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Enantioseparation of a-substituted proline analogs with macrocyclic glycopeptide-based chiral stationary phases immobilized on superficially porous particles of silica applying liquid chromatography with ultraviolet and mass spectrometric detection

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ABSTRACT

In this study, the liquid chromatography-based direct enantioseparation of the stereoisomers of α-substituted proline analogs has been investigated utilizing chiral stationary phases with UV and/or mass spectrometric (MS) detection. Macrocyclic antibiotics, such as vancomycin, teicoplanin, modified teicoplanin, and teicoplanin aglycone, all covalently immobilized to 2.7 μm superficially porous silica particles have been applied as stationary phases. Mobile phases utilizing mixtures of methanol and acetonitrile with different additives (polar-ionic mode) were optimized during method development. Best separations were achieved with mobile phases of 100% MeOH containing either 20 mM acetic acid or 20 mM triethylammonium acetate. Special attention was given to the applicability of MS-compatible mobile phases. Acetic acid was found to be advantageous as a mobile phase additive for MS detection.

Enantioselective chromatographic behaviors are interpreted based on the explored correlations between the analytes' structural features and those of the applied chiral stationary phases. For the thermodynamic characterization, separations were studied in the temperature range of 5–50 °C. Generally, retention and selectivity decreased with increasing temperature, and in most cases, enthalpy-driven enantiorecognition was observed, but entropic contributions also were present. Unexpectedly, unusual shapes for the van Deemter curves were registered in the kinetic evaluations. General trends could be observed in the enantiomeric elution orders: $S < R$ on VancoShell and NicoShell, and opposite $R < S$ on TeicoShell and TagShell columns.

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1. Introduction

One of the most abundant biological macromolecules in all living cells is proteins, an important class of natural compounds with diverse biological functions. Many possess pharmacological activity, *e.g.*, hormones, enzyme inhibitors, receptor ligands, antibiotics, neurotransmitters, *etc*. Lower molecular weight peptides, *e.g.*, peptidomimetics and peptide-based therapeutics, are compounds of high importance in drug discovery research [1]. However, peptide therapies can be problematic when limited by poor absorption, proteolytic instability, undesirable side effects, and rapid elimination from the body. Generally, the all L-stereochemistry of the peptide's constituent amino acids can accentuate such problems [1,2]. Chemical modifications of peptides, *e.g.*, the substitution of an L-amino acid by the corresponding D-amino acid during peptide synthesis or incorporation of sterically constrained amino acids, are convenient options to enhance metabolic stability and modify pharmacological activity [3–5]. Among protein amino acids, proline is an attractive target moiety. It contains an α-imino group in a five–membered ring. When incorporated into peptides or peptidomimetics, the unique ring structure may develop special properties, such as conformational rigidity and enhanced chemical stability. Moreover, proline within a peptide chain may lead to the appearance of *cis/trans* isomerization [6,7] and induces strong type-II β-turns [8]. The biological importance of proline, especially the role of D-proline and its metabolism in living organisms, has recently been reviewed in several scientific papers and book chapters [9–13].

Since, in all living systems, the biological activity of chiral compounds strongly depends on their steric arrangement, identifying

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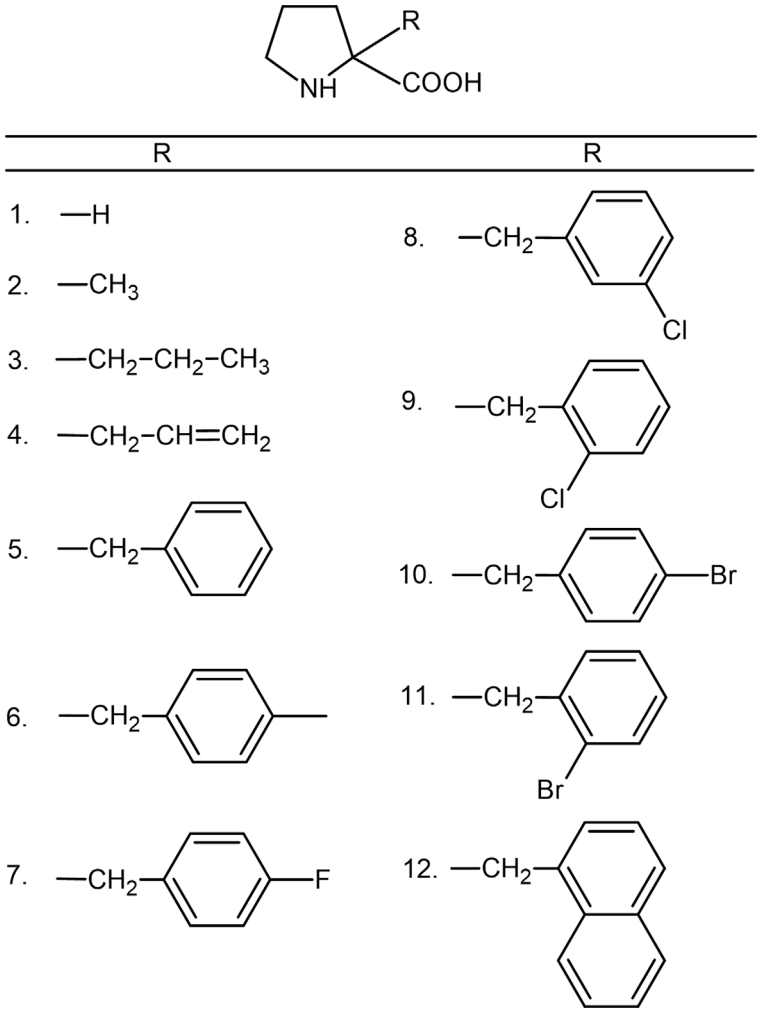


Fig. 1. Structure of α-substituted proline analogs.

the absolute configuration of the chiral building blocks is required. Many scientific papers deal with separating amino acid stereoisomers and determining their location and configuration in the peptide sequence. In most analytical procedures, the stereochemistry of proteinic amino acids, after peptide hydrolysis, is determined by applying chiral or non-chiral derivatization before chromatographic separation [14–20]. It is worth noting that chiral stationary phases (CSPs) applied with subsequent mass spectrometric (MS) detection (*e.g.*, HPLC-MS/MS) offer the advantage of enantiomeric separation and determination without any derivatization process thus, the pitfalls of the derivatization process (*e.g.,* incomplete derivatization, racemization, kinetic resolution, *etc.*) can be avoided [19]. However, developing MS-compatible mobile phases is still challenging in "chiral chromatography".

Despite the unique structural features of proline, relatively few papers have focused on the enantiomeric separation of its α-substituted analogs. Chiral analysis of hydroxyproline [20] and different α-substituted proline analogs [21,22] was performed on its *N*-derivatized form. The present paper focuses on the direct enantioseparation of the free form of highly constrained α-substituted proline analogs possessing either alkyl or aromatic side chains. The structures of the studied analytes are shown in Fig. 1.

