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Physico-chemical profiling of α-lipoic acid and related compounds

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ABSTRACT

Lipoic acid, the biomolecule of vital importance following glycolysis, shows diversity in its thiol-disulfide equilibria and also in its eight different protonation forms of the reduced molecule. In this paper, lipoic acid, lipoamide and their dihydro derivatives were studied to

quantify their solubility, acid-base, and lipophilicity properties at a submolecular level. The acid-base properties are characterized in terms of 6 macroscopic, 12 microscopic protonation constants and 3 interactivity parameters. The species-specific basicities, the pH-dependent distribution of the microspecies and lipophilicity parameters are interpreted by means of various intramolecular effects, and contribute to understanding the antioxidant, chelate-forming and enzyme cofactor behavior of the molecules observed.

KEY WORDS

Lipoic acid, Microspeciation; NMR-pH titration

1. INTRODUCTION

α-Lipoic acid (LA), also known as 6,8-thioctic acid or 1,2-dithiolane-pentanoic acid is an open-chain carboxylic acid that contains a disulfide bond connecting the carbons in the sixth and eighth positions in a five-membered ring. In its reduced form (dihydrolipoic acid, DHLA), the disulfide bond is cleaved, and two thiol groups are formed.

The protein-bound lipoic acid/dihydrolipoic acid system (LA/DHLA) is important in a number of biochemical reactions [1]. The LA/DHLA system is known for its cofactor function in mitochondrial enzyme complexes such as the pyruvate dehydrogenase complex, and the α-ketoglutarate dehydrogenase enzyme complex [2-4]. The LA/DHLA couple also functions as an antioxidant system [5], redox regulator of proteins (e.g. myoglobin, prolactin) [6-7], heavy metal chelator [8-10], and gene expression regulator in certain cell lineages [11]. Its therapeutic importance has been demonstrated in diabetes [12], neurodegenerative diseases [13], and vitamin C and E deficiencies [14].

It is a key feature of any thiol-disulfide redox system, that only the deprotonated thiol is active in the redox process, i.e. only the anionic thiolate can be oxidized directly [15], making

the redox and acid-base behavior interfering and co-dependent. In fact, the redox potential in most biological thiol-disulfide systems is double pH-dependent. Primarily, the deprotonated fraction of every thiol depends on the pH of the solution, with strong dependence typically in the 6-11 pH range. Secondarily, the thiolate oxidizability in most biomolecules is multiply modulated by adjacent basic or acidic groups, since protonation of any such group exerts an electron-withdrawing effect on the thiolate [16].

This is of particular importance for DHLA, the most basic form of which contains a carboxylate and two nearby thiolate sites, with specific propensities for protonation. Therefore, macroscopic physico-chemical parameters will not characterize the thiolate moieties specifically. For an exact/detailed elaboration, the site-specific, so-called microscopic parameters are needed [17-18]. There is no convincing set of data in connection with the physical properties of LD and DHLA, much less for their species-specific equilibrium constants. In this work the solubility and lipophilicity parameters along with the macroscopic and microscopic protonation constants of lipoic acid, dihydrolipoic acid, lipoamide, and dihydrolipoamide were determined using mainly NMR techniques, including ^{1}H NMR- and ^{1}H/^{13}C HSQC NMR-pH titrations.

2. RESULTS

2.1 Determination of acid-base parameters

Figure 1 represents the protonation schemes of dihydrolipoic acid. Macroequilibria (top line) indicate the stoichiometry of the successively protonating ligand and the stepwise macroscopic protonation constants. In the microspeciation scheme, 8 microspecies with their one-letter symbols (a, b … h), and 12 microscopic protonation constants are depicted (k^{S6}, $k_{S6}{}^{S8}$, …, $k_{S6S8}{}^{O}$). The superscript at k for any microconstant indicates the protonating group while the subscript (if any) shows the site(s) already protonated. S6, S8 and O symbolize the

thiolate at C6, thiolate at C8, and carboxylate sites, respectively. Some examples of macro- and microconstants of dihydrolipoic acid are:

(1) $K_1 = \beta_1 = \frac{[\mathrm{HL^{2-}}]}{[\mathrm{L^{3-}}][\mathrm{H^+}]}$ $K_2 = \frac{[\mathrm{H_2L^-}]}{[\mathrm{HL^{2-}}][\mathrm{H^+}]}$ $K_1K_2 = \beta_2 = \frac{[\mathrm{H_2L^-}]}{[\mathrm{L^{3-}}][\mathrm{H^+}]^2}$

(2) $k^{\mathrm{S6}} = \frac{[\mathrm{b}]}{[\mathrm{a}][\mathrm{H^+}]}$ $k_{\mathrm{S6}}^{\mathrm{S8}} = \frac{[\mathrm{e}]}{[\mathrm{b}][\mathrm{H^+}]}$ $k_{\mathrm{S6S8}}^{\mathrm{O}} = \frac{[\mathrm{h}]}{[\mathrm{e}][\mathrm{H^+}]}$

Concentrations of the various macrospecies comprise the sum of the concentration of those microspecies that contain the same number of protons. For example:

(3) $[\mathrm{HL^{2-}}] = [\mathrm{b}] + [\mathrm{c}] + [\mathrm{d}]$

(4) $[\mathrm{H_2L^-}] = [\mathrm{e}] + [\mathrm{f}] + [\mathrm{g}]$

The following equations show the relationships between the macro- and microconstants of dihydrolipoic acid [19]:

(5) $K_1 = k^{\mathrm{S6}} + k^{\mathrm{S8}} + k^{\mathrm{O}}$

(6) $K_1K_2 = \beta_2 = k^{\mathrm{S6}}k_{\mathrm{S6}}^{\mathrm{S8}} + k^{\mathrm{S6}}k_{\mathrm{S6}}^{\mathrm{O}} + k^{\mathrm{S8}}k_{\mathrm{S8}}^{\mathrm{O}} = \cdots$

(7) $K_1K_2K_3 = \beta_3 = k^{\mathrm{S6}}k_{\mathrm{S6}}^{\mathrm{S8}}k_{\mathrm{S6S8}}^{\mathrm{O}} = \cdots$

Equations (6) and (7) can be written in 6 different, equivalent ways depending on the path of protonation from microspecies 'a'.

