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PAPER

Damage of aromatic amino acids by the atmospheric free radical oxidant $NO_3^{\bullet}$ in the presence of $NO_2^{\bullet}$, N_2O_4, O_3 and O_2†

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Analysis of the products formed in the reaction of $NO_3^{\bullet}$ with the *N*- and *C*-protected aromatic amino acids **1–5**, which was performed under conditions that simulate exposure of biosurfaces to environmental pollutants, revealed insight how this important atmospheric free-radical oxidant can cause irreversible damage. In general, $NO_3^{\bullet}$ induced electron transfer at the aromatic ring is the exclusive initial pathway in a multi-step sequence, which ultimately leads to nitroaromatic compounds. In the reaction of $NO_3^{\bullet}$ with tryptophan **5** tricyclic products **12** and **13** are formed through an intramolecular, oxidative cyclization involving the amide moiety. In addition to this, strong indication for formation of *N*-nitrosamides was obtained, which likely result from reaction with N_2O_4 through an independent non-radical pathway.

The adverse health effects of ambient air pollutants have been addressed in numerous epidemiological studies.[1] In particular, a link has been proposed between increases in respiratory morbidity and hospital admissions for asthma and increased concentrations of atmospheric ozone, O_3, and nitrogen oxides, NO_x (NO_x is a generic term for the free radical species nitrogen oxide and nitrogen dioxide, $NO^{\bullet}$ and $NO_2^{\bullet}$, respectively), but the underlying mechanism is not clear.[2] It has been suggested that O_3 and/or $NO_2^{\bullet}$ 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] Interestingly, an investigation of the pulmonary effects of a combined exposure to O_3 and $NO_2^{\bullet}$ in mice revealed that changes in the lung metabolism were synergistic.[4] This might suggest that not $NO_2^{\bullet}$ and O_3 as individual species are the actual culprit behind these adverse health effects, but instead highly reactive nitrate radicals, $NO_3^{\bullet}$, which are formed through the reaction of $NO_2^{\bullet}$ with O_3 (eqn (1)):

$$NO_2^{\bullet} + O_3 \rightarrow NO_3^{\bullet} + O_2 \quad (1)$$

$NO_3^{\bullet}$ is the most important oxidant in the atmosphere at night (where it is also formed through the reaction in eqn (1)), and its reactivity towards most organic compounds is many orders of magnitude higher than that of either $NO_2^{\bullet}$ or O_3, respectively.[5] Since $NO_3^{\bullet}$ can react *via* oxidative electron transfer (ET; E^0 ($NO_3^{\bullet}/NO_3^-$) = 2.3 V *vs.* NHE),[6] hydrogen abstraction (HAT), or addition to π-systems, every organic molecule is principally susceptible to attack by this free radical species.[5] In the absence of organic reaction partners, $NO_3^{\bullet}$ can recombine with $NO_2^{\bullet}$ to form dinitrogen pentoxide, (N_2O_5) in an equilibrium reaction, which serves as a reservoir for $NO_3^{\bullet}$.

Although the chemistry of $NO_3^{\bullet}$ in the atmosphere has been extensively studied during the past decades,[5] only very few links between this free radical oxidant and oxidative damage in biomolecules that are exposed to the atmospheric environment have been made. The most important constituents of the respiratory tract surface lining fluid, which covers the respiratory tract epithelial cells, are, besides antioxidants such as glutathione, vitamin C and uric acids, metal binding proteins.[7] A recent investigation showed that birch pollen proteins are efficiently nitrated in polluted air.[8] This suggests 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, especially since its reaction with water, which is the major constitutent of the respiratory tract lining fluid, is slow.[5] In a recent product study, we have shown for the first time that aromatic amino acids are irreversibly oxidatively damaged by reaction with $NO_3^{\bullet}$, which was generated *in situ* by irradiation of ceric(IV) ammonium nitrate (CAN) at λ = 350 nm.[9] Although this is in principle a very convenient methodology to produce $NO_3^{\bullet}$ on preparative scale in solution, because of the considerable oxidation power of CAN

