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3-(Phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole: a fascinating molecular framework to study the enantioseparation ability of the amylose (3,5-dimethylphenylcarbamate) chiral stationary phase. Part I. Structure-enantioselectivity relationships.

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Highlights

- This paper describes the HPLC enantioseparation of a set of six chiral pyrazolines on the IA-3 CSP.
- By using pure methanol as a mobile phase an $\alpha = 50$ was observed for one of them.
- HPLC data suggested the involvement of solvophobic interactions in the unusual recognition mechanism.

Abstract

Chiral stationary phases (CSPs) based on amylose (3,5-dimethylphenylcarbamate) (ADMPC) exhibit a wide-range of enantioselectivity in high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Although this class of CSPs has been extensively used, chiral discriminations at receptorial level, which are useful to develop predictive molecular models, have been rarely reported in the literature.

Herein, we describe the results obtained in the enantioselective HPLC of a set of six C5-chiral 4,5-dihydro-(1*H*)-pyrazole derivatives on the ADMPC-based Chiralpak AD-3 CSP (CSP) under normal-phase and polar organic conditions. Using pure methanol as a mobile phase the exceptional enantioseparation factor value of 50 at 25 °C was found for one of the investigated analytes. To the best of our knowledge, the enantiomeric bias represents the most outstanding enantioseparation ever recorded on ADMPC-based CSPs.

Systematic variations in chemical groups in specific positions of the 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole molecular framework resulted in peculiar changes in retention and enantioselectivity. A careful analysis of the chromatographic data permitted to advance some hypotheses concerning the role played by the individual chemical groups in determining the exceptional enantioseparation.

In particular, under methanol-rich mode, the prenyl moiety of the second eluted enantiomer of the better resolved analyte was recognized as a critical structural element to establish direct and favorable solvophobic interactions with apolar portions of selector.

Keywords: Enantiomer separation; Receptorial-like enantioselectivity; Chiral stationary phase; Chiralpak AD-3; Structure-enantioselectivity relationships; solvophobic interaction.

1. Introduction

HPLC on chiral stationary phase (CSP) is the most widely used technique for separation of enantiomers of chiral compounds. Method development for a chiral separation involves the selection of an appropriate CSP. Despite the tremendous advances over the past few years in many aspect of chiral column technology, the strategy adopted to achieve the desired enantioseparation is remained essentially the same, being it again based on empirical approaches. This is mainly due to a limited knowledge of chiral recognition mechanism of the most CSPs.

Consequently, in order to minimize time-consuming and unproductive studies, it is convenient to screen a limited number of CSPs with recognized high chiral discrimination ability.

Although a number of enantioselective stationary phases have been described in the literature or are commercially available, only a few of them have demonstrated broad chiral selectivity and effectiveness in a wide range of eluent conditions. These include polysaccharide CSPs prepared by conversion of hydroxyl groups of monosaccharide units of cellulose or amylose to arylcarboxylate or arylcarbamate moieties and their fixing (by physical coating or chemical immobilization) onto macroporous silica particles [1-4].

A survey by Okamoto et al. on the HPLC determination of the enantiomeric excess (ee) published in Angewandte Chemie International Edition in 2012 highlights that in 97% of cases the enantioselective analyses were carried out on polysaccharide-based CSPs [5]. Moreover, the 40% of enantioseparations were performed using the Chiralpak IA and Chiralpak AD CSPs which both contain amylose (3,5-dimethylphenylcarbamate) (ADMPC) as a chiral selector.

The field of application of the polysaccharide-based CSPs is not only restricted to non-racemic compounds obtained by asymmetric synthesis but extends to other important classes of chiral analytes. Huybrechts et al. have reported a survey on the HPLC resolution of a set of 150 chiral analytes of pharmaceutical interest variously functionalized (117 basic, 27 neutral and 6 acid compounds) [6]. In 85% of the investigated chiral drugs the HPLC enantioseparation was achieved using only four coated-type polysaccharide-based CSPs (Chiralcel OJ-H, Chiralcel OD-H, Chiralpak AD-H and Chiralpak AS-H CSPs). Once again, the ADMPC-based Chiralpak AD-H CSP produced the best enantioselectivity.

