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# Substituent effect on superoxide elimination from peroxyl radicals of adenine and methylated derivatives

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**Abstract**

The hydroxyl radical, generated by UV photolysis of 4-mercaptopyridine-N-oxide, was made to react with adenine and several methylated derivatives. The formation of the corresponding 8-hydroxy derivatives was observed in all cases and is the result of the oxidation of the respective 8-hydroxyl adducts. When this oxidation is carried out by molecular oxygen, an intermediate peroxyl radical is formed by addition of $O_2$ at C(4). Superoxide anion is eliminated from the peroxyl radical, followed by deprotonation, resulting in the formation of 8-hydroxyadenines (8-oxo-7,8-dihydroadenines) as the final stable products. The extent of superoxide elimination, as compared to other processes by which peroxyl radicals decay, was found to increase with the number of methyl substituents at either $N^6$ or $N(9)$. This indicates that positive charge is developed in the transition state, as suggested by the negative $\rho$ value of the Hammett relationship for this reaction. 

*Keywords:* Superoxide elimination; Peroxyl radicals; Hammett relationship

## 1. Introduction

The hydroxyl radical $HO^{\bullet}$ generated after water radiolysis, has for a long time been pointed out to be one of the major species responsible for the deleterious effects of ionising radiation on living tissues (for a review see [1–3]) The reaction of this radical with the deoxyribonucleic acid (DNA) is particularly important because it is a potential source of genetic mutations, or cell death. Since $HO^{\bullet}$ tends to react faster with unsaturated than with saturated systems, its reaction with the purine and the pyrimidine bases in DNA has been the subject of a large number of studies, employing kinetic methods and final product analysis [4–16]. In the case of the purine bases, the formation of 8-hydroxy derivatives, also referred in the literature as 8-oxo-7, 8-dihydro derivatives, is a frequently observed permanent chemical change. Such compounds result from the oxidation of the hydroxyl adducts at C(8) (Ade-8-$OH^{\bullet}$ Scheme 1). Molecular oxygen, which is the most important biological oxidant, is, however, relatively inefficient in promoting that oxidation, at least in the case of adenine (Ade), as we have reported before [16]. This was attributed to the fact that oxygen reacts with Ade-8-$OH^{\bullet}$ by addition, forming a peroxyl radical. The subsequent superoxide elimination, which is required for the formation of 8-hydroxyadenine (8-OH-Ade), occurs to an extent of only about 34%. If the superoxide anion is not eliminated, other products are likely to be formed [17].

We report now the effect of oxygen in the formation of the 8-hydroxy derivatives from $N^6$- and $N(9)$-methylated adenine derivatives, by using UV photolysis of 4-mercaptopyridine-*N*-oxide (4-MPNO) as a source of the $HO^{\bullet}$ radical [18] (Scheme 2). This method has several advantages over other radiomimetic methods to produce $HO^{\bullet}$, such as its relative simplicity and ease to set up experimentally, as compared to the Fenton or the Udenfriend systems. In particular, we found out that, under the very same conditions, 4-MPNO is photolysed much faster than $H_2O_2$ [19], which is a major advantage when large amounts of unidentified final products are needed for their identification, as is the case with the 8-hydroxy derivatives of all methylated adenines studied. The products that result from the 4-MNPO photolysis (mainly dissulfides) are unlikely to interfere with the 8-OH-Ade formation since, apart the faster photolysis, no other differences have been observed by using this method or $H_2O_2$ photolysis to produce the $HO^{\bullet}$ radical.

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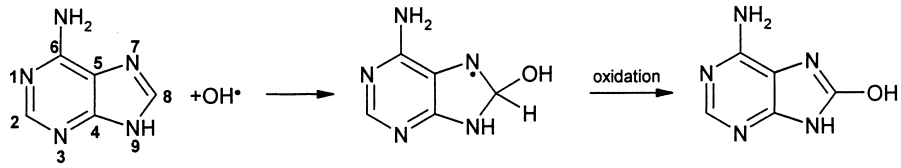
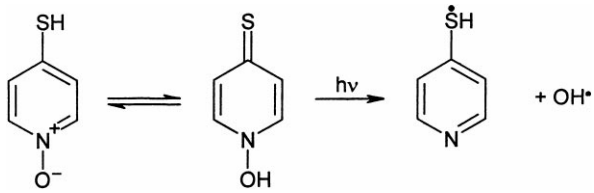



Scheme 1.




Scheme 2.

