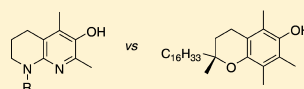


Besting Vitamin E: Sidechain Substitution is Key to the Reactivity of Naphthyridinol Antioxidants in Lipid Bilayers

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S Supporting Information

ABSTRACT: A series of naphthyridinol analogs of α -tocopherol (α -TOH, right) with varying sidechain substitution was synthesized to determine how systematic changes in the lipophilicity of these potent antioxidants impact their radical-trapping activities in lipid bilayers, regenerability by water-soluble reductants, and binding to human tocopherol transport protein (TTP). The activities of the naphthyridinols were assayed in phosphatidylcholine unilamellar liposomes using a recently developed high-throughput assay that employs a boron dipyrromethene conjugate of α -TOH (H_2B -PMHC) that undergoes fluorescence enhancement upon oxidation. The naphthyridinols afforded a dose-dependent protection of H_2B -PMHC consistent with unprecedented peroxy radical-trapping activity in lipid bilayers. While sidechain length and/or branching had no effect on their apparent reactivity, it dramatically impacted reaction stoichiometry, with more lipophilic compounds trapping two peroxy radicals and more hydrophilic compounds trapping significantly less than one. It is suggested that the less lipophilic compounds autoxidize rapidly in the aqueous phase and that preferential partitioning of the more lipophilic compounds to the bilayer protects them from autoxidation. The cooperativity of a lipophilic naphthyridinol with water-soluble reducing agents was also studied in liposomes using H_2B -PMHC and revealed superior regenerability by each of ascorbate, *N*-acetylcysteine, and urate when compared to α -TOH. Binding assays with human TTP, a key determinant of the bioavailability of the tocopherols, reveal that the naphthyridinols can be very good ligands for the protein. In fact, naphthyridinols with sidechains of eight or more carbons had affinities for TTP which were similar to, and in one case 10-fold better than, α -TOH.

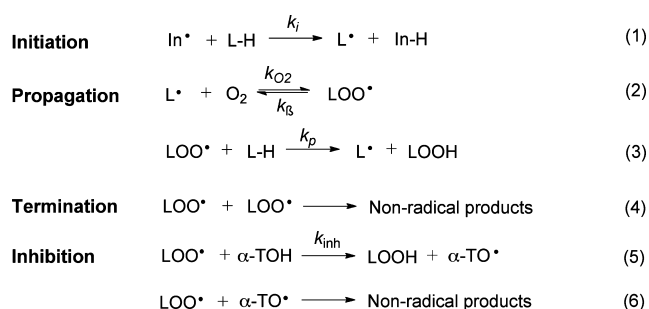


~30x more reactive in solution;
≥30x more reactive in lipid bilayers;
derived radical is ~2 kcal/mol more stable;
regenerable by a variety of water-soluble antioxidants;
when R=C₁₅H₃₁, 10-fold better binding to TTP;
prepared in 6 steps from piperidinone

INTRODUCTION

The oxidative modification of proteins and DNA as a result of polyunsaturated lipid peroxidation has been implicated in the onset of essentially all degenerative diseases, including atherosclerosis,^{1,2} neurodegenerative disease,^{3,4} and cancer.^{5,6} Peroxidation of polyunsaturated lipids proceeds by the prototypical free radical chain reaction (eqs 1–4, Scheme 1)^{7,8} and can be inhibited by radical-trapping antioxidants, such as α -tocopherol (α -TOH, Chart 1), which interrupt the chain

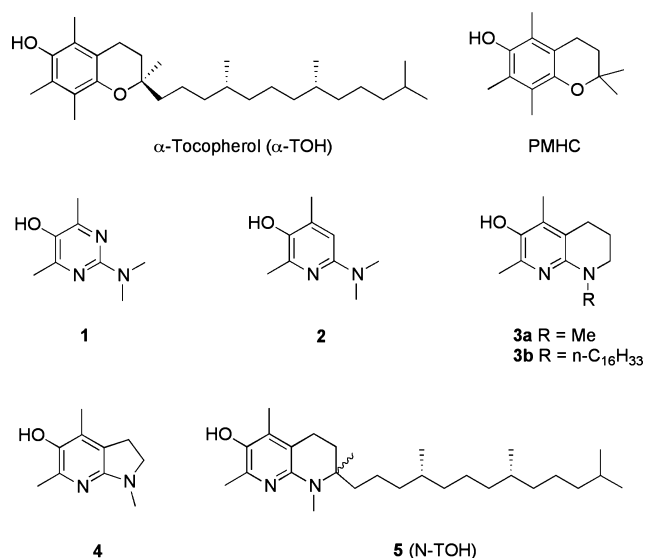
sequence by reaction with chain-carrying peroxy radicals (eqs 5 and 6, Scheme 1).⁹ α -TOH, the most biologically active congener of vitamin E ($k_{inh} = 3.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at 30 °C),⁹ is the major lipid-soluble radical-trapping antioxidant in human plasma and circulating lipoproteins. It is well established as one of the most reactive radical-trapping antioxidants *in vitro* and is commonly used as the standard against which others are evaluated.¹⁰ Given its high reactivity as an inhibitor of lipid peroxidation and the implication of this process in the pathogenesis of degenerative disease, a staggering number of clinical trials employing α -TOH in a therapeutic and/or preventive capacity have been conducted over the past 30 years (the NIH clinical trials database currently lists well over 300 relating to cancer and cardiovascular and neurodegenerative diseases alone). The results have been largely disappointing, leading researchers to question whether oxidative damage has a causal or consequential role in the onset and development of degenerative disease.^{11,12} Perhaps a more appropriate question is: Is α -TOH the best compound to study, or is it simply convenient to do so?

Scheme 1. Mechanism of Lipid Peroxidation and Its Inhibition by Phenolic Antioxidants (e.g., α -TOH)

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Chart 1. Highly Reactive Radical-Trapping Antioxidants



Some time ago we and our collaborators demonstrated that incorporation of nitrogen atoms in the aromatic rings of phenolic antioxidants at the 3 and/or 5 positions relative to the phenolic hydroxyl greatly stabilizes the compounds to autoxidation.^{13,15} As a result, the phenol-like 3-pyridinol and 5-pyrimidinol compounds can be substituted with very strong electron-donating (e.g., *N,N*-diakylamino) groups to weaken the O–H bond¹⁴ that must be broken in the transfer of the labile H-atom to peroxy radicals (as in eq 5, Scheme 1). For example, pyrimidinol **1** and pyridinol **2** are stable in aerated solutions and react two- and five-fold faster with peroxy radicals in organic solution than α -TOH.^{13,15,25} The bicyclic pyridinol compounds **3a** and **4** are even more reactive, with rate constants measuring 28- and 88-fold, respectively, that determined for α -TOH under the same conditions.^{15,25} While **4** has limited stability in aerated solutions, **3a** can be more easily manipulated, suggesting that it is a better compromise between activity and stability. Its lipophilic analog **3b**¹⁶ and the tocopherol analog **5** (which was dubbed *N*-tocopherol)¹⁷ are both excellent inhibitors of cholesterol ester oxidation in human low-density lipoprotein (LDL), and the latter was found to bind to recombinant human tocopherol transport protein (TTP) at least as well as α -TOH.¹⁷ Despite these exciting results, further experimentation with **5** has been all but impossible due to its lengthy synthesis, which required 17 chemical steps. Since **3b** has similar activity to **5** in LDL, but is more synthetically accessible, it is a much better candidate for further studies.

It is well-known that the localization and mobility of tocopherols and their analogs can play at least as important a role in their antioxidant activity as their inherent chemical reactivity upon moving from homogeneous organic solvents to the heterogeneous environment of a lipid bilayer.¹⁸ While we have worked hard over the years to optimize the chemical reactivity of the phenolic headgroup toward peroxy radicals in homogeneous solution in our development of **3** (and **5**), we have yet to undertake a detailed study of their reactivities in lipid bilayers and to determine how changes in their physical properties will impact their activities. Furthermore, since the pyridinoxyl radicals derived from **3** (and **5**) are more stable than the tocopheroxyl radical derived from α -TOH,^{15,17} it is

unknown whether they will be regenerated by co-antioxidants in the aqueous medium surrounding the lipid bilayers. Indeed, the cooperativity displayed between water-soluble antioxidants, such as ascorbate (vitamin C), and α -TOH has long been thought to be key to the activity of both compounds *in vivo*.^{19–21} Furthermore, the impact of changes in the lipophilic sidechain of tocopherols (or any tocopherol-like ligands) on their affinity for the TTP has never been systematically surveyed, and it remains to be determined whether the simpler naphthyridinol **3** will bind at all, given the removal of the quaternary carbon (whose stereochemistry is known to be important in binding the tocopherols)^{22–24} and the relocation of the lipophilic sidechain to a position α to the aromatic ring.

