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Short communication

Direct high-performance liquid chromatographic separation of unusual secondary amino acids and a comparison of the performances of Chirobiotic T and TAG columns

Antal Péter[a,*], Roland Török[a], Daniel W. Armstrong[b]

[a] *Department of Inorganic and Analytical Chemistry, University of Szeged, Dóm tér 7, H-6720 Szeged, Hungary*
[b] *Department of Chemistry, Iowa State University, Gilmann Hall, Ames, IA 50011, USA*

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Abstract

Two macrocyclic glycopeptide antibiotic-type chiral stationary phases (CSPs) based on native teicoplanin and teicoplanin aglycone, Chirobiotic T and TAG, respectively, were evaluated with regard to the high-performance liquid chromatographic separation of the enantiomers of 10 secondary α-amino acids (imino acids). The chromatographic results are given as the retention, separation and resolution factors, together with the enantioselective free energy difference corresponding to the separation of the enantiomers. By application of these two CSPs, excellent resolutions were achieved for the investigated compounds by using reversed-phase mobile mode systems. The separation conditions were optimized by variation of the mobile phase composition. The difference in enantioselective free energy between the aglycone CSP and the teicoplanin CSP for these particular amino acids ranged between 0.70 and $-1.83\,\mathrm{kJ\,mol^{-1}}$. It was established that better enantioseparations of the secondary α-amino acids were attained in most cases on the aglycone CSP.

Keywords: High-performance liquid chromatography; Direct enantiomeric separation; Macrocyclic antibiotic-type stationary phases; Chirobiotic T and TAG; Secondary amino acids (“imino acids”)

1. Introduction

Over the past few years, it has been demonstrated that chiral stationary phases (CSPs) based on macrocyclic antibiotics are extremely useful for the enantiomeric separation of biological and synthetic amino acids [1–5], food flavor components [6], reagents and catalysts advertised as being enantiomerically pure [7,8], and a wide variety of compounds with various polarities [9–13]. The vancomycin-related antibiotics bind to the bacterial cell-wall D-Ala–D-Ala terminal group, blocking the process of cell-wall growth. More recently, it has emerged that teicoplanin and vancomycin molecules without the attached carbohydrate (sugar) moieties, consisting of only an aglycone peptide “basket”, are more effective in the enantioseparation of certain types of analytes [9,11–14].

The bioactive conformations of peptides are usually not known, but the incorporation of rigid, sterically hindered unusual amino acids into peptides generally leads to a better knowledge of the three-dimensional requirements for molecular recognition [15]. Several unusual α-amino acids have been designed recently with a view to constraining the side-chain functional groups of natural α-amino acids [16].

The presence of secondary α-amino acids (imino acids), where the nitrogen is a ring-atom, has profound consequences for the conformation of peptides and peptidomimetic agents since the nitrogen is unable to act as a hydrogen-bond donor unless it is located in the terminal position of the peptide. These types of amino acids have proven to be very useful in biological studies. Their synthesis, incorporation into

* Corresponding author. Tel.: +36 62 544 000/3656; fax: +36 62 420 505.
E-mail address: apeter@chem.u-szeged.hu (A. Péter).

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peptides, and the biological properties of peptides containing such imino acids have been surveyed in several reports [17–19].

The chirality of such compounds is of the utmost importance. Peptide diastereomers can have different biological properties (agonistic or antagonistic). Therefore, there is great interest in the methods developed for the separation and identification of these unusual amino acid enantiomers.

In the present work two, macrocyclic glycopeptide CSPs based on native teicoplanin and teicoplanin aglycone, Chirobiotic T and TAG, respectively, were used for the high-performance liquid chromatographic (HPLC) separation of enantiomers of 10 secondary α-amino acids (imino acids). The chromatographic results presented here include retention, separation and resolution factors, and the enantioselective free energy differences. The conditions affording the best resolution were determined and the differences in the separation capabilities of the two related CSPs are discussed. The sequence of elution of the enantiomers was determined in most cases.

