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# Indirect high-performance liquid chromatographic separation of stereoisomers of $\beta$ -alkyl-substituted amino acids by the application of (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester as chiral derivatizing agent

Erika Vékes<sup>a</sup>, Gabriella Török<sup>a</sup>, Antal Péter<sup>a,\*</sup>, János Sápi<sup>b</sup>, Dirk Tourwé<sup>c</sup>

<sup>a</sup>Department of Inorganic and Analytical Chemistry, University of Szeged, PO Box 440, Dóm tér 7, H-6720 Szeged, Hungary

<sup>b</sup>Faculté de Pharmacie, Université de Reims-Champagne-Ardenne, 51 rue Cognacq Jay, F-51096 Reims Cedex, France

<sup>c</sup>Eenheid Organische Chemie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

## Abstract

The indirect high-performance liquid chromatographic enantioresolution of  $\beta$ -alkyl-substituted analogues of tyrosine, phenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and tryptophan is reported. (*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester, a recently developed chiral derivatizing agent, was used for pre-column derivatization of the investigated analytes. The diastereoisomers formed were analysed under reversed-phase conditions. The effects of parameters such as the amount and type of the organic modifier and the type of the stationary phase on the resolution and retention of the derivatives were investigated. Chromatographic conditions were found for the separation of all four stereoisomers of each analyte. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Enantiomer separation; Derivatization, LC; Amino acids; Nitrophenoxycarbonylphenylalanine methoxyethyl ester

## 1. Introduction

Besides their primary structure (amino acid sequence), the conformation (secondary and tertiary structures) of peptides and proteins is crucial as concerns their biological activity. The chi space, the three-dimensional structure of the side-chain moieties, which can be characterized by chi torsional angles, is also important, because these angles together with the backbone angles are of key importance for an understanding of the mode of action of peptides. Since these molecules are structurally

flexible, they can adopt numerous conformations under physiological conditions [1]. Determination of the biologically active conformation of peptides is an important goal in modern biology. The introduction of conformational constraints into peptides is one of the frequently used possible approaches for the conformational restriction of peptides, which can lead to enhanced receptor affinity and selectivity, and stability towards proteolytic degradation [2–4]. Increasing attention is currently being paid to aromatic amino acids since the aromatic groups on a peptidic ligand are believed to play an important role in the interactions with the receptor. Analogues of tyrosine, phenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and tryptophan have recently been designed by stereospecific  $\beta$ -alkyl-substitution with

\*Corresponding author. Tel.: +36-62-544-000, ext. 3656; fax: +36-62-420-505.

E-mail address: apeter@chem.u-szeged.hu (A. Péter).

the aim of constraining the aromatic side-chain. These  $\beta$ -alkyl-substituted aromatic unusual  $\alpha$ -amino acids are obtained synthetically either as a mixture of stereoisomers, or via asymmetric synthetic strategies as stereochemically pure or enriched products [5]. However, even when the latter strategy is applied, the optical purity of the isomers never reaches 100%. Since the biological and physicochemical properties of peptides are strongly related to the stereochemistry of the incorporated amino acids, the separation and identification of the amino acid stereoisomers are very important.

Among the effective analytical methods, high-performance liquid chromatography (HPLC) is widely used for the discrimination of stereoisomers. The procedures used for chromatographic enantioseparations can generally be classified as either direct or indirect methods. Direct methods involve two techniques: separation on chiral stationary phases (CSPs) and the use of chiral mobile phase additives (CMPAs) in conjunction with achiral phases. In indirect methods, the enantiomers are converted into diastereoisomers by reaction with an optically pure chiral derivatizing agent (CDA), followed by their separation on achiral columns. For the enantioseparation of chiral amino compounds, both direct and indirect methods are frequently used [6–13]. The popularity of indirect chiral HPLC separations has not decreased, despite the existence of effective direct methods. On the contrary, there is a constant search for new CDAs [14–23]. A new CDA, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester or (*S*)-NIFE, developed by Peptisyntha, a wholly owned affiliate of Solvay [24], was recently commercialized. This CDA has proved to be efficient in the enantiomeric separation of proteinogenic amino acids [25], several ring- and  $\alpha$ -substituted phenylalanine and phenylalanine amide analogues [26] and unnatural sterically constrained secondary  $\alpha$ -amino acids, “imino acids” [27].

$\beta$ -Substituted  $\alpha$ -amino acids contain two asymmetric carbon atoms, and four stereoisomers (two pairs of enantiomers) are therefore possible. In our earlier work, different CSPs and CDAs were applied for the separation of the stereoisomers of these amino acids [28–37]. The indirect analysis was achieved by applying pre-column derivatization with optically pure chiral reagents, 1-fluoro-2,4-dinitro-

phenyl-5-L-alanine amide (FDAA or Marfey's reagent) and 2,3,4,6-tetra-*O*-acetyl- $\beta$ -D-glucopyranosyl isothiocyanate (GITC). In most cases, the FDAA derivatives of the stereoisomers could be separated with better enantioselectivity, but even this reagent was not effective in the separation of all of the stereoisomers of most of the amino acids [32,36].

On a chiral crown ether-based Crownpak CR(+) column the (*2S,3S*)- and (*2R,3R*)-*erythro* stereoisomers were separated in most cases, whereas the attempted separations of (*2S,3R*)- and (*2R,3S*)-*threo* stereoisomers and stereoisomers of amino acids containing a secondary amino group were unsuccessful [36]. (*R,S*)-2-Hydroxypropyl ether- and (*S*)-naphthylethylcarbamate-derivatized  $\beta$ -cyclodextrin-bonded CSPs were used for the analysis of  $\beta$ -substituted Trp, but the attempted resolution of the stereoisomers was completely unsuccessful [36]. The Chirobiotic T column, containing the macrocyclic antibiotic teicoplanin as chiral selector, was very effective in most cases for separation of the *erythro* and the *threo* enantiomers, but (with the exception of  $\beta$ -MeTic) separation of the four stereoisomers could not be achieved [32,36]. An analogous column, the Chirobiotic R column, which contains the macrocyclic glycopeptide ristocetin A as chiral selector, has also been applied in the enantioseparation of different unusual amino acids [37]. This CSP was very effective in the separation of the four stereoisomers of  $\beta$ -MePhe and  $\beta$ -MeTrp in the reversed-phase (RP) mode, but for all stereoisomers of  $\beta$ -MeTic and  $\beta$ -MeTyr only partial separation was achieved in the polar organic mode and in RP mode, respectively. A quinine-derived weak anion-exchange CSP has been used for the separation of *N*-protected  $\beta$ -Me-substituted amino acid stereoisomers [35]. This CSP displayed good enantioselectivity, but poor diastereoselectivity was observed.

No one general method, direct or indirect, was found where the baseline separation of all four stereoisomers of the different  $\beta$ -substituted  $\alpha$ -amino acids could be achieved. The applied direct and indirect methods were complementary to each other.

Besides our own work, a few articles have been published on the chiral chromatographic separation of  $\beta$ -substituted amino acids [38,39].

The aim of the present work was to develop an efficient indirect HPLC method for the resolution of

the enantiomers and possibly of all four stereoisomers of racemic  $\beta$ -alkyl-substituted aromatic  $\alpha$ -amino acids, using the recently developed CDA, (*S*)-NIFE. The diastereoisomers formed in the reaction with the CDA were analysed under RP conditions, using different octadecyl-modified silica-based columns. The effects of parameters such as the amount and type of the organic modifier on the resolution and retention of the derivatives were investigated. Chromatographic conditions were found for the separation of all four isomers of each analyte.

