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ORIGINAL ARTICLE

Direct chromatographic enantioresolution of fully constrained β-amino acids: exploring the use of high-molecular weight chiral selectors

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Abstract To the best of our knowledge enantioselective chromatographic protocols on β-amino acids with polysaccharide-based chiral stationary phases (CSPs) have not yet appeared in the literature. Therefore, the primary objective of this work was the development of chromatographic methods based on the use of an amylose derivative CSP (Lux Amylose-2), enabling the direct normal-phase (NP) enantioresolution of four fully constrained β-amino acids. Also, the results obtained with the glycopeptide-type Chirobiotic T column employed in the usual polar-ionic (PI) mode of elution are compared with those achieved with the polysaccharide-based phase. The Lux Amylose-2 column, in combination with alkyl sulfonic acid containing NP eluent systems, prevailed over the Chirobiotic T one, when used under the PI mode of elution, and hence can be considered as the elective choice for the enantioseparation of this class of rigid β-amino acids. Moreover, the extraordinarily high α (up to 4.60) and R_S (up to 10.60) values provided by the polysaccharidic polymer, especially when used with camphor sulfonic acid containing eluent systems, make it also suitable for preparative-scale enantioisolations.

Keywords Polysaccharide-based stationary phase · Glycopeptide-based stationary phase · Alkyl sulfonic acid additives · Fully constrained β-amino acids · Mechanism of enantiorecognition

Abbreviations

AcOH	Acetic acid
CSA	Camphorsulfonic acid
CSPs	Chiral stationary phases
DCM	Dichloromethane
ESA	Ethanesulfonic acid
EtOH	Ethanol
MeOH	Methanol
MSA	Methanesulfonic acid
NP	Normal-phase
PI	Polar-ionic
PO	Polar-organic
SAX	Strong anion exchange
TFA	Trifluoroacetic acid

Introduction

β-Amino acids play a central role in modern chemical research with important implications in synthetic and medicinal chemistry. β-Proline analogs are known as organocatalysts (Terakado et al. 2005; Mitsumori et al. 2006; Zhang et al. 2008; Armstrong et al. 2009) while several β-amino acids are commonly employed as intermediates in the synthesis of more complex natural and biologically active products (Liu and Sibi 2002; Steer et al. 2002; Weiner et al. 2010), to cite but a few examples. Several synthetic methods for the construction of conformationally constrained bicyclic and polycyclic α- and β-aminoacidic scaffolds have appeared in the recent literature (Yu et al. 2009; Fustero et al. 2010; Mitsunuma and Matsunaga 2011; Zheng et al. 2011; Biggs-Houck et al. 2012; Li et al. 2012). Cyclic amino acids have attracted considerable interest as

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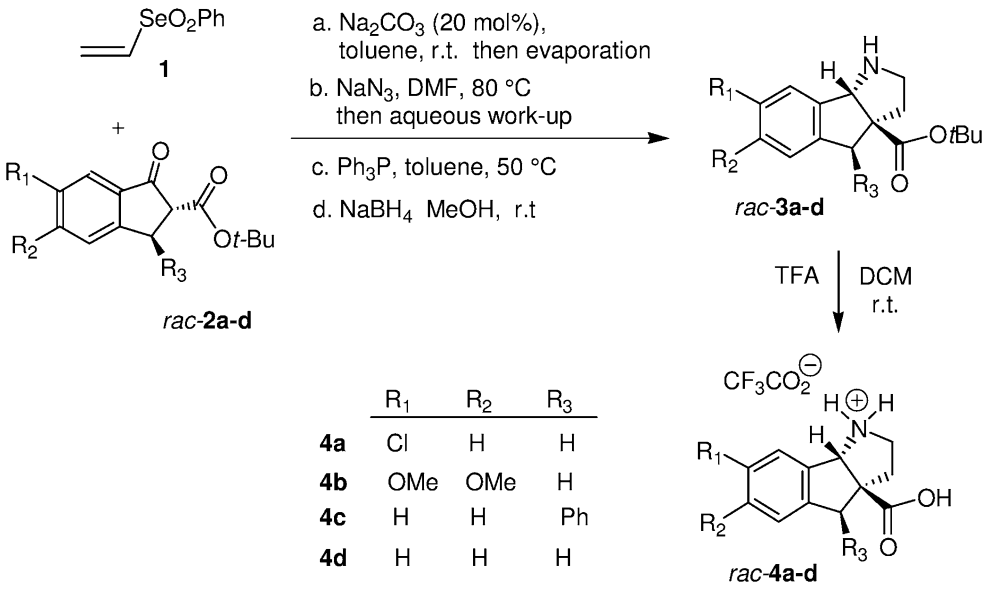
Scheme 1 Synthesis of the investigated compounds



