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Application of a new chiral derivatizing agent to the enantioseparation of secondary amino acids

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Abstract

A new chiral derivatizing agent, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester, (*S*)-NIFE, was applied for the high-performance liquid chromatographic separation of enantiomers of 19 unnatural secondary amino acids: proline, pipecolic acid analogues, piperazine-2-carboxylic acid, morpholine-3-carboxylic acid, thiomorpholine-3-carboxylic acid and analogues containing the 1,2,3,4-tetrahydroisoquinoline, 1,2,3,4-tetrahydronorharmane, 1,2,3,4-tetrahydro-2-carboline and 2-benzazepine skeletons. Excellent resolutions were achieved for most of the investigated compounds by using a reversed-phase mobile phase system. The conditions of separation were optimized by variation of the mobile phase composition.

Keywords: Enantiomer separation; Mobile phase composition; Derivatization, LC; Amino acids; Nitrophenoxycarbonylphenylalanine methoxyethyl ester; Imino acids

1. Introduction

Determination of the biologically active conformations of peptide hormones is an important goal in modern biology. Since most peptide hormones are highly flexible molecules with numerous possible conformations under physiological conditions, one highly useful approach involves the introduction of conformational constraints [1]. In this approach, mimetics of secondary structures such as an α-helix, β-turns, γ-turns, etc. are built into the peptides in order to stabilize their structures. Several unusual α-amino acids have recently been designed with a view to constraining the side-chain functional groups of natural α-amino acids [1]. In the secondary α-amino acids (''imino acids''), the nitrogen is contained in a ring. This may have profound consequences for the conformation of the peptide, peptidomimetic, etc. into which the amino acid is incorporated, since nitrogen is unable to act as a hydrogen bond donor unless it is located in a terminal position of the molecule. Nevertheless, this type of amino acid has proved very useful in biological studies. The synthesis, the incorporation into peptides and the biological properties of the peptides containing these type of ''imino acids'' have been surveyed in several papers [2–4].

Most of these new ''imino acids'' are produced synthetically. The syntheses lead either to a mixture of stereoisomers or, via asymmetric synthesis strategies, to enantiomerically enriched products. When incorporated into peptides, such stereochemically

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and/or enantiomerically impure components lead to mixtures of compounds with different biological properties. It is therefore very important to have available enantiomerically pure and defined substances and analytical methods for the separation and identification of the different stereoisomers.

For this purpose, chromatographic methods are widely used. Successful high-performance liquid chromatographic (HPLC) methods for the resolution of amino acids include indirect and direct methods. Indirect methods involve precolumn derivatization with chiral derivatizing agents (CDAs), with subsequent separation of the diastereoisomers on an achiral column [5,6]. Direct methods are performed by ligand-exchange chromatography [7,8], or by application of chiral stationary phases [9–11].

In the present paper, an indirect HPLC method is described for the separation of enantiomers of 19 unnatural sterically constrained ''imino acids''. A new CDA, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester, (*S*)-NIFE, was applied. This CDA has been successfully used for the enantioseparation of proteinogenic amino acids [12] and for different ring- and α-methyl-substituted phenylalanine and phenylalanine amide analogues [13]. The derivatization was performed under mild conditions, and the diastereomers formed were separated in the reversed-phase (RP) mode. The effect of the mobile phase composition on the separation was investigated, and the conditions affording the best resolution were determined. The sequence of elution of the enantiomers was in most cases determined by spiking the racemic samples with enantiomers with known absolute configurations.

2. Experimental

2.1. Chemicals and reagents

With the exceptions of (2*R*,4*R*)- and (2*S*,4*S*)-*cis*-4-hydroxypyrrolidine-2-carboxylic acid (D- and L-*cis*-4-hydroxyproline) (**1**) and D,L-piperazine-2-carboxylic acid (**7**), which were purchased from Aldrich (Steinheim, Germany), the amino acids were synthesized in our laboratories (for their structures, see Tables 1 and 2). The nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [14]. Enantiopure or enantiomerically enriched L-1,2,3,6-tetrahydropyridine-2-carboxylic acid (L-baïkaïne) (**3**) [15], L- and D-piperidine-2-carboxylic acid, (pipecolic acid, Pip) (**4**) [16], (2*S*,4*R*)-*cis*-4-hydroxypipecolic acid (L-*cis*-4-hydroxy-Pip) (**5**) [15], (2*S*,5*R*)-*trans*-5-hydroxypipecolic acid (L-*trans*-5-hydroxy-Pip) (**6**) [15], L-morpholine-3-carboxylic acid (**9**) [16], L-thiomorpholine-3-carboxylic acid (**10**) [17], D- and L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**12**) [4], D- and L-3-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**13**) [4], D- and L-5-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**14**) [18], D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**16**) [4], D- and L-1,2,3,4-tetrahydro-3-carboxy-2-carboline (Tcc) (**19**) [4], racemic D,L-α-methylproline (**2**) [19], (2*S*,4*R* and 2*R*,4*S*)-*cis*-4-hydroxypipecolic acid (**5**) [20], (2*S*,5*R* and 2*R*,5*S*)-*trans*-5-hydroxypipecolic acid (**6**) [21], (2*S*,5*R* and 2*R*,5*S*)-*cis*-5-methylpiperazine-2-carboxylic acid (**8**) [22], d,l-morpholine-3-carboxylic acid (**9**) [23], D,L-thiomorpholine-3-carboxylic acid (**10**) [24], D,L-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (**11**) [25], D,L-6-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**15**) [4], D,L-1,2,3,4,5-pentahydro-2-benzazepine-3-carboxylic acid (Pac) (**17**) [26] and D,L-1,2,3,4-tetrahydronorharmane-1-carboxylic acid (**18**) [27] were synthesized by literature methods. D-Baïkaïne (**3**) was produced by partial racemization of L-baïkaïne by refluxing in 2 *M* NaOH.

