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Bioorganic & Medicinal Chemistry Letters 15 (2005) 67–71
Bioorganic & Medicinal Chemistry Letters

Possible role of hydroxyl radicals in the oxidative degradation of folic acid

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Received 11 August 2004; accepted 12 October 2004
Available online 5 November 2004

Abstract—Hydroxyl radicals have been found to cause oxidative *N*-dealkylation of amines including folic acid via a hydrogen atom transfer mechanism.

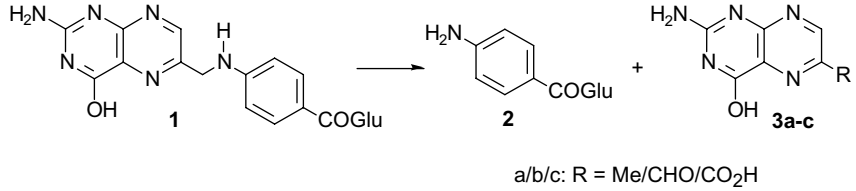
1. Introduction

Oxidative *N*-dealkylation of amines, catalyzed by several oxidative hemin-containing enzymes and hemin-hydroperoxides combinations, is of growing interest[1a–e] due to its central role in xenobiotic metabolism. One of the important examples of this biochemical reaction is the C9–N10 bond cleavage (Scheme 1) of folic acid (**1**) leading to the formation of the amine **2** and 6-substituted pterins **3a**–**c**. For this, participation of the superoxide radicals ($O_2^{-\bullet}$)[2a,b] or a ferryl–hydroxo (Fe^{4+}–OH) complex[2c,d] has been reported. Herein, we report the possible role of the ubiquitous hydroxyl radicals in the reaction, and provide insights about the reaction mechanism.

2. Results and discussion

Exposure of **1** to the hydroxyl radical generating Fenton system (Fe^{2+}–EDTA–H_2O_2) furnished **2** and **3b** as revealed by the Bratton–Marshall (BM) procedure[2c] and 2,4-DNPH test respectively. Thus, cleavage of **1** by $^{\bullet}$OH radicals was apparent. However, the above Fenton system can generate the Fe^{4+}–OH complex[3] which is also implicated in the hemin-catalyzed dealkylation of various amines[1a] including **1**.[2c] Consequently, **1** was reacted with $^{\bullet}$OH, generated by an iron independent, radiolytic process, which also led to the same amine **2** and aldehyde **3b**. The time dependent growths of **2** and **3b** were followed spectrophotometrically over a period of 0–7 h, by recording the absorbances at 550 nm for the BM derivative of **2** and at 497 nm for the DNPH derivative of **3b** (Fig. 1). The product distribution and characterization were also carried out by paper chromatography and HPLC by comparing with authentic samples. The time dependent HPLC analyses showed **3b** as the major pterin product, while a linear relationship between the depletion of **1** and production of **2** was also observed.

The possible involvement of the $^{\bullet}$OH radicals in amine dealkylation was ascertained further by carrying out



Scheme 1.

Keywords: Folic acid; *N*-Dealkylation; Pulse radiolysis; Radicals.
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doi:10.1016/j.bmcl.2004.10.038

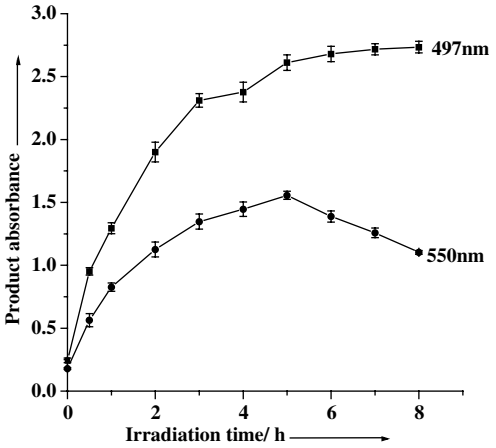


Figure 1. Time dependent growth of **2** and **3b** formed during oxidation of **1** by radiolytically generated ·OH. (–■–) DNPH derivative of **3b** (absorbance at 497 nm); (–●–) BM derivative of **2** (absorbance at 550 nm). Values are mean ± SE ($n = 4$).

