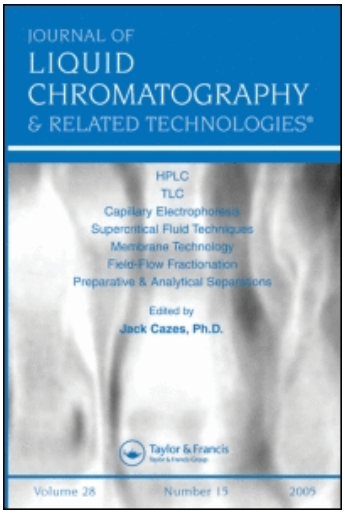

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HPLC Separation of Enantiomers of α-Substituted Proline Analogues by the Application of (*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine Methoxyethyl Ester as Chiral Derivatizing Agent

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ABSTRACT

Indirect high-performance liquid chromatographic enantioresolution of highly constrained α-substituted proline analogues as (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester derivatives is reported.

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The diastereoisomers formed were analysed under reversed-phase conditions by means of gradient elution. Baseline separation was achieved for all of the derivatives of each investigated analyte. The elution sequence was determined, and this allows identification of the configuration of the α-substituted proline analogues in the peptide epimers, resulting from use of the racemates of proline analogues in peptide synthesis.

Key Words: HPLC separation; Enantiomers; α-Substituted proline analogues; Peptides; (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester, ((*S*)-NIFE).

INTRODUCTION

The use of peptides as potential drugs is restricted by their poor absorption, proteolytic instability, unwanted side-effects, and fast clearance from the body. Therefore, research is directed towards the development of peptidomimetics. In the design of peptidomimetics, the bioactive conformation of peptides is often used as lead structure. However, in consequence of the conformational flexibility of linear peptides, identification of the bioactive conformation is difficult. A valuable tool can be the incorporation of conformational constraints into a peptide.[1–3]

Proline occupies a special place among the amino acids, as its presence in peptides can give rise to *cis*/*trans* isomerization.[4–6] Moreover, the D-Pro-L-Pro motif has been shown to be a strong type-II β-turn inducer.[7] α-Substituted proline analogues are members of an important class of conformationally constrained amino acids. The substitution of proline for (*S*)-α-methylproline has been shown to stabilize a type-I β-turn.[8] Prolines substituted at the α position by substituents that correspond to the side-chains of other amino acids are regarded as "proline chimeras." These chimeras combine the conformational constraint of proline with the side-chain of another amino acid that might be important for biological recognition.[9] Since the biological and physicochemical properties of peptides are strongly related to the stereochemistry of the incorporated amino acids, it is very important to have, at hand, effective analytical methods that are suitable for the separation and identification of the amino acid enantiomers. Among the possible methods, high-performance liquid chromatography (HPLC) is routinely used for the discrimination of enantiomers. For chromatographic enantioseparation of chiral amino compounds, direct and indirect methods are frequently used.[10–17] In indirect methods, the enantiomers are converted into diastereoisomers by reaction with a chirally pure derivatizing agent (CDA), followed by their separation on an achiral column.

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Several α-substituted proline analogues, including alkyl, aryl, and diverse benzyl substituents are obtained synthetically, either as racemic mixtures or via asymmetric synthesis.[18–20] The application of racemic α-substituted proline analogues in peptide synthesis needs the identification of stereoisomers in epimeric peptides. The asymmetric synthesis requires an analytical method for the determination of enantiomeric purity. In both cases, in qualitative and quantitative analysis of α-substituted proline analogues, the starting step is the separation of stereoisomers. The aim of the present work was to develop an efficient, simple, indirect HPLC method for the resolution of enantiomers of α-substituted proline analogues. For this purpose, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester ((*S*)-NIFE) as CDA was used, which was earlier successfully applied in the indirect HPLC enantioseparation of unnatural amino acids.[21–24] The diastereoisomers formed were analyzed under reversed-phase (RP) conditions by means of gradient elution. The elution sequence was determined, and this allowed identification of the configuration of the α-substituted proline analogues in the peptide epimers resulting from use of the racemates of proline analogues in peptide synthesis.

EXPERIMENTAL

Chemicals and Reagents

The investigated α-substituted proline analogues (Fig. 1) were produced in our laboratory (BioQuadrant Inc., Quebec, Canada). The racemic samples were synthesized in *N-tert*-butyloxycarbonyl (Boc) protected form (**1-11a**), while the enantiopure samples in one enantiomeric form were free amines (**1-11b**) and were synthesized according to the Seebach method.[10] Enantiomeric purity was determined by the present method. (*S*)-NIFE was a gift from Solvay-Peptisyntha (Brussels, Belgium), but now is available from Fluka (Buchs, Switzerland).

