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Peptide enantiomer separations: Influence of sequential isomerism and the introduction of achiral glycine moieties on chiral recognition

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Abstract

The influence of sequential isomerism and the introduction of achiral, conformationally flexible glycine moieties into a peptide chain on the chiral recognition mechanism of a cinchona alkaloid based chiral selector has been evaluated. For this purpose, enantiomers of N-terminally protected alanine–glycine di- and tripeptides were separated by liquid chromatography–mass spectrometry on a corresponding chiral stationary phase (CSP). To obtain complementary information, the reversed phase retention behaviour of the various peptides was also evaluated and subsequently used to further elucidate the chromatographic characteristics of the CSP. For peptides that contained glycines in the N-terminal region chiral recognition was compromised, while glycines located at the C-terminus had no or little negative effect.

Keywords: Peptides; Chiral recognition; Enantiomers; Sequence isomers; Glycine; Liquid chromatography–mass spectrometry

1. Introduction

α-Amino acids and peptides as their oligomers form an important group of analytes in the area of enantiomer separations. While the only structural variability of α-amino acids lies in their side chains, peptides offer an additional possibility for variation based upon sequential isomerism, i.e. identical amino acid composition but different arrangement of the amino acid residues within the peptide chain. Each sequence isomer may influence the chiral recognition process in a specific way and consequently affect the respective enantiomer separation result.

This dependence of the enantiomer discrimination on the arrangement of given amino acid residues in the peptide sequence has been noted and discussed in several studies dealing with the separation of peptide enantiomers. In two early investigations using gas chromatography and Chirasil-Val® as chiral stationary phase (CSP) the enantiomer elution order was reversed between Leu-Gly and Gly-Leu [1,2]. Regarding peptide enantiomer separations by HPLC, a pronounced influence of sequential isomerism on the chiral recognition process has been described in several publications. Markedly differing enantioselectivities were observed for the two pairs of enantiomers of Ala-Leu and Leu-Ala on a crown ether based CSP [3] and for the Ala-Gly/Gly-Ala and Leu-Gly/Gly-Leu isomers on teicoplanin [4], teicoplanin aglycone [5] and crown ether [6] based CSPs. In enantiomer separation studies of di- and tripeptides consisting of one or two glycine moieties, respectively, plus an alanine or leucine residue employing different cyclodextrin derived CSPs separability and elution order varied depending on the sequences [7,8]. In a study of the pairs of enantiomers of Leu-X and X-Leu, where X was Phe, Tyr or Trp, on a chymotrypsin CSP the magnitude of enantiomer resolution was affected depending on the location of the aromatic amino acid and a reversal of the enantiomer elution order occurred in some cases [9]. Turning to investigations where capillary zone electrophoresis (CZE) was used as separation technique, differences between sequence isomers regarding enantiomer separation results were observed in a number of studies by

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Scriba and co-workers. They employed various native and modified cyclodextrins and studied the chiral recognition of Gly-Asp-Pro/Pro-Asp-Gly [10], Ala-Phe/Phe-Ala [11–14], Ala-Leu/Leu-Ala [13] and Gly-Ala-Phe/Ala-Gly-Phe [14]. In the studies dealing with Ala-Phe/Phe-Ala and Ala-Leu/Leu-Ala the (*R*,*R*)- and (*S*,*S*)-enantiomers were resolved less often for the peptides with Ala at the C-terminus than for those with Ala at the N-terminus [11–13]. Moreover, a reversal in the enantiomer migration order was observed in some cases for Ala-Phe/Phe-Ala [11,14] and Gly-Ala-Phe/Ala-Gly-Phe [14].

