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DNA-Damaging Disinfection Byproducts: Alkylation Mechanism of Mutagenic Mucohalic Acids

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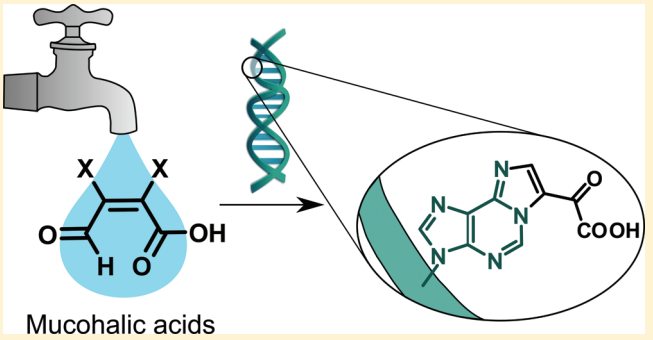
Supporting Information

ABSTRACT: Hydroxyhalofuranones form a group of genotoxic disinfection byproduct (DBP) of increasing interest. Among them, mucohalic acids (3,4-dihalo-5-hydroxyfuran-2(5H)-one, MXA) are known mutagens that react with nucleotides, affording etheno, oxaloetheno, and halopropenal derivatives. Mucohalic acids have also found use in organic synthesis due to their high functionalization. In this work, the alkylation kinetics of mucochloric and mucobromic acids with model nucleophiles aniline and NBP has been studied experimentally. Also, the alkylation mechanism of nucleosides by MXA has been studied *in silico*. The results described allow us to reach the following conclusions: (i) based on the kinetic and computational evidence obtained, a reaction mechanism was proposed, in which MXA react directly with amino groups in nucleotides, preferentially attacking the exocyclic amino groups over the endocyclic aromatic nitrogen atoms; (ii) the suggested mechanism is in agreement with both the product distribution observed experimentally and the mutational pattern of MXA; (iii) the limiting step in the alkylation reaction is addition to the carbonyl group, subsequent steps occurring rapidly; and (iv) mucoxyhalic acids, the hydrolysis products of MXA, play no role in the alkylation reaction by MXA.



INTRODUCTION

Water disinfectants such as chlorine, ozone, chlorine dioxide, or chloramines can react with naturally occurring organic matter, pollutants, or anions such as bromide and iodide during the production of drinking water.[1] Disinfection byproducts (DBPs) formed in these reactions, such as halomethanes or haloacetic acids, are responsible for most of the observed mutagenicity of chlorinated tap water[2−5] and have been linked to the incidence of bladder[6] and colorectal[7] cancer.

A group of DBPs of rising interest and yet to be regulated are the halofuranones, which are formed in the chlorination of organic matter, such as humic substances. Their genotoxic and carcinogenic properties have been studied thoroughly and are well-known.[8,9] Among these halofuranones formed in chlorination are mucochloric (MCA, 3,4-dichloro-5-hydroxyfuran-2(5*H*)-one), and mucobromic acids (MBA, 3,4-dibromo-5-hydroxyfuran-2(5*H*)-one), both of which are direct genotoxins and potential carcinogens.[10−16] Aside from their role as anthropogenic pollutants, mucohalic acids are gaining use in organic synthesis, due to their high functionalization and availability.[17−25]

Both mucohalic acids (MXA) are known to alkylate the DNA bases guanosine, adenosine, and cytidine equally in the form of monomers and forming part of DNA, giving rise to etheno, oxalo etheno, and halopropenal derivatives.[26−33]

The reaction of mucohalic acids with hydroxide ions to form mucoxyhalic acids (MOXA) has been proposed in some works as the initial step in the reaction of MXA with DNA bases.[26,29−31] However, our previous work suggests that the rate of formation of MOXA in the cellular conditions is negligible.[34] This is in agreement with the alkylation mechanism proposed in more recent works,[31,33] shown in Scheme 1. Identical reaction paths are expected for adenosine, guanosine, and cytidine: alkylation of the exocyclic secondary amine followed by elimination, hydrolysis, cyclization, and decarboxylation.

Additional minor pathways exist, yielding α-hydroxy chlorohydrins from guanosine[31] and also dialkylated products in an excess of adenosine.[28]

In this work, a kinetic study of the alkylation reactivity of MXA using monofunctional model nucleophiles was undertaken. Since mucohalic acids react with the exocyclic primary amino groups of guanosine, cytidine, and adenosine, aniline (AN) was chosen as a model for those groups. 4-(*p*-nitrobenzyl)pyridine (NBP) is a known trap for alkylating agents and gives its name to a simple colorimetric assay and was chosen to model the reactivity of endocyclic nitrogen atoms. Its use as a nucleophilicity model for the aromatic nitrogen atoms of nucleotides has allowed us to gain insight into the alkylation mechanisms of both strong[35−40] and weak electrophiles.[41−44]

