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Monitoring of optical isomers of some conformationally constrained amino acids with tetrahydroisoquinoline or tetraline ring structures. Part II

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Abstract

Conformationally constrained amino acids were synthesized in chiral or racemic forms: D- and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), the *erythro*-D,L-4-methyl analogue, D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D- and L-7-hydroxy-6,8-diiodo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, D,L-2-aminotetraline-2-carboxylic acid, D,L-6-hydroxy-2-aminotetraline-2-carboxylic acid and D,L-6-methoxy-2-aminotetraline-2-carboxylic acid. The optical isomers were characterized and identified by applying precolumn derivatization with chiral reagents (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) and enzymatic digestion with L-amino acid oxidase, carboxypeptidase A and α-chymotrypsin. The HPLC conditions (pH, eluent composition and different buffers) were varied to obtain optimum separations.

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 synthesis of these sterically constrained or biased unusual amino acids either leads to a racemic form, while the asymmetric synthesis may lead to a product which contains only a minor amount of the enantiomeric form. Furthermore, the originally pure amino acids can racemize to some extent in the course of the N^{α}-Boc-protection or during the synthesis of biologically active peptides, resulting in the diastereomers of the peptides. 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.

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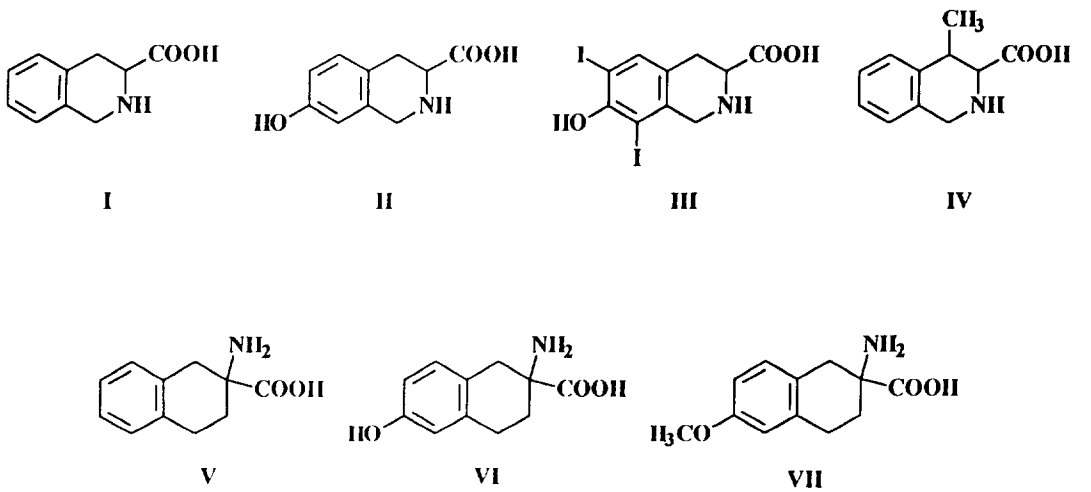

I II III IV

V VI VII

Fig. 1. Structures of the compounds used. I = D- and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic); II = D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (HOTic); III = D- and L-7-hydroxy-6,8-diiodo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (I_2HOTic); IV = [*erythro*, (*SS*, *RR*)]-D,L-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (β-MeTic); V = D,L-2-aminotetraline-2-carboxylic acid (Atc); VI = D,L-6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat); VII = D,L-6-methoxy-2-aminotetraline-2-carboxylic acid (Matc).

Many attempts have been made to resolve amino acid enantiomers by liquid chromatographic techniques. The enantioselective separations performed by high-performance liquid chromatographic (HPLC) methods can be divided into three main groups: direct separation on chiral stationary phases [3–5], separation on achiral columns with chiral eluents [5–7] and separation of the diastereomers formed by precolumn derivatization with chiral reagents [5,8–19].

