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Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase

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Abstract

The glycopeptide antibiotic teicoplanin is shown to be a highly effective stationary phase chiral selector for the resolution of underivatized amino-acid and imino-acid enantiomers. Fifty four of these compounds (including all chiral protein amino acids) as well as a number of dipeptides were resolved. Hydro-organic mobile phases are used and no buffers or added salts are needed in most cases. Hence the purified analytes are easily isolated in pure form, if needed, by evaporating of the solvent. The effect of pH, organic modifier type and amount are discussed. The enantioselective separation mechanism is examined using both molecular modeling and retention data. The strongest stereoselective interaction is for carboxy-terminated D-amino-acids. In the case of peptides, it is not necessary for these to be a D-, D-, terminal sequence for strong interactions. In some cases, including Ala–Ala, the L-, D- terminal sequence showed greater interaction with the teicoplanin chiral stationary phase.

Keywords: Enantiomer separation; Enantioselectivity; Chiral stationary phases, LC; Amino acids; Peptides; Teicoplanin; Imino acids

1. Introduction

Most amino acids are chiral. To understand the properties and disposition of these substances, it is necessary to separate and to quantitate the enantiomers individually. The diverse vital functions of amino acids in living organisms make it important to determine the ratios of the enantiomers in various areas such as pharmacology, protein or peptide analysis and synthesis, geochemical dating, food chemistry, and so on [1].

Amino acids can be resolved either indirectly by derivatizing with a chiral agent to form diastereoisomers, or directly by using a chiral stationary phase (CSP) or a chiral mobile phase additive (CMA) [2]. Direct methods are most often used today because of possible problems involving trace enantiomeric impurities, kinetic discrimination, racemization and non-identical detection response factors in the indirect methods. Even when using direct methods for the resolution of amino acid enantiomers, achiral derivatization is sometimes employed to enhance the separation or detection properties of the analyte. For example, volatile derivatives of amino acids must be made prior to analysis by gas chromatographic methods [3,4]. When doing HPLC analyses, fluorescent tagged amino acids are frequently easier to

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isolate, detect and resolve when analyzing "real world" samples [5–9].

The direct LC resolution of native, unaltered amino acids has been limited mainly to two approaches. The first is a ligand-exchange method originally developed by Davankov [10–12]. In this technique, a chiral bidentate ligand (such as proline or a number of such compounds) capable of complexing with Cu^{2+} is the active component of the stationary phase. Cu^{2+} (or occasionally, another appropriate transition metal) is added to the water-based, buffered mobile phase. The analyte amino acid in solution then completes the coordination sphere [10–12]. The second approach was developed by Cram and co-workers. It used a chiral crown ether as the stationary phase chiral selector [13,14]. A variation of this method was later commercialized [15] and evaluated [16,17]. The crown ether moiety can complex protonated primary amines [13–17]. Consequently the mobile phase must be acidic and secondary amines (imino acids) cannot be resolved. The commercial version of this column works only when perchloric acid is used in the mobile phase. This poses a safety problem when evaporating to dryness and isolating samples, particularly from preparative separations. Finally aromatic amino acids can be resolved on an α-cyclodextrin stationary phase [18].

Recently, macrocyclic antibiotics were introduced as a new class of chiral selectors for the chromatographic [19] and electrophoretic [20] separation of enantiomers. Antibiotics of the *ansa* family such as rifamycin B and rifamycin SV are able to resolve neutral and positively charged compounds [20,21]. Glycopeptide antibiotics, such as vancomycin [22], ristocetin [23] and teicoplanin [24,25], are able to resolve neutral and negatively charged compounds.

Teicoplanin is naturally produced by the *Actinoplanes teicomycetus* mildew. It is active against aerobic and anaerobic Gram positive bacteria [26]. Its structure, shown in Fig. 1, has a number of unique features. The aglycone has the form of a semi-rigid basket with four fused macrocyclic rings. It contains seven aromatic rings, two of which have chlorosubstituents and four of which bear phenolic moieties. Six amido groups and three ether groups link the aromatic rings. Two important features are that the aglycone basket contains a single primary amine and a single carboxylic acid group (Fig. 1). These two groups, of which one is basic (pK ~9.2) with a cationic tendency, and the other is acidic (pK ~2.5) with an anionic tendency, control the teicoplanin zwitterionic charge at pHs normally used in HPLC (i.e., ~3.5–8.0). The central basket has three carbohydrate moieties consisting of two D-glucosamines and one D-mannose. One D-glucosamine has a substituted nonyl hydrocarbon chain which gives the molecule surface-active properties. Five teicoplanin glycopeptides have been identified. They differ only in the nature of the hydrocarbon chain. The most common teicoplanin glycopeptide is referred as A_2-2 (Fig. 1) with a molecular mass of 1877.

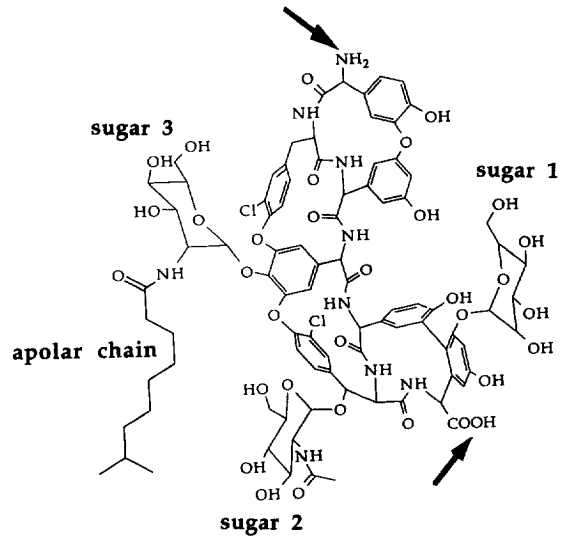


Fig. 1. The teicoplanin structure. The two chlorine atoms, the three sugar units and the hydrophobic side chain can be located. The most important functional groups for amino acid chiral recognition are the $-NH_2$ and the –COOH group (arrows) ionized over the 3.5–8.0 pH range.

In this work, teicoplanin was covalently linked to 5-μm spherical silica particles as previously reported for other glycopeptides [19]. This material was then evaluated as a chiral stationary phase for the HPLC resolution of a variety of native amino acids and imino acids using the classical reversed-phase mode. The mobile phases were methanol–water solutions. As far as possible, added buffer salts and/or any other additives were avoided. The mechanism for the enantioselective retention of amino acids is studied.

