Synthesis and characterization of oligodeoxynucleotides containing the two 5*R* and 5*S* diastereomers of (5′*S*,6*S*)-5′,6-cyclo-5,6-dihydrothymidine; radiation-induced tandem lesions of thymidine

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The insertion of the (5′*S*,5*R*,6*S*) and (5′*S*,5*S*,6*S*) diastereomers of 5′,6-cyclo-5,6-dihydrothymidine into oligonucleotides is described. Due to the poor reactivity of the 5′-OH group of this modified nucleoside, the preparation of its 3′-phosphoramidite synthon **8** required the use of the non-standard 5′-levulinyl protecting group. It was successfully applied to the solid-phase synthesis of various oligonucleotides ranging from 3 to 22 bases long in combination with conventional dimethoxytrityl mononucleoside phosphoramidite chemistry. Characterization of these modified DNA fragments by enzymic digestions and mass spectroscopic analyses confirmed the designated sequences and compositions.

Introduction

In the last decade, many efforts have been devoted to the synthesis of modified oligodeoxynucleotides (ODNs) containing specific DNA lesions at selected sites in defined sequences.[1] Indeed, they are powerful tools to investigate the biological consequences of such DNA lesions through mutagenesis and enzymic repair studies. Among the numerous modified nucleosides chemically inserted, those caused by free radicals and ionizing radiation have received some attention, particularly in our laboratory.[1,2]

Thus, our research is currently focused on the preparation of DNA fragments containing carbon-bridged cyclonucleosides. Such modified nucleosides, which may be considered as products of tandem DNA lesions, the base and the 2-deoxyribose residues both being altered, have been found to be generated in both purine and pyrimidine nucleic acid monomeric components and in DNA polymers upon exposure to ionizing radiation.[3–5] Several authors speculated that the conformational distortions of DNA generated by the additional covalent bond between the base and the sugar-phosphate backbone might have a significant biological impact especially toward enzymic repair processes.[4,6] To investigate the effects of such anhydronucleosides on the structure and biological functions of the nucleic acids in which they are present, we have recently synthesized ODNs ranging from 3 to 22 bases long and containing each of the 5′*R* and 5′*S* diastereomers of 5′,8-cyclo-2′-deoxyadenosine and 5′,8-cyclo-2′-deoxyguanosine.[7,8] Interestingly, enzymic studies and repair experiments have shown that these modified DNA fragments were not completely hydrolyzed by mixtures of endo- and exonucleases and were not substrates for DNA N-glycosylases.

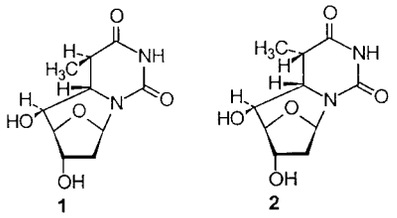
In the present paper, we describe the chemical insertion of a pyrimidine analogue of the latter compounds which has been detected in γ-irradiated frozen aqueous solutions of thymidine: *viz*., 5′,6-cyclo-5,6-dihydrothymidine (CyclodHThd). Six of the eight possible diastereomers have been isolated and fully characterized by extensive mass spectrometry and ^{1}H NMR analyses.[5] However, the significant epimerization at C-5 occurring under basic conditions used for oligonucleotide deprotection prevents the insertion of a unique 5*R* or 5*S* diastereomer. Consequently, a mixture of 5*R* and 5*S* diastereomers of (5′*S*,6*S*)-CyclodHThd **1** and **2** was used in this work. Furthermore, the loss of aromaticity of the modified nucleosides **1** and **2** and the poor reactivity of their secondary 5′-OH alcohol function (*vide infra*) required the development of a synthetic strategy entirely different from that used for the preparation and subsequent incorporation of the phosphoramidite synthons of 5′,8-cyclopurine-2′-deoxyribonucleosides. The method reported here which involves the use of the non-standard 5′-*O*-levulinyl (Lev) protecting group for the CyclodHThd 3′-phosphoramidite, provided the expected modified ODNs in high yields. The CyclodHThd-containing oligonucleotides have been used for enzymic studies.

Results and discussion

Prior to the preparation of the phosphoramidite synthon **8**, the stability of CyclodHThd **1** and **2** was checked at room temperature (rt) under the three main experimental conditions used in the course of the solid-support synthesis. These include treatments with 30% aq. ammonia, 80% acetic acid and a commercial oxidizing solution of iodine. Authentic samples of (5′*S*,5*R*,6*S*)-CyclodHThd **1** and (5′*S*,5*S*,6*S*)-CyclodHThd **2**, obtained by desilylation of 3′-*O*-(*tert*-butyldiphenylsilyl)-CyclodHThd **5** (*vide infra*), were used in the stability studies. Aliquots of the reaction mixtures were taken up at increasing periods of time and analyzed by reversed-phase HPLC (system A), using thymidine as an internal standard. No detectable degradation of CyclodHThd was observed after 24 h of incubation. Only the isomerization between the two diastereomers **1** and **2** occurred under the basic conditions used. Such epimerization at C-5, which has been already reported by Schulhof *et al.* with 5,6-dihydrothymidine,[9] prevents the single chemical insertion of the two diastereomers into DNA fragments. The main consequence of the high stability of CyclodHThd is the choice of the standard amino-protecting groups for the natural nucleoside: benzoyl (Bz) for 2′-deoxyadenosine (dAdo) and isobutyryl (iBu) for 2′-deoxycytidine (dCyd) and 2′-deoxyguanosine (dGuo).

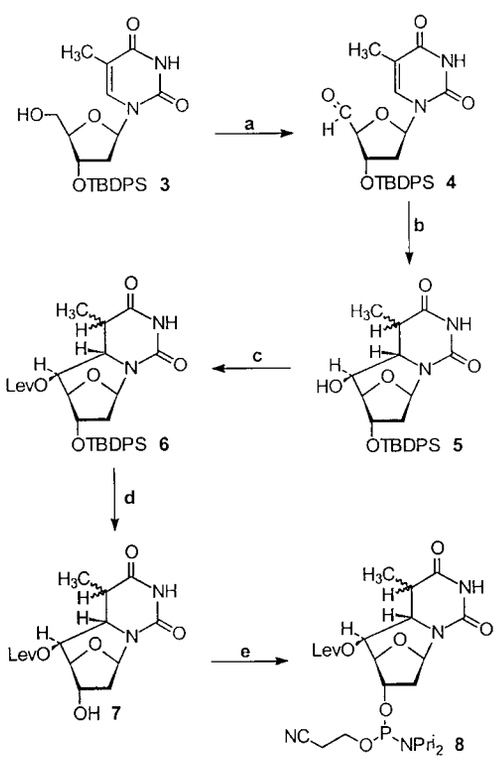
Preparation of the phosphoramidite building block of CyclodHThd

