Volume 12 Number 13 1984 Nucleic Acids Research

# Two rotameric forms of open ring 7-methylguanine are present in alkylated polynucleotides

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Received 26 April 1984; Accepted 1 June 1984

## ABSTRACT

High performance liquid chromatography analysis of imidazole open ring 7-methylguanine, 2-6 diamino-4-hydroxy-5N-methyl-formamidopyrimidine (rom$^7$G), showed two well-separated peaks (fI and fII) of the same magnitude. Rechromatography of each isolated component indicated that they are slowly interconverted to give a 1:1 mixture. NMR analysis demonstrated that the two species observed on reversed phase HPLC are rotational isomers. Thermodynamic measurements strongly suggested that the equilibrium can be assigned to rotation around the N-methyl formamido bond. The two species, fI and fII, separated by HPLC were identified as rotamers E and Z, respectively. The structures of fI and fII were also determined.

A polynucleotide containing rom$^7$G was obtained by alkaline treatment of poly(dGC) containing 7-methylguanine. In order to study its structure within the polynucleotide, rom$^7$G was enzymatically excized by E.coli rom$^7$G-DNA glycosylase. The analysis of the products released by the enzyme showed a 1:4 mixture of the two rotamers favoring the Z form (fII).

## INTRODUCTION

Treatment of cells by alkylating agents such as alkylsulfates, alkyl methanesulfonates or nitrosoureas can result in cell death, mutagenesis and carcinogenesis (1-2). These effects are believed to be due to the alkylation of nucleic acids. The main DNA targets of such compounds are the N3 of adenine, the O6 of guanine and the N7 of guanine (1-2). Whereas mutagenesis is currently attributed to O6-methylguanine (3), killing is associated with purines alkylated at the N3 position (4,5). Guanine, methylated at the N7 position, is by far the main product of DNA alkylation. It does not seem to be harmful for the cell as it persists in DNA for generations (6), although it has also been shown to be enzymatically excized (7). However, quaternization of ring nitrogen resulting from alkylation reaction destabilizes the glycosylic bond leading to depurination (1). In addition, N7 alkylation favors cleavage of the imidazole ring by alkali (8,9,10) yielding 2-6 diamino-4-hydroxy-5N-methylformamidopyrimidine (rom$^7$G). This compound exists under the hydroxy and the oxo tautomers.

Several observations suggest that rom$^7$G might play an important role in cellular processes leading to carcinogenesis and/or death. Formation of rom$^7$G occurs in vivo in the liver of rats treated with carcinogens such as N-N dimethyl nitrosamine or 1-2 dimethyl hydrazine (11). In vitro, rom$^7$G inhibits DNA chain elongation (12) and is specifically excized from DNA by a DNA-glycosylase in both prokaryots (10,13) and in eukaryots (14).

Therefore, we have undertaken the characterization of products resulting from alkaline treatment of 7-methylguanosine and of a polynucleotide containing 7-methylguanine residues. We show that, at the base level, rom$^7$G is an equimolecular mixture of two rotameric forms, fI and fII (the E and Z forms respectively). In a polynucleotide containing rom$^7$G, a mixture of the two rotamers was also detected. However, the equilibrium was different from that observed with the free base because the amount of Z form was four times greater than that of the E form.

## MATERIALS AND METHODS

### Preparation of rom$^7$G

The opening of the imidazole ring of 7-methylguanosine was done according to Chetsanga and Lindahl (10). 7-methylguanosine (Sigma) was alkali treated, and the modified base was released from the nucleoside by formic acid treatment. The rom$^7$G was purified by chromatography on Biogel-P2 (200-400 mesh, Biorad) as already described (7). The column was equilibrated and eluted with 20 mM potassium tetraborate adjusted to pH 10.55. Optical density at 254 nm was continously monitored and 1 ml fractions were collected. The major peak of absorbance which was free of ribose was concentrated and rechromatographed using the HPLC system described in the next section. The material eluting between 3 and 6 min was collected and concentrated. This fraction co-eluted with the rom$^7$G marker molecule (a gift of Dr. T.Lindahl) and was more than 99 % pure. This material was used in all further studies reported.