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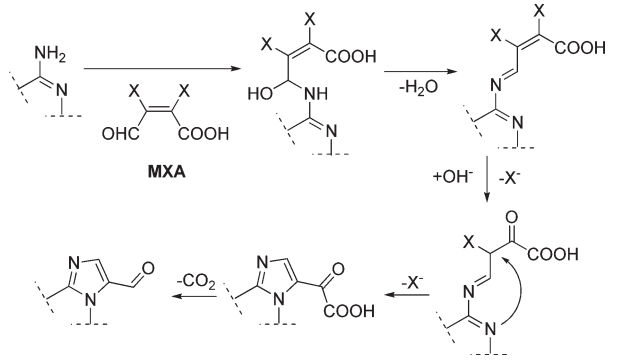
Scheme 1. Proposed Reaction Mechanism for the Alkylation of Nucleotides by MXA



In addition, the alkylation reaction of adenosine by mucohalic acids has been modeled *in silico* to gain insight into the alkylation mechanism and the order in which the successive reaction steps take place.

EXPERIMENTAL SECTION

Reactions were monitored by UV−vis spectroscopy in a Shimadzu UV2401 PC with a thermoelectric six-cell holder temperature control system (±0.1 °C). The reactions were carried out in an excess of nucleophile, and thus the pseudo-first-order approximation was applied. pH was kept constant by using buffer solutions: 0.020 M CH_3COOH/CH_3COO^- for 4 < pH < 6 and 0.020 M $H_2PO_4^-/HPO_4^{2-}$ for 6 < pH < 8. Electrospray ionization mass spectra were recorded on a Waters ZQ4000 spectrometer by direct injection.

Reaction with Aniline (AN). Reactions were carried out in water, and in 7:3 (vol) water/dioxane (*w*/*d*) mixtures. The kinetic species was monitored at λ = 320 nm for both MBA and MCA, where aniline shows almost null absorption. The concentration of MXA was in the 7−50 μM range and that of aniline was in the 1−20 mM range.

Reaction with 4-(*p*-nitrobenzyl)pyridine (NBP). Because the adduct formed in the alkylation of NBP by MXA shows significant absorption in the visible region of the spectrum, a variation of the NBP test was used in which basification of the reaction media to obtain color was not necessary. Thus, the alkylation reaction was monitored continuously in the reaction medium at λ = 485 nm for MBA and λ = 510 nm for MCA. The MXA concentration was in the 30−200 μM and that of NBP in the 5−20 mM range. Because NBP is insoluble in water, reactions were carried out in 7:3 (vol) *w*/*d* mixtures.

Computational Calculations. Geometries were optimized at the B3LYP/6-31G(d) level, and were characterized as either minima or transition states by harmonic analysis. Solvent effects were included using the IEF-PCM method with default parameters. Calculations were performed using the Gaussian 03 suite of programs.

Safety. Mucohalic acids have tested positive in a number of genotoxicity assays, including the *Salmonella* assay. They should be handled with care and disposed properly.

RESULTS AND DISCUSSION

Reaction with Aniline. Figure 1 in Supporting Information (SI) depicts the variation in the UV−vis spectrum of the reaction mixture along time during the reaction of MXA with excess aniline. The variation in absorbance (*A*) with time at the wavelength of maximum absorption of the adduct, λ = 320 nm, (Figure 2, SI),

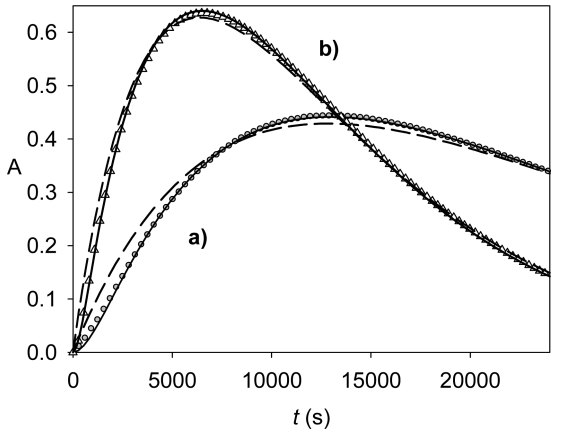


Figure 1. Typical kinetic profile of the alkylation reaction of NBP by MXA. Dashed line (− − −): fit to a two-reaction mechanism; solid line (— — —): fit to a three-reaction mechanism. Symbols: experimental, (○) MCA, (Δ) MBA. [NBP] = 0.015 M, *T* = 25.0 °C. (a) [MCA] = 2.0 × 10^{-5} M, (b) [MBA] = 2.0 × 10^{-5} M, pH = 7.00, 7:3 water/dioxane (vol).

complies with a pseudo-first-order rate equation (eq 1)

$$A = \varepsilon_{\text{AN-MXA}} l [\text{MXA}]_o (1 - e^{-k_{1\text{alk}}^{\text{AN}} t}) \quad (1)$$

where $\varepsilon_{\text{AN-MXA}}$is the molar absorption coefficient of the adduct; *l* is the optic path, and $k_{1\text{alk}}^{\text{AN}}$ is the alkylation pseudoconstant.

