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Effect of temperature on retention of enantiomers of β-methyl amino acids on a teicoplanin chiral stationary phase

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Abstract

The isocratic retention of enantiomers of β-methyl amino acids (β-methyltyrosine, β-methylphenylalanine, β-methyltryptophan and β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) was studied on a teicoplanin-containing chiral stationary phase at different temperatures and at different mobile phase compositions, using the reversed-phase mode. With variation of both mobile phase composition and temperature, almost baseline separations could be achieved for all four enantiomers of sterically hindered amino acids. The retention factors and selectivity factors for the enantiomers of all investigated compounds decreased with increasing temperature. The natural logarithms of the retention factors ($\ln k$) of the investigated compounds depended linearly on the inverse of temperature ($1/T$). van 't Hoff plots afforded thermodynamic parameters, such as the apparent change in enthalpy (ΔH°), the apparent change in entropy (ΔS°) and the apparent change in Gibbs free energy (ΔG°) for the transfer of analyte from the mobile to the stationary phase. The thermodynamic constants (ΔH°, ΔS° and ΔG°) were calculated in order to promote an understanding of the thermodynamic driving forces for retention in this chromatographic system.

Keywords: Enantiomer separation; Thermodynamic parameters; Temperature effects; Methyl amino acids; Amino acids

1. Introduction

Conformationally constrained β-methyl amino acids play an important role in the design of new opioid peptides [1]. These amino acids have two stereogenic centres, and therefore four stereoisomers (two pairs of enantiomers) are possible. The direct separation of these isomers is a difficult task and, additionally the effect of temperature on teicoplanin-based enantioseparations has not been thoroughly examined.

The temperature effect on retention in typical reversed-phase high-performance liquid chromatography (RP-HPLC) has been considered by many groups [2–22]. The results suggest that there are at least two completely different effects of temperature, and both can affect resolution. One effect changes the separation factor (α), the peak-to-peak separation distance. The separation factor usually decreases as the temperature is increased. This occurs because the partition coefficients and therefore the free energy change (ΔG°) of transfer of the analyte between the stationary phase and the mobile phase vary with temperature. This is the *thermodynamic effect*. In the case of multicomponent or ionizable mobile phases or an ionizable solute, both the distribution of the solvent components and the pK_a of the ionizable compound can also vary with temperature.

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Another completely different effect of temperature is the influence on viscosity and on diffusion coefficients. This is largely a *kinetic effect*, which improves efficiency (i.e. peak width). There are two different mass transfer effects here. One is mobile phase mass transfer. An increase of temperature reduces the viscosity of the mobile phase. However an increase of temperature also increases the diffusion coefficient of the solute in both the mobile phase and the stationary phase, and it decreases the viscosity of the stationary phase (enhancing stationary phase mass transfer).

A temperature increase often produces a trade-off for resolution. The increased efficiency is good for resolution, while the lessening of the peak-to-peak separation is bad for resolution. In highly efficient separations (in regular achiral HPLC), there is usually little to be gained by increasing the temperature. In less efficient separations, however, where there is an adequate peak-to-peak distance (i.e. many chiral separations), the gain in efficiency sometimes outweighs the loss in peak-to-peak separation when the temperature is increased. However, whether raising temperature is beneficial or not, its effect must be determined on a case-by-case basis.

Enantioselective retention mechanisms are sometimes influenced by temperature to a greater extent than ordinary reversed-phase separations. The degree of chiral recognition of amino acid enantiomers by a chiral crown ether stationary phase depends strongly on the temperature of the separation process, with greater selectivity at lower temperature [23–34]. Similar behavior was observed for other chiral stationary phases (CSP), such as those based on cyclodextrins, α_1-glycoprotein and chiral macrocyclic antibiotics [35–37]. The aim of the present paper was to investigate the effect of temperature on enantioselective separations with a teicoplanin containing chiral stationary phase, the Chirobiotic T column, in order to obtain more information on the retention mechanism.

The model compounds investigated were the enantiomers of different β-methyl amino acids, β-methyltyrosine (β-MeTyr), β-methylphenylalanine (β-MePhe), β-methyltryptophan (β-MeTrp) and β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (β-MeTic) (Fig. 1). The separation of all four stereoisomers in a single run is a difficult task. Considerable variation of the mobile phase composition and of the temperature was necessary in order to find optimal conditions for enantioresolution. The separation of some of these compounds by RP-HPLC on an achiral column was discussed previously [38,39].

Retention data obtained at different temperatures allow an evaluation of the variations in enthalpy (ΔH°) and entropy (ΔS°), which govern the free energy of transfer (ΔG°) of the analyte from the mobile to the stationary phase. These thermodynamic constants were determined in order to better understand the thermodynamic driving forces affecting retention in this chromatographic system.

2. Experimental

2.1. Apparatus

HPLC measurements were performed on a Waters system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode array detector and a Millenium 2010 Chromatography Manager Data System (Waters Chromatography, Milford, MA, USA), and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 20 μl sample loop.

The column used for analytical separation was a teicoplanin-containing Chirobiotic T column, 250× 4.6 mm I.D., 5 μm particle size (Astec, Whippany, NJ, USA). The column was thermostated with an MK 70 thermostat (Mechanik Prüfgeräte, Medlingen, Germany). The accuracy of temperature adjustment was ±0.1°C.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal and reproducible retention factors were obtained for the subsequent injections. This procedure was always followed when a new mobile phase or temperature was chosen.

