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High-performance liquid chromatographic methods for separation of the isomers of β-amino acids possessing bicyclo[2.2.1]heptane or heptene skeletons

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Abstract

Reversed-phase high-performance liquid chromatographic methods were developed for separation and identification of the enantiomers of bicyclic β-amino acids: racemic *diendo*-(1*S*,2*S*,3*R*,4*R* and 1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R* and 1*R*,2*S*,3*R*,4*S*)-3-amino-bicyclo[2.2.1]heptane-2-carboxylic acids and racemic *diendo*-(1*S*,2*S*,3*R*,4*R* and 1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R* and 1*R*,2*S*,3*R*,4*S*)-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acids. The enantioselective separations were carried out in two ways: separation of the diastereomers formed by precolumn derivatization with the chiral derivatizing reagents: 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, and direct separation by the application of a chiral stationary phase, Crownpak CR(+). The results of the methods were compared and optimal conditions were developed in systematic chromatographic examinations.

Keywords: Enantiomer separation; Derivatization, LC; Mobile phase composition; Amino acids; Bicycloheptane amino acids

1. Introduction

Compounds possessing 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 [1–4]. Bicyclo[2.2.1]heptane skeleton-containing β-amino acids and their derivatives are often used as starting materials of various heterocycles. Several alicycle-condensed 1,3-heterocycles were recently synthesised from racemic mixtures of *diendo*- and *diexo*-3-aminobicyclo[2.2.1]heptane-2-carboxylic acids and *diendo*- and *diexo*-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acids in order to examine their biological activities and to study their reactions [5]. Thromboxane A_2 (TXA_2) receptor antagonists can be prepared from one of the enantiomers of *diendo*-3-amino-bicyclo[2.2.1]heptane-2-carboxylic acid [6]. Such pharmacons may be useful in the treatment of circulatory disorders (angina pectoris, thrombosis) and asthma.

In the synthesis of such compounds, chirality is often of the utmost importance. For example, in the case of the TXA_2 receptor antagonist (5*Z*)-7-(3-*endo*-(phenyl-sulfonyl)amino)bicyclo[2.2.1]hept-2-*exo*-yl)heptenoic acid the (+)-isomer was found to be more potent than the (−)-isomer [7].

The β-amino acids are not only important pharmacologically, but are also used as building blocks for

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the preparation of modified (unusual) analogues of biologically active peptides. These investigations are applied to determine the fine structures of receptors [8–13]. With the increasing appreciation that the isomers of a chiral drug can differ pharmacokinetically and/or pharmacodynamically, the interest in methods developed for the resolution and quantification of isomers is rapidly growing. The separation of enantiomers requires an asymmetric or chiral environment allowing diastereomeric interactions. For this purpose, high-performance liquid chromatography (HPLC) is widely used.

Our present aim is to develop HPLC methods for separation and identification of the elution sequence of the enantiomers of *diendo*- and *diexo*-3-amino-bicyclo[2.2.1]heptane-2-carboxylic acids (*diendo*-ABHC and *diexo*-ABHC, respectively) and of *diendo*- and *diexo*-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acids (*diendo*-ABHC-ene and *diexo*-ABHC-ene, respectively) (Fig. 1) by using two reversed-phase (RP) HPLC methods: direct separation on a chiral stationary phase (Crownpak CR(+) contains a chiral crown ether as a chiral selector), and precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as chiral reagents. The separations were carried out in different eluent systems. The effects of the mobile phase composition, organic modifier content, temperature and pH on the resolution were also investigated.

2. Experimental

2.1. Apparatus

The HPLC measurements were carried out on a Waters chromatographic system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode array detector and a Millennium 2010 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA). The columns used for analytical separations were Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5 μm particle size (The Separations Group, Hesperia, CA, USA), and Crownpak CR(+) (150×4 mm I.D.), 5 μm particle size (Daicel, Tokyo, Japan).

A Rheodyne injector Type 7125 (Cotati, CA, USA) with a 20-μl loop was used for sample injections.

The Crownpak CR(+) column was thermostated with an MK70 thermostat (Mechanik Prüfgeräte, Medlingen, Germany). The accuracy of temperature adjustment was ±0.1°C.

