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Chromatographic methods for monitoring the optical isomers of unusual aromatic amino acids

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Abstract

Unusual aromatic amino acids (phenylalanine, tyrosine and tryptophan analogues, and analogues containing tetraline, 1,2,3,4-tetrahydroisoquinoline or 1,2,3,4-tetrahydro-2-carboline skeletons) were synthesized in racemic or chiral form. The enantiomers of these unusual aromatic amino acids were separated by different chromatographic methods. The gas chromatographic analyses were based on separation on a Chirasil-L-Val column, using N-trifluoroacetyl-isobutyl esters of amino acids, while high-performance liquid chromatography was carried out either on a Crownpak CR(+) chiral column, or on an achiral column for the separation of diastereomeric derivatives formed with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide or 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate.

Keywords: Enantiomer separation; Amino acids

1. Introduction

Conformationally constrained or topographically biased amino acids have been shown to provide an important new approach in the design of selectively acting hormone analogues [1,2]. The syntheses of these sterically constrained or biased unusual amino acids lead to a racemic form, while the asymmetric syntheses may afford a product which contains only a minor amount of the enantiomeric form [3]. The originally pure amino acids can racemize to some extent in the course of N^{α}-Boc protection or during the synthesis of biologically active peptides.

Endogenous opioid peptides are small, flexible compounds and are bonded to different opioid receptors (μ, δ, κ). The synthesis of peptide analogues with conformationally constrained aromatic amino acids can result in less flexible compounds. The conformationally constrained Phe- and Tyr-analogues are essential in this field. The opioid peptides, enkephalins, deltorphins, with L-aromatic amino acids are potent in the binding and in vitro assays, but sometimes change of the configuration of amino acids also changes the agonistic or antagonistic properties [4]. In the TIPP-NH_2 family Tyr-D-Tic-Phe-Phe-NH_2 is a δ-opioid receptor selective agonist

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while Tyr-Tic-Phe-Phe-NH_2 has antagonistic properties [5]. Atc^3-deltorphins are more potent and selective for δ-opioid receptors compared to parent peptides. Interestingly the D-Atc derivatives in these peptides have similar affinity as L-Atc derivatives [6]. Peptides with unusual aromatic amino acids or with D-amino acids are more stable in the biological systems against proteolytic enzymes.

The difficulties in obtaining many of the uncommon amino acids in homochiral form underline the importance of having at hand effective chromatographic methods for the characterization and identification of their enantiomers. Many attempts have been made to resolve amino acid enantiomers by chromatographic techniques. Enantioselective separations by chromatography can be divided into three main groups: direct separation on a chiral stationary phase [7–9], separation on an achiral column with a chiral eluent [9–11] and separation of the diastereomers formed by precolumn derivatization with a chiral reagent [9,12–22].

Separation of amino acid enantiomers using gas chromatography (GC) is based on precolumn derivatization and analysis on a chiral capillary column [23–26].

For the synthesis of receptor-selective opioid peptides, a number of conformationally constrained aromatic amino acids have been prepared (Fig. 1) (nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [27]), and indirect methods for the separation of some of these isomers were published earlier [28–31].

The present paper deals with the separation of enantiomers of these amino acids by using three different chromatographic methods. GC–MS analysis was performed with the N-trifluoroacetylated isobutyl ester derivatives of the enantiomers. HPLC was carried out either as direct separation on a Crownpak CR(+) chiral stationary phase, or as indirect separation, applying precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) or 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). The effects of various parameters (temperature of column, flow-rate, mobile phase composition, pH, etc.) on the separations were investigated and optimized.

2. Experimental

2.1. Chemicals and reagents

The amino acids shown in Fig. 1 were synthesized in our laboratory by methods from literature. The syntheses of 1, 5, 8, 14 [32], 2 [33], 6, 18 [34], 13 [35,36], 15 [37] and 21 [38] led to one enantiomer of the substrate, depending on the configuration of the starting material, whereas 3, 4 [39,40], 9, 10 [41], 11 [42], 12 [43], 16, 17 [36], 19 [6] and 20 [44] were obtained in racemic form. The identity of the compounds was checked by means of melting point determination, fast atom bombardment (FAB) mass spectrometry and nuclear magnetic resonance (^{1}H NMR) spectroscopy.

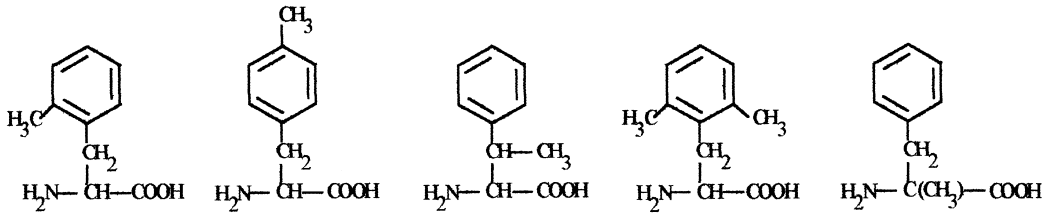
GITC and *m*-tyrosine (7) were purchased from Aldrich (Steinheim, Germany), FDAA from Pierce (Rockford, IL, USA), L-amino acid oxidase and carboxypeptidase A from Sigma (St. Louis, MO, USA) and trifluoroacetic acid, trifluoroacetic anhydride, perchloric acid of analytical reagent grade, acetonitrile and methanol of HPLC grade and other reagents of analytical reagent grade from Merck (Darmstadt, Germany). Buffers were prepared with Milli-Q water and further purified by filtration on a 0.45-μm filter type HV (Millipore, Molsheim, France).

