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Preparation and Investigation of Vitamin B_6-Derived Aminopyridinol Antioxidants

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Abstract: 3-Pyridinols bearing amine substitution *para* to the hydroxylic moiety have previously been shown to inhibit lipid peroxidation more effectively than typical phenolic antioxidants, for example, α-tocopherol. We report here high-yielding, large-scale syntheses of mono- and bicyclic aminopyridinols from pyridoxine hydrochloride (i.e., vitamin B_6). This approach provides straightforward, scaleable access to novel, potent, molecular scaffolds whose antioxidant properties have been investigated in homogeneous solutions and in liposomal vesicles. These molecular aggregates mimic cell membranes that are the targets of oxidative damage in vivo.

Keywords: antioxidants • lipid peroxidation • pyridinols • radical scavengers • radicals

Introduction

The generation of reactive oxygen species and the corresponding initiation of lipid peroxidation has been associated with the onset and progressions of cancer, cardiovascular disease, and neurodegeneration.[1] This radical-mediated process may be inhibited by radical-trapping chain-breaking antioxidants that transfer a phenolic hydrogen atom to a lipid peroxyl radical ($LOO^•$) at a rate faster than that of chain propagation [Eqs. (1)–(5)].[2] Substituted phenols (ArOH, Ar = aryl), for example, α-tocopherol (**1**, α-TOH)—the most potent form of vitamin E—are the most common lipid peroxidation inhibitors in the biological milieu.

$$\text{Initiation: } In^{\bullet} + LH \rightarrow InH + L^{\bullet} \tag{1}$$

$$\text{Propagation: } L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \tag{2}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \tag{3}$$

$$\text{Inhibition: } LOO^{\bullet} + ArOH \rightarrow LOOH + ArO^{\bullet} \tag{4}$$

$$ArO^{\bullet} + LOO^{\bullet} \rightarrow \text{non-radical products} \tag{5}$$

Previous studies have shown that 6-aminopyridin-3-ols, such as **2**, possess comparable or higher reactivity towards chain-carrying peroxyl radicals than α-TOH (i.e., higher k_4, commonly referred to as the inhibition rate constant, k_{inh}).[3] These compounds also show better air stability than their phenolic counterparts due to the presence of a nitrogen atom in the aromatic ring.[3,4] The O–H bond dissociation enthalpy (BDE) of bicyclic compounds, such as **3** and **4**, is decreased compared to the acyclic compound **2** due to more electron density in the aromatic ring from the substituted methylene unit. In fact, **3a** showed a 28-fold higher k_{inh} than α-TOH whereas maintaining acceptable air stability in organic solvents.[3] Increased lipophilicity of **3b** makes it an excellent antioxidant in isolated human low-density lipoprotein (LDL) where it not only inhibits the oxidation of cholesterol fatty acid esters but also prevents consumption of endogenous α-TOH.[5a] N-Tocopherol (**4**, N-TOH),[5b] an aminopyridinol analogue of α-TOH, is further optimized for

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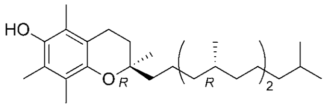
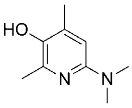
in vivo activity; its binding affinity, as a mixture of epimers, to human α-tocopherol transfer protein, was marginally higher than that of the active α-tocopherol isomer [K_d(α-TOH, *R*,*R*,*R* isomer) = 25 nm], and its lateral diffusion in a model lipid membrane was about 1.5-fold greater than that of α-TOH.[5b]



1 (α-Tocopherol)



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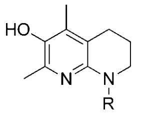
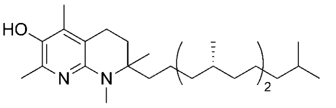


3a: R = Me
3b: R = n-$C_{16}H_{33}$



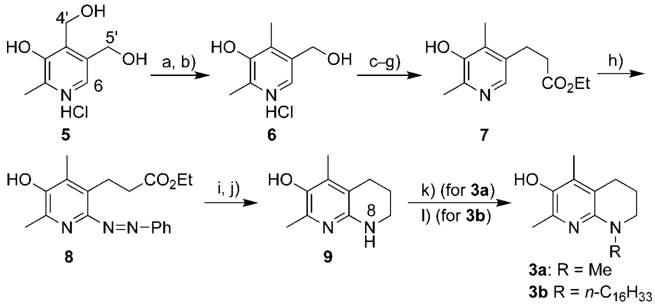
4 (N-Tocopherol)

The early synthetic strategy for the preparation of **2**, **3**, and several analogues thereof utilized electrophilic aromatic hydroxylation as a key step.[3,5a,6] Although this approach provided access to these novel compounds, low yields for the hydroxylation step limited its use. Although we have since reported an improved Cu-catalyzed procedure,[8] it remains a challenge to easily obtain large quantities of these compounds for further study. We report here a convenient and efficient synthetic route for the preparation of both 6-aminopyridin-3-ols and 5,6,7,8-tetrahydro-1,8-naphthyridin-3-ols, together with kinetic studies that support the notion that these compounds have excellent antioxidant activities in both homogeneous and heterogeneous media.

Results and Discussion

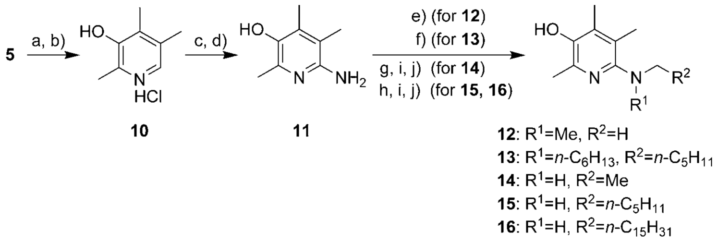
The new approach (Scheme 1) starts from pyridoxine hydrochloride (**5**), vitamin B_6•HCl, a strategy that avoids the hydroxylation protocol of previous approaches. Compound **5** was deoxygenated with Zn/AcOH followed by acid hydrolysis to afford **6**. This reaction was carried out on a 300 g scale to give essentially pure pyridinol **6** (HPLC > 95 %) without chromatographic purification. This route to **6** appears to be safer and more appropriate for large-scale syntheses than previous methods for 4′-deoxygenation that utilize hydrazine.[9]

