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Separation of enantiomeric β-methyl amino acids and of β-methyl amino acid containing peptides

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Abstract

Erythro-D,L- and *threo*-D,L-β-methylphenylalanine, -β-methyltyrosine and -β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid were synthesized. High-performance liquid chromatographic methods were developed for the separation and identification of the enantiomers of the β-methyl amino acids, with the application of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate as derivatizing reagents. These amino acids were incorporated into the μ-agonist/δ-antagonist opioid peptides H-β-MeTyr-Tic-Phe-Phe-NH_2, H-Tyr-Tic-β-MePhe-Phe-NH_2 and H-Tyr-Tic-Phe-β-MePhe-NH_2, and the δ-antagonist H-Tyr-β-MeTic-Phe-Phe-OH, by solid-phase peptide synthesis. Each peptide has four stereoisomers. The peptide stereoisomers were separated on different columns and in different eluent systems and the elution order of the peptide epimers was determined.

Keywords: Enantiomer separation; Derivatization, LC; Amino acid, β-methyl; Peptide

1. Introduction

In the synthesis of receptor-selective peptides, unusual amino acids are often used. These unnatural amino acids can be prepared either in racemic form (which is usually the fastest method) or by asymmetric synthesis. Even when the latter strategy is applied, the amino acid is often not chirally pure and small amounts of the other stereoisomers can be present.

After the incorporation of these amino acids into the peptides, the resulting epimers may have similar or different physicochemical or biological properties. Diastereoisomers of peptides (the peptide epimers) with biological activity often exhibit antagonistic or agonistic properties which differ greatly. It is therefore very important to have chirally pure substances. The crude peptides are purified by reversed-phase high-performance liquid chromatographic (RP-HPLC) methods. In many cases, these purifications are difficult, and chromatographic analysis of the epimeric peptides leads to overlapping peaks. Another problem is the determination of the configurations of the asymmetric centres of the unusual amino acids in the diastereomeric (epimeric) peptides. The latter underlines the importance of having at hand effective chromatographic methods for the separation and identification of enantiomeric amino acids.

Many attempts have been made to resolve amino

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acid enantiomers by liquid chromatographic techniques. The enantioselective separations performed by RP-HPLC methods can be divided into three main groups: direct separation on chiral stationary phases [1–3]; separation on achiral columns with chiral eluents [3–5]; and separation of the diastereoisomers formed by precolumn derivatization with chiral reagents [3,6–17].

For the study of analogues of H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic-Phe-Phe-NH_2 (TIPP–NH_2) opioid peptide, three conformationally constrained aromatic amino acids have been prepared for their syntheses (Fig. 1) [nomenclature and abbreviations are according to IUPAC-IUB JCBN recommendations [18]. The amino acids have L-configuration unless otherwise indicated. In addition, Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid]: (I) *erythro*-[(2*S*,3*S*),(2*R*,3*R*)]- and *threo*-[(2*S*,3*R*), (2*R*,3*S*)]-β-methylphenylalanine (β-MePhe); (II) *erythro*-[(2*S*,3*S*),(2*R*,3*R*)]-, and *threo*-[(2*S*,3*R*), (2*R*,3*S*)]-β-methyltyrosine (β-MeTyr); and (III) *erythro*-[(2*S*,3*S*),(2*R*,3*R*)]- and *threo*-[(2*S*,3*R*), (2*R*,3*S*)]-1,2,3,4-tetrahydroisoquinoline-β-methyl-3-carboxylic acid (β-MeTic). All these amino acids have two chiral centres, and four stereoisomers are possible.

The present paper deals with the separation of all four stereoisomers of these amino acids by using precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), and with the separation of the peptides. β-MeTyr was incorporated in position 1 into H-β-MeTyr-Tic-Phe-Phe-NH_2 [(β-MeTyr^1)TIPP–NH_2]. β-MePhe was incorporated in position 3 into H-Tyr-Tic-β-MePhe-Phe-NH_2 [(β-MePhe^3)TIPP–NH_2] and in position 4 into H-Tyr-Tic-Phe-β-MePhe-NH_2 [(β-MePhe^4)TIPP–NH_2]. β-MeTic was incorporated in position 2 into H-Tyr-β-MeTic-Phe-Phe-OH, [(β-MeTic^2)TIPP–OH]. The syntheses were carried out with *erythro*-D,L-or *threo*-D,L-isomers, and thus two pairs of diastereoisomers of the peptides were formed. Methods were developed for separation of the pairs of diastereomers