So, according to literature, ADMPC may be included among the chiral selectors with the broadest spectrum of enantioselectivity.

Despite the experimental works on ADMPC-based CSPs, not much is known about the nature of the selectand-selector interactions at molecular level, thus making their chromatographic behavior difficult to predict.

As demonstrated in our previous works, investigations on chiral discrimination mechanism of polysaccharide-based CSPs can be facilitated by designing and analyzing chiral probes whose enantiomers show large differences in the free energy of interaction with the CSP [7-9]. Selectand-selector systems that display receptor-like enantioselectivity [10,11] (i.e. α>15 corresponding to difference in free energy of interaction larger than 1.5 kcal mol^{-1} [12]) can be promising references for the *in silico* construction of molecular models of polysaccharide selectors able to account the observed discrimination and to elucidate at molecular level the interactions operating in the enantioseparation process [7,9].

Besides providing a very high enantioselectivity, an ideal molecular framework for developing reliable predictive models should meet the following requirements:

i) be easily synthesizable and functionalizable;

ii) its functionalization should lead to a large fluctuation in enantioselectivity;

iii) the introduction of elements of molecular diversity should produce in one or more terms of the series of chiral compounds an inversion of the chiral discrimination and, therefore, of the enantiomer elution order.

In this article, the chromatographic behavior of a series of chiral compounds incorporating the 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole scaffold on the coated-type ADMPC-based Chiralpak AD-3 CSP is shown. The functionalization of the molecular framework in the insertion points O, C5, and N1 is quite easy to achieve and offers the possibility of placing different functional groups in key positions for chiral recognition. The analytes were chromatographed under normal-phase (NP) and polar organic (PO) conditions and their enantiomer elution order was determined. Based on the experiential retention and enantioselectivity data, some hypothesis concerning the molecular fragments responsible for enantioselectivity and the nature of interactions occurring in the enantiodiscrimination process have been formulated.

2. Experimental

2.1. Enantioselective HPLC

Analytical HPLC analysis of **1**–**6** was performed using the commercially available 100 mm x 4.6 mm I.D. Chiralpak AD-3 column (Chiral Technologies Europe, Illkirch, France). HPLC solvents were purchased from Aldrich (St. Louis, MO, USA). HPLC apparatus consisted of a Perkin-Elmer (Norwalk, CT, USA) 200 Lc pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 1000-μL sample loop, a HPLC Perkin-Elmer oven and a Perkin-Elmer detector. The signal was acquired and processed by Clarity software (DataApex, Prague, Czech Republic).

The standard solutions were prepared by dissolving about 1.0 mg of sample into 25 mL of ethanol. The injection volume was 20–50 μL.

*2.2. Synthesis of **6***

Racemates **1**-**5** were synthesized by a chemical pathway reported elsewhere [8,13]. The synthetic procedure and ^{1}H and ^{13}C NMR characterization data for the novel pyrazoline **6** are reported in Fig. S1 of Supporting Information (SI).

2.3. Absolute configuration and enantiomeric elution order

Semipreparative HPLC separations of the enantiomers of **1**-**4** and **6** were carried out on the commercially available 250 mm x 10 mm I.D. Chiralpak AD column using pure methanol (in the case of **2**, **3**, **4** and **6**) and ethanol (in the case of **1**) elution modes. The temperature was set at 25 °C. The flow rates were 2.5 mL min^{-1} with ethanol and 4.5 min^{-1} with methanol. The amounts of racemic samples resolved for single chromatographic runs ranged from 2 to 10 mg.

The absolute configuration of the enantiomers isolated on a semipreparative scale was empirically established by CD correlation method using as references the enantiomers of **1** [7,9,14] and a structurally related pyrazoline of known stereochemistry previously published by our group [15]. The CD spectra (Fig. S2 of SI) were recorded in ethanol at 25 °C by using a Jasco Model J-700 spectropolarimeter. The optical path was 0.1 mm. The spectra are average computed over three instrumental scans and the intensities are presented in terms of ellipticity values (mdeg).