## 2. Experimental

### 2.1. Chemicals and reagents

The structures of the investigated amino acids are illustrated in Fig. 1. The nomenclature and abbreviations are in accordance with the IUPAC-IUB JCBN recommendations [40,41]. Racemic *erythro*-(2*S*,3*S* and 2*R*,3*R*)- and *threo*-(2*S*,3*R* and 2*R*,3*S*)- $\beta$ -methyltyrosine (*erythro*- and *threo*- $\beta$ -MeTyr, **1a–d**) [42],  $\beta$ -methylphenylalanine (*erythro*- and *threo*- $\beta$ -MePhe, **2a–d**) [43], -4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*- and *threo*- $\beta$ -MeTic, **3a–d**) [44],  $\beta$ -methyltryptophan (*erythro*- and *threo*- $\beta$ -MeTrp, **4a–d**) [45],  $\beta$ -2-propyltryptophan (*erythro*- and *threo*- $\beta$ -*i*-PrTrp, **5a–d**) [45],  $\beta$ -3-pentyltryptophan (*erythro*- and *threo*- $\beta$ -*i*-PentTrp, **6a–d**) [45],  $\beta$ -phenyltryptophan (*erythro*- and *threo*- $\beta$ -PhTrp, **7a–d**) [45] and  $\beta$ -2,5-dimethoxyphenyltryptophan (*erythro*- and *threo*- $\beta$ -diMeOPhTrp, **8a–d**) [45] and the pure enantiomers or enantiomerically enriched mixtures of the stereoisomers were synthesized in our laboratories according to literature methods. For the  $\beta$ -substituted tryptophans, enzymatic degradation with L-amino acid oxidase was used to obtain enantiomerically enriched isomers [36].

(*S*)-NIFE was obtained from Solvay-Peptisyntha (Brussels, Belgium). HPLC-grade methanol (MeOH) and acetonitrile (MeCN) and analytical reagent-grade trifluoroacetic acid (TFA), triethylamine (TEA) and dioxane were purchased from Merck (Darmstadt, Germany).

### 2.2. Apparatus and chromatography

The HPLC system used for isocratic chromatographic analyses consisted of an L-6000 liquid chromatographic pump (Merck–Hitachi, Tokyo, Japan) equipped with an SPD-6AV variable-wavelength UV detector (Shimadzu, Tokyo, Japan) and an HP3395 integrator (Agilent Technologies, formerly Hewlett-Packard, Waldbronn, Germany). Gradient elutions were performed with a Waters HPLC system, using an M-600 low-pressure gradient pump with an M-996 photodiode array detector and Millennium<sup>32</sup> Chromatography Manager software for data processing (Waters, Milford, MA, USA). Both HPLC systems were equipped with Rheodyne Model 7125 manual injectors with 20- $\mu$ l sample loops (Rheodyne, Cotati, CA, USA).

All the RP stationary phases used to perform the analyses were octadecyl-modified, spherical and endcapped silica based phases. The Vydac 218TP54 C<sub>18</sub> 250 $\times$ 4.6 mm I.D. column was from The Separations Group (Hesperia, CA, USA) and had 5- $\mu$ m particle size, 300- $\text{Å}$  pore size, 66–80 m<sup>2</sup> g<sup>-1</sup> surface area, 0.4–0.5 ml g<sup>-1</sup> pore volume and 7–8.5% carbon content. The Discovery C<sub>18</sub> 250 $\times$ 4.6 mm I.D. column was from Sigma (St. Louis, MO, USA) and had 5- $\mu$ m particle size, 180- $\text{Å}$  pore size, 200 m<sup>2</sup> g<sup>-1</sup> surface area, 1.0 ml g<sup>-1</sup> pore volume and 12.5% carbon content. The technical data for Nova-Pak C<sub>18</sub> 150 $\times$ 3.9 mm I.D. column (Waters, Milford, MA, USA) were 5- $\mu$ m particle size, 60- $\text{Å}$  pore size, 120 m<sup>2</sup> g<sup>-1</sup> surface area, 0.3 ml g<sup>-1</sup> pore volume and 7.3% carbon content.

Mobile phases used in isocratic analyses were prepared by pre-mixing the components volume by volume (v/v). Exact compositions are given in the tables and figures. Gradient elutions were performed by using a binary solvent system, where component A was 0.1% aqueous TFA and component B was methanol containing 0.1% TFA. The gradient program consisted of three steps. The content of component B was increased linearly from 5 to 40% within 35 min, kept at 40% during the next 15 min, and then again increased linearly to 95% within the next 30 min. Both the isocratic and the gradient runs were performed at a flow-rate of 1.0 ml min<sup>-1</sup>.

Derivatization of the investigated analytes with (*S*)-NIFE was performed according to a literature

