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Comparison of the separation efficiencies of Chirobiotic T and TAG columns in the separation of unusual amino acids

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Abstract

Two macrocyclic antibiotic type chiral stationary phases (CSPs), based on native teicoplanin and teicoplanin aglycone, Chirobiotic T and Chirobiotic TAG, respectively, were evaluated for the high-performance liquid chromatographic separation of enantiomers of 15 unnatural conformationally constrained α-amino acids, Phe and Tyr analogs, and 12 β-amino acids having cycloalkane or cycloalkene skeletons. The chromatographic results are given as the retention, separation and resolution factors along with the enantioselective free energy difference corresponding to the separation of the enantiomers. It is clearly established that in most cases the aglycone is responsible for the enantioseparation of amino acids. The difference in enantioselective free energy between the aglycone CSP and the teicoplanin CSP was between 0.02 and 0.30 kcal mol^{-1} for these particular amino acids. The resolution factors are higher with the aglycone CSP. Although the sugar units generally decrease the resolution of amino acid enantiomers, they can contribute significantly to the resolution of some unusual amino acid analogs. By application of these two CSPs excellent resolutions were achieved for most of the investigated compounds by using reversed phase or polar organic mobile mode systems. The separation conditions were optimized by variation of the mobile phase composition.

Keywords: Efficiency; Chiral stationary phases, LC; Enantiomer separation; Thermodynamic parameters; Amino acids; Phenylalanine; Tyrosine

1. Introduction

Currently, the antibiotics of last resort are glycopeptides of the vancomycin family. The vancomycin-related antibiotics bind to the bacterial cell-wall D-alanyl-D-alanine terminal group, blocking the process of wall building. It turned out that chiral stationary phases (CSPs) based on these macrocyclic antibiotics were extremely useful in the chiral separation, not only of native and unusual amino acids [1–5], but also in the resolution of food flavors [6], reagents and catalysts advertised as being enantiomerically pure [7,8], and a wide variety of compounds of various polarities [9–13]. More recently it was found that teicoplanin and vancomycin molecules without the attached carbohydrate (sugar) moieties, consisting of only an aglycone peptide "basket", were more effective in the enantioresolution of some types of analytes [9,11–14].

The importance of conformationally constrained α-amino acids and alicyclic β-amino acids as target compounds in peptide syntheses is increasing. Understanding of the biologically active conformations of peptide hormones is an important goal in modern biology. Most peptide hormones are highly flexible molecules with numerous possible conformations under physiological conditions. One synthetic approach to limit these conformations to the most favorable ones involves the introduction of conformational constraints [15]. In this approach, mimetics of secondary structures such as an α-helix, β-turns, γ-turns, etc., are built into the peptides in order to stabilize their structures. Several unusual α-amino acids have recently been designed with a view toward constraining the side-chain functional groups of natural α-amino acids including the different ring- and α-substituted aromatic amino acids, i.e. phenylalanine and tyrosine analogues [15–20].

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Alicyclic β-amino acids have played an important role in chemistry and biology. *cis*-(1*R*,2*S*)-Aminocyclopentanecarboxylic acid (*cis*-pentacin) is an antifungal antibiotic [21–24]. *cis*- and *trans*-2-Aminocyclohexanecarboxylic acids and the analogues with norbornane and norbornene skeleton were used in the synthesis of heterocycles with the aim of preparing potential pharmacons [25,26].

The β-amino acids are not only important pharmacologically but are also used as building blocks for the preparation of peptidomimetics [27–29]. Also, these amino acids have been used for the determination of the fine structures of receptors [26,30].

In the synthesis of such compounds, chirality is of the utmost importance. Peptide diastereomers may have different biological properties (agonistic or antagonistic), therefore there is a great interest in methods developed for the separation and identification of enantiomers. The separation of optical isomers requires an asymmetric or chiral environment allowing enantiorecognition through diastereomeric interactions. For this purpose, high-performance liquid chromatography (HPLC) is widely applied.

In the present paper, two macrocyclic antibiotic type CSPs, based on native teicoplanin and teicoplanin aglicone, Chirobiotic T and Chirobiotic TAG, respectively, were used for the high-performance liquid chromatographic separation of enantiomers of 15 unnatural conformationally constrained α-amino acids, Phe and Tyr analogs and 12 β-amino acids having cycloalkane or cycloalkene skeletons. The chromatographic results presented include: retention, separation and resolution factors along with the enantioselective free energy difference corresponding to the separation of the enantiomers. Conditions affording the best resolution were determined and the difference between the separation capability of two related chiral stationary phases was discussed. The elution sequence of the enantiomers was determined in most cases by spiking the racemic samples with enantiomers with known absolute configurations.

2. Experimental

2.1. Chemicals and reagents

D- and L-Phe (**1**) and D- and L-Tyr (**13**) were obtained from Sigma (St. Louis, MO, USA). The unusual α-amino acids were synthesized in our laboratories in racemic or in enantiopure form [31–35]: 2-amino-3-*o*-tolylpropionic acid (**2**, 2′-MePhe); 4′-methylphenylalanine (**3**, 4′-MePhe); 2′,4′-dimethylphenylalanine (**4**, 2′,4′-diMePhe); 2′,6′-dimethylphenylalanine (**5**, 2′,6′-diMePhe); 2′,4′,6′-trimethylphenylalanine (**6**, 2′,4′,6′-triMePhe); 2-amino-3-(3-methoxyphenyl)propionic acid (**7**, 3′-MeOPhe); α-methylphenylalanine (**9**, α-MePhe); 2-amino-2-methyl-3-*o*-tolylpropionic acid (**10**, α-Me-2′-MePhe); 2-amino-3-(3-methoxyphenyl)-2-methylpropionic acid (**11**, α-Me-3′-MeOPhe); 2-amino-3-(2-cianophenyl)-2-methylpropionic acid (**12**, α-Me-2′-CNPhe); 2′-methyltyrosine (**14**, 2′-MeTyr); 2′,6′-dimethyltyrosine (**15**, Dmt); (for their structures, see Table 1). The nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [36].

Monocyclic β-amino carboxylic acids in racemic form, *cis*- and *trans*-2-aminocyclopentane carboxylic acids (*cis*- and *trans*-Acpc, **16** and **17**) [37], *cis*- and *trans*-2-aminocyclohexane carboxylic acids (*cis*- and *trans*-Achc, **18** and **19**) and *cis*- and *trans*-6-aminocyclohex-3-ene-1-carboxylic acids (*cis*- and *trans*-Achc-ene, **20** and **21**) [38] and bicyclic β-amino carboxylic acids also in racemic form *diendo*- and *diexo*-3-amino-bicyclo[2.2.1]heptane-2-carboxylic acids (*diexo*- and *diendo*-Abhc, **22** and **23**), *diendo*- and *diexo*-3-aminobicyclo[2.2.1]hept-5-ene-2-carboxylic acids (*diexo*- and *diendo*-Abhc-ene, **24** and **25**) [39], *cis*-2-aminocycloheptane carboxylic acids (*cis*-Ach7c, **26**) and *cis*-2-aminocyclooctane carboxylic acids (*cis*-Acoc, **27**) were prepared in our laboratory [40] (for their structures, see Table 2). Enantiopure or enantiomerically enriched analytes were prepared by enzymatic resolution applying *lipase PS* enzymes [37–40].