2. Experimental

2.1. Materials

Most of the racemic amino acids and pure amino-acid enantiomers were obtained from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA). Methanol and acetic acid were HPLC grade solvents obtained from Fisher (Pittsburgh, PA, USA). Ethanol contained 1% (v/v) ethyl acetate, 1% hexane and 1% methyl isobutyl ketone as denaturant agents, it was the OmniSolv® Ethyl Alcohol solvent obtained from EM Science (Gibbstown, NJ, USA). Teicoplanin was the generous gift of the Marion Merrell Dow Research Institute (Cincinnati, OH, USA). The 250×4.6 mm I.D. teicoplanin CSP column, named Chirobiotic T, can now be obtained from Astec (Whippany, NJ, USA).

2.2. Methods

A Shimadzu LC 6A chromatograph was used to perform all separations. It included two LC-6A pumps, a SPD-6A UV detector, all three driven by a SCL-6A controller. A CR 601 integrator was used to obtain the chromatograms and produce the peak retention time and area. All amino acids analyzed were not derivatized. The mobile phases were prepared by mixing the indicated volumes of deionized and filtered water and methanol or ethanol. When a low mobile phase pH was necessary, drops of glacial acetic acid were added to the hydro-organic mixture. The pH was monitored using a classical water-based pH electrode (ATI Orion Research, Boston, MA, USA). 1 mg/ml amino acid solutions were prepared in the mobile phase. Unless otherwise indicated, the mobile phase flow-rate was 1 ml/min and the UV detection wavelength was 215 nm.

2.3. Molecular modelling

The software *Alchemy III* from Tripos Associates (St. Louis, MO, USA) was used on a Macintosh Centris 610. The software is able to compute the molecule conformation corresponding to the minimal energy taking into account the atom size, the electrostatic and dipole–dipole interactions, the bond lengths and bond angles. Once the minimum energy conformation is obtained, a powerful 3-D tool allows rotation of the molecule around the X, Y and Z axis.

3. Results and discussion

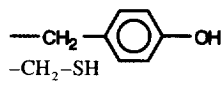
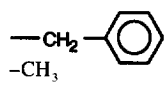
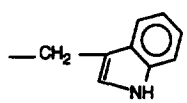
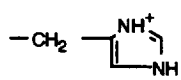
Table 1 lists the chromatographic parameters for enantioresolution of the twenty native protein amino acids. Most of the separations were obtained with an unbuffered reversed-phase mobile phase of 60–40 (v/v), methanol–water. All amino acids are easily resolved and detected without pre- or post-column derivatization. Fig. 2 shows the separation of the L- and D-enantiomers of methionine and alanine with selectivity factors of 2.2 and 1.8, respectively. The resolution factors were as high as 3.3 and 2.9, respectively. Table 2 lists the results obtained for 36 additional amino acids not found in proteins. All were also analyzed without any chemical modification and separated with classical hydro-organic reversed-phase mobile phases. Note that for comparison purposes, all separations utilize the same volume ratio of methanol and water. By altering this ratio the retention and enantioresolution can be enhanced in some cases (particularly for the less retained amino acids).

3.1. Stationary phase selectivity

The amino acids are listed in Table 1 and Table 2 in order of the size of the capacity factor, k', of the first eluting enantiomer. Solute polarity seems to be one factor that affects retention, but not necessarily enantioselectivity. Threonine, aspartic acid, glutamic acid and serine are polar amino acids. The capacity factor of their first eluting enantiomer is below 0.33 (Table 1). The next group of compounds in Table 1 (isoleucine, glutamine and glycine) have the same capacity factor, 0.40. However, isoleucine is far less polar than glycine which means that solute polarity is not the only factor affecting solute retention. This fact is also shown by the retention of glutamine ($k'=0.40$) which is less polar than the more retained asparagine ($k'=0.60$).

With a mobile phase of 60–40 (v/v), methanol–water, the charged amino acids were either unretained (anionic: aspartic and glutamic acids) or did not elute after passing more than fifty column

Table 1
Chromatographic parameters for the enantioresolutionof the 20 naturally occuring amino acids[a]

Amino acid	R-moiety[b]	k'_1[c]	k'_2[c]	α[d]	R_s[d]
Aspartic acid[f]	$-CH_2-COOH$	0.20	0.34	1.7	1.2
Threonine[e]	$-CHOH-CH_3$	0.28	0.39	1.4	1.1
Glutamic acid[f]	$-CH_2-CH_2-COOH$	0.30	0.57	1.9	1.5
Serine	$-CH_2OH$	0.33	0.45	1.4	1.2
Isoleucine[e]	$-CH(CH_3)-CH_2-CH_3$	0.40	0.80	2.0	2.5
Glutamine	$-CH_2-CH_2-CO-NH_2$	0.40	0.72	1.8	1.6
Glycine	$-H$	0.41	achiral	–	–
Tyrosine		0.42	0.64	1.5	1.9
Cysteine	$-CH_2-SH$	0.45	0.72	1.6	1.6
Valine[e]	$-CH(CH_3)-CH_3$	0.46	0.75	1.6	1.9
Leucine[e]	$-CH_2-CH(CH_3)-CH_3$	0.48	1.01	2.1	3.5
Methionine[e]	$-CH_2-CH_2-S-CH_3$	0.53	1.16	2.2	3.3
Phenylalanine[e]		0.56	0.83	1.5	2.0
Alanine	$-CH_3$	0.56	1.03	1.8	2.9
Proline	$-CH_2-CH_2-CH_2-$	0.58	1.46	2.5	2.5
Asparagine	$-CH_2-CO-NH_2$	0.60	0.98	1.6	2.1
Tryptophan[e]		0.77	1.17	1.5	2.2
Lysine[e,f]	$-(CH_2)_4-NH_3^+$	6.12	9.18	1.5	2.2
Arginine[f]	$-(CH_2)_3-NH-C(NH_2)_2^+$	6.48	8.96	1.4	2.1
Histidine[e,f]		6.60	7.60	1.2	0.8

[a] These data were generated with a 250 × 4.6 mm Chirobiotic T (5-μm Teicoplanin bonded silica particles) column, methanol–water (60–40, v/v) mobile phase, 1 ml/min, 210 nm UV detection of underivatized solutes.
[b] The general structure of amino acids is $^+NH_3-CHR-COO^-$ with the R group stucture listed.
[c] k'_1 and k'_2 are the capacity factor of the first eluting L-enantiomer and the second eluting D-enantiomer, respectively. Note that all of these values can be enhanced by using a different organic modifier and/or varying the modifier–water ratio. However, for comparison purposes, all were run under the same conditions.
[d] α and R_s are the selectivity factor and the resolution factor, respectively.
[e] Essential amino acid.
[f] Mobile phase methanol–water (60–40, v/v) adjusted to pH 3.80 by acetic acid.