(*S*)-NIFE was obtained from Peptisyntha (Brussels, Belgium). MeCN and MeOH of HPLC grade were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA), trifluoroacetic acid (TFA) and other reagents of analytical reagent grade were also from Merck.

The starting mobile phases, water (**A**), MeCN (**B**) or MeOH (**C**), all containing 0.1% TFA, were prepared by adding 1.0 ml TFA to 1 l Milli-Q water, MeCN or MeOH, respectively, and were purified by passing through a 0.45-μm Millipore filter, type HV (Molsheim, France). Gradient elutions were run with mobile phases **A** and **B** (or **C**); the gradient slopes were: (**a**) **A**–**B** (95–5%) at 0 min, increased at 1.4% **B** min^{-1} to 60% **B**; (**b**) **A**–**C** (95–5%) at 0 min, increased at 1.65% **C** min^{-1} to 80% **C**.

2.2. Apparatus

Two HPLC systems were used. The Waters chro-

Table 1
Retention factors (k), separation factors (α) and resolutions (R_s) for the separation of enantiomers of unusual secondary amino acids in MeCN-containing mobile phase

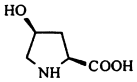
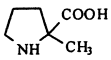
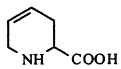
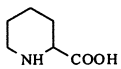
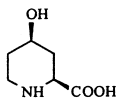
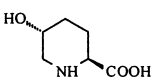
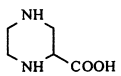
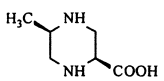
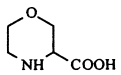
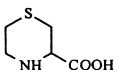
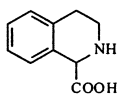
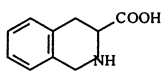
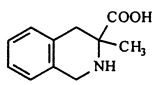
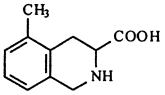
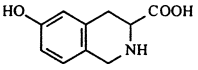
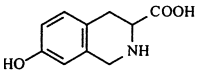
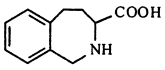
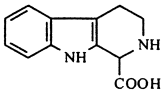
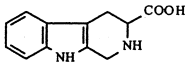
Compound		Eluent composition TFA–MeCN (v/v)	k_L	k_D	α	R_s
1		70:30	0.26	0.30	1.15	<0.40
		75:25	2.20	2.44	1.11	0.88
		80:20	4.79	5.46	1.14	1.83
2[a]		70:30	4.16	5.11	1.23	3.22
3		70:30	4.68	5.77	1.23	1.92
4		70:30	5.19	5.87	1.23	2.97
5		70:30	1.49	1.65	1.11	0.86
		75:25	3.44	3.96	1.15	1.45
		80:20	7.28	8.85	1.21	3.81
6		70:30	1.23	1.36	1.10	0.88
		75:25	2.41	2.80	1.16	1.88
		80:20	5.15	6.23	1.21	2.98
7[a]		60:40	2.84	3.06	1.08	0.82
		65:35	6.81	7.47	1.20	1.48
		70:30	16.29	18.32	1.16	2.31
8[a]		60:40	4.82	4.98	1.03	0.57
		65:35	9.43	9.85	1.04	0.86
		70:30	18.46	19.26	1.05	0.95
9		70:30	2.33	2.46	1.06	0.32
		75:25	6.51	7.05	1.08	1.46
10		70:30	4.64	5.12	1.10	1.50
11[a]		60:40	3.85	4.41	1.14	1.86
12		65:35	9.04	11.22	1.24	4.10
		60:40	3.92	4.58	1.17	2.23
13		60:40	6.33	6.86	1.08	0.71
		70:30	8.59	10.94	1.27	2.72