2.2. Chemicals and reagents

Racemic *diexo*-3-amino-5-bicyclo[2.2.1]heptene-2-carboxylic acid (**IIc,d**) and *diexo*-3-amino-bicyclo[2.2.1]heptane-2-carboxylic acid (**Ic,d**) (Fig. 1) were synthesised by chlorosulfonyl isocyanate addition to the corresponding cycloalkane, followed by acidic hydrolysis [14]. Compounds **IIa,b** were

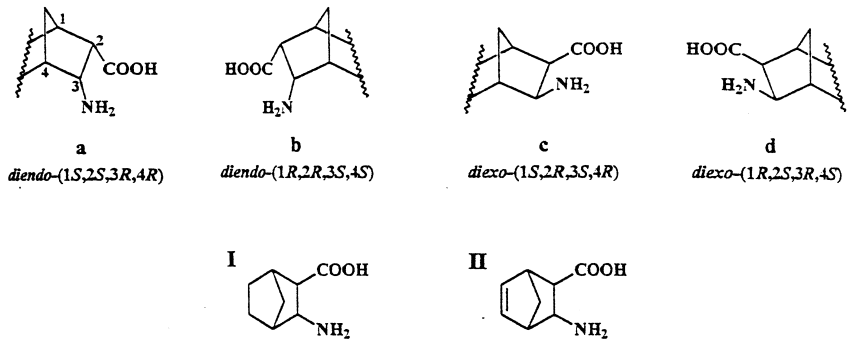

Fig. 1. Structures of the four enantiomers of ABHC and ABHC-ene. (**I**) ABHC; (**II**) ABHC-ene; (**a**) *diendo*-(1*S*,2*S*,3*R*,4*R*) isomer; (**b**) *diendo*-(1*R*,2*R*,3*S*,4*S*) isomer; (**c**) *diexo*-(1*S*,2*R*,3*S*,4*R*) isomer; (**d**) *diexo*-(1*R*,2*S*,3*R*,4*S*) isomer.

prepared in racemic forms from bicyclo[2.2.1]hept-5-ene-2,3-*endo*-dicarboxylic anhydride by ammonolysis, followed by Hoffmann degradation and ion-exchange purification [15]. Catalytic reduction of **IIa,b** resulted in **Ia,b**. For preparation of the enantiomers of **Ic** and **IIc** lipase-catalysed selective N-acylation of the ethyl esters of racemic **Ic,d** and **IIc,d** was carried out. The esters were then hydrolysed to the amino acids [16]. The same procedure was applied for the preparation of **IIb** from racemic **IIa,b**. The hydrochloride of the ethyl ester of **Ib** was obtained by palladium charcoal reduction of **IIb**. The **Ib** and **IIb** enantiomers of the free amino acids were obtained after small scale hydrolysis.

The identity of the compounds was checked by means of melting point determination, FAB mass spectrometry, ^{1}H NMR spectroscopy and optical rotation measurement.

Lipase PS (*Pseudomonas cepacia*) was obtained from Amano Pharmaceuticals (Nagoya, Japan) and was immobilized on Celite [17].

FDAA was purchased from Sigma (St. Louis, MO, USA) and GITC from Aldrich (Steinheim, Germany). Perchloric acid, potassium dihydrogenphosphate, sodium acetate, trifluroacetic acid (TFA), phosphoric acid and acetic acid of analytical-reagent grade and HPLC-grade acetonitrile, methanol and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany).

Buffers were prepared with Milli-Q water purified further by filtration on a 0.45-μm Millipore filter, type HV (Molsheim, France). The phosphate and acetate buffers were prepared by dissolving 0.01 mol potassium dihydrogenphosphate or sodium acetate in water, adjusting the pH with phosphoric or acetic acid to pH 3.0 and diluting to a final volume of 1 l.

2.3. Sample preparation and derivatization procedure

For the direct separation, 1–10 m*M* solutions of the investigated β-amino acids were made in 0.01 *M* perchloric acid and injected after filtration on a 0.45-μm Millipore filter.

For the derivatization with FDAA by the modified method of Marfey [18,19], and with GITC by the method of Nimura et al. [20], 1 mg/ml solutions of the β-amino acids were used.

3. Results and discussion

Our earlier work on the separation of derivatized amino acids showed that the resolution of isomers can be improved by using a buffered, acidic mobile phase [21], e.g. maintenance of the ionization of the molecules at a constant level along the column needs the control of pH. In these investigations a 0.1% aqueous solution of trifluoroacetic acid, a 0.01 *M* aqueous solution of sodium acetate (pH 3.0) and a 0.01 *M* aqueous solution of phosphate buffer (pH 3.0) were used.

3.1. Separation of GITC derivatives

The results of the separation of the enantiomers of ABHC derivatives are summarized in Table 1. With methanol as organic modifier, relatively good resolution could be achieved for all the four enantiomers if the hydrophilic part of the eluent was aqueous TFA. The addition of a third component, THF, improved the resolution of the *diexo* and *diendo* isomers ($R_{s;d,a}$) in spite of the decrease in the retention time due to the sharper peak shape. Any other variation of the eluent composition, with phosphate buffer–methanol or sodium acetate buffer–methanol did not lead to success in the separation of the *diexo-diendo* isomers.