2.2. Chemicals and reagents

The racemic *threo-(*2*S*,3*R* and 2*R*,3*S)*- and *erythro-(*2*S*,3*S* and 2*R*,3*R*)-β-MePhe, -β-MeTyr, -β-MeTrp and -β-MeTic (Fig. 1) were prepared in our laboratory. For identification of the *threo*-(2*R*,3*S*) and *erythro*-(2*R*,3*R*) enantiomers of β-methyl amino

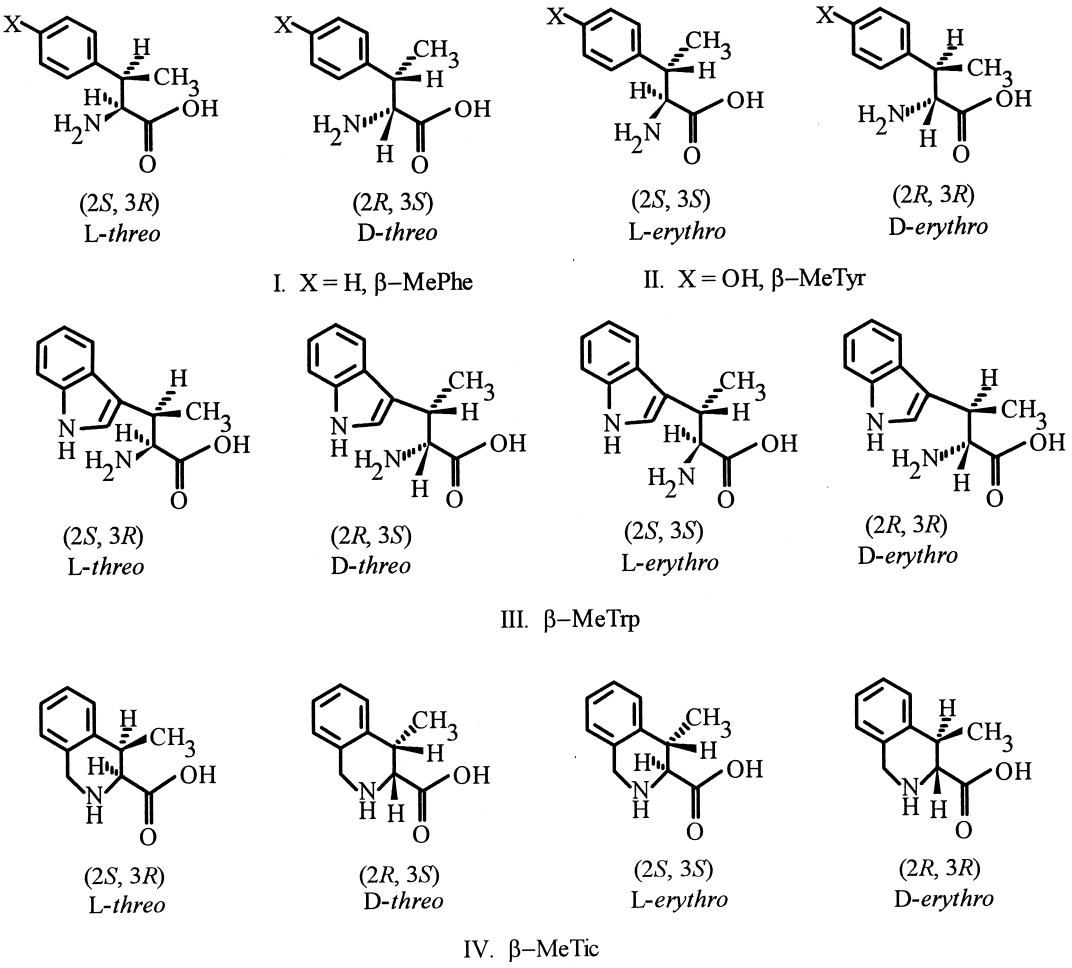

Fig. 1. Structures of four enantiomers of β-methyl amino acids **I**, β-Methylphenylalanine (β-MePhe); **II**, β-methyltyrosine (β-MeTyr); **III**, β-methyltryptophan (β-MeTrp); **IV**, β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (β-MeTic).

acids, L-amino acid oxidase digestion of *erythro* or *threo* compounds was used [19].

Methanol of HPLC grade and other chemicals of analytical reagent grade were purchased from Merck (Darmstadt, Germany). Ultrapure water from a Millipore Milli-Q system (Milford, MA, USA) was used for the preparation of all the solutions.

3. Results and discussion

The investigated conformationally constrained β-methyl amino acids differ from each other in hydrophobicity, bulkiness and rigidity. The direct separation of some of their enantiomers was investigated earlier [40]. The chiral separation on this CSP can be affected by varying the mobile phase composition. With water–methanol as mobile phase, Table 1 provides data on the separation of D and L enantiomers of the *erythro* and *threo* isomers of the β-methyl amino acids at different ratios of water–methanol, at a constant temperature of 20°C. It can be seen that the retention of the different β-methyl amino acids depends on the structure of the molecule. The hydrophilic, β-MeTyr had the smallest retention and an increase in hydrophobicity, bulkyness and rigidity increased the retention. As regards the sequence of elution of the enantiomers, the L

Table 1
Retention factors (k), separation factors (α) and resolutions (R_S) as a function of mobile phase composition for four enantiomers of β-MeTyr, β-MePhe, β-MeTrp and β-MeTic

	Mobile phase composition, water–MeOH (v/v)		
	50:50	30:70	10:90
erythro-β-MeTyr			
k_L	0.32	0.35	0.62
k_D	0.69	0.81	1.49
α	2.15	2.31	2.40
R_S	1.80	2.40	2.89
threo-β-MeTyr			
k_L	0.40	0.48	0.83
k_D	0.63	0.83	1.67
α	1.57	1.73	2.01
R_S	1.15	1.60	2.86
erythro-β-MePhe			
k_L	0.42	0.43	0.71
k_D	0.88	0.98	1.64
α	2.10	2.28	2.31
R_S	2.30	2.25	2.65
threo-β-MePhe			
k_L	0.58	0.58	0.99
k_D	0.85	1.00	1.98
α	1.48	1.72	2.00
R_S	1.65	2.10	3.10
erythro-β-MeTrp			
k_L	0.56	0.58	1.04
k_D	0.96	1.04	1.97
α	1.71	1.79	1.89
R_S	1.35	1.50	1.80
threo-β-MeTrp			
k_L	0.75	0.72	1.16
k_D	1.10	1.20	2.19
α	1.46	1.67	1.89
R_S	1.45	1.70	2.85
erythro-β-MeTic			
k_L	1.40	1.55	3.00
k_D	4.97	6.64	15.83
α	3.55	4.28	5.28
R_S	4.70	6.90	7.45
threo-β-MeTic			
k_L	0.74	0.82	1.62
k_D	2.08	2.45	4.96
α	2.82	2.99	3.06
R_S	3.30	4.05	4.15

Column, Chirobiotic T; temperature 20°C; flow-rate, 1 ml/min.

