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High-performance liquid chromatographic separation of enantiomers of unusual amino acids on a teicoplanin chiral stationary phase

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Abstract

A glycopeptide antibiotic, teicoplanin, was used as chiral stationary phase for the high-performance liquid chromatographic (HPLC) separation of enantiomers of more than 30 unnatural amino acids, such as phenylalanine and tyrosine analogues and analogues containing 1,2,3,4-tetrahydroisoquinoline, tetraline, 1,2,3,4-tetrahydro-2-carboline, cyclopentane, cyclohexane, cyclohexene, bicyclo[2.2.1]heptane or heptene skeletons. Excellent resolutions were achieved for most of the investigated compounds by using a hydro-organic mobile-phase system. The effects of organic modifier content, temperature and flow-rate on the resolution were investigated and the conditions of separation were optimized. © 1998 Elsevier Science B.V.

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

1. Introduction

The increasing demands for the production of enantiomerically pure compounds in the fields of pharmacology, peptide research, etc., have led to chiral separations becoming one of the most important analytical tasks. For this purpose, chromatographic methods are widely used. Amino acids, being chiral compounds, play an important role in the design of new pharmacons. Successful high-performance liquid chromatographic (HPLC) methods for the resolution of amino acids include ligand-exchange chromatography [1–3], chiral crown ether stationary phases [4,5] and cyclodextrin-bonded stationary phases [6,7]. Indirect methods include precolumn derivatization reaction with chiral reagents, with subsequent separation on an achiral column [8–13]. Through the application of chiral stationary phases, the occurrence of racemization during the derivatization can be avoided. Macrocyclic antibiotics are a recent class of chiral stationary phases and have been used successfully to resolve enantiomers in liquid chromatography, thin-layer chromatography, capillary electrophoresis, etc. [14–21].

The present paper describes the separation of enantiomers of more than 30 unusual amino acids on a chiral stationary phase containing the glycopeptide antibiotic teicoplanin, used in a reversed-phase (RP) mode. The effects of organic modifier content, temperature and flow-rate on the separation were

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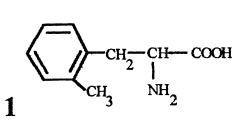
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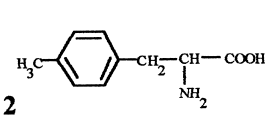
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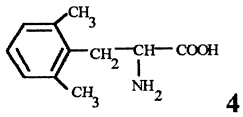
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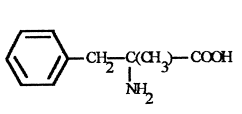
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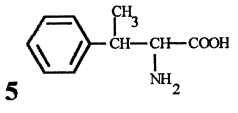
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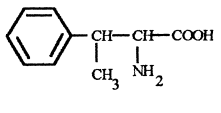
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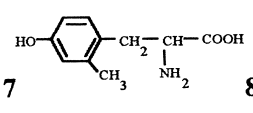
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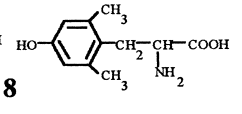
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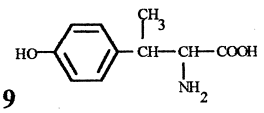
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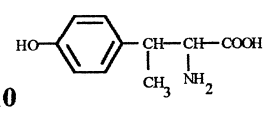
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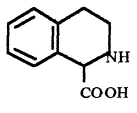
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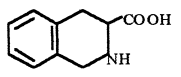
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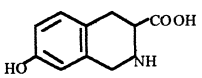
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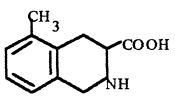
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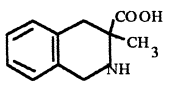
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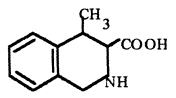
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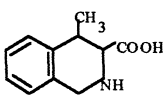
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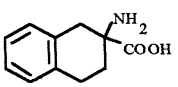
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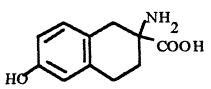
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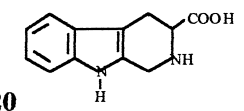
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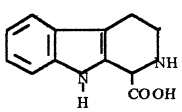
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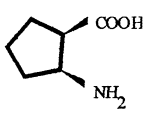
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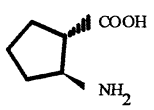
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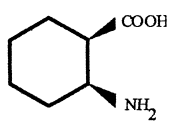
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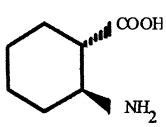
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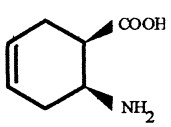
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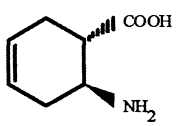
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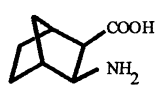
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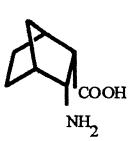
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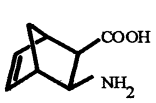
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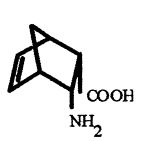
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investigated, and the conditions affording the best resolution were determined.