Lipoic acid, dihydrolipoic acid, lipoamide, and dihydrolipoamide were titrated under conditions near physiological. The macroscopic protonation constants are compiled in Table 1. Lipoamide was only titrated to demonstrate the effect of deprotonation of the amide group. Because of the complexity of the proton spectra, in addition to ^{1}H NMR-pH titration, ^{1}H/^{13}C HSQC-pH titrations were also performed with dihydrolipoic acid, to aid the chemical shift assessment. To characterize the microscopic basicities of dihydrolipoic acid, the introduction and utilization of an auxiliary compound is necessary [20] to mimic the minor microspecies. Such minor microspecies are those in which the less basic carboxylate is protonated (-COOH) while one or two of the more basic thiolates are unprotonated (-S^-) [21]. Substituting the –COOH by –$CONH_2$ moiety keeps the carboxylic end of the compound in neutral, almost identical form. In our case, dihydrolipoamide is the appropriate, isoelectronic model compound for the complete elucidation of the protonation scheme. The microconstants and microspecies of dihydrolipoamide are thus regarded as constituents of the dihydrolipoic acid microspeciation scheme, i.e. microspecies 'd', 'f', 'g', and 'h'. Another method of evaluating site-specific protonation constants is the utilization of a spectroscopic pH-response phenomenon that is selectively sensitive to the protonation of certain basic sites only [17]. Therefore, one auxiliary compound proves to be enough to elucidate the triprotic system of dihydrolipoic acid, since appropriately chosen NMR nuclei can provide selective signals of the mid-chain thiolate, both for dihydrolipoic acid and dihydrolipoamide.

The titration curves of lipoic acid (Figure 2A) show that the protonation shift decreases drastically as the covalent distance increases between the observed proton and the basic moiety: $\Delta\delta_{C2} = 0.218 \pm 0.002$, $\Delta\delta_{C3} = 0.046 \pm 0.002$, $\Delta\delta_{C4} = 0.038 \pm 0.002$, $\Delta\delta_{C5} = 0.003 \pm 0.002$. In fact, reasonable titration curves could not be fitted to the chemical shifts of the C6,

C7, and C8 protons. Therefore it is safe to assume, that in the case of dihydrolipoic acid and dihydrolipoamide the C3 and C2 protons will be selective for the S6 moiety of the two thiolates. The C3 protons are, in fact, the best-conditioned protons for the evaluation of the S6 thiolate basicities, since apart from selectivity they also have sufficient sensitivity for the mid-chain thiolate protonation. Therefore, the titration data of dihydrolipoamide (Figure 2B, 2C) was evaluated as such: the C6, C7a, and C8 proton chemical shifts were fitted using equation (22) keeping n=2, while simultaneously, the C3 proton chemical shifts were fitted to the following equation:

$$(8)\quad \delta_{\mathrm{obs(pH)}} = \frac{(\delta_{\mathrm{HL}}-\delta_{\mathrm{L}})\times(10^{\log k_{\mathrm{O}}^{\mathrm{S6}}-\mathrm{pH}}+10^{\log\beta_2-2\times\mathrm{pH}})}{1+10^{\log\beta_1-\mathrm{pH}}+10^{\log\beta_2-2\times\mathrm{pH}}}+\delta_{\mathrm{L}}$$

where δ_{L} is the C3 chemical shift of the unprotonated dihydrolipoamide, δ_{HL} is the C3 chemical shift of the dihydrolipoamide species protonated at S6, β_1 and β_2 are the cumulative macroconstants of dihydrolipoamide. It is clear from the chemical shift-pH profile of lipoamide (Figure 2B) that the protonation shift due to deprotonation of the amide moiety will not perturb the C3 proton chemical shift considerably in dihydrolipoamide up to pH 13.5. Having the value of $k_{\mathrm{O}}^{\mathrm{S6}}$, the remaining dihydrolipoamide microconstants could be calculated with equations analogous to equations (5) and (7):

$$(9)\quad k_{\mathrm{O}}^{\mathrm{S8}} = K_1^{\mathrm{dihydrolipoamide}} - k_{\mathrm{O}}^{\mathrm{S6}}$$

$$(10)\quad \log k_{\mathrm{S6O}}^{\mathrm{S8}} = \log\beta_2^{\mathrm{dihydrolipoamide}} - \log k_{\mathrm{O}}^{\mathrm{S6}}$$

$$(11)\quad \log k_{\mathrm{S8O}}^{\mathrm{S6}} = \log\beta_2^{\mathrm{dihydrolipoamide}} - \log k_{\mathrm{O}}^{\mathrm{S8}}$$

