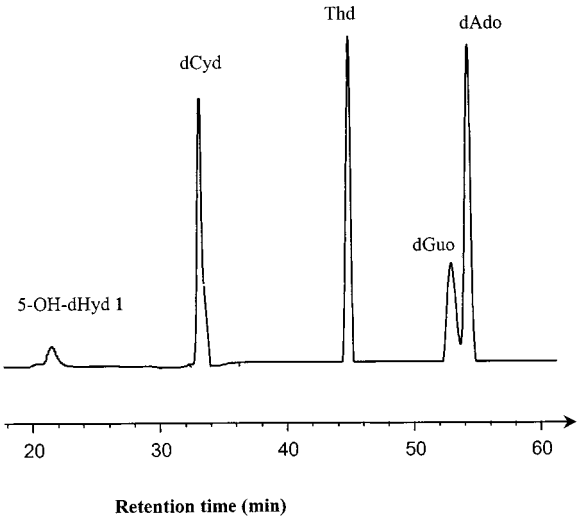


Figure 4. Reversed-phase HPLC profile of the enzymatic digestion mixture of 14-*mer*-modified oligonucleotide **9** by nuclease P_1 (2 h) and alkaline phosphatase (1 h) using a porous graphitized Hypercarb carbon column

the exonucleases was followed by withdrawal of aliquots from the digestion mixtures over increasing periods of time. The resulting DNA fragments were then analyzed by MALDI-TOF MS.[21,22] The different molecular ions observed when using this powerful technique correspond to digested DNA fragments, which differ in mass as the consequence of successive loss of nucleotides. The difference in mass between two successive fragments enables the released nucleotide to be identified, and hence the overall sequence of the oligonucleotides to be determined. Thus, MALDI-TOF MS analysis allows the location of the lesion within the DNA strand to be assessed. In addition, relevant information on the integrity of **1** and the processing of the damage by the exonucleases was gained.

The unmodified 14-*mer* oligonucleotide 5′-d(ATC GTG ACT GAT CC)-3′, used first as a control, was totally hydrolyzed both by 5′-exonucleases and by 3′-exonucleases in less than 10 min, allowing the complete sequence to be determined (data not shown). In contrast, the presence of 5-OH-dHyd (**1**) in ODN induces total resistance to digestion by both 3′-exonucleases and 5′-exonucleases at the site of the lesion. Bovine intestinal mucosa phosphodiesterase sequentially degraded the oligonucleotide **9** from the 3′-end until it reached 5-OH-dHyd (**1**), the phosphodiester bond of which is resistant to further cleavage even after prolonged treatment (Figure 5). This was inferred from observation, after 1 h of incubation, of a single peak at *m*/*z* = 2414.2 Da, corresponding to the positive ion $[M + H]^+$ of the 8-*mer* 5′-d(ATC GTG A**X**)-3′ (calcd. mass = 2414.6 Da). Different behavior was observed for the calf spleen phosphodiesterase mediated digestion of **9**, which starts from the opposite end (Figure 6, A and B). The enzymatic hydrolysis after 5 min shows sequential cleavage of the normal nucleosides (Figure 6, A). After 2 h of incubation (Figure 6, B), the mass spectrum exhibited a major peak at 2373.9 Da, corresponding to the positive ions $[M + H]^+$ of the 8-*mer* 5′-d(A**X**T GAT CC)-3′ (calcd. mass = 2374.6 Da). This indicates that the 5′-exonuclease is able to digest the 14-*mer* oligonucleotide sequentially from the 5′-end until it reaches the 2′-deoxyadenosine nucleoside before the 5-OH-dHyd (**1**) lesion, the phosphodiester bond of which is resistant to further cleavage. Even after longer periods of enzymatic treatment, the 5′-exonuclease failed to release 5-OH-dHyd (**1**). Similar results have previously been observed for 5-OH-5-Me-dHyd.[15] It has already been shown that the enzymatic activity of exonucleases is affected by the presence of modifications within the DNA chain. This is the case for tandem lesions including formylamine and 8-oxo-7,8-dihydro-2′-deoxyguanosine and the opposite