volumes of mobile phase (cationic: lysine, arginine and histidine). This means that there are anionic sites on the stationary phase that tend to repel the negatively charged amino acids and strongly attract the cationic amino acids. In all cases, the charged amino acids can be resolved by acidifying the mobile phase to pH 3.80 using glacial acetic acid. The acetic acid concentration was in the $\sim 8\times10^{-4}$ M range. The carboxylic acid groups of aspartic and glutamic acid (pK=3.9 and 4.3, respectively) were shifted toward their molecular forms which greatly facilitated retention and analysis (Table 1). The anionic sites on the stationary phase also were protonated somewhat thereby allowing the cationic amino acids to be eluted and resolved. The retention volumes and capacity factors were high however (k'>6, Table 1).

The teicoplanin molecule has several characteristic parts that seem to be involved in amino acid interactions (Fig. 1). They are: (1) those of ionic character including a cationic site ($-NH_3^+$) and an

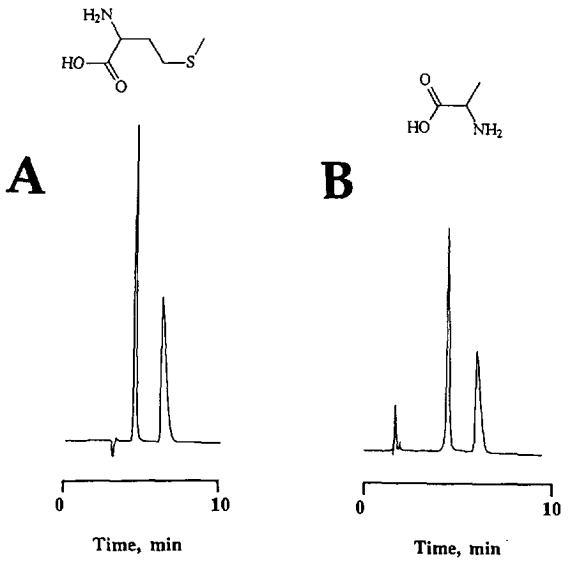


Fig. 2. Reversed-phase enantiomeric separations of naturally occurring amino acids. (A) methionine; (B) alanine. Mobile phase: methanol–water (60–40, v/v); flow-rate, 1 ml/min. Column: 25 cm × 0.46 cm I.D., Chirobiotic T (5-μm silica particle bonded with teicoplanin). Injection volume: 20 μl; injection mass: ~20 μg; UV detection at 215 nm.

anionic site ($-COO^-$); (2) additional polar groups with three sugar moieties (10 primary or secondary hydroxyl groups) and four phenolic groups (Fig. 1); and (3) apolar character of the aglycone "basket" and the nine carbon side chain. The strong charge–charge interactions are responsible for the amino acids net retention behavior. This can be affected by the mobile phase pH. The polar, apolar and steric interactions with the amino acid **R** group affect both retention and selectivity. From a practical point of view, the twenty naturally occurring amino acids cannot be all analyzed in one run since many of their capacity factors are too close. The interest of the new teicoplanin column is its enantioselectivity. It may resolve a greater number and variety of native amino acids and peptides than any other CSP.

3.2. Enantioselectivity

The high enantioselectivity of the teicoplanin bonded phase is supported by many of the resolution factors, R_s, listed in Table 1 and Table 2. R_s values as high as 11 or 9.1, for 4-chlorophenylalanine and 3-thiophenylglycine, respectively, are among the higher R_s values found in the LC literature. Baseline resolutions (R_s>1.5) or better were obtained for 49 amino acids out of the 55 studied (Table 1 and Table 2). These resolution factors could be increased considerably, if desired, by going to less commonly used organic modifiers such as ethanol and propanol or by altering the modifier–water volume ratio of the mobile phase. Table 3 shows the effect of the organic modifier on enantioselectivity and retention. Methanol, ethanol and 2-propanol were compared. The selectivity factor increased by 30% (pipecolic acid) to 110% (*o*-thiophenylglycine) by changing from methanol to 2-propanol. The resolution factor was enhanced even more dramatically as the result of an increase in separation efficiency. However the mobile phase viscosity also increased with the alcohol chain length. For example, the back pressure with 60:40 (v/v) 2-propanol–water mobile phase was almost four times that of the same flow-rate and same volume ratio of methanol–water. The use of ethanol as the organic modifier appears to be a good compromise. The selectivities are higher than with methanol and acceptable back pressure and flow-rates are easily obtained.

The naturally occurring L-enantiomers were available for all chiral amino acids found in proteins (Table 1). This allowed identification of the retention order of the L- and D-amino acids. With no exception, the first eluting peak was the L-enantiomer.

The strong energy difference between the interactions of the L and D forms of the amino acids and the teicoplanin chiral selector renders the method less sensitive to changes in ionic strength, pH and organic modifier concentration than other reversed-phase CSPs. Using five representative amino acids, namely asparagine, alanine, methionine, phenylalanine and tryptophan; the chromatographic retention and enantioselectivity were studied as a function of the buffer and buffer concentrations in the mobile phase. The first mobile phase consisted of unbuffered 60:40 (v/v) methanol–water. The second and the third were 60:40 (v/v) methanol–water mobile phases buffered using 20 m*M* and 50 m*M* of $NH_4H_2PO_4$ salt, respectively. The k', α, and R_s values were identical with a standard deviation below 5%.