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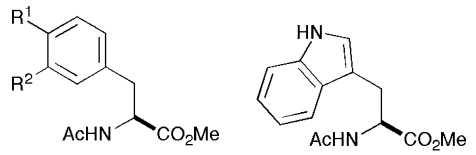
† Electronic supplementary information (ESI) available: Experimental conditions and spectroscopic data for all reaction products, X-ray data for **12a**. CCDC reference number 810818. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c0ob01186j

itself,‡ its thermal reaction with some more easily oxidizable amino acids can complicate analysis of the products formed in the reaction with $NO_3^{\bullet}$. In addition, under environmental conditions $NO_3^{\bullet}$ is not an isolated reactant, but is always accompanied by other radicals and non-radical oxidants, such as $NO_2^{\bullet}$ (which is in equilibrium with its dimer dinitrogen tetroxide, N_2O_4), O_3, and O_2, respectively, which principally could all trap reactive intermediates. Thus, in order to explore the role of such additional reactants on the products formed during the $NO_3^{\bullet}$ induced oxidative damage of aromatic amino acids, we have performed detailed product studies on the reaction of *N*- and *C*-protected§ L-phenylalanine (**1a**), L-tyrosines (**2a**–**4a**) and L-tryptophan (**5**) with $NO_3^{\bullet}$ (Fig. 1), which was *in situ* generated through the reaction shown in eqn (1). These experiments could be considered as model to simulate exposure of proteins lining the respiratory tract surface to the atmospheric environment.



1a: $R^1 = R^2 = H$
2a: R = OH, $R^2 = H$
3a: R = OAc, $R^2 = H$
4a: R = OH, $R^2 = NO_2$

5

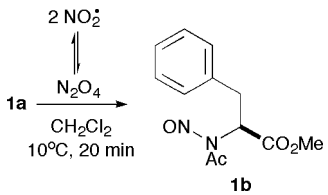
Fig. 1

Results and Discussion

The reactions were performed at +10 °C under exclusion of moisture in dichloromethane by adding a measured excess amount of liquid $NO_2^{\bullet}$ to the amino acid, with a stream of O_3 in O_2 passing through the solution.[10] Consumption of the amino acids was usually complete after 20 min of reaction time.¶ After work-up the reaction products were isolated and purified by preparative HPLC, followed by NMR and ESI-MS analysis. Unless stated otherwise, product ratios were obtained from the ^{1}H NMR spectrum of the reaction mixture through integration of the acetyl proton signals, which showed sufficiently different chemical shifts for the starting amino acids and the reaction products. Detailed spectroscopical data are given in the ESI.† In addition, control reactions were also performed where the amino acids were separately treated with either $NO_2^{\bullet}/N_2O_4$ or an O_2/O_3 mixture, respectively. It should be noted that, due to the required repeated purifications by HPLC, yields could not be determined for any of the reactions under investigation. However, since this study is aimed at obtaining insight into the nature of the products in order to assess the potential impact of such chemical modifications for biological systems, exact yields are only of minor importance.