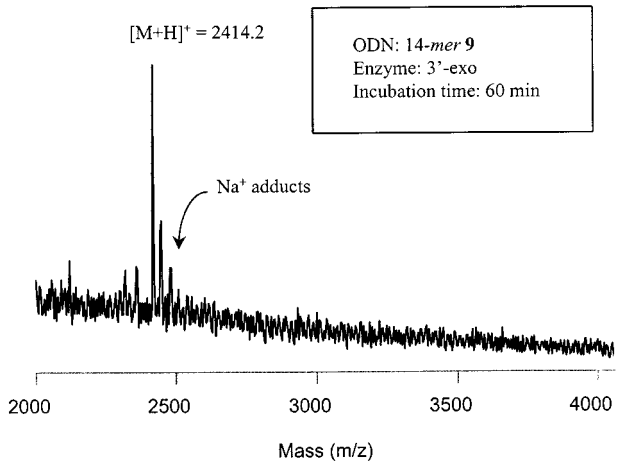


Figure 5. MALDI-TOF mass spectrum, in positive mode, of the products resulting from the digestion of the 14-*mer*-modified oligonucleotide **9** upon incubation with the 3′-exonuclease for 60 min

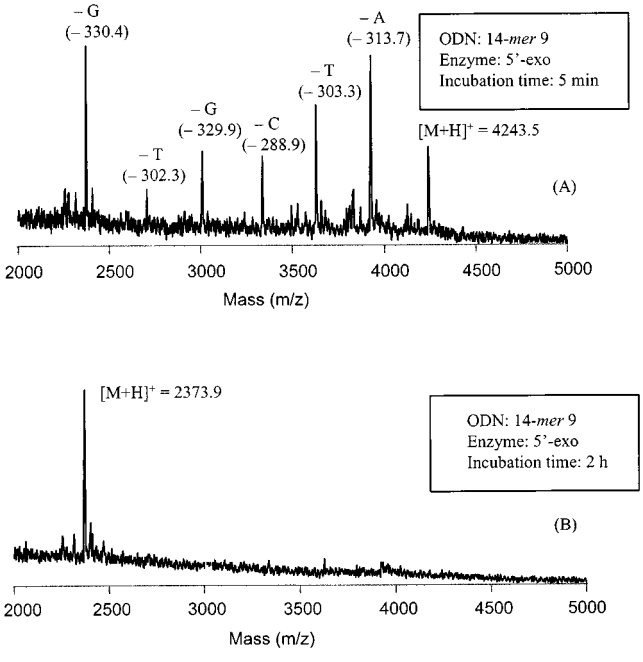


Figure 6. MALDI-TOF mass spectrum, in positive mode, of the products resulting from the digestion of the 14-*mer*-modified oligonucleotide **9** upon incubation with the 5′-exonuclease for 5 min (A) and 120 min (B)

sequence[24] together with 5′,8-cyclopurine nucleosides[25,26] that are refractory to the hydrolytic activity of 3′-exonucleases and 5′-exonucleases.

Conclusion and Perspectives

The synthesis of 5-OH-dHyd (**1**) and its incorporation into several oligonucleotides with the aid of the phosphoramidite approach were achieved using mildly alkaline deprotection conditions. The synthetic oligonucleotides were isolated in good yields and characterized by several complementary techniques, demonstrating the integrity of the incorporated modified nucleoside. The piperidine experiment performed on the modified oligonucleotides indicated an instability in the 5-OH-dHyd (**1**) lesion, which leads to a few strand cleavages. The processing of **1** by several nucleases was also investigated. Thus, it was shown that nuclease P_1 is able to cleave the 5-OH-dHyd (**1**) residue from the oligonucleotides, while both bovine intestinal mucosa phosphodiesterase and calf spleen phosphodiesterase failed to release this modified moiety from the DNA fragments. These results should be taken into account for the development of assays geared towards measuring the level of formation of **1** either in isolated oxidized DNA or in cellular oxidized DNA. Therefore, the use of nuclease P_1 together with Micrococcus nuclease and alkaline phosphatase appears to be a suitable approach to achieve quantitative release of 5-OH-dHyd (**1**) from oxidized DNA. Moreover, these modified DNA fragments are suitable tools for further biochemical studies, including DNA repair specificity assessment and mutagenic potential evaluation. The oligonucleotides will also be used to determine the conformational changes induced by the modified nucleosides.

