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2D NMR of the Metabolic Antioxidant Dihydrolipoic Acid and its Derivatives

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Dihydroplipoate and lipoate are physiological thiols which in addition to their coenzyme functions exhibit antioxidant activity. For NMR investigations of their protective mechanism in biological and model systems it is very important to know the full assignment of proton and carbon spectra of these molecules in water (D_2O). An unambiguous assignment of proton and carbon NMR spectra has been made for dihydrolipoate and its short chain derivatives bisnor-and tetranor-lipoic acid in D_2O and $CDCl_3$ solutions using 2D NMR methods.

Oxidation of dihydrolipoic acid produces substantial electron density deshielding of the carbons nearest to the SH groups with the largest shift found at the inner SH group (17.79 ppm in D_2O, 16.93 in $CDCl_3$) and almost no changes in the tail portion of the molecule. However, bisnor-dihydrolipoic acid and especially tetranor-dihydrolipoic acid have more carbon deshielding near the outer SH group of the molecule which correlates with their known diminished ion chelating activity.

Moreover, the proton triplet at position 2 of lipoic acid has strong pH dependence (pK = 4.58) due to the close proximity to the carboxylic group and this feature may be used for monitoring pH.

Keywords: Dihydrolipoic acid, lipoic acid, bisnor dihydrolipoic acid, tetranor dihydrolipoic acid, NMR assignment

INTRODUCTION

Dihydrolipoic acid (6,8-dimercaptooctanoic acid, DHLA) and its oxidized form lipoic (thioctic) acid (LA) have recently received attention as molecules displaying both a coenzyme function in the mitochondrial enzyme complexes and important antioxidant features. Its protective activities have significant applications for the treatment of chronic disorders and toxicity related to oxidative stress[1,2]. This preventive and therapeutic action is largely governed by interaction with proteins, DNA, other antioxidants and transition metal ions.

For non-invasive measurements of the redox state and a detailed study of the protective mechanism in biological and model systems, it is of essential to have the full assignment of proton and carbon NMR spectra for these molecules. To date only the carbon spectra of LA have been studied in $CDCl_3$ at 25 MHz[3]. For DHLA also

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only 13-carbon was presented in methanol-D4[4]. Our goal was to make the necessary assignment for proton and carbon NMR spectra of DHLA and LA primarily in water (D_2O) solution, since most biological reactions occur in an aqueous milieu. The main difficulty in water measurements is the low solubility of dihydrolipoic and lipoic acids. To our knowledge this report provides the first NMR data about dihydrolipoate-lipoate in water.

The assignment was done using 2D NMR (COSY, DQF-COSY, HETCOR) and DEPT pulse sequences. It was of interest to compare the proton and carbon chemical shift changes occurring in dihydrolipoic acid upon oxidation in both solutions in $CDCl_3$ and water. In $CDCl_3$ solution the above methods were applied to dihydrolipoate, and also to its short chain derivatives bisnor dihydrolipoic acid (4,6-dimercaptohexanoic acid, DHBA) and tetranor dihydrolipoic acid (2,4-dimercaptobutanoic acid, DHTA). These derivatives are products of beta-oxidation of DHLA and thus are of biological interest. These results have given a better understanding of the dihydrolipoate structural features, especially in connection with a search[5–7] to find the structural uniqueness of individual antioxidant molecules which could correlate with their activities in biological systems. For example, it was found that α-tocopherol derivatives with a shorter side chain can better protect membranes *in vitro* from lipid peroxidation[7].

MATERIALS AND METHODS

Lipoic acid and its derivatives bisnor (BA) and tetranor (TA) lipoic acids were the racemic mixtures from ASTA Medica (Germany). The reduced forms of these molecules were made by reduction of the disulfide bond of the dithiolane ring using sodium borohydride according to the procedure of Kawabata *et al.*[6]. For NMR measurements in D_2O, a high concentration of lipoate and dihydrolipoate was achieved by dissolving lipoate at high pH with a subsequent adjustment of pH afterwards using NaOD and DCl bubbled with N_2 or Ar to protect from oxidation. For HETCOR NMR the DHLA and LA water solutions were prepared at the maximum possible concentrations ~30 mM. The final pH of water solutions for carbon and proton water measurements was 7.4. The concentration of dihydrolipoic acid and its derivatives in $CDCl_3$ were 10 mM and 100 mM for proton and carbon NMR experiments respectively. All molecules had a natural abundance of ^{13}C carbon.

