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Original Contribution

Radicals in the reaction between peroxynitrite and uric acid identified by electron spin resonance spectroscopy and liquid chromatography mass spectrometry

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ABSTRACT

Peroxynitrite is a reactive oxidant produced in vivo in response to oxidative and other stress by the diffusion-limited reaction of nitric oxide and superoxide. This article is focused on the identification of free radical intermediates of uric acid formed during its reaction with peroxynitrite. The experimental approach included the ESR spin trapping of the radical generated from the reaction between uric acid and peroxynitrite at pH 7.4 and mass spectrometry studies of the trapped radicals. Using PBN (*N-tert*-butyl-α-phenylnitrone) as the spin trapping agent, a six-line ESR spectrum was obtained and its hyperfine coupling constants, $a_N = 15.6$ G and $a_H = 4.4$ G, revealed the presence of carbon-based radicals. Further structural identification of the PBN–radical adducts was carried out using liquid chromatography–mass spectrometry. After comparison with the control reactions, two species were identified that correspond to the protonated molecules (M + 1) at m/z 352 and 223, respectively. The ions of m/z 352 were characterized as the PBN–triuretcarbonyl radical adduct and the m/z 223 ion was identified as the PBN–aminocarbonyl radical adduct. Their mechanism of formation is discussed.

During primate evolution, about 5–20 million years ago, two parallel but distinct mutations occurred in early humanoids that rendered the uricase gene nonfunctional [1]. As a result, humans and the great apes have higher uric acid (**1,** Fig. 1) levels (in the range between 3 and 14 mg/dl) compared with most mammals (1 mg/dl) [2]. Thus, uric acid, a metabolic product of purine, has been assumed to replace vitamin C as the major antioxidant in humans [3]. Many studies have investigated the role of uric acid and its ability to scavenge reactive oxygen and nitrogen species. Among these reactive species, peroxynitrite, formed by the fast reaction ($k = 6.7–19 \times 10^9\ M^{-1}\ s^{-1}$) between nitric oxide and superoxide [4,5], has received great interest in toxicology as a potent oxidant in vivo. Moreover, uric acid was found to scavenge peroxynitrite very effectively in vivo [6]. Urate ($\mathbf{1}^-$, Fig. 10), the conjugate base of uric acid, has been observed to inhibit tyrosine nitration [7] by scavenging the radical intermediates generated in the decomposition of peroxynitrite [8], which are responsible for tyrosine nitration.

Despite its role as a major antioxidant in plasma, there is growing evidence of uric acid being a true risk factor for the development of obesity, hypertension, and cardiovascular disease, all conditions associated with oxidative stress [2,9]. Uric acid can form free radicals in various reactions [10], including the interaction with peroxynitrite [8,11]. Reacting with peroxynitrite, urate decomposes to form radicals, which are responsible for the amplification of lipid oxidation products found in liposomes and low-density lipoprotein [12]. In fact, one such radical was identified as the aminocarbonyl radical [12]. Thus the reaction between uric acid and oxidants such as peroxynitrite may initiate and propagate a radical chain reaction and damage the cells [13].

The reactivity of peroxynitrite is strongly pH dependent [14]. Once protonated peroxynitrous acid (ONOOH; $pK_a = 6.8$) is formed (Eq. (1)). It can rapidly undergo homolysis to yield nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl ($^{\bullet}OH$) radicals (Eq. (2)) [14,15]. Furthermore, peroxynitrite can react with carbon dioxide, leading to the formation of the carbonate radical anion ($CO_3^{\bullet -}$) and nitrogen dioxide radical

Abbreviations: ESR, electron spin resonance; PBN, *N-tert*-butyl-α-phenylnitrone; LC–MS, liquid chromatography–mass spectrometry; DTPA, diethylenetriaminepentaacetic acid.

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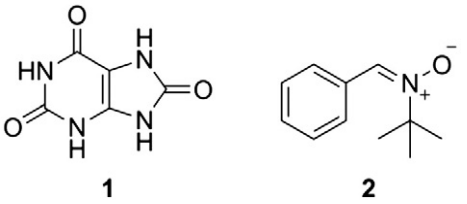


Fig. 1. Structures of uric acid (**1**) and *N-tert*-butyl-α-phenylnitrone (PBN) (**2**).

($^{\bullet}NO_2$) (Eq. (3)) [14,15]. These peroxynitrite-derived radicals can initiate one-electron oxidation:

$$ONOO^- + H^+ \leftrightarrows ONOOH \quad (1)$$

$$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \quad (2)$$

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet -} \quad (3)$$

Because of the complexity of the peroxynitrite-mediated oxidation of uric acid, its mechanism has not yet been established. During our investigation to unfold the chemistry of uric acid when treated with peroxynitrite at pH 7.4, we discovered a novel urate-derived radical—triuretcarbonyl—which could be an intermediate for the production of the aminocarbonyl radical. The radicals were studied by electron spin resonance spectroscopy using the spin trapping method; *N-tert*-butyl-α-phenylnitrone (PBN)[1] (**2,** Fig. 1) was used as a spin trapping agent, and the structures of the PBN–radical adducts were characterized by liquid chromatography–mass spectrometry (LC–MS).

