Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 2445–2452

BIOORGANIC & MEDICINAL CHEMISTRY

Site-Specific Incorporation of the 1-Hexanol-1,N^6-etheno-2′-deoxyadenosine Adduct into Oligodeoxyribonucleotides

Valdemir M. Carvalho,[a] Didier Gasparutto,[b] Paolo Di Mascio,[a] Marisa H. G. Medeiros[a] and Jean Cadet[b,*]

[a]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26 077, CEP 05513-970, São Paulo, Brazil*
[b]*Laboratoire Lésions des Acides Nucléiques, DRFMC/SCIB-FRE 2600, CEA/Grenoble, 38054 Grenoble Cedex 9, France*

Received 18 October 2002; accepted 18 February 2003

Abstract—Modified oligonucleotides that contain the hydrophobic 1-hexanol-1,N^6-etheno-2′-deoxyadenosine adduct have been synthesized using a mild solid phase phosphoramidite chemistry. The presence and the integrity of the modified nucleoside in the synthetic oligomers were confirmed by electrospray ionization and MALDI mass spectrometry measurements together with analysis of the complete enzymatic hydrolysate by high performance liquid chromatography coupled to UV and fluorescent detection techniques.

Introduction

Ethenoadducts (ε) to DNA appear to be key lesions to understand the association between many carcinogenic compounds and their ominous effects. They were initially reported to be the main adducts arising from the reaction of chloroacetaldehyde with amino substituted nucleobases.[1] Later, it was shown that metabolites from occupational carcinogens such as vinyl halides, vinyl carbamate and urethane also react with DNA to form the latter exocyclic adducts. These findings motivated a number of studies aimed at evaluating the mutagenic properties of these DNA lesions (see ref 2 for a review). For this purpose, 1,N^6-etheno-2′-deoxyadenosine adduct (εdAdo) and 3,N^4-etheno-2′-deoxycytidine adduct (εdCyd) were transfected in host cells as site-specifically modified plasmids within shuttle vectors. Then, after replication in mammalian cells, high frequent mutagenic events were found to occur, with yields of 81 and 70%, respectively, at defined positions.[3,4]

The detection of background levels of εdAdo and εdCyd in tissues from unexposed rats and humans suggested an endogenous pathway for the formation of these adducts.[5] Suggestion that lipid peroxidation (LP) is the endogenous source of ethenoadduct formation is supported by several studies.[6,7] Thus, *trans*-4-hydroxy-2-nonenal (HNE), one of the most studied breakdown LP products, is able to react with peroxides to give rise to an epoxide derivative. The latter compound may rearrange prior to generate 1,N^6-εdAdo, 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdGuo) and their respective dihydroxyheptyl derivatives.[8,9] We have also demonstrated that *trans*-2-octenal and *trans*,*trans*-2,4-decadienal (DDE) are efficient DNA alkylating agents. Thus, DDE is indeed very versatile being able to produce, after epoxidation, the non substituted DNA adducts formed by HNE, together with three other products, namely decan-1,2,3-triol, 2,3-epoxy-1-decanol and 1-hexanol-1,N^6-εdAdo.[10,11] It has been recently shown that *trans*-4-oxo-2-noneal, another lipid peroxidation product, also induces the formation of substituted ethenoadducts.[12] Each α,β-unsaturated aldehyde arising from the breakdown of lipid peroxides appears to form one or more different substituted ethenoadducts. Overall, the adducts share some common features: they are bulky modifications and the alkyl side chain attached to the etheno ring confers high hydrophobicity. They differ mostly in terms of side-chain length and the presence of hydroxyl groups. Among the already characterized substituted ethenoadducts, 1-[3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo-[2,1-*i*]-purin-7-yl]-1-hexanol (1-hexanol-εdAdo) **1** generated from the reaction of 2′-deoxyadenosine with either *trans*-2-octenal or DDE[11] has the simplest structure.

*Corresponding author. Tel.: +33-4-3878-4987; fax: +33-4-3878-5090; e-mail: cadet@drfmc.ceng.cea.fr

0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00125-1

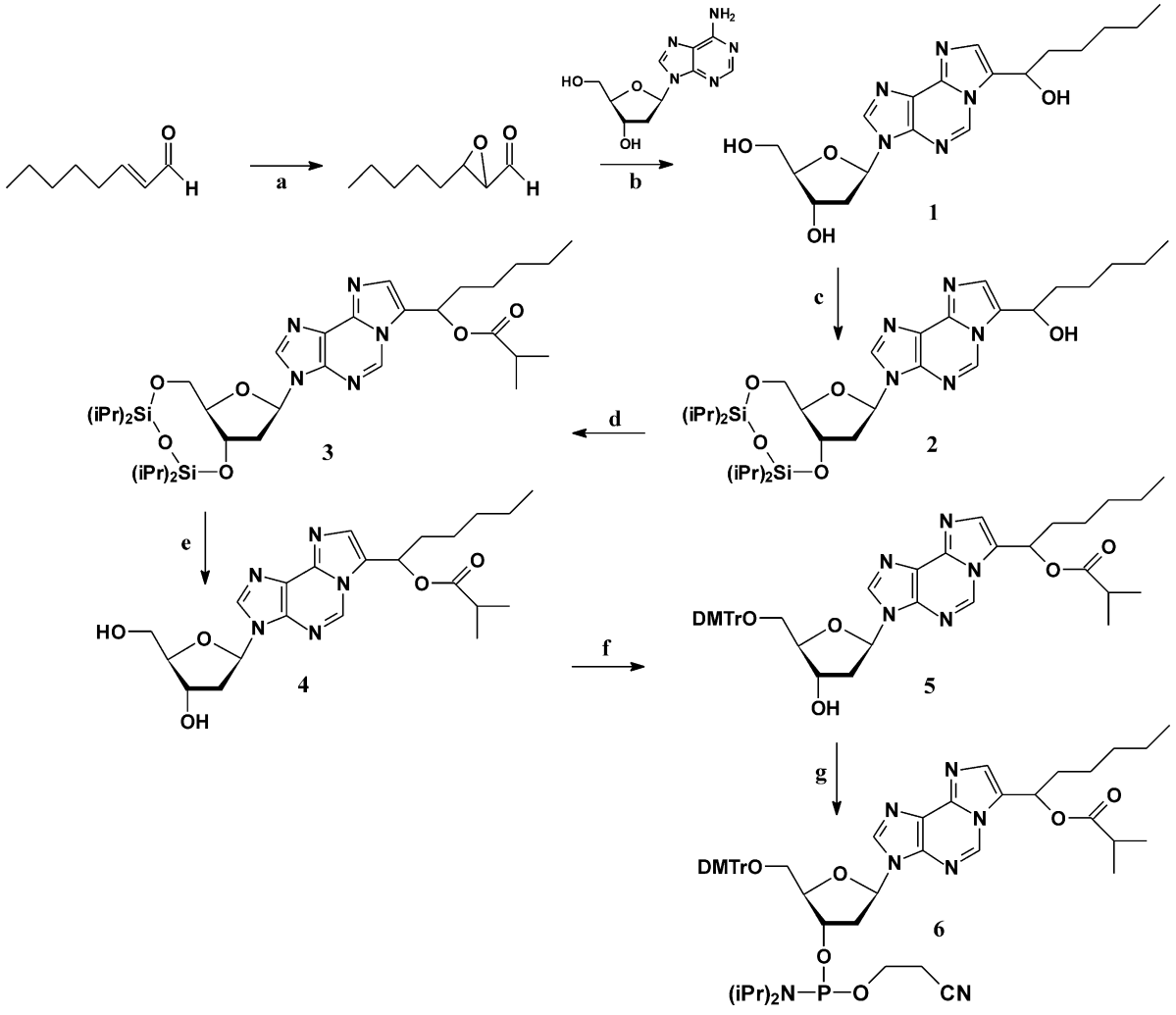
In order to investigate the biological features of this kind of DNA damage, site-specific incorporation of the targeted modification into oligonucleotides is necessary. Therefore, we reported here an efficient synthesis of the 1-hexanol-εdAdo adduct **1** together with a protective strategy to prepare its phosphoramidite building block (Scheme 1). The latter phosphoramidite derivative was successfully site-specifically incorporated into defined oligodeoxynucleotides by mild chemical DNA synthesis on solid support.

