ChemPubSoc Europe

DOI: 10.1002/chem.201501850

CHEMISTRY A European Journal Full Paper

Peptides

Fragmentation–Rearrangement of Peptide Backbones Mediated by the Air Pollutant $NO_2^{\bullet}$

Luke F. Gamon,[a] Joses G. Nathanael,[a] Bethany I. Taggert,[a] Fraser A. Henry,[a] Jana Bogena,[a, b] and Uta Wille*[a]

Abstract: The fragmentation–rearrangement of peptide backbones mediated by nitrogen dioxide, $NO_2^{\bullet}$, was explored using di-, tri-, and tetrapeptides **8**–**18** as model systems. The reaction, which is initiated through nonradical N-nitrosation of the peptide bond, shortens the peptide chain by the expulsion of one amino acid moiety with simultaneous fusion of the remaining molecular termini through formation of a new peptide bond. The relative rate of the fragmentation–rearrangement depends on the nature of the amino acids and decreases with increasing steric bulk at the α carbon in the order Gly > Ala > Val. Peptides that possessed consecutive aromatic side chains only gave products that resulted from nitrosation of the sterically less congested N-terminal amide. Such backbone fragmentation–rearrangement occurs under physiologically relevant conditions and could be an important reaction pathway for peptides, in which sections without readily oxidizable side chains are exposed to the air pollutant $NO_2^{\bullet}$. In addition to $NO_2^{\bullet}$-induced radical oxidation processes, this outcome shows that ionic reaction pathways, in particular nitrosation, should be factored in when assessing $NO_2^{\bullet}$ reactivity in biological systems.

Introduction

Recent data from the World Health Organisation suggest that air pollution is responsible for the premature death of about seven million people every year.[1] Promotion of oxidative stress has been identified as one of the most important mechanisms that arises from poisonous air pollutant effects.[2] Nitrogen dioxide, $NO_2^{\bullet}$, is one of the most important toxic, gaseous air pollutants, which is produced through fossil fuel combustion both outdoors (e.g., traffic exhaust) and indoors (e.g., gas stoves, cigarette smoke, etc.). Animal exposure studies showed that high concentrations of $NO_2^{\bullet}$ can lead to damage of the lung, whereas experiments with cell cultures demonstrated that the presence of $NO_2^{\bullet}$ increases the permeability, injury, and death of cells.[3,4] Furthermore, recent studies revealed a correlation between the concentrations of environmental $NO_2^{\bullet}$ and the occurrence of postmenopausal breast cancer and prostate cancer.[5]

The airway surface fluids (ASF) are the first biological fluids that come into contact with air pollutants. In vivo studies showed that exposure to $NO_2^{\bullet}$ resulted in a significant reduction of antioxidant levels in ASF.[6] A weakened defence shield could provide a pathway for environmental oxidants to directly attack proteins and lipids that are present on cell surfaces or in the ASF. This could lead to highly reactive protein and lipid oxidation products that may subsequently damage the underlying epithelial cells, thereby causing inflammation. However, despite considerable efforts, the processes that occur between the initial encounter of ASF constituents with $NO_2^{\bullet}$ and the acute disease state are not fully understood at the molecular level.

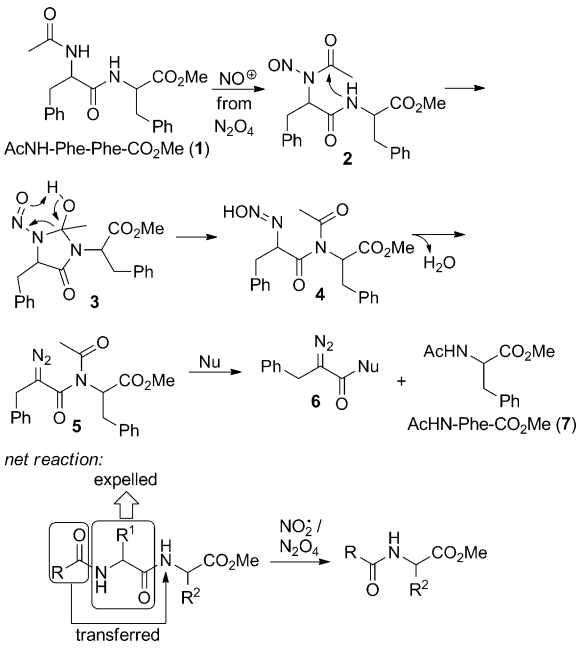
$NO_2^{\bullet}$ is a moderately powerful free-radical oxidant [E^0 ($NO_2^{\bullet}$/NO_2^-) = 1.03 V][7] and is capable of damaging amino acids that contain readily oxidisable side chains.[8] For example, $NO_2^{\bullet}$-induced oxidation of tyrosine or tryptophan leads to the formation of nitrotyrosines or pyrroloindolines, respectively.[9] $NO_2^{\bullet}$ is also an efficient radical trap, whereas its reactions with closed-shell systems through addition or hydrogen atom transfer are actually comparably slow.[10] In lipids that contain activated allylic C–H bonds, hydrogen abstraction is more favourable, which could promote lipid peroxidation.[11]

We recently found that exposure of aromatic dipeptides to $NO_2^{\bullet}$ in acetonitrile could lead to backbone fragmentation through scission of the peptide bond.[9b] This appeared to be the major reaction pathway when the dipeptide side chain was less prone to oxidation, for example phenylalanine.[9b] In dipeptides with reactive aromatic ring systems, such as tyrosine or tryptophan, aromatic oxidation provided a rapid and efficient "sink" for $NO_2^{\bullet}$, with which peptide backbone cleavage could not compete. The proposed mechanism for the $NO_2^{\bullet}$-mediated peptide scission is outlined in Scheme 1 for the dipeptide AcNH–Phe–Phe–CO_2Me (**1**).[9b]

[a] L. F. Gamon, J. G. Nathanael, B. I. Taggert, F. A. Henry, J. Bogena, Prof. U. Wille
School of Chemistry, Bio21 Institute
The University of Melbourne
30 Flemington Road, Parkville, VIC 3010 (Australia)
E-mail: uwille@unimelb.edu.au

[b] J. Bogena
Department of Chemistry
Ludwig-Maximilian Universität München
Butenandtstr. 5-13, 81377 München (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201501850.



