Capillary Electrophoresis, ROESY NMR and Molecular Modelling Study of the Inclusion Complex β-Cyclodextrin/Lipoic Acid

Tommaso Carofiglio,*[a] Roberto Fornasier,*[b] Laszlo Jicsinszky,[c] Giacomo Saielli,[b] Umberto Tonellato,[b] and Rachele Vetta[b]

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The interaction of lipoic acid with β-cyclodextrin (β-CD) has been studied by capillary electrophoresis in aqueous phosphate buffer (20 mM, pH = 9.0) at 20 °C. The results allowed us to detect the formation of an inclusion complex with a 1:1 lipoic acid/β-CD stoichiometry and an association constant of 1645 M^{-1}. The geometry of the inclusion complex in D_2O was investigated by means of 2D-ROESY (Rotating-frame Overhauser Enhancement Spectroscopy) NMR experiments, which showed the occurrence of a bimodal complexation of lipoic acid inside the β-CD cavity. Semi-empirical calculations using the PM3 method show that the orientations of the included lipoic acid are very close in energy, thus confirming the experimental observations.

Introduction

Lipoic acid (1,2-dithiolane-3-pentanoic acid) (Figure 1), a naturally occurring compound, is an essential cofactor of the pyruvate dehydrogenase multienzyme complex.[1]

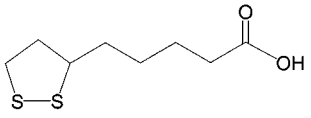

Figure 1. Structure of lipoic acid

Recently, free lipoic acid has been demonstrated to be effective in preventing in vivo oxidative stresses.[1] Its protective properties are ascribed to the ability of the sulfur atoms to scavenge reactive oxygen species such as superoxide, peroxyl, hydroxyl radicals, hypochlorous acid and peroxynitrite, thus breaking the chain reaction of the oxidation of biomolecules. Furthermore, exogenous lipoic acid is being widely investigated in view of its potential therapeutic properties against a variety of diseases, such as diabetes, neurodegenerative disorders, radiation damage, heavy-metal poisoning and acquired immunodeficiency syndrome.[1]

The 1,2-dithiolane ring is the source of the antioxidant activity of lipoic acid, but also of its instability to light, heat and alkaline conditions. Cyclodextrins are well-known for their ability to protect the substrates that are included in their cavity and such a property is exploited by the pharmaceutical industry to preserve drugs from degradation and to control their release. This holds true also in the case of a cyclodextrin-based formulation of the easily degradable lipoic acid so that a study of the properties of its inclusion complex is of great importance. As a matter of fact, the ability of β-CD to form an inclusion complex with lipoic acid has already been discussed in the literature.[3,4] In particular, the value of the binding constant and some of the thermodynamic parameters of the host-guest interaction have been studied by pH-potentiometric techniques and UV/Vis spectroscopy.[4] The present study was undertaken in order to better define the main features of the complex with β-CD and, particularly, the inclusion mode of lipoic acid.

Herein we report the results of an experimental procedure for the determination of the binding constant of the lipoate anion with β-CD based on the use of capillary electrophoresis. More importantly, we also report the results of an NMR spectroscopic investigation and of molecular modelling calculations (using the PM3 method), which allow us to reasonably define the solution structure of the lipoic acid/β-CD inclusion complex and the existence of two possible orientations of the substrate molecule inside the receptor cavity.

[a] Dipartimento di Chimica Inorganica, Metallorganica ed Analitica, Università di Padova,
Via Marzolo 1, 35100 Padova, Italy
Fax: (internat.) + 39-049/827-5239
E-mail: tommaso.carofiglio@unipd.it
[b] Dipartimento di Chimica Organica, and Centro Meccanismi Reazioni Organiche del CNR, Università di Padova,
Via Marzolo 1, 35131 Padova, Italy
[c] Cyclolab R&D Laboratory,
Dombóvári út 5−7, 1117 Hungary

Results and Discussion

Capillary Electrophoresis

In recent years, capillary electrophoresis[5] (CE), a technique that achieves separation of charged solutes inside a narrow-bore capillary filled with a buffer by differential migration in an electric field, has gained acceptance as a probe for molecular interactions. The CE approach to estimate a binding constant relies on measuring the shifts in the electrophoretic mobility of a solute through buffer solutions containing dissolved complexation agents or ligands.[6]