Developing columns packed with highly efficient particles is one of the most challenging areas in "chiral chromatography". The advantageous features of CSPs based on superficially porous silica particles have already been proved [23–25]. In this study liquid chromatographic analyses were carried out with macrocyclic glycopeptide-based selectors such as vancomycin (VancoShell), teicoplanin (TeicoShell), teicoplanin aglycone (TagShell), and functionalized teicoplanin (NicoShell), all covalently bonded to 2.7 μm high-efficiency superficially porous particles (SPPs).

Experiments were performed in the polar-ionic mode (PIM) utilizing columns with 3.0 and 2.1 mm internal diameters (i.d.). Effects of the nature and concentration of the mobile phase components, acid, and salt additives under various conditions were studied. Due to the lack of a chromophore in α-substituted proline analogs possessing alkyl side chains, optimizing conditions for chiral separation with MS detection was necessary. Since the configurations of all samples are known, the elution sequences were determined in all cases.

2. Materials and methods

2.1. Chemicals and reagents

(*R*)- and (*S*)-proline were purchased from Sigma-Aldrich (Steinheim, Germany). Racemic and enantiopure α-substituted proline analogs were obtained from BioQuadrant Inc. (Montreal, Quebec, Canada). Methanol (MeOH), acetonitrile (MeCN), and water of LC-MS grade, triethylamine (TEA), formic acid (FA), glacial acetic acid (AcOH), triethylammonium acetate (TEAA), trifluoroacetic acid (TFA), ammonium acetate (NH_4OAc), and ammonium formate (NH_4HCOO) of analytical reagent grade were from VWR International (Radnor, PA, USA).

2.2. Apparatus

The applied Waters® ACQUITY UPLC® H–Class PLUS UHPLC System (Waters Incorporation, Milford, MA, USA) consisted of a quaternary solvent manager, sample manager FTN–H, column manager, photodiode array (PDA) detector, and MS (QDa) detector. The QDa detector parameters were set: positive ion mode, probe temperature, 600 °C, capillary voltage, 1.5 V, cone voltage, 20 V. The UHPLC system was controlled with Empower 3 software.

Chiral selectors employed in this study are attached covalently to 2.7 μm silica-based SPPs. The core diameter and shell thickness of the SPPs were 1.7 μm and 0.5 μm, respectively. All columns have 100 × 3.0 mm i.d. or 100 × 2.1 mm i.d. dimensions. (Abbreviations for dimensions are: 3.0 mm i.d. and 2.1 mm i.d., respectively). Selectors of the macrocyclic glycopeptide-based columns are vancomycin (VancoShell, V-3.0, and V-2.1), teicoplanin (TeicoShell, T-3.0, and T-2.1), teicoplanin aglycone (TagShell, TAG-3.0, and TAG-2.1) and modified teicoplanin (NicoShell, N-3.0). All columns were obtained from AZYP LLC (Arlington, TX, USA).

Analyte stock solutions (1.0 mg ml^{-1}) were prepared in MeOH and diluted with the mobile phase. The columns' hold-up time (t_0) was determined with 0.1% AcOH dissolved in MeOH and detected at 210 or 256 nm. The flow rate and the column temperature were set at 0.3 ml min^{-1} and 20 °C, respectively, if not otherwise stated.

3. Results and discussion

The investigated α-substituted proline analogs can be divided into two sub-groups: (i) those that contain an aliphatic moiety (**2**–**4**) with different chain lengths and (ii) those possessing an aromatic moiety (**5**–**12**) differing in nature and position of substituents on the aromatic ring. All these different structural features may affect the molecular characteristics (*e.g.*, size and polarity of the solute), thus influencing the selector–analyte interactions.

For the preliminary investigations, six analytes were selected: proline (**1**), analytes **2** and **4** from α-substituted proline analogs possessing aliphatic side chains, and analytes **5, 6**, and **10** from molecules possessing an aromatic moiety. Thus, all the critical structural features were represented, and the effects of different mobile phase additives, their concentration, the bulk solvent composition of the mobile phase, and the geometric size of the columns could be explored.

3.1. Effect of nature of mobile phase additives

Chiral separation of amino acids on macrocyclic glycopeptide-based CSPs can be performed in different chromatographic modes. The best performances are usually achieved in PIM with MeOH/MeCN as a bulk solvent in the presence of TEAA or in reversed-phase mode (RPM), applying MeOH or MeCN with aqueous TEAA [26–29]. In preliminary experiments, the teicoplanin-based **T-3.0** column exhibited only moderate enantiorecognition ability by the variation of mobile phase composition either in RPM or PIM (data not shown). Under the same conditions, the teicoplanin aglycone-based **TAG-3.0** CSP and the vancomycin-based **V-3.0** CSP provided better results. Mobile phase additives may significantly affect enantiorecognition, therefore, the nature of different additives was first studied on **V-3.0** and **TAG-3.0** CSPs applying a mobile phase composition of MeOH/MeCN 80/20 (*v/v*) containing 20 mM additives. As examples of the observed effects, chromatograms obtained with analyte **5** are presented in Figure S1 in the Supplementary Materials. Based on these results, taking the peak shape, retention, resolution, and detection mode into account, applying TFA was less advantageous. The effects of FA, TEAA, NH_4HCOO, and NH_4OAc were found to strongly depend on the bulk solvent composition of the mobile phase, the nature of the analyte, and the selector. From a chromatographic point of view, TEAA is a goodchoice, however, due to its ion suppression effect in the case of MS detection, applying AcOH as an additive is more advantageous. Based on these results, TEAA (with UV detection) or AcOH (with MS detection) was applied as a mobile phase additive in all further experiments.

3.2. Mobile phase selection

To study the influence of MeOH/MeCN ratio on the PIM enantioseparation, mobile phases composed of 100/0 – 20/80 (*v/v*) MeOH/MeCN containing 20 mM AcOH were employed, and the chromatographic properties of the preselected analytes (**1, 2, 4, 5, 6**, and **10**) were recorded on **V-3.0, TAG-3.0**, and **N-3.0** CSPs. Effective separations could not be observed on the **N-3.0** column. Fig. 2 shows the results obtained with the **V-3.0** and **TAG-3.0** columns. On both columns, k_1 increases with increasing MeCN content; however, the increase in retention factor is most prominent at or above a 60/40 (*v/v*) MeOH/MeCN ratio. The change of mobile phase polarity can explain the retention behavior observed. The solvation of the polar amino acids decreases with increasing MeCN content (*i.e.*, with increasing apolarity), leading to higher retentions, while increasing MeOH content results in a more polar mobile phase and better solvation, accompanied by decreased retentions. Additionally, all amino acids are less soluble in MeCN.

