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Study of mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-based chiral stationary phase

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Abstract

The behavior of a series of amino acids and some of their methyl ester hydrochloride, *N*-acetyl and *N*-*tert*-butyloxycarbonyl derivatives has been investigated on a teicoplanin-based chiral stationary phase by changing the chromatographic conditions, namely, the type and amount of mobile phase organic modifier and the ionic strength of the solutions. By using species with significantly different characteristics and chemical reactivity, some general conclusions regarding the chiral recognition process on this kind of stationary phase have been formulated. The importance of the carboxylic moiety for the formation of the complex between enantiomers and the aglycone basket of teicoplanin has been demonstrated via chromatography. Additionally, the increased possibility to make an hydrogen bond between the amidic hydrogen of the acetylated compounds and an amidic group on the stationary phase has been proposed to be pivotal for the stability of the complex aglycone D-enantiomer. Phenomena leading to the exclusion from the chiral stationary phase of one or both enantiomers have been rationalized by considering the ionic interactions between stationary phase, molecules to be separated and the surrounding medium and/or steric hindrance effects. The understanding of some of the observed phenomena may be important for optimizing the performance of the separation on aglycone-based media.

Keywords: Chiral stationary phases, LC; Donnan effect; Teicoplanin; Amino acids

1. Introduction

Macrocyclic antibiotics (teicoplanin, vancomycin, ristotecin A, etc.) are glycopeptide structures extensively used in recent years as chiral selectors for liquid chromatography [1–10]. They have many of the characteristics of protein-based stationary phases but can be used under very different mobile phase (MP) conditions (normal-phase, reversed-phase and organic mode). Macrocyclic antibiotics have shown an excellent ability to separate different classes of racemic compounds (such as underivatized amino acid, imino acid, acidic and basic drugs, etc.) and appear particularly suitable for preparative chromatography because of their high capacities.

All the known glycopeptide structures are made of a backbone constituted by seven amino acids. The aglycone of teicoplanin is a semi-rigid basket that includes seven aromatic rings (two with chloro substituents and four with phenolic moiety, see Fig. 1). It contains a single primary amine ($pK_a \simeq 9.2$) and a single carboxylic group ($pK_a \simeq 2.5$). It has three sugar moieties that introduce additional polar groups; one of these bears a C_{11} acyl side-chain, which has a marked apolar character and is responsible for several specific pharmacological properties of the teicoplanin antibiotic [11,12]. There are more than 20 chiral centers surrounding four cavities.

The structure of teicoplanin is characterized by a large variety of possible interaction sites. This causes the reach of the diastereomeric equilibrium between enantiomeric compounds and teicoplanin chiral stationary phase (CSP) to be possibly controlled or influenced by several distinct phenomena, such as Van der Waals interactions, ionic forces, hydrogen bonding, dipole stacking, π–π aromatic stacking,

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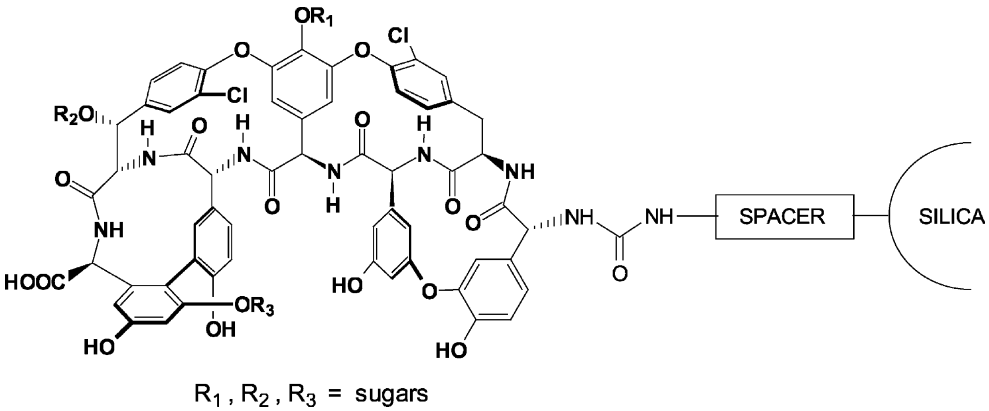

Fig. 1. Chiral stationary phase structure.

enthalpic–entropic compensation effect, chelate effect, hydrophobic and steric effects, etc. [2,4,5,7,13–16]. These effects can often be simultaneous, involving secondary interactions [4,5,11–13,17,18] or originate multi-step kinetics [8]. The surrounding medium (in particular, its pH and the type and amount of organic modifier) also has a basic role in determining the structure of the phase.

In 1969, before the structure of antibiotics was elucidated, Perkins proposed that Vancomycin binded to bacterial cell wall mucopeptide precursors that terminated in the sequence L-lysyl-D-alanyl-D-alanine [19]. A few years later, teicoplanin was also shown to be able to recognize D-Ala-D-Ala-terminating peptides [18]. Significant contributions to the present understanding of the molecular mechanisms leading to the recognition process between macrocyclic antibiotics and peptides came from Williams and his colleagues. They studied the interactions between small peptides and macrocyclic antibiotics in solution, specifically by means of NMR techniques [11,12,16–18,20,21]. The dominant role played by hydrogen bonding in cooperation with weak hydrophobic interactions as the driving force in the molecular recognition process was pointed out. It was shown that ligands such as *N*-acetyl-D-alanine make a 1:1 complex with the aglycone-basket of the macrocyclic antibiotics [22]. Armstrong et al., was the first to understand the importance of macrocyclic antibiotics as CSP [1,4,7,23]. In cooperation with the Gasparrini group [2], the role of the carbohydrate moieties on teicoplanin-based CSP was also evaluated. Recently, ion-exclusion effects leading to the elution of Dansyl amino acids at unusually small retention volumes were observed on CSPs based on teicoplanin aglycone [15].

In this work, the behavior of a series of amino acids and some of their methyl ester hydrochloride (MEH), *N*-acetyl (*N*-Ac) and *N*-*tert*-butyloxycarbonyl (*t*-BOC) derivatives on a teicoplanin-based CSP (TE-SP-100) has been investigated. The purpose is to give a further contribution to the elucidation of some factors that may affect the chiral separation process on this kind of CSP. A set of chiral compounds (amino acids and their derivatives) with a variety of chemical functionalities, and which is characterized by different properties in terms of acid–base character, steric hindrance, polarity, solubility, hydrogen bonding ability, etc., was chosen to exploit possible specific interactions arising between CSP and the *probe*-compound itself. Additionally, the role of the surrounding medium (namely, MP composition and MP ionic strength) and its effect on the separation of the different classes of compounds has been considered. The better understanding and the control of some of the observed effects may be useful for the design of new CSPs on which the separation performance might possibly be improved. To use an expression from Horváth et al. [24], *HPLC goes beyond the usual analytical or preparative applications and falls in the domain of molecular chromatography.*

2. Experimental

2.1. Chemicals

The α-amino acids glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), *tert*-leucine (*tert*-Leu), proline (Pro), phenylglycine (Φ-Gly), phenylalanine (Phe) were purchased from Sigma (St. Louis, MO, USA), Aldrich (Steinheim, Germany), Serva (New York, NY, USA) and Fluka (Buchs, Switzerland). Their structures are reported in Fig. 2.

N-Acetyl amino acids (*N*-Ac-Gly, *N*-Ac-Ala, *N*-Ac-Val, *N*-Ac-Leu, *N*-Ac-Pro, *N*-Ac-Φ-Gly, *N*-Ac-Phe, see Fig. 3) and amino acids methyl ester hydrochlorides (Ala-MEH, Val-MEH, Leu-MEH, see Fig. 4) were synthesized in the laboratory.

