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A Simple Helical Macrocyclic Polyazapyridinophane as a Stereoselective Receptor of Biologically Important Dicarboxylates under Physiological Conditions

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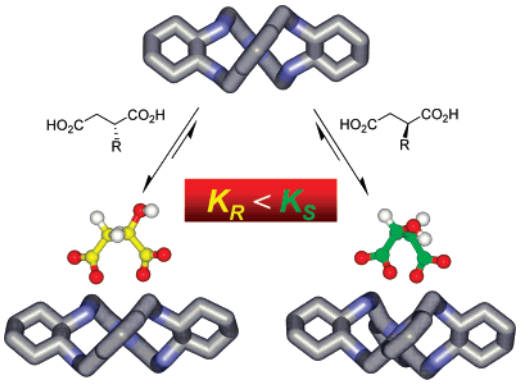

The interaction of a synthetic enantiopure azamacrocyclic receptor (**L**) with biologically important chiral dicarboxylates (A, **1**−**7**) has been studied by means of potentiometric titrations in 0.15 M NaCl aqueous solution in a wide pH range. This macrocycle forms strong complexes of the type $[H_n\mathbf{L}A]^{(n-2)}$ (with n = 0−5). As a general trend, the binding is much tighter at basic or neutral pH than in acidic medium. Interestingly, nonprotected excitatory amino acids (Asp and Glu) are strongly bound even at acidic pH. Regarding selectivity, the receptor showed stereoselective binding toward those substrates bearing an H-bonding donor at Cα, being *S*-selective in most of the cases, except for glutamic acid. Thus, **L** displayed an excellent enantioselectivity for (*S*)-malate dianion (K_S/K_R = 11.50 at pH 10.0 and K_S/K_R = 6.86 at pH 7.0) and exhibited moderate enantiopreference for (*S*,*S*)-tartrate (K_{SS}/K_{RR} = 3.01 at pH 10 and K_{SS}/K_{RR} = 1.70 at pH 7.0). For this last anion, a very good diastereopreference was also observed (K_{SS}/K_{RS} = 8.46 at pH 10 and K_{SS}/K_{RS} = 4.99 at pH 7.0). On the contrary, **L** is smoothly *R*-selective toward (*R*)-Glu (K_R/K_S = 3.22 at pH 10 and K_R/K_S = 2.05 at pH 7.0) due to its longer and more flexible molecular structure. The stereoselectivity of the corresponding complexes decreased when decreasing pH values. For the hydroxy derivatives, mass spectrometry also reflected the trends observed by potentiometry and confirmed the receptor:dicarboxylate 1:1 stoichiometry of the supramolecular complexes. Additional experimental techniques were used to study the most stereoselective example. Solution studies by NMR suggested a good geometrical complementarity between the malate dianion and the receptor, which showed a predominant helical conformation in solution. Besides, self-diffusion rates (PGSE) of the diastereomeric complexes with malate also agree with binding data. Circular dichroism was also used in this case at different pH values, showing a very good correlation between the helical content of the receptor and the stereoselectivity of the molecular recognition process.

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Introduction

Despite the ubiquitous presence of negatively charged entities in living systems, the molecular recognition of biologically active polyanions still constitutes a difficult task facing supramolecular chemists, because the features responsible for their vital role (*eg*. charge density and complex shapes) also make their recognition difficult by simple abiotic receptors.[1] This goal is particularly challenging in aqueous solution due to the strong solvation capabilities of water, which disfavor the electrostatic attractions and the formation of hydrogen bonds between host and guest species.[2] Besides, anions are usually pH-dependent charged species and molecular recognition in water requires the adequate control of protonation states. Additionally, other anionic species present in large concentration in biological systems (such as chloride anions) could also compete with the target anion in binding with the receptor.

In this paper we concretely address the recognition of dicarboxylates, which form one of the most relevant families of organic anions in biological terms.[3] Among others, polyamine receptors are able to interact with dicarboxylic acids in both organic solvents and aqueous medium.[4] However, to obtain a good selectivity in aqueous medium is usually complicated, especially under physiological conditions (pH and ionic strength).[5] On the other hand, although most of the dicarboxylates present in biological systems are chiral and their activity depends on the absolute configurations of their chiral centers,[6] only a few enantioselective receptors for chiral dicarboxylates have been described.[7] Here again, the number of receptors able to exert enantiodiscrimination in water at physiological conditions is very scarce.[8] In this sense, we have recently reported that the enantiopure polyazamacrocycle **L** (Chart 1) with (*R*,*R*,*R*,*R*) absolute configuration displays unprecedented enantioselectivity toward malate dianion in aqueous solution.[9] Following this work, we tested related chiral dicarboxylates and binding studies were carried out by different techniques. Here we describe in detail the results of this study.

The structures of the dicarboxylates (**1**−**7**, Chart 1) were selected by mapping different possible residues attached to the chiral center (aliphatic, aromatic, charged/uncharged H-bonding). Moreover, many of them present important biological activities; for instance, malate is an intermediate in the citric acid and glyoxylate cycles,[10] tartrate regulates the acidity of some fruits,[11] and aspartate and glutamate behave as excitatory amino acid neurotransmitters.[12] Unusual concentrations of either the natural or non-natural enantiomer of some of these dicarboxylates are related to some methabolic disorders,[13] or can be used to detect frauds in alimentary industry.[14] Accordingly, the discovery of new and simple abiotic stereoselective receptors for these diacids in biomimetic medium has many potential applications in biomedicine and biotechnology.