## 2. Experimental details

### 2.1. Chemicals

Adenine (p.a., Sigma), $N^6$-methyladenine and $N^6$, $N^6$-dimethyladenine (p.a., Fluka), 9-methyladenine (Chemalog), $KH_2PO_4$, $Na_2HPO_4$ and potassium ferricyanide (p.a., Merck) were used without further purification. $N^6$, $N^6$, 9-trimethyladenine was synthesised according to the method of Itaya [20]. 8-hydroxyadenine was synthesised by the method of Cavalieri and Bendich [21]. 4-MPNO was synthesised by combining the methods of Shaw [22] and Barton [23].

### 2.2. Apparatus

Solutions were photolysed during 10 min, using UV light emitted from a Hanau 150 W high pressure Hg lamp and 2.5 ml quartz cells placed at 2.5 cm from the lamp. Analysis of the irradiated solutions were performed by a HPLC system consisting of a Shimadzu LC-10AS pump, a Reodhyne model 5125 injector fitted with a 100 μl loop, a reversed phase (octadecylsilane) Merck analytical column, a Shimadzu SPD-M10A diode array optical detector set to the range 200–300 nm, and a PARC EG and G model 400 electrochemical detector at +650 mV vs. NHE. This potential was optimized for a sensitive detection of 8-OH-Ade (down to the $10^{-7}$ M scale) without introducing unwanted currents such as eluent oxidation. For this reason it was also chosen for detecting the other hydroxylated adenines. Electrochemical chromatographic data was acquired by a Merck Hitachi D-2500 integrator. Optical data was acquired via specific software through an IBM compatible computer coupled to the diode array detector. The eluent was an aqueous solution, buffered at pH 7 with both 1 mM $KH_2PO_4$ and $Na_2HPO_4$, containing methanol in the range 10–30% (v/v), the higher concentrations having been used to elute the solutions containing $N^6$, $N^6$, 9-trimethyladenine.

### 2.3. Procedure

Solutions to be irradiated were prepared immediately before use in water purified by a Millipore Milli-Q system. The concentration of the adenines was 1 mM, while that of 4-MPNO was 0.1 mM, in order to minimise its reaction with $HO^{\bullet}$. The pH was adjusted at 7 with both 5 mM $KH_2PO_4$ and $Na_2HPO_4$. When required, oxygen was removed from the solutions by bubbling with argon. Potassium ferricyanide was added with a micropipette from a 0.5 mM solution. After being irradiated, the solutions were immediately analysed by HPLC. The peak height of 8-OH-Ade in the electrochemical chromatograms was found to be linear to its concentration within the range 0–50 μM, therefore, this was used as calibration method. The absolute concentrations of methylated 8-hydroxyadenines were not determined, due to the absence of authentic standards. This was not a major inconvenience, since the relevant data calculated from the experiments were ratios of two concentrations and not their absolute values. The only assumption made was that these concentrations were directly proportional to the respective heights of the electrochemical peaks, as was observed for 8-OH-Ade.

## 3. Results and discussion

### 3.1. Effect of oxidants on the formation of 8-hydroxyadenine

In a first set of experiments, we studied the effects of the ferricyanide anion and molecular oxygen in promoting the oxidation of the Ade-8-$OH^{\bullet}$ adduct to 8-OH-Ade, using a procedure similar to the one we have described before, by using $H_2O_2$ as a photolytical source of $HO^{\bullet}$ [16]. As was mentioned above, 4-MPNO is photolysed much faster than $H_2O_2$: under the same photolytic conditions (10 min at 2.5 cm from the lamp), 4-MPNO is quantitatively photolysed, but $H_2O_2$ is only about 2% decomposed. Adenine degradation was not detected in either case. This did not allow the study of the formation of 8-OH-Ade at time intervals where the rate of $HO^{\bullet}$ production could be assumed to be constant. Instead, the concentrations of 8-OH-Ade (and of the corresponding compounds derived from methylated adenines) were measured only after complete photolysis of 4-MPNO. Therefore, the results from these experiments were obtained by plotting the concentrations of 8-OH-Ade formed as a function of added ferricyanide in range

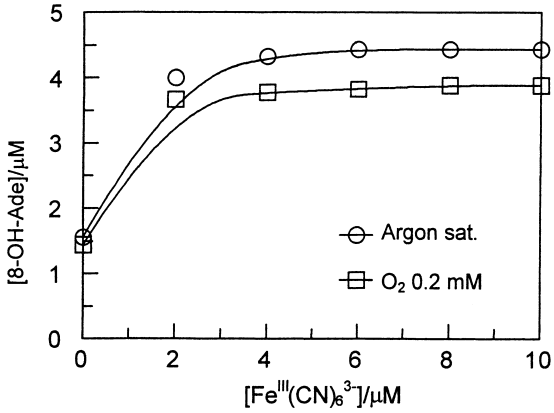



Fig. 1. Effect of ferricyanide on the formation of 8-OH-Ade after 10 min. UV photolysis, at 2.5 cm from the lamp, of a solution containing 1 mM adenine, 0.1 mM 4-MPNO, at pH 7, either aerated or deaerated.