$k_{1\text{alk}}^{\text{AN}}$ is proportional to the concentration of aniline and hence

$$k_{1\text{alk}}^{\text{AN}} = k_{\text{alk}}^{\text{AN}} [\text{AN}] \quad (2)$$

From these results the following experimental rate equation (eq 3) can be derived:

$$\frac{d[\text{AD}_{\text{AN-MXA}}]}{dt} = k_{\text{alk}}^{\text{AN}} [\text{MXA}][\text{AN}] \quad (3)$$

The fit to eq 1 affords the $k_{1\text{alk}}^{\text{AN}}$ and $\varepsilon_{\text{AN-MXA}}$values ($\varepsilon_{\text{AN-MCA}}$= (30.3 ± 0.2) × 10^3 M^{-1} cm^{-1}; $\varepsilon_{\text{AN-MBA}}$= (27.3 ± 0.3) × 10^3 M^{-1} cm^{-1} at λ = 320 nm). The reaction rate was observed to be pH-independent in the 5.5 < pH < 8.0 range. Table 1 shows the alkylation rate constants obtained in water and aquo-organic reaction media at different temperatures. The two mucohalic acids react at very similar rates, with the reaction with MBA being slightly faster. The decrease in the dielectric constant when switching to 7:3 (vol) water/dioxane (*w*/*d*) medium elicits approximately a 6-fold decrease in the rate constant.

The reaction rate for the alkylation of aniline is very fast compared to the rate of formation of mucoxyhalic acids (MOXA) from mucohalic acids.[34] The hydrolysis half-life of MXA at 25.0 °C and pH = 8.0 is of the order of years,[34] whereas the alkylation half-life in the same conditions ranges from minutes to hours. This confirms that the role of MOXA in the alkylation by MXA is very small or inexistent, and thus the presence of hydroxyl groups in the earlier position of halogen atoms must be due to a reaction with water or hydroxide ions succeeding, and not preceding, the alkylation.

The fit of the $k_{\text{alk}}^{\text{AN}}$ (Table 1) values to the Eyring−Wynne−Jones equation (eq 4), gives the activation parameters for the alkylation reaction (Table 2).

$$\ln \frac{k}{T} = \ln \frac{\mathbf{k}}{h} + \frac{\Delta^{\ddagger} S^{\circ}}{R} - \frac{\Delta^{\ddagger} H^{\circ}}{R} \frac{1}{T} \quad (4)$$

The low values for the activation enthalpies are consistent with the high reactivity of the aldehyde group of mucohalic acids,

Table 1. Rate Constants of Aniline Alkylation by Mucohalic Acids as a Function of Temperature and the Reaction Medium

	k_{alk}^{AN} (M^{-1} s^{-1})[a]			
	MCA		MBA	
T (°C)	water	7:3 w/d (vol)	water	7:3 w/d (vol)
25.0	0.162	0.0304	0.242	0.0454
27.5	0.190	0.0329	0.273	0.0490
30.0	0.210	0.0368	0.302	0.0529
32.5	0.235	0.0392	0.335	0.0559
35.0	0.275	0.0430	0.370	0.0610
37.5	0.300	0.0475	0.404	0.0640

[a] Values are reproducible to within 5%.

Table 2. Activation Parameters for k_{alk}^{AN} as a Function of the Reaction Medium

	MCA		MBA	
activation parameters for k_{alk}^{AN}	water	7:3 w/d (vol)	water	7:3 w/d (vol)
$\Delta^{\ddagger}H°$ (kJ mol^{-1})	35 ± 1	25 ± 1	29 ± 1	19 ± 1
$-\Delta^{\ddagger}S°$ (J K^{-1} mol^{-1})	141 ± 191	191 ± 2	159 ± 2	207 ± 2
$\Delta^{\ddagger}G°$ (kJ mol^{-1}) 35 °C	78 ± 2	84 ± 2	78 ± 2	83 ± 2

whereas the negative activation entropies are consistent with the idea of alkylation being an addition reaction.

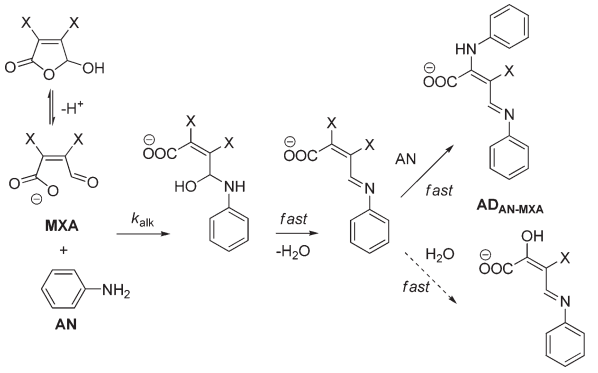
The reaction products ($AD_{AN\text{-}MXA}$) were identified as the 2:1 aniline-MXA adducts by mass spectrometry, with m/z = 301.1 for MCA and m/z = 345.1 for MBA (calcd 301.1 and 345.1). This is consistent with the observation that mucohalic acids react with a stoichiometric amount of aniline to form the Schiff base, whereas in an excess of aniline the diadduct is formed by substitution of the halogen atom α to the carboxylic group.[24,45] Minor amounts of a secondary product showing monoalkylation and substitution of the halogen in α by a hydroxyl group were also detected (m/z = 226.1 for MCA and m/z = 270.0 for MBA).