2.1.1. N-Ac-amino acids synthesis

A solution of the corresponding α-amino acid (5.0 mmol in 15.0 ml of aq Na_2CO_3 10%), was dropwise added to a cold (ice-bath) solution of acetyl chloride (6.0 mmol in 15.0 ml of tetrahydrofuran). The mixture was vigorously stirred at room temperature for 24 h, acidified to pH 2.0, and extracted with ethyl acetate. The organic extract was dried (Na_2SO_4) and

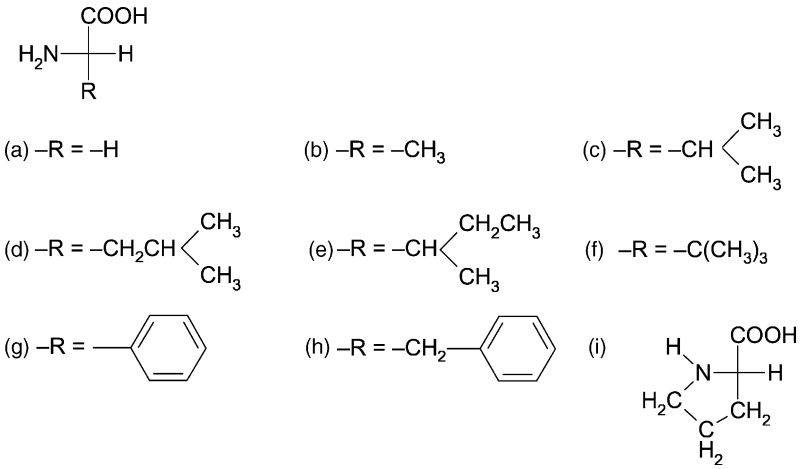

Fig. 2. α-Amino acids: (a) glycine; (b) alanine; (c) valine; (d) leucine; (e) isoleucine; (f) *tert*-leucine; (g) Φ-glycine; (h) Φ-phenylalanine; (i) proline (complete structure).

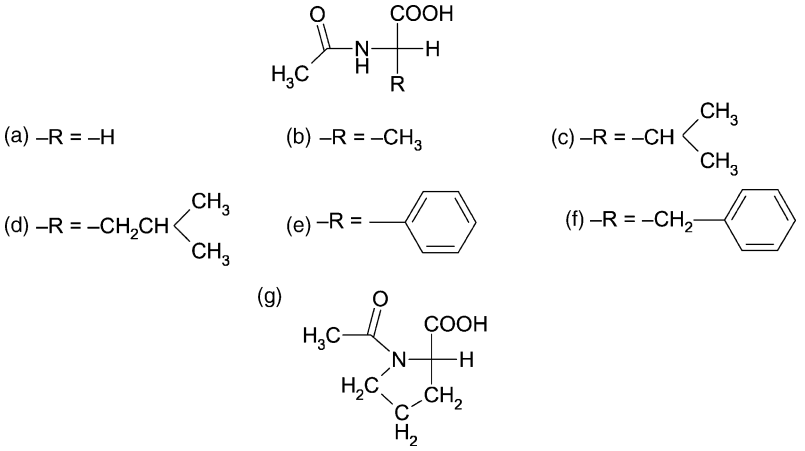

Fig. 3. *N*-Acetyl-amino acids: (a) *N*-Ac-glycine; (b) *N*-Ac-alanine; (c) *N*-Ac-valine; (d) *N*-Ac-leucine; (e) *N*-Ac-Φ-glycine; (f) *N*-Ac-phenylalanine; (f) *N*-Ac-proline (complete structure).

evaporated. *N*-Ac-Pro was prepared according to Ref.[25]. Acetic anhydride (20.0 mmol) was added to a suspension of Pro (10.0 mmol in 20.0 ml of acetonitrile). After 2 h at room temperature, the mixture was diluted with water and extracted with dichlorometane. The organic extract was dried (Na_2SO_4) and evaporated.

2.1.2. Amino acids-MEH synthesis

A 2.1 ml of freshly distilled acetyl chloride were added dropwise to 14.0 ml of cooled (ice-bath) anhydrous MeOH.

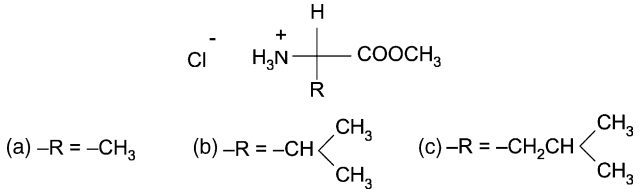

Fig. 4. Amino acid-methyl ester hydrochlorides: (a) alanine-MEH; (b) valine-MEH; (c) leucine-MEH.

The solution was stirred for 10 min. A 11.0 mmol of the amino acid were added in one portion and the solution was slowly heated to reflux for 2 h. The solvent was removed under reduced pressure.

N-*tert*-Butyloxycarbonyl amino acids (*t*-BOC-Val, *t*-BOC-Pro, *t*-BOC-Tyr, *t*-BOC-Phe, see Fig. 5) were purchased from Sigma. Acetonitrile (ACN) and MeOH, as well as ammonium acetate were purchased by Fluka-Riedel de Haën (Buchs, Switzerland). Buffer solutions were filtered before use with 0.22 μm pore size membrane (Durapore GVWP04700, Millipore, Bedford, MA, USA). The pH of the buffer solutions, measured before the addition of the MP modifier, was 7.0.

2.2. Preparation of the CSP

A 5.96 g sample of aminopropyl silica gel Kromasil Si 100 5 mm (C, 3.95%;H, 1.05%;N, 1.20%, corresponding to 658 μmol of Aminopropyl groups per gram of final matrix or 2.11 μmol/m^2 based on the C percentage) was

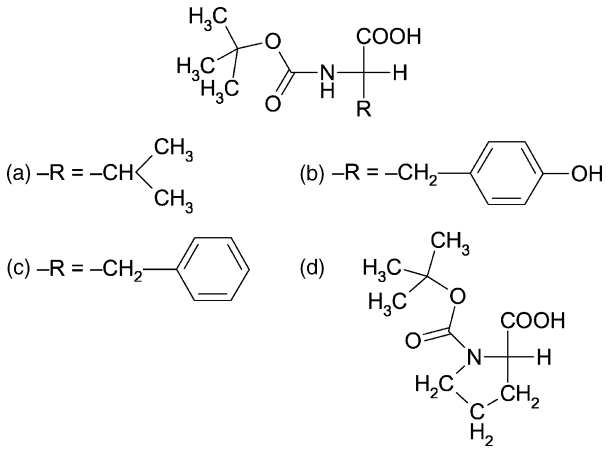

Fig. 5. *N*-*tert*-Butyloxycarbonyl amino acids: (a) *t*-BOC-valine; (b) *t*-BOC-proline; (c) *t*-BOC-tyrosine; (d) *t*-BOC-phenylalanine.

dried at 150 °C for 1 h under vacuum in a round-bottom flask. A 80 ml portion of dry toluene was added, the mixture cooled to 0 °C in an ice-bath, and a 5 ml portion of 1,6-diisocyanatohexane (31 mmol) was added at once. The ice-bath was removed and the slurry was heated to 70 °C in an oil bath for 2 h. After cooling to room temperature, the supernatant toluene phase was removed under an argon atmosphere by suction filtration and excess reactant was removed by dry toluene washing. A suspension of 1.98 g of teicoplanin in 50 ml of a dry pyridine mixture was added dropwise to the wet activated silica, and the mixture was heated at 70 °C for 12 h with gentle stirring under an argon atmosphere. After cooling to room temperature, the teicoplanin-bonded silica was isolated by filtration and washed with 50 ml sequential portions of pyridine, water, MeOH, ACN, dichloromethane and dried under vacuum (70 °C, 10 Pa, 2 h). Elemental analysis: C, 14.44%;H, 2.16%;N, 3.61%, corresponding to 97.8 μmol of teicoplanin per gram of final matrix or 0.39 μmol/m^2 based on the C percentage.

2.3. Column preparation

A 3.3 g sample of the bonded Kromasil was suspended in 60 ml of a acetone–chloroform mixture (1:1) with 15% acetic acid. After 5 min of ultrasonication, the slurry was packed in a 250 × 4.0 mm stainless steel column at 7 × 10^7 Pa with a Haskel DSTV-122 pump using MeOH as the pressurizing agent. The column efficiencies were larger than 4×10^4 plates/m, checked with a hexane–chloroform (90:10) mobile phase and acetophenone ($k' \simeq 11$).

2.4. Chromatographic system

An Agilent-1100 series HPLC system, equipped with a two-pump delivery system, a multiple-wavelength detector, a manual injector (Rheodyne 7725i, Rheodyne, Cotati, CA, USA) and a data acquisition system was used (Chemstation, Rev. A.09.01, Copyright Agilent Technologies). Peak parameters (namely, peak maxima) were estimated through this software. It is well known that this is not the most accurate approach for measuring chromatographic parameters, and this simplification becomes increasingly more critical when the peak shapes move away from an ideal Gaussian profile (also when the goal of researcher is not, as in the present case, the gathering of any kinetic information [26], see below). All the measurements were performed at 25 °C, at 1 ml/min flow rate, and repeated at least twice. Data reproducibility was, in all cases, better than 2%. The wavelength used was 214 nm.

