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Oxidative damage of aromatic dipeptides by the environmental oxidants $NO_2^{\bullet}$ and O_3†

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Irreversible oxidative damage at both aromatic side chains and dipeptide linkage occurs in the aromatic N- and C-protected dipeptides **7–11** upon exposure to the environmental pollutants $NO_2^{\bullet}$ and O_3. The reaction proceeds through initial oxidation of the aromatic ring by *in situ* generated $NO_3^{\bullet}$, or by $NO_2^{\bullet}$, respectively, which leads to formation of nitroaromatic products. The indole ring in Phe-Trp undergoes oxidative cyclization to a pyrroloindoline. An important reaction pathway for dipeptides with less oxidisable aromatic side chains proceeds through fragmentation of the peptide bond with concomitant acyl migration. This process is likely initiated by an ionic reaction of the amide nitrogen with the $NO_2^{\bullet}$ dimer, N_2O_4.

Introduction

According to the World Health Organization, air pollution is responsible for the premature death of about seven million people each year. Nitrogen dioxide ($NO_2^{\bullet}$) and ozone (O_3) are amongst the most important gaseous pollutants in the outdoor and indoor environment, and their inhalation is associated with the development of respiratory tract inflammation.[1,2] The respiratory tract lining fluids (RTLF) are the first biological fluids that come into contact with environmental free radical and non-radical oxidants. It has been shown in *in vivo* studies that exposure to O_3 and $NO_2^{\bullet}$ in isolation results in significant reduction of RTLF antioxidant levels.[1] This weakened defence shield could provide a pathway for environmental oxidants to directly attack proteins and lipids present on cell surfaces or in the RTLF, which could lead to highly reactive protein and lipid oxidation products that may subsequently damage the underlying epithelial cells, thereby causing inflammation.

The effects of ground-level O_3 and $NO_2^{\bullet}$ on lung function, both in isolation and in combination, has been explored in exposure studies.[1–3] Unfortunately, there is only limited knowledge of the individual mechanistic steps that lead from the initial damage to the acute disease state. However, a detailed understanding of the chemical pathways by which gaseous air pollutants damage the constituents of the RTLF is essential for the development of effective strategies to combat deleterious impacts of air pollution.

$NO_2^{\bullet}$ is moderately oxidizing [E^0 ($NO_2^{\bullet}/NO_2^-$) = 1.03 V][4a] and reacts only with the most reactive amino acids through oxidative electron transfer (ET).[4b] $NO_2^{\bullet}$ is also a potent radical trap, but reactions with closed-shell systems through addition or hydrogen abstraction are comparably slow.[4c,d] The biological effect of O_3 is attributed to its high reactivity with π systems, and O_3 reacts with lipids exclusively at the C=C double bonds in unsaturated fatty acids.[5a] Reaction of O_3 with proteins occurs preferably at easily oxidisable side chains.[5b]

In the polluted ambient atmosphere O_3 and $NO_2^{\bullet}$ always coexist. It was found that birch pollen proteins are rapidly nitrated upon exposure to traffic pollution (which is characterised by large concentrations of O_3 and $NO_2^{\bullet}$).[6a] In fact, significant synergistic effects were observed in various biological molecules when these were subjected to $O_3/NO_2^{\bullet}$ mixtures, such as increased lipid peroxidation and protein nitration.[3,6] In particular, 3-nitrotyrosine concentrations are increased in exhaled breath condensates of patients with asthma[7] or cystic fibrosis,[8] and a strong immunoreactivity for nitrotyrosine in the airway epithelium and inflammatory cells in the airways of asthmatic patients has been found.[9] The more than additive response to exposure of $O_3/NO_2^{\bullet}$ mixtures suggests that nitrate radicals, $NO_3^{\bullet}$, might be involved in these processes, which are *in situ* generated *via* eqn (1).[10]

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

It was proposed that the observed synergistic effects are due to the toxicity of dinitrogen pentoxide, N_2O_5, which is formed

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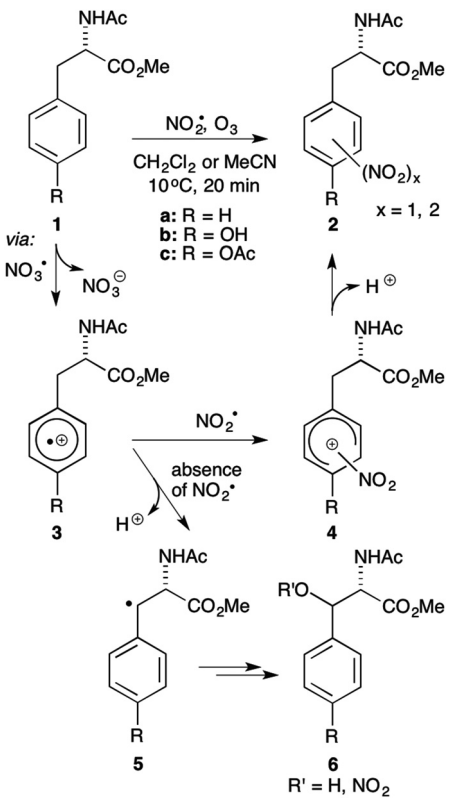
†Electronic supplementary information (ESI) available: Synthetic procedures and spectroscopic data for dipeptides **7–11**, experimental and spectroscopic details for reaction of all dipeptides with $NO_2^{\bullet}$ and O_3; crystallographic data for compound **16**. CCDC 1016028. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c4ob01577k