	R_1	R_2	R_3
4a	Cl	H	H
4b	OMe	OMe	H
4c	H	H	Ph
4d	H	H	H

key structural element in the design of peptidomimetics and enzyme inhibitors (Hanessian et al. 2003, 2004; Kuhl et al. 2005; Hanessian and Auzzas 2008).

In this field, and as part of a broader research program aimed at obtaining highly enantioenriched, privileged scaffolds containing densely functionalized quaternary stereocenters (Marini and Sternativo 2013), we recently reported the synthesis of a series of polycyclic pyrrolidine derivatives (Sternativo et al. 2012). Racemic β-amino esters **3a–d** were prepared from vinyl selenone **1** and the indanone carboxylates *rac*-**2a–d**.

For the scope of the present work, the racemic β-amino acidic salts **4a–d** were then obtained as described in Scheme 1 by treatment with trifluoroacetic acid (TFA) in dichloromethane (DCM) (detailed information is reported in the Online Resource).

So far, the direct enantioresolution of β-amino acids has been successfully achieved with both low (Hyun et al. 2002, 2003; Péter 2002; Berkecz et al. 2006; Hoffmann et al. 2009) and high (D'Acquarica et al. 2000; Árki et al. 2004; Péter et al. 2004) molecular weight chiral selectors, with the glycopeptide-type chiral stationary phases (CSPs) exhibiting the widest versatility. However, apart from a singular case we recently described (Sardella et al. 2014), to the best of our knowledge no other focused applications with polysaccharide-based CSPs have appeared in the literature.

A number of notable drawbacks are usually encountered in the analysis of ionic compounds with polysaccharide-type CSPs, essentially due to their ability to undergo unwanted interactions with some of the stationary phase functionalities (Ye and Stringham 2001; Ye et al. 2002b). In this regard, it is worthwhile mentioning the excessive analysis times along with peak broadening and distortion phenomena (Ye and Stringham 2001; Ye et al. 2002b). Nevertheless, the incorporation of strong alkyl sulfonic acid additives into the eluent was proven to be very effective for the enantioseparation of underivatized α-amino acids with polysaccharide-based CSPs by other authors (Ye and Stringham 2001; Ye et al. 2002b). Therefore, our primary objective was the development of chromatographic methods based on the use of an amylose derivative CSP (Lux Amylose-2, Fig. 1), enabling the direct normal-phase (NP) enantioresolution of *rac*-**4a–d** (Scheme 1).

Also, the results obtained with the glycopeptide-type Chirobiotic T column based on teicoplanin (Fig. 2), employed in the more usual polar-ionic (PI) mode of elution have been compared with those achieved with the polysaccharide-based one.

Chemicals and reagents

Rac-**4a–d** were synthesized in our laboratories according to Scheme 1. Analytical grade ethanol (EtOH), methanol (MeOH), *n*-hexane, acetic acid (AcOH), ethanesulfonic acid (ESA), methanesulfonic acid (MSA), (1*S*)-(+)-camphorsulfonic acid (CSA), trifluoroacetic acid (TFA), dichloromethane (DCM), and ammonium formate were purchased from Sigma-Aldrich (Milano, Italy). The compound 1,3,5-tri-*tert*-butylbenzene was used as the unretained marker in the analysis with the polysaccharide-based column and purchased from Sigma-Aldrich (Milano, Italy). The compound $NaNO_2$ was used as the unretained marker in the analysis with the glycopeptide-based column and