(a) The reaction of $NO_2^{\bullet}/N_2O_4$ with amino acids

Analysis of the reactions of all amino acids with $NO_2^{\bullet}/N_2O_4$ in both presence and absence of O_3 revealed formation of small to considerable amounts of new products, which underwent fragmentation upon ionization (ESI-MS) to reveal a molecular mass that was apparently identical with the respective parent amino acid, but which showed longer retention times on the HPLC column. Assignment of these compounds will be exemplified for the reaction of phenylalanine **1a** with $NO_2^{\bullet}$, where isolation by HPLC gave a yellowish material, which we propose is *N*-nitroso phenylalanine **1b** (Scheme 1). ^{1}H NMR analysis of this compound revealed characteristic differences compared to the spectrum of **1a**, such as the absence of the amide proton signal, and significant downfield shifts of the proton at the α-carbon from $\delta = 4.88$ ppm (in **1a**) to $\delta = 5.55$ ppm in **1b**, of one of the benzylic protons from $\delta = 3.14$ ppm (in **1a**) to $\delta = 3.42$ ppm in **1b**, and of the *N*-acetyl protons from $\delta = 1.98$ ppm (in **1a**) to $\delta = 2.60$ ppm in **1b**. The magnitude of the observed downfield shifts are in excellent agreement with ^{1}H NMR data of *N*-nitrosated peptide bonds in literature.[11] In addition to this, the IR spectrum of **1b** showed the absence of the N–H stretching vibration and a significant shift of the carbonyl stretching vibration to higher wavenumbers, which is in accordance with an electron-withdrawing substituent at the amide nitrogen (see ESI†). We believe that **1b** is formed by reaction of **1a** with N_2O_4, which has been shown to act as effective non-radical nitrosating agent for amides.‖ The mechanism of this reaction has been explored in detail and will not be discussed here.[12] *N*-Nitrosamide **1b** is expected to undergo cleavage of the N–N bond in the mass spectrometer, and the lability of **1b** was demonstrated by the observation that decomposition to the parent amino acid **1a** occurred upon standing in solution at room temperature. The finding that the atmospheric pollutant system $NO_2^{\bullet}/O_3$ leads to rapid formation of *N*-nitrosoamides is significant, since these species are the most broadly acting and among the most potent carcinerogens.[13] It should be noted that,



Scheme 1 Suggested *N*-nitrosation of phenylalanine **1a**.

‡ E^0 (Ce^{4+}/Ce^{3+}) = 1.61 V *vs.* NHE [V. Nair and A. Deepthi, *Chem. Rev.*, 2007, **107**, 1862].

§ Protection of the N- and C-terminus in the amino acids was performed to simulate the peptide bond in the protein.

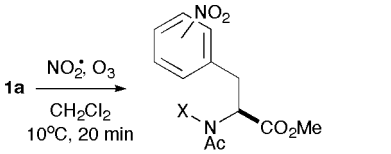
¶ Typical experimental procedure: To a stirred mixture of the amino acid (1.00 mol) in anhydrous dichloromethane (15 mL) at 10 °C was added liquid $NO_2^{\bullet}$ (0.5 mL, 15 mmol), and ozonized O_2 was bubbled through the mixture at a low flow rate. After 20 min, the reaction was quenched by addition of aq. $NaHCO_3$ (10 mL), the phases were separated and the aqueous phase was extracted with dichloromethane. The combined organic fractions were dried over $MgSO_4$, concentrated and the reaction products isolated and purified by repeated preparative HPLC (see also ESI†). It is not possible to state the exact [$NO_2^{\bullet}$] in our experiments, since an undeterminable amount of $NO_2^{\bullet}$ evaporated prior to its reaction with O_3.

‖ It cannot be excluded that this new product is derived from *N*-nitration through reaction of the amide moiety with $NO_2^{\bullet}/N_2O_4$ [T. Kaiya, K. Nakamura, M. Tanaka, N. Miyaka and K. Kohda, *Chem. Pharm. Bull.*, 2004, **52**, 570; B. C. Challis, D. E. C. Shuker, D. H. Fine, E. U. Goff and G. A. Hoffman, *Acta Cient. Compostel.* 1982, **19**, 153]. Compound **10b** was the only case where we were able to obtain high resolution ESI-MS data, which support formation of an *N*-nitroso amide. Unfortunately, all *N*-nitroso amides obtained in this work were too unstable to allow identification through chemical derivatization.

in contrast to other studies,[14] a reaction of $NO_2^{\bullet}$ and/or N_2O_4 with the aromatic residue in the amino acids **1–5** was not observed under our experimental conditions.