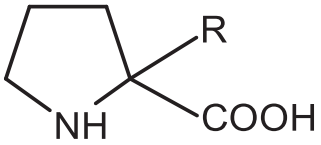
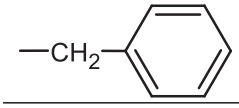
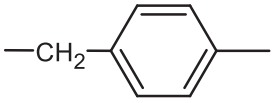
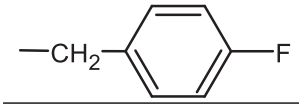
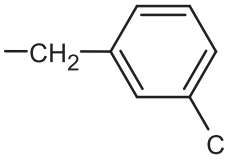
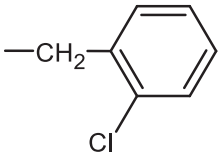
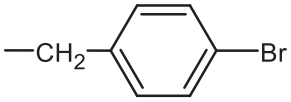
By examining the α and R_S values, it can be seen that they decrease (or are unchanged when there is no enantioseparation) with increasing MeCN content (Fig. 2). This indicates that the increased retentive interactions between the analyte and the selector with increasing MeCN content are not enantioselective. The highest α and R_S values were obtained at 100% MeOH concentration. Interestingly, comparing the two CSPs, k_1 values were higher on the **TAG-3.0** column, while higher α and R_S values were registered on the **V-3.0** column. It should be noted that under the applied conditions, enantiomers of **2** and **4** on the **V-3.0** CSP, plus enantiomers of **1** on the **TAG-3.0** CSP, could not be enantioresolved (Fig. 2).

For the purpose of comparison, the effect of the MeOH/MeCN ratio on chiral recognition of analytes **4, 5, 6**, and **10** was studied by applying 20 mM TEAA instead of 20 mM AcOH with PDA detection. (Enantioseparation of **1** and **2** due to the lack of chromophore moiety could not be monitored with PDA detection). Results, depicted in Figure S2, indicate that the general trends observed in the change of k_1, α, and R_S values as a function of MeOH/MeCN ratio are very similar to the ones observed in the presence of AcOH (Fig. 2), *i.e.*, k_1 increases, while α and R_S decrease with increasing MeCN content. Interestingly, retention was much less in the presence of TEAA than in the case of AcOH, while selectivity and resolution were comparable. Contrary to the results obtained with AcOH, the **N-3.0** CSP exhibited some separation ability for compounds **4, 5, 6**, and **10** in the presence of TEAA (Fig. S2). Comparing the results obtained with the two additives, it can be concluded that the applicability of a mobile phase additive is usually a compromise between detection capability and chromatographic separation properties.

3.3. Structure–retention (selectivity) relationships

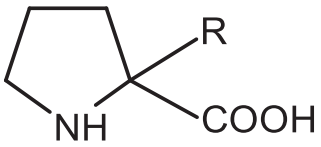
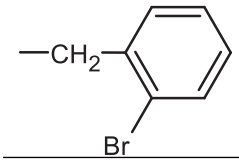
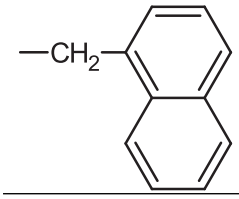
The sterically demanding structures of the macrocyclic glycopeptide-based CSPs and constrained α-substituted proline analogs (Fig. 1) influence the retention and the chiral recognition in several ways. Table 1 and Table S1 report the k_1, α, and R_S values observed on **V-3.0, T-3.0, TAG-3.0**, and **N-3.0** CSPs with the most effective two mobile phases applied, *i.e.,* 100% MeOH containing either 20 mM AcOH or 20 mM TEAA. Investigation of the structure of the chiral selector in both applied eluent systems

Table 1
Chromatographic data for the separation of α-substituted proline analogs on macrocyclic glycopeptide-based chiral stationary phases in the presence of AcOH as mobile phase additive.

Analyte (R)	Column	k_1	α	R_s	e.e.o.
					
1 —H	**V-3.0**	1.04	1.27	1.26	$R < S$
	T-3.0	7.56	1.00	0.00	–
	TAG-3.0	9.74	1.00	0.00	–
	N-3.0	0.64	1.00	0.00	–
2 —CH_3	**V-3.0**	0.57	1.00	0.00	–
	T-3.0	8.15	1.46	1.65	$S < R$
	TAG-3.0	9.19	1.41	2.52	$S < R$
	N-3.0	0.38	1.00	0.00	–
3 —CH_2–CH_2–CH_3	**V-3.0**	0.31	1.00	0.00	–
	T-3.0	7.64	1.00	0.00	–
	TAG-3.0	2.54	2.32	1.90	$S < R$
	N-3.0	0.25	1.00	0.00	–
4 —CH_2–CH=CH_2	**V-3.0**	0.37	1.00	0.00	–
	T-3.0	4.59	1.31	1.62	$R < S$
	TAG-3.0	4.56	1.07	0.21	$R < S$
	N-3.0	0.16	1.62	0.92	$S < R$
5 	**V-3.0**	0.51	3.03	3.62	$S < R$
	T-3.0	1.93	1.08	< 0.10	$R < S$
	TAG-3.0	2.24	1.53	1.90	$R < S$
	N-3.0	0.26	1.71	1.67	$S < R$
6 	**V-3.0**	0.40	2.96	3.68	$S < R$
	T-3.0	1.88	1.13	0.71	$R < S$
	TAG-3.0	2.31	1.67	2.16	$R < S$
	N-3.0	0.15	1.98	1.96	$S < R$
7 	**V-3.0**	0.52	1.52	1.49	$S < R$
	T-3.0	1.74	1.10	0.15	$R < S$
	TAG-3.0	2.04	1.34	1.22	$R < S$
	N-3.0	0.29	1.50	1.32	$S < R$
8 	**V-3.0**	0.55	2.34	3.36	$S < R$
	T-3.0	1.49	1.13	0.18	$R < S$
	TAG-3.0	2.10	1.18	0.60	$R < S$
	N-3.0	0.30	1.59	1.80	$S < R$
9 	**V-3.0**	0.48	1.52	0.96	$S < R$
	T-3.0	1.70	1.00	0.00	–
	TAG-3.0	3.49	1.17	0.55	$R < S$
	N-3.0	0.28	1.43	0.89	$S < R$
10 	**V-3.0**	0.74	1.54	1.66	$S < R$
	T-3.0	1.69	1.14	0.40	$R < S$
	TAG-3.0	2.66	1.36	1.28	$R < S$
	N-3.0	0.37	1.44	1.37	$S < R$

(*continued on next page*)

Table 1 (*continued*)

