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Chemical and Biochemical Properties of Oligonucleotides that Contain (5′*S*,6*S*)-Cyclo-5,6-dihydro-2′-deoxyuridine and (5′*S*,6*S*)-Cyclo-5,6-dihydrothymidine, Two Main Radiation-Induced Degradation Products of Pyrimidine 2′-Deoxyribonucleosides

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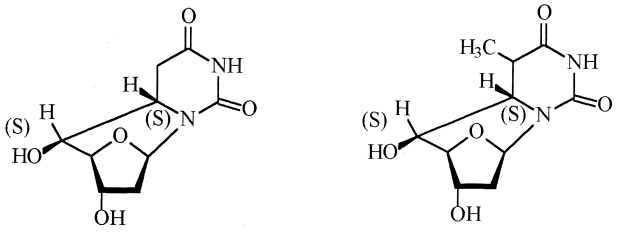
Abstract—The first chemical synthesis of (5′*S*,6*S*)-cyclo-5,6-dihydro-2′-deoxyuridine [(5′*S*,6*S*)-CyclodHdUrd], a major product of gamma irradiation of oxygen free aqueous solution of 2′-deoxycytidine is reported. Subsequently, the latter cyclonucleoside was incorporated in defined sequence oligodeoxyribonucleotides. The chemical composition of the modified DNA fragments was assessed by enzymatic digestions and mass spectrometry measurements. The latter analyses confirmed the presence and the integrity of the lesion within the synthesised DNA fragments. Replication and repair studies showed that (5′*S*,6*S*)-CyclodHdUrd together with (5′*S*,6*S*)-CyclodHThd [(5′*S*,6*S*)-cyclo-5,6-dihydrothymidine] inserted into DNA oligomers act as blocks for DNA polymerases and are not excised by DNA *N*-glycosylases.

Introduction

Cyclonucleosides constitute a class of modified nucleosides, which can be generated from both purine and pyrimidine nucleic acid components upon exposure to ·OH radicals.[1–5] They are expected to have a potential biological impact especially toward enzymatic repair processes.[2,6] Biological features of the 5′*S* and 5′*R* diastereoisomers of 5′,8-cyclo-2′-deoxyadenosine and 5′,8-cyclo-2′-deoxyguanosine were assessed upon insertion into oligodeoxyribonucleotides (ODNs).[7,8] Enzymatic and repair experiments have shown that the modified DNA fragments were not completely hydrolysed by mixtures of endo- and exonucleases and were not substrates for DNA *N*-glycosylases. However the 5′*S* and 5′*R* diastereoisomers of 5′,8-cyclo-2′-deoxyadenosine were found to be repaired by the human nucleotide excision-repair enzymes.[9] Moreover, the insertion of (5′*S*,6*S*) and (5′*S*,6*S*) diastereoisomers of 5′,6-cyclodihydrothymidine [(5′*S*,6*S*)-CyclodHThd] **2** into oligonucleotides, was recently reported.[10] In order to assess the biochemical features of cyclopyrimidine nucleosides, (5′*S*,6*S*)-cyclo-5,6-dihydro-2′-deoxyuridine [(5′*S*,6*S*)-CyclodHdUrd] **1** has been synthesised following a similar chemical approach used for the synthesis of (5′*S*,6*S*)-CyclodHThd **2** (Schemes 1 and 2).[10] It has to be pointed out, that cyclonucleoside 1 is the stable decomposition product of related cytosine derivative which is likely to readily deaminate in aqueous solution.

In the present paper, we report the first chemical synthesis of (5′*S*,6*S*)-CyclodHdUrd **1** and its insertion into DNA oligomers. Particularly, the stability of **1** under acidic,



1 **2**

Scheme 1. Chemical structure of (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2**.

Keywords: cyclonucleosides; synthetic oligonucleotides; DNA *N*-polymerases; DNA-*N*-glycosylases.

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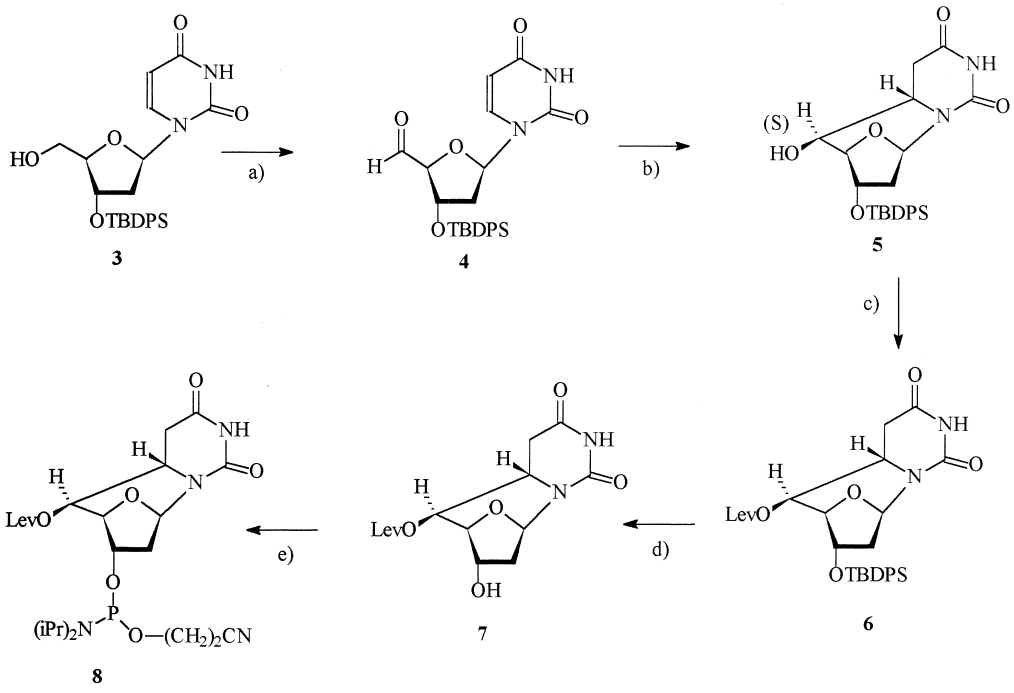


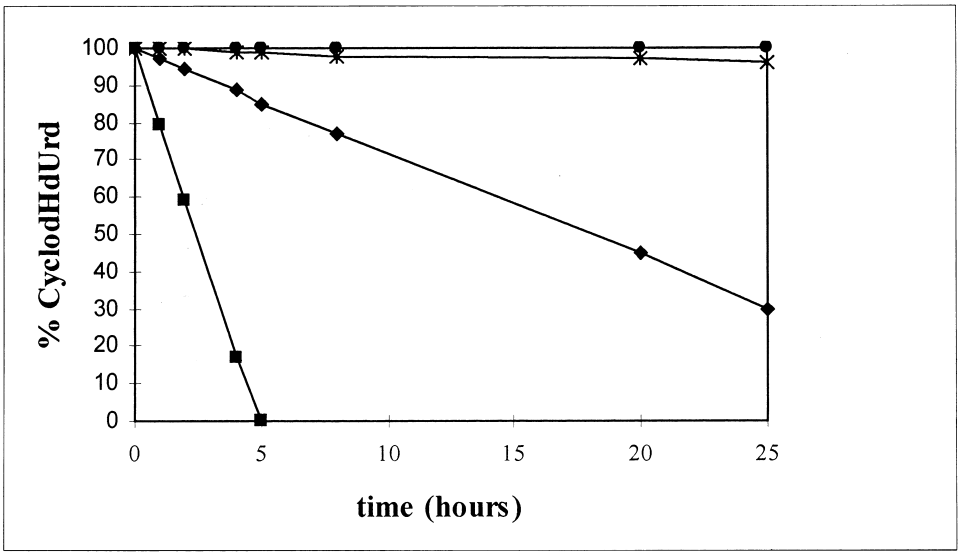
Scheme 2. Synthetic pathway used for the preparation of the phosphoramidite synthon **8** of (5′*S*,6*S*)-CyclodHdUrd. *Reagents*: (a) Dess–Martin periodinane, CH_2Cl_2, 2 h, room temperature; (b) Bu_3SnH, AIBN, benzene, 6 h, reflux; (c) levulinic acid, DCC, DMAP, tetrahydrofuran, 2 h 30 min, room temperature; (d) TBAF, THF, 40 min, room temperature; (e) 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite, DIEA, CH_2Cl_2/THF, 1 h, room temperature.

oxidising and alkaline conditions, and the piperidine stability of modified oligonucleotides containing (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** have been studied. Then, the site-specific insertion of **1** into several oligonucleotides using the Pac phosphoramidite chemistry has been performed. The thermal denaturation has been studied in order to determinate the structural effect of the incorporation of (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** into DNA. Then the modified DNA oligomers were used to study the properties of **1** and **2** towards several endonucleases, exonucleases and DNA *N*-glycosylases. Finally, the coding properties of **1** and **2** during the replication of the modified ODNs mediated by two bacterial DNA polymerases, namely Klenow fragment of *E. coli* polymerase I and *Taq* DNA polymerase, were also investigated.

