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DNA Damage by Genotoxic Hydroxyhalofuranones: An in Silico Approach to MX

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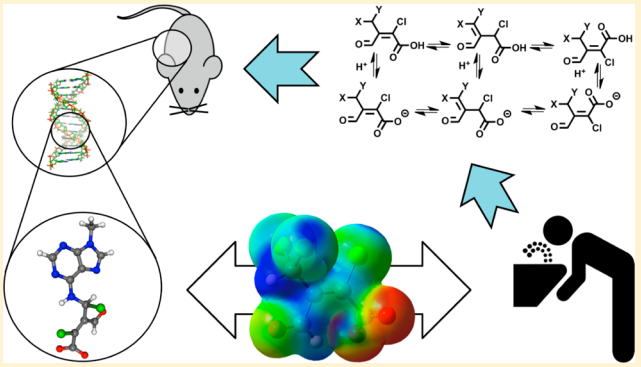
Supporting Information

ABSTRACT: MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone), a disinfection byproduct present in chlorinated drinking water, is one of the most potent mutagens known. Whereas its genotoxic effects are well documented, the mechanism by which MX exerts such an intense biological effect is still unclear. To gain further insight into both the general reactivity of hydroxyhalofuranones, and especially as regards their genotoxicity, here we report an in silico study of the aqueous reactivity of MX and two less powerful analogues (MXY, in general): (3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone −CMCF− and 3-chloro-4-(methyl)-5-hydroxy-2(5*H*)-furanone −MCF−). The following aspects were investigated: (i) the acid dissociation and isomerization equilibria of MXY, i.e. the species distribution among the possible isomers; (ii) the one-electron reduction potential of MXY; (iii) the guanosine and adenosine alkylation mechanism by MXY, which leads to covalent-DNA adducts; and (iv) the redox properties of the adducts. No significant differences were observed between MCF, CMCF, and MX, with a single exception: the unimolecular carbon−chlorine cleavage of some MX−nucleotide adducts may afford highly oxidative intermediates, which could be able to remove an electron from contiguous nucleotides directly, especially guanosine. This reaction would provide a pathway for the hypothesized ability of some hydroxyhalofuranones to oxidize DNA.

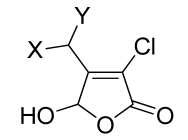


INTRODUCTION

Because of their potential impact on human health, genotoxic hydroxyhalofuranones arising from water chlorination have attracted widespread attention in recent years. Among these compounds,[1] 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone − also known as MX or Mutagen X − ranks among the most powerful mutagens known, affording record-high results in a variety of genotoxicity assays: 5600 to 13 000 revertants per nanomole were obtained in the *Salmonella* strain TA100;[2−5] MX proved twice as potent as aflatoxin B1 in the 8-azaguanine resistance assay in *Salmonella* TM677[6] and was seen to have the highest potency in inducing deletions, interchromosomal recombination, and aneuploidy of all carcinogens tested in the deletion-events assay in yeast (*S. cerevisae* strain RS112).[7]

Although its concentration is 100- to 1000-fold lower than that of other genotoxic halocompounds, such as trihalomethanes or haloacetic acids,[1,8] owing to its intrinsically higher genotoxicity MX is responsible for most of the observed mutagenicity of tap water. MX and many of its analogues (MCF and CMCF in Scheme 1) afford consistent positive results in a number of in vitro and in vivo assays, such as the comet assay, chromosomal aberrations, sister chromatid exchange, and so forth,[9−13] and they react covalently with the nucleotides adenosine, guanosine, and cytosine, affording

Scheme 1. MX and Some Analogues (MXY)



MCF: X = H, Y = H
CMCF: X = Cl, Y = H
MX: X = Cl, Y = Cl

polycyclic adducts.[14−16] Of these, only the adenosine-nucleotide adduct has been detected in the reaction of MX with calf-thymus DNA. Such adducts, however, are not consistent with the mutational pattern of this compound, which suggests guanosine lesions.[1,10−12]

Whereas many authors have addressed the alkylating capacity, mutagenicity, genotoxicity, and carcinogenicity of MX and its analogues (MXY, in general), the mechanism by which MX exerts such an intense biological effect − several orders of magnitude larger than that of the analogues MCF and CMCF, or mucohalic acids − is still unclear. In addition to the