2.2. Apparatus

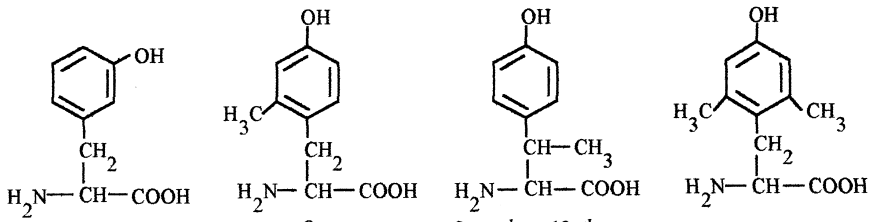
The HPLC system consisted of an M-600 low-pressure gradient pump equipped with an M-996 photodiode array detector, a Millennium 2010 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA), an L-6000 liquid chromatographic pump (Merck Hitachi, Tokyo, Japan) equipped with a variable-wavelength UV detector Model 2550 (Varian Instruments, Walnut Creek, CA, USA) and an HP 3395 integrator (Hewlett–Packard, Waldbronn, Germany).

The columns used were Vydac 218TP54 C18 (250×4.6 mm I.D.), 5 μm particle size (The Separations Group, Hesperia, CA, USA), and Crownpak CR(+) (150×4 mm I.D.), 5 μm particle size (Daicel, Tokyo, Japan). The Crownpak CR(+) column was thermostated with an MK 70 thermostat

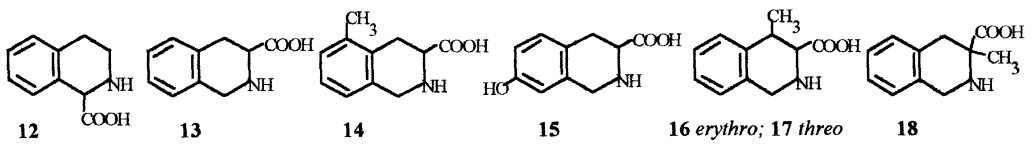
Phenylalanine analogues



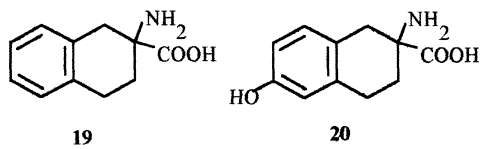
Tyrosine analogues



Tic analogues



Tetraline analogues



Tryptophan analogue

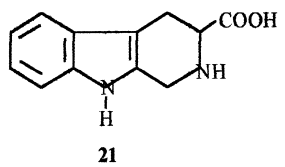


Fig. 1. Structures of the compounds analysed. 1. 2′-Methylphenylalanine (2′-MePhe); 2. 4′-methylphenylalanine (4′-MePhe); 3. *erythro*-β-methylphenylalanine (*erythro*-β-MePhe); 4. *threo*-β-methylphenylalanine (*threo*-β-MePhe); 5. 2′,6′-dimethylphenylalanine (2′,6′-diMePhe); 6. α-methylphenylalanine (α-MePhe); 7. *meta*-tyrosine (*m*-Tyr); 8. 2′-methyltyrosine (2′-MeTyr); 9. *erythro*-β-methyltyrosine (*erythro*-β-MeTyr); 10. *threo*-β-methyltyrosine (*threo*-β-MeTyr); 11. 2′,6′-dimethyltyrosine (2′,6′-diMeTyr); 12. 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (Tic-1); 13. 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic-3); 14. 5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (5-MeTic-3); 15. 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (HO-Tic-3); 16. *erythro*-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic); 17. *threo*-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*threo*-β-MeTic); 18. 3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (α-MeTic); 19. 2-aminotetraline-2-carboxylic acid (Atc); 20. 6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat); 21. 1,2,3,4-tetrahydro-3-carboxy-2-carboline (Tcc).

(Mechanik Prüfgeräte, Medlingen, Germany). The accuracy of temperature adjustment was ±0.1°C.

GC was performed on a Model MD800 EI 250 GC–MS system (Fisons Instruments, Manchester, UK). Ionization was carried out by electron impact and the mass spectrometer was scanned in a multiple ion detection mode (MID). This means that specific ions corresponding with specific fragments were monitored. For our analysis generally we used a full scan in a range of masses 50 to 500. By using MID we used a dwell time of 0.1 s. The samples were analysed on a Chirasil-L-Val capillary column, 50 m×0.32 mm I.D. (Alltech, Helifex, Deerfield, IL, USA). The operating conditions were: injection port temperature 250°C, carrier gas helium [13 p.s.i. inlet pressure (1 p.s.i.=6755 Pa)] and splitting ratio 1:30. The column temperature was kept in the range of 80–200°C, and the rate of temperature change was varied to achieve good resolution.

2.3. Derivatization of amino acids

For GC–MS analysis, the N-trifluoroacetylated isobutyl (TAB) esters of the D,L-amino acids were prepared by reaction with isobutanol in 3 *M* HCl following reaction with trifluoroacetic anhydride [45].