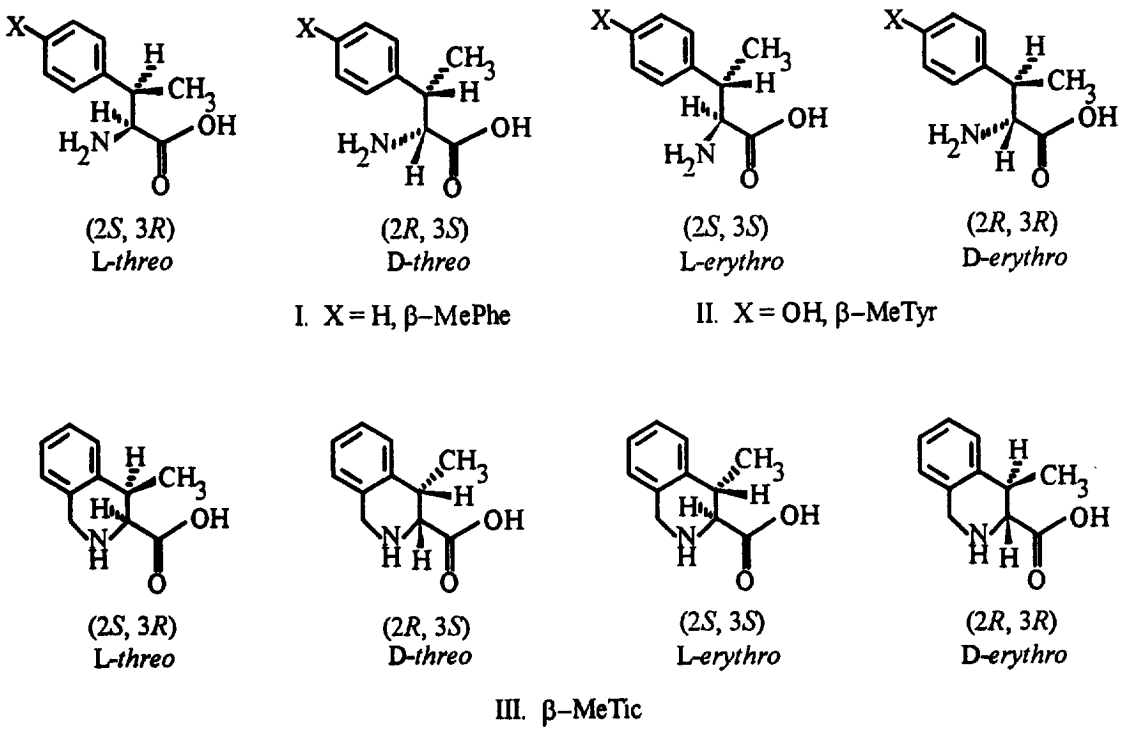

Fig. 1. Structures of 4 stereoisomers of β-methyl amino acids: (I) β-methylphenylalanine (β-MePhe); (II) β-methyltyrosine (β-MeTyr); (III) 1,2,3,4-tetrahydroisoquinoline-β-methyl-3-carboxylic acid (β-MeTic).

and for establishment of the configurations of the chiral centres in these stereoisomers.

2. Experimental

2.1. Chemicals and reagents

β-MePhe in *erythro* and *threo* form and standard (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)- and (2*R*,3*S*)-β-MePhe for chromatographic identification were prepared by a slight modification of the method of Kataoka et al. [19,20].

β-MeTic in *erythro* and *threo* form was prepared in our laboratory by means of the Pictet–Spengler reaction, using β-MePhe and formaldehyde under acidic conditions [21,22].

β-MeTyr in *erythro* or *threo* forms was prepared by a modified literature procedure [23,24]. Pure *erythro*-[(2*S*,3*S*),(2*R*,3*R*)] and *threo*-[(2*S*,3*R*), (2*R*,3*S*)]-β-Me amino acids were obtained by the chromatographic separation method described in this paper.

The identities of the β-methyl amino acids were checked by means of melting point measurement, FAB mass spectrometry, ^{1}H NMR spectroscopy and chiral TLC [25].

TIPP and TIPP–NH_2 analogues were synthesized by solid-phase methods reported previously for TIPP by Schiller et al. [26], using Merrifield resin for β-MeTic-TIPP analogues and *p*-methylbenzhydrylamine resin for the peptides with a C-terminal amide. The configurations of the β-methyl amino acids in the peptides were determined by means of enzymatic digestion, chiral TLC, and derivatization with GITC or FDAA reagents (see later). Details of the syntheses and biological activities will be reported elsewhere.

GITC was purchased from Aldrich Chemie (Steinheim, Germany), FDAA from Pierce Chemical Company (Rockford, IL, USA), L-amino acid oxidase type I from Sigma (St. Louis, MO, USA), and trifluoroacetic acid (TFA), potassium dihydrogenphosphate 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 doubly distilled water and further purified by pumping through a 0.45-μm filter Type HV Millipore (Molsheim, France).

2.2. Apparatus

HPLC measurements were performed with three chromatographic systems: (A) the Waters system consisted of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA); (B) the Spectra-Physics system consisted of an SP-4000 low-pressure gradient pump with an SP-2000 variable-wavelength detector and an SP-4600 integrator (Spectra-Physics Analytical, Fremont, CA, USA); and (C) the Gilson Auto-Prep system contained type 302 and 303 pumps, a type 115 detector and a type 712 system controller (Gilson Medical Electronics, Villiers le Bel, France).

The columns used for analytical separations were: (I) Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5 μm particle size (The Separations Group, Hesperia, CA, USA); (II) Nova Pak C_{18} (150×3.9 mm I.D.), 4 μm particle size (Waters Chromatography); and (III) Hyperpep 300 C_{18} (250×4.6 mm I.D.), 5 μm particle size (Shandon Scientific, Astmoor, Runcorn, Cheshire, UK). For semi-preparative separations, Vydac 218TP1010 C_{18} (250×10 mm I.D.), 10 μm particle size, and Vydac 218TP101522 C_{18} (250×22 mm I.D.), 10–15 μm particle size (The Separations Group) columns were used.

