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ANTIOXIDANT ACTIVITIES OF DIHYDROLIPOIC ACID AND ITS STRUCTURAL HOMOLOGUES

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The relationships between structure and antioxidant activity of dihydrolipoic acid (DHLA) were studied using homologues of DHLA: bisonor-DHLA (a derivative which lacks two carbons in the hydrophobic tail), tetranor-DHLA (which lacks four carbons) and a methyl ester derivative. It was observed that: i) DHLA homologues with shorter hydrocarbon tails (i.e., bisnor- and tetranor-DHLA) had greater ability to quench superoxide radicals ($O_2^{\cdot-}$); ii) no differences among homologues with different chain lengths were found for peroxyl radical (ROO·) scavenging in aqueous solution, and iii) DHLA was the best membrane antioxidant in terms of ROO· scavening and lipid peroxidation inhibition. Differences among the DHLA homologues in their antioxidant properties in polar and apolar environments generally agreed with differences in their partition coefficients. The methyl ester was the least effective antioxidant both in aqueous phase and in membranes. Tetranor-DHLA was found not only to be less effective in preventing ROO·-induced lipid peroxidation, but also to induce lipid peroxidation in the presence of residual iron. Thus, the complexity of biological systems seems to complicate generalizations on the correlation of molecular structure with antioxidant activity of DHLA.

KEY WORDS: α-lipoic acid, antioxidant, dihydrolipoic acid, oxy-radicals, peroxyl radicals, superoxide radicals, thioctic acid.

INTRODUCTION

Structure-activity relationships are the heart of the understanding of the molecular mechanisms of biochemical processes. The approach of using structure-activity relationships to evaluate or engineer new compounds has led to tremendous success in molecular pharmacology, for example in designing enzyme inhibitors. Such concepts may also be applied to the development of effective biological antioxidants, the importance of which is emerging from increasing evidence of the role of oxygen-derived free radicals in many acute and chronic degenerative diseases.[1] In our laboratory, we have examined the structure-activity relationships of antioxidant homologues with different hydrocarbon chain lengths with regard to their abilities to protect against lipid peroxidation. In previous work, it has been shown that vitamin E, ubiquinol and butylated hydroxytoluene homologues with shorter hydrocarbon chains are better antioxidants against membrane lipid peroxidation, but the shorter chain homologues also have a more pronounced perturbing effect on membrane organization.[2,3]

α-Lipoic acid (thioctic acid) is an essential cofactor, as lipoamide, in the

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decarboxylation of pyruvate by the pyruvate dehydrogenase complex. Its reduced form, dihydrolipoic acid (DHLA), has recently gained attention as an effective antioxidant. It was found that DHLA protected membranes by inhibiting lipid peroxidation.[4,5] We have reported that DHLA also possesses the ability to scavenge superoxide anion radicals (O_2^-) in solution.[6] As is the case for other thiol compounds, DHLA was shown to react with free radicals by means of its sulfhydryl groups.[6] The contribution of other portions of the molecular structure of DHLA to its antioxidant activity, however, has not been well understood.

The catabolic metabolites of α-lipoic acid include a variety of derivatives.[7] Reduction of these compounds forms a variety of structural homologues of DHLA. In the present study, we used these homologues to examine how alterations of the structure of DHLA may influence its antioxidant activities. DHLA, bisnor-DHLA (a derivative which lacks two carbons in the hydrophobic tail), tetranor-DHLA (which lacks four caarbons) and methyl ester derivative were studied with respect to their ability to scavenge superoxide anions in solution and peroxyl radicals (ROO·) in solution and in membranes.