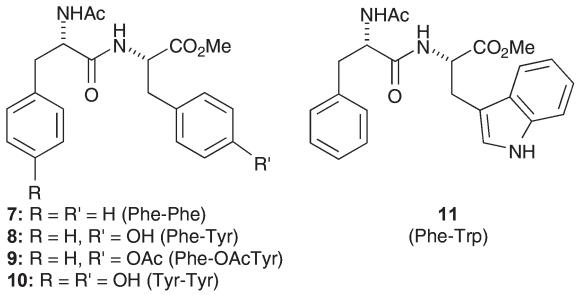
through (reversible) recombination of $NO_2^{\bullet}$ with $NO_3^{\bullet}$, and also the hydrolysis product nitric acid (HNO_3), but detailed mechanistic studies were not performed.[1,3] In fact, formation of $NO_3^{\bullet}$ through reaction of $NO_2^{\bullet}$ with O_3 is of significant environmental relevance, since $NO_3^{\bullet}$ is the most important tropospheric free radical oxidant at night and responsible for initiating chemical transformation processes in the atmosphere. Although the atmospheric concentration of $NO_3^{\bullet}$ is less than that of $NO_2^{\bullet}$ or O_3, its high reactivity towards most organic compounds outweighs its lower abundancy.[10] Thus, $NO_3^{\bullet}$ is highly electrophilic and strongly oxidising [E^0 ($NO_3^{\bullet}/NO_3^-$) = 2.5 V],[11] and also reacts through addition to π systems and hydrogen abstraction.[10] $NO_3^{\bullet}$ has not only a higher solubility in water than $NO_2^{\bullet}$ [K_H ($NO_3^{\bullet}$) = 6.0×10^{-1} M atm^{-1}; K_H ($NO_2^{\bullet}$) = 1.2×10^{-2} M atm^{-1}, at 298 K],[12] but also maintains its high reactivity in water,[10] with which it actually reacts only slowly, according to: $H_2O + NO_3^{\bullet} \rightarrow HO^{\bullet} + HNO_3$ ($k = 3 \times 10^2$ M^{-1} s^{-1}).[13] On this basis, it appears highly likely that also biological molecules in the RTLF are susceptible to attack by $NO_3^{\bullet}$.

We have recently shown in an *in vitro* product study that exposure of various N- and C-protected aromatic amino acids **1** to $O_3/NO_2^{\bullet}$ mixtures in either dichloromethane or acetonitrile leads to rapid formation of nitroaromatic products **2** (Scheme 1).[14a]

The reaction likely proceeds through initial $NO_3^{\bullet}$ induced oxidation of the aromatic ring,[15] which is followed by trapping



Scheme 1 Reaction of $NO_3^{\bullet}$ with aromatic amino acids **1**.



7: R = R' = H (Phe-Phe)
8: R = H, R' = OH (Phe-Tyr)
9: R = H, R' = OAc (Phe-OAcTyr)
10: R = R' = OH (Tyr-Tyr)

11 (Phe-Trp)

Fig. 1 Aromatic dipeptides **7–11** studied in this work.

of the aryl radical cation **3** with $NO_2^{\bullet}$ and subsequent deprotonation of **4**. In the absence of $NO_2^{\bullet}$, benzylic deprotonation in **3** occurs first,[15b]‡ and the resulting radical **5** is ultimately transformed to β-oxygenated amino acids **6**.[14b] The high oxidation power of $NO_3^{\bullet}$ causes irreversible damage to all aromatic amino acids, even those that are inert to oxidation by free radical and non-radical oxidants produced through natural biochemical pathways, such as phenylalanine (**1a**). In the fast reaction with tyrosine (**1b**) consecutive formation of 3-nitro tyrosine (**2b**, with $x = 1$) and 3,5-dinitrotyrosine (**2b**, with $x = 2$) occurs. Tryptophan reacts through multiple pathways that result in both aromatic nitration and oxidative cyclization through involvement of the amino acid functionality (not shown).[14a] A recent multiphase kinetic study by Pöschl *et al.* proposes that uptake of $NO_2^{\bullet}$ by proteins in the gas phase and in the presence of O_3 is a combination of a direct reaction of $NO_3^{\bullet}$ and a sequential process, where reaction of O_3 with the protein leads first to reactive oxygen intermediates, which react with $NO_2^{\bullet}$ in a second step.[6c] The employed kinetic model considered tyrosine as the only target for reaction with $NO_2^{\bullet}/O_3$ mixtures, and did not include potential multiple attacks at one site or at other protein residues, for example the protein backbone. In light of our previous findings that $NO_3^{\bullet}$ attacks not only tyrosine, it is not unlikely that the direct involvement of $NO_3^{\bullet}$ in oxidative protein damage may have been underestimated.

