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Journal of Chromatography A, 1021 (2003) 1–10
JOURNAL OF CHROMATOGRAPHY A
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Liquid chromatographic enantioseparation of spin-labelled β-amino acids

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Received 29 April 2003; received in revised form 11 August 2003; accepted 3 September 2003

Abstract

Direct and indirect high-performance liquid chromatographic (HPLC) methods were developed for the enantioseparation of spin-labelled, cyclic, chiral β-amino acids containing nitroxide free radicals, *trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (*trans*-POAC), *cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (*cis*-β-TOAC) and their *N*-Fmoc-protected analogues, synthesized in racemic and enantiomerically pure forms. The direct method involved the use of a Chiralcel OD-RH column, while indirect separation was carried out by application of either 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate or (*S*)-*N*-(4-nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester as chiral derivatizing agent. Use of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) as chiral derivatizing agent failed because of the low of yield of the derivatization reaction. Selection and variation of the mobile phase was restricted by the sensitivity of the spin-labelled amino acids to acidic conditions. Conditions affording the best resolution were found and the differences in separation capability of the methods were noted. The sequence of elution of the enantiomers was determined by different methods and, in the case of the β-TOAC analogues, the absolute configurations of the enantiomers corresponding to each peak were identified.

Keywords: Enantiomer separation; Derivatization, CC; Amino acids; Spin-labelled β-amino acids; 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl isothiocyanate; (*S*)-*N*-(4-Nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester

1. Introduction

The β-amino acids have been the subject of much synthetic effort in recent years, particularly after it was demonstrated that their oligomers may fold into stable helical conformations [1–5]. Alicyclic β-amino acids play important roles in chemistry and biology. *cis*-(1*R*,2*S*)-Aminocyclopentanecarboxylic acid (cispentacin) is an antifungal antibiotic [6,7]. *cis*- and *trans*-2-aminocyclohexanecarboxylic acids have been used in the synthesis of heterocycles with the aim of preparing potential pharmacophores [8]. Gellman and co-workers [9] successfully developed asymmetric routes to *trans*-3-aminopyrrolidine-4-carboxylic acid and *trans*-2-aminocyclopentanecarboxylic acid, while Xu et al. [10] reported a similar method for *cis*-2-aminocyclohexanecarboxylic acid. Compounds containing the bicyclo[2.2.1]heptane skeleton are versatile building blocks, e.g. in the synthesis of numerous naturally occurring compounds, such as prostanoids, alkaloids and nucleosides [11–13]. The β-amino acids are not only important pharmacologically, but are also used as building blocks for the preparation of modified (unusual) analogues of biologically active peptides. These amino acids have been utilized for determination of the fine structures of receptors [14–17].

Stable nitroxide free radicals are of continuing interest for use as spin labels in studies of the conformations and structural mobilities of biological systems [18–21], as spin traps of other radical species [22–25] and as oxidizing agents [26–28]. Moreover, optically active nitroxides have been applied as enantioselective oxidizing agents and for stereoselective coupling with prochiral radicals [29–31]. As far as amino acids are concerned, the nitroxide-bearing achiral $C^{\alpha,\alpha}$-disubstituted glycine, 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-4-carboxylic acid residue (TOAC) [29–31], has been widely used to label peptides at N-terminal and internal positions for biological studies and for conformational analysis by means of ESR methods [32–37]. We are interested in the prospect

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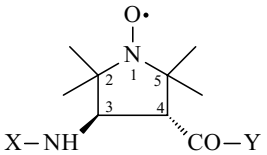
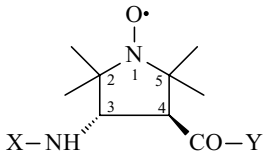
doi:10.1016/j.chroma.2003.09.015

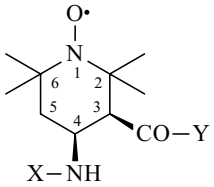


(3*R*,4*R*)

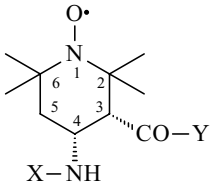


(3*S*,4*S*)

	X	Y	
1	H–	–OH	H-*trans*-POAC-OH
2	Fmoc–	–OH	Fmoc-*trans*-POAC-OH



(3*S*,4*S*)



(3*R*,4*R*)

	X	Y	
3	H–	–OH	H-*cis*-β-TOAC-OH
4	H–	$–OCH_3$	H-*cis*-β-TOAC-OMe
5	Fmoc–	–OH	Fmoc-*cis*-β-TOAC-OH

Fig. 1. Structures of *trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (POAC) and *cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (β-TOAC) analogues.

of creating spin-labelled cyclic β-amino acids that can be obtained in enantiomerically pure form, and we have recently reported the synthesis of enantiomerically pure *cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (*cis*-β-TOAC) [38] and *trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (*trans*-POAC) [39] (Fig. 1). Racemic *trans*-POAC, first described by Rassat and Rey [29], was later incorporated by solid-phase synthesis into an angiotensin II analogue [40].