MATERIALS AND METHODS

Synthesis of Dihydrolipoic Acid Homologues

Racemic mixtures of α-lipoic acid, bisnorlipoic acid, tetranorlipoic acid and methyl ester of α-lipoic acid [see Figure 1 for structures] were obtained from ASTA Medica (Frankfurt). Reduced forms were synthesized using sodium borohydride as described as Bonomi and Pagani.[8] Structures of compounds were confirmed and success in the synthetic procedures was verified by analyzing the 1D and 2D COSY ^{1}H NMR spectra. Samples (50 mg) were dissolved in 600 μl chloroform-*d* (Sigma), placed in 5 mm NMR tubes, and spectra were obtained on a Bruker AM300 spectrometer. Concentrations of reduced forms were estimated by measuring the sulfhydryl content using 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB].[9] An extinction coefficient of 13,600 M^{-1} cm^{-1} for *p*-mercaptonitrobenzoic acid at the absorbance of 412 nm was used for calculation.

Partition Coefficient Determination

Partition coefficients for DHLA homologues were determined in an octanol/water system.[10] DHLA homologues (final concentration of 200 μM) were dissolved in a 1:1 (v/v) octanol:HCl (10 mM) mixture, vortexed for 2 hrs at 25°C and centrifuged at 3000 × g for 30 min. concentrations in each phase were determined with a Shimadzu UV 160U spectrophotometer using appropriate reference blanks. Partition coefficients were calculated according to the formula: $P = C_{org}/C_{aq}$, and expressed as log P.

Determination of Second Order Kinetic Rate Constants for Reaction of Homologues with Superoxide Anion Radicals

Apparent second order kinetic rate constants for reactions of DHLA homologues with O_2^- were determined from the competition between DHLA homologues and epinephrine for O_2^-. The oxidation of epinephrine (0.5 mM), by xanthine (0.1 mM)

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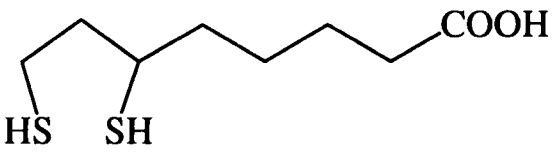
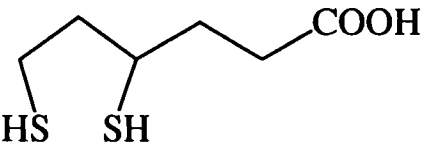
REDUCED FORM OF α-LIPOIC AICD (dihydrolipoic acid, DHLA)



REDUCED FORM OF BISNORLIPOIC ACID



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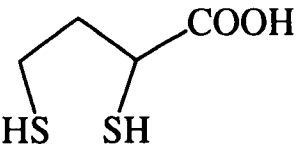

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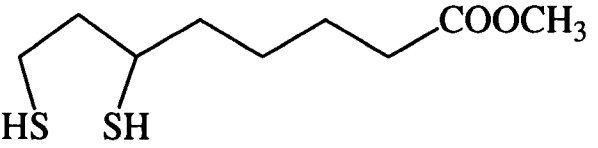

Figure 1 Structure of dihydrolipoic acid homologues.

plus xanthine oxidase (40 mU/ml), was followed by monitoring the increase of adrenochrome product at an absorbance of 480 nm in 100 mM KH_2PO_4-KOH (pH 7.4) at 25°C. The concentrations of DHLA homologues at which 50% of O_2^--induced epinephrine oxidation was inhibited were determined, and the second order kinetic rate constants for reactions between O_2^- and DHLA homologues (k_{DHLA}) were computed according to the equation: $[DHLA]k_{DHLA} = [epinephrine]k_{epinephrine}$, where $k_{epinephrine}$ is the second order kinetic rate constant for the reaction between

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O_2^- and epinephrine (i.e., 4.0×10^4 M^{-1} s^{-1}).[11] The direct effect of DHLA homologues on xanthine oxidase was determined by monitoring the increase in uric acid at 295 nm.[12]

Fluorescence-based Assay for Peroxyl Radical Scavening Activity in the Aqueous Phase and Membranes