### HPLC chromatography

Chromatography was performed with an HPLC system model 6000 Waters Associate (Milford, MA). A dual piston pump equipped with a UGK universal liquid chromatograph injector was used. Products were detected by monitoring UV absorption at 254 nm and by scintillation counting of fractions containing labeled material. Separation of the species was obtained on a $C_{18}$µBondapak column (Waters) with a guard column (Waters) packed with $C_{18}$µBondapak inserted between the injector and the column. The mobile phase was 50 mM $NH_4H_2PO_4$ pH

4.5 containing 5% methanol (v/v). The column was isocraticaly run at 1.5 ml/min.

**NMR studies**

NMR samples were prepared by dissolution of rom$^7$G in $H_2O$ or $^2H_2O$, and then lyophilized. The dried rom$^7$G was dissolved in $2H_2O$ (99.9%) or $(C^2H_3)_2SO$ (100%) at a concentration of about $5x10^{-2}M$. The pH values of aqueous solutions were measured with a microelectrode (Ingold 405 M3) without correction for the deuterium effect. Spectra were run in the Fourier transform mode at 270 MHz on a Brüker WH 270 spectrometer equipped with aspect 2000 computer and Brüker temperature controller (± 1°C). Resolution enhancement was achieved using a gaussian multiplication of the F.I.D. Chemical shifts are given in ppm ± 0.01 ppm from hexamethyldisiloxane ($Me_6OSi_2$) as internal reference in a $(C^2H_3)_2SO$ solution and from $Me_4Si$ as internal reference in a $^2H_2O$ solution.

Thermodynamic parameters were determined as described (15) from linear regression analysis of the data.

**Preparation of poly(dGC) containing [$^3$H]7-methylguanine or[$^3$H]rom$^7$G residues**

The polynucleotide (PL. Biochemical product) was alkylated as already described (15) with 5 mCi of [$^3$H]-dimethylsulfate (New England Nuclear, 4.7 Ci/mmol). The reaction was carried out at 37°C for 1h. Under these conditions, about 0.5% of the guanine residues are methylated. The specific activity was $4x10^4$ cpm/µg of poly(dGC). Imidazole ring opening of 7-methylguanine was obtained by incubating DMS-treated poly(dGC) in 50 mM $Na_2HPO_4$-NaOH (pH 11.4) for 48 h at 25°C (11).

**Hydrolysis of poly(dGC) containing [$^3$H]7-methylguanine or [$^3$H] rom$^7$G.**

Three different protocols were used : 1) Neutral hydrolysis at 100°C for increasing lengths of time in 5 mM $KPO_4$ buffer (pH 7.5). 2) 0.1N hydrochloric acid hydrolysis at 37°C for 17 h. 3) 75% (v/v) formic acid hydrolysis at 37°C for 17 h.

The bases were separated and analyzed by HPLC chromatography as described above.

**Enzymatic removal of rom$^7$G**

1) Preparation of E.coli rom$^7$G-DNA glycosylase. The rom$^7$G-DNA glycosylase was purified from extracts of E.coli HB 1100 endo$^-$ (14). The purification procedure involved chromatography on Biogel AO.5 to separate nucleic acids from proteins and DEAE-cellulose. The enzyme was further purified by Fast Protein Liquid Chromatography using mono-Q and mono-S columns (Pharmacia). The purified enzyme preparation used was essentially free of detectable

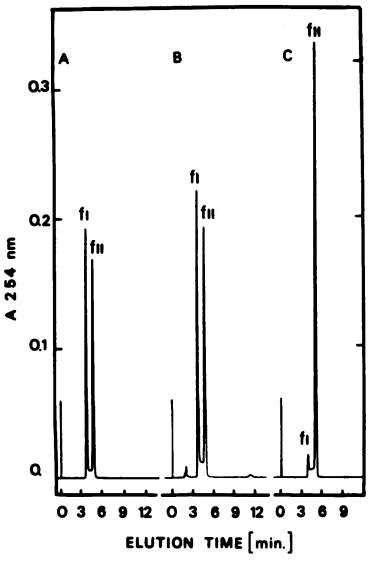


Figure 1 : Reversed phase HPLC analysis of rom$^7$G. A) Elution profile of rom$^7$G prepared as described in Material and Methods. B) Elution profile of rom$^7$G prepared according to Chetsanga and Lindahl (9). C) Elution profile of rom$^7$G lyophilyzed and dissolved in water immediately before HPLC analysis. 7-methylguanine and 7-methylguanosine eluted at 9.5 min. and 8 min. respectively.

non-specific DNase activity as well as other DNA-glycosylases (7,16). One unit of enzyme released 1pmol of rom$^7$G in 15 min at 37°C. The detailed purification and properties of the enzyme will be published elsewhere.

