Reactions of Peroxynitrite with γ-Tocopherol

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Received December 9, 1996⊗

The reaction of peroxynitrite with γ-tocopherol (γ-TH) in a methanol/potassium phosphate buffer solution results in the formation of four major products, which were identified as 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5-nitro-6-chromanol (NGT), 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5,6-chromaquinone (tocored), and two diastereomers of 8a-(hydroxy)-γ-tocopherone. NGT was the major product formed in these reactions, and its formation was modestly increased by increasing amounts of Fe^{3+}−EDTA. Tocored and NGT also were formed when γ-TH was exposed to 3-morpholinosydnonimine (SIN-1), a compound that decomposes to form peroxynitrite. When γ-TH reacted with the nitrating agent $NO_2^+BF_4^-$ in acetonitrile or methanol/potassium phosphate buffer, NGT and tocored also were formed, but the major product detected was γ-tocopherol quinone (γ-TQ). This product was not detected in reactions involving peroxynitrite. Oxidation of γ-TH by peroxynitrite involves nitration and electron transfer reactions. Since the product distribution in oxidations with $NO_2^+BF_4^-$ differed substantially from that in oxidations with peroxynitrite and SIN-1, NO_2^+ appeared not to be the principal species involved in NGT formation. Nitration of γ-TH may involve either peroxynitrite or some peroxynitrite-derived oxidant other than NO_2^+. Because of its stability and formation as a novel product of the reaction between γ-TH with peroxynitrite, NGT may be a useful *in vivo* marker for peroxynitrite interactions with lipid structures that contain γ-TH.

Introduction

A family of lipid soluble tocopherols and tocotrienols comprises vitamin E, which is the major chain-breaking antioxidant within biological membranes. Four major forms of the tocopherols are α, *β*, *γ*, and *δ*-tocopherol, which differ only in number and location of methyl substitutents on the aromatic chromanol ring (*1*). Of the tocopherols, α- and γ-tocopherols (α-TH, and γ-TH,[1] **1**, respectively) are the principal forms found in human and animal diets and comprise most of the vitamin E content of tissues (*2*). α-TH is the most effective antioxidant and exhibits more bioactivity and reactivity toward reactive oxygen species than any of the other tocopherols (*3, 4*). However, Cooney *et al*. recently found γ-TH to be a more effective cellular protectant against nitrogen dioxide (NO_2) and demonstrated that γ-TH suppressed NO_2-induced neoplastic transformation more effectively than α-TH *in vitro* (*5*). Their data suggest that γ-TH **1** may be more important in protecting organisms from reactive nitrogen species than α-TH.

One such reactive nitrogen species is peroxynitrite, a potent oxidizing agent formed both *in vivo* and *in vitro* by the reaction of nitric oxide and superoxide anion (*6, 7*). The rate constant for formation of peroxynitrite (6.7×10^9 M^{-1} s^{-1}) exceeds that for the degradation of both superoxide anion and nitric oxide (*8*). Cells capable of generating both superoxide anion and nitric oxide (e.g., macrophages, neutrophils) can thus produce peroxynitrite, especially when activated (*9, 10*). Peroxynitrite has been shown to react with a number of biomolecules including DNA, proteins, and lipids (*11*−*13*) and has been implicated in a number of disease processes including atherosclerosis, inflammation, sepsis, and tumor promotion (*14*−*17*). In aqueous solutions, peroxynitrite anion ($ONOO^-$) is rapidly protonated to peroxynitrous acid (ONOOH, $pK_a = 6.8$), which has a half-life of about 1 s at pH 7.4 (*7*). The decomposition chemistry of peroxynitrite/peroxynitrous acid is complex and has recently been reviewed (*18*). Peroxynitrite has been postulated to decompose via two distinct mechanisms. First, peroxynitrite has been postulated to undergo homolytic cleavage to yield two highly reactive free radicals, namely, NO_2 and the hydroxyl radical (•OH) (*19*). However, Koppenol *et al*. suggested that homolytic cleavage is energetically unfavorable and proposed instead that peroxynitrite may isomerize to an activated complex with reactivity similar to •OH (*20*). Increasing evidence for an activated form of peroxynitrous acid with the capability of undergoing one- or two-electron oxidations has been shown (*18, 21, 22*). An alternative reaction pathway is heterolytic cleavage of peroxynitrite to nitronium (NO_2^+) and hydroxide (OH^-) ions, which is catalyzed by coordination with metal ions (*23*). This reaction was implicated in the nitration of tyrosine residues near the active site of Cu/Zn superoxide dismutase by nitric oxide in the presence of superoxide (*24*). This heterolytic pathway contributes to the metal-catalyzed nitration of low molecular weight phenols by peroxynitrite (*24*). 3-Nitrotyrosine, the pre-

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⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.
[1] Abbreviations: DTPA, diethylenetriaminepentaacetic acid; Fe^{3+}−EDTA, ferric−sodium ethylenediaminetetraacetic acid; SIN-1, 3-morpholinosydnonimine; NO_2, nitrogen dioxide; NO_2^+, nitronium cation; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; α-TH, α-tocopherol; γ-TH, γ -tocopherol; γ-TQ, γ-tocopherolquinone; $\gamma\text{-T}^+$, γ-tocopherone cation; 8a-γ-TOH, 8a-(hydroxy)-γ-tocopherone; $\gamma\text{-T}^\bullet$, γ-tocopheroxyl radical; tocored, 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5,6-chromaquinone; NGT, 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5-nitro-6-chromanol; 4-HPA, 4-hydroxyphenylacetic acid.

