^{15}N CIDNP investigations of the peroxynitric acid nitration of L-tyrosine and of related compounds

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Peroxynitric acid (O_2NOOH) nitrates L-tyrosine and related compounds at pH 2–5. During reaction with $O_2{}^{15}NOOH$ in the probe of a ^{15}N NMR spectrometer, the NMR signals of the nitration products of L-tyrosine, *N*-acetyl-L-tyrosine, 4-fluorophenol and 4-methoxyphenylacetic acid appear in emission indicating a nitration *via* free radicals. Nuclear polarizations are built up in radical pairs [$^{15}NO_2^{\bullet}$, $PhO^{\bullet}$]^{F} or [$^{15}NO_2^{\bullet}$, $ArH^{\bullet+}$]^{F} formed by diffusive encounters of $^{15}NO_2^{\bullet}$ with phenoxyl-type radicals $PhO^{\bullet}$ or with aromatic radical cations $ArH^{\bullet+}$. Quantitative ^{15}N CIDNP investigations with *N*-acetyl-L-tyrosine and 4-fluorophenol show that the radical-dependent nitration is the only reaction pathway. During the nitration reaction, the ^{15}N NMR signal of $^{15}NO_3^-$ also appears in emission. This is explained by singlet–triplet transitions in radical pairs [$^{15}NO_2^{\bullet}$, $^{15}NO_3^{\bullet}$]^{S} generated by electron transfer between $O_2{}^{15}NOOH$ and $H^{15}NO_2$ formed as a reaction intermediate. During reaction of peroxynitric acid with ascorbic acid, ^{15}N CIDNP is again observed in the ^{15}N NMR signal of $^{15}NO_3^-$ showing that ascorbic acid is oxidized by free radicals. In contrast to this, $O_2{}^{15}NOOH$ reacts with glutathione and cysteine without the appearance of ^{15}N CIDNP, indicating a direct oxidation without participation of free radicals.

Introduction

Peroxynitric acid (O_2NOOH/O_2NOO^-; pK_a 5.9) is known as an unstable intermediate during reaction of H_2O_2 with either N_2O_5 or HNO_2 since about 100 years.[1] It has found growing interest after detection in the Earth's atmosphere as the recombination product of free radicals $HO_2^{\bullet}$ and $NO_2^{\bullet}$.[2] Furthermore, it may be generated under physiological conditions as the recombination product of superoxide, $O_2^{\bullet-}$ (HO_2/O_2^-; pK_a 4.8), and $NO_2^{\bullet}$.[3] In living organisms, this reaction has been suggested to be an effective detoxification pathway for $NO_2^{\bullet}$.[4] During the last years, its formation and decomposition have been studied in greater detail, see Scheme 1.[5–9]

$$2H_2O_2 + HNO_2 \rightarrow 2H_2O + O_2NOOH$$
$$O_2NOOH \rightarrow HO_2^{\cdot} + NO_2^{\cdot} \quad (k = 0.025\ s^{-1}\ [8b])$$
$$HO_2^{\cdot} + NO_2^{\cdot} \rightarrow O_2NOOH \quad (k = 1.8\cdot10^9\ M^{-1}s^{-1}\ [9c])$$
$$2NO_2^{\cdot} + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (k = 6.5\cdot10^7\ M^{-1}s^{-1}\ [10])$$
$$2HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad (k = 8.6\cdot10^5\ M^{-1}s^{-1}\ [11])$$
$$O_2NOOH + HNO_2 \rightarrow 2HNO_3$$

Scheme 1 Formation and decay reactions of peroxynitric acid (see ref. 8*b*, 9*c*, 10 and 11).

Peroxynitric acid is expected to exhibit nitrating as well as oxidizing properties. Tyrosine nitration and the nitration of tyrosine residues in proteins are used as markers for the activity of reactive nitrogen species (RNS) in living systems.[12] The most important RNS seem to be peroxynitrite and nitrite in the presence of peroxydase or hypochlorite.[12,13] Concerning peroxynitrite, an indirect nitration pathway *via* $NO_2^{\bullet}$ and tyrosinyl radicals is generally accepted.[14] Peroxynitric acid might also be considered as a possible nitration agent of tyrosine residues in biological systems.[4c,15]

Nitration of L-tyrosine (Tyr) with peroxynitric acid has not been observed at pH 7.[4b] The purpose of the present paper is to look for nitration reactions with peroxynitric acid at lower pH values. ^{15}N CIDNP investigations during the nitration of *N*-acetyl-L-tyrosine (Tyrac), 4-fluorophenol (4-F–C_6H_4OH) and 4-methoxyphenylacetic acid (4-MeO–C_6H_4–CH_2–COOH) will be described for the study of the nitration mechanism of peroxynitric acid. In preceding communications, ^{15}N CIDNP has been applied in proving the radical mechanism of the nitration reactions of L-tyrosine and *N*-acetyl-L-tyrosine with peroxynitrite at pH 4–5 as well as with nitrite in the presence of peroxydase or hypochlorite at physiological pH values.[16]

A few oxidizing reactions with peroxynitric acid have been reported.[9a,b] They were proposed to occur *via* different mechanisms, a direct one and an indirect one *via* free radicals formed during the decomposition of peroxynitric acid (Scheme 1). For demonstrating the possibility of different mechanisms, ^{15}N CIDNP studies during oxidation of ascorbic acid, glutathione and cysteine will be described, too. These compounds act as scavengers for oxidants in biological systems, especially for free radicals.[17]

CIDNP is used for evaluating reaction mechanisms in organic chemistry. It leads to emission (E) and/or enhanced absorption (A) signals in the NMR spectra of products formed during fast radical reactions running in the probe of an NMR spectrometer and proves the occurrence of free radicals.[18–21] Especially, ^{15}N CIDNP has been applied to study nitration reactions of activated aromatics by nitric acid, nitrous acid and peroxynitrous acid.[16,22,23]

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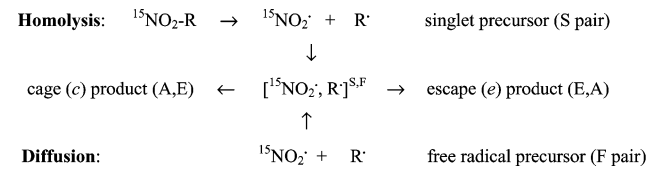
CIDNP is generated in radical pairs formed by the homolysis of diamagnetic compounds from singlet states (S pairs) or by diffusive encounters of independently generated free radicals (F pairs). Free radicals reacting within the pairs give cage (*c*) products. Free radicals which do not react within the pairs form escape (*e*) products. If ^{15}N nuclei are observed, the phase of CIDNP effects (E, A) in the reaction products of free radicals generated by homolysis of diamagnetic compounds $^{15}NO_2$–R and by reactions of $^{15}NO_2^{\bullet}$ with free radicals R$^{\bullet}$ is given in Scheme 2 assuming $g(R^{\bullet}) > g(NO_2^{\bullet})$.[22]



Scheme 2 ^{15}N CIDNP effects in the reaction products of free radicals $^{15}NO_2^{\bullet}$ and R$^{\bullet}$ generated by homolysis of diamagnetic compounds $^{15}NO_2$–R (S pairs) and free radical encounters (F pairs) assuming $g(R^{\bullet}) > g(NO_2^{\bullet})$. E: emission, A: enhanced absorption.