The titration data of dihydrolipoic acid (Figure 3) were evaluated analogously: utilizing only the first two macroconstants, the C6, C7a (^{1}H and ^{13}C), and C8 chemical shifts were fitted to equation (22) keeping n=2, while simultaneously, the C2 (^{1}H and ^{13}C) and C3 chemical shifts were fitted to the following function:

$$(12)\quad \delta_{\mathrm{obs(pH)}} = \frac{(\delta_{\mathrm{HL}}-\delta_{\mathrm{L}})\times(10^{\log k^{\mathrm{S6}}-\mathrm{pH}}+10^{\log\beta_2-2\times\mathrm{pH}})+(\delta_{\mathrm{H_2L}}-\delta_{\mathrm{L}})\times 10^{\log\beta_3-3\times\mathrm{pH}}}{1+10^{\log\beta_1-\mathrm{pH}}+10^{\log\beta_2-2\times\mathrm{pH}}+10^{\log\beta_3-3\times\mathrm{pH}}} + \delta_{\mathrm{L}}$$

where δ_{L} is the chemical shift (C2, C3) of the unprotonated dihydrolipoic acid, δ_{HL} is the chemical shift (C2, C3) of the dihydrolipoic acid species where S6 is protonated and O is unprotonated (microspecies 'b' and 'e'), δ_{H2L} is the chemical shift (C2, C3) of the dihydrolipoic acid species where S6 and O are protonated (microspecies 'h'), β_1, β_2 and β_3 are the cumulative protonation macroconstants of dihydrolipoic acid. Since C2 and C3 chemical shifts are completely insensitive to the protonation state of the C8 thiolate, and microspecies 'd', 'g', and 'f' are too minor to tangibly influence any chemical shifts, the formulation of equation (12) and the related assignments are perfectly valid for all practical purposes. The ^{13}C chemical shifts of the C7 carbon were chosen, because this nucleus is the most sensitive to the protonation of both thiolate moieties. Protonation of the thiolates and the carboxylate take place in separate pH regions, the K_3 and $k_{\mathrm{S6S8}}{}^{\mathrm{O}}$ values of dihydrolipoic acid are therefore practically identical. The remaining microconstants of dihydrolipoic acid could be calculated with equations analogous to equations (5) and (7):

$$(13)\quad k^{\mathrm{S8}} = K_1 - k^{\mathrm{S6}}$$

$$(14)\quad \log k_{\mathrm{S6}}^{\mathrm{S8}} = \log\beta_2 - \log k^{\mathrm{S6}}$$

(15) $\log k_{S8}^{S6} = \log\beta_2 - \log k^{S8}$

(16) $\log k_{S6}^{O} = \log\beta_3 - \log k^{S6} - \log k_{S6O}^{S8}$

(17) $\log k_{S8}^{O} = \log\beta_3 - \log k^{S8} - \log k_{S8O}^{S6}$

(18) $\log k^{O} = \log\beta_3 - \log\beta_2^{\text{dihydrolipoamide}}$

These data also afford the interactivity parameters between the basic moieties in dihydrolipoic acid. The interactivity parameter shows to what extent the protonation of site A reduces the basicity of site B, and vice versa. In general, the interactivity parameter is widely considered to be the most invariant quantity in analogous moieties of different compounds and also in various protonation states of the neighboring moiety in the same molecule [22]. For instance, the interactivity parameter between the two thiolate sites can be defined as follows:

(19) $\log\Delta E_{S6/S8} = \log k^{S6} - \log k_{S8}^{S6} = \log k^{S8} - \log k_{S6}^{S8}$

Since the pieces of information used to elucidate the dihydrolipoic acid microspeciation scheme exceeds that of the number of independent variables in the system (the equation system is redundant), the interactivity parameters could be calculated by two methods, such as:

(20) $\log\Delta E_{S6/S8} = \log k^{S6} - \log k_{S8}^{S6} = \log k_{O}^{S6} - \log k_{S8O}^{S6}$

All microconstants and interactivity parameters (by both calculation methods) of dihydrolipoic acid are presented in Table 2. The excellent agreement between the variously calculated interactivity parameters justifies the choice of the model compounds and the custom-tailored evaluation method. Even though each interactivity parameter could be calculated in two different ways, the better-conditioned ones were taken into consideration to draw conclusions.

2.2 Determination of thermodynamic solubility and lipophilicity

The thermodynamic solubilities and the octanol/water distribution coefficients of lipoic acid, dihydrolipoic acid, lipoamide, and dihydrolipoamide were measured in triplicates, using the stir-flask method at selected pH values. Determining the solubility of the studied compounds proved to be a difficult task, because the solubility of ionized species was unquantifiable. For example the solubility of lipoic acid at pH < 3 is of a low, quantifiable value, but as the pH increases above the the log*K* of lipoic acid the solubility increases without bound, even above the molar limit. Eventually, after adding excessive amounts of lipoic acid to a small amount of solvent (pH kept above 10), a viscous syrup is formed. Keeping in mind therefore, that all ionized species of the studied compounds have practically infinite solubility, only the intrinsic solubilites of the compounds are reported: lipoic acid log*S* = -2.33 ±0.02, dihydrolipoic acid log*S* = -2.13 ±0.02, lipoamide log*S* = -2.51 ±0.03, dihydrolipoamide log*S* = -1.75 ±0.01.

The pH dependence of log*D* is depicted in Figure 4. The distribution coefficient data (except for lipoamide) were fitted to functions expressing the molar ratio weighted average of the species-specific lipophilicity values (partition coefficients, *P*) of the major microspecies:

(21) $D_{(\mathrm{pH})} = \sum_{\mathrm{i}=1}^{\mathrm{m}} P_{\mathrm{i}} f_{\mathrm{i}}$

where f is the mole fraction and m is the serial number assigned to the various species involved. The model fits of log$D$ provided the limiting log$P$ values. These limiting values reflect the species-specific lipophilicites of the major species, i.e. for the case of dihydrolipoic acid the limiting log$D$ values (at pH 13, 7, and 1) will essentially characterize the log$P$ values of the completely deprotonated ligand (microspecies 'a'), the ligand protonated at the two thiolates (microspecies 'e'), and the completely protonated ligand (microspecies 'h'), respectively. The log$P$ values of selected species obtained from the model fits are compiled in Figure 5.