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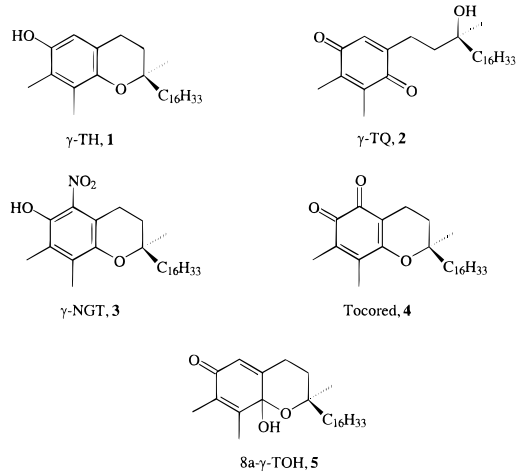


Figure 1. Structures of compounds referred to in the text.

dominant product formed from peroxynitrite/protein interactions, has therefore been used as a marker of *in vivo* protein alteration by peroxynitrite (*14*).

The antioxidant activity of α-TH toward peroxynitrite has been previously investigated (*25*, *26*). Hogg *et al.* demonstrated that successive one-electron oxidations of α-TH by peroxynitrite in methanol/water led to the formation of 8a-methoxytocopherone and α-tocopherolquinone (*26*). Because γ-TH possesses an unsubstituted carbon at C-5 of the chromanol ring *ortho* to the phenol (Figure 1), we postulated that nitro substitution may occur at C-5 upon exposure to peroxynitrite. However, the ability of peroxynitrite to act as a one-electron oxidant and as a nitrating agent suggests that other reactions may occur. This study therefore was undertaken to investigate the reactions of peroxynitrite with γ-TH **1**, to identify the major products formed, and to examine the possible mechanisms of formation of the major products.

Experimental Procedures

Chemicals. HPLC grade acetonitrile, hexane, and methanol were obtained from Fisher Scientific (Fairlawn, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated in the text. Peroxynitrite was prepared in a quenched-flow reactor with sodium nitrite and hydrogen peroxide and concentrated by freeze fractionation as previously described (*27*). Unreacted hydrogen peroxide was eliminated by passing the peroxynitrite solution through a manganese dioxide column prewashed with H_2O and 1 N NaOH. Concentrations of peroxynitrite were determined spectrophotometrically at 302 nm in 1 N NaOH, and peroxynitrite was stored as stock solutions in 0.1 N NaOH. Authentic standards of γ-TQ **2** and tocored **4** were generated by the reaction of γ-TH with $FeCl_3$ in ethanol (*28*).

Analytical Methods. Products of reactions were analyzed by HPLC with a Spectra Physics 8800 solvent delivery system (Spectra Physics, San Jose, CA), a Spherisorb ODS2 5-μm reverse-phase column (Alltech Associates, Deerfield, IL), and a Spectra Physics 100 variable wavelength detector. Compounds were eluted with a mobile phase consisting of methanol/water (98/2, v/v) at a flow rate of 1.5 mL min^{-1} and detected at a wavelength of 286 nm. UV−vis spectra of reaction products were obtained using a Hewlett Packard 1040A diode array detector (DAD) operated by an HP79994A analytical work station.

GC−MS analyses were performed with a Fisons MD800 instrument (Fisons Instruments, Beverly, MA). Samples for GC−MS analysis were derivatized to trimethylsilyl (TMS) derivatives using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, IL). Samples were analyzed in the positive electron impact mode at 70 eV. LC−MS analyses were done with a Finnigan TSQ 7000 instrument (Finnigan-MAT, San Jose, CA) equipped with an atmospheric pressure ionization source. ^{1}H- and ^{13}C-NMR spectroscopy were performed on either a Varian Gemini 200-MHz NMR or a Varian Unity 300-MHz NMR operating at 200−300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR as described in the Results.

Table 1. Quantification of Product Formation from Reaction of Peroxynitrite with γ-Tocopherol[a]

ratio of peroxynitrite/γ-TH	additions	nmol of product formed	
		NGT	tocored
1:4	none	86.0 ± 1.2	3.9 ± 0.2
	10 μM Fe^{3+}−EDTA	89.3 ± 1.1[b]	4.0 ± 0.4
	100 μM Fe^{3+}−EDTA	100.9 ± 1.4[b]	4.0 ± 0.2
	100 μM DTPA	91.1 ± 5.3	3.8 ± 0.3
1:2	none	171.0 ± 4.0	7.6 ± 0.7
	10 μM Fe^{3+}−EDTA	196.2 ± 2.3[b]	8.7 ± 0.4
	100 μM Fe^{3+}−EDTA	224.8 ± 6.9[b]	8.5 ± 0.6
	100 μM DTPA	215.7 ± 5.4[b]	8.9 ± 0.5[b]
1:1	none	229.6 ± 10.2	15.4 ± 0.4
	10 μM Fe^{3+}−EDTA	265.8 ± 12.6[b]	17.1 ± 0.9
	100 μM Fe^{3+}−EDTA	345.5 ± 8.1[b]	18.5 ± 0.9[b]
	100 μM DTPA	287.0 ± 6.3[b]	16.7 ± 0.9

