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Epimeric N-substituted L-proline derivatives as chiral selectors for ligand-exchange chromatography

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ABSTRACT

Mainly N-alkyl derivatives of L-proline have proved useful as chiral selectors, particularly in the field of chiral ligand-exchange chromatography (CLEC). This paper describes the synthesis of new N-alkyl derivatives of L-proline, containing a second centre of chirality, and their immobilization on silica gel. The applicability of these chiral stationary phases in CLEC with either copper(II) or nickel(II) as complexing ion was investigated. Particular attention was paid to the effects of the organic modifier, buffer concentration and pH on stereoselectivity and retention. The aim of this study was also to elucidate the influence of the second centre of chirality, which contains a π-basic, space-filling phenyl group and a polar amide function, on enantioseparation and elution order. Based on simple molecular modelling studies, recognition mechanisms and the possibility of the amide oxygen coordinating with either copper(II) or nickel(II) are discussed. Both epimeric chiral selectors (either *R,S*- or *S,S*-configuration) resolved the enantiomers of various amino acids and derivatives thereof.

INTRODUCTION

Since the first attempts by Rogozhin and Davankov [1] to separate enantiomers via ligand-exchange chromatography, this technique has become increasingly popular. Davankov and co-workers made early systematic studies with chiral chelating resins containing immobilized amino acids (AAs) [2,3]. Subsequently, many ligand-exchange systems using different chemically bonded ligands and various kinds of transition metals have been studied. To overcome the limiting pressure resistance of polymeric soft resins, silica-bonded chiral stationary phases have been introduced in chiral ligand-exchange chromatography (CLEC), offering increased column efficiency, resolution and detection sensitivity [4–7]. Apart from the commonly separated enantiomers of amino acids and derivatives thereof, it is also possible to resolve enantiomers

of carboxylic acids, hydroxy acids, amino alcohols and amino alcohols as their Schiff bases, barbiturates, hydantoins and succinimide derivatives. The various techniques of CLEC, including the use of chiral stationary phases (CSPs), chiral coated phases and chiral mobile phases, have been surveyed in several publications [8–12].

Mainly N-alkyl derivatives of L-proline have proved useful as chiral selectors (CSs) in CLEC. In this paper, two new chiral selectors (CSs) derived from L-proline are presented (see Fig. 1, CS-I and CS-II). These epimeric N-alkyl derivatives of L-proline, containing a second centre of chirality, were synthesized, fully characterized and immobilized on silica gel. The applicability of these CSPs in CLEC with either copper(II) or nickel(II) as complexing ion was investigated. Particular attention was paid to the effects of the organic modifier, buffer concentration, temperature and pH on resolution. The aim of this study was also to elucidate the influence of the second centre of chirality, containing a π-basic, space-
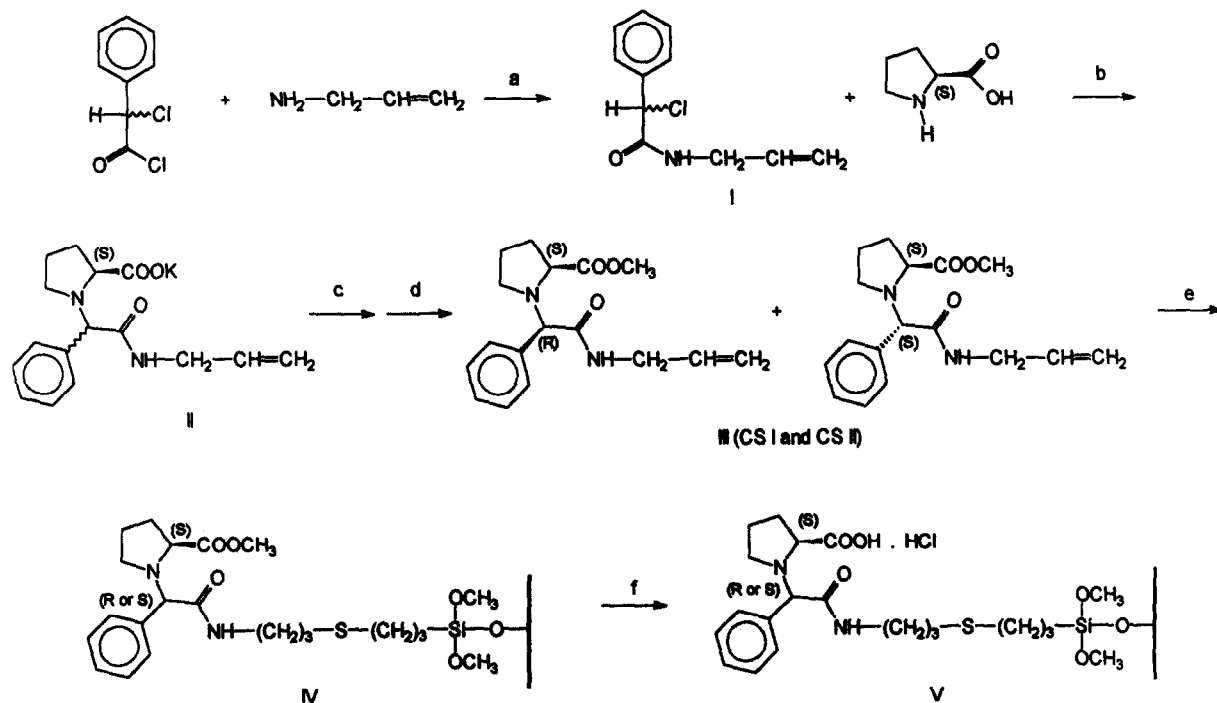
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Fig. 1. Preparation of CSPs derived from L-proline. (a) Light petroleum, 25°C for 2 h; (b) potassium carbonate, ethanol, reflux for 20 h; (c) acetyl chloride, methanol, reflux for 5 days; (d) preparative resolution of diastereomers; (e) 3-mercaptosilane, AIBN, chloroform, reflux for 43 h; (f) Na₂CO₃–NaHCO₃ solution (pH 8.5), 25°C for 20 h, 50°C for 4 h.

filling phenyl group and a polar amide function, on chiral recognition and elution order. Models to explain the differing chromatographic properties of the epimeric CSPs based on the opposite configuration of the second centre of chirality are discussed.

EXPERIMENTAL

Apparatus

Liquid chromatography (LC) was performed with a modular liquid chromatograph (Merck–Hitachi, Darmstadt, Germany), equipped with a Model L-6200 intelligent pump, a Model AS-2000A autosampler with a 100-μl loop and a Model L-4250 UV–Vis detector, controlled via Model D-6000 chromatography data station software, HPLC Manager Vers. 2.09. Temperature control was provided with a column thermostat (B.O. Electronics, Lang-Enzersdorf, Austria). Standard operating conditions were a flow-rate of 1 ml/min and a column temperature of 30°C.

Preparative LC was performed with a modular liquid chromatograph (Merck–Hitachi), equipped with a Model L-6000 pump (preparative pump head) and a Model L-4000 UV detector (2-mm flow cell unit) with the wavelength set at 230 nm. Model D-6000 chromatography data station software, HPLC Manager Vers. 2.09, was used for data management. Solutes were injected via a Rheodyne Model 7125 injector (3-ml loop) and resolution was obtained on a Hibar column RT, 250 × 25 mm I.D., LiChrosorb Si 60 (7 μm) (Merck); the column temperature was maintained with a water-bath (Haake, Karlsruhe, Germany); a Model A-330 Semi-prep filter (Upchurch, Oak Harbor, WA, USA) was placed between injector and column to protect the latter from plugging.

Infrared spectra were recorded on a Perkin-Elmer (Beaconsfield, UK) Model 225 grating infrared spectrophotometer. Diffuse reflectance infrared spectra were recorded on a Perkin-Elmer Model 1720-x Fourier transform infrared spectrometer.

^{1}H NMR spectra were recorded at 60 MHz on a Hitachi–Perkin-Elmer R-248 High-resolution NMR spectrometer using tetramethylsilane as internal standard. Chemical shifts are given in parts per million.

Melting points were measured on a Büchi-Tottoli (Flawil, Switzerland) apparatus and are uncorrected.

Molecular modelling studies were undertaken with Hyperchem Release 2 for Windows software from Autodesk (Sausalito, CA, USA), using the MM+ force field for molecular mechanics calculations.

Chemicals

Heptane and methanol were of HPLC grade and methylene chloride and 2-propanol of analytical-reagent grade, all from Merck. Water obtained from a Milli-Q system (Millipore) was used. pH was adjusted with either glacial acetic acid or aqueous ammonia and buffer solutions were prepared with ammonium acetate, copper(II) acetate and nickel(II) acetate tetrahydrate, all of analytical-reagent grade from Merck. Mobile phases were filtered and degassed in an ultrasonic bath before use.

L-Proline was obtained from Bachem (Basle, Switzerland), (±)-2-chloro-2-phenylacetyl chloride and 3-mercaptopropyltrimethoxysilane from Aldrich (Steinheim, Germany) and LiChrospher Si 100 (5 μm) from Merck. Other reagents and solvents were purchased from Fluka (Buchs, Switzerland).

Racemic and optically pure 3,4-dehydroproline was obtained from Fluka and L, D- and DL-amino acids and derivatives therefore were purchased from Sigma or prepared from commercially available materials using conventional methods.

2-(R,S)-N-Allyl-(2-chloro-2-phenyl)acetyl-amide (I). N-Allylamine (6.32 ml, 84 mmol) in 40 ml of light petroleum was added dropwise to a cooled solution of 2-(R,S)-2-chloro-2-phenylacetyl chloride (6.43 ml, 40 mmol) in 200 ml of light petroleum. After stirring for 2 h, the yellow precipitate that formed was filtered and washed successively with water, dilute ammonia solution, water and dilute hydrochloric acid. The white solid showed impurities by thin-layer chro-

matography [mobile phase toluene–acetone (3:1, v/v)] and was recrystallized from cyclohexane. Chemical purity was monitored by HPLC on a C_{18} column with methanol–0.01 M ammonium acetate buffer (30:70, v/v) as the mobile phase. The final product was dried in air at 40°C to give 5.66 g (67%) of I, m.p. 79°C. ^{1}H NMR [C^{2}HCl$_3$, (C^{2}H$_3$)$_2$SO]: δ 3.70–4.01 (m, 2H, NCH$_2$CH), 4.90–5.35 (m, 2H, CH$_2$=CH), 5.49 (s, 1H, ClCHAr), 5.52–6.20 (m, 1H, CH$_2$=CH), 7.21–7.68 (m, 5H, Ar), 7.75–8.28 (s, 1H, CONHCH$_2$).

Potassium N-{(R,S)-[(2-propen-1-yl)amino-carbonyl]phenylmethyl}-L-prolinate (II). This was prepared in a similar manner to the procedure given by Lochead and Proctor [13] for N-benzylproline. L-Proline (1.15 g, 10 mmol), I (2.31 g, 11 mmol), potassium carbonate (1.38 g, 10 mmol) and ethanol (50 ml) were stirred at reflux for 20 h. To speed up the reaction a small portion of 18-crown-6 was added. After cooling, the solution was filtered and the solvent was removed under reduced pressure. The residue was dissolved in acetone (to remove potassium chloride and non-alkylated L-proline), filtered again and evaporated to dryness. The residue was dissolved in water and extracted several times with diethyl ether to recover unreacted amide I. After evaporating the aqueous phase, a yellow oil (3,7 g, 113%) was obtained, containing L-proline, amide, potassium chloride and other possible side-products, hence a theoretical yield above 100% was calculated.

N-{(R,S)-[(2-Propen-1-yl)aminocarbonyl]-phenylmethyl}-L-proline methyl ester hydrochloride (III). The crude prolinate II (4.5 g, 0.014 mol) was dissolved in methanol (100 ml) and 25 ml of a mixture of acetyl chloride in methanol (10:90, v/v, forming a dry solution of hydrochloric acid in methanol) were added. After refluxing for 5 days, the white precipitate that formed was filtered off and the yellow solution was evaporated to dryness. The residue was dissolved in acetone, filtered and evaporated twice. The amount and speed of ester formation were determined during the reaction by RP-HPLC, using methanol–0.01 M ammonium acetate buffer (10:90, v/v) as the mobile phase. A yellow, non-crystalline product (3.86 g, 81%) was ob-

tained, obviously consisting of the desired diastereomeric methyl esters and unesterified N-alkyl-L-proline II.

Preparative resolution of the epimeric L-proline derivatives

Further purification and resolution of the diastereomers was carried out by preparative HPLC using a 250×25 mm I.D. RP-8 column with methanol–0.01 M ammonium acetate buffer (50:50, v/v) as the mobile phase at a flow-rate of 15 ml/min, a column temperature of 40°C and a detection wavelength of 230 nm. The amount of III injected per run was about 100 mg. The fractions collected from four runs containing the same diastereomer were combined, methanol was evaporated, then the solution was made alkaline with ammonia solution (pH ≈ 8) and extracted with diethyl ether. The organic layer was dried over magnesium sulphate, filtered and after evaporation under reduced pressure the pure diastereomeric bases were obtained as slightly yellow, viscous oils, with a yield of CS-I of *ca.* 20% (99.2% diastereomeric purity) and of CS-II of *ca.* 32% (99.3% diastereomeric purity). All attempts to crystallize the chromatographically pure product (following ion-exchange chromatography and freeze-drying of the free amines or of the hydrochloride, sulphate, tartrate and phosphate salts) failed. The physico-chemical characterization of the two diastereomers was realized on the basis of [1] H NMR and IR spectra and the results were in agreement with the proposed structures.

CS-I. ^{1}H NMR (C^2HCl_3): δ 1.65–2.03 (m, 4H, CH_2), 2.65–2.95 (m, 2H, CH_2CH_2N), 3.20–3.45 (m, 1H, $COCHCH_2$), 3.65 (s, 3H, OCH_3), 3.75–4.08 (d, 2H, NCH_2CH), 4.39 (s, 1H, NCHAr), 4.95–5.40 (m, 2H, $CH_2 = CH$), 5.60–5.95 (m, 1H, $CH_2 = CH$), 7.20–7.38 (m, 5H, Ar), 7.80 (s, 1H, NH).

CS-II. ^{1}H NMR (C^2HCl_3): δ 1.65–2.03 (m, 4H, CH_2), 2.65–2.95 (m, 2H, CH_2CH_2N), 3.40–3.55 (m, 1H, $COCHCH_2$), 3.63 (s, 3H, OCH_3), 3.75–4.08 (d, 2H, NCH_2CH), 4.39 (s, 1H, NCHAr), 4.95–5.35 (m, 2H, $CH_2 = CH$), 5.60–5.95 (m, 1H, CH_2–CH), 7.20–7.38 (m, 5H, Ar), 8.0 (s, 1H, NH).

3-Mercaptopropylsilica gel

A 10-g amount of LiChrospher Si 100 (5 μm) and 140 ml of distilled toluene (b.p. 109°C) were introduced into a 250-ml Quickfit flask equipped with a mechanical stirrer and a distilling apparatus. To remove traces of water, the toluene was azeotropically evaporated until the distillate had a boiling temperature of 108°C. After *ca.* 30 ml of toluene had been removed the distillation apparatus was replaced with a reflux condenser. Freshly distilled pyridine (16.7 ml) and 3-mercaptopropyltrimethoxysilane (16.7 ml, 87.5 mmol) were added dropwise under nitrogen. After refluxing for 40 h and sedimentation of the silica gel particles, the solvent was exhausted and the stationary phase was washed successively with toluene, diethyl ether and light petroleum. The modified silica (12.08 g) was dried in air at 40°C to give a 11.27 g yield. Elemental analysis indicated 970 μmol of bonded 3-mercaptopropyldimethoxysilyl residues per gram of modified silica gel (based on carbon elemental analysis). Analysis: found, 5.95% C; starting material, 0.12% C. DRIFT spectrum (KBr): 3649, 3346, 2946, 2848, 2582, 2362, 2340, 2000, 1881, 1633, 1457, 1412, 1345, 1094, 1082, 956, 808, 693. cm^{-1}.

CSP-I

CS-I (474.5 mg, 1.75 mmol), 3.3 g of 3-mercaptopropylsilica gel, 60 mg of AIBN [2,2′-azobis(2-methylpropionitrile), 0.4 mmole] and 100 ml of azeotropically dried chloroform were stirred and refluxed under nitrogen for 43 h. The derivatized silica was washed thoroughly with chloroform and methanol. To cleave the methyl ester, a solution of sodium carbonate and sodium hydrogencarbonate (pH 8.5) was added and the slurry was stirred for 20 h at room temperature and additionally for 4 h at 50°C. The reaction was stopped by adding 70 ml of water and adjusting the pH to 2.9 with dilute hydrochloric acid. The modified silica gel was washed with methanol–water (1:1) (twice), methanol (three times), acetone (twice) and light petroleum. After drying in air at 40°C, 2.92 g of white silica gel were obtained. Elemental analysis of CSP-I afforded 7.97% C, 1.57% H, 0.39% N. The calculated coverage was 139 μmol of chiral

selector bonded per gram (based on N analysis) and 105 μmol of chiral selector per gram g (based on C analysis). DRIFT spectrum (KBr): 3650, 3316, 2946, 2848, 2577, 2362, 2340, 2001, 1882, 1743, 1664, 1526, 1455, 1412, 1346, 1097, 1084, 957, 804, 705, 692 cm^{-1}.

CSP-II

CSP-II was similarly obtained from CS-II (426 mg, 1.41 mmol), 3 g of 3-mercaptopropylsilica gel, AIBN (50 mg, 0.3 mmol) and chloroform (100 ml), yield 2.64 g. Elemental analysis resulted in 7.24% C, 1.53% H, 0.31% N. From these data the coverage was calculated to be 110.6 μmol of chiral selector per gram (based on N analysis) and 67 μmol chiral selector per gram (based on C analysis). DRIFT spectrum (KBr): 3649, 3319, 2938, 2849, 2577, 2001, 1882, 1745, 1665, 1526, 1455, 1412, 1346, 1079, 957, 812, 694 cm^{-1}.

Both CSP-I and CSP-II were packed into 100 × 4.0 mm I.D. and 150 × 4.0 mm I.D. analytical columns by a conventional slurry technique and coupled in series for chromatography.

"End-capping" (reaction of accessible free thiol groups with 1-hexene)

Before end-capping, each column was flashed with heptane (30 ml) and chloroform (50 ml). End-capping of the remaining sulphydryl groups was performed by recycling a solution containing 1-hexene (1.17 ml, 10 mM) and AIBN (50 mg, 0.3 mM) in chloroform (150 ml) at a flow-rate of 0.3 ml/min and a water-bath temperature of 70°C for 24 h.

To calculate the coverage of "end-capping" judged by elemental analysis, small portions of CSP-I and CSP-II were treated with excess of 1-hexene and AIBN in glass tubes to give a loading of 369 μmol hexyl residues per gram for CSP-I (based on C analysis) and 422 μmol per gram for CSP-II (based on C analysis).

RESULTS AND DISCUSSION

Synthesis of chiral selectors, purification and immobilization

The preparation of CSP-I and CSP-II is outlined in Fig. 1. As mentioned under Experimen-

tal, the N-alkylation of sodium L-prolinate gave only impure compound II. Several attempts were made to optimize the alkylation reaction. To obtain better yields and a shorter reaction time, different carbonates were tested. Potassium carbonate seems to be superior to sodium carbonate because of forming better soluble prolinates and neutralizing the formed hydrochloric acid. A crown ether was additionally added to afford better phase-transfer catalysis. Tetrabutylammonium hydroxide did not speed up the alkylation via ion-pair formation resulting in enhanced solubility of L-proline. Further, applying ultrasound instead of refluxing resulted in poor yields. The reaction speed and amount of remaining amide I were measured via RP-HPLC, using methanol–10 mM ammonium acetate buffer (30:70, v/v) as the mobile phase. Formation of N-allyl-2-phenyl-2-ethoxyacetamide did not occur in the ethanolic solution, as was confirmed by a comparable test under identical conditions but without L-proline in the reaction mixture. A further possible by-product, O-[(allylaminocarbonyl)methylphenyl]-L-proline ester, was obviously not formed because of the lack of an ester band in the IR spectra. However, degradation of adduct II, forming a biscarboxylate by cleaving the amide bond, could not be excluded. As was also pointed out before, the epimeric L-proline derivatives in the form of their methyl esters were well resolvable by RP-HPLC. CS-I and CS-II exhibited satisfactory chemical purity.

In the literature several different possibilities have been described for the covalent binding of a chiral selector to a silica gel surface. However, based on our experience, it seems that the basic nitrogen of CS-I and CS-II may result in inactivation of the hexachloroplatinic acid or (cyclooctadiene)platinum chloride commonly used for the classical immobilization procedure via hydrosilylation of a vinyl group followed by immobilization of the siloxane to silica gel. To overcome this obvious limitation, the allylic residues of the CSs were reacted according to a radical anti-Markownikoff addition of a vinyl group with the thiol groups of 3-mercaptopropylsilica gel, as was described by Rosini et al. [14]. However, only 139 μmol of 970 μmol of mercapto groups available per gram of 3-mercap-

topropylsilica gel reacted with the CS-I to give CSP-I and 111 μmol with CS-II to give CSP-II. The remaining 831 μmol/g CSP-I and 859 μmol/g CSP-II, respectively, of mercapto groups represent polar residues being able to interact with polar solutes. Hence they may influence the overall selectivity of the chromatographic system, as was discussed recently by Pirkle and co-workers [15,16]. To reduce the number of remaining polar sulfhydride groups a new "end-capping" procedure was developed. 1-Hexene was reacted under the same conditions as the chiral selectors with the CSPs to produce a more lipophilic stationary phase, covering about 50% of the remaining free sulphydride groups. Despite the fact that the residual silanols are not alkylated under these reaction conditions, the accessibility of these groups to solute molecules should be decreased. Further, Feibush *et al.* [17] demonstrated the enhanced stereoselectivity of "diluted CSPs" compared with concentrated ones, owing to a decreased interference of neighbouring chelating ligands. Additional experiments dealing with the "end-capping" of mercapto residues are in progress and further studies with such "mixed" or "diluted phases" will be published elsewhere [18]. To summarize, there are at least three different covalently bound residues on the final CSPs, probably providing different retention and selectivity characteristics (Fig. 2). These CSPs, as almost all other CSPs described in the literature, represent randomly scattered surface structures and it is not clear how homogeneous the surface will be and/or how much "clustering" will take place.

Chromatographic properties

Tables I and II are giving a general view of the resolved solutes used in this study and the applicability of the epimeric selectors in CLEC with Cu(II) and Ni(II) as complexing metal ions. As can be seen, CSP-I and -II resolve a broad range of amino acids and amino acid derivatives. Representative chromatograms are shown in Figs. 3 and 4.

Underivatized AAs show similar capacity factors on both stationary phases with copper(II) as complexing ion. Simple molecular modelling studies were undertaken to elucidate the spatial
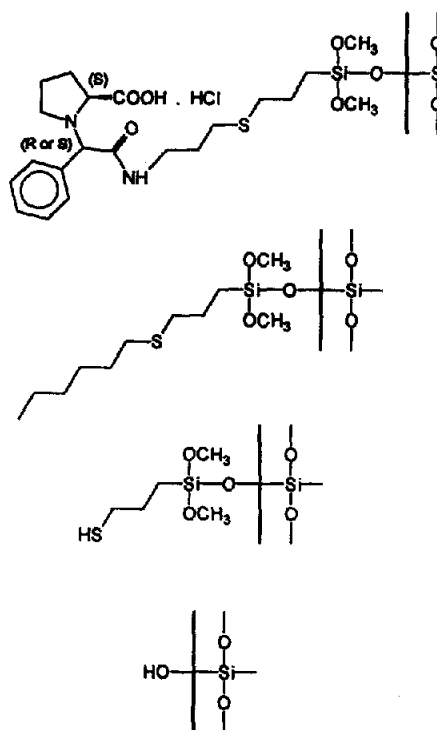

Fig. 2. Three different covalently bound residues on CSP-I and CSP-II.

arrangement of the chiral selectors. An outcome of such calculations is shown in Fig. 5. Formation of mixed-ligand sorption complexes with the silica-bonded chiral ligands is the most important retention mechanism for the amino acid solutes and seems to be the same for CSP-I and CSP-II. In contrast, the lipophilicity of the epimeric CSPs is different, as can be seen from the capacity factors of aromatic AAs and dansyl-AAs. These solutes are more strongly retained on the obviously more "lipophilic" stationary phase CSP-II. Although there is a slightly altered density of the CSs on CSP-I and CSP-II, the different retention characteristics are probably due to the different spatial arrangements of the chiral selectors and therefore altered Van der Waals and π–π type interaction sites combined with chelation possibilities. This was also supported by the diastereoseparation of the two proline esters CS-I and CS-II (CS-I eluted before CS-II) on a reversed-phase column even on a preparative scale.

TABLE I

RESOLUTION OF AMINO ACIDS AND DERIVATIVES ON CSP-I AND CSP-II USING COPPER(II) AS COMPLEXING ION

Flow-rate, 1 ml/min; temperature, 30°C; detection at 240 nm. MP (mobile phases): $1 = 50$ mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$, (pH 4.5), $2 = 90\%$ (v/v) 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ + 10% (v/v) methanol (pH 5.5); $3 \approx 70\%$ (v/v) 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ + 30% (v/v) methanol (pH 5.5); $4 = 50$ mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ (pH 6.5); $6 = 100$ mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ (pH 6.5); $7 = 100$ mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ (pH 5.5); $9 = 70\%$ (v/v) 100 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ + 30% (v/v) methanol (pH 7.5); $10 = 70\%$ (v/v) 100 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ + 30% (v/v) methanol (pH 4.5). Capacity factor $k_2' = (t_R - t_0)/t_0$; selectivity $\alpha = k_2'/k_1'$.

Solute[a]	CSP-I			CSP-II		
	MP	k_2'	α	MP	k_2'	α
Asn	2	0.79	1.00	7	0.57	1.09
Ile	2	2.09	1.08	6	3.94	1.15
Met	4	6.53	1.08	7	3.50	1.22
Nleu	2	2.62	1.12	6	4.73	1.21
Nval	2	1.58	1.09	7	1.19	1.21
Phe	2	6.77	1.00	7	6.82	1.28
p-Amino-Phe	2	6.82	1.00	7	4.94	1.10
Pro	2	1.52	1.39	8	3.42	1.19
Allo-4-OHPro	2	1.93	1.74	8	1.61	1.37
3,4-Dehydro-Pro	1	0.86	1.53	6	3.28	2.03
N-Hexyl-Pro	2	1.78	1.40	9	13.35	1.31
Ser	2	0.73	1.07	7	0.47	1.12
Thr	2	0.86	1.08	7	0.66	1.10
Trp	3	13.82	1.18	10	2.63	1.00
α-Methyl-Trp	3	18.94	1.44	10	2.62	1.00
5-OHTrp	3	8.90	1.19	10	1.49	1.00
Tyr	2	5.27	1.11	6	5.69	1.00
α-Methyl-m-Tyr	2	8.24	1.35	7	5.86	1.08
α-Methyl-Tyr	2	7.46	1.43	7	5.20	1.22
O-Methyl-Tyr	2	11.26	1.06	7	11.24	1.17
3-Methoxy-Tyr	2	7.22	1.21	6	9.27	1.00
Val	2	1.27	1.00	7	1.11	1.20
2-Aminolactic acid	4	1.63	1.00	7	0.65	1.12
DNS-Phe	3	12.21	1.12	10	9.31	1.00
DNS-Thr	2	10.10	1.06	10	2.06	1.00

[a] Commonly used abbreviations of amino acids.

TABLE II

RESOLUTION OF AMINO ACIDS AND DERIVATIVES ON CSP-I AND CSP-II USING NICKEL(II) AS COMPLEXING ION

Flow-rate, 1 ml/min; temperature, 30°C; detection at 254 nm. MP (mobile phases): $1 = 70\%$ (v/v) 50 mM NH$_4$OAc–0.5 mM Ni(OAc)$_2 \cdot 4$H$_2$O + 30% (v/v) methanol (pH 5.5); $2 = 70\%$ (v/v) 50 mM NH$_4$OAc–0.5 mM Ni(OAc)$_2 \cdot 4$H$_2$O + 30% (v/v) methanol (pH 6.5); $3 = 70\%$ (v/v) 100 mM NH$_4$OAc–0.5 mM Ni(OAc)$_2 \cdot 4$H$_2$O + 30% (v/v) methanol (pH 7.5); $4 = 70\%$ (v/v) 100 mM NH$_4$OAc–0.5 mM Ni(OAc)$_2 \cdot 4$H$_2$O + 30% (v/v) methanol (pH 6.5).

| Solute	CSP-I			CSP-II		
	MP	k_2'	α	MP	k_2'	α
Tyr	1	4.67	1.25	3	3.16	1.76
α-Methyl-m-Tyr	1	5.24	1.73	3	1.99	1.70
α-Methyl-Tyr	1	5.41	1.87	3	1.50	1.73
3-Methoxy-Tyr	1	5.99	1.39	3	3.37	1.84
O-Methyl-Tyr	1	7.49	1.17	3	7.77	1.79
Trp	1	14.41	1.28	4	2.26	1.29
α-Methyl-Trp	1	20.67	1.70	3	5.62	1.35
5-Methyl-Trp	1	21.12	1.22			
6-Methyl-Trp	1	26.16	1.29	3	16.22	1.53
7-Methyl-Trp				3	14.77	1.47
5-OHTrp	1	9.77	1.30	3	5.15	1.52
DNS-Met	2	4.42	1.05	4	4.52	1.00
DNS-Phe	2	6.97	1.11	4	8.56	1.00
DNS-Thr	2	1.69	1.05	4	1.71	1.00
DNS-Trp	2	11.17	1.19			
p-Amino-Phe	1	4.44	1.11	3	2.75	1.63
Phenyl-Gly	1	2.05	1.28	3	1.62	1.00
β-Phenyllactic acid	1	0.74	1.00	4	0.31	1.44
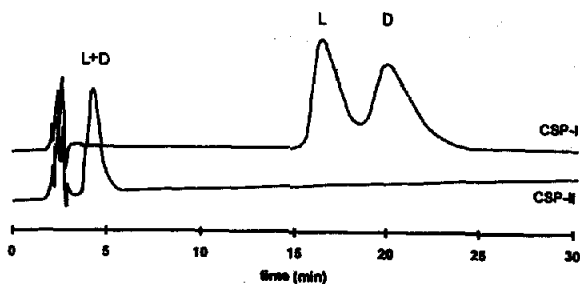
Fig. 3. Different capacity factors and enantioselectivity of CSP-I and CSP-II for D,L-phenylglycine. Mobile phase, 70% (v/v) 50 mM NH$_4$OAc–0.5 mM Ni(OAc)$_2 \cdot 4$H$_2$O + 30% (v/v) methanol (pH 6.5).

Remarkably, using nickel(II) as complexing ion the two diastereomeric CSPs show opposite behaviour in terms of stereoselectivity and retention. Depending on the epimeric form of the chiral selector used, the capacity factors of AAs
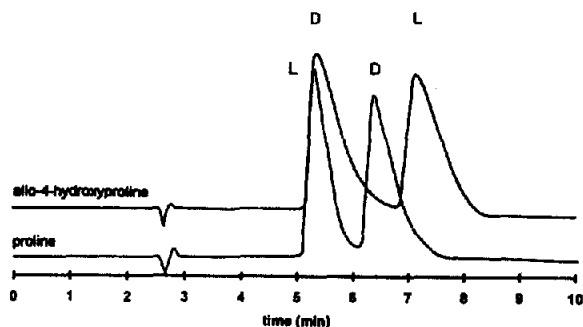
Fig. 4. Different capacity factors and enantioselectivity of CSP-I for D,L-proline and D,L-allo-4-hydroxyproline. Mobile phase, 90% (v/v) 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ + 10% (v/v) methanol (pH 5.5).

and methylated derivatives thereof differ widely from one another (Fig. 3). The solutes are much more strongly retained on CSP-I, by a factor of 10–20. Despite the different amounts of chiral selector loaded on to CSP-I and CSP-II, there
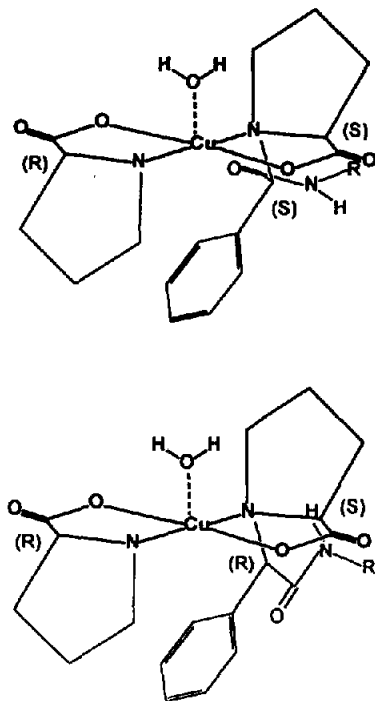

Fig. 5. Proposed mixed-ligand complexes of CS-I and CS-II using copper(II) as complexing ion and D-proline as guest molecule. The commonly used presentation of mixed-ligand planar copper(II) complexes of L-proline-derived CSPs (introduced by Davankov and co-workers) was used in combination with the outcome of the molecular modelling studies.

must be an additional reason for the altered capacity factors. As can be seen in Fig. 5, the phenyl residue occupies the lower axial position of the mixed-ligand complex of CSP-I and CSP-II. The chiral selector with an S,S-configuration may possibly form a hydrogen bond between its amide and carboxylate groups, causing hindered rotation of the prolyl residue and additional rigidity of the chelate complex. Subsequently, the CSs have different possibilities of coordinating with their carbonyl oxygen in the lower axial position of the octahedral nickel(II) complex. As a consequence, altered mixed ligand complexes will be formed. This assumption is supported by the similar retention behaviours of the solutes on CSP-I and CSP-II using copper(II) as complexing ion, because of its square-planar distribution of coordinate valences.

Particular attention was paid to the effects of the buffer concentration, temperature, organic modifier and pH on enantioselectivity and resolutions, as discussed below.

Influence of buffer concentration. As was demonstrated for CSP-II (Tables III and IV), the buffer concentration plays an important role on retention, enantioselectivity and resolution with either copper(II) or nickel(II) as complexing ion. Doubling the concentration of the ammonium acetate buffer resulted in decreased capacity factors (about 40% smaller) and enhanced α-values. Different buffer concentrations and type of buffer ions can be used to optimize resolution and retention.

Influence of temperature. Ligand-exchange chromatographic systems based on covalently bonded ligands are commonly used at elevated temperatures in order to improve mass transfer and column efficiency. As a rule, but with exceptions, the selectivity decreases with increasing temperature, *e.g.*, the resolution of N-benzylproline on an L-proline-containing polystyrene-type ligand exchanger described by Davankov *et al.* [19]. The temperature dependence of retention, enantioselectivity and resolution on CSP-I in the range of 30–60°C is shown in Figs. 6 and 7. As expected, the α-values decreased with increase in temperature, but there was also an exception, namely allo-4-hydroxyproline, where the highest enantioselec-

TABLE III

INFLUENCE OF BUFFER CONCENTRATION ON CAPACITY FACTOR, ENANTIOSELECTIVITY AND RESOLUTION ON CSP-II USING COPPER(II) AS COMPLEXING ION

Mobile phase, 0.5 mM copper(II) acetate–ammonium acetate buffer (pH 5.5); other conditions as in Table I.

Solute	50 mM ammonium acetate			100 mM ammonium acetate		
	k'_2	α	R_s [a]	k'_2	α	R_s
Asn	0.74	1.00	–	0.57	1.09	n.c.[b]
Ile	2.11	1.00	–	1.68	1.11	n.c.
Nval	1.80	1.14	0.72	1.19	1.21	0.89
Phe	9.75	1.25	1.22	6.82	1.28	1.50
Phenyl-Gly	3.81	1.20	1.19	2.07	1.12	–[c]
Pro	1.61	1.41	1.11	1.06	1.56	1.36
Ser	0.68	1.00	–	0.47	1.12	n.c.
Thr	0.87	1.00	–	0.66	1.10	n.c.
Val	1.63	1.14	0.65	1.11	1.20	n.c.
2-Aminobutyric acid	0.95	1.00	–	0.65	1.12	n.c.
3,4-Dehydro-Pro	2.09	1.37	–[c]	1.76	1.62	1.82
α-Methyl-m-Tyr	8.20	1.06	n.c.	5.86	1.08	n.c.
α-Methyl-Tyr	7.68	1.20	0.99	5.20	1.22	1.09
O-Methyl-Tyr	17.46	1.17	1.32	11.24	1.17	1.16
p-Amino-Phe	7.86	1.10	n.c.	4.94	1.10	n.c.

[a] $R_s = 1.18 \, (t_{R_2} - t_{R_1} / w_{1/2_1} + w_{1/2_2})$.
[b] n.c. = Not calculated.
[c] Co-eluted with system peak.

TABLE IV

INFLUENCE OF BUFFER CONCENTRATION ON CAPACITY FACTOR AND ENANTIOSELECTIVITY ON CSP-II USING NICKEL(II) AS COMPLEXING ION

Mobile phase, 0.5 mM nickel(II) acetate–ammonium acetate buffer (pH 6.5); other conditions as in Table II.

Solute	50 mM ammonium acetate		100 mM ammonium acetate	
	k'_2	α	k'_2	α
Trp	2.52	1.00	2.26	1.29
Phe	1.51	1.00	1.26	1.32
6-Methyl-Trp	4.53	1.00	4.11	1.33
7-Methyl-Trp	4.29	1.00	3.82	1.27
O-Methyl-Tyr	1.96	1.20	1.90	1.58
β-Phenyllactic acid	0.22	1.46	0.31	1.44

tivity was obtained at 40°C. The resolution of the racemate of norvaline was, however, not influenced by temperature.

Influence of methanol content on the mobile phase. The effect of the organic component in the mobile phase was studied. Of various possible modifiers used in CLEC, *e.g.*, methanol, ethanol, acetonitrile and tetrahydrofuran, methanol was chosen in this study. The influence of the methanol content of the mobile was investigated between 0% and 30% (data not shown) and was found to be of minor importance with regard to retention and enantioselectivity.

Influence of mobile phase pH. In CLEC, retention and enantioselectivity are also optimized by varying the pH of the mobile phase. The common pH range examined with silica-bonded CSPs is between 4 (lower pH values result in dissociation of the copper–amino acid
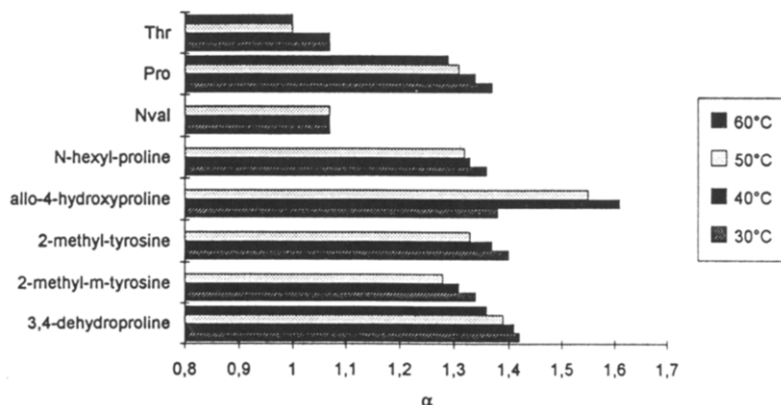
Fig. 6. Influence of temperature on enantioselectivity. Stationary phase, CSP-I; mobile phase, 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ (pH 5.5); flow-rate, 1 ml/min; detection at 240 nm.

complexes) and 9 (higher pH values induce column degradation, especially in combination with elevated temperatures), assuming ammonium ions are present in the mobile phase. Raising the pH usually increases the capacity factors of the solutes as a consequence of the enhanced formation of mixed-ligand sorption complexes. CSP-I and -II were tested between pH 4.5 and 7.5 with regard to retention times of the solutes and enantioselectivity (see Tables V–VII). Employing copper(II) as the complexing ion, the highest enantioselectivity was observed on CSP-I at pH 5.5, except for 3,4-dehydroproline and 3-methoxytyrosine (pH 4.5). As an exception, allo-4-hydroxyproline showed a different behaviour, as can be seen in Fig. 8. The highest enantioselectivity with CSP-II was ob-

tained between pH 5.5 and 6.5. No resolution was obtained at an eluent pH of 4.5 owing to the small capacity factors. A similar difference between CSP-I and CSP-II was observed when using nickel(II) as the complexing metal. CSP-I gave the best results at lower pH, except for dansylamino acids (see Table V), which are known to be better resolved at alkaline pH. In contrast, CSP-II displayed higher enantioselectivity at higher pH values.

Elution order and chiral recognition mechanism. When searching for simple recognition models, one must bear in mind the complex combination of hydrophobic, electrostatic and complexation interactions of the chiral analytes with the (inhomogenous) stationary phase. However, the elution order on a given stationary
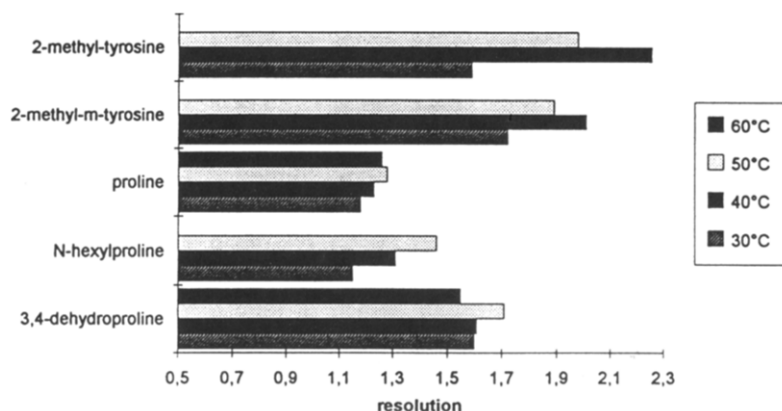

Fig. 7. Influence of temperature on resolution. Stationary phase, CSP-I; mobile phase, 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$ (pH 5.5); flow-rate, 1 ml/min; detection at 240 nm.

TABLE V

INFLUENCE OF pH ON RETENTION AND ENANTIO-SELECTIVITY ON CSP-I USING NICKEL(II) AS COMPLEXING ION

Mobile phase, 70% (v/v) 50 mM ammonium acetate–0.5 mM nickel(II) acetate + 30% (v/v) methanol; flow-rate, 1 ml/min; temperature, 30°C; detection at 254 nm.

Solute	Mobile phase pH					
	5.5		6.5		7.5	
	k_2'	α	k_2'	α	k_2'	α
DNS-Thr	1.47	1.00	1.69	1.05	7.22	1.09
DNS-Trp	9.50	1.08	11.17	1.19	12.27	1.18
Phenyl-Gly	2.05	1.28	6.85	1.26		
Tyr	4.67	1.25	12.58	1.20		
3-Methoxy-Tyr	5.99	1.39	21.26	1.31		
O-Methyl-Tyr	7.49	1.17	22.64	1.11		
α-Methyl-m-Tyr	5.24	1.73	6.85	1.26		
α-Methyl-Tyr	5.41	1.87	17.82	1.62		

TABLE VI

INFLUENCE OF pH ON RETENTION AND ENANTIO-SELECTIVITY ON CSP-II USING NICKEL(II) AS COMPLEXING ION

Mobile phase, 70% (v/v) 100 mM ammonium acetate–0.05 mM nickel(II) acetate + 30% (v/v) methanol; flow-rate, 1 ml/min; temperature, 30°C; detection at 254 nm.

Solute	Mobile phase pH			
	6.5		7.5	
	k_2'	α	k_2'	α
Phe	1.26	1.32	5.37	1.57
p-Amino-Phe	0.44	1.00	2.75	1.63
Tyr	0.45	1.00	3.16	1.76
3-Methoxy-Tyr	0.54	1.00	3.37	1.84
α-Methyl-m-Tyr	0.36	1.00	1.99	1.70
α-Methyl-Tyr	0.57	1.00	1.50	1.73
O-Methyl-Tyr	1.90	1.58	7.77	1.79
α-Methyl-Trp	1.20	1.00	6.62	1.35
6-Methyl-Trp	4.11	1.33	16.22	1.53
7-Methyl-Trp	3.82	1.27	14.77	1.47
5-OHTrp	1.06	1.00	5.15	1.52
β-Phenyllactic acid	0.31	1.44	0.41	1.42

TABLE VII

ELUTION ORDER ON CSP-I AND CSP-II WITH EITHER COPPER(II) OR NICKEL(II) AS COMPLEXING ION

First eluted enantiomer indicated.

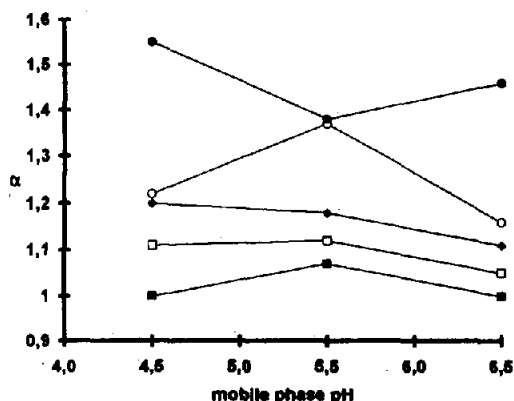| Solute	Copper(II)		Nickel(II)	
	CSP-I	CSP-II	CSP-I	CSP-II
β-Phenyllactic acid	–	L = D	L = D	L
Met	L	L		
Phe	L = D	L	L = D	L
Phenyl-Gly	–	L	L	L = D
Pro	L	L		
Allo-4-OHPro	D	D		
3,4-Dehydro-Pro	L	L		
N-Hexyl-Pro	L	L		
Tyr	D	D = L	L	L
α-Methyl-Tyr	D	D	D	L
3-Methoxy-Tyr	L	L	L	L
Val	L = D	L		

Fig. 8. Influence of pH on enantioselectivity. Stationary phase, CSP-I; mobile phase, 50 mM NH$_4$OAc–0.5 mM Cu(OAc)$_2$; flow-rate, 1 ml/min; temperature, 30°C; detection at 240 nm. ■ = Nval + Thr; ○ = Pro; □ = Tyr; ◆ = 3-methoxy-Tyr; ● = allo-4-OHPro.

phase is often the same for a series of amino acids or dansylated amino acids. L-Proline-containing polystyrene-type resins, for instance, show a stronger retention for the D-enantiomers of bidentate AAs and for the L-antipode of tridentate AAs. Polacrylamide-type and poly(2,3-epoxypropyl methacrylate)-type L-proline stationary phases display a higher affinity

towards the L-enantiomers, indicating that additional binding groups fixing the conformation of the chelate structure of the chiral selector together with space-filling barriers (including the surface of the described gel) may play an important role.

Davankov and Kurganov [20] and Boue et al. [21] proposed a chiral recognition model with donating OH and C=O groups of the immobilized selectors in the lower axial positions of the Cu(II) coordinating sphere. A similar behaviour has been observed on silica-based phases carrying N-alkyl-L-proline residues [4] or L-proline fixed via an amide bond [5]. Testing dansylated AAs on the latter CSP with copper(II) as complexing ion resulted in an elution order of L-before D-enantiomers, but the elution order was reversed on using cadmium(II) ions. This, however, clearly indicates that the different coordination and chelation characteristics of transition metal ions can become a dominant factor.

On CSP-I and -II the D-enantiomers of AAs are in general more retained than their antipodes (see Table VII), except for the D-antipodes of allo-4-hydroxyproline, tyrosine and α-methyltyrosine. The commonly used presentation of mixed-ligand planar copper(II) complexes of L-proline-derived CSPs (introduced by Davankov and co-workers) was used in the proposed chiral recognition models for proline, allo-4-hydroxyproline, α-methyltyrosine and 3-methoxytyrosine in Fig. 9. Reversal of the elution order from proline to allo-4-hydroxyproline seems to be due to the additional coordination of the hydroxy group of L-allo-4-hydroxyproline in the upper axial position of the planar copper(II) complex. The elution order of D- before L-enantiomer of α-methyltyrosine can be explained by the low stability of the D-α-methyltyrosine complex with the CSP due to steric hindrance. In contrast, the reversal of the elution order of the 3-methoxy-tyrosine antipodes compared with α-methyltyrosine cannot be fully explained by the stronger π-basic phenyl residue due to the additional methoxy group, and in this instance other influences must be dominating.

As can be seen in Table I, the methylation of the chiral centre of tyrosine enhanced the observed enantioselectivity (the α-values increase at similar capacity factors from tyrosine to α-
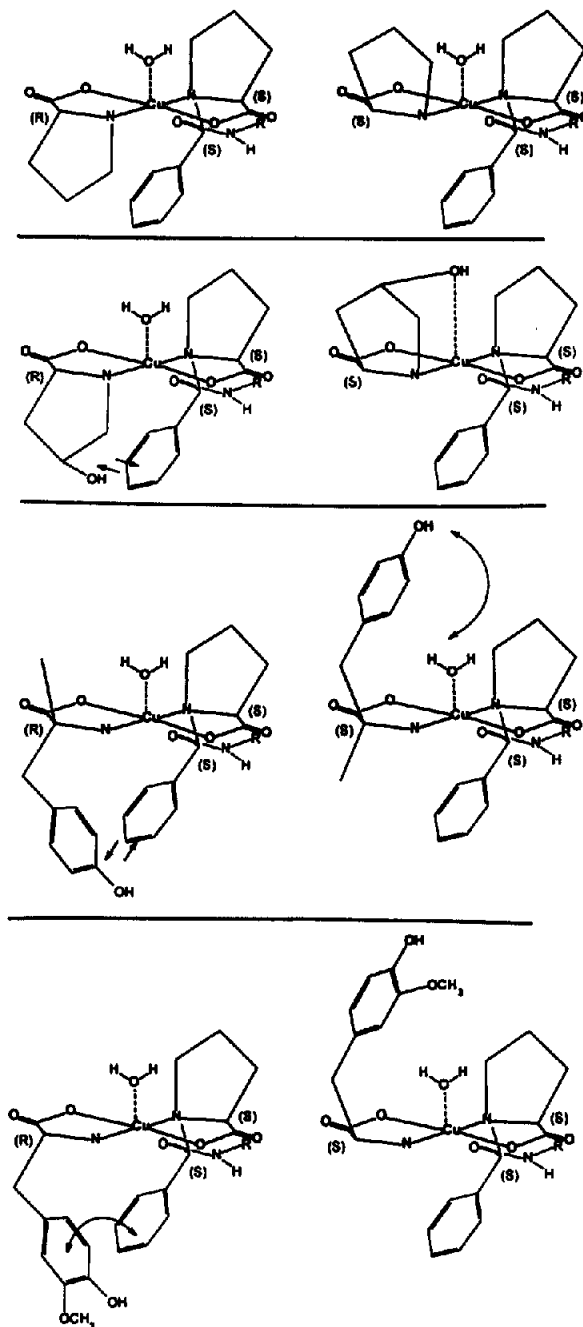

Fig. 9. Proposed chiral recognition mechanisms for Pro, allo-4-OHPro, α-methyl-Tyr and 3-methoxy-Tyr enantiomers using copper(II) as complexing ion.

methyl-m-tyrosine and α-methyltyrosine). Reversal of the elution order on changing the metal ion from copper(II) to nickel(II) was observed for tyrosine (CSP-I) and α-methyltyrosine (CSP-

II). Varying the mobile phase pH did not cause a reversal of elution order.

To point out another interesting phenomenon, the resolutions of phenylglycine, phenylalanine and 2-phenyllactic acid using nickel(II) as complexing ion were compared. Phenylglycine is resolvable on CSP-I but the additional methylene group of phenylalanine induces a loss of stereoselectivity. In contrast, phenylalanine and its hydroxy homologue 2-phenyllactic acid are well resolvable on CSP-II but no resolution was obtained for phenylglycine. The chiral recognition mechanism on CSP-I seems to need the small and rigid structure of phenylglycine for discrimination between the two enantiomers, and CSP-II differentiates only the solutes with the mobile and more space-filling benzyl group.

At this point, and without having exact data from X-ray crystallographic measurements, it is speculative to define exactly the nature of the interactions between the solute molecules, chiral resolving agents and the non-chiral components of the chromatographic system. However, being aware of these shortcomings, and considering the unique elution order of the AAs studied, it seems evident that the influence of the second centre of chirality of the new chiral selectors derived from L-proline may be of minor importance for the enantioselectivity of the whole system and the general enantioselective recognition mechanism of the presented CSPs. The configuration of the epimeric phases affects the overall selectivity in a moderate manner and the two CSPs show approximately the same resolving power when using copper(II) as the complexing ion. Using nickel(II) as the central chelating ion the situation is, however, changed significantly.

CONCLUSION

Two novel chiral stationary phases based on epimeric N-alkyl derivatives of L-proline for chiral ligand-exchange chromatography were synthesized and the applicability of these CSPs to resolve chelating selectands was investigated. Because of the difficult synthesis of CSP-I and CSP-II, their practical applicability will be limited, but they offer excellent possibilities for

investigating chiral recognition mechanisms in CLEC.

The CSPs showed good enantioselectivity for amino acids and derivatives thereof with either copper(II) or nickel(II) as the central transition metal ion. Buffer concentration, temperature and pH of the mobile phase influenced the chromatographic behaviour in a large scale. The epimeric stationary phases showed comparable enantioselectivity using copper(II) as complexing ion, the configuration of the second center of chirality had a moderate effect, due to the spacefilling, aromatic phenyl residue in vicinity of the five-membered ring of the metal complex. Using nickel(II) as chelating ion in the diastereomeric chiral selectors showed, however, different retentions for amino acids as a consequence of altered octahedral mixed-ligand sorption complexes.

The changes in chromatographic behaviour within a series of tyrosine analogues demonstrate effectively the great influence of slight variations of the solute structure on chiral discrimination mechanisms and it becomes complicated to explain the complex simultaneous interactions of matrix fragments, solvent molecules, solutes and immobilized selectors by simplified complex formation models.

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