Results and Discussion

Synthesis of the modified phosphoramidite building block and its insertion into defined sequence oligonucleotides

Synthetic procedure for the preparation of the phosphoramidite synthon of (5′*S*,6*S*)-CyclodHdUrd 1. The modified nucleoside (5′*S*,6*S*)-CyclodHdUrd **1** was prepared according to the method developed by Romieu et al.[10] and further applied to the synthesis of (5′*S*,6*S*)-CyclodHThd **2** with the appropriate modifications. The synthesis of the targeted phosphoramidite **8** (Scheme 2) started with the 3′-*O*-*tert*-butyldiphenylsilyl (TBDPS) ether of 2′-deoxyuridine **3**.[11] Compound **3** was then oxidised using the Dess–Martin periodinane in anhydrous dichloromethane under an argon atmosphere. This afforded the 5′-aldehyde nucleoside **4** in 81% yield. Cyclisation of **4** was performed by treatment with tributyltin hydride (Bu_3SnH) and 2,2′-azobis(2-methylpropionitrile) (AIBN) in benzene under reflux. The formation of **3** was prevented by working under an argon atmosphere together with a dropwise addition of an excess of the reagents over a period of 6 h. Thus, the desired cyclonucleoside **5** was obtained in a 70% yield. The structure of **5** was confirmed by ^1H and ^{13}C NMR analyses together with ESI-MS measurements in both the positive and negative mode. Interestingly, only the (5′*S*,6*S*) diastereoisomer was obtained. As shown for (5′*S*,6*S*)-CyclodHThd,[10] attempts to convert **5** into the 5′-*O*-DMTr-protected derivative failed. Therefore, the levulinyl (Lev-) group, a non-standard 5′-OH protecting group, was chosen. This has already been successfully applied to the solid phase synthesis of oligoribonucleotides by the phosphoramidite and phosphotriester approaches.[12] The levulinyl protecting group can be easily introduced on secondary alcohol functions. Moreover the removal of the latter ether group under neutral conditions (0.5 M hydrazine monohydrate in 3:2 pyridine–acetic acid at room temperature for 10 min) is compatible with the stability of (5′*S*,6*S*)-CyclodHdUrd **1** together with that of the acyl and the cyanoethyl groups that protected the amino and phosphate residues, respectively of the non modified nucleotides. Thus, **6** was obtained in 84% yield. The 3′-*O*-TBDPS ether **6** was selectively desilylated upon treatment with tetrabutylammonium fluoride (TBAF) in THF at room temperature for 40 min, giving **7** in 67% yield. Only 10% of the deacylation side-reaction were observed. The phosphoramidite **8** was finally synthesised in a 80% yield by the reaction of the secondary alcohol of the compound **7** with 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite [$NCCH_2CH_2OP(Cl)NPr^i_2$] in the presence of *N*,*N*-diisopropylethylamine (DIEA).



Scheme 3. Stability studies of (5′*S*,6*S*)-CyclodHdUrd **1** in an 0.1 M oxidising solution of iodine at room temperature (×); in an 80% acid acetic aqueous solution at room temperature (●); in a 30% ammonia aqueous solution at room temperature (◆) and 55°C (■).

Stability studies of (5′*S*,6*S*) CyclodHdUrd 1. The stability of (5′*S*,6*S*)-CyclodHdUrd **1** was studied under the main experimental conditions used in the course of the solid-support synthesis. Samples of (5′*S*,6*S*)-CyclodHdUrd **1** obtained by desilylation of the 3′-*O*-(*tert*-butyldiphenylsilyl)-CyclodHdUrd **5** were used for the stability studies. Thus, **1** was treated with either 80% acidic acetic, a commercial oxidising solution of either iodine at room temperature or ammonia under two temperature conditions including room temperature and 55°C. Aliquots of the reaction mixtures were taken up at increasing periods of time and subsequently analysed by reversed-phase HPLC. (5′*S*,6*S*)-CyclodHdUrd **1** was found to be stable under both the acidic and oxidising conditions applied. However, unlike (5′*S*,6*S*)-CyclodHThd **2**,[10] (5′*S*,6*S*)-CyclodHdUrd **1** was found to be unstable under the alkaline conditions (Scheme 3). Indeed, after 4 h at room temperature, approximately 5% of **1** was degraded; the yield of decomposition reached 55% after 24 h at room temperature. In all cases, epimerisation of **1** was not observed. The main consequence of the unstability of **1** under alkaline conditions was to substitute the usual amino protecting groups (benzoyl for dAdo, isobutiryl for dCyd and dGuo) by those used in the Pac-protected phosphoramidite chemistry: the phenoxyacetyl, the isopropyl-phenoxyacetyl and the acetyl groups for dAdo, dGuo and dCyd respectively.

Table 1. Sequences and molecular masses of the modified oligonucleotides and primers synthesised and used in this study [**X**=(5′*S*,6*S*)-CyclodHdUrd **1**; **Y**=(5′*S*,6*S*)-CyclodHThd **2**]; the oligonucleotide masses were obtained by ESI-MS measurements in the negative mode

Name	Sequences (5′-3′)	Length	Mass calcd	Mass found
11	A**X**T	3	845.6	845.7
12	ATC GTG A**X**T GAT CT	14	4254.8	4255.6
13	CAC TTC GGA T**X**G TGA CTG ATC T	22	6702.4	6701.2
14	CAC TTC GGA **Y**CG TGA CTG ATC T	22	6701.2	6700.5
15	AGA TCA GTC AC	11	3325.2	3324.3
16	AGA TCA GTC ACG	12	3654.4	3653.5

Solid-phase synthesis and characterisation of (5′*S*,6*S*)-CyclodHdUrd 1 containing oligonucleotides. Several oligonucleotides (Table 1) bearing a (5′*S*,6*S*)-CyclodHdUrd residue **1** were synthesised on solid support using the 'Pac chemistry', with the modifications previously described in the experimental section. The modified oligonucleotides were then cleaved from the solid support and deprotected with 30% ammonia at room temperature for 4 h. The HPLC elution profile of the crude reaction mixture of 3-mer **11** indicates a good coupling yield for compound **8** (data not shown). The crude 5′-tritylated-ODNs were purified by RP-HPLC on polymeric support using an on-line detritylation-purification procedure.[13] The purity and homogeneity of the modified oligonucleotides were controlled first by HPLC and then by polyacrylamide gel electrophoresis of the 5′-[^{32}P]-labelled fragments. Finally, mass measurements by MALDI-TOF-MS in the positive mode and ESI-MS in the negative mode were performed. As a result, they confirmed the presence and the integrity of **1** into the ODN fragments. It should be added that more than 30 $AU_{260\ nm}$ of the purified oligonucleotides **12** and **13** were obtained using the 1 μmol-scale synthesis. No detectable degradation of the cyclonucleoside was observed, within the limit of detection, whereas 5% of decomposition was found to occur for the free lesion **1** upon treatment under the same conditions (see above) (data not shown). Moreover only 10% of the modified nucleoside was decomposed, when ODN **12** or **13** was treated for 5 h with 30% ammonia at 55°C under the same conditions, whereas the free lesion **1** was fully degraded. This indicates that the lesion **1**, when inserted into oligonucleotide, is more stable than the free cyclonucleoside **1**.

Piperidine stability of modified oligodeoxyribonucleotides that contained (5′*S*,6*S*)-CyclodHdUrd 1 or (5′*S*,6*S*)-CyclodHThd 2

The availability of modified oligonucleotides that contained either (5′*S*,6*S*)-CyclodHdUrd **1** or (5′*S*,6*S*)-CyclodHThd **2** lesion allowed the determination of the stability of the

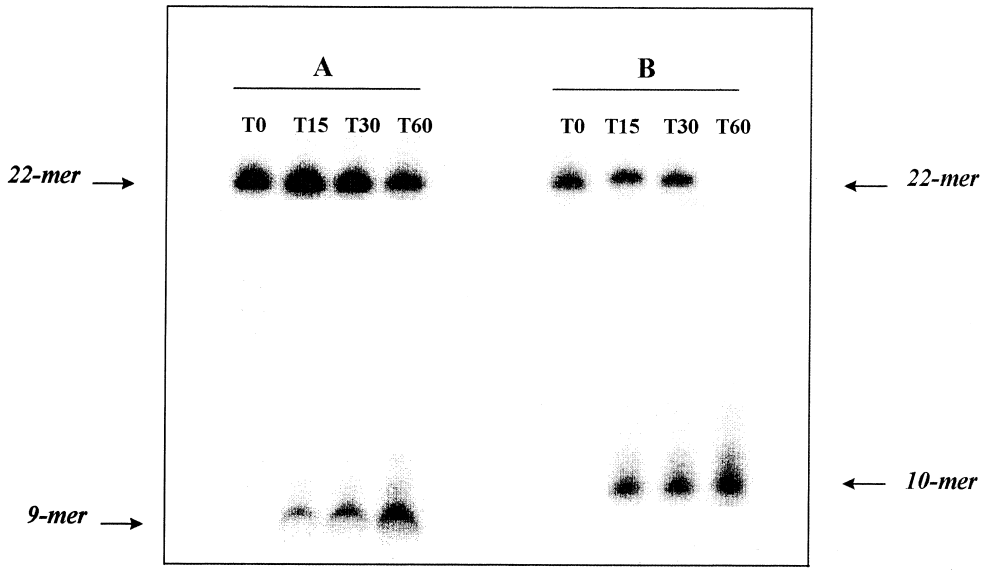


Figure 1. PAGE analysis of the 5′-end-labeled 22-mer ODNs containing: (A) (5′*S*,6*S*)-CyclodHThd **1** (ODN **14**); (B) (5′*S*,6*S*)-CyclodHdUrd **2** (ODN **13**), after treatment with a 1 M piperidine aqueous solution at 90°C for 0, 15, 30 and 60 min, respectively.

