Chemical Synthesis and Biochemical Properties of Oligonucleotides that Contain the (5′*S*,5*S*,6*S*)-5′,6-Cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine DNA Lesion

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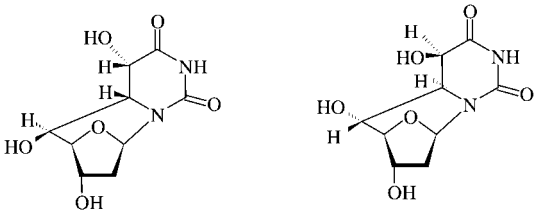
The first chemical synthesis of (5′S,5S,6S)-5′,6-cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine [(5′S,5S,6S)-cyclo-5-OH-dHdU], a radiation-induced decomposition product of 2′-deoxycytidine in aerated solution, is reported. Subsequently, 2′-deoxycytidine was incorporated into oligodeoxyribonucleotides with defined sequences by using an optimized system of protection that takes into account the reactivity and stability of the modified building blocks. After deprotection and purification, the chemical composition of the modified DNA fragments was assessed by enzymatic digestions and mass spectrometry measurements. The MS analyses confirmed the presence and integrity of the lesion within the synthesized DNA fragments. In vitro replication and repair studies showed that (5′S,5S,6S)-cyclo-5-OH-dHdU acts as a block for DNA polymerases when inserted into DNA oligomers and is not excised by any of the tested DNA N-glycosylases. Therefore, (5′S,5S,6S)-cyclo-5-OH-dHdU may represent a potential lethal lesion within the cell if it is not removed by the nucleotide excision repair machinery.

KEYWORDS:

DNA damage · DNA polymerases · glycosylases · nucleosides · oligonucleotides

Introduction

Two diastereoisomers of 5′,6-cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine (cyclo-5-OH-dHdU) have recently been identified as radiation-induced decomposition products of 2′-deoxycytidine in aerated aqueous solution (Scheme 1).[1] The formation of the cyclonucleosides may be described in terms of initial abstraction of a hydrogen atom from the C-5′ atom of the sugar moiety in 2′-deoxycytidine by an OH radical, followed by intramolecular addition of the resulting radical to the C-6 atom of the cytosine moiety and subsequent fixation of molecular oxygen onto the resulting C-5-centered radical.[1] Analogous purine cyclonucleosides were found to be generated through the intramolecular addition of the C-5′ radical that is formed by OH-radical-mediated abstraction to the C-8 atom of either an adenine or a guanine moiety.[2–4] Interesting biological features of DNA repair excision and mutagenesis were recently inferred from several studies[5–8] that involved the use of site-specific modification to form oligonucleotides that contained 5′,8-cyclo-2′-deoxyadenosine and 5′,8-cyclo-2′-deoxyguanosine. In order to assess the structural features and the biological role of the cyclopyrimidine nucleoside cyclo-5-OH-dHdU in DNA, it is necessary to prepare oligonucleotides that contain this lesion at defined sites. This procedure also allows a comparison of the biochemical properties of cyclo-5-OH-dHdU with those of (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT lesions previously studied.[9, 10]



(5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) (5′*R*,5*R*,6*R*)-cyclo-5-OH-dHdU

Scheme 1. *Structure of the two diastereoisomers of 5′,6-cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine (cyclo-5-OH-dHdU), which are radiation-induced decomposition products of 2′-deoxycytidine in aerated aqueous solution.*

We report herein the first chemical synthesis of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) and its site-specific incorporation into oligonucleotides by an original protection strategy. In particular, the stability of **1** under acidic, oxidizing, and several different alkaline conditions, which included hot piperidine treatment of modified oligonucleotides containing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**), was studied. The site-specific insertion of **1** into several oligonucleotides was then performed by using highly labile protecting groups. The modified oligodeoxyribonucleotides (ODNs) were used to study the behavior of **1** towards

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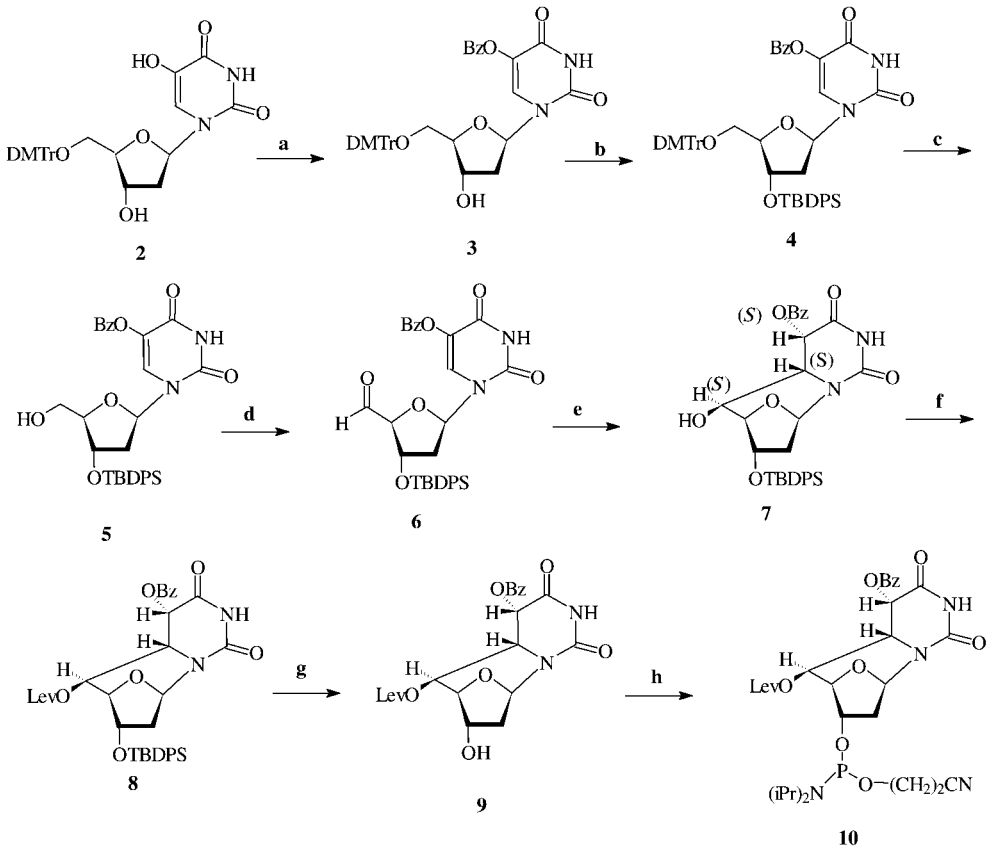
several endonucleases, exonucleases, and DNA *N*-glycosylases. Finally, the coding properties of **1** were also investigated during the replication of the modified ODNs, which was mediated by two bacterial DNA polymerases, namely the Klenow fragment of *Escherischia coli* polymerase I and *Taq* DNA polymerase. The primer extension results produced with *Taq* DNA polymerase suggest that the lesion may induce mutations and cell death in vivo.