2. Experimental

2.1. Chemicals and reagents

All of the investigated unusual amino acids were synthesized in our own laboratories or those of our collaborators (see Acknowledgements). Their structures, names and abbreviations are shown in Fig. 1. Methanol was obtained from Merck (Darmstadt, Germany), and ethanol from Carlo Erba (Milan, Italy); both were of HPLC grade. The inorganic component of the mobile phase was prepared from Milli-Q water and further purified by filtering on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

2.2. Apparatus

HPLC measurements were performed on a Waters chromatography system, consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millennium 2010 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA).

The column used was a Chirobiotic T chiral stationary phase, 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 analysed compounds were free (underivatized) unusual amino acids. Solutions (1 mg/ml) were prepared by dissolving the analytes in methanol, ethanol or methanol–water mixtures.

All the analyses were carried out in the RP mode with hydro-organic mobile phases. The mobile phases were prepared by mixing Milli-Q water with methanol or ethanol. The eluent was degassed in an ultrasonic bath, and during the analysis helium gas was sparged through the solution.

3. Results and discussion

The unusual amino acids were analysed on the teicoplanin-containing stationary phase (Table 1). The teicoplanin molecule has several characteristic features that make it suitable for amino acid analysis. These features are: a cationic ($-NH_3^+$) site, an anionic (COO^-) site, three additional polar groups

Fig. 1. Structures of compounds investigated. The names of the investigated compounds (nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [22]): (**1**) 2′-methylphenylalanine (2′-MePhe); (**2**) 4′-methylphenylalanine (4′-MePhe); (**3**) 2′,6′-dimethylphenylalanine (2′,6′-diMePhe); (**4**) α-methylphenylalanine (α-MePhe); (**5**) *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*erythro*-β-MePhe); (**6**) *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methylphenylalanine (*threo*-β-MePhe); (**7**) 2′-methyltyrosine (2′-MeTyr); (**8**) 2′,6′-dimethyltyrosine (2′,6′-diMeTyr); (**9**) *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine (*erythro*-β-MeTyr); (**10**) *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltyrosine (*threo*-β-MeTyr); (**11**) 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic1); (**12**) 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic3); (**13**) 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (HO-Tic3); (**14**) 5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (5′-MeTic3); (**15**) 3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (α-MeTic3); (**16**) *erythro*-(2*S*,3*S* and 2*R*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic3); (**17**) *threo*-(2*S*,3*R* and 2*R*,3*S*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*threo*-β-MeTic3); (**18**) 2-aminotetraline-2-carboxylic acid (Atc); (**19**) 6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat); (**20**) 1,2,3,4-tetrahydro-3-carboxy-2-carboline (Tcc); (**21**) 1,2,3,4-tetrahydronorharmane-1-carboxylic acid (norharmane); (**22**) *cis*-(1*S*,2*R* and 1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid (*cis*-ACPC); (**23**) *trans*-(1*S*,2*S* and 1*R*,2*R*)-2-aminocyclopentane-1-carboxylic acid (*trans*-ACPC); (**24**) *cis*-(1*S*,2*R* and 1*R*,2*S*)-2-aminocyclohexane-1-carboxylic acid (*cis*-ACHC); (**25**) *trans*-(1*S*,2*S* and 1*R*,2*R*)-2-aminocyclohexane-1-carboxylic acid (*trans*-ACHC); (**26**) *cis*-(1*S*,2*R* and 1*R*,2*S*)-2-amino-4-cyclohexene-1-carboxylic acid (*cis*-ACHC-ene); (**27**) *trans*-(1*S*,2*S* and 1*R*,2*R*)-2-amino-4-cyclohexene-1-carboxylic acid (*trans*-ACHC-ene); (**28**) *diexo*-(1*S*,2*R*,3*S*,4*R* and 1*R*,2*S*,3*R*,4*S*)-3-aminobicyclo[2.2.1]heptane-2-carboxylic acid (*diexo*-ABHC); (**29**) *diendo*-(1*S*,2*S*,3*R*,4*R* and 1*R*,2*R*,3*S*,4*S*)-3-aminobicyclo[2.2.1]heptane-2-carboxylic acid (*diendo*-ABHC); (**30**) *diexo*-(1*S*,2*R*,3*S*,4*R* and 1*R*,2*S*,3*R*,4*S*)-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acid (*diexo*-ABHC-ene); (**31**) *diendo*-(1*S*,2*S*,3*R*,4*R* and 1*R*,2*R*,3*S*,4*S*)-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acid (*diendo*-ABHC-ene).

Table 1
Retention factor (k), separation factors (α) and resolutions (R_S) for the separation of enantionmers of unusual amino acids on teicoplaninbounded stationary phase