Scheme 1. Proposed mechanism for the $NO_2^{\bullet}$-mediated fragmentation–rearrangement of peptide backbones.

It is suggested that the reaction does not directly involve $NO_2^{\bullet}$, but instead the dimer N_2O_4,[12] which is a known non-radical nitrosating agent in solution, is present and exists in both the covalent ($ONONO_2$) and the ionic form ($NO^+NO_3^-$).[13–15] Thus, in the initial step, a (likely reversible)[16] nitrosation of the *N*-acetyl terminus in dipeptide **1** produces *N*-nitrosamide **2**. The electron-withdrawing effect of the *N*-nitroso group[9a] leads to activation of the adjacent acyl moiety and enables nucleophilic, intramolecular cyclization of the peptide nitrogen atom. The resultant five-membered ring adduct **3** rearranges to give the diazotic acid **4** through a concerted hydrogen transfer/fragmentation sequence, which was previously confirmed by computational studies.[9b] We proposed that the latter would subsequently rearrange into the diazo intermediate **5**. The actual peptide bond cleavage would occur in the final step through selective attack of a nucleophile (Nu e.g., water) at the imide carbonyl group in intermediate **5**, which is activated by the neighbouring diazo substituent. This would lead to the release of the *N*- and *C*-protected phenylalanine AcNH–Phe–CO_2Me (**7**) and the diazo compound **6** as a byproduct. Under our experimental conditions, the latter would likely undergo subsequent reactions, which were not further explored in our previous studies.[17]

Overall, the exposure of dipeptides to $NO_2^{\bullet}/N_2O_4$ results in the shortening of the peptide chain by one amino acid moiety with simultaneous acyl migration in the N→C direction. This sequence should, therefore, be considered as a fragmentation–rearrangement. A similar peptide fragmentation has previously been reported by Hood and Johnson et al., who observed multiple backbone scission in polylysine and polyarginine by $NO_2^{\bullet}$ in water that was buffered at pH 7.4.[18] Therefore, we can conclude that $NO_2^{\bullet}$-mediated peptide backbone damage is probably a very general process as it occurs in both organic and aqueous environments, and it could be highly relevant for airway systems that are exposed to traffic-related air pollution.

Owing to the potential implications for respiratory health, a detailed understanding of the fragmentation–rearrangement of peptide backbones by $NO_2^{\bullet}/N_2O_4$ is required, in particular, how this process is affected by steric hindrance at the peptide bond. Therefore, we prepared a series of di-, tri-, and tetrapeptides **8**–**18** (Figure 1), in which the side chains at the α-carbon were: 1) not susceptible to attack by $NO_2^{\bullet}$[8,19] and 2) have different steric demands, and we studied the products that resulted from exposure to the $NO_2^{\bullet}/N_2O_4$ system. All amino acids had L-configuration (where relevant), which is not shown here for clarity.

a) Dipeptides
AcNH-Gly-Phe-CO_2Me (**8**)
AcNH-Ala-Phe-CO_2Me (**9**)
AcNH-Val-Phe-CO_2Me (**10**)

b) Tripeptides
AcNH-Gly-Gly-Phe-CO_2Me (**11**)
PhthN-Gly-Gly-Phe-CO_2Me (**12**)
AcNH-Gly-Ala-Phe-CO_2Me (**13**)
AcNH-Tyr-Phe-(4-AcO)Phe-CO_2Me (**14**)
PhthN-Tyr-Phe-(4-AcO)Phe-CO_2Me (**15**)

c) Tetrapeptides
AcNH-Gly-Gly-Gly-Phe-CO_2Me (**16**)
PhthN-Gly-Gly-Gly-Phe-CO_2Me (**17**)
AcNH-Val-Val-Gly-Phe-CO_2Me (**18**)

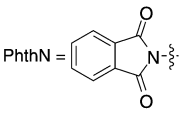


Figure 1. Peptides studied in this work.

As the $NO_2^{\bullet}$-mediated peptide backbone rearrangement proceeds in N→C direction, phenylalanine or 4-acetoxy phenylalanine were installed as the C-terminal amino acids to provide an inert, UV active "marker tag", which enabled detection of the cleavage products by diode array HPLC. To increase their solubility in acetonitrile, all peptides were protected at the C-terminus as methyl esters and at the N-terminus as acetamides or phthalimides.

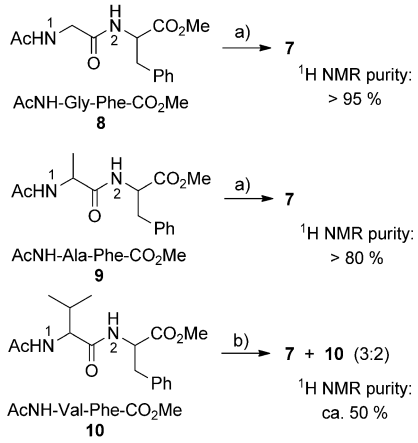
Results and Discussion

The reactions of dipeptides **8**–**18** were carried out in acetonitrile (HPLC grade) in a sealed vessel at 10 °C, to which a measured, excess amount (ca. 10 equiv) of liquid $NO_2^{\bullet}/N_2O_4$ was added.[20,21] After 20 min, the reaction was neutralized with aqueous sodium bicarbonate solution followed by extraction with ethyl acetate.[22] In most cases, consumption of the starting material was complete by this time. Part one of our research focused on exploring the chemoselectivity of the peptide fragmentation–rearrangement; in the second part, we performed mechanistic studies to identify the excised amino acid fragment (see Scheme 1). The composition of the reaction mixtures was analyzed by comparing the ^{1}H NMR spectra and HPLC retention times with authentic samples. In addition, the molecular masses of the reaction products were confirmed by ESI-MS analysis. Product ratios were determined from the

