A rapid and simple screening test for potential inhibitors of tocopherol-mediated peroxidation of LDL lipids

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Abstract We report a rapid and convenient method for screening potential inhibitors of the initiation of low density lipoprotein (LDL) lipid peroxidation. The method uses positively and negatively charged micelles of either cetyltrimethyl ammonium chloride or sodium dodecyl sulfate with added α-tocopherol. It is based on the capacity of an antioxidant to attenuate α-tocopheroxyl radicals, generated by irradiation of the α-tocopherol-containing micelles with UV light, and measured directly by electron spin resonance spectroscopy. To establish the reliability of the method, we compared the α-tocopheroxyl radical attenuating ability (TRAA) of 53 natural and synthetic potential antioxidants with their respective ability to inhibit the early stages of LDL lipid peroxidation initiated by a low flux of water-soluble peroxyl radicals. The relationship between the measured TRAA and corresponding LDL antioxidation activity was highly significant ($P < 0.00005$, Rank test). Thus, the potency of a co-antioxidant for LDLs α-tocopherol could be predicted with > 98% probability by the TRAA test alone. The results suggest that this relatively simple method represents an effective and simple screening test that can be used for large numbers of potential inhibitors of the early stages of LDL lipid oxidation.—**Witting, P. K., C. Westerlund, and R. Stocker.** A rapid and simple screening test for potential inhibitors of tocopherol-mediated peroxidation of LDL lipids. *J. Lipid Res.* 1996. **37:** 853–867.

Supplementary key words antioxidant • atherosclerosis • electron spin resonance spectroscopy (ESR) • α-tocopheroxyl radical

Free radical-mediated oxidation of low density lipoprotein (LDL) has become a widely studied model for atherosclerosis research (1). If LDL peroxidation contributes to the initiation of atherogenesis, increasing the resistance of LDL to oxidative modification may mitigate or even prevent the development of the disease. In support of this, supplementation of animals with antioxidants increases the resistance of the respective LDLs to lipid peroxidation and also reduces the extent of atherogenesis in most (2–5), though not all (6) cases. Hence, there is interest in LDL antioxidants and in vitro tests assessing the efficacy of compounds for their potential anti-atherogenic activity.

The susceptibility of LDL to oxidation is most commonly tested by the method of Esterbauer et al. (7) where the isolated lipoprotein is exposed to a strongly oxidizing environment, in the form of non-physiological and high concentrations of Cu^{2}+, i.e., Cu^{2+}: LDL ≈ 16. In this method LDL 'oxidizability' is gauged by the duration of the inhibited or 'lag phase' of peroxidation, the maximal rate of peroxidation during the uninhibited phase, and the maximal accumulation of primary lipid oxidation products (7). The relevance of this test and the parameters measured to the putative in vivo oxidation of LDL, however, remain unknown. For example, testing the anti-atherogenic effect of probucol in monkeys fed a high-cholesterol diet, Sasahara et al. (8) observed a "trend towards an inverse relationship between intimal lesion size and oxidation resistance" (as measured by the length of 'lag phase' observed during in vitro Cu^{2+} oxidation of plasma LDL), but this did not reach statistical significance. Also, a 'lag phase' such as that observed during the Cu^{2+}/LDL oxidation test (7) is not observed when LDL is exposed to different types of comparatively milder oxidizing conditions, including aqueous and lipophilic peroxyl radicals ($ROO^{\bullet}$), the transition metal-containing Ham's F-10 medium (9), Cu^{2+}:LDL ratios ≤ 2, (10), Fe^{3+}, low fluxes of $^{\bullet}OH$ radicals, and15-lipoxygenase (J. Neuzil, S. Thomas, J.

Abbreviations: AAPH, 2,2′-azobis(2-amidopropane); BHT, butylated hydroxytoluene; HTAC, cetyltrimethyl ammonium chloride; LDL, low density lipoprotein; LOOH, lipid hydroperoxide; SDS, sodium dodecyl sulfate; TMP, tocopherol-mediated peroxidation; α-TOH, α-tocopherol; α-$TO^{\bullet}$,-tocopheroxyl radical; TRAA, tocopheroxyl radical attenuating ability; XH, co-antioxidant.

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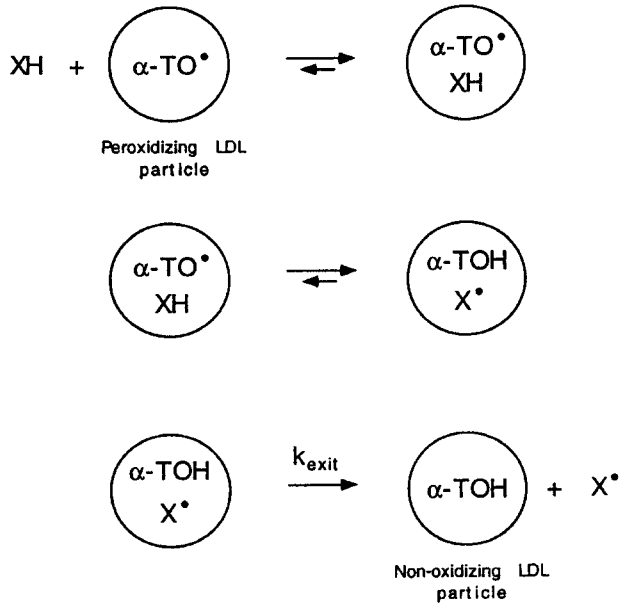
Upston, and R. Stocker, unpublished results). Furthermore, under such mild oxidizing conditions, substantial portions of LDL lipids are oxidized in the presence of α-tocopherol (α-TOH, the most abundant lipid-soluble antioxidant in LDL extracts) (9–12). As a result of detailed investigations on the molecular mechanism(s) of LDL lipid peroxidation and antioxidation, and the role α-TOH plays in this, we have proposed tocopherol-mediated peroxidation (TMP) as a novel model to explain these and many other anomalies of radical-mediated LDL lipid peroxidation and antioxidation (9, 11, 13).

In the TMP model, α-TOH acts as a pro-oxidant for LDL due to its ability to draw active oxygen radicals from the aqueous phase into the lipoprotein particle and the ability of the resulting α-tocopheroxyl radicals (α-TO•) to initiate LDL lipid peroxidation via reaction with bis-allylic methylene groups of polyunsaturated fatty acids. Examining selected natural and synthetic antioxidants for their ability to inhibit the early stages of LDL peroxidation initiated by ROO•, we recently observed (14) that an effective antioxidant (termed co-antioxidant, XH in **Scheme 1**), acts by neither sparing α-TOH nor by competitively scavenging ROO•. Rather, XH attenuates the pro-oxidant activity of α-TO• via its ability to first, reduce the chain propagating α-TO• radical and second, export the radical center from the LDL particle into the aqueous phase, thereby providing anti-TMP activity.

A disadvantage of the 'anti-TMP assay' described in reference 14 is its complexity and work intensity which prevents rapid determination of the LDL antioxidant efficacy of a large number of compounds. As an in vitro screening test for LDL antioxidants is desirable, we decided to develop a simple and rapid method to determine anti-TMP activity as a test for potential in vivo LDL oxidation inhibitors. The method is based on the ability of a putative antioxidant to eliminate α-TO• inside micelles and is assessed directly by electron spin resonance spectroscopy (ESR). The results obtained with this method were in excellent agreement with results obtained from the LDL anti-TMP assay, suggesting that the novel α-TO• attenuating ability (TRAA) test described here may indeed be useful for the screening of in vivo LDL antioxidants.



Scheme 1.

