Experimental and Theoretical Studies of Vacuum-Ultraviolet Electronic Circular Dichroism of Hydroxy Acids in Aqueous Solution

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ABSTRACT   The electronic circular dichroism (ECD) spectra of three L-hydroxy acids (L-lactic acid, (+)-(S)-2-hydroxy-3-methylbutyric acid, and (−)-(S)-2-hydroxyisocaproic acid) were measured down to 160 nm in aqueous solution using a vacuum-ultraviolet ECD spectrophotometer. To assign the two positive peaks around 210 and 175 nm and the one negative peak around 190 nm in the observed spectra, the ECD spectrum of L-lactic acid was calculated using time-dependent density functional theory (DFT) for the optimized structures by DFT and a continuum model. The observed ECD spectrum was successfully reproduced as the average spectrum for four optimized structures with seven water molecules that localized around the COO^- and OH groups of L-lactic acid. The positive peak around 210 nm and the negative peak around 185 nm in the calculated spectrum were attributable to the $n\pi^\star$ transition of the carboxyl group, with the latter peak also being influenced by the $\pi\pi^\star$ transition of the carboxyl group; however, the positive peak around 165 nm involved unassignable higher energy transitions. The comparison of the calculated ECD spectra for L-lactic acid and L-alanine revealed that the network with loose hydrogen bonding around the COO^- and OH groups is responsible for the flexible conformation of hydroxy acids and complicated side-chain dependence of ECD spectra relative to amino acids. *Chirality 00:000–000, 2011.*   © 2011 Wiley-Liss, Inc.

KEY WORDS: assignment of electronic circular dichroism; L-lactic acids; time-dependent density functional theory; hydrated structures; synchrotron-radiation spectroscopy

INTRODUCTION

Electronic circular dichroism (ECD) spectroscopy is very useful for analyzing the structure of chiral molecules such as organic materials and biomolecules, because it is very sensitive to the conformation of optically active compounds. ECD spectroscopy cannot provide structural information at the atomic level, but combining it with theoretical analysis methods such as the octant rule, π-SCF-CI-DV method, and Tinoco's method has revealed the absolute configurations of various organic compounds with aromatic hydrocarbons[1] and the conformation of large molecules such as polypeptides and proteins.[2] However, such combination methods have been limited to the far- and near-ultraviolet (UV) regions of the ECD spectrum, as conventional ECD spectrophotometers cannot make measurements in the vacuum-UV (VUV) region below 190 nm, where detailed novel information based on the higher energy transitions ($\pi\pi^\star$ and $n\sigma^\star$) of chromophores such as COO^-, NH_3^+, and OH groups could be obtained.

To extend the ECD measurements into the VUV region, several synchrotron-radiation facilities have developed ECD spectrophotometers that use synchrotron radiation as an intense light source.[3–6] We constructed the VUV-ECD spectrophotometer at Hiroshima Synchrotron Radiation Center (HiSOR),[7] with which we have successfully measured the ECD spectra of amino acids,[8] proteins,[9] and saccharides[10] in aqueous solution down to 160 nm.