The effect of organic modifier concentration on enantioselectivity was studied as well. The mobile phase composition was varied from 100% water

Table 2
Chromatographic parameters for the enantioresolution of amino acids not found in proteins[a]

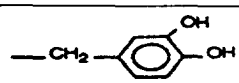
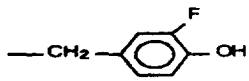
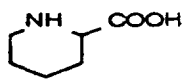
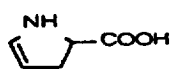
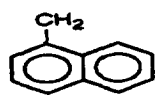
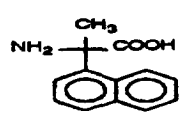
Amino acid	R-moiety[b]	k'_1[c]	k'_2[c]	α[d]	R_s[d]
3,4-Dihydroxyphenyl alanine (DOPA)		0.25[e]	0.62[e]	2.5[e]	2.9[e]
		0.31[f]	0.47[f]	1.5[f]	1.2[f]
Phenylglycine	–Ph	0.37[f]	1.13[f]	3.1[f]	2.9[f]
Homoserine	CH_2–CH_2–OH	0.39	0.59	1.5	1.3
α-Amino-butyric acid	–CH_2–CH_3	0.41	0.80	1.9	1.9
Norleucine	–CH_2–CH_2–CH_2–CH_3	0.44	1.16	2.6	4.0
		0.29[e]	0.63[e]	2.2[e]	3.0[e]
β-Phenylalanine	–CH(CH_3)–Ph	0.46	0.89	1.9	2.6
m-Tyrosine	–CH_2–Ph(*m*)OH	0.47	1.20	2.6	4.2
		0.57[g]	1.88[g]	3.3[g]	6.9[g]
α-Methyl-*m*-tyrosine	–CH_2–Ph(*m*)OH,CH_3	0.48	0.76	1.6	1.8
Ethionine	–CH_2–CH_2–S–CH_2–CH_3	0.48	0.97	1.6	1.6
m-Fluorotyrosine		0.49	0.89	1.8	2.5
Norvaline	–CH_2–CH_2–CH_3	0.49	1.35	2.8	4.4
		0.25[e]	0.59[e]	2.4[e]	3.0[e]
2-Fluorophenylglycine	–Ph(*o*)F	0.49	1.78	3.6	7.0
4-Chlorophenylalanine	–CH_2–Ph(*p*)Cl	0.52	4.25	8.2	11
2-Thiophenylglycine	–Ph(*o*)SH	0.52[g]	2.15[g]	4.1[g]	7.6[g]
Citrulline	–$(CH_2)_3$–NH–CO–NH_2	0.53	1.02	1.9	2.5
		0.33[e]	0.55[e]	1.7[e]	2.6[e]
Pipecolic acid		0.60	0.93	1.6	1.6
Isoserine	NH_2–CH_2–CHOH–COOH	0.61	0.80	1.3	1.1
o-Tyrosine	–CH_2–Ph(*o*)OH	0.61	0.87	1.4	1.8
		0.62[g]	1.07[g]	1.7[g]	2.4[g]
2-Fluorophenylalanine	–CH_2–Ph(*o*)F	0.61	1.08	1.8	2.5
4-Fluorophenylalanine	–CH_2–Ph(*p*)F	0.63	0.93	1.5	2.1
3-Fluorophenylalanine	–CH_2–Ph(*m*)F	0.63	0.96	1.5	2.1
3-Thiophenylglycine	–Ph(*m*)SH	0.63	3.91	6.2	9.1
5-Fluorotryptophan	–	0.70	1.25	1.8	2.7
3,4-Dehydroproline		0.70	2.33	3.3	3.9
6-Fluorotryptophan	–	0.71	1.08	1.5	1.9
α-Methyltryptophan	–	0.76	1.04	1.4	1.6
7-Methyltryptophan	–	0.77	1.10	1.4	1.7
2-Methyltyrosine	–CH_2–Ph(*o*)CH_3(*p*)OH	0.80	1.19	1.5	2.3
2,6-Dimethyltyrosine	–	0.81	1.00	1.2	1.4
4-Bromophenylalanine	–CH_2–Ph(*p*)Br	0.83	1.13	1.4	1.7
6-Methyltryptophan	–	0.83	1.23	1.5	1.9
3-(1-Naphthyl)alanine		1.12	1.57	1.4	1.9
5-Benzyloxytryptophan	–	1.12	1.59	1.4	2.0
2-(1-Naphthyl)alanine		1.44	1.92	1.3	1.4

Table 3
Comparison of four different amino acids using different mobile phases

Compound	k'_1	k'_2	α	R_s	Alcohol
o-Thiophenylglycine	1.63	3.91	2.4	4.7	Methanol
o-Thiophenylglycine	0.57	2.79	4.9	5.4	Ethanol
o-Thiophenylglycine	0.80	3.92	4.9	10.1[a]	2-Propanol[a]
3-(1-Naphthyl)alanine	2.79	3.63	1.3	1.8	Methanol
3-(1-Naphthyl)alanine	1.0	1.6	1.6	1.3	Ethanol
3-(1-Naphthyl)alanine	1.04	1.87	1.8	3.8[a]	2-Propanol[a]
Pipecolic acid	2.25	3.60	1.6	1.7	Methanol
Pipecolic acid	1.12	2.24	2.0	1.9	Ethanol
Pipecolic acid	1.32	2.90	2.2	4.0[a]	2-Propanol[a]
Threonine	1.40	1.54	1.1	1.0	Methanol
Threonine	0.99	1.49	1.5	1.6	Ethanol
Threonine	0.62	0.84	1.3	1.6[a]	2-Propanol[a]

Mobile phase consisted of alcohol–water (60:40, v/v). See Table 1 and Table 2 for all other experimental conditions.
[a] Flow-rate was 0.5 ml/min due to the viscosity of the propanol–water mobile phase that produced a high back pressure.

(unbuffered) to 100% methanol by 10% (v/v) steps. Fig. 3 shows the results obtained for methionine (top) and phenylalanine (bottom). In both cases, the increase of the water content in the mobile phase produced a decrease of the capacity factors, k'. It is unusual, in RPLC, for water-rich mobile phases to produce shorter solute retention times. Most amino acids are more soluble in water than in methanol which explains the retention decrease observed when the water content in the mobile phase increases. The interesting point is that the selectivity factor is not very sensitive to the mobile phase composition. It decreases in water-rich mobile phase compositions. This is due to the high dielectric constant of water. The electrostatic forces, responsible for the chiral recognition of the amino acids are decreased in water-rich phases. There is a small maximum in both the selectivity and resolution at about 90:10 (v/v), methanol–water (Fig. 3). Note that more hydrophobic analytes give a more typical reversed-phase retention profile on this column [25].