MATERIALS AND METHODS

Reagents

Phosphate buffer (pH 7.4, 50 mM in phosphate) was prepared from Nanopure water and the highest purity reagents commercially available and stored over Chelex-100 (Bio-Rad Laboratories, Richmond, CA) at 4°C for at least 24 h. This treatment effectively removed contaminating transition metals, as verified routinely by the ascorbate autoxidation method (15). Fatty acid-free human serum albumin and all of the putative antioxidants were obtained from Sigma (Sydney, Australia), except 3,5-di-tert-butyl-4-hydroxyanisole, *tert*-butylhydroquinone, 2-aminophenol, 3-aminophenol, *L*-ascorbic, caffeic, and protocatechuic acids, bilirubin and Trolox® which were obtained from Aldrich (Milwaukee, WI), *L*-adrenalin and pyrroloquinoline quinone which were from Fluka (Buchs, Switzerland), and desferrioxamine (desferal) which was obtained from Ciba-Geigy (Basel, Switzerland). α-TOH and ebselen were obtained as gifts from Henkel Corporation (Sydney, Australia) and Dr. Nicolas Hunt (University of Sydney, Australia), respectively. Probucol was a gift from Marion-Merrel Dow Inc. (Cincinnati, OH), while propyl bis(3,5-di-*tert*-butyl-4-hydroxyphenyl)ether (BM15.0639) was generously given to us by Dr. Daniel Steinberg (University of California, San Diego). The compounds H 290/51, H 301/92, H 327/74, H 328/73 (referred to henceforth as *N,N'*-diphenyl-diphenylenediamine, or DPPD), H 330/40 and 43 (the latter also designated as S-12340, Servier, France) as well as 2,6-di-*tert*-amyl-hydroquinone were obtained from Astra-Hässle (Sweden). The α-TOH homologues 2-methyl- (C1OH), 2-hydroxymethyl- (HC1OH), 2-(penten-1-yl)- (C5OH), 2-hexyl- (C6OH), 2-undecyl- (C11OH), 2-tridecyl- (C13OH), and 2-pentadecyl-2,5,7,8-tetramethylchroman-6-ol (C15OH), 2,2',5,7,8-pentamethylchromanol (PMC), 2,2',5,7,8-pentamethylbenzoquinone (PMQ), and the dimers derived from PMC and Trolox were synthesized by Dr. Cacang Suarna (Biochemistry Group, Heart Research Institute). Ubiquinone homologues CoQ_1 and CoQ_{10} were ob-

tained from Mitsubishi (Japan) while α-tocopherol quinone (α-tQ) was obtained from Kodak (Sydney, Australia). The corresponding hydroquinones of CoQ10 ($CoQ_{10}H_2$), α-tQ (α-tQH_2) and PMQ ($PMQH_2$) were prepared by reduction of the respective quinones as described in (16). Ethanolic stock solutions of the hydroquinones were standardized by UV spectroscopy using extinction coefficients of ε_{286} = 4011 ($Q_{10}H_2$) and ε_{285} = 3933 (α-tQH_2) M^{-1} cm^{-1}, respectively, while an estimated extinction value of ε_{288} = 4000 M^{-1} cm^{-1} was used to standardize solutions of $PMQH_2$. All hydroquinone stock solutions were degassed with argon and used within 30 min of preparation. 2,2′-Azobis (2-amidopropane) (AAPH) was purchased from Polysciences (Warrington, PA)[2] and was diluted in freshly prepared phosphate buffer. The nitroxide spin label 2,2′,5,5′-tetramethyl-3-phenyl-4*N*-oxo-3,4-dehydroimidazolin-1-yl oxyl (TPDI) and its corresponding hydroxylamine form 2,2′,5,5′-tetramethyl-3-phenyl-4*N*-hydroxy-3,4-dehydroimidazolin-1-yl oxyl (TPDHI) were received in crystallized forms as gifts from Dr. Vitaly Roginsky, (Russian Academy of Sciences, Moscow) and were used without further purification.

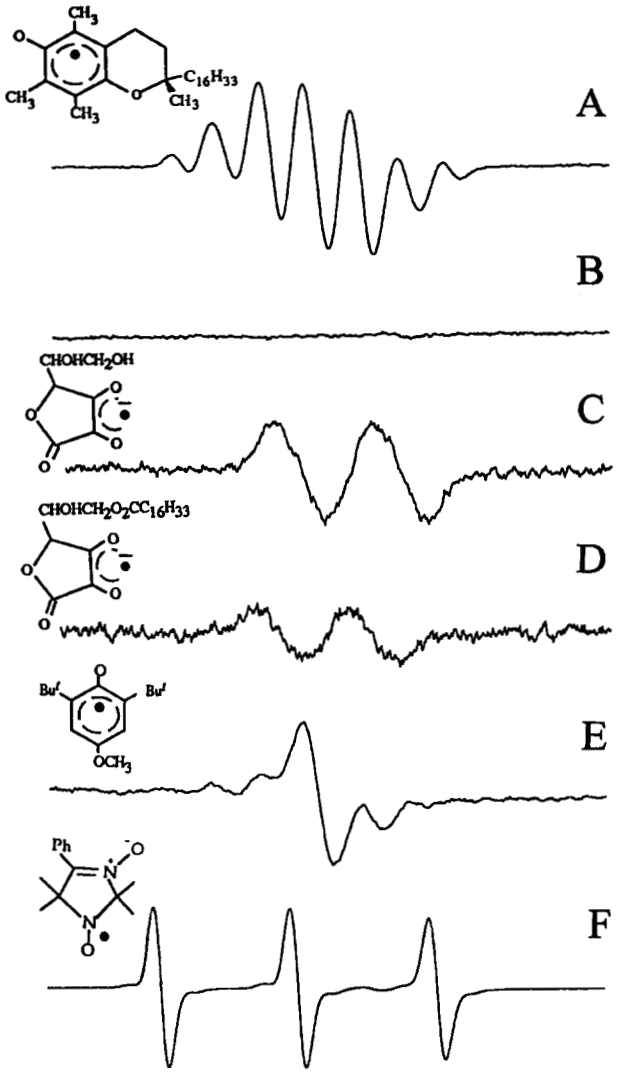


Fig. 1. ESR spectra obtained from UV-irradiation of 500 μM α-TOH dispersed in 100 mM SDS micelles at 37°C to give (A) α-TO•. (B) Identical region of field after addition of 10 μM 3-hydroxyanthranilic acid to a solution containing α-TO• as an example of immediate decay of α-TO•. Residual signals obtained after addition of 10 μM of either AH^- (C), APH^- (D), DBHA (E), and TPDHI (F) (refer to Table 1 for list of abbreviations), respectively, to α-TO•-containing micelles. ESR spectra were measured at 9.41 GHz with modulation amplitude 1.0 G and modulation frequency 12.5 kHz. In all cases the total field sweep represents 60 G, except in C and D where the field sweep was set to 10 G. ESR signals represent the average of three successive accumulations with sweep time of 20.5 sec.

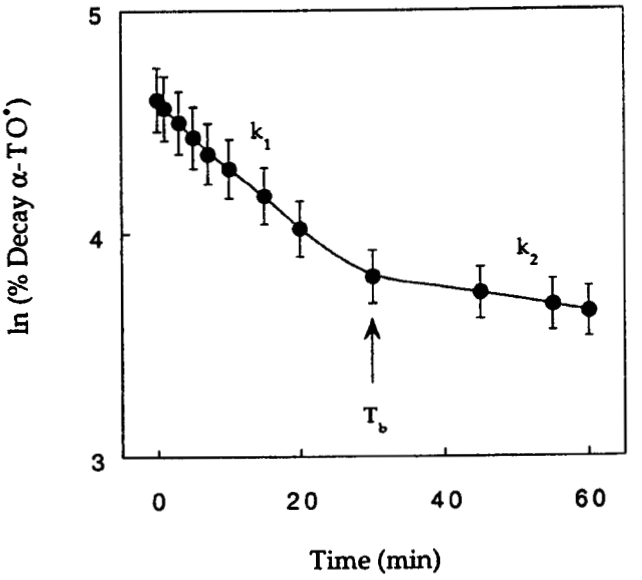


Fig. 2. First order decay treatment of the time-dependent decay of α-TO• radicals measured by ESR spectroscopy at 37°C in 100 mM HTAC micelles in the absence of added co-antioxidants. Results shown are the mean ± SD of three separate experiments. Values k_1 and k_2 represent the observed rate constants for the biphasic decay, while T_b represents the break point time between the two phases of decay. ESR parameters are as for Fig. 1.