Herein we describe the preparation of a small library of naphthyridinols with sidechains of varying length and branching, the results of high-throughput competitive kinetic studies of their reactivities in phosphatidylcholine liposomes under various conditions, which make use of a fluorescence-based microplate assay (recently developed by one of us),³⁰ and the determination of their abilities to bind to recombinant human TTP. Together, these data provide a clear picture of the vital role of the sidechain on the reactivity and potential bioavailability of naphthyridinol antioxidants and set the stage for experiments *in vivo*.

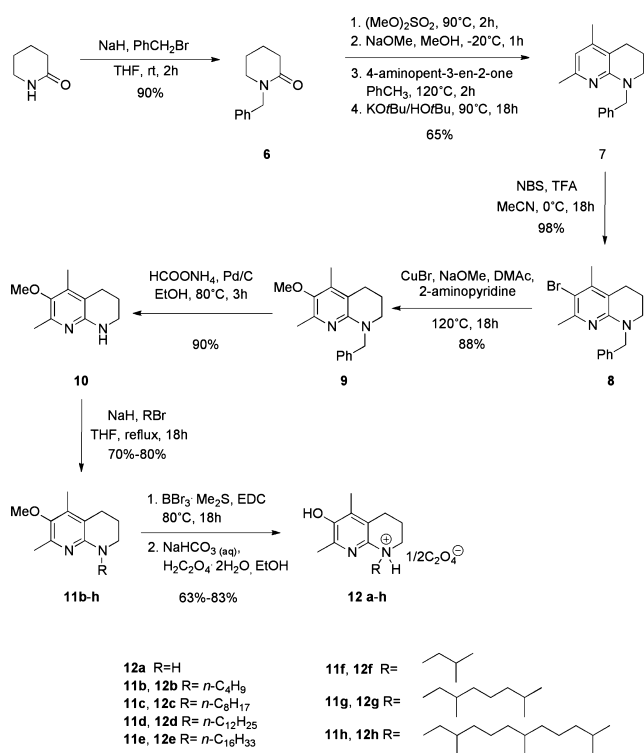
RESULTS

Synthesis of Naphthyridinols with Different Sidechain Substitution. In previous work, we (and others) have accessed the tetrahydronaphthyridinol core structure of **3** (and **5**) starting from either 2-amino-4,6-lutidine^{15,26} or pyridoxine (vitamin B₆).²⁷ The former approach (6 steps) featured two very low-yielding reactions: the (initial) ring annulation (23%) and the (final) installation of the pyridinol moiety via a low-yielding lithiation/oxidation sequence (25%). This served as motivation for the development of the latter approach (11 steps), which carried the pyridinol moiety through the synthesis, and although longer, did not suffer a single low-yielding step. In parallel, we found that a Cu-mediated alkoxylation/hydrogenolysis sequence was a much better option than the lithiation/oxidation approach.²⁸ In addition, Hecht's group provided a concise route to the tetrahydronaphthyridinol core from 2-piperidinone—a shorter, higher-yielding approach than our earlier annulation strategy.²⁹ This prompted us to consider a combination of these two developments to prepare our small library.

Since we desired a variety of substitution at the amine nitrogen, we wanted to carry out the alkylation in the final step. Therefore, we elected to benzylate the piperidone to protect this nitrogen through the annulation and aryl substitution steps, after which it could be removed and the amine alkylated with different sidechains. The target compounds are shown as **12a–h**, the oxalic acid addition salts of naphthyridinols (**3**). We chose increments of four carbons for the linear sidechains (*n*-butyl, *n*-octyl, *n*-dodecyl, *n*-hexadecyl) and increments of five carbons for the branched sidechains, including one, two, and three of the isoprene units found in the phytyl sidechain of α -TOH, in addition to the derivative lacking a sidechain. This range of sidechain lengths provides a range of over 7 orders of magnitude in predicted values of log*P* (see Supporting Information for values). The eight compounds were prepared as in Scheme 2.

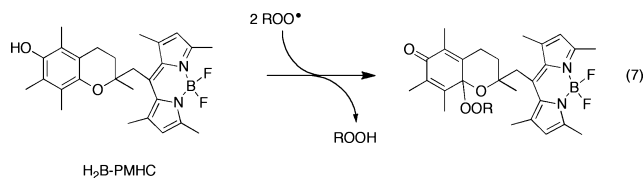
Briefly, 2-piperidinone was first protected with a benzyl moiety to provide **6**, which was ketalized with dimethyl sulfate/

Scheme 2. Synthesis of Naphthyridinol Antioxidants



sodium methoxide and then condensed with 4-aminopent-3-en-2-one to give 7. The naphthyridine was halogenated with *N*-bromosuccinimide and then methoxylated to provide 9. While we had initially attempted the alkoxylation with benzyl alcohol, as we had in the preparation of 1 and 2,²⁸ we found much lower yields for benzylolation of 8 presumably due to its greater electron richness, unless we pushed the reactions with large concentrations of CuI and Cs₂CO₃. The benzyl-protecting group was then removed by hydrogenolysis, and the key intermediate 10 was alkylated with various alkyl bromides to afford the *O*-methylated naphthyridinols 11b–h. Removal of the *O*-methyl group using the dimethylsulfide complex of boron tribromide (BBr₃·Me₂S) followed by addition of oxalic acid provided the ammonium oxalate salts of the naphthyridinols 12a–h. Unlike the free naphthyridinols 3, these derivatives are indefinitely stable at room temperature in an aerobic atmosphere.

Peroxy Radical Trapping by Naphthyridinols in Liposomes. The peroxy radical-trapping activities of the naphthyridinols were assayed in unilamellar liposomes prepared from egg phosphatidylcholine using the fluorogenic H₂B-PMHC (eq 7) probe (λ_{ex} = 485 nm; λ_{em} = 520 nm).³⁰ H₂B-



PMHC features a boron dipyrromethene (BODIPY) fluorophore conjugated to 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC), an analog of α -TOH which lacks the lipophilic phytyl sidechain. The function of the probe is straightforward: In the 'off' state, the fluorescence of the BODIPY moiety is

quenched by photoinduced electron transfer from the electron-rich PMHC group, whereas upon reaction of the phenol with peroxy radicals (eq 7), the fluorescence of the probe is turned 'on' since the resultant chromanone is not sufficiently electron rich to reduce the BODIPY moiety upon excitation.^{31,32} The probe was recently used in a high-throughput approach utilizing a microplate reader to monitor the antioxidant status (α -TOH and PMHC) in liposomes of different phospholipid composition. The probe, the fluorescence assay, and the data analysis provide a new method to obtain, in a rapid parallel format, relative antioxidant activities in phospholipid membranes.³⁰

Representative results of oxidations of solutions of H₂B-PMHC-supplemented liposomes containing different concentrations of added antioxidant are shown in Figure 1. These results were obtained from oxidations mediated by the hydrophilic azo-compound 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) (2.7 mM), which yields water-soluble peroxy radicals with a reported rate of $R_i = 2ek_d[\text{AAPH}] = 3.1 \times 10^{-9} \text{ M s}^{-1}$ under these conditions.³³ Representative data corresponding to oxidations inhibited by the highly lipophilic derivative 12e the more hydrophilic derivative 12b are shown in Figure 1A,C, respectively, while data for oxidations inhibited by α -TOH and its truncated analog PMHC are shown for comparison in Figure 1B,D, respectively (data for the other naphthyridinols are included in the Supporting Information).

Five concentrations of each antioxidant were examined from which a clear dose–response relationship is evident. That is, with the addition of increasing amounts of antioxidant, the time required to achieve the maximum rate of fluorescence increase was extended proportionately. The 'inhibited period', where the oxidation of H₂B-PMHC is inhibited (or retarded) by the added antioxidant, is analogous to the 'inhibited period' that is observed in inhibited autooxidations of hydrocarbons,³⁴ which are usually monitored by either O₂ consumption or the formation of product hydroperoxides—much more laborious experiments not amenable to high-throughput study. Throughout, we have determined the length of the inhibited period (τ) from the intersection of the lines of best fit to the maximum rate of increase in fluorescence intensity (due to oxidation of the chromanol moiety of H₂B-PMHC) and the slower subsequent increase in fluorescence intensity (due to follow-up reactions of the fluorophore), as this is the time at which all of the added antioxidant as well as the phenolic moiety of the probe has reacted (see Supporting Information).⁵⁷ The inhibited period provides important insight into the reactivities of the antioxidant under investigation, as its duration reflects the relative stoichiometry of the reaction of the antioxidant with peroxy radicals, since the probe is present in a constant amount in all experiments.³⁰

The inhibited periods observed in Figure 1 are very similar for the same concentration of 12e, α -TOH, and PMHC (Figure 1A,B,D, respectively), becoming longer with increasing antioxidant concentration. In contrast, the inhibited periods observed for 12b (Figure 1C) are comparably shorter and do not increase to the same extent with increasing antioxidant concentration. This is representative of the trends observed with all of the naphthyridinols (see Supporting Information). The more lipophilic derivatives (C₁₀H₂₁ or longer) have similar inhibited periods to α -TOH and PMHC, and the more hydrophilic compounds (C₅H₁₁ or shorter) have much shorter inhibited periods (less than half at higher concentrations), and the octylated derivative is roughly intermediate between them.