[a] Peroxynitrite reacted with γ-TH in a methanol/50mM potassium phosphate buffer (3/1, pH 7.6) in the absence or presence of either Fe^{3+}−EDTA (10−100 μM) or DTPA (100 μM). After 10 min, reactions were spiked with internal standard, extracted with hexane, and quantified by HPLC as described in Experimental Procedures using standard curves generated from authentic NGT and tocored. [b] Indicates significant difference from reactions with no additions ($p < 0.05$, ANOVA).

Oxidation of γ-TH. Most reactions were carried out in 600 μL of a methanol/50 mM potassium phosphate buffer (3/1 v/v, pH 7.6) containing 2 mM γ-TH (1.2 μmol). Peroxynitrite in 0.1 N NaOH as a 100× concentrated stock was quickly added to the buffer containing γ-TH with mixing. pH changes after the addition of peroxynitrite were minimal in the buffer system used. After 10 min, samples were spiked with internal standard (α-tocopheryl acetate, i.s.), extracted three times with hexane (2 mL), evaporated, and dissolved in methanol prior to HPLC analysis. Milligram quantities of NGT were prepared by the reaction of γ-TH with peroxynitrite in acetonitrile/50 mM potassium phosphate buffer (3/1 v/v, pH 7.6) for 1 h, followed by extraction with hexane and purification by HPLC. Negative controls consisted of the addition of degraded peroxynitrite in 0.1 N NaOH. In some reactions, diethylenetriaminepentaacetic acid (DTPA) or ferric−sodium ethylenediamine tetraacetic acid (Fe^{3+}−EDTA) were added at concentrations indicated in Table 1.

Reactions with γ-TH and 3-morpholinosydnonimine (SIN-1) (Alexis Corporation, San Diego, CA) were carried out in methanol/50 mM potassium phosphate buffer (3/1, v/v, pH 7.6). SIN-1 was freshly prepared in H_2O as a 100× concentrated stock and added to 2 mM solutions of γ-TH. The reaction was carried out for 4 h in the dark and then extracted and analyzed as above.

Reactions with γ-TH and $NO_2^+BF_4^-$ (Aldrich, Milwaukee, WI) were carried out in a total volume of 1 mL of either acetonitrile or a methanol/50 mM potassium phosphate buffer (3/1 v/v, pH 7.6) containing 2 mM γ-TH. Reactions in acetonitrile were performed by the addition of freshly prepared $NO_2^+BF_4^-$ as a 100-fold concentrated stock solution with vortex mixing and allowed to react for 1 h at 25 °C in the dark. Methanol was added to quench the reaction, and the sample was extracted with hexane and analyzed as described above. For reactions in methanol/50 mM phosphate buffer (pH 7.6), a 40-fold excess $NO_2^+BF_4^-$ was added as a 100× stock, followed by immediate extraction with hexane to eliminate possible product degradation by nitric acid.

The reduction of 8a-γ-TOH **5** with ascorbic acid was performed in a sodium formate/ethanol buffer as previously de-

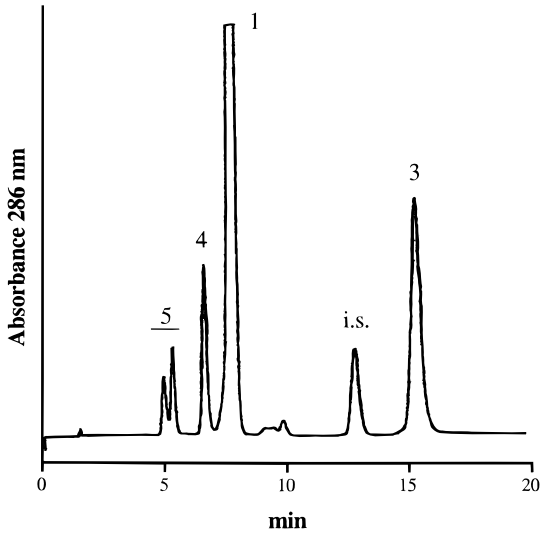


Figure 2. HPLC analysis of products formed from peroxynitrite and γ-TH (1:1 molar ratio) in a methanol/50 mM potassium phosphate buffer (3/1 v/v, pH 7.6).

scribed (*29*). Reactions were carried out for up to 4 h in the dark at 25 °C, extracted as described above, and analyzed by DAD−HPLC.

