Cross-Linked Thymine-Purine Base Tandem Lesions: Synthesis, Characterization, and Measurement in γ-Irradiated Isolated DNA

Sophie Bellon, Jean-Luc Ravanat, Didier Gasparutto, and Jean Cadet*

Laboratoire des Lésions des Acides Nucléiques, Service de Chimie Inorganique et Biologique, UMR 5046, Département de Recherche Fondamentale sur la Matière Condensée, CEA-Grenoble, F-38054 Grenoble Cedex 9 (France)

Received December 20, 2001

5-(Phenylthiomethyl)-2′-deoxyuridine has been recently shown to be a specific photolabile precursor of 5-(2′-deoxyuridilyl)methyl radical that is involved in the formation of tandem base lesion with vicinal guanine in oxygen-free aqueous solution. The thionucleoside was incorporated by either liquid or solid-phase phosphoramidite synthesis into dinucleoside monophosphates with a 2′-deoxyadenosine residue as the vicinal nucleoside located either at the 3′ or 5′-extremity. UV-C irradiation of the modified dinucleoside monophosphate under anaerobic conditions gives rise to cross-linked thymine^{CH2-C8}adenine tandem base lesions which were isolated and characterized by ^{1}H NMR and mass spectrometry analyses. The formation of the latter tandem lesions involved an intramolecular addition of the 5-(2′-deoxyuridilyl)methyl radical to the C8 of the adenine moiety. A sensitive and specific assay aimed at monitoring the formation of the four thymine^{CH2-C8}purine adducts, namely d(T∧G), d(G∧T), d(T∧A), d(A∧T), within DNA, was designed. This was based on a liquid chromatography analysis coupled to tandem mass spectrometry (HPLC-MS/MS) detection of the dinucleoside monophosphates which were quantitatively released from γ-irradiated DNA and oligodeoxyribonucleotides by enzymatic hydrolysis. The four lesions were detected in both single-stranded oligodeoxyribonucleotide and isolated DNA upon exposure to γ-radiation in oxygen-free aqueous solution. It was found that the tandem guanine-thymine lesions were produced more efficiently than the adenine-thymine cross-links. Moreover, a significant sequence effect was observed. Thus, the yield of formation of the tandem lesions is higher when the purine base is located at the 5′ position of the 5-(2′-deoxyuridilyl)methyl radical.

Introduction

Ionizing radiation, high-intensity laser UV photolysis, and UV-A photosensitization that generate reactive oxygen species and/or mediate one-electron oxidation processes are known to induce oxidative damage to nucleic acids. This includes altered bases, strand breaks, abasic sites, DNA−DNA, and DNA−protein cross-links (*1*−*3*). During the last two decades, major efforts were made to isolate and characterize modified nucleobases within isolated DNA including tandem base lesions. Two different classes of vicinal base damage were identified by Box et al. (*4*−*7*). They are constituted by two adjacent modified bases, such as in 8-oxo-7,8-dihydro-2′-deoxyguanosylyl-(3′→5′)-*N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)-formylamine (8-oxodG-dβF). The lesion 8-oxodG-dβF that contains an oxidized guanine and an adjacent pyrimidine base remnant was first identified in short oligodeoxyribonucleotides upon exposure to X-rays in aerated aqueous solution. Then, the characterization and the measurement of the latter modification within isolated γ-irradiated DNA have been reported (*4*−*6*, *8*). The second type of double lesion involves a covalent bridge between a thymine and a vicinal guanine (*4*, *6*, *7*). The adduct has been found to be generated by •OH radical reaction in oxygen-free aqueous solution of DNA (*7*). The structure of the latter vicinal base lesion involves a covalent bond between the C8 carbon atom of a guanine residue and the carbon atom of the methyl group of an adjacent thymine. The tandem lesion was initially described in a GT containing tetramer oligonucleotide following exposure to X-radiation in deaerated aqueous solution (*7*). Interestingly, both tandem base lesions result from a single free-radical initiating event which leads to the formation of the double modifications.

The d(T∧G)[1] and d(G∧T) cross-linked tandem base lesions were isolated from UV photolysis under anaerobic conditions of dinucleoside monophosphates that contained a photolabile precursor of 5-(2′-deoxyuridilyl)-methyl radical (*9*, *10*). The latter bridged thymine-guanine adducts have been characterized by NMR and MS analyses. Recently, the reactivity of 5-(2′-deoxyuridilyl)methyl radical, was investigated by Greenberg et al (*11*, *12*). The latter radical was generated by Norrish Type I photocleavage of the 5-(1-(3-phenyl-2-oxopropyl))-2′-deoxyuridine motif, which implies abstraction of the hydrogen atom from the methyl group. It was concluded that the generation of the radical may induce the damage transfer from the nucleobase to an adjacent nucleotide in DNA under hypoxic conditions.

One of the two main objectives of the present work was to extend our previous studies on the formation of d(G∧T)

* To whom the correspondence should be addressed. Phone: +33 4-38-78-49-87. Fax: +33 4-38-78-50-90. E-mail: cadet@drmfc.ceng.cea.fr.

10.1021/tx015594d CCC: $22.00
Published on Web 04/15/2002

and d(T∧G) adducts to other vicinal tandem bridged thymine-purine base in γ-irradiated isolated DNA. Thus, two new tandem base lesions were isolated, namely thymine-adenine adduct d(T∧A) and its reversed sequence d(A∧T). The latter tandem modifications were generated using a similar approach than that previously described for the thymine-guanine vicinal base lesions (*9*, *10*). The UV-C reactive precursor of 5-(2′-deoxyuridilyl)methyl radical was incorporated into ODN located either 3′ or 5′ to a 2′-deoxyadenosine nucleoside.

In addition, an assay aimed at measuring the four tandem base lesions d(G∧T), d(T∧G), d(A∧T), and d(T∧A) within isolated DNA was designed. The four lesions were efficiently separated by high-performance liquid chromatography, and the detection of the damage was achieved using the highly accurate electrospray ionization tandem mass spectrometry (ESI-MS/MS) technique. The four vicinal base lesions were produced in both ODNs and isolated DNA upon exposure to γ-rays in oxygen-free aqueous solution. Interestingly, it was found that the tandem lesions were produced in a much higher yield in the purine(5′→3′)thymine compounds than in the reversed sequence within both oligonucleotides and calf thymus DNA. Moreover, the two tandem base lesions including the guanine residue are generated more efficiently than the respective tandem base damage bearing the adenine moiety.

Experimental Section

General Procedures and Materials. The silica gel (70−200 μm) used for the low-pressure column chromatography was purchased from SDS (Peypin, France). Thin-layer chromatography was performed on DC Kieselgel Polygram SilG/UV254 (0.2 mm) plastic sheets from Macherey-Nagel (Dueren, Germany). Deuterated solvents were purchased from Acros (Geel, Belgium). Buffers for high performance liquid chromatography (HPLC) were prepared using water purified with a Milli-Q apparatus (Milford, MA).