(b) The reaction of $NO_3^{\bullet}$ with phenylalanine 1a

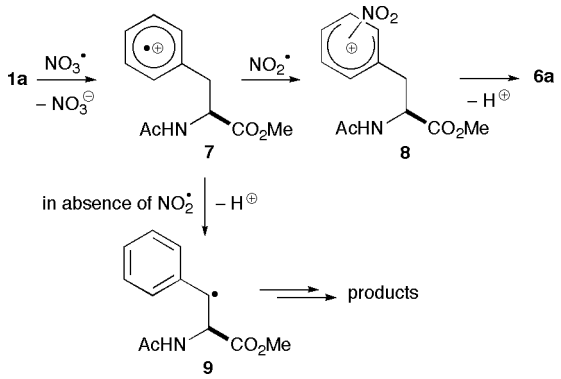
The reaction of $NO_3^{\bullet}$ with phenylalanine **1a** led to formation of the isomeric ring-nitrated products **6a** (Scheme 2). NMR and GC analysis revealed that the *para* and *ortho* substituted isomers were the major products, but very minor amounts of the *meta* nitrated product were also obtained. In addition to this, *N*-nitrosation was also found to occur, leading to both *ortho*- and *para*-**6b**, whereas *meta*-**6b**, if formed, was below the detection limit of the HPLC. The *N*-nitrosamides **6b** were assigned on the basis of their ^{1}H NMR data, which showed similar characteristic downfield shifts for the protons adjacent to the amide moiety as observed for **1b**. No reaction occurred between **1a** and an O_2/O_3 mixture in the control experiment.



Scheme 2 Products of the reaction of $NO_3^{\bullet}$ with phenylalanine **1a**.

This reaction outcome strongly contrasts the findings from our previous experiments, where $NO_3^{\bullet}$ was generated in the absence of $NO_2^{\bullet}$ by photolysing CAN.[9] Under the latter conditions, the reaction involving **1a** only resulted in products with an oxidized benzylic position, whereas the aromatic ring remained apparently intact. We will restrict the mechanistic discussions to reactions involving $NO_3^{\bullet}$, since the observed *N*-nitrosation likely results from an independent non-radical side reaction of the amide moiety with N_2O_4, which could principally occur before and/or after the $NO_3^{\bullet}$ reaction with the amino acid.

The different reaction pathways in dependence of the $NO_3^{\bullet}$ source can be rationalized by the mechanism proposed in Scheme 3, where $NO_3^{\bullet}$ induced oxidation (oxidative ET) of the aromatic ring leads to the radical cation **7**,[9] which is trapped by $NO_2^{\bullet}$ to give the σ-complex **8**. Rearomatization through deprotonation yields nitrophenylalanine **6a**.** A mechanism proceeding through an intermediate of type **8** is supported by the observed strong preference for *ortho*- and *para*-substituted products, which is in line with the selectivity of electrophilic aromatic substitutions involving alkylaromatic compounds and which proceed *via* the same intermediate.[10] In contrast to this, in the absence of $NO_2^{\bullet}$ (for example, when $NO_3^{\bullet}$ is generated by CAN photolysis) the



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

radical cation **7** is stabilized through loss of a benzylic proton, thus leading to the benzyl radical intermediate **9** with a restored aromatic ring, which undergoes further reactions (not shown).†† From these findings, we conclude that the radical cation **7** must have a certain lifetime that enables trapping by suitable reagents, for example $NO_2^{\bullet}$, prior to benzylic deprotonation. However, a reaction of **7** (or any other radical intermediates) with either O_2 or the solvent, which were present in excess in our experiments, was not observed.

Interestingly, under biochemical conditions the aromatic ring in phenylalanine is practically inert to oxidation. Our finding that this amino acid is readily damaged by the environmental free radical oxidant $NO_3^{\bullet}$ has a potentially considerable biological impact for proteins. Thus, since the radical cation intermediate **7** is in turn also a strong oxidant, it might trigger an electron transfer cascade within the protein that could potentially lead to oxidative damage at remote positions.[15]