Results and Discussion

Protonation Studies. The first point to be considered when determining the aqueous binding abilities of a polyamine receptor must be the study of its acid−base behavior, which has been performed for **L** by potentiometric titrations at 298.1 K. The measurements were carried out in pure water with NaCl (0.15 M) as supporting electrolyte in order to maintain constant ionic strength. Although the presence of extraneous cations and anions can interfere with the binding of the targeted substrates,

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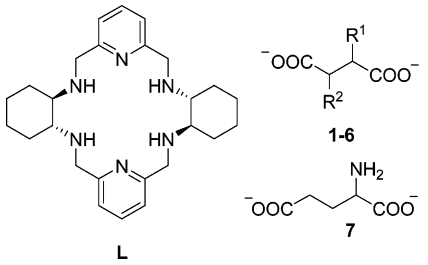
CHART 1. Molecular Structures of the Receptor (*R*,*R*,*R*,*R*)-L and the Dicarboxylates Studied in This Work.



1 (R^1=Me, R^2=H); Methylsuccinate, MeSuc
2 (R^1=Ph, R^2=H); Phenylsuccinate, PhSuc
3 (R^1=OH, R^2=H); Malate, Mal
4 (R^1=OH, R^2=OH); Tartrate, Tar
5 (R^1=NH_2, R^2=H); Aspartate, Asp
6 (R^1=NHAc, R^2=H); *N*-acetylaspartate, AcAsp

7 Glutamate, Glu

TABLE 1. Protonation Constants[a] for L, Determined in 0.15 M NaCl at 298.1 K

reaction[b]	log *K*	Δ
$L + H \leftrightarrows HL$	9.07(1)	
$HL + H \leftrightarrows H_2L$	8.53(1)	0.54
$H_2L + H \leftrightarrows H_3L$	4.97(1)	3.56
$H_3L + H \leftrightarrows H_4L$	3.06(1)	1.91

[a] Values in parentheses correspond to standard deviation in the last significant figure. [b] Charges have been omitted for clarity.

these conditions were chosen due to their similarity with physiological medium. Table 1 shows the stepwise basicity constants (log *K*) and the difference (Δ) between successive protonation processes, a parameter that describes the ease of proton binding at each consecutive step.[15] The higher the Δ value, the more disfavored is the corresponding protonation process. Macrocycle **L** has two highly basic nitrogen atoms and two less basic amine groups, the pyridine nitrogen atoms remaining unprotonated through the whole pH range. This behavior can be explained by electrostatic reasons. This fact is also reflected in the species distribution as a function of pH (Figure S1 in the Supporting Information), which shows the diprotonated ligand as the major species at pH ∼5−8.

By comparing our data with those previously reported by Jackels et al.[16] for the same compound but using 0.2 M KNO_3 as the supporting electrolyte, a significant disagreement was found for the fourth protonation constant. We repeated the measurements in KNO_3 and we obtained the same results than those published in the literature. Then, we suspected that the anion present in the electrolyte could bind the tetraprotonated species of **L**. To check this hypothesis, the protonation constants in 0.15 M $NaClO_4$ were determined and used for the estimation of the binding of the tetraprotonated ligand with both nitrate and chloride anions, rendering log $K(NO_3^-) = 1.8(1)$ and log $K(Cl^-) = 1.2(1)$. Although our data demonstrate that chloride can compete with the target dicarboxylates at acidic pH, we decided to keep NaCl as the supporting electrolyte for our measurements, in order to mimic physiological medium. Therefore, all the binding constants reported in this paper are relative to the competing presence of chloride.

Binding Studies. The binding constants between **L** and the dicarboxylates (Tables 2 and 3) were determined by means of potentiometric titrations. The results collected in Tables 2 and 3 show the formation of H_x**L**A adducts with protonation degrees varying from 0 to 5 for all the tested substrates. Since both the substrates and the receptor participate in overlapping proton-transfer processes, translating the cumulative stability constants at the top entries of both tables into representative stepwise constants is not always straightforward. To do so, one has to consider the basicities of **L** and of the different substrates and assume that the interaction will not much affect the pH ranges of existence of the protonated species of receptor and substrates. If this is taken into account, the stepwise constants shown in the bottom entries of Tables 2−4 can be deduced. However, in these systems, due to the existence of overlapping equilibria involving different species of the receptor and the substrate, the use of conditional stability constants (K_{cond})[17] is particularly useful for comparing different systems. This parameter is defined in eq 1 as the ratio between the total amounts of adducts formed ($\sum[H_{i+j}\mathbf{L}A]$) and the free species of ligand ($\sum[H_j\mathbf{L}]$) and substrate ($\sum[H_iA]$):

$$K_{cond} = \sum[H_{i+j}\mathbf{L}A]/\sum[H_iA]\sum[H_j\mathbf{L}] \qquad (1)$$

Thus, any kind of selectivity of the receptor can be simply calculated by dividing the conditional constants obtained for each isomer of the substrate at a selected pH (see below). As can be deduced from the values in Tables 2 and 3 and the plots of the logarithms of K_{cond} vs pH (Figure 1), all the carboxylates form stable 1:1 supramolecular complexes with **L** throughout the whole pH range studied (log K_{cond} varying from ca. 2 to 6). As a general trend, the interaction is weaker at low pH values. This behavior can be ascribed to the fact that the protonation of the dicarboxylates hampers the electrostatic attractions with the azamacrocycle. Particularly surprising is the strong interaction observed at basic pH, where the $[\mathbf{L}A]^{2-}$ species predominates (Figure 2 for malate, the rest are given in Figure S3 in the Supporting Information), ruling out Coulombic attractions as the only driving force for the process. For a better comparison and an easier discussion of the results, we have grouped the dicarboxylic acids attending to the nature of their substituents in nonpolar (Figure 1A), amino acids (Figure 1B), and hydroxy acids (Figure 1C). The substitution of a methyl by a phenyl group in the diacid structure (Figure 1A, Table 2) produced a slight decrease of the binding to **L**, which can be explained by simple steric hindrance, and discarded any kind of attractive aryl−aryl interaction between receptor and substrate.[18] Comparison of the results obtained with the amino acid derivatives (Figure 1B, Table 3) rendered very interesting observations. First of all, acetylation of the free amino group unexpectedly decreased the binding, even considering that this nitrogen of the aspartic substrate remains protonated at almost the whole pH range under study (see Figure S2 in the Supporting