oligonucleotides **13** or **14**, respectively in the presence of piperidine used to reveal alkali-labile sites in oxidised DNA. The latter studies were performed by treating the 5′-[^{32}P]-labelled oligonucleotides 22-mer **13** and **14** with piperidine at 90°C for 15, 30 and 60 min, respectively. In a subsequent step, the resulting DNA fragments were analysed by denaturing polyacrylamide gel electrophoresis (Fig. 1A and B). It was found that (5′*S*,6*S*)-CyclodHdUrd **1** upon insertion into oligonucleotide was unstable under piperidine treatment. Indeed, after a 30 min heating period, approximately 50% of the oligonucleotide **13** was cleaved at the site of **1**. Unlike (5′*S*,6*S*)-CyclodHdUrd **1**, (5′*S*,6*S*)-CyclodHThd **2** containing oligonucleotide **14** was shown to be more stable. Under these conditions, only 20% of breaks were observed after 30 min at 90°C. As a conclusion, (5′*S*,6*S*)-CyclodHdUrd **1** can be considered as an alkali-labile lesion, whereas (5′*S*,6S)-CyclodHThd **2** shows a weak alkali-lability.

Thermal denaturation studies

In order to determine the structural effect of the incorporation of (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** into DNA, the thermal stability of respectively the **X**.dGuo and **Y**.dAdo base pair, where **X**=(5′*S*,6*S*)-CyclodHdUrd **1** and **Y**=(5′*S*,6*S*)-CyclodHThd **2**, was evaluated. Thus, 5′-d(ATC GTG A**X**T GAT CT)-3′ and 5′-d(ATC GTG AC**Y** GAT CT)-3′ were annealed with their complementary DNA strand 5′-d(AGA TCA GTC ACG AT)-3′. The melting temperature (T_m) of the duplex was determined by UV measurements at 260 nm. It was found that the T_m of the duplex that contained either (5′*S*,6*S*)-CyclodHdUrd **1** (T_m=49±1°C) or (5′*S*,6*S*)-CyclodHThd **2** (T_m=50±1°C) was lower than that of the unmodified duplex (T_m=56±1°C) (data not shown). The decrease in the T_m (ΔT_m=7 or 6°C, respectively) suggests that the incorporation of either (5′*S*,6*S*)-CyclodHdUrd **1** or (5′*S*,6*S*)-CyclodHThd **2** induces a local destabilisation of the duplex DNA structure. A similar behaviour was already observed for the related purine cyclonucleosides.[7]

Enzymatic digestion of (5′*S*,6*S*)-CyclodHdUrd 1 containing oligonucleotides by nuclease P_1 and alkaline phosphatase

Aliquots of the modified 14-mer oligonucleotide **12** were submitted to the action of nuclease P_1 over a period of either 2 or 24 h, followed by bacterial alkaline phosphatase. In both cases, the resulting hydrolysate, which was analysed by reversed-phase HPLC, shows a similar content. It was found to consist of dCyd, dGuo, Thd, dAdo and the trinucleotide [5′-d(A**X**T)-3′] in a 2:3:4:2:1 ratio. This provides support to the structure of modified 14-mer **12** (Fig. 2A). The observed lack of the free modified nucleoside **1**, even after 24 h of incubation, received further confirmation upon co-injection of the enzymatic digestion with an authentic sample of (5′*S*, 6*S*)-CyclodHdUrd **1** and HPLC analysis (Fig. 2B). Moreover, the structure of the trinucleotide was established by ESI-MS analysis in the negative mode of the collected peak (calcd 845.7 Da, found 845.2 Da) and by co-injection of the enzymatic digestion with the trinucleotide **11** previously synthesised. Alternatively, incubation of **11** with nuclease P_1 during either 2 or 24 h, followed by alkaline phosphatase, did not provide the free 2′-deoxyribonucleosides dAdo, Thd and (5′*S*,6*S*)-CyclodHdUrd **1** (data not shown). Similar results were obtained with the modified 22-mer oligonucleotide **13**. In addition, the unmodified 14-mer oligonucleotide 5′-d(ATC GTG ACT GAT CT)-3′ was used as a reference. It was completely hydrolysed under the same enzymatic conditions described above. The results are consistent with previous studies, particularly those concerning (5′*S*,6*S*)-CyclodHThd **2**.[10] Indeed, nuclease P_1 mediated hydrolysis of the phosphodiester bonds between normal and altered 2′-deoxyribonucleosides was inhibited.[7,14,15]

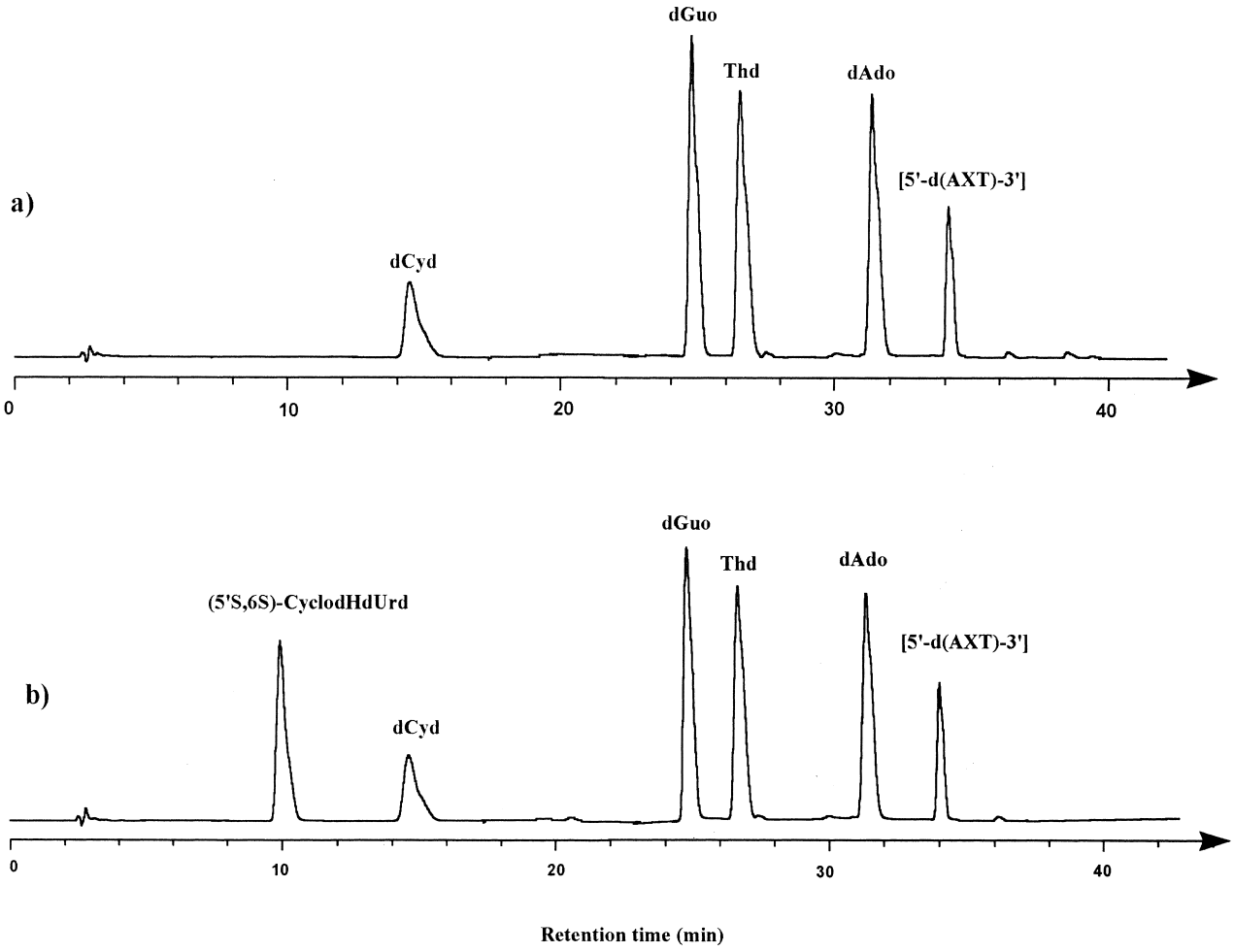


Figure 2. (A) Reversed-phase HPLC profile of the enzymatic digestion mixture of 14-mer modified oligonucleotide **12** by nuclease P_1 (24 h) and phosphatase alkaline (1 h) (the chromatographic conditions are reported in the Experimental section); (B) Co-injection with cyclonucleoside **1**.