Several groups[7] have discussed the mathematical derivation of the binding isotherms for a number of ligand-solute stoichiometries. Restricting the discussion to the ligand-solute 1:1 interaction, which is pertinent to our study, the theoretical treatment considers a system containing a solute, S, which reversibly binds to a ligand, L, according the equilibrium:

$$S + L \rightleftarrows SL$$

Thus, the experimentally measured electrophoretic mobility (μ_I) of the solute S in a solution containing the ligand L is the weighted average of the mobilities of the solute in the free (μ_f) and complexed (μ_c) states:

$$\mu_i = x_f\,\mu_f + x_c\,\mu_c \qquad (1)$$

where x is the molar fraction of the solute in free (x_f) and complexed (x_c) forms. Equation (1) can also be expressed in terms of the equilibrium concentrations of free solute, [S], and complexed solute, [SL], using the proper mass balances and the equilibrium constant (K = [SL]/([S][L]), to give Equation (2):

$$\mu_i = \frac{\mu_f + \mu_c K[L]}{1 + K[L]} \qquad (2)$$

This equation relates the experimental electrophoretic mobility of the solute to the concentration of the ligand. Equation (2) can be rearranged into the following linear form:

$$\frac{1}{\mu_i - \mu_f} = \frac{1}{(\mu_c - \mu_f)K[L]} - \frac{1}{(\mu_c - \mu_f)} \qquad (3)$$

Equation (3) yields a linear plot, and the value of the binding constant K can be calculated by the quotient of the intercept and the slope of the straight line obtained by plotting $1/(\mu_i - \mu_f)$ versus 1/[L] (double-reciprocal plot).

The electrophoretic mobility of lipoic acid (0.48 mM) was measured under an applied potential of 15 kV (normal mode polarization, detector at the cathode side) as a function of the β-CD concentration (range 0 to 5 mM) in the background electrolyte (20 mM phosphate buffer, pH = 9.0). Figure 2 shows the binding plot (a) and the corresponding double reciprocal plot (b). From the linear regression a value for the binding constant of 1645 M^{-1} can be calculated. Lipoic acid, whose pK_a is 4.8,[1] is completely dissociated under the analysis conditions so that the above value of the binding constant refers to the inclusion of the lipoate anion in the β-CD. As a comparison, Junquera[4] and co-workers have reported a slightly larger value of the binding constant (2390 M^{-1}) from pH potentiometric experiments. Furthermore, they estimated that the affinity of β-CD for the undissociated form of lipoic acid is approximately 1.4 times higher than that of lipoate anion.

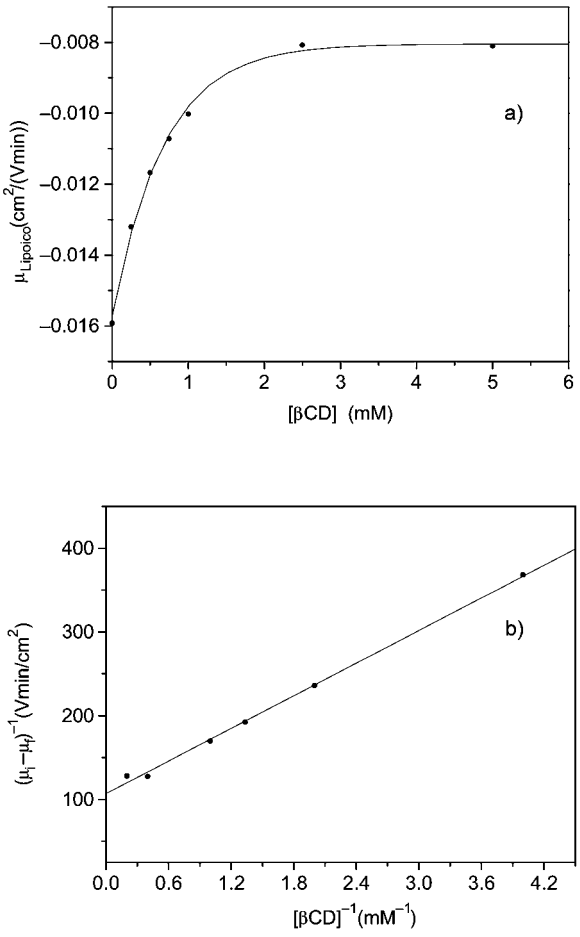

Figure 2. Binding curve (a) and double-reciprocal plot (b) for the titration of lipoic acid with β-CD