3. DISCUSSION

A species-specific acid-base evaluation of lipoic acid and related compounds allows deeper insight compared to the conventional macroscopic pKa values, especially when minor microspecies are also considered. Several straightforward conclusions can be drawn from the macro- and microspeciation of LA and DHLA. Special emphasis will be laid on the thiolate basicities, since it is proportional to oxidizability [15].

The carboxylate protonation constant of lipoic acid (4.65) is a trivial one, in agreement with the most recent potentiometric determination (pKa = 4.82) [23]. It has been demonstrated [16] that the disulfide forms of thiol-containing compounds and the protonated thiol moieties have similar inductive, etc. effects on the rest of the molecule. Thus, the third macroconstant of dihydrolipoic acid (4.64), which corresponds to the microscopic protonation constant $k_{\mathrm{S6S8}}{}^{\mathrm{O}}$ due to the separate protonation regions, is expected to be close in value to that of lipoic acid. The first two protonation steps of dihydrolipoic acid therefore, correspond to the overlapping protonation of the two thiolates, followed by the separate third protonation step

overwhelmingly belonging to the carboxylate. The basicity of the S6 thiolate is 1.3 times greater than that of the terminal thiolate (S8), due to the greater inductive effects on the C6 secondary carbon compared to the primary C8 carbon. Such effect is well-known for amino sites. The interactivity parameter is a good indicator of the basicity-reducing effect on one of the sites when the other site protonates. The examination of dihydrolipoamide as model compound, afforded the remaining parameters to the dihydrolipoic acid microspeciation scheme. In dihydrolipoic acid the interactivity parameters are consistent with the general assumption that in small molecules interactivity decreases along with the covalent distance between the moieties. The consistency of our model is supported by the fact that the interactivity parameters could be calculated by two independent methods, which were in agreement with each other.

At physiological pH the microspecies required for redox equilibria (microspecies ‘a’ and ‘d’) are at least seven orders of magnitude less abundant compared to the major microspecies: the abundance of ‘e’ is 99.4 %, while ‘b’, ‘c’, and ‘h’ have roughly 0.2-0.2 % abundance each (Figure 6). To participate in redox processes, both thiolate groups of dihydrolipoic acid must be in deprotonated form, therefore in enzyme-catalyzed reactions the local electron density conditions of the protein and the appearing, stable five-membered ring in lipoic acid must be important factors in the thiol-disulfide interchange processes. Previous studies have shown that the dihydrolipoyl dehydrogenase (E3) enzyme subunit of mitochondrial enzyme complexes (PDHc, αKGDHc) that oxidize dyhidrolipoic acid to lipoic acid, change their function with decreasing pH, from the normal physiological to inverse, or diaphorase activity [24-27]. Based on our findings, at the pH optimum of diaphorase activity (4.8-5.7) [28] 8-10 % of the species bear two, oxidizable thiolate moieties. This tendency is not observed with increase of pH above the physiological value, therefore the pH-induced conformation change of the enzyme or various mutations may inflict this problem [29].

The low intrinsic solubility of the studied molecules in their neutral form can be explained by the long aliphatic chain, while ionization brings about an extreme increase in solubility. Surprisingly, the intrinsic solubility of lipoamide is lower than that of lipoic acid, contrary to what would be anticipated. The solubility increasing effect of reducing the disulfide ring is the consequence of the presence of the more polar thiol functional groups. Perhaps the most extraordinary characteristic of lipoic acid and dihydrolipoic acid is the large difference between the lipophilicity of the fully protonated and unprotonated species. The ionization of lipoic acid results in some 4 orders of magnitude decrease in lipophilicity. This is consistently observed in dihydrolipoic acid, escalated by only 1.5 fold further decrease brought on by the protonation of the thiolates. Interestingly, the ionization of the thiolates has the same effect on solubility as does the carboxylate, however in the case of lipophilicity the effect of the thiolates is minor compared to that of the carboxylate. As expected, both amide derivatives have lower lipophilicities compared to the acid counterparts, however the lipophilicity of lipoamide is closer to that of unprotonated dihydrolipoamide as opposed to fully protonated dihydrolipoamide (the fully protonated lipoic acid and dihydrolipoic acid are close in lipophilicity as expected).

4. CONCLUSIONS

The 12 site-specific basicities determined for lipoic acid are a significant dataset and a *sine qua non* precondition to quantify and interpret the redox and enzyme cofactor behavior of the LA/DHLA system at the submolecular level. Since thiolate basicities are related to their redox and chelating properties, the 8 different thiolate protonation constants provide sound means to explore thiolate oxidizabilities, a key parameter to understand and influence oxidative stress and heavy metal chelation.

5. ACKNOWLEDGEMENT

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6. EXPERIMENTAL PART

6.1 Materials

α-Lipoic acid, α-lipoamide, and all other chemicals were purchased from Sigma-Aldrich and used without further purification.

