

# Alkylating potential of *N*-phenyl-*N*-nitrosourea

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Alkylating agents are considered to be archetypical carcinogens. Since the decomposition of alkylnitrosoureas gives rise to alkyldiazonium ions without any need for metabolic activation, they offer an ideal series of compounds with which to examine the effect of variations in chemical structure on alkylating potential. Due to its instability *N*-phenyl-*N*-nitrosourea is easily hydrolyzed to a benzenediazonium ion. Since *N*-phenyl-*N*-nitrosourea shows a particular behavior when compared with other nitrosoureas, here its alkylating potential was studied. The nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents, was used as an alkylation substrate. Conclusions were drawn as follows: (i) the mechanism of the alkylation reaction of NBP by phenylnitrosourea is different from that caused by other nitrosoureas; (ii) the formation of the NBP-*N*-phenyl-*N*-nitrosourea adduct occurs about 20,000-fold slower than with *N*-methyl-*N*-nitrosourea, one of the most effective carcinogenic nitrosoureas; and, (iii) a correlation was found between the alkylating potential of nitrosoureas and their tumorigenicity. Copyright © 2008 John Wiley & Sons, Ltd.

**Keywords:** reactivity of *N*-phenyl-*N*-nitrosourea; alkylation reactions

## INTRODUCTION

Alkylating agents are considered to be archetypical carcinogens.<sup>[1,2]</sup> Since the decomposition of alkylnitrosoureas (ANU) gives rise to alkyldiazonium ions ( $\text{RN}_2^+$ ) without any need for metabolic activation, they offer an ideal series of compounds with which to examine the effect of variations in chemical structure on alkylating potential.

Previous studies (Chapter 3<sup>[3]</sup>) have shown that some ANUs induce tumors in different organs of rats, but does not have a favorable partition coefficient into organic solvents from water. If physical properties such as solubility and partition coefficient play any role, it must be minor and perhaps limited to effects on potency. Some results<sup>[4]</sup> have indicated the structure of the alkyl group adjacent to the nitroso group as the most important determinant of the carcinogenic effect.

We tested the alkylating potential of various *N*-alkyl-*N*-nitrosoureas. We found that these alkylation reactions occur mainly through steric control.<sup>[5]</sup>

Due to its instability, *N*-phenyl-*N*-nitrosourea<sup>[6]</sup> (PhNU) is easily hydrolyzed to a benzenediazonium ion ( $\text{PhN}_2^+$ )<sup>[7]</sup> and induces local tumors in rats.<sup>[8]</sup> However, painting PhNU on mouse skin failed or induced few skin tumors.<sup>[9]</sup> The reason for its failure to induce skin tumors is, therefore, not clear.

Despite its chemical relevance, to our knowledge, the chemical reactivity of *N*-phenyl-*N*-nitrosourea as an alkylating agent has not been investigated in a comparative quantitative way, and accordingly, we were prompted to address this issue.

## RESULTS AND DISCUSSION

The nucleophile 4-(*p*-nitrobenzyl)pyridine (NBP), a trap for alkylating agents<sup>[10]</sup> with nucleophilic characteristics similar to DNA bases,<sup>[11]</sup> was used as the alkylation substrate. We had

previously used this method to investigate the alkylating potential of strong alkylating reagents such as lactones<sup>[12–16]</sup> as well as to determine the reactivity of much weaker alkylating molecules, such as sorbates.<sup>[17,18]</sup>

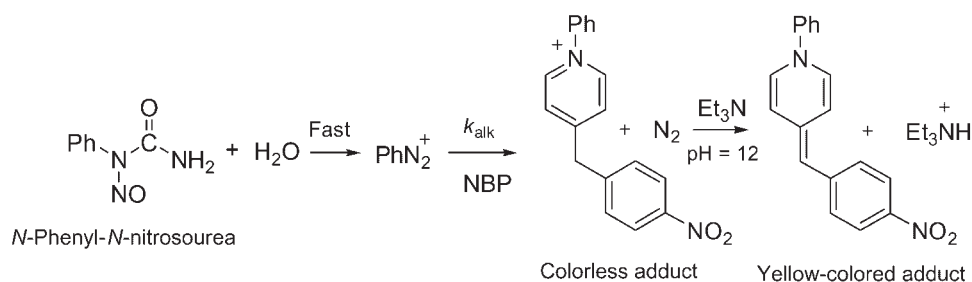
The NBP alkylation reaction by *N*-phenyl-*N*-nitrosourea was investigated. The effective alkylating agent resulting from the decomposition of PhNU is the benzenediazonium ion, giving rise to NBP-PhNU adduct (Scheme 1).