A lot of the peptides investigated in enantiomer separation studies contain a glycine residue. The incorporation of an achiral glycine moiety into a peptide chain with chiral amino acid units does not only reduce the number of possible stereoisomers but also adds an increment of conformational flexibility due to the lack of a side chain. Consequently, this change in the peptide's steric properties and behaviour can be expected to also affect its chiral recognition. Therefore, a systematic variation of the position(s) and the number of glycine residues that are introduced into a peptide chain offers the possibility of assessing the role a specific amino acid residue within a peptide plays in the stereoselective recognition process of the latter. The effects of replacing a chiral amino acid by glycine or inserting/adding a glycine moiety within/at the end of a peptide chain have been investigated in some HPLC and CZE studies of peptide enantiomer separations. The elongation of Ala-Gly to Ala-Gly-Gly was found to increase enantioselectivity in HPLC using a crown ether based CSP [6]. In HPLC investigations using cyclodextrin derived CSPs the addition of one or two glycine moieties to an amino acid or dipeptide increased the retention of the analytes and the separation of the enantiomers was often enhanced [7,8,15]. In some cases, a reversal of elution order occurred. The position(s) of the glycine residue(s) relative to that of the chiral amino acid significantly altered the chiral recognition process [8]. Pirkle et al. utilised the introduction of a glycine moiety between the amino acids of a dipeptide to assess whether the stereochemistry of the second amino acid residue still manifested itself in the chromatographic behaviour of the peptide enantiomers on various "Pirkle-type" CSPs, if it was moved away from the principle interaction site [16]. Finally, the effects of either replacing a chiral amino acid by glycine or adding/inserting an extra glycine residue in Ala-Phe on the migration order of the (all-*S*)- and (all-*R*)-enantiomers in CZE were studied by Sabbah and Scriba and marked differences were observed [11].

The family of cinchona alkaloid derived chiral selectors has been shown to be highly suited for the discrimination of N-protected peptide enantiomers [17–23]. Several studies showing successful HPLC separations, where CSPs containing immobilised versions of the selectors were used, have been reported in the recent years [18,19,21,22]. In HPLC applications that employ a reversed-phase type (hydro-organic) mobile phase the enantioselective discrimination of this class of selectors is based on a weak anion-exchange functionality, which makes acidic compounds the prime analyte target group. The primary ion-pairing process is non-enantioselective and mainly serves the purpose of bringing the selector and the analyte close enough together so that secondary enantioselective interactions that include hydrogen-bonding, π–π-stacking and steric interactions can come into effect and yield the desired enantiomer separation [18]. Besides their ion-exchange characteristic and the resultant retention behaviour, the cinchona alkaloid based CSPs also exhibit a reversed phase (hydrophobic) retention increment, which is non-enantioselective [24]. Therefore, the overall retention of an N-terminally protected peptide (and of any other acidic analyte) is a combination of the ion-exchange process and the hydrophobic interactions. Thus, an examination of the differences between the enantiomer separation behaviours of peptides with different sequences on a cinchona alkaloid based CSP will be aided and enhanced by a deconvolution of these two contributions, which can be accomplished by measuring the reversed phase retentions of the peptides.

In the present study, the influence of introducing one or two glycine residue(s) into an oligoalanine di- or tripeptide chain at different positions on the chiral recognition by a cinchona alkaloid chiral selector is investigated. To this end, the enantiomers of the various peptides were separated by HPLC employing an immobilised version of the selector in the form of a CSP. Arising from the considerations discussed above, these experiments were accompanied by an analysis of the reversed phase behaviour of the peptide set.

2. Experimental

2.1. Materials

The amino acids and most of the peptides were purchased from Sigma–Aldrich (Steinheim, Germany) or Bachem (Bubendorf, Switzerland). Those peptides that were not commercially available were synthesized as *N*-3,5-dinitrobenzoyl derivatives by piChem (Graz, Austria) according to standard protocols. 3,5-Dinitrobenzoyloxysuccinimide was prepared from 3,5-dinitrobenzoyl chloride (Sigma–Aldrich) and hydroxysuccinimide (Fluka, Buchs, Switzerland) by Hünig base coupling. Ammonium acetate, acetic acid, sodium hydrogencarbonate and sodium carbonate were obtained from Fluka. HPLC grade methanol (Merck, Darmstadt, Germany) and doubly distilled water were used throughout.

The preparation of the *tert*-butylcarbamoylquinine chiral selector has been described elsewhere [25]. The corresponding chiral stationary phase was obtained by subsequent coupling of the selector to thiol-modified silica gel (Kromasil 100-5 μm, Eka Chemicals, Bohus, Sweden) [25]. The 5 μm CSP particles were slurry-packed into a 150 mm × 4.0 mm I.D. stainless steel column (Austrian Research Centers, Seibersdorf, Austria). A 150 mm × 4.6 mm I.D. BetaBasic-18 column containing a C_{18} stationary phase with 3 μm

particle size (Thermo Hypersil-Keystone, Runcorn, UK) was used for the reversed phase (RP) separations.