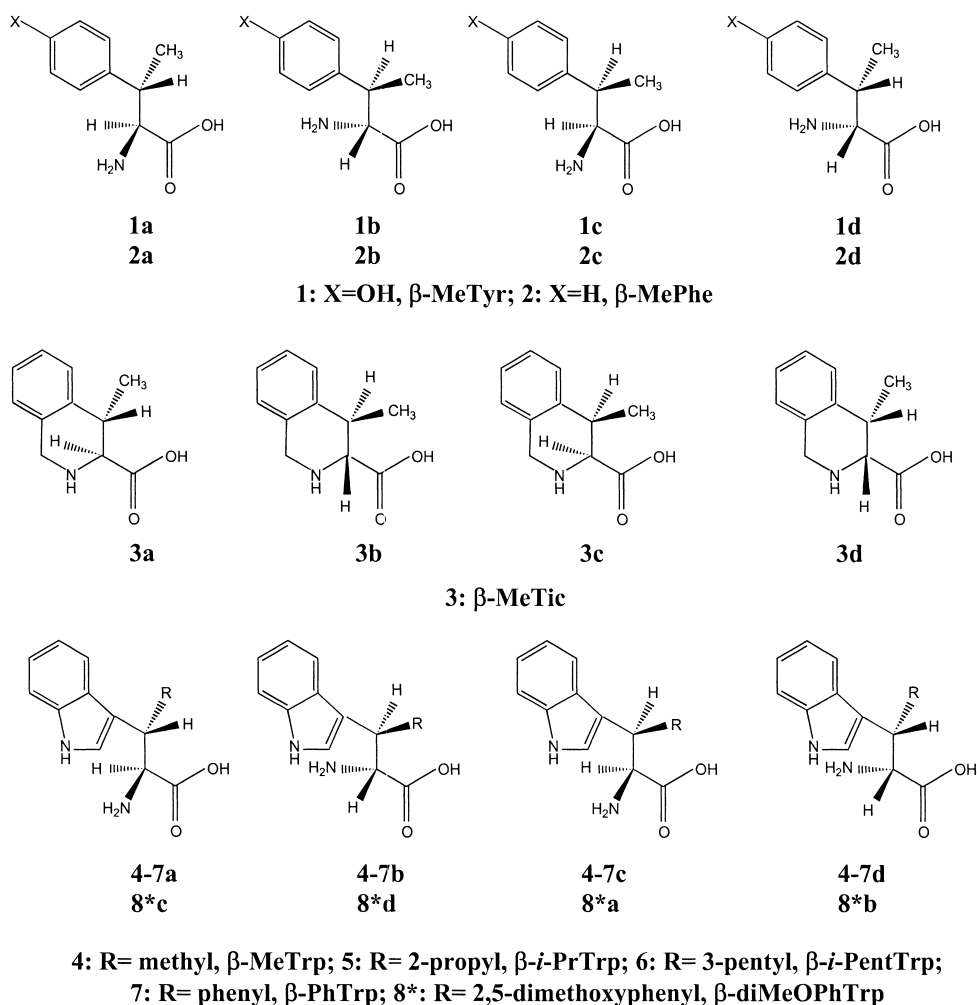


Fig. 1. Structures of the investigated compounds. **1:** *erythro*- and *threo*- $\beta$ -methyltyrosine ( $\beta$ -MeTyr); **1a:** *erythro*-(2*S*,3*S*)- $\beta$ -methyltyrosine, **1b:** *erythro*-(2*R*,3*R*)- $\beta$ -methyltyrosine, **1c:** *threo*-(2*S*,3*R*)- $\beta$ -methyltyrosine, **1d:** *threo*-(2*R*,3*S*)- $\beta$ -methyltyrosine; **2:** *erythro*- and *threo*- $\beta$ -methylphenylalanine ( $\beta$ -MePhe); **2a:** *erythro*-(2*S*,3*S*)- $\beta$ -methylphenylalanine, **2b:** *erythro*-(2*R*,3*R*)- $\beta$ -methylphenylalanine, **2c:** *threo*-(2*S*,3*R*)- $\beta$ -methylphenylalanine, **2d:** *threo*-(2*R*,3*S*)- $\beta$ -methylphenylalanine; **3:** *erythro*- and *threo*-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid ( $\beta$ -MeTic); **3a:** *erythro*-(2*S*,3*S*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, **3b:** *erythro*-(2*R*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, **3c:** *threo*-(2*S*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, **3d:** *threo*-(2*R*,3*S*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; **4:** *erythro*- and *threo*- $\beta$ -methyltryptophan ( $\beta$ -MeTrp); **4a:** *erythro*-(2*S*,3*S*)- $\beta$ -methyltryptophan, **4b:** *erythro*-(2*R*,3*R*)- $\beta$ -methyltryptophan, **4c:** *threo*-(2*S*,3*R*)- $\beta$ -methyltryptophan, **4d:** *threo*-(2*R*,3*S*)- $\beta$ -methyltryptophan; **5:** *erythro*- and *threo*- $\beta$ -2-propyltryptophan ( $\beta$ -i-PrTrp); **5a:** *erythro*-(2*S*,3*S*)- $\beta$ -2-propyltryptophan, **5b:** *erythro*-(2*R*,3*R*)- $\beta$ -2-propyltryptophan, **5c:** *threo*-(2*S*,3*R*)- $\beta$ -2-propyltryptophan, **5d:** *threo*-(2*R*,3*S*)- $\beta$ -2-propyltryptophan; **6:** *erythro*- and *threo*- $\beta$ -3-pentyltryptophan ( $\beta$ -i-PentTrp); **6a:** *erythro*-(2*S*,3*S*)- $\beta$ -3-pentyltryptophan, **6b:** *erythro*-(2*R*,3*R*)- $\beta$ -3-pentyltryptophan, **6c:** *threo*-(2*S*,3*R*)- $\beta$ -3-pentyltryptophan, **6d:** *threo*-(2*R*,3*S*)- $\beta$ -3-pentyltryptophan; **7:** *erythro*- and *threo*- $\beta$ -phenyltryptophan ( $\beta$ -PhTrp); **7a:** *erythro*-(2*S*,3*S*)- $\beta$ -phenyltryptophan, **7b:** *erythro*-(2*R*,3*R*)- $\beta$ -phenyltryptophan, **7c:** *threo*-(2*S*,3*R*)- $\beta$ -phenyltryptophan, **7d:** *threo*-(2*R*,3*S*)- $\beta$ -phenyltryptophan; **8\*:** *erythro*- and *threo*- $\beta$ -2,5-dimethoxyphenyltryptophan ( $\beta$ -diMeOPhTrp); **8a\*:** *threo*-(2*S*,3*R*)- $\beta$ -2,5-dimethoxyphenyltryptophan, **8b\*:** *threo*-(2*R*,3*S*)- $\beta$ -2,5-dimethoxyphenyltryptophan, **8c\*:** *erythro*-(2*S*,3*S*)- $\beta$ -2,5-dimethoxyphenyltryptophan, **8d\*:** *erythro*-(2*R*,3*R*)- $\beta$ -2,5-dimethoxyphenyltryptophan. \*According to the Cahn-Ingold-Prelog (CIP) rule, the presence of the oxygen attached to the aromatic ring changes the priority of the groups surrounding asymmetric carbon C-3, and thus the absolute configurations are different from those for the other analogues investigated.

method [25]. All derivatives were detected at 205 nm.

The 0.1% aqueous TFA and methanol and acetonitrile containing 0.1% TFA were prepared as follows: 1.0 ml TFA was added to 1 l Milli-Q water or to HPLC-grade MeOH or MeCN, and the mixture was filtered on a 0.45- $\mu\text{m}$  filter of type HV (Millipore, Molsheim, France). Mobile phases were degassed in an ultrasonic bath prior to analysis, and during chromatographic analyses, helium was sparged through them. The dead-time ( $t_M$ ) of the used RP columns was determined by injecting 20  $\mu\text{l}$  of a 0.01 M solution of KBr. To establish the sequence of elution of the amino acids, enantiomerically pure or enriched isomers of amino acids were co-injected with the corresponding racemic mixture.

### 3. Results and discussion

For indirect separation, pre-column derivatization of the investigated amino acids with the applied CDA, (*S*)-NIFE, was carried out. The amino acids were derivatized via their amino groups in basic media and were transformed to the corresponding urea diastereoisomers [25]. During the derivatization, besides the desired urea diastereoisomers, three side-products are also formed. One of them is 4-nitrophenol, another is phenylalanine methoxyethyl ester (“Phe ester”) and the third is a “urea dimer”, *N,N'*-bis(3-phenylpropionic acid methoxyethyl ester 2-yl)urea [25]. For some of the investigated analytes, the separation of the stereoisomers, either from 4-nitrophenol or from the “urea dimer”, was only partial, especially in a MeOH-containing mobile phase. The separation could be slightly improved by changing the organic modifier content. This co-elution is indicated (+ sign as superscript) in the tables.

$\beta$ -MeTyr exhibited interesting behavior towards the CDA. Not only the primary amine function, but also the hydroxy group attached to the aromatic ring was derivatized; thus, mono and bis derivatives were also formed.

Derivatives were analysed under RP conditions on different octadecyl-modified silica-based columns. Since each investigated analyte contains functional groups that can be protonated, ionization of the molecules was kept at a constant level along the

columns by control of the pH. For this purpose, the mobile phase systems in all cases contained a 0.1% aqueous solution of TFA. Additionally, the applied organic modifier, MeOH or MeCN, also contained 0.1% TFA. Selected results are presented in Tables 1–3. In most cases, one particular mobile phase composition is indicated for comparison purposes, but for some analytes other conditions are also given. These data allow conclusions concerning the effects of organic modifiers and different stationary phases on the retention and separation efficiency of the (*S*)-NIFE derivatives of the investigated amino acids. Illustrative chromatograms are presented in Figs. 2 and 3.

#### 3.1. General chromatographic behavior of the derivatives, enantioselectivity and resolution on different stationary phases

Of the three stationary phases, the Discovery  $C_{18}$  column exhibited the greatest carbon content (12.5%), surface area (200  $\text{m}^2 \text{g}^{-1}$ ) and pore volume (1  $\text{ml g}^{-1}$ ). For the Vydac  $C_{18}$  and Nova-Pak  $C_{18}$  columns these values were lower: 7–8%, 80–120  $\text{m}^2 \text{g}^{-1}$  and 0.3–0.5  $\text{ml g}^{-1}$ , respectively. As concerns the pore size, the Vydac  $C_{18}$  column exhibited the greatest value (300  $\text{\AA}$ ), the Discovery  $C_{18}$  column had a somewhat smaller pore size (180  $\text{\AA}$ ), while the Nova-Pak  $C_{18}$  column had the smallest size (60  $\text{\AA}$ ). At a fixed mobile phase composition, either in MeOH or in MeCN, analysis on the Vydac  $C_{18}$  column is associated with the lowest  $k$  values, and that on the Discovery  $C_{18}$  column with the highest (Tables 1 and 2). This elution behavior can be explained by the higher carbon content of the Discovery  $C_{18}$  column, which resulted in higher retention due to the greater extent of hydrophobic interaction between the stationary phase and solute molecules. The lower carbon content of Vydac  $C_{18}$  resulted in weaker hydrophobic interaction, and thus smaller retention factors. The  $k$  values for the Nova-Pak  $C_{18}$  column are similar to those on the Discovery  $C_{18}$  column, despite the similar carbon content to the Vydac  $C_{18}$  column (Table 3). These results revealed the importance of the pore size of the stationary phase used in the separation. Since the pore size of the Nova-Pak  $C_{18}$  column was very

Table 1  
Retention factors ( $k$ ), separation factors ( $\alpha$ ) and resolutions ( $R_s$ ) of stereoisomers of  $\beta$ -alkyl amino acids on Vydac 218TP54 column