HPLC grade acetonitrile (MeCN) and analytical reagent grade trifluoroacetic acid (TFA), triethylamine (TEA), dioxane, and other chemicals, were purchased from Merck (Darmstadt, Germany).

Derivatization

Derivatization of the investigated analytes with the applied CDA was based on literature method.[21–24] Since derivatization with CDA occurs on

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R_1: -Boc

1-11 a

R_1: -H

1-11 b#

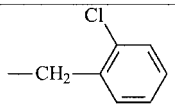
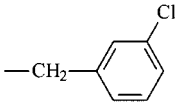
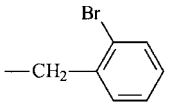
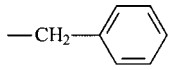
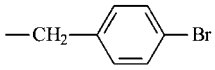
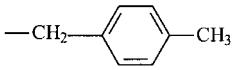
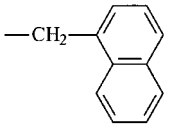
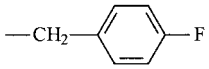
Compound	R_2	Compound	R_2
1	$-CH_3$	7	
2	$-CH_2-CH_2-CH_3$	8	
3	$-CH_2-CH=CH_2$	9	
4		10	
5		11	
6			

Figure 1. Structures of the investigated α-substituted proline analogues. **1a:** (*R*,*S*)-Boc-α-methylproline, **1b**#: (*S*)-α-methylproline; **2a:** (*R*,*S*)-Boc-α-propylproline, **2b**#: (*S*)-α-propylproline; **3a:** (*R*,*S*)-Boc-α-allylproline, **3b:** (*R*)-α-allylproline; **4a:** (*R*,*S*)-Boc-α-benzylproline, **4b:** (*R*)-α-benzylproline; **5a:** (*R*,*S*)-Boc-α-(4-methylbenzyl) proline, **5b:** (*R*)-α-(4-methylbenzyl)proline; **6a:** (*R*,*S*)-Boc-α-(4-fluorobenzyl)proline, **6b:** (*R*)-α-(4-fluorobenzyl)proline; **7a:** (*R*,*S*)-Boc-α-(2-chlorobenzyl)proline, **7b:** (*R*)-α-(2-chlorobenzyl)proline; **8a:** (*R*,*S*)-Boc-α-(3-chlorobenzyl)proline, **8b:** (*R*)-α-(3-chlorobenzyl)proline; **9a:** (*R*,*S*)-Boc-α-(2-bromobenzyl)proline, **9b:** (*R*)-α-(2-bromobenzyl)proline; **10a:** (*R*,*S*)-Boc-α-(4-bromobenzyl)proline, **10b:** (*R*)-α-(4-bromobenzyl)proline; **11a:** (*R*,*S*)-Boc-α-(1-naphthylmethyl)proline, **11b:** (*R*)-α-(1-naphthylmethyl)proline. #According to the Cahn–Ingold–Prelog rule, the priority of the groups surrounding the asymmetric carbon is different with methyl and propyl substituents from that with other substituents, and thus, the absolute configurations are different from those of the other analogues investigated.

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the amino group, racemic samples were Boc-deprotected prior to derivatization, according to the usual method.[25]

Completion of derivatization was checked by analysing samples taken from these reaction mixtures as a function of time. Reactions were considered to be complete when the integrated peak areas of the derivatives reached a maximum, or to follow disappearance of the peak of amino acid on the chromatogram detected at 205 nm, by application of a PDA detector. Derivatization with (*S*)-NIFE was complete within 1 hour at room temperature. The excess of (*S*)-NIFE was removed by adding a 20-fold molar excess of Gly to the reaction mixtures after derivatization was complete.[22]

Apparatus and Chromatography

Chromatographic analyses were carried out with a Waters HPLC system, equipped with an M-600 low-pressure gradient pump, an M-996 photodiode array (PDA) detector, and an M-717 Autosampler. Waters Millennium^{32} Chromatography Manager software was used for acquisition and for data handling (Waters, Milford, MA).

Gradient elutions were performed on RP columns, Vydac 218TP54 C_{18}, 250 × 4.6 mm I.D., 5-μm particle size (The Separations Group, Hesperia, CA) and Discovery® BIO Wide Pore C_{18}, 250 × 4.6 mm I.D., 5-μm particle size (Sigma-Aldrich, Bornem, Belgium) using a binary solvent system. Component A was 0.1% aqueous TFA and component B was MeCN containing 0.1% TFA. The gradient program consisted of the following steps. Component B was increased linearly from 3% (v/v) to 80% (v/v) within 30 min, kept at 80% (v/v) during the next 10 min, and then decreased to the starting value of 3%. Runs were performed at a flow rate of 1.0 mL min^{-1}. (*S*)-NIFE derivatives were detected at 205 nm.

