Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 10, pp 1747–1753, 1996

PII: S0968-0896(96)00191-5
0968-0896/96 $15.00+0.00

Nitric Oxide-Induced Oxidation of α-Tocopherol

Marco d'Ischia* and Luisa Novellino
Department of Organic and Biological Chemistry, University of Naples Federico II, via Mezzocannone 16, I-80134 Naples, Italy

Abstract—Exposure of α-tocopherol (α-T) to nitric oxide under aerobic conditions resulted in a complex oxidation process whose final outcome was dictated by the nature of the reaction medium. In a cyclohexane solution, a prevailing route led to a mixture of relatively unstable polar products positive to Griess reagent. On standing at room temperature these were partially converted to the novel 2,3-dimethyl-4-acetyl-4-hydroxy-5-nitroso-2-cyclopentenone derivative. Reaction of α-T via a secondary oxidation path led to the formation of α-tocopherylquinone (α-TQ) as well as of little amounts of the corresponding nitrite ester. A quite different product pattern was observed when the reaction was carried out on a suspension of α-T in 0.1 M phosphate buffer, pH 7.4. Besides a significant formation of α-TQ and its nitrite ester, product analysis revealed a characteristic pattern of apolar compounds consisting of a yellow dimer and a series of related oligomers. These results provide an improved chemical background to inquire into the role of α-T in nitric oxide-induced tissue injury. Copyright © 1996 Elsevier Science Ltd

Introduction

Current reports on the biological functions of nitric oxide (nitrogen monoxide, NO) in the central nervous system place increasing emphasis on the potential role of this unconventional neurotransmitter as an etiological agent in a variety of pathological processes, including neuronal degeneration in Parkinson's and Alzheimer's diseases,[1] demyelination in multiple sclerosis[2] and, especially, secondary tissue damage in cerebral ischemia and reperfusion.[3,4] If produced in abnormally high fluxes by hyperstimulated glutamate neurones, astrocytes, microglial cells, as well as by macrophages and other cells of the immune system,[5,6] nitric oxide appears to act as a mediator and an amplifier of oxidative stress-induced free radical processes in response to the primary injury.[7]

Because of the elusive nature of the intermediate products and the broad spectrum of molecular events implicated, the detailed dissection of the mechanisms of nitric oxide-induced neurotoxicity remains at present a most challenging task. Chemical evidence indicates that, though relatively inert per se, nitric oxide is readily converted in the presence of oxygen and oxygen-derived species to a range of oxidized species, including nitrogen dioxide and peroxynitrite, with a longer half-life and a larger diffusion radius than active oxygen radicals.[8,9] Such nitric oxide-derived species would exert damaging effects at remote sites from the actual location of oxygen-radical generation, whereby a cascade of neurochemical and pathophysiological processes is set in motion.

A crucial event in this scenario is thought to be a depletion of the lipid-soluble antioxidant α-tocopherol (α-T), the major component of natural vitamin E. A recent report by de Groot et al.[10] shows that in the presence of oxygen nitric oxide is capable of provoking a rapid oxidation of α-T. Similar effects are produced by nitrogen dioxide[11] and peroxynitrite, the latter inducing oxidation of α-T in low-density lipoprotein.[12] Complementary evidence for a central role of the α-T-nitric oxide interaction comes from a relevant paper by Burkart et al.[13] showing that this phenolic chain-breaking antioxidant efficiently protects eukaryotic cells from nitric oxide-induced cytoxicity, and therefore qualifies as a member of the cellular nitric oxide defense system, a property which is not shared by other important biological antioxidants like ascorbic acid and glutathione.

In spite of the keen interest raised by α-T as a prime target in nitric oxide-induced neurotoxicity, the chemistry of this reaction has remained largely unexplored. The only available information[14] would point to tocopherylquinone (α-TQ) as the major, if not the sole, product derived by oxidation of α-T with nitric oxide and related species.

In the course of our studies on the reactivity of nitric oxide toward potential biochemical targets in oxidative stress-induced cell damaging processes,[15,16] we were recently prompted to investigate the effects of this free radical on α-T. In contrast to previous reports, we found that the reaction follows a more complex path than implied by formation of the sole α-TQ. It is the aim of the present paper to report the isolation and

Key words: vitamin E; tissue injury; oxidative stress; nitrosation; α-tocopherylquinone.
Abbreviations: α-T, α-Tocopherol; α-TQ, α-tocopherylquinone; α-TQE, α-tocopherylquinone-2,3-epoxide; TLC, thin layer chromatography.

characterization of the main products arising by reaction of nitric oxide with α-T in vitro.

