3660–3669 *Nucleic Acids Research, 2006, Vol. 34, No. 13*
doi:10.1093/nar/gkl496

Formation of isodialuric acid lesion within DNA oligomers via one-electron oxidation of 5-hydroxyuracil: characterization, stability and excision repair

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Received April 13, 2006; Revised June 28, 2006; Accepted June 29, 2006

ABSTRACT

5-Hydroxyuracil is a major oxidized nucleobase that can be generated by the action of •OH radical and one-electron oxidants. The latter modified base that exhibits a low ionization potential is highly susceptible to further degradation upon exposure to various oxidants. Emphasis was placed in this work on the formation and characterization of one-electron oxidation products of 5-hydroxyuracil within DNA fragments of defined sequence. For this purpose, 5-hydroxyuracil containing single- and double-stranded oligonucleotides of various lengths were synthesized and then exposed to the oxidizing action of iridium salts. Isodialuric acid was found to be formed almost quantitatively by a one-electron oxidation mechanism for which relevant information was inferred from a freeze-quenched ESR study. Information on the stability of isodialuric acid thus formed and its conversion products in aqueous solutions was also gained from experiments performed at acidic, neutral and alkali pH's. Moreover, biochemical features dealing with the substrate specificity of several bacterial and yeast base excision repair enzymes to remove isodialuric acid from site-specifically modified DNA fragments were determined.

INTRODUCTION

Oxidatively generated damage to DNA bases by reactive oxygen species, which is either formed endogenously in cells during aerobic metabolism or following exposure of cells to chemical agents or radiations, can lead to cellular lethality, mutations and diseases such as cancer (1–4). Until now, ~100 oxidized bases and nucleosides have been isolated and characterized in numerous model studies (5,6). The most frequently investigated oxidized lesion is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a highly mutagenic nucleoside that is formed almost ubiquitously by one-electron oxidants and most of the reactive oxygen and nitrogen species (7–14). 8-OxodGuo, currently used as a biomarker of DNA oxidation, is highly susceptible to further reaction with one-electron oxidants agents and singlet oxygen. 8-OxodGuo-related oxidation products and their potential biological impact, in terms of mutagenicity and repairability, have been extensively studied over the last decade [for a recent review see (15) and references cited therein]. Pyrimidine-oxidation products also present a major interest and therefore have received a large attention in the recent years. Thus, cytosine is an efficient target for chemical oxidants such as $KMnO_4$, hydroxyl radical or type I photosensitizers that produce a wide set of base lesions. Among them, 5-hydroxycytosine and, to a lesser extent, 5-hydroxyuracil (5-ohUra) are abundant degradation products (5,6,16–19). The toxicity and mutagenicity of 5-hydroxy-2′-deoxyuridine (5-ohdUrd) are now well established. Indeed, mutations, mainly C>T transitions, are induced when the latter oxidized nucleoside is bypassed by DNA polymerases *in vitro* and *in vivo*, proving its high miscoding feature (20–23). On the other hand 5-ohdUrd is known to be an efficient substrate for several repair enzymes, namely DNA *N*-glycosylases, both in eukaryotic and prokaryotic organisms (12,24).

Similar to 8-oxodGuo, 5-ohdUrd exhibits a lower oxidation potential than its unmodified initial nucleoside precursor (6). This property suggests that oxidation of 5-ohdUrd may occur

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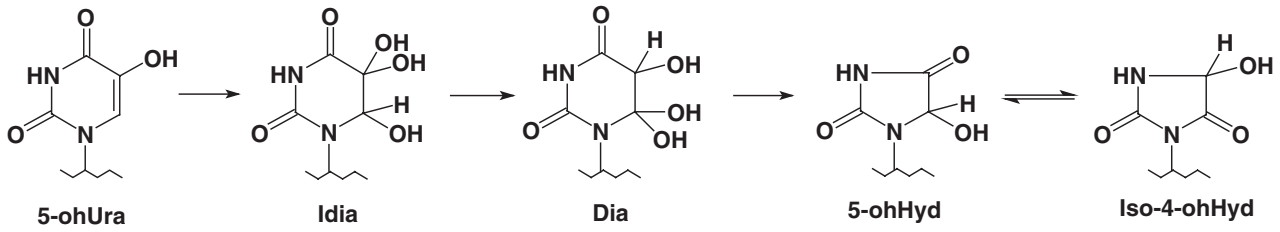


Scheme 1. Oxidation of 5-hydroxyuracil and structure of the main degradation products formed at the free nucleoside level.

in cellular DNA even if the expected frequency of the event is low. If this is the case, it is likely that the resulting degradation products may have biological effects. Recently, Wagner and co-workers (25) reported that oxidation of 5-ohdUrd by Br_2, Na_2IrBr_6 or type I photosensitizers exclusively generates diastereomers of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl) isodialuric acid (dIdial) as the primary degradation products. Subsequently, diastereoisomeric isodialuric acid nucleosides are converted into 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-dialuric acid (dDial), 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxyhydantoin (5-ohdHyd) diastereomers and 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-hydroxyimidazolidine-2,5-dione(iso-4-ohdHyd) diastereomers (25,26) (Scheme 1). Until now, there was a paucity of information on the formation of Idial and on biochemical features of this oxidatively generated base lesion within DNA. Thus, isodialuric acid base lesion has been detected at significant levels in O_sO_4-treated isolated DNA, γ-irradiated isolated DNA and H_2O_2-treated cells, by gas chromatography-mass spectrometry measurements (27–29). The same group has reported that two *Escherichia coli* enzymes, Nth and Ung, as well as human Ung are able to recognize and excise isodialuric acid from oxidized isolated DNA (29–31). Recently, Lindahl and co-workers (32) provided some evidence on the toxicity of radiation-induced dIdial in the DNA of mammalian cells deficient in Smug and Ung proteins.

In this article, we report the results of a study dealing with the one-electron oxidation-mediated conversion of 5-ohdUrd residues into dIdial within synthetic single- and double-stranded oligonucleotides. The conversion of dIdial into several degradation products was also investigated in order to further establish the chemistry of the successive steps together with the stability of the resulting modified nucleosides formed within DNA strands. Pulse ESR experiments were performed using the freeze-quench approach in order to gain further insights into the mechanisms of 5-ohdUrd oxidation. Finally, information is also provided on the abilities of several prokaryotic and eukaryotic DNA *N*-glycosylases, involved in the base excision repair (BER) pathway, to remove isodialuric acid from site-specifically modified oligonucleotides.

