Can the night-time atmospheric oxidant $NO_3^\bullet$ damage aromatic amino acids?†

Duanne C. E. Sigmund and Uta Wille*

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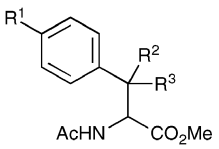
Reaction of nitrate radicals, $NO_3^\bullet$, with aromatic amino acids leads to irreversible oxidative functionalization at the β-position or at the aromatic ring, suggesting that this important atmospheric oxidant could potentially cause damage to peptides lining the respiratory tract and may contribute to pollution-derived diseases.

Adverse health effects of ambient air pollutants have been addressed by numerous epidemiological studies.[1] In particular, an association between increased concentrations of ozone and nitrogen oxides, and increases in respiratory morbidity and hospital admissions for asthma in both adults and children has been proposed, but the underlying mechanism is not clear.[2] It has been suggested that O_3 and $NO_2^\bullet$ may modulate airway diseases, such as asthma, by increasing the release of inflammatory mediators from bronchial epithelial cells and that the cells of asthmatic patients may be more susceptible to the adverse effects of these pollutants.[3] Quite remarkably, to our knowledge, neither of these studies considered that in the atmosphere $NO_2^\bullet$ and O_3 form highly reactive nitrate radicals, $NO_3^\bullet$:

$$NO_2^\bullet + O_3 \rightarrow NO_3^\bullet + O_2$$

$NO_3^\bullet$ is rapidly photolyzed during the daytime, but after sunset tropospheric concentrations of $NO_3^\bullet$ rise to ppt levels, and this radical is responsible for the initiation of atmospheric transformations at night.[4] Although during the past decades the role of $NO_3^\bullet$ in atmospheric chemistry was addressed by many studies,[4] virtually no link between this oxidant and oxidative damage in biomolecules exposed to the atmosphere has been made. A recent investigation showed that birch pollen proteins are efficiently nitrated in polluted air, suggesting that protein nitration may play a central role in the promotion of allergies by air pollutants and a direct involvement of $NO_3^\bullet$ in this process.[5] Although the atmospheric concentration of $NO_3^\bullet$ is less than that of $NO_2^\bullet$ or O_3, $NO_3^\bullet$ is many orders of magnitude more reactive, and this reactivity can outweigh its lower abundance.[4,6] Also, since $NO_3^\bullet$ can react *via* different pathways that include electron transfer (ET), hydrogen abstraction (HAT), or addition to π-systems, every organic molecule is principally susceptible to attack by $NO_3^\bullet$.[4]

In our effort to obtain fundamental insight into the $NO_3^\bullet$ induced oxidative damage of proteins, we studied the products formed in the reaction of $NO_3^\bullet$ with the N- and C-end protected aromatic amino acids **1–4**[7] using mass spectrometry.



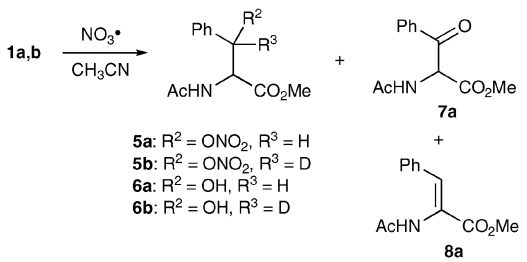
1a: $R^1 = R^2 = R^3 = H$
1b: $R^1 = H$, $R^2 = R^3 = D$
2: $R^1 = OMe$, $R^2 = R^3 = H$
3: $R^1 = NO_2$, $R^2 = R^3 = H$
4: $R^1 = OH$, $R^2 = R^3 = H$