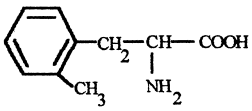
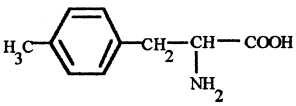
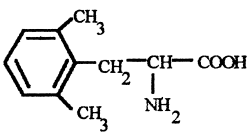
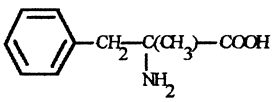
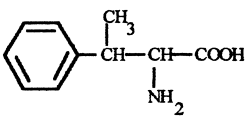
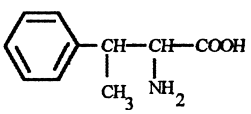
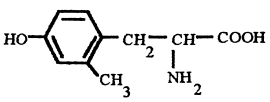
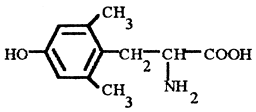
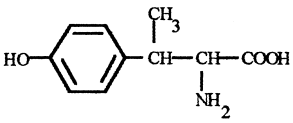
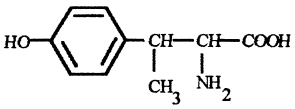
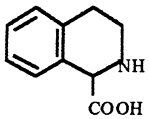
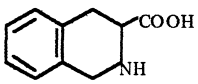
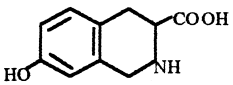
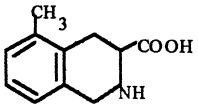
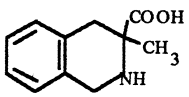
Compound	k_L	k_D	α	R_S	Detection wavelength λ (nm)	Eluent composition H_2O–CH_3OH (v/v)	Conditions of elution Flow-rate (ml/min)
Phenylalanine analogues							
1 2′-MePhe 	1.36	2.17	1.59	2.10	200	10:90	1.00
	0.68	0.89	1.31	1.12	200	50:50	1.00
	2.01	2.92	1.41	1.64	200	20:80	0.60
2 4′-MePhe 	1.19	2.11	1.77	1.71	215	10:90	1.00
	0.61	0.78	1.28	0.90	215	50:50	1.00
3 2′,6′-diMePhe 	1.40	1.53	1.09	<0.40	196	10:90	1.00
	0.71	1.28	1.80	2.55	196	50:50	1.00
4 α-MePhe 	1.63	1.93	1.18	0.52	207	0:100	1.00
	0.83	0.97	1.16	<0.40	207	10:90	1.00
	1.48	1.73	1.17	0.90	207	0:100	0.60
5 *erythro*-β-MePhe 	0.80	1.94	2.42	2.50	202	10:90	1.00
6 *threo*-β-MePhe 	1.09	2.04	1.87	1.53	202	10:90	1.00
Tyrosine analogues							
7 2′-MeTyr 	1.28	2.21	1.73	2.17	200	10:90	1.00
	0.44	0.65	1.48	1.42	200	50:50	1.00

Table 1. Continued

Compound	k_L	k_D	α	R_S	Detection wavelength λ (nm)	Eluent composition H_2O–CH_3OH (v/v)	Conditions of elution Flow-rate (ml/min)
8 2′,6′-diMeTyr							
	1.44	1.65	1.14	0.47	200	10:90	1.00
	0.63	0.73	1.16	0.61	200	40:60	1.00
	0.58	0.79	1.36	1.23	200	50:50	1.00
	0.55	0.91	1.65	1.83	200	60:40	1.00
9 *erythro-β*-**MeTyr**							
	0.73	1.88	2.57	1.53	200	10:90	1.00
10 *threo-β*-**MeTyr**							
	0.95	1.88	1.98	1.88	200	10:90	1.00
Tic analogues							
11 Tic1							
	4.15	11.0	2.65	4.61	200	10:90	1.00
	0.65	1.03	1.58	1.52	200	50:50	1.00
12 Tic3							
	3.44	8.35	2.43	5.66	200	10:90	1.00
	1.25	2.72	2.18	4.72	200	50:50	1.00
13 HO-Tic3							
	2.75	5.59	2.03	4.45	200	10:90	1.00
	0.79	1.59	2.01	4.33	200	50:50	1.00
14 5′-MeTic3							
	2.73	7.84	2.87	5.00	200	10:90	1.00
	1.01	2.79	2.76	4.80	200	50:50	1.00
15 *α*-**MeTic3**							
	8.90	10.79	1.21	1.34	200	10:90	1.00

(*continued*)

Table 1. Continued

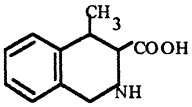
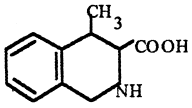
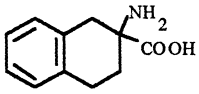
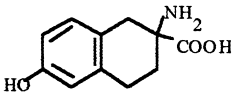
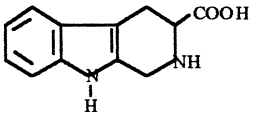
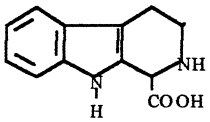
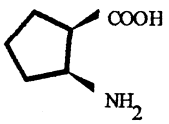
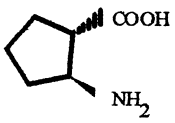
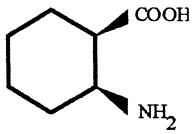
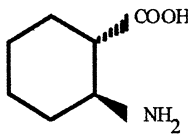
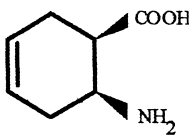
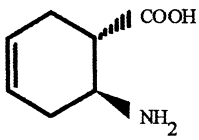
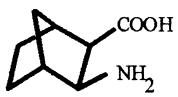
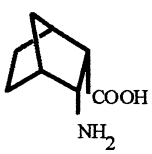
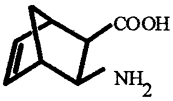
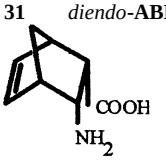
Compound	k_L	k_D	α	R_S	Detection wavelength λ (nm)	Eluent composition H_2O–CH_3OH (v/v)	Conditions of elution Flow-rate (ml/min)
16 *erythro-β*-**MeTic3** 	3.08	15.82	5.13	9.40	200	10:90	1.00
	0.81	2.64	3.26	5.40	200	30:70	1.00
17 *threo-β*-**MeTic3** 	1.61	5.72	3.55	6.40	200	10:90	1.00
	1.57	7.48	4.76	9.30	200	30:70	1.00
Tetraline analogues							
18 **Atc** 	1.60	2.81	1.76	2.47	198	10:90	1.00
	0.79	1.30	1.64	1.55	198	30:70	1.00
	0.78	1.32	1.69	2.37	198	30:70	0.80
19 **Hat** 	1.61	2.82	1.75	2.37	201	10:90	1.00
	0.63	1.12	1.78	1.35	201	30:70	1.00
	0.64	1.16	1.80	2.65	201	30:70	0.80
Tryptophan analogues							
20 **Tcc** 	2.15	2.56	1.19	0.50	221	10:90	1.00
	0.85	1.10	1.29	0.60	221	50:50	1.00
	2.19	2.61	1.20	0.90	221	10:90	0.60
21 **Norharmane** 	7.29	23.6	3.23	6.84	221	10:90	1.00
Cycloalkane analogues							
22 *cis*-**ACPC** 	2.34	2.34	1.00	0.00	200	10:90	1.00
	6.37	6.87	1.08	<0.40	200	0:100*	1.00