The enantiomeric elution order on the Chiralpak AD-3 CSP was established by analyzing non-racemic samples enriched by the (*S*)-enantiomer and modulating the elution of second eluted enantiomer using the stopped-flow based procedure as previously described [14].

3. Results and discussion

*3.1 High enantioselectivity of **1** under methanol mode*

The preliminary step of our study was to analyze a series of chiral compounds with 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole framework using a 100 mm x 4.6 mm ID column packed with 3-μm particles of Chiralpak AD-3 CSP and pure methanol as a mobile phase.

The highest value of enantioselectivity was achieved for the compound **1** (Fig. 1) which showed the exceptional enantioseparation factor of 50.3 at 25 °C (corresponding to a difference in free energy of interaction between the two enantiomers of 2.3 kcal mol^{-1}). Such a high value is uncommon in enantioselective HPLC on ADMPC-based CSPs which exhibits very broad spectrum of enantioselectivity but rarely α-values at receptorial level.

3.2 Structure-enantioselectivity relationships under methanol mode

Starting from the 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole scaffold of **1**, the substituents linked to the O, C5 and N1 atoms were changed one at a time in order to obtain a series of pyrazolines useful for the study on structure-enantioselectivity relationships.

Fig. 1 shows the most significant pyrazolines obtained and the relevant chromatographic data on AD-3 CSP using pure methanol as a mobile phase.

Comparing the values of the retention and enantioselectivity factors of **1** with those of the corresponding analogues **2** and **3**, it is clear that the prenyl substituent at the oxygen atom of the phenolic moiety is one of the structural elements responsible for the exceptionally high enantioselectivity of **1** on the AD-3 CSP. The replacement of the alkenyl fragment with a phenyl group (compound **2**) or a hydrogen atom (compound **3**) led to a dramatic decrease in enantioselectivity (from 50.3 to 2.1 in the case of compound **2** and 7.2 in the case of compound **3**).

Inspection of retention data highlights that the lowering in enantioselectivity recorded for the compound **2** is essentially due to a severe decrease in retention factor of the more retained enantiomer (from 170.4 to 10.1). In the case of compound **3** the reduction of retention involves also the first eluted enantiomer, although much less pronounced respect to that observed for the more retained enantiomer. These data lead to hypothesize a critical engagement of the prenyl chain both in chiral recognition and retention mechanisms.

The second functional group removed from the molecular framework of **1** was the thiocarbamoyl portion. This group is potentially capable of establishing various types of significant attractive electrostatic interactions with complementary CONH sites of ADMPC [16]. Despite the presence of a polar group such as the acetyl substituent, the replacement at N1 (compound **6**) gave a significant decrease in resolution of the derivative (α = 2.0) due to a strong drop in retention of the second eluting enantiomer (k_2 changed from 170.4 to 7.2). This chromatographic effect, which is quantifiable as a loss of 1.9 kcal mol^{-1} in the global amount of selector-selectand association free energy (ΔG_{ass}), may be imputable to the loss of a N-H site capable to establish a H-bond as the donor towards a carbamic group of the CSP. Indeed, the above quoted reduction of the ΔG_{ass} quantity, relevant to the retention of the (*R*)-enantiomer, appears widely compatible with the one typically involved in a single C=O$\cdots$H-N interaction, established between amide groups [17].

Keeping fixed the prenyl group, the substitution of the thiocarbamoyl moiety with a methyl group (compound **4**) again led to a less resolved compound (α = 2.3). Furthermore, in the latter case, the drop in enantioselectivity was accompanied by an inversion of the enantiomer elution order. It is worth noting that, although the methyl group is unable to establish interactions with polar portions of the CSP, the retention of both the enantiomers of **4** was higher than those of the acetyl analogue **6**. This strongly supports the hypothesis that a change of chiral discrimination mechanism takes place in this case, evidently induced by the complete loss of possible H-bond interactions at level of the group bound to N1.

With the exception of the compound **4**, the elution order of the enantiomers of all the other compounds resolved in this study was invariant, with the (*S*)-enantiomer eluting first.