The appearance of CIDNP proves the occurrence of radical reactions, which does not exclude non-radical reactions leading to the same product. To prove this, quantitative experiments have been performed. CIDNP intensities are proportional to the product rate formation. For quantitative investigations, the dependence of reaction time and product concentrations is eliminated by determining an enhancement factor *E* which is the ratio between the intensity of the NMR signal immediately after formation of the polarized product and the intensity of the NMR signal of the product after finishing the reaction.[24] The value is compared with ^{15}N CIDNP data obtained from various nitrating systems[17,23] and calculations based on quantitative formulations of the radical pair theory.[25] This procedure will be applied during reactions of peroxynitric acid-^{15}N with *N*-acetyl-L-tyrosine and with 4-fluorophenol, not with L-tyrosine and 4-methoxyphenylacetic acid because of low product concentrations.

Experimental

^{15}N CIDNP experiments

Authentic peroxynitric acid-^{15}N or a mixture of $Na^{15}NO_2$ and H_2O_2 was dissolved in H_2O/D_2O containing phosphoric acid (0.3 M) and $NaHCO_3$ (0.05 M) at pH 2. After putting the reactants into the 10 mm NMR tube, it was transferred into the probe of the ^{15}N NMR spectrometer (Bruker DPX 300) within 5 s and then locked. A single pulse spectrum of the peroxynitric acid was then taken with a pulse angle of 90° 2 or 3 min later. After that, the tube was replaced, and the reactant was added to the solution. ^{15}N NMR spectra were then taken every 2 or 3 min until the reaction was completed. For detecting the reaction products, ^{15}N NMR spectra were taken with several hundred pulses. ^{15}N NMR intensities *I* were taken directly from the spectra. During single runs, signal intensities are proportional to concentrations within about 5%. Signal intensities taken during different runs differ within about 20%. An *E* value was determined from eqn (1).[23]

$$E = \Sigma I_i \Delta t(i, i+1)/I_o T_1 \quad (1)$$

I_i is the intensity of the CIDNP signal during the i^{th} measurement and $\Delta t(i, i+1)$ is the time interval (2 or 3 min) between the i^{th} and the $(i+1)^{th}$ measurement. I_o is the intensity of the ^{15}N NMR signal of the reaction product after finishing the reaction and T_1 is the longitudinal relaxation time of the nucleus investigated. For determining *E*, eqn (1) is a good approximation if the reaction time exceeds T_1 by more than about one order of magnitude. This procedure cancels differences between various runs which can therefore be compared directly. Values determined from different runs differ by about 15%. Chemical shifts are given in δ values relative to nitrobenzene-^{15}N dissolved in acetonitrile as an external reference. ^{15}N CIDNP experiments using nitric acid or nitrous acid as nitrating agents were performed in an analogous manner.[23]

Materials

$O_2{}^{15}NOOH$ solutions (1.57 M) were freshly prepared prior to use as described.[4a] $O_2{}^{15}NOOH$ was also prepared *in situ* by addition of H_2O_2 (1 M) to a solution of $Na^{15}NO_2$ (0.3 M or 0.15 M) in H_2O.[8c,15b] All the other compounds and solvents were commercial. Nitric acid was 0.4 M in H_2O and labelled with 60.3 atom% ^{15}N (Isotec Inc.). $NaNO_2$ was labelled with 99.3 atom% ^{15}N (Isotec Inc.).

Solutions

For preparing the buffer solutions, doubly distilled water was bubbled (2 L min^{-1}) with synthetic air at room temperature for 20 min. Traces of transition metal ions were removed from the buffer solutions by treatment with the heavy metal scavenger resin Chelex 100 by gentle shaking for 18 h in the dark.[26] The pH value was adjusted with sulfuric acid and sodium hydroxide using a pH Meter CG 825.

Capillary zone electrophoresis measurement

L-Tyrosine and 3-nitro-L-tyrosine were quantified on a Beckman P/ACE 5000 apparatus. Separation conditions for L-tyrosine and 3-nitro-L-tyrosine were as follows: fused silica capillary (50 cm effective length, 75 m internal diameter), hydrodynamic injection for 5 s, temperature 30 °C, voltage 18 kV, normal polarity, UV detection at 214 nm. A mixture of 50 mM sodium phosphate, 25 nM sodium borate, and 50 mM sodium dodecyl sulfate (pH 9.0) was used as the electrolyte system. To each sample, 0.2 mM of *p*-hydroxybenzoic acid was added as an internal standard.

Quantum chemical calculations

Complete basis set (CBS-Q) computations were carried out with the Gaussian 03 (Revision A.11.3) suite of programs.[27] Molecular interactions were evaluated on the optimized gas-phase geometries with the PCM[28a] procedure incorporated in Gaussian 03. Both the PCM/(U)HF/6-31(+)G(d) and the CBS-Q methodology are known to provide estimates within "chemical accuracy" (±1 kcal mol^{-1}), as has also been demonstrated for O_2NOOH-derived reactions.[28b] Isotropic absolute shielding constants of the nitrogen nucleus in a couple of compounds were calculated with the gauge-including atomic orbital (GIAO) protocol[29] at the DFT/aug-cc-pVDZ (DFT = B1LYP and B3LYP) level of theory. The optimization of the structure and molecular interactions with the solvent were respected at the same level of theory.

Table 1 Effect of pH on nitration of L-tyrosine (1 mM) with peroxynitric acid (1 mM)

pH	NO_2–Tyr (μM)[a]
7	0[b]
6	0
5	13 ± 1.2
4	101.5 ± 5.6
3	118.5 ± 6.2

[a] Determined using capillary zone electrophoresis (detection limit 8 μM). [b] Recovery of L-tyrosine 96.1%.

Results and discussion

Nitration of L-tyrosine with peroxynitric acid

During reaction of peroxynitric acid with L-tyrosine (Tyr) in acidic solution, the nitration product 3-nitro-L-tyrosine (3-NO_2–Tyr) is formed [eqn (2)]. The product yield increases with decreasing pH values from zero at pH 7 to 118.5 mM at pH 3, see Table 1.

$$\text{Tyr} + \text{O}_2\text{NOOH} \rightarrow \text{3-NO}_2\text{–Tyr} + \text{HOOH} \quad (2)$$

The unprotonated form, which is present at pH 7, is not able to nitrate Tyr.

^{15}N CIDNP during decomposition of peroxynitric acid-^{15}N at pH 2

Peroxynitric acid decomposes to nitric acid in acidic solution (Scheme 1), half-life times of 30–60 min have been found.[8a,c] A typical ^{15}N NMR spectrum taken during the decay of $O_2{}^{15}$NOOH in H_2O is given in Fig. 1a. The time dependence of ^{15}N NMR signal intensities I of $O_2{}^{15}$NOOH (0.54 M) and $^{15}NO_3^-$ and details of this reaction are given in the Tables 2 and 3. The assignment of