Results

The reaction of α-T with nitric oxide was investigated mainly under two experimental conditions, namely with the substrate dissolved in cyclohexane or finely suspended in phosphate buffer. Such conditions were chosen to represent opposed extremes for what concerns solvation of the substrate and polarity of the medium.

In preliminary experiments, purified nitric oxide gas was slowly bubbled through either a solution of α-T in cyclohexane or a fine suspension of the antioxidant in 0.1 M phosphate buffer at pH 7.4 rigorously under an oxygen-free atmosphere. In both cases, HPLC analysis revealed no detectable consumption of α-T. By contrast, when air was allowed into the reaction mixture, a fast oxidation reaction took place, as apparent by the rapid decay of α-T both in cyclohexane and in phosphate buffer. The rate of decay of α-T was found to vary significantly depending on such experimental parameters as nature of the solvent, volume of the solution, shape and size of the flask, rate of stirring. Throughout this study, the reaction of α-T with nitric oxide was therefore carried out in air-equilibrated media.

Reaction of nitric oxide with α-T in cyclohexane

When aerated solutions of α-T (1.0×10^{-5} M) in cyclohexane were reacted with variable amounts of nitric oxide over a period of 15 min at 25 °C, complete substrate consumption was observed with initial NO concentrations higher than 1.5×10^{-4} M. Under these conditions, HPLC analysis of the reaction mixtures revealed the formation, besides some α-TQ, of complex patterns of products whose chromatographic properties did not match with those of any of the known oxidation products of α-T.

Table 1 reports α-T consumption and the yields of formation of α-TQ with various concentrations of nitric oxide in cyclohexane. It appears that α-TQ formation decreases with increasing initial NO concentration, suggesting that alternative reaction paths become prevailing at high NO concentration. To gain insight into the nature of the reaction products, in further experiments the reaction of nitric oxide with α-T was carried out by directly bubbling NO gas into the reaction mixture with the substrate at higher concentration.

Figure 1(A) shows a typical HPLC elution profile of the reaction mixture obtained by slowly bubbling nitric oxide into a cyclohexane solution of 1 mM α-T over a period of 10 min at 25 °C. Under such conditions, α-TQ, though clearly detectable, was only a minor component of the reaction mixture. In four different experiments the yield of α-TQ, determined both by

Table 1. Substrate consumption and α-TQ formation by reaction of α-T (1.0×10^{-5} M) with different amounts of nitric oxide in cyclohexane

NO concentration (μM)	α-T consumption (%)	α-TQ yield (%)[a]
7500	100	10–13
3750	100	25–28
150	99	39–42
37	97	49–51
19	42	56–61

[a]Determined by HPLC based on reacted α-T (range for three experiments). All analyses were run at least in duplicate. Reaction time was 10 min.

HPLC analysis and on the isolated material, was found to vary in the range 10–15% based on reacted α-T. Other known products of α-T oxidation, such as the quinone epoxide (α-TQE) and the tocopherones, were apparently formed in low or negligible yields. Similar HPLC traces were obtained with α-T at lower concentrations (e.g. 1.0×10^{-5} M) provided that NO concentration was in large excess.

TLC analysis of the reaction mixture revealed a consistently complex pattern of UV-absorbing

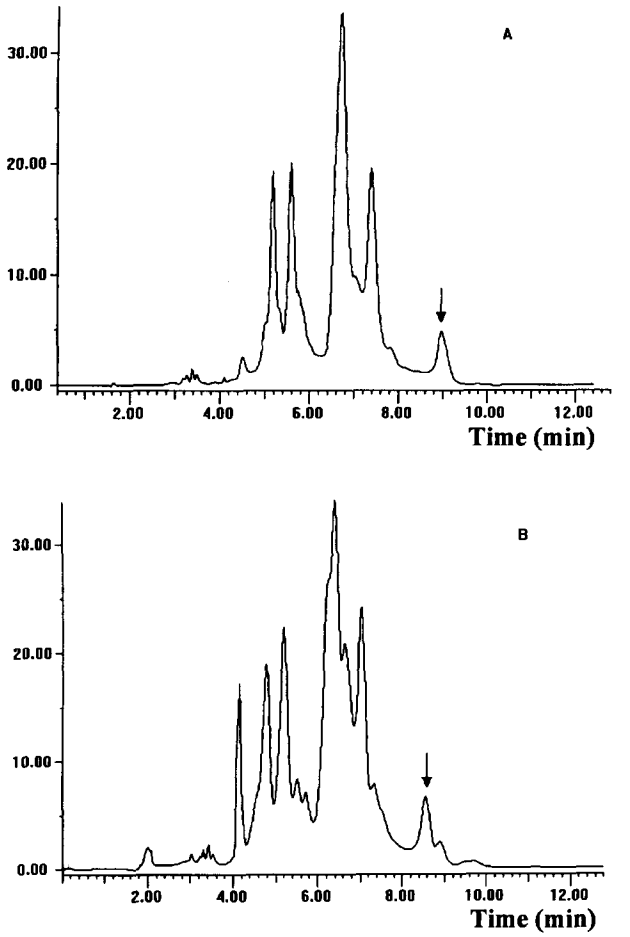

Figure 1. HPLC elution profile of the mixture obtained by reaction of nitric oxide with α-T in cyclohexane. Detection was at 240 nm. (A) Immediately after removal of nitric oxide with argon; (B) after 5 h. Compound **1** is eluted at about 4 min. The arrow indicates α-TQ.