The oxidation of B-phycoerythrin (0.5 nM; Sigma) by ROO· generated by 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH; 25 mM; Polysciences Inc.) in 20 mM Tris-HCl (pH 7.4) solution was monitored by following the decay of fluorescence at an excitation wavelength of 540 nm and an emission wavelength of 575 nm at 40°C using a Perkin-Elmer MPF-44A fluorescence spectrophotometer.[13] Oxidation of *cis*-parinaric acid (80 μM; Molecular Probes) by ROO· generated by 2,2'-azobis(2,4-dimethylvaleronitril) (AMVN; 0.3 μM; Polysciences Inc.) in dioleoylphosphatidylcholine liposomes (DOPC; 0.8 mM; Sigma) was monitored by following the decay of fluorescence at an excitation wavelength of 328 nm and an emission wavelength of 415 nm at 40°C.[13] DHLA homologues were incorporated into DOPC liposomes by sonication of DOPC dispersions with homologues in 20 mM Tris-HCl (pH 7.4) solution under nitrogen gas at 4°C. Experimental values are represented as the ratio V/V_A where V and V_A represent the rate of decay of fluorescence without and with antioxidants, respectively.

Determination of Lipid Peroxidation

The effects of DHLA homologues on AMVN-induced lipid peroxidation of rat liver microsomal membranes were determined by monitoring the increase in thiobarbituric acid reaction substances (TBARS).[14] Liver microsomes (1.0 mg protein/ml) were incubated in 10 ml of 0.1 M K,Na-phosphate buffer (pH 7.4) with 0.5 mM DHLA homologues at 42°C in the presence or absence of 50 μM deferoxamine (Sigma). ROO· were generated by the addition of AMVN at final concentration of 10 mM. 1 ml samples were taken from the reaction mixture at 60 min after the addition of AMVN, mixed with 2.0 ml 1:1 (v/v) 30% TCA:0.67% TBA and 150 μl of 0.2 M butylated hydroxytoluene. The samples were heated for 20 min at 100°C and centrifuged for 15 min. The absorbance of the supernatant was determined at 535 nm using a Shimadzu UV160U spectrophotometer. A molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} for TBARS was used for calculation.[15]

RESULTS

Partition coefficients of DHLA homologues in octanol and 10 mM HCl are listed in Table 1. The order of the molecules in terms of hydrophobicity was DHLA = methyl ester derivative > bisnor-DHLA > tetranor-DHLA.

Second order kinetic rate constants for reactions between different DHLA homologues and O_2^- are listed in Table 2. The order of the molecules in terms of their rate constants is bisnor-DHLA > tetranor-DHLA > methyl ester derivative = DHLA. The shorter chain homologues (i.e., bisnor- and tetranor-DHLA) were particularly more reactive to O_2^- than the longer chain length homologues. DHLA and the methyl ester of DHLA, which have the same hydrocarbon chain length, were

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Table 1 Partition coefficients for dihydrolipoic acid homologues

Dihydrolipoic acid homologues	log P
Native molecule	2.41 ± 0.12*
Methyl ester	2.27 ± 0.16*
Bisnor	1.40 ± 0.02
Tetranor	0.82 ± 0.01

Values represent mean ± S.E. of 3 replicates.
*Denote that these values are not significantly different from each other at $P < 0.05$. All other values are significantly different from each other at $P < 0.05$.

Table 2 Second order kinetic constants of dihydrolipoic acid homologues with superoxide radical

Dihydrolipoic acid homologues	kinetic constant ($M^{-1} \cdot s^{-1}$)
Native molecule	$7.30 \pm 0.24 \times 10^5$*
Methyl ester	$7.79 \pm 0.61 \times 10^5$*
Bisnor	$17.7 \pm 2.30 \times 10^5$
Tetranor	$10.9 \pm 0.60 \times 10^5$

Values represent mean ± S.E. of 9 replicates at 3 different concentrations of homologues. pH = 7.4; 25°C.
*Denote that these values are not significantly different from each other at $P < 0.05$. All other values are significantly different from each other at $P < 0.05$.