Results and Discussion

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

Stability studies of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1): Prior to the preparation of the phosphoramidite **10** (Scheme 2), the stability of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) was studied under the main experimental conditions used in the course of the solid-support synthesis. It was found that **1** is stable in the presence of trichloroacetic acid, tetrazole, iodine/pyridine/tetrahydrofuran (THF)/H_2O, and acetic anhydride/*N*-methylimidazole, the reagents that are used in automated chemical DNA synthesis. However, **1** was fully decomposed under standard basic deprotection conditions (30% ammonium hydroxide, 55 °C, 12 h). About 15% of the modified monomer was degraded when left in a 30% ammonium hydroxide solution at room temperature for 4 h. This was circumvented by using the highly alkali-labile amino-protecting groups developed by Schulhof et al.[11] ("Pac phosphoramidite" chemistry), which allow complete deprotection of synthetic oligonucleotides in a solution of K_2CO_3 (0.05 M) in methanol at room temperature within 4 h. Under these mild conditions, neither degradation nor isomerization of the modified nucleoside **1** was detected.

Synthetic procedure for the preparation of the phosphoramidite building block of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1): The major source of difficulty in the preparation of oligonucleotides that contain (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) is the presence of an additional hydroxy group at the C-5 position of the modified base. Firstly, the hydroxy group must be introduced and protected before the cyclization step. Moreover, this secondary hydroxy group (5-OH) may interfere with the solid-phase DNA synthesis, either by providing a starting point for the attachment of nucleosides or by giving rise to other undesired byproducts. Consequently, it was deemed necessary to mask the 5-OH group with a protecting group that is stable under the conditions of solid-phase DNA synthesis. However, this protecting group has to be easily removable during the final deprotection step, which



Scheme 2. *Synthesis pathway used for the preparation of the phosphoramidite building block* ***10*** *for the synthesis of (5′S,5S,6S)-cyclo-5-OH-dHdU. Conditions: a) Bz_2O, pyridine, 5 h, RT; b) TBDPS-Cl, imidazole, pyridine, 5 h, RT; c) TFA, dichloromethane, 45 min, RT; d) Dess–Martin periodinane, CH_2Cl_2, 2 h, RT; e) AIBN, Bu_3SnH, benzene, 5 h, reflux; f) Levulinic acid, N,N′-dicyclohexylcarbodiimide, 4-dimethylaminopyridine (DMAP), THF, 1 h 30 min, RT; g) TBAF, THF, 1 h, RT; h) 2-Cyanoethyl-N,N-diisopropylphosphoramidochloridite, DIEA, CH_2Cl_2/THF, 1 h, RT. For abbreviations, see the text and the Experimental Section.*

requires the use of a solution of K_2CO_3 (0.05 M) in methanol at room temperature.

The synthesis of the targeted phosphoramidite **10** (Scheme 2) started with 5′-*O*-(4,4′-dimethoxytrityl)-5-hydroxy-2′-deoxyuridine (**2**), which was prepared according to the method designed by Essigmann et al.[12] This method allowed us to introduce the hydroxy group at the C-5 position. Compound **2** was then converted into ester **3** by treatment of **2** with benzoic anhydride in pyridine at room temperature to give a 71% yield. The ester protecting group was found to be inert during the cyclization step and was easily removed during deprotection with K_2CO_3 (0.05 M) in methanol. Silylation of the 3′-hydroxy group of **3** with *tert*-butylchlorodiphenylsilane (TBDPS-Cl) and imidazole in pyridine afforded compound **4**. The 5′-dimethoxytrityl (DMTr) group of **4** was then selectively removed by treatment with trifluoroacetic acid in dichloromethane to yield the 5′-hydroxy derivative **5** (74%). Subsequently, **5** was oxidized by using Dess–Martin periodinane[13] in anhydrous dichloromethane under an argon atmosphere, to afford the 5′-aldehyde nucleoside **6** in a 75% yield. Cyclization of **6** was achieved upon treatment with tributyltin hydride (Bu_3SnH) and 2,2′-azobis(2-methylpropionitrile) (AIBN) in benzene under reflux and under an argon atmosphere over a period of 5 h. The desired cyclonucleoside **7** was obtained in a yield of only 21%. This poor yield could be explained by the presence of the bulky benzoyl group, which induces steric hindrance. The structure of **7** was inferred by ^1H and ^{13}C NMR spectroscopy analyses. Additional structural insights were gained from ESI MS measurements in the negative mode. Interestingly, only the (5′*S*,5*S*,6*S*) diastereoisomer was obtained. The structure of this cyclonucleoside was confirmed by comparison of the coupling constants with those reported by Shaw and Cadet for the diastereoisomers of cyclodHT and cyclodHdU.[14] Thus, the values of $^3J_{4'5'}$ (4.5 Hz), $^3J_{5'6'}$ (9.8 Hz), and $^3J_{56}$ (3.3 Hz) for **7** are consistent with the 5′*S*,5*S*,6*S* configuration. Further structural information was inferred from 1D NOESY experiments. The dipolar interaction observed between H-6 and H-5 is in agreement with a *cis* configuration of these protons. In contrast, irradiation of H-6 did not lead to any detectable NOE enhancement of the H-5′ signal. This is strongly indicative of an antiperiplaner relationship between the two concerned protons. As previously observed for (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT,[2, 3] attempts to convert **7** into the 5′-*O*-DMTr-protected derivative failed. Therefore, the levulinyl (Lev) group, a non-standard hydroxy protecting group already used in the synthesis of (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT, was chosen.[9, 10, 15] Thus, the protected cyclonucleoside **8** was obtained in a 94% yield. The 3′-*O*-TBDPS ether **8** was selectively desilylated upon treatment with tetrabutylammonium fluoride (TBAF) in THF at room temperature for 1 h to give **9** in 72% yield. The phosphoramidite **10** was finally synthesized in 81% yield by reaction of the secondary alcohol in compound **9** with 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite ($NCCH_2CH_2OP(Cl)NPr^i_2$) in the presence of *N*,*N*-diisopropylethylamine (DIEA).

Solid-phase synthesis and characterization of oligonucleotides that contain (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1): Modified ODNs **11**–**13** (for details, see the Experimental Section)[16] were synthesized according to the solid-phase phosphoramidite method (1 μmol scale) on an automated DNA synthesizer by using **10** and commercially available phenoxyacetyl protecting group (PAC)-dA-, *i*Pr-PAC-dG-, and acetyldC-cyanoethyl phosphoramidites ("Pac chemistry").[11] Interestingly, the levulinyl protecting group of the modified nucleoside can be easily removed under neutral conditions (hydrazine monohydrate (0.5 M) in pyridine/acetic acid (3:2) at room temperature for 10 min) after cleavage of the oligonucleotides from the column of the synthesizer. The efficiency of the coupling of the cyclonucleoside cannot be measured directly because there is no trityl group present. However, the estimated yield, based upon the subsequent insertion of a DMTr-containing phosphoramidite nucleoside, was 85%. After cleavage from the support and removal of the alkali-labile groups by treatment with a solution of K_2CO_3 (0.05 M) in methanol at room temperature for 4 h, the 5′-DMTr-oligomers were purified by reversed-phase HPLC on a polymeric support by an on-line detritylation purification procedure.[17] The purity and homogeneity of the modified oligonucleotides were controlled first by HPLC and then by polyacrylamide gel electrophoresis of 5′-[^{32}P]-labeled fragments. Approximately 5–25 absorbance units (AU_{260}) modified oligonucleotides were obtained. ESI MS measurements of the modified ODNs confirmed the incorporation of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**).