Results

Addition of peroxynitrite to γ-TH in methanol/50 mM potassium phosphate buffer generated a stable, light yellow-orange color within seconds. Reverse-phase HPLC analysis separated four major products (Figure 2). The major product eluting at 15.5 min had an intense yellow color and was identified as 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5-nitro-6-chromanol (NGT), **3**: UV−vis λ_{max} (EtOH) at 264 nm (ϵ = 2483), 312 nm (ϵ = 2464), and 415 nm (ϵ = 904); GC−MS (TMS derivative), *m/z* 533 ($M^{\bullet+}$, 100%), 518 (35), 292 (19), 276 (22), 238 (14), 222 (13), and 202 (14); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.81−0.85 (12H, m), 1.06−1.54 (21H, m), 1.24 (3H, s), 2.17 (3H, s), 2.21 (3H, s), 2.99 (2H, t, J = 6.3 Hz), 10.7 (1H, s). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 12.1 (C7′), 13.3 (C8′), 19.7 (C4), 20.9, 21.8, 22.6, 22.7, 23.6, 24.5, 24.8, 28.0, 29.7, 30.9, 32.7, 32.8 (C2′), 37.3 (C3), 37.4, 39.4, 39.6, 75.5 (C2), 113.4 (C4a), 125.3 (C7), 131.9 (C5), 145.2 (C8a), 148.3 (C6). These data are consistent with assignment as NGT previously characterized by Cooney *et al*. from the reaction of γ-TH with NO_2 (*30*). The ^{13}C-NMR spectrum had not been previously reported.

The product eluting at 6.8 min was a deep red colored oil and was identified as 2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5,6-chromaquinone (tocored), **4:** UV−vis (MeOH/H_2O) 282, 465 nm; LC−MS (APCI): *m/z* 431 [M + H]$^+$; MS−MS (dau *m/z* 431): 413, 291, 207, 167; ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 0.82 (3H, d, J = 6.2 Hz), 0.84 (9H, d, J = 6.6 Hz), 0.97−1.55 (2H, m), 1.30 (3H, s), 1.67−1.75 (2H, m), 1.93 (3H, s), 2.00 (3H, s), 2.39 (2H, q, J = 5.9 Hz). These data are consistent with previously published data for compound **4** (*30*−*32*).

Products eluting at 4.8 and 5.5 min were identified as diasteromers of 8a-γ-TOH, **5**. These compounds were only moderately stable, the major breakdown product being γ-TQ. Although the peaks could not be assigned to specific epimers, virtually identical spectra were obtained for both compounds: UV λ_{max} (MeOH/H_2O) 236, 261 nm. LC−MS (APCI): *m/z* 431 [M + H]$^+$; MS−MS (dau *m/z* 431) 403, 207, 167. ^{1}H-HMR analysis of these yielded spectra identical to that for γ-TQ, indicating rearrangement prior to or during analysis. The UV−vis spectra are similar to those previously reported for 8a-(hydroxy)-α-tocopherone (*33*, *34*), which readily arranges to α-tocopherylquinone. Because the 8a-substituted tocopherones readily undergo reduction by ascorbic acid to the corresponding tocopherols (*28*, *29*, *33*, *34*), we treated both products with ascorbic acid in sodium formate buffer (*29*). HPLC analysis revealed the disappearance of both products, with a concomitant increase in the formation of γ-TH. After 1-h incubation, both compounds were reduced to γ-TH by about 25%, and after 4 h, the reduction to γ-TH was nearly complete (data not shown).

Because nitration of phenols by peroxynitrite is reportedly enhanced by ferric chelates (*24*), we carried out some reactions in the presence of Fe^{3+}−EDTA. Increasing concentrations of Fe^{3+}−EDTA increased the amount of NGT formed when peroxynitrite was added to γ-TH at three different ratios (Table 1). The addition of 100 μM Fe^{3+}−EDTA to the reactions increased the formation of NGT by 15, 24, and 34% at molar ratios of 1:4, 1:2, and 1:1 peroxynitrite/γ-TH, respectively. The addition of 10 μM Fe^{3+}−EDTA also increased the formation of NGT by 13% and 14% at molar ratios of 1:2 and 1:1, respectively. Furthermore, the presence of 100 μM Fe^{3+}−EDTA modestly but significantly increased the formation of tocored by 17% when equimolar concentrations of peroxynitrite and γ-TH were used (Table 1). The effect of Fe^{3+}−EDTA on formation of the diastereomers of 8a-γ-TOH was minimal. DTPA was added to some reactions to evaluate the possible impact of trace metal contamination. DTPA did not reduce product formation in any of the reactions studied, which indicates a lack of significant trace metal contamination. However, DTPA at a concentration of 100 μM significantly increased the amount of NGT formation in both the 1:2 and 1:1 peroxynitrite/γ-TH reactions as compared to peroxynitrite with γ-TH alone (Table 1). No product formation was detected when decomposed peroxynitrite was added to γ-TH under similar conditions. Product yields as a percent of γ-TH oxidized ranged from approximately 60 to 80%, whereas those for tocored were approximately 3−5%.

The reaction of SIN-1 with γ-TH also was investigated. SIN-1 decomposes with the release of both nitric oxide and superoxide, which react to form peroxynitrite (*35*). This study was done to compare products formed from a brief exposure of peroxynitrite and a sustained release of peroxynitrite over time. Reactions with SIN-1 and γ-TH slowly produced a yellow-orange color over 4 h. DAD−HPLC analysis confirmed the formation of two products with identical retention times and UV−vis spectra for NGT and tocored (Figure 3). The major product formed in reactions with all molar ratios of SIN-1/γ-TH was NGT (Table 2), with smaller amounts of tocored. The ratio of NGT formation to tocored was greater when γ-TH was exposed briefly to peroxynitrite than to SIN-1. In the reaction with SIN-1 and γ-TH at a 5:1 ratio, the yield of NGT was 53% of γ-TH oxidized, whereas yields of tocored and γ-TQ were both 5%.

