Radiation-induced formation of purine 5′,8-cyclonucleosides in isolated and cellular DNA: high stereospecificity and modulating effect of oxygen

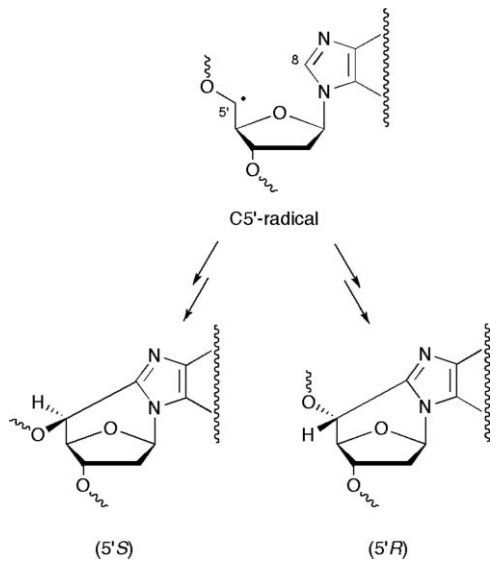
Nourreddine Belmadoui,[a] Fabien Boussicault,[b] Maurizio Guerra,[b] Jean-Luc Ravanat,[a] Chryssostomos Chatgilialoglu*[b] and Jean Cadet*[a]

Received 23rd March 2010, Accepted 5th May 2010
First published as an Advance Article on the web 25th May 2010
DOI: 10.1039/c004531d

The present work is aimed at gaining conclusive mechanistic insights into the radiation-induced formation of the 5′*R* and 5′*S* diastereomers of both adenine and guanine 5′,8-cyclo-2′-deoxyribonucleosides, with emphasis on the delineation of the inhibitory effect of O_2 in isolated and cellular DNA. The levels of purine 5′,8-cyclo-2′-deoxyribonucleosides as assessed by HPLC-MS/MS were found to decrease steadily with the increase of O_2 concentration, the 5′,8-cyclo-2′-deoxyguanosine being produced more efficiently than the 5′,8-cyclo-2′-deoxyadenosine for low O_2 concentrations. A high stereoselectivity was observed in the intramolecular addition of the C5′ radical to the C8 of the purine leading, after the creation of the C5′–C8 bond and a subsequent oxidation step, to the predominant formation of the 5′*R* diastereomer for both purine 5′,8-cyclonucleosides. The reduced formation yield of the 4 tandem lesions in the presence of O_2 explains, at least partly, the low efficiency of radiation-induced yields of the purine 5′,8-cyclo-2′-deoxyribonucleosides in cellular DNA, which are about two orders of magnitude lower than the previously reported data obtained from HPLC-MS analysis.

1. Introduction

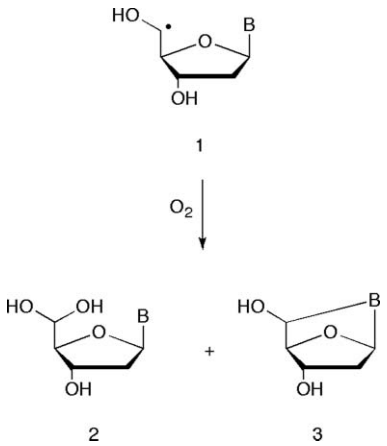
Cellular components, in particular nucleic acids, are the targets of a wide set of endogenous or exogenous genotoxic agents. Among them one may quote reactive oxygen species (ROS) that are generated as the result of respiration burst in mitochondria, metabolism of xenobiotics and exposure to physical agents such as ionizing radiation and UVA light. Two of the main ROS, including hydroxyl radical (•OH) and singlet oxygen ($^1O_2$), have been shown to damage both isolated and cellular DNA.[1] Many efforts have been devoted during the last two decades to the identification and measurement of the main classes of oxidatively generated damage to DNA. Thus •OH-mediated reactions have been shown to give base alterations, strand breaks, abasic sites and DNA-protein cross-links.[1,2] There is increasing interest devoted to the chemistry and biochemical processing of tandem lesions, another class of oxidatively generated DNA damage, whose formation involves only one radical hit. These include, among others, adjacent base lesions[3] and purine 5′,8-cyclonucleosides in which both the base and the 2-deoxyribose moiety are modified.[2,4] Evidence was provided from earlier model studies for the formation of both 5′*R* and 5′*S* diastereomers of adenine and guanine 5′,8-cyclonucleosides as the result of initial •OH-mediated hydrogen abstraction from the exocyclic 5′-hydroxymethyl group of the 2-deoxyribose moiety and subsequent intramolecular cyclization of the 5′-yl radical thus formed to the purine ring at C8 (Scheme 1).[5] It was reported that



Scheme 1 Radical reactions for the formation of purine 5′,8-cyclonucleosides in DNA.

the presence of oxygen in a gamma-irradiated solution of DNA prevented the formation of the adenine and guanine 5′,8-cyclonucleosides.[5d,6] The reactivity of purine-substituted C5′ radicals **1** under aerobic conditions has been recently elucidated at the nucleoside level.[7] Using an oxygen concentration in the range of 13–266 μM, conditions that are typical for oxygenated tissues, two main degradation products including the hydrated 5′-aldehyde **2** and the purine 5′,8-cyclo-2′-deoxyribonucleoside **3** (Scheme 2) were found to be generated. The formation of 5′,8-cyclonucleosides is relevant in all experiments, and the yields of the latter degradation products increase by decreasing O_2

[a]*Laboratoire "Lésions des Acides Nucléiques", DSM/INAC/SCIB UMR-E 3 CEA/UJF & FRE CNRS 3200CEA-Grenoble, 38054 Grenoble Cedex 9, France. E-mail: jean.cadet@cea.fr; Fax: +33 4 38 78 50 90; Tel: +33 4 38 78 49 87*
[b]*I.S.O.F. – Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129 Bologna, Italy. E-mail: chrys@isof.cnr.it; Fax: +39 051 639 8349; Tel: +39 051 639 8309*



Scheme 2 The C5′ radical **1** at single nucleoside level in the presence of oxygen is partitioned between two reaction channels with formation of products **2** and **3**.

concentration. One may point out that the role of molecular oxygen is far from being understood either as the trap of the C5′ radical prior to cyclization at DNA level or as the oxidant of the aminyl radical after cyclization.

It was hypothesized that both 5′,8-cyclo-2′-deoxyadenosine (cdAdo) and 5′,8-cyclo-2′-deoxyguanosine (cdGuo), whose removal from damaged DNA is achieved by the nucleotide excision repair (NER) and not the base excision repair (BER) pathway,[8] may be involved in the etiology of several human diseases.[9] This could be the case for the neurological disorders that are associated with complementation groups A, C and D of *xeroderma pigmentosum* patients. It was hypothesized that a possible accumulation of both cdAdo and cdGuo lesions may take place with age in the brain as the result of NER-deficiency; one may add that indirect evidence was provided for a higher cytotoxicity of the 5′*S* diastereomer of cdAdo with respect to the 5′*R* form.[10] However, there is so far only one example of the accumulation of (5′*S*)-5′,8-cyclo-2′-deoxyadenosine with age in the DNA of brain, kidney and liver of Cockayne syndrome complementation group B gene knockout mice.[11] This is likely due, at least partly, to the lack of accurate and highly sensitive methods capable of detecting very low amounts of cdAdo and cdGuo in cellular DNA, despite recent claims that have involved GC-MS and HPLC-MS measurements.[12] The present work constitutes the first attempt of a global investigation that includes the design and optimization of robust and highly specific HPLC-MS/MS method together with the assessment of oxygen effect on the ˙OH-mediated formation of purine 5′,8-cyclonucleosides. Information is also provided on the very low efficiency for the indirect effects of ionizing radiation that implicate ˙OH as the main ROS to generate (5′*R*)-5′,8-cyclo-2′-deoxyadenosine in the DNA of human cells.