0.1% aqueous TFA and MeCN containing 0.1% TFA, were prepared as follows: 1.0 mL TFA was added to 1 L Milli Q water or to HPLC grade MeCN, and the mixtures were filtered through a 0.45-μm filter of type HV (Millipore, Molsheim, France). Mobile phases were degassed in an ultrasonic bath, and during chromatographic analyses helium was sparged through them.

Twenty microliters aliquots of derivatized samples were injected onto the RP HPLC column after a 10-fold dilution with the starting mobile phase. The dead-time (t_M) of the RP column was determined by injecting 20 μL of a 0.01 M solution of KBr.

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RESULTS AND DISCUSSION

Chemistry of Derivatization

The derivatization with (*S*)-NIFE took place quantitatively under mild conditions (room temperature), within a reasonable reaction time (1 hr). In the course of chromatographic analysis, some stereoisomers may co-elute with the excess of reagent. The best solution of this problem (besides change of the slope of the gradient), was to add a 20-fold molar excess of Gly to the reaction mixtures after the completion of derivatization. The peak of the excess of reagent no longer interfered, since the (*S*)-NIFE-Gly derivative eluted at a different retention time. The derivatized samples could be stored in a refrigerator for several weeks without significant decomposition.

Besides (*S*)-NIFE, other derivatizing agents were applied too (data not shown). Derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-*L*-alanine amide) was not complete even within 5 days, and kinetic resolution was observed during derivatization, although a large excess of the CDA was applied. Reaction with 2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl isothiocyanate (GITC) at room temperature did not result in the formation of derivatives. At elevated temperature (40°C), the desired thiourea derivatives were formed quantitatively after 5 days of reaction. For the derivatization of highly constrained α-substituted proline analogues, therefore, (*S*)-NIFE was the most applicable.

Chromatographic Separation of the Derivatives

In order to ensure the reproducibility of the RP analyses, the degree of ionization of the carboxylate group should be kept at a constant level through the use of an acidic mobile phase. In our case, 0.1% TFA was used both in the aqueous and in the organic part of the mobile phase, to ensure a constant ionic strength and to reduce the baseline drift and asymmetric peak shape.

Separation of Stereoisomers as (*S*)-NIFE Derivatives

The results of the chromatographic separation of the derivatives formed with (*S*)-NIFE are given in Table 1. Of the two stationary phases, the Discovery BIO Wide Pore C18 column resulted in a higher resolution than that attained with the Vydac column. The separation factors (α) did not differ significantly, but the R_S factors were larger on the Discovery stationary phase. The solutes and stationary phases exhibited a reversed-phase system: increase

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Table 1. Retention factors (k), separation factors (α) and resolutions (R_S) for separations of enantiomers of α-substituted proline analogues as (*S*)-NIFE derivatives.

Compound	Column (a, b)	$k_{(R)}$	$k_{(S)}$	α	R_s
1[a]	**a**	5.00	4.81	1.04	3.84
	b	4.42	4.63	1.05	3.04
2[a]	**a**	6.06	5.82	1.04	4.20
	b	5.51	5.74	1.04	3.33
3	**a**	5.57	5.78	1.04	4.00
	b	5.25	5.46	1.04	2.87
4	**a**	6.31[b]	6.44	1.02	2.50
	b	6.04[b]	6.16	1.02	1.47
5	**a**	6.78	6.91	1.02	2.73
	b	6.44	6.55	1.04	1.47
6	**a**	6.44	6.53	1.01	1.83
	b	6.19	6.26	1.01	1.18
7	**a**	6.67	6.83	1.02	2.72
	b	6.42	6.53	1.02	1.73
8	**a**	6.81	7.01	1.03	4.09
	b	6.50	6.66	1.02	2.34
9	**a**	6.86	7.00	1.02	2.87
	b	6.52	6.64	1.02	1.59
10	**a**	7.03	7.16	1.02	1.95
	b	6.74	6.83	1.01	1.40
11	**a**	6.99	7.15	1.02	2.83
	b	6.73	6.83	1.01	1.43

Notes: Column, **a**, Discovery® BIO Wide Pore C18; **b**, Vydac 218TP54; mobile phase, 0.1% aqueous trifluoroacetic acid/acetonitrile containing 0.1% trifluoroacetic acid, gradient elution (see "Experimental: Apparatus and Chromatography"); flow rate, $1\,mL\,min^{-1}$; detection, 205 nm; $k_{(R)}$ and $k_{(S)}$ are the retention factors of the enantiomers; dead-volume of the column: $V_M = 3.62\,mL$.

