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Reactivity of the Mutagen 1,4-Dinitro-2-methylpyrrole as an Alkylating Agent

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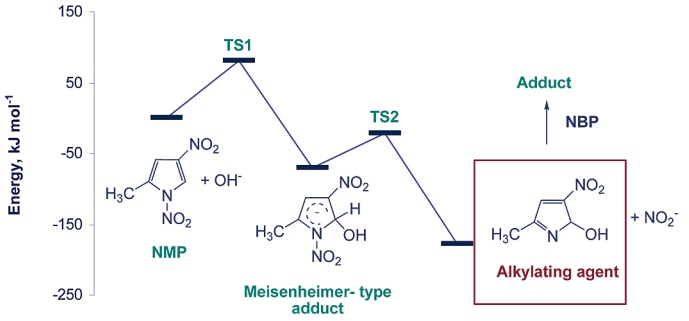

The formation of chemical species with DNA-damaging and mutagenic activity for bacterial test systems was detected in sorbic acid-nitrite mixtures. 1,4-Dinitro-2-methylpyrrole (NMP), one the main products resulting from the reaction between sorbic acid and nitrite, has mutagenic properties, and here its alkylating capacity was investigated. The conclusions drawn are as follows: (i) In aqueous medium, after the addition of a hydroxide ion and the subsequent loss of nitrite, NMP affords 5-methyl-3-nitro-1*H*-pyrrol-2-ol. This species is in equilibrium with 5-methyl-3-nitro-1*H*-pyrrol-2(5*H*)-one, the effective alkylating agent responsible for the genotoxic capacity of NMP; (ii) 5-methyl-3-nitro-1*H*-pyrrol-2(5*H*)-one alkylates 4-(*p*-nitrobenzyl)pyridine (NBP), a molecule with nucleophilic characteristics similar to those of DNA bases, forming an adduct (AD; $\varepsilon = 1.14 \times 10^4\ M^{-1}\ cm^{-1}$); (iii) The calculated energy barrier for the alkylation of NBP for NMP and the value of the fraction of alkylating agent forming the adduct are consistent with the observed mutagenicity of NMP; (iv) The reactivity of NMP can be explained in terms of the instability of the *N*-NO_2 bond as well as the effect of this group on aromaticity.

Introduction

Sorbic acid and potassium sorbate are widely used food additives that are generally recognized as safe[1] (GRAS), with an extremely high (25 mg/kg) acceptable daily intake level.[2] They inhibit the growth of fungi and yeasts and have antibacterial activity.[3,4] Although most studies have failed to reveal any mutagenic properties for these compounds, some results have indicated that they exhibit a weak genotoxic potential.[5−9]

One of the main uses of sorbic acid as a food preservative is in the curing of meat where, in conjunction with sodium nitrite, it inhibits the growth of *Clostridium botulinum* and also reduces the formation of nitrosamines.[10]

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The formation of chemical species with DNA-damaging and mutagenic activity for bacterial test systems has been detected in sorbic acid−nitrite mixtures.[11−14] In the 3.5−4.2 pH range, the main mutagens have been proposed to be ethylnitrolic acid (ENA) and 1,4-dinitro-2-methylpyrrole (NMP).[11,15,16]

ENA is at least 40-fold more active than sorbic acid alone, and at least 30-fold more active than sodium nitrite alone in the bacterial *rec*-assay.[17] It also is a direct-acting mutagen in the *Salmonella* forward-mutation assay.[18] A kinetic study of its alkylation mechanism has revealed that acetonitrile oxide, an ENA decomposition product,[19,20] is the active alkylating agent.[21]

NMP shows strong activity in the *rec*-assay. The Ames assay with strains TA98 and TA100 without metabolic activation also reveals high mutagenic activity.[11]

Since (i) NMP shows an unusual degree of reactivity toward nucleophiles[11] in comparison with pyrroles without the nitro group in the ring, and (ii) to our knowledge, the mechanism through which NMP acts as an alkylating agent is not known, here we were prompted to investigate these issues. The nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents[22] with nucleophilic characteristics similar to those of DNA bases,[23] was used to determine the alkylating capacity of NMP.