In the synthesis of such compounds, chirality is often of the utmost importance. Peptide diastereomers (epimers) may have different biological properties (agonistic or antagonistic), and there is therefore great interest in methods devised for the separation and identification of enantiomers. The separation of optical isomers requires an asymmetric or chiral environment that allows diastereomeric interactions. For this purpose, high-performance liquid chromatography (HPLC) is widely applied.

Few examples are to be found in the literature on the enantioseparation of optically active compounds containing nitroxide radicals. Benfaremo et al. [41] achieved partial separation of the stereoisomers of 2,5-dimethyl-2,5-diphenylpyrrolidin-1-oxyl on an (*S*,*S*)-Whelk-01 column, as did Einhorn et al. [42] for the stereoisomers of *trans*-2,6-dimethyl-2,6-diphenylpiperidin-1-oxyl on a Chiralcel OD-H column, a normal-phase mode being applied in both cases, with hexane/2-propanol as the mobile phase. No examples have been found in the field of amino acids.

The present paper describes direct and indirect methods for the enantioseparation of spin-labelled β-amino acids containing cycloalkane skeletons. The chromatographic results are given as the retention, separation and resolution factors. Conditions affording the best resolution were determined and the differences in separation capability of the methods are discussed. The sequence of elution of the enantiomers was determined by spiking the racemic samples with enantiomers with known absolute configurations.

2. Experimental

2.1. Chemicals and reagents

The unusual spin-labelled β-amino acids were synthesized in our laboratories in racemic or in enantiopure form [38,39].

The chiral derivatizing agents (CDAs) 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent, FDAA) were from Sigma (St. Louis, MO, USA), and (*S*)-*N*-(4-nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester [(*S*)-NIFE] was from Solvay-Peptisyntha, Brussels, Belgium (but is now available from Fluka, Buchs, Switzerland). Methanol (MeOH), acetonitrile (MeCN), hexane and 2-propanol (IPA), all of HPLC grade, were obtained from Merck (Darmstadt, Germany). Triethylamine (TEA), glacial acetic acid (HOAc) and other reagents of analytical-reagent grade were also from Merck. Potassium hexafluorophosphate (KPF_6) was from Aldrich (Steinheim, Germany). The inorganic component of the mobile phase used in the reversed-phase method was prepared from Milli-Q water, which was further purified by filtering on a 0.45 μm filter, type HV, Millipore (Molsheim, France).

Triethylammonium acetate (TEAA, 0.1%) buffers were prepared by titration of 0.1% (by volume) aqueous solutions of TEA with AcOH to a suitable pH. Mobile phases for normal phase, reversed phase and polar-organic chromatography were prepared by mixing the indicated volumes of buffers and/or solvents and were further purified by filtration through a 0.45 μm Millipore filter, type HV. The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses.

Stock solutions of amino acids (1 mg ml^{-1}) were prepared by dissolution in water or in the starting mobile phase.

2.2. Apparatus

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium[32] Chromatography Manager data system; the alternative Waters Breeze system consisted of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, a column thermostat and Breeze data manager software (both systems from Waters Chromatography, Milford, MA, USA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20 μl loops.

The columns used for indirect analytical separation were Vydac 218TP54, 5 μm particle size, 250 mm × 4.6 mm i.d. (The Separations Group, Hesperia, USA) and Hyperpep 300, 5 μm particle size, 250 mm × 4.6 mm i.d. (Shandon, Rucaron, Anglia). For direct separation, Cyclobond I 2000 SN, 5 μm particle size, 250 mm × 4.6 mm i.d. (Astec, Whippany, NJ, USA), Chiralcel OD-H, 5 μm particle size, 150 mm × 4.0 mm i.d., Chiralcel OD-RH, 5 μm particle size, 150 mm × 4.0 mm i.d. (both Daicel, Tokyo, Japan) and Prontosil AX-QN-2, 5 μm particle size, 150 mm × 4.6 mm i.d. (Bishoff, Leonberg, Germany) columns were applied.

The columns were thermostated at 20–40 °C; the precision of temperature adjustment was ±0.1 °C.

2.3. Derivatization

The derivatization of the investigated analytes with the applied CDAs was based on literature methods [43–45], which were somewhat modified. For derivatization with GITC and (*S*)-NIFE, analytes were dissolved in 0.4% TEA [in MeCN/water 1/1 (v/v)] and in 0.4% aqueous TEA (v/v), respectively, while for derivatization with FDAA analyte solutions were made in 1 M $NaHCO_3$. The concentration of each analyte varied between 1 and 10 mM. The reagent stock solutions were prepared as follows: GITC was dissolved in MeCN (10 mM), (*S*)-NIFE was dissolved in dry dioxane (50 mM) that was kept on a molecular sieve (5 Å), and FDAA was dissolved in acetone (20 mM). Aliquots of 10–50 μl of analyte and reagent solutions were mixed to achieve different molar ratios of reagent to analyte (generally 4 to 1 or 2 to 1). Blank reaction mixtures containing only the solvents and the reagent in the same volumes as in the real samples were also prepared. Completion of derivatization as a function of time was checked by analysing samples taken from these reaction mixtures. Reactions were considered to be complete when the signal of the free amino acid had disappeared from the chromatogram or the integrated peak areas of the derivatives had reached a maximum.