Nuclease P1 (*Penicillium citrinium*), bovine intestinal mucosa phosphodiesterase (3′-exo), and calf thymus DNA were obtained from Sigma (St. Louis, MO). Calf spleen phosphodiesterase (5′-exo) and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim (Mannheim, Germany).

The aqueous solutions of DNA and oligonucleotides were deaerated by bubbling for 15 min with a nitrogen stream prior to UV or gamma irradiation.

NMR Measurements. ^{1}H NMR spectra of 400 MHz were recorded on a U400 (Varian) operating in the Fourier transform mode. The chemical shifts are reported in ppm (parts per million), using the residual proton signal of D_2O ($\delta_H = 4.65$) as the external reference.

Mass Spectrometry Measurements. All modified and unmodified oligonucleotides were characterized by electrospray ionization mass spectrometry measurement (ESI-MS) using a LCQ ion-trap model mass spectrometer from Thermo-Finnigan (San Jose, CA). Typically, for the analysis in the negative mode, 0.1 AU_{260nm} of the dinucleoside monophosphates was dissolved in a mixture of acetonitrile and water (50/50, v/v) that contained 1% of triethylamine. For the measurements performed in the positive mode, the samples were dissolved in a water/methanol mixture (50/50, v/v).

High-Performance Liquid Chromatography. System A: Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm) with a mixture of acetonitrile and 25 mM ammonium formate buffer (AF, pH 6.2) as the eluents [100% AF (2 min), linear gradient from 0 to 20% of acetonitrile (50 min)] at a flow rate of 1 mL min^{-1}. UV detection at 260 nm. System B: Reversed-phase HPLC (Hamilton PRP_3, polymeric phase column, 10 μm, 305 × 7.0 mm) with a mixture of acetonitrile and 10 mM TEAA buffer as the eluents [100% TEAA (5 min), linear gradient from 0 to 8% of acetonitrile (10 min), then isocratic TEAA-acetonitrile (92/8) v/v (10 min); after isocratic 100% TFA (1%) (10 min) and finally a gradient from 0 to 10% acetonitrile (40 min)] with a flow rate of 2.5 mL min^{-1}. UV detection set at 260 nm. System C: Reversed-phase HPLC (Hypersil C_{18} column, 5 μm, 250 × 4.6 mm) with a mixture of acetonitrile and 25 mM ammonium formate buffer (AF, pH 6.2) as the eluents [100% AF (2 min), linear gradient from 0 to 20% of acetonitrile (30 min)] at a flow rate of 1 mL min^{-1}, UV detection at 260 nm. System D: Reversed-phase HPLC (Uptisphere ODB, 3 μm, 150 × 2 mm); elution with acetonitrile and TEAA (5 mM) [linear gradient: from 0 to 40% of a 20% acetonitrile solution in TEAA (10 min) and then from 40% to 60% (20 min) at a flow rate of 0.2 mL/min].

Synthetic Procedures (Scheme 2). 3′-*O*-Acetyl-5-(phenylthiomethyl)-2′-deoxyuridine (**2**) was prepared as previously described (*10*). Then, the modified nucleoside was coupled by the phosphoramidite liquid-phase synthesis method to commercial ^{pac}dAdo building block (**1**); subsequently, the deprotection and purification of the dinucleoside monophosphate **4** [5′-d(AT^{SPh})-3′] were achieved as reported below.

Synthesis of Product 3. Commercially available 5′-*O*-dimethoxytrityl-N^6-phenoxyacetyl-2′-deoxyadenosine-3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite **(1)** (1 g, 1.12 mmol) was dissolved in 30 mL of dry acetonitrile and 3′-*O*-acetyl-5-(phenylthiomethyl)-2′-deoxyuridine (**2**) (486 mg, 1.24 mmol) dissolved in 30 mL dry acetonitrile and 427 mg of tetrazole (6 mmol) were added. The resulting solution was stirred at room temperature for 20 min and then the reaction mixture was partly reduced. Then, 40 mL of 0.1 M iodine solution in THF-water-pyridine was added and the reaction mixture was stirred for 45 min. Finally the reaction was quenched by addition of 10 mL of 1 M $Na_2S_2O_3$ and 100 mL of chloroform. The organic layer was washed with water and dried by addition of Na_2SO_4 prior to be evaporated to dryness under vacuum. The resulting yellow oil was dissolved in 1% TFA in methylene chloride solution and stirred for 30 min. Then, the organic solution was evaporated to dryness under vacuum. The residue thus obtained was purified by chromatography on a silica gel column using a step gradient of methanol in methylene chloride (from 0 to 5%). Evaporation to dryness of the appropriate fractions yielded **3** (1.12 g, 53%). R_f ($CHCl_3$/CH_3OH 95/5): 0.32. ESI-MS (positive mode): *m*/*z* 892.9 [M + H]$^+$, 915.1 [M + Na]$^+$.

The dinucleoside monophosphate **4** was obtained after deprotection of **3** (480 mg, 0.54 mmol), by treatment with concentrated aqueous ammonia (30%, 30 mL) at room temperature for 2 h. After evaporation of the solvent to dryness under vacuum, the crude 2-mer **4** [5′-d(AT^{SPh})-3′] was purified by reversed-phase HPLC (system A) with a retention time of 42 min.

ESI-MS (positive mode): *m*/*z* 664.0 [M + H]$^+$, 686.0 [M + Na]$^+$, 702.0 [M + K]$^+$. ^{1}H NMR in D_2O (400 MHz): δ (in ppm)

[1] Abbreviations: MMTrCl, 4-monomethoxytrityl chloride; TFA, trifluoroacetic acid; DCM, dichloromethane; ESI-MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MRM, multiple reaction monitoring; ODN, oligodeoxyribonucleotide; AF, ammonium formate buffer; AU, absorbance unit; TEAA, triethylammonium acetate; pac, phenoxyacetyl; T^{SPh}, 5-(phenylthiomethyl)-2′-deoxyuridine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DHT, 5,6-dihydrothymine; d(T∧G), 2′-deoxy-8-[[1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl]methyl]-3′-guanylic acid intramol. 3′,5‴-ester; d(G∧T), 2′-deoxy-8-[[1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl]methyl]-5′-guanylic acid intramol. 5′,3‴-ester; d(T∧A), 2′-deoxy-8-[[1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl]methyl]-3′-adenylic acid intramol. 3′,5‴-ester; d(A∧T), 2′-deoxy-8-[[1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl]methyl]-5′-adenylic acid intramol. 5′,3‴-ester; 5′-d(AT^{SPh})-3′, 2′-deoxyadenylyl-(3′→5′)-5-(phenylthiomethyl)-2′-deoxyuridine; 5′-d(GT^{SPh})-3′, 2′-deoxyguanylyl-(3′→5′)-5-(phenylthiomethyl)-2′-deoxyuridine; 5′-d(AT)-3′, 2′-deoxyadenylyl-(3′→5′)-thymidine; 5′-d(A^{HM}U)-3′, 2′-deoxyadenylyl-(3′→5′)-5-(hydroxymethyl)-2′-deoxyuridine.