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TABLE 2. Logarithms of the Stability Constants[a] for the Interaction of L with the Dicarboxylates 1−4, Determined in 0.15 M NaCl at 298.1 K

reaction[b]	(*R*)-**1**	(*S*)-**1**	(*R*)-**2**	(*S*)-**2**	(*R*)-**3**	(*S*)-**3**	(*R*,*R*)-**4**	(*R*,*S*)-**4**	(*S*,*S*)-**4**
A + L ⇆ AL	5.12(2)	5.09(2)	4.86(4)	4.81(5)	4.40(2)	5.47(3)	3.77(6)	3.32(7)	4.27(3)
A + H +L ⇆ HAL	14.17(2)	14.22(2)	13.80(4)	13.75(6)	13.41(2)	14.37(3)	12.86(7)	12.42(7)	13.11(3)
A+ 2H + L ⇆ H_2AL	22.42(2)	22.55(2)	22.14(4)	22.12(4)	21.74(1)	22.57(2)	21.19(5)	20.72(7)	21.42(3)
A + 3H +L ⇆ H_3AL	27.65(2)	27.84(2)	27.14(4)	27.10(5)	26.52(2)	27.22(3)	25.55(7)	25.58(7)	25.76(3)
A + 4H +L ⇆ H_4AL	31.98(2)	32.18(2)	31.37(4)	31.35(4)	30.59(2)	31.28(3)	29.40(1)	29.70(1)	29.65(4)
A + 5H +L ⇆ H_5AL	35.56(3)	35.72(3)	34.65(9)	34.62(1)	33.62(5)	34.19(7)			
A + HL ⇆ HAL	5.1	5.2	4.7	4.7	4.4	5.3	3.8	3.5	4.0
A + H_2L ⇆ H_2AL	4.8	5.0	4.5	4.5	4.1	5.0	3.6	3.1	3.8
A + H_3L ⇆ H_3AL	5.1	5.3	4.6	4.5	4.0	4.7	3.0	3.0	3.2
HA + H_2L ⇆ H_3AL	4.7	4.9	4.5	4.4	4.2	4.9	3.9	3.6	4.1
HA + H_3L ⇆ H_4AL	4.1	4.3	3.7	3.7	3.3	3.9	2.8	2.7	3.1
HA + H_4L ⇆ H_5AL	4.6	4.7	3.9	3.9	3.2	3.8			
H_2A + H_3L ⇆ H_5AL	3.7	3.9	3.3	3.3	3.0	3.6			

[a] Values in parentheses correspond to standard deviation in the last significant figure. [b] Charges omitted for clarity.

TABLE 3. Logarithms of the Stability Constants[a] for the Interaction of L with the Dicarboxylates 5−7, Determined in 0.15 M NaCl at 298.1 K

reaction[b]	(*R*)-**5**	(*S*)-**5**	(*R*)-**6**	(*S*)-**6**	(*R*)-**7**	(*S*)-**7**
A + L ⇆ AL	5.67(3)	5.68(5)	5.38(3)	5.55(4)	6.13(5)	5.58(3)
A + H +L ⇆ HAL	15.10(3)	15.19(5)	14.30(3)	14.44(4)	15.38(5)	15.03(3)
A+ 2H + L ⇆ H_2AL	24.09(3)	24.22(6)	22.48(2)	22.60(3)	24.47(5)	24.05(3)
A + 3H +L ⇆ H_3AL	32.09(3)	32.22(5)	27.01(2)	27.12(3)	32.37(4)	32.06(3)
A + 4H +L ⇆ H_4AL	36.28(3)	36.40(5)	31.03(3)	31.12(4)	36.69(4)	36.44(3)
A + 5H +L ⇆ H_5AL	39.70(3)	39.84(9)			40.43(6)	40.20(4)
A + HL ⇆ HAL			5.2	5.4		
HA + L ⇆ HAL	5.4	5.5			5.8	5.5
A + H_2L ⇆ H_2AL			4.9	5.0		
HA + HL ⇆ H_2AL	5.4	5.5			5.8	5.4
A + H_3L ⇆ H_3AL			4.4	4.6		
HA + H_2L ⇆ H_3AL	4.8	5.0	4.7	4.8	5.2	4.9
HA + H_3L ⇆ H_4AL	4.1	4.2	3.7	3.8	4.5	4.3
H_2A + H_2L ⇆ H_4AL	5.3	5.4			5.3	5.1
HA + H_4L ⇆ H_5AL	4.4	4.6			4.3	4.1
H_2A + H_3L ⇆ H_5AL	3.7	3.9			4.1	3.9

[a] Values in parentheses correspond to standard deviation in the last significant figure. [b] Charges omitted for clarity.

TABLE 4. Hydrodynamic Parameters of the Samples Containing 1:1 Mixtures of L and (*R*)- or (*S*)-3 (500 MHz, D_2O, pD = 6.5, 303 K)

entry	sample	signal	D ($\times 10^{-10}$ m^2/s)	r_H (Å)[a]
1	**L** + (*S*)-**3**	C3H (**3**)	5.80 (±0.08)	3.9
2	**L** + (*S*)-**3**	C2Heq[b] (**L**)	5.13 (±0.06)	4.4
3	**L** + (*R*)-**3**	C3H (**3**)	7.65 (±0.11)	3.0
4	**L** + (*R*)-**3**	C2Heq[b] (**L**)	5.95 (±0.05)	3.8
5	calculated[c]		4.38[a,c]	5.2[c]

[a] Calculated by using the Stokes−Einstein equation.[24] [b] The signal C2Heq corresponds to the proton in equatorial at position C2 of the cyclohexane moiety of **L**. [c] Estimated upper limit value from a model geometry.