In the present study we explored the reaction of the aromatic dipeptides **7–11** (Fig. 1) with $NO_2^{\bullet}$ and O_3 in both isolation and combination to simulate exposure of peptides lining the respiratory tract surface to the atmospheric environment.

Analysis of the reaction mixtures and identification of major products revealed the sites in dipeptides most susceptible to damage by these environmental oxidants. While *O*-acetylated tyrosine (OAcTyr) is not a naturally occurring amino acid, dipeptide **9** was included in this work to examine how electron density at the aromatic system directs the reaction outcome.

‡ In the case of very electron-rich aromatic compounds, a concerted electron and proton transfer process is likely (see ref. 15*b*).

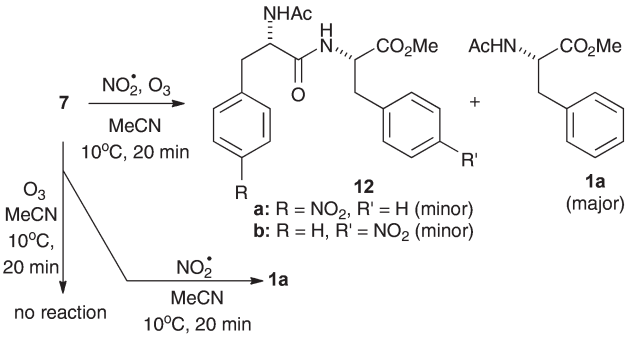
Results and discussion

The dipeptides were protected at both the N-terminus (as acetamide) and C-terminus (as methyl ester; see Fig. 1). All reactions were carried out at 10 °C for 20 min under exclusion of moisture in acetonitrile by adding a measured excess amount of liquid $NO_2^{\bullet}$ (which is in equilibrium with its dimer dinitrogen tetroxide, N_2O_4) to the dipeptide **7**–**11**, with a stream of ozonized oxygen passing through the solution.[14a]§ The reactions of the dipeptides with $NO_2^{\bullet}$ and O_3 in isolation were also explored under similar conditions. Reaction products were isolated and purified by preparative HPLC, followed by HR-ESI-MS and ^{1}H NMR analysis. ^{13}C NMR data were also obtained for major products, where sufficient material could be isolated. Full experimental and spectroscopic details are given in the ESI.† Although in our experiments O_3 was always supplied as a mixture with O_2, we will simply refer to these as O_3 experiments, since a direct involvement of O_2 was not observed for any reaction. It should be noted that, due to the required repeated HPLC purifications, yields could not be determined. Qualitative assignments for major and minor products were made on the basis of the UV absorptions in the HPLC runs at different wavelengths. The aim of the present study was the identification of 'hot spots' in dipeptides for attack by $NO_2^{\bullet}$ and O_3 in order to assess the potential impact for biological systems. In the biological context even seemingly minor chemical changes often have serious pathological consequences and, therefore, knowledge of explicit yields is not required.

Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Phe (7)

Of the naturally occurring aromatic amino acids, phenylalanine contains the least electron-rich aromatic ring and is therefore comparably difficult to oxidise. The outcome of the reaction of the Phe-Phe dipeptide **7** with O_3 and $NO_2^{\bullet}$ in isolation and combination is shown in Scheme 2.

Exposure of **7** to a mixture of $NO_2^{\bullet}$ and O_3 for 20 min resulted in incomplete consumption of the starting material and formation of three products. The isomeric *para*-nitrated dipeptides **12a** and **12b** were generated in a 1 : 1 ratio (according to HPLC analysis at λ = 280 nm, where the UV absorption of **7** is negligible), which indicates unselective oxidative attack at both the N- and C-terminal amino acid. The aromatic nitration likely proceeds through the mechanism in Scheme 1,



Scheme 2 Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Phe (**7**).

e.g. $NO_3^{\bullet}$ induced oxidation of the aromatic ring, followed by trapping of the intermediate aryl radical cation by $NO_2^{\bullet}$. The observed *para* selectivity for this radical mediated nitration (confirmed by ^{1}H NMR) is in agreement with previous findings for the reaction of **1a** with $NO_2^{\bullet}/O_3$ mixtures[14a] and can be rationalized by the stability of the intermediate σ complex of type **4a**. According to the UV absorption of the reaction mixture at λ = 220 nm, where only small signals from **12a/b** were present,¶ the major product of this reaction was the *N*-acetylated phenylalanine methyl ester **1a**. This assignment was confirmed through comparison of the spectroscopic data with those of an authentic sample. The presence of the ester group indicates that **1a** originates from the C-terminal amino acid in **7**, suggesting cleavage of the peptide bond linking the two amino acids with concomitant migration of the N-terminal acetyl group. The counterpart of the peptide cleavage, *e.g.* the N-terminal amino acid (or possible subsequent reaction products), could not be obtained. Remarkably, a similar fragmentation of the peptide bond also occurred in the reaction of **7** with $NO_2^{\bullet}$ in isolation. Qualitatively, the rate of peptide fragmentation was slow and not affected by the presence of O_3. It is therefore reasonable to suggest that peptide cleavage in **7** is caused by reaction with $NO_2^{\bullet}$ (and not $NO_3^{\bullet}$). In contrast to this, a reaction between $NO_2^{\bullet}$ and the aromatic moieties in **7** did not occur in the absence of O_3. The possible mechanism for the $NO_2^{\bullet}$ induced fragmentation of the peptide backbone will be discussed below.