Experimental Section

General: The silica gel, Geduran® Si 60, used for low-pressure column chromatography was purchased from Merck (Darmstadt, Germany). Thin-layer chromatography was carried out on Macherey−Nagel DC Kieselgel Polygram® Sil G /UV_{254} (0.2 mm) plastic sheets (Düren, Germany). Deuterated solvents were purchased from Acros (Geel, Belgium). − All reagents were of the highest available purity. Anhydrous solvents for synthesis were obtained from SDS. Acetonitrile and methanol (HPLC grade) were purchased from Carlo Erba (Milan, Italy). Buffers for high performance liquid chromatography (HPLC) were prepared using water purified with a Milli-Q system (Milford, MA). [γ-^{32}P]ATP was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). [γ-^{32}P]ATP, NAP-25 Sephadex and Microspin G-25 columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Ozone was generated using a Labo 76 ozonizer (Trailigaz, Garges-les-Gonesse, France).

Enzymes: Nuclease P_1 (*Penicillium citrinium*) and bovine intestinal mucosa phosphodiesterase (3′-*exo*) were obtained from Sigma (St Louis, MO). Calf spleen phosphodiesterase (5′-*exo*) and calf intestinal alkaline phosphatase were purchased from Boehringer-Mannheim (Mannheim, Germany). T_4 polynucleotide kinase was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

NMR Measurements: 200-MHz ^{1}H NMR and 100-MHz ^{13}C NMR spectra were recorded with AC 200 and AM 400 Bruker spectrometers, respectively (Bruker, Wissembourg, France) operating in the Fourier transform mode. The chemical shifts are reported in ppm (parts per million), using the residual proton signal of TMS ($\delta_H = 0$), [D_6]acetone ($\delta_H = 2.17$), or [D_6]DMSO ($\delta_H = 2.62$) as the external reference. The ^{31}P NMR measurements were performed using a Unity 400 Varian; H_3PO_4 (85%) was chosen as the external standard.

Mass Spectrometry Measurements: All modified and unmodified oligonucleotides were characterized by electrospray ionization mass spectrometry measurement (ESI-MS) using a Platform 3000 model spectrophotometer from Micromass (Manchester, UK). Typically, 0.1 AU_{260nm} of the sample was dissolved in a solution of acetonitrile and water (50:50, v/v) containing 1% triethylamine, prior to analysis in the negative mode. The modified nucleosides were analyzed by ESI-MS in both the positive and the negative modes. For the measurements performed in the positive mode, the samples were dissolved in an acetonitrile/water (50:50, v/v) solution containing 0.5% formic acid. − MALDI mass spectra were obtained with a commercially available, time-of-flight mass spectrometer (Voyager-DE, Perseptive Biosystems, Framingham, MA) equipped with a 337-nm nitrogen laser and a pulsed delay source extraction as previously described.[18]

High-Performance Liquid Chromatography Separations. − System A: Reversed-phase HPLC (porous graphitized Hypercarb carbon column, 98.5% carbon, 5 μm, 250 Å, 10053 mm i.d.) with a mixture of acetonitrile and 25 mM ammonium formate buffer (AF, pH = 6.2) as the eluents [100% AF (5 min), linear gradient from 0 to 20% of acetonitrile (30 min)] at a flow rate of 0.4 mL/min. UV detection at 230 nm. − **System B:** Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm i.d.) with a mixture of acetonitrile and 25 mM AF buffer (pH = 6.2) as the eluents [linear gradient from 0 to 70% of acetonitrile (30 min)] at a flow rate of 1 mL/min. UV detection at 230 nm. − **System C:** Reversed-phase HPLC (Hamilton PRP_3, polymeric phase column, 10 μm, 305 × 7.0 mm i.d.) with a mixture of acetonitrile and 10 mM triethylammonium acetate buffer (TEAA, pH = 7) as the eluents [100% TEAA (5 min), then isocratic TEAA/acetonitrile (92:8 v/v) (13 min); after, isocratic 100% TFA (1%) (10 min) and finally a gradient from 0 to 10% acetonitrile (40 min)]; flow rate: 2 mL·min^{-1}; UV detection at 260 nm. − **System D:** Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm i.d.) with a mixture of acetonitrile and 10 mM TEAA buffer (pH = 7) as the eluents [100% TEAA (5 min), linear gradient from 0 to 10% of acetonitrile (30 min)] at a flow rate of 1 mL·min^{-1}; UV detection at 260 nm. − **System E:** Reversed-phase HPLC (porous graphitized Hypercarb carbon column, 98.5% carbon, 5 μm, 250 A, 100 × 3 mm i.d.) with a mixture of acetonitrile and 25 mM ammonium formate buffer (AF, pH = 6.2) as the eluents [linear gradient from 0 to 28% of acetonitrile (60 min)] at a flow rate of 0.4 mL/min. UV detection was performed at 230 nm during the first 30 min and then at 260 nm.