Enzymatic digestion of (5′*S*,6*S*)-CyclodHdUrd 1 containing oligonucleotides by bovine intestinal mucosa phosphodiesterase (3′-*exo*) and calf spleen phosphodiesterase (5′-*exo*)

Additional enzymatic digestion experiments were performed on the modified 14-mer **12** using two exonucleases including bovine intestinal mucosa phosphodiesterase (3′-*exo*) and calf spleen phosphodiesterase (5′-*exo*). Thus, the course of the hydrolysis of the DNA strand by nucleases was followed by withdrawing aliquots from the digestion mixtures at increasing periods of time. Then, the resulting DNA fragments were analysed by MALDI-TOF MS.[16,17] Using the latter powerful technique, the different molecular ions observed correspond to digested DNA fragments which differ in mass by a successive loss of nucleotides. The difference in mass between two successive fragments allows to identify the released nucleotide and thus to determine the overall sequence of the oligonucleotides. Thus, MALDI-TOF MS analysis allows the determination of the location of the lesion within the DNA strand. In addition, relevant information was provided on the integrity of the modified nucleoside and the processing of the damage by the exonucleases.

The 2′-deoxycytidine containing unmodified 14-mer oligonucleotide 5′-d(ATC GTG ACT GAT CT)-3′, used first as control, was totally hydrolysed by both 5′-and 3′-exonucleases in less than 10 min, allowing the determination of the complete sequence (data not shown). In contrast, the presence of (5′*S*,6*S*)-CyclodHdUrd **1** in ODN induces a total resistance to digestion by both 3′- and 5′-exonucleases at the site of the lesion.

Bovine intestinal mucosa phosphodiesterase sequentially degraded the oligonucleotide **12** from the 3′-end until it reached (5′*S*,6*S*)-CyclodHdUrd **1**, which is resistant to further cleavage even after a prolonged treatment (Fig. 3A and B). This was inferred from the observation of a single peak at *m/z*=2409.01 Da after 1 h of incubation corresponding to the positive ion $[M+H]^+$ of the 8-mer 5′-d(ATC GTG A**X**)-3′ (mass calcd=2411 Da). In order to confirm these results, the enzymatic digestion experiment was performed with the trinucleotide 5′-d(A**X**T)-3′ **11**. Then, the mixture was analysed by reversed-phase HPLC (system E) (data not shown). Two peaks were detected and collected. One corresponds to thymidine (Thd) while the second was attributed to the dimer 5′-d(A**X**)-3′; the latter structure was confirmed by ESI-MS analysis in the negative mode (calcd 541 Da, found 541.1 Da).

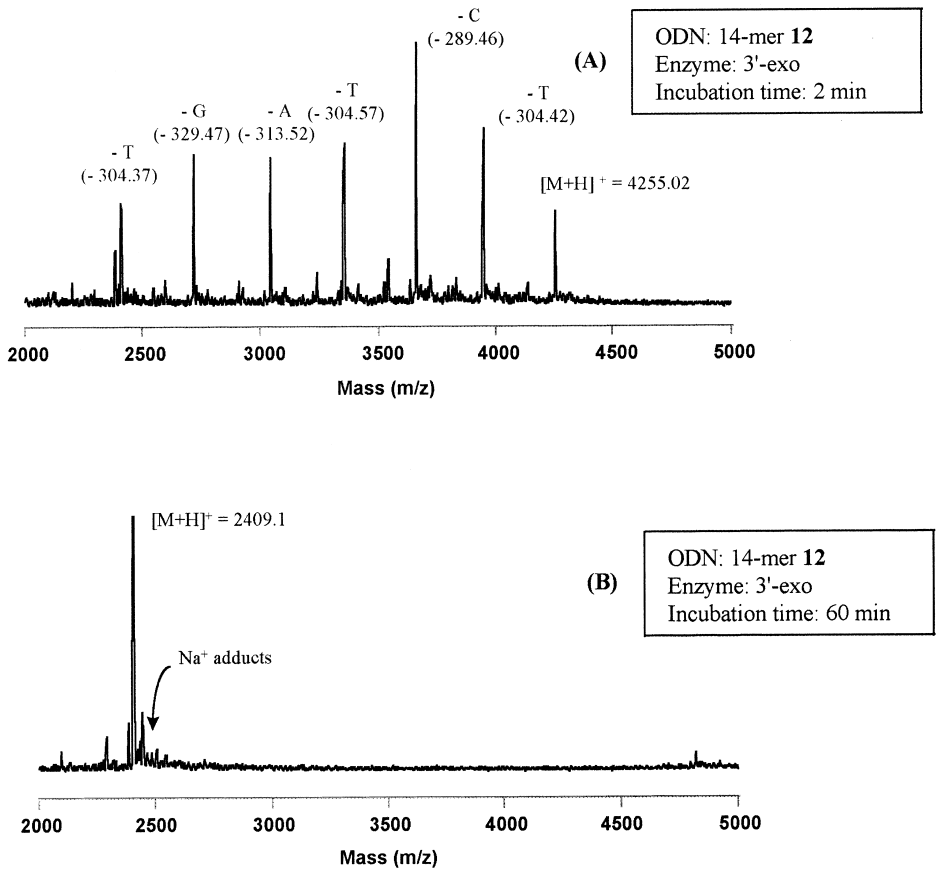


Figure 3. MALDI-TOF mass spectrum in the positive mode of the products resulting from the digestion of the 14-mer modified oligonucleotide **12** by the 3′-exonuclease after incubation for: (A) 2 min; and (B) 60 min.

A different behaviour was observed for the calf spleen phosphodiesterase-mediated digestion of **12**, which starts from the opposite end (Fig. 4A and B). The enzymatic hydrolysis after 5 min shows a sequential cleavage of the normal nucleosides (Fig. 4A). After 1 h of incubation (Fig. 4B), the mass spectrum exhibited a major peak at 2383.95 Da, corresponding to the positive ions $[M+H]^+$ of the 8-mer 5′-d(AXT GAT CT)-3′ (mass calcd=2385 Da). This indicates that the 5′-exonuclease is able to digest sequentially the 14-mer oligonucleotide from the 5′-end until it reaches the 2′-deoxyadenosine nucleoside before the (5′*S*,6*S*)-CyclodHdUrd **1** lesion, whose phosphodiester bond is resistant to further cleavage. Even, after longer enzymatic treatments, the 5′-exonuclease failed to release the (5′*S*,6*S*)-CyclodHdUrd **1**. In addition, the trinucleotide 5′-d(A**X**T)-3′ **11** was submitted to the action of the 5′-exonuclease at 37°C over a period of 24 h (data not shown). As expected, no peak corresponding to the cyclonucleoside **1** was detected on the reverse-phase HPLC elution profile. Only the peak of 5′-d(A**X**T)-3′ was observed after analysis by ESI-MS in the negative mode. It was also shown that the modified oligonucleotide could not be fully digested to the monomer level by a combination of 3′-*exo* and 5′-exonucleases. Similar results have been already obtained for (5′*S*,6*S*)-CyclodHThd **2**[10] and the 5′,8-cyclopurine-2′-deoxyribonucleosides.[7,8]

Repair assays of (5′*S*,6*S*)-CyclodHdUrd 1 and (5′*S*,6*S*)-CyclodHThd 2 containing oligonucleotides with Fpg, endo III and endo VIII proteins

To investigate the biological significance of (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** in DNA, attempts were made to assess whether **1** and **2** may be substrates for three base excision repair enzymes including formamidopyrimidine DNA *N*-glycosylase (Fpg), endonuclease III (endo III) and endonuclease VIII (endo VIII) proteins. The *E. coli* Fpg protein is a well-known repair enzyme which is able to excise several modified purine bases from DNA duplexes, through both a *N*-glycosylase and an AP-endonuclease activities.[18,19] Substrates recognised and excised by Fpg include 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine), 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Me-Fapy-guanine), 4,6-diamino-5-formamidopyridine (Fapy-adenine)[20–25] and 2,2,4-triamino-5-(2*H*)-oxazolone.[26] It was also found that several modified pyrimidine bases, including 5-hydroxycytosine (5-OHC), 5,6-dihydrothymine (DHT), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol: Tg), formylamine, *N*-3-[2-hydroxyisobutyric acid] urea and 5-hydroxy-5-methylhydantoin,[27–33] are recognised and excised by Fpg. The same enzyme cleaves the DNA backbone through a β–δ elimination reaction, thus leading to the release of the sugar residue from the DNA strand.[33]

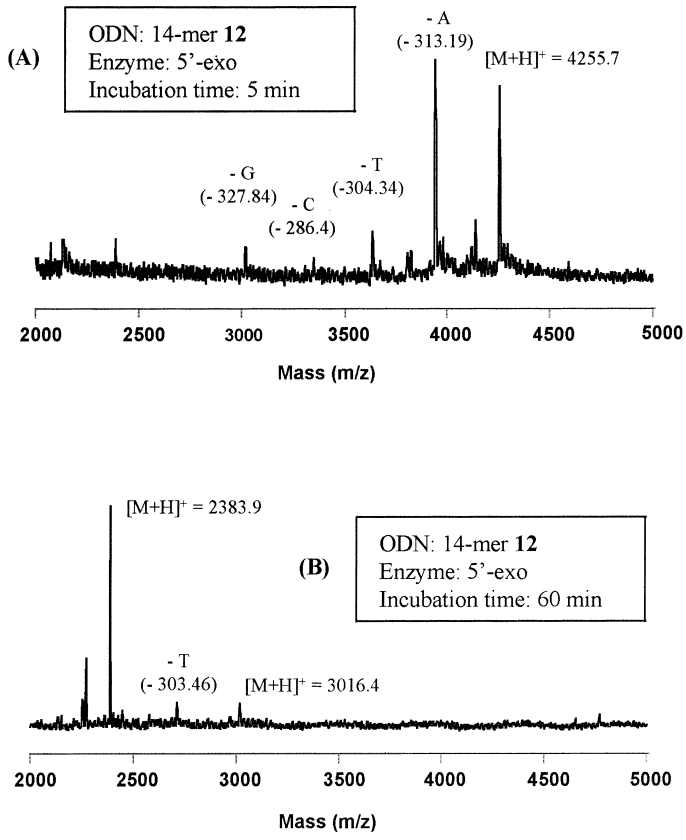


Figure 4. MALDI-TOF mass spectrum in the positive mode of the products resulting from the digestion of the 14-mer modified oligonucleotide **12** by the 5′-exonuclease after incubation for: (A) 5 min; and (B) 60 min.