2.2. Derivatisation procedure

The *N*-3,5-dinitrobenzoyl DNB derivatives of the peptides were prepared as follows: 5 μmol of the amino acid and peptides, respectively, were dissolved in 900 μl carbonate buffer (0.1 mol/l sodium hydrogencarbonate/0.1 mol/l sodium carbonate, 2/1, v/v). 300 μl of a saturated solution of 3,5-dinitrobenzoyloxysuccinimide in acetonitrile were added and the reaction was carried out at 60 °C over night.

2.3. Liquid chromatography–mass spectrometry (LC–MS) separations

LC–MS separations were carried out using an HP1100 series high-performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PE Sciex API 365 triple quadrupole mass spectrometer (MDS Sciex, Concord, Canada) equipped with an electrospray ion source. The LC effluent was split in an approximately 1:100 ratio before entering the mass spectrometer. MS detection was performed in the selected ion mode employing negative ionisation with an ionspray voltage of −4200 V.

The mobile phase for enantiomer separations was composed of a mixture of 80% methanol and 20% 0.1 mol/l ammonium acetate (v/v), to which 0.3% (v/v) acetic acid were added to obtain an apparent pH of 6.0. The mobile phase for RP separations was made up of 25% methanol and 75% water (v/v), to which 0.5% (v/v) acetic acid were added. All separations were performed at a flow rate of 1 ml/min. The derivatisation reaction mixtures were diluted five times with mobile phase prior to injection. Aliquots of 50 μl were injected and the columns were thermostated at 25 °C.

2.4. Calculations

log *D* values were calculated for an aqueous system using the ACD/log *D* 7.09 software (Advanced Chemistry Development, Toronto, Canada).

3. Results and discussion

The structures of the alanine–glycine peptides investigated in this study are shown in Fig. 1. The parent amino acids alanine and glycine were included in the sample set as references as were those peptides containing solely alanine or glycine residues (Ala-Ala and Ala-Ala-Ala as well as Gly-Gly and Gly-Gly-Gly, respectively). All possible alanine–glycine sequences of di- and tripeptides were investigated. This set of analytes on the one hand offered the possibility to study the effect of the various sequence isomers on the chiral recognition process, while on the other it allowed the evaluation of the "glycine effect", i.e. the influence of the introduction of one or two achiral, conformationally flexible moieties into the peptide chain. Of all chiral analytes the (all-*R*)- and (all-*S*)-enantiomers were separated and investigated in terms of their chiral recognition by the cinchona alkaloid based chiral selector (Fig. 2a). The other stereoisomers of those peptides that contain two or three stereocentres were not included as no analogues exist for the peptides with only one stereocentre and thus no comparison based upon the "glycine effect" would have been possible.

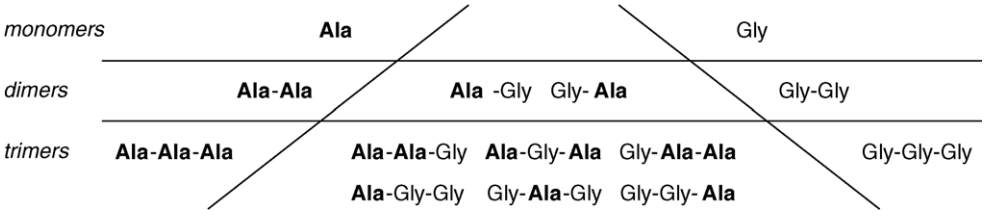

Fig. 1. Investigated alanine–glycine peptides. The blocks in the middle show the shift of a glycine–alanine residue through chains of alanines–glycines.

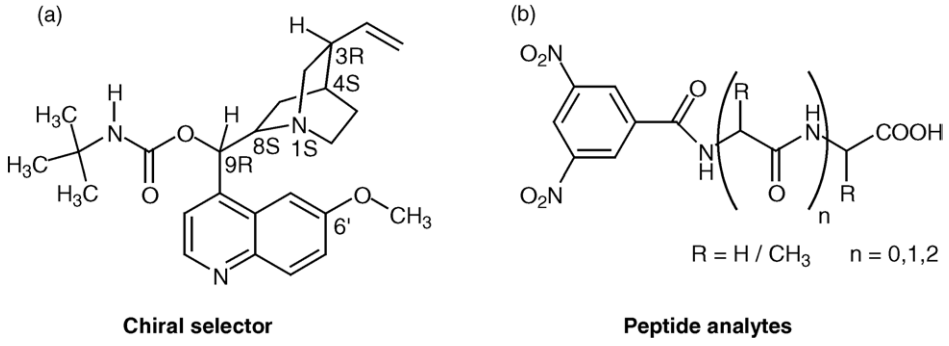

Fig. 2. Structures of (a) the chiral selector and (b) the peptide analytes.