MATERIALS AND METHODS

General procedures and materials

Na_2IrCl_6, β-mercaptoethanol and piperidine were obtained from Aldrich (Saint Quentin Fallavier, France). Buffers for high performance liquid chromatography (HPLC) were prepared using water purified with a Milli-Q system (Millipore, Milford, MA). Modified oligonucleotides were analyzed on a Hypersil (Interchim, Montluçon, France) ODS column (5 μm − 250 × 4.6 mm i.d.) with a gradient of acetonitrile (from 0 to 12% in 45 min) in 10 mM triethylammonium acetate (pH 7) at a flow rate of 1 ml/min. Detection at the output of the HPLC column was provided by UV absorbance at 260 nm. Oligonucleotides **1**–**4** were synthesized by standard phosphoramidite chemistry using highly labile phenoxyacetyl protective groups (33) on an Applied Biosystems Inc. 392 DNA synthesizer. The phosphoramidite monomer of 5-ohdUrd was purchased from GlenResearch (Sterling, VA). Modified oligonucleotides were deprotected by treatment in a concentrated 32% NH_4OH solution for 4 h at room temperature. β-Mercaptoethanol (0.25 M) was added to prevent further oxidation of the modified base during the latter deprotection step. After NH_4OH deprotection, the crude 5′-tritylated oligomers were purified and detritylated online by reverse phase HPLC (RP-HPLC) following a method reported previously (34). The integrity and purity of modified oligodeoxynucleotides were verified by HPLC together with MALDI-TOF and electrospray ionization mass spectrometry (ESI-MS) analyses.

Enzymes

Fpg, endo III, yNtg1, yNtg2 and yOgg1 were kindly provided by Dr Serge Boiteux (CEA/Fontenay-aux-Roses, France). Endo VIII and ecUng proteins were obtained from Trevigen (Gaithersburg, MD). Nuclease P_1 (*penicillium citrium*) and acid phosphatase (*white potatoes*) were purchased from Sigma (St Louis, MO). T_4 polynucleotide kinase was obtained from Pharmacia Biotech (Uppsala, Sweden).

Mass spectrometry analyses

MALDI mass spectra were obtained on a time-of-flight Biflex mass spectrometer (Bruker, Wissembourg, France) equipped with a 337 nm nitrogen laser and pulsed delay source extraction. The matrix was prepared by dissolving 3-hydroxypicolinic acid in 10 mM ammonium citrate buffer and a small amount of Dowex-50W 50 × 8-200 cation exchange resin (Sigma). Sample (1 μl) was added to matrix (1 μl) and the resulting solution was made homogeneous by stirring. The resulting sample was placed on the target plate and allowed to dry. Spectra were calibrated using reference oligonucleotides of known masses.

ESI-MS measurements were performed on a Platform 3000 model spectrophotometer (Micromass, Manchester, UK). Typically, oligonucleotides were dissolved in a solution

of acetonitrile and water (50:50, v/v) containing 1% triethylamine prior to be analyzed in the negative mode.

Oxidation and characterization of 5-ohdUrd-containing oligonucleotides

Typically, 0.5 AU_{260nm} of 5-ohdUrd-containing oligonucleotides were treated with 30 µl of an aqueous solution of 10 mM Na_2IrCl_6 for 5 min at room temperature. Oxidized oligonucleotides were purified by HPLC, as described above. Oxidation of 5-ohdUrd in duplex DNA was performed by incubating 50 pmol of oligonucleotide **4** with 100 pmol of the complementary strand in 10 µl of a 50 mM phosphate buffer, 100 mM KCl, pH 7. After heating for 5 min at 90°C, the solution was slowly cooled to 20°C and then 10 µl of a solution of 10 mM Na_2IrCl_6 was added. After 5 min at room temperature, Na_2IrCl_6 was removed by purification of the oligonucleotide on a MicroSpin™ G-25 exclusion column (Pharmacia, Uppsala, Sweden).

Labeling of modified oligonucleotides

Oligonucleotides (20 pmol) were labeled at the 5′-terminus with 10 µCi of [γ-^{32}P]ATP (2 pmol and 10 mCi/ml) Amersham (Buckinghamshire, UK) using T_4 polynucleotide kinase (3 U) in 10 µl of supplied buffer at 37°C for 30 min. Unincorporated [γ-^{32}P]ATP was removed by purification of the oligonucleotide on a MicroSpin™ G-25 column.

Piperidine treatment of lesion-containing oligodeoxyribonucleotides

Oligonucleotides were treated with a freshly made aqueous piperidine solution (1 M) at 90°C for 15 or 30 min. The reactions were carried out on 5 pmol of 5′-^{32}P-labeled modified oligodeoxyribonucleotides in 100 µl of piperidine solution in sealed tubes. After cooling, the samples were co-evaporated twice with water, dissolved in formamide loading buffer and then loaded onto a 20% polyacrylamide denaturing gel. Electrophoresis was carried out at 1300 V for 3 h and, subsequently, analysis of radiolabeled bands was achieved by phosphorimagery (Personnal FX phosphorimager from Bio-Rad).

Digestion of modified ODNs by nuclease P1 and acid phosphatase

Oxidized oligonucleotide **4** (1 AU_{260nm}), prepared and purified by MicroSpin™ exclusion column as described previously, was placed in 35 µl water and 15 µl acetate buffer (300 mM sodium acetate, pH 5.3, and 1 mM $ZnSO_4$). Nuclease P1 (3 U) and acid phosphatase (1 U) were added before incubation of the resulting mixture at 37°C for 2 h. The enzymatic hydrolysate was then centrifuged before analysis by RP-HPLC on a highly hydrophobic Hypercarb (Thermo-Hypersil, Les Ulis, France) porous carbon column (5 µm – 100 × 3 mm i.d.). Separation was achieved using a linear gradient of acetonitrile (from 0 to 30% over 45 min) in water, at a flow rate of 0.5 ml/min. The released nucleosides were detected by monitoring absorption at 230 nm.