It should be noted that dipeptide **7** did not react with O_3 in the absence of $NO_2^{\bullet}$.

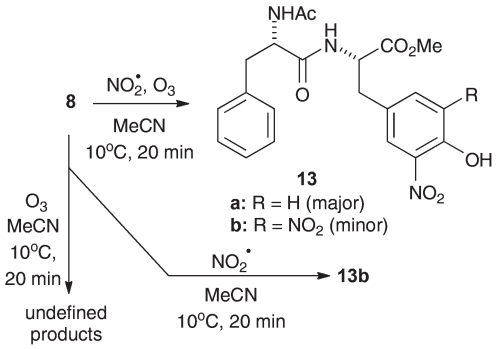
Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Tyr (8)

Reaction of dipeptide **8** with a mixture of O_3 and $NO_2^{\bullet}$ occurred highly selectively at tyrosine, resulting in complete consumption of **8** after 20 min of reaction time and formation of dipeptides consisting of an intact phenylalanine moiety that is linked to either 3-nitrotyrosine (**13a**) or 3,5-dinitrotyrosine (**13b**), respectively (Scheme 3).

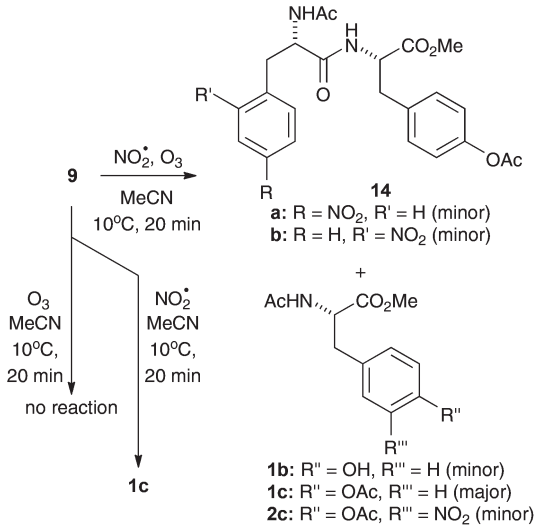
According to UV analysis of the reaction mixture at λ = 250, 280 and 350 nm, the mono-nitrated dipeptide **13a** was formed

§ Typical experimental procedure: To a stirred mixture of the dipeptide (1.00 mmol) in acetonitrile (100 mL) at 10 °C was added under exclusion of moisture liquid $NO_2^{\bullet}$ (0.5 mL, 15 mmol), and a stream of ozonized O_2 was passed through the mixture at a low flow rate. Consumption of the substrates was usually complete after 20 min of reaction time. The reaction was quenched by addition of aq. $NaHCO_3$ (50 mL), acetonitrile removed *in vacuo* and the aqueous phase extracted with ethyl acetate. The combined organic fractions were dried over $MgSO_4$, concentrated and the reaction products isolated and purified by repeated preparative HPLC (see ESI† for details). It is not possible to state the exact [$NO_2^{\bullet}$] in the experiments, since an undeterminable amount of $NO_2^{\bullet}$ evaporated prior to its reaction with O_3. The experiments with $NO_2^{\bullet}$ and O_3 in isolation were performed analogously.

¶ It is estimated that the UV absorbance of the nitrated dipeptides **12a/b** at λ = 220 nm is approximately 1000 times higher than that of **7**.



Scheme 3 Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Tyr (**8**).



Scheme 4 Reaction of $NO_2^{\bullet}$ and O_3 with Phe-OAcTyr (**9**).

as major product,‖ which was accompanied by smaller amounts of the di-nitrated dipeptide **13b**. Formation of dimeric products of type Phe-Tyr-Tyr-Phe, which are linked through a covalent bond *ortho* to the phenolic hydroxyl group in tyrosine (such dityrosines are commonly produced during tyrosine oxidation in the absence of radical traps),[4b] was not observed. The reaction of **8** with $NO_2^{\bullet}$ in the absence of O_3 led to formation of **13b** as the major pathway, while the mono-nitrated product **13a** was not obtained. The higher degree of aromatic nitration in the absence of O_3 suggests a higher concentration of dissolved $NO_2^{\bullet}$ under these conditions, which can be rationalized by the fact that the partial pressure of $NO_2^{\bullet}$ is higher in the absence of O_2/O_3.