This allows us to propose the following reaction mechanism (Scheme 2): mucohalic acids exist as an equilibrium between the closed-chain acetal lactone and the open-chain aldehyde.[34,46] The attack of aniline on the aldehyde group is the limiting step, whereas the subsequent elimination to form the Schiff base and further reaction with aniline or water take place rapidly.

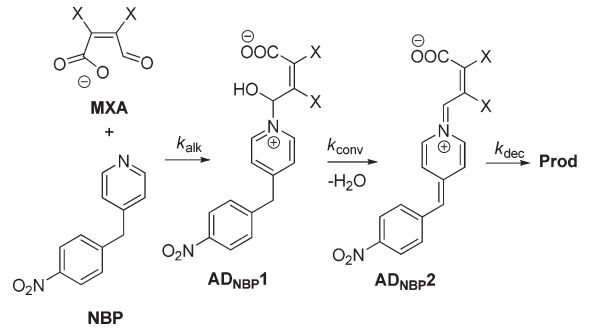
It is known that no substitution of the halogen atom by aniline is observed prior to formation of the Schiff base[24,45] and that substitution of α-halogens by water to form mucoxyhalic acids is very slow.[34] This, together with our results, suggests that the reactivity of the halogen α to the carboxyl group in the Schiff base is increased with respect to that in mucohalic acids. This enhanced electrophilicity is also consistent with the formation of products with hydroxyl substituents α to the carboxylate group in adducts of MXA with adenosine, guanosine, and cytidine.

Cyclic adducts are the main product in the alkylation of cytidine, adenosine, and guanosine in water, as shown in Scheme 1. They are formed by the attack of an aromatic nitrogen in the nucleobases on the carbon β to the carboxylate group after alkylation of the exocyclic amine group.

Scheme 2. Proposed Reaction Mechanism for the Alkylation of Aniline by MXA



Scheme 3. Proposed Reaction Mechanism for the Alkylation of NBP by MXA



The fact that the intramolecular reaction takes place at the β instead of the more reactive α position is possibly due to a combination of various factors: (a) the potential six-ring products obtained by intramolecular attack on the carbon α are disfavored; (b) the endocyclic nitrogen atoms are not nucleophilic enough to react with the sp^2 α-carbon, while stronger nucleophilic hydroxide ions do react at that site; and (c) substitution in α by water or hydroxide is relatively fast and takes place *before* cyclization in α can occur.

Because the position β to the carboxylate shows almost no reactivity prior to alkylation—MXA undergo substitution by hydroxide only in the α position—it may be concluded that the electrophilicity of this carbon must also be enhanced before cyclization. Substitution of chlorine in α by a hydroxyl group yields an enol, whose corresponding keto form would show a strongly increased reactivity on the β-carbon, since it would acquire a more electrophilic sp^3 hybridization and yield a favored five-membered ring.

Reaction with NBP. The variation in absorbance along time is shown in Figure 3 of the Supporting Information. The adducts show maximum absorption at λ = 510 nm in the case of MCA and λ = 485 nm for MBA.

The kinetic profiles of typical reactions are shown in Figure 1. The adduct is seen to be unstable, decomposing quite rapidly in the reaction medium, which suggests a biexponential mechanism.

However, closer scrutiny reveals the presence of an induction period, which imposes the need to take into account an extra reaction. When this third step is considered (Scheme 3), the rate eqs 5−7 are obtained. If k_{alk}^{NBP} is the alkylation rate constant, k_{1alk}^{NBP}

Table 3. Rate Constants of NBP Alkylation by Mucohalic Acids as a Function of Temperature at pH = 7.0

T (°C)	$10^3 k_{alk}^{NBP}$ (M^{-1} s^{-1}) 7:3 w/d (vol)	
	MCA	MBA
25.0	8.0 ± 0.2	4.7 ± 0.1
27.5	9.1 ± 0.2	5.6 ± 0.1
30.0	10.3 ± 0.5	6.5 ± 0.2
32.5	11.6 ± 0.5	7.5 ± 0.2
35.0	12.6 ± 0.4	8.6 ± 0.1
37.5	14.0 ± 0.3	10.0 ± 0.2