In order to render the NBP soluble, the PhNU + NBP alkylation mixtures were prepared in a 7:3 (vol) water/dioxane medium. Reactions were carried out in the 5.0–6.0 pH range. Acetic/acetate buffer was used to maintain pH constant. 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 ( $\text{Et}_3\text{N}$ ), which stopped the alkylation process and generated a yellow color, the absorbance of which was measured at the wavelength of maximum absorption (see below). The observation of  $\text{N}_2$  bubbles along the reaction shows that this occurs through a dediazoniation mechanism.

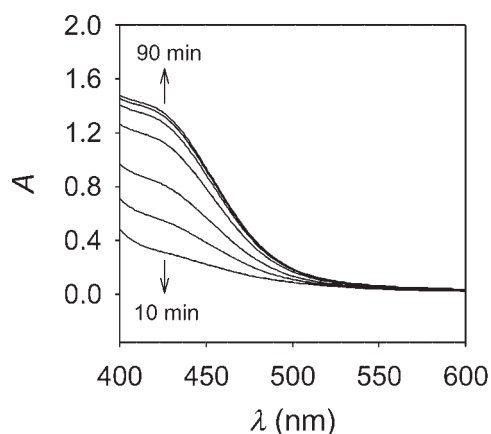
The NBP-PhNU adduct showed maximum absorption at  $\lambda = 425$  nm. As an example, Fig. 1 shows the increase in absorption caused by the formation of the NBP-PhNU adduct over time until no change in absorbance, *A*, was observed. Because the NBP was in large excess, it can be assumed that all the nitrosourea was consumed.

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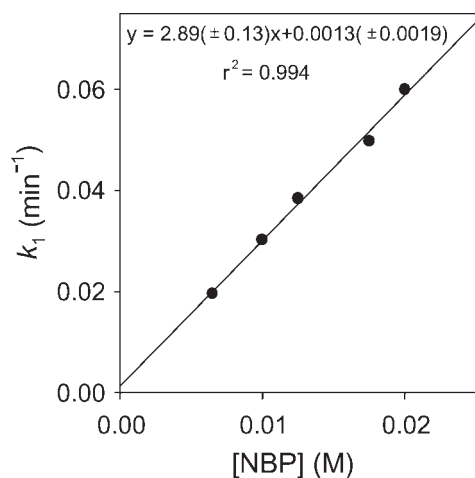
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Scheme 1.



**Figure 1.** Spectrograms showing the formation of the NBP-Ph adduct over time in a 7:3 water/dioxane medium. Variation in absorbance in the 10–90 min interval.  $[\text{PhNU}]_0 = 2 \cdot 10^{-4} \text{ M}$ ;  $[\text{NBP}] = 0.01 \text{ M}$ ;  $T = 35^\circ \text{C}$ ; and  $\text{pH} = 5.72$

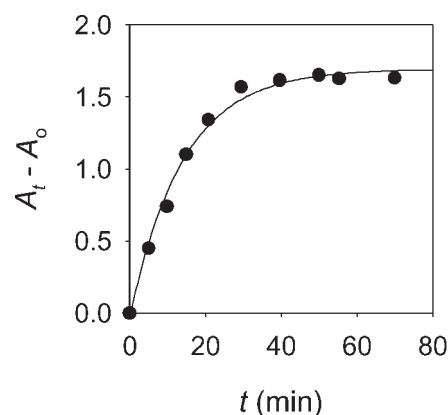


**Figure 2.** Influence of  $[\text{NBP}]$  in the alkylation reaction of NBP by PhNU at  $35^\circ \text{C}$  in a 7:3 water/dioxane medium.  $[\text{PhNU}]_0 = 2 \cdot 10^{-4} \text{ M}$  and  $\text{pH} = 5.72$