Endonuclease III is the product of the *n*th gene.[34,35] The enzyme exhibits both *N*-glycosylase and 3′-apurinic/apyrimidinic (AP) endonuclease activities. Previous studies have shown that endonuclease III and endonuclease VIII recognise and excise modified thymine and cytosine residues, including 5,6-dihydrothymine (DHT), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil, 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol), 5-hydroxy-5,6-dihydrouracil, 5-hydroxyuracil, 6-hydroxy-5,6-dihydrocytosine, 5-hydroxycytosine (5-OHC), urea, methyltartronyl-*N*-urea, 5-hydroxy-5-methylhydantoin and, alloxan.[27,32,36–39] Recently, it was reported that 2,2,4-triamino-5-(2*H*)-oxazolone, a modified purine nucleobase was recognised and excised by endo III.[26] In contrast to Fpg, the removal of a lesion is believed to be followed by a β-elimination step catalysed by the enzyme.[40–44]

Thus, the modified 22-mer ODNs **13** and **14** that contained, respectively (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** were incubated with each of the three repair enzymes. This was achieved after 5′-[^{32}P]-end labelling of the modified oligomers and subsequent hybridisation with their complementary sequence 5′-d(AGA TCA GTC ACG ATC CGA AGT G)-3′, which contains a guanine in front of **1** and an adenine in front of **2**. Then, the excision of the damaged base by the repair enzymes was probed by searching for strand breakage of the ODN using polyacrylamide gel electrophoresis. It was found that both endo III and endo VIII proteins, which act primarily at modified pyrimidine base, are not able to cleave the modified DNA duplex at the site of **1** or **2** (data not shown). The same results were found for the Fpg protein (data not shown). It should be added that alkali treatment of the incubated **13** or **14**, did not lead to any detectable cleavage of the corresponding oligonucleotide. This may be rationalised in term of the lack of excision of the modified base.

In vitro replication experiments with DNA polymerases

The ability for the *Taq* polymerase and the Klenow exo$^-$ fragment to extend a primer annealed with a template bearing either (5′*S*,6*S*)-CyclodHdUrd **1** or (5′*S*,6*S*)-CyclodHThd **2** was investigated. The primer was ^{32}P-labelled at its 5′-end so that extension by nucleotide incorporation could be observed upon sequencing polyacrylamide gel electrophoresis (PAGE). The intensity of each band is proportional to the number of molecules that terminates the synthesis at a given position of the template.

Fig. 5A shows the denaturing PAGE bands obtained by elongation of the 5′-[^{32}P]-labelled 11-mer primer 5′-d(AGA TCA GTC AC)-3′ annealed with 5′-d(CAC TTC GGA T**X**G TGA CTG ATC T)-3′ **13** where **X**=(5′*S*,6*S*)-CyclodHdUrd **1**, in the presence of the DNA polymerases. In addition, similar replication assays were performed using the unmodified 2′-deoxycytidine containing 22-mer ODN as the template in order to assess the activity and the specificity of the polymerases (data not shown). It was found that *Taq* polymerase-mediated polymerisation exclusively incorporates dAMP opposite (5′*S*,6*S*)-CyclodHdUrd **1**

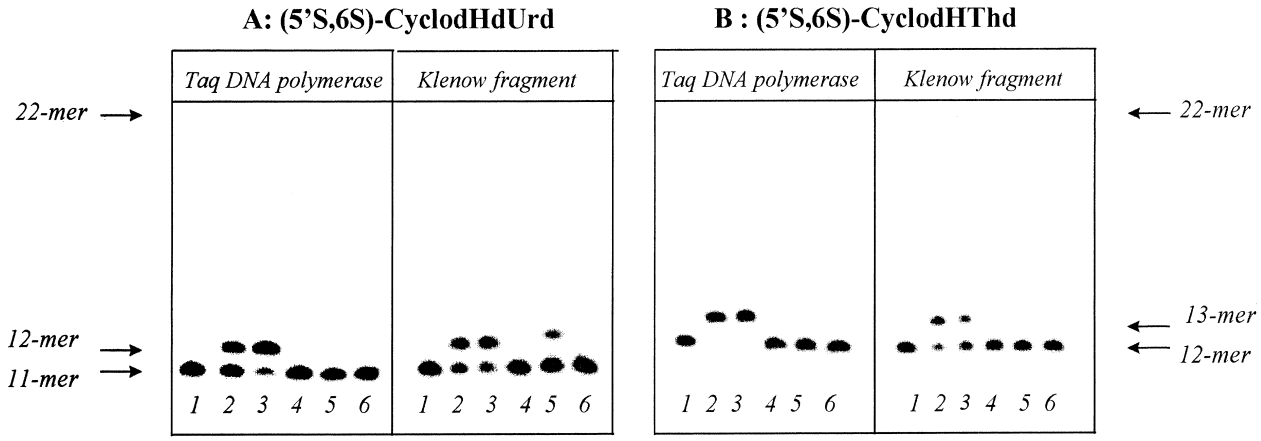


Figure 5. (A) Modified 22-mer template 5′-d(CAC TTC GGA T**X**G TGA CTG GTG ATC T)-3′, where **X**=(5′*S*,6*S*)-CyclodHdUrd **1** annealed with a 5′-[^{32}P]-labelled 11-mer 5′-d(AGA TCA GTC AC)-3′ (line 1). (B) Modified 22-mer template 5′-d(CAC TTC GGA **Y**CG TGA CTG GTG ATC T)-3′, where **Y**=(5′*S*,6*S*)-CyclodHThd **2** annealed with a 5′-[^{32}P]-labelled 12-mer 5′-d(AGA TCA GTC ACG)-3′ (line 1). In both cases, primer extension reactions were catalysed by the *Taq* DNA polymerase and the Klenow fragment, in the presence of 100 μM of dNTP (lane 2), dATP (lane 3), dCTP (line 4), dGTP (line 5) and dTTP (line 6) as described in the Experimental section. Then the reaction mixtures were subjected to denaturing 20% PAGE and the extended products were visualised by phosphorimaging (Molecular Dynamics Phosphorimager) using Image Quan T software.

lesion. Moreover, the enzyme was not able to extend the (5′*S*,6*S*)-CyclodHdUrd **1**/dAdo pair beyond the damage in the presence of the four dNTPs. Thus, **1** appeared to strongly inhibit *Taq* polymerase. Using Klenow fragment, the primer extension reactions led mainly to the same dAMP incorporation opposite lesion **1**. In addition, small amounts of dGMP were also incorporated. As observed with *Taq* polymerase, Klenow fragment was blocked in front of **1** since only small amounts of nucleotides, likely to be dAMP and dGMP, were inserted opposite **1** with no fully extended primer.

The mutagenic properties of the (5′*S*,6*S*)-CyclodHThd **2** lesion incorporated into oligonucleotides were also evaluated. Similar replication experiments were performed with **2** as the lesion using the 5′-[^{32}P]-labelled 12-mer primer 5′-d(AGA TCA GTC ACG)-3′ annealed with 5′-d(CAC TTC GGA **Y**CG TGA CTG ATC T)-3′ where **Y**=(5′*S*,6*S*)-CyclodHThd **2**. The results are reported in Fig. 5B. It was found that *Taq* polymerase and Klenow fragment-mediated polymerisation exclusively incorporate dAMP opposite the (5′*S*,6*S*) CyclodHThd **2** lesion. In addition, it was observed that the two enzymes were not able to extend the (5′*S*,6*S*)-CyclodHThd **2**/dAdo pair beyond the damage in the presence of the four dNTPs. The primer extension results suggest that both (5′*S*,6*S*)-CyclodHdUrd **1** and (5′*S*,6*S*)-CyclodHThd **2** act as a blocking lesion in the course of in vitro DNA synthesis by bacterial DNA polymerase. These results are indication of a possible lethal action of these lesions. In contrast, both (5′*S*) and (5′*R*) diastereoisomers of 5′,8-cyclo-2′-deoxyguanosine were found to exhibit an important miscoding potential during the replication step leading to the mutagenic G>T transversion.[45]