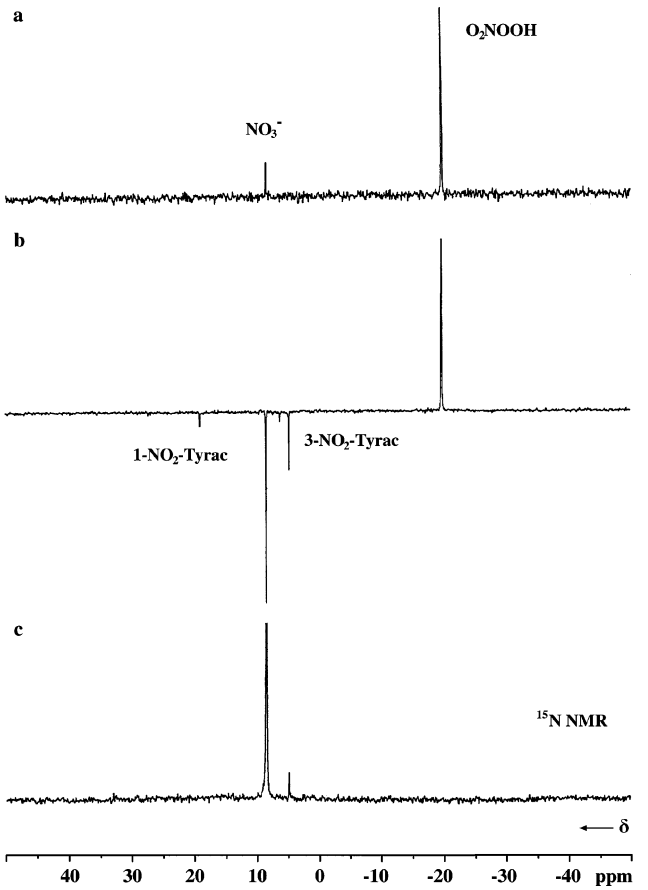


Fig. 1 ^{15}N NMR spectra of solutions of peroxynitric acid-^{15}N in H_2O at pH 2 and 298 K taken (a) 3 min after putting the tube in the probe (1 pulse), (b) 3 min after adding *N*-acetyl-L-tyrosine (1 pulse), (c) 300 min after adding *N*-acetyl-L-tyrosine (500 pulses).

Table 2 ^{15}N CIDNP during reaction of $O_2{}^{15}$NOOH with organics at pH 2 and 295 K

Reactant	Assignment	δ (ppm)[a]	CIDNP[b]	Yield (%)[c]	$t_{\frac{1}{2}}$ (min)[d]
None[15b] (Fig. 1a)	$O_2{}^{15}$NOOH (0.54 M)	−18	A	—	20
	$^{15}NO_3^-$	9	N	100	
N-Acetyl-L-tyrosine (0.2 M) (Fig. 1b,c)	$O_2{}^{15}$NOOH (0.3 M)	−18	A	—	4
	3-$^{15}NO_2$–Tyrac	4	E	2.0	
	(?)	6	E	—	
	$^{15}NO_3^-$	9	E	98.0	
	1-$^{15}NO_2$–Tyrac	18	E	—	
4-Fluorophenol (0.1 M) (Fig. 3)	$O_2{}^{15}$NOOH[e]	−18	A	—	4
	2-$^{15}NO_2$-4-F–C_6H_4OH	3	E	0.2	
	4-$^{15}NO_2$-4-F–C_6H_4=O	13/14	E	—	
	3-NO_2-4-F–C_6H_4OH	−2	E	—	
	$^{15}NO_3^-$	9	E	100	
	(?)	4	E	—	
Ascorbic acid (0.15 M) (Fig. 2a)	$O_2{}^{15}$NOOH[f]	−18	A	—	3
	$^{15}NO_3^-$	9	E	100	
Glutathione (0.1 M)	$O_2{}^{15}$NOOH[f]	−18	—	—	N. o.[g]
	$^{15}NO_3^-$	9	N	100	
Cysteine (0.1 M)	$O_2{}^{15}$NOOH[f]	−18	A	—	0.15
	$^{15}NO_3^-$	9	N	100	
4-Methoxyphenylacetic acid (0.02 M) (Fig. 2b)	$O_2{}^{15}$NOOH[f]	−18	A	—	8
	3-$^{15}NO_2$-4-MeO-C_6H_4–CH_2–COOH	3	E	<0.1	
	$^{15}NO_3^-$	8	E	100	

[a] δ values against $Ph^{15}NO_2$, positive δ values downfield. [b] E: emission, A: enhanced absorption, N: no CIDNP. [c] Product yields determined from ^{15}N NMR spectra taken after reaction. [d] Half-life time of the ^{15}NMR signal decay of $O_2{}^{15}$NOOH. [e] Generated *in situ* from $Na^{15}NO_2$ (0.3 M) and H_2O_2 (1 M). [f] Generated *in situ* from $Na^{15}NO_2$ (0.15 M) and H_2O_2 (1 M). [g] Not observed.

Table 3 ^{15}N NMR intensities I^a (a) during decay of $O_2{}^{15}NOOH$ (0.54 M), (b) during reaction of $O_2{}^{15}NOOH$ (0.3 M) with *N*-acetyl-L-tyrosine (0.2 M), (c) during reaction of $O_2{}^{15}NOOH^b$ with 4-fluorophenol (0.1 M), (d) during reaction of $O_2{}^{15}NOOH^c$ with ascorbic acid (0.15 M) at pH 2 and 295 K

(a)											
t^d	0	3	6	12	18	28	40	60	100	300	
$I(O_2{}^{15}NOOH)$	400	400	360	300	240	150	80	33	17	0	
$I(^{15}NO_3^-)$	10	14	17	25	41	53	55	67	67	70	
(b)											
t^d	0	3	6	9	12	15	18	21	30	300	
$I(O_2{}^{15}NOOH)$	750	500	330	125	100	60	30	20	3	0	
$I(^{15}NO_3^-)$	−310	−200	−150	−70	−55	−22	−5	2	20	40	
$I(3\text{-}^{15}NO_2\text{–Tyrac})$	−50	−26	−19	−10	−7	−4	−2	0	0	0.8[e]	
$I(1\text{-}^{15}NO_2\text{–Tyrac})$	−17	−12	−12	−3	−3	−2	0	0	0	0	
(c)											
t^d	0	2	4	6	8	10	12	14	18	22	300
$I(O_2{}^{15}NOOH)$	60	200	150	100	60	30	15	9	3	0	0
$I(^{15}NO_3^-)$	10	−140	−150	−70	−30	−10	4	15	25	40	40
$I(2\text{-}^{15}NO_2\text{-}4\text{-}F\text{–}C_6H_4OH)$	—	−32	−26	−12	−5	−3	−2	0	0	0	0.08[f]
$I(4\text{-}^{15}NO_2\text{-}4\text{-}F\text{–}C_6H_4{=}O)$	—	−4	−3	−2	0	0	0	0	0	0	0
(d)											
t^d	0	2	4	6	8	10	14	22			
$I(O_2{}^{15}NOOH)$	40	250	150	30	7	2	—	—			
$I(^{15}NO_3^-)$	5	−75	−30	−4	7	12	20	20			

[a] *I* values determined from the signal-to-noise ratios using single 90° pulses. [b] $O_2{}^{15}NOOH$ generated *in situ* from $Na^{15}NO_2$ (0.3 M) and H_2O_2 (1 M). [c] $O_2{}^{15}NOOH$ generated *in situ* from $Na^{15}NO_2$ (0.15 M) and H_2O_2 (1 M). [d] *t*: time after starting the reaction (in min). The spectrum at $t = 0$ has been taken before adding the reactant to the solution of $O_2{}^{15}NOOH$. [e] Determined from ^{15}N NMR spectra taken after reaction (625 scans, 90° pulses, delay time 2 min). [f] Determined from ^{15}N NMR spectra taken after reaction (400 scans, 90° pulses, delay time 3 min).

the ^{15}NMR signals is supported by results of quantum-chemically calculated ^{15}N chemical shifts, see Table 4.