NMR measurements were performed at room temperature (293K) on a AM 300 WB (Bruker) spectrometer using a 5 mm probehead for proton and a 10 mm probehead for ^{13}C nucleus. Chemical shifts are given relative to TSP (sodium 3-trimethyl [2, 2, 3, 3-$^2H_4$] propionate) in water or TMS (tetramethylsilane) in $CDCl_3$ solutions.

Typical parameters for 2D NMR experiments were as follows. COSY spectra[8,9] were acquired by 8 scans after 4 dummy scans with 770 μs increments in the F1 direction and collected as 128 × 1024 data points and then zeroed to 256 × 1024. The spectral width in the F2 direction was 5600 Hz and relaxation delay was 1 sec. During Fourier transformation the data for COSY were processed with a zero shifted sine-bell function in both directions. DQF-COSY[10] were done at the same parameters as COSY. The DQF-COSY pulse sequence usually gave us better resolution. The interval enhancing intensity of multiple quantum transition was ~50 ms. To choose this parameter, 1D double quantum filtered spectra were obtained at different delays before the DQF-COSY experiment.

HETCOR spectra were acquired with a data spectra matrix 256 × 2048 and NS = 64[11]. Relaxation delay was 2 sec and the increment time 0.8 msec. The data was processed in the F2 direction by Lorentzian function with a broadening factor of 2 Hz and in the F1 direction with a sine-bell function shifted by π/10.

DEPT experiments were used to determine the position of CH lines, and to setup acquisition parameters before each HETCOR measurement.

Carbon ^{13}C NMR spectra were acquired with composite pulse decoupling by single pulse 30° (~3 μs), the relaxation delay was 1.5 sec.

Proton spectra were measured by a single pulse of 5 μs. In water solutions measurements were done by a jump-return pulse sequence with an interpulse delay of 0.4 msec.

Experiments at different pH have been done for 7 mM lipoic acid in normal water solution. The proton spectra were measured by jump-return pulse sequence for water suppression. The coaxial glass insert containing D_2O with 0.75% TSP was used for spin lock and chemical shift reference.

RESULTS

The assignment of NMR spectra in $CDCl_3$ was started beginning with the simplest derivative molecule, tetranor dihydrolipoic acid. Spectra are not shown, results are given in Tables I and II.

2D NMR spectra of bisnor dihydrolipoic acid in $CDCl_3$ are given in Figure 1. Line 4 corresponds to the CH group of the molecule. The assignment was confirmed by DEPT NMR spectra and is supported by the HETCOR spectrum (Figure 1). Proton 4 has cross peaks with lines 3-3′, 5-5′ and also with the proton of SH(4) in the COSY spectrum (Figure 1). The SH(4) proton is a doublet with J = 8.53 Hz. Further proton 6 is having subsequent cross peaks with 5-5′ and its nearest proton of SH(6) group (triplet with J = 8.04 Hz). The protons 3-3′ have cross peaks corresponding with proton 2. Note that the protons 3-3′ are strongly magnetically nonequivalent and have intensive cross peaks between each other. The same situation to a lesser extent can be observed for 5-5′ protons.

Results of 2D NMR measurements of dihydrolipoic acid in $CDCl_3$ are given on Figure 2. The only proton CH(6) is situated in the low field part of the proton spectrum. Its carbon line has the lowest down field position among the protonated carbons and assignment of this proton is also confirmed by the corresponding DEPT spectrum. Proton CH(6) has cross peaks with 7-7′ protons and with the nearest proton of SH(6). Proton SH(6) is visualized by the doublet with J = 7.67 Hz. Protons in position 8 can be assigned as they have strong cross peaks with 7-7′ protons and with subsequent SH(8) proton (triplet with J = 7.99 Hz). Protons at position 2 have a distinct triplet (J = 7.28 Hz) due to interaction with the nearest CH_2 group of the molecule and in the

TABLE I NMR ^{13}C Chemical Shifts of DHLA/LA and its Derivatives in $CDCl_3$.