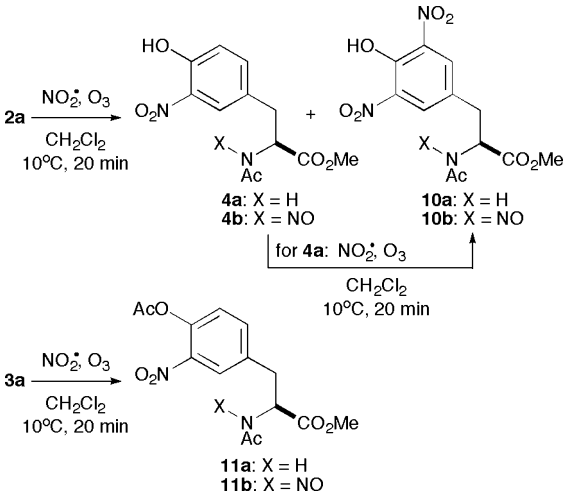
(c) The reaction of $NO_3^{\bullet}$ with tyrosines 2a–4a

Tyrosine **2a**, which is very easily oxidized under biological conditions, rapidly reacts with $NO_3^{\bullet}$ to give a mixture of 3-nitrotyrosine **4a**, 3,5-dinitrotyrosine **10a** and their respective *N*-nitroso compounds **4b** and **10b** with a ratio of **4a** : **4b** : **10a** : **10b** = 16 : 1.4 : 1 : 1.8 (Scheme 4). Through an independent experiment, where **4a** was reacted with $NO_3^{\bullet}$ in the presence of $NO_2^{\bullet}$ and O_3/O_2, it was established that the bis-nitrated tyrosine **10a** results from $NO_3^{\bullet}$ induced oxidation of 3-nitrotyrosine **4a**. From a synthetic point of view, this is an interesting finding, since nitration of such strongly deactivated aromatic rings requires usually much harsher conditions.[16]

Formation of the nitroaromatic ring in both **4** and **10** is likely to proceed through an oxidative ET/radical recombination/deprotonation mechanism in analogy to that shown in Scheme 3. Trapping of any radical intermediates by O_2 was not observed. Similarly, reaction of the *O*-acetylated tyrosine **3a** with $NO_3^{\bullet}$ leads to the 3-nitrotyrosine derivative **11a**, in addition to the corresponding *N*-nitroso product **11b** (**11a** : **11b** = 1 : 1.35). In contrast to the reaction of tyrosine **2a**, formation of an *O*-acetylated 3,5-dinitrotyrosine derivative was not observed in our experiments. It is further important to note that none of the

** It should be noted that the $NO_2^{\bullet}/N_2O_4$ equilibrium constant favours the dimer in solution [T. F. Redmond and B. B. Wayland, *J. Phys. Chem.*, 1968, **72**, 1626], and N_2O_4 can be oxidized with O_3 to give N_2O_5. Kinetic studies in the gas-phase revealed that the latter reacts with unsaturated compounds by several orders of magnitude slower than $NO_3^{\bullet}$ [C. Pfrang, R. S. Martin, C. E. Canosa-Mas and R. P. Wayne, *Phys. Chem. Chem. Phys.*, 2006, **8**, 354], and N_2O_5 does not nitrate deactivated aromatic compounds in solution (see ref. 13). The inert and nonpolar solvent dichloromethane was used in these experiments to avoid ionic dissociation of any N_2O_5 present in the system.

†† We have shown that formation of **9** does not occur in a direct step through benzylic hydrogen abstraction in **1a** by $NO_3^{\bullet}$ (see ref. 9).



Scheme 4 Products of the reaction of $NO_3^{\bullet}$ with tyrosines **2a–4a**.