enantiomers always eluted before the D enantiomers, which is typical for this stationary phase, while the *erythro* compounds (except β-MeTic) exhibited smaller retention factors than the *threo* ones at all investigated mobile phase compositions. An increase of the methanol content in the mobile phase increased the retention factor, k, the selectivity factor, α, and the resolution, R_S (Table 1). Increase of the methanol content in the mobile phase from 50% to 90% (v/v) doubled the retention factor for the L enantiomers, while the increase in k for the D enantiomers was more than twofold. α was better for the *erythro* compounds than for the *threo* ones. The increase in α with increasing methanol content was higher for the *threo* compounds (except *threo*-β-MeTic) than for the *erythro* isomers. The separation of four stereoisomers in one chromatographic run was successful for *erythro*- and *threo*-β-MeTic at all investigated mobile phase compositions. *Erythro*- and *threo*-β-MeTyr and β-MePhe displayed two pairs of unresolved peaks at water–methanol mobile phase compositions of 50:50 (v/v) and 30:70 (v/v). Increase of the methanol content to 90% (v/v) somewhat improved the resolution of the diastereomers. Partial separation of the diastereomers of β-MeTrp was observed for all investigated mobile phase compositions at 20°C, too. In these cases, the peak shapes were better with a mobile phase containing 50% methanol.

Increase of the methanol content in the mobile phase improved the separation of the four stereoisomers at 20°C, but baseline resolution of all isomers could be obtained only for β-MeTic. The enantiomers were, therefore chromatographed at three different mobile phase compositions, e.g. water–methanol 50:50, 30:70 and 10:90 (v/v), at different temperatures in the temperature range 1.5–50°C.

The temperature dependence of the retention and separation data obtained at a water–methanol eluent composition of 30:70 (v/v) are presented in Table 2. Similar tendencies in the retention and separation data as a function of temperature were observed at two other mobile phase compositions: water–methanol 50:50 and 10:90 (v/v). Thermodynamic parameters calculated from these data are presented in Tables 3–5. For the investigated analytes, k and α decreased with increasing temperature (Table 2). The

Table 2
Retention factors (k), separation factors (α) and resolutions (R_S) as a function of temperature for four enantiomers of β-MeTyr, β-MePhe, β-MeTrp and β-MeTic

	Temperature (°C)					
	1.5	10	20	30	40	50
erythro-β-MeTyr						
k_L	0.37	0.37	0.35	0.34	0.33	0.32
k_D	1.11	0.91	0.81	0.72	0.63	0.55
α	3.00	2.45	2.31	2.11	1.91	1.68
R_S	3.05	2.60	2.40	1.90	1.80	1.50
threo-β-MeTyr						
k_L	0.58	0.51	0.48	0.46	0.41	0.37
k_D	1.12	0.91	0.83	0.73	0.64	0.55
α	1.93	1.78	1.73	1.58	1.56	1.48
R_S	1.70	1.65	1.60	1.50	1.30	1.25
erythro-β-MePhe						
k_L	0.50	0.50	0.43	0.41	0.38	0.36
k_D	1.38	1.22	0.98	0.87	0.74	0.64
α	2.76	2.06	2.28	2.12	1.95	1.78
R_S	2.60	2.35	2.25	1.65	1.45	1.50
threo-β-MePhe						
k_L	0.76	0.70	0.58	0.56	0.49	0.44
k_D	1.44	1.21	1.00	0.88	0.76	0.66
α	1.89	1.73	1.72	1.57	1.43	1.50
R_S	2.20	2.15	2.10	1.65	1.55	1.40
erythro-β-MeTrp						
k_L	0.71	0.66	0.58	0.53	0.48	0.44
k_D	1.44	1.28	1.04	0.91	0.80	0.70
α	2.02	1.94	1.79	1.72	1.67	1.58
R_S	1.90	1.50	1.50	1.25	0.95	0.90
threo-β-MeTrp						
k_L	0.96	0.84	0.72	0.67	0.58	0.52
k_D	1.75	1.46	1.20	1.04	0.89	0.77
α	1.82	1.74	1.67	1.55	1.53	1.32
R_S	2.05	1.85	1.70	1.50	1.25	1.05
erythro-β-MeTic						
k_L	2.42	1.90	1.55	1.28	1.08	0.89
k_D	12.60	8.92	6.64	4.80	3.55	2.70
α	5.20	4.69	4.28	3.75	3.29	3.03
R_S	6.40	6.75	6.90	6.50	6.40	5.70
threo-β-MeTic						
k_L	1.19	0.98	0.82	0.71	0.65	0.56
k_D	3.76	2.94	2.45	1.97	1.67	1.39
α	3.16	3.00	2.99	2.77	2.57	2.48
R_S	4.05	4.10	4.05	4.30	4.35	4.45

Column, Chirobiotic T; mobile phase, water–methanol 30:70 (v/v); flow-rate, 1 ml/min.

decrease in k for the more retained D enantiomer was substantial as compared to that for the less retained L enantiomer. The resolution, R_S, of the L and D enantiomers decreased when the temperature was increased. β-MeTic underwent baseline separation at all investigated temperatures, with better peak shapes at elevated temperature. Fig. 2 depicts the chromatograms of the β-methyl amino acid enantiomers under optimized conditions. The separation of the enantiomers (peaks 1 from 2, and 3 from 4) are usually substantial. Overlapping peaks were observed for the diastereomers (peaks 1 and 3, and 2 and 4), with the exception of β-MeTic. In spite of the loss in k and α at elevated temperatures, the application of higher temperatures was beneficial when the goal of the analysis was the separation of the L and D enantiomers. It seems that many of the enantiomeric separations on the teicoplanin-based CSP are fairly efficient as compared to many chiral separations; the gain in efficiency at higher temperature is therefore not enough to completely counteract the decrease in α.