3. Results and discussion

3.1. Direct separation of spin-labelled amino acids

Table 1 contains results relating to the separation of the enantiomers of the free and *N*-Fmoc-protected spin-labelled β-amino acids on a Chiralcel OD-RH column. The chiral selector of this stationary phase was cellulose tris(3,5-dimethylphenylcarbamate). On this stationary phase, the enantiomers of the free amino acid [H-*trans*-POAC-OH (**1**)] or the free amino acid ester [H-*cis*-β-TOAC-OMe (**4**)] exhibited only a partial separation. The chromatographic system behaved as a real reversed-phase system, i.e. the retention factors decreased with increasing organic modifier content. The application of aqueous KPF_6 instead of water in the mobile phase improved the separation. With increasing salt concentration, the retention factor decreased and an improved peak shape generally resulted, with improved resolution (data not shown). Decrease of the column temperature or flow rate had little effect on the resolution, and for compounds **1** and **4** none of these modifications improved the separation. A significant improvement in the separation was achieved by application of the *N*-Fmoc-protected stereoisomers of the spin-labelled β-amino acids. This reveals the importance of the interaction of the fluorenylmethyloxycarbonyl group of the selectand and the tris(3,5-dimethylphenylcarbamate) group of the selector in the mechanism of chiral recognition. The increased interac-

Table 1
Retention factors (k), separation factors (α) and resolutions (R_S) for the direct separation of enantiomers of spin-labelled β-amino acids on a Chiralcel OD-RH column

Compound	Mobile-phase H_2O/MeCN[a], KPF_6/MeCN[b,c,d] (v/v)	k_1	k_2	α	R_S	Elution sequence
(**1**) H-*trans*-POAC-OH	90/10[a]	0.17	0.26	1.50	<0.40	(+) < (−)[i]
	98/2[b,e,g]	0.40	0.42	1.05	<0.40	(+) < (−)[i]
	98/2[b,f,h]	0.41	0.47	1.15	0.66	(+) < (−)[i]
(**2**) Fmoc-*trans*-POAC-OH	70/30[a]	13.12	18.00	1.38	0.60	(+) < (−)[i]
	75/25[b]	5.75	7.17	1.22	0.42	(+) < (−)[i]
	75/25[c]	4.54	5.60	1.26	0.75	(+) < (−)[i]
	80/20[d]	14.09	16.88	1.20	1.11	(+) < (−)[i]
(**4**) H-*cis*-β-TOAC-OMe	80/20[a]	1.30	1.85	1.43	0.50	(−)-(3*R*,4*R*) < (+)-(3*S*,4*S*)
	90/10[d]	2.53	2.95	1.17	0.55	(−)-(3*R*,4*R*) < (+)-(3*S*,4*S*)
(**5**) Fmoc-*cis*-β-TOAC-OH	70/30[d,e]	10.09	12.92	1.28	1.04	(−)-(3*S*,4*S*) < (+)-(3*R*,4*R*)
	70/30[d]	6.38	8.28	1.30	1.78	(−)-(3*S*,4*S*) < (+)-(3*R*,4*R*)

Column, Chiralcel OD-RH; detection 205 and 254 nm; mobile phase.
[a] Water/MeCN.
[b] 0.01 M KPF_6/MeCN.
[c] 0.05 M KPF_6/MeCN.
[d] 0.1 M KPF_6/MeCN; temperature 30 °C.
[e] 20 °C.
[f] 8 °C; flow rate, 0.5 ml min^{-1}.
[g] 0.2 ml min^{-1}.
[h] 0.1 ml min^{-1}.
[i] The absolute configuration has yet not been assigned.

tion between the selector and the selectand resulted in almost baseline separation of the *N*-Fmoc-protected stereoisomers of *trans*-POAC-OH (**2**) and *cis*-β-TOAC-OH (**5**) (Table 1 and Fig. 2).

The sequence of elution of the enantiomers was determined. For the POAC analogues **1** and **2** the (+) enantiomers eluted before the (−) enantiomers, while for the β-TOAC analogues **4** and **5** the reverse elution sequence was observed. In the latter case, the previously established [38] absolute configurations of the enantiomers were confirmed (Table 1).

In the course of the direct separation, other chiral columns were tested. With the Chiralcel OD-H column, which has the same selector as Chiralcel OD-RH but used in normal-phase mode, the application of hexane/IPA or hexane/ethanol eluent systems resulted in unresolved peaks of the enantiomers of **1**–**5**. The β-cyclodextrin-based Cyclobond SN 2000 column also contains a carbamate-type group (naphthylethylcarbamate) and offers the possibility for inclusion complex formation inside the β-cyclodextrin cavity. Despite the different possibilities of interactions, no conditions were found for the separation of the enantiomers, either in the normal phase or in a polar organic mode. The same held true for the separation of these enantiomers on a Prontosil AX-QN-2 column, where the chiral selector was a 2,5-di-2-propylphenylcarbamate-derivatized quinine analogue. This selector was successfully applied earlier with *N*-2,4-dinitrophenyl-derivatized β-amino acids [46], but for the enantiomers of these spin-labelled β-amino acids no separation could be observed.

