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Reaction of Amino Acids, Di- and Tripeptides with the Environmental Oxidant NO_3^\cdot : A Laser Flash Photolysis and Computational Study

Joses G. Nathanael, Amber N. Hancock and Uta Wille*^[a]

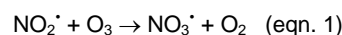
Abstract: Absolute rate coefficients for the reaction between the important environmental free radical oxidant NO_3^\cdot and a series of *N*- and *C*-protected amino acids, di- and tripeptides were determined using 355 nm laser flash photolysis of cerium(IV) ammonium nitrate in the presence of the respective substrates in acetonitrile at 298 ± 1 K. Through combination with computational studies it was revealed that the reaction with acyclic aliphatic amino acids proceeds through hydrogen abstraction from the α -carbon, which is associated with a rate coefficient of about $1.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ per abstractable hydrogen atom. The considerably faster reaction with phenylalanine [$k = (1.1 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$] is indicative for a mechanism involving electron transfer. An unprecedented amplification of the rate coefficient by a factor of 7-20 was found with di- and tripeptides that contain more than one phenylalanine residue. This suggests a synergistic effect between two aromatic rings in close vicinity, which makes such peptide sequences highly vulnerable to oxidative damage by this major environmental pollutant.

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^\cdot) and ozone (O_3) are amongst the most important gaseous pollutants both out- and indoor, which mainly result from combustion processes, for example in automotive engines. Inhalation of these gases, which is the primary route of exposure, is associated with the development of respiratory tract inflammation.^[1,2] The airway surface fluids (ASF), which are the first biological material that come into contact with environmental pollutants, contain small molecular-weight antioxidants, i.e., glutathione, ascorbic and uric acid, that protect the underlying epithelial cells against oxidative damage. However, when exposure to environmental oxidants exceeds the capacity of the antioxidant defense system, proteins and lipids present on cell surfaces or in the ASF are attacked.^[3] The resulting oxidation products could damage the epithelial cells through secondary reactions that may lead to inflammation in a subsequent step.

Generally, reactions of NO_2^\cdot or O_3 with lipids occur at the π

system of unsaturated fatty acids through allylic hydrogen atom abstraction, HAT (by NO_2^\cdot), or oxidation (by O_3), respectively. With peptides both NO_2^\cdot and O_3 attack readily oxidizable side chains, for example those present in tyrosine, tryptophan or methionine.^[4-7] In the polluted ambient atmosphere NO_2^\cdot and O_3 always coexist, and significant synergistic effects upon exposure to $\text{NO}_2^\cdot/\text{O}_3$ mixtures have been found, such as increased lipid peroxidation and protein nitration.^[8,9] The more than additive response suggests that highly electrophilic nitrate radicals, NO_3^\cdot , might be involved in these processes, which are formed through reaction of NO_2^\cdot with O_3 (eqn. 1).^[10]



The observed synergism was originally rationalized by the toxicity of dinitrogen pentoxide (N_2O_5) that results from recombination of NO_2^\cdot and NO_3^\cdot , as well as its hydrolysis product nitric acid (HNO_3), but detailed mechanistic studies were not performed.^[1,11] However, since NO_3^\cdot is strongly oxidising [$E^0(\text{NO}_3^\cdot/\text{NO}_3^-) = 2.3 - 2.5 \text{ V vs. NHE}$]^[12] and reacts with organic compounds also through HAT and addition to π systems, it is reasonable to suggest that the synergistic effects could be due to reactions directly involving NO_3^\cdot .^[9,10] Because of the complex network of reactions occurring between the numerous components in the atmosphere, identification of adverse health effects caused by individual pollutants is a challenging task. In fact, despite considerable efforts, the mechanism that leads from the initial pollutant exposure to the actual disease state is still not well understood.

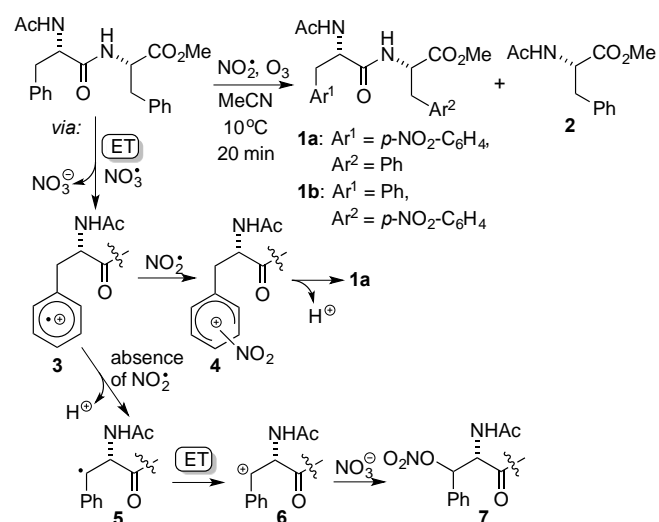
We recently initiated a research program to investigate the reaction of NO_3^\cdot with biological molecules present in the ASF. Our *in vitro* model studies revealed that exposure of *N*- and *C*-protected aromatic amino acids and dipeptides, for example diphenylalanine (AcNH-Phe-Phe-OMe), to $\text{NO}_2^\cdot/\text{O}_3$ mixtures leads to nitration of the aromatic ring and formation of 4-nitrophenylalanine residues in dipeptide **1** (Scheme 1).^[13]