The resolution factor for methionine was not very sensitive to the mobile phase composition in the range 90:10 (v/v) to 30:70 (v/v) methanol–water. However, baseline resolution of the two methionine enantiomers was obtained over the whole mobile phase composition range, with R_s values higher than 2. The resolution factor of phenylalanine continuously decreased when the water content of the mobile phase increased (Fig. 3, bottom). However, baseline resolution was obtained as long as the water content was less than 60% (v/v). The enantioselectivity factor, α, depends on the thermodynamics of the chiral interaction. The resolution factor depends on

Footnotes to Table 2
[a] These data were generated with a 250 × 4.6 mm Chirobiotic T (5-μm Teicoplanin bonded silica particles) column, methanol–water (60:40, v/v) mobile phase, unless otherwise indicated, 1 ml/min, 210 nm UV detection of underivatized solutes.
[b] The general structure of aminoacids is $^+NH_3$–CH**R**–COO^- with the R group stucture listed, *o*, *m*, *p* stand for *ortho*, *meta* and *para* substituted benzene ring (ph), respectively; a dash (–) means the structure can be easily related to the parent amino acid.
[c] k'_1 and k'_2 are the capacity factor of the first eluting L-enantiomer and the second eluting D-enantiomer, respectively. Note that all of these values can be enhanced by using a different organic modifier and/or varying the modifier–water ratio. However, for comparison purposes, all were run under the same conditions.
[d] α and R_s are the selectivity factor and the resolution factor, respectively.
[e] Mobile phase methanol–water (40:60, v/v).
[f] Mobile phase methanol–water (20:80, v/v).
[g] Mobile phase ethanol–water (70:30, v/v).

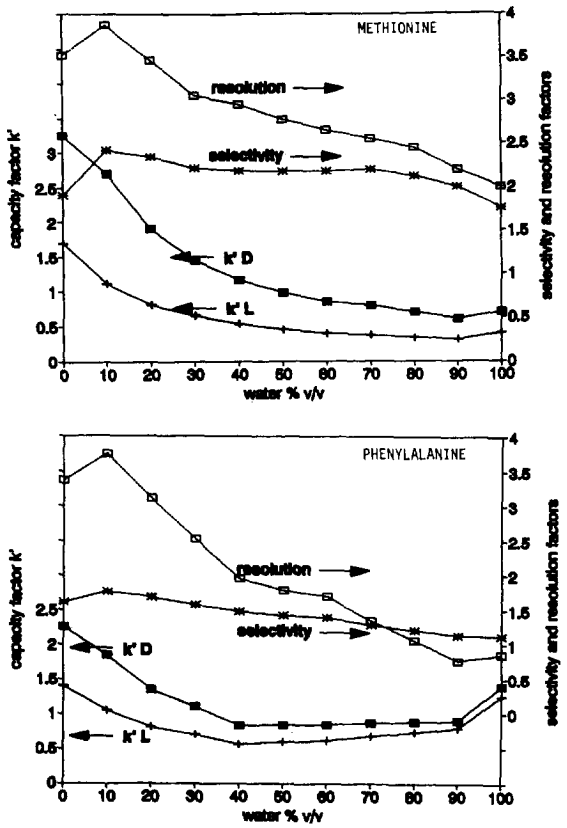


Fig. 3. Effect of the mobile phase composition on the enantiomeric separation of methionine (top) and phenylalanine (bottom). *Y* scale on the left for the *k'* capacity factors; *Y* scale on the right for the selectivity and resolution factors.

both the selectivity factor and the efficiency which is controlled by the kinetics of the interactions. The efficiency, measured using the peak of the second eluting enantiomer, was always lower than 3000 plates (Fig. 2). This value is low for a 25-cm column. The HETP is as high as 85 μm or 17 times the particle diameter. This means that the kinetics of the teicoplanin-D-amino acid exchange are slow, resulting in poor mass transfer.

3.3. Chiral recognition mechanism

In the working pH range (3.5<pH<8) of the column, the teicoplanin and most of the amino acids exist in the zwitterionic form. Thus the carboxylic acid moieties are in the anionic $-COO^-$ form and the amino groups are in the cationic $-NH_3^+$ form. Chiral recognition requires a three-point interaction [27]. The primary point of interaction appears to be electrostatic in nature. There are two possibilities: the teicoplanin ammonium group can interact with the carboxylate group of the amino acid or the ammonium group of the amino acid interacts with the teicoplanin carboxylate. Since electrostatic forces are among the stronger interactions in solution, it is tempting to imagine that both interactions can occur simultaneously thereby orienting the amino acid. Molecular modeling can be used to evaluate possible binding scenarios as well as the three-dimensional structure of teicoplanin. As will be shown, simultaneous dual electrostatic interactions, as described above, are not possible for common amino acids.

Fig. 4 shows the aglycone portion of the teicoplanin molecule as well as the molecule L-phenylalanine. Note that in this space-filling model, the hydrophilic groups are colored: the hydroxyl groups in blue, the ammonium groups in green and the carboxylate groups in red. The aromatic rings, amido linkages and other apolar connecting carbons are in black. By comparing the structure shown in Fig. 1 with the energy minimized, space-filling model in Fig. 4, several things are apparent. First, the four fused macrocyclic rings are skewed and twisted with respect to one another such that the phenolic –OH group at one end of the aglycone is less than 6 Å away from the phenolic group at the opposite end (Fig. 4). The profile of the aglycone is almost circular, however there is an access to the inner surface from either side. The larger opening (~8 × 11 Å) is shown in this profile. The distance between the amine (in green) and carboxylate (in red) groups is ~12 Å. This distance and their relative positions on the aglycone would prevent their simultaneous ionic interaction with the corresponding groups of an amino acid (Fig. 4).

Fig. 5 shows the entire teicoplanin molecule. Fig. 5A is the same profile as for the aglycone in Fig. 4. However, the mannopyranosyl unit and two glucosamide units (in blue) are now present. Also the C_9-alkyl hydrophobic tail is attached to the requisite glucosamine moiety (see lower left side of Fig. 5A). Fig. 5A also shows that the carboxylate moiety (in red) is between two bulky sugar moieties (in blue). The ammonium group (in green) remains accessible,

Fig. 4. The aglycone part of the teicoplanin molecule is shown to the left and the L-phenylalanine molecule is on the right. Black atoms, apolar; blue atoms, polar hydroxyl groups; green atoms, NH_3^+; red atoms, COO^-.