For HPLC analysis, D,L-amino acids were derivatized with FDAA or GITC by the slightly modified method of Marfey [18] or Nimura et al. [15–17]. Generally, 90 min was used for the reaction instead of the 30 min originally proposed [18,15–17]. 2′,6′-diMePhe (5), 2′,6′-diMeTyr (11) and α-MePhe (6) contain the methyl groups near the primary amino group, and the reaction of the derivatizing reagent with the amino group was therefore sterically hindered. With the usual reaction time (90 min), no reaction product was observed, and the time of the reaction was increased to 4–5 h. With the long reaction time, racemization or a side-reaction might occur, and new peaks appeared in the chromatogram. The racemization yield was controlled by derivatization of the pure enantiomers, and was found to be negligible: less than 2%.

For HPLC analysis the free or derivatized amino acids were dissolved in the mobile phase except 2′,6′-diMePhe (5) which was insoluble in the eluent (perchloric acid). 1 mg of 2′,6′-diMePhe was dissolved in 200 μl glacial acetic acid and further diluted with perchloric acid pH 2.0.

2.4. Enzymatic digestion of D,L-amino acids

The elution sequence of D,L-*erythro*- and D,L-*threo*-β-methyl amino acids was determined either by enzymatic digestion of the amino acids with L-amino acid oxidase (3, 4, 9, 10 and 17) or with a standard of *erythro*-D-β-Me-Tic (16) made from *erythro*-D-β-methyl-phenylalanine by the method given in reference [35].

The configurations of amino acids containing a tetraline ring were determined by means of digestion with carboxypeptidase A. For digestion of Atc and Hat (see Fig. 1 for full names and structures) with carboxypeptidase A, the N-trifluoroacetyl derivative of the amino acid was first prepared [6].

3. Results and discussion

The unusual aromatic amino acids were analysed by three different chromatographic methods: GC and HPLC using direct or indirect separation. Chromatograms representing the three methods are shown in Figs. 2 and 3.

3.1. Gas chromatography of enantiomers

For GC, all the amino acids were converted into the N-trifluoroacetylated isobutyl esters. With a GC–MS system, an electron impact ionization mass spectrum was obtained to confirm the expected structure of the derivatives. The derivatives of the enantiomers were separated on a Chirasil-L-Val column. Typical chromatogram and mass spectrum are shown in Fig. 4 for the separation of 2′-MePhe (1) and the results of GC–MS analysis are collected in Table 1. On the Chirasil-L-Val column, the D enantiomer eluted before the L enantiomer. To increase the resolution of D and L enantiomers, the rate of temperature change was varied between 10 C°/min and 1 C°/min. The initial and final temperatures of the column were 80 and 200°C, respectively. The decrease of the rate of temperature change in most cases had a slight effect on the resolution and