NO_2^+ has been proposed to be a nitrating species responsible for phenol nitrations by peroxynitrite (*23*). The reaction of γ-TH with $NO_2^+BF_4^-$ was thus examined to determine what products are formed by NO_2^+. γ-TH reacted with $NO_2^+BF_4^-$ in acetonitrile to form a yellow-red color over 1 h. The reaction yielded three major products: NGT, tocored, and a yellow product eluting at 5.2 min (Figure 4). This latter product was identified as

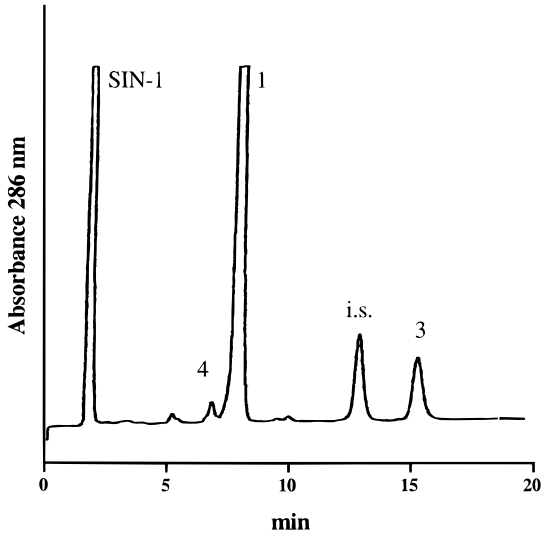


Figure 3. HPLC analysis of products formed from SIN-1 and γ-TH (1:1 molar ratio) in a methanol/50 mM potassium phosphate buffer (3/1 v/v, pH 7.6).

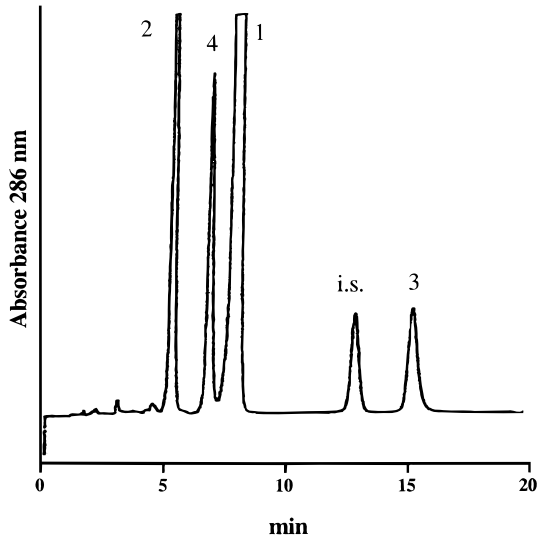


Figure 4. HPLC analysis of products formed from $NO_2^+BF_4^-$ and γ-TH (1:1 molar ratio) in acetonitrile.

Table 2. Quantification of Product Formation from Reaction of SIN-1 with γ-TH[a]

ratio of SIN-1/γ-TH	nmol of product formed		
	NGT	tocored	γ-TQ
1:2	39.0 ± 6.2	4.0 ± 0.3	ND
1:1	74.2 ± 16.7	4.4 ± 1.1	trace
5:1	484.9 ± 5.6	41.6 ± 2.9	45.7 ± 5.8

[a] SIN-1 was added to a 1 mM solution of γ-TH in a methanol/50mM potassium phosphate buffer (3/1 v/v, pH 7.6) to a final concentration of 1.0−10.0 mM. Samples were mixed and allowed to react for 4 h. After 4 h, an internal standard (α-TH acetate) was added, and the reactions were extracted with hexane and analyzed by HPLC as described in Experimental Procedures. Amounts of each product were calculated based on standard curves for each product. Data are expressed as the mean ± SD of 3−5 separate reactions. ND, not detected.

γ-TQ: UV−vis (MeOH) 255 nm (ϵ = 18 035), 330 nm (ϵ = 639); GC−MS (TMS derivative) m/z 504 ($M^{\bullet+}$, not detected) 416 (4%), 341 (29), 279 (100), 189 (20), and 151 (19); ^{1}H-NMR (200 MHz, $CDCl_3$): δ (ppm), 0.82 (3H, d, J = 6.2 Hz), 0.84 (9H, d, J = 6.6 Hz), 1.20 (3H, s), 1.00−1.50 (21H, m), 1.54−1.63 (2H, m), 1.98 (3H, s), 2.00 (3H, s), 2.43−2.51 (2H, m), 6.51 (1H, t, J = 1.2 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$): δ (ppm) 12.0, 12.4, 19.7, 19.8, 21.4, 22.6, 22.7, 24.0, 24.5, 24.8, 26.7, 28.0, 32.8, 37.3, 37.4, 37.6, 39.4, 39.9, 42.4, 72.5, 132.1, 140.6, 141.1, 149.4, 187.6.

