

Alkylating potential of α,β -unsaturated compounds†

José A. Manso,‡ Isaac F. Céspedes Camacho,‡ Emilio Calle and Julio Casado*

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Alkylation reactions of the nucleoside guanosine (Guo) by the α,β -unsaturated compounds (α,β -UC) acrylonitrile (AN), acrylamide (AM), acrylic acid (AA) and acrolein (AC), which can act as alkylating agents of DNA, were investigated kinetically. The following conclusions were drawn: i) The Guo alkylation mechanism by AC is different from those brought about the other α,β -UC; ii) for the first three, the following sequence of alkylating potential was found: AN > AM > AA; iii) A correlation between the chemical reactivity (alkylation rate constants) of AN, AM, and AA and their capacity to form adducts with biomarkers was found. iv) Guo alkylation reactions for AN and AM occur through Michael addition mechanisms, reversible in the first case, and irreversible in the second. The equilibrium constant for the formation of the Guo-AN adduct is K_{eq} (37 °C) = 5×10^{-4} ; v) The low energy barrier (≈ 10 kJ mol⁻¹) to reverse the Guo alkylation by AN reflects the easy reversibility of this reaction and its possible correction by repair mechanisms; vi) No reaction was observed for AN, AM, and AA at pH < 8.0. In contrast, Guo alkylation by AC was observed under cellular pH conditions. The reaction rate constants for the formation of the α -OH-Guo adduct (the most genotoxic isomer), is 1.5-fold faster than that of γ -OH-Guo. vii) a correlation between the chemical reactivity of α,β -UC (alkylation rate constants) and mutagenicity was found.

Introduction

The world's population is especially exposed to alkylating agents generated endogenously¹ and those present in the environment.² Almost all heteroatoms in the DNA double helix have the potential to become alkylated and the sites of alkylation in duplex DNA depend on the nature of the alkylating agent.³

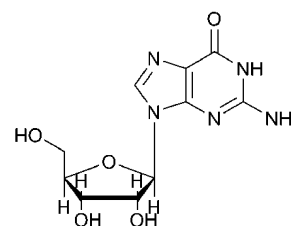
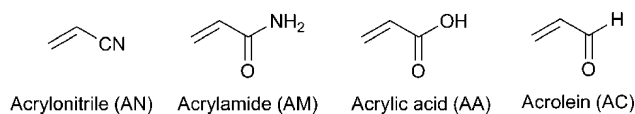
In recent decades, the *in vitro* alkylating activity of many potentially mutagenic and carcinogenic substances has been investigated,^{4,5} often through their reactions with 4-(*p*-nitrobenzyl) pyridine (NBP).⁶⁻¹⁵

Since to our knowledge no investigations have been carried out to analyze the alkylating capacity of α,β -unsaturated compounds (α,β -UC) over nucleosides such as guanosine (Guo) from a kinetic mechanistic perspective, here we were prompted to address this issue. Among other facts, the following recommended this investigation: a) α,β -UC, which can act as alkylating agents, are important substances used in the polymer industries and are present as environmental pollutants and as components in certain foodstuffs;¹⁶ b) these compounds are particularly reactive and interact with biological macromolecules, resulting in a

variety of adverse effects, including toxicity, mutagenicity and carcinogenicity;¹⁷ c) acrolein (AC) has recently been correlated with lung tumors.^{18,19}

Results and discussion

The current investigation correlates and discusses the results from a kinetic study of the alkylation reactions of the nucleoside Guo, with four relevant α,β -UC: namely, acrylamide (AM), acrylonitrile (AN), acrylic acid (AA) and acrolein (AC) (Fig. 1).



Guanosine (Guo)

Fig. 1 Alkylating agents and alkylation substrate investigated in this work.

Departamento de Química Física, Universidad de Salamanca, Plaza de los Caidos s/n, E-37008, Salamanca, (Spain). E-mail: jucali@usal.es; Fax: (+34) 923 294574; Tel: (+34) 923 294486

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‡ These authors contributed equally to this work

Alkylation of guanosine by acrylonitrile, acrylamide and acrylic acid

The reaction rates of Guo with AN, AM and AA were measured in aqueous sodium hydroxide solutions in the pH = 8–11 and the $T = 15\text{--}40\text{ }^{\circ}\text{C}$ ranges. Guo solutions in $[\text{NaOH}] = 10^{-3}$ to 10^{-6} M range are stable at room temperature.²⁰ pH was adjusted with 0.1 M NaOH and kept constant throughout the reaction time. Because the hydrolysis of the amide group requires high temperatures,²¹ the hydrolysis of AM was not taken into account. $\alpha,\beta\text{-UC}$ were in large excess with respect to Guo. To monitor the Guo-alkylation reactions, the change in the peak area of the adducts was used to determine the rate of alkylation.

Ultra Fast Liquid Chromatography (UFLC) analysis resolved the major peaks of the reaction mixture whose retention times were 19.5 min and 25.5 min for Guo and the Guo-AN adduct, respectively (the chromatographic methods and chromatograms are shown in the Experimental Section and the Supplementary Information,† respectively).