Results and Discussion

Synthesis of 1-hexanol-εdAdo 1

Recently, we have reported that 2′-deoxyadenosine (dAdo) was able to react with *trans*-2-octenal in the presence of peroxides to produce a main fluorescent product that was characterized as 1-hexanol-εdAdo **1**.[11] In the latter study, *trans*-2-octenal was shown to be an efficient alkylating agent yielding ethenoadduct levels comparable to HNE. Taking into account its high formation yield, 1-hexanol-εdA **1** appears to be a good candidate to delineate the biological properties of a substituted ethenoadduct in term of replication, mutagenesis, and repair. Thus, we have focused our attention on the development of a strategy aimed at incorporating **1** into oligonucleotide sequences.

The first requirement to reach this purpose is to synthesize large amounts of the adduct **1** to allow a wide set of stability assays and to define protection steps. In order to improve the formation yields of **1**, it was necessary to use a more efficient epoxidation method. Thus, *trans*-2-octenal epoxidation derivative was prepared by a method previously described by Yang[13] with some modifications. The approach is well suited for unsaturated carbonyl compounds through the in situ generation of the strong epoxidizing trifluoromethyldioxirane agent. Reaction of an excess of the unpurified *trans*-2-octenal oxidation product with dAdo allows the formation of 1-hexanol-εdAdo **1** in a 60% yield. Then, **1** was purified by preparative HPLC and comparison of its spectroscopic properties with those of an authentic sample[11] proved the identity of the product.



Scheme 1. Synthesis pathway used for the preparation of the phosphoramidite building block of 1-hexanol-εdAdo **1**: (a) trifluoroacetone, Oxone®, CH_3CN, aqueous EDTA, 4 h; (b) sodium phosphate buffer, 50 °C, 120 h, 58%; (c) $TIPDSCl_2$ (1.1 equiv), imidazol (4.4 equiv), triethylamine (2 equiv), DMF, 2 h, 70%; (d) isobutyric anhydride (3.6 equiv), DMAP (6.2 equiv), pyridine, 30 min, 83%; (e) CsF (10 equiv), pyridine, 24 h, 85%; (f) DMTrCl (1.1 equiv), pyridine, 1 h, 85%; (g) Cl–P(N–*i*Pr$_2$)O(CH$_2$)$_2$CN (1.3 equiv), diisopropylethylamine (2.4 equiv), CH_2Cl_2, argon, 1 h, 62%.

Stability studies of 1-hexanol-εdAdo 1

In order to estimate the stability of 1-hexanol-εdAdo **1** during the different oligonucleotide solid phase synthesis steps, the modified nucleoside was treated with acid (80% acetic acid), oxidizing (0.1 M iodine solution in THF/water) and alkali solutions (32% aqueous ammonia solution and 0.05 M potassium carbonate in methanol). Then, the degradation of **1** was quantified by reversed phase HPLC using UV and fluorescence detections. Thus, 1-hexanol-εdAdo **1** was found to be quite stable under acid and oxidizing conditions. However, as numerous DNA exocyclic adducts, **1** showed a high unstability in ammonia solutions (complete degradation after 2 h at room temperature). An alternative deprotection reagent for alkali-labile DNA component-containing oligonucleotides is methanolic K_2CO_3.[14] The latter treatment was already successfully applied as a mild basic deprotection agent for the incorporation of malonaldialdehyde-2′-deoxyguanosine[15,16] and *p*-benzoquinone-2′-deoxyguanosine adducts[17] into DNA fragments. 1-Hexanol-εdAdo **1** appeared stable under the K_2CO_3 treatment (less than 10% degradation after 24 h at room temperature), allowing the use of this deprotection strategy in combination with the phenoxyacetyl protecting groups[18] for the amino functions of the adduct **1** and unmodified nucleosides.