2. Experimental

2.1. Chemicals and reagents

4-Hydroxypyrrolidine-2-carboxylic acid [**1**, enantiopure (2*R*,4*R*) and (2*S*,4*S*)] and piperazine-2-carboxylic acid (**7**, racemic D,L) were purchased from Aldrich (Steinheim, Germany). α-Methylproline (**2**, racemic [23]), 1,2,3,6-tetrahydropyridine-2-carboxylic acid (**3**, baïkaïne, enantiopure L [20]; D-isomer was produced via the partial racemization of L-baïkaïne by refluxing in 2 M NaOH), piperidine-2-carboxylic acid (**4**, pipecolic acid, Pip, enantiopure L and D [21]), 4-hydroxypipecolic acid {**5**, enantiopure (2*S*,4*R*) [20]; racemic [24]}, 5-hydroxypipecolic acid {**6**, enantiopure (2*S*,5*R*) [20]; racemic [25]}, 5-methylpiperazine-2-carboxylic acid (**8**, racemic [26]), morpholine-3-carboxylic acid (**9**, enantiopure L [21]; racemic [27]), thiomorpholine-3-carboxylic acid (**10**, enantiopure L [22]; racemic [28]) were synthetized by using the indicated reported literature methods.

The physico-chemical data on the synthetized amino acids were determined and were found to be identical with the data cited in [20–28]. The nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [29].

Methanol (MeOH) and acetonitrile (MeCN) were from Merck (Darmstadt, Germany); both were of HPLC grade. Triethylamine (TEA), glacial acetic acid (HOAc), trifluoroacetic acid (TFA) and other analytical-grade reagents were also from Merck. The inorganic component of the mobile phase used in the reversed-phase (RP) method was prepared from Milli-Q water, which was further purified by filtering on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

Adjustment of the pH of the mobile phase (inorganic part) and to study the effect of pH on the separations, 0.1% (v/v) of aqueous solutions of TEA was titrated with AcOH to a suitable pH. For investigation of the effect of ionic strength, the concentration of TEAA in the total volume of mobile phase (inorganic + organic part) was adjusted from 0.1 to 1.0%. Mobile phases were prepared by mixing the indicated volumes of buffers and/or solvents and were further purified by filtration through a 0.45-μm Millipore filter, type HV. The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses. Stock solutions of amino acids (1 mg ml^{-1}) were prepared by dissolution in water or in the starting mobile phase.

Dead-times (t_0) of the columns were measured by injecting 10^{-4} mol l^{-1} aqueous solution of KBr into the mobile phase of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v), at a flow-rate of 1.0 ml min^{-1}. The t_0 values for Chirobiotic T and TAG columns were 1.98 and 2.00 min, respectively.

2.2. Apparatus

The HPLC separations were carried out on a Waters LC system consisting of an M-600 low-pressure gradient pump, a M-996 photodiode-array detector and a Millenium^{32} Chromatography Manager data system; the alternative Waters Breeze system consisted of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, a column thermostat Model 5CH and Breeze data manager software (both systems from Waters Chromatography, Milford, MA, USA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20-μl loops.

The columns used for analytical separation were a teicoplanin-containing Chirobiotic T and a teicoplanin aglycone-containing Chirobiotic TAG column, 250 mm × 4.6 mm i.d., 5-μm particle size (Astec, Whippany, NJ, USA). The column was thermostated at 25 °C.

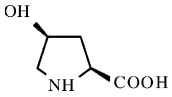
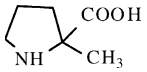
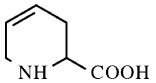
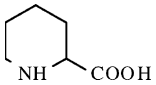
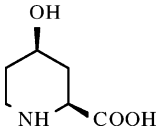
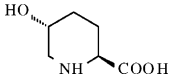
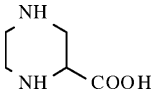
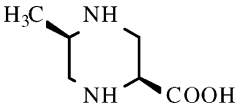
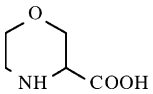
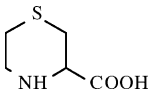
3. Results and discussion

3.1. Analyte, mobile phase and stationary phase selection

The structures of analytes investigated are highly constrained due to the position of the nitrogen in the ring. Relevant separation data on these compounds are given in Table 1. The data include the retention factors, separation factors, resolutions and enantioselective energy differences for each analyte. For comparison purposes, the compounds listed in Table 1 were evaluated with a mobile phase of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v), but for optimized separations other mobile phase compositions are included.