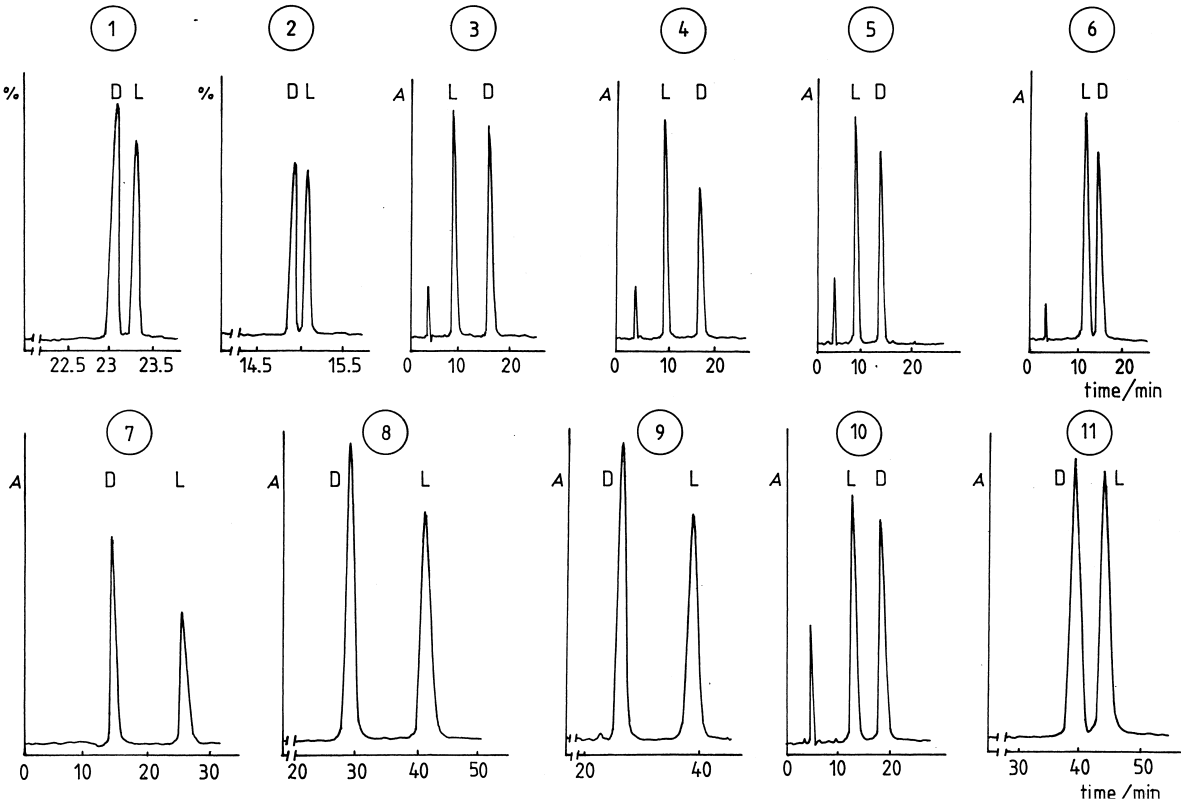


Fig. 2. Representative chromatograms of phenylalanine and tyrosine analogues. Compounds 1–11 (see legend to Fig. 1); methods, GC–MS for compounds 1 and 2; column, Chirasil-L-Val, temperature program of column thermostat 80–200°C at 5 C°/min for compound 1, and 80–200°C at 10 C°/min for compound 2; for detection and other conditions for compounds 1 and 2, see Section 2; HPLC direct separation for compounds 7, 8, 9 and 11; column, Crownpak CR(+); flow-rate, 0.75 ml/min; column temperature, 5°C; UV detection, 195 nm for compounds 7 and 9, 198 nm for compound 8 and 200 nm for compound 11; mobile phase, perchloric acid pH 2.0; HPLC indirect separation as FDAA derivatives for compounds 3, 4, 5 and 6 and HPLC indirect separation as GITC derivative for compound 10; column, Vydac 218TP54 C_{18}; flow-rate, 0.80 ml/min; column temperature, ambient; UV detection, 340 nm for compounds 3, 4, 5 and 6, 250 nm for compound 10; mobile phase, 0.1% aqueous TFA–methanol (40:60, v/v) for compounds 3, 4 and 6, 0.1% aqueous TFA–methanol (35:65, v/v) for compound 5 and 0.1% aqueous TFA–methanol (55:45, v/v) for compound 10.

the increased retention time was disadvantegeous (see compounds 2, 13 in Table 1).

Good resolution ($R_S > 1.4$) was obtained for the Phe and Tyr analogues, with relatively short retention times. Unfortunately, the enantiomers of *erythro*-β-MePhe (3), *erythro*-β-MeTyr (9) and α-MePhe (6) were not separated on the Chirasil-L-Val column.

The Tic analogues exhibited high retention times (large *k* values) and poor resolutions ($R_S < 1.0$). The GC method needed a long analysis time for the separation of Tic analogues. The enantiomers of *erythro*-β-MeTic (16), like enantiomers of other *erythro*-β-methyl-amino acids, were not separated under any conditions of analysis mentioned in the footnote of Table 1. The same held for HO-Tic-3 (15), Atc (19), Hat (20) and Tcc (21).

3.2. HPLC separation of enantiomers

3.2.1. Direct separation on chiral stationary phase

For direct separation by HPLC, a Crownpak CR(+) column was used. This column contains a chiral crown ether as chiral selector and can resolve compounds bearing a primary amino group near the chiral centre. Chiral recognition is achieved when a

