A Chromatographic Approach to Analyze Dansyl Amino Acid–HP-β-CD Association Using Macrocyclic Antibiotic as the Stationary Phase

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Abstract

The retention mechanism for a series of D,L-dansyl amino acids in high-performance liquid chromatography is investigated using a teicoplanin stationary phase and hydroxypropyl-β-cyclodextrin (HP-β-CD) as the mobile phase additive (0–16mM). A theoretical treatment is developed to determine the HP-β-CD influence on the equilibrium between the teicoplanin phase and the aqueous medium, respectively. From the experimental data, the association constants of the D,L-dansyl amino acids–HP-β-CD inclusion complexes are determined and discussed in relation to the enantiomer structure. A thermodynamic study confirms that both the retention and complexation mechanisms are independent of the dansyl amino acid molecular structure and its absolute carbon configuration.

Introduction

Cyclodextrins (CDs) are a series of torus-shaped oligosaccharides composed of six to twelve α-1,4-linked D-glucopyranose units per molecule. The most widely used CDs are α-, β-, γ-, and δ-CD containing six, seven, eight, and nine glucose monomers, respectively (1). The rigidity of the CD structure and its relativity nonpolar central cavity (2) are characteristics that enable the formation of inclusion complexes with various solutes. These reversible "host–guest" inclusion complexes can change the physicochemical properties of the guest molecule. Therefore, CDs are used in many application fields to take full advantage of the encapsulation to improve solubility (3), stability (4), and the dissolution rate of therapeutics (1). The addition of CDs to the mobile phase modifies the solute retention according to its size, functional group position, or the strength and the stoichiometry of its complex with CD. Mohseni et al. (5) reported retention data and apparent complex dissociation constants of several compounds classes on a C_{18} column with methanol– or ethanol–water mobile phases containing various concentrations of CD. They also measured changes in enthalpy and entropy with and without CDs in order to explore the thermodynamic aspects of the separation process. Nowakowski et al. (6) demonstrated, using thermodynamic data and molecular modelling, that the calculation of inclusion equilibrium constants was dependent on the stationary phase–mobile phase system.

More recently, Guillaume et al. (7) showed that the main parameter governing retention of imidazole derivatives on an RP_{18} column with hydro-organic mobile phases containing β-CD was the distribution of the solute in the mobile phase, rather than the interactions with stationary phase. Usually, workers had to add co-modifier to the mobile phase in order to define the stoichiometry and formation constant of CD–guest molecule complexes. The presence of these co-modifiers led to a modification in the interaction between the CD and the guest molecule so that the "true" inclusion mechanism could not be observed. An alternative method can be explored via the use of a more polar stationary phase than the C_{18} phase. In this study, the retention data of a dansyl amino acid family were analyzed using macrocyclic antibiotic (8,9) as the stationary phase, an organic modifier (OM)–water mixture with a very low OM fraction as the mobile phase, and hydroxypropyl-β-CD (HP-β-CD) as the complexing agent.

Experimental

Apparatus

The high-performace liquid chromatography (HPLC) system consisted of a Merck Hitachi pump L7100 (Nogent sur Marne, France), an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a Merck L4500 diode array detector. An Astec 150- × 4.6-mm chirobiotic T HPLC column (Interchim) (packed with a stationary phase produced by chemically bonding the macrocyclic glucopeptide teicoplanin to

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5-µm silica gel) was used at a controlled temperature in an Interchim oven (no. 701).

Solvents and samples

Methanol (OM) was used without further purification (Merck). Water was obtained from an Elgastat water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge. Tri-sodium citrate and citric acid were from Prolabo (Paris, France). R-2 HP-β-CD was supplied by C.E.A. (Fontenay-aux-Roses, France) and was used in the bulk solvent from 0 to 16mM. All the dansyl amino acids were obtained from Sigma Aldrich (Saint Quentin, France). The mobile phase consisted of a 0.05M citrate buffer–methanol (95:5, v/v) mixture. The value of the buffer pH fraction was equal to 6.

The asymmetry factor of all peaks calculated from measurements made at 10% of the total peak height was in the range of $1.00 < As < 1.10$, indicating that the peaks had a gaussian shape. To examine the concentration fraction dependency of solute retention corresponding to the binding capacity of the dansyl amino acids, retention measurements were related to varying amounts of injected solute. Solute samples were prepared at different concentrations in the mobile phase (1–10 µg/mL). Each solute (20 µL) was injected in triplicate and retention times measured. The plots of retention factor versus sample concentration were < 5 µg/mL, followed by a decrease at higher solute concentrations. Therefore each solute was injected at a concentration of 2.5 µg/mL when the retention was sample-concentration independent (i.e., in linear elution condition).