The preparation of the phosphoramidite synthon **8** has required the development of an efficient method for the synthesis of CyclodHThd. The methodology used for the synthesis of 5′,8-cyclopurine-2′-deoxyribonucleosides (photochemical



Chemical structures of (5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-CyclodHThd.

cyclization followed by chemical functionalization at C-5′) is not applicable to the pyrimidine nucleosides. Indeed, preliminary experiments (data not shown) and several works in the literature have shown that intramolecular cyclizations involving C-5′ alkyl radicals of pyrimidine nucleosides, upon generation by photolysis or chemical homolysis, afford non-aromatic 5′,6-cyclopyrimidine-2′,5′-dideoxyribonucleosides.[10] Consequently, the C-5′ position of these cyclonucleosides cannot be functionalized by the oxidation–reduction procedure involving the use of selenium dioxide (SeO_2) as reagent for the selective benzylic oxidation.[11] Therefore, we have investigated other synthetic routes leading to the formation of 5′,6-cyclopyrimidines exhibiting a hydroxy function at C-5′. A survey of the literature revealed a variety of available chemical methods for the preparation of such compounds, particularly in the RNA series.[12] The methodology developed by Sugawara *et al.* for the preparation of (5′*S*,6*S*)-5′,6-cyclo-5,6-dihydrouridine[12c] was chosen. In fact, the tin radical-mediated intramolecular cyclization proceeded in a highly stereospecific manner together with good yield. In addition, the nucleoside 5′-aldehyde used as a precursor in this chemical cyclization can be easily obtained from a 3′-*O*-protected derivative of thymidine. Thus, the synthesis of the targeted phosphoramidite **8** (Scheme 1) required for the incorporation of compounds **1** and **2**, started with the available 3′-*O*-(*tert*-butyldiphenylsilyl) (TBDPS) ether **3**.[13] The TBDPS group was chosen because it is a suitable marker for detecting UV products such as CyclodHThd derivatives on TLC and



Scheme 1 Synthetic reactions used for the preparation of the phosphoramidite synthon of CyclodHThd. *Reagents*: a) Dess–Martin periodinane, CH_2Cl_2; b) Bu_3SnH, AIBN, benzene; c) levulinic acid, DCC, DMAP, THF; d) TEA·3HF, THF; e) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, DIEA, CH_2Cl_2.

column chromatography. Subsequent oxidation of **3** using the Dess–Martin periodinane in anhydrous CH_2Cl_2 affords the 5′-aldehyde **4** in 70% yield.[14] Cyclization of **4** was accomplished by treatment with a benzene solution of tributyltin hydride (Bu_3SnH) and AIBN at refluxing temperature. Dropwise addition (over 5 h) of an excess of these reagents led to the formation of two main products which were identified as 3′-*O*-(TBDPS)-thymidine **3** and 3′-*O*-(TBDPS)-5′,6-cyclo-5,6-dihydrothymidine **5**. The 5′,6-cyclonucleoside was isolated by silica gel chromatography (yield 61%) and its structure was confirmed by detailed measurements including fast-atom bombardment (FAB) mass spectrometry in the positive mode ($m/z = 481.0 \pm 0.1$ Da) and NMR analyses. The ^{1}H NMR spectrum of **5** in $CDCl_3$ showed the presence of two diastereomers in a 2.7 : 1 ratio as determined by integration of the well separated anomeric proton resonances (minor: δ 6.32, major: δ 6.21). Selective decoupling experiments allowed us to assign all the signals. A common feature to both diastereomers of **5** is the lack of detectable coupling between H-1′ and H-2′ on the one hand and between H-3′ and H-4′ on the other hand which is characteristic of 5′,6-cyclopyrimidine nucleosides.[5] This is due to the occurrence of a severe distortion of the sugar moiety upon the formation of the (C-5′, C-6) covalent bond. The stereochemistry of both diastereomers was determined by comparison of the coupling constants with those reported by Shaw and Cadet for six of the eight possible diastereomers of CyclodHThd.[5] Thus, the values of $^3J_{4'5'}$ (4.4 Hz), $^3J_{5'6'}$ (9.4 Hz) and $^3J_{56}$ (3.8 Hz) for the major diastereomer are consistent with the 5′*S*,5*S*,6*S* configuration and those of $^3J_{4'5'}$ (4.4 Hz) and $^3J_{5'6}$ (8.7 Hz) for the minor diastereomer are indicative of the 5′*S*,5*R*,6*S* configuration. Attempts to convert **5** into the 5′-*O*-DMTr-protected derivative failed. Several methods described in the literature for the tritylation of hindered secondary or tertiary alcohols have been applied, *i.e.* (i) the use of an excess of DMTr chloride under refluxing pyridine[15] (ii) the use of DMTr chloride in combination with a strong base (DBU)[16] (iii) the use of DMTr triflate in pyridine at room temperature.[17] In all cases, no tritylation of the secondary 5′-OH group was observed. To overcome this major difficulty in the preparation of the phosphoramidite synthon of CyclodHThd, a non-standard 5′-OH protecting group which is less sterically hindered than the substituted trityl groups was used. We have chosen the Lev group which has already been successfully applied to the solid-phase synthesis of oligoribonucleotides by the phosphoramidite and phosphotriester approaches.[18,19] It can be easily introduced on secondary alcohol functions and its removal under neutral conditions (0.5 M hydrazine monohydrate in 3 : 2 pyridine–acetic acid at rt) is compatible with the stability of the acyl and cyanoethyl groups that protect the amino and phosphate residues respectively of the non-modified nucleosides. Acylation of the sterically hindered secondary 5′-OH group of **5** with an excess of levulinic acid in anhydrous THF in the presence of DCC and DMAP furnished the fully protected cyclonucleoside **6** in 77% yield. Selective desilylation of the 3′-*O*-TBDPS ether was achieved by a treatment with triethylamine trishydrofluoride (TEA·3HF) in THF.[20] This protocol allowed almost quantitative recovery of 5′-*O*-(Lev)-CyclodHThd **7** in 90% yield, whereas the usual tetrabutylammonium fluoride (TBAF) silyl-deprotection procedure gave rise to a deacylation side-reaction.[21] The desired phosphoramidite **8** was finally synthesized in 74% yield after column chromatographic purification, by reaction of the secondary alcohol of **7** with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite [$NCCH_2CH_2OP(Cl)NPr^i_2$] in the presence of *N*,*N*-diisopropylethylamine (DIEA).[22]

Solid-phase synthesis and characterization of oligodeoxynucleotides containing CyclodHThd

The modified ODNs **9**–**12** (Table 1) were synthesized according

Table 1 Sequences and relative molecular masses (Da) of the modified ODNs synthesized

	Sequences	Length	Mass (calc.) (Da)	Mass (found) (Da)
9	5′-d(C**X**G)-3′ (first diastereomer)	3	860.59	861.25
9	second diastereomer	3	860.59	860.55
10	5′-d(A**X**C)-3′ (first diastereomer)	3	844.59	845.39
10	second diastereomer	3	844.59	844.44
11	5′-d(ATCGTGAC**X**GATCT)-3′	14	4253.71	4254.21
12	5′-d(CACTTCGGA**X**CGTGACTGATCT)-3′	22	6701.23	6700.50

X = CyclodHThd. All the oligonucleotides' masses have been obtained by MALDI-TOF-MS in the positive mode.