2.4.1. Hold-up volume determination

The determination of the column hold-up volume requires the choice of a probe compound that does not interact with the stationary phase and that is able to visit both the inner and the *extra* pores [27,28]. When the adsorbing surface presents multiple interaction points (as in the case of teicoplanin-based CSP, see Fig. 1), the choice of such a compound is not trivial and the accurate determination of the hold-up volume may become difficult. Compounds such as KI, KNO_3, NH_4NO_3 and $NaNO_3$ have often been used [3,5,13] as hold-up volume markers for teicoplanin-based CSPs. In this work, hold-up volumes were estimated by comparing the results obtained by injections of plugs of pure ACN and MeOH [29]. Hold-up volume was assumed in correspondence with the first signal recorded (disturbance), whose value was found to be substantially the same both with MeOH and ACN. Estimation of hold-up volumes through KNO_3, instead, showed a significant dependence on the ionic strength of the medium [30]. This is because of ionic interactions arising between nitrate ion and CSP, the intensity of which changes by changing the amount of salt in the MP (see later). The following hold-up volumes (ml) were obtained for the different MP conditions exploited (see below): 1.85 ± 0.10 for 40% of organic-modifier (MeOH or ACN); 2.00 ± 0.10 for 60% of organic-modifier.

3. Results and discussion

3.1. Compounds

The amount of data measured in this work is significant. The chromatographic behavior (under linear conditions) of a series of nine amino acids (see Section 2 for details), three amino acids-MEH, seven *N*-Ac-amino acids and four *t*-BOC-amino acids was investigated by considering two different MP modifiers (MeOH and ACN at different concentrations) and various ionic strength conditions. In the case of amino acids-MEH, only MeOH-modified MPs were employed. For the sake of clarity, the data are organized in seven tables in which the retention factor value, k':

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the retention time (or volume) and t_0 the hold-up time, and the selectivity value, α:

$$\alpha = \frac{k'_2}{k'_1} \quad (2)$$

where k'_2 is the retention factor of the more retained enantiomer and k'_1 that of the less retained, are reported. In the case of enantiomeric separations, k' includes the contribution coming both from non-enantioselective and enantioselective interactions [31]:

$$k' = k'_{ns} + k'_s \quad (3)$$

and, accordingly, α is defined as:

$$\alpha = \frac{(k'_{ns} + k'_s)_2}{(k'_{ns} + k'_s)_1} \quad (4)$$

3.2. Stationary phase

The teicoplanin-based CSP used in this work was prepared by linking teicoplanin macrocyclic to an isocyanate functionalized-silica gel via ureidic linkage (as described in Section 2). The ureidic nitrogen is significantly less basic than the nitrogen of the amino group originally present on the teicoplanin. The zwitterionic character of the macrocylic [4] becomes lost and, under the conditions exploited in this work (pH 7.0), the phase bears a negative charge because of the deprotonation of the carboxylic group.

When a surface with an ionizable group is kept in contact with an electrolytic solution, a charged surface is created. Because of the thermal motion (entropic effect), the counter-ions are not bound in a stoichiometric 1:1 ratio with the charges of opposite sign present on the surface. They instead occupy a diffuse layer around the surface (double layer), the composition of which depends on several factors such as the electrostratic attraction to the charged surface, the way the counter-ions shield each other and the thermal motion [32]. In chromatography, a simplified model, the Donnan model, has been introduced to account for the complexity of the physical system. In the Donnan model, a distinct boundary between the electrolyte solution and the stationary phase [resin] is assumed. They are considered as two independent phases. When in contact, a redistribution of ions will occur between these two phases as a result of the thermal motion. After an equilibrium is reached, the resin will be characterized by an excess of charges of the same sign as those borne by the functional groups of the resin itself, while the opposite situation will be observed in the bulk phase. The result is the onset of a potential between the two phases, the Donnan potential. The further tendency of ions to level-out the differences of concentrations between phases is balanced by the Donnan potential [33]. Some important results of the Donnan theory, when applied to chromatographic separations, are [32]: (1) the ions experience an energy difference between the resin and electrolyte phases that is proportional both to the Donnan potential and ion charge; (2) the Donnan potential decreases as the concentration of the counter-ions in the electrolyte solution increases; (3) when the eluite species and the counter-ions have charges of opposite signs, the eluite will be excluded by the phase [34].

3.3. Amino acids

The amino acids investigated in this work can be qualitatively divided into different groups. (a) Ala, Val, Leu, Ile, and *tert*-Leu bear neutral R groups (see Fig. 2); moreover, Leu, Ile and *tert*-Leu are structural (constitutional) isomers, which may have different steric hindrances depending on the way their atoms are joined together. (b) Pro is a secondary amine with a cyclic structure, which is responsible for a much higher molecular rigidity than that of the other amino acids considered; structural rigidity is recognized as an important property for increasing chiral discrimination [35]. (c) Φ-Gly and Phe bear aromatic rings, potentially able to make π–π interactions with the numerous aromatic rings of the CSP. Finally Gly, which is the only non-chiral amino acid, was also included in the list of compounds investigated (see below).

Table 1 reports the retention data of the amino acids in MeOH–buffer MPs. Two organic modifier compositions were considered (40 and 60%, v/v) as were several different conditions of ionic strength (solutions at 10, 20, 30 and 50 mM CH_3COONH_4 in the case of MeOH–40% and only 20 and 50 mM CH_3COONH_4 for MeOH 60%). Table 2 reports, instead, the retention data of amino acids in the case of ACN modifier (two MP modifier concentrations were used while changing the ionic strength of the solutions).

By looking at the data reported in Tables 1 and 2 some general observations can be made. First, L-amino acids are always eluted before D-amino acids [3,6,7,14]. Secondly, the retention of amino acids shows an extremely small dependence on the ionic strength of the solution, both with MeOH and with ACN MP modifiers, as shown by the k' values, whose values are almost constant even when the buffer composition is changed (see Tables 1 and 2). Finally, the retention of amino acids increseas dramatically when the MP modifier concentration is increased, especially in the case of ACN-based MPs (Table 2) [3–5].

Phenomena leading to chiral separation may originate from many different effects and interactions. In the case in which the process takes place in a cavity (as, for instance, on cellulose [36] or on macrocyclic antibiotics-based CSPs [16]), it is the "ability" of one enantiomer (with respect to the other) to reach a better steric fit in the cavity that induces the separation. With simple chromatographic data, obviously, only qualitative hypotheses about specific molecular enantioselective interactions can be made. At pH 7.0, the anphoteric character of α-amino acids induces the molecules to exist as dipolar ions. COO^- is a strong hydrogen-bond acceptor that can interact with the numerous amide groups (hydrogen-bond donors) of the aglycone (see Fig. 1) [16]. The ammonium group, instead, has a

Table 1
Retention factors (k') and selectivity values (α) of amino acids and their dependence on the MP composition (MeOH as organic modifier)

	Buffer–MeOH (60:40)								Buffer–MeOH (40:60)			
	10 mM		20 mM		30 mM		50 mM		20 mM		50 mM	
	k'	α	k'	α	k'	α	k'	α	k'	α	k'	α
Gly	0.34	–	0.34	–	0.32	–	0.32	–	0.47	–	0.46	–
Ala												
L	0.32	2.00	0.32	1.97	0.32	1.80	0.32	1.86	0.45	2.25	0.41	2.30
D	0.65	2.00	0.64	1.97	0.58	1.80	0.59	1.86	1.00	2.25	0.95	2.30
Val												
L	0.36	1.58	0.36	1.50	0.35	1.57	0.34	1.56	0.37	1.95	0.34	1.91
D	0.56	1.58	0.54	1.50	0.55	1.57	0.53	1.56	0.71	1.95	0.65	1.91
Leu												
L	0.48	2.06	0.48	2.03	0.47	1.94	0.46	1.85	0.45	2.25	0.42	2.33
D	0.98	2.06	0.97	2.03	0.91	1.94	0.86	1.85	1.00	2.25	0.97	2.33
Ile												
L	0.44	2.40	0.43	2.23	0.43	2.25	0.42	2.39	0.40	2.94	0.38	2.84
D	1.05	2.40	0.95	2.23	0.96	2.25	0.99	2.39	1.18	2.94	1.07	2.84
tert-Leu												
L	0.35	1.72	0.34	1.68	0.33	1.64	0.34	1.69	0.30	2.14	0.29	2.11
D	0.59	1.72	0.56	1.68	0.54	1.64	0.57	1.69	0.63	2.14	0.60	2.11
Pro												
L	0.76	2.57	0.75	2.41	0.72	2.48	0.74	2.58	1.06	2.88	1.00	2.94
D	1.96	2.57	1.80	2.41	1.79	2.48	1.90	2.58	3.04	2.88	2.94	2.94
Φ-Gly												
L	0.78	4.22	0.73	4.25	0.71	4.18	0.70	4.19	0.68	5.51	0.65	5.65
D	3.29	4.22	3.10	4.25	2.96	4.18	2.92	4.19	3.75	5.51	3.65	5.65
Phe												
L	1.09	1.05	1.07	1.08	1.05	1.06	1.04	1.07	0.92	1.17	0.87	1.18
D	1.15	1.05	1.15	1.08	1.11	1.06	1.11	1.07	1.08	1.17	1.02	1.18