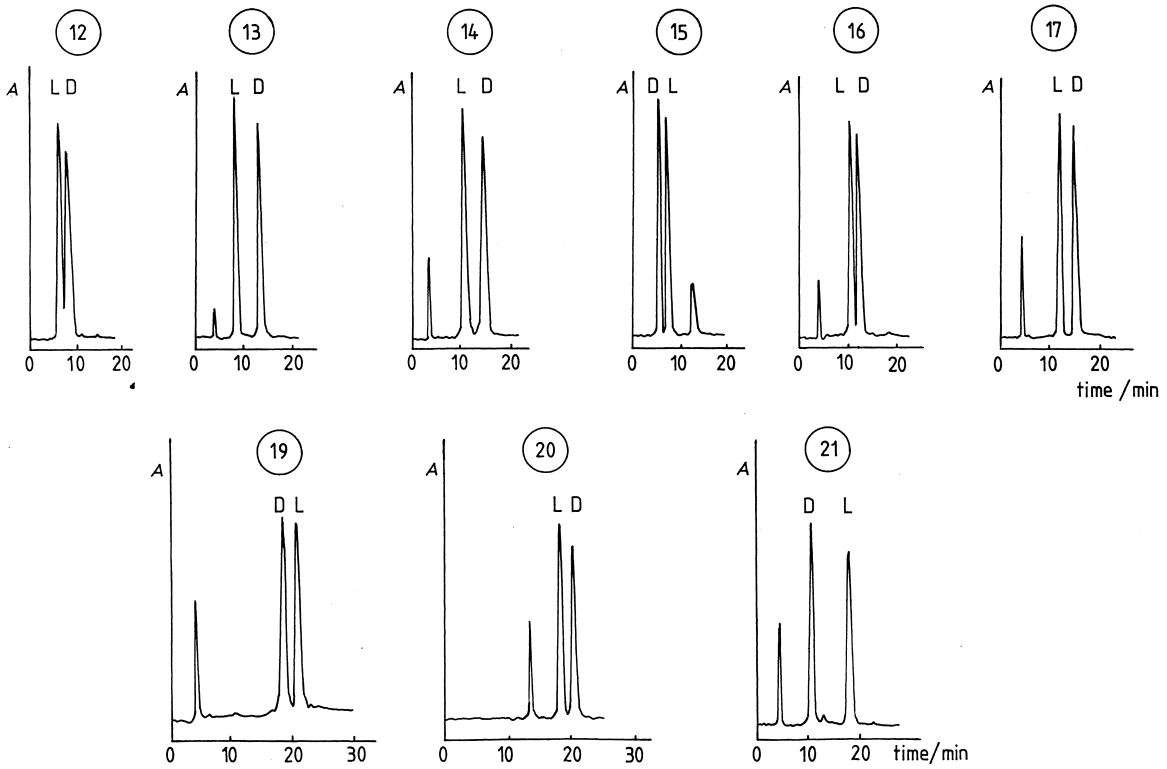


Fig. 3. Representative chromatograms of Tic, tetraline and tryptophan analogues. Compounds 12–21 (see legend to Fig. 1); methods, HPLC indirect separation as FDAA derivatives for compounds 13, 15, 16, 17, 20 and 21 and HPLC indirect separation as GITC derivatives for compounds 12, 14 and 19; column, Vydac 218TP54 C_{18}; flow-rate, 0.80 ml/min; column temperature, ambient; UV detection, 340 nm for compounds 13, 15, 16, 17, 20 and 21, 250 nm for compounds 12, 14 and 19; mobile phase, 0.01 *M* aqueous solution of sodium acetate (pH 3.0)–methanol (70:30, v/v) for compound 20 (see Ref. [30]), 0.01 *M* aqueous solution of sodium acetate (pH 3.0)–methanol (50:50, v/v) for compound 19 (see refernce [30]), 0.1% aqueous TFA–methanol (45:55, v/v) for compounds 12 and 14, and 0.1% aqueous TFA–methanol (40:60, v/v) for compounds 13, 15, 16, 17 and 21.

complex is formed between the crown ether and the ammonium ion derived from the sample.

Since Tic and tryptophan analogues contain a secondary amino group, for the separation of these compounds the Crownpak CR(+) column cannot be applied.

The enantiomers of aromatic amino acids undergo strong complex formation with the chiral selector, therefore the retention factors are relatively high (Table 2). In most cases, baseline separation (R_S> 1.5) was achieved at 20°C (perchloric acid at pH 2.0, flow-rate 0.75 ml/min). The decrease of the temperature improved the separation as it can be seen in the two sets of results, but this involved an increased analysis time. By temperature decrease, baseline separation could be achieved for 2′,6′-diMeTyr (11), but no conditions were found for separation of the enantiomers of *threo*-β-MePhe (4), *threo*-β-MeTyr (10), α-MePhe (6), Atc (19) or Hat (20).

3.2.2. Indirect separation of enantiomers

For the indirect separation of enantiomers, pre-column derivatization was applied. The enantiomers of phenylalanine analogues could be separated either as GITC or as FDAA derivatives (Table 3). The two derivatizing reagents have different separation capabilities: the FDAA derivatives exhibited larger enantioseparation, and higher R_S values were obtained for similar analysis times. The hydrophobicity of the molecule was the main factor governing the retention: the more hydrophobic species had larger