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

Triethylammonium acetate (0.1%) (TEAA) buffers were prepared by titration of 0.1% (by volume) aqueous solutions of TEA with AcOH to a suitable pH. Mobile phases for reversed-phase and polar-organic chromatography 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 starting mobile phase.

2.2. Apparatus

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an 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 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-

Table 1
Chromatographic results obtained at 20 °C on the two chiral stationary phases, Chirobiotic T and TAG, for the Phe and Tyr analogs

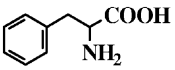
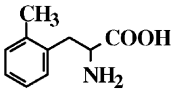
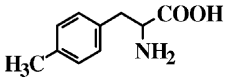
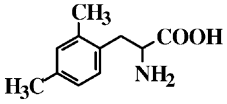
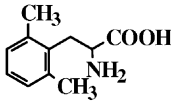
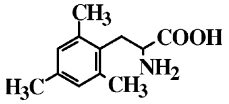
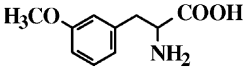
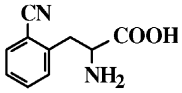
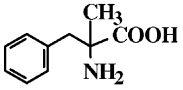
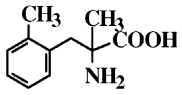
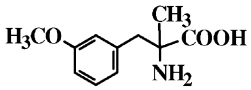
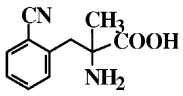
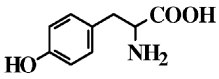
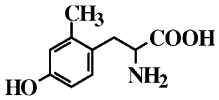
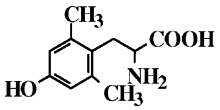
Compound	CSP	Mobile phase (a, b, c)	k_S	k_R	α	R_S	$-\Delta(\Delta G^\circ)$ (kcal mol^{-1})
1 (Phe) 	T	40:60 a, e	1.45	1.84	1.27	1.60	0.14
	TAG	40:60 a, e	2.14	3.65	1.71	2.83	0.31
	T	40:60 b, f	1.49	1.59	1.07	2.25	0.15
	TAG	40:60 b, f	2.18	3.95	1.81	3.50	0.35
2 (2′-MePhe) 	T	10:90 a, f	4.16	5.40	1.29	1.00	0.15
	TAG	10:90 a, f	2.36	3.08	1.31	1.07	0.16
	T	40:60 a, f	1.73	2.03	1.17	0.86	0.09
	TAG	40:60 a, f	3.12	4.06	1.30	2.13	0.15
	T	40:60 b, f	1.77	2.03	1.15	0.80	0.09
	TAG	40:60 b, f	3.07	4.02	1.31	1.84	0.16
3 (4′-MePhe) 	T	10:90 a, f	2.22	2.54	1.14	0.50	0.08
	TAG	10:90 a, f	3.27	5.11	1.55	1.75	0.26
	T	40:60 a, e	1.54	1.78	1.16	0.71	0.09
	TAG	40:60 a, e	2.75	3.93	1.43	1.24	0.13
	T	40:60 b, f	1.61	1.87	1.16	0.56	0.09
	TAG	40:60 b, f	2.97	4.49	1.51	2.45	0.23
4 (2′,4′-diMePhe) 	T	10:90 a, f	2.54	3.27	1.29	1.69	0.15
	TAG	10:90 a, f	4.14	4.74	1.14	0.71	0.08
	T	10:90 b, e	2.60	3.37	1.30	2.94	0.15
	TAG	10:90 b, e	4.05	4.76	1.17	0.89	0.07
	T	40:60 a, f	2.22	2.22	1.00	0.00	0.00
	TAG	40:60 a, f	4.18	4.63	1.10	0.75	0.06
	T	40:60 b, f	2.21	2.21	1.00	0.00	0.00
	TAG	40:60 b, f	4.44	4.70	1.06	0.40	0.03
5 (2′,6′-diMePhe) 	T	40:60 a, f	1.92	2.33	1.21	1.20	0.11
	TAG	40:60 a, f	2.95	3.86	1.31	2.00	0.16
	T	40:60 b, f	1.83	2.28	1.25	1.45	0.13
	TAG	40:60 b, f	2.78	4.02	1.45	2.29	0.21
6 (2′,4′,6′-triMePhe) 	T	40:60 a, f	2.66	4.50	1.69	3.43	0.30
	TAG	40:60 a, f	3.31	5.65	1.71	3.13	0.31
	T	40:60 b, f	1.92	3.55	1.85	2.09	0.36
	TAG	40:60 b, f	3.14	6.24	1.99	3.62	0.40
7 (3′-MeOPhe) 	T	40:60 a, f	1.60	2.18	1.36	1.71	0.18
	TAG	40:60 a, f	2.41	5.45	2.26	5.23	0.48
	T	40:60 b, f	1.54	2.13	1.38	1.67	0.19
	TAG	40:60 b, f	2.35	5.75	2.45	4.42	0.52
8 (2′-CNPhe) 	T	40:60 a, f	1.52	1.87	1.23	1.75	0.12
	TAG	40:60 a, f	2.46	3.79	1.54	3.18	0.25
	T	40:60 b, f	1.56	1.95	1.25	1.78	0.13
	TAG	40:60 b, f	2.55	4.06	1.59	2.72	0.27
9 (α-MePhe) 	T	0:100 f	2.89	2.89	1.00	0.00	0.00
	TAG	0:100 f	2.86	2.86	1.00	0.00	0.00
	T	100:0.1:0.1 c, e	2.08	2.08	1.00	0.00	0.00
	TAG	100:0.1:0.1 c, e	2.67	2.67	1.00	0.00	0.00
10 (α-Me-2′-MePhe) 	T	0:100 f	1.90	2.35	1.24	0.32	0.13
	TAG	0:100 f	2.85	3.75	1.32	0.82	0.16
	T	10:90 a, e	1.44	1.59	1.10	0.40	0.06
	TAG	10:90 a, e	2.36	2.76	1.17	0.91	0.09
	T	40:60 a, e	1.49	1.49	1.00	0.00	0.00
	TAG	40:60 a, e	2.27	2.58	1.14	0.67	0.08
	T	40:60 b, f	1.48	1.48	1.00	0.00	0.00
	TAG	40:60 b, f	2.32	2.62	1.13	0.67	0.07
11 (α-Me-3′-MeOPhe) 	T	0:100 f	2.58	2.82	1.09	0.40	0.05
	TAG	0:100 f	2.59	3.61	1.39	0.89	0.19
	T	10:90 a, e	1.87	1.90	1.07	0.40	0.04
	TAG	10:90 a, e	2.14	2.52	1.18	0.67	0.09
	T	10:90 b, e	1.78	1.92	1.08	0.40	0.04
	TAG	10:90 b, e	2.23	2.64	1.18	0.67	0.23