Compound	Carbon Number							
	1	2	3	4	5	6	7	8
DHLA	180.02	33.89	24.25	26.43	38.68	39.26	42.72	22.28
LA	180.04	33.77	24.27	28.52	34.45	56.19	40.13	38.45
Oxidized minus reduced	0.02	−0.12	0.02	2.09	−4.23	16.03	−2.59	16.17
BDHLA	179.54	31.60	33.65	38.88	42.90	22.20		
BLA	179.19	33.09	29.47	55.12	40.05	38.61		
Oxidized minus reduced	−0.35	1.49	−4.18	16.24	−2.85	16.41		
TDHLA	179.23	39.10	38.31	21.85				
TLA	177.24	52.47	36.06	41.14				
Oxidized minus reduced	−1.99	13.37	−2.25	19.29				

Chemical shifts are given in ppm relative to TMS at 293K.

TABLE II NMR ^1H Chemical Shifts of DHLA/LA and its Derivatives in $CDC1_3$.

Compound	Proton Number								
	2	3, 3′	4, 4′	5, 5′	6	7, 7′	8	SH_a	SH_b
DHLA	2.38	1.65	1.61 1.43	1.69 1.51	2.92	1.92 1.76	2.71	1.33	1.38
LA	2.39	1.66	1.48	1.70	3.57	2.47 1.93	3.14		
Oxidized minus reduced	+0.01	+0.01	−0.04*	+0.1*	+0.65	+0.36*	0.43		
DHBA	2.61 1.74	2.06	2.96 1.80	1.94	2.73			1.27	1.38
BA	2.55	1.99	3.67	2.49 1.98	3.18				
Oxidized minus reduced	−0.06	+0.09*	+0.71	+0.37*	+0.35				
DHTA	3.65	2.22 2.02	2.72					2.14	1.42
TA	4.23	2.67 2.41	3.37 3.20						
Oxidized minus reduced	+0.58	+0.42*	+0.58*						

Chemical shifts are given in ppm relative to TMS at 293K; SH_a, SH_b - proton of SH group at inner and end positions of the molecule; * - for average chemical shift of two non equivalent protons.

COSY spectrum it has a cross peak seen only with protons at position 3. Protons in position 7, 5 and 4 are substantially magnetically nonequivalent as can be seen from the HETCOR spectrum (Figure 2). The assignment of protons in 5 and 4 is supported by the fact that upon oxidation of DHLA to lipoic acid protons 5 being nearer to the S-S bridge experience a larger chemical shift than protons in positions 4.

The D_2O 2D NMR spectra of dihydrolipoic acid are given on Figure 3. There are no lines for the protons of the SH group as they are quickly substituted by deuterons of water. Even in H_2O solution sulfhydryl protons are unobservable because of quick exchange conditions with the water protons. The proton of CH(6) is again in the low field part of the proton spectrum. It has strong cross peaks with 7-7′ and 5-5′ protons. Proton 2 has strong coupling to the protons in the position 3. A similar measurement performed with lipoic acid is given in Figure 5. Thus 2D NMR measurements provide an unambiguous assignment of the proton and carbon spectra.

Protons at position 2 (and protons in 3, 4 positions but in a smaller range) of DHLA and LA in D_2O have pH dependence because of the nearby carboxylic group. The pH dependence for line 2 at 23°C was approximated by the function:

$$F = (a-d)/(1+(x/c)^b + d$$

Using nonlinear regression the following values of the parameters have been obtained:

$$a = 2.408 \pm 0.001$$

$$b = 10.08 \pm 0.4$$

$$c = 4.58 \pm 0.02$$

$$d = 2.192 \pm 0.002$$

Hence the pH in this region can be determined by NMR using the formula

$$pH = 4.58 + \log[(2.408-\beta)/(\beta-2.192)]$$

where β is the chemical shift for the proton triplet at position 2 of the LA molecule.