6.2 Determination of thermodynamic solubility by the saturation stir-flask method

The solid or liquid substance of lipoic acid, dihydrolipoic acid, lipoamide, or dihydrolipoamide was added to 2 ml of an aqueous buffer recommended by the IUPAC, suitable for the desired pH, until a heterogeneous system was obtained. The exact pH was adjusted with 0.2 mol/l NaOH or 0.2 mol/l HCl, the pH was measured using a Metrohm 6.0204.100 combined pH glass electrode and a Radiometer pHM93 reference pH meter. The solution containing excess of the sample was stirred for a period of 4 h at a temperature of 25.0 ± 0.1°C allowing it to achieve thermodynamic equilibrium. The experiments and the NMR samples pertaining to the dihydro derivatives were prepared in an *815-PGB* glove box (*Plas-Labs Ic.*, Lansing, MI, USA) under N_2 atmosphere to preclude oxidation. In principle, the concentration of lipoic acid can be determined using UV spectrophotometry (λ_{max} ~ 330 nm); however this method cannot be applied to the dihydro derivatives on the account of oxidation. After further 20 h of sedimentation the concentration of the saturated solution was therefore measured by quantitative NMR spectroscopy for all samples, using pyrazine as a concentration standard.

6.3 Lipophilicity measurements by the stir-flask method

Stock solutions of the molecules were prepared using octanol-saturated water (0.15 mol/l ionic strength). A 0.6 ml volume of these stock solutions was diluted to 6 ml with an aqueous buffer recommended by the IUPAC, suitable for the desired pH. The exact pH was adjusted with 0.2 mol/l NaOH or 0.2 mol/l HCl, the pH was measured using a Metrohm 6.0204.100 combined pH glass electrode and a Radiometer pHM93 reference pH meter. After collecting samples from the aqueous phase for determining the initial aqueous concentraion, water-saturated octanol was added. Next, the two phases were intensively stirred for 2 h in thermostated double-walled glass cells at constant temperature (25.0 ± 0.1 °C). After separation of the equilibrated phases the final concentration of the solute was determined in the aqueous phase by quantitative NMR spectroscopy using pyrazine as a concentration standard. The octanol-water distribution coefficients were calculated from the concentration of the molecules in the aqueous phase before and after partitioning. The experiments and the NMR samples pertaining to the dihydro derivatives were prepared in an 815-PGB glove box (Plas-Labs Ic., Lansing, MI, USA) under N_2 atmosphere to preclude oxidation.

6.4 NMR measurements

NMR spectra were recorded on a *Varian 600* MHz spectrometer at 25.0 °C. The solvent in every case was an aqueous solution with H_2O:D_2O, 95:5, v/v (0.15 mol/l ionic strength), using DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as the reference compound. The samples that contained dihydro derivatives were titrated in the presence of excess sodium tetrahydroborate to preclude oxidation. The sample volume was 600 µl, pH values were determined by internal indicator molecules optimized for NMR [30-31]. ^{1}H NMR spectra were recorded with the WET solvent suppression sequence (for titration measurements: number of transients = 16, number of points = 65536, acquisition time = 3.407 s, relaxation

delay = 1.5 s; for quantitative measurements: number of transients = 64, number of points = 65536, acquisition time = 3.407 s, relaxation delay = 15 s). $^{1}H/^{13}C$ HSQC spectra were recorded using a WET solvent-suppressed band-selective gradient-enhanced heteronuclear single quantum coherence sequence with adiabatic pulses (number of transients = 16, number of points = 1186, number of increments = 64, $^{1}J_{C,H}$ coupling constant = 146 Hz, acquisition time = 150 ms, relaxation delay = 1 s, spectral width in ^{1}H dimension = 3955.7 Hz, spectral width in ^{13}C dimension = 10556.9 Hz).

6.5 Data analysis

For the analysis of quantitative NMR measurements, the AMARES [32] time domain fit algorithm (without apodization) of the jMRUI v. 5.1 software package [33] was used. For the analysis of NMR titration curves of chemical shifts versus pH, the non-linear curve fitting regression analysis of the Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) software was used with the following function [17] for general cases (special cases of NMR-pH profiles were evaluated with equations (8) and (12), detailed in the Results section):

$$(22)\quad \delta_{\mathrm{obs(pH)}} = \frac{\delta_{\mathrm{L}} + \sum_{i=1}^{n} \delta_{\mathrm{H_iL}} \times 10^{\log\beta_i - i\times pH}}{\sum_{i=0}^{n} 10^{\log\beta_i - i\times pH}}$$

where δ_{L} is the chemical shift of the unprotonated ligand (L), δ_{HiL} values stand for the chemical shifts of successively protonated species of L, n is the maximum number of protons that L can bind, and β is the cumulative protonation macroconstant. The standard deviations of protonation constant values obtained from the regression analyses were used to calculate the Gaussian propagation of uncertainty for the microscopic protonation constants derived in the Results section. The standard deviations of Gaussian-Lorentzian curve fits were used to

calculate the Gaussian propagation of uncertainty for solubility and lipophilicity parameters derived in the Results section.

6.6 TOF MS measurements

The exact masses of the synthesized and isolated compounds were determined with an Agilent 6230 time-of-flight mass spectrometer equipped with a JetStream electrospray ion source in positive ion mode. JetStream parameters: drying gas (N_2) flow and temperature: 10.0 l/min and 325 °C; nebulizer gas (N_2) pressure: 10 psi; capillary voltage: 4000 V; sheath gas flow and temperature: 7.5 l/min and 325 °C. TOF MS parameters: fragmentor voltage: 170 V; skimmer potential: 170 V; OCT 1 RF Vpp: 750 V. Samples (0.1-0.3 µl) were introduced by the Agilent 1260 Infinity HPLC system (flow rate = 0.5 ml/min, 0.1% formic acid in 70% v/v methanol-water mixture). Reference masses of *m/z* 121.050873 and 922.009798 were used to calibrate the mass axis during analysis. Mass spectra were acquired over the *m/z* range 100-1000 at an acquisition rate of 250 ms/spectrum and processed using Agilent MassHunter B.02.00 software.