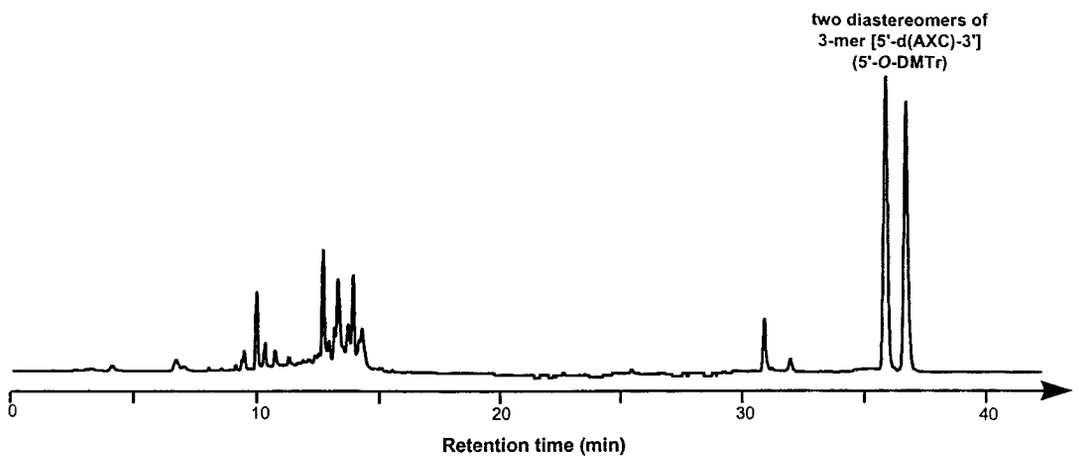


Fig. 1 Reversed-phase HPLC profile of the crude reaction mixture of 3-mer [5′-d(AXC)-3′] **10** which mostly consists of two diastereomers with X = (5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-CyclodHThd.

to the solid-phase phosphoramidite method (1 μmol scale) on an automated DNA synthesizer using **8** and commercially available 2′-deoxyribonucleosides as 2-cyanoethyl phosphoramidite derivatives. The incorporation of the modified monomer required the following modifications: its coupling time was slightly extended with respect to that used for normal nucleoside phosphoramidites and the automatic detritylation step was replaced by manual hydrazine treatment (see Experimental section). Due to the poor reactivity of the 5′-secondary alcohol function of **8**, the duration of the coupling of the next nucleoside involved in the oligonucleotide sequences [*i.e.*, 2′-deoxyadenosine (dAdo) or 2′-deoxycytidine (dCyd)] was also increased. After chain elongation (trityl-on mode), the modified ODNs were cleaved from the Controlled Pore Glass (CPG) solid support, deprotected with aq. ammonia at rt for 25 h and analyzed by reversed-phase HPLC (system B). The HPLC elution profile of the crude reaction mixture of 3-mer [5′-d(AXC)-3′] **10** (Fig. 1) illustrates the good coupling yield of compound **8**. Indeed, it exhibits two main peaks (~50 : 50 ratio) corresponding to the two diastereomers of (trityl-on) modified ODNs. The crude 5′-*O*-DMTr oligomers were purified by reversed-phase HPLC, detritylated on the HPLC column and then further purified by reversed-phase HPLC (system C). The purity and homogeneity of the collected fractions were controlled by HPLC (system D) and gel electrophoresis. Generally, more than 30 AU_{260nm} of the purified oligomers **11** and **12** were obtained following a 1 μmol-scale synthesis. Determination of the relative molecular masses of the modified ODNs was accomplished by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Table 1). These results confirmed the incorporation of CyclodHThd **1** and **2** and the purity of oligomers **9**–**12**. A fraction of **12** was enzymically hydrolyzed by successive incubation with nucleases P_1 and bacterial alkaline phosphatase. The resulting mixture of 2′-deoxyribonucleosides was analyzed by reversed-phase HPLC (system E). This provided dCyd, dGuo, Thd, dAdo and a trimer [5′-d(AXC)-3′] in 5 : 5 : 6 : 3 : 1 proportions, confirming the structure of the modified 22-mer (Fig. 2a). The absence of any detectable amounts of the free modified nucleosides **1** and **2** was further confirmed by co-injection of the enzymic digestion products with authentic samples of (5′*S*,5*R*,6*S*)-CyclodHThd **1** and (5′*S*,5*S*,6*S*)-CyclodHThd **2**. The structure of the trimer was confirmed by MALDI-TOF-MS analysis of the collected peaks and by co-injection of the enzymic digestion with the two diastereomers of **10** previously synthesized (Fig. 2b). Further treatment of **10** with nuclease P_1 and alkaline phosphatase did not afford the free 2′-deoxyribonucleosides dCyd, dGuo and CyclodHThd **1** and **2** even after 24 h of incubation. Similar results were obtained with the other CyclodHThd oligonucleotides. They are consistent with previous studies that clearly showed the inhibition of nuclease P_1 towards the hydrolysis of phosphodiester bonds between normal and altered 2′-deoxyribonucleosides.[7,23,24] In order to obtain additional information about the effect of the tandem DNA lesions **1** and **2** on the behavior of nucleases, we have also studied the stability of the modified ODNs towards exonucleases. The enzymic digestions of **11** by snake venom phosphodiesterase (3′-exo) and calf spleen phosphodiesterase (5′-exo) were monitored by MALDI-TOF-MS according to the methodology developed by Pieles *et al.*[25] Snake venom phosphodiesterase sequentially degraded the oligonucleotide **11** from the 3′-end until it reached CyclodHThd **1** and **2**, which is resistant to further cleavage (Fig. 3). Indeed, the mass spectrum after 2 h of 3′-exo enzymic digestion exhibited a single peak at $m/z = 2713.47 \pm 0.1$ Da, corresponding to the positive ion $[M + H]^+$ of the 9-mer [5′-d(ATCGTGACX)-3′] **13** (Calc. 2713.75 Da). A different behavior was observed for the calf spleen phosphodiesterase-mediated digestion of **11**, which starts from the opposite end (Fig. 4). Within 5 min of incubation, the enzyme induced the release of the first seven nucleotides. Thereafter, the presence of the 5′,6-cyclonucleoside **1** and **2** dramatically reduced the rate of digestion. The mass spectrum of the enzymic hydrolysate, after 1 h of incubation, exhibited two main peaks at $m/z = 2385.70 \pm 0.1$ Da and 2072.53 ± 0.1 Da corresponding to the positive ions $[M + H]^+$ of the 8-mer [5′-d(ACXGATCT)-3′] **14** and the 7-mer [5′-d(CXGATCT)-3′] **15** (calc. 2071.35 Da).