Eluent composition, v/v	$k$				$\alpha$		$R_s$				
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )							
<i><math>\beta</math>-MeTyr</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	10.99 <sup>a</sup>	12.94 <sup>a</sup>	9.96 <sup>a</sup>	10.99 <sup>a</sup>	1.18	1.10	1.41	0.00	2.32	2.32	1.41
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	0.74 <sup>b</sup>	1.18 <sup>b</sup>	1.03 <sup>b</sup>	1.56 <sup>b</sup>	1.59	1.51	1.28	0.74	1.92	2.00	2.40
45:55	7.11 <sup>a</sup>	12.48 <sup>a</sup>	6.07 <sup>a</sup>	12.08 <sup>a</sup>	1.76	1.99	2.23	10.55	0.75	10.08	12.18
<i><math>\beta</math>-MePhe</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	1.79	2.60	1.70	2.51	1.45	1.48	<0.40	2.45	<0.40	3.60	2.57
TFA–MeOH							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	1.66	4.42	1.29	3.63	2.66	2.81	1.00	6.85	1.25	8.28	7.71
47.5:52.5	2.58	6.68	2.19	5.94	2.59	2.71	1.00	7.60	1.45	8.36	10.50
<i><math>\beta</math>-Me-Tic</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	2.49	3.29	2.24	2.95	1.32	1.32	1.00	1.67	1.33	3.00	2.67
67.5:32.5	3.97	5.29	3.54	4.69	1.33	1.32	1.25	2.00	1.75	3.75	3.25
TFA–MeOH							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	1.98 <sup>+</sup>	3.29	1.89	3.15	1.66	1.67	<0.40	4.33	<0.40	5.00	4.67
<i><math>\beta</math>-MeTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	1.55	2.12	1.55	2.12	1.37	1.37	0.00	2.33	0.00	2.33	2.33
TFA–MeOH							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	0.94	2.43 <sup>+</sup>	0.80	1.90 <sup>+</sup>	2.58	2.38	1.00	5.50	2.40	6.80	6.50
<i><math>\beta</math>-i-PrTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	6.55	9.24	6.18 <sup>+</sup>	8.89	1.41	1.44	1.00	5.78	0.73	6.00	6.67
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	4.53	10.18	4.72	10.30	2.25	2.18	<0.40	10.40	<0.40	10.50	10.50
<i><math>\beta</math>-i-PenTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	18.12	27.75	16.87	25.75	1.53	1.53	1.09	6.77	1.33	7.71	8.33
TFA–MeOH							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	19.12	22.82	17.25	21.26	1.19	1.23	2.62	2.65	1.75	6.87	7.50

Table 1. Continued

Eluent composition, v/v	<i>k</i>				$\alpha$		$R_S$					
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$	
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )								
<i><math>\beta</math>-PhTrp</i>												
TFA–MeCN												
65:35	7.28	9.08	7.87	9.80	1.25	1.25	1.17	2.33	1.23	3.33	3.39	
TFA–MeOH												
45:55	4.14	7.00	5.14	8.04	1.69	1.56	2.42	4.00	1.85	6.50	5.28	
<i><math>\beta</math>-diMeOPhTrp</i>												
TFA–MeCN												
65:35	7.97	9.76	8.58	10.56	1.22	1.23	1.17	1.73	1.13	3.08	2.93	
TFA–MeOH												
45:55	3.78	6.68	5.38	6.95	1.77	1.29	3.26	2.63	0.51	6.60	2.77	

TFA: 0.1% aqueous solution of trifluoroacetic acid; flow-rate: 1 ml/min; dead-time of the column: Vydac,  $t_M = 2.80$  min; detection: 205 nm.  $\alpha_{b-a}$  and  $\alpha_{d-c}$  represent the separation factors of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-b}$  and  $R_{S; c-d}$  represent the resolution of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-c}$ ,  $R_{S; c-b}$ ,  $R_{S; b-d}$ , and  $R_{S; c-a}$ ,  $R_{S; a-d}$ ,  $R_{S; d-b}$  represent the resolution of four consecutive stereoisomers; <sup>+</sup>partial separation from the “urea dimer”; in the case of  $\beta$ -diMeOPhTrp, according to the Cahn-Ingold-Prelog (CIP) rule, the presence of the oxygen attached to the aromatic ring changes the priority of the groups surrounding asymmetric carbon C-3, and thus the absolute configurations are different from those for the other analogues investigated.

<sup>a</sup> bis derivatives.

<sup>b</sup> mono derivatives.

small (60 Å), the diffusion and mass-transfer in the small pores was hindered. The relatively high surface area (pore size is inversely related to surface area) of the Nova-Pak C<sub>18</sub> column could also contribute to higher retention.

The solutes and stationary phases exhibited a reversed-phase system: the increase of organic modifier content decreased the retention factor, selectivity factor and resolution.

Comparison of the chromatographic data for analogous compounds under the same chromatographic conditions [e.g. 0.1% aqueous TFA–MeCN (65:35, v/v)] permit observations relating to the structure–retention relationship. Within the series of  $\beta$ -methyl-substituted amino acids, the isomers of  $\beta$ -MeTyr were the least retained, while those of  $\beta$ -MeTic eluted last, due to the difference in hydrophobicity. The effect of the  $\beta$ -alkyl substituents of Trp on the retention of the analogues was more pronounced than that of the rigidity or the backbone structure (hydrophobicity) of the molecules, either under isocratic conditions or in gradient elution. Retention of the Trp analogues increased with increasing chain

length. The aromatic substituents also contributed to an increased retention.

For each analyte, the CDA applied revealed similar enantioselectivity for the *erythro* and *threo* stereoisomers ( $\alpha_{b-a}$  and  $\alpha_{d-c}$  in Tables 1–3). The  $\alpha_{b-a}$  and  $\alpha_{d-c}$  values observed under the same conditions differ to only a slight extent. The different RP stationary phases used did not exert a significant effect on the enantioselectivity (Tables 1–3). Similar  $\alpha$  values were obtained for a given analyte on the different RP columns in identical mobile phase systems [0.1% aqueous TFA–MeCN (65:35, v/v)]. The range of the  $\alpha_{b-a}$  and  $\alpha_{d-c}$  values for  $\beta$ -MeTyr was 1.10–1.26, for  $\beta$ -MePhe 1.33–1.48, for  $\beta$ -MeTic 1.26–1.32, for  $\beta$ -MeTrp 1.21–1.37, for  $\beta$ -*i*-PrTrp 1.41–1.54, for  $\beta$ -PhTrp 1.21–1.31 and for  $\beta$ -diMeOPhTrp 1.22–1.26.  $\beta$ -*i*-PentTrp was the only exception. A slightly higher enantioselectivity was observed on the Nova-Pak C<sub>18</sub> column (1.97–2.08) (Table 3) than on the other two phases (1.39–1.41) (Tables 1 and 2). On a given stationary phase, a better enantioselectivity was generally observed in the MeOH-containing mobile phases under isocratic

Table 2

Retention factors ( $k$ ), separation factors ( $\alpha$ ) and resolutions ( $R_S$ ) of stereoisomers of  $\beta$ -alkyl amino acids on Discovery C<sub>18</sub> column

Eluent composition, v/v	$k$				$\alpha$		$R_S$				
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )							
<i><math>\beta</math>-MeTyr</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	1.75 <sup>b</sup>	2.06 <sup>b</sup>	1.99 <sup>b</sup>	2.50 <sup>b</sup>	1.18	1.26	1.20	<0.40	2.27	1.60	2.80
TFA–MeOH Gradient	18.47 <sup>b</sup> 22.20 <sup>a</sup>	20.02 <sup>b</sup> 22.70 <sup>a</sup>	18.31 <sup>b</sup> 21.18 <sup>a</sup>	19.90 <sup>b</sup> 22.36 <sup>a</sup>	1.08 1.02	1.09 1.02	1.43 2.00	9.71 2.20	2.46 3.20	12.77 5.40	9.88 4.75
<i><math>\beta</math>-MePhe</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	5.06	7.06	4.80	6.86	1.40	1.43	<0.40	6.57	<0.40	7.14	10.40
TFA–MeOH Gradient	21.00	21.96	20.57	21.74	1.05	1.06	2.25	11.50	1.35	11.31	13.50
<i><math>\beta</math>-Me-Tic</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	6.30	8.10	5.69	7.22	1.29	1.27	1.75	3.25	2.50	5.75	5.00
TFA–MeOH Gradient	21.11 <sup>+</sup>	21.72	21.03 <sup>+</sup>	21.60	1.03	1.03	0.62	5.00	<0.40	5.81	5.71
<i><math>\beta</math>-MeTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	4.81	6.30	4.49	5.57	1.31	1.24	1.05	2.40	1.25	5.43	3.25
TFA–MeOH Gradient	6.17 20.41	13.74 21.45	5.55 20.26	11.25 21.19	2.23 1.05	2.03 1.05	1.78 2.00	10.83 7.40	4.27 2.60	14.92 10.42	16.22 8.00
<i><math>\beta</math>-i-PrTrp</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	11.56	17.85	12.39	18.54	1.54	1.50	1.73	9.56	1.10	11.65	10.78
TFA–MeOH Gradient	21.59	22.55	21.83	22.66	1.04	1.04	2.20	7.33	1.25	8.71	8.33
<i><math>\beta</math>-i-PentTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
55:45	6.05	8.41	5.83	8.23	1.39	1.41	1.00	9.56	0.73	9.40	10.44
TFA–MeOH Gradient	22.81	23.63	22.79	23.40	1.04	1.03	0.63	7.85	1.33	9.14	9.00
<i><math>\beta</math>-PhTrp</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	13.05	17.05	14.33	18.60	1.31	1.30	2.46	4.67	2.35	7.29	6.88
TFA–MeOH Gradient	21.75	22.43	21.75	23.38	1.03	1.03	0.00	7.71	<0.40	8.34	7.71