Table 1
LC–MS separation results for the (all-*R*)- and (all-*S*)-enantiomers of alanine–glycine peptides on a cinchona alkaloid based chiral stationary phase

Peptide	Configurations	k_1	k_2	α	R_S	Elution order
Ala	(*R*) + (*S*)	8.86	61.18	**6.91**	19.55	(*R*) < (*S*)
Gly	n/a[a]	24.27		n/a	n/a	n/a
Ala-Ala	(*R*,*R*) + (*S*,*S*)	6.97	37.44	**5.37**	21.25	(*R*,*R*) < (*S*,*S*)
Ala-Gly	(*R*) + (*S*)	8.53	29.13	**3.42**	17.24	(*R*) < (*S*)
Gly-Ala	(*R*) + (*S*)	13.08	16.53	**1.26**	3.12	(*R*) < (*S*)
Gly-Gly	n/a	13.68		n/a	n/a	n/a
Ala-Ala-Ala	(*R*,*R*,*R*) + (*S*,*S*,*S*)	4.83	7.59	**1.57**	4.90	(*R*,*R*,*R*) < (*S*,*S*,*S*)
Ala-Ala-Gly	(*R*,*R*) + (*S*,*S*)	5.78	9.62	**1.66**	5.27	(*R*,*R*) < (*S*,*S*)
Ala-Gly-Ala	(*R*,*R*) + (*S*,*S*)	6.96	8.52	**1.22**	2.39	(*R*,*R*) < (*S*,*S*)
Gly-Ala-Ala	(*R*,*R*) + (*S*,*S*)	6.41	6.76	**1.05**	0.59	(*R*,*R*) < (*S*,*S*)
Ala-Gly-Gly	(*R*) + (*S*)	7.22	11.36	**1.57**	6.12	(*R*) < (*S*)
Gly-Ala-Gly	(*R*) + (*S*)	7.59	7.59	**1.00**	0.00	(*R*) = (*S*)
Gly-Gly-Ala	(*R*) + (*S*)	7.09	7.83	**1.10**	1.17	(*S*) < (*R*)
Gly-Gly-Gly	n/a	7.99		n/a	n/a	n/a

Separation conditions: *tert*-butylcarbamoylquinine based chiral stationary phase; mobile phase: 80% methanol/20% 0.1 mol/l aqueous ammonium acetate + 0.3% acetic acid (v/v); flow rate 1 ml/min; 25 °C; MS detection by selected ion monitoring of the respective $[M-H]^-$ ion.
[a] Not applicable.

All amino acid and peptide analytes were protected at the N-terminus with a 3,5-dinitrobenzoyl (DNB) group (Fig. 2b), which served two purposes: first, it avoided zwitter-ion formation, which would severely disturb or even inhibit the ion-exchange process. Secondly, the π-acidic DNB group offers a site for additional stereoselective interactions with the chiral selector; specifically, the π-basic quinoline group [18,21]. In a previous study the DNB group was found to be the best among a wide variety of protecting groups in terms of achieving high enantioselectivities for peptide analytes [21]. In the present study a maximisation of enantioselectivity was desirable to be able to pick up also minute differences in the chiral recognition of different analytes.

As chiral selector *tert*-butylcarbamoylquinine (Fig. 2a) was employed, which provides a hydrogen bond donor/acceptor system as well as a sterically bulky group, which both enhance the quinine's chiral recognition capabilities [18].

3.1. Influence of the sequence of alanine–glycine peptides on their chiral recognition by a cinchona alkaloid derived chiral selector

The chromatographic results for the various analytes (Fig. 1) are presented in Table 1. Identical hydro-organic mobile phase conditions were employed throughout, which were highly compatible with mass spectrometric detection. The use of MS as detection technique allowed the unequivocal identification of the analytes, which was especially important for those two cases where a coelution with the reagent hydrolysate occurred (data not shown).