products. These comprised, as the most noticeable feature, a group of relatively polar compounds positive to the Griess reagent, suggesting incorporation of nitric oxide. Attempts to characterize such Griess-positive products were hampered by difficulties encountered during isolation and/or by the marked instability during acquisition of NMR spectra.

Notably, however, when the reaction of α-T with nitric oxide was stopped by flushing the solution with argon, and the mixture was allowed to stand for some hours at 25 °C, a significant change in the product pattern occurred, leading to the formation of a relatively more polar compound (TLC, R_f 0.4 in cyclohexane:ethyl acetate, 6:4) positive to the Griess reagent. Figure 1(B) shows a typical HPLC elutogram of the reaction mixture recorded after 5 h, highlighting the polar Griess-positive product. The compound was eventually isolated by preparative TLC and was identified as the novel 2,3-dimethyl-4-acetyl-4-hydroxy-5-nitroso-2-cyclopentenone derivative (**1**) arising by nitrosation and oxidative ring contraction of α-T. A detailed discussion of the arguments supporting such a structural assignment has been reported in a preliminary communication.[16] The average yield of **1** after complete decomposition of the reaction mixture was more than 10% based on reacted α-T.

Time course studies using both HPLC and TLC did not show significant changes in product distribution during the early stages of the reaction, when most of the α-T was still present in the mixture. No significant variation was observed when different concentrations of α-T were used. Significant, yet variable amounts of **1** were also detected by HPLC in the reaction mixtures obtained by reacting 1.0×10^{-5} M α-T with excess nitric oxide in cyclohexane.

To get some insight into the mechanism of formation of **1**, in separate experiments α-TQ and α-TQE were exposed to nitric oxide in cyclohexane under the usual conditions, but in neither case could any **1** be detected. These results indicate that neither α-TQ nor α-TQE are precursors to **1**.

Another characteristic feature of the TLC product pattern was the presence of a relatively apolar, Griess-positive compound (R_f 0.45 in cyclohexane:ethyl acetate, 95:5) that could not be detected by HPLC, probably due to decomposition in the eluant. This could eventually be obtained in small amounts by preparative TLC as an oily residue (λ_{max} 260, 268 nm) which, however, readily decomposed to α-TQ both in solution and when stored in the absence of solvent. The marked instability of the product prevented acquisition of meaningful ^1H and ^{13}C NMR spectra. The FT-IR spectrum, taken on a partially decomposed sample containing some α-TQ, displayed as the most salient feature strong bands at 1642 cm^{-1}, for the quinone moiety, and 1603 cm^{-1}, suggesting a nitrite group. Overall, these data were strongly suggestive of the nitrous ester of α-TQ (**2**). Support to this view was obtained in separate experiments, in which the synthesis of **2** was carried out by a straightforward route involving transesterification between *n*-butylnitrite and α-TQ. Although the O-nitrosation product could not be isolated in pure form due to its marked instability, its chromatographic properties (TLC) and behaviour to Griess reagent were identical to those of the product obtained from reaction of α-T with nitric oxide.

In further experiments, α-T was allowed to react with *n*-butylnitrite in cyclohexane solution. Product analysis revealed a relatively rapid consumption of the substrate and the formation of substantial amounts of α-TQ and its nitrite ester **2**, but no detectable **1** was formed. Attempts to isolate and characterize other minor reaction products met with failure.

Reaction of nitric oxide with α-T in phosphate buffer

When α-T (1.0×10^{-5} M) was suspended in a saturated NO solution (ca. 1.8×10^{-3} M) in 0.1 M phosphate buffer at pH 7.4 at 25 °C, and air was then allowed into the mixture, a smooth reaction occurred, as apparent from the substrate decay after 15 min (more than 70%) and the concomitant formation of α-TQ (ca. 50% yield based on reacted α-T) as the major product.