Piperidine stability of modified oligodeoxyribonucleotides that contain (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1): The availability of modified oligonucleotides that contained **1** allowed the determination of the stability of the oligonucleotide **12** in the presence of piperidine, which is used to reveal alkali-labile sites in oxidized DNA. For this purpose, the oligonucleotide 5′-d(ATC GTG A**X**T GAT CC)-3′ (**12**; **X** = **1** = (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU) was labeled with [^{32}P] at the 5′ end and then treated with a 1 M piperidine solution at 90 °C for 15, 30, and 60 min. The DNA oligomers were finally analyzed by denaturing PAGE. It was shown that **1** forms only a weakly alkali-labile lesion (Figure 1) since only 35% strand cleavage was observed after 1 h of piperidine treatment at 90 °C.

Thermal denaturation studies: In order to determine the structural effect of the incorporation of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU into DNA, the thermal stability of the **X**·dG base pair was evaluated. Thus, 5′-d(ATC GTG A**X**T GAT CC)-3′ was annealed with the complementary DNA strand 5′-d(GGA TCA GTC ACG AT)-3′. The melting temperature (T_m) of the duplex was determined by measurement of UV absorbance at 260 nm. It was found that the T_m value of the duplex that contained (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU ($T_m = 33 \pm 1$ °C) was lower than that of the unmodified duplex ($T_m = 44 \pm 1$ °C; data not shown). The decrease in the T_m value ($\Delta T_m = 11$ °C) suggests that the incorporation of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU induces a local destabilization of the duplex DNA structure. A similar behavior was previously observed for (5′*S*,6*S*)-cyclodHdU[9] and (5′*S*,6*S*)-cyclodHT.[10]

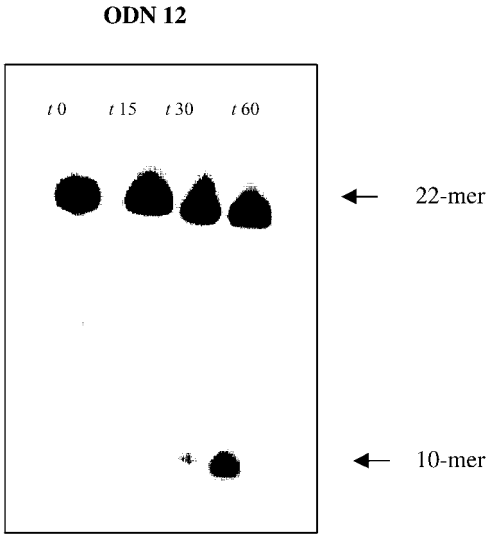


Figure 1. *PAGE analysis of the 5′-labeled 14-mer ODN that contains* ***1*** *(ODN* ***12****) after treatment with a 1 M piperidine solution at 90 °C for 0, 15, 30, and 60 min.*

Enzymatic digestion of oligonucleotides that contain (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1) by nuclease P_1 and alkaline phosphatase: Aliquots of the modified 14-mer oligonucleotide **12** were submitted to the action of the single-strand specific endonuclease nuclease P_1 over a period of either 2 h or 24 h, followed by incubation with bacterial alkaline phosphatase. In both cases, the resulting hydrolysate, which was analyzed by reversed-phase HPLC, shows a similar content. This hydrolysate was found to consist of the nucleotides dC, dG, T, and dA and the trinucleotide [5′-d(A**X**T)-3′] in a 3:3:3:2:1 ratio. This provides support for the suggested structure of modified 14-mer **12** (Figure 2). The observed lack of free modified nucleoside **1**, even after 24 h of incubation, received further confirmation upon co-injection of the enzymatic digestion products with an authentic sample of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU **1** and subsequent HPLC analysis. Moreover, the structure of the trinucleotide was established by negative-mode ESI MS analysis of the material collected by HPLC, and by co-injection of the enzymatic digestion products with the trinucleotide 5′-d(A**X**T)-3′ (**11**) previously synthesized. In contrast, incubation of **11** with nuclease P_1 over either 2 h or 24 h followed by treatment with alkaline phosphatase did not provide the free 2′-deoxyribonucleosides dA, T, and **1** (data not shown). Similar results were obtained with the modified 22-mer oligonucleotide **13**. These observations are consistent with those of previous studies on enzymatic digestion of modified oligonucleotides into which (5′*S*,6*S*)-cyclodHT[10] and (5′S,6S)-cyclodHdU[9] were inserted. Nuclease P_1 was found to be unable to cleave the phosphodiester bonds between normal and altered 2′-deoxyribonucleosides.[18, 19]

Enzymatic digestion of oligonucleotides containing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1) by bovine intestinal mucosa phosphodiesterase (3′-exo) and calf spleen phosphodiesterase (5′-exo): Additional enzymatic digestion experiments were performed on the modified 3-mer **11** by using the two exonucleases bovine intestinal mucosa phosphodiesterase (3′-

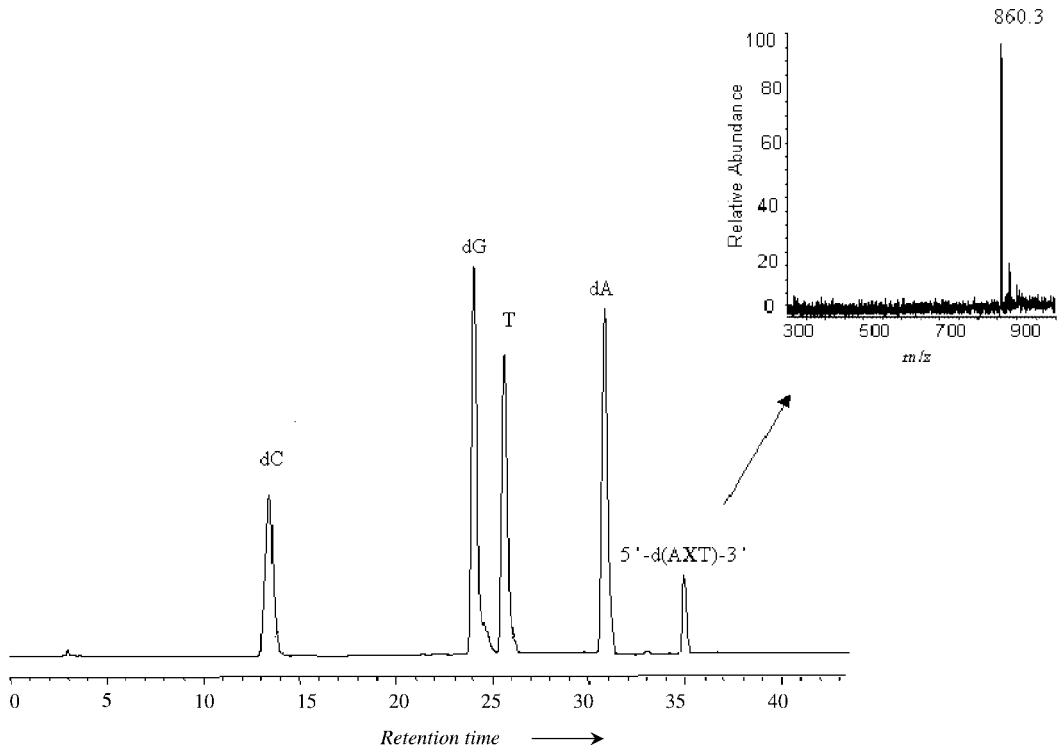


Figure 2. *HPLC profile (Hypersil C_{18} column) of the enzymatic digestion mixture that results from the 14-mer oligonucleotide* ***12*** *after digestion by nuclease P1 (2 h) and alkaline phosphatase (1 h). Inset: ESI MS (negative mode) of the trinucleotide 5′-d(A**X**T)-3′.*

exo) and calf spleen phosphodiesterase (5′-exo). It was shown that both enzymes failed to cleave the phosphodiester bond on either side of the (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) residue, as already observed for (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT.[9, 10]