Results and Discussion

Reaction Mechanism for the Alkylation of NBP. The experimental observations (i) that the NMP molecule decomposes in slightly alkaline aqueous solution[19,20] and (ii) that the alkylation reaction of NBP for NMP shows an induction period in its kinetic profile (Figure 1) suggest that NMP does not react directly with the NBP molecule, the alkylating agent being one of the NMP decomposition products that forms an adduct (AD) with NBP.

In the NMP decomposition reaction, hydroxide ions react with the compound to form a Meisenheimer-type adduct (**1**),[19,20] which then undergoes nitrite ion loss. The resulting species (**2**) exist as an equilibrium among different isomers, one of which would be the effective alkylating species (Scheme 1).

To check the reactivity of NMP toward OH^-, isodensity surfaces of NMP were drawn. The calculations revealed a minor charge density on the C-atom situated in α position with respect to the pyrrolic nitrogen. The high electrophilicity of this site helps to understand the mechanism involved in the addition of OH^- to the NMP molecule. Moreover, while NMP reacts with nucleophiles, such as the methoxide ion,[15] the lack of reactivity of pyrrole toward nucleophiles is well-known.[24] Here we investigated how aromaticity and variations in it affect the reactivity of NMP.

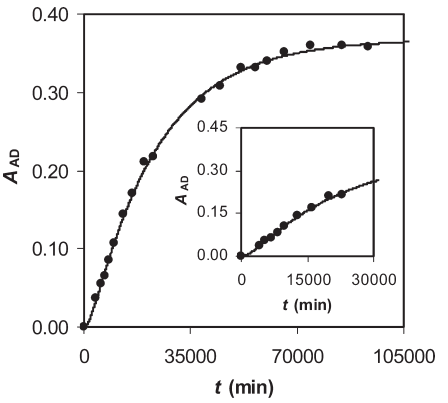
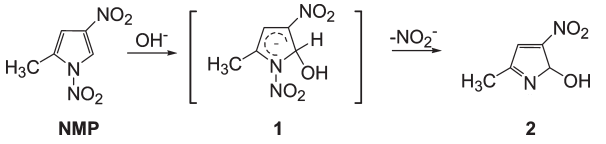


FIGURE 1. Kinetic profile of the NBP alkylation reaction for NMP. Variation in the absorbance of the adduct (A_{AD}) in 7:3 water/dioxane medium; λ = 575 nm; pH = 4.77; $[NMP]_o = 8.0 \times 10^{-4}$ M; $[NBP]_o = 1.5 \times 10^{-2}$ M; T = 37.5 °C.

SCHEME 1. NMP Decomposition Reaction



The most widely used index to measure aromaticity involves Nucleus-Independent Chemical Shifts (NICS).[25,26] NICS data were calculated at the center of the rings (NICS (0)) and at 1 Å above the molecular plane (NICS (1)). Negative values of NICS reveal points in or near an aromatic system.[25] Antiaromatic systems have strongly positive NICS values.[26]

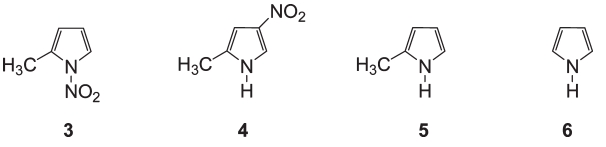


FIGURE 2. Pyrroles investigated in this work.