In addition, cleavage of the peptide bond also occurred. Closer inspection showed that latter reaction, which leads to release of the *C*-terminal amino acid **2**, is a fragmentation-rearrangement sequence that can be accounted to the NO_2^\cdot dimer N_2O_4 , which acts as a non-radical nitrosating agent.^[14] On the other hand, NO_2^\cdot does not react with the aromatic ring in the absence of O_3 . Conversely, when AcNH-Phe-Phe-OMe was treated with O_3 in the absence of NO_2^\cdot , no reaction took place. Thus, the nitrophenylalanine residues in **1** clearly result from combined $\text{NO}_2^\cdot/\text{O}_3$ exposure, suggesting *in situ* formation of NO_3^\cdot . The experimental findings to date support the mechanism shown in Scheme 1, which involves NO_3^\cdot initiated oxidative electron transfer (ET) at one of the aromatic rings in AcNH-Phe-

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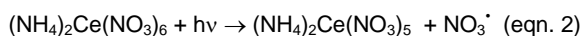
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Phe-OMe, followed by trapping of the aryl radical cation **3** with NO_2^+ and subsequent deprotonation of **4**. In the absence of NO_2^+ (see below), **3** undergoes loss of a benzylic proton, which restores the aromatic π system. The resulting radical **5** undergoes a number of transformations, which lead to a β -nitrooxy amino acid **7** and other oxidation products (not shown).^[15] However, it should be noted that a possible alternative pathway to **5** may proceed through benzylic hydrogen abstraction by $\text{NO}_3^{\cdot-}$ (not shown).



Scheme 1. Reaction of *N*- and *C*-protected diphenylalanine (AcNH-Phe-Phe-OMe) with NO_2^+/O_3 mixtures in acetonitrile.

While identification of reaction products enables to evaluate the toxicity of the particular environmental pollutant, kinetic data provide the opportunity to assess the potential relevance of individual reactions in biological systems. In the only existing kinetic study of the reaction of $\text{NO}_3^{\cdot-}$ with peptide constituents to date absolute rate coefficients were determined for a series of α -, β - and γ -amino acids.^[16] $\text{NO}_3^{\cdot-}$ was generated through flash photolysis of cerium(IV) ammonium nitrate (CAN) in 6 M HNO_3 via a photo-induced electron transfer at $\lambda > 300$ nm, according to eqn. 2.^[10,16,17]



The considerably higher rate coefficients obtained for the reaction of $\text{NO}_3^{\cdot-}$ with aromatic amino acids compared to those determined for aliphatic amino acids [$k = (1.5 - 3.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ vs. $k = (1.5 - 6500) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$] were taken as an indication for different reaction mechanisms, i.e., initial ET in the case of aromatic amino acids, whereas the aliphatic amino acids react through a notably less facile HAT.^[16]

In order to consolidate our previous product analyses, we have now performed a laser flash photolysis study to obtain absolute rate coefficients for the reaction of $\text{NO}_3^{\cdot-}$ with a series of L-amino acids, di- and tripeptides in acetonitrile (Figure 1). All substrates used in this work were protected at the *C*-terminus as

methyl ester and acetylated at the *N*-terminus in order to mimic an extended peptide structure. The experimental kinetic studies are augmented by density functional theory (DFT) computations to gain additional mechanistic understanding of these reactions.

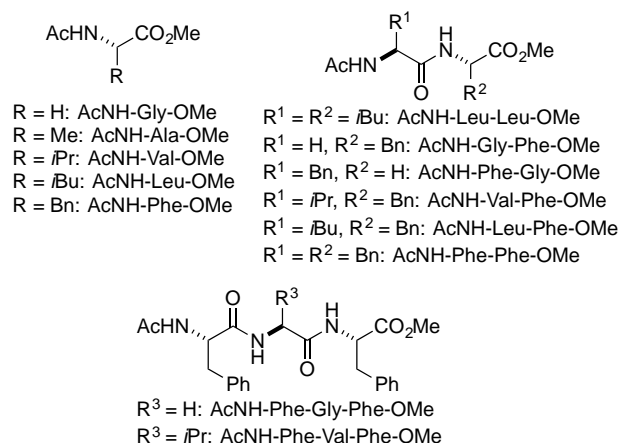


Figure 1. Amino acids, di- and tripeptides studied in this work.

Results and Discussion

Synthesis of the starting materials

The *N*- and *C*-protected L-amino acids, di- and tripeptides were obtained using standard solution-phase synthetic methodology, followed by purification through column chromatography and characterisation by ^1H and ^{13}C NMR spectroscopy and HRMS analysis. The purity was checked by analytical HPLC prior to the laser flash photolysis experiments. Experimental conditions and spectroscopic details are provided in the Supporting Information.

Laser flash photolysis studies

All experiments were performed at 298 ± 1 K on an Edinburgh Instruments LP920 spectrometer using the third harmonic of a Quantel Brilliant B Nd:YAG laser (10 ns pulse, 10 – 17 mJ, $\lambda = 355$ nm)^[18] to generate the reaction transient. Details are given in the Supporting Information and in ref. [19].