Repair assays of oligonucleotides containing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1) with purified DNA repair proteins: Attempts were made to assess whether the (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU lesion may be a substrate for the base excision repair enzymes formamidopyrimidine DNA *N*-glycosylase (Fpg), endonuclease III (endo III), endonuclease VIII (endo VIII), Ntg1 protein, Ntg2 protein, and yOgg1 protein. The substrate specificity of these repair enzymes is well documented.[20] The 5′-[^{32}P]-labeled 22-mer ODN **13**, which contains compound **1**, was hybridized with its complementary strand, which has a guanine opposite **1**. The resulting duplex was incubated with each of the repair enzymes mentioned above. The excision of the cyclonucleoside by the repair enzymes was probed by searching with PAGE. for the occurrence of ODN cleavage It was found that endo III, endo VIII, Ntg1 protein, and Ntg2 protein, which act primarily on altered pyrimidine bases, are not able to cleave the modified DNA duplex at the site of **1** (data not shown). The same results were obtained for the Fpg and yOgg1 proteins (data not shown). Moreover, the glycosylase AlkA was not able to excise the damage from the oligonucleotide. It should be added that alkali treatment of the DNA duplex that contained **13** and was incubated with AlkA did not lead to any detectable cleavage of the corresponding oligonucleotide. This may be rationalized in terms of the lack of cleavage of the *N*-glycosidic bond of the cyclic nucleoside **1**, as already observed for (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT with endo III, endo VIII, or Fpg proteins.[9, 10]

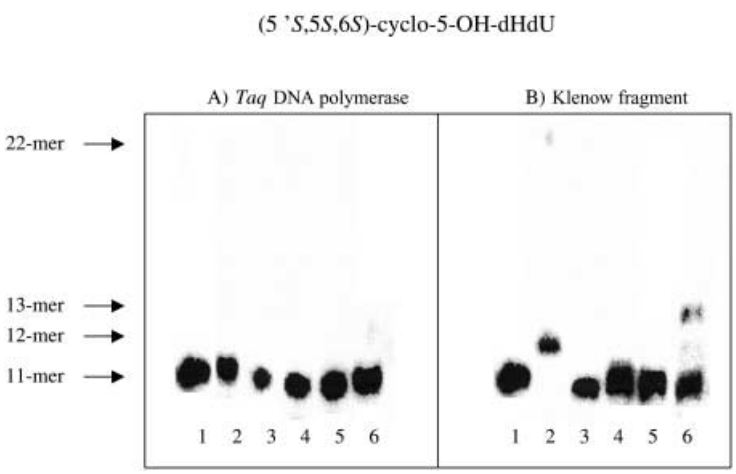


Figure 3. *Modified 22-mer template 5′-d(CAC TTC GGA T**X**G TGA CTG ATC C)-3′ annealed with a 5′-[^{32}P]-labeled 11-mer 5′-d(GGA TCA GTC AC)-3′ (line 1). Primer extension reactions were catalyzed by the* Taq *DNA polymerase (**A**) and the Klenow fragment (**B**), in the presence of 100 μM of dNTP (lane 2), dCTP (lane 3), dGTP (line 4), dTTP (line 5), and dATP (line 6), as described in the Experimental Section. The reaction mixtures were subjected to denaturing 20% PAGE and the extended products were visualized by phosphorimaging (Molecular Dynamics Phosphorimager) with the Image Quan T software.*

In vitro replication experiments with DNA polymerases: The ability of the *Taq* polymerase and the Klenow exo$^-$ fragment to extend a primer annealed with a template bearing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU **1** was investigated. The primer was [^{32}P]-labeled at its 5′ end so that extension by nucleotide incorporation could be observed by sequencing PAGE. The intensity of each band is proportional to the number of molecules that terminates the synthesis at a given position of the template. Figure 3 shows the denaturing PAGE bands obtained by elongation of the 5′-[^{32}P]-labeled 11-mer primer 5′-d(GGA TCA GTC AC)-3′ annealed with the oligonucleotide **13** in the presence of each of the two DNA polymerases. In addition, similar replication assays were performed with the unmodified 22-mer ODN, which contained 2′-deoxycytidine, as the template in order to assess the activity and the specificity of the polymerases (data not shown). It was found that for *Taq*-polymerase-mediated polymerization the extension was inhibited and no deoxynucleotide insertion occurred when all the deoxynucleoside triphosphates (dNTPs) were present (Figure 3 A, lane 2). When the Klenow fragment was used, the primer extension reactions led mainly to the same deoxyadenosine monophosphate (dAMP) incorporation opposite lesion **1**. Nevertheless, when the four dNTPs were present, a transient inhibition was observed opposite the lesion and only small amounts of the fully extended primer were formed (Figure 3 B, lane 2). Similar results were obtained for the (5′*S*,6*S*)-cyclodHdU and (5′*S*,6*S*)-cyclodHT lesions.[9, 10] These preliminary in vitro results are indicative of a possible lethal action of these cyclopyrimidine lesions within cells.

Conclusion

The synthesis of the nucleoside (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) and its incorporation into several oligonucleotides by the phosphoramidite approach were achieved by using an adapted set of protecting groups and mild alkaline deprotection conditions. The synthetic oligonucleotides were isolated in good yields and characterized by several complementary techniques, which showed the integrity of the incorporated modified nucleoside. Studies of the stability of the modified oligonucleotides in the presence of piperidine prove the weak lability of the (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU lesion when it is inserted into DNA strands. The processing of **1** by different nucleases was then studied. It was found that nuclease P1, calf spleen phosphodiesterase, and bovine intestinal mucosa phosphodiesterase failed to cleave the (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) residue. 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 oxidized DNA. The ability of repair enzymes such as Fpg, endo III, endo VIII, Ntg1, Ntg2, and yOgg1 and AlkA proteins to excise the lesion **1** was also investigated. It was found that **1** was not a substrate for any of the repair enzymes. The biological study was extended to the evaluation of the coding properties of the lesion **1**. This involved the determination of base-specific incorporation directed by **1** during in vitro replication by the Klenow exo$^-$ fragment and

Taq DNA polymerase. Thus, **1** was found to act as a block for both procaryote DNA polymerases. Therefore, **1** may represent a potentially lethal lesion within the cell if it is not removed by the nucleotide excision repair machinery.

Experimental Section

General: See Muller et al.[9] In addition, Fpg, endonuclease III, Ntg1, Ntg2, yOgg1, and AlkA were kind gifts from Dr. Serge Boiteux (CEA Fontenay-aux-Roses, France).