The results of the separation of ABHC derivatives in the acetonitrile-containing system showed that the TFA-containing mobile phase had the highest separation capability. With regard to the mobile phases, TFA–methanol and TFA–acetonitrile, the larger peak broadening in the latter system resulted in poorer resolution. The addition of THF to the TFA–acetonitrile system shortened the retention time, but the resolution achieved was not as good as with the TFA–methanol–THF system. The application of phosphate and acetate buffer instead of TFA at the same acetonitrile content did not improve the separation of the *diexo-diendo* diastereomer pairs in comparison with the TFA system.

The elution sequence was independent of the

Table 1
Dependence of retention factor (k), separation factor (α) and resolution (R_s) of ABHC-GITC derivatives on eluent system

Eluent composition (v/v)	k				$\alpha_{c,d}$	$\alpha_{b,a}$	$R_{S;c,b}$	$R_{S;b,d}$	$R_{S;d,a}$
	diexo (**c**)	*diendo* (**b**)	*diexo* (**d**)	*diendo* (**a**)					
$TFA–CH_3OH$									
(55:45)	5.18	5.69	7.04	7.63	1.36	1.34	1.15	2.45	1.10
(60:40)	10.95	12.20	13.38	18.95	1.22	1.55	1.44	3.03	1.35
$TFA–CH_3OH–THF$									
(55:45:2)	4.08	4.44	5.22	5.75	1.28	1.30	1.10	1.80	1.30
(60:40:2)	7.01	7.68	9.04	10.14	1.30	1.32	1.35	2.24	1.58
$KH_2PO_4–CH_3OH$									
(55:45)	5.16	5.16	6.80	6.80	1.32	1.32	0.00	2.75	0.00
$NaOAc–CH_3OH$									
(55:45)	4.41	4.41	5.70	5.70	1.29	1.29	0.00	2.80	0.00
$TFA–CH_3CN$									
(70:30)	6.00	6.39	7.08	7.42	1.18	1.16	<0.40	0.90	<0.40
(75:25)	14.15	15.34	17.42	18.47	1.23	1.27	1.40	2.24	1.08
$TFA–CH_3OH–THF$									
(72.5:27.5:2)	6.90	7.40	8.09	8.55	1.17	1.16	1.17	1.53	0.95
$KH_2PO_4–CH_3CN$									
(75:25)	14.20	14.20	16.90	17.30	1.22	1.22	0.00	2.60	<0.40
$NaOAc–CH_3CN$									
(70:30)	4.77	4.77	5.30	5.40	1.11	1.13	0.00	1.02	<0.40
(75:25)	10.71	10.71	13.18	13.30	1.23	1.24	0.00	2.80	<0.40

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 250 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3.0); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3.0). $\alpha_{c,d}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $\alpha_{b,a}$ represents separation of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; $R_{S;c,b}$ represents resolution of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diendo*-(1*R*,2*R*,3*S*,4*S*) isomers; $R_{S;b,d}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; and $R_{S;d,a}$ represents resolution of *diexo*-(1*R*,2*S*,3*R*,4*S*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers.

nature of the buffers or of the organic modifiers: **c**<**b**<**d**<**a**. It seems that the configuration of the carbon atom bearing the amino group, at which the derivatization reaction takes place, is the determining factor in the elution sequence of the isomers. In the case of two *diendo* or two *diexo* isomers, the enantiomers with the *S* configuration at this carbon atom eluted before the enantiomers with the *R* configuration there.

The GITC derivatives of the enantiomers of ABHC-ene could be separated in most of the eluent systems (Table 2). With methanol as organic modifier, an acceptable resolution (R_s>1.1) could be achieved within a short time (k<6). The addition of THF as second organic modifier resulted in chromatograms with shorter retention times and sharper peak shapes. As concerns the two organic modifiers, in the acetonitrile-containing systems the analysis time was longer, especially with phosphate buffer as mobile phase additive, and the resolution was poorer. The addition of THF to the TFA–acetonitrile or sodium acetate–acetonitrile systems resulted in a sharper peak, but the resolution of isomers **c** and **a** did not improve; they were shifted to the same extent in the chromatogram. The elution sequence differed from that observed for ABHC derivatives: the *diendo* isomer **b** eluted before the *diexo* isomer **c**, and isomer **a** before isomer **d**. The enantiomers with the *S* configuration at carbon 3 eluted first in the series of *diendo-diendo* and *diexo-diexo* isomers, as observed for the ABHC derivatives.