Preparation of the protected phosphoramidite building block of 1-hexanol-εdAdo 1

Besides its unstability, another potential difficulty for 1-hexanol-εdAdo **1** incorporation into oligonucleotides is the presence of an additional hydroxyl group on the adduct side-chain (Scheme 1). This could lead to undesirable elongation during the oligonucleotide synthesis cycle. Therefore, it was necessary to protect selectively this reactive function. The secondary alcohol groups within the 2-deoxyribose moiety (3′-OH) and the etheno ring (13-OH) showed very similar reactivities. A number of protecting groups such as acetyl, *tert*-butyldimethylsilyl and levulinyl were unsuccessfully tested while a mixture of the products substituted at both positions was obtained (data not shown). This difficulty was overcome by the simultaneous protection of both 2-deoxyribose hydroxyl groups using the bifunctional silyl protecting group, namely 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPDS).[19] By adding TIPDS chloride, carefully and in small portions, it was possible to obtain the desired nucleoside **2**, with a protection at the 3′,5′-position as the major product (70% yield), along with a very small amount of the completely silyl-protected derivative. After achieving the 2-deoxyribose protection, it was possible to block the 13-OH function with the isobutyryl group to give rise to derivative **3** in 83% yield. Quantitative removal of the 3′,5′-*O*-disiloxane group with cesium fluoride afforded compound **4**. Then, the primary 5′-OH of **4** was efficiently protected with a dimethoxytrityl group, yielding product **5**. Finally, the latter nucleoside was phosphitylated using the standard methodology to afford the expected phosphoramidite building block **6**, after preparative RP-HPLC purification. HPLC analysis with fluorescence monitoring and UV detection at λ_{278nm} confirmed the homogeneity of **6**. The ^{31}P NMR features of purified **6** showed the presence of a 1:1 mixture of diastereoisomers (δ 149.3 and 149.2 ppm, respectively). The latter analysis showed also the presence of a very small amount (less than 5%) of the phosphonate derivatives (δ 8.4 ppm), indicating that the phosphoramidite is stable during the HPLC purification process.

Solid-phase synthesis and characterization of oligonucleotides containing 1-hexanol-εdA 1

The phosphoramidite synthon was incorporated into three oligonucleotide sequences (Table 1) following the solid-phase phosphoramidite method. Commercially available unmodified 2′-deoxyribonucleoside phosphoramidite derivatives protected with the phenoxyacetyl (dAdo and dGuo) and acetyl (dCyd) groups were chosen with regard to their high alkali lability.[15,18] By increasing the coupling time of phosphoramidite synthon **6** from 30 s to 10 min, it was possible to obtain a coupling yield up to 90% for the modified monomer, as monitored by the release of the DMTr cation during the detritylation step. The cleavage and complete deprotection of the oligonucleotides were accomplished upon incubation with 50 mM K_2CO_3 in dry methanol[14] for 12 h. The crude 5′-DMTr-oligomers were purified and deprotected on-line with 1% aqueous trifluoroacetic acid solution by reversed phase HPLC.[20] Finally, the synthetic modified DNA oligomers were further purified by preparative denaturating polyacrylamide gel electrophoresis (PAGE).

The structure of the 5-mer, 14-mer and 22-mer oligonucleotides was assessed by electrospray ionization (ESI) and MALDI-TOF mass spectrometry measurements (Fig. 1; Table 1). The molecular masses obtained by both techniques were in agreement with the calculated molecular masses and therefore confirmed the presence and the integrity of the base adduct within the DNA fragments. The presence of the adduct 1-hexanol-εdAdo **1** in the oligonucleotide sequences received further support from the reversed phase HPLC analysis coupled to UV and fluorescent detection of the enzymatic digestion mixture of the modified DNA fragments as shown in Figure 2. Thus, it clearly appears that, besides the four normal 2′-deoxyribonucleosides, 1-hexanol-εdAdo **1** that eluted at 37 min was present. The identity and integrity of **1** were confirmed by co-injection with a standard together with ESI-MS measurement of the content of the collected peak.

Table 1. Sequences and relative molecular masses (Da) of the modified ODNs synthesized (ESI MS and MALDI-TOF MS measurements were performed in the negative mode)

Sequences	Mass calcd	ESI-MS	MALDI-MS
5′-d(T**[1]**GCA)-3′	1611.2	1611.3	1611.1
5′-d(ATCGTG**[1]**CTGATCT)-3′	4378.0	4376.0	4377.4
5′-d(CACTTCGG**[1]**TCGTGACTGATCT)-3′	6825.6	6825.1	6825.0

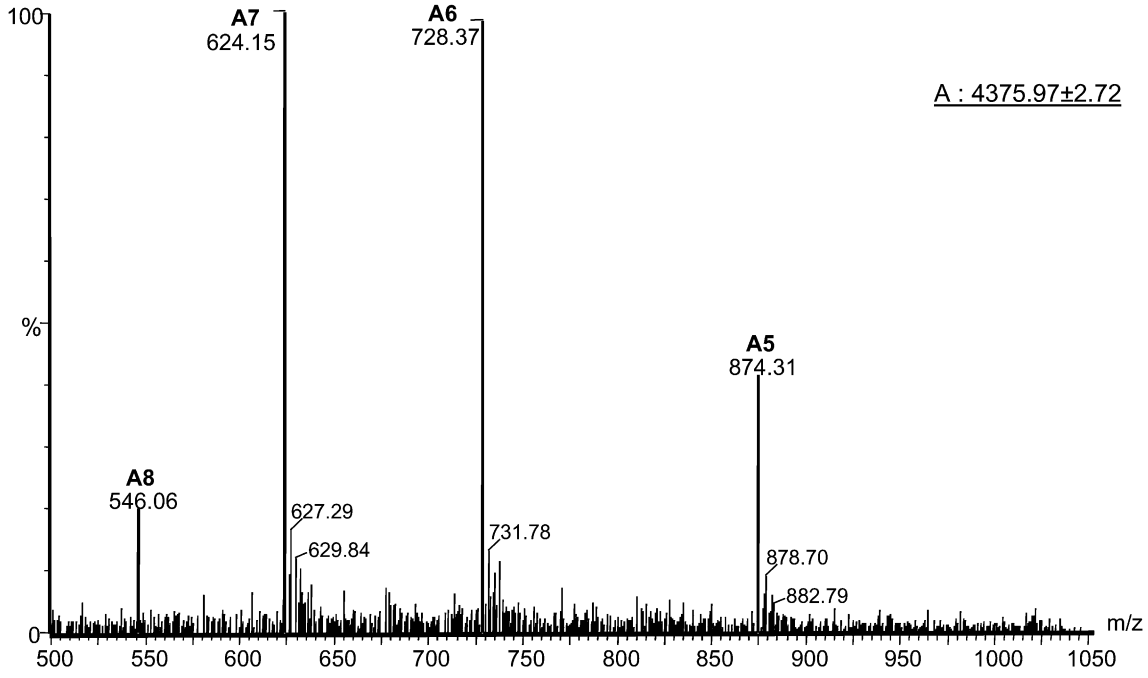


Figure 1. Electrospray ionization mass spectrum, in the negative mode, of the modified 14-mer oligonucleotide [seq: 5′-d(ATCGTG**[1]**CTGATCT)-3′; $M_r = 4378.0$ Da).