3.2. Chemistry of derivatization

Derivatization with (*S*)-NIFE took place quantitatively under mild conditions within a reasonable reaction time [45]. At room temperature, with the (*S*)-NIFE/amino acid ratio kept at 4 to 1, after 2 h of reaction the yields of derivatization for (+)-H-*trans*-POAC-OH and (−)-H-*trans*-POAC-OH (**1**) were 98.2 and 98.9%, respectively, and for (+)-H-*cis*-β-TOAC-OH and (−)-H-*cis*-β-TOAC-OH (**3**) were 99.6 and 99.3%, respectively. In all cases, the yield of derivatization with (*S*)-NIFE could be kept above 98%. Under these conditions, no racemization of these spin-labelled amino acids was observed.

Derivatization with GITC required some modifications of the original procedure [44], despite which ambiguous results were obtained. The reaction of GITC with (+)-H-*trans*-POAC-OH or with (−)-H-*trans*-POAC-OH (**1**) furnished a 99.5% or 99.7% yield, and the extent of racemization remained below 0.1%. These yields were measured at a GITC/H-*trans*-POAC-OH molar ratio of 2 to 1, after a reaction time of 4 h. The results of the reactions of GITC with (+)-H-*cis*-β-TOAC-OH and (−)-H-*cis*-β-TOAC-OH (**3**) were not so straightforward (Table 2). For (+)-H-*cis*-β-TOAC-OH (**3**) at a constant GITC/β-TOAC molar ratio of 1.5 the yield of derivatization reached its maximum value (>98%) after 4 h and the degree of racemization increased from 1.5 to 8.9% with increasing reaction time. At a constant reaction time of 90 min, increase of the GITC/(+)-H-β-TOAC molar ratio from 1.5 to 10 resulted in a 9% increase in the yield of derivatization and an

