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RESEARCH ARTICLE

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Inherently chiral dialkyloxy-calix[4]arene acetic acids as enantiodiscriminating additives for high-performance liquid chromatography separation of D,L-amino acids

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Abstract

Inherently chiral dialkyloxy-calix[4]arene acetic acids with asymmetric placement of substituents on the lower rim of the macrocycle were first studied as enantiodiscriminating additives to the mobile phase MeCN/H_2O/HCOOH (75/25/0.02 by volume) in the high-performance liquid chromatography (HPLC) separation of D,L-alanine and D,L-valine on the achiral stationary phase ZORBAX Original CN. The dependence of enantio-binding properties on the position of alkyl groups is demonstrated. The highest resolution (1.65) and enantioselectivity (1.80) were obtained for the 1,2-dipropyloxy-calix[4]arene acetic acid.

KEYWORDS

amino acids, calix[4]arene acetic acids, chiral mobile phase additive, complexation, enantioseparation, HPLC

1 | INTRODUCTION

Amino acids play an important role in the nature. They are the key structural units of proteins, enzymes, and hormones. Many drugs widely used in medical practice are based on them.[1,2] It was believed that all living organisms contain and use in their lives only L-amino acids. However, recent studies have shown that D-amino acids are also widely present in biological fluids and tissues of higher organisms, including humans. D-Amino acids enter the body of mammals with food, during the metabolism of the intestinal flora as well as a result of racemization during aging and biosynthesis.[3–6] The specific functions of individual D-amino acids in a healthy body and in pathological conditions were determined. Thus, D-serine and D-aspartate play an important role in neuroplasticity, memory processes, and learning.[7–10] D-Aspartate is also involved in developmental processes and endocrine functions of the body.[11] Racemization of L-amino acids with their transition to the D-form plays an important role in aging, and aspartic acid is most prone to racemization.[12,13] All these facts stimulate development of highly sensitive and rapid methods for the determination of enantiomeric forms of amino acids in biological samples, which can be used for early diagnosis and monitoring of a number of diseases.

Currently, the main method for determining the chiral form of amino acids is chiral liquid chromatography,[6,14–19] a key element of which is a chiral receptor capable of distinguishing D,L-stereoisomers. The promising chiral receptors are calixarenes—three-dimensional cup-shaped macrocyclic compounds which due to various nonvalent interactions (hydrogen bonds, π–π, CH–π, cation–π, anion–π, Van der Waals interactions, solvatophobic interactions, etc.) able to recognize with high selectivity and separate similar in properties cations, anions, neutral organic molecules,

and biomolecules. Such chiral calixarenes can recognize the optical antipodes of chiral molecules, including amino acids.[20,21]

There are two types of chiral calixarene receptors: calixarenes modified by chiral substituents (Figure 1) and inherently chiral calixarenes with asymmetrical placement of achiral substituents on the macrocyclic platform. As a result of the asymmetrical substitution, the calixarene molecule loses symmetry plane and becomes chiral.

Thus, the calix[4]arene **1** possessing chiral cyclopeptide moiety at the upper rim binds enantiomers of D,L-phenylalaninate anion with low enantioselectivity 1.3 in terms of binding constants.[22] Carbamido-calix[4]arene **2** bearing L-alanine fragment on the lower rim effectively recognizes D- and L-enantiomers of *N*-acetylphenylalanine anion. The binding constant of the D-enantiomer in acetone solution is four times higher than that one for the L-enantiomer.[23] Calixarene **3** bearing L-tryptophan fragments forms with *N*-Boc-D-alanine anion fluorescent complexes (stability constant 53 M^{-1}), whereas with *N*-Boc-L-alanine anion, the complexation is not observed.[24] Electron spectroscopy and molecular modeling showed that the *cS*-stereoisomer of inherently chiral *N*-(*S*)-1-phenylethylamide dipropyloxy-*tert*-butylcalix[4]arene acetic acid **4** discriminates D,L-enantiomers of valine in methanol solution. The D/L ratio for logarithms of the stability constants of their complexes is 1.26.[25] The calixarenes modified on the upper rim with fragments of the alkaloids quinine, C9-epiquinine, 9-aminoquinine, and 9-amino-epiquinine were used in manufacturing of chiral stationary phases **5** for liquid chromatography, which were tested for the separation of *N*-acylated amino acids.[26,27] Phases **5b**,**d** showed selectivity for *S*-enantiomers of noncyclic amino acids. Phases **5a**,**c** separated both linear and cyclic amino acids and better bound the *R*-enantiomers. The separation enantioselectivity for *N*-Boc-D,L-proline exceeded 5.0 when chloroform was used as the mobile phase.