2. Experimental

2.1 Chemicals and biochemicals

Calf thymus DNA, 2′-deoxyadenosine (dAdo), 2′-deoxyguanosine (dGuo), *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), nuclease P1 from *Penicillium citrium*, phosphodiesterase II, phosphodiesterase I from *Crotalus adamentus* venom, DNAse II and alkaline phosphatase were obtained from Sigma (St Quentin-Fallavier, France). The preparation of the two sets of 5′*R* and 5′*S* diastereomers of both purine 5′,8-cyclo-2′-deoxyribonucleosides and the oligonucleotides in which the latter tandem lesions were site-specifically inserted was achieved using previously reported protocols.[13] The isotopically [$^{15}N_5$]-labeled internal standards of (5′*R*)- and (5′*S*)-cdAdo were prepared by gamma irradiation[5b] of an oxygen-free aqueous solution of [$^{15}N_5$]-dAdo (Cambridge Isotope Laboratories, Andover, MA) and subsequently purified by HPLC analysis.

2.2 Computational details

Hybrid meta DFT calculations with the BB1K (Becke88[14]–Becke95[15] 1-parameter model for thermochemistry) functional[16] were carried out using the Gaussian 03 system of programs.[17] This HMDFT model was tailored to give good reaction barrier heights.[15] Unrestricted wave function was used for radical species. Optimized geometries and total energies were obtained employing the valence double-ζ basis set supplemented with polarization functions and augmented with diffuse functions on heavy atoms (6-31+G**). The nature of the ground (one imaginary frequency) and transition (zero imaginary frequency) states was verified by frequency calculations. Total energies were corrected for the zero point vibrational energy (ZPVE) computed from frequency calculations using a scaling factor of 0.9561 to account for anharmonicity.[16] The effect of water solution on the total energies was computed with the polarizable continuum model[18] (PCM) carrying out calculations at the optimum BB1K/6-31+G** geometries (PCM (solvent = water)/BB1K/6-31+G**//BB1K/6-31+G**).

2.3 Gamma irradiation of isolated DNA

Aqueous solutions of calf thymus DNA (0.5 mg mL^{-1}) were γ-irradiated using a ^{60}Co-Gamma cell, at room temperature (22 ± 2 °C). The dose rate was determined with the Fricke chemical dosimeter by taking $G(Fe^{3+}) = 1.61$ μmol J^{-1}.[19] The preparation of O_2/N_2 and/or N_2O saturated aqueous solutions was achieved by bubbling gas(es) continuously through the samples with a flow rate ranging from 60 to 100 mL min^{-1}, for 5 min before the irradiation and maintained during exposure to the gamma ray source.

2.4 Radiolytic production of transients[20]

Radiolysis of neutral water leads to the species e_{aq}^-, ˙OH and H˙, as shown in eqn (1) where the values in parentheses represent the chemical radiation yields (G) expressed in μmol J^{-1}. The presence of oxygen transforms efficiently e_{aq}^- and H˙ into $O_2^{\bullet-}$ (eqn (2) and (3)). The rate constants for the reaction of e_{aq}^- and H˙ with O_2 are $k_2 = 1.9 \times 10^{10}$ M^{-1} s^{-1} and $k_3 = 1.2 \times 10^{10}$ M^{-1} s^{-1}, respectively. The pK_a value of HOO˙ is 4.8 (eqn (4/–4)).[21]

$$H_2O \rightsquigarrow e_{aq}^-(0.27), {}^{\bullet}OH\ (0.28), H^{\bullet}\ (0.062) \quad (1)$$

$$e_{aq}^- + O_2 \rightarrow O_2^{\bullet-} \quad (2)$$

$$H^{\bullet} + O_2 \rightarrow HOO^{\bullet} \quad (3)$$

$$HOO^{\bullet} \rightleftarrows O_2^{\bullet-} + H^+ \quad (4/-4)$$

The presence of N_2O allows the efficient conversion of e_{aq}^- into the $O^{\bullet-}$ species (eqn (5)), $k_5 = 9.1\times 10^9$ M^{-1} s^{-1}). The $^{\bullet}OH$ radical ($pK_a(^{\bullet}OH) = 11.9$) is in equilibrium with its conjugated base $O^{\bullet-}$ (eqn (6/−6)), $k_6 = 1.2 \times 10^{10}$ M^{-1} s^{-1}, $k_{-6} = 1 \times 10^8$ s^{-1}).

$$e_{aq}^- + N_2O \rightarrow O^{\bullet-} + N_2 \quad (5)$$

$$^{\bullet}OH + HO^- \rightleftarrows O^{\bullet-} + H_2O \quad (6/-6)$$

Two sets of irradiation experiments were carried out: the first one by bubbling N_2/O_2 at different proportions and the second one by bubbling N_2O/O_2 at different ratios. The solubility of O_2 and N_2O in water is 1.33×10^{-3} M and 0.02 M, respectively, at 22 °C.[22] The air-saturated, N_2 (80%)/O_2 (20%), solution (corresponds to 2.66×10^{-4} M of O_2, which is *ca.* 6 times higher than typical well-oxygenated tissues, *i.e.* $[O_2] \cong 4 \times 10^{-5}$ M (the oxygen concentration is even lower in the nucleus).[23] Therefore, we performed experiments in the range 1.33×10^{-5}–2.66×10^{-4} M of oxygen concentration. In the absence of N_2O, oxygen quenches nearly all the primary reducing species, producing $^{\bullet}OH$ and $O_2^{\bullet-}$ radicals in 45% and 55% yields, respectively. In the presence of N_2O, $^{\bullet}OH$ and $O_2^{\bullet-}$ radicals accounted for 90% and 10%, respectively, of the reactive species.

2.5 Enzymatic DNA digestion

To 100 µL of the DNA solution was added 5 µL of 2.5 mM EHNA, 5 µl of nuclease P1 (5 U), 1 µL of phosphodiesterase II (0.004 U), 0.025 µL (0.25 U) of DNAse II together with 10 µL of buffer P1 10x (200 mM succinic acid, 100 mM $CaCl_2$, pH 6.0). The resulting solution was incubated for 2 h at 37 °C. Thereafter, 10 µL of alkaline phosphatase buffer 10x (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.2 µL (0.003 U) of phosphodiesterase I and 0.25 µL (5 U) of alkaline phosphatase. The samples were then incubated at 37 °C for 2 h. Thereafter, the first step of digestion with nuclease P1, DNAse II and phosphodiesterase 1 was repeated (without adding EHNA) for 2 h at 37 °C to achieve complete digestion of the purine 5′,8-cyclo-2′-deoxyribonucleosides. Then, the samples were centrifuged for 5 min at 5000 *g* prior to HPLC-MS/MS analysis.