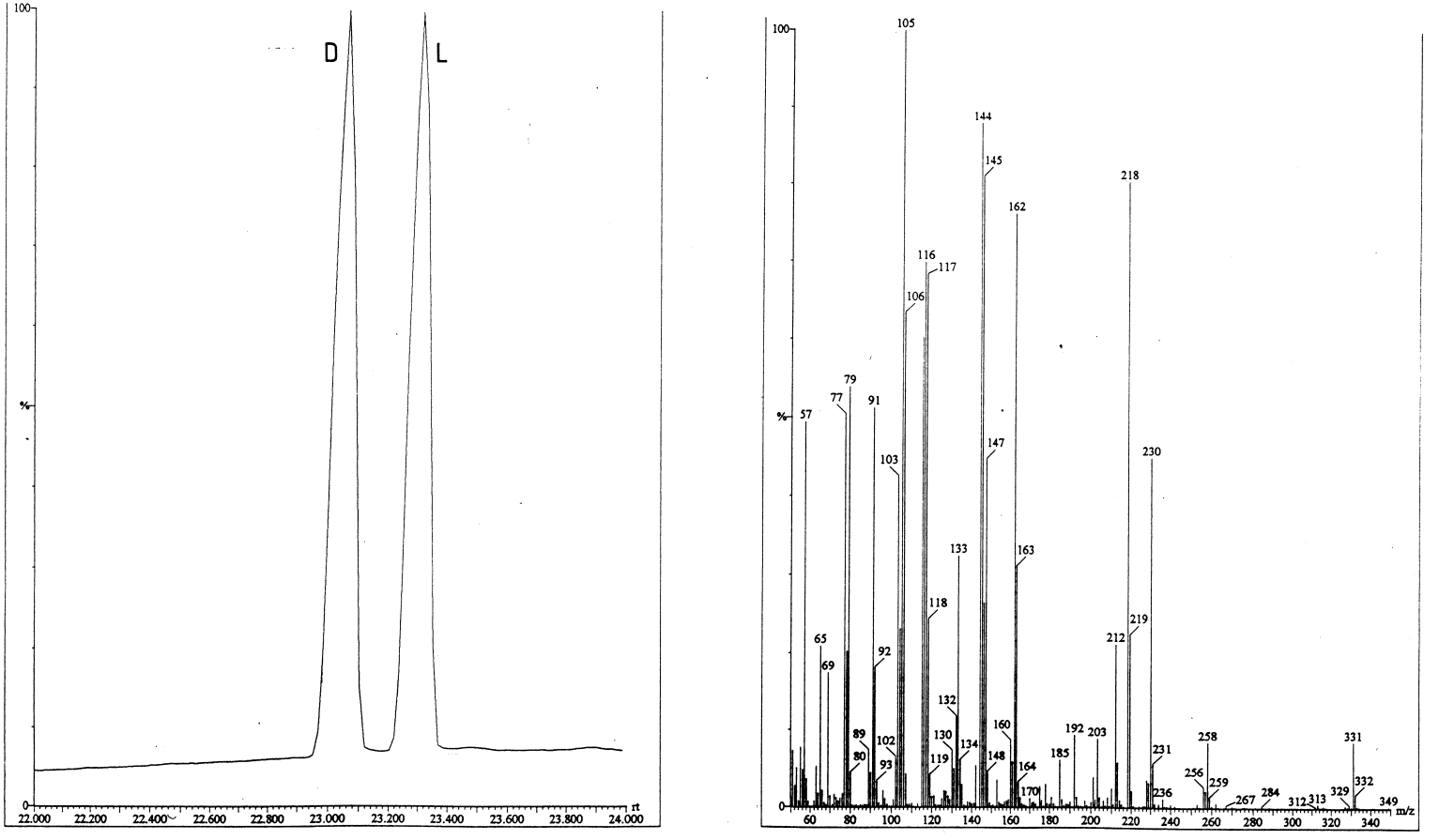


Fig. 4. GC chromatogram and electron impact ionization mass spectrum of 2′-MePhe (1). Column, Chirasil-L-Val; temperature program of column thermostat: 80–200°C at 5 C°/min; for detection and other conditions, see Section 2.

Table 1
Retention factors (k), separation factors (α) and resolutions (R_S) measured on the Chirasil-L-Val column

	Compound	k_D	k_L	α	R_S	Conditions of analysis
	Phe analogues					
1	2′-MePhe	8.38	8.48	1.01	1.86	b
2	4′-MePhe	8.55	8.64	1.01	1.57	b
		5.07	5.16	1.02	1.41	a
3	*erythro*-β-MePhe	7.75	7.75	1.00	0.00	b
4	*threo*-β-MePhe	7.93	8.01	1.01	2.50	b
5	2′,6′-diMePhe	8.71	8.79	1.01	1.92	b
6	α-MePhe	6.71	6.71	1.00	0.00	b
	Tyr analogues					
8	2′-MeTyr	9.66	9.79	1.01	2.70	b
9	*erythro*-β-MeTyr	8.65	8.65	1.00	0.00	b
10	*threo*-β-MeTyr	8.86	8.96	1.01	1.95	b
11	2′,6′-diMeTyr	9.44	9.54	1.01	2.24	b
	Tic analogues					
12	Tic-1	38.40	38.54	1.00	1.02	d
13	Tic-3	26.83	26.98	1.01	0.95	b
		41.36	41.50	1.01	0.95	d
14	5′-MeTic-3	20.48	20.55	1.00	0.95	c
15	HO-Tic-3	21.28	21.28	1.00	0.00	c
16	*erythro*-β-MeTic	24.90	24.90	1.00	0.00	e
17	*threo*-β-MeTic	28.89	29.00	1.01	0.70	e
	Tetraline analogues					
19	Atc	9.05	9.05	1.00	0.00	b
20	Hat	13.05	13.05	1.00	0.00	b
	Tryptophan analogue					
21	Tcc	27.96	27.96	1.00	0.00	d

Temperature programme of column thermostat: [a] 80–200°C at 10 C°/min; [b] 80–200°C at 5 C°/min; [c] 80–200°C at 2 C°/min; [d] 80–200°C at 1 C°/min; [e] 80–100°C at 5 C°/min, 100–200°C at 1 C°/min; dead time of column, t_0=2.40 min.

retention factors, e.g. 2′-MePhe, 4′-MePhe and 2′,6′-diMePhe. The enantiomers of *erythro*- and *threo*-β-MePhe have the same retention factors under these analysis conditions and therefore separation of the epimers, the four enantiomers, in one chromatogram, needs special conditions [31]. α-MePhe as the GITC derivative showed no resolution in 0.1% aqueous TFA–methanol (45:55, v/v) as eluent. Decrease of the methanol content to 50% increased the resolution slightly (Table 3), and the separation of the α-MePhe enantiomers was therefore more favourable as the FDAA derivatives. Surprisingly, the elution sequence was different for the GITC derivative: the elution sequence observed was D before L.

The results of the separation of enantiomers of tyrosine analogues are shown in Table 4. Most of the enantiomers could be separated with $R_S>1.5$, keeping the retention factor of the component eluted second below 5. As regards a comparison of the phenylalanine and tyrosine analogues, to obtain similar retention factors the tyrosine analogues should be chromatographed in a water-rich mobile phase system, indicating the difference in hydrophobicity of the two homologous series.