2.3. Identification of enantiomers of β-methyl amino acids

The elution sequence of *erythro*-D- and L-β-MePhe, and *threo*-D- and L-β-MePhe was determined either by enzymatic degradation of the amino acids with L-amino acid oxidase or with standards of β-MePhe synthesized by the method of Kataoka et al. [19]. For enzymatic digestion, 1 mg of *erythro*-D,L- or *threo*-D,L-β-MePhe was dissolved in 200 μl of 0.1 *M* Tris buffer (pH 7.2) in a test-tube and 10 μl of L-amino acid oxidase was added. The test-tube was filled with oxygen, tightly capped and incubated

for 24 h at 37°C. The reaction product was evaporated by a flow of argon and was used for derivatization reactions.

The elution order of *erythro*-D,L-, and *threo*-D,L-β-MeTyr and *erythro*-D,L-β-MeTic was determined similarly, by enzymatic digestion with L-amino acid oxidase. An example for the chromatogram of product of enzymatic digestion is shown in Fig. 2. This enzyme was ineffective in the enzymatic digestion of *threo*-D,L-β-MeTic. The peaks of *threo*-D,L- β-MeTic were identified via a standard of *threo*-L-β-MeTic made from *threo*-L-β-MePhe by the Pictet–Spengler method [21].

2.4. Derivatization of β-methyl amino acids

An amount of 0.5–1 mg of *erythro*-D,L- or *threo*-D,L-amino acids was derivatized with FDAA by the method of Marfey [10], or with GITC by the method of Nimura et al. [7].

2.5. Peptide hydrolysis

The TIPP analogues containing different stereoisomers of β-MeTyr in position 1, of β-MeTic in position 2 and of β-MePhe in positions 3 and 4 were hydrolysed under argon pressure in 6 *M* HCl in teflon bombs in a microwave oven [27]. The solvent was removed by flushing with argon. The dried samples were used for different types of derivatization. The racemization at the α-carbon of the β-methyl amino acids under these conditions was below 1%.

3. Results and discussion

3.1. Separation of erythro and threo isomers of β-methyl amino acids

The syntheses of β-methyl amino acids I and II led to mixtures of both diastereoisomers (*threo* and

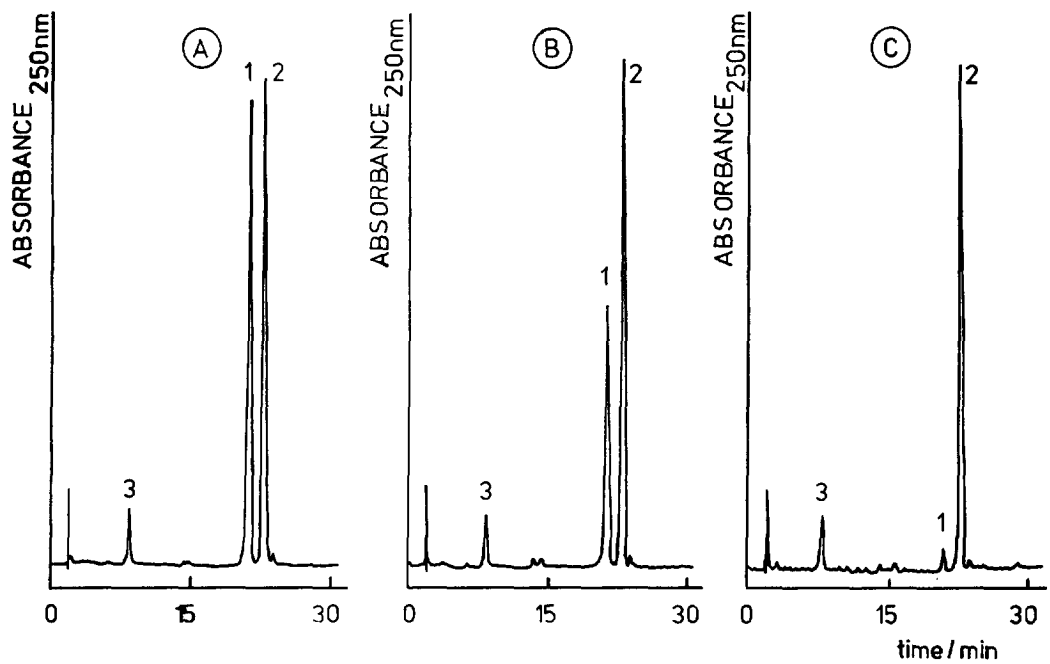

Fig. 2. Chromatographic pattern of D,L-*erythro*-β-MeTyr after digestion with L-amino acid oxidase. Digestion time: (A) blank; (B) 10 h; (C) 20 h. The degradation product of enzymatic digestion was reacted with GITC (see Experimental section). Column, Vydac 218TP54 C_{18}; flow-rate, 1.5 ml/min; detector, 250 nm. Composition of eluent: (A) 0.1% TFA; (B) 0.1% TFA–acetonitrile 50:50; gradient elution, 30–70% B in 30 min. Peaks: 1=L-*erythro*-β-MeTyr; 2=D-*erythro*-β-MeTyr; 3=unreacted GITC.

