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Reaction of Vitamin E Compounds with *N*-Nitrosated Tryptophan Derivatives and Its Analytical Use

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Abstract: We recently showed that nitrosated tryptophan residues may act as endogenous nitric oxide storage compounds. Here, a novel reaction of nitrosotryptophan derivatives is described, in the form of the release of nitric oxide from *N*-nitrosotryptophan derivatives initiated either by α-tocopherol or by its water-soluble form trolox. α-Tocopherol and trolox were found to release stoichiometric amounts of nitric oxide from *N*-acetyl-*N*-nitrosotryptophan as well as from the nitrosotryptophan residue in albumin. The reaction proceeds both in water and in lipophilic solution and reconstitutes the indole moiety of the tryptophan molecule quantitatively. During this reaction, α-tocopherol- and trolox-derived phenoxyl-type radicals were identified as intermediates by ESR spectrometry. The chemical mechanism of the NO-releasing process was established. Since *S*-nitrosothiols do not react under the applied conditions, it is suggested that the trolox-dependent release of nitric oxide may be utilizable for the detection of *N*-nitrosotryptophan residues in biological samples. Furthermore, as *N*-nitrosotryptophan derivatives do not undergo spontaneous decay in lipophilic environments, vitamin E may have the so far unrecognized function of preventing the accumulation of *N*-nitrosotryptophan residues to toxic concentrations in biological systems.

Keywords: nitric oxide · nitrosothiol · tryptophan · vitamins

Introduction

In the human body, endogenous nitric oxide reacts rapidly with a variety of metalloproteins, preferably with heme enzymes, and may in this way be inactivated.[1] In order to maintain its physiological functions under such conditions, NO• is believed to be protected by the formation of so-called "storage compounds", which may act as NO• donors when needed. Accordingly, interest in nitric oxide storage compounds has strongly increased in recent years.

Various forms of storage compounds that may prevent the artificial scavenging of NO• in intra- and extracellular milieux have been proposed. Beside low-molecular-weight *S*-nitroso compounds, such as GSNO,[2,3] *S*-nitroso and *N*-nitroso residues in albumin[4] have also been investigated in this context. Zhang et al.[5] compared the NO_2^-/HCl-dependent nitrosation of bovine serum albumin with the nitrosation of carboxymethyl–bovine serum albumin (CM-BSA) and found a regiospecific nitrosation at Trp214 in CM-BSA. In physiological environments, different modes of nitrosation have been described in the literature. N_2O_3, generated by autoxidation of NO•,[6,7] rapidly nitrosates thiols/thiolates,[8] as well as tryptophan derivatives.[9] A peroxynitrite-driven nitrosation of thiols[10] and *N*-terminal-blocked tryptophan derivatives has also been reported.[11–13]

In aqueous solution around pH 7, *N*-nitroso derivatives undergo slow hydrolysis to give the parent amine and nitrite in a proton-catalysed process, followed by nucleophilic attack of OH^-/H_2O (Scheme 1).[14]

In accord, the rate of hydrolysis is accelerated in the presence of various nucleophiles Y^-, which in essence represents a transnitrosation reaction. Nitroso compounds (YNO) from halide or pseudohalide nucleophiles are hydrolysed rapidly,[15] but formation of hydrolytically more stable transnitrosation products is well known: for example, for *n*-butanol (*n*BuONO),[16] H_2O_2 (ONOOH)[17] or thiols (RSNO).[18] However, in cases in which a stabilized radical Y• can be produced, the intermediate YNO may also undergo facile homolysis to release NO• [Eq. (1)].

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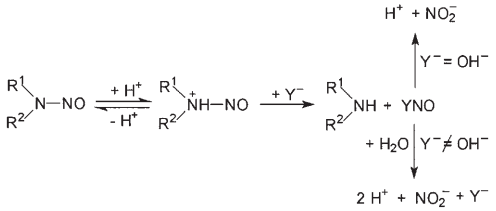
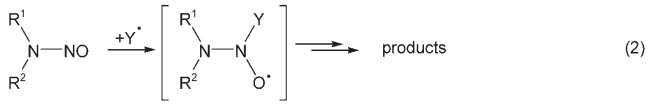
$$YNO \rightleftharpoons Y^{\bullet} + NO^{\bullet} \quad (1)$$



Scheme 1. Proton-catalyzed and nucleophile-accelerated hydrolysis of *N*-nitrosamines.

Further, for sufficiently reactive and sterically unhindered radicals $Y^{\bullet}$, the decay of the N-nitroso compound might then be further accelerated by attack of $Y^{\bullet}$ on the nitroso group [Eq. (2)].



(2)

This has recently been demonstrated to be the case for Y^{-} = ascorbate and certain phenolic compounds (that is, catechol derivatives).[19,20]

Among the naturally occurring phenolic compounds, vitamin E plays a major role. α-Tocopherol is the major constituent of natural vitamin E and is the most important lipid-soluble antioxidant. It is essential for the prevention of lipid peroxidation[21,22] (termination of radical chain reactions) and for cellular signalling.[23] It has been reported that vitamin E additionally inhibits smooth muscle cell proliferation,[24,25] decreases protein kinase C activity[23] and increases $NO^{\bullet}$ levels through regulation of eNOS.[26]

Here we demonstrate that α-tocopherol and its water-soluble analogue trolox release $NO^{\bullet}$ quantitatively from *N*-terminal blocked *N*-nitrosotryptophan derivatives, not only from the isolated molecules but also from *N*-nitrosotryptophan residues in proteins. This reaction is not catalysed by heavy metal ions and occurs both in aqueous and in nonaqueous solution.

Results

Kinetics of the α-tocopherol/trolox–NANT reaction: In order to corroborate the existence of a reaction between α-tocopherol and *N*-acetyl-*N*-nitrosotryptophan (NANT), the decay of NANT (1 mM) in lipophilic medium (ethyl acetate), was monitored spectrophotometrically at 335 nm and 37 °C in the absence and in the presence of α-tocopherol (1 mM). In the absence of α-tocopherol, the concentration of NANT decreased by 7% within 60 min, corresponding to a first-order rate constant of $k_{obs} = (1.9 \pm 0.1) \times 10^{-5}\,s^{-1}$. In the presence of α-tocopherol, the decay of NANT was accelerated by a factor of 4.5 with an apparent rate constant of $k_{obs} = (8.4 \pm 0.1) \times 10^{-5}\,s^{-1}$ (Figure 1), thus confirming an α-tocopherol-induced decomposition of NANT. During this reaction, α-tocopheryl radicals and nitric oxide are being produced, as indicated by ESR experiments (see below).