Table 1. Continued

Compound	k_L	k_D	α	R_S	Detection wavelength λ (nm)	Eluent composition H_2O–CH_3OH (v/v)	Conditions of elution Flow-rate (ml/min)
23 *trans*-**ACPC** 	3.05	3.05	1.00	0.00	200	10:90	1.00
	7.18	7.47	1.04	<0.40	200	0:100*	1.00
24 *cis*-**ACHC** 	1.84	1.97	1.07	0.45	203	10:90	1.00
	3.19	3.43	1.07	0.50	203	0:100	1.00
25 *trans*-**ACHC** 	2.11	2.29	1.08	0.55	203	10:90	1.00
	3.51	4.00	1.14	0.85	203	0:100	1.00
26 *cis*-**ACHC-ene** 	2.05	2.41	1.17	1.25	202	10:90	1.00
	5.70	7.82	1.37	1.75	202	0:100*	1.00
27 *trans*-**ACHC-ene** 	1.98	2.15	1.09	0.73	202	10:90	1.00
	5.19	6.09	1.17	0.85	202	0:100*	1.00
28 *diexo*-**ABHC** 	2.21	2.45	1.11	0.50	203	10:90	1.00
	3.14	3.32	1.06	0.55	203	0:100	1.00
29 *diendo*-**ABHC** 	1.75	1.81	1.03	<0.40	203	10:90	1.00
	2.34	2.55	1.09	0.60	203	0:100	1.00

(*continued*)

Table 1. Continued

Compound	k_L	k_D	α	R_S	Detection wavelength λ (nm)	Eluent composition H_2O–CH_3OH (v/v)	Conditions of elution Flow-rate (ml/min)
30 *diexo*-**ABHC-ene**							
	2.05	2.33	1.14	1.00	203	10:90	1.00
	3.09	3.75	1.21	1.10	203	0:100	1.00
31 *diendo*-**ABHC-ene**							
	2.32	3.74	1.61	1.95	203	10:90	1.00
	3.19	5.80	1.82	3.70	203	0:100	1.00

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, respectively; for elution sequence of β-methyl and of β-amino acids: see text; *analyses were carried out at 1°C.

with sugar moieties (bearing primary and secondary hydroxyl groups), phenolic groups and the apolar character of the fused macrocyclic rings and the side-chain [21].

Most of the separations were carried out with an unbuffered RP mobile phase containing water–methanol. All amino acids were analysed and detected without pre- or post-column derivatization. For comparison purposes, the results are given for one particular eluent composition (water–methanol (10:90, v/v)), but in some cases data for various other eluent compositions are provided.

3.1. Retention behaviour of amino acids

The retention and selectivity can be controlled by altering the concentration and nature of the organic modifier, but variation of the temperature or flow-rate sometimes has a beneficial effect on the resolution. Change of the water–methanol ratio strongly affects the retention factors. Increase of the water content in the mobile phase led to a decrease in the retention factor (k). It is unusual in RP-HPLC for a water-rich mobile phase to produce shorter retention times. The explanation appears to be that amino acids are more soluble in water than in methanol, and the retention time therefore decreases with increasing water content. It has to be mentioned that the more hydrophobic the solutes are the more typical reversed-phase retention behaviour could be observed [23]. It seems that the solute polarity is not only the factor governing solute retention. At the same eluent composition, the homologous series of phenylalanine and tyrosine analogues [2′-MePhe (**1**) and 2′-MeTyr (**7**), β-MePhe (**5**,**6**) and β-MeTyr (**9**,**10**), etc.] exhibit almost the same retention factor, in spite of the different polarities of the molecules. The same holds for Atc (**18**) and Hat (**19**).

At the mobile-phase composition water–methanol (10:90, v/v), the Tic analogues (**11**–**17**) and norharmane (**21**) exhibit high retention factors, $k>5$ for the second eluting enantiomer. For this reason, the analysis of these compounds is favourable in an eluent containing less than 90% methanol.