2.6 HPLC-MS/MS assay

Purine 5′,8-cyclo-2′-deoxyribonucleosides were detected in hydrolyzed DNA samples using HPLC coupled to tandem mass spectrometry. Separations were performed using a 2 × 150 mm octadecylsilyl silica gel (3 µm particle size) Uptispher column (Interchim, Montluçon, France) as described previously for the measurement of other oxidatively generated DNA lesions.[24] The HPLC system consists of a complete Accela system (Thermo Corporation) equipped with an HPLC pump, a thermostated autosampler and a UV detector set-up at 260 nm for monitoring normal nucleosides. DNA tandem lesions were detected using a TSQ Quantum Ultra mass detector equipped with an electrospray ion source. Separations of 2′-deoxyribonucleosides were performed using a linear gradient of 2 mM ammonium formate and acetonitrile: the concentration of acetonitrile was increased from 0 up to 15% in ammonium formate within 30 min. Under these conditions, the 5′*R* and 5′*S* diastereomers of cdGuo elute at 6.8 and 10.6 min, respectively, whereas the retention times of (5′*R*)- and (5′*S*)-cdAdo are 9.3 and 14.3 min, respectively (Fig. 3). Detection of purine 5′,8-cyclo-2′-deoxyribonucleosides was performed in the multiple reaction monitoring mode after optimization of the parameters of electrospray ionization and fragmentation. This was achieved by infusing diluted solutions of pure standards. The most intense transitions used to detect and quantify cdGuo and cdAdo were 250→166 and 266→180, respectively using a collision energy of 18 eV. In addition, transitions 250→134 and 266→160 (collision energy of 38 ev), corresponding to the loss the 2-deoxyribose moiety, which are significantly less intense (20% relative intensity) were used as a qualifier in order to improve the specificity of detection of the tandem lesions. The quantification of each of the 5′*R* and 5′*S* diastereomers of the two pairs of purine 5′,8-cyclo-2′-deoxyribonucleosides formed upon gamma irradiation of aqueous solutions of DNA was achieved by external calibration. For the measurement of cdAdo in cellular DNA, samples were spiked with known amounts of the 5′*R* and 5′*S* diastereomers of [$^{15}N_5$]-cdAdo that were used as internal standards. The isotope dilution technique thus used allowed an accurate quantification of very low amounts of cdAdo. The transition 255→171 was used for the detection of [$^{15}N_5$]-cdAdo.

2.7 Cell culture

THP1 monocytes were grown following the protocol that was previously reported.[25]

3. Results and discussion

3.1 Formation and rearomatization of aminyl radical arising from intramolecular cyclization of $^{\bullet}OH$-mediated C5′ radical to C8 of purine bases

It has been shown that the C5′ radical generated by $^{\bullet}OH$-mediated hydrogen atom abstraction intramolecularly attacks the C8,N7 double bond of the adenine or guanine moieties to form purine 5′,8-cyclonucleosides as the final products (Scheme 1).[1a,26,27] However, the intermediate aminyl radical **4** derived from the cyclization (Scheme 3) should be further oxidized in order to afford the final product. Information on this elementary step is limited, in particular, on the oxidation induced by molecular oxygen.

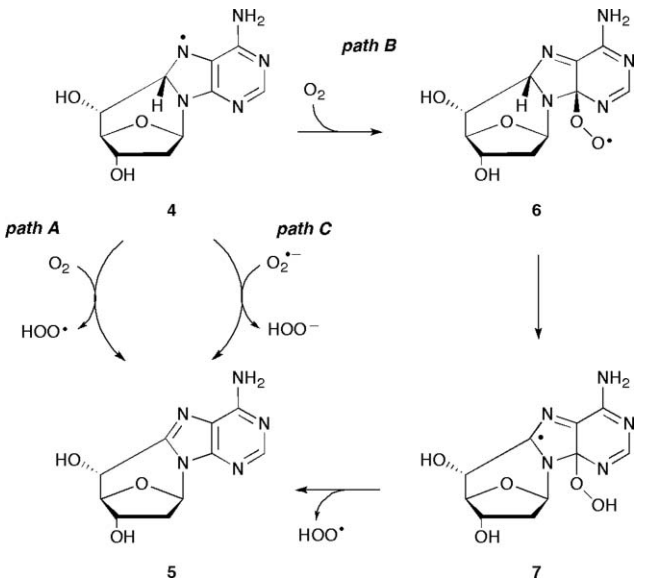
Hybrid meta DFT calculations were carried out to determine the energy profiles for the rearomatization of aminyl radical **4** to **5** by the action of molecular triplet oxygen (Scheme 3). *Path A* involves a direct hydrogen abstraction, whereas *path B* involves addition of O_2 at C4 to afford peroxyl radical **6**, followed by hydrogen shift from C8 to give **7** and release of the $HOO^{\bullet}$ radical to give the final product. The energy profiles in water solution were computed with the polarizable continuum model[27] (PCM) carrying out calculations at the optimum BB1K/6-31+G** geometries[18] (PCM (solvent = water)/BB1K/6-31+G**//BB1K/6-31+G**).

Energy barriers and reaction energies are reported in Table 1. The whole oxidation process is computed to be highly exothermic (ΔE_r = −24.9 kcal mol^{-1}). Hydrogen abstraction ($\Delta E^{\ddagger}$ = 11.6 kcal mol^{-1}) is slightly favored *versus* addition (15.4 kcal mol^{-1}). Interestingly, peroxyl radical **6** is highly unstable, since a low barrier (0.2 kcal mol^{-1}) is computed for the backward reaction (O_2 release) so that initially an equilibrium between radicals **4** and **6** should exist. However, the energy barrier of 22.9 kcal mol^{-1} for

Table 1 Energy barriers $\Delta E^{\ddagger}$ and reaction energies ΔE_r (kcal mol^{-1}) for the various steps in Scheme 3 computed at the PCM/BB1K/6-31+G**//BB1K/6-31+G** level[a]

Pathway	Step	$\Delta E^{\ddagger}$	ΔE_r
Path A[b]	**4** → **5**	11.3 (7.5)	−24.9 (−23.3)
Path B[b]	**4** → **6**	15.4 (15.4)	15.2 (11.7)
	6 → **7**	22.9 (23.5)	−21.6 (−18.1)
	7 → **5**	4.6 (4.6)	−18.5 (−16.9)
Path C	**4** → **5**	20.4	−33.6

[a] Energies are corrected for zero point vibrational energy (ZPVE). [b] In parentheses, the computed energies are reported employing explicitly one water molecule (BB1K/6-31+G**), see text.



Scheme 3 Three possible pathways for the rearomatization of aminyl radical **4**.

the second step (**6**→**7**) in *path B* (Scheme 3) is computed to be as large as about twice that for hydrogen abstraction. The final release of the peroxyl radical is computed to be fast, as expected for the rearomatization. The intramolecular hydrogen shift is, hence, the rate determining step for *path B*. On the basis of these findings, *path A* should be the preferred one.

In our experiments, a large fraction of superoxide radical anions ($O_2^{\bullet -}$) are also produced, so the oxidation process could be also generated by this reactive species (*path C* in Scheme 3). The energy barrier and the reaction energy for *path C* are also reported in Table 1.[28] It is noticeable that the energy barrier is computed to be as large as about twice than that computed for hydrogen abstraction by molecular oxygen (*path A*).

The trend in the relative energies of the optimized structures (BB1K/6-31+G**) indicates that the effect of water as the solvent simulated with the PCM model is small, as expected. However, hydrogen-bonding in the transition state for the direct hydrogen atom abstraction could lower the energy barrier significantly; thus, the reaction mechanisms of paths A and B were computed in the presence of one water molecule (water-assisted reactions) at the BB1K/6-31+G** level. The effect due to a polarizable continuum solvent was not estimated with the PCM method in this case, since it was shown that the use of the PCM method in addition to the hydrogen-bonding model decreases the agreement between theory and experiment, not only sizably with respect to the hydrogen-bonding model but also significantly with respect to the PCM approach in reactions where hydrogen-bonding interactions with water molecules are important.[29] Table 1 shows that hydrogen-bonding interaction with one water molecule lowers sizably only the energy barrier for the hydrogen atom abstraction by molecular oxygen.

