CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200600405

On the Mechanism of the Ascorbic Acid-Induced Release of Nitric Oxide from *N*-Nitrosated Tryptophan Derivatives: Scavenging of NO by Ascorbyl Radicals

Anna Kytzia,[a] Hans-Gert Korth,[b] Reiner Sustmann,[b] Herbert de Groot,[a] and Michael Kirsch*[a]

Abstract: During the past years, there has been increasing interest in endogenous nitric oxide storage compounds. Recently, we briefly reported on the ascorbate-dependent release of nitric oxide (•NO) from *N*-nitrosotryptophan derivatives. In the present study, the underlying mechanism of •NO release is studied in more detail, primarily utilizing *N*-acetyl-*N*-nitrosotryptophan (NANT) as a model compound. The initial rate of the ascorbate-induced release of nitric oxide has been found to correspond to the rate of NANT decay. In this process, *N*-acetyltryptophan (NAT) is produced almost quantitatively. The final yield of nitrite amounted to around 90% with respect to the applied amount of NANT. However, the total release of nitric oxide was only 60%, as determined by using an FNOCT-4(fluorescent nitric oxide cheletropic trap number 4) assay. Besides nitric oxide, a second volatile product, dinitrogen oxide (N_2O), has been identified by using ^{15}N NMR spectrometry, strongly indicating the intermediacy of nitroxyl (HNO). The formation of intermediate ascorbyl radical anions during the NANT–ascorbate reaction has been monitored by using ESR spectrometry. Unexpectedly, it was found that the primary oxidized product of vitamin C, dehydroascorbic acid (DHA), efficiently consumes nitric oxide. Since ESR spectrometry further revealed that ascorbyl radical anions are also generated during the spontaneous decay of DHA, the DHA–nitric oxide reaction is related to recombination of •NO with the thus formed ascorbyl radical anions. A conclusively established mechanism of the NANT–ascorbate reaction is presented, with *O*-nitrosoascorbate as a key intermediate, as additionally supported by CBS-QB3 calculations. The present study suggests that vitamin C and its oxidation products can chemically counterbalance endogenous nitric oxide levels.

Keywords: ascorbate · EPR spectroscopy · nitrogen oxides · nitrosotryptophan · radicals

Introduction

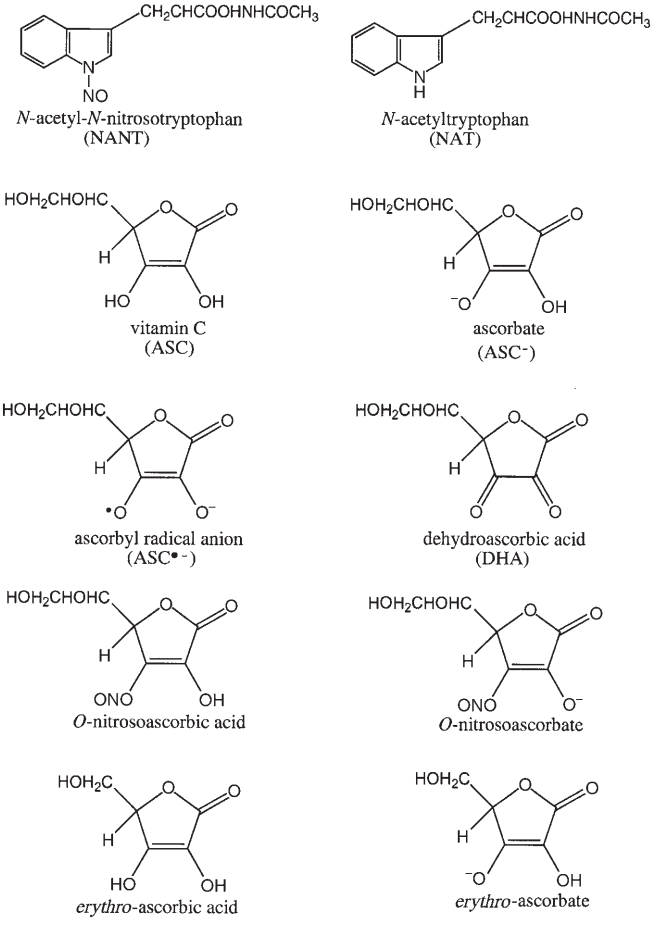
Endogenous nitric oxide acts as a vasodilatative compound, as a neurotransmitter, and is involved in the immune response of activated macrophages.[1] Nitric oxide reacts rapidly with a variety of iron proteins, such as, methemoglobin, metmyoglobin, oxidized cytochrome c, and catalase.[2] These effective reactions limit the lifetime of •NO in physiological environments to a few seconds, and so there is increasing interest in identifying natural stores of nitric oxide. In particular, low-molecular weight *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO) seem to be involved in intracellular •NO storage.[3,4] *S*-Nitrosated proteins such as albumin have been postulated as extracellular transporters of nitric oxide.[5,6] Besides cysteine, tryptophan may additionally function as a storage compound and transporter of nitric oxide. Zhang et al.[5] reported on the *N*-nitrosation of Trp-214 in both bovine serum albumin and carboxymethyl bovine serum albumin (CM-BSA) by nitrite in acidic media. Evidence has been presented that at physiological pH values the strong nitrosating agent N_2O_3 rapidly *N*-nitrosates ($k = 4.4\times10^7\,M^{-1}s^{-1}$)[7] *N*-terminal-blocked tryptophan derivatives in a strictly regiospecific manner.[7–9] From the pioneering work of Zhang et al.,[5] it is additionally understood that nitrosated tryptophan residues such as NO-GlyTrp and NO-CM-BSA mediate vasodilatation of aortic vessel rings

[a] A. Kytzia, Prof. Dr. H. de Groot, Priv.-Doz. Dr. M. Kirsch
Institut für Physiologische Chemie, Universitätsklinikum Essen
Hufelandstrasse 55, 45122 Essen (Germany)
Fax. (+49) 201-723-5943
E-mail: michael.kirsch@uni-essen.de

[b] Dr. H.-G. Korth, Prof. Dr. R. Sustmann
Institut für Organische Chemie, Universität Duisburg-Essen
Universitätsstrasse 5, 45117 Essen (Germany)

in a manner comparable to *S*-nitrosothiols. Later, Harohalli et al.[6] and Blanchard-Fillion et al.[10] reported on the slow, spontaneous release of ·NO from nitrosated tryptophan in human serum albumin and from *N*-nitrosomelatonin. However, de Biase et al.[11] recently demonstrated that such a "spontaneous" release of nitric oxide is driven by incident light. In fact, the N–NO bond dissociation enthalpy in *N*-nitrosated indoles is about 29 kcal mol^{-1},[12] which is too high for spontaneous dissociation at 37 °C in aqueous solution. In a recent paper, we briefly reported that ascorbate strongly accelerates the release of nitric oxide from *N*-terminal-blocked *N*-nitrosotryptophan derivatives.[7] This finding was verified by de Biase et al.,[11] who attributed the release of ·NO from *N*-nitrosomelatonin in the presence of ascorbate to an initial thermal homolysis of the N–NO bond.

In the present study, we analyze in more detail the potentially important reaction of ascorbate with an *N*-nitrosotryptophan derivative, namely, *N*-acetyl-*N*-nitrosotryptophan (NANT), in order to elucidate the underlying mechanism of ·NO release. It is demonstrated that the total yield of ·NO is only 60 % with respect to the applied NANT because the intermediary *O*-nitrosoascorbate can either be hydrolyzed to nitrite and ascorbate or may reversibly dissociate to nitric oxide and the ascorbyl radical anion. The reversibility of the latter reaction has been independently demonstrated through consumption of the ascorbyl radical anion by added nitric oxide.



Results

·NO release: The ability of ascorbate to release nitric oxide from either NANT or *N*-nitrosomelatonin was monitored with an ·NO-sensitive electrode at 25 °C and at 37 °C, respectively. Figure 1 shows that the steady-state concentration of

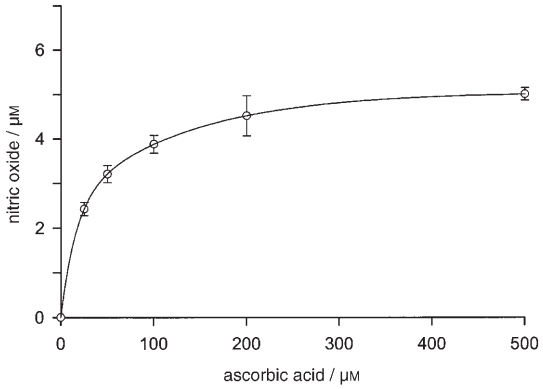
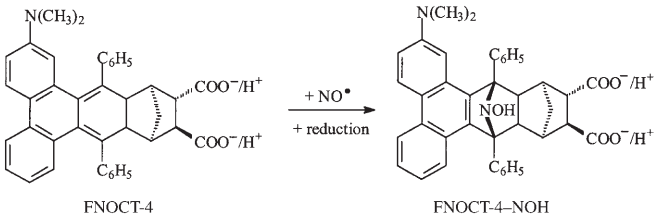


Figure 1. Steady-state concentrations of nitric oxide from the reaction of L-ascorbic acid with *N*-nitrosomelatonin. The steady-state concentrations of nitric oxide generated from reaction of *N*-nitrosomelatonin (100 μM) with various amounts of ascorbic acid in phosphate buffer (50 mM, pH 7.4, 37 °C) were monitored by using an ·NO-sensitive electrode. Each value represents the results of at least four independently performed experiments.

nitric oxide, generated from 100 μM *N*-nitrosomelatonin, followed the vitamin C concentration in an exponential manner, leveling off at about 5 μM ·NO at ascorbate concentrations greater than 100 μM. Similar results were obtained with 100 μM NANT as the *N*-nitrosotryptophan derivative (data not shown). Thus, both NANT and *N*-nitrosomelatonin released substantial amounts of ·NO in the presence of ascorbate.