A closer look to the data collected in Fig. 1 permits other considerations about the role played by the phenyl substituent linked to the stereogenic center C5 in the enantiodiscrimination mechanism. In fact, when the aromatic ring was replaced by a methyl group, giving rise to the formation of compound **5**, the chiral discrimination was completely lost. Thus, the phenyl group must be certainly responsible for one of the interactions needed for the exceptional chiral recognition of **1**, such as solvophobic, C-H···π, π···π or steric interactions. According to these considerations, the racemate **5** was unresolved in any of the analytical conditions compatible with the AD-3 CSP (e.g. normal and polar organic- mobile phases containing *n*-hexane, ethanol, IPA, methanol and acetonitrile).

*3.3 Influence of mobile phase composition on retention and enantioselectivity of **1** and **3***

Qualitative conclusions achievable through the comparison of chromatographic data pertinent to compounds **1**-**6** suggest that the exceptionally high enantioselectivity of **1** on the AD-3 CSP cannot be attributed to only one pivotal group but to the synergistic action of the chemical groups at N1, O and C5 positions.

However, keeping our focus on the high enantioselectivity of **1** and considering the lack of attention usually dedicated to non-polar groups of selectands, in this work it was mainly investigated the influence of the prenyl group on the enantiorecognition process. Based on the evidence that the absence of this moiety in **3** led to a dramatic drop in retention, especially of the second eluting (*R*)-enantiomer, further investigations on the chromatographic behavior of the two pyrazolines **1** and **3** using different alcoholic elution modes were set. The idea was that if the prenyl group was involved in attractive interactions with complementary apolar portions of CSP, such interactions would critically depend on the nature of the mobile phase just for **1**. To test this hypothesis besides methanol, the pyrazolines **1** and **3** were analyzed on the AD-3 CSP also using pure ethanol and 2-propanol (IPA) as mobile phases. A comparison of the retentions and enantioselectivities obtained

in the three analyzed alcoholic elution modes is shown in Fig. 2. As predicted, a strong dependence of both retention of the selectands and chiral recognition ability of the AD-3 CSP was found to be related to the employed eluting alcohol. More in particular, passing from methanol to ethanol, and then to IPA, the enantioselectivity decreased in a strong and progressive way. In the case of **1** the values of α lowered from 50.3 to 11.7 in ethanol and to 2.7 in IPA, while the same factor ranged between 7.2 and 2.2 for compound **3**. Interestingly, the trend of the enantioseparation factor of **1** turned out to be closely related to the retention of the second eluting enantiomer. As an example, the value of k'_2 of **1** dramatically diminished from 170.4 to 46.8 and 1.2 in ethanol and in IPA, respectively. This trend is in sharp contrast to what is usually reported in the literature, where the eluting power of methanol is commonly recognized to be significantly higher than that of IPA. Thus, the unusual trend observed for k'_2 of **1**, by passing from methanol to IPA, strengthens the hypothesis that the prenyl portion may be actually involved in the establishment of solvophobic forces with non-polar molecular portions placed at the surface or in suitable clefts of the CSP structure. Differently, and coherently with the exclusion of the prenyl moiety the fluctuations in k_2 of **3** under the three alcoholic conditions of analysis were much less significant.

However, as evidenced by solid-state NMR studies, it should be taken into consideration that different conformational distributions of ADMPC can exist, depending on the type of alcohol used as/in the mobile phase [18]. So, the particular chromatographic effects observed for **1** in different alcoholic elution modes could also be partially attributed to conformational alterations of the chiral environment characterizing surface and cavities of the CSP.

Although the change in solvent may impact not only the analytes but also the structure of the stationary phase, it is plausible that solvophobic effects, under methanol mode, may participate in the unusual enantioseparation and retention of **1**. However, it is unclear whether such effects can take place in binary systems in which the alcohol is mixed with non-polar solvents.

Under conditions of a progressive increase of alcohol percentage, the enantioselectivity and retention of the chromatographic system were expected to change linearly just in a limited range of

alcohol concentrations, that is, long as the used mixture maintains a permittivity (ε) high enough to allow the establishment of appreciable selector-selectand solvophobic interactions.