Analyte (R)	Column	k_1	α	R_s	*e.e.o.*
					
11 	**V-3.0**	0.59	1.33	0.80	*S* < *R*
	T-3.0	2.68	1.00	0.00	–
	TAG-3.0	4.22	1.24	0.85	*R* < *S*
	N-3.0	0.29	1.60	1.22	*S* < *R*
12 	**V-3.0**	0.77	2.31	2.87	*S* < *R*
	T-3.0	2.89	1.00	0.00	–
	TAG-3.0	5.18	1.42	1.39	*R* < *S*
	N-3.0	0.38	1.56	1.62	*S* < *R*

Chromatographic conditions: columns, VancoShell (**V-3.0**), TeicoShell (**T-3.0**), TagShell (**TAG-3.0**) and NicoShell (**N-3.0**); mobile phase, 100% MeOH containing 20 mM AcOH; flow rate, 0.3 ml min^{-1}; detection, MS; ***e.e.o.***, enantiomeric elution order.

showed that on **V-3.0** and **N-3.0** CSPs, the interactions between selector and selectand are particularly weak, resulting in very low retention factors (Tables 1 and S1). Despite the short retention times, the differences in interactions between the two enantiomers with the chiral selector are significant enough to result in baseline separation in several cases, especially for analytes possessing aromatic side chains. The TEAA-containing mobile phase system proved less effective for the enantioseparation of α-substituted proline analogs, especially in the case of **N-3.0** CSP (Table 1 *vs.* Table S1).

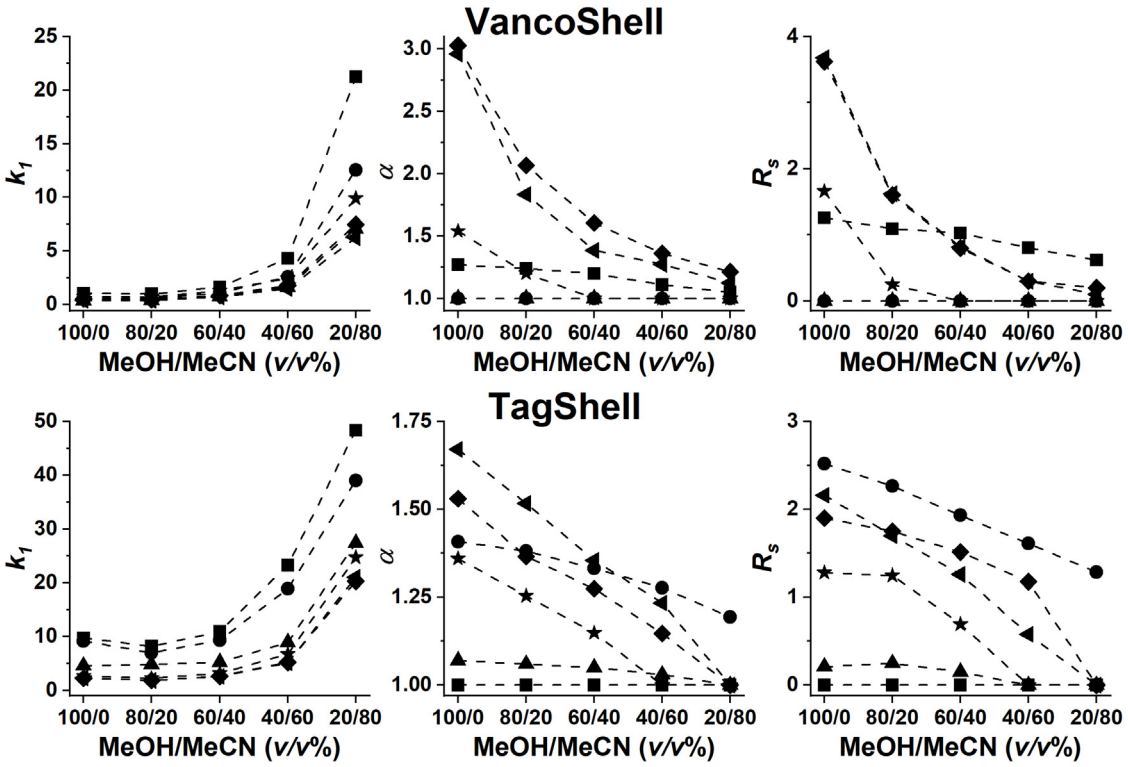


Fig. 2. Effect of MeCN content on the chromatographic parameters of analytes **1, 2, 4, 5, 6**, and **10**. Chromatographic conditions: columns, VancoShell (**V-3.0**) and TagShell (**TAG-3.0**); mobile phase, MeOH/MeCN 100/0, 80/20, 60/40, 40/60 and 20/80 (*v/v*) all containing 20.0 mM AcOH; flow rate, 0.3 ml min^{-1}; detection, MS; symbols, for analyte **1** ■, for **2** ●, for **4** ▲, for **5** ◆, for **6** ◀, and for **10** ★.

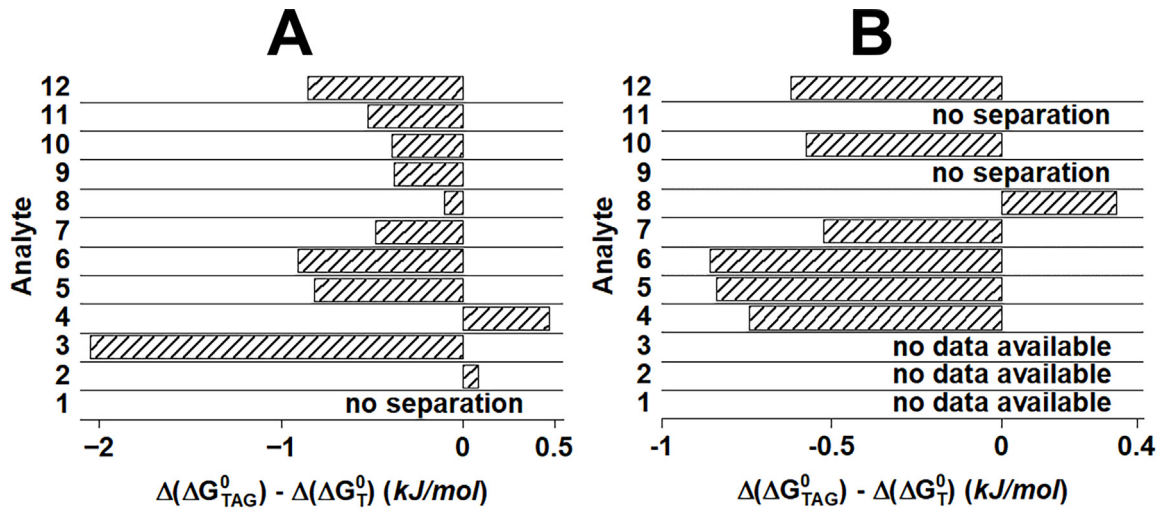


Fig. 3. Enantioselective energy differences between the aglycone and the native teicoplanin CSPs. Chromatographic conditions: column, TeicoShell (**T-3.0**) and TagShell (**TAG-3.0**); mobile phase, **A**, 100% MeOH containing 20.0 mM AcOH, **B**, 100% MeOH containing 20.0 mM TEAA; flow rate, 0.3 ml min^{-1}; detection, PDA, 215 nm.