(weaker) hydrogen-bond donor character through which it can interact with one of the carbonyl groups present in the cavity. Alternatively, it could also give dipole–dipole interactions. The combination of hydrogen-bond, dipole–dipole and short-distance Van der Waals interactions may to varying degrees favor the stabilization of the enantiomer–CSP complexes. Obviously, these effects strictly depend how the enantiomers fit the aglycone cavity. A deeper analysis of the results reported in Tables 1 and 2 shows that the k' values for L-Ala and L-Val are similar to each other (both with MeOH and ACN MP modifiers and at different ionic strengths) and only slightly smaller than the k's measured for L-Leu. They are also similar to the k' values measured for Gly and to those of L-Ile and L-*tert*-Leu, when these compounds were investigated (i.e. in the case of MeOH modifier, see Table 1). The corresponding D-enantiomers are instead retained at different degrees and the selectivity of the CSP is significant in all the cases, as demonstrated by the α values obtained. A possible explanation for these data is the assumption of an enantioselective recognition mechanism in which L-enantiomers do not experience selective interactions and are, accordingly, eluted at retention volumes similar to those of Gly. D-Amino acids, instead, may have enantioselective interactions at higher affinity [2,14,37–40,42,43].

Through hindrance effects (i.e. by occupying space inside the aglycone basket of the CSP), carbohydrate moieties present on teicoplanin may have an important role in the separation of amino acids, as they block possible interaction sites on the aglycone itself, and offer competing interaction sites (the sugars themselves are chiral) [2]. This is particularly important for α-amino acids, which are thought to "dock" and bind inside the cleft of the aglycone near its amine (or ureido) functional group [4,23]. In this work, Leu and the constitutional isomers Ile and *tert*-Leu (see Table 1) have been chosen to exploit the possible steric hindrance effect arising between CSP and different R substituents (see Fig. 2). In extreme cases, large R-groups can cause the exclusion of the amino acid from the aglycone basket. This fact is associated to the loss of selectivity by the CSP (see below). Additionally, they can favor (or hinder) specific orientations of the molecules in the pocket through weak hydrophobic interactions with the aglycone walls. The ligand in the binding site will have a different degree of motion that may improve (or decrease) the strength of existing interactions with different effects on the α value [44,45]. According to the k' values reported in Table 1, it is evident that the *tert* group has a negative role in the stabilization of the molecule–CSP complex, causing a

Table 2
Retention factors (k') and selectivity values (α) of amino acids and their dependence on the MP composition (ACN as organic modifier)

	Buffer–ACN (60:40)				Buffer–ACN (40:60)			
	10 mM		30 mM		10 mM		30 mM	
	k'	α	k'	α	k'	α	k'	α
Gly	0.34	–	0.32	–	1.18	–	1.09	–
Ala								
L	0.34	1.41	0.33	1.36	1.13	1.35	1.04	1.35
D	0.48	1.41	0.45	1.36	1.53	1.35	1.40	1.35
Val								
L	0.36	1.33	0.35	1.31	1.01	1.25	0.94	1.26
D	0.48	1.33	0.46	1.31	1.26	1.25	1.19	1.26
Leu								
L	0.40	1.47	0.39	1.46	0.93	1.36	0.87	1.40
D	0.59	1.47	0.57	1.46	1.27	1.36	1.22	1.40
Pro								
L	0.49	1.71	0.49	1.69	1.39	1.64	1.34	1.63
D	0.84	1.71	0.83	1.69	2.28	1.64	2.18	1.63
Φ-Gly								
L	0.50	2.38	0.55	2.04	1.06	2.23	1.04	2.18
D	1.19	2.38	1.12	2.04	2.36	2.23	2.27	2.18
Phe								
L	0.60	1.10	0.59	1.10	1.10	1.10	1.08	1.10
D	0.66	1.10	0.65	1.10	1.21	1.10	1.19	1.10

significant loss of enantioselectivity respect to the other two isomers.

Pro represents an exception to the amino acids previously considered. First of all, it is a secondary amine with a cyclic structure that gives the molecule a much higher rigidity. The cyclic structure of Pro stabilizes the complex between the amino acid and the cavity, possibly facilitating the onset of interactions between the N–H group of the secondary amine and an amidic group of the aglycone (as reflected by the fact that α for Pro is larger than the α observed for other amino acids under the same conditions).

Phe and Φ-Gly possess aromatic rings. This significantly changes the chemical–physical properties of the molecules. Additionally, they may participate in π–π interactions with the aromatic rings present on the CSP. In spite of the apparent similarity between their molecular structures, a large difference in the chromatographic behavior of these enantiomers has been observed in this work. In particular, for Phe the enantioselectivity appears almost completely lost in MeOH and extremely low in ACN (see Tables 1 and 2), while the α values of Φ-Gly are the largest observed among all the amino acids. According to our results, the presence of an extra CH_2 group in the structure of Phe seems enough to deny the D-enantiomer the ability to properly fit the aglycone basket of the teicoplanin, which causes the loss of enantioselectivity. The high k' values observed for Φ-Gly and Phe (L and D forms) suggest that the molecules have additional non-enantioselective interactions with the numerous Phenyl groups of the CSP through their aromatic rings. Again, involving π–π interactions, once in the aglycone pocket, Φ-Gly may further stabilize its complex with the CSP (which justifies the largest αs). Fig. 6 represents an example of a chromatographic separation of D,D-Φ-Gly (see figure caption for experimental details). As it is evident by this figure, the peak corresponding to the more retained compound (D) is characterized by a stronger asymmetry factor (3.3, the asymmetry value as classically calculated at 10% of peak height) than the peak corresponding to the less retained enantiomer (D, asymmetry: 2.3). Under these conditions, the evaluation of physical–chemical quantities via chromatographic measurements needs a careful analysis of peak shapes. The use of peak maxima (apparent retention time) instead of first moments (thermodynamic quantity) can be misleading. For these reasons, the data presented in this work should be considered as a preliminary study. Further empirical and theoretical investigations are being planned to investigate these aspects through the use of a recently developed stochastic microscopic-molecular approach [14,40–43]. The complexity of the subject requires a specific discussion that goes beyond the scope of this paper.