[a]Apparent change of elution sequence arising from the Cahn–Ingold–Preloge rule.

[b]Co-elution with the "urea dimer."

of the organic modifier content decreased the retention factor, selectivity factor, and resolution (data not shown). Comparison of the chromatographic data for analogous compounds under the same chromatographic conditions permit observations relating to the structure–retention relationship (Table 1). Within the series of α-alkyl-substituted Pro analogues, the isomers of α-methylproline were the least retained, while those of α-propylproline eluted later, due to the difference in hydrophobicity. α-Allylproline, which is more polar than α-propylproline, eluted before them. The same held true for α-aryl-substituted Pro analogues. The isomers of α-benzylproline were the least

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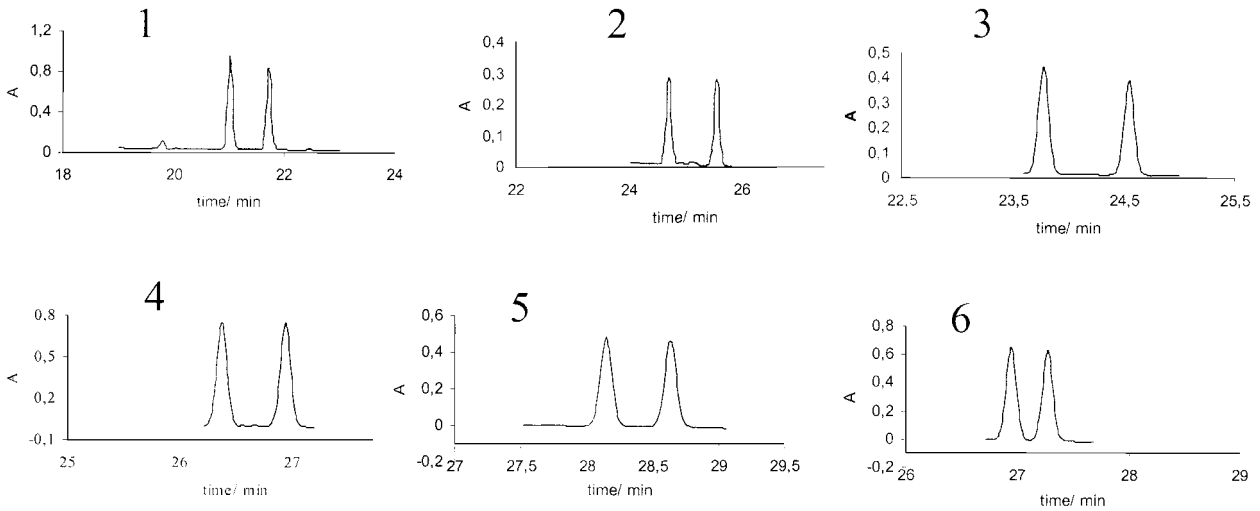