NMR measurements: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Unity 400 Varian spectrometer in the Fourier transform mode. The residual proton signal of tetramethylsilane (TMS; $\delta_H = 0$ ppm), $CDCl_3$ ($\delta_H = 7.26$ ppm), acetone-d_6 ($\delta_H = 2.17$ ppm), D_2O ($\delta_H = 4.92$ ppm), or dimethyl sulfoxide (DMSO)-d_6 ($\delta_H = 2.62$ ppm) was used as the external reference. $CDCl_3$ ($\delta_H = 77$ ppm) and acetone-d_6 (29 ppm) were used as references for the calibration of the ^{13}C NMR spectra. ^{31}P NMR spectroscopy measurements were performed on a Unity 400 Varian spectrometer and H_3PO_4 (85%) was chosen as the external standard. NOESY spectra (500 MHz) were recorded on AM 500 Brucker spectrometers (Brucker, Wissembourg, France). NOE experiments were carried out with a delay time of 1 s between the end of the data acquisition and the beginning of the following pulse, with the decoupling field gating off during data acquisition. Difference NOE spectra were obtained by subtracting the irradiated spectra, which had a 10330 Hz width (65000 data points), from the reference spectrum, which was usually recorded with the decoupling fields set symmetrically on the opposite side from the carrier frequency.

Mass spectrometry measurements: All modified and unmodified oligonucleotides were characterized by ESI MS by using an LCQ model spectrometer from Finnigan (San Jose, CA). Typically, 0.1 AU_{260nm} sample was dissolved in a solution of acetonitrile and water (50/50, v/v) that contained 1% triethylamine prior to analysis in the negative mode. The modified nucleosides were analyzed by ESI MS in both the positive and 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. FAB HRMS was carried out on a ZAB2-SEQ spectrometer (VG-Analytical) with thioglycerol as a matrix (C.N.R.S., Echangeur de Solaise, Vernaison, France).

HPLC separations:

System A: Reversed-phase HPLC (porous graphitized Hypercarb carbon column, 98.5% carbon, 5 μm, 250 Å, 100 × 3 mm) with a mixture of acetonitrile and ammonium formate buffer (AF; 25 mM, pH 6.2) as the eluent (100% AF for 5 min followed by a linear gradient of acetonitrile from 0–20% over 30 min) at a flow rate of 0.4 mL min^{-1}; UV detection at 230 nm.

System B: Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm) with a mixture of acetonitrile and triethylammonium acetate buffer (TEAA; 10 mM, pH 7) as the eluent (100% TEAA for 5 min followed by a linear gradient of acetonitrile from 0–30% over 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 inside diameter) with a mixture of acetonitrile and TEAA buffer (10 mM) as the eluent (100% TEAA for 5 min, isocratic TEAA/acetonitrile (92:8 v/v) for 13 min, isocratic TFA (1%) for 10 min, and finally a gradient from 0–10% acetonitrile for 40 min) at a flow rate of 2 mL min^{-1}; UV detection at 260 nm.

System D: Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm inside diameter) with a mixture of acetonitrile and TEAA buffer (10 mM, pH 7) as the eluent (100% TEAA for 5 min followed by a linear gradient from 0–10% of acetonitrile for 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 inside diameter) with a mixture of acetonitrile and AF buffer (25 mM, pH 6.2) as the eluent (100% AF for 10 min followed by a linear gradient from 0–10% of acetonitrile for 30 min) at a flow rate of 1 mL min^{-1}; UV detection at 230 nm during the first 12 min and then at 260 nm.

Synthetic procedures:

5′-*O*-(4,4′-Dimethoxytrityl)-5-hydroxy-2′-deoxyuridine (2): Compound **2** was prepared according to the method developed by Essigmann et al.[12] $R_f = 0.61$ (CH_2Cl_2/MeOH 90:10); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 9.48$ (s, 1H; NH), 7.39–7.13 (m, 10H; arom H (DMTr), H-6), 6.81 (m, 4H; arom H (DMTr)), 6.31 (t, ^3J(H,H) = 6.1 Hz, 1H; H-1′), 4.38 (m, 1H; H-3′), 3.95 (m, 1H; H-4′), 3.75 (s, 6H; OCH_3-DMTr), 3.37–3.33 (m, 2H; H-5′, H-5″), 2.41–2.15 (m, 2H; H-2′, H-2″) ppm; ESI MS (positive mode) *m/z*: calcd: 547.2 $[M+H]^+$; found: 547.1; ESI MS (negative mode) *m/z*: calcd: 545.2 $[M-H]^-$; found: 545.3.

5′-*O*-(4,4′-Dimethoxytrityl)-5-benzoyloxy-2′-deoxyuridine (3): Benzoic anhydride (Bz_2O; 6.4 g, 28.3 mmol) was added to a stirred solution of **2** (12 g, 21.9 mmol) in pyridine (206 mL). After 3 h at room temperature, additional Bz_2O (3.2 g, 14.15 mmol) was added. After 5 h, the solvent was removed in vacuo without heating and the residue was redissolved in CH_2Cl_2 (500 mL) then washed with a saturated aqueous solution of $NaHCO_3$ (400 mL). The organic solution was dried (Na_2SO_4), filtered, and concentrated. The residue was purified by chromatography on a silica gel column by using a step gradient of methanol (0–5%) in dichloromethane as the mobile phase. Evaporation to dryness of the collected chromatographic fractions provided **3** as a white foam (10.2 g, 71%). $R_f = 0.26$ (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 9.45$ (s, 1H; NH), 8.08 (s, 1H; H-6), 7.75–7.13 (m, 14H; arom H (DMTr, Bz), 6.72 (m, 4H; arom H (DMTr)), 6.39 (t, ^3J(H,H) = 6.2 Hz, 1H; H-1′), 4.62 (m, 1H; H-3′), 4.06 (m, 1H; H-4′), 3.81 (s, 6H; OCH_3-DMTr), 3.37–3.34 (m, 2H; H-5′, H-5″), 2.48–2.25 (m, 2H; H-2′, H-2″) ppm; ESI MS (positive mode) *m/z*: calcd: 673.2 $[M+Na]^+$; found: 673.1 $[M+Na]^+$; 303.2 $[DMTr]^+$; ESI MS (negative mode) *m/z*: calcd: 649.3 $[M-H]^-$; found: 649.3.

3′-*O*-(*tert*-Butyldiphenylsilyl)-5-benzoyloxy-2′-deoxyuridine (5): Imidazole (9.8 g, 144.4 mmol) and then *tert*-butylchlorodiphenylsilane (TBDPS-Cl; 18.5 mL, 72.2 mmol) were added to a stirred solution of **3** (10.2 g, 15.7 mmol) in pyridine (130 mL). After 2 h at room temperature, additional imidazole (4 g, 58.8 mmol) and TBDPS-Cl (6 mL, 23.4 mmol) were added. After 3 h, the reaction was quenched by addition of water (50 mL) and the solvents were removed in vacuo. The resulting residue was redissolved in CH_2Cl_2 (500 mL) and washed with an aqueous solution of saturated $NaHCO_3$ (400 mL). The organic solution was dried (Na_2SO_4), filtered, and concentrated. The crude residue **4** was then redissolved in dichloromethane (130 mL). Trifluoroacetic acid (TFA; 4 mL) was added to the solution. After 45 min at room temperature, the reaction mixture was cooled to 5 °C and then neutralized by dropwise addition of an aqueous ammonia solution (30%). Finally, water (300 mL) and then dichloromethane (200 mL) were added to the mixture. The organic layer was dried by addition of Na_2SO_4 and then concentrated under vacuum. Chromatography of the crude product on an open silica gel column, which was achieved by using methanol (0–3%) in dichloromethane, afforded **5** as a white foam (6.87 g, 74%). $R_f = 0.57$ (CH_2Cl_2/MeOH 95:5); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 8.79$ (s, 1H; NH), 7.78 (s, 1H; H-6), 7.66–7.36 (m, 15H; arom H (TBDPS, Bz)), 6.32