Table 4. Activation Parameters for k_{alk}^{NBP}

activation parameters for k_{alk}^{NBP}	7:3 w/d (vol)	
	MCA	MBA
$\Delta^{\ddagger}H°$ (kJ mol^{-1})	32 ± 1	43 ± 1
$-\Delta^{\ddagger}S°$ (J K^{-1} mol^{-1})	177 ± 4	144 ± 3
$\Delta^{\ddagger}G°$ 35 °C (kJ mol^{-1})	86 ± 2	88 ± 2

is the alkylation pseudoconstant, k_{conv} is the rate constant for the conversion of $AD_{NBP}1$ into $AD_{NBP}2$, and k_{dec} is the rate constant for the decomposition of $AD_{NBP}2$, we have

$$\frac{d[MXA]}{dt} = -k_{alk}^{NBP}[NBP][MXA] = -k_{1alk}^{NBP}[MXA] \quad (5)$$

$$\frac{d[AD_{NBP}1]}{dt} = k_{1alk}^{NBP}[MXA] - k_{conv}[AD_{NBP}1] \quad (6)$$

$$\frac{d[AD_{NBP}2]}{dt} = k_{conv}[AD_{NBP}1] - k_{dec}[AD_{NBP}2] \quad (7)$$

Integration of eqs 5−7 affords eq 8:

$$[AD_{NBP}2] = [MXA]_o \frac{k_{1alk}^{NBP} k_{conv}((-k_{1alk}^{NBP} - k_{conv})e^{-k_{dec}t} + (k_{conv} - k_{dec})e^{-k_{1alk}^{NBP}t} - (-k_{1alk}^{NBP} - k_{dec})e^{-k_{conv}t})}{(k_{1alk}^{NBP} + k_{conv})(k_{1alk}^{NBP} + k_{dec})(k_{dec} - k_{conv})} \quad (8)$$

The quality of the fit of the experimental data to eq 8 is shown in Figure 1. To confirm the need for the inclusion of the three steps, the fit of the kinetic profile to a simplified biexponential mechanism with only two successive reactions is also shown in Figure 1. It can be seen that the quality of the fit is insufficient, and that there is significant deviation from the curve described by the experimental points.

Fitting to eq 8 affords values for the three rate constants and the molar absorption coefficients of the adducts. $AD_{NBP}2$ shows $\varepsilon_{NBP\text{-}MCA\text{-}2} = (25.6 \pm 0.4) \times 10^3$ M^{-1} cm^{-1} at $\lambda = 510$ nm and $\varepsilon_{NBP\text{-}MBA\text{-}2} = (30.1 \pm 1.4) \times 10^3$ M^{-1} cm^{-1} at $\lambda = 485$ nm. $AD_{NBP}1$ does not show significant absorption at the wavelengths of measurement.

Alkylation Reaction. k_{1alk}^{NBP} was observed to be linear in the concentration of NBP and pH-independent in the working conditions. The values for the second-order alkylation rate constants (k_{alk}^{NBP}) are shown in Table 3.

The alkylation rate constants are higher for MCA, which is coherent with the electronegativity of chlorine and also with the greater steric repulsion of bromine atoms. The values are between 5- and 10-fold lower than those observed for the alkylation of aniline, which is in keeping with the observation that mucohalic acids react primarily at the exocyclic amino groups of DNA bases rather than at the sp^2 nitrogen atoms in the ring. As a matter of fact, no nucleobase adducts of MXA with thymine—which lacks exocyclic nitrogen atoms—have been detected. However, the reactivity of MXA toward endocyclic nitrogen atoms is not completely negligible; a minor alkylation of aromatic nitrogen has also been observed in the reaction of MXA and guanosine,[31] for whose endocyclic nitrogen atoms NBP is a nucleophilicity model.

Table 4 shows the activation parameters—from the fit to eq 4—for the alkylation of NBP.

The activation parameters parallel those obtained with aniline: low activation enthalpies, consistent with the electrophilicity of the aldehyde, and high negative activation entropies, consistent with an addition mechanism.

The values of the activation free energies are slightly higher than the values obtained for the alkylation of aniline in water/dioxane medium. This is consistent with the lower reactivity of aromatic nitrogen atoms with mucohalic acids.

Comparison of the activation parameters with those obtained for the alkylation of NBP by other alkylating agents such as diketene[36] ($\Delta^{\ddagger}G° = 71 \pm 2$ kJ mol^{-1}), β-propiolactone[35] ($\Delta^{\ddagger}G° = 87 \pm 2$ kJ mol^{-1}), potassium sorbate[42] ($\Delta^{\ddagger}G° = 99 \pm 6$ kJ mol^{-1}), or acrylamide[43] ($\Delta^{\ddagger}G° = 104 \pm 2$ kJ mol^{-1}) in the same reaction conditions suggests that mucohalic acids are strong alkylating agents when attacking endocyclic nitrogen atoms, and even more so when reacting with exocyclic amino groups, as is suggested by the higher rate constants obtained in the reaction with aniline.