The initial reaction of $NO_3^{\bullet}$ or $NO_2^{\bullet}$ with tyrosine likely proceeds through oxidative ET, but it is not clear whether trapping of the resulting tyrosyl radical cation of type **3a** (see Scheme 1) by $NO_2^{\bullet}$ occurs faster than its deprotonation to give the tyrosyl radical (not shown), which could subsequently be quenched by $NO_2^{\bullet}$. It can also not be excluded that, at least in the reaction involving the highly reactive $NO_3^{\bullet}$, direct abstraction of the phenolic hydrogen atom occurs. However, knowledge of the mechanism to such detail is not essential in order to assess the potential biological impact of the damage. Tyrosine nitration has, in fact, serious consequences for biological systems, since each nitro substituent lowers the pK_a of tyrosine by about 3 units.[16] Under physiological conditions nitrated tyrosine is deprotonated and generates negatively charged hydrophilic sites in peptides, which could induce structural changes that may lead to disruption of subunit interactions.[16] Indeed, it was found that nitration of tyrosine results in inactivation of a number of biologically significant proteins.[17]

It should be noted that in the reaction of **8** with $NO_2^{\bullet}$, both in the presence and absence of O_3, products arising from scission of the peptide backbone could not be identified. This shows that the fast reaction of $NO_2^{\bullet}$ with the tyrosine moiety in **8** acts as efficient "sink" for $NO_2^{\bullet}$ with which the slower reaction with the peptide bond (leading to peptide fragmentation) could not compete to a measurable extent under our experimental conditions. On the other hand, the reaction of O_3 with dipeptide **8** led to rapid consumption of the starting material, resulting in decomposition to many different products, which could not be isolated and identified. This process is likely triggered by initial attack of O_3 at the tyrosine moiety in **8**,[18] since phenylalanine does not react with O_3 (see above).

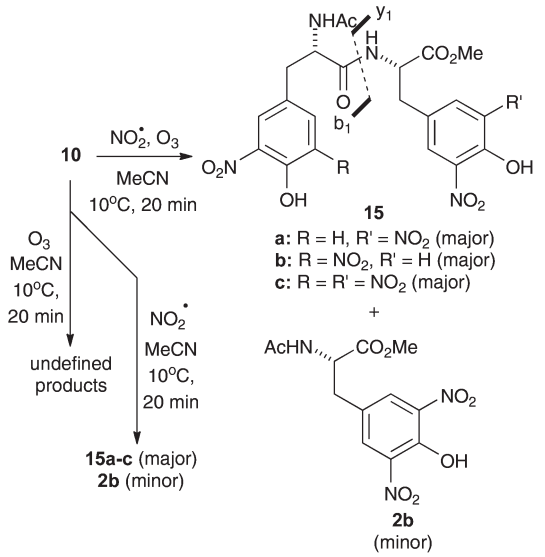
‖ The absorbance of **13a** at these wavelengths is approximately 5–10 times lower than that of **13b**.

Reaction of $NO_2^{\bullet}$ and O_3 with Phe-OAcTyr (9)

Protection of the tyrosine hydroxyl group through *O*-acetylation reduced significantly the susceptibility of this amino acid to oxidative damage. Similar to the reaction of **7**, Phe-OAcTyr (**9**) was only incompletely consumed after 20 min exposure to a mixture of $NO_2^{\bullet}/O_3$, which led, according to HPLC analysis, to a number of different products. Only the most abundant could be isolated and identified, which are shown in Scheme 4.

UV analysis at λ = 220 nm revealed the *O*-acetylated tyrosine **1c**, which is the C-terminal amino acid in **9**, as major reaction product, indicating that fragmentation of the peptide bond is the dominant pathway. Minor amounts of *O*-acetyl-3-nitrotyrosine **2c** and tyrosine **1b** were also formed. It is reasonable to assume that **1b** is a secondary product that results from **1c**, presumably post-reaction through cleavage of the O–Ac bond during aqueous work-up. If formed in the presence of $NO_2^{\bullet}/O_3$, rapid oxidation to nitrotyrosine **2b** would be expected. On the other hand, **2c** could be obtained *via* first nitration of the *O*-acetylated tyrosine in **9**, followed by peptide fragmentation, or in inverse order where nitration follows fragmentation (not shown). An additional reaction pathway for dipeptide **9** with $NO_2^{\bullet}/O_3$ mixtures involves oxidative damage at the aromatic ring of the phenylalanine moiety to produce the isomeric *ortho* and *para* nitrated dipeptides **14a/b**.

The reaction of dipeptide **9** with $NO_2^{\bullet}$ in the absence of O_3 was slow and led predominantly to the fragmentation product **1c**, but formation of very minor amounts of **1b** and **2c** cannot be excluded. On the other hand, no reaction occurred between **9** and O_3 in the absence of $NO_2^{\bullet}$.



Scheme 5 Reaction of $NO_2^{\bullet}$ and O_3 with Tyr-Tyr (**10**).