(t, ^3J(H,H) = 6.3 Hz, 1H; H-1′), 4.42 (m, 1H; H-3′), 3.98 (m, 1H; H-4′), 3.55 and 3.22 (dd, ^3J(H,H) = 2.4 Hz, ^2J(H,H) = 11.9 Hz, 2H; H-5′, H-5″), 2.33 – 2.05 (m, 2H; H-2′, H-2″), 1.18 (s, 9H; C(CH_3)$_3$) ppm; ^{13}C NMR (100 MHz, acetone-d$_6$, 25 °C, TMS): δ = 164.1 (1C; C-4), 149.8 (1C; C-2), 135.9 – 127.9 (18C; arom C (TBDPS, Bz), C-6, C-5), 88.6 (1C; C-4′), 85.9 (1C; C-1′), 74.5 (1C; C-3′), 61.9 (1C; C-5′), 41.3 (1C; C-2′), 28.6 (3C; C(CH$_3$)$_3$), 18.9 (1C; C(CH$_3$)$_3$) ppm; ESI MS (positive mode): *m/z*: calcd: 587.2 [*M*+H]$^+$, 609.2 [*M*+Na]$^+$; found: 587.0 [*M*+H]$^+$, 609.0 [*M*+Na]$^+$.

1-[3-*O*-(*tert*-Butyldiphenylsilyl)-5-benzoyloxy-2-deoxy-*β*-D-*erythro*-pento-5-dialdo-1,4-furanosyl]uracil (6): Fresh Dess – Martin periodinane (5.64 g, 13.3 mmol) was added to a stirred solution of **5** (4.20 g, 7.2 mmol) in dry dichloromethane (130 mL) under an argon atmosphere. After 2 h at room temperature, the reaction mixture was cooled to 5 °C and diluted with dichloromethane (70 mL). Finally the reaction was quenched by addition of 5% aq NaHCO$_3$/saturated aq Na$_2$S$_2$O$_3$ (120 mL; 1:1 v/v). The organic layer was dried by addition of Na$_2$SO$_4$ and then concentrated under vacuum. Chromatography of the crude product on a silica gel column with a step gradient of methanol (0 – 4%) in dichloromethane afforded the aldehyde **6** as a white foam (3.14 g, 75%). R_f = 0.55 (CH$_2$Cl$_2$/MeOH 95:5); ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C, TMS): δ = 9.05 (s, 1H; H-5′), 8.52 (s, 1H; NH), 7.75 (s, 1H; H-6), 7.67 – 7.29 (m, 15H; arom H (TBDPS, Bz)), 6.28 (t, ^3J(H,H) = 6.1 Hz, 1H; H-1′), 4.41 (m, 1H; H-3′), 3.97 (s, 1H; H-4′), 2.49 – 2.11 (m, 2H; H-2′, H-2″), 1.15 (s, 9H; C(CH_3)$_3$) ppm; ^{13}C NMR (100 MHz, acetone-d$_6$, 25 °C, TMS): δ = 196.6 (1C; C-5′), 162.8 (1C; C-4), 147.6 (1C; C-2), 138.4 – 129.7 (18C; arom C (TBDPS, Bz), C-6, C-5), 90.2 (1C; C-4′), 83.9 (1C; C-1′), 74.5 (1C; C-3′), 42.5 (1C; C-2′), 29.1 (3C; C(CH$_3$)$_3$), 19.3 (1C; C(CH$_3$)$_3$); ESI MS (positive mode): *m/z*: calcd: 585.3 [*M*+H]$^+$; found: 585.8; λ_{max} (acetonitrile) = 271 nm.

(5′*S*,5*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5-benzoyloxy-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (7): Compound **6** (3.14 g, 5.37 mmol) was co-evaporated with dry dichloromethane (2 × 20 mL) and then redissolved in benzene (162 mL) under an argon atmosphere prior to heating under reflux. A mixture of AIBN (440 mg, 2.68 mmol) and Bu$_3$SnH (2.89 mL, 10.72 mmol) in benzene (162 mL) was added dropwise to the heated solution with vigorous stirring over a period of 5 h. When the reaction was complete, the mixture was cooled to room temperature in an ice – water bath, and the solvent was removed under reduced pressure. The resulting yellow oil was purified by chromatography on a silica gel column with a step gradient of methanol (0 – 4%) in dichloromethane as the mobile phase. Evaporation to dryness of the collected chromatographic fractions provided the cyclonucleoside **7** as a white foam (640 mg, 21%). R_f = 0.66 (CH$_2$Cl$_2$/MeOH 95:5); ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C, TMS): δ = 7.82 – 7.62 (m, 15H; H-TBDPS, H-Bz), 6.28 (d, ^3J(H,H) = 6.0 Hz, ^3J(H,H) < 0.4 Hz, 1H; H-1′), 5.65 (d, ^3J(H,H) = 3.2 Hz, 1H; H-5), 4.74 (dd, ^3J(H,H) = 2.7, 6.8 Hz, 1H; H-3′), 4.28 (d, ^3J(H,H) = 4.5, < 0.6 Hz, 1H; H-4′), 3.58 (dd, ^3J(H,H) = 4.7, 9.9 Hz, 1H; H-5′), 3.19 (dd, ^3J(H,H) = 3.3, 9.8 Hz, 1H; H-6), 2.43 – 2.04 (m, 2H; H-2′, H-2″), 1.15 (s, 9H; C(CH_3)$_3$) ppm; ^{13}C NMR (100 MHz, CDCl$_3$, 25 °C, TMS): δ = 166.5 (1C; C-4), 149.3 (1C; C-2), 135.9 – 127.8 (18C; arom C (TBDPS, Bz)), 85.5 (1C; C-4′), 84.1 (1C; C-1′), 70.2 (1C; C-3′), 67.5 (1C; C-5′), 56.4 (1C; C-6), 43.4 (1C; C-5), 32.5 (1C; C-2′), 27.1 (3C; C(CH$_3$)$_3$), 19.3 (1C; C(CH$_3$)$_3$); ESI MS (negative mode): *m/z*: calcd: 585.3 [*M* − H]$^-$; found: 585.5; λ_{max} (acetonitrile) = 226 nm.