Synthetic Procedures

(5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (1):** Commercial 2′-deoxycytidine (227 mg, 1 mmol) was dissolved in water (50 mL) and subjected to the action of ozone (3−4% in O_2) at room temperature over 1 h, 30 min. The course of the reaction was followed by HPLC (system A). The solvent was then removed by evaporation under reduced

pressure. After lyophilization (20 h), the crude product was purified by chromatography on a silica gel column with a step gradient of methanol (0−20%) in dichloromethane as the mobile phase. The appropriate fractions were pooled and then concentrated to dryness, giving 50 mg of compound **1** as a white foam (yield 20%). The analytical values found for **1** were the same as those described in the literature.[11,12] − 200-MHz ^{1}H NMR ([D$_6$]DMSO; *second diastereoisomer): δ = 6.02 (t, 0.6 H, *J* = 6.1 Hz, H-1′), 5.94 (t, 0.4 H, *J* = 6.1 Hz, H-1′*), 5.37 (s, 1 H, H-5, H-5*), 4.29 (m, 1 H, H-3′, H-3′*), 3.73 (m, 1 H, H-4′, H-4′*), 3.27−3.22 (m, 2 H, H-5′, H-5′*, H-5′′, H-5′′*), 2.48 (m, 1 H, H-2′, H-2′*), 2.05 (m, 1 H, H-2′′, H-2′′*). − ESI-MS (positive mode): *m*/*z* = 233.1 [M + H]$^+$ (calcd. 233.2), 250.1 [M + Na]$^+$ (calcd. 250.2), 117 [2-deoxy-D-*erythro*-pentose]; ESI-MS (negative mode): *m*/*z* = 231.1 [M − H]$^−$ (calcd. 231.2).

3′-*O*-(*tert*-Butyldimethylsilyl)-2′-deoxycytidine (2): Compound **2** was prepared according to the method developed by Yang et al.[23] − R_f = 0.22 (CH_2Cl_2/MeOH, 95:5); λ_{max} = 231 nm. − 200-MHz ^{1}H NMR ([D$_6$]DMSO) δ = 7.72 (d, 1 H, *J* = 7.52 Hz, H-6), 7.10 (s, 2 H, NH_2), 6.10 (t, 1 H, *J* = 6.6 Hz, H-1′), 5.68 (d, 1 H, H-5), 4.97 (t, 1 H, OH-5′), 4.32 (m, 1 H, H-3′), 3.71 (m, 1 H, H-4′), 3.49 (m, 2 H, H-5′, H-5′′), 1.99 (m, 2 H, H-2′, H-2′′), 0.83 [s, 9 H, $C(CH_3)_3$], 0.07 [s, 6 H, $Si(CH_3)_2$]. − ESI-MS (positive mode): *m*/*z* = 342.1 [M + H]$^+$ (calcd. 342.2).