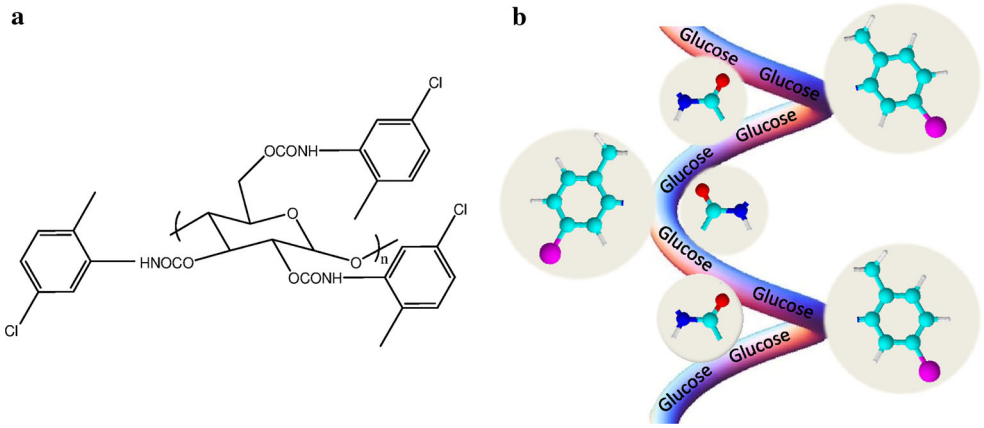


Fig. 1 **a** Structure of the chloromethylated amylose-based chiral selector employed in the study; **b** cartoon depiction of the polymer morphology and disposition of the carbamate substructural motifs (chlorine in *purple*, hydrogen in *grey*, carbon in *cyano*, nitrogen in *blue*, oxygen in *red*) (color figure online)

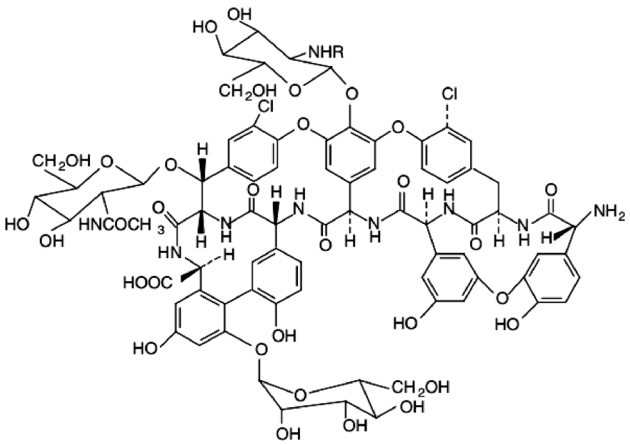


Fig. 2 Structure of teicoplanin

purchased from Sigma-Aldrich (Milano, Italy). Water for HPLC analysis was purified with a New Human Power I Scholar water purification system (Human Corporation, Seoul, Korea).

All the employed mobile phases were degassed with 10 min sonication before use. Compounds to be injected were solubilized in the selected mobile phase and analyzed at the approximate concentration of 0.5–1.0 mg/ml.

The "apparent" pH [${}^{s}_{w}$pH, that is the one measured in the employed hydro-organic mobile phase (s), while the calibration of the pH-meter system was done in water (w)] of the PI eluents was measured with a conventional pH-meter, and then opportunely adjusted.

Instrumentation

The enantioseparation analyses were carried out with the following two columns: the column Lux Amylose-2 (250 × 4.6 mm I.D., containing amylose tris(5-chloro-2-methylphenylcarbamate) adsorbed onto a 5 µm silica gel) was purchased from Phenomenex (Torrance, CA, USA); the column Chirobiotic T (250 × 4.6 mm I.D., containing the glycopeptide teicoplanin covalently bonded to a high purity 5 µm spherical silica gel) was purchased from Sigma-Aldrich (Milano, Italy). Before the use, the selected column was conditioned by flowing through it about 40 ml of the selected mobile phase. The HPLC measurements were made on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 20 µl stainless steel loop. Column temperature was controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R) thermostat. The chromatographic trace was obtained and handled with the LC Solution Software from Shimadzu (Kyoto, Japan).