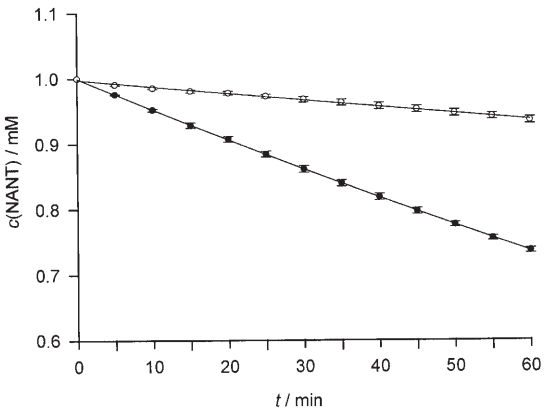


Figure 1. Decay of NANT (1 mM) in ethyl acetate, monitored spectrophotometrically at 335 nm in the absence (open symbols) and in the presence (closed symbols) of α-tocopherol (1 mM) at 37 °C. Each value represents the average (±s.d.) of at least three independent runs.

Since α-tocopherol is a lipophilic compound, the influence of the polarity of the environment on the rate of the α-tocopherol–NANT reaction (100 μM each) was analysed by employing various organic solvents with a range of dielectric constants. However, the decay rate of NANT was only weakly affected by the polarity (dielectric constant) of the solvent, varying inversely by just a factor of two between ethyl acetate ($\varepsilon = 6.4$) and dimethyl sulfoxide ($\varepsilon = 46.7$) (Table S1, Supporting Information). This observation clearly argues against the formation of charged intermediates in the α-tocopherol-mediated decomposition of NANT.

In order to investigate the foregoing reaction in aqueous solution, we employed the water-soluble α-tocopherol analogue trolox [(*R*)-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid]. As mentioned in the Introduction, NANT undergoes slow, proton-catalysed hydrolysis in aqueous solution to give exclusively NAT and nitrite. In previous papers,[17,19,20,27–30] this process was reported to be of first order, and from the data of Meyer et al.[14] an apparent room-temperature rate constant of $k_{obs} = (6.6 \pm 0.1) \times 10^{-5}\,s^{-1}$ at pH 7.0 can be extrapolated. This value was reproduced well in this study [$k_{obs} = (6.1 \pm 0.1) \times 10^{-5}\,s^{-1}$]. However, close inspection of the decay traces revealed that the decomposition of NANT—both in the absence and in the presence of trolox—could not satisfactorily be described by a first-order rate law, nor by a second-order rate law (Figure 2). Excellent empirical fits ($r^2 \geq 0.998$ in all cases) were achieved by assuming a (minor) contribution of a linear (apparent zeroth order) change in absorbance to a first-order decay. In the absence of trolox, the kinetic analysis gave a ca. 50% lower first-order rate constant for the dominant first-order

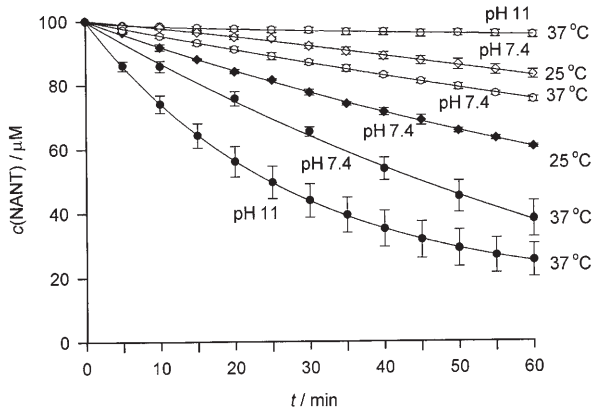


Figure 2. Decay of NANT (100 μM) in phosphate buffer at pH 7.4 and 11, monitored spectrophotometrically at 335 nm in the absence (open symbols) and in the presence (closed symbols) of trolox (100 μM) at 25 and 37 °C, respectively. Each value represents the average (±s.d.) of at least three independent runs. Continuous lines are empirical fits to a superposition of a first- and zeroth-order rate law.

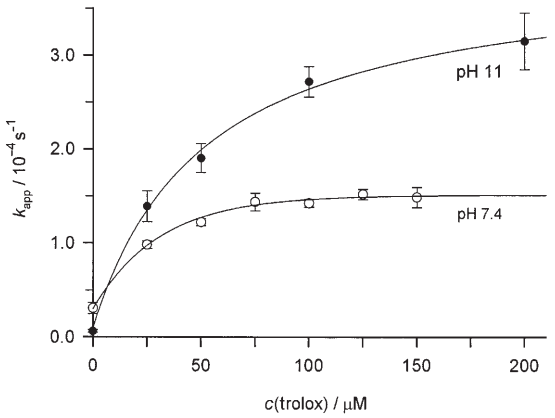


Figure 3. Dependence of the apparent first-order rate constants for decay of NANT (100 μM) on the concentration of trolox in phosphate buffer at pH 7.4 and 11, $T = 25$ °C. Each value represents the average (±s.d.) of at least three independent experiments. The continuous lines are empirical fits to a hyperbolic function.

process at pH 7.4 and 25 °C [$k_{app}^{298\,K} = (3.1 \pm 0.6) \times 10^{-5}\,s^{-1}$]. At 37 °C, the decay is about two times faster [$k_{app}^{310\,K} = (6.5 \pm 0.6) \times 10^{-5}\,s^{-1}$]. In accord with the reported proton catalysis of hydrolysis,[14,27] NANT was found to be more stable at pH 11, its concentration decreasing by only 5% within 60 min with a rate constant of $k_{app}^{310\,K} = (8.0 \pm 1.1) \times 10^{-6}\,s^{-1}$ at 37 °C (Figure 2), in line with our previously reported data.[30] (At this temperature, evaluation in terms solely of a first-order process gave a slightly higher k_{obs} of $(1.2 \pm 0.1) \times 10^{-5}\,s^{-1}$.) At pH 7.4 and 25 °C, addition of trolox (100 μM) accelerated the decay of NANT by a factor of five [$k_{app}^{298\,K} = (1.5 \pm 0.1) \times 10^{-4}\,s^{-1}$]. At these concentrations, the decay at 37 °C was about 1.5 times faster [$k_{app}^{310\,K} = (2.1 \pm 0.3) \times 10^{-4}\,s^{-1}$]. Notably, and differently to what was observed in the absence of trolox, decay of NANT in the presence of an equimolar amount of trolox was further increased at pH 11 [$k_{app}^{310\,K} = (5.3 \pm 0.1 \times 10^{-4}\,s^{-1}$], to give a final concentration of 25.2 ± 5.1 μM after 60 min (Figure 2).

For a fixed concentration of NANT (100 μM), the dependence of the decay rate constants on the concentration of trolox (0–200 μM) was determined at 25 °C at pH values 7.4 and 11 (Figure 3). (The data for Figure 3 were evaluated by taking a minor contribution from a zeroth-order decay into account. However, evaluation in terms solely of a first-order process gave very similar values, with less than 20% difference.) As is evident from Figure 3, the decay rate constants increased nonlinearly (apparently hyperbolically) with increasing trolox concentration, to approach limiting values of about $3.5 \times 10^{-4}\,s^{-1}$ at pH 11 and $1.5 \times 10^{-4}\,s^{-1}$ at pH 7.4 for trolox concentrations greater than 200 μM. Hence, excess trolox accelerates the decay of NANT about 50-fold at pH 11, but only an about five times rate enhancement is observed at pH 7.4.