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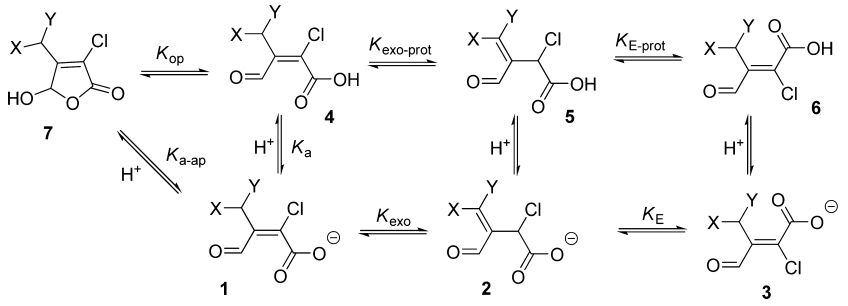
Scheme 2. Equilibria of MX and Analogues in Solution; X, Y = H, Cl



formation of covalent DNA adducts, it has been suggested that MX removes an electron from DNA, generating a charged radical, which leads to an abasic site or other type of lesion.[17] This hypothesis is also supported by the ability of MX to induce oxidative stress.[18]

Hydroxyhalofuranones are known to exist in equilibrium (Scheme 2) as a mixture of a cyclic lactone-lactol (**7**) and an open-chain aldehyde acid form (**4**), which can in turn undergo deprotonation and isomerization.[19−21] In its open-chain form, MX is also known to undergo base-catalyzed isomerization between the *Z* (**1** and **4**) and *E* (**3** and **6**) forms.[22] Also, on the basis of the structures of the DNA adducts, the reaction of MX and CMCF with DNA bases has been suggested to occur through an *α*,*β*-unsaturated MX isomer (**2** and **5**), in which the double bond is in an exocyclic terminal position, this species being the proposed effective alkylating agent.[14−16,23−25]

How MXY are distributed among all possible species in aqueous solution is of interest, especially because the amount of the highly electrophilic species **2** and **5**, could be very influential in the ability to react with DNA. Also, comparison with other halofuranones could shed some light onto the origins of the higher genotoxicity of MX. The high number of different species, their low relative concentrations, and the rates at which they interconvert make the experimental determination of the equilibrium constants for the reactions in question extremely complex, and we therefore approached the issue using ab initio and DFT computational methods. The low availability and the biological hazards of hydroxyhalofuranones also encouraged an in silico approach.

MXY have been proposed to cause oxidative damage to DNA in what can be called a redox hypothesis.[17] Accordingly, we studied the one-electron reduction potentials of MXY to check whether they were able to oxidize DNA directly.

In addition, because a good correlation between the theoretical results and experimental evidence has been found in the alkylation of nucleobases by mucohalic acids[26] − a group of halofuranones also formed in chlorination − we also performed a computational study of the alkylation reaction of nucleobases by these hydroxyhalofuranones. To explore the redox hypothesis, special attention was paid to the ability of MXY−nucleotide adducts to undergo one-electron reduction.

METHODOLOGY AND COMPUTATIONAL DETAILS

Determination of Equilibrium Constants. The free energies in solution of **1**−**7** at different levels of theory were calculated and, using the definition of equilibrium constant (eq 1), the values for the equilibrium constants of the processes shown in Scheme 2 were computed.

$$\log K = -\frac{\Delta G^\circ}{RT\ln 10} = -\frac{G_{\text{products}} - G_{\text{reactants}}}{RT\ln 10} \quad (1)$$

Calculation of pK_a Values. Because short-range solvent−solute interaction such as hydrogen bonding were not taken into account, the above approach was likely to introduce systematic errors in *G*, mainly in the solvation energies. If free energy differences are computed between similar species, such as, for instance **1**, **2**, **3** or **4**, **5**, **6**, these systematic deviations tend to cancel out. However, errors are not favorably compensated if the species on both sides of the chemical equation have very different solvation patterns, such as when one species is charged and the other is not.[27]

This is a very common problem in the calculation of pK_a values (AF ⇌ A^- + H^+), and suitable solutions exist: the use of relative methods, or the addition of explicit solvent molecules for accurate determination of solvation energies. Here, we opted for the use of a relative approach, using a homodesmic reaction of the type AH + B^- ⇌ A^- + BH. Inclusion of an appropriate reference or anchor (BH), whose experimental pK_a is well-known, and use of eq 2, allowed accurate calculation of pK_a values to within ±1 log units.[28−30] Trichloroacetic acid (pK_a = 0.7) was used as a reference owing to its structural similarities with open-chain halofuranones.

$$\text{p}K_a(\text{AH}) = -\log K_a(\text{AH}) = \text{p}K_a(\text{BH}) + \frac{\Delta G^\circ}{RT\ln 10} \quad (2)$$

A very similar approach has been used successfully to study the reaction equilibria of mucohalic acids in aqueous solution.[20]

Reaction Paths. The alkylation mechanism of adenosine and guanosine by MXY was modeled using density functional theory. All minima on the potential energy hypersurface were characterized by harmonic analysis (zero imaginary frequencies for reactants, and one for transition states) and the frequencies computed were used to obtain the thermodynamic parameters.