Excision of isodialuric acid lesion from double-stranded oligonucleotides by repair enzymes

The modified ODN **4** (20 pmol) was 5′ end labeled using [γ-^{32}P]ATP and then purified using MicroSpin™ G-25 columns. The complementary DNA strand **5** (50 pmol) was added in order to generate a double-stranded DNA fragment containing the 5-ohUra lesion. Hybridization was performed in 30 µl of 50 mM phosphate buffer, pH 7, and 100 mM KCl. The oligonucleotide mixture was then heated for 5 min at 90°C before slow cooling to 4°C. DNA oxidation was carried out by adding 10 µl of a solution of Na_2IrCl_6 (10 mM) and incubated for 5 min at room temperature. After purification on a MicroSpin™ G-25 column, the oxidized solution (30 µl) was aliquoted (3 µl per reaction). Of a 2× buffer solution 5 µl (40 mM Tris–HCl and 200 mM KCl, pH 7.5) and one of the following enzymes (Fpg, 250 ng; endo III, 300 ng; endo VIII, 3 U; yNtg1, 300 ng; yNtg2, 350 ng or yOgg1, 800 ng) were added to each aliquot (10 µl final). The resulting solutions were incubated at 37°C for 15 min and the reactions were stopped by addition of formamide (10 µl). Samples were electrophoresed on a 20% polyacrylamide–7 M urea gel at 1300 V for 30 min in TBE buffer (50 mM Tris–HCl, 50 mM boric acid and 5 mM EDTA, pH 8). The reaction products were visualized by phosphorimagery.

RPE analyses of 5-ohdUrd one-electron oxidation

An Update Instrument System 715 freeze-quench apparatus was used to prepare samples for EPR spectroscopy measurements. The solution concentrations before mixing were 5 mM for both the iridium salt and the 5-ohdUrd sample. The 5-ohdUrd and iridium solutions were transferred to 2 ml syringes and pushed in a 1:1 ratio through the mixer and reactor (ageing) hose before spraying into a funnel containing 2-methylbutane immersed in the quench bath maintained at −130°C. The 'dead time' of the instrument was 15 ms.

EPR measurements were performed on a Bruker EMX X-band continuous wave spectrometer with a Bruker ER 4116 DM rectangular cavity and an Oxford Instrument ESR 900 continuous flow He cryostat.

RESULTS AND DISCUSSION

Synthesis and characterization of isodialuric acid containing oligonucleotides

Oxidation of 5-ohdUrd in single-stranded oligonucleotides. Oxidation of 5-ohdUrd in single-stranded DNA (ssDNA) fragments (Table 1) was performed using Na_2IrCl_6, a one-electron oxidizing agent which does not react with normal

Table 1. Oligonucleotide sequences used in the current study (the corresponding lengths and masses are mentioned)

Name	Sequences (5′–3′)	Length	Mass calculated	Mass found
1	T(**5-ohUra**)T	3	852.6	852.1
2	AC(**5-ohUra**)TG	5	1479.0	1478.8
3	ATC T(**5-ohUra**)T AGT	9	2705.8	2705.5
4	CTC CTC T(**5-ohUra**)T CAC TTC	15	4407.0	406.9
5	GAA GTG AGA GAG GAG	15	4754.2	4753.1

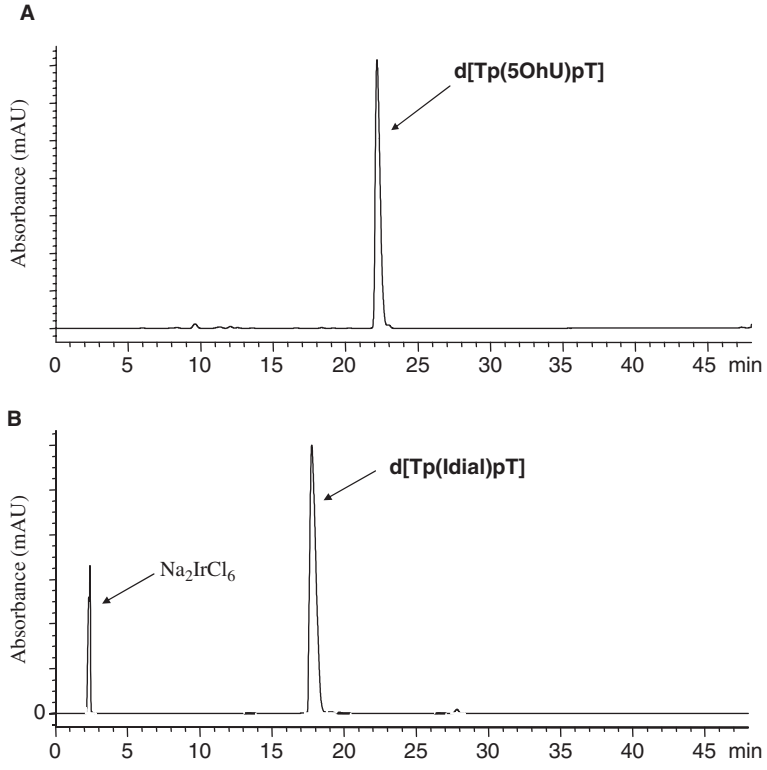


Figure 1. RP-HPLC chromatogram (UV detection at 260 nm) of trimer **1** (**A**) and after oxidation for 5 min at room temperature, in 10 mM Na_2IrCl_6 aqueous solution (**B**).