It should be pointed out that, as in the case of Aincart, we used racemic D,L-lipoic acid, which is commercially available, for the determination of the binding constant with β-CD. Since β-CD is a chiral molecule, the inclusion complexes of the two enantiomeric forms of lipoic acid are a pair of diastereoisomers and they should have different stabilities. This is the basis for the use of cyclodextrins as selectors in chiral capillary electrophoresis.[8] However, in the case of lipoic acid, we did not observe any splitting of the peaks in the electropherograms so that we can safely infer that the binding constants for the two enantiomers are almost indistinguishable. The reported binding constant should therefore be correctly defined as the averaged value

of the very close binding constants of the two lipoic acid enantiomers with β-CD.

NMR Experiments

In order to obtain detailed information about the solution structure of the lipoic acid/β-CD inclusion complex, we carried out an NMR spectroscopy study in D_2O. Figure 3 shows the numbering scheme used for the carbon atoms of both β-CD and lipoic acid used below. The protons are named as belonging to the carbon atom to which they are attached and a prime is added to the β-CD protons to distinguish them from the lipoic acid ones. The protons of β-CD pointing inside the cavity are H3′, H5′ and H6′ whereas the outer protons are H1′, H2′, and H4′.

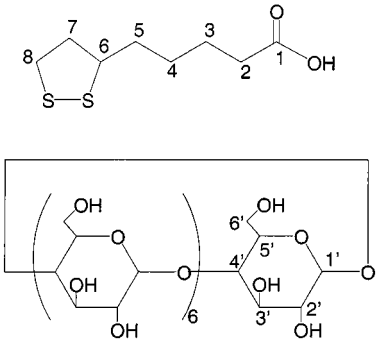

Figure 3. Numbering scheme for lipoic acid and β-CD

Lipoic acid possesses two different hydrophobic regions, namely the dithiolane ring and the aliphatic chain, whereas the carboxyl group is relatively hydrophilic in character. Furthermore, we can expect two different orientations of the lipoic acid inside the β-CD cavity, with the carboxyl group protruding either from the primary or from the secondary side of the macrocycle (Figure 4); these two isomeric structures are referred to as *carboxyl-up* and *carboxyl-down*, respectively. The ^1H and ^{13}C NMR spectra of lipoic acid and β-CD have already been assigned in the literature[9] and we have confirmed them by ^1H-^1H COSY spectroscopy.

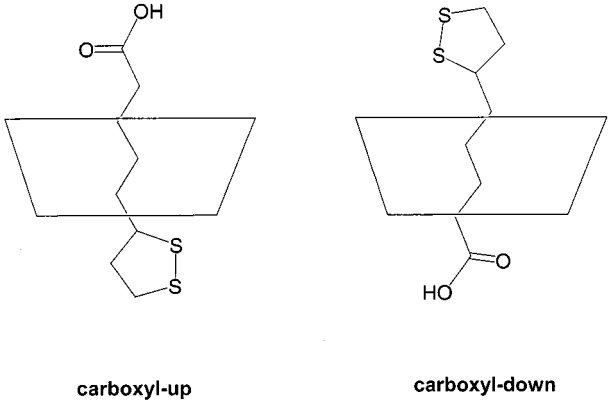

Figure 4. Inclusion modes of lipoic acid into the β-CD cavity

In order to infer the geometry of the β-CD/lipoic acid inclusion complex from the NOE intensity, we recorded the 400 MHz ROESY spectrum of a 2.4 mM solution of lipoic acid in D_2O in the presence of 5.5 equivalents of β-CD to ensure a shift of the inclusion equilibrium toward the formation of the complex and obtain experimentally detectable species signals (Figure 5). The NOE values were used qualitatively and no quantitative conclusions on intermolecular distances were extracted due to the large dynamics in the complexation process.