Fig. 2 shows typical kinetic runs for the formation of the AN-Guo adduct at position N1, as well as the influence of pH on its stability. This adduct is stable at pH < 10.0.

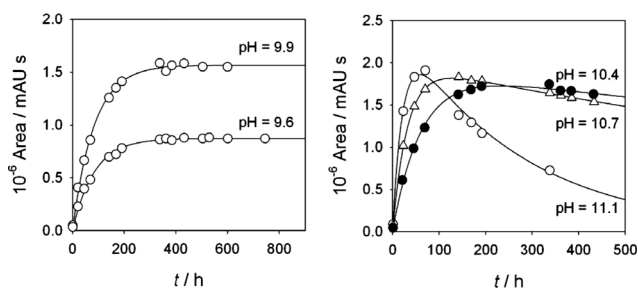


Fig. 2 Variation of [Guo-AN] with time; $[\text{Guo}]_0 = 10^{-4}$ M; $[\text{AN}]_0 = 0.05$ M; $T = 37.5\text{ }^{\circ}\text{C}$.

Since the reaction time was very long (~3 weeks), the initial rate method²² was used.

Because: a) the initial rate, v_0 , is proportional to the $K_a/(K_a + [\text{H}^+])$ quotient, K_a being the deprotonation constant of Guo, and b) no reaction was observed at pH < 8, the active form of Guo to form adducts must be the deprotonated one (Fig. 3). In order to confirm this, the $\text{p}K_a$ values were determined: $\text{p}K_a = 10.1$ and $\text{p}K_a = 9.8$ ($T = 37.5\text{ }^{\circ}\text{C}$) for Guo alkylation by AN and AM, respectively (Fig. 4). These values, when corrected for ionic strength and temperature effects, agree well with the value reported in the literature²³ ($\text{p}K_a = 9.25$; $T = 25.0\text{ }^{\circ}\text{C}$).

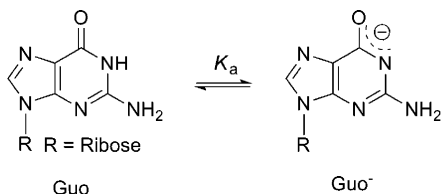


Fig. 3 Deprotonation at position N1 in guanosine.

The $\text{p}K_a$ value can be modulated by a variety of modifications in Guo. *Ab initio* calculations²⁴ were utilized to predict the nucleobase acidity. Alkylation at position N7²⁵ increases the acidity of the N1

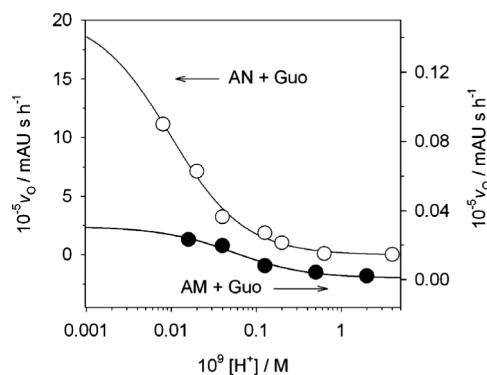


Fig. 4 pH titration curve in Guo alkylation reactions by $\alpha,\beta\text{-UC}$; $[\text{Guo}]_0 = 10^{-4}$ M; $[\text{AN}]_0 = [\text{AM}]_0 = 0.05$ M; $T = 37.5\text{ }^{\circ}\text{C}$.

atom, decreasing the $\text{p}K_a$ value from 9.2 to 7.1. As a consequence, alkylation processes are favoured at physiological pH.

Since the enhanced nucleophilicity of Guo at higher pH is due to the presence of the conjugate-base anion^{26,27} and since Michael addition of Guo^- to the double bond of the $\alpha,\beta\text{-UC}$ is favoured,²⁸ this means that alkaline media (not infrequent in human organs such as the intestine or pancreas, and in some human fluids such as saliva and urine) are particularly favourable to the course of alkylation reaction for AN and AM.