In order to quantify the ascorbate-mediated production of nitric oxide during the entire reaction period, its formation was monitored by means of the ·NO scavenger FNOCT-4, which produces the stable fluorescent product FNOCT-4–NOH [Eq. (1)].[13] After 1 h of reaction under aerobic conditions, 20 μM NANT and 500 μM L-ascorbic acid yielded 11.8 ± 0.9 μM FNOCT-4–NOH. Under hypoxic conditions, 14.0 ± 0.7 μM FNOCT-4–NOH was formed from 23.1 μM NANT. The related maximum fluorescence intensity was unchanged after 3 h, proving that under the applied conditions the reaction was complete after 1 h (data not shown). The yields of



nitric oxide, 59.0% under normoxic and 60.5% under oxygen-free conditions with respect to the applied NANT, confirmed that the ˙NO-producing reaction is independent of the presence of oxygen, and also indicated that either the nitric oxide is not generated stoichiometrically or it is consumed by an as yet unidentified reaction. Unfortunately, nitric oxide release from *N*-nitrosomelatonin could not be quantified by using the FNOCT assay because the fluorescence properties of the reaction product melatonin interfered with the detection of FNOCT-4–NOH.

^{15}N NMR measurements: Since nitric oxide was only produced to about 60% of the theoretical yield, the question arose as to whether the formation of other nitrogen products might account for the missing nitrogen content. To identify possible products, the reaction between ^{15}N-enriched NANT and ascorbate was monitored by using ^{15}N NMR spectrometry under normoxic conditions. After a reaction period of 24 h in DMSO, two nonvolatile compounds, namely, ^{15}N-NANT (δ = 170.7, 185.7 ppm) and $^{15}NO_3^-$ (δ = −5.8 ppm), and two volatile products, namely, $^{15}N_2O$ (δ = −146.6, −229.6 ppm) and $^{15}N_2$ (δ = −69.7 ppm), were identified (Figure 2A).

In the absence of ascorbic acid, only the NMR resonance of ^{15}N-NANT was recorded, reflecting its thermal stability in nonaqueous solution (data not shown). To establish the influence of water, similar experiments with ascorbate and ^{15}N-NANT (100 mM each) were performed in phosphate buffer (Figure 2B). The latter spectrum revealed the presence of ^{15}N-NANT (δ = 165.7, 180.2 ppm), $^{15}NO_2^-$ (δ = 228.9 ppm), and $^{15}N_2O$ (δ = −146.7, −230.1 ppm) in the reaction mixture, but neither $^{15}N_2$ nor $^{15}NO_3^-$ could be detected. In the absence of ascorbate, only the ^{15}N NMR resonances of ^{15}N-NANT and $^{15}NO_2^-$ appeared in the spectrum (data not shown), in line with observations of Meyer et al.[14] Hence, $^{15}N_2O$ derives solely from the ^{15}N-NANT–ascorbate reaction. The formation of N_2O strongly indicates the intermediate generation of nitroxyl, HNO [Eqs. (2) [15] and (3)].[16,17]

$$2\,HNO \rightleftharpoons N_2O + H_2O \quad (2)$$

$$HNO + 2\,^{\cdot}NO \rightleftharpoons N_2O + HNO_2 \quad (3)$$

Nitrite measurements: Since nitrite was found to be a major product of the reaction of NANT with ascorbate in aqueous solution, its yield was determined by means of the Griess assay. In the absence of ascorbic acid, 98.2 ± 2.1 μM NO_2^- was produced from 100 μM NANT within 24 h, in accordance with a stoichiometric hydrolysis to NAT and NO_2^-.[14] When 100 μM ascorbic acid was added to a 100 μM solution of NANT, the yield of nitrite was reduced to 87 ± 3 μM, that is, by about 13%, in accordance with the above ^{15}N NMR experiments, in which a substantial amount of N_2O was detected. However, the yield of nitrite was about 30% higher than the yield of nitric oxide. This additional fraction of nitrite is most likely formed through an N_2O_3-independent pathway because autoxidation of ˙NO and subsequent hydrolysis of the corresponding product N_2O_3[18] would only account for a yield of nitrite of around 60%.

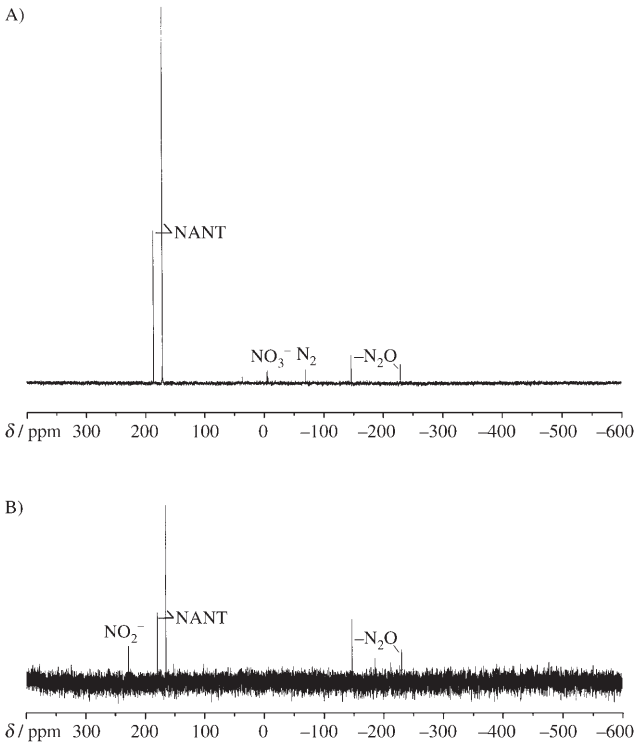


Figure 2. ^{15}N NMR spectra from the reaction of ascorbic acid with *N*-acetyl-^{15}*N*-nitrosotryptophan. A) The reaction between 1 M L-ascorbic acid and 1 M *N*-acetyl-^{15}*N*-nitrosotryptophan (^{15}N-NANT) was performed in DMSO at RT. B) Similar measurements were carried out with 100 mM ascorbic acid and 100 mM ^{15}N-NANT in phosphate buffer (50 mM, pH 7.4) at 37 °C. ^{15}N NMR (DMSO): δ = 185.7 (^{15}N-NANT, *Z* isomer), 170.7 (^{15}N-NANT, *E* isomer), −5.8 ($^{15}NO_3$), −69.7 ($^{15}N_2$), −146.6 ($^{15}N_2O$), −229.6 ppm ($^{15}N_2O$); ^{15}N NMR (phosphate buffer): δ = 228.9 ($^{15}NO_2^-$), 180.2 (^{15}N-NANT, *Z* isomer), 165.7 (^{15}N-NANT, *E* isomer), −146.7 ($^{15}N_2O$), −230.1 ppm ($^{15}N_2O$).

Formation of NAT: In contrast to NANT, which is nonfluorescent, NAT fluoresces at λ_{em} = 358 ± 5 nm (λ_{exc} = 270 nm), but shows no significant absorbance at 335 nm, the UV/Vis maximum of NANT. Therefore, the decay of NANT and the formation of NAT during the reaction of ascorbate with NANT (200 μM each) could be monitored simultaneously (Figure 3).

The data clearly indicate that, in the presence of air, NANT is (with the same time dependence) almost stoichiometrically converted to NAT. This finding is at variance with the view that the N–NO bond of NANT undergoes homolysis to yield ˙NO and aminyl radicals, as has been reported for *N*-nitrosomelatonin,[11] because tryptophan-centered radicals effectively react with oxygen to give the corresponding peroxyl radical at the C-3 carbon of the indole ring.[19] This irreversible process would significantly reduce the yield of NAT.

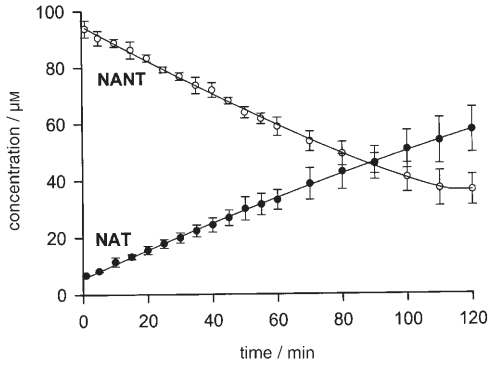


Figure 3. Fluorometrically determined formation of *N*-acetyltryptophan (NAT) and photometrically determined decay of *N*-acetyl-*N*-nitrosotryptophan (NANT). The formation of NAT from reaction of 100 μM NANT with 200 μM ascorbate was monitored spectrofluorophotometrically in phosphate buffer (50 mM, RT, pH 7.4). The fluorescence intensity was determined from a tenfold diluted aliquot of the reaction mixture. The decay of NANT was determined simultaneously by recording the absorption at 335 nm from a twofold diluted aliquot of the reaction mixture. Each value represents the mean of the results from at least four independent experiments.

In order to substantiate the formation of NAT from the NANT–ascorbate reaction, the reaction mixture was analyzed by means of capillary zone electrophoresis. NAT was identified by spiking with authentic material. From the reaction of 0.9 mM NANT with 5 mM L-ascorbic acid, a final concentration of NAT of 0.52 ± 0.03 mM and a NANT concentration of 0.42 ± 0.03 mM were determined after 1 h (Figure 4A), in agreement with the above experiments.

After 3.5 h, the yield of NAT had increased to 0.86 ± 0.02 mM and NANT could no longer be detected (detection limit $\leq$ 5 μM) (Figure 4B). In the absence of ascorbate, 0.44 ± 0.01 mM NANT remained and 0.71 ± 0.09 mM NAT was formed within 4 h from the hydrolysis of 1.1 mM NANT. Thus, as already deduced from the experiments that yielded Figure 3, ascorbate reacts with NANT to yield NAT almost quantitatively.