The ^{15}N NMR signal intensity of $^{15}NO_3^-$ increases from 10 to 70 units during reaction showing that it is proportional to the $^{15}NO_3^-$ concentration. The half-life time of 20 min taken from the spectra is smaller than the values given in the literature (30–60 min). We think that this is of no importance for the nitration experiments. It follows that 15% of $O_2{}^{15}NOOH$ decomposed before taking the first spectrum, and furthermore, that the intensity of the ^{15}N NMR signal of $O_2{}^{15}NOOH$ should be about 60 units which is much less than the 400 units observed, indicating that it shows enhanced absorption (CIDNP of A type, Scheme 2). It decreases with a half-time of about 20 min too; the magnitude of the effect is about the same during reaction. The reaction is finished, 95% complete, in 100 min. The ^{15}N CIDNP effect has also been observed at higher pH values and is built up in radical pairs $[^{15}NO_2^{\bullet},{}^{\bullet}O_2H]^S$ (Scheme 3) formed during homolysis of $O_2{}^{15}NOOH$ (Scheme 1).[15b]

The formation of $O_2{}^{15}NOOH$ by recombination of $^{15}NO_2^{\bullet}$ and $HO_2^{\bullet}$ *via* radical pairs $[^{15}NO_2^{\bullet},{}^{\bullet}O_2H]^F$ leads to an E type effect[16b] which is not observed under the given conditions. The ^{15}N NMR signal of $^{15}NO_3^-$ appears in emission at pH 3.1.[15b] This has been explained by electron transfer between $O_2{}^{15}NOOH$ and $O_2{}^{15}NOO^-$ (Scheme 3), which has no importance at pH 2. The reaction of

Table 4 Quantum-chemically calculated isotropic absolute shielding constants and ^{15}N chemical shifts (δ, in ppm)

	Isotropic shielding constants[a]		Isotropic chemical shifts		
Molecule	B1LYP	B3LYP	B1LYP	B3LYP	Exp[b]
Nitrobenzene	−125.2	−121.5	0.0	0.0	0
NO_3^-	−134.9	−130.2	9.7	8.7	9
O_2NOOH	−105.6	−105.8	−19.6	−15.7	−18
trans-$ONONO_2$	−90.6	−92.0	184.2	180.0	
	−309.4	−301.5	−34.6	−29.5	
$2\text{-}NO_2\text{-}4\text{-}F\text{–}C_6H_4OH$	−125.9	−121.5	0.7	0.0	3
$4\text{-}NO_2\text{-}4\text{-}F\text{–}C_6H_4{=}O$	−142.2	−141.8	17.0	20.3	13/14
$3\text{-}NO_2\text{-}4\text{-}F\text{–}C_6H_4OH$	−121.0	−117.0	−4.2	−4.5	−2
$4\text{-}F\text{–}C_6H_4\text{–}O\text{–}NO_2$	−103.3	−102.8	−21.9	−18.7	
$2\text{-}ONO\text{-}4\text{-}F\text{–}C_6H_4OH$	−343.6	−344.3	218.4	222.8	
$3\text{-}ONO\text{-}4\text{-}F\text{–}C_6H_4OH$	−344.6	−339.3	219.4	217.8	
$4\text{-}F\text{–}C_6H_4\text{–}O\text{–}ONO$	−291.6	−283.1	166.4	161.6	
N_2O_5	−72.3	−71.4	−52.9	−50.1	

[a] Isotropic absolute shielding constants were calculated using the GIAO protocol at the DFT/aug-cc-pVDZ/DFT/aug-cc-pVDZ level of theory. During these calculations, solvation corrections (CH_3CN for nitrobenzene, H_2O for all others) with the PCM solvation model were performed at the same level of theory. [b] Experimental values, see Table 2 and Fig. 1–3.

$$O_2{}^{15}NOOH \rightarrow [{}^{15}NO_2^{\cdot}, {}^{\cdot}O_2H]^S \rightarrow {}^{(A)}O_2{}^{15}\underline{N}OOH$$

$${}^{15}NO_2^{\cdot} + HO_2^{\cdot} \rightarrow [{}^{15}NO_2^{\cdot}, {}^{\cdot}O_2H]^F \rightarrow {}^{(E)}O_2{}^{15}\underline{N}OOH$$

$$H^{15}NO_2 + O_2{}^{15}NOOH \xrightarrow{-H_2O} ({}^{15}N_2O_5) \rightarrow [{}^{15}NO_2^{\cdot}, {}^{15}NO_3^{\cdot}]^S \rightarrow {}^{15}NO_2^+ + {}^{(E)}{}^{15}\underline{N}O_3^-$$

$${}^{15}NO_2^+ + H_2O \rightarrow H^{15}NO_3 + H^+$$

$$O_2{}^{15}NOO^- + O_2{}^{15}NOOH \xrightarrow{-O_2} [{}^{15}NO_2^{\cdot}, O_2{}^{15}NOOH^{\cdot}]^S \rightarrow {}^{15}NO_2^{\cdot} + HO^{\cdot} + {}^{(E)}{}^{15}\underline{N}O_3^-$$

Scheme 3 ^{15}N CIDNP during formation and decay of peroxynitric acid-^{15}N.

$O_2{}^{15}NOOH$ with $H^{15}NO_2$ (Schemes 1 and 3) might lead to emission in the ^{15}N NMR signal of $^{15}NO_3^-$ too, which is not observed under the applied conditions either.

Reaction of peroxynitric acid-^{15}N with *N*-acetyl-L-tyrosine and 4-fluorophenol

For elucidating the mechanism of the nitration reaction, ^{15}N CIDNP studies have been performed at pH 2. During the reaction of $O_2{}^{15}NOOH$ with Tyr, emission has been observed in 3-$^{15}NO_2$–Tyr, but the product yield is too low for quantitative studies. Therefore, *N*-acetyl-L-tyrosine (Tyrac) has been used. ^{15}N NMR spectra taken during the reaction of $O_2{}^{15}NOOH$ (0.3 M) with Tyrac (0.2 M) at 295 K and after reaction are given in Fig. 1b,c, details of the reaction in Table 2. The ^{15}N NMR signal of $O_2{}^{15}NOOH$ shows enhanced absorption, as described, signals at $\delta = 4$ ppm and $\delta =$ 18 ppm are due to 3-nitro-*N*-acetyl-L-tyrosine (3-$^{15}NO_2$–Tyrac) and 1-nitro-*N*-acetyl-L-tyrosine (1-$^{15}NO_2$–Tyrac) and appear in emission. Additionally, the ^{15}N NMR signal of $^{15}NO_3^-$ shows E, in contrast to the results during the decay of $O_2{}^{15}NOOH$. A signal at $\delta = 6$ ppm could not be assigned. After reaction, only the ^{15}N NMR signals of 3-$^{15}NO_2$–Tyrac and $^{15}NO_3^-$ are observed indicating that 1-$^{15}NO_2$–Tyrac and the unassigned product are unstable reaction intermediates.