Temperature study

Compound retention factors were determined over the temperature range of 20–45°C. The chromatographic system was left to equilibrate at each temperature for at least 1 h before each experiment. To study this equilibration, the retention time of D-dansyl tryptophan was measured after 22, 23, and 24 h. The maximum relative difference between retention times of this compound was never more than 0.6%, meaning that after 1 h the chromatographic system was sufficiently equilibrated for use. All the solutes were injected three times at each temperature and for each HP-β-CD concentration.

Theory

It is known that the solute retention factor (k') at temperature T is related to the change in free energy $(\Delta G^\circ)_T$ of the solute transfer between the mobile phase to the teicoplanin stationary phase. This relationship is expressed by (7,10–12):

$$\ln k' = \frac{-(\Delta G^\circ)_T}{RT} + \ln \phi \qquad \text{Eq. 1}$$

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). When the dead volume of the column and the technical data were taken into account (13–14), ϕ was calculated as 0.098.

The free energy $(\Delta G^\circ)_T$ from equation 1 can be broken into enthalpic and entropic terms to give the following equation (7,11,12,15):

$$\ln k' = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \qquad \text{Eq. 2}$$

where ΔH° and ΔS° were the enthalpy and entropy of the solute transfer from the mobile to teicoplanin stationary phase. With an invariant retention mechanism over the temperature range being studied, the enthalpy of transfer ΔH° remained constant, and a plot of $\ln k'$ in relation to $1/T$, which is commonly described as a van't Hoff plot, led to a straight line with an enthalpic slope and entropic origin (15). For the complexation mechanism, assuming that the HP-β-CD was not retained with the teicoplanin phase when its concentration changed in the mobile phase, the solute retention factor k' is affected, as described by the well-known equation (16):

$$\frac{1}{k'} = \frac{1}{k'_0} + \frac{K_f}{k'_0}[(CD)_m] \qquad \text{Eq. 3}$$

where k'_0 is the retention factor without CD, $(CD)_m$ is the equilibrium concentration of HP-β-CD in the mobile phase, and K_f is the apparent formation constant of the inclusion complex. The corresponding inclusion complex formation enthalpy and entropy (ΔH°_f, ΔS°_f) between the solute molecule and the CD in the mobile phase are determined by plotting the logarithm of the apparent complex formation (K_f) versus the temperature reciprocal (16):

$$\ln K_f = -\frac{-\Delta H^\circ_f}{RT} + \frac{\Delta S^\circ_f}{R} \qquad \text{Eq. 4}$$

A further thermodynamic approach to the analysis of physicochemical data is enthalpy-entropy compensation. This investigation tool has been previously used in chromatographic procedures to analyze and compare the retention mechanism for a group of compounds (15,17–19). This enthalpy–entropy compensation can be described by the following equation (19,20):

$$Z^\circ_1 = \beta Z^\circ_2 + Z^\circ_\beta \qquad \text{Eq. 5}$$

For the solute retention mechanism, Z°_1, Z°_2, and Z°_β corresponded, respectively, to the enthalpy (ΔH°), entropy (ΔS°), and Gibbs free energy (ΔG°_β) at the compensation temperature β.

For the complexation process, these values were, respectively, the inclusion formation enthalpy (ΔH°_f), entropies (ΔS°_f), and Gibbs free energy ($\Delta G^\circ_{f\beta}$) at the compensation temperature β between the solute molecule and the CD in the mobile phase.

According to equation 5, when enthalpy–entropy compensation is observed for a compound group in a particular chemical transformation, all of the compounds have the same Z°_β at

temperature β. For example, if enthalpy–entropy compensation is observed on liquid chromatography for a compound group, all the solutes will have the same net retention at the temperature β, although their temperature dependences may be differ (5,17–19).