3.1. Thermodynamic parameters for enantiomeric resolution

In order to calculate thermodynamic parameters and to acquire information of value for an understanding of the enantiomeric retention, selectivity and mechanism on this CSP, van't Hoff plots were constructed. The chromatographic retention, expressed by k, was related to the thermodynamic equilibrium constant (K) according to the following equation:

$$k = K\phi \quad (1)$$

in which ϕ is the phase ratio of the column (the volume of the stationary phase divided by the volume of the mobile phase). The free energy change for the process is expressed by

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K = -RT \ln (k/\phi) \quad (2)$$

in which ΔG° is the standard free energy of transfer of the analyte from the mobile phase to the CSP, ΔH° is the enthalpy of transfer of the solute from the mobile phase to the CSP, ΔS° is the entropy of

Table 3
Thermodynamic parameters [$-\Delta H°$, $\Delta S°$, $-\Delta G°$, $-\Delta(\Delta H°)$, $-\Delta(\Delta S°)$ and $-\Delta(\Delta G°)$] and correlation coefficients of β-methyl amino acids at a water–methanol mobile phase composition of 50:50 (v/v)

Compound	$-\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1}K^{-1})	$-\Delta G°_{1.5°C}$ (cal mol^{-1})	$-\Delta G°_{50°C}$ (cal mol^{-1})	$-\Delta(\Delta H°)$ (kcal mol^{-1})	$-\Delta(\Delta S°)$ (cal mol^{-1}K^{-1})	$-\Delta(\Delta G°)_{1.5°C}$ (cal mol^{-1})	$-\Delta(\Delta G°)_{50°C}$ (cal mol^{-1})	Correlation coefficient, r^2
β-MeTyr									
erythro-L	1.23	−1.59	790	710	1.87	4.81	550	320	0.998
erythro-D	3.10	−6.40	1340	1030					0.998
threo-L	1.71	−2.70	970	840	0.90	2.29	270	160	0.958
threo-D	2.61	−4.99	1240	1000					0.998
β-MePhe									
erythro-L	1.42	−1.65	970	890	1.71	4.32	500	290	0.972
erythro-D	3.11	−5.97	1470	1180					0.996
threo-L	2.03	−3.13	1170	1020	0.99	1.42	270	140	0.994
threo-D	3.02	−5.75	1440	1160					0.998
β-MeTrp									
erythro-L	2.61	−5.09	1210	970	1.00	2.40	340	220	0.976
erythro-D	3.61	−7.49	1550	1190					0.978
threo-L	3.29	−6.91	1400	1060	0.51	1.08	230	180	0.994
threo-D	3.82	−7.99	1630	1240					0.996
β-MeTic									
erythro-L	4.01	−8.13	1780	1380	2.03	4.45	800	600	0.998
erythro-D	6.04	−12.58	2580	1980					0.998
threo-L	2.75	−5.07	1360	1110	1.25	2.26	630	520	0.994
threo-D	4.00	−7.33	1990	1630					0.998

Column, Chirobiotic T; mobile phase, water–methanol, 50:50 (v/v); flow-rate, 1 ml/min; ϕ was calculated as 0.086. 1 cal = 4.184 J.

transfer of the solute from the mobile phase to the CSP, R is the gas constant, and T is the temperature.

Therefore

$$\ln k = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} + \ln \phi \quad (3)$$

This expression shows that a plot of ln k versus $1/T$ has a slope of $-\Delta H°/R$ and an intercept of $\Delta S°/R + \ln \phi$ if $\Delta H°$ is invariant with temperature (i.e. a linear van't Hoff plot is obtained). This provides a convenient way of calculating the thermodynamic constants $\Delta H°$ and $\Delta S°$ for a chromatographic system if the phase ratio is known or can be calculated.

The corresponding $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values can be obtained as the differences $\Delta H°_D - \Delta H°_L$ and $\Delta S°_D - \Delta S°_L$, or can be estimated from the selectivity factor (α), which is related to the difference in Gibbs free energy of association for an enantiomeric pair, $\Delta(\Delta G°)$:

$$-\Delta(\Delta G°) = RT \ln \alpha \quad (4)$$

and

$$R \ln \alpha = -\Delta(\Delta H°)/T + \Delta(\Delta S°). \quad (5)$$

If $\Delta(\Delta H°)$ is constant within the temperature range, a straight line should be obtained when the natural logarithms of the α values of a given enantiomeric pair at different temperatures ($R \ln \alpha$) are plotted versus $1/T$. The slope is $-\Delta(\Delta H°)$ and the intercept is $\Delta(\Delta S°)$. The $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values obtained by the two different methods should be identical within experimental error.

Linear [2–21] and nonlinear [9,12,19,22] van't Hoff plots have been observed in temperature studies on reversed-phase stationary phases. Nonlinear van't Hoff behavior *may possibly be* indicative of a change in the mechanism of retention. Linear van't Hoff behavior was earlier observed on chiral chro-

Table 4
Thermodynamic parameters [$-\Delta H°$, $\Delta S°$, $-\Delta G°$, $-\Delta(\Delta H°)$, $-\Delta(\Delta S°)$ and $-\Delta(\Delta G°)$] and correlation coefficients of β-methyl amino acids at a water–methanol mobile phase composition of 30:70 (v/v)

Compound	$-\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1}K^{-1})	$-\Delta G°_{1.5°C}$ (cal mol^{-1})	$-\Delta G°_{50°C}$ (cal mol^{-1})	$-\Delta(\Delta H°)$ (kcal mol^{-1})	$-\Delta(\Delta S°)$ (cal mol^{-1}K^{-1})	$-\Delta(\Delta G°)_{1.5°C}$ (cal mol^{-1})	$-\Delta(\Delta G°)_{50°C}$ (cal mol^{-1})	Correlation coefficient, r^2
β-MeTyr									
erythro-L	0.56	0.87	800	840	1.87	4.74	580	360	0.970
erythro-D	2.43	−3.81	1380	1200					0.992
threo-L	1.51	−1.72	1040	950	0.92	2.08	350	250	0.972
threo-D	2.43	−3.80	1390	1200					0.988
β-MePhe									
erythro-L	1.29	−1.17	970	910	1.51	3.51	540	380	0.962
erythro-D	2.80	−4.68	1515	1290					0.996
threo-L	1.97	−2.84	1190	1050	0.81	1.74	330	250	0.982
threo-D	2.78	−4.58	1520	1300					0.996
β-MeTrp									
erythro-L	1.76	−2.22	1150	1040	0.89	1.81	400	310	0.996
erythro-D	2.65	−4.03	1550	1350					0.996
threo-L	2.18	−3.17	1310	1160	0.76	1.60	320	240	0.994
threo-D	2.94	−4.77	1630	1400					0.998
β-MeTic									
erythro-L	3.53	−6.30	1800	1500	2.01	3.99	915	720	0.996
erythro-D	5.54	−10.29	2715	2220					0.998
threo-L	2.64	−4.48	1410	1190	0.90	0.94	640	600	0.990
threo-D	3.54	−5.42	2050	1780					0.996

Column, Chirobiotic T; mobile phase, water–methanol, 30:70 (v/v); flow-rate, 1 ml/min; ϕ was calculated as 0.086.

matography of β-amino acids with cycloalkane or cycloalkene skeletons on a crown ether-containing stationary phase, Crownpak CR(+) [41].