Materials and methods

Chemicals

Uric acid was purchased from Sigma. Diethylenetriaminepentaacetic acid (DTPA) was from Fluka. PBN was obtained from Alexis Biochemicals. Peroxynitrite was synthesized following the method reported by Uppu and Pryor [16]. The peroxynitrite concentration was measured spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$).

ESR experiments

Stock solutions of urate (100 mM) were prepared in 0.3 M potassium hydroxide. The reactions, typically conducted in 0.3–0.5 M potassium phosphate buffer at pH 7.4, contained a final concentration of 3 mM urate, 30 mM PBN, 0.1 mM DTPA, and 9 mM peroxynitrite. A small aliquot of the reaction mixture was transferred into a quartz capillary of approximately 1×3 mm i.d.×o.d. for ESR measurement. After 2 min, the ESR spectrum was recorded at room temperature, using a commercial Bruker Elexsys E580 spectrometer, employing the high-Q cavity (ER 4123SHQE). Instrumental parameters were typically 100 kHz modulation frequency, 1 or 0.5 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 82 ms time constant, and 164 ms conversion time/point.

Sample preparation for LC–MS

The reaction mixtures were prepared at room temperature in 0.3 M potassium phosphate buffer, pH 7.4, and contained a final concentration of 10 mM urate, 30 mM PBN, 0.1 mM DTPA, and 30 mM peroxynitrite in a final volume of 10 ml. The reaction mixtures were subsequently extracted by 2×20 ml of CH_2Cl_2, dried under nitrogen gas, and resuspended in 1 ml CH_3CN.

LC–MS analysis

The LC–MS analyses were carried out with an Agilent 1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) and a TSQ 7000 triple-quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an APCI interface and operated in positive-ion mode detection. In the TSQ 7000 instrument, nitrogen was used as both the sheath and the auxiliary gas. The second quadrupole was used as a collision chamber, with argon as a collision gas, at a pressure in the vicinity of 2.5×10^{-3} Torr. The operation of the LC–MS and data analyses was performed using the ThermoFinnigan Xcalibur 1.4 software.

Full-scan LC–MS

Liquid chromatography analyses were performed in a gradient elution mode using a Phenomenex Luna 5 μ C18(2) 100 Å (150×4.6 mm) column (Phenomenex, Torrance, CA, USA) coupled with a Phenomenex Luna C18(2), 5-μm particle size guard column. The mobile phase used included 5 mM ammonium acetate/0.1% acetic acid (A) and methanol (B) as the gradient. The mobile phase flow was 0.6 ml min^{-1}, and the injection volume was 20 μl. The gradient began at 90% A. Composition was linearly ramped to 95% B over the next 10 min, remained constant for 3 min, and then reversed to the original composition of 90% A over 1 min, after which it was kept constant for 1 min to reequilibrate the column. The extracted reaction products, control samples and standard samples, were analyzed in the full-scan mode at a mass range of m/z 90–450.

LC–MS tandem mass spectrometry analysis

LC analysis was performed as outlined above. The tandem mass spectrometry (MS/MS) analysis was performed for the M+1 ions with m/z of 223 and 352 in the positive mode at a collision energy of 20 V, with a mass scan range of m/z of 40–230 for the ion at 223 and a mass scan range of m/z of 45–360 for the 352 ion.

Results

Electron spin resonance spin trapping

To probe the generation of the PBN–radical adducts at pH 7.4, the reaction between urate with peroxynitrite was monitored by ESR using the spin trapping method. The reaction between urate and peroxynitrite resulted in a six-line ESR spectrum when the modulation amplitude was set to 1 G (Fig. 2A). The trapped radical adducts displayed average hyperfine coupling constants of $a_N = 15.6$ G and $a_H = 4.4$ G. No trapped radicals were observed when the reactions were conducted without urate or peroxynitrite. PBN alone, or mixed with urate or peroxynitrite, did not yield any ESR signal (Fig. 2B).

Furthermore, the ESR intensities increased when the urate concentration was increased (Fig. 3). These experiments confirmed that the observed radicals were derived from urate, not artifacts. Moreover, when the experiment was performed at higher resolution (lower modulation amplitude), we observed additional line splitting (data not shown) indicating that PBN might trap more than one different carbon-based radical. The radical formation increased with the concentration of peroxynitrite, but yielded a maximum at a fourfold molar excess of peroxynitrite over urate (Fig. 4).