In subsequent experiments, the reaction of nitric oxide with α-T in phosphate buffer at pH 7.4 was carried out by bubbling purified NO gas through a fine suspension of the substrate at 1 mM concentration and at 25 °C under vigorous stirring. Determination of the levels of nitrite ions provided a rough estimate of the amount of NO absorbed by the mixtures in each experiment. Typically, bubbling of nitric oxide for 10 min converted more than 90% α-T and gave an average nitrite ion concentration of 4.4×10^{-2} M $\pm 1.1 \times 10^{-2}$ M (6 experiments).

HPLC analysis of the products obtained after extraction of the reaction mixture with cyclohexane showed the presence of α-TQ as the major product. Isolated or estimated (HPLC) yields were found to vary in the range 20–40% with respect to reacted α-T. Such variations in the product yield were apparently due to the heterogeneous character of the mixture and difficulties to ensure reproducible concentrations of nitric oxide versus oxygen. Significant amounts of the nitrite ester **2** were also observed by TLC analysis (estimated yield based on recovered α-TQ about 5–10%), though rapid hydrolysis occurred when the aqueous mixture was allowed to stand for some time at room temperature prior to extraction with cyclohexane. Neither **1** nor any of the other Griess positive products were apparently formed.

Notably, careful analysis of the reaction mixture by TLC (cyclohexane:ethyl acetate, 95:5) revealed the presence, besides α-TQ and its nitrite ester, of a characteristic set of relatively apolar compounds (R_f 0.5–0.7), which escaped detection by HPLC due to unusually long elution times on the reverse-phase column. The majority of these compounds, bright-

yellow in colour, displayed a typical absorption spectrum with a maximum centred at 294 nm and a less intense absorption at 334 nm. Scrutiny of the FT-IR, ^{1}H and ^{13}C NMR spectra and comparison with literature data allowed identification of the compound as the spirodimer **3**. Support to this structural assignment came from oxidation of α-T with potassium ferricyanide according to Skinner et al.,[17] a reaction known to produce substantial amounts of **3**. Chromatographic analysis revealed formation of a yellow product indistinguishable in all respects from that obtained by nitric oxide oxidation. By similar arguments, the presence of smaller amounts of related oligomers could be inferred, but their nature was not investigated further. No significant reaction apparent took place when α-T was suspended in an air-equilibrated solution containing sodium nitrite in 0.1 M phosphate buffer, pH 7.4.

In another experiment, 1 mM α-T in 0.1 M phosphate buffer pH 7.4 was allowed to react at 25 °C with sodium nitroferricyanide (sodium nitroprusside) a widely used donor of nitric oxide at 10 mM concentration. In spite of relatively long reaction times (about 72 h), due to the slow kinetics of decomposition of the donor,[18] the resulting pattern of products proved to be closely similar to that obtained by bubbling of nitric oxide gas, with the notable exception of the lack of nitrite ester **2**, which probably did not survive hydrolysis in the aqueous reaction medium. Quantitative determination of the nitrite ions which accumulated after 16 h indicated about 50% release of nitric oxide from the donor.

A summary of the main products identified in the reaction of α-T with nitric oxide in cyclohexane and in phosphate buffer, along with their yields is provided in Table 2.

Discussion

Although a coordinated production of oxygen- and nitric oxide-derived reactive species is now recognized as one of the inciting events for tissue injury in ischemia-reperfusion and other pathological processes in the central nervous system, the actual biochemical sequels have thus far escaped detailed characterization. Besides difficulties connected with the identification of the target molecules and the complex balance of competing pathways involved, the problem is compounded by the broad spectrum of factors which ultimately modulate the expression of nitric oxide reactivity in vivo. A very insightful analysis of such factors has recently been done by Rubbo et al.,[19] who emphasized the importance of the cellular site and rate of nitric oxide production, the partitioning of nitric oxide into different tissue compartments, the extent and rate of diffusion of nitric oxide, the effects of the molecular environment, and shifts in the liquid–gas equilibria of nitric oxide and dioxygen, to mention only a few.

Similar factors are expected to affect the reaction of nitric oxide with α-T in processes of oxidative stress. An additional, crucial point to be considered, which is probably the key finding to emerge from the present study, is the marked dependence of the mode of decomposition of α-T on the nature of reaction medium. As apparent from the observed product distribution, a lipophilic environment, as in cyclohexane solution, enhances coupling of nitric oxide with α-T, provided that NO is present in sufficiently high concentrations, whereas in aqueous suspension the reaction is much less efficient and oxidative processes normally prevail.