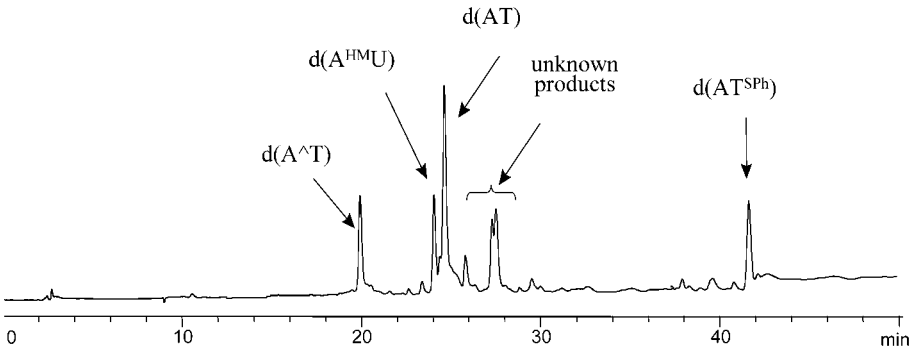
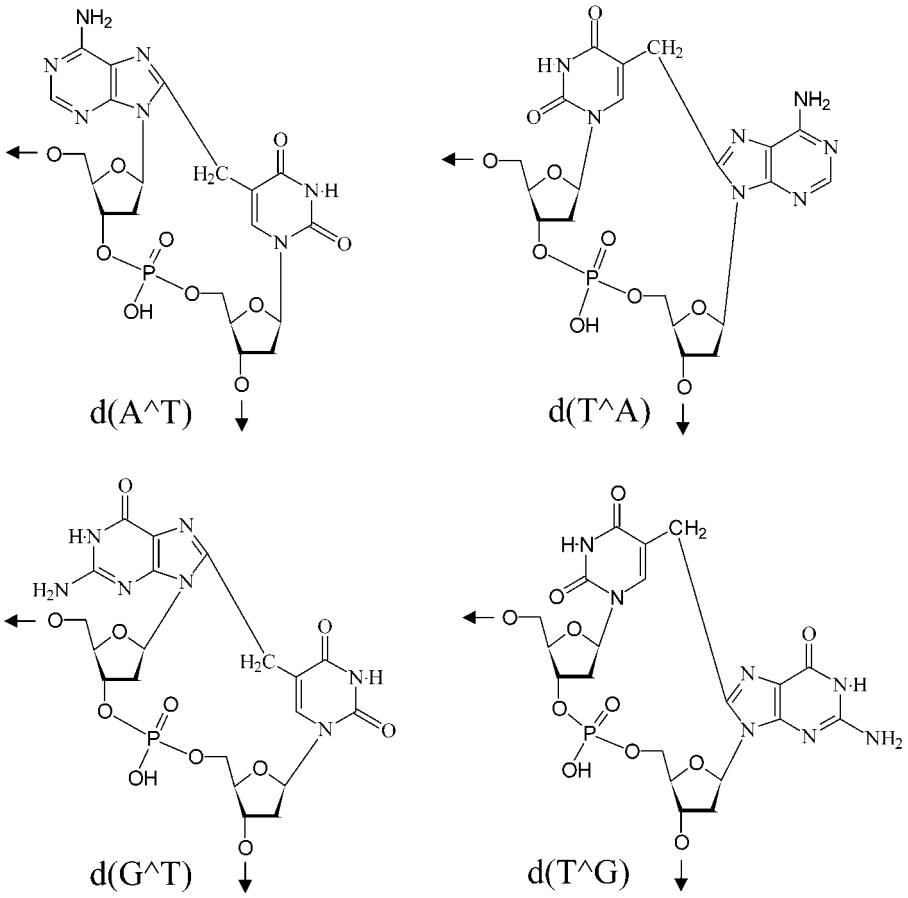


Figure 1. HPLC elution profile of the UV-C irradiated **4** on a C_{18} Hypersil analytical column (system A).

Scheme 1. Structure of the Thymine^{CH2-C8}Purine Tandem Base Lesions Studied in This Work



8.0 (s, 1H, H_6T); 7.9 (s, 1H, H_2A); 7.1 (m, 5H, H-arom PhS); 7.0 (s, 1H, H_8A); 6.20 (t, 1H, $H_{1'}$T); 5.92 (t, 1H, $H_{1'}$A); 4.17 (m, 2H, $H_{3'}$T, $H_{3'}$A); 4.08 (m, 1H, $H_{4'}$T); 3.97 (m, 1H, $H_{4'}$A); 3.86 (m, 2H, $H_{5'5''}$T); 3.68 (m, 2H, $H_{5'5''}$A); 3.51 (m, 2H, CH_2-T); 2.64 (m, 2H, $H_{2'}$T, $H_{2'}$A); 2.06 (m, 1H, $H_{2''}$T); 1.70 (m, 1H, $H_{2''}$A).

Solid-Phase Synthesis of Oligodeoxyribonucleotides. The synthesis of oligodeoxyribonucleotides was performed at 1 μmol scale using the "Pac phosphoramidite" chemistry (*13*), with retention of the 5′ terminal DMTr group ("trityl-on" mode). The standard 1 μmole DNA cycle was used on a 392 DNA synthesizer (Applied Biosystems Inc, Palo Alto, CA) with a few modifications. First, the duration of the condensation was increased 4 times for the modified nucleoside phosphoramidite (120 s instead of 30 s for a normal nucleoside phosphoramidite). Second, a 0.3 M solution of phenoxyacetic acid in anhydrous tetrahydrofuran was used for the capping step.

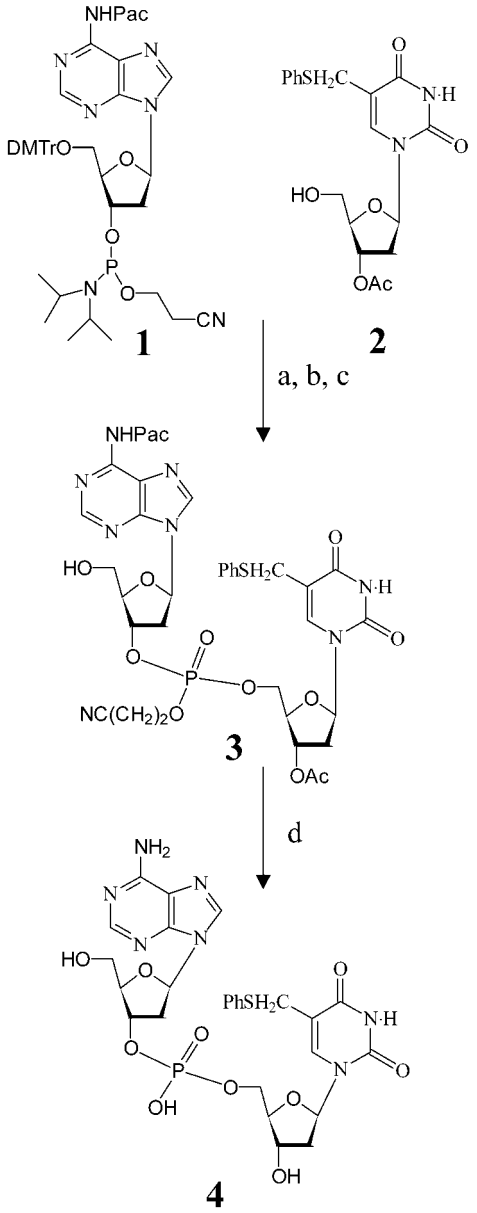
Deprotection and Purification of Oligodeoxyribonucleotides. Upon completion of the synthesis, the alkali-labile protecting groups of the oligodeoxyribonucleotides were removed by treatment with concentrated aqueous ammonia (30%) at room temperature for 4 h. Thereafter, solvents were removed by evaporation under vacuum. Then, the crude 5′-DMTr-oligomers were purified and deprotected on-line by reversed-phase HPLC (system B) (*14*). The purity of the collected fractions was controlled by HPLC analyses (system C) and ESI mass spectrometry measurements.