^{1}H NMR spectra of the reaction mixtures by integrating the signals of the amide N–H protons, except for the reaction that involved the tetrapeptide AcNH–Gly–Gly–Gly–Phe–CO_2Me (**16**), in which the product ratios were determined by integration of the methyl protons of the N-terminal acetyl group. Although chemical yields were not determined, we were able to assess the "purity" of the product mixture by ^{1}H NMR spectroscopy. It is important to emphasize that in biological systems even seemingly minor chemical changes can have serious pathological consequences.

1. Fragmentation studies

Exposure of the dipeptides AcNH–Gly–Phe–CO_2Me (**8**) and AcNH–Ala–Phe–CO_2Me (**9**) to $NO_2^{\bullet}/N_2O_4$ resulted in complete consumption of the starting material after 20 min and formation of AcNH–Phe–CO_2Me (**7**) as the only product (Scheme 2).

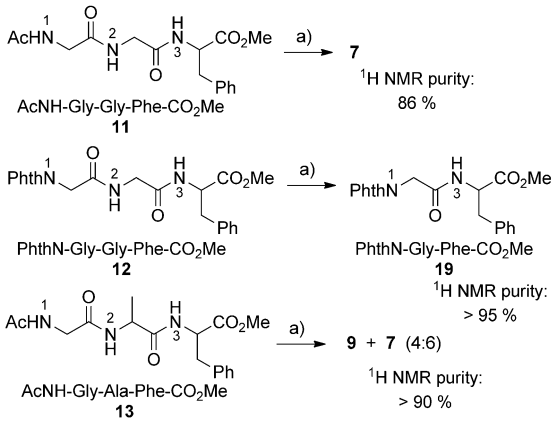


Scheme 2. Reaction of dipeptides **8**–**10** with $NO_2^{\bullet}/N_2O_4$. a) $NO_2^{\bullet}/N_2O_4$, CH_3CN, 10 °C, 20 min, then aq. $NaHCO_3$; b) $NO_2^{\bullet}/N_2O_4$,CH_3CN, 10 °C, 4.5 h, then aq. $NaHCO_3$.

The fragmentation–rearrangement of the peptides **8** and **9** proceeded with high efficiency to give the expected product **7**, which constituted >95% and >80% of the product mixture, respectively, according to ^{1}H NMR analysis. Small amounts of byproducts were formed in the reaction of dipeptide **9**, which could not be identified. The fragmentation was initiated by N-nitrosation of the N-terminus (N-1), followed by nucleophilic cyclization of the N-2 atom onto the acetyl carbonyl group (see Scheme 1). Compared with the sterically unhindered glycine-containing dipeptide **8**, the overall cleavage process in dipeptide **9** was not palpably influenced by the α-methyl group adjacent to the N-1 atom.

In contrast, the bulky isopropyl substituent at the α-carbon that is adjacent to the N-1 atom in AcNH–Val–Phe–CO_2Me (**10**) slows down the cleavage process poconsiderably; no conversion was found after a reaction time of 20 min. Only after the exposure time was extended to 4.5 h did cleavage of dipeptide **10** occur to some extent to give protected phenylalanine **7**. Calculations at the M06-2X/cc-pVDZ level of theory revealed that the branched side chain in valine shields one side of the amide N-1 atom in dipeptide **10** (data not shown). This indicates that steric effects that arise during the initial N-nitrosation most likely control the overall rate of the fragmentation–rearrangement (see below). It should be noted that, under such extended reaction conditions, additional unidentified side reactions occurred to a significant extent, and compounds **7** and **10** only constituted 50% of the product mixture. It is possible that acidification of the reaction system, due partly to the ionic nature of N_2O_4, promoted decomposition of the peptide; in particular, this was observed for the reactions in which the $NO_2^{\bullet}$-mediated peptide fragmentation was slow.

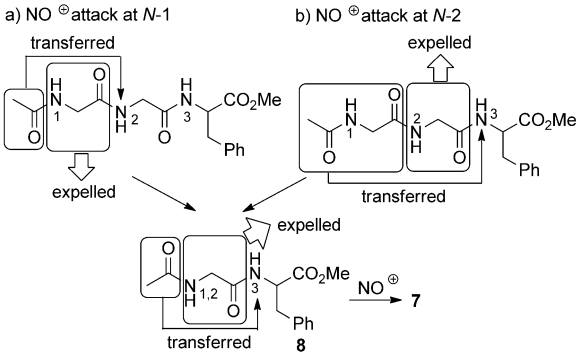
Reaction of $NO_2^{\bullet}/N_2O_4$ with the tripeptide AcNH–Gly–Gly–Phe–CO_2Me (**11**) also led to extensive cleavage of the peptide backbone (Scheme 3). Protected phenylalanine **7** was identified as the major compound in the product mixture (>86% by ^{1}H NMR analysis); the remaining 14% was comprised of three other amide-containing products, which were not identified.



Scheme 3. Reaction of tripeptides **11**–**13** with $NO_2^{\bullet}/N_2O_4$. a) $NO_2^{\bullet}/N_2O_4$,CH_3CN, 10 °C, 20 min, then aq. $NaHCO_3$.