The analyses were generally carried out at a flow-rate of 1 ml/min. In some cases, besides variation of the mobile-phase composition, the 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, flow-rate does not affect enantioselectivity (α) but does affect the separation efficiency. This is reflected by the inverse relationship between resolution (R_S) and flow-rate. Decreasing the flow-rate from 1 to 0.8 or 0.6 ml/min enhanced the resolution of enantiomers of 2′-MePhe (**1**), α-MePhe (**4**) and Tcc (**20**) and baseline resolution could be achieved, e.g. for

Atc (**18**), Hat (**19**) and Tcc (**20**) even at a 30% (v/v) methanol content. Further decreases in the flow-rate did not produce any increase in the resolution of Tcc (**20**).

3.2. Enantioselectivity of the stationary phase

With the aim of improving the enantioselectivity of the separation, parameters influencing the resolution, e.g. the concentration and nature of the organic modifier, the effect of temperature, were studied.

Increasing the methanol content the retention factor (k), the selectivity factor (α) and the resolution (R_S) of two enantiomers generally also increased. The behaviour of 2′,6′-diMePhe (**3**), 2′,6′-diMeTyr (**8**) and Tcc (**20**) proved to be exceptions, relating to the selectivity factor (α) and resolution (R_S). The α and R_S values for these compounds were higher in the water-rich mobile phase in spite of the smaller retention factors. Both 2′,6′-diMePhe (**3**) and 2′,6′-diMeTyr (**8**) were baseline resolved in water-rich mobile phases.

For the series of β-methyl amino acids separated at high methanol contents (90% methanol by volume) the *erythro* compounds showed larger enantioselectivities on the teicoplanin column than the *threo* counterparts (compounds **5**,**6**, **9**,**10** and **16**,**17**). Especially large α values were observed for *erythro*- and *threo*-β-MeTic3 (**16**,**17**). In this case, the α values showed an inverse trend depending on the water–methanol ratio. The *erythro* stereoisomers had higher α values in methanol-rich mobile phases while the α values of the *threo* stereoisomers were significantly larger in water-rich mobile phases.

Somewhat different behaviour was observed for β-amino acids containing cycloalkane or cycloalkene skeletons. The enantioselectivity (α) for β-amino acids with cycloalkane skeletons (ACPC, ACHC and ABHC) was unsatisfactory at the 90% methanol mobile-phase content and underwent only a small improvement even when 100% methanol was used. Change of the organic component of the mobile phase from methanol to ethanol did not improve the selectivity or resolution. No variation of the mobile-phase composition between 50 and 100% methanol or ethanol content yielded a resolution better than $R_S=0.9$. The teicoplanin column exhibited better enantioselectivity for β-amino acids with cycloalkene skeletons, however. The enantiomers of *cis*-ACHC-ene (**26**) and *diendo*-ABHC-ene (**31**) could be separated with $R_S>1.5$, while for *trans*-ACHC-ene (**27**) and *diexo*-ABHC-ene (**30**) almost baseline separation could be achieved.

The enantiomeric elution order 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 D enantiomer. This is in agreement with earlier observations of the more common protein amino acids [21].

Interesting observations were made relating to the elution sequence of the stereoisomers of β-methyl α-amino acids having two stereogenic centres. It was found that for enantiomeric pairs (L and D enantiomers) the component eluting second had the *R* configuration at the carbon atom adjacent to the carboxyl group. The observed elution orders were: *erythro*-(2*S*,3*S*)-L-βMePhe eluted before *erythro*-(2*R*,3*R*)-D-βMePhe and *threo*-(2*S*,3*R*)-L-βMePhe eluted before *threo*-(2*R*,3*S*)-D-βMePhe. The same elution sequences were obtained for *erythro*-βMeTyr or -βMeTic and for *threo*-βMeTyr or -βMeTic. In all cases a stronger interaction was observed for the D-stereoisomers with teicoplanin column.

Berthod et al. [21] found that in the case of dipeptides bearing two stereogenic centres, the D-D or L-D dipeptides showed the strongest interaction with the teicoplanin column. It means that compounds having D-configuration at the carbon atom adjacent to the carboxyl group had the greatest affinity for the stationary phase.

The retention behaviour of cyclic β-amino acids was more ambiguous. The *cis*- and *trans*-ACHC (**24**,**25**), *cis*-ACHC-ene and (**26**) and *diendo*-ABHC-ene (**31**) showed the same behaviour mentioned above, while in the case of *trans*-ACHC-ene (**27**), diexo-ABHC (**28**) and *diexo*-ABHC-ene (**30**) the component eluting second was that which had *S* configuration at the carbon atom adjacent to the carboxyl group. Elution sequences for compounds having $R_S<0.5$ were not identified (*cis*- and *trans*-ACPC (**22**,**23**), *diendo*-ABHC (**29**)). Further investigations are in progress to verify the relationship between elution sequence and carbon atom configuration.

3.3. Separation of β-methyl amino acid stereoisomers

β-Methyl amino acids containing two stereogenic centres consist of two pairs of enantiomers and their separation needs special conditions. Routine synthesis leads to racemic forms (*erythro* or *threo* isomers). But even in the case of stereoselective synthesis, to produce one enantiomer, the product is always contaminated by the opposite enantiomer. Further, peptide synthesis, frequently causes epimerization (racemization). There is therefore a great need for separation methods that can achieve the separation of all four isomers.