Table 2. Continued

Eluent composition, v/v	<i>k</i>				$\alpha$		$R_S$				
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; c-d}$	$R_{S; a-b}$
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )							
<i><math>\beta</math>-diMeOPhTrp</i>											
TFA–MeCN											
60:40	10.57	11.94	10.99	12.52	1.13	1.14	1.43	2.80	1.63	4.13	4.53
TFA–MeOH											
Gradient	21.59	22.20	21.70	22.35	1.03	1.03	0.78	4.67	1.06	7.00	6.91
45:55	4.78	6.23	5.48 <sup>+</sup>	7.15	1.30	1.30	1.79	1.76	1.95	3.45	3.82

TFA: 0.1% aqueous solution of trifluoroacetic acid; flow-rate: 1 ml/min; dead-time of the column: Discovery,  $t_M = 3.20$  min; detection: 205 nm;  $\alpha_{b-a}$  and  $\alpha_{d-c}$  represent the separation factors of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-b}$  and  $R_{S; c-d}$  represent the resolution of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-c}$ ,  $R_{S; c-b}$ ,  $R_{S; b-d}$ , and  $R_{S; c-a}$ ,  $R_{S; a-d}$ ,  $R_{S; d-b}$  represent the resolution of four consecutive stereoisomers; <sup>+</sup>partial separation from the "urea dimer"; in the case of  $\beta$ -diMeOPhTrp, according to the Cahn-Ingold-Prelog (CIP) rule, the presence of the oxygen attached to the aromatic ring changes the priority of the groups surrounding asymmetric carbon C-3, and thus the absolute configurations are different from those for the other analogues investigated; gradient is specified in the Experimental section.

<sup>a</sup> bis derivatives.

<sup>b</sup> mono derivatives.

conditions. Of the two organic modifiers, MeOH, but not MeCN, can participate in hydrogen-bonding processes and this interaction between solute and mobile phase can contribute to better selectivity. The Discovery  $C_{18}$  column exhibited a lower enantioselectivity when it was operated in gradient elution mode with MeOH as organic modifier, but in most cases this was not accompanied by a poorer resolution.

The structures (rigidities) of the analytes did not have a significant influence on the enantioselectivity or on the resolution: similar values were obtained for the different analytes. However, the different  $\beta$ -alkyl substituents of Trp did exert a slight effect on the selectivity of the enantiomers. Under identical chromatographic conditions, increasing chain length of the substituent (Me, *i*-Pr, *i*-Pent) led to higher  $\alpha$  values and better resolution of the enantiomers. Aromaticity had similar effect, although to a lesser extent. While the  $\alpha$  values were decreased,  $R_S$  values improved for  $\beta$ -PhTrp and  $\beta$ -diMeOPhTrp compared with those for  $\beta$ -MeTrp.

The enantiomers of each of the investigated amino acids were baseline separated on all three RP columns in both organic modifiers (except  $\beta$ -MeTyr on Nova-Pak  $C_{18}$  column), with  $R_{S; a-b}$  and  $R_{S; c-d}$  values ranging up to 16.22, with an average value of  $\sim 3$ –5.

Using the same chromatographic conditions [e.g. either 0.1% aqueous TFA–MeCN (65:35, v/v) or 0.1% aqueous TFA–MeOH (45:55, v/v)] the highest  $R_S$  values were obtained on the Discovery  $C_{18}$  column (Table 2). The Vydac  $C_{18}$  column exhibited somewhat lower values (Table 1), while the resolutions on the Nova-Pak  $C_{18}$  column were the poorest (Table 3). The high  $R_S$  values on the Discovery  $C_{18}$  and Vydac  $C_{18}$  columns were attributed to the large pore size (180 or 300 Å) of these phases, which exhibited a fast mass-transfer between mobile and stationary phases and resulted in narrow peak shapes. The Nova-Pak  $C_{18}$  column had small pore size (60 Å) and exhibited slow mass-transfer kinetics and resulted in broad peak shapes.

On a given stationary phase the enantiomers were better resolved with MeOH as organic modifier than with MeCN, but the separation from the reaction side-products was sometimes only partial. In the MeOH-containing mobile phases co-elution with the side-products was observed for  $\beta$ -MeTic on all three phases,  $\beta$ -MeTrp on Vydac  $C_{18}$  and Nova-Pak  $C_{18}$  columns,  $\beta$ -*i*-PrTrp on Vydac  $C_{18}$  column and for  $\beta$ -*i*-PentTrp on Nova-Pak  $C_{18}$  and for  $\beta$ -diMeOPhTrp on Discovery  $C_{18}$  column.

For all of the enantiomeric pairs, the *erythro*-(2*S*,3*S*)- and *threo*-(2*S*,3*R*) isomers eluted first.

Table 3  
Retention factors ( $k$ ), separation factors ( $\alpha$ ) and resolutions ( $R_S$ ) of stereoisomers of  $\beta$ -alkyl amino acids on Nova-Pak C<sub>18</sub> column

Eluent composition (v/v)	$k$				$\alpha$		$R_S$				
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )							
<i><math>\beta</math>-MeTyr</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	1.29 <sup>a</sup>	1.52 <sup>a</sup>	1.29 <sup>a</sup>	1.52 <sup>ba</sup>	1.18	1.18	0.00	0.84	0.00	0.84	0.84
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
55:45	9.84 <sup>b</sup>	11.63 <sup>b</sup>	9.84 <sup>b</sup>	13.54 <sup>b</sup>	1.18	1.38	0.00	1.43	1.36	1.43	2.86
<i><math>\beta</math>-MePhe</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	4.21	5.61	4.26	5.89	1.33	1.38	<0.40	1.90	<0.40	2.00	2.25
TFA–MeOH							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	33.82	8.67	3.51	8.05	2.27	2.29	0.86	4.89	0.67	5.20	4.80
<i><math>\beta</math>-MeTic</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	5.34	6.16	4.93	6.21	1.27	1.26	0.67	0.89	0.89	1.78	1.56
72.5:27.5	24.34	33.89	21.15	29.17	1.39	1.38	1.31	1.86	1.39	3.00	3.31
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	4.80 <sup>+</sup>	7.38	5.02 <sup>+</sup>	7.59	1.54	1.51	<0.40	2.18	<0.40	2.55	2.33
<i><math>\beta</math>-MeTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	3.80	4.92	3.80	4.60	1.29	1.21	0.00	1.33	<0.40	1.50	1.33
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	2.66	5.98 <sup>+</sup>	2.66	4.95 <sup>+</sup>	2.25	1.86	0.00	2.18	0.92	2.83	2.18
<i><math>\beta</math>-i-PrTrp</i>											
TFA–MeCN							$R_{S; c-a}$	$R_{S; a-d}$	$R_{S; d-b}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	11.59	16.61	10.97	16.19	1.43	1.48	0.67	4.17	<0.40	4.73	4.31
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
45:55	8.16	19.86	8.36	20.36	2.43	2.44	<0.40	5.40	<0.40	5.56	5.56
<i><math>\beta</math>-i-PentTrp</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	9.00 <sup>+</sup>	18.75	9.76	19.25	2.08	1.97	0.60	7.60	<0.40	8.00	7.85
<i><math>\beta</math>-PhTrp</i>											
TFA–MeCN							$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; a-b}$	$R_{S; c-d}$
65:35	12.94	16.16	14.21	17.21	1.25	1.21	1.27	1.61	0.75	2.83	2.43
TFA–MeOH							$R_{S; a-c}$	$R_{S; c-d}$	$R_{S; d-b}$	$R_{S; a-b}$	
45:55	7.82	15.96	8.07	15.02	2.04	1.86	<0.40	6.17	0.67	8.00	