Depending on pH, both uric acid and peroxynitrite will exist in either their neutral or their anionic forms. Therefore, the radical formation should be affected by pH as well. Indeed, we observed that the urate–peroxynitrite reaction yielded more trapped radicals when the pH increased (Fig. 5). This indicates that the radical production depended on the availability of peroxynitrite in one of its anionic forms. The simulated titration curve shows an inflection point at pH 8.1. Our value is different from that reported previously [12];

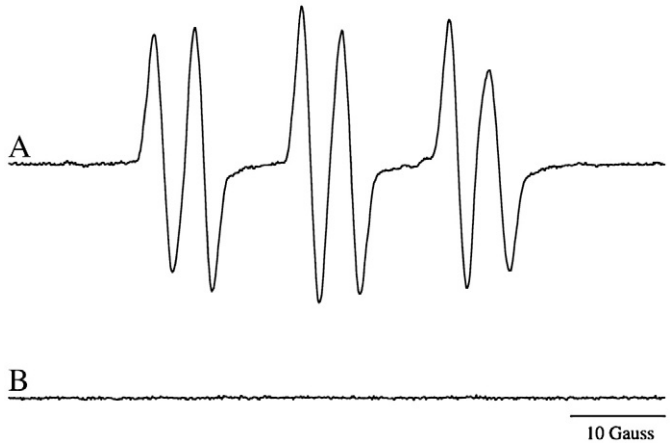


Fig. 2. ESR spectra of PBN–radical adducts obtained from the incubation of 3 mM urate, 30 mM *N-tert*-butyl-α-phenylnitrone (PBN), 0.1 mM DTPA, and 9 mM peroxynitrite in phosphate buffer, pH 7.4. At room temperature, the ESR spectrum was recorded 2 min after addition of peroxynitrite. (A) The ESR spectrum of the PBN–radical adducts using the instrumental parameters of 100 kHz modulation frequency, 1 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 82 ms time constant, and 164 ms conversion time/point. (B) The control reaction conducted under the same conditions as described for (A) but without urate.

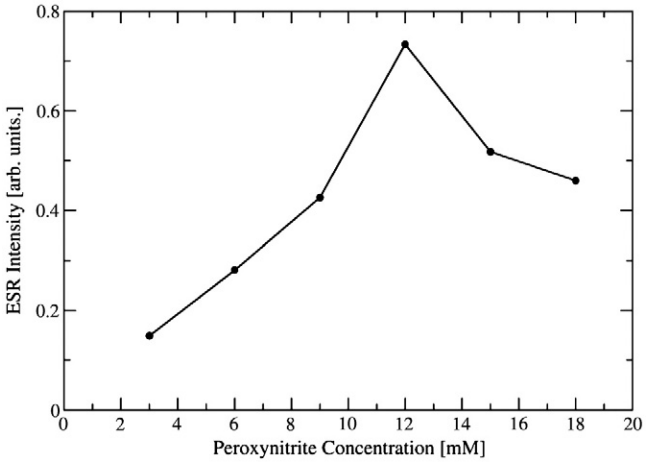


Fig. 4. Effect of peroxynitrite concentration on the production yield of the PBN–radical adduct derived from urate obtained from the oxidation of urate by peroxynitrite. The ESR spectra were recorded after 2 min of incubation of 3 mM urate, 30 mM PBN, 0.1 mM DTPA, and various concentrations of peroxynitrite in 0.5 M phosphate buffer, pH 7.4. The instrumental parameters were 100 kHz modulation frequency, 1 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 20 ms time constant, and 82 ms conversion time/point.

however, it is still in the range of the pK_a of peroxynitrous acid, which varies from 6.5 to 8.6 depending on the composition of the solution, determined previously by Kissner et al. [4]. We suspected that the use of a higher concentration in our reaction mixture may cause the shift of the pH profiles as well as the pK_a of peroxynitrous acid. In addition to the pH effect, we have investigated the effect of CO_2 concentration on the production of PBN–radical adducts. Increased [CO_2] decreased the observed ESR signal intensity (Fig. 6).

Product identification of the radical adducts by LC–MS analysis

From the reaction between urate and peroxynitrite in phosphate buffer, pH 7.4, the extracted radical adducts were separated and characterized by LC–MS. After comparison with the control reaction (Fig. 7B)—the reaction without urate—the full-scan LC–MS showed two products, at the retention times of 10:56 and 13:13 min. These peaks correspond to the ions of m/z 223 and m/z 352, respectively (Fig. 7A). As displayed in Figs. 7A and B, the large area under the total ion current chromatography starting at a retention time of 11:12 min up to 15:00 min arises from ions derived from PBN (M + 1 = 178), and the small peak at 9 min (m/z 122), which was observed in the control reaction as well, could be a degradation product of PBN, which is tentatively identified as benzaldehyde oxime.