Interestingly, all analytes exhibit much higher retention in both mobile phase systems on **T-3.0** and **TAG-3.0** CSPs. However, these stronger interactions usually do not contribute to improved enantioselectivities. Higher α and R_S only in the case of analytes **2, 3**, and **4** were observed in the AcOH-containing eluent (Table 1). Comparing retention behaviors of the studied analytes on the teicoplanin-based CSPs reveals that stronger interactions are formed with **TAG-3.0** than with **T-3.0**. Generally, the improved interactions resulted in higher α and R_S values. These two CSPs differ in the presence (or absence) of three saccharide units which may intervene in the chiral recognition process in at least three distinct ways [30]. They may (i) sterically hinder the access of the molecules to binding sites on the aglycone basket; (ii) block the two phenol hydroxyl groups on the aglycone where two sugar units are attached; (iii) and block the alcohol moiety on the aglycon where the third sugar unit is linked. To quantify the effect of the three saccharide moieties in chiral recognition, the differences in enantioselective free energies obtained on **TAG-3.0** and **T-3.0** $[\Delta(\Delta G°)_{TAG} - \Delta(\Delta G°)_T]$ were calculated. A negative number means that the separation of enantiomers of a specific analyte is more effective on **TAG-3.0**, while a positive number indicates better enantiorecognition on **T-3.0**. Data in Fig. 3 show that enantioseparations, in most cases, were more effective on **TAG-3.0**, indicating that the sugar units hinder the stereoselective interactions between the selector and the selactand. Exceptions were analytes **2** and **4** in AcOH-containing eluent and analyte **8** in TEAA-containing eluent, where slightly higher selectivities were registered on **T-3.0** CSP. Of the studied macrocyclic glycopeptide-based CSPs the **V-3.0** and **TAG-3.0** CSPs proved to be the most effective for the enantioseparation of α-substituted proline analogs. One possible reason may be that vancomycin structurally resembles teicoplanin aglycon. While teicoplanin contains three saccharide units, vancomycin possesses only one, and as discussed above, enantioseparation is more advantageous in the absence of sugar moieties for the studied proline analogs.

The nature of the proline substitute (Fig. 1) also has a profound effect on chiral recognition and must be explored. Based on the obtained results (Table 1), it can be seen that for the **V-3.0** CSP employing AcOH-containing mobile phases, retention is significantly reduced by a substituent in the α position. Only a slight difference can be seen in the chromatographic behaviors comparing the aliphatic and aromatic substituents; the presence of the aromatic moiety may induce π-π interactions in the basket of the selector, ideally leading to higher selectivity and retention.

On both **T-3.0** and **TAG-3.0** CSPs, compounds with aliphatic substituents (**2**–**4**) were more strongly retained than compounds with aromatic substituents (**5**–**12**). In other words, substituting the H atom with an aromatic ring substantially reduced the retention. Despite the strong retention, no enantioselectivity was observed in the case of proline. Interestingly, the methyl substitution had no substantial effect on the retention but resulted in a very efficient chiral recognition (compound **2** *vs.* **1**). To explain the observed changes in enantioselectivities, a strong steric effect is assumed, and the importance of molecular geometry (*i.e.*, the shape and size of the molecule) is likely in the case of the teicoplanin-based selectors, even in the absence of saccharide substituents.

The effect of the nature and position of the substituent on the aromatic ring (**5** *vs.* **6, 7**, and **10; 8** *vs.* **9** and **10** *vs.* **11**) can be explored in both eluent systems on the two most effective CSPs **V-3.0** and **TAG-3.0**. The electron-donating methyl substitution on the benzyl ring (**5** *vs.* **6**) does not markedly change the chromatographic properties. In contrast, substitution with an electron-withdrawing atom (**5** *vs.* **7** and **10**) resulted in more significant variations of the chromatographic values, however, these variations depend on the applied conditions (*i.e.*, on the mobile phase and the CSP).

The position of the halogen atom in **8** *vs*. **9**, and **10** *vs.* **11** was found to affect chiral recognition markedly. Both the chlorine (**8** and **9**) and the bromine (**10** and **11**) placed in *ortho* position led to significantly longer retentions but reduced, or in most cases eliminated enantiorecognition on both **V-3.0** and **TAG-3.0** CSPs in both eluent systems. The halogen substitution of the aromatic ring in an *ortho* position may sterically hinder effective chiral recognition in the case of the studied α-substituted proline analogs.

All the studied analytes are available in racemic and enantiomerically pure forms, thus the enantiomeric elution order was determined in all cases. A general trend could be observed: on **V-3.0** and **N-3.0** CSPs where the elution order was $S < R$. However, on the **T-3.0** and **TAG-3.0** CSPs, it was opposite: $R < S$ (Table 1 and Table S1 and Fig. 4).

Selected chromatograms for the enantioseparation of the α-substituted proline analogs are depicted in Fig. 4.

3.4. Thermodynamic characterization

Optimizing column temperature to achieve more efficient enantioseparations is worthwhile [31–33]. In addition to gaining shorter analysis time and/or higher resolution, temperature effects on