Table 3. Quantification of Product Formation from Reaction of $NO_2^+BF_4^-$ with γ-TH[a]

ratio of $NO_2^+BF_4^-$/γ-TH	nmol of product formed		
	NGT	tocored	γ-TH quinone
	ACN		
1:4	26.7 ± 2.2	5.4 ± 1.1	45.8 ± 3.1
1:1	125.3 ± 21.2	38.5 ± 6.4	243.9 ± 22.5
	MeOH/KPO_4^-		
40:1	16.0 ± 1.9	4.7 ± 0.4	46.5 ± 9.1

[a] $NO_2^+BF_4^-$ was added to a 2 mM solution of γ-TH in either 100% ACN or MeOH/KPO_4^- buffer to a final concentration indicated. Reactions in ACN were carried out for 1 h, while reactions in MeOH/KPO_4^- buffer were extracted immediately after the addition of reagents. All samples were quenched with MeOH, followed by the addition of an internal standard (α-TH acetate). Samples were extracted with hexane, dried, and analyzed by HPLC as described in Experimental Procedures. Amounts of each product were calculated based on standard curves for each γ-TH derivative. Data are expressed as mean ± SD of three separate reactions.

In these reactions, the formation of γ-TQ was almost 2-fold greater than NGT (Table 3). In the reaction with $NO_2^+BF_4^-$ and γ-TH in a 1:1 ratio, the yield of NGT was 28% of γ-TH oxidized, whereas those for tocored and γ-TQ were 9% and 55%, respectively. The reaction was also performed in methanol/50 mM phosphate buffer (3/1 v/v, pH 7.6) to determine if the product distribution was altered in the presence of water. In methanol/50 mM phosphate buffer, a 40-fold molar excess $NO_2^+BF_4^-$ was added because of rapid competing hydrolysis of NO_2^+ to nitric acid. The reaction mixture was immediately extracted to prevent product decomposition by nitric acid. As in the acetonitrile system, γ-TQ was the major product formed, in addition to NGT and tocored (Table 3). The formation of γ-TQ was over 4-fold greater than NGT under these reaction conditions.

Discussion

This study was conducted to determine the major products formed from the reaction of peroxynitrite with γ-TH. Because γ-TH contains an unsubstituted carbon at C-5 (*ortho* to the phenol), we hypothesized that nitration at this position by peroxynitrite would occur. Indeed, we found that NGT was the major product formed from the reaction of peroxynitrite with γ-TH. This product was characterized by Cooney *et al.* in studies on the reaction of γ-TH with NO_2 in hexane (*30*). In addition to NGT, tocored and 8a-γ-TOH were identified as products of peroxynitrite oxidation of γ-TH.

The formation of all the observed products in peroxynitrite oxidations reflects the complex chemistry of peroxynitrite as an oxidant. Peroxynitrite and its conjugate acid, peroxynitrous acid, can act as one-electron oxidants, whereas an activated form of peroxynitrous acid ([ONOOH]*) can act as a one-electron oxidant or as a "hydroxyl radical-like" hydroxylating reagent (*20, 18*). Alternatively, some metal complexes, such as Fe^{3+}−EDTA can catalyze the heterolysis of peroxynitrous acid to water and NO_2^+. Both NO_2^+ and its one-electron reduction product NO_2 may act as nitrating agents or one-electron oxidants. Therefore, in addition to oxidations with peroxynitrite and SIN-1, we also conducted

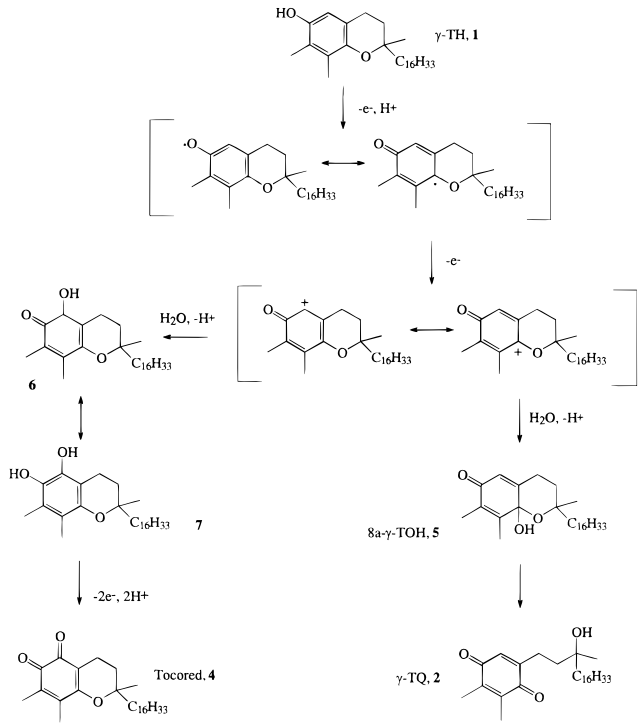
reactions with $NO_2^+BF_4^-$ to assess the role of NO_2^+ in peroxynitrite-mediated γ-TH oxidation.