Degradation of tripeptide **11** to give protected phenylalanine **7** required two sequential N-nitrosation–fragmentation–rearrangement sequences via the intermediate dipeptide AcNH–Gly–Phe–CO_2Me (**8**) (Scheme 4). Thus, N-nitrosation could occur either at the N-1 atom (pathway (a)), followed by nucleophilic cyclization of the N-2 atom onto the acetyl carbonyl group, or at the N-2 atom (pathway (b)), followed by nucleophilic cyclization of the N-3 atom onto the glycyl carbonyl group. The latter pathway involves transfer of an *N*-acetyl glycyl moiety onto the C-terminal amino acid through expulsion of the central glycine residue. It should be noted that degradation of tripeptide **11** to give the protected phenylalanine **7** in one step, in which the N-3 atom cyclizes onto the terminal acetyl carbonyl group followed by expulsion of a dipeptide moiety, is unlikely (not shown). Such a process would involve formation of an eight-membered ring intermediate, which is usually energetically unfavourable.

Experimental evidence that the $NO_2^{\bullet}$-mediated backbone fragmentation does not require an accessible N-terminus in the peptide was obtained from exposure of the tripeptide



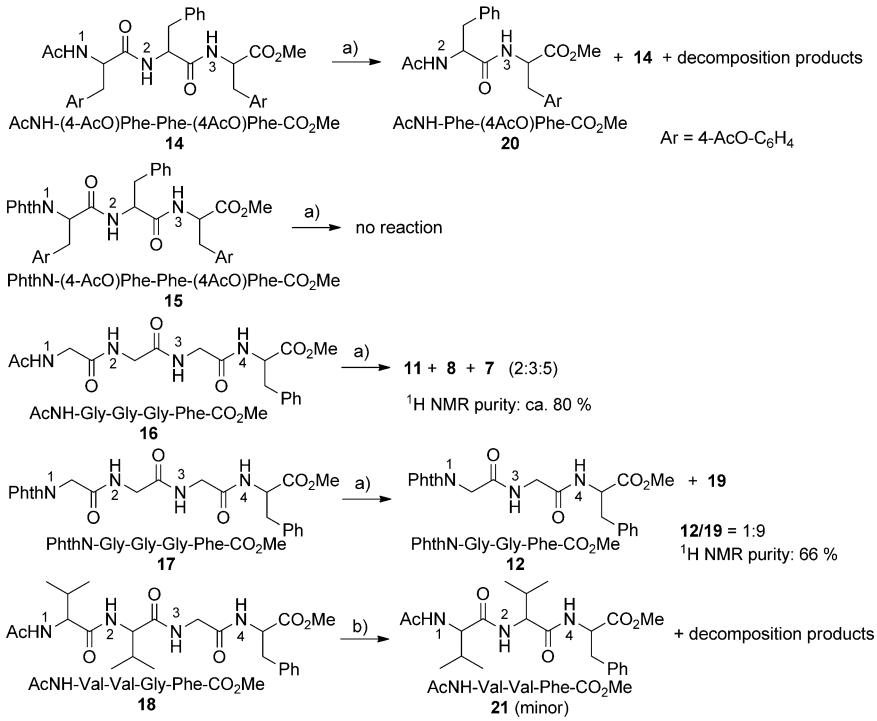
Scheme 4. Degradation pathway for AcNH–Gly–Gly–Phe–CO_2Me (**11**).

PhthN–Gly–Gly–Phe–CO_2Me (**12**) to $NO_2^{\bullet}/N_2O_4$. The N-terminus (N-1) in **12** was protected as a phthalimide group, which cannot be nitrosated. However, fragmentation–rearrangement occurred with high efficiency to yield the dipeptide PhthN–Gly–Phe–CO_2Me (**19**) as the only product (>95% by ^{1}H NMR spectroscopy; crude **19** was isolated in 82% yield); this results from nitrosation of the N-2 atom and transfer of the PhthN–Gly fragment onto the N-3 atom, with expulsion of the central glycine moiety. The finding that entire amino acid residues can be rearranged supports our assumption that, in aliphatic peptides, the rate-determining step is the initial N-nitrosation, which is controlled by steric hindrance, and not the actual rearrangement process, according to **2**→**6**+**7** (see Scheme 1 and the mechanistic studies below). It should also be noted that a further fragmentation–rearrangement of dipeptide **19** by $NO_2^{\bullet}/N_2O_4$ is not possible.

Exposure of the tripeptide AcNH–Gly–Ala–Phe–CO_2Me (**13**) to $NO_2^{\bullet}/N_2O_4$ led to the dipeptide AcNH–Ala–Phe–CO_2Me (**9**) and protected phenylalanine **7** in a 4:6 ratio. Both compounds constituted about 90% of the product mixture (determined by ^{1}H NMR spectroscopy) alongside two unidentifiable byproducts (10%). Dipeptide **9** was formed from N-nitrosation of the N-1 atom (according to pathway (a) in Scheme 4). The presence of the latter in the product mixture could indicate that the α-methyl group of the central alanine residue in tripeptide **13** causes a degree of steric hindrance about the N-2 atom, which slows down the rate of degradation.

We believe that increased steric bulk and/or reduced flexibility (likely due to intra- and intermolecular interactions, such as π stacking) could be the reason for the enhanced stability of the aromatic tripeptides AcNH–(4-AcO)Phe–Phe–(4-AcO)Phe–CO_2Me (**14**) and PhthN–(4-AcO)Phe–Phe–(4-AcO)Phe–CO_2Me (**15**) towards $NO_2^{\bullet}$-mediated backbone fragmentation–rearrangement (Scheme 5). According to HPLC analysis, tripeptide **14** undergoes incomplete degradation to give the dipeptide AcNH–Phe–(4-AcO)Phe–CO_2Me (**20**), which results from elimination of the N-terminal amino acid residue ((4-AcO)Phe) following N-nitrosation of the less hindered amide, for example the N-1 atom (pathway (a) in Scheme 4). Degradation products that result from nitrosation of the less accessible N-2 atom (pathway (b) in Scheme 4) were not found. The product ratio could not be determined by ^{1}H NMR spectroscopy because of the incomplete consumption of substrate **14** and the significant amount of decomposition products that were formed during this reaction.