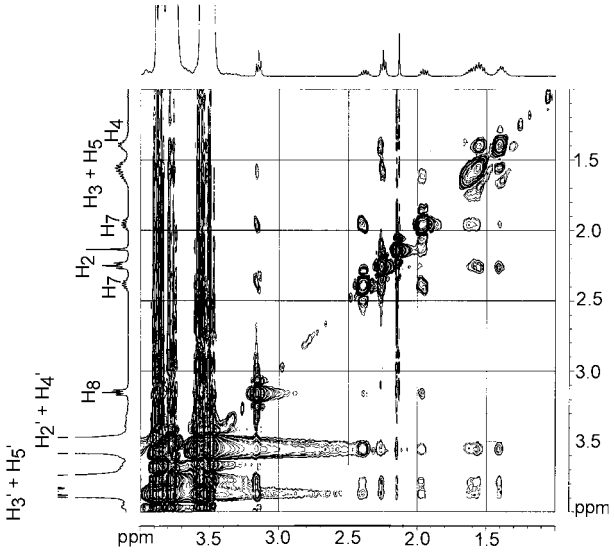

Figure 5. 2D-ROESY NMR spectrum of lipoic acid/β-CD inclusion complex in D_2O

All the protons belonging to the lipoic acid give rise to cross-peaks with H3′, H5′ and H6′, confirming the inclusion. However, the simultaneous presence of NOEs for all the protons of lipoic acid with all the inter-cavity protons of β-CD indicates that both the two geometries of the inclusion complexes are possible in solution, with no appreciable preference for either of the two.

Furthermore, cross-peaks were observed between the lipoic acid protons and the H2′ and H4′ protons that lie outside the cavity of the β-CD. This result is rather common and is generally explained as being due to the occurrence of a dipolar transfer via their *J* coupling to H3′ and H5′ rather than to the formation of an external host-guest complex.

Molecular Modelling Calculations

We used the semiempirical PM3 method[10] to calculate the energy minimum for the host/guest complexes that can be formed by the inclusion of the lipoic acid with β-CD. Only recently have semiempirical methods — in particular the PM3 Hamiltonian — been used to study host-guest complexes involving cyclodextrins and the results appear to be promising.[11] Whilst acknowledging that the solvent plays an important role in the inclusion process, as will be discussed below, no solvent effects have been included in the calculations, since at this level it would result in very high computational efforts and cost.

As stated above, two different inclusion geometries can be expected, as shown in Figure 4. Furthermore, considering the two enantiomeric forms of lipoic acid, a total of four possible complexes have to be considered. The (*R*)- and (*S*)-enantiomers of lipoic acid were built with the Hyperchem[12] program with the alkyl chain in the *all-trans* conformation. The geometry was then optimized with Gaussian 98[13] at the PM3 level. β-CD was also optimized with Gaussian 98 at the PM3 level starting from the crystal structure reported in the literature.[14]

A full optimization of the inclusion compound was expected to lead to a local minimum whose final energy is strongly dependent on the starting configuration selected for the host-guest complex. Thus, we adopted a step-by-step insertion method widely used in the literature[15] to simulate the docking process of the guest inside the host molecule. We prepared a set of starting configurations each one made from the isolated optimized configurations of β-CD and lipoic acid. The two molecules were oriented so that their principal axes of inertia and their mass centres were coincident. For the CD this results in the *xy* plane being roughly the plane containing the glycosidic oxygens and the *z*-axis being the pseudo C_7 symmetry axis. For the lipoic acid, the *z*-axis is roughly oriented as the long molecular axis. After aligning the two molecules the starting configurations were generated by displacing the lipoic acid with respect to the β-CD by ±1 Å, ±2 etc. A full optimization at the PM3 level was then performed.

In Figure 6 we report, as an example, the energy curve for the inclusion process of the lipoic acid *S*-enantiomer inside the β-CD. The squares represent the *carboxyl-up* geometry and the circles the *carboxyl-down* geometry.

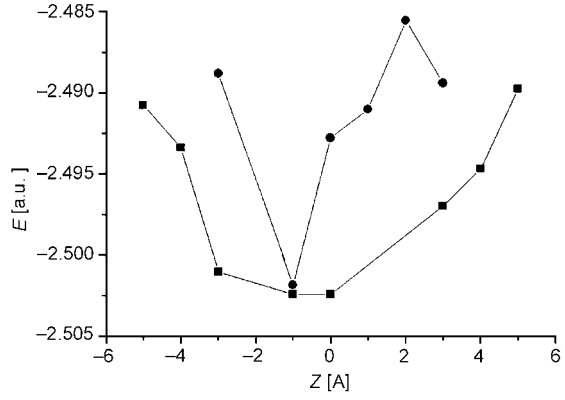

Figure 6. Emulation of the docking process of (*S*)-lipoic acid into the β-CD; : carboxyl-down geometry; •: carboxyl-up geometry; see text for the definition of the energy and *Z* coordinate

The *Z* coordinate represents the starting displacement between the centre of mass of the two molecules. The energy is defined in atomic units as in the PM3 Hamiltonian. The difference in energy between the minima for the *carboxyl-up* and *carboxyl-down* orientation of the guest is only 0.4 kcal/mol. The optimized geometries of the *carboxyl-up* and *carboxyl-down* complexes are shown in Figure 7.