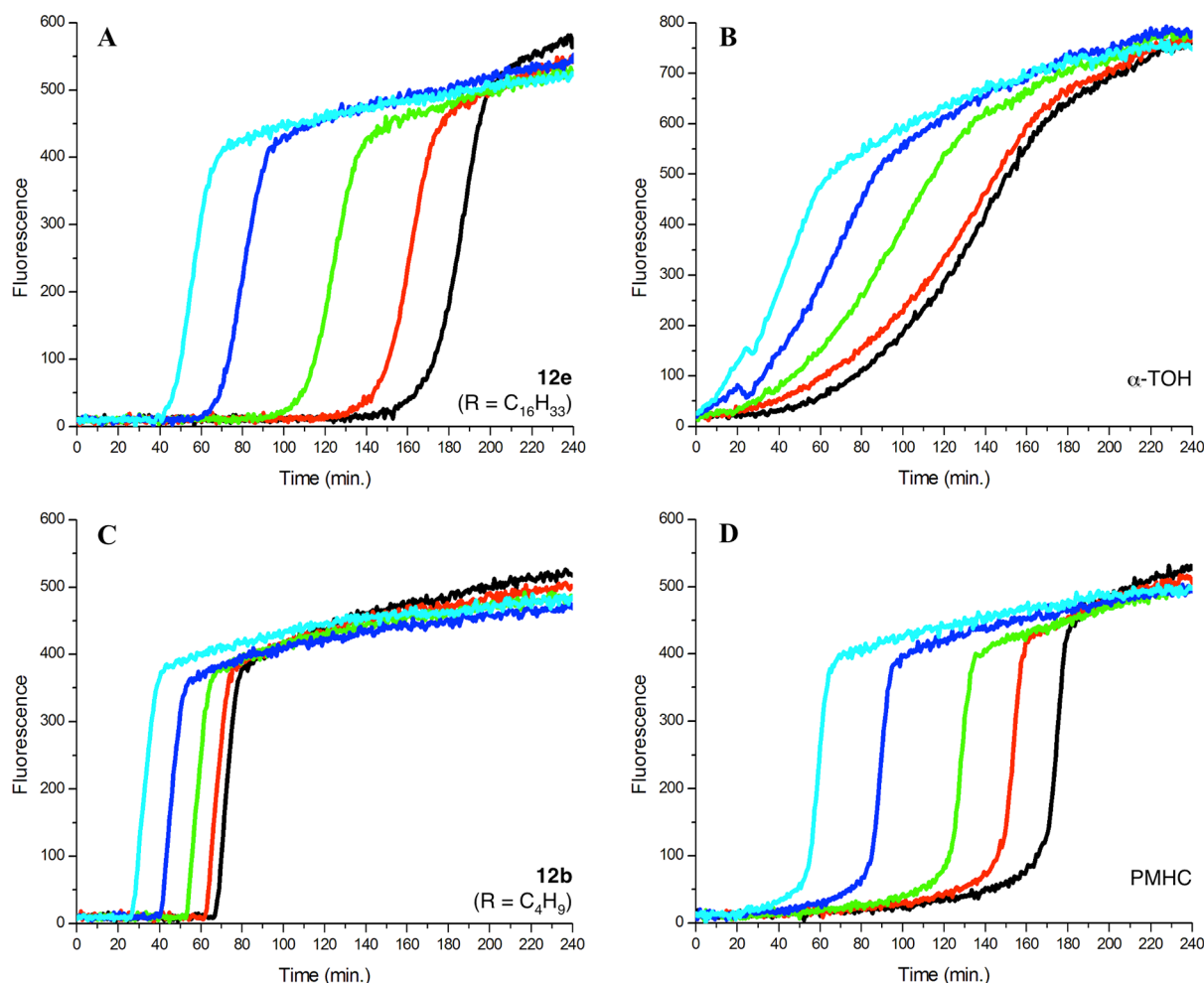


Figure 1. Representative fluorescence (at 520 nm) intensity–time profiles from AAPH-mediated (2.7 mM) oxidations of egg phosphatidylcholine liposomes (1 mM in PBS buffer, pH 7.4) containing 0.15 μM $\text{H}_2\text{B-PMHC}$ and increasing concentrations (1.5 μM , cyan; 3.0 μM , blue; 4.5 μM , green; 6.0 μM , red; and 7.5 μM , black) of **12e** (A), $\alpha\text{-TOH}$ (B), **12b** (C), and PMHC (D).

In addition to relative stoichiometry, the data provide insight into the relative rates for the reactions of peroxy radicals with the different antioxidants vs the fluorogenic $\text{H}_2\text{B-PMHC}$, since the rate of fluorescence increase during the inhibited period represents the competition of the added antioxidant and $\text{H}_2\text{B-PMHC}$ for peroxy radicals. Focusing on this part of the data, it is clear that $\alpha\text{-TOH}$ is by far the least reactive compound (Figure 1B), as it barely retards $\text{H}_2\text{B-PMHC}$ oxidation, except perhaps in the first few minutes of the experiment when the highest antioxidant concentration is used (7.5 μM). PMHC is more reactive (Figure 1D), as is clear from the more obvious inhibited periods. A kinetic analysis based on the initial rates of $\text{H}_2\text{B-PMHC}$ oxidation in the presence and absence of added antioxidant has been carried out to provide an expression useful for the quantification of the relative rate constants in this competition (eq 8):³⁰

$$-\ln\left(\frac{I_\infty - I_t}{I_\infty - I_0}\right) = -\frac{k_{\text{inh}}^{\text{H}_2\text{B-PMHC}}}{k_{\text{inh}}^{\text{unknown}}}\ln\left(1 - \frac{t}{\tau}\right) \quad (8)$$

Thus, from a plot of $-\ln[(I_\infty - I_t)/(I_\infty - I_0)]$ vs $-\ln(1 - t/\tau)$, the relative rate constant can be determined (see Supporting Information). This analysis indicates a ~ 10 -fold increase in the rate constant for the reaction of PMHC and hydrophilic radicals compared to $\alpha\text{-TOH}$ under these conditions.³⁰

Interestingly, the naphthyridinols **12e** and **12b** (Figure 1A,C, respectively) clearly display much faster rates of reaction with peroxy radicals than both $\alpha\text{-TOH}$ and PMHC, as they completely suppress $\text{H}_2\text{B-PMHC}$ oxidation throughout the inhibited periods. In fact, the same is true of all of the other substituted naphthyridinols (see Supporting Information). Based on the lack of any emission enhancement during the inhibited periods observed in the presence of the naphthyridinols, the relative rate constants for H-atom transfer from the naphthyridinols to peroxy radicals cannot be derived from eq 8. However, a lower bounds can be estimated of ≥ 30 times the rate constant for $\text{H}_2\text{B-PMHC}$ (which has essentially the same rate constant for reaction with peroxy radicals as does $\alpha\text{-TOH}$). Analogous results were obtained when experiments were carried out using the hydrophobic azo-compound 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVN) (see Supporting Information).³⁵ The inhibited periods for all of the naphthyridinols as well as $\alpha\text{-TOH}$ and PMHC are summarized in Figure 2 for the five concentrations that were studied, alongside the corresponding data for the oxidations with lipophilic peroxy radicals. Presented in this way it is clear that increasing the concentration of the less lipophilic naphthyridinols has very little effect on the observed inhibited periods, while increasing the concentration of the more