At the chemical level, this behaviour is likely to reflect a competition between different autoxidation routes of nitric oxide, as well as concentration and medium effects on the half-life and redox-potential of secondary oxidants. In this connection, it is known, for example, that changes in concentration and reaction medium can basically modify the reactivity of nitrogen dioxide, a central intermediate in nitric oxide autoxidation, altering the balance of hydrogen abstraction versus free radical addition processes.[20,21]

No definite evidence is available about the species that actually brings about α-T oxidation in the early stages. Aerial oxidation of nitric oxide involves a complex sequence of reactions that are triggered by coupling with oxygen, to give a peroxynitrite radical intermediate which evolves to afford nitrogen dioxide.[22,23] The subsequent fate of nitrogen dioxide is dictated

Table 2. Main products obtained by reaction of α-T with nitric oxide and related compounds

Reagent	Solvent or medium	Products (% yield)[a]				
		α-TQ	α-TQE	**1**[b]	**2**[c]	**3**
Nitric oxide	cyclohexane	10–15	less than 5	10–15	5	n.d.[d]
Nitric oxide	0.1 M phosphate buffer, pH 7.4	20–40	n.d.	n.d.	5–10	10–25
n-Butylnitrite	cyclohexane	20–30	less than 5	n.d.	20–30	n.d.
Sodium nitroprusside	0.1 M phosphate buffer, pH 7.4	25–35	n.d.	n.d.	n.d.	20–30

[a]Product yields (%) refer to reacted substrate and were determined on the basis of at least three experiments. All analyses were run at least in duplicate. Reaction times did not exceed 10 min.
[b]Determined after 5 h.
[c]Estimated after complete conversion to α-TQ.
[d]n.d.: not detected.

mainly by its rate of formation and the relative concentrations of nitric oxide and oxygen in the medium: normally, dinitrogen trioxide (nitrous anhydride) represents an important intermediate with nitrosating properties, but its reactivity is influenced by the presence of water or moisture, causing extensive hydrolysis to nitrous acid or nitrite ions.

Effects of solvation on the rate and mode of decomposition of α-T and the relative stability of transient oxidation products, like the phenoxy radical and the tocopherone cation, should also be considered. Thus, it could be argued that in a lipophilic environment the trapping of radical intermediates of α-T oxidation by nitric oxide becomes a relatively efficient process, on account of a higher time of survival of those species.

From the transesterification experiments with *n*-butylnitrite, converting α-T to its nitrite ester, it seems reasonable to rule out the involvement of this ester in the coupling route leading eventually to **1**. Since formation of **1** does not seem to proceed through the intermediacy of α-TQ or α-TQE, it should follow from reaction of nitric oxide with an epoxytocopherone intermediate, or a related species, leading to a nitroso derivative, as schematically outlined in Figure 2. This latter would eventually suffer ring contraction by a mechanism akin to that described in the reaction of α-T with superoxide ion.[24]

Whether the ester **2** is formed via direct ring opening of an intermediate like **8a**-hydroxytocopherone nitrous ester, as suggested by Cooney et al.[11] or derives from a posteriori nitrosation of α-TQ is difficult to assess on the basis of available evidence, though we favour the latter possibility on the basis of the observed reactivity of α-TQ with nitric oxide. It is also likely that both α-TQ and **2** arise from decomposition of the initially formed nitrite ester of α-T, as observed in the relevant experiments. Less uncertainties surround the oxidative pathway leading to dimer **3** and its congeners. These arise by coupling of a quinonemethide intermediate derived by dismutation of the phenoxy radical of α-T.[25]

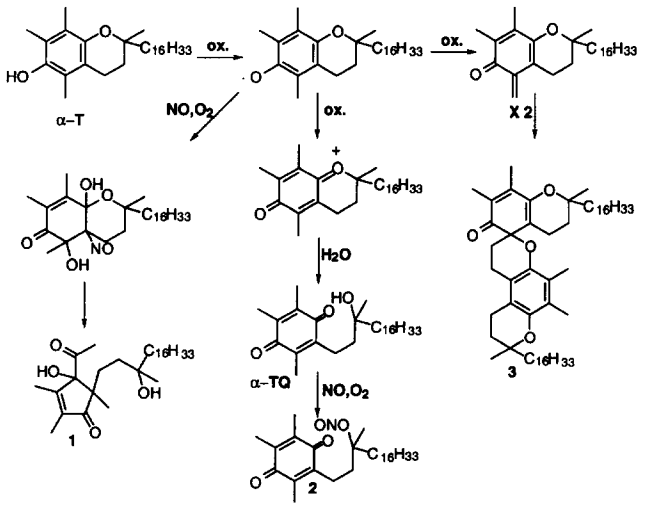

Figure 2. Proposed mechanism of reaction of α-T with nitric oxide.