0–10 μM, in both aerated and deaerated solutions (Fig. 1). The trends observed in both the cases are similar to the ones described before [16] and consequently confirm the conclusions that had previously been drawn, though the effect of ferricyanide is somewhat more pronounced, especially when compared to the results obtained in $\gamma$-radiolysis experiments [12]. Moreover, the fraction of peroxyl radicals formed by the reaction of $O_2$ with Ade-8-OH$^{\bullet}$ that eliminate superoxide, resulting in the formation of 8-OH-Ade, was also found to be identical (34%) to the value previously reported [16]; this provides good evidence that the mechanism of the formation of 8-OH-Ade is similar in both cases. This value was calculated by taking the ratio of the concentrations of 8-OH-Ade formed in aerated solutions ($[Fe^{III}(CN)_6^{3-}] = 0$) and in the presence of $Fe^{III}(CN)_6^{3-}$ 10 μM ($[O_2]$=0), for which the concentration of 8-OH-Ade formed is maximised.

### 3.2. Separation and identification of methylated 8-Hydroxyadenines

Similar experiments were performed using $N^6$-methyladenine ($N^6$-MA), $N^6$, $N^6$-dimethyladenine (DMA), 9-methyladenine (9-MA) and $N^6$, $N^6$, 9-trimethyladenine (TMA). In all these cases, the formation of the 8-hydroxy derivatives was observed as quite distinctive peaks in the electrochemical chromatograms, as is shown in Fig. 2 for the case of DMA. Just as in the case of adenine, none of the other methylated adenines was electrochemically detected. Although, this detection method is very efficient for detecting this particular class of purine oxidation products [24,25], the major obstacle to their unambiguous identification was the absence of authentic standards. In order to correctly assign the peaks to the 8-hydroxyadenines, their UV spectra were also traced and are shown in Fig. 3(A), together with the spectra of the parent compounds (Fig. 3 (B)), as a comparison. The separation between each adenine and the corresponding 8-hydroxyadenine was complete in all cases, thus allowing to obtain uncontaminated UV spectra of these

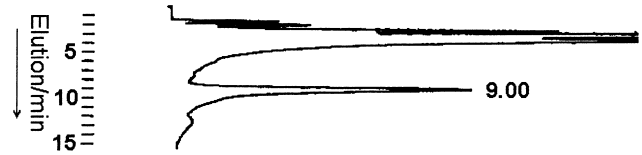



Fig. 2. Electrochemical chromatogram at +650 mV vs. NHE of an aerated solution containing initially 1 mM DMA, 0.1 mM MPNO, at pH 7, after 10 min. UV photolysis. The eluent contained 25% (v/v) MeOH, at pH 7, as described in the experimental section. The first broad peak is due to unretained solutes, mostly 4-MPNO photolysis by-products. The peak at 9.00 min. is 8-hydroxy-DMA, whose UV spectrum is shown in Fig. 3(A) as a dash-point-dash line.

products, except in the case of adenine/8-OH-Ade, where the peak of 8-OH-Ade is completely overlapped by that of adenine. This overlap was found to occur always to a certain extent in this kind of columns, but we found out that the separation was much improved by using methylsilane columns. For the aim of the electrochemical detection and quantification of 8-OH-Ade this was not a problem because adenine is invisible at the electrochemical potential used. Therefore, for illustrative purposes, the spectrum of 8-OH-Ade is shown in Fig. 3(A) as obtained from a solution of synthesised 8-OH-Ade (see experimental details). The similarity between the spectra of the 8-hydroxyadenines, and the parallelism between this set of spectra and that of the respective adenines is quite meaningful and permits to attribute their structures with reasonable confidence.