Table 1. Continued

Compound		Eluent composition TFA–MeCN (v/v)	k_L	k_D	α	R_s
14		60:40	4.83	5.66	1.17	2.55
15[a]		65:35 60:40[b]	2.98 11.42	3.36 12.82	1.12 1.12	1.48 1.90
16		65:35 60:40[b]	2.88 9.36	3.44 10.19	1.19 1.08	2.15 1.18
17[a]		60:40 70:30	5.33 8.63	5.74 16.45	1.07 1.89	1.20 4.38
18[a]		60:40	5.54	6.22	1.12	1.65
19		60:40	4.28	4.83	1.13	1.80

Chromatographic conditions: column, Lichrospher RP-18; flow-rate, 0.8 ml/min; detection, 205 nm; mobile phase, TFA, 0.1% aqueous solution of trifluoroacetic acid; MeCN, acetonitrile, containing 0.1% TFA; k_L and k_D are the retention factors of the L isomer (eluting first) and the D isomer (eluting second) of the α-amino acid, respectively.

[a] Elution sequence not determined.

[b] Bis derivative (analyte derivatized on the hydroxy group, too); void volume, 1.18 ml.

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

The mass spectrometric (MS) measurements were carried out on a VG Quattro II apparatus (VG Analytical, Manchester, UK) with electrospray ionization, coupled to an HPLC system. The HPLC system consisted of a low-pressure gradient pump of type 325, a UV detector of type 332 and an Autosampler of type 465, all from Kontron (Milan, Italy). A Vydac 218TP54 C_{18} 250×4.6 mm I.D., 5-μm particle size column (The Separations Group, Hesperia, CA, USA) was used.

RP analyses were performed on a LiChrospher RP-18 150×4.0 mm I.D., 5-μm particle size column (Merck).

2.3. Derivatization procedure

Stock solutions of amino acids (1 mg/ml) were prepared by dissolution in water. To 25 μl stock solution in a 1-ml reaction vial, 0.5 μl TEA and 10–25 μl CDA (1 mg/100 μl, dissolved in water-

Table 2
Retention factors (k), separation factors (α) and resolutions (R_s) for the separation of enantiomers of unusual secondary amino acids in MeOH-containing mobile phase

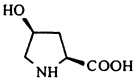
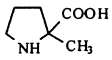
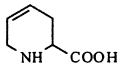
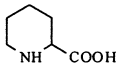
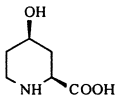
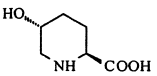
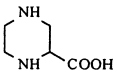
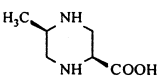
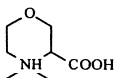
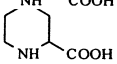
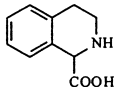
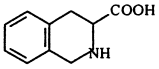
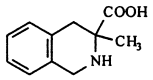
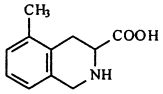
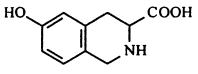
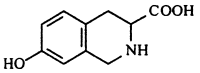
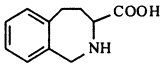
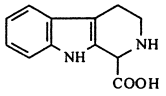
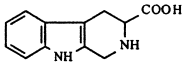
Compound		Eluent composition TFA–MeOH (v/v)	k_L	k_D	α	R_s
1						
		60:40	3.70[c]	4.34	1.17	1.38
		70:30	10.09	12.39	1.22	3.03
2[a]						
		40:60	1.47	1.97	1.34	2.37
		50:50	3.80	5.34	1.40	4.84
3		50:50	3.88	5.55	1.43	4.45
4		50:50	5.09	6.43	1.26	3.63
5						
		50:50	2.15	2.30	1.07	0.56
		55:45	3.27	3.52	1.07	0.73
		60:40	5.56	6.55	1.18	2.53
6						
		50:50	2.73	2.98	1.08	0.68
		55:45	3.72	4.09	1.10	1.18
		60:40	4.72	5.27	1.12	1.85
7[a]						
		35:65	1.69	1.96	1.16	1.05
		40:60	2.69	3.06	1.13	1.45
8[a]						
		35:65	2.16	2.30[d]	1.07	<0.40
		40:60	4.67	5.04	1.08	1.02
		50:50	22.21	24.62	1.11	1.78
9						
		50:50	2.12[c]	2.40	1.13	1.26
		55:45	3.38	3.90	1.15	1.52
10		50:50	3.87	5.55	1.43	4.13
11[a]		35:65	1.94	2.94	1.52	2.51
12						
		35:65	1.80	2.52[d]	1.40	2.30
		40:60	4.72	6.89	1.46	3.95
13		35:65	6.12	8.09	1.32	4.92
14						
		35:65	2.93[c]	4.16	1.40	4.26
		40:60	6.07	8.64	1.42	4.80