The NICS values of some pyrroles (Figure 2) were calculated (Table 1). Nonsubstituted pyrrole **6** was by far the most aromatic, the presence of the 2-methyl substituent decreasing the aromaticity of the ring (**5**). The nitro groups had very different effects, depending on their position: 4-NO_2 increased the aromaticity slightly, as in **4** with respect to **5** and NMP with respect to **3**, while 1-NO_2 decreased

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TABLE 1. NICS(0) and NICS(1) Values for Pyrroles[a]

compound	NICS(0)	NICS(1)
NMP	−13.04	−8.02
3	−10.85	−7.85
4	−12.88	−8.81
5	−12.47	−8.45
6	−13.62	−10.09

[a]Calculated at DFT-B3LYP 6-311+G(d,p) level.

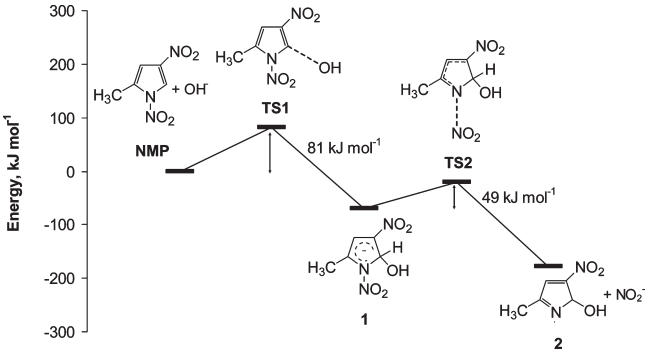


FIGURE 3. Energy barriers calculated for Meisenheimer-type adduct (**1**) formation and elimination of the NO_2 group.

aromaticity very significantly, as in NMP with respect to **4** or **3** with respect to **5**.

The low aromaticity of the nitropyrrole NMP with respect to other pyrroles without nitro groups on the pyrrolic nitrogen appears to be the cause of the different reactivity of this molecule.

The energy barriers calculated for the Meisenheimer-type adduct formation and the elimination of the NO_2 group are shown in Figure 3. As can be observed, the attack by hydroxide ions is the limiting step, the barrier being quite significant (81 kJ mol^{-1}), while the loss of nitrite is favored kinetically, with a barrier of only 49 kJ mol^{-1}. The **TS1** transition state is quite low in energy, which allows the somewhat unexpected addition of the hydroxide ion. This could be partly due to the remaining aromaticity (NICS(0) and NICS(1) being −10.59 and −6.35, respectively). The calculations show that the charge in **1** is conjugated in the large π system connecting the two nitro groups.

The results show that the reactivity of NMP can be understood in terms of the instability of the *N*-NO_2 bond and its effect on aromaticity: the low aromaticity as well as the acceptor character of the nitro group favor the addition of OH^- to the unsubstituted α carbon. The charged intermediate (**1**) formed is highly conjugated, although nonaromatic, and it provides a low-energy path for the elimination of the unstable NO_2 group (transition state **TS2**). The product of this nitrite loss (**2**), although more stable than **1**, is still nonaromatic and very high in energy. Product **2** can be interconverted, by deprotonation and reprotonation, into several isomers (Figure 4). The isomerization energies ($\Delta G_{isom} = G_n - G_7$), with **7** as a reference, are reported in Table 2.

Among these isomers, **7** recovered a large part of the aromaticity (NICS(0) and NICS(1) being −13.23 and −7.55, respectively) and, thus, showed the minimum value of energy dominating the isomer mixture. Owing to its greater aromaticity, **7** is sparingly reactive toward nucleophiles. The more stable isomers (**8** and **9**) have a common characteristic in their