In perfect agreement with such a prediction, as shown in Fig. 3, when a growing volume of methanol is added to *n*-pentane, the retention of (*R*)-**1** (*i.e.*, the second eluting enantiomer of **1**) progressively decreases, but just until a critical alcohol content, corresponding to 70%, is reached. Under this range of alcohol percentages, methanol acts in a classical way, manifesting growing eluting power with increasing of its concentration. Beyond such a critical mobile phase composition, the trend reversed, so that any further additions of methanol produced an increase in retention, with the maximum value of k_2 reached under pure methanol condition. Evidently, under this second highly polar range of mobile phase composition (ε changes from about 23 to 33), methanol acts promoting the establishment of solvophobic interactions that increase their strength on increasing of the solvent permittivity. As a consequence of such a bimodal behavior, the retention-plot of (*R*)-**1**, expressed as a function of methanol percentage in mobile phase, assumes a characteristic U-shape pattern. Differently, for the enantiomer (*S*)-**1** the U-shape pattern is barely visible in the zone of the highly polar mobile phase composition.

Also in the case of (*R*)-**3** (*i.e.*, the second eluting enantiomer of **3**) the retention trend was not monomodal but the right branch of the U-shape retention map appeared much more flattened than that of (*R*)-**1**, and quite similar to that observed for (*S*)-**1**.

Similar but less pronounced trends of enantioselectivity and retention of **1** and **3** were observed using *n*-hexane-ethanol mixtures (see Fig. S3 of SI).

Thus, by reassuming, the anomalous retention patterns shown in Fig. 3 and S3 clearly delineate two retention domains.

On the left side of the minimum, the alcohol acts as the strongest eluting solvent and its percentage increase leads to a sharp decrease in retention of the (*R*)-enantiomer. This area is commonly established for NPLC, *i.e.* methanol and ethanol work as typical organic modifiers in normal phase eluent mixtures weakening attractive interactions between polar portions of selector and selectand.

On the right side of the minimum, alcohol modifier acts as the least eluting solvent and the increase of its content results in longer retention. It is therefore clear that interactions of different nature respect to those normally experienced in normal-phase mode were operative and played a predominant role in the retention mechanism.

A possible explanation for interpreting the difference in retention trends between the (*R*)-enantiomers of **1** and **3** is to hypothesize the involvement of apolar areas of contact between CSP and the prenyl portion of the (*R*)-**1** in establishing solvophobic interactions, modulated in their intensity by the different alcoholic nature of the eluent.

An interesting manifestation of the unusual retention behavior can be observed in Fig. 4 in which are compared the chromatograms pertinent to the resolution of **1** under NP and solvophobic elution modes. It can be noted that, despite their very different polarity, methanol-poor (containing only 20% of methanol) and pure methanol eluent conditions produced similar retention of the most retained enantiomer.

As a conclusive remark on the effects exercised by the prenyl group on the exceptional enantioresolution of compound **1**, it can be observed that only within the region of solvophobic interactions the α factor increases its values. These, in fact, become higher as the methanol content increases over 70% (see the right branch of the graph α vs. %MeOH shown in Fig. 3), following the anomalous retentive behavior of the (*R*)-enantiomer (*i.e.*, the α values changed from about 20 with the 20-70% methanol content to 50 with pure methanol). In the same region of the plot, the change in enantioselectivity for **3** was much smaller because of the lack of contribution coming from the prenyl group to the retention of the second eluted enantiomer.

