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High-performance liquid chromatographic separation of enantiomers of synthetic amino acids on a ristocetin A chiral stationary phase

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Abstract

A macrocyclic glycopeptide, ristocetin A, was used as chiral stationary phase for the high-performance liquid chromatographic separation of enantiomers of 28 unnatural amino acids, such as analogues of phenylalanine, tyrosine and tryptophan, and analogues containing 1,2,3,4-tetrahydroisoquinoline, tetraline or 1,2,3,4-tetrahydro-2-carboline skeletons. Excellent resolutions were achieved for most of the investigated compounds by using reversed-phase or a new polar-organic mobile phase system. The conditions of separation were optimized by variation of the mobile phase composition, temperature and flow-rate.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Amino acids; Ristocetin A

1. Introduction

Determination of the biologically active conformation of peptide hormones is an important goal in modern biology. Since most peptide hormones are highly flexible molecules with numerous possible conformations under physiological conditions, one highly useful approach involves the introduction of conformational constraints [1]. 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. The incorporation of conformational constraints into biologically active peptides derived from α-amino acids is a well-known approach for enhancement of their receptor selectivity and modulation of their efficacy. Several unusual α-amino acids have recently been designed with a view to constraining the side-chain functional groups of natural α-amino acids [1].

These new α-amino acids are produced synthetically. The syntheses lead either to a mixture of stereoisomers or, via asymmetric synthesis strategies, to enantiomerically enriched products, whose enantiomeric purity rarely approaches 100%. Such stereochemically and/or enantiomerically impure components should not be incorporated into peptides since this will lead to a mixture of compounds with different biological properties. It is therefore very important to have available enantiomerically pure and defined substances and analytical methods for

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the separation and identification of the different stereoisomers.

For this purpose, chromatographic methods are widely used. Successful high-performance liquid chromatographic (HPLC) methods for the resolution of amino acids include indirect and direct methods. Indirect methods involve pre-column derivatization reaction with chiral reagents, with subsequent separation of the diastereoisomers on an achiral column [2,3]. Direct methods are performed by ligand-exchange chromatography [4–7], or by application of other chiral stationary phases (CSPs) [8–10]. Through the application of CSPs, the occurrence of racemization during the derivatization can be avoided. Macrocyclic antibiotics are a recent class of CSPs that have been used successfully to resolve enantiomers in liquid chromatography [11–19], capillary electrophoresis [20–34], thin-layer chromatography [35], supercritical fluid chromatography [36,37] and capillary electrochromatography [38,39].

In the present paper, a direct HPLC method is described for the separation of enantiomers of 28 unnatural amino acids, most of which are sterically constrained. The CSP containing the macrocyclic glycopeptide, ristocetin A, was used in a reversed-phase mode (RPM) or a new polar-organic mode (POM). The effects of mobile phase composition, temperature and flow-rate on the separation were investigated, and the conditions affording the best resolution were determined. The sequence of elution of the enantiomers was in most cases determined by spiking of racemic samples with enantiomers with known absolute configuration.

2. Experimental

2.1. Chemicals and reagents

With the exceptions of **7**, *m*-tyrosine (*m*-Tyr) and **11**, α-methyltyrosine (α-MeTyr), which were purchased from Aldrich (Steinheim, Germany), the amino acids were synthesized in our laboratories by literature methods. The names of the investigated compounds, nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [40]. The synthesis of **1**, 2′-methylphenylalanine (2′-MePhe) [41], **2**, 4′-methylphenylalanine (4′-MePhe) [42], **3**, 2′,6′-dimethylphenylalanine (2′,6′-diMePhe) [41], **4**, α-methylphenylalanine (α-MePhe) [43], **8**, 2′-methyltyrosine (2′-MeTyr) [41], **15**, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic3) [44], **16**, 6′-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (6′-HO-Tic3) [44], **17**, 7′-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (7′-HO-Tic3) [44], **18**, 5′-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (5′-MeTic3) [41], **19**, 3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (α-MeTic3) [44], and **27**, 1,2,3,4-tetrahydro-3-carboxy-2-carboline (Tcc) [44] led to one enantiomer of the substrate, depending on the configuration of the starting material, whereas **5**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*erythro*-β-MePhe) [45], **6**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methylphenylalanine (*threo*-β-MePhe) [45], **9**, 2′,6′-dimethyltyrosine (2′,6′-diMeTyr) [46], **10**, 2′,6′-dimethyltyrosineamide (2′,6′-diMeTyrNH_2) [47], **12**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine (*erythro*-β-MeTyr) [45], **13**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltyrosine (*threo*-β-MeTyr) [45], **14**, 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic1) [48], **20**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic3) [44], **21**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*threo*-β-MeTic3) [44], **22**, 2-aminotetraline-2-carboxylic acid (Atc) [49], **23**, 6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat) [50], **24**, 6-methoxy-2-aminotetraline-2-carboxylic acid (MeOAtc) [50], **25**, *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltryptophan (*erythro*-β-MeTrp) [45], **26**, *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltryptophan (*threo*-β-MeTrp) [45], and **28**, 1,2,3,4-tetrahydronorharmane-1-carboxylic acid (norharmane) [51] were obtained as racemates. Methanol (MeOH) and acetonitrile (MeCN) were obtained from Merck (Darmstadt, Germany); both were of HPLC grade. Triethylamine (TEA), glacial acetic acid (HOAc) and other reagents of analytical-reagent grade were also obtained from Merck. The inorganic component of the mobile phase used in the RPM was prepared from Milli-Q water, which was further purified by filtering on a 0.45 μm filter, type HV, Millipore (Molsheim, France).