With the same RP mobile phase composition, the retention factors of the first-eluted enantiomers were lower on the teicoplanin stationary phase than on the aglycone phase

Table 1
Chromatographic results obtained on the chiral stationary phases Chirobiotic T and TAG

Compound number	Compound	Column	Eluent composition (v/v)	k_1'	k_2'	α	R_S	$-\Delta(\Delta G^\circ)$ (kJ mol^{-1})
1		T	60:40	0.16	0.26	1.63	1.21	1.21
			20:80	2.77	3.62	1.31	1.87	0.67
		TAG	60:40	1.11	1.39	1.25	1.19	0.55
			40:60	1.96	2.51	1.28	1.55	0.61
2[a]		T	60:40	1.12	1.36	1.21	1.43	0.47
		TAG	60:40	2.09	2.21	1.05	0.60	0.12
			70:30	2.36	3.18	1.35	1.55	0.74
3		T	60:40	1.51	1.72	1.14	<0.40	0.32
			20:80	3.29	4.22	1.28	1.52	0.61
		TAG	60:40	2.21	4.41	1.99	2.38	1.70
4		T	60:40	1.51	2.20	1.45	2.00	0.92
		TAG	60:40	2.34	7.08	3.03	2.90	2.75
5		T	60:40	1.03	1.17	1.14	0.80	0.32
			20:80	2.23	2.99	1.34	1.81	0.73
		TAG	60:40	1.07	1.93	1.80	2.00	1.45
6		T	60:40	0.27	0.34	1.26	0.65	0.57
			20:80	0.75	1.01	1.35	1.42	0.74
		TAG	60:40	1.85	2.03	1.10	0.70	0.24
			40:60	2.78	3.13	1.13	1.00	0.30
			20:80	5.63	6.12	1.09	0.90	0.21
7[a]		T	60:40	1.80	2.08	1.16	1.10	0.37
			20:80	6.96	9.21	1.32	1.70	0.69
		TAG	60:40	4.99	6.53	1.31	1.30	0.67
			30:70	6.63	11.14	1.68	2.40	1.29
8[a]		T	60:40	2.33	3.25	1.39	1.70	0.82
		TAG	60:40	2.54	5.07	2.00	2.30	1.72
9		T	60:40	0.21	0.58	2.76	2.56	2.52
		TAG	60:40	1.86	4.17	2.24	2.80	2.00
10		T	60:40	0.45	0.88	1.96	2.24	1.67
		TAG	60:40	2.80	8.79	3.14	3.26	2.83

Chromatographic conditions: column, Chirobiotic T and TAG; flow rate, 1.0 ml min^{-1}; detection, 205 nm; temperature, 25 °C; mobile phase, 0.1% TEAA (pH 6.5)–MeOH (v/v); $R_S = 1.18(t_2 - t_1)/(w_{1;1/2} + w_{2;1/2})$.

[a] Elution sequence not determined.

(Table 1). Berthod et al. [9] observed that the retention factors of compounds of intermediate polarity were relatively similar on the two CSPs with the same mobile phase, or were somewhat lower on the aglycone phase. However, for polar amino acids, in some cases they found higher retention factors on the aglycone phase. Since the retention factors for the first-eluted components differed considerably on the two stationary phases in our study, it can be stated that the overall polarity of the aglycone stationary phase used here differed from that of the corresponding teicoplanin stationary phase. The retention factors of the second-eluted isomers differ much more widely.