Kinetic measurements: The initial rates of nitric oxide release (Figure 5) increased exponentially with increasing ascorbate concentration to approach constant values of about 33 nM s^{-1} (25 °C) and 60 nM s^{-1} (37 °C), respectively, at an ascorbate concentration of 100 μM.

Ascorbate-induced nitric oxide release from NANT was found to be slightly faster than that from *N*-nitrosomelatonin (Table 1).

An obvious explanation for the difference in the yields of nitric oxide and nitrite may be that the hydrolysis of NANT produces nitrite in about 30 % yield even in the presence of ascorbate. To investigate this possibility, the initial decay rates of NANT (100 μM), that is, over the first 300 s of decay, were monitored at various ascorbate concentrations (Figure 6A).

In the absence of ascorbate, NANT was found to decompose hydrolytically in a pseudo-first-order process with a rate constant of $k_{hyd} = 6.6 \times 10^{-5}$ s^{-1}, in good agreement with

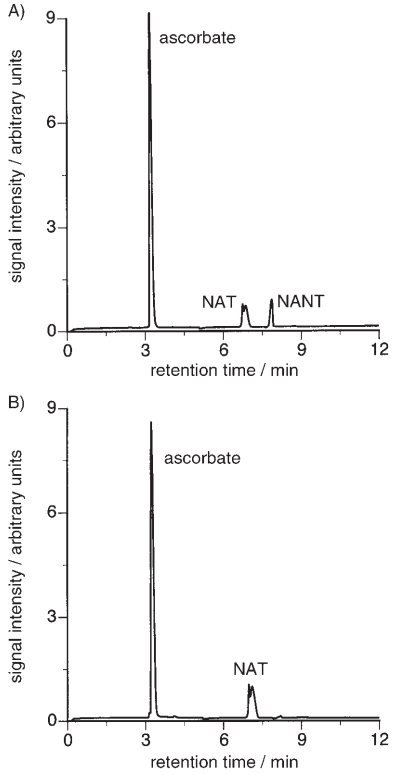


Figure 4. Capillary zone electrophoresis of the *N*-acetyl-*N*-nitrosotryptophan/ascorbic acid reaction mixture. Ascorbic acid (5 mM) and *N*-acetyl-*N*-nitrosotryptophan (0.9 mM) were mixed in 50 mM phosphate buffer at pH 7.4 at RT. The formation of *N*-acetyltryptophan is shown: A) after 1 h; B) after 3.5 h.

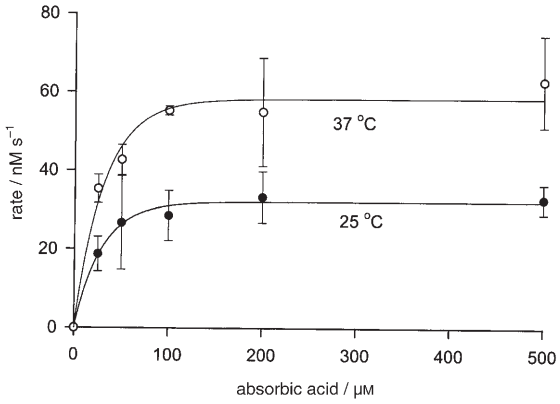


Figure 5. Rates of nitric oxide release from the reaction of *N*-acetyl-*N*-nitrosotryptophan with ascorbic acid. Rates of ·NO release from reaction of NANT (100 μM) with various ascorbic acid concentrations in phosphate buffer (50 mM, pH 7.4, 37 °C) were determined by using an ·NO-sensitive electrode. Each value represents the average of the results of at least four independently performed experiments.

the literature data.[7,11,20] The linear approximation provided an initial rate of 12.3 ± 0.5 nM s^{-1} (Figure 6A, trace a). Addition of ascorbate accelerated the decay of NANT. The fivefold increased initial rate at equimolar concentrations of NANT and ascorbic acid (100 μM each) (56.5 ± 1.4 nM s^{-1}; Figure 6A, trace b) is in excellent agreement with the corre-

Table 1. Rates of ascorbate-induced •NO release from *N*-terminal-blocked tryptophan derivatives.[a]

Compound	*T* [°C]	Rate [nM^{-1}s^{-1}]
N-acetyl-*N*-nitrosotryptophan	25	33±4
N-acetyl-*N*-nitrosotryptophan	37	63±11
N-nitrosomelatonin	25	24±4
N-nitrosomelatonin	37	52±3

[a] *N*-Acetyl-*N*-nitrosotryptophan and *N*-nitrosomelatonin (each 100 μM), respectively, were added to 500 μM ascorbic acid in 50 mM phosphate buffer, pH 7.4. The release of nitric oxide was monitored by using an •NO-sensitive electrode. The •NO release rate was evaluated from the linear approximation of the concentration–time profile over the first 300 s after mixing. Each result represents the mean value obtained from at least four experiments.

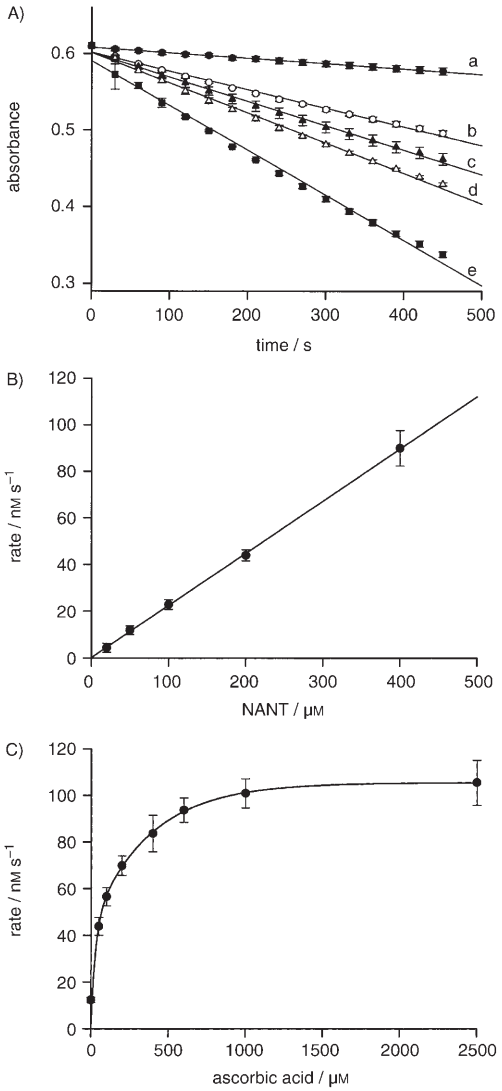


Figure 6. Ascorbic acid-induced decomposition of *N*-acetyl-*N*-nitrosotryptophan. The decay of NANT in phosphate buffer (50 mM, pH 7.4, 37 °C) was monitored spectrophotometrically at 335 nm in the absence and in the presence of ascorbate. A) 100 μM NANT and various ascorbate concentrations (trace a = 0 μM, trace b = 50 μM, trace c = 100 μM, trace d = 200 μM, trace e = 2500 μM). B) The dependence of the NANT decay rate at 200 μM ascorbate. C) The dependence of the NANT (100 μM) decay rate on the ascorbate concentration. Each value represents the average result of at least four independently performed experiments.

sponding initial rate of nitric oxide release as displayed in Figure 5. This fact strongly suggests that nitric oxide production proceeds quantitatively in the early stage of the NANT–ascorbate reaction. At a 25 times higher ascorbate concentration (100 μM NANT and 2.5 mM ascorbate), that is, at a concentration ratio similar to that in the FNOCT experiments (see above), the decay rate (105.4±1.5 nM s^{-1}, Figure 6A, trace e) was 8.6 times faster than in the case of the hydrolysis reaction (Figure 6A, trace a). Thus, the significantly higher production of nitrite relative to nitric oxide cannot be satisfactorily explained by competing hydrolysis of NANT. On the other hand, hydrolysis of N_2O_3, formed by autoxidation of •NO, would only account for a nitrite yield of about 60%. Hence, a second •NO-derived intermediate, most likely a hydrolysis-labile alkyl nitrite, was suspected to be a major source of nitrite.

De Biase et al.[11] proposed that the indole-type aminyl radical that would be generated from reversible homolysis of the N–NO bond of *N*-nitrosomelatonin should be reduced by ascorbate to yield tryptophan on protonation. As a consequence of this proposal, de Biase et al.[11] analyzed their decay data in terms of an overall first-order rate law. Indeed, the decay trace of NANT in the presence of an equimolar concentration of ascorbate (Figure 6A, 100 μM each, trace b) could be satisfactorily fitted to a first-order reaction. However, the data could also be fitted to an overall second-order rate law, with an even slightly improved accuracy ($r^2 \geq 0.999$) (fits not shown). To resolve this discrepancy, the influence of both the NANT and ascorbate concentrations on the decay rate of NANT was studied in more detail (Figure 6B, C). At a constant ascorbate concentration of 200 μM, the decay rate of NANT increased 20-fold on increasing the NANT concentration from 20 to 400 μM (Figure 6B). This observation confirmed that the NANT–ascorbate reaction is first order with respect to the NANT concentration. The decay rate of NANT (100 μM) was found to be about 3.6-fold accelerated to 43.9±2.2 nM s^{-1} in the presence of 50 μM ascorbate (Figure 6C). Doubling the ascorbate concentration to 100 μM increased the NANT decay rate by only about 23% (56.5±2.4 nM s^{-1}). An identical relative increase of the NANT decay rate (69.9±2.5 nM s^{-1}) was observed at 200 μM ascorbate. Thus, the NANT–ascorbate reaction appeared to be of order 1/3 with respect to the ascorbate concentration. The occurrence of a fractional reaction order implies the involvement of a reactive intermediate,[21,22] which in the present case should be an ascorbate-derived radical. With increasing ascorbate concentration, the decay rate of NANT leveled off to about 105 nM s^{-1} at [ascorbate] > 1 mM. Hence, the proposal of de Biase et al.[11] that solely an ascorbate-independent, reversible homolysis of the N–NO bond would account for the decay of *N*-nitrosotryptophan derivatives is at variance with the data presented in Figure 6C. Initial N–NO homolysis is also incompatible with the fact that replacement of ascorbate (100 μM) by an excess amount of the known spin-trapping compound 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; 10 mM), which efficiently scavenges tryptophan-derived radicals,[23] did not

accelerate the spontaneous decay of NANT; rather, an identical decay rate as displayed by trace a in Figure 6A was observed in the presence of DMPO.