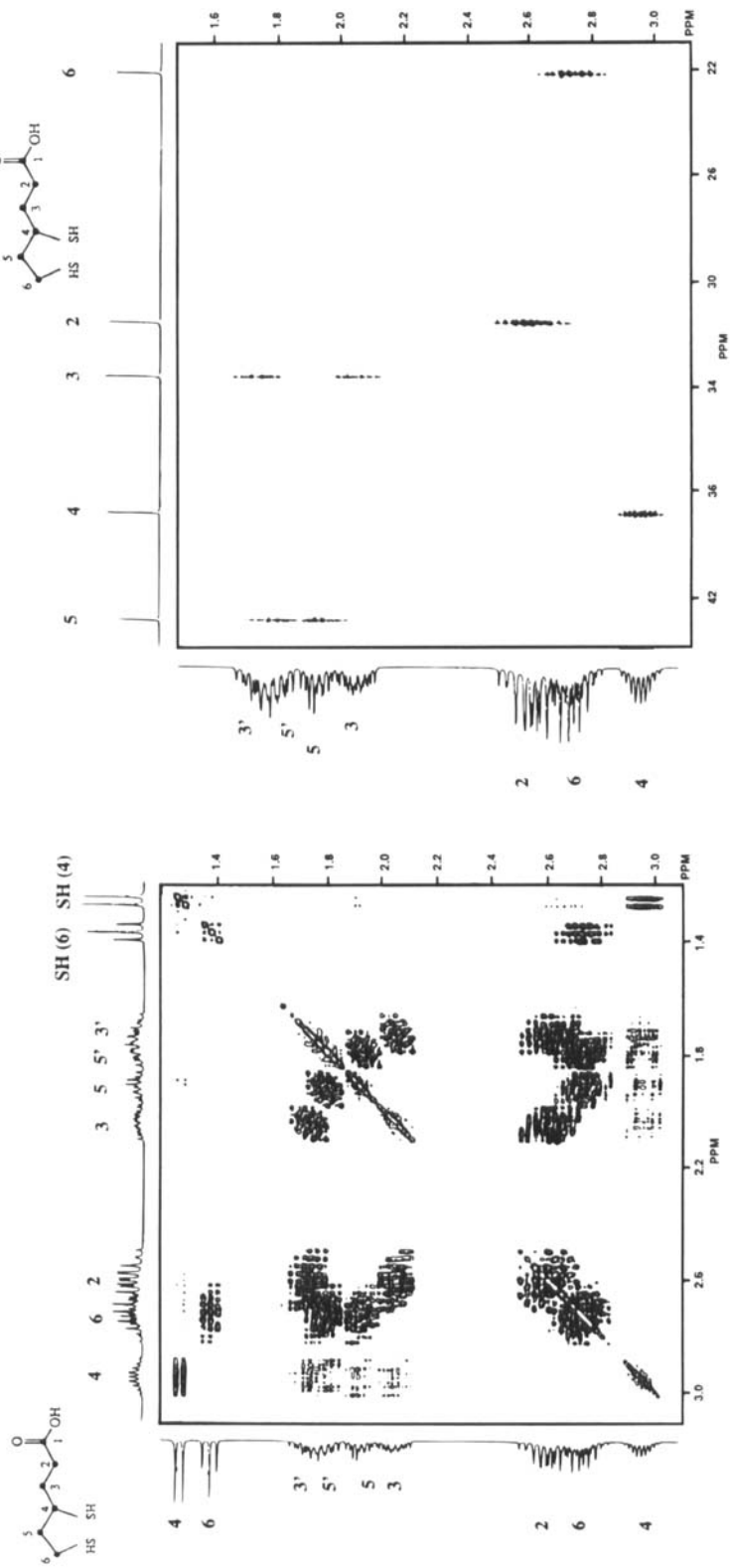

FIGURE 1 COSY and HETCOR 2D NMR spectra of bisnor dihydrolipoic acid (DHBA) in $CDCl_3$ solution. The sharp proton lines of the SH groups are split into 2 or 3 lines due to the interaction with nearby CH and CH_2 groups of the molecule respectively.