6.7 Synthetic protocols

Dihydrolipoic Acid (= **(6*R*)-6,8-Disulfanyloctanoic Acid**; **1**) was synthesized by dissolving 0.104 g (0.50 mmol) lipoic acid in 5 ml of methanol and adding 2.1 eqv sodium tetrahydroborate (40 mg). After stirring at ambient temperature for 1 h, the reaction mixture was evaporated *in vacuo* to yield a clear glassy solid. ^{1}H NMR and HPLC-HRMS measurements showed residual excess sodium tetrahydroborate, and that the reduction of α-lipoic acid commenced with quantitative yield. This product was used directly in NMR-pH titration and solubility/lipophilicity measurements. ^{1}H-NMR: (600 MHz, H_2O:D_2O 95:5): 1.36 – 1.44 (*m*, 1 H of CH_2(4)); 1.44 – 1.50 (*m*, 1 H of CH_2(4)); 1.48 – 1.60 (*m*, 1 H of

CH_2(5)); 1.50 – 1.58 (*m*, CH_2(3)); 1.66 – 1.74 (*m*, 1 H of CH_2(5)); 1.74 – 1.82 (*m*, 1 H of CH_2(7)); 1.90 – 1.98 (*m*, 1 H of CH_2(7)); 2.18 (*t*, *J* = 7.5, CH_2(2)); 2.63 – 2.71 (*m*, 1 H of CH_2(8)); 2.68 – 2.76 (*m*, 1 H of CH_2(8)); 3.00 – 3.04 (*m*, CH(6)). HR-MS: 209.0655 ([*M* + H]$^+$; calc. 209.0670).

Dihydrolipoamide (= **(6*R*)-6,8-Disulfanyloctanamide**; **2**) was synthesized in a similar fashion as (**1**), using lipoamide as starting material. ^{1}H-NMR: (600 MHz, H_2O:D_2O 95:5): 1.40 – 1.48 (*m*, 1 H of CH_2(4)); 1.47 – 1.55 (*m*, 1 H of CH_2(4)); 1.56 – 1.62 (*m*, 1 H of CH_2(5)); 1.58 – 1.64 (*m*, CH_2(3)); 1.64 – 1.72 (*m*, 1 H of CH_2(5)); 1.74 – 1.82 (*m*, 1 H of CH_2(7)); 1.89 – 1.97 (*m*, 1 H of CH_2(7)); 2.28 (*t*, *J* = 7.7, CH_2(2)); 2.63 – 2.71 (*m*, 1 H of CH_2(8)); 2.67 – 2.75 (*m*, 1 H of CH_2(8)); 2.99 – 3.03 (*m*, CH(6)); 6.22 (*s*, 1 H of NH_2); 7.54 (*s*, 1 H of NH_2). HR-MS: 208.0838 ([*M* + H]$^+$; calc. 208.0830).

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Table 1. The macroscopic protonation constants of lipoic acid, dihydrolipoic acid, and dihydrolipoamide (0.15 mol/l ionic strength) in log values ± s.d.

	Lipoic acid	Dihydrolipoic acid	Dihydrolipoamide
$\log K_1$	4.65 ± 0.01	11.17 ± 0.01	11.05 ± 0.02
$\log K_2$	–	9.79 ± 0.03	9.69 ± 0.03
$\log K_3$	–	4.64 ± 0.01	–

Table 2. The microscopic protonation constants and interactivity parameters of dihydrolipoic acid in log values ± s.d.

Microscopic protonation constants					
Mid-chain thiolate (S6)		End-chain thiolate (S8)		Carboxylate (O)	
Symbol	Value	Symbol	Value	Symbol	Value
$\log k^{S6}$	10.97 ± 0.01	$\log k^{S8}$	10.74 ± 0.03	$\log k^{O}$	4.87 ± 0.05
$\log k_{S8}{}^{S6}$	10.23 ± 0.04	$\log k_{S6}{}^{S8}$	9.99 ± 0.03	$\log k_{S6}{}^{O}$	4.74 ± 0.05
$\log k_{O}{}^{S6}$	10.85 ± 0.01	$\log k_{O}{}^{S8}$	10.62 ± 0.06	$\log k_{S8}{}^{O}$	4.76 ± 0.01
$\log k_{S8O}{}^{S6}$	10.12 ± 0.07	$\log k_{S6}{}^{S8}$	9.89 ± 0.04	$\log k_{S6S8}{}^{O}$	4.64 ± 0.01
Interactivity parameters					
$\log \Delta E_{S6/S8}$	0.74 ± 0.04	$\log \Delta E_{S6/O}$	0.12 ± 0.01	$\log \Delta E_{S8/O}$	0.10 ± 0.05
$\log \Delta E_{S6/S8}$	0.73 ± 0.07	$\log \Delta E_{S6/O}$	0.10 ± 0.08	$\log \Delta E_{S8/O}$	0.12 ± 0.06

Captions:

Fig. 1. The protonation scheme of dihydrolipoic acid in terms of stepwise macroscopic protonation constants (K_1, K_2, K_3) where L^{3-} is the unprotonated ligand (top); below the site-specific protonation scheme of dihydrolipoic acid, in terms of microspecies (a, b, c, … h) and microscopic protonation constants (k^{S6}, $k_{S6}{}^{S8}$, $k_{S6S8}{}^{O}$, … *etc*.); O, S6, and S8 denote the carboxylate and the thiolate groups bound to the carbons numbered, respectively.