Table 2. Continued

Compound		Eluent composition TFA–MeOH (v/v)	k_L	k_D	α	R_s
15		40:60	1.13	1.53	1.35	1.95
		50:50	3.73	5.35	1.43	4.43
		35:65[b]	3.47	4.46	1.28	2.63
16		40:60	0.92[c]	1.22	1.32	1.39
		50:50	4.05	6.69	1.65	6.33
		35:65[b]	3.84	4.80	1.25	2.42
17[a]		35:65	3.54	4.38	1.24	2.56
18[a]		35:65	5.05	6.26	1.24	3.05
19		35:65	2.35	3.48	1.48	4.17

Chromatographic conditions: column, LiChrospher RP-18; flow-rate, 0.8 ml/min; detection, 205 nm; mobile phase, TFA, 0.1% aqueous solution of trifluoroacetic acid; MeOH, methanol, containing 0.1% TFA; k_L and k_D are the retention factors of the L isomer (eluting first) and the D isomer (eluting second) of the α-amino acid, respectively.

[a] Elution sequence not determined.

[b] Bis derivative (analyte derivatized on the hydroxy group, too).

[c] Partial separation from 4-nitrophenol.

[d] Partial separation from the ''urea dimer''; void volume, 1.18 ml.

free dioxane) were added; the molar ratio of CDA to amino acid was 2:1 or 5:1. The latter ratio was applied when the amino acids (**7**, **8**, **15** and **16**) form bis derivatives. The vial was tightly capped, vortexed and stored at ambient temperature for 20 min. After complete derivatization, the reaction mixture was diluted with dioxane (25 μl) and acidified by addition of 0.1% aqueous TFA (5–10-fold dilution). The derivatized samples were stable during storage in a refrigerator for several weeks. The derivatized amino acids were detected at 205 nm.

3. Results and discussion

3.1. Reactions of amino acids with (S)-NIFE

In the presence of a substrate containing an amino group (amino acids), the ''reactive ester type'' CDA (**I**) reacts with the amino group to form the desired urea diastereomer (**II**), which is accompanied by the formation of an equimolar amount of 4-nitrophenol (**III**), as depicted in Fig. 1. The excess CDA (**I**) in aqueous–organic solution under basic conditions decomposes and forms three major side-products, according to the reactions detailed in Fig. 1. The self-decomposition results in the formation of 4-nitrophenol (**III**), and the reactions of the ''carbamic acid intermediate'' explain the presence of the phenylalanine methoxyethyl ester (''Phe ester'') (**IV**) and a ''urea dimer'': *N,N′*-bis(3-phenylpropionic acid methoxyethyl ester 2-yl)urea (**V**), all of which were identified by HPLC–MS. The optimum conditions of derivatization were determined earlier [12,13]. For of α-amino acids, the highest yield of derivatization was obtained at ambient temperature