Table 1 (*Continued*)

Compound	CSP	Mobile phase (a, b, c)	k_S	k_R	α	R_S	$-\Delta(\Delta G^\circ)$ (kcal mol^{-1})
12 (α-Me-2′-CNPhe) 	T	0:100 f	1.17	1.85	1.58	2.86	0.27
	TAG	0:100 f	4.74	4.74	1.00	0.00	0.00
	T	10:90 a, f	1.00	1.26	1.26	1.40	0.13
	TAG	10:90 a, f	1.62	1.86	1.15	1.51	0.24
	T	40:60 a, f	1.12	1.12	1.00	0.00	0.00
	TAG	40:60 a, f	1.92	1.92	1.00	0.00	0.00
	T	40:60 b, e	0.51	0.63	1.23	1.00	0.12
	TAG	40:60 b, e	1.07	1.67	1.56	2.00	0.26
13 (Tyr) 	T	10:90 a, f	2.24	3.46	1.54	1.07	0.25
	TAG	10:90 a, f	3.02	5.66	1.87	1.78	0.36
	T	40:60 a, f	1.36	1.73	1.27	1.20	0.14
	TAG	40:60 a, f	4.79	7.77	1.58	2.62	0.27
	T	40:60 b, f	1.34	1.72	1.28	1.33	0.14
	TAG	40:60 b, f	1.97	3.38	1.72	2.00	0.32
14 (2′-MeTyr) 	T	10:90 a, f	3.42	4.63	1.35	1.78	0.17
	TAG	10:90 a, f	3.60	4.89	1.36	1.13	0.18
	T	40:60 a, f	1.46	1.75	1.20	0.86	0.11
	TAG	40:60 a, f	2.68	3.48	1.30	1.20	0.15
	T	40:60 b, f	1.49	1.80	1.21	0.83	0.11
	TAG	40:60 b, f	2.47	3.42	1.38	1.78	0.19
15 (2′,6′-diMeTyr) 	T	40:60 a, f	0.63	0.73	1.16	0.68	0.09
	TAG	40:60 a, f	2.62	3.51	1.34	1.40	0.17
	T	40:60 b, f	1.56	1.70	1.09	0.40	0.05
	TAG	40:60 b, f	2.36	3.18	1.35	1.67	0.17
	T	60:40 b, f	1.59	1.92	1.21	1.00	0.11
	TAG	60:40 b, f	2.51	3.51	1.40	1.07	0.20
	T	10:90 a, f	1.44	1.65	1.14	0.47	0.08
	TAG	10:90 a, f	3.21	4.00	1.25	0.83	0.13

Columns (CSPs), Chirobiotic T (T), Chirobiotic TAG (TAG); eluent, a: water–MeOH (v/v); b: 0.1% TEAA (pH 4.1)–MeOH (v/v), c: MeOH–AcOH–TEA (v/v/v); flow rate, d: 0.5 ml min^{-1}, e: 0.8 ml min^{-1} and f: 1.0 ml min^{-1}; detection, 205 nm.

containing Chirobiotic TAG column, 250×4.6 mm i.d., 5 μm particle size (Astec, Whippany, NJ, USA). The column was thermostated in a water bath, a cooling-heating thermostat (MK 70, Mechanik Prüfgeräte, Medlingen, Germany) being used. The precision of temperature adjustment was ±0.1 °C.

3. Results and discussion

3.1. Analyte selection

The analytes in this study (Tables 1 and 2) can be arranged into three classes. The classes A and B compounds are α-amino acids. The class A compounds are Phe analogs which differ in their substitution on the aromatic ring. This influences the hydrophobicity, bulkiness and rigidity of these molecules. The same holds true for the α-methyl- and ring-substituted analogs. α-Methyl substitution makes the molecules conformationally highly constrained. The compounds in class B consist of Tyr analogs, which differ from the Phe analogs by the presence of an –OH group on the aromatic ring. All class C molecules are monocyclic and bicyclic β-amino carboxylic acids, mostly with cycloalkane skeletons. However, a few analogs with cycloalkene skeletons (providing some π-character) are included (Table 2). All relevant separation data on these three classes of compounds are given in Tables 1 and 2. This includes the retention factors, separation factors, resolutions and enantioselective energy differences for each analyte for several mobile phases.

3.2. Mobile phase selection

All compounds in Tables 1 and 2 were evaluated with a minimum of five reversed-phase mobile phases plus a 100% MeOH mobile phase. One additional polar organic mobile phase (MeOH–AcOH–TEA) was evaluated, when the other mobile phases produced only partial resolution. To simplify the presentation, Tables 1 and 2 list only the chromatographic results obtained when enantiomeric separation was achieved on at least one CSP (in some cases, for purposes of comparison, results obtained at the same eluent composition, but not leading to separation, are also included).

3.3. Solute retention and stationary phase polarity

As regards the nature of the bonded chiral selectors on the silica surface, it is apparent that different functional

Table 2
Chromatographic results obtained at 20 °C on the two chiral stationary phases, Chirobiotic T and TAG, for the β-amino acids containing cycloalkane or cycloalkene skeletons