2) Enzymatic excision of rom$^7$G. The standard incubation mixture (50 µl) contained 70 mM Hepes KOH, 100 mM KCl, 2 mM $Na_2$-EDTA, 10% glycerol (pH 7.6) and 2500 cpm of [$^3$H] rom$^7$G-poly(dGC) and 1unit of rom$^7$G-DNA glycosylase. The reaction was carried out at 20°C for 15 min. When the incubation was completed, the reaction mixture was immediately injected in the HPLC system without further processing. The column was run as described above. The retention time of the marker molecules was not modified under these conditions. The proteins and the non-reacted DNA which were strongly adsorbed onto the column could be further eluted with methanol.

## RESULTS

Ring-opened 7-methylguanine was prepared from 7-methylguanosine by alkali cleavage of the imidazole ring and further elimination of the ribosyl residue by formic acid treatment. It was purified by Biogel $P_2$ chromatography and HPLC. When rom$^7$G was analyzed on a reversed phase column, two peaks (fI and fII) of the same magnitude were resolved, eluting at 4 and 5 min respectively (Fig. 1A). When rom$^7$G, prepared according to Chetsanga and Lindahl (10) (a gift from Dr. Lindhal), was analyzed under the same conditions, the same profile was obtained (Fig. 1B). When rom$^7$G is lyophylized then dissolved

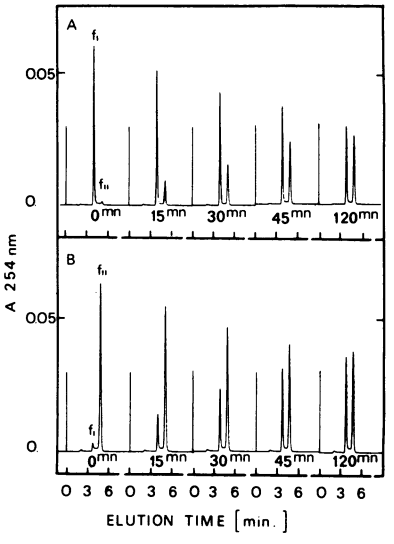


Figure 2 : Kinetics of interconversion at 30°C of the two species of rom$^7$G. A) The material eluting at 4 min (peak fI from fig. 1A) was isolated and stored at 0°C. It was further analyzed by HPLC after incubation at 30°C for 0, 15, 30, 45, 120 min. B) The material eluting at 5 min (peak fII from fig. 1A) was isolated, stored, analyzed as in A.

in cold water and immediately analyzed, only peak fII was observed eluting at 5 min (Fig. 1C). The two species were isolated by HPLC and were stable for more than 6h. at 0°C and for weeks at -20°C (data not shown).

In order to study the interconversion of the two forms, each was incubated at 30°C for increasing lengths of time. At the end of each incubation period, the sample was analyzed by HPLC. The profiles presented in Fig. 2A show the time-dependent conversion of peak fI in a mixture of peak fI and fII. The same result was obtained with peak fII which was converted in a mixture of fI and fII (Fig. 2B). The kinetics of interconversion of the two isolated species were studied. The rate of conversion of fI → fII and fII → fI increased with increasing temperature. The half-life of fI or fII was 120 min, 35 min, 22 min and 8 min at 20°C, 25°C, 30°C and 37°C respectively. These results suggest that the fI and fII species are isomeric forms of the same molecule rather than two distinct compounds.

To support this hypothesis NMR studies were performed taking advantage of the fact that after lyophilization rom$^7$G is in form II (Fig. 1C). At 289°K and in DMSO-$d_6$, the $^1$H NMR spectrum of the rom$^7$G showed two methyl singlets at 2.7 ppm and 2.81 ppm of respective intensity 88 % and 12 % with the corresponding formamido signals at 7.61 (88 %) and 7.88 ppm (12 %). The protons of the 2 and 6 amino groups exhibited distinct chemical shifts at 6.08 and 6.16 ppm whereas the signal of the oxo NH group was downfield-shifted (9.96 ppm) (Fig. 3). As expected, these latter resonances disappeared with the addition