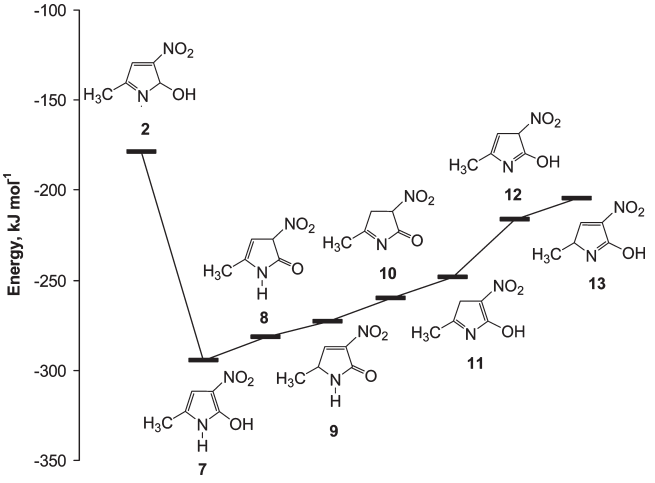
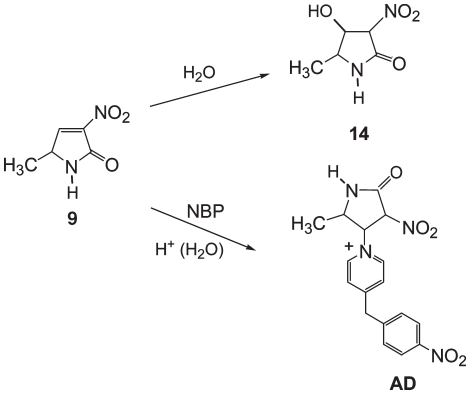


FIGURE 4. Energy diagram for the isomers of **7**.

TABLE 2. ΔG_{isom} Values for the Different Isomers of 7

compound	ΔG_{isom} (kJ mol^{-1})
8	12.8
9	21.8
10	34.4
11	46.1
12	78.3
13	90.1
2	116.2

SCHEME 2. Reactivity of 9 towards Water and NBP Molecules



structure: a hydrogen atom at the pyrrolic nitrogen and the lactame group.

Isomer **9** is more electrophilic at carbon 4, and because of this, a low-energy path for nucleophilic attacks does exist. Nucleophiles such as water or NBP will react with this compound to form 4-hydroxy-5-methyl-3-nitro-pyrrolidin-2-one (**14**) and the NBP-isomer **9** adduct (AD), respectively (Scheme 2). The reaction barrier for the addition of NBP was calculated to be 71 kJ mol^{-1}; this value is very low and in keeping with the most active alkylating agents, such as β-propiolactone,[27] β-butyrolactone,[27] or diketene.[28]

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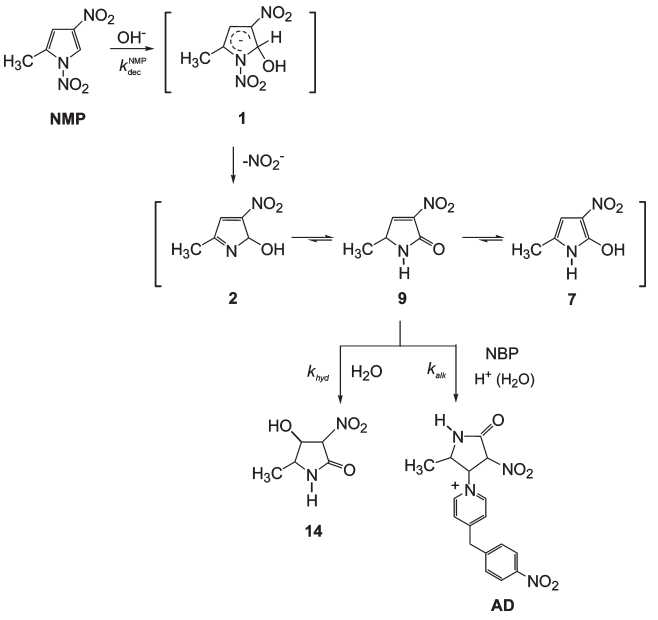
SCHEME 3. Mechanism of NBP Alkylation for NMP



The above energy difference, together with ΔG_{isom} = 21.8 kJ mol^{-1}, affords a global free energy barrier of 93 kJ mol^{-1}, which is very much in keeping with species of a lower alkylating potential, such as sorbic acid and sorbates,[8,9] acrylamide,[29] and styrene oxides, among which NMP can be included.