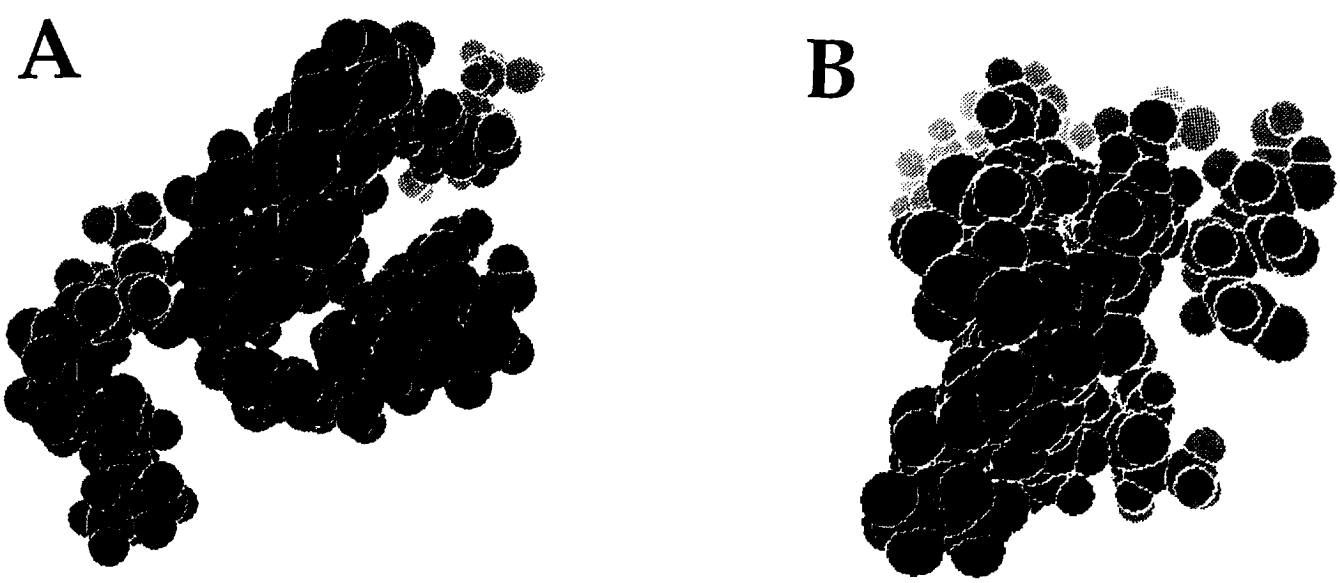


Fig. 5. (A) The complete energy minimized, molecular model of the teicoplanin molecule, corresponding to the Fig. 1 structure. View (B) corresponds to a 90° rotation to the right compared to view (A). Color coding is the same as for Fig. 4.

however. This is clear in Fig. 5B where the teicoplanin is rotated 90° to the right. Note that the sugar moieties and alkyl chain are free to rotate and can assume a variety of positions.

The results of this and previous CE teicoplanin studies [24] indicate that chiral compounds with acidic groups (i.e., carboxylate, sulfonate, phosphate, etc.) are most easily resolved. The teicoplanin ammonium group is the most available and logical site for initial docking and enantioselective retention. Also available at or near that site is a hydrophobic cleft on the aglycone, as well as additional hydrogen bonding and dipolar groups associated with the aglycone peptide bonds and the pendant sugar moieties (Fig. 5). It is known that teicoplanin binds to bacteria cell walls via peptides terminating in a D-Ala–D-Ala sequence [28]. The binding of an N-blocked tripeptide (N-acetyl-α-N-dansyl-L-Lys–D-Ala–D-Ala) was previously shown to be 28.4 kJ/mol which is significant [29]. Other studies have shown that while the aglycone is essential, the pendant sugar moieties also play a role. For example, the binding energy of Acetyl-D-Ala–D-Ala to teicoplanin was 31.2 kJ/mol, but it dropped to 23.9 kJ/mol for the aglycone "basket" alone [30]. Unfortunately, the binding energies of L-amino acids to teicoplanin were not found in literature. Given the low toxicity of teicoplanin [27,29], they are probably very low. These results and the modeling studies indicate the importance of electrostatic interactions as well as the secondary and tertiary structure of the teicoplanin molecule in chiral recognition.

3.4. Dipeptide separations

Table 4 gives the chromatographic data for the separation of some di- and tripeptides. Fig. 6 and Fig. 7 show the chromatograms obtained. When the dipeptide has only one chiral center, for example Ala–Gly, there are only two enantiomers and the selectivity and resolution factors can be calculated. For most di- and tripeptides, there are two chiral centers or more, producing four or more stereoisomers. The selectivity and resolution factors can be calculated only if the peak for each pure enantiomer can be identified.

Polypeptides are formed by reaction of the carboxylic acid group of one amino acid with the amino group of another amino acid. Dipeptides are also in the zwitterionic form in the HPLC mobile phase pH range used in this study. However, the anionic carboxylate group is no longer located on the same carbon atom as the cationic ammonium group. There are four atoms or more in between the two charged groups. The four carbon atoms in between the ionized groups can rotate, modifying the interactions. Consequently, predicting and understanding retention behavior and enantioselectivity may not be straightforward. For example, the Ala–Gly dipeptide is formed by the reaction of the carboxylic acid of alanine with the glycine amino group. The $-CH(CH_3)-CO-NH-CH_2-$ five-atom sequence is in between the two charged groups. Because glycine is not chiral, the carboxylate group of the Ala–Gly dipeptide is attached to a non-chiral CH_2- group and is four atoms away from the stereogenic center of the dipeptide. The Ala–Gly selectivity factor is an unimpressive 1.15, producing a 1.4 resolution factor. The Gly–Ala isomeric form is obtained when the amino group of alanine reacts with the carboxylic acid group of glycine. The $-CH_2-CO-NH-CH(CH_3)-$ five atom sequence is located between the two charged groups. The sequence looks similar to that of Ala–Gly, however the –CO–NH– amido group is reversed. The carboxylate group is now directly attached to the stereogenic center. This difference allows the Gly–Ala dipeptide to be much better recognized and resolved by the teicoplanin phase than the Ala–Gly isomer. The selectivity and the resolution factors jump to 10 and 2.5, respectively. Analogous trends were found for other dipeptides. The DL-Leu–Gly dipeptide is not listed in Table 4 because it was not resolved by the teicoplanin column ($\alpha = 1$, $R_s = 0$). The Gly–DL-Leu was easily separated with a 2.9 α factor and an $R_s = 4.2$. Again, the carboxylate group of Gly–DL-Leu is attached to the stereogenic center while that of the DL-Leu–Gly dipeptide is five atoms away from the stereogenic center. These results clearly show the essential role of the teicoplanin ammonium group as well as the analyte carboxylate group (and its proximity to the stereogenic center) in the enantiorecognition mechanism.