The energy profiles for the direct hydrogen abstraction from the aminyl radical by molecular triplet oxygen (*path A*), in the absence and presence of a water molecule, are displayed in Fig. 1. The two insets also show the two transition states in the absence and presence of a water molecule. In the latter case, the reaction occurs with a relatively low energy barrier of 7.5 kcal mol^{-1}. The rate constant for this process should be about 10^3 M^{-1} s^{-1} at room temperature assuming a "normal" frequency factor ($\log A/\mathrm{M}^{-1}\ \mathrm{s}^{-1} = 8.5$) for the bimolecular process.[30]

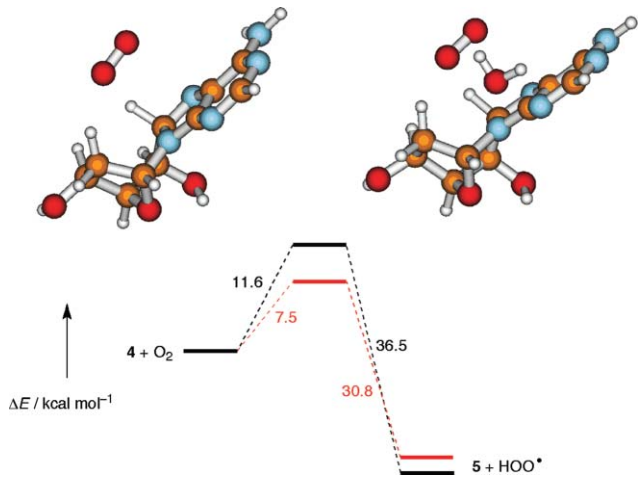


Fig. 1 Relative energies for the hydrogen abstraction from aminyl radical **4** by molecular oxygen computed at the PCM/BB1K/6-31+G**//BB1K/6-31+G** level. The red line represents the energies computed employing explicitly one water molecule (BB1K/6-31+G**). Insets: the transition states in the absence (left) and in the presence (right) of a water molecule.

3.2 Optimization of enzymatic digestion of purine 5′,8-cyclo-2′-deoxyribonucleosides from DNA

To obtain a quantitative assessment of the levels of DNA lesions, complete hydrolysis of DNA into normal and modified 2′-deoxyribonucleosides is required.[25] Since it has been reported previously that the release of purine 5′,8-cyclonucleosides from DNA as free nucleosides could be incomplete,[13,32] efforts have been made in the present work to optimize conditions for a quantitative enzymatic digestion of DNA. Attempts were first made to use the conditions we have previously designed[25,31,33] for the measurement of several oxidized 2′-deoxyribonucleosides, including the four *cis* and *trans* diastereomers of 5,6-dihydroxy-5,6-dihydrothymidine,[34] lesions that are known to be resistant to nuclease P1 hydrolysis.[35] Using non-irradiated DNA samples spiked with increasing amounts of cdAdo containing oligonucleotides prepared as previously described,[13] it was shown that under such conditions the release of cdAdo is not quantitative. In agreement

with the conclusions of an early work,[32] it was found that the lesion was mostly released as a dinucleoside monophosphate that was detected by HPLC-MS/MS (not shown). This particularly applies when a thymine base is located 3′ to bulky cdAdo. The increase in the time of incubation from 2 h up to 24 h had only a limited effect on the efficacy of digestion. Several conditions were tested and finally optimal conditions were obtained using a three-step procedure (see experimental section). First, DNA is digested using a mixture of two endonucleases (nuclease P1 and DNAse II) to which a 3′-exonuclease (phosphodiesterase II) is added. In a second step, a 5′-exonuclease is used (phosphodiesterase I) together with alkaline phosphatase in order to convert nucleotides into the corresponding nucleosides. Finally, step I using nuclease P1, DNAse II and phosphodiesterase I is resumed for 2 h at 37 °C. Under these conditions, the recovery of cdAdo in DNA samples spiked with cdAdo-containing oligonucleotides was found to be nearly quantitative, even when the lesion was located 5′ to a thymine nucleoside (recovery > 85%). The enzymatic conditions were also found to be suitable for complete hydrolysis of DNA samples spiked with cdGuo containing oligonucleotides. It should be added that, since some of the enzymes used to digest DNA were found to be contaminated with adenine deaminase, EHNA was added to prevent deamination of adenine nucleosides.[36]

3.3 HPLC-MS/MS measurement of the (5′*R*) and (5′*S*) diastereomers of cdAdo and cdGuo

Tandem mass spectrometry was used to detect simultaneously the 5′*R* and 5′*S* diastereomers of cdAdo and cdGuo in digested DNA samples. First, optimization of the parameters for electrospray ionization and fragmentation was performed by infusing a micro-molar solution of pure standards, as previously reported for the detection of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo).[24] In the positive ionization mode, the collision-induced dissociation mass spectra of the pseudo-molecular ion (m/z = 250 amu) of cdAdo exhibits an intense fragment at m/z 164 amu, corresponding to the loss of 86 amu (Fig. 2). A similar fragmentation pattern was also observed for cdGuo with an intense fragment at m/z = 180 amu upon decomposition of the pseudomolecular ion at m/z = 266 amu. Such a fragmentation corresponds to the splitting of the 2-deoxyribose moiety, as the result of a concomitant bond cleavage between C1′ and N1 on the one hand and between C4′ and C5′ on the other hand; it may be added that the base moiety remains attached to the C5′-hydroxymethyl group in agreement with recent similar observations.[6] One may note a similar fragmentation pattern for the two diastereomers of either cdAdo or cdGuo (not shown). Therefore, mass spectrometry could not be used to distinguish between the 5′*R* and 5′*S* diastereomers. However, any of the purine cyclic nucleosides could be detected individually by HPLC-MS/MS, since each of them exhibits significantly different retention times and, as discussed below, the 5′*R* diastereomer is eluted more rapidly than the 5′*S* form. To improve the sensitivity of detection, the so-called multiple reaction monitoring (MRM) mode was applied and the most intense transitions, *i.e.* 250→166 and 266→180 for cdAdo and cdGuo, respectively, were used. A similar sensitivity was obtained for the two diastereomers, whereas the threshold of detection was found to be significantly lower for cdAdo compared to cdGuo. The limit of quantification for either

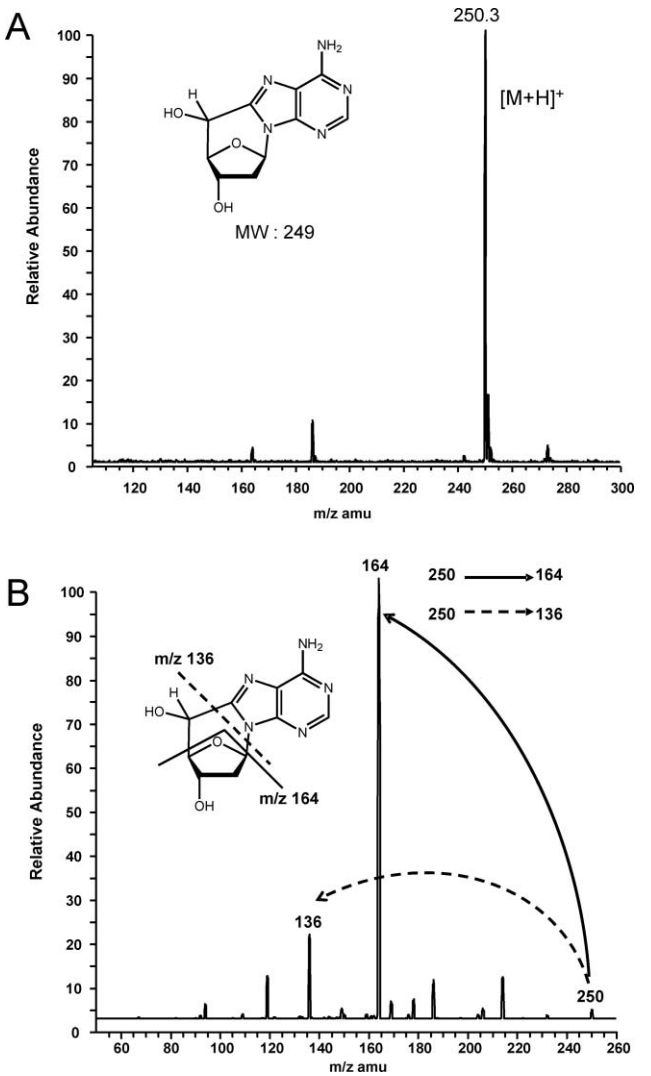


Fig. 2 Pseudomolecular ion of (5′*S*)-cdAdo in the positive mode of ESI-MS/MS spectrum (A). Collision-induced dissociation mass spectra of ion at m/z = 250 corresponding to the $[M + H]^+$ (B).

the 5′*R* or 5′*S* diastereomer of cdAdo was found to be 5 fmol. In contrast, the detection of cdGuo is far less sensitive, with a limit of detection estimated to be around 100 fmol. It should be noted that, in our hands and as expected, the detection of the purine 5′,8-cyclonucleosides using the selected ion monitoring mode (HPLC-MS) was found to be about two orders of magnitude less sensitive than the HPLC-MS/MS method in the MRM mode. This explains why HPLC-MS assay cannot be used to accurately detect such minor classes of oxidatively generated tandem lesions in cellular DNA. One may note that HPLC with MS^3 detection[37] has been recently employed to detect radiation-induced vicinal base lesions that are generated with an efficiency similar to that of purine 5′,8-cyclonucleosides in cellular DNA.