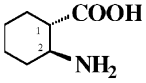
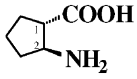
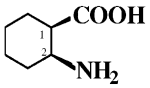
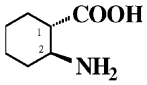
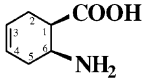
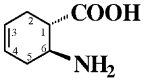
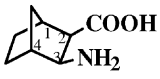
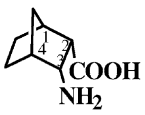
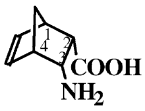
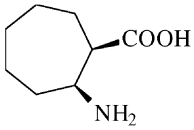
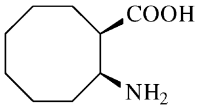
Compound	CSP	Mobile phase a, b, c	k_S	k_R	α	R_S	$-\Delta(\Delta G^\circ)$ (kcal mol^{-1})
16 (*cis*-Acpc) 	T	0:100 f	6.37	6.87	1.08	0.40	0.04
	TAG	0:100 f	10.89	12.34	1.13	0.50	0.07
	T	30:70 a, e	1.84	1.84	1.00	0.00	0.00
	TAG	30:70 a, e	4.34	4.69	1.08	0.56	0.03
	T	30:70 b, e	1.95	1.95	1.00	0.00	0.00
	TAG	30:70 b, e	2.86	3.27	1.14	0.77	0.08
	T	100:0.1:0.1 c, e	3.52	4.25	1.21	0.93	0.04
	TAG	100:0.1:0.1 c, e	8.77	8.77	1.00	0.00	0.00
17 (*trans*-Acpc) 	T	0:100 f	6.70	6.70	1.00	0.00	0.00
	TAG	0:100 f	18.38	18.38	1.00	0.00	0.00
	T	30:70 a, d	2.21	2.21	1.00	0.00	0.00
	TAG	30:70 a, d	3.99	4.68	1.17	0.75	0.09
	T	30:70 b, e	2.20	2.20	1.00	0.00	0.00
	TAG	30:70 b, e	1.81	2.46	1.36	2.40	0.17
18 (*cis*-Achc) 	T	0:100 f	3.19	3.49	1.07	0.80	0.04
	TAG	0:100 f	9.93	11.33	1.14	0.59	0.08
	T	30:70 a, d	1.77	1.77	1.00	0.00	0.00
	TAG	30:70 a, d	4.04	4.04	1.00	0.00	0.00
	T	30:70 b, e	1.95	1.95	1.00	0.00	0.00
	TAG	30:70 b, e	2.29	2.58	1.13	0.82	0.07
	T	100:0.1:0.1 c, e	3.18	3.42	1.08	0.63	0.05
	TAG	100:0.1:0.1 c, e	5.22	6.45	1.24	0.87	0.13
19 (*trans*-Achc) 	T	0:100 f	4.64	5.34	1.15	0.56	0.08
	TAG	0:100 f	10.38	11.99	1.16	0.60	0.08
	T	30:70 a, d	1.88	1.88	1.00	0.00	0.00
	TAG	30:70 a, d	3.38	4.49	1.33	1.68	0.17
	T	30:70 b, e	1.98	1.98	1.00	0.00	0.00
	TAG	30:70 b, e	2.06	2.98	1.45	2.10	0.22
20 (*cis*-Achc-ene) 	T	0:100 f	4.78	5.40	1.13	0.56	0.07
	TAG	0:100 f	10.48	12.80	1.22	0.72	0.12
	T	30:70 a, d	1.86	1.86	1.00	0.00	0.00
	TAG	30:70 a, d	3.46	4.39	1.27	1.68	0.14
	T	30:70 b, e	1.98	2.10	1.06	0.75	0.03
	TAG	30:70 b, e	2.44	3.21	1.32	2.00	0.16
21 (*trans*-Achc-ene) 	T	0:100 f	5.81	5.81	1.00	0.00	0.00
	TAG	0:100 f	12.38	12.38	1.00	0.00	0.00
	T	30:70 a, d	1.90	1.90	1.00	0.00	0.00
	TAG	30:70 a, d	3.86	4.92	1.27	1.83	0.14
	T	30:70 b, e	1.94	2.02	1.04	0.89	0.02
	TAG	30:70 b, e	2.80	3.79	1.35	2.13	0.17
22 (*diexo*-Abhc)[a] 	T	0:100 f	3.14	3.32	1.06	0.55	0.03
	TAG	0:100 f	9.19	10.01	1.09	0.64	0.05
	T	30:70 a, d	1.92	1.92	1.00	0.00	0.00
	TAG	30:70 a, d	3.76	3.76	1.00	0.00	0.00
	T	30:70 b, e	1.91	1.91	1.00	0.00	0.00
	TAG	30:70 b, e	2.48	2.48	1.00	0.00	0.00
	T	100:0.1:0.1 c, e	2.70	2.94	1.09	0.71	0.05
	TAG	100:0.1:0.1 c, e	4.87	5.49	1.13	0.75	0.07
23 (*diendo*-Abhc) 	T	0:100 f	2.34	2.55	1.09	0.60	0.05
	TAG	0:100 f	6.76	7.93	1.17	0.86	0.09
	T	30:70 a, d	1.73	1.73	1.00	0.00	0.00
	TAG	30:70 a, d	2.63	3.10	1.18	0.84	0.10
	T	30:70 b, e	1.68	1.68	1.00	0.00	0.00
	TAG	30:70 b, e	1.70	2.10	1.23	1.33	0.12

Table 2 (*Continued*)

Compound	CSP	Mobile phase a, b, c	k_S	k_R	α	R_S	$-\Delta(\Delta G^\circ)$ (kcal mol^{-1})
24 (*diexo*-Abhc-ene)[a] 	T	0:100 f	3.09	5.75	1.21	1.10	0.11
	TAG	0:100 f	8.30	10.31	1.24	0.67	0.13
	T	30:70 a, d	1.78	1.89	1.06	0.40	0.03
	TAG	30:70 a, d	3.62	3.84	1.07	0.40	0.04
	T	30:70 b, e	1.81	1.93	1.07	0.80	0.04
	TAG	30:70 b, e	2.57	2.80	1.09	0.40	0.05
	T	100:0.1:0.1 c, e	3.67	4.24	1.16	0.80	0.09
	TAG	100:0.1:0.1 c, e	6.21	7.12	1.15	0.69	0.08
25 (*diendo*-Abhc-ene)[b] 	T	0:100 f	3.19	5.80	1.82	3.70	0.35
	TAG	0:100 f	8.71	17.09	1.96	3.05	0.39
	T	30:70 a, d	1.75	1.75	1.00	0.00	0.00
	TAG	30:70 a, d	3.07	3.07	1.00	0.00	0.00
	T	30:70 b, e	1.81	2.55	1.41	3.00	0.20
	TAG	30:70 b, e	1.98	4.97	2.51	5.88	0.54
26 (*cis*-Ach7c) 	T	0:100 f	5.38	5.54	1.03	0.40	0.02
	TAG	0:100 f	7.98	9.38	1.18	0.73	0.10
	T	30:70 a, d	1.80	2.60	1.44	1.68	0.21
	TAG	30:70 a, d	4.56	4.56	1.00	0.00	0.00
	T	30:70 b, e	3.24	3.24	1.00	0.00	0.00
	TAG	30:70 b, e	3.94	3.94	1.00	0.00	0.00
27 (*cis*-Acoc) 	T	0:100 f	3.07	3.49	1.14	0.57	0.08
	TAG	0:100 f	6.47	8.54	1.32	1.03	0.16
	T	30:70 a, d	1.86	2.22	1.19	0.76	0.10
	TAG	30:70 a, d	3.71	4.59	1.24	1.00	0.13
	T	30:70 b, e	2.02	2.02	1.00	0.00	0.00
	TAG	30:70 b, e	3.45	4.08	1.18	1.00	0.10

Column (CSP), Chirobiotic T (T) and Chirobiotic TAG (TAG); eluent, a: water–MeOH (v/v), b: 0.1% TEAA (pH 4.1)–MeOH (v/v), c: MeOH–AcOH–TEA (v/v/v); flow rate, d: 0.5 ml min^{-1}, e: 0.8 ml min^{-1} and f: 1.0 ml min^{-1}; detection, 205 nm.