In the present study, all the plots of ln k versus $1/T$ can be fitted by straight lines. Fig. 3 shows the plot of ln k versus $1/T$ for β-MeTrp at a water–methanol mobile phase composition of 30:70 (v/v). For the other three amino acids and at two other mobile phase compositions, similar straight lines were obtained with good correlation coefficients (Tables 3–5).

The $-\Delta H°$ values calculated from the slopes of the plots at three different water–methanol ratios (50:50, 30:70 and 10:90) are listed in Tables 3–5. Calculation of $\Delta S°$ from the intercept needs a knowledge of the phase ratio (ϕ). Determination of ϕ is relatively easy in pure liquid–liquid chromatography, but the situation is much more complex in reversed-phase chromatography with chemically bonded materials. In any case, the choice of ϕ must be in agreement with the definition of K. For reversed-phase chromatography, Melander and Horváth [42] suggested the expression of ϕ per surface area of the adsorbent (m^2). Davydov et al. divided the mass of material (g) in the column by the column dead volume (cm^3), as is usual in adsorption chromatography [43]. Dorsey et al. [18,44] proposed different equations for the evaluation of ϕ. These equations make use of technical data relating to the stationary phase packing materials, such as the percentage carbon load, the bonded packing weight, the bonded alkyl chain density and the number of carbons in the alkyl ligand. Any uncertainty in the phase ratio affects the $\Delta S°$ values equally, and trends in $\Delta S°$ as a function of molecular structure are therefore unaffected.

Since the technical data on the Chirobiotic T column were available, ϕ could be calculated. When

Table 5
Thermodynamic parameters [$-\Delta H°$, $\Delta S°$, $-\Delta G°$, $-\Delta(\Delta H°)$, $-\Delta(\Delta S°)$ and $-\Delta(\Delta G°)$] and correlation coefficients of β-methyl amino acids at a water–methanol mobile phase composition of 10:90 (v/v)

Compound	$-\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1}K^{-1})	$-\Delta G°_{1.5°C}$ (cal mol^{-1})	$-\Delta G°_{50°C}$ (cal mol^{-1})	$-\Delta(\Delta H°)$ (kcal mol^{-1})	$-\Delta(\Delta S°)$ (cal mol^{-1}K^{-1})	$-\Delta(\Delta G°)_{1.5°C}$ (cal mol^{-1})	$-\Delta(\Delta G°)_{50°C}$ (cal mol^{-1})	Correlation coefficient, r^2
β-MeTyr									
erythro-L	0.17	3.33	1080	1240	1.33	2.77	570	440	0.758
erythro-D	1.50	0.56	1650	1680					0.992
threo-L	1.14	0.64	1310	1350	0.75	1.219	420	360	0.980
threo-D	1.89	−0.57	1730	1710					0.998
β-MePhe									
erythro-L	0.48	2.53	1170	1300	1.11	1.90	540	430	0.916
erythro-D	1.59	0.43	1710	1730					0.982
threo-L	1.25	0.59	1410	1440	0.86	1.46	430	360	0.992
threo-D	2.11	−0.97	1840	1800					0.996
β-MeTrp									
erythro-L	0.80	2.21	1400	1510	1.00	2.20	400	300	0.998
erythro-D	1.80	0.01	1800	1810					0.998
threo-L	1.32	0.62	1490	1520	0.57	0.69	370	350	0.990
threo-D	1.89	−0.07	1860	1870					0.994
β-MeTic									
erythro-L	2.68	−2.05	2120	2020	2.21	4.43	1030	820	0.996
erythro-D	4.93	−6.48	3150	2840					0.998
threo-L	1.99	−0.91	1740	1700	0.75	0.37	650	630	0.992
threo-D	2.74	−1.28	2390	2330					0.998

Column, Chirobiotic T; mobile phase, water–methanol, 10:90 (v/v); flow-rate, 1 ml/min; ϕ was calculated as 0.086.

the dead volume of the column and the technical data were taken into account, ϕ was calculated as 0.086, which is much smaller than the smallest ϕ value (~0.2) mentioned in the literature for reversed-phase columns [45]. The $\Delta S°$ values calculated from the intercepts of the plots via Eq. (3) are listed in Tables 3–5.

The $\Delta H°$ values calculated from the slopes of the plots by using Eq. (3) for all enantiomers are negative, which indicates that the transfer of the enantiomers from the mobile to the stationary phase is enthalpically favored (Tables 3–5). The $-\Delta H°$ values are in the range 0.17–6.04 kcal/mol. Similar $-\Delta H°$ values (2.3–7.5 kcal/mol) were obtained on a chiral crown ether stationary phase for the enantiomers of β-amino acids with cycloalkane or cycloalkene skeletons [41]. For all the investigated compounds, the enthalpy change, $-\Delta H°$, for the enantiomers with the D configuration (2*R*,3*S* or 2*R*,3*R*) is always greater than that for the L enantiomers (2*S*,3*R* or 2*S*,3*S*). This means that the interaction between the D enantiomer and the CSP is more favorable than that with the L enantiomer, and the D enantiomers therefore always elute later.

As regards the $\Delta S°$ values, both positive and negative values were observed. For the enantiomeric pairs, the $\Delta S°$ values for the L enantiomers are always more positive than those for the D enantiomers. The $\Delta S°$ values must relate to the entire system, including the release of solvating solvent molecules that surround the CSP and surround the analyte in the mobile phase.