Influence of •NO on the decay of NANT: The above data strongly suggest that an ascorbate-derived radical is involved in the NANT–ascorbate reaction. Furthermore, because •NO is released under the applied conditions (Figures 1 and 5), and considering the fact that an •NO-derived species accelerates the decomposition of *S*-nitrosothiols,[24] the influence of nitric oxide on the decay rate of NANT was studied in the absence and in the presence of ascorbate (Figure 7).

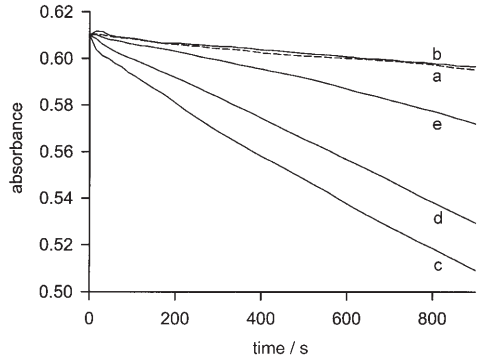


Figure 7. The influence of nitric oxide on the NANT–ascorbate reaction under hypoxic conditions. The decomposition of NANT (100 μM) in hypoxic phosphate buffer (50 mM, pH 7.4, 25 °C, 1–2 μM O_2) in the absence and in the presence of either ascorbate or MAHMA/NO (100 μM each) was monitored spectrophotometrically at 335 nm. The traces show the results from solutions containing 100 μM NANT with a) nothing else added, b) 100 μM MAHMA/NO, c) 100 μM ascorbate, d) 100 μM ascorbate + 10 μM MAHMA/NO, and e) 100 μM ascorbate + 100 μM MAHMA/NO. Each value represents the average result of four independently performed experiments.

These experiments were performed under hypoxic conditions (ca. 1–2 μM residual O_2) in order to limit the autoxidation of •NO so that NAT would not be artificially nitrosated by way of an N_2O_3-driven reaction.[7] Inspection of Figure 7 (traces a and b) clearly shows that in the absence of ascorbate, nitric oxide, as released from MAHMA/NO ((*Z*)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}-diazen-1-ium-1,2-diolate) (100 μM), did not affect the decomposition of NANT. This experiment clearly ruled out the possibility that an ascorbate-independent and rate-determining, reversible homolysis of the N–NO bond takes place at a significant rate, because if this were the case excess nitric oxide would retard the decomposition of NANT. (A possible renitrosation of the produced NAT by the added MAHMA/NO was excluded by control experiments, that is, addition of MAHMA/NO to a deoxygenated, buffered solution of NAT did not lead to N-nitrosation (data not shown).) Thus, the slow decomposition of NANT in the absence of ascorbate is an ordinary, nonradical hydrolysis process. Nevertheless, •NO was found to inhibit rather than accelerate the ascorbate-mediated decay of NANT (Figure 7, traces c–e). This observation implies that a radical other than •NO governs the ascorbate-dependent decay of NANT.

Ascorbyl radical anion: The above experiments strongly suggest that the ascorbyl radical anion is produced in the NANT–ascorbate reaction. In fact, after mixing aqueous solutions of NANT and ascorbic acid (7.5 mM each) under aerobic conditions, a strong ESR signal of the ascorbate radical anion was promptly detected (i.e., within 2 min) (Figure 8A). In accordance with the above-described kinetic experiments, the ESR signal decayed within 60 min (Figure 8B).

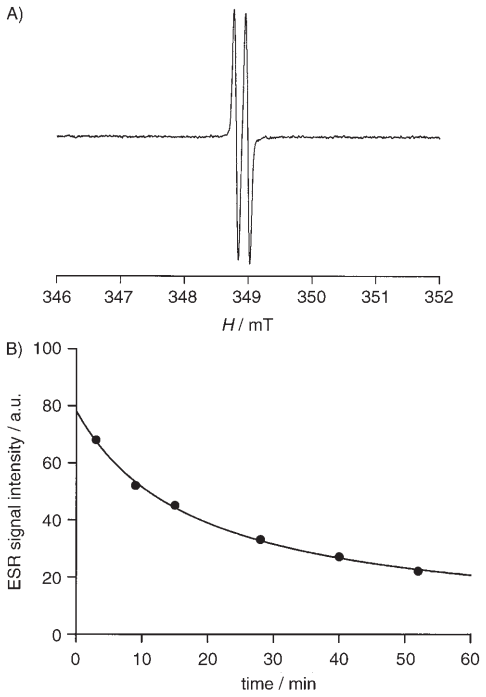


Figure 8. ESR spectrum of the ascorbate radical anion. A) The formation of the ascorbate radical anion from the reaction of NANT with ascorbate (7.5 mM each) was monitored by using ESR spectrometry in phosphate buffer (50 mM, pH 7.4, 18 °C) 4 min after mixing of the reactants. B) Time dependence of the ESR signal intensity. The solid line is a least-squares fit ($r^2 = 0.998$) to a second-order rate law.

Since the bimolecular self-decay of the ascorbate radical anion is quite fast ($k = 6\times10^7\,M^{-1}s^{-1}$),[25] the rather long lifetime of the ESR signal reflects a continuous production of this radical. When the aforementioned experiment was repeated under oxygen-free conditions, the ESR signal intensity of the ascorbyl radical anion significantly increased. Reaction of the ascorbate radical anion with molecular oxygen ($k = 5.0\times10^2\,M^{-1}s^{-1}$)[25] would account for this observation.

Effect of ascorbate and ascorbate derivatives on nitric oxide production: The occurrence of the ascorbyl radical anion under oxygen-free conditions is suggestive of its formation upon homolysis of short-lived *O*-nitrosoascorbate. The latter

compound might be generated by a nonradical transnitrosation reaction between NANT and ascorbate. The 30% lower yield of nitric oxide with respect to the yield of nitrite (see above) would then imply that homolytic O–NO cleavage of *O*-nitrosoascorbate is accompanied by its competitive hydrolysis. In fact, it is well known that alkyl nitrites in aqueous solution are generally prone to proton-catalyzed hydrolysis.[26] Unfortunately, no procedure for the preparation of *O*-nitrosoascorbate could be found in the literature, evidently due to its instability. To overcome this problem, reactions of nitric oxide with ascorbate as well as with common oxidation products of ascorbate were additionally performed, employing the frequently used •NO donor compound SPE/NO (SPE/NO = (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}-diazen-1-ium-1,2-diolate).

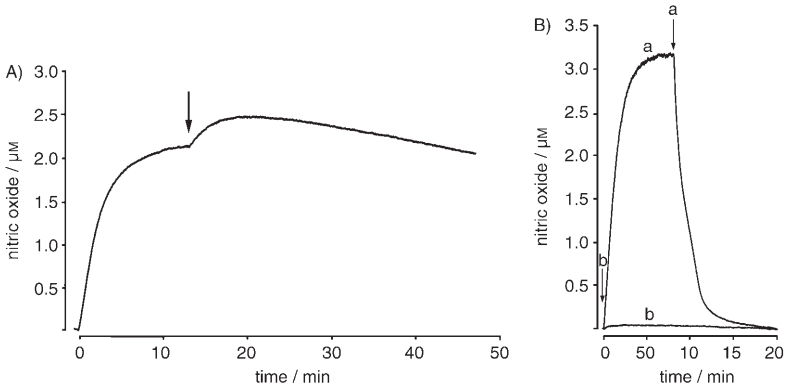


Figure 9. Effects of ascorbic acid and dehydroascorbic acid on the steady-state level of nitric oxide. The effects of addition of: A) ascorbate (1 mM), and B) dehydroascorbic acid (10 mM) on the steady-state concentration of nitric oxide produced from the decomposition of SPE/NO (100 μM) in phosphate buffer (50 mM, pH 7.4, 37 °C). The arrows indicate the point of addition of the ascorbic acid derivative. In B, DHA was added 10 min (trace a) and 10 s (trace b), respectively, after the beginning of the SPE/NO decay. The displayed traces are representative of four similar experiments.

On inspection of Figure 9A it can clearly be seen that under normoxic conditions the addition of ascorbate slightly increases the steady-state level of nitric oxide produced from SPE/NO. This increase of the steady-state concentration of •NO can reasonably be explained in terms of highly effective trapping of the nitrogen dioxide formed by autoxidation of nitric oxide [Eq. (4)].[27]

$$ASC^- + {}^{\bullet}NO_2 \rightarrow ASC^{\bullet -} + H^+ + NO_2^- \quad (k_4 = 3.6 \times 10^7\ M^{-1}\,s^{-1})^{[25]} \qquad (4)$$

This reaction depletes nitrogen dioxide, thereby diminishing •NO consumption through the formation of N_2O_3 and its subsequent hydrolysis [Eqs. (5) and (6)].

$${}^{\bullet}NO_2 + {}^{\bullet}NO \rightleftharpoons N_2O_3 \quad (k_5 = 1.1 \times 10^9\ M^{-1}\,s^{-1})^{[25]} \qquad (5)$$

$$N_2O_3(+H_2O) \rightleftharpoons 2\,NO_2^- + 2\,H^+ \quad (k_6 = 2000\ s^{-1})^{[28]} \qquad (6)$$

However, since reactions according to Equations (5) and (6) provide no satisfactory explanation for the observed difference in the yields of nitric oxide and nitrite, an additional nitric oxide-consuming pathway must be operative. Most surprisingly, we found that dehydroascorbic acid (DHA) effectively consumes the nitric oxide released from SPE/NO (Figure 9B). This unexpected observation seems unlikely to be due to intermediate formation of an adduct between •NO and DHA because density functional theory calculations performed at the MPW1B/6-31+G(d,p) level of theory predict that the formation of an •NO–DHA adduct would be highly endergonic by about 30 kcal mol^{-1} (calculations not shown). In 1998, Jung and Wells[29] reported that at physiological pH DHA spontaneously converts to both ascorbate and *erythro*-ascorbate. Both compounds have similar UV/Vis spectra.