CAN (Ajax Finechem, 98.5%) was purified by dissolving in acetonitrile (Honeywell B&J, HPLC grade) and filtering through neutral alumina. The solvent was removed under reduced pressure and the resulting light orange solid was stored in the freezer, protected from ambient light, until used. Stock solutions of 0.66 ± 0.06 mM of CAN and of the amino acids and peptides (in the range 2 – 99 mM, depending on the nature of the substrate) in HPLC grade acetonitrile were prepared. These amino acid/peptide stock solutions were diluted to obtain a series of five reactant solutions with different concentrations. For the laser flash photolysis experiments 5 mL of the CAN stock solution were mixed in an amber volumetric flask (for ambient light protection) with 5 mL of the substrate solution.^[20]

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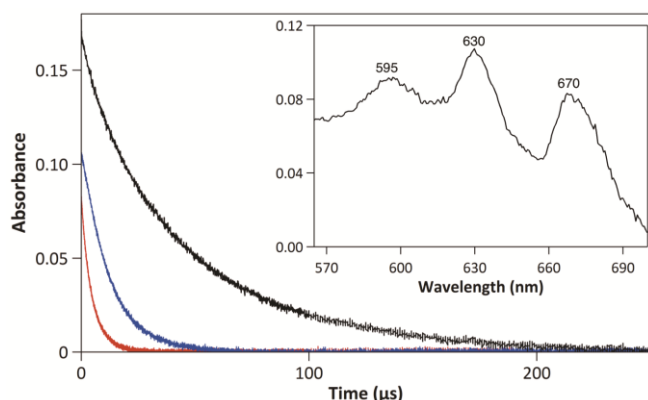


Figure 2. Time-dependent decay of the NO_3^\bullet signal at 630 nm in acetonitrile ($[\text{CAN}] = 0.33 \text{ mM}$) in the absence (black line) and presence of 15.8 mM AcNH-Gly-OMe (blue line) and 16.5 mM AcNH-Phe-OMe (red line), respectively. Inset: NO_3^\bullet absorption spectrum 10 μs after the laser flash.

The absorption spectrum of the transient species formed through photolysis of a solution of $0.33 \pm 0.03 \text{ mM}$ CAN in acetonitrile showed three peaks in the visible region at $\lambda = 595$, 630 and 670 nm with the largest absorbance at 630 nm (see inset in Figure 2). This spectrum agrees well with the literature and indicates successful formation of NO_3^\bullet through the reaction in eqn. 2.^[16,17,21] Using the extinction coefficient of $\epsilon = 1350 \text{ M}^{-1} \text{ cm}^{-1}$ at 630 nm,^[17b] NO_3^\bullet concentrations of $80 \pm 20 \mu\text{M}$ were obtained. All rate data in this work were determined from the time-dependent decay of the signal at 630 nm. In order to minimise effects resulting from photobleaching [the Ce(III) formed upon generation of NO_3^\bullet is colourless], the laser was operated in a single-shot mode. After each laser pulse the cuvette was shaken manually to restore homogeneity. Each kinetic data point was obtained by averaging five of such measurements. All experiments were performed at least in triplicate.

Figure 2 shows that the NO_3^\bullet signal decayed in the absence of any substrate (black line), most likely due to a reaction with the solvent acetonitrile through hydrogen abstraction (eqn. 3).^[22]



Using the concentration of pure acetonitrile of 19.1 M, the second-order rate coefficient for this reaction was determined as $k = 0.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, which agrees well with the literature value of $(1 - 2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1})$.^[2]

The bimolecular rate coefficients for the reaction of NO_3^\bullet with the various substrates were obtained under pseudo-first order conditions by measuring the rate of the NO_3^\bullet signal decrease as function of substrate concentration, with $[\text{substrate}]:[\text{CAN}]$ in the range of 10 – 100. However, due to the low solubility of AcNH-Phe-Val-Phe-OMe in acetonitrile, the experiments with this tripeptide could only be performed with $[\text{substrate}]:[\text{CAN}] = 0.6 - 3$. It was confirmed that even at the lowest substrate concentration pseudo-first order behaviour was maintained. The pseudo-first order rate coefficients, k_{obs} , were determined from decay profiles at each of five different substrate concentrations.

Bimolecular rate coefficients k were obtained from linear regression, plotting k_{obs} vs. substrate concentrations and the absolute rate constants were given by the slope. An exemplary selection of k_{obs} versus $[\text{substrate}]$ plots is presented in Figure 3. The plots for the remaining reaction systems are given in the Supporting Information.

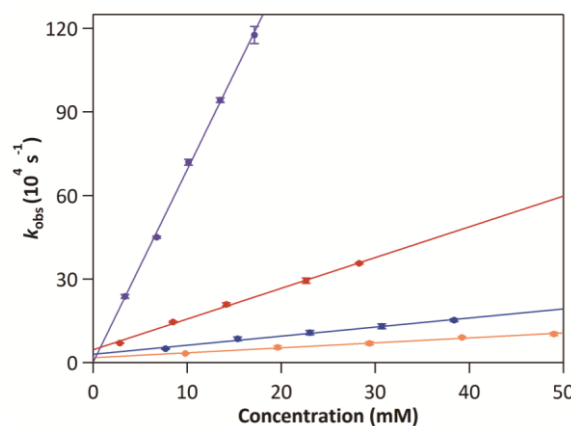


Figure 3. Plot of the pseudo-first order rate coefficient (k_{obs}) versus substrate concentration. Purple: AcNH-Phe-Phe-OMe, red: AcNH-Phe-OMe, blue: AcNH-Gly-OMe, orange: AcNH-Val-OMe. Error bars shown are 2σ statistical uncertainties.