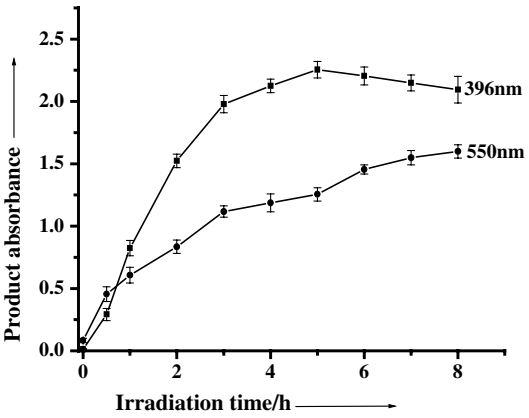


Figure 2. Time dependent growth of **5** and **6** formed during oxidation of **4** by radiolytically generated ·OH. (–■–) DNPH derivative of **6** (absorbance at 396 nm); (–●–) BM derivative of **5** (absorbance at 550 nm). Values are mean ± SE ($n = 4$).

the reaction of the radiolytically generated ·OH radicals with **4**, a simpler analogue of **1**. This also produced the expected cleavage products **5** and **6** (Scheme 2), which were quantified spectrophotometrically (Fig. 2) and identified from their ^{1}H NMR spectra.

Given the reported[2a,b] involvement of the $O_2^{-\cdot}$ in the above reaction, its reactions with **1** and **4** were also studied. The degradations of **1** and **4** were significantly less (40–50%) in these cases that was consistent with the less reactivity of the $O_2^{-\cdot}$ compared to the ·OH radicals. Between **1** and **4**, more dealkylation products were formed with the latter (Fig. 3). Besides hydrogen atom transfer (HAT), the $O_2^{-\cdot}$ radicals can react with **1** in various other pathways including reduction of its pterin moiety. With **4**, however, only a HAT is possible, resulting in higher amount of its oxidized metabolites.

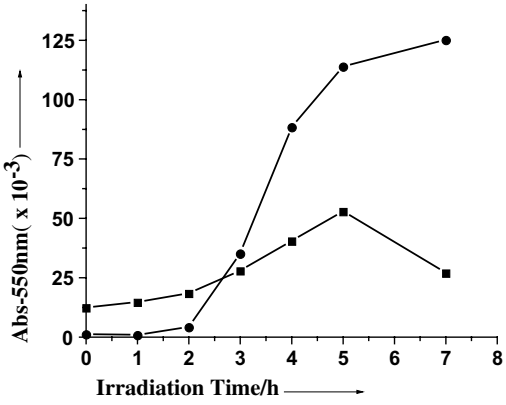
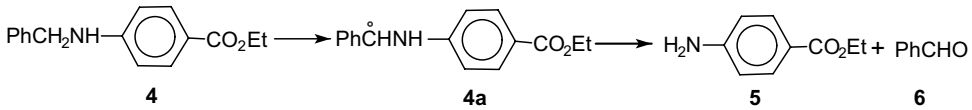


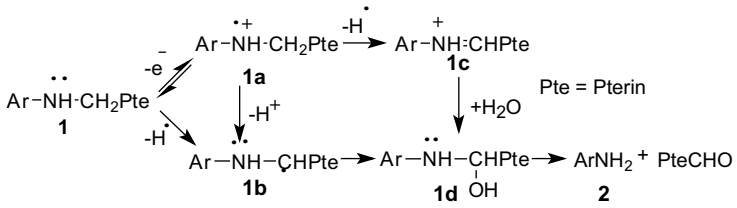
Figure 3. Time dependent growth of **2** and **5** formed during oxidation of **1** and **4** by $O_2^{-\cdot}$. (–■–) BM derivative of **2** (for **1**); (–●–) BM derivative of **5** (for **4**). Values are mean ± SE ($n = 4$).