The comparison of the retentions of ABHC and ABHC-ene derivatives in the same eluent revealed that the enantiomers of the unsaturated compounds had shorter retention times (lower k values) than those of the saturated ones. This behaviour reflects the decreased hydrophobicity of the unsaturated amino acids. Representative chromatograms illustrating the separation of the enantiomers as GITC derivatives are shown in Fig. 2.

Table 2
Dependence of retention factor (k), separation factor (α) and resolution (R_s) of ABHC-ene-GITC derivatives on eluent system

Eluent composition (v/v)	k				$\alpha_{b,a}$	$\alpha_{c,d}$	$R_{S;b,c}$	$R_{S;c,a}$	$R_{S;a,d}$
	diendo (**b**)	*diexo* (**c**)	*diendo* (**a**)	*diexo* (**d**)					
$TFA–CH_3OH$ (55:45)	4.06	4.61	5.52	6.28	1.36	1.36	1.40	2.15	1.50
$KH_2PO_4–CH_3OH$ (55:45)	3.78	4.38	5.19	5.97	1.37	1.36	1.25	1.70	1.40
$NaOAc–CH_3OH$ (55:45)	3.26	3.71	4.36	4.91	1.34	1.32	1.10	1.50	1.20
$NaOAc–CH_3OH–THF$ (55:45:2)	2.50	2.86	3.15	3.57	1.26	1.25	1.40	1.10	1.40
$TFA–CH_3CN$ (72.5:27.5)	6.78	7.62	8.08	9.10	1.19	1.19	1.90	1.00	1.90
$TFA–CH_3CN–THF$ (72.5:27.5:2)	5.38	6.24	6.24	7.11	1.16	1.14	1.75	0.00	1.65
$KH_2PO_4–CH_3CN$ (75:25)	12.55	13.03	15.36	15.90	1.22	1.22	<0.40	2.75	<0.40
$NaOAc–CH_3CN$ (75:25)	8.34	9.52	10.06	11.41	1.20	1.20	2.10	0.90	2.05
$NaOAc–CH_3CN–THF$ (75:25:2)	5.86	6.78	6.78	7.81	1.15	1.15	1.80	<0.40	2.00

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 250 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3.0); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3.0). $\alpha_{b,a}$ represents separation of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; $\alpha_{c,d}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;b,c}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R*) isomers; $R_{S;c,a}$ represents resolution of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; and $R_{S;a,d}$ represents resolution of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers.

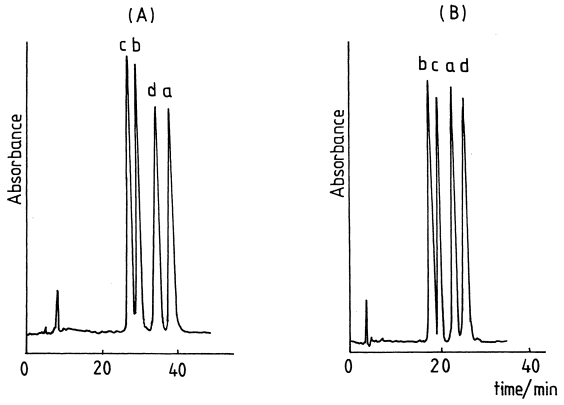

Fig. 2. Chromatograms of GITC derivatives of ABHC and ABHC-ene. (A) ABHC-GITC derivative; (B) ABHC-ene-GITC derivative. Column, Vydac 218TP54 C_{18}; mobile phase, (A) 0.1% TFA–CH_3OH–THF (60:40:2, v/v/v), (B) 0.1% TFA–CH_3OH (55:45, v/v); flow-rate, 0.8 ml/min; detection at 250 nm; Peaks: (**a**) *diendo*-(1*S*,2*S*,3*R*,4*R*) isomer; (**b**) *diendo*-(1*R*,2*R*,3*S*,4*S*) isomer; (**c**) *diexo*-(1*S*,2*R*,3*S*,4*R*) isomer; (**d**) *diexo*-(1*R*,2*S*,3*R*,4*S*) isomer.

3.2. Separation of FDAA derivatives

Variation of the derivatizing reagent sometimes afforded conditions allowing an improved separation of the enantiomers. Results on the separation of ABHC derivatives are given in Table 3. Variation of the derivatizing reagent changed the elution sequence of the isomers, but the same rule was observed for the enantiomer pairs as for the GITC derivatives. The enantiomers with the *S* configuration at carbon atom 3 eluted before those with the *R* configuration there. The elution sequence was **b**<**c**<**a**<**d**.