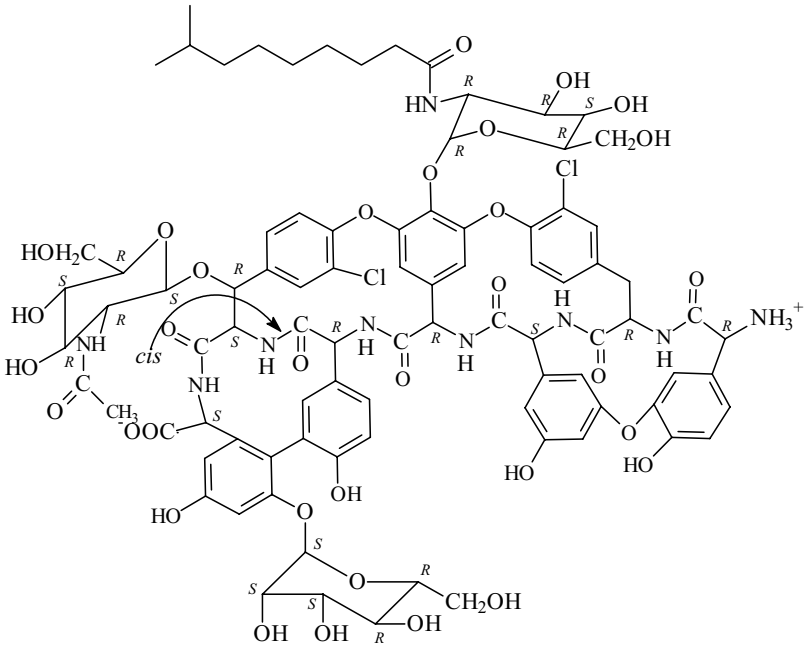
[a] Reverse elution sequence, (*R*) < (*S*).

[b] Elution sequence has yet not been assigned.

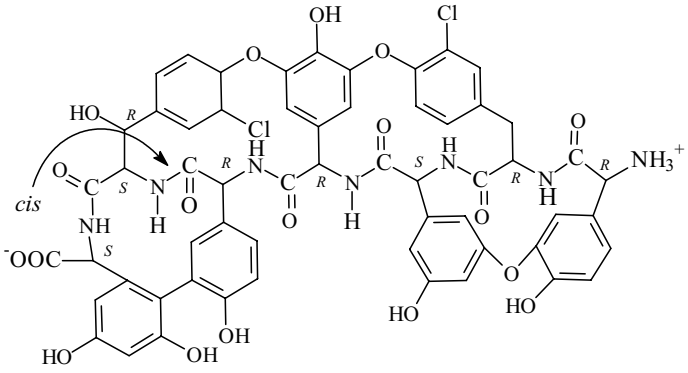
groups are present in the two CSPs. The linkage between the silica surface and the antibiotic involves nine apolar methylene units and either a ureido or a carbamate linkage [41]. The polar groups of the free teicoplanin antibiotic are 14 hydroxy groups (of which four are phenolic groups), one free amino group, and one free carboxylic group. Its apolar groups are the nine methylene units of its sugar alkyl chain, the row of six amide linkages in the macrocyclic portion of the molecule and seven benzene rings attached to it (Fig. 1A). For the aglycone molecule, the basket itself has only seven polar hydroxy groups (of which six are phenols), and it lacks the apolar alkyl chain connected to the sugar. It has the free amino and carboxylic acid groups, the apolar row of six amide linkages, and the seven aromatic rings (Fig. 1B). However, it is difficult to evaluate the polarities of these stationary phases by considering simply their molecular structures. The retention factors of the different test molecules should give a better idea of the relative polarities of these stationary phases.

With the same reversed-phase and polar organic mobile phase, the retention factors of the first-eluted enantiomers of the α- and β-amino acids are lower on the teicoplanin phase than on the aglycone phase [with exception for analyte **2** at a mobile phase composition of water–MeOH (10:90, v/v) and for analyte **17** at a mobile phase composition of 0.1% TEAA–MeOH (30:70, v/v)] (Tables 1 and 2). 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. On a CSP containing an A-40,926 glycopeptide (differing from teicoplanin mainly in the lack of the β-D-*N*-acetylglucosamine), D'Acquarica et al. [14] observed somewhat higher retention factors for alicyclic- and cyclic-β-amino acids than on teicoplanin CSP. Since the retention factors for the first-eluted components differed considerably on the two phases in this 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. This will be discussed in the section of this paper dealing with enantioselectivity.

The retention factors for the first-eluted enantiomers on the same stationary phase and at the same mobile-phase



(A) **Teicoplanin (Chirobiotic T)**



(B) **Teicoplanin aglycone (Chirobiotic TAG)**

Fig. 1. Structures of the macrocyclic antibiotic chiral stationary phases: (A) teicoplanin complex; (B) teicoplanin aglycone.

composition but with different pH (mobile phases with water or with 0.1% TEAA pH 4.1) are relatively similar for the whole set of compounds at different pHs. It seems that the degree of ionization of the carboxyl and amino groups above pH 4.1 were similar at the pHs tested.

Fig. 2A and B show the retention factors of two compounds versus the mobile-phase composition. When the MeOH content of the mobile phase was increased for α-Me-2′-MePhe (**10**), a U-shaped curve was observed, while for *diendo*-Abhc-ene (**25**) there was an increase in the retention factor. In both cases, at higher MeOH concentration, the retention factor increased with increasing MeOH content; this was due to the reduced solubility of the amino acids in the MeOH-rich mobile phase. This behavior has been observed on other stationary phases [42]. The increase in the retention factor with increasing water content was due to enhanced hydrophobic interactions in the water-rich mobile phase. In this study, as for most amino acids, a U-shaped curve was observed, but the inflection point and the slope of the curve at higher MeOH concentrations differed somewhat for each compound.

