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Article

Actions and Interactions of Mirror-Image Cyclodextrins

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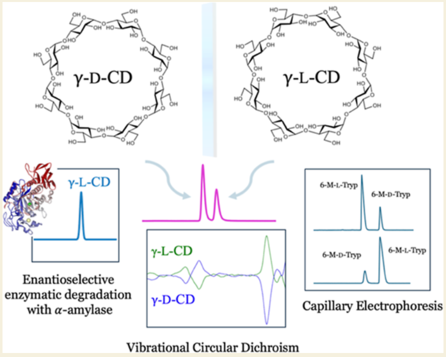
ABSTRACT: Cyclodextrins (CDs) were the first identified molecules and arguably the most consequential molecules in the areas of macrocyclic science and technology. As carbohydrates, their intrinsic architecture and chirality have played a fundamental role in their response to and effect on guest molecules. Indeed, the nature of inclusion complexation was formalized with CD-based hosts. A recent report on the first synthesis of unnatural mirror-image L-cyclodextrins foreshadows a new era of stereochemical research involving macrocyclic entities. Here, we show the inherently difficult, but rapid and sensitive, separation of enantiomeric CDs. The absolute configurations of CD antipodes have been investigated and distinguished by vibrational circular dichroism (VCD). Results indicated that the most intense VCD band at 1150 cm^{-1} arises from the C−O stretching of the glucosidic bonds lining the CD torus. It follows that chiroptical radiation can excite either D- or L-CD selectively. The enzymatic susceptibilities of mirror-image CDs are vastly different, suggesting the possibility of different biochemical and medicinal uses. The electrophoretic migration of small chiral molecules, e.g., amino acids is easily and predictably reversed with D- and L-CDs.



KEYWORDS: *macrocyclic enantiomers, chiral recognition, enzymatic reaction, absolute configuration, separation*

INTRODUCTION

Macrocycles [e.g., cyclodextrins (CDs),[1] crown ethers,[2] cryptands,[3] cyclophanes,[4] calixarenes,[5] cucurbiturils,[6] pillararenes,[7] etc.] that can form host−guest complexes represent vast, diverse, and consequential areas of science and technology. Their role in supramolecular chemistry and molecular recognition has led to artificial molecular machines[8−15] and a myriad of other scientific and practical applications.[16−23] In comparison to other macrocyclic entities, CDs and their derivatives are the most ubiquitous and widely used in host−guest chemistry research and technology. As a class, CDs dwarf all other macrocycles in terms of publications, patents, applications, and amounts of materials produced (Supporting Information, Figure S1). CDs have played an important role in supramolecular organo-catalysis in water,[1,24,25] chiral recognition and separations[26−30] (that in turn played an integral role in the Food & Drug Administration's issuance of new guidelines for the development of stereometric drugs),[31] as well as development of mechanically interlocked compounds,[22,32] drug delivery or excipients,[33,34] pharmaceutical compounds such as Sugammadex,[35−37] medical imaging,[38] metal−organic frameworks,[21,39] cosmetics,[40,41] volatile molecule sequestration and release, e.g., fragrance, flavors, and colors,[42−44] and much more.[45−47]

Native CDs consist of α-1,4-linked D-glucopyranose units that are produced enzymatically as a macrocyclic homologous series of which α, β, and γ-CDs (with six, seven, and eight units, respectively) dominate most studies. Additionally, native CDs are the starting materials for the vast number of derivatized or semisynthetic analogues.[21,22,29,30,33−36,38] The concept and proof of inclusion complexation was developed by Cramer[47] and others[1] from the 1950s through the 1960s, a concept subsequently utilized for a plethora of synthetic macrocyclic entities.[23,48,49] Likewise, the early use of CDs as enzyme models and catalysts for a variety of reactions[24,25,50] continues with a variety of other macrocyclic entities to this day.

Recently a new frontier in CD chemistry was breeched with the first synthesis of mirror-image L-cyclodextrins (L-CDs) by Wu et al.[51] (Figure 1). This synthesis was achieved by one-pot glycosylation of linear oligosaccharide precursors followed by diastereoselective cyclization and removal of the protecting groups.[51] This effective stereoselective gram-scale unnatural product synthesis[52] occurred 133 years after the initial discovery of naturally occurring D-cyclodextrins (D-CDs) by Villers.[53]

The broad applicability of CDs encompasses both stereoselective and achiral functions and uses. Given their inherent chiralities, however, it is not surprising that CDs have had a pronounced impact on research involving stereochemical systems. The development of a synthetic approach to produce