For the synthesis of receptor-selective opioid peptides, a number of conformationally constrained aromatic amino acids have been prepared (Fig. 1). The separation of some of these isomers was described earlier [20]. The present paper deals with the separation of enantiomers 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). The eluent system contains methanol as organic modifier instead of the earlier-used acetonitrile. This change in eluent composition proved to be very effective: the analysis time is shortened and the separation is improved.

2. Experimental

2.1. Chemicals and reagents

The amino acids shown in Fig. 1 were synthesized in our laboratory by our own or literature methods. The syntheses of I [21], II and III [22] lead to one enantiomer of the substrate, depending on the configuration of the starting material, whereas IV [23], V [24], VI and VII [25] were obtained in racemic form. The identity of the compounds was checked by means of melting point determination, FAB mass spectrometry, ^{1}H NMR spectroscopy and chiral TLC [26].

GITC was purchased from Aldrich (Steinheim, Germany), FDAA from Pierce Chemical Company (Rockford, IL, USA), L-amino acid oxidase, carboxypeptidase A and α-chymotrypsin Type II from Sigma (St. Louis, MO, USA) and trifluoroacetic acid, sodium

acetate, 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 twice distilled water and further purified by filtration on a 0.45-μm filter Type HV (Millipore, Molsheim, France). The pH was adjusted with acetic acid (acetate buffer), phosphoric acid (phosphate buffer) and sodium hydroxide.

Chiral plates (Macherey Nagel, Düren, Germany) were used for chiral TLC, and spots were detected with ninhydrin reagent.

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, Division of Millipore, Milford, MA, USA), an L-6000 liquid chromatographic pump (Merck Hitachi, Tokyo, Japan) equipped with a UV 308 detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany).

The columns used were Vydac 218TP104 C_{18} and Vydac 218TP54 C_{18} (250 × 4.6 mm I.D.) with 10 and 5 μm particle size, respectively (The Separations Group, Hesperia, CA, USA).

^{1}H NMR spectroscopy was performed on an AM 400 spectrometer (Brucker, Zug, Switzerland).

FAB mass spectra were obtained on a double focussing MS 902 S spectrometer with xenon at 8 kV as bombarding gas (A.E.I. Scientific Apparatus Division, Manchester, UK).

2.3. Derivatization of amino acids

Amino acids (0.5–1 mg) were derivatized with FDAA or GITC by the method of Marfey [14] or Nimura et al. [11].

2.4. Enzymatic digestion of D,L-amino acids

The elution sequence of *erythro*-D- and L-β-MeTic (see Fig. 1 for full name and structure) was determined either by enzymatic degradation of the amino acid with L-amino acid oxidase by the method described in Ref. [20] or with a standard of *erythro*-D-β-MeTic made from *erythro*-D-β-methylphenylalanine by method [21].

The configurations of amino acids containing a tetraline ring were determined by means of enzymatic digestion with carboxypeptidase and α-chymotrypsin. For enzymatic degradation of Atc and Hat (see Fig. 1 for full names and structures), with carboxypeptidase A first the N-trifluoroacetyl derivative of the amino acid was prepared by the method described in Ref. [27]. A 1-mg amount of N-trifluoroacetyl derivative of the amino acid was dissolved in 200 μl 0.1 mol/l Tris buffer (pH 7.2) in a test-tube and 10 μl carboxypeptidase A was added. The test-tube was tightly capped and incubated for 1–10 h at 37°C. The carboxypeptidase A was inefficient in the enzymatic digestion of Matc. For the identification of configuration of Matc the α-chymotrypsin catalysed hydrolysis of D,L-6-methoxy-2-aminotetraline-2-carboxylic acid methyl ester was applied [28]. A 1-mg amount of D,L-amino acid methyl ester was dissolved in 200 μl 0.1 mol/l Tris buffer (pH 7.2) in a test-tube and 1 mg α-chymotrypsin was added. The incubation time was 48 h at 37°C and the pH was checked from time to time. The reaction mixtures were used for derivatization with GITC or FDAA.