4. Conclusions

In summary, the retention and enantioselectivity factors of six structurally similar chiral analytes incorporating the 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1*H*)-pyrazole scaffold obtained by HPLC on the ADMPC-based AD-3 CSP are reported. Among these chiral compounds, only **1** showed

receptorial-like enantioresolution under methanol-rich and pure methanol elution modes. Through a systematic chemical variation at three critical points of molecular framework, we have established a coherent structure−enantioselectivity relationship around this molecule class. A careful assessment of chromatographic data pointed out the importance of the simultaneous presence of the following chemical groups to obtain a high degree of chiral recognition: (i) a prenyl group at O position; (ii) a thiocarbamoyl group at N1; (iii) a phenyl group linked to C5 stereogenic center. This evidence supports the hypothesis that the general recognition mechanism of **1** is not driven by the pivotal intervention of a leading group or atom but, differently, by the synergic participation of more sites. It was also demonstrated through a meticulous study of the influence of mobile phase composition on retention, that the uncommon solvophobic forces between the prenyl group of the second eluting (*R*)-enantiomer of **1** and apolar moieties of AD-3 CSP under methanol-rich eluent conditions occurred.

Although statistically significant structure-enantioselectivity relationships need relatively larger series of data; the purely empirical approach for chiral recognition presented in this work can be considered as a useful reference to establish strategy and methods of property predictions.

Further investigations aimed at developing a deeper understanding of the role played by the prenyl group in the exceptionally high enantioselectivity of compound **1** will be the subject in our next paper.

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CAPTIONS

Fig. 1. Structures, enantioseparation and retention factors of **1**–**6**.

Structural fragments necessary for a high enantioselectivity are shown in red. The color blue is used to visualize fragments reducing enantioselectivity. The notations (*R*)<(*S*) and (*R*)>(*S*) indicate the elution sequence of enantiomers. Chromatographic conditions: column, Chiralpak AD-3 100 mm × 4.6 mm I.D.; eluent, pure methanol; flow-rate, 3.5 mL min^{-1}; temperature, 25 °C; detection, UV at 325 nm.

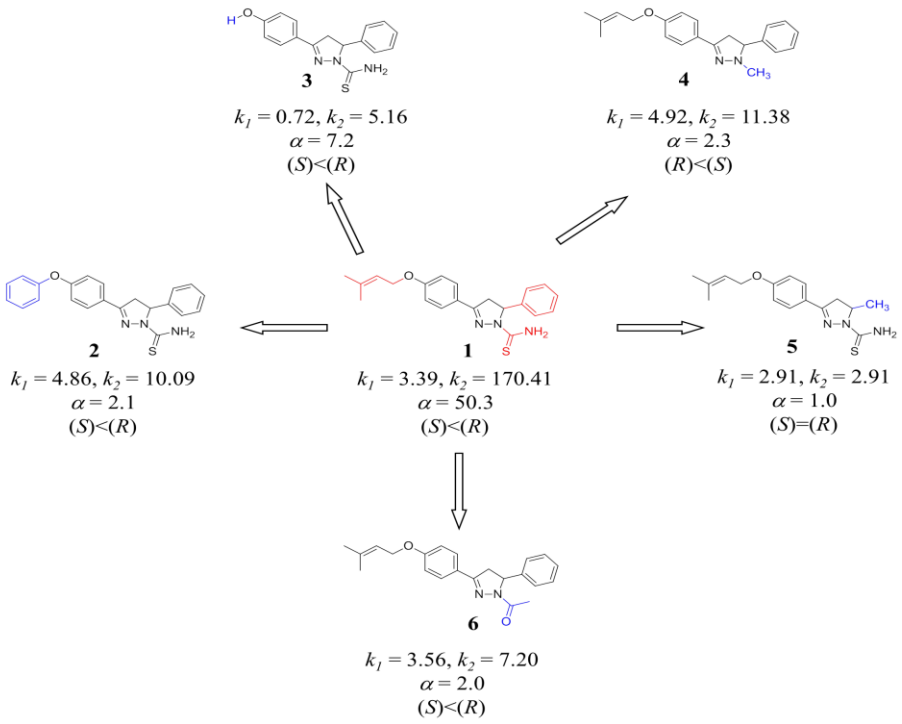

Fig. 2. Enantioseparation and retention factors of **1** and **3** using pure methanol (MeOH), ethanol (EtOH) and 2-propanol (IPA) as eluents.