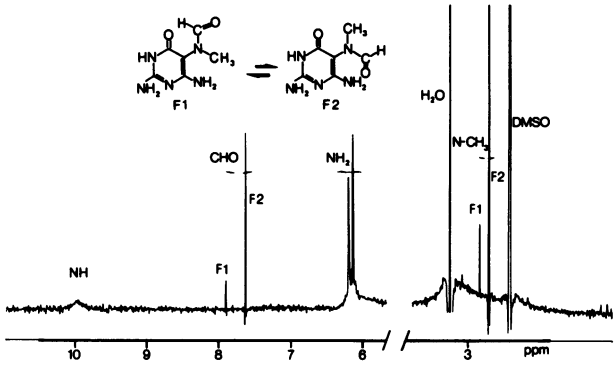


Figure 3 : $^1H$ NMR spectrum of the rom$^7$G in DMSO-$d_6$.

of $D_2O$. Increasing the temperature of the preceding solution from 289°K to 352°K does not induce significant changes in either chemical shifts or relative intensities of non-exchangeable protons.

A nuclear Overhauser enhancement (23%) was observed on the formamido proton at 7.61 ppm by irradiation of methyl signal at 2.7 ppm whereas this proton remained unchanged after irradiation on the other methyl peak at 2.81 ppm. This result clearly indicates that the resonances at 2.7 ppm and 7.61 ppm are in close proximity and therefore belong to the Z conformer of the N-methyl formamido group. On the other hand, irradiation of the methyl at 2.81 ppm (E isomer) led to a slight but unambiguous NOE effect (4.5 %) on the $NH_2$ signal at 6.08 ppm. Because this peak corresponds to only 12% of the E isomer, the actual NOE effect produced on the corresponding $NH_2$ group can be approximated at about 30%.

Taken together, these results suggest the occurence of two distinct but structurally related molecules or, more probably, to a mixture of two conformers in slow exchange. This latter hypothesis was clearly confirmed by an NMR study at 297°K in $D_2O$ (pH 7.8). In this solvent a slow equilibration process indeed occured (Fig. 4) with changes in the intensity of the methyl peaks at 3.02 ppm and the singlet at 8.06 ppm (fI), from 16 % to 53 % at equilibrium. Obviously the corresponding resonances at 2.9 ppm and 7.8 ppm (fII) decrease in the reverse manner. Such NMR equilibration allows calculations of both kinetics and thermodynamic parameters as already described in the isomerization of proline-containing derivatives (15). The plots of $Ln[^1/(fI)e - (fI)t]$ versus time (in sec.) for the $^1H$ methyl allows the calculation of the following value for the fII $\rightleftarrows$ fI equilibrium : $k_1$ (fII → fI) = 1.04 x $10^{-4} s^{-1}$;

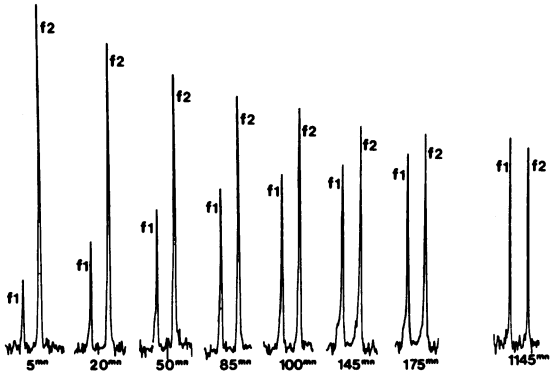


Figure 4 : Evolution of the $^1H$ methyl signals of rom$^7$G ($5.10^{-2}$M in $D_2O$) in the two rotamers as a function of time.

$k_{-1}$ (fI → fII) = 0.92 x $10^{-4}s^{-1}$; $\Delta G$ (fII → fI) = 22.9 Kcal/mole; $\Delta G$ (fI → fII) = 23.0 Kcal/mole; $\Delta G_0$ (fI → fII) = 0.07 Kcal/mole.