In addition to the full scan, the ions at m/z 223 and 352 were selected and analyzed by MS/MS. At m/z 223, the following fragment ions were identified: m/z (% intensity) 223 (4), 167 (30), 149 (29), 134 (12), 132

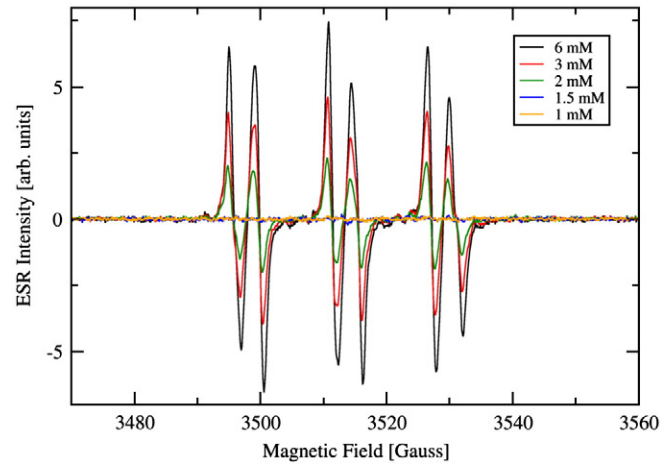


Fig. 3. Effect of urate concentration on the production yield of the PBN–radical adduct derived from urate obtained from the oxidation of urate by peroxynitrite. The ESR spectra were recorded after 2 min of incubation with various urate concentrations, 19 mM PBN, 0.1 mM DTPA, and 23 mM peroxynitrite in 0.3 M phosphate buffer, pH 7.4. The instrumental parameters were 100 kHz modulation frequency, 0.5 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 82 ms time constant, and 164 ms conversion time/point.

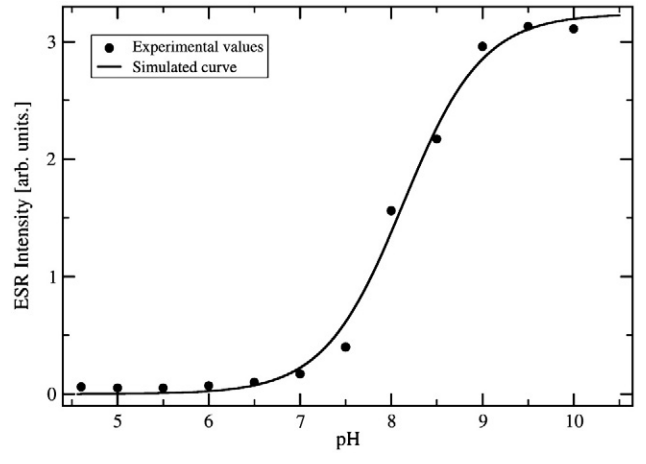


Fig. 5. Effect of pH on the yield of the PBN–radical adduct derived from urate obtained from the oxidation of urate by peroxynitrite. The ESR spectra were recorded after 2 min of incubation of 3 mM urate, 30 mM PBN, 0.1 mM DTPA, and 9 mM peroxynitrite in 0.5 M phosphate buffer at various pH's. The instrumental parameters were 100 kHz modulation frequency, 1 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 20 ms time constant, and 82 ms conversion time/point. The measured ESR intensities corresponded to the low-field PBN–radical adduct peak-to-peak heights.

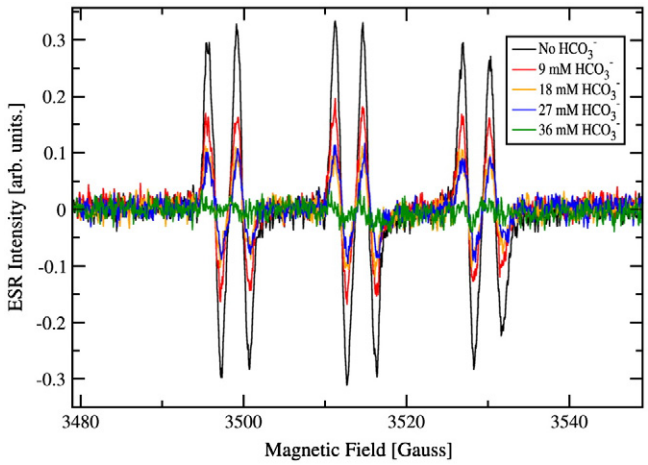


Fig. 6. Effect of [CO_2] on the yield of the PBN–radical adduct derived from urate obtained from the oxidation of urate by peroxynitrite. The ESR spectra were recorded after 2 min of incubation of 3 mM urate, 30 mM PBN, 0.1 mM DTPA, and 9 mM peroxynitrite in 0.5 M phosphate buffer at pH 7.4 in the presence of various concentrations of bicarbonate (HCO_3^-). The instrumental parameters were 100 kHz modulation frequency, 1 G modulation amplitude, 20 mW microwave power, 9.87 GHz microwave frequency, 82 ms time constant, and 164 ms conversion time/point.