Chromatographic conditions: column, Chiralpak AD-3 100 mm × 4.6 mm I.D.; eluents, pure methanol (MeOH), ethanol (EtOH) and 2-propanol (IPA); flow-rate, 1 (IPA), 2.0 (EtOH) and 3.5 (MeOH) mL min^{-1}; temperature, 25 °C; detection, UV and CD at 325 nm.

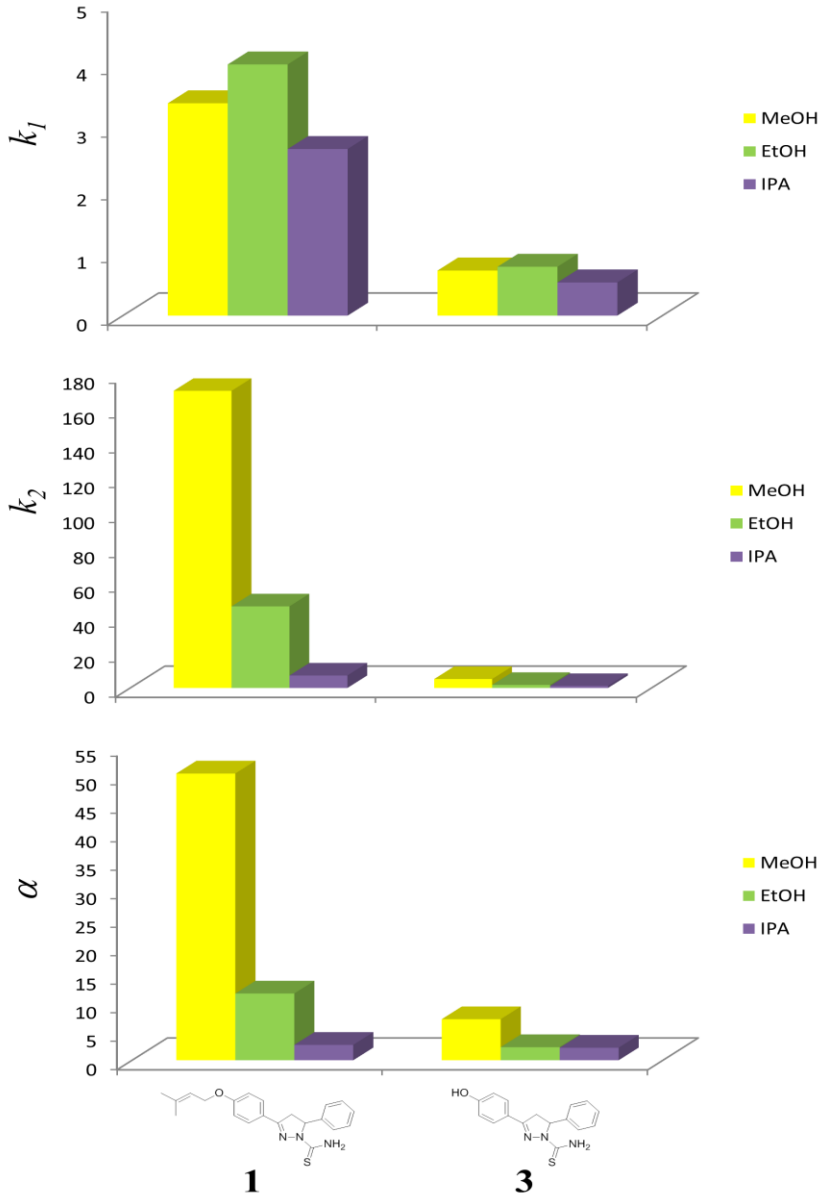

Fig. 3. Plots of the retention and enantioselectivity factors of **1** and **3** as a function of the methanol content in *n*-pentane–methanol mixtures.

Chromatographic conditions: column, Chiralpak AD-3 100 mm × 4.6 mm I.D.; eluent, *n*-pentane-methanol mixtures; flow-rate, 3.5 mL min^{-1}; temperature, 25 °C; detection, UV at 325 nm.