erythro), which can be partially separated by crystallization, but the product is always contaminated with the opposite diastereoisomer, i.e., the *erythro* isomer with the *threo* isomer and vice versa. Purification via the crystallization procedure is very time-consuming, and is especially difficult for the *threo* isomers. An HPLC method was developed for the purification of these amino acids and the results are shown in Fig. 3. D,L-*threo*- and D,L-*erythro*-β-MePhe were separated on a Vydac 218TP54 column in water containing 0.1–0.2% acetonitrile as eluent under isocratic conditions. D,L-*threo*- and D,L-*erythro*-β-MeTic were separated on the same column by means of gradient elution, the acetonitrile content in the water being raised from 0 to 3% within 15 min, while the separation of D,L-*threo*- and D,L-*erythro*-β-MeTyr was carried out in pure water as eluent. The procedure is suitable for the separation of diastereoisomers of these amino acids on a preparative scale; the purity of the product is better than 99.5%.

3.2. Separation of enantiomers of β-methyl amino acids

The separation of the D- and L- isomers of the *erythro*- and *threo*-β-methyl amino acids was carried out with the compounds in derivatized form, applying GITC or FDAA as derivatizing reagent. The mobile phase systems used were an aqueous solution of 0.1% TFA or 0.01 *M* potassium dihydrogenphosphate (pH 3) (phosphate buffer) containing methanol or acetonitrile as organic modifier.

The most important results relating to the separation of the β-MePhe isomers are shown in Table 1. The GITC derivatives of the β-MePhe isomers gave only a partial separation. In the TFA–methanol system, the L-*erythro* and L-*threo* isomers eluted very close to one another or in one peak, whereas in the acetonitrile-containing system, this was the case for the D-*erythro* and D-*threo* isomers. Even the ternary system with different ratios remained ineffec-

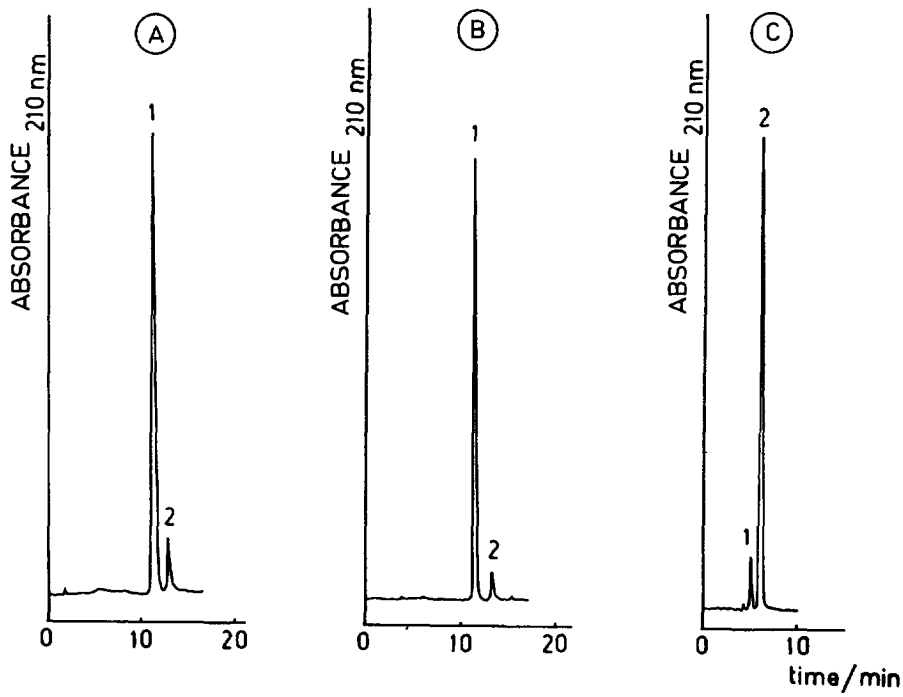

Fig. 3. Separation of D,L-*threo* and D,L-*erythro* isomers of β-methyl amino acids: (A) β-MePhe; (B) β-MeTic; (C) β-MeTyr. Column, Vydac 218TP54 C_{18}; flow-rate, 1.5 ml/min; detector, 210 nm. Eluent: (A) 0.1% acetonitrile in water; (B) gradient elution, 0–3% acetonitrile in water within 15 min; (C) water. Peaks: 1=D,L-*threo* isomer; 2=D,L-*erythro* isomer.