Preparation of micelles

One hundred mM stock solutions of cetyltrimethyl ammonium chloride (HTAC) and sodium dodecyl sulfate (SDS, both obtained from Aldrich) were prepared in phosphate buffer principally as described in (17). Micellular dispersions of α-TOH were prepared by diluting an ethanolic solution of α-TOH (0.2 M) into such micelles (17) at a final vitamin concentration of 500 μM. This resulting solution was sonicated for 15 sec at which time it was completely homogeneous.

[2]We are aware that Polyscience no longer sells AAPH, though the azo-initiator can be obtained, e.g., from Wako Pure Chemicals, Osaka, Japan or ACROS Chemica supplier for Fisher Scientific.

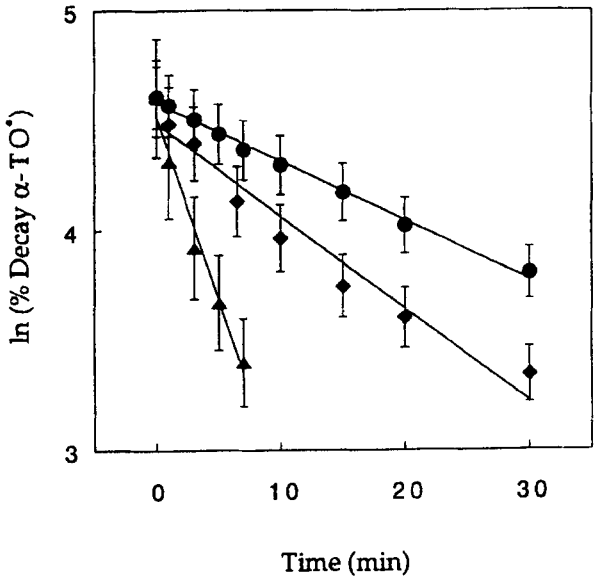


Fig. 3. First order decay treatment of the time-dependent decay of α-TO• radicals measured by ESR spectroscopy at 37°C. Results shown are the mean ± SD of three separate experiments and symbols correspond to (▲) 5 mM, (◆) 1 mM, and (●) 0.5 mM α-TOH each dispersed in 100 mM HTAC micelles. ESR parameters are as for Fig. 1.

Isolation of LDL

LDL was isolated by ultracentrifugation (18) of freshly heparinized plasma obtained from a non-fasted healthy male donor (27 years of age). LDL was obtained by direct aspiration and stored at 4°C for 16 h before use, resulting in the loss of most of the endogenous ubiquinol-10. Immediately prior to use, excess KBr and remaining low molecular weight water-soluble antioxidants were removed from the LDL by gel filtration chromatography, using a PD-10 column (Pharmacia, Uppsala, Sweden) and the concentration of LDL was determined as described in (19).

Determination of antioxidant efficiency by the anti-TMP test

The anti-TMP test has been described recently in detail (14). Briefly, ascorbate and ubiquinol-10-free LDL (1 μM in apoB) was supplemented with an aliquot of a stock solution of either the lipophilic (dissolved in ethanol) or water-soluble antioxidant to be tested; the mixture was incubated at 37°C for 10 min and subsequently oxidized at 37°C with a low flux of water-soluble ROO• generated from AAPH (4 mM). The time-dependent consumption of LDL α-TOH and accumulation of cholesteryllinoleate hydroperoxide (Ch18:2-OOH) were monitored by HPLC with electrochemical and post-column chemiluminescence detection, respectively (18). The effectiveness of an antioxidant, assigned as its anti-TMP index, was defined (14) as the relative amounts of Ch18:2-OOH formed with versus without the added antioxidant measured after 20% consumption of the endogenous α-TOH in the control sample (i.e., in the absence of the antioxidant). Anti-TMP indices were expressed as the percentage of the lipid peroxidation in the control. Where possible, values were taken from our previous study (14); for compounds not tested previously, anti-TMP indices were determined in the current study as mean values (± SD) from three separate experiments. Butylated hydroxytoluene (BHT) was used concurrently as an internal control for each batch of LDL and a given series of compounds, and anti-TMP indices were deemed valid only when the corresponding anti-TMP index for BHT (x) was in the range 8% < x < 12% (see ref. 14).

Generation and detection of α-tocopheroxyl radicals (α-TO•)

Aliquots of the α-TOH-containing micelles were placed into the neck of an ESR flat cell (100 μL, Wilmad Glass Co., Buena, NJ) and placed 0.5 m from a 125 W Osram HQL-Mercury fluorescent bulb (GEC distributors, Roseberry, Sydney) used as a UV light source. To increase the light intensity, the frosted casing of the bulb was removed. Samples were irradiated for 3 min, followed by thorough mixing and subsequent transfer of the flat cell to the corresponding temperature-controlled Dewar insert (Wilmad) in the ESR cavity, where the sample was allowed to equilibrate to 37°C. This procedure afforded α-TO• between 1 and 2 μM as estimated against a 10 μM TPDI nitroxide standard. Unless specified otherwise, ESR spectra were obtained at 9.41 GHz

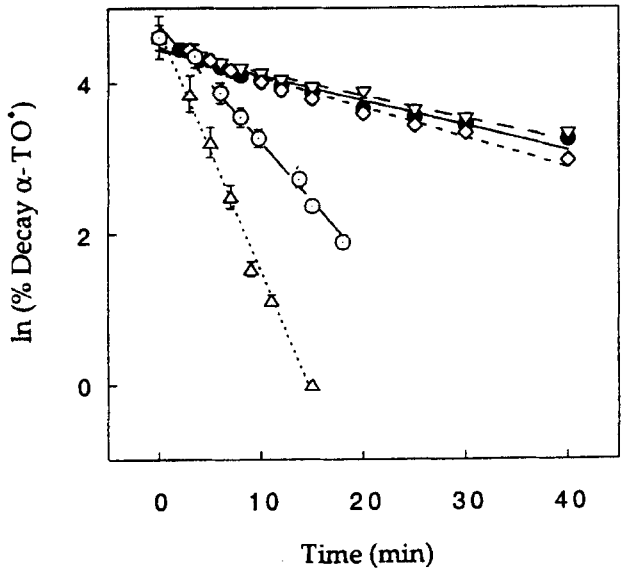


Fig. 4. First order decay treatment of the time-dependent decay of α-TO• radicals dispersed in 100 mM HTAC micelles, measured by ESR spectroscopy at 37°C. Results shown are the mean ± SD of three separate experiments and symbols correspond to (●) control decay in the absence of added co-antioxidant and 10 μM addition of (△) bilirubin, (open circle dot) ebselen, (▽) 2-hydroxyl salicylic acid (2-OH), and (◇) asprin. Where error bars are not visible the symbol is larger than the SD measured. ESR parameters are as for Fig. 1.