Nitrosation of the phthaloyl-protected tripeptide **15** at the N-1 position was not possible, and **15** was found to be inert to $NO_2^{\bullet}$-mediated fragmentation under the experimental conditions. It is not possible to state whether the high stability of **15** towards backbone rearrangement is due to the steric hindrance at the peptide bonds, which prevents N-nitrosation, and/or to a reduced conformational flexibility, which could inhibit the actual rearrangement process. However, it is possible that, by elongating the exposure time, some fragmentation might occur, which is similar to what was found for the valine-containing dipeptide **10** (see Scheme 2).



Scheme 5. Reaction of tripeptides **14**–**15** and tetrapeptides **16**–**18** with $NO_2^{\bullet}/N_2O_4$. a) $NO_2^{\bullet}/N_2O_4$,CH_3CN, 10 °C, 20 min, then aq. $NaHCO_3$; b) $NO_2^{\bullet}/N_2O_4$, CH_3CN, 10 °C, 4.5 h, then aq. $NaHCO_3$.

Exposure of tetrapeptide AcNH–Gly–Gly–Gly–Phe–CO_2Me (**16**) to $NO_2^{\bullet}/N_2O_4$ produced three cleavage products: tripeptide AcNH–Gly–Gly–Phe–CO_2Me (**11**), dipeptide AcNH–Gly–Phe–CO_2Me (**8**), and protected phenylalanine **7**, in a 2:3:5 ratio, respectively (Scheme 5). ^{1}H NMR analysis showed that these contributed to about 80% of the product mixture, whereas the remaining 20% constituted various degradation products, which could not be identified.

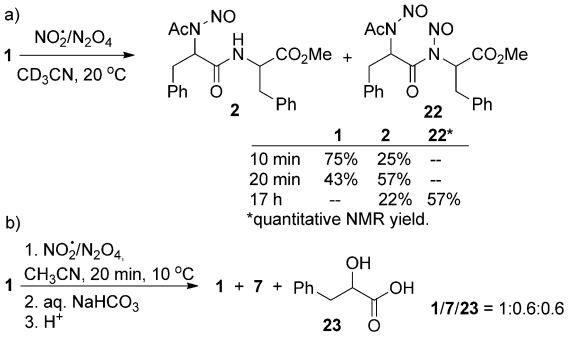
Tripeptide **11** resulted from one single N-nitrosation process (at either the N-1, N-2, or N-3 atom), whereas the shorter products required several successive N-nitrosation and fragmentation–rearrangement sequences. This caused depletion of the $NO_2^{\bullet}/N_2O_4$ concentration before the fragmentation of tetrapeptide **16** to give protected phenylalanine **7** was quantitative, and it explains the mixture of shorter peptides that was obtained in this reaction.

As expected, protection of the N-terminus as phthalimide reduced the number of fragmentation–rearrangement sequences. Exposure of tetrapeptide PhthN–Gly–Gly–Gly–Phe–CO_2Me (**17**) to $NO_2^{\bullet}/N_2O_4$ led to the tripeptide PhthN–Gly–Gly–Phe–CO_2Me (**12**), which resulted from nitrosation of the N-2 or N-3 atom, and dipeptide PhthN–Gly–Phe–CO_2Me (**19**), which was formed through nitrosation of product **12** at the N-3 atom.

Steric hindrance at the N-1 and N-2 positions increases the resistance of the tetrapeptide AcNH–Val–Val–Gly–Phe–CO_2Me (**18**) towards backbone rearrangement; consequently, no reaction occurred within 20 min of exposure to $NO_2^{\bullet}/N_2O_4$. However, the high-resolution mass spectrum of the crude reaction mixture, which was obtained after a reaction time of 4.5 h, indicated the formation of the tripepeptide AcNH–Val–Val–Phe–CO_2Me (**21**) as a minor component of a complex mixture of decomposition products (HRMS, ESI) for tripeptide **21**: *m/z* calcd. for $C_{22}H_{34}N_3O_5$ $[M+H]^+$: 420.2493, found: 420.2498; *m/z* calcd. for $C_{22}H_{33}NaN_3O_5$ $[M+Na]^+$: 442.2312, found: 442.2312). Tripeptide **21** was formed from tetrapeptide **18** by N-nitrosation of the sterically least hindered peptide bond (N-3) and expulsion of the glycine residue.

2. Mechanistic studies

To identify the chemical nature of the excised amino acid moiety, we performed independent mechanistic investigations on the previously studied reaction of dipeptide **1**[9b] with an excess of $NO_2^{\bullet}/N_2O_4$ in [D_3]acetonitrile, but the reaction mixture was not neutralized. Quantitative ^{1}H NMR analysis of the reaction progress at different times (at 20 °C, using dimethyl terephthalate as internal standard) revealed that N-nitrosation of **1** occurred rapidly at the less hindered N-terminal amide and led to the mono-nitrosated dipeptide **2** in 57% yield (determined by ^{1}H NMR spectroscopy) after 20 min (Scheme 6a). No peptide fragmentation to produce the protected phenylalanine **7** occurred; elongation of the reaction time only resulted in exhaustive N-nitrosation, which included reaction of the sterically more hindered "internal" amide bond. Thus, the reaction mixture, after 17 h of exposure to $NO_2^{\bullet}/N_2O_4$, contained only the bis-nitrosated dipeptide **22** and the mono-nitrosated **2** in 57 and 22% yield, respectively (determined by ^{1}H NMR spectroscopy), in addition to some unidentifiable decomposition products. The complete absence of protected phenylalanine **7** in the ^{1}H NMR spectrum of the product mixture, even after prolonged reaction with $NO_2^{\bullet}/N_2O_4$, is a clear indication of the high stability of N-nitrosated peptides in an acidic, non-aqueous environment, which is provided by $NO_2^{\bullet}/N_2O_4$ in acetonitrile. Therefore, we can conclude that fragmentation of the N-nitrosated peptide backbone requires an aqueous environment at a physiologically-relevant[22] near-neutral pH, which can be achieved by neutralizing the reaction system with aqueous sodium bicarbonate.