2.2. Apparatus

Two HPLC systems were used. The Waters chromatographic system consisted of an M-600 low-pressure gradient pump, equipped with an M-996 photodiode-array detector and a Millennium 32 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA). The system working under isocratic conditions included an L-6000 Merck–Hitachi pump (Tokyo, Japan) with a Shimadzu SPD-6AV variable-wavelength UV–Vis detector. For data processing, a Hewlett-Packard HP 3395 integrator (Waldbronn, Germany) was applied. The Model 7125 injectors with a 20-μl loop were from Rheodyne (Cotati, CA, USA).

The column used was a Chirobiotic R CSP (bonded with ristocetin A), 250×4.6 mm I.D., 5 μm particle size (Astec, Whippany, NJ, USA). The column was thermostated with a water-bath. The temperature was regulated and controlled by a heating-cooling circulator system, type MK-70 (Mechanik Prüfgeräte, Medlingen, Germany).

2.3. Methods

All the analyzed compounds were free (underivatized) unusual amino acids. Solutions of 1 mg/ml were prepared by dissolving the analytes in methanol, or in the mobile phase.

The RP mobile phases were prepared by mixing Milli-Q water with MeOH (by volume). The PO mobile phases were prepared in a similar way by mixing MeOH, HOAc and TEA (by volume). The eluents were degassed in an ultrasonic bath, and during the analysis helium gas was sparged through the solution.

3. Results and discussion

The synthetic amino acids were analyzed on the ristocetin A-containing stationary phase working in RPM or POM. All the analytes were chromatographed and detected without pre- or post-column derivatization. Ristocetin A is one of the more complex molecules evaluated as a CSP chiral selector [16]. Ristocetin A has 38 chiral centres, seven aromatic rings surrounding four pockets or cavities, six amide linkages, 21 hydroxyl groups, two primary amino groups, one methyl ester group, a pendant tetrasaccharide and two monosaccharide moieties. These saccharides include D-arabinose, D-mannose, D-glucose and D-rhamnose. The ionizable primary amine groups control their charge and are thought to play a role in their association with and chiral recognition of chiral analytes. The only other ionizable groups are the phenolic moieties. At operational conditions these are generally protonated and probably serve mainly as hydrogen-bonding sites [16].

3.1. Retention and enantioselectivity in RPM

The separations were carried out with an unbuffered RP mobile phase containing water–MeOH. For comparison purposes, the results are given for one particular eluent composition (water–MeOH, 80:20, v/v), but in some cases data for various other eluent compositions are provided (Table 1).

The retention and selectivity can be controlled by altering the concentration and nature of the organic modifier, but variation of the flow-rate or temperature sometimes has a beneficial effect on the resolution. Change of the water–MeOH ratio affects the retention factors. Increase of the MeOH content in the mobile phase led to a decrease in the retention factor (Table 1), but this decrease was not so pronounced as in the case of RP C_{18} phases. 10–20% (v/v) changes in MeOH content resulted in changes of only a few percent in the retention factors, which was far from the expected 2–3-fold changes. On decrease of the MeOH content, the selectivity factor (α) and the resolution (R_s) of two enantiomers generally increased in parallel with the retention factor (k). This was true, for instance for the tetraline analogues (**22**–**24**), but the β-methyl-substituted α-amino acids proved to be exceptions. The α and R_s values for these compounds were higher in the MeOH-rich mobile phase, in spite of the smaller retention factors. Especially high R_s values were obtained for β-MeTic3 (**20**, **21**).

It seems that the polarity of analyte is one of the factors governing retention. At the same eluent composition, the more hydrophobic 2′,6′-diMePhe (**3**) has a higher retention factor than that of 2′-MePhe (**1**), 2′,6′-diMeTyr (**9**) has a higher value than that of 2′-MeTyr (**8**), and the Phe analogues

Table 1
Retention factors (k), separation factors (α) and resolutions (R_s) for the separation of enantiomers of unusual α-amino acids on ristocetin A-bonded stationary phase in RPM[1]

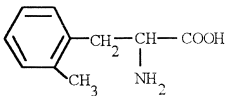
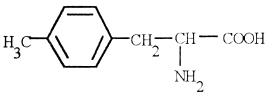
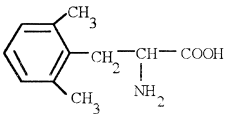
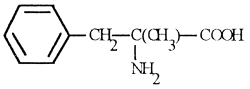
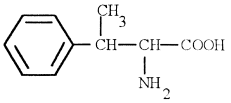
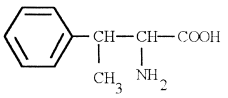
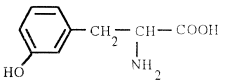
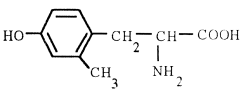
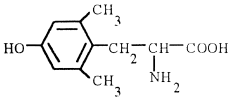
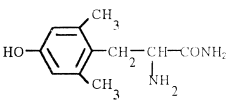
Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, water–MeOH (%, v/v)	Flow-rate (ml/min) and temperature[a] (°C)
Phenylalanine analogues							
1. 2′-MePhe							
	1.30	1.80	1.38	1.52	205	80:20	1.00
2. 4′-MePhe							
	1.57	2.25	1.43	1.63	205	80:20	1.00
3. 2′,6′-diMePhe							
	1.20	2.31	1.92	3.84	205	80:20	1.00
4[b]. α-MePhe							
	0.97	0.97	1.00	0.00	205	80:20	1.00
	1.22	1.10	0.90	0.80	205	70:30	0.75; 10°C
5. *erythro*-β-MePhe	0.74	1.34	1.85	2.16	205	90:10	1.00
	0.62	1.30	2.10	2.95	205	80:20	1.00
	0.72	1.72	2.38	3.82	205	75:25	0.75; 15°C
	0.79	2.03	2.09	4.28	205	70:30	0.75; 10°C
6. *threo*-β-MePhe	1.00	1.13	1.13	<0.40	205	90:10	1.00
	0.86	1.06	1.24	0.90	205	80:20	1.00
	1.05	1.40	1.33	1.35	205	75:25	0.75; 15°C
	1.18	1.64	1.39	1.45	205	70:30	0.75; 10°C
Tyrosine analogues							
7[c]. *m*-Tyr							
	0.83	1.52	1.83	2.33	205	80:20	1.00
8. 2′-MeTyr							
	0.88	1.30	1.47	2.00	205	80:20	1.00
9. 2′,6′-diMeTyr							
	0.74	1.72	2.32	2.25	205	80:20	1.00
10. 2′,6′-diMeTyrNH$_2$							
	1.90	2.08	1.09	<0.20	205	70:30	0.75; 10°C