No conditions were found for the separation of all four enantiomers; isomers **b** and **c** coeluted. Decrease of the content of organic modifier, methanol or acetonitrile, was not accompanied by any change in the resolution of these isomers ($R_{s,b,c}\approx 0$). The addition of THF as second organic modifier to the mobile phase did not improve the resolution. FDAA derivatization resulted in much higher α values for the *diendo-diendo* and the *diexo-diexo* isomers than those observed for the GITC derivatives, e.g. $\alpha_{a,b}$

Table 3
Dependence of retention factor (k), separation factor (α) and resolution (R_s) of ABHC-FDAA derivatives on eluent system

Eluent composition (v/v)	k				$\alpha_{b,a}$	$\alpha_{c,d}$	$R_{S;b,c}$	$R_{S;c,a}$	$R_{S;a,d}$
	diendo (**b**)	*diexo* (**c**)	*diendo* (**a**)	*diexo* (**d**)					
TFA–CH_3OH									
(50:50)	1.67	1.67	5.11	6.80	3.05	4.07	0.00	7.80	3.30
(55:45)	3.24	3.24	11.35	13.54	3.50	4.20	0.00	12.66	2.53
KH_2PO_4–CH_3OH									
(55:45)	3.19	3.19	11.50	13.70	3.60	4.30	0.00	12.90	2.50
NaOAc–CH_3OH									
(55:45)	2.73	2.73	9.08	11.04	3.32	4.04	0.00	10.00	2.66
TFA–CH_3CN									
(70:30)	2.27	2.27	4.89	5.51	2.15	2.42	0.00	9.10	1.70
KH_2PO_4–CH_3CN									
(70:30)	2.13	2.13	4.49	5.18	2.11	2.43	0.00	7.60	1.50
NaOAc–CH_3CN									
(70:30)	1.89	1.89	4.18	4.78	2.21	2.53	0.00	13.50	2.50

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 340 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3.0); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3.0). $\alpha_{b,a}$ represents separation of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; $\alpha_{c,d}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;b,c}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R*) isomers; $R_{S;c,a}$ represents resolution of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; and $R_{S;a,d}$ represents resolution of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers.

and $\alpha_{d,c}$ were between 2 and 4.3, while in the latter case the corresponding values were lower than 1.4. The application of FDAA as derivatizing reagent for ABHC seems to be favourable if the task is the separation of the enantiomers of the *diendo* or *diexo* compounds. Table 3 reveals that there was no pronounced difference between the organic modifiers, methanol and acetonitrile, in the separation of the FDAA derivatives of ABHC, as observed for the GITC derivatives.

The results of the separation of the ACHC-ene FDAA derivatives are listed in Table 4. The sequence of elution remained the same as for the ABHC-ene-GITC derivatives. All four isomers could be separated in one chromatographic run. In methanol-containing systems at 45% (v/v) organic modifier content, almost the same resolutions were observed in all three buffer systems, and the resolutions were higher than those for the GITC derivatives. Good resolutions could also be achieved in acetonitrile-containing systems. This behaviour differed greatly from that for the ABHC-ene-GITC derivatives (Table 2), where total separation was not achievable in the acetonitrile-containing system. Chromatograms showing the best resolution for the four enantiomers as FDAA derivatives are presented in Fig. 3.

3.3. Application of chiral chromatography

For the direct separation, a Crownpak CR(+) column was used. The chiral selector of this column is a chiral crown ether and it can resolve compounds bearing a primary amino group near the chiral centre. Chiral recognition is achieved when a complex is formed between the ammonium ion derived from the analyte and the crown ether stationary phase. The isomers could be separated by varying the pH and the temperature influencing the stability of the complex formed.

The results for the ABHC enantiomers can be seen in Table 5. The separation was started at 25°C with perchloric acid of pH 2.0 as eluent, at a flow-rate of 0.5 ml/min. Under these conditions isomers **a–b** and **d–c** coeluted. The effect of pH was more significant, due to the stronger complex formation under acidic conditions. On decrease of the pH (pH 1.5 and 1.0) the separation of enantiomers **a–b** and **d–c** improved, but was not complete.