The experiments were performed in the absence of oxygen in the largely inert solvent acetonitrile to exclude potential side-reactions of reactive intermediates with the solvent, which could complicate the interpretation of the experiments. $NO_3^\bullet$ was generated in the presence of the required amino acid through photoinduced ET from ceric(IV) ammonium nitrate, CAN, at $\lambda = 350$ nm:[8]

$$(NH_4)_2Ce^{IV}(NO_3)_6 \rightarrow NO_3^\bullet + (NH_4)_2Ce^{III}(NO_3)_5$$

Irradiation of three equivalents of CAN with phenylalanine **1a** resulted in four new products (Scheme 1), which were identified by LC/HR-MS(ESI). The major product β-nitrooxy phenylalanine **5a** showed the protonated molecular ion $[M+H^+]$ at $m/z = 283.09225$ (calcd. 283.09246). Collision-induced dissociation (CID) performed on the $[M+H^+]$ signal revealed a fragment ion at $m/z = 220.15$, resulting from loss of the ONO_2 group. As minor products, we observed the formation of β-hydroxy phenylalanine **6a**[9] ($[M+H^+]$: calcd. 238.10738; found 238.10732), β-keto aminoacid **7a**[10] ($[M+H^+]$: calcd. 236.09173; found 236.09164) and the alkene **8a**[9] ($[M+H^+]$: calcd. 220.09682; found 220.09676).‡

In order to unequivocally identify the site of oxidation, the β-bisdeuterated phenylalanine **1b**[11] was treated with $NO_3^\bullet$,

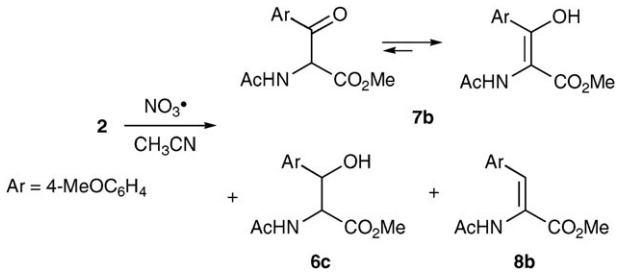


5a: $R^2 = ONO_2$, $R^3 = H$
5b: $R^2 = ONO_2$, $R^3 = D$
6a: $R^2 = OH$, $R^3 = H$
6b: $R^2 = OH$, $R^3 = D$

Scheme 1 Identified products in the reaction of phenylalanine **1** with $NO_3^\bullet$.

ARC Centre of Excellence for Free Radical Chemistry and Biotechnology, School of Chemistry, BIO21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia

† Electronic supplementary information (ESI) available: ^1H NMR spectra for **1b**, **5a**, **7b** and **14b**; experimental procedures, LC/HR-MS(ESI) spectra for all products of the reaction of $NO_3^\bullet$ with **1**, **2** and **4**. See DOI: 10.1039/b803456g



Scheme 2 Identified products in the reaction of 4-methoxyphenylalanine **2** with $NO_3^{\bullet}$.

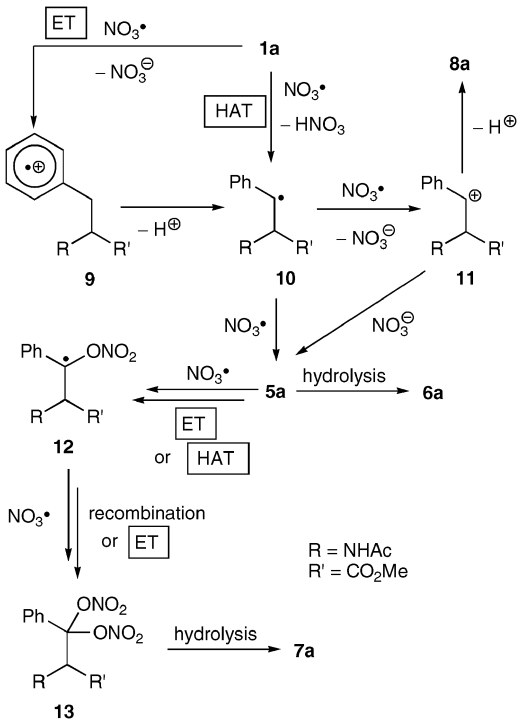
which resulted in formation of the monodeuterated nitrate ester **5b** and alcohol **6b**.§ In addition, the ^{1}H NMR spectrum of a sample of **5a**, which could be isolated by preparative HPLC, showed the remaining benzylic proton as a doublet with a strong downfield shift at $\delta = 6.33$ ppm caused by the electron-withdrawing nitrate substituent.¶