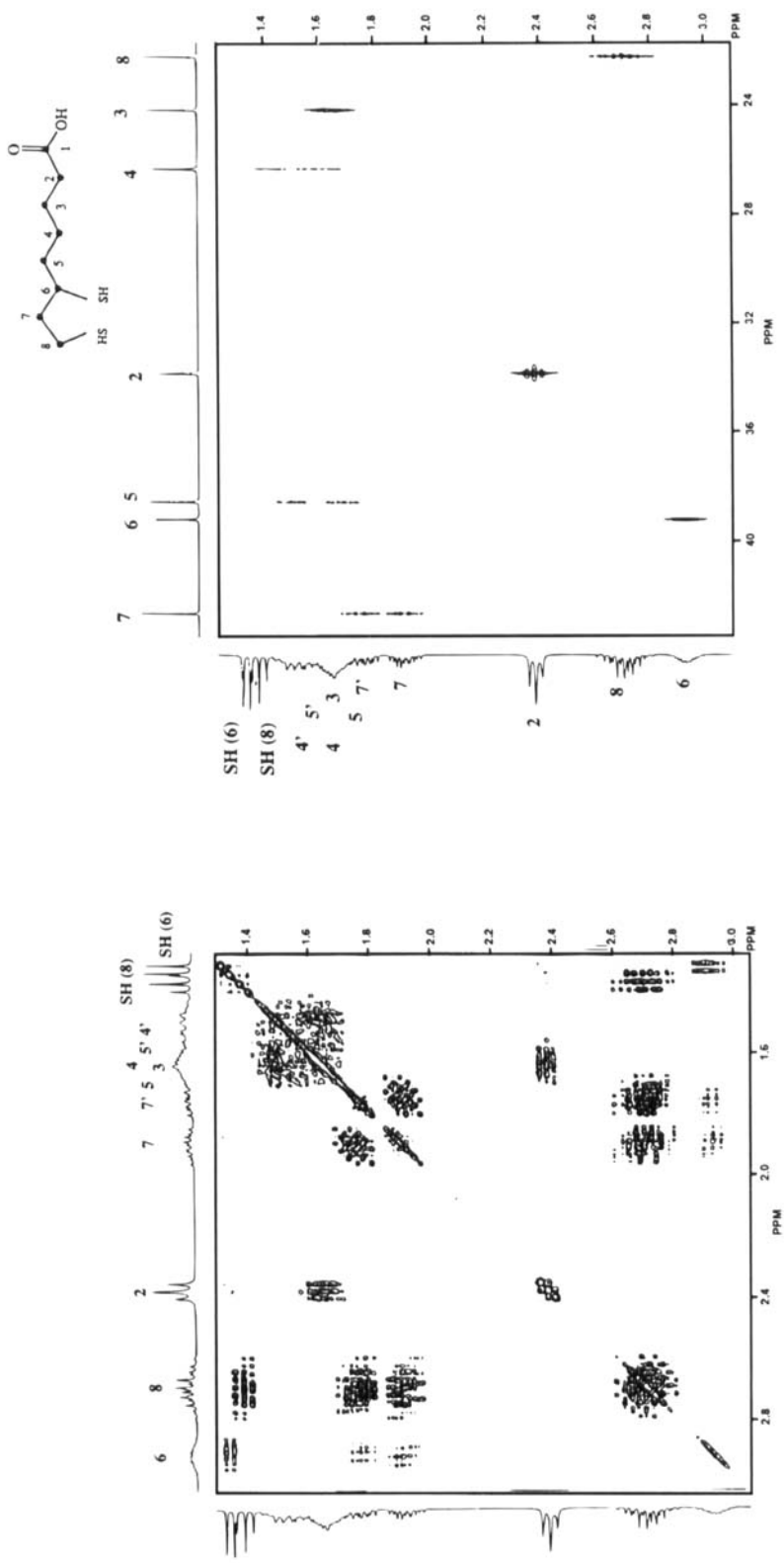

FIGURE 2 2D NMR spectra (COSY and HETCOR) of dihydrolipoic acid in $CDCl_3$.