γ-Irradiation of Isolated DNA and ODN. Deaerated aqueous solutions of either calf thymus DNA or ODNs (0.5 mg mL^{-1}) were exposed to the γ rays of a ^{60}Co source immersed in a pool. The dose rate was 18 Gy.min^{-1}. The selected irradiation times ranged from 0 to 16 min.

UV-C Irradiation of Oligonucleotides. Oxygen-free aqueous solutions of oligonucleotides containing the thiophenyl nucleoside (1 AU_{260nm} mL^{-1} solution) were exposed to UV-C light provided by 8 lamps (λ_{max} = 254 nm) from a Rayonet photoreactor (The Southern New England Ultraviolet Company, Hamden, CT). The irradiation times ranged from 5 to 10 min.

Photochemical Synthesis of d(A^T). 1 AU_{260nm} mL^{-1} of **4** in 10 mM sodium phosphate oxygen-free buffer (pH 7) that contained 100 mM NaCl, was exposed to UV-C radiation. The

Scheme 2. Synthetic Pathway Used for the Preparation of the Dinucleoside Monophosphate 5′-[AT^{SPh}]-3′ 4[a]



[a] Reagents: (a) tetrazole, CH_3CN, 20 min, RT; (b) I_2, THF/H_2O/pyridine, 45 min, RT; (c) 1% TFA, CH_2Cl_2, 30 min, RT; (d) NH_4OH 30%, 2H, RT.

main products were isolated by analytical HPLC (system A). The d(A∧T) tandem base lesion was obtained in a 10% yield.

ESI-MS (Negative Mode) of d(A∧T) Tandem Base Lesion. (Figure 3) *m*/*z* 552.2 [M − H]$^-$, 574.3 [M − 2H + Na]$^-$; **MS^2** *m*/*z* 454.2 [M − H]$^-$; **MS^3** *m*/*z* 258.3 [M − H]$^-$.

^{1}H NMR (400 MHz, D_2O) of d(A∧T) Tandem Base Lesion. δ (in ppm): 8.0 (s, 1H, H_2A); 7.8 (m, 1H, H_6T); 6.3 (q, 1H, $H_{1'}A$); 6.2 (t, 1H, $H_{1'}T$); 4.8 (m, 1H, $H_{3'}A$); 4.3 (m, 2H, $H_{3'}T$, $H_{4'}A$); 4.0 (m, 5H, CH_2T,$H_{5'5''}T$, $H_{4'}T$); 3.7 (m, 2H, $H_{5'5''}A$); 3.1 (m, 1H, $H_{2'}A$); 2.4 (q, 1H, $H_{2''}A$); 2.2 (q, 2H, $H_{2'2''}T$).

ESI-MS (negative mode) of d(AT) Dinucleoside Monophosphate: *m*/*z* 554.3 [M − H]$^-$.

^{1}H NMR (400 MHz, D_2O) of d(AT) Dinucleoside Monophosphate. δ (in ppm): 8.2 (s, 1H, H_2A); 8.0 (s, 1H, H_8A); 7.3 (m, 1H, H_6T); 6.3 (t, 1H, $H_{1'}A$); 6.0 (t, 1H, $H_{1'}T$); 4.7 (m, 2H, $H_{3'}A$); 4.4 (q, 1H, $H_{3'}T$); 4.2 (q, 1H, $H_{4'}A$); 4.0 (m, 1H, $H_{4'}T$); 3.9 (m, 2H, $H_{5'5''}T$); 3.7 (m, 2H, $H_{5'5''}A$); 2.7 (m, 2H, $H_{2'2''}A$); 1.5 (d, 3H, CH_3T).

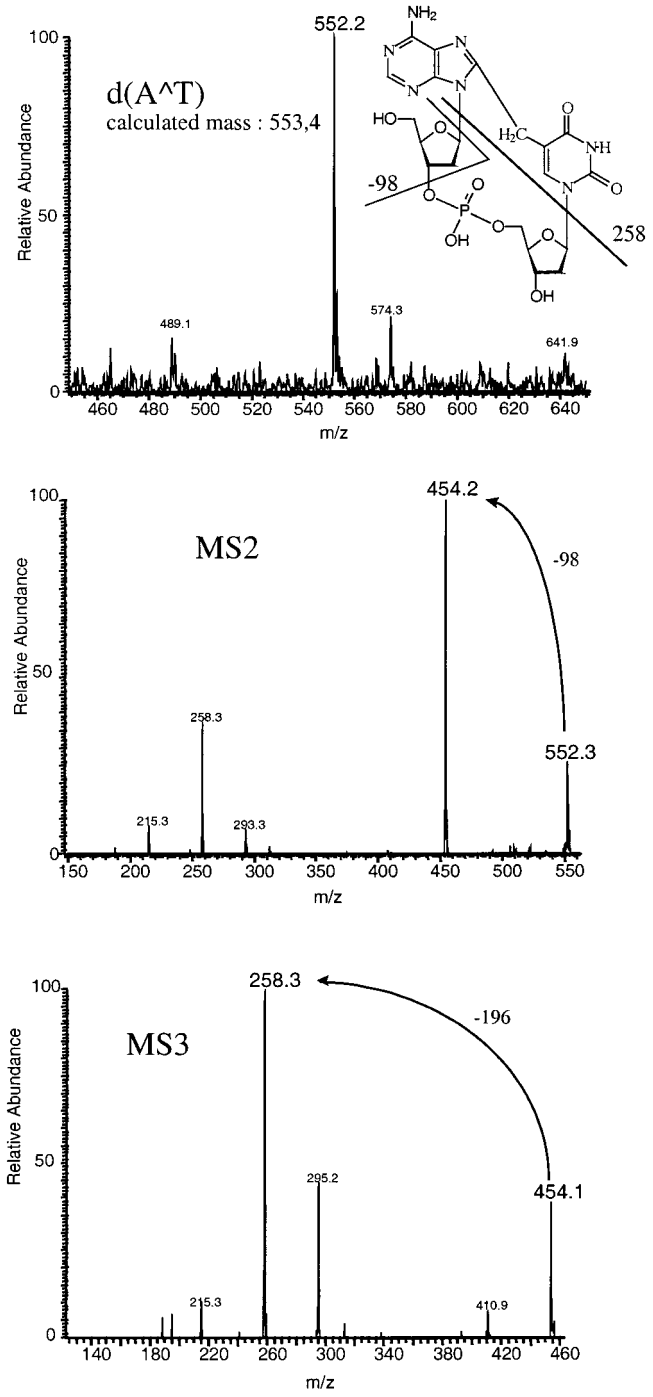


Figure 2. ESI-MS and ESI-MS/MS spectra (in the negative mode) of the tandem base damage d(A∧T).