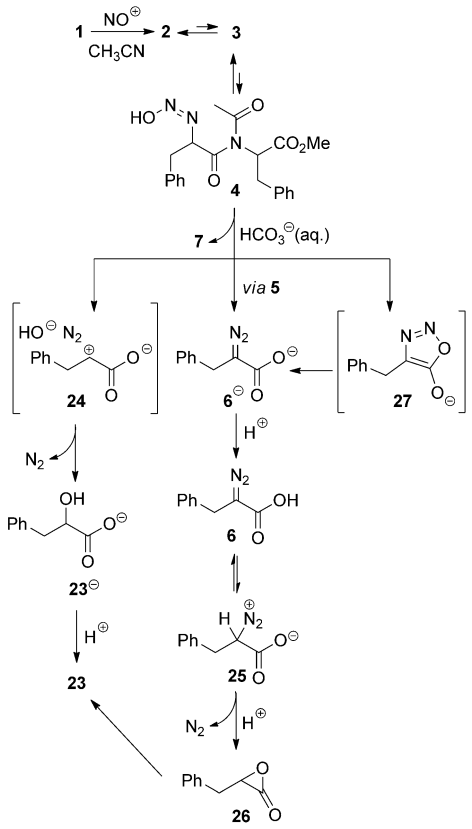


Scheme 6. Mechanistic studies of the reaction of dipeptide **1** with $NO_2^{\bullet}/N_2O_4$.

Because the ^{1}H NMR data of the fragmentation studies in part one of our research did not reveal any product signals that could be traced back to the excised amino acid, it is likely that this byproduct was extracted into the aqueous phase upon neutralization. Therefore, we performed a reaction in which dipeptide **1** was treated with excess $NO_2^{\bullet}/N_2O_4$ in acetonitrile for 20 mins. The reaction mixture was neutralized with aqueous sodium bicarbonate and subsequently acidified with hydrochloric acid (1 M), followed by extraction with ethyl acetate (Scheme 6b). ^{1}H NMR analysis of the resultant product mixture showed, as well as unconsumed dipeptide **1**, the expected phenylalanine derivative **7** and a second product formed in equal amount, which could be unequivocally identified as 2-hydroxy-3-phenyl propionic acid (**23**) by comparison with literature data.[23] The latter byproduct likely relates to the expelled amino acid moiety.

Based on these findings, we have proposed a revised mechanism for the $NO_2^{\bullet}$ mediated fragmentation–rearrangement, which is shown in Scheme 7, by using the reaction of dipeptide **1** as an example.

Thus, under non-aqueous conditions, the *N*-nitroso peptide **2**, the cyclized nitroso amide **3**, and the rearranged diazotic acid **4** exist in equilibrium, which lies on the side of peptide **2**. Upon neutralization in an aqueous environment, irreversible decomposition of compound **4** to give the protected phenylalanine **7** and the α-hydroxylated acid **23** occurs, which could proceed through various pathways. Thus, as outlined in Scheme 1, intermediate **4** could rearrange to give α-diazo imide **5**; subsequent regioselective imide hydrolysis and release of the protected phenylalanine **7** produced the α-diazo acid anion **6**$^-$. The latter could also be formed through intra-



Scheme 7. Revised mechanism for the $NO_2^{\bullet}$-mediated fragmentation–rearrangement of peptide backbones.

molecular acyl substitution in **4** via a (likely highly unstable) 1,2,3-oxadiazole intermediate **27** to give **7**, followed by tautomerization.[24] The α-diazo acid anion **6**$^-$ would be expected to remain in the aqueous phase during extraction of the reaction mixture with ethyl acetate. However, under the conditions that are shown in Scheme 6 b, acidification liberates the diazo acid **6**, which tautomerizes to the zwitterionic diazonium carboxylate **25**. Protonation of the latter initiates a cyclization to give a strained α-lactone **26** and the release of nitrogen,[25] which is followed by ring opening to give compound **23**. Alternatively, in protic solvents, diazotic acids of type **4** are known to undergo hydrolytic decomposition to the corresponding alcohols through a nitrogen-separated ion pair.[26] Activation by the positive charge in the α position relative to the imide carbonyl group enables regioselective cleavage of the imide C–N bond and formation of the ion pair **24**, which collapses/hydrolyses to the hydroxyphenyl propionate **23**$^-$. The latter is water-soluble and would be separated from the peptide products during extraction of the neutralized reaction mixture with ethyl acetate, in accordance with our experimental observations.

Conclusion

The air pollutant $NO_2^{\bullet}$ mediates the cleavage of peptide backbones through a fragmentation–rearrangement mechanism, which is strongly influenced by the steric hindrance at the peptide bond. The reaction proceeds by initial nitrosation of the peptide nitrogen atom by an ionic reaction, in which the dimer of $NO_2^{\bullet}$ (i.e., N_2O_4) acts as the nitrosating agent. By using various di-, tri-, and tetrapeptide model systems, it was shown that the relative rate of the fragmentation–rearrangement depends on the nature of the amino acids and decreases with increasing steric bulk at the α carbon in the order Gly > Ala > Val. This suggests that N-nitrosation of the peptide bond controls the overall rate of the rearrangement process in peptides with aliphatic side chains. In the case of peptides with a number of consecutive aromatic side chains, their considerable resistance towards $NO_2^{\bullet}$ mediated backbone cleavage could be due to an increased steric hindrance and/or conformational rigidity that might be caused by intra- and intermolecular interactions. This likely affects both the N-nitrosation and the subsequent nucleophilic cyclization that leads to the rearranged molecular framework. It could be hypothesized that, in larger polypeptides, activation of a peptide bond through N-nitrosation might also trigger intramolecular nucleophilic attack of even remote acyl groups that become proximate through the secondary or tertiary structure. Future work that involves more complex polypeptides, for example gramicidin, will reveal further insight into how the structure of the peptide influences this rearrangement process.