Reaction of $NO_2^{\bullet}$ and O_3 with Tyr-Tyr (10)

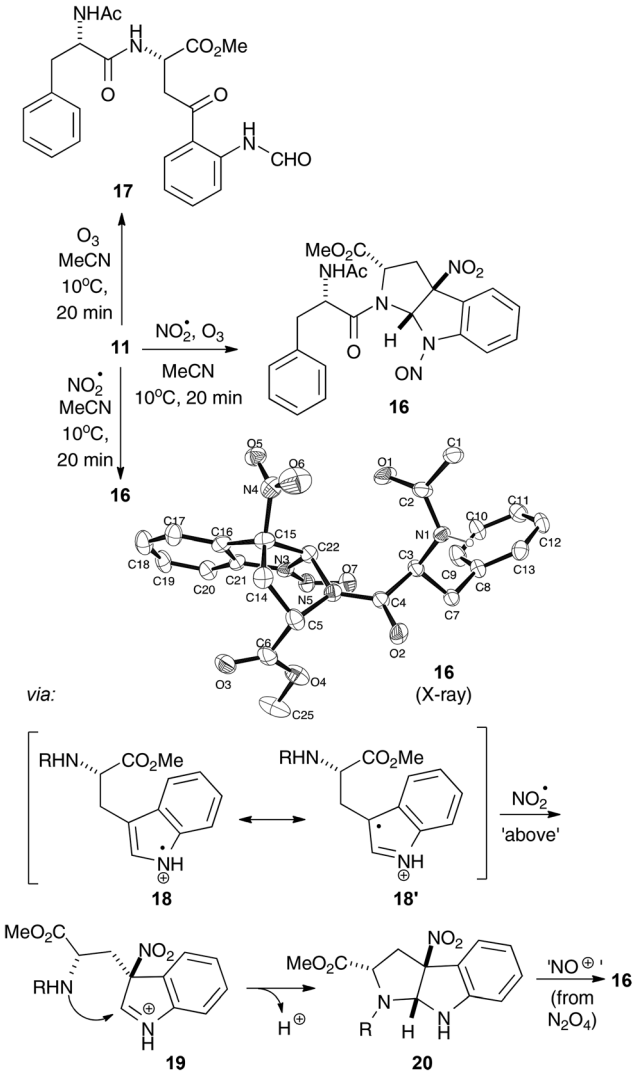
Reaction of Tyr-Tyr (**10**) with a mixture of $NO_2^{\bullet}$ and O_3 resulted in complete consumption of the dipeptide after 20 min. The major products are the isomeric tri-nitro compounds **15a**/**b** and the tetra-nitrated dipeptide **15c** (Scheme 5).

In addition to this, LC/MS analysis of the reaction mixture reveals formation of a product with a high-resolution mass of *m*/*z* 491.1409, which corresponds to a dinitrated dityrosine dipeptide ($C_{21}H_{22}N_4O_{10}$ + H^+: calcd 491.1250). Due to the small amount, isolation and further characterization of this product was not possible.

The site of nitration in the tri-nitro dipeptides **15a** and **15b** was unequivocally confirmed by ESI mass spectrometric sequencing using collision-induced dissociation (b_x–y_z pathway).[19] Thus, for **15a** the fragment at *m*/*z* 251.08 corresponds to the mono-nitrated N-terminal amino acid (b_1: $C_{11}H_{11}N_2O_5^+$, calcd 251.07). The fragment at *m*/*z* 286.08 could be assigned to the C-terminal dinitrated amino acid (y_1: $C_{10}H_{11}N_3O_7^+$, calcd 286.07). Likewise, the y_1 fragment of the protonated C-terminal amino acid in **15b** appears at *m*/*z* 241.08 ($C_{10}H_{13}N_2O_5^+$, calcd 241.08), confirming mononitration of the tyrosyl moiety. The corresponding b_1 fragment could not be obtained.

In addition to this, fragmentation of the peptide backbone also occurred, but only as a very minor reaction pathway, to yield 3,5-dinitrotyrosine **2b**, which is the former C-terminal amino acid moiety. From the UV absorption of the reaction mixture at λ = 280 nm a product ratio for **15c**/**2b** of *ca.* 5 could be estimated. Given the high reactivity of tyrosine towards oxidative aromatic nitration by $NO_2^{\bullet}/O_3$ mixtures, it is reasonable to assume that the comparably slow fragmentation of the peptide bond occurred only after nitration of the phenolic ring.

Reaction of **10** with $NO_2^{\bullet}$ in the absence of O_3 also led to the various tri- and tetra-nitrated dipeptides **15a–c**, which further confirms the high susceptibility of tyrosine to $NO_2^{\bullet}$ induced oxidation. Peptide cleavage to yield **2b** occurred too, but, however, only to a very minor extent under these conditions.



Scheme 6 Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Trp (**11**).

Similar to the reaction of the phenylalanine-tyrosine dipeptide **8**, reaction of **10** with O_3 resulted in complete decomposition, and isolation of reaction products was not possible.

Reaction of $NO_2^{\bullet}$ and O_3 with Phe-Trp (11)

Tryptophan is, similar to tyrosine, prone to oxidation by radical and non-radical oxidants. The reaction of O_3 with the phenylalanine-tryptophan dipeptide **11** occurred rapidly and proceeded selectively at tryptophan through oxidative cleavage of the indole ring. The resulting dipeptide **17** consists of an *N*′-formyl kynurenine residue and an intact phenylalanine moiety (Scheme 6).[14a,20]

Tryptophan was also exclusively targeted when **11** was exposed to a mixture of $NO_2^{\bullet}$ and O_3. Under these conditions formation of the O_3 cleavage product **17** was largely sup-

pressed, and the indole ring underwent oxidative cyclization involving the nitrogen atom of the dipeptide linkage, to yield a mixture of, presumably, isomeric products. Only the major product could be isolated through crystallisation. Its structure was confirmed by X-ray analysis as dipeptide **16**, which consists of an intact phenylalanine moiety and a *cis*-fused *N*-nitroso pyrroloindoline framework possessing an angular nitro substituent.[14a] While pyrroloindolines are structural motifs in many biologically active alkaloids,[21] *N*-nitrosamines are known for their highly carcinogenic effects.[22] The reaction of **11** with $NO_2^{\bullet}$ in the absence of O_3 also leads to formation of pyrroloindoline **16**, which demonstrates the ease by which tryptophan is damaged by abundant environmental oxidants.