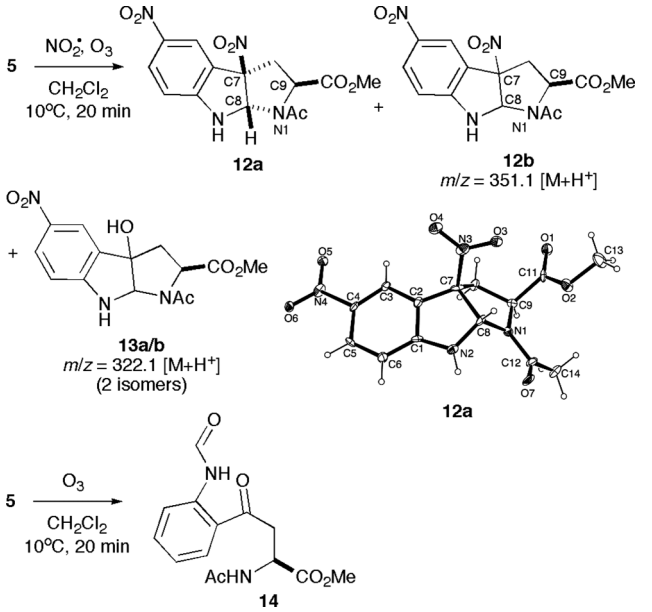
tyrosines **2a–4a** underwent a reaction with the O_3/O_2 mixture in the absence of $NO_2^{\bullet}$.

Nitration of the aromatic ring caused by environmental free radicals might have serious consequences, in particular, if it occurs on tyrosine residues in proteins. Since the pK_a, which is 10 in tyrosine, decreases to about 7.5 in 3-nitrotyrosine, under physiological conditions 3-nitrotyrosine exists in its anionic form. Formation of a negatively charged hydrophilic site in proteins has severe structural implications, since subunit interactions can be disrupted.

(d) The reaction of $NO_3^{\bullet}$ with tryptophan 5

In the reaction of tryptophan **5** with $NO_3^{\bullet}$ four products were formed. According to the respective protonated molecular ions [M + H$^+$] in the LC/ESI-MS of the crude reaction mixture, the two major products were isomers possessing formally two additional NO_2 moieties, compared to the starting amino acid **5**. The two minor compounds were also isomeric species containing one additional NO_2 and OH group (Scheme 5). After isolation and purification by preparative HPLC, we were delighted that one of the major products readily crystallized and could be analysed by X-ray. This revealed the tricyclic compound **12a**, which contained a nitrated aromatic ring and two *cis*-fused cyclopentyl rings featuring a nitro substituent at the bridgehead carbon of the bicyclo[3.3.0] system, which is *cis* to the ester group at C9. On the basis of the available spectroscopic data and mechanistic considerations (see below), we believe that the other isomer possessing two NO_2 substituents (*e.g.* **12b**) must be a diasteroisomer of **12a**, but it was not possible from the ^{1}H NMR data to deduce the stereochemistry at C7 and C8.‡‡ In the case of the two minor products, unfortunately only one isomer could be isolated in an amount sufficient for NMR analysis. Based on the general similarity of its NMR spectra with those for **12a/b** (see ESI†), however, we assume that both minor isomers should have a similar tricyclic framework possessing also a nitroaromatic ring,

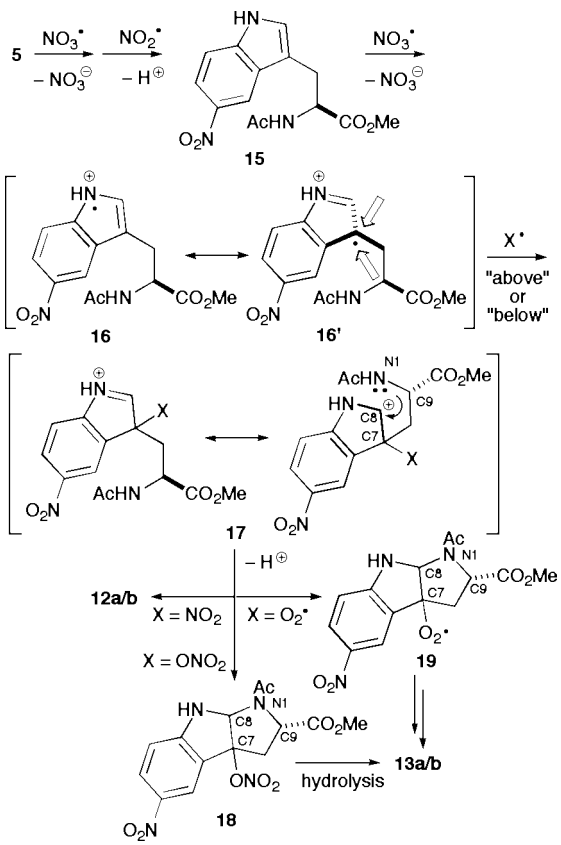
‡‡ A change of the stereochemistry at the α-carbon of the amino acid as a result of the reaction with $NO_3^{\bullet}$ can in principle not be excluded, but seems to be not very likely.