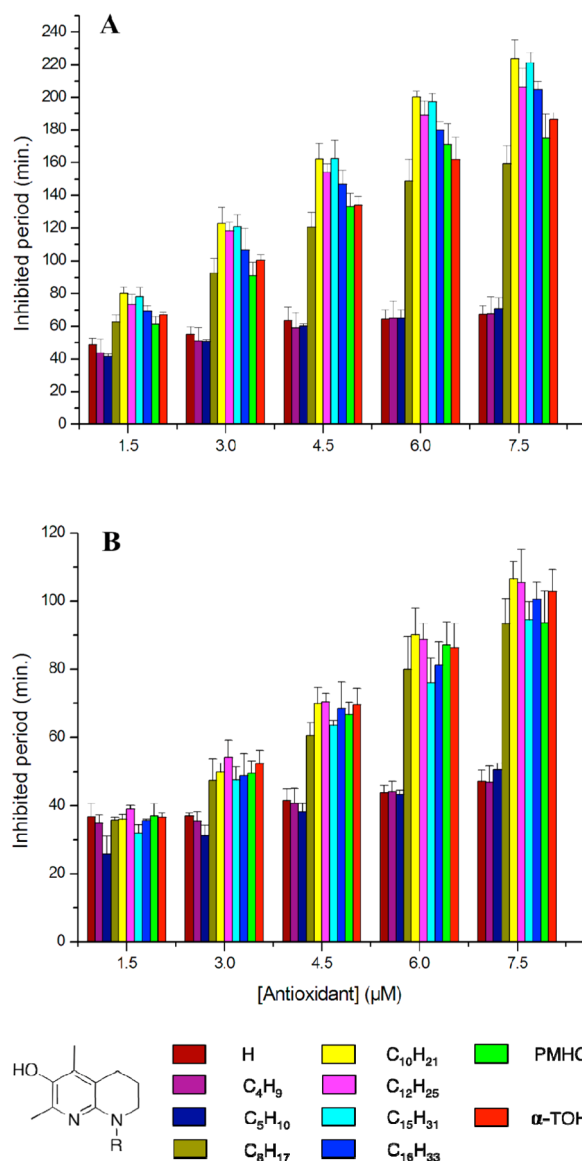


Figure 2. Inhibited periods (averages of at least three measurements) observed for naphthyridinols **12a–h**, PMHC, and α -TOH as a function of antioxidant concentration when egg phosphatidylcholine liposomes were oxidized with hydrophilic (A) or lipophilic (B) peroxyl radicals.

lipophilic naphthyridinols, PMHC, or α -TOH leads to a substantial increase in inhibited periods.

In an attempt to shed light on the origin of the shorter inhibited periods for the less hydrophobic naphthyridinols, additional oxidations were carried out in a buffer of different pH. In parallel, both AAPH- and MeOAMVN-mediated oxidations were carried out at pH 5.8 and 7.4 with two different concentrations of either naphthyridinol **12b** or PMHC (4.5 and 7.5 μ M). Representative results are shown in Figure 3 for the AAPH-mediated oxidations with 7.5 μ M of either **12b** or PMHC (others are included in the Supporting Information). It is important to note that the change in pH had no effect on the rate of H_2B -PMHC oxidation either alone (not shown) or in the presence of PMHC. However, a marked increase in the inhibition period was clear for at more acidic pH. The increase was almost 2-fold when the oxidation was carried out with

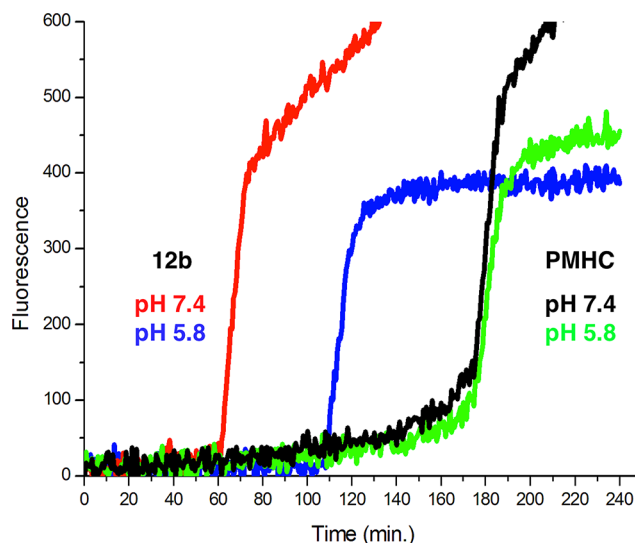


Figure 3. Representative fluorescence (at 520 nm) intensity–time profiles from oxidations of egg phosphatidylcholine liposomes (1 mM in PBS buffer, pH 7.4) containing 0.15 μ M H_2B -PMHC with 2.7 mM AAPH in the presence of 7.5 μ M of **12b** (blue, pH 5.8; red, pH 7.4) or PMHC (green, pH 5.8; black, pH 7.4).

hydrophilic radicals and 1.5-fold when the oxidation was carried out with hydrophobic radicals (see Supporting Information).

Peroxy Radical Trapping by Pyri(mi)dinols in Liposomes. To provide further structure–reactivity data to help understand the origin of the shorter inhibited periods for the less hydrophobic naphthyridinols, analogous liposome oxidations inhibited by the less reactive pyrimidinol **1** and pyridinol **2** were carried out with both hydrophilic and lipophilic peroxy radicals (see Supporting Information for the fluorescence intensity–time traces). The inhibited periods, determined as a function of antioxidant concentration, are presented in Figure 4, alongside those for naphthyridinol **12a** and PMHC for comparison. Clearly, the naphthyridinol has the shortest inhibition periods, followed by the pyridinol **2**, the pyrimidinol **1**, and finally PMHC. The inhibited periods follow the trend in the standard potentials (E°) of each of the compounds, which were determined by cyclic voltammetry (see Supporting Information) and are given alongside the structures in Figure 4.

Cooperativity with Water-Soluble Antioxidants. In order to evaluate the interaction of the naphthyridinols with water-soluble reductants, oxidations of H_2B -PMHC-loaded liposomes supplemented with a relatively small and constant concentration (1.5 μ M) of the most lipophilic (hexadecylated) naphthyridinol **12e** were carried out in buffered solutions to which were added increasing amounts of water-soluble antioxidants (ascorbate, *N*-acetylcysteine or urate; 1.5–15 μ M). Representative results are shown in Figure 5. The oxidations were carried out using only the lipid-soluble radical initiator MeOAMVN, since the water-soluble antioxidants react directly with hydrophilic peroxy radicals generated from the water-soluble initiator AAPH and therefore do not provide any information on potential cooperativity between them and the naphthyridinols. In addition to these series of experiments, oxidations of liposomes to which no lipophilic antioxidant was added were carried out in parallel in order to account for either the reaction of the water-soluble reductants with lipophilic peroxy radicals or the probe-derived phenoxyl radical (formed from reaction of H_2B -PMHC with lipophilic peroxy radicals) at

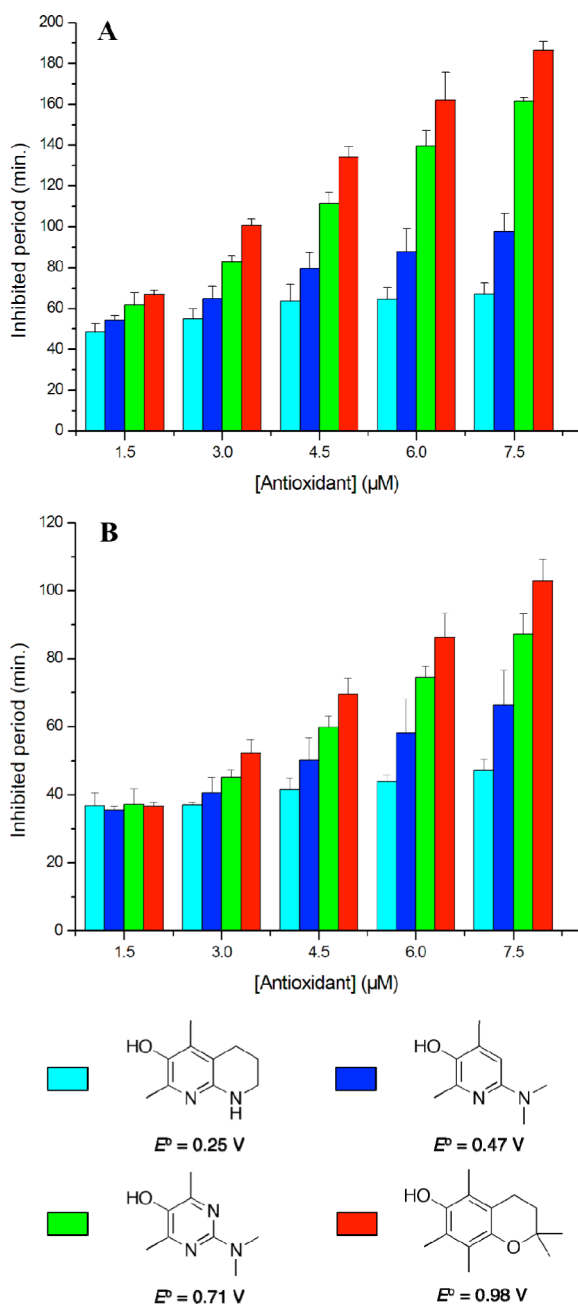


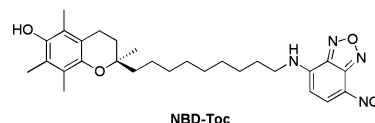
Figure 4. Inhibited periods (averages of at least three measurements) observed for pyrimidinol **1**, pyridinol **2**, naphthyridinol **12a**, and PMHC as a function of antioxidant concentration when egg phosphatidylcholine liposomes were oxidized with hydrophilic (A) or lipophilic (B) peroxyl radicals. Standard potentials (vs NHE at 298 K in CH_3CN) are given in the legend.