The teicoplanin molecule has several characteristic features that make it suitable for amino acid analysis [1,30]. The retention and selectivity on the teicoplanin stationary phase could be controlled by altering the nature and concentration of the organic modifier. The retention factor versus organic modifier content curves exhibited different shapes depending on the nature of the analyte and the teicoplanin-based selector. It was earlier demonstrated [9,30,31] that, on Chirobiotic T column for primary α-amino acids separated in the RP mode, a U-shaped curve was characteristic for the retention factor plot versus the MeOH content of the mobile phase. On Chirobiotic T column this type of curve was obtained for the analytes (with exception of **2**, **7** and **8**) but the inflection point and the slope of the curve at higher MeOH concentrations differed somewhat for each compound. The increase in the retention factor with increasing water content was due to enhanced hydrophobic interactions in the water-rich mobile phase, while the increase in the retention factor with increasing MeOH content was due to the decreased solubility of polar amino acid in the MeOH-rich mobile phase. On the Chirobiotic TAG column a somewhat different type of retention curve was observed for analytes investigated (with exception of **2**), where the retention factor continuously increased with increasing MeOH content. The teicoplanin aglycon, without sugar units and a hydrophobic alkyl chain was less capable of hydrophobic interactions than the native teicoplanin. On both CSPs for **2**, there was a decrease in the retention factor with increasing MeOH content, i.e., typical RP retention behavior was observed. On both columns for **7** and **8**, the retention factor continuously increased with increasing MeOH content, i.e., on Chirobiotic T column the increase in retention factor in water-rich mobile phase here was not observed.

For α-amino acids on a native teicoplanin stationary phase, the more hydrophobic the solute, the more typical the RP retention behavior observed [1,3,30]. For most of the analytes investigated, a structure-retention relationship could be observed on both stationary phases. At an eluent composition of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v), the more hydrophobic α-MePro (**2**) of the Pro analogs **1** and **2** exhibited a higher k' value, however, this higher retention was not accompanied by better α and R_S values (Table 1). The more apolar **2** exhibited a typical RP retention behavior, i.e., the retention factor decreased with increasing MeOH content. In the series of the Pip analogs **3–6**, the more hydrophilic **3**, **5** and **6** were less retained than **4** on both stationary phases. The incorporation of –S– or –O– atoms into the ring instead of a $–CH_2–$ group in position 4 (**4** versus **9**, **10**) led to a decreased k' value (with exception of **10** on TAG column), as a consequence of the decreased hydrophobicity of the molecules.

The incorporation of an additional –NH– into the ring in position 4 (**4** versus **7**, **8**) led to higher retention factors on both columns in spite of the decreased hydrophobicity of the molecules. The retention on the teicoplanin based stationary phases is critically dependant on the ionization state of both the analyte and the CSP. To compare behavior of **4** versus **7** and **8**, a study of the effect of pH was carried out (Fig. 1). The free selectors, teicoplanin and teicoplanin aglycone contain a single primary amine and a single carboxylic group. The respective pK_a values are around 9.2 and 2.5 as reported previously [1]. The amino group may be an ureido, when attached to a linkage chain. The pK_a values for Pip (**4**) are approximately 10.5 (–NH–) and 2.5 (–COOH) [32] and the values for **7** are 9.53 and 5.41 (–NH–, respectively) and 1.5 (–COOH) [33]. There are no data for pK_a values for **8**, but probably they are similar to the aforementioned related compounds.