Scheme 5 Products of the reaction of tryptophan **5** with $NO_3^{\bullet}$ and with O_3.

but with an OH substituent at the bridgehead carbon so that the structure can be tentativly assigned as **13a/b**.

Formation of compounds **12/13** obviously proceeds under involvement of the amide functionality in **5**. Scheme 6 shows the



Scheme 6 Proposed mechanism for the reaction of $NO_3^{\bullet}$ with tryptophan **5** in the presence of $NO_2^{\bullet}$.

proposed reaction mechanism, where nitration of the aromatic ring occurs *para* to the dominating activating amino substituent through a pathway similar to that outlined in Scheme 3. The resulting nitrotryptophan **15** can be further oxidized by $NO_3^{\bullet}$ at the indole-nitrogen to give radical cation **16**, which is in resonance with the distonic isomer **16′**. Recombination with $X^{\bullet}$ ($X^{\bullet}$ = $NO_2^{\bullet}$, $NO_3^{\bullet}$ or O_2, see below) from either above or below the molecular plane of **16′** yields cation **17**, which acts as acceptor in the subsequent nucleophilic cyclization of the amide-nitrogen by which the tricyclic framework is established. The major pathway involves trapping of **16′** by $NO_2^{\bullet}$, which gives the observed dinitro compounds **12a/b**. The minor products **13** could be formed through two different pathways. Trapping of **16′** by $NO_3^{\bullet}$ gives nitrate ester **18**, which may undergo hydrolysis to the corresponding alcohol **13** during work-up and/or purification of the reaction products. If trapping of **16′** occurs through reaction with O_2, subsequent decomposition of the resulting peroxyl radical **19** could also lead to **13**. The latter pathway would represent the only case in the $NO_3^{\bullet}$ induced oxidative damage of aromatic amino acids so far, where residual oxygen would be involved in product formation. It should be noted that the reactions outlined in Scheme 6, *e.g.* those leading to nitration of the aromatic ring and those resulting in oxidative cyclization involving the amide moiety could also proceed in reverse order. Interestingly, the finding that in the reaction of tryptophan **5** with $NO_2^{\bullet}/O_3$ *N*-nitroso amides were not formed as by-products, can be taken as indication that the radical-induced oxidative cyclization of the amide moiety must occur much faster than *N*-nitrosation.

The stereochemistry in **12a**, which features a *cis* arrangement between the ester group at C9 and the NO_2 substituent, can be rationalized through attack of the amide nitrogen N1 at C8 from the less sterically hindered site *anti* to the NO_2 substituent at C7 (which in turn results from bottom face recombination of $NO_2^{\bullet}$ with **16′**). It is likely that the isomeric product **12b** could possess inverted configurations at C7 and C8, which result from recombination of $NO_2^{\bullet}$ with **16′** from the top face.§§

The observed participation of the amide group in the $NO_3^{\bullet}$ induced oxidation of tryptophan **5** could be unique to our experimental system, where the reaction with the isolated amino acid was studied. In a protein the less flexible backbone may prevent the conformational change required for such a cyclization. On the other hand, a positive charge similar to that of intermediate **17** in proteins is a potential target for attack by any nucleophile present in the system through both intra- and intermolecular pathways, which could lead to significant structural modificatios in the protein.

Tryptophan **5** was the only amino acid in this study, which reacted rapidly with a mixture of O_3/O_2. The product of this reaction was identified as dicarbonyl compound **14** (Scheme 5),[17] which results from ozonolysis of the more electron-rich N-heterocycle. This product was not observed when the reaction was performed in the presence of $NO_2^{\bullet}$, which indicates that formation of $NO_3^{\bullet}$ and its reaction with **5** occurred significantly faster than reaction of **5** with O_3.