Table 1
Retention factors (k') and resolutions (R_S) of 4 diastereoisomers of β-MePhe–GITC and β-MePhe–FDAA derivatives

Composition of eluent	k'				R_S		
	L-*erythro*	L-*threo*	D-*erythro*	D-*threo*	L–L	L–D	D–D
β-MePhe–GITC derivatives							
$TFA–CH_3CN–CH_3OH$							
70 : 30 : 0	9.21	9.48	11.86	11.86	0.70	3.75	0
55 : 0 : 45	9.22	9.42	15.79	16.75	<0.40	9.10	1.32
60 : 6 : 34	9.12	9.43	15.17	15.78	0.65	7.85	0.75
β-MePhe–FDAA derivatives							
$KH_2PO_4–CH_3CN–CH_3OH$							
70 : 30 : 0	5.93	6.20	11.80	11.80	0.95	9.72	0
50 : 0 : 50	3.54	3.94	9.93	10.84	1.06	9.68	1.48
53 : 7 : 40	6.06	6.65	15.35	16.60	1.40	14.20	1.54

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detector, 250 nm (GITC derivatives), 340 nm (FDAA derivatives); TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogen phosphate (pH 3). $R_{S,L-L}$, $R_{S,L-D}$ and $R_{S,D-D}$ represent separation of 4 successive isomers.

tive. Change of the TFA in the mobile phase to the phosphate buffer did not improve the separation. The FDAA derivatives gave a better separation when the phosphate buffer was used with methanol as organic modifier. Quantitative separation of the four isomers was achieved through the application of a few percent of acetonitrile in a phosphate buffer–methanol mobile phase (Fig. 4A). The elution sequence,

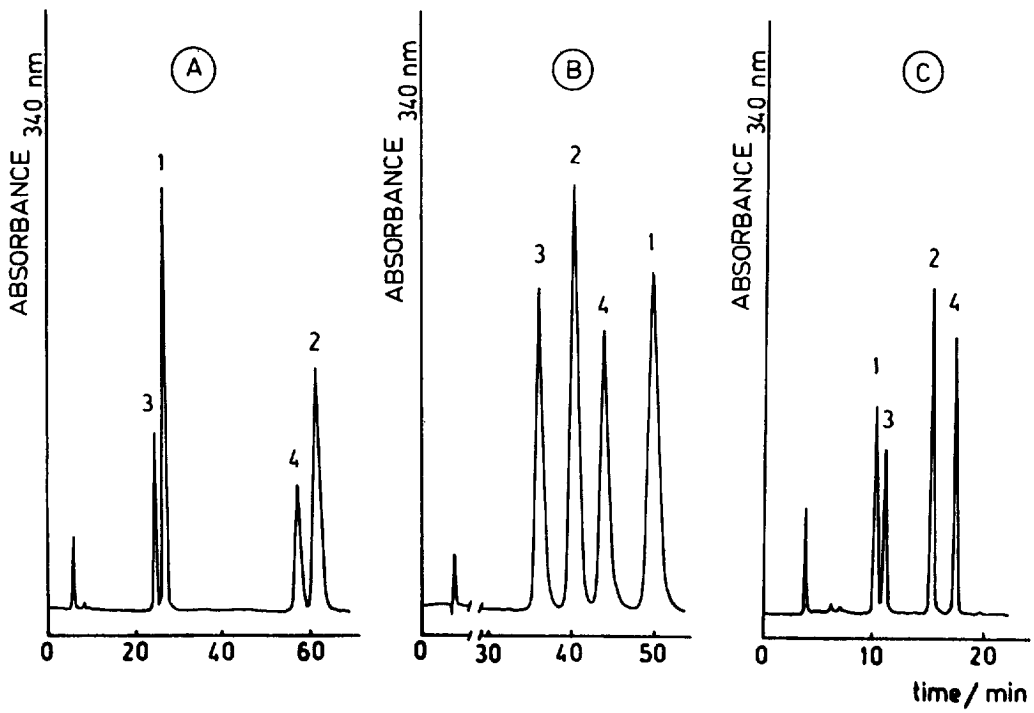

Fig. 4. Chromatograms of FDAA derivatives of β-methyl amino acids: (A) β-MePhe; (B) β-MeTic; (C) β-MeTyr. Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detector, 340 nm. Composition of eluent: (A) phosphate buffer (pH 3)–acetonitrile–methanol=53:7:40 v/v; (B) 0.1% TFA–acetonitrile–methanol=52:11.5:36.5 v/v; (C) phosphate buffer (pH 3)–methanol=36:64 v/v. Peaks: 1=L-*threo* isomer; 2=D-*threo* isomer; 3=L-*erythro* isomer; 4=D-*erythro* isomer.

Table 2
Retention factors (k') and resolutions (R_s) of four diastereoisomers of β-MeTic–GITC and β-MeTic–FDAA derivatives

Composition of eluent	k'				R_s		
	L-*threo*	D-*threo*	L-*erythro*	D-*threo*	L–D	D–L	L–D
β-MeTic–GITC derivatives							
TFA–CH_3CN–CH_3OH							
65:35:0	3.71	3.71	4.37	5.57	0	1.70	1.90
45:0:55	1.92	1.92	2.27	3.21	0	1.55	3.45
	L-*erythro*	D-*threo*	D-*erythro*	L-*threo*	L–D	D–D	D–L
β-MeTic–FDAA derivatives							
TFA–CH_3CN–CH_3OH							
65:35:0	5.35	6.04	6.95	5.35	–[a]	–[a]	–[a]
45:0:55	4.30	6.40	5.09	5.09	–[a]	–[a]	–[a]
51:12:37	9.36	10.55	11.60	13.28	2.25	1.74	1.28

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detector, 250 nm (GITC derivatives), 340 nm (FDAA derivatives); TFA 0.1% aqueous solution of trifluoroacetic acid. $R_{s,L-D}$, $R_{s,D-L}$, $R_{s,L-D}$ (GITC derivatives) and $R_{s,L-D}$, $R_{s,D-D}$, $R_{s,D-L}$ (FDAA derivatives) represent separation of four successive isomers, in the order indicated in the table headings.
[a] Because the elution order is different and the separation is not complete, the R_s values are not indicated.

determined either by enzymatic digestion or by standard addition, was: L-*erythro*<L-*threo*<D-*erythro*<D-*threo*.