With the exception of Gly-Ala-Gly successful enantiomer separation was achieved for all pairs of enantiomers. The enantiomer elution order was identical for all peptides ((all-*R*) < (all-*S*)), except for Gly-Gly-Ala. This phenomenon will be discussed in detail later. Overall, retention factors and enantioselectivities decreased with increasing analyte length, independent of the peptide sequence. An especially pronounced decrease in enantioselectivity was observed upon elongating the peptide chain from two to three amino acid residues. This behaviour has also been noted in previous studies and can be explained by a loss of enantiomer discriminating interactions [18,20]. Its independence from the peptide sequence suggests that this is a general characteristic of the chiral selector that is not limited to certain peptides.

Turning now to the influences of sequential isomerism and the introduction of one or two glycine moieties, some pronounced effects can be seen for both the retention behaviour and the enantioselectivity values. Regarding the retention factors of a pair of enantiomers the respective achiral all-glycine analyte can be regarded as a "neutral" control marker, since it will undergo neither attractive nor repulsive steric interactions with the chiral selector that are based on the configuration(s) of the α-carbon(s) of the chiral amino acid(s). Therefore, one may expect the retention factor of the corresponding all-glycine analyte to be located between the retention factors of the weakly and the strongly retained enantiomer of a certain peptide. An inspection of Table 1 shows that this behaviour holds true for the amino acid and the dipeptides. The agreement of the prediction with the experimental results is also shown in Fig. 3, which depicts the relative locations of the enantiomers of the three chiral dipeptides and of the "reference" Gly-Gly on the chromatographic timescale. For the tripeptides the "neutral" control marker role of Gly-Gly-Gly is fulfilled only for three of the six pairs of enantiomers. It seems that additional effects come into play for the tripeptides, which lead to a different behaviour compared to the amino acid and the dipeptides. Moreover, the relatively low enantioselectivities obtained for the tripeptides bring the respective enantiomers into close proximity on the chromatographic timescale, while at the same time the retentions of the different peptide sequences vary considerably. Thus, it becomes impossible that the retention factor of

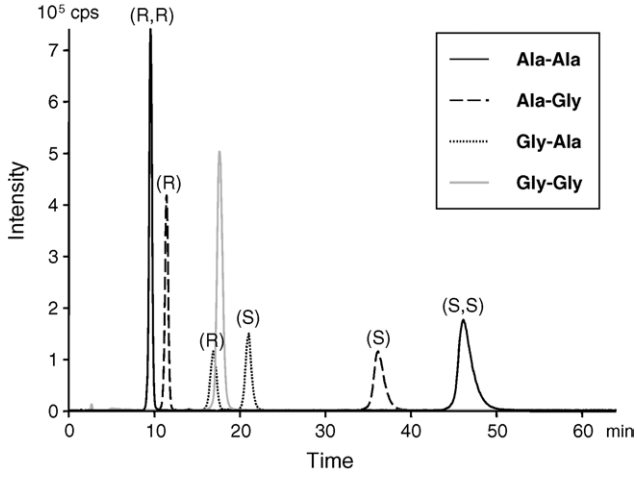

Fig. 3. Overlaid LC–MS chromatograms of the (all-*R*)- and (all-*S*)-enantiomers of Ala-Ala, Ala-Gly and Gly-Ala and of Gly-Gly obtained by selected ion monitoring of the respective $[M-H]^-$ ion. *Conditions*: *tert*-butylcarbamoylquinine based chiral stationary phase; mobile phase: 80% methanol/20% 0.1 mol/l aqueous ammonium acetate + 0.3% acetic acid (v/v), 1 ml/min; 25 °C.

a neutral control marker lies between the retention factors of the enantiomers of all peptides.

A comparison of the enantioselectivities observed for the different peptides shows some well-defined trends, which yield some insights into the chiral recognition process. An investigation of the results for the sequence isomers resulting from shifting a glycine residue through an oligoalanine peptide chain from the C- to N-terminus, i.e. Ala-Gly/Gly-Ala and Ala-Ala-Gly/Ala-Gly-Ala/Gly-Ala-Ala, respectively, shows that enantioselectivity is gradually decreased during this shift of glycine from the carboxyl to the amino end of the peptide (Figs. 3 and 4). A corresponding behaviour was found for the tripeptide series Ala-Gly-Gly/Gly-Ala-Gly/Gly-Gly-Ala, where an alanine residue is shifted through an oligoglycine chain from the N- to C-terminus (Fig. 4). In this case, the magnitude of discrimination of the (*S*)- against the (*R*)-enantiomer, represented by $\alpha = k_S/k_R$, decreases from 1.57 over 1.00 to 0.91, which leads to a reversal of the enantiomer elution order (Table 1). It