Decrease of the temperature of a crown ether

Table 4
Dependence of retention factor (k), separation factor (α) and resolution (R_s) of ABHC-ene-FDAA derivatives on eluent system

Eluent compostion (v/v)	k				$\alpha_{b,a}$	$\alpha_{c,d}$	$R_{S;b,c}$	$R_{S;c,a}$	$R_{S;a,d}$
	diendo (**b**)	*diexo* (**c**)	*diendo* (**a**)	*diexo* (**d**)					
$TFA–CH_3OH$									
(50:50)	1.40	1.77	4.56	5.55	3.25	3.13	1.50	9.75	2.40
(55:45)	2.41	3.10	8.68	10.71	3.60	3.45	2.20	11.15	3.10
$KH_2PO_4–CH_3OH$									
(55:45)	2.40	3.07	9.52	11.84	3.96	3.85	1.45	10.00	2.90
$NaOAc–CH_3OH$									
(55:45)	2.04	2.57	7.39	8.94	3.62	3.48	1.55	10.45	2.55
$TFA–CH_3CN$									
(70:30)	2.06	2.40	4.25	5.03	2.06	2.10	1.45	6.20	2.50
$KH_2PO_4–CH_3CN$									
(70:30)	1.86	2.15	3.88	4.54	2.09	2.11	1.15	6.10	2.25
$NaOAc–CH_3CN$									
(70:30)	1.65	1.85	3.63	4.08	2.13	2.20	0.95	5.10	1.10
(75:25)	3.57	4.09	9.54	11.11	2.67	2.71	1.70	12.60	2.70

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 340 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3.0); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3.0). $\alpha_{b,a}$ represents separation of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; $\alpha_{c,d}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;b,c}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R*) isomers; $R_{S;c,a}$ represents resolution of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diendo*-(1*S*,2*S*,3*R*,4*R*) isomers; and $R_{S;a,d}$ represents resolution of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers.

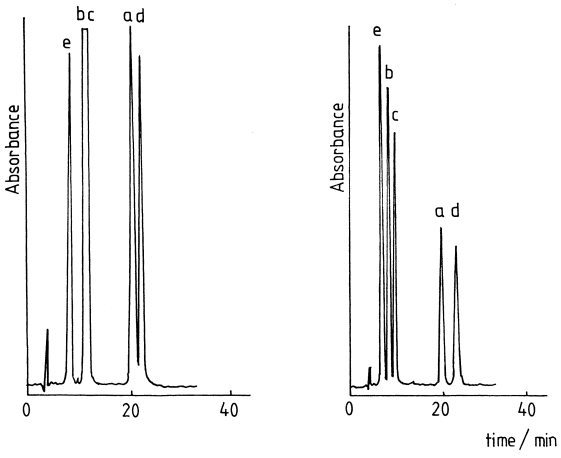

Fig. 3. Chromatograms of FDAA derivatives of ABHC and ABHC-ene. (A) ABHC-FDAA derivative; (B) ABHC-ene-FDAA derivative. Column, Vydac 218TP54 C_{18}; mobile phase, (A) 0.1% TFA–CH_3CN (70:30, v/v), (B) 0.1% TFA–CH_3OH (50:50, v/v); flow-rate, 0.8 ml/min; detection at 340 nm; Peaks: (**a**) *diendo*-(1*S*,2*S*,3*R*,4*R*) isomer; (**b**) *diendo*-(1*R*,2*R*,3*S*,4*S*) isomer; (**c**) *diexo*-(1*S*,2*R*,3*S*,4*R*) isomer; (**d**) *diexo*-(1*R*,2*S*,3*R*,4*S*) isomer; (**e**) unreacted FDAA.

column has been reported [22–25] to lead improved enantioselectivity. Table 5 shows that a decrease of temperature increased the enantioselectivity at all investigated pH values. $R_{S;a,b}$ increased from <0.4 to 1.15 and $R_{S;d,c}$ from <0.4 to 1.0 on decrease of the temperature from 25 to 5°C, while the resolution of isomers **b–d**, $R_{S;b,d}$, increased to a small extent, from 2.05 to 2.40. A decrease of the flow-rate for ABHC was not favourable. The decrease from 0.5 to 0.25 ml/min doubled the retention time, and thus the analysis time, but the increase in R_S was not significant.

The elution sequence differed greatly from that observed for the derivatized compounds. The *diendo* compounds eluted before the *diexo* ones, and in the *diendo-diendo* and *diexo-diexo* pairs the first peaks related to the variants with the *R* configuration at carbon atom 3. The literature data show that with a Crownpak CR(+) column (+)-*R* enantiomers elute before (−)-*S* ones for α-amino acids with one chiral centre, and dipeptides containing two chiral centres [24,26].