Enzymatic Digestion of Dinucleoside Monophosphates by Nuclease P1 and Alkaline Phosphatase. A total of 0.5 AU_{260nm} of oligonucleotide in water (45 μL) was digested at 37 °C by incubation for 2 h with 5 units of nuclease P_1 (1 unit/μL) in a 30 mM NaOAc and 0.1 mM $ZnSO_4$ aqueous solution (pH 5.5). Then, 5 μL of Tris 500 mM, 1 mM EDTA (pH 8.5), and 2 units of alkaline phosphatase were added. The resulting mixture was subsequently incubated at 37 °C for 1 h and then diluted in 50 μL of 25 mM AF (pH 6.2). After centrifugation, the mixture was finally resolved by reversed-phase HPLC (system A). The different products were identified by co-injection with synthetic standards and electrospray ionization mass spectrometry analysis in the negative mode.

Enzymatic Digestion of Dinucleoside Monophosphates by Calf Spleen Phosphodiesterase (5′-exo). A total of 0.2 AU_{260nm} of oligonucleotide in 20 μL of 0.02 M ammonium citrate

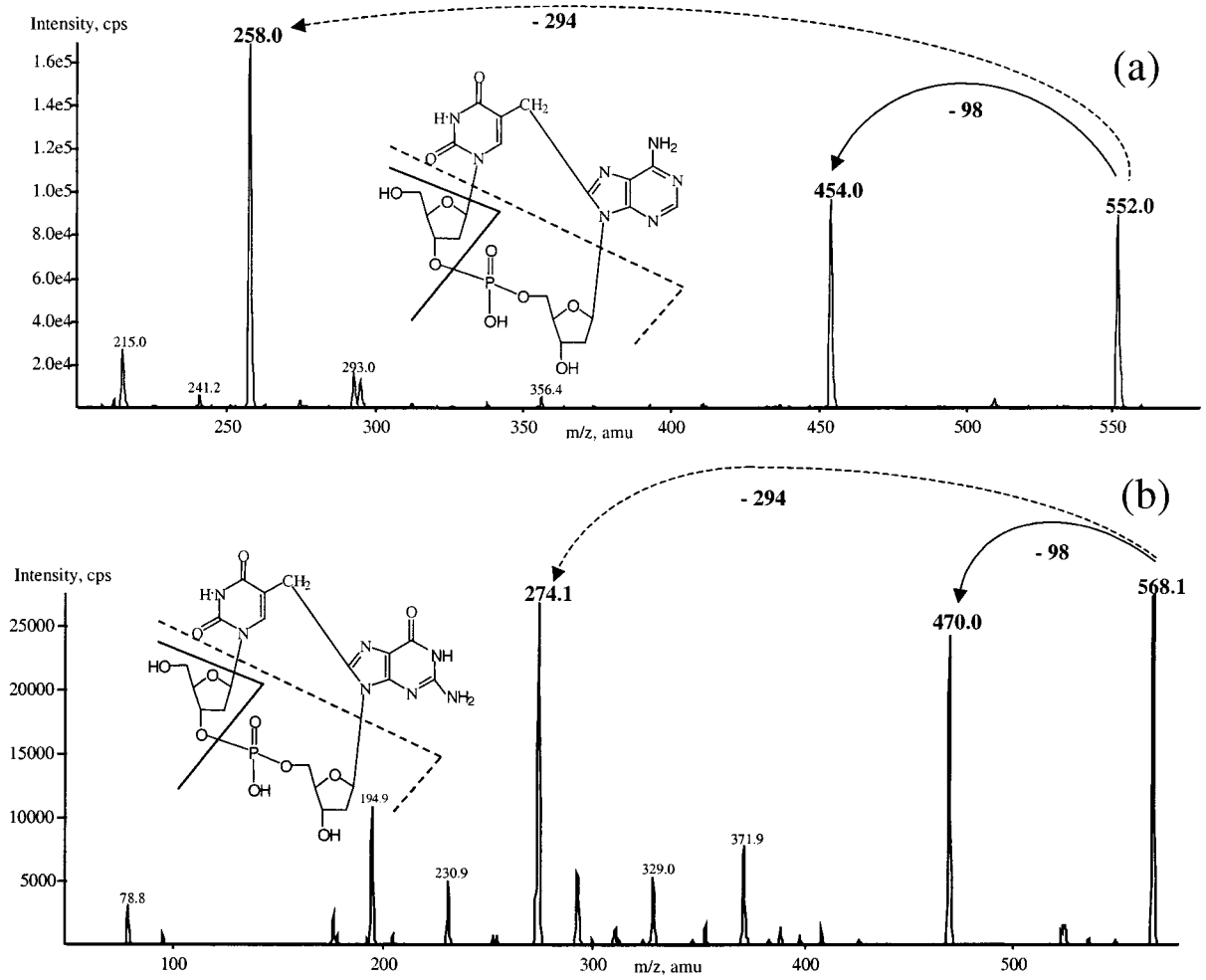


Figure 3. Collision-induced dissociation mass of the pseudo-molecular ion of 5′-d(A∧T)-3′ and 5′-d(T∧A)-3′ (a), 5′-d(G∧T)-3′ and 5′-d(T∧G)-3′ (b) obtained in the negative electrospray ionization mode.

(pH 5) was digested at 37 °C by incubation with 10^{-3} units of calf spleen phosphodiesterase for 1 h and the resulting mixture was directly submitted to reversed-phase HPLC analysis (system A). The different products were collected and analyzed by electrospray ionization mass spectrometry in the negative mode.

Enzymatic Digestion of Dinucleoside Monophosphates by Bovine Intestinal Mucosa Phosphodiesterase (3′-exo). Similarly, enzymatic digestion of oligonucleotides was performed using 10^{-5} units of bovine intestinal mucosa phosphodiesterase in 0.02 M ammonium citrate buffer (pH 9).

Enzymatic Digestion of Isolated DNA. Fractions of DNA samples (100 μL, 50−100 μg) were incubated for 2 h at 37 °C with 5 μL of nuclease P_1 (1 unit/μL), 1 μL of calf spleen phosphodiesterase (0.004 unit), and 10 μL of enzymatic buffer (200 mM succinic acid, 100 mM $CaCl_2$, 0.2 M ammonium citrate, pH 5). Then, 10 μL of alkaline phosphatase buffer (500 mM TRIS, 1 mM EDTA, pH 8.5) was added together with 5 units of alkaline phosphatase and 0.003 unit of bovine intestinal mucosa phosphodiesterase and the incubation was resumed for 2 h. The enzymatic reaction was quenched by addition of 10 μL of HCl 0.1 M. Then, chloroform (100 μL) was added, and the resulting solution was centrifuged. The aqueous layer was collected, and its content was analyzed by HPLC-MS/MS.