TABLE 1. Anti-TMP indices and corresponding TRA

	Compound	Ant-TMP Index(%)[a]	TRAA[a,b] HTAC	TRAA[a,b] SDS
1	Butylated hydroxytoluene (BHT)	10 (2)[c]	ID	ID to ≤ 7% I(0)
2	Butylated hydroxyanisole	0.2 (0.2)[c]	ID	ID
3	3,5-Di-*tert*-butylhydroxyanisole (DBHA)	8.1 (0.3)[c]	ID	ID
4	Estradiol	103 (6)[c]	1.04 (0.02)	1.04 (0.04)
5	Probucol	98 (0.5)[c]	0.92 (0.01)	0.76 (0.01)
6	2,2′,5,7,8-Pentamethylchroman-6-ol dimer	0.9 (1.6)[d]	ID	ID
7	Trolox dimer	0 (1.1)[d]	ID	ID
8	Methyl 2-hydroxybenzoate	100 (8)[d]	0.97 (0.02)	1.06 (0.02)
9	2-Hydroxybenzoic acid	91.5 (2.6)[d]	1.02 (0.03)	1.16 (0.02)
10	4-Hydroxybenzoic acid	84 (3.5)[d]	1.0 (0.05)	1.22 (0.04)
11	2-Aminophenol	0.9 (0.1)[c]	ID	ID to ≤ 5% I(0)
12	3-Aminophenol	111 (3)[d]	1.03 (0.07)	0.97 ± 0.03
13	4-Aminophenol	0.43 (0.4)[d]	ID	ID to ≤ 5% I(0)
14	3-Hydroxyanthranilic acid (3HAA)	1.1 (0.2)[d]	ID	ID to ≤ 18% I(0)
15	Anthranilic acid	100 (0)[d]	1.07 (0.20)	1.11 (0.30)
16	*E*-3,4′,5-Trihydroxystilbene	123 (3.9)[d]	1.0 (0.12)	0.97 (0.2)
17	1,2-Benzisoselenazol-3(2H)-one (ebselen)	0.08 (0)[d]	6.21 (0.07)	5.98 (0.02)
18	*Tert*-butylhydroquinone	0.1 (0.1)[c]	ID	ID
19	2,6-Di-*tert*-butylhydroquinone	0 (0)[d]	ID	ID
20	2,5-Di-*tert*-butylhydroquinone	0 (0)[d]	ID	ID
21	2,5-Di-*tert*-amylhydroquinone	0.26 (0.1)[d]	ID	ID
22	α-Tocopherol hydroquinone (α-tQH_2)	0 (0)[d]	ID	ID
23	2,2′,5,7,8-Pentamethylhydroquinone ($PMQH_2$)	0 (0.5)[d]	ID	ID
24	Ubiquinol-10	< 5[e]	ID	ID
25	Adrenalin	0.8 (0.4)[c]	ID	ID
26	2-Hydroxyestradiol	1.9 (0.5)[c]	ID	ID to ≤ 8% I(0)
27	Caffeic acid	0 (1.9)[d]	ID	3.97 (0.18)
28	Protocatechuic acid	1.4 (1.28)[d]	ID	2.6 (0.10)
29	*L*-Ascorbate (AH^-)	0.1 (0.1)[c]	ID	ID[f]
30	Ascorbyl palmitate (APH^-)	0.2 (0.2)[d]	ID	ID[g]
31	Bilirubin (BR)	3.5 (0.1)[c]	12.75 (0.4)	ID to 20% I(0)
32	Human serum albumin-bilirubin (HSA-BR)	40 (2)[c]	2.8 (0.1)	0.95 (0.02)
33	2,2′,5,5′-Tetramethyl-3-phenyl-4*N*-hydroxy-3,4-dehydroimidazolin-1-yl oxyl (TPDHI)	30.2 (0.1)[c]	ID	ID[h]
34	Hydroxylamine	99 (6)[c]	ID to 20% I(0)[i]	0.97 (0.02)
35	Desferrioxamine	97 (1)[c]	1.16 (0.02)	0.98 (0.03)
36	Pyroloquinoline quinone	121 (6)[c]	0.99 (0.08)	1.01 (0.05)
37	Ubiquinone-1	100 (0)[d]	1.01 (0.07)	0.95 (0.04)
38	Ubiquinone-10	103 (0)[d]	10.98 (0.09)	0.92 (0.07)
39	H 290/51	4.3[d]	ID	ID
40	H 301/92	132 (6)[d]	0.81 (0.12)	1.03 (0.05)
41	H 327/74	76 (1.3)[d]	1.15 (0.06)	1.04 (0.04)
42	Diphenylphenylenediamine (DPPD)	3.4[d]	ID	ID
43	H 330/40	80.8 (2.1)[d]	1.08 (0.04)	1.07 (0.03)
44	H 330/43	3.4[d]	ID	ID
45	Propyl bis(3,5-di-*tert*-butyl-4-hydroxyphenyl)ether (BM15.0639)	0.06 (0)[d]	ID	ID

46	Trolox	1.0 (0.1)[c]	ID	ID
47	2-Hydroxymethyl-2′,5,7,8-pentamethylchroman-6-ol (HC1OH)	0.9 (0.2)[c]	ID	ID
48	2,2′,5,7,8-Pentamethylchroman-6-ol (PMC)	4.6 (0.2)[c]	ID	ID
49	2-(Penten-1-yl)-2′,5,7,8-pentamethylchroman-6-ol (C5OH)	15.7 (0.7)[c]	ID to 23% I(0)	ID to 20% I(0)
50	2-Hexyl-2′,5,7,8-pentamethylchroman-6-ol (C6OH)ITC-NewBaskerville"	19.1 (0.8)[c]	ID to 12% I(0)	ID to 26% I(0)
51	2-Undecyl-2′,5,7,8-pentamethylchroman-6-ol (C11OH)	66.4 (11)[c]	0.99 (0.09)	1.23 (0.01)
52	2-Tridecyl-2′,5,7,8-pentamethylchroman-6-ol (C13OH)	100 (1)[c]	0.98 (0.10)	1.06 (0.06)
53	2-Pentadecyl-2′,5,7,8-pentamethylchroman-6-ol (C15OH)	107 (4)[c]	1.02 (0.06)	0.99 (0.03)

Compound abbreviations list: Butylated hydroxytoluene (BHT); 2,6-di-*tert*-butyl-hydroxyanisole (DBHA); 3-hydroxy-anthranilic acid (3HAA); 1,2-benzisoselenazol-3(2H)-one (ebselen); α-tocopherol hydroquinone (α-tQH$_2$); 2,2′,5,7,8-pentamethyl-hydroquinone (PMQH$_2$); *L*-ascorbate (AH$^-$); ascobyl palmitate (APH$^-$); bilirubin (BR); human serum albumin-bilirubin (HSA-BR); 2,2′,5,5′-tetramethyl-3-phenyl-4*N*-hydroxy-3,4-dehydroimidazolin-1-yl oxyl (TPDHI); 2,2′,5,5′-tetramethyl-3-phenyl-4*N*-oxo-3,4-dehydroimidazolin-1-yl oxyl (TDPI); N,N′-diphenylphenylenediamine (DPPD); propyl bis(3,5-di-*tert*-butyl-4-hydroxyphenyl)ether (BM15.0639); 2-hydroxymethyl-2′,5,7,8-pentamethylchroman-6-ol (HC1OH); 2,2′,5,7,8-pentamethylchroman -6-ol (PMC); 2-(penten-1-yl)-2′,5,7,8-pentamethylchroman-6-ol (C5OH); 2-hexyl-2′,5,7,8-pentamethylchroman-6-ol (C6OH); 2-undecyl-2′,5,7,8-pentamethylchroman-6-ol (C11OH); 2-tridecyl-2′,5,7,8-pentamethylchroman-6-ol (C13OH); and 2-pentadecyl-2′,5,7,8-pentamethylchroman-6-ol (C15OH).

[a]Results presented are the mean (± SD) of triplicate experiments, immediate decay (ID) is defined as decay to < 20% initial intensity I(0) on addition of the test compound, while compound structures are listed in Appendix.