In agreement with the mechanism in Scheme 1, and  $[\text{PhN}_2^+]_0 \cong [\text{PhNU}]_0$ , the alkylation reaction rate of NBP by phenylnitrosourea is

$$\text{rate} = \frac{d[\text{AD}]}{dt} = k_{\text{alk}}[\text{PhN}_2^+][\text{NBP}] = k_1[\text{PhN}_2^+], \quad (1)$$

where,  $[\text{AD}]$  represents the concentration of the NBP-PhNU adduct, and  $k_1 = k_{\text{alk}}[\text{NBP}]$  is the pseudo-first-order rate constant. In the 5.0–6.0 pH range, no dependence on the acidity of the



**Figure 3.** Formation of the NBP-Ph adduct in a 7:3 water/dioxane medium;  $[\text{PhNU}]_0 = 2 \cdot 10^{-4} \text{ M}$ ;  $[\text{NBP}] = 0.02 \text{ M}$ ;  $\text{pH} = 5.72$ ;  $T = 37.5^\circ \text{C}$ .  $A_0$  and  $A_t$  designate the absorbance values of the adducts at times, respectively, of 0 and  $t$

**Table 1.** Alkylation rate constants as a function of temperature for benzenediazonium ion in a 7:3 water/dioxane medium

$T$ ( $^\circ \text{C}$ )	$k_{\text{alk}}$ ( $\text{M}^{-1} \text{min}^{-1}$ ) <sup>a</sup>
25.0	$1.0 \pm 0.2$
27.5	$1.4 \pm 0.2$
30.0	$1.7 \pm 0.2$
32.5	$2.2 \pm 0.4$
35.0	$3.0 \pm 0.4$
37.5	$3.5 \pm 0.4$

<sup>a</sup> Values are given with their standard deviations

medium was observed, unlike other nitrosoureas.<sup>[5]</sup> Therefore, the mechanism of the NBP alkylation by PhNU, through the formation of the benzenediazonium ion, which is more stable than aliphatic alkyldiazonium ions, must be different from that of other nitrosoureas. Since the intercept of the  $k_1/[\text{NBP}]$  plot is not significantly different from 0 (Fig. 2), the possibility of some competitive reactions, such as solvolytic dediazoniations,<sup>[19]</sup> was discarded in the current conditions.

By designating as  $A_0$ ,  $A_t$ , and  $A_\infty$ , the absorbance values of the NBP-PhNU adduct at times, respectively, of 0,  $t$ , and infinity (i.e., when the plateau is reached; see Fig. 3),  $\epsilon_{\text{PhN}_2^+}$ ,  $\epsilon_{\text{NBP}}$ , and  $\epsilon_{\text{AD}}$  being the molar absorption coefficients of  $\text{PhN}_2^+$ , NBP, and

**Table 2.** Alkylating potential of *N*-phenyl-*N*-nitrosoarene and *N*-methyl-*N*-nitrosoarene and their tumorigenicity

<i>N</i> -alkyl- <i>N</i> -nitrosoarene	Alkylating potential (Table 1) (35 °C)	Tumorigenicity <sup>[9]</sup>
	$k_{\text{alk}}$ (M <sup>-1</sup> min <sup>-1</sup> ) <sup>a</sup>	Number of Swiss mice with skin tumors
<i>N</i> -methyl- <i>N</i> -nitrosoarene	62,000 ± 2,000	19
<i>N</i> -phenyl- <i>N</i> -nitrosoarene	3.0 ± 0.4	1

<sup>a</sup> Values are given with their standard deviations.

adducts, respectively, and  $x$  the adduct concentration, Eqns (2)–(4) can be written.

$$A_o = \varepsilon_{\text{PhN}_2^+} [\text{PhN}_2^+]_o + \varepsilon_{\text{NBP}} [\text{NBP}] \quad (2)$$

$$A_t = \varepsilon_{\text{PhN}_2^+} ([\text{PhN}_2^+]_o - x) + \varepsilon_{\text{NBP}} [\text{NBP}] + x\varepsilon_{\text{AD}} \quad (3)$$

$$A_\infty = \varepsilon_{\text{AD}} [\text{PhN}_2^+]_o + \varepsilon_{\text{NBP}} [\text{NBP}] \quad (4)$$

Integration of Eqn (1) gives

$$A_t - A_o = (A_\infty - A_o)(1 - e^{-k_1 t}) \quad (5)$$

Figure 3 shows the fitting of the experimental data to Eqn (5).