The epimers of 2*S*,3*R*-L-*threo*- and 2*S*,3*S*-L-*erythro*-β-MeTyr or 2*R*,3*S*-D-*threo*- and 2*R*,3*R*-D-*erythro*-β-MeTyr exhibited better separation than that observed for *threo*-L- and *erythro*-L-β-MePhe or for

Table 2
Retention factors (k), separation factors (α) and resolutions (R_S) of unusual amino acids measured on the Crownpak CR(+) column

	Compound	k_D	k_L	α	R_S	Detection wavelength λ (nm)	Conditions of analysis
	Phe analogues						
1	2′-MePhe	17.20	21.04	1.22	2.10	195	a
		24.66	35.80	1.45	3.80	195	b
2	4′-MePhe	22.26	30.08	1.35	3.35	195	a
		33.14	50.67	1.53	4.70	195	b
3	*erythro*-β-MePhe	13.15	20.90	1.59	3.50	193	a
		21.67	35.98	1.66	6.20	193	b
4	*threo*-β-MePhe	14.98	14.98	1.00	0.00	195	a
		17.78	18.63	1.05	<0.40	195	b
5	2′,6′-diMePhe	31.42	33.60	1.07	0.80	201	a
6	α-MePhe	14.21	14.21	1.00	0.00	193	b
	Tyr analogues						
7	*meta*-Tyr	4.78	7.40	1.55	3.35	195	a
		9.25	16.84	1.82	6.06	195	b
8	2′-MeTyr	8.60	11.48	1.35	2.30	198	a
		18.49	27.10	1.47	3.54	198	b
9	*erythro*-β-MeTyr	9.05	11.90	1.31	3.10	195	a
		17.09	25.05	1.47	4.20	195	b
10	*threo*-β-MeTyr	7.60	7.60	1.00	0.00	195	a
		13.15	13.15	1.00	0.00	195	b
11	2′,6′-diMeTyr	12.10	13.41	1.11	1.00	200	a
		26.06	29.47	1.13	1.50	200	b
	Tetraline analogues						
19	Atc	21.90	21.90	1.00	0.00	194	a
20	Hat	8.11	8.11	1.00	0.00	198	a
	Tryptophan analogue						
21	Tcc	45.70	48.10	1.05	0.95	220	a

Column, Crownpak CR(+); mobile phase, perchloric acid; condition of analysis: [a] pH 2.0, flow-rate, 0.75 ml/min, temperature 20°C; [b] pH 2.0, flow-rate, 0.75 ml/min, temperature 5°C; dead-time of column, t_o=1.40 min.

threo-D- and *erythro*-D-β-MePhe. The L enantiomers could be separated as the FDAA derivatives, while the D enantiomers separated better as the GITC derivatives.

The results of the separation of the enantiomers of Tic and tryptophan analogues are given in Table 5. The Tic analogues could be separated well with relatively small k values, in contrast with GC separation, where large retention factors were characteristic. *threo*-β-MeTic could not be separated as the GITC derivative at any mobile phase composition, whereas a good resolution was achieved as the FDAA derivative. The enantiomers of α-MeTic as the GITC derivative were coeluted, but no reaction product was observed with FDAA, probably due to the steric hindrance. Tcc (21) could be separated well as the FDAA derivative, while as the GITC derivative no reaction was observed, we could detect only the excess of the reagent on the chromatogram.

The sequence of elution of the enantiomers of all the investigated amino acids was checked by co-chromatography of amino acids with one enantiomer and was found to be L isomer before D isomer, with the exception of the FDAA derivatives of HO-Tic-3 (15) and Tcc (21), and the GITC derivative of

Table 3
Retention factors (k), separation factors (α) and resolutions (R_S) of phenylalanine analogues measured as GITC and FDAA derivatives

	Composition of eluent TFA–CH_3OH	k_L	k_D	α	R_S
GITC derivatives					
1	2′-MePhe				
	45:55	2.61	4.01	1.54	1.78
2	4′-MePhe				
	45:55	3.04	4.88	1.61	2.08
3	*erythro*-β-MePhe				
	45:55	2.00	3.17	1.59	2.60
4	*threo*-β-MePhe				
	45:55	2.00	3.17	1.59	2.60
5	2′,6′-diMePhe				
	45:55	3.62	5.39	1.49	4.15
6	α-MePhe				
	45:55	3.13	3.13	1.00	0.00
	50:50	7.50[a]	7.90[a]	1.05	<0.40
FDAA derivatives					
1	2′-MePhe				
	40:60	1.69	3.67	2.17	3.37
	35:65	1.02	2.08	2.04	3.27
2	4′-MePhe				
	40:60	1.94	4.43	2.28	4.12
	35:65	1.02	2.11	2.07	2.72
3	*erythro*-β-MePhe				
	40:60	1.90	4.13	2.17	6.30
	35:65	0.98	1.88	1.92	3.60
4	*threo*-β-MePhe				
	40:60	1.90	4.13	2.17	6.30
	35:65	0.98	2.00	2.04	3.90
5	2′,6′-diMePhe				
	40:60	3.86	7.62	1.97	9.30
	35:65	1.65	3.17	1.92	4.56
6	α-MePhe				
	40:60	2.44	3.20	1.31	1.65
	35:65	1.17	1.50	1.28	1.05

Column, Vydac 218TP54; flow-rate, 0.8 ml/min; detection, 250 nm for GITC derivatives, 340 nm for FDAA derivatives; TFA, 0.1% aqueous solution of trifluoroacetic acid; dead-time of column, t_o=3.50 min; [a] elution sequence D before L.

α-Me-Phe (6), where the enantiomers were eluted in the sequence D before L. (In the case of Tic-1, the elution sequence was presumed on analogy).

The results obtained by application of the different methods show that there was no method that met all the requirements. The methods differ in the times needed for analysis and for precolumn derivatization, in the difficulty and accuracy of the procedures before the analysis, etc. Table 6 lists the methods proposed for enantiomeric separation.