HPLC-ESI-MS/MS Detection of the Multiple Base Lesions. The HPLC apparatus consisted of a 7100 Hitachi-Merck pump (Merck, Darmstadt, Germany) connected to a SIL-9A automatic injector (Shimadzu, Tokyo, Japan) and equipped with an octadecylsilyl silica gel column Uptisphere 3 μm ODB (150 × 2 mm) (system D). Detection of the tandem lesions was performed using a API 3000 tandem mass spectrometer (Perkin-Elmer, Toronto, Canada). Electrospray ionization in the negative mode was performed at −3300 V using a turbospray ionization source (SCIEX, Thornill, Canada) with the turbospray gas heated at 500 °C. The multiple reaction monitoring technique (MRM) using the four transitions 552→454, 552→258, 568→470, and 568→274 allowed a sensitive detection of d(A∧T), d(T∧A), and d(G∧T), d(T∧G). The collision energy 45 eV was applied to the pseudo-molecular ion. Accurate quantification was obtained by external calibration.

Results and Discussion

Preparation and Characterization of Thymine^{CH2-C8}Adenine Tandem Base Lesions. The thiophenyl derivative of 3′-monoacetylthymidine **2** was prepared according to the multiple-step procedure reported by Romieu et al. (*10*). This consisted in the 5-hydroxymethylation of 2′-deoxyuridine followed by a specific monoacetylation and the substitution of the acetyl group by thiophenol (*15*, *16*). The latter compound was condensed by liquid-phase synthesis with dA-cyanoethyl phosphoramidite **1**. After deprotection in an ammoniac solution, [5′-d(AT^{SPh})-3′] was obtained and purified by HPLC. The dinucleoside monophosphate **4** was characterized by electrospray ionization mass spectrometry in the negative mode and ^{1}H NMR analysis. As described previously for [5′-d(GT^{SPh})-3′], the 5-(2′-deoxyuridilyl)methyl radical was generated into DNA fragments by UV-C irradiation (λ_{max} = 254 nm); the isolation and the characterization of the covalent bridged tandem

base damage generated under anaerobic conditions have been previously performed (*10*).

UV irradiation of 2-mer [5′-d(AT^{SPh})-3′] in oxygen-free aqueous solution gave rise to several photoproducts including simple and multiple base lesions as observed by HPLC separation with UV detection (Figure 1). The major products were isolated and characterized. Thus, the predominant peak eluted at 25 min was found to be [5′-d(AT)-3′], which is likely to be generated by atom hydrogen trapping. The dinucleoside monophosphate eluted at 24 min contains 5-(hydroxymethyl)uracil as a modified thymine base [5′-d($A^{HM}U$)-3′]. The slower eluting compound is the starting dinucleoside monophosphate. The fastest eluting photoproduct in the chromatogram is the cross-linked adenine-thymine that is searched. The other compounds in the elution profile have not been yet identified.

The targeted tandem damage, with the methyl carbon atom of thymine linked to the C-8 carbon of adenine, [5′-d(A∧T)-3′] eluted at 20 min, was isolated and characterized. The structure of this vicinal DNA lesion was confirmed using tandem mass spectrometry (ESI-MS/MS, Figure 2): the pseudo-molecular ion at *m/z* 552.2 obtained in the negative mode is in agreement with the calculated mass of 553.4. Collision-induced dissociation of the pseudo-molecular ion provided additional structural information. The main fragment at *m/z* 454 results from the loss of the sugar residue at the 5′-end of the dinucleoside monophosphate. Further fragmentation of the latter ion (*m/z* 454) gives rise to a fragment at *m/z* 258 corresponding to the thymine residue covalently linked to adenine through the methylene bridge.

The ^{1}H NMR spectra of [5′-d(A∧T)-3′] and [5′-d(AT)-3′] dinucleoside monophosphates, that were collected from HPLC runs, provided further support to the structure of the photoproducts. As striking features, both the singlet resonance of the thymine methyl group and the adenine H-8 signal are lacking in the ^{1}H NMR spectrum of [5′-d(A∧T)-3′]. Moreover, the signal corresponding to the methylene bridge (CH_2) in [5′-d(A∧T)-3′] resonates as a multiplet pattern ($\delta = 4.0$ ppm). Further confirmation of the assignment was inferred from ^{1}H NMR TOCSY experiments (see Supporting Information).

The dinucleoside monophosphate [5′-d(T^{SPh}A)-3′] of opposite sequence was synthesized using solid-phase phosphoramidite method and a commercially available adenine support. The tandem base lesion [5′-d(T∧A)-3′] was isolated after UV-C irradiation under anaerobic conditions. As described above, the fragmentation observed by ESI-MS/MS confirmed the formation of a methylene bridge between the C8 of the adenine base on one hand and the methyl group of the thymine base on the other hand.

The two new tandem base lesions d(A∧T) and d(T∧A) were used as standards for the design and optimization of an analytical method aimed at measuring their formation upon γ-irradiation of isolated DNA in oxygen-free aqueous solution.

Enzymatic Hydrolysis of DNA Fragments That Contain the Tandem Lesions. The ability for the exonuclease to cleave the phosphodiester bond of the four tandem base lesions, d(T∧A), d(A∧T), d(T∧G), d(G∧T) (Scheme 1), obtained by UV-C irradiation of the corresponding photoreactive dinucleoside monophosphate precursor was checked under different enzymatic hydrolysis conditions (*17*, *18*).

Table 1. Sequences and Relative Molecular Mass (Da) of the ODNs Synthesized and Used in This Study

		mass (Da)	
sequences	length	calcd	found
5′-d(GT^{SPh}G)-3′	3	1008.8	1008.5
5′-d(AT^{SPh}A)-3′	3	976.8	977.3
5′-d(ATT CCG GTG GAA GCC)-3′	15	4593.0	4592.6
5′-d(CTT CCG ATA GAA GGA)-3′	15	4601.0	4600.8

Enzymatic processing by either a mixture of nuclease P1 and alkaline phosphatase, or bovine intestinal mucosa phosphodiesterase followed by calf spleen phosphodiesterase (3′-and 5′-exonucleases respectively), was investigated. HPLC and mass spectrometry analyses of the reaction mixtures showed that no hydrolysis of the phosphodiester bond of the dinucleoside monophosphate occurred under the two enzymatic digestion conditions. Moreover, to confirm that the 3′ or 5′-adjacent nucleoside of the tandem lesion was hydrolyzed, the 3-mer ODNs 5′-[AT^{SPh}A]-3′ and 5′-[GT^{SPh}G]-3′, prepared by the solid-phase phosphoramidite method (Table 1), were exposed to UV-C radiation under anaerobic conditions. In a subsequent step, the irradiated mixture was analyzed by HPLC-MS/MS before and after an enzymatic treatment by the phosphodiesterase (3′-and 5′-exonucleases). Before hydrolysis of the irradiated mixture of 5′-[AT^{SPh}A]-3′, a pseudo-molecular ion $[M - H]^-$ was found at *m/z* 866 using the HPLC-MS method. The latter ion was not observed after the action of the 3′- and 5′-exonucleases. In fact, a pseudo-molecular ion $[M - H]^-$ at *m/z* 552 corresponding to the dinucleoside monophosphates was detected. Then, the release of the lesions was quantitatively assessed by the action of both the 3′-and 5′-exonucleases either on ODN or DNA.