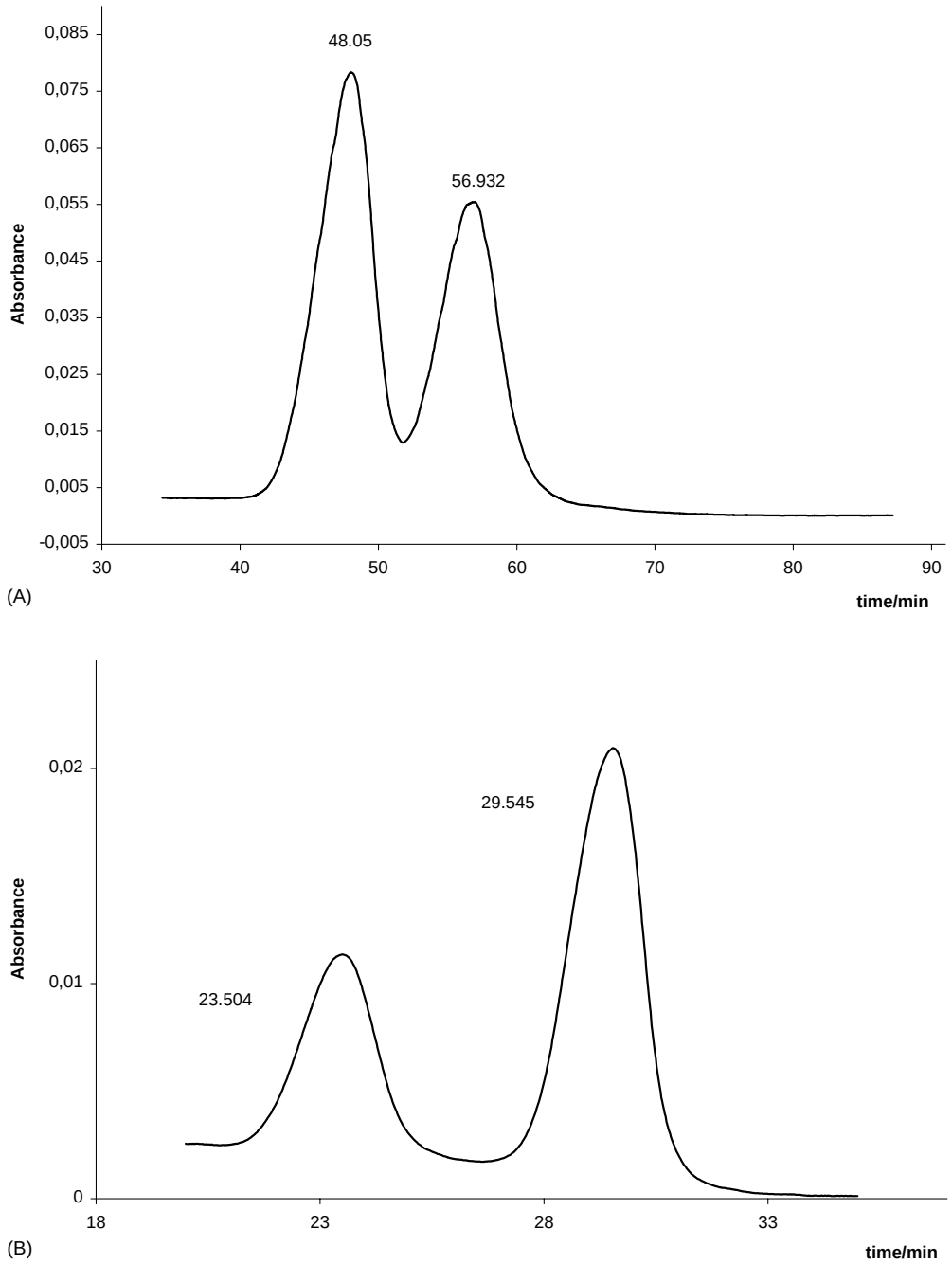


Fig. 2. Direct chromatographic separation of enantiomers of POAC (A) and β-TOAC (B) analogues. Compounds: (A) *N*-Fmoc-*trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (Fmoc-*trans*-POAC-OH) and (B) *N*-Fmoc-*cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (Fmoc-*cis*-β-TOAC-OH). Chromatographic conditions: column, Chiralcel OD-RH; temperature, 30 °C; flow rate, 0.5 ml min^{-1}; detection, 254 nm; mobile phase. (A) 0.1 M aqueous KPF_6/MeCN = 80/20 (v/v), (B) 0.1 M KPF_6/MeCN = 70/30 (v/v); peaks, artificial mixture of (+) and (−) enantiomers.

almost 100% increase in the yield of racemization. Under optimized conditions, i.e. a GITC/(+)-H-β-TOAC molar ratio of 2 and a reaction time >4 h, the yield of derivatization reached >98% and the yield of racemization could be kept below 3%.

Derivatization of (−)-H-*cis*-β-TOAC-OH with GITC at a constant GITC/(−)-H-β-TOAC molar ratio of 1.5 exhibited a low yield of derivatization and a higher degree of racemization with increasing reaction time (Table 2). Increase of the reaction time to 24 h resulted in a high yield of derivatiza-

Table 2
Dependence of the yield of derivatization (%) and the yield of racemization (%) of (+)-H-*cis*-TOAC-OH + GITC and (−)-H-*cis*-TOAC-OH + GITC (in parenthesis) on reaction time and of GITC/amino acid molar ratio

GITC/amino acid molar ratio: 1.5				
Reaction time (min)	20	60	90	>240
yield of derivatization (%)	79.4 (4.3)	89.2 (6.1)	90.4 (8.2)	98.1 (96.3[a])
Yield of racemization (%)	1.5 (<1.0)	2.0 (3.2)	2.8 (3.4)	8.9 (16.7[a])
Reaction time: 90 min				
GITC/amino acid molar ratio	1.5	2.0	5.0	10.0
Yield of derivatization (%)	90.4 (8.2)	94.5 (24.0)	99.5 (40.7)	99.3 (98.9)
Yield of racemization (%)	2.9 (3.4)	2.8 (5.1)	6.1 (5.3)	5.8 (6.2)

[a] Reaction time: >24 h.

tion (>96%), but unfortunately the yield of racemization also increased (16.7%). At a constant reaction time of 90 min, increase of the GITC/(−)-H-β-TOAC molar ratio from 1.5 to 10 drastically increased the yield of the reaction, while the yield of racemization also increased. No condition was found at which a high yield (>98%) of derivatization could be attained and the degree of racemization could be kept low (<1%). In summary, no explanation has been found for this difference in racemization of the POAC (**1**) and β-TOAC (**3**) analogues.

Derivatization and separation of the enantiomers of **1**, **3** and **4** were also carried out by the application of FDAA [43] (data not shown). At an FDAA/amino acid molar ratio of 2 to 1 and at 40 °C, this derivatization did not lead to the formation of a derivative: complete decomposition of the reagent was detected after 8 h. On periodical addition of the reagent, after reaction for 18 h, a small extent of derivative formation was detected (<5%). Increase of the FDAA/amino acid molar ratio to 5 to 1 or increase of the temperature to 50 °C did not result in higher yields, but at 50 °C the rate of decomposition of FDAA increased. In summary, increase of the FDAA/amino acid molar ratio, the reaction time and the reaction temperature did not improve the yield of derivatization: the rate of reaction with FDAA remained low. Application of FDAA for the derivatization of spin-labelled β-amino acids is therefore suggested only for qualitative purposes.

4. Indirect separation of spin-labelled amino acids

The enantiomers of **1**, **3** and **4** were separated as (*S*)-NIFE or GITC derivatives on Vydac 218TP54 C_{18} or Hyperpep 300 C_{18} columns and some of these results are presented in Table 3. Since spin-labelled β-amino acids containing nitroxide free radicals are sensitive to acidic conditions, selection of the mobile phase was a crucial point of the analysis. The inorganic part of the mobile phase contained 0.1 M TEAA at pH 6.5 and MeOH was applied as organic modifier. Under these conditions, no β-amino acid decomposition products were observed during the analysis (extra peaks in the chromatograms originated from the decomposition of the CDAs and were the same as obtained for blank solutions of the CDAs). Separations were carried out both in isocratic mode and in gradient mode, but better peak shapes and

Table 3
Retention factors (k), separation factors (α) and resolutions (R_S) for the indirect separation of enantiomers of spin-labelled β-amino acids as (*S*)-NIFE and GITC derivatives