After two-carbon elongation by a five-step sequence from **6**, the amino functionality was introduced at the C(6) position of **7** by an electrophilic aromatic azo coupling to yield **8**.[10] Reductive cyclization with zinc in hot acetic acid followed by reduction of the intermediate lactam with BH_3•THF afforded 5,6,7,8-tetrahydro-1,8-naphthyridin-3-ol (**9**), which was transformed into N(8)-alkyl derivatives such as **3a** and **3b** under reductive alkylation conditions. This synthetic pathway has significant advantages in terms of efficiency (63 % yield over ten steps to produce the key intermediate **9** from inexpensive **5**) and convenience over the previously reported methods.[3,5,6,8]



Scheme 1. Pyridoxine-based route for the synthesis of 5,6,7,8-tetrahydro-1,8-naphthyridin-3-ols. a) Zn, AcOH (Ac = acetyl), reflux, 3 h; b) 2 м HCl in ether, MeOH, reflux, 1 h, 93 % (for 2 steps); c) Ac_2O, $NaHCO_3$, H_2O, RT, 10 min, 99 %; d) NaOCl, 2,2,6,6-tetramethylpyridine-1-oxyl (TEMPO), $NaHCO_3$, CH_2Cl_2, RT, 1 h, 94 %; e) $(EtO)_2P(O)CH_2CO_2Et$, NaH, CH_2Cl_2, RT, 1 h, 93 %; f) H_2, 10 % Pd/C, EtOH, RT, 3 h, 98 %; g) K_2CO_3, EtOH, RT, 2 h, 97 %; h) $PhNH_2$, 6 м HCl, $NaNO_2$, 2.5 м NaOH, H_2O/THF (1:1), 0 °C to RT, 1 h, 96 %; i) Zn, AcOH, reflux, 1 h, 92 %; j) BH_3•THF, THF, reflux, 2 , 93 %; k) HCHO, $NaBH_3CN$, AcOH, MeOH, reflux, 6 h, 91 %; l) $CH_3(CH_2)_{14}CHO$, $NaBH(OAc)_3$, AcOH, THF, RT, 6 h, 85 %.

Pyridoxine also serves as starting material for a practical synthetic pathway to a new series of monocyclic aminopyridinols, as described in Scheme 2. The key intermediate, 6-amino-2,4,5-trimethylpyridin-3-ol (**11**), was prepared in four steps from **5** in 72 % overall yield. It should be noted that this four-step process was reproduced on a 100 g scale with no need for chromatographic purification. Alkyl groups could be attached to the C(6) NH_2 group of **11** by either reductive alkylation (for the *N*,*N*′-dialkyl analogues **12**, **13**) or a sequential *N*,*O*-diacylation, selective *O*-acyl hydrolysis, and reduction of the intermediate amide with BH_3•THF (for the *N*-monoalkyl analogues **14**–**16**). Because the hydrophobic character of a radical scavenger may be crucial for its activity in vivo, the approach offers the advantage of the alkylation of **9** and **11** in the last step of the sequence, allowing



Scheme 2. Synthesis of 6-amino-2,4,5-trimethylpyridin-3-ols. a) $SOCl_2$, DMF (cat.), reflux, 30 min, 99 %; b) Zn, AcOH, reflux, 3 h, 90 %; c) $PhNH_2$, 6 м HCl, $NaNO_2$, 2.5 м NaOH, H_2O, 0 °C, 1 h, 87 %; d) Zn, HCO_2H, MeOH, reflux, 2 h, 85 %; e) HCHO, HCO_2H, reflux, 48 h, 85 %; f) hexanal, $Na(OAc)_3BH$, AcOH, THF, RT, 6 h, 80 %; g) Ac_2O, Et_3N, THF, RT, 12 h; h) R^2COCl, $ClCH_2CH_2Cl$, reflux, 4 h; i) NaOMe, MeOH, RT, 30 min; j) BH_3•THF, THF, reflux, 2 h, 57–75 % (for 3 steps).

for easy modulation of the lipophilicity of the target molecule. In both organic and aqueous solvents the monocyclic pyridinols were more stable than the bicyclic compounds. In aerated benzene, the latter started to gradually decompose after nearly 3 h of non-detectable decay. However, in aqueous media the difference was more profound. Thus, in phosphate-buffered saline (PBS, pH 7.4) at room temperature **3a** and **3b** noticeably begin to decompose immediately, leading to a significant decomposition within few hours. The maximal aryl–N overlap in **3a**, lowers its ionization potential (IP), leading to increased reactivity towards molecular oxygen.[4]

The reactivities of the new, vitamin B_6-derived pyridinols towards peroxyl radicals were studied by both the venerable inhibited autoxidation of styrene methodology[2a] and a new peroxyl radical clock methodology.[11] The results, given in Table 1, are in reasonable qualitative agreement, with rate constants differing by a factor of two or less where comparison is possible.[12,13] Most interestingly, both methods predict that the vitamin B_6-derived pyridinol **12** reacts with peroxyl radicals much slower than the previously studied and highly similar analogue **2**, which differs only in its lack of a ring methyl group. The same observation applies for the dihexylated analogue **13**. Whereas removal of both alkyl groups on the amine substituent (i.e., **11**) partially restores activity, the removal of just one of the alkyl groups (i.e., **14**) leads to the vitamin B_6-derived pyridinol with kinetics that most closely match the previously studied compound **2**.

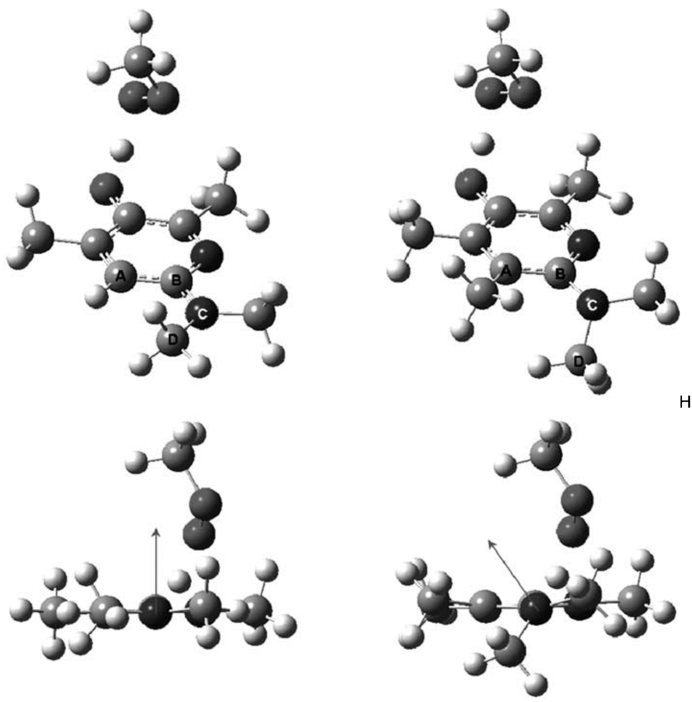
Table 1. Rate constants (k_{inh}) and stoichiometric factors (n) for the reactions of aminopyridinols with peroxyl radicals.