The time dependence of the ^{15}N NMR signals reveals further details of the reaction (Table 3b). After addition of Tyrac, the ^{15}N NMR signal of $O_2{}^{15}NOOH$ is enhanced, the half-time of the reaction is shortened from 20 to 4 min and the overall reaction time (95% yield progress) from 100 to 18 min. After reaction, the signal due to 3-$^{15}NO_2$–Tyrac can only be observed by taking a large number of scans (Fig. 1c). By taking 170 scans, a yield of 2.0% has been determined in relation to the $^{15}NO_3^-$ yield.

Nitric acid and nitrous acid are effective nitration agents of Tyrac in acidic solution.[16b,22d] Both are formed during the decomposition of peroxynitric acid (Scheme 1). With the aim of excluding them as nitrating agents, experiments were performed with $H^{15}NO_3$ (0.1 M) and $Na^{15}NO_2$ (0.3 M) at pH 2. Tyrac is not nitrated under these conditions, and ^{15}N CIDNP effects are not observed either.

The occurrence of ^{15}N CIDNP indicates that the nitration proceeds *via* free radicals. The ^{15}N CIDNP effects are identical to those using peroxynitrous acid-^{15}N as the nitrating agent and are explained as described earlier by reactions of radical pairs $[{}^{15}NO_2^{\cdot}, \text{Tyrac}^{\cdot}]^F$ formed by diffusive encounters of $^{15}NO_2^{\cdot}$ and phenoxyl-type radicals Tyrac$^{\cdot}$, (Scheme 4).[16] $NO_2^{\cdot}$ is known to generate radicals GlyTyr$^{\cdot}$ very efficiently from GlyTyr;[30] $HO_2^{\cdot}$ might readily be oxidized by phenolic compounds.[31a] The conclusions are supported by calculations of Gibbs energies of the reaction of $NO_2^{\cdot}$ with phenol (8.7 kcal mol^{-1}) and of $HO_2^{\cdot}$ with phenol (1.4 kcal mol^{-1}) (Table 5, entries 1 and 2).

$${}^{15}NO_2^{\cdot} + \text{Tyrac} \rightarrow H^{15}NO_2 + \text{Tyrac}^{\cdot}$$

$$HO_2^{\cdot} + \text{Tyrac} \rightarrow H_2O_2 + \text{Tyrac}^{\cdot}$$

$${}^{15}NO_2^{\cdot} + \text{Tyrac}^{\cdot} \rightarrow [{}^{15}NO_2^{\cdot}, \text{Tyrac}^{\cdot}]^F \rightarrow {}^{(E)}1\text{-}{}^{15}\underline{N}O_2\text{-Tyrac}, \; {}^{(E)}3\text{-}{}^{15}\underline{N}O_2\text{-Tyrac}$$

Scheme 4 ^{15}N CIDNP during reaction of peroxynitric acid-^{15}N with *N*-acetyl-L-tyrosine.

The enhancement factor E of the nuclear polarization has been determined using eqn (1), see Table 6. An E value of -1100 is derived from the ^{15}N NMR signals of 3-$^{15}NO_2$–Tyrac (Table 3b). It is comparable with that found during nitration of Tyrac with peroxynitrous acid-^{15}N in the presence of sodium bicarbonate ($E = -1350$).[16b]

Table 5 Quantum-chemically calculated Gibbs energies and aqueous solvation energies

Entry	Reaction[a]	$\Delta_R G_g$ [b]	$\Delta_R E_{solv}$ [c]	$\Delta_R G_{aq}$ [d]
1	$PhOH + NO_2^{\cdot} \rightarrow PhO^{\cdot} + HNO_2$	7.6	1.1	8.7
2	$PhOH + HO_2^{\cdot} \rightarrow PhO^{\cdot} + H_2O_2$	−1.3	2.7	1.4
3	$O_2NOOH + HNO_2 \rightarrow H_2O + N_2O_5$	−25.9	7.2	−18.6
4	$O_2NOOH + HNO_2 \rightarrow H_2O + NO_2^{\cdot} + NO_3^{\cdot}$	−8.4	7.3	−1.1
5	$NO_2^{\cdot} + NO_3^{\cdot} \rightarrow NO_2^+ + NO_3^-$	127.7	−139.8	−12.1
6	$O_2NOOH + N_2O_3{}^e \rightarrow HNO_2 + NO_2^{\cdot} + NO_3^{\cdot}$	−7.4	3.7	−3.7
7	$O_2NOOH + N_2O_3{}^f \rightarrow HNO_2 + NO_2^{\cdot} + NO_3^{\cdot}$	−11.8	5.6	−6.2
8	$O_2NOOH + HNO_2 + H_2O \rightarrow O_2NOOH^{\cdot -} + NO_2^{\cdot} + H_3O^+$	196.6	−146.6	50.0
9	$PhOCH_3 + HO_2^{\cdot} \rightarrow PhOCH_2^{\cdot} + H_2O_2$	8.1	−2.6	5.4
10	$PhOCH_3 + NO_2^{\cdot} \rightarrow PhOCH_2^{\cdot} + HNO_2$	18.5	−4.1	14.4
11	$O_2NOOH + PhOCH_3 \rightarrow O_2NOOH^{\cdot -} + PhOCH_3^{\cdot +}$	159.7	−95.0	64.7
12	$PhOCH_3 + NO_2^{\cdot} \rightarrow PhOCH_3^{\cdot +} + NO_2^-$	138.6	−109.1	29.6
13	$PhOCH_3 + NO^+ \rightarrow PhOCH_3^{\cdot +} + NO^{\cdot}$	−25.2	38.2	12.9
14	$PhOCH_3 + N_2O_5 \rightarrow PhOCH_3^{\cdot +} + NO_3^- + NO_2^{\cdot}$	111.0	−106.3	4.7
15	$PhOCH_3 + NO_3^{\cdot} \rightarrow PhOCH_3^{\cdot +} + NO_3^-$	93.6	−106.4	−12.9

[a] Thermodynamic properties were calculated using the complete basis set (CBS-Q) methodology. [b] Gas phase data (kcal mol^{-1}). [c] Solvation corrections from (U)HF/6-31(+)G(d)//CBS-Q single point calculations with the PCM-UAHF solvation model for water (kcal mol^{-1}). [d] $\Delta_R G_{aq} = \Delta_R G_g + \Delta_R G_{solv}$ (kcal mol^{-1}). [e] *trans-trans*-ONONO. [f] *cis-trans*-ONONO.

Table 6 Enhancement factors E of ^{15}N CIDNP in nitration products of *N*-acetyl-L-tyrosine and 4-fluorophenol

Compound	Nitrating agent	T_1 value (s)	E value	Reference
3-NO_2–Tyrac	$O^{15}NOOCO_2^-$	24	−1350	16*b*
	$O_2{}^{15}NOOH$	24	−1100	This work
2-NO_2-4-F–C_6H_4OH	$H^{15}NO_2$	96	−1090	23*d*
	$H^{15}NO_3$	96	−1202	23*d*
	$O^{15}NOOH$	96	−890	16*a*
	$O^{15}NOOCO_2^-$	96	−1110	16*a*
	$O_2{}^{15}NOOH$	96	−1250	This work
			−1222[a]	23*d*

[a] Calculated following Pedersen's treatment of the radical pair theory.[25d]

The nitration of 4-fluorophenol (4-F–C_6H_4OH) has been investigated by ^{15}N CIDNP using different nitrating agents like nitrous acid and nitric acid,[23d] peroxynitrous acid and its CO_2 adduct.[16a] Additionally, an E value has been calculated using quantitative treatments of the radical pair theory.[23] Furthermore, the nitration reaction of 4-F–C_6H_4OH by nitrous acid has been thoroughly investigated.[31b]