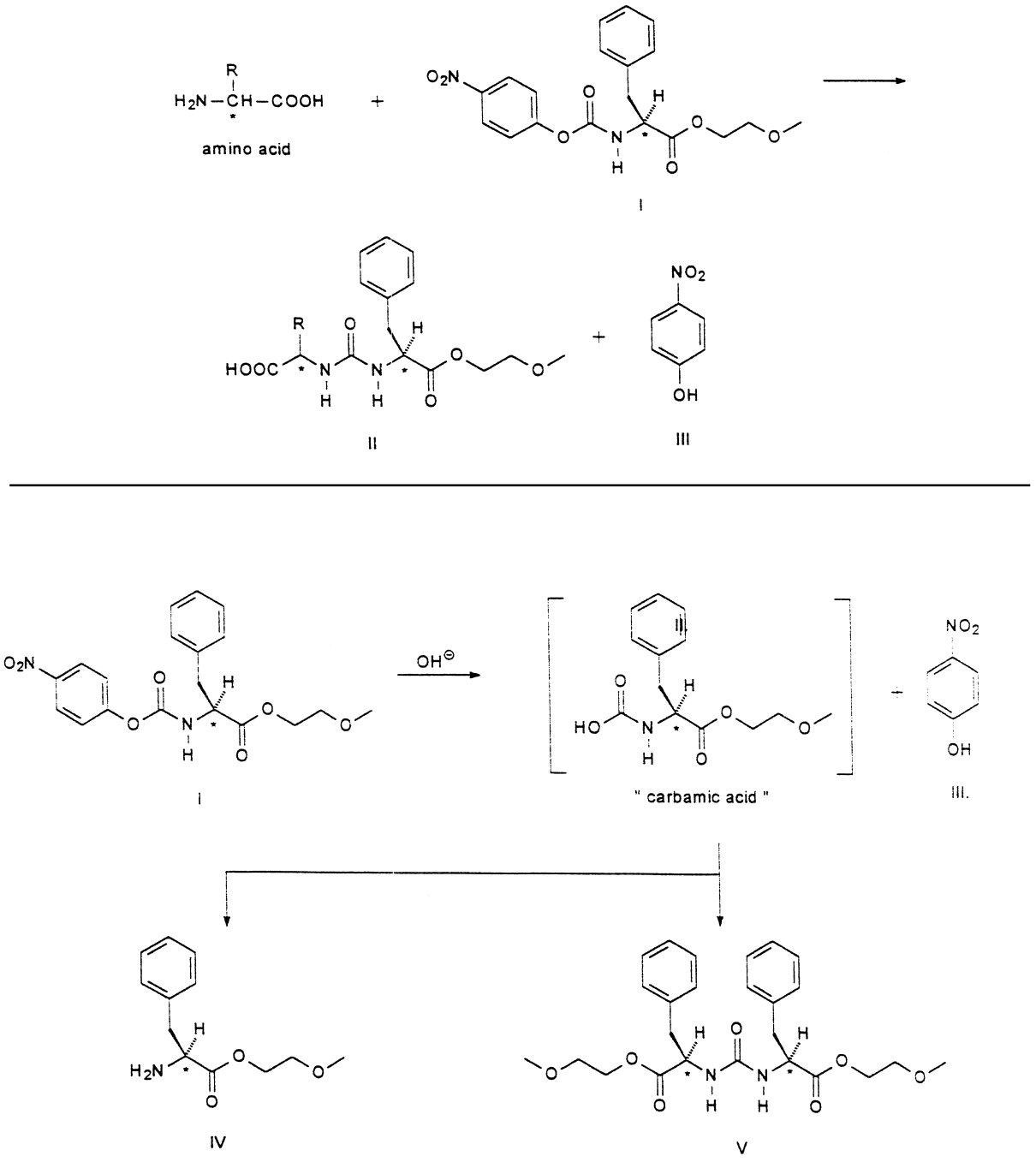


Fig. 1. Scheme of self-decomposition and derivatization of (*S*)-NIFE.

in alkaline aqueous solution at pH 11 (the pH was adjusted with TEA). The reaction time was 20 min and the molar ratio of CDA:analyte was kept at 2:1 in the formation of mono derivatives. Analytes forming bis derivatives need a 5-fold excess of CDA. Figs. 2 and 3 demonstrate the full chromatograms of

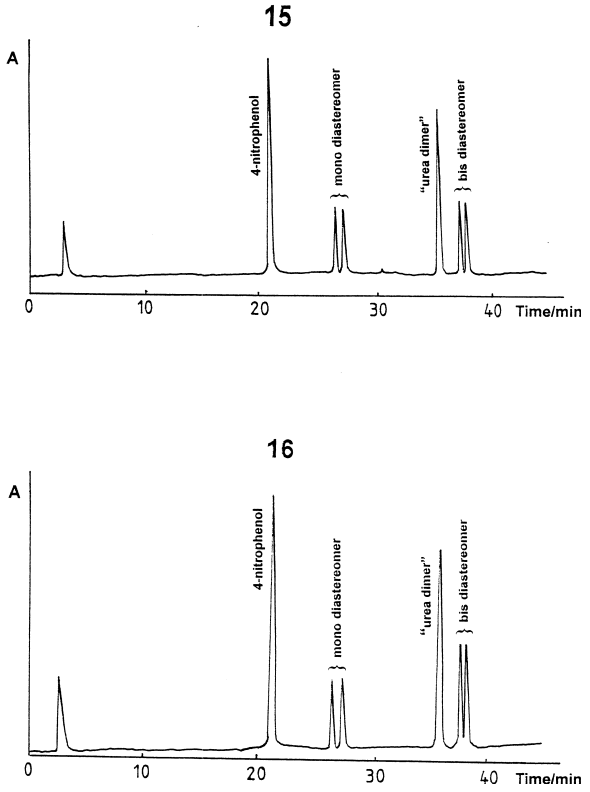


Fig. 2. Full chromatograms in MeCN-containing mobile phase of D,L-6-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**15**) and D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**16**) derivatized with (*S*)-NIFE. Conditions of derivatization: amount of amino acid, 0.1 μmol; molar ratio CDA:amino acid: 5:1; pH 11; temperature, ambient; duration of reaction, 20 min; chromatographic conditions: column, LiChrospher RP-18; flow-rate, 0.8 ml/min; detection, 205 nm; mobile phase, 0.1% aqueous TFA–MeCN (with 0.1% TFA); type of gradient, (**a**) (see Experimental); elution sequence for **16**, L<D for both mono and bis derivatives; elution sequence not determined for **15**.