To theoretically assign the ECD spectrum of L-alanine down to 140 nm, Fukuyama et al. optimized the structure of L-alanine with nine hydrated water molecules using density functional theory (DFT) and calculated the ECD spectrum by time-dependent DFT (TDDFT).[11] The obtained VUV-ECD spectrum of L-alanine reproduced the peak positions and intensities of the experimentally observed spectrum, which could be assigned to $n\pi^\star$ and $\pi\pi^\star$ transitions of the carboxyl group, thereby elucidating the detailed hydrated structure of L-alanine in aqueous solution. Šebek et al. compared the ECD spectra of di-alanine measured down to 170 nm at three pH values (1.43, 6.17, and 12.1) with those theoretically calculated by the TDDFT method, and found that the anionic, cationic, and zwitterionic forms of di-alanine were hydrated with 10 water molecules.[12] Nielsen et al. also applied such a combination method to investigate the fingerprints of bonding motifs in DNA duplexes of adenine and thymine.[13] However, theoretical analyses of ECD spectra of biomolecules have remained limited, and further developments of the ECD theory are necessary to confirm the validity of VUV-ECD spectroscopy in structural biology.
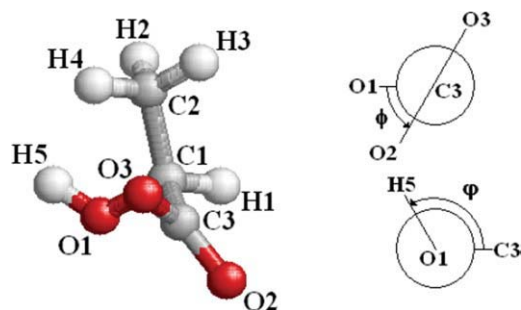
Fig. 1. Model structure and definition of dihedral angles of LA. Oxygen, hydrogen, and carbon atoms are colored red, white, and gray, respectively. ϕ and φ indicate the dihedral angles of the COO^- ($O1-C1-C3-O2$) and OH ($O3-C1-O1-H5$) groups, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In this study, we measured the VUV-ECD spectra of three ʟ-hydroxy acids—lactic acid (LA), (+)-(S)-2-hydroxy-3-methylbutyric acid (HMBA), and (−)-(S)-2-hydroxyisocaproic acid (HICA)—in aqueous solution, and theoretically calculated the ECD spectrum of LA using the TDDFT method. ʟ-Hydroxy acids have a hydroxyl group (OH) instead of the amino group (NH_3^+) that is present in ʟ-amino acids, and hence the theoretical calculation of ECD spectra of ʟ-hydroxy acids would be helpful for understanding the relationships between the ECD spectrum and structure of both hydroxy acids and amino acids. The differences in ECD spectra between LA and ʟ-alanine are discussed here based on the theoretically determined hydration structures of the two compounds.

EXPERIMENTAL AND THEORETICAL METHODS
Materials

Three hydroxy acids (LA, HMBA, and HICA) were purchased from Sigma-Aldrich (St. Louis, MO). All hydroxy acids were of high purity (>98%) and were used without further purification. The sample solutions were freshly prepared by dissolving the hydroxy acids in double-distilled water at concentrations of 5.0–10.0 (w/v %) and adjusting them to pH 5.1–5.4 with sodium hydroxide.

ECD Measurements

The ECD spectra of the hydroxy acids were measured from 250 to 160 nm in aqueous solution at 25°C using the VUV-ECD spectrophotometer at HiSOR. The details of the optical devices of the spectrophotometer are given in previous papers.[14,15] The ECD measurements were made using an assembled-type optical cell with MgF_2 windows.[16] The path length of the cell was adjusted with a Teflon spacer to 50 μm for measurements from 250 nm to 175 nm. To reduce the effect of light absorption by water, no spacer was used for measurements below 175 nm. The spectra obtained without the spacer (path lengths of 1.4–2.7 μm) were calibrated by normalizing the ellipticities to the spectra measured using a 50-μm spacer in the overlapping wavelength region from 250 nm to 175 nm. All of the spectra were recorded with a 1.0-mm slit, a 16-sec time constant, a 4-nm/min scan speed, and 4–9 accumulations. A commercial spectrophotometer (J-720W, Jasco) was also used for comparisons of the ECD spectra in the far-UV region from 250 nm to 190 nm. The ellipticity was reproducible within an error of ±5%, which was mainly attributable to signal noise and inaccuracy in the light path length.

Initial Structures of LA

The 16 model structures of LA were constructed with the standard molecular parameters for bond lengths (C−C, 1.53 Å; C−O, 1.25 Å; C−H, 1.09 Å; and O−H, 0.97 Å) and bond angles (O−C−O, 125.00°; and

C−C−N, C−C−H, and C−O−H, 120.00°)[17–21] by changing the dihedral angles of the COO^- group ($\phi = 0°$ and 90°) and the OH group ($\varphi = -120°$ to 180°). The model structure of LA and the definition of the dihedral angles are shown in Figure 1.

Optimization of LA Structure

In a previous study, we successfully optimized the hydrated structure of ʟ-alanine with nine water molecules using the DFT method at the B3LYP/6-31G* level.[11] The DFT method has also been used for optimizing the LA structure in aqueous solution.[22,23] Therefore, we adopted the DFT method to optimize the LA structure at the B3LYP/6-31G* level. The effect of the solvent surrounding LA was calculated using a continuum model (Onsager) with the dielectric constant of water (78.39) and a recommended cavity radius for a solute volume. Optimization was performed with the Gaussian 98 program (Gaussian)[24] on an HPC 3000 computer (HPC Systems, Japan).