Information). This suggests that a possible interaction between the protonated amine of the amino acid substrates and the pyridine group of **L** could be playing a role, especially at acidic pH, where the differences in the binding of protected and unprotected aspartic are larger. On the other hand, the more flexible and longer glutamic acid is bound stronger than the aspartic derivative. Anyway, the fact that a simple receptor like **L** displays very strong binding to excitatory amino acids at physiological conditions is highly remarkable.

The best structural stereoselectivity was observed with hydroxy acids, malic and tartaric (Figure 1C, Table 2). It is noteworthy that the presence of a second OH group largely decreased the binding to **L**, which can be due to a more efficient solvation of tartaric derivatives in aqueous solution. The binding constants vary within this family in ca. 2 orders of magnitude for subtle differences in the molecular structure of the substrates, in the order (*S*)-Mal ≫ (*R*)-Mal > (*S*,*S*)-Tar > (*R*,*R*)-Tar > *meso*-Tar.

Concerning enantioselectivity (see Figure 1D for a free-energy representation), the best result was obtained with malate, for which **L** forms much more stable complexes with (*S*)-**3** than with the (*R*)-enantiomer over the pH interval tested (see Figure 2 for species distribution of both). This selectivity is very high at basic pH (at pH 10, $K_S/K_R = 11.50$), but decreases at acidic pH (at pH 2, $K_S/K_R = 3.89$). Interestingly, at neutral pH, where the uncharged diprotonated supramolecular complex [H_2**L**A] predominates (Figure 2), the interaction is also highly enantioselective (at pH 7, $K_S/K_R = 6.86$). **L** also displayed a moderate preference for the (*S*,*S*) enantiomer of tartrate (**4**), going from $K_{SS}/K_{RR} = 3.01$ at pH 10 to $K_{SS}/K_{RR} = 1.70$ at pH 7, showing practically no complexation at acidic pH. Additionally, it is interesting to note the excellent diastereoselectivity exhibited by the receptor toward the diastereoisomers of **4**. The *meso*-**4** isomer showed the weakest binding under our experimental conditions and, once again, the selectivity diminishes on decreasing the pH. For instance, comparing with the strongest bound chiral isomer, compound **L** showed relative binding constants of $K_{SS}/K_{RS} = 8.46$ at pH 10 and $K_{SS}/K_{RS} = 4.99$ at pH 7.

Negligible or absent enantioselectivity was found toward the substrates bearing aliphatic (**1**) and aromatic (**2**) substituents at

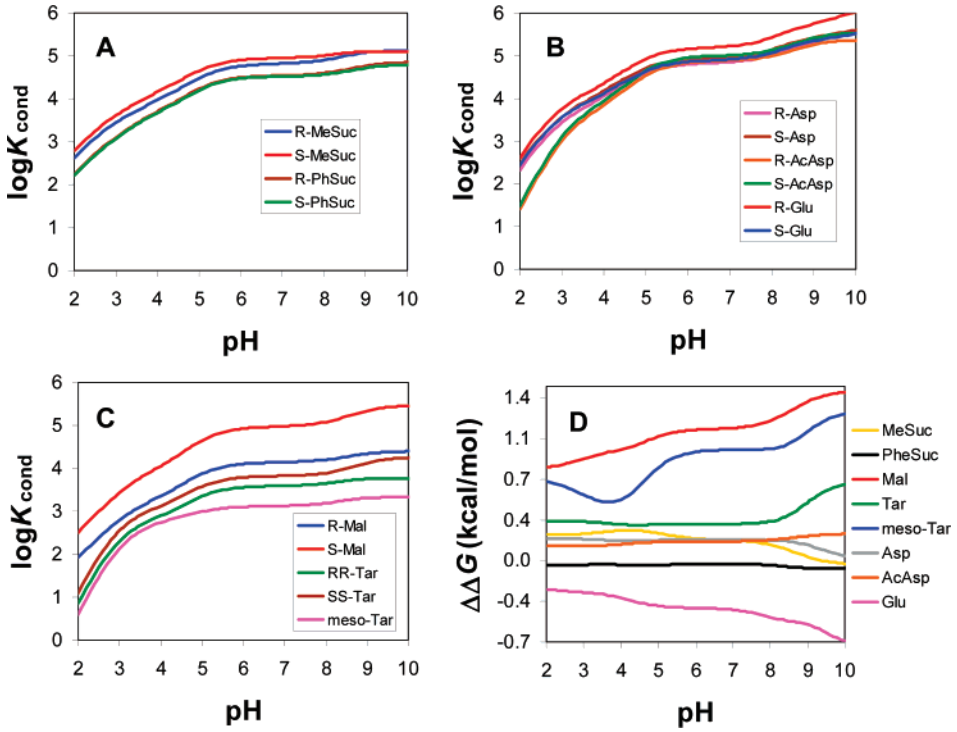

FIGURE 1. Plots of log K_{cond} vs pH for the binding of **L** with all the stereoisomers of the dicarboxylic acids: (A) methyl- and phenylsuccinic; (B) aspartic, *N*-acetylaspartic and glutamic; and (C) malic and tartaric. (D) Plot of the enantioselectivity, defined as the Gibbs energy difference (in kcal/mol), vs pH value. Positive $\Delta\Delta G$ means *S*-selectivity and for *meso*-tartaric acid this value corresponds to its ΔG difference with the (*S*,*S*)-isomer.