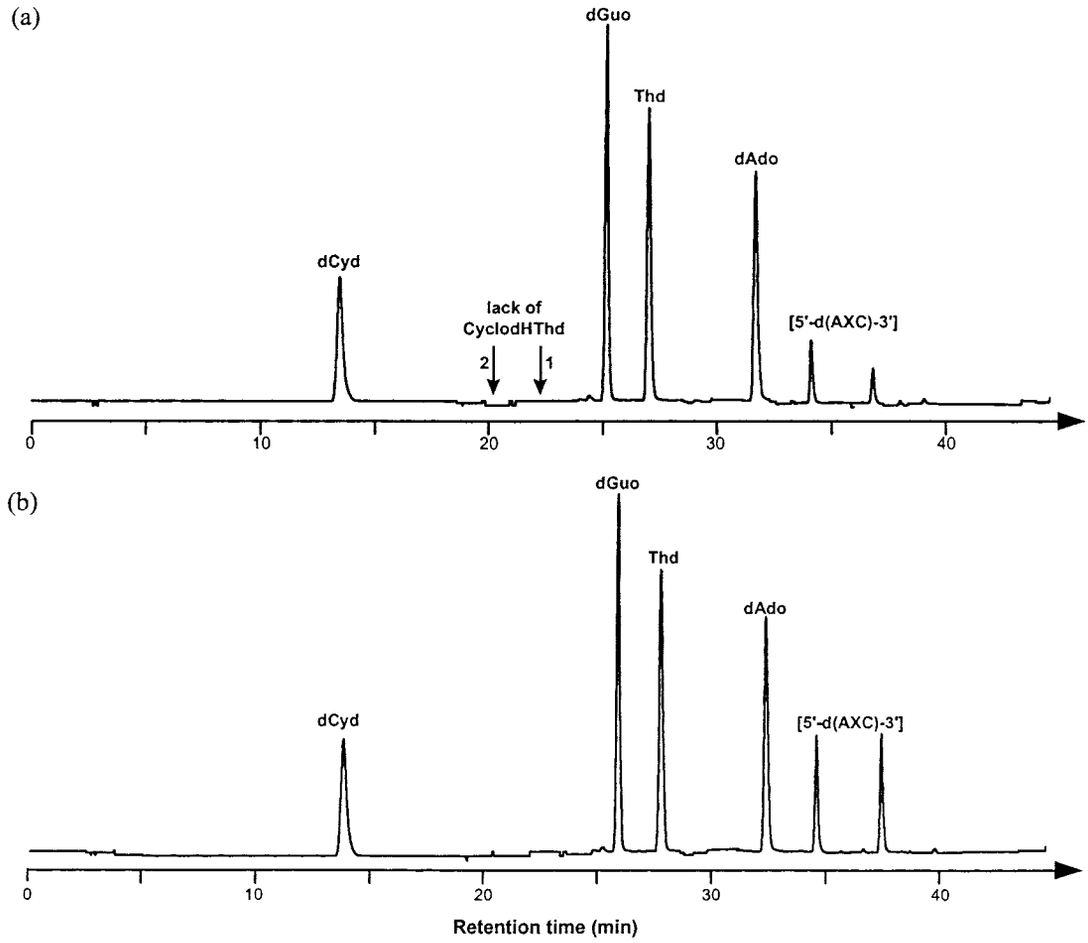


Fig. 2 (a) Reversed-phase HPLC profile of the enzymic digestion mixture of 22-mer **12**. (b) Co-injection with the two diastereomers of trimer **10**.

Thus, calf spleen phosphodiesterase failed to cleave the phosphodiester linkages between CyclodHThd and dCyd on the 5′-side and dGuo on the 3′-side, respectively. Consequently, the modified ODNs could not be fully digested to the monomer level by a combination of 3′-exo and 5′-exonucleases. Similar results have been already obtained with the 5′,8-cyclopurine-2′-deoxyribonucleosides.[7,8]

Conclusion and perspectives

In conclusion, we have developed a convenient and efficient method for the synthesis of CyclodHThd-containing oligonucleotides. These modified DNA fragments are suitable probes for further investigations aimed at assessing the structural and biochemical effects of cross-link formation between the thymine base and the 2-deoxyribose moieties. Furthermore, this versatile synthetic methodology can be used for the incorporation, into oligonucleotides, of other radiation-induced 5′,6-cyclopyrimidines such as 5′,6-cyclo-5,6-dihydro-2′-deoxyuridine and 5′,6-cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine.[5,26]

Experimental

General

The silica gel (70–200 μm) used for the low-pressure column chromatography was purchased from SDS (Peypin, France). TLC was carried out on Merck DC Kieselgel 60 F-254 plastic sheets. Deuterated solvents (acetone-d_6, $CDCl_3$ and D_2O) were purchased from Acros (Geel, Belgium). $CDCl_3$ was passed through a small plug of Al_2O_3 immediately prior to use. All reagents used were of the highest available purity. Anhydrous solvents were purchased from SDS. The HPLC-grade solvents

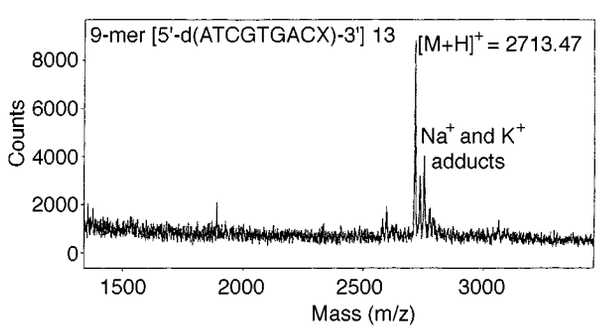


Fig. 3 MALDI-TOF mass spectrum, in the positive mode, of 14-mer **11** after 2 h of incubation with snake venom phosphodiesterase (3′-exo).