DNA bases, since its redox potential is 0.9 V (35). The RP-HPLC elution profile of the reaction mixture arising from the oxidation of trinucleotide **1** by Na_2IrCl_6 shows a quantitative conversion of the starting material, within a few minutes, giving rise to a new product eluting as a single peak (Figure 1). The latter peak was collected and its content analyzed by MALDI-TOF mass spectrometry measurements. The pseudo-molecular ion of the related modified oligonucleotide exhibits an *m*/*z* ratio 34 amu higher than that of the starting oligomer **1**, as determined by MALDI-MS (Figure 2A). The overall data are in agreement with the formation of isodialuric acid base due to selective one-electron oxidation of the 5-ohUra residue within the oligonucleotide, following the mechanism reported in Scheme 2. Similar results were obtained using ESI-MS analyses of the latter oxidized oligonucleotide (data not shown). Moreover, the fragmentation pattern in the MS/MS analysis mode is indicative of an initial 18 amu mass loss from the pseudo-molecular ion, most probably as the result of a dehydration process. Indeed, isodialuric acid was shown in a previous work to be susceptible to dehydration under MS/MS analysis conditions (25). The oxidation of **1** is quantitative and selective as shown by the absence of starting material or any byproducts after 1 h reaction. It is important to mention here that the presence of buffer (typically a 50 mM phosphate buffer, pH 7, was used) does not significantly influence the efficiency and the nature of the products formed during the present oxidation reaction, in contrast to iridium-mediated oxidation of 8-oxo-7,8-dihydroguanine. In the same way, the pH dependence of the oxidation step was investigated by performing the reaction at three different pH values (pH 6, 7 and 8, respectively). The results obtained clearly show that the pH has no effect on the nature of the products formed, the isodialuric acid lesion being generated as the sole product. The only significant change observed was a higher rate of degradation of the isodialuric acid lesion at pH 8 by comparison with acidic and neutral pHs (*vide infra*). Similarly, oxidation of the 5-ohUra residue within ssDNA fragments **2**, **3** and **4** leads selectively to related isodialuric acid-containing oligonucleotides, as detected by RP-HPLC analyses (data not shown). However a second product with a lower molecular weight by 18 mass units with respect to the previous one is detected by MALDI-TOF mass measurement (Figure 2B–D). The possibility that the second product was generated through a dehydration event was investigated by nuclease P1 and acid phosphatase treatment of oligonucleotide **4** oxidation mixture, in which both hydrated and dehydrated products are present in equal amounts. The resulting HPLC chromatogram shows four peaks corresponding to 2′-deoxycytidine, 2′-deoxyadenosine, thymidine and a product with $m/z = 581$ that consists of a non-digested thymine-hydrated isodialuric acid dinucleoside monophosphate (Supplementary Data). No thymine-dehydrated isodialuric acid dinucleoside monophosphate was detected at the dinucleotide level, thus confirming the predominance of the hydrated form of the base lesion within short DNA oligomers and the presence of the dehydrated species when the length of the DNA fragment increases. The two oxidation products are likely to be in a hydratation–dehydratation equilibrium which is displaced to the hydrated form at the nucleoside level and in short single-stranded oligonucleotides (Scheme 2). On the other hand, the percentage of the dehydrated form rises with the

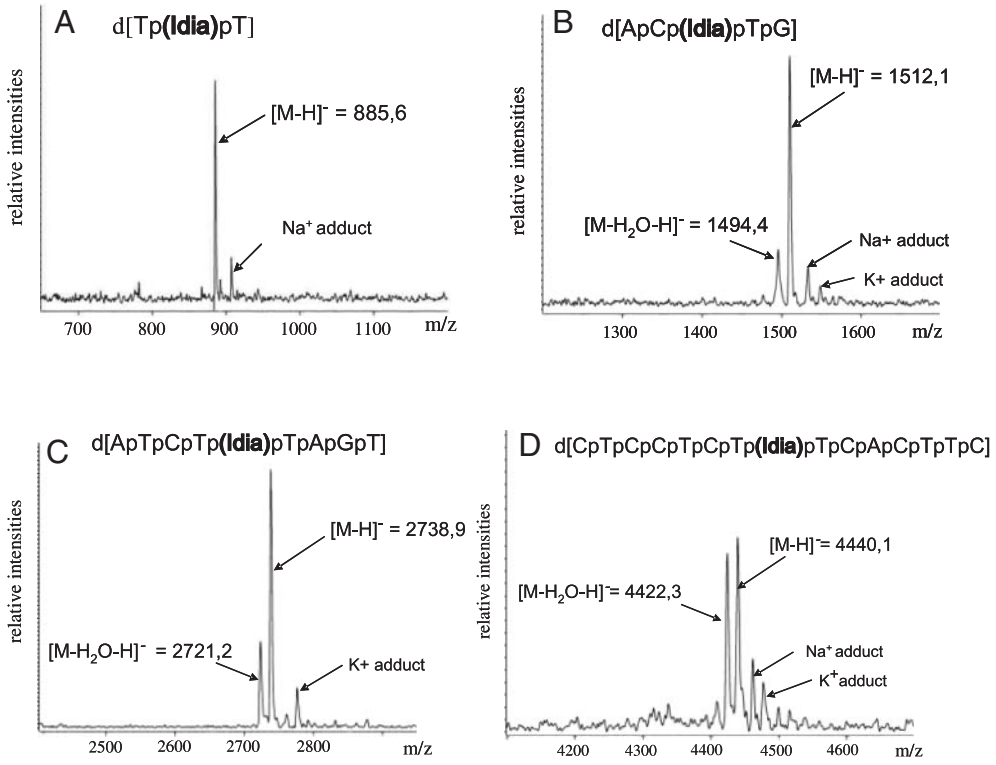
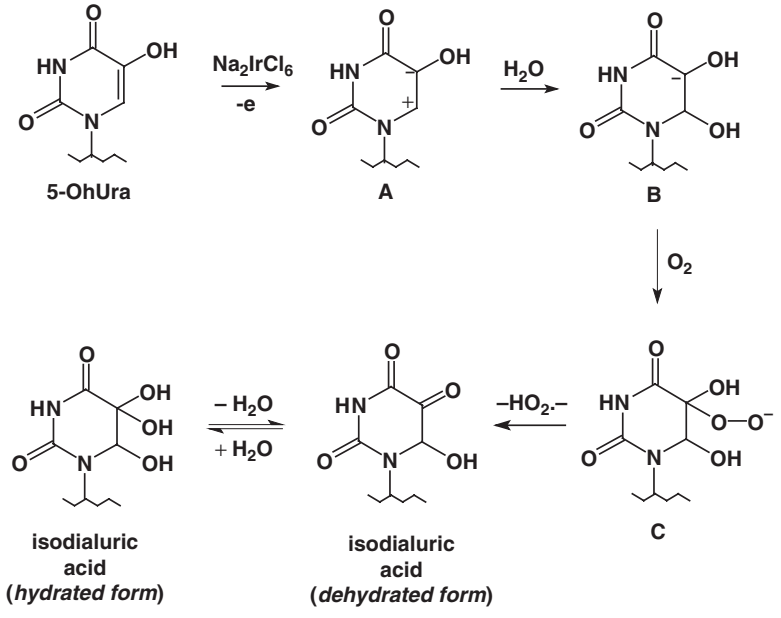


Figure 2. MALDI-TOF mass spectra (negative mode) of oligonucleotides **1** (**A**), **2** (**B**), **3** (**C**) and **4** (**D**) after selective Ir(IV)-mediated one-electron oxidation of 5-ohUra residue.