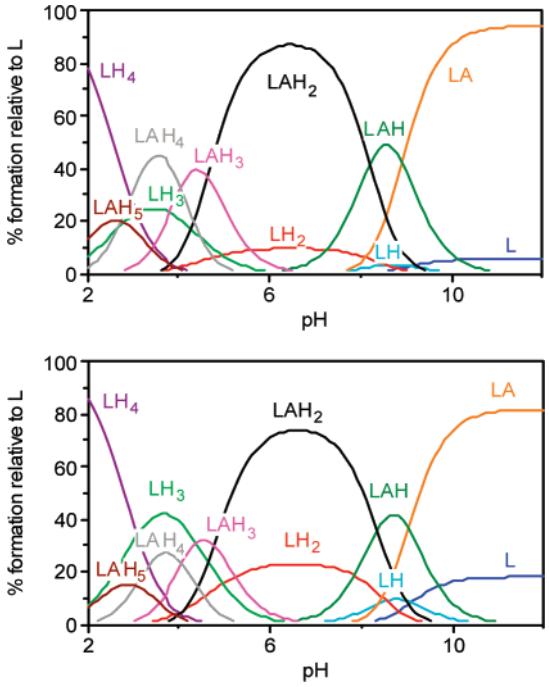

FIGURE 2. Distribution diagram for the [H_n**L**A] complexes of *S* (up) and *R* (down) malate as a function of pH ([**L**] = [A] = 10^{-3} M). Charges were omitted for clarity.

Cα. Regarding the amino acids derivatives, *N*-acetylation of aspartic acid produced a tiny increase of the enantioselectivity at basic pH (K_S/K_R = 1.48 at pH 10) that was absent with the free compound. Interestingly, **L** was found to exert a moderate and reversed enantioselectivity with free glutamic acid, being *R*-selective in this case (K_R/K_S = 3.22 at pH 10 and K_R/K_S = 2.05 at pH 7.0). The anomalous behavior with this substrate could be due to a more flexible structure that would lead to a different binding mode than those attained with the C4-dicarboxylates.

Therefore, the receptor is able to preferentially bind to substrates bearing an uncharged hydrogen bonding donor attached at C2 with an *S* configuration. Intrigued by this observation, we decided to go deeply into the study of the recognition process by means of different techniques.

Mass spectrometry (ESI-MS) can serve as an alternative to study the molecular recognition properties of **L**, since it has been recently used to detect the formation of diastereomeric supramolecular complexes with chiral selectors, showing a good correlation with the enantiodiscrimination found in solution.[19] However, to clearly reflect the selectivity found in solution, the experimental conditions must be carefully planned. We decided to carry out the mass spectra experiments at a concentration able to discriminate between the diastereomeric complexes formed by **L** and malate dianion at neutral pH. By looking at the data in Table 2 and Figure 1C, the binding to the *R* isomer is around 4 log units, while for the *S* isomer it is almost 5 log units. Accordingly, at a concentration close to 10^{-5} M we should be able to distinguish between both enantiomers using **L** as the selector. Thus, the ESI-MS spectrum of a 5×10^{-5} M aqueous solution of **L** and (*S*)-**3** (1:1 mixture, pH 6.4) showed peaks corresponding to the species $[H_3LA]^+$ (*m*/*z* 569) and $[H_4LA]^{2+}$ (*m*/*z* 285). Both full isotopic pattern analysis and tandem MS/MS experiments supported the assignation of the peaks. However, when the ESI-MS was acquired with (*R*)-**3** in exactly

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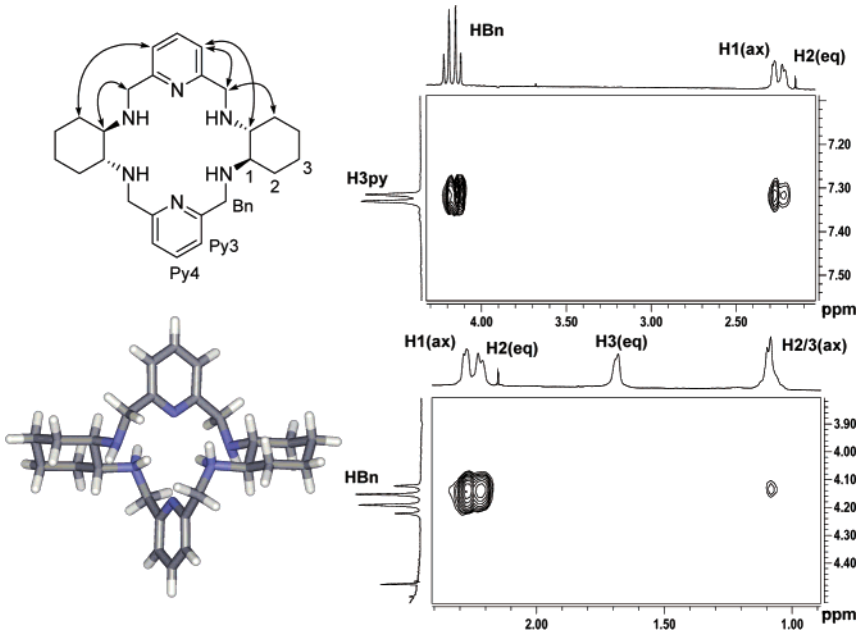

FIGURE 3. Selected sections of the 2D NOESY spectrum of **L** (500 MHz, D_2O, pD = 6.5, 303 K) and optimized molecular geometry for the diprotonated species.

the same experimental and operational conditions, the peaks corresponding to the supramolecular species were absent, supporting the higher stability of the supramolecular complex with the (*S*) enantiomer. Besides, for tartrate derivatives, a concentration of 7×10^{-4} M in **L** and a fivefold excess of the substrates ($\sim 4 \times 10^{-3}$ M in diacids) were necessary to detect the corresponding supramolecular complexes, namely $[H_3LA]^+$ (*m*/*z* 585) and $[H_4LA]^{2+}$ (*m*/*z* 283). This observation also correlates with the much lower binding of tartrate isomers (log $K \approx 3$, Table 2). Thus, ESI-MS allowed us to corroborate the stoichiometry and binding trends measured for the hydroxy acids by potentiometry.

Finally, the ESI-MS spectra at pH 11 (NaOH) of **L** and either malate or tartrate (negative mode) showed the presence of ternary complexes of the type $[NaLA]^-$ at *m*/*z* 589 (Mal) and 605 (Tar). Although sodium-complexed species are frequently formed in ionization chambers of ESI-MS equipments, the observation of ternary species could give an explanation for the large binding constants observed at basic pH.