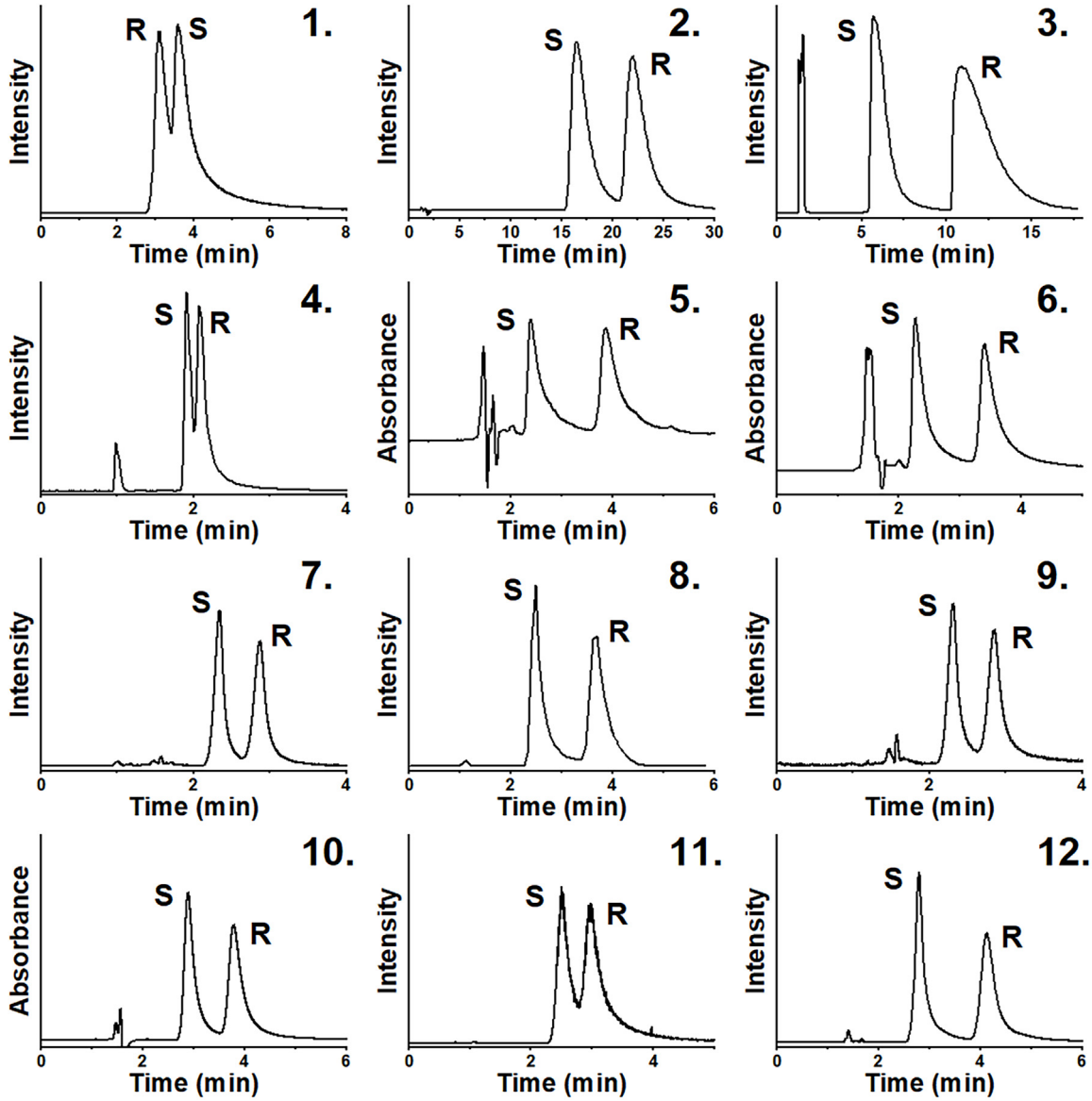


Fig. 4. Selected chromatograms of analytes **1**–**12.** Chromatographic conditions: columns, for analytes **1, 5**–**10** and **12** VancoShell (**V-3.0**), for **2** and **3** TagShell (**TAG-3.0**), and for **4** and **11** NicoShell (**N-3.0**); mobile phase, for analytes **1**–**4, 7**–**9, 11**, and **12** 100% MeOH containing 20.0 mM AcOH, for analytes **5, 6**, and **10** 100% MeOH containing 20.0 mM TEAA; flow rate, 0.3 ml min^{-1}; temperature, for analytes **1**–**6, 8** and **12**, 20 °C, for **7** and **9**–**11**, 5.0 °C.

chromatographic parameters may provide information about the mechanism of chiral recognition. Enantioselectivity is related to the difference in free energy changes of adsorption of the enantiomers [$\Delta(\Delta G°)$], and the van't Hoff equation (plotting R ln α *vs. 1/T)* is frequently applied to determine thermodynamic parameters,

$$ln\ \alpha = -\frac{\Delta(\Delta H°)}{RT} + \frac{\Delta(\Delta S°)}{R} \quad (1)$$

where α is the selectivity factor, $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ are the differences in standard enthalpy and standard entropy, R is the universal gas constant, and T is the temperature in degrees Kelvin. Applying Eq. (1), the problems related to the determination of the phase ratio can be excluded, however, contributions of enantioselective and non-selective sites to the free energy cannot be distinguished [34].

The effects of temperature on the chromatographic parameters for all the analytes were studied on **V-3.0** and **TAG-3.0** CSPs over the temperature range of 5–50 °C. Experimental data on both columns with a mobile phase of 100% MeOH containing 20 mM AcOH are presented in Table S2.

In all cases, the k values on **V-3.0** and **TAG-3.0** CSPs decreased with increasing temperature. As expected, α and R_S decreased with increasing temperature, except for **4** on the **TAG-3.0** column, where α slightly increased with increasing temperature in the temperature range of 10–50 °C. For analytes possessing an aliphatic side chain (**2**–**4**), some improvement in R_S could be registered with increasing temperature on **TAG-3.0** CSP, probably due to the favorable kinetic effects observed at higher temperatures.

The differences in the changes in standard enthalpy and entropy [-$\Delta(\Delta H°)$ and -$\Delta(\Delta S°)$] calculated from the ln α *vs. 1/T* curves are presented in Table 2. The $\Delta(\Delta H°)$ values on **V-3.0** CSP ranged from −10.80 to −2.43 kJ mol^{-1}, and on **TAG-3.0** CSP from −6.45 to +1.15 kJ mol^{-1}. Comparing the $\Delta(\Delta G°)$ values determined for the two CSPs, more efficient bindings are found on the **V-3.0** CSP, as reflected by the more negative $\Delta(\Delta G°)$ values. The trends in the change in $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ were similar. The relative contribution to the free energy of adsorption can be characterized by the calculation of the enthalpy/entropy ratio, $Q = \Delta(\Delta H°)/[298 \times \Delta(\Delta S°)]$. As indicated in Table 2, the enantios-

Table 2
Thermodynamic parameters, -Δ(Δ*H*°), -Δ(Δ*S*°), -Δ(Δ*G*°), correlation coefficient (R^2), $T_{iso}$and *Q* values of analytes **1**–**12** on **VancoShell (V-3.0)** CSP.