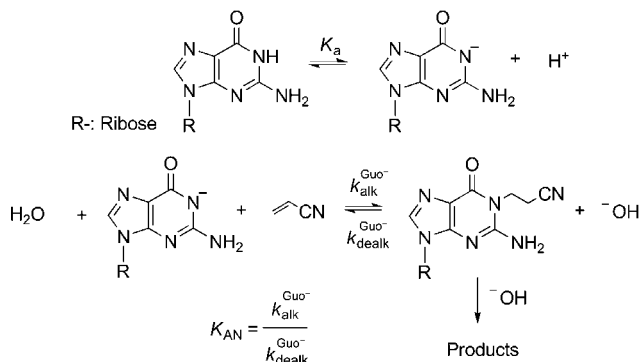
The protonated and sodium molecular ion peaks were observed at m/z 337.2 and m/z 359.2, respectively, corresponding to 1-(2-cyanoethyl)-guanosine. Sugar fragmentation was observed at the expected masses of m/z 117.8 (ribose minus one molecule of water) and m/z 101.8 (ribose minus two molecules of water), indicating that no cyanoethylation of the 3'- and/or 5'-hydroxyl groups of the sugar had occurred. Glycosidic fragmentation with hydrogen transfer from sugar afforded an m/z 204.9, which further fragmented by losing AN (m/z 151.8). Also, the $^1\text{H-NMR}$ signals of the reaction products revealed the presence of the expected adduct. The absence of the signal at 10.6 ppm, corresponding to the proton at position N1, is good evidence of Guo alkylation at this position. Moreover, the UV spectra of the adduct have features that are consistent with an N1-substituted guanosine adduct.²⁹ All this is in agreement with the fact that transition states of Michael additions or $\text{S}_{\text{N}}2$ reactions are sufficiently short for covalent interactions and hence N -alkylation is favoured. In contrast, O -alkylation processes are expected in $\text{S}_{\text{N}}1$ reactions, such as with N -alkyl- N -nitrosoureas.^{30,31}

Experiments performed to determine the influence of the concentrations of $\alpha,\beta\text{-UC}$ and Guo revealed the reactions to be first-order with respect to each reactant.

Since: (i) AN was in large excess with respect to the alkylation substrate (more than 50-fold); (ii) Guo was not fully consumed; (iii) there is evidence in the literature of a reversible alkylation reaction at position N1 of deoxynucleosides,³² a chemical equilibrium (K_{AN}) between Guo^- and alkylated-Guo should exist (see below, Scheme 1).

From the mechanism depicted in Scheme 1, the following rate equation at pH < 10.0 can be deduced:

$$\begin{aligned} \frac{d[\text{AD}]}{dt} &= k_{\text{alk}}^{\text{Guo}^-} \frac{K_a}{K_a + [\text{H}^+]} [\text{AN}]_0 ([\text{Guo}]_0 - [\text{AD}]) - k_{\text{dealk}}^{\text{Guo}^-} [\text{OH}^-] [\text{AD}] = \\ &= k_{\text{alk}}^{\text{Guo}^-} ([\text{Guo}]_0 - [\text{AD}]) - k_{\text{dealk}}^{\text{Guo}^-} [\text{AD}] \end{aligned} \quad (1)$$



Scheme 1 Guo alkylation by AN.

where $[AD]$ is the concentration of the Guo-AN adduct, $k_{\text{alk}}^{\text{Guo}^-} = k_{\text{alk}}^{\text{Guo}^-} K_{\text{a}} / (K_{\text{a}} + [\text{H}^+]) [\text{AN}]_0$, $k_{\text{dealk}}^{\text{Guo}^-} = k_{\text{dealk}}^{\text{Guo}^-} [\text{OH}^-]$ being the pseudo first-order rate constant.

Integrating eqn (1) and expressing $[AD]$ in terms of the peak area³³ (Area) affords eqn (2):

$$\text{Area (mAU s)} = 60000 \varepsilon l \Delta t_{\text{elut}} [\text{Guo}]_0 \times \frac{k_{\text{alk}}^{\text{Guo}^-} (1 - \exp[-(k_{\text{alk}}^{\text{Guo}^-} + k_{\text{dealk}}^{\text{Guo}^-})t])}{k_{\text{alk}}^{\text{Guo}^-} + k_{\text{dealk}}^{\text{Guo}^-}} \quad (2)$$

where ε is the molar absorption coefficient of the Guo-AN adduct in $\text{AU M}^{-1} \text{cm}^{-1}$ units, l the pathlength, and Δt_{elut} the elution time (~ 0.4 min).

Fig. 5 shows the excellent fit of the results to eqn (2).

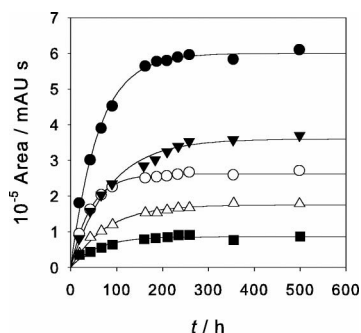


Fig. 5 Kinetic profiles at different AN concentrations; (●) 0.1 M; (▼) 0.05 M; (○) 0.03 M; (△) 0.01 M; (■) 0.005 M; $T = 37.5$ °C; pH = 9.6. (mAU = mili arbitrary units)

To monitor the formation of the Guo-AN adduct, its molar absorption coefficient ($\lambda = 254$ nm) must be known. Experiments designed to measure this coefficient were performed using $[\text{Guo}]_0 = 1 \times 10^{-4}$ M and $[\text{AN}]_0$ concentrations in the 0.005–0.5 M range.