γ-TH underwent two types of reactions in oxidations with peroxynitrite. In the first, nitration at C-5 formed NGT. In the second, γ-TH was oxidized to 8a-γ-TOH and tocored, most likely via electron transfer and hydrolysis reactions (Scheme 1). Electron transfer may be mediated by any of several peroxynitrite-associated oxidants, including peroxynitrite/peroxynitrous acid, [HOONO]*, NO_2^+, or NO_2. Sequential removal of two electrons from γ-TH may be followed by hydrolysis at either C5 or C8a of the resulting γ-T^+. Hydrolysis at C-8a yields 8a-γ-TOH, which can subsequently rearrange to γ-TQ. On the other hand, hydrolysis at C5 yields a hydroxydienone (**6**), which rearranges to the catechol (**7**). Further two-electron oxidation forms the more stable *o*-quinone, tocored. We propose one-electron oxidation via the tocopheroxyl radical for three reasons. First, tocopherols react with a variety of oxidants by initial electron or hydrogen atom transfer (*36*, *37*). Second, peroxynitrite oxidizes various substrates by either one- or two-electron routes (reviewed in ref 18). Finally, Hogg et al. (*26*) recently reported that peroxynitrite oxidized α-TH to 8a-substituted tocopherone products and that the tocopheroxyl radical was detected under the reaction conditions. Although these authors concluded that one-electron oxidation was a minor reaction pathway based on low levels of the tocopheroxyl radical detected, it should be noted that tocopheroxyl radicals would not be expected to accumulate under the reaction conditions employed in that work or those in our study, as further oxidation by peroxynitrite or peroxynitrite-derived oxidants (e.g., NO_2 or NO_2^+) should readily occur. Thus, although it is possible that a concerted two-electron oxidation formed γ-T^+ in our studies, sequential one-electron oxidations appear to be a more likely reaction sequence.

The formation of 8a-γ-TOH is analogous to the oxidation of α-TH, which also yields an 8a-hydroxytocopherone via electron transfer and hydrolysis (*28*). However, peroxynitrite oxidation of α-TH in aqueous ethanol (*25*) or in acetonitrile (*16*) yielded α-tocopherolquinone. Although the 8a-hydroxytocopherones of both α-TH and γ-TH rearrange to the corresponding quinones, the 8a-γ-TOH appeared to be somewhat more stable than 8a-α-TOH. The 8a-γ-TOH was stable to HPLC analysis and approximately 4 h was required to achieve complete reduction to γ-TH by ascorbate, whereas 8a-α-TOH is reduced within minutes under the same conditions and rearranges to α-tocopherolquinone during HPLC analysis. Hogg et al. (*16*) also reported that 8a-methoxytocopherone was formed by α-TH oxidation by peroxynitrite in methanol, apparently by methanolysis of the tocopherone cation. Such 8a-alkoxytocopherones are considerably more stable than 8a-hydroxy derivatives (*37*). The appearance of γ-TQ rather than 8a-γ-TOH in the $NO_2^+BF_4^-$ oxidations reflected the ease of rearrangement of 8a-γ-TOH to γ-TQ in the presence of NO_2^+ or nitric acid generated in the reactions. In the peroxynitrite oxidations, similarly reactive Lewis acids were not present in high concentrations, and 8a-γ-TOH was comparatively stable.

Because of the unavailability of sufficiently stable standards, we were unable to perform accurate quantification of 8a-γ-TOH and thus we cannot account completely for the partitioning of γ-TH between electron transfer and nitration pathways of oxidation. We also

Scheme 1. Reaction Mechanism of 8a-γ-TOH, Tocored, and γ-TQ Formation from γ-TH by Peroxynitrite



cannot evaluate the partitioning between oxidation at C-5 and C-8a. However, comparison of the integrated peak areas for 8a-γ-TOH in peroxynitrite oxidations with or without Fe^{3+}−EDTA indicates little variation in yield, as was also observed for formation of tocored. In reactions with both peroxynitrite and SIN-1, NGT was the major product formed.

The nitration of phenolic compounds such as tyrosine has been studied previously (*23*, *24*, *38*, *39*), but the exact mechanisms remain unclear and may vary depending on the substrate (*39*). Interpretation of the reaction mechanism for NGT formation is complicated by the ability of peroxynitrite to form different oxidants. The formation of NGT may result, at least in part, from NO_2^+ generated by peroxynitrous acid heterolysis. The NO_2^+ could nitrate the C-5 position of γ-TH by either of two mechanisms. The first is an electrophilic aromatic substitution reaction, in which NO_2^+ could add to γ-TH to form a cationic intermediate, which then deprotonates to NGT (eqs 1 and 2).

$$\gamma\text{-TH} + NO_2^+ \rightarrow [\gamma\text{-TH-}NO_2]^+ \quad (1)$$

$$[\gamma\text{-TH-}NO_2]^+ \rightarrow \text{NGT} + H^+ \quad (2)$$

Alternatively, NO_2^+ could react with γ-TH by initial electron transfer, followed by recombination of the tocopheroxyl and NO_2 radical intermediates (eqs 3 and 4).

$$\gamma\text{-TH} + NO_2^+ \rightarrow \gamma\text{-T}^\bullet + NO_2 + H^+ \quad (3)$$

$$\gamma\text{-T}^\bullet + NO_2 \rightarrow \text{NGT} \quad (4)$$

The electron transfer step (reaction 3) would be consistent with the high reduction potential of the NO_2^+ (1.6 V) (*20*).