Compound	k_{inh} [$10^6\,\text{M}^{-1}\,\text{s}^{-1}$]	n	
	C_6H_5Cl; C_6H_6	C_6H_5Cl; C_6H_6	PBS[a]
2	16 ± 2;[b] 17 ± 2[c]	1.9;[b] 2.0[d]	0.9
11	6.2 ± 1.5[b]	1.2–2.0;[b] 2.1[d]	1.4
12	3.3 ± 1.1;[b] 1.6 ± 0.2[c]	2.0;[b] 1.9[d]	1.4
13	2.2 ± 1.5[b]	2.1[b]	–
14	8.7 ± 2.6;[b] 14 ± 2[c]	1.7–2.1;[b] 2.0[b,d]	0.9
3a	88 ± 32[b]	1.1–1.7;[b] 1.7[d]	0.2

[a] Calculated with respect to Trolox[14] ($n = 2.0$) in PBS buffer (pH 7.4) based on HPLC-electrochemical detection (ECD) measurements. An excess amount (20 equiv) of AIPH[14] (in PBS) was used to maintain a zero-order reaction over the course of the experiment. [b] Measured by the inhibited autoxidation of styrene in chlorobenzene at 30 °C.[3] [c] Measured by the peroxyl radical clock method in benzene at 37 °C.[11] [d] Calculated with respect to PMC[14] ($n = 2.0$) based on HPLC-ECD measurements. An excess amount (20 equiv) of MeOAMVN[14] (in benzene) was used to maintain a zero-order reaction over the course of the experiment.

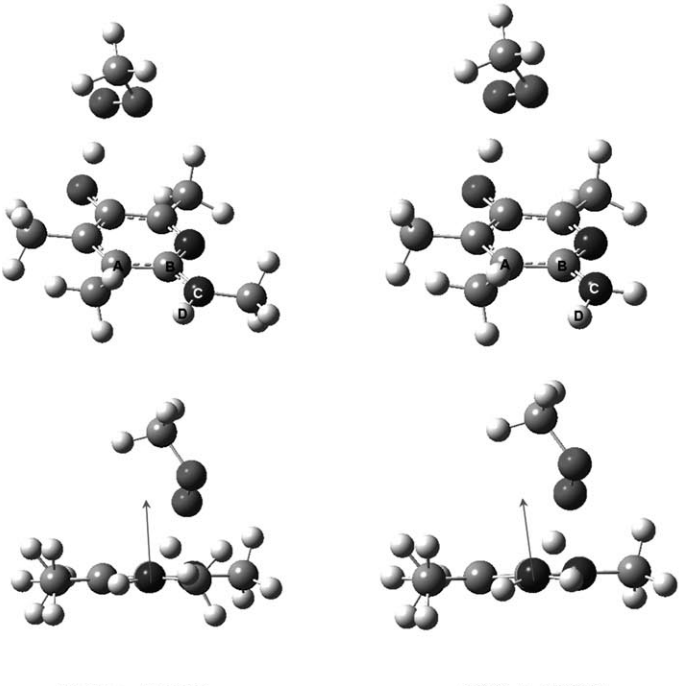
To better understand the basis for the reduced reactivity of pyridinol **12** (and its longer chain analogue **13**) relative to the previously studied pyridinol **2**, we undertook a series of theoretical calculations. By using density functional theory methods, we determined the low-energy transition state (TS) structures for the reactions of **2** and **12** with methylperoxyl—a model for the chain-carrying peroxyl radicals in the autoxidation. The TS structures are shown in Figure 1 a and b . At first glance, they have very similar overall structures. Both display a *syn* relationship of the groups bound to the oxygen atoms between which the hydrogen atom is being transferred. This provides the maximum overlap between the π HOMO of the pyridinol and the π* SOMO of the peroxyl radical, and is consistent with a proton-coupled electron transfer mechanism.[15] However, whereas the conformation of the *N*,*N*-dimethylamino group is ideal to make a contribution of the nitrogen lone pair to the π HOMO in **2** (i.e., it is orthogonal to the plane of the aromatic ring, as depicted by the arrow in Figure 1 a), the same orientation of the *N*,*N*-dimethylamino group in **12** is not possible owing to a steric interaction with the ring methyl group. This leads to a lesser contribution of the nitrogen lone pair to the π HOMO in **12**, thereby leading to a reduced interaction with the peroxyl compared to **2**. This translates into a higher calculated enthalpy of activation for the reaction of **12** with methylperoxyl of almost 2 $\text{kcal}\,\text{mol}^{-1}$ relative to **2**, which is in good agreement with the experimental observations. If one of the methyl groups on the amine nitrogen of **12** is replaced with an hydrogen atom, such that the amine group can adopt the optimal orientation for interaction with the aromatic ring such as in pyridinol **14**, the activation enthalpy drops back down to within a couple of tenths of a kilocalorie to what is calculated for **2**—again, in good agreement with the experiment. The experimental results also show that the free amine **11** reacts with peroxyls at a rate intermediate to those of **2** and **12**, and just a bit slower than the monoalkylamine **14**. Again, theory nicely accounts for this trend, and the activation enthalpy calculated for the reaction of **11** with methylperoxyl falls in between those of **2** and **12**, and is slightly larger than that of **14**. This is presumably due to the fact that the free amine group in **11** is less electron donating than the *N*-ethylamine in **14**, which is less electron donating than the *N*,*N*-dimethylamine in **2**.[16]

From the length of the inhibited autoxidation period we obtained the stoichiometric factor n[2a]—the number of peroxyl radicals trapped by each pyridinol. The n values are typically close to 2.0 for most phenols, the first peroxyl being scavenged by the formal hydrogen atom transfer (HAT) depicted above [Eq. (4)], and the second by peroxyl-phenoxyl radical coupling [Eq. (5)]. The values of n determined for pyridinols **12** and **13** are close to 2.0, similar to that previously determined for **2**, suggesting that the mechanism of chain breaking is basically the same as for phenols, as we have noted before.[3] In contrast, the values of n we determined for **11** and **14** were found to be both smaller and dependent on the concentration of the pyridinol, similar to what was observed previously for **3**.[3b] The low values of n for pyridinol **3** have been ascribed to its lability in air, that is, its own ability to autoxidize. Pyridinols **11** and **14**, however, would not be expected to be as oxidizable as **3**. In fact, we would expect them to be less oxidizable than even **2**, owing to the fact that the free amine group of **11** and the *N*-ethylated amine group of **14** are less electron donating than the *N*,*N*-dimethylamine group of **2**. Instead, we believe that