Vertical electron affinities were calculated as the free energy difference of the starting molecule and a radical anion with the same geometry. Adiabatic electron affinities were obtained using the relaxed geometry of the radical anion.

Computational Details. For the calculation of equilibrium constants, the geometries were optimized and the free energies were computed using the B3LYP hybrid functional with the 6-31++G(d,p) and 6-31++G(2df,2pd) basis sets, at the MP2/6-31++G(d,p) level, and also using the CBS-QB3 and G3MP2B3 compound methods. Regarding the reaction pathway, the geometries were optimized at the DFT-B3LYP 6-31G(d) level. Solvent was taken into account using the integral equation formalism polarizable continuum model (IEF-PCM). The *Gaussian 03* suite of programs was used for all calculations.

Table 1. Constants for Some MXY Equilibria

		B3LYP 6-31++G (d,p)	B3LYP 6-31++G (2df,2pd)	MP2 6-31++G (d,p)	CBS-QB3	G3MP2B3	mean
pK_{op}	MCF	4.27	4.00	4.11	6.24	5.68	4.86
	CMCF	4.01	3.22	3.49	5.95	5.40	4.41
	MX	4.11	4.41	3.70	6.20	5.66	4.82
pK_a	MCF	2.61	2.21	2.01	1.98	1.93	2.15
	CMCF	1.14	1.34	1.26	1.90	1.90	1.51
	MX	1.06	0.58	0.77	0.92	0.94	0.85
$pK_{a\text{-app}}$	MCF	6.88	6.20	6.12	8.22	7.61	7.01
	CMCF	5.15	4.56	4.75	7.55	7.30	5.86
	MX	5.17	4.98	4.47	7.12	6.60	5.67

RESULTS AND DISCUSSION

Equilibrium Constants and Species Distribution. *Equilibrium Constants.* The equilibrium constants (pK_a, pK_{op} and $pK_{a\text{-app}}$) computed for the most significant reactions are reported in Table 1. Table S1 of the Supporting Information reports the equilibrium constant for the other reactions.

Consideration of all negatively charged species and all noncharged species for the calculation of $pK_{a\text{-app}}$ afforded values within ±0.01 of those reported in Table 1, and hence a simplified definition of $pK_{a\text{-app}}$ was used (eq 3).

$$K_{a-app} = \frac{[\mathbf{1}] + [\mathbf{2}] + [\mathbf{3}]}{[\mathbf{4}] + [\mathbf{5}] + [\mathbf{6}] + [\mathbf{7}]}[H^+] \approx \frac{[\mathbf{1}]}{[\mathbf{7}]}[H^+] = K_a K_{op} \quad (3)$$

It was observed that, as occurs with other hydroxyhalofuranones, the cyclic form was far more stable than all the open-chain undissociated counterparts. Because the inductive effect of the CHXY group simultaneously modifies the nucleophilicity of the carboxylate group and the electrophilicity of the aldehyde group in opposite directions, little variation in pK_{op} should be observed between MCF, CMCF, and MX as, in fact, occurred.

The most stable open-chain form, **4**, had a pK_a value between approximately 1 and 2. This suggests that MCF, CMCF, and MX are strong acids, and justifies the choice of trichloroacetic acid as a reference for pK_a calculation. Given the inductive effect of the halogen atoms and the conjugative effect of the α,β-unsaturation, this result was not unexpected. For the same reasons, the decreasing order of pK_a was as follows: MCF > CMCF > MX. Previous theoretical estimates for the pK_a of MCF and MX (3.27 and 1.85)[6] were found to be rather high, especially when compared to the pK_a of chloroacetic acid (2.85), which lacks the conjugative effect of the double bond and the carbonyl group.

Because of this high acidity, halofuranones exist either as the cyclic form in acidic media (**7**), or as the open-chain anions at higher pH values (mostly the *Z*-form, **1**), the intermediate protonated open-chain species always being minor.

Thus, the apparent dissociation constants of **7** to **1**, (which correspond to those measured experimentally either by UV−vis or NMR spectroscopy or by titration), are about 10^{-6} for the more halogenated furanones and around 10^{-7} for MCF. These values are in good agreement with the experimental results reported in the literature: $pK_{a\text{-app}} = 5.25$ for MX[15,22] and $pK_{a\text{-app}} \sim 6$ for MCF.[25]

Species Distribution. Because the pH values of tap and natural waters as well as that of the cellular medium are close to neutral, the equilibrium concentrations of **1**−**7** at pH 7.00 were determined. Tables S2 and S3 of the Supporting Information report these values as the negative logarithms (p[**i**]) of the relative concentrations at pH 7.00 and 4.00 with respect to the major species (**1** and **7**, respectively).