§§ It cannot be excluded, however, that the NO_2 substituent in **13b** occupies a different position on the aromatic ring.

Conclusions

This first detailed product analysis of the reaction of $NO_3^{\bullet}$ with aromatic amino acids, which was performed under conditions that simulate exposure of biosurfaces to atmospheric pollutants, revealed clear pathways how this important atmospheric free-radical oxidant can cause irreversible damage to biolocially important molecules. All amino acids under investigation reacted rapidly with $NO_3^{\bullet}$, which was *in situ* generated by treating $NO_2^{\bullet}$ with an O_3/O_2 mixture, leading ultimately to aromatic ring nitration. There are no indications that the initial reaction of $NO_3^{\bullet}$ occurs at any other site of the amino acid except the aromatic ring. No influence of residual O_2 on product formation in the reactions involving phenylalanine **1** and tyrosines **2a–4a** was found. In the reaction with tryptophan **5** trapping of radical intermediates by O_2 cannot be excluded, but this would represent only a very minor pathway. Also, in none of the reactions under investigation products arising from a reaction involving the solvent were observed.

In the reaction of $NO_3^{\bullet}$ with tryptophan **5** the damage was not limited to the aromatic ring system. Oxidation occurred also at the nitrogen atom of the pyrrol ring leading to formation of an intermediate carbocation **17**, which is quenched by intramolecular nucleophilic cyclization involving the amino acid functionality. Because of the reduced flexibility of the backbone, a similar cyclization is not very likely to occur in proteins. On the other hand, a cationic intermediate of type **17** in a protein could be trapped by any nucleophile available in the reaction system, for example water or hydroxyl- and thiyl groups of cysteine, threonine or serine residues, respectively, which could lead to significant structural changes in the protein.

Another important outcome of this study is the finding that $NO_3^{\bullet}$ can even damage phenylalanine, which is usually inert to oxidation by free radical and non-radical oxidants produced through natural biochemical pathways. Thus, since the initially formed aryl radical cation **7** has a certain lifetime, it would represent a very strong oxizing site in a protein, which might trigger an oxidative ET along the peptide chain that could lead to damage at positions remote from the initial reaction site at the interface between biological surface and environment. Our future work is aimed at exploring this hypothesis in detail, using specially designed peptides as model substrates.

Whereas $NO_2^{\bullet}$ does not undergo a noticeable chemical reaction with the aromatic amino acids investigated in this study, we have strong experimental indications that *N*-nitrosamides are formed through an independent, non-radical pathway by reaction with N_2O_4, which is in equilibrium with $NO_2^{\bullet}$. Although we have not further explored this reaction, our results reveal a "dual mode of action" of the atmospheric pollutant system $NO_2^{\bullet}/O_3$ in its reactions with aromatic amino acids, *e.g.* (i) $NO_3^{\bullet}$ initiated nitration of the aromatic ring, and (ii) *N*-nitrosation of the peptide bond by N_2O_4, thus generating highly labile and carcinogenic species.

It should be noted that formation of nitrated aromatic amino acids is a general feature observed in a wide range of inflammatory-immune responses, which are conditions associated with increased levels of oxidative stress.[18] *In vivo* studies revealed that 3-nitrotyrosine concentrations are increased in exhaled breath condensate of patients with asthma[19] or cystic fibrosis,[20] and a

strong immunoreactivity for nitrotyrosine in the airway epithelium and inflammatory cells in the airways of asthmatic patients has been found.[21] Since nitration of proteins has been observed to occur in polluted air, our results suggest that $NO_3^{\bullet}$ formed through reaction of $NO_2^{\bullet}$ and O_3 in close vicinity to biological surfaces, such as the respiratory tract lining fluid, could potentially be the real causer of certain pollution-derived diseases.

Acknowledgements

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