The mass spectra of the most stable isomer (**7**) and the hydrolysis product (**14**) show m/z = 144 and 161, respectively, in agreement with the suggested structures. The latter was identified through ^{1}H and ^{13}C NMR spectroscopy (see Supporting Information).

Based on the above results, Scheme 3 shows the proposed mechanism for the reaction between NMP and NBP.

Kinetics of the NBP Alkylation Reaction. From the mechanism in Scheme 3, eq 1 can be deduced:

$$\text{rate} = \frac{\mathrm{d}[\mathrm{AD}]}{\mathrm{d}t} = k_{\mathrm{alk}}[\mathbf{9}][\mathrm{NBP}] \quad (1)$$

Because pH was kept constant and the experiments were performed with [NBP] and [H_2O] in large excess (see Experimental Section), in agreement with the general kinetic scheme for consecutive first-order reactions[30] and the boundary condition $[\mathbf{9}]_o = 0$, the concentration of the alkylating agent at time t is given by eq 2:

$$[\mathbf{9}] = \frac{k_{\mathrm{obs}}^{\mathrm{NMP}}[\mathrm{NMP}]_o}{(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}} - k_{\mathrm{obs}}^{\mathrm{NMP}})}\left(e^{-k_{\mathrm{obs}}^{\mathrm{NMP}}t} - e^{-(k_{\mathrm{hyd\ obs}}+k_{\mathrm{alk\ obs}})t}\right) \quad (2)$$

where $k_{\mathrm{obs}}^{\mathrm{NMP}}$, $k_{\mathrm{hyd\ obs}}$, and $k_{\mathrm{alk\ obs}}$ are the pseudofirst order rate constants defined as $k_{\mathrm{obs}}^{\mathrm{NMP}} = k_{\mathrm{dec}}^{\mathrm{NMP}}[\mathrm{OH}^-]$, $k_{\mathrm{hyd\ obs}} = k_{\mathrm{hyd}}[H_2O]$, and $k_{\mathrm{alk\ obs}} = k_{\mathrm{alk}}[\mathrm{NBP}]$, respectively.

(29) Céspedes-Camacho, I. F.; Manso, J. A.; Pérez-Prior, M. T.; Gómez-Bombarelli, R.; González-Pérez M.; Calle, E.; Casado, J. *J. Phys. Org. Chem.* **2010**, *23*, 171.

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Therefore, eq 1 can be converted to eq 3.

$$\text{rate} = \frac{\mathrm{d}[\mathrm{AD}]}{\mathrm{d}t} = k_{\mathrm{alk\ obs}}\frac{k_{\mathrm{obs}}^{\mathrm{NMP}}[\mathrm{NMP}]_o}{(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}} - k_{\mathrm{obs}}^{\mathrm{NMP}})}\left(e^{-k_{\mathrm{obs}}^{\mathrm{NMP}}t} - e^{-(k_{\mathrm{hyd\ obs}}+k_{\mathrm{alk\ obs}})t}\right) \quad (3)$$

Equation 4 shows the result of the integration of eq 3, A_{AD}, ε_{AD}, and l, respectively, being the absorbance of the adduct at time t, the molar absorption coefficient, and the cuvette light path.

$$A_{\mathrm{AD}} = \frac{k_{\mathrm{alk\ obs}}[\mathrm{NMP}]_o\varepsilon_{\mathrm{AD}}l}{(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}} - k_{\mathrm{obs}}^{\mathrm{NMP}})(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}})}\left[(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}})(1 - e^{-k_{\mathrm{obs}}^{\mathrm{NMP}}t}) - k_{\mathrm{obs}}^{\mathrm{NMP}}(1 - e^{-(k_{\mathrm{hyd\ obs}}+k_{\mathrm{alk\ obs}})t})\right] \quad (4)$$