Accordingly, Figure 10 shows that ascorbate and *erythro*-ascorbate are formed at about 25 μM from dissolved DHA (1 mM) within 30 min. However, the formation of ascorbate and *erythro*-ascorbate cannot account for the consumption of •NO by DHA because the steady-state concentration of nitric oxide is increased rather than decreased by the addition of ascorbate (see Figure 9A). Kimoto et al.[30] reported that at alkaline pH as well as in nonaqueous solution only *erythro*-ascorbate, and no ascorbate, is formed from DHA via the intermediates 2,3-diketogluconic acid and xylosone [Eq. (7)].

$$DHA \rightarrow\!\rightarrow \textit{erythro}\text{-ascorbate} \qquad (7)$$

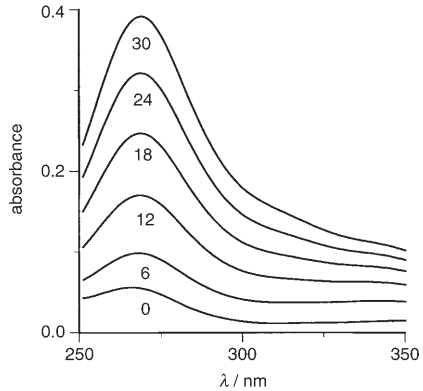


Figure 10. Formation of ascorbate and *erythro*-ascorbate by spontaneous decay of dehydroascorbic acid (1 mM) in phosphate buffer (50 mM, pH 7.4, 37 °C), as monitored spectrophotometrically for the times (min) indicated. The shown traces are representative of four similar experiments.

Considering this finding and bearing in mind that the ascorbyl radical anion is in equilibrium with DHA and ascorbate [Eq. (8)],[31]

$$DHA + ASC^- \rightleftharpoons 2\,ASC^{\bullet -} + H^+ \quad (8)$$

it can reasonably be concluded that the ascorbyl radical anion may also be generated at physiological pH from a similar reaction between DHA and *erythro*-ascorbate [Eq. (9)],

$$DHA + \textit{erythro}\text{-}ASC^- \rightleftharpoons ASC^{\bullet -} + \textit{erythro}\text{-}ASC^{\bullet -} + H^+ \quad (9)$$

so that ascorbate is ultimately generated via the sequence Equation (7)→Equation (9)→Equation (8). In fact, after dissolution of DHA in phosphate buffer, a doublet signal similar to that of the ascorbyl radical anion was instantaneously detected by means of ESR spectrometry (Figure 11a).

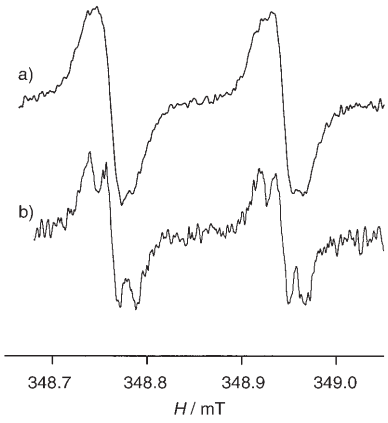


Figure 11. ESR detection of the formation of the ascorbyl and *erythro*-ascorbyl radical anions by spontaneous decomposition of dehydroascorbic acid. Radical anion formation during the spontaneous decomposition of dehydroascorbic acid (50 mM) in phosphate buffer (50 mM, pH 7.4, 23 °C) was monitored by using ESR spectrometry 2 min (a) and 30 min (b) after dissolution of DHA. ESR hyperfine splittings were evaluated by simulation. ESR data: ascorbyl radical anion, a(H) = 1.79 (1 H), a(H) = 0.018 (2 H), a(H) = 0.007 mT (1 H); *erythro*-ascorbyl radical anion, a(H) = 1.84 (1 H), a(H) = 0.038 (1 H), a(H) = 0.024 mT (1 H), g = 2.0053.

Close inspection of this spectrum reveals a superposition of the signals of the ascorbyl radical anion and the *erythro*-ascorbyl radical anion[32] in a relative 1:1 ratio, in accordance with the mechanism outlined above. The fraction of the *erythro*-ascorbyl radical anion decreased with time (Figure 11b), but the steady-state intensity of the sum of the two signals decreased only slightly in the absence of $^{\bullet}NO$ (Figure 12, trace a), indicating that both species are permanently regenerated under the applied conditions.

Reactions according to Equations (7)–(9) thus represent a simple route for the generation of the ascorbyl radical anion in the absence of either transition metals, reactive oxygen species, or reactive nitrogen oxides. Provided that the ascorbyl radical reacts with nitric oxide in a reversible manner to produce the intermediate *O*-nitrosoascorbate [Eq. (10)]

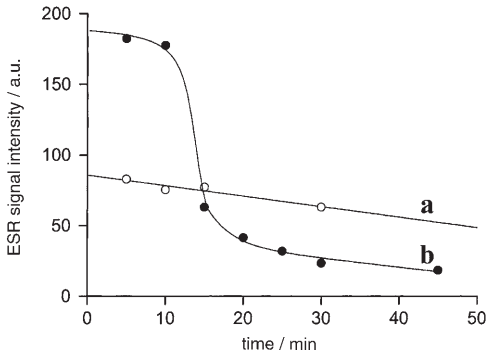


Figure 12. Effect of nitric oxide on the combined ESR signal intensity of the ascorbyl and *erythro*-ascorbyl radical anions obtained by spontaneous decomposition of dehydroascorbic acid. The formation of the ascorbyl and *erythro*-ascorbyl radical anions during the spontaneous decomposition of dehydroascorbic acid (50 mM) in phosphate buffer (50 mM, pH 7.4, 23 °C) was monitored by using ESR spectrometry in the absence (open symbols) and in the presence (filled symbols) of SPE/NO (2 mM).

$$ASC^{\bullet -} + {}^{\bullet}NO \rightleftharpoons [NO(ASC)]^- \quad (10)$$

and this is followed by rapid hydrolysis of the latter [Eq. (11)],

$$[NO(ASC)]^- + H_2O \rightarrow ASC^- + NO_2^- + H^+ \quad (11)$$

then the steady-state concentration of the DHA-derived ascorbyl/*erythro*-ascorbyl radical anion would be expected to be strongly diminished in the presence of nitric oxide. This indeed turned out to be the case, as is evident from Figure 12. In the presence of SPE/NO, the ascorbyl/*erythro*-ascorbyl radical anion concentration at the beginning of the reaction was about twice as high (Figure 12, trace b) as in the absence of nitric oxide (Figure 12, trace a). This is because initially these radicals are additionally generated by attack of nitrogen dioxide on ascorbate/*erythro*-ascorbate [Eq. (4)]. After this initial phase, the ascorbyl/*erythro*-ascorbyl radical anion concentration decreases in an apparently exponential fashion, similar to the decrease of nitric oxide, giving rise to a sigmoidal concentration–time profile (Figure 9B).

Discussion

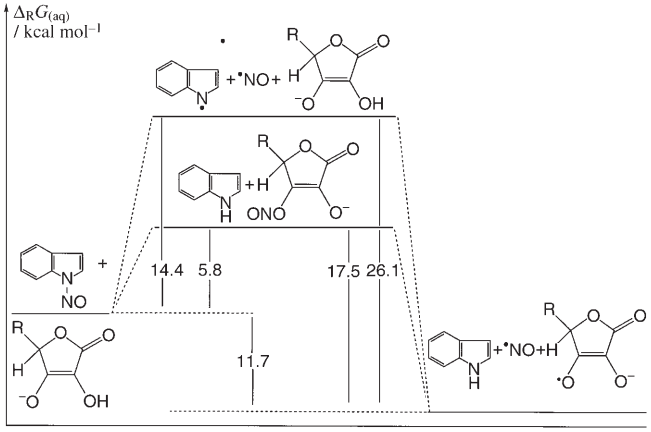
In past years, it has been demonstrated that vitamin C indirectly accelerates $^{\bullet}NO$ formation by enforcing the affinity of tetrahydrobiopterin for NOS,[33] by re-reducing oxidized tetrahydrobiopterin,[34] or by stabilizing the reduced state of the heme iron in guanylate cyclase.[35] Our experiments suggest that ascorbate additionally counterbalances nitric oxide levels through various nonenzymatic pathways, that is, by releasing $^{\bullet}NO$ from *N*-nitrosated tryptophan derivatives, by

scavenging •NO-depleting reactive nitrogen-oxygen species, and through trapping of •NO by ascorbate-derived products.