Product analysis: The trolox–NANT reaction mixtures were analysed by ^{13}C NMR spectrometry and comparison with expected, preformed products. Inspection of Figure 4a,b (only carbonyl region shown) reveals the formation of the trolox-

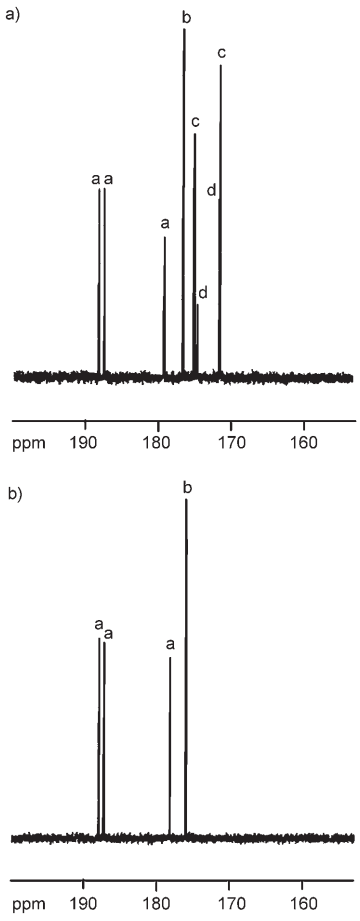


Figure 4. ^{13}C NMR spectra (carbonyl region) from: A) reaction of trolox (100 mM) with NANT (100 mM), and B) reaction of trolox (100 mM) with Fremy's salt (100 mM). Spectra were recorded in a mixture (80:20 *v/v*) of [D_6]DMSO and phosphate buffer (50 mM, pH 7.4) after a reaction period of 18 h at room temperature. Spectral assignments: a) trolox quinone, b) trolox, c) NAT, and d) NANT.

derived benzoquinone ($\delta = 188.3$, 187.6, 179.3 ppm) and of NAT ($\delta = 175.2$, 171.5 ppm) as final products from the trolox–NANT reaction. No other products could be detected (see Table S2, Supporting Information for a complete listing of the resonances).

The yields of NAT and the fraction of unconverted NANT from the NANT–trolox (90 and 100 μM, respectively) reaction were quantified in independent experiments. As demonstrated in Figure 5, NAT was stoichiometrically produced at the expense of NANT, because the sum of NAT and NANT during the reaction was constant at 88.8 ± 1.0 μM. After a reaction period of 60 min, 59.9 ± 2.7 μM NANT had been decomposed, thereby yielding 62.4 ± 4.7 μM NAT. Thus, NANT was quantitatively converted into NAT.

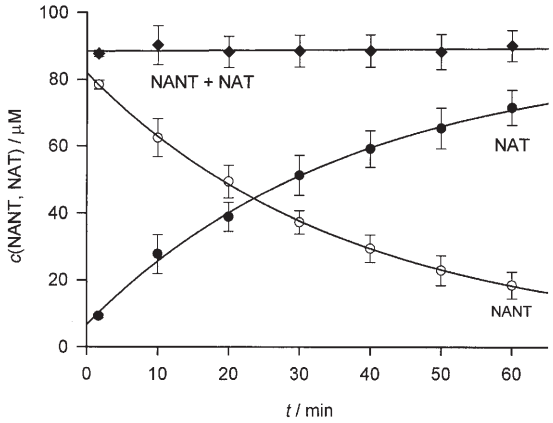


Figure 5. Decomposition of NANT and production of NAT during the trolox–NANT reaction. Decomposition of NANT (90 μM) was read at 335 nm after addition of trolox (100 μM) in phosphate buffer (50 mM, pH 11, 25 °C). NAT production was fluorimetrically determined at $\lambda_{em} = 355$ nm ($\lambda_{exc} = 270$ nm) from a 10-fold diluted aliquot of the reaction mixture. Each value represents the mean (± s.d.) of at least three independent experiments.

ESR measurements: As depicted in Equation (1), it was proposed that the transnitrosation products YNO (Y = trolox, α-tocopherol) existed in equilibrium with NO• and the corresponding phenoxyl-type radicals. Since the latter radicals are known to decay by disproportionation to the parent phenol and the related *p*-quinone and *o*-quinone methide, respectively, with rate constants of $k_3 = 1180\ M^{-1}s^{-1}$ at 37 °C[31] (Scheme 2), they would be expected to attain (temporarily) stationary concentrations. This assumption was confirmed by monitoring the reactions between NANT and trolox or α-tocopherol by ESR spectrometry. The characteristic ESR patterns of the trolox radical and the α-tocopheryl radical could be instantaneously detected after mixing of the reactants (Figures 6, 7).

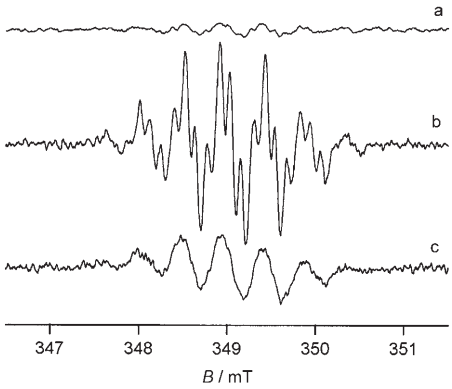


Figure 6. ESR spectrum of the trolox phenoxyl radical produced from the reaction between trolox (7.5 mM) and *N*-acetyl-*N*-nitrosotryptophan (7.5 mM) in deoxygenated phosphate buffer (50 mM, pH 7.4, 18 °C): a) prior to addition of NANT, b) 10 min, and c) 24 min after addition of NANT. All spectra were recorded with identical instrument settings.

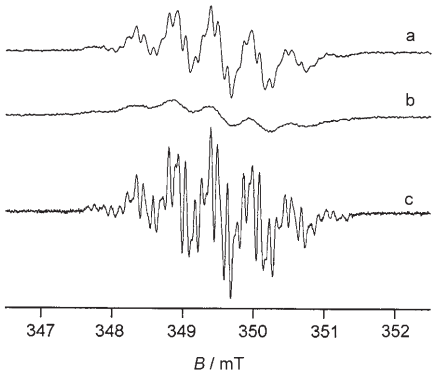
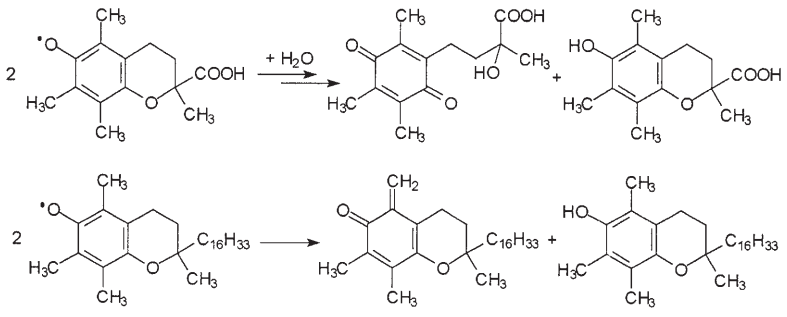


Figure 7. ESR spectrum of the α-tocopheryl radical produced from the reaction between α-tocopherol (7.5 mM) and *N*-acetyl-*N*-nitrosotryptophan (7.5 mM) in deoxygenated ethyl acetate at 18 °C: a) 2 min, and b) 27 min after mixing of the reactants. c) Spectrum from (b) after 1 min purging of the sample with argon. All spectra were recorded with identical instrument settings.