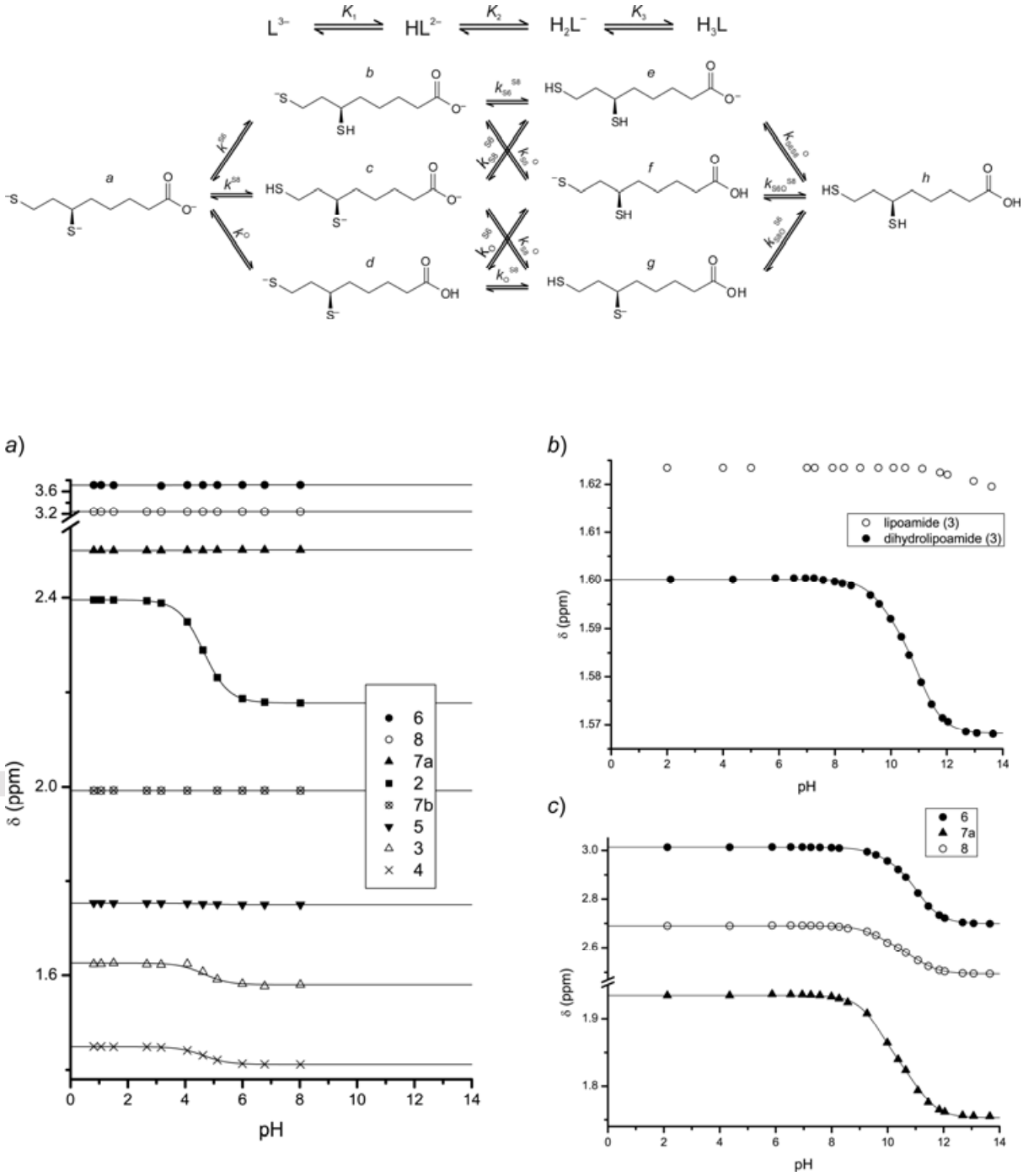
Fig. 2. The plot of ^{1}H chemical shifts versus pH for lipoic acid (A), the C3 protons of dihydrolipoamide and lipoamide (B), and the C6, C7a, C8 protons of dihydrolipoamide.