Scheme 2. Proposed mechanism of one-electron oxidation of 5-ohUracil within DNA fragments.

length of oligonucleotide. This oligonucleotide size dependence (Figure 2) may be explained by enhanced stability of the dehydrated form in longer oligonucleotides. This equilibrium has been suggested previously in the literature (30). It is interesting to note that under our current digestion conditions, the oligonucleotide was not cleaved at the isodialuric acid site by nuclease P1, reflecting significant structural changes within the DNA molecule. Indeed, the resistance of Idial nucleoside to enzymatic digestion may be the result of structural modifications at the damaged site together with the possible sensitivity of nuclease P1 to some alterated DNA bases.

Oxidation of 5-ohdUrd in a double-stranded oligonucleotide. To study the oxidation of 5-ohdUrd in a double-stranded

structure, the reaction was performed after hybridization of oligonucleotide **4** with the complementary DNA strand **5**. As shown in Figure 3, the 5-ohdUrd residue of the targeted double-stranded oligonucleotide was quantitatively converted into an overoxidized nucleoside. The modified oligomer was found to exhibit a molecular weight of 4423 amu, i.e. 16 mass units higher than that of the starting DNA fragment, as inferred from MALDI-TOF mass spectrometry analysis. This may be rationalized in terms of a structure corresponding to dehydrated isodialuric acid for the oxidation product of 5-ohdUrd. This strongly suggests that the latter form of dIdial is much more stable than the hydrated one in a DNA double-helix structure. As it was observed for single-stranded oligonucleotides **2**, **3** and **4**, a quantitative oxidation of 5-ohdUrd into dIdial without the formation of any detectable side-products was found to take place within the DNA duplex. This clearly shows that the reactivity of 5-hydroxypyrimidine bases toward one-electron oxidants and the specificity of the oxidation reaction are similar, irrespective of the single- or double-stranded nature of the DNA fragment. This also indicates that isodialuric acid may be formed in isolated double-stranded DNA as a secondary oxidation product of cytosine, suggesting that it could also be generated in cellular DNA. These results underline the interest for assessing biological features and the DNA repairability of the isodialuric acid lesion.

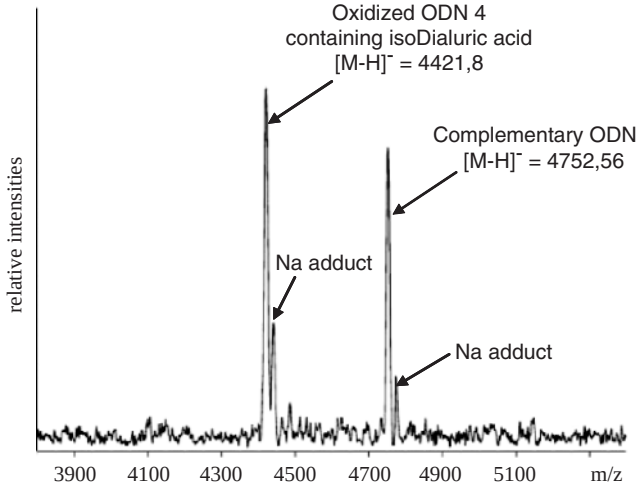


Figure 3. MALDI-TOF mass spectra (negative mode) of duplex **4/5** after one-electron oxidation by Na_2IrCl_6.

Stability of dIdial-containing oligonucleotides

Post-synthetic preparation of isodialuric acid containing oligonucleotides has allowed the determination of the stability of the dIdial residue in 50 mM phosphate buffer at three pH values. This was achieved by RP-HPLC analysis of the solution of dIdial-containing trimer oligonucleotide **1** at increasing periods of time. Conversion of dIdial with time at pH 7 and 25°C is shown in Figure 4. Degradation of dIdial (**a**) leads to two products (**b**) and (**c**) after 10 h of incubation. The ESI-MS analysis of both products shows a pseudo-molecular ion at *m*/*z* of 885. By homology with a recent study performed on free dDial (25), compounds **b** and **c** could be identified as DNA trimers that contain the two diastereomers of dialuric acid (dDial). After a 15 h period, dDial evolves into three other products. MS analysis of two