For the solute retention mechanism, combining equtions 2 and 5 the following equation is obtained (21,22):

$$\ln k' = \frac{-\Delta H^\circ}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G^\circ_\beta}{R\beta} + \ln \phi \qquad \text{Eq. 6}$$

For the complexation process, combining equations 4 and 5, the following equation is obtained:

$$\ln K_f = \frac{-\Delta H^\circ_f}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G^\circ_{\beta f}}{R\beta} + \ln \phi \qquad \text{Eq. 7}$$

Equations 6 and 7 show that if a plot $\ln k'_T$ or $\ln K_f$ versus Z°_1 (i.e., ΔH° or ΔH°_f) evaluated at a constant temperature T is a linear function, a compensation temperature β can therefore be evaluated from the slope. The similarity of the values for the compensation temperature β suggested that the solutes are retained or included in HP-β-CD cavity by essentially identical mechanisms, and the compensation study was therefore a useful tool for comparing the retention mechanism or complexation for different compounds.

Results and Discussion

Solute transfer from the mobile phase to the teicoplanin phase when the HP-β-CD concentration was nil

From the determined solute retention times, k' of the height solute molecules under study was determined with the teicoplanin phase in the entire range of temperatures (i.e., 20–45°C). For all solutes, when the temperature increased there was a decrease in the retention factor, as expected for a reversed-phase system (23). Linear van't Hoff plots were obtained with correlation coefficients (r) higher than 0.97 for all fits. This behavior demonstrated that the invariant retention mechanism in the full range of temperatures was observed. According to equation 2, these linear van't Hoff plots provided a conventional way of calculating the thermodynamic parameters. Table I reports a complete list of ΔH° and $T\Delta S^\circ$ values (T = 30°C) when the HP-β-CD was nil. Negative enthalpies indicated that it was energetically more favorable for the solute to be in the teicoplanin phase (Table I). Negative entropies showed a freedom loss for the amino acid when the solute was transferred from the mobile to teicoplanin phase. For all solutes, this transfer was enthalpi-

Table I. ΔH° and $T\Delta S^\circ$ Values for All Amino Acids at T = 30°C when HP-β-CD was Nil

Solute denomination	ΔH° (kJ/mol)	$T\Delta S^\circ$ (kJ/mol)
L-Valine	–29.49	–2.16
L-Norvaline	–32.03	–2.34
L-Leucine	–32.48	–2.36
L-Phenylalanine	–35.52	–2.52
L-Tryptophane	–36.24	–2.72
D-Valine	–30.72	–2.40
D-Norvaline	–32.66	–2.31
D-Leucine	–33.44	–2.53
D-Phenylalanine	–35.10	–2.72
D-Tryptophane	–36.98	–2.73

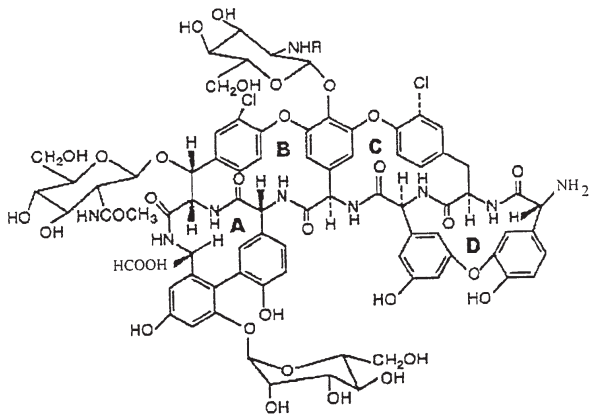

Figure 1. Teicoplanin structure.

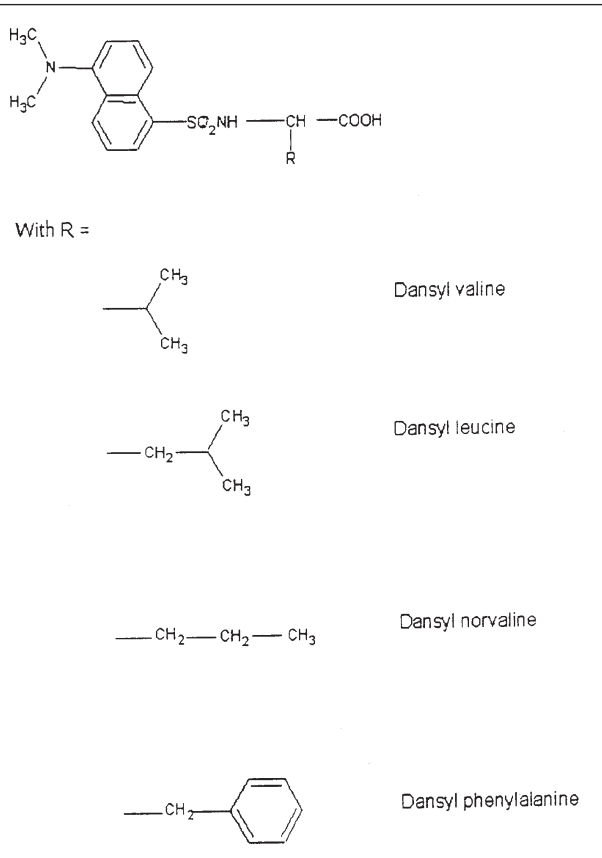

Figure 2. Dansyl amino acid structures.