A typical elution profile of HPLC-MS/MS analysis of both cdAdo and cdGuo in a digested DNA sample is shown in Fig. 3. It appears that the 5′*R* diastereomer of either cdAdo or cdGuo is eluted before the related (5′*S*)-cyclonucleoside under the HPLC conditions used. Therefore, each compound of the two pairs of diastereomers could be detected and quantitatively measured

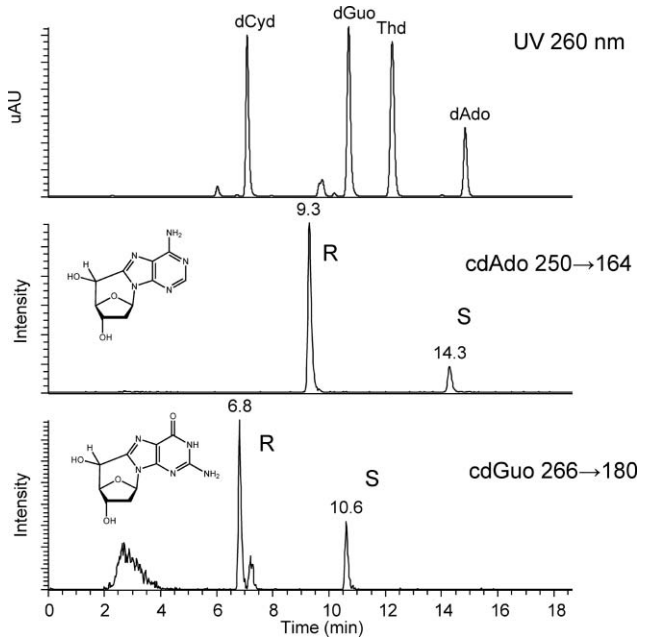


Fig. 3 Typical HPLC elution profile of the two diastereomers of cdAdo (middle chromatogram) and cdGuo (bottom chromatogram) in an enzymatically digested DNA sample. UV detection (260 nm, top chromatogram) was used for the determination of the amount of normal nucleosides.

individually. UV detection was used to quantify in each sample the amount of normal nucleosides (dAdo and dGuo) and the results were expressed as the number of modifications per 10^6 normal nucleosides.

3.4 Radiation-induced formation of 5′*R* and 5′*S* diastereomers of purine cyclo-2′-deoxyribonucleosides in oxygen-free aqueous solutions of isolated DNA

Initially, γ-radiolysis experiments coupled with DNA damage analysis were carried out under oxygen-free conditions. For this purpose, N_2- or N_2O-saturated aqueous solutions of calf thymus DNA (0.5 mg mL^{-1}) were γ-irradiated at neutral pH under steady-state conditions with a dose rate of *ca.* 8 Gy min^{-1} followed by enzymatic DNA digestion and HPLC-MS/MS analysis, as reported above. Both 5′*R* and 5′*S* diastereomers of cdAdo and cdGuo were found to be generated linearly with the applied dose within the range 0–60 Gy under conditions of very low DNA degradation that preserve the native conformation of the biopolymer. The levels of DNA lesions per Gy with respect to oxygen concentrations varying from 0 up to 20% are reported in Fig. 4. The yield of radiation-induced cdGuo in oxygen-free aqueous solutions of DNA was found to be higher than that of cdAdo, the ratio of formation between cdGuo and cdAdo being ~1.7. It may be added that irrespective of the purine 5′,8-cyclo-2′-deoxyribonucleosides, the 5′*R* diastereomer is formed predominantly leading to a ratio 5′*R*/5′*S* of ~3 and ~4 for cdGuo and cdAdo, respectively (data not shown). As expected from the doubling of •OH by saturation of the γ-irradiated solutions of DNA with N_2O, an about two-fold increase in the levels of radiation-induced 5′*R* and 5′*S* diastereomers of 5′,8-cyclo-2′-deoxyribonucleosides was observed. In addition, the formation of

Table 2 Radiation-induced formation[a] of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodAdo) and the 5′*R* and 5′*S* diastereomers of 5′,8-cyclo-2′-deoxyguanosine (cdGuo) and 5′,8-cyclo-2′-deoxyadenosine (cdAdo) in aqueous solution of DNA saturated with various amounts in N_2/O_2 and N_2O/O_2 mixtures by varying the amount of O_2

	N_2/O_2 (% O_2)			N_2O/O_2 (% O_2)		
Modified nucleosides	0	10	20	0	10	20
8-OxodGuo	38	58	40	78	88	110
8-OxodAdo	1.3	3.2	3.0	7.2	7.8	9.9
cdGuo	11.1	0.9	0.5	20.1	2.1	0.9
cdAdo	6.5	0.8	0.6	14.2	1.9	0.8

[a] Expressed in number of modified nucleosides per 10^6 nucleosides and per Gy.

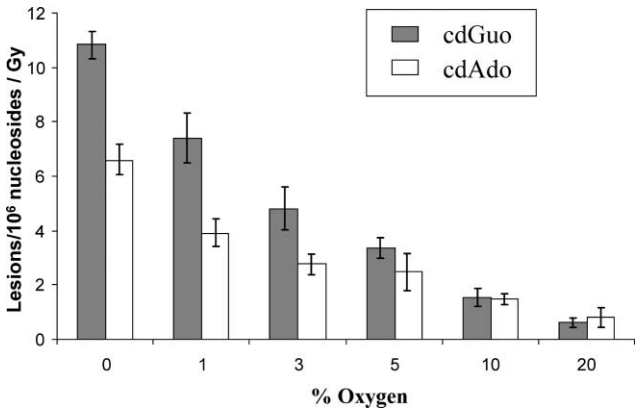


Fig. 4 Radiation-induced formation of cdAdo and cdGuo in N_2/O_2 saturated aqueous solutions of DNA with increasing amounts of oxygen. The purine 5′,8-cyclonucleosides were measured as single diastereomers by HPLC-MS/MS in enzymatically digested DNA samples. Each point corresponds to the average of three independent measurements in two distinct experiments.

8-oxodGuo and 8-oxodAdo that have been previously detected as relevant degradation products of both indirect and direct effects of ionizing radiation in isolated and cellular DNA,[38] was monitored by HPLC-MS/MS (Table 2). Thus it was shown that the efficiency of •OH-induced formation of (5′*R*)-cdAdo was about 4-fold higher and 7.3-fold lower than that of 8-oxodAdo and 8-oxodGuo, respectively, in N_2-saturated aqueous solution of DNA.