(5), 122 (24), and 104 (100) (see Fig. 8). The fragments of m/z 352 exhibited the following m/z ratios (% intensity): 352 (2), 335 (13), 296 (33), 279 (25), 263 (100), 246 (83), 218 (7), 193 (12), 177 (24), 175 (22), 167 (9), 147 (24), 122 (8), 118 (6), 104 (9), and 61 (1) (see Fig. 9).

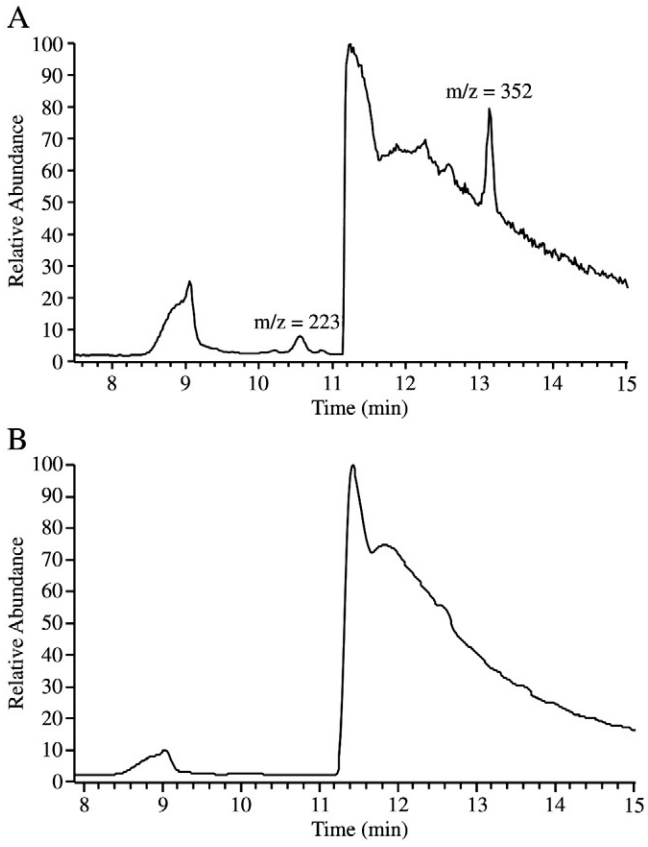


Fig. 7. LC–MS study of the reaction between urate and peroxynitrite in phosphate buffer, pH 7.4. Before being subjected to LC, the reaction products were extracted by methylene chloride (CH_2Cl_2), dried under nitrogen gas, and resuspended in acetonitrile. (A) LC chromatogram of 10 mM urate treated with 30 mM peroxynitrite in the presence of 30 mM PBN and 0.1 mM DTPA. (B) LC chromatogram of the control reaction in which every reagent had the same concentration as described in (A) but without urate.

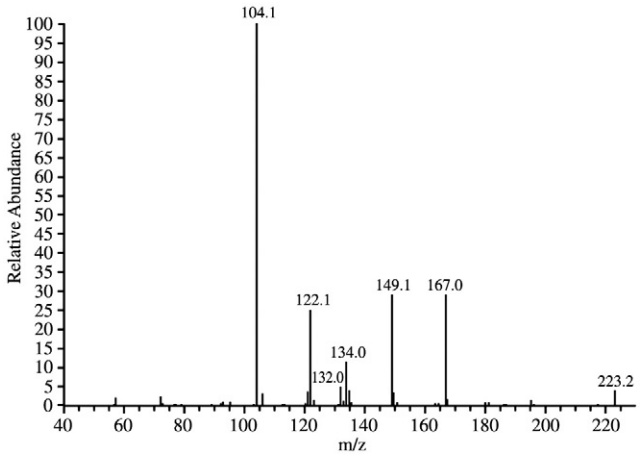


Fig. 8. MS/MS spectrum of the m/z 223 (M + 1) ion identified as the PBN–aminocarbonyl radical adduct.

Discussion

Identification of the free radicals

Spin trapping allows the trapping of short-lived radicals to form a more stable radical adduct amenable to ESR analysis [17]. However, it is usually not possible to obtain detailed structural information about the trapped radical from ESR alone. The identification of the radical adducts therefore relies on the interpretation of the LC–MS experiments. Their proposed formation mechanism in the peroxynitrite–urate reaction will be discussed in the following paragraphs.