For all reactions studied in this work k_{obs} increased linearly with substrate concentration. The non-zero intercept of about $1 \times 10^4 \text{ s}^{-1}$ in most of these plots (excluding for the very fast reactions of the di- and tripeptides with two phenylalanine residues), coincides approximately with the pseudo-first order rate coefficient for the reaction of NO_3^\bullet with the solvent. The second order rate coefficients for the reactions with all amino acids, di- and tripeptides studied in this work are compiled in Table 1.

The reaction of NO_3^\bullet with the aliphatic amino acids glycine, alanine, valine and leucine (entries 1-4) most likely proceeds through hydrogen abstraction at the α -carbon atom to form a capto-datively stabilised radical (see computational studies below). The determined rate coefficients are in the range of $(1.6 - 2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for the α -substituted amino acids, whereas the value obtained for glycine of $3.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ is about twice as high. This is presumably a consequence of the doubled number of successful collisions rather than lower steric hindrance at the reaction centre (*vide infra*). Likewise, due the presence of two α -carbon centres the rate coefficient for the reaction of NO_3^\bullet with the dipeptide AcNH-Leu-Leu-OMe is higher by a factor of about two than that measured for the reaction of NO_3^\bullet with AcNH-Leu-OMe (entry 6 vs. 4). The linear dependence of the rate coefficients with increasing number of available α -hydrogen atoms is a clear indication for HAT from the α -C–H bond to NO_3^\bullet , similar to the reaction of the O-centred cumyloxyl radicals ($\text{PhCMe}_2\text{O}^\bullet$) with *N*-Boc-protected acyclic aliphatic amino acids.^[23] Such linear correlation would not be found if NO_3^\bullet would react through abstraction of the hydrogen atoms from the aliphatic side chains. Latter appears

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to be the kinetically preferred pathway in the reaction of electrophilic chlorine radicals (Cl^\bullet) with aliphatic amino acids, which has been rationalised by deactivating polar effects at the α -position.^[24] It should be noted that NO_3^\bullet -induced hydrogen abstraction could principally also occur from the methyl groups of the protecting groups at the C- and N-terminus. If this were the case, however, the same rate coefficients would be expected for all of the acyclic aliphatic amino acids and dipeptides studied in this work. The high selectivity of NO_3^\bullet for the α -hydrogen atom is also supported by the computational studies, which are outlined below.

Table 1. Second-order rate coefficients for the reaction of NO_3^\bullet with amino acids, di- and tripeptides in acetonitrile at $298 \pm 1 \text{ K}$.^[a]

Entry ^[a]	Substrate	$k/\text{M}^{-1} \text{ s}^{-1}$
1	AcNH-Gly-OMe	$(3.3 \pm 0.5) \times 10^6$
2	AcNH-Ala-OMe	$(1.6 \pm 0.3) \times 10^6$
3	AcNH-Val-OMe	$(1.8 \pm 0.3) \times 10^6$
4	AcNH-Leu-OMe	$(2.0 \pm 0.2) \times 10^6$
5	AcNH-Phe-OMe	$(1.1 \pm 0.1) \times 10^7$
6	AcNH-Leu-Leu-OMe	$(3.7 \pm 0.7) \times 10^6$
7	AcNH-Gly-Phe-OMe	$(2.0 \pm 0.3) \times 10^7$
8	AcNH-Phe-Gly-OMe	$(3.6 \pm 0.3) \times 10^7$
9	AcNH-Val-Phe-OMe	$(1.1 \pm 0.1) \times 10^7$
10	AcNH-Leu-Phe-OMe	$(1.1 \pm 0.1) \times 10^7$
11	AcNH-Phe-Phe-OMe	$(6.9 \pm 0.4) \times 10^7$
12	AcNH-Phe-Gly-Phe-OMe	$(8.8 \pm 0.5) \times 10^7$
13 ^[b]	AcNH-Phe-Val-Phe-OMe	$(2.6 \pm 0.4) \times 10^8$

[a] Quoted uncertainties are 2σ . [b] Smaller concentration range due to low solubility of the substrate in acetonitrile (see text).

The rate coefficients for the reaction of NO_3^\bullet with acyclic aliphatic amino acids in acetonitrile are about one order of magnitude larger than those for the reaction of the cumyloxyl radical in the same solvent.^[23] The higher reactivity of NO_3^\bullet can be rationalized by the strong electrophilicity of the O-radical centre caused by the electron-withdrawing NO_2 'substituent'.

In contrast, the rate coefficient obtained for the reaction of NO_3^\bullet with phenylalanine is nearly one order of magnitude higher than that for the acyclic aliphatic amino acids (entry 5). This clearly supports the assumption that the reaction does not occur through HAT but proceeds *via* initial ET at the aromatic ring, in agreement with our previous product studies (Scheme 1), the computational data in this work (see below) and earlier kinetic investigations of the reaction of NO_3^\bullet with aromatic compounds.^[16,17b] However, in contrast to the previous laser flash photolysis study of the reaction of NO_3^\bullet with alkylbenzenes in acetonitrile by Baciocchi and Steenken *et al.*,^[17b] we did not observe the value of k_{obs} leveling off at high concentration of any