Conclusion and Perspectives

The synthesis of (5′*S*,6*S*)-CyclodHdUrd **1** and its incorporation into several oligonucleotides by the phosphoramidite approach were achieved using mild alkali deprotection conditions. The synthetic oligonucleotides were isolated in good yields and characterised by several complementary techniques, showing the integrity of the incorporated modified nucleoside. Studies of the stability of the modified oligonucleotides in the presence of piperidine prove the high lability of the (5′*S*,6*S*)-CyclodHdUrd **1** lesion inserted into DNA strands. In contrast (5′*S*,6*S*)-CyclodHThd **2** appears as a rather stable damage. A decrease in the melting temperature of the double stranded 14-mer DNA fragment that contained a (5′*S*,6*S*)-CyclodHdUrd **1**/G or (5′S,6S)-CyclodHThd **2**/A base pair was observed. This suggests that the presence of either (5′*S*,6*S*)-CyclodHdUrd **1** or (5′*S*,6*S*)-CyclodHThd **2** induces a local destabilisation of the duplex DNA structure. Then, the processing of **1** by different nucleases was studied. It was found that nuclease P_1, calf spleen phosphodiesterase and bovine intestinal mucosa phosphodiesterase failed to cleave the (5′*S*,6*S*)-CyclodHdUrd **1** residue. Similar observations were made for the (5′*S*,6*S*)-CyclodHThd **2** lesion. These results have to be taken into account for the development of assays aimed at measuring the level of formation of such damage in either isolated or cellular oxidised DNA. On the other hand, the ability for three repair enzymes, namely the Fpg, endo III and endo VIII proteins, to excise the lesions **1** and **2** was investigated. It was found that both **1** and **2** were not substrates for either repair enzymes. The biological study was extended to the evaluation of the mutagenic properties of the lesions **1** and **2**. This involved the determination of the base-specific incorporation directed by the latter residues during in vitro replication using Klenow exo$^-$ fragment and *Taq* DNA polymerase. Thus, both **1** and **2** act as a block for the two DNA polymerases. Therefore, **1** and **2** may represent potential lethal lesions within the cell if the latter lesions are not removed by the nucleotide excision repair machinery.

Experimental

General

The silica gel (70–200 μm) used for the low-pressure

column chromatography was purchased from SDS (Peypin, France). Thin-layer chromatography was carried out on Macherey–Nagel DC Kieselgel Polygram® Sil G/UV_{254} (0.2 mm) plastic sheets (Dueren, Germany). Deuterated solvents were purchased from Acros (Geel, Belgium). All reagents used 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, dNTPs, NAP-25 Sephadex and MicroSpin G-25 columns were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

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*), *Taq* DNA polymerase from *Thermus aquaticus* and calf intestinal alkaline phosphophatase were purchased from Boehringer Mannheim (Mannheim, Germany). T_4 polynucleotide kinase and Klenow fragment (exo^-) of *E. coli* DNA polymerase I were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Endonuclease VIII was from Trevingen–Interchim (Montlucon, France). Fpg and endonuclease III were kind gifts from Dr Serge Boiteux (CEA, Fontenay-aux-Roses, France).

NMR measurements

200 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were recorded on AC200 and AM400 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 ((δ_H=O), $CDCl_3$ (δ_H=7.26), acetone-d_6 (δ_H=2.17), D_2O (δ_H=4.92) or DMSO-d_6 (δ_H=2.62) as the external reference. $CDCl_3$ (δ_H=77) was used as reference for the 100 MHz ^{13}C NMR spectrum. 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 characterised by electrospray ionisation mass spectrometry measurement (ESI-MS) using a Platform 3000 model spectrophotometer from Micromass (Manchester, UK). Typically, 0.1 $AU_{260\ nm}$ of the sample was dissolved in a solution of acetonitrile and water (50/50, v/v) that contained 1% triethylamine prior to be analysed in the negative mode. The modified nucleosides were analysed by ESI-MS using both the positive and the negative modes. For the measurements performed in the positive mode, the samples were dissolved in a solution of acetonitrile and water (50/50, v/v) that contained 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. Spectra were recorded from 256 laser shots with an accelerating voltage of 25 kV in both the linear and positive modes. The matrix consisted of a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4/1 (w/w) ratio, which was dissolved in an aqueous acetonitrile solution (50%) that contained 0.1% TFA and a small amount of Dowex-50W 50X8-200 cation-exchange resin (Sigma). Typically, 1 μL of a 0.1% trifluoroacetic acid aqueous solution of the sample was added to 1 μL of the matrix and the resulting solution was stirred. Then, the sample was placed on the top of the target plate and allowed to dry by itself. The spectra were calibrated with a 1 pmol/μL solution of myoglobin (*m/z* 16952), using the same conditions that were described for the analysis of oligonucleotides.

High-performance liquid chromatography separations

System A: Reversed phase HPLC (Hypersil C_{18} column, 5 μm, 250×4.6 mm id) with a mixture of acetonitrile and 25 mM ammonium formiate buffer (AF, pH=6.2) as the eluents [100% AF (5 min), linear gradient from 0 to 10% of acetonitrile (40 min)] at a flow rate of 1 mL min^{-1}. UV detection at 230 nm. *System B:* Reversed phase HPLC (Hypersil C_{18} column, 5 μm, 250×4.6 mm id) with a mixture of acetonitrile and 10 mM triethylammonium acetate buffer (TEAA, pH=7) as the eluents [100% TEAA (5 min), linear gradient from 0 to 30% of acetonitrile (35 min)] at a flow rate of 1 mL min^{-1}; UV detection at 260 nm. *System C:* Reversed phase HPLC (Hamilton PRP_3, polymeric phase column, 10 μm, 305×7.0 mm id) with a mixture of acetonitrile and 10 mM TEAA buffer 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 id) 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 (Hypersil C_{18} column, 5 μm, 250×4.6 mm id) with a mixture of acetonitrile and 25 mM AF buffer (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 during the first 12 min and then at 260 nm.

Synthetic procedures

1-[3-*O*-(*tert*-Butyldiphenylsilyl)-2-deoxy-β-D-*erythro*-pento-5-dialdo-1,4-furanosyl]uracil (4). Fresh Dess–Martin periodinane (4.36 g, 10.3 mmol) was added to a stirred solution of the 3′-*O*-(*tert*-butyldiphenylsilyl) (TBDPS) ether of 2′-deoxyuridine **3** (2.65 g, 5.68 mmol) in dry dichloromethane (80 mL) under an argon atmosphere. After 2 h at room temperature, the reaction mixture was cooled to 5°C and diluted with dichloromethane (50 mL). Finally the reaction was quenched by the addition of 5% aq. $NaHCO_3$-satured aq. $Na_2S_2O_3$ (80 mL 1:1 v/v). The organic layer was dried by addition of Na_2SO_4 and then concentrated under vacuum. Chromatography of the crude product on silica gel and elution with EtOAc/hexane (70/30) afforded

the aldehyde **4** as a white foam (2.14 g, 81%). R_f=0.26 (EtOAc/hexane 75/25); 200 MHz ^{1}H NMR ($CDCl_3$) δ: 9.05 (s, 1H, H-5′), 8.52 (s, 1H, NH), 7.97 (d, 1H, J_{56}=8.2 Hz, H-6), 7.67–7.43 (m, 10H, H arom., TBDPS), 6.53 (dd, 1H, $J_{1'2'}$=5.1 Hz, $J_{1'2''}$=8.7 Hz, H-1′), 5.76 (d, 1H, J_{56}=8.2 Hz, H-5), 4.59 (m, 1H, H-3′), 4.44 (s, 1H, H-4′), 2.51–2.14 (m, 2H, H-2′, H-2″), 1.09 (s, 9H, $C(CH_3)_3$); ESI-MS (positive mode) *m/z*=465.2 ($[M+H]^+$) (calculated $[M+H]^+$=465.6).

(5′*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (5). Compound **4** (2.14 g, 4.60 mmol) was co-evaporated with dry dichloromethane (2×10 mL) and then re-dissolved in benzene (114 mL) under an argon atmosphere prior to be heated at reflux. A mixture of AIBN (375 mg, 2.28 mmol) and Bu_3SnH (2.48 mL, 9.20 mmol) in benzene (95 mL) was added dropwise to the heated solution under a vigorous stirring over a period of 6 h. The course of the reaction was monitored by TLC (EtOAc/hexane 75/25). After the reaction was completed, the mixture was cooled in an ice-water bath to room temperature, and the solvent was removed under reduced pressure. The resulting yellow oil was purified by chromatography on a silica gel column using EtOAc/hexane 1/1, as the mobile phase. Evaporation to dryness of the collected chromatographic fractions provided the cyclonucleoside **5** as a white foam (1.48 g, 69%). R_f=0.45 (EtOAc/hexane 75/25); 200 MHz ^{1}H NMR ($CDCl_3$) δ: 7.82–7.62 (m, 10H, H arom., TBDPS), 6.26 (d, 1H, $J_{1'2'}$=6.0 Hz, H-1′), 4.54 (dd, 1H, $J_{3'2'}$=3 Hz, $J_{3'2''}$=7.1 Hz, H-3′), 4.28 (d, 1H, $J_{4'5'}$=4.3 Hz, $J_{4'3'}$<0.6 Hz, H-4′), 3.40 (dd, 1H, $J_{5'4'}$=4.5 Hz, $J_{5'6}$=8.6 Hz, H-5′), 3.04–2.92 (m, 3H, H-5 and H-6), 2.43–2.04 (m, 2H, H-2′ and H-2″) 1.11 (s, 9H, $C(CH_3)_3$); 100 MHz ^{13}C NMR ($CDCl_3$), δ: 168.2 (1C, C-4), 150.4 (1C, C-2), 135.9-127.9 (12C, C arom., TBDPS), 85.7 (1C, C-4′), 83.5 (1C, C-1′), 69.9 and 69.2 (2C, C-3′ and C-5′), 50.3 (1C, C-6), 42.5 (1C, C-5), 34.7 (1C, C-2′), 26.8 (3C, $C(CH_3)_3$), 18.6 (1C, $C(CH_3)_3$); ESI-MS (positive mode) *m/z*=489.1 ($[M+Na]^+$) (calculated $[M+Na]^+$=490.5); ESI-MS (negative mode) *m/z*=465.1 ($[M-H]^-$) (calculated $[M-H]^-$=465.5).