Table 3. Continued

Eluent composition (v/v)	<i>k</i>				$\alpha$		$R_S$				
	<i>erythro</i>		<i>threo</i>		$\alpha_{b-a}$	$\alpha_{d-c}$	$R_{S; a-c}$	$R_{S; c-b}$	$R_{S; b-d}$	$R_{S; c-d}$	$R_{S; a-b}$
	<b>a</b> (2 <i>S</i> ,3 <i>S</i> )	<b>b</b> (2 <i>R</i> ,3 <i>R</i> )	<b>c</b> (2 <i>S</i> ,3 <i>R</i> )	<b>d</b> (2 <i>R</i> ,3 <i>S</i> )							
<i><math>\beta</math>-diMeOPhTrp</i>											
TFA–MeCN											
65:35	14.14	17.84	13.11	16.58	1.26	1.26	0.56	1.24	0.64	1.82	1.73
TFA–MeOH											
45:55	7.52	14.98	8.73	15.48	1.99	1.77	1.08	4.77	<0.40	5.71	5.29

TFA: 0.1% aqueous solution of trifluoroacetic acid; flow-rate: 1 ml/min; dead-times of column: Nova-Pak,  $t_M = 1.34$  min; detection: 205 nm;  $\alpha_{b-a}$  and  $\alpha_{d-c}$  represent the separation factors of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-b}$  and  $R_{S; c-d}$  represent the resolution of *erythro* and *threo* enantiomers, respectively;  $R_{S; a-c}$ ,  $R_{S; c-b}$ ,  $R_{S; b-d}$ , and  $R_{S; c-a}$ ,  $R_{S; a-d}$ ,  $R_{S; d-b}$  represent the resolution of four consecutive stereoisomers; <sup>a</sup>partial separation from the “urea dimer”; in the case of  $\beta$ -diMeOPhTrp, according to the Cahn-Ingold-Prelog (CIP) rule, the presence of the oxygen attached to the aromatic ring changes the priority of the groups surrounding asymmetric carbon C-3, and thus the absolute configurations are different from those for the other analogues investigated.

<sup>a</sup> bis derivatives.

<sup>b</sup> mono derivatives.

### 3.2. Diastereoselectivity of the method

Since  $\beta$ -MeTyr formed both mono and bis derivatives, the separation of all derivatives in one chromatographic run appeared to be difficult. When the mono derivatives eluted within a reasonable analysis time, the bis derivatives were either retained very strongly (a too long analysis time) or not eluted from the column at all under the applied conditions. When the bis derivatives were forced to elute by the use of stronger eluents with higher organic modifier content, the mono derivatives were hardly retained, or not at all, and eluted in the solvent front. However, on the Vydac  $C_{18}$  column under isocratic conditions with a MeOH-containing mobile phase [0.1% aqueous TFA–MeOH (45:55, v/v)], all eight diastereomeric derivatives were separated (Table 1). It is worth noting that the sequence of elution of the mono and bis derivatives of the *erythro* and *threo* stereoisomers is different under these conditions. A change in the sequence of elution of the *erythro* and *threo* stereoisomers may also be observed when the analyses on the different RP columns are compared. Separation of all eight diastereomeric derivatives of  $\beta$ -MeTyr could likewise be achieved on the Discovery  $C_{18}$  column with gradient elution, again with a MeOH-containing mobile phase (Table 2).

Besides this gradient elution, all stereoisomers of

$\beta$ -MePhe were also separated under isocratic conditions. The best results were achieved on the Vydac  $C_{18}$  column with a MeOH-containing eluent (Table 1). The resolution was worse in MeCN on all three RP phases at 0.1% aqueous TFA–MeCN (65:35, v/v) eluent composition, but the decrease of organic modifier content somewhat improved the resolutions (data not shown). It is interesting that the sequences of elution of the *erythro* and *threo* stereoisomers differ in the different organic modifiers on the Vydac  $C_{18}$  and Nova-Pak  $C_{18}$  columns. For most of the amino acids, change of the stationary phase or the use of different organic modifiers was accompanied by a change in the elution of the stereoisomers. However, such changes did not influence the sequence of elution of the enantiomers.

In the separation of the four stereoisomers of  $\beta$ -MeTic, all the RP columns applied seemed to be effective, but especially so with mobile phases containing MeCN (Tables 1–3). MeOH seemed to be less effective in the separation of diastereomers of  $\beta$ -MeTic. On the Nova-Pak  $C_{18}$  column, a change in the sequence of elution of the *erythro*–*threo* stereoisomers was again observed in the different organic modifiers.

$\beta$ -MeTrp was the only investigated amino acid whose stereoisomers did not change their elution sequence in the different mobile phases or on the