Table 1. Continued

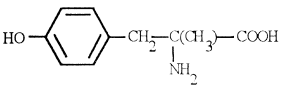
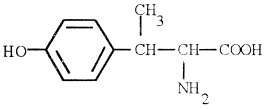
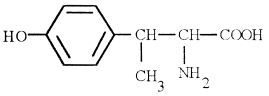
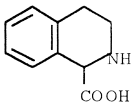
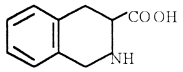
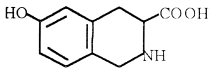
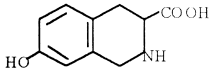
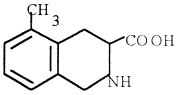
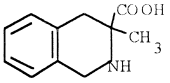
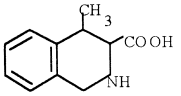
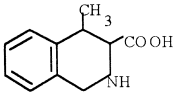
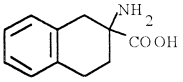
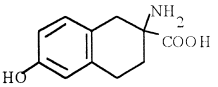
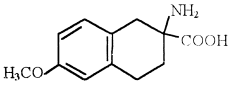
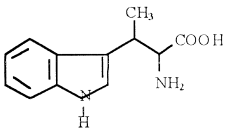
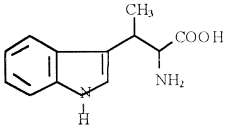
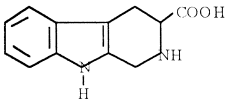
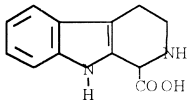
Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, water–MeOH (%, v/v)	Flow-rate (ml/min) and temperature[a] (°C)
11. α-MeTyr 	0.65	0.65	1.00	0.00	205	90:10	1.00
	0.56	0.56	1.00	0.00	205	80:20	1.00
	0.75	0.75	1.00	0.00	205	80:20	1.00; 20°C
	1.04	1.04	1.00	0.00	205	70:30	0.75; 10°C
12. *erythro*-β-MeTyr 	0.43	0.97	2.25	3.05	205	90:10	1.00
	0.38	0.98	2.58	3.15	205	80:20	1.00
	0.55	1.64	2.98	2.28	205	70:30	0.75; 10°C
	1.03	3.24	3.14	5.45	205	65:35	0.75; 5°C
13. *threo*-β-MeTyr 	0.72	0.72	1.00	0.00	205	90:10	1.00
	0.70	0.70	1.00	0.00	205	80:20	100
	1.01	1.22	1.20	0.90	205	70:30	0.75; 10°C
	1.93	2.39	1.25	1.05	205	65:35	0.75; 5°C
Tic analogues							
14[c]. Tic1 	1.38	1.75	1.27	1.55	205	80:20	1.00
15. Tic3 	1.60	2.95	1.85	4.00	205	80:20	1.00
16. 6′-HO-Tic3 	1.03	2.74	1.38	7.50	205	80:20	1.00
17. 7′-HO-Tic3 	0.86	2.08	2.42	3.30	205	80:20	1.00
18. 5′-MeTic3 	1.98	4.23	2.14	4.66	205	80:20	1.00
19. α-MeTic3 	2.89	2.35	0.81	<0.20	205	90:10	1.00
	2.23	2.18	0.97	<0.20	205	80:20	1.00
	2.97	2.76	0.93	<0.20	205	75:25	0.75; 5°C
20. *erythro*-β-MeTic3 	1.77	2.50	1.41	1.75	205	90:10	1.00
	1.65	2.54	1.54	2.35	205	80:20	1.00
	1.67	2.74	1.64	3.05	205	75:25	0.75; 15°C
	1.76	3.02	1.72	3.07	205	70:30	0.75; 10°C
21. *threo*-β-MeTic3 	1.67	3.67	2.19	3.84	205	90:10	1.00
	1.76	3.97	2.25	4.00	205	80:20	1.00
	1.74	3.37	1.93	2.50	205	75:25	0.75; 15°C
	1.62	3.33	2.05	4.00	205	70:30	0.75; 10°C

Table 1. Continued

Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, water–MeOH (%, v/v)	Flow-rate (ml/min) and temperature[a] (°C)
Tetraline analogues							
22. Atc 	1.46	1.76	1.21	1.20	205	90:10	1.00
	1.08	1.28	1.19	1.10	205	80:20	1.00
	1.08	1.29	1.19	1.10	205	70:30	1.00
23. Hat 	0.90	1.43	1.59	2.22	205	90:10	1.00
	0.72	1.00	1.38	1.57	205	80:20	1.00
	0.75	1.05	1.37	1.50	205	70:30	1.00
24. MeOAtc 	2.25	2.88	1.28	1.45	205	90:10	1.00
	1.48	1.87	1.25	1.30	205	80:20	1.00
	1.38	1.74	1.25	1.25	205	70:30	1.00
Tryptophan analogues							
25. *erythro*-β-MeTrp 	1.71	2.56	1.50	0.95	205	90:10	1.00
	1.24	2.05	1.65	1.42	205	80:20	1.00
	1.43	2.70	1.89	1.87	205	75:25	0.75; 15°C
	1.45	2.97	2.04	2.48	205	70:30	0.75; 10°C
26. *threo*-β-MeTrp 	1.82	3.98	2.19	2.85	205	90:10	1.00
	1.70	3.42	2.01	3.37	205	80:20	1.00
	2.14	4.35	2.03	3.60	205	75:25	0.75; 15°C
	2.33	4.52	1.94	3.75	205	70:30	0.75; 10°C
27. Tcc 	2.18	2.50	1.14	<0.60	220	90:10	1.00
28[c]. Norharmane 	4.06	5.62	1.38	2.22	220	80:20	1.00