In enantioselective chromatography, the application of a stationary phase coated with a crown ether, and the use of subambient temperatures, increase the chiral recognition of the crown ether and also the retention. The combination of these two effects causes an increase in enantioselective retention [24,25].

The four stereoisomers of β-MePhe, β-MeTyr and β-MeTic were analysed in the temperature range 1–50°C. Fig. 2 depicts the temperature dependence of the separation of the β-MePhe enantiomers. The poor resolution observed at ambient temperature was increased at low and at elevated temperatures. Similar chromatograms were obtained for β-MeTyr, while all isomers of β-MeTic were baseline resolved at all investigated temperatures, although better peak shapes were obtained at elevated temperatures.

The improvement in the resolution of the L-L and D-D isomers of the *erythro* and *threo* forms of β-MePhe and of β-MeTyr at lower or higher temperatures than ambient temperature was significant. The resolution of the L enantiomers, *threo*-L and *erythro*-L ($R_{S,L\text{-}L}$), and the resolution of the D enantiomers, *threo*-D and *erythro*-D ($R_{S,D\text{-}D}$), showed minimum values at room temperature, while at 1 and 50°C these values increased. At higher temperature this behaviour can be explained by the increased efficiency of the column. The efficiency of the column for the peak of the fourth eluting stereoisomer was very low at 1°C (for β-MePhe and β-MeTyr, N~300, and for β-MeTic, N~700), whereas at 50°C the corresponding values were about N=1000 and N=1600, respectively. These values are low for a 25-cm column, and this means that the kinetics of the teicoplanin D-amino acid exchange is slow, and the mass transfer is poor. Fig. 3 shows chromatograms

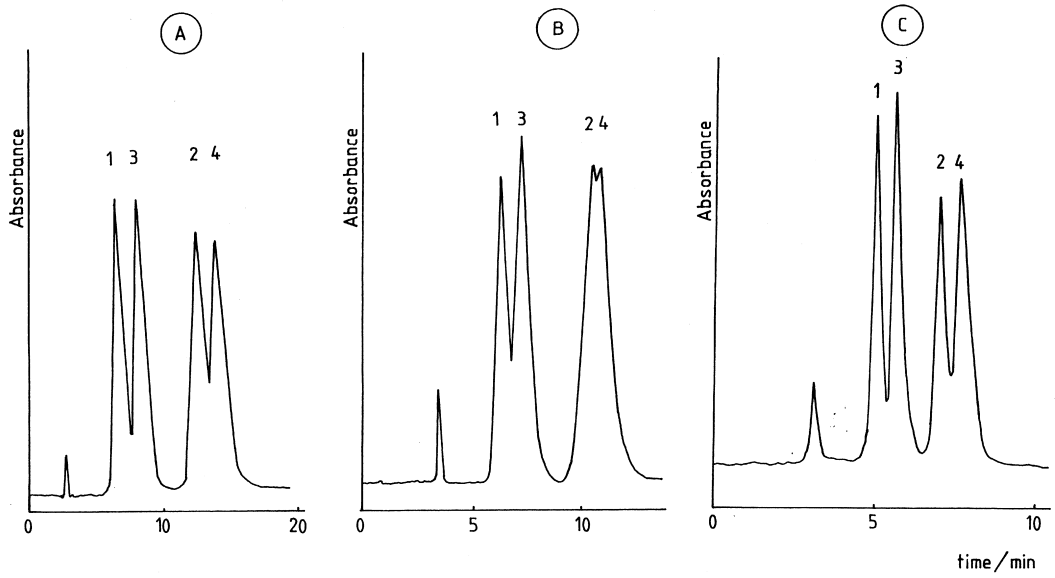


Fig. 2. Effects of temperature on separation of β-MePhe enantiomers. Column, Chirobiotic T; mobile phase, water–methanol (10:90, v/v); detection, 202 nm; flow-rate, 1 ml/min; temperature, (A) 1, (B) 20 and (C) 50°C; peaks, (1) erytho-L isomer, (2) erytho-D isomer, (3) threo-L isomer, (4) threo-D isomer.