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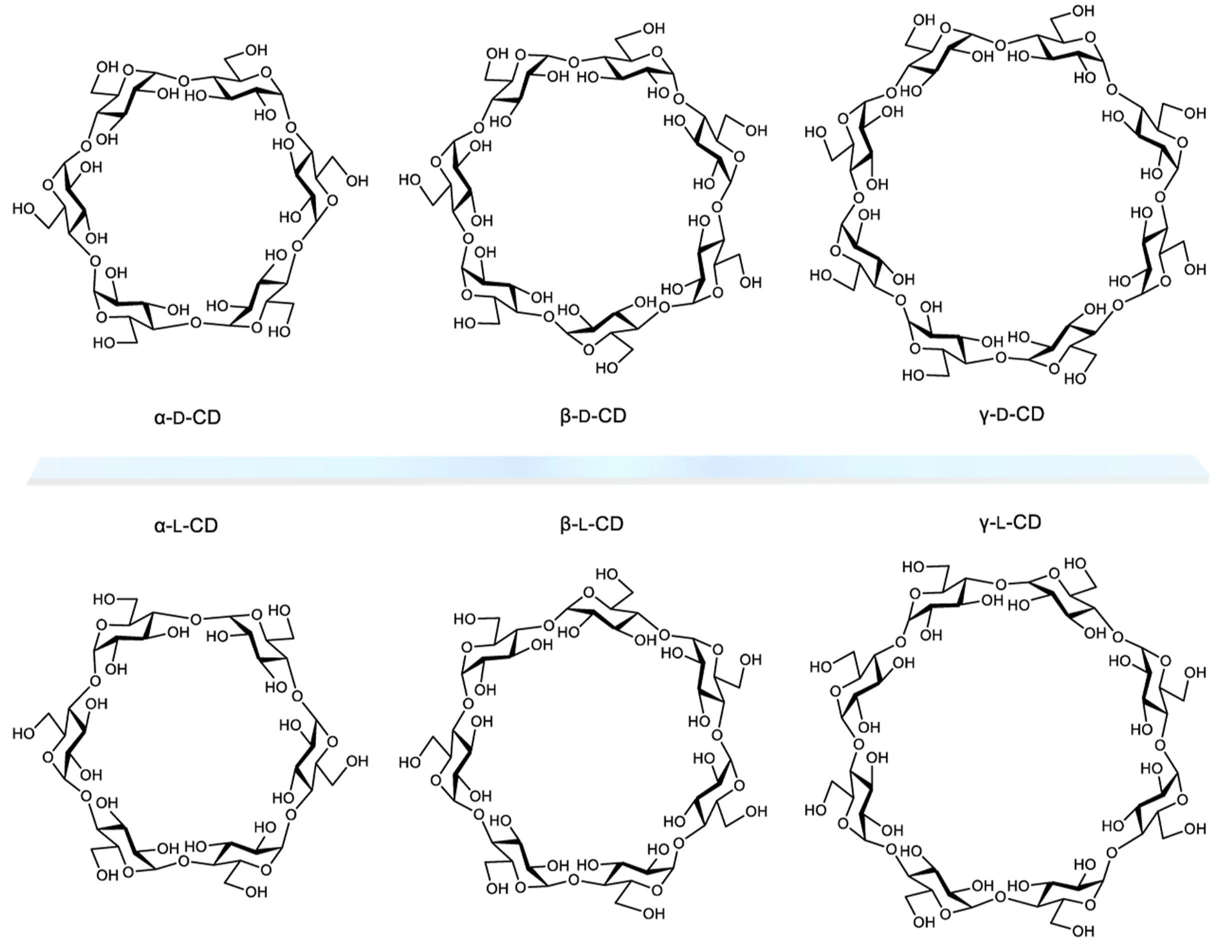

Figure 1. Structures of *α*, *β*, and *γ*-cyclodextrin (CD) enantiomers.

significant quantities of L-CDs can be anticipated to have an impact on multiple areas of stereochemical, biochemical, and medicinal research. The advent of L-CDs adds a new and important dimension to the most widely utilized class of macrocyclic molecules. While the properties and actions in isotropic environments must be identical to their mirror-image D-CDs, it is their ability to provide and manipulate stereoselective interactions with chiral molecules and electromagnetic radiation that is most intriguing.

Herein, we compare and contrast the properties and behavior of mirror-image CDs in selected chemical, biochemical, and spectroscopic systems. This report is the first one on their highly enantioselective interactions in enzymatic reactions, the separation of their individual enantiomers, their use in effective resolution strategies for included chiral molecules, and unique vibrational circular dichroism (VCD) studies that could have important ramifications in the manipulation of the strength and selectivity of inclusion complexes.