It is very significant that the D enantiomers always have the larger $-\Delta H°$ values and at the same time the smaller $\Delta S°$ values. The D and L enantiomers must be solvated identically in the mobile phase and release the same number of solvent molecules when they associate with the CSP. This contribution to $\Delta S°$ is therefore the same for the two enantiomers. Since the D enantiomers have smaller $\Delta S°$ values, they may

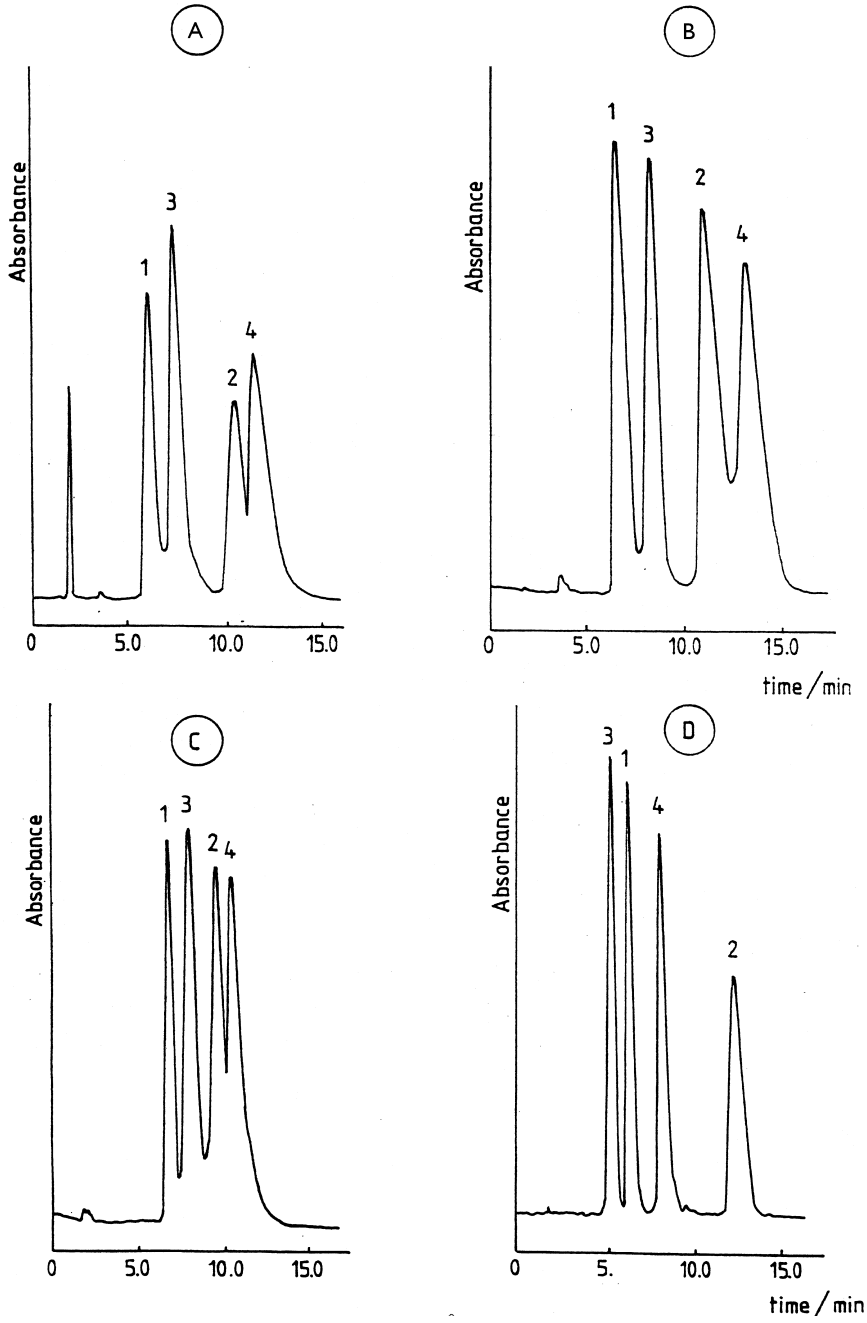

Fig. 2. Chromatograms of β-methyl amino acids A, β-MeTyr; B, β-MePhe, C, β-MeTrp, D, β-MeTic. Column, ChirobioticT; mobile phase, water–methanol, A, B 10:90 (v/v), C, 50:50 (v/v), D, 30:70 (v/v); detection, 200 nm; flow-rate, 1 ml/min; temperature, A, B, C, 1.5°C, D, 50°C; peaks, 1, *erythro*-L isomer, 2, *erythro*-D isomer, 3, *threo*-L isomer, 4, *threo*-D isomer.

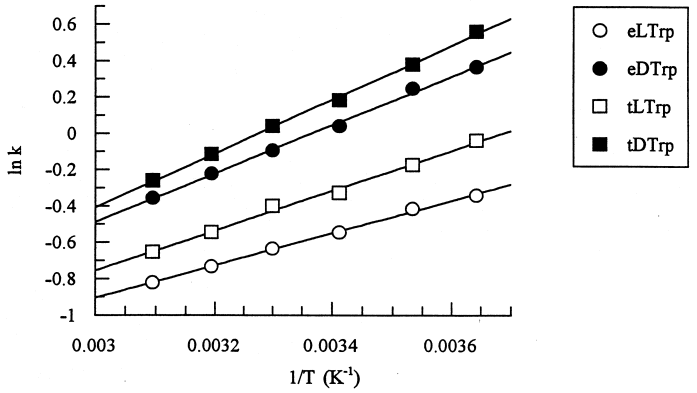

Fig. 3. Plots of natural logarithms of retention factors (ln k) of β-MeTrp enantiomers as a function of the inverse of temperature ($1/T$) Column, Chirobiotic T; mobile phase, water–methanol 30:70 (v/v); detection, 200 nm; flow-rate, 1 ml/min. ○, *erythro*-L isomer; ●, *erythro*-D isomer; □, *threo*-L isomer; ■, *threo*-D isomer.

have fewer degrees of freedom on the CSP (i.e. they are held at more points or are less able to move or rotate). Enantioselective recognition requires three or more points of interaction between the CSP and the chiral analyte. This appears likely for the D enantiomers and somewhat less likely for the L enantiomers in this study.