Elimination Reaction. NBP has been shown to be a reasonable nucleophilicity model of guanosine *N*-7 and other nitrogen atoms in the cycle of nucleobases. However, its structure is very different from that of nucleotides and hence the subsequent transformations undergone by the MXA-NBP adduct after alkylation are hardly extrapolable to DNA. Therefore, they will only be discussed briefly.

The values for k_{conv} are shown in Table 1 of the Supporting Information. k_{conv} was seen to be pH-independent in the $5.5 < pH < 8.0$ range. Because water elimination reactions occur through a base-catalyzed pathway, the possibility of general base catalysis by the phosphate buffer cannot be ruled out.

It can be seen that the values for MBA are almost 4-fold higher than those of MCA.

The UV−vis spectrum of $AD_{NBP}2$ (SI, Figure 3) deserves some attention: it shows absorption in the visible part of the spectrum, without the need for basification; the wavelengths of maximum absorption are significantly blue-shifted when compared to NBP adducts with other alkylating agents and the molar absorption coefficients are high as compared to those of other NBP adducts, which are about 5×10^3 M^{-1} cm^{-1}. This suggests that AD2 is the highly conjugated elimination product shown in Scheme 3.

Computational Modeling of Nucleobase Alkylation. To bring together the kinetic evidence obtained with the model substrates, and the products identified by Kronberg et al. in the reaction of MXA and nucleotides, we modeled the proposed reaction mechanism of nucleobase alkylation by MXA *in silico* (Scheme 4).

As shown in Scheme 4, transition states were computed for the following reactions: the alkylation reaction (TS1); the putative ring closure reactions of AD1, affording both five- or six-membered rings (TS2 and TS3); substitution of the α and β halogen atoms in AD1 by hydroxide (TS4 and TS5); the ring-closure reaction of AD2 in both keto and enol forms (TS6 and TS8); the hydrolysis of AD2 in TS7 and TS9.

The energy barriers for the reactions and energy differences between different possible isomers are reported in Table 5. Guanosine adducts can exist both as keto and enol tautomers. The results reported correspond to the lower in energy.

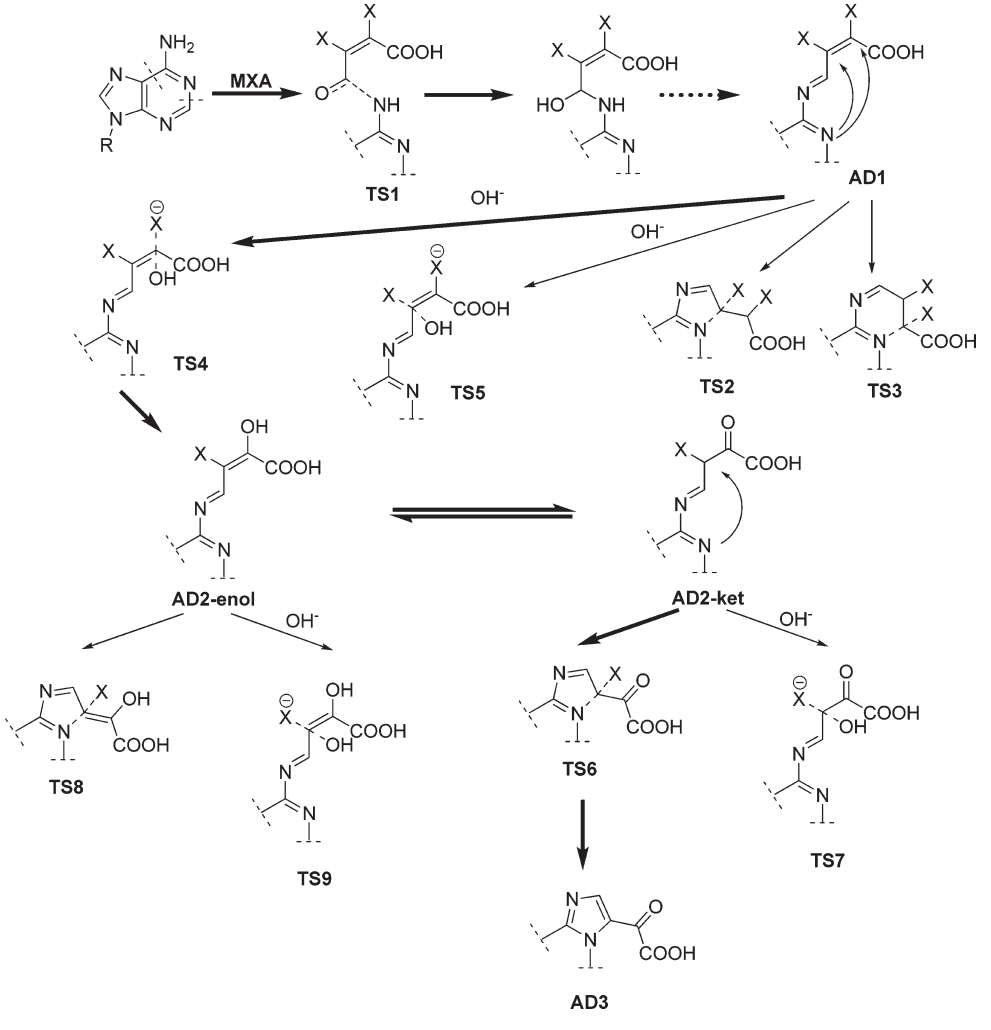
The energy barriers for the alkylation reaction (TS1) suggest that this is the rate-limiting step for the complete alkylation pathway. Of the three nucleobases studied, guanosine shows the lowest alkylation barrier, which is quite similar to that of aniline (Table 2), with the calculated activation free energies for adenosine being about 10−15 kJ mol^{-1} higher. The initial barrier for the alkylation of cytidine is higher than those of the purine bases (MCA $\Delta^{\ddagger}H^{\circ}$ = 95.6 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 99.8 kJ mol^{-1}), and thus no further steps in the alkylation path were computed for this compound.