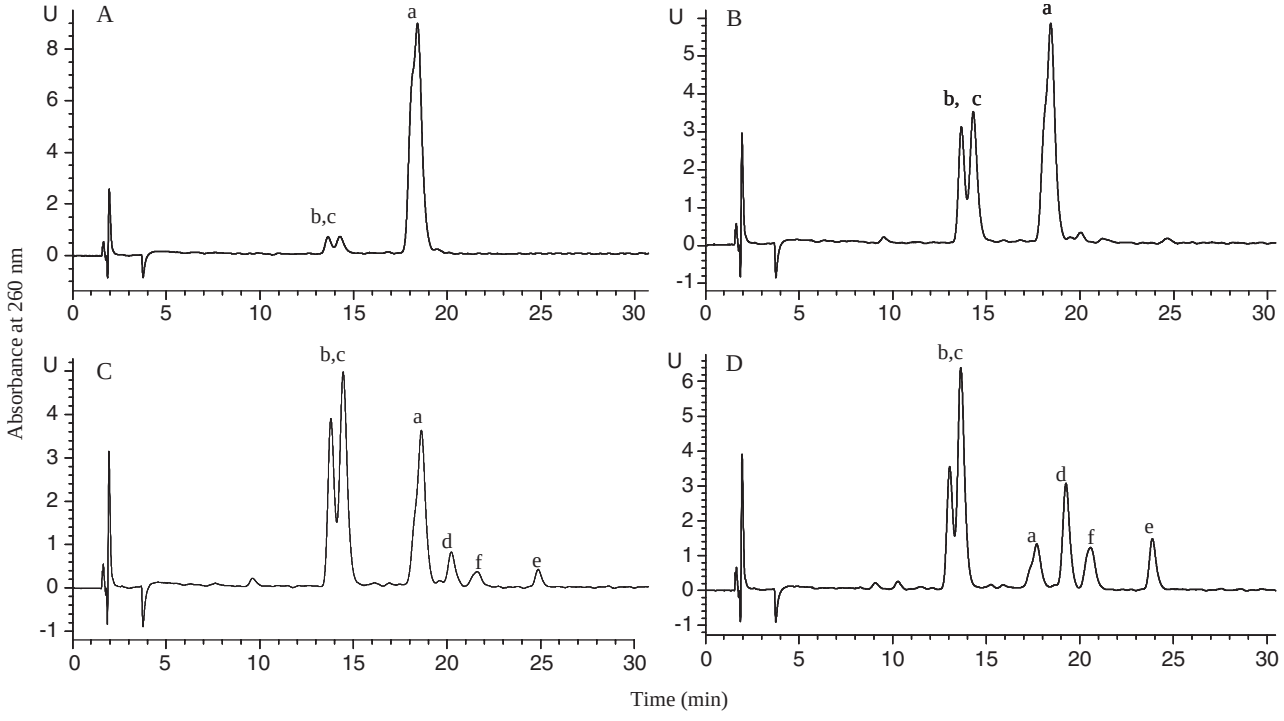


Figure 4. RP-HPLC analyses (UV detection at 260 nm) of oxidized oligonucleotide **1** after incubation in 50 mM phosphate buffer, pH 7, for 1 h (**A**), 10 h (**B**), 15 h (**C**) and 30 h (**D**). (a) Isodialuric acid-containing trimer; (b and c) dialuric acid-containing trimers (two isomers); (d and e) 5-ohHyd- and iso-4-ohHyd-containing trimers.

of them, namely (**d**) and (**e**), show pseudo-molecular ion at $m/z = 839$ indicating that they are probably the two diastereomers of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxyhydantoin (5-ohdHyd) (25). It should be noticed that the structure of hydantoin diastereomeric residues needs to be further investigated. Indeed, a recent report (26) indicates that dIdial can lead not only to the hydantoins but also to 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-4-hydroxyimidazolidine-2,5-dione (iso-4-ohdHyd), resulting from an α-hydroxyketone rearrangement. In the present case, compound **d** could tentatively be assigned to 5-ohdHyd diastereomers and peak **e** would contain the likely iso-4-ohdHyd compounds.

The last product formed, namely (**f**), was collected and then assigned as urea by ESI-MS measurement ($m/z = 783$). 783). The presence of urea byproducts could be explained by the formation of iso-4-ohdHyd diastereomeric intermediates that undergo opening of the five-membered ring with subsequent hydrolysis of the ureid thus formed.

Degradation studies of dIdial at pH 7 and 25°C, have allowed determination of the half-life of the modified base, estimated at 10 h in a ssDNA fragment (Figure 4B). The relativity fair stability of dIdial at neutral pH, that is expected to be significantly increased in the double-stranded DNA molecule, would suggest that this oxidized cytosine base would be a target for different biochemical processes such as DNA replication and transcription that are expected to give rise to potential mutagenic effects. Stability studies of Idial base within DNA oligomers show very low degradation in acidic buffer (pH 6) and a high instability in basic aqueous solutions (pH 8). Thus, 19% of dIdial was found to be decomposed after a 2 h period under the latter alkali conditions. The nature of the byproducts thus formed and the pH susceptibility are in agreement with the recently reported results on the free nucleoside (25,26). Information is also provided on the alkali-lability of dIdial-containing oligonucleotide as inferred from the results of a hot piperidine cleavage assay (Figure 5). In this experiment, total strand cleavage was shown to occur within 30 min at the Idial site (Figure 5, lane 6). This has to be compared with the 60% cleavage observed at the 5-ohUra site under the same pH and time conditions (Figure 5, lane 3). Then, the latter study clearly shows the higher alkali-lability of the Idial nucleoside than its parent 5-ohdUrd nucleoside within DNA.

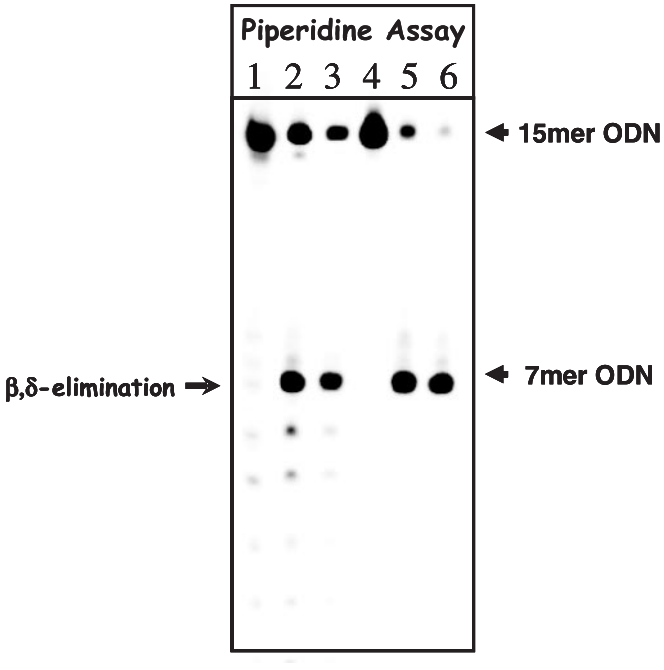


Figure 5. Incubation of 5-ohUra containing oligonucleotide **4** (lanes 1–3) and Idial containing ssDNA pentadecamer (lanes 4–6) with 1 M piperidine for 0 min (lanes 1 and 4), 15 min (lanes 2 and 5) and 30 min (lanes 3 and 6) at 90°C.