aromatic amino acid or peptide studied in this work. This unusual finding was rationalized by (reversible) formation of a π complex between the aromatic compound and NO_3^\bullet . It was suggested that dissociation of this complex into the reaction products (i.e., a benzyl radical and HNO_3) becomes the rate-determining step at high alkylbenzene concentrations, which determines the disappearance of NO_3^\bullet . Ramamurthy *et al.* also proposed formation of an intermediate reactant π complex in the reaction of β -phenylalanine with NO_3^\bullet in 6 M HNO_3 based on the observed concentration-independent growth and decay of a transient at 550 nm, which was assigned as the phenylalanine radical cation.^[16] Our experimental conditions did not enable us to monitor transient radical cations of type **3** (see Scheme 1) due to their short lifetime in aprotic solvents.^[25] In contrast, the linear correlation between k_{obs} and substrate concentration over the large concentration range used in this work is a clear indication for a bimolecular reaction without intermediate formation of a *longer lived* NO_3^\bullet -substrate complex, which is also supported by our computational studies shown below.

Not unexpected, the rate coefficients obtained for the reaction of the dipeptides AcNH-Gly-Phe-OMe, AcNH-Val-Phe-OMe and AcNH-Leu-Phe-OMe are largely determined by the higher reactivity of the phenylalanine residue (entries 7, 9 and 10). A slight increase of the rate coefficient by about 50% was found for the reaction of AcNH-Phe-Gly-OMe, compared to the inverted sequence with C-terminal phenylalanine (entry 8 vs. 7). Whether this indicates that N-terminal amino acid residues react faster with NO_3^\bullet is difficult to assess from this single example, given the only moderate difference between the measured rate data. It is worth noting that a similar observation was made in the reaction of sulphate radicals with dipeptides containing glycine and tyrosine or tryptophan residues, which showed an increase of the rate coefficient by a factor of 1.5 – 2.3 when the aromatic amino acid was the N-terminus.^[26] On the other hand, a recent mass spectrometric product study of the reaction of excess hydroxyl radicals with dipeptides consisting of lysine and tyrosine indicated a more rapid oxidation of tyrosine in the C-terminal position.^[27] However, since rate data were not obtained and multiple oxidations were possible under the experimental conditions in this study, these findings cannot be directly compared with the results from the present investigation.

We were very surprised to discover that the rate coefficient increased by a factor of at least seven for the reaction of NO_3^\bullet with di- and tripeptides that contained two phenylalanine residues. This enhancement was found not only when the two phenylalanines were adjacent (entry 11), but also when they were separated by glycine (entry 12). When valine was used as 'spacer' between the phenylalanine residues the rate coefficient increased further by a factor of about three (entry 13). Currently, we have no plausible explanation for this unexpected and unprecedented amplification of the electron transfer rate when two aromatic amino acids are present in close vicinity in the peptide chain. Due to the poor solubility in acetonitrile it was not possible (without drastically changing the reaction conditions) to further extend the peptide chain and explore the kinetics in systems with more than two adjacent phenylalanine residues, or where two phenylalanines are separated by more than one non-

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aromatic amino acid. Despite this, this finding suggests a synergistic effect between two aromatic rings in close vicinity (possibly caused by π stacking), which makes such peptides highly vulnerable to oxidative damage by NO_3^\cdot .

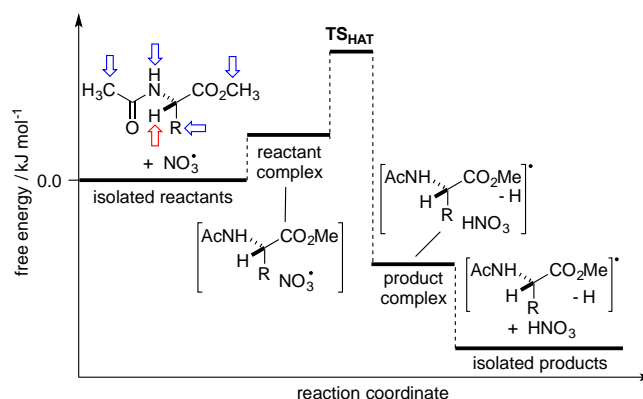
Comparison of the rate coefficients obtained in this work with the earlier data measured in 6 M HNO_3 ^[16] show that the reactivity of NO_3^\cdot with aliphatic amino acids is significantly higher in acetonitrile by about three orders of magnitude. A similar trend has been reported previously, which has been rationalised by hydrogen bonding between the solvent and NO_3^\cdot decreasing the rate for HAT processes.^[28] However, the practically identical rate coefficient obtained for phenylalanine in this work and that previously measured in aqueous solution (using β -phenylalanine)^[16] indicates that hydrogen bonding with the solvent has no noticeable effect on NO_3^\cdot reactions proceeding through electron transfer.

Computational studies

In order to explore the site selectivity of the reaction of NO_3^\cdot with the *N*- and *C*-protected amino acids, we performed DFT calculations of the potential energy surface. The computations were carried out with the Gaussian 09 program^[29] using the BHandHLYP, wB97XD and M06-2X methods, which have been employed previously to investigate radical induced α -hydrogen abstraction in amino acids.^[30] Calculations in acetonitrile were performed for selected reactions using the Conductor-like Polarizable Continuum Model (CPCM).^[31] All ground and transition structures were verified by vibrational frequency analysis at the same level of theory, and all identified transition structures showed only one imaginary frequency. The spin expectation value, $\langle s^2 \rangle$, was very close to 0.75 after spin annihilation. The Gaussian archive entries for all optimized ground and transition structures, including the imaginary frequencies of the transition structures, are given in the Supporting Information.