3. Results and discussion

The HPLC separations of the derivatized amino acids were carried out in the three different aqueous buffer systems containing methanol as organic modifier: 0.1% trifluoroacetic acid (0.1% TFA), 0.01 mol/l potassium dihydrogenphosphate (pH 3) (phosphate buffer) and 0.01 mol/l sodium acetate (pH 3) (acetate buffer). Comparison with earlier results obtained with acetonitrile [20] reveals that the change of the organic modifier from acetonitrile to methanol improves the separation and the peak shapes.

The results of the separation of Tic derivatives are listed in Table 1. Decrease of the methanol

Table 1
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Tic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
50:50	3.33	4.85	1.45	3.23
55:45	7.25	11.81	1.63	4.42
KH_2PO_4–CH_3OH				
50:50	3.08	4.36	1.42	2.34
55:45	6.66	9.72	1.46	3.86
NaOAc–CH_3OH				
50:50	2.28	2.95	1.29	1.32
52.5:47.5	3.82	5.38	1.40	2.29
55:45	5.49	8.00	1.46	3.07
FDAA derivatives				
TFA–CH_3OH				
35:65	1.62	2.96	1.82	3.84
40:60	2.73	5.25	1.92	4.41
KH_2PO_4–CH_3OH				
30:70	0.86	1.34	1.56	1.66
35:65	1.30	2.15	1.65	2.78
40:60	2.33	4.17	1.79	3.20
NaOAc–CH_3OH				
40:60	1.86	3.26	1.75	4.11
45:55	3.09	5.72	1.85	5.00
50:50	5.92	11.87	2.01	5.47

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 2
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of HOTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
50:50	1.45	2.35	1.60	1.58
KH_2PO_4–CH_3OH				
50:50	1.66	2.51	1.51	1.92
52.5:47.5	2.05	3.31	1.61	2.37
NaOAc–CH_3OH				
50:50	1.45	2.08	1.43	3.12
52.5:47.5	1.98	3.07	1.55	3.71
FDAA derivatives				
TFA–CH_3OH				
35:65	0.76	1.46	1.92	2.73
45:55	1.46	2.61	1.79	3.50
50:50	2.18	5.34	2.45	8.90
KH_2PO_4–CH_3OH				
50:50	1.34	2.58	1.93	4.00
52.5:47.5	1.48	3.06	2.07	6.06
NaOAc–CH_3OH				
40:60	0.96	1.03	1.08	0.50
50:50	1.82	3.02	1.66	4.00
60:40	2.60	10.33	3.97	13.30

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

content increases the retention times, and the values of α and R_s. Comparison of the three buffer systems demonstrates that sodium acetate is a slightly superior, with relatively low k' values. The GITC derivatives have lower k' values than the FDAA derivatives at the same eluent composition.

The HOTic derivatives exhibit very good separation, with short retention times (Table 2). There is little difference between the three buffer systems, and all appear useful. The GITC derivatives have shorter retention times than the FDAA compounds.

The I_2HOTic derivatives separate very well within a short time in a methanol-containing system (Table 3). At a given percentage of organic modifier, the k' values are 5–10 times lower with methanol as organic modifier than with acetonitrile, while the R_s values improve [20]. To achieve the same R_s values for the separation of GITC and FDAA derivatives, the GITC derivatives should be analyzed at a lower methanol content and thus they require a longer analysis time.

The separation of *erythro*-β-MeTic derivatives can be carried out with good resolution in a

Table 3
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of I_2HOTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
45:55	3.35	5.44	1.62	3.53
50:50	7.09	13.01	1.83	4.92
KH_2PO_4–CH_3OH				
45:55	1.59	1.89	1.13	1.16
47.5:52.5	2.30	2.92	1.27	1.92
50:50	4.60	8.82	1.92	6.66
NaOAc–CH_3OH				
47.5:52.5	1.99	2.55	1.28	1.33
50:50	3.67	5.98	1.63	4.15
FDAA derivatives				
TFA–CH_3OH				
35:65	1.42	3.10	2.18	5.60
40:60	1.45	3.91	2.70	6.44
KH_2PO_4–CH_3OH				
35:65	1.05	2.21	2.10	4.65
40:60	1.83	5.02	2.74	6.72
NaOAc–CH_3OH				
35:65	1.28	2.36	1.84	3.21
40:60	1.85	3.60	1.95	4.66