Calculation of ECD Spectra

Circular dichroism (CD) is induced by the interaction between electric and magnetic dipole transition moments of chromophores. As for the relationship between absorption and the dipole strength, CD is related to the rotational strength, R, which is theoretically defined by[1,25]

$$R_{0a} = Im\left\{ \langle \Psi_0|\hat{\boldsymbol{\mu}}|\Psi_{\mathbf{a}}\rangle \cdot \langle \Psi_{\mathbf{a}}|\hat{\mathbf{m}}|\Psi_{\mathbf{0}}\rangle \right\} \tag{1}$$

where R_{0a} is the rotational strength of the electric transition from the "0" to "a" states, $\hat{\boldsymbol{\mu}}$ and $\hat{\mathbf{m}}$ are the electric and magnetic dipole moments, respectively, and Im{} is the imaginary part of a complex number. The rotational strength is expressed in cgs units (erg cm³), which are conveniently transformed into a Debye-Bohr magnetron (1 DBM = 0.9273×10^{-38} erg cm³ = 0.9273×10^{-51} J m³).

The final CD spectrum can be calculated using the following equations:

$$R_i = 1.23 \times 10^{-42} \frac{[\theta]_i \Delta\lambda}{\lambda_i} \tag{2}$$

$$[\theta](\lambda) = \sum_i [\theta]_i \exp\left[-\left(\frac{\lambda - \lambda_i}{\Delta\lambda}\right)^2 \right] \tag{3}$$

where $[\theta]$ is the molar ellipticity, λ_i is the wavelength of the ith transition, and $\Delta\lambda$ is the half bandwidth of a spectrum calculated assuming that it conforms to the Gaussian distribution. The ECD spectra were cal-
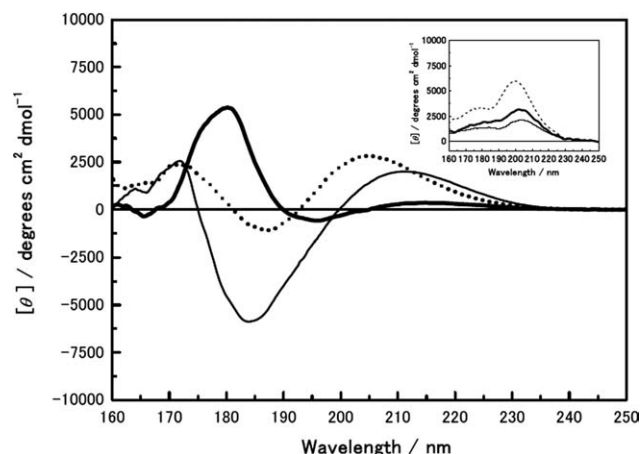

Fig. 2. Experimentally observed VUV-ECD spectra of ʟ-hydroxy acids: LA, solid line; HMBA, bold line; and HICA, dotted line. For comparison, the insert shows spectra of ʟ-alanine (solid line), ʟ-valine (bold line), and ʟ-leucine (dotted line).[8]

TABLE 1. Peak wavelengths (λ) and molar ellipticities ([θ]) of the VUV-ECD spectra of L-hydroxy acids and L-amino acids in aqueous solution

Side chain		L-Hydroxy acids		L-Amino acids[a]		
		λ (nm)	[θ] (degrees cm^2 dmol^{-1})		λ (nm)	[θ] (degrees cm^2 dmol^{-1})
Methyl	LA	211.2	2000	L-Alanine	203.3	2200
		183.8	−5900		184.1	1400
		171.9	2600			
Isopropyl	HMBA	214.8	360	L-Valine	202.3	3200
		196.1	−600		181.4	1900
		180.2	5400			
Isobutyl	HICA	205.0	2800	L-Leucine	200.1	6200
		187.2	−1100		179.4	3500
		171.9	2300			

[a]Ref. 8.

culated using Gaussian 98. The rotational strength, R_i, was calculated using the TDDFT method at the B3LYP/6-31+G** level and a polarized continuum model (PCM)[26] to account for the effect of the solvent. From the obtained rotational strength, ECD spectra were calculated using eqs. 2 and 3 with a $\Delta\lambda$ value of 12.5 nm.