To handle eq 4 in a simpler form, it can be written as in eq 5.

$$y = a[b(1 - e^{-cx}) - c(1 - e^{-bx})] \quad (5)$$

where a, b, and c are defined in the form $a = k_{\mathrm{alk\ obs}}[\mathrm{NMP}]_o\varepsilon_{\mathrm{AD}}l/(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}} - k_{\mathrm{obs}}^{\mathrm{NMP}})(k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}})$, $b = (k_{\mathrm{hyd\ obs}} + k_{\mathrm{alk\ obs}})$, and $c = k_{\mathrm{obs}}^{\mathrm{NMP}}$. The value of the latter (c) has been determined by us previously.[24,25]

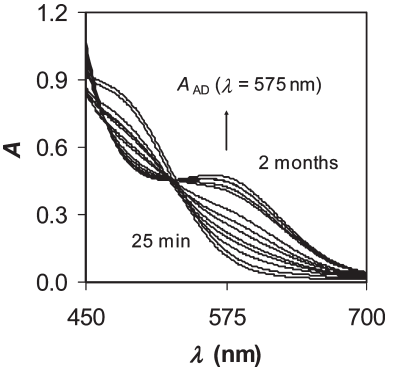


FIGURE 5. Formation of the NBP-isomer **9** adduct over time in 7:3 water/dioxane medium; pH = 4.77; $[\mathrm{NMP}]_o = 8.0 \times 10^{-4}$ M; $[\mathrm{NBP}]_o = 1.5 \times 10^{-2}$ M; T = 37.5 °C.

Figure 5 shows the increase in absorption caused by the formation of the adduct (λ = 575 nm) over time. The measured value of the molar absorption coefficient of the adduct in 7:3 (v/v) water/dioxane medium is $\varepsilon_{AD} = 1.14 \times 10^4$ M^{-1} cm^{-1}.

Figure 1 shows the good fit of the results to eq 4.

The quotient between the NBP alkylation rate constant by **9** and the hydrolysis rate constant of **9**, (k_{alk}/k_{hyd}), was used as a reference for correlations between mutagenic activity and alkylating potential. To determine its value, we proceeded as follows.

The definitions of parameters a, b, and c (eq 5) allow one to write eq 6.

$$\frac{1}{a(b-c)} = \frac{[\mathrm{H_2O}]}{[\mathrm{NMP}]_o\varepsilon_{\mathrm{AD}}l}\left(\frac{k_{\mathrm{hyd}}}{k_{\mathrm{alk}}}\right)\frac{1}{[\mathrm{NBP}]_o} + \frac{1}{[\mathrm{NMP}]_o\varepsilon_{\mathrm{AD}}l} \quad (6)$$

Figure 6 shows the good fit of the experimental results to eq 6.

The values of (k_{alk}/k_{hyd}) were calculated with the slope and intercept of eq 6.

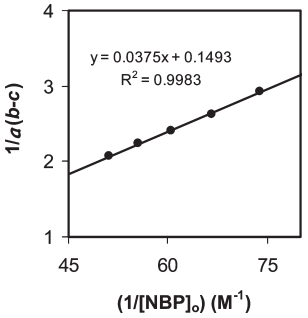


FIGURE 6. Fitting of the results to eq 6 in 7:3 water/dioxane medium; pH = 4.77; $[NMP]_o = 8.0 \times 10^{-4}$ M; $T = 37.5$ °C.

Another parameter of interest regarding the influence of the hydrolysis reaction of **9** in the alkylating capacity of this compound toward the NBP molecule in chemical quantitative terms is the fraction of alkylating agent (**9**) forming the adduct (*f*). It is defined as follows:

$$f = \frac{k_{alk}[NBP]}{k_{alk}[NBP] + k_{hyd}[H_2O]} \quad (7)$$

Equation 7 can be written in the form

$$\frac{1}{f} = 1 + \frac{1}{(k_{alk}/k_{hyd})}\frac{[H_2O]}{[NBP]} \quad (8)$$

Equation 8 shows that the value of *f* can be known once the quotient (k_{alk}/k_{hyd}) has been calculated.