HPLC-ESI-MS/MS Assay. Our objective was to develop a quantitative method for the measurement of the tandem base lesions d(A∧T), d(T∧A), d(G∧T), and d(T∧G), within isolated DNA upon exposure to gamma radiation in oxygen-free aqueous solution. In that respect, HPLC separation combined with electrospray ionization tandem mass spectrometry operating in the multiple reaction monitoring (MRM) mode constitutes a specific and sensitive detection method (*4*, *19*−*22*). The full mass spectra of both lesions d(T∧A) and d(A∧T) in the negative mode exhibited a pseudo-molecular ion $[M - H]^-$ at *m/z* 552. Two main fragments were observed in the MS/MS spectra at *m/z* 454 and 258, respectively (Figure 3a). The transitions 552→454 and 552→258 were monitored to detect the tandem lesions in the MRM mode. The full scan spectra of both d(T∧G) and d(G∧T) cross-linked lesions recorded in the negative mode exhibited a pseudo-molecular ion $[M - H]^-$ at *m/z* 568. Two main fragments were observed in the MS/MS spectra of d(G∧T) similar to d(T∧G) at *m/z* 470 and 274 (Figure 3b). Therefore, the two major transitions 568→470 and 568→270 were selected to detect such lesions. In addition, the HPLC conditions used allow an efficient separation of the sequence isomer on the ODB column (Figure 4). It may be noted that the purine-thymine lesions are eluted slower than the reversed sequence whereas the cross-linked guanine-thymine adducts are eluted more rapidly than the adenine-thymine tandem base lesions.

Measurement of the Tandem Lesions within Isolated DNA Exposed to γ-Radiation. A deaerated aqueous solution of calf thymus DNA was exposed to doses of γ-radiation ranging from 0 to 280 Gy. The

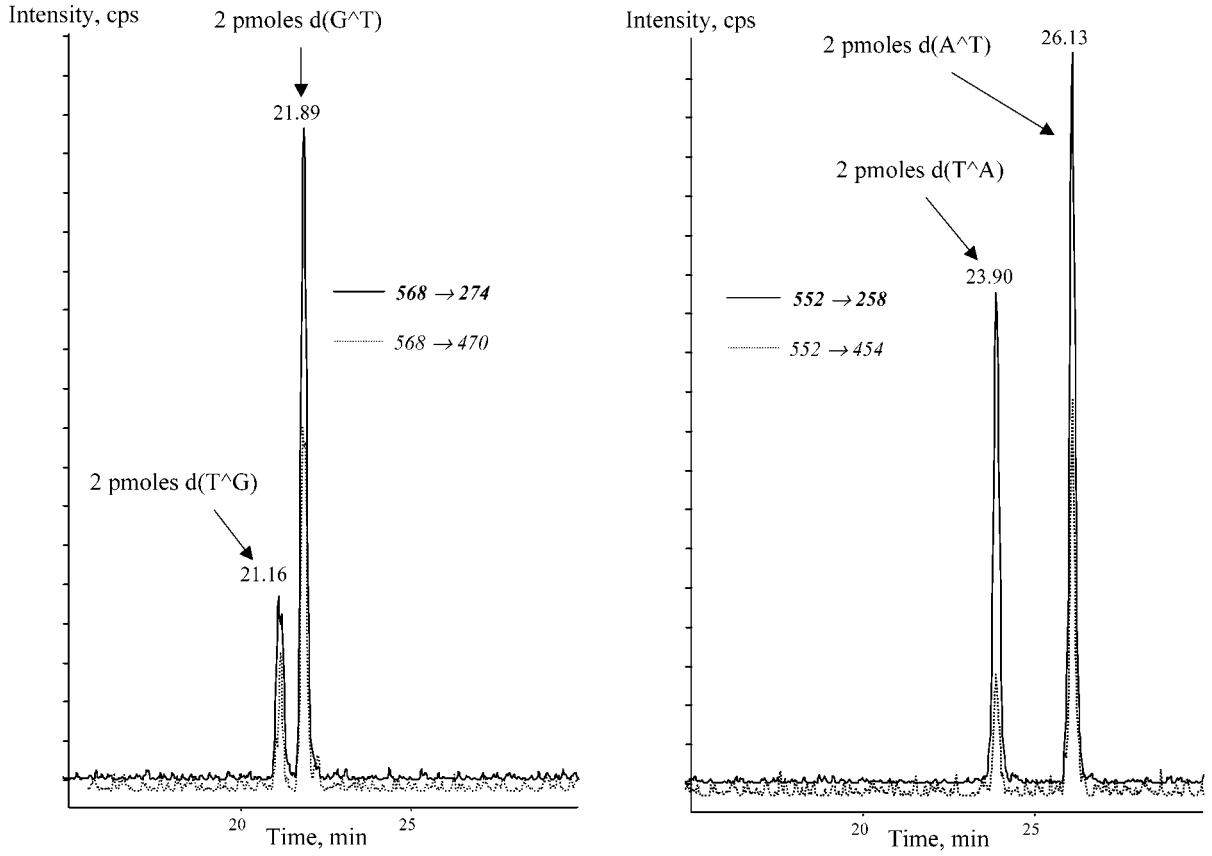


Figure 4. HPLC-MS/MS chromatograms of the four tandem base lesions (system D).

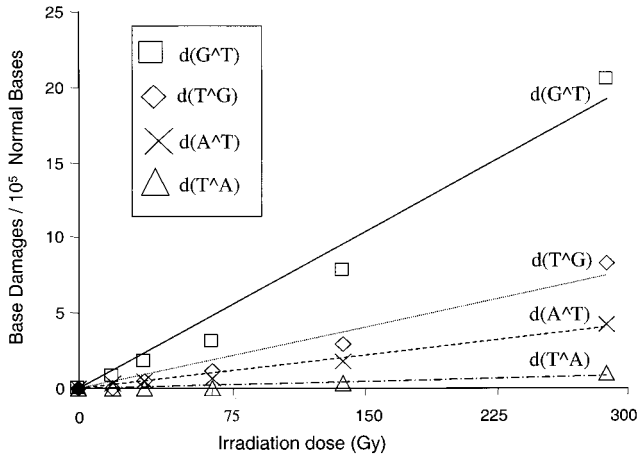


Figure 5. Radiation-induced formation of the four tandem base lesions measured by HPLC-MS/MS (MRM mode) in calf-thymus DNA upon exposure in oxygen-free aqueous solution to various doses of γ-radiation.