Chemical mechanism: The ascorbate-mediated decomposition of NANT can be satisfactorily described in terms of a radical chain mechanism with the ascorbyl radical anion as the chain-propagating intermediate. The initiation reaction should be a transfer of the nitroso function from NANT to ascorbate to yield *O*-nitrosoascorbate [Eq. (12)]. Similar reactions between NANT and H_2O_2[20] or *n*-butanol[36] are well known. The $ASC^{\bullet-}$-producing reaction should then be homolytic fragmentation of $[NO(ASC)]^-$ [Eq. (13)].

$$NANT + ASC^- \rightarrow NAT + [NO(ASC)]^- \quad (12)$$

$$[NO(ASC)]^- \rightarrow {}^{\bullet}NO + ASC^{\bullet-} \quad (13)$$



Scheme 1. Comparison of the homolysis of the N–NO bond with the chain-initiating transnitrosation pathway.

In order to elucidate the thermodynamics of possible pathways for the initiation reaction, quantum chemical calculations were performed at the CBS-QB3 level of theory utilizing *N*-nitrosoindole as a model for *N*-nitrosotryptophan derivatives. In Scheme 1, the proposed overall pathway of de Biase et al.,[11] that is, homolysis of the N–NO bond to give •NO and the corresponding aminyl radical, is compared with the chain-initiating transnitrosation pathway.

Table 2. Data from quantum chemical calculations.

Entry	Reaction	$\Delta_R G_{(g)}$[a]	$\Delta_R G_{solv}$[b] [kcal mol^{-1}]	$\Delta_R G_{(aq)}$[c]
1	(*E*)-nitrosoindole + ASC^- → (*E*)-nitrosoindole$^{\bullet-}$ + $ASC^{\bullet}$	44.7	−7.0	37.6
2	(*Z*)-nitrosoindole + ASC^- → (*Z*)-nitrosoindole$^{\bullet-}$ + $ASC^{\bullet}$	45.3	−6.7	38.6
3	$ASC^{\bullet-}$ + •NO → DHA + $^3NO^-$	66.8	−38.2	28.6
4	*O*-nitrosoascorbate → DHA + $^3NO^-$	50.4	−40.2	10.2
5	*O*-nitrosoascorbate → $ASC^{\bullet-}$ + •NO	−15.4	−2.0	−17.5
6	*O*-nitrosoascorbic acid → DHA + HNO	3.6	−8.9	−5.3
7	*O*-nitrosoascorbic acid → $ASC^{\bullet}$ + •NO	−6.0	−0.9	−6.9
8	*O*-nitrosoascorbate + H_2O → ASC + NO_2^-	21.5	−8.9	12.7
9	*O*-nitrosoascorbate + H_2O → ASC^- + HNO_2	−2.5	−0.9	−3.4
10	*O*-nitrosoascorbic acid + H_2O → ASC + HNO_2	−1.3	−3.9	−5.2
11	(*E*)-nitrosoindole + ASC^- → indole + *O*-nitrosoascorbate	9.9	−2.5	7.3
12	(*E*)-nitrosoindole + ASC^- → indole + $ASC^{\bullet-}$ + •NO	−7.1	−4.6	−11.7
13	(*E*)-nitrosoindole → indole$^{\bullet}$ + •NO	15.5	−1.1	14.4
14	indole$^{\bullet}$ + ASC^- → indole + $ASC^{\bullet-}$	−22.6	−3.4	−26.1
15	(*E*)-nitrosoindole + ASC^- + $ASC^{\bullet-}$ → indole + 2 $ASC^{\bullet-}$ + •NO	−7.1	−4.6	−11.7

[a] Gas-phase data. [b] Change of aqueous solvation free energies. [c] Aqueous-phase data, $\Delta_R G_{(aq)} = \Delta_R G_{(g)} + \Delta_R G_{solv}$.

As expected from our experiments, the initiation reaction involving *O*-nitrosoascorbate is thermochemically favored by 8.6 kcal mol^{-1} (5.8 vs. 14.4 kcal mol^{-1}). It is noteworthy that as early as 1959 Bunton et al.[37] proposed the formation of *O*-nitrosoascorbate from attack of nitrite on ascorbate under acidic conditions (pH 3–4), in line with the present results. These authors also suggested the rapid release of •NO with formation of the ascorbate radical anion. The alternative outer-sphere electron-transfer mechanism with formation of an intermediate NANT radical anion can safely be excluded. Since ascorbate has an oxidation potential of $E_{ox} = 0.282$ V,[38] it is unlikely to reduce *N*-nitrosotryptophan derivatives to the corresponding *N*-nitrosoindole radical anions ($E_{red} = -0.587$ V[11]). The latter conclusion was confirmed by means of CBS-QB3 calculations (Table 2, entries 1 and 2).

In any case, as the overall kinetics of the NANT–ascorbate reaction does not conform to a reaction order of two, the transnitrosation reaction according to Equation (12) cannot be the rate-determining step.

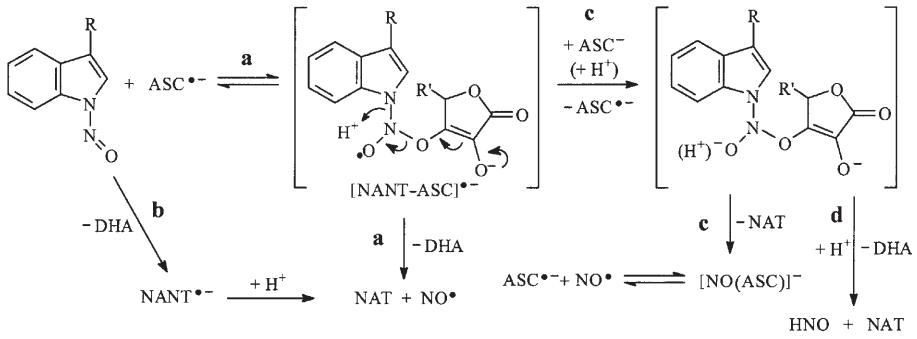
As noted above, for the ascorbate–NANT reaction, the overall reaction order with respect to the ascorbate concentration is a fractional order of about 1/3. This fact indicates the involvement of one or more reactive intermediates, most likely radical species. Since nitric oxide does not accelerate the decomposition of NANT (Figure 7B), $ASC^{\bullet-}$ is likely to be the key intermediate. Its action is experimentally verified by both the efficacy of •NO in decreasing the concentration of $ASC^{\bullet-}$ (Figure 12) as well as the DHA-dependent consumption of nitric oxide (Figure 9B). The low steady-state concentration of $ASC^{\bullet-}$ (Figure 8A, B) is consistent with its role as a chain-propagating intermediate [Eqs. (14)–(16)]:

$$NANT + ASC^{\bullet-} \rightleftharpoons [NANT - ASC]^{\bullet-} \quad (14)$$

$$[NANT - ASC]^{\bullet-} + H^+ \rightleftharpoons [HNANT - ASC]^{\bullet} \quad (15)$$

$$[HNANT - ASC]^{\bullet} + ASC^- \rightarrow NAT + {}^{\bullet}NO + H^+ + 2\,ASC^{\bullet-} \quad (16)$$

According to our CBS-QB3 calculations, the propagation chain [Eqs. (14)–(16)] is exergonic by 11.7 kcal mol^{-1} (Table 2, entry 15). Nitroso compounds, such as *S*-nitrosothiols, generally react with a variety of radicals with formation of the corresponding nitroxide radicals,[39] in our case the adduct between NANT and ASC$^{\bullet-}$, NANT–ASC$^{\bullet-}$. Nitroxide radicals of this kind must be expected to be rather short-lived,[40] and fragmentation would give, after protonation, NAT, DHA, and $^{\bullet}$NO (Scheme 2, path **a**).



Scheme 2. Suggested mechanism showing the hydrolysis reaction competing with both homolysis of *O*-nitrosoascorbate and fragmentation of *O*-nitrosoascorbic acid.

It should be noted that this reaction is mechanistically equivalent to an outer-sphere electron transfer from ASC$^{\bullet-}$ to NANT followed by fragmentation/protonation of the radical anion of NANT (Scheme 2, path **b**). However, [NANT–ASC]$^{\bullet-}$ and its protonated form, [NANT–ASCH]$^{\bullet}$, might be reduced by a second ascorbate molecule (Scheme 2, path **c**). Homolysis of either [NO(ASC)]$^{-}$ or *O*-nitrosoascorbic acid would then yield $^{\bullet}$NO and a second ASC$^{\bullet-}$. Hence, the concentration of the chain-carrying ASC$^{\bullet-}$ radical will be formally doubled per propagation cycle; this is typical of an explosive reaction, whereby the reaction rate increases rapidly. However, the occurrence of highly effective termination reactions controls the concentration of ASC$^{\bullet-}$ so that the reaction proceeds smoothly at physiological concentrations.