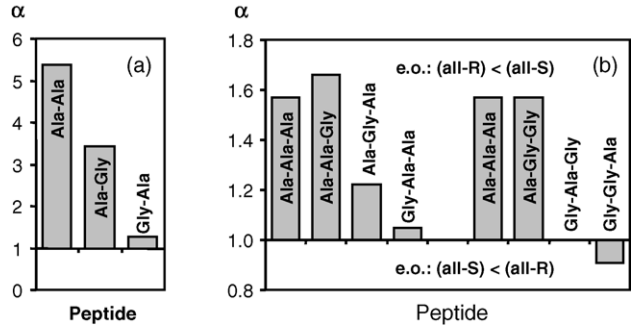

Fig. 4. Comparison of enantioselectivities obtained for the (all-*R*)- and (all-*S*)-enantiomers of alanine–glycine (a) dipeptides and (b) tripeptides, depending on the position(s) of the glycine(s).

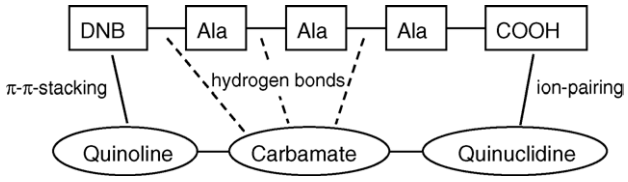

Fig. 5. Envisaged interactions between the various parts of the selector and a peptide analyte, shown for DNB-Ala-Ala-Ala as example.

seems that a pronounced change in the relative contributions of the various enantiomer discriminating increments occurs. A switch-over of the interaction that dominates the enantiomer discrimination process can be envisioned to lead to the change of the enantiomer elution order.

Several interactions can be envisaged between the various parts of the selector and a peptide analyte, as are shown in Fig. 5 and were discussed in full detail elsewhere [18]: ion-pairing between the peptide's analyte group and the selector's quinoline moiety, hydrogen bonding between the peptide's amide bonds and the selector's carbamate group and π–π-stacking between the DNB group of the analyte and the quinoline ring of the selector. Depending on the magnitude of the binding increments that the various interactions exert, a certain group will dominate the overall chiral recognition process. The experimentally observed enantioselectivities and elution orders suggest that the presence of an alanine residue at the N-terminus, next to the DNB group, unanimously effects an (all-*R*) < (all-*S*) elution order. If the *N*-terminal position is occupied by a non-chiral glycine residue and the alanine(s) are moved away from the DNB group, enantioselectivity is strongly diminished. Thus, the experimental findings seem to suggest that the DNB group in combination with the adjacent amino acid residue, i.e. the N-terminus, dominates the enantiomer discrimination process.

The comparison of the enantioselectivities achieved for the alanine–glycine peptides with those obtained for the respective all-alanine references with provides information whether a "glycine effect" on the chiral recognition process exists or not. In Fig. 4, the enantiomer separation results for the related peptides are juxtaposed. The replacement of alanine residues by glycine moieties has no (tripeptides) or a medium (dipeptides) effect on enantioselectivity when it occurs at the C-terminus. In contrast, if this substitution takes place at the N-terminus, enantioselectivity is drastically reduced. It seems that the loss of a chiral centre at the C-terminus does not diminish the chiral recognition capabilities of the selector, while, on the other hand, it is highly sensitive towards the removal of an N-terminal stereocentre. This finding agrees well with the conclusion from the comparison of the sequence isomers (see above). It is interesting to note that while the glycine substitution at the C-terminus maintains the absolute level of enantioselectivity in the tripeptides (or even slightly increases it), this type of amino acid replacement reduces the magnitude of enantioselectivity for the dipeptide somewhat. This observation may be explained by the general loss of enantiomer discrimination between the di- and tripep-