The first spin trapping studies on urate-derived radicals were reported by Santos et al. on the peroxynitrite–urate system [12]. More than one DMPO–radical adduct were observed from the ESR spectrum. However, only one radical was identified as the aminocarbonyl radical by MS (**13,** Fig. 10). Our experiments also showed that PBN could trap at least two carbon-based radicals. The assignment to carbon-based radicals was made by comparison with the literature [18]. The full-scan LC–MS study showed two distinctive products with m/z 223 (M + 1) and 352 (M + 1), which were absent in the control reaction (Fig. 7).

The analysis of the LC–MS/MS spectra (Figs. 8 and 9) revealed that both ions showed the same initial fragment loss of a neutral compound with a molecular weight (MW) of 56, corresponding to the loss of 2-methylpropene ($[M + H–C_4H_8]^+$). This fragment loss can be explained by the cleavage of the *tert*-butyl group from the PBN moiety. Moreover,

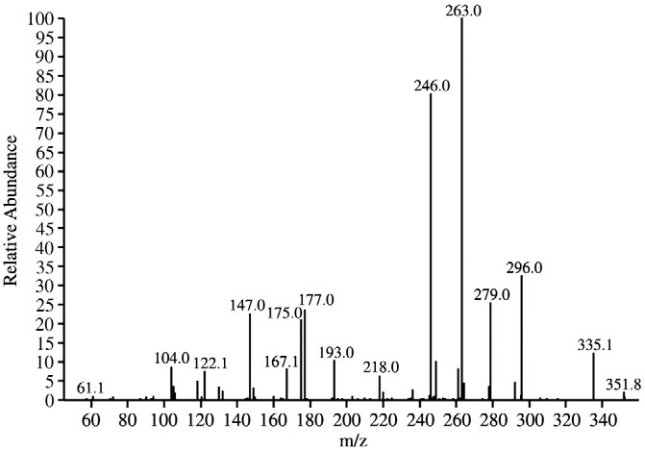


Fig. 9. MS/MS spectrum of the m/z 352 (M + 1) ion identified as the PBN–triuretcarbonyl radical adduct.