(acetonitrile and methanol) were obtained from Carlo Erba (Milan, Italy). Alkaline phosphatase, nuclease P_1 (*Penicilium citrinium*) and 0.3 M sodium acetate–$ZnCl_2$ buffer (pH = 5.3) were purchased from Sigma (St Louis, MO). Snake venom phosphodiesterase and calf spleen phosphodiesterase were obtained from Boehringer Mannheim (Mannheim, Germany). Buffers for HPLC were prepared using water purified with a Milli-Q system (Milford, MA).

Instrumental

^{1}H NMR (200 and 400 MHz), ^{13}C NMR (101 MHz) and ^{31}P NMR (101 MHz) spectra were recorded with AC200, WM250 and AM400 Bruker spectrometers operating in the Fourier transform mode (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from acetone-d_6 ($\delta_H = 2.17$), $CDCl_3$ ($\delta_H = 7.25$) or D_2O ($\delta_H = 4.92$) for ^{1}H NMR spectra and calibration of ^{31}P NMR was achieved with 85%

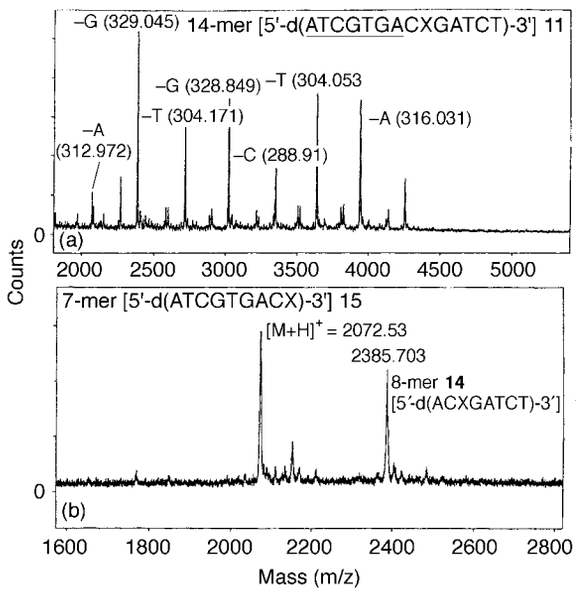


Fig. 4 MALDI-TOF mass spectra, in the positive mode, of partial calf spleen phosphodiesterase (5′-exo) digestion of 14-mer **11** (a) after 5 min and (b) after 64 min.

H_3PO_4 as an external standard. *J*-Values are in Hz. Fast-atom bombardment (FAB) mass spectra were recorded on a TRIO.2 spectrometer (Micromass), Electrospray ionization (ESI) mass spectra were recorded on an LCQ spectrometer (Thermoquest) and matrix-isolated laser desorption ionization (MALDI) mass spectra were obtained with a Voyager-DE time-of-flight (TOF) mass spectrometer (Perseptive Biosystems, Framingham, MA).

High-performance liquid chromatography separations

Several liquid chromatographic systems were used for the analytical experiments and the purification steps. **System A**: reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm id) with acetonitrile and ammonium formate buffer (AF, 25 mM, pH = 6.2) as the eluents [100% AF (10 min), linear gradient from 0 to 10% of acetonitrile (30 min)] at a flow rate of 1 ml min^{-1}. The UV detection was achieved at 230 nm. **System B**: reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm id) with acetonitrile AF (25 mM, pH = 6.2) as the eluents [100% AF (1 min), linear gradient from 0 to 40% of acetonitrile (34 min)] at a flow rate of 1 ml min^{-1}. UV detection was achieved at 260 nm. **System C**: purification of 5′-[4,4′-dimethoxytrityl (DMTr)]oligomers by reversed-phase HPLC (Hamilton PRP3, polymeric phase column, 10 μm, 305 × 7.0 mm id) with acetonitrile and triethylammonium acetate buffer (TEAA, 25 mM, pH = 7) as the eluents using a non-linear gradient [100% TEAA (5 min), then isocratic TEAA–acetonitrile (92:8) v/v (10 min)]; in order to remove the DMTr group, the oligomers were treated with 1% aq. trifluoroacetic acid [isocratic 100% TFA (1%) (6 min)]. The resulting deprotected oligomers were again purified using the same eluents [gradient from 2 to 12% of acetonitrile over a 22 min period]. The flow rate was 2.5 ml min^{-1} and the UV detection was achieved at 254 nm. **System D**: System B with a new gradient of acetonitrile and FA (25 mM, pH = 6.2) [100% AF (2 min), linear gradient from 0 to 12% of acetonitrile (25 min)]. **System E**: System A but the UV detection was achieved at 260 nm during the first 20 min, then at 230 nm for 5 min and back to 260 nm for the remainder of the HPLC analyses.

(5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-5′,6-Cyclo-5,6-dihydrothymidine 1 and 2

Compound **5** (116 mg, 0.24 mmol) was dissolved in dry THF (5 cm^3). A solution of TBAF (0.48 cm^3, 0.48 mmol) in THF (1 M) was added and the resulting mixture was stirred at rt for 14 h. The reaction was checked for completion by TLC and the solvent was evaporated under reduced pressure. Chromatography of the crude product on silica gel (30 g) and elution with a gradient of methanol 0–12% in dichloromethane afforded a mixture of two diastereomers of CyclodHThd as a powder (1:1; 50 mg, 95%), R_f 0.30 on TLC in dichloromethane–methanol (90:10 v/v); $\delta_H(D_2O)$ 6.45 (1 H, d, *J* 6.5, H-1′), 6.36 (1 H, d, *J* 6.2, H-1′), 4.76 (2 H, m, H-3′), 4.46 (1 H, d, *J* 4.8, H-4′), 4.39 (1 H, d, *J* 4.8, H-4′), 4.11 (1 H, q, J_1 4.8, J_2 9.9, H-5′), 3.94 (1 H, q, J_1 5.1, J_2 9.1, H-5′), 3.36–3.26 (1 H, m, H-6), 3.02 (2 H, m, H-5), 2.66–2.54 (2 H, m, J_1 7.5, J_2 −14.5, H-2′), 2.32–2.20 (2 H, m, H′-2″), 1.47 (3 H, d, *J* 7.0, CH_3), 1.38 (3 H, d, *J* 7.3, CH_3); ESI-MS *m*/*z* 242.4 $[M + H]^+$.