Table 2
Reversed phase retentions and log *D* values of alanine–glycine peptides

Peptide	*k*	log *D*[a]
Ala	8.67	0.27
Gly	4.53	−0.09
Ala-Ala	8.43	0.37
Ala-Gly	5.20	0.01
Gly-Ala	5.18	0.02
Gly-Gly	3.24	−0.34
Ala-Ala-Ala	8.36	0.53
Ala-Ala-Gly	5.69	0.18
Ala-Gly-Ala	9.28	0.18
Gly-Ala-Ala	6.33	0.18
Ala-Gly-Gly	4.97	−0.17
Gly-Ala-Gly	3.89	−0.17
Gly-Gly-Ala	5.79	−0.17
Gly-Gly-Gly	3.10	−0.52

Separation conditions: stationary phase: C_{18}; mobile phase: 25% methanol/75% water + 0.3% acetic acid (v/v); flow rate 1 ml/min; 25 °C; MS detection by selected ion monitoring of the respective $[M-H]^-$ ion.
[a] For a pH of 2.9 (see text).

tide [20]. The binding of the chiral selector with a tripeptide occurs in a less stereodiscriminative fashion and the resulting complex can thus more easily accommodate side-chain manipulations and the loss of one chiral centre than it is the case for the binding process and the complex between the selector and a dipeptide.

3.2. Reversed phase characteristics of the alanine–glycine peptides and deconvolution of their reversed phase increments on the chiral stationary phase

The reversed phase characteristics of the various alanine–glycine peptides were measured on a C_{18} stationary phase under isocratic conditions. The obtained retention factors were compared with a calculated hydrophobicity parameter (log *D*) and used to assess the influence of the reversed phase retention increment of the CSP on the enantiomer separations of the various peptides.

The retention factors measured on the reversed phase column are presented in Table 2. An increasing peptide chain length led to a decrease in retention for both the oligoalanine and oligoglycine series, with a more pronounced effect for the latter. As expected, an increasing content of glycine, which is less hydrophobic than alanine, resulted in a decrease of the retention factors. Only Ala-Gly-Ala was an exception to this rule. The reason for the deviating behaviour of this peptide will be discussed below.

The experimental hydrophobicities of the various peptides (chromatographic retention factors) were also compared with the corresponding theoretical ones. For this purpose log *D* values, which represent the combined log *P* values for the different analyte species (protonated and deprotonated forms) at a certain pH value, were calculated. The obtained values are given in Table 2. The computation was performed for a pH of 2.9, which was calculated from the experimentally employed acetic acid concentration in pure water. The methanol contained in the mobile phase (25%, v/v) will certainly shift the pH; however, due to the main component of the mobile phase being water, this effect is estimated to be rather small.

The relationships found by comparing the experimental retention factors and the theoretical log *D* values are shown in Fig. 6. For the amino acids and dipeptides a good correlation between retention factors and log *D* values was established (Fig. 6a), however, for the tripeptides pronounced deviations from the expected trend were noted (Fig. 6b). This difference between the experimental and the theoretical hydrophobicities can be ascribed to the fact that the log *D* values represent the hydrophobicities of the entire molecules, while the retention factors mirror the hydrophobic interactions of the stationary phase with the analytes, which include conformational aspects that are effected by the molecules' shapes. The order of the retention factors of the tripeptide sequence isomers can be explained by the relative orientations of the amino acids' side chains. It has been shown that the side chains of adjacent amino acid residues that possess the same absolute configuration (e.g., (*S*,*S*)) point towards opposite sides of the peptide molecule [26]. Consequently, the methyl groups of the two alanine residues in Ala-Gly-Ala (which have the same absolute configuration) are located on the same side of the peptide molecule where they form a large hydrophobic area, resulting in enhanced retention, while in Ala-Ala-Gly

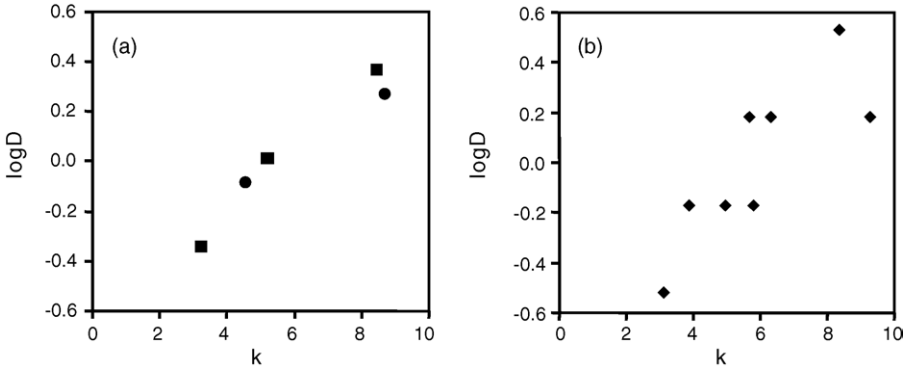

Fig. 6. Plots of log *D* values (for a pH of 2.9) versus reversed phase retention factors for alanine–glycine (a) amino acids (●) and dipeptides (■) and (b) tripeptides (♦).