(a) **2** + •$OOCH_3$
$\Delta\Delta H^{\ddagger} = 0.0$ kcal mol^{-1}
Φ(ABCD) = 2.1°

(b) **12** + •$OOCH_3$
$\Delta\Delta H^{\ddagger} = 1.9$ kcal mol^{-1}
Φ(ABCD) = 56.3°

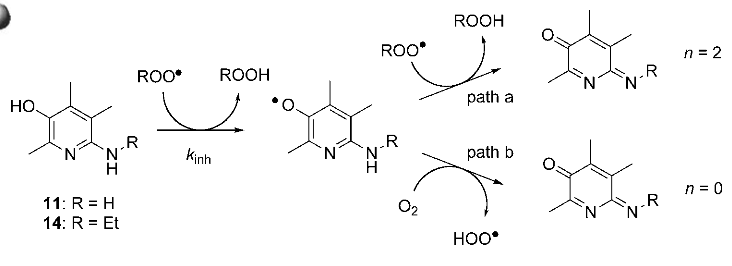


(c) **14** + •$OOCH_3$
$\Delta\Delta H^{\ddagger} = 0.2$ kcal mol^{-1}
Φ(ABCD) = 11.3°

(d) **11** + •$OOCH_3$
$\Delta\Delta H^{\ddagger} = 0.7$ kcal mol^{-1}
Φ(ABCD) = 23.6°

Figure 1. Lowest-energy transition state structures for the reactions of pyridinol **2** and the vitamin B_6-derived analogues **11**, **12**, and **14** with a methylperoxyl radical. Two views are shown for each; the top to make all atoms clear, the bottom to depict the orientation of the amine lone pair (normal shown as the arrow) relative to the aromatic ring. The activation enthalpies are given relative to the most reactive pyridinol **2**, and the torsion angle of the labeled atoms. a) **2** + $^{\bullet}OOCH_3$; b) **12** + $^{\bullet}OOCH_3$, c) **14** + $^{\bullet}OOCH_3$, and d) **11** + $^{\bullet}OOCH_3$.

the presence of a free N–H, in addition to the reactive O–H, allows the compounds to react as hydroquinones do.[17] This is shown in Scheme 3.



Scheme 3. Two fates of the aryloxyl radicals derived from pyridinols **11** and **14** during the inhibited hydrocarbon autoxidation in non-polar organic solvents.

Thus, at low pyridinol concentrations path a dominates, and the pyridinol traps two peroxyl radicals. However, increasing the pyridinol concentration leads to an increasing contribution from path b, because all peroxyl radicals are trapped by pyridinol in the first reaction and the aryloxyl radicals derived from **11** or **14** react with O_2 to yield a hydroperoxyl radical, which can carry on the chain reaction. This leads to a concentration-dependent diminution in the stoichiometric factor. In fact, under our experimental conditions, concentrations of **11** of only 12.5 μM already gave $n = 1.2$. Of course, because the partitioning of the intermediate radical along path a and path b depends on the steady state concentration of peroxyl radicals, a variety of factors, including the rate of initiation and the concentration of the antioxidant and/or oxidizable substrate are relevant.

A short while ago, we reported that the lipophilic pyridinols **3b** and **4** were excellent inhibitors of lipid peroxidation in human LDL.[5] Despite these exciting results, we had yet to follow them up with kinetic studies of pyridinol antioxidants in biphasic media, such as lipoproteins, which are of far more physiological relevance than the homogenous organic solution studies that we present above. Here, we present such studies, which we have carried out in multi-lamellar vesicles (MLV) (i.e., liposomes)—a convenient alternative to the investigation of lipid peroxidation in cells or isolated lipoproteins.[18] In the current study, pyridinols **2** and **11**–**16**, as well as the reference radical scavengers uric acid (UA) and α-TOH were used to inhibit lipid peroxidation. The heterogeneous system consisted of oxidizable 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) liposomes suspended in PBS (pH 7.4) at 37 °C. Oxidation was initiated by the water-soluble radical initiator AIPH.

In the early stage of reactions when antioxidants were present in the system, relatively low rates of formation of

PLPC oxidation products (PLPC-OOHs) were typically observed, followed by a rapid increase in the peroxidation rate upon consumption of antioxidants (Figure 2). It is important to note that the phospholipid peroxidation rates in the presence of pyridinols (except for **11**) are similar to or lower than the corresponding rate determined for α-TOH (Table 2).[19] The rates of oxidation in the presence of the inhibitors, $R_{\mathrm{ox,inh}}$, are given in Table 2, along with the associated stoichiometric factors (n). Interestingly, the relative efficacies of the pyridinols in inhibiting the autoxidation of PLPC liposomes do not mirror the results in homogenous organic solution. In fact, where the order of reactivity in chlorobenzene is **2** > **14** > **11** > **12** ≈ **13**, the order of reactivity in liposomes is **2** > **12** ≈ **13** ≈ **14** > **11**. Furthermore, the reactivity of **15** and **16** in liposomes is even superior to that of **2**. Although the inhibition rate constants of these two lipophilic pyridinols were not determined in chlorobenzene, it can be expected that they are essentially the same as the one determined for **14**, because all three bear a monoalkylated amine—and as such would be expected to activate the pyridinol to the formal hydrogen-atom transfer to the same extent.

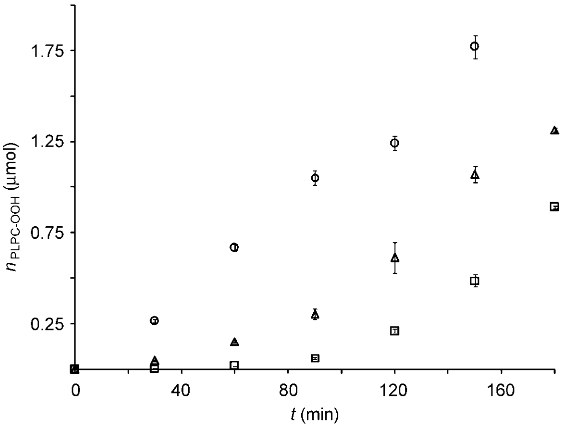


Figure 2. Formation of PLPC-OOH during oxidation of 26.6 μmol of PLPC (MLV) suspended in PBS (pH 7.4, 2 mL) at 37 °C initiated by 1.06 μmol of AIPH: uninhibited reaction (circles); inhibited by 26.6 nmol of **11** (triangles) or α-TOH (squares).