Next, we turned our attention to the mechanism of the ·OH-mediated oxidation. In spite of being long known, the exact mechanism of oxidation of **1** remains considerably speculative. The reaction can proceed either through an initial HAT or a single electron transfer (SET) process as shown in Scheme 3. For example, the key intermediate **1d** of the reaction can be produced either via the (i) HAT intermediate **1b**, or (ii) SET intermediate **1a** through **1c** which also involves a HAT as the secondary process. The third process involving a proton transfer from **1a** to **1b** can be excluded in the present case, due to the poor basicity of the system.

Alternate views involving HAT[1a,d,e,c] and SET mechanisms[1b,c] have been proposed by different groups for oxidative *N*-dealkylation. Further, distinction between the intermediacy of **1b** and **1c** could not be made as generation of both the intermediates involve a hydrogen atom transfer. The mechanistic controversies possibly arose because of overemphasis on indirect evidences, which were not applicable universally, and recognition of the minor reactions as the major ones. Very recently, in a more direct approach, two groups have established[1d,e] the operation of a HAT mechanism in the cytochrome P_{450}-catalyzed dealkylation of cyclopropyl amines, by characterizing the oxidized metabolites. However, these evidences could only be generated because of the presence of the cyclopropyl group in



Scheme 2.



Scheme 3.

the chosen substrates and may not be used for all kinds of substrates such as **1**.

We envisaged that looking into the transients formed in the oxidation reaction and following their reaction dynamics by pulse radiolysis technique might provide more direct evidence. Earlier, using the pulse radiolytic studies, we have shown[4a] that the reaction between ·OH radical and **1** leads to the bleaching in the ground state absorption around 300 nm and a transient absorption maximum at 425 nm at 10 μs after electron pulse, which shifted to 390 nm at 800 μs. The absorption peak at 425 nm, formed with a bimolecular rate constant of $1.13 \times 10^{10}\,dm^3\,mol^{-1}\,s^{-1}$ was attributed to a 'pseudo-phenoxyl' radical. The absorption at 425 nm decayed in μs time scale, while that at 390 nm grew steadily and remained stable even up to seconds. Thus, generation of the transient (λ_{max} 390 nm) both directly from **1** as well as from the 'pseudo-phenoxyl' intermediate was inferred. However, presence of multiple reactive centres in **1** precluded its exact identification and we proposed it to be a molecular radical. It was envisaged that the pulse radiolysis studies of the reaction using **4** as a simpler congener of **1** would be better suited for the characterization of the intermediate.

Reaction of **4** with ·OH radicals furnished two transient absorption peaks at ~310 and 390 nm (Fig. 4). Kinetic studies on the formation (bimolecular formation rate constant of $9 \times 10^9\,dm^3\,mol^{-1}\,s^{-1}$) and decay of the transients matched exactly and hence these were assigned to the same radical species. Given that **4** is nonphenolic, the absence of any transient absorption peak at ~430 nm was anticipated. The transient absorption at 390 nm decayed faster in the presence of oxygen (Fig. 4, inset), revealing it to be a carbon-centred radical. The oxygen effect on transient was studied only with the signal at 390 nm in order to avoid the possible interference from the bleaching signal at ~310 nm. Moreover, compound **1** also showed the transient absorption at 390 nm only. The above results, in conjunction with those obtained[4a] by us with **1**, clearly revealed that the ·OH radical induced oxidation of **1** or **4** proceeds via the HAT mechanism. Absence of any absorption due to a N-centred radical excluded the possibility of any SET mechanism in the reaction.