RESULTS AND DISCUSSION
ECD Spectra of Hydroxy Acids

Figure 2 shows the VUV-ECD spectra of LA, HMBA, and HICA down to 160 nm in aqueous solution. The ECD spectrum of LA exhibits two positive peaks around 211 nm and 172 nm, and one negative peak around 184 nm, and these peaks shift to around 215 nm, 180 nm, and 196 nm, respectively, for HMBA, and to around 205 nm, 172 nm, and 187 nm, respectively, for HICA. The peak positions and molar ellipticities for the three L-hydroxy acids are summarized in Table 1. These are the first VUV-ECD data for L-hydroxy acids to be reported, although some ECD data down to 200 nm have been reported for LA and D-lactic acid.[27–29]

The ECD spectra of the three hydroxy acids exhibited similar peak positions but significantly different intensities. The structures of these hydroxy acids differ only in the sizes of side chains (methyl, isopropyl, and isobutyl groups for LA, HMBA, and HICA, respectively). Table 1 indicates that there is no correlation between the CD intensity and the bulkiness of the side chains, although the CD intensities of L-alanine, L-valine, and L-leucine—corresponding to LA, HMBA, and HICA in the side chains, respectively—increase with the side-chain bulkiness.[8] The mechanism underlying this difference in the side-chain dependence of ECD spectra between amino acids and hydroxy acids is unclear, as the absorptions of the OH and NH$_3^+$ groups are affected in a complicated manner not only by the bulkiness but also by the charge and hydration around the side chains. However, theoretically assigning ECD spectra is important to characterizing the spectral differences between hydroxy acids and amino acids.

ECD Spectra of the Initial Structures of LA

To elucidate the effects of the dihedral angles of the COO$^-$ and OH groups on the ECD spectrum of LA, the ECD spectra of the 16 model structures (see "Experimental and Theoretical Methods" Section) were calculated by the TDDFT method without optimization. Figure 3 shows the ECD spectra of LA with the OH group having one of eight different dihedral angles ($\varphi = -120°$ to 180°) and the COO$^-$

group having one of two different dihedral angles ($\phi = 0°$ or 90°). The peak positions and intensities of ECD spectra are markedly affected by the dihedral angle of the OH group when $\phi = 0°$ but not when $\phi = 90°$, which suggests that this angle strongly affects the ECD spectrum of LA when the O2 (or O3) atom of the COO$^-$ group is close to the O1 atom of the OH group (Fig. 1).
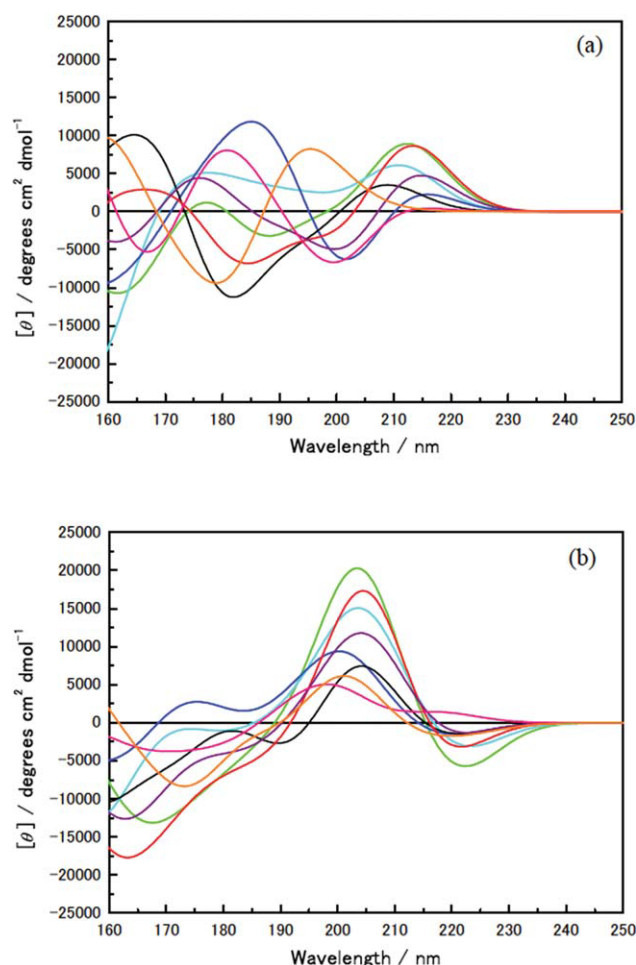