RESULTS AND DISCUSSION

The enzymatic reactions of proteins and carbohydrates are generally stereoselective for L-amino acid-containing peptides/proteins[54] and D-carbohydrates.[55] There are, however, exceptions where peptidases will more slowly hydrolyze some D-amino acid peptide bonds.[56] Furthermore, the enzyme L-galactose dehydrogenase was identified early on in plants.[57] All CDs are several times more stable to acid hydrolysis than their linear dextrin counterparts.[58] D-CDs are susceptible to hydrolysis by specific enzymes such as *α*-amylase from *Aspergillus oryzae*.[59] Also, it is known that the larger *γ*-D-CD is more susceptible to enzymatic hydrolysis than the smaller *α*- and *β*-D-CDs.[59] All three CDs are resistant to stomach acid, but *γ*-D-CD is digestible in the small intestine, while *α*- and *β*-D-CDs are mainly degraded by intestinal flora.[60]

Table 1 compares the enzymatic degradation of D-CDs and L-CDs by four different enzymes. All synthetic L-CDs are resistant to enzymatic degradation. As expected from previous studies, *γ*-D-CD is the most susceptible to *α*-amylase hydrolysis, but not all *α*-amylases are equally effective, e.g.,

Table 1. Percent Degradation of CDs Using Various Enzymes (±3%)[a]

compound	*α*-amylase from aspergillus	taka diastase	*α*-amylase from human saliva	*α*-amylase from B. subtilis
α-D-CD	15	15	<3[b]	<3[b]
β-D-CD	100	100	<3[b]	<3[b]
γ-D-CD	100	100	100	<3[b]
α-L-CD	<3[b]	<3[b]	<3[b]	<3[b]
β-L-CD	<3[b]	<3[b]	<3[b]	<3[b]
γ-L-CD	<3[b]	<3[b]	<3[b]	<3[b]

[a] Percent degradation was calculated based on the disappearance of the CD peak. [b] These values were reported as less than 3% since the reproducibility and experimental error of this method was ±3%. Therefore, no degradation below this limit was observable.

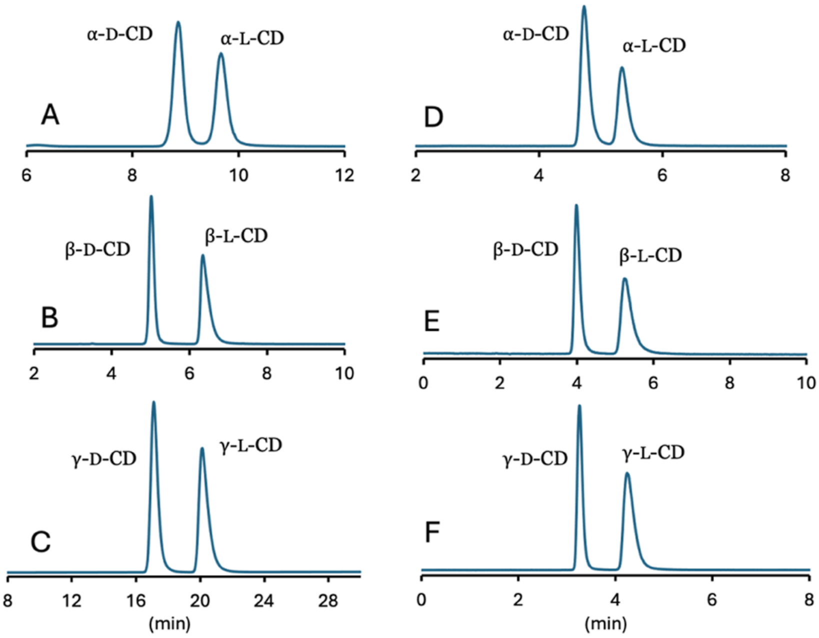

Figure 2. Separation of α, β, and γ-cyclodextrin enantiomers. (A) 80/20/0.1, acetonitrile/water/formic acid, 0.425 mL/min. (B,C) 70/30/0.1, acetonitrile/water/formic acid, 0.425 mL/min. (D) 95/5/0.1 methanol/water/formic acid, 0.225 mL/min, (E,F) 90/10/0.1 methanol/water/formic acid, 0.325 mL/min. All separations were performed on a TeicoShell column, 15 cm × 3 mm i.d. See the Materials and Methods Section.