The synthesis of inherently chiral derivatives of calixarenes is rather difficult[28,29] and their enantiorecognition properties are not studied up to date. Recently, we have developed the preparative methods for the synthesis of enantiomerically pure inherently chiral dialkyloxy-calix[4]arene acetic acids.[30–32] It was shown by an electron spectroscopy method the enantiodiscrimination for complexation of D,L-valine by dipropyloxy-calix[4]arene acetic acid *cR*-$(DP)_{prox}$CA (Figure 2) with stability constants ratio 1.11 in methanol solution.[25]

The aim of this work was to study the enantiomerically pure inherently chiral calixarene acetic acids as enantiodiscriminating additives to the mobile phase for the high-performance liquid chromatography (HPLC) separation of D,L-alanine and D,L-valine on the achiral stationary phase ZORBAX Original CN.

2 | MATERIALS AND METHODS

The inherently chiral calix[4]arene acetic acids were synthesized by methods described early in our works.[30,31]

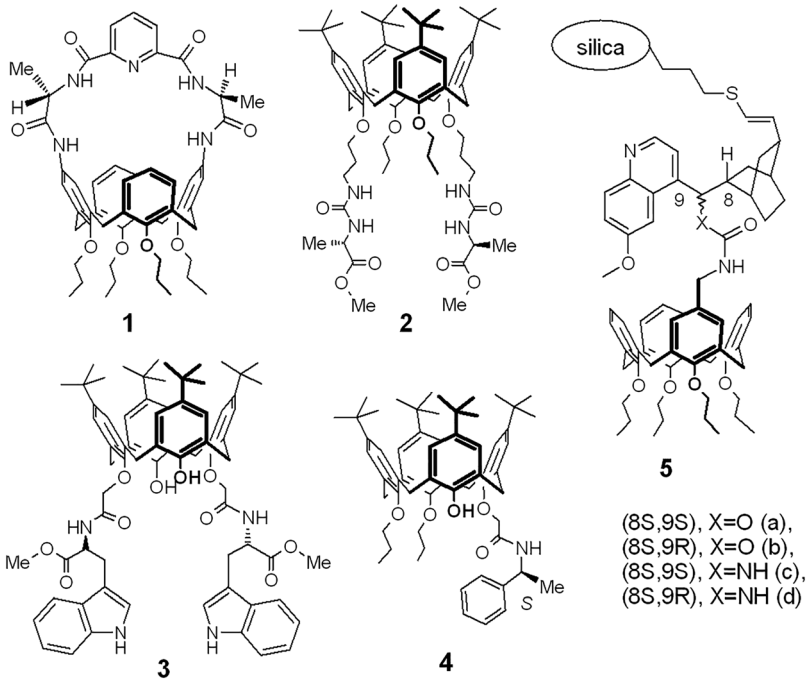

FIGURE 1 Chiral calix[4]arenes 1–5 capable of enantiodiscrimination of D,L-amino acids