[b]Relative ratio of observed rate constants $k_{(+)}/k_{(-)}$ (± SD).

[c]Value obtained from reference (14).

[d]Value obtained from current study.

[e]Anti-TMP index estimated from LDL enriched with ubiquinol-10 (final concentration 5.02 μM), by in vivo enrichment with ubiquinone-10 as described in (32), and subsequently oxidized with 2 mM AAPH. Addition of ubiquinol-10 to LDL in vitro by the method outlined in (14) only increased ubiquinone content due to the rapid oxidation of the quinol on addition to LDL.

[f]A^H 2.26 ± 0.20 and 1.74 ± 0.2 G in HTAC and SDS micelles, respectively.

[g]A^H 1.78 ± 0.20 and 1.65 ± 0.2 G in HTAC and SDS micelles, respectively.

[h]A^N 15.2 ± 0.2 and 15.1 ± 0.2 G in HTAC and SDS micelles, respectively.

[i]Subsequent to initial rapid decay, α-TO• regenerated to 40–45% I(0).

with modulation amplitude 1.0 G, microwave power 20 mW, and modulation frequency 12.5 kHz using a Bruker ESP 300 ESR spectrometer fitted with an X-band cavity. Temperature control was obtained using a Bruker Temperature Control Unit and temperatures were accurate to ± 0.5°C.

Determination of α-TO• attenuating ability (TRAA)

After accumulation of the T = 0 min spectrum, the flat cell arrangement containing α-TO• was removed from the ESR cavity and the solution was gently coaxed into the neck of the flat cell under positive pressure. The compound of choice (or the appropriate volume of water or ethanol for the controls) was then added to give a final concentration of 10 μM and the treated sample was replaced in the cavity and allowed to equilibrate to standard conditions, and sampling was resumed. Preliminary experiments showed that this method of addition, which normally required 3 min in total, did not affect the rate of decay of α-TO• as verified by identical decays in control samples treated in identical fashion with or without an appropriate volume ethanol. The time-dependent decay of ESR signal intensity for α-TO• was measured both in the presence and absence of the added co-antioxidant (10 μM) using a sweep time of 20.5 sec, averaging the output from three successive sweeps at each time point, and averaging the results of three separate experiments. Control decay curves (i.e., in the absence of added antioxidant) were run periodically between separate experiments and averaged over the sample set to afford observed rate constants in the range $k_1 = 4.62 \pm 0.3 \times 10^{-4}$ sec^{-1}(see below).

Oxidation of antioxidants with ferricyanide

Authentic antioxidant radicals were prepared by treatment of the appropriate antioxidant (10 μM, dispersed in 100 mM HTAC or SDS micelles) with an equimolar concentration of ferricyanide. The radical-containing solutions were transferred to the flat cell arrangement and ESR measurements were obtained as described above. Where additional hyperfine structure was evident, ESR spectra were accumulated with the modulation amplitude set at 0.1 G to improve signal resolution.

RESULTS

Generation and decay of α-TO• in HTAC and SDS micelles

Bisby and Parker (17) reported detectable concentrations of α-TO• generated by nanosecond laser flash

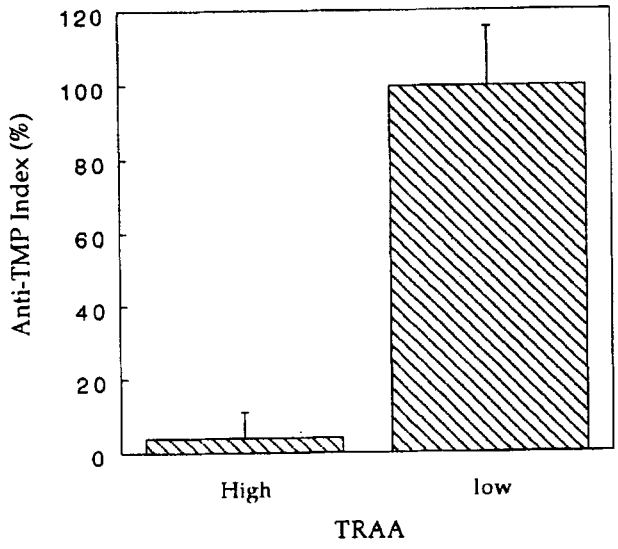


Fig. 5. Illustration of the correlation between TRAA and corresponding anti-TMP indices for a total of 53 synthetic and naturally occurring potential inhibitors of LDL lipid peroxidation. Compounds that show a high ability to attenuate α-TO• show corresponding low anti-TMP values in the range (mean ± SD) 4 ± 7%, while compounds with low TRAA show corresponding anti-TMP indices in the range 99 ± 16%.

photolysis in both HTAC and SDS micellular solutions containing α-TOH. α-TO• formed by this technique were relatively stable, but absorbed only weakly in the regions 420–440 and 600–700 nm. By way of contrast, we utilized a far more sensitive probe, namely ESR spectroscopy, to observe and quantify α-TO• in aqueous micellular solutions. Also, as lasers are expensive and not commonly available, we used an inexpensive and commercially available UV source to generate α-TO•. **Figure 1A** shows that irradiation of a solution of α-TOH solubilized in 100 mM HTAC or SDS micelles with UV light afforded α-TO• at 1–2 μM; these radicals were readily quantified by using ESR spectroscopy.

The decay kinetics of α-TO• generated under these conditions consisted of two phases, a rapid followed by a slower, linear exponential decay, with observed rate constants k_1 and k_2, respectively (**Fig. 2**). This biphasic decay profile is consistent with decay kinetics of α-TO• generated in either phosphatidylcholine liposomes, submitochondrial particles, or rat liver microsomes (20), although no explanation has been offered by these authors. One possible explanation may be that the initial, fast decay is due to bi-molecular termination reactions of radicals within the same micelle particles, whereas the second, slower phase of decay is due to bi-molecular termination of radicals within different micelle particles. The initial rate of decay and the breakpoint time (T_b), distinguishing the two phases of α-TO• decay, were similar in both HTAC and SDS micelles (not shown) indicating that the decay process was independent of the charge of the micelles. However, the rate constant k_1 for the decay of α-TO• (or corresponding half-life, T½ for the initial decay process) decreased as the initial concentration of α-TOH was decreased (**Fig. 3**).

Optimizing the experimental conditions for the initial phase of α-TO• decay, we obtained a working system where the ESR signal decayed exponentially with T½ = 25 ± 2 min and $T_b \approx 30$ min. Importantly, even though the initial concentration of α-TO• obtained in different experiments varied by 100% (i.e., 1–2 μM, see above), the variation in T½ for a given set of experiments was ≤ 8%, as determined by periodic accumulation of standard decay curves (measured in the absence of added antioxidant) over a given series of analyses. Thus, analyses were performed with the optimized system consisting of a 100 mM micellular solution containing 500 μM α-TOH (as described in Materials and Methods).

Screening for antioxidant potency by monitoring TRAA

Irradiation of HTAC or SDS micelles containing 500 μM α-TOH afforded ESR spectra for α-TO• with excellent signal to noise (Fig. 1A). Treatment of such solution with an antioxidant resulted in either: *a)* immediate decay of the ESR signal, defined here as < 20% residual signal by the time the sample was re-introduced into the ESR cavity (e.g., Fig. 1B); *b)* little or no acceleration of the decay of α-TO• compared with the control sample (e.g., **Fig. 4**); or *c)* formation of a new, antioxidant-derived, radical signal (Fig. 1C–F). For *b)*, relative ratios of measured rate constants were expressed as $k_{(+)}/k_{(-)}$, where $k_{(+)}$ and $k_{(-)}$ represented the observed rate constants for the decay of α-TO• in the presence and absence of the added antioxidant, respectively. Where the relative rate value was close to unity ($k_{(+)}/k_{(-)} \approx 1$) the antioxidant showed very low TRAA.