At pH levels close to neutral MXY are predicted to exist in equilibrium, mostly in the dissociated *Z* form (**1**), with significant equilibrium concentrations of the *E* form (**3**): about 10% for MCF and CMCF and 1% for MX. This observation can be understood in terms of the steric bulk of the CXY group: Steric hindrance forces the carboxylate moiety to deviate from the more conjugated planar conformation. Because the chlorine atom in CMCF is oriented outward, similar effects are observed for MCF and CMCF, whereas repulsion is stronger in MX.

The cyclic form (**7**) is also a major species, with an equilibrium concentration of about 10% for CMCF and MX. In the case of MCF, whose somewhat low acidity displaces the **7** ⇌ **4** ⇌ **1** + H^+ equilibrium toward the closed-chain form, the equilibrium concentration of **7** is higher and accounts for more than 25% of the total concentration.

For all three halofuranones, the relative equilibrium concentration of the putative alkylating intermediate **2** was very low (1:10^4 approx.). No significant differences within the accuracy of the method were observed in the concentration of **2** for MCF, CMCF, and MX, which suggests that the extraordinary behavior of MX does not arise from an abnormally high concentration of **2**. Thus, the unmatched genotoxicity of MX could lie in the inherent reactivity of MX-**2**, rather than in its high concentration, or, as has been proposed, it could be due to a mechanism other than DNA alkylation, such as oxidative damage.[17]

All undissociated open-chain forms were present in very minor amounts, with concentrations about six logarithmic units below that of the major species. However, the species featuring an exocyclic double bond (**5**) was much more significant among the neutral forms than among the anions, the concentrations of **4**, **5**, and **6** being very similar. The reasons for this different behavior probably lie in the fact that the negative charge is stabilized in **1** and **3** by the extended conjugation, as compared to **2**. Because no charges are present, loss of this conjugation by species **4** and **6** resulted in a less dramatic destabilization.

Owing to its high acidity, MX had the lowest concentration of undissociated species of the three compounds studied, which further ruled out the high concentration of **5** as a source of the high mutagenicity.

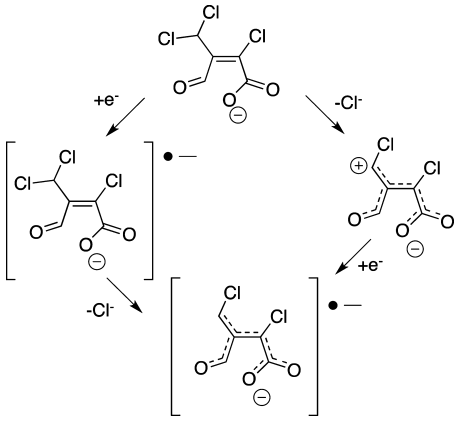
One-Electron Reduction. It has been suggested that MX can remove an electron from DNA, especially from guanosine oxidation hot-spots. This would afford a radical cation within DNA, which is likely to lead to abasic lesions.[17] This mechanism of DNA damage would be in better agreement

with the observed mutational pattern of MX than the nucleotide adducts found so far. In addition, some evidence for the ability of MXY to cause oxidative damage exists.[18] However, no proof of MXY being such powerful oxidants is available.

We computed the electron affinities of MXY in their open-chain anionic and closed forms (**1** and **7**). The results (Table S4 of the Supporting Information) suggested that MXY had quite high electron affinities and thus can be reduced quite easily.

The radical anion forms of CMCF and MX are unstable and expel a chloride anion affording a neutral radical. This stabilized the system to a considerable extent and hence was responsible for the large difference between the vertical and adiabatic electron affinities (Scheme 3).

Scheme 3. One-Electron Reduction of MX



The ionization potentials of nucleobases and (poly)-nucleotides, and the closely related one-electron oxidation potentials, have been determined experimentally. In general, guanine and guanine-containing oligonucleotides show the lowest ionization potential, which is around 5 eV in solution.[31] Several computational studies have also addressed the issue, good agreement being found between high-level theoretical and empirical values.[32,33] For comparison with the electron affinities of MXY, we obtained somewhat overestimated values of 6.3 eV for adenosine and 6.0 eV for guanosine (similar results to within 0.05 eV were obtained with the 6-31G(d)) and 6-31++G(d,p) basis sets).

The differences between the IP of the nucleotides and the EA of the MXY are conclusive: The vertical reduction potentials of MXY are too small for the MXY, both in their open-chain and closed forms, to oxidize nucleotides directly.