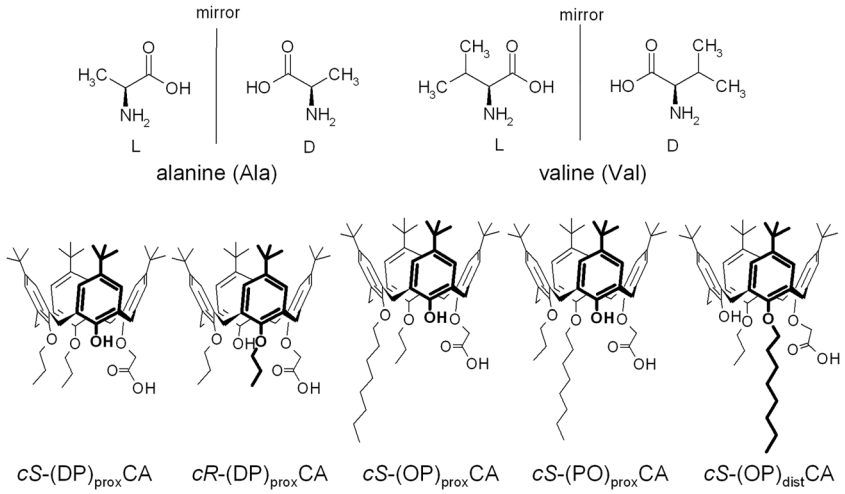

FIGURE 2 D,L-Amino acids and inherently chiral dialkyloxy-*tert*-butyl-calix[4]arene acetic acids

D- and L-enantiomers of alanine and valine were provided by "Enamine Ltd" (Kyiv, Ukraine; http://enamine.net). D,L-mixtures of amino acids for HPLC enantioseparation were obtained by mixing individual enantiomeric forms of alanine and valine.

HPLC analysis was performed on a liquid chromatograph Hitachi (Japan), equipped with chromatographic column Zorbax CN (250 × 4.6 mm, stationary phase Original CN) in mobile phase MeCN/H_2O/HCOOH (75/25/0.02 by volume). The wavelength of the UV detector was 220 nm for amino acids analysis and 254 nm for calixarenes analysis. The experiment was performed under isocratic conditions. The mobile phases contained the calixarene additives in concentrations of 0.1–0.55 mM. Amino acids for the analysis were prepared in a solution identical to the mobile phase. The concentrations of the amino acids in chromatographic solutions were 0.14–0.28 × 10^{-4} M, and the amount of the sample injected was 25 µl. The flow rate was 0.8 ml/min.

All chromatograms were obtained at a temperature of 30°C. The column was equilibrated in the stream of mobile phase for 3 h before analysis. Under such conditions, the column was saturated with calixarenes and was prepared for further amino acid analysis.

The resolution of D,L-amino acids (R_S) was calculated by the Equation (1):

$$R_S = 2(t_{R2} - t_{R1})/(\omega_{R1} + \omega_{R2}), \quad (1)$$

where t_{R2} and t_{R1} are the retention times of D- and L-enantiomers; ω_{R1} and ω_{R2} are the width of the chromatographic peaks of these enantiomers, which are determined at the points of intersection of the tangent peaks with the baseline.

The separation factor for D- and L-amino acids (α) was determined by the Equation (2):

$$\alpha = (t_{R2} - t_{R0})/(t_{R1} - t_{R0}), \quad (2)$$

where t_{R0} is the retention time for the sodium salt of ethylenediamine tetraacetic acid, which was used as a referred compound not retained in the column.

Stability constants K_A of the calixarene complexes with the amino acids were determined by the HPLC method described early.[33] The calixarene additive to the HPLC mobile phase decrease capacity coefficients k' of the amino acids due to the "host–guest" complexes formation. The linear character plots of the amino acid's $1/k'$ *v*ersus the calixarene concentration testifies formation of the 1:1 host–guest complexes and allows to calculate the stability constants K_A using the Equation (3):

$$1/k' = 1/k_0' + K_A \times [CA]/k_0' \quad (3)$$

where k_0' and k' are the capacity coefficients of the amino acid molecule determined in the absence and the presence of the calixarene additive to the mobile phase; [CA] is the concentration of calixarene additive in the mobile phase.