3.4. Enantioselectivity of enantiomers of amino acids on antibiotic phases

Tables 1 and 2 list the separation factors (α) and resolution (R_S) of the stereoisomers of unusual α- and β-amino acids. The highest separation factors obtained on the teicoplanin CSP were $\alpha = 1.85$ and 1.58 for 2′,4′,6′-triMePhe (**6**) and α-Me-2′-CNPhe (**12**), respectively. The highest separation factors obtained on the aglycone CSP were $\alpha = 2.45$ and 2.51 for 3′-MeOPhe (**7**) and *diendo*-Abhc-ene (**25**), respectively. The α-values for the conformationally constrained α-amino acids (Table 1) were lower than those reported for

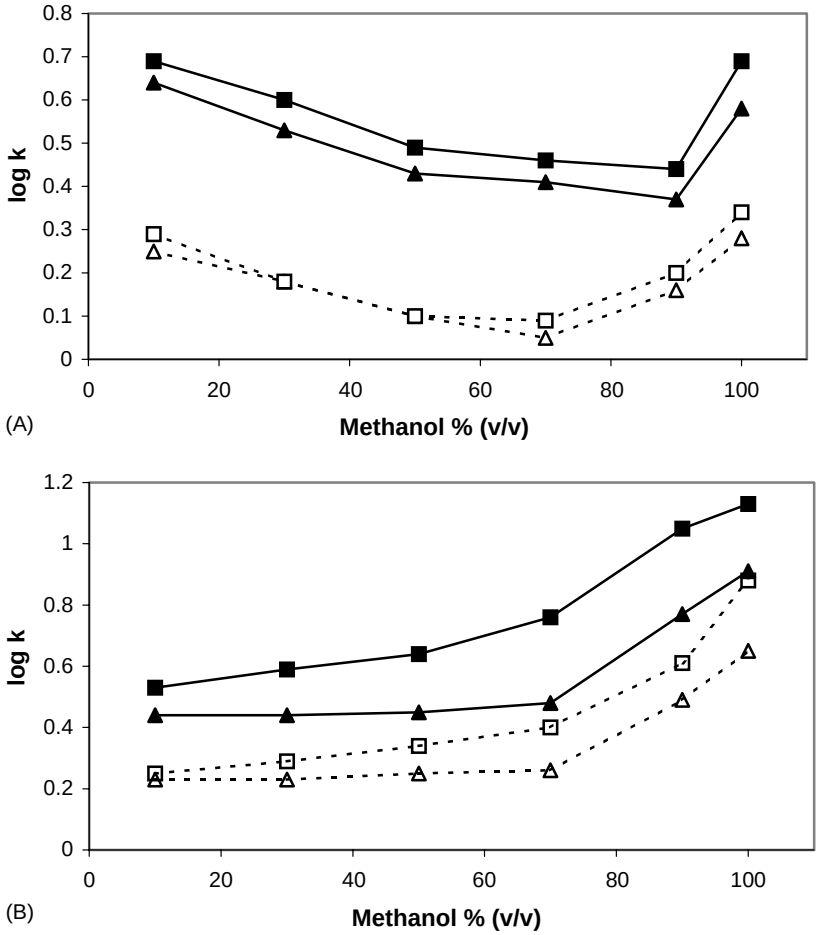


Fig. 2. Retention factors (k) vs. MeOH content of the mobile phase on Chirobiotic T and Chirobiotic TAG columns. (A) α-Methyl-2′-methylphenylalanine (**10**); (B) *diendo*-3-aminobicyclo[2.2.1]hept-5-ene-2-carboxylic acid (**25**); chromatographic conditions: columns, Chirobiotic T and Chirobiotic TAG; mobile phase: (A) water–MeOH (v/v), (B) 0.1% aqueous TEAA (pH 4.1)–MeOH (v/v); flow rate, 0.8 ml min^{-1}; detection, 205 nm; (△) k_1 for teicoplanin; (□) k_2 for teicoplanin; (▲) k_1 for aglycone; (■) k_2 for aglycone.

the common proteinogenic and other α-amino acids having simple structures [9]. The β-amino acid *cis*-Achc (**18**) in this study had a higher α value. The highest α values observed correspond to a difference in enantioselective free energy in the 0.5 kcal mol^{-1} range, which is indicative of the good enantiorecognition capability of these chiral selectors. Tables 1 and 2 show that the resolution factors associated with these separation factors can be as high as 5.9. These stationary phases are sometimes so selective in resolving these particular enantiomers that it may take several minutes after the first enantiomer appears for the peak of the second enantiomer to be seen. Fig. 3A and B show the chromatograms for 3′-MeOPhe (**7**) on the two CSPs with the 0.1% aqueous TEAA–MeOH (30:70, v/v) buffered mobile-phase system. The 1.2 min span separated the first eluting (*S*)-3′-MeOPhe from the (*R*) enantiomer on the teicoplanin CSP. This time difference increased to 6.0 min for these enantiomers on the aglycone CSP. A change in the mobile-phase composition or the use of buffer in the mobile phase produced relatively small variations in separation factors and enantioselective free energies for the amino acids in this study (Tables 1 and 2).

3.5. The role of carbohydrate units in enantiorecognition

The carbohydrate units are themselves chiral, which can help in the enantiorecognition process. 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, $\Delta_{\text{TAG-T}}\Delta(\Delta G^\circ)$, listed in Tables 1 and 2, were used ($-\Delta(\Delta G^\circ) = RT\ln\alpha$). The $\Delta(\Delta G^\circ)$ values obtained for a given compound were summed for every mobile phase, and the rounded average values for the compounds $\{[\Sigma(\Delta(\Delta G^\circ_{\text{aglycone}})) - \Sigma(\Delta(\Delta G^\circ_{\text{teicoplanin}}))]/$ number of mobile phases$\}$ were plotted as shown in Fig. 4. A negative number means that the stereoisomers are better separated on the aglycone CSP. A positive number means

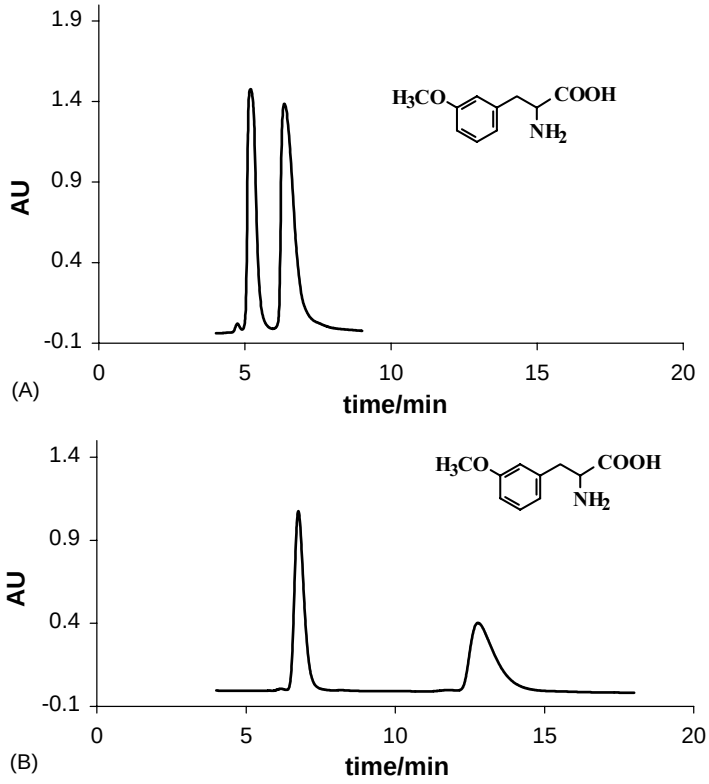


Fig. 3. Chromatograms of 2-amino-3-(3-methoxyphenyl)propionic acid (**7**) on Chirobiotic T (A) and on Chirobiotic TAG (B) columns. Chromatographic conditions: column, (A) Chirobiotic T, (B) Chirobiotic TAG; mobile phase, 0.1% aqueous TEAA (pH 4.1)–MeOH (30:70, v/v); flow rate, 0.8 ml min^{-1}; detection, 205 nm.

that the stereoisomers are better separated on the native teicoplanin CSP, which contains the carbohydrate units.