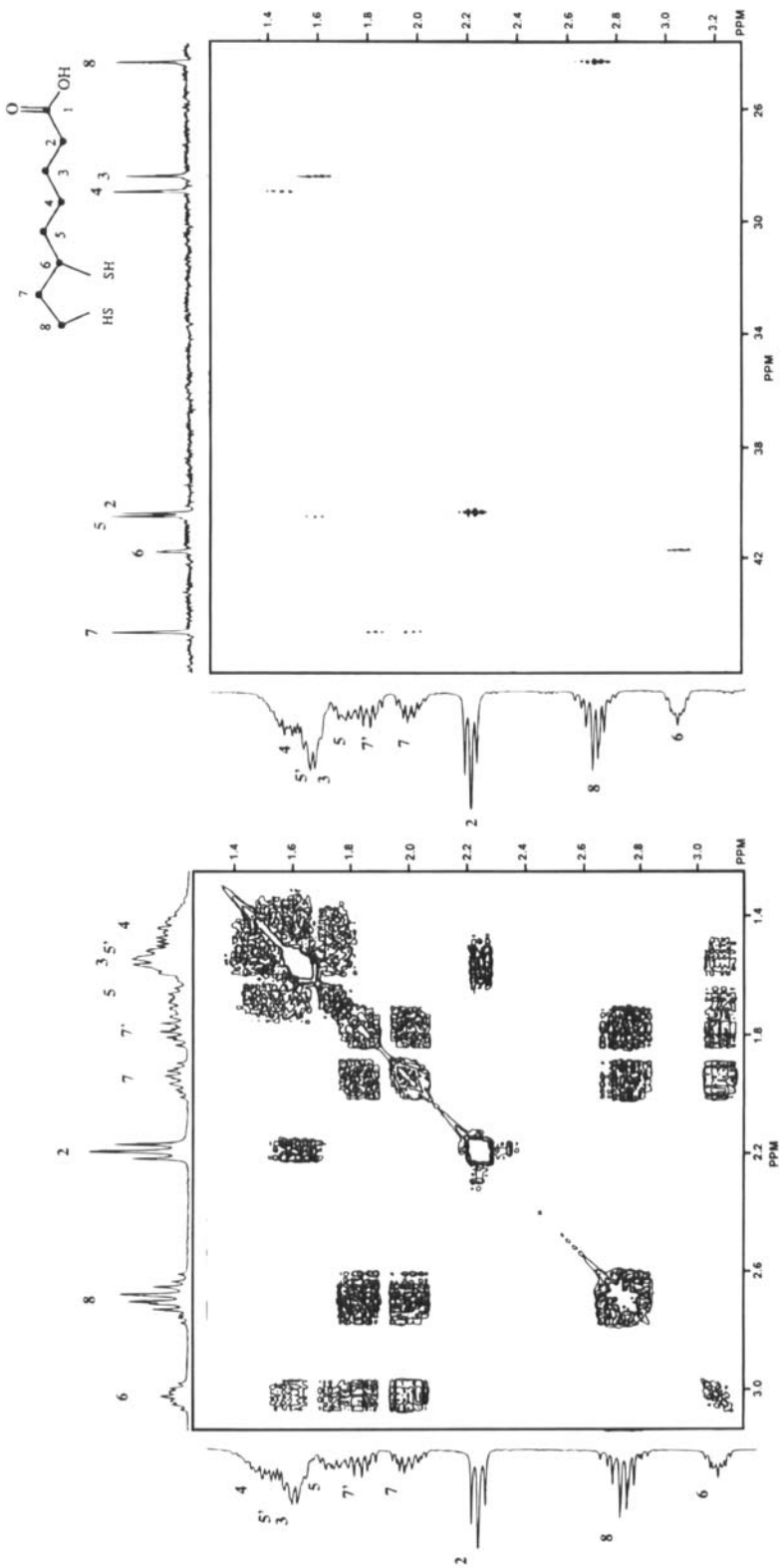

FIGURE 3 COSY and HETCOR 2D NMR spectra of dihydrolipoic acid (DHLA) in D_2O (30 mM, pD 7.0).