NP-based enantioseparation with the Lux Amylose-2 column

Owing to their very strong acidic character, alkyl sulfonic acids are particularly suitable to form stable ion-pairs (Ye and Stringham 2001; Ye et al. 2002b; Stringham and Ye 2006). Maintaining stable ion-pairs is of fundamental importance for the analysis of compounds in salified form (Stringham and Ye 2006). Indeed, in a recent work (Sardella et al. 2014) we demonstrated TFA being not sufficiently strong to produce stable ion-pairs with **4d** enantiomers, thus favoring an on-column equilibrium between the salt and the free base form ("salt breaking" effect) (Stringham and Ye 2006). Differently, excellent chromatographic performances were achieved for all the investigated compounds, when the three alkyl sulfonic acidic additives MSA, ESA and CSA were used at the same concentration (15 mM) (Table 1).

In the following discussion, tentative interpretations of the retention and enantiorecognition processes are done

Table 1 Chromatographic performance obtained with the three employed alkyl sulfonic acids

Cpd	MSA				ESA				CSA			
	k_1	k_2	α	R_S	k_1	k_2	α	R_S	k_1	k_2	α	R_S
4a	8.75	12.01	1.38	3.91	6.26	11.12	1.78	5.46	9.15	34.62	3.79	7.54
4b	15.26	24.33	1.60	4.54	10.27	16.90	1.65	4.71	18.45	82.68	4.48	7.34
4c	8.22	11.62	1.41	3.80	5.65	9.58	1.70	4.45	16.63	18.14	1.09	nc
4d	12.10	21.57	1.78	6.66	7.38	20.89	2.83	9.75	13.00	52.80	4.60	10.60

Experimental conditions: column, Lux Amylose-2; flow rate, 1.0 ml/min; column temperature, 35 °C; detection wavelength, 220 nm; mobile phase, 15 mM alkyl sulfonic acid additive in *n*-hexane/EtOH-90/10 (v/v)

nc not calculated by the LC Solution Software

Table 2 Physico-chemical parameters of the three employed alkyl sulfonic acid additives

Acidic additive	pK_a	$\log P_n^a$	$\log P_c^b$	Vol (Å^3)
MSA	−1.89 ± 0.15	−1.89 ± 0.39	−5.39 ± 1.0	64.0
ESA	−1.61 ± 0.15	−1.36 ± 0.39	−4.86 ± 1.0	82.4
CSA	−2.38 ± 0.18	−0.58 ± 0.50	−4.08 ± 1.0	199.9