Table 5. Relative Energies of Transition States and Isomeric Forms (kJ mol^{-1})

	dA				dG			
	MCA		MBA		MCA		MBA	
	$\Delta^{\ddagger}H^{\circ}$	$\Delta^{\ddagger}G^{\circ}$	$\Delta^{\ddagger}H^{\circ}$	$\Delta^{\ddagger}G^{\circ}$	$\Delta^{\ddagger}H^{\circ}$	$\Delta^{\ddagger}G^{\circ}$	$\Delta^{\ddagger}H^{\circ}$	$\Delta^{\ddagger}G^{\circ}$
TS1[a]	74.4	89.3	71.3	84.5	64.3	78.7	62.8	77.3
TS2[b]	158.8	168.3	142.4	149.1	272.5	285.3	269.2	284.1
TS3[b]	119.9	130.9	112.8	122.1	193.1	200.9	185.2	191.8
TS4[b]	33.4	42.4	30.7	38.5	57.3	68.1	49.8	68.7
TS5[b]	95.2	105.4	93.5	106.3	102.4	126.1	100.2	120.7
AD2-en[c]	0	0	0	0	0	0	0	0
TS8[c]	187.6	189.5	182.7	185.7	194.3	192.6	189.7	191.4
TS9[c]	172.8	177.7	164.7	170.4	182.5	186.9	175.5	179.4
AD2-ket[c]	20.1	12.5	14.0	8.2	14.2	11.0	6.8	4.5
TS6[c]	75.1	77.5	67.2	69.7	77.0	83.0	75.5	79.5
TS7[c]	29.2	34.8	20.9	29.3	28.5	33.7	26.5	31.5

[a] Referred to the reactants. [b] Referred to AD1. [c] Referred to AD2.

Scheme 4. Computed Alkylation Pathway for Nucleobases



Computational calculation of the energy barrier for the elimination step is complex, since the reaction is susceptible to general base catalysis by a number of species that are present in both experimental conditions and in vivo, such as phosphate. Experimental evidence from the reaction of aniline suggests that this elimination reaction is very fast, and thus that it has little influence on the global reaction rate. For these reasons, the elimination step in the reaction pathway was not computed.

AD1 can undergo a variety of reactions that cleave the halogen−carbon bonds: substitution by hydroxide or by endocyclic nitrogen atoms, which yields cyclic products. Of these reactions, the one corresponding to the hydrolysis of the halogen atom α to the carboxylate group (TS4) is by far the most favored. This is consistent with the final reaction products and the proposed reaction pathway (Scheme 1). It also confirms that hydrolysis precludes cyclization, as was the case with aniline. We also computed the barriers for the noncatalyzed hydrolysis reaction, with water as a nucleophile. The barrier is comparatively higher than that for hydroxide ions ($\Delta^{\ddagger}H^{\circ}$ = 82.1 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 89.7 kJ mol^{-1} for adenosine-MCA), but the reaction is still faster than the intramolecular alternatives or the hydrolysis at the β-position. Thus, the alkylation pathway proceeds through TS4, or its neutral counterpart, depending on the reaction conditions. Since the amino group in aniline is a stronger nucleophile than the *N*-1 atom in adenosine or guanosine, double alkylation is observed instead of hydrolysis in the reaction of aniline and MXA.

The *enol* form of AD2 is slightly more stable than the *keto* form. This is possibly due to the formation of a hydrogen bond between the *enol* hydrogen and the carboxylate oxygen, and to the extended conjugation that connects the aromatic system and the carboxylate unit. Reactivity, however, is favored in the *keto* form. The sp^2 carbon in the *enol* form is less reactive in electrophilic reactions, such as hydrolysis or displacement by cyclic nitrogen atoms, as suggested by the high activation free energies for TS8 and TS9, as compared to TS6 and TS7.