α-amylase from human saliva and *Bacillus subtilis* were less active under the utilized experimental conditions. The smaller α- and β-D-CDs are more slowly hydrolyzed (Table 1). For comparative studies of mixtures of D- and L-CD, it was necessary to develop an effective methodology to separate these unique and large enantiomers.

The ability to identify, separate, and quantitate enantiomeric CDs rapidly and effectively from one another and their homologues is fundamental. While great strides have been made in all manner of enantiomeric separations over the past four decades, carbohydrates remain a notable exception. Interestingly, a few L-monosaccharides occur naturally in some plants although the D-enantiomers predominate in all living systems.[61] Hemicelluloses reportedly contain some L-arabinose and very small amounts of L-rhamnose and L-fucose.[62] The few reported separations of enantiomeric monosaccharides generally used chiral derivatizing agents[63] until the advent of enantioselective gas chromatography. Even then, the very limited reports involved complete derivatization of the small monosaccharides for volatility purposes.[64−66] A single instance of a liquid-based monosaccharide separation has been disclosed.[67] The separation of carbohydrate enantiomers that are not monosaccharides has not been reported to our knowledge. The inherent properties of higher carbohydrates (>disaccharides) can pose difficulties for liquid-based separations like HPLC or electrophoresis. They tend to be insoluble in most nonaqueous mobile-phase solvents, are uncharged, have no chromophore, and have poor volatility even if derivatized. Upon screening a variety of chiral stationary phase classes, only one had appreciable selectivity for mirror-image CDs, i.e., the macrocyclic glycopeptides. Notably, TeicoShell[68] and NicoShell[69] chiral stationary phases provided high selectivity and short retention times and allowed sensitive and selective MS/MS detection of all α, β, and γ-CD enantiomers (Figure 2 and Supporting Information, Tables S1 and S2). The use of methanol-containing mobile phases greatly reduced retention times while maintaining excellent selectivity. The naturally occurring D-CDs elute before the synthetic L-CDs in all cases, providing the first effective and quantitative separations.

Chiral selectors for enantiomeric separations by LC, supercritical fluid chromatography (SFC), GC, and capillary electrophoresis (CE) are dominated by larger naturally occurring chiral molecules (e.g., CDs, macrocyclic glycopeptides, cellulose, amylose, and proteins). Only for small synthetic chiral selectors are both pure enantiomers available such that absolute and predictable reversing of all enantiomeric elution orders can be achieved under the same conditions, which can be essential when doing stereoselectivity tuning and quantifying low levels of enantiomeric impurities.[70,71] The advent of L-CDs provides a unique advantage to this important class of chiral selectors. The electropherograms of Figure 3 show the enantiomeric reversal in the migration order of 6-fluoro-D,L-tryptophan and 6-methyl-D,L-tryptophan under identical conditions. Note that the L-enantiomers in these samples were deliberately spiked so that they had peak areas greater than those of the D-antipode. Thus, the migration order could be easily discerned. Additional separations with reversals are recorded in Table S3 of the Supporting Information. Indeed, such reversals for mirror-image CDs must be maintained in all of the aforementioned separation techniques. This ability greatly extends the capability of CD-based chiral selectors, effectively bridging the gap between small totally synthetic entities and larger oligomeric and polymeric natural molecules. No other class of chiral selectors has such broad applicability in all separations as well as such reversibility.

Vibrational circular dichroism (VCD) has developed into an indispensable and often facile approach for the determination of the absolute configuration of molecules.[72−75] It can be used on solutions, oils, or neat samples of chiral molecules.[72] There are no requirements for “heavy” atoms, and often a milligram or less of sample is sufficient. Notably, VCD has often provided the only means of determining the absolute configuration of enantiomeric entities that cannot be crystallized.[76,77] Con-

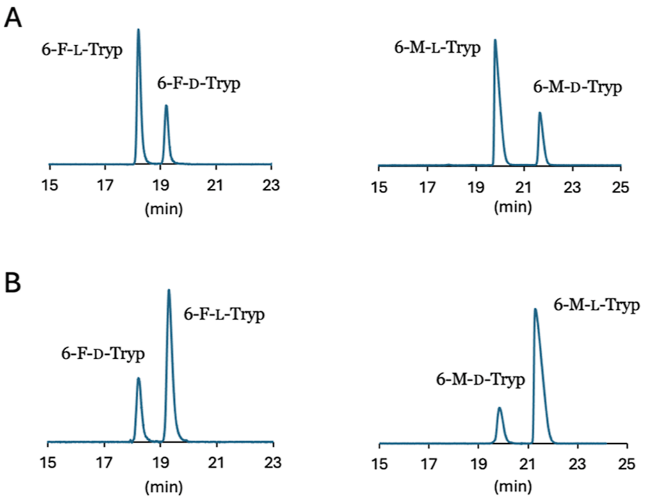

Figure 3. Capillary electropherograms of the enantiomeric separation of 6-fluoro-D,L-tryptophan (6 F-D,L-Tryp) and 6-methyl-D,L-tryptophan (6 M-D,L-Tryp) using a 10 mM segment of (A) α-D-CD and (B) α-L-CD as chiral selectors in a run buffer consisting of 10 mM sodium dihydrogen phosphate, pH 2.2. The separation voltage was 15 kV. See the Materials and Methods Section for further details.