The reaction of NO_3^\cdot with aliphatic amino acids should proceed through HAT.^[10,16] Although intuitively abstraction of the α -hydrogen might be expected as the dominant pathway, the hydrogen atoms of the α -side chains as well as of the protecting groups at the *N*- and *C*-terminus may possibly also be targeted by NO_3^\cdot . The potential energy surface is shown in Scheme 2 and Table 2 compiles the computed free energies of stationary points for different reaction pathways relative to the sum of the free energies of the isolated reactants.

The abstraction of the α -hydrogen in glycine ($R = \text{H}$, red arrow) was used to assess the performance of different DFT methods to enable a qualitative evaluation of the most important reaction pathway. Thus, the energies obtained with M06-2X and wB97XD did not depend significantly on the size of the basis set (entries 1 vs. 9 and 7 vs. 8), but wB97XD predicts a slower reaction, as indicated by the slightly higher barrier TS_{HAT} , as well as a smaller exothermicity (i.e., higher energy of the product complex and isolated products, respectively) compared with M06-2X. Of the various methods studied, BHandHLYP calculates the highest barrier but similar reaction energies as wB97XD (entry 6 vs. 7).



Scheme 2. Potential energy surface for the hydrogen abstraction in *N*- and *C*-protected α -amino acids by NO_3^\cdot . The arrows indicate potential sites for HAT.

Table 2. Calculated potential energy surface for alternative HAT processes in the reaction of NO_3^\cdot with *N*- and *C*-protected amino acids.^[a]

Entry	R	Reactant complex	TS_{HAT}	Product complex	Isolated products
1 ^[b]	H	30.8	46.3	-53.3	-90.1
2 ^[b,c]			70.4	-43.1	-25.5
3 ^[b,d]			n.d. ^e	n.d.	-19.3
4 ^[b,f]			n.d.	n.d.	11.5
5 ^[g]		37.8	40.3	-72.7	-102.0
6 ^[h]		23.1	59.8	-34.1	-75.7
7 ^[i]		34.5	51.4	-22.2	-61.7
8 ^[k]		19.6	51.3	-34.1	-71.5
9 ^[m]		18.8	46.1	-66.9	-99.2
10 ^[b]	Me	28.2	44.7	-79.2	-92.0
11 ^[b,n]			62.6	n.d.	-8.2
12 ^[b]	<i>i</i> Pr	39.8	47.7	-74.3	-77.6
13 ^[b,o]			n.d.	n.d.	-44.1
14 ^[b]	<i>i</i> Bu	13.8	37.3	-82.4	-97.4
15 ^[b]	Bn	21.0	39.5	-78.8	-84.1
16 ^[b,p]			50.7	-51.2	-52.6
17 ^[g]			30.6	36.5	-86.8

[a] Free energies (kJ mol^{-1}) relative to the isolated reactants. HAT from α -carbon, unless stated otherwise. [b] M06-2X/6-31+G* (gas phase). [c] HAT from *N*-acetyl group. [d] HAT from methyl ester. [e] n.d. = not determined. [f] HAT from NH group. [g] M06-2X/6-31+G* (in acetonitrile). [h] BHandHLYP/6-31+G* (gas phase). [i] wB97XD/6-31+G* (gas phase). [j] wB97XD/6-31+G** (gas phase). [k] wB97XD/6-311+G** (gas phase). [m] M06-2X/6-311+G** (gas phase). [n] HAT from β -methyl group. [o] HAT from β -methine group. [p] HAT from benzyl group.

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Because of the very broad applicability^[32] of the M06-2X^[31] functional in conjunction with its moderate average mean absolute errors,^[33] we decided to use this method in combination with the less expensive 6-31+G* basis set to explore the reaction mechanism.

α -Hydrogen abstraction is the most exothermic pathway for the reaction of NO_3^\cdot with glycine (entry 1), followed by HAT from the *N*-acetyl group (entry 2) and the methyl ester (entry 3). In contrast to this, abstraction of the amide hydrogen is endothermic (entry 4) and can be excluded. Calculation of the activation barrier (TS_{HAT}) associated with the two thermodynamically most favourable processes clearly confirms that HAT from the α -carbon is also the kinetically preferred pathway (entry 1 vs. 2). Furthermore, the calculations predict for the reaction involving alanine ($R = \text{Me}$) that formation of the α -radical is both kinetically and thermodynamically also more favourable than HAT from the methyl side chain (entry 10 vs. 11). Although alkyl substitution at the β -carbon, for example in valine ($R = i\text{Pr}$), leads to a considerable increase of the stability of the β -radical, the capto-datively stabilized α -radical is still the most favourable product (entry 12 vs. 13).

The reaction of NO_3^\cdot with the amino acids proceeds through an initial association complex, which is located 14–40 kJ mol^{-1} above the free reactants. Because of its endothermic nature this complex likely has no significance^[24] for the course of the reaction other than being a short-lived steppingstone for the subsequent HAT *via* TS_{HAT} , which leads to the α -amino acid radical. The latter is initially obtained as association complex with HNO_3 , which is exothermic by 53 – 83 kJ mol^{-1} . Dissociation of the latter to give the isolated products is associated with a further energy gain by 46 kJ mol^{-1} for the smallest amino acid glycine and 3 – 15 kJ mol^{-1} for amino acids with alkyl side chains.