Termination of the chain process occurs primarily through two reactions, bimolecular self-decay of ASC$^{\bullet-}$ [Eq. (17)] and recombination of ASC$^{\bullet-}$ with $^{\bullet}$NO [Eq. (18)], that is, the reverse of Equation (13).

$$2\,ASC^{\bullet-} + H^{+} \rightarrow DHA + ASC^{-} \quad (17)$$

$$^{\bullet}NO + ASC^{\bullet-} \rightarrow [NO(ASC)]^{-} \quad (18)$$

At high ASC$^{\bullet-}$ concentrations, the moderately fast self-decay reaction [Eq. (17)] ($k = 6\times10^{7}$ M^{-1}s^{-1})[41] competes with the propagation reactions [Eqs. (14)–(16)]. Therefore, the overall reaction rate cannot be systematically increased by increasing the rate of the initiation reaction [Eqs. (12) and (13)]. The recombination of $^{\bullet}$NO with ASC$^{\bullet-}$ [Figure 12, Eq. (18)] preferentially proceeds when the concentration of $^{\bullet}$NO approaches the concentration of ASC$^{\bullet-}$. The ensuing decomposition of the thus formed [NO(ASC)]$^{-}$ appears to be quite complex because CBS-QB3 calculations predict the feasibility of three reasonable reaction channels. The fragmentation of *O*-nitrosoascorbate or *O*-nitrosoascorbic acid can explain both the difference in the yields of $^{\bullet}$NO and NO_2^{-} as well as the intermediacy of nitroxyl (^{3}NO^{-}, HNO), which is clearly indicated by the detection of N_2O after the reaction of NANT with ascorbate (Figure 2A, B). According to the experimental redox potentials, $E°(^{\bullet}NO/^{3}NO^{-}) = -0.81$ V[42] and $E°(ASC^{\bullet-}/DHA) = +0.17$ V,[38] the ASC$^{\bullet-}$ radical should be incapable of reducing nitric oxide to ^{3}NO^{-} by way of an outer-sphere electron-transfer mechanism; this is also supported by the CBS-QB3 data (Table 2, entry 3). Likewise, heterolytic fragmentation of *O*-nitrosoascorbate to ^{3}NO^{-} and DHA can be ruled out as an alternative reaction path (Table 2, entry 4 vs. entry 5). However, protonation of *O*-nitrosoascorbate to give *O*-nitrosoascorbic acid would explain the formation of N_2O because fragmentation to HNO and DHA is predicted to be in competition with the nitric oxide-yielding homolysis reaction (Table 2, entry 6 vs. entry 7). The difference in the yields of $^{\bullet}$NO and nitrite can then be easily related to the hydrolyses of *O*-nitrosoascorbate and *O*-nitrosoascorbic acid because both reactions are predicted to be exergonic (Table 2, entries 8–10). Hence, hydrolysis competes with both homolysis of *O*-nitrosoascorbate and fragmentation of *O*-nitrosoascorbic acid. The suggested mechanism is outlined in Scheme 2.

Physiological implications: One may ask whether the unexpected chemical reactivities of ASC$^{\bullet-}$ in accelerating both the release of $^{\bullet}$NO from nitroso compounds and the trapping of $^{\bullet}$NO (at elevated levels) may have some (patho)physiological significance. It is known that the physiological level of the ascorbyl radical anion is generally enhanced under conditions of oxidative stress.[43] Indeed, elevated levels of this radical have been found in rat homogenates of lung, spleen, liver, kidney, testis,[44] muscle, tumor tissues,[45] in rat plasma, and in human sera[46] following the action of reactive oxygen species. Since a physiological $^{\bullet}$NO concentration outcompetes vitamin E in the trapping of chain-carrying peroxyl radicals,[47] and because low amounts of $^{\bullet}$NO can protect cultured cells against reactive oxygen species,[48] it may be advantageous to slightly increase the amount of nitric oxide in vivo during oxidative stress through ASC$^{\bullet-}$-dependent pathways. Besides enhanced in vivo production of ASC$^{\bullet-}$ as a result of oxidative stress, the concentration of this radical should also be enhanced by increasing fluxes of reactive nitrogen-oxygen species because ascorbate is highly effective in counteracting peroxynitrite,[49] nitrogen diox-

ide,[27] and N_2O_3.[50] On the other hand, enhanced generation of such reactive nitrogen-oxygen species is generally caused by increased endogenous nitric oxide concentrations. Hence, since ascorbate is readily oxidized to $ASC^{\bullet-}$ by reactive nitrogen-oxygen intermediates,[25] subsequent reaction of the latter with $^{\bullet}NO$ would then cause a down-regulating feedback of the nitric oxide level. In other words, the antioxidative action of vitamin C may not only be due to direct trapping of reactive nitrogen-oxygen species, but may also be due to an attenuation of pathophysiological nitric oxide levels by way of its radical anion.

Experimental Section

Materials: *N*-Acetyl-D,L-tryptophan, sulfanilamide, dehydroascorbic acid, dodecyltrimethylammonium bromide, naphthylethylenediamine, diethylenetriaminepentaacetic acid, Chelex 100, and melatonin were obtained from Sigma (Taufkirchen, Germany). Ascorbic acid was purchased from Merck (Darmstadt, Germany). MAHMA/NO, SPE/NO, and PAPA/NO (PAPA/NO = (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl)amino]-diazen-1-ium-1,2-diolate) were from Situs (Düsseldorf, Germany). *N*-Acetyl-*N*-nitrosotryptophan, *N*-nitrosomelatonin, and FNOCT-4 were prepared as described in the literature.[9,13]. Stock solutions were freshly prepared on a daily basis and their concentrations were determined spectrophotometrically as described below. All other chemicals were of the highest purity commercially available.

Experimental system: Since 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). After gently shaking, the solution was stored overnight over the resin and was then carefully decanted off. Afterwards, the pH of all solutions was readjusted to 7.4±0.1 by the addition of 50 mM H_3PO_4 or 50 mM K_3PO_4. L-Ascorbic acid solutions were treated with Chelex 100 (1.5 g in 10 mL) for only about 2 h to avoid decomposition of the ascorbic acid. To avoid any influence of remaining amounts of transition metals, DTPA (100 μM) was added to all reaction solutions.

Determination of nitric oxide with an $^{\bullet}NO$-sensitive electrode: Nitric oxide formation was determined by using an $^{\bullet}NO$-sensitive electrode (ISO-NO; World Precision Instruments, Sarasota, Florida) as described in ref. [51]. The reaction mixtures were continuously stirred throughout the measurements, and the temperature was maintained at 25±1 °C or 37±1 °C as required. The electrode was calibrated daily, and $^{\bullet}NO$ production was quantified according to the manufacturer's instructions 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. A standard concentration of 100 μM of the nitrosated tryptophan derivative was applied to various concentrations of L-ascorbic acid. Nitric oxide production from the nitric oxide donor PAPA/NO was additionally observed, and the influence of dehydroascorbic acid on the steady-state concentration of nitric oxide was studied.

$^{\bullet}NO$ measurement with a fluorescent nitric oxide cheletropic trap: The amount of $^{\bullet}NO$ produced by the NANT–ascorbate reaction was quantified by using the fluorescent nitric oxide cheletropic trap FNOCT-4, which directly traps $^{\bullet}NO$.[13] FNOCT-4 fluorescence at λ_{em} = 460±5 nm was recorded with a Shimadzu spectrofluorophotometer at an excitation wavelength of λ_{exc} = 320±5 nm. The fluorescence intensity was calibrated by mixing 200 μM L-ascorbic acid and 50 μM FNOCT-4 in the presence and in the absence of 200 μM MAHMA/NO and reading the fluorescence after an incubation period of 30 min at 37±1 °C. In the subsequent experiments, 20 μM NANT was added to a mixture of ascorbic acid and FNOCT-4 (concentrations as above). The stability of the nitric oxide–FNOCT-4 reaction product, FNOCT–NOH, was verified over a period of 6 h. Experiments were performed under normoxic conditions as well as under hypoxic conditions using a glove bag (Roth, Karlsruhe, Germany) flushed with nitrogen. In the latter case, only 100 μM MAHMA/NO was used for the calibration, and the concentration of NANT was varied between about 10 and 30 μM.

Nitrite determination: The amounts of nitrite formed from both the reaction of ascorbic acid with NANT (100 μM each) and the hydrolysis of NANT were quantified 24 h after mixing of the reactants. An aliquot (50 μl) of the reaction mixture (NANT and L-ascorbic acid) and phosphate buffer (pH 7.4) (250 μl) were added to freshly prepared Griess reagent (750 μl). After a reaction period of 10 min, the optical density at 542 nm was recorded, and the nitrite concentration was evaluated by means of a calibration curve.

Kinetic experiments: The ascorbic acid-induced decay of NANT and *N*-nitrosomelatonin was monitored on a SPECORD S 100 spectrophotometer from Analytic Jena (Jena, Germany), using the absorption coefficients ε_{265} = 14500 $M^{-1}cm^{-1}$ for L-ascorbic acid,[52] ε_{335} = 6100 $M^{-1}cm^{-1}$ for NANT,[53] and ε_{346} = 7070 $M^{-1}cm^{-1}$ for *N*-nitrosomelatonin.[54] The reaction between ascorbic acid and the nitrosated tryptophan derivative (100 μM each) was monitored for 30 min by taking recordings every 30 s. Similar experiments (100 μM NANT and 100 μM ascorbic acid) were performed under hypoxic conditions in an argon-flushed glove box at 30 °C.

Determination of NAT and NANT: The production of NAT and the decay of NANT were monitored by using capillary zone electrophoresis apparatus (Beckman P/ACE 5000; Beckman, Munich, Germany). The separation conditions for capillary zone electrophoresis were as follows: fused silica capillary (50 cm effective length, 75 μm internal diameter); hydrodynamic injection for 5 s; temperature, 23 °C; voltage, 20 kV; normal polarity; UV detection at 280 nm; electrolyte system: 10 mM Tris, 10 mM NaH_2PO_4, and 25 mM dodecyltrimethylammonium bromide (pH 7.4); *I* = 65 μA, outlet (+). Migration times in capillary zone electrophoresis were 14 min. The reaction solution containing 1 mM NANT and 5 mM L-ascorbic acid was examined after 0.5, 1, and 3.5 h of incubation.

In addition, the formation of NAT and the decay of NANT were simultaneously monitored during the reaction of NANT with L-ascorbic acid (200 μM each) in phosphate buffer at room temperature. The formation of NAT was determined by tenfold dilution of the sample and recording the fluorescence with a Shimadzu spectrofluorophotometer (λ_{exc} = 270± 5 nm, λ_{em} = 358±5 nm). The decomposition of NANT was determined spectrophotometrically by 1:2 dilution of the samples and reading the optical density at 335 nm.