ceptually, VCD simply involves a comparison of the measured and calculated VCD spectra. Neither of these tasks, however, were straightforward until recently[78,79] and there are limitations, particularly on the computational side.[75,78,79] An analyte's molecular weight and particularly its conformational flexibility can be limiting factors in VCD calculations.[72−75,78,79]

The advent of mirror-image CDs has provided a way to test and extend the limits of the VCD approach. Figure 4 shows the VCD spectra of γ-D-CD and γ-L-CD (MWs = 1297 Da, 168 atoms). The experimental CD spectra pose no problems for VCD (also see Supporting Information, Figures S2 and S3 for α- and β-CDs), and they are the highest molecular weight enantiomeric compounds yet analyzed with VCD. The VCD calculations for even the largest and most flexible γ-CD, while time-consuming, provided an excellent match with the experimental VCD (Figures 5 and Supporting Information, S4, S5 for α- and β-CDs), significantly extending the range and applicability of the VCD method. The calculations for the ten lowest energy conformations of all three CDs are provided in the Supporting Information.

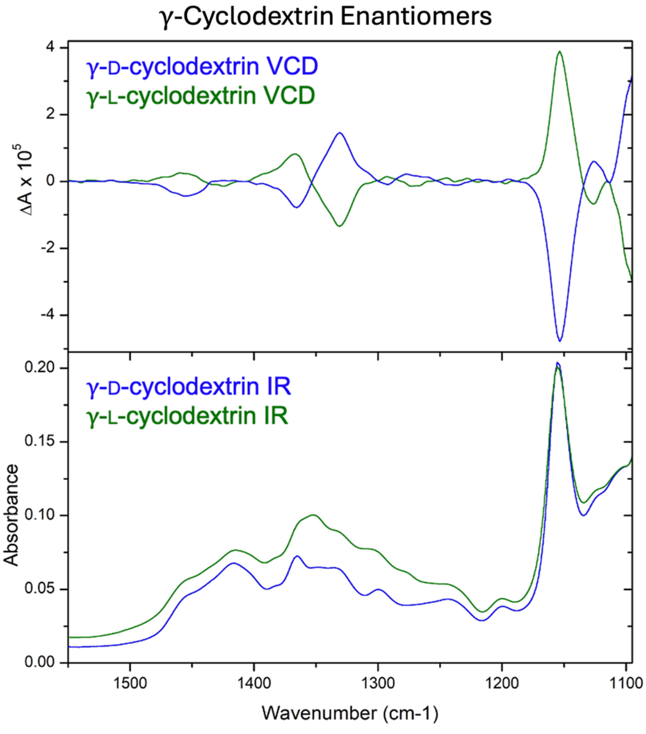

Figure 4. VCD and infrared spectra (IR) for γ-D-CD and γ-L-CD. See the Materials and Methods Section for details.

Note the strong VCD absorbance at ~1150 cm^{-1}, which is the C−O stretching of the α-1,4-glucosidic linkage of the CD torus (Figure 4). Irradiating at this frequency causes the greatest vibrational variations within the torus of the CD molecule (Figure 6). The hydrophobic effect and the structure/size of an associated molecule relative to the CDs' internal diameters are important factors that affect inclusion complex formation. These results indicate that specific chiroptical radiation can excite selectively the C−O glucosidic bonds that line the D or L-CD cavities, potentially affecting the strength and/or nature of an inclusion complex. Consequences of such directed VCD approaches are currently being considered. However, to investigate this effect and its possible uses requires a steady-state circularly polarized light of a single handedness as opposed to what is used in VCD, which is a rapidly oscillating crystal (via a photoelastic modulator) that produces alternating left and right circularly polarized light.[79] Such a source would present the possibility of expanding the research space involving and controlling selective inclusion complexation using intense steady-state circularly polarized luminescence and mirror-image CDs.