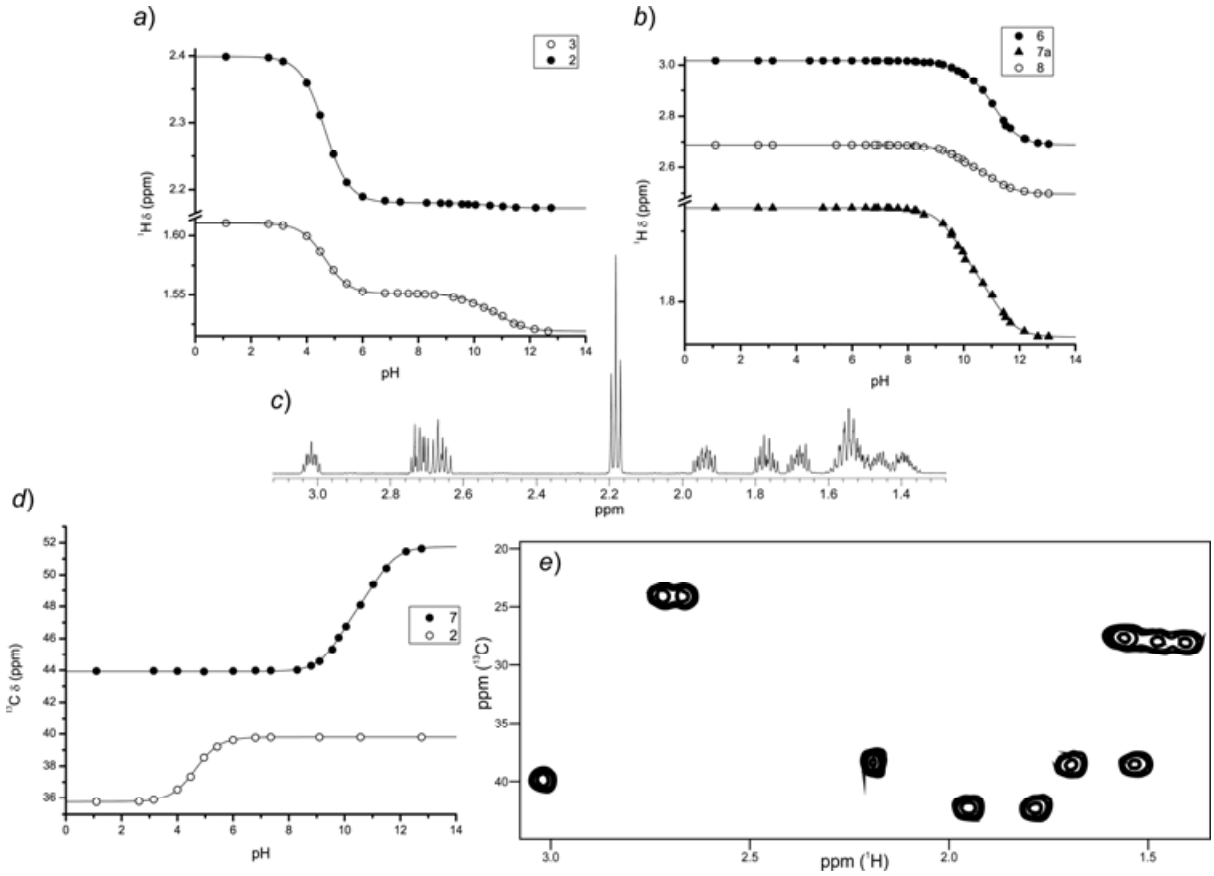
Fig. 3. The plot of ^{1}H chemical shifts versus pH for dihydrolipoic acid (A, B); the ^{1}H NMR spectrum of dihydrolipoic acid at pH~7 (C4 1.3-1.5 ppm, C3 C5a 1.5-1.6 ppm, C5b 1.64-1.68 ppm, C7a 1.76-1.8 ppm, C7b 1.9-2 ppm, C2 2.2 ppm, C8 2.6-2.8 ppm, C6 3.0-3.4 ppm) (C); the plot of ^{13}C chemical shifts versus pH for dihydrolipoic acid (D); the $^{1}H/^{13}C$ HSQC spectrum of dihydrolipoic acid at pH~7 (E).

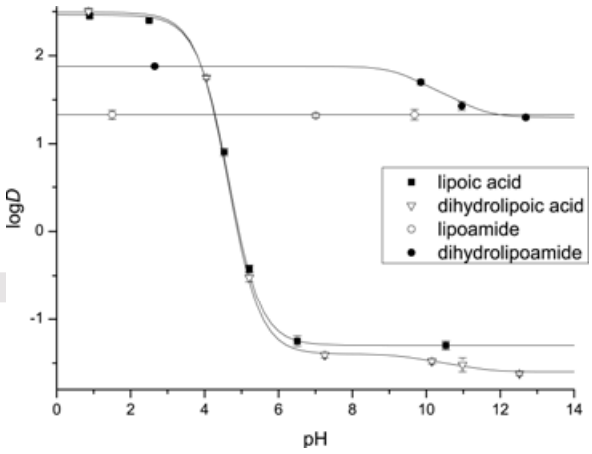
Fig. 4. The plot of log*D* (B) versus pH for lipoic acid, dihydrolipoic acid, lipoamide, and dihydrolipoamide.

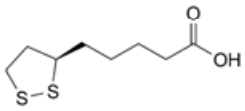
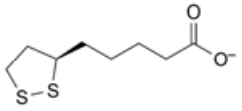
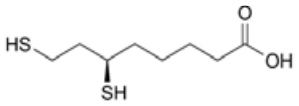
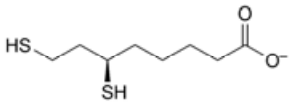
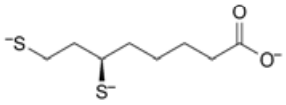
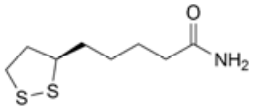
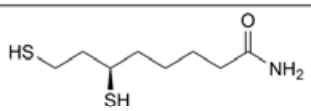
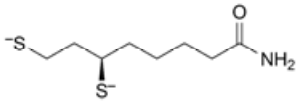
Fig. 5. Partition coefficient values of lipoic acid, dihydrolipoic acid, lipoamide, and dihydrolipoamide in log values ± s.d.

Fig. 6. The relative concentrations, in logarithmic scale, of the various microspecies of dihydrolipoic acid, as a function of pH. The one-letter symbols of the microspecies are defined in *Fig. 1*.







Name	Formula	Log*P*
Lipoic acid		2.46 ± 0.04
(Lipoate)		−1.30 ± 0.02
Dihydrolipoic acid		2.49 ± 0.03
		−1.40 ± 0.02
		−1.60 ± 0.03
Lipoamide		1.33 ± 0.01
Dihydrolipoamide		1.88 ± 0.01
		1.30 ± 0.01