the aqueous/lipid interface. The results of these control experiments reveal that neither *N*-acetylcysteine nor urate are particularly effective at inhibiting H_2B -PMHC oxidation under these conditions (see Supporting Information). In contrast, ascorbate is quite effective (Figure 5D), yielding a clear inhibited period, which increases with increasing concentration of ascorbate. The addition of a small (constant) amount of **12e** to the oxidations carried out in the presence of increasing amounts of either *N*-acetylcysteine and urate (Figures 5B and 5C) led to a complete suppression in the rate of probe oxidation similar to what is observed when **12e** is used alone in increasing amounts under these conditions (see Supporting

Information). Smaller, but reproducible, differences were also observed upon addition of a small (constant) amount of **12e** to the oxidations in the presence of increasing ascorbate concentration, in that the inhibited period was elongated and the rate of inhibited oxidation of the probe was nil when both **12e** and ascorbate were present (compare Figure 5A,D), as is the case for **12e** alone.

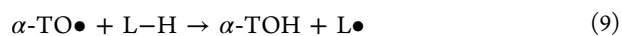
The inhibited periods can be plotted as a function of coantioxidant concentration in the presence of naphthyridinol **12e** (see Supporting Information) from which it is clear that the inhibited periods increase linearly with coantioxidant (i.e., water-soluble reductant) concentration. Furthermore, the magnitudes of the correlations indicate that ascorbate extends the inhibited periods more effectively ($11.8 \text{ min}/\mu\text{M}$) than both *N*-acetylcysteine ($7.6 \text{ min}/\mu\text{M}$) and urate ($6.8 \text{ min}/\mu\text{M}$). All three correlations have smaller magnitudes than that between the inhibited period and the concentration of **12e** alone ($18.2 \text{ min}/\mu\text{M}$).

Binding to the TTP. The binding of the naphthyridinols **12b–h** to the TTP was assayed using the fluorescent tocopherol analogue NBD-Toc.³⁶ The fluorescence of NBD-Toc ($\lambda_{\text{ex}} = 470 \text{ nm}$; $\lambda_{\text{em}} = 535 \text{ nm}$) diminishes upon its displacement from TTP's binding site by competitive ligands due to intramolecular quenching of the NBD fluorophore by the benzochroman moiety. From the fluorescent titration of TTP with NBD-Toc, a dissociation constant (K_d) of $45 \pm 15 \text{ nM}$ is obtained. By way of comparison, a radio ligand binding assay with ^3H - α -TOH gives a K_d of 25 nM for α -TOH binding to TTP, which demonstrates the validity of the fluorescence assay. Effective dissociation constants ($K_{d,\text{eff}}$) for the various naphthyridinols are given in Table 1 as is the value obtained for α -TOH, which is provided for comparison.



DISCUSSION

Excitement surrounding the promise of radical-trapping antioxidants for degenerative disease prevention has receded somewhat in recent years due in large part to the disappointing results of clinical trials with α -TOH, ascorbate, and β -carotene, among other less-trumpeted natural products.^{11,12} However, it must be pointed out that these compounds have well-documented shortcomings as radical-trapping antioxidants. For instance, in the absence of water-soluble reductants (or reduced coenzyme Q_{10}), α -TOH can mediate the oxidation of polyunsaturated lipids in circulating LDLs,^{37–39} via the thermodynamically favorable chain transfer reaction:



Hence, under some (physiologically relevant) conditions, α -TOH can mediate the peroxidation of lipids, rather than inhibit the process. Ascorbate, which is water-soluble, does not inhibit lipid peroxidation directly, but can do so only in a cooperative manner with a lipid-soluble radical-trapping antioxidant such as α -TOH.²¹ Furthermore, since ascorbate is such a powerful reductant, it can act as a prooxidant through reactions with product hydroperoxides⁴⁰ or O_2 .⁴¹ The problems with β -carotene are even more significant; it can trap peroxyl radicals at low partial pressures of O_2 , but acts as a prooxidant at normal

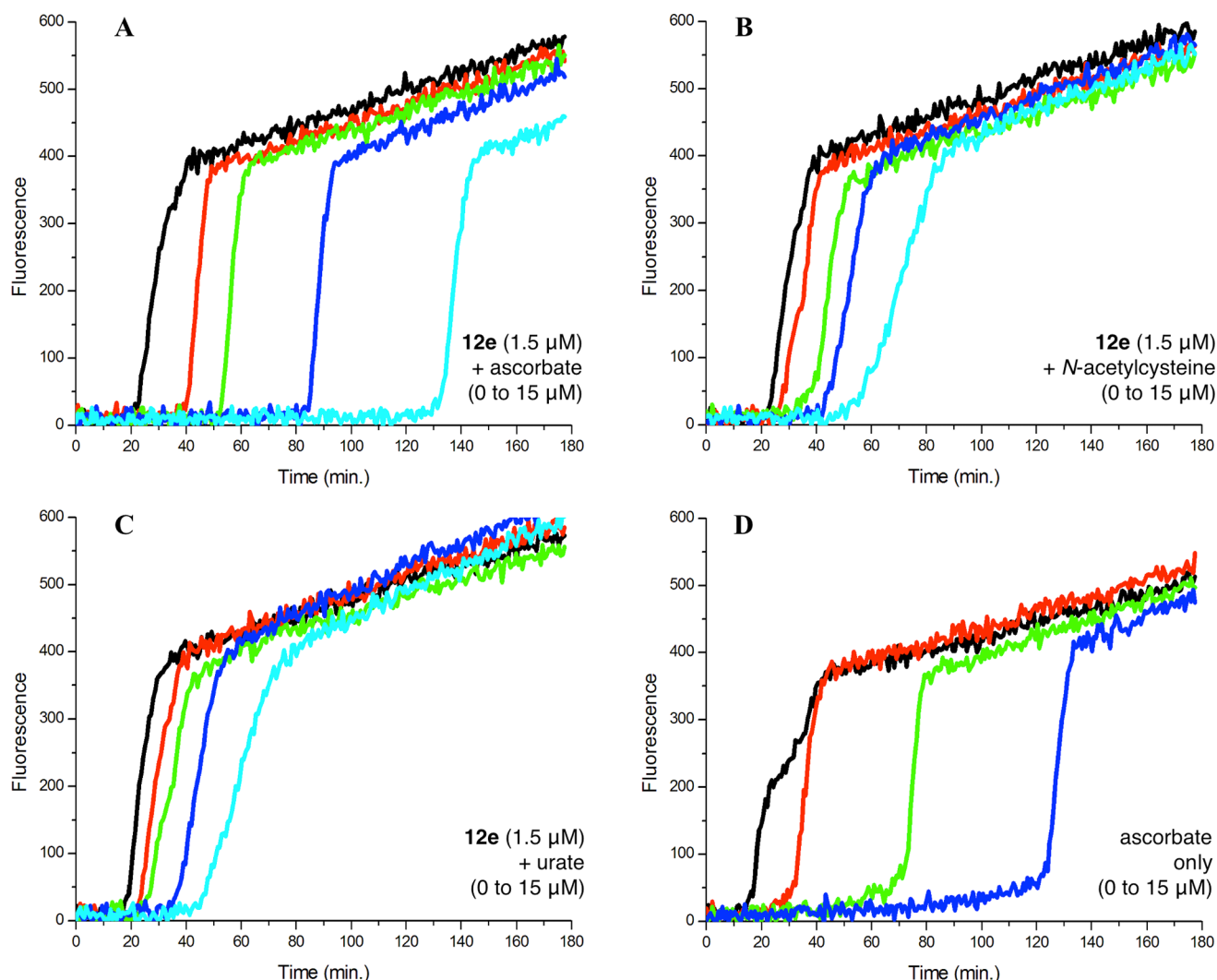


Figure 5. Representative fluorescence (at 520 nm) intensity–time profiles from MeOAMVN-mediated (0.68 mM) oxidations of egg phosphatidylcholine liposomes (1 mM in PBS buffer, pH 7.4) containing 0.15 μM H₂B-PMHC and 1.5 μM of naphthyrindinol **12e** in the presence of various concentrations (0 μM , black; 1.5 μM , red; 3.0 μM , green; 7.5 μM , blue; and 15 μM , cyan) of (A) ascorbate, (B) *N*-acetylcysteine, and (C) urate. Results of corresponding experiments lacking **12e** are shown for ascorbate in (D), whereas those for *N*-acetylcysteine and urate, which show no interaction between them and H₂B-PMHC, are included in the Supporting Information.