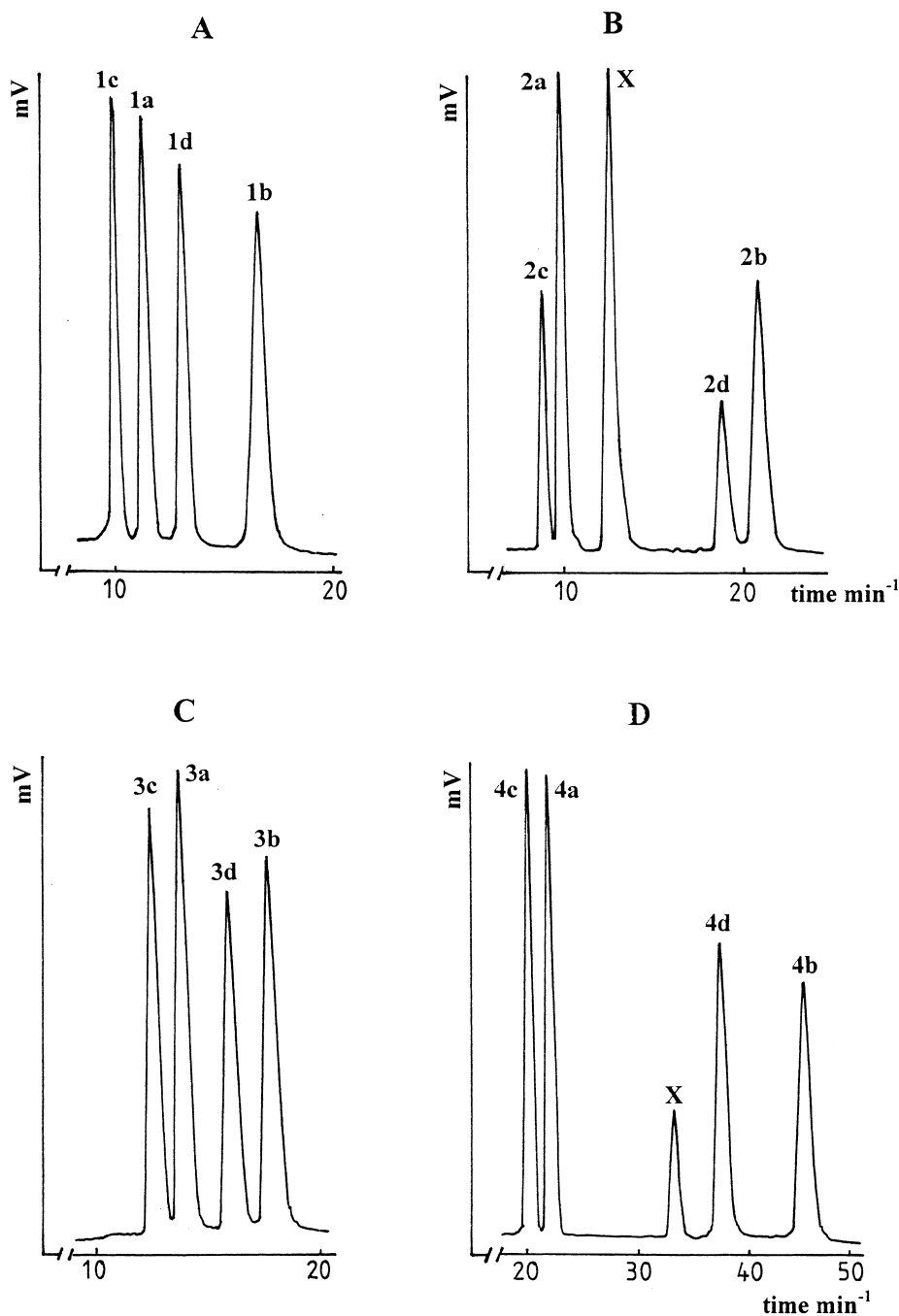


Fig. 2. Representative chromatograms of the separation of all four stereoisomers of the  $\beta$ -methyl-substituted amino acids as (*S*)-NIFE derivatives. (A) **1**,  $\beta$ -MeTyr\*; (B) **2**,  $\beta$ -MePhe; (C) **3**,  $\beta$ -MeTic; (D) **4**,  $\beta$ -MeTrp. Column: (A–C) Vydac 218TP54 C<sub>18</sub>, (D) Discovery C<sub>18</sub>; flow-rate, 1 ml min<sup>-1</sup>; mobile phase: (A) 0.1% aqueous TFA–MeOH (40:60, v/v), (B) 0.1% aqueous TFA–MeOH (47.5:52.5, v/v), (C) 0.1% aqueous TFA–MeCN (67.5:32.5, v/v), (D) 0.1% aqueous TFA–MeOH (50:50, v/v); detection at 205 nm; for identification of the peaks see legend to Fig. 1; \*bis derivatives; X denotes the “urea dimer”.

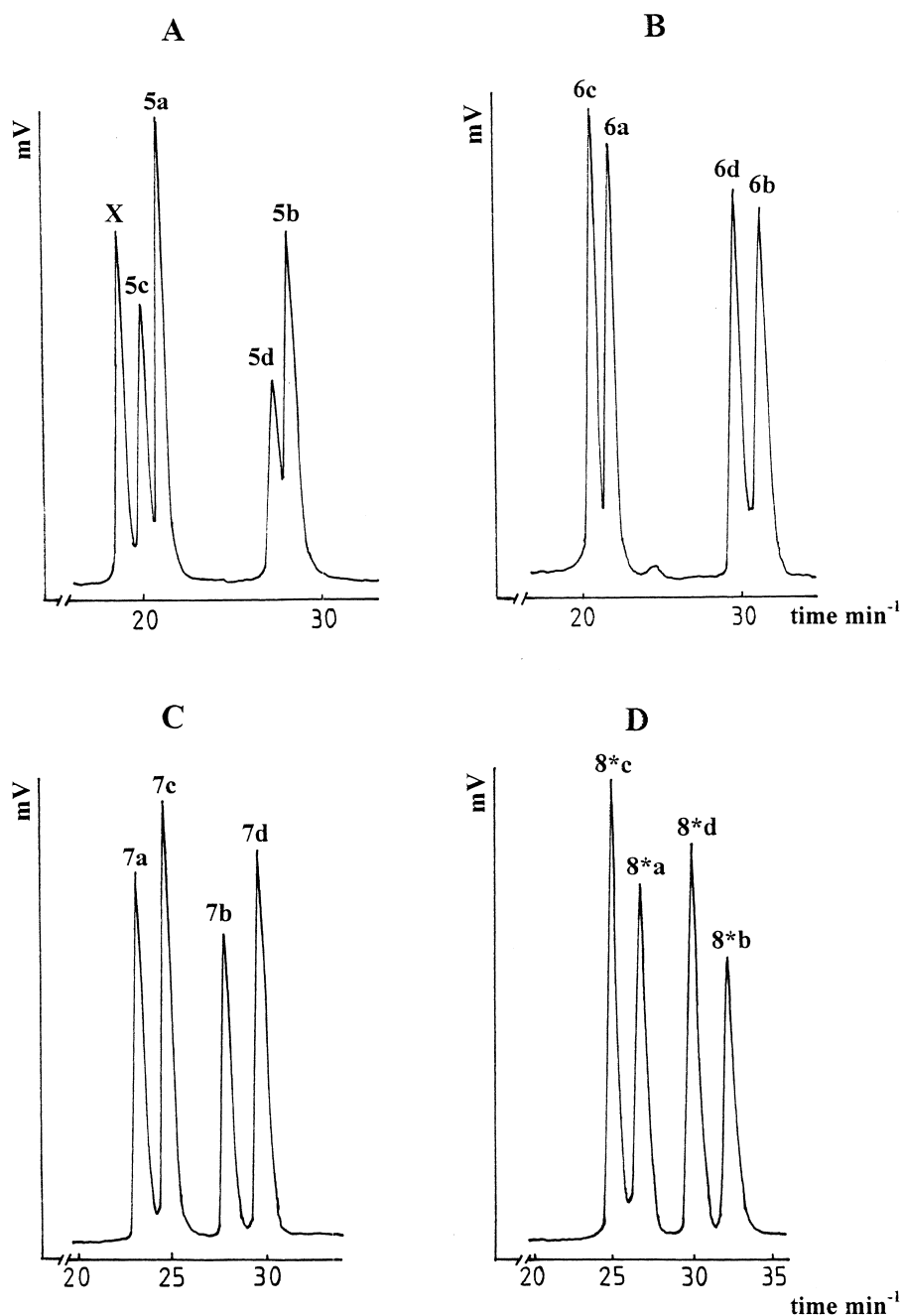


Fig. 3. Representative chromatograms of the separation of all four stereoisomers of the  $\beta$ -alkyl-substituted tryptophan analogues as (*S*)-NIFE derivatives. (A) **5**,  $\beta$ -*i*-PrTrp; (B) **6**,  $\beta$ -*i*-PentTrp; (C) **7**,  $\beta$ -PhTrp; (D) **8**,  $\beta$ -diMeOPhTrp. Column: (A–D) Vydac 218TP54  $C_{18}$ ; flow-rate,  $1 \text{ ml min}^{-1}$ ; mobile phase: (A, C, D) 0.1% aqueous TFA–MeCN (65:35, v/v), (B) 0.1% TFA–MeCN (60:40, v/v); detection at 205 nm; for identification of the peaks, see legend to Fig. 1; X denotes the “urea dimer”.

different RP columns. However, baseline separation could be achieved only in a MeOH-containing mobile phase on the Discovery C<sub>18</sub> column with either gradient or isocratic elution (Table 2). Similarly in MeOH, good separation was observed on the Vydac C<sub>18</sub> column. MeCN did not prove efficient in most cases except with the Discovery C<sub>18</sub> column. Of the three RP phases, the Nova-Pak C<sub>18</sub> column seemed to be the least applicable for the separation of these derivatives (Table 3).

The Discovery C<sub>18</sub> column proved to be the most efficient in the separation of all four isomers of  $\beta$ -*i*-PrTrp in either the MeOH or the MeCN-containing systems (Table 2). For the other two columns, better resolution was achieved in MeCN (Tables 1 and 3). A change in the sequence of elution of the stereoisomers was observed, as mentioned above.

Relatively good resolution of all the stereoisomers of  $\beta$ -*i*-PentTrp was achieved on all the RP columns. The best results were obtained on the Vydac C<sub>18</sub> column with MeOH (Table 1). Unfortunately, the analysis on the Nova-Pak C<sub>18</sub> column in MeOH was not acceptable, in consequence of the long retention time and broad peaks (data not shown).

As concerns the two aromatic-substituted Trp analogues,  $\beta$ -PhTrp and  $\beta$ -diMeOPhTrp, the stereoisomers of the former were better resolved and very good resolutions were achieved in all the chromatographic systems applied. For  $\beta$ -PhTrp MeCN proved to be more efficient on the Discovery C<sub>18</sub> column (Table 2), while on the Vydac C<sub>18</sub> column better resolution within a shorter analysis time was observed in MeOH (Table 1). The stereoisomers exhibited very interesting elution behavior on the Nova-Pak C<sub>18</sub> column in MeOH. The *threo* stereoisomers eluted between the *erythro* stereoisomers.

Conditions were also found where the stereoisomers of  $\beta$ -diMeOPhTrp could be baseline separated. The best results were achieved on the Discovery C<sub>18</sub> column with either organic modifier (Table 2). Separation on the Vydac C<sub>18</sub> column was also successful, but resolution on the Nova-Pak C<sub>18</sub> column was not satisfactory.