ESR measurements: The ascorbyl radical anion was identified by means of 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 1 mL of buffer solution (pH 7.4) containing either L-ascorbic acid (7.5 mM) and NANT (7.5 mM) in the absence and in the presence of oxygen or L-dehydroascorbic acid (50 mM) under normoxic conditions in the absence and in the presence of SPE/NO (2 mM). The reaction solutions were quickly transferred to an aqueous solution quartz cell (Willmad, Buena, NY). 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 were simulated using the WinSim program.[55]

^{15}N NMR measurements: ^{15}N NMR experiments were performed on a Bruker AVANCE DRX 500 instrument (Bruker Biospin, Germany) at 50.67 MHz. ^{15}N NMR chemical shifts (δ) are given in ppm relative to neat nitromethane (δ = 0) as an external standard. Spectra were acquired from a mixture of 1 M ascorbic acid and 1 M ^{15}N-NANT in DMSO. To elucidate the effect of water, similar measurements were performed with 100 mM ascorbic acid and 100 mM ^{15}N-NANT in 50 mM phosphate buffer solution in the presence of 10 % D_2O.

Quantum chemical calculations: Complete basis set (CBS-QB3) computations were carried out with the Gaussian 03 (revision AM64L-G03 RevC0.2) suite of programs.[56] Basis set superposition errors (BSSE) were estimated by performing counterpoise calculations at the MP2/CBSB3 level of theory. Gibbs free energies of solvation for water were estimated for the optimized gas-phase geometries by means 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 pro-

vide thermochemical estimates within "chemical accuracy" (± 1 kcal mol^{-1}).[57,58]

[1] S. Moncada, R. M. J. Palmer, E. A. Higgs, *Pharmacol. Rev.* **1991**, *43*, 109–142.
[2] M. Hoshino, K. Ozawa, H. Seki, P. C. Ford, *J. Am. Chem. Soc.* **1993**, *115*, 9568–9575.
[3] D. Nikitovic, A. Holmgren, *J. Biol. Chem.* **1996**, *271*, 19180–19185.
[4] Y. C. Hou, Z. M. Guo, J. Li, P. G. Wang, *Biochem. Biophys. Res. Commun.* **1996**, *228*, 88–93.
[5] Y. Y. Zhang, A.-M. Xu, M. Nomen, M. Walsh, J. F. Keaney, Jr., J. Loscalzo, *J. Biol. Chem.* **1996**, *271*, 14271–14279.
[6] K. Harohalli, C. E. Petersen, C.-E. Ha, J. B. Feix, N. V. Bhagavan, *J. Biomed. Sci.* **2002**, *9*, 47–58.
[7] M. Kirsch, A. Fuchs, H. de Groot, *J. Biol. Chem.* **2003**, *278*, 11931–11936.
[8] T. Suzuki, H. F. Mower, M. D. Friesen, I. Gilbert, T. Sawa, H. Ohshima, *Free Radical Biol. Med.* **2004**, *37*, 671–681.
[9] M. Kirsch, H. de Groot, *J. Pineal Res.* **2005**, *38*, 247–253.
[10] B. Blanchard-Fillion, C. Servy, C. Ducrocq, *Free Radical Res.* **2001**, *35*, 857–866.
[11] P. M. de Biase, A. G. Turjanski, D. A. Estrin, F. Doctorovich, *J. Org. Chem.* **2005**, *70*, 5790–5798.
[12] X.-Q. Zhu, J.-Q. He, Q. Li, M. Xian, J. Lu, J.-P. Cheng, *J. Org. Chem.* **2000**, *65*, 6729–6735.
[13] P. Meineke, U. Rauen, H. de Groot, H.-G. Korth, R. Sustmann, *Chem. Eur. J.* **1999**, *5*, 1738–1747.
[14] T. A. Meyer, D. L. H. Williams, R. Bonnett, S. L. Ooi, *J. Chem. Soc. Perkin Trans. 2* **1982**, 1383–1387.
[15] M. N. Hughes, *Biochim. Biophys. Acta* **1999**, *1411*, 263–272.
[16] W. A. Seddon, J. W. Fletcher, F. C. Sopchyshyn, *Can. J. Chem.* **1973**, *51*, 1123–1130.
[17] W. A. Seddon, M. J. Young, *Can. J. Chem.* **1970**, *48*, 393–394.
[18] L. J. Ignarro, J. M. Fukuto, J. M. Griscavage, N. E. Rogers, R. E. Byrns, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8103–8107.
[19] M. R. Gunther, D. J. Kelman, J. T. Corbett, R. P. Mason, *J. Biol. Chem.* **1995**, *270*, 16075–16081.
[20] M. Kirsch, M. Lehnig, *Org. Biomol. Chem.* **2005**, *3*, 2085–2090.
[21] P. Atkins, *Physical Chemistry*, Oxford University Press, Oxford, **1994**.
[22] J. W. Moore, R. G. Pearson, *Kinetics and Mechanism*, Wiley, New York, **1981**.
[23] M. R. Gunther, R. A. Tschirret-Guth, H. E. Witkowska, Y. C. Fann, D. P. Barr, P. R. Ortiz de Montellano, R. P. Mason, *Biochem. J.* **1998**, *330*, 1293–1299.
[24] Y.-L. Zhao, P. R. McCarren, K. N. Houk, B. Y. Choi, E. J. Toone, *J. Am. Chem. Soc.* **2005**, *127*, 10917–10924.
[25] A. B. Ross, W. G. Mallard, W. P. Helman, G. V. Buxton, R. E. Huie, P. Neta, *NDRL/NIST Solution Kinetics Database 3.0*, NDRL/NIST, Gaithersburg, MD, **1998**.
[26] D. L. H. Williams, *Nitrosation Reactions and the Chemistry of Nitric Oxide*, Elsevier, Amsterdam, **2004**.
[27] M. Kirsch, H.-G. Korth, R. Sustmann, H. de Groot, *Biol. Chem.* **2002**, *383*, 389–399.
[28] A. Treinin, E. Hayon, *J. Am. Chem. Soc.* **1970**, *92*, 5821–5828.
[29] C.-H. Jung, W. W. Wells, *Arch. Biochem. Biophys.* **1998**, *355*, 9–14.
[30] E. Kimoto, S. Terada, T. Yamaguchi, *Methods Enzymol.* **1997**, *279*, 3–12.
[31] J. van der Zee, P. J. A. van den Broek, *Free Radical Biol. Med.* **1998**, *25*, 282–286.
[32] M. Fitchett, B. C. Gilbert, *J. Chem. Soc. Perkin Trans. 2* **1986**, 1169–1177.
[33] H. Hofmann, H. H. H. W. Schmidt, *Biochemistry* **1995**, *34*, 13443–13452.
[34] J. M. May, *Free Radical Biol. Med.* **2000**, *28*, 1421–1429.
[35] J. N. Burstyn, A. E. Yu, E. A. Dierks, B. K. Hawkins, J. H. Dawson, *Biochemistry* **1995**, *34*, 5896–5903.
[36] R. Bonnett, P. Nicolaidou, *Heterocycles* **1977**, *7*, 637–659.
[37] C. A. Bunton, H. Dahn, L. Loewe, *Nature* **1959**, *183*.
[38] G. R. Buettner, *Arch. Biochem. Biophys.* **1993**, *300*, 535–543.
[39] W. Chamulitrat, *Arch. Biochem. Biophys.* **1998**, *355*, 206–214.
[40] J. Fossey, D. Lefort, J. Sorba, *Free Radicals in Organic Chemistry*, Wiley, Chichester, **1995**, 90–91.
[41] C. von Sonntag, H. P. Schuchmann, *Angew. Chem.* **1991**, *103*, 1255–1279; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 1229–1253.
[42] V. A. Benderskii, A. G. Krivenko, E. A. Ponomarev, *Sov. Electrochem.* **1989**, *25*, 154–161.
[43] G. R. Buettner, B. A. Jurkiewicz, *Free Radical Biol. Med.* **1993**, *14*, 49–55.
[44] K. Minakata, O. Suzuki, F. Horio, S. I. Saito, N. Harada, *Free Radical Biol. Med.* **2000**, *33*, 179–185.
[45] H. Isoda, K. Akagi, T. Murata, Y. Aoki, S. Ikeda, Y. Tanaka, T. Kihara, M. Ikeda, *Cancer Res.* **1996**, *56*, 5741–5744.
[46] K. Minakata, O. Suzuki, S. Saito, N. Harada, *Arch. Toxicol.* **1993**, *67*, 126–130.
[47] V. Darley-Usmar, H. Wiseman, B. Halliwell, *FEBS Lett.* **1995**, *369*, 131–135.
[48] D. A. Wink, Y. Vodovotz, J. Laval, F. Laval, M. W. Dewhirst, J. B. Mitchell, *Carcinogenesis* **1998**, *19*, 711–721.
[49] M. Kirsch, H. de Groot, *J. Biol. Chem.* **2000**, *275*, 16702–16708.
[50] M. G. Espey, K. M. Miranda, D. D. Thomas, D. A. Wink, *J. Biol. Chem.* **2001**, *276*, 30085–30091.
[51] Z. Taha, F. Kiechle, T. Malinski, *Biochem. Biophys. Res. Commun.* **1992**, *188*, 734–739.
[52] G. R. Buettner, *J. Biochem. Biophys. Methods* **1988**, *16*, 27–40.
[53] R. Bonnett, R. Holleyhead, *J. Chem. Soc. Perkin Trans. 1* **1974**, 962–964.
[54] A. G. Turjanski, F. Leonik, D. A. Estrin, R. E. Rosenstein, F. Doctorovich, *J. Am. Chem. Soc.* **2000**, *122*, 10468–10469.
[55] D. R. Duling, *J. Magn. Reson. Ser. B* **1994**, *104*, 105–110.
[56] Gaussian 03, Revision C.02, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople, Gaussian, Inc., Wallingford CT, **2004**.
[57] V. Barone, M. Cossi, *J. Phys. Chem.* **1998**, *102*, 1995–2001.
[58] V. Barone, M. Cossi, J. Tomasi, *J. Chem. Phys.* **1997**, *107*, 3210–3221.

Received: March 22, 2006
Published online: September 1, 2006