All the parameters are calculated with the ACD/Labs Software version 7.0

[a] Value calculated with the acid in its neutral form

[b] Value calculated with the acid in its charged form

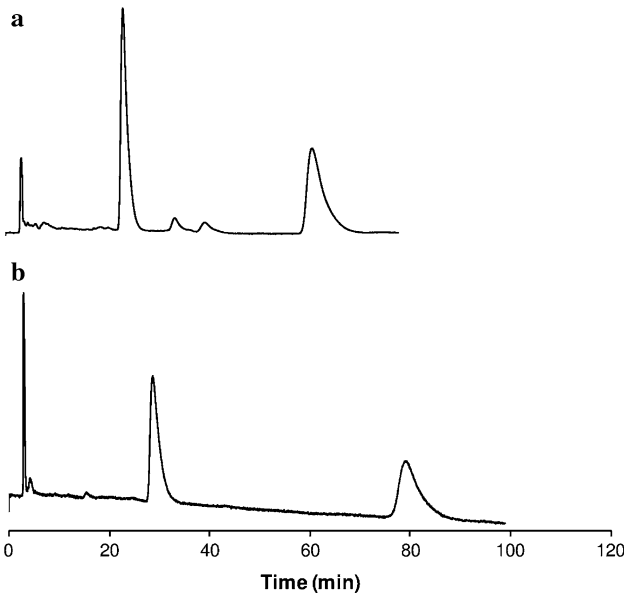


Fig. 3 Chromatographic traces of **4d**. Experimental conditions: column, Lux Amylose-2; flow rate, 1.0 ml/min; column temperature, 35 °C; detection wavelength, 220 nm; mobile phase, **a** 15 mM ESA in *n*-hexane/EtOH-90/10 (v/v), **b** *n*-hexane/EtOH-90/10 (v/v) but after having flowed (**a**) for 2 h at 1.0 ml/min

according to literature data (Ye and Stringham 2001; Ye et al. 2002b; Stringham and Ye 2006). However, it should be remarked that the enantiorecognition mechanism with polysaccharide-based stationary phases has not yet been fully clarified at a molecular level (Chankvetadze 2012).

Consistent with the carbon number in the acidic additive side-chain (Ye and Stringham 2001) for all compounds, retention was greater with MSA than with ESA (Table 1). Instead, the greatest retention always produced by CSA can be explained by invoking additional H-bond contacts and/or dipole–dipole interactions by the keto group on the bicyclic scaffold (Ye et al. 2002b) with the enantioselective carbamate residues located inside the "chiral grooves" lying along the main axis of the polysaccharide chain (Fig. 1b). IR studies (Chankvetadze et al. 1995) demonstrated that the simultaneous presence of a methyl (electron donating) group and a chlorine (electron withdrawing) atom on the phenyl ring of the carbamate moiety (Fig. 1a) increases the number of interior –NH and –C=O groups available for stereoselective intermolecular contacts at expenses of those occurring intramolecularly between adjacent carbamate motifs along the polymer chain.

It is worth noting that the trend of retention runs counter to the alkyl sulfonic acid additive pK_a value (Tables 1, 2). This evidence furthermore confirms that alkyl sulfonic acids not only act as ion suppressors of residual silanols and analyte carboxylic acid moiety, and as part of ion-pair associates, but are also involved in the chromatographic event at different levels (Sardella et al. 2014).

Accordingly, it was demonstrated that the beneficial effects of alkyl sulfonic acids on enantioselectivity primarily originate from their stable adsorption onto the arsenal of stationary phase functionalities (Ye et al. 2002a; Sardella et al. 2014). Also in the present study, the persistence of the ESA effect even after its removal out from the eluent system (known as "memory effect") was observed. In Fig. 3, the chromatographic performance on **4d** obtained with ESA into the eluent (Fig. 3a) and after its removal from it (Fig. 3b) is compared.

Depending on the structural features of the additive, a different adsorption onto the stationary phase motifs takes place, which can lead to a peculiar winding of the polymer chain and hence a different access to the stereoselective

Table 3 Physico-chemical parameters of the investigated compounds

Cpd	pK_a-I[a]	pK_a-II[b]	logP_n
4a	3.68 (±0.20)	10.22 (±0.20)	2.63 (±0.68)
4b	3.81 (±0.20)	10.17 (±0.20)	1.77 (±0.69)
4c	3.23 (±0.40)	10.31 (±0.40)	4.00 (±0.81)
4d	3.58 (±0.20)	10.63 (±0.20)	2.03 (±0.67)

All the parameters are calculated with the ACD/Labs Software version 7.0

[a] Value referred to the acidic moiety

[b] Value referred to the basic moiety

binding sites (Sardella et al. 2014). Moreover, the adsorbed additive itself can contribute with further interaction points. On this basis, the improved enantioselectivity observed for three out of four compounds (namely **4a**, **4b** and **4d**) with an increase in the acidic additive bulkiness can be explained. Steric effects by the additional aromatic ring on **4c** could be invoked to account for its counter current behavior (Table 1). Accordingly, the phenyl ring in **4c** can be hypothesized to limit the access to the enantioselective binding sites on the polymer chain, even if non-traditional π-interactions with the flanking aromatic groups (Fig. 1b) or other functionalities cannot be ruled out (Steiner and Koellner 2001). It can be speculated that the relevant enantioselectivity generated by CSA can be also due to its chiral nature.