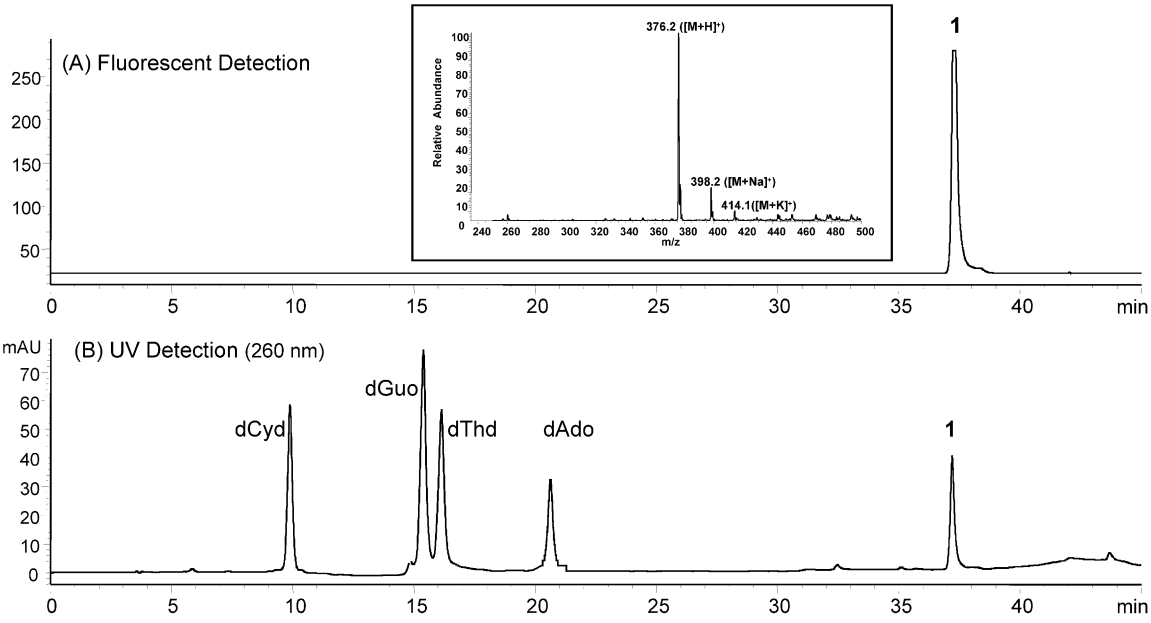


Figure 2. HPLC profile of the enzymatic digestion mixture of ODN [$_{5'}$-d(T**[1]**GCA)-$_{3'}$] with nuclease P1, calf spleen phosphodiesterase, bovine intestinal mucosa phosphodiesterase and calf intestine alkaline phosphatase. The digestion mixture was analyzed by RP-HPLC (see Experimental for conditions). The detection was achieved by UV measurement at 260 nm (**B**) together with specific fluorescence measurement of 1-hexanol-εdAdo ($\lambda_{ex} = 310$ nm/$\lambda_{em} = 420$ nm) (**A**). The inset shows the electrospray ionization mass spectrum, in the positive mode, of the collected 1-hexanol-εdAdo residue **1**, eluted at 37 min.

Conclusion

We report herein the first incorporation into synthetic oligonucleotides of a hydrophobic 1,N^6-εdAdo adduct **1**, resulting from lipid peroxidation processes. The adduct can be obtained in a high yield from a *trans*-2-octenal epoxidized derivative generated in situ. The strategy of protection developed to prepare the phosphoramidite derivative took into account the unstability of the nucleoside adduct **1** under alkali conditions. After solid-phase assembling, the synthetic oligonucleotides were isolated in good yields and characterized by mass spectrometry measurements and complete enzymatic digestion, showing the integrity of the modified nucleoside **1** within the synthetic DNA oligomers. This methodology, that may be applied to the incorporation of numerous adducts including oxidized and alkylated DNA modifications,[21,22] should allow the assessment of the biological and structural properties of this family of genetic modifications.

Experimental

Chemicals

All reagents were of the highest available purity. Buffers were prepared using water purified with a Milli-Q system (Milford, MA, USA). Anhydrous solvents (dichloromethane, dimethylformamide, tetrahydrofuran, acetonitrile and pyridine) were purchased from SDS (Peypin, France) and stored over 3 Å molecular sieves. HPLC grade acetonitrile was obtained from EM Science (Gibbstown, NJ, USA). Dichloromethane, methanol and the silica gel (70–200 μm) used for the low-pressure column chromatography were from SDS. 2′-Deoxyadenosine, formic acid, silica gel 60 F-254 TLC and preparative silica gel 60 F-254 TLC (2 mm) plates were acquired from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), triethylamine (TEA) and diisopropylethylamine (DIEA) were from Acros (Geel, Belgium). 4-Dimethylamine-pyridine was from Fluka (Buchs, Switzerland). *Trans*-2-octenal, 3-(trimethylsilyl)-1-propanesulfonic acid, D_2O 99,9%, Oxone®, 1,1,1-trifluoroacetone and chloroacetaldehyde were from Aldrich (Milwaukee, WI, USA). Phenoxyacetyl-dAdo and phenoxyacetyl-dGuo phosphoramidites were purchased from Pharmacia Biotech (Uppsala, Sweden). All other unmodified deoxyribonucleoside phosphoramidites, DNA synthesis reagents as well as CPG supports were from Glen Research (Sterling, VA, USA). Calf intestinal alkaline phosphatase was acquired from Amershan Pharmacia Biotech (Uppsala, Sweden). Nuclease P1 (*Penicillium citrium*) was from USB Corporation (Cleveland, OH, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

HPLC apparatus

Preparative HPLC was conducted using a Shimadzu system (Kyoto, Japan) with two LC-6AD, a Rheodyne injector, a SPD-M10AV photodiode array and a RF-10AXL fluorescence detector controlled by a SCL-10A*VP* module and Class-*VP* software. Semi-preparative and analytical HPLC were performed on a system composed of a Merck L-6200 pump, a Rheodyne injector, a Waters 990 photodiode array and a Merck F1000 fluorescence detector. Several liquid chromatographic columns and gradients were used for the purification steps and analytical experiments (vide infra).