Structural Studies in Aqueous Solution. To obtain more information about the conformation of **L**, we performed a complete NMR study in aqueous solution at pD = 6.5, where $[\mathbf{L}H_2]^{2+}$ is the major species. The receptor showed an effective D_2 symmetry in the NMR time scale, suggesting a very symmetrical conformation and a very fast proton movement within the macrocyclic cavity. Besides, the protons of the methynes attached to the chiral centers of the cyclohexane frameworks resonate at higher field compared to those present in similar structures,[8a] due to the shielding effect of the anisotropy cone of the pyridine ring. This observation suggests that the pyridine ring sets on apical positioning, directing its shielding anisochrony cone toward the region of axial protons of the cyclohexane. Additionally, NOESY experiments showed cross-peaks between Py-C*H*$_2$NH− and both methyne and H2 in the equatorial position of cyclohexane moeity (see Figure 3). More surprisingly, the proton at position 3 of the pyridine ring also showed NOESY cross-peaks with those same hydrogen atoms of the cyclohexane (Figure 3). All these experimental observations (chemical shifts and NOEs) agree with a twisted helical conformation of the macrocycle. Molecular modeling calculations[20] rendered the geometry also depicted in Figure 3, which is in very good agreement with that of the observed NOEs. The receptor showed a D_2 symmetrical helical conformation with the six nitrogen atoms at the vertices of an octahedron. The protonation takes place in two secondary nitrogen atoms and the structure is stabilized by several intramolecular hydrogen bonds.[21] This orientation of the pyridine and cyclohexane rings had been previously proposed for the free receptor and some of its metal complexes.[16]

Once we had determined the structure of the receptor in aqueous solution, we decided to do the same with the supramolecular complexes with the highest enantioselectivity. Analyses of ^{1}H, ^{13}C, and ^{15}N NMR experiments of a 1:1 mixture of **L** and each enantiomer of malate in D_2O (pD = 6.5, H_2LA supramolecular species are predominant) revealed that there are no significant chemical shift differences between the spectra of the diastereomeric complexes and free receptor or free substrate at the same pH. This suggests a very good fitting between host and guests structures. Besides, the effective D_2 symmetry of

(20) Geometry optimization was performed at the HF/3-21G* level of theory as implemented in Spartan 04 software.

(21) The microscopic protonation sites for macrocyclic polyamines is a controversial topic, as protons can freely move around the macrocyclic structure, especially in a polar protic solvent. In this particular system, we have calculated two different protonation scheme possibilities, the one depicted in Figure 3 and that one with alternated ammonium groups (given in Figure S4 in the Supporting Information). Both optimized structures showed very similar geometries and energies, although the one in Figure 3 is more stable, probably because the distance between positive charges in the twisted helical geometry is actually larger (4.52 vs. 4.28 Å). Thus, we followed the criteria of setting the protons maximizing the distance between protonation sites, within the helical conformation. For a deeper discussion on this topic, see refs 8a and 15. A more accurate description of the real situation would be the equilibrium between all the possible protonation sites. However, as the geometry of the macrocycle is not changed by this fast prototropic process, our conformational rationale is valid using one of the structures, and we have done it for simplicity. We thank the comments from a referee who drove our attention to the need for explaining this topic.

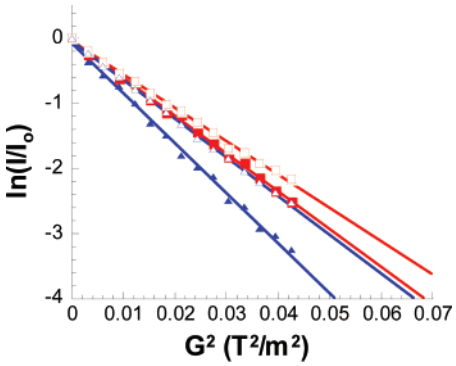

FIGURE 4. Plots of $\ln(I/I_o)$ vs G^2 for samples containing [(*S*)-**3** + **L**] (red) or [(*R*)- **3** + **L**] (blue). Open symbols correspond to signals from the receptor and solid symbols to signals from the substrate.

the receptor within the complex implies fast complexation−decomplexation equilibrium on the NMR time scale.

Unfortunately, there were no unambiguous intermolecular NOEs which could give us additional structural information. Nevertheless, the receptor showed the same intramolecular NOEs when forming the supramolecular complexes, as an indication of the retention of the helical conformation in solution. Definitive proofs for the solution behavior of both diasteromeric complexes were obtained by PGSE measurements.[22] By the accurate measurement of the dependence of NMR signal intensities with gradient strength, some hydrodynamic parameters can be extracted (Figure 4, Table 4). As complexation−decomplexation equilibrium would change the size of the species in solution, the observed self-diffusion rate (D)[23] of a given signal would be the time average of those of the interconverting species implicating that signal. Thus, the smaller the D value, the larger the size (hydrodynamic radius, r_H)[24] of the effective diffusing species and, thus, the more stable and with longer existence would be the supramolecular structure. When we compare the self-diffusion rates measured using one diastereotopic proton at position C3 of **3** (Table 4, entries 1 and 3), we observed that, under the same experimental conditions, the sample containing (*R*)-**3** seems to diffuse faster than the one containing (*S*)-**3**. More impressively, this trend was also observed comparing signals of the receptor in both samples (Table 4, entries 2 and 4), although the difference is smaller due to a closer size of free and complexed receptor. Finally, for the sample containing (*S*)-**3**, the measured D values with either receptor or substrate signals (Table 4, entries 1 and 2) are closer to one another than those measured in the sample with (*R*)-**3** (Table 4, entries 3 and 4). This implies that, in the case of (*S*)-**3**, the dianion diffuses longer and in higher