[1] k_L and k_D are the retention factors of the L isomer (eluting first) and the D isomer (eluting second) of α-amino acids; [a]if not indicated, analyses were carried out at 25°C; [b]reverse elution sequence; [c]the elution sequence was not determined.

have higher *k* values than Tyr analogues. The same holds for the β-methyl-α-amino acid series β-MePhe (**5**, **6**), β-MeTyr (**12**, **13**), β-MeTic3 (**20**, **21**) and β-MeTrp (**25**, **26**), for the tetraline analogues, Atc (**21**), Hat (**22**), MeOAtc (**23**), etc.

For the series of β-methyl-α-amino acids, the *erythro* stereoisomers of β-MePhe (**5**) and β-MeTyr (**12**) exhibited larger enantioselectivities on the ristocetin A column than those of the *threo* counterparts (**6** and **13**, respectively). This is similar to our earlier results relating to the separation of these stereoisomers on a teicoplanin-containing CSP [18]. The stereoisomers of β-MeTic3 (**20**, **21**) and β-MeTrp (**25**, **26**) displayed an inverse trend: the *threo* stereoisomers had the higher α and R_s values.

The analyses were generally carried out at a flow-rate of 1.0 ml/min. In some cases, besides variation of the mobile phase composition, decrease of the

flow-rate might be a possible way to improve the resolution. This procedure increases the retention time, but the increased resolution sometimes compensates for the longer analysis time. As expected, the flow-rate does not affect the enantioselectivity (α), but it does affect the separation efficiency (data not shown).

Significant effects can sometimes be attained by altering the temperature. A lower temperature usually enhances chiral separation (i.e., increases in α and R_s). In our case, the effect of lower temperature was utilized in the separation of the stereoisomers of β-methyl amino acids (Table 1). Lowering the temperature increased the α and R_s values and rendered possible the separation of four stereoisomers in one chromatographic run (see Section 3.4).

The enantiomeric elution sequence of these amino acids could readily be identified by co-chromatography of single pure enantiomers of each amino acid. The elution sequence for the α-amino acids was found to be the L enantiomer before the D enantiomer (except for the α-methyl-α-amino acids, if they were separable). This is similar to our earlier observations on the more common protein amino acids [16].

Interesting observations were made concerning the elution sequence of the stereoisomers of β-methyl-α-amino acids with two stereogenic centres. It was found for the enantiomeric pairs that the component eluting second had the *R* configuration at the carbon atom adjacent to the carboxyl group. The observed elution sequence were: *erythro*-(2*S*,3*S*)-β-MePhe before *erythro*-(2*R*,3*R*)-β-MePhe, and *threo*-(2*S*,3*R*)-β-MePhe before *threo*-(2*R*,3*S*)-β-MePhe. The same elution sequences were obtained for *erythro*-β-MeTyr, -β-MeTic3 or -β-MeTrp and for *threo*-β-MeTyr, -β-MeTic3 or -β-MeTrp. In all cases, a stronger interaction with the ristocetin A column was observed for the 2*R* stereoisomers.

In the case of dipeptides bearing two stereogenic centres, Berthod et al. [13] found that the D–D or L–D dipeptides underwent the strongest interaction with the teicoplanin column, and Ekberg-Ott et al. [16] reported that the D-carboxy-terminal dipeptides were retained longer than the L-terminal ones on ristocetin A CSP; however three groups of compounds deviated from this trend (D,L-Ala, D,L-Leu-containing dipeptides and dansyl-amino acids). It seems that compounds with the D configuration at the carbon atom adjacent to the carboxyl group had the greatest affinity for the stationary phase.

3.2. Structure–enantioselectivity relationship

The main molecular interactions thought to take place in the RPM are (i) electrostatic (charge–charge) interactions, occurring between the racemate and the free amino group of the ristocetin A molecule, and (ii) hydrophobic associations, occurring with the chiral analyte and the hydrophobic ''pocket'' of the ristocetin A CSP [16,19]. Secondary interactions most probably involve hydrogen-bonding and steric effects. In the applied water–MeOH mobile phases the actual pH, pH_a was about 6. The isoelectric point of ristocetin A is 7.5 [52], ristocetin A and most of the amino acids exist in ionic form. Chiral recognition requires a minimum of three points of interaction [53]. The cationic ammonium group of ristocetin A is the most available and logical site for initial docking and retention. The secondary and tertiary structures of the ristocetin A molecule play an additional important role in chiral recognition by supplying appropriate hydrogen-bonding, hydrophobic and steric interaction sites [16,19]. This mechanism is supported by the analysis of 2′,6′-diMeTyr (**9**) and 2′,6′-diMeTyrNH$_2$ (**10**). The former, has a free carboxyl group that may dock to the cationic ammonium group ($-NH_3^+$) of the chiral selector, and the enantiomers were separated with high α and R_s values. In the latter case, the lack of a free carboxyl group inhibits the primary charge–charge interaction, and the observed retention and resolution were due to the secondary and hydrophobic interactions.