(5′*S*,5*S*,6*S*)-3′-*O*-(*tert*-Butyldiphenylsilyl)-5′-*O*-levulinyl-5-benzoyloxy-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (8): Compound **7** (600 mg, 0.85 mmol) was dried by repeated co-evaporation with dry dichloromethane and then dissolved in dry THF (30 mL) under an argon atmosphere. *N*,*N*′-dicyclohexylcarbodiimide (DCC; 500 mg, 2.42 mmol), DMAP (10 mg, 0.44 mmol), and levulinic acid (0.20 mL, 21.1 mmol) were added to the stirred solution. After 1 h 30 min 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 (10 mL). The resulting filtrate was evaporated under reduced pressure. Chromatography of the crude product on a silica gel column and subsequent elution with a step gradient of methanol (0 – 4%) in dichloromethane afforded **8** as a white foam (660 mg, 94%). R_f = 0.73 (CH$_2$Cl$_2$/MeOH 95:5); ^{1}H NMR (400 MHz, acetone-d$_6$, 25 °C, TMS): δ = 7.85 – 7.54 (m, 15H; arom H (TBDPS, Bz)), 6.27 (d, ^3J(H,H) = 6.0 Hz, 1H; H-1′), 5.74 (d, ^3J(H,H) = 2.8 Hz, 1H; H-5), 4.78 (dd, ^3J(H,H) = 4.7, 9.9 Hz, 1H; H-5′), 4.55 (m, 1H; H-3′), 4.32 (d, ^3J(H,H) = 4.6 Hz, 1H; H-4′), 4.15 (dd, ^3J(H,H) = 3.2, 10.0 Hz, 1H; H-6), 2.92 – 2.28 (m, 6H, CH$_2$CH$_2$; H-2′, H-2″), 2.17 (s, 3H; COCH_3), 1.02 (s, 9H; C(CH_3)$_3$) ppm; ESI MS (negative mode): *m/z*: calcd: 683.3 [*M* − H]$^-$; found: 683.4; HRMS (FAB): calcd: 691.2663; found: 691.2664 [*M*+Li]$^+$.

(5′*S*,5*S*,6*S*)-5′-*O*-Levulinyl-5-benzoyloxy-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (9): Compound **8** (310 mg, 0.45 mmol) was dissolved in dry THF (17 mL). A solution of TBAF (2.1 mL) in THF (1 M) was added and the resulting mixture was stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the resulting red oil was purified by low pressure chromatography on a silica gel column. Elution was achieved with a step gradient of MeOH (0 – 5%) in dichloromethane. The appropriate fractions were pooled and then evaporated to dryness to give **9** (145 mg) as a white foam in a yield of 72%. R_f = 0.32 (CH$_2$Cl$_2$/MeOH 90:10); ^{1}H NMR (400 MHz, acetone-d$_6$, 25 °C, TMS): δ = 8.06 – 7.54 (m, 5H, arom H (Bz)), 6.28 (d, ^3J(H,H) = 6.0 Hz, 1H; H-1′), 5.80 (d, ^3J(H,H) = 2.8 Hz, 1H; H-5), 4.86 (dd, ^3J(H,H) = 4.8, 10.0 Hz, 1H; H-5′), 4.67 (m, 1H; H-3′), 4.34 (d, ^3J(H,H) = 4.6 Hz, 1H; H-4′), 4.19 (dd, ^3J(H,H) = 3.1, 10.1 Hz, 1H; H-6), 3.05 – 2.41 (m, 6H; CH$_2$CH$_2$, H-2′, H-2″), 2.18 (s, 3H; COCH$_3$) ppm; ESI MS (positive mode): *m/z*: calcd: 469.2 [*M*+Na]$^+$; found: 469.1; ESI MS (negative mode): *m/z*: calcd: 445.2 [*M* − H]$^-$; found: 445.3.

(5′*S*,5*S*,6*S*)-5′,6-Cyclo-5-hydroxy-5,6-dihydro-2′-deoxyuridine (1): Compound **9** was treated with a solution of K$_2$CO$_3$ (0.05 M) in methanol over a period of 3 h. The resulting mixture was evaporated under reduced pressure and then redissolved in water. The mixture was then purified by several injections onto a Hypercarb column (system A) to give **1** in a 90% yield. ^{1}H NMR (400 MHz, D$_2$O, 25 °C, TMS): δ = 6.29 (d, ^3J(H,H) = , < 0.4 Hz, 1H; H-1′), 4.72 (dd, ^3J(H,H) = 2.8, 7.1 Hz, 1H; H-3′), 4.39 (d, ^3J(H,H) = 4.8, < 0.6 Hz, 1H; H-4′), 4.36 (d, ^3J(H,H) = 2.8 Hz, 1H; H-5) 4.07 (dd, ^3J(H,H) = 4.7, 9.6 Hz, 1H; H-5′), 3.30 (dd, ^3J(H,H) = 2.9, 9.7 Hz, 1H; H-6), 2.52 – 2.32 (m, 2H; H-2′, H-2″) ppm; ^{13}C NMR (100 MHz, D$_2$0, 25 °C, TMS), δ = 168.3 (1C; C-4), 147.2 (1C; C-2), 83.1 (1C; C-4′), 82.2 (1C; C-1′), 71.1 (1C; C-3′), 68.6 (1C; C-5′), 53.2 (1C; C-6), 41.8 (1C; C-5), 33.5 (1C; C-2′) ppm; ESI MS (negative mode): *m/z*: calcd: 244.1 [*M* − H]$^-$; found: 244.3; λ_{max} (H$_2$O, pH 7) = 220 nm.

(5′*S*,5*S*,6*S*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphine]-5′-*O*-levulinyl-5-benzoyloxy-5′,6-cyclo-5,6-dihydro-2′-deoxyuridine (10): Compound **9** (100 mg, 0.22 mmol) was dissolved in dry dichloromethane (7 mL) and then evaporated to dryness. This process was repeated twice and the resulting residue was redissolved in CH$_2$Cl$_2$ (2 mL) and THF (2 mL) under an argon atmosphere. Dry DIEA (85 μL, 0.5 mmol) and then 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochloridite (55 μL, 0.2 mmol) were added. After 1 h at room temperature, the reaction mixture was cooled to 5 °C in 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 (0 – 2%) in dichloromethane/TEA (99:1, v/v). The appropriate fractions were pooled and then evaporated to dryness to give the phosphoramidite **10** (115 mg, 0.18 mmol) as a white foam in a yield of 81%. R_f = 0.68 (CH$_2$Cl$_2$/TEA/

MeOH 97:1:2); ^{1}H NMR (400 MHz, acetone-d_6, 25 °C, TMS): $\delta = 7.98 - 7.45$ (m, 5H; arom H (Bz)), 6.28 (br, ^3J(H,H) = 6.2 Hz, 1H; H-1′), 5.85 (d, ^3J(H,H) = 3.1 Hz, 1H; H-5), 4.80 (m, 1H; H-5′), 4.45 (m, 1H; H-3′), 4.32 (m, 1H; H-4′), 4.17 (dd, ^3J(H,H) = 3.2, 10.0 Hz, 1H; H-6), 3.86 – 3.18 (m, 4H; 2NC*H*$(CH_3)_2$, CH_2CH_2OP), 2.88 – 2.51 (m, 9H; CH_2CH_2CN, $CH_2CH_2COCH_3$, H-2′, H-2″), 1.95 (s, 3H; $COCH_3$), 1.26 – 1.18 (m, 12H, 2NCH(CH_3)$_2$) ppm; ^{31}P NMR (100 MHz, acetone-d_6, 25 °C, H_3PO_4 85%): $\delta = 150.62, 150.36$ (s, 1P) ppm; ESI MS (negative mode): *m/z*: calcd: 645.0 $[M-H]^-$; found: 645.2.