An alternative pathway, in which chloride is lost *before* reduction, could also be possible. However, the energy barrier for the unimolecular cleavage is quite high (120 kJ mol^{-1} for MX at the B3LYP/6-31++G(d,p) level of theory) and the intermediates are very high in energy (~90 kJ mol^{-1}), strongly favoring the back-reaction.

Alkylation Mechanism of Adenosine and Guanosine. Certain evidence suggests that the mode of action of MX is very different from that of other halohydroxyfuranones and hence that the formation of covalent DNA adducts is not the cause of MX mutagenicity but rather that some other unknown mechanism would be involved.

For instance, the mutational pattern of MX, consisting mainly of GC→TA transversions, is different from that of other halofuranones, such as MCF (but quite similar to that of CMCF) or mucohalic acids, which are known to alkylate adenine residues.[1,10−12] In addition, only adenosine adducts have been observed in the reaction of MX with purified double-stranded DNA. No guanosine adducts, which would be more consistent with the observed mutational pattern of MX, have been detected. This suggests that lesions in guanine bases may occur through some mechanism other than the formation of stable DNA adducts with guanine.

Whereas the presence of high concentrations of **2** or **5** can be dismissed as the source of the exceptional behavior of MX, the inherent reactivity of these electrophiles could still be related to the particular biological effects of MX.

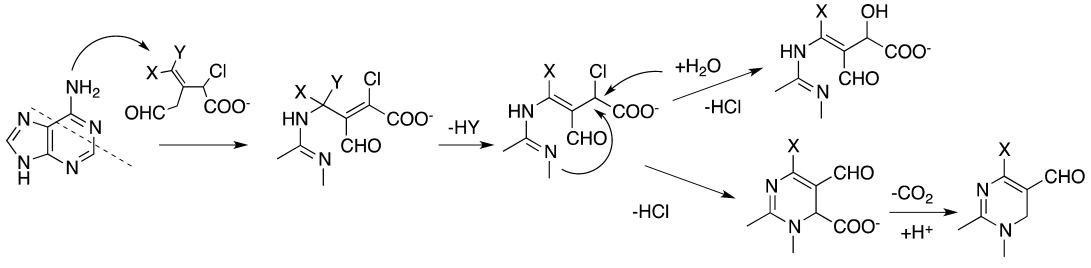
The reaction products of nucleotide alkylation by MXY reported in the literature are shown in Scheme S1 of the Supporting Information. Where possible, the nomenclature used in the literature has been maintained.

Briefly, the alkylation reaction of nucleotides by MXY has been proposed to occur as depicted in Scheme 4: The exocyclic amino group reacts with the terminal double bond of the **2** isomer, and a chlorine atom from the terminal methylene is expelled. CMCF and MX, but not MCF,[25] undergo further isomerization, cyclization, and decarboxylation reactions (Supporting Information for a detailed description).[14−16]

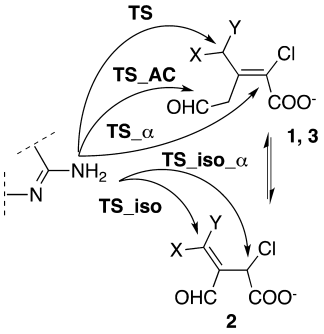
To gain insight into how the adducts reported in the literature are formed, and to assess whether MX in any of its isomeric forms shows exceptional reactivity as an alkylating agent, we modeled several modes of nucleophilic attack of the nucleobases on the halofuranones (Scheme 5).

The energy barriers for the direct displacement of a halogen atom on the methyl group by the exocyclic amino group in adenosine and guanosine (**TS**) in either the *Z* (**TS_Z**) or *E* (**TS_E**) isomeric forms were determined. Because MCF lacks any substitution on the methyl group, this pathway is only possible for CMCF and MX. The formation of the hemiaminal (**TS_AC**), the addition of the nucleophile on the terminal double bond in the iso form of the halofuranones (**iso_TS**), and the substitution of the chlorine atom α with respect to the carboxylate group, both in the major form (**TS_α**) and in the

Scheme 4. Proposed General Alkylation of Adenosine and Guanine by MXY



Scheme 5. Modes of Nucleophilic Attack of the Nucleobases on the Halofuranones Studied. X, Y = H, Cl



terminally double-bonded isomer (**TS_iso_*α***), were considered. Table 2 shows the computed free energy differences for the nucleophilic attacks shown in Scheme 5. Although guanosine can exist as both keto and enol isomers, the keto isomer was found to be more stable in all cases.