$O_2{}^{15}NOOH$ has been generated *in situ* using $Na^{15}NO_2$ (0.3 M) and H_2O_2 (1 M). After that, 4-F–C_6H_4OH has been added to the solution. The ^{15}N CIDNP effects are shown in Fig. 2 and described in the Tables 2 and 3. They are similar to those observed during the reaction of $O_2{}^{15}NOOH$ with Tyrac. After adding 4-F–C_6H_4OH, the intensity of the ^{15}N NMR signal of $O_2{}^{15}NOOH$ is enhanced by a factor of 3, the half-life time for the decay of peroxynitric acid is 4 min and the reaction time 15 min (at 95% completion). The enhancement is smaller than during reaction with authentic peroxynitrite-^{15}N which might be the consequence of a $O_2{}^{15}NOOH$ formation yield of less than 50% under the given conditions. The ^{15}N NMR signal of $^{15}NO_3^-$ also appears in emission. The ^{15}N NMR spectra of the stable product 2-nitro-4-fluorophenol (2-$^{15}NO_2$-4-F–C_6H_4OH) and of the intermediate 4-nitro-4-fluorocyclohexadien-1-one (4-$^{15}NO_2$-4-F–C_6H_4=O) show emission as was observed earlier.[16,23] Additionally, two small emission lines at $\delta = -2$ and $\delta = 4$ appear. The first one is tentatively assigned to 3-$^{15}NO_2$-4-F–C_6H_4OH. After reaction, only the ^{15}N NMR signals due to $^{15}NO_3^-$ and 2-$^{15}NO_2$-4-F–C_6H_4OH are observed. The assignment of the ^{15}N NMR signals is supported by calculating the ^{15}N NMR chemical shifts with the gauge-including atomic orbitals method performed at the DFT/aug-cc-pVDZ level of theory (Table 4).

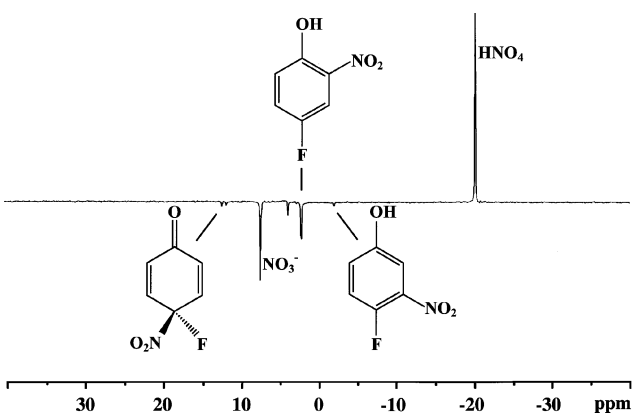


Fig. 2 ^{15}N NMR spectrum of peroxynitric acid-^{15}N in H_2O at pH 2 and 298 K taken 4 min after adding 4-fluorophenol to the solution (1 pulse).

From ^{15}N NMR spectra taken after reaction, the yield of 2-$^{15}NO_2$-4-F–C_6H_4OH has been determined to be 0.2% in relation to $^{15}NO_3^-$ indicating that nitration of 4-F–C_6H_4OH with O_2NOOH is only a side reaction.

The ^{15}N CIDNP effects in the nitration products are explained in an analogous manner as described[23d] (Scheme 5). From the time dependence of the ^{15}N NMR signal of 2-$^{15}NO_2$-4-F–C_6H_4OH, an E value of −1250 is deduced which agrees well with those found during nitration reactions with nitrous acid, nitric acid and peroxynitrous acid with and without bicarbonate as well as a calculated one using Pedersen's formulation of the radical pair theory[25d] (Table 6).

$$^{15}NO_2^{\bullet} + \text{4-F-}C_6H_4OH \rightarrow H^{15}NO_2 + \text{4-F-}C_6H_4O^{\bullet}$$

$$HO_2^{\bullet} + \text{4-F-}C_6H_4OH \rightarrow H_2O_2 + \text{4-F-}C_6H_4O^{\bullet}$$

$$^{15}NO_2^{\bullet} + \text{4-F-}C_6H_4O^{\bullet} \rightarrow [^{15}NO_2^{\bullet}, \text{4-F-}C_6H_4O^{\bullet}]^F \rightarrow \text{2-}^{15}\underset{(E)}{\underline{N}}O_2\text{-4-F-}C_6H_4OH,$$
$$\text{3-}^{15}\underset{(E)}{\underline{N}}O_2\text{-4-F-}C_6H_4OH,\ \text{4-}^{15}\underset{(E)}{\underline{N}}O_2\text{-4-F-}C_6H_4{=}O$$

Scheme 5 ^{15}N CIDNP during reaction of peroxynitric acid-^{15}N with 4-fluorophenol.

Recombination of free radicals $NO_2^{\bullet}$ and 4-F–$C_6H_4O^{\bullet}$ might lead to 4-F–C_6H_4–O–NO_2 or nitrite-type products like 4-F–C_6H_4–O–ONO, 2-ONO-4-F–C_6H_4OH, 3-ONO-4-F–C_6H_4OH and 4-ONO-4-F–C_6H_4=O. The unassigned signal at $\delta = 4$ ppm might be due to one of these products. Calculations of the ^{15}N chemical shift values show that this can be excluded, see Table 4.

The enhancement of the A type effect observed in the ^{15}N NMR signal of $O_2{}^{15}NOOH$ in the presence of Tyrac and 4-F–C_6H_4OH is explained by scavenging of free radicals $^{15}NO_2^{\bullet}$ and $HO_2^{\bullet}$ (Schemes 4 and 5) which cancels the E type polarization built up in radical pairs $[^{15}NO_2^{\bullet},.O_2H]^F$ formed by free radical encounters of $^{15}NO_2^{\bullet}$ and $HO_2^{\bullet}$ (Scheme 3).

The E type effect in the ^{15}N NMR signal of $^{15}NO_3^-$ has not been observed in the absence of phenolic compounds and must therefore be due to a reaction of $O_2{}^{15}NOOH$ with Tyrac and 4-F–C_6H_4OH or one of the reaction products (Schemes 4 and 5). It will be explained as a consequence of the reaction of $O_2{}^{15}NOOH$ with $H^{15}NO_2$ which is a reaction intermediate. This reaction is described in the literature.[8a] Nuclear polarization is generated in radical pairs $[^{15}NO_2^{\bullet}, {}^{15}NO_3^{\bullet}]^S$ formed by disproportionation between $H^{15}NO_2$ and $O_2{}^{15}NOOH$ followed by electron transfer between radicals $^{15}NO_2^{\bullet}$ and $^{15}NO_3^{\bullet}$ (Scheme 3). $^{15}N_2O_5$ might be an intermediate of the reaction, since it has been reported that the dissociation

of N_2O_5 in aqueous solution to NO_2^+ and NO_3^- ($k > 10^4$ s^{-1}) is about 5 times faster than the direct hydrolysis reaction.[32] However, no signal could be observed which might be assigned to N_2O_5 (Table 4). In any case, NO_2^+ is not stable under these conditions.[33] Following this interpretation, the nuclear polarization in the ^{15}N nuclei of $^{15}NO_3^-$ is of *c* type giving emission (Scheme 2, R = $^{15}NO_3^{\bullet}$) because of $g(NO_3^{\bullet}) > g(NO_2^{\bullet})$.[34] An *e* type polarization built up in radical pairs $[^{15}NO_2^{\bullet},.O_2H]^S$ might be transferred to $^{15}NO_3^-$ by radicals $^{15}NO_2^{\bullet}$ leading to emission in the ^{15}N NMR signal of $^{15}NO_3^-$ too. ^{15}N CIDNP effects of this type have been observed.[16c] However, they are much weaker than *c* type effects.[16c,24b]