The TRAA and corresponding anti-TMP values obtained for a range of synthetic and natural compounds are shown collectively in **Table 1.** As can be seen, where the compound tested showed a 'high' TRAA, its corresponding anti-TMP activity was most often also high, as indicated by a relatively low anti-TMP index, with mean ± SD of 4 ± 7%. Importantly, this range of anti-TMP indices included all compounds (n = 33) that caused an immediate decay of α-TO• and/or a > 5 times accelerated rate of α-TO• decay measured in one or both of the micellular systems compared with that measured in the absence of the added antioxidant. Compounds with 'low' TRAA (i.e., compounds with $k_{(+)}/k_{(-)} < 5$ for each micellular system, n = 19), in general, also showed low anti-TMP activity (i.e., anti-TMP indices in the range 99 ± 16%). This resulted in the majority of compounds falling into either of two categories, i.e., compounds with high or low TRAA (**Fig. 5**). The relationship be-

tween the two indices corresponded to > 98% success in predicting the antioxidant potency of a potential inhibitor of LDL lipid peroxidation, based upon its TRAA alone and using the arbitrary value $k_{(+)}/k_{(-)} > 5$ as the marker for 'high' activity. Furthermore, the correlation was highly significant ($P < 0.00005$, Rank test). The only compound tested that showed a relatively poor correlation (i.e., albumin-bound bilirubin) exhibited moderate anti-TMP activity (i.e., 40%).

Interestingly, probucol and its low molecular weight analogue H 327/74 and the probucol-derived synthetic compound H 330/40 were all poor inhibitors of LDL lipid peroxidation, as judged from our in vitro model results, and showed corresponding low TRAA ($k_{(+)}/k_{(-)} \approx 1$). In contrast, H 330/43 (also designated S-12340, Servier, France) containing a carbamate moiety was highly efficient in both test systems. A number of the synthetic derivatives, namely H 290/51 and H 328/73, phenols, catechols, and hydroquinones, inhibited LDL lipid peroxidation significantly in our in vitro model and also showed corresponding high TRAA (see Table 1).

Addition of hydroxylamine to α-TO• in the positively charged HTAC micelles resulted in initially significant decay of α-TO• with subsequent regeneration of the radical signal over 30 min. This regeneration of α-TO• presumably resulted from the reversible reaction of an unstable nitroxide radical with α-TOH. However, at no stage was a putative nitroxide species detected by ESR. In contrast, addition of hydroxylamine to α-TO• in the negatively charged SDS micelles afforded no acceleration of decay, consistent with its high anti-TMP index.

In all cases where the introduction of an antioxidant to a solution of α-TO• resulted in the immediate decay of the ESR signal, no other free radical signal was observed in the region of the ESR spectrum with g value ≈ 2.004, except for the case of ascorbate (AH^-), ascorbyl palmitate (APH^-), the hydroxylamine TPDHI, 3,5-di-*tert*-butylhydroxyanisole (DBHA) or BM15.0639 (Fig. 1C–F and **Fig. 6A,** respectively). More specifically, the new radicals formed corresponded to the one-electron oxidation products of the respective co-antioxidants, i.e., X•, as confirmed by separate experiments where the authentic radicals were generated using ferricyanide as a mild one-electron oxidant. For example, the phenoxyl radical generated from BM 15.0639 by ferricyanide oxidation (Fig. 6B) is clearly identical with that obtained from the corresponding interaction with α-TO• (Fig. 6A). In addition, for this compound assignment of the radical structure was confirmed by digital simulation (Bruker ESP 300 Simulation Software) of the ESR spectrum (not shown), which indicated that the delocalized radical showed hyperfine couplings of 1.835 G (n = 2 H atoms) and 0.940 G (n = 2 H atoms). Investigations of the broad singlet response corresponding to DBHA• at lower modulation amplitude (0.1 G) again resolved hyperfine couplings consistent with a delocalized system, however, this was not pursued. The hyperfine couplings (A^H) determined for ascorbyl and ascorbyl palmitate radicals (Table 1) were in good agreement with literature values (21, 22). The hyperfine coupling value for the residual triplet signal generated by treatment of α-TO• with the hydroxylamine TPDHI (Fig. 1F, $A^N = 15.2 \pm 0.2$) also corresponded with the coupling value of authentic TPDI (not shown).

DISCUSSION

There are several methods used to assess the ability of compounds to inhibit LDL oxidation in vitro (14, 18, 23, 24) (for a review see ref. 25). All these methods require human blood as a source of LDL and more or less sophisticated analytical methods, making them potentially hazardous, expensive, and time consuming, and therefore not suitable for the rapid testing of large numbers of compounds as LDL antioxidants. The present test for the first time describes a simple and rapid in vitro test for LDL antioxidants, which act as co-antioxidants for α-TOH, that does not require blood or other biological material. The test is based on the ability of a putative antioxidant to quench α-TO• in micelles and predicts with 98% probability whether a compound can efficiently inhibit the initiation of peroxyl radical-mediated LDL lipid peroxidation, as verified by testing 53 different compounds. An additional feature of the assay is that it most often clearly distinguishes reactive from unreactive compounds. Together, these features

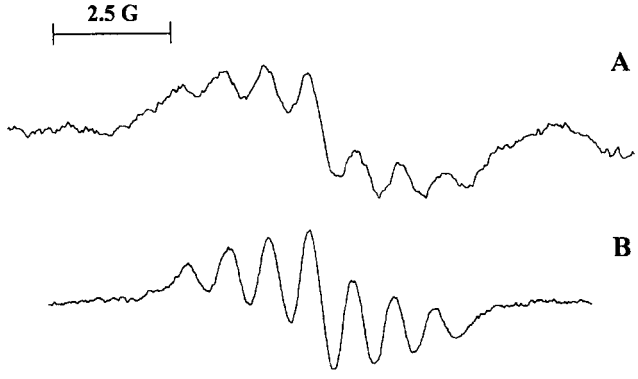


Fig. 6. ESR spectra for radicals generated on the addition of (A) 10 μM BM 15.0639 to α-TO• radicals in 100 mM HTAC micelles, and (B) authentic BM 15.0639 phenoxyl radicals generated on treatment of 10 μM BM 15.0639 with equimolar amounts of ferricyanide in 100 mM HTAC micelles. ESR parameters are as for Fig. 1, except that modulation amplitude was set to 0.1 G.

TABLE 2. Advantages and disadvantages of TRAA test

Advantages	Disadvantages
Simplicity	Requirement for ESR machine (expense)
Rapidly, allowing testing of large number of potential LDL antioxidants	Qualitative data obtained only; quantitative data requires modification of procedure
Reproducibility	
High selectivity, screening active from inactive compounds	Active compounds identified require additional in vitro and in vivo testing
High probability that TRAA reflects anti-TMP activity	Lack of direct link between TRAA and LDL lipid peroxidation inhibitory activity
Lack of requirement for biological materials	
Suitability for hydro- and lipophilic compounds	Lack of consideration for potential in vivo metabolites of test compound with TRAA

make our simple test suitable for an initial screening of a large number of compounds as potential LDL antioxidants.

Pryor and coworkers (26) recently described a simple general test for antioxidants. The method used peroxidation of linoleic acid dispersed in SDS or HTAC micelles where the antioxidant efficacy of compounds was determined largely by their ability to scavenge lipid peroxyl radicals. In this system, negatively charged antioxidants such as AH^-, other 2-hydroxytetronic acids, and Trolox were less effective in negatively charged SDS than positively charged HTAC micelles, whereas the micellular charge did not affect neutral inhibitors (26). In contrast to these findings, our results indicate that the ability of compounds to react with α-TO• in SDS or HTAC micelles was largely independent of the micellular charge, as all neutral and most charged compounds (e.g., AH^-, APH^-, 2- and 4-hydroxybenzoic acid, 3-HAA and Trolox) showed effectively the same TRAA, independent of which micelles were used. Possible exceptions to this were NH_2OH, bilirubin, caffeic and protocatechueic acids.