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 4
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of *erythro*-β-MeTic derivatives on eluent composition

Eluent composition	k'_L	k'_D	α	R_s
GITC derivatives				
TFA–CH_3OH				
40:60	1.48	1.92	1.30	1.26
45:55	2.96	4.15	1.39	2.26
KH_2PO_4–CH_3OH				
45:55	2.21	2.82	1.28	1.16
47.5:52.5	3.54	4.67	1.32	1.56
NaOAc–CH_3OH				
50:50	5.25	7.23	1.38	3.83
FDAA derivatives				
TFA–CH_3OH				
40:60	3.73	4.30	1.15	1.38
42.5:57.5	5.49	6.42	1.17	2.11
KH_2PO_4–CH_3OH				
30:70	1.14	1.41	1.25	1.60
35:65	1.69	2.16	1.28	3.00
40:60	2.77	3.73	1.35	3.75
45:55	5.39	7.41	1.37	4.55
NaOAc–CH_3OH				
35:65	1.50	1.95	1.30	2.43
40:60	1.87	2.63	1.40	3.00
45:55	3.83	5.43	1.42	4.36
50:50	6.78	9.94	1.47	6.33

Column: Vydac 218TP104 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihidrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

relatively short time in all three buffer systems (Table 4). The peak shapes are best in sodium acetate, the band broadening being larger in trifluoroacetate and phosphate. The GITC derivatives have lower k' values than the FDAA derivatives at the same eluent composition, but the shorter analysis time means a worse resolution.

The four amino acids containing a tetrahydroisoquinoline ring which are investigated can be separated from each other conveniently as GITC derivatives in the acetate buffer–methanol (50:50) system. The elution sequence is HOTic < Tic < I_2HOTic < *erythro*-β-MeTic. The GITC derivatives in all cases have shorter retention times than the corresponding FDAA derivatives at the same eluent composition, but not better R_s values.

The elution sequence of the D- and L-forms of the amino acids I–IV was checked by enzymatic digestion of the L-forms with L-amino acid oxidase, followed by derivatization of the reaction mixture with GITC or FDAA. The results show that, for the GITC and FDAA derivatives of tetrahydroisoquinoline ring-containing unusual

amino acids, the first peak corresponded to the L-form and the second to the D-form.

The uncommon amino acids containing a tetraline ring (Atc, Hat and Matc) were investigated in the same buffer systems. The results of the separation of the Atc derivatives are listed in Table 5. Comparison of the three buffer systems shows that the acetate system is the best in the series of GITC derivatives, while for the FDAA derivatives all three systems are suitable. Otherwise, the FDAA derivatives give better peak shapes and R_s values than the GITC analogues.

The Hat derivatives exhibit good chromatographic results in the acetate buffer system (Table 6). The analyses in the trifluoroacetic acid and phosphate buffer systems take a longer time, but the R_s values do not improve and peaks are broad.

The Matc derivatives can be separated well in all buffer systems, but the chromatograms in sodium acetate display the best peak shapes (Table 7).