Table 2. Calculated Energy Barriers for the Alkylation of Nucleotides by MXY

	Reaction Barrier (ΔG kJ mol^{-1})[a]					
	dA			dG		
	MCF	CMCF	MX	MCF	CMCF	MX
TS_Z		96.5	109.6		111.3	131.2
TS_E		75.6	98.1		81.5	109.8
TS_*α*		111.6	110.3		120.4	131.9
TS_AC	115.5	131.3	126.6	112.4	128.7	129.9
TS_iso	87.1	107.4	135.9	86.6	101.5	137.6
TS_iso_*α*	109.5	109.2	109.7	110.3	111.4	130.9

[a]With respect to the nucleotide +*E*−MXY system.

In the case of CMCF and MX, attacks **TS_Z** and **TS_E**, which involve species **1** and **3**, show lower energy barriers than **TS_iso** and **TS_iso_*α***, which involve **2**. This suggests that the alkylation pathway proceeds through the major open-chain species (**3** and **1**) and not through the very minor terminally double-bonded **2**, contrary to what has been proposed in the literature.[14−16,23−25] Moreover, in the less-favored reaction of 2, it is the *α* chlorine atom with respect to the carboxyl group that acts as a leaving group − as observed experimentally for MCF − and not the chlorine atom on the methyl group, as observed experimentally for MX and CMCF. This confirms that the alkylation of both adenosine and guanosine by CMCF and MX most likely proceed through **3**. The reaction with adenosine shows a lower barrier, in keeping with the fact that no guanosine adducts have been detected in the reaction of MX with double-stranded DNA. Interestingly, the barrier for the alkylation by the *Z* form was ∼20 kJ mol^{-1} lower, in keeping with some observations suggesting that the *Z* form may be more strongly mutagenic.[8] It should be kept in mind that the *Z* isomers are around 5−10 kJ mol^{-1} less stable than *E*, as evidenced by their equilibrium concentrations.

The favored pathway for CMCF and MX does not exist for MCF, because it lacks any halogen atoms in the methyl group, and hence the nucleophilic attack on MCF takes place at the terminal double bond of **2**, or at the carbonyl group, in accordance with the products isolated experimentally.[25]

These results are coherent with the known final products of MXY−nucleotide reactions and suggest that MXY are moderately strong alkylating agents. Nevertheless, they are weaker (even in their more active *E* form) than similar compounds, for example mucohalic acids,[26] or other genotoxic compounds,[34] which, however, exhibit lower activity in genotoxicity assays. Also, CMCF has a much lower alkylation barrier, which suggests an increased alkylating ability, contrary to the trends observed in biological assays.

Scheme 6 depicts the subsequent reactions undergone by the MXY−nucleobase adduct after the initial nucleophilic attack (depicted as TS in Scheme 5).

The reaction sequence commences in **AD1**, which is rapidly formed from the initial product of the alkylation reaction through loss of a proton. **AD1** may undergo an isomerization reaction in the position of the double bond, affording **AD2**. Both isomers may undergo nucleophilic additions: (i) ring closure of **AD2** (**TS_Cycl**), affording a fairly stable six-membered ring (**AD3**); (ii) hydrolysis of the allyl chlorine substituent in **AD2** (**TS_Hyd**); and (iii) ring closure of **AD1** to afford a five-membered cycle (**TS_Cycl_5**).

There is experimental evidence suggesting the existence of reactions occurring through **TS_Cycl** and **TS_Hyd**: products arising from the reaction of **AD2**-CMCF have been observed experimentally (pfA-dR and OH-fbA-dR, Scheme S1 of the Supporting Information). Whereas reaction products including a five-membered ring have been reported in the reaction of guanosine with MX − but not in any other combination − no transition states leading to the five-membered species (**TS_Cycl_5**) could be found for any of the halofuranones, and hence the products can be expected to arise from another intermediate (below).

Scheme 6. Evolution of MXY−Nucleotide Adducts; X = H (CMCF), X = Cl (MX)