3.5 Inhibitory effects of oxygen on the radiation-induced formation of purine 5′,8-cyclo-2′-deoxyribonucleosides in isolated DNA

In order to investigate the effect of molecular oxygen on the competitive reactions of radiation-induced C5′ sugar radicals, two different sets of experiments were designed by bubbling aqueous solutions of DNA with mixtures of N_2/O_2 or N_2O/O_2 in different proportions. For example, a N_2(80%)/O_2(20%) saturated aqueous solution of calf thymus DNA (0.5 mg mL^{-1}), which corresponds to 2.66×10^{-4} M of O_2, was irradiated under stationary state conditions with a dose rate of *ca.* 8 Gy min^{-1}, while the mixture of gases was continuously bubbled throughout the reaction time. In all cases, the formation of the 5′*R* and 5′*S* diastereomers of both cdGuo and cdAdo was found to increase with the dose of γ-rays

within the dose-range 0–60 Gy. The numbers of lesions per Gy *versus* oxygen concentration are shown in Fig. 4. The following observations can be made: first, the formation of both cdGuo and cdAdo decreases with the increase of the oxygen concentration, indicating competition paths for C5′ radical between cyclization and oxygen addition whose rate was found to be 1.9×10^9 M^{-1} s^{-1} at the nucleoside level;[26] secondly, the formation of cdGuo decreases faster than cdAdo with the increase in oxygen concentration suggesting different rates of cyclization for the corresponding C5′ radicals; thirdly, the 5′*R*/5′*S* ratio is constant within experimental errors for both purine 5′,8-cyclo-2′-deoxyribonucleosides, the 5′*R* diastereomer being predominant as previously observed in the absence of oxygen (data not shown).

The order of reactivity of ˙OH towards the various hydrogen atoms of the 2-deoxyribose moiety is currently under debate.[39] A proposed order that also parallels the exposure to solvent of the 2-deoxyribose hydrogen atoms (*i.e.*, H5′ > H4′ > H3′ ≈ H2′ ≈ H1′) is generally accepted.[40] Experimental data on the reaction of ˙OH radical with simple nucleosides like 2′-deoxyadenosine and 2′-deoxyguanosine indicated that 20–25% of these reactions occur at the sugar moiety as H-atom abstraction, 40–50% of which is at H5′ positions.[7] A rate constant of $k_c = 1.6 \times 10^5$ s^{-1} for the cyclization of 2′-deoxyadenosin-5′-yl radical has also been reported[26] and strong evidence exists that the cyclization of 2′-deoxyguanosin-5′-yl radical is even faster.[41] However, our findings showing that the formation of cdGuo decreases faster than that of cdAdo by increasing oxygen concentration suggest that the rate of cyclization of the 2′-deoxyguanosin-5′-yl radical is lower than that of the 2′-deoxyadenosin-5′-yl radical in DNA. We believe that local conformations due to the supramolecular organization of DNA should influence considerably (either accelerating or reducing) the cyclization rates of the two radicals. Local conformational changes may also modulate the rate of O_2 addition to the C5′-yl radical and, therefore, may explain the lower efficiency of cdGuo formation with the increase in oxygen concentration.

Depending on the substrate and the experimental conditions, the ratio of the 5′*S* and 5′*R* diastereomers changes substantially. The (5′*R*)/(5′*S*) ratios of 6 : 1 and 8 : 1 were obtained in water for 5′,8-cyclo-2′-deoxyadenosine and 5′,8-cyclo-2′-deoxyguanosine, respectively, upon irradiation of free nucleosides.[7,26,27] The situation in the literature regarding single- and double-stranded DNA is quite confusing. In early studies based on GC-MS analysis,[42] it was reported that the 5′*R* diastereomer of both compounds predominates in single-stranded DNA, whereas 5′*S* diastereomers slightly prevail in double-stranded DNA. More recently, (5′*R*)/(5′*S*) ratios of approximately 2 and 0.3 have been reported for 5′,8-cdAdo and 5′,8-cdGuo lesions, respectively, using HPLC/MS analysis.[12a,43] Based on the present results, previously reported quantification of purine 5′,8-cyclonucleosides in DNA achieved by GC-MS,[42] HPLC-MS[12a,43] or HPLC-MS/MS[6] analyses appear to be questionable.

3.6 Radiation-induced formation of (5′*R*)-5′,8-cyclo-2′-deoxyadenosine in cellular DNA

As discussed above for the HPLC-MS/MS assay designed in the present work for monitoring the formation purine 5′,8-cyclonucleosides in DNA, the detection threshold of both 5′*R* and 5′*S* diastereomers of cdAdo is 20-fold lower than that of (5′*R*)- and (5′*S*)-cdGuo. In addition, it was found that the radiation-induced formation of the 5′*R* diastereomer in aqueous solutions of DNA was 4-fold more efficient than that of the related (5′*S*)-cyclonucleoside (Table 2). This explains why our efforts have focused on the search of cdAdo with emphasis on the more abundant 5′*R* diastereomer in the DNA of human monocytes exposed to ionizing radiation using (5′*R*)- and (5′*S*)-[$^{15}N_5$]-cdAdo as the isotopically labeled internal standard. Using such a sensitive and accurate isotope dilution technique in combination with HPLC-MS/MS, the background level of either the 5′*R* or 5′*S* diastereomer of cdAdo determined in untreated cells was found to be lower than our limit of detection, estimated to be around 0.1 lesion per 10^9 nucleosides. This contrasts with the much higher steady-state values of either (5′*R*)- or (5′*S*)-cdAdo which were measured mostly by HPLC-MS analysis in previous studies. Thus, it was reported that the basal level of (5′*R*)-cdAdo was estimated to be 0.133 and 0.22 per 10^7 normal nucleosides in the DNA of mouse liver as measured by HPLC-MS/MS[6] and HPLC-MS,[44] respectively. One may note higher levels of (5′*S*)-cdAdo, whose frequencies per 10^7 normal nucleosides were found to be 0.482,[6] 1.92[44] and 0.40[11] in mouse liver DNA, and 2.0[45] and 2.5[46] in the DNA of human keratinocytes. Another piece of information concerning the steady-state level of purine 5′,8-cyclonucleosides was provided using a highly sensitive[32] P-postlabeling assay that has been designed for monitoring the formation of the so-called "type II I-compounds", a class of bulky oxidatively generated DNA lesions that were tentatively identified as tandem base lesions and base-sugar cross-links.[47] The levels of 5′,8-cyclo-2′-deoxyadenosine, that was shown to be one of these DNA modifications, were found to be between 180 and 320 lesions per cell in fetal and 24 h newborn rat liver DNA.[48] This is about 3 orders of magnitude lower than the above HPLC-MS values of cdAdo, thus questioning the validity of the latter measurements.

Ionizing radiation is a suitable physical agent capable of inducing oxidatively generated damage to cellular DNA in a quantitative manner. Thus oxidized bases and sugar residues were shown to be formed in a linear dose dependent manner under acute irradiation conditions where the contribution of DNA repair is minimized. It may be stressed that the detection of a small peak of (5′*R*)-cdAdo (Fig. 5) has required a high dose of ionizing radiation (2 kGy) which was delivered during a very short time period. This, although biologically irrelevant, is suitable from a chemical point of view to induce molecular damage to cellular DNA. The yield of radiation-induced formation of the 5′*R* diastereomer of 5,8-cyclo-2′-deoxyadenosine was estimated to be 0.2 cdAdo/10^9 nucleosides/Gy. This is much lower, by at least two orders of magnitude, than the radiation-induction formation yields of both cdAdo and cdGuo that were measured by either HPLC-MS or GC-MS in cellular DNA.[45,46] It may be reminded that the levels of (5′*S*)-cdAdo and both 5′*R* and 5′*S* diastereomers of cdGuo were found to be significantly increased in the DNA of human keratinocytes upon exposure to a dose of X-rays as low as 5 Gy! Thus, the yields of radiation-induced cyclonucleosides per 10^9 normal nucleosides and per Gy were found to be 14, 100 and 30 for (5′*S*)-cdAdo and the 5′*S* and 5′*R* diastereomers of cdGuo, respectively.[45] It is also informative to compare the radiation-induced formation efficiency of purine 5′,8-cyclo-2′-deoxyribonucleosides with that of oxidized nucleosides such as 8-oxodGuo and 8-oxodAdo, whose yields in the DNA of