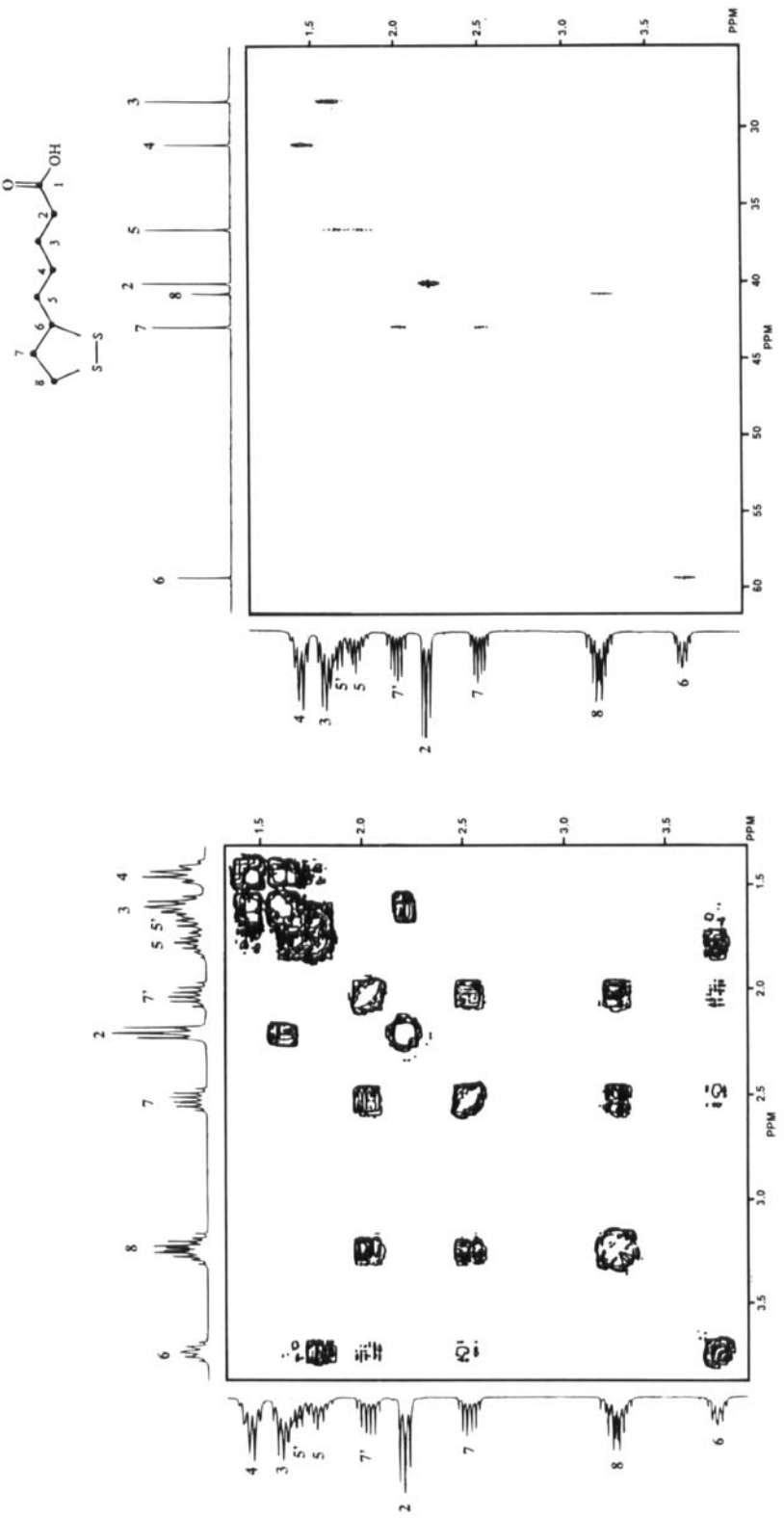

FIGURE 4 COSY and HETCOR NMR spectra of lipoic acid (LA) in D_2O (30 mM, pD 7.0). Note the large magnetic nonequivalence of protons in position 7 of the molecule.

DISCUSSION

DHLA and its oxidized form, lipoic acid, have been shown to have potent antioxidant effects, and hold promise for treatment of numerous conditions which have an oxidative stress component[12]. In this study, an unambiguous assignment of proton and carbon NMR spectra has been made for dihydrolipoate and its short chain derivatives bisnor and tetranor lipoic acid in D_2O and $CDCl_3$ solutions using 2D NMR methods. This is an essential first step for studies of how these compounds interact with oxidants and with other antioxidants. For example, NMR studies may prove useful in resolving the chelating abilities of these compounds, as well as the possible pro-oxidant effects, about which there is some uncertainty[13].

Comparisons of ^{13}C and ^{1}H NMR chemical shifts of dihydrolipoic acid and derivatives are given in Table I, II. An increase of chemical shift indicates a deshielding of the nuclei and, respectively, a decreasing of electron density at this position. The largest changes upon oxidation are observed at the carbons nearest to the SH groups. These changes are slightly asymmetric for DHLA. The change of chemical shift for the carbon nearest the inner SH group is the largest. However, DHBA oxidation produces the largest changes for the carbon nearest to the outer SH group. In the shortest (chain) homologue, DHTA, this change for the outer carbon is the largest (19.29 ppm). The average changes of the two sulfhydryl carbon chemical shifts are approximately the same, 16.55 ppm for DHLA and 16.33 for DHBA, DHTA, but the distribution of electron density detected by the chemical shift is different, especially for DHTA. It is remarkable that this structural feature correlates with a lesser stability constant of binary metal-ion complex formation for tetranor lipoic acid[14] and with substantially less peroxyl radical activity of DHTA in the absence of chelating agents[5].

The protons of the CH_2 group between the sulfhydryls of DHLA and the homologues studied are always non-equivalent. This non-equivalence can be assessed by the NMR spectral splitting of these protons: in $CDCl_3$ solution the DHLA/LA pair have respectively 0.16 ppm/0.53 ppm (in D_2O 0.14 ppm/0.55 ppm), for DHBA/BA pair 0.14 ppm/0.51 ppm and for DHTA/TA 0.2 ppm/0.26 ppm. Consequently, the oxidized form of these molecules always exhibits a larger CH_2 group proton magnetic non-equivalence. This splitting is substantially different in DHTA as compared to DHLA and DHBA. It points out on the largest strain of dithiolane ring of lipoic acid, especially in water solution.