1-[3′-*O*-(*tert*-Butyldiphenylsilyl)-2′-deoxy-β-D-*erythro*-pento-5-dialdo-1,4-furanosyl]thymine 4

Nucleoside **3**[13] (0.96 g, 2 mmol) was coevaporated with dry dichloromethane (2 × 5 cm^3) and re-dissolved in dry dichloromethane (30 cm^3). Dess–Martin periodinane (1.017 g, 2.4 mmol) was added at rt. After stirring for 1 h, the mixture was poured into 5% aq. $NaHCO_3$–saturated aq. $Na_2S_2O_3$ (70 cm^3; 1:1 v/v), extracted with dichloromethane (30 cm^3), and the extract was dried (Na_2SO_4) and evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (75 g). Elution was achieved with a step gradient of ethyl acetate from 10 to 65% in hexane [10% (100 cm^3), 30% (100 cm^3), 45% (200 cm^3), 50% (100 cm^3) and 65% (300 cm^3)]. Aldehyde **4** was obtained as a foam (0.677 g, 70%), R_f 0.39 on TLC in ethyl acetate–hexane (75:25 v/v); $\delta_H(CDCl_3)$ 9.08 (1 H, s, H-5′), 8.52 (1 H, br s, NH), 7.71–7.39 (11 H, m, H-6, Ph), 6.53 (1 H, q, J_1 5.3, J_2 9.1, H-1′), 4.60 (1 H, m, H-3′), 4.50 (1 H, s, H-4′), 2.44–1.94 (5 H, m, H-2′, H-2″, CH_3), 1.10 [9 H, s, $C(CH_3)_3$]; $\delta_C(CDCl_3)$ 197.48 (C-5′), 163.48 and 159.98 (C-2 and -4), 135.88, 135.62, 135.54, 132.36, 132.16, 130.36, 130.28, 130.06, 128.05, 128.01 and 127.85 (C-6, Ph), 111.20 (C-5), 91.02 (C-4′), 87.09 (C-1′), 72.44 (C-3′), 38.97 (C-2′), 26.68 [$C(CH_3)_3$], 18.90 [$C(CH_3)_3$], 12.46 (CH_3); FAB-MS *m*/*z* 479.0 $[M + H]^+$.

(5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5′,6-cyclo-5,6-dihydrothymidine 5

A mixture of Bu_3SnH (0.76 cm^3, 2.82 mmol) and AIBN (115 mg, 0.70 mmol) in benzene (30 cm^3) was added dropwise to a solution of **4** (0.677 g, 1.41 mmol) in benzene (35 cm^3) under reflux over a period of 5 h. The reaction was checked for completion by TLC and the solvent was evaporated under reduced pressure. The resulting oil was deposited on a silica gel column (75 g), which was eluted with a step gradient of ethyl acetate from 10 to 70% in hexane (see above). Evaporation to dryness of the (EtOAc–hexane, 50:50) chromatographic fractions yielded a mixture of two diastereomers of cyclonucleoside **5** as a foam (2.7:1; 0.414 g, 61%); $\delta_H(CDCl_3)$ 7.72–7.36 (10 H, m, Ph), 6.32* (1 H, d, *J* 5.9, H-1′), 6.21 (1 H, d, *J* 5.6, H-1′), 4.59* (1 H, dd, J_1 3.2, J_2 7.0, H-3′), 4.53 (1 H, dd, J_1 3.2, J_2 7.4, H-3′), 4.12 (1 H, d, *J* 4.4, H-4′), 4.06* (1 H, d, *J* 4.4, H-4′), 3.66 (1 H, q, *J* 9.4, H-5′), 3.50* (1 H, q, *J* 8.7, H-5′), 3.01 (1 H, q, *J* 3.8, H-6), 2.75 (3 H, m, H-5*, H-6), 2.41 (1 H, m, H-2′), 2.34* (1 H, m, H-2′), 2.20 (1 H, m, *J* −15.8, H-2″), 2.13* (1 H, m, *J* −15.8, H-2″), 1.26* (3 H, d, *J* 7.0, CH_3), 1.17 (3 H, d, *J* 7.3, CH_3), 1.06 [9 H, s, $C(CH_3)_3$]; FAB-MS *m*/*z* 481.0 $[M + H]^+$.

* Minor diastereomer: (5′*S*,5*R*,6*S*).

(5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5′-*O*-levulinyl-5′,6-cyclo-5,6-dihydrothymidine 6

Compound **5** (0.3 g, 0.62 mmol) was coevaporated with dry dichloromethane (2 × 5 cm^3) and re-dissolved in dry THF (15 cm^3). DCC (322 mg, 1.56 mmol), DMAP (7 mg, 0.06 mmol)

and levulinic acid (0.13 cm^3, 1.26 mmol) were added at rt. After stirring for 3 h, the reaction was stopped by addition of methanol (0.1 cm^3). The precipitate of 1,3-dicyclohexylurea (DCU) was removed by filtration and washed with dichloromethane (50 cm^3). The filtrate was evaporated under reduced pressure and the resulting yellow oil was purified by chromatography on a silica gel column (50 g). Elution was achieved with a gradient of methanol 0–3% in dichloromethane. Evaporation to dryness of the (CH_2Cl_2–MeOH, 97:3) chromatographic fractions provided a mixture of two diastereomers of cyclonucleoside **6** as an oil (3.5:1; 0.277 g, 77%); R_f 0.61 on TLC in dichloromethane–methanol (94:6 v/v); $\delta_H(CDCl_3)$ 7.67–7.33 (10 H, m, Ph), 6.32* (1 H, d, *J* 5.9, H-1′), 6.22 (1 H, d, *J* 5.6, H-1′), 5.00 (1 H, q, J_1 4.8, J_2 9.5, H-5′), 4.81* (1 H, q, J_1 5.1, J_2 9.1, H-5′), 4.58–4.46 (4 H, m, H-3′, H-3′*, H-4′, H-4′*), 3.24 (1 H, q, *J* 3.5, H-6), 3.01* (1 H, t, *J* 9.9, H-6), 2.89–2.21 (10 H, m, H-5, H-5*, CH_2CH_2, H-2′, H-2′*, H-2″, H-2″*), 2.15 and 2.14 (3 H, 2 s, $COCH_3$), 1.24* (3 H, d, *J* 7.0, CH_3), 1.15 (3 H, d, *J* 7.3, CH_3), 1.02 [9 H, s, $C(CH_3)_3$]; ESI-MS *m*/*z* 601.2 $[M + Na]^+$.

* Minor diastereomer: (5′*S*,5*R*,6*S*).