It is interesting that nitration of the γ-TH 8a-position did not occur. This position is frequently the site of

radical substitution and solvolysis reactions to generate 8a-substituted tocopherones (see above). However, nitration at C-8a may be disfavored by steric constraints that limit accomodation of a nitro group. Whereas bulky 8a-alkyldioxytocopherones may be formed from α-TH (*40*, *41*), bulky 8a-alkoxytocopherones are not (*42*).

Beckman and co-workers demonstrated that Fe^{3+}−EDTA catalyzed the nitration of 4-hydroxyphenylacetic acid (4-HPA) as well as other phenolic compounds, apparently by enhancing heterolytic cleavage of peroxynitrous acid to NO_2^+ (*23*, *24*). We investigated the effect of Fe^{3+}−EDTA on the formation of NGT in our reaction system. Fe^{3+}−EDTA modestly increased the nitration of γ-TH. The increase in γ-TH nitration was maximal at 100 μM Fe^{3+}−EDTA in reactions with equimolar γ-TH and peroxynitrite, as compared to reactions with or without 10 μM addition of Fe^{3+}−EDTA. The fact that only modest (i.e., 20−30%) increases in NGT yield were induced by Fe^{3+}−EDTA in our studies may reflect the ability of NO_2^+ formed by peroxynitrous acid heterolysis to act as an electron transfer agent as well as a nitrating agent. This is consistent with the production of γ-TQ and tocored along with NGT in reactions with the NO_2^+ donor $NO_2^+BF_4^-$.

Our data therefore suggest that than NO_2^+ is not the principal nitrating species involved in the formation of NGT. This conclusion is further supported by the observation that, in our studies with NO_2^+ BF_4^-, the combined yield of γ-TQ and tocored exceeded that of NGT (Table 3). This is in contrast to reactions with peroxynitrite and SIN-1, in which NGT was by far the major product (Tables 1 and 2). Thus, NO_2^+ was a less efficient nitrating agent for γ-TH than was peroxynitrite. In comparing oxidations with $NO_2^+BF_4^-$ to those with peroxynitrite, it is possible that differences in concentrations of NO_2^+ generated by these reagents may account for differences in nitration versus electron transfer oxidation pathways.

Peroxynitrous acid itself rather than NO_2^+ may nitrate γ-TH. Isomerization of peroxynitrous acid yields a highly reactive oxidant that is capable of carrying out hydroxyl radical-like hydroxylation reactions (*20*, *17*). It is not clear whether such an intermediate also can act directly as an NO_2 donor. However, Ramezanian *et al.* (*39*) recently proposed that phenol nitration reactions could involve initial electron transfer from the phenol to peroxynitrous acid with release of water and formation of NO_2, which then may recombine with the phenoxyl radical (eqs 5 and 6).

$$\gamma\text{-TH} + \text{ONOOH} \rightarrow \gamma\text{-T}^\bullet + NO_2 + H_2O \quad (5)$$

$$\gamma\text{-T}^\bullet + NO_2 \rightarrow \text{NGT} \quad (6)$$

Because NO_2 is a strong one-electron oxidant (*20*), electron transfer from γ-$T^\bullet$ to form γ-T^+ (eq 7) may compete with reaction 6.

$$\gamma\text{-T}^\bullet + NO_2 \rightarrow \gamma\text{-T}^+ + NO_2^- \quad (7)$$

The competition between these reaction pathways most likely would depend on properties of the reaction medium.

Recent reports from several laboratories indicate that the presence of dissolved carbon dioxide may accelerate peroxynitrite-dependent nitration reactions, possibly via the formation of a nitrosoperoxycarbonate intermediate (eq 8), which may yield a nitrocarbonate anion as the ultimate nitrating species (eq 9; *43*−*46*).

$$\text{ONOO}^- + CO_2 \rightarrow \text{ONOOCO}_2^- \quad (8)$$

$$\text{ONOOCO}_2^- \rightarrow O_2\text{N-CO}_3^- \quad (9)$$

Adventitious carbon dioxide/bicarbonate contamination in our system is likely to be well below the millimolar concentrations of bicarbonate used to catalyze nitration in these other studies. Nevertheless, we made no attempt to exclude ambient carbon dioxide from the reactions, and thus these carbon dioxide-derived intermediates could contribute to the formation of NGT.

In summary, we have demonstrated that the reaction of γ-TH with peroxynitrite yields several products, including NGT. Because γ-TH is associated with biological lipids, NGT may provide a novel marker for peroxynitrite interaction with lipid membranes and lipoproteins. NGT appears to be sufficiently stable to permit extraction from biological samples and analysis. Although NGT also is formed by the reaction of NO_2 with γ-TH (*5*), appropriate experimental controls could exclude this reaction. NGT thus may be a useful marker for the formation of peroxynitrite in biological systems.

Acknowledgment. This research was supported by USPHS Grants ES 05675 and CA 59585 and by Southwest Environmental Health Sciences Center Grant ES 06694. The authors also acknowledge Jeanne A. Burr and Kathy L. Kaysen for technical assistance and Dr. Thomas D. McClure of the Southwest Environmental Health Sciences Center Analytical Core Laboratory for LC−MS analyses.

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