The separation of ABHC-ene isomers is surveyed in Table 6. Variation of temperature, pH and flow-

Table 5
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ABHC isomers on temperature, pH and eluent flow-rate

Temp. (°C)	pH	flow-rate (ml/min)	k				$\alpha_{a,b}$	$\alpha_{d,c}$	$R_{S;a,b}$	$R_{S\ b,d}$	$R_{S;d,c}$
			diendo (**a**)	*diendo* (**b**)	*diexo* (**d**)	*diexo* (**c**)					
25	2.0	0.50	2.48	2.48	3.22	3.22	1.00	1.00	0.00	1.55	0.00
	1.5	0.50	3.23	3.28	4.18	4.18	1.02	1.00	<0.40	1.70	0.00
	1.0	0.50	3.66	3.85	4.72	4.85	1.05	1.03	0.40	2.05	<0.40
20	1.0	0.50	3.85	4.08	4.97	5.18	1.06	1.04	0.60	2.10	0.60
15	1.0	0.50	4.18	4.46	5.38	5.65	1.07	1.05	0.75	2.20	0.70
10	1.0	0.50	4.48	4.84	5.78	6.11	1.08	1.06	0.75	2.30	0.75
5	2.0	0.50	3.41	3.62	4.43	4.45	1.06	1.01	0.70	1.90	<0.40
		0.25	3.44	3.66	4.50	4.71	1.06	1.05	0.85	2.45	<0.40
	1.5	0.50	4.40	4.73	5.70	6.00	1.07	1.05	1.05	2.05	0.80
		0.25	4.50	4.84	5.81	6.14	1.07	1.06	1.10	2.60	0.90
	1.0	0.50	4.81	5.26	6.21	6.65	1.09	1.07	1.15	2.40	1.00
		0.25	4.78	5.23	6.17	6.61	1.09	1.07	1.20	2.30	1.05

Column, Crownpak CR(+); eluent, aqueous perchloric acid; detection, 210 nm; $\alpha_{a,b}$ represents separation of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diendo*-(1*R*,2*R*,3*S*,4*S*) isomers; $\alpha_{d,c}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;a,b}$ represents resolution of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diendo*-(1*R*,2*R*,3*S*,4*S*) isomers; $R_{S;b,d}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;d,c}$ represents resolution of *diexo*-(1*R*,2*S*,3*R*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R*) isomers.

rate did not reveal any conditions suitable for the total separation of enantiomers **a** and **b**; only a small degree of resolution was observed ($R_S \approx 0.5$) at a relatively high retention time. The elution sequence on the chiral crown ether column was the same for the enantiomers of ABHC and ABHC-ene. The ABHC-ene compounds gave sharper peak shapes than ABHC. Representative chromatograms for ABHC and ABHC-ene isomers are shown in Fig. 4.

3.4. Temperature dependence of the direct separation

Measurement of the temperature dependence of the separation affords a possibility for estimation of the enthalpy and entropy of complex formation. Supposing a stoichiometry of 1:1 between the amines and the crown ether, the free energy difference can be calculated from the equation $\Delta(\Delta G^\circ) = -RT \ln \alpha$

Table 6
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ABHC-ene isomers on temperature, pH and eluent flow-rate

Temp. (°)	pH	flow-rate (ml/min)	k				$\alpha_{a,b}$	$\alpha_{d,c}$	$R_{S;a,b}$	$R_{S;b,d}$	$R_{S;d,c}$
			diendo (**a**)	*diendo* (**b**)	*diexo* (**c**)	*diexo* (**d**)					
25	1.0	0.50	2.86	2.86	3.80	4.03	1.00	1.06	0.00	2.00	0.80
20	1.0	0.50	3.08	3.08	4.10	4.39	1.00	1.07	0.00	2.00	1.05
15	1.0	0.50	3.18	3.18	4.39	4.76	1.00	1.08	0.00	2.20	1.10
10	1.0	0.50	3.55	3.55	4.73	5.18	1.00	1.09	0.00	2.50	1.10
5	2.0	0.50	2.80	2.80	3.85	4.13	1.00	1.07	0.00	2.30	1.00
	1.5	0.50	3.56	3.56	4.82	5.24	1.00	1.09	0.00	2.50	2.00
	1.0	0.50	3.82	3.82	5.12	5.67	1.00	1.11	<0.40	2.80	1.30
		0.25	3.93	4.08	5.27	5.86	1.04	1.11	0.50	3.50	1.40

Column, Crownpak CR(+); eluent, aqueous perchloric acid; detection, 210 nm; $\alpha_{a,b}$ represents separation of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diendo*-(1*R*,2*R*,3*S*,4*S*) isomers; $\alpha_{d,c}$ represents separation of *diexo*-(1*S*,2*R*,3*S*,4*R*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;a,b}$ represents resolution of *diendo*-(1*S*,2*S*,3*R*,4*R*) and *diendo*-(1*R*,2*R*,3*S*,4*S*) isomers; $R_{S;b,d}$ represents resolution of *diendo*-(1*R*,2*R*,3*S*,4*S*) and *diexo*-(1*R*,2*S*,3*R*,4*S*) isomers; $R_{S;d,c}$ represents resolution of *diexo*-(1*R*,2*S*,3*R*,4*S*) and *diexo*-(1*S*,2*R*,3*S*,4*R*) isomers.