By comparing the data concerning the separation of α-amino acids in MeOH and ACN, it is evident that the selectivity is enhanced when the former MP organic modifier is employed. A possible explanation for this effect is related to the competition for the adsorption to the CSP exercised by *strong* species (i.e. species able to interact with the stationary phase) present in the MP. In particular, acetate ions coming from the dissociation of the salt may

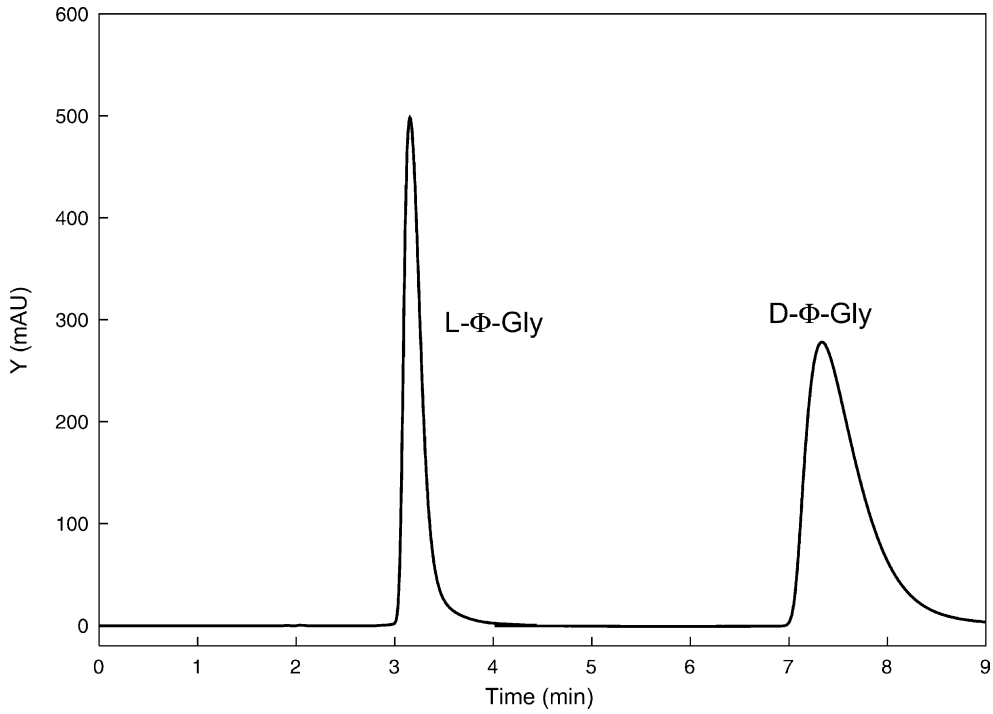

Fig. 6. Separation of (D,L)-Φ-Gly. Experimental conditions: flow-rate, 1 ml/min; MP: MeOH–buffer (40:60, v/v) (buffer concentration: 30 mM); $T = 25\,^{\circ}C$.

be adsorbed in the aglycone of teicoplanin [16]. Their competitive effect, which is certainly also present in the case of MeOH-modified MPs, appears, however, stronger in ACN because of the poorer solubility of acetate in this medium. As previously discussed, amino acid retention showed quite a small dependence on the ionic strength of the medium. The qualitative conclusions proposed in this work substantially ignored these effects. For some compounds, however, a systematic trend can be observed (for instance: Φ-Gly, Table 1). Making hypotheses about the causes that lead to these phenomena is not easy: Donnan effects (amino acid, whose iso-electric point is different than 7, bear a net charge); competition effects played by acetate for the adsorption on the aglycone pocket; structural changes in the CSP because of composition changes of the surrounding medium [1,46] could all represent plausible explanations.

3.4. Amino acids-MEH

The esterification of the carboxylic group of the amino acids completely changes the chemical properties of the molecules. Under the experimental conditions employed in this work, esterified amino acids bring a positive charge (protonated amino group) and the zwitterionic equilibrium is unattainable. The disappearance of the carboxylate group has important consequences. COO^- represents the most important moiety through which amino acids can interact with the aglycone basket via hydrogen bonding. This has been demonstrated via NMR investigations [18,47–50] and, more recently, by X-ray cristallography (although, in that case, for vancomycin macrocyclic antibiotic) [51–53]. In particular, Williams et al. [16] proposed that, in the binding of acetylated dipeptides to macrocyclic antibiotics, a possible reaction mechanism would include the following steps: (1) binding of the carboxylate anion of the C-terminal amino acid in the pocket of the three amide NH groups of residues 2–4 of the antibiotic (see Fig. 1); (2) formation of amide–amide hydrogen bonds between the acetylated dipeptide and the antibiotic; (3) hydrophobic interactions formed by the methyl groups of the amino acid in their contacts with hydrocarbon portions of the antibiotic.

Table 3 reports the retention data measured for three amino acids-MEH, in an MP composed of MeOH–buffer (40:60, v/v), at different ionic strengths. The retention factors of amino acids-MEH were found to be significantly larger than those observed for the corresponding underivatized α-amino acids, under the same conditions (compare Tables 1 and 3). Additionally, k' values for amino acids-MEH change dramatically when the ionic strength of the MP is varied. The rationale of these observations lies in the effect of the ionic interactions arising between the molecules (positively charged) and the CSP, which is instead negatively charged. The simple Donnan model may be used to interpret the trend. Amino acids-MEH experience attractive interactions with the CSP surface, which increases their retention. On the other hand, the Donnan potential decreases with increases in the amount of salt in the MP, which also causes the attractive intensity to decrease and, consequently, the retention.

The other important aspect that can be observed from the data of Table 3 is that the selectivity of the CSP has almost completely disappeared. This is indicated by the α values that are roughly equal to 1 for all the cases considered.

Table 3
Retention factors (k') and selectivity values (α) of amino acids methyl ester hydrochloride (MEH) and their dependence on the medium composition (MeOH as organic modifier)

	Buffer–MeOH (60:40)							
	10 mM		20 mM		30 mM		50 mM	
	k'	α	k'	α	k'	α	k'	α
Ala-MEH								
L	4.28	1.10	2.59	1.01	1.88	1.01	1.32	1.02
D	4.69	1.10	2.61	1.01	1.90	1.01	1.34	1.02
Val-MEH								
L	4.51	1.04	2.56	1.05	2.36	1.03	1.40	1.01
D	4.69	1.04	2.68	1.05	2.42	1.03	1.41	1.01
Leu-MEH								
L	5.50	1.07	3.29	1.02	2.02	1.00	1.72	1.02
D	5.88	1.07	3.34	1.02	2.01	1.00	1.76	1.02

This dramatic change in the chromatographic behavior of the modified amino acids can have different origins. A possible explanation could be the protection of the carboxylic group (and the consequent loss of the carboxylate moiety). It is, in fact, mainly through this group that molecules can interact with the CSP via hydrogen bonds [18]. According to Eqs. (3) and (4), however, an increase of the non-enantioselective contribution (namely, $k'_{ns,1}$ and $k'_{ns,2}$), instead keeping constant the selective contribution, would also cause a decrease in α. Because the contribution to the retention coming from non-enantioselective interactions is the same for both the enantiomers, the difference between the retention factors of the L-, k'_l, and the D-, k'_d, species (see Eq. (3)):

$$\Delta k' = k'_D - k'_L = \Delta k'_s \quad (5)$$

only accounts for the selective part of the retention. For instance, by considering the case of Val, $\Delta k'_s$ for the unprotected amino acid roughly holds 0.2 (see Table 1, first column), while $\Delta k'_s$ for Val-MEH is about 0.18 (Table 3, first column). This means that, in the case of Val, only the so-called "apparent selectivity" [31], which corresponds to the conventional separation factor, significantly changes (from 1.58 to 1.04) when the carboxylic group is derivatized. Conversely, the "true selectivity" (that only includes selective contributions) does not dramatically vary. It is important to realize that the amount of the individual contributions, coming from the two types of sites (selective and non-selective), to the retention cannot be easily estimated by data measured under linear conditions. Only the determination of the equilibrium adsorption isotherms will allow the gathering of this information [54–58] and further investigations to fully clarify this point have been planned.

3.5. *N*-Ac-amino acids

The formation of an amide bond by acetilation of the Amino group increases the acidity of the N–H hydrogen. As in the case of amino acids-MEH, the amphoteric character is lost and, at pH 7.0, *N*-Ac-amino acids are negatively charged (carboxylic group deprotonated). Because the CSP bears a negative charge as well, an electrostatic repulsive interaction occurs between *N*-Ac-amino acids and CSP. Tables 4 and 5 report the retention data observed for *N*-Ac-amino acids under the different experimental conditions exploited in this work. The most evident observation is that L-enantiomers are in many cases *excluded* by the CSP, i.e. their elution volumes (or retention times) are smaller than the column hold-up volume (see Section 2 for details about hold-up volume determination). According to Eq. (1), excluded species have a negative k' value (being $t_R < t_0$). Accordingly, negative retention factors reported in Tables 4 and 5 represent species excluded by the CSP. D-Enantiomers show, instead, significantly large k' values. (The symbol ∞ has been used in Tables 4 and 5 to underline the fact that the CSP selectivity is, in principle, infinite under these conditions). Exclusion phenomena for dansyl-amino acids on teicoplanin-aglycone-based and (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic-acid-based CSPs have been recently observed [15], as well. In that case, however, both L- and D-forms of the amino acids showed small k's and α values around 1. The case of *N*-Ac-amino acids is, in our opinion, quite different. The D-forms of *N*-Ac-amino acids can penetrate the aglycone basket [22]. Therein, they are strongly stabilized through the creation of multiple hydrogen bonds that overcome the repulsion effect due to ionic forces. Exclusion of L-forms, instead, means that these compounds are not able to enter the aglycone cavity. Unable to form strong interactions inside the cavity (through hydrogen bonds), they only experience repulsive forces that exclude these compounds from the possibility of any other interaction with the CSP. The reason for the exclusion of L-*N*-Ac-amino acids is most likely of steric hindrance nature. A molecule such as *N*-Ac-Gly, whose characteristics are noticeably similar to those of the other L-*N*-Ac-amino acids but without any potentially hindering moiety is, in fact, not excluded by the CSP (Tables 4 and 5).