Table 2. Antioxidant properties of pyridinols in PLPC liposomes (MLV).

Antioxidant	$R_{\mathrm{ox,inh}}$ [nmol min^{-1}][a]	n[b]
2	0.18	1.4
11	1.90	1.3
12	0.23	1.6
13	0.24	2.0
14	0.24	1.4
15	0.10	2.2
16	0.12	2.1
UA	1.90	2.0
α-TOH	0.24	2.0
none	17.0	n/a[c]

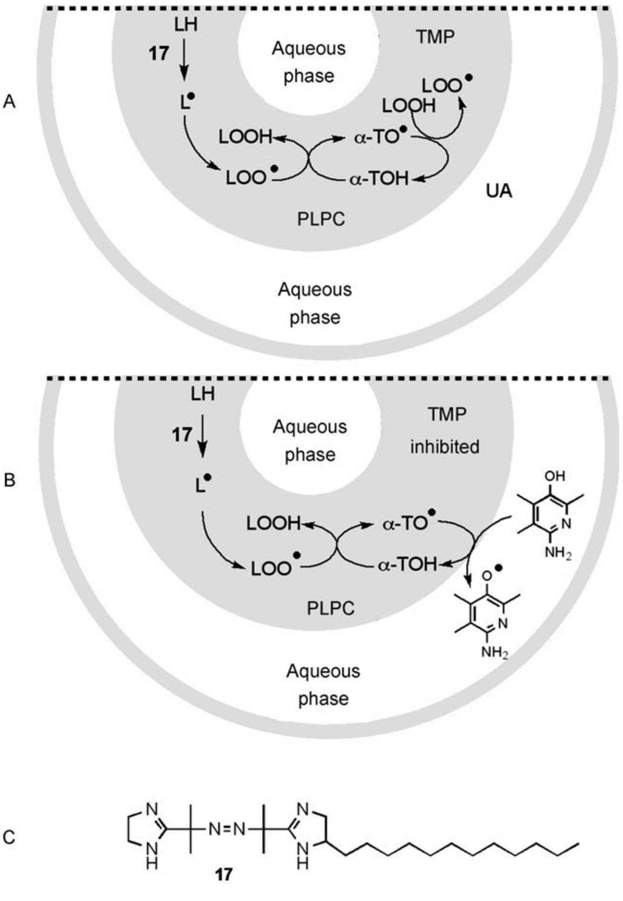
[a] The rate of oxidation during the inhibited phase based on initial time points (≥ 4) obtained by measurements of formation of PLPC-OOHs analyzed as methyl esters of linoleyl alcohols (MeLin-OHs) by UV-HPLC. Composition of the mixture: PLPC (26.6 μmol), AIPH (1.06 μmol), antioxidant (26.6 nmol), PBS (pH 7.4, 2 mL). [b] Stoichiometric factors in PLPC (MLV)/PBS (pH 7.4) suspension at 37 °C calculated with respect to α-TOH (n=2.0) based on the formation of PLPC-OOHs. [c] Not applicable.

Clearly, the origin of the differences in the reactivities of these compounds in the biphasic micellar system as compared to the homogenous system should be due to their phase separation. In fact, we found that the distribution between PBS and the MLV lipid phase was 92:8 for **11** and roughly 1:1 for **14**. Although partitioning studies were not conducted for **2** and **12**, these compounds, being structurally related to **14**, are expected to partition between the phases to a similar extent, that is, 1:1. Hence, **11** is not a good radical scavenger in liposomes because it does not spend any significant amount of time there, and can scavenge only the initiator-derived radicals. Pyridinols **2** and **12**–**14** can intercept both the initiator-derived and lipid-derived radicals, accounting for the good reactivity. Lastly, the highly lipophilic **15** and **16**, which are expected to partition essentially exclusively to the lipid phase of the liposome, exhibit the highest activity. From these data, it would appear that physical factors are most important rather than chemical ones.[18e] A further consequence of the phase separation behavior of the pyridinols is clearly evident upon consideration of the stoichiometric factors that are obtained from the length of the inhibited period of the PLPC autoxidation. The pyridinols that partition between both the lipid and aqueous phases (**2**, **11**, **12**, and **14**) were found to have lower n values (1.3–1.6), whereas those pyridinols that will partition more or less exclusively to the lipid phase had n values around the ideal value of 2.0 (**13**, **15**, and **16**). It has been noted by others that UA has little impact on radical chain oxidation processes of lipid bilayers,[20,21] although more lipophilic radical scavengers protect oxidizable substrates more efficiently,[18b,22] as we observe for the pyridinols (see above).

In order to provide further insight into the behavior of the water-soluble pyridinols in the aqueous phase, we measured the stoichiometries of the reactions of **2**, **11**, **12**, and **14** with peroxyl radicals independently in PBS (see Table 1). In all cases, values of n that were lower than those measured in chlorobenzene—and PLPC MLVs—were obtained. This can be explained by the combination of two factors. The formal hydrogen-atom transfer reaction between the pyridinol and peroxyl radical will be suppressed in water owing to both the hydrogen bond donor interaction between the water and the pyridine N atom and the hydrogen bond acceptor interaction between the water and the reactive O–H moiety.[23] This allows the direct oxidation of the pyridinol (likely as the pyridinoxide anion, because pyridinols have pK_a values that are lower than those in phenols by ≈1.5–2 log units),[24,25] by molecular oxygen to compete an electron transfer reaction whose rate is now greatly enhanced by the more polar environment. Hence, overall, the more lipophilic pyridinols **15** and **16** display the best radical-trapping activities in liposomes because they partition favorably to the lipid phase, allowing them to trap the maximum numbers of radicals and slowing their consumption through undesired reactions with O_2.

Although **11** is not an effective antioxidant on its own, we wondered if it might still serve as a co-antioxidant in the presence of α-TOH, similar to hydroquinones (such as coenzyme Q_{10}) or catechols (such as plant polyphenols). To evaluate this possibility, experiments were conducted in a PLPC (MLV)/PBS system, where lipid peroxidation was initiated by an unsymmetrical azo radical initiator **17** (see Scheme 4c for the chemical structure).[20,21] Initiator **17** partitions almost exclusively into the lipid phase of MLVs but it generates one hydrophilic and one hydrophobic radical upon decomposition.[21] Under these conditions, it is anticipated that **11** should be consumed before α-TOH, if it is able to reduce α-TO$^\bullet$ back to α-TOH, thus preventing undesired oxidation of lipids promoted by α-TO$^\bullet$, that is, tocopherol-mediated peroxidation (TMP)[26,27] (Scheme 4). In one experiment a mixture of α-TOH and UA was treated with **17** and as shown in Figure 3A, α-TOH was indeed consumed before UA. The water-soluble UA does not effectively reduce the tocopheryl radical. On the other hand, as shown in Figure 3B, aminopyridinol **11** spared α-TOH in the PLPC (MLV)/PBS system.