The mechanistic studies clearly showed that the $NO_2^{\bullet}$-mediated peptide fragmentation–rearrangement requires an aqueous environment at a physiologically relevant, near-neutral pH. In the absence of water and at the acidic pH caused by the $NO_2^{\bullet}/N_2O_4$ system, only N-nitrosation of the peptide bonds occurs. Thus, the experimental conditions that we employed in this work act as a suitable model to simulate biological conditions, in which local acidification in the ASF, which was caused by exposure to environmental $NO_2^{\bullet}/N_2O_4$ would be neutralized by the extracellular buffer system, such as the bicarbonate buffer.

The fragmentation–rearrangement sequence contracts the peptide chain in the N→C direction by expelling one amino acid moiety with simultaneous fusion of the remaining molecular termini, thereby forming a new peptide bond. Basically, this process could repeat over and over until "accessible" peptide bonds are no longer available and/or the supply of $NO_2^{\bullet}/N_2O_4$ is exhausted, thereby successively shortening the peptide chain. Therefore, apart from the various biological implications of such peptide fragmentation-rearrangements by $NO_2^{\bullet}/N_2O_4$, this reaction potentially provides new pathways to peptide sequences that cannot otherwise be readily accessed. We will report on synthetic applications of this method in due course.

The outcomes from this work provide clear evidence that the environmental pollutant $NO_2^{\bullet}$ can principally damage peptides through multiple pathways. In addition to the well-known $NO_2^{\bullet}$-induced radical oxidation of peptide side chains,[8,9] exposure to $NO_2^{\bullet}/N_2O_4$ also damages the peptide backbone through a non-radical process. This is contrary to the generally accepted view that oxidative stress that results from environmental free radical oxidants, in particular damage that leads to peptide backbone cleavage, proceeds largely through radical pathways.[27]

Our earlier findings showed that oxidation of aromatic side chains by $NO_2^{\bullet}$ is considerably faster than the $NO_2^{\bullet}$-mediated peptide cleavage.[9b] Therefore, we propose that, in the ASF, the latter pathway could potentially be highly relevant for peptides in which segments of "non-vulnerable" side chains are exposed to the environment. Because $NO_2^{\bullet}$ has only moderate solubility in water [$K_H(NO_2^{\bullet}) = 1.2 \times 10^{-2}\,M\,atm^{-1}$, at 298 K),[28] there is considerable interest in understanding why $NO_2^{\bullet}$ has such a pronounced toxicity in vivo. In fact, it has recently been suggested that $NO_2^{\bullet}$ absorption in the ASF is supported by antioxidants, which catalyze its hydrolytic disproportionation to NO^+ and NO_3^-.[29] This demonstrates that ionic reaction pathways, in particular nitrosation, need to be included in the considerations about the reactivity of $NO_2^{\bullet}$ with biological molecules.

Experimental Section

General

The *N*- and *C*-protected peptides **8**–**18** were obtained according to literature coupling procedures.[9b] $NO_2^{\bullet}$ was prepared by the reaction of Cu metal with concentrated nitric acid (70%) under a stream of oxygen,[15] and it was collected at −10 °C. The reactions were carried out at 10 °C in a sealed vessel by adding liquid $NO_2^{\bullet}$/N_2O_4 (0.5 mL) to a solution of the peptide in acetonitrile. Consumption of the substrates was usually complete after 20 min. The reaction was neutralized by the addition of aqueous sodium bicarbonate solution, the acetonitrile was removed in vacuo, and the aqueous phase was extracted with ethyl acetate. The combined organic fractions were dried over magnesium sulphate and concentrated, and the crude mixture was analyzed by reverse-phase HPLC, ^{1}H NMR spectroscopy, and ESI-MS. Full experimental and spectroscopic details are given in the Supporting Information.

Cautionary Note: Many nitrosamines are potent carcinogens. Proper precautions against inhalation of the vapours and contact with skin should always be maintained.

Acknowledgements

Financial support by the Australian Research Council (Centre of Excellence Scheme) and The University of Melbourne is gratefully acknowledged. We thank the Australian Government for an Australian Postgraduate Award (LFG) and an International Postgraduate Research Scholarship (JGN).

Keywords: nitrogen oxides · peptides · radicals · reaction mechanism · rearrangement

[1] a) R. T. Burnett, C. A. Pope III, M. Ezzati, C. Olives, S. S. Lim, S. Mehta, H. H. Shin, G. Singh, B. Hubbell, M. Brauer, H. R. Anderson, K. R. Smith, J. R. Balmes, N. G. Bruce, H. Kan, F. Laden, A. Prüss-Ustün, M. C. Turner, S. M. Gapstur, W. R. Diver, A. Cohen, *Environ. Health Perspect.* **2014**, *122*, 397–403; b) K. R. Smith, N. Bruce, K. Balakrishnan, H. Adair-Rohani, J. Balmes, Z. Chafe, M. Dherani, H. D. Hosgood, S. Mehta, D. Pope, E. Rehfuess, *Ann. Rev. Publ. Health* **2014**, *35*, 185–206 .

[2] a) M. Lodovici, E. Bigagli, *J. Toxicol.* **2011**, *2011*, 1–9; b) U. Pöschl, M. Shiraiwa, *Chem. Rev.* **2015**, *115*, 4440–4475.