The proton triplet at position 2 of lipoic acid has strong pH dependence (pK = 4.58) due to the close proximity to carboxylic group and this feature can be used for monitoring pH.

In D_2O (Table III), in comparison with spectra taken in $CDCl_3$, the inner carbon of dihydrolipoic acid at position 6 has the largest change of chemical shift upon oxidation and, subsequently, more electron density occurs at the inner sulfur. This indicates that in water the molecule has the highest changes of electron density in this region, which is an important feature of the molecule in the process of interaction with metal ions during redox reactions.

The chemical shift changes of DHLA and LA as the result of solvent alteration between D_2O and $CDCl_3$ (Table IV) show that in the D_2O solution all lines of the carbon spectra of DHLA and LA appear as deshielded, i.e., shifted to the down field region of the spectra. The largest ^{13}C chemical shift changes (~6.5 ppm) occur for the carboxyl group of the molecules. Comparison of data between different solvents also reveals the large ^{13}C chemical shift changes for LA in the thiol region in comparison with DHLA. This fact correlates with the greater hydrophobity of LA. It is noteworthy that the change of solvent again reveals that inner carbon (position 6) of DHLA/LA has a larger susceptibility to surrounding media than outer carbon (position 8). All the above mentioned structural peculiarities

TABLE III NMR ^{13}C and ^{1}H Chemical Shifts of DHLA/LA in D_{20}

Compound	Carbon Number							
	1	2	3	4	5	6	7	8
DHLA	186.69	40.29	28.36	28.90	40.37	41.70	44.51	24.29
LA	186.60	40.24	28.46	31.26	36.74	59.49	43.19	40.92
Oxidized minus reduced	−0.09	−0.05	0.1	2.36	−3.49	17.79	−1.32	16.63

Compound	Proton Number						
	2	3	4	5, 5′	6	7, 7′	8
DHLA	2.20	1.56	1.44	1.70 1.57	3.03	1.95 1.81	2.70
LA	2.20	1.58	1.43	1.80 1.67	3.73	2.51 2.01	3.23
Oxidized minus reduced	0	+0.02	−0.01	+0.1*	+0.7	+0.38*	+0.53

Where chemical shifts are given in ppm relative to TSP at 293K and pH* 7.4;
*- for average chemical shift of two non equivalent protons

of the inner sulfhydryl group of the molecule correlate with the fact that the breaking apart of the disulfide bond in the strained dithiolane ring begins with the involvement of the sulfur at position 6. This feature may also be important in the formation of complexes with metal ions[14].

Oxidation of dihydrolipoic acid produces substantial electron density deshielding of the carbons nearest to the SH groups with the largest shift found at the inner SH group (17.79 ppm in D_2O, 16.93 in $CDCl_3$) and almost no changes in the tail portion of the molecule. However, bisnor dihydrolipoic acid and especially tetranor dihydrolipoic acid have more carbon deshielding near the outer SH group of the molecule which correlates with their diminished ion chelating activity.

In conclusion, the present study will allow future researchers to use NMR to examine interactions of dihydrolipoic acid and lipoic acid with compounds of biological interest, with precise knowledge of line assignments for NMR in water.

Table IV Effect of Solvent Exchange from $CDCl_3$ to D_2O on NMR ^{13}C and ^{1}H Chemical Shifts of DHLA and LA.

Compound	Proton Number							
	2	3	4	5	6	7	7′	8
Δ(DHLA)	−0.18	−0.09	−0.08*	+0.04*	+0.11	+0.03	0.05	−0.01
Δ(LA)	−0.19	−0.08	−0.05	+0.04*	+0.16	+0.04	+0.08	+0.09

Compound	Carbon number							
	1	2	3	4	5	6	7	8
Δ(DHLA)	+6.67	+6.40	+4.11	+2.47	+1.62	+2.44	+1.79	+2.0
Δ(LA)	+6.54	+6.47	+4.19	+2.74	+2.29	+3.30	+3.06	+2.47

Where Δ = $d(D_2O) - d(CDCl_3)$, d - chemical shift in ppm.
*- for average chemical shift of two non equivalent protons.

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