Table 1. Effective Dissociation Constants ($K_{\text{d,eff}}$) for Naphthyrindinol Binding to Recombinant hTTP Measured by Competition with Fluorogenic NBD-Toc (1 μM) in SET Buffer, pH 7.4 at 20°C

compound	$K_{\text{d,eff}}$ (μM)
12b	nc ^a
12c	3.7 $\pm$ 0.4
12d	1.3 $\pm$ 0.2
12e	1.0 $\pm$ 0.2
12f	nc ^a
12g	1.0 $\pm$ 0.1
12h	0.1 $\pm$ 0.1
α -TOH	1.1 $\pm$ 0.2

^anc = noncompetitive with NBD-Toc.

partial pressures.⁴² In light of these chemical facts, one has to wonder if it is wise to downplay the idea that radical-trapping antioxidants may have a preventive role to play *in vivo* on the basis of the clinical data collected to date.

Given the foregoing, the ideal peroxyl radical-trapping antioxidant must, at a minimum, be a readily accessible lipophilic compound that: (1) is more reactive to peroxyl radicals than α -TOH; (2) is less reactive to chain transfer than α -TOH; (3) maintains these reactivity differences when embedded in lipid bilayers; (4) is at least as regenerable by phase-separated (i.e., water-soluble) reductants as α -TOH; and (5) has good bioavailability and limited metabolism in the liver, which likely requires that it have high affinity for the tocopherol transport protein. If such a compound were available for *in vivo* study, its use would almost certainly help in shedding definitive light on whether lipid peroxidation has a causal or consequential role in degenerative disease pathogenesis.

Our work to date on the naphthyrindinols reveals that they meet the first two of these criteria. The rate constant for the reaction of the naphthyrindinols **3** with peroxyl radicals is ~ 30 -fold greater than that measured for α -TOH in homogeneous organic solution and likely takes place with a negligible E_a , since $\log k = 7.9$ is very close to the expected $\log A = 8$ for this reaction.⁴³ The O–H BDE of the naphthyrindinol **3a** (75.2 kcal/mol)¹⁵ is ~ 2 kcal/mol lower than the O–H BDE of α -TOH

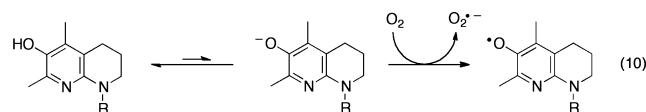
(77.2 kcal/mol),⁴⁴ implying that the chain transfer reaction of the naphthyridinol-derived aryloxy radical and a polyunsaturated lipid can be expected to be thermoneutral at best (the bis-allylic C–H BDE is ~76 kcal/mol).⁴⁵ Indeed, naphthyridinols **3b**¹⁶ and **5**¹⁷ did not appear to mediate lipid peroxidation to a significant extent in our preliminary studies of LDL oxidation under conditions where α -TOH did so. While these results have been highly encouraging, we had yet to address the other three criteria identified above. The recent development of the chromanol-BODIPY conjugates as indicators of the antioxidant status (and indirectly the extent of lipid peroxidation) in lipid bilayers,^{30–32} in combination with some synthetic advances,^{28,29} prompted us to quantify how effective the naphthyridinols are at trapping peroxy radicals in lipid bilayers and to understand the role of sidechain substitution on their reactivity and regenerability in these systems.

Oxidations of H₂B-PMHC-supplemented liposomes with either hydrophilic or lipophilic peroxy radicals were completely suppressed by the naphthyridinols, regardless of the length or branching of the sidechain. This is in contrast to the results obtained with α -TOH and PMHC, which reveal an obvious sidechain dependence in that the latter is much more efficient at preventing probe oxidation than the former. The relative rate constants can be determined from the initial rates of fluorescence intensity increase and reveal a difference of roughly 1 order of magnitude in the case of hydrophilic radicals and ~3-fold in the case of lipophilic radicals.³⁰ The former can be explained simply by the greater access of PMHC to hydrophilic peroxy radicals owing to the lack of a lipophilic sidechain,⁵⁷ while the latter may be approaching the reproducibility of the measurement. While it could be expected that a corresponding difference should exist for the naphthyridinols of different sidechain substitution, it could not be observed using the H₂B-PMHC probe, presumably because it is not sufficiently reactive to compete for peroxy radicals with any of the naphthyridinols. As a result, only an estimate of the lower bounds of the reactivity of the naphthyridinols toward peroxy radicals of at least 30-fold (relative to the probe) can be derived from these experiments. Since the reactivity of the probe toward peroxy radicals is essentially the same as that of α -TOH,³⁰ this provides a direct comparison with the reactivity of α -TOH as well. The much greater activity of the naphthyridinols is consistent with their much higher inherent chemical reactivities (*vide supra*), which may be further bolstered by the greater polarity of the naphthyridinol moiety compared to the benzochromanol moiety found in α -TOH and PMHC. While the naphthyridinol is not expected to be either deprotonated (pK_a of the O–H of **2** is 10.1)⁴⁶ or protonated (pK_a of pyridinium derived from **2** is less than 6)⁴⁶ to a significant extent at pH 7.4, the two nitrogen atoms are expected to increase the polarity and H-bond accepting ability of the naphthyridinol compared to the benzochromanol.

Although liposomal oxidations of the H₂B-PMHC probe did not reveal any differences in the apparent kinetics of the reactions of the naphthyridinols of different sidechain substitution with peroxy radicals, the differences in the inhibited periods are consistent with significantly different reaction stoichiometries. This, again, is in contrast with α -TOH and PMHC, which gave approximately equal inhibited periods for the various concentrations that were studied, and is fully consistent with previous work, which has demonstrated that both of these phenols react with two peroxy radicals to form

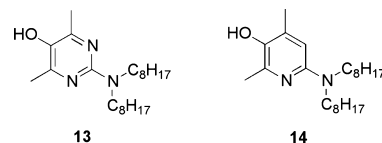
nonradical products (i.e., eqs 5 and 6).^{9,21} The fact that the inhibited periods of the less lipophilic naphthyridinols approach one-third of those observed for PMHC and α -TOH (at the upper-end of the concentrations we studied) implies that only a corresponding fraction of peroxy radicals are trapped by these derivatives.

The reduced stoichiometries of the more hydrophilic naphthyridinols can be explained by their direct reaction with O₂ (eq 10), which deplete the antioxidant. Since this reaction



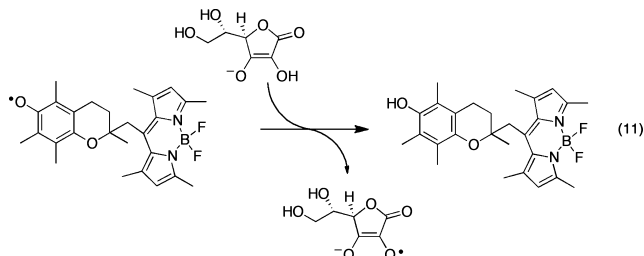
can be expected to be faster in aqueous solution, which better supports charge development in the electron transfer reaction, it should be more apparent in the reactions of the more hydrophilic compounds which can partition there.²⁷ Since the oxidation of phenols in aqueous solution is greatly accelerated upon deprotonation of the phenol to yield its corresponding phenoxide,⁴⁷ follows that this pre-equilibrium should contribute to the rate. Indeed, results of liposomal oxidations carried out at slightly depressed pH (of 5.8), which would shift any unfavorable, but kinetically relevant, pre-equilibrium and slow the rate of competing autoxidation, support the latter mechanism. Indeed, while there was no change in the inhibited periods observed for PMHC (which has a higher pK_a and is less oxidizable, *vide infra*), there was a significant increase in the inhibited periods observed for the naphthyridinol (**12b**) at the lower (more acidic) pH.

Further evidence for the contribution of naphthyridinol autoxidation to the shorter inhibited periods observed for the less lipophilic derivatives was provided from the results of liposome oxidations that were carried out on in the presence of pyrimidinol **1** and pyridinol **2**. While these compounds have log *P* values in the same range as naphthyridinol **12a** and PMHC (see Supporting Information), they have redox potentials which span the range between **12a** ($E^\circ = 0.25$ V) and PMHC ($E^\circ = 0.98$ V) at $E^\circ = 0.71$ and 0.47 V, respectively. Indeed, the inhibited periods for each of the five concentrations we studied follow the trend PMHC > pyrimidinol **1** > pyridinol **2** > naphthyridinol **12a**, which is clearly consistent with the trends in E° ; hence, the greater the one-electron oxidizability of the compound, the lesser the inhibited period. Therefore, it would appear that the longer inhibited periods for oxidations inhibited by the more lipophilic compounds arise simply because their partitioning to the lipid region protects them from autoxidation, which proceeds much faster in the aqueous phase. Consistent with this, additional experiments carried out with lipophilic analogs of pyrimidinol **1** and pyridinol **2** (compounds **13** and **14**, respectively, see Supporting Information for details) show significantly longer inhibited periods in corresponding liposome oxidations.