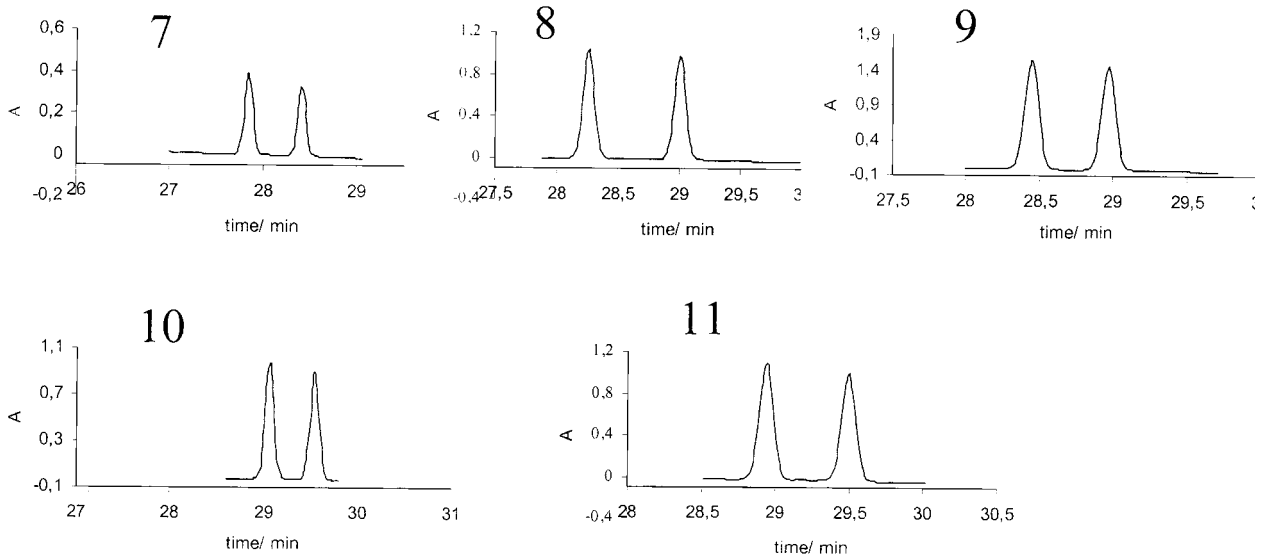

Figure 2. Separation of α-substituted proline analogues **1–11** as (*S*)-NIFE derivatives. Column: Discovery® BIO Wide Pore C18; gradient elution, component **A**, 0.1% aqueous TFA and component **B**, MeCN containing 0.1% TFA; gradient program, **B** was increased linearly from 3% (v/v) to 80% (v/v) within 30 min [for compound **4**, **B** was increased linearly from 3% (v/v) to 80% (v/v) within 40 min]; flow rate, 1.0 mL/min; detection 205 nm.

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retained, while α-fluorobenzyl, α-chlorobenzyl, α-bromobenzyl, α-methylbenzyl, and α-(1-naphthylmethyl) substitution caused slight increases in retention, due to the increase in hydrophobicity.

The structures (rigidities and bulkiness) of the analytes did not have a significant influence on the enantioselectivity or on the resolution: similar values were obtained for the various analytes. However, the different α-alkyl substituents did exert slight effects on the resolution of the enantiomers as compared with α-aryl substitution. Under identical chromatographic conditions, α-alkyl substitution led to a better resolution of the enantiomers than did α-aryl substitution (with exception of **8**). With the applied CDA, baseline separation was achieved for all of the investigated stereoisomers.

Under these chromatographic conditions, the first-eluting stereoisomer of the (*S*)-NIFE derivative of compound **4** co-eluted with the side-product of the derivatization, the "urea dimer": *N*, *N*′-bis(3-phenylpropionic acid methoxyethyl ester 2-yl)urea, which eluted at the end of the chromatogram.[21,24] (Table 1); this problem could easily be solved by change of the slope of the gradient. Chromatograms illustrating the separation of the enantiomers **1–11** as (*S*)-NIFE derivatives are shown in Fig. 2.

Determination of Elution Sequence, Detection Limit of the Procedure

The elution sequence was determined by co-injection of the derivatized enantiopure samples with the derivatives of racemic samples. The first-eluting enantiomers of **1** and **2** had the (*S*) configuration, while the less strongly retained enantiomers of **3–11** had the (*R*) configuration. This is only an apparent change of elution sequence arising from the Cahn–Ingold–Prelog rule. The knowledge of the elution sequence allows the identification of the configurations of the α-substituted proline analogues in the peptide epimers, resulting from use of the racemates of **1a-11a** in the peptide synthesis.

The limit of detection (LOD) of the second-eluting minor enantiomer in the presence of the major enantiomer was also determined. Two analytes, α-propyl- and α-(4-bromobenzyl)-substituted prolines (**2** and **10**, respectively), were selected for this study. The LOD was determined at a signal-to-noise ratio of 3 : 1. For the (*S*)-NIFE derivatives, the LOD for the second-eluting minor enantiomer in the presence of the major one was 0.1%.

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CONCLUSIONS

(*S*)-NIFE was applied for the indirect resolution of enantiomers of highly constrained α-alkyl- and α-benzyl-substituted proline analogues. The short reaction time (1 hour) of derivatization makes this CDA suitable for the chiral analysis of highly constrained α-alkyl- and α-benzyl-substituted proline analogues.

As regards the chromatographic properties, baseline separation of the enantiomers of each analyte was achieved. The apparent change in elution sequence observed between α-alkyl- or α-alkene- and α-aryl-substituted proline analogues can be explained by the Cahn–Ingold–Prelog rule.

The present method, based on (*S*)-NIFE derivatization, allows determination of enantiomeric purity after asymmetric synthesis, and identification of the configuration of the α-substituted proline analogues in the peptide epimers resulting from use of racemates in the peptide synthesis.

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