Table 4
Retention factors (k') and selectivity values (α) of *N*-acetyl-amino acids (*N*-Ac-) and their dependence on the MP composition (MeOH as organic modifier)

	Buffer–MeOH (60:40)								Buffer–MeOH (40:60)			
	10 mM		20 mM		30 mM		50 mM		20 mM		50 mM	
	k'	α	k'	α	k'	α	k'	α	k'	α	k'	α
N-Ac-Gly	0.08	–	0.26	–	0.36	–	0.50	–	0.46	–	0.76	–
N-Ac-Ala												
L	−0.22	∞	−0.16	∞	−0.11	∞	0.02	400	−0.01	∞	0.02	546
D	3.32	∞	5.19	∞	6.37		8.00	∞	7.63		10.93	546
N-Ac-Val												
L	−0.19	∞	−0.14	∞	−0.08	∞	0.08	75	0	∞	0	∞
D	2.46	∞	3.62	∞	4.63	∞	5.98	75	5.23		7.18	∞
N-Ac-Leu												
L	−0.18	∞	−0.10	∞	0.03	33	0.14	27	0.02	139	0.06	67
D	1.52	∞	2.34	∞	3.02	33	3.77	27	2.79	27	4.01	67
N-Ac-Pro												
L	−0.16	/	−0.12	/	−0.11	/	0.04	1	−0.02	/	0.02	1
D	−0.19	/	−0.12	/	−0.07	/	0.05	/	0	1	0.03	1
N-Ac-Φ-Gly												
L	0	∞	0.09	34	0.17	18	0.26	12	0.08	49	0.16	23
D	3.26	∞	3.05	34	3.12	18	3.08	12	3.90	49	3.64	23
N-Ac-Phe												
L	0.22	8	0.43	7	0.59	6	0.79	6	1.09	2	0.66	6
D	1.82	8	2.98	7	3.67	6	4.53	6	−2.72	2	3.90	6

Negative k' values refer to excluded compounds (see text for details).

Table 5
Retention factors (k') and selectivity values (α) of *N*-acetyl-amino acids (*N*-Ac-) and their dependence on the MP composition (ACN as organic modifier)

	Buffer–ACN (60:40)				Buffer–ACN (40:60)					
	10 mM		30 mM		10 mM		20 mM		30 mM	
	k'	α	k'	α	k'	α	k'	α	k'	α
N-Ac-Gly	0.10	–	0.10	–	0.63	–	0.75	–	0.89	–
N-Ac-Ala										
L	−0.23	∞	−0.15	∞	0.20	28	0.32	24	0.41	23
D	1.36	∞	2.58	∞	5.60	28	7.81	24	9.47	23
N-Ac-Val										
L	−0.23	∞	0.01	185	0.14	21	0.27	16	0.35	15
D	0.84	∞	1.85	185	3.01	21	4.39	16	5.21	15
N-Ac-Leu										
L	−0.22	∞	0.02	57	0.14	12	0.26	9	0.32	9
D	0.56	∞	1.15	57	1.70	12	2.34	9	2.78	9
N-Ac-Pro										
L	−0.23	/	0.01	1	0.19	1	0.34	1	0.38	1
D	−0.23	/	0.01	1	0.18	1	0.33	1	0.39	1
N-Ac-Φ-Gly										
L	−0.21	∞	0.06	18	0.14	17	0.28	8	0.36	6
D	1.18	∞	1.08	18	2.40	17	2.35	8	2.26	6
N-Ac-Phe										
L	−0.15	∞	0.19	5	0.27	4	0.46	3	0.58	3
D	0.38	∞	0.95	5	1.08	4	1.61	3	1.91	3

Negative k' values refer to excluded compounds (see text for details).

By increasing the amount of salt, the Donnan potential decreases. L-*N*-Ac-amino acids, which were excluded under the previous conditions, may exhibit small k' values in the new media (see Tables 4 and 5). They have the possibility to arrive closer to the adsorptive phase where the onset of weak interactions is responsible for the observed k's. In this work, the limit case is represented by ACN 60% (Table 5). Regardless of the ionic strength of the solution, no L-compound is excluded, although the k' values observed under these conditions are small (often near the exclusion limit and substantially smaller than those of *N*-Ac-Gly). In the case of MeOH modifier, instead, such a condition was not observed (Table 4). A possible explanation for the different behavior of these two media could be related to their dielectric constant values. The dielectric constant of CH_3CN (36.64 at 293.2 T/K [59]) is larger than that of MeOH (33.00 at 293.2 T/K [59]). This inversely affects the intensity of the repulsive ionic forces between molecules and CSP. The solubility of compounds should not be responsible for the observed effects, particularly considering that *N*-Ac-amino acids are easily soluble in ACN-enriched MPs.

By comparing the data for D-enantiomers reported in Table 4 with those of Table 1 and the data in Table 5 with those of Table 2, it is evident that the presence of an acetyl moiety has dramatic effects on the stabilization of D-enantiomers-aglycone complexes. A significant increase in the retention of D-*N*-Ac-compounds with respect to the corresponding unprotected amino acids is evident, despite the repulsive forces the former must undergo. This common trend that can be, in quantitative terms, more or less pronounced (see, for instance, the case of D-*N*-Ac-Φ-Gly) has only one significant exception. In fact, D-*N*-Ac-Pro was found in some cases to be excluded by the CSP (as indicated in Tables 4 and 5). Additionally, in those cases in which exclusion does not occur, the selectivity of the CSP for this compound has almost completely disappeared. (In Tables 4 and 5, αs were rounded to integer numbers to emphasize cases characterized by enormous selectivity and cases with almost no selectivity). The enhanced possibility of making hydrogen bonds between the hydrogen of the amidic group of the acetylated amino acids (which has an increased acidity with respect to the unprotected compounds) and the amidic group of the aglycone (through residue 5, see Fig. 1) is pivotal for increasing the chiral separation ability of the CSP. This opportunity is common to all the amides obtained from primary amino acids. Amides derived by secondary amino acids, such as L-*N*-Ac-Pro obviously do not have the possibility of making this extra amide–amide hydrogen bond. The fact that the selectivity of the CSP for *N*-Ac-Pro is lost could be a consequence of this. However, the data reported in Tables 4 and 5 show that the exclusion or, in any case, an extremely small retention of both L- and D-*N*-Ac-Pro is present. This could indicate that the impossibility to fit the aglycone cavity (for steric hindrance effects), rather than the lack of the extra amide–amide hydrogen bond, is the reason for the loss of selectivity.

3.6. *t*-BOC-amino acids

The last class of compounds investigated in this work was chosen to exploit the effect of a group the *tert*-group, whose hindrance is significant. With both MeOH and with ACN MP-modifiers, the k's for *N*-*tert*-butyloxycarbonyl amino acids are small and, in some cases, the compounds (D and L) are excluded by the CSP (negative k's in Tables 6 and 7). The ratios between extremely small values of k', whose differences are also often of the same magnitude as the experimental errors, may give the erroneous impression that the CSP selectivity is satisfactory (being αs reasonably larger than 1). This is, for instance, the case of *t*-BOC-Val (see

Table 6
Retention factors (k') and selectivity values (α) of *N*-*tert*-butyloxycarbonyl-amino acids (*t*-BOC) and their dependence on the MP composition (MeOH as organic modifier)

	Buffer–MeOH (60:40)								Buffer–MeOH (40:60)			
	10 mM		20 mM		30 mM		50 mM		20 mM		50 mM	
	k'	α	k'	α	k'	α	k'	α	k'	α	k'	α
t-BOC-Val												
L	0	/	0.10	1.00	0.13	1.15	0.22	1.04	0	/	0.02	2.00
D	0	/	0.10	1.00	0.15	1.15	0.23	1.04	0	/	0.04	2.00
t-BOC-Pro												
L	−0.20	/	−0.12	/	0.01	1.00	0.09	1.00	−0.03	/	0.06	1.18
D	−0.20	/	−0.12	/	0.01	1.00	0.09	1.00	−0.04	/	0.07	1.18
t-BOC-Tyr												
L	0.15	1.07	0.31	1.00	0.42	1.02	0.59	1.37	0.07	1.16	0.16	1.00
D	0.16	1.07	0.31	1.00	0.43	1.02	0.81	1.37	0.08	1.76	0.16	1.00
t-BOC-Phe												
L	0.23	1.04	0.43	1.04	0.56	1.04	0.79	1.02	0.07	1.14	0.23	1.04
D	0.24	1.04	0.45	1.04	0.58	1.04	0.81	1.02	0.08	1.14	0.24	1.04

Negative k' values refer to excluded compounds (see text for details).