When time tends to infinity (*i.e.* when the plateau is reached; Fig. 5), eqn (2) can be written as:

$$\frac{\text{Area (mAU s)}_{t \rightarrow \infty}}{\Delta t_{\text{elut}} (\text{min})} = 60000 \varepsilon l [\text{Guo}]_0 \times \frac{[\text{AN}]_0 K_{\text{a}} / (K_{\text{a}} + [\text{H}^+])}{[\text{AN}]_0 K_{\text{a}} / (K_{\text{a}} + [\text{H}^+]) + k_{\text{dealk}}^{\text{Guo}^-} / k_{\text{alk}}^{\text{Guo}^-}} \quad (3)$$

Eqn (3) can be written in a simpler form as:

$$y = \frac{ax}{b+x} \quad (4)$$

a and b being parameters obtained by non-linear fitting of the results, defined as in eqn (5) and (6):

$$a = 60000 \varepsilon l [\text{Guo}]_0 \quad (5)$$

$$b = k_{\text{dealk}}^{\text{Guo}^-} / k_{\text{alk}}^{\text{Guo}^-} = \frac{k_{\text{dealk}}^{\text{Guo}^-} [\text{OH}^-]}{k_{\text{alk}}^{\text{Guo}^-}} = \frac{[\text{OH}^-]}{K_{\text{AN}}} \quad (6)$$

To test the mechanism proposed for the alkylation of Guo by AN, the value of K_{AN} was obtained from: i) fitting the results to eqn (4) (Fig. 6): $\varepsilon = (5.1 \pm 0.3) \times 10^5 \text{ AU M}^{-1} \text{cm}^{-1}$ ($\lambda = 254$ nm) gave a value of $K_{\text{AN}} = (5.5 \pm 0.6) \times 10^{-4}$; ii) direct experimental determination: $K_{\text{AN}} = k_{\text{alk}}^{\text{Guo}^-} / k_{\text{dealk}}^{\text{Guo}^-} = ([\text{AN-Guo}]_{\text{eq}} [\text{OH}^-]) / ([\text{Guo}]_{\text{eq}} [\text{AN}]_0) = (6.2 \pm 1.4) \times 10^{-4}$. This agreement supports the proposed mechanism.

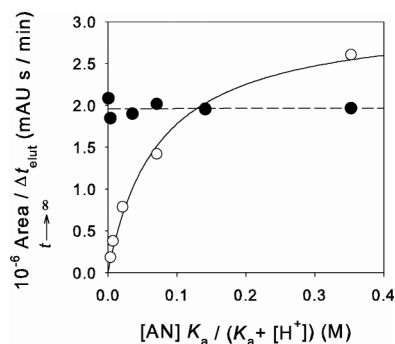


Fig. 6 Influence of the α,β -UC concentration on the formation of the Guo-AN adduct (○) and Guo-AM adduct (●); pH = 9.6; $T = 37.5$ °C.

The values of the alkylation rate constants calculated (eqn (7)) are shown in Table 1.

$$v_o = \left(\frac{d(\text{Area})}{dt} \right)_{t \rightarrow 0} = 60000 \varepsilon l \Delta t_{\text{elut}} k_{\text{alk}}^{\text{Guo}^-} [\text{AN}]_0 \times \frac{K_{\text{a}}}{K_{\text{a}} + [\text{H}^+]} [\text{Guo}]_0 \quad (7)$$

With the Eyring equation,³⁴ the values of free energy of activation, ΔG^\ddagger (Table 2), and the activation enthalpy, $\Delta H^\ddagger = 58 \pm 2$ kJ mol⁻¹, were calculated.

With the values of the equilibrium rate constant at different temperatures, the enthalpy of reaction, $\Delta H^\circ = 47 \pm 5$ kJ mol⁻¹, was calculated with the van't Hoff equation.³⁴

The low energy barrier (≈ 10 kJ mol⁻¹; Scheme 2) to reverse the reaction of alkylation by AN reflects the easy reversibility of this reaction. This is consistent with the fact that alkylated-Guo at position N1 by AN can be easily corrected by repair mechanisms (oxidative dealkylation is a mechanistic pathway to remove alkylation damage, generally by *N*-alkylations, from DNA bases and regenerate nucleobases to their native state^{35,36}).

Guo alkylation by AM is slower than that brought about by AN. Fig. 7 depicts two typical kinetic runs. UFLC analysis resolved the major peaks of the reaction mixture, whose retention times were 11.2 min and 20 min for Guo and the Guo-AM adduct, respectively (the chromatographic methods and chromatograms are shown in the Experimental Section and the Supplementary Information,† respectively). The adducts of Guo with AM are stable in the pH range studied.³⁷

Table 1 Rate constants as a function of temperature for the alkylation of Guo⁻ by AN and AM

<i>T</i> /°C	AN		AM
	$10^5 \times k_{\text{alk}}^{\text{Guo}^-}$ (M ⁻¹ s ⁻¹) ^a	$10^4 \times K_{\text{AN}}^a$	$10^5 \times k_{\text{alk}}^{\text{Guo}^-}$ (M ⁻¹ s ⁻¹)
15.0	1.33 ± 0.08	1.1 ± 0.1	— ^b
20.0	2.1 ± 0.2	2.2 ± 0.2	0.090 ± 0.001
25.0	3.5 ± 0.3	2.5 ± 0.1	0.18 ± 0.01
30.0	5.7 ± 0.4	2.5 ± 0.1	0.28 ± 0.01
35.0	6.8 ± 0.8	4.7 ± 0.2	0.58 ± 0.03
37.0	8.5 ± 0.6	5.3 ± 0.3	0.69 ± 0.05
37.5	8.7 ± 0.6	5.5 ± 0.3	0.72 ± 0.05
40.0	10.0 ± 0.7	7.1 ± 0.4	0.92 ± 0.08

^a Values are given within the 95% confidence interval. ^b Reaction too slow to be measured.