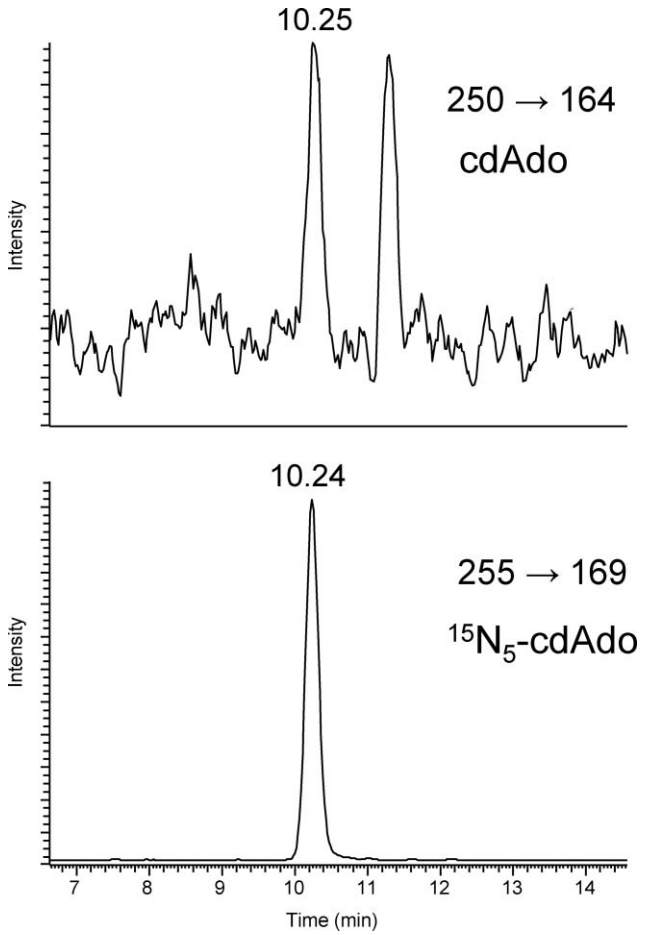


Fig. 5 HPLC-MS/MS chromatograms showing the detection of cyclodAdo in 2kGy gamma irradiated cells. The upper trace (transition 250→166) exhibits a peak at 10.25 min that corresponds to the amount of detected cdAdo (about 3 fmol detected). The lower trace (transition 255→169) corresponds to the detection of the internal standard.

human monocytes have been determined by HPLC-MS/MS to be 20 and 4 lesions, respectively, per 10^9 normal nucleosides and per Gy.[38] The much lower yield of radiation-induced formation of (5′*R*)-cdAdo established in the present investigation is consistent with the fact that ˙OH-mediated generation of purine 5′,8-cyclonucleosides is far less efficient than that of 8-oxodGuo in isolated DNA upon exposure to gamma rays in partially deaerated aqueous solutions (Table 2). The observed decrease, by about three orders of magnitude, of the radiation yields of (5′*R*)-cdAdo, 8-oxodGuo and 8-oxodAdo in cellular DNA with respect to isolated DNA is in agreement with the similar shielding effect exerted by cellular content on the base and sugar.[38,39]

4. Conclusions

Clarifications have been provided in this study on several key chemical reactions implicated in the formation of purine 5′,8-cyclo-2′-deoxyribonucleosides following initial ˙OH-mediated H atom abstraction from the C5′-hydroxymethyl group that may represent a significant biochemical event under oxidative stress conditions. This concerns the delineation of the role of molecular oxygen that, in contrast to previous claims, only partly prevents the intramolecular cyclization from occurring, suggesting that formation of both cdAdo and cdGuo may occur in aerobic cells. In addition, a detailed mechanism is available on the implication of molecular oxygen in the rearomatization of the radical intermediate arising from the latter reaction. Another major observation deals with the low efficiency of the intramolecular cyclization reaction in cellular DNA that is at least 2 orders of magnitude lower than that inferred[45,46] from the use of the questionable HPLC-MS method[49] for monitoring these tandem DNA lesions. It is clear that in light of these data there is a strong need for reconsidering several studies in which the biological role of purine 5′,8-cyclonucleosides was highlighted.

Acknowledgements

We thank Dr Didier Gasparutto for his contribution on the preparation of cdAdo and cdGuo containing oligonucleotides and Benoît Duroux for his help concerning the HPLC-MS/MS measurements. The work was supported by European Union Marie Curie Training and Mobility Project Number MRNT-CT2003 "CLUSTOXDNA". The support and sponsorship concerned by COST Action CM0603 on "Free Radicals in Chemical Biology (CHEMBIORADICAL)" are kindly acknowledged.

References

1 (*a*) C. J. Burrows and J. G. Muller, *Chem. Rev.*, 1998, **98**, 1109–1151; (*b*) J. Cadet, T. Douki, D. Gasparutto and J.-L. Ravanat, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 2003, **531**, 5–23.
2 J. Cadet, T. Douki and J.-L. Ravanat, in *The Chemical Biology of DNA Damage*, ed. N. E. Geacintov, and S. S. Broyde, Wiley-VCH Verlag, Veinheim, 2010, 53–80.
3 (*a*) H. C. Box, H. G Freund, E. E. Budzinski, J. C. Wallace and A. E. MacCubbin, *Radiat. Res.*, 1995, **141**, 91–94; (*b*) T. Douki, J. Rivière and J. Cadet, *Chem. Res. Toxicol.*, 2002, **15**, 445–454; (*c*) I. S. Hong, K. N. Carter, K. Sato and M. M. Greenberg, *J. Am. Chem. Soc.*, 2007, **129**, 4089–4098.
4 (*a*) P. Jaruga and M. Dizdaroglu, *DNA Repair*, 2008, **7**, 1413–1425; (*b*) Y. Wang, *Chem. Res. Toxicol.*, 2008, **21**, 276–281.
5 (*a*) Z. Keck, *Naturforsch.*, 1968, **23b**, 1034–1043; (*b*) N. Mariaggi, J. Cadet and R. Téoule, *Tetrahedron*, 1976, **32**, 2385–2387; (*c*) J. A. Raleigh, W. Kremers and R. Whitehouse, *Radiat. Res.*, 1976, **65**, 414–422; (*d*) M. Dizdaroglu, *Biochem. J.*, 1986, **238**, 247–254.
6 P. Jaruga, Y. Xian, B. C. Nelson and M. Dizdaroglu, *Biochem. Biophys. Res. Commun.*, 2009, **386**, 656–666.
7 F. Boussicault, P. Kaloudis, C. Caminal, Q. G. Mulazzani and C. Chatgilialoglu, *J. Am. Chem. Soc.*, 2008, **130**, 8377–8385.
8 (*a*) P. J. Brooks, D. S. Wise;, D. A. Berry;, J. V. Kosmoski;, M. J. Smerdon, R. L. Somers, H. Mackie, A. Y. Spoonde, E. J. Ackerman, K. Coleman, R. E. Tarone and J. H. Robbins, *J. Biol. Chem.*, 2000, **275**, 22355–22362; (*b*) I. Kuraoka, C. Bender, A. Romieu, J. Cadet, R. D. Wood and T. Lindahl, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 3832–3837.
9 (*a*) P. J. Brooks, *Neuroscience*, 2007, **145**, 1407–1417; (*b*) P. J. Brooks, *DNA Repair*, 2008, **7**, 1168–1179.
10 I. Kuraoka, P. Robins, C. Masutani, F. Hanaoka, D. Gapsarutto, J. Cadet, R. D. Woods and T. Lindahl, *J. Biol. Chem.*, 2001, **276**, 49283–49288.
11 G. Kirkali, N. C. de Souza-Pinto, P. Jaruga, V. A. Bohr and M. Dizdaroglu, M., *DNA Repair*, 2009, **8**, 274–278.
12 (*a*) M. Dizdaroglu, P. Jaruga and H. Rodriguez, *Free Radical Biol. Med.*, 2001, **30**, 774–784; (*b*) P. Jaruga, M. Birincioglu, H. Rodriguez and M. Dizdaroglu, *Biochemistry*, 2002, **41**, 3703–3711.
13 A. Romieu, D. Gasparutto and J. Cadet, *Chem. Res. Toxicol.*, 1999, **12**, 412–421.
14 A. D. Becke, *Phys. Rev. A: At., Mol., Opt. Phys.*, 1988, **38**, 3098–3100.
15 A. D. Becke, *J. Chem. Phys.*, 1996, **104**, 1040–1046.