Compound	Mobile phase, TEAA/MeOH[a,b]	k_1	k_2	α	R_S	Elution sequence
(*S*)-NIFE						
(**1**) H-*trans*-POAC-OH	a	6.07	7.07	1.17	7.20	(+) < (−)[c]
(**3**) H-*cis*-β-TOAC-OH	a	7.10	7.89	1.10	2.90	(+)-(3*S*,4*S*) < (−)-(3*R*,4*R*)
(**4**) H-*cis*-β-TOAC-Ome	a	10.35	10.41	1.01	<0.40	(+)-(3*S*,4*S*) < (−)-(3*R*,4*R*)
GITC						
(**1**) H-*trans*-POAC-OH	a	6.38	7.05	1.10	2.80	(+) < (−)[c]
(**3**) H-*cis*-β-TOAC-OH	b	7.70	8.85	1.15	6.03	(+)-(3*S*,4*S*) < (−)-(3*R*,4*R*)
(**4**) H-*cis*-β-TOAC-Ome	a	12.86	12.86	1.00	0.00	–

Column, Vydac 218TP54; temperature 25 °C; detection, (*S*)-NIFE 205 nm, GITC 250 nm; mobile phase, 0.1 M aqueous TEAA (pH 6.5)/MeOH (v/v), gradient elution.

[a] Linear gradient, 0 min 0.1 M TEAA/MeOH = 90/10 (v/v), 60 min 0.1 M TEAA/MeOH = 10/90 (v/v).

[b] Linear gradient, 0 min 0.1 M TEAA/MeOH = 90/10 (v/v), 60 min 0.1 M TEAA/MeOH = 30/70 (v/v); absolute configuration, (+)-Fmoc-*cis*-β-TOAC-OH: (3*R*,4*R*), (−)-Fmoc-*cis*-β-TOAC-OH: (3*S*,4*S*), (+)-H-*cis*-β-TOAC-OH: (3*S*,4*S*), (−)-H-*cis*-β-TOAC-OH: (3*R*,4*R*).

[c] The absolute configuration has yet not been assigned.