Table 2 Correlation between chemical reactivity of α,β-unsaturated compounds and their capacity to form adducts with hemoglobin

Alkylating agent	N1 Guo ⁻ alkylation		Hemoglobin adducts in smokers ⁴²
	$10^4 \times k_{\text{alk}}^{\text{Guo}^-}$ (37 °C) (M ⁻¹ s ⁻¹) ^a	ΔG^\ddagger (37 °C) (kJ mol ⁻¹) ^b	20 cigarettes/day (pmol g ⁻¹)
Acrylonitrile	0.85 ± 0.08	100 ± 2	168 ± 4
Acrylamide	0.069 ± 0.001	107 ± 2	144 ± 5
Acrylic acid	No reaction was observed		IARC-classified as not carcinogenic to humans ⁴³

^a Values of rate constants are given within the 95% confidence interval. ^b Values are given with their standard deviations.

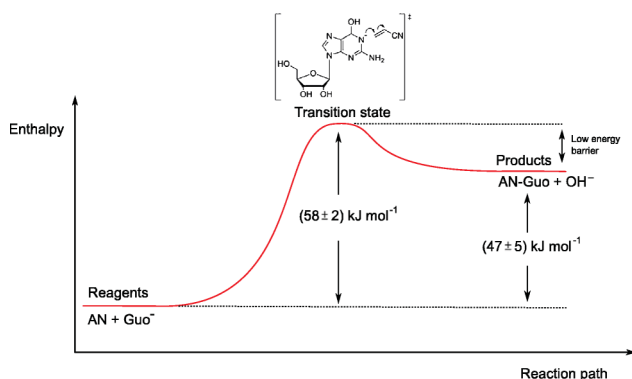
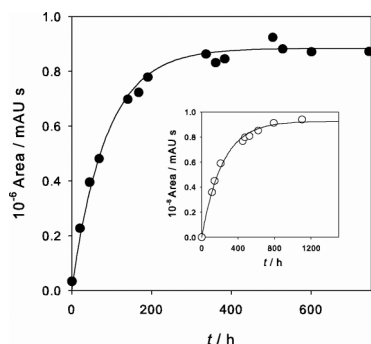
**Scheme 2** Enthalpy diagram for Guo alkylation by AN.

Fig. 7 Formation of the Guo-AN adduct (●): [Guo]₀ = 10⁻⁴ M; [AN]₀ = 0.05 M and Guo-AM adduct (○): [Guo]₀ = 10⁻⁴ M; [AM]₀ = 0.1 M. Variation in peak area with time. pH = 9.6; *T* = 37.5 °C.

Contrary to the alkylation of Guo by AN, in the alkylation by AM the $\text{Area}_{t \rightarrow \infty} / \Delta t_{\text{elut}}$ quotient does not vary with [AM]

(Fig. 6) *i.e.* the reaction is not reversible (Scheme 3). Moreover, no formation of 1-(2-formamidoethyl)guanosine was observed, probably due to the ready hydrolysis of the amide group to form 1-(2-carboxyethyl)guanosine.³⁸ The protonated molecular ion peak observed at *m/z* 302 together with the absence of the 1-(2-carboxyethyl)guanosine peak in the negative ion electrospray mass spectra confirm the cyclization of this adduct into the 1, *N*²-cyclic Guo adduct (Scheme 3). These results are consistent with the fact that α,β-UC form adducts with DNA components through Michael addition of one of the N1 nitrogens or *N*² atoms to the activated double bond, with cyclization through the reaction of one of the nitrogen atoms with the carbonyl function.³⁹ The formation of this kind of adduct can obstruct Watson–Crick base pairing⁴⁰ and possibly generate genotoxic effects in humans, which is consistent with previous studies addressing the toxicity of acrylamide.