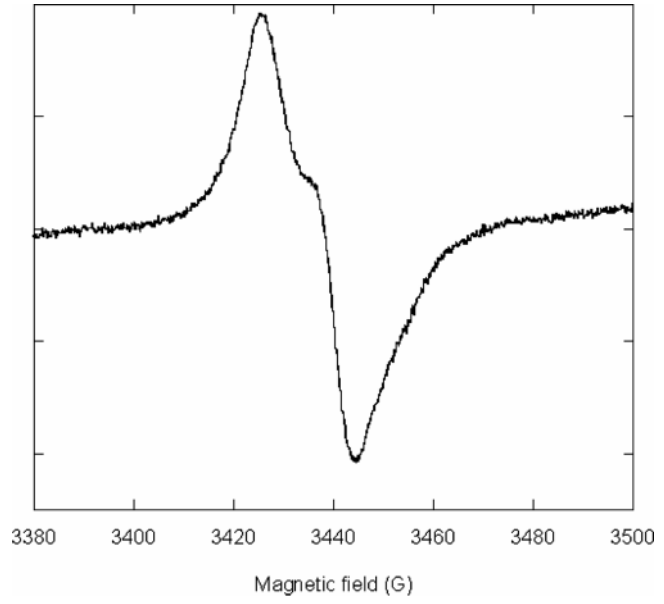


Figure 6. The g ≈ 2 region EPR spectrum of a Na_2IrCl_6/5oh-dUrd freeze-quenched reaction mixture. Experimental parameters: temperature, 40 K; modulation amplitude, 5G; frequency, 9.654 GHz; hyperfrequency power, 4 mW; conversion time, 40 ms; 200 accumulations.

RPE analyses of one-electron oxidation of free 5-ohdUrd and proposed mechanism

As a first remark it may be pointed out that freeze-quenched samples showed a brown color reminiscent of the color of Na_2IrCl_6 salt. Among the two different types of EPR signals, one was attributed to the Na_2IrCl_6 salt (data not shown) by comparison with the EPR spectrum of a freeze-quenched sample containing only the iridium salt. The second EPR signal was observed at g_av = 2.0063 and exhibited a narrow (~20 G peak to peak) spectrum characteristic of free radicals (Figure 6). Albeit not well resolved, the shape of the signal was dominated by the presence of a strong hyperfine coupling ($a = 20$ G) with an $I = 1/2$ nucleus and by a low g tensor anisotropy. Upon thermal annealing at 200 K for 1 min, the brown coloration of the sample disappeared and the EPR signals could no longer be detected. We conclude from these observations that the previously quenched reactions had now run to completion. EPR results are strongly indicative of the existence of at least one short-lived organic radical formed during the oxidation of 5-ohdUrd by the iridium salt. However, the chemical nature of this intermediate could not be determined unambiguously. The g_av value of 2.006 and the low g tensor anisotropy precluded the attribution of the spectrum to either an alkoxy or a peroxy radical such as **C**

(Scheme 2), since the latter species are known to possess a g tensor principal value higher or equal to 2.03 (36–38). Carbon centred radicals that exhibit typical lower g_av value and g tensor anisotropy would be better candidates. By comparison with previously studied nucleic acid components, namely thymine and isoorotic acid, a radical cation such as **A** would exhibit an important N1 nitrogen hyperfine coupling constant and only a very weak hydrogen hyperfine coupling constant (H6) which in fact are not observed (39,40). Radical **B** is a better candidate since it should exhibit a strong proton hyperfine coupling. For example, Close *et al*. (41,42) determined β proton hyperfine couplings in a radical similar to **B**, derived from a thymine derivative, to be 38 G by ENDOR experiments. Unfortunately, these constants are strongly dependent on the chemical nature and local geometry of the radical centre; therefore, it was not possible to determine the exact nature of the radical intermediate. The information inferred from the EPR measurements thus provides further support for the proposed one-electron oxidation mechanism of 5-ohdUrd (Scheme 2).

DNA repair studies of isodialuric acid containing oligonucleotides

The possibility to specifically generate a dIdial-containing double-stranded oligonucleotide **4/5**, that shows enough stability, has allowed us to study the ability of several DNA repair enzymes to excise this pyrimidine lesion. It is now well documented that BER is the major DNA repair mechanism involved in the removal of structurally non-disturbing and non-bulky lesions such as oxidized bases (12,24,43). BER is a multi-enzymatic pathway initiated by specific DNA *N*-glycosylases that are able to remove the alterated base by cleaving the N-glycosidic bond. The hydrolytic cleavage by monofunctional DNA glycosylases leads to the release of the damaged base and the formation of an AP-site, subsequently converted to a single-strand break by an AP-endonuclease. Bifunctional DNA N-glycosylases present an associated lyase activity that catalyzes the cleavage of the DNA phosphodiester backbone via β– or β, δ–elimination eactions. Only a few DNA *N*-glycosylases are specific for a dedicated substrate, whereas others are able to recognize several different types of damaged nucleobases with a considerable degree of overlap (24,43).

In the current study, the major DNA *N*-glycosylases involved in the removal of oxidatively generated DNA base damage (*E.coli* endo III, endo VIII and Fpg together with *Saccharomyces cerevisiae* Ntg1, Ntg2 and Ogg1 enzymes) were selected for the excision repair experiments. A short reaction time (15 min) was used to avoid potential degradation of isodialuric acid within the oxidized duplex **4/5** which would complicate data interpretation. We have shown that under the experimental conditions applied, dIdial was the predominant lesion within the DNA duplex probe since <5% side products, mainly dialuric acid derivatives, were detected (data not shown). As illustrated on the PAGE analysis depicted in Figure 7, isodialuric acid is a substrate for both endo III and endo VIII proteins (lanes 3 and 4, respectively), two DNA *N*-glycosylases that show high excision activities for several oxidized pyrimidine bases. These data are in agreement with a previous result obtained from