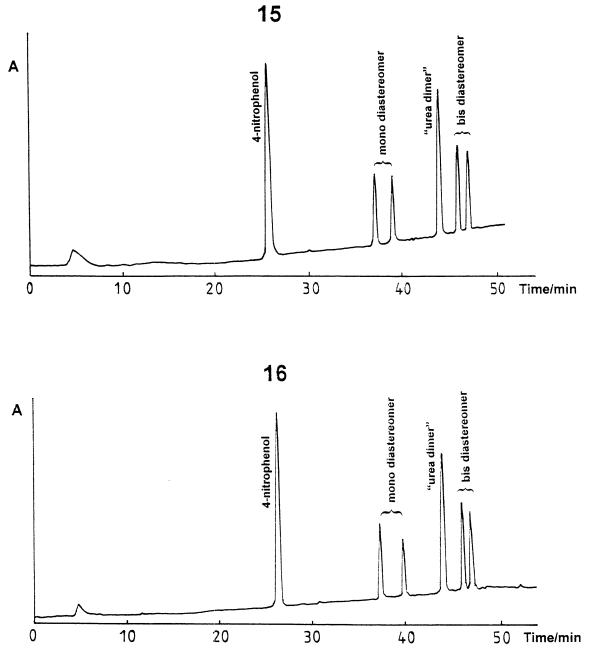


Fig. 3. Full chromatograms in MeOH-containing mobile phase of D,L-6-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**15**) and D- and L-7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**16**) derivatized with (*S*)-NIFE. Conditions of derivatization: amount of amino acid, 0.1 μmol; molar ratio CDA:amino acid: 5:1; pH 11; temperature, ambient; duration of reaction, 20 min; chromatographic conditions: column, LiChrospher RP-18; flow-rate, 0.8 ml/min; detection, 205 nm; mobile phase, 0.1% aqueous TFA–MeOH (with 0.1% TFA); type of gradient, (**b**) (see Experimental); elution sequence for **16**, L<D for both mono and bis derivatives; elution sequence not determined for **15**.

racemic **15** and **16**, which form parallel mono and bis derivatives, and also show the relative amounts of the desired urea diastereomers and side-products **III** and **V** (for details, see below).

3.2. Separation of "imino acid"–(S)-NIFE derivatives

Separations were carried out on a LiChrospher RP-18 column. The inorganic part of the mobile phase contained 0.1% aqueous TFA and the organic modifiers were MeCN or MeOH, also containing 0.1% (v/v) TFA. Mobile phases without TFA resulted in chromatograms with poor reproducibility of retention and asymmetric peaks having a heading.

3.2.1. Separation of diastereomers of "imino acid"–(S)-NIFE derivatives in MeCN-containing mobile phase

Selected data on the separation of the stereoisomers of **1–19** are reported in Table 1. It is seen that decrease of the MeCN content of the mobile phase resulted in the expected increase in the retention factor (k), but the separation factor (α) and resolution (R_s) also improved. These data indicate that the hydrophobicity of the stereoisomers investi-

gated plays an important role in the retention. A comparison of the chromatographic data, which reflect the behavior of these analogous compounds, can be made under the same chromatographic conditions. Of the two Pro analogues (**1** and **2**), the stereoisomers of the more hydrophobic **2** were separated nicely in the TFA–MeCN (70:30, v/v) mobile phase, while the separation of the less hydrophobic **1** required a lower MeCN content (80:20, v/v) for baseline separation. Of the Pip analogues (**3–6**), at the same eluent composition (TFA–MeCN (70:30, v/v) the unsaturated (**3**) and the two hydroxy analogues (**5**, **6**) eluted earlier than Pip (**4**), in agreement with the hydrophobic characters of the molecules, and parallel decreases in α and R_s were also observed. These decreases for **5** and **6** were so high that the ratio of MeCN in the mobile phase had to be decreased to 25 or 20% (v/v) in order to obtain a reasonable resolution. Compounds **7** and **8** exhibited high retention in TFA–MeCN (70:30, v/v) as compared to **4**. This finding indicates that in analytes **7** and **8** both ''imino'' groups were derivatized and the large ''bis diastereomers'' were retarded more strongly on the C-18 column. In spite of the stronger retardation, the resolution for the stereoisomers of **8** was only partial. The analytes **7** and **8** differ by a methyl group. The methyl substitution in **8**, which increased the hydrophobic character, resulted in an increased retention. For analytes **9** and **10**, the incorporation of oxygen or sulfur atoms into the ring decreased the hydrophobicity of the molecules as compared to **4**. Therefore, in the TFA–MeCN (70:30 v/v) eluent system they eluted earlier and the α and R_s values were also decreased. To achieve baseline resolution for **9**, a lower MeCN content was applied.