Scheme 2. Disproportionation of the phenoxyl-type trolox radical in aqueous solution (top) and of the α-tocopheryl radical in nonaqueous solution (bottom).

The good hyperfine resolutions of the initial spectra are increasingly lost with reaction duration (compare Figures 6b,c and 7a,b). This observation can easily be attributed to the accumulation of released nitric oxide, the spin exchange interaction of which with the phenoxyl-type radicals leads to the well known phenomenon of line broadening.[32] This inter-

pretation was confirmed by the recording of a well resolved spectrum of the α-tocopheryl radical after removal of NO• by briefly flushing the sample with argon (Figure 7c). Because of the continued production of NO•, this spectrum was again subject to line broadening within the next four minutes (not shown). Note that the integrated intensities of the spectra shown in Figure 6b,c and 7a–c were found to be identical within the error limits of the ESR experiment, confirming the above proposal of the attainment of steady-state levels of the radicals.

Nitric oxide release: The release of nitric oxide from the trolox–NANT reaction was first monitored with a NO•-sensitive electrode. These measurements were carried out in an open system, so the NO• level after mixing of the reactants readily advances to a steady state, controlled by the rate of NO• production versus diffusion into the gas phase and decay by autoxidation (Figure 8).

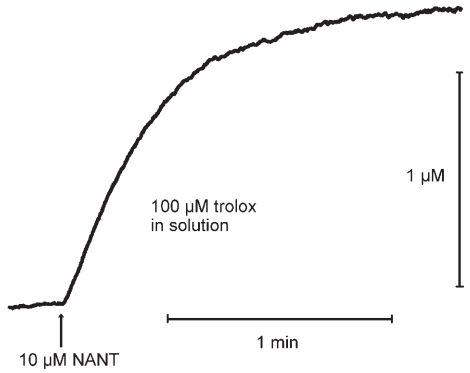


Figure 8. Trolox-induced NO•-release from *N*-acetyl-*N*-nitrosotryptophan. The steady state concentration of liberated NO• was monitored with a NO•-sensitive electrode after addition of NANT (10 μM) to trolox (100 μM) in phosphate buffer (50 mM, pH 7.4, 25 °C).

At a fixed trolox concentration of 100 μM, the steady-state NO• concentration increased apparently exponentially with increasing NANT concentration, to level off at about 2.5 μM (Figure 9).

Similar experiments were performed with α-tocopherol in an ethanol/phosphate buffer mixture (1:1). Here, a steady-state concentration of about 1.2 μM was achieved at equimolar concentrations of the reactants (Figure 9): that is, only about 50% relative to the trolox–NANT reaction in pure buffer. In any case, nitric oxide was unequivocally confirmed as a product in aqueous solution.

In order to quantify the total yield of nitric oxide from the trolox/α-tocopherol–NANT reaction, the FNOCT-4 assay was applied.[33] In contrast with the electrochemical (NO-electrode) method, which only measures the momentary concentration of liberated NO•, the FNOCT assay is an integrating method (i.e., it scavenges all nitric oxide and yields, after a rapid reduction step, stoichiometric amounts of the fluorescent dye FNOCT-4-NOH). As shown in Figure 10, the reaction between NANT and trolox (100 μM

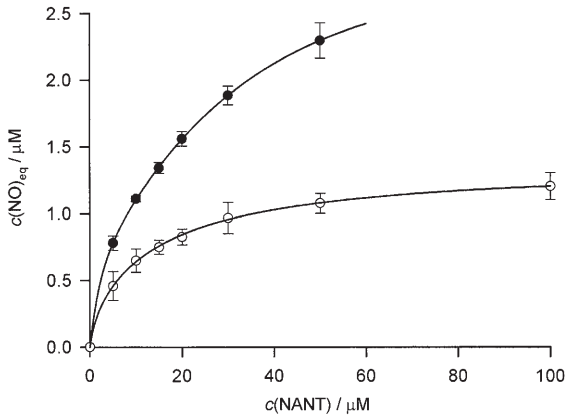


Figure 9. Steady-state concentrations of nitric oxide from the reactions between *N*-acetyl-*N*-nitrosotryptophan and trolox (closed circles) or α-tocopherol (open circles). NO• steady-state concentrations were measured at 37 °C with a NO• electrode after addition of NANT (5–100 μM) to fixed concentrations either of trolox or of α-tocopherol (100 μM each) in phosphate buffer (50 mM, pH 7.4) or 50% ethanol/phosphate buffer. Each value represents the mean (±s.d.) of at least three independent experiments.

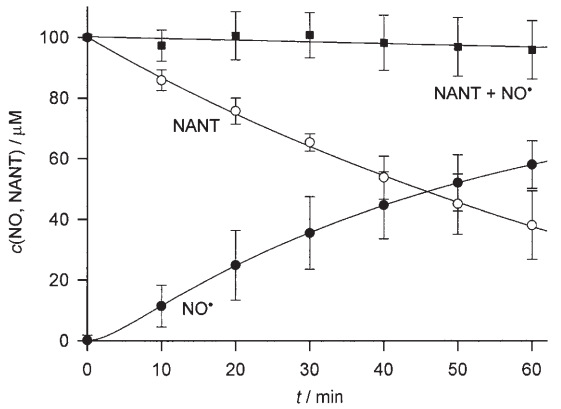


Figure 10. Quantification of nitric oxide from the reaction between *N*-acetyl-*N*-nitrosotryptophan and trolox (100 μM each) in oxygen-free phosphate buffer (50 mM, pH 7.4, 37 °C). NO• was trapped by FNOCT-4 (100 μM) and FNOCT-4-NOH was quantified fluorimetrically at $\lambda_{em}=460$ nm ($\lambda_{exc}=320$ nm). Simultaneously, residual NANT was monitored spectrophotometrically at 335 nm. Each value represents the mean (±s.d.) of at least three independent experiments.

each) at 37 °C yielded FNOCT-4-NOH (57.9±6.6 μM) after a reaction period of 60 min. A similar amount of NANT (62.0±11.2 μM) was consumed during the reaction. The sum of residual NANT and produced NO• amounted to about 100±5 μM for the whole reaction period. Thus, the trolox–NANT reaction produces NAT and NO• in a stoichiometric 1:1 ratio. It is worth noting that no increase in the fluorescence of FNOCT-4-NOH exceeding the basal decay[33] was observed from NANT in the absence of trolox. This observation clearly rules out the possibility that decomposition of NANT in the presence of reductants proceeds through initial homolysis of the N–NO bond, as has been proposed by de Biase et al.[29] According to the characteristics of the

Fischer–Ingold persistent radical effect (i.e., transient and persistent radicals from the same precursor dominantly yield the cross-reaction product),[34] decay of NANT through initial N–NO bond homolysis should not be observed in the absence of an efficient radical trap for NO• (persistent radical) and/or the simultaneously produced NANT aminyl radical (transient radical). Therefore, stoichiometric and essentially quantitative production of both nitric oxide and NAT, as observed here, contradicts homolytic cleavage of the N–NO bond of NANT as the initial step.