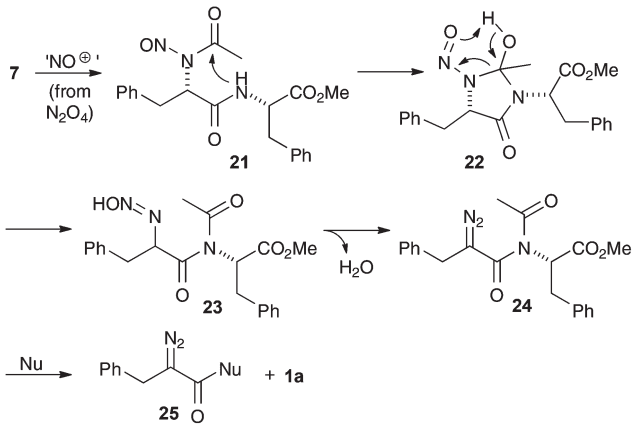
A mechanism for formation of **16** is proposed in the lower part of Scheme 6,[14a] which consists of initial oxidation by either $NO_2^{\bullet}$ or $NO_3^{\bullet}$ to produce indole radical cation **18**.** The distonic radical cation resonance isomer **18′** is the likely intermediate involved in the subsequent steps that include radical trapping by $NO_2^{\bullet}$ and nucleophilic cyclization of the amide nitrogen, which leads to the pyrroloindoline **20**. It is obvious that formation of the pyrroloindoline framework in **16** requires some conformational flexibility of the peptide backbone. We believe that N-nitrosation occurs only in the final step through reaction of **20** with N_2O_4 (resulting from $NO_2^{\bullet}$ dimerisation), which is a known non-radical nitrosating agent.[23] On the other hand, if N-nitrosation would occur as initial step, the reduced electron density at the indole ring would prevent such oxidative cyclisation. This hypothesis was confirmed in independent reactions of $NO_2^{\bullet}/O_3$ mixtures with N- and C-protected tryptophan, where the indole nitrogen was deactivated by acetylation, and which did not lead to pyrroloindolines (not shown).

Peptide cleavage by reaction of **11** with $NO_2^{\bullet}$ did not occur, which can be rationalized by the fact that $NO_2^{\bullet}$ was rapidly consumed through the reaction with the indole ring in tryptophan.

$NO_2^{\bullet}$ induced peptide cleavage: mechanistic considerations

To our knowledge, the $NO_2^{\bullet}$ induced cleavage of a peptide bond, which was the major pathway in the reaction of dipeptides possessing less oxidisable aromatic side chains, has never been reported before. We believe that this reaction, which releases the C-terminal amino acid with concomitant acyl transfer from the N-terminal amino acid, is not a radical but an ionic process. Scheme 7 outlines a mechanistic rationale for this fragmentation/rearrangement sequence, using the reaction of Phe-Phe (**7**) as example.

N-nitrosation by N_2O_4 at the sterically less hindered *N*-acetyl terminus in **7** occurs in the first step to give *N*-nitrosamide **21**. The electron-withdrawing effect caused by the *N*-nitroso group[14a] leads to considerable activation of the adjacent acetyl moiety and enables intramolecular nucleophilic attack by the nitrogen atom of the dipeptide linkage.



Scheme 7 Proposed mechanism for $NO_2^{\bullet}$ induced peptide cleavage.

The resulting 5-membered ring adduct **22** can rearrange to imide **23** through a concerted hydrogen transfer/fragmentation sequence, followed by formation of the diazo succinimide **24**. M06-2X/cc-pVDZ calculations using *N*-Ac-*N*-NO-Gly-Gly-OMe as simplified model for **21** (data not shown) revealed an activation barrier of some 67 kJ mol^{-1} for the hetero-ene type process **22** → **23**, with the sequence **22** → **24** being overall exothermic by about 55 kJ mol^{-1}, showing that this process is energetically highly feasible. Subsequent hydrolysis of **24** through selective nucleophilic attack at the imide carbonyl group, which is activated by the neighbouring diazo substituent, for example during aqueous work-up, leads to release of the *N*-acetylated amino acid **1a**. Although we were not able to isolate diazo compounds of type **25** as by-products to confirm the proposed mechanism, formation of the latter has been observed in the cleavage of *N*-nitrosopeptides with amines and α-amino esters.[24] Given the high abundance of nucleophilic sites in peptides, our findings show that activation of peptide bonds through reaction with the environmental pollutant $NO_2^{\bullet}$ (or N_2O_4, respectively) may lead to significant structural changes, which could range from rearrangement of the peptide framework to backbone fragmentation.