Compounds containing two or three rings (**11–19**) were nicely separated as (*S*)-NIFE derivatives, but the optimum elution required a higher organic modifier content than that for compounds **1–10**. Analytes **11**, **12** and **18**, **19** differ structurally in the position of the carboxy group. At an eluent composition of TFA–MeCN (60:40, v/v), no substantial difference in elution behavior was observed for the stereoisomers of **11**, **12** or **18**, **19**, but **18**, **19** exhibited somewhat higher retention than **11**, **12**, probably in consequence of the higher carbon content. The differences in the *k* values of **11** and **18** or **12** and **19** were far from that observed for **4** and **7**, which indicates that in these cases the indole ''imino'' groups remained underivatized. A comparison of the *k* values for the stereoisomers of **12**, **13** and **14** at a mobile phase composition of TFA–MeCN (60:40, v/v) and of **12**, **15** and **16** in the eluent system TFA–MeCN (65:35, v/v) supported the hydrophobicity rule of retention. In the first case, the increased retention of **13**, **14** as compared to **12** was due to the methyl substitution, while the hydroxy substitution decreased the hydrophobicity and retention of the stereoisomers of **15**, **16** as compared to those of **12**.

Interesting behavior was observed for analytes **15** and **16**. In this case, not only the ''imino'' group was derivatized, but the hydroxy group coupled to the aromatic ring, too. In the mobile phase with TFA–MeCN (65:35, v/v), the stereoisomers of mono derivatives were separated within a reasonable time, while the bis derivatives exhibited very high *k* values (data not shown). In the eluent TFA–MeCN (60:40, v/v), the stereoisomers of the mono derivatives appeared in one peak at the beginning of the chromatogram and the bis derivatives were separated. The separations of the stereoisomers of the mono and bis derivatives of **15** and **16** in one chromatographic run are depicted in Figs. 2 and 3; these chromatograms were recorded in gradient mode in mobile phases containing either MeCN or MeOH. The applied gradient systems ensure baseline separations for both mono and bis derivatives. Analytes **1**, **5** and **6** also contain a hydroxy group, but in these cases the hydroxy group was not derivatized, because it was coupled to an alicyclic ring. The reactivity of this type of hydroxy group is very low [28], which is probably the reason why the formation of bis derivatives was not observed during the reactions of the analytes with (*S*)-NIFE.

The sequence of elution of the stereoisomers was in most cases determined by spiking the racemates with stereoisomers of known configurations. In all the cases investigated, elution sequence was found to be L<D.

3.3. Separation of diastereomers of ''imino acid''–(S)-NIFE derivatives in MeOH-containing mobile phase

The structure–retention relationships observed in a

MeCN-containing mobile phase hold true for the TFA–MeOH eluent system (Table 2). Thus, analytes with one ring (**1–10**) required a lower MeOH content for an acceptable resolution than did analytes with two or three rings (**11–19**). Methyl substitution in the molecule increased the retention, while incorporation of a hydroxy group resulted in a lower hydrophobicity and retention at the same or similar eluent composition. A comparison of the two eluent systems revealed that the separation of stereoisomers of analyte–(*S*)-NIFE derivatives was more efficient in MeOH-containing mobile phases than in eluents containing MeCN. In TFA–MeOH, the stereoisomers were separated with higher R_s, while at the same time the *k* values were similar or lower than in the TFA–MeCN system, except for compounds **5** and **6**, where better resolutions were obtained in MeCN-containing mobile phases. The stereoisomers of **8**, which were partially resolved in TFA–MeCN, exhibited a baseline separation in TFA–MeOH. The diastereomers of analytes forming bis derivatives (**7** and **8**) were nicely separated. The stereoisomers of mono derivatives of **15** and **16** were separated in the TFA–MeOH (40:60, v/v) or (50:50, v/v) eluent systems. In these eluent systems, the bis derivatives exhibited high *k* values ($k>20$, data not shown) and they were separated in TFA–MeOH (35:65, v/v) within a reasonable time. Fig. 3 depicts the resolution of the stereoisomers of the mono and bis derivatives of **15** and **16** in one chromatographic run in a MeOH-containing mobile phase by gradient elution. In spite of the higher selectivity, the MeOH-containing mobile phase system had the disadvantage in some cases, that one of the stereoisomers exhibited only partial separation from 4-nitrophenol or from the ''urea dimer'' (Table 2, *k* denoted by asterisks). This partial separation was improved by changing the MeOH content of the mobile phase.

The elution sequence was determined in the same manner as in MeCN-containing mobile phases and was found to be L<D.