In addition, the apparent first-order rate constant of NANT decomposition [$k_{app} = (2.1 \pm 0.3) \times 10^{-4}\,s^{-1}$] (see above) corresponds well with the pseudo-first-order rate constant of NO• production [$k_{NO} = (3.3 \pm 0.3) \times 10^{-4}\,s^{-1}$], as extracted from the trace of Figure 10. These data exclude any significant NO•-consuming pathways, differently to what has been observed for the ascorbate–NANT reaction.[20]

α-Tocopherol–nitrosoalbumin reaction: In order to demonstrate that the trolox-/α-tocopherol-mediated release of NO• is not restricted to the model compound NANT, additional experiments were performed with *N*-nitrosated protein albumin. Only one tryptophan residue is present in albumin, thus allowing straightforward quantification. The degree of nitrosation of albumin was determined spectrophotometrically to be 82.5%. Varying concentrations of trolox (5–50 μM) were added to nitrosated albumin (100 μM) in phosphate buffer (pH 7.4, 37 °C), and the release of NO• was monitored with the NO• electrode. Trolox-induced liberation of NO• from the *N*-nitrosated tryptophan residue of the protein indeed took place (Figure 11). In analogy with the data obtained from NANT, the steady-state concentration of released NO• approached a constant value with increasing concentrations of trolox (ca. 1.1 μM NO• at ≥50 μM trolox).

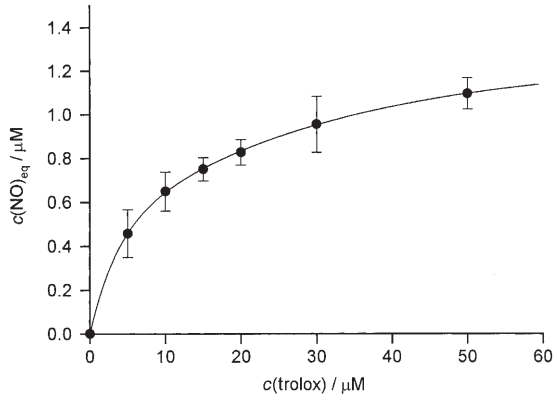


Figure 11. Release of nitric oxide from the reaction between trolox and *N*-nitrosated albumin. Steady-state concentrations of NO• were measured with the NO•-sensitive electrode after addition of trolox (5–50 μM) to *N*-nitrosated albumin (100 μM) in phosphate buffer (50 mM, pH 7.4, 37 °C). Each value represents the mean (±s.d.) of at least three independent experiments. The solid line is an empirical fit to the sum of two exponentials.

Quantification of the NO• production from the trolox–NO-albumin reaction was carried out by the FNOCT method by addition of varying amounts of NO-albumin (0–100 μM) and trolox (400 μM) to a solution of FNOCT-4 (100 μM) in phosphate buffer (pH 7.4, 25 °C). The excess of trolox was employed not only to enhance the rate of NO• release, but also to ensure rapid and complete reduction of the initial FNOCT-4-nitric oxide adduct (a nitroxide radical) to the fluorescent dye FNOCT-4-NOH.[33] The build-up of the FNOCT-4-NOH fluorescence followed a clean first-order process; initial rates of NO• production were evaluated from the first 300 s of fluorescence growth. Constant fluorescence intensities of FNOCT-4-NOH were achieved after a reaction period of 90 min, indicating completion of NO• release. The final NO• concentration, as well as the initial rates of NO• production, both showed a linear dependence on the nitrosoalbumin concentration (Figure 12), confirming that the reaction is of first order in nitrosoalbumin. With allowance for the 82.5% degree of nitrosation of albumin, a yield of 89.5±7.2% of NO• was evaluated from the regression line.

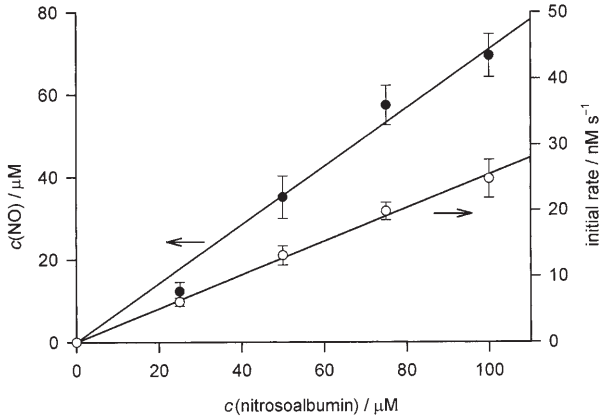


Figure 12. Quantification and initial rates of NO• release from the reaction between trolox and *N*-nitrosated-albumin. *N*-Nitroso-albumin (25–100 μM; 82.5% nitrosation) was added to a mixture of FNOCT-4 (100 μM) and trolox (400 μM) in phosphate buffer (50 mM, pH 7.4, 25 °C), and the fluorescence of FNOCT-4-NOH was monitored at $\lambda_{em} = 460$ nm ($\lambda_{exc} = 320$ nm). NO• production was evaluated from the constant fluorescence intensity after 90 min. Each value represents the mean (±s.d.) of at least three independent experiments.

Although the trolox–*N*-nitrosoalbumin reaction was slightly less effective than the trolox–NANT reaction in releasing NO•, the foregoing data clearly demonstrate that the reaction proceeds with high efficiency for *N*-nitroso-tryptophan residues in proteins.