Figure 4 shows the optimized geometries for the reactant association complex, TS_{HAT} and the product association complex for the exemplary reaction of NO_3^\cdot with glycine. In the reactant complex both α -hydrogen atoms are loosely coordinating to two different oxygen atoms of NO_3^\cdot . In fact, the lowest-energy association complexes for all amino acids studied in this work exhibit a similar coordination between NO_3^\cdot and the α -hydrogen atom (not shown). TS_{HAT} is an early transition state for all reactions under investigation, which is characterized by a $\alpha\text{-C-H}$ distance of $1.163 \pm 0.007 \text{ \AA}$ (for comparison: the computed $\alpha\text{-C-H}$ bond in glycine is 1.097 \AA) and $1.577 \pm 0.041 \text{ \AA}$ for the $\text{O}_2\text{NO-H}$ distance (computed O-H bond in $\text{HNO}_3 = 0.977 \text{ \AA}$). The product association complex shows a hydrogen bond between HNO_3 and the carbonyl oxygen of the *N*-protecting group. In general, TS_{HAT} is located $43 \pm 5 \text{ kJ mol}^{-1}$ above the free reactants in the gas phase, which indicates that the side chain at the α -carbon has no significant influence on the rate coefficient. This agrees well with the experimental data shown in Table 1 and confirms our suggestion that the higher rate coefficient obtained for the reaction of NO_3^\cdot with glycine is

due to the presence of two abstractable hydrogen atoms and not a steric effect.^[34]

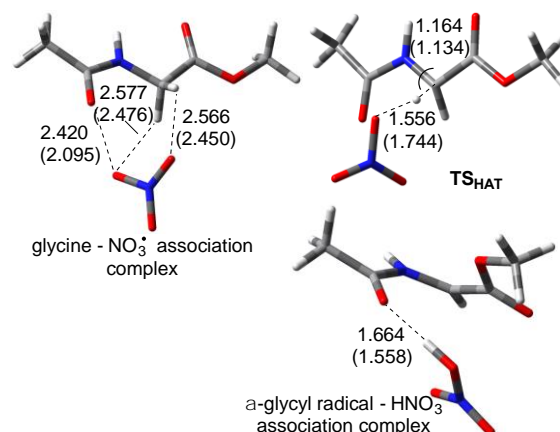
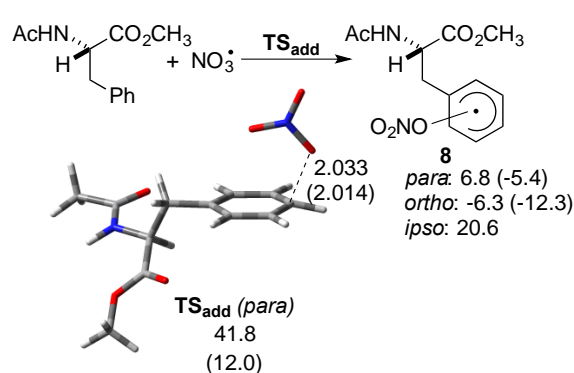


Figure 4. Optimized geometries for the α -hydrogen abstraction in the reaction of NO_3^\cdot with *N*- and *C*-protected glycine. Selected M06-2X/6-31+G* distances in \AA in the gas phase and in acetonitrile (in brackets).

The reaction of NO_3^\cdot with phenylalanine could principally also proceed through HAT from either the α - or β -carbon atom. Interestingly, the calculations revealed that abstraction of a β -hydrogen by NO_3^\cdot and formation of a resonance stabilized benzyl radical is both kinetically and thermodynamically less favourable than the reaction leading to the α -carbon radical (entry 15 vs. 16). In fact, the latter pathway is associated with energies comparable to those predicted for the aliphatic amino acids. Exemplary calculations in acetonitrile were performed for the reaction of NO_3^\cdot with glycine and phenylalanine, which revealed only slightly more ‘compact’ structures in acetonitrile (see Figure 4 for glycine), but not a significant effect on the barrier height compared to the gas phase (entries 1 vs. 5 and 15 vs. 17). Thus, if the reaction of NO_3^\cdot with phenylalanine would proceed through HAT from the α - or β -carbon atom, respectively, a rate coefficient similar or even smaller to those obtained for the reaction with aliphatic amino acids would be expected in acetonitrile. This is not the case.

This finding supports our conclusion that NO_3^\cdot reacts with phenylalanine through ET (see Scheme 1). The oxidation could proceed through an inner-sphere mechanism^[17b] initiated by NO_3^\cdot addition to the aromatic π system, followed by heterolytic dissociation of the covalent radical adduct (σ -complex or ‘bridged intermediate’)^[35] into an arylradical cation and NO_3^- . This is similar to the addition/elimination pathway proposed for the oxidation of tyrosine residues in dipeptides by sulphate radicals.^[26] We explored this hypothesis by computing the energies of selected stationary points for the NO_3^\cdot addition to the aromatic ring, which should be the rate-determining step (because it breaks the aromatic π system up), but must be faster than HAT from the α -carbon (Scheme 3).^[36]



Scheme 3. Addition of NO_3^\bullet to the aromatic ring in phenylalanine. M06-2X/6-31+G* gas phase energies in kJ mol^{-1} relative to isolated reactants. Distances in Å. Data in acetonitrile in brackets.