The limited number of model systems examined and their poor mimicry of the actual situation in vivo do not permit any safe generalization about the biological relevance of the results described in the present paper. Yet, a plausible inference from this study is that the ability of α-T to counteract noxiuos effects induced by nitric oxide may vary significantly depending on the nature of the medium. Thus, in a lipophilic microenvironment protected from water a prominent process would involve covalent coupling with nitric oxide, which would account for a true scavenging action. On the other hand, at sites exposed to aqueous media, oxidative routes would prevail, whereby nitric oxide is mainly converted to inorganic nitrite ions. Such differences may be of particular relevance since nitric oxide is known to concentrate mainly in lipophilic cell compartments with a lipid:water partition coefficient of 8:1.[19] In this scenario, the observed generation of a potential nitrosating agent, like **2**, would somewhat affect the current perception of α-T as a potent antioxidant toward nitric oxide-induced neurotoxicity.

Experimental

Chemicals and reagents

dl-α-Tocopherol (α-T) and sodium nitroprusside were purchased from Aldrich. Nitric oxide gas (electronic grade, 99.99%) was from Air Liquide. It was purified from higher nitrogen oxides by passage through a solution of concentrated NaOH previously purged with Ar for 1 h and then through NaOH pellets. All other chemicals and solvents were of the highest purity available and were used as received. Reference samples of α-tocopherylquinone (α-TQ) and α-tocopherylquinone-2,3-epoxide (α-TQE) were prepared by standard procedures.[26,27] Reaction of α-T with alkaline potassium ferricyanide, to prepare dimer **3**, was performed as described by Skinner et al.[17] *n*-Butylnitrite was prepared fresh before use according to a known procedure.[28] All compounds were identified by analysis of spectral data and comparison of their chromatographic behaviour with literature data. Silica gel plates (0.25 and 0.5 mm, F254) were from Merck. Saturated nitric oxide solns (typically 1.5×10^{-2} M in apolar solvents[29] and 1.8×10^{-3} M in aqueous solvents[30]) were prepared at 25 °C by bubbling purified NO for 30 min through the deoxygenated solvent in a septum-capped vial by means of hypodermic needles. The solvents were deoxygenated with Ar for 45 min prior to the bubbling with NO. Aqueous buffers were prepared in glass distilled, deionized water.

Equipment

UV spectra were determined with a Perkin–Elmer Lambda 7 spectrophotometer. FT-IR spectra were determined on a Perkin–Elmer 1760-X spectrophotometer. EI-MS spectra were usually determined with a Trio 2000 Fisons spectrometer. Samples were ionized with a 70 eV electron beam. ^{1}H NMR (270 or 400 MHz) and ^{13}C NMR (67.9 or 100 MHz) were carried

out on a Bruker AC 270 or on a Bruker WM 400 spectrometer equipped with an Aspect 3000 computer. Long range 2-D carbon-proton shift correlation experiments were performed at 100 MHz using a delay of 50 ms.

Analytical HPLC was performed by using a Gilson model 302 pump, a Gilson 316 UV detector and a Spherisorb S50DS2 (4.6 × 250 mm, Phase separation, Ltd.) column. Detection was carried out at 295, 266, or 240 nm to monitor α-T, α-TQ, or reaction products, in that order. The flow rate was maintained at 1 mL/min. Quantification was carried out by comparing integrated peak areas with external calibration curves. All analyses were run at least in triplicate. CAUTION: All operations with nitric oxide were carried out under an efficient hood.

Reaction of α-T with nitric oxide in cyclohexane

In a typical experiment, appropriate volumes of freshly prepared NO-satd solns in cyclohexane were transferred via syringe into air-equilibrated solutions of the substrate in the same solvent in a septum-capped vial. The final α-T concentration was 1.0×10^{-5} M. The mixtures were left under stirring for 15 min at 25 °C, to complete the reactions, then were flushed with argon, evapd to dryness, taken up in methanol (4 mL), and injected into the HPLC. HPLC analysis was carried out using methanol:water (97:3 v/v) as the mobile phase. TLC (silica) was carried out using mixtures of cyclohexane:ethyl acetate in proportions varying in the range 95:5–60:40.

For product isolation purposes, purified nitric oxide gas was slowly bubbled for 10 min into a vigorously stirred solution of α-T (100 mg, 1 mM concentration) in anhydrous cyclohexane at 25 °C. Argon was then flushed through the solution for 10 min to remove excess nitric oxide and the solvent carefully evapd to dryness at room temperature in a rotary evaporator. The yellow residue was taken up in cyclohexane and subjected to chromatography.

Isolation of products 1 and 2

The mixture obtained after bubbling nitric oxide into a cyclohexane solution of α-T (1 mM) for 10 min was thoroughly purged with argon and allowed to stand for 4 h at room temperature in air. The residue obtained after evaporation of the solvent was chromatographed on silica gel plates (0.5 mm) using cyclohexane:ethyl acetate, 60:40 v/v as the eluant. For a satisfactory separation, no more than 30 mg of the mixture were loaded on each plate.