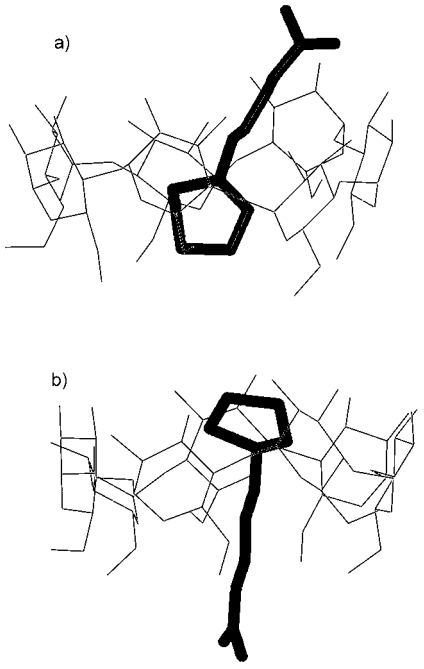

Figure 7. Optimised geometries of the lipoic acid/β-CD inclusion complex in the carboxyl-up (a) and carboxyl-down (b) structure

The two minimum energy configurations have been tested by calculating their vibrational frequencies at the PM3 level: no negative eigenvalues have been found for these structures. We also extended the search for the global minimum by performing an optimization starting from structures obtained by rotating the lipoic acid around the *z* axis in the range ±10° up to ±50°, with steps of 10°, for a value of the *Z* coordinate of −1.0 Å, corresponding to that of the minimum. None of these optimizations resulted in a lower value of the energy for the optimized structure.

The complexation energies, calculated as the difference between the energy of the complex and the sum of the energies for the lipoic acid and β-CD optimized separately, are reported in Table 1 for the four possible cases. For both enantiomers there is a relatively high stabilization (greater than 16 kcal/mol) due to the formation of the complex. However, since the difference between the *carboxyl-up* and *carboxyl-down* geometries is small, both complexes are of similar energy and therefore both are likely to be formed, as verified experimentally in solution by NMR experiments.

Table 1. Stabilization energies for the various complexes

Compound	E_{tot} [kcal/mol]	ΔE[a] [kcal/mol]
β-CD	−14364.7	–
Lipoic acid	−2447.6	–
(*S*)-carboxyl down	−16828.8	−16.5
(*S*)-carboxyl up	−16828.4	−16.1
(*R*)-carboxyl down	−16830.1	−17.8
(*R*)-carboxyl up	−16829.2	−16.9

[a] Calculated as $E_{complex} - (E_{\beta\text{-CD}} + E_{acid})$.

The results obtained from the calculations shows that the stable complexes formed by lipoic acid and β-CD have a structure where the dithiolane ring is included into the cavity while the alkyl chain and the carboxylic end are found outside. This is true for both geometries shown in Figure 7. If we optimize a starting configuration whereby the carboxylic end is inside the cavity we often get the formation of hydrogen bonds between the lipoic acid and the cyclodextrin; however, the resulting structure is always more than 5 kcal/mol higher in energy than the conformation at the minimum. Therefore, it seems that van der Waals interactions between the larger and more polarizable dithiolane ring play a dominant role in stabilizing the complexes.

At this point a consideration of the role of the solvent is in order. The calculations were performed assuming in vacuo conditions, even though the solvent effect may be very important. However, as an a posteriori justification, we observe that, due to the very similar structure of the *carboxyl-up* and *carboxyl-down* complexes, in which the carboxylic part of the lipoic acid remains outside the β-CD cavity, the effect of the solvent is likely to be very similar in both cases. Moreover, the presence of a solvent, namely water, would lead to a larger stabilization of the outer chain because of the hydrogen bonds formed with the carboxylic end. Thus, a semiquantitative account of the inclusion process can be drawn from the consideration of the interaction energies alone.

In Table 1 we also report the results for the complexation of the (*R*)-enantiomer of the lipoic acid with β-CD, obtained in a similar way as for the (*S*)-enantiomer. Again we notice that the complex appears to be very stable and that the difference in energy between the *carboxyl-up* and *carboxyl-down* geometries is relatively small. In this case the similarity between the two enantiomers is even more pronounced: the optimized structures of the (*R*)-lipoic acid/β-CD inclusion complex are similar to those found for the (*S*)-enantiomer, with the dithiolane moiety inside the cavity and the carboxylic end outside (data not reported here).