16 Y. Zhao, B. J. Lynch and D. G. Truhlar, *J. Phys. Chem. A*, 2004, **108**, 2715–2719.

17 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, *Gaussian 03, Revision D.2*, Gaussian, Inc., Wallingford CT, 2004.

18 J. Tomasi, B. Menucci and E. Cances, *THEOCHEM*, 1999, **464**, 211–226.

19 J. W. T. Spink and R. J. Woods, in *An Introduction to Radiation Chemistry*, Wiley, New York, 3rd edn, 1990, p. 100.

20 (*a*) G. V. Buxton, C. L. Greenstock, C. L., W. P. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1988, **17**, 513–886; (*b*) Ross, W. G. Mallard, W. P. Helman, G. V. Buxton, R. E. Huie and P. Neta, *NDRL-NIST Solution Kinetic Database - Ver. 3*, Notre Dame Radiation Laboratory, Notre Dame, IN and NIST Standard Reference Data, Gaithersburg, MD, 1998.

21 B. H. T. Bielski, D. E. Cabelli and R. L. Arudi, *J. Phys. Chem. Ref. Data*, 1985, **14**, 1041–1100.

22 R. Battino, T. R. Rettich and T. Tominaga, *J. Phys. Chem. Ref. Data*, 1983, **12**, 163–178.

23 (*a*) R. Zander, *Z. Naturforsch.*, 1976, **31C**, 339–352; (*b*) R. Zander, *Adv. Exp. Med. Biol.*, 1976, **75**, 463–467.

24 J.-L. Ravanat, B. Duretz, A. Guiller, T. Douki and J. Cadet, *J. Chromatogr., B: Biomed. Sci. Appl.*, 1998, **715**, 349–356.

25 S. Frelon, T. Douki, J.-L. Ravanat, J.-P. Pouget, C. Tornabene and J. Cadet, *Chem. Res. Toxicol.*, 2000, **13**, 1002–1010.

26 C. Chatgilialoglu, M. Guerra and Q. G. Mulazzani, *J. Am. Chem. Soc.*, 2003, **125**, 3839–3848.

27 C. Chatgilialoglu, R. Bazzanini, L. B. Jimenez and M. A. Miranda, *Chem. Res. Toxicol.*, 2007, **20**, 1820–1824.

28 The energy barrier ($\Delta E^{\ddagger}$) and reaction energy (ΔE_r) for the addition of $O_2^{\bullet -}$ to aminyl radical **4** are computed to be 25.3 and 4.7 kcal mol^{-1}, respectively.

29 M. Guerra, R. Amorati and G. F. Peduli, *J. Org. Chem.*, 2004, **69**, 5460–5467.

30 S. W. Benson, in *Thermochemical Kinetics*, Wiley, New York, 2nd edn, 1976.

31 T. Douki, J.-L. Ravanat, J.-P. Pouget, I. Testard and J. Cadet, *Int. J. Radiat. Biol.*, 2006, **82**, 119–127.

32 P. Jaruga, J. Theruvathu, M. Dizdaroglu and P. J. Brooks P., *Nucleic Acids Res.*, 2004, **32**, e87.

33 J.-L. Ravanat, T. Douki, P. Duez, E. Gremaud, K. Herbert, T. Hofer, J. Lasserre, C. Saint-Pierre, A. Favier and J. Cadet, *Carcinogenesis*, 2002, **23**, 1911–1918.

34 P. Regulus, S. Spessotto, M. Gateau, J. Cadet, A. Favier and J.-L. Ravanat, *Rapid Commun. Mass Spectrom.*, 2004, **18**, 2223–2228.

35 J. M. Falcone and H. C. Box, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1997, **1337**, 267–275.

36 K. S. Lim, A. Jenner and B. Halliwell, *Nat. Protoc.*, 2006, **1**, 1995–2002.

37 H. Hong, H. Cao and Y. Wang, *Nucleic Acids Res.*, 2007, **35**, 7118–7127.

38 J. Cadet, T. Douki and J.-L. Ravanat, *Acc. Chem. Res.*, 2008, **41**, 1075–1083.

39 (*a*) P. C. Dedon, *Chem. Res. Toxicol.*, 2008, **21**, 206–219; (*b*) C. Chatgiglialoglu, in *Radical and Radical Ion Reactivity in Nucleic Acid Chemistry*, ed. M. M. Greenberg, Wiley, New Jersey, 2009, pp. 99-133.

40 (*a*) D. Sy, C. Savoye, M. Begusova, V. Michalik, M. Charlier and M. Spotheim-Maurizot, *Int. J. Radiat. Biol.*, 1997, **72**, 147–155; (*b*) W. K. Pogozelski and T. D. Tullius, *Chem. Rev.*, 1998, **98**, 1089–1108; (*c*) C. von Sonntag, in *Free-Radical-Induced DNA Damage and Its Repair: A Chemical Perspective*, Springer-Verlag, Berlin, 2006.

41 (*a*) A. Manetto, D. Georganakis, T. Gimisis, L. Leondiadis, P. Mayer, T. Carell and C. Chatgilialoglu, *J. Org. Chem.*, 2007, **72**, 3659–3666; (*b*) M. Russo, L. B. Jimenez, Q. G. Mulazzani, M. D'Angelantonio, M. Guerra, M. A Miranda and C. Chatgilialoglu, *Chem.–Eur. J.*, 2006, **12**, 7684–7693.

42 M.-L. Dirksen, W. F. Blakely, E. Holwitt and M. Dizdaroglu, *Int. J. Radiat. Biol.*, 1988, **54**, 195–204.

43 P. Jaruga, M. Birincioglu, H. Rodriguez and M. Dizdaroglu, *Biochemistry*, 2002, **41**, 3703–3711.

44 R. A. Egler, E. Fernandes, K. Rothermund, S. Sereika, N. de Souza-Pinto, P. Jaruga, M. Dizdaroglu and E. V. Prochownick, *Oncogene*, 2005, **24**, 8038–8050.

45 M. D'Errico, E. Parlanti, M. Teson, B. M. Bernardes de Jesus, P. Degan, A. Calcagnile, P. Jaruga, M. BjØrås, M. Crescenzi, A. M. Pedrini, J.-M. Egly, G. Zambruno, M. Stefanini, M. Dizdaroglu and E. Dogliotti, *EMBO J.*, 2006, **25**, 4305–4315.

46 M. D'Errico, E. Parlanti, M. Teson, P. Degan, T. Lemma, A. Calcagnile, I. Iavarone, P. Jaruga, M. Ropolo, A. M. Pedrini, D. Orioli, G. Frosina, G. Zambruno, M. Dizdaroglu, M. Stefanini and E. Dogliotti, *Oncogene*, 2007, **26**, 4336–4343.

47 K. Randerath, E. randerath, G.-D. Zhou and D. Li, *Mutat. Res., Fundam. Mol. Mech. Mutagen.*, 1999, **424**, 183–194.

48 K. Randerath, G.-D. Zhou, R. L. Somers, J. H. Robbins and P. J. Brooks, *J. Biol. Chem.*, 2001, **276**, 36051–36057.

49 (*a*) J. Cadet and H. Poulsen, *Free Radic. Biol. Med.*, 2010, **48**, 1457–1459; (*b*) J. Cadet, T. Douki and J.-L. Ravanat, *Free Radic. Biol. Med.*, 2010, **49**, 9–21.