(5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-5′-*O*-Levulinyl-5′,6-cyclo-5,6-dihydrothymidine 7

Compound **6** (0.277 g, 0.48 mmol), dissolved in dry THF (5 cm^3), was treated with TEA·3HF (0.55 cm^3, 3.32 mmol) and stirred at rt for 23 h. The reaction was checked for completion by TLC and the solvent was evaporated under reduced pressure. The resulting yellow oil was purified by column chromatography on silica gel (30 g) using a gradient of methanol 0–6% in dichloromethane as the eluent. Chromatographic fractions were evaporated to dryness, yielding a mixture of two diastereomers of cyclonucleoside **7** as a foam (2.7:1; 0.146 g, 90%); R_f 0.53 on TLC in dichloromethane–methanol (90:10 v/v); $\delta_H(CDCl_3)$ 6.35* (1 H, d, *J* 5.6, H-1′), 6.27 (1 H, d, *J* 5.6, H-1′), 5.00 (1 H, q, J_1 4.7, J_2 9.5, H-5′), 4.85* (1 H, q, J_1 5.0, J_2 8.8, H-5′), 4.75–4.60 (2 H, m, H-3′, H-3′*), 4.44 (1 H, d, *J* 4.4, H-4′), 4.42* (1 H, d, *J* 4.4, H-4′), 3.56 (1 H, q, *J* 3.8, H-6), 3.30* (1 H, t, *J* 10.3, H-6), 3.35–2.23 (10 H, m, H-5, H-5*, CH_2CH_2, H-2′, H-2′*, H-2″, H-2″*), 2.20 (3 H, s, $COCH_3$), 1.39* (3 H, d, *J* 7.3, CH_3), 1.29 (3 H, d, *J* 7.3, CH_3); ESI-MS *m*/*z* 341.0 $[M + H]^+$.

* Minor diastereomer: (5′*S*,5*R*,6*S*).

(5′*S*,5*R*,6*S*)- and (5′*S*,5*S*,6*S*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphine]-5′-*O*-levulinyl-5′,6-cyclo-5,6-dihydrothymidine 8

Cyclonucleoside **7** (0.146 g, 0.43 mmol) was coevaporated with dry dichloromethane (2 × 5 cm^3), dissolved in dichloromethane (5 cm^3) and kept under an argon atmosphere. DIEA (0.165 cm^3, 0.95 mmol) was added followed by dropwise addition of 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (0.105 cm^3, 0.47 mmol) at rt. After stirring for 30 min, the reaction mixture was cooled to 5 °C with an ice-bath and quenched by addition of DIEA (0.165 cm^3) and methanol (0.1 cm^3). The solvent was evaporated under reduced pressure and the resulting yellow oil was purified by column chromatography on silica gel (30 g) and using a gradient of methanol 0–4% in dichloromethane–TEA (99:1 v/v) as the eluent. Evaporation to dryness of the chromatographic fractions yielded a mixture of four diastereomers for the phosphoramidite derivative **8** as a foam (0.172 g, 74%); R_f 0.89 on TLC in dichloromethane–methanol–TEA (92:8:1 v/v/v); δ_H(acetone-d_6) 6.42* (1 H, d, *J* 6.2, H-1′), 6.31 (1 H, d, *J* 5.9, H-1′), 5.13 (1 H, m, H-5′), 4.92 (3 H, m, H-5′*, H-3′, H-3′*), 4.58 (2 H, m, H-4′, H-4′*), 4.36–3.53 [6 H, m, H-6, H-6*, 2NC*H*$(CH_3)_2$, CH_2CH_2OP], 3.05–2.37 (12 H, m, H-5, H-5*, CH_2CH_2CN, $CH_2CH_2COCH_3$, H-2′, H-2′*, H-2″, H-2″*), 2.27–2.23 (3 H, s, $COCH_3$), 1.40–1.30 [15 H, CH_3, NCH$(CH_3)_2$]; δ_P(acetone-d_6) 151.83, 151.71, 151.53, 151.36 (1 P, s); ESI-SM *m*/*z* 563.2 $[M + Na]^+$, 539.3 $[M - H]^-$.

* Minor diastereomers: (5′*S*,5*R*,6*S*).

Solid-phase synthesis of oligonucleotides

Oligonucleotides containing CyclodHThd **1** and **2** were prepared by phosphoramidite solid-phase synthesis using the Bz group for dAdo and the iBu group for dGuo and dCyd. The synthesized phosphoramidite **8** was found to be insoluble in acetonitrile. Consequently, **8** (94 mg, 0.17 mmol) was dissolved in 1.1 cm^3 of dry dichloromethane and placed in the additional port of a model 392 DNA synthesizer (ABI). The standard 1 μmol synthesis scale with retention of the 5′-terminal DMTr group (trityl-on mode) was used with the following modifications. The duration of the condensation of the modified monomer **8** and the next nucleoside involved in the oligonucleotide sequences was increased with respect to that of the standard monomers (5 min instead of 40 s for normal nucleoside phosphoramidites). After incorporation of **8**, the machine was stopped and the CPG column was disconnected. With two gastight syringes, one on each end of the column, 3 cm^3 of a freshly prepared solution of hydrazine monohydrate (0.5 M; pyridine–acetic acid 3:2 v/v) were passed over the resin by slowly pushing the solution from one syringe to the other. This was performed for 5 min. After washing with dry acetonitrile (3 × 1 cm^3), the CPG column was placed again in the DNA synthesizer, and the remaining nucleotides were added.

Deprotection and purification of oligonucleotides

Following synthesis, the solid supports were placed in conc. aq. ammonia (30%) in sealed vials at rt for 25 h. The crude 5′-DMTr oligomers were purified and deprotected on-line by reversed-phase HPLC (System C).

Enzymic digestions of oligomers 11 and 12

0.5 AU_{260nm} of purified oligomers **11** and **12** were taken up in 45 μl of water. Then 5 μl of a solution of nuclease P_1 in acetate buffer (1 unit μl^{-1}) was added. The resulting mixture was incubated at 37 °C for 2 h (or 24 h). Subsequently, 5 μl of Tris-HCl buffer (10×) and 2 units of alkaline phosphatase were added, and the resulting mixture was incubated for a further 1 h at 37 °C. Subsequently, the mixture was taken up in 50 μl of FA (25 mM, pH = 6.2) and analyzed by reversed-phase HPLC (system E). The 2′-deoxyribonucleosides were quantified on the basis of the integrals of the absorption peak areas at 260 nm. The following molar extinction coefficients (260 nm, $\times 10^{-3}$) were used: 7.05 (dCyd), 12.01 (dGuo), 8.40 (Thd), 15.20 (dAdo). Due to the lack of absorption of CyclodHThd at 260 nm, sums of epsilon-values of normal 2′-deoxyribonucleosides were used for the trimers [5′-d(CXG)-3′] and [5′-d(AXC)-3′].