Since the reaction is not reversible, $k_{\text{dealk}}^{\text{Guo}^-} = 0$, and hence eqn (2) and (3) are converted into eqn (8) and (9), respectively.

$$\text{Area (mAU s)} = 60000 \varepsilon l \Delta t_{\text{elut}} (\text{min}) \times [\text{Guo}]_0 (1 - \exp[-(k_{\text{alk}}^{\text{Guo}^-})t]) \quad (8)$$

$$\frac{\text{Area (mAU s)}_{t \rightarrow \infty}}{\Delta t_{\text{elut}} (\text{min})} = 60000 \varepsilon l [\text{Guo}]_0 \quad (9)$$

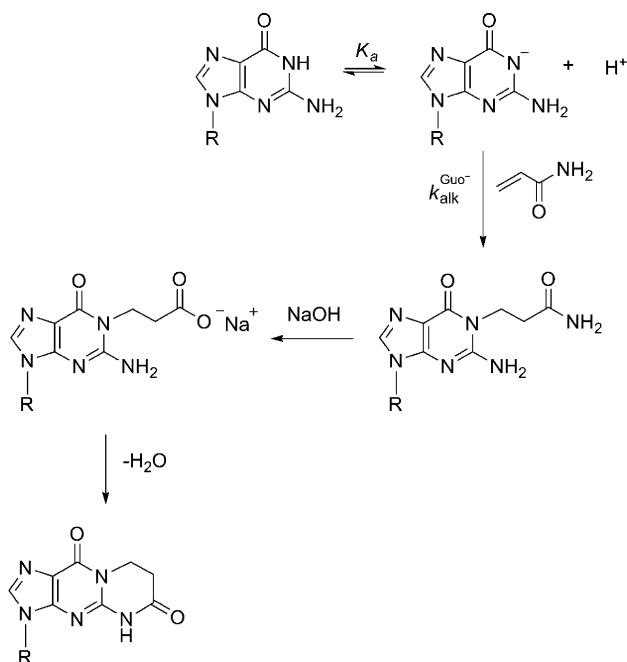
Fig. 7 shows the good fit of the results to eqn (8).

The value of ε was calculated by adjusting the experimental data to eqn (9): ε (37 °C) = (3.3 ± 0.1) × 10⁵ AU M⁻¹ cm⁻¹.

The values of the alkylation rate constants and those of the free energy of activation are given in Tables 1 and 2, respectively.

In the case of AA no alkylation was observed after 3 weeks.

Because the p*K*_a of acrylic acid is 4.25, at pH >7 the Michael addition of nucleophiles such as guanosine to AA cannot occur



Scheme 3 Alkylation of Guo by AM.

through the anion (AA^-). Nucleophilic addition to the vinyl double bond in AA^- generates a significant amount of negative charge on the carbon adjacent to the carbonyl carbon, which cannot participate in further charge delocalization. The net effect is to disfavour Michael addition to AA^- .⁴¹

In order to gain deeper insight into a possible correlation between chemical reactivity of α,β -UC and their capacity to form adducts with biomarkers, the values of the free energy of activation (ΔG^\ddagger) were measured.

Table 2 shows that the values of the ΔG^\ddagger for alkylation reactions can be considered as an indicator of their efficiency to form adducts with nucleophilic substrates (according to the Eyring Activated Complex Theory,³⁴ the alkylation rate constant (k) correlates exponentially with ΔG^\ddagger : $k = (kT/h) \times \exp(-\Delta G^\ddagger/RT)$).

Alkylation of guanosine by acrolein

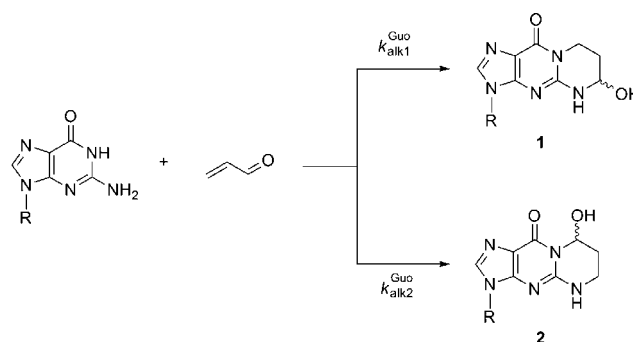
Contrary to AN, AM and AA the Guo alkylation reaction occurs at pH 7.2–7.4 (cellular conditions) in its neutral form, which indicates the higher alkylating capacity of AC compared with the other three α,β -UC.

The retention times obtained were 20.0 for Guo, 22.6 min and 24.8 min for adduct **1** (γ -OH-Guo), and 27.3 min for **2** (α -OH-Guo) (See Supporting information†). The chemical structures of the products are known.^{19,44}

From the mechanism depicted in Scheme 4, the following equation can be deduced to follow the peak area of **1** and **2** over time:

$$\text{Area}_{AD} \text{ (mAU s)} = 60000 \varepsilon_{AD} k_{\text{alk}(AD)}^{\text{Guo}} l \Delta t_{\text{elut}} \text{ (min)} [\text{Guo}]_0 \times \frac{(1 - \exp[-(k_{\text{alk1}}^{\text{Guo}} + k_{\text{alk2}}^{\text{Guo}})t])}{k_{\text{alk1}}^{\text{Guo}} + k_{\text{alk2}}^{\text{Guo}}} \quad (10)$$

The quotient between the peak areas of **1** and **2** when time tends to infinity yields eqn (11):