3 | RESULTS AND DISCUSSION

In our HPLC studies, five optically pure *cS*- or *cR*-forms[34] of inherently chiral dialkyloxy-calix[4]arene acetic acids with an asymmetric arrangement of substituents on the lower rim of the macrocycle were used (Figure 2): *cS*- and *cR*-enantiomers of dipropyloxy-calix[4]arene acetic acids with proximal placement of propyl groups (*cS*-$(DP)_{prox}$CA and *cR*-$(DP)_{prox}$CA); *cS*-enantiomers of propyloxy-octyloxy-calix[4]arene acetic acids with proximal arrangement of propyl and octyl groups in different sequences (*cS*-$(PO)_{prox}$CA and *cS*-$(OP)_{prox}$CA); *cS*-enantiomer of propyloxy-octyloxy-calix[4]arene acetic

acid with distal arrangement of the alkyl groups (*cS*-$(PO)_{dist}$CA).

All investigated calixarenes were registered on chromatograms by rather narrow peaks with retention times depended on the length of alkyl substituents and their mutual location on the macrocyclic platform. The enantiomers of dipropyloxy-calixarenes *cS*-$(DP)_{prox}$CA and *cR*-$(DP)_{prox}$CA had the same retention times ($t_R = 16.0$ min). The presence in the molecules of *cS*-$(PO)_{prox}$CA and *cS*-$(OP)_{prox}$CA proximal lipophilic octyl group increased their retention times t_R to 17.5 min. The retention time t_R of *cS*-$(OP)_{dist}$CA stereoisomer with distal location of propyl and octyl group was extended to 22.0 min. The isotherms of adsorption of calixarenes on the surface of the stationary phase are presented in Figure 3. The linear nature of the isotherms indicates the inverse of this interaction.

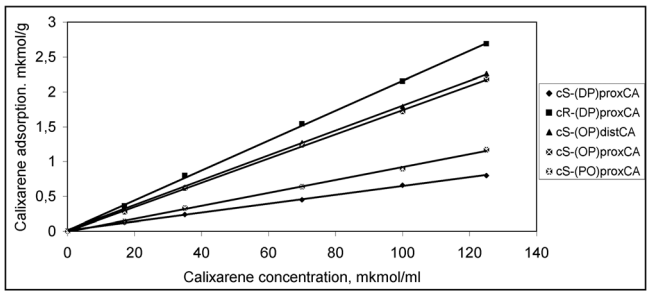

FIGURE 3 Adsorption isotherms of calixarenes on the surface of the stationary phase ZORBAX original CN

The D,L-racemic mixtures of alanine and valine were recorded by single peaks with retention times t_R 3.9 and 5.4 min, respectively. When chiral calixarenes were added to the mobile phase the single peaks of D,L-mixtures of both alanine and valine were separated into two peaks (Figures 4 and 5). The resolutions R_S and separation factors α for D,L-isomers of the amino acid by the inherently chiral calixarenes are presented in Table 1.

The resolutions R_S depend on the nature of the amino acid, the length of the alkyl substituents, and their relative position on the macrocyclic platform (Table 1). The resolutions for alanine stereoisomers decrease from 1.65 to 0.68 in the row cS-$(DP)_{prox}$CA > cS-$(OP)_{prox}$CA > cS-$(PO)_{prox}$CA > cS-$(OP)_{dist}$CA. At the same time, for bulk and lipophilic valine, the resolutions are less different and are in the range of 1.47–0.89. The