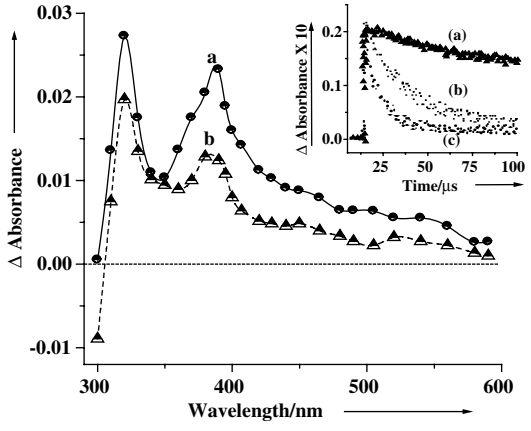


Figure 4. Transient absorption spectra obtained from an N_2O-saturated aqueous solution containing **4** (100 μM) at pH 8.0 after the electron pulse at (a) 5 μs and (b) 40 μs. Dose = 12 Gy. Inset: absorbance versus time plots for the traces recorded at 390 nm obtained from the above experiment. Different ratios of air and N_2O were used. (a) without air, (b) 1:4 air–N_2O and (c) 1:1 air–N_2O.

The transients obtained with **1** and **4** were similar both in terms of their λ_{max} and formation rate constants. Hence, these can be safely attributed to similar methylenic radical intermediates, **1b** (for **1**) and **4a** (for **4**). The hydrogen abstraction from the designated CH_2 group is expected, as the radical **1b** or **4a** will be resonance stabilized by the adjacent, pteryl/phenyl and amino groups. Formation of a similar methylenic radical absorbing at 490 nm was earlier reported for the reaction of curcumin, a β-diketone with the $^{\cdot}CH_3$ radical.[4b] Unlike the curcumin-derived radical, **4a** is devoid of extended conjugation, explaining the blue shift of its absorption. The other C-centred radicals that may be produced by ·OH addition to the double bonds in **1** or **4** would not furnish the *N*-dealkylated products, without getting transformed into intermediates like **1b** or **4a**. Such a possibility of radical interconversion could be excluded as the pulse radiolysis oscilloscope traces of the 390 nm transient with **4** were smooth without any shoulder and did not show any evolution within the small time scale of the study. With **1** also, the intermediate **1b** was generated by a direct hydrogen abstraction at C-9 as well as via evolution from the 'pseudo-phenolic' intermediate, but not through any N-centred radical.

3. Conclusion

Overall, the present study revealed the participation of freely diffusible hydroxyl radicals in the oxidative degradation of secondary amines including **1**. Based on direct evidences, the involvement of a HAT mechanism in the reaction has been shown unambiguously. Given the high cellular concentrations of free iron and H_2O_2, the

biotoxic hydroxyl radicals may play a major role in oxidative degradation of folic acid in the cellular systems. Although the physiological implication of the process is not clear, this may partly explain the increased need of folic acid supplementation in case of iron-overload diseases.

4. Experimental

4.1. Materials

Folic acid (Aldrich) was used as received. All solutions were made with triply distilled water. High purity N_2O, from BOC India Pvt. Ltd, was used for all radiolytic experiments. Compounds **3b**,**c** and **4** were synthesized as reported.[5a,b]

4.2. Cleavage of 1 with Fenton (Fe^{2+}–EDTA–H_2O_2) reagent

The reaction mixture (1 mL) contained **1** (2.8 mM), $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (80 μM), EDTA (100 μM) [EDTA and $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ were mixed prior to the addition of **1**], H_2O_2 (200 μM), KH_2PO_4–KOH (10 mM) pH 7.4 buffer and **1** (250 μM). After incubating the mixture at 37 °C for 1–7 h in absence or presence (300 μM) of vitamin C, a solution of TBA in 50 mM NaOH (1 mL, 1% w/v) and trichloroacetic acid (1 mL, 2.8% w/v aqueous solution) was added. The reaction mixture was heated for 15 min in boiling water bath and the amount of chromogen produced was spectrophotometrically measured[4a] at 532 nm.