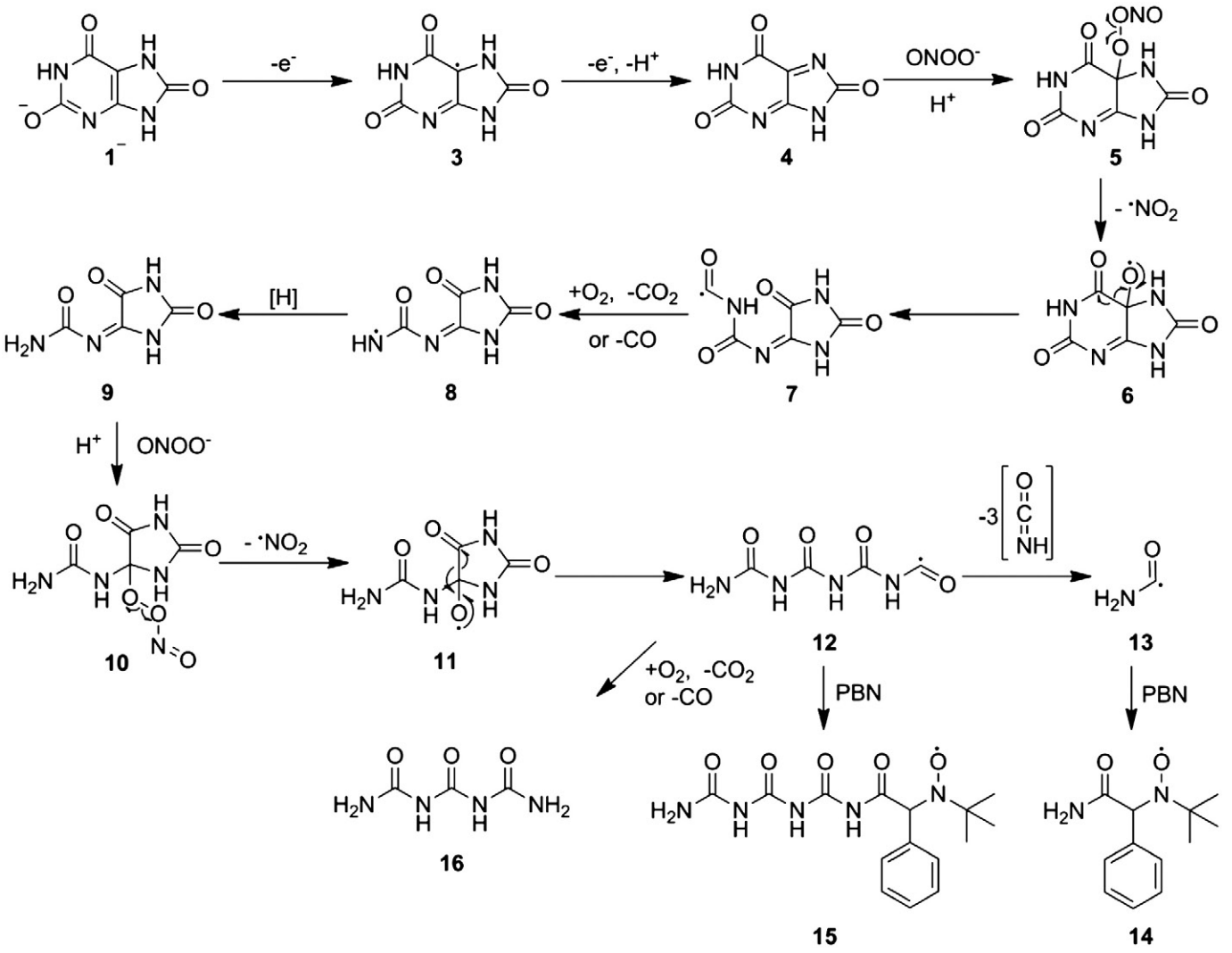


Fig. 10. Proposed radical formation mechanism of the reaction between urate and peroxynitrite.

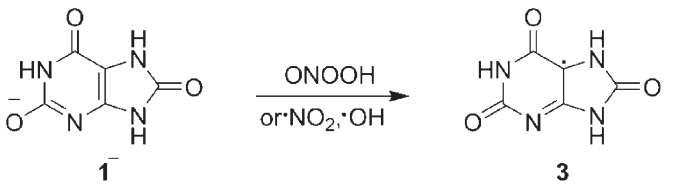
the fragmentation pattern of both parent ions contained the same fragment daughter ion at m/z 122. This ion was identified as protonated benzaldehyde oxime (Supplementary Figs. 1 and 2), which was derived from PBN as well. These results indicated that both parent ions contained PBN in their structure. Furthermore, the loss of formamide (MW 45), from both 223 and 352 ions, and isocyanic acid (HNCO; MW 43), in the case of the 352 ion, indicated that at least one amide group was present in both compounds. This assignment is further supported by the loss of ammonia (MW 17) from both 223 and 352 ions, which reflects the fact that the parent compounds have a functional group that contains a primary amine such as an amide. Based on these observations, the MS/MS fragmentation analysis confirmed that the structure of the product corresponding to m/z 223 was consistent with the hydroxylamine form of the PBN–aminocarbonyl radical adduct **14** (Fig. 10), and the structure corresponding to m/z 352 is proposed to be the hydroxylamine form of the PBN–triuretcarbonyl radical adduct **15** (Fig. 10). The existence of the triuret moiety in compound **15** is further confirmed by the fragment ions at m/z 147, 104, and 61 (Supplementary Fig. 2), which is a signature fragmentation pattern of triuret (M+1 = 147) [19].

Mechanism of the radical formation

Although the aminocarbonyl radical has been previously characterized, little is known about its formation mechanism. Two possible pathways have been proposed for its generation. Santos et al. suggested that the aminocarbonyl radical could be produced from one of the products of the urate–peroxynitrite reaction, such as alloxan, parabanic acid, or allantoin, even though they did not observe any trapped radical when these compounds were incubated with peroxynitrite [12]. Robinson et al. later suggested that triuret (**16,** Fig. 10), a novel product from peroxynitrite-induced oxidation of urate, could account for the detection of the aminocarbonyl radical [19]. We have tested the reaction of the proposed intermediates, alloxan, parabanic acid, allantoin, and triuret, with peroxynitrite under the same conditions as the oxidation of urate, but did not observe any radical adduct formation. We therefore conclude that the aminocarbonyl radical was not derived from these known urate-derived products. In our study, the trapping of radical adduct **15** led us to postulate that the triuretcarbonyl radical **12** is an intermediate in the production of the aminocarbonyl radical **13**, as proposed in Fig. 10.

How is the oxidation of urate initiated? It is not clear whether the oxidant is peroxynitrite itself or one of its possible derivatives. Depending on the pH of the reactions, a variety of peroxynitrite-derived compounds can coexist. At pH 7.4, a mixture of the peroxynitrite anion ($ONOO^-$) and ONOOH or its decomposition products is present [14,15,19]. It is highly unlikely that the negatively charged peroxynitrite anion reacts with the negatively charged urate anion. Rather, at pH 7.4, it is more feasible for urate to

be oxidized by neutral uncharged oxidants, for example, ONOOH or its decomposition radicals, $^{\bullet}NO_2$ and $^{\bullet}OH$ [14,15]. These oxidants can initiate a one-electron oxidation [14,15,20,21]. Moreover, the initial step of the oxidation of many purine-based compounds is considered to be a one-electron oxidation as well [10,22,23]. Thus, it is likely that urate is initially oxidized via one-electron transfer, forming the urate radical **3** (Eq. (4)).