Fig. 3. Theoretical VUV-ECD spectra of 16 model structures for dihedral angles (ϕ) of the COO$^-$ group of 0° (a) and 90° (b). The dihedral angles (φ) of the OH group were −120° (green), −90° (light blue), −60° (dark blue), 0° (black), 60° (red), 90 (purple), 120° (pink), and 180° (orange). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
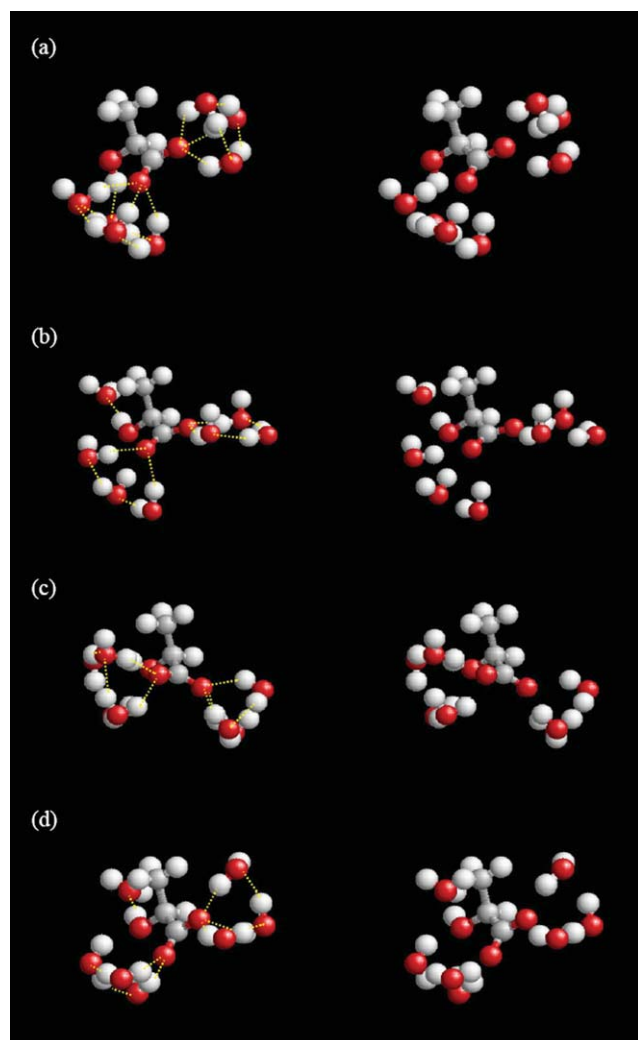
Fig. 4. Stereographs of the optimized structures of LA with seven hydrated water molecules. **(a–d)** represent the optimized structures of (ϕ, φ) pairs of $(0°, -90°)$, $(0°, 180°)$, $(90°, 90°)$, and $(90°, 180°)$, respectively. Oxygen, hydrogen, and carbon atoms are colored red, white, and gray, respectively. The lengths of hydrogen bonds between oxygen and hydrogen atoms (dashed lines) are optimized to within 2.1 Å. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Optimization of the Hydrated Structures of LA

There have been experimental and theoretical evaluations of the amounts of hydration of COO^- and OH groups.[21,30–32] The hydrated structure of L-alanine could be optimized with six water molecules around the COO^- group and three water molecules around the NH_3^+ group.[11] On the other hand, Sadlej et al. successfully optimized the hydrated structure of D-lactic acid with one water molecule around the OH group using the DFT method.[22] Therefore, we constructed eight initial structures of LA with one and six water molecules around the OH and COO^- groups, respectively, for dihedral-angle pairs (ϕ, φ) of $(0°, -90°)$, $(0°, 0°)$, $(0°, 90°)$, $(0°, 180°)$, $(90°, -90°)$, $(90°, 0°)$, $(90°, 90°)$, and $(90°, 180°)$. The following molecular parameters were used for the water molecules that were bonded via hydrogen to LA: O—H, 0.948 Å; H—O—H, 106.6°; $COO^-\cdots$H—O, 2.011 Å; and O—H$\cdots$H—O, 2.011 Å.