Influence of NO• on the NANT–trolox reaction: The above experiments demonstrated that nitric oxide is released in the trolox/α-tocopherol-mediated decay of NANT as depicted in Equation (1). With regard to possible reversibility of

the NANT–trolox/α-tocopherol transnitrosation reaction, an external supply of nitric oxide would be expected to exert a retarding effect on the decay of NANT. We therefore examined the effect of additional NO• by monitoring the decay of NANT in the presence of the NO•-releasing compound MAHMA/NO. In order to exclude possible nitrosation of NAT by N_2O_3 (formed by autoxidation of NO•), these experiments were performed under hypoxic conditions (1–2 μM residual O_2). In the absence of trolox, decomposition of NANT was not significantly inhibited by NO•, because after addition of MAHMA/NO (100 μM) to NANT (100 μM, phosphate buffer pH 7.4, 37 °C) a residual NANT concentration of 97.4 ± 0.2 μM was monitored after a reaction period of 900 s [$k_{app}^{310\,K} = (1.4 \pm 0.4) \times 10^{-4}\,s^{-1}$] (Figure 13). This value

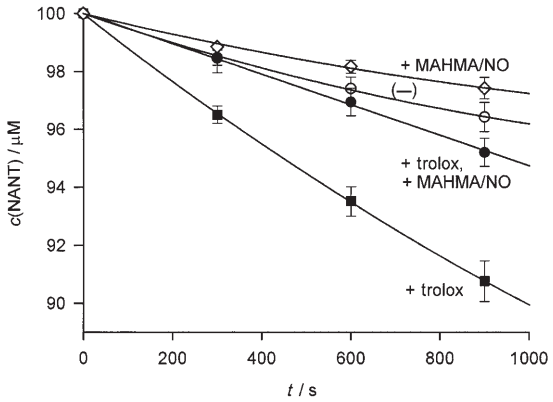


Figure 13. Influence of NO• on the decay of *N*-acetyl-*N*-nitrosotryptophan. The decay of NANT (100 μM) was followed spectrophotometrically in deoxygenated phosphate buffer (50 mM, pH 7.4, 37 °C) in the absence of any additives (open circles), in the presence of trolox (100 μM; closed squares), in the presence of the NO• donor MAHMA/NO (100 μM; open diamonds), and in the presence of trolox and MAHMA/NO (100 μM each; closed circles). Each value represents the mean (± s.d.) of at least three independent experiments.

compares well with the 96.4 ± 0.5 μM concentration of NANT detected in the absence of MAHMA/NO under otherwise identical conditions [$k_{app}^{310\,K} = (1.8 \pm 0.3) \times 10^{-4}\,s^{-1}$]. The above results again rule out the proposal of de Biase et al.[29] mentioned above that N–NO bond homolysis of NANT is the initial process of NANT decomposition. In the presence of trolox (100 μM), NANT (9.2 ± 1.4 μM) was decomposed within 900 s in the absence of the NO• donor compound [$k_{app}^{310\,K} = (2.1 \pm 0.1) \times 10^{-4}\,s^{-1}$]; see above. In the presence of both trolox and MAHMA/NO (100 μM each), the decay was reduced by about 50% (4.8 ± 0.3 μM consumption of NANT; Figure 13). Note, however, that in the latter case the time dependence of NANT decay for the first 15 min is strictly linear [$k_{app}^{310\,K} = (5.1 \pm 0.1) \times 10^{-2}\,M\,s^{-1}$], whereas in the other cases the superposition of a linear and an exponential decay mentioned above (see Figure 2) is followed.

Use of trolox/α-tocopherol for analytical purposes: The characteristics of the trolox-/α-tocopherol-mediated release of nitric oxide from NANT and *N*-nitrosoalbumin described above suggest that this process might be useful for selective detection of *N*-nitrosated tryptophan residues in proteins, because in the absence of Cu^+ ions *S*-nitrosothiols do not react in a similar manner. Figure 14 demonstrates the high

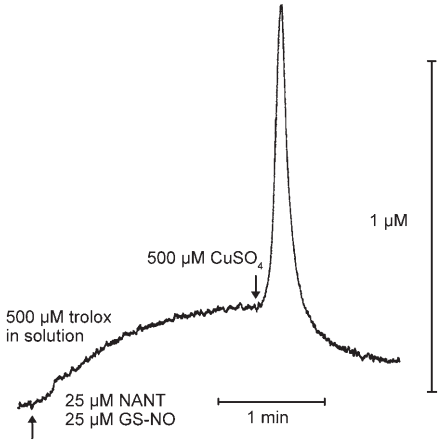


Figure 14. Stepwise release of nitric oxide from an *N*-acetyl-*N*-nitrosotryptophan/GSNO mixture. A mixture of GSNO (25 μM) and NANT (25 μM) was treated consecutively with trolox and $CuSO_4$ (500 μM each) in phosphate buffer (50 mM, pH 11, 37 °C), and release of nitric oxide was monitored with the NO•-sensitive electrode. The trace shown is representative of three independent measurements.

selectivity of trolox in the liberation of NO• only from *N*-nitrosated tryptophan compounds. A solution containing both GSNO (*S*-nitrosoglutathione) and NANT (25 μM each) was supplemented with trolox (500 μM), with the effect of a slow release of NO• resembling the time profile of NO• production from low concentrations of NANT only. After the NANT-dependent NO• equilibrium level had been achieved, addition of $CuSO_4$ (500 μM) led to an instantaneous burst of NO•, indicating its rapid liberation from GSNO. A reduced production rate of nitric oxide was observed when only 150 μM $CuSO_4$ were added, and at Cu^{2+} concentrations lower than the applied concentration of EDTA (100 μM), liberation of NO• from GSNO could no longer be detected, due to complete complexation of the copper ions (data not shown). These observations suggested that it should be possible to quantify both types of nitroso compounds in nitrosated proteins by use of just one reducing agent: namely trolox. To validate this hypothesis, additional experiments with fully *N*- and *S*-nitrosated albumin (150 μM) were performed. *N*- and *S*-Nitrosoalbumin was first mixed with trolox (150 μM) to release NO• from the nitrosotryptophan residues, and Cu^{2+} (150 μM) was then added to decompose the nitrosocysteine residues (Figure 15). In fact, as is evident from Figure 14, behaviour similar to that seen for the GSNO–NANT mixture (see above) could be observed when both posttranslational modifications were present in the protein.

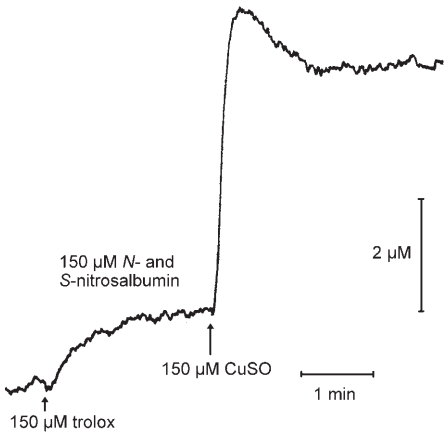


Figure 15. Consecutive release of NO• from *N*-nitrosotryptophan and *S*-nitrosocysteine in *N*- and *S*-nitrosated albumin. trolox and Cu^{2+} ions (150 μM each) were added to *N*- and *S*-nitrosoalbumin (150 μM, phosphate buffer, pH 7.4, 37 °C), and NO• release was determined with the NO•-sensitive electrode.