In addition to the advantages referred to above, and like any other method, the current screening test has a number of disadvantages (summarized in **Table 2**). One limitation of the present test is the requirement for access to an ESR spectroscopic facility, though these facilities are generally available in chemistry departments of most large universities. Attempts to use UV or fluorometric instead of ESR detection for α-TO• failed due to the inherent sensitivity limitations of the former two detection methods. The method also distinguishes between two groups of compounds only (Fig. 5). This potential limitation could possibly be overcome by lowering the decay rates of α-TO• by performing the ESR measurements at lower temperatures and by titrating the co-antioxidant such that accurate rate constants for their reaction with α-TO• may be obtained. In doing so, however, the system becomes more work intensive and may also become more vulnerable to the micellular charge effects described above, thereby reducing its suitability as a rapid screening test for potential LDL antioxidant. Another potential disadvantage of our method, as for most in vitro methods, is that it does not test for the possibility that a metabolite of a compound may be the biologically active antioxidant (see also below).

While predicting with very high probability, the present screening test does not demonstrate that a compound with high TRAA activity is a good anti-TMP agent; to verify the latter, additional in vitro LDL oxidation/antioxidation test(s) are required. In addition to these in vitro tests, in vivo tests, such as the ability of a compound to enter the blood stream and associate with LDL, are ultimately required to ascertain the antiatherogenic activity of an anti-TMP agent.

The current study extends our previous work on the prevention of TMP in ubiquinol-10-free human LDL (14). From the results shown (Table 1) it is clear that among the phenolic antioxidants tested, those compounds with high peroxyl radical scavenging activity are also efficient in quenching α-TO• in the micellular systems. This is consistent with studies showing that compounds with high ROO• scavenging activity also show high reactivities toward phenoxyl radicals (27, 28). From the data presented in Table 1 several observations are novel and worthy of further discussion.

Hydroquinones generally are potent co-antioxidants as measured in each in vitro test system. In particular, α-tocopherol hydroquinone, which may be produced in vivo from α-tocopherol quinone (29) which itself is an oxidation product of α-TOH, is an extremely potent inhibitor of LDL lipid peroxidation and warrants further study. Also, the results (Table 1) show that ebselen is capable of reacting with α-TO•, indicating that this drug may spare α-TOH during peroxyl radical-mediated oxidation of biological lipids in addition to its well-established glutathione peroxidase-like activity. In addition to α-tocopherol hydroquinone and the previously described AH^- (9, 30), ubiquinol-10 (31, 32), BR (33, 34), 3-HAA, and adrenalin (14), the dietary catechols caffeic and protocatechuic acid are shown here to be natural co-antioxidants for LDL α-TOH and hence are of potential physiological importance. The ability of the latter two to efficiently inhibit LDL lipid peroxidation is in agreement with a previous report by others (35). From these studies it appears that biological systems are endowed with an array of co-antioxidants implying that in

most in vivo situations α-TO• is unlikely to act as a lipid peroxidation chain-transfer agent (9) for LDL. This does not necessarily mean, however, that the phase transfer activity of LDL α-TOH that causes formation of α-TO• (9) is unimportant in vivo.

The physiological relevance of results obtained with the present test system is largely dependent on the relative contribution of the phase- and chain-transfer activity of LDL α-TOH to the putative in vivo oxidation of the lipoprotein. As pointed out earlier, there is increasing evidence that these two TMP-determining activities of α-TOH are important for many different types of radical-mediated LDL oxidation. The relative importance of TMP as a mechanism of LDL oxidation increases with the reduction of the reactivity of the radical oxidants and radical flux to which LDL is exposed; conversely, it decreases with greater reactivity of the radical oxidant and increased radical flux conditions. As we presently do not know to what fluxes and kinds of radical oxidants LDL becomes exposed in the subendothelial space, we cannot directly assess the relevance of our test systems to the screening of in vivo LDL antioxidants. What is possible is to compare results obtained with the present tests and the three compounds that have been tested for anti-atherogenic capacity in different animal models of the disease, i.e., probucol (2–4, 8, 35–37), *N,N′*-diphenyl-phenylenediamine (DPPD) (5), and BM 15.0639 (6).

Probucol and other synthetic derivatives with a sulfur atom in a *para* position to the phenoxyl group were very poor inhibitors of LDL lipid peroxidation in our in vitro anti-TMP test system and exhibited corresponding poor TRAA (Table 1). Also, and in sharp contrast to the highly effective 2,6-di-*tert*-butyl hydroquinone, its sulfur analog H 327/74 was almost totally ineffective in each of our in vitro tests. Together, these findings suggest that a sulfur atom in a *para* position to the phenoxyl group of a compound lowers its activity in the in vitro tests used here. The only compound that acted contrary to this was H 330/43, suggesting that the antioxidant activity may be due to the carbamate moiety itself or perhaps the activation of the phenoxyl group by the nitrogen-containing heterocycle. The discrepancy between our findings and the known in vivo anti-atherogenic activity of probucol in most though not all (38) animal studies on atherosclerosis suggests that probucol itself most likely owes its in vivo anti-atherogenic activity to some mechanism other than co-antioxidation of α-TO•. As probucol has been shown to inhibit LDL oxidation (39–43) under more severe oxidizing conditions than those used here, the in vivo oxidizing conditions may also be severe. Alternatively, probucol may act as a prodrug where its effectiveness as an anti-atherogenic compound is dependent on the in vivo formation of metabolites (40) that are more potent antioxidants. Finally, the anti-atherogenic activity of probucol may be independent of LDL antioxidation (36, 44–46). We conclude that as the precise molecular action of probucol in vivo remains unknown, the suitability of our method cannot be dismissed on the basis of the failure of the drug to show TRAA. In contrast, our finding that DPPD has high TRAA supports the possible importance of anti-TMP activity and hence our assay for in vivo LDL antioxidants and anti-atherogenic activity.

In support of a previous report (6), we also observed that the structural analog of probucol, propyl bis(3,5-di-*tert*-butyl-4-hydroxyphenyl)ether (BM15.0639) is a most efficient in vitro LDL antioxidant, as judged by the anti-TMP assay and the corresponding high TRAA (Table 1). The reasons for the reported absence of anti-atherogenic activity of BM15.0639 are not clear, though its lower ability to both associate with LDL in vivo and increase the 'lag phase' during its Cu^{2+} oxidation in vitro (compared to probucol) may be important. Clearly, the former is a feature not assessed by the screening test described here or by any other in vitro LDL antioxidation method, as discussed above.