Scheme 4 Alkylation of guanosine by acrolein.

$$\left(\frac{\text{Area}_1}{\text{Area}_2} \right)_{t \rightarrow \infty} = \frac{k_{\text{alk1}}^{\text{Guo}} \varepsilon_1}{k_{\text{alk2}}^{\text{Guo}} \varepsilon_2} \quad (11)$$

Knowing the value of $\varepsilon_1/\varepsilon_2$, (1.07¹⁹) and $(k_{\text{alk1}}^{\text{Guo}} + k_{\text{alk2}}^{\text{Guo}})$ (from the fit of the experimental data to eqn (10) (Fig. 8)), the values of the formation rate constants of each adduct can be calculated. These values are given in Table 3. As can be observed, the formation of adduct **2** was faster than **1** (approximately 1.5 fold). Since adduct **2** is more genotoxic than **1**,⁴⁵ this result is significant.

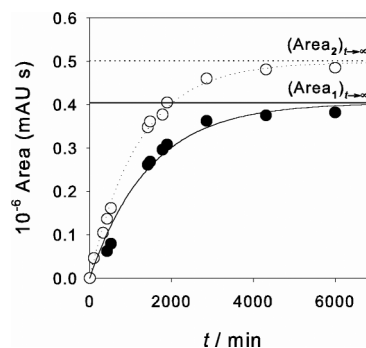


Fig. 8 Variation in peak area over time of adduct **1** (●) and adduct **2** (○) in the Guo-AC reaction mixture: $[\text{Guo}]_0 = 10^{-4}$ M; $[\text{AC}]_0 = 0.1$ M; pH = 7.2; $T = 37.5$ °C.

Table 4 shows the correlation between chemical reactivity and biological activity. As can be observed there is a good correlation between the Guo alkylation rate constants and the mutagenicity of

Table 3 Rate constants as a function of temperature for the alkylation of Guo by AC

$T/^\circ\text{C}$	$10^5 \times k_{\text{alk1}}^{\text{Guo}} \text{ }^a \text{ (M}^{-1} \text{ s}^{-1}\text{)}$	$10^5 \times k_{\text{alk2}}^{\text{Guo}} \text{ }^a \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
15.0	0.40 ± 0.08	0.70 ± 0.05
20.0	0.67 ± 0.05	1.3 ± 0.1
25.0	1.3 ± 0.1	2.9 ± 0.2
30.0	2.5 ± 0.1	4.2 ± 0.2
35.0	5.2 ± 0.4	7.2 ± 0.4
37.0	5.0 ± 0.5	7.6 ± 0.5
37.5	5.2 ± 0.4	8.0 ± 0.6
40.0	5.4 ± 0.5	11.3 ± 0.7

^a Values are given within the 95% confidence interval.

Table 4 Correlation between Chemical Reactivity of α,β -Unsaturated Compounds and Mutagenicity

Alkylating agent	Chemical reactivity		Mutagenicity ⁴⁷⁻⁴⁹
	$10^4 \times k_{\text{alk1}}^{\text{Guo}}$ (37 °C) ($\text{M}^{-1} \text{s}^{-1}$)	$10^4 \times k_{\text{alk2}}^{\text{Guo}}$ (37 °C) ($\text{M}^{-1} \text{s}^{-1}$)	Ames test
Acrolein	0.50 ± 0.05	0.76 ± 0.05	+++
Acrylonitrile	No reaction ^b		–
Acrylamide	No reaction ^b		–
Acrylic acid	No reaction ^b		–

^a Values of rate constants are given within the 95% confidence interval. ^b No reaction was observed at pH < 8.0.

the alkylating substances. As is known,⁴⁶ AN and AM are weakly mutagenic agents giving “Ames test negative”, although their oxidative metabolites –glycidamide and cyanoethylene oxide– are mutagenic at relatively high doses. In contrast, AC has a high mutagenic capacity relative to other α,β -UC.

Conclusions

i) The alkylation reaction of the nucleoside guanosine (Guo) by acrolein (AC) occurs through a mechanism different from that by acrylonitrile (AN), acrylamide (AM) and acrylic acid (AA).

ii) For the latter three, the following sequence of alkylating potential was found: AN > AM > AA.

iii) A correlation between the chemical reactivity (alkylation rate constants) of AN, AM, and AA and their capacity to form adducts with biomarkers was found.

iv) Guo alkylation reactions for AN and AM occur through Michael addition mechanisms, reversible in the first case and irreversible in the second. The equilibrium constant for the formation of the adduct Guo-AN is K_{eq} (37 °C) = 5×10^{-4} .

v) The low energy barrier ($\approx 10 \text{ kJ mol}^{-1}$) to reverse the Guo alkylation reaction by AN reflects the easy reversibility of this reaction and its possible correction by repair mechanisms.

vi) No reaction was observed for AN, AM, and AA at pH < 8.0. In contrast, Guo alkylation for AC under cellular pH conditions was observed. The reaction rate constants for the formation of the adduct α -OH-Guo (the most genotoxic isomer), is 1.5-fold than that of γ -OH-Guo.

vii) A correlation between chemical reactivity (alkylation rate constants) of α,β -UC and mutagenicity was found.