The four initial structures of LA having (ϕ, φ) pairs of $(0°, -90°)$, $(0°, 180°)$, $(90°, 90°)$, and $(90°, 180°)$ were successfully optimized with seven water molecules. The stereographs of

the optimized structures are shown in Figure 4, and their molecular parameters are listed in Table 2 and Supporting Information (Table S1). The hydrogen bonds between solute and water molecules (yellow dotted lines in Fig. 4) had lengths of 1.73–2.13 Å, which is very consistent with results previously observed for L-alanine (1.75–1.95 Å).[11] Previous optimization studies of the structures of LA and D-lactic acid with a few water molecules in vacuo and PCM suggested that both compounds take the most stable conformation when intramolecular hydrogen bonds are formed between the OH and COO^- groups.[22,23,33] However, no such intramolecular hydrogen bond was formed in any of the optimized structures with seven water molecules, and the COO^- and OH groups formed hydrogen bonds with the water molecules only. This result suggests that seven hydrated water molecules around COO^- and OH groups play an important role in the optimized structures of LA in aqueous solution.

ECD Spectra of the Hydrated Structures of LA

Figure 5 shows the rotational strengths and ECD spectra calculated for the four optimized structures of LA with seven hydrated water molecules. These hydrated water molecules were ignored when calculating the ECD spectra due to computational limitations. All of the calculated spectra were greatly influenced by the dihedral angles of the COO^- and OH groups and were not consistent with the experimentally observed spectrum in terms of peak positions and/or intensities. The total energies of the four optimized structures as estimated by the DFT method were very close to each other, suggesting that these four structures would coexist in almost

TABLE 2. Bond lengths, bond angles, and dihedral angles of four optimized structures of LA

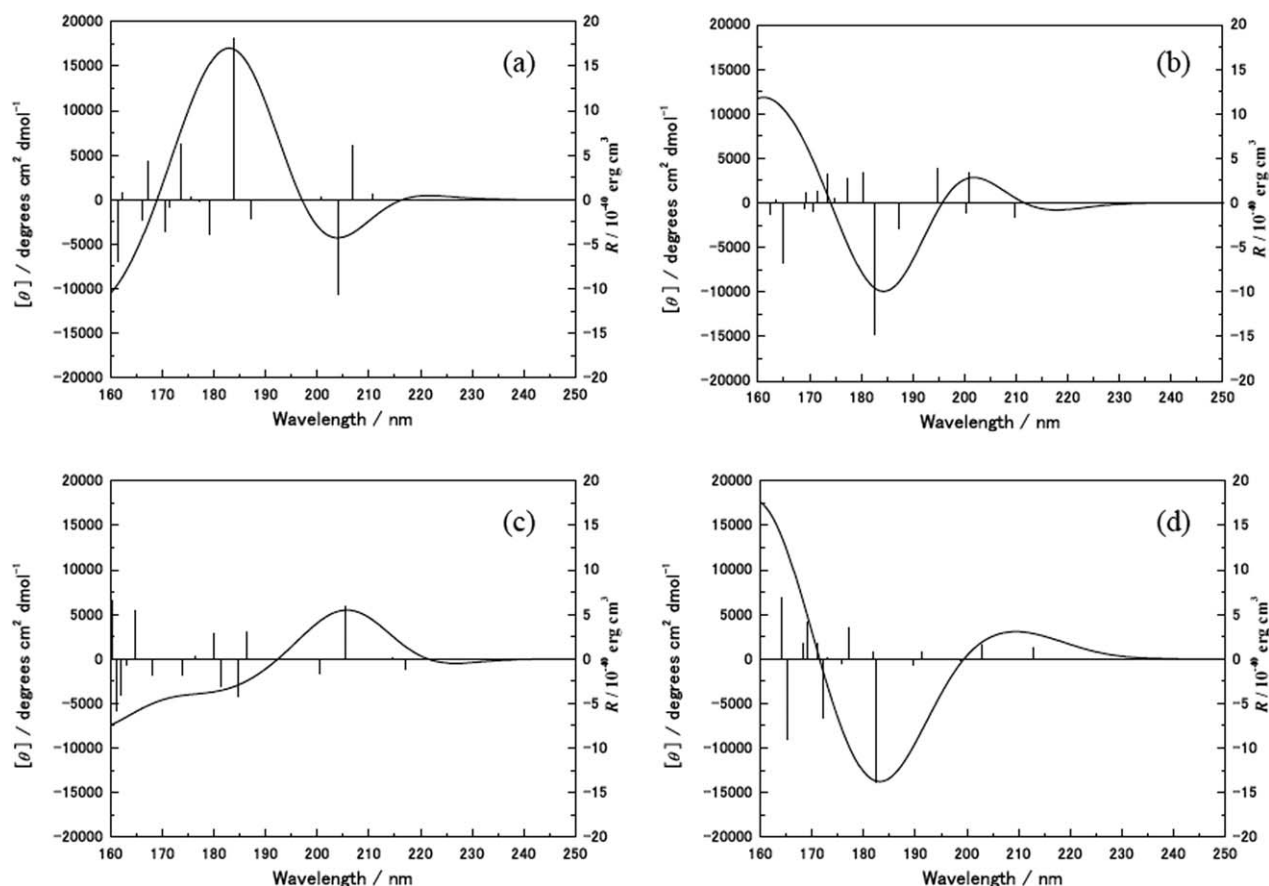| (ϕ, φ)	$(0°, -90°)$	$(0°, 180°)$	$(90°, 90°)$	$(90°, 180°)$
Bond length (Å)				
C1—C2	1.530	1.535	1.529	1.535
C1—H1	1.104	1.100	1.096	1.103
C1—O1	1.410	1.430	1.428	1.429
C1—C3	1.550	1.546	1.550	1.551
C2—H2	1.095	1.095	1.095	1.095
C2—H3	1.093	1.095	1.097	1.093
C2—H4	1.096	1.096	1.095	1.096
O1—H5	0.989	0.982	0.994	0.984
C3—O2	1.265	1.252	1.267	1.276
C3—O3	1.266	1.276	1.265	1.254
Bond angle (degrees)				
C2—C1—H1	108.3	109.0	109.5	108.5
C2—C1—O1	107.5	111.1	112.1	110.3
C2—C1—C3	110.4	109.8	112.5	111.9
C1—C2—H2	109.8	110.4	110.1	109.6
C1—C2—H3	110.7	110.4	111.1	111.7
C1—C2—H4	110.0	110.0	110.7	110.1
C1—O1—H5	109.9	107.5	110.8	106.8
C1—C3—O2	118.3	120.4	117.2	115.7
C1—C3—O3	116.5	114.7	118.2	120.0
Dihedral angle (degrees)				
H1—C1—C2—H2	62.5	61.8	61.5	61.8
H1—C1—C2—H3	−58.4	−58.5	−58.8	−58.6
H1—C1—C2—H4	−178.8	−178.5	−178.8	−179.1
H4—C2—C1—O1	61.3	60.5	63.7	60.9
H4—C2—C1—C3	−63.4	−61.8	−60.5	−62.4
O1—C1—C3—O2	−5.6	−24.8	108.3	−178.1
O1—C1—C3—O3	173.7	158.3	−69.7	2.2
C3—C1—O1—H5	−59.1	176.6	93.2	−176.8