Table 3 Comparison of peroxyl radical scavening activity of dihydrolipoic acid homologues in aqueous solution and in liposomes

	V/V_A	
Dihydrolipoic acid homologues	aqueous solution	liposomes
Native molecule	4.18 ± 0.33	3.32 ± 0.26**
Methyl ester	3.27 ± 0.06**	2.07 ± 0.20
Bisnor	4.61 ± 0.15	1.85 ± 0.12
Tetranor	4.48 ± 0.38	2.11 ± 0.11

Values represent mean ± S.E. of 3–5 replicates. pH = 7.4; 40°C.
Concentrations of homologues were 1 μM for aqueous solution and 15 μM for liposomes.
V/V_A represents the ratio of rates of fluorescence decay without and with DHLA homologues.
**Denotes that the value is significantly differet from other values at $P < 0.05$.

found to possess very similar kinetic constant values. None of the DHLA homologues inhibited the xathine oxidase activity as determined by monitoring uric acid.

In aqueous solution, all the DHLA homologues exhibited similar scavening capabilities gainst ROO·, except that the methyl ester derivative was significantly less effective than others at $P < 0.05$ (Table 3). In liposomes, DHLA was found to be a significantly better scavenger for ROO· than the shorter homologues or methyl ester derivatives at $P < 0.05$ (Table 3).

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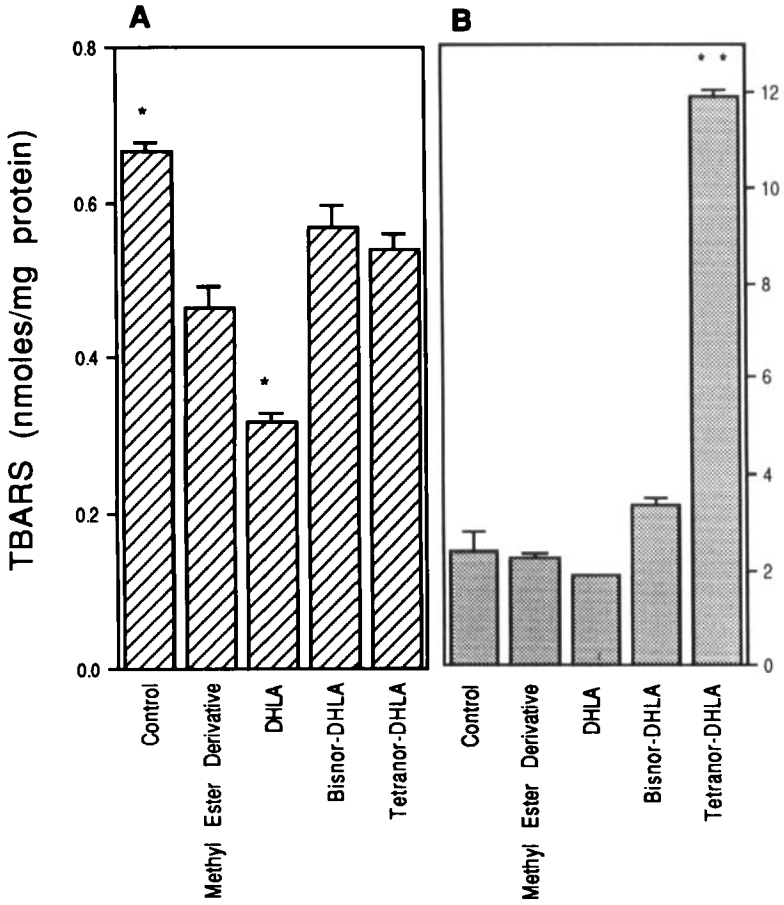

Figure 2 Comparisons of different dihydrolipoic acid homologues for their effect on lipid peroxidation inhibition. Lipid peroxidation was induced in rat liver microsomes by 10 mM AMVN in the presence or absence of 0.5 mM of various dihydrolipoic acid homologues (A) with or (B) without 50 μM deferoxamine. Values represents mean $\pm$ S.E. of 4 replicates. Values denoted by (*) are different from all other values at $P < 0.05$. The value denoted by (**) is the only value different from control value, and different from any other values at $P < 0.05$.