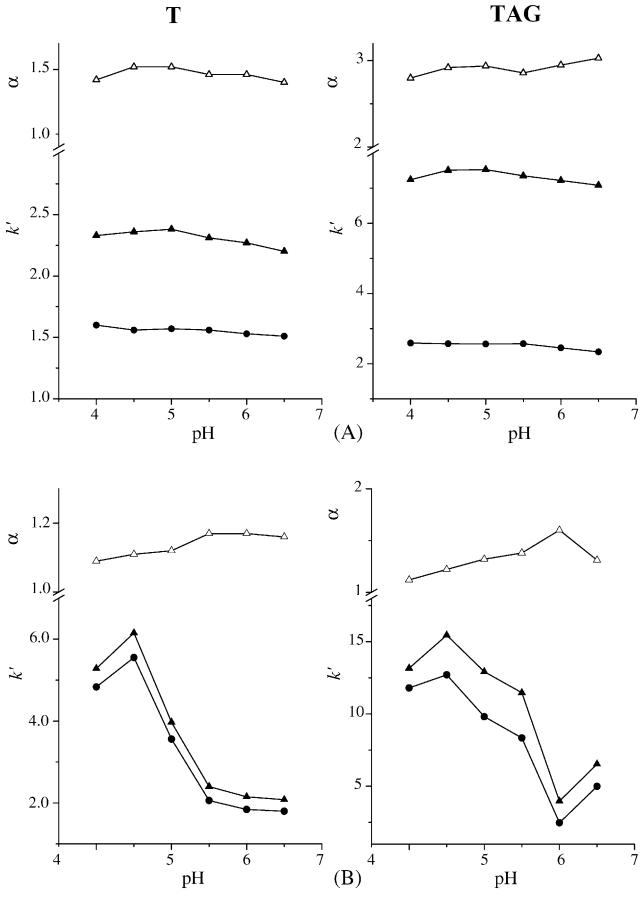


Fig. 1. Retention factors (k') vs. pH value of the mobile phase on Chirobiotic T and TAG columns. (**A**), D,L-pipecolic acid (**4**); (**B**), D,L-piperazine-2-carboxylic acid (**7**); chromatographic conditions: columns, Chirobiotic T and TAG; mobile phase, 0.1% aqueous TEAA (pH 4–6.5)–MeOH (60:40, v/v); flow rate, 1.0 ml min^{-1}; detection, 205 nm; (●) retention factor, k_1'; (▲) retention factor, k_2'; (△) separation factor, α.

A slight decrease in the retention factor for Pip (4) was observed on both CSPs with increasing pH, while a very small increase in its α-value was observed (Fig. 1). The change in ionic strength had a small effect on retention factors and α values. With increases in the buffer concentration from 0.1% (7.2×10^{-3} mol l^{-1}) to 1.0% (7.2×10^{-2} mol l^{-1}) of TEAA (pH 6.5, TEAA–MeOH, 60:40, v/v) on the Chirobiotic T column, k'_1 decreased from 1.51 to 1.48 and α exhibited a small change between 1.45 and 1.57. On the Chirobiotic TAG column k'_1 was around 2.40 and the α exhibited a small decrease from 3.03 to 2.67. It seems that the ionization state of columns and of stereoisomers of Pip (**4**) had little effect on the efficiency of the separation. A more pronounced change in k' and α values versus pH was detected for **7** (Fig. 1) and **8** (data not shown) with an inflexion point around pH 5. In the pH range (pH 4–6.5) Pip (**4**) was in its zwitterionic form, while beyond pH 5.5 both –NH– groups were protonated and the –COOH groups were deprotonated for **7** and **8**. The stronger interaction of stereoisomers of **7** and **8** with the CSPs seems to be due to the strong interaction between the protonated imino group of the analyte and the carboxylate of the teicoplanin. Moreover, the inflexion point, observed for the k' versus pH plots near the pK_a value of the analyte imino group confirmed this hypothesis. The dissimilarities of the k' versus MeOH content plots observed for **7** and **8** on the Chirobiotic T column (no U-shaped curves) support our contention that the retention and separation mechanism for these compounds differed from **1**–**6** to **9**–**10**.

3.2. Enantioselectivity of enantiomers and the role of carbohydrate units in enantiorecognition on antibiotic phases

Table 1 lists the separation factors (α), resolutions (R_S) and differences in Gibbs free energy [$\Delta(\Delta G^\circ)$] for the two stereoisomers of the unusual secondary α-amino acids. The highest separation factors obtained on the teicoplanin CSP were $\alpha = 2.76$ and 1.96 for **9** and **10**, respectively. The highest separation factors obtained on the aglycone CSP were $\alpha = 3.14$ and 3.03 for **10** and **4**, respectively. The α values for the conformationally constrained imino acids (Table 1) were in the same range as those reported for the common proteogenic and other α-amino acids having simple structures [9]. The highest α values observed correspond to a difference in enantioselective free energy of 2.83 kJ mol^{-1}, which is indicative of the good enantiorecognition capability of these chiral selectors. Table 1 shows that the resolution factors associated with these separation factors can be as high as 3.26 (**10**).