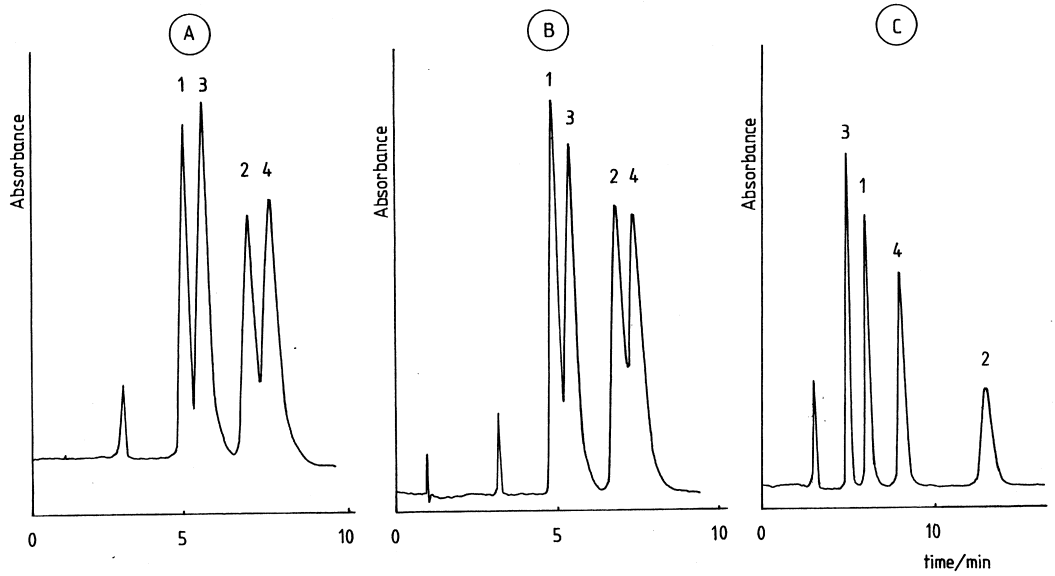


Fig. 3. Separation of enantiomers of β-methyl amino acids at elevated temperature. (A) β-MePhe; (B) β-MeTyr; (C) β-MeTic. Column, Chirobiotic T; flow-rate, 1 ml/min; temperature, 50°C; mobile phase, water–methanol 10:90 (v/v) (A,B), 30:70 (v/v) (C); detection, (A) 202, (B) 200, (C) 200 nm; peaks, (1) erytho-L isomer, (2) erytho-D isomer, (3) threo-L isomer, (4) threo-D isomer.

of best separation for the stereoisomers of β-MePhe, β-MeTyr and β-MeTic at elevated temperatures.

3.4. Structure–enantioselectivity relationship

In the working pH range of the column (water–methanol or water–ethanol mobile phase) the teicoplanin and most of the amino acids exist in the zwitterionic form. Thus the carboxylic acid moieties are in the anionic $-COO^-$ forms and the amino groups are in the cationic $-NH_3^+$ forms. Chiral recognition requires a minimum of three-points of interaction [26]. Earlier studies showed [21] that the teicoplanin ammonium group is the most available and logical site for initial docking and enantioselective retention. The secondary and tertiary structure of the teicoplanin molecule play an additional important role in chiral recognition by supplying appropriate hydrogen bonding, hydrophobic and steric interaction sites [21].

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 to the α- or β-position increases the hydrophobicity of the molecule. In the first case the methyl group is near to the carboxylate group and sterically hinders the interaction of carboxylate group with the ammonium moiety of the teicoplanin chiral selector. Therefore the chiral recognition is weakened and smaller α and R_S values were observed for α-MePhe (**4**) and α-MeTic (**15**) than for β-MePhe (**5**,**6**) and β-MeTic (**16**,**17**).

The rigidity of the molecule is one of the governing factors of enantioselectivity. Most of the investigated unnatural aromatic α-amino acids showed good separation on teicoplanin column. Especially Tic analogues exhibited large α and R_S values. The Tic analogues (**11**–**17**) are cyclic and have a more rigid structure than the phenylalanine or tyrosine analogues. In addition to the sterically constrained Tic analogues (**11**–**17**), Atc (**18**), Hat (**19**), Tcc (**20**) and norharmane (**21**) are also conformationally constrained and, with the exception of Tcc, showed good α and R_S values. It is interesting that the α and R_S values for norharmane (**21**) were among the highest in this study, while those for the closely related Tcc (**20**) were low. 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 teicoplanin chiral selector is through their carboxyl groups, it is clear that these two compounds must have a very different orientation relative to the teicoplanin. It is likely that the Tcc (**20**)–teicoplanin complex suffers from adverse steric interactions and/or inadequate hydrogen bonding to its heterocyclic amine moiety.

The investigated β-amino acids (**22**–**31**) were all cyclic molecules. In spite of the ring structure of the molecules the *cis*- (**22**) and *trans*-ACPC (**23**) did not show appreciable resolution. The increase of the ring number makes the molecule more bulky and the larger analogues tend to separate better. Larger α and R_S values were obtained for ACHC (**24**,**25**) or ABHC (**28**,**29**) than for ACPC.

The separation of the poorly resolved cyclic β-amino acids was enhanced by increasing the rigidity of the molecules. The incorporation of a double bond to the ACHC and ABHC caused a positive change in α and R_S values. The *cis*-ACHC-ene (**26**) and *diendo*-ABHC-ene (**31**) had good α and R_S values while these separation parameters for *trans*-ACHC-ene (**27**) and *diexo*-ABHC-ene (**30**) also improved to the extent that baseline separation nearly could be achieved.

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 teicoplanin.

Any hindrance of this interaction 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 teicoplanin stationary phase.

3.5. Comparison of the methods used for the separation of investigated unusual amino acids

Five different HPLC methods are summarized and compared in Table 2, including that of the teicoplanin column. The gas chromatographic method was based on the separation of *N*-trifluoroacetyl-iso-butyl ester derivatives of amino acids on a Chirasil-L-Val column. This method requires two derivatization steps and produces large retention factors for the unusual aromatic amino acids in this study. Indeed most of them are not separable on the Chirasil-L-Val column.

Indirect HPLC methods include precolumn derivatization either with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [8–10] or with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) [11] followed by separation on achiral C_{18} column.