The explanation is supported by the results of quantum chemical calculations (Table 5). The reactions of HNO_2 with O_2NOOH leading to either N_2O_5 or $NO_2^{\bullet}$ and $NO_3^{\bullet}$ are predicted to be exergonic as well as the reaction of $NO_2^{\bullet}$ with $NO_3^{\bullet}$ (entries 3–5). From an energetical point of view, the reaction of O_2NOOH with N_2O_3, which is always present in solutions containing nitrous acid, might give radical pairs $[NO_2^{\bullet}, NO_3^{\bullet}]^S$ too (entries 6 and 7). A possible contribution of N_2O_3 to the radical pair formation cannot be proven but it follows from the equilibrium constant of the reaction between nitrous acid and its corresponding anhydride ($3^{\bullet}10^{-3}$ M^{-1}) that the concentration of N_2O_3 is some orders of magnitude lower than the concentration of HNO_2.[35] The reaction of $O_2{}^{15}NOOH$ with $H^{15}NO_2$ might form radicals $O_2{}^{15}NOOH^{\bullet-}$ and $^{15}NO_2^{\bullet}$ leading to radical pairs $[^{15}NO_2^{\bullet}, O_2{}^{15}NOOH^{\bullet-}]^S$ and emission in the ^{15}N NMR signal of $^{15}NO_3^-$. We reject this possibility because of energetic reasons (entry 8).

The ^{15}N NMR signal of $^{15}NO_3^-$ is generally expected to show emission during reaction of $O_2{}^{15}NOOH$ with reducing agents if free radicals $^{15}NO_2^{\bullet}$ and/or $HO_2^{\bullet}$ are involved. For proving this, reactions of $O_2{}^{15}NOOH$ with the radical scavengers ascorbic acid, glutathione and cysteine have been studied. Qualitative ^{15}N CIDNP investigations during reactions of these compounds with $O_2{}^{15}NOOH$ will be described. Nitration reactions of activated non-phenolic aromatics might occur *via* free radicals too.[22,23] To set about proving this, the reaction of $O_2{}^{15}NOOH$ with 4-methoxyphenylacetic acid has been performed in the probe of a ^{15}N NMR spectrometer too.

Reaction of peroxynitric acid-^{15}N with ascorbic acid, glutathione and cysteine

Ascorbic acid AH_2 and its anion AH^- (pK_a 4.25) are known to react with $HO_2^{\bullet}$ as well as with $NO_2^{\bullet}$ to give the dehydrogenated radical $AH^{\bullet}$ (pK_a −0.45) and dehydroascorbic acid A (Scheme 6).[36] It should therefore be oxidized by peroxynitric acid in an indirect manner. After adding ascorbic acid to a solution of $O_2{}^{15}NOOH$ in H_2O at pH 2, the ^{15}N NMR signals of $O_2{}^{15}NOOH$ and of $^{15}NO_3^-$ show A and E as described during reaction of $O_2{}^{15}NOOH$ with phenolic compounds. A ^{15}N NMR spectrum taken 3 min after starting the reaction of $O_2{}^{15}NOOH$ with ascorbic acid is shown in Fig. 3a, the assignment of the signals and their time dependencies in Tables 2 and 3. Additional ^{15}N CIDNP signals are not observed.

$$HO_2^{\bullet} + AH_2 \rightarrow H_2O_2 + A^{\bullet-} + H^+ \quad (k = 1.6{\cdot}10^4\ M^{-1}s^{-1}\ [36a])$$
$$NO_2^{\bullet} + AH^{\bullet} \rightarrow HNO_2 + A^{\bullet-} \quad (k = 1.8{\cdot}10^7\ M^{-1}s^{-1}\ [36b])$$
$$HO_2^{\bullet} + A^{\bullet-} \rightarrow HO_2^- + A \quad (k = 5.9{\cdot}10^9\ M^{-1}s^{-1}\ [36a])$$

Scheme 6 Reaction of peroxynitric acid-^{15}N with ascorbic acid.

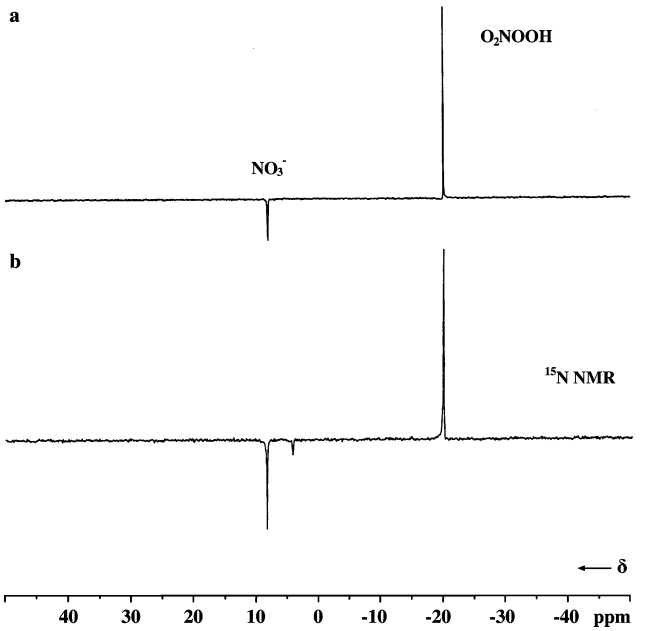


Fig. 3 ^{15}N NMR spectra of solutions of peroxynitric acid-^{15}N in H_2O at pH 2 and 298 K taken (a) 3 min after adding ascorbic acid (1 pulse), (b) 3 min after adding 4-methoxyphenylacetic acid (1 pulse).

After 6 min, the ^{15}N NMR signal of $^{15}NO_3^-$ changes from emission to absorption. 9 min later, the ^{15}N NMR signal of $O_2{}^{15}NOOH$ disappears. The ^{15}N CIDNP effects observed in the presence of ascorbic acid are explained as described (Schemes 2 and 6).

The kinetic constants of the scavenging reactions are known (Scheme 6). This allows us to compare the ^{15}N CIDNP effects with the progress of the reaction. $HO_2^{\bullet}$ should be scavenged efficiently by ascorbic acid at pH 2, and the half-life time of O_2NOOH should therefore be about 0.7 min (Schemes 1 and 6). The reaction should be finished before taking the first spectrum after adding ascorbic acid to the solution. The time behaviour of the ^{15}N NMR signal of $^{15}NO_3^-$ is therefore caused by nuclear relaxation ($T_1 = 140$ s [37]) and not by the reaction. We think that this is also the case for the decay of the signal of $O_2{}^{15}NOOH$. The relaxation time of the ^{15}N nucleus is unknown; it should be of the same order of magnitude ($T_1 = 140$ s).