1^{-} **3** (4)

When the pH of the reaction is increased, the chemistry of peroxynitrite is changed. At high pH, both peroxynitrite and urate are present in their anion or dianion forms, and their direct reaction is unfavorable because of the charge repulsion. The only oxidant that reacts with urate under basic conditions should be $^{\bullet}NO_2$ generated from the reaction between peroxynitrite and carbon dioxide [14,15].

In this case the diimine **4,** an oxidative intermediate of uric acid described in many urate oxidation studies [12,24], can be produced by one-electron and one-proton transfer from the urate radical **3** to another peroxynitrous acid or its derived radicals as described above. The diimine **4** is susceptible to nucleophilic addition and can rapidly react with nucleophiles such as water or ammonia [24] and, in our case, the peroxynitrite anion. The peroxynitrite anion has been reported to undergo nucleophilic addition in many reactions [25–29]. Moreover, both the observed pH dependence and the dependence on CO_2 concentration suggest one important conclusion, which is that $ONOO^-$ is necessary for the production of radicals. Our observation confirms the results reported by Santos et al. in which urate-derived radical formation was dependent on peroxynitrite anion [12]. At high pH, peroxynitrite is monoanionic and we observed higher yields of radical production (Fig. 5). In the presence of CO_2, which can rapidly react with $ONOO^-$ and reduce its concentration [30], the radical formation was decreased (Fig. 6). These observations suggest that peroxynitrite anion can act as a nucleophile and react with the diimine **4** to produce the peroxo adduct **5**.

After undergoing homolysis of the weak peroxo bond (O–O bond) [22,25], the peroxo adduct **5** decomposes to yield $^{\bullet}NO_2$ and the radical intermediate **6,** which then rearranges to form a carbonyl radical, **7**. The carbonyl radical **7** can be converted to radical **8** by either losing carbon monoxide (CO) or reacting with oxygen (O_2) and releasing carbon dioxide (CO_2). We have performed the reactions under anaerobic conditions with the ESR spin trapping experiments (data not shown). Interestingly, we found that the yield of the trapped radicals under anaerobic conditions was higher than under aerobic conditions. On the other hand, when reactions were carried out under O_2, we observed less or no ESR signal from the reaction. But the work by Santos et al. has shown that the urate–peroxynitrite reaction consumed oxygen [12]. We rationalized that oxygen may either quench the radical adducts or quickly react with radical **12** to form triuret, which will diminish the probability of trapping the radicals. From this observation we therefore proposed that it could be either carbon monoxide (when oxygen is absent) or carbon dioxide that could be released from radicals **7** and **12.** Indeed, a similar reaction path has been reported recently for the peroxynitrite-mediated oxidation of 8-oxoguanine [22]. Another molecule of peroxynitrite anion may react with intermediate **9,** which is produced by the reduction of compound **8,** to generate the allantoin–peroxo adduct **10.** The multimolar equivalent consumption of peroxynitrite leading to radical formation was supported by our observation that the yield of radical formation was at a maximum when a fourfold excess of peroxynitrite over urate was used (Fig. 4). By analogy, similar to the degradation of **5,** the triuretcarbonyl radical **12** is obtained. The β-cleavage of triuretcarbonyl radical **12** yields isocyanic acids and the aminocarbonyl radical **13,** which is subsequently trapped by PBN (Fig. 10).

Aside from being the precursor of the aminocarbonyl radical, the triuretcarbonyl radical could also be an intermediate candidate for the generation of triuret **16,** which could be achieved by eliminating carbon monoxide or carbon dioxide by reaction with oxygen (Fig. 10). Given the high concentrations of both urate and peroxynitrite used in this study, which were necessitated by the limited sensitivity of the ESR spin trap method, one can question the relevance of the proposed mechanism to the intracellular fate of urate. However, we point out that triuret is a unique product of the reaction between urate and peroxynitrite that not only was observed in vitro [19] but also has been detected in urine samples of hypertensive patients [31].

Conclusion

The observation of a novel intermediate, triuretcarbonyl radical, sheds light on the formation mechanism of triuret [19] and the aminocarbonyl radical [12] in the reaction between urate and peroxynitrite. In contrast to other known oxidants that can react with uric acid, triuret is observed only in the peroxynitrite–urate reaction [32]. This mechanism rationalizes the uniqueness of peroxynitrite-mediated oxidation of uric acid to produce triuret as a major product.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2010.04.010.

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