As expected, the higher the structural complexity of the acidic additive, the lower the impact of the analyte physico-chemical properties on the retention behavior. Indeed, with MSA, the analyte retention strictly respected the trend of analyte hydrophobicity (measured as log*P* values) (Table 3).

The presence of additional sources of H-bond contacts in **4b** (that is the two methoxy groups) can justify the almost always highest retention recorded for this compound. While, a steric hindrance produced by the extra phenyl ring in **4c** could explain its almost always lowest retention.

In Fig. 4, the chromatograms obtained in the best experimental conditions are shown.

PI mode-based enantioseparation with the Chirobiotic T column

Due to the limited samples amount, a tuning of the eluent composition was first carried out with **4b** enantiomeric couple (Table 4). Excessive retention along with a poor peak shape (trace not shown) was obtained by running the analyses in the polar-organic (PO) mode of elution with a H_2O/MeOH-10/90 (v/v)-based mobile phase (Table 4, entry A).

Oppositely, to fix the eluent $^{s}_{w}$pH at the value of 4.50 with a 20 mM ammonium formate buffer (polar-ionic, PI, mode of elution), allowed us to sensitively ameliorate the overall chromatographic performance ($R_S = 2.70$, $\alpha = 1.37$), within usable analysis time (<20 min) (Table 4, entry B; Fig. 5). Very interestingly, the replacement of the buffer system with AcOH, while keeping the mobile phase $^{s}_{w}$pH unmodified, revealed the outstanding role of the anionic site (–COO^-) on the chiral selector in governing the analyte retention (Berthod et al. 1996; Tesařová et al.

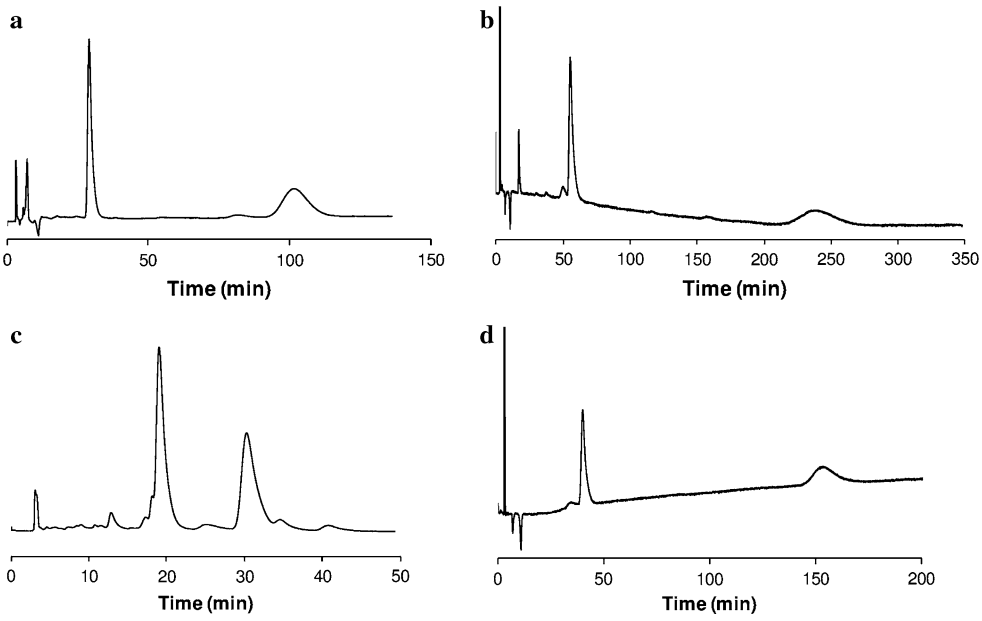


Fig. 4 Chromatographic traces of **4a** (**a**), **4b** (**b**), **4c** (**c**) and **4d** (**d**) obtained in the best eluent conditions. Experimental conditions: column, Lux Amylose-2; flow rate, 1.0 ml/min; column temperature, 35 °C; detection wavelength, 220 nm; mobile phase, **a**, **b** and **d** 15 mM CSA in *n*-hexane/EtOH-90/10 (v/v), **c** 15 mM ESA in *n*-hexane/EtOH-90/10 (v/v)