cally driven (magnitude of $\Delta H°$ was always < $T\Delta S°$) (Table I) and can be described as the replacement of weak solute–solvent interactions by strong solute–teicoplanin-phase interactions. This is consistent with results reported in the literature about various chromatographic separations (24,25). On this teicoplanin stationary phase, the elution order of the five L-amino acids was: dansyl valine < dansyl norvaline < dansyl leucine < dansyl norvaline ~ dansyl tryptophane. The D-dansyl amino acid elution order was similar. Teicoplanin (Figure 1) is a molecule consisting of an aglycone peptide basket with three attached carbohydrate moieties and four cavities denoted A, B, C, and D (26–28). Moreover, teicoplanin used as a stationary phase, contains a single primary amine and a single carboxylic group (26–29). As well, dansyl amino acids contain two ionizable groups, a carboxylic group, and a tertiary amino group from the dimethylaminonaphtyl moiety. The pH of the mobile phase aqueous fraction was, in this study, equal to 6. In this pH, the protonation of the carboxylic group on both the teicoplanin and the dansyl amino acids can, thus, be neglected. Therefore, in this pH (= 6), the teicoplanin surface is fully ionized ($-COO^-$ and NH_3^+), and the dansyl amino acid is also ionized (28,29). It appeared that, in this study, the hydrophobic effect mainly determined the dansyl amino acid elution order. Figure 2 shows the molecular structure of these compounds. The apolar groups [dimethylaminonaphtyl of the dansyl group and side-chains (R)] acted as factors governing the analyte degree solvation in the bulk solvent. When the R group's hydrophobicity increased, the tendency for the solute to be excluded from the bulk solvent with a high water fraction (0.95, v/v), and to go toward the teicoplanin, increased. Thus, dansyl valine was eluted first, followed by dansyl norvaline and dansyl leucine, which contained a second CH_2 group. These results were corroborated by the fact that the highest values of $\Delta H°$ and $\Delta S°$ were observed for dansyl valine. Concerning the dansyl phenylalanine and dansyl tryptophane, the side-chains (R) were more highly polarizable than they were for the aliphatic dansyl amino acids. Dansyl tryptophan was then eluted more slowly from the teicoplanin column than dansyl leucine because the hydrophobicity of the indoyl methyl group was greater than the isopropyl group. Among the dansyl amino acids, dansyl tryptophan exhibited the lowest thermodynamic data because of the interactions involving delocalized electrons of the aromatic ring system. Thus, dansyl tryptophan had the weakest interactions with the teicoplanin phase. As well, it appeared that in all cases, the L-enantiomers were eluted before the D-enantiomers. It has been recently demonstrated that the chiral discrimination was principally governed by short-range interactions between the ionic form of the dansyl amino acid and the teicoplanin macrocycle (30). Figure 3 represents the chromatogram obtained for dansyl valine enantiomers at 30°C. Such behavior has been previously reported by Guillaume et al. for the same D,L-amino acids' retention on human serum albumin (30). Moreover, it was also demonstrated that dansyl amino acids bound on the most hydrophobic cycle of teicoplanin phase (i.e., A-cycle) (28). As can be inferred from Figure 4, binding of the L-enantiomer to teicoplanin was stabilized by steric repulsion between the R group and the rim of the binding site (29). In contrast, the D-enantiomer bound more tightly because the R group did not contact the rim. It was confirmed by the fact that the D-dansyl amino acid thermodynamic data was always lower than L-dansyl amino acid $\Delta S°$ and $\Delta H°$ values (28,29).