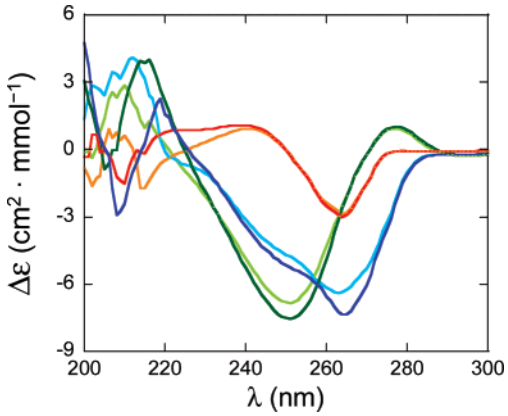

FIGURE 5. Normalized CD spectra of a 1:1 mixture of **L** and (*R*/*S*)-Mal at acidic (pH 2, red), neutral (pH 6.5, green), and basic (pH 10, blue) conditions. Dark and light colors correspond to *S* and *R* malate isomers, respectively.

proportion within the receptor. It is also noteworthy that the hydrodynamic radius calculated in all the cases is within the theoretical limit obtained with model structures. Once again, these measurements clearly correlate with the enantioselectivity observed by other techniques.

Since we suspected that the enantioselectivity displayed by our receptor (**L**) was related to its helical conformation, we decided to use circular dichroism spectroscopy (CD)[25] to monitor the differences between the diastereomeric complexes in solution for the most enantioselective example. With this aim, CD spectra of equimolecular mixtures of **L** and both enantiomers of **3** were acquired in water at different pH values. Results are shown in Figure 5. The CD spectra at the wavelength range 230−300 nm can be attributed to the chiral environment of the pyridine chromophore, which is produced not only by the chirality of the diaminocyclohexane moieties, but also by the helical macrocyclic conformation. The CD spectra showed a less pronounced signal for the complex of **L** with (*R*)-**3** than with (*S*)-**3**, suggesting that the binding of (*R*)-**3** produces an averaged larger distortion in the D_2 symmetric conformation of the receptor and, thus, a lower helical content in solution. Besides, the relationship between the state of protonation and the conformation of the macrocyclic structure is also evident in the CD spectra obtained at different pH values. It has been reported that a decrease in the pH results in a lower CD signal for the free receptor,[16] and this is also true for the supramolecular complexes. We suggest that at acidic pH values the protonation of the ligand produces Coulombic repulsions, which destabilize its helical conformation (see Figure S4 in the Supporting Information). At neutral pH, the diprotonated ligand dominates and $|\Delta\epsilon|$ is still high because the adjacent amine nitrogen atoms can share a proton in a hydrogen-bonded ring stabilizing the macrocycle in the helical conformation. Moreover, comparing our CD experiments with the reported data for the free receptor,[16] we can conclude that the presence of the dianion stabilizes the helical conformation of **L**, as $|\Delta\epsilon|$ is almost double. The interaction with the substrate will counterbalance the repulsion between the ammonium groups of **L** leading to stabilization of its helical conformation.

We also tried to analyze the CD spectra in a more detailed way, by comparison with the potentiometric data. Thus, the

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(23) For the accurate measurement of self-diffusion coefficients (D), the value of the HDO signal in deuterium oxide at 303 K ($D_{HDO} = 1.9 \times 10^{-9}$ m^2 s^{-1}) was used as an internal standard for every sample. Although D values are dependent on solvent, temperature, and concentration of the sample, as the measurements were performed under the same experimental conditions for samples containing either (*R*)-**3** or (*S*)-**3**, a direct comparison should be suitable to extract conclusions.

(24) The hydrodynamic radius (r_H) values were estimated by approximating the shape of the molecule to a sphere and using the Stokes−Einstein equation: $D = k_bT/6\pi\eta r_H$, where k_b is the Boltzmann constant and η is the viscosity of deuterated water at 303 K (0.976×10^{-3} kg m^2 s^{-2}). Also see: Edward, J. T. *J. Chem. Educ.* **1970**, *47*, 261.

(25) (a) Berova, N.; Di Bari, L.; Pescitelli, G. *Chem. Soc. Rev.* **2007**, *36*, 914. (b) Berova, N.; Nakanishi, K.; Woody, R. W. *Circular Dichroism. Principles and Applications*; Wiley-VCH: New York, 2000.

difference between the helicities of the diastereomeric supramolecular complexes decreased when lowering the pH, as happened with the ratio between the corresponding stability constants. Thus, the CD spectra at different pH values seem to correlate with the helical content difference between diastereomeric complexes and the stereoselectivity of the binding. Our interpretation of this fact is that the helicity of the receptor in the supramolecular structures dominates the enantioselectivity of the recognition process.

Conclusions

The simple macrocyclic polyazapyridinophane **L** forms supramolecular complexes with chiral dicarboxylates of the type $[H_n\mathbf{L}A]^{n-2}$ with n ranging from 0 to 5, depending on the anion and the pH value. The macrocycle **L** is especially selective in the interaction with hydroxyacid derivatives, despite that water solvation makes molecular recognition of these diacids much more problematic. However, a highly selective trend was found in the binding within the series (*S*)-Mal ≫ (*R*)-Mal > (*S*,*S*)-Tar > (*R*,*R*)-Tar > *meso*-Tar, with the very high enantioselectivity obtained for malate dianion being particularly outstanding. Both the stoichiometry and binding selectivity, measured by potentiometry, have been additionally observed by ESI-MS experiments, showing a very good qualitative agreement.