Fig. 5. Rotational strengths and VUV-ECD spectra of four optimized structures with seven hydrated water molecules. (**a–d**) represent the rotational strengths and ECD spectrum for the optimized structures of (ϕ, φ) pairs of $(0°, -90°)$, $(0°, 180°)$, $(90°, 90°)$, and $(90°, 180°)$, respectively.

the same proportions in aqueous solution. We then averaged the four spectra to obtain the ECD spectrum of LA in aqueous solution. As shown in Figure 6, the average spectrum exhibited two positive peaks around 210 nm and 165 nm, and one negative peak around 185 nm. These peaks were consistent with the experimentally observed ones, demonstrating that the four optimized structures of LA are in equilibrium in aqueous solution.

Furthermore, we used the TDDFT method (B3LYP/6-31+G** level) and PCM to calculate the molecular orbitals that participate in the electronic transitions to assign the peaks in the ECD spectrum at 250–160 nm (Supporting Information Table S2). There were $n\pi^*$ transitions at 220–200 nm mainly from the lone-pair orbital (n-orbital) to the π^*-orbital of two oxygen atoms of the COO^- group. In addition to these $n\pi^*$ transitions, there were $\pi\pi^*$ transitions of the COO^- group at 190–160 nm, but it was difficult to accurately assign all of the peaks in this VUV region of the CD spectrum. Anand and Hargreav reported a very small CD-spectrum peak at 250–240 nm due to the $n\pi^*$ transition, which has the opposite sign to the peak around 205 nm due to the $\pi\pi^*$ transition.[28] However, Toniolo suggested that both peaks could be assigned to the $n\pi^*$ transition since their ellipticities decreased with the ionization of the carboxyl group, as expected from the greater symmetry of the carboxylate ion.[29] As shown in Figures 5a–5c, we also detected a small CD-spectrum peak around 220–230 nm and a large peak around 205 nm in the calculated ECD spectra.