Compound	**-Δ(Δ*H*°)** (kJ mol^{-1})	**-Δ(Δ*S*°)** (J mol^{-1} K^{-1})	**-Δ(Δ*G*°)**298 K (kJ mol^{-1})	***Corr. coeff.*** ***R²***	***T*$_{iso}$** (°C)	***Q***
			V-3.0			
1	2.43	6.29	0.56	0.992	112.5	1.29
2			**no separation**			
3			**no separation**			
4			**no separation**			
5	9.89	24.71	2.52	0.995	127.2	1.34
6	10.80	27.91	2.48	0.992	113.8	1.30
7	4.21	10.97	0.94	0.989	110.4	1.29
8	8.60	22.31	1.95	0.993	112.3	1.29
9	6.14	17.65	0.88	0.970	74.8	1.17
10	5.19	14.22	0.95	0.992	92.1	1.23
11	5.36	15.86	0.63	0.985	64.6	1.13
12	8.92	23.64	1.87	0.991	104.2	1.27
Compound	**-Δ(Δ*H*°)** (kJ mol^{-1})	**-Δ(Δ*S*°)** (J mol^{-1} K^{-1})	**-Δ(Δ*G*°)**298 K (kJ mol^{-1})	***Corr. coeff.*** ***R²***	***T*$_{iso}$** (°C)	***Q***
			TAG-3.0			
1			**no separation**			
2	2.15	4.69	0.75	0.993	184.2	1.53
3	4.00	6.70	2.00	0.996	323.4	2.00
4	−1.15	−4.46	0.16	0.994	−15.4	0.86
5	5.65	15.74	0.96	0.998	86.0	1.21
6	6.45	17.68	1.18	0.999	91.5	1.22
7	3.80	10.50	0.67	0.998	89.1	1.22
8	4.32	13.35	0.34	0.999	50.2	1.09
9	2.93	8.81	0.31	0.999	60.1	1.12
10	4.05	11.28	0.68	0.999	85.4	1.20
11	3.06	8.76	0.45	0.993	76.1	1.17
12	4.35	12.03	0.77	0.991	86.7	1.21

Chromatographic conditions: columns, VancoShell (V-3.0) and TagShell (TAG-3.0); mobile phase, MeOH containing 20 mM AcOH; detection, MS-QDa; R^2, correlation coefficient of van't Hoff plots, ln *α vs. 1/T* curves; $T_{iso,}$ temperature, where the enantioselectivity cancels; $Q = \Delta(\Delta H°)/298 \text{ x } \Delta(\Delta S°)$; temperature range, 5–50 °C.

elective discriminations are enthalpically-driven ($Q>1$), except for analyte **4** on **TAG-3.0** CSP, which is probably related to the unique structural features of the allyl-group in the α-position of proline. (Analytes **2**–**4** on **V-3.0**, while **1** on **TAG-3.0** was not separable under the conditions applied in the thermodynamic study.)

Based on the $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values, correlations between the structure of the analyte and the thermodynamic parameters can be explored. Compared to analyte **5**, analyte **6**, containing an electron-donating methyl group, possesses more negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values. In contrast, analytes **7** and **10** containing electron-withdrawing atoms (F or Br atom at the same position) possess less negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values. This is also true for analytes **8, 9**, and **11** possessing Cl or Br substituents on the aromatic ring. The electron density of the aromatic moiety seems to be correlated to the energetics of sorption. The comparison of the thermodynamic parameters of **7** *vs.* **10** further supports this assumption; in the case of analyte **7**, the presence of the F atom results in lower electron density on the aromatic moiety compared to analyte **10**, resulting in less negative $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ values.

Comparing the thermodynamic parameters of **8** *vs.* **9** and **10** *vs.* **11** may shed light on the possible effects of the halogen atom's position on the aromatic ring. The less negative $\Delta(\Delta G°)$ values determined for the analytes possessing the halogen atom in the *ortho* position clearly show a less favorable binding to the selector.

The thermodynamic analysis can also be applied to calculate the "isoeluotropic" temperature (T_{iso}), at which the entropy and enthalpy changes compensate each other, and the enantiomers coelute [34,35]. Applying temperatures higher than T_{iso}, the enantiomeric elution order should be reversed. As shown in Table 2, $T_{iso}$was out of the applied temperature range for most of the studied analytes, while for analyte **5**, it was around 50 °C. However, due to the column's limitations, higher temperatures could not be applied to prove the reversal in the enantiomeric elution order.

3.5. Kinetic studies

The kinetics of the selector–selectand interactions are commonly studied by preparing plate height (*H*) *vs.* linear velocity (*u*) curves (van Deemter plots) [25,36–39]. To gather data for the characterization of column efficiencies, experiments were performed with analytes **1, 2, 4, 5, 6**, and **10** applying a mobile phase composition of MeOH/MeCN 80/20 (*v/v*) containing 20 mM AcOH on VancoShell and TagShell columns, possessing 3.0 and 2.1 mm internal diameters. (As mentioned earlier, best performances were obtained with pure MeOH. However, to avoid high back-pressures at higher flow rates, the mixture of MeOH and MeCN was applied in the kinetic study, resulting in a significantly lower viscosity eluent.) The flow rate was varied between 0.1–1.0 ml min^{-1} (0.24–2.36 mm sec^{-1}) on the column with a 3.0 mm i.d. and 0.05–0.5 ml min^{-1} (0.24–2.41 mm sec^{-1}) on the column with a 2.1 mm i.d. (data are depicted in Fig. 5).

Interestingly, under the applied conditions, the shape of van Deemter curves for the first eluting enantiomer is unusual; no *H* minima *vs.* linear velocity can be identified for the analytes studied. Consequently, the curves do not fit the classical van Deemter equation ($H = A + B/u + Cu$), as reported in some cases [25,39]. At higher flow rates in a few cases (*e.g.*, analytes **1** and **2** on VancoShell columns), lower *H* values are obtained, caused probably by improved stationary phase mass transfer processes triggered by frictional heating. It is important to highlight that the *H*–*u* plots