Importantly, in both cases the length of the lag-phase in PLPC-OOH formation was closely correlated with the disappearance of α-TOH. The observed small protective effect of UA on α-TOH was associated with its ability to scavenge a portion of hydrophilic radicals generated by the unsymmetrical initiator **17** that diffused out of the lipid bilayer.[20,21] In direct contrast to the situation with UA, α-TOH maintained its initial concentration until **11** was essentially depleted (Figure 3B), suggesting strong co-antioxidant activity of **11**.



Scheme 4. Proposed reactivity of α-TOH in the presence of UA (A), and **11** (B) in the PLPC (MLV)/PBS system, where oxidation is initiated by a lipid associated radical initiator **17** (C).

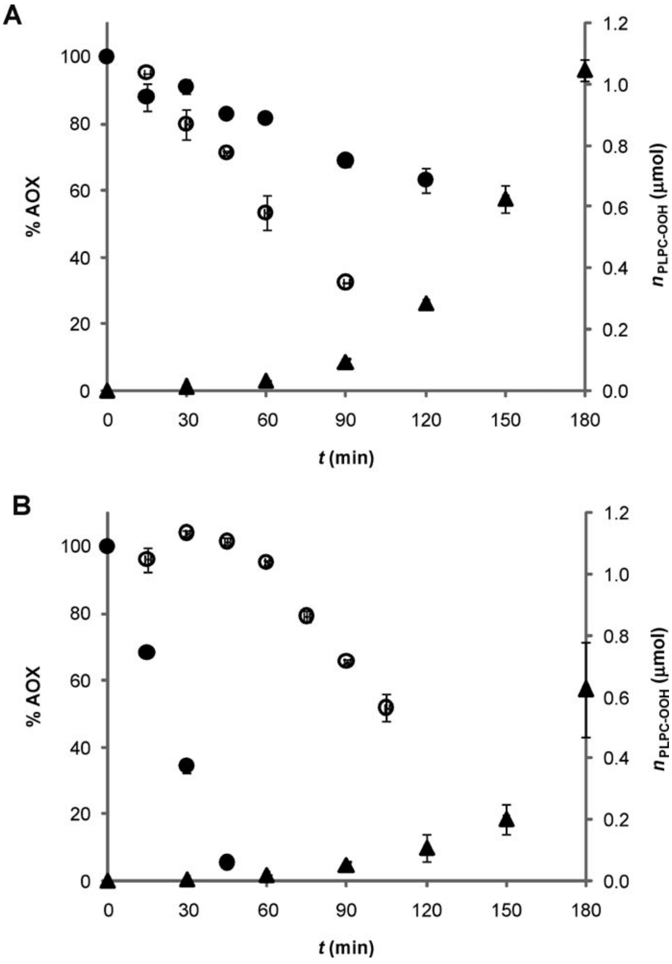


Figure 3. The disappearance of antioxidants from mixtures of α-TOH with UA (A) or **11** (B): 13.3 nmol of α-TOH (open circles) and 13.3 nmol of UA/**11** (closed circles); and the formation of PLPC-OOH (triangles) during peroxidation of 26.6 μmol of PLPC (MLV) in PBS (pH 7.4, 2 mL) at 37 °C initiated by 2.12 μmol of lipid-associated **17** (see Scheme 4c).

Analysis of the stoichiometric factors and lipid peroxidation rates obtained for the antioxidant mixtures is in further support of the notion that **11** acts as a co-antioxidant with α-TOH (Table 3). The unusually low *n* value found for **11** is likely associated with its increased autoxidizability under the reaction conditions (Table 3), where hydrophilic radicals generated from **17** must first undergo diffusion across the interface to produce radicals in the aqueous phase. Only a fraction of **11** (≈8 %) encounters lipophilic radicals derived from **17** in the bilayer because of its phase separation, whereas the majority of the compound undergoes the super-

Table 3. Oxidizibility of PLPC (MLV) in the presence of antioxidant mixtures.

Antioxidant	$R_{\mathrm{ox,inh}}$ [nmol min^{-1}][a]	n[b]
11	1.4	0.9
UA	17	4.5
α-TOH + UA	0.27	2.5[c]
α-TOH	0.28	2.0
α-TOH + **11**	0.17	3.3
none	22	n/a

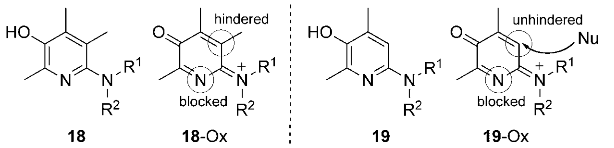
[a] The rate of oxidation during the inhibited part of the autoxidation, based on initial time points (≥ 4) obtained by measurements of formation of PLPC-OOHs (HPLC-UV analysis of MeLin-OHs). Composition of the mixture: PLPC (26.6 μmol), **17** (2.12 μmol), hydrogen-atom donor (13.3 nmol of a single antioxidant or 13.3 nmol of each in the mixture of two), PBS (2 mL). [b] Stoichiometric factors are based on the disappearance of antioxidants from the system, reported with respect to α-TOH (n=2.0), by HPLC-ECD. [c] The number given for the consumption of α-TOH only (the disappearance of UA was not monitored).

oxide-mediated consumption described in Scheme 3, path b. The addition of α-TOH to the system diminishes the path b oxidation of **11** as reflected in the n factor measured for the mixture (n=3.3; exceeding by 1.3 the value n=2.0 due to α-TOH itself). Presumably α-TO$^{\bullet}$ in the liposome can be accessed by **11** at the lipid/water interface (Scheme 4 A),[28] thereby inhibiting TMP in the lipid phase. Such a synergistic effect between natural α-TOH and vitamin B$_6$-derived aminopyridinols suggests that they may serve to extend the potency of vitamin E,[22] as shown previously for vitamin C.[29]

In previous work, we reported that the lipophilic 3-pyridinols **3b** and **4** showed co-antioxidant behavior with α-TOH in human LDL.[5] As is the case for pyridinol **11** above, we observed that either **3b** or **4** was consumed before α-TOH in the oxidation, and that α-TOH was only consumed to a significant extent, after all of either **3b** or **4** had been consumed. We explained this observation by a combination of the fact that **3b** and **4** are approximately 20-fold more reactive towards peroxyl radicals than α-TOH,[5] and would be expected to be consumed first, and also by the fact that **3b** and **4** have O–H BDEs that are 2 kcal mol^{-1} weaker than the O–H BDE in α-TOH,[3] allowing α-TO$^{\bullet}$ to equilibrate with **3b** or **4** and reform α-TOH, whereas the more reactive **3b** or **4** was still present in the system.