Table 4 Optimization of the mobile phase composition in the analysis of **4b**

Mobile phase	Chromatographic parameters			
	k_1	k_2	α	R_S
A[a]	17.41	22.80	1.31	2.43
B[b]	2.56	3.51	1.37	2.70
C[c]	11.93	15.28	1.28	2.37

Experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm; mobile phase, see the footnotes

[a] H_2O/MeOH-10/90 (v/v)

[b] H_2O/MeOH-10/90 (v/v), $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

[c] H_2O/MeOH-10/90 (v/v), $^{s}_{w}$pH 4.50, fixed with concentrated AcOH

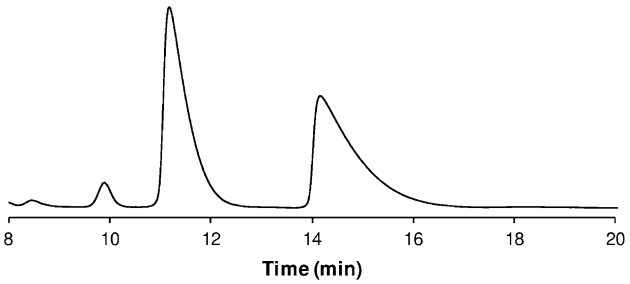


Fig. 5 Chromatographic traces of **4b**. Experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm; eluent, H_2O/MeOH-10/90 (v/v), $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

1999; Berthod 2009) (Table 4, entry C). The exclusive use of the acidic modifier caused an excessively long retention (about 80 min). In addition, also the peak shape resulted negatively affected by such a change (trace not shown).

At $^{s}_{w}$pH 4.50, both **4b** (Table 3) and the chiral selector feature zwitterionic characteristic (Berthod et al. 1996; Tesařová et al. 1999; Berthod 2009). Therefore, in this setting, ammonium ions of the buffer system behave as competitive species towards the anionic sites of the chiral selector units, thus shortening the analyte retention by virtue of their higher concentration.

The established PI mode conditions were then applied to the remaining compounds. According to the data listed in Table 5 and the chromatographic traces shown in Fig. 6, only **4c** did not experience enantioresolution. Quite predictably for PI mode systems (Beesley and Lee 2007), the most hydrophobic **4c** eluted first while, oppositely, **4b** resulted the most tightly bonded, maybe owing to its ability to undergo additional H-bond contacts with complementary functionalities decorating the glycopeptidic phase (Beesley and Lee 2007). However, from data reported in Tables 3 and 5 it is evident that the analyte polarity is not the only factor contributing to rule the solute retention.

Table 5 Chromatographic performance obtained with experimental conditions optimized on **4b**

Cpd	Chromatographic parameters			
	k_1	k_2	α	R_S
4a	2.40	2.96	1.23	2.12
4b	2.56	3.51	1.37	2.70
4c	0.73	0.77	1.05	nc
4d	2.32	2.83	1.22	2.23

Experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm; eluent, H_2O/MeOH-10/90 (v/v), $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

nc not calculated by the LC Solution Software

Indeed, despite their different hydrophobicities (Table 3), comparable retention profiles were experienced by **4a** and **4d**. A role of π–π stacking interactions can be hypothesized to explain this chromatographic behavior.

The reduction of the water content into the eluent (from 10 down to 5 %, in volume) produced a slight increase of the α value in the analysis of **4c** (Table 6, entry D). In parallel, the enantiomeric retention was observed to slightly increase as a result of the lower solubility of the submitted compound in the water-poorer mobile phase system (Berthod et al. 1996; Péter et al. 1998).