The difference in enantioselectivity of α- or β-substituted α-amino acids may be attributed to a steric hindrance effect. The incorporation of a methyl group in the α-position weakened the chiral recognition and smaller α and R_s values or no resolution was observed for α-MePhe (**4**), α-MeTyr (**11**) and α-MeTic3 (**19**) than for β-MePhe (**5**, **6**), β-MeTyr (**12**, **13**) and β-MeTic (**20**, **21**). The low resolutions of the α-methyl-α-amino acids were probably due to a different chiral discrimination mechanism; this is supported by a reverse in the sequence of elution, too.

The rigidity of the molecule is one of the factors

Table 2
Retention factors (k), separation factors (α) and resolutions (R_s) for the separation of enantiomers of unusual α-amino acids on ristocetin A-bonded stationary phase in POM[1]

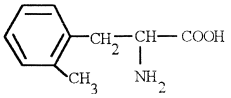
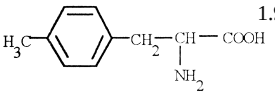
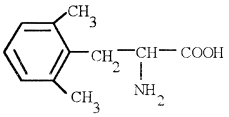
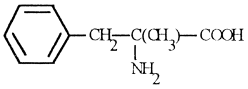
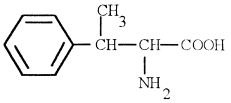
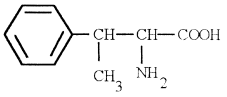
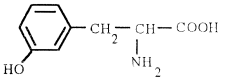
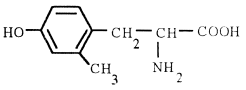
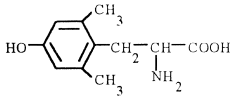
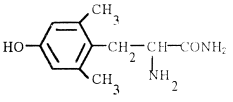
Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, MeOH–HOAc–TEA (%, v/v/v)	Flow-rate (ml/min) and temperature[a] (°C)
Phenylalanine analogues							
1. 2′-MePhe 	1.87	3.10	1.66	1.45	205	100:0.1:0.1	1.00
2. 4′-MePhe 	1.96	3.58	1.83	2.63	205	100:0.1:0.1	1.00
3. 2′,6′-diMePhe 	2.77	2.77	1.00	0.00	205	100:0.1:0.1	1.00
4. α-MePhe 	1.31	1.57	1.20	0.45	205	100:0.1:0.1	1.00
	1.70	1.70	1.00	0.00	205	100:0.01:0.02	1.00
5. *erythro*-β-MePhe 	0.94	2.27	2.41	1.60	205	100:0.1:0.1	1.00
	1.03	2.60	2.52	2.70	205	100:0.01:0.02	1.00
6. *threo*-β-MePhe 	1.28	2.07	1.62	1.00	205	100:0.1:0.1	1.00
	1.41	2.40	1.70	1.20	205	100:0.01:0.02	1.00
Tyrosine analogues							
7[b]. *m*-Tyr 	2.47	5.36	2.17	2.88	205	100:0.1:0.1	1.00
8. 2′-MeTyr 	1.78	2.86	1.61	1.50	205	100:0.1:0.1	1.00
9. 2′,6′-diMeTyr 	1.66	1.66	1.00	0.00	205	100:0.1:0.1	1.00
10. 2′,6′-diMeTyrNH$_2$ 	0.60	0.69	1.15	<0.40	205	100:0.1:0.1	1.00

Table 2. Continued

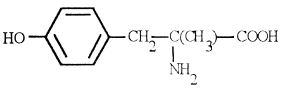
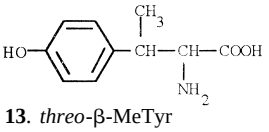
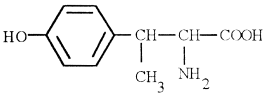
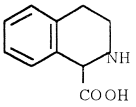
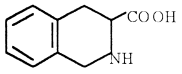
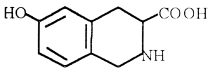
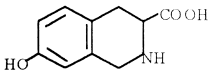
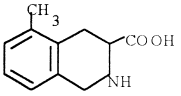
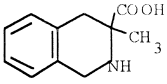
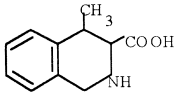
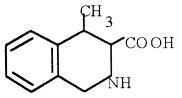
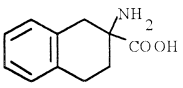
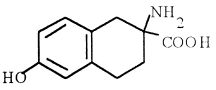
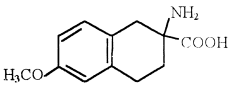
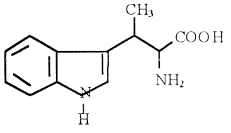
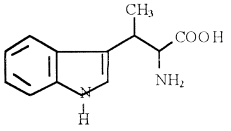
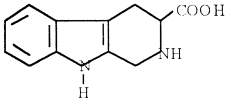
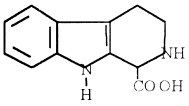
Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, MeOH–HOAc–TEA (%, v/v/v)	Flow-rate (ml/min) and temperature[a] (°C)
11. α-MeTyr 	1.14	1.14	1.00	0.00	205	100:0.1:0.1	1.00
12. *erythro*-β-MeTyr 	1.03	2.30	2.22	2.18	205	100:0.1:0.1	1.00
13. *threo*-β-MeTyr 	1.26	1.95	1.55	1.50	205	100:0.1:0.1	1.00
Tic analogues							
14[b]. Tic1 	6.20	9.74	1.57	2.20	205	100:0.1:0.1	1.00
15. Tic3 	4.91	8.11	1.65	2.50	205	100:0.1:0.1	1.00
16. 6′-HO-Tic3 	4.46	8.00	1.79	3.14	205	100:0.1:0.1	1.00
17. 7′-HO-Tic3 	5.07	7.27	1.43	1.35	205	100:0.1:0.1	1.00
18. 5′-MeTic3 	3.82	7.82	2.05	2.44	205	100:0.1:0.1	1.00
19. α-MeTic3 	4.54	5.14	1.13	0.75	205	100:0.1:0.2	1.50
20. *erythro*-β-MeTic3 	3.53	8.20	2.32	3.74	205	100:0.1:0.05	1.00
21. *threo*-β-MeTic3 	2.70	4.20	1.55	1.35	205	100:0.1:0.05	1.00