As shown in Figure 2A, in the presence of the iron chelator, deferoxamine, all homologues exerted some protective effect against AMVN-induced lipid peroxidation; DHLA had the greatest protective effect. DHLA was more effective than the methyl ester derivative. In the absence of deferoxamine, tetranor-DHLA induced lipid peroxidation (Figure 2B).

DISCUSSION

The advantages of using DHLA for investigation of structure-activity relationships of antioxidants is that DHLA has been found to be an effective antioxidant both in

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membranes and in solution, and that several natural homologues exist. Using these advantages, we found, in the present study, that shorter chain length homologues (i.e., tetranor- and bisnor-DHLA) exhibited greater O_2^- scavening activities in a polar environment, while DHLA is a better antioxidant against ROO· in an apolar environment.

Several studies in our laboratory using α-tocopherol, ubiquinol and butylated hydroxytoluene homologues with different hydrocarbon chain lengths have demonstrated superior antioxidant activities in shorter homologues. In these studies, it was shown that a shorter hydrocarbon tail confers better protection from (Fe^{2+} + ascorbate)-induced membrane lipid peroxidation. These studies are not consistent with the present results obtained using DHLA homologues, showing that shorter homologues are not better antioxidants in membranes. Hence, the idea that shorter homologues possess better antioxidant properties appears not to be a general rule for membrane antioxidants. Rather, our results are consistent with the partition properties of these compounds. The observations that methyl ester derivative does not have a similar ability in scavening ROO· in membrane as DHLA, which should be expected from the partition coefficients, are probably due to the difference in chemical reactivity between the two compounds as can be seen in a poorer reactivity of methyl ester derivative with ROO· in solution.

There is, at present, little information on whether the hydrocarbon portion of DHLA plays a role in influencing the nature of antioxidant activity, in addition to its role in determining its solubility properties. Our results from the determination of scavening activities for O_2^- may provide such information, in that bisnor-DHLA was found to have a higher kinetic constant for O_2^- than tetranor-DHLA. In contrast, ROO· scavening activity in solution appears not to depend on the hydrocarbon chain length. This could be due to an electrostatic interactions of O_2^- with the carboxylate structure of the reacting antioxidant which is absent in ROO· reactions. The proximity of carboxylate structure to O_2^- reacting site appears to dictate such effects induced by electrostatic interactions in that the native DHLA did not have a significantly lower kinetic constant than the uncharged methyl ester derivative for reaction with O_2^-. Despite the absence of a clear understanding of the mechanisms for the observed phenomenon, the two shorter homologues of DHLA, which possess low values for octanol/water partition coefficients, certainly were better scavengers for O_2^- in solution than other longer homologues. Thus, these results in solution are also, to a degree, consistent with the partition properties of these compounds although such a rule does not apply to the ROO· scavening activity in solution.

The observations that tetranor-DHLA is not only less effective as membrane antioxidant, but also induces lipid peroxidation in the presence of residual iron suggest that the notion that the shorter homologues may be more powerful antioxidants is simplistic. From the observations that these homologues do not enhance lipid peroxidation in the presence of an iron chelator, we suggest that the mechanism involves the reduction of Fe^{3+} to Fe^{2+} which in turn leads to lipid peroxidation. Since only the shorter homologue seemed to promote this reduction, it indicates interaction btween the SH groups, the iron, and the carboxylate structure, which makes it efficient in stabilizing the iron ion in close proximity to the reducing SH groups which in turn induces the redox reaction. It is an unequivocal demonstration of how radically a relatively small structural change can alter the balance between a substance's antioxidant and pro-oxidant tendencies.

The present study attempted to provide an undertanding of the structure-activity

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relationships of DHLA with regard to its physiologically important free radical scavening activities. We have learned that different behaviors governed by hydrophobic and electrostatic environments preclude the determination as to which molecule is the best antioxidant. Furthermore, other factors may also influence the choice of antioxidants as observed in the lipid peroxidation-inducing capabilities of shorter length (bisnor and tetranor) DHLA homologues. As the complexity of biological systems seems to preclude the ease of generalizations on the correlation of molecular structures with antioxidant activities, further work is necessary in order to define the best "antioxidant structure".

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