The UV absorbing band at R_f 0.4 was scraped off and eluted with ethyl acetate to give **1** as an oily residue (about 10% yield with respect to reacted α-T), homogeneous on TLC and HPLC. UV (cyclohexane) λ_{max} 239 nm (log ε = 3.9), 275 nm (sh, log ε = 3.1). FT-IR ($CHCl_3$) 3428, 1719, 1656, 1547, 1380, 1365 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.42 (1H, m), 2.18 (1H, m), 2.02 (3H, s), 1.87 (2 × 3H, s) 1.12 (3H, s) (other resonances were obscured by those of the phytyl side chain, which are not reported). ^{13}C NMR ($CDCl_3$): δ 202.8 (C), 192.9 (C), 163.5 (CH), 138.6 (CH), 99.2 (C), 89.3 (C) 72.2 (C), 11.8 (CH_3), 8.7 (CH_3). The resonances of the phytyl chain are not reported. EI-MS *m/z* 494 ($(M+1)^+$, 1%, exact mass calcd. for $C_{29}H_{52}NO_5$: 494.3848; found: 494.3840), 476 (2%), 419 (15%) 403 (20%), 266 (60%), 238 (35%), 195 (60%), 167 (65%), 153 (80%), 151 (85%), 137 (100%).

For isolation of nitrite ester **2**, the reaction mixture purged with argon was immediately evapd to dryness as above and chromatographed on silica gel plates (0.5 mm) using cyclohexane:ethyl acetate (95:5 v/v) as the eluant. The band at R_f 0.45 was scraped off, eluted with ethyl acetate and evaporated to dryness to give a mixture of **2** and α-TQ. UV (cyclohexane) λ_{max} 260, 268 nm; FT-IR ($CHCl_3$) 1642, 1603, 1559 cm^{-1}. No meaningful mass spectrum was obtained in spite of several attempts under different conditions.

Reaction of α-T with nitric oxide in phosphate buffer

A solution (43 μL) of α-T (4.6 mM) in deoxygenated methanol was added to a vigorously stirred satd NO solution (vol 20 mL, 4 mL of headspace) in 0.1 M phosphate buffer, pH 7.4 in a septum-capped vial at 25 °C using a hypodermic syringe. Air was then allowed into the resulting suspension by means of three hypodermic needles and the mixture was vigorously stirred at 25 °C for 15 min. The reaction mixture remained virtually unchanged after this time. The mixture was then flushed with argon and extracted with cyclohexane (2 × 20 mL). The organic phase was carefully evapd to dryness and taken up in methanol (4 mL) for HPLC analysis.

For large scale reactions, nitric oxide gas was slowly bubbled through a fine suspension of α-T (1 mM) in 0.1 M phosphate buffer, pH 7.4, obtained by adding dropwise a small volume (1–2%) of a concentrated methanol solution of the substrate to the appropriate volume of buffer, under vigorous stirring at 25 °C. Usually, about 100 mg of α-T were reacted for preparative purposes.

Use of a relatively concentrated buffer, such as 0.1 M or higher, prevented the marked drop in pH caused by the oxidative conversion of nitric oxide to nitrous acid, provided that the bubbling of the gas was rather slow and was not prolonged for more than 10 min. Under such conditions, the pH of the buffer was 6.5 or higher. The turbid reaction mixture was purged with argon and extracted three times with equal vols of cyclohexane. The aq phase was analyzed spectrophotometrically for nitrite concentrations using the Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in 5% phosphoric acid) to form a chromophore absorbing at 543 nm. The organic phase was dried over anhydrous sodium sulphate and carefully evaporated to dryness at room temperature in a rotary evaporator. Product analysis was carried out as described above. HPLC

analysis was carried out using methanol:water (95:5 v/v) as the mobile phase. Dimer **3** was identified by comparison of its chromatographic and spectral data with those of an authentic sample obtained by ferricyanide oxidation of α-T according to Skinner et al.[17] The presence of related oligomers was inferred on the basis of the spectrophotometric properties and the close similarities of the product pattern with that obtained by ferricyanide oxidation of α-T as described in the literature.[31]

Reaction of α-T with *n*-butylnitrite

An excess of *n*-butylnitrite was added to a soln of α-T (1 mM) in cyclohexane and the mixture allowed to stand at 25 °C under stirring for about 20 min. Work up and product analysis was carried out as described above for the reaction with nitric oxide in cyclohexane.