As regards the $-\Delta(\Delta H°)$ and $-\Delta(\Delta S°)$ values, the data can be arranged in two groups. The *erythro* isomers always have larger $-\Delta(\Delta H°)$ and $-\Delta(\Delta S°)$ values than the *threo* isomers. The smaller $-\Delta(\Delta H°)$ and more positive $\Delta(\Delta S°)$ values for the *threo* isomers resulted in a poorer separation of the *threo*-L and *threo*-D enantiomers than that for the *erythro*-L and *erythro*-D isomers. The more negative entropy change, $-\Delta(\Delta S°)$, in the series of *erythro* compounds can be explained by the fact that the difference in freedom of *erythro*-D and *erythro*-L enantiomers on the CSP is larger than that in the case of the *threo* isomers, probably in consequence of the sterically favorable structure of the *erythro* isomers.

The differences in Gibbs free energy, $-\Delta(\Delta G°)$, are in the same range as the data observed on chiral crown ether stationary phases [24,25,33,41]. The temperature dependence of $-\Delta(\Delta G°)$ indicates that the retention of the more retained *erythro*-D and *threo*-D enantiomers depends greatly on the enthalpic term, whereas the retention of the less retained *erythro*-L and *threo*-L enantiomers is more dependent on the entropic term. When the temperature is decreased from 50°C to 1.5°C, discrimination of the *erythro* isomers takes place, with larger differences in $-\Delta(\Delta G°)$. This indicates that the retention of the *erythro* isomers is more dependent on the enthalpic term than that of the *threo* isomers.

The effects of the mobile phase composition on the thermodynamic parameters are presented in Tables 3–5. The enthalpy change for transfer of analyte from the mobile phase to the CSP, $-\Delta H°$, decreased with increasing methanol content. The enthalpy change was greater in water-rich eluent, whereas the retention was higher in methanol-rich eluent. The smaller enthalpy change in the methanol-rich mobile phase was compensated by the more positive entropy change, $\Delta S°$; the total free energy change, $\Delta G°$, was therefore more negative at higher methanol content. The larger $-\Delta G°$ resulted in stronger retention of the compounds.

The more positive entropy change, $\Delta S°$, at higher methanol content might be attributed to different degrees of solvation in the mobile phase. The solvation of the enantiomers in methanol-rich eluent yielded a more ordered structure as compared to water-rich mobile phase. Accordingly the transition of analyte from methanol-rich mobile phase to the CSP has a more positive entropy change (if it is supposed that the analytes have the same freedom on the CSP). The $-\Delta(\Delta H°)$ and $-\Delta(\Delta S°)$ values decreased with increasing methanol content, particularly for the *threo* isomers. However, in parallel with the decreases in $-\Delta(\Delta H°)$ and $-\Delta(\Delta S°)$, the difference in Gibbs free energy, $-\Delta(\Delta G°)$, increased

with increasing methanol content in the mobile phase. When the temperature was decreased from 50°C to 1.5°C, the differences between $-\Delta(\Delta G^\circ)_{50°C}$ and $-\Delta(\Delta G^\circ)_{1.5°C}$ were higher in water-rich mobile phase than in methanol-rich mobile phase. This indicates that the retention in water-rich eluent enthalpically favored.

The primary dominating step in chiral recognition on a teicoplanin-containing CSP is the strong charge–charge interaction between the carboxylate group of the amino acid and the ammonium group of teicoplanin. The secondary and tertiary structures of the teicoplanin molecule play additional important roles in chiral recognition, by supplying appropriate hydrogen-bonding, hydrophobic and steric interaction sites [46]. Any hindrance of these interactions weakens the chiral recognition. The bulkyness and rigidity of the molecule influence the secondary and tertiary interactions necessary for chiral recognition. In most cases, increasing rigidity and bulkyness improve the enantioselectivity on the teicoplanin CSP. Our results on the temperature dependence of the retention of the β-methyl amino acids support these facts. As concerns the four amino acids, β-MeTyr exhibited the lowest $-\Delta H^\circ$. An increase in hydrophobicity (β-MePhe) increased the enthalpy change, indicating an increased interaction between the analyte and the CSP. β-MeTrp and β-MeTic, which are more bulky than β-MeTyr or β-MePhe, exhibited greater enthalpy changes. The effect of the rigidity is illustrated by the higher $-\Delta H^\circ$ for β-MeTic than for β-MePhe. ΔS° is more positive for β-MeTyr than for β-MePhe. The higher hydrophobicity of β-MePhe resulted in a more ordered system on the CSP than in the mobile phase. The more negative ΔS° values for β-MeTrp and for β-MeTic showed that the bulkyness and rigidity of the molecule tend to enhance the interaction with the CSP.

In the working pH range of the column (water–methanol or water–ethanol mobile phase), teicoplanin and most of the amino acids exist in the zwitterionic form. Thus, the carboxylic acid moieties are in the anionic form $-COO^-$, and the amino groups are in the cationic form $-NH_3^+$. Chiral recognition requires a minimum of three points of interaction [47]. Earlier studies showed [46] that the teicoplanin ammonium group is the most available and logical site for initial docking and enantioselective retention. It was found for amino acids and dipeptides that isomers with *R* configuration at the carbon atom adjacent to the carboxyl group underwent a stronger interaction with the stationary phase. The situation with amino acids having two stereogenic centres was similar to that with dipeptides. The isomer that eluted second had the *R* configuration at the carbon atom adjacent to the carboxyl group. The configuration of the carbon atom adjacent to the carboxyl group is one of the most important factors affecting enantioselectivity and retention order.

3.2. Enthalpy–entropy compensation

A further thermodynamic approach to the analysis of physicochemical data is enthalpy–entropy compensation [48]. The enthalpy–entropy compensation method was used by Horváth in a study of hydrophobic interactions and separation mechanism in reversed-phase HPLC [49]. Mathematically, enthalpy–entropy compensation can be expressed by the formula

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G_\beta^\circ \quad (6)$$

where ΔG_β° is the Gibbs free energy of a physicochemical interaction at the compensation temperature, β (β and ΔG_β° are constants). According to Eq. (4), when enthalpy–entropy compensation is observed for a group of compounds in a particular chemical transformation (or interaction in the case of chromatographic retention), all of the compounds have the same free energy change, ΔG_β°, at temperature β. For example, if enthalpy–entropy compensation is observed in LC or GC for a group of compounds, all the compounds will have the same net retention at the compensation temperature β, although their temperature dependences may differ. In order to express the free energy change, ΔG_T°, measured at a given temperature, *T*, the Gibbs–Helmholz relationship can be written with the use of Eq. (4) as

$$\Delta G_T^\circ = \Delta H^\circ(1 - T/\beta) + (T\Delta G_\beta^\circ)/\beta \quad (7)$$