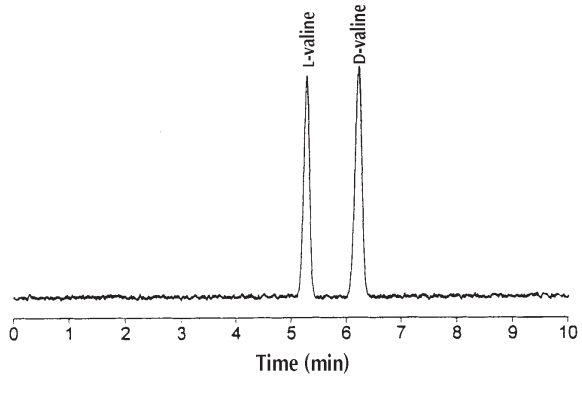

Figure 3. Chromatogram obtained for a dansyl valine racemic mixture.

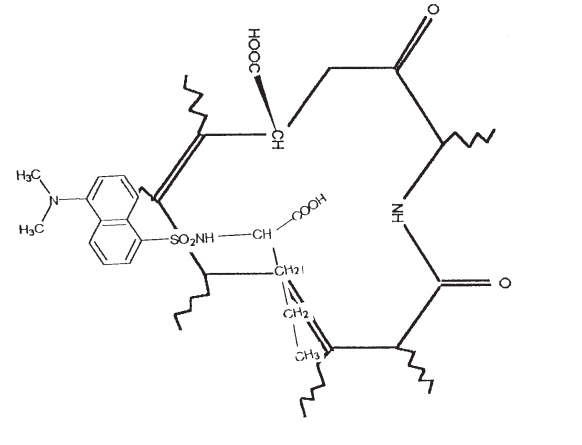

Figure 4. Binding of dansyl norvaline to teicoplanin A-cycle.

HP-β-CD–dansyl amino acid complexation process

The plot ln k' versus $\Delta H°$ at 30°C (equation 6) was drawn for

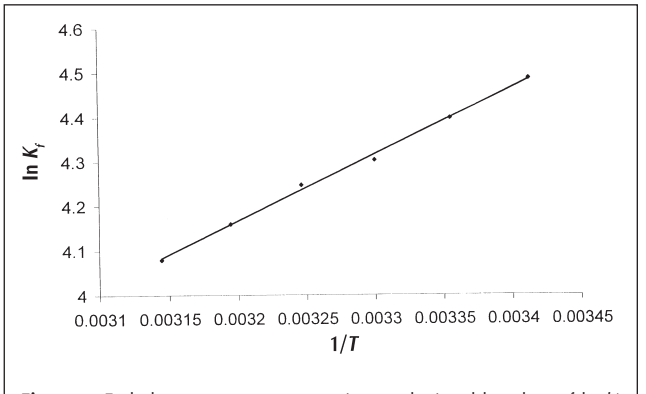

Figure 5. Enthalpy–entropy compensation as depicted by plots of ln k' versus $\Delta H°$ at 30°C and at different values of HP-β-CD concentration for all D,L-dansyl amino acids.

the height amino acids and all the HP-β-CD concentration in the bulk solvent (Figure 5). The correlation coefficient for the linear fit (r = 0.95) can be considered adequate to verify enthalpy–entropy compensation. Consequently, the solute transfer mechanism was independent of both the dansyl amino acid structure and carbon absolute configuration. Additionally, this revealed that the eventual interactions of the HP-β-CD with teicoplanin phase and the possible complexation of these solutes with HP-β-CD bound on teicoplanin seem to be negligible. The apparent formation constant (K_f) of dansyl amino acid–HP-β-CD complexation was calculated according to equation 3. The correlation coefficients obtained were over 0.97. These complex formation constants were calculated for different temperatures. Table II presents K_f values for all D,L-dansyl amino acids at six temperatures (T). For all solutes, when T increased, there was a decrease in the K_f, values, as usually expected (7). The K_f, of L-dansyl amino acids were always greater than for the D-enantiomers, indicating that the HP-β-CD–L-dansyl amino acid complex was more favorable than the HP-β-CD–D-dansyl amino acid complex. The van't Hoff plots (equation 4) were drawn for all solutes. For all dansyl amino acids, a linear graph was observed (r^2 over 0.98). For example, Figure 6 represents the plot ln K_f versus 1/T for D-dansyl norvaline. The resulting thermodynamic parameters are given in Table III (equation 4). The negative formation enthalpy values were caused by the favorable interactions that the compounds can establish with the hydrophobic HP-β-CD cavity. For all the compounds, the solute complexation mechanism with HP-β-CD was enthalpically driven (magnitude of ΔH°_f was always > $T\Delta S^\circ_f$), compared with the classical results generally observed in the literature with an RP_{18} stationary phase (7). An enthalpy–entropy compensation study was used as well, in order to gain further insight into the solute-HP-β-CD complexation process (equation 7). The linear variation (r^2 = 0.98) verified the enthalpy–entropy compensation. This confirmed that this association mechanism was both identical for the D- and L-enantiomers and principally took place in the bulk solvent. The ΔH°_f and ΔS°_f values were smaller for L-dansyl amino acids than for D-enantiomer. Therefore, CD complexation was more ordered and more energetically stabilized with L-enantiomer than with D-dansyl amino acids, confirming well the role of absolute carbon configuration. Moreover, in this study, the hydrophobic effect seems to determine the amino acids complexation with HP-β-CD. The apolar groups (dimethylaminonaphtyl of the dansyl group and R) acted as factors governing the degree of inclusion of the analyte in the HP-β-CD cavity. When the R group's hydrophobicity increased (Figure 2), the dansyl amino acid transfer from the bulk solvent to the HP-β-CD cavity was more favorable, leading a thermodynamic data (ΔH°_f and ΔS°_f) decrease (Table III). Dansyl valine with a propyl group had the weakest van der Waals and hydrogen interaction with the HP-β-CD cavity. These were associated with the greatest entropy value (Table III), classically attributed to the release of the water molecules surrounding the solute when they were transferred towards the HP-β-CD. For dansyl tryptophane, the side-chains increased the dipolar–dipolar interactions between the solute and the HP-β-CD cavity and thus decreased its degree of freedom in this cavity leading to the lowest values of