As can be seen in Fig. 4, the amino acid enantiomers are much better resolved by the aglycone CSP than by the native teicoplanin CSP. Fig. 3 depicts the enantioseparation of 3′-MeOPhe (**7**) on the two CSPs. The 0.32 kcal mol^{-1} average energy difference corresponds to almost a two times higher α value on the aglycone CSP as compared to the teicoplanin CSP. This energy difference means that the sugar units decrease the amino acid enantiorecognition. It also indicates that the aglycone basket of the teicoplanin molecule is solely responsible for the enantiorecognition of the common amino acids. The aglycone is not soluble in water; the sugars make it soluble and dispersible in biological systems (which is necessary if it is to be an effective antibiotic). The sugar units may be present to decrease the affinity for amino acids other than D-Ala, since the teicoplanin target is the D-Ala-D-Ala peptide termination in the Gram(+) bacterial cell wall [43]. The role of sugar units was more pronounced in the case of *trans*-Achc-ene (**21**) (Fig. 5). With the 0.1% aqueous TEAA–MeOH (30:70, v/v) buffered mobile phase system the selectivity and resolutions on the native teicoplanin were 1.04 and 0.89, respectively, and on the aglycone stationary phase were 1.35 and 2.13, respectively. From the chiral separation point of view, the sugar moieties of the native teicoplanin may intervene in the chiral recognition process in at least three ways [9]: (i) steric hindrance, where the sugar units occupy space inside the "basket", which limits the access of other molecules to binding sites; (ii) the blocking of possible interaction sites on the aglycone, where two sugars are linked through phenol hydroxy groups and the third sugar is linked through an alcohol moiety (Fig. 1); and (iii) the offer of competing interaction sites, where the three sugars are themselves chiral and have hydroxy, ether and amido functional groups.

The free energy difference between the two related CSPs may be due to the effect of steric hindrance, but other possibilities should be considered as well. The conformationally constrained amino acids **2**–**5** exhibited lower free energy differences as compared to Phe (**1**). Despite the conformational constraints, methoxy and cyano group substitution (**7** and **8**) promoted enantiorecognition, probably due to the possibility of hydrogen donor–acceptor effects. As compared to the highly sterically constrained α-methyl-substituted analogs **9**–**12**, α-MePhe (**9**) did not exhibit any enantiorecognition, whereas the aryl-substituted analogs **10**–**12** did exhibit some. Especially good enantiorecognition could be observed for compound **12**, probably because of the hydrogen bonding effect.

It was previously observed that the hydroxy groups on the aromatic ring of 3,4-dihydroxyphenylalanine (DOPA) enhanced its chiral recognition relative to that of Phe on the aglycone CSP [9]. This was thought to be due to the presence of an additional interaction with a hydroxy group of

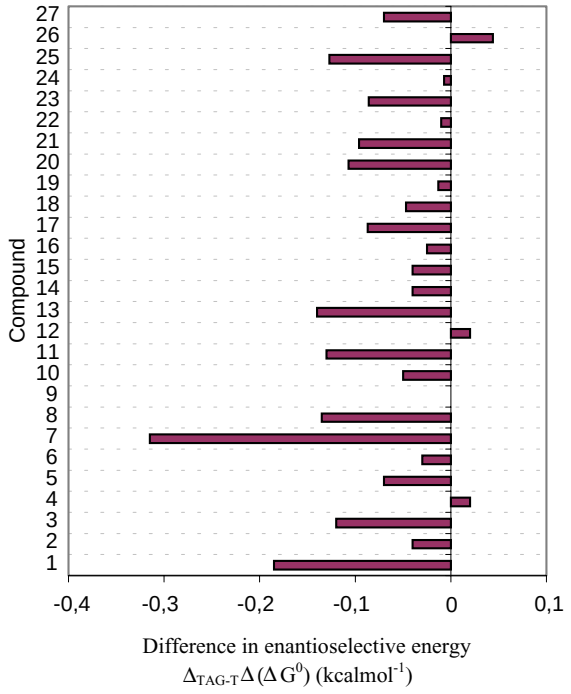


Fig. 4. Average enantioselectivity differences, $\Delta_{TAG-T}\Delta(\Delta G^{\circ})$, between the aglycone and native teicoplanin stationary phases. A negative value corresponds to better enantioresolution by the aglycone column. A positive value corresponds to better enantioresolution by the teicoplanin column. For compounds, see Tables 1 and 2.

the aglycone that is occupied by an attached sugar moiety in teicoplanin. In this study, if we compare the Tyr analogs **13**–**15** with the appropriate Phe analogs **1**, **2** and **5**, the one hydroxy group of the Tyr analogs has no or only a limited effect on enantiorecognition. In general, it seems that the steric hindrance effect of the sugar moieties was predominant for 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 enantioreaction and better enantioselectivity. Besides steric hindrance, the two phenols and the hydroxy group on the aglycone seem to further enhance the interaction with the amino acids.

For β-amino acids with cycloalkane or cycloalkene skeletons **16**–**27**, the steric arrangement of the molecule had a great influence on the enantioselectivity and resolution. The *trans* and *diendo* isomers exhibited higher enantioselectivity than the *cis* or *diexo* isomers. For the *trans* and *diendo* isomers with higher separation factors (α values), higher resolutions could generally be observed. Among β-amino acids with cycloalkane skeletons, *cis*-Ach7c (**26**) was the only compound that exhibited higher enantioselectivity and resolution on the native teicoplanin stationary phase as