MATERIALS AND METHODS

Materials

All D-CDs and enzymes were purchased from MilliporeSigma (formerly Sigma-Aldrich, St. Louis, MO). The L-cyclodextrins were synthesized as described elsewhere.[51] Poly(ether sulfone) centrifugal filters were purchased from VWR (Atlanta, GA, USA). LC−MS grade acetonitrile and methanol were purchased from Fisher Scientific (Waltham, MA, USA), and ultrapure water (18.2 MΩ cm) was obtained using a Barnstead system (Thermo Scientific, USA). Formic acid (Optima, LC−MS grade) was purchased from Fisher Scientific (Waltham, MA, USA) and used as a mobile phase additive. DMSO-d_6 solvent from Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA was used without further purification. TeicoShell, NicoShell, TagShell, VancoShell, and FructoShell-N columns with 2.7 μm superficially porous particles (SPPs) were acquired from AZYP, LLC (Arlington, TX, USA). Chiralpak IA, ID, IE, and IF columns with 5 μm fully porous particles (FPP) and Chiralpak-IC columns with 3 μm FPP particles were acquired from Daicel Corp. (Kita-Ku, Osaka, Japan). The fused silica capillary for electrophoresis experiments was obtained from Beckman Coulter (Palo Alto, CA, USA).

Enzyme Studies

α-Amylase from *A. oryzae* (200 U/ml) and taka diastase (100 U/ml) enzymes were dissolved in 50 mM phosphate buffer at pH 5.3. The pH of the phosphate buffer for α-amylase from *B. subtilis* (200 U/ml) and human salivary α-amylase (200 U/ml) was 6 and 7, respectively. The optimum pHs were selected based on the optimum enzyme activities reported previously.[80−83] For degradation studies, 20 μL of the enzyme solution was added to a 200 μL solution (0.1 mM) of each CD. In the case of taka diastase and human salivary α-amylase, the volume of enzyme solution was increased to 100 μL to ensure full degradation of at least one of the six CDs. The control samples were prepared for each CD by adding the same volume of buffer instead of the enzyme solution. The mixtures were held at 37 °C for 5 h and

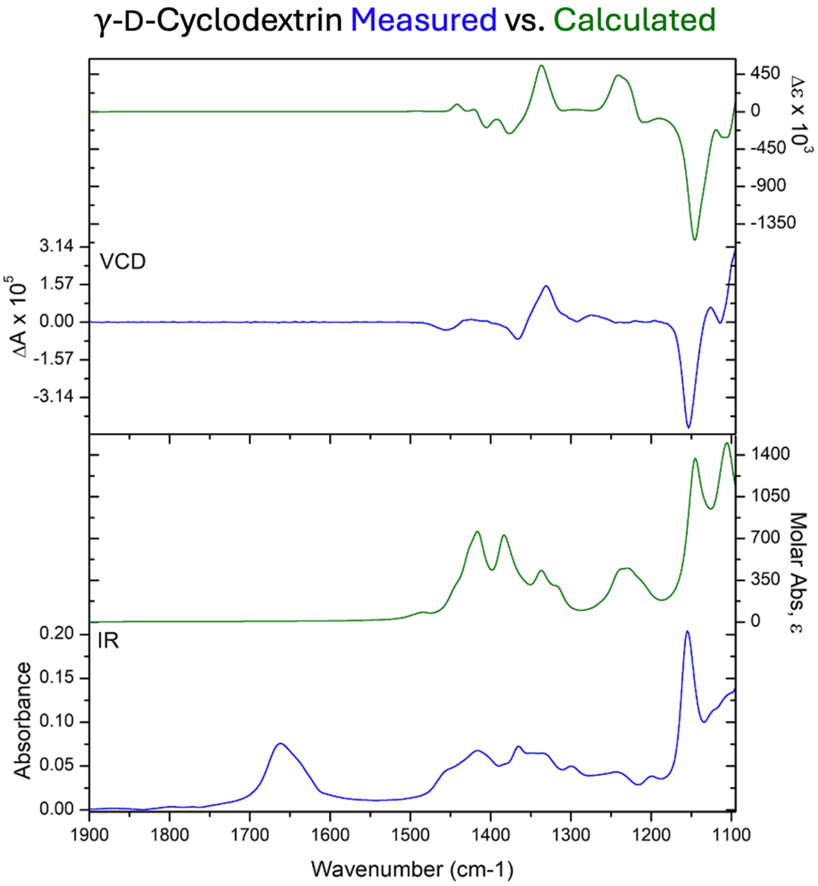

Figure 5. Comparison of the measured VCD spectrum (in blue) for γ-D-CD vs its calculated spectrum (in green) as well as its IR spectra (bottom).