Nuclear magnetic resonance spectroscopy

^1H NMR (300 MHz) and ^{13}C NMR spectra were recorded either with a Bruker Avance Series DPX-300 or a DRX-500 MHz apparatus (Rheinstetten, Germany). ^{31}P NMR (101 MHz) spectra were acquired on a Varian INOVA 400 MHz spectrometer (Palo Alto, CA, USA) calibrated with 85% H_3PO_4 as an external standard. Chemical shifts are expressed in parts per million (ppm) from 2,2′-dimethyl-2-silapentane-5-sulfonate (DSS) when spectra were acquired in D_2O or DMSO-d_6 or from tetramethylsilane (TMS) when they were recorded in $CDCl_3$, methanol-d_4 or acetone-d_6.

Mass spectrometry measurements

Electrospray Ionization (ESI) mass spectrometry analyses of the adducts and derivatives together with those of the modified oligonucleotides were conducted on either a LCQ Ion Trap spectrometer (ThermoFinnigan, San Jose, CA, USA) or a Platform 3000 spectrometer (Micromass, Manchester, UK). Samples were diluted in a solution of acetonitrile and water (50/50, v/v) that contained either 0.1% formic acid (positive mode) or 1% triethylamine (negative mode) at a concentration of 10 μmol/L. The samples were introduced into the electrospray ion source by a syringe pump at a flow rate of 10 μL/min.

MALDI mass spectra were obtained with either a time-of-flight mass spectrometer Voyager-DE (Perseptive Biosystems, Framingham, MA, USA) or a time-of-flight mass spectrometer Biflex (Bruker Daltonik, Bremen, Germany) equipped with a 337 nm nitrogen laser and a pulsed delay source extraction. For the matrix, a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4:1 (w/w) ratio was dissolved in a 50% acetonitrile aqueous solution and a small amount of Dowex-50W 50X8-200 (Sigma) cation exchange resin. Then, 1 μL of an aqueous solution of the sample was added to 1 μL of the matrix and the resulting solution was stirred. The sample was subsequently placed on 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* 16 952), using the same assay conditions as described for the oligonucleotides.

Preparation of 1-[3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]-1-hexanol (1-hexanol-εdAdo) (1). To an acetonitrile solution (70 mL) of *trans*-2-octenal (7.614 g, 60 mmol) an aqueous Na_2EDTA solution (60 mL, of 0.4 mM) was added. The resulting homogenous solution was cooled down to 0–1 °C, followed by addition of trifluoroacetone (12 mL) via a precooled syringe. To this solution, a mixture of sodium bicarbonate (7.8 g, 98 mmol) and Oxone® (36.9 g, 60 mmol) was added in portions over a period of 1 h. After 3 h, the reaction mixture was poured into water (150 mL) and extracted with methylene chloride (2 × 50 mL). The combined organic extracts were dried over anhydrous magnesium sulfate and the solvent was subsequently removed under a N_2 flow. Then, the resulting residue was diluted in acetonitrile (100 mL) and added to a 2′-deoxyadenosine solution (3 g, 12 mmol) in 0.1 M pH 7.4 sodium phosphate buffer (100 mL) and the resulting mixture was stirred for 120 h at 50 °C. Unreacted aldehyde was removed by extraction with methylene chloride (2 × 50 mL). The aqueous solution was lyophilized and the residue was dissolved in 250 mL of a solution of acetonitrile and water (60/40, v/v). The resulting solution was filtered through 0.22 μm Durapore membranes and purified by preparative RP-HPLC [System: a Prodigy C_{18} preparative column (Phenomenex, Torrance, CA—250 × 21.2 mm i.d., 10 μm). The elution was performed with 40% acetonitrile in water at a flow rate of 9 mL/min. The fluorescence detection was achieved at the following wavelengths: 310 nm (excitation) and