Fig. 6A and Fig. 7B show the resolution of the four stereoisomers of DL-Ala–DL-Ala and DL-Leu–DL-Leu. The availability of standards allowed the verifi-

Table 4
Dipeptides and tripeptides separated using reversed-phase on teicoplanin

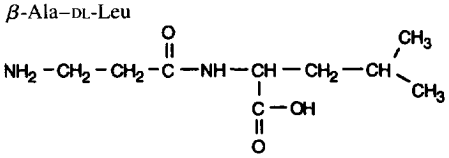
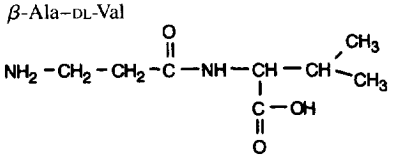
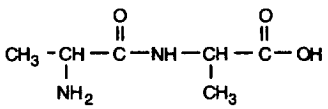
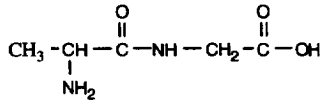
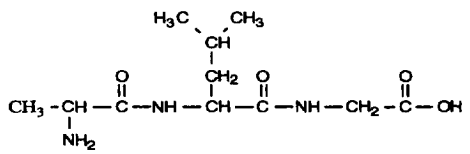
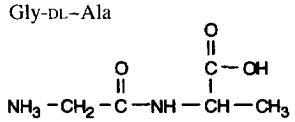
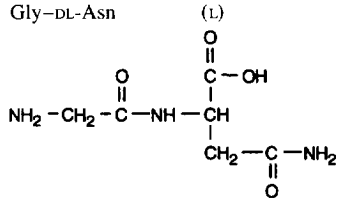
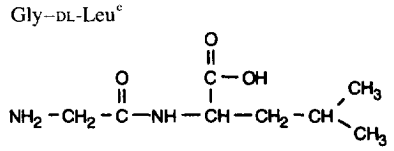
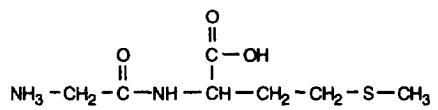
Compounds	k'^{a}	α	R_s
β-Ala–DL-Leu 	4.00	1.71	2.0
β-Ala–DL-Val 	4.52	1.38	1.8
DL-Ala–DL-Ala 	1.94 (D–L) 8.68 (D–D)	1.76[b] 1.20[b]	4.5[b] see Fig. 6A 1.6[b]
DL-Ala–Gly 	2.73	1.15	1.4
DL-Ala–DL-Leu–Gly 	2.82 (1–2) 4.75 (3–4)	1.2[c] 1.35[c]	2.0[c] see Fig. 6C 2.8[c]
Gly-DL-Ala 	0.53 (L)	10.0	2.50
Gly–DL-Asn (L) 	1.58	1.76	2.4
Gly–DL-Leu[c] 	3.19	2.90	4.24
Gly–DL-Met 	0.53 (L)	10.75	4.60

Table 4
Continued

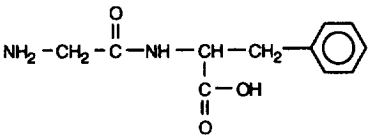
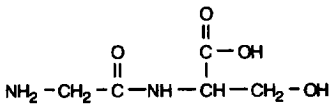
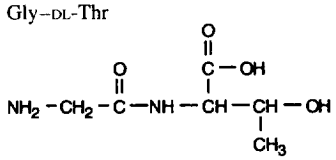
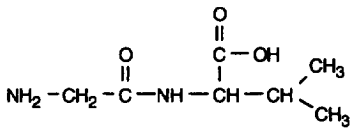
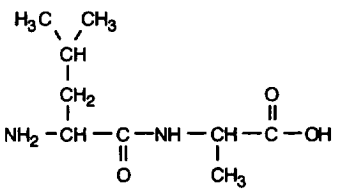
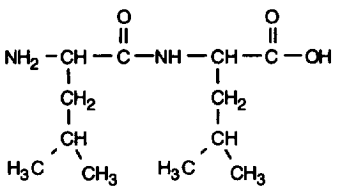
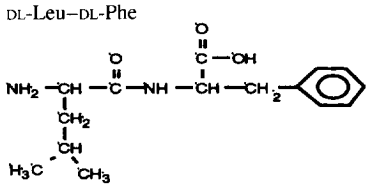
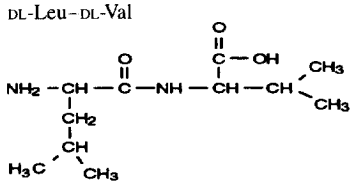
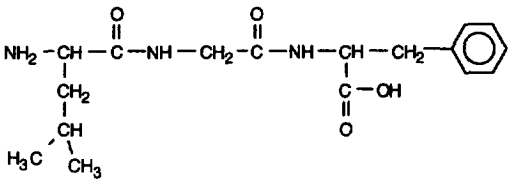
Compounds	k'^{a}	α	R_s
Gly–DL-Phe 	2.22 (L)	4.28	8.2
Gly–DL-Ser 	1.42	1.95	3.8
Gly–DL-Thr 	1.62	1.46	1.5
Gly–DL-Val 	4.00 (L)	1.97	4.6
DL-Leu–DL-Ala 	1.70 (1,2) 4.30 (3,4)	1.60[c] 1.28[c]	2.4[c] see Fig. 6B 2.3[c]
DL-Leu–DL-Leu 	0.79 (D–L) 2.33 (D–D)	2.20[d] 1.56[d]	3.9[d] see Fig. 7A 2.9[d]
DL-Leu–DL-Phe 	0.65 (1,2) 2.16 (3,4)	1.69[c] 1.32[c]	1.7[c] see Fig. 7C 1.5[c]
DL-Leu–DL-Val 	0.75 (1,3) 1.24 (2,4)	2.04[c] 1.68[c]	3.1[c] see Fig. 7B 2.1[c]

Table 4
Continued

Compounds	k'^{a}	α	R_s
DL-Leu–Gly–DL-Phe	1.60 (1,2)	1.30[c]	1.4[c] see Fig. 7D
	6.80 (2,3)	2.60[c]	3.3[c]

[a] Capacity factor of the first eluted enantiomer. The letters in parentheses indicate the configuration of this enantiomer if it is known. The numbers in parentheses indicate which two peaks were used to calculate α and R_s. This was only done when standards were not available. These peaks were chosen on the basis of their similar efficiencies as discussed in Section 3.
[b] fully determined absolute configuration, peaks: 1, D–L; 2, L–L; 3, D–D; 4, L–D.
[c] configuration not determined. The numbers between parenthesis refer to the peak order of the chromatogram.
[d] fully determined absolute configuration, peaks: 1, D–L; 2, L–L; 3, L–D; 4, D–D.
[e] Note that DL-Leu–Gly was not resolved.