In order to calculate the alkylation rate constant,  $k_{\text{alk}}$  (Scheme 1), experiments at different [NBP] were carried out (Fig. 2). The intercept, not significantly different from 0, revealed first order with respect to the concentration of [NBP] according to Eqn (1).

Table 1 gives the values of  $k_{\text{alk}}$  for the alkylation reaction as a function of temperature ( $T$ ).

The value of the rate constants for NBP alkylation by phenylnitrosoarene is consistent with its biological activity in comparison with other nitrosoarenes.<sup>[9]</sup> The NBP alkylation rate constant by the methyldiazonium ion (previously calculated with data from<sup>[5]</sup> and<sup>[20]</sup>) is about 20,000-fold greater than that of the phenyldiazonium ion. This result is consistent with their relative biological activities, can be observed in Table 2.

The structural characteristics of alkylnitrosoarenes, such as molecular size, are important as regard to their biological activity, hindered alkylnitrosoarenes being relatively inactive biologically.<sup>[3,21]</sup> This is consistent with the sequence of the alkylating rate constants found here for the respective methyldiazonium and benzenediazonium ions.

The calculated activation energy is  $E_a = 75 \pm 2 \text{ kJ mol}^{-1}$ .

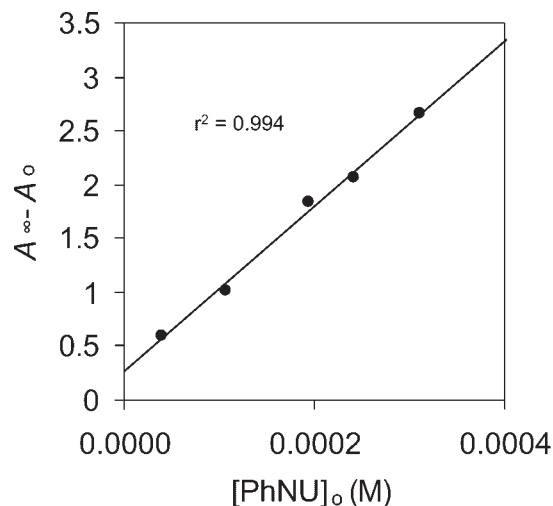
The kinetic results here observed are consistent with the fact that the biological activity of alkylnitrosoarenes decreases when their molecular size increases hindered alkylnitrosoarenes being biologically inactive.<sup>[3]</sup>

### Molar absorption coefficient of the NBP-PhNU adduct

We were also interested in knowing the molar absorption coefficient ( $\varepsilon$ ) of NBP-PhNU adduct. Knowledge of these values should permit easy determination of the concentration of adduct by simply measuring the absorbance.

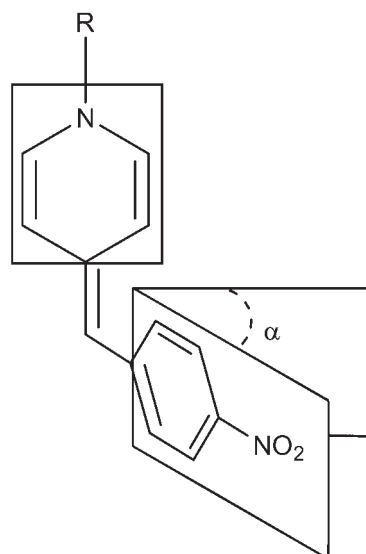
Experiments were performed with [NBP] = 0.02 M and PhNU concentrations in the  $4 \times 10^{-5}$ – $3.5 \times 10^{-4}$  M range.

The plot in Fig. 4 shows the absorption coefficients to be  $\varepsilon_{\text{NBP-PhNU}} = 7,674 \pm 343 \text{ M}^{-1} \text{ cm}^{-1}$  ( $\lambda = 425 \text{ nm}$ ).