TABLE 3. Alkylating Capacity of 9 Compared to that of Other Alkylating Agents[a]

alkylating agent	(k_{alk}/k_{hyd})	*f*
β-propiolactone[b]	5395	0.76
β-butyrolactone[b]	3158	0.62
9[c]	155	0.07

[a] $[NBP]_o = 2.0 \times 10^{-2}$ M; 7:3 water/dioxane. [b] Ref 27; $T = 35.0$ °C. [c] pH = 4.77; $T = 37.5$ °C.

Comparison of the (k_{alk}/k_{hyd}) and *f* values for NBP alkylation by **9** with those determined with other alkylating agents should be of interest. Table 3 shows the values obtained here together with those measured previously by us for NBP alkylation by lactones such as β-propiolactone and β-butyrolactone.

The low value of *f* for **9** in comparison with that of other alkylating agents (Table 3) reveals a smaller fraction of alkylating agent forming the adduct, and a greater hydrolyzed fraction.

Table 4 shows some of the results from the literature on the biological activity of some mutagens with the Ames test and *rec*-assay, pointing to the greater mutagenic activity of NMP. On the basis of the above results, this NMP mutagenic potential can be explained in terms of stability. Thus, although only a small fraction of the alkylating agent (~7%) reacts with the NBP molecule, the adduct formed persists for some time (approximately 2 months; see Figures 1 and 5), and as a consequence of this its mutagenic activity would probably be relevant.

Conclusions

The results obtained about the reactivity of the mutagen 1,4-dinitro-2-methylpyrrole as an alkylating agent allow us to draw the following conclusions: (1) In aqueous medium, 1,4-dinitro-2-methylpyrrole affords 5-methyl-3-nitro-1*H*-pyrrol-2-ol after addition of a hydroxide ion and subsequent loss of nitrite. This species is in equilibrium with 5-methyl-3-nitro-1*H*-pyrrol-2(5*H*)-one, the effective alkylating agent, responsible for the genotoxic capacity of NMP. (ii) 5-Methyl-3-nitro-1*H*-pyrrol-2(5*H*)-one alkylates 4-(*p*-nitrobenzyl)pyridine (NBP), a molecule with nucleophilic characteristics similar to those of DNA bases, forming an adduct (AD; $\varepsilon = 1.14 \times 10^4$ M^{-1} cm^{-1}). The alkylation of NBP by NMP complies with the rate equation

$$\text{rate} = d[AD]/dt = k_{alk\ obs}k_{obs}^{NMP}[NMP]_o\left(e^{-k_{obs}^{NMP}t} - e^{-(k_{hyd\ obs}+k_{alk\ obs})t}\right)/(k_{hyd\ obs} + k_{alk\ obs} - k_{obs}^{NMP})$$

where $k_{alk\ obs}$ and $k_{hyd\ obs}$ are the observed NBP alkylation rate constant and the observed rate constant for the hydrolysis of 5-methyl-3-nitro-1*H*-pyrrol-2(5*H*)-one, respectively. (iii) The calculated energy barrier for the alkylation of NBP for NMP, as well as the value of the fraction of alkylating agent forming an adduct are consistent with the observed mutagenicity of NMP. (iv) The reactivity of NMP can be explained in terms of the instability of the *N*-NO_2 bond as well as its effect on aromaticity.

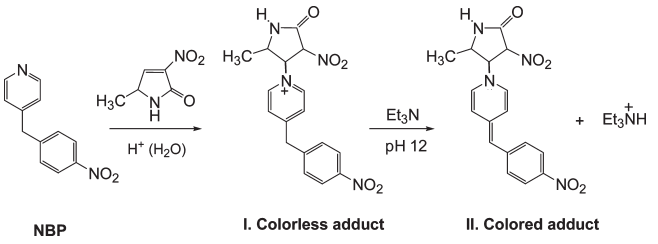
Methodology and Computational Details. As said before, to check the reactivity of NMP toward OH^-, as well as to gain deeper insight into the Meisenheimer-type adduct formation and the elimination of the NO_2 group, some additional computational chemistry was performed.