Discussion

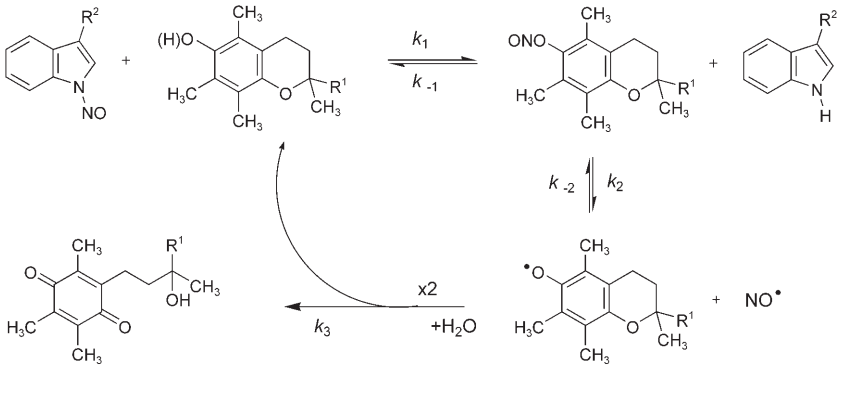
α-Tocopherol, the vitamin E derivative with the highest biological activity, acts in human beings as a supremely effective lipid-soluble antioxidant. It is believed that α-tocopherol functions as a chain-breaking antioxidant[35] and scavenges reactive nitrogen oxide species,[36] thereby preventing the formation of nitroso compounds.[37] To the best of our knowledge, a novel chemical capability of α-tocopherol and of its water-soluble derivative trolox is introduced here. Both compounds react with *N*-terminal blocked *N*-nitrosotryptophan derivatives through restoration of the indole ring of the tryptophan moiety and release of stoichiometric amounts of nitric oxide.

Chemical mechanism: Our data are in accordance with the view that the α-tocopherol/trolox denitrosated *N*-nitrosotryptophan derivatives by a stepwise mechanism. The first reaction should be the transfer of the nitroso function from NANT to α-tocopherol/trolox to yield the corresponding aryl nitrite (Scheme 3).

Similar *O*-nitrosation reactions between NANT and either H_2O_2,[30] ascorbate,[20] catechols[19] or *n*-butanol,[16] as well as between *N*-nitrosoamides and alcohols,[38] are known. Generally, aryl nitrites are unstable compounds, undergoing rearrangement and transnitrosation.[15] *O*-Nitrosophenols have short lifetimes as they exist in equilibrium with NO• and the corresponding phenoxyl-type radical.[39–41] The intermediary aryl nitrite should therefore exist in equilibrium with the parent phenol and the nitrosotryptophan derivative, as well as with the phenoxyl-type radical and nitric oxide (Scheme 3).

In fact, no products other than nitric oxide, α-phenoxyl-type radicals, and the *para*-quinone of trolox were observed in the course of the reaction (Figures 4, 6–11). Quantum chemical calculations with 1,4-dihydroxy-2,3,5,6-tetramethylbenzene as a model for trolox, performed at the CBS-QB3 level of theory, predict that the deprotonated (phenolate) form of trolox/α-tocopherol should be the reacting entity in aqueous solution (data not shown). This prediction is strongly supported by the pH dependence of the decay of NANT as depicted in Figure 2. Scheme 3 also explains why the overall kinetics of the trolox-/α-tocopherol–NANT reaction do not obey a second-order rate law as expected for a rate-limiting first step (Figure 3). This is due to the regeneration of one trolox/α-tocopherol molecule from the bimolecular disproportionation of the corresponding phenoxyl radical. The bimolecular self-decay of the vitamin E radical is a relatively slow reaction ($k_2 = 1180\,\text{M}^{-1}\text{s}^{-1}$),[31] so that the overall kinetics depend both on the recombination of the vitamin E-type radical with nitric oxide and on the aryl nitrite-dependent transnitrosation of the *N*-terminal blocked tryptophan molecule. Facilitated decomposition of the *N*-nitrosotryptophan derivative by a radical chain mechanism, as has recently been established for the ascorbyl radical,[20] seems to be largely prevented by steric hindrance of the trolox/α-tocopheryl radical. Thus, the α-tocopherol-/trolox-mediated decomposition of *N*-nitrosotryptophan derivatives can be fully explained by Scheme 3.

The addition of α-tocopherol/trolox for analytical purposes: Thanks to the capability of the vitamin E derivatives α-tocopherol and trolox to liberate nitric oxide selectively and stoichiometrically from *N*-nitrosated tryptophan compounds both in aqueous solution and in lipophilic solvents, one may suggest the use of the trolox–NANT reaction for the deter-



$R^1 = CH_2CH(COOH)NHOCH_3$: NANT
R^2 = COOH: trolox
$R^2 = C_{16}H_{33}$: α-tocopherol

Scheme 3. Suggested mechanism showing *O*-nitrosated α-tocopherol derivatives as key intermediates.

mination of *N*-nitrosotryptophan residues in proteins. Since the trolox–NANT reaction proceeds even under oxygen-free conditions, the liberated nitric oxide can easily be detected with a NO•-sensitive electrode as demonstrated here, but it should additionally be possible to transport NO• into the gas phase, where it can be detected by the sensitive ozone chemiluminescence method.[15] Although these methods can detect low amounts of nitric oxide, it is often impossible to extrapolate the concentration of the nitric oxide-donating compound (in our case *N*-nitrosotryptophan residues in peptides and proteins) from the detected amount of gaseous nitric oxide. For quantitative NO• monitoring, the cheletropic compound FNOCT-4 is best applied, because of its ability to accumulate the released NO• in a reaction product that can be easily detected fluorimetrically. In addition to the capability to detect small amounts of *N*-nitroso residues, it is additionally possible to differentiate between *N*-nitroso and *S*-nitroso residues. In accord with our findings, trolox does not react with *S*-nitroso compounds, but trolox does reduce Cu^{2+} ions to cuprous ions, which are well known to catalyse the release of nitric oxide from *S*-nitroso derivatives such as GSNO.[42] Consecutive and selective trolox-mediated nitric oxide release either from *N*-nitrosotryptophan residues in the absence of Cu^{2+} or from S-nitrosocysteine residues in the presence of Cu^{2+} ions is therefore possible (Figures 14 and 15).

Physiological implications: Various physiological aspects can be related to the function of α-tocopherol. Although Desideri et al. noticed significant increases in nitric oxide production in hypercholesterolemic patients mediated by vitamin E supplementation,[43] its benefit in vasodilatation and prevention of myocardial infarction is disputed.[44,45]

Rubbo et al. noted that the reaction between nitric oxide and α-tocopherol inhibited membrane lipid peroxidation in a more potent manner than the one-electron α-tocopherol plus ascorbate redox cycle system.[36] Since nitric oxide is known to protect cultured cells against reactive oxygen species,[46] it is suggested (and very likely) that nitric oxide preferentially scavenges lipid hydroperoxyl radicals irreversibly, rather than scavenging the α-tocopheroxyl radical in a reversible manner. Although the α-tocopheroxyl radical has a limited capability to propagate radical chain reactions,[47] the dismutation of this radical in part regenerates the parent α-tocopherol (Scheme 2). The reaction between vitamin E derivatives and *N*-nitrosotryptophan residues therefore increases the general antioxidative power of vitamin E towards oxidizing radicals.