According to the high value of the free energy of activation and to the positions of the different signals, this equilibrium can be unambiguously assigned to a slow rotation around an amide bond. Such a process is expected for the four rotamers of 2-6diamino-3(H)oxo-4-N-methylformamido-5-pyrimidine arising from the ring opening of the 7-methylguanine. rom$^7$G exists under the two tautomeric forms hydroxy and oxo. This latter form is shown in Fig. 5. Irradiation of the methyl signal at 2.9 ppm produced a large increase (35%) of the signal at 7.8 ppm. As already discussed for the spectrum performed in

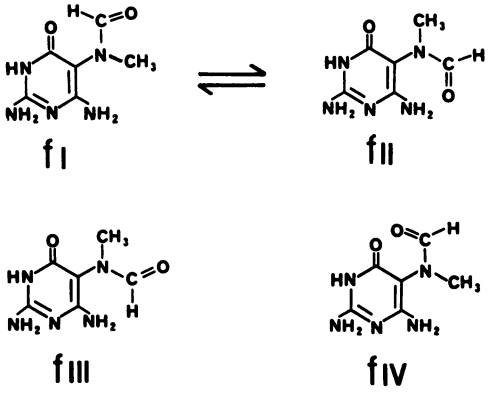


Figure 5 : Four rotamers of rom$^7$G.

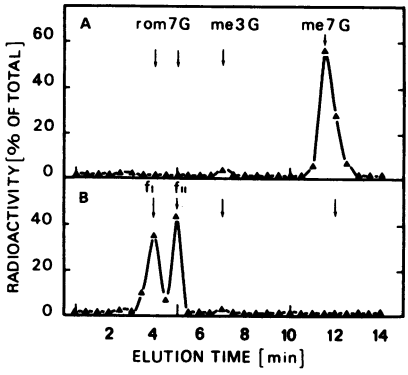


Figure 6 : HPLC analysis of methylated products released from [$^3$H] DMS-poly(dGC) with or without treatment under alkaline conditions. The substrates (2000 cpm) were hydrolyzed under formic acid conditions. A) [$^3$H]-DMS poly(dGC), O$^6$meG elutes at 23 min and less than 0.5% of the total radioactivity was recovered at this position. me$^3$G : 3-methylguanine ; m$^7$G : 7-methylguanine. B) [$^3$H]-DMS poly(dGC) treated under alkaline conditions : [$^3$H] rom$^7$G poly(dGC).

DMSO, this NOE effect related to a spatial proximity between N-methyl and formamido protons allows us to assign these resonances to one of the two Z-conformers fII or fIV. The fIV form can be eliminated, however, owing to the unfavourable electrostatic repulsion between the oxygen atoms. The structure of the E rotamer could be assigned to the form fI since, in this conformer, the $CH_3$ signal and the $NH_2$ group in position 6 are in proximity thus accounting for the NOE effect observed in DMSO-$d_6$ (see above). Finally, addition to 50% DMSO-$d_6$ in the equilibrated aqueous solution of 2,6 diamino 3(H) oxo-4, N-methylformamido-5-pyrimidine led to a slow decrease of the FI isomer without significant changes in the different chemical shifts.

What is the structure of rom$^7$G when this modified base is inserted into a polynucleotide chain? To answer this question, we have analyzed the nature of the products obtained after alkaline treatment of methylated poly(dGC). The polymer was alkylated with tritiated dimethylsulfate yielding poly(dGC)-containing methylated guanine residues [DMS-poly(dGC)]. Formic acid hydrolysis of DMS-poly(dGC) showed that 97% of the radioactivity eluted with 7-methylguanine, 2-3% with 3-methylguanine. O$^6$-methylguanine and methylated pyrimidine accounted for less than 1% (Fig. 6A). Hydrolysis at 100°C under neutral or acidic (0.1 N HCl) conditions gave identical results.

The [$^3$H]DMS-poly(dGC) was further treated under alkaline conditions in order to induce the cleavage of the imidazole ring yielding rom$^7$G-poly(dGC).

Formic acid hydrolysis and HPLC analysis of rom$^7$G poly(dGC) (Fig. 6B) showed that the 7-methylguanine residues completely disappeared whereas 3-methylguanine remains unchanged. Most of the radioactivity coeluted with rom$^7$G fI and fII (Fig. 6B). Complete release of rom$^7$G was also obtained by heating rom$^7$G-poly(dGC) for two hours at 100°C under neutral conditions. It should be noted that 0.1 N HCl hydrolysis of rom$^7$G-poly(dGC) showed in addition to the two peaks of rom$^7$G, a third peak of radioactivity eluting at 3 min. This new compound corresponds to the deformylated form of rom$^7$G as it can be reformylated by incubation with formic acid (9 and data not shown).