Table 7
Retention factors (k') and selectivity values (α) of *N*-*tert*-butyloxycarbonyl-amino acids (*t*-BOC) and their dependence on the MP composition (ACN as organic modifier)

	Buffer–ACN (60:40)				Buffer–ACN (40:60)			
	10 mM		30 mM		10 mM		30 mM	
	k'	α	k'	α	k'	α	k'	α
t-BOC-Val								
L	−0.20	/	0.07	1.14	0.09	1.11	0.25	1.04
D	−0.20	/	0.08	1.14	0.10	1.11	0.26	1.04
t-BOC-Pro								
L	−0.22	/	−0.21	/	0.12	1.00	0.30	1.00
D	−0.21	/	−0.20	/	0.12	1.00	0.30	1.00
t-BOC-Phe								
L	−0.16	/	0.19	1.05	0.11	1.37	0.26	1.00
D	−0.15	/	0.20	1.05	0.08	1.37	0.26	1.00

Negative k' values refer to excluded compounds (see text for details).

Table 6, last column) or *t*-BOC-Phe (Table 7, case corresponding to ACN 60% and 10 mM buffer concentration), for which k' values equal to 1.14 and 1.37 were observed, respectively. These small differences, when statistically significant, could be associated to the presence of secondary chiral recognition mechanisms (due to the large number of potential adsorptive sites present on the teicoplanin surface [2]), while L- and D-*t*-BOC-amino acids would, in any case, be excluded from aglycone because of their steric hindrance.

3.7. Donnan effect and retention: a closer look

Because under the experimental conditions exploited in this work the CSP is negatively charged, the onset of the Donnan potential is connected to the differences in the ammonium ions' concentrations (coming from the buffer dissociation) in the resin and in the bulk phase [32,33]. The CSP, in fact, can be considered a cationic exchanger.

At equilibrium, the chemical potential of a species in the two phases is the same. By assuming the Donnan potential, Ψ, equal to zero in the resin phase (as is usually done in the literature concerning the Donnan potential), simple thermodynamic considerations lead to the following equation for the ammonium ions:

$$\mu_{0,NH_4^+} + RT \ln \{NH_4^+\}_{resin} = \mu_{0,NH_4^+} + RT \ln \{NH_4^+\}_{bulk} + Z_{NH_4^+} F\Psi \quad (6)$$

where the symbol { } represents the activity of the species, μ_{0,NH_4^+} is the standard-state chemical potential of the ammonium, R the gas constant, T the temperature and the product $[Z_{NH_4^+} F\Psi]$ (last term of the RHS of Eq. (6)) is the contribution to the chemical potential due to the Donnan potential (F being the Faraday and $Z_{NH_4^+}$ the effective charge of the species NH_4^+). From Eq. (6), the Donnan potential can be expressed as:

$$\Psi = \frac{RT}{Z_{NH_4^+} F} \ln \frac{\{NH_4^+\}_{resin}}{\{NH_4^+\}_{bulk}} \quad (7)$$

and since the term $\{NH_4^+\}_{resin}$ can be considered constant and fixed by the structure of the matrix [60], from Eq. (7) it follows that:

$$\Psi \propto \ln \{NH_4^+\}_{bulk}^{-1} \quad (8)$$

The effect of the Donnan potential on a charged species can be derived through the following considerations. Let us assume an A species bearing an electrostatic charge (positive or negative) that undergoes the following equilibrium:

$$A_{bulk} \rightleftharpoons A_{resin}$$

At the equilibrium, the chemical potentials in the two phases are equal:

$$\mu_{0,A} + RT \ln \{A\}_{resin} = \mu_{0,A} + RT \ln \{A\}_{bulk} + Z_A F\Psi \quad (9)$$

where Z_A is the effective charge of the A species. By introducing Eq. (7) in Eq. (9), one has:

$$\ln \frac{\{A\}_{resin}}{\{A\}_{bulk}} = \ln K_D = \frac{Z_A}{Z_{NH_4^+}} \ln \frac{\{NH_4^+\}_{resin}}{\{NH_4^+\}_{bulk}} \quad (10)$$

where K_A is the partition coefficient related to k' through [54,61]:

$$k' = K_A \frac{V_s}{V_0} \quad (11)$$

being V_s and V_0 the stationary-phase and the hold-up volumes, respectively. Finally, by using Eqs. (8) and (11), Eq. (10) can be written as:

$$\ln k' \propto \frac{Z_A}{Z_{NH_4^+}} \ln \{NH_4^+\}_{bulk}^{-1} \quad (12)$$

Eq. (12) suggests that the plot of $\ln k'$ versus $\ln \{NH_4^+\}$ activity of the MP modifier) should be linear. Additionally,

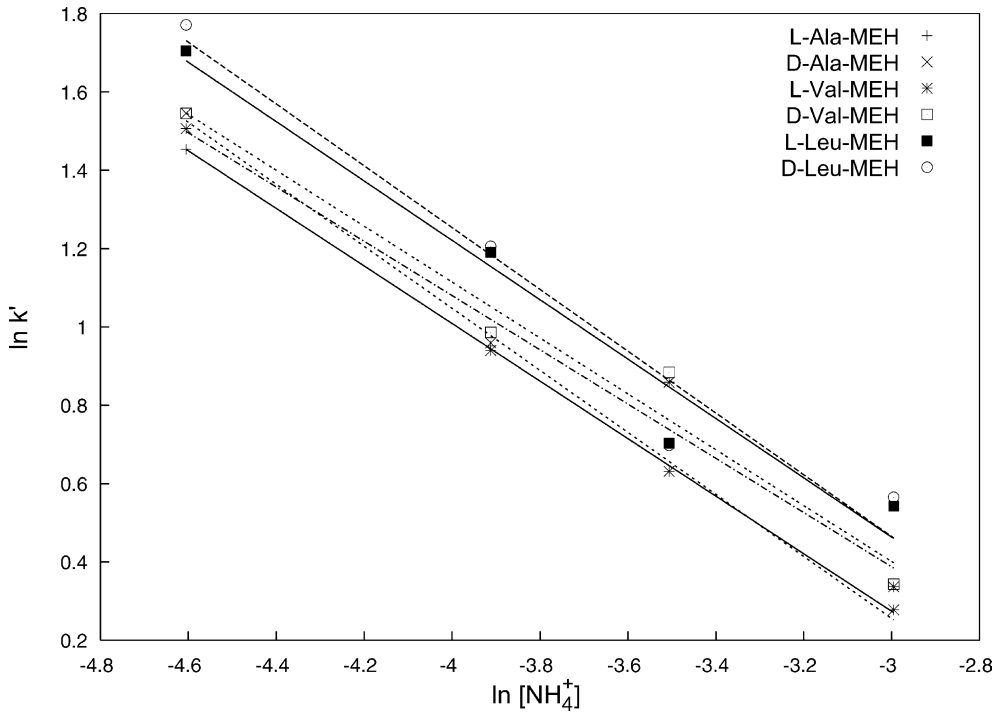

Fig. 7. ln k' vs. ln[NH_4^+] plots for amino acids-MEH (see Table 8). Mobile phase composition, buffer–MeOH (60:40).