Table 2. Continued

Compound	k_L	k_D	α	R_s	Detection wavelength (λ, nm)	Eluent composition, MeOH–HOAc–TEA (%, v/v/v)	Flow-rate (ml/min) and temperature[a] (°C)
Tetraline analogues							
22. Atc 	1.98	1.98	1.00	0.00	205	100:0.1:0.1	1.00
23. Hat 	2.11	2.11	1.00	0.00	205	100:0.1:0.1	1.00
24. MeOAtc 	2.38	2.38	1.00	0.00	205	100:0.1:0.1	1.00
Tryptophan analogues							
25. *erythro*-β-MeTrp 	1.41	2.67	1.90	1.22	205	100:0.1:0.1	1.00
	1.44	2.87	2.00	0.85	205	100:0.01:0.02	1.00
26. *threo*-β-MeTrp 	1.74	2.27	1.30	0.57	205	100:0.1:0.1	1.00
	1.90	2.51	1.32	0.66	205	100:0.01:0.02	1.00
27. Tcc 	4.46	5.44	1.14	0.80	220	100:0.1:0.2	1.00
28[b]. Norharmane 	12.48	17.23	1.38	1.88	220	100:0.1:0.1	1.00

[1] k_L and k_D are the retention factors of the L isomer (eluting first) and the D isomer (eluting second) of α-amino acids; [a]if not indicated, analyses were carried out at 25°C; [b]the elution sequence was not determined.

governing the enantioselectivity. Most of the investigated conformationally constrained aromatic α-amino acids displayed good separation on a ristocetin A column. The separation of the *erythro* and *threo* stereoisomers of β-methyl-substituted amino acids furnished interesting results. For β-MePhe (**5**, **6**) and β-MeTyr (**12**, **13**), the *erythro* stereoisomers have higher α and R_s values than the *threo* ones. β-MeTic3 (**20**, **21**) and β-MeTrp (**25**, **26**) have more rigid structures than those of the Phe and Tyr analogues, and it was probably the different secondary interactions that resulted in the difference in stereoselectivity. The *threo* stereoisomers exhibited higher α and R_s values than those of the *erythro* compounds.

The tetraline analogues are also α,α-disubstituted

compounds, but with more rigid structure than those of the α,α-disubstituted Phe, Tyr and Tic analogues. This rigidity resulted probably in the enhanced stereoselectivity. In the enantioseparation of Tcc (**27**) and norharmane (**28**), the former displayed low, while the latter high α and R_s values. The position of the carboxylic acid moiety is the only difference between these two compounds (Table 1). Since the primary attractive interaction between these amino acids and the ristocetin A chiral selector is through their carboxyl groups, it is clear that these two compounds must have very different orientations relative to ristocetin A. It is likely that the Tcc–ristocetin A complex suffers from adverse steric interactions and/or inadequate hydrogen-bonding to its heterocyclic amine moiety.

In conclusion, the primary dominating step in chiral recognition is the strong charge–charge interaction between the carboxylate group of the amino acid and the ammonium group of ristocetin A. Any hindrance of this interaction such as α,α-disubstitution weakens the chiral recognition. The bulkiness and rigidity of the molecule influence the secondary and tertiary interactions that are necessary for chiral recognition. In most cases, increased rigidity and bulkiness improve the enantioselectivity on the ristocetin A stationary phase.

3.3. Retention and enantioselectivity in POM

PO separations were introduced a few years ago as an alternative approach to unusual enantioselective separations on cyclodextrin-bonded phases [54–56]. This mode is more closely related to the normal-phase mode (NPM) than to the RPM; however, no nonpolar organic solvents (e.g., hexane) are used. The main component in the PO mobile phase is generally, acetonitrile. However, other more polar solvents (such as MeOH) and organic acids and bases can also be added to decrease retention and alter selectivity, respectively [54–56]. A different type of PO mobile phase has recently been used with the macrocyclic antibiotic CSPs [19]. This mobile phase mainly contains MeOH, together with smaller amounts of an organic acid (0.1% HOAc) and base (0.1% TEA). If the analyte is eluting too fast, the concentration of acid/base is reduced. The key factor in obtaining complete resolution is still the acid-to-base ratio. The ratios generally run from 2:1 to 1:2.

The main interactions in the POM are hydrogen-bonding, π–π and steric interactions. To some extent, charge–charge interactions may be seen between the macrocyclic molecule and chiral analyte, similar to the RPM.

Table 2 presents the separation data observed in the POM. For comparison purposes, the results are given for one particular eluent composition MeOH–HOAc–TEA (100:0.1:0.1, v/v/v), but in the cases of partial separations, data for other eluent compositions are also provided. With a few exceptions, compounds were baseline-resolved, but there are some interesting results as compared to the RPM. No separation was found for 2′,6′-diMePhe (**3**) and 2′,6′-diMeTyr (**9**) at any mobile phase composition (data not shown). The tetraline analogues also displayed poor resolution in POM.