The R_S value underwent a small improvement when 100 % MeOH was used (Table 6, entry E). Very profitably, change of the alcoholic eluent system from MeOH to EtOH noticeably improved selectivity ($\alpha = 1.32$) and resolution ($R_S = 1.79$) (Table 6, entry F; Fig. 7). This finding is in line with the observations by other authors (Péter et al. 1998) who highlighted the crucial importance of the concentration and nature of the alcoholic mobile phase component in the control of retention and selectivity with glycopeptide-type CSPs.

Conclusions

Both the polysaccharide- and the glycopeptide-based CSP effectively enantioresolved *rac*-**4a**–**d**. However, the Lux Amylose-2 column, in combination with alkyl sulfonic acid containing NP eluent systems, prevailed over the Chirobiotic T one, when used under the PI mode of elution, and hence can be considered as the elective choice for the enantioseparation of this class of rigid β-amino acids. Moreover, the extraordinarily high α (up to 4.60) and R_S (up to 10.60) values produced by the polysaccharidic polymer make it also suitable for preparative-scale enantioisolation issues. In this framework, the "racemic approach" would be facilitated for the presence of easy-to-

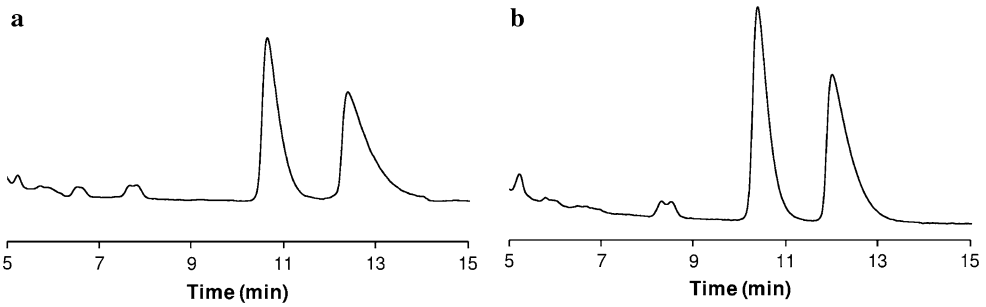


Fig. 6 Chromatographic traces of **4a** (**a**) and **4d** (**b**) obtained with the eluent H_2O/MeOH-10/90 (v/v), $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate. Other experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm

Table 6 Optimization of the mobile phase composition in the analysis of **4c**

Mobile phase	Chromatographic parameters			
	k_1	k_2	α	R_S
D[a]	0.79	0.86	1.10	nc
E[b]	0.88	0.98	1.11	1.03
F[c]	3.24	4.28	1.32	1.79

Experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm; mobile phase, see the footnotes

nc not calculated by the LC Solution Software

[a] H_2O/MeOH-5/95 (v/v), apparent pH 4.50, fixed with 20 mM ammonium formate

[b] MeOH, $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

[c] EtOH, $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

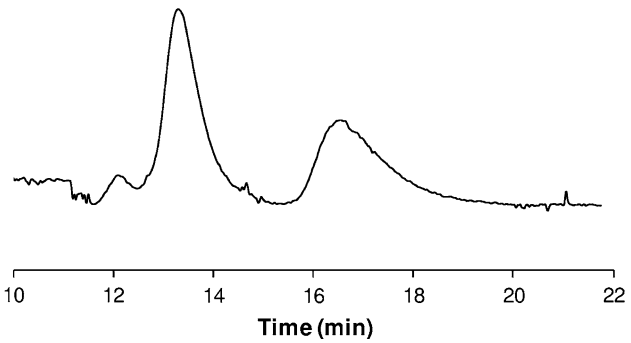


Fig. 7 Chromatographic traces of **4c** in the optimized conditions. Experimental conditions: column, Chirobiotic T; flow rate, 1.0 ml/min; column temperature, 25 °C; detection wavelength, 220 nm; eluent, EtOH, $^{s}_{w}$pH 4.50, fixed with 20 mM ammonium formate

evaporate NP eluent components. Then, the removal of the alkyl sulfonic acid additive could be achieved through the use of conventional strong anion exchange (SAX) resins.

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Conflict of interest The authors declare no conflict of interest.

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