and Gly-Ala-Ala the methyl groups of the alanine residues are located on opposite sides of the molecule, which results in isolated areas of hydrophobicity leading to a lower retention [26]. The higher retention of Ala-Gly-Ala compared to Ala-Ala-Ala can be explained by the presence of a methyl group in the middle amino acid of the latter peptide, which "counteracts" the hydrophobic interactions of the other two alanines to some extent.

Finally, a comparison of the reversed phase retention behaviour and the enantiomer separation results of the various peptides was carried out to assess to which extent the differences in enantioselectivity are caused by differing stereoselective interactions, i.e. varying chiral recognition, and to which degree by differences in the peptide's hydrophobicities. The overall retention factors of the enantiomers of a certain peptide on the CSP can be viewed as a sum of retention causing increments resulting from interactions with the chiral selector moiety (Fig. 5) plus a reversed phase (hydrophobic) retention increment (see above): $k = k_{\text{selector}} + k_{\text{hydrophobic}}$. The latter term will be equal for both enantiomers. Enantioselectivity is then defined as the ratio of the overall retention factors of the enantiomers: $\alpha = k_S/k_R = k_{\text{selector},S} + k_{\text{hydrophobic}}/k_{\text{selector},R} + k_{\text{hydrophobic}}$, whereby *S* and *R* denote the opposite configurations of the enantiomers. Consequently, if the non-enantioselective reversed phase retention increment dominates the overall retention, an increase in the hydrophobic retention increment should result in a reduced enantioselectivity. The comparison of the respective chromatographic results shows that the RP and CSP data exhibit no consistent correlation. For example, Ala-Ala has a much higher RP retention than Ala-Gly and Gly-Ala but supersedes the latter two peptides in terms of enantioselectivity. On the other hand, Ala-Ala-Gly has the lowest RP retention amongst the Ala_2Gly_1 sequence isomers accompanied by the highest enantioselectivity. Apparently, the overall retention behaviour of the CSP is quite complex and a simple partitioning into specific increments is not possible. Nevertheless, it can be stated that the reversed phase retention increment does not dominate the overall retention behaviour of the CSP, which is primarily determined by the ion-exchange process in combination with the hydrogen bonding and π–π-stacking increments.

4. Conclusions

Chromatographic separations of the (all-*R*)- and (all-*S*)-enantiomers of series of N-terminally protected alanine–glycine di- and tripeptides on a chiral stationary phase derived from a cinchona alkaloid based selector elucidated the susceptibility of the chiral recognition mechanism towards sequential isomerism and the introduction of conformationally flexible achiral glycine moieties into the peptide chain ("glycine effect"). Regarding the first aspect, it was found that shifting the glycine residue(s) from the C- to N-terminus diminished the obtained enantioselectivities. A mechanistic explanation for these observations was proposed, ascribing the DNB-group in conjunction with the N-terminal amino acid residue a dominating influence towards the chiral recognition process. An increasing number of glycines in the peptide structure generally reduced enantioselectivity, with this effect being more pronounced for the dipeptides than for the tripeptides.

Reversed phase retention data on the whole showed the expected trends for the various peptide sequences. A comparison of the retention factors with calculated hydrophobicity ($\log D$) values showed a good agreement for the amino acids and dipeptides but for the tripeptides the theoretical model used for the computations could not account for the experimental intricacy. Finally, the application of the reversed phase retention data towards a deconvolution of the overall CSP retentions showed that the latter are composed in quite a complex way.

As another interesting aspect of stereoselectivity, the investigation of the influence of peptide sequential isomerism on diastereoselectivity combined with the deconvolution of stereoselective and hydrophobic retention increments of peptide diastereomers on cinchona alkaloid derived chiral stationary phases constitutes a study of its own, which is currently in preparation in our laboratory.

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