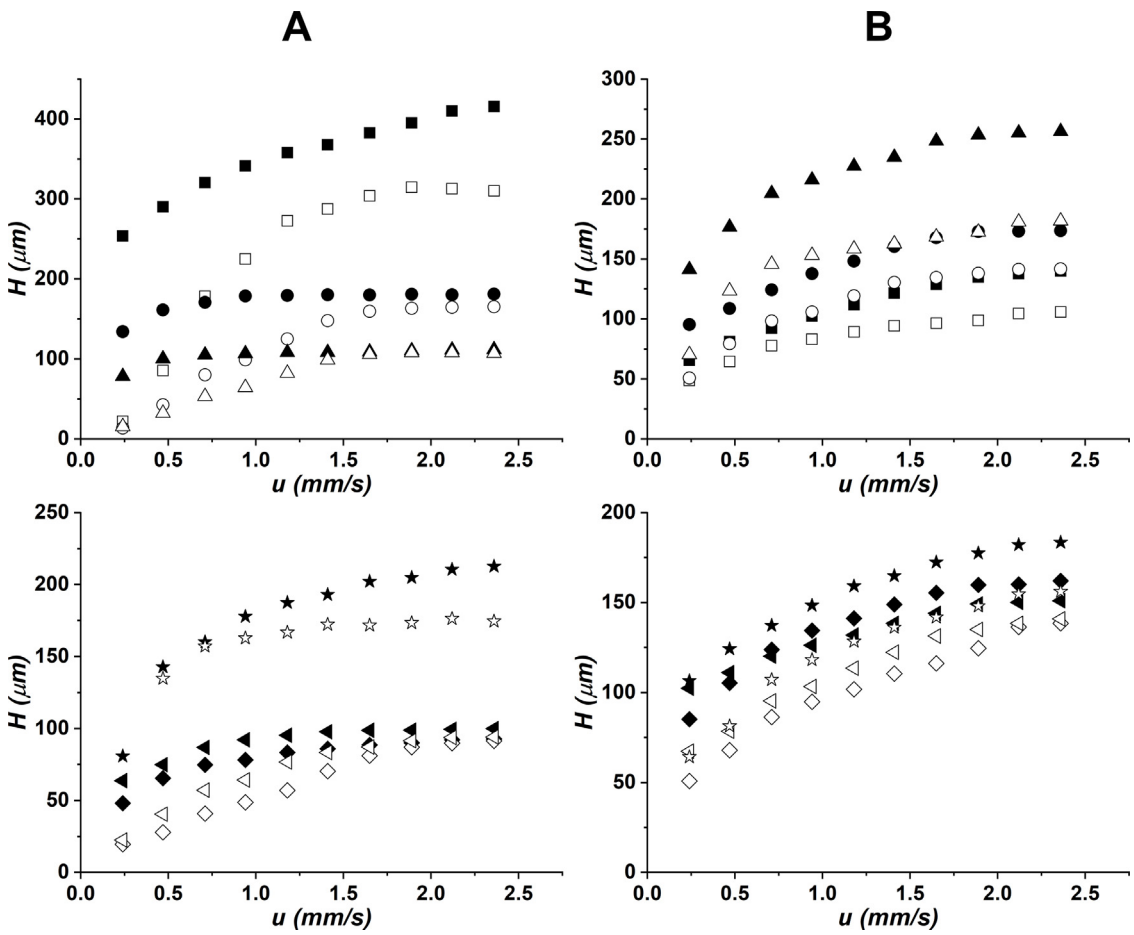


Fig. 5. van Deemter plots for analytes **1, 2, 4, 5, 6**, and **10** on macrocyclic glycopeptide-based CSPs. Chromatographic conditions: columns, **A**, VancoShell (**V-3.0;** full symbols) and VancoShell (**V-2.1;** empty symbols); **B,** TagShell (**TAG-3.0,** full symbols) and TagShell (**TAG-2.1**; empty symbols)**;** mobile phase, MeOH/MeCN (80/20 *v/v*) containing 20 mM AcOH; flow rate, 0.05 – 1.0 ml min^{-1}; detection, MS; temperature, 20 °C; symbols for analyte **1**, ■ and □; **2**, ● and ○; **4**, ▲; and △; **5**, ◆ and ◇; **6**, ◀ and ◁; **10**, ★ and ☆.

measured on 2.1 mm i.d. columns run below those obtained with the same column type with a larger 3.0 mm i.d. for all the studied analytes. This founding partly contradicts our previous results. Applying the same core-shell particle-based columns earlier, the narrow bore columns (2.1 mm i.d.) in most cases possessed decreased efficiency compared to their counterparts with 3.0 mm i.d. [40,41]. However, the narrow bore columns offered more efficient kinetics in some cases (*e.g.*, for $ß^2$-amino acids with aromatic side chains and certain fluorinated phenylalanine analogs) [40,41]. All these findings draw attention to an important fact; the kinetic performance of a column depends not only on the geometric size and the mobile phase composition, as described often in the literature, but also on the nature of analytes. It should be noted here that data were handled without corrections for the extra-column volume of the instrument. Physically modifying the UHPLC (*i.e.*, reducing the extra-column effects) would probably result in lower plate heights.

4. Conclusions

In this study, macrocyclic glycopeptide-based chiral stationary phases immobilized on superficially porous particles of silica were successfully applied for the direct enantiomeric separation of α-substituted proline analogs. The most effective CSPs were the vancomycin- and the teicoplanin aglycone-based ones, used in the polar-ionic mode. Effects of mobile phase additives (acids and salts) were found to strongly depend on the applied conditions, the nature of the analyte, and the chiral selector. Depending on the applied detection type (MS or UV), best performances were obtained using 100% MeOH with 20 mM acetic acid or 20 mM triethylammonium acetate.

Details of the chiral recognition mechanism could be explored by comparing the chromatographic and the thermodynamic parameters of the structurally strongly related α-substituted proline analogs. Regarding the structure of the teicoplanin-based selectors, the sugar units were found to hinder the enantioselective interactions in most cases. Regarding the structure of the analytes, the electron density of the aromatic moiety was found to be correlated to the energetics of sorption. The position of the halogen substituent affects chiral recognition; the *ortho* position resulted in a less favorable binding to the chiral selector.

Interestingly, the kinetic study revealed a different shape for the van Deemter plots than usual, *i.e.*, no *H* minima were recorded for the studied analytes. Further, at higher flow rates, the efficiencies began to improve somewhat, most likely due to frictional heating improving stationary phase mass transfer. Contrary to the results usually obtained, a more efficient kinetic performance of columns with 2.1 mm i.d. was found.

Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Dániel Tanács: Investigation, Writing – original draft, Visualization. **Róbert Berkecz:** Conceptualization, Writing – original draft, Writing – review & editing. **Daniel W. Armstrong:** Conceptualization, Writing – original draft, Writing – review & editing. **Antal Péter:** Conceptualization, Writing – original draft, Writing – review & editing. **István Ilisz:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

Figure S1. Effect of mobile phase additives on chromatographic behavior of analyte **5**. Chromatographic conditions: columns, VancoShell (**V-3.0**) and TagShell (**TAG-3.0**); mobile phase, MeOH/MeCN 80/20 (*v*/*v*) containing 20 mM additives; additives, FA, TFA, AcOH, TEAA, NH_4HCOO, NH_4OAc; flow rate, 0.3 ml min^{-1}; detection, PDA, 215 nm

Figure S2. Effect of MeCN content on chromatographic parameters of analytes **4, 5, 6**, and **10**. Chromatographic conditions: columns, VancoShell (**V-3.0**), TagShell (**TAG-3.0**) and NicoShell (**N-3.0**); mobile phase, MeOH/MeCN 100/0, 75/25, 50/50 and 25/75 (*v*/*v*) all containing 20.0 mM TEAA; flow rate, 0.3 ml min^{-1}; detection, PDA, 215 nm; symbols for analyte **4,** ▲; **5,** ♦; **6,** ◄ and **10,** ⋆

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.463997.

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