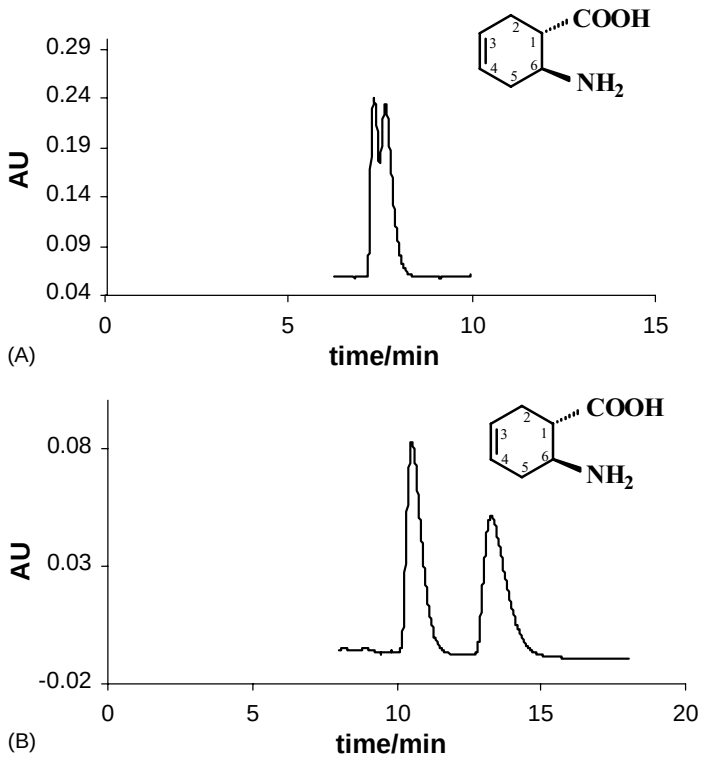


Fig. 5. Chromatograms of *trans*-6-aminocyclohex-3-ene-1-carboxylic acids (**21**) on Chirobiotic T (A) and Chirobiotic TAG (B) columns. Chromatographic conditions: column, (A) Chirobiotic T, (B) Chirobiotic TAG; mobile phase, 0.1% aqueous TEAA (pH 4.1)–MeOH (30:70, v/v); flow rate, 0.8 ml min^{-1}; detection, 205 nm.

compared to the aglycone phase. Of the 15 α-amino acids, 2′,4′-diMePhe (**4**) and α-Me-2′-CNPhe (**12**) were the only analytes that had a positive value for the difference in their relative enantioselective energies (Fig. 4), i.e. better α and R_S values were obtained on the native teicoplanin phase.

It is well known that a small change in a molecule can cause a large change in its enantiorecognition and resolution by a CSP [42]. This effect was observed among Phe analogs **1**–**6**, α-methyl-substituted Phe analogs **9**–**12** and Tyr analogs **13**–**15**. The steric hindrance or conformational constraints may contribute to or inhibit the enantioselectivity or resolution, while the possibility of hydrogen bonding may result in an increased enantioselectivity. For β-amino acids, on both CSPs, an increase in the ring number of a molecule makes it more bulky, and these larger analogs tend to separate better [Acpc (**16**, **17**) versus Achc (**18**, **19**)], but the improvement in enantioselectivity for even higher ring numbers **22**, **23**, **26** and **27** diminished. At the same time, the unsaturated analogs of β-amino acids **20**, **21** and **24**, **25** exhibited better enantioselectivity and resolution than the saturated ones **18**, **19** and **22**, **23**, respectively. This may be explained by the π–π interactions, which are weak in polar-aqueous solvents and are more pronounced on the aglycone phase than on the native teicoplanin phase.

3.6. Kinetic effects

In our earlier work on macrocyclic antibiotic stationary phases [9,31], a significant difference in efficiency was observed between the first-eluted enantiomer and the second. In the present study, e.g. for *trans*-Achc-ene (**21**) separated with a 0.1% aqueous TEAA–MeOH (30:70, v/v) mobile phase (Fig. 5), the efficiency on the teicoplanin CSP was 2800 plates for the first-eluted enantiomer and 2100 plates for the second-eluted enantiomer, a 25% drop. This trend was similar on the aglycone CSP, with 1700 and 1350 plates for the first- and second-eluted enantiomers, respectively, a 20% drop. For the more polar 3′-MeOPhe (**7**), a more pronounced decrease in efficiency was observed (Fig. 3). At 0.1% aqueous TEAA–MeOH (30:70, v/v) eluent composition, the efficiency on the teicoplanin CSP was 1300 and 900 plates for the first- and second-enantiomers, a 30% drop. The theoretical plate numbers on the aglycone stationary phase were 2200 and 1050, respectively, a 52% drop. In all cases in this investigation, according to the observed plate numbers, the mass transfer of the more-retained enantiomer was much slower than that of the first-eluted enantiomer. The interaction between the second-eluted stereoisomer and the CSP is thermodynamically strong, producing higher retention times and slow adsorption–desorption kinetics, which resulted in poor mass transfer.

3.7. Elution sequence on macrocyclic antibiotic phases

Biological investigations [43] suggest elution sequence the L < D [(*S*) < (*R*)]. In the present work, this held true for the α-amino acids, Phe and Tyr analogs **1**–**15**, having one stereogenic center. For β-amino acids with two stereogenic centers, the elution sequence (*S*) < (*R*) was observed in most cases, where the elution sequence was determined by the configuration about the carbon atom adjacent to the carboxyl group. In the cases of *diexo*-Abhc (**22**) and *diexo*-Abhc-ene (**24**) on both the native teicoplanin and teicoplanin aglycone CSPs, the component eluted second was that which had the (*S*) configuration at the carbon atom adjacent to the carboxyl group. These findings indicate the importance of identifying the elution sequence, especially for β-amino acids or for α-amino acids with two stereogenic centers (the elution sequence of *diendo*-Abhc-ene (**25**) was not determined).

4. Conclusions

It was found that the carbohydrate moieties on teicoplanin are not needed for the enantioresolution of unusual α- and β-amino acids. The cleft near the amine end (or ureido group, if attached to a linkage chain) of the aglycone basket is an important part of the receptor site for amino acid chiral recognition. Amino acids appear to have easier access to this site on the aglycone CSP, which produces much higher enantioselectivities and resolution factors for these compounds as compared to those attained on the native teicoplanin CSP. It is clearly established that in most cases the aglycone is responsible for the enantioseparation of amino acids. The difference in enantioselective free energy between the aglycone CSP and the teicoplanin CSP was between 0.02 and 0.3 kcal mol^{-1} for these unusual amino acid separations (1 cal = 4.184 J). Although the sugar units decrease the resolution of most amino acid enantiomers, they can contribute significantly to the resolution of some unusual amino acid analogs. By application of these two CSPs, excellent resolutions were achieved for most of the investigated compounds in the reversed-phase mode or polar organic mobile phase systems. The elution sequence of the stereoisomers of unusual amino acids was determined and in most cases was found to be L < D [(*S*) < (*R*)], according to the configuration about the carbon atom adjacent to the carboxyl group [only exceptions were some β-amino acids with two stereogenic centers, *diexo*-Abhc (**22**) and *diexo*-Abhc-ene (**24**), where a reversal in the elution sequence (*R*) < (*S*) could be observed].

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