The ability of ascorbate to recycle α -TOH via reduction of the α -tocopheroxyl radical (α -TO•) at the interfacial region of the bilayer is believed to be key to the lipophilic peroxy radical-

trapping antioxidant activity of both compounds *in vivo*. Consistent with this, oxidations of liposome-embedded H₂B-PMHC with lipophilic peroxy radicals could be inhibited in a dose-dependent manner by added ascorbate, presumably due to scavenging of the probe-derived aryloxy radical (eq 11). Upon



incorporation of a small, constant amount (1.5 μ M) of lipid-soluble naphthyridinol (e.g., the hexadecylated derivative **12e**) into the liposomes, probe oxidation was completely suppressed, and the inhibited period could be increased in a dose-dependent manner with increasing ascorbate concentration. In fact, the data were almost indistinguishable from those obtained from oxidations carried out in the presence of increasing concentration of the naphthyridinol **12e** alone. This provides the first evidence that the naphthyridinols can indeed be recycled with ascorbate as is the case for α -TOH, despite the fact that the reaction is less exothermic by 2 kcal/mol (*vide supra*). However, it should be pointed out that the magnitude of the correlation of the inhibited period and coantioxidant concentration was only $\sim 65\%$ of what is observed for the naphthyridinol alone (cf. Figure 6). Since the naphthyridinol

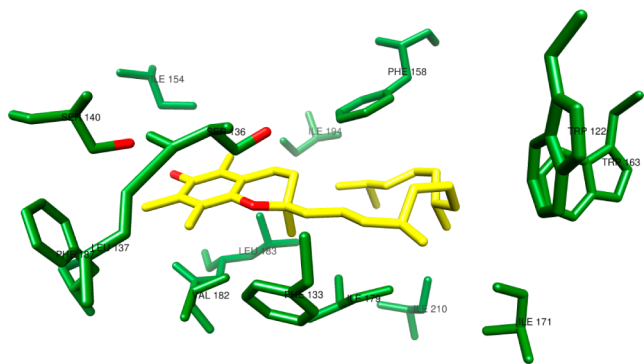


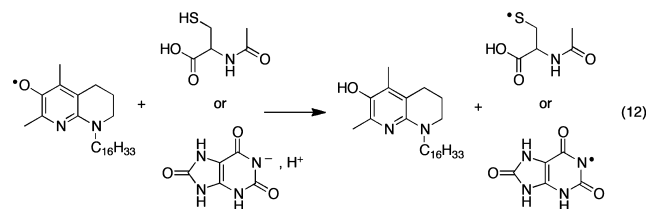
Figure 6. Crystallographic positions of amino acid sidechains in hTTP in the vicinity of its ligand, α -TOH.⁵²

traps 2 peroxy radicals, as does PMHC and α -TOH, this means that each molecule of ascorbate is supplying less than 2 reducing equivalents to regenerate the naphthyridinol ($\sim 0.65 \times 2 = 1.3$). Ingold,²¹ Barclay,⁴⁸ and others have found that while ascorbate can indeed regenerate α -TOH, this process often proceeds without complete fidelity; a fact that has been explained by the intervention of other competing reactions, such as oxidation of ascorbate or the ascorbyl radical anion by O₂ in competition with either the disproportionation of the ascorbyl radical anion or its reaction with an aryloxy (such as α -TO $\bullet$), thereby effectively wasting reducing equivalents.

When analogous experiments were carried out with small amounts (1.5 μ M) of the other naphthyridinols (see Supporting Information), we found the same proportional increase in inhibited period with added ascorbate. However, unlike liposome oxidations inhibited by the naphthyridinol

alone, the inhibited periods were almost independent of the sidechain length. For example, where the inhibited periods decreased from 155 to 125 to 62 min as the sidechain of the naphthyridinol was shortened from *n*-hexadecyl to *n*-octyl to *n*-butyl in oxidations inhibited by 7.5 μ M of **12e**, **12c**, and **12b**, respectively, when any of the three were used at 1.5 μ M along with 6 μ M of ascorbate, the inhibited periods were all within a few minutes of each other (104 ± 5). At first glance, it would appear that ascorbate is protecting the more hydrophilic naphthyridinol from autoxidation. However, this would deplete the ascorbate, which would give rise to shorter inhibited periods. However, careful consideration of the data obtained in the absence of ascorbate reveals that under these conditions and at a concentration of 1.5 μ M, autoxidation of the hydrophilic naphthyridinols is not yet competitive with peroxy radical trapping (see Supporting Information). Therefore, since the maximum concentration of **12b** cannot exceed 1.5 μ M throughout the experiment regardless of ascorbate concentration, autoxidation is not a problem, and the dependence of the inhibited period on sidechain length is minimized.

Analogous experiments were carried out with *N*-acetylcysteine and urate. Glutathione, as well as its biosynthetic precursor cysteine, are very poor co-antioxidants with α -TOH, and urate has not demonstrated any cooperativity at all.⁵¹ Consistent with this, both *N*-acetylcysteine and urate were not effective scavengers of the H₂B-PMHC-derived aryloxy radical in liposomal oxidations. However, when a small amount of lipophilic naphthyridinol was incorporated, as was the case with ascorbate, probe oxidation was completely suppressed, and the inhibited periods increased in a dose-dependent fashion with increasing NAC or urate concentration. In this case, the magnitude of the correlation of the length of the inhibited period and coantioxidant concentration was only ~ 42 and 38% (NAC and urate, respectively) of what is observed with the naphthyridinol alone, as compared to 65% for ascorbate (cf. Figure 6). This is consistent with the fact that *N*-acetylcysteine and urate are expected to be only one-electron reductants (eq 12), so maximum regeneration would yield a correlation of only



half that possible for ascorbate, which is a two-electron reductant. We can only speculate that the increased polarity of the naphthyridinol is responsible for its greater regenerability by NAC and urate, since the thermodynamics are less favorable than the corresponding reactions for α -TOH (*vide supra*).

Secretion of α -TOH from hepatic cells for delivery to peripheral tissues and circulating lipoproteins is facilitated by the tocopherol transport protein (TTP), which displays a clear preference for α -TOH ($K_d = 25$ nM) over the other tocopherol congeners (β -, γ -, and δ -TOH), with relative $1/K_d$'s determined to be 1:0.2:0.09:0.04 for α : β : γ : δ , respectively.²⁴ The three-dimensional structure of recombinant human TTP with α -TOH bound has been determined to a resolution of 1.5 Å⁵² and clearly shows that the binding pocket has evolved to bind α -TOH, with close contacts all along the periphery of the fully methylated phenolic ring (Figure 6). Although it is known that

replacement of the 16-carbon sidechain in α -TOH with a single carbon carboxylic acid moiety (to give the water-soluble tocopherol analog, trolox) increases the K_d over 40-fold,²⁴ there has never been a systematic study of sidechain length on binding to the TTP.

In previous work, we demonstrated that replacement of the benzochroman core of α -TOH with the tetrahydronaphthylidol core in **5** did not impair its binding to human TTP (hTTP).¹⁷ In fact, it bound (marginally) better, a result we ascribed to the potential for a strong H-bond between the tertiary amine and Ser136. From the outset of the current study, we were concerned that the attachment of the lipophilic sidechain to the tertiary amine (in lieu of the methyl group in **5**) and the corresponding removal of the quaternary center, known to be a key determinant in why synthetic (racemic) and natural (*R*)- α -TOH differ significantly in their bioavailabilities,²⁴ would disrupt binding. However, when we examined the binding of our new compounds to recombinant hTTP, we were gratified to learn that these changes did not significantly affect binding. While the butyl- and isopentyl-substituted naphthylidols were noncompetitive with the fluorogenic NBD-Toc probe for hTTP's active site (implying very poor affinity) and the octyl-substituted derivative was only a fair competitor, all other derivatives had very similar or better binding than α -TOH itself (cf. Table 1). The most lipophilic linear alkyl-substituted naphthylidol (hexadecylated **12e**) had essentially equivalent binding to hTTP when compared to α -TOH ($K_{d,eff} = 1.0 \pm 0.2$ vs 1.1 ± 0.2 μ M), whereas the most lipophilic branched alkyl-substituted compound (the farnesol-derived, C₁₅H₃₁-substituted **12h**) is roughly 10-fold better ($K_{d,eff} = 0.1 \pm 0.1$ μ M). The latter is the best ligand for hTTP reported to date and presumably demonstrates superior binding to the linear analog because of advantageous interactions between the sidechain methyl substituents and the binding pocket residues evolved to bind the phytyl tail of the tocopherols.⁵² Of course, whether superior binding is actually a desirable characteristic of these analogs for *in vivo* studies is unclear, since the ligand must eventually be released to peripheral tissues and circulating lipoproteins in order to serve its purpose as a radical-trapping antioxidant, and the mechanism of ligand release from TTP remains unknown.⁵³

Although the high affinity of the naphthylidol derivatives for the TTP is likely critical to ensure their appropriate systemic distribution in animal models, it may also have implications on any potential cytotoxicity of these compounds. The strong binding of α -TOH to TTP is believed to contribute to its slower metabolism compared to the other members of the vitamin E family, which are rapidly metabolized by hepatic CYP4F2 in order to improve their water-solubility and facilitate excretion.^{54–56} Thus, the high affinity of the naphthylidols to TTP may also help protect them from metabolism in the liver.