Stability studies of (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1) under the alkaline, acidic, and oxidizing conditions used for chemical synthesis of oligonucleotides: Aqueous ammonia (30%, 1 mL), 80% acetic acid solution, or a 0.1 M oxidizing solution of iodine in THF was added to compound **1** (0.2 AU_{230nm}) and the resulting solutions were kept in sealed tubes at room temperature and, under alkaline conditions, also at 55 °C. A similar type of experiment was performed with a solution of K_2CO_3 (0.05 M) in methanol instead of ammonia. 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 lyophilization. Samples were then analyzed by reversed-phase HPLC (system A).

Solid-phase synthesis of oligonucleotides: Oligonucleotides containing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (**1**) were prepared by phosphoramidite solid-phase synthesis[21] on a model 392 DNA synthesizer (Applied Biosystem) by using Pac chemistry[11], with retention of the 5′ terminal DMTr group (trityl-on mode). The duration of the condensation was increased by a factor of four for the modified nucleoside phosphoramidite **10** (120 s instead of the 30 s used for normal nucleoside phosphoramidites). After incorporation of the building block **10**, the levulinyl group was deprotected as previously described.[9]

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 a solution of K_2CO_3 (0.05 M) in methanol at room temperature for 4 h. After evaporation of the solvent under vacuum, the crude 5′-DMTr oligonucleotides were first analyzed by reversed-phase HPLC (system B) and then purified and deprotected on-line by reversed-phase HPLC (system C). The purity and homogeneity of the collected fractions were checked by HPLC analysis (system D). Three oligonucleotides were thus obtained: a trimer, 5′-d(A**X**T)-3′ (**11**), a 14-mer, 5′-d(ATC GTG A**X**T GAT CC)-3′ (**12**), and a 22-mer, 5′-d(CAC TTC GGA T**X**G TGA CTG ATC C)-3′ (**13**), where **X** = (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU. The modified 14- and 22-mer oligonucleotides **12** and **13** used for the enzymatic studies were further purified by PAGE with a 20% polyacrylamide/7 M urea gel and then desalted by using a NAP-25 Sephadex column. ESI MS (negative mode): *m/z*: found (calcd): **11**, 860.3 (860.6); **12**, 4253.5 (4252.8); **13**, 6704.3 (6703.4).

Thermal denaturation studies: Either 5′-d(ATC GTG ACT GAT CC)-3′ or 5′-d(ATC GTG A**X**T GAT CC)-3′ (0.25 AU_{260nm}) was mixed together with the complementary sequence (5′-d(GGA TCA GTC ACG AT)-3′; 0.3 AU_{260} nm) in a buffer (200 μL, pH 7) that contained sodium phosphate (0.01 M), NaCl (0.1 M), and ethylenediaminetetraacetate (EDTA; 0.001 M). 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 hybridization solutions were diluted in the same buffer (600 μL) and then UV absorbance was measured in a quartz cell (0.8 mL, 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 of 15 – 80 °C at the heating rate of 1 °C min^{-1}. The reported data are the average of three melting curves for each oligonucleotide duplex.

Labeling of oligonucleotides: The 5′ ends of the DNA fragments were labeled by treatment with [γ-^{32}P] ATP and T4 polynucleotide kinase according to the standard procedure.[22]

Stability studies of modified oligonucleotides that contained (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdU (1) in hot piperidine solution: See Muller et al.[9] for the general procedure.

Enzymatic digestion of modified oligonucleotides by nuclease P_1 and alkaline phosphatase: The protocol used here is the same as that described previously.[9] System E was used for HPLC analysis.

Enzymatic digestion of modified oligonucleotides by calf spleen phosphodiesterase (5′-exo) or bovine intestinal mucosa phosphodiesterase (3′-exo): The trinucleotide 5′-d(A**X**T)-3′ (**11**) was incubated with bovine intestinal mucosa phosphodiesterase (0.1×10^{-4} U) in ammonium citrate buffer (pH 9) or calf spleen phosphodiesterase (10^{-3} U) over a period of 2 h. The resulting mixture was submitted directly to reversed-phase HPLC analysis (system E). The different products thus separated were collected and analyzed by ESI MS in the negative mode.

Studies of the repair of DNA oligomers containing (5′*S*,5*S*,6*S*)-cyclo-5-OH-dHdUrd (1) by DNA *N*-glycosylases: DNA repair experiments were carried out with Fpg, endo III, endo VIII, Ntg1, Ntg2, Ogg1, and AlkA proteins by using modified double-stranded DNA fragments that contained the modified nucleotide **1** as the substrate. Typically, 1 pmol of the 5′-[^{32}P]-labeled modified 22-mer oligonucleotide **13** was annealed to 2 pmol of the nonlabeled complementary strand 5′-d(CGA TCA GTC ACG ATC CGA AGT G)-3′ by heating at 80 °C for 5 min and subsequent slow cooling to a temperature no greater than 4 °C over 3 h. The enzymatic reactions were performed in solutions (10 μL) of tris(hydroxymethyl)aminomethane (Tris)-HCl (20 mM; pH 7.5), EDTA (1 mM), and KCl (100 mM) for Fpg and endo III, Tris-HCl (25 mM; pH 7.6), NaCl (50 mM), and EDTA (2 mM) for Ntg1, Ntg2, and Ogg1 proteins, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes; 10 mM; pH 7.5), EDTA (1 mM), and NaCl (50 mM) for endo VIII protein, and Hepes-KOH (70 mM; pH = 7.8), EDTA (1 mM), and 2-mercaptoethanol (5 mM) for AlkA, at 37 °C for 30 min with increasing concentrations of enzymes. After an additional alkali treatment with 1 M piperidine for 5 min at 90 °C, the reactions were stopped by addition of formamide dye (5 μL). Samples were then loaded onto a 20% polyacrylamide/7 M urea gel in tetrabromoethane (TBE) buffer. Electrophoresis was carried out at 1600 V for 2 h. The products of the reaction were then analyzed by phosphorimaging.

Primer extension catalyzed by the Klenow exo$^-$ fragment and *Taq* polymerase: Reactions catalyzed by the Klenow exo$^-$ fragment were carried out in Tris-HCl (10 μL, 50 mM; pH 7.5), $MgCl_2$ (10 mM), bovine serum albumin (BSA; 0.05 mg mL^{-1}), and dithiothreitol (DTT; 1 mM). Primer extension reactions with *Taq* polymerase were conducted in 10 μL solutions of Tris-HCl (10 mM; pH 8.3), $MgCl_2$ (1.5 mM), and KCl (50 mM). Buffered solutions that contained the oligonucleotide template **13** (1 nM) and the 5′-[^{32}P]-labeled 11-mer 5′-d(GGA TCA GTC AC)-3′ (1.5 nM) were heated at 80 °C for 5 min and then cooled to 4 °C over a period of 3 h. DNA polymerization reactions were carried out with 100 μM solutions of either a single dNTP or a mixture of all four dNTPs. The solutions were maintained at 37 °C for 30 min in the presence of either the Klenow exo$^-$ fragment or *Taq* polymerase (0.2 U). Reactions were stopped by addition of formamide dye (5 μL). Samples were then loaded onto a 20% polyacrylamide/7 M urea gel in TBE buffer. Electrophoresis was carried out at 1600 V for 2 h. The products of the reaction were finally analyzed by phosphorimaging. A similar experiment was performed with an unmodified 22-mer template as a control.

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