Reaction of three equivalents of $NO_3^{\bullet}$ with the methoxy-substituted phenylalanine **2** led to formation of the β-keto aminoacid **7b** as the major product, which, according to ^{1}H NMR data obtained from an isolated sample, exists practically exclusively in the isomeric enol form (Scheme 2). **7b** is highly labile to ionization (calcd. m/z of $[M+H^+] = 266.1$), and we were only able to identify a fragment ion at $m/z = 248.09140$ ($[M^+ - 17]$: calcd. 248.09173), resulting from formal loss of a hydroxyl group. A β-nitrate ester of type **5** was not found, but small amounts of its hydrolysis product, **6c**, and the alkene **8b**[12] were identified by LC/HR-MS(ESI).‖

The proposed mechanism for the reaction of $NO_3^{\bullet}$ with **1a** (as an example) is shown in Scheme 3.

The key intermediate is presumably the benzylic radical **10**, which could be formed through two pathways, either (i) by HAT or (ii) by ET** to yield at first the radical cation **9** that undergoes deprotonation in the benzylic position. The nitrate ester **5a** could be formed either through radical recombination of **10** with $NO_3^{\bullet}$ or stepwise *via* a second ET†† and trapping of the benzyl cation **11**[13] by nitrate ions. Loss of a proton in the latter could give the alkene **8a**. We believe that the hydroxylated compound **6a** is a by-product resulting from hydrolysis of **5a** during the LC/MS procedure. The carbonyl compound **7a** could be formed from **5a** through a similar sequence *via* the benzyl radical **12** and subsequent hydrolysis of the resulting dinitrate **13**.

In order to explore the mechanism of the initial step, competition experiments were performed by reacting $NO_3^{\bullet}$ with mixtures of electron rich and electron deficient phenylalanines, *e.g.* **1a**, **2** and **3**, respectively. Different electron density at the aromatic ring should only have an effect on the rate of an ET process, whereas the rate for a HAT from the benzylic position should not be significantly affected. Indeed, the experiments revealed that the nitroaromatic compound **3** did not noticeably react with $NO_3^{\bullet}$, whereas the methoxy compound **2** reacted much faster with $NO_3^{\bullet}$ than the unsubstituted phenylalanine **1a**. This finding clearly supports an initial ET mechanism, which is also in accordance with rate data obtained for the reaction of phenylalanine with $NO_3^{\bullet}$ in 6 M nitric acid.[14]



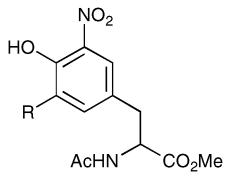
Scheme 3 Proposed pathway for the reaction of phenylalanine **1** with $NO_3^{\bullet}$.

It is not clear how the benzyl radical **10** is transformed into nitrate ester **5a**, because our experimental observations are contradictory. If this reaction proceeds *via* cation **11**, trapping with a nucleophile should result in new products, but no different outcome was observed when the reaction was performed in the presence of small amounts of water or methanol. Also, no products resulting from a Ritter reaction with the solvent acetonitrile were observed. On the other hand, the observed formation of alkene **8a** would support a pathway *via* benzyl cation **11**. Clearly, further experimental and computational work is required to fully elucidate the mechanism of these transformations.

The finding that, in contrast to the unsubstituted phenylalanine **1a**, the methoxy-substituted derivative **2** reacts with $NO_3^{\bullet}$ to preferably yield the more oxidized carbonyl product **7b** (see Scheme 2) is in accordance with the proposed mechanism, because the more electron-rich aromatic ring is likely to be oxidized again after the first ET/deprotonation sequence, if sufficient oxidizing agent, *e.g.* $NO_3^{\bullet}$, is present. The suggestion that the carbonyl products **7** could result from hydrolysis of a geminal β-dinitrate **13** is supported by the observation of a partially hydrolyzed product possessing both a hydroxyl and nitrate substituent in the β-position (not shown)‡‡ in the reaction of **2** with $NO_3^{\bullet}$. According to the suggested mechanism, formation of the nitrate esters of type **5** would require two equivalents of $NO_3^{\bullet}$, whereas formation of carbonyl compounds of type **7** would require four equivalents of $NO_3^{\bullet}$. It should be noted that experiments performed with a 1 : 1 ratio of $NO_3^{\bullet}$ and **1a** still led to formation of the nitrate ester **5a**, but much less **1a** was consumed under these conditions. Surprisingly, reaction of equimolar amounts of the methoxyphenylalanine **2** with $NO_3^{\bullet}$ did not lead to a β-nitrate ester of type **5**,