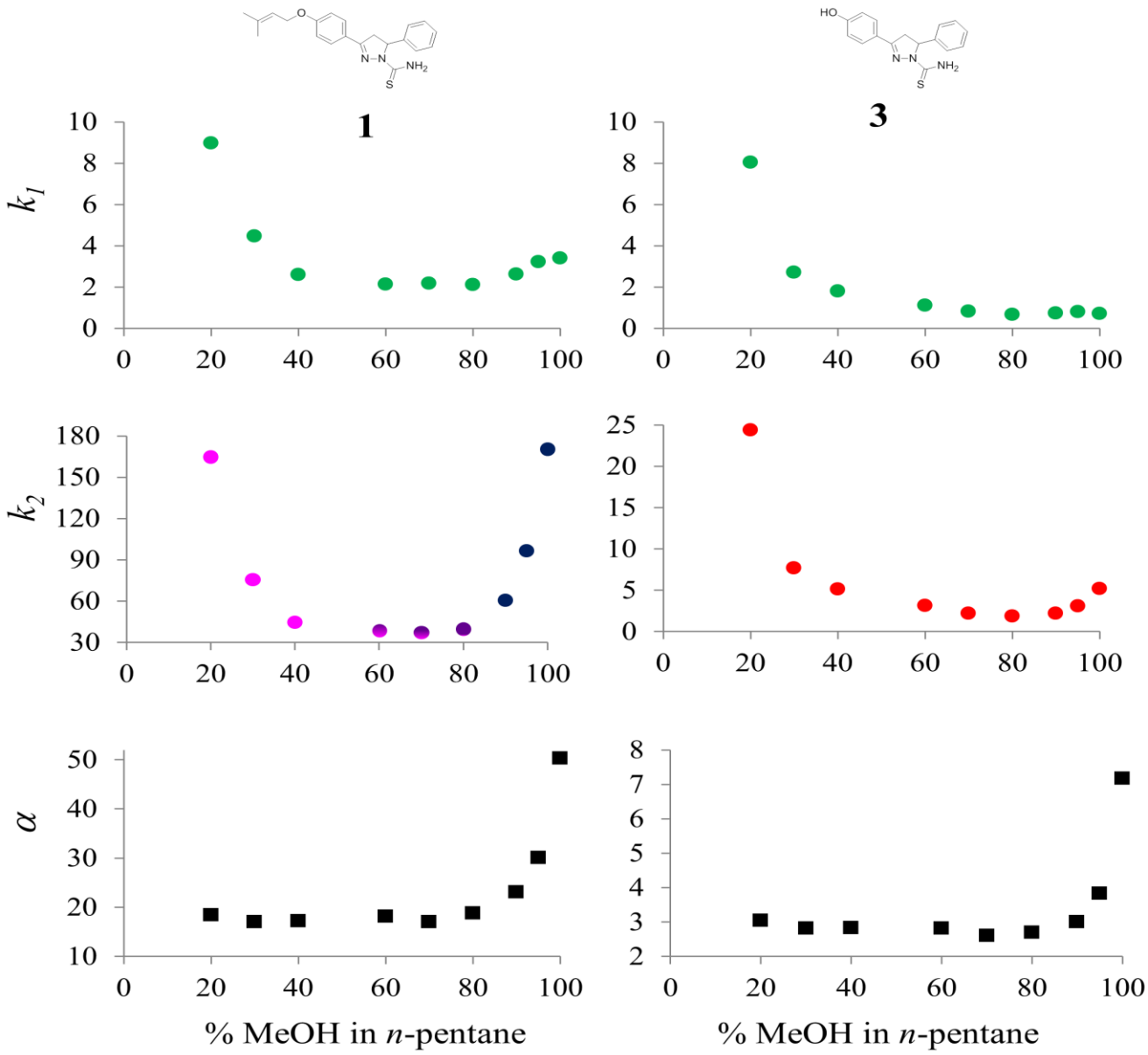

Fig. 4. Typical HPLC chromatograms illustrating the separation of enantiomers of **1** under NP and solvophobic elution modes.

Chromatographic conditions: column, Chiralpak AD-3 100 mm × 4.6 mm I.D.; flow-rate, 3.5 mL min^{-1}; temperature, 25 °C; detection, UV at 325 nm.