On the other hand, the reaction of vitamin E with *N*-nitrosotryptophan residues (Scheme 3) would limit the concentration of such kinds of nitroso compounds in a lipophilic environment. *N*-Nitrosotryptophan derivatives do not spontaneously decay in hydrophobic solutions,[18,30] so their concentration would continuously increase in the absence of vitamin E. Noticeably, NANT is a mutagenic nitrosamine at high concentrations (>120 μM).[48] We thus believe that vitamin E has the previously unnoticed function of preventing the accumulation of *N*-nitrosotryptophan residues to toxic levels in lipophilic environments.

Since human skin is very lipophilic and both nitrosoamine and α-tocopherol concentrations are relatively high,[47,49] it appears reasonable that this reaction between α-tocopherol and *N*-nitrosotryptophan residues may play a major role in this tissue.

Experimental Section

Materials: *N*-Acetyl-D,L-tryptophan, trolox, bovine serum albumin (BSA), EDTA and Chelex 100 were purchased from Sigma (Taufkirchen, Germany). $NaNO_2$, Me_2SO, acetonitrile and ethyl acetate were obtained from Merck (Darmstadt, Germany). α-Tocopherol was from Fluka Chemie (Steinheim, Germany), ethanol from J.T. Baker (Deventer, Netherlands) and MAHMA/NO from Situs (Düsseldorf, Germany). *N*-Acetyl-*N*-nitrosotryptophan, the fluorescent nitric oxide cheletropic trap (FNOCT-4) and NO-albumin were prepared as described in refs. [30,33] Stock solutions were freshly prepared on a daily basis and their concentrations were spectrophotometrically determined.

Experimental system: As nitrosation reactions are highly sensitive to the presence of metal ions, phosphate buffer solutions (50 mM) were treated with the heavy metal scavenger resin Chelex 100 (0.5 g in 15 mL). The solution was gently shaken, stored overnight and then carefully decanted from the resin. Afterwards, the pHs of all solutions were readjusted to pH 7.4±0.1 by addition of either H_3PO_4 (50 mM) or K_3PO_4 (50 mM). In order further to avoid any interference from traces of transition metals, EDTA (100 μM) was added to all reaction mixtures.

Determination of nitric oxide with a NO•-sensitive electrode: Nitric oxide formation was determined by use of a NO• sensitive electrode (ISO-NO; World Precision Instruments, Sarasota, Florida), as described in Ref. [50] The reaction mixtures were continuously stirred throughout the measurements, and temperatures were maintained at 25±1 and 37±1 °C. The electrode was calibrated daily and NO• production was quantified according to the manufacturer's instructions, by employing potassium iodide (100 mM) in H_2SO_4 (0.1 M) as a calibration solution to which various amounts of $NaNO_2$ (0.5 mM) were added. The concentrations of α-tocopherol/trolox, as well as of NANT and NO-albumin, were varied.

NO• measurement with the fluorescent nitric oxide cheletropic trap (FNOCT-4): The amounts of NO• from the α-tocopherol/trolox–NANT reaction, as well as from the corresponding one with NO-albumin, were quantified with FNOCT-4, which directly traps NO• to yield the fluorescence dye FNOCT-4-NOH ($\lambda_{em}=460\pm5$ nm, $\lambda_{ex}=320\pm5$ nm). A two-point calibration of FNOCT-4 (100 μM) was performed by mixing with α-tocopherol (200 μM) in the absence and in the presence of MAHMA/NO (200 μM) and reading the fluorescence intensity after an incubation period of 30 min at 37±1 °C. The stability of the nitric oxide–FNOCT-4 reaction product, FNOCT-4-NOH, was verified for a period of 4 h.

Kinetic experiments: The α-tocopherol- and trolox-induced decay of NANT was monitored on a SPECORD S100 spectrophotometer from Analytic Jena (Jena, Germany), with use of an absorption coefficient of $\varepsilon_{335}=6100\,M^{-1}cm^{-1}$ for NANT.[51] The reaction between trolox and NANT in aqueous solution was monitored for 60–120 min by taking recordings every 5 min. Measurements were performed in phosphate buffer at pH 7.4 and pH 11 and at 25 and 37 °C. The effect of α-tocopherol on the decomposition of NANT (100 μM) was also studied in dimethyl sulfoxide (DMSO), ethanol, ethyl acetate and acetonitrile.

NAT determination: The formation of NAT and the decay of NANT were simultaneously monitored during the reaction between NANT and trolox (100 μM each) in phosphate buffer at pH 11 and room temperature. The formation of NAT was determined by tenfold dilution of the sample and subsequent recording of the fluorescence ($\lambda_{exc}=270\pm5$ nm, $\lambda_{em}=355\pm5$ nm). The decomposition of NANT was determined by reading the optical density at 335 nm.

ESR measurements: The trolox radical was identified by ESR spectrometry. ESR spectra were recorded at 18 °C on a Bruker ESP-300E X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with a TM_{110} wide-bore cavity. Solutions were prepared from the buffer solution (pH 7.4, 1 mL) containing trolox and NANT (7.5 mM each) under normoxic conditions. Additional recordings in order to visualize the α-tocopherol radical were performed in ethyl acetate with α-tocopherol and NANT (75 mM each) under both normoxic and hypoxic conditions. Recording conditions were as follows: microwave frequency, 9.79 GHz; modulation, 0.04 mT; signal gain, 5×10^5; sweep range, 10 mT; microwave power, 2 mW; sweep time, 2.8 min. Spectra simulation was performed with the WinSim program.[52]

^{13}C NMR measurements: NMR experiments were performed on a Bruker ADVANCE DRX 500 instrument (Bruker Biospin, Rheinstetten, Germany) at 125.71 MHz. Chemical shifts (δ) are given in ppm relative to TMS ($\delta=0$) as external standard. ^{13}C NMR spectra were obtained from NANT (100 mM) added to trolox (100 mM), as well as from NANT (100 mM) mixed with Fremi's salt (100 mM) in [D_6]DMSO/phosphate buffer pH 7.4 (8:2). The oxidation of 2,3,5,6-tetraalkylphenols such as trolox with Fremy's salt represents a clean synthetic method for the preparation of the corresponding *p*-benzoquinones.[53] In addition, NANT (100 mM) or trolox (100 mM) were monitored in the absence of any other reactants. All samples were measured after a reaction period of 18 h.

Quantum-chemical calculations: Complete basis set (CBS-QB3) computations were carried out with the Gaussian 03 (revision AM64 L-G03 RevC0.2) suite of programs.[54] Gibbs free energies of solvation for water were estimated for the optimized gas-phase geometries by use of the CPCM-UAHF procedure incorporated in Gaussian 03. Both the CBS-QB3 and CPCM/(U)HF/6-31+G (d) methodologies are known to provide thermochemical estimates within "chemical accuracy" (±1 kcal mol^{-1}).[55,56]

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