Decrease of the acid–base content increased the retention factor, which resulted in higher α and R_s. In this way, better resolutions were obtained for the enantiomers of β-methyl-α-amino acids, and especially for *threo*-β-MeTyr (**13**), for which there was no baseline resolution in the RPM. The enantioselectivity for β-methyl-α-amino acids differed in the RPM and POM. In the POM higher enantioselectivities were obtained for the *erythro* stereoisomers in all cases, while in the RPM the *threo* stereoisomers of the more constrained β-MeTic3 (**21**) and β-MeTrp (**25**) had higher α values. Especially low resolution was obtained for β-MeTrp (**25**, **26**) in the β-methyl-α-amino acid series, which was due to the low efficiency of the column for these stereoisomers in the POM.

As regards the elution sequence of the enantiomers and stereoisomers, it was found that for enantiomeric pairs (L and D enantiomers) the component eluting second had the D (*R*) configuration. For the enantiomers of the α-methyl-amino acids, in the RPM, the reverse elution sequence was found, if they were separable.

3.4. Separation of β-methyl-amino acid stereoisomers

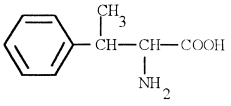
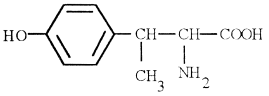
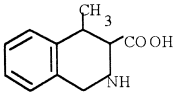
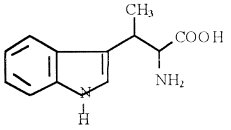
β-Methyl-α-amino acids contain two stereogenic centers and therefore consist of two pairs of dia-

stereomers (*erythro* and *threo*). Non-selective synthesis leads to the racemic forms of these *erythro* and *threo* isomers, and their separation demands special conditions. Even in the case of stereoselective synthesis, to produce one enantiomer, the product is always contaminated with its stereoisomers. Further, peptide synthesis, frequently causes epimerization. There is therefore a great need for methods that can achieve the separation of all four isomers.

In enantioselective chromatography, the application of subambient temperature and a lower flow-rate causes an increase in enantioselective retention [16,18]. The four stereoisomers of β-MePhe (**5**, **6**), β-MeTyr (**12, 13**), β-MeTic3 (**20**, **21**) and β-MeTrp (**25**, **26**) were analyzed in the temperature range 5-25°C at a flow-rate of 0.6–1.0 ml/min; some of the results are listed in Table 3 and depicted in Fig. 1. The poor resolution of the four stereoisomers observed at ambient temperature, was improved at low temperatures.

The diastereoselectivity of ristocetin A depended on the nature of the analyte and also on the method used (RPM or POM). In the cases of β-MePhe (**5**, **6**) and β-MeTyr (**12**, **13**), the *threo* isomers always eluted between the *erythro* ones in both separation methods, and nearly baseline separation was achieved for the four consecutive peaks with the RPM (Table 3). β-MeTrp (**25**, **26**) displayed different stereoselectivities. In the POM (Table 2) the two *threo* isomers eluted between the two *erythro* ones; however, in the RPM, the following sequence was observed *erythro*-(2*S*,3*S*)<*threo*-(2*S*,3*R*)<*erythro*-(2*R*,3*R*)<*threo*-(2*R*,3*S*). The more rigid and bulky β-MeTic3 (**20**, **21**), which had a good enantioselectivity in the RPM (Table 1), exhibited poor diastereoselectivity. The *erythro*-(2*S*,3*S*) and *threo*-

Table 3
Retention factors (k), separation factors (α) and resolutions (R_s) for the separation of four stereoisomers of β-methyl-α-amino acids on ristocetin A-bonded stationary phase[1]

Compound	k_1	k_2	k_3	k_4	$\alpha_{1,2}$	$\alpha_{2,3}$	$\alpha_{3,4}$	$R_{s;1,2}$	$R_{s;2,3}$	$R_{s;3,4}$	Method
5, **6**. β-MePhe	*erythro*-L	*threo*-L	*threo*-D	*erythro*-D							
	0.72	1.05	1.40	1.72	1.46	1.33	1.22	1.33	1.35	1.33	RP[a]
	0.79	1.18	1.64	2.03	1.50	1.89	1.23	1.54	1.43	1.41	RP[b]
12, **13**. β-MeTyr	*erythro*-L	*threo*-L	*threo*-D	*erythro*-D							
	0.55	1.01	1.22	1.64	1.83	1.20	1.34	1.66	0.85	1.38	RP[a]
	1.03	1.93	2.39	3.24	1.87	1.25	1.35	3.00	1.20	1.65	RP[c]
20, **21**. β-MeTic3	*threo*-L	*erythro*-L	*threo*-D	*erythro*-D							
	2.70	3.53	4.20	8.20	1.30	1.18	1.95	0.95	1.05	2.96	PO[d]
25, **26**. β-MeTrp	*erythro*-L	*threo*-L	*erythro*-D	*threo*-D							
	1.43	2.14	2.70	4.35	1.49	0.79	1.61	1.07	0.95	2.10	RP[a]
	1.45	2.33	2.97	4.52	1.60	1.27	1.52	1.91	1.05	2.25	RP[b]

[1] k_1, k_2, k_3 and k_4 are the retention factors of the four consecutive peaks; $\alpha_{1,2}=k_2/k_1$, $\alpha_{2,3}=k_3/k_2$, $\alpha_{3,4}=k_4/k_3$; $R_{s;1,2}$ relates to resolution between peaks 1 and 2, $R_{s;2,3}$ relates to resolution between peaks 2 and 3, $R_{s;3,4}$ relates to resolution between peaks 3 and 4; conditions of analysis: mobile phase composition, [a]water–MeOH (75:25, v/v), [b]water–MeOH (70:30, v/v), [c]water–MeOH (65:35, v/v), [d]MeOH–HOAc–TEA (100:0.1:0.05, v/v/v); flow-rate, [a, b]0.75 ml/min, [c]0.65 ml/min, [d]1.00 ml/min; temperature, [a]15°C, [b]10°C, [c]5°C, [d]25°C; absolute configurations, *erythro*-L: (2*S*,3*S*), *erythro*-D: (2*R*,3*R*), *threo*-L: (2*S*,3*R*), *threo*-D: (2*R*,3*S*).