Reaction of α-T with sodium nitroprusside

α-T (1 mM final concn) was suspended in 0.1 M phosphate buffer, pH 7.4 as above and treated with sodium nitroprusside (10 mM) under stirring at 25 °C in the dark. After about 72 h, extraction of the mixture with cyclohexane afforded a yellowish residue that was analysed as above. Usually about 30% α-T was recovered unreacted.

Acknowledgments

This work was supported by grants from MURST and CNR (Rome). We thank Miss Silvana Corsani for technical assistance. Mass spectra were performed by the Servizio di Spettrometria di Massa del CNR e dell'Universita' di Napoli. The assistance of the staff is gratefully acknowledged. We thank CIMCF (Naples) for NMR spectra.

References

1. Bruhwyler, J.; Chleide, E.; Liegeois, J. F.; Carreer, F. *Neurosci. Biobehav. Rev.* **1993**, *17*, 373.

2. Mitrovic, B.; Ignarro, L. J.; Montestruque, S.; Smoll, A.; Merrill, J. E. *Neuroscience* **1994**, *61*, 575.

3. Beckman, J. S.; Crow, J. P. *Biochem. Soc. Trans.* **1993**, *21*, 330.

4. Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.

5. Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6368.

6. Darley-Usmar, V.; Wiseman, H.; Halliwell, B. *FEBS Lett.* **1995**, *369*, 131.

7. Huang, Z.; Huang, P. L.; Panahian, N.; Dalkara, T.; Fishman, M. C.; Moskowitz, M. A. *Science* **1994**, *265*, 1883.

8. Saran, M.; Bors, W. *Method Enzymol.* **1994**, *233*, 20.

9. Lewis, R. S.; Tamir, S.; Tannenbaum, S. R.; Deen, W. M. *J. Biol. Chem.* **1995**, *270*, 29350.

10. de Groot, H.; Hegi, U.; Sies, H. *FEBS Lett.* **1993**, *315*, 139.

11. Cooney, R. V.; Franke, A. A.; Harwood, P. J.; Hatch-Pigott, V.; Custer, L. J.; Mordan, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1771.

12. Hogg, N.; Darley-Usmar, V. M.;Wilson, M. T.; Moncada, S. *FEBS Lett.* **1993**, *326*, 199

13. Burkart, V.; Gross-Eick, A.; Bellmann, K; Radons, J.; Kolb, H. *FEBS Lett.* **1995**, *364*, 259.

14. Hogg, N.; Joseph, J.; Kalyanaraman, B. *Arch. Biochem. Biophys.* **1994**, *314*, 153.

15. d'Ischia, M.; Costantini, C. *Bioorg. Med. Chem.* **1995**, *3*, 923.

16. d'Ischia, M. *Tetrahedron Lett.* **1995**, *36*, 8881.

17. Skinner, W. A.; Alaupovic, P. *J. Org. Chem.* **1963**, *28*, 2854.

18. Feelisch, M. J. *Cardiovasc. Pharmacol.* **1991**, *17* (Suppl. 3), S25

19. Rubbo, H.; Radi, R.;Trujillo, M.; Telleri, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B. A. *J. Biol. Chem.* **1994**, *269*, 26066.

20. Pryor, W. A.; Lightsey, J. W. *Science* **1981**, *214*, 435.

21. Rathore, R.; Bosch, E.; Kochi, J. K. *Tetrahedron* **1994**, *50*, 6727.

22. Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. *J. Biol. Chem.* **1994**, *269*, 5881.

23. Lewis, R. S.; Deen, W. M. *Chem. Res. Toxicol.* **1994**, *7*, 568.

24. Matsuo, M.; Matsumoto, S.; Iitaka, Y. *J. Org. Chem.* **1987**, *52*, 3514.

25. Matsuo, M.; Matsumoto, S.; Iitaka, Y.; Niki, E. *J. Am. Chem. Soc.* **1989**, *111*, 7179.

26. Grams, G. W.; Eskins, K.; Inglett, G. E. *J. Am. Chem. Soc.* **1972**, *94*, 866.

27. Grams, G. W. *Tetrahedron Lett.* **1971**, 4823.

28. Blatt, A. H., Ed.: *Organic Syntheses*; John Wiley and Sons: New York, 1957; Vol II, pp 108–109.

29. Korth, H.-G.; Sustmann, R.; Lommes, P.; Paul, T.; Ernst, A.; de Groot, H.; Hughes, L.; Ingold, K. U. *J. Am. Chem. Soc.* **1994**, *116*, 2767.

30. Butler, A.; Williams, D. L. H. *Chem. Soc. Rev.* **1993**, *22*, 233.

31. Skinner, W. A.; Parkhurst, R. M. *J. Org. Chem.* **1964**, *29*, 3601.

(*Received in U.S.A.* 15 *March* 1996; *accepted* 9 *July* 1996)