TABLE 4. Biological Activity of Some Mutagens[a]

	rec-assay with *B. subtilis*		Ames test
	growth inhibition		revertants/μg per plate
compound	*rec*$^+$	*rec*$^-$	TA100
caffeine[b,c]	−	±	< 70/6000
mitomicine C[b,c]	++	+++	< 70/1
N-nitroso-*N*-methyl urethane[b,c]	++	++++	
N-methyl-*N*′-nitro-*N*-nitrosoguanidine[b,c]	±	++	18700/2
N-oxide 4-nitroquinoline[b,c]	+	++	7640/0.5
styrene oxide[c]			1292/500
β-propiolactone[c]			6460/114
ethylnitrolic acid[d]	−	++	583/150
1,4-dinitro-2-methyl pyrrole[d]	−	++	1704/100

[a] − no inhibition zone; ± length of inhibition zone is less than 5 mm; + 5−10 mm of inhibition zone; ++ more than 10 mm of inhibition zone; +++ more than 20 mm of inhibition zone; ++++ more than 30 mm of inhibition zone. [b] *rec*-Assay with *B. subtilis* (ref 31). [c] Ames test (ref 32). [d] Ref 11.

SCHEME 4. Method for Monitoring NBP Alkylation by 9



All calculations were carried out using Gaussian 03W. Free-energy differences were calculated from the free energies of the corresponding molecules obtained from the vibrational analysis at the DFT-B3LYP 6-31++G(2df,2pd) level, using the IEF-PCM method with default parameters for solvation. Molecules were checked to have either one (transition states) or zero vibration modes with imaginary frequencies.

NICS calculations were performed as gas-phase GIAO at the DFT-B3LYP 6-311+G(d,p) level.[33]

Experimental Section

Acetate buffer was used to maintain pH constant. To render NBP soluble, the alkylation mixtures (NMP + NBP) were prepared in 7:3 (v/v) water/dioxane medium. To monitor the alkylation reactions, 2.4 mL aliquots of the alkylation mixture were removed at different times and added to a cuvette containing 0.6 mL of 99% triethylamine reagent (Et_3N) to stop the alkylation process (Scheme 4), after which absorbance was measured at the wavelength of maximum absorption (λ = 575 nm). Detailed reaction conditions are given in the figure and table legends. A spectrophotometer with a thermoelectric six-cell holder temperature control system (±0.1 °C) was used. Detailed reaction conditions are given in the figure and table legends. The reaction temperature was kept constant (±0.05 °C). All kinetic runs were performed in triplicate.

Synthesis of 1,4-Dinitro-2-methylpyrrole. 1,4-Dinitro-2-methylpyrrole was obtained from the reaction between sorbic acid and sodium nitrite in aqueous solution at pH = 3.5.[19]

A solution of sodium nitrite (11.0 g) was added to a partially suspended solution of sorbic acid (2.2 g) in distilled water (200 mL). While the pH of the mixture was kept constant at 3.5 with dilute H_2SO_4, the mixture was stirred at 60 °C for 2 h. The mixture was extracted with four 50 mL portions of CH_2Cl_2. The combined extracts were washed with water, dried over Na_2SO_4, and evaporated to dryness in vacuo to give the residue. The residue was recrystallized in chloroform−ether.

Identification of the product NMP was carried out by UV-spectroscopy, mass spectroscopy, and ^1H NMR spectroscopy (see Supporting Information).

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Supporting Information Available: Free energies for all optimized structures and Cartesian coordinates. This material is available free of charge via the Internet at http://pubs.acs.org.

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