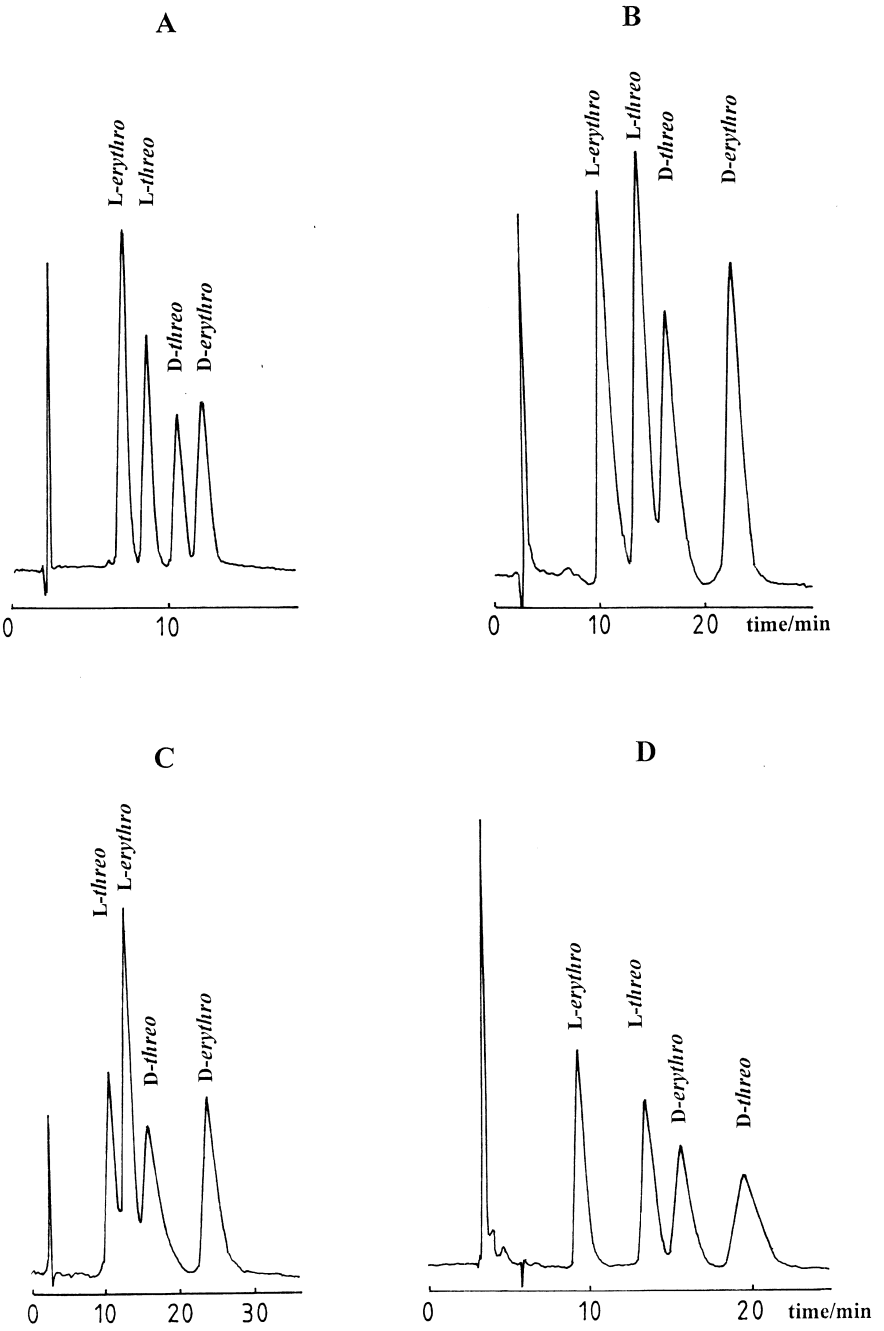


Fig. 1. Separation of stereoisomers of β-methyl-α-amino acids (A) β-MePhe; (B) β-MeTyr; (C) β-MeTic3, (D) β-MeTrp; column, Chirobiotic R; mobile phase, water–MeOH, (A, D) (70:30, v/v), (B) (65:35, v/v), (C) MeOH–HOAc–TEA (100:0.1:0.1, v/v/v); flow-rate, (A, D) 0.75 ml/min, (B) 0.65 ml/min, (C) 1.0 ml/min; temperature, (A, D) 10°C, (B) 5°C, (C) 25°C; detection, (A, C, D) 205 nm, (B) 200 nm; chromatograms relate to artificial mixtures of *erythro* and *threo* stereoisomers.

(2*S*,3*R*) stereoisomers practically coeluted. Better diastereoselectivity was observed in the POM, where the *threo* stereoisomers eluted before the *erythro* ones. In conclusion, ristocetin A as a chiral selector displayed good enantioselectivity and diastereoselectivity for β-methyl-amino acids [except for the bulky β-MeTic3 (**20**, **21**)]. The diastereoselectivity of this type of CSP for conformationally constrained, rigid amino acids containing two stereogenic centers will be the subject of future study.

4. Conclusions

The ristocetin A-based CSP is readily applicable for the separation of unnatural conformationally constrained primary and secondary amino acids. A baseline separation could be achieved for most of the investigated amino acids and even the four stereoisomers (or two pairs of enantiomers) of β-methyl-α-amino acids containing two chiral carbon atoms displayed good resolution. The effects of the mobile phase composition, flow-rate and temperature on the enantioselectivity and diastereoselectivity of the column were studied, and the conditions affording the best resolution were determined.

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