Snake venom phosphodiesterase (3′-exo) and calf spleen phosphodiesterase (5′-exo) digestions

The enzymic digestions of oligomer **11** by exonucleases were followed by MALDI-TOF mass spectrometry according to a methodology previously described.[7,24]

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References

1 S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, **49**, 6123.
2 (*a*) A. Guy, J. Dubet and R. Téoule, *Tetrahedron Lett.*, 1993, **34**, 8101; (*b*) L. C. Sowers and G. P. Beardsley, *J. Org. Chem.*, 1993, **58**,

1664; (*c*) A. Matsuda, M. Inada, H. Nara, E. Ohtsuka and A. Ono, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 2751; (*d*) A. Ono, T. Okamoto, M. Inada, H. Nara and A. Matsuda, *Chem. Pharm. Bull.*, 1994, **42**, 2231; (*e*) T. J. Matray and M. M. Greenberg, *J. Am. Chem. Soc.*, 1994, **116**, 6931; (*f*) T. Berthod, Y. Pétillot, A. Guy, J. Cadet, E. Forest and D. Molko, *Nucleosides, Nucleotides*, 1996, **15**, 1287; (*g*) T. Berthod, Y. Pétillot, A. Guy, J. Cadet and D. Molko, *J. Org. Chem.*, 1996, **61**, 6075; (*h*) H. Sugiyama, S. Matsuda, K. Kino, Q.-M. Zhang, S. Yonei and I. Saito, *Tetrahedron Lett.*, 1996, **37**, 9067; (*i*) S. Tardy-Planechaud, J. Fujimoto, S. S. Lin and L. C. Sowers, *Nucleic Acids Res.*, 1997, **25**, 553; (*j*) A. Romieu, D. Gasparutto, D. Molko and J. Cadet, *Tetrahedron Lett.*, 1997, **38**, 7531; (*k*) J. Fujimoto, L. Tran and L. C. Sowers, *Chem. Res. Toxicol.*, 1997, **10**, 1254; (*l*) M. L. Morningstar, D. A. Kreutzer and J. M. Essingmann, *Chem. Res. Toxicol.*, 1997, **10**, 1345; (*m*) M. Kotera, A.-G. Bourdat, E. Defrancq and J. Lhomme, *J. Am. Chem. Soc.*, 1998, **120**, 11810; (*n*) A.-G. Bourdat, D. Gasparutto and J. Cadet, *Nucleic Acids Res.*, 1999, **27**, 1015.

3 J. Cadet and M. Berger, *Int. J. Radiat. Biol.*, 1985, **47**, 127.

4 M. Dizdaroglu, *Biochem. J.*, 1986, **238**, 247.

5 A. A. Shaw and J. Cadet, *Int. J. Radiat. Biol*, 1988, **54**, 987.

6 T. Lindahl, *Nature* (*London*), 1993, **362**, 709.

7 A. Romieu, D. Gasparutto, D. Molko and J. Cadet, *J. Org. Chem.*, 1998, **63**, 5245.

8 A. Romieu, D. Gasparutto and J. Cadet, *Chem. Res. Toxicol.*, in press.

9 J. C. Schulhof, D. Molko and R. Téoule, *Nucleic Acids Res.*, 1988, **16**, 319.

10 (*a*) Y. Yamagata, S. Fujii, T. Fujiwara, K.-I. Tomita and T. Ueda, *Biochim. Biophys. Acta*, 1981, **654**, 242; (*b*) T. Ueda and S. Shuto, *Heterocycles*, 1982, **17**, 95; (*c*) T. Ueda, H. Usui, S. Shuto and H. Inoue, *Chem. Pharm. Bull.*, 1984, **32**, 3410; (*d*) Y. Suzuki, A. Matsuda and T. Ueda, *Chem. Pharm. Bull.*, 1987, **35**, 1085.

11 A. Matsuda, M. Tezuka, K. Niizuma, E. Sugiyama and T. Ueda, *Tetrahedron*, 1978, **34**, 2633.

12 (*a*) J. A. Rabi and J. J. Fox, *J. Org. Chem.*, 1972, **37**, 3898; (*b*) B. A. Otter, E. A. Falco and J. J. Fox, *J. Org. Chem.*, 1976, **41**, 3133; (*c*) T. Sugawara, B. A. Otter and T. Ueda, *Tetrahedron Lett.*, 1988, **29**, 75; (*d*) K. Haraguchi, H. Tanaka, S. Saito, K. Yamaguchi and T. Miyasaka, *Tetrahedron Lett.*, 1994, **35**, 9721; (*e*) K. Haraguchi, H. Tanaka, S. Saito, S. Kinoshima, M. Hosoe, K. Kanmuri, K. Yamaguchi and T. Miyasaka, *Tetrahedron*, 1996, **52**, 9469.

13 C.-O. Yang, H. Y. Wu, E. B. Fraser-Smith and K. A. M. Walker, *Tetrahedron Lett.*, 1992, **33**, 37.

14 D. B. Dess and J. C. Martin, *J. Am. Chem. Soc.*, 1991, **113**, 7277.

15 M. Sekine and T. Hata, *J. Org. Chem.*, 1983, **48**, 3011.

16 S. Colin-Messager, J.-P. Girard and J.-C. Rossi, *Tetrahedron Lett.*, 1992, **33**, 2689.

17 M. Tarköy, M. Bolli and C. Leumann, *Helv. Chim. Acta*, 1994, **77**, 716.

18 (*a*) S. Iwai and E. Ohtsuka, *Nucleic Acids Res.*, 1988, **16**, 9443; (*b*) S. Iwai, T. Sasaki and E. Ohtsuka, *Tetrahedron*, 1990, **46**, 6673.

19 (*a*) J. H. van Boom and P. M. J. Burgers, *Tetrahedron Lett.*, 1976, 4875; (*b*) J. H. van Boom, P. M. J. Burgers, C. H. M. Verdegaal and G. Wille, *Tetrahedron*, 1978, **34**, 1999.

20 D. Gasparutto, T. Livache, H. Bazin, A.-M. Duplaa, A. Guy, A. Khorlin, D. Molko, A. Roget and R. Téoule, *Nucleic Acids Res.*, 1992, **20**, 5159.

21 J. H. Clark, *Chem. Rev.*, 1980, **80**, 429.

22 N. D. Sinha, J. Biernat and H. Köster, *Tetrahedron Lett.*, 1983, **24**, 5843.

23 J. M. Falcone and H. C. Box, *Biochim. Biophys. Acta*, 1997, **1337**, 267.

24 A. Romieu, D. Gasparutto, D. Molko, J.-L. Ravanat and J. Cadet, *Eur. J. Org. Chem.*, 1999, 49.

25 U. Pieles, W. Zürcher, M. Schär and H. E. Moser, *Nucleic Acids Res.*, 1993, **21**, 3191.

26 J. R. Wagner, C. Decarroz, M. Bergen and J. Cadet, *J. Am. Chem. Soc.*, in press.

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