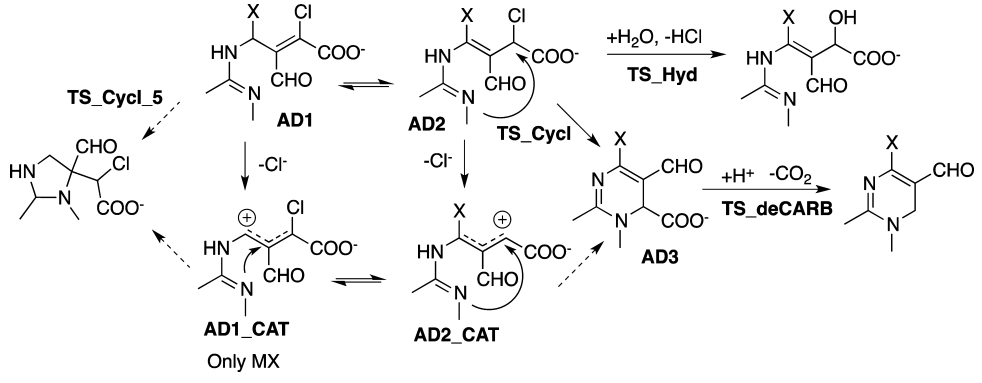


Table 3. Energy Barriers for Further Reactions of MXY−Nucleotide Adducts

	ΔG (kJ mol^{-1})								
				GUA					
	ADE			Keto			Enol		
	CMCF	MX		CMCF	MX		CMCF	MX	
	Op−H	Op−H	Cl	Op−H	Op−H	Cl	Op−H	Op−H	Cl
AD1	38.6	0.0	0.0	58.7	0.0	0.0	93.6	11.8	15.6
AD1_S_N1_CAT		−13.2	14.2		−16.8	31.3		−12.6	10.3
AD2	0.0	1.5		0.0	31.2		42.5	40.8	
AD2_S_N1_CAT	16.4	44.1		20.6	23.3		−23.3	30.4	
TS_HID	86.3	59.7		40.7	76.7				
TS_Cycl	85.7	81.3		162.7	155.4		156.9	225.3	
AD3	−47.5	−53.1							
TS_deCARB	120	109							

Table 4. Electron Affinities in Aqueous Solution for the MXY−Nucleotide Adducts

		EA (eV)					
		vertical			adiabatic		
		CMCF	MX		CMCF	MX	
		Op−H	Op−H	Cl	Op−H	Op−H	Cl
dA	**AD1**	1.5	2.0	2.2	3.5	4.4	4.7
	AD2	2.3	1.9		3.9	4.3	
	AD1_S_N1_CAT		3.9	4.6		4.2	4.9
	AD2_S_N1_CAT	3.6	3.4		3.9	4.8	
dG	**AD1_ket**	1.1	1.7	2.5	3.3	4.3	4.7
	AD1_en	1.1	2.9	2.5	3.2	4.2	4.6
	AD2_ket	1.4	2.1		3.8	4.1	
	AD2_en	1.6	1.8		4.0	3.7	
	AD1_ket_S_N1_CAT		3.7	4.7		4.1	5.0
	AD1_en_S_N1_CAT		3.5	4.4		3.9	4.7
	AD2_ket_S_N1_CAT	4.0	3.6		4.0	4.0	
	AD2_en_S_N1_CAT	3.3	3.4		3.8	3.6	

Table 3 reports the relative energies along the reaction paths depicted in Scheme 6. Because the adducts themselves feature an aldehyde and acid moieties, a ring-closure equilibrium between open-chain (Op-H) and cyclic (Cl) forms still exists within the adduct molecules. The adducts formed by CMCF and MX show comparable energy barriers for the subsequent reactions, the only appreciable difference observed in the relative stability of **AD1** and **AD2**: **AD2** was clearly favored by CMCF, whereas for MX there was a preference for **AD1**. Although this observation may be consistent with the stronger tendency of MX to form adducts containing five-membered rings, it can hardly account for the ability of MX to damage guanosine positions.

Unimolecular Cleavage. The interatomic distance in the allylic C−Cl bond in **AD1**, and to a lesser extent in **AD2**, is exceptionally long (∼205 pm and ∼195 pm, respectively) as compared with a common value around 170−195 pm, which is suggestive of a very labile bond. In addition, adenosine adducts of MX that have no any chlorine atoms have been observed experimentally (pfA-dR in Scheme S1 of the Supporting Information).[15] Therefore, unimolecular cleavage of the allyl C−Cl bond in the adducts is considered in this section. This reaction readily affords chloride ion and a cationic adduct, which can be expected to be a highly reactive electrophile/electron acceptor.

Our calculations showed that, contrary to the results obtained for the parent halohydroxyfuranones, the S_N1-formation of the cation is highly favored, possibly because of the conjugation with the extended π systems of the nucleobases: The cleavage has low positive reaction free energies and is even exoergonic in some cases (Table 3). Important differences between CMCF and MX can be observed: CMCF can only yield the **AD2**-like cation, whereas the **AD1** adduct formed by MX is more prone to heterolytic cleavage.

These carbocationic adducts may react in several different ways: trapping nucleophiles such as water or nucleophilic nitrogen positions, or undergoing elimination. In fact, the polycyclic final products isolated in nucleotide-alkylation experiments (Scheme S1 of the Supporting Information) may be formed by the reaction of the cations after S_N1 cleavage rather than by S_N2 displacement of **AD1** and **AD2**.