The results of the separation of the β-MeTic isomers are summarized in Table 2. A partial separation was achieved in the case of the GITC derivatives: the L-*threo* and D-*threo* isomers are not separable, either in methanol or in the acetonitrile system. Application of the phosphate buffer instead of TFA in the mobile phase does not improve the separation. The β-MeTic–FDAA derivatives in TFA–acetonitrile or TFA–methanol mobile phase systems undergo a partial separation. In the eluent containing acetonitrile, the elution sequence is L-*erythro*=L-*threo*<D-*threo*<D-*erythro*, while in the methanol system it is L-*erythro*<L-*threo*=D-*erythro*-<D-*threo*. A combination of the two organic modifiers leads to a quantitative separation of the β-MeTic–FDAA derivatives, with the elution sequence L-*erythro*<D-*threo*<D-*erythro*<L-*threo*. The elution sequence was determined by means of standard addition. The R_s values for all four components are better than 1.5 (Fig. 4B).

The isomers of β-MeTyr can be separated as

Table 3
Retention factors (k') and resolutions (R_s) of four diastereoisomers of β-MeTyr–GITC and β-MeTyr–FDAA derivatives

Composition of eluent	k'				R_s		
	L-*threo*	L-*erythro*	D-*threo*	D-*erythro*	L–L	L–D	D–D
β-MeTyr–GITC derivatives							
TFA–CH_3CN–CH_3OH							
80:20:0	10.60	11.33	14.95	16.05	1.32	5.50	1.39
55:0:45	6.60	7.07	9.53	10.02	1.15	4.77	0.85
β-MeTyr–FDAA derivatives							
TFA–CH_3CN–CH_3OH							
60:0:40	10.03	10.47	12.22	13.06	1.67	7.00	3.42
KH_2PO_4–CH_3CN–CH_3OH							
36:0:64	1.88	2.13	3.34	3.92	1.38	6.25	3.05

Column, Vydac 218TP54 C_{18}; detector and flow-rate, 250 nm and 0.4 ml/min (GITC derivatives), 340 nm and 0.8 ml/min (FDAA derivatives); TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogen phosphate (pH 3); $R_{s,L-L}$, $R_{s,L-D}$ and $R_{s,D-D}$ represent separation of four successive isomers.

either GITC or FDAA derivatives (Table 3). The GITC derivatives gave the best separation in the TFA–acetonitrile mobile phase system, at a flow-rate of 0.4 ml/min. Because of the low flow-rate and the long analysis time (2 h), this method is disadvantageous. Some experiments were carried out in phosphate buffer–acetonitrile or phosphate buffer–methanol systems, without attainment of any improvement in the separation. The elution sequence, determined by enzymatic assay, is L-*threo*<L-*erythro*<D-*threo*<D-*erythro*. For FDAA derivatives, the best separation can be achieved in TFA–methanol or phosphate buffer–methanol systems, with a considerably shorter time in the latter. The elution sequence, determined by enzymatic assay, is L-*threo*<L-*erythro*<D-*threo*<D-*erythro* (Fig. 4C). The application of acetonitrile as mobile phase component for FDAA derivatives was ineffective because of the lack of separation of the D-*erythro* and D-*threo* isomers.

3.3. Separation of epimers of TIPP analogues

The pure D,L-*erythro*- and D,L-*threo*-β-methyl amino acids were incorporated into TIPP analogues with free carboxylic acid or amide, in separate syntheses which resulted in two pairs of stereoisomers of the peptides. The crude peptide epimers were separated by HPLC in one of the chromatographic methods described below. The configurations of the β-methyl amino acids in each peptide were determined. Since racemization or epimerization (conversion of the L-*erythro* to the D-*threo* and the L-*threo* to the D-*erythro* isomer) may occur in the course of the synthesis of a peptide, it is important to have at hand chromatographic methods suitable for the separation and identification of all four stereoisomers.