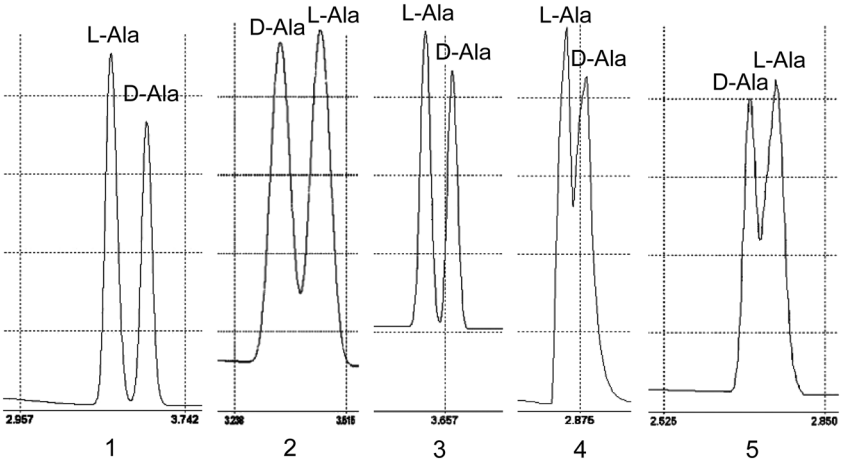

FIGURE 4 High-performance liquid chromatography (HPLC) enantioseparation of D,L-alanine by inherently chiral calixarenes: *cS*-$(DP)_{prox}$CA (1), *cR*-$(DP)_{prox}$CA (2), *cS*-$(OP)_{prox}$CA (3), *cS*-$(PO)_{prox}$CA (4), and *cS*-$(OP)_{dist}$CA (5). Mobile phase: MeCN-H_2O-HCOOH (75/25/0.02 v/v) with 0.1 mM of the corresponding calixarene additive. Flow rate 0.8 µl/min

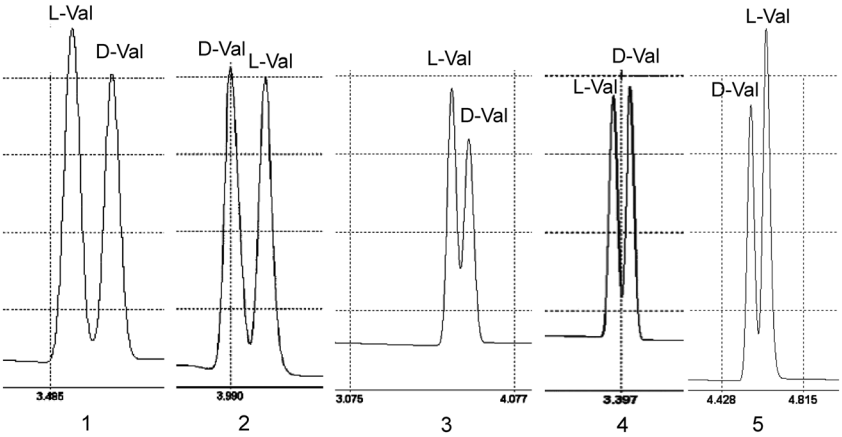

FIGURE 5 High-performance liquid chromatography (HPLC) enantioseparation of D,L-valine by inherently chiral calixarenes: *cS*-$(DP)_{prox}$CA (1), *cR*-$(DP)_{prox}$CA (2), *cS*-$(OP)_{prox}$CA (3), *cS*-$(PO)_{prox}$CA (4), *cS*-$(OP)_{dist}$CA (5). Mobile phase: MeCN-H_2O-HCOOH (75/25/0.02 v/v) with 0.1 mM of the corresponding calixarene additive. Flow rate 0.8 µl/min

TABLE 1 The resolutions (R_S) and separation factors (α) of D,L-isomers of alanine and valine by inherently chiral calixarenes

	Calixarenes									
	cS-(DP)$_{prox}$CA		*cR*-(DP)$_{prox}$CA		*cS*-(OP)$_{prox}$CA		*cS*-(PO)$_{prox}$CA		*cS*-(OP)$_{dist}$CA	
Amino acids	R_S	α	R_S	α	R_S	α	R_S	α	R_S	α
D-Ala	1.65	1.06	1.00	1.04	1.50	1.01	0.69	1.01	0.68	1.03
L-Ala										
D-Val	1.47	1.05	1.44	1.03	0.89	1.04	1.05	1.04	1.05	1.02
L-Val										