#### 4. Conclusions

(*S*)-*N*-(4-Nitrophenoxy-carbonyl)phenylalanine m-

ethoxyethyl ester, (*S*)-NIFE, proved to be applicable for the indirect resolution of the stereoisomers of  $\beta$ -alkyl-substituted aromatic amino acids. The CDA applied displayed similar enantioselectivity for the *erythro* and *threo* stereoisomers. Better selectivity was generally observed in MeOH-containing mobile phases. As regards the RP phases applied, under the same chromatographic conditions the analysis was the longest on the Discovery C<sub>18</sub> column due to the high carbon content and surface area of the stationary phase and this was accompanied by the best resolution of the enantiomers. This can be explained by the narrow peak shapes resulting from fast mass-transfer kinetics due to large pore size. However, the other two columns (Vydac C<sub>18</sub> and Nova-Pak C<sub>18</sub>) were likewise very efficient in the enantioresolution of the derivatives. As concerns the sequence of elution of the enantiomers, the *erythro*-(2*S*,3*S*) and *threo*-(2*S*,3*R*) isomers always eluted first.

Conditions were found that allowed the separation of all four stereoisomers of each investigated analyte in one chromatographic run. In most cases, this was achieved in MeOH-containing mobile phases on either the Discovery C<sub>18</sub> or the Vydac C<sub>18</sub> column. For some of the investigated analytes, separation of one of the stereoisomers from the reaction-side products (4-nitrophenol or “urea dimer”) was only partial, but this problem could be eliminated by optimization of the mobile phase composition.

With the exception of  $\beta$ -MeTrp, a change in the sequence of elution of the *erythro*–*threo* stereoisomers was observed on change of the stationary phase on the use of different organic modifiers. However, this change did not influence the sequence of elution of the enantiomers.

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#### References

- [1] V.J. Hruby, G. Li, C. Haskell-Luevano, M. Shenderovich, *Biopolymers* 43 (1997) 219.

- [2] S.E. Gibson, N. Guillo, M.J. Tozer, *Tetrahedron* 55 (1999) 585.
- [3] R. Kaul, P. Balam, *Bioorg. Med. Chem.* 7 (1999) 105.
- [4] S. Hanessian, G. McNaughton-Smith, H.G. Lombart, W.D. Lubell, *Tetrahedron* 53 (1997) 12854.
- [5] D.A. Evans, T.C. Britton, J.A. Ellman, R.I. Dorow, *J. Am. Chem. Soc.* 112 (1990) 4011.
- [6] R. Bhushan, S. Joshi, *Biomed. Chromatogr.* 7 (1993) 235.
- [7] G. Subramanian, *A Practical Approach To Chiral Separations By Liquid Chromatography*, VCH, Weinheim, 1994.
- [8] K. Jinno, *Chromatographic Separations Based On Molecular Recognition*, Wiley–VCH, New York, 1997.
- [9] T.E. Beesley, R.P.W. Scott (Eds.), *Chiral Chromatography*, Wiley, Chichester, 1998.
- [10] G. Lunn, L.C. Hellwig (Eds.), *Handbook For Derivatization Reaction For HPLC*, Wiley, New York, 1998.
- [11] T. Toyo'oka, *Modern Derivatization Methods For Separation Sciences*, Wiley, Chichester, 1999.
- [12] H. Engelhardt, T. Koenig, S. Kromidas, *Fresenius' Z. Anal. Chem.* 317 (1984) 670.
- [13] S. Görög, M. Gazdag, *J. Chromatogr. B* 659 (1994) 51.
- [14] E. Martin, K. Quinke, H. Spahn, E. Mutschler, *Chirality* 1 (1989) 223.
- [15] J. Kondo, T. Imaoka, N. Suzuki, T. Kawasaki, A. Nakanishi, Y. Kawahara, *Anal. Sci.* 10 (1994) 697.
- [16] Y. Nishida, E. Itoh, M. Abe, H. Ohru, H. Meguro, *Anal. Sci.* 11 (1995) 213.
- [17] T. Toyo'oka, Y.-M. Liu, *Analyst* 120 (1995) 385.
- [18] O.P. Kleidernigg, K. Posch, W. Lindner, *J. Chromatogr. A* 729 (1996) 33.
- [19] T. Toyo'oka, *Biomed. Chromatogr.* 10 (1996) 265.
- [20] O.P. Kleidernigg, W. Lindner, *Chromatographia* 44 (1997) 465.
- [21] O.P. Kleidernigg, W. Lindner, *J. Chromatogr. A* 795 (1998) 251.
- [22] S. Al-Kindy, T. Santa, T. Fukushima, H. Homma, K. Imai, *Biomed. Chromatogr.* 12 (1998) 276.
- [23] M. Péter, A. Péter, F. Fülöp, *Chromatographia* 50 (1999) 373.
- [24] Solvay (Société Anonyme), patent pending.
- [25] A. Péter, E. Vékes, G. Török, *Chromatographia* 52 (2000) 821.
- [26] E. Olajos, A. Péter, R. Casimir, D. Tourwé, *Chromatographia* 54 (2001) 77.
- [27] A. Péter, E. Vékes, G. Tóth, D. Tourwé, F. Borremans, *J. Chromatogr. A* 948 (2002) 283.
- [28] A. Péter, G. Tóth, G. Török, D. Tourwé, *J. Chromatogr. A* 728 (1996) 455.
- [29] A. Péter, G. Tóth, *Anal. Chim. Acta* 352 (1997) 335.
- [30] A. Péter, G. Török, G. Tóth, W. Van den Nest, G. Laus, D. Tourwé, *J. Chromatogr. A* 797 (1998) 165.
- [31] Zs. Darula, Gy. Török, G. Wittmann, E. Mannekens, K. Iterbeke, G. Tóth, D. Tourwé, A. Péter, *J. Planar Chromatogr.* 11 (1998) 346.
- [32] A. Péter, G. Török, D.W. Armstrong, *J. Chromatogr. A* 793 (1998) 283.
- [33] A. Péter, G. Török, D.W. Armstrong, G. Tóth, D. Tourwé, *J. Chromatogr. A* 828 (1998) 177.
- [34] A. Péter, G. Török, D.W. Armstrong, G. Tóth, D. Tourwé, *J. Chromatogr. A* 904 (2000) 1.
- [35] A. Péter, G. Török, G. Tóth, W. Lindner, *J. High Resolut. Chromatogr.* 23 (2000) 628.
- [36] G. Török, A. Péter, E. Vékes, J. Sápi, M. Laronze, J.Y. Laronze, D.W. Armstrong, *Chromatographia* 51 (2000) S165.
- [37] G. Török, A. Péter, D.W. Armstrong, D. Tourwé, G. Tóth, J. Sápi, *Chirality* 13 (2001) 648.
- [38] M. Schlauch, A.W. Frahm, *J. Chromatogr. A* 868 (2000) 197.
- [39] M. Schlauch, F.J. Volk, K.P. Fondekar, J. Wede, A.W. Frahm, *J. Chromatogr. A* 897 (2000) 145.
- [40] IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), *Nomenclature and Symbolism for Amino Acids and Peptides. Recommendations 1983*, *Eur. J. Biochem.* 138 (1984) 9.
- [41] IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), *Nomenclature and Symbolism for Amino Acids and Peptides. Corrections to recommendations 1983*, *Eur. J. Biochem.* 213 (1993) 2.
- [42] D. Tourwé, E. Mannekens, T.N. Thi Diem, P. Verheyden, H. Jaspers, G. Tóth, A. Péter, I. Kertész, G. Török, N.N. Chung, P.W. Schiller, *J. Med. Chem.* 41 (1998) 5167.
- [43] V.J. Hruby, G. Tóth, C. Gehrig, L.-F. Kao, R. Knapp, G.K. Lui, H.I. Yamamura, T.H. Kramer, P. Davis, T.F. Burks, *J. Med. Chem.* 34 (1991) 1823.
- [44] D. Tourwé, K. Iterbeke, W.M. Kazmierski, G. Tóth, in: W.M. Kazmierski (Ed.), *Peptidomimetics Protocols, Methods in Molecular Medicine*, Vol. 23, Humana Press, Totowa, NJ, 1999, p. 321.
- [45] C. Nemes, L. Jeannin, J. Sápi, M. Laronze, H. Seghir, F. Augé, J.-Y. Laronze, *Tetrahedron* 56 (2000) 5479.