(5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-3-*O*-(*tert*-butyldimethylsilyl)-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (4):** Compound **2** (5 g, 14.7 mmol) was dissolved in CH_3CN/H_2O (1:1 v/v, 600 mL) and the resulting solution was subjected to the action of ozone (3−4% in O_2) for 4 h at room temperature. The reaction was checked for completion by HPLC (system B). The solvents were then removed by evaporation under reduced pressure. After lyophilization (6 h), 4.1 g of a white solid that contained 3′-*O*-silylated-5-OH-hydantoin **3** was obtained. The crude product was then redissolved in dry pyridine (30 mL) and the resulting solution was concentrated to dryness. The operation was repeated twice and the resulting residue was dissolved in 100 mL of dry pyridine. 4-(Dimethylamino)pyridine (DMAP) (60 mg, 0.49 mmol) and 4,4′-dimethoxytrityl chloride (6.4 g, 18.92 mmol) were added to the solution. After 20 h at room temperature, the reaction mixture was cooled to 5 °C (ice bath) and methanol (2 mL) was then added. The mixture was concentrated to dryness after 10 min. The resulting red oil was then redissolved in dichloromethane (300 mL), washed with saturated aq. $NaHCO_3$ (300 mL), dried with Na_2SO_4, and concentrated under vacuum. The residue thus obtained was purified by chromatography on a silica gel column with a step gradient of methanol (0−5%) in dichloromethane as the mobile phase. The appropriate fractions were pooled and then concentrated to dryness, giving 2.7 g of compound **4** as a yellow foam [yield 28% (two steps)]. − R_f = 0.45 and 0.43 (CH_2Cl_2/MeOH, 95:5); λ_{max} = 232 nm. − 200-MHz ^{1}H NMR ([D$_6$]DMSO, *second diastereoisomer): δ = 7.52−7.36 (m, 9 H, aromatic H of DMTr), 7.05−6.95 (m, 4 H, aromatic H of DMTr), 6.07 (t, 0.6 H, *J* = 6.1 Hz, H-1′), 5.96 (t, 0.4 H, *J* = 6.1 Hz, H-1′*), 5.35 (s, 1 H, H-5,H-5*), 4.29 (m, 1 H, H-3′, H-3′*), 3.84 (s, 6 H, C*H*$_3$O DMTr), 3.78 (m, 1 H, H-4′, H-4′*), 3.27−3.22 (m, 2 H, H-5′, H-5′*, H-5′′, H-5′′*), 2.48 (m, 1 H, H-2′, H-2′*), 2.05 (m, 1 H, H-2′′, H-2′′*), 0.91 [s, 9 H, $C(CH_3)_3$], 0.10 [s, 6 H, $Si(CH_3)_2$]. − ESI-MS (positive mode): *m*/*z* = 649.2 [M + H]$^+$ (calcd. 649.3), 671.4 [M + Na]$^+$ (calcd. 671.3), 303.3 [DMTr]$^+$; ESI-MS (negative mode): *m*/*z* = 647.4 [M − H]$^−$ (calcd. 647.3).

(5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-3-*O*-(*tert*-butyldimethylsilyl)-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-5-*O*-levulinylhydantoin (5):** Compound **4** (2.7 g, 4.16 mmol) was dried by repeated coevaporation with anhydrous dichloromethane and then dissolved in dry THF (100 mL) under argon. *N*,*N*′-dicyclohexylcarbodiimide (DCC) (2.15 g, 10.40 mmol), DMAP (50 mg, 0.41 mmol), and finally levulinic acid (870 μL, 8.50 mmol) were added to the stirred solution. After 20 h at room temperature, the reaction mixture was cooled to 5 °C in an ice bath and quenched by addition of methanol (0.6 mL). The white precipitate of 1,3-dicyclohexylurea (DCU) was removed by filtration, and washed twice with dichloromethane (40 mL). The resulting filtrate was then concentrated under reduced pressure. The resulting dry residue was redissolved in dichloromethane (150 mL), washed with saturated aq. $NaHCO_3$ (100 mL), dried with Na_2SO_4, and concentrated under vacuum. Chromatography of the crude product on a silica gel column, eluting with a step gradient of methanol from 0 to 3% in dichloromethane, afforded **5** as a white foam (2 g, 64%). − R_f = 0.64 (CH_2Cl_2/MeOH, 95:5); λ_{max} = 232 nm. − 200-MHz ^{1}H NMR ([D$_6$]DMSO, *second diastereoisomer): δ = 7.49−7.36 (m, 9 H, aromatic H of DMTr), 7.05−6.98 (m, 4 H, aromatic H of DMTr), 6.24 (s, 1 H, H-5, H-5*), 5.93 (t, 0.6 H, H-1′, *J* = 6.1 Hz), 5.77 (t, 0.4 H, H-1′*, *J* = 6.1 Hz), 4.24 (m, 1 H, H-3′, H-3′*), 3.85 (s, 6 H, C*H*$_3$O DMTr), 3.82 (m, 1 H, H-4′, H-4′*), 3.29−3.21 (m, 2 H, H-5′, H-5′*, H-5′′, H-5′′*), 2.61−2.09 (m, 6 H, CH_2−CH_2, H-2′, H-2′*, H-2′′, H-2′′*), 2.21 (s, 3 H, $COCH_3$), 0.92 [s, 9 H, $C(CH_3)_3$], 0.12 [s, 6 H, $Si(CH_3)_2$]. − ESI-MS (negative mode): *m*/*z* = 745.1 [M − H]$^−$ (calcd. 745.3).