(5′*S*,6*S*)-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (1) [(5′*S*,6*S*)-CyclodHdUrd]. Tetrabutylammonium fluoride (0.22 mL, 0.22 mmol) was added to a solution of compound **5** (35 mg, 0.11 mmol) in THF (2 mL). The resulting reaction mixture was stirred at room temperature over a period of 30 min. Then, the reaction mixture was diluted in methanol (4 mL) and concentrated under reduced pressure. The resulting oil was purified by chromatography on a silica gel column using a step gradient of methanol from 0 to 15% in dichloromethane. Evaporation to dryness of the appropriate fractions yielded (5′*S*,6*S*)-CyclodHdUrd **1** as a white foam (24 mg, 98%). R_f=0.21 (CH_2Cl_2/MeOH 80/20); 200 MHz ^{1}H NMR (D_2O) δ: 6.18 (d, 1H, $J_{1'2'}$=6.44 Hz, H-1′), 4.52 (dd, 1H, $J_{3'2'}$=3.0 Hz, $J_{3'2''}$=7.5 Hz, H-3′), 4.21 (d, 1H, $J_{4'5'}$=4.3 Hz, H-4′), 3.59 (dd, 1H, $J_{5'4'}$=4.6 Hz, $J_{5'6}$=9.4 Hz, H-5′), 3.37 (m, 1H, H-6), 2.95–2.67 (m, 2H, H-5), 2.59–2.32 (m, 2H, H-2′, H-2″); ESI-MS (negative mode) *m/z*=227.0 ($[M-H]^-$) (calculated $[M-H]^-$=227.1).

(5′*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5′-*O*-levulinyl-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (6). Compound **5** (1.48 g, 3.17 mmol) was dried by repeated co-evaporation with dry dichloromethane and then dissolved in dry THF (70 mL) under an argon atmosphere. To the stirred solution, *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.64 g, 7.95 mmol), 4-dimethylaminopyridine (DMAP) (23 mg, 0.19 mmol) and levulinic acid (0.66 mL, 6.39 mmol) were added. After 2 h 30 at room temperature, the reaction mixture was cooled down to 5°C in an ice-bath and quenched by addition of methanol (0.5 mL). The white precipitate of 1,3-dicyclohexylurea (DCU) was removed by filtration and washed twice with dichloromethane (20 mL). Then, the resulting filtrate was evaporated under reduced pressure. Chromatography of the crude product on a silica gel column and elution with EtOAc/hexane 1/1 afforded **5** as a white foam (1.50 g, 84%). R_f=0.54 (CH_2Cl_2/MeOH 97/3); 200 MHz ^{1}H NMR ($CDCl_3$) δ: 7.66–7.62 (m, 10H, H arom., TBDPS), 6.29 (d, 1H, $J_{1'2'}$=6 Hz, H-1′), 4.74 (dd, 1H, $J_{5'4'}$=4.6 Hz, $J_{5'6}$=9.3 Hz, H-5′), 4.55 (dd, 1H, $J_{3'2'}$=2.8 Hz, $J_{3'2''}$=7.1 Hz, H-3′), 4.46 (d, 1H, $J_{4'5'}$=4.6 Hz, H-4′), 3.19 (m, 1H, H-6), 2.89–2.28 (m, 8H, H-5, CH_2CH_2, H-2′, H-2″), 2.17 (s, 3H, $COCH_3$), 1.02 (s, 9H, $C(CH_3)_3$); ESI-MS (positive mode) *m/z*=587.1 ($[M+Na]^+$) (calculated $[M+Na]^+$=587.5).

(5′*S*,6*S*)-5′-*O*-Levulinyl-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (7). Compound **6** (1.50 g, 2.66 mmol) was dissolved in dry THF (75 mL). A solution of TBAF (5.62 mL) in THF (1 M) was added and the resulting mixture was stirred at room temperature for 40 min. The solvent was removed under reduced pressure and the resulting red oil was purified by chromatography on a silica gel column. Elution was achieved with a step gradient of MeOH from 0 to 5% in dichloromethane. The appropriate fractions were pooled and then concentrated to dryness giving 580 mg of compound **7** as a white foam (yield of 67%). R_f=0.25 (CH_2Cl_2/MeOH 90/10); 200 MHz ^{1}H NMR (DMSO-d_6) δ: 6.09 (d, 1H, $J_{1'2'}$=5.9 Hz, H-1′), 5.22 (m broad, 1H, –OH), 4.54 (dd, 1H, $J_{5'4'}$=4.6 Hz, $J_{5'6}$=9.7 Hz, H-5′), 4.33 (m, 1H, H-3′), 4.09 (d 1H, $J_{4'5'}$=4.3 Hz, H-4′), 3.53 (m, 1H, H-6), 3.08–2.24 (m, 8H, H-5, CH_2CH_2, H-2′, H-2″), 2.17 (s 3H, $COCH_3$); ESI-MS (positive mode) *m/z*=327.0 ($[M+H]^+$) (calculated $[M+H]^+$=327.3).

(5′*S*,6*S*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphine]-5′-*O*-levulinyl-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (8). Compound **7** (90 mg, 0.28 mmol) was dissolved in dry dichloromethane (7 mL) and then evaporated to dryness. The operation was repeated twice. Then, the resulting residue was re-dissolved in CH_2Cl_2 (4 mL) and THF (1 mL) under an argon atmosphere. Dry DIEA (106 μL, 0.6 mmol) and then 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (68 μL, 0.3 mmol) were added under a vigorous stirring. After 1 h at room temperature, the reaction mixture was cooled to 5°C with an ice-bath and then quenched by addition of DIEA (150 μL) and methanol (100 μL). After 10 min, the mixture was evaporated to dryness. 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 (99/1, v/v). The appropriate fractions were pooled and then concentrated to dryness giving 115 mg (0.2 mmol) of the phosphoramidite synthon **8** as a white foam (yield of 80%). R_f=0.61 (CH_2Cl_2/TEA/MeOH97/1/2); 200 MHz ^{1}H NMR

(acetone-d_6) δ: 6.23 (d, 1H, J=6.2 Hz, H-1′), 4.73 (m, 1H, H-5′), 4.43 (m, 1H, H-3′), 4.14 (m, H-4′), 3.86–3.18 (m, 5H, H-6, 2NC*H*(CH_3)$_2$, CH_2CH_2OP), 2.88–2.51 (m, 10H, H-5, CH_2CH_2CN, $CH_2CH_2COCH_3$; H-2′, H-2″), 2.03 (s, 3H, $COCH_3$), 1.26–1.18 (m, 12H, 2NCH(CH_3)$_2$); ^{31}P NMR (acetone-d_6) δ: 147.73, 147.42 (1P, s) ESI-MS (negative mode) m/z=525.1 ([M−H]$^-$) (calculated [M−H]$^-$=525.0).

Stability studies of (5′*S*,6*S*)-CyclodHdUrd (1) under the alkaline conditions used for chemical synthesis of oligonucleotides

Aqueous ammonia (30%, 1 mL) was added to 0.2 $AU_{230\ nm}$ of compound **1** in sealed tubes and the resulting solutions were kept at either room temperature or 55°C. Then, the reactions were quenched at increasing time intervals (0, 1, 2, 4, 16 and 24 h) by freezing the samples in liquid nitrogen and subsequent lyophilisation. Samples were subsequently analysed by reverse-phase HPLC (system A).

Stability studies of (5′*S*,6*S*)-CyclodHdUrd (1) under the acid conditions used for chemical synthesis of oligonucleotides

A similar procedure as described above for the alkali stability assays was applied to study the stability 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 of (5′*S*,6*S*)-CyclodHdUrd (1) under oxidising conditions used for chemical synthesis of oligonucleotides

Similarly, compound **1** was incubated in a 0.1 M oxidising solution of iodine for 1, 2, 4, 8, 16, 24 h at room temperature.