The $-\Delta(\Delta G^\circ)$ values for the compounds in Table 1 differ considerably. The highest $-\Delta(\Delta G^\circ)$ values were obtained for **4**, **9** and **10**. With mobile phase of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v) for the imino acids, higher $-\Delta(\Delta G^\circ)$ values were obtained on the Chirobiotic TAG column (with exception of **1**, **2**, **6** and **9**, Table 1). Comparison of the results obtained on the two CSPs may contribute to an understanding of the role of the pendant sugar moieties in chiral recognition. To quantify the effects of the sugar units, the differences in enantioselective free energies between the two CSPs, $\Delta_{TAG-T}\Delta(\Delta G^\circ)$, listed in Table 1 were used [$-\Delta(\Delta G^\circ) = RT \ln \alpha$]. The $\Delta(\Delta G^\circ)$ value obtained on the native teicoplanin CSP with mobile phase of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v) was subtracted from the $\Delta(\Delta G^\circ)$ value obtained for a given compound on the teicoplanin aglycone CSP [$\Delta(\Delta G^\circ)_{aglycone} - \Delta(\Delta G^\circ)_{native\ teicoplanin} = \Delta_{TAG-T}\Delta(\Delta G^\circ)$] and the results were plotted as shown in Fig. 2. A negative number means that the stereoisomers separated better on the aglycone CSP. A positive number means that the stereoisomers separated better on the native teicoplanin CSP, which contains the carbohydrate units.

Of the stereoisomers of the imino acids, the sterically highly constrained Pro analogs (**1** and **2**), the *trans*-stereoisomer of **6** and the cyclic compound **9** with the oxygen heteroatom exhibited better separation on the Chirobiotic T column (Fig. 2). The probable explanation is that, in the case of Pro analogs and **6**, the highly constrained structure or the *trans*-position of substituents do not fit the active sites of the aglycone, while in the case of **9,** additional interactions could be achieved through the extra heteroatom and sugar units of the teicoplanin (Fig. 2).

From the aspect of enantiomeric separation, the sugar moieties of the native teicoplanin may intervene in the chiral recognition process [9]. In general, appears that the steric hindrance effect of the sugar moieties was predominant for the α-amino acids, which are thought to "dock" and bind inside the cleft of the aglycone, near its amine (or ureido, if attached to a linkage chain) functional group. It appears that D-α-amino acids [(*R*)-α-amino acids] can associate more strongly and easily with this active binding site of the aglycone than they can on native teicoplanin molecules. This closer approach produces a stronger diastereomeric complex and better enantioselectivity. The two phenols and the OH

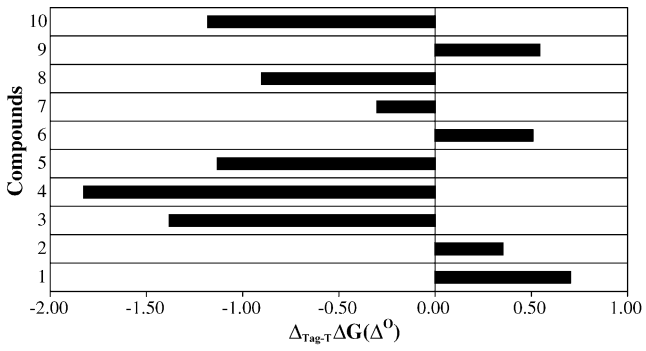


Fig. 2. Enantioselectivity differences, $\Delta_{TAG-T}\ \Delta(\Delta G^\circ)$, between the aglycone and native teicoplanin stationary phases. A negative value corresponds to better enantioresolution by the aglycone column. A positive value corresponds to better enantioresolution by the teicoplanin column. For compounds, see Table 1. Columns, Chirobiotic T and TAG; mobile phase, 0.1% aqueous TEAA (pH 6.5)–MeOH (60:40, v/v); flow rate, 1.0 ml min^{-1}; detection, 205 nm.