### 3.3. Reaction of oxygen with the C(8) hydroxylic adducts

The ratio between the concentrations of the 8-hydroxyadenines, formed by oxidising the C(8) hydroxylic adducts by either oxygen or ferricyanide, was calculated for each methylated adenine, as was described above for adenine. The values were then plotted as a function of the number of methyl substituents (Fig. 4(A)). In the case of TMA it has been reported before, after pulse radiolysis studies, that superoxide elimination from the peroxyl radical derived from TMA-8-OH$^{\bullet}$ occurs with a low yield, though it was not quantified [26]. Although there is no theoretical basis for the linearity observed, it is clear that the consecutive methylation increases the efficiency of oxygen in oxidising the C(8) hydroxylic adducts to the respective 8-hydroxy derivative. In other terms, the methylation increases the fraction $f$ of peroxyl radicals, formed after reaction of oxygen with these adducts, that eliminate the superoxide anion, followed by deprotonation from C(8). The effect of the methyl groups is exerted in stabilising the incipient cation formed after superoxide elimination, which is illustrated in Scheme 3 for the effect of substitution on $N^6$. It should be noted that $f$ can be viewed as the relative rate of superoxide elimination, measured against the rates of all reactions undergone by the peroxyl radicals. Therefore, by considering the set formed by adenine, $N^6$-MA and DMA, it

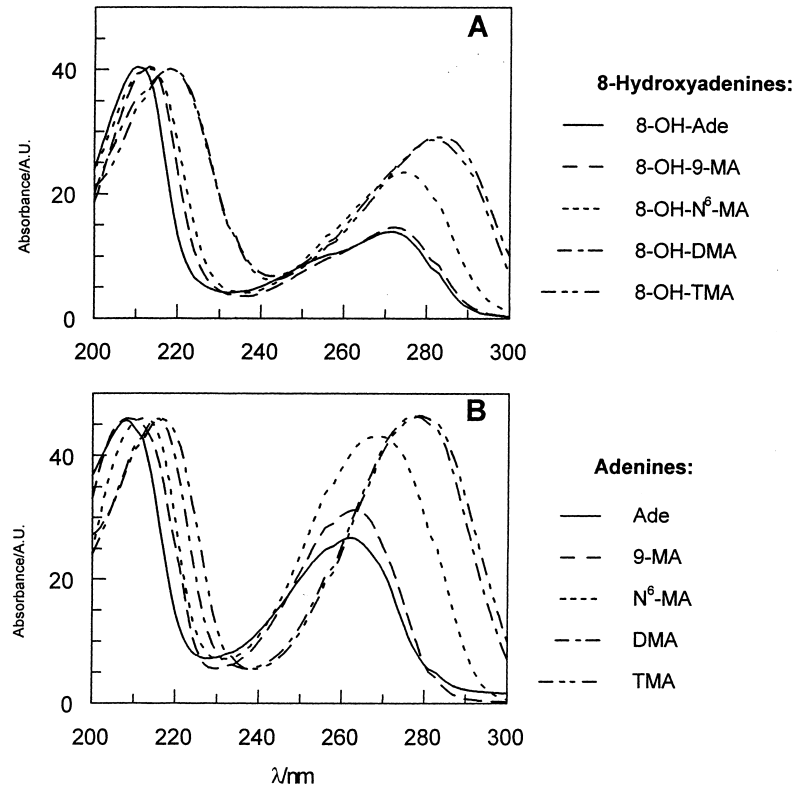



Fig. 3. (A) UV spectra of the 8-hydroxy derivatives of the methylated adenines studied, as determined by diode array detection, after photolysis of their aqueous solutions in the presence of 0.1 mM 4-MPNO. The spectrum of 8-OH-Ade was obtained from a solution of the authentic standard. (B) Spectra of adenine and its methylated derivatives, determined in the optical chromatograms of the solutions referred to in (A), before irradiation.

is licit to try to build a Hammett relationship between log $f$ and the $\sigma^+$ constant [27,28] for the substituent at C(6), keeping $N(9)$ unsubstituted (Fig. 4 (B)). The parameter $\sigma^+$ was chosen because it is possible to write down contributors in which there is direct resonance between the $N^6$ or $N(9)$ non-bonding electron pairs and the incipient positive charge that is formed after superoxide elimination (Scheme 3). In spite of the low number of points, the linearity found is quite acceptable. The value found for $\rho$, $-0.79\pm0.05$, is an unequivocal indication that, during the transformation of the peroxyl radicals derived from the hydroxylic adducts at C(8) into each methylated 8-hydroxyadenine, positive charge is developed in the purine system. It is also very interesting to compare this effect to the one exerted by the