3.4. Separation of (β-MeTyr[1])TIPP–NH_2 epimers

The results of the separation, carried out on a Vydac column, of (β-MeTyr[1])TIPP–NH_2 stereoisomers are summarized in Table 4. With acetonitrile as organic modifier in a TFA-containing mobile phase, only a partial separation was observed. The D-*erythro* and L-*erythro* stereoisomers of the peptide are not separable even at an acetonitrile content of 20%. Change of the organic modifier from acetonitrile to

Table 4
Retention factors (k') and resolutions (R_s) of four diastereoisomers of (β-MeTyr[1])TIPP-NH_2, (β-MePhe[3])TIPP-NH_2 and (β-MePhe[4])TIPP-NH_2 in different eluent systems

Composition of eluent	k'				R_s		
	D-*threo*	L-*threo*	D-*erythro*	L-*erythro*	D–L	L–D	D–L
(β-MeTyr[1])TIPP–NH_2							
TFA–CH_3CN–CH_3OH							
70:30:0	2.64	3.37	4.45	4.45	2.00	2.17	0
50:0:50	2.06	2.96	3.46	4.27	4.00	2.00	2.77
	L-*erythro*	L-*threo*	D-*threo*	D-*erythro*	L–L	L–D	D–D
(β-MePhe[3])TIPP–NH_2							
TFA–CH_3CN–CH_3OH							
67.5 : 32.5 : 0	1.84	2.15	6.31	7.44	1.28	11.06	1.76
70 : 30 : 0	2.95	3.51	11.11	12.38	1.49	14.20	3.15
	D-*threo*	L-*erythro*	L-*threo*	D-*erythro*	D–L	L–L	L–D
(β-MePhe[4])TIPP–NH_2							
TFA–CH_3CN–CH_3OH							
72.5:27.5:0	3.90	6.06	11.15	11.15	2.70	4.87	0
50:0:50	3.00	4.12	6.02	6.71	3.80	4.47	1.43

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detector, 210 nm; TFA, 01% aqueous solution of trifluoroacetic acid. $R_{s,D-L}$, $R_{s,L-D}$ and $R_{s,D-L}$ [(β-MeTyr[1])TIPP–NH_2], $R_{s,L-L}$, $R_{s,L-D}$ and $R_{s,D-D}$ [(β-MePhe[3])TIPP–NH_2] and $R_{s,D-L}$, $R_{s,L-L}$ and $R_{s,L-D}$ [(β-MePhe[4])TIPP–NH_2] represent separation of four successive peaks.

methanol improves the separation of the *erythro* isomers, and a good separation for all four epimers can be achieved within 20 min (Fig. 5A). The elution sequence for the four stereoisomers is D-*threo*<L-*threo*<D-*erythro*<L-*erythro*. The identification procedure is described in the Experimental section.

3.5. Separation of (β-MeTic²)TIPP–OH epimers

The results concerning the chromatographic behaviour of (β-MeTic²)TIPP–OH stereoisomers are summarized in Table 5. At a 50% acetonitrile content of the mobile phase, only two peaks were observed, while at a content of 30%, three peaks were observed on the Vydac column. A further decrease of the acetonitrile content does not improve the separation, but the increase in the k' values is disadvantageous. In the acetonitrile system, the peptide epimers containing L-isomers of β-MeTic are not separated (they eluted in the first peak), while peptides containing D-isomers can be separated well.