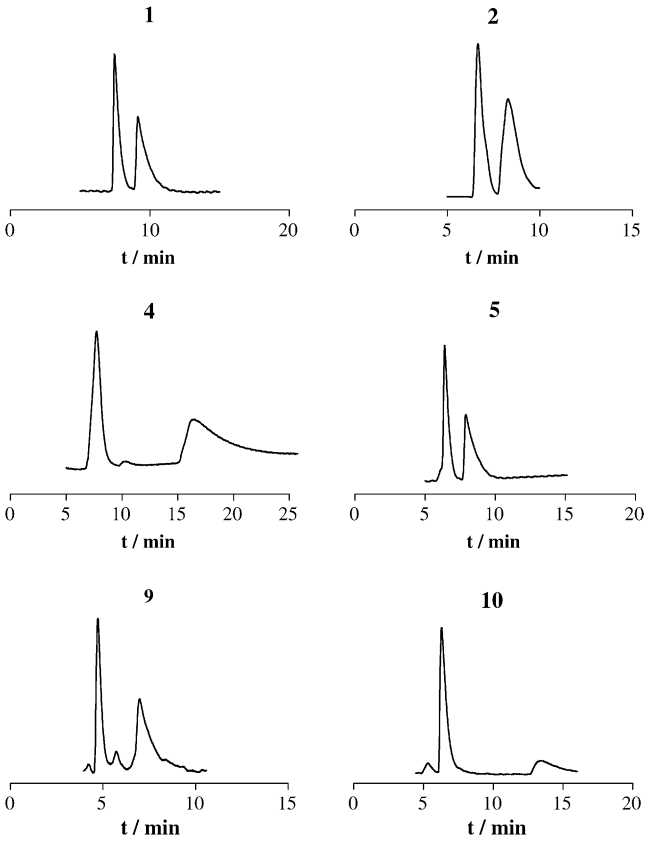


Fig. 3. Selected chromatograms for the enantioseparation of imino acids. Chromatographic conditions: column Chirobiotic T for compounds **1** and **5** and Chirobiotic TAG for compounds **2**, **4**, **9** and **10**; mobile phase, 0.1% aqueous TEAA (pH 6.5)–MeOH (20:80, v/v) for compounds **1** and **5**, 0.1% TEAA (pH 6.5)–MeOH (60:40, v/v) for compound **4**, 0.1% TEAA (pH 6.5)–MeOH (70:30, v/v) for compound **2**, and 0.1% TEAA (pH 6.5)–MeOH (90:10, v/v) for compounds **9** and **10**; flow rate, 1.0 ml min^{-1}; detection, 205 nm.

group on the aglycone may contribute to the interaction with the amino acids.

For imino acids the steric arrangement (a highly constrained structure), the nature of heteroatoms had a considerable influence on the enantioselectivity and resolution. For imino acids **1**, **2**, **6** and **9**, the rigidity of the molecule or *trans*-position of substituents or the incorporation of oxygen heteroatom into the ring promoted chiral recognition on the native teicoplanin, while in other cases behavior similar to that of primary α-amino acids was observed, i.e., the aglycone CSP was more effective in the separation of the stereoisomers of imino acids (**3–5**, **7**, **8** and **10**).

3.3. Optimization of separation and elution sequence

In cases, when separation of enantiomers [using a mobile phase of 0.1% TEAA–MeOH (pH 6.5) (60:40, v/v)] led to poorly separated peaks, the resolution was increased by optimization of the mobile phase (Table 1). Proper optimization has to take into account that the trends for the change in buffer/organic modifier ratio depend on the shape of the k' versus MeOH content plots. That is, e.g. in the cases of **1**, **3**, **5**, **6** and **7** the increase of MeOH content, while in the case of **2** the increase of buffer content, led to higher k' values and better enantioresolution. Unfortunately, for **6**, any variation of the mobile phase composition or pH failed to improve the resolution, which was $R_S \sim 1.0$. Selected chromatograms are depicted in Fig. 3.

The sequence of elution was determined in most cases. A general rule could be established for the sequence of elution of the stereoisomers: L < D [(*S*) < (*R*)].

4. Conclusions

By application of these two CSPs, excellent separations were achieved for most of the investigated compounds in the RP mobile phase systems. It was found that the carbohydrate moieties on teicoplanin are not always needed for the enantioresolution of unusual secondary α-amino acids, in most cases the aglycone was responsible for the enantioseparation. Although the sugar units decrease the resolution of most α-amino acid enantiomers, they can contribute significantly to the resolution of some unusual secondary α-amino acid analogs. The sequence of elution of the stereoisomers of unusual amino acids was determined and a general rule could be established for the sequence of elution of the stereoisomers on both CSPs. It was found to be L < D [(*S*) < (*R*)].

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