Eq. (7) shows that a plot of ΔG_T° for different compounds at a constant temperature *T*, is a linear function of the corresponding ΔH°, and compensa-

tion temperature β can be evaluated from the slope. From Eq. (7) and (2):

$$\ln k = -(\Delta H^\circ/R)(1/T - 1/\beta) + \Delta G_\beta^\circ/R\beta + \ln \phi \quad (8)$$

which shows that plots of ln k versus ΔH° can be used to obtain the compensation temperature, β. Similarity of the values for the compensation temperature suggests that the solutes are retained by essentially identical interaction mechanisms, and the

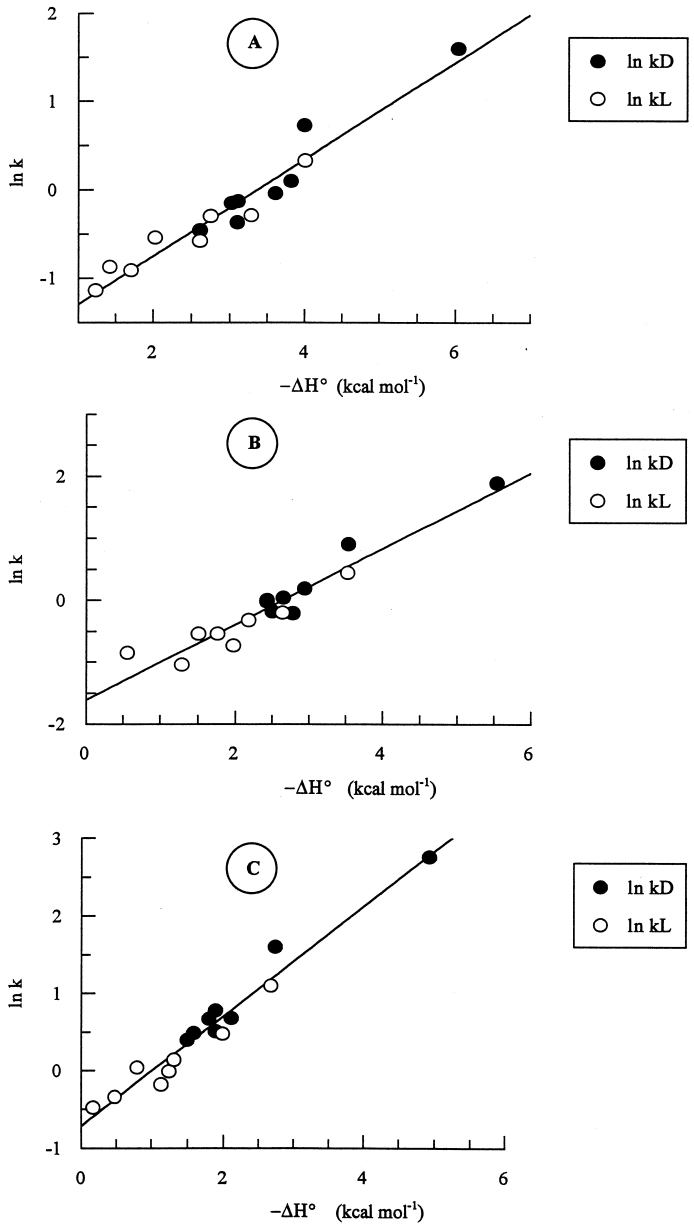

Fig. 4. Plots of enthalpy–entropy compensation for L and D enantiomers of β-methyl amino acids. Column, Chirobiotic T; mobile phase, water–methanol, A, 50:50 (v/v), B, 30:70 (v/v), C, 10:90 (v/v); detection, 200 nm; flow-rate, 1 ml/min; temperature, A, C, 20°C, B, 1.5°C. ○, L enantiomer; ●, D enantiomer.

compensation study is therefore a useful tool for comparing the retention mechanisms for different compounds. However, the results obtained with this method can not be used alone: they can be misleading, due to the cumulative errors associated with the determination of enthalpy [50,51].

Fig. 4 shows enthalpy–entropy compensation plots for the L and D enantiomers of β-methyl amino acids at three different mobile phase compositions on the Chirobiotic T CSP. The regression lines for the enantiomers at three different water–methanol mobile phase ratios were:

Water–methanol 50:50 (v/v)

$$\ln k_{L,D} = -1.841 + 0.547(-\Delta H^\circ) \quad r = 0.967$$

Water–methanol 30:70 (v/v)

$$\ln k_{L,D} = -1.607 + 0.610(-\Delta H^\circ) \quad r = 0.956$$

Water–methanol 10:90 (v/v)

$$\ln k_{L,D} = -0.714 + 0.710(-\Delta H^\circ) \quad r = 0.978$$

The compensation data (plots) for the L and D enantiomers are not significantly different from one another. This indicates that the two enantiomers are retained via similar retention mechanisms. Indeed, the initial docking and charge–charge interactions between the carboxylate group of the amino acid and the ammonium group of the teicoplanin selector [46] may be identical for the two enantiomers. Hence, enantioselectivity occurs as a result of the different secondary interactions (i.e. hydrogen-bonding, steric and hydrophobic interactions) that must occur when two spatially distinct enantiomers are located at the same teicoplanin binding site or cleft. There is no evidence that a change of the mobile phase conditions (i.e. methanol to water) significantly alters the retention mechanism of the compounds studied.

4. Conclusions

The effect of temperature on retention demonstrated that the enantiomers of β-methyl amino acids can be separated by using either subambient or elevated temperatures. Linear van't Hoff plots were observed in the studied temperature range, 1.5–50°C, and the apparent changes in enthalpy, ΔH°, entropy, ΔS°, and Gibbs free energy, ΔG°, were calculated. The values of the thermodynamic parameters depend on the structures of the compounds. The primary step in chiral interactions leading to the retention of both enantiomers on the teicoplanin-containing CSP is the charge–charge interaction between the carboxylate group of the amino acid and the ammonium group of the teicoplanin molecule. Chiral recognition and enantioselective retention results from the distinct hydrogen-bonding, steric and hydrophobic interactions that occur when two amino acid enantiomers are retained at the same CSP site. The thermodynamic data obtained support the proposed mechanism.

Acknowledgements

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