3.4. Separation of diastereomers of ''imino acid''–(S)-NIFE derivatives by gradient elution

Gradient elutions were carried out with mobile phase systems containing 0.1% aqueous TFA as inorganic moiety and MeCN or MeOH (both with 0.1% TFA) as organic modifier. The gradient slope was 1.4% **B** min^{-1} for MeCN and 1.65% **C** min^{-1} for MeOH (see Experimental). With these gradient profiles, all the stereoisomers were separated with $R_s>1.5$, except for **8**, for which only a partial resolution (R_s~0.8) was obtained (data not shown). The *k* values obtained on gradient elution were much higher than those observed under isocratic conditions. In a MeCN-containing mobile phase with gradient slope (**a**), typical *k* values for the stereoisomers of **1–6** and **9**, **10** were $12<k<18$, while for the stereoisomers of **8**, **9** (bis derivatives) and **11–19** they were $20<k<28$. The corresponding values in MeOH-containing eluents were $18<k<26$ and $20<k<32$, respectively, with gradient profile (**b**). In spite of the high *k* values, gradient elution was advantageous when the stereoisomers of two or more amino acids or mono and bis derivatives were separated. Figs. 2 and 3 illustrate the separation of mono and bis derivatives of **15** and **16** in MeCN- or MeOH-containing mobile phases, respectively. In both eluent systems, a baseline resolution was obtained for both mono and bis derivatives. The chromatograms clearly show the relative intensities of the desired ''urea diastereomers'' (**II**) and the main side-products **III** and **V**. In the course of derivatization for compounds **15** and **16**, a five-fold excess of CDA was applied and the unreacted CDA formed **III** and **V** in the amounts depicted in Figs. 2 and 3. The side-product **IV** was formed in very low concentration and exhibited practically the same retention time as that of **III**; its position is therefore not indicated in the chromatograms. Another example of the separation of a complex mixture by gradient elution is presented in Fig. 4, where the resolution of the stereoisomers of Pip analogues may be seen. The enantiomers of *cis*-4-hydroxy-Pip (**5**), *trans*-5-hydroxy-Pip (**6**) and Pip (**4**) were baseline-separated by gradient elution in a MeCN-containing mobile phase. Under isocratic conditions the enantiomers of *cis*-4-hydroxy-Pip (**5**) and *trans*-5-hydroxy-Pip (**6**) separated only partially. In Fig. 4, the diastereomers of analytes **4**, **5**, and **6** are seen to be eluted between 4-nitrophenol and the ''urea dimer''. Similar elution behavior was observed for most of the analytes in the isocratic mode. Under gradient conditions, the diastereomers of analytes with two or three rings (**11–19**) in a MeOH-containing mobile phase and the bis

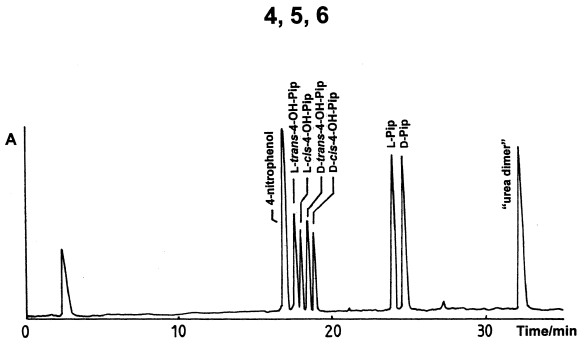


Fig. 4. Chromatogram of an artificial mixture of derivatized L- and D-piperidine-2-carboxylic acid (**4**), L- and D-*cis*-4-hydroxypipecolic acid (**5**) and L- and D-*trans*-5-hydroxypipecolic acid (**6**). Conditions of derivatization: amount of amino acid, 0.1 μmol (each); molar ratio CDA:amino acid: 2:1; pH 11; temperature, ambient; duration of reaction, 20 min; chromatographic conditions: column, LiChrospher RP-18; flow-rate, 0.8 ml/min; detection, 205 nm; mobile phase, 0.1% aqueous TFA–MeCN (with 0.1% TFA); type of gradient, (**a**) (see Experimental).

derivatives of **15** and **16** in both MeCN- and MeOH-containing mobile phases eluted close to or after the ''urea dimer'' (data not shown).

4. Conclusions

(*S*)-NIFE is well suited for the indirect separation of stereoisomers of imino acids by means of conventional RP-HPLC. Derivatization was carried out under mild reaction conditions and the derivatives formed were stable for several weeks. Separations were performed in water–MeCN or in water–MeOH mobile phases containing 0.1% TFA. Slight differences in selectivity were observed between the organic modifiers. In general, a higher resolution was achieved with smaller retention factors (k) with MeOH as organic modifier than with MeCN, but this advantage was sometimes counterbalanced by co-elution of one of the diastereomers with 4-nitrophenol or the ''urea dimer''. The elution sequence was determined in most cases and was found to be L<D.

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