**Figure 4.** Absorption of NBP-PhNU adduct in a 7:3 water/dioxane medium

The structures of the NBP-PhNU adduct obtained by geometry optimization (refer to Experimental Section) revealed (Fig. 5) a planar adduct, with an angle between the phenyl rings,  $\alpha$ , approximately equal to  $0^\circ$ . Thus, the  $\varepsilon$  value should be greater



**Figure 5.** Lack of coplanarity in the NBP-PhNU adducts

**Table 3.** Correlation between the structure of NBP-ANU adducts and their molar absorption coefficients,  $\epsilon_{AD}$ .

<i>N</i> -alkyl- <i>N</i> -nitrosourea	$\alpha$ (°)	$\epsilon_{AD}$ (M <sup>-1</sup> cm <sup>-1</sup> )
<i>N</i> -phenyl- <i>N</i> -nitrosourea	≈ 0	7,674 ± 343
<i>N</i> -methyl- <i>N</i> -nitrosourea <sup>a</sup>	29.0	521 ± 30
<i>N</i> -allyl- <i>N</i> -nitrosourea <sup>a</sup>	31.0	282 ± 20
<i>N</i> -ethyl- <i>N</i> -nitrosourea <sup>a</sup>	31.2	204 ± 8
<i>N</i> -propyl- <i>N</i> -nitrosourea <sup>a</sup>	31.6	93 ± 11
<i>N</i> -butyl- <i>N</i> -nitrosourea <sup>a</sup>	31.6	65 ± 8

<sup>a</sup> Values obtained from Reference [5].

than that of the other nitrosoureas studied.<sup>[5]</sup> The greater the non-planarity in the NBP-ANU adducts, disrupting the  $\pi$ -electron cloud to interlink the two phenyl rings, the smaller the values of  $\epsilon$ , as can be seen in Table 3.

A similar argument can be made to rationalize the fact that the biological activity of ANUs decreases when their molecular size increases:<sup>[3]</sup> the smaller the alkyl group of the alkyldiazonium ion, the stronger the linkage between DNA nucleophile sites and the electrophilic alkyldiazonium ions.

## CONCLUSIONS

- The mechanism of the alkylation reaction of NBP by *N*-phenyl-*N*-nitrosourea is different from that caused by other nitrosoureas.
- The formation of the NBP-*N*-phenyl-*N*-nitrosourea adduct occurs about 20,000-fold slower than with *N*-methyl-*N*-nitrosourea, one of the most effective carcinogenic nitrosoureas.
- A correlation was found between the alkylating potential of nitrosoureas and their tumorigenicity.

## EXPERIMENTAL

### General remarks

A Shimadzu UV-2401-PC spectrophotometer with a thermoelectric six-cell holder temperature control system ( $\pm 0.1$  °C) was used.

The reaction temperature was kept constant ( $\pm 0.05$  °C) with a Lauda Ecoline RE120 thermostat.

A Crison Micro pH 2000 pH-meter was used to perform pH measurements ( $\pm 0.01$ ).

Water was deionized with a MilliQ-Gradient (Millipore).

All kinetic runs were performed in triplicate.

Numerical treatment of data was performed using the 7.1.44 Data Fit software. Geometry optimization of the NBP-alkyldiazonium adducts was carried out with the Chem3D Ultra Molecular Modeling and Analysis software, version 9.0, and with Gaussian 03W Client Pro 9.0. The PM3 semiempirical method was used.

### Procedures: synthesis of *N*-phenyl-*N*-nitrosourea

PhNU were prepared from the respective *N*-phenylurea (this urea was obtained from Fluka). PhNU was prepared as by Werner.<sup>[22]</sup> *N*-phenylurea (approx. 1 g) was dissolved in water and 10 g of sodium nitrite was added. The mixture was cooled to below 0 °C and ice-cold sulphuric acid (10%) was added dropwise during continuous stirring. The nitrosourea precipitated as yellow crystals. After vacuum filtration, the crystals collected were repeatedly washed with cool water and then desiccated.

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