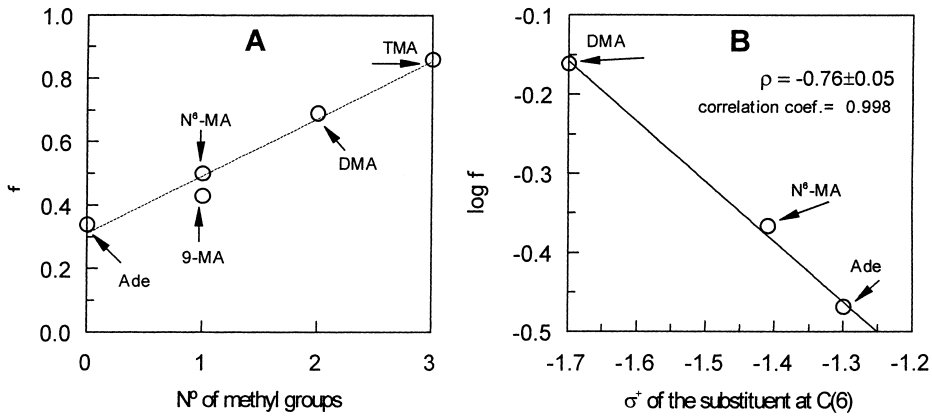
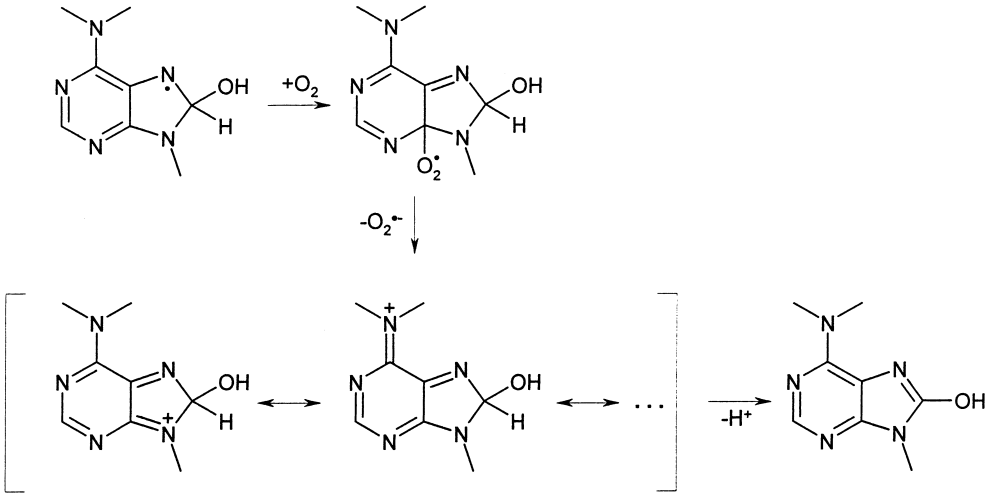



Fig. 4. A: Yields ($f$) of superoxide elimination from the peroxyl radicals formed after the reaction of $O_2$ with the hydroxylic adducts at C(8) of adenine and its methylated derivatives, as a function of methyl substituents. B: Hammet relationship between log f and the $\sigma^+$ constant of the substituent at C(6) for adenine, $N^6$-MA and DMA. The values of $\sigma^+$ for $-NH_2$ and $-N(CH_3)_2$ were taken from ref. [27]; the value for $-NHCH_3$ was taken from [29].



Scheme 3.

same groups on the elimination of $HO^-$ from the C(4) hydroxyl adducts of purines [29].

The slight difference between the values of $f$ for the peroxyl radicals derived from $N^6$-MA-8-OH$^\bullet$ and 9-MA-8-OH$^\bullet$ (Fig. 4(A)) may be attributed to the different environments of the methylated nitrogen atoms, respectively $N^6$ and $N(9)$. In the latter, $N(9)$, besides the methyl group, is attached to the electrodeficient carbon atom C(8), thus making its non-bonding electron pair less available than that of $N^6$ in the peroxyl radical derived from $N^6$-MA, and, therefore, decreasing the rate of superoxide elimination.

## 4. Conclusions

The reaction of the HO$^\bullet$ radical produced by UV photolysis of 4-MPNO with adenine and some of its methylated derivatives results in the hydroxylation at the C(8) position of the purine moiety. In addition to 8-hydroxyadenine itself, similar compounds have been isolated for $N^6$-MA, 9-MA, DMA and TMA. These compounds result from the oxidation of the intermediate hydroxylic adducts at C(8). When this oxidation is carried out by oxygen, the concentrations of the 8-hydroxyadenines obtained depend on the yield of superoxide elimination from the peroxyl radical formed after reaction of oxygen with those adducts. This yield was found to increase with methylation at $N^6$ and $N(9)$, indicating that positive charge is developed during the transformation of the peroxyl radical into the 8-hydroxy derivative. The negative value of $\rho$ calculated for the Hammett relationship found for log (yield) as a function of $\sigma^+$ of the substituent is in good agreement with the mechanism proposed for the formation of the 8-hydroxy derivatives, i.e., elimination of superoxide anion from a peroxyl radical concerted with deprotonation of the incipient cation.

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