Solid-phase synthesis of oligonucleotides

(5′*S*,6*S*)-CyclodHdUrd **1** containing oligonucleotides were prepared by phosphoramidite solid-phase synthesis using the 'Pac chemistry', with retention of the 5′ terminal DMTr group (trityl-on mode). Therefore phenoxyacetyl, isopropyl-phenoxyacetyl and acetyl were used to protect the amino function of dAdo, dGuo and dCyd, respectively. Due to the insolubility in acetonitrile, the phosphoramidite synthon **8** was dissolved in dry dichloromethane and then placed in the additional port of a model 392 DNA synthesiser (Applied Biosystem), using the standard 1 μmol synthesis scale. The duration of the condensation was increased by a factor of 4 for the modified nucleoside phosphoramidite **8** (120 s instead of 30 s for normal nucleoside phosphoramidites). After incorporation of the synthon **8**, the synthesiser was stopped and the CPG column was disconnected. Using two gastight syringes, one at each extremity of the column, 2×3 ml of a freshly prepared solution of hydrazine monohydrate (pyridine–acetic acid 3:2 v/v) was passed over the resin by pushing slowly the solution from one syringe to the other. This was performed for 2×3 min. After washing with 3×3 mL of dry acetonitrile, the CPG column was placed again in the synthesiser and the remaining nucleotides were added.

Deprotection and purification of oligonucleotides

Upon completion of the synthesis, the oligonucleotides were detached from the solid support and the amino functions were 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 reverse-phase HPLC (system C). Then, the purity and homogeneity of the collected fractions were controlled by HPLC analysis (system D). Five oligonucleotides including **11**, **12**, **13**, **15**, **16** were thus obtained. The modified 22-mer oligonucleotide **13**, used in repair and replication studies, was further purified by PAGE using a 20% polyacrylamide/7 M urea gel and then desalted using a NAP-25 Sephadex columns.

Stability studies of modified oligonucleotides that contained (5′*S*,6*S*)-CyclodHdUrd 1 and (5′*S*,6*S*)-CyclodHThd 2 in piperidine solution

Modified 22-mer oligonucleotides that contained (5′*S*,6*S*)-CyclodHdUrd **1** [ODN **13**; 5′-d(CAC TTC GGA T**X**G TGA CTG ATC T)-3′, **X**=**1**] or (5′*S*,6*S*)-CyclodHThd **2** [ODN **14**; 5′-d(CAC TTC GGA **Y**CG TGA CTG ATC T)-3′, **Y**=**2**] were treated with a freshly made 1 M piperidine aqueous solution at 90°C for 15 min, 30 min and 1 h, respectively. Typically, the reactions were carried out on 0.5 pmol of 5′-[^{32}P]-labelled modified oligonucleotides in 50 μL of the piperidine solution in sealed tubes. The mixtures were then cooled and co-evaporated twice with water, before to be 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)]. The electrophoresis was carried out at 1500 V for 2 h. Subsequently, analysis of the radiolabelled bands was achieved by phosphorimaging (Molecular Dynamics Phosphorimager) using Image Quan T software.

Thermal denaturation studies

0.25 $AU_{260\ nm}$ of either 5′-d(ATC GTG ACT GAT CT)-3′ or 5′-d(ATC GTG A**X**T GAT CT)-3′ where **X**=(5′*S*,6*S*)-CyclodHdUrd **1**, or 5′-d(ATC GTG AC**Y** GAT CT)-3′ where **Y**=(5′*S*,6*S*)-CyclodHThd **2** together with 0.3 $AU_{260\ nm}$ of their complementary sequence [5′-d(AGA TCA GTC ACG AT)-3′] were mixed in 200 μL of a buffer that contained 0.01 M sodium phosphate, 0.1 M NaCl, 0.001 M EDTA, pH=7. The DNA fragments were annealed by heating the solutions at 90°C for 3 min, followed by slow cooling to 4°C (3 h). The hybridisation solutions were diluted in 400 μL of buffer. Then, measurements of UV absorbance were made in a 0.8 mL quartz cell (0.2 cm path length) with a UV/vis spectrophotometer equipped with a Peltier temperature controller. The absorbance of the samples was monitored at 260 nm over a temperature range comprised between 15 and 80°C at a heating rate of 1°C/min. The reported data are the average of three melting curves per oligonucleotide duplexes.

Labelling of oligonucleotides

Oligonucleotides (50 pmol) were labelled 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. Then, the reaction was 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 column G-25 columns.

Enzymatic digestion of modified oligonucleotides by nuclease P_1 and alkaline phosphatase

0.5 $AU_{260\ nm}$ of modified 14-mer oligonucleotide **12** 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 (1U/μL) in a 30 mM NaOAc and 0.1 mM $ZnSO_4$ aqueous solution (pH=5.5). Then, 5 μL of Tris-HCl buffer (10 X) and 2 U of calf intestinal alkaline phosphatase were 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 resolved by reversed-phase HPLC (system E). The different products, which were thus obtained, were identified by both co-injection with synthetic standards and electrospray ionisation mass spectrometry analysis in the negative mode. The same enzymatic digestion procedure was performed with the synthetic trinucleotide [5′-d(A**X**T)-3′] **11** and the modified 22-mer oligonucleotide **13**.

Enzymatic digestion of modified oligonucleotides by calf spleen phosphodiesterase (5′-*exo*)

0.2 $AU_{260\ nm}$ of modified 14-mer oligonucleotide **12** in 20 μL of 0.02 M ammonium citrate (pH=5) was digested by incubation at 37°C with 10^{-3} U of calf spleen phosphodiesterase. Aliquots (2 μL) were withdrawn at increasing periods of time and the reactions were stopped by addition of 50 μL of water. Then, the solution was frozen in liquid nitrogen and lyophilised. The resulting dry residues were taken up in 20 μL of 0.1% aqueous solution of trifluoroacetic acid and subsequently analysed by MALDI-TOF spectrometry following the procedure described above. The trinucleotide [5′-d(A**X**T)-3′] **11** was incubated with 10^{-3} U of calf spleen phosphodiesterase over a period of 24 h. Then, the resulting mixture was directly submitted to reversed-phase HPLC analysis (system E). The different products thus separated were collected and analysed by electrospray ionisation mass spectroscopy in the negative mode.

Enzymatic digestion of modified oligonucleotides by bovine intestinal mucosa phosphodiesterase (3′-*exo*)

Similarly, enzymatic digestions of modified oligonucleotides were performed using 0.1×10^{-4} U of bovine intestinal mucosa phosphodiesterase in ammonium citrate buffer (pH=9). Then, MALDI-TOF spectrometry analyses were performed on the separated compounds as described above.

Repair studies of (5′*S*,6*S*)-CyclodHdUrd 1 and (5′*S*,6*S*)-CyclodHThd 2 containing DNA oligomers by repair enzymes namely Fpg, endo III and endo VIII

DNA repair experiments were carried out with Fpg, endo III and endo VIII on modified double-stranded DNA fragments that contained the modified lesion **1** or **2**. Typically, 5 pmol of 5′-[^{32}P]-end-labelled modified 22-mer oligonucleotide **13** or **14** was annealed to 10 pmol of the nonlabelled complementary strand [5′-d(AGA TCA GTC ACG ATC CGA AGT G)-3′]. This was achieved by heating the resulting solutions at 80°C for 5 min and subsequent slow cooling to 4°C, at least, for 3 h. The integrity of the modified duplex was then assessed by MALDI-TOF-MS. Then, the enzymatic reactions were performed in 10 μL solutions of 20 mM Tris-HCl (pH=7.5), 1 mM EDTA and 100 mM KCl at 37°C for 30 min with increasing concentrations of enzymes (1–200 ng/μL final concentration). The enzymatic reaction was quenched by adding 5 μL of formamide dye (95% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol). Samples were then loaded onto a 20% polyacrylamide/7 M urea gel in TBE buffer. The electrophoresis was carried out at 1600 V for 2 h. The products of the reaction were then analysed by phosphorimaging. A similar assay using 8-oxodGuo containing ODN was performed as a control.

Primer extension catalysed by Klenow exo$^-$ fragment and *Taq* polymerase

Reactions catalysed by Klenow exo$^-$ were carried out in 10 μL of 50 mM Tris-HCl (pH=7.5), 10 mM $MgCl_2$, 0.05 mg/mL bovine serum albumin (BSA) and 1 mM dithiothreitol (DTT). Primer extension reactions using *Taq* polymerase were conducted in 10 μL solutions of 10 mM Tris-HCl (pH=8.3), 1.5 mM $MgCl_2$ and 50 mM KCl. Buffered solutions that contained the oligonucleotide template **13** or **14** and the 5′-[^{32}P]-end-labelled 11- or 12-mer primer (**15** or **16**), at the, respectively concentration 1 to 1.5 nanomolar, were heated at 80°C for 5 min and then cooled to 4°C over a period of 3 h. DNA polymerisation reactions were carried out with either 100 μM solutions of a single dNTP or a mixture of all four dNTPs. The solutions were maintained at 25°C for 30 min in the presence of 0.2 U of either Klenow exo$^-$ or *Taq* polymerase. Reactions were stopped by addition of 5 μL formamide dye. Samples were then loaded onto a 20% polyacrylamide/7 M urea gel in TBE buffer. The electrophoresis was carried out at 1600 V for 2 h. The products of the reaction were finally analysed by phosphorimaging. A similar experiment using an unmodified 22-mer template was performed as a control.

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