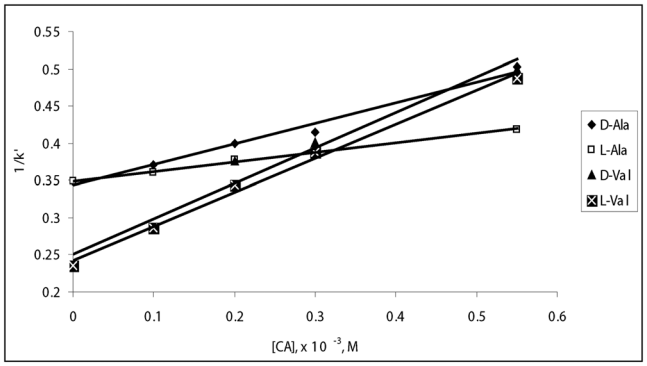

FIGURE 6 The dependence of $1/k'$ of amino acids on the concentration of calixarene *cS*-(DP)$_{prox}$CA in the mobile phase

separation factor of enantiomers α is almost independent of the structure of both amino acid and calixarene and is in the range of 1.01–1.06.

The next stage of the study was the determination of stability constants for complexes of the chiral calixarenes with individual D- and L-forms of the amino acids. During chromatographic analysis of the amino acids in the presence of the chiral calixarene additives, the diastereomeric supramolecular host–guest complexes were formed in the mobile phase. The complexation reduces the retention time and the capacity coefficients of amino acids and allows to determine the stability constants of the complexes formed. In all experiments, the dependences of the inverse capacity coefficients of amino acids $1/k'$ on

TABLE 2 The stability constants (K_A, M^{-1}) of diastereomeric complexes of calixarenes with D- and L-amino acids and selectivity of complexation (S)

	Calixarenes									
	cS-(DP)$_{prox}$CA		*cR*-(DP)$_{prox}$CA		*cS*-(OP)$_{prox}$CA		*cS*-(PO)$_{prox}$CA		*cS*-(OP)$_{dist}$CA	
Amino acids	K_A	S	K_A	S	K_A	S	K_A	S	K_A	S
D-Ala	388 ± 35	(L/D)	583 ± 52	(D/L)	400 ± 34	(L/D)	790 ± 86	(L/D)	1461 ± 185	(D/L)
L-Ala	699 ± 67	1.80	448 ± 49	1.30	525 ± 43	1.31	1078 ± 115	1.36	1121 ± 143	1.30
D-Val	1092 ± 103	(L/D)	1225 ± 121	(D/L)	1011 ± 80	(L/D)	1502 ± 165	(L/D)	346 ± 43	(D/L)
L-Val	1190 ± 107	1.09	1068 ± 99	1.15	1157 ± 91	1.14	1503 ± 168	1.00	333 ± 42	1.04

TABLE 3 The calculated values of binding energies (ΔG, kcal/mol) of diastereomeric complexes of calixarenes with D- and L-amino acids in vacuum (ΔG_vac.) and in solution (ΔG_PCM)

	Calixarenes									
	cS-(DP)$_{prox}$CA		*cR*-(DP)$_{prox}$CA		*cS*-(OP)$_{prox}$CA		*cS*-(PO)$_{prox}$CA		*cS*-(OP)$_{dist}$CA	
Amino acids	**ΔG_vac.**	**ΔG_PCM**	**ΔG_vac.**	**ΔG_PCM**	**ΔG_vac.**	**ΔG_PCM**	**ΔG_vac.**	**ΔG_PCM**	**ΔG_vac.**	**ΔG_PCM**
D-Ala	−13.97	−24.27	−9.84	−26.66	−10.07	−25.55	−10.79	−22.86	−13.97	−24.27
L-Ala	−13.20	−35.08	−15.67	−35.29	−13.71	−24.31	−9.85	−23.70	−13.20	−35.08
D-Val	−12.43	−31.37	−9.52	−26.91	−12.01	−23.37	−15.87	−30.57	−12.43	−31.37
L-Val	−13.77	−36.07	−17.90	−36.25	−14.63	−25.45	−15.19	−21.64	−13.77	−36.07