(5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]-5-*O*-levulinylhydantoin (6):** Compound **5** (2 g, 2.68 mmol) was dissolved in dry THF (100 mL). A solution of TBAF (22 mL) in THF (1 M) was added, and the resulting mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the resulting oil was redissolved in dichloromethane (100 mL). The organic layer was then washed with saturated aq. $NaHCO_3$ (100 mL), dried with Na_2SO_4, and concentrated under vacuum. The resulting yellow oil was purified by chromatography on a silica gel column. Elution was performed with a step gradient of MeOH from 0 to 3% in dichloromethane. The appropriate fractions were pooled and then concentrated to dryness giving 900 mg of compound **6** as a white foam (yield 53%). − R_f = 0.36 and 0.34 (CH_2Cl_2/MeOH, 90:10); λ = 234 nm. − 200-MHz ^{1}H NMR ([D$_6$]acetone, *second diastereoisomer): δ = 7.59−7.28 (m, 9 H, aromatic H of DMTr), 7.02−6.94 (m, 4 H, aromatic H of DMTr), 6.34 (s, 1 H, H-5, H-5*), 6.16 (t, 0.6 H, H-1′, *J* = 6.2 Hz), 6.10 (t, 0.4 H, H-1′*, *J* = 6.1 Hz), 4.30 (m, 1 H, H-3′, H-3′*), 3.90 (s, 6 H, C*H*$_3$O DMTr), 3.75 (m, 1 H, H-4′, H-4′*), 3.37−3.26 (m, 2 H, H-5′, H-5′*, H-5′′, H-5′′*), 2.50−2.20 (m, 6 H, CH_2−CH_2, H-2′, H-2′*, H-2′′, H-2′′*), 2.24 (s, 3 H, $COCH_3$). − ESI-MS (positive mode): *m*/*z* = 655.1 [M + Na]$^+$ (calcd. 655.2), 303.3 [DMTr]$^+$; ESI-MS (negative mode): *m*/*z* = 631.1 [M − H]$^−$ (calcd. 631.2).

(5*R) and (5*S**) Diastereoisomers of 1-{2-Deoxy-3-*O*-[2-cyanoethoxy(diisopropylamino)phosphanyl]-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl}-5-*O*-levulinylhydantoin (7):** The nucleoside **6** (100 mg, 0.16 mmol) was coevaporated twice with dry dichloromethane and dried under vacuum for 1 h. The dry residue was then redissolved in 4 mL of dry CH_2Cl_2 under argon. Dry DIPEA (122 μL, 0.70 mmol), followed by chloro-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (78 μL, 0.35 mmol), were added with vigorous stirring. After 2 h, the reaction mixture was quenched by addition of methanol (100 μL). The resulting mixture was diluted 10 min later with dichloromethane (30 mL), washed successively