This is in agreement with the formation of polycyclic adducts and accounts for the existence of a five-membered MX adduct, whose formation cannot be explained in terms of bimolecular reactions: In the case of CMCF, **AD1** isomerizes to the more stable **AD2** and the reaction affording the five-membered ring is avoided. However, MX favors **AD1** over **AD2**, especially in the case of guanosine (by a difference of 30 kJ mol^{-1}, Table 3), which may explain why only the MX−guanosine combination affords five-membered adducts. This also explains the loss of all chlorine atoms initially present in MX in the formation of certain final products (pfA-dR, Scheme S1 of the Supporting Information): the first one in the nucleophilic attack; the

second in the unimolecular cleavage reaction, and the third one in the final ring-closure reaction. The unimolecular cleavage pathway also helps to understand the formation of adducts showing the substitution of chlorine by hydroxide, since the barrier for the S_N2 reaction is very high (especially when considering that hydroxide ions are present at a concentration of 10^{-7} M in vivo, which roughly corresponds to a 40 kJ mol^{-1} increase in the reaction barrier).

Because MX and CMCF clearly show different patterns as regards cationic products of S_N1 cleavage, this is very probably related to the greater biological effects of MX. However, the issue of guanosine residues being damaged cannot be accounted for by these covalent adducts, either cationic or neutral. An oxidation scheme would indeed explain the damage to guanosine − the most easily oxidized base. Nevertheless, the calculated vertical electron affinities of MXY suggest that this cannot be a direct reaction. Thus, we have addressed the ability of the MXY−nucleotide adducts and their cations to undergo one-electron reduction.

Reduction. The results obtained at the DFT-B3LYP/6-31G(d) level of theory (Table 4) suggest that MXY−nucleotide adducts have high adiabatic electron affinities, which is due to the loss of the chloride anion, as occurred in the parent halofuranones. Vertical electron affinities are moderate and, in general, seem to be insufficient to oxidize the bases directly.

Despite the above, there is one clear exception: the cations arising from the unimolecular cleavage of the adducts, both in their open and closed-chain furanone isomeric forms, show very high vertical electron affinities.

Electron affinities depend strongly on the choice of method and basis set, and thus we have improved the results for the most interesting species: that is the cations, using the 6-31++G(d,p) and 6-311+G(2df,2p) basis sets. These superior basis sets afford larger electron affinities, by about 0.25−0.50 eV (Table S5 of the Supporting Information).

The electron affinities of the cations are thus quite high, and the highest values overlap with the experimental ionization potential of guanosine (adiabatic electron-detachment energies are around 5.0 ± 0.1 eV for mono-, di-, and trinucleotides containing at least one guanosine).[31]

Electrons are known to be able to migrate along the DNA polymer,[35] which would be consistent with the lesions taking place in mutational hot-spots for oxidation, somewhat independently of the position of the alkylated nucleotide. The $\pi-\pi$ stacking of the bases in DNA, and the orbital overlap would allow ready electron transfer from the guanine nucleotides to the MXY−nucleotide cationic adducts, affording two radicals, whose further reactivity could produce a variety of DNA lesions.

Whereas the precise determination of the redox properties of the species involved would require a complex approach, including higher levels of theory and modeling of the adjacent bases, the results obtained here do support the redox hypothesis. Our findings suggest that MXY may be responsible for oxidative DNA damage, albeit indirectly, through the formation of cationic DNA adducts, and they suggest targets for further research, both experimental and theoretical.

The following conclusions can be drawn from this work:

i. The equilibrium composition of the hydroxyhalofuranones studied (MCF, CMCF, and MX) at neutral pH is mostly a mixture of the open-chain *E* and *Z* carboxylates, and the closed-chain furanone (Table 1).
ii. The equilibrium concentration of the terminally double-bonded electrophilic isomer is equally low for the three MXY (Table S1 of the Supporting Information).
iii. The lowest-energy alkylation mechanism by CMF and MX takes place through direct nucleophilic attack on the (di)chloromethyl group, rather than at the terminal double-bond of a minor, highly electrophilic isomer (Table 3).
iv. CMCF and MX show very similar alkylation pathways.
v. MX is not a stronger alkylating agent than MCF or CMCF as regards their reactivity with nucleotides (Table 3).
vi. The unimolecular carbon-chlorine bond cleavage in MX−nucleotide adducts may lead to the formation of strong oxidants within the DNA double helix (Tables 4 and Table S5 of the Supporting Information). The in situ generation of these species would be in agreement with the redox hypothesis advanced to explain MX genotoxicity.

■ ASSOCIATED CONTENT

Supporting Information

Isomerization equilibrium constants, and concentrations of MXY at pH 7.00 and 4.00, and calculated electron affinities, and schemes depicting known MXY−nucleotide adducts. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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