the concentration of calixarene in the mobile phase were linear (see Figure 6 as an example), which indicated the formation of 1:1 complexes and allowed to use Equation (3) to calculate the stability constants. The values of the stability constants K_A and the selectivity of complexation S ($S = K_{A(D)}/K_{A(L)}$ or $S = K_{A(L)}/K_{A(D)}$) are shown in Table 2.

The stability constants (K_A = 333–1503 M^{-1}) are depended on the nature of the amino acid and its D- or L-stereochemical configuration, as well as on the length and position of the alkyl substituents on the lower rim of the calixarene. All proximally substituted *cS*-calixarene acetic acids form stronger complexes with L-alanine or L-valine. In contrast, the *cR*-calixarene acetic acid prefers D-amino acids. The replacement in *cS*-calixarenes the proximal propyl group on octyl one gives little effect on stability of the complex. At the same time, replacing distal propyl to octyl increases the stability constant almost 1.5 times.

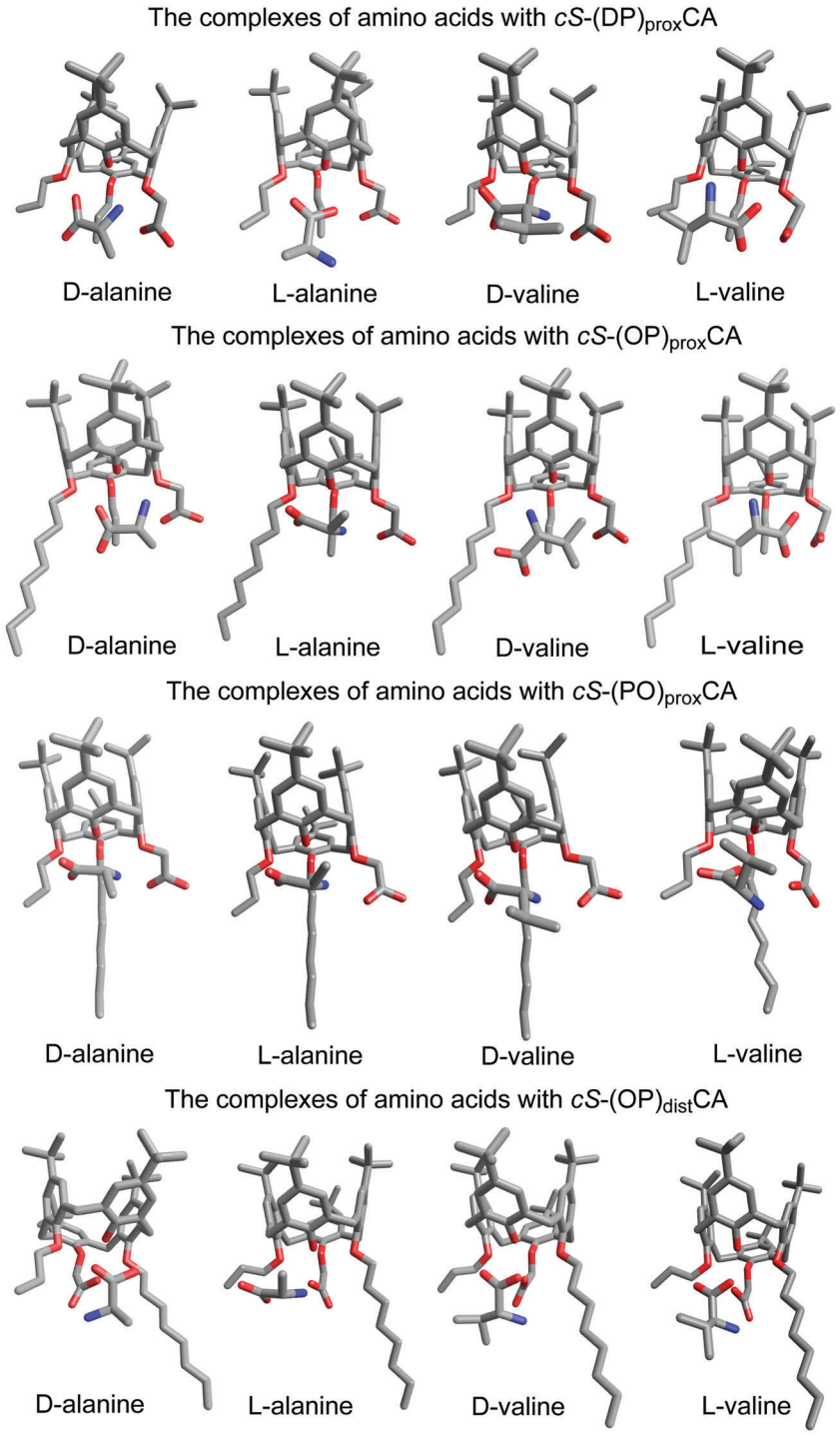

FIGURE 7 The calculated molecular structures of complexes of calixarene acetic acids with D- and L-forms of amino acids

All calixarene acetic acids (except *cS*-$(OP)_{dist}$CA) form more stable complexes with more lipophilic valine; however, D,L-selectivity of complexation is lower compared with alanine (Table 2). It should be noted that the similar D,L-selectivity ($S = 1.11$) was observed for complexes of *cR*-$(DP)_{prox}$CA with valine determined by a UV spectroscopy method.[25] The highest D,L-enantioselectivity was observed for complexation of alanine with *cS*-$(DP)_{prox}$CA. It should be noted that the enantioselectivity of complexation does not correlate with R_S and α parameters of chromatographic separation of D,L-enantiomers.

To understand the nature of the enantioselectivity of the HPLC separation, molecular modeling of calixarene complexes with amino acids by an m062x/cc-pvdz method[35,36] were performed. The calculations were made in two approximations: in vacuum and in the environment of solvent molecules (PCM model). The minimal binding energies for such complexes are given in Table 3, and the optimized structures are shown at Figure 7.