500 nm (emission). The emission wavelength was shifted up to 90 nm from the maximum in order to attenuate the detection. The latter HPLC purification yielded **1** as a white solid (2.6 g, 58%). UV: λ_{max} 232, 261, 268, 278 and 300 nm (pH $\geq$4); λ_{max} 224 and 277 nm (pH $<$4). R_f (CH_2Cl_2/CH_3OH 80/20): 0.1; ESI-MS (positive mode—cone voltage = 15 V): *m/z* 398 (15) $(M+Na)^+$, 376 (100) $(M+H)^+$, 282 (5) $(BH+Na)^+$, 260 (35) BH_2^+; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.18 (H-2, s, 1H), 8.52 (H-8, s, 1H), 7.41 (H-11, s, 1H,), 6.48 (H-1′, *t*, 1H, $J_{1',2'}$ = 6.9 Hz, $J_{1',2''}$ = 6.3 Hz), 5.48 (13-OH, d, 1H, exch., $J_{13,13\text{-OH}}$ = 6.1 Hz), 5.34 (3′-OH, d, 1H, exch., $J_{3',3'\text{-OH}}$ = 4.2 Hz), 5.01 (H-13, q, 1H, $J_{13,14}$ = 6.6 Hz), 4.95 (5′-OH, t, 1H, $J_{5',5'\text{-OH}}$ = 5.5 Hz), 4.43 (H-3′, m, 1H, $J_{3',4'}$ = 3.0 Hz), 3.89 (H-4′, m, 1H, $J_{4',5'}$ = 3.7 Hz, $J_{4',5''}$ = 4.7 Hz), 3.60 (H-5′, m, 1H, $J_{5',5''}$ = −12.5 Hz), 3.55 (H-5″, *m*, 1H), 2.73 (H-2′, m, 1H, $J_{2',2''}$ = −14.0 Hz, $J_{2',3'}$ = 6.8 Hz), 2.36 (H-2″, m, 1H, $J_{2'',3'}$ = 3.9 Hz), 1.92 (H-14, td, 1H), 1.51 (H-15, m, 1H), 1.29 (H-16, m, 2H), 1.23 (H-17, m, 2H), 0.85 (H-18, t, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 141.0 (C-6), 139.9 (C-8), 138.1 (C-4), 136.0 (C-2), 129.6 (C-11), 127.7 (C-12), 123.2 (C-5), 88.1 (C-4′), 84.1 (C-1′), 70.8 (C-3′), 63.6 (C-13), 61.8 (C-5′), 40.0 (C-2′), 34.9 (C-14), 31.2 (C-15), 25.3 (C-16), 22.2 (C-17), 14.1 (C-18).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-[3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]-purin-7-yl]-1-hexanol (2). Compound **1** (261 mg, 0.70 mmol) was combined with imidazol (379 mg, 3.10 mmol) and the mixture was coevaporated twice in dry pyridine. The residue was dissolved in dry dimethylformamide (4 mL) with triethylamine (197 μL, 1.4 mmol) and the resulting solution was cooled to 0 °C and kept under stirring. $TIPDSCl_2$ (468 μL, 0.76 mmol) was added in two portions over 1 h. Then, the mixture was allowed to warm to room temperature while stirring was maintained. After 2 h, TLC analysis showed that less than 10% of the starting material was left. The reaction was stopped by the addition of water (2 mL). The mixture was neutralized by the addition of 5% sodium bicarbonate solution (5 mL) and the products were extracted with methylene chloride (2 × 10 mL). The combined organic extracts were dried over anhydrous magnesium sulfate, evaporated to dryness and coevaporated twice with anhydrous methylene chloride. TLC on silica gel (80/20 CH_2Cl_2/CH_3OH, as eluent) showed the formation of one major product and a more hydrophobic additional compound. Column chromatography on silica gel using 1–10% MeOH in CH_2Cl_2, as the eluent, was performed for the purification of the products. MS analysis (vide infra) showed that the main product was the expected protected nucleoside whereas the formation of the second product is explained by the addition of two $TIPDSCl_2$ groups. After combining and evaporating the corresponding fractions, the pure desired product **2** was obtained as a white powder (350 mg, 70%). R_f (80/20 CH_2Cl_2/CH_3OH): 0.58; ESI-MS (positive mode—cone voltage = 15 V): *m/z* 618 (15) $(M+H)^+$, 260 (35) BH_2^+; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.08 (H-2, s, 1H), 8.15 (H-8, s, 1H), 7.40 (H-11, s, 1H,), 6.39 (H-1′, t, 1H), 4.94 (H-13, m, 1H), 4.95, 4.93 (H-3′, m, 1H) 3.94 (H-4′, m, 1H), 4.53 (H-5′, H-5″, m, 2H), 2.64–2.80 (H-2′,H-2″, m, 2H), 2.06 (H-14, m, 2H), 1.66 (H-15, m, 2H), 1.37 (H-16, m, 2H), 1.25 (H-17, m, 2H), 1.05–1.13 (iPr-TIPDS, m, 28H), 0.91 (H-18, t, 3H).

3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-[3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]-purin-7-yl]-1-hexyl-2-methylpropanoate (3). Compound **2** (356 mg, 0.56 mmol) and 4-(dimethylamino)pyridine (392 mg, 3.5 mmol) were coevaporated three times from dry pyridine (5 mL). The mixture was redissolved in dry pyridine (5 mL) and isobutyric anhydride (332 μL, 2 mmol) was added dropwise. After 30 min, the reaction was completed as checked by TLC on silica gel plates (80/20 CH_2Cl_2/CH_3OH). The reaction was stopped by the addition of methanol (2 mL) and the solvent was removed under reduced pressure. A solution of 5% $NaHCO_3$ was added to the residue and the product was extracted with dichloromethane (5 mL). Then, the extract was dried over Na_2SO_4 and evaporated under reduced pressure. Column chromatography on silica gel (1–7.5% MeOH in CH_2Cl_2, as the eluent) yielded a white powder product (331 mg, 83%); R_f (80/20 CH_2Cl_2/CH_3OH): 0.77; ESI-MS (positive mode—cone voltage = 15 V): *m/z* 618 (15) $(M+H)^+$, 260 (35) BH_2^+; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.95 (H-2, s, 1H), 8.16 (H-8, s, 1H), 7.63 (H-11, s, 1H,), 6.39 (H-1′, t, 1H), 6.28 (H-13, m, 1H), 4.93 (H-3′, m, 1H), 3.94 (H-4′, m, 1H), 4.06 (H-5′, H-5″, m, 2H), 2.66–2.76 (H-2′, H-2″, m, 2H), 2.56 (CH-iBu, m, 1H) 2.14 (H-14, m, 2H), 1.41 (H-15, m, 2H), 1.25–1.28 (H-16, H-17 m, 4H), 1.10–1.19 (CH_3-iBu, d, 6H), 1.04–1.10 (iPr-TIPDS, m, 28H), 0.88 (H-18, t, 3H).

1-[3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo-[2,1-*i*]purin-7-yl]-1-hexyl-2-methylpropanoate (4). Compound **3** (234 mg, 0.34 mmol) was dried by coevaporation from dry pyridine (3 × 2 mL) and redissolved in dry dimethylformamide (5 mL). To this solution, CsF (500 mg, 3.3 mmol) was added and the mixture was stirred for 24 h. TLC analysis on silica gel plates with a mixture of CH_2Cl_2/CH_3OH (80/20, v/v) showed that the starting product was fully consumed. The mixture was poured into 5% aq $NaHCO_3$ (10 mL), extracted with dichloromethane (3 × 5 mL), and the organic solution was dried over Na_2SO_4 and finally evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (1–15% MeOH in CH_2Cl_2, as the eluent) yielding a white powder product (129 mg, 85%). R_f (80/20 CH_2Cl_2/CH_3OH): 0.32; ESI-MS (positive mode—cone voltage = 15 V): *m/z* 618 (15) $(M+H)^+$, 260 (35) BH_2^+; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 9.21 (H-2, s, 1H), 8.48 (H-8, s, 1H), 7.59 (H-11, s, 1H), 6.65 (H-1′, t, 1H), 5.25 (H-13, *m*, 1H), 4.64 (H-3′, m, 1H), 4.21 (H-4′, m, 1H), 3.81–3.87 (H-5′, H-5″, m, 2H), 2.98 (H-2′, m, 1H), 2.65 (H-2″, m, 1H), 2.39 (CH-*i*Bu, m, 1H), 2.13 (H-14, m, 2H), 1.55 (H-15, m, 2H), 1.29–1.44 (H-16, H-17, m, 4H), 1.06 (CH_3-*i*Bu, d, 6H), 0.88 (H-18, t, 3H).