The calculations revealed that NO_3^\bullet addition to the aromatic ring is thermodynamically considerably less favourable than the hydrogen abstraction pathway. While in the gas phase formation of the *ortho* radical adduct **8** is weakly exothermic, the *para* and *ipso* adducts are endothermic. Acetonitrile leads to stabilization of these adducts by about 6 – 12 kJ mol^{-1} . Exemplary calculations of the barrier for the *para*-attack of NO_3^\bullet in the gas phase showed that TS_{add} is actually located some 2 kJ mol^{-1} above TS_{HAT} for the α -hydrogen abstraction, (see Table 2, entry 15).

Remarkably, in acetonitrile the barrier TS_{add} drops to just 12 kJ mol^{-1} , indicating a very fast reaction. The reason for the stabilizing effect of this polar aprotic solvent was revealed from analysis of the Mulliken charges, which showed development of a negative charge on the NO_3 fragment of $-0.53e$ in TS_{add} in acetonitrile ($-0.37e$ for the gas phase). This correlates with the significantly reduced spin density on NO_3 (14% in acetonitrile, 30% in the gas phase) and indicates advanced electron redistribution in TS_{add} . These data suggest that the lifetime of radical adduct **8** must be extremely short, and it is likely that NO_3^\bullet adds to the aromatic ring and leaves as NO_3^- during the course of a single vibration. Similar to the HAT pathway, this inner-sphere ET should also proceed through formation of an intermediate association complex. We were able to locate a stationary point where NO_3^\bullet coordinates to the aromatic ring system (ca. 2.2 Å distance in acetonitrile), which is energetically ca. 6 kJ mol^{-1} above the isolated reactants (not shown).

Conclusions

NO_3^\bullet reacts in acetonitrile rapidly with acyclic aliphatic amino acids through HAT from the α -carbon with a rate coefficient of about $1.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ per abstractable hydrogen atom. This value is about three orders of magnitude greater than that obtained for the reaction in aqueous solution,^[16] where hydrogen bonding with the solvent lowers the reactivity of NO_3^\bullet . In acyclic aliphatic dipeptides with more than one reaction centre the rate coefficient correlates with the sum of the rate data for the

individual reactions. Computational studies supported our observation that NO_3^\bullet -induced hydrogen abstraction from sites other than the α -carbon in acyclic aliphatic amino acids is not competitive on both kinetic and thermodynamic grounds. This contrasts the recent findings for the reaction of aliphatic amino acids with Cl^\bullet , where HAT occurred preferably from side chain sites distal to the α -carbon, despite being thermodynamically less favourable pathways.^[24] In comparison with other O-centred radicals, the reactivity of NO_3^\bullet falls between that observed for the alkoxy radicals $\text{PhCMe}_2\text{O}^\bullet$, which are about one order of magnitude less reactive,^[23] and hydroxyl radicals (OH^\bullet), whose reactivity is ca. three orders of magnitude higher.^[37] Thus, the latter react non-selectively with acyclic aliphatic amino acids through hydrogen abstraction and oxidation,^[24,37,38] whereas both NO_3^\bullet and $\text{PhCMe}_2\text{O}^\bullet$ ^[23] exhibit a strong regioselectivity for α -hydrogen abstraction. It is worth noting that the rate coefficients for reactions of NO_2^\bullet are considerably lower. In fact, only amino acids with readily oxidizable amino acids, such as tyrosine or tryptophan, are damaged by NO_2^\bullet (as a *radical species*) through one-electron oxidation of the side chain.^[14,39]

The rate coefficient for the reaction of phenylalanine with NO_3^\bullet is about one order of magnitude higher than for the aliphatic amino acids, which indicates a reaction proceeding through ET. Computational studies suggest that oxidation of the aromatic ring likely proceeds through an inner-sphere ET mechanism, which is characterized by a transition state with significant charge separation. This transition state is stabilized in polar aprotic solvents, for example acetonitrile. In the gas phase, however, HAT from the α -carbon and ET are predicted to occur with similar rates. An unprecedented amplification of the rate coefficient was found in di- and tripeptides containing two phenylalanine residues, which was independent of whether they were adjacent or separated by one (aliphatic) amino acid. Although we currently have no explanation for this unexpected synergism, this suggests that peptide sequences, which contain more than one phenylalanine residue in close vicinity, are more prone to oxidative damage by NO_3^\bullet than would be expected based on the reactivity of the individual components. In future work we will further explore the mechanism through molecular dynamics studies to gain detailed insights into the origin of this unprecedented rate amplification.

Acknowledgements

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Keywords: nitrate radical • peptides • oxidative damage • kinetics • reaction mechanisms • density functional calculations

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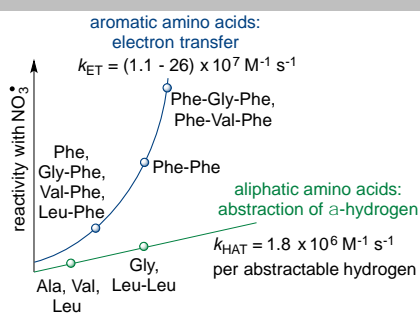
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Entry for the Table of Contents

FULL PAPER

Unexpected synergism: Kinetic studies revealed an unprecedented amplification of the reactivity of di- and tripeptides with two phenylalanine residues in close vicinity towards nitrate radicals, NO_3^\bullet . This suggests that such peptide sequences are highly prone to oxidative damage by this important environmental radical oxidant.



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