4.3. Cleavage of 1 or 4 with ·OH radicals and spectrophotometric analyses of products

An N_2O purged aqueous solution (1.0 mL) of **1** (500 μM) or **4** (500 μM) was irradiated with γ-ray (^{60}Co source, dose rate 560 Gy/h) for 0–8 h. At different time intervals, a fixed volume of the solution was withdrawn, diluted with an equal volume of water and treated with HCl (40 μL, 0.5 N) followed by $NaNO_2$ (100 μL, 0.1% aqueous solution). After 5 min, ammonium sulfamate (100 μL, 0.5% aqueous solution) was added, the mixture shaken and kept for a further 5 min at room temperature. Finally, the Bratton–Marshall reagent, NEDD (100 μL, 0.1% aqueous solution) was added and the chromogen was read spectrophotometrically at 550 nm.[2c]

For detecting the aldehyde **3b**, 2,4-DNPH (50 μL, 25 mM in 2 N HCl) was added to the irradiated solution of **1** (250 μL) followed by NaOH (200 μL, 1 N) after 15 min, and the absorbance of its hydrazone derivative was monitored at 497 nm.

For assaying **6**, 2,4-DNPH (50 μL, 25 mM in 2 N HCl) was added to the irradiated solution of **4** (250 μL), the mixture kept for 20 min and the precipitate formed was centrifuged at 13,000 rpm for 20 min. The pellet was washed twice with 2 N HCl, dissolved in DMSO (500 μL) and the absorbance at 396 nm was read.

Table 1. HPLC data for the oxidative degradation of folic acid (**1**)

Peak no.	Retention time (min)	Product
1	1.26	*p*-Aminobenzoic acid and **2**
2	2.28	**3c**
3	4.37	**3b**
4	15.98	**1**
5	22.26	Unknown

4.4. HPLC analysis of the reaction products from 1 and ·OH

The tubes containing the solution (each of 1 mL) of **1** (500 μM) in N_2O purged H_2O were irradiated with γ-ray for different periods (0–7 h). The products obtained, were analyzed by HPLC (Bruker HPLC instrument) under the following conditions: RP-18 column (E. Merck, Germany); eluent flow rate: 1 mL/min; detection: 255 nm and injection volume: 20 μL. The eluent was prepared by mixing $NaC_2O_4{\cdot}H_2O$ (35.1 g), KH_2PO_4 (1.36 g), aqueous KOH (6.94 mL, 1 N) and methanol (40 mL), making-up the volume to 1 L and adjusting the pH to 7.2 with aqueous KOH. The peak areas were calculated by using a Bruker HPLC software. The results presented in Table 1 correlated well with those reported.[6]

The paper chromatography was carried out using butanol–acetic acid–water (4:1:5) mixture as the solvent system.

4.5. Cleavage of 1 or 4 with $O_2^{-\cdot}$ radicals

The aqueous solution (1.0 mL) containing **1** (500 μM) or **4** (500 μM) and formate (0.1 M) was irradiated with γ-ray (^{60}Co source, dose rate 560 Gy/h) for 0–7 h.

4.6. Pulse radiolysis studies

The pulse radiolysis system using 7 MeV electrons has been described earlier.[7] The dosimetry was carried out using an air-saturated aqueous solution containing 5×10^{-2} mol dm^{-3} KSCN ($G\varepsilon$ = 23,889 dm^3 mol^{-1} cm^{-1} per 100 eV at 500 nm.[8] The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. The optical path length of the cell was 1.0 cm. The width of the electron pulse was 50 ns and the dose was 16 Gy per pulse. The pH of the solution was adjusted by adding NaOH or $HClO_4$. The ·OH radicals were generated by pulse radiolysis of N_2O saturated aqueous solution as described earlier.[4a]

The bimolecular rate constants were calculated by plotting the pseudo-first order rate of formation of the transient against the solute concentration. The uncertainty in the measurement in bimolecular rate constant was ±10%.

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