depending on the sign (positive or negative) of the charge of the A species involved in the equilibrium, the slope of the plot can be positive (for negatively charged species) or negative (for positevely charged species). As discussed in the previous sections, the k' values of the amino acids-MEH and *N*-Ac-amino acids show a strong dependence on the amount of the buffer concentration (Tables 3 and 4). For some of these compounds (and under the same experimental conditions of Tables 3 and 4), the plots of the ln k' values versus the logarithm of the analytical concentrations of the buffer were evaluated. (Only those compounds for which there were at least four experimental data points available were

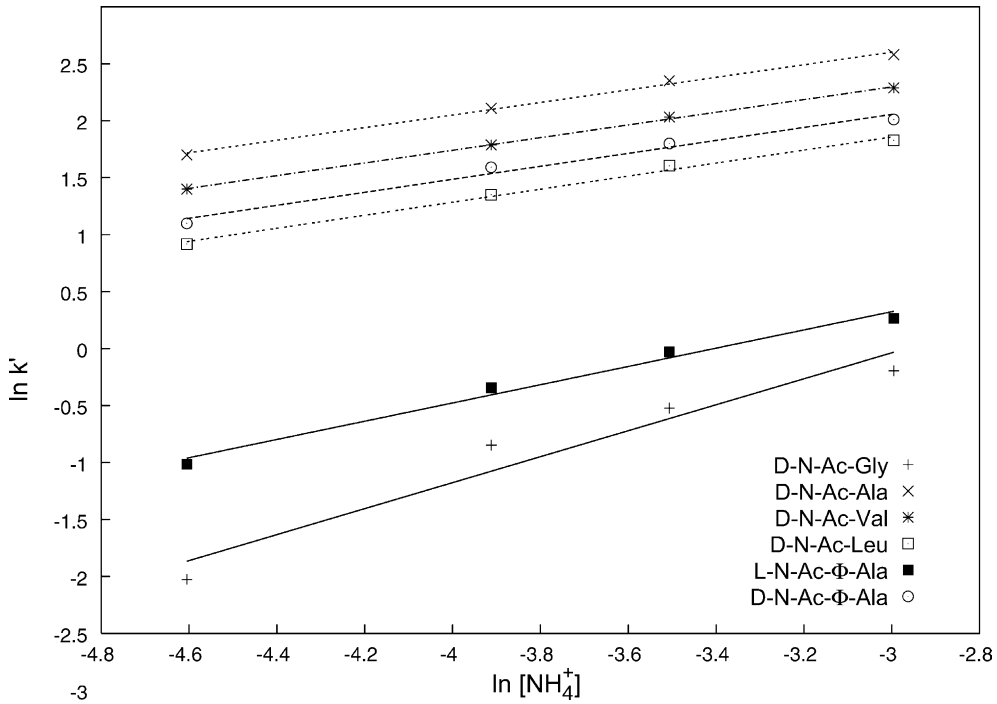

Fig. 8. ln k' vs. ln[NH_4^+] plots for *N*-Ac-amino acids (see Table 9). Mobile phase composition, buffer–MeOH (60:40). Φ-Ala: phe.

Table 8
Slopes of the ln k' vs. ln[NH_4^+] plots for amino acids-MEH and corresponding squares of the correlation coefficients

	Slope	R^2
Ala-MEH L	−0.74	0.99
Ala-MEH D	−0.78	0.99
Val-MEH L	−0.69	0.97
Val-MEH D	−0.71	0.97
Leu-MEH L	−0.76	0.96
Leu-MEH D	−0.79	0.95

Mobile phase composition, buffer–MeOH (60:40).

considered). Figs. 7 and 8 report the ln k' vs. ln {NH_4^+} plots for amino acids-MEH and *N*-acetyl-amino acids, respectively (buffer–MeOH, 60:40). Tables 8 and 9 list the values of the slopes of the linear regressions, as well as the squares of the correlation coefficients (R^2), obtained in the different cases. These data show that the slopes of the plots for compounds bearing positive charges (amino acids-MEH, Fig. 7 and Table 8) have negative values (as expected by Eq. (12)). The opposite is true for the slopes of the plots of negatively charged species (*N*-Ac-amino acids, Fig. 8 and Table 9). Much more critical is the interpretation of the values of these slopes, which according to Eq. (12) should be proportional to the ratio of the effective charges ($Z_A/Z_{NH_4^+}$). The data reported in Tables 8 and 9 were obtained by plotting the ln k' values versus the logarithm of the analytical concentrations of the buffer. When the ionic strength of the medium is low, it is common to substitute activity with concentration. Under the conditions used in this work, however, the presence of an organic mixed solvent (with a dielectric constant different than that of water) may dramatically change the equilibria involved in the system. For a 40% MeOH–buffer mixture, for instance, the dielectric constant is 60.94 at 25 °C [62], which is markedly smaller than in water (80). In these media, phenomena such as the onset of ion-pairing are likely to happen. Substituting activities with analytical concentrations can be incorrect and the values listed in Tables 8 and 9 must be considered merely as empirical slopes. Admittedly, there is another important simplification that was implicitly assumed in this treatment. Eq. (11) should be more correctly written in terms of number of active sites present on the stationary phase (m_L) instead that V_s: $k' = K_D m_L / V_0$. This is common in affinity chromatography, when receptor (here the chiral selector) is immobilized on the chromatographic particles. This means that if m_L changed with the ionic strength, then also the retention factor would vary (without any variation of K_D). Systematic studies of the dependence of the saturation capacity on the ionic strength of the medium are, therefore, necessary in order to better validate the Donnan model. Further investigations are being carried out in this respect.

Table 9
Slopes of the ln k' vs. ln[NH_4^+] plots for *N*-Ac-amino acids and corresponding squares of the correlation coefficients

	Slope	R^2
N-Ac-Gly	1.17	0.93
N-Ac-Ala *D*	0.55	0.99
N-Ac-Val *D*	0.56	0.99
N-Ac-Leu *D*	0.57	0.99
N-Ac-Φ-Gly *D*	1.12	0.97
N-Ac-Phe *L*	0.82	0.98
N-Ac-Phe *D*	0.57	0.98

Mobile phase composition, buffer–MeOH (60:40).

4. Conclusions

Chiral separation on teicoplanin-based CSP is a complex process that involves a relevant number of different kinds of phenomena, including specific interactions between enantiomers and CSP and bulk effects due to the composition of the surrounding medium. The HPLC investigation of possible enantioselective recognition mechanisms for amino acids was achieved by comparing the behavior of the unprotected amino acids with that of amino acids protected in different ways. The effect of the MPs was investigated both for that which concerns the organic modifier kind and the ionic strength of the medium.

It was shown via HPLC that the (deprotonated) carboxylic group is pivotal in the interaction process between molecules and CSP. Compounds able to fit the aglycone basket may create herein several hydrogen bonds (through COO^-) with the aglycone walls. Once in the cavity, the onset of weak hydrophobic interactions stabilizes, at different extents, the specific complexes. The esterification of the carboxylic group has dramatic effects on the separation of enantiomers, causing the complete loss of the “apparent” enantioselectivity. The understanding of the reasons leading to this phenomenon, however, requires the systematic investigation of the equilibrium adsorption isotherms of these compounds on teicoplanin-based CSPs. On the other hand, the acetylation of the amino group leads to apparently unexpected results. L-Enantiomers are excluded by the CSP, while the corresponding D-enantiomers are strongly retained. The rationale of these phenomena is related to the

presence of repulsive ionic forces arising between negatively charged molecules and CSP, which is also negatively charged. L-Enantiomers cannot enter the aglycone basket and only experience repulsive interactions. D-Enantiomers are, instead, able to penetrate the cavity, where they are strongly stabilized (through the creation of hydrogen bonds). Accordingly, they exhibit large retention volumes, significantly greater than those of the corresponding unprotected amino acids. The amidic hydrogen of acetylated compounds is more acidic than the corresponding hydrogen of the original amino acid. It can then be involved in an additional amide–amide hydrogen bond with the CSP, which justifies the significant large retention volumes observed for this kind of compounds.

The effect of the ionic strength of the medium on the behavior of the different classes of compounds investigated has been explained (and controlled) in light of the simple Donnan model.

The exclusion from the aglycone basket of amino acids modified with the introduction of large substituents (BOC group) has been also shown. This case, however, is due to a *real* hindrance effect and substantially differs from the exclusion of *N*-Ac compounds, for which the definition of "enantioselective ion-exclusion chromatography" can be properly used [15].

Finally, different MP modifiers have been shown to have different effects on the separation of amino acids. In particular, selectivity appears larger with MeOH than with ACN modifier.

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