[3] *Air Pollution and Health* (Eds.: S. T. Holgate, J. M. Samet, H. S. Koren, R. L. Maynard), Academic Press, London, **1999**.

[4] a) M. G. Mustafa, N. M. Elsayed, F. M. von Dohlen, C. M. Hassett, E. M. Postlethwait, C. L. Quinn, J. A. Graham, D. E. Gardner, *Toxicol. Appl. Pharmacol.* **1984**, *72*, 82–90; b) T. T. Gelzleichter, H. P. Witschi, J. A. Last, *Toxicol. Appl. Pharmacol.* **1992**, *112*, 73–80; c) P. Rajini, T. R. Gelzleichter, J. A. Last, H. P. Witschi, *Toxicol. Appl. Pharmacol.* **1993**, *121*, 186–192; d) J. A. Last, W. M. Sun, H. P. Witschi, *Environ. Health Perspect.* **1994**, *102*, 179–184; e) R. L. Persinger, M. E. Poynter, K. Ckless, Y. M. Janssen-Heininger, *Mol. Cell. Biochem.* **2002**, *234–235*, 71–80; f) R. L. Persinger, W. M. Blay, N. H. Heintz, D. R. Hemenway, Y. M. Janssen-Heininger, *Am. J. Respir. Cell Mol. Biol.* **2001**, *24*, 583–590.

[5] a) D. L. Crouse, M. S. Goldberg, N. A. Ross, H. Chen, F. Labrèche, *Environ. Health Perspect.* **2010**, *118*, 1578–1583; b) M. E. Parent, M. S. Goldberg, D. L. Crouse, N. A. Ross, H. Chen, M. F. Valois, A. Liautaud, *Occup. Environ. Med.* **2013**, *70*, 511–518.

[6] F. J. Kelly, *Occup. Environ. Med.* **2003**, *60*, 612–616.

[7] R. E. Huie, P. Neta, *J. Phys. Chem.* **1986**, *90*, 1193–1198.

[8] M. J. Davies, R. T. Dean, in: *Radical-Mediated Protein Oxidation*. Oxford University Press, Oxford, **1999**.

[9] a) C. Goeschen, N. Wibowo, J. M. White, U. Wille, *Org. Biomol. Chem.* **2011**, *9*, 3380–3385; b) L. F. Gamon, J. M. White, U. Wille, *Org. Biomol. Chem.* **2014**, *12*, 8280–8287.

[10] R. E. Huie, *Toxicology* **1994**, *89*, 193–216.

[11] W. A. Pryor, J. W. Lightsey, *Science* **1981**, *214*, 435–437.

[12] N. S. Bryan, T. Rassaf, R. E. Maloney, C. M. Rodriguez, F. Saijo, J. R. Rodriguez, M. Feelisch, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4308–4313.

[13] a) R. W. Darbeau, R. S. Pease, E. V. Perez, *J. Org. Chem.* **2002**, *67*, 2942–2947; b) M. Shiri, M. A. Zolfigol, H. G. Kruger, Z. Tanbakouchian, *Tetrahedron* **2010**, *66*, 9077–9106.

[14] *S*-Nitrosation was reported in the reaction of N_2O_3 with sulphur containing amino acids: L. J. Marnett, J. N. Riggins, J. D. West, *J. Clin. Invest.* **2003**, *111*, 583–593.

[15] P. Gray, A. D. Yoffe, *Chem. Rev.* **1955**, *55*, 1069–1154.

[16] T. P. Suntsova, N. V. Beda, A. A. Nedospasov, *Life* **2002**, *54*, 281–292.

[17] J. Garcia, J. Gonzalez, R. Segura, J. Vilarrasa, *Tetrahedron* **1984**, *40*, 3121–3127.

[18] D. B. Hood, P. Gettins, D. A. Johnson, *Arch. Biochem. Biophys.* **1993**, *304*, 17–26.

[19] M. J. Davies, *BBA Proteins Proteom.* **2005**, *1703*, 93–109.

[20] It cannot be excluded that small amounts of N_2O_3 were also present under our experimental conditions.

[21] *CRC Handbook of Chemistry and Physics* (Ed. W. M. Haynes), 95th edition, CRC Press, **2014**, p. 4–79.

[22] This procedure mimics conditions in biological systems, where a pH of 7.65 ± 0.20 has been determined in airway surface fluids of healthy humans: J. F. Hunt, K. Fang, R. Malik, A. Snyder, N. Malhotra, T. A. Platts-Mills, B. Gaston, *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 694–699.

[23] G. DeSantis, Z. Zhu, W. A. Greenberg, K. Wong, J. Chaplin, B. Farwell, L. W. Nicholson, C. L. Rand, D. P. Weiner, D. E. Robertson, M. J. Burk, *J. Am. Chem. Soc.* **2002**, *124*, 9024–9025.

[24] M. T. Nguyen, A. F. Hegarty, J. Elguero, *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 713–715; *Angew. Chem.* **1985**, *97*, 704–705.

[25] R. W. Lamson, *J. Bacteriol.* **1924**, *9*, 307–313.

[26] a) B. Gold, A. Deshpande, W. Linder, L. Hines, *J. Am. Chem. Soc.* **1984**, *106*, 2072–2077; b) J. I. Finneman, J. Ho, J. C. Fishbein, *J. Am. Chem. Soc.* **1993**, *115*, 3016–3017.

[27] a) B. S. Berlett, E. R. Stadtman, *J. Biol. Chem.* **1997**, *272*, 20313–20316; b) M. J. Davies, *Aust. Biochem.* **2012**, *43*, 8–12.

[28] W. L. Chameides, *J. Geophys. Res. Atmos.* **1984**, *89*, 4739–4755.

[29] S. Enami, M. R. Hoffmann, A. J. Colussi, *J. Phys. Chem. A* **2009**, *113*, 7977–7981.

Received: May 11, 2015
Published online on September 1, 2015