The activation free energy for the ring-closure reaction (TS6) is quite low—the activation entropy being close to null, as expected for unimolecular reactions—and hence the reaction is expected to proceed rapidly. The activation free energies for the competing hydrolysis are also very low, which suggests that hydrolysis by hydroxide ions is very fast. However, the concentration of hydroxide ions in neutral medium is very low (10^{-7} M). Because the effect of reducing the concentration of hydroxide from the reference standard state of 1 M to 10^{-7} M is approximately equivalent to an increase of 40 kJ mol^{-1} in the activation free energy, both ring-closure and base-catalyzed hydrolysis could be expected to occur similar at similar rates.

The hydrolysis of AD2 by the more abundant water molecules has a higher energy barrier (adenosine: $\Delta^{\ddagger}H^{\circ}$ = 59.7 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 81.3 kJ mol^{-1} for MCA; $\Delta^{\ddagger}H^{\circ}$ = 52.6 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 79.8 kJ mol^{-1} for MBA) and is somewhat higher than that observed for the formation of the cycle. Thus, it may be concluded that hydrolysis by both hydroxide and water might pose some competition to intramolecular cyclization. However, the formation of nucleotide-adducts showing substitution of the second halogen atom by a hydroxyl group has not been observed experimentally.

Analysis of the reported free energy barriers shows that the nucleophilic attack of the primary amine at the carbonyl carbon is the rate-limiting step of the alkylation reaction, and that the rest of the steps in the reaction path take place rapidly. However, since the free energy barriers are very similar for all the steps, especially in the case of guanosine, the indetermination in the calculated energies is compatible with more than one step being rate-limiting, rather than a single specific reaction being a bottleneck.

When the experimental alkylation reactions of nucleotides with MXA are carried out at high temperatures (∼90 °C), decarboxylated and even deoxalo products are encountered. However, these are hardly detectable when alkylation reactions are carried out at temperatures closer to biological conditions. Because they lack a keto group to assist the reaction, no transition states were found for the decarboxylation of MXA or AD1, which is consistent with the fact that no decarboxylated products have been observed in the alkylation reaction, prior to the formation of AD2. The decarboxylation reactions of the AO2 (and analogue protonated MOXA) somewhat unfavored with free energies of activation about 150−160 kJ mol^{-1} (MOCA: $\Delta^{\ddagger}G^{\circ}$ = 161 kJ mol^{-1}; MOBA: $\Delta^{\ddagger}G^{\circ}$ = 156 kJ mol^{-1}; adenosine-AD2: $\Delta^{\ddagger}H^{\circ}$ = 161 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 156 kJ mol^{-1} for MCA; $\Delta^{\ddagger}H^{\circ}$ = 162 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 158 kJ mol^{-1} for MBA). The decarboxylation of AD3 is more favored, consistently with the increased amount of decarboxylated AD3 adduct observed in the experiments (adenosine-AD3: $\Delta^{\ddagger}H^{\circ}$ = 113 kJ mol^{-1}, $\Delta^{\ddagger}G^{\circ}$ = 116 kJ mol^{-1}).

These reactions show high activation enthalpies and positive activation entropies (consistent with a unimolecular cleavage that affords two product molecules) which imply a large increase in the decarboxylation rate at higher temperatures; this is in keeping with the observation of decarboxylated products in these conditions.

The mutational pattern of MXA—which consists mainly of GC→AT transitions—has been explained by the formation of guanosine and, possibly to a lesser extent, cytidine-etheno derivatives.[14] Our results are in good agreement with these conclusions: guanosine shows the lower theoretical free energy barrier for alkylation and thus it is expected to be the main target of mucohalic acids. The formed adducts have shown no tendency to lead to abasic sites and it is difficult to envision such bulky tricyclic adducts mispairing with large purine residues, be they A or G. Of the two pyrimidine nucleobases, only pairing with thymidine leads to a net mutation, and thus, GC→G*C→G*T→AT (G* being the modified guanosine) would be the expected sequence of base pairs, which is in keeping with the results reported in the literature.

The combined experimental and theoretical results presented here allow us to conclude the following:

(i) Mucohalic acids (MXA) are strong alkylating agents that react directly with amino groups in nucleotides, as suggested by the high reaction rates and low activation free energies measured in the alkylation of aniline and NBP.
(ii) MXA preferentially attack the exocyclic amino groups over the endocyclic aromatic nitrogen atoms.
(iii) Mucoxyhalic acids, the hydrolysis products of MXA, play no role in the alkylation reaction by MXA.
(iv) The limiting step in the alkylation reaction by mucohalic acids is the addition to the carbonyl group. Subsequent elimination, hydrolysis, and cyclization occur rapidly.
(v) Kinetic and computational evidence suggests an alkylation mechanism in agreement with both the experimental product distribution and the mutagenic spectra of mucohalic acids.

■ ASSOCIATED CONTENT

Supporting Information. Figures showing the variation in the UV−vis spectra of the AN-MXA and NBP-MXA alkylation mixtures and also fit of the experimental data to eq 1. This information is available free of charge via the Internet at http://pubs.acs.org.

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