CONCLUSIONS

Herein we have presented a series of naphthylidol analogs of α -TOH with varying sidechain substitution, which were synthesized in an expeditious manner in order to determine how systematic changes in the lipophilicity of these potent antioxidants impact both their radical-trapping activities in lipid bilayers, regenerability by water-soluble reductants and binding to the human TTP. Liposomes supplemented with the different naphthylidols were oxidized using either hydrophilic or lipophilic peroxy radicals and consistently revealed a dose-dependent protection of the fluorogenic H₂B-PMHC. In fact,

no detectable oxidation of H₂B-PMHC took place in the presence of any of the naphthylidols under conditions where either α -TOH or its truncated analog PMHC were effective only in retarding the rate of oxidation, indicating an unprecedented peroxy radical-trapping activity in lipid bilayers and suggesting that the same difference in reactivity may be expected *in vivo*. While sidechain length and/or branching did not have an effect on the apparent reactivity of the naphthylidols to either hydrophilic or lipophilic peroxy radicals, it had a dramatic effect on their stoichiometry, with more lipophilic compounds trapping about two peroxy radicals and more hydrophilic compounds trapping significantly fewer than one. It is suggested that the more hydrophilic compounds autoxidize in the aqueous phase and that the preferential partitioning of the more lipophilic compounds to the lipid phase protects them from this deleterious pro-oxidative reaction. Studies at more acidic pH, as well as with hydrophilic and hydrophobic pairs of less electron-rich pyridinols, and related pyrimidinols support this assertion. The cooperativity of the most lipophilic naphthylidol with water-soluble reducing agents was also studied in liposomes using H₂B-PMHC. Despite the fact that the naphthylidol-derived radical is more stable than the radical derived from α -TOH, it appeared to have better regenerability by each of ascorbate, *N*-acetylcysteine and urate, suggesting that this may be expected *in vivo*. It is suggested that the greater polarity of the naphthylidol moiety makes regeneration more efficient than for α -TOH. Binding assays with recombinant human TTP, a key determinant of the bioavailability of the tocopherols, reveal that these compounds have very high affinities. In fact, naphthylidols with sidechains of eight or more carbons had similar affinities (and in one case, 10-fold better) for the protein than α -TOH, suggesting that similar bioavailabilities may be expected *in vivo*.

EXPERIMENTAL SECTION

Synthesis. Complete details are provided in the Supporting Information.

Liposome Oxidations. Liposome Preparation. Egg phosphatidylcholine was weighed (75 mg) in a dry vial and dissolved in a minimum volume of chloroform. The solvent was then evaporated under argon to yield a thin film on the vial wall. The film was left under vacuum to remove any remaining solvent for 1 h. The lipid film was then hydrated with 4.83 mL of a 10 mM phosphate buffered-saline (PBS) solution containing 150 mM NaCl (pH 7.4), yielding a 20 mM lipid suspension. The lipid suspension was subjected to 10 freeze–thaw–sonication cycles, where each cycle involved storing the vial with the solution in dry ice for 4 min, thawing at rt for 4 min, followed by 4 min of sonication. The lipid suspension was then extruded 20–25 times using a mini extruder equipped with a 100 nm polycarbonate membrane.

Inhibited Oxidations. To individual 21.4 μ L aliquots of the 20 mM liposome solution were added increasing amounts (5, 10, 15, 20, and 25 μ L, respectively) of a solution of the test antioxidant in either aqueous acetonitrile (129 μ M) or 5 μ L of a solution of H₂B-PMHC in acetonitrile (12.9 μ M). Each resultant solution was then diluted to 400 μ L with PBS, from which 280 μ L of each was loaded into a well of a 96-well microplate. The solution was equilibrated to 37 °C for 5 min, after which 20 μ L of a solution of azo compound (40.5 mM in 2,2'-azobis-(2-amidinopropane)monohydrochloride (AAPH) in PBS or 10.1 mM in 2,2'-azobis-(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) in acetonitrile) was added to each well using the reagent dispenser of the microplate reader. The fluorescence was then monitored for 6 h at 50 s time intervals ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 520$ nm). The final solutions in each well were 1 mM in lipids, 0.15 μ M in H₂B-PMHC, 2.7 mM in AAPH, or 0.68 mM in MeOAMVN and either 1.5, 3.0, 4.5, 6.0, or 7.5 μ M in antioxidant. Each liposome contained, on

average, 15 fluorophores with an egg phosphatidylcholine/fluorophore molar ratio of 6700:1. Under these conditions, no fluorescence self-quenching was expected to occur within the liposome bilayer.³⁰ Control experiments wherein the liposome solutions were purified by size exclusion chromatography following supplementation with H₂B-PMHC and lipophilic antioxidants (e.g., α -TOH, **12e**) revealed that they are indeed incorporated into the liposomes using the above procedure (see Supporting Information), as the fluorescence profiles are essentially indistinguishable.

Co-Inhibited Oxidations. To individual 21.4 μ L aliquots of the 20 mM liposome solution were added 5 μ L of a solution of **12e** in aqueous acetonitrile (129 μ M) or simply 5 μ L of acetonitrile only, followed by 5 μ L of a solution of H₂B-PMHC in acetonitrile (12.9 μ M). To the aliquots of the two sets of samples were then added increasing amounts (0, 5, 10, 25, and 50 μ L) of solutions of water-soluble reducing agent (ascorbate, urate, or N-acetylcysteine) in PBS (129 μ M). Each resultant solution was then diluted to 400 μ L with PBS, from which 280 μ L was loaded into a well of a 96-well microplate. The microplate was equilibrated to 37 °C for 5 min, after which 20 μ L of a solution of MeO-AMVN in acetonitrile (10.1 mM) was added to each well using the reagent dispenser of the microplate reader. The fluorescence was then monitored for 6 h at 50 s time intervals (λ_{ex} = 485 nm; λ_{em} = 520 nm). The final solutions in each well were 1 mM in lipids, 0.15 μ M in H₂B-PMHC, 0.68 mM in MeOAMVN, 0 or 1.5 in **12e** μ M and either 0, 1.5, 3.0, 7.5, or 15 μ M in water-soluble reducing agent.

Voltammetry. Standard potentials for the oxidation of **1**, **2**, **12a**, and PMHC were determined from cyclic voltammograms measured using a three-electrode cell equipped with a glassy carbon working electrode, a platinum counter electrode, and an Ag/AgNO₃ reference electrode. Voltammograms were obtained at a scan rate of 100 mV/s in dry acetonitrile using Bu₄N·PF₆ (0.1 M) as electrolyte at 25 °C. All potentials are reported vs the normal hydrogen electrode (NHE) via reference to the ferrocene/ferrocenium couple.

hTTP Binding Affinity by Competition Studies. Recombinant hTTP (0.35 μ M) was incubated with 1.0 μ M NBD-Toc in SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris, pH 7.4) at approximately 20 °C. After reaching equilibrium, the maximum fluorescence was recorded, and a small aliquot of competitor dissolved in EtOH was added. Approximately 10 min was required for the competitor to reach equilibrium again before the new fluorescence value was recorded. The competitor aliquots were added in increasing concentrations, and the total volume of the competitor added did not go above 1% of the total volume of the solution in the cuvette. Data were plotted using the Prism software package (Prism GraphPad v4.0) and analyzed using nonlinear regression for a one-site competition model, from which effective dissociation constants, $K_{\text{d,eff}}$ were calculated for competing ligands **12c–e**, **12g**, and **12h** as well as α -TOH.

■ ASSOCIATED CONTENT

■ Supporting Information

Complete synthetic details and compound characterization, supplemental fluorescence intensity–time profiles referred to in the text but not shown, representative cyclic voltammograms and binding curves used to determine effective dissociation constants in the TTP assays. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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