The calculated binding energies for the complexes of calixarene acetic acids with valine both in vacuum and in solution are consistent with stability constants obtained by the HPLC method. In the case of alanine, the stability constants of the complexes for dipropyloxy-calixarenes correspond to the binding energies calculated in solution, and for propyloxy-octyloxy-calixarenes—calculated in vacuum.

Analysis of the calculated structures (Figure 7) shows that the interaction energy of the carboxyl group of the amino acid with the functional groups of calixarene is significantly greater than the acid–base interaction of the amino group and is decisive in the formation of complexes. As a result, there are different orientations of the amino acid in relation to the calixarene molecule: it can bind both an amino group and a carboxyl group to calixarene carboxyl. This can be explained only by the fact that the amino acid in the complex exists in the zwitterionic form.

4 | CONCLUSION

In this work, the use of enantiomerically pure inherently chiral dialkyloxy-calix[4]arene acetic acids as additives to the mobile phase in HPLC was investigated for the first time. It was shown that in the water-organic phase, these compounds form supramolecular diastereomeric complexes of the host–guest type with D- and L-forms of alanine or valine with the stability constants in the range of 333–1503 M^{-1}. Different sorption properties of such complexes on the surface of the stationary phase promote the chromatographic separation of D,L-enantiomers of the amino acids. The molecules of valine bind better to inherently chiral calixarenes, but the separation of enantiomers is worse than for alanine. The obtained values for resolutions (0.68–1.65) and selectivity of complexation (1.3–1.8) allow us to consider such compounds promising for use in chiral chromatography, including the analysis of D/L-isomers of amino acids.

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DATA AVAILABILITY STATEMENT

Data are available from the authors, upon reasonable request.

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