Conclusions

Capillary electrophoresis measurements clearly indicate that, in water, lipoic acid forms 1:1 inclusion complexes with β-CD with an association constant of 1645 M^{-1}. 2D ROESY NMR experiments, combined with semiempirical molecular modelling calculations (PM3 Hamiltonian), supply strong evidence concerning the mode of inclusion, which may be either *carboxyl-up* or *carboxyl-down* without any appreciable difference between the two enantiomeric forms of the lipoic acid. In our view, one of the most important results of this study, clearly emphasised by the modelling calculations, is that concerning the location of the dithiolane moiety, which is in each case inserted within the cavity of the complex. The sulfur-containing ring, the part of the molecule that is most sensitive to chemical or physical attacks leading to the degradation of lipoic acid in water and in most biological environments, appears, therefore, to be sufficiently well protected to be able to justify the use of its CD complexes.

Experimental Section

Chemicals: Lipoic acid and D_2O were purchased from Fluka. β-CD was obtained from Cyclolab.

Capillary Electrophoresis: CZE experiments were performed on a BioFocus3000 capillary electrophoresis system from $^{bio\text{-}rad}$ (Richmond, CA, USA) equipped with a multi-wavelength UV/Vis detector. An uncoated fused silica capillary (Composite Metal Services LTD, UK) of 50 μm I.D. with a total length of 50 cm (45.4 cm to the UV/Vis detector window) mounted on a user-assembled cartridge was used. A voltage of 15 kV was used for the separation and the detection was set at 214 nm. Sample injection was achieved using the pressure mode set at 5 psi. Both carousels and cartridge were thermostated at 20 °C with air and a circulating liquid system, respectively. Preliminary to each set of experiments, the capillary was thoroughly rinsed with 1 M NaOH solution for 15 min and then with deionized water and running buffer for 15 min. During the interval between two runs the capillary was rinsed with the running buffer for 3 min. A solution containing high concentrations of β-CD became viscous so it was important to make viscosity corrections by measuring the current during capillary electrophoresis experiments. Thus, the relative viscosity was determined by taking the ratio of the currents I_0 at [β-CD] = 0 and I at [β-CD] = C and the viscosity correction was obtained by multiplying the experimental electrophoretic mobility for such a ratio.

NMR Spectroscopy: NMR spectra were obtained on a Bruker Avance DRX-400 spectrometer fitted with a BBI probe. ROESY experiments were recorded using a 2.4 mM solution of lipoic acid in D_2O in the presence of 5.5 equivalents of β-CD. The experiments were carried out at 750 ms mixing time. NMR computations were performed on an SGI-Indy Data Station.

Calculations: Lipoic acid [(*R*)- and (*S*)-enantiomer] was built with the Hyperchem[12] program with the alkyl chain in the *all-trans* conformation. The geometry was then optimized with Gaussian 98[13] at the PM3 level. β-CD was also optimized with Gaussian 98 at the PM3 level starting from the crystal structure reported in the literature. The calculations were run on a DEC/OSF1 workstation. In order to build the starting geometry for the inclusion complex, both molecules are oriented in their principal axes of inertia system. The inertia tensor **I** is defined as

$$I_{ij} = \sum_n m_n \left(\mathbf{r}^2 \delta_{ij} - r_{i,n} r_{j,n} \right)$$

where n is the number of atoms in the molecule, m_n the mass of the n-th atom, $r_{i,n}$ the i-th cartesian component of the n-th atom, with $i = x, y, z$ and δ_{ij} the Kronecker symbol. The inertia tensor was diagonalized and the matrix of the eigenvectors used as a Euler matrix to rotate the molecule so that the principal axes of inertia were coincident with the axes of the reference system. For the CD this results in the xy plane being roughly the plane containing the glycosidic oxygens and the z-axis being the pseudo C_7 symmetry axis. For the lipoic acid, the z-axis is roughly oriented as the long molecular axis defined by the alkyl chain. After aligning the two molecular reference systems, the starting configurations were generated by displacing the lipoic acid with respect to the CD by ±1Å, ±2Å, ±3Å along the z-axis. A full optimization at the PM3 level was then performed on each starting configuration.

Acknowledgments

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