The reaction of $O_2{}^{15}NOOH$ with glutathione is finished before it is possible to take a ^{15}N NMR spectrum. It is much faster than the radical decay of $O_2{}^{15}NOOH$. It follows that a direct oxidation without participation of free radicals occurs. During reaction of $O_2{}^{15}NOOH$ with cysteine, a spectrum could be taken before peroxynitric acid-^{15}N completely disappeared. The ^{15}N NMR signal of $^{15}NO_3^-$ does not show emission during this reaction. It is concluded that there is a direct oxidation of glutathione and cysteine by peroxynitric acid, too. More detailed conclusions concerning the reaction mechanism cannot be drawn. Radical reactions as described before seem not to be of any importance.

Reaction of peroxynitric acid-^{15}N with 4-methoxyphenylacetic acid

During reaction of $O_2{}^{15}NOOH$ with 4-methoxyphenylacetic acid ($MeO–C_6H_4–CH_2–COOH$) the ^{15}N NMR signals of $O_2{}^{15}NOOH$ and $^{15}NO_3^-$ appear in A and E, respectively, and the decay rate of $O_2{}^{15}NOOH$ is enhanced (half-life time 8 min) as was observed

during reaction of peroxynitric acid with Tyrac, 4-F–C_6H_4OH and ascorbic acid. Additionally, an emission signal is observed at $\delta = 3$, see Fig. 3b and Table 2, which is assigned to 3-nitro-4-methoxyphenylacetic acid (3–$^{15}NO_2$-4-MeO–C_6H_4–CH_2–COOH). After reaction, the product could not be observed by ^{15}N NMR spectroscopy, indicating a product yield <0.1% in relation to $^{15}NO_3^-$. At the beginning, the concentration of MeO–C_6H_4–CH_2–COOH is much smaller than that of peroxynitric acid. Nevertheless, the emission in the ^{15}N NMR signal of $^{15}NO_3^-$ is observed throughout the reaction, indicating that the nitration of the aromatic is only a side reaction. This is confirmed by the low yield of the nitration product.

The generation of the nuclear polarization in the ^{15}N NMR signals of $O_2{}^{15}NOOH$ and $^{15}NO_3^-$ is explained as above. Hydroperoxy radicals $HO_2^\bullet$ are trapped by 4-methoxyphenylacetic acid leading to the accelerated decay of $O_2{}^{15}NOOH$ and the formation of $H^{15}NO_2$ (Schemes 1 and 7). To our knowledge, the reactions are not described in the literature. Calculations concerning the reaction of $HO_2^\bullet$ with anisole show that the reaction might be exergonic (Table 5, entry 9). The reaction of $NO_2^\bullet$ with MeO–C_6H_4–CH_2–COOH which might inhibit the decay of peroxynitric acid, too, seems to be less probable (Table 5, entry 10).

$$HO_2^\bullet + \text{MeO-C}_6\text{H}_4\text{-CH}_2\text{-COOH} \rightarrow H_2O_2 + \text{MeO-C}_6\text{H}_4\text{-CH}^\bullet\text{-COOH}$$
$$HO_2^\bullet + \text{MeO-C}_6\text{H}_4\text{-CH}_2\text{-COOH} \rightarrow H_2O_2 + {}^\bullet\text{CH}_2\text{O-C}_6\text{H}_4\text{-CH}_2\text{-COOH}$$

$$\text{ArH} - e^- \rightarrow \text{ArH}^{+}$$
$$^{15}NO_2^\bullet + \text{ArH}^{+} \rightarrow [^{15}NO_2^\bullet, \text{ArH}^{+}]^F \rightarrow {}^{15}\underset{(E)}{N}O_2\text{-Ar} + H^+$$

Scheme 7 ^{15}N CIDNP during reaction of peroxynitric acid-^{15}N with 4-methoxyphenylacetic acid (ArH).

The E type effect observed in the nitration product is explained by analogy with the ^{15}N CIDNP effects observed during nitration reactions of activated aromatic compounds with nitric acid or nitrous acid.[22a,b,23] The nuclear polarization is built up in radical pairs formed by diffusive encounters of $^{15}NO_2^\bullet$ and radical cations of MeO–C_6H_4–CH_2–COOH, ArH^+ (Scheme 7).

Radical cations $ArH^{\bullet+}$ are generated by oxidation of the aromatic compound with reactive nitrogen species. Oxidation by O_2NOOH is excluded because of energetical reasons (Table 5, entry 11). Additionally, the possibility of nitration reactions with $H^{15}NO_2$ and $H^{15}NO_3$ has been investigated. At pH 1.5, no reaction has been found using $Na^{15}NO_2$ (0.3 M) or $H^{15}NO_3$ (0.1 M). Under strong acidic conditions (~10% sulfuric acid), 4-methoxyphenylacetic acid is nitrated with $Na^{15}NO_2$ (0.3 M) as well as with $H^{15}NO_3$ (0.1 M), in a similar manner as has been observed with anisole.[23b] The nitration product also shows ^{15}N CIDNP. It follows that reactive nitrogen species other than O_2NOOH and HNO_2 must be responsible for the formation of the aromatic radical cations at pH 2.

Aromatic radical cations $ArH^{\bullet+}$ might be formed by intermediate nitrogen species like $NO_2^\bullet$, NO_2^+, NO^+, N_2O_5 or $NO_3^\bullet$ which are discussed as electron acceptors in the literature.[22b] The oxidation by $NO_2^\bullet$ is energetically not favoured which follows from the reported oxidation potentials of anisole (E_o = 1.76 V[38a]) and $NO_2^\bullet$ (E_o = 0.9–1.0 V[38b]) as well as from our calculations (Table 5, entry 12). The oxidation by NO_2^+ might be energetically possible (E_o = 1.51 V[39]), but is unlikely as the addition of NO_2^+ to aromatic systems is favoured over the electron transfer.[22b,23a] NO^+, N_2O_5 and $NO_3^\bullet$ should be capable of oxidizing anisole (Table 5, entries 13–15) and one of them might be responsible for the formation of ArH^+. NO^+ is the oxidating species under strong acidic conditions using nitrous acid or nitric acid as nitrating agents.[22a] It might also be formed in slow concentrations at pH 2. N_2O_5 and $NO_3^\bullet$ are postulated to be present under our conditions, see Scheme 3, and are the most probable electron acceptors.

Conclusions

Reactions of peroxynitric acid with activated aromatic compounds and with ascorbic acid, glutathione and cysteine have been described at pH 2 showing nitration and oxidation. They may occur without or with participation of free radicals which has been demonstrated by ^{15}N CIDNP. L-Tyrosine, *N*-acetyl-L-tyrosine, 4-fluorophenol and 4-methoxyphenylacetic acid are nitrated. The quantitative analysis of the ^{15}N CIDNP effects shows that nitration occurs exclusively *via* free radicals. ^{15}N CIDNP is observed during reaction with ascorbic acid indicating an indirect oxidation *via* free radicals. In contrast to this, the reaction with glutathione and cysteine is faster than the decay of peroxynitric acid and does not show ^{15}N CIDNP indicating a direct oxidation without the involvement of free radicals.

At pH 7, the deprotonated peroxynitric acid does not nitrate *in situ* which makes it unlikely that peroxynitric acid has any pathophysiological importance at pH 7. However, the pH value is significantly lower in both the stomach and the lysosomes, and the reaction with glutathione might be fast enough to occur in living cells. In any case, peroxynitric acid must be discussed as a "reactive oxygen species (ROS)" as well as a "reactive nitrogen species (RNS)".[40]

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