but resulted only in formation of the carbonyl product **7b** with significant amounts of unreacted **2**. Thus, it appears from these findings that these phenylalanines, once damaged, are even more prone to further oxidation than the undamaged amino acids.

Finally, the reaction of $NO_3^\bullet$ with the tyrosine derivative **4** was studied. Three equivalents of $NO_3^\bullet$ led to complete consumption of the amino acid, and dinitrotyrosine **14b** was identified as the only product in the LC/HR-MS(ESI) through a $[M+H^+]$ signal at m/z = 328.07712 (calcd. 328.07754). The ^{1}H NMR spectrum obtained after isolation of a sample of **14b** was identical to that of an authentic sample.[15] Reaction of equimolar amounts of $NO_3^\bullet$ with tyrosine **4** resulted in incomplete consumption of **4** and only nitrotyrosine **14a**[16] was obtained as product, as identified by LC/HR-MS(ESI) through a $[M+H^+]$ signal at m/z = 283.09229 (calcd. 283.09246) and by comparison of the fragmentation pattern with that of an authentic sample. At present, we have no mechanistic rationale that could explain formation of **14a** and **14b**, and in-depth experimental and computational studies are currently underway.



14a: R = H
14b: R = NO_2

To conclude, this first detailed product analysis of the reaction of $NO_3^\bullet$ with amino acids shows clearly that this important atmospheric oxidant can damage aromatic amino acids in an irreversible way. β-Nitrate esters, β-carbonyl compounds or aromatic nitro compounds are the major or exclusive products in these reactions, and since nitration of proteins has been observed to occur in polluted air, our results suggest that atmospheric $NO_3^\bullet$ could potentially be the real culprit in certain pollution-derived diseases. Even if only a small fraction of atmospheric $NO_3^\bullet$ is actually involved in the oxidative damage of peptides lining the respiratory tract, this damage, if not repaired by the natural defence system, could have serious consequences for human health. We are currently investigating the reaction of amino acids and small peptides with $NO_3^\bullet$ under atmosphere-relevant conditions, *e.g.* in the presence of $NO_2^\bullet$ and O_2 in both nonaqueous and aqueous environments.

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Notes and references

‡ Since not all products in this study could be isolated, their respective structures were verified in all cases by CID.
§ HR-MS(ESI): **5b** ($[M+H^+]$); calcd. 284.09874, found 284.09833. **6b** ($[M+H^+]$); calcd 239.11366, found 239.11337.
¶ Treatment of L-phenylalanine with $NO_3^\bullet$ leads to the diastereomeric *threo*- and *erythro*-β-nitrate esters in a ratio of 11 : 1.‖ **6c** ($[M+H^+]$; calcd. 268.11795, found 268.11755); **8b** ($[M+H^+]$; calcd. 250.10738, found 250.10704).
** The ET could proceed through an addition/elimination process, where $NO_3^\bullet$ first undergoes addition to the aromatic ring, followed by elimination of NO_3^- to give an aryl radical cation.
†† This second ET could principally be promoted by either CAN or $NO_3^\bullet$. We limit the discussion to $NO_3^\bullet$, because it is the stronger oxidant: E^0 ($NO_3^\bullet/NO_3^-$) = 2.3 V *vs.* NHE [see ref. 8*b* and refs. 17*a*, *b*]. E^0 (Ce^{4+}/Ce^{3+}) = 1.61 V *vs.* NHE [ref. 17*c*].

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