with saturated aq. $NaHCO_3$ (30 mL) and saturated aq. NaCl (30 mL), dried with Na_2SO_4, and concentrated under vacuum. The resulting yellow oil was deposited on a silica gel column, which was eluted with a step gradient of methanol from 0 to 2% in dichloromethane/TEA (99.9:0.1, v/v). The appropriate fractions were pooled and then concentrated to dryness, giving 67 mg (0.08 mmol) of the phosphoramidite synthon **7** as a white foam (yield of 51%). − R_f = 0.45 and 0.43 (CH_2Cl_2/TEA/MeOH, 95:0.1:5). − 200-MHz ^{1}H NMR ([D$_6$]acetone, *second diastereoisomer): δ = 7.64−7.30 (m, 9 H, aromatic H of DMTr), 7.05−6.98 (m, 4 H, aromatic H of DMTr), 6.38 (s, 1 H, H-5, H-5*), 6.10 (t, 0.6 H, *J* = 6.3 Hz, H-1′), 5.92 (t, 0.4 H, *J* = 6.2 Hz, H-1′*), 4.33 (m, 1 H, H-3′, H-3′*), 3.91 (s, 6 H, C*H*$_3$O DMTr), 3.75 (m, 1 H, H-4′, H-4′*), 3.90−3.31 (m, 6 H, H-5′, H-5′*, H-5′′, H-5′′*, 2 NC*H*(CH$_3$)$_2$, CH$_2$C*H*$_2$OP), 2.89−2.37 (m, 8 H, H-2′, H-2′*, H-2′′, H-2′′*, C*H*$_2$C*H*$_2$COCH$_3$, CH$_2$C*H*$_2$CN), 2.25 (s, 3 H, COC*H*$_3$), 1.45−1.28 (m, 12 H, 2 NCH(C*H*$_3$)$_2$]. − ^{31}P NMR ([D$_6$]acetone) δ = 149.41, 149.14 (1 P, s). − ESI-MS (negative mode): *m*/*z* = 831.1 [M − H]$^-$ (calcd. 832.2).

Stability Studies on the (5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (1) under the Alkaline Conditions Used for the Chemical Synthesis of Oligonucleotides:** Aqueous ammonia (30%, 1 mL) was added to 0.2 AU_{230nm} of compound **1** in sealed tubes, and the resulting solutions were kept either at room temperature or at 55 °C. The reactions were then quenched at increasing time intervals (0, 1, 2, 4, 16, and 24 h) by freezing the samples in liquid nitrogen and subsequent lyophilization. The samples were then analyzed by reversed-phase HPLC (system A).

Stability Studies on the (5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (1) under the Acidic Conditions Used for the Chemical Synthesis of Oligonucleotides:** A similar procedure to that described above for the alkali stability assays was applied to study the behavior of **1** in 80% acetic acid aqueous solution after incubation for 0, 1, 2, 4, 8, 16, and 24 h, respectively, at room temperature.

Stability Studies on the (5*R) and (5*S**) Diastereoisomers of 1-[2-Deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (1) under Oxidizing Conditions Used for the Chemical Synthesis of Oligonucleotides:** Similarly, compound **1** was incubated in a 0.1 M oxidizing solution of iodine for 1, 2, 4, 8, 16, and 24 h at room temperature.

Solid-Phase Synthesis of Oligonucleotides: The oligonucleotides containing the (5*R**) and (5*S**) diastereoisomers of 1-[2-deoxy-β-D-*erythro*-pentofuranosyl]-5-hydroxyhydantoin (**1**) were prepared by phosphoramidite solid-phase synthesis using "Pac chemistry", with retention of the 5′-terminal DMTr group ("trityl-on" mode). Therefore, phenoxyacetyl, isopropylphenoxyacetyl, and acetyl groups were used to protect the amino functions of dAdo, dGuo, and dCyd, respectively. Because of its insolubility in acetonitrile, the phosphoramidite synthon **7** was dissolved in dry dichloromethane and then placed in the addition port of a model 392 DNA synthesizer (Applied Biosystem), using the standard 1-μmol synthesis scale. The duration of the condensation was increased by a factor of 10 for the modified nucleoside phosphoramidite **7** (300 s instead of 30 s for normal nucleoside phosphoramidites).