Table 4
Retention factors (k), separation factors (α) and resolutions (R_S) of tyrosine analogues measured as GITC and FDAA derivatives

	Composition of eluent TFA–CH_3OH	k_L	k_D	α	R_S
GITC derivatives					
7	*meta*-Tyr				
	55:45	2.98	4.74	1.59	1.80
8	2′-MeTyr				
	55:45	3.14	4.88	1.55	3.20
9	*erythro*-β-MeTyr				
	55:45	3.25	4.86	1.50	3.48
10	*threo*-β-MeTyr				
	55:45	3.01	4.65	1.54	3.54
11	2′,6′-diMeTyr				
	55:45	3.61	4.93	1.36	3.10
FDAA derivatives					
7	*meta*-Tyr				
	55:45	2.81	5.63	2.01	6.00
	50:50	1.53	2.85	1.86	2.35
8	2′-MeTyr				
	55:45	4.02	7.06	1.76	6.10
	50:50	1.82	3.03	1.66	3.90
9	*erythro*-β-MeTyr				
	55:45	3.03	8.44	2.78	10.35
	50:50	1.45	3.65	2.51	6.20
10	*threo*-β-MeTyr				
	55:45	5.06	8.44	1.67	5.55
	50:50	2.38	3.65	1.53	3.30
11	2′,6′-diMeTyr				
	55:45	7.33	9.38	1.28	3.15
	50:50	3.31	4.05	1.22	2.05

Column, Vydac 218TP54; flow-rate, 0.8 ml/min; detection, 250 nm for GITC derivatives, 340 nm for FDAA derivatives; TFA, 0.1% aqueous solution of trifluoroacetic acid; dead-time of column, t_o=3.50 min.

4. Conclusions

The described procedures can be applied for the separation and identification of conformationally constrained unusual aromatic amino acids. The methods permit a check of the configurations of amino acids after synthesis and their incorporation into peptides. The GC and direct HPLC separation methods are useful for the separation of phenylalanine

Table 5
Retention factors (k), separation factors (α) and resolutions (R_S) of Tic and tryptophan analogues measured as GITC and FDAA derivatives

	Composition of eluent TFA–CH_3OH	k_L	k_D	α	R_S
GITC derivatives					
12	Tic-1				
	45:55	1.15	1.74	1.52	1.38
13	Tic-3				
	45:55	1.31	1.95	1.49	1.61
14	5′-MeTic-3				
	45:55	2.36	3.46	1.47	2.60
15	HO-Tic-3				
	45:55	0.59	0.98	1.66	2.10
16	*erythro*-β-MeTic				
	45:55	1.95	2.85	1.46	2.95
17	*threo*-β-MeTic				
	45:55	1.62	1.62	1.00	0.00
18	α-MeTic				
	45:55	6.83	6.83	1.00	0.00
21	Tcc				
	45:55	–	–	–	–
FDAA derivatives					
12	Tic-1				
	40:60	1.92	2.32	1.16	1.02
13	Tic-3				
	40:60	1.54	3.05	1.98	4.65
14	5′-MeTic-3				
	40:60	2.67	5.77	2.16	6.80
15	HO-Tic-3				
	40:60	1.43[a]	0.80[a]	1.78	2.15
16	*erythro*-β-MeTic				
	40:60	2.39	2.85	1.20	1.40
17	*threo*-β-MeTic				
	40:60	2.90	3.65	1.25	2.05
18	α-MeTic				
	40:60	–	–	–	–
21	Tcc				
	40:60	4.49[a]	2.23[a]	2.01	5.95

Column, Vydac 218TP54; flow-rate, 0.8 ml/min; detection, 250 nm for GITC derivatives, 340 nm for FDAA derivatives; TFA, 0.1% aqueous solution of trifluoroacetic acid; dead-time of column, t_o=3.50 min; [a] elution sequence D before L.

and tyrosine analogues, while most of the enantiomers of Tic, tetraline and tryptophane analogues can be separated as GITC or FDAA derivatives on an achiral column. The application of direct and indirect methods ensures the availability of effective chromatographic procedures for chiral purity control.

Table 6
Methods proposed for the separation of enantiomers of unusual amino acids

	Compound	Methods			
		a	b	c	d
1	2′-MePhe	+	+	+	+
2	4′-MePhe	+	+	+	+
3	*erythro*-β-MePhe	−	+	+	+
4	*threo*-β-MePhe	+	−	+	+
5	2′,6′-diMePhe	+	(+)	+	+
6	α-MePhe	−	−	−	+
7	*meta*-Tyr	+	+	+	+
8	2′-MeTyr	+	+	+	+
9	*erythro*-β-MeTyr	−	+	+	+
10	*threo*-β-MeTyr	+	−	+	+
11	2′,6′-diMeTyr	+	+	+	+
12	Tic-1	(+)	−	+	+
13	Tic-3	(+)	−	+	+
14	5′-MeTic-3	(+)	−	+	+
15	HO-Tic-3	−	-	+	+
16	*erythro*-β-MeTic	−	−	+	+
17	*threo*-β-MeTic	−	−	−	+
18	α-MeTic	−	−	−	−
19	Atc	−	−	+[e]	+[e]
20	Hat	−	−	+[e]	+[e]
21	Tcc	−	(+)	−	+

Condition of analysis: [a] GC, Chirasil-L-Val column; [b] HPLC, direct separation on Crownpak CR(+) column; [c] HPLC, indirect separation as GITC derivative; [d] HPLC, indirect separation as FDAA derivative; +, baseline separation, R_S>1.5; (+) partial separation, 1.5>R_S>0.8; −, no separation, R_S<0.8; [e] see reference [30].

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