These peaks had opposite signs and were assigned to the $n\pi^*$ transition of the carboxyl group from the molecular orbitals for ground and excited states calculated by the TDDFT method. This assignment was consistent with the findings of Toniolo[29] that the two CD peaks of LA at 240–250 and 210 nm are pH-dependent due to the modified ionization state or $n\pi^*$ transition of the carboxyl group.
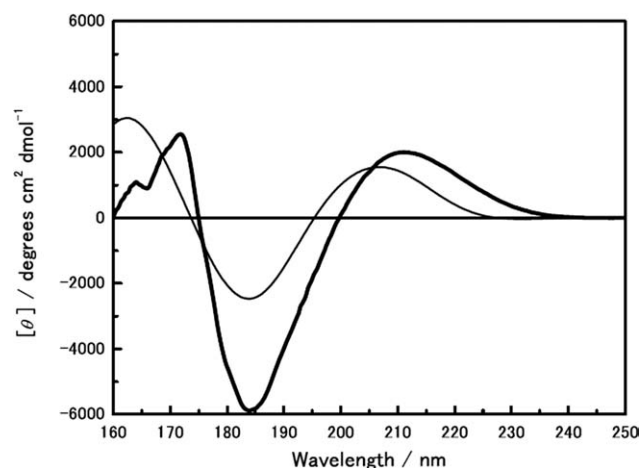

Fig. 6. Theoretical and experimental VUV-ECD spectra of LA. Solid line shows the average spectrum for four optimized structures with seven water molecules. Bold line shows the observed VUV-ECD spectrum.

Comparisons Between L-Hydroxy Acids and L-Amino Acids

As indicated in Figure 2 and Table 1, the ECD spectra of L-hydroxy acids differ markedly from those of the corresponding L-amino acids. The intensities of the ECD spectra of L-alanine, L-valine, and L-leucine increase and their peaks exhibit a small blue shift as their side chains become bulky, whereas no such systematic side-chain dependence was observed for LA, HMBA, and HICA, whose spectra were more complicated with different signs and peak positions. This suggests that the ability to fix the conformation in aqueous solution is weaker for the OH group in hydroxy acids than for the NH_3^+ group in amino acids. A typical finding from comparisons between LA and L-alanine is that the observed ECD spectrum of LA requires four optimized structures with seven hydrated water molecules for reproduction, whereas that of L-alanine can be reproduced with only one optimized structure with nine hydrated water molecules.[11] Such diverse structures of LA relative to L-alanine might be due to the hydration effects being weaker for the OH group than for the NH_3^+ group. The networks of hydrogen bonds of LA form around only the OH and COO^- groups (Fig. 4), whereas those of L-alanine extend not only around the NH_3^+ and COO^- groups but also throughout the molecule (eight of nine hydrated water molecules were bonded via hydrogen to each other).[11] A network with loose hydrogen bonding would facilitate the rotation of the dihedral angle of the COO^- group of LA, with this rotation being strongly restricted for L-alanine due to the network exhibiting extended hydrogen bonding. As the ECD spectra of the four optimized structures of LA are very different (Fig. 5), the modified proportions of such flexible conformations in equilibrium are likely to be responsible for the complicated side-chain dependence of ECD spectra of hydroxy acids.

CONCLUSIONS

This study has been the first to apply TDDFT to the theoretical assignment of VUV-ECD spectra of L-hydroxy acids. The average ECD spectrum for four optimized structures with seven hydrated water molecules successfully reproduced the peak positions and intensities of the experimentally observed VUV-ECD spectra of LA, although TDDFT has some limitations as a method for computing excited states, all relevant minima, and the influence of conformational dynamics. This demonstrates that hydration is indispensable for determining the equilibrium conformation of L-hydroxy acids and L-amino acids in aqueous solution. The obtained results suggest that combining VUV-ECD spectroscopy and theoretical assignments based on higher energy transitions will open a new field in the structural analysis of biomolecules in aqueous solutions.

ACKNOWLEDGMENTS

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