Deprotection and Purification of Oligonucleotides: Upon completion of the synthesis, the oligonucleotides were cleaved from the solid support and deprotected by treatment with concentrated aqueous ammonia (30%) at room temperature for 4 h. After evaporation of the solvent under vacuum, the crude 5′-DMTr oligonucleotides were purified and deprotected on-line by reversed-phase HPLC (system C). The purity and the homogeneity of the collected fractions were then checked by HPLC analysis (system D). Three oligonucleotides comprising **8**, **9**, and **10** were thus obtained. The modified 14-*mer* oligonucleotide **9** used for the enzymatic studies and the piperidine assay was further purified by PAGE, using a 20% polyacrylamide/7 M urea gel, and then desalted using a NAP-25 Sephadex column.

^{32}P-Labeling of the Oligonucleotides: The modified 14-*mer* oligonucleotide **9** (50 pmol) was labeled at the 5′-end with 5 μCi of [γ-^{32}P]ATP (2 pmol, 10 mCi/mL) upon incubation with T_4 polynucleotide kinase (9.5 units) in 10 μL of supplied buffer at 37 °C for 30 min. The reaction was then stopped by addition of 1 μL of a 0.5 M EDTA solution (pH = 8). Non-incorporated [γ-^{32}P]ATP was removed by purification of the oligonucleotides on MicroSpin G-25 columns.

Stability Studies on Oligonucleotides Containing 5-OH-dHyd (1) in Piperidine Solutions: Modified 14-*mer* oligonucleotide **9**, containing 5-OH-dHyd (**1**), was treated with a freshly prepared 1 M aqueous piperidine solution at 90 °C for 15 min, 30 min, and 1 h. Typically, the reactions were carried out on 0.5 pmol of 5′-[^{32}P]-labeled modified oligonucleotides in 50 μL of the piperidine solution in sealed tubes. The mixtures were then cooled and coevaporated twice with water, before being loaded onto a 20% polyacrylamide/7 M urea gel in TBE buffer [50 mM tris, 50 mM boric acid, and 50 mM EDTA (pH = 8)]. Electrophoresis was carried out at 1500 V for 2 h. Subsequently, detection of the radiolabeled bands was achieved by autoradiography of the gels.

Enzymatic Digestion of Modified Oligonucleotides by Nuclease P_1 and Alkaline Phosphatase: Modified 14-*mer* oligonucleotide **9** (0.5 AU_{260nm}) in water (45 μL) was digested into nucleosides at 37 °C by incubation for 2 h (or 24 h) with 5 U nuclease P_1 (1 U/μL) in an aqueous solution containing NaOAc (30 mM) and $ZnSO_4$ (0.1 mM, pH = 5.5). Tris-HCl buffer (5 μL, 10 X) and calf intestinal alkaline phosphatase (2 U) were then added. The resulting mixture was subsequently incubated at 37 °C for 1 h and then diluted in 50 μL of 25 mM AF (pH = 6.2). After centrifugation, the mixture was finally analyzed by reversed-phase HPLC (system E). The digestion products were identified by HPLC after co-injection with synthetic standards. Further confirmation was obtained by electrospray ionization mass spectrometry analysis of HPLC-collected peaks in the negative mode. The same enzymatic digestion procedure was applied to the synthetic trinucleotide **8** and the modified 22-*mer* oligonucleotide **10**.

Enzymatic Digestion of Modified Oligonucleotides by Calf Spleen Phosphodiesterase (5′-*exo*): Modified 14-*mer* oligonucleotide **9** (0.2 AU_{260nm}) in 0.02 M ammonium citrate (20 μL, pH = 5) was digested by incubation at 37 °C with calf spleen phosphodiesterase (10^{-3} U). Aliquots (2 μL) were withdrawn at increasing periods of time and the reactions were stopped by addition of 50 μL of water. The solutions were then frozen in liquid nitrogen and lyophilized. The resulting dry residues were taken up in 20 μL of 0.1% aqueous solution of trifluoroacetic acid and subsequently analyzed by MALDI-TOF spectrometry, following the procedure described above.

Enzymatic Digestion of Modified Oligonucleotides by Bovine Intestinal Mucosa Phosphodiesterase (3′-*exo*): Similarly, enzymatic digestions of modified 14-*mer* oligonucleotide **9** was performed using bovine intestinal mucosa phosphodiesterase (10^{-5} U) in ammonium citrate buffer (pH = 9). MALTI-TOF spectrometric analyses were then performed as described above.

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