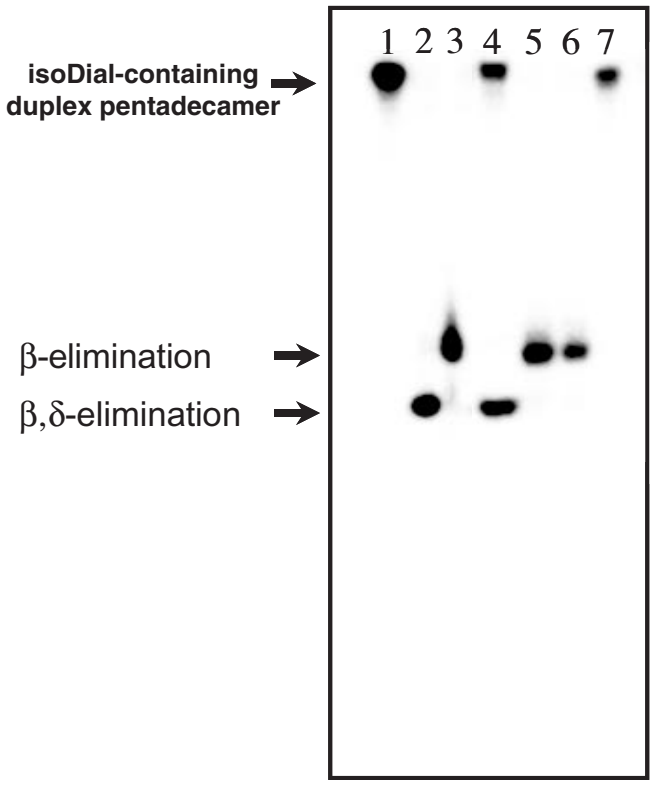


Figure 7. Denaturating PAGE analysis of Ir(IV)-oxidized duplex **4/5** incubated with Fpg (lane 2), endo III (lane 3), endo VIII (lane 4), yNtg1 (lane 5), yNtg2 (lane 6) and yOgg1 (lane 7) proteins. Lane 1 shows the control, without repair enzyme.

repair studies using oxidized isolated DNA (29). Further information on the capacity of other prokaryote DNA N-glycosylases to cope with the excision of isodialuric acid was gained from additional investigations. Thus it was shown that Fpg is able to remove isodialuric acid from **4/5** (Figure 7, lane 2). This original result confirms recent experiments which have shown clearly that the Fpg protein has a wider specificity than initially proposed, being able to efficiently remove several ring-saturated, ring-contracted and ring-fragmented pyrimidine and purine bases (44–50). Insights into the mechanisms of AP-lyase activity of the enzymes tested were gained from PAGE patterns concerning the migration of enzymatically cleaved oligonucleotides. Further confirmation of the implication of a β, δ–elimination mechanism in the repair mode of Fpg and endo VIII proteins was thus obtained. It may be reminded that cleavage products resulting from the latter pathway show a faster migration than those generated by endo III. Moreover, incubation of **4/5** with *S.cerevisiae* Ntg1 and Ntg2 proteins showed that isodialuric acid was efficiently removed by both eukaryote enzymes following a β-elimination mechanism (Figure 7, lanes 5 and 6). This was also the case with the monofunctionnal *E.coli* Ung repair enzyme which is able to excise the isodialuric acid base from single- and double-stranded DNA oligomers. This gives rise to an abasic site that may be cleaved by endonuclease IV (data not shown). The latter activity is in agreement with previous findings reported in the literature (30). It was also found in a recent study by Lindahl and co-workers (32) that in mammalians cells, Ung

and Smug are able to recognize and excise isodialuric acid. In contrast, as shown in Figure 7 (lane 7), isodialuric acid is not a substrate for yOgg1 protein. This is in conformity with the much narrower substrate specificity of the yeast analog of bacterial Fpg protein (51). Moreover, it is widely assumed that the excision activity of yOgg1 and hOgg1 proteins is highly dependent on the presence of a cytosine opposite the lesion (52). In the present experiment, the isodialuric acid lesion, an oxidized cytosine residue, was paired to a guanine residue, a situation which is not favorable to Ogg1 activity.

Finally, the presence of several DNA *N*-glycosylases capable of repairing the isodialuric acid lesion within prokaryotic and eucaryotic organisms highlights the biological significance and the possible impact of this type of base damage within cells. Therefore, it would be of interest in forthcoming repair studies to assess the capability of *E.coli*, yeast and mammalian total cell extracts to eliminate isodialuric acid and to determine which BER proteins may have relevant activity in cells. Attempts should be made to check for the possible existence of backup enzymes for the repair of Idial.

CONCLUSION AND PERSPECTIVES

In this work, evidence is provided that the one-electron oxidation of 5-ohdUrd-containing oligonucleotides by iridium salts induces the formation of dIdia nucleoside with a high yield within both single- and double-stranded DNA fragments. Strikingly, the 5-ohUra lesion was found to be highly susceptible to overoxidation within DNA, confirming previous findings observed with nucleosides. Then, the specific oxidation of 5-ohUra was used to prepare, for the first time, a set of site-specific modified oligonucleotides containing the isodialuric acid base lesion. Indeed, stability studies of dIdia within the synthetic DNA oligomers show that this oxidatively generated base damage has a lifetime compatible with several biological processes that take place in cells, such as DNA replication, transcription and repair. The data thus obtained allow us to suggest that this lesion could be present *in vivo* and may have potential deleterious effects for cells. A search for the formation of dIdia lesions in cellular DNA is currently under investigation using the highly accurate ESI-MS technique coupled to HPLC (53). Further information should also be gained on biological features of Idia base damage in terms of mutagenic, toxic and lethal potential during cellular replication. One possibility to assess the mutagenic features of dIdia would be to use site-specifically modified oligonucleotides as specific probes to extend mutagenesis studies using host cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The contributions of Dr Serge Boiteux (CEA Fontenay-aux-Roses, France) by providing DNA repair enzymes and Colette Lebrun for ESI mass spectrometry measurements are gratefully acknowledged. We thank Prof. J. R. Wagner, University of Sherbrooke for fruitful discussions. The partial financial support from CLUSTOXDNA EU Research Training Network is also acknowledged. Funding to pay the Open Access publication charges for this article was provided by the Commissariat à l'Energie Atomique.

Conflict of interest statement. None declared.

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