Table II. K_f Values of the Height Solutes at Six Temperatures

T (°C)	20	25	30	35	40	45
L-Valine*	71.52	68.90	54.30	49.32	47.89	44.23
L-Norvaline	94.65	76.74	76.10	54.12	48.91	42.10
L-Leucine	105.98	80.47	70.55	63.59	49.41	40.44
L-Phenylalanine	211.56	142.82	87.87	80.08	78.04	72.96
L-Tryptophane	247.44	183.23	141.33	114.04	73.56	62.23
D-Valine	86.84	84.20	70.40	68.70	62.17	57.39
D-Norvaline	88.89	81.36	78.34	69.99	64.07	59.14
D-Leucine	114.78	104.58	94.98	86.97	79.04	72.96
D-Phenylalanine	232.43	178.87	102.30	97.98	87.89	81.23
D-Tryptophane	278.78	267.87	166.45	142.12	97.81	88.98

* See Figure 2.

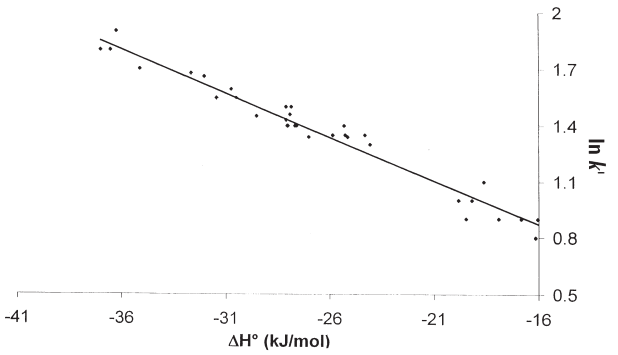

Figure 6. Ln K_f vs. 1/T for D-dansyl norvaline.

Table III. Values of Formation Enthalpy ΔH°_f and Entropy ΔS°_f of the Height Complexes Between the Dansyl Amino Acids with HP-β-CD

Solute denomination	ΔH°_f (kJ/mol)	ΔS°_f (J/mol/K)
L-Valine	–16.40	–18.91
L-Norvaline	–25.42	–48.84
L-Leucine	–28.26	–57.83
L-Phenylalanine	–39.30	–90.04
L-Tryptophane	–42.90	–102.86
D-Valine	–11.74	–2.19
D-Norvaline	–12.47	–5.24
D-Leucine	–14.15	–8.81
D-Phenylalanine	–33.17	–68.84
D-Tryptophane	–41.88	–97.36

ΔH°_f = –41.88 (kJ/mol) and ΔS°_f = –97.36 (J/mol/K) (Table III).

Conclusion

The use of a teicoplanin stationary phase favors the host–guest complexation mechanism study in a very high aqueous mobile phase. In addition, the model developed in this paper allows linking both the retention and complexation processes with the dansyl amino acid steric hindrance. This treatment could be applied to other host–guest complex types, in which steric effects play a preponderant role.

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