Conclusion

The present model study revealed that exposure of aromatic N- and C-protected dipeptides to the atmospheric pollutants $NO_2^{\bullet}$ and O_3 results in irreversible oxidative damage at both aromatic side chains as well as the dipeptide linkage. Whereas both $NO_2^{\bullet}$ and O_3 in isolation could not induce oxidation of the aromatic rings in Phe-Phe (**7**) and Phe-OAcTyr (**9**), these two gases in combination led to formation of the isomeric dipeptides **12a/b** and **14a/b** possessing nitrophenylalanine residues. No preference for attack at either the N- or C-terminus was found for the reaction involving dipeptide **7**. The observed strong synergistic effects caused by the combination

** It should be noted that hydrogen abstraction has recently been proposed as initial step in the reaction of indole with $NO_2^{\bullet}$; see: P. Astolfi, M. Panagiotaki, C. Rizzoli and L. Greci, *Org. Biomol. Chem.*, 2006, **4**, 3282.

of $NO_2^{\bullet}$ and O_3 suggests *in situ* generation of $NO_3^{\bullet}$. This highly oxidizing radical is capable to attack even those peptide moieties that are usually inert to damage by endogenous free radical and non-radical oxidants, such as phenylalanine. The $NO_3^{\bullet}$ induced oxidation leads first to a phenylalanyl radical cation of type **3**, which is subsequently trapped by $NO_2^{\bullet}$. If formed in peptides, the phenylalanyl radical cation, which has been shown in transient absorption spectroscopy studies to have a certain lifetime in the absence of $NO_2^{\bullet}$,[15c] would represent a very strong oxidizing site. This might trigger secondary inter- or intramolecular oxidation processes, for example oxidative ET along the peptide chain,[25] by which damage could be induced at positions remote from the initial reaction site.

In the case of dipeptides that consist of two aromatic amino acids with different electron density, oxidative attack occurred selectively at the more electron rich aromatic ring. The reactions of $NO_2^{\bullet}$ with dipeptides containing a tyrosine residue were rapid, both in the presence and absence of O_3, and led to multiple aromatic nitration. It is known from previous work that this radical mediated nitration occurs stepwise *via* formation of 3-nitrotyrosine, which is subsequently transformed into 3,5-dinitrotyrosine.[14a] This clearly shows that the deactivating effect of one nitro group in 3-nitrotyrosine is not sufficient to prevent further oxidation by the $NO_2^{\bullet}/O_3$ system. The reaction involving the tryptophan containing dipeptide **11** with $NO_2^{\bullet}$, both with O_3 present or absent, was also fast and led to the rearranged dipeptide **16**, in which the indole ring system has undergone oxidative cyclization to a pyrroloindoline. This process involves the nitrogen atom of the peptide linkage and leads to a considerable structural change in the dipeptide. In future work we will explore the impact of the reduced flexibility in larger oligopeptides on the reaction outcome. Nevertheless, even if conformational constraints prevent such cyclization, radical cations of type **18**/**18′** formed through exposure of peptides to environmental radical oxidants are easily attacked by any nucleophile present in the system (both intra- and intermolecular), which could lead to significant changes of the peptide structure.

This work also revealed important and, to our knowledge, new aspects of the chemistry of peptides with the pollutant $NO_2^{\bullet}$ and its dimer N_2O_4. While we have found previously that N-nitrosation of the amide nitrogen occurred in isolated amino acids,[14a] this study showed for the first time that amide N-nitrosation triggers peptide bond fragmentation through a non-radical pathway. The extent to which peptide cleavage occurred was dependent on the relative rate of radical oxidation of the aromatic side chains by $NO_2^{\bullet}/O_3$ (or $NO_2^{\bullet}$ alone, respectively) and ionic peptide N-nitrosation by $NO_2^{\bullet}/N_2O_4$. Thus, in the case of dipeptides possessing comparatively unreactive aromatic side chains, such as phenylalanine or *O*-acetyl tyrosine, peptide cleavage was a major pathway, whereas with reactive amino acids (tyrosine, tryptophan), in which the aromatic ring system acts as efficient 'sink' for $NO_2^{\bullet}$, peptide cleavage was largely suppressed. Preliminary studies revealed that $NO_2^{\bullet}/N_2O_4$ induced peptide fragmentation also occurs in tripeptides. These findings clearly indicate that the environmental oxidants $NO_2^{\bullet}$ and O_3, in both isolation and combination, can principally damage peptides to a larger extent than previously believed. It is therefore not unreasonable to suggest that in biological systems oxidative damage in peptides by $NO_2^{\bullet}$ and O_3 may also occur even when only amino acids with 'non-vulnerable' side chains are exposed to these environmental oxidants. We are currently exploring the reaction of the $NO_2^{\bullet}/O_3$ system with different oligopeptides to reveal a deeper understanding of the relative importance of peptide bond cleavage *versus* side chain oxidation in peptide damage, in particular in dependence of the amino acid sequence and concentration of the oxidizing pollutants.

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