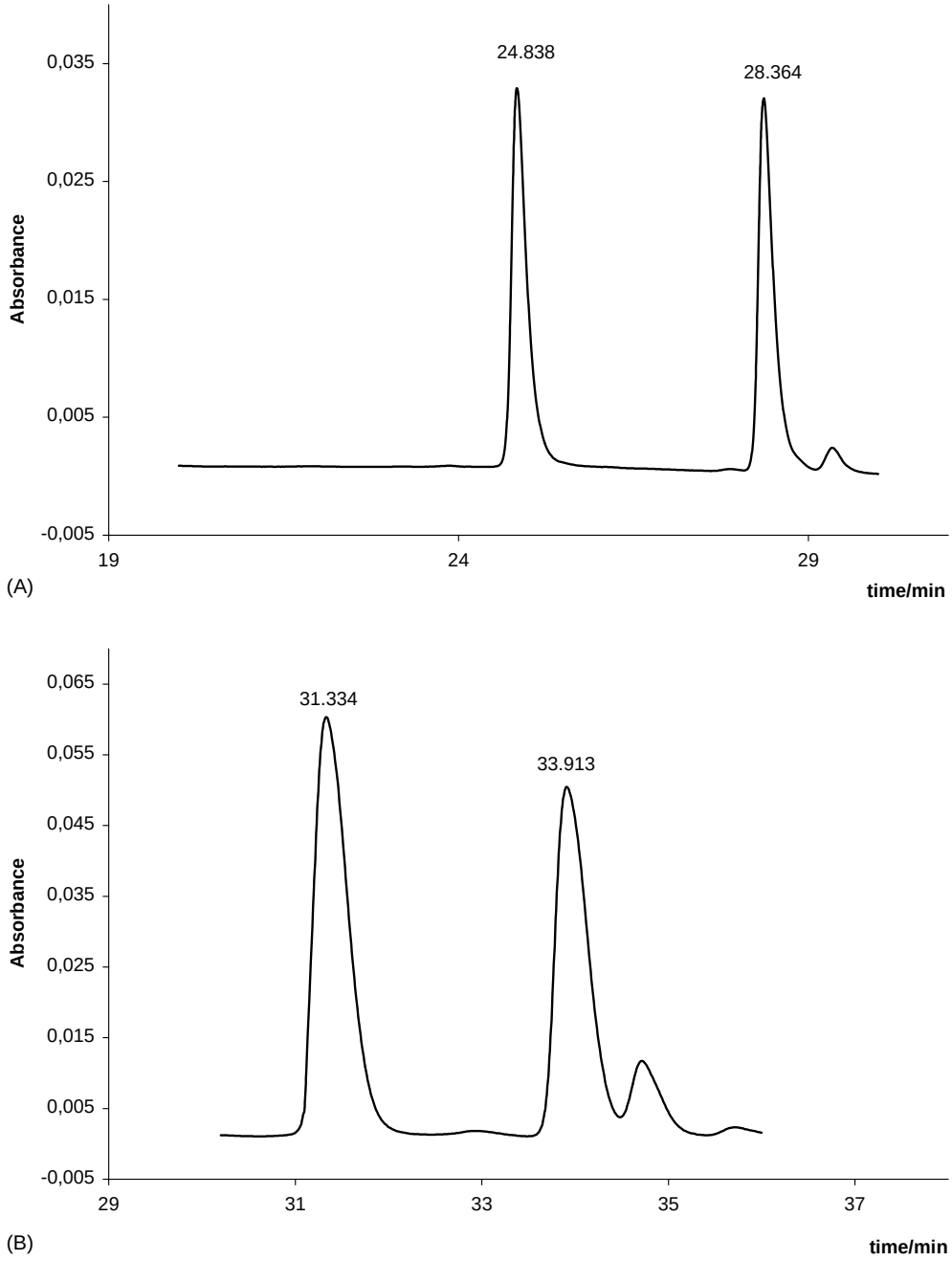


Fig. 3. Indirect chromatographic separation of enantiomers of POAC (A) and β-TOAC (B) analogues as *N*-(*S*)-NIFE derivatives. Compounds: (A) *N*-(*S*)-NIFE derivative of *trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (*N*-(*S*)-NIFE-*trans*-POAC-OH) and (B) *N*-(*S*)-NIFE derivative of *cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (*N*-(*S*)-NIFE-*cis*-β-TOAC-OH). Chromatographic conditions: column, Vydac 218TP54 C_{18}; temperature, 25 °C; flow rate, 0.8 ml min^{-1}; detection, 205 nm; mobile phase. (A and B) 0.1 M aqueous TEAA (pH 6.5)/MeOH, linear gradient elution, 0 min 0.1 M TEAA/MeOH = 90/10 (v/v), 60 min 0.1 M TEAA/MeOH = 10/90 (v/v); peaks, artificial mixture of (+) and (−) enantiomers.

resolutions were achieved on gradient elution. Table 3 therefore shows results obtained in gradient runs. Both (*S*)-NIFE and GITC derivatives resulted in high resolutions (Figs. 3 and 4), but a significant difference in efficiency of derivatization was observed for the two CDAs. Since derivatization with GITC sometimes led to ambiguous results, the work with this CDA needs higher circumspection. The same held true for the application of FDAA, where the rate of derivatization was low. In summary, (*S*)-NIFE was the most appropriate of the three CDAs for the derivatization and separation of spin-labelled β-amino acids.

Chromatographic methods developed in this investigation were applied to check on the enantiomeric purity