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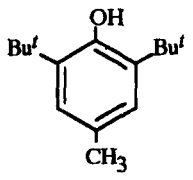
APPENDIX



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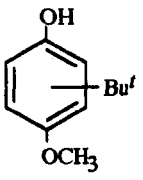

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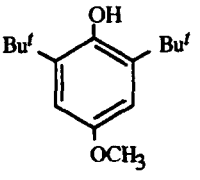

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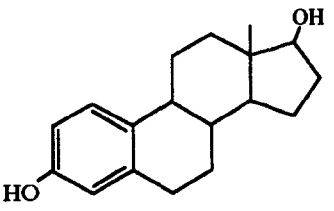

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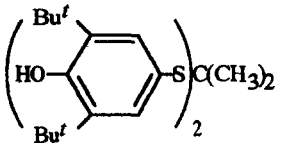

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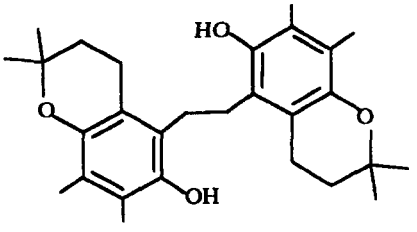

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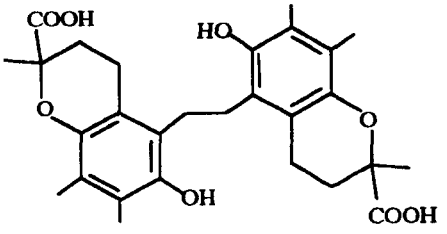

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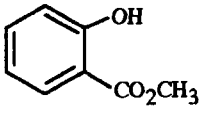

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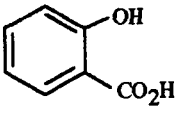

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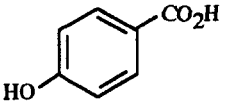

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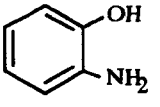

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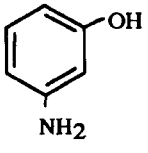

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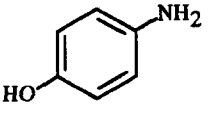

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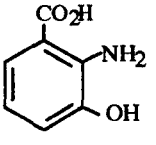

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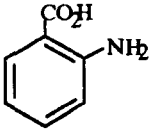

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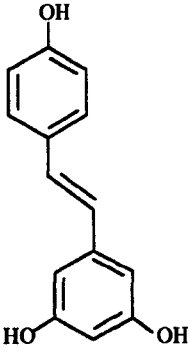

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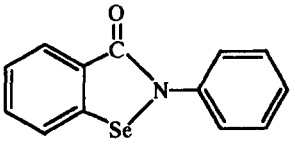

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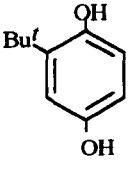

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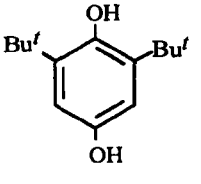

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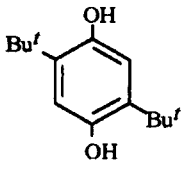

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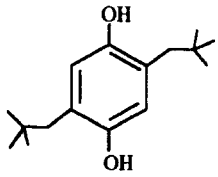

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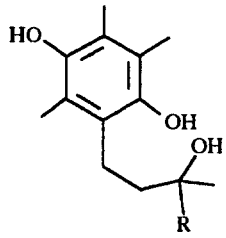

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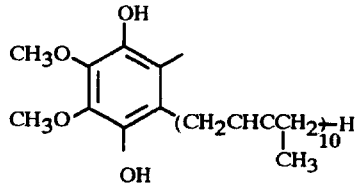

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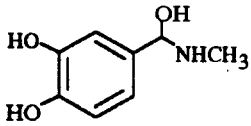

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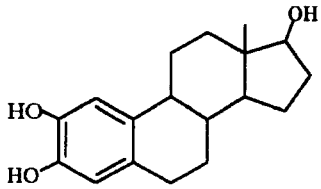

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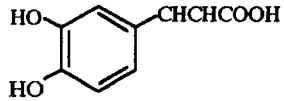

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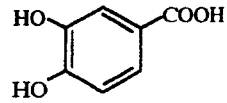

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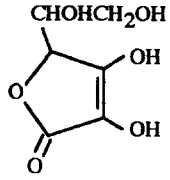

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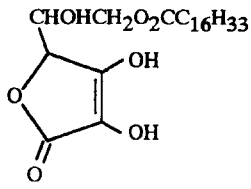

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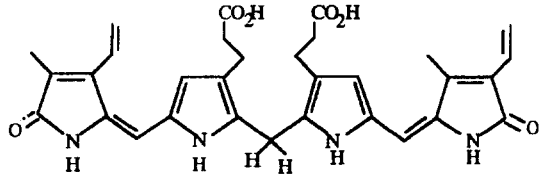
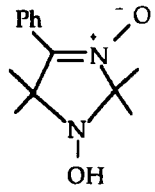


31 BR

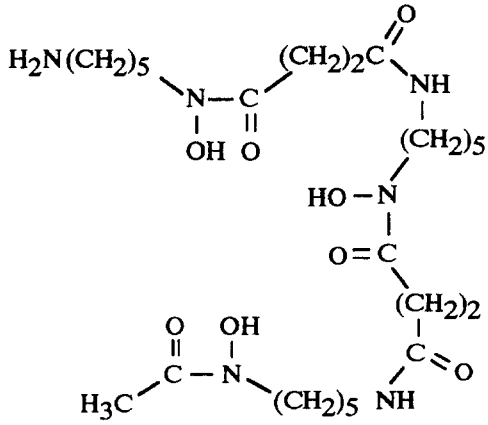
32 HSA - BR



33

NH_2OH

34



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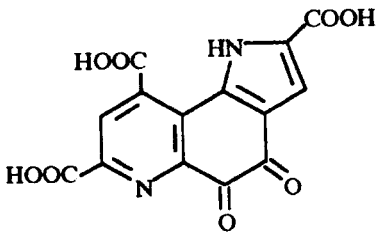

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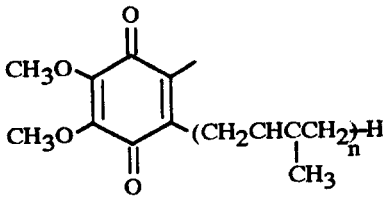
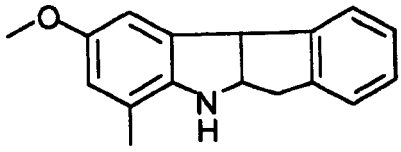


37 (n = 1)
38 (n = 10)



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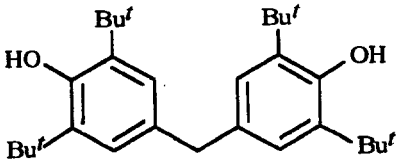

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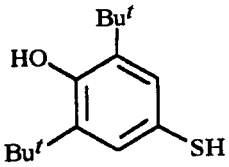
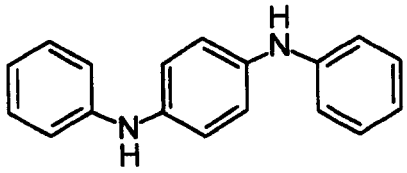


41



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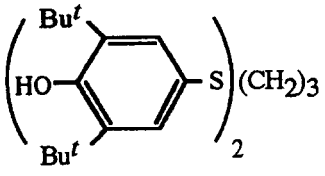

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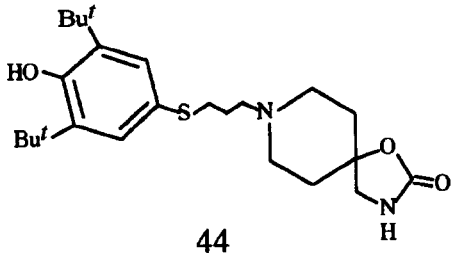

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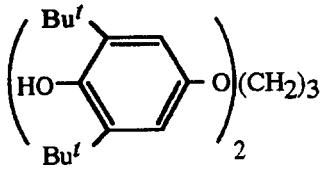

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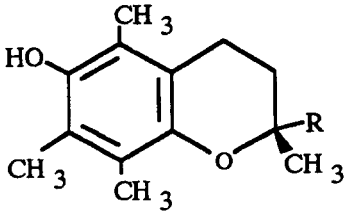

R

46	CO_2H	50	n -C_6H_{13}
47	CH_2OH	51	n -$C_{11}H_{23}$
48	CH_3	52	n -$C_{13}H_{27}$
49	$CH{=}CHC_3H_7$	53	n - $C_{15}H_{31}$

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