1-[3-(2-Deoxy-5-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]-1-hexyl-2-methylpropanoate (5). Compound **4** (125 mg, 0.28 mmol) was dried by coevaporation from dry pyridine (3 × 2 mL)

and then redissolved in dry pyridine (5 mL). The solution was cooled in an ice/water bath and kept under an argon atmosphere. Then, 4,4′-dimethoxytrityl chloride (DMTrCl, 108 mg, 0.32 mmol) was added under stirring. The cooling bath was removed. After 1 h, it was found that the reaction was completed as inferred from TLC analyses on silica gel plates with a mixture of CH_2Cl_2/CH_3OH (80/20, v/v). The reaction was stopped by the addition of methanol (1 mL) and the resulting solution was evaporated to dryness. The oily residue was dissolved in ethyl acetate (5 mL) and washed with 5% aq $NaHCO_3$ (5 mL). The organic extract was dried over Na_2SO_4 and evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel (1–8% MeOH in CH_2Cl_2, as the eluent) yielding a yellowish foam product (172 mg, 85%). R_f (80/20 CH_2Cl_2/CH_3OH): 0.66; ESI-MS (positive mode—cone voltage = 15 V): m/z 748.9 (100) $(M+H)^+$, 330.4 (35) BH_2^+; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.89 (H-2, s, 1H), 8.52 (H-8, s, 1H), 7.61 (H-11, s, 1H,), 6.77–7.41 (aromatic H of DMTr, m, 13H), 6.52 (H-1′, t, 1H), 6.26 (H-13, m, 1H), 4.43 (H-3′, m, 1H), 3.89 (H-4′, m, 1H), 3.76 and 3.77 (CH_3–O–DMTr, s, 6H), 3.55–3.60 (H-5′, H-5″, m, 2H), 2.82 (H-2′, m, 1H), 2.55 (H-2″, m, 1H), 2.53 (CH–*i*Bu, m, 1H), 2.13 (H-14, m, 2H), 1.55 (H-15, m, 2H), 1.25–1.38 (H-16, H-17, m, 4H), 1.13 (CH_3–*i*Bu, d, 6H), 0.88 (H-18, t, 3H).

1-{2-Deoxy-3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-5-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]-1-hexyl-2-methylpropanoate (6). Compound **5** (157 mg, 0.21 mmol) was dried by coevaporation from dry pyridine (3 × 2 mL) and then dissolved in dry dichloromethane (2.5 mL). The solution was cooled in an ice/water bath and kept under argon atmosphere. Diisopropylethylamine (88 μL, 0.50 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (60 μL, 0.27 mmol) were added to the solution under stirring at room temperature. The course of the reaction was monitored by TLC with a mixture of CH_2Cl_2/CH_3OH/TEA (89/10/1, v/v/v). After 1 h, a total consumption of **5** was observed. The reaction was then stopped by the addition of methanol (2 mL). The mixture was diluted with ethyl acetate (5 mL) and subsequently washed with 5% aq $NaHCO_3$ (5 mL). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. Then, the products were purified by RP-HPLC [System: a Bondclone C_{18} semi-preparative column (Phenomenex—300 × 7.8 mm i.d., 10 μm) eluted with 20% acetonitrile in 10 mM TEAA, pH 7.0, at a flow rate of 5 mL/min]. The collected fractions were lyophilized yielding **6** as a light yellowish foam (123 mg, 62%). The latter product was kept under argon atmosphere at −20 °C. R_f (89/10/1 CH_2Cl_2/CH_3OH/TEA): 0.77; ESI-MS (positive mode—cone voltage = 15 V): m/z 948 (100) $(M+H)^+$, 330 (10) BH_2^+, 303 (5) $(trityl)^+$; ^{31}P NMR (acetone-d_6, 101.21 MHz): δ 149.31 and 149.20 ppm (two diastereoisomers).

Stability studies of 1-[3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3*H*-imidazo[2,1-*i*]purin-7-yl]-1-hexanol (1) under the alkaline, acid and oxidizing conditions used for chemical synthesis of oligonucleotides. 30 μg of compound **1** was placed in 0.5 mL of the following solutions: 32% aqueous ammonia, 80% acetic acid aqueous solution, 0.1 M oxidizing solution of iodine and 0.05 M K_2CO_3 methanolic solution. The solutions were kept at room temperature and the reactions were stopped at increasing time intervals (0, 1, 2, 4, 8 and 24 h) by freezing the samples in liquid nitrogen with subsequent lyophilization. Then, samples were analyzed by HPLC [system: a Luna C_{18} analytical column (Phenomenex—150 × 4.6 mm i.d., 3 μm) eluted with a linear binary gradient of acetonitrile in 10 mM pH 7.0 TEAA ranging acetonitrile concentration from 2 to 10% in 15 min, then from 10 to 45% in 15 min at a flow rate of 1 mL/min].

Solid-phase synthesis of oligonucleotides

The synthesis of all oligonucleotides was performed at a 1 μmol scale using the phosphoramidite chemistry on an Applied Biosystems 392 automated Nucleic Acids synthesizer. The Pac-protection system[18] for the nucleobase amino functions was used with regard to the high lability of **1** under alkali deprotection conditions. The synthesis was performed on controlled pore glass (CPG) supports following the standard protocol, at the exception of two modifications. First, the duration of coupling of the modified nucleoside phosphoramidite was extended to 10 min, instead of 30 s for normal nucleosides. Under the latter conditions, an adduct coupling efficiency up to 90% was achieved. Moreover, the capping step was carried out with a solution of phenoxyacetic anhydride in THF to prevent possible transacylation reactions.[23] Finally, the 5′-terminal DMTr group was kept at the end of the synthesis (trityl-on mode).