Table 5
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Atc derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH				
55:45	10.22	11.92	1.17	1.64
KH_2PO_4–CH_3OH				
55:45	9.14	10.64	1.16	1.42
NaOAc–CH_3OH				
50:50	5.25	5.99	1.14	2.22
FDAA derivatives				
TFA–CH_3OH				
45:55	8.62	9.64	1.12	1.46
KH_2PO_4–CH_3OH				
50:50	9.28	11.30	1.22	2.86
NaOAc–CH_3OH				
55:45	14.42	17.36	1.20	3.14

Column: Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

Table 6
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Hat derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH				
65:35	15.33	16.94	1.10	1.12
KH_2PO_4–CH_3OH				
65:35	15.83	18.43	1.16	1.49
NaOAc–CH_3OH				
60:40	4.72	5.88	1.25	1.78
FDAA derivatives[a]				
TFA–CH_3OH				
60:40	8.93	7.81	1.14	1.18
65:35	16.44	14.22	1.16	1.64
KH_2PO_4–CH_3OH				
65:35	11.12	9.79	1.14	1.53
NaOAc–CH_3OH				
50:50	2.15	1.96	1.10	1.20
60:40	5.64	5.04	1.12	1.27
65:35	9.41	8.29	1.14	1.43

Column: Vydac 218TP104 C_{18} and Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).
[a] FDAA derivatives were analysed on Vydac 218TP54 C_{18}.

The configurations of the D,L-amino acids containing a tetraline ring were checked by enzymatic digestion of the N-trifluoroacetyl amino acid with carboxypeptidase A and the amino acid methyl ester with α-chymotrypsin. The elution sequence of the isomers was D followed by L, except for Hat, where an interesting reversal of the elution sequence between the GITC and FDAA derivatives was observed.

In Fig. 2 selected chromatograms are shown of the separation of amino acids investigated in this work.