A detailed structural NMR analysis of the receptor showed a preferred twisted D_2 symmetrical helical conformation in aqueous solution at neutral pH. On the other hand, the NMR study of the corresponding supramolecular complexes formed by **L** and both enantiomers of malate rendered a perfect structural complementarity and the retention of the receptor helical conformation. Self-diffusion rates of the complexes yielded values in a very good agreement with the data obtained by potentiometric titrations. More importantly, circular dichroism spectra of the corresponding [**L**-Mal] supramolecular complexes at different pH values showed a clear correlation between the helical content of the receptor and the strength of the interaction with the chiral substrate. Thus, the dicarboxylate binding stabilizes the macrocyclic helical conformation and, on the other hand, this stabilization is more efficient with *S* malate than with the *R* enantiomer.

In summary, the deep multidisciplinary study of the anion binding abilities of **L** allowed us to propose a new mechanism for the enantiomeric recognition of anions in aqueous solution. To date, *the three points of interaction rule* seemed to be the mostly used explanation for enantioselective recognition processes.[26] Our study demonstrates that other sources of chirality, such as helicity, could be playing a vital role in the enantioselective binding of chiral susbtrates.[27] Besides, to the best of our knowledge, **L** displays the higher enantioselectivity for the binding of a chiral dicarboxylate at physiological conditions. We hope that our efforts to understand this supramolecular process will help us to better design more efficient systems for the selective molecular recognition of biologically important anions in their natural environment.

Experimental Section

Chemicals. The macrocycle **L** (all-*R* isomer) was prepared as previously described[28] and purified by recrystallization of the corresponding HCl salt in cold ethanol, rendering the correct spectroscopic and analytical data. All the chiral dicarboxylates were purchased in enantiopure forms and analytical grade, and were used without purification.

Electromotive Force (emf) Measurements. Potentiometric titrations were carried out with use of a reaction vessel water-thermostated at 25.0 ± 0.1 °C in an argon atmosphere. As it is present in biological systems in similar concentrations, NaCl 0.15 mol dm^{-3} was used as the supporting electrolyte. The titrant was delivered by a precision microburette. The potentiometric measurements were made with a pH-mV meter. The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen ion concentration probe by titration of previously standardized amounts of HCl with CO_2-free NaOH solutions and determining the equivalent point by Gran's method, which gives the standard potential, $E°'$, and the ionic product of water [$pK_w = 13.73(1)$]. The acquisition of the emf data was performed with the computer program PASAT. The HYPERQUAD program was used to process the data and calculate both the protonation and stability constants. The pH range investigated was 2.5−11.0, and the concentration of the anions and of the ligand ranged from 1×10^{-3} to 5×10^{-3} M with L:A molar ratios from 3:1 to 1:3. The different titrations for each system (at least two) were treated either as a single set or as separate curves without significant variations in the values of the stability constants. Finally, the sets of data were merged together and treated simultaneously to give the final stability constants.

Electrospray Ionization Mass Spectrometry (ESI-MS) Analysis. A quadrupole−hexapole−quadrupole mass spectrometer with an orthogonal Z-spray electrospray interface was used. Weighted amounts of the corresponding compounds in MeOH:water 1:1 were infused via syringe pump directly to the interface at a flow rate of 10 μL/min. The temperature of the source block was set to 120 °C and the interface to 150 °C. A capillary voltage of 3.5 kV was used in the positive scan mode and the cone voltage was kept at 15 V. The drying gas as well as nebulizing gas was nitrogen at flow rates of 400 and 80 L/h, respectively. The CID spectra were obtained at various collision energies (typically varied from E_{lab} 0 to 10 eV) by selecting the precursor ion of interest with MS1 and scanning MS2 at a cone voltage kept at 15 V. Argon was used as collision gas and the pressure in the collision cell was maintained at 1×10^{-3} mbar.

NMR Measurements. ^{1}H, ^{13}C, ^{1}H−^{15}N HSQC, NOESY, and PGSE experiments were preformed in an apparatus operating at 500 MHz for proton. Samples were prepared by mixing equimolar amounts of **L** and either (*R*)- or (*S*)-**3** (10^{-2} M and pD = 6.5). For ^{1}H, ^{13}C, ^{1}H−^{15}N HSQC, and NOESY experiments, the standard sequences implemented in the equipment software were used. For PGSE measurements, the Dbppste (DOSY bipolar pulse pair stimulated echo) sequence was applied and the gradient strength increments (typically an array of 15) calculated depending on the actual signal attenuation. For the accurate value of D, a linear regression of $\ln(I/I_o)$ vs G^2 was used for selected non-overlapped signals of either receptor or substrate, following the Stejskal−Tanner equation: $\ln(I/I_o) = -\gamma_x^2\delta^2(\Delta - \delta/3)DG^2$. All the linear fittings showed excellent correlation coefficients ($R^2 > 0.99$). For the calibration of self-diffusion coefficients, the value of the HDO signal in deuterium oxide at 303 K ($D_{HDO} = 1.9 \times 10^{-9}$ m^2/s) was used

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as an internal standard for every sample. The measurements were repeated at least three times to ensure reproducibility.

Circular Dichroism. Stock solutions of receptor (**L**) and both enantiomers of malic acid were prepared in pure water (0.05 M). Starting from these solutions, samples containing equimolecular amounts of receptor and either *R* or *S* malic acid were prepared. Final concentration was obtained by correcting the volume with pure water and the pH value was adjusted by adding either 1 M NaOH or 1 M HCl. The CD spectra were then recorded in a CD spectrophotometer equipped with a Peltier temperature controller. The measurements were performed at 298.1 K and at three different concentrations (0.1, 0.4, and 2 mM). The normalized spectra were obtained by transforming the data in the molar circular-dichroic absortion ($\Delta\epsilon$, $cm^2 \cdot mmol^{-1}$), using the formula $\Delta\epsilon = \theta/(32980Cl)$ where θ is the measured ellipticity (in mdeg), C is the concentration (M), and l is the path length (in cm). No changes were observed for normalized spectra at different overall concentration.

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Supporting Information Available: Tables for the protonation constants of dicarboxylates, species distribution diagrams for all the studied systems not given in the paper, and *XYZ* coordinates for the optimized structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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