cation of stereochemistry for the compound represented by each peak. Table 4 gives the capacity factors, selectivities and resolutions of the four isomers. In all cases, the D-terminated dipeptides were more retained than the L-terminated ones. As mentioned previously, the interaction between the teicoplanin molecule and the D-terminated dipeptide is thermodynamically strong producing high retention times and slow adsorption–desorption kinetics resulting in poor mass transfer and low efficiencies. Fig. 6A and Fig. 7B show that the peak efficiency was in the ~3000 plate range for the L-terminated peptides that eluted first. The measured efficiency for the D-terminated peptides was in the low 500 plate range. The D-Ala–D-Ala dipeptide was expected to have the higher affinity for teicoplanin given the previously published mechanism of teicoplanin antibiotic activity [28]. However, it was found that the

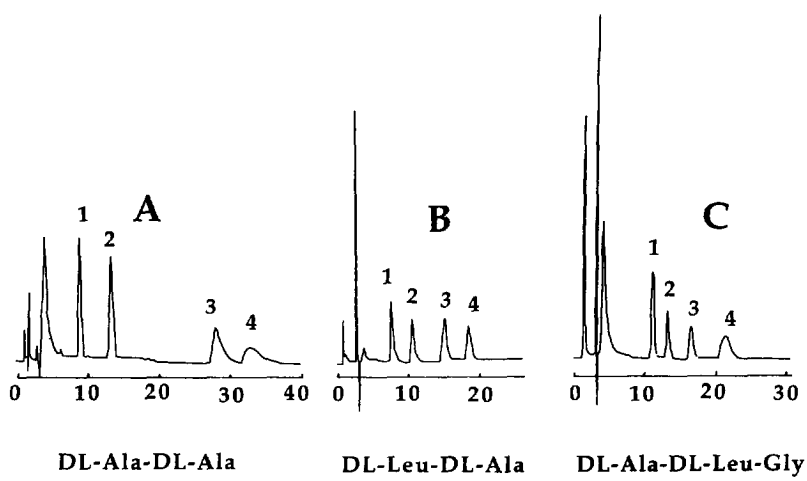


Fig. 6. Dipeptide and tripeptide separations. Column: teicoplanin bonded 5-μm silica particles, Chirobiotic T, 25 × 0.46 cm; mobile phase: ethanol–water (60:40, v/v); flow-rate: 1 ml/min; UV detection at 215 nm; time in minutes.

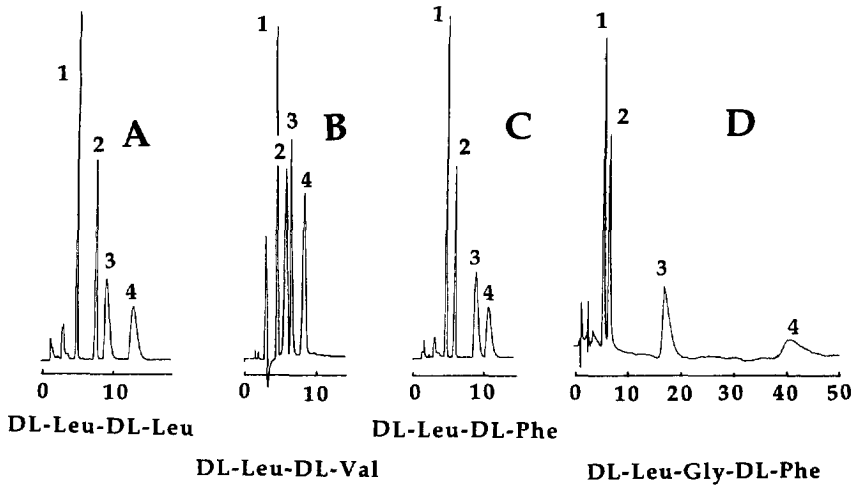


Fig. 7. Dipeptide and tripeptide separations. Operating conditions: see Fig. 6 legend.

L-Ala–D-Ala isomer was the most retained. For the Leu–Leu dipeptide, the D-Leu–D-Leu isomer was the most retained. Clearly it is the stereochemistry of the carboxy-terminal amino acid that is the most important factor in binding and selectivity.

The chromatograms of Fig. 6 and Fig. 7 show that the observed variations of peak efficiencies in chromatograms of DL-Ala–DL-Ala and DL-Leu–DL-Leu are generally true for most resolved dipeptides. Among the four peaks obtained with fully resolved dipeptides, one pair of peaks always has a lower efficiency than the other pair. It is likely that the broader "less efficient" peaks correspond to the two D-carboxylic terminated dipeptides. The faster eluting efficient peaks would correspond to the L-carboxylic terminated dipeptides. The selectivity and resolution factors listed in Table 4 for unidentified enantiomers were calculated between peaks of comparable efficiency. The corresponding peak numbers are given in Table 4. For all chromatograms except Fig. 7B (DL-Leu–DL-Val), the broader peaks were also the most retained. Fig. 7B shows that Peaks 1 and 3 of DL-Leu–DL-Val are thinner than Peaks 2 and 4. An extreme case was obtained with the tripeptide DL-Leu–Gly–DL-Phe (Fig. 7D). Two peaks elute first with k' values around 2 and a high efficiency (~3000 plates). They are likely the D- and L-Leu–Gly–L-Phe. Then, two broad peaks were obtained with k' values of ~7 and ~18 and efficiencies lower than 500 plates. They are likely the D- and L-Leu–Gly–D-Phe.

4. Conclusion

The teicoplanin based chiral stationary phase has a unique capability to resolve primary and secondary amino acids in their native state. Generally, the antibiotic prefers the D-form of amino acids. We have shown that the primary associative interaction involves a strong charge–charge interaction between the carboxylate group of the amino acid and the ammonium group of teicoplanin. Dipole orientation, hydrogen bonding and hydrophobic interactions made the interaction energy between the D-form of amino acids differing greatly from the similar interaction energy of the L-form. It is not necessary to have a terminal D-, D-dipeptide for a strong interaction as reported in earlier work. Sometimes the L-, D-dipeptide shows stronger interaction. The amino acid enantioselectivity is not sensitive to small mobile phase changes in organic modifier content, ionic strength, pH or buffer capacity. The teicoplanin CSP is able to resolve all 20 naturally occurring amino acids as well as many other amino acids and small peptides with high resolution factors. Most separations can be accomplished with hydro-organic

solvent systems containing no buffer or salts of any kind.

Acknowledgments

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