Because **11** is not significantly more reactive towards peroxyl radicals than α-TOH (see above), its O–H BDE is not expected to be significantly lower than the O–H BDE of α-TOH,[3] and as **11** partitions preferentially to the aqueous phase (see above), the co-antioxidant activity of **11** with α-TOH must be driven by some other factor(s). Although the reaction between α-TO$^{\bullet}$ and **11** (as shown in Scheme 4 A) is not particularly exothermic, the subsequent reaction of the aryloxyl radical derived from **11** with either a water-soluble peroxyl or O$_2$ to form the p-iminoquinone can be expected to be highly exothermic and irreversible. This effectively removes a radical from the lipid region of the liposome, exporting it to the aqueous phase, precluding the possibility for TMP. This co-antioxidant activity of **11** with α-TOH is similar to those of catechols or hydroquinones,[30] in that radical character is irreversibly exported from the liposome, driven by the formation of either o- or p-quinones, respectively.[17]

In closing, we note that all of the vitamin B$_6$-derived compounds contain a C(5)-methyl group that may have significant toxicological implications.[31] After oxidation, the iminoquinone or its cationic iminium ion (**18**-Ox, Scheme 5) are expected to be more resistant to nucleophilic conjugate addition by endogenous nucleophiles, for example, glutathione or proteins,[32] than the previously studied monocyclic aminopyridinol antioxidants (i.e., **2**), which have demonstrated significant cytotoxcities in recent preliminary studies.[24] Studies along these lines are underway.



Scheme 5. The enhanced resistance of oxidized 6-amino-2,4,5-trimethylpyridin-3-ols (**18**-Ox) with respect to 2,4-dimethyl analogues (**19**-Ox) towards a nucleophilic attack.

Conclusion

The development of a practical synthetic route to previously described bicyclic aminopyridinols from vitamin B$_6$ prompted the consideration of a novel class of monocyclic aminopyridinol antioxidants that contain three methyl groups on the aromatic ring in lieu of the two methyl groups found in previously described compounds. Whereas the hydrophilic monocyclic compounds are readily autoxidized in aqueous media and liposomes, their lipophilic analogues are stable under such conditions and outperform the archetypical antioxidant, α-tocopherol. The autoxidation of water-soluble aminopyridinols in heterogeneous systems is, however, diminished in the presence of α-TOH, which is in turn spared by a co-antioxidant action of these synthetic radical scavengers during the inhibited phase of lipid peroxidation.

Experimental Section

For the general section concerning reported analytical procedures, and for synthetic protocols applied to prepare pyridinolic antioxidants, please see the Supporting Information.

Inhibited autoxidation studies: Autoxidation experiments were performed in a two-channel oxygen uptake apparatus, based on a Validyne DP 15 differential pressure transducer, described elsewhere.[33] The entire apparatus was immersed in a thermostated bath ensuring a constant temperature within ±0.1 °C. In a typical experiment, an air-saturated solution of styrene (4.3 M, 50 % v/v) in chlorobenzene containing azobisisobutyronitrile (AIBN, $0.1–1\times10^{-2}$ M) was equilibrated with a reference solution containing an excess of 2,2,5,7,8-pentamethyl-chroman-6-ol (PMC, 1×10^{-4} M) at 30 °C. When a constant oxygen consumption rate was reached, a small amount of a solution of the aminopyridinols (antioxidants) was added to the autoxidizing mixture and the oxygen consumption in the sample was measured from the differential pressure between the two channels recorded as a function of time. The initial concentration of the antioxidant in the autoxidizing mixture was in the range 0.6–12 μM and was adjusted so to have a kinetic chain length > 4. Induction (inhibition) period lengths (τ) were determined by the intersection between the regression lines to the inhibited and the uninhibited traces. Initiation rates, R_i, were determined in preliminary experiments by the inhibitor method by using PMC as reference antioxidant: $R_i = 2[\text{PMC}]/\tau$. The absolute rate constant for inhibition k_{inh} was obtained by Equation (6),[33] where k_p is the propagation rate constant, which in the case of styrene is $41\,\text{M}^{-1}\text{s}^{-1}$.[2a]

$$-\Delta[O_2]_t = \frac{k_p[\text{styrene}]}{k_{inh}}\ln(1 - t/\tau) \tag{6}$$

Theoretical calculations: All calculations were carried out by using the B3LYP density functional[34] and CBSB7 basis set[35] as implemented in the Gaussian 03 suite of programs,[36] compiled to run on Sun Microsystems SunFire 25000 or Enterprise M9000 servers with UltraSPARC-IV+ or Sparc64 VII CPUs, respectively. Briefly, geometries were first optimized and then vibrational frequencies calculated to characterize the stationary points as either minima or transition states on the surface. Following thermochemical corrections to 298 K, the enthalpies of activation were calculated relative to the separated reactants, and given relative to that calculated for the reaction of **2** and methylperoxyl as shown in Figure 1.

Stability of antioxidants in aerated solutions: Antioxidant solutions (1.00 μmol) in benzene (10 mL) were placed in a sand bath at 37 °C. Aliquots (400 μL) were withdrawn every 20 min over 3 h (**3a**) or every 8 h over 48 h (**2**, **11**, **12**, and **14**) and added to an ice-cold mixture of BHT and $P(OMe)_3$ (2.0 μmol each) in benzene (100 μL). Internal standards were added in benzene (80 μL), mixtures were vortexed for 30 s and the solvent was blown off under a stream of argon. The residue was dissolved in $MeOH/H_2O$ (65 %, 150 μL) and placed in the autosampler at 5 °C prior to sample injection on HPLC. For aqueous mixtures, aliquots were drawn every hour over eight hours (**2**, **11**, **12**, and **14**) or every 15 min over two hours (**3a**). Compounds were incubated in PBS (pH 7.4, 50 mM) and a mixture of BHT and $P(OMe)_3$ (2.0 μmol each) was added in MeOH (500 μL). Internal standards were also added in MeOH. Information about stability was extracted from plots of [antioxidant] versus time.