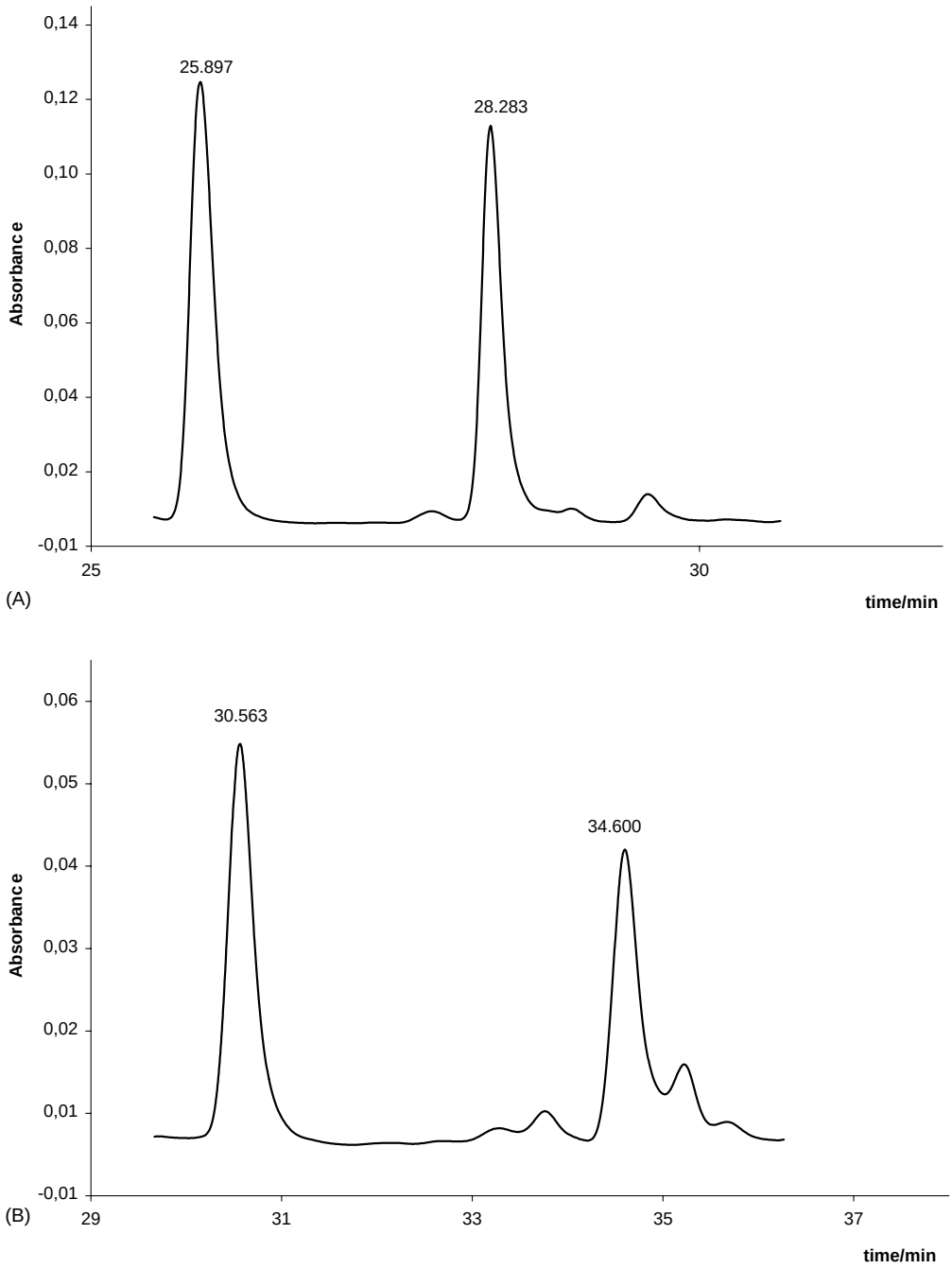


Fig. 4. Indirect chromatographic separation of enantiomers of POAC (A) and β-TOAC (B) analogues as *N*-GITC derivatives. Compounds: (A) *N*-GITC derivative of *trans*-3-amino-1-oxyl-2,2,5,5-tetramethylpyrrolidine-4-carboxylic acid (*N*-(*S*)-NIFE-*trans*-POAC-OH) and (B) *N*-GITC derivative of *cis*-4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine-3-carboxylic acid (*N*-GITC-*cis*-β-TOAC-OH). Chromatographic conditions: column, Vydac 218TP54 C_{18}; temperature, 25 °C; flow rate, 0.8 ml min^{-1}; detection, 250 nm; mobile phase. (A and B) 0.1 M aqueous TEAA (pH 6.5)/MeOH, linear gradient elution, 0 min 0.1 M TEAA/MeOH = 90/10 (v/v), 60 min 0.1 M TEAA/MeOH = 30/70 (v/v); peaks, artificial mixture of (+) and (−) enantiomers.

of the stereoisomers after synthesis. The results listed in Table 4 revealed that the extent of contamination of the enantiomers as determined by the indirect (*S*)-NIFE method was comparable with that obtained by direct separation on a Chiralcel OD-RH column. Of the two indirect methods, derivatization with (*S*)-NIFE indicated a low degree of racemization, while the application of GITC sometimes led to a higher degree. The relative standard deviation (R.S.D.%) for the direct and (*S*)-NIFE methods lay in the range 5–15%. The higher values were obtained when the minor component eluted after the major one.

Table 4
Chiral impurity (%) of spin-labelled β-amino acid enantiomers, determined by direct and indirect methods

Compound	Contamination (%)			Elution sequence
	Direct method	Indirect method		
		(*S*)-NIFE	GITC	
(**2**) (+)-Fmoc-*trans*-POAC-OH[a,c]	0.15	–	–	(+) < (−)[c]
(**2**) (−)-Fmoc-*trans*-POAC-OH[b,c]	0.40	–	–	(+) < (−)[c]
(**5**) (+)-Fmoc-*cis*-β-TOAC-OH[a]	0.17	–	–	(−) < (+)
(**5**) (−)-Fmoc-*cis*-β-TOAC-OH[b]	0.21	–	–	(−) < (+)
(**1**) (+)-H-*trans*-POAC-OH[a,c]	–	0.42	0.10	(+) < (−)[c]
(**1**) (−)-H-*trans*-POAC-OH[b,c]	–	0.52	0.69	(+) < (−)[c]
(**3**) (+)-H-*cis*-β-TOAC-OH[a]	–	0.13	>3.0	(+) < (−)
(**3**) (−)-H-*cis*-β-TOAC-OH[b]	–	0.12	>5.0	(+) < (−)

[a] (−) Contamination in (+) enantiomer.
[b] (+) Contamination in (−) enantiomer; absolute configurations, (+)-Fmoc-*cis*-β-TOAC-OH: (3*R*,4*R*), (−)-Fmoc-*cis*-β-TOAC-OH: (3*S*,4*S*), (+)-H-*cis*-β-TOAC-OH: (3*S*,4*S*), (−)-H-*cis*-β-TOAC-OH: (3*R*,4*R*).
[c] The absolute configuration has not yet been assigned; direct method, column: Chiralcel OD-RH; indirect method, (*S*)-NIFE, derivatization with (*S*)-*N*-(4-nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester, GITC, derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate.

5. Conclusions

Direct and indirect high-performance liquid chromatographic methods were developed for the enantioseparation of spin-labelled, cyclic, chiral β-amino acids containing nitroxide free radicals. The direct method involved the use of a Chiralcel OD-RH column, while indirect separations were carried out by the application of either 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate or (*S*)-*N*-(4-nitrophenoxycarbonyl)-phenylalanine methoxyethyl ester as chiral derivatizing agent. Application of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) failed because of the low reactivity of the analytes towards the reagent. The methods were compared and the applicability of the best methods for chiral purity analysis was suggested. Conditions affording the best resolution were determined, and the differences between the separation capabilities of the methods were noted. The sequences of elution in the different methods were determined and for the β-TOAC analogues the absolute configuration of the enantiomer corresponding to each peak was identified.

Acknowledgements

This work was supported by OTKA grant T 042451.

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