Experimental

General

Acrylonitrile (99%) was obtained from Aldrich. Acrylamide (98%) was purchased from Fluka and guanosine (98%) and acrolein (90%) were from Sigma. HPLC grade acetonitrile (ACN) and acrylic acid (99%) were Panreac reagents. *Caution: Because AN and AM are probably and possibly carcinogenic to humans, respectively,⁴³ they should be handled carefully.*

The reaction temperature was kept constant (± 0.05 °C) with a Lauda Ecoline RE120 thermostat. A Crison Micro pH 2000 pH-meter was used to perform pH measurements (± 0.01). Water was deionized with a MilliQ-Gradient device (Millipore). Numerical treatment of the data was performed using the Wolfram Mathematica[®] 7 software.

UFLC Separation of α,β -UC-guanosine adducts

UFLC separations of the reaction mixtures were performed with a 100- μL injection volume on a mediterranea sea18TM reversed-phase C18 column 5 μm , 250 \times 10 mm, attached to a mediterranea sea18TM guard C18 column, using a Shimadzu prominence gradient-controlled UFLC system (LC-20AD) equipped with a Shimadzu diode array detector (SPD-M20A) and channel UV light detection at 254 nm. The column oven (CTO-10AS) was set at 20 °C and the temperature of the diode array cell was 40 °C. H₂O and ACN were used as mobile phases and the flow rate was 1 mL min⁻¹. The chromatographic methods were as follows: Guo-AN Gradient: initially 90% H₂O; a 25 min linear gradient to 100% CH₃CN; isocratic at 100% CH₃CN for 5 min, followed by a 5 min linear gradient to the initial conditions. Guo-AM Gradient: initially 90% H₂O; a 5 min linear gradient to 50% CH₃CN; isocratic at 50% CH₃CN for 5 min; 5 min linear gradient to 100% CH₃CN; isocratic at 100% CH₃CN for 15 min, followed by a 5 min linear gradient to the initial conditions. Guo-AC Gradient: initially 90% H₂O; a 25 min linear gradient to 100% CH₃CN; isocratic at 100% CH₃CN for 5 min, followed by a 5 min linear gradient to the initial conditions.

Characterization of Guo-adducts

Purified alkylated guanosines were isolated from larger-scale reactions by semipreparative UFLC using a Shimadzu FRC-10A fraction collector. The samples were concentrated with a Genevac miVac Duo concentrator coupled to a miVac quattro pump. The Guo-adducts were redissolved and characterized according to their UV absorbance, ¹H NMR, and mass spectrometric features.

Positive and negative ion electrospray mass spectra were obtained using a Waters ZQ4000 apparatus. The samples were dissolved in ACN (200 μL).

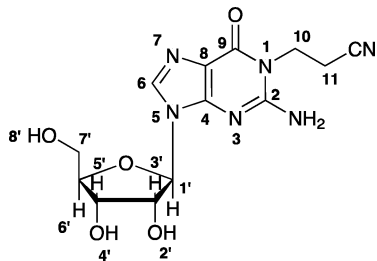
Proton NMR spectra of the adducts were obtained on a Bruker Avance 400 MHz apparatus. The samples were dissolved in Me₂SO-*d*₆ and the solvent was used as the internal reference standard.

The UV spectra of each peak were obtained with a Shimadzu diode array detector at pH 7.0 in the mobile phase, as described in the chromatographic methods.

The isolated adducts have the following spectroscopic characteristics:

Guo-AN adduct: UV λ_{max} (H₂O/CH₃CN)/nm 253sh and 223; δ_{H} (400 MHz, DMSO-*d*₆) 2.75 (dt, 2H, $J = 2.8, 9.0$ Hz, CH₂ H11), 3.60 (dt, 2H, $J = 3.0, 9.0$ Hz, CH₂ H10), 3.80 (dd, 2H, $J = 4.4, 12.1$ Hz,

CH₂ H^{7'}), 4.20 (t, 1H, *J* = 4.5 Hz, H^{5'}), 4.30 (t, 1H, *J* = 5.1 Hz, H^{3'}), 4.40 (dd, 1H, *J* = 3.7, 9.4 Hz, H^{6'}), 5.05 (bs, 1H, OH^{4'}), 5.10 (bs, 1H, OH^{8'}), 5.25 (bs, 1H, OH^{2'}), 5.80 (d, 1H, *J* = 6.0 Hz, H^{1'}), 7.95 (s, 1H, H⁶). **Guo-AM adduct:** UV λ_{\max} (H₂O/CH₃CN)/nm 257sh and 229. **Guo-AC adduct: 1.** UV λ_{\max} (H₂O/CH₃CN)/nm 258sh and 229; **2.** UV λ_{\max} (H₂O/CH₃CN)/nm 259sh and 229.



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