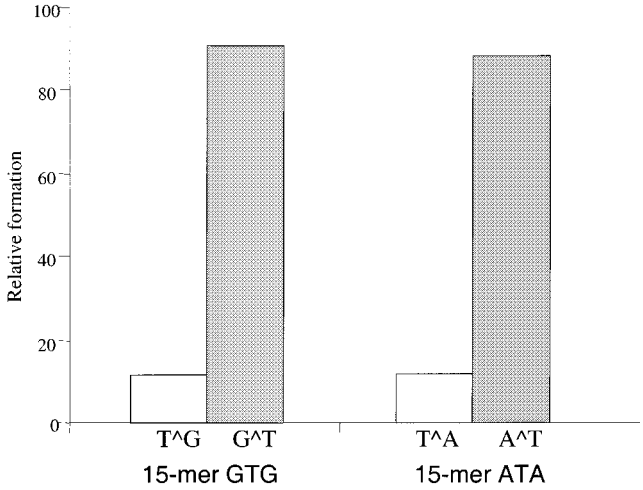


Figure 6. Relative formation of the tandem base lesions d(Purine∧T) and d(T∧Purine) in the 15-mer oligonucleotides containing either the GTG sequence or the ATA sequence at a central position upon exposure to gamma rays (dose: 288 Gy). The measurement was achieved by HPLC-MS/MS in MRM mode.

measurement of the vicinal lesions including thymine on one hand and each of the two purine bases on the other hand was performed as described above. It was found that the tandem lesions are formed approximately linearly with the radiation doses (Figure 5). In addition, the HPLC-ESI-MS/MS analysis clearly showed that d(G∧T) and d(T∧G) tandem base lesions are produced preferentially. A likely mechanism for the radiation-induced formation of the cross-linked thymine-purine base tandem lesions is accounted for by the initial formation of 5-(2′-deoxyuridilyl)methyl radical by •OH-mediated hydrogen abstraction from the thymine methyl group. Then, intramolecular addition to the C8 of the vicinal purine base takes place as shown from model studies involving the photolabile thiophenyl precursor of the latter thymine radical. Interestingly, a strong sequence effect was observed for the formation of both thymine−adenine and thymine−guanine cross-linked lesions. Thus, the tandem damage is generated in a higher yield when the purine base is located on the 5′-end. This sequence effect may be explained within DNA by the distances between the 5-(2′-deoxyuridilyl)methyl radical and the carbon 8 of the purine base. The two concerned carbons are separated by 6.30 Å when the purine base (either guanine or adenine) is located at the 3′-extremity (*23*). This dis-

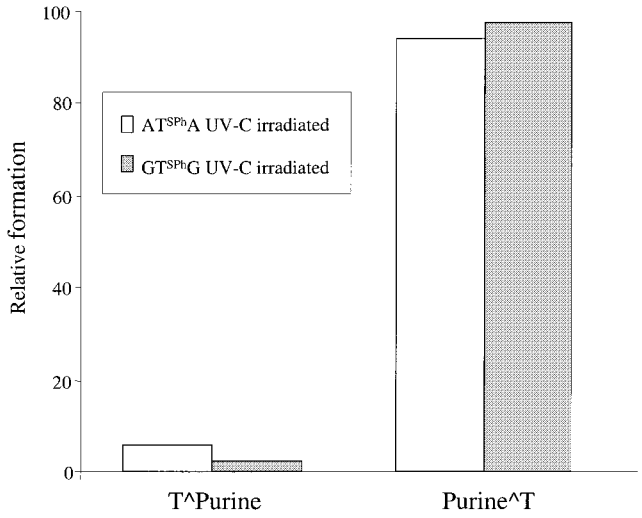


Figure 7. Relative formation of d(G∧T), d(T∧G) and d(A∧T), d(T∧A) in the 3-mer oligonucleotides $GT^{SPh}G$ and $AT^{SPh}A$, respectively, upon UV-C irradiated in oxygen-free aqueous solution (measured by HPLC-MS/MS in MRM mode).

tance is reduced to 3.58 Å when the purine base is situated at the 5′-extremity. Such a sequence effect was also observed in single-stranded 15-mer ODNs containing the GTG or ATA sequences at a central position (Table 1). There is a 4-fold preferential formation of the 5′-(purine-thymine)-3′ compared to the reversed sequence (Figure 6).

In addition, the 3-mer 5′-d[AT^{SPh}A]-3′ and 5′-d[GT^{SPh}G]-3′, were submitted to UV-C irradiation in deaerated solution and the enzymatic hydrolysis was applied. The HPLC-ESI-MS/MS analysis confirmed that the formation of the vicinal lesion with the purine located on 5′-position was produced in a 95% yield (Figure 7), compared to the reversed sequence.

Conclusion and Perspectives

The two cross-linked lesions that involved the formation of a covalent bond between the methyl group of thymine and the C8 of adenine were generated following the methodology previously described for the formation of thymine-guanine adducts. After isolation, the latter tandem base lesions were characterized by electrospray mass spectrometry and ^{1}H NMR measurements. The synthetic modified dinucleoside monophosphates d(A∧T) and d(T∧A) together with the two other previously described d(G∧T) and d(T∧G) lesions have been used as standards for their measurement in ODNs and isolated DNA following γ-irradiation under anaerobic conditions. This was achieved by high-performance liquid chromatography combined with electrospray ionization tandem mass spectrometry in the multiple reaction monitoring mode, subsequently to DNA enzymatic digestion. The HPLC-MS/MS assay is an accurate and powerful method to separate and measure the four radiation-induced thymine-purine d(T∧A), d(A∧T), d(T∧G) and d(G∧T) cross-linked base damage within single-stranded oligodeoxyribonucleotides and isolated DNA in oxygen-free aqueous solutions after their enzymatic release as dinucleoside monophosphate. Furthermore, an important sequence effect was observed for the formation of both lesion families. Finally, the tandem base lesions appear to be important °OH-mediated lesions in isolated DNA. Future work is aimed at delineating the biochemical features of the latter damage as it was done for the vicinal oxidative lesions 8-oxodG-dβF or 8-oxodG-DHT (*24*−*26*). Emphasis will be placed on the evaluation of the mutational potential of the cross-linked tandem lesion and the ability for repair enzymes to excise such a damage.

Acknowledgment. The contributions of Colette Lebrun (LRI/SCIB/CEA-Grenoble) to the electrospray mass spectrometry measurements of synthetic compounds and Pierre-Alain Bayle (RMN/SCIB/CEA-Grenoble) to the NMR analysis are gratefully acknowledged. We thank Dr. Thierry Douki for helpful discussions on the HPLC-MS/MS experiments. Financial support from the Comité de Radioprotection (Electricité de France) and the French Research Ministry (PhD fellowship to S.B.) is acknowledged.

Supporting Information Available: ESI-MS and ESI-MS/MS spectra (in the negative mode) of 5′-d(AT)-3′, ^{1}H NMR 1D, and 2D TOCSY spectra of 5′-d(A∧T)-3′ tandem base lesion and 5′-d(AT)-3′, radiation-induced formation of the cross-linked thymine-purine base tandem lesions. This material is available free of charge via the Internet at http://pubs.acs.org.

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