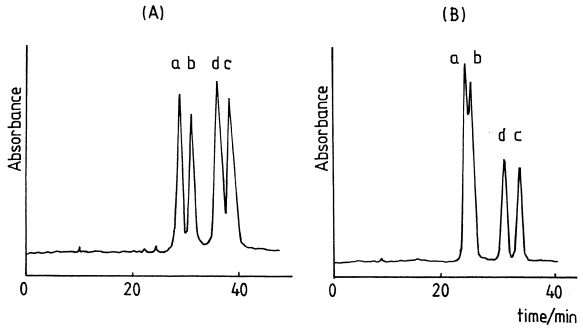

Fig. 4. Chromatograms of ABHC and ABHC-ene. (A) ABHC; (B) ABHC-ene. Column, Crownpak CR(+); mobile phase, 0.1 *M* perchloric acid, pH 2.0; flow-rate, 0.25 ml/min; temperature, 5°C; detection at 210 nm; Peaks: (**a**) *diendo*-(1*S*,2*S*,3*R*,4*R*) isomer; (**b**) *diendo*-(1*R*,2*R*,3*S*,4*S*) isomer; (**c**) *diexo*-(1*S*,2*R*,3*S*,4*R*) isomer; (**d**) *diexo*-(1*R*,2*S*,3*R*,4*S*) isomer.

[27]. The calculated values of $\Delta(\Delta G°)$ for the complex formation of ABHC and ABHC-ene with the crown ether are listed in Table 7. The first observation is that the $-\Delta(\Delta G°)$ values are much smaller than those observed in the literature [28]. The temperature dependence of $\Delta(\Delta G°)$ indicates that the complex formation of the more retained compound (having the *S* configuration at carbon atom 3) with the crown ether depends to a large extent on the enthalpy term, whereas the process that results in the formation of the less-stable complex (with the less-retained compound having the *R* configuration at carbon atom 3) is more dependent on the entropy term. When the temperature is decreased from ambient to 5°C, discrimination of the *diexo*-ABHC-ene isomers takes place with larger

Table 7
Free energy differences $\Delta(\Delta G°)$ for complex formation of ABHC and ABHC-ene with the crown ether at different temperatures

Temp. (K)	$\Delta(\Delta G°)$ (cal/mol)		
	diendo-ABHC	*diexo*-ABHC	*diexo*-ABHC-ene
298	−28.9	−17.5	−34.5
293	−33.9	−22.8	−39.4
288	−38.7	−27.9	−44.1
283	−43.3	−32.8	−48.5
278	−47.6	−37.4	−64.0

Column, Crownpak CR(+); eluent, perchloric acid, pH 1.0; flow-rate, 0.5 ml/min.

Table 8
Detection limits for ABHC and ABHC-ene analysis

Method	Detection, λ (nm)	Detection limit (pmol)	
		ABHC	ABHC-ene
Chiral	210	2500	33
Achiral			
GITC	250	48	12
FDAA	340	17	12

Columns, Crownpak CR(+) and Vydac 218TP54 C18; volume injected, 20 μl; signal-to-noise ratio, 3.

$\Delta(\Delta G°)$. This indicates that the formation of the crown ether amine complexes with the *diexo*-ABHC-ene isomers is more dependent than that for the saturated isomers on the enthalpy term.

3.5. Detection limits of measurement

The limit of detection was determined at a signal-to-noise ratio of 3. Table 8 shows the detection limits for ABHC and ABHC-ene in the direct and indirect separations. The detection limit for the derivatized amino acids is one or two orders of magnitude lower than for the underivatized ones, due to the much higher molar absorptivity of the former. This calls attention to pre- or postcolumn derivatization (e.g. using a highly absorbent or fluorescent reagent). Naturally it is not easy to find an appropriate column and conditions for this kind of separation.

4. Conclusions

The enantiomers of ABHC could be separated with good resolution as GITC derivatives, mainly when THF was applied as second organic modifier together with methanol. The enantiomers of ABHC-ene were separable either as GITC or as FDAA derivatives. Methanol organic modifier was generally more efficient than acetonitrile, ensuring better resolution.

The direct determination on the chiral crown ether column Crownpak CR(+) proceeded with good resolution for the ABHC enantiomers, whereas the *diendo* isomers of ABHC-ene were not separable.

The direct and indirect methods complement each other very well.

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