The conditions under which chemical hydrolysis was performed (length of time and temperature used), are not suitable to study the conformation of rom$^7$G in polynucleotides. To overcome this drawback we have analyzed the products released by an excess of E.coli rom$^7$G-DNA-glycosylase. In order to minimize the conversion of the rom$^7$G rotamers after their excision from the polynucleotide, the enzymatic reaction was performed at 20°C for 15 min and the products analyzed immediately after the end of the incubation. HPLC analysis of the products enzymatically released from rom$^7$-poly(dGC) is shown in fig. 7 : 27% of the radioactivity eluted as rom$^7$G rotamer fI and 73% with rotamer fII (see Fig. 5). Isolated rotamer fII incubated under the same conditions yielded 12% fI and 88% fII (Fig. 7, insert). Therefore, the amount of rotamer fI released by the rom$^7$G-DNA-glycosylase was significantly higher than expected from the conversion of pure rotamer fII only (12%).

## DISCUSSION

Analysis of the products resulting from alkaline fission of imidazole ring of 7-methylguanine have shown puzzling results. The differences might be due to the various ways of preparation and to the various chromatographic systems used to characterize the products (9,17,18). In this work both HPLC chromatography and NMR analysis showed that, in our conditions of preparation, rom$^7$G consists of two species corresponding to rotameric forms of pure 2-6 diamino-4-hydroxy-5N-methylformamidopyrimidine (Fig. 5). It should be noted that the two interconverting forms do not correspond to isomers resulting from a slow chemical exchange of the formyl group between the 5 and 6 positions of the pyrimidine ring as has been stated (18). Such an equilibrium would lead to one isomer bearing a basic secondary methylamino group but the lack of change in the chemical shifts of the methyl signals following addition of DCl in the aqueous solution allows us to rule out the occurrence of this isomer. Moreover, the kinetics and thermodynamic parameters of the equilibrium process are

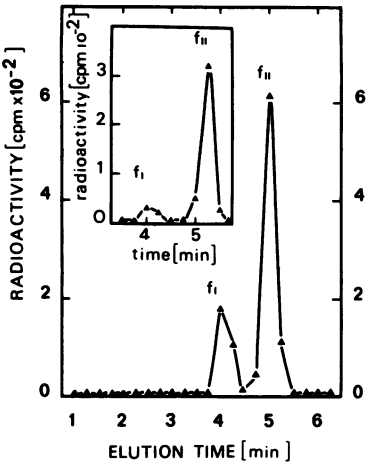


Figure 7 : HPLC analysis of the products released by E.coli rom$^7$G DNA glycosylase from rom$^7$G poly(dGC). The reaction mixture was incubated for 15 min at 20°C in the presence of 1.0 units of purified rom$^7$G-DNA glycosylase. The products of the reaction were directly analysed by HPLC. For details see Materials and Methods. INSERT : Mixture of fI and fII rotamers obtained after incubation of pure isolated fII rotamer for 15 min at 20°C in the enzyme assay mixture without substrate.

in the range usually found for rotational barriers around amide groups.

Finally, the structure of the two rotamers has been unambiguously established through NOE experiments and seems to indicate that, at least in fI, the N-methyl formamido group and the pyrimidine remains almost coplanar.

The nature of the equilibrium between the two rotamers into poly(dGC) chains cannot be directly studied. The analysis of the products excized by purified rom$^7$G-DNA glycosylase was used as an indirect approach. The HPLC analysis of the reaction products showed a large excess of the Z rotamer, (73%). However, the amount of E rotamer, fI was higher (27%) than expected from the interconversion of Z rotamer fII only (less than 12%). This result implies the occurence of both rotamers in rom$^7$G-poly(dGC) chains and that both are, to some extent, substrates for the rom$^7$G-DNA glycosylase. The bias in favor of fII might be due to either a preferential excision of fII by the enzyme, or a real excess of this rotamer in the poly(dGC) chains. This latter hypothesis is the more probable since formation of the fI rotamer requires rotation around both the C-N bond and the C=O bond : such process are probably hindered by base-pair stacking.

## ACKNOWLEDGMENTS

We thank C. Lagravère who prepared the rom$^7$G-DNA glycosylase used in this work. This work was supported by the Centre National de la Recherche Scientifique (LA 147 and ERA 613), the Institut National de la Santé et de la Recherche Médicale (U.140 and SC 21) and grant from the Association pour le Développement de la Recherche sur le Cancer.

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