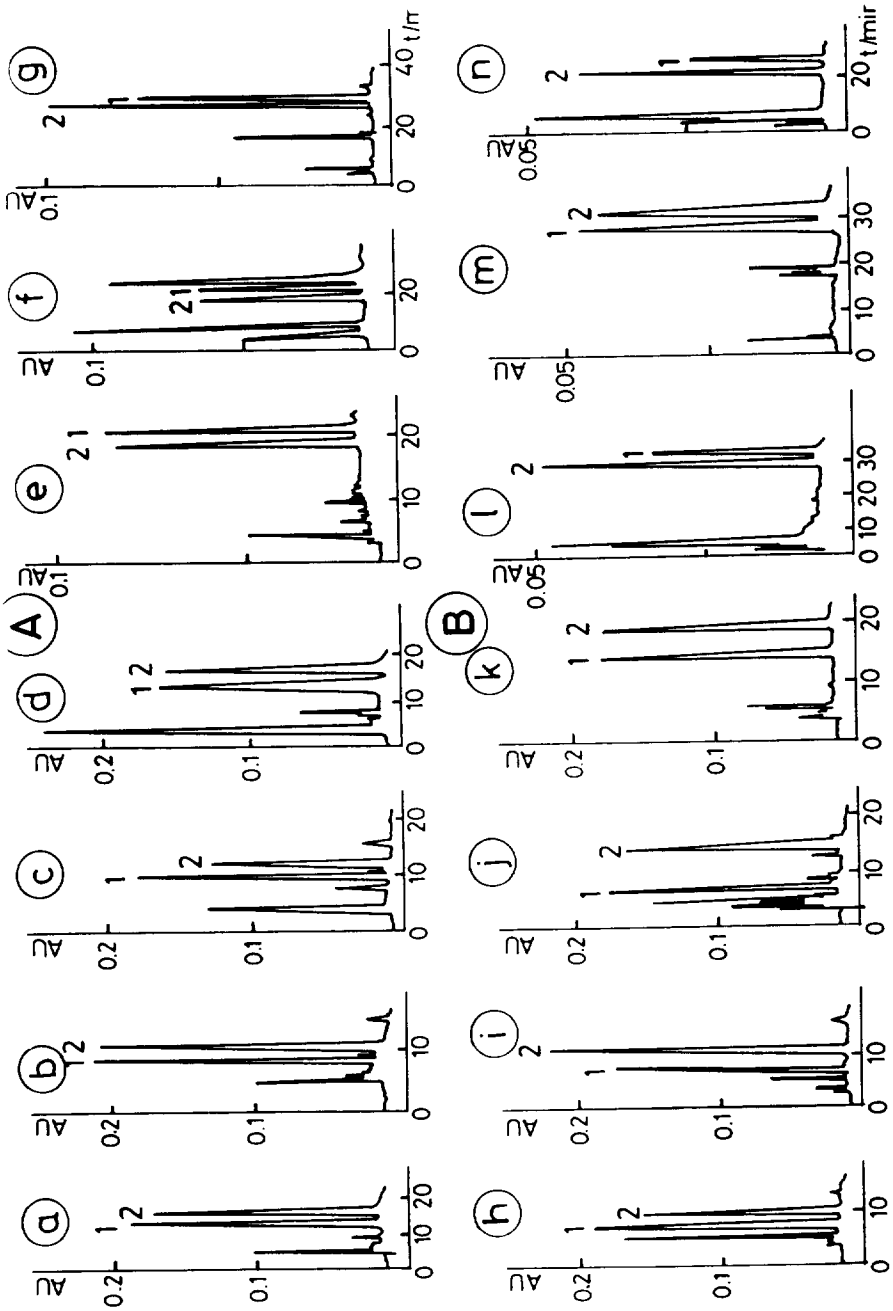

Fig. 2. Selected chromatograms of GITC (A) and FDAA (B) derivatives of amino acids. Amino acids: (a,h) Tic; (b,i) HOTic; (c,j) I_2HOTic; (d,k) *erythro*-β-MeTic; (e,l) Atc; (f,m) Hat; (g,n) Matc. Column, Vydac 218TP104 C_{18}; flow-rate, 0.8 ml/min; detection, (A) at 250 nm, (B) at 340 nm; mobile phase: (a,b) 0.01 *M* phosphate buffer–methanol (50:50), (c,d) 0.01 *M* phosphate buffer–methanol (47.5:52.5), (e,n) 0.01 *M* acetate buffer–methanol (50:50), (f,g) 0.01 *M* acetate buffer–methanol (60:40), (h) 0.01 *M* phosphate buffer–methanol (35:65), (i,l) 0.1% TFA–methanol (45:55), (j) 0.1% TFA–methanol (40:60), (k) 0.01 *M* acetate buffer–methanol (45:55), (m) 0.01 *M* acetate buffer–methanol (65:35); Peaks: 1 = L-isomer, 2 = D-isomer, unlabelled peaks originated from GITC or FDAA reagents.

Table 7
Dependence of retention factor (k'), separation factor (α) and resolution (R_s) of Matc derivatives on eluent composition

Eluent composition	k'_D	k'_L	α	R_s
GITC derivatives				
TFA–CH_3OH				
55:45	9.69	11.94	1.23	2.42
KH_2PO_4–CH_3OH				
60:40	4.83	5.78	1.20	2.12
NaOAc–CH_3OH				
50:50	7.76	8.50	1.10	2.08
FDAA derivatives				
TFA–CH_3OH				
45:55	6.91	8.16	1.18	1.82
KH_2PO_4–CH_3OH				
50:50	8.87	11.43	1.29	3.57
NaOAc–CH_3OH				
50:50	6.45	8.14	1.26	2.23

Column: Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; TFA: 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4: 0.01 mol/l aqueous solution of potassium dihydrogen phosphate (pH 3); NaOAc: 0.01 mol/l aqueous solution of sodium acetate (pH 3).

4. Conclusions

The described procedures can be applied for the separation and identification of conformationally constrained unusual aromatic amino acids. The method permits to check the configuration of amino acids after synthesis and their incorporation into peptides, and hence optimization of the conditions of synthesis of amino acids and peptides. Methanol-containing mobile phase systems seem to be more efficient than acetonitrile-containing ones. The sodium acetate–methanol system is generally more efficient than the TFA or phosphate system. In general the GITC derivatives have lower k' values than the FDAA derivatives. As general rule relating elution sequence to configuration of the amino acid does not seem to be warranted since reversal of elution sequence may occur.

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