On change of the organic modifier from acetoni-

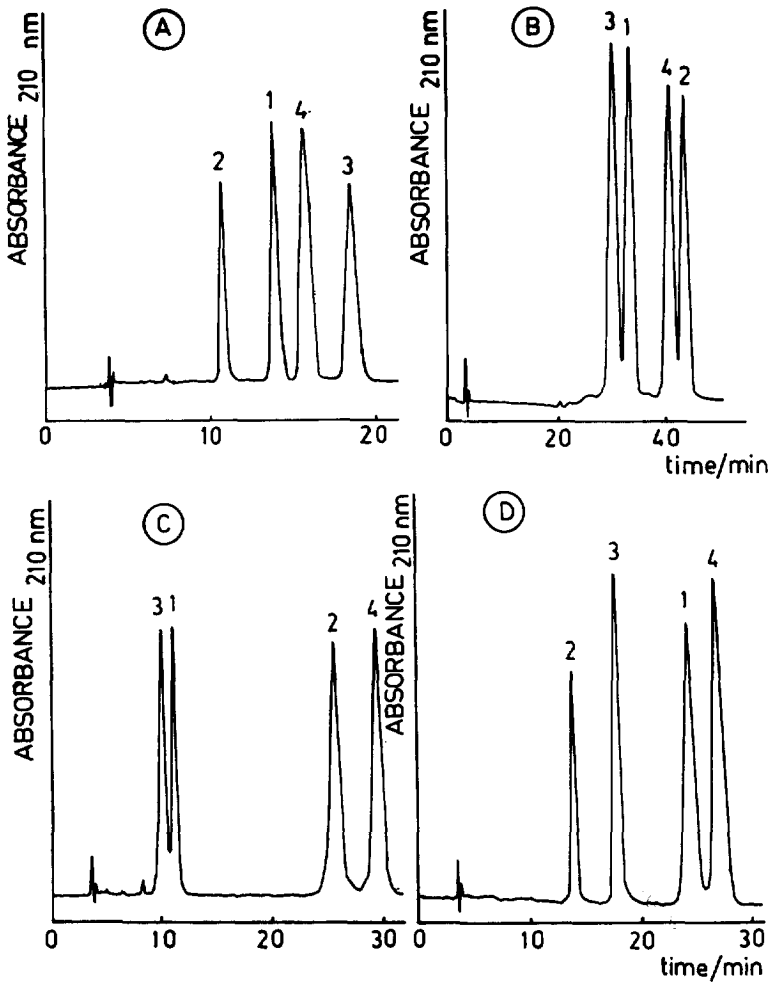

Fig. 5. Chromatograms of TIPP analogues: (A) (β-MeTyr¹)TIPP–NH_2; (B) (β-MeTic²)TIPP–OH; (C) (β-MePhe³)TIPP–NH_2; (D) (β-MePhe⁴)TIPP–NH_2. Columns: A, C and D, Vydac 218TP54 C_{18}; B, Hyperpep 300 C_{18}. Flow-rate, 0.8 ml/min; detector, 210 nm. Composition of eluent: (A) 0.1% TFA–methanol=50:50 v/v; (B) 0.1% TFA–acetonitrile–methanol=45:14:41 v/v; (C) 0.1% TFA–acetonitrile=57.5:32.5 v/v; (D) 0.1% TFA–methanol=50:50 v/v. Peaks: 1=L-*threo* isomer; 2=D-*threo* isomer; 3=L-*erythro* isomer; 4=D-*erythro* isomer.

Table 5
Retention factors (k') and resolutions (R_s) of four diastereoisomers of (β-MeTic^2)TIPP–OH in different eluent systems and columns

Column	Composition of eluent	k'				R_s		
	TFA–CH_3CN–CH_3OH	L-*erythro*	L-*threo*	D-*erythro*	D-*threo*	L–L	L–D	D–D
I	65:35:0	2.30	2.30	3.69	4.11	0	2.82	1.25
I	50:0:50	5.05	5.57	6.50	6.50	1.35	1.45	0
I	62:16:22	16.35	17.72	26.42	27.60	0.85	5.07	0.75
II	65:35:0	7.15	7.15	11.39	13.35	0	2.71	1.63
II	50:0:50	21.42	24.63	28.30	28.30	1.20	1.45	0
II	59:21:20	23.60	25.94	40.73	44.90	0.70	3.75	1.35
III	55:45:0	1.87	1.87	2.60	3.00	0	1.92	1.40
III	40:0:60	4.50	5.14	5.79	5.79	1.42	1.30	0
III	46:40:14	6.46	7.24	9.11	9.72	1.25	2.60	1.20

Columns: I, Vydac 218TP54 C_{18}; II, Nova Pak C_{18}; and III, Hyperpep 300 C_{18}. Flow-rate, 0.8 ml/min; detector, 210 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid. $R_{s,L-L}$, $R_{s,L-D}$ and $R_{s,D-D}$ represent separation of four successive peaks.

trile to methanol, the reverse situation was observed: peptides containing L-isomers were separated, while peptides containing D-isomers were not. The combination of the two organic modifiers resulted in only a partial separation of the four diastereoisomers. The elution sequence, determined as described earlier, is L-*erythro*<L-*threo*<D-*erythro*<D-*threo*.

Application of a Nova Pak C_{18} column was not successful, but the separation of D-*erythro*- and D-*threo*-β-MeTic containing peptides was better than observed on the Vydac column.

The best separation of the four diastereomers can be obtained on a Hyperpep 300 C_{18} column with a ternary solvent mixture. The R_s values for all components are better than 1.2 (Fig. 5B).

3.6. Separation of (β-MePhe^3)TIPP–NH_2 epimers

The results of the separation of (β-MePhe^3)TIPP–NH_2 stereoisomers are shown in Table 4. The quantitative separation of all four isomers in one chromatogram can be achieved on the Vydac column in a TFA–acetonitrile system. The elution sequence for the four stereoisomers is L-*erythro*<L-*threo*<D-*threo*<D-*erythro* (Fig. 5C). The procedure of identification of the isomers is the same as described earlier.

3.7. Separation of (β-MePhe^4)TIPP–NH_2 epimers

The results of the separation experiments are summarized in Table 4. Application of acetonitrile as organic modifier in the TFA-containing mobile phase was unsuccessful: only 3 peaks appeared in the chromatogram, even below an acetonitrile content of 20%. The change of acetonitrile to methanol in the mobile phase results in a good separation of all four isomers within a short time. The elution order is different from that observed in the case of (β-MePhe^3)TIPP–NH_2: D-*threo*<L-*erythro*<L-*threo*<D-*erythro* (Fig. 5D).

4. Conclusions

The described procedures can be applied for the preparation of pure D,L-*erythro* or D,L-*threo*-β-methyl amino acids and for the separation and identification of their enantiomers and peptides containing them. For the separation of enantiomers of β-methyl amino acids, FDAA as derivatizing reagent proved more effective than GITC.

Our results demonstrate that the RP-HPLC separation of L- from D-β-methyl amino acid-containing peptides is especially good. However, separation of the *erythro* from the *threo* isomers (epimers at the β-carbon) may be difficult. A synthetic strategy which applies racemic *erythro*- or *threo*-β-methyl amino acids for incorporation into peptides is therefore acceptable in view of the good separation of the L- and D-stereoisomers. Since small amounts of the β-carbon epimers (e.g., L-*threo* in L-*erythro*) in the final product are generally difficult to remove, par-

ticular attention should be paid to the diastereomeric purity of the *threo* or *erythro* amino acids.

There is no general rule for the elution sequence for derivatized amino acid enantiomers or for peptide epimers. This fact underlines the importance of the identification of individual peaks.

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