Most of the investigated unusual amino acids can be separated by indirect methods as GITC or FDAA derivatives. These methods are very useful for analytical purposes and have high sensitivity due to the high molar absorptivity of the reagent–amino acid adducts. However for preparative purposes the precolumn derivatzitaion is not a useful approach. The recovery of amino acids is difficult and another problem is the racemization occurring during the derivatization step. The control of the degree of racemization needs precise analytical procedures.

Direct HPLC separations include analysis on two chiral stationary phases, on a chiral crown ether-containing column (Crownpak CR(+)) and on a teicoplanin-containing stationary phase (Chirobiotic T).

It should be mentioned that resolution of the majority of unusual amino acids under consideration using chiral ligand exchange chromatography has not been reported in the literature, with the exception of few β-amino acids and several α-methyl amino acids, which were all resolved well [27]. To compare the application of the two chiral stationary phases the teicoplanin excels in good separation capability and could be applied for both primary and secondary amino acids. For the separation of Tic analogues, and indeed all secondary amino acids, the Crownpak CR(+) column cannot be used. In our experience the teicoplanin column is the only chiral stationary phase that can resolve the stereoisomers of α-MeTic3 (**15**).

Interesting behaviour was observed for β-methyl amino acids. The stereoisomers of *threo*-βMePhe (**6**) and *threo*-βMeTyr (**10**) were separable by a GC method, while *erythro* isomers (**5**,**9**) were separated on a Crownpak CR(+) column.

The teicoplanin-containing chiral stationary phase separated both *threo* and *erythro* stereoisomers even for the Tic compounds (**16**,**17**). The teicoplanin-containing stationary phase showed good separation capability for aromatic amino acids. Amino acids bearing cycloalkane skeletons were poorly resolved

Table 2
Methods used for the separation of enantiomers of unusual amino acids

Compound	Methods					Ref.
	a	b	c	d	e	
(**1**) 2′-MePhe	+	+	+	+	+	[28]
(**2**) 4′-MePhe	+	+	+	+	+	[28]
(**3**) *erythro*-β-MePhe	+	+	−	+	+	[28–31]
(**4**) *threo*-β-MePhe	+	+	+	−	+	[28–31]
(**5**) 2′,6′-diMePhe	+	+	+	(+)	+	[28]
(**6**) α-MePhe	−	+	−	−	(+)	[28]
(**7**) 2′-MeTyr	+	+	+	+	+	[28]
(**8**) 2′,6′-diMeTyr	+	+	+	+	+	[28]
(**9**) *erythro*-β-MeTyr	+	+	−	+	+	[28,31]
(**10**) *threo*-β-MeTyr	+	+	+	−	+	[28,31]
(**11**) Tic-1	+	+	(+)	−	+	[28]
(**12**) Tic-3	+	+	(+)	−	+	[28,32,33]
(**13**) HO-Tic-3	+	+	−	−	+	[28,32,33]
(**14**) 5′-MeTic-3	+	+	(+)	−	+	[28]
(**15**) α-MeTic-3	−	−	−	−	+	[28]
(**16**) *erythro*-β-MeTic-3	+	+	−	−	+	[28,31,32]
(**17**) *threo*-β-MeTic-3	−	+	−	−	+	[28,31,32]
(**18**) Atc	+	+	−	−	+	[28,33]
(**19**) Hat	+	+	−	−	+	[28,32,33]
(**20**) Tcc	−	+	−	(+)	(+)	[28]
(**21**) Norharmane	*	*	*	*	+	[28]
(**22**) *cis*-ACPC	+	+	*	+	−	[34]
(**23**) *trans*-ACPC	+	+	*	+	−	[34]
(**24**) *cis*-ACHC	+	+	*	+	−	[35]
(**25**) *trans*-ACHC	+	+	*	+	(+)	[35]
(**26**) *cis*-ACHC-ene	+	+	*	−	+	[35]
(**27**) *trans*-ACHC-ene	+	+	*	+	(+)	[35]
(**28**) *diexo*-ABHC	+	+	*	(+)	−	[36]
(**29**) *diendo*-ABHC	−	+	*	(+)	−	[36]
(**30**) *diexo*-ABHC-ene	+	+	*	+	(+)	[36]
(**31**) *diendo*-ABHC-ene	+	+	*	−	+	[36]

Condition of analysis: [a]HPLC, indirect separation as GITC derivative; [b]HPLC, indirect separation as FDAA derivative; [c]GC, Chirasil-L-Val column; [d]HPLC, direct separation on Crownpak CR(+) column; [e]HPLC, direct separation on Chirobiotic T column; +, baseline separation; $R_S > 1.5$; (+), partial separation $1.5 > R_S > 0.8$; −, no separation; * no data available.

on this column, but incorporation of a double bond to these molecules improved the separation. The separation of the cycloalkane group of amino acids was usually better on the Crownpak CR(+) column, but some of these compounds were only partially resolved as well.

4. Conclusions

The teicoplanin-based chiral stationary phase is readily applicable for the separation of unusual conformationally constrained primary and secondary amino acids. A baseline separation could be achieved for most of the investigated amino acids and even the four enantiomers of α-amino acids containing two chiral carbon atoms showed good resolution. The effects of mobile phase composition, flow-rate and temperature on enantioselectivity of the column were studied, and the conditions affording the best resolution were optimized.

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