Oxidation of antioxidants in homogenous solutions: Experiments were conducted similar to the stability measurements, except antioxidants were incubated in the presence of a radical initiator (20 μmol), MeOAMVN (in benzene) or AIPH (in PBS), and aliquots were withdrawn every 5 min over 20 min (**2**, **3a**, **11**, **12**, and **14**) for benzene solutions, whereas aliquots from the PBS solutions were taken every 15 min over 90 min (**2**, **11**, **12**, and **14**) or every 5 min over 25 min (**3a**). The half-life times for antioxidants in the presence of radical initiators were extracted from plots of [antioxidant] versus time. Stoichiometric factors were calculated as shown in the Supporting Information.

Oxidation of PLPC (MLV) in PBS in the presence of AIPH: Antioxidants (26.6 nmol) in methanol (100 μL) were added to PLPC (20 mg) in chloroform (800 μL). Solvents were blown off under a stream of argon to form thin films. PBS (1.90 mL) was added to those films followed by an addition of AIPH (1.06 μmol) in PBS (100 μL). Samples were vortexed for 2 min and incubated at 37 °C for 4 h. Every 30 min, aliquots (200 μL) were drawn, vortexed (20 s) with an ice-cold $BHT/PPh_3/MeOH$ (1.0 mg:1.2 mg:1 mL), then vortexed (20 s) with KOH/MeOH (0.50 M, 1 mL) and left for 15 min at RT. After that time, mixtures were vortexed (20 s) with aqueous NH_4Cl (5 %, 1 mL) extracted with hexane (2×1 mL). Organic layers were combined and dried over Na_2SO_4, and solvents were blown off under a stream of argon. The residual material was dissolved in cinnamyl alcohol/hexane (12.5 μM, 100 μL) and placed in the HPLC autosampler at 5 °C prior to sample injection on HPLC. The lag-phase duration periods were extracted from plots of [PLPC-OOHs] versus time. Data points (at least four per reaction) obtained at early stages of PLPC oxidation were fit to linear equations from which $R_{ox,inh}$ values were calculated.

Oxidation of PLPC (MLV) in PBS in the presence of 17: α-Tocopherol (13.3 nmol) in MeOH (100 μL) was added (if applicable) to PLPC (20 mg) in chloroform (800 μL). Solvents were blown off under a stream of argon to form thin films. PBS (1.85 mL) was added to those films followed by an addition of an aqueous antioxidant (13.3 nmol) in PBS (100 μL), if applicable, or additional PBS (100 μL) in case aqueous antioxidant was not used. Samples were vortexed for 2 min. Compound **17** (2.12 μmol) in MeOH (50 μL) was added, and milky mixtures were vortexed for an additional 20 s and incubated at 37 °C for 120 min (single antioxidant) or 210 min (two antioxidants).

To monitor disappearance of antioxidants, aliquots were withdrawn every 15 min over at least 1 h. In case of aqueous antioxidants, aliquots (100 μL) were vortexed (20 s) with $BHT/PPh_3/MeOH$ (0.25 mg:0.30 mg:400 μL) and an internal standard in MeOH (50 μL). Samples were centrifuged (2 min at 10000 rpm), supernatants (300 μL) were withdrawn, and solvents were blown off under a stream of argon. Residues were dissolved in aqueous MeOH (70 %, 70 μL, for **11**) or aqueous MeOH (50 %, 100 μL, for uric acid), and mixtures were placed in the autosampler at 5 °C prior to sample injection on HPLC.

For α-TOH analysis, an aliquot (200 μL) was vortexed (20 s) with $BHT/PPh_3/MeOH$ (0.50 mg:0.60 mg:700 μL) containing an internal standard in MeOH (100 μL). Highly lipophilic compounds were extracted with hexane (2×1 mL), organic layers were combined and dried over Na_2SO_4. Solvents were blown off under a stream of argon, residues were dissolved in hexanes (60 μL) and placed in the autosampler at 5 °C prior to sample injection on HPLC.

Analyses of MeLin-OHs were performed as for oxidations initiated by AIPH. Antioxidant consumption profiles were plotted on charts: [antioxidant] versus time, whereas the lag-phase duration periods and $R_{ox,inh}$ values were extracted from [PLPC-OOHs] versus time.

Partition of antioxidants between PLPC (MLV) and PBS: Antioxidants (52.2 nmol) in MeOH (100 μL) were added to PLPC (20 mg) in chloroform (800 μL). Solvents were blown off under a stream of argon to form thin films. Degassed PBS (2.00 mL) was added to those films, mixtures were vortex (2 min) and further degassed for 5 min with bubbling argon. Vials containing milky suspensions were then carefully closed and incubated for 2 h at 37 °C. The aqueous phase was separated from lipid aggregates by centrifugation (3×5 min at 3500 rpm) in Amicon centrifugal filter units (10 kDa cut off). Filtrates obtained after the initial 5 min of centrifugation were discarded, an aliquot of the remaining supernatants (400 μL) was vortexed with an internal standard in MeOH (400 μL) and mixtures were placed in the HPLC autosampler at 5 °C prior to sample injection on HPLC. The extent of partitioning of an antioxidant into liposomes was assigned by comparison of $[\text{antioxidant}]_{aqueous}$ found in samples incubated with and without PLPC and otherwise identically following the above procedure.

Reproducibility of the reported results: All experiments described were repeated at least three times and an average value (stoichiometric factor, rate of lipid peroxidation, rate of hydrogen atom donor autoxidation, percent distribution in aqueous and lipid phase) was reported for each measurement. In homogeneous systems, the repeated values were within a 10 % margin of the reported value. In heterogeneous systems, the highest standard deviation was less than 13 %. Stability tests in aerated solvents were also repeated at least three times consistent with all other results.

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[12] It should be noted that the autoxidations were carried out at 30°C in chlorobenzene, whereas radical clock experiments were carried out at 37°C in benzene. Although the higher temperatures should lead to slightly larger rate constants, because benzene is a better hydrogen bond accepting solvent than chlorobenzene, rate constants in benzene should be slightly slower. Overall, it would appear that these effects cancel each other out and the rate constants are highly comparable.

[13] The rate constant for **11** could not be determined by the peroxyl radical clock method because it relies on the aryloxyl radical derived from the antioxidant to carry the chain reaction. The aryloxyl radical derived from **11** is likely too reactive to oxygen to be persistent long enough to carry the chain.

[14] For chemical structures see the Supporting Information.

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