Deprotection and purification of oligonucleotides

The solid supports were treated with 50 mM K_2CO_3 in anhydrous methanol (1.5 mL). After 12 h, the methanolic solution was transferred to another tube and the resin was washed with water (2 × 1 mL). The combined extracts were immediately and carefully neutralized with acetic acid. The solvent was removed under reduced pressure and the residue was dissolved in water (200 μL). The crude synthetic mixture was purified and detritylated on-line by RP-HPLC following a protocol previously described.[20] Finally, the 14- and 22-mer oligonucleotides were further purified by preparative 20% polyacrylamide gel electrophoresis, to afford 24 AU_{260nm} and 31 AU_{260nm}, respectively.

Enzymatic digestion of the modified oligonucleotides by nuclease P1, calf spleen phosphodiesterase, bovine intestinal mucosa phosphodiesterase and alkaline phosphatase

The modified oligonucleotides (0.4 UA_{260nm}) were taken up in 45 μL of water and completely digested into nucleotides upon incubation with 3 μL of a solution of nuclease P_1 (1 U/μL) and 1 μL of calf spleen phosphodiesterase (0.004 U) in acetate buffer (0.3 M sodium acetate/1 mM $ZnCl_2$, pH = 5.3) for 2 h at 37 °C. Then, 5 μL of Tris buffer (1 M Tris–HCl, 2 mM EDTA,

pH = 8.5) together with 2 units of alkaline phosphatase and 0.003 U of bovine intestinal mucosa phosphodiesterase were added and the resulting solution was further incubated for 1 h at 37 °C. The resulting digestion mixture of 2′-deoxyribonucleosides was centrifuged and analyzed by RP-HPLC [system: a Hypersil 5 μm C18 column (4.6 × 250 mm) eluted with TEAA (10 mM, pH 7) and acetonitrile (linear gradient from 0 to 18% of acetonitrile in 40 min at a flow rate of 1 mL/min)].

Acknowledgements

The authors thank Colette Lebrun, Christine Saint-Pierre (SCIB/CEA-Grenoble) and Dr. Michel Jaquinod (LSMP, IBS-Grenoble) for their contributions to the mass spectrometry measurements. The assistance of Pierre-Alain Bayle (SCIB/CEA-Grenoble) and Miriam Uemi (IQ-USP, São Paulo) for the NMR analyses is gratefully acknowledged. This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (Brazil), the Comité de Radioprotection (Electricité de France), the Conselho Nacional para o Desenvolvimento Científico e Tecnológico, CNPq (Brazil) and the Comité Français d'Evaluation de la Coopération Universitaire avec le Brésil (USP-COFECUB/UC-23/96). V.M.C. was a recipient of a FAPESP fellowship.

References and Notes

1. Kochetkov, N. K.; Shibaev, V. N.; Kost, A. A. *Tetrahedron Lett*. **1971**, *15*, 1993.
2. Barbin, A. *Mutat. Res*. **2000**, *462*, 55.
3. Moriya, M.; Zhang, W.; Johnson, F.; Grollman, A. P. *Proc. Natl. Acad. Sci. U.S.A*. **1994**, *91*, 11899.
4. Pandya, G. A.; Moriya, M. *Biochemistry* **1996**, *35*, 11487.
5. Nair, J.; Barbin, A.; Guichard, Y.; Bartsch, H. *Carcinogenesis* **1995**, *16*, 613.
6. Marnett, L. J. DNA Adducts of α,β-Unsaturated Aldehydes and Dicarbonyl Compounds. In *DNA Adducts: Identification and Biological Significance*. *IARC Scientific Publications no. 125*, Hemminki, K., Dipple, A., Shuker, D. E. G., Kadlubar, F. F., Segerbäck, D., Bartsch, H. Eds.; IARC: Lyon, France, 1994; p 151.
7. el Ghissassi, F.; Barbin, A.; Nair, J.; Bartsch, H. *Chem. Res. Toxicol*. **1995**, *8*, 278.
8. Sodum, R. S.; Chung, F. L. *Cancer Res*. **1988**, *48*, 320.
9. Sodum, R. S.; Chung, F. L. *Cancer Res*. **1991**, *51*, 137.
10. Carvalho, V. M.; Di Mascio, P.; de Arruda Campos, I.; Douki, T.; Cadet, J.; Medeiros, M. H. *Chem. Res. Toxicol*. **1998**, *11*, 1042.
11. Carvalho, V. M.; Asahara, F.; Di Mascio, P.; de Arruda Campos, I.; Cadet, J.; Medeiros, M. H. *Chem. Res. Toxicol*. **2000**, *13*, 397.
12. Rindgen, D.; Lee, S. H.; Nakajima, M.; Blair, I. A. *Chem. Res. Toxicol*. **2000**, *13*, 846.
13. Yang, D.; Wong, M. K.; Yip, Y. C. *J. Org. Chem*. **1995**, *60*, 3887.
14. Kuijpers, W. H.; Kuyl-Yeheskiely, E.; van Boom, J. H.; van Boeckel, C. A. *Nucleic Acids Res*. **1993**, *21*, 3493.
15. Reddy, G. R.; Marnett, L. J. *J. Am. Chem. Soc*. **1995**, *117*, 5007.
16. Schnetz-Boutaud, N. C.; Mao, H.; Stone, M. P.; Marnett, L. J. *Chem. Res. Toxicol*. **2000**, *13*, 90.
17. Chenna, A.; Singer, B. *Chem. Res. Toxicol*. **1997**, *10*, 165.
18. Schulof, J. C.; Molko, D.; Téoule, R. *Nucleic Acids Res*. **1987**, *15*, 397.
19. Markiewicz, W. T. *J. Chem. Res. (S)* **1979**, 24.
20. Romieu, A.; Gasparutto, D.; Molko, D.; Cadet, J. *Tetrahedron Lett*. **1997**, *38*, 7531.
21. Butenandt, J.; Burgdorf, L. T.; Carell, T. *Synthesis* **1999**, *7*, 1085.
22. Gasparutto, D.; Bourdat, A. G.; D'Ham, C.; Duarte, V.; Romieu, A.; Cadet, J. *Biochimie* **2000**, *82*, 19.
23. Chaix, C.; Molko, D.; Téoule, R. *Tetrahedron Lett*. **1989**, *30*, 71.