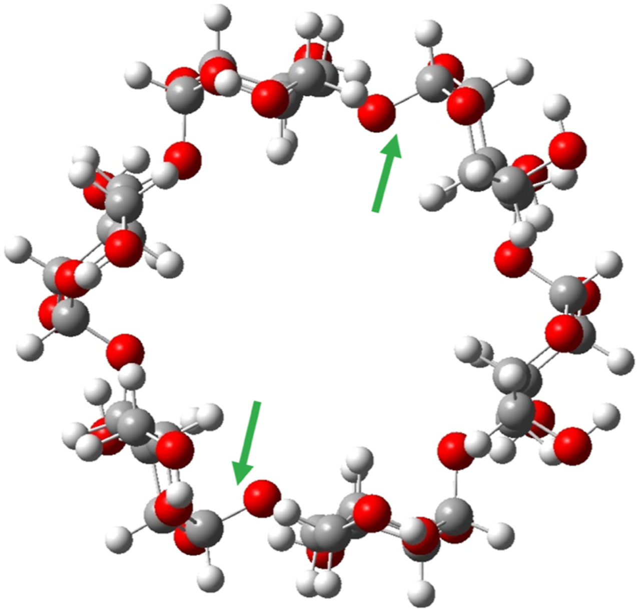

Figure 6. Model of α-cyclodextrin with the green arrows indicating two of the six α-1,4-glucosidic linkages (12 C−O bonds) of the CD torus. These C−O bonds strongly absorb VCD and IR light in the 1150 cm^{-1} range (see Figure 4). The size and shape of this toroidal cavity affect the inclusion complex formation of a variety of molecules. Red: oxygen, gray: carbon, white: hydrogen.

shaken occasionally. The mixtures were then added to a 10,000 Da molecular weight cutoff filter with poly(ether sulfone) membrane and centrifuged at 13,200 rpm for 15 min to remove the enzymes from the mixtures. The concentration of CDs in the filtrate was then measured by ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) using single reaction monitoring (SRM) mode and was compared with the concentration of the control sample.

UHPLC-MS/MS Studies

The UHPLC-MS/MS instrument was a TSQ Quantis Plus triple quadrupole mass spectrometer from Thermo Scientific. The instrument was controlled with Chromeleon 7 software. The SRM methods for α-, β-, and γ-cyclodextrins, as well as for maltose and maltotriose, were developed using the optimization feature in Chromeleon software. The detailed parameters regarding each method are provided in the Supporting Information, Table S1. The mobile phase condition for quantitation of CDs and maltooligosaccharides in enzyme studies was 60/40/0.1 acetonitrile/water/formic acid at a flow rate of 0.425 mL/min. The reported percentages of mobile phases are listed as volume/volume (v/v). The chromatography column was FructoShell-N (15 cm × 3 mm i.d., on 2.7 μm SPPs). For the enantiomeric separations of CDs, the mobile phase conditions were various combinations of acetonitrile/water/formic acid or methanol/water/formic acid based on the stationary phases and compounds. For detailed chromatographic conditions, see the captions of the figures and Table S2 of the Supporting Information. The MS data presented in Table S2 of the Supporting Information were collected in positive mode (selected ion monitoring) at m/z values of 973, 1135, and 1297. Chromatograms of γ-D-CD and γ-L-CD using the acetonitrile-containing mobile phase (Figure 2C) employed PeakFit (software version 4.12), using an exponentially modified Gaussian model.[84]

Capillary Electrophoresis

The capillary electrophoresis system was a P/ACE MDQ (Beckman Coulter, Palo Alto, CA, USA) with a 52 cm (42 cm to the detector) ×

75 μm i.d. fused silica capillary. The run buffer was sodium dihydrogen phosphate with a concentration of 10 mM and pH was adjusted to 2.2 using phosphoric acid. Before each run, the capillary was conditioned with 0.1 M sodium hydroxide for 2 min at 20 psi. It was then purged by Milli-Q water for 2 min at 20 psi followed by the run buffer for 5 min at 20 psi. The capillary was then filled with a segment of 10 mM α-cyclodextrin with 20 psi pressure for 30 s. The analytes were injected at 0.3 psi for 2 s, and the separation was done by applying a voltage of 15 kV. The temperature was maintained at 25 °C during conditioning and analysis procedures. Nonparametric baseline correction was applied, postanalysis, using Peak Lab software (V1.07.01).

Vibrational Circular Dichroism

To a small vial containing CD (D or L/α, β, or γ: 2 to 8 mg) was added 95 to 160 μL of deuterated dimethyl sulfoxide, CD_3SOCD_3. The D- and L-CDs were all measured at lower concentrations due to the limited amount of L-enantiomers available: the D-CDs were also measured at higher concentrations. In each case, the resulting solution was transferred to a liquid IR cell (BaF_2, 100 μm cell path) and placed in the measurement chamber. The instrumentation was a BioTools, Inc. (Jupiter, FL) ChiralIR 2X Dual PEM FT-VCD spectrometer, set to 4 cm^{-1} resolution, with the PEM (both 1 and 2) maximum frequency set to 1400 cm^{-1}. Each sample was then measured for 8 to 16 h in 1 h blocks. The IR data from the first block was solvent and water vapor subtracted and then offset to zero at 2000 cm^{-1}. The VCD data blocks were averaged, and a previously measured solvent baseline (16 h average) was subtracted. Finally, the VCD spectrum was offset to zero at 2000 cm^{-1}. The VCD noise data were block averaged and used without further processing.

VCD Calculations

The D-enantiomer of each CD (α, β, or γ) was constructed using ComputeVOA (BioTools, Jupiter, FL) with all alpha linkages and all chair conformations for each sugar. A thorough conformational search was then performed at the MM level using the MMF94 force field in a 10 kcal/mol energy window. The ~200 lowest energy conformers for each compound were then subjected to DFT level optimization and frequency calculation with Gaussian ′09 (Wallingford, CT)[85] at the B3LYP/6-31G(d)/CPCM (DMSO) level. The large size of the molecules, particularly γ-CD with 168 atoms/688 electrons, took a reasonably large amount of computing resources to calculate. The resulting spectra were Boltzmann-averaged using free energy, plotted at 10 cm^{-1} resolution, and then x-axis scaled by a factor of 0.970 for comparison to the experimental IR and VCD spectra. The complete VCD calculations for the D-CDs are provided in the Supporting Information.

CONCLUSIONS

A direct result of the recently synthesized mirror-image α, β, and γ-L-cyclodextrins is the creation of the three largest carbohydrate enantiomers and macrocyclic enantiomers known to exist. Given the broad usefulness of native D-CDs, it would be expected that their L-enantiomers could provide unique and tunable properties in chiral environments. In some instances, it was observed that L-CDs can provide reciprocal actions such as those involving chiral separations. In other areas, such as enzymatic susceptibility, antithetical actions dominate, which raises the question as to whether microbial degradation of L-CDs can occur given the plethora of such organisms and the ability of some to make use of almost any carbon source. The first separation and quantitation of D- and L-cyclodextrins was possible only using specific macrocyclic glycopeptide chiral selectors, which will allow future chemical, biochemical, and biological studies involving mixtures of CD enantiomers and homologues to be carried out more easily. D- and L-cyclodextrins are the largest molecules to be completely analyzed and characterized—in terms of absolute configuration—by VCD. While obtaining the experimental VCD spectra was straightforward, the calculated VCD spectra, especially for the largest γ-cyclodextrin, were quite time-consuming but accurate. The strongest VCD band was at 1150 cm^{-1}, which is for the C−O stretching of the α-1,4-glucosidic linkage of the CD torus. Stereoselective excitation at 1150 cm^{-1} suggests the possibilities of chiral modulation of inclusion complexation and other interactions using circularly polarized infrared radiation.

DECLARATION OF COMPETING INTEREST

Dr. Daniel W. Armstrong has a potential research conflict of interest due to a financial interest in the company AZYP, LLC. A management plan has been created to preserve objectivity in research in accordance with the UTA policy.

ASSOCIATED CONTENT

Data Availability Statement

Data will be made available on request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00962.

> Comparison of number of publications and patents related to the widely used macrocyclic molecules; calculated and experimental VCD spectra of CDs; SRM optimized parameters for determination of CDs, maltose, and maltotriose; screening information for enantiomeric separation of CDs; capillary electrophoresis data using α-CDs as chiral selectors; and VCD calculations (PDF)

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Author Contributions

CRediT: **Daniel W. Armstrong** conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, writing - original draft, writing - review & editing; **Saba Aslani** conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing - original draft, writing - review & editing; **Jordan Nafie** formal analysis, investigation, methodology, visualization, writing - review & editing; **Yong Wu** investigation, resources, visualization, writing - review & editing; **J. Fraser Stoddart** funding acquisition, resources, supervision, writing - review & editing.

Notes

The authors declare the following competing financial interest(s): Dr. Daniel W. Armstrong has a